# FUNGAL METABOLITE CHEMISTRY:
# SPORIDESMIN, CREPIDOTINE

A thesis
submitted in partial fulfilment
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of
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by

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# ABSTRACT

This thesis was concerned with the chemistry of two fungal products, sporidesmin and crepidotine. The structure of sporidesmin was known but aspects of its chemistry, such as how it could be modified to function as an antigen, were not. The structure of crepidotine was unknown but was elucidated in the course of this work.

Since only 50 mg of crepidotine was available, non-destructive methods were used to arrive at the structure, 2-phenyl-1,6-naphthyridin-4-one. Of these methods, $^{1}H$ nuclear magnetic resonance spectroscopy was the most important. This spectrum ($^{1}H$ n.m.r.) of crepidotine was compared with known or expected spectra of possible isomers most of which were unknown.

Sporidesmin is the toxin which causes Facial Eczema. One approach for combating the problem of Facial Eczema is to develop antibodies in the animals against sporidesmin. Sporidesmin is too small a molecule *per se* to induce antibody formation so one of the aims of this thesis was to produce derivatives of sporidesmin and to complex them to proteins. Proteins with many molecules of sporidesmin covalently bound to them were produced to be used as antigens. When animals were treated with these antigens, antibodies were detected in their sera. The titre was low for sporidesmin complexed to albumins but four times higher when sporidesmin was complexed to bovine thyroglobulin.

The method of complexing sporidesmin to the proteins was to open the -S-S- bridge (in sporidesmin) and alkylate the sulphur atoms with iodoacetic esters. When protein was treated with these sporidesmin derivatives the ε-amino-groups of the lysyl residues (in the protein) were transacylated yielding modified sporidesmin covalently bound to the protein, synthetic or natural.

The new compounds formed from sporidesmin in these syntheses were: diethyl and dimethyl secosporidesmin-*S*,*S*'-diacetate, ethyl and methyl 11a-mercaptosecosporidesmin-3-*S*-acetate, methyl 3-mercaptosecosporidesmin-11a-*S*-acetate; and one of the antigens was cross-linked poly(ethoxy)-poly(secosporidesmin-*S*,*S*'-diacetyl)poly-(L-lysine). Of other new derivatives of sporidesmin that were synthesized, 3,11a-dimercaptoseco-sporidesmin, *S*,*S*'-homosporidesmin, and sporidesmin 11-(methyl glutarate), the first was the most difficult to prepare. When attempted in the normal way (opening the -S-S- bridge as for the above esters) only sporidesmin was recovered. But the dimercapto-compound was obtained when oxygen was eliminated in the reaction.

Because the toxicity of the sporidesmin molecule resides in the epidithiodioxopiperazine ring, means of covalently linking sporidesmin to protein without altering the -S-S- bridge were sought, e.g. acylation or diazonium coupling with suitable bridging groups. Having success-fully acylated with the chloroacetyl group (forming sporidesmin di(chloro-acetate) it may be possible to complex this ester to protein through the halogen atom.

In the diazonium coupling reaction, the sole aromatic hydrogen resisted coupling. Diazonium salts eliminated the epidithiodioxopiper-azine ring by rupturing the pyrrolidine ring: the products were the strongly coloured substituted indoleazobenzenes, 5-chloro-2-hydroxy-6,7-dimethoxy-1-methyl-4'-nitroindole-3-azobenzene and the 2'-chloro- and 2'-nitro-analogues. From anhydrodethiosporidesmin 5-chloro-6,7-dimethoxy-8-methyl-2',4'-dinitropyrrolo[2,3-*b*]indole-2-azobenzene was obtained. These indoleazobenzenes are new compounds.

In the search for methods of diazonium coupling to *o*-dihydroxy-benzene, veratrole, the following new compounds were synthesized: 2-chloro-3',4'-dimethoxy-4-nitrobiphenyl, 2-chloro-4'-hydroxy-3'-methoxy-

4-nitroazobenzene, 4'-hydroxy-3'-methoxy-2,4-dinitroazobenzene, 3',4'-dimethoxy-2,4-dinitroazobenzene, 2-chloro-3',4'-dimethoxy-4-nitroazobenzene and 2-chloro-4,5-dimethoxy-2',4'-dinitroazobenzene.

In the $^{13}C$ nuclear magnetic resonance spectra of sporidesmin and sporidesmin-D (dimethylthiosecosporidesmin) (decoupled and undecoupled) the peaks were assigned to the appropriate carbons. Where a resonance seemed anomalous, a reasonable explanation of the anomaly has been put forward. Comparison of the spectra of the two compounds indicated the resonances of those carbons which were involved in the strained epidithiodioxopiperazine ring system. Having assigned the peaks for sporidesmin, the structure of unknown analogues may now be more readily elucidated.

An analogue of sporidesmin, sporidesmin-E, of comparable toxicity, having an -S-S-S- bridge was synthesized from sporidesmin for field studies. It was an unstable molecule readily decomposing to sporidesmin plus sulphur, e.g. pure sporidesmin-E always showed three spots in thin layer chromatography. The mixed melting point between these two compounds was undepressed (they melted within $3.5^{o}$ of each other). That sporidesmin-E had been formed was confirmed by the $M^{+}$ being 32 mass units greater than sporidesmin but this was still no criterion of purity. In the hydrogen-bonding investigation of the two compounds sporidesmin absorbed in the $\nu$OH region where sporidesmin-E did not so the degree that sporidesmin-E was contaminated with sporidesmin could be determined. Comparison of the infra-red spectra of sporidesmin and sporidesmin-E prepared in different ways (in KBr from MeOH or EtOEt, in nujol or in halocarbon oil) showed there were only minor differences between them.

The hydrogen bonding analysis revealed that the C-S bonds of 3,11a-dimercaptosecosporidesmin and sporidesmin-E were similarly orientated (i.e. diverging) and not more or less parallel as in sporidesmin-D. The monomercapto-derivative (methyl 11a-mercaptosecosporidesmin-3-*S*-acetate) showed no $\nu$SH peak: the elements of the thiol group are present (m.s.) but the hydrogen is ionic and with the adjacent amido-group is apparently in hyperconjugation.

# PREFACE

This thesis is concerned with the chemistry of two fungal metabolites, sporidesmin and crepidotine. The structure of sporidesmin was known but facets of its chemistry were not, such as how it could be modified to function as an antigen, its $^{13}C$ nuclear magnetic resonance spectroscopy, and the infrared spectroscopy (OH stretching region particularly) of it and of some of its analogues and derivatives. On the other hand the structure of crepidotine was unknown: a structure has been deduced using the non-degradative methods of spectroscopy.

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# CHAPTER 1. SOME CHEMISTRY OF SPORIDESMIN

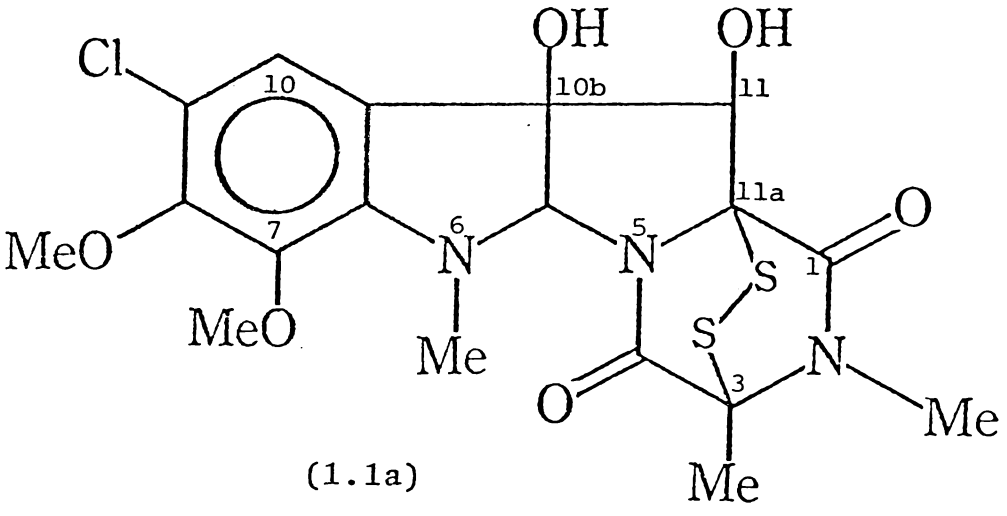
*Introduction*

The sheep industry of New Zealand has, since before the beginning of this century, been troubled by a disease called Facial Eczema.

A history of this disease has been outlined in reviews by Mortimer, *et al.* (1977), di Menna, *et al.* (1977) and Atherton, *et al.* (1974) in which they described how the liver is affected by the disease.

In 1959 (Thornton and Percival) it was shown that facial eczema was associated with a high-sporing strain of a fungus, *Pithomyces chartarum* (Berk. and Curt.) M.B. Ellis. From cultures of this organism, Synge and White (1959) isolated an active compound, having the molecular formula $C_{18}H_{20}ClN_3O_6S_2.CCl_4$ which they named sporidesmin (henceforth abbreviated to sdm). (The fungus was, at first, named *Sporidesmium bakeri* Syd. The name, pithomycin, was avoided in case the substance might be considered an antibiotic like the many '-mycins' being isolated at the time.) Feeding experiments have amply proved that sdm is implicated in the disease facial eczema (Mortimer and Taylor, 1962).

As sheep numbers have increased with the country's more intensive agriculture so have the number of animals that have been affected by facial eczema. In 1935 and 1938 the weather conditions were right for the disease to reach epidemic proportions: millions of sheep died. Since then farmers have been awaiting a practical cure for the disease. Immunisation is one of the possibilities. The main purpose of this study is to produce a large molecule e.g. a protein with sdms covalently bound to it in the hope of stimulating animals to produce antibodies to the toxin.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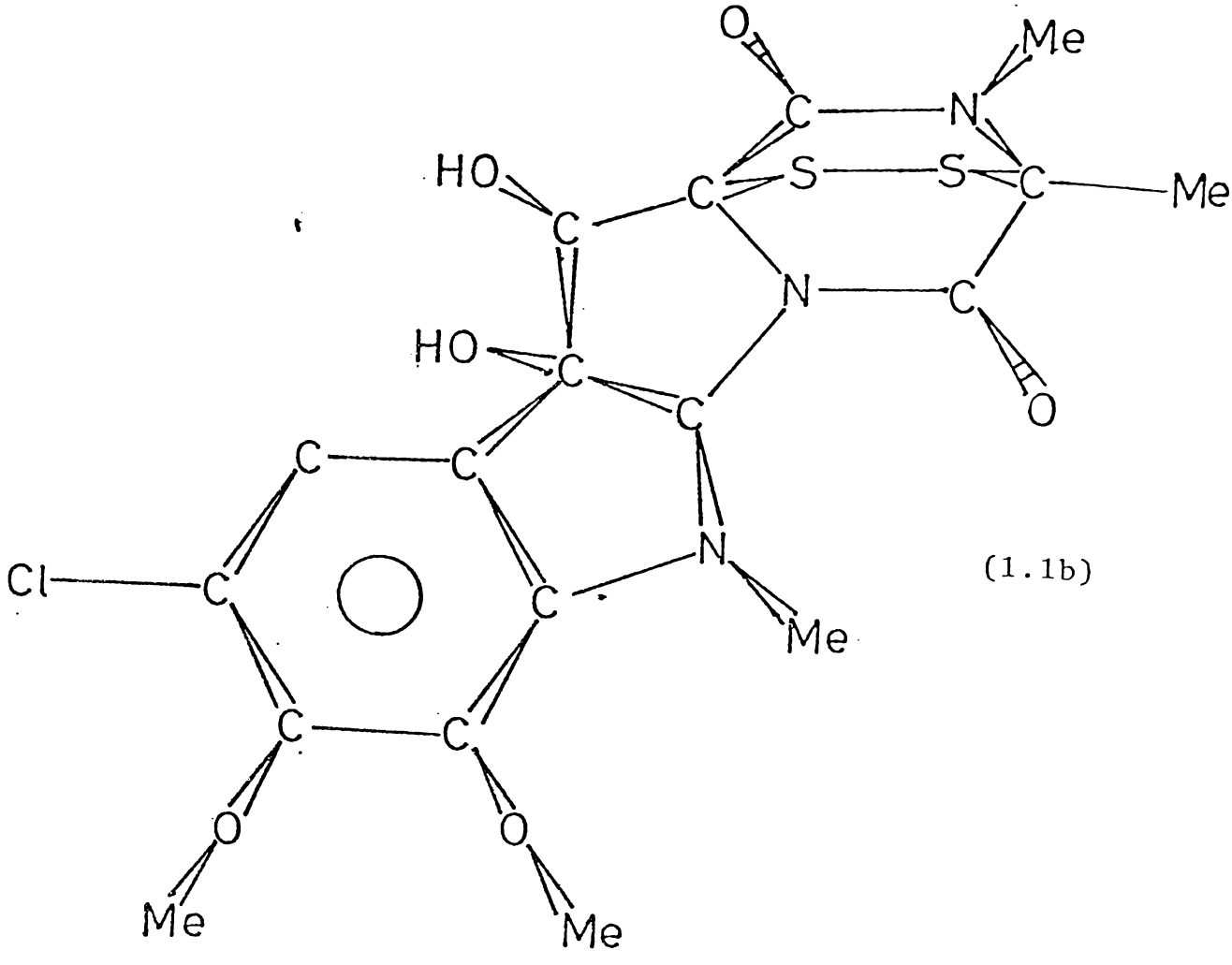
OH
Cl
10
10b
11
11a
O
MeO
7
6
5
1
N
N
S
MeO
Me
S
N
3
O
Me
Me

(1.1a)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Me
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HO
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N
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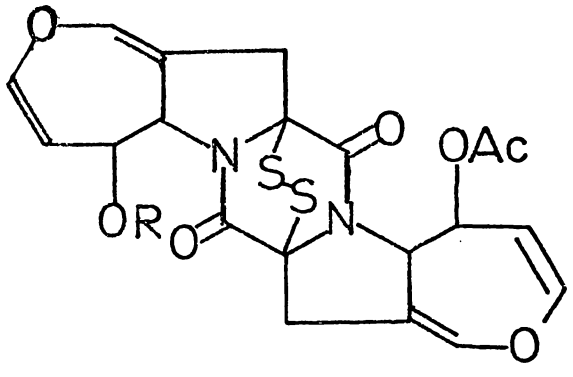
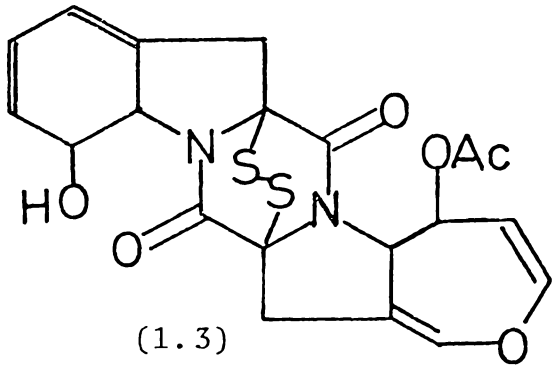
O
Me
Me

(1.1b)

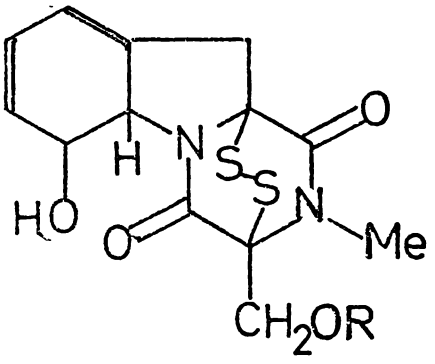


(1.2a) R=H

(1.2b) R=Ac

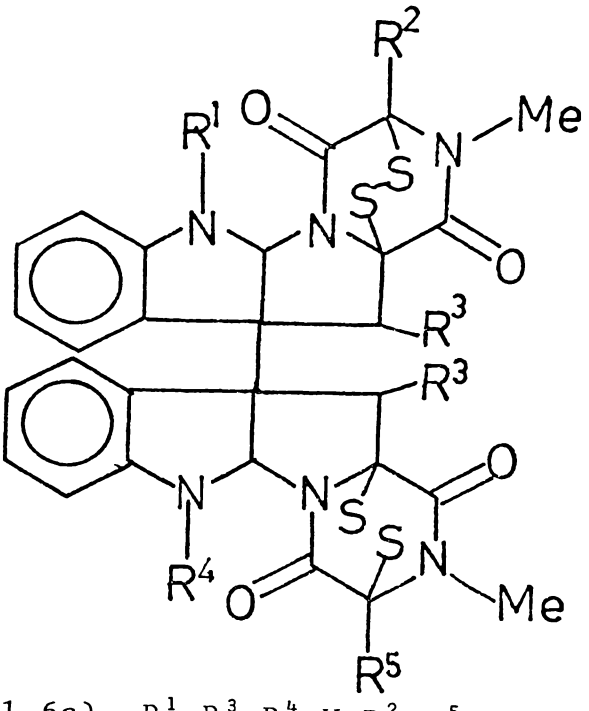


(1.3)



(1.4a) R=H

(1.4b) R=Ac

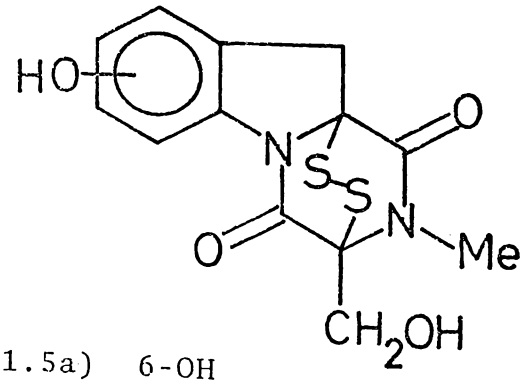


(1.6a) $R^1=R^3=R^4=H$, $R^2=R^5=CH_2OH$

(1.6b) $R^1=R^4=H$, $R^2=R^5=Me$, $R^3=OH$

(1.6c) $R^1=R^4=H$, $R^2=R^5=CH_2OH$, $R^3=OH$

(1.6d) $R^1=R^4=H$, $R^2=Me$, $R^3=OH$, $R^5=CH_2OH$



(1.5a) 6-OH

(1.5b) OH position unknown

The structure of sdm was elucidated by X-ray analysis (Fridrichsons and Mathieson, 1962) and later refined (Beecham, *et al.*, 1966) to the structure (1.1). This structure was substantiated chemically (Ronaldson, *et al.*, 1963, Hodges, *et al.*, 1963a and b).

The crystals which were used in the X-ray analysis were the methylene dibromide solvate. Other solvates also occur and those that have been described are the carbon tetrachloride, methyl iodide, and benzene solvates (Ronaldson, *et al.*, 1963). Sdm is usually stored and used as the sdm benzene solvate because it can be readily prepared without decomposition and because the benzene solvate appears to be quite stable when stored in a refrigerator.

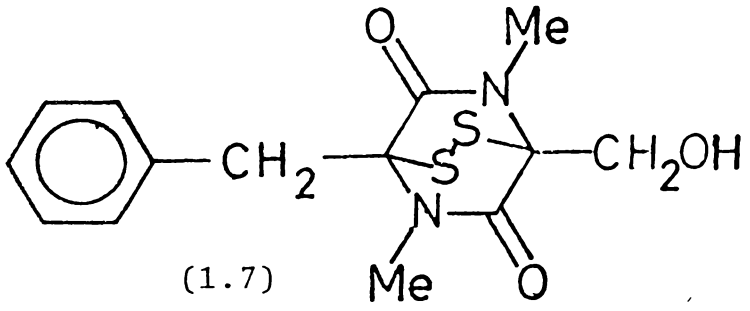
The chemistry of sdm has been reviewed by Taylor (1971), Atherton, *et al.* (1974) and White, *et al.* (1977).

The following 'Rationale' sets out the thinking which led to the various experiments. The main object was to complex sdm to protein for use in immunological studies in association with other scientists (Jonas and Ronaldson, 1974; Fairclough, pers. comm.).

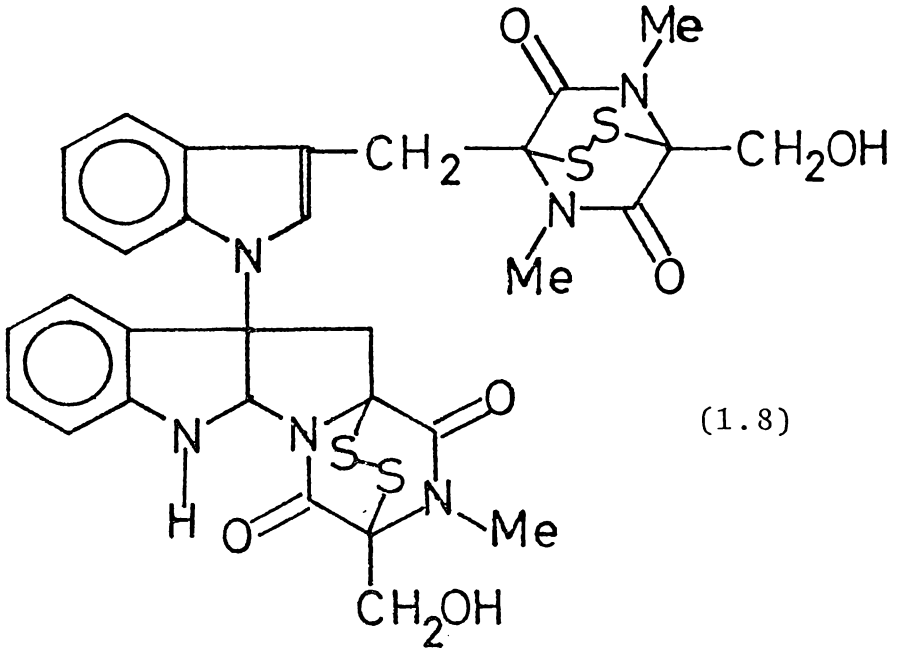
Some other chemical experiments with sdm are also described.

*Rationale*

With time more and more naturally occurring epidithiodioxopiperazine ring compounds are being discovered as metabolites or toxins. They all have toxicological properties (to at least one class of organism). The structural diversity of this important group of toxins is indicated by the following list of epidithiodioxopiperazine ring compounds: sdm (1.1), aranotin (1.2a), acetylaranotin (1.2b), apoaranotin (1.3), gliotoxin (1.4a), gliotoxin acetate (1.4b), dehydrogliotoxin (1.5a), isodehydrogliotoxin (1.5b), chaetocin (1.6a), and verticillin-A (1.6b) (all listed by Taylor, 1971), oryzachlorin (Kato, *et al.*, 1969), dioxychaetocin (1.6c) (Hauser, *et al.*, 1972), melinacidins I to IV (Argoudelis, 1972), verti-



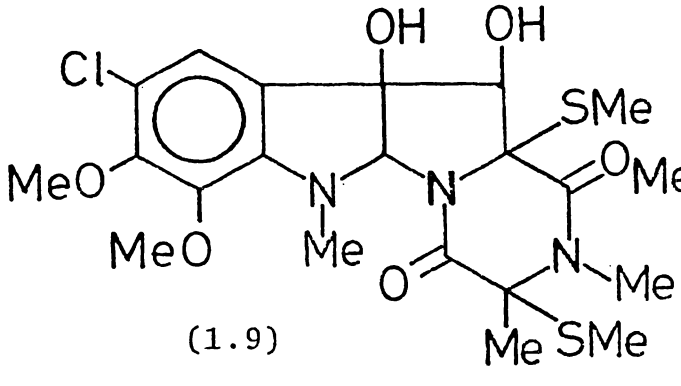
(1.7)



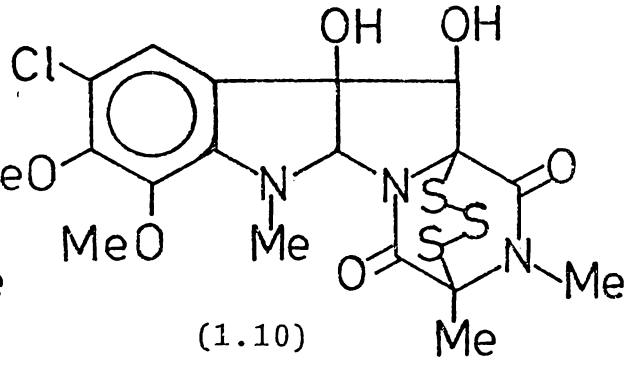
(1.8)

cillins-B (1.6d) and -C (= -B with 5 sulphurs) (Minato, *et al.*, 1973), hyalodendrin (1.7) (Strunz, *et al.*, 1973), and chetomin (1.8) (McInnes, *et al.*, 1976). As well, with these compounds there may be naturally occurring related substances with variable numbers of sulphur atoms (0, 1, 3 or 4) or methylthio-groups. There is also the growing group of naturally occurring dioxopiperazines: Sammes (1975) listed at least 22 simple dioxopiperazines and 13 echinulins and related compounds, and bicyclomycin, and dibromophakellin; Yamazaki, *et al.* (1975) reported fumitremorgin A; Cole, *et al.* (1972), verruculogen; Shiba and Nunami (1974), BP-II; and Dorn and Arigoni (1974), gliovictin. Related to the dioxopiperazine compounds may be the mono- and/or di-(methylthio)-analogues.

Because of this importance of the dioxopiperazine series and because no $^{13}C$ nuclear magnetic resonance ($^{13}C$ n.m.r.) spectroscopy had been published on epidithiodioxopiperazine compounds, the $^{13}C$ n.m.r. spectroscopy of sdm was undertaken, to facilitate the elucidation of the structure of such new compounds as they are discovered. A few simple dioxopiperazines had been examined (Ottnad, *et al.*, 1975; Deslauriers, *et al.*, 1975). The sdm benzene solvate (sdm.$C_6H_6$) as prepared according to Ronaldson, *et al.* (1963) and Ronaldson and Fyvie (1973), always contained a small amount of sdm-D (1.9) and sdm-E (1.10). Therefore, for a $^{13}C$




(1.9)




(1.10)

n.m.r. study of sdm, sdm free from sdm-D and sdm-E was prepared (A-A)*.

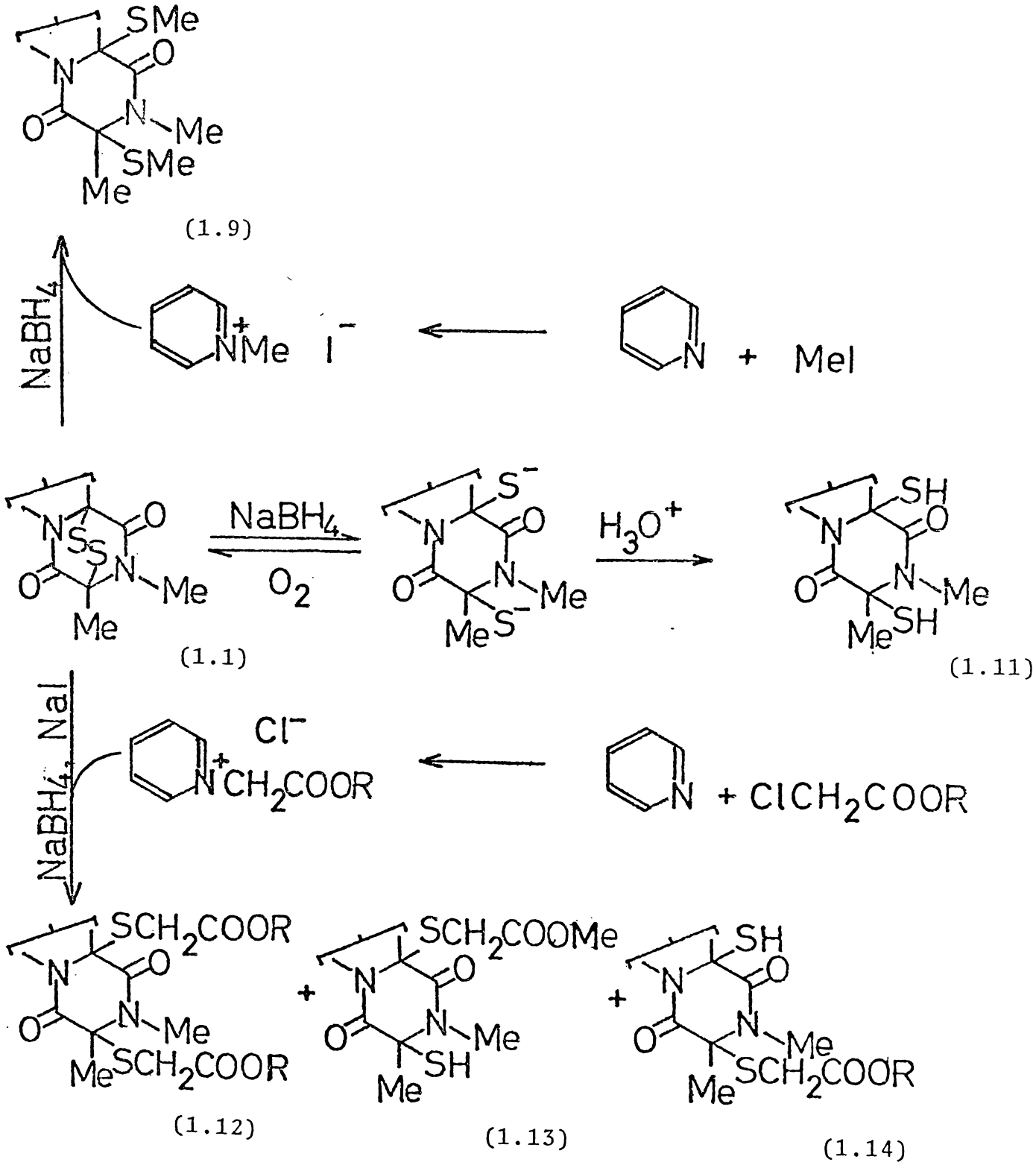
* The brackets refer to the particular experiment or experiments in the Experimental.

For comparison, the already known compound sdm-D was prepared (A-C1) and its $^{13}C$ n.m.r. spectrum examined. In chapter 3, 'The $^{13}C$ nuclear magnetic resonance spectra of sporidesmin and sporidesmin-D,' the two spectra are discussed and the peaks assigned.

Because sdm-E (1.10) was reported (Brewer, *et al.*, 1968; Francis, *et al.*, 1972) to be 10 times more toxic than sdm, attempts were made to prepare it. It had already been prepared by a laborious method (Rahman, *et al.*, 1969). The simpler method of Murdock and Angier (1970) was successfully applied (A-B3). Sdm-E proved to be a rather labile substance hence procedures were developed (Chapter 4) for the characterization of the compound and the differentiation of it from sdm ('Sporidesmin-E, its synthesis, and comparison of its infra-red and $^{13}C$ nuclear magnetic resonance spectra with those of sporidesmin').

To provide a bridge by which sdm might be covalently linked to a protein, acylation was considered. Sdm was acylated with acetic anhydride (Ronaldson, *et al.*, 1963) but attempts at acylation with succinic anhydride (White, pers. comm.) or with chloroacetyl chloride (A-D3) were unsuccessful. Therefore, other points of entry into the molecule were sought.

Jamieson, *et al.* (1969) isolated the analogue sdm-D (1.9) (mentioned above) from cultures of *P. chartarum* and devised a synthesis for the compound from sdm. The synthesis showed that the -S-S- bridge of the epidithiodioxopiperazine ring system could be opened and alkylated with little decomposition occurring in the rest of the molecule. [The name epidithiodioxopiperazine is used in preference to the name dioxoepidithiopiperazine (I.U.P.A.C., 1965, 515.4) because so often this ring system will be compared and contrasted with the dioxopiperazine ring of which the former system is a derivative.]


SMe
O
N
O
N
Me
SMe
Me
(1.9)
NaBH4
NMe I⁻
N + MeI
S⁻
S
S
Me
NaBH4
O2
H3O+
SH
Me
(1.1)
(1.11)
NaBH4, NaI
Cl⁻
N CH2COOR
N + ClCH2COOR
SCH2COOR
SCH2COOMe
SH
MeSCH2COOR
(1.12)
(1.13)
(1.14)
(a) R = Me
(b) R = Et

Scheme 1.1

The problem with the chemistry of sdm has been that the molecule broke down readily when treated with seemingly mild reagents e.g. repeated recrystallization from methanol-water resulted at each step in a product which became more green, from the formation of more and more of the substituted indigotin, 5,5'-dichloro-6,6',7,7'-tetramethoxy-1,1'-dimethylindigotin.

Several unsuccessful attempts were made to obtain the dimercapto-compound but when air was excluded in the work-up of the reaction products then the 3,11a-dimercaptosecosdm (1.11) was obtained (A-B1).

Seeing that the sulphurs could be so alkylated it was reasoned that by alkylation with a compound having a group which would readily condense with a protein, sdm could then be complexed to protein. The immunologist suggested complexing to poly-L-lysine (pll). It is well known that ethyl and methyl esters condense at room temperature with primary aliphatic amino-groups (as in pll) (Ansell and Gigg, 1965). So in the sdm-D synthesis (A-C1) ethyl chloroacetate (and sodium iodide) was used instead of methyl iodide (A-C4) (Scheme 1.1). This alkylation with an ethyl haloacetate yielded a product which did not crystallize. Because diethyl secosdm-*S*,*S*'-diacetate (1.12b) would not crystallize, the possibility of partial transesterification between the ethyl group of the ester and the methyl of the methanol solvent under the influence of sodium borohydride, was considered. So the methyl ester of chloroacetic acid was tried (A-C5) instead of the ethyl ester but crystallization still did not eventuate.

The synthesis required the use of a large excess (20 fold) of chloro-compound: when lesser amounts were used then the monoacetates, methyl 3-mercaptosecosdm-11a-*S*-acetate (1.13a) (A-C5c) and (m)ethyl 11a-mercaptosecosdm-3-*S*-acetate (1.14) (A-C4b) and (A-C5b), tended to occur.

The structures of these two isomers (3-mercapto and 11a-mercapto) (1.13, 1.14) were resolved largely by mass spectrometry. The infra-red (i.r.) spectrum of the former showed a well-defined peak in the νSH region but that of the other compound showed no such peak.

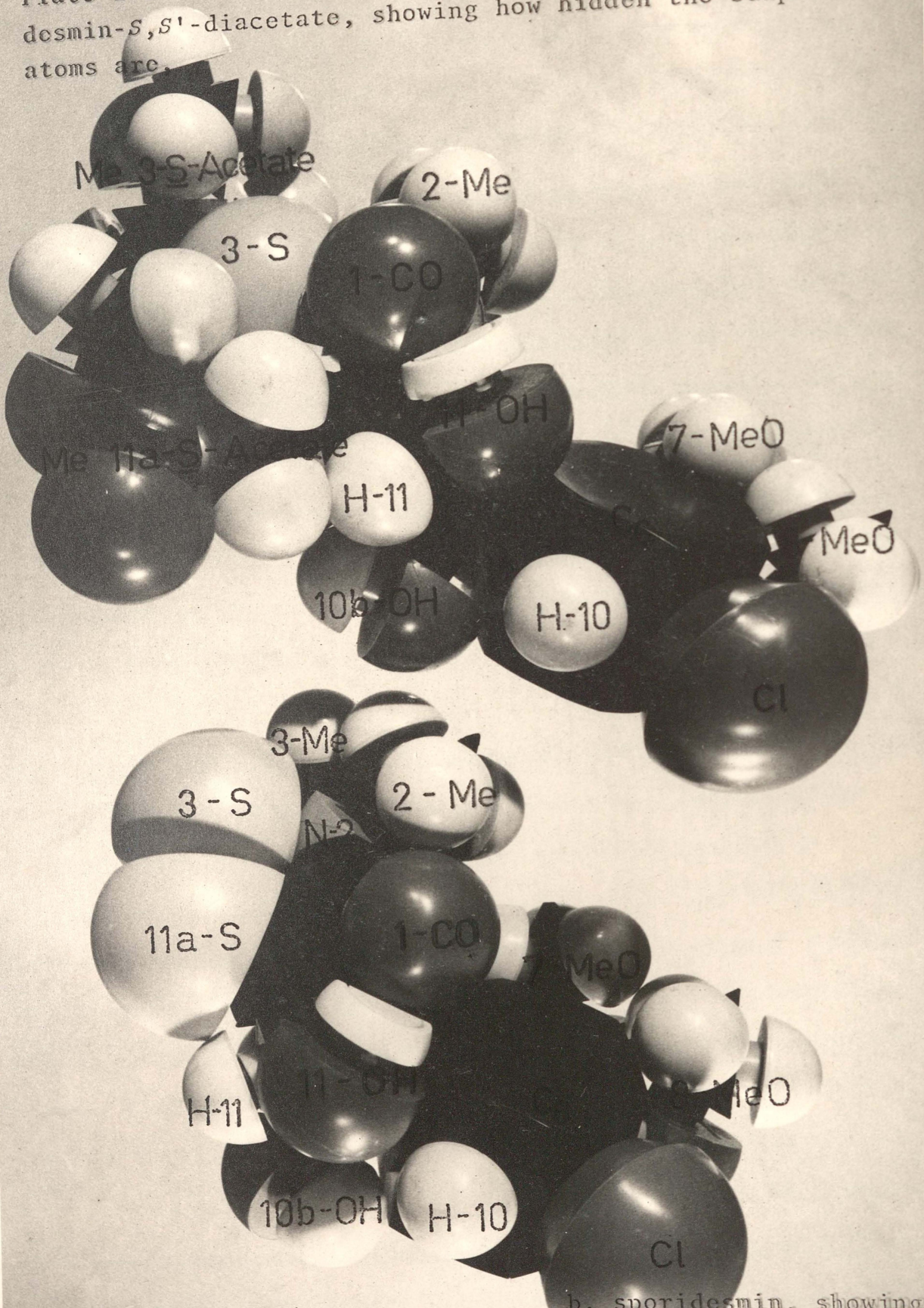
This absence of a νSH peak in a mercapto-compound prompted a study in hydrogen bonding in these compounds. There was also the possibility that the high frequency i.r. spectra of sdm (1.1) and sdm-E (1.10) (above) might assist in distinguishing the two compounds, hence the chapter (5) on 'The infra-red solution spectra (4000—2700 $cm^{-1}$) of sporidesmin and its derivatives'.

Complexations to several different proteins were requested, beginning with pll, then bovine plasma albumin (bpa), rabbit serum albumin (rsa), ovalbumin (ova) and latterly bovine thyroglobulin (btg). Since they all contained lysyl residues, the same synthetic procedure was used with each (A-C6a—f).

In order to estimate the degree of substitution of the ε-amino-groups in the pll preparations and so show that covalent bonding had actually occurred rather than protein binding of the acetate, ninhydrin estimation (A-C6aiv) of the ε-amino-groups of the lysyls was used (Slobodian, *et al.*, 1962). By applying the estimation to equal quantities of pll in the unsubstituted and the substituted state a ratio could be obtained indicating the degree of substitution in the complex. For the natural proteins, that covalent bonding had taken place was shown by a change in the electrophoretic pattern for the substituted compared with the unsubstituted protein (A-C6c—f).

Considerable difficulty was experienced in obtaining consistent results in the ninhydrin estimations. Other workers have also experienced similar difficulties (Wolff, pers. comm.). Although a number of

Plate 1.1 Courtauld models of a. dimethyl secosporidesmin-*S*,*S*'-diacetate, showing how hidden the sulphur atoms are.



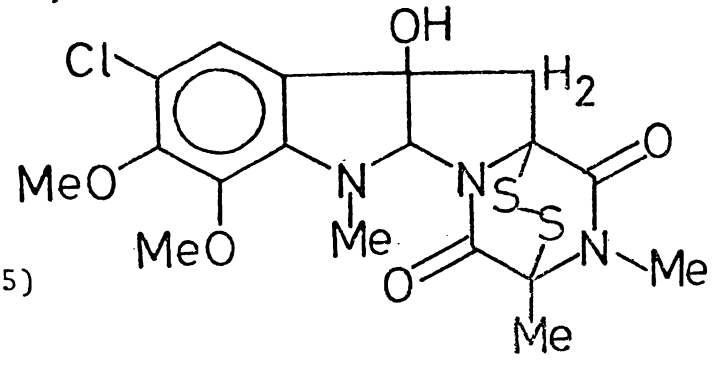

b. sporidesmin, showing how exposed the sulphur atoms are. In a. 10b-OH is shown in the position of hydrogen bonding to the 11a-S while in b. it is shown in that of π-bonding.

refinements to the method were applied none gave consistent results.

When the -S-S- bridge of sdm was modified by opening and alkylating (MeI) (A-ClaiI) then the substance ceased to be so toxic (Hela cells, Taylor, 1971). The antibodies produced by the animals which have been treated with some of the protein complexes are only of low titre (Jonas and Ronaldson, 1974). Nevertheless, in immunological terms it was believed to matter little whether the actual hapten used in complexation was as toxic as the original molecule or not, so long as it retained the general shape of the toxic molecule.

A Courtauld model of sdm shows how prominent on the surface of the molecule the two sulphur atoms are (Plate 1.1). But when the *S*-alkylated sdm was complexed to protein these sulphur atoms ceased to be prominent: in fact, they tended to be hidden by the rest of the molecule. Therefore, it is reasonable to consider that a more specific immunological response (than reported by Jonas and Ronaldson, 1974) might be generated if the prominent sulphur atoms of sdm could be retained and the sdm complexed to protein by other routes than through opening the disulphide bridge. Greater specificity has been obtained in the steroid field by coupling the steroid to protein through pathways other than through the functional groups already present on the hormone (e.g. Hillier, *et al.*, 1973). The functional groups in steroid hormones are usually involved in the hormonal activity. Therefore, a search was made for other points of entry into the sdm molecule. In tissue culture (Taylor, 1971) sdm-B (1.15) which has only the tertiary hydroxy-group (10b-OH), has 1/3 of the

(1.15)



toxicity of sdm so a derivative through the secondary hydroxy-group (11-OH) would, perhaps, lose a small amount of specificity. Whether the aromatic hydrogen in sdm is involved in the toxicity is unknown.

In the sdm molecule there is one aromatic hydrogen. It was not possible to sulphonate sdm because of its instability to sulphuric acid. Diazonium coupling was therefore a possibility but *para* to this aromatic hydrogen is a methoxy-group. It was discovered (Fieser and Fieser, 1961; Turner and Harris, 1960) that anisole or veratrole would diazonium couple with diazonium salts of the very weakly basic anilines, e.g. *p*-nitroaniline.

Because sdm was in short supply and what was available was very costly, diazonium coupling was attempted first to veratrole because sdm is a derivative of veratrole and because veratrole was readily available.

Bunnett and Hoey (1958) performed *p*-nitrobenzene diazonium coupling to 1-methoxynaphthalene and observed that with time the product lost the methyl group to yield 4-(4'-nitrobenzeneazo)-1-naphthol. Should diazonium coupling to sdm occur, it may then be possible thus to demethylate the 7-methoxy-group of sdm. Such a compound is of interest to the Experimental Pathologists (in the study of the pathology of sdm) and of use in preparing sdm labelled in that position for biochemical studies. Furthermore, an azo-compound can be reduced to two anilines, so the amino-group that would be on sdm (in the 10-position) could be diazotized for coupling directly to proteins (containing tyrosines), hence the interest in diazonium coupling to sdm.

*p*-Nitroaniline (A-F1aiii) diazotized readily in concentrated hydrochloric acid but the reaction with veratrole was far from complete, no azo-compound precipitated out and a complex mixture was recovered by solvent extraction. Diazotized *p*-nitroaniline was reacted with sdm dissolved in ethanol and produced nitrobenzene by the Sandmeyer reaction

(Cowdrey and Davies, 1952) and a compound of *m/e* 594 instead of 622 (which would have been the 4'-nitrobenzeneazo-10-sdm). Use of acetone instead of alcohol did not yield a sdm derivative either.

2,4-Dinitroaniline is a weaker base than *p*-nitroaniline, and therefore, according to Schoutissen (1933c) the diazonium salt from 2,4-dinitroaniline would be a more powerful coupling agent in conc. acids, hence the attempts at 2,4-dinitrobenzene diazonium coupling to veratrole and to sdm (A-F2).

In order to recognize the products from the diazonium coupling to veratrole, and since the direct synthesis was not as straightforward as textbooks inferred, diazonium coupling to guaiacol was carried out. It gave satisfactory products which were then methylated with diazomethane.

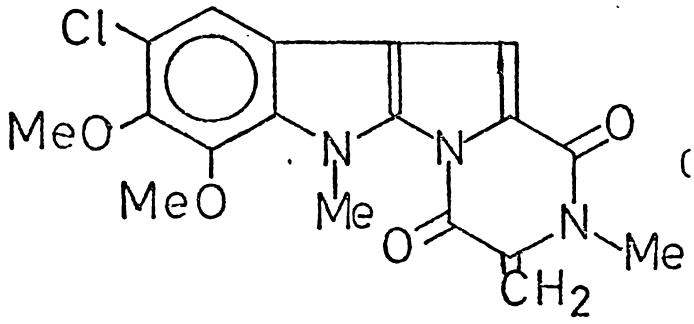
Mass spectrometry of products from coupling with 2,4-dinitrobenzene diazonium chloride (prepared in concentrated hydrochloric acid) showed that a chlorine radicle had substituted a nitro-group. To decide which nitro-group had been substituted the Sandmeyer reaction with potassium iodide was carried out (A-F2Id).

Because of this substitution of the nitro-group diazotization was attempted in other concentrated acids such as orthophosphoric acid ($pK_a$ = 2.12, Chemical Rubber, 1964). In this acid (A-F2III), diazotization took place but there was no reaction with sdm (A-F2IIIc).

This finding led to the choice of the more powerful acid, saturated trichloroacetic acid ($pK_a$ = 0.70, Chemical Rubber, 1964) (A-F2IV) in which the diazotized 2,4-dinitroaniline reacted with sdm (A-F2IVc), again with decomposition of the sdm molecule.

*p*-Nitrobenzene, 2-chloro-4-nitrobenzene, or 2,4-dinitrobenzene diazonium salts in aqueous acids promoted decomposition of sdm in the pyrroloepidithiodioxopiperazine ring system, so non-aqueous acids were turned up in the literature. Sdm has an N-C-N group which hydrolyzes

in aqueous acid. Trifluoroacetic acid is an anhydrous liquid acid (pK = 0.23, Ansell and Gigg, 1965), in which trifluoroacetic anhydride (tfaa) could be used to take up water as it is formed. Tedder (1955) reviewed the chemistry of tfaa and indicated that it 'catalyzes' difficult esterificationsunder mild conditions. This paper indicated that esterification with strong acids, e.g. chloroacetic acid, was unsatisfactory, as was succinic acid; while adipic acid gave good results, so monomethyl glutarate was selected. The main product of the reaction with sdm (A-D1a) was anhydrodethiosdm (1.16). Only a few milligrams of the




(1.16)

monomethyl glutarate ester were obtained from a number of syntheses. Since tfaa dehydrated and dethionated sdm to anhydrodethiosdm, no acid mixture containing tfaa could be used as a solvent in any reaction involving sdm. No esterification of sdm was achieved with monomethyl glutarate in the presence of a substituted carbodiimide (A-D1b).

Since, then, anhydrodethiosdm was obtained as a stable byproduct of the above reaction, it was reasoned that it might be stable to diazonium coupling. It also decomposed but in a different way from that of intact sdm (A-F2IVb).

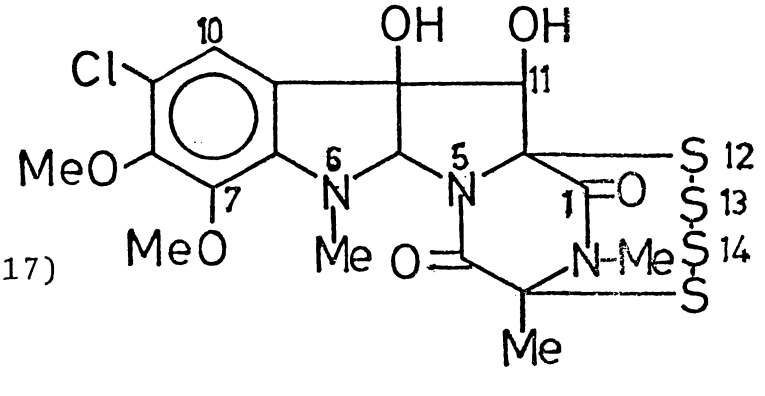
Seeing that sdm could be acetylated with acetic anhydride and pyridine it was reasoned that chloroacetic anhydride and pyridine should be effective (A-D4). In order to link the sdm chloroacetate onto a protein another bridging group was needed i.e. to link the methylenes of the chloromethyl groups to the ε-amino-groups. For this purpose *S*-acetylmercaptosuccinic anhydride (samsa) (A-D4v) was used (Marks, 1967). The anhydride acylates the protein, and the mercapto-group from hydro-

lysis of the acetylmercapto-moiety, should react with the halogenated hapten. Linking the sdm to protein through the secondary hydroxy-group does not expose the sulphur atoms as markedly as a linkage through the remaining aromatic site would do.

Under the acid conditions used only 2,4-dinitrobenzene diazonium salt coupled to veratrole (A-F2IIIaii, A-F2IVa). Therefore coupling to sdm at the aromatic hydrogen was expected. Instead coupling produced rather interesting elimination reactions (A-F2IVc). The same reactions were also observed with the less reactive diazonium salts, e.g. 4-nitrobenzene diazonium chloride (A-F1ciii). Nevertheless, although it had not been possible to link sdm through an azo-group to a protein, it had been possible to link it through modifying the -S-S- bridge (A-C6). Only a partial success in producing antibodies to sdm was achieved when animals were treated with poly-L-lysine covalently complexed to this modified sdm (Table 2.5) (Jonas and Ronaldson, 1974). A greater titre (*c.* 4 times) was obtained when modified sdm was complexed to bovine thyroglobulin (Table 2.5).

The system of numbering the atoms in the sdms was that adopted by Chemical Abstracts (see 1.1a) with the following extension. For convenience of comparing the epipolythio-groups of sdm-E and sdm-G (1.17) with the epidithio-group in sdm the sulphur atoms were numbered this way:

(1.17)




the one on C-3 was 3-S but the others were 12 (on C-11a) *et seq.* (Chemical Abstracts numbers the atoms in sdm-E in a different order from the order in which it numbers the atoms in sdm. For sdm-E it numbers

the sulphur atoms, 1, 2, 3 whereas in sdm the sulphur atoms are unnumbered but referred to as the 3,11a-epidithio-group.) Arising from this system of numbering the sulphur atoms, 11a-S an S-12 are used interchangeably for the same *S*-atom which is on C-11a.

To name the ester derivatives of sdm the conjunctive nomenclature in Rule C-52 and examples under Rule C-463 were followed (I.U.P.A.C., 1965). In naming the protein complexes the syllable (m)ethoxy is in brackets because it was uncertain whether the methoxy or ethoxy had been completely substituted.

# CHAPTER 2. RESULTS AND DISCUSSION

In this chapter is discussed in the first Sections (§§ 1,2,3) the sporidesmin (sdm) derivatives which were prepared and used in linking modified sdm to protein. Briefly, the discussion begins with dimercaptosecosdm (1.11) which is the simplest sdm derivative with an opened -S-S- bridge. This is followed by the derivatives obtained by alkylation of the opened -S-S- bridge and then the complexing to protein of this modified sdm.

The final sections (§§ 4,5) are concerned with derivatives obtained by reaction at other positions in the molecule such as acylation of the secondary hydroxy-group and attempts at diazonium coupling at the aromatic hydrogen.

## §1. *Derivatives from the Opening of the -S-S- Bridge*

### 3,11a-*Dimercaptosecosporidesmin* (1.11) (A-B1)

Since sodium borohydride reduction of sdm followed by alkylation (methyl iodide) produced the alkylated dimercaptosecosdm, sdm-D (1.9), attempts were made to obtain the intermediate compound, dimercaptosecosdm (1.11). The first two attempts (A-B1i, ii) using the same methods as were used in the alkylating experiments described later, failed. When the acidified residue from the reaction was extracted with chloroform, the product by i.r. analysis and by the mass spectrum (*m/e* 473) was sdm itself.

For the third preparation (A-B1iii) cold methanol was added to sdm and sodium borohydride in a small flask. When warmed the suspension dissolved and before effervescence ceased chloroform and water were added to exclude air. Upon shaking, an emulsion was obtained which was filtered from the chloroform. No peaks (νSH) were observed in this ($CHCl_3$) extract. When the raffinate was acidified, it became turbid

(i.e. the two $S^-$s had been protonated to SHs and so the substance was insoluble) and was extracted with chloroform (not an emulsion this time). Again air was excluded during shaking. Although the i.r. spectrum of this solution ($CHCl_3$) showed promise there was a chance that the peak at 2535 $cm^{-1}$ was not real because of the absorbances of chloroform in that region. Increasing the concentration of sdm (A-B1iv) in the reaction showed that the peak observed at 2535 $cm^{-1}$ was, in fact, due to the synthesized solute. When the compound was eventually obtained (A-B1iv, v; *m/e* 475), it was then clear why the first two attempts had been unsuccessful. Because the thiolate ion ($-S^-$) is so reactive (Fontana and Toniolo, 1974) and because of the close proximity of the two ionic sulphur atoms (*c.* 3.2 Å, Courtauld model) when they were formed in the presence of dissolved oxygen (Scheme 1.1) there was oxidation to the -S-S- bridge again (see also under Chapter 5, 'The infra-red solution spectra (4000—2700 $cm^{-1}$) of sporidesmin and its derivatives'). So when oxygen was excluded in the work-up by performing the extraction while hydrogen was still being evolved, then the dimercapto-compound was obtained. It would appear from the experiments under (A-B1vα, A-C5bivα) and from washing chloroform extracts (of mercaptosecosdm derivatives) with alkaline solutions (*c.* pH 10) (A-B1v, A-C5cvi) that the -SH group once it was formed on the dioxopiperazine ring (Scheme 1.1) was inert to reaction either with alkaline solutions or with alkyl iodide. On the other hand, when the $-S^-$ compound was formed (A-B1iii) from reaction of sdm with sodium borohydride, nothing was extractable till the solution was made acid, and while still in the newly formed ionic condition, reaction (with iodo-compounds or dissolved oxygen) took place (A-C1, 4, 5, 8).

The $^1H$ n.m.r. spectrum of this compound was, for the common protons (apart from the positions of the 2 hydroxy-*H*s) similar to that of sdm-D (1.9) (neglecting the ethyl ether peaks for the sdm-D etherate). It

was desired to know what happened to the SH resonances when the adjacent CH protons were irradiated but because of the overlapping of the 2 methoxy-peaks and the 2 *N*-methyl peaks the result could not be clearly observed. Because the 3-SH proton split the 3-Me peak (d) in methyl 3-mercaptosecosdm-11a-*S*-acetate (1.13) it was expected that there would here be the same phenomenon in dimercaptosecosdm. However, it transpired that the 3-Me peak in dimercaptosecosdm was not split but only slightly broadened. Although the C-Me peak was not split, it was irradiated which resulted in the disappearance of the major peak in the SH multiplet at δ 3.66. Because of the overlapping peaks, it could not be observed where this SH resonance now occurred. From the work under 'The infra-red solution spectra (4000—2700 $cm^{-1}$) of sporidesmin and derivatives' (chapter 5) it would appear that perturbing the methyl adjacent to the mercapto-group (3-SH) had upset the dynamic equilibrium which existed between the 2 mercapto-groups. In that chapter (5) it is suggested that the relationship between the 2 C-S bonds (3-CS, 11a-CS) in dimercaptosecosdm was similar to that between the same 2 bonds in sdm-E. In line with this was the shift in the resonances of the adjacent 3-Me protons. For sdm-E (Safe and Taylor, 1971) there are 2 conformers for which the 3-Me protons resonate at δ 2.00 and 1.95. The former is comparable with that of sdm (δ 2.03, Ronaldson, *et al.*, 1963) while the latter with that of dimercaptosecosdm (δ 1.96). Upon reducing the temperature (to $-40^{o}$) at which the $^{1}H$ n.m.r. spectrum of sdm-E was determined, the 3-Me peak occurs as a broad singlet (δ *c.* 1.7) (Safe and Taylor, 1971).

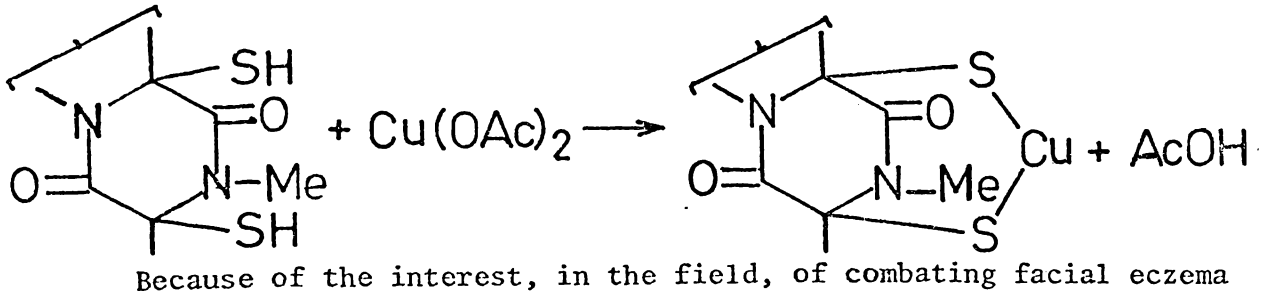
*A copper derivative of sporidesmin* (A-B2)

The first attempt (A-B2i) at synthesizing this substance was made in the hope of arresting 3,11a-dimercaptosecosdm before the -S-S- bridge reformed. A red brown precipitate of copper was obtained from the reaction of the sodium borohydride with the cupric chloride consequently

only sdm was recovered.

After obtaining the 3,11a-dimercaptosecosdm crystalline another attempt was made to prepare the copper derivative (A-B2ii). That reaction between the dimercapto-compound and the cupric acetate had taken place was indicated by the marked change in colour from the green-blue of the cupric acetate to an olive green and further, upon evaporation of the methanol, there was the smell of acetic acid. The complex, of unknown structure, was non-crystalline, insoluble in water but soluble in methanol. The mass spectrum showed no definite parent peak (*m/e* 536) corresponding to the copper derivative.

The i.r. spectrum of the amorphous product showed no νSH peak (2535 $cm^{-1}$) nor the pattern of the dimercaptosecosdm (Fig. IR2.4) between 940 and 600 $cm^{-1}$. Instead it showed a spectrum similar to that of sdm (Fig. IR2.3) except in the 1400 to 1300 $cm^{-1}$ region (Fig. IR2.2). Where sdm had a medium peak at 1380, a very strong peak at 1350 and a medium one at 1310 $cm^{-1}$ the copper derivative had a strong peak at 1385, a less strong one at 1345 and a medium to weak peak at 1310 $cm^{-1}$ comparable with those of 3,11a-dimercaptosecosdm. Hence the cupric ions had not oxidized the dimercaptosecosdm back to sdm but had formed a new substance, for which this interim structure is suggested.




Because of the interest, in the field, of combating facial eczema with zinc salts, the zinc derivative (A-B2iii) was attempted but it did not crystallize either.

*Sporidesmin-E* (1.10)

The discussion of the chemistry of sdm-E (A-B3) is set out under 'Sporidesmin-E, its synthesis and comparison of its infra-red and $^{13}C$ nuclear magnetic resonance spectra with those of sporidesmin' (see Chapter 4).

[13-$^{35}S$]*Sporidesmin-E* (A-B3vi)

To assay the serum from animals which have been treated with the protein which has had modified sdm complexed to it, it was necessary to have some form of sdm which was similar to the sdm hapten and which bore a radioactive atom(s). Since sulphur could be incorporated into sdm fairly simply it was considered that using radiosulphur ($^{35}S$) would produce the desired compound.

Immediately the $^{35}S$ was received it was diluted ($C_6H_6$) and 1/3 of it taken and evaporated to dryness. The sdm.$C_6H_6$ and radiosulphur were redissolved in pyridine; the vessel, wrapped in foil, was heated ($32^{o}$, 3 h). This method of synthesis was the same as that used to synthesize sdm-E (see Chapter 4). To remove excess pyridine the reaction mixture (in $CHCl_3$) was washed (dil. HCl, pH 3 then $H_2O$). In order not to cause decomposition by drying and yet to remove the chloroform, the chloroform solution was diluted with benzene, evaporated to a small volume, re-diluted with benzene and again evaporated to a small volume. This benzene solution was chromatographed in a microcolumn (1 g $SiO_2$ gel G in 7 mm tube) with chloroform-benzene (1:4). Each fraction was collected when the free surface of the solvent had moved 10 cm and the radioactivity of each was determined. After the radioactive front and the relatively inactive trough, fractions 6 and 7 had considerable activity. Fraction 8 which had only half the activity of either fractions 6 or 7, was chosen for the radioassay work because it was farther from any $^{35}S$ which may be 'tailing'.

For the immunologist it was necessary to achieve the following:

1. complete elimination of unreacted radiosulphur,
2. complete elimination of unreacted sdm, and
3. the highest incorporation of radioactivity possible.

These goals were achieved by the following procedures:

1. In the solvent mixture ($C_6H_6$-$CHCl_3$ 7:13) the $R_F$ (t.l.c. $SiO_2$ gel G $F_{254}$; for elemental sulphur was 0.76 which meant that it would be eluted from a column of the same adsorbent, near the front, hence the high radioactivity of fraction 1 (A-B3vi).
2. Sdm, in the same solvent system and adsorbent, had an $R_F$ (0.44) somewhat higher than for sdm-E (0.3). Therefore, fraction 8 (A-B3vi) was chosen because it contained a reasonable level of radioactivity and was sufficiently behind the point where unreacted sdm might be expected to occur.
3. On occasions, for radiotracer studies the radioactive element is diluted with non-radioactive material for the reaction. This procedure simplifies the work but lowers the specific activity. For the reaction described here, then, the elemental radiosulphur had to be used as received. The quantities were so small that there was insufficient to use t.l.c. for monitoring even the arrival of the sdm front.

The ratio of atoms of sulphur to molecules of sdm (A-B3vi) was greater than unity so as to reduce the percentage of unreacted sdm. That the extra sulphur should appear as sdm-G (1.17) was of no concern because the latter compound would have a higher specific activity ([13,14-$^{35}S_2$]-sdm-G) and some may have been present in fraction 8.

The fractions were not evaporated to dryness and weighed, because as observed above evaporation to dryness led to decomposition of sdm-E with the formation of sdm and sulphur.

For the results of using this radioactive sdm derivative, see later (this Chapter under §3, 'The results of treating animals with the modified sporidesmin-protein complexes').

*Sdm-E diacetate*

This compound was synthesized (A-B3a) according to Rahman, *et al.* (1969) to confirm that sdm-E had been synthesized. As they recorded, the compound has not crystallized, having remained a gum for more than three years.

The ultimate analysis was satisfactory for a gum. The mass spectrum was interesting in that the parent peak, *m/e* 589, was strong but there was a minor peak present at *m/e* 589 + 32 = 621. This was the parent peak for the diacetate of sdm-G (1.17), which may have been present in the gum or probably was synthesized on the instrument probe because free sulphur arose in the fragmentation. The other peaks were 557 (589-32), 525 (589-2.32), 493 (589-3.32), 433 (493-60, $CH_3COOH$), 391 (433-42, $CH_2CO$) and 373 (391-18, $H_2O$). 373 Is the molecular weight for anhydrodethiosdm (1.16) (Hodges, *et al.*, 1964).

Later, the instability of sdm-E will be discussed (Chapter 4) and how evaporation to a gum caused decomposition to sdm and sulphur. Such a decomposition process opens up the possibility that the non-crystalline sdm-E diacetate material might have been contaminated with sdm diacetate. The *m/e* for sdm diacetate is 557. There was a minor peak at *m/e* 557 in the sdm-E diacetate spectrum and although some of it might be attributable to sdm diacetate it was as much as would be expected from the loss of one S from sdm-E diacetate under the conditions of mass spectrometry. Because the $M^+$-32 peak in sdm-E acetate was less than 10% of the $M^+$ peak while that in sdm-E was 50% (of the sdm-E $M^+$ peak), it would appear that the sdm-E, acetate was somewhat more stable than the alcohol (sdm-E). There was no peak at either *m/e* 505 or 473 which would have suggested unaltered sdm-E and sdm.

## §2. *The Alkylation of the Sulphur Atoms*

1. *With methyl iodide.*

*Sporidesmin-D* (1.9)

As a model reaction sdm-D (1.9) was synthesized as described by Jamieson, *et al.* (1969). No product was obtained (A-C1ai) because the reducing properties of the sodium borohydride solution in methanol (3 ml, 2 mM) were quickly exhausted at room temperature. But when the sodium borohydride was dissolved in methanol which had been cooled (dry ice-AcMe) (A-C1aii, iii) the evolution of hydrogen was slow and upon mixing and allowing the reaction mixture to warm slowly, reduction of the -S-S- bridge resulted and methylation of the sulphur atoms followed.

This opening of the -S-S- bridge without decomposition of the rest of the molecule, followed by alkylation is the key to the following syntheses (Scheme 1.1).

The $^{13}C$ n.m.r. of sdm-D is discussed in Chapter 3, 'The $^{13}C$ nuclear magnetic resonance of sporidesmin and sporidesmin-D' and the hydrogen bonding and C-H stretching vibrations are discussed in Chapter 5 on 'The infra-red solution spectra (4000—2700 $cm^{-1}$) of sporidesmin and its derivatives'.

Until these $^{13}C$ n.m.r. spectra were obtained for sdm and sdm-D it was considered that the 3-proton singlet peak at $\delta$ 3.30—3.37 in the $^{1}H$ n.m.r. spectra of sdm (Ronaldson, *et al.*, 1963) and sdm-D was due to the methyl on the lactam-*N* of the dioxopiperazine ring and that the 3-proton, singlet peak at $\delta$ 3.07 was due to the methyl on the indoline-*N*. The reason for assigning these two methyls in this way was that the amido-*N* methyl in 1,3-dimethyluracil (Varian, 1963) resonates at $\delta$ 3.30, 3.37 or 3.43 whereas in the saturated pentacyclic ring the indoline-*N* methyl of eserine, 1,3,3-trimethyl-2-*exo*methyleneindole and 2-*exo*formylmethylene-1,3,3-trimethylindole resonate at $\delta$ 2.92, 3.02 and 3.22.

resp. (Varian, 1963). Even though the dimethyluracil was not strictly comparable with the dioxopiperazine ring of sdm yet there was some similarity. In Chapter 3 the basis for reversing these assignments is set out.

*The function of pyridine in the reactions.*-From experiment (A-C5ai) no alkylation took place when the cold methanolic solution of sodium borohydride was added to the methanolic solution of sdm and alkylating agent (methyl chloroacetate + NaI). But after the addition of pyridine, alkylation of the sulphur atoms took place. The presence of pyridine was thus essential but its function was more than that of an acid scavenger (Allinger, *et al.*, 1971) for the hydriodic acid as it was formed. Because of the decomposition of the sodium borohydride during the reaction there was an excess of hydroxide ions. The contribution of the pyridine here may be comparable with its effective function in acylations using acyl chlorides (*N*-acylpyridinium chloride, Allinger, *et al.*, 1971). Stahmann, *et al.* (1946) found that bis-(β-pyridiniumethyl)sulphone dichloride alkylated cysteine to the biscystenyl derivative of divinyl sulphone. Borrows, *et al.* (1949) observed the same phenomenon with *N*-substituted pyridinium salts in alkylating phenols to a diphenyl ether. Hence, it was that alkylating function of pyridine that was observed here and in the reactions described subsequently. The pyridine reacted first with the haloacetic ester to form *N*-alkoxycarbonylmethylpyridinium halide (Scheme 1.1) which then was the alkylating agent.

[3,11a-($SC^3H_3$)$_2$]*Sporidesmin-D* (A-C1b)

To assay the sera from sheep which had been treated with a sdm antigen, some radioactive sdm derivative was required. Sdm-D was a reasonably stable sdm analogue compared with sdm-E (for [13-$^{35}$S]sdm-E see above, §1) so an attempt was made to prepare [3,11a-($SC^3H_3$)$_2$]sdm-D. The method of synthesis followed the outline used to prepare sdm-D from sdm but using

[$^3H_3$]methyl iodide instead of methyl iodide. Microgram amounts of compounds tend to decompose when chromatographed on a t.l.c. plate: this decomposition of the solute seems to occur, when the plate is drying. Therefore instead of purifying the *c.* 40 μg of product by t.l.c. the whole product of the reaction was chromatographed, without evaporation to dryness. To change from chloroform to benzene solution the second solvent was added and the lower boiling chloroform evaporated off: this process was repeated. The final small volume of solvent-solute was transferred to a column.

After the dead volume had emerged, the radioactive counts (x $10^{-6}$) for the respective fractions were: 34, 3.5, 1.6, 1.2, 4.7, 1.8, 1.9, 0.9 and 0.5. Thus it appeared that fraction 5 was the one required. Fraction 1 on examination by t.l.c. showed a very strong radioactive peak at the origin and a strong one just faster ($R_{\text{sdm-D}}$ 1.15) than sdm-D while fraction 5 also showed (after concentrating to 1/10 vol) a peak at the origin somewhat larger than that at sdm-D. The large peak at the origin from the t.l.c. of fraction 5 suggested that [3,11a-(*SC*$^3H_3)_2$]sdm-D was unstable (owing to either the treatment, the t.l.c. spotting or radiolysis). Fraction 5 developed an amber colour when it was concentrated. For the immunoassay, fractions 6 and 7 (of the tailing peak) were combined and used because they had not been concentrated). For its use in the immunological experiment see §3.

*Sporidesmin-D diacetate* (A-C2)

Only the molecular weight of this acetate, synthesized from sdm-D.-EtOEt, acetic anhydride and pyridine in the usual way (Jamieson, *et al.*, 1969), was checked. The mass spectrum showed $M^+$ at *m/e* 587, and this was consistent with the conversion of sdm-D ($C_{20}H_{26}ClN_3O_6S_2$, *M* 503) to the diacetate ($C_{24}H_{30}ClN_3O_8S_2$, *M* 587).

2. *With chloroacetic acid.*

*Secosporidesmin*-S,S'-*diacetic acid*

The two attempts (A-C3i, ii) to produce this acid by alkylation of the opened -S-S- bridge with sodium chloroacetate or chloroacetic acid were unsuccessful perhaps for the same reason that bis-(β-pyridiniumethyl)-sulphide (Stahmann, *et al.*, 1946, reason not given) did not alkylate cysteine, whereas the sulphone did.

A third attempt by treating sdm with sodium sulphide (after Dutcher, *et al.*, 1945) and alkylating with chloroacetic acid also failed (A-C3iii). Dutcher, *et al.* also failed to alkylate gliotoxin this way when using benzyl chloride as the alkylating agent. The sdm dissolved in the aqueous solution of sodium sulphide suggesting that a reaction had taken place to form the disodium mercaptide (Dutcher, *et al.*, 1945).

A fourth attempt (A-C5d) was made beginning with one of the non-crystalline esters (methyl, 1.12a) described below. Since sdm and its derivatives are labile to acid and alkaline hydrolytic action (Ronaldson, *et al.*, 1963), the less drastic process of hydrolysis with an ion exchange resin (Amberlite IR-100 in the acid form) at room temperature was used. Since water was the medium in which this hydrolysis takes place and since the ester was water insoluble the latter was spread as a film over the surface of the flask to which the ion exchange resin and water were added. After shaking for 20 days the filtrate was extracted first with chloroform then with ether. Only the residue from the ether extract showed any suggestion of the formation of carboxylic acids by the three i.r. spectral peaks: two broad ones in the carboxylic acid hydrogen-bonding area (2700—2500 $cm^{-1}$) and one very strong one in the carboxy-carbonyl region (1720vs $cm^{-1}$, not at 1730s $cm^{-1}$ for the ester, Nakanishi, 1964). Because the yields were low, these syntheses were not pursued further.

Table 2.1. Reagents used in the syntheses (A-C4a, b) of diethyl secosporidesmin-*S*,*S*'-diacetate.

(Solvent system for t.l.c.: ether-benzene 2:3.)

| Expt | Sdm.$C_6H_6$ (µmol) | Pyridine (µℓ) | $ClCH_2COOEt$ (mmol) | NaI (mmol) | MeOH (ml) | $NaBH_4$ (mg) | Product | $R_{sdm}$ | Crude Yield (%) |
|---|---|---|---|---|---|---|---|---|---|
| a i. | 190 | 400 | 20 | - | 2.4 | 30 | (sdm) | | |
| a ii. | 59 | 130 | 1 | 1.2 | 1.5 | 15 | (1.12b) | 0.8 | 100 |
| a iii. | 200 | 550 | 4 | 4.1 | 4.6 | 76 | (sdm) | 1.0 | 1 |
| | | | | | | | (1.12b) | 0.8 | 86 |
| b iv. | 1000 | 2750 | 20 | 21 | 18 | 292 | (1.12b) | 0.8 | 38 |
| | | | | | | | (1.14b) | 0.4 | 21 |

Table 2.2. Reagents used in the syntheses (A-C5a—c) of dimethyl secosporidesmin-*S*,*S*'-diacetate.

(Solvent system for t.l.c.: ether-benzene 2:3.)

| Expt | Sdm.$C_6H_6$ (µmol) | Pyridine (µl) | $ClCH_2COOMe$ (mmol) | NaI (µmol) | MeOH (ml) | $NaBH_4$ (mg) | Product | $R_{sdm}$ | Crude Yield (%) |
|---|---|---|---|---|---|---|---|---|---|
| a i. | 1000 | (1200)* | 5.7 | 270 | 12 | 193 | (1.1) | 1.00 | 11 |
| | | | | | | | (1.12a) | 0.59 | 68 |
| a ii. | 500 | 600 | 2.9 | 135 | 6 | 96 | - | - | - |
| a iii. | 430 | 500 | 2 | 110 | 4 | 56 | (1.1) | 1.00 | 34 |
| | | | | | | | (1.12a) | 0.59 | 39 |
| | | | | | | | (1.14a) | 0.22 | 24 |
| b iv. | 400 | 500 | 2 | 125 | 4 | 61 | (1.12a) | 0.59 | 70 |
| | | | | | | | (1.14a) | 0.22 | 20 |
| c v. | 1000 | 1200 | 5.7 | 270 | 12 | 193 | (1.1) | 1.00 | 5 |
| | | | | | | | (1.13) | 0.70 | 86 |
| | | | | | | | (1.12a) | 0.59 | 10 |
| | | | | | | | (1.14a) | 0.22 | 1 |
| c vi. | 2000 | 2400 | 11.5 | 650 | 24 | 320 | (1.1) | 1.00 | 20 |
| | | | | | | | (1.13) | 0.70 | 27 |
| | | | | | | | (1.12a) | 0.59 | 27 |
| | | | | | | | (1.14a) | 0.22 | 18 |

* The pyridine was not added at the beginning of the synthesis.

3. *With chloroacetic esters.*

*Di(m)ethyl secosporidesmin*-S,S'-*diacetates* (1.12),

*(M)ethyl 11a-mercaptosecosporidesmin*-3-S-*acetates* (1.14), and

*Methyl 3-mercaptosecosporidesmin*-11a-S-*acetate* (1.13)

As set out in the 'Rationale' (Chapter 1) these syntheses (A-C4, 5) were attempted to furnish the hapten (sdm) with a group which may be used to link it to lysine-containing proteins.

The first attempt at this synthesis failed (A-C4ai) (Table 2.1) because both sodium iodide and cooling were absent. In the alkylation of thiols an iodo-alkylating reagent is required (Jocelyn, 1972), hence the use of sodium iodide in each reaction. For the ethyl ester reactions, *c.* a mole (NaI) for each mole of alkylating agent was used while for the methyl esters only *c.* 1/20 mole was used. The latter ratio was satisfactory, because, as reaction proceeded, more chloroacetate would be transiodinated. It is well-known that the transiodination equilibrium favours the formation of the iodide.

A cold solution of sodium borohydride in methanol was added to a cold solution of sdm and sodium iodide in pyridine, (m)ethyl chloroacetate and methanol. The reagents dissolved freely. When the product was acidified in work-up the quantity of *N*-alkoxycarbonylmethylpyridinium salt was negligible (compare A-C4aii with A-C4aiii). Chromatography in benzene with increasing concentrations of ether yielded after unchanged sdm, methyl 3-mercaptosecosdm-11a-*S*-acetate (1.13) (from methyl chloroacetate only), di(m)ethyl secosdm-*S*,*S*'-diacetate (1.12) as a gum and finally (m)ethyl 11a-mercaptosecosdm-3-*S*-acetate (1.14) (for $R_F$s see Tables 2.1, 2.2). Because the $R_F$s of dimethyl secosdm-*S*,*S*'-diacetate and methyl 3-mercaptosecosdm-11a-*S*-acetate were so close they were not completely separated (e.g. compound 1.13 not detected in experiment A-C5aiii) in the above solvent system. T.l.c. of the mixture with chloroform ($R_F$ 0.64), ethyl acetate ($R_F$ 0.53), acetone ($R_F$ 0.83), t-butyl alcohol-benzene

(1:9, $R_F$ 0.61), methanol-benzene (1:9, $R_F$ 0.72) or with acetone-benzene (3:17, $R_F$ 0.41) showed no separation. From this latter system it was deduced that ether alone was the solvent that effected the separation in ether-benzene mixtures. The solvents that effected separations were: ether ($R_F$ 0.70, 0.80), acetone-light petroleum (40—60°) (1:3, $R_F$ 0.25, 0.36), ether-light petroleum (1:1 $R_F$ 0.045, 0.09), (4:1 $R_F$ 0.18, 0.25), and (23:2 $R_F$ 0.49, 0.60). The greatest percentage apparent separation was with ether-light petroleum (40—60°) (1:1), hence those fractions containing both compounds were rechromatographed in light petroleum (40—60°) and developed with ether. At the 1 mmol level (A-C5cv) this process effectively separated these substances but at the 2 mmol level (A-C5cvi) it was necessary to chromatograph a third time ($C_6H_6$ with more slowly increasing concentrations of EtOEt).

The ratio of (m)ethyl chloroacetate to sdm was *c.* 20:1 for the ethyl esters and *c.* 5:1 for the methyl esters, yet even with the high ratio some monosubstituted derivatives (ethyl 11a-mercaptosecosdm-3-*S*-acetate, 1.14b) were formed (Table 2.1, biv). The other monosubstituted (methyl 3-mercaptosecosdm-11a-*S*-acetate,1.13a) compound appeared in quantity only under the methyl ester syntheses (Table 2.2, cv, cvi).

In the attempt to obtain the secosdm-*S*,*S*'-diacetates crystalline, the following crystallizing systems were tried on the gum: acetone-light petroleum (80—100°), benzene-light petroleum, acetone-water, ethyl acetate-light petroleum. An ether solution (100 µl) of the gum (1 mg) was applied across a t.l.c. plate and developed (EtOEt-$C_6H_6$ 2:3). Elution of the quenching zone yielded a gum which would not crystallize.

Another approach was to obtain a diacetate derivative which might crystallize readily and at the same time have a 'good leaving group' for the acylation process. Attempts were made to prepare dipicryl secosdm-*S*,*S*'-diacetate and then di-(*p*-nitrophenyl) secosdm-*S*,*S*'-diacetate because the picryl synthesis failed.

In attempting to synthesize the picryl chloroacetate from chloroacetyl chloride and sodium picrate or pyridine and picric acid (A-C7i), no product was obtained having ester carbonyl (>1700 $cm^{-1}$) or chloroacetate C-O-C (*c.* 1150 $cm^{-1}$) i.r. absorption.

*p*-Nitrophenyl chloroacetate (A-C7ii) (Auwers, *et al.*, 1927) was synthesized and the i.r. spectrum of the crystals showed absorptions at 1770 $cm^{-1}$ (ester C=O) and 1140 $cm^{-1}$ (chloroacetate C-O-C). This ester was used in reaction with sdm (A-C7iii) in the same way as methyl chloroacetate was used in the previous syntheses. Only *p*-nitrophenol and dimethyl secosdm-*S*,*S*'-diacetate were obtained. This was not surprising since basic (pyridine)methanolysis of *p*-nitrophenyl acetate readily occurs (Kirkien-Konasiewicz, *et al.*, 1967) i.e. the *p*-nitrophenyl group was such a good leaving group that it was readily replaced by the methyl of methanol (in presence of pyridine).

After this, sublimation (0.001 mmHg) was attempted: the gum commenced darkening at 74° and was dark brown at 120° but nothing had sublimed. Since trimethylsilyl ethers are more volatile, the ester (1.12a) was trimethylsilylated with hexamethyldisilazane (A-C5e), after Safe and Taylor (1972). Although it was possible to wash the product ($CHCl_3$ solution) with water, it was not possible to chromatograph it either on t.l.c. or in a column, without it hydrolyzing. That the two hydroxy-groups of the ester had been trimethylsilylated was shown by the $^1H$ n.m.r. spectrum where there were no peaks corresponding to the hydroxy-*H*s, the CH (δ 4.61) of the secondary hydroxy-group was unsplit, and the two trimethylsilyl peaks appeared as two equally intense peaks at different frequencies (δ 0.05, -0.17). Further the aromatic proton had shifted upfield by 0.30 ppm. It is suggested that this shielding of the aromatic proton resonance upon trimethylsilylation resulted from the close proximity (Courtauld model) of both groups to this proton. For the impurities in this uncrystallized substance there were only 3

extraneous peaks (δ 4.7, 3.5, 3.1) whose intensities were little more than that of the background.

When this substance was sublimed some hairlike cystals appeared, which did not increase with time although the sublimation was prolonged (2 d). The hairlike cyrstals had the same i.r. spectrum as the unsublimed product so they were not some volatile extraneous material.

Also when diethyl secosdm-*S*,*S*'-diacetate (1.12b) would not crystallize, it was suspected that the sodium borohydride was effecting sufficient transesterification of the ethyl ester to the methyl ester in the methanol, to hinder crystallization. Hence to avoid the chance of partial transesterification methyl chloroacetate was used: even so crystallization again (after chromatography) did not occur. By contrast the monoalkyl mercaptosecosdm-*S*-acetate esters crystallized quite readily. When the gum of methyl 11a-mercaptosecosdm-3-*S*-acetate from experiment (A-C5aiii) was being prepared for complexation to p11 (A-C6aii) it dissolved freely in the alcohol then suddently crystallized out and would not redissolve.

Because repeated crystallization did not give a sharp melting point for methyl 11a-mercaptosecosdm-3-*S*-acetate (1.14a) (A-C5biv) further t.l.c. on the crystals was investigated: acetone-ethyl acetate (1:1) $R_F$ 0.9, t-butyl alcohol-light petroleum (40—60°) (1:9) $R_F$ 0.6, acetone-chloroform (1:1) $R_F$ 0.6 one spot in iodine vapour, t-butyl alcohol-benzene (1:4) $R_F$ 0.74 one spot in iodine vapour, and methyl cyanide-ether (7:43) $R_F$ 0.4 one spot in iodine vapour, on silica gel sheets. On polyamide sheets, water gave an $R_F$ of 0.0; methanol, $R_F$ >0.9 with tailing; and methanol-water (1:1), $R_F$ <0.1 with tailing. But none of these systems separated any exogenous material. On sublimation (0.01 mmHg) the ester was stable to 118° then browned (120°) and turned black (126°) without subliming. Upon ultimate analysis it was found that the molecule

crystallized with a molecule of water hence the indefinite melting point (150—156°). The ester did not decompose when boiled in ethanol. It was freely soluble in benzene, acetone, acetic acid and chloroform but not in ether and only partially in ethanol, methanol and ethyl acetate.

The following chemical tests for the presence of a mercapto-group in ethyl (1.14b) (A-C4biv) and methyl 11a-mercaptosecosdm-3-*S*-acetate (1.14a) (A-C4aiii) and methyl 3-mercaptosecosdm-11a-*S*-acetate (1.13) (A-C5cv) were applied:

1. Though Grote's (1931) reagent, modified sodium nitroprusside, gave a fleeting red positive with dithiothreitol, there was no positive red coloration with either the 11a- or the 3-mercapto-esters, perhaps, because of the insolubility of the esters in the reagent. Although acetone is a good solvent for these derivatives, it could not be used to dissolve the esters for this reaction, since acetone alone gave a stable deep red colour, a false positive.
2. Acetone solutions of the latter compound gave a positive yellow colour with Ellman's reagent [after Ellman (1959), Beutler, *et al.* (1963), 5,5'-dithiobis-(2-nitrobenzoic acid)]. But a similar solution of the former compound gave no yellow coloration. (A slight excess of ammonium ions gave a false positive.)
3. Sdm, 3- and 11a-mercaptosecosdm esters, sdm-D and both methyl and ethyl secosdm-*S*,*S*'-diacetates (20 μg each) were disc chromatographed ($H_2O$) and sprayed with azide-iodine (Feigl, 1966) solution. This showed both sdm and methyl 3-mercaptosecosdm-11a-*S*-acetate as colourless spots (starch) at the same $R_F$, sdm-D as a colourless mark which quickly disappeared and the others could not be detected.

Consistent with methyl 3-mercaptosecosdm-11a-*S*-acetate (1.13) giving the positive thiol reactions above, its i.r. spectrum (KBr) showed a peak at 2535 $cm^{-1}$, in the S-H stretching region (see Fig. IR2.1). The $^1H$ n.m.r. spectrum indicated the position of this thiol group because the

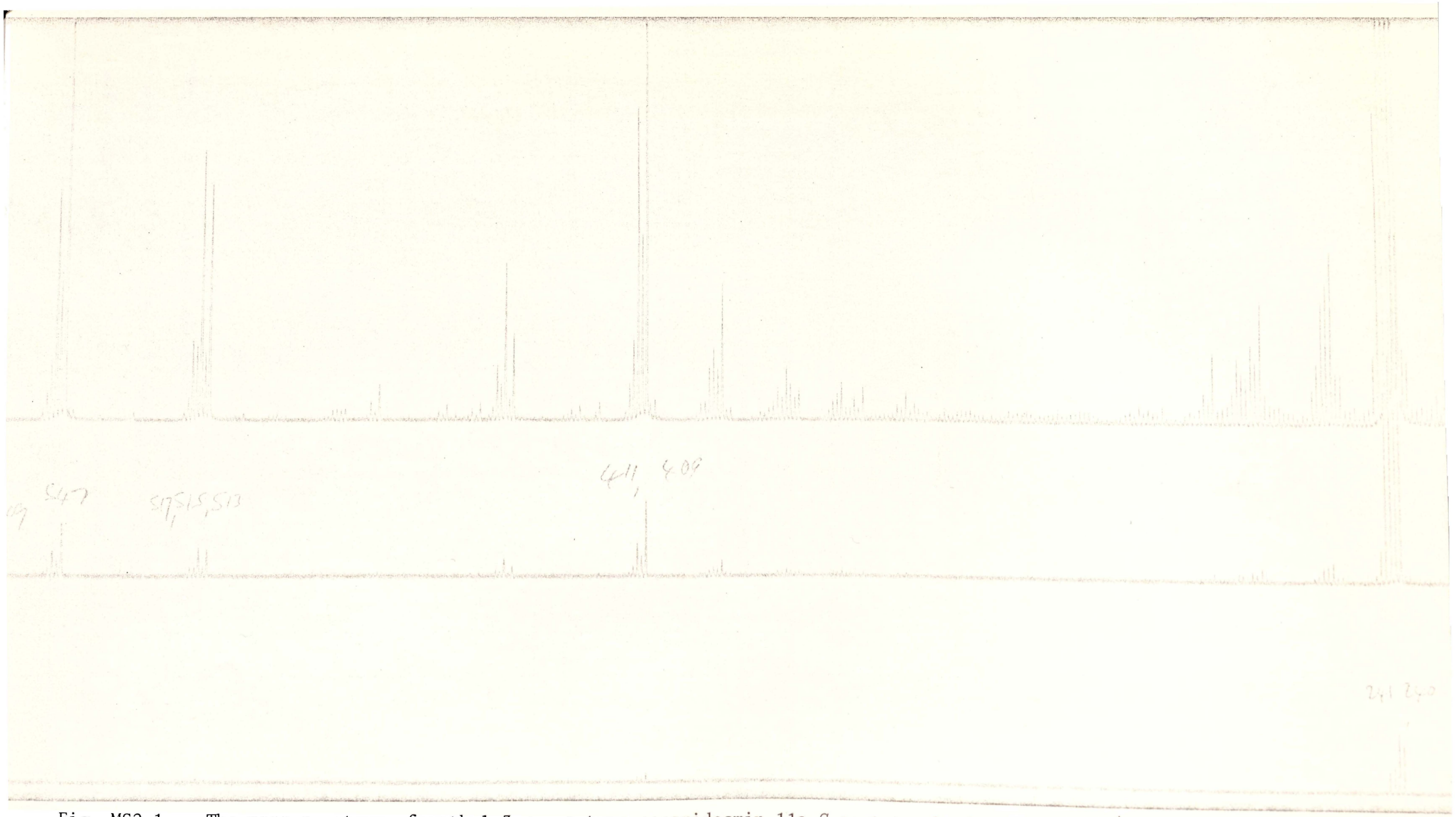


Fig. MS2.1. The mass spectrum of methyl 3-mercaptosecosporidesmin-11a-*S*-acetate showing that the $M^+$ - $H_2S$ peak (*m/e* 513) is less intense than the $M^+$ peak (*m/e* 547); compare Fig. MS2.2.

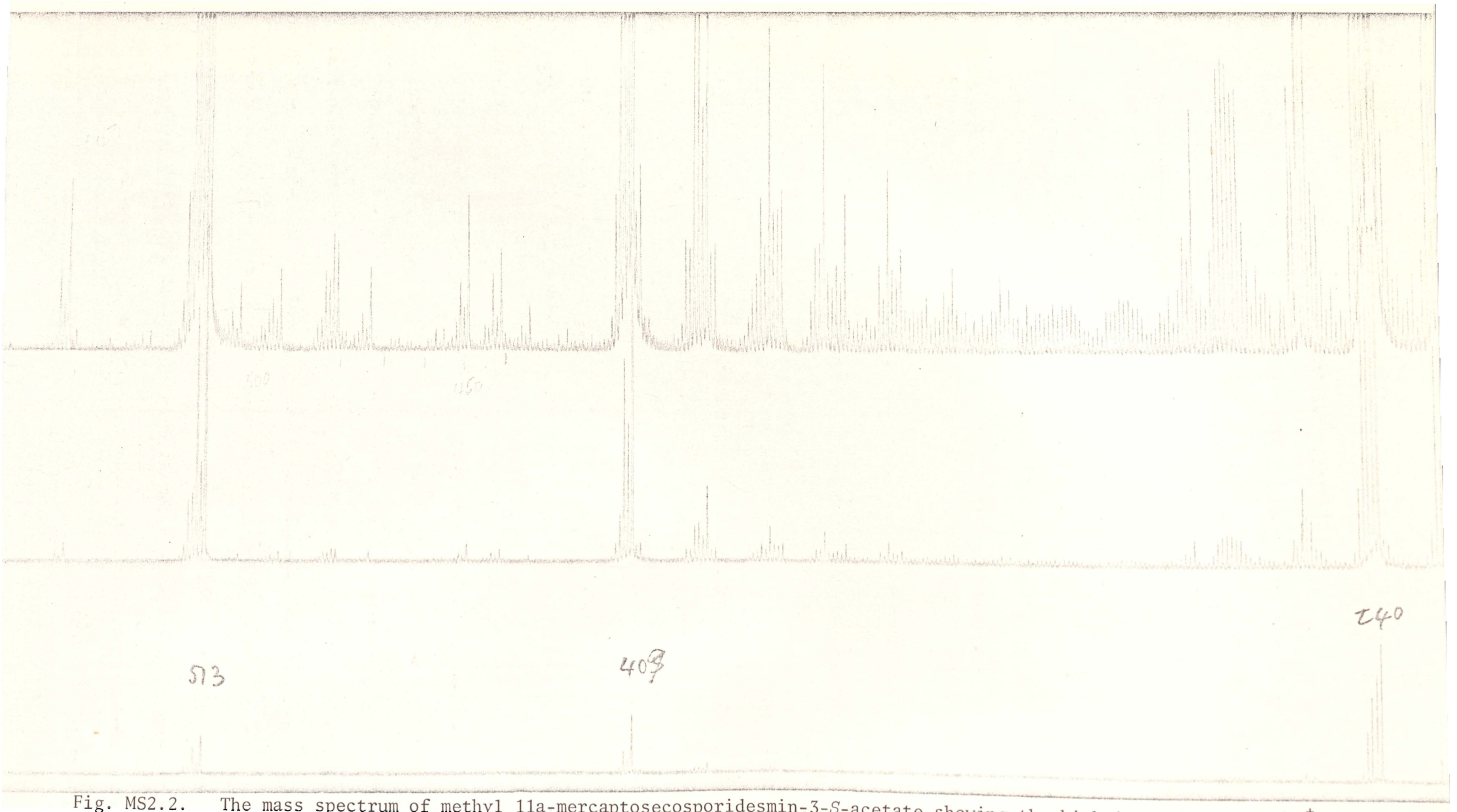



Fig. MS2.2. The mass spectrum of methyl 11a-mercaptoseco-sporidesmin-3-*S*-acetate showing the high intensity of the $M^+ - H_2S$ peak (*m/e* 513) compared with that of the $M^+$ peak (*m/e* 547); compare Fig. MS2.1.

single proton quartet at δ 3.98 collapsed to a singlet when the 3-proton doublet at δ 1.89 was irradiated. The $J$ value of *c.* 2 Hz was low for the possible alternative of the methyl being split by a methine ($J$ 6—8 Hz) but was consistent with long-range coupling, e.g. $^4J_{HSCCH_3}$. Jochims, *et al.* (1969) have observed long-range couplings of $^4J$ *c.* 2 Hz in several deoxypyranoses. They point out that a coplanar arrangement of the two protons and of the connecting atoms was necessary and that long-range coupling rapidly diminished at even slight deviation from coplanarity. In a Courtauld model of compound (1.13) coplanarity obtained when the S-H bond was more or less parallel to the $CH_3$-C bond (see 'The infra-red solution spectra (4000—2700 $cm^{-1}$) of sporidesmin and its derivatives', Chapter 5, for further discussion of this structure). Consideration of the mass spectral results (Fig. MS2.1) for the 3-mercapto-compound suggested that, under the conditions of fragmentation, the mercapto-group formed $H_2S$ at the expense of one of the protons of the adjacent methyl group, comparable with the synthesis of anhydrodethiosdm from sdm di-acetate (Hodges, *et al.*, 1964).

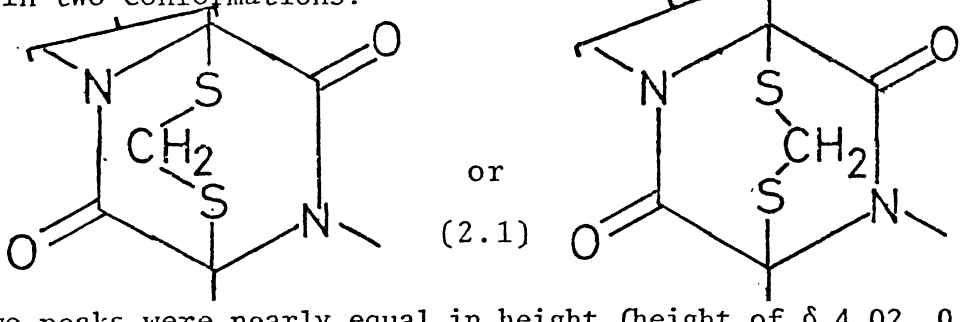
Again for the isomeric compound (1.14), that the chemical tests for thiol were negative (azide-iodine was positive in the test tube but the test is non-specific) was consistent with its i.r. spectrum (KBr) showing no S-H stretching peak (see 'The infra-red solution spectra (4000—2700 $cm^{-1}$) of sporidesmin and its derivatives'). The $^1H$ n.m.r. spectrum showed no single proton peak which could be assigned to an SH. Conclusive proof of the presence of an SH group came from this mass spectral data (Fig. MS2.2) that there was an $M^+$-34 peak and that it was about 6 times (in relation to the $M^+$ peak) as intense as that in the mass spectrum of compound (1.13). The great ease of formation of $H_2S$ in the mass spectrometer could arise from the close proximity of the thiol to the exchangeable hydrogen, hence structure (1.14a), methyl 11a-mercaptoseco-sporidesmin-3-*S*-acetate.

4. *With methylene dichloride* (A-C8)

S,S'-*Homosporidesmin* (2.1)

Since it was possible both to alkylate the sulphur atoms of sdm (above) or to incorporate another sulphur atom between the two in sdm (sdm-E) without the molecule decomposing to either anhydrodethiosdm or to the substituted indigotin, it may be possible to separate the sulphurs and link them together with a methylene. By alkylating the opened -S-S- bridge with the bifunctional methylene diiodide (A-C8i—iii) instead of the monofunctional iodoacetate ($ClCH_2CO$, NaI, as before) it was possible to obtain crystalline the *S,S'*-homo-derivative of sdm.

The protons of the new S-$CH_2$-S group appeared either as two singlets 0.04 ppm apart or as a doublet (*J* 2 Hz) of intensity 2 at δ 4.00. Since the unstrained divalent-sulphur bond-angle is 105°, the molecule can exist in two conformations:




The two peaks were nearly equal in height (height of δ 4.02, 0.9 that of δ 3.98) suggesting that the concentration (in $CDCl_3$) of each conformer was about equal. If on the other hand there was only one of these conformers, the two protons would be in quite different environments. But apparently they were resonating at nearly the same frequency. Since the coupling constant for unstrained geminal hydrogen atoms (bond angle *c.* 110°) is *c.* 12 Hz (Emsley, *et al.*, 1965a), the $^{\Delta\nu}/_{J}$ would be so small that lines 1 and 4 of the AB pattern would not be detected (Silverstein and Bassler, 1968). The other protons of the molecule resonated at the same frequencies as for other sdm derivatives.

This *S,S'*-homosdm derivative was comparable with the 12a-*p*-anisyl-*S,S'*-homosdm synthesized by Kishi, *et al.* (1973) from 1,6-dimethyl-2,5-dioxo-

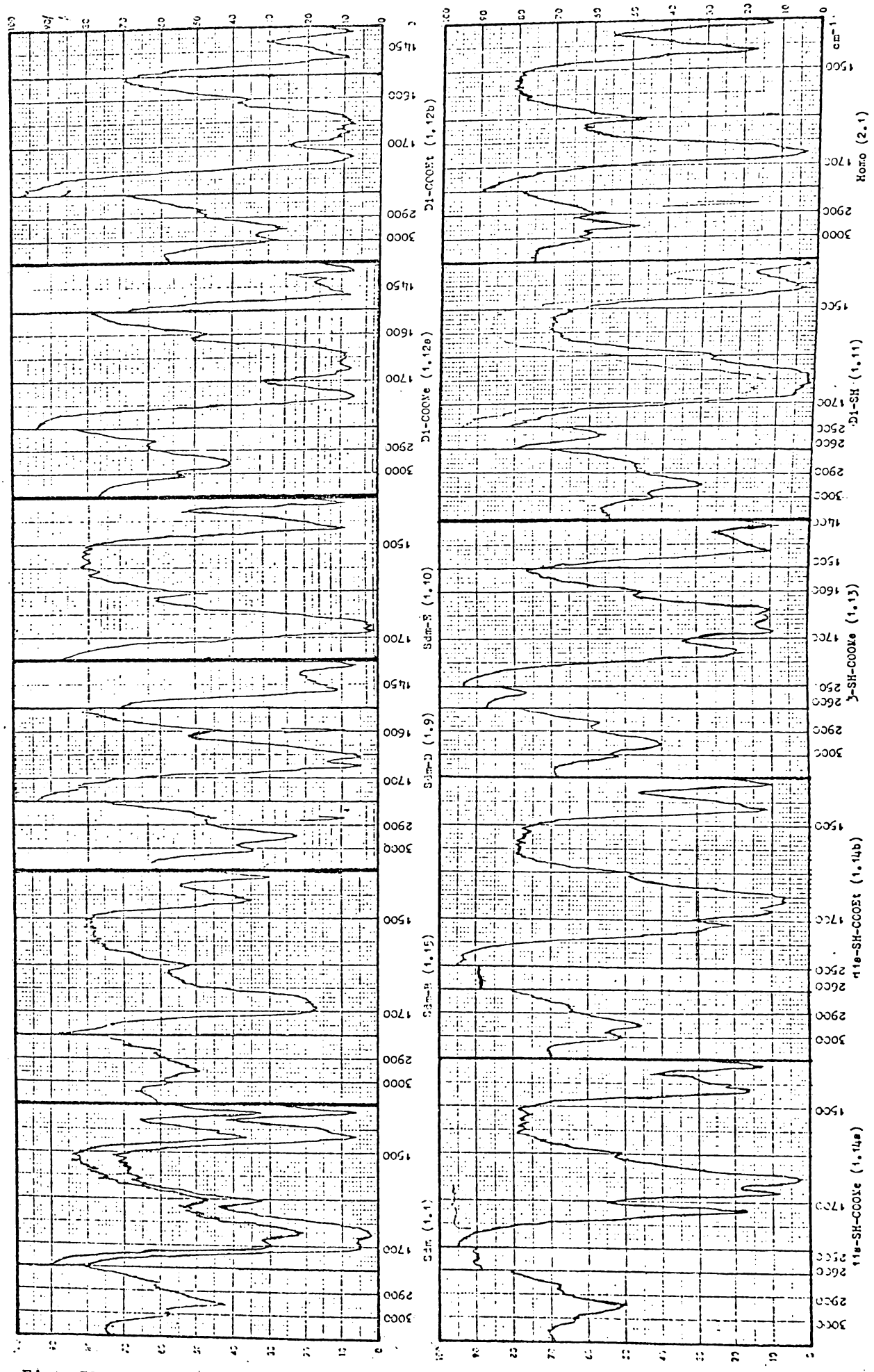
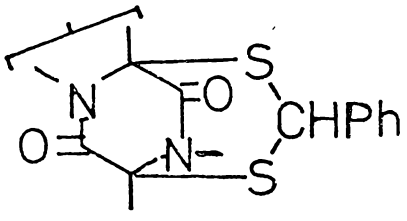


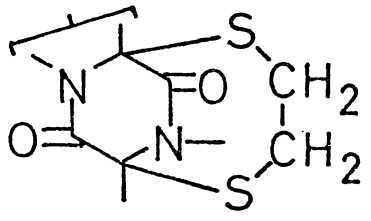

Fig. IR2.1. The νCH, νSH, νCO and δCH (3100—1300 $cm^{-1}$) peaks of sporidesmin, some analogues and derivatives.

piperazine. They were able to remove the *p*-methoxybenzylidene group and form the -S-S- bridge as the last step in their synthesis of sdm.

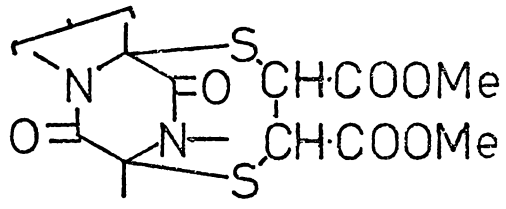
The syntheses of 12a-phenyl-*S*,*S*'-homosdm (2.2) (A-C9) (from benzylidene dichloride), *S*,*S*'-dihomosdm (2.3) (A-C10) (from ethylene dibromide), and 12a,12b-di(methoxycarbonyl)-*S*,*S*'-dihomosdm (2.4) (A-C11) (from dimethyl



(2.2)



(2.3)



(2.4)

2,3-dibromosuccinate) were also attempted but in each case the expected substance was not detected in the column effluent. Even so, there was no decomposition to orange (anhydrodethiosdm) or blue-green (substituted indigotin) colours.

*Some observations on the infra-red (KBr) and* $^1H$ *nuclear magnetic resonance spectra of the preceding sporidesmin derivatives.*-The basic sdm molecule was so large in comparison with the size of the modifications in the sulphur area that it is not surprising that all the spectra present a generic similarity. Only the νOH, νSH and νCO regions were useful structurally; the following observations are mostly subjective.

(a) *The C-H stretching region to the C-H bending region* (3000—1400 $cm^{-1}$) (Fig. IR2.1)

Sdm-D (1.9), diethyl secosdm-*S*,*S*'-diacetate (1.12b) and ethyl 11a-mercaptosecosdm-3-*S*-acetate (1.14b) showed stronger peaks (than the others) at *c.* 3000 $cm^{-1}$ which may be attributed to the methyl of the S-Me and $CH_2$-Me (see the analysis of the solution spectra in Chapter 5, 'The infra-red solution spectra (4000—2700 $cm^{-1}$) of sdm and derivatives'). The other compounds (1.1; 1.10—1.12a, 1.13, 1.14a, 1.15, 2.1) showed only well defined weak peaks or shoulders.

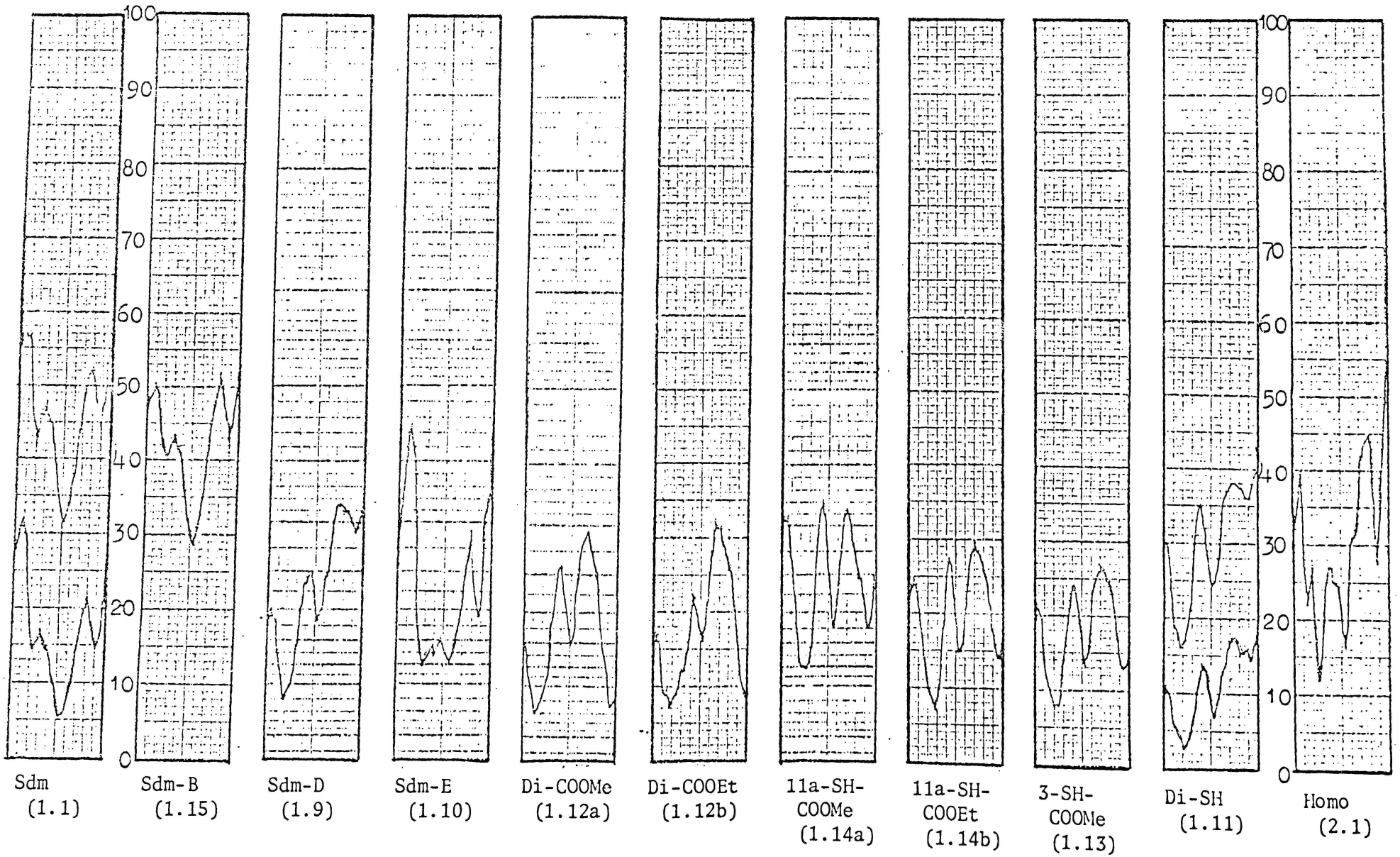



Fig. IR2.2. The infra-red peaks between 1400 and 1300 $cm^{-1}$ (KBr) for sporidesmin, some analogues and derivatives.

In Fig. IR2.1 for methyl 3-mercaptosecosdm-11a-*S*-acetate (1.13) and 3,11a-dimercaptosecosdm (1.11) there were the νSH peaks at *c.* 2530 $cm^{-1}$: for both methyl and ethyl 11a-mercaptosecosdm-3-*S*-acetates the same νSH region (2600—2500 $cm^{-1}$) was displayed showing the absence of any peaks. For discussion of this phenomenonsee Chapter 5, 'The infra-red solution spectra (4000—2700 $cm^{-1}$) of sporidesmin and its derivatives'.

The acetates (1.12—1.14) had peaks at 1740—1710 $cm^{-1}$ for the ester carbonyls as well as the lactam carbonyl peaks (1700—1630 $cm^{-1}$) which were common to all the spectra.

In the asymmetrical methyl C-H bending (1465 $cm^{-1}$) and the 'perturbed' methylene (1410 $cm^{-1}$) region they all had the characteristic pattern; though methylenes were absent in sdm, sdm-B, sdm-D, sdm-E and 3,11a-dimercaptosecosdm yet the 'perturbed' methylene (1410 $cm^{-1}$) peak was always present.

(b) *The peaks between 1400 and 1300 $cm^{-1}$* (Fig. IR2.2)

In both sdm and sdm-B the peaks at 1380 $cm^{-1}$ were weak to medium and those at 1345 $cm^{-1}$ were strong to very strong while in all the other compounds where the -S-S- bridge has been opened (including 3,11a-dimercaptosecosdm), the relative intensities were reversed. (Sdm-E was anomalous: these two peaks were of *c.* equal intensity.) In the same compounds where the peaks at 1380 $cm^{-1}$ were strong there were one or two extra Me groups (SMe, COOMe, $CH_2Me$, particularly in diethyl secosdm-*S*,*S*'-diacetate) which might contribute to the increased intensity but there was this increased intensity at 1380 $cm^{-1}$ in 3,11a-dimercaptosecosdm where there were no extra Me groups. This suggests that the peak at 1380 $cm^{-1}$ for the symmetrical deformation mode of the hydrogen atoms of the $C-CH_3$ group (Bellamy, 1975a) shifted from this normal position to the lower frequency at 1345 $cm^{-1}$. This shift was the result of the strain that the -S-S- bridge applied to the dioxopiperazine ring in sdm and sdm-B.

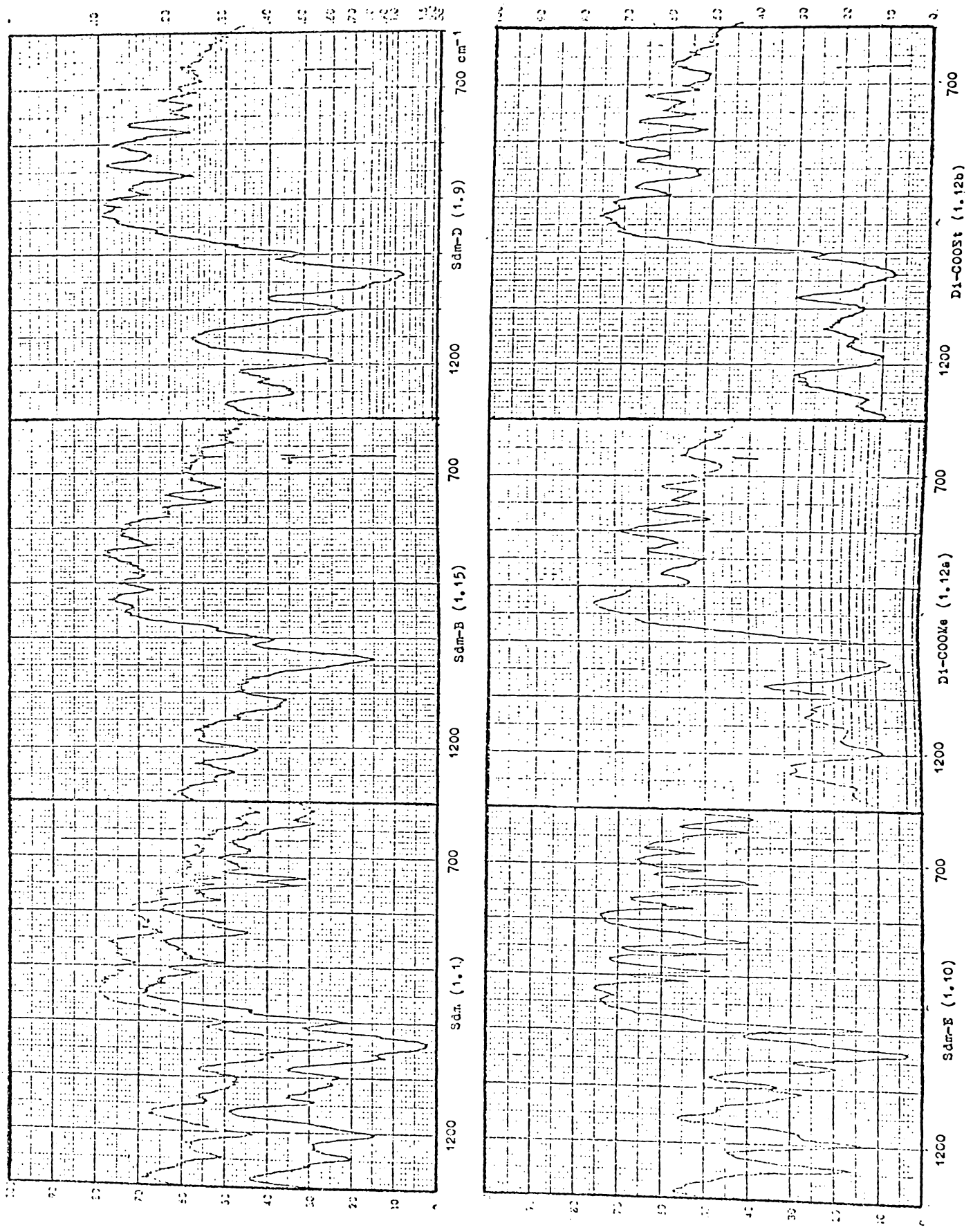



Fig. IR2.3. The infra-red spectra (1300—600 $cm^{-1}$, KBr) for sporidesmin, some analogues and derivatives.

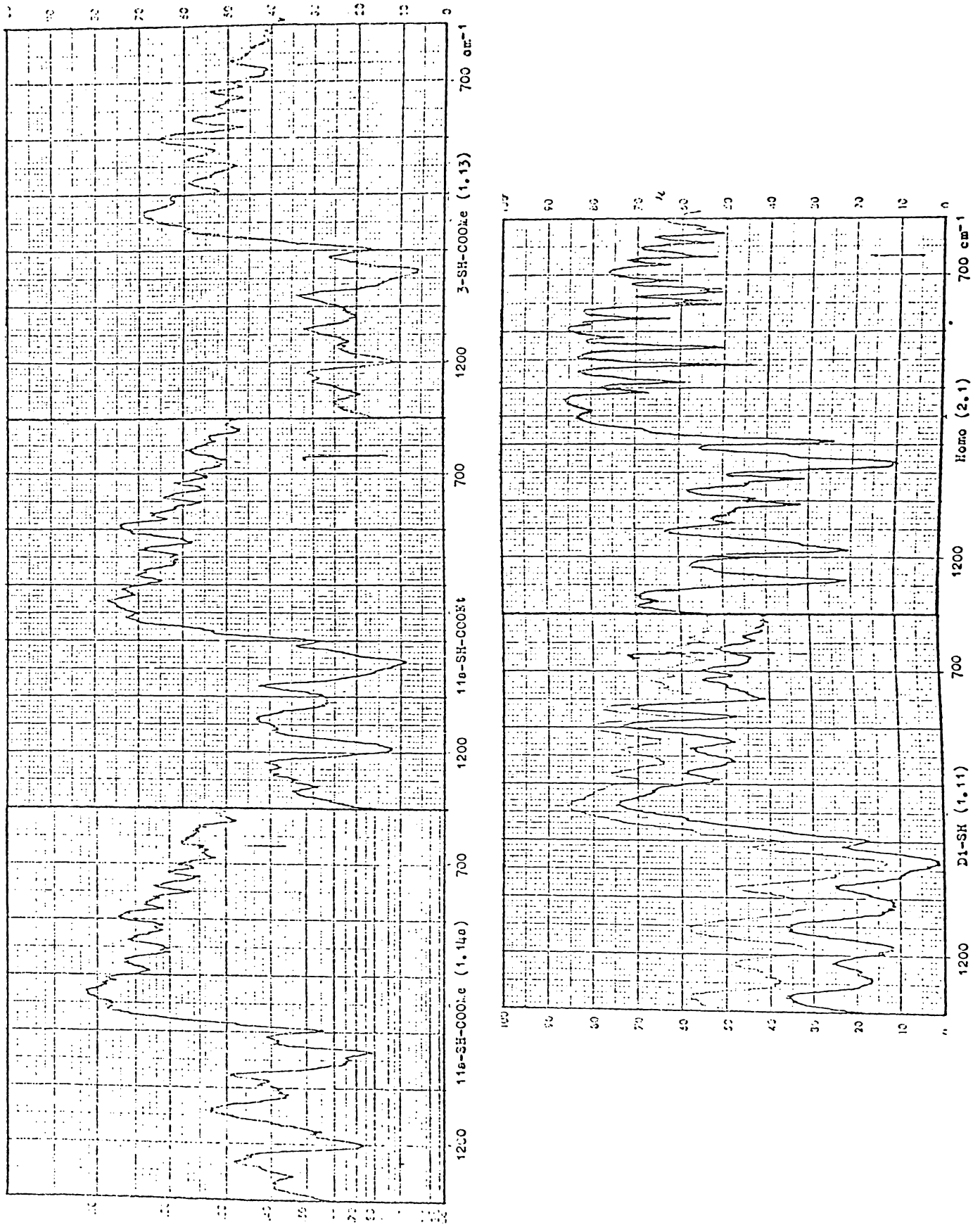



Fig. IR2.4. The infra-red spectra (1300—600 $cm^{-1}$, KBr) for some derivatives of sporidesmin.

At the same time there was no evidence for a comparable shift in the asymmetrical deformation.

The medium to strong peak at *c.* 1310 $cm^{-1}$ attributed by Bellamy (1975d) to primary and secondary alcohol vibrations, was weak in 3,11a-dimercaptosecosdm.

(c) *The peaks between 1300 and 600 $cm^{-1}$* (Figs IR2.3, IR2.4)

The spectra for dimethyl and diethyl secosdm-*S,S'*-diacetates (1.12) are almost indistinguishable from each other and similarly for methyl and ethyl 11a-mercaptosecosdm-3-*S*-acetates (1.14) except for the shape and position of the strong peaks at *c.* 1200 $cm^{-1}$. For the methyl homologue the peak tended to be sharp (at 1200 $cm^{-1}$) while that for the ethyl homologue was broad with its apex at 1185 $cm^{-1}$. Perchard (1970) observed a comparable shift in going from $MeOC_2D_5$ (1192s) to $CD_3OEt$ (1159m, KBr). In other sdm derivatives where the COOAlk group was absent there tended to be medium peaks at *c.* 1200 $cm^{-1}$. In *S,S'*-homosdm (2.1) there was a weak, well defined peak at 1200 $cm^{-1}$ and a strong one at 1190 $cm^{-1}$.

Mooney (1963) attributed a peak in the spectrum of *o*-chloroanisole at 1247 $cm^{-1}$ to aryl-O-Me vibrations. This function is common to all the sdm derivatives. For the Figs IR2.3 and IR2.4 the non-ester derivatives all have medium to strong peaks at 1240—1250 $cm^{-1}$ while the esters have weaker ones at *c.* 1260 $cm^{-1}$.

On the whole the C-O vibrations at *c.* 1040 $cm^{-1}$ were among the strongest of the peaks, sometimes stronger than the νCO peaks notably in sdm-B. With this peak was always the one at *c.* 1000 $cm^{-1}$ which was weakest in the two ethyl esters (of 11a-mercaptosecosdm-*S*-acetate, 1.14b, and secosdm-*S,S'*-diacetate, 1.12b), weak to medium in 3,11a-dimercaptosecosdm (1.11), sdm-D (1.9) and sdm-B (1.15), medium in sdm and the three methyl esters (of 3- and 11a-mercaptosecosdm-11a-*S*-acetate, 1.13, 1.14a, and secosdm-*S,S'*-diacetate, 1.12a), and strong in *S,S'*-homosdm

(2.1) and sdm-E (1.10). No suggestion is made as to assignment except that this vibration was very sensitive to the structural environment: it is strong where the epithio-bridge was 3-membered (e.g. the latter 2 compounds 2.1 and 1.10) (Fig. IR2.4).

The spectrum of sdm had an outstanding (medium) doublet at 752, 740 $cm^{-1}$. The peak at 752 $cm^{-1}$ was present (slightly less intense) in sdm-B but the one at 740 was absent. The peak at 740 $cm^{-1}$ in sdm may be attributed to an out-of-plane bonded OH deformation frequency (Bellamy, 1975e). As is discussed in Chapter 5, 'The infra-red solution spectra (4000—2700 $cm^{-1}$) of sdm and derivatives', the hydrogen bonding in sdm was quite different from that in the other derivatives.

(d) *$^1H$ nuclear magnetic resonance*

In each of the $^1H$ n.m.r. spectra there were a set of overlapping peaks, more or less unresolved in the region of δ 3.9—3.7, which included the two aromatic methoxy-groups and (for the esters) the ester methoxy-groups and the methylenes occurring between the sulphur atoms and the carboxy-groups. Compound (1.13, 3-mercaptosecosdm-11a-*S*-acetate) showed a split (d) proton peak (δ 4.65, *J* 4 Hz) but there was no sign of the other split peak; the latter was the peak of a hydroxy-group and consequently broad and of indefinite position, perhaps under the overlapping methyl peaks.

The aliphatic 3-methyl in sdm (1.1) was deshielded (δ 2.03) (Ronaldson, *et al.*, 1963) by the strained condition of the epidithiodioxopiperazine ring system. Because of the planarity of the two amido-groups, a dioxopiperazine ring is compelled to be boat shaped. In an epidithiodioxopiperazine ring system the C-S-S-C group constrains the dioxopiperazine ring to bend even further from planarity (see Plate 5.1). Added to this the dioxopiperazine ring forces the epidithio-group (C-S-S-C) to have a dihedral angle of only 10-20° (Rahman, *et al.*, 1970; Safe and

Taylor, 1971), compared with its normal orientation of 90°. When this strained condition was released as in sdm-D (1.9) and the esters, seco-sdm-*S*,*S*'-diacetate (1.12) and 3-mercaptosecosdm-11a-*S*-acetate (1.13), the *C*-methyl peak shifted upfield to δ 1.90—1.86. But under the environment as suggested for methyl 11a-mercaptosecosdm-3-*S*-acetate (1.14) in Chapter 5, there was deshielding comparable with that in sdm. There was a similar deshielding in dimercaptosecosdm (1.11) and sdm-E (1.10), which deshielding was discussed under '3,11a-Dimercaptosecosdm', above.

Possibly it was this strained condition that accounted for the intensity of the band at 308 nm in the u.v. spectrum of sdm (log ε 3.96) A-A) for it was double (or more) that in the compounds where the sulphurs were alkylated, viz. the log εs were for diethyl secosdm-*S*,*S*'-diacetate, 3.29 (A-C4aiii); dimethyl secosdm-*S*,*S*'-diacetate, 3.34 (A-C5ai); methyl 11a-mercaptosecosdm-3-*S*-acetate, 3.65 (A-C5biv); and methyl 3-mercapto-secosdm-11a-*S*-acetate, 3.64 (A-C5cv).

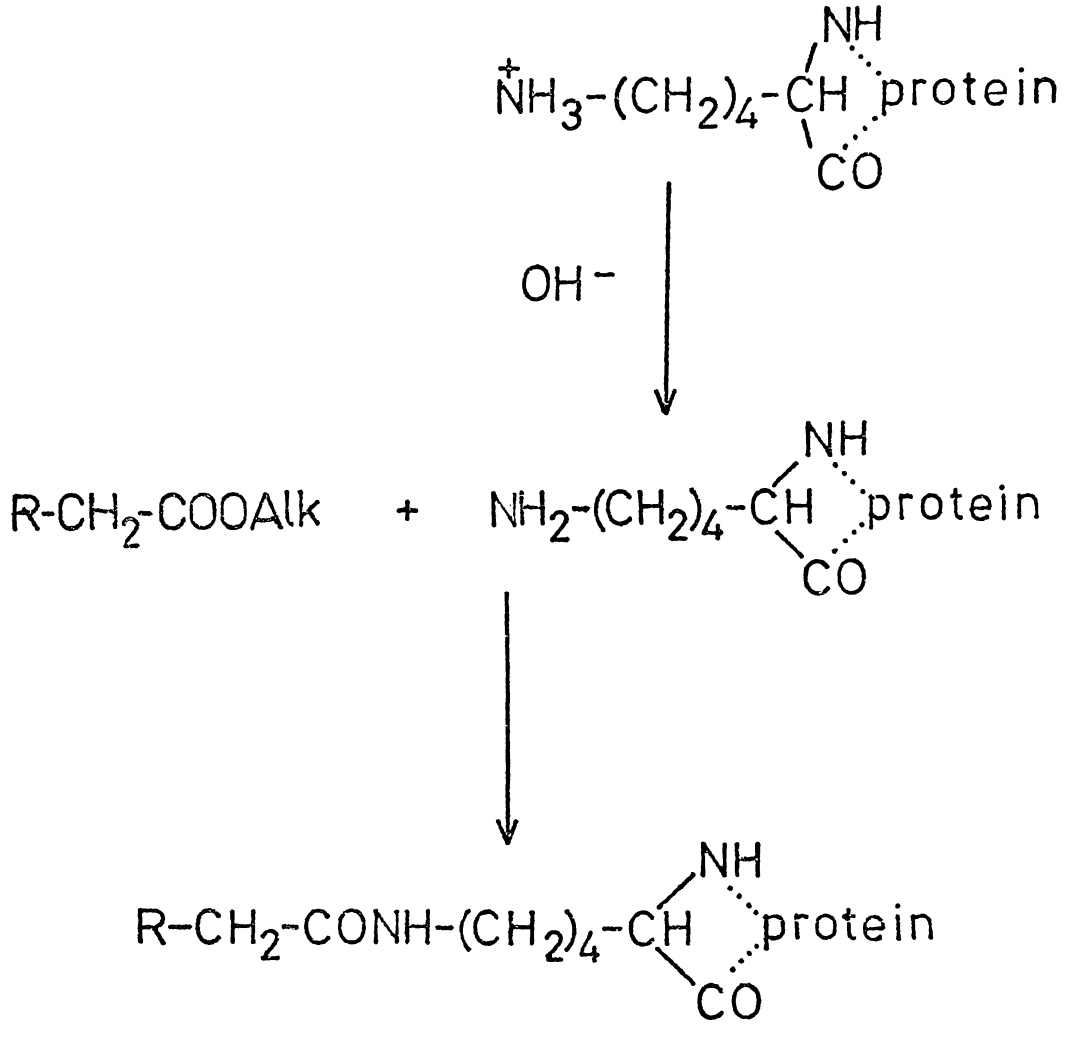
## §3. *The Antigens*

These syntheses (based on Ansell and Gigg, 1965) were undertaken in order to produce high molecular weight (*c.* 100,000 or more) complexes, the antigens, (containing sporidesmin (sdm) molecules covalently bound to protein) so that when the complex was administered to animals it would stimulate the production of antibodies to sdm.

*The formation of the modified-sporidesmin protein complex.*-The proteins were dissolved in small amounts of water. They all (synthetic or natural) dissolved freely. Then alcohol was added (EtOH-$H_2O$ 2:3). Poly-(L-lysine) (p11) formed a clear aqueous-alcohol solution. Bovine plasma albumin (bpa), rabbit serum albumin (rsa) and bovine thyroglobulin (btg) all turned slightly cloudy upon additon of the alcohol but became clear when the pH was raised to 8. Ovalbumin (ova) (A-C6ei) came out of solution at low concentrations of alcohol (EtOH-$H_2O$ *c.* 1:6) and did not redissolve on dilution ($H_2O$) nor upon raising the pH to 9, so the first synthesis with this protein had to be abandoned. This concentration of alcohol was too low to bring sufficient sdm ester derivative into solution for reaction.

To the solution of protein was added the alcoholic solution of the modified-sdm ester after it had been diluted with water (EtOH-$H_2O$ 2:3). If the ester came out of solution upon addition of water, dilution with alcohol (and $H_2O$) or with the protein solution redissolved the ester. On the whole it was not difficult to obtain an aqueous-alcohol solution of the esters.

The gum of (m)ethyl 11a-mercaptosecosdm-3-*S*-acetate (1.14) dissolved in alcohol, but immediately it crystallized out. Boiling the alcoholic solution did not redissolve it nor did the compound decompose at that temperature. What could be dissolved in the aqueous alcohol was complexed to protein but gave only a low concentration of hapten on the


NH
$\overset{+}{N}H_3-(CH_2)_4-CH$
protein
CO
$OH^-$
$R-CH_2-COOAlk$
+
$NH_2-(CH_2)_4-CH$
NH
protein
CO
$R-CH_2-CONH-(CH_2)_4-CH$
NH
protein
CO

Scheme 2.1

protein (A-C6aii).

After mixing the protein and the ester solutions, the pH was raised to *c.* 9—10. The protein in the solution became cloudy as the pH passed through its isoelectric point but it redissolved at higher pH. As reaction (Scheme 2.1) between the ester group and the lysyl ε-amino-groups of the proteins took place (room temperature), the pH of the solution fell to *c.* neutral point where reaction ceased till the pH was raised again.

Upon completion of reaction the complex was concentrated (reduced pressure) to remove most of the alcohol. When the slightly cloudy aqueous suspension was extracted ($CHCl_3$) it would become a dense white suspension which often did not redissolve on dilution ($H_2O$ or EtOH). Apparently the chloroform denatured the protein. An aqueous solution of protein (bpa) was unaltered by extraction with chloroform but when alcohol was present then a dense white suspension resulted from chloroform extraction. When bpa precipitated in this way or treated with ether (which did not cause the dense white precipitate) was compared electrophoretically with natural protein, there was no difference between the treated and untreated proteins in the distance that they moved. Chloroform-precipitated bpa redissolved in water.

Since ether did not appear to cause this precipitation it was used to remove excess unreacted ester from the aqueous suspension. This was done in order to obtain the u.v. spectrum of the complex and to obtain (by difference) an estimate of the substition that had taken place. The u.v. spectrum showed the characteristic peaks of the modified sdm (217, 251 and 302 nm).

Since the samples were solvent extracted to remove any unreacted modified-sdm ester, the presence of modified-sdm ester peaks in the u.v. spectrum suggested that the hapten (sdm) was covalently bound to the pro-

tein. Nevertheless, it was not overlooked that some of the ester may be 'protein bound' to the protein.

The complexed protein product tended to be less water soluble than the natural protein, because hydrophilic groups (ε-aminos) had been replaced by the hydrophobic modified sdms. In most (A-C6ai—v) of the preparations the product was alcohol-water soluble. Diluting the alcohol-water (2:3) solution of complexed rsa (A-C6dii) with water precipitated the complex. This radical alteration in solubility was itself evidence that the hapten had been covalently bound to the protein. In this case, poly-(3-mercaptosecosporidesmin-11a-*S*-acetyl)rabbit serum albumin, there was no cross-linking (see below) since the ester (3-mercaptosecosdm-11a-*S*-acetate) was monofunctional.

For the preparation containing the complex, poly-(11a-mercaptosecosporidesmin-3-*S*-acetyl)poly-(L-lysine) (A-C6aii), when the ethanol-water solution was being evaporated (reduced pressure), as the alcohol evaporated so white amorphous material separated from solution. This material redissolved upon addition of ethanol. Similar properties to this on evaporation were observed for poly(methoxy)poly(secosporidesmin-*S*,*S*'-diacetyl)poly-(L-lysine) (A-C6aiii).

But insolubility in other experiments (A-C6avi—viii) using higher concentrations of the bifunctional diesters showed itself by a gel being precipitated which it was suggested was the cross-linked product i.e. each of the two functional groups on the diacetate esters had complexed to lysine residues in separate protein molecules and to each protein molecule there would be a number of bifunctional (secosdm-*S*,*S*'-diacetyl) groups so this cross-linking condition would be compounded throughout the gel. The gel, cross-linked poly(ethoxy)poly(secosporidesmin-*S*,*S*'-diacetyl)poly-(L-lysine), was insoluble not only in the reaction medium but also in water over the pH range of 2—10, in methanol, chloroform, *N*,*N*-dimethylformamide or trifluoroacetic acid. Heating in the latter solvent

decomposed the modified sdm moiety to the substituted indigotin (dark blue).

The presence of this gel was particularly marked in the experiment (A-C6aviii) where an automatic titrator was used to maintain the pH constant. From the ultimate analysis of the material and the molecular formulae of *N,N'*-disubstituted lysyl (-NH-$(CH_2)_4$-CH(CO-)NH-) and seco-sdm-*S,S'*-di(acetyllysyl) this equation was arrived at:

x(56.4C + 9.4H + 21.8N + 12.4O) + (1-x)(50.3C + 5.7H + 4.4Cl + 12.1N + 19.7O + 7.9S) is nearest to (46.9C + 6.3H + 3.7Cl + 12.2N + 25.6O + 5.4S)

So for carbon where 56.4x + (1-x)50.3 - 46.9 was made = 0

| | | |
|---|---|---|
| was the | value | x = -0.55 |
| and for | hydrogen | = 0.16 |
| | chlorine | = 0.16 |
| | nitrogen | = 0.01 |
| | oxygen | = -0.81 |
| | sulphur | = 0.32. |

Since the values for x for both hydrogen and chlorine were equal at 0.16 (= the average for the positive values), it is suggested that *c.* 84% of the gel was secosdm-*S,S'*-di(acetyllysyl). Since 31.3% of secosdm-*S,S'*-di(acetyllysyl) is lysyl and 84% of the whole was secosdm-*S,S'*-di(acetyl-lysyl) then (0.313 x 84) 26.3% of the whole was substituted lysyl. 16% Of the whole was unsubstituted lysyl (100 - 84%) therefore the total lysyl in the gel was 42.3% and hence 26.3/42.3 = 62% of the ε-amino groups of the lysyl moieties were substituted. If 62% were substituted, it would require: C, 51.2; H, 6.3; Cl, 3.7; N, 13.6; S, 6.6% compared with found: C, 46.9; H, 6.3; Cl, 3.7; N, 12.2; S, 5.4%. Further, since 58.3% of secosdm-*S,S'*-di(acetyllysyl) is the elements of sdm then 58.3% of 84 = 49% of the gel, cross-linked poly(methoxy)poly(seco-sporidesmin-*S,S'*-diacetyl)poly-(L-lysine), came from the elements of sdm.

Table 2.3. Quantities of reagents in complexing modified sporidesmin to a synthetic protein, poly-(L-lysine)HBr (A-C6a).

| Expt | Protein | | Sdm derivatives | | Reaction time | Unchanged sdm derivative | Calculated secosdm in complex | Product solubility in EtOH-$H_2O$ | Calculated % ε-amino-groups substituted: No of ester groups | |
|---|---|---|---|---|---|---|---|---|---|---|
| | (mg) | (*M*) | (No) | (mg) | (days) | (mg) | (%) | | one | two |
| i. | 22 | 139,000 | (1.12b) | 19.2 | 1 | 7.6 | 36 | Soluble | 17 | 34 |
| ii. | (62.5<br>(82 | (139,000<br>( 70,000 | (1.14a) | 121 | 3 | 74 | 31 | do. | 12.5 | - |
| iii. | 148 | 70,000 | (1.12a) | 139 | 2 | 30 | 42 | do. | 22 | 44 |
| iv. | 103 | 180,000 | (1.12a) | 30 | 4 | 6 | 22 | do. | 8 | 16 |
| v. | 21 | 180,000 | (1.12a) | 47 | 4 | 10 | 62 | do. | 61 | 122* |
| vi. | 21 | 180,000 | (1.12a) | 53 | 7 | - | - | gel (23 mg) | - | - |
| vii. | 20 | 180,000 | (1.12b) | 50 | 3 | 28 | - | gel (28 mg) | - | - |
| viii. | 53 | 180,000 | (1.12a) | 300 | 5 h | - | 49 | gel (63 mg) | - | 62 |
| ix. | 105 | 70,000 | control | - | 3 | - | - | - | - | - |

* Apparently most of the ε-amino groups of the lysyls had reacted but for many of the di-ester molecules (>18%) only one ester group had reacted.

Table 2.4. Names of Antigens, Complexes between modified Sporidesmin (sdm) and proteins.

| Expt | Modified protein | Product moving as a discrete protein (electrophoretically) (%) | $R_{protein}$ |
|---|---|---|---|
| A-C6ai. | Poly(ethoxy)poly(secosdm-*S*,*S*'-diacetyl)poly-(L-lysine) | | |
| aii. | Poly-(11a-mercaptosecosdm-3-*S*-acetyl)poly-(L-lysine) | | |
| aiii. | Poly(methoxy)poly(secosdm-*S*,*S*'-diacetyl)poly-(L-lysine) | | |
| avii. | Cross-linked poly(ethoxy)poly(secosdm-*S*,*S*'-diacetyl)poly-(L-lysine) | | |
| aviii. | Cross-linked poly(methoxy)poly(secosdm-*S*,*S*'-diacetyl)poly-(L-lysine) | | |
| aix. | Poly(chloroacetyl)poly-(L-lysine) | | |
| c. | Poly(methoxy)poly(secosdm-*S*,*S*'-diacetyl)bovine plasma albumin | 33% | 0.77 |
| dii. | Poly-(3-mercaptosecosdm-11a-*S*-acetyl)rabbit serum albumin | 91% | 0.80 |
| aiv. | Poly(ethoxy)poly(secosdm-*S*,*S*'-diacetyl)rabbit serum albumin | 100% | 0.80 |
| eii. | Poly(ethoxy)poly(secosdm-*S*,*S*'-diacetyl)ovalbumin | | |
| f. | Poly(ethoxy)poly(secosdm-*S*,*S*'-diacetyl)bovine thyroglobulin | 99% | 1.2 |

For a control in the immunological work p11 substituted with chloroacetyl groups, a mixture containing poly(chloroacetyl)poly-(L-lysine), was prepared (A-C6aix) from ethyl chloroacetate in the same way as the modified-sdm complexes were.

In order to show chemically that the modified sdm was covalently bound to the p11 and not 'protein bound' it was necessary to assay the solutions for the concentration of ε-amino-groups in relation to those of untreated p11 of the same concentration. Boyd, *et al.* (1972) and Slobodian, *et al.* (1962) used the ninhydrin estimation to show that they had acetylated the ε-amino-groups of protein. For experiment (A-C6aiv) the assay showed that 12.6% of the lysyl groups in the p11 had been substituted. This result was comparable with the result (16%, Table 2.3) derived from the weight of the reagents which appeared to enter into reaction; the difference suggests *c.* 40% of the ester was monosubstituted (assuming that all the p11 chains were acylated at least once). Thus this evidence of substitution of ε-amino-groups in synthetic p11 agreed with electrophoretic observations on natural proteins. Electrophoresis (A-C6c—f, Table 2.4) showed that new proteins had been formed from the natural proteins.

In the second experiment (A-C6av) where the ninhydrin assay was used the result suggested that there was 15% substitution. In this experiment the absorbance readings were so variable that no reliance could be placed upon the result. Even though a number of refinements had been applied to the method, no reproducible results could be obtained. It is well-known that it is difficult to obtain reproducible results in the ninhydrin assay, so assaying by ninhydrin was abandoned. There was, in addition, no agreement between the ninhydrin assay (15%) and the result (122% of reacted and unreacted functional groups, -COOMe, Table 2.3) calculated from the amounts of the reagents which appeared to enter into reaction and supposing all the ε-amino-groups (100 μmol) had re-

Table 2.5. The immunologist's results from applying to animals the antigens synthesized from sporidesmin (sdm).

| Prepa-ration | Expt | Condition of hapten | Sdm (%) | Degree of substitution (%) | Animals | Results IHA* | Results Warm CFT** with ACDMBA-KLH*** |
|---|---|---|---|---|---|---|---|
| Immunologist: Jonas, pers. comm. | | | | | | | |
| 1 | A-C6ai. | diethyl diacetate-p11 | 36 | 17—34 | 6 rabbits | +ve IHA* Titre 1:160 | - |
| 2 | aii. | methyl 3-S-acetate-p11 | 31 | 12.5 | 6 rabbits | doubtful +ve | -ve |
| 3 | aiii. | dimethyl diacetate-p11 | 42 | 22—44 | 3 rabbits<br>guinea pigs | -ve<br>-ve | -ve<br>- |
| 3 | do. | mixed with methylated bsa and phosphorylated bsa | do. | do. | guinea pigs | -ve | - |
| 4 | c. | dimethyl diacetate-bpa | 2.5 | 6—12 | 4 rabbits | -ve | - |
| 5 | dii. | methyl 11a-S-acetate-rsa | 22 | 69—100 | 10 guinea pigs | -ve | - |
| 6 | aiv. | dimethyl diacetate-p11 | 22 | 8—16 | 4 rabbits | -ve | - |
| 7 | av. | dimethyl diacetate-p11 | 62 | 61—100 | not yet tested | | |
| 8 | aviii. | dimethyl diacetate-p11 | 49 | 62 | 4 rabbits | doubtful +ve | one animal +ve |
| 9 | div. | diethyl diacetate-rsa | 27 | 100 | not yet tested | | |
| 10 | eii. | diethyl diacetate-ova | 7.3 | 39—78 | not yet tested | | |
| Immunologist: Fairclough, pers. comm. | | | | | | | |
| 11 | A-C6f. | diethyl diacetate-btg | 35 | - | 4 sheep<br>2 rabbits | see Table 2.8 | |

* Where IHA represents indirect haemagglutination test, see Jonas and Ronaldson (1974).

** Where CFT represents Complement fixation test.

*** Where ACDMBA-KLH represents 2-amino-5-chloro-3,4-dimethoxybenzyl alcohol-keyhole limpet haemocyanin.

Table 2.6. Quantities of reagents in complexing modified sporidesmin to natural proteins (A-C6).

| Expt | Protein | | | Sporidesmin derivative | | | | | | Reaction time | Calculated apparent secosdm in complex | Calculated ε-amino groups substituted | No. of lysyl groups substituted/ molecule where only one COOAlk reacted | Protein binding (mol of ester/mol protein) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | (name) | (mg) | (*M*) | (/mol) | (μmol) | (No) | (mg) | (recovered) | (μmol) | (days) | (%) | (%) | | |
| c. | bpa | 300 | 65,000 | 58 | 266 | (1.12a) | 26 | 16 | 16 | 2 | 2.5 | 6 | 10 | nil* |
| di. | rsa | 60 | 65,000 | 58 | 54 | (1.12a) | 21 | 21 | - | 2 | - | - | - | - |
| dii. | rsa | 56 | do. | do. | 50 | (1.13) | 23 | 4 | 34.5 | 2.5 | 22 | 69 | 44 out of 58 | nil* |
| diii. | rsa | 61 | do. | do. | 55 | (1.13) | 23 | 23 | - | 6 | - | - | - | - |
| div. | rsa | 54 | do. | do. | 48 | (1.12b) | 53 | 4 | 76 | 3 | 36 | 76 | 44 out of 58 | 49 |
| ei. | ova | 712 | 46,000 | 20 | 310 | (1.12b) | 100 | 87 | 20 | 1 h | - | - | - | *c.* 1.3 |
| eii. | ova | 620 | do. | do. | 270 | (1.12b) | 87 | 19 | 105 | 12 | 7.3 | 39** | <8 | - |
| f. | btg | 22 | 660,000 | 137 | 4.6 | (1.12b) | 19 | 1 | 28 | 7 | 35 | - | - | >685 |

* Assuming all the acylating agent was covalently bound.

** Assuming that only one -COOAlk group reacted.

acted. Of the 122 μmol of functional groups of the bifunctional modified sdm (dimethyl secosdm-*S*,*S*'-diacetate) at least 22 μmol had not reacted. But since the ninhydrin test showed a positive reaction (even though the reading was not reproducible) it indicated that at least some of the lysyl ε-amino-groups were unsubstituted hence less than 100 μmol of the 122 (and more than 61 μmol) had entered into reaction (neglecting 'protein binding') to form the poly(methoxy)poly(secosporidesmin-*S*,*S*'-diacetyl)poly-(L-lysine): rather than only 15% as assayed by ninhydrin.

In addition to the evidence of the major change in solubility and the change in the number of unsubstituted amino-groups (relative to untreated p11), (and the altered electrophoretic pattern in natural proteins) the proof that covalent bonding between the modified sdm and the synthetic protein (p11) had been achieved was in the fact that antibodies to sdm had been raised in the animal as the result of injecting the mixture containing the p11 complex, poly(ethoxy)poly(secosporidesmin-*S*,*S*'-diacetyl)poly-(L-lysine), (A-C6ai, Table 2.5) (Jonas and Ronaldson, 1974).

For the non-synthetic proteins (Table 2.6) the method of synthesis (A-C6c—f) was the same as for the synthetic protein, p11. No difficulty was experienced in dissolving these proteins in the ethanol-water (2:3) except in the case of ovalbumin mentioned above. For the synthesis (A-C6eii) with this protein, a film of the diethyl secosdm-*S*,*S*'-diacetate was formed over the wall of the reaction flask and the ovalbumin in as high a concentration of alcohol (EtOH-$H_2O$ 1:4.9) as possible without denaturing it, was added. Since less than a quarter of the original ester was recovered at the end of the experiment, it was assumed that some, if not most, was covalently bound. Protein binding is a rapid reaction (Last, 1969; Mester, *et al.*, 1970) and with the first ovalbumin experiment (A-C6ei) 87% modified sdm ester was recovered in less than 1 h so it is suggested that no more than *c.* 13% would be 'protein bound' in

Table 2.7. Molecular weights ($M$) for the -S-acetyl esters of modified sporidesmin and lysine for calculations.

| | |
|---|---|
| Sporidesmin (sdm) | 473.5 |
| Mercaptosecosdm-S-acetyl | 516.5 |
| Secosdm-S,S'-diacetyl | 557.5 |
| Methyl mercaptosecosdm-S-acetate | 547.5 |
| Ethyl mercaptosecosdm-S-acetate | 561.5 |
| S-Methoxycarbonylmethylsecosdm-S-acetyl | 588.5 |
| S-Ethoxycarbonylmethylsecosdm-S-acetyl | 602.5 |
| Dimethyl secosdm-S,S'-diacetate | 619.5 |
| Diethyl secosdm-S,S'-diacetate | 647.5 |
| Lysine | 146 |
| $N_{\alpha}$-substituted lysyl ($NH_2(CH_4)_4CH(CO\text{-})NH\text{-}$) | 128 |
| Lysine. HBr | 227 |
| $N_{\alpha}$-substituted lysyl.HBr | 209 |
| N,N'-disubstituted lysyl | 127 |

the second experiment, where some poly(ethoxy)poly(secosporidesmin-*S*,*S*'-diacetyl)ovalbumin was obtained.

Since non-covalent binding ('protein binding' of compounds to proteins) is rapid, e.g. tetracycline is bound to ribosomes in 8 min (Last, 1969) or with oestradiol added to rabbit uterine cytoplasm, 90% is bound in less than 30 min (Mester, *et al.*, 1970), the slowness of the reaction between the modified-sdm esters and the protein (5 h with a titrator, A-C6aviii) suggests that, although some protein binding of the ester may have taken place, the major reaction had been a transacylation. This was further evidence that a covalent bond had been formed. This transacylation is comparable with that of lysine residues in serum albumin when the latter is treated *in vivo* or *in vitro* with acetylsalicyclic acid (Pinckard, *et al.*, 1970).

*The calculation of the percentage of sdm in the protein complexes.*-For Experiment (A-C6ai) (assuming no losses during preparation)

From Table 2.3, 22 mg p11.HBr ≡ 22 x 127/209 = 13.5 mg *N*,*N*'-disubstituted lysyl residues (for *M* see Table 2.7)

In 11.6 mg of the ester 11.6 x 558/648 = 10.0 mg secosdm-*S*,*S*'-diacetyl

23.5 mg complex

10.0 mg modified sdm ≡ 10.0 x 473/558 = 8.5 mg of final product was derived from the elements of sdm

therefore 8.5/23.5 x 100 = 36% of the complex was the elements of sdm.

*The calculation of the percentage of substituted lysyl residues in the complexes.*-For Experiment (A-C6ai) above

11.6 mg diethyl secosdm-*S*,*S*'-diacetate appeared to enter into the reaction

11.6/648 = 18 μmol of the ester

13.5 mg of *N*,*N*'-disubstituted lysyls

13.5/127 =105 μmol of lysyls

If only one functional group of the ester reacted in each case there would be:

$$18/105 \times 100 = 17\%$$

Hence between 17 and 34% of the lysyl groups were substituted. (The number of terminal α-amino-groups was negligible.)

The corresponding results for the other preparations (A-C6aii—A-C6f) were calculated in the same way and presented in Tables 2.3 and 2.6. For the experiments involving natural proteins where electropherograms and the number of lysyl residues/molecule were known (Peters, 1970; Tristram and Smith, 1963), an attempt was made to suggest the number of lysyl groups which had been substituted in the new protein. From the electrophoretic result for (A-C6dii) where substitution was with the monofunctional ester, 3-mercaptosecosdm-11a-*S*-acetate, 91% of the product migrated as a discrete new protein ($R_{rsa}$ 0.80). Since the staining of electropherograms is a function of the protein only, then (Table 2.6):

91% of the 50 μmol of ε-amino-groups were in the new compound.

i.e. 45.7 μmol " " " " " " " " .

From the Table 2.6, 34.5 μmol of 3-mercaptosecosdm-11a-*S*-acetate appeared to enter into reaction.

Hence in the new compound (assuming that each rsa molecule was equally substituted)

34.5/45.7 x 100 = 75% of the ε-amino-groups in the new protein were substituted:

or 34.5/45.7 x 58 = 44 of the 58 ε-amino-groups were substituted in each of the new rsa molecules, poly-(3-mercaptosecosporidesmin-11a-*S*-acetyl)rabbit serum albumin.

It is suggested that since in acylation of rsa with the monofunctional ester, 3-mercaptosecosdm-11a-*S*-acetate, 44 of the 58 lysyl residues were substituted, acylation of rsa, therefore, with the bifunctional ester, diethyl secosdm-*S*,*S*'-diacetate, (A-C6div) would substitute the same number

of lysyl groups. (In this preparation, from electrophoresis, there was no protein which was unaltered: the new protein moved as a discrete entity as for A-C6dii.)

Hence from Table 2.6, 44/58 x 48 μmol of ε-amino-groups

= 36 μmol of *S*-ethoxycarbonylmethylsecosdm-*S'*-acetyl if each molecule was monosubstituted.

But 76 μmol of diester were not solvent extracted (EtOEt).

Hence the new protein, poly(ethoxy)poly(secosporidesmin-*S*,*S'*-diacetyl)-rabbit serum albumin, had adsorbed or 'protein bound' 40 μmol of diethyl secosdm-*S*,*S'*-diacetate, i.e. at most 44 mol of monosubstituted diester were covalently bonded/mol of rsa and in this preparation, at least 49 mol/mol of diester were 'protein bound'.

Similarly for bpa ($R_{bpa}$ 0.77) where at least 33% moved as the discrete band (A-C6c) for poly(methoxy)poly(secosporidesmin-*S*,*S'*-diacetyl)-bovine plasma albumin, between 10 (where one of each pair of the ester functional groups had reacted) and 21 (where both had reacted) of the 58 ε-amino-groups had been substituted.

The stained electropherogram of the new protein derived from bovine thyroglobulin (btg) and diethyl secosdm-*S*,*S'*-diacetate (A-C6f) showed, as with bpa and rsa, a new discrete band which moved faster ($R_{btg}$ 1.2, under the same conditions as used for bpa and rsa) than the untreated btg. Both the new protein which showed *c.* 1% residual natural btg and the btg, showed tails to the origin.

From the Table 2.6, all the 4.6 μmol of ε-amino-groups were in the new compound, and 28 μmol of diethyl secosdm-*S*,*S'*-diacetate, appeared to enter into reaction.

Hence in this new complex, poly(ethoxy)poly(secosporidesmin-*S*,*S'*-diacetyl)-bovine thyroglobulin, and adsorbed diethyl secosdm-*S*,*S'*-diacetate, there is 28/4.6 x 100 = 600% of adsorbed or 'protein bound' and covalently bound material.

If all (which is not expected) the lysyl groups in the btg were substituted with *S*-ethoxycarbonylmethylsecosdm-*S'*-acetyl (137 mol/mol) then 5 times as many more (685 mol/mol btg) were adsorbed or 'protein bound'.

Table 2.8. The antibody titre for the animals treated with the antigen, modified sporidesmin complexed to bovine thyroglobulin (Fairclough, pers. comm.).

A. Sheep: plasma assayed with [3,11a-($SC^3H_3$)$_2$]sporidesmin-D.

| Sheep | Plasma dilution | Counts in precipitate | % Bound |
|---|---|---|---|
| 1 | 1:500 | 730 | 17.3 |
| | 1:2500 | 480 | 11.3 |
| 2 | 1:500 | 2079 | 49.5 |
| | 1:2500 | 264 | 6.3 |
| 3 | 1:500 | 1856 | 44.1 |
| | 1:2500 | 709 | 16.8 |
| 4 | 1:500 | 909 | 21.6 |
| | 1:2500 | 866 | 20.7 |
| Total counts | | 4200 | |

B. Rabbits: plasma assayed with [13-$^{35}S$]sporidesmin-E.

| Rabbit | Plasma dilution | % Bound |
|---|---|---|
| A | 1:10 | 60.2 |
| | 1:20 | 58.2 |
| | 1:40 | 33.6 |
| | 1:100 | 12.5 |
| B | 1:10 | 96.2 |
| | 1:20 | 55.4 |
| | 1:40 | 34.2 |
| | 1:100 | 20.0 |

For natural proteins, substituted with bifunctional esters, there was no insoluble ($H_2O$, $H_2O$-EtOH, EtOH) gel as had occurred in similar experiments (A-C6avi—viii) with p11. Therefore it is suggested that whereas cross-linkage was suspected with p11, it was absent in the natural protein complexes. Whether both alkoxycarbonyls in bifunctional esters had reacted with two separate ε-amino-groups in any one molecule of the natural proteins is unknown. Only those ε-amino-groups which are within a certain interatomic distance of each other and which are not sterically hindered would be able to react. This interatomic distance is the limit of the range of the second acylating group in the secosdm-*S,S'*-diacetate, when the first one has already reacted.

*The results of treating animals with the modified sporidesmin-protein complexes.*-The results from treating animals with the antigens, prepared above are set out in Table 2.5. Preparations 1 and 8 produced some positive antibody response in the respective animals, but at only a low titre. The results from using Preparation 1 have been published by Jonas and Ronaldson (1974).

For Preparation 11 (Table 2.5) in which 4 sheep and 2 rabbits were treated (Fairclough, pers. comm.), the plasmas of the former were tested with [3,11a-($SC^3H_3$)$_2$]sdm-D (A-C1b) and counted at two dilutions, see Table 2.8, while the latter were assayed with [13-$^{35}$S]sdm-E (A-B3vi), see also Table 2.8. From the results the antibody titre, defined by Abraham (1975) as the dilution at which 50% of the radioactivity was bound, was for the sheep <1:200, 1:500, *c.* 1:400, *c.* 1:200 and for the rabbits 1:30 and 1:28 respectively. At least, two of the 4 sheep showed a promising result (1:400 and 1:500). By treating a larger number of animals, others may be found which will give a higher antibody titre than these two. So immunization against facial eczema might yet be achieved by using modified sdm complexed to proteins of high molecular weight (>600,000).

*Attempts at condensing lysine with secosporidesmin*-S,S'-*diesters*.-Since dimethyl secosdm-*S*,*S*'-diacetate condensed so readily with poly-(L-lysine) without warming, the similar acylation of the ε-amino-group of lysine with the secosdm-*S*,*S*'-diacetate was attempted (A-C6b). If this acylation could be achieved it was proposed to hydrolyze the modified sdm-protein complex with the enzyme, pronase, and look for the acylated lysine in the product, as a further proof of covalent bonding.

It appeared from Ansell and Gigg (1965) that the reaction took place readily at room temperature and since the condensation of the above esters with p11 (A-C6a) was satisfactory at that temperature there should be no problem with lysine itself. In order to prevent reaction occurring with the α-amino-group, this group was protected by forming the copper chelate (A-C6bi) (with the amino-acid moiety) thus leaving the ε-amino-group free (Taniyama, *et al.*, 1971). Acylation of the copper chelate with either modified sdm ester or with ethyl acetate was unsuccessful (the ester peak at 1725 $cm^{-1}$ was apparently undiminished in the product). Since the ε-amino-group is more basic ($pK_b$ 5.05, Chemical Rubber, 1964) than the α-amino-group ($pK_b$ 11.8) direct acylation (without chelation) should react preferentially (Okawa and Hase, 1965) with the ε-amino-group: this was attempted first at close to neutrality (pH 7.5—8) (A-C6bii) then at a higher pH (*c.* 9) (A-C6biii). In these two experiments only starting materials were detected (t.l.c.). The lysine was visualized (on t.l.c.) with Folin's (1922) reagent and in the last experiment the plates were also sprayed with either ninhydrin or with bromocresol purple. These two latter sprays were used to check whether the u.v. quenching spots (sdm derivatives) on the plates were amino-acids but they were not.

In acylations of amines with esters (Hayes and Gever, 1951; Rebstock, *et al.*, 1951) it is necessary to heat the reaction mixture to 90—100°, at which temperature most sdm derivatives decompose. The derivative,

($N_{\varepsilon}$,$N'_{\varepsilon}$-(secosdm-*S*,*S*'-diacetyl)di-(L-lysine), may have been prepared by alkylating the opened -S-S- bridge of sdm with $N_{\varepsilon}$-chloroacetyl-L-lysine (Birnbaum, *et al.*, 1952).

Since acylation of the ε-amino-group of lysine with the sdm ester was unsuccessful, the other methods (above) of demonstrating that a covalent link had been formed were used.

## §4. *Derivatives of Sporidesmin by Esterification (A-D) or Quaternization (A-E)*

These syntheses were undertaken in order to produce a sdm derivative in which the -S-S- bridge was retained. Since sdm-D (1.9) was less than 1/1000th the toxicity (by tissue culture, Taylor, 1971) of sdm itself, it was considered the molecule was toxic as long as the -S-S- bridge was intact. Hence, in order to stimulate animals to produce antibodies to the toxic sdm rather than to the non-toxic modified sdm it was necessary to retain the toxigenic epidithiodioxopiperazine ring system by synthesizing sdm derivatives which have the bridging (between sdm and protein) group covalently bonded at a functional group other than the -S-S- bridge. Sdm (1.1) has two hydroxy-groups, (one, 11-OH, a secondary-hydroxy; the other, 10b-OH, tertiary-) an indoline-*N*, and an aromatic hydrogen.

*Acylation of sporidesmin.*-Attempts to acylate sdm met with mixed success. Acetylation with acetic anhydride and pyridine (Ronaldson, *et al.*, 1963) was successful, but acylation in the usual way with succinic anhydride failed (White, pers. comm.). Therefore, in order to acylate with a compound which would provide a functional group (Cl-) for bridging to protein, acylation with chloroacetyl chloride (and *N*,*N*-dimethylalanine) (A-D3) was attempted but the yield was negligible (14% in A-D3i).

Similarly with tosylation (with *p*-toluenesulphonyl chloride) (A-D2) there was the formation of a small amount of a substance other than sdm (*c.* 20% by t.l.c.) after 48 d. The i.r. spectrum of the crude product showed unchanged sdm with no intense $R\text{-}SO_2\text{-}OR'$ peak between 1420 and 1330 $cm^{-1}$. There was a small peak (1175 $cm^{-1}$) in the 1200—1145 $cm^{-1}$ $R\text{-}SO_2\text{-}OR'$ region (Nakanishi, 1964).

*Sporidesmin* 11-*(methyl glutarate)* (2.5)

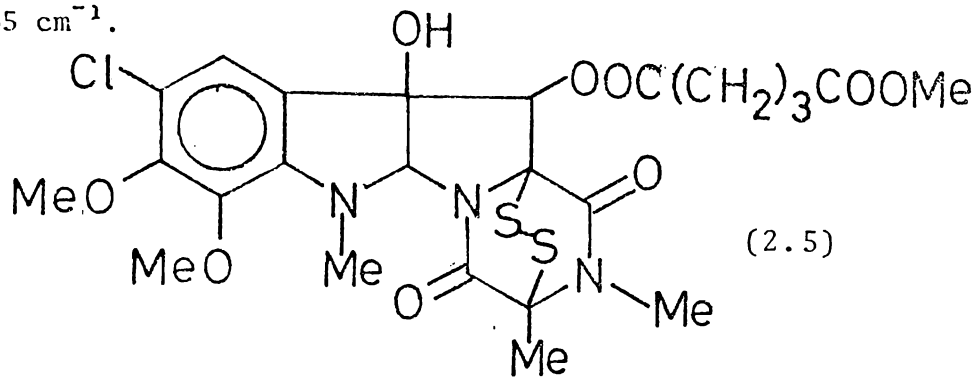
Since the above acylations failed, other avenues of acylation were sought.

In order to have methoxycarbonyl groups so that they would acylate the lysyl ε-amino-groups of proteins and in order that the sdm would be held away from the surface of the protein, 'snail-eye like', and so be exposed for antibody induction and recognition, monomethyl glutarate (mmg) was chosen. Because White (pers. comm.) failed to acylate sdm with succinic anhydride and pyridine and because Tedder's (1955) review suggested that trifluoroacetic anhydride (tfaa) 'catalyzed' difficult acylations tfaa was adopted to 'catalyze' acylation with mmg. The work was done in a dry box.

In synthesis (A-D1ai) mmg and tfaa were mixed immediately before adding the mixture to the dry sdm. The latter did not dissolve but immediately turned dark orange. This orange material was the major product and by i.r. spectroscopy was anhydrodethiosdm (adsdm, 1.16). This compound is usually synthesized from the diacetate of sdm by the dehydrating action of boron trifluoride etherate (Hodges, *et al.*, 1964) but here tfaa functioned as the acylating, dehydrating and desulphurizing agent simultaneously.

Because of this dehydration and desulphurization, a mixture (mol for mol) of mmg and tfaa was made up (A-D1aii) and stored. The mixture developed a light amber suggesting that a reaction had taken place perhaps to the mixed anhydride. The reaction of this mixture with sdm.-$C_6H_6$ (0.1 mmol, incompletely dissolved in AcMe) (A-D1aiiα,β) produced an orange solution only slowly. Even so chromatography yielded no more than *c.* 27% of a gum. In the 0.2 mmol experiment (A-D1aiiγ) where more of the benzene solvate of sdm was first dissolved in the acetone the reaction product, a gum (80 μmol), crystallized ($C_6H_6$). The *m/e* (601, $M^+$) was that of sporidesmin 11-(methylglutarate) (2.5) whose i.r. spectrum

showed peaks at 3600 (OH), 1750, 1740 (methyl and sdm ester C=O), 1710 and 1665 $cm^{-1}$.




(2.5)

Because of the difficulty in dissolving the sdm benzene solvate in small amounts of acetone the system of completely dissolving the solvate first in acetone and mmg was investigated (A-D1aiii). After the tfaa was added the solution immediately turned dark amber. During the work-up, acetone was used to obtain the ester solution free from as much of the acetone-insoluble adsdm as possible. By maintaining the solute in solution but changing the solvent from acetone to ether, crystals formed. Their i.r. spectrum showed a strong peak at *c.* 1800 $cm^{-1}$ which is characteristic of trifluoroacetates. Upon chromatography of these crystals no esters were obtained. In the chromatographic process the labile trifluoroacetate hydrolyzed back to sdm. In this experiment where the mixed anhydride was not used no ester resulted. Apparently the tfaa forms a mixed anhydride with the acylating acid, which anhydride then reacts with the substrate to yield trifluoroacetic acid and the acylated substance. The trifluoroacetyl group therefore is a good leaving group.

When the sdm solvate was completely dissolved in acetone (A-D1aiv) and the mixed anhydride (1:1 ratio sdm to tfaa) added the crude yield was 17%. Repeating this experiment (A-D1av) with more tfaa (2.3 times) and with amorphous sdm (benzene-free, from evaporating an AcMe solution) resulted in only a 10% crude yield. Similarly in (A-D1avi) upon further increasing the concentration of the tfaa (in the mixed anhydride) to sdm (5 to 1) resulted in a similar percentage yield of the expected ester.

It appears that tfaa has fallen into disrepute as 'catalyst' in acylations and that the substituted carbodiimides are more widely used. Hence one attempt was made (A-D1b) to form sdm 11-(methyl glutarate) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride but no ester peaks appeared in i.r. spectra even after prolonged treatment (7 months).

Upon reconsidering the original reasons for attempting to prepare sdm 11-(methyl glutarate) the following may be said. Monomethyl glutarate was chosen because it was hoped that the methoxycarbonyl group would readily acylate the lysyl ε-amino-groups of proteins. There is no reason why the sporidesmin-*O*-carbonyl (sdm-OOC-)group should not function similarly as an acylating group. The rate of acylation is inversely proportional (Ansell and Gigg, 1965) to the molecular weight of the alkoxy-group but this resistance to acylation is defeated by the time it takes for the acylation reaction to complete. Further, the 3 methylene groups might place the sdm on a stalk as it were but it is more likely that such a group would readily bend round so that the sdm would 'dissolve' in the body of a natural protein.

Arising out of the finding that tfaa in one experimental step converts sdm to anhydrodethiosdm has come the basis upon which other workers have produced a method for estimating sporidesmin in culture extracts and in biochemical reactions with sdm. Unfortunately it is not specific for sdm alone: sdm-D (1.9), sdm-E (1.10) and sdm-G (1.17) will also give the same reaction. In cultures these three compounds are minor constituents.

*Sporidesmin di(chloroacetate)*

Since acylation with monomethyl glutarate-trifluoroacetic anhydride gave a poor yield (A-D1) and a glutaryl bridge between the sdm and protein would be of doubtful value, and since acylation with chloroacetyl chloride failed (A-D3) it was considered that a reinvestigation of chloroacetylation

along the lines of the successful acetylation (Ronaldson, *et al.*, 1963) might be profitable.

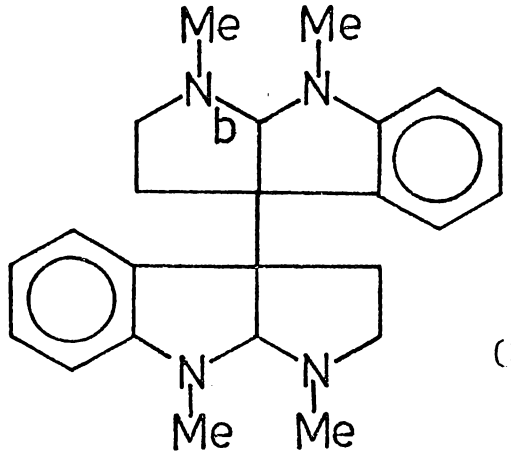
In the first synthesis (A-D4iii) it appeared (t.l.c.) that by the end of 30 min the whole of the sdm had been acylated to the chloroacetate, but upon leaving overnight as would be done with acetic anhydride, the product decomposed.

When chloroacetic anhydride (*c.* 2 mmol) and sdm (*c.* 1 mmol) were dissolved in pyridine, there was no sdm left after 10 min (t.l.c.). There was a 70% yield of crude ester, upon chromatography (A-D4iv).

*Complexing sporidesmin di(chloroacetate) to protein* (A-D4v).-It was not possible to link the chloro- (or iodo-) compound directly with a protein without first forming sulphhydryl groups in the protein. It was better (because of the larger number involved) first to react an acetylated mercapto-compound with the protein's lysyl groups then regenerate the mercapto-groups in the presence of the iodinated sdm derivative (provided that the halo-group of the halo-acetic ester does not first hydrolyze). In bpa there are more available lysyl-*N*s than sulphur atoms to which to link haptens. The method of Marks (1967) was adapted to this work. He used *S*-acetylmercaptosuccinic anhydride (samsa) to acylate the protein. The modified protein was then separated from small molecules (unreacted samsa, *etc.*) on a sephadex column. This modified protein was then treated with hydroxylamine hydrochloride to hydrolyze the acetyl off the mercapto-group so that the latter could react with the iodinated sdm derivative. In this latter reaction there was no pH change registered by the automatic titrator: as each mercapto-group is alkylated with the sdm derivative there should be an $H^+$ formed. There was no evidence of this occurring. Further after the aqueous solution had been ether extracted and the raffinate examined in the u.v. (against bpa) there were no sdm peaks. Perhaps in this preparation, for there to be

no reaction, the SHs were as inert as those in the 11a-mercaptosecosdms described above: there was no reaction with iodoacetamide. Or it may be that the halo-group in both the halo-acetic ester and the iodoacetamide had hydrolyzed.

*Quaternization of sporidesmin* (A-E).-As a first step in exploring the possibility of entering the sdm molecule this way, sdm was treated with methyl iodide in a sealed tube. This was heated in boiling water (1.5 d). Upon treating the product with Reinecke salt no derivative of sdm (i.r.) was formed. This is not surprising, because, although the sdm is a 1-methylindoline derivative, sdm has another nitrogen forming a 1,3-diaza group. Hodson and Smith (1957) pointed out, in respect of folicanthine (2.6) (Mason, 1966) that for the 1,3-diaza-group in contrast to the 1,4(or more)-diaza-groups, when the $N_b$ is protonated (in dil. acid), the $N_a$ was rendered virtually non-basic. So with sdm the $N_b$ is an amide which may have the structure $\rangle N\text{-}C{=}O \rightleftarrows \rangle N^{+}{=}C\text{-}O^{-}$ hence similarly rendering the indoline-*N* virtually non-basic.




(2.6)

## §5. *Diazonium Coupling to Sdm*

As set out in the Rationale (Chapter 1) attempts at diazonium coupling to sdm were made in order 1. to retain the -S-S- bridge intact 2. to attach a functional group at the remaining aromatic hydrogen (position 10) by reducing the new azo-group to an amino-group so that it could then be diazotized for coupling to protein or 3. to diazonium couple with a diazonium compound which already has a group which will condense with protein and 4. having coupled then perhaps to hydrolyze off the methyl of the 7-OMe group.

A number of attempts were made to diazonium couple to veratrole because sdm is a substituted veratrole. Further, it was considered that these attempts with a model compound might indicate the optimum conditions for coupling to sdm. But the work of diazonium coupling (A-F) to the dimethyl ether of *o*-dihydroxybenzene (veratrole) showed that it occurred to only a limited extent and when applied to the sdm no diazonium coupling at its remaining aromatic hydrogen took place.

The reaction which was looked for with the model compound was the one which would give a reasonable yield (*c.* 50% or more) of a (no more than 2) product which could be readily recovered by extraction or preferably filtration.

*The media in which diazonium coupling was attempted:*

The weaker the aniline base the stronger the acid needed to effect diazotization (Zollinger, 1961a) and then, similarly, the weaker the aniline base the stronger the diazotized aniline is as a coupling reagent (Meyer, *et al.*, 1914; Schoutissen, 1933c). [2,4-Dinitroaniline is such a weak base that it forms no salts (Heilbron, 1965).] The diazonium group is by far the most strongly electron-attractive substituent known (Zollinger, 1973).

(a) *Sulphuric acid*

Hence the commonest strong acid, conc. sulphuric, was tried; unfortunately sdm was not stable in this acid but was more stable dissolved in conc. hydrochloric acid.

Although *p*-nitroaniline was diazotized in sulphuric acid, veratrole resisted diazonium coupling to it (A-F1ai, ii). No precipitate could be obtained from the intensely coloured reaction mixture after diluting with water (50 volumes). 3,4-Dimethoxy-4-nitroazobenezene (A-F1bii), the expected product, was later found to be, for practical purposes, insoluble in water or dilute acid, so if it had formed it would have appeared as a precipitate. Although there was negligible reaction with veratrole, the diazonium salt was unspent, since there was immediate reaction with phenols (β-naphthol) producing a red precipitate (A-F1aii).

Haginiwa, *et al.* (1958) reported *c.* 100% yield of hydroxymethoxy- plus dimethoxy-2,4-dinitroazobenzene from coupling 2,4-dinitrobenzene diazonium sulphate with veratrole in acetic acid. But because of the instability of sdm in sulphuric acid this reaction was not followed up. To make an authentic preparation of this azo-derivative (3',4'-dimethoxy-2,4-dinitroazobenzene) the following system of synthesis was pursued with guaiacol.

The 2,4-dinitroaniline was diazotized in conc. sulphuric acid-acetic acid and coupled to guaiacol (A-F2IIai); the reaction formed a deep red dye. Because crystals were observed to be floating on the mixture, concentration of the acetic acid solution was attempted but this did not produce more crystals, instead it produced only tars (from $H_2SO_4$ digestion). The experiment was repeated (A-F2IIaii) using the conventional method of pouring into water. This again produced tar which contained at least 5 substances.

(b) *Conc. hydrochloric acid*

Taylor (Hodges, *et al.*, 1963b) noted that sdm was soluble in conc. hydrochloric acid. Such solutions fairly rapidly (15 min) turn a green-blue colour (substituted indigotin) but sdm can be recovered unaltered from solutions in hydrochloric acid by dilution and extraction ($CHCl_3$). So if the sdm was left in acid for no longer than 30 min *c.* 80% could be recovered and thus in a reaction a reasonable yield could be expected.

(i) p-*Nitroaniline*

Hence diazotization and coupling were investigated in this acid. Diazotization of *p*-nitroaniline in dil. hydrochloric acid was carried out by Nietzki, in 1887. In this work (A-F1aiii) the *p*-nitroaniline was suspended in conc. hydrochloric acid and aqueous sodium nitrite added. When the two were mixed the *p*-nitroaniline dissolved as diazotization took place. As with *p*-nitroaniline in sulphuric acid, insufficient reaction with veratrole (in methanol) took place to produce a precipitate (A-F1ai). From the lowness of the yield of 3,4-dimethoxy-4'-nitroazobenzene it was clear that diazonium coupling of *p*-nitroaniline to veratrole was not a satisfactory means of entering the *o*-dimethoxybenzene ring.

4-*Hydroxy*-3-*methoxy*-4'-*nitroazobenzene* (A-F1bi)

For comparison and for identification of the azo-compound in the extracts of reactions between *p*-nitrobenzenediazonium chloride and veratrole, *p*-nitroaniline was diazotized in hydrochloric acid (*c.* 0.8 M) and guaiacol (in methanol) added (A-F1bi). This reaction immediately deposited a dark orange mass (yield *c.* 40%). Hence it was reasoned an acid medium was no hindrance to this diazonium coupling. Raising the pH (to 5.5) did not produce a Gomberg reaction (Grieve and Hey, 1938) (see later) but increased the yield (*c.* 10%) of the crystalline compound.

For the melting point, Colombano and Leonardi (1908) recorded

125—135° which is widely different from that observed here (170.5—173°). Palkin and Wales (1924) and Wales and Palkin (1926) claimed they obtained the compound and reported its u.v. spectrum but not its melting point. But since the following compound (3,4-dimethoxy-4'-nitroazobenzene) was obtained by simple methylation of this compound, and since its melting point (157.5—160°) agrees with a recently published value (158°) the structure implied in the heading is confirmed. It is generally accepted that diazonium coupling takes place *para* to the hydroxy-group (Zollinger, 1961d).

3,4-*Dimethoxy*-4'-*nitroazobenzene* (A-F1bii)

This compound was synthesized from the above compound by methylation with diazomethane. The melting point (157.5—160°) agreed with that reported by Kokkinos and Wizinger (1971) who also synthesized it from 4-hydroxy-3-methoxy-4'-nitroazobenzene but used methyl iodide as the methylating agent.

5-*Chloro*-2-*hydroxy*-6,7-*dimethoxy*-1-*methyl*-4'-*nitroindole*-3-*azobenzene*

This compound, derived from diazotized *p*-nitroaniline and sdm, and its 2-chloro-4-nitro- and 2,4-dinitro- analogues are dealt with together, see later. It was formed when a solution of sdm was added to the *p*-nitrobenzene diazonium chloride solution in conc. hydrochloric acid. When sdm, dissolved in methanol or ethanol (A-F1ci, iii) was added, there was the smell of nitrobenzene. This was a Sandmeyer reaction (Cowdrey and Davis, 1952); but occurred in the cold (ice bath) in contrast to the deamination of *sym*-tribromoaniline which occurred in the hot (boiling alcohol) as described by Vogel (1966).

*Solvents in which sporidesmin was added to the diazonium solution.*-To avoid the Sandmeyer reaction with alcohols, sdm was dissolved in acetone (A-F1cii) but this modification produced only low molecular weight materials (*m/e* 293 and 254). Indeed, (under experiments with 2,4-dinitroaniline) acetone-conc. hydrochloric acid alone (A-F2Iciv) produced

coloured compounds (yellow–deep orange) and mesityl oxide (Schmidt, 1932). After this the sdm was added, dissolved in chloroform, which formed two phases with the diazonium chloride solution. The chloroform phase was purplish but the yield of pigment was negligible.

Since sdm was soluble in conc. hydrochloric acid and that acid was the medium which had been used to form the diazonium chloride, sdm was taken up in it and added quickly (A-F2Icv). After one hour chloroform gave a coloured extract which was mainly sdm (i.r.) but when the reaction mixture was allowed to stand (20 h, $5^{o}$) (A-F2Icvi) and the extract chromatographed sufficient sulphur was recovered to equal that which was added as sdm, and the quantity of any azo-compound was minimal (0.5 mg).

(b) 2,4-*Dinitroaniline*

In diazotizing 2,4-dinitroaniline in conc. hydrochloric acid (A-F2I) it was observed that a pressure built up (A-F2Iai) in the flask used in the reaction. Further, when excess nitrous acid was discharged with urea (A-F2Iaiii) a rather large amount of urea had to be used (*c.* 1 g for a 2 mmol reaction). When it was attempted to couple 2,4-dinitroaniline to guaiacol (A-F2Ibi) then it was discovered (mass spectrum) that a nitro-group was being replaced by a chlorine (Meldola's work summarised in Saunders 1949b). 4'-Hydroxy-3'-methoxy-2,4-dinitroazobenzene required an *m/e* for $M^{+}$ of 318. Instead, the $M^{+}$ values were *m/e* 309 and 307, e.g. 318 minus 307 = 11 = 46 - 35 (*m/e* $NO_2$ - Cl).

This finding explained the above observations. The increased pressure arose from the excess of unstable nitrous acid not being used up in diazotization and so excess urea was needed to discharge the nitrous acid. In the synthesis (A-F2Icv) only a small amount of nitrite was used because of the 'self diazotizing' effect (from the displacement of the $NO_2$ group from the 2,4-dinitroaniline) once diazotization had commenced (Saunders, 1949b).

2-*Chloro*-1-*iodo*-4-*nitrobenzene* (A-F2Id)

In order to ascertain which nitro-group had been substituted by chlorine the chloroiodonitrobenzene derivative was synthesized. By commencing with the diazotization of 2,4-dinitroaniline in conc. hydrochloric acid (A-F2Id) and replacing the diazonium group with iodine (by adding potassium iodide to the diazonium chloride solution, a Sandmeyer reaction) a compound melting at 96—98.5$^{o}$ was obtained. Of the two possible isomers which would form, one, the 4-chloro-1-iodo-2-nitrobenzene, melted at 63$^{o}$ (Korner, 1875) and the other, 2-chloro-1-iodo-4-nitrobenzene, melted at 99$^{o}$ (Wallagh and Wibaut, 1936). So it was established that the nitro-group *ortho* to the diazonium group was substituted. In the mass spectrum the $M^{+}$ peak was *m/e* 283 which is what is calculated for $C_6H_3{}^{35}ClINO_2$.

Therefore those products from the diazotization of 2,4-dinitroaniline in conc. hydrochloric acid were all derivatives of 2-chloro-4-nitrobenzene diazonium chloride.

2-*Chloro*-4'-*hydroxy*-3'-*methoxy*-4-*nitroazobenzene* (A-F2Ibi)

This compound was obtained from coupling the diazotized 2,4-dinitroaniline (conc. HCl and AcOH) to guaiacol. As observed above, from the synthesis of 2-chloro-1-iodo-4-nitrobenzene it was 2-chloro-4-nitrobenzene diazonium chloride which formed in conc. hydrochloric acid. It is accepted, as stated by Zollinger (1961d), that 98% of the coupling occurred *para* to the hydroxy-group.

2-*Chloro*-3',4'-*dimethoxy*-4-*nitroazobenzene* (A-F2Ibii)

This compound was obtained from that above by simple methylation ($CH_2N_2$).

The $^{1}H$ n.m.r. spectra of the above two compounds were essentially the same except that, in that of the former was the proton peak (δ 6.17)

for the hydroxy-group and in the latter was the second methoxy-peak (δ 3.98). The down field resonances (δ 8.41, 8.39), showing *meta*-splitting (2 Hz), were attributed to those between the chloro- and nitro-groups (H-3) (δ 8.3 in 3,4-dichloronitrobenzene, Pouchert and Campbell, 1974). The corresponding resonances which showed the same *meta*-splitting were the downfield doublets (δ 8.20, 8.16) of the AB pattern for the coupling of the *ortho*-protons (H-5, -6) (δ 8.05 in 3,4-dichloronitrobenzene, Pouchert and Campbell, 1974). In these azo-compounds the doublets for the H-6 protons were well upfield (compared with the biphenyl compound, below) at δ 7.07 and 7.02 (resp.). In neither case were the splitting patterns (at 60 MHz) for the hydroxymethoxy- or dimethoxy-moieties readily interpretable. For the guaiacol derivative there were 6 lines; each of the 3 intense lines had a less intense one (1/5 intensity) upfield from it by *c.* 2 Hz. The two upfield intense lines were *c.* 6.5 Hz apart. But, for the dimethoxy-derivative there were 5 lines: the major downfield one was broadened (higher resolution may have split it). The other 4 lines appeared to form an AB pattern of 2 Hz, *c.* 0.13 ppm apart: these spectra were in the class called 'deceptively simple spectra' Bishop, 1968). Two hertz was too low for *ortho*-splitting (6—9 Hz, Emsley, *et al.*, 1965b): *c.* 8 Hz was expected, as was observed above for the nitrochloro-moiety. There were 8 Hz between the upfield minor peak (for the 4 upfield lines) and the downfield major one and vice versa. It was suggested that the patterns were between that of ABC and AA'A": they did not indicate the substitution pattern. Diazonium coupling takes place *para* to the hydroxy-group (Zollinger, 1961d) except where the position *para* to the hydroxy-group is blocked which latter condition was not so for guaiacol. Hence the 1,2,4-substitution pattern suggested in the headings (above).

*2-Chloro-4,5-dimethoxy-2',4'-dinitroazobenzene* (A-F2Iai)

In the four attempts, (A-F2Iai—iv), to couple 2-chloro-4-nitrobenzene diazonium chloride to veratrole, no 2-chloro-3',4'-dimethoxy-4-nitroazobenzene was detected only a 'red tar', 2-chloro-4,5-dimethoxy-2',4'-dinitroazobenzene, and two substituted biphenyls (below).

The substance, 2-chloro-4,5-dimethoxy-2',4'-dinitroazobenzene, was unexpected and was obtained as a result of adding neat veratrole to the diazotized 2,4-dinitroaniline in conc. hydrochloric acid. It formed immediately as a 'red tar' on the stirring rod and its production did not increase with time. After crystallizing, the compound had an $M^+$ of *m/e* 366 which agreed with the molecular formula $C_{14}H_{11}ClN_4O_6$. In analyzing its mass spectrum it was expected to find the chlorine on the nitrobenzene moiety of the molecule as in the previous compounds synthesized from 2,4-dinitroaniline diazotized in conc. hydrochloric acid. The mass spectrum showed a metastable peak at *m/e* 108.3, for the transition 366→199. The difference between the parent ion (366) and the ion of mass 199 was 167 which equals the mass of the dinitrophenyl group; while 199 equals the mass of the chlorodimethoxyphenylazo-group. So, it appeared that in the conc. hydrochloric acid solution of the diazonium chloride there was some 2,4-dinitrobenzene diazonium salt which had not converted to the 2-chloro-4-nitrobenzene diazonium chloride and which reacted immediately with the veratrole. At the same time, because of the reaction: $2NO_2^- + 2Cl^- + 4H^+ \rightarrow 2NO + Cl_2 + 2H_2O$ (Zollinger, 1961a) the veratrole was chlorinated.

The $^1H$ n.m.r. spectrum of this compound showed, apart from the methoxy-peaks at δ 3.96 and 3.87, these aromatic proton peaks: a doublet (*J* 2 Hz) at δ 8.77 for the proton between the two nitro-groups (H-3'; in eight 2,4-dinitrobenzene compounds, this proton resonates between δ 8.88 and 8.68, Pouchert and Campbell, 1974), split by long-range coupling to the H-5' proton; two doublets at δ 8.48 and 7.88, *J* 10 Hz, (the former

a doublet of doublets, *J* 2 Hz, therefore assigned to H-5') forming an AB pattern for the two adjacent protons (splitting each other) between the 4'-nitro-group and the azo-group (protons *ortho* to the azo-group in azobenzene resonate at δ 7.88, Simons and Zanger, 1972); and two singlet peaks at δ 7.33 and 7.03 for the two protons on the *o*-dimethoxy-aromatic ring. The azo- and nitro-groups shift proton signals downfield. For the other ring the influence of the azo-group was modified by the adjacent methoxy-groups: in 3,4-dimethoxyacetophenone (an X=Y-phenyl compound) the 2-proton resonates at δ 7.37 (Pouchert and Campbell, 1974) which suggests that the resonance at δ 7.33 be attributed to the 6-proton. In *m*-chloroanisole (Pouchert and Campbell, 1974) the aromatic proton envelope is centred on *c.* δ 7.0 which therefore suggests that the resonance at δ 7.03 be attributed to the 3-proton. Were the structure of the dimethoxy-benzene group such that the two protons were adjacent as in a 2-chloro-3,4-dimethoxyazo-group then there would be an upfield AB pattern which was not present.

2-*Chloro*-3',4'-*dimethoxy*-4-*nitrobiphenyl and*

2,2'-*Dichloro*-4',5'-*dimethoxy*-4-*nitrobiphenyl* (A-F2Iaiv)

These two yellow substances were unexpected products from the addition of veratrole dissolved in acetic acid to diazotized 2,4-dinitroaniline (in conc. hydrochloric acid) diluted with acetic acid (1-fold). The mass spectrum of the crystalline material which was sublimed from the extract ($CHCl_3$) showed the $M^+$ peaks (*m/e* 295, 293) for 2-chloro-3',4'-dimethoxy-4-nitrobiphenyl and another set of $M^+$ peaks 34 mass units more (*m/e* 331, 329, 327). These extra peaks whose isotopic ratios indicated two chlorines suggested the 2,2'-dichloro-4',5'-dimethoxy-4-nitrobiphenyl. This position for the extra chlorine atom was indicated on the basis of the proven position of the chlorine atom in 2-chloro-4,5-dimethoxy-2'4'-dinitroazobenzene, above. This statement implies that in the latter case the veratrole was chlorinated before coupling, or in the former case

before substitution by the 2,4-dinitrophenyl radicle.

When the excess nitrous acid was discharged with urea (A-F2Iaiii) so that the reaction, $2NO_2^- + 2Cl^- + 4H^+ \rightarrow 2NO + Cl_2 + 2H_2O$ (Zollinger, 1961a), was absent, there was no formation of the dichloro-substance with the 2-chloro-3',4'-dimethoxy-4-nitrobiphenyl.

In the $^1H$ n.m.r. spectrum of 2-chloro-3',4'-dimethoxy-4-nitrobiphenyl there was the downfield peak (δ 8.36) for the proton (H-3) between the nitro-group and the chlorine (δ 8.3 in 3,4-dichloro-1-nitrobenzene, Pouchert and Campbell, 1974) showing *meta*-splitting (*c*. 2.5 Hz). The downfield half of the AB pattern (δ 8.18) for the two *ortho*-protons (H-5, -6) was a doublet of doublets (2.5, 8 Hz) so it was attributed to the proton (H-5) *meta* to the one resonating at δ 8.36 (above) (δ 8.05 in 3,4-dichloro-1-nitrobenzene, Pouchert and Campbell, 1974). The upfield half of the AB pattern (δ 7.53) was then attributed to the other proton (H-6) (δ 7.78 in 4,4'-dinitrobiphenyl, Pouchert and Campbell, 1974). For the dimethoxyphenyl moiety all three protons resonated at the same frequency (δ 7.02). Since each proton was in a different environment it is suggested that it was an AA'A" condition. Since there was no splitting pattern, the orientations, 1,2,3 or 1,2,4 could not be distinguished explicitly. The latter orientation was preferred, on steric grounds and because for dialkoxy-1,2,4-orientations a single peak is not unusual (δ 6.7—6.8, 4-ethoxy-3-methoxy-, 2,5- and 3,4-dimethoxy-phenylacetic acid and 3-(3,4-dimethoxyphenyl)propionic acid, Pouchert and Campbell, 1974). (Some 1,2,3-orientation compounds e.g. 2,3-dihydroxytoluene and 2,3-dimethoxyphenylcyanide, also have a single aromatic peak, Pouchert and Campbell, 1974.) The remaining upfield signal (δ 3.93) of 6 protons was that for the two methoxy-groups.

*2',5-Dichloro-2-hydroxy-6,7-dimethoxy-1-methyl-4'-nitroindole-3-azobenzene*

This compound, obtained from 2,4-dinitroaniline diazotized in hydrochloric acid and sdm, and its 4-nitro- and 2,4-dinitro-analogues are dealt with together, see below.

*Acetic acid.*-In a number of the syntheses acetic acid was used, after Bunnett and Hoey (1958), with the mineral acid in the diazotization process e.g. with sulphuric acid (A-F1ai, ii; A-F2IIai), or with conc. hydrochloric acid (A-F2Iaiii, iv; A-F2Ibi). At other times just the veratrole was in acetic acid, when it was added to the diazonium salt prepared in mineral acid (A-F2Iaii) alone. The presence or absence of acetic acid did not seem to alter the result. In one series (A-F2Ia) there was a different result when acetic acid was present (A-F2Iaii, iii) from that when it was absent (A-F2Iai). For the latter there was a 'red tar' (2-chloro-4,5-dimethoxy-2',4'-dinitroazobenzene, see above) which formed immediately on the addition of the veratrole to the diazonium salt in conc. hydrochloric acid but which did not form when veratrole was added in acetic acid.

*Compounds resulting from the raising of the pH of the acid solution of the diazonium salt and dimethoxy substrate.*-Since the yield of 3,4-dimethoxy-4'-nitroazobenzene (A-F1ai, iii) was almost negligible when the syntheses were carried out in either sulphuric or hydrochloric acids (Schoutissen, 1933a,c), the effect of raising the pH was investigated. Since guaiacol was successfully coupled in an acid medium (A-F1bi), acid conditions are not a hindrance to coupling. Saunders (1949c) and Zollinger (1961b) both suggested that coupling took place in acid media between energetic diazo-compounds e.g. the *p*-nitrobenzene diazonium salts and arylalkyl ethers. When the pH of the reaction mixture (A-F1aii) in sulphuric acid-acetic acid was raised (to *c.* 3) orange material was extracted. The amount of this orange material seemed to increase with increase in pH up to more than 7 when the amount formed made the mixture

a rusty red colour. The mass spectrum of the crude product showed no peaks at *m/e* 287 (the $M^+$ peak for 3,4-dimethoxy-4'-nitroazobenzene) but peaks at *m/e* 259 and 380 which corresponded to those for 3,4-dimethoxy-4'-nitrobiphenyl and 4',5'-dimethoxy-4,4''-dinitroterphenyl (resp.). This latter name assumes that the nitrophenyl groups entered *para* to the methoxy-groups to form an *o*-terphenyl.

A similar result was obtained when the same (i.e. addition of alkali) reaction was carried out in conc. hydrochloric acid (after Nietzki, 1887) (A-F1aiii). Here the product was chromatographed and the leading zone sublimed. The mass spectrum of this sublimate showed $M^+$ peaks at *m/e* 501 and 622 as well as at 259 and 380. These *m/e* peaks are 121 mass units apart, i.e. nitrophenyl minus the substituted hydrogen (*m/e* 122-1); a Gomberg-like (Grieve and Hey, 1938) type of reaction in which tris- and tetrakis-(*p*-nitrophenyl)veratroles had been formed. (This main fraction from chromatography had the same $R_F$ of 0.88 ($Al_2O_3$ T, $CHCl_3$) as for 3,4-dimethoxy-4'-nitroazobenzene, which was confusing.) There was much froth associated with the reaction (A-F1aiii). This was not surprising because in the Gomberg (Saunders, 1949d) reaction to produce the mono- and poly-phenyls (*p*-nitrophenyldimethoxybenzene, bis-, tris- and tetrakis-(*p*-nitrophenyl)dimethoxybenzenes) nitrogen gas was evolved leaving the *p*-nitrophenyl radical to react at the aromatic hydrogens of the veratrole. The limitation to the number of *p*-nitrophenyl groups which would react with one molecule of veratrole was its number of aromatic hydrogens (*m/e* 622 was equivalent to 4 substituents). It is plausible to consider that the reaction could produce two *p*-nitrophenylveratroles, four bis-(*p*-nitrophenyl)veratroles, two tris-(*p*-nitrophenylveratroles, and one tetrakis-(*p*-nitrophenyl)veratrole. Since the possible number of isomers was so great no further attempt was made to obtain any pure compound.

10-(4-*Nitrophenyl)sporidesmin*

Although it was a methanol solution of guaiacol that was added to the *p*-nitrobenzene diazonium salt there was no smell of nitrobenzene detected about the reaction mixture. But when sdm, dissolved in methanol (as mentioned before), was added to the diazonium salt in hydrochloric acid (1.1 M) (A-F1ci) there was no immediate reaction (coupling): there was the evolution of tiny bubbles ($N_2$) and the smell of nitrobenzene was much in evidence. Similarly with sdm dissolved in ethanol and treated with diazonium salt in hydrochloric acid, (5 M) (Schwalbe, 1905) (A-F1ciii) there was this Sandmeyer reaction but in spite of this reaction with the ethanol there was an intensely coloured product in very low yield, see 5-chloro-2-hydroxy-6,7-dimethoxy-1-methyl-4'-nitroindole-3-azobenzene, later.

In the mass spectrometry of the red crystalline product of the reaction of *p*-nitrobenzene diazonium chloride with sdm dissolved in methanol (A-F1ci) a weak $M^+$ peak of 594 was detected. Such an $M^+$ value corresponded to that of 10-(*p*-nitrophenyl)sdm. For sdm there was only one aromatic hydrogen to substitute and assuming that the reaction (A-F1ci) took place at the *para*-position on the nitrophenyl group, 10-(*p*-nitrophenyl)sdm would be formed. Strictly a reaction of *p*-nitrobenzene diazonium chloride with sdm (A-F1ci) in this way in acid was not a Gomberg reaction (Saunders, 1949). Though it occurred at the pH of the conc. hydrochloric acid used in diazotizing the aniline, it was Gomberg-like. Gomberg reactions usually occur at pHs greater than 7. The Gomberg reaction has been recorded as taking place in strong acid ($H_2SO_4$-AcOH) in the reaction of 2,4-dinitrobenzenediazonium salts with thiophen (Bartle, *et al.*, 1974). The experiment has been repeated (A-F1ciii) in the search for this substance but no fraction has appeared whose i.r. spectrum showed the characteristic pattern of sdm between 1700 and 1300 $cm^{-1}$.

Even when the pH was raised rapidly (A-F1civ) in the hope of forming the *p*-nitrophenyl radicle in presence of sdm no sdm derivative was detected. There was no violent evolution of gas in this reaction. Perhaps the pH was not taken high enough to cause a Gomberg reaction as occurred in experiment (A-F1aiii).

The formation of 10-(*p*-nitrophenyl)sdm which could be expected from the occurrence of the biphenyl in the reaction of diazonium salts with veratrole would have been a satisfactory achievement. Further experiments may elucidate how this type of compound may be achieved.

(c) *Orthophosphoric acid*

The substitution of chlorine for the nitro-group in 2,4-dinitroaniline lessened the strength of the diazonium salt so other media were looked for in which to do the diazotization. Sulphuric acid was an obvious choice (A-F2IIai, ii) but was not persisted with, for reasons stated above.

Schoutissen (1933b) successfully tetrazotized the weak base *p*-phenylenediamine in orthophosphoric acid (with nitrite dissolved in sulphuric acid). Hence phosphoric acid was tried (A-F2III). Although 2,4-dinitroaniline was not freely soluble in the phosphoric acid, it dissolved during diazotization after the addition of (dry) sodium nitrite, so very little dinitroaniline was unreacted at the end of one hour. Upon reaction with veratrole (A-F2IIIai, ii) this preparation of diazonium phosphate was sufficiently strong to yield *c.* 10% of product (by $CHCl_3$ extraction). Sublimation of this product yielded both 3',4'-dimethoxy-2,4-dinitroazobenzene and 3',4'-dimethoxy-2,4-dinitrobiphenyl in separate bands in the gradient sublimation tube. The higher-melting compound, the biphenyl (177-178°) sublimed to the cooler part of the tube while the azo-compound (152-152.5°) sublimed the least distance.

When this diazonium phosphate had sdm, dissolved in phosphoric acid, added to it (A-F2IIIc) there was no reaction since, although there were green colours, the sdm was recovered almost quantitatively. This unaltered sdm crystallized spontaneously. That there was no reaction in this medium, suggested that orthophosphoric acid was not a strong enough acid ($pK_a$ 2.12, Chemical Rubber, 1964).

4'-*Hydroxy*-3'-*methoxy*-2,4-*dinitroazobenzene* (A-F2IIIbi)

To identify the product of reaction between 2,4-dinitrobenzene diazonium phosphate and veratrole, 3',4'-dimethoxy-2,4-dinitroazobenzene was synthesized via the conventional route through coupling to the phenolic compound, guaiacol, and then methylating. Coupling to guaiacol (in $H_3PO_4$) in the acid medium (conc. $H_3PO_4$) yielded 54% of crystalline compound.

Its $^1H$ n.m.r. spectrum ($CD_3COCD_3$) showed the phenolic peak as a singlet at δ 2.98, the methoxy-singlet at δ 3.91 (δ 3.80 for anisole, Pouchert and Campbell, 1974) and the aromatic peaks (δ 7—9). These formed two groups (δ 7.0—7.65; 7.9—8.9) each consisting of a split singlet (long-range coupling) and an AB pattern. The upfield group showed an *ortho*-doublet (H-5', δ 7.03, 9 Hz) comparable with the position of the aromatic proton resonances in 4-hydroxyazobenzene (δ 6.93, $CDCl_3$, Pouchert and Campbell, 1974). The other *ortho*-doublet (H-6', 9 Hz) was at δ 7.63. This resonance with that of H-2' (δ 7.45) was shifted downfield (from that of benzene, δ 7.3) because of being adjacent to the azo-group. Being *meta* to each other, each showed *meta*-splitting (2 Hz). The lower field group showed deshielding because of the two electron-withdrawing nitro-groups and the azo-group. Within this lower field group the upfield doublet (of the AB pattern, δ 7.94, 9 Hz) showed no long-range coupling so it was attributed to the proton *ortho* to the azo-group (H-6). The adjacent proton (H-5, δ 8.63) showed a doublet (9 Hz, *ortho*-splitting) of doublets (2 Hz, *meta*-splitting). The singlet (for

H-3, *meta* to H-5), split by long-range coupling (2 Hz, *meta*-splitting), was further downfield (δ 8.85), because of being between an azo-group and a nitro-group.

If the coupling to guaiacol had taken place *ortho* to the hydroxy-group, the upfield group would have shown two doublets (both showing long-range coupling) and a triplet comparable with that shown by 3-methyl-salicylic acid (Pouchert and Campbell, 1974); or it would have shown some modification thereof. Haginiwa, *et al.* (1958) reacted 2,4-dinitrobenzene diazonium sulphate (in AcOH) with veratrole but claimed no more than $C_{13}H_{10}N_4O_6$ m.p. 173—175° for 70% of the product. The Chemical Abstracts (1958) abstracted the paper (in Japanese) to claim 3-hydroxy-2-methoxy-2',-4'-dinitroazobenzene as the structure of this compound. Since as shown above (from $^1$H n.m.r. evidence) this is not the structure for $C_{13}H_{10}N_4O_6$ m.p. 174.5—176.5°, therefore 4'hydroxy-3'-methoxy-2,4-dinitroazobenzene is claimed, in this work, to be a 'new compound'. Further it is suggested that Haginiwa, *et al.* (1958) synthesized not 3-hydroxy-2-methoxy-2',4'-dinitroazobenzene but 4'-hydroxy-3'-methoxy-2,4-dinitro-azobenzene.

3',4'-*Dimethoxy*-2,4-*dinitroazobenzene* (A-F2IIIbii)

This compound was synthesized from the compound above by simple methylation ($CH_2N_2$). Because the 4'-hydroxy-3'-methoxy-2,4'dinitroazo-benzene was not freely soluble in ether, for methylation with diazomethane, it was dissolved (AcMe) and evaporated to a film onto the wall of the flask. Since upon addition of the ether some of the dye crystallized, the ethereal solution was poured off after what was still amorphous (film) had dissolved and reacted. Then the crystalline material was redissolved and spread again as a film. The 3',4'-dimethoxy-2,4-di-nitroazobenzene melted at 151—152°.

The same compound was also obtained from 2,4-dinitrobenzene diazo-

nium phosphate and veratrole (A-F2IIIaii) or 2,4-dinitrobenzene diazonium trichloroacetate and veratrole (A-F2IVa) in <8% and *c.* 25% yield (resp.). The mixed melting point of the latter preparation with that synthesized above was 150—151.5°.

Again, Chemical Abstracts (1958) claimed that Haginiwa, *et al.* (1958) obtained 2,3-dimethoxy-2',4'-dinitroazobenzene (m.p. 135°) from veratrole and 2,4-dinitroazobenzene diazonium sulphate. That compound may melt at that temperature but it is doubtful whether they obtained it in their synthesis: 2,4-dinitrobenzene diazonium sulphate in acetic acid is unlikely to react differently (i.e. *ortho* to a methoxy-group of veratrole) from 2,4-dinitrobenzene diazonium phosphate in orthophosphoric acid, which reacted *para* to a methoxy-group (as shown above).

3',4'-*Dimethoxy*-2,4-*dinitrobiphenyl* (A-F2IIIaii)

These yellow crystals (a few mg by sublimation) appeared with 3',-4'-dimethoxy-2,4-dinitroazobenzene, in the product from the reaction of 2,4-dinitrobenzene diazonium phosphate with veratrole in orthophosphoric acid. The yield was low (<5%). Since the $M^+$ was 304, 3',4'-dimethoxy-2,4-dinitrobiphenyl (*M* 304) is suggested

(d) *Trichloroacetic acid* (tca)

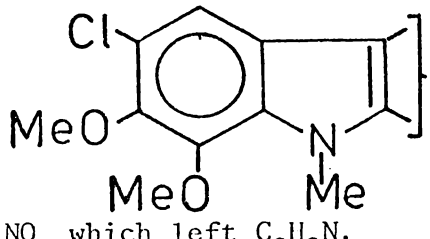
Trichloroacetic acid was a stronger acid ($pK_a$ 0.70, Chemical Rubber, 1964) than orthophosphoric acid but it is a solid. A saturated solution in water contained very little water (100 g of a saturated solution contains 92.3 g tca at 25°, Seidell, 1941). Although after the 2,4-dinitroaniline was dissolved in the saturated tca the solution became a suspension of crystals on cooling, this did not inhibit diazotization on addition of sodium nitrite solution (A-F2IVa).

*5-Chloro-6,7-dimethoxy-8-methyl-2',4'-dinitropyrrolo[2,3-b]indole-2-azobenzene* (A-F2IVb)

In several experiments using trifluoroacetic anhydride (tfaa) as a 'catalyst' in the esterification of sdm (see §4, above), the main product was anhydrodethiosdm (adsdm), a yellow to yellow-orange derivative, in which sdm had lost the two sulphur atoms and two molecules of water. Adsdm was considered to be fairly stable since it had aromatic-like properties (Hodges, pers. comm.). Since it was stable to tfaa it might be possible to diazonium couple at its remaining aromatic site.

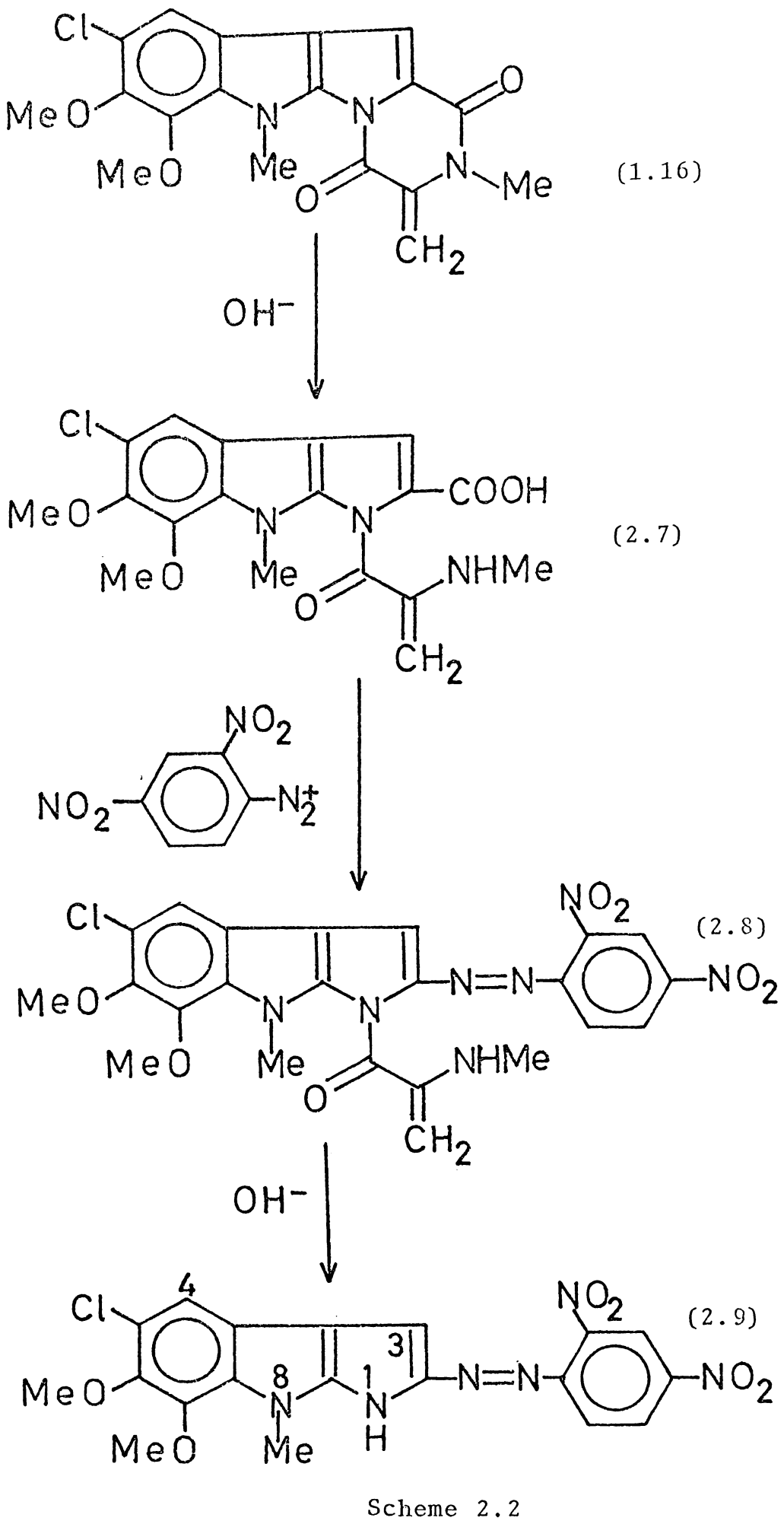
At the pH of the 2,4-dinitrobenzene diazonium salt in saturated tca solution there was no reaction with adsdm (A-F2IVb). Binks and Ridd (1957), in *p*-nitrobenzene diazonium coupling to indole, made the indole solution alkaline (with 0.11 M KOH) before adding the diazotized aniline (in 0.9 M hydrochloric acid). When the solution of adsdm in saturated tca was neutralized the adsdm came out of solution (i.e. it was water insoluble) but redissolved when the solution was made alkaline (with 10 M NaOH). When the diazonium salt in tca was added there was an immediate reaction to products of a dark red colour. There was a 50% yield upon chloroform extraction and crystallization.

The molecular formula for these crystals was $C_{19}H_{15}ClN_6O_6$. The i.r. spectrum (Table 2.9) showed that there were nitro-groups present. When the elements for the dinitroazobenzene ($C_6H_3N_4O_4$) were subtracted there was $C_{13}H_{12}ClN_2O_2$ left. The stable indole moiety of partial structure:




accounted for $C_{11}H_{10}ClNO_2$ which left $C_2H_2N$.

The i.r. spectrum of the crystals did not show the carbonyl stretching peaks of adsdm (1710, 1655 $cm^{-1}$, Hodges, *et al.*, 1964), so the


Cl
MeO
MeO
N
Me
N
O
O
N
Me
CH2
(1.16)
OH−
Cl
MeO
MeO
N
Me
N
COOH
O
NHMe
CH2
(2.7)
NO2
NO2
N2+
Cl
MeO
MeO
N
Me
N
N=N
NO2
NO2
O
NHMe
CH2
(2.8)
OH−
4
Cl
MeO
MeO
8
N
Me
1
N
H
3
N=N
NO2
NO2
(2.9)

Scheme 2.2

product was not a substituted adsdm. This absence of carbonyl absorption (i.r. spectrum) indicated that the CO-C(=$CH_2$)-N($CH_3$)-CO group of the dioxopiperazine ring had been hydrolyzed off in the reaction. It was known that the dioxopiperazine ring of adsdm would rapidly hydrolyze to a colourless amino-acid in 5% methanolic potash but not to the complete removal of the CO-C(=$CH_2$)-NMe-CO group. This amino-acid from adsdm upon methylation (with $CH_2N_2$) reformed the adsdm (unpublished data). Scharwin and Kaljanoff (1908) reported that diazobenzenesulphonic acid eliminated the carboxy-group from *p*-dimethylaminobenzoic acid. Hence this mechanism is suggested: either a. the alkali hydrolyzed the 1,2-lactam (locants as for sdm) to the amino-acid (2.7) (Scheme 2.2) from which the 2,4-dinitrobenzene diazonium salt eliminated the carboxy-group (2.8) followed by further hydrolysis of the 4,5-amide to the -NH- (2.9); or b. the hydrolyses (and elimination) took place in reverse order; or c. the diazonium salt ruptured the 11a-C-CO bond (sdm locant) first, to be followed by hydrolysis of the 4,5-amido-bond. The i.r. spectrum in nujol of the compound (2.9) showed a hydrogen-bonded NH peak (*c.* 3300 $cm^{-1}$). There was also the medium peak at 1650 $cm^{-1}$ (nujol) for a pyrrolo-double bond which was not conjugated with the indole-double bond system but was conjugated to that of the azobenzene system. For 5-chloro-2-hydroxy-6,7-dimethoxy-1-methyl-2',4'-dinitroindole-3-azobenzene (see later) the i.r. absorption (nujol) for the tautomeric oxindole carbonyl was at a higher frequency (1710 $cm^{-1}$) than those for the mono-nitro-analogues (1660—1665 $cm^{-1}$) which suggested that it may be that the dinitroazobenzene group in (2.8) was causing the shift of the pyrrolo-double bond absorption from its usual frequency (<1600 $cm^{-1}$, Bellamy, 1975f) to that observed here (1645 $cm^{-1}$). From this reasoning it is suggested that the remaining $C_2H_2N$ (above) completed the pyrrolo-ring, hence the structure, 5-chloro-6,7-dimethoxy-8-methyl-2',4'-dinitropyrrolo[2,3-*b*]indole-2-azobenzene, is plausible.

The $^1$H n.m.r. did not contribute any conclusive structural data. It showed a broad peak well downfield (δ 12.4) for the pyrrolo-*NH:* the typical pattern (δ 9.05, d *J* 2 Hz; 8.18, dd *J* 9.5, 2 Hz; 8.03, d *J* 9.5 Hz) for the 2,4-dinitroazobenzene group as was observed above for 4'-hydroxy-3'-methoxy-2,4-dinitroazobenzene (δ 8.9—7.9): a singlet (1 H) on the side of the $CHCl_3$ peak (δ 7.30, no spectrum to confirm this was obtained in either AcMe-$d_6$ or $CCl_4$ because of insolubility) attributed to the aromatic proton (H-4) (the same proton in adsdm resonated at either δ 7.65 or 7.41, Hodges, *et al.*, 1964): a singlet (δ 6.65) attributed to the pyrrolo-*H* (H-3); the resonances of protons on aromatic rings adjacent to azo-groups usually shift downfield by *c.* 0.5 ppm but this pyrrolo-*H* had shifted upfield by more than 0.75 ppm compared with its resonance in adsdm but compared with the resonance of the corresponding proton in 2-methylindole and substituted 2-methylindoles (Pouchert and Campbell, 1974) it was downfield by *c.* 0.45 ppm: and two singlets of 6 (δ 4.27) and 3 (δ 3.62) protons each, for the two methoxy-groups and the *N*-methyl-group (resp.).

5-*Chloro*-2-*hydroxy*-6,7-*dimethoxy*-1-*methyl*-4'-*nitroindole*-3-*azobenzene* (2.11a) (A-F1ciii)

2',5-*Dichloro*-2-*hydroxy*-6,7-*dimethoxy*-1-*methyl*-4'-*nitroindole*-3-*azobenzene* (2.11b) (A-F2Icii)

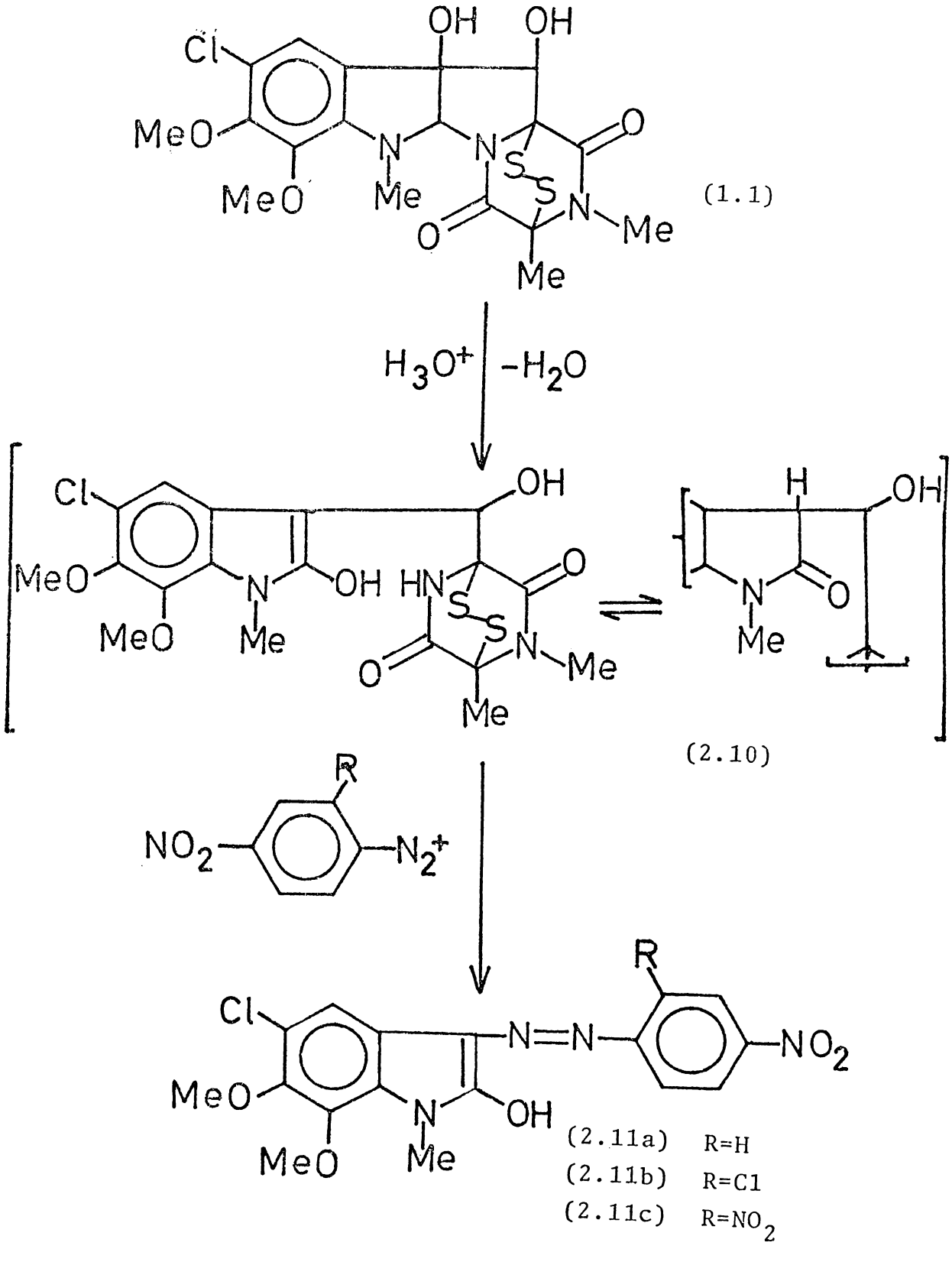
5-*Chloro*-2-*hydroxy*-6,7-*dimethoxy*-1-*methyl*-2',4'-*dinitroindole*-3-*azobenzene* (2.11c) (A-F2IVc)

Although the foregoing reactions of coupling nitrobenzene or chloronitrobenzene diazonium salts with veratrole yielded biphenyl derivatives and not azo-compounds, sdm was treated with these diazonium salts. Before it was known that it was the 2-chloro-derivate of the diazonium chloride which had been formed, the diazotized 2,4-dinitroaniline (in conc. HCl) was reacted with sdm (in AcMe). Reaction for 15 min yielded a trace of a dark purple substance plus unreacted dinitroaniline and sdm

(t.l.c. of $CHCl_3$ extract) (A-F2Ici) but on reaction overnight purple crystals were filtered out (*c.* 24%). Although the filtrate darkened on exposure to air, the extract ($CHCl_3$) of the darkened filtrate did not show any spot corresponding to the purple crystals (A-F2Icii). Similarly *p*-nitrobenzene diazonium chloride yielded 8% of purple crystals.

Because 2,4-dinitrobenzene diazonium salts (trichloroacetate) yielded *c.* 25% of 2,4-dinitro-3',4'-dimethoxybenzene from veratrole, it might so react with sdm. When the 2,4-dinitroaniline was diazotized in orthophosphoric acid forming 2,4-dinitrobenzene diazonium phosphate and sdm added, there was no reaction (as mentioned above). Sdm was recovered unaltered. But when 2,4-dinitrobenzene diazonium trichloroacetate in saturated trichloroacetic acid was reacted with sdm (dissolved in saturated trichloroacetic acid) then a purple compound (8%) was slowly formed.

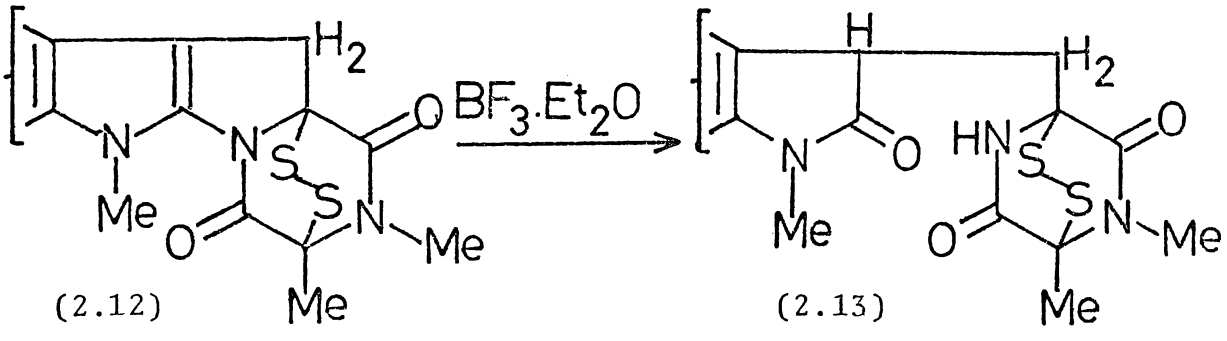
The i.r. spectra of these compounds (in nujol) showed a hydrogen-bonded OH peak (*c.* 3400 $cm^{-1}$) and medium peaks at 1660, 1665 and 1710 $cm^{-1}$ (resp.). This is consistent with the molecules being tautomeric and existing mainly in the hydroxy-state. Both the characteristic sdm-carbonyl peaks (1700 and 1675 $cm^{-1}$) (and the pattern for sdm between 1500 and 1300 $cm^{-1}$) were absent, which suggested that in the reaction the epidithiodioxopiperazine ring had been lost. The respective molecular formulae were $C_{17}H_{15}ClN_4O_5$, $C_{17}H_{14}Cl_2N_4O_5$ and $C_{17}H_{14}ClN_5O_7$. Since there were no dioxopiperazine-*N*s in these compounds (i.r.) and since there were 4—5 nitrogen atoms present it is suggested that 2 of them were azo-*N*s i.e. part of each molecule was respectively $C_6H_4N_3O_2$, $C_6H_3ClN_3O_2$ and $C_6H_3N_4O_4$ derived from the coupling reagents. This left $C_{11}H_{11}ClNO_3$. The molecular formula for 5-chloro-6,7-dimethoxy-1-methylindolyl, derived from sdm, was $C_{11}H_{11}ClNO_2$ which left one oxygen atom; hence the suggested hydroxy-groups in the headings.


OH
OH
Cl
MeO
MeO
N
Me
N
S
S
N
Me
O
O
Me
(1.1)
$H_3O^+$
$-H_2O$
Cl
MeO
MeO
N
Me
OH
OH
HN
S
S
N
Me
O
O
Me
H
OH
N
Me
O
(2.10)
R
$NO_2$
$N_2^+$
Cl
MeO
MeO
N
Me
OH
N=N
R
$NO_2$
(2.11a) R=H
(2.11b) R=Cl
(2.11c) $R=NO_2$

Scheme 2.3

A $^1H$ n.m.r. spectrum of any or all these compounds would not help in deciding the position of the substituted azobenzene group and the hydroxy-group because if they were adjacent to each other on the pyrrolo-ring there would be no protons (H-2; H-3). The aromatic proton (H-4) would be shifted from its resonance position (δ 7.08) for the indoline ring system of sdm: the resonance of that proton shifts downfield when sdm is converted into the indole derivative anhydrodethiosdm (1.16) (δ 7.65 or 7.41 ppm, Hodges, *et al.*, 1964). Even supposing the azo-group were in the 4 position, the H-2 or H-3 protons would still be in an aromatic environment and therefore not readily assignable.

Chemically a structure could be deduced. The acid unstable 1,3-diaza-group hydrolyzed and dehydrated in the strong acid (Robinson, 1963). A similar reaction has been recorded by Hodges, *et al.* (1966): when anhydrosdm-B (2.12) was treated with boron trifluoride ether complex, the product was isosdm-B (2.13). In this reaction the elements of water




(2.12) (2.13)

had been added across the 5-N-C-5a bond. Zollinger (1961c) reviewed work where diazonium coupling took place at the α-carbon (to the phenyl group) of vinologues of dialkylanilines. The structure (2.10) was a similar vinologue i.e. an *N*-aryl-*N*-methyl-β-aminostyrene, in which it is suggested, the diazonium salt eliminated the 3-hydroxymethylene-1,6-dimethyl-2,5-dioxopiperazine group as in Scheme 2.3. The sulphur bridge was probably lost early in the dehydration step as in the formation of anhydrodethiosdm (1.16) (Hodges, *et al.*, 1964), (c.f. prolonged action of conc. hydrochloric acid on sdm, above).

Table 2.9. Infra-red spectra (KBr) of nitrobenzene compounds.

| Compounds | Phenyl nucleus | The nitro group | | | | Substitution pattern | | In-plane bending intensified by -ve group |
|---|---|---|---|---|---|---|---|---|
| | | $\nu_{as}$ | $\nu_{s}$ | C-N stretching | CNO bending | Two *ortho*-Hs | Isolated Hs | |
| 2-Chloro-1-iodo-4-nitrobenzene | 1590m (1510s)* 1445m | 1510vs | 1330vvs | 880s | - | 825m | 880s | - |
| 4-Hydroxy-3-methoxy-4'-nitroazobenzene | 1600m, 1585s, 1510vs, 1450m. | 1515vs | 1340vs | 860m | 615w | 850s | 865s | 1270vs |
| 3,4-Dimethoxy-4'-nitroazobenzene | 1595s (1500vs) 1450m. | 1525—1500vs | 1340vs | (860s) | 615w | 805s | 860s | 1265brvs |
| 2-Chloro-3',4'-dimethoxy-4-nitrobiphenyl | 1600m, 1580s, 1505vs, 1450sh | 1515vs | 1335vsbr | 870m | - | 800s | 860s | 1250s |
| 2-Chloro-4'-hydroxy-3'-methoxy-4-nitroazobenzene | 1590s, (1500vs), 1450m | 1500vs | 1340vs | (865s) | 620w | 820m | 865s | 1270vs |
| 2-Chloro-3',4'-dimethoxy-4-nitroazobenzene | 1590m, (1510vs) 1455m | 1510vs | 1340vs | 860w | - | 800m | 870m | 1270vs |
| 4'-Hydroxy-3'-methoxy-2,4-dinitroazobenzene | 1610s, 1595s, 1580s, (1500s), 1450sh. | 1500s | 1340vs | (865m) | 620m | 830—840m | 865m | 1255m |
| 3',4'-Dimethoxy-2,4-dinitroazobenzene | 1590s, 1505s, 1460m. | 1530s | 1340vs | (860s) | 620m | 835m | 860s | 1270vs |
| 2-Chloro-4,5-dimethoxy-2',4'-dinitroazobenzene | 1595s, 1500s, 1445m. | 1530s | 1335vs | (860s) | - | 830m | 860s | 1270vs |
| 5-Chloro-2-hydroxy-6,7-dimethoxy-1-methyl-4'-nitroindole-3-azobenzene | (1600vs), 1505m, 1455msh. | 1600vs | 1330s | (840s) | 625m | 840s | - | 1270vs |
| 2',5-Dichloro-2-hydroxy-6,7-dimethoxy-1-methyl-4'-nitroindole-3-azobenzene | (1600vs), 1580m, 1500s, 1450m. | 1600vs | 1330vs | 840m | 620w | 800m | 890m | 1260s |
| 5-Chloro-2-hydroxy-6,7-dimethoxy-1-methyl-2',4'-dinitroindole-3-azobenzene | 1610s, 1595s, 1505msh, 1455m. | 1610s 1595s | 1340vs | (835m) | 610w | 835m | 915m | 1265s |
| 5-Chloro-6,7-dimethoxy-8-methyl-2',4'-dinitropyrrolo[2,3-*b*]indole-2-azobenzene | 1610vs, 1585vs, 1500s, 1445m. | 1600vs 1585vs | 1330vs | (830m) | 600w | 830m | 855m | 1260m |

*Those frequencies inside brackets ( ), represent those which could be hidden by other group absorptions.

Therefore the structures, 5-chloro-2-hydroxy-6,7-dimethoxy-1-methyl-4'-nitroindole-3-azobenzene (2.11a), 2',5-dichloro-2-hydroxy-6,7-dimethoxy-1-methyl-4'-nitroindole-3-azobenzene (2.11b) and 5-chloro-2-hydroxy-6,7-dimethoxy-1-methyl-2',4'-dinitroindole-3-azobenzene (2.11c) are suggested for the compounds derived respectively from *p*-nitro-, 2-chloro-4-nitro- and 2,4-dinitro-benzene diazonium salts.

*The infra-red spectra of the foregoing azo- and nitro-compounds.*-Apart from the very strong nitro-group peaks at *c.* 1510 and 1340 $cm^{-1}$, and the phenyl group peaks at *c.* 1590 and 1450 $cm^{-1}$, any assignment to other peaks was purely subjective. These peaks and some others (substitution pattern) are set out in Table 2.9. Though a number (9) of the compounds had C-Cl functions, none showed strong peaks at <650 $cm^{-1}$ attributable to C-Cl vibration. Plyler (1950) reported that the C-Cl absorption for chlorobenzene was weak, which observation is consistent with that observed in the foregoing compounds, for sdm, and for all those compounds derived from sdm. In respect of these weak absorptions it is noted that 4-hydroxy-3-methoxy-4'-nitroazobenzene and 3,4-dimethoxy-4-nitroazobenzene had weak absorptions at 612 $cm^{-1}$ without any C-Cl function while 3-chloro-4-iodo-1-nitrobenzene, 2-chloro-3',4'-dimethoxybiphenyl and 2-chloro-4,5-dimethoxy-2',4'-dinitroazobenzene showed no absorption at less than 630 $cm^{-1}$.

## §6. *Conclusion*

1 - The -S-S- bridge of sdm may be opened and alkylated without the rest of the molecule decomposing.

2 - By using alkoxycarbonylalkyl groups the sdm molecule was successfully complexed to proteins (through the lysyl residues) to form antigens.

3 - By the antibody titre (using the precipitation of a radioactive derivative of sdm) these antigens show some activity in stimulating sheep to produce antibodies to sdm.

4 - Attempts to diazonium coupling to sdm did not produce compounds coupled at the aromatic hydrogen but compounds coupled elsewhere by an elimination process. The elimination eliminated the epidithiodioxopiperazine ring moiety which it was hoped to preserve.

5 - By *O*-acylation with a suitable bridging reagent it may be possible to link sdm to protein while preserving the -S-S- bridge.

# CHAPTER 3. THE $^{13}C$ NUCLEAR MAGNETIC RESONANCE OF SPORIDESMIN AND SPORIDESMIN-D

*Purification of Sporidesmin*

Because of the close similarity in polarity of sdm, sdm-D and sdm-E, the latter two, after all the partitioning, column chromatography (Ronaldson and Fyvie, 1973) and crystallizations, were not readily separated from sdm. A system of chromatography to separate sdm (1.1) from sdm-D (1.9) and sdm-E (1.10) was arrived at thus: on t.l.c. ($SiO_2$ gel G $F_{254}$) acetone alone moved sdm, sdm-D, sdm-E and sdm-G (1.17) all the same distance ($R_F$ 0.61); benzene alone moved them less than $R_F$ 0.1; but acetone-benzene (1:9) moved sdm to $R_F$ 0.52, sdm-E 0.50, sdm-D and sdm-G 0.35; chloroform alone moved sdm to $R_F$ 0.51, sdm-D and sdm-E to 0.40 and sdm-G to 0.27. From a number of combinations of chloroform and benzene, the best separation was obtained with the mixture ($CHCl_3$-$C_6H_6$ 13:7). Under the conditions of the t.l.c. plates at the time, this mixture gave the following $R_F$ values: sdm 0.31, sdm-E 0.24, sdm-D 0.18 and sdm-G 0.16 and separated all four when they were all applied at the same point on the t.l.c. plate.

When, therefore, sdm as prepared by Ronaldson and Fyvie (1973), was chromatographed on a column of silica gel G and developed with benzene containing increasing concentrations of chloroform, it was possible to elute the sdm free (by t.l.c.) of either sdm-D or sdm-E. It was found advisable, as a general rule, to keep the concentration of the more polar solvent considerably lower than that used in t.l.c. The appropriate fractions were crystallized from methanol-water to give unsolvated sdm ready for $^{13}C$ n.m.r. (A-A).

If having crystallized the sdm (unsolvated), as produced above, it was found necessary to recrystallize from methanol-water, each crystallization in the conventional way produced a decomposition product which

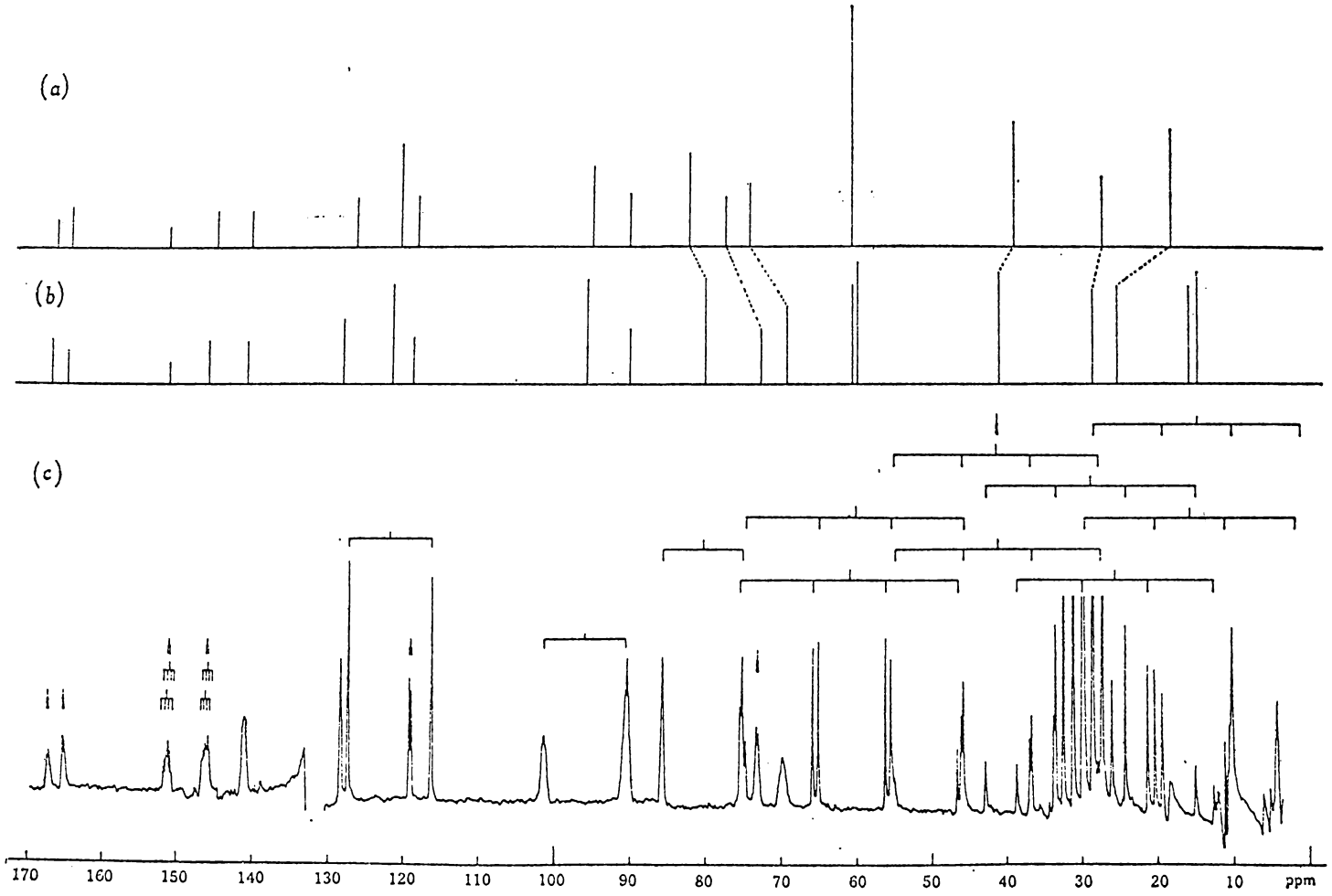



Fig. CM3.1 (*a*) Decoupled spectrum of sdm; (*b*) decoupled spectrum of sdm-D; (*c*) undecoupled spectrum of sdm-D, showing the multiplicities. The peaks in (*c*) between 140 and 155 ppm have been doubled in intensity. The five truncated peaks in the (*c*) spectrum, between 27 and 33 ppm, are those of $CD_3COCD_3$.

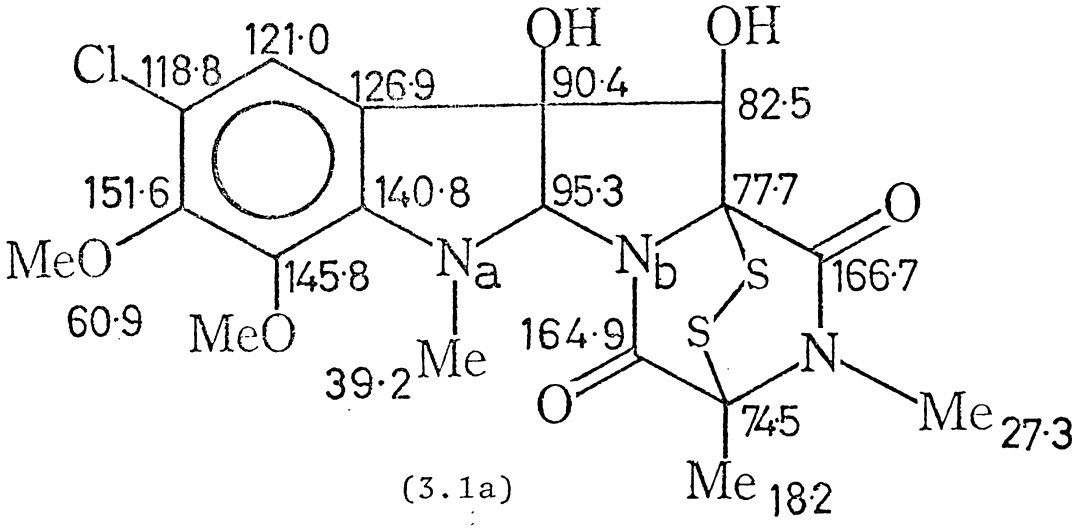
was greener (akin to the green produced by hot, dilute alkali, Ronaldson, *et al.*, 1963) at each step. Charcoal had to be used each time. Melting point determinations were no criteria of ultimate purity because mixtures with sdm-E did not give the usual depression in melting point (A-B3iv).

When sdm-D (A-C1aiii) was treated with acetone to remove ether of crystallization, and redissolved several times there was not the decomposition to greens as with sdm, so it was considered to be more stable and hence satisfactory for the overnight treatment required to obtain the undecoupled $^{13}C$ n.m.r. spectrum.

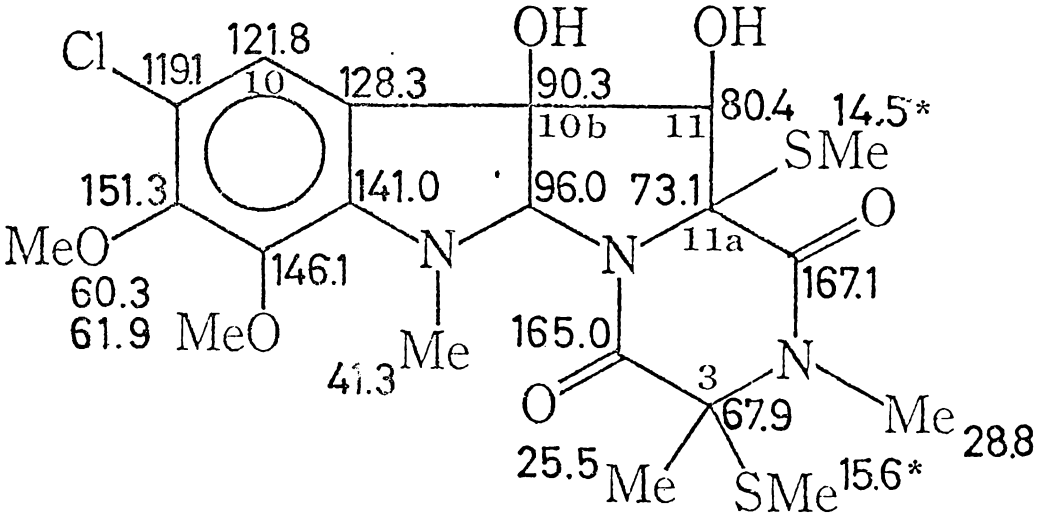
$^{13}C$ *Nuclear Magnetic Resonance*

In the following discussion where a resonance shows a long-range multiplicity and no one-bond carbon-hydrogen splitting, it was considered, in the first instance, as a singlet and $W_{\frac{1}{2}}$ was the width at half height.

Fig. CM3.1 sets out the proton-noise-decoupled spectra of the two compounds, sdm and sdm-D, and the undecoupled spectrum of sdm-D. In this figure the spectral width for the two decoupled spectra was 4 kHz and for the undecoupled spectrum 2.5 kHz, which gives 1.221 Hz between any two data points, hence the coupling constants quoted in this discussion are multiples of this value and are ± 1.2 Hz or better. To correct for the difference in intensity between the two decoupled spectra of sdm and sdm-D, that for sdm-D was increased by a factor (1.18), determined by making the average height of the three least-shifted resonances (at 90.3, 119 and 141 ppm) in sdm-D equal to that of the same three in sdm. The peaks fell naturally into four groups: (a) the methyl carbon peaks from 10 to 65 ppm, (b) the methine and quaternary carbon peaks from 65 to 100 ppm, (c) the aromatic peaks from 115 to 155 ppm and (d) the two lactam carbonyl peaks about 165 ppm.




(3.1a)




(3.1b)

* Values interchangeable.

The following discussion deals with the carbon-13 shieldings (3.1a, b) and $^1J$ spin-spin couplings. The discussion of the long range coupling relevant to sections (a) and (b), is at the end of section (b).

(a) *The Methyl Peaks*

(i) *At* 14.5 *and* 15.6 *ppm (q).* J 140 *Hz (sdm-D).*-Although these two *S*-methyl resonances were more shielded than those in dimethyl sulphide (19.5 ppm, Stothers, 1972i), their positions were consistent with those of the *S*-methyls in methionine (15.0 ppm) and thioanisole (15.6 ppm, Johnson and Jankowski, 1972). In the Dreiding model the two sulphurs were *c.* equidistant from their adjacent lactam carbonyl oxygens. The proximity of carbonyl groups (see later) was a factor in the shielding.

By contrast the proton peaks of the *S*-methyl (δ 2.32, 2.40) were downfield (by *c.* 0.5 ppm) from that of the *C*-methyl (δ 1.87, Jamieson, *et al.*, 1969), while these *S*-methyl carbon peaks were upfield. The coupling constants in sdm-D were comparable with those in dimethyl sulphide (138 Hz).

(ii) *At* 18.2 *ppm (sdm) and* 25.5 *ppm (q),* J 131.2 *Hz (sdm-D).*-In sdm there was no ambiguity in assigning the peak at 18.2 ppm to the carbon of the *C*-methyl; the 2-methyl peak of 1,2,4-trimethylpiperazine absorbed at 17.8 ppm (Ellis and Jones, 1972) and the 2- and 5-methyls of *cis*-2,5-dimethylpiperazine absorbed at 18.3 ppm (Johnson and Jankowski, 1972).

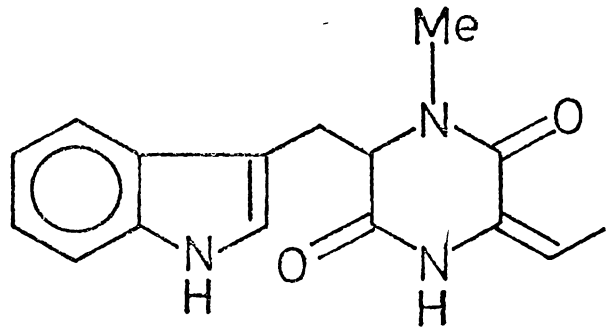
By irradiating the *C*-methyl protons at δ *1.79* in the $^1$H spectrum of sdm-D (acetone), the carbon peak observed to be at 18.2 ppm in sdm was observed to be downfield by 7.3 ppm in sdm-D. This deshielding of the carbon stood in contrast to what was observed for the *C*-methyl protons (δ 1.87 C-$CH_3$, 2.32 and 2.40 S-$CH_3$, Jamieson, *et al.*, 1969).

Dorman and Bovey (1973) have observed the strong carbon-13 shielding effect of amido-carbonyl oxygens on *N*-alkyl carbons and they concluded that a similar effect could occur at the β-carbon. In a Dreiding model

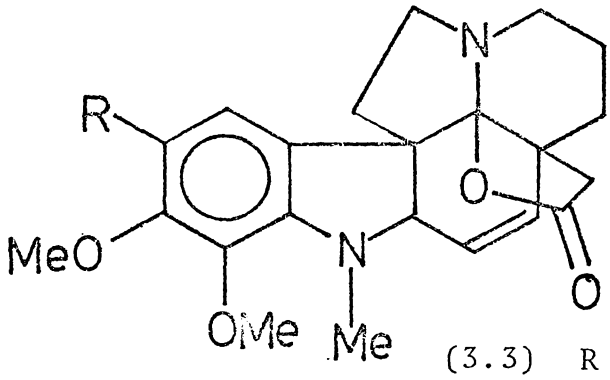
of sdm-D the methyl carbon (β to the nitrogen) was not coplanar with the 1-carbonyl oxygen, hence the deshielding (7.3 ppm) observed by releasing the strain upon the dioxopiperazine ring by opening the -S-S- bridge. Therefore, it might be that there were a shielding and an equal and opposite deshielding which made the methyl-*C* of the *C*-methyl resonance in sdm appear at 18.2 ppm. In the piperazines quoted above (chair conformation, Ellis and Jones, 1972) there were no strains in the rings (comparable with those in sdm) whereas in sdm there were deshielding strains imposed upon the piperazine ring (boat conformation) by the fused pyrrolidine ring, the -S-S- bridge and by the two amido-groups. All these strains were opposed by the shielding arising from the near coplanarity of the methyl-carbon with the 1-carbonyl oxygen in sdm.

According to Haake *et al.* (1964) increased electronegativity of the atom upon which a methyl resided increased the coupling constant of that methyl group. The coupling constant for the four methyl groups on the quaternary carbon of 2,2-dimethylpropane, (Stothers, 1972ℓ) was 124 Hz (25% 's' character, Jackman and Sternhell, 1969) but in sdm-D the value was 131 Hz (greater 's' character), hence the sulphur, nitrogen and carbonyl adjacent to the C-3 in the di(methylthio)dioxopiperazine ring conferred electronegativity on this carbon atom, which electronegativity accounted for the increased one-bond coupling constant of the methyl.

(iii) *At 27.3 ppm (sdm) and 28.8 ppm (q), J 140.4 Hz (sdm-D).*-This methyl resonance of the *N*-methyl lactam in sdm was strongly shielded compared with that of the *N*-methyls in *1*,2,4-trimethylpiperazine (42.9 ppm, Ellis and Jones, 1972), dibenzyl methylamine (42.1 ppm), N,N-dimethylpropanediamine (45.4 ppm) and 3-dimethylamino-2,2-dimethylpropionaldehyde (47.2 ppm), (Johnson and Jankowski, 1972). This shielding (>13 ppm) was attributed to the strong carbon-13 shielding effect of the lactam-carbonyl oxygen (Dorman and Bovey, 1973), as was observed for the *N*-methyls in *1*,3-dimethyluracil (27.5 ppm) and ethyl methylcarbamate

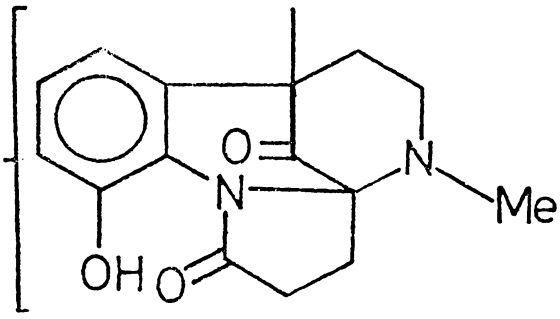


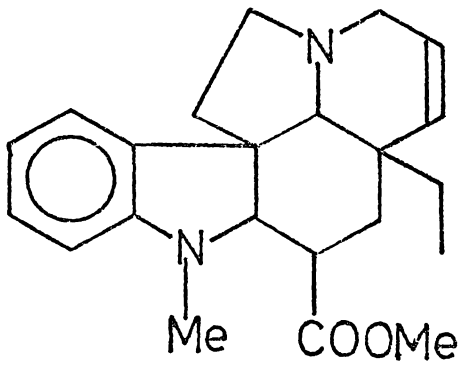
(3.2)



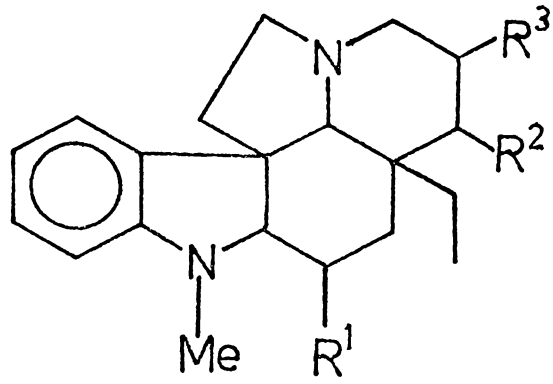
(3.3) R = H

(3.4) R =





(3.5a)



(3.5b) $R^1$=COOMe, $R^2$=$R^3$=H

(3.5c) $R^1$=COOMe, $R^2$=OH, $R^3$=H

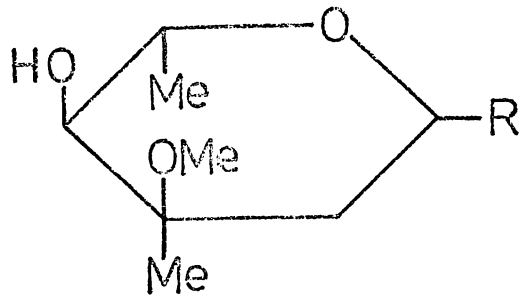
(3.5d) $R^1$=COOMe, $R^2$=H, $R^3$=OH

(3.5e) $R^1$=$CH_2OH$, $R^2$=H, $R^3$=OH

(27.4 ppm) (Johnson and Jankowski, 1972), 3-ethyl-1-methyl-3-phenyl-glutarimide (26–27 ppm, Dorlet and Van Binst, 1973). In tryptophan-dehydrobutyrine dioxopiperazine (3.2) (Kakinuma and Rinehart, 1974) the corresponding *N*-methyl resonates at 32.8 ppm. This deshielding may be attributed to the influence of the *exo*-double bond upon the dioxopiperazine ring. Models of this ring system did not conform to those in sdm or sdm-D. In the sdms, the shielding in sdm-D was 1.5 ppm less than that in sdm because, when the strain of the -S-S- bridge was relaxed, the coplanarity with the oxygen of the lactam and with the 3-methyl was lessened; in a model where a pyrrolidine ring was fused to a dioxopiperazine ring the lactam group tended to be less planar. In sdm-D the coupling constant was comparable with that of the *N*-methyl acetamides (138 Hz, Stothers, 1972ℓ).

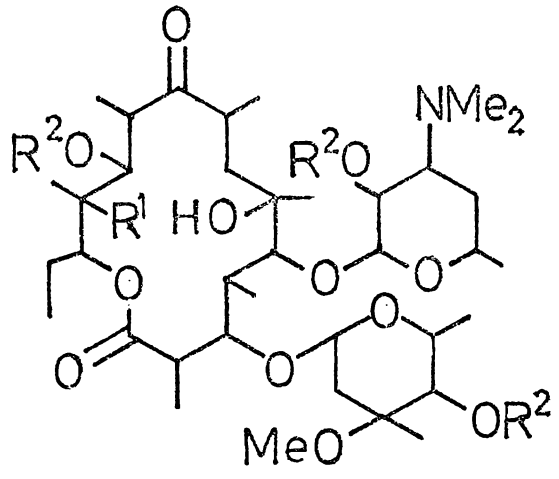
(iv) *At* 39.2 *ppm (sdm) and* 41.3 *ppm (q)*, J 137.9 *Hz (sdm-D)*.-Yates, *et al.* (1973) studied the carbon-13 spectrum of aspidophytine (3.3) which contained a 6,7-dimethoxy-1-methylindoline moiety, and assigned the peak at 35.4 ppm to the *N*-methyl carbon. Similarly, Lukacs, *et al.* (1974), studying tabersonine (3.5a) and 1-methyl-2,16-dihydrovincadifformines (3.5b—e) containing a 1-methylindoline moiety, found that the *N*-methyl carbon resonated at 35–36 ppm. In both sdm and sdm-D the *N*-methyl carbon absorbed at lower fields and in both compounds the methyl was sterically crowded by a carbonyl of the dioxopiperazine ring, producing a deshielding of 5.9 ppm in sdm-D. The strain of the -S-S- bridge in sdm tended to move the carbonyl oxygen away from the *N*-methyl, hence the absorption at higher field.

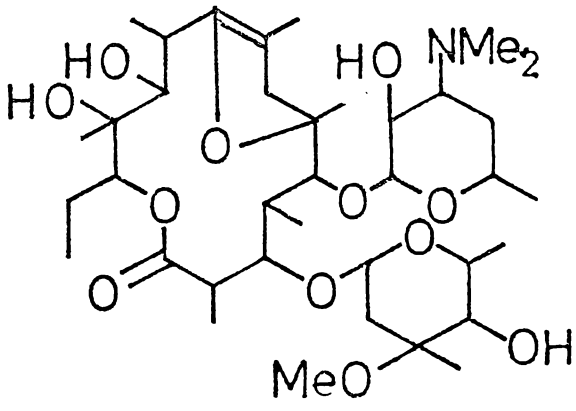
By irradiating the protons of the *N*-methyl group (in sdm-D) at δ 3.01 the carbon resonance at 28.8 ppm remained a singlet while that at 41.3 ppm was split (q). Conversely, when the protons of the *N*-methyl group at δ 3.3 were irradiated the peak at 41.3 ppm was unsplit while that at 28.8 ppm was split (q).




(3.6a) R=α-OMe

(3.6b) R=β-OMe



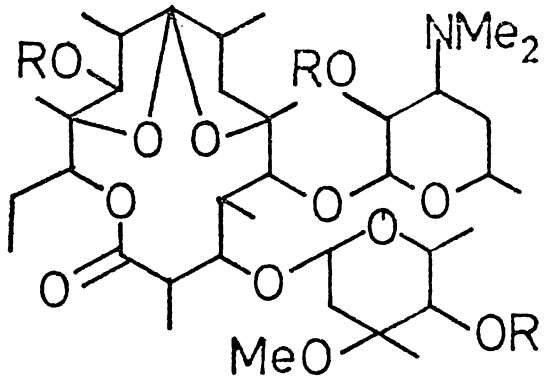




(3.7d)

(3.7a) $R^1$=OH, $R^2$=H

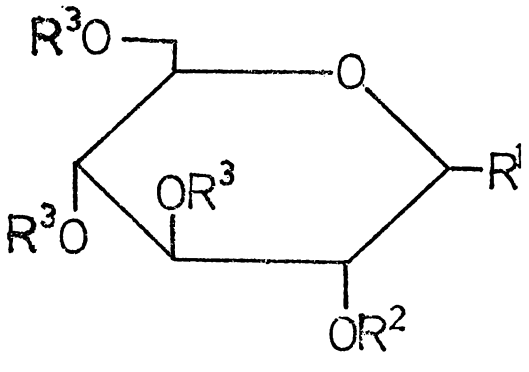
(3.7b) $R^1$=$R^2$=H

(3.7c) $R^1$=H, $R^2$=Ac




(3.7e) R=H

(3.7f) R=Ac




(3.8) $R^1$=α or β OMe

$R^2$= $R^3$=Ac or Bz

The coupling constant (137.9 Hz) exceeded that reported (Stothers, 1972ℓ) for methylamine (133 Hz). The significantly greater value for this constant in sdm-D was attributed to enhancement of the electronegativity (reflecting greater 's' character) of the indoline nitrogen by the benzene ring.

(v) *At* 60.9 *ppm (sdm) and* 60.3 *and* 61.0 *ppm (q)*, J 144.9 *Hz (sdm-D)*.- No ambiguity exists in the assignment of these resonances in either compound, for the intensity of the singlet in sdm about equalled the sum of the two peaks in sdm-D and these occurred within the chemical shift range of comparable aromatic methoxy-groups in aspidophytine (3.3) and haplophytine (3.4) (55.8—61.1 ppm, Yates, *et al.*, 1973).

The use of the shieldings of the methyl-*C*s of methylated alcohols and phenols as a diagnostic tool is fraught with difficulties. The following analysis of the recorded values (ranging from 48.9 to 65.6 ppm) demonstrates the problems.

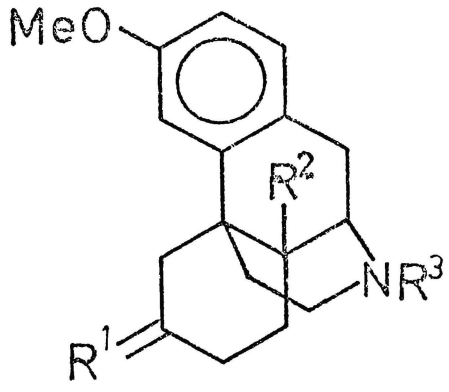
The carbons of the methoxy-groups of the simplest ether, dimethyl ether, resonate at 59.4 ppm (Stothers, 1972h). When one methyl is substituted by an ethylene as in 1,2-dimethoxyethane, methoxyethyl thioglycolate and bis-(2-methoxyethyl)phthalate the carbon of the methoxy-group is shielded to 58.6—58.7 ppm (Johnson and Jankowski, 1972). Further, substitution on the α-carbon as in 3-methoxybutan-1-ol produces greater shielding (55.8 ppm, Johnson and Jankowski, 1972). In a series of α- and β-substituted L-cladinosides (3.6a,b)(3.7a—f) the 3-methoxy-groups (carbohydrate numbering system) are on methylated-*C*s in tetrahydropyran rings and their resonances occur between 48.9 and 49.6 ppm (Terui, *et al.*, 1975b). (Within this group of erythromycins (3.7a—f) there is the same phenomenon as is observed in the aromatic series, see below.)

Methyl pyranosides (e.g. 3.8) show, with increasing substitution, a similar series of shieldings. The series is anomalous, however,

because in at least two of the many possible monomethyl pyranosides the 2- or 3-methoxy-methyl-*C*s of the 2- or 3-methyl pyranosides, resonate (59.6—62.8 ppm, Bock and Pedersen, 1974, Dorman and Roberts, 1970) at values more deshielded than that in dimethyl ether (59.4 ppm, Stothers, 1972h). But the methoxy-methyl-*C*s of the 1-methylpyranosides resonate at 55.1—58.7 ppm (Burton, *et al.*, 1971; Dorman and Roberts, 1970; Uchida, *et al.*, 1975). Acetylation of the methyl pyranosides almost invariably shields the methyl-*C*s of the 1-methoxy-groups. A 4-amino-group shields the methyl-*C* of the 1-methoxy-group even more (52.4 ppm Uchida, *et al.*, 1975).

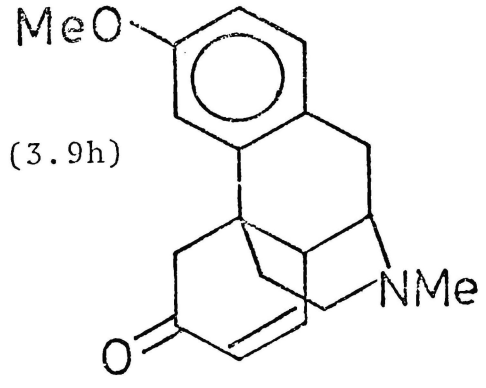
Against these increased shieldings with increasing substitution in aliphatic molecules are the phenomena observed in the aromatic series. Beginning with simplest aromatic methoxy-compound, anisole (54.7 ppm, Johnson and Jankowski, 1972), increasing substitution on carbons becoming less remote from the methoxy-group (except the anomalous *o*-methyl, *o*-ethyl, *m*-methyl and 2,5-dimethyl, 54.5—54.4 ppm, Dhami and Stothers, 1966) deshields the methyl-*C* of the aromatic methoxy-group as in Table 3.1. [Johnson and Jankowski (1972) set out a number (*c.* 20) of methoxy-aromatic compounds in which all the methyl-*C*s of the methoxy-groups resonated between 55.0 and 57.3 ppm.] Crowding of the anisole-methoxy-group (54.7 ppm) by 2,6-di-isopropyl deshields that methyl-*C* by 7 ppm (62.0 ppm), 2,6-di-t-butyl deshields by nearly 10 ppm (64.5 ppm) and electron-withdrawing by a 4-nitro as well deshields the methyl-*C* by nearly 11 ppm (2,6-di-t-butyl-4-nitroanisole, 65.6 ppm, Dhami and Stothers, 1966). These latter structures are not strictly comparable with what is observed in sdm.

The methyl-*C*s of the methoxy-groups of substituted 2-methoxycyclohex-2-enones formed another series with similarity to that of the aromatic series above. The methyl-*C* of the methoxy-group of 2-methoxycyclohex-2-enone resonated at 53.7 ppm (Polonsky, *et al.*, 1975) (more shielded than

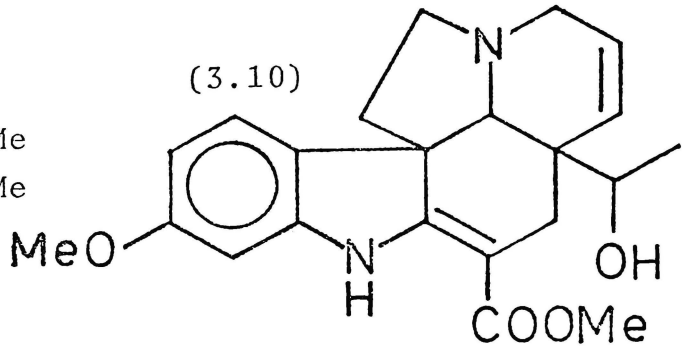



(3.9a) $R^1=H_2$, $R^2=R^3=H$
(3.9b) $R^1=H_2$, $R^2=H$, $R^3=Me$
(3.9c) $R^1=H$, $OH$, $R^2=H$, $R^3=Me$
(3.9d) $R^1=OH$, $H$, $R^2=H$, $R^3=Me$
(3.9e) $R^1=O$, $R^2=R^3=H$
(3.9f) $R^1=O$, $R^2=H$, $R^3=Me$
(3.9g) $R^1=O$, $R^2=OH$, $R^3=Me$

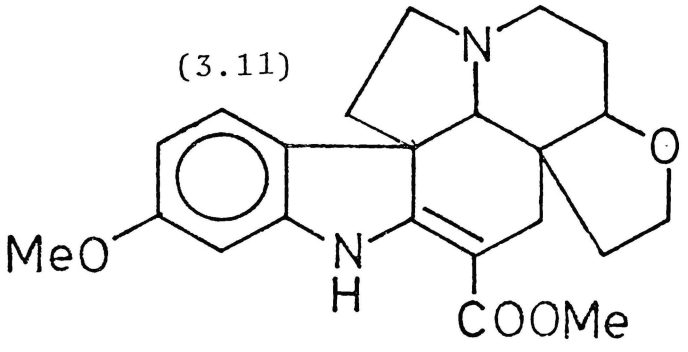
(3.9h)



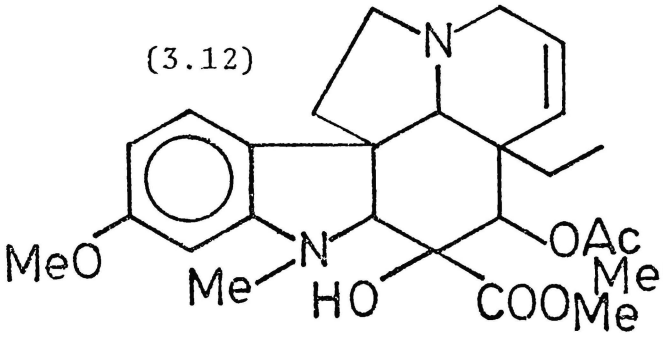

(3.10)




(3.11)




(3.12)

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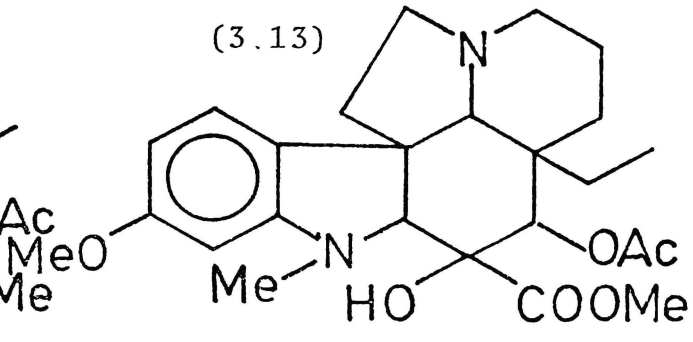
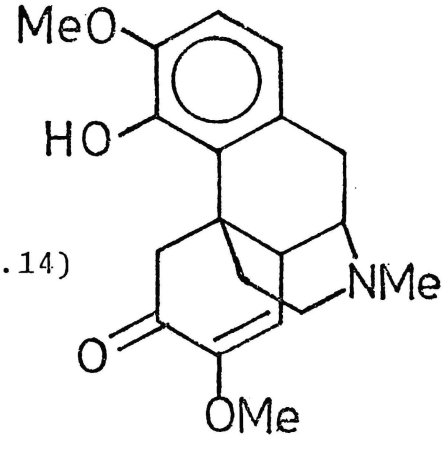
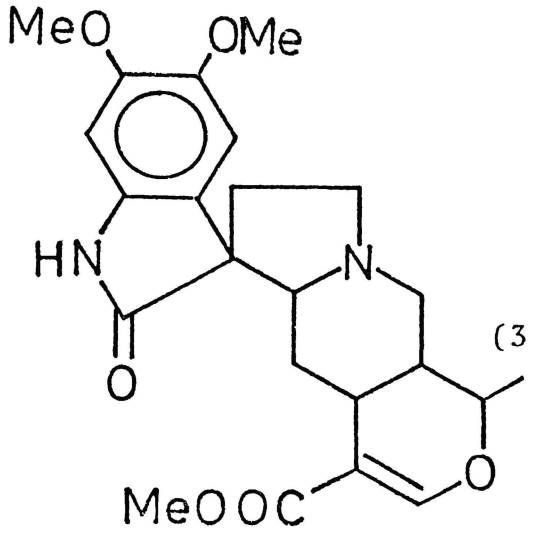


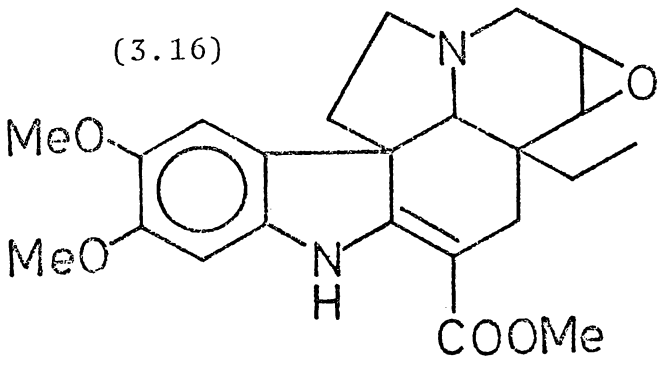



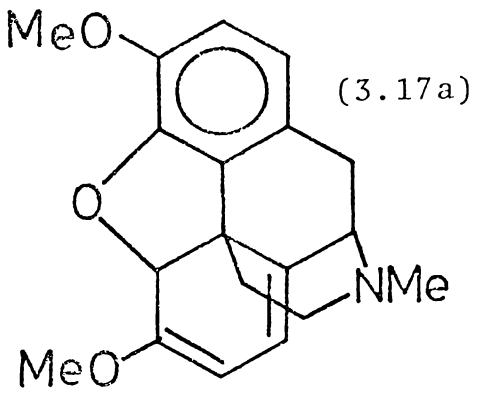

(3.14)



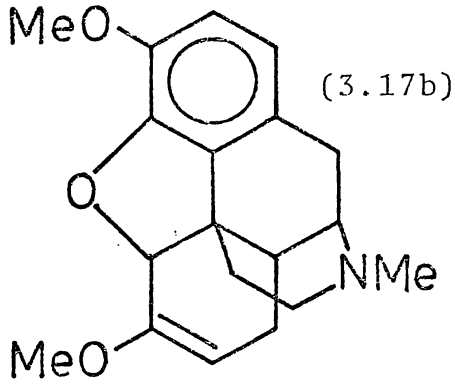

(3.15)

(3.16)

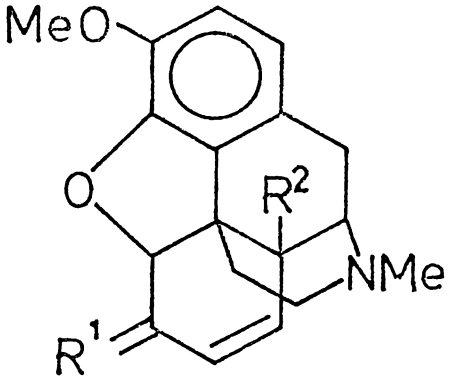


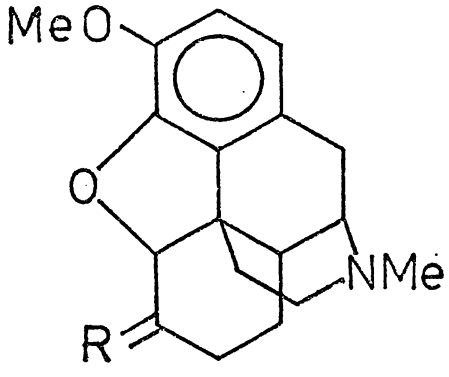

(3.17a)



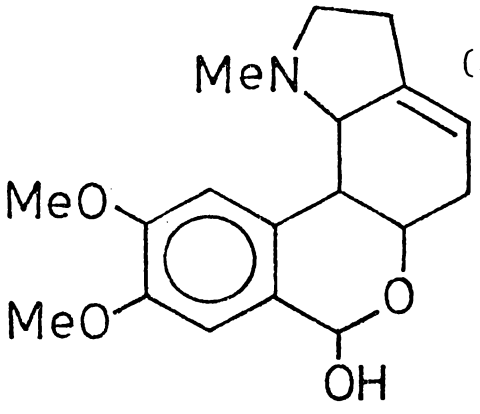

(3.17b)




(3.18a) $R^1$=H, OH; $R^2$=H
(3.18b) $R^1$=O; $R^2$=H
(3.18c) $R^1$=O; $R^2$=OH

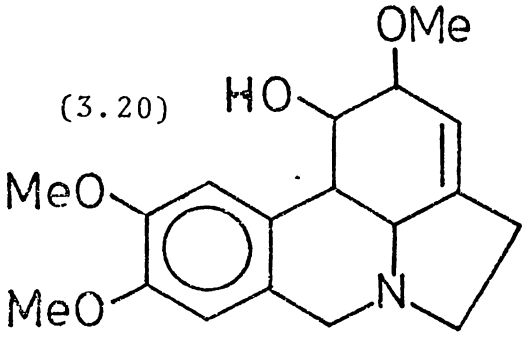



(3.18d) R=$H_2$
(3.18e) R=H, OH
(3.18f) R=O




(3.19)

(3.20)

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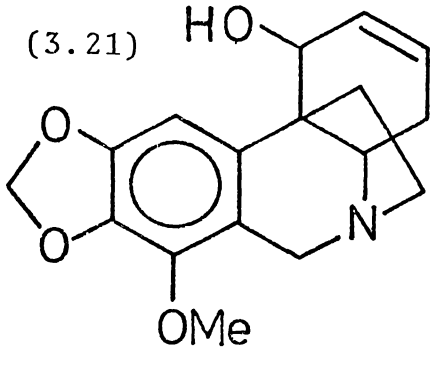
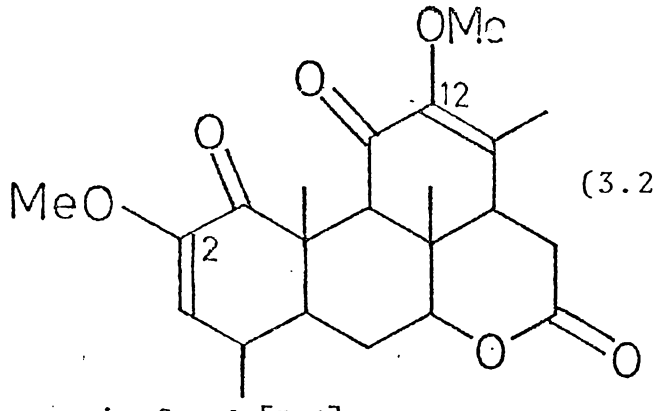


Table 3.1. Resonance frequencies of aromatic methoxy-*C*s (in increasing order of frequency).

| Chemical shift (ppm) | Relation to and nearest substituent | Aromatic ring structure | Derivative or type of derivative | Structure | Reference |
|---|---|---|---|---|---|
| 54.9 - 55.2 | *m*- to -$CC_3$ | 6-Methoxytetralin | 3-Methoxymorphinane derivatives | (3.9a—h) | Terui, *et al.*, 1975a. |
| 55.0 - 55.3 | *m*- to -NH | 6-Methoxyindoline | Vandrikidine<br>Vandrikine | (3.10)<br>(3.11) | Wenkert, *et al.*, 1973b. |
| | *m*- to -NMe | 6-Methoxy-1-methylindoline | Vindoline<br>Dihydrovindoline | (3.12)<br>(3.13) | |
| 55.3 | *p*- to -Cl | Methoxybenzene | 4-Chloroanisole | | Johnson & Jankowski, 1972. |
| 55.8 | *o*- to -OH | 5-Hydroxy-6-methoxytetralin | Sinomenine | (3.14) | Terui, *et al.*, 1975a. |
| 55.8 | *o*- to -OMe | *6*,7-Dimethoxy-1-methylindoline | Aspidophytine | (3.3) | Yates, *et al.*, 1973. |
| 55.8, 55.9 | *o*- to -OMe | 1,2-Dimethoxybenzene | 2-(3,4-Dimethoxyphenyl)ethylamine | | Johnson & Jankowski, 1972. |
| 55.9 - 56.8 | *o*- to -OMe | 5,6-Dimethoxyindoline | Carapanaubine<br>Hazuntinine | (3.15)<br>(3.16) | Wenkert, *et al.*, 1973b. |
| 56.2 - 57.0 | *o*- to epoxide ring | 5-Epoxy-6-methoxytetralin | Thebaine derivs<br>Codeine derivs | (3.17a,b)<br>(3.18a—f) | Terui, *et al.*, 1975a. |
| 57.1 - 57.5 | *o*- to -OMe and both *m*- to -CH | 6,7-Dimethoxy-2-oxatetralin*<br>6,7-Dimethoxy-2-azatetralin** | Lycorenine<br>An amaryllidaceous alkaloid | (3.19)<br>(3.20) | Crain, *et al.*, 1971. |
| 60.3 | *o*- to -$OCH_2O$- | 8-Methoxy-6,7-methylenedioxy-2-azatetralin** | Buphanamine | (3.21) | Crain, *et al.*, 1971. |
| 58.5—60.9 | adj. to -OMe and/or -NMe | 6,7-Dimethoxy-1-methylindoline | Haplophytine | (3.4) | Yates, *et al.*, 1973. |
| 60.3—61.0 | | | Sdm-D | (1.9) | this work. |
| 60.9 | | | Sdm | (1.1) | this work. |
| 61.1 | | | Aspidophytine | (3.3) | Yates, *et al.*, 1973. |

* And ** represent isochroman and tetrahydroisoquinoline (resp.). They have been named above as derivatives of tetralin for ease of comparison.

in anisole). Both in quassin (3.22) where a 2-methoxy-4,6-dimethyl-

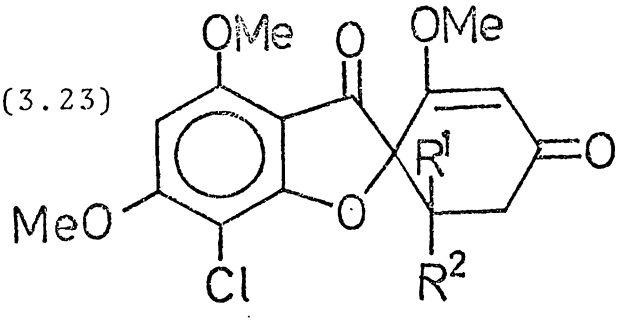


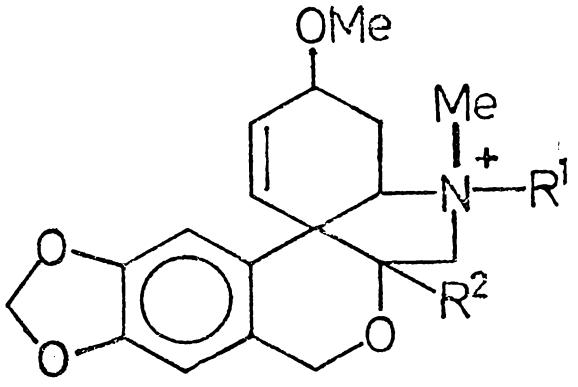

(3.22)

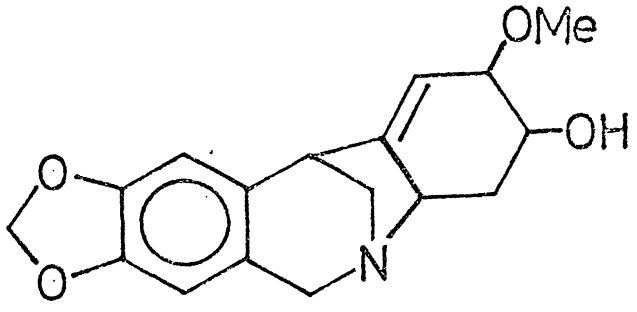
cyclohex-2-enone is fused [5,6] to a saturated hexacyclic ring, and in sinomenine (3.14) where a 2-methoxycyclohex-2-enone ring is fused [4,5-1',2'] to a saturated hexacyclic ring and a 5-*N*-methylpiperidine ring, the methyl-*C*s of the methoxy-groups are deshielded by 0.9 ppm (54.6 ppm, Polonsky, *et al.*, 1975; Terui, *et al.*, 1975a). In quassin (3.22) there is a second α-methoxy-group (12-) whose 2-methoxy-3,5-dimethylcyclohex-2-enone ring is fused [4,5] and [5,6] to a δ-lactone and a saturated hexacyclic ring. The methyl-*C* of this methoxy-group is deshielded by 5.2 ppm (58.9 ppm, Polonsky, *et al.*, 1975). This similarity described here between substituted anisoles and 2-methoxycyclohex-2-enones is hinted at by Polonsky, *et al.*, (1975) when they said "the shift of the 2-methoxy-group (of quassin) is normal...., the 12-methoxy-group is deshielded by *c.* 4 ppm, reminiscent of the deshielding effect on the methoxy-shift of anisoles by two ortho-substituents".

In the 'mixed multitude' of methoxycyclohexenes (1-methoxycyclohexenes, 3-methoxycyclohexenes and 3-methoxycyclohex-2-enones) in Table 3.2, there appears (without the knowledge of the shieldings in the simplest appropriate methoxycyclohexenes) to be increased shielding of the methyl-*C*s of the methoxy-groups with increased substitution. Although this statement stands in contrast to that above concerning the 2-methoxycyclohex-2-enone, there is a suggestion from Torri and Azzaro (1974) that 3-substituted cyclohex-2-enones differ from the 2-substituted ones. They reported that the methyl-*C* of 2-methylcyclohex-2-enone

(3.23)

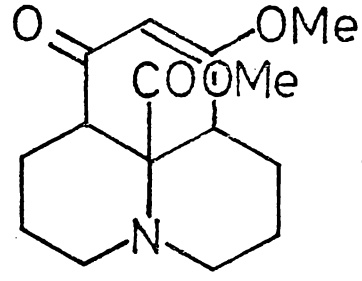


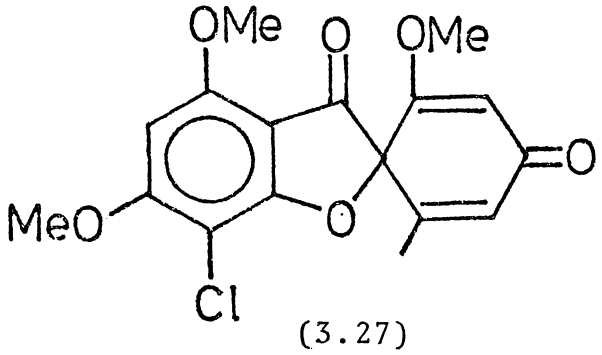




(3.25)

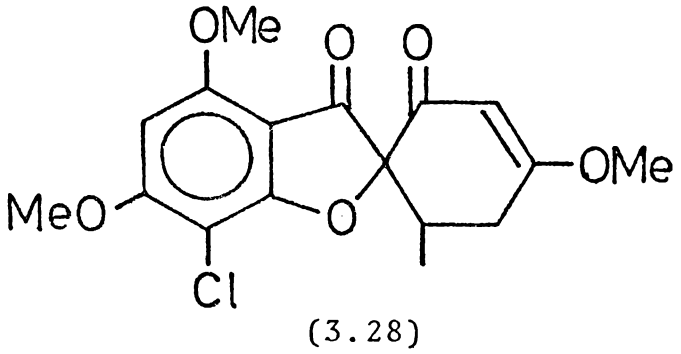
(3.24a) no $R^1$, $R^2$=OH
(3.24b) no $R^1$, $R^2$=H
(3.24c) $R^1$=H$^1$, $R^2$=OH
(3.24d) $R^1$=Me, $R^2$=OH




(3.26)




(3.27)



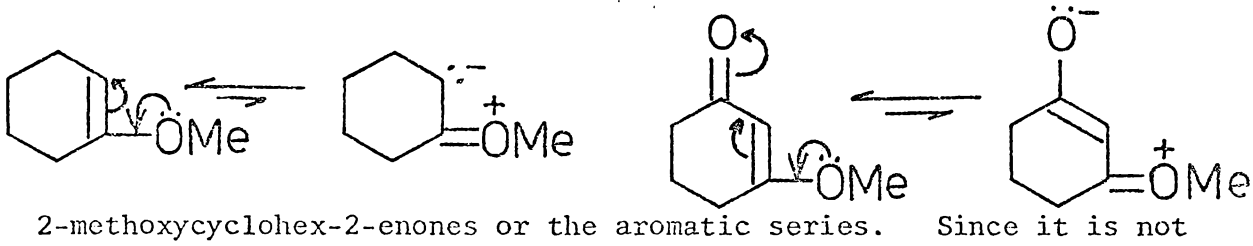

(3.28)

Table 3.2. The resonance frequencies of methoxy-*C*s in some methoxycyclohexenes

| Chemical shift (ppm) | Derivative or type of derivative | Structure | Cyclohexene ring structure and environment | Reference |
|---|---|---|---|---|
| 57.1 & 56.8 | Griseofulvin and Epigriseofulvin | (3.23a,b) | 3-Methoxy-5-methylcyclohex-2-enone linked [4-2'] spiro to a 3-coumaranone | Levine, *et al.*, 1975. |
| 56.9 — 56.7 | Tazettine and derivatives | (3.24a—d) | 3-Methoxycyclohexene fused [5,6-1',7a'] to a tetrahydropyrano[2,3-*c*]pyrrolidine | Crain, *et al.*, 1971. |
| 56.9 & 56.3 | Amaryllidaceous alkaloids | (3.20) (3.25) | 4-Hydroxy-3-methoxycyclohexene fused [1,6,5-1',8a',8'] to an indolizidine or [1,6-6',7'] to a 1-azabicyclo[3.2.1]octane resp. | Crain, *et al.*, 1971. |
| 56.8 — 55.4 | 10-Methoxy-10b-methoxycarbonyl-hydrojulolidines | (3.26) | 3-Methoxy-5-methoxycarbonylcyclohex-2-enone fused [4,5,6-1',9a',9'] to a quinolizidine | Wenkert, *et al.*, 1973a. |
| 56.7 | Dehydrogriseofulvin | (3.27) | 3-Methoxy-5-methylcyclohexa-2,5-dienone linked spiro [4-2'] to a 3-coumaranone | Levine, *et al.*, 1975. |
| 56.2 | Isogriseofulvin | (3.28) | 3-Methoxy-5-methylcyclohex-2-enone linked spiro [6-2'] to a 3-coumaranone | Levine, *et al.*, 1975. |
| 54.7 | Thebaine | (3.17a) | 1-Methoxycyclohexa-1,3-diene fused [4,5,6-12',5',6'] to a 2-aza-7-oxatricyclo-[6.3.1.$0^{5,9}$]dodec-9-ene | Terui, *et al.*, 1975a. |
| 54.2 | 8,14-Dehydro-thebaine | (3.17b) | 1-Methoxycyclohexene fused [4,5,6-12',5',6'] to a 2-aza-7-oxatricyclo[6.3.1.$0^{5,9}$]dodec-9-ene | Terui, *et al.*, 1975a. |

resonated at 16.1 ppm while that of the 3-methyl isomer at 24.4 ppm. Since the methyl-*C* of the methoxy-group of 2-methoxycyclohex-2-enone resonated at 53.7 ppm it could be argued that the one for the 3-methoxy-isomer would resonate *c.* 60 ppm.

For the 1-methoxycyclohexenes and the 3-methoxycyclohex-2-enones canonical structures can be written which are not possible for the



2-methoxycyclohex-2-enones or the aromatic series. Since it is not possible to write a canonical structure for the 3-methoxycyclohexenes, their methoxy-methyl-*C*s may belong to the same group as those for the 2-methoxycyclohex-2-enones.

Seeing that with aromatic compounds the carbon bearing the methoxy-group is completely substituted, further substitution can occur only on adjacent carbons. Stothers (1972a) lists the following alkanes in which an aliphatic methyl is on a methylene-*C* adjacent to the carbon bearing the increasing substitution: $MeCH_2\mathit{C}H_3$, 15.6 ppm; $EtCH_2\mathit{C}H_3$, 13.2; $Pr^iCH_2\mathit{C}H_3$, 11.5; $Bu^sCH_2\mathit{C}H_3$, 11.3; $Bu^tCH_2\mathit{C}H_3$, 8.7; and $Am^tCH_2\mathit{C}H_3$, 6.8 ppm, where shielding of the terminal methyl-*C* increases with increasing substitution at C-3.

There is a similar series of shieldings in the polymethyl benzenes but it arises from steric interactions between the methyl groups (Stothers, 1972e).

The conclusion drawn from this study is that, in general, the methoxy-methyl-*C*s resonate, in the aliphatic series, upfield (to *c.*48 ppm) from that of dimethyl ether (59.4 ppm) except for the 2- or 3-methoxy-methyl-*C*s of the 2- or 3-methylpyranosides (59.6— *c.* 63 ppm) and in the aromatic series downfield (to *c.* 66 ppm, when crowded) from that of anisole

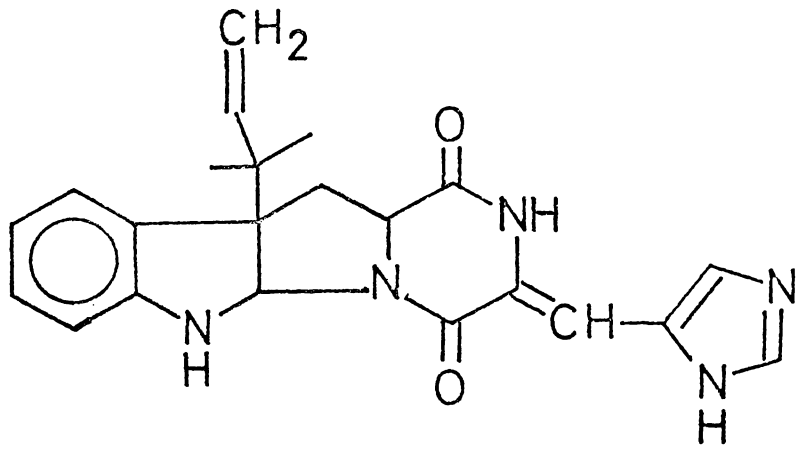
(54.7 ppm) except for some methylanisoles (2,5-dimethylanisole, 54.4 ppm). And further that the methoxy-methyl-*C*s of the sdms resonate at about the lowest field observed for methoxy-groups (not crowded by bulky alkyls).

The coupling constant for these (sdm-D) methoxy-groups (144.9 Hz) could be compared with that reported (Stothers, 1972ℓ) for methanol (142 Hz); the greater value in the former case may be attributed to the aromatic enhancement of the electronegativity of the oxygen atoms.

(b) *The Peaks from Quaternary and Methine Carbons, and Long-Range Coupling*

(i) *At 74.5 ppm (sdm) and 69.7 (s) (sdm-D) and at 77.7 ppm (sdm) and 73.1 ppm (s) (sdm-D).*-The intensity ratio of these two peaks in sdm was comparable with the ratio of the two in sdm-D but both peaks in going from sdm to sdm-D have shifted to higher field by a similar amount (4.8 and 4.6 ppm). Hence these two peaks were assigned to those quaternary carbons in the dioxopiperazine ring which were affected by the opening of the -S-S- bridge. These assignments were confirmed by long-range coupling (see below). The former (C-3, sdm-D) resonance was strongly $^{14}N$-quadrupole broadened ($W_{\frac{1}{2}}$ *c.* 14.6 Hz) while the latter (for C-11a) was less broadened ($W_{\frac{1}{2}}$ *c.*11 Hz). This broadening was indicative of adjacent nitrogen atoms (Levy and Nelson, 1972a).

Some dioxopiperazine ring systems (Deslauriers, *et al.*, 1975; Kakinuma and Rinehart, 1974; Ottnad, *et al.*, 1975) have been investigated and in each of the 9 synthesized rings the carbons (C-3 and C-6) are either a methylene or methine. The methylene-*C*, derived from glycine, resonates between 43.7 and 45.5 ppm; the methines with adjacent methylenes, derived from cystine or leucine, resonate between 53.8 and 54.5; those methines which, though adjacent to a methylene, are β to an aromatic group e.g. derived from tryptophan, phenylalanine or tyrosine, resonate between 56.7 and 57.3 ppm; and the methine adjacent to a methine,




(3.29)

derived from valine resonates at 60.8 ppm. Though the methine in tryptophan-dehydrobutyrine dioxopiperazine (3.2) is β to an indole it is deshielded (to 63.3 ppm by *c.* 6.5 ppm) by the adjacent *N*-methyl (Ellis and Jones, 1972) and by the strain imposed by the *exo*-double bond upon the configuration of the dioxopiperazine ring (referred to under a,iii above). In the neurotoxin, roquefortine (3.29), the corresponding methine is part of a 1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole ring system (as in the sdms) and is shielded (*c.* 20 ppm compared with sdm) to 58.8 ppm (Scott, *et al.*, 1976). The dioxopiperazine ring of this compound is also strained by an *exo*-double bond (at C-3) which contributes some deshielding to the methine. Although the methine is not adjacent to an *N*-methyl it is adjacent to an *N*-methine which might also contribute some deshielding. So the observed upfield shift for this methine (to 58.8 ppm) is attributed to the shielding effect of a fused hexahydropyrroloindole ring system. (Scott, *et al.*, 1976, have not indicated the conformation of the roquefortine molecule.) In the sdms these methine carbons were quaternary and were strongly deshielded (by 11—19 ppm). Although being quaternary deshielded them *c.* 2 ppm more than methines (as in 1,1,3-trimethylcyclohexane, Stothers, 1972c), the observed shift of C-11a (to 73.1 ppm, sdm-D) was accounted for by the deshielding effect of the adjacent sulphur atom and by being β to an oxygen atom (5—10 ppm, Stothers, 1972f) in a pyrrolidinediol. Alkylated sulphur atoms deshield adjacent methylenes by 0—6 ppm (DeSimone, *et al.*, 1974) compared with corresponding alkanes (Stothers, 1972a,b,i). Although the carbon, C-3, is not β to a hydroxy-group, nevertheless it was deshielded (to 69.7 ppm, sdm-D) from the frequency observed for the methine (54 ppm, Deslauriers, *et al.*, 1975) in the dioxopiperazine from leucine by being adjacent to an *N*-methyl (8—9 ppm, Ellis and Jones, 1972) and an S-methyl (0—5 ppm). But replacing the isobutyl group of leucine with the methyl group shields the carbon bearing this methyl by 4.6—5.6 ppm (going from $Bu^{i}CH_2Me$ to

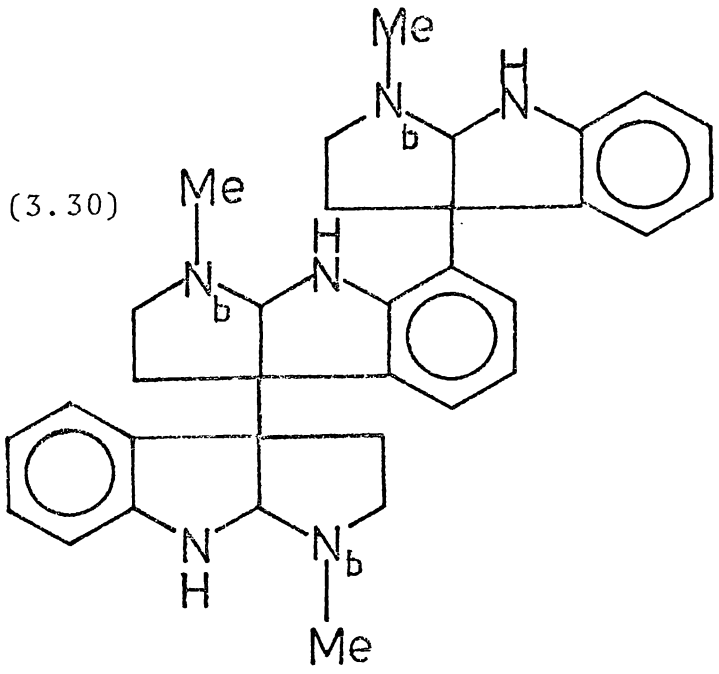
$MeCH_2Me$ shows a shielding of 4.6 ppm and from $Bu^iCH_3$ to $MeCH_3$, 5.6 ppm, Stothers, 1972a). The further deshielding was accounted for by the interaction of the two adjacent methyl groups, which in a Dreiding model were almost coplanar. The dioxopiperazine ring was rigidly held in the boat configuration by the planarity of the amido-groups.

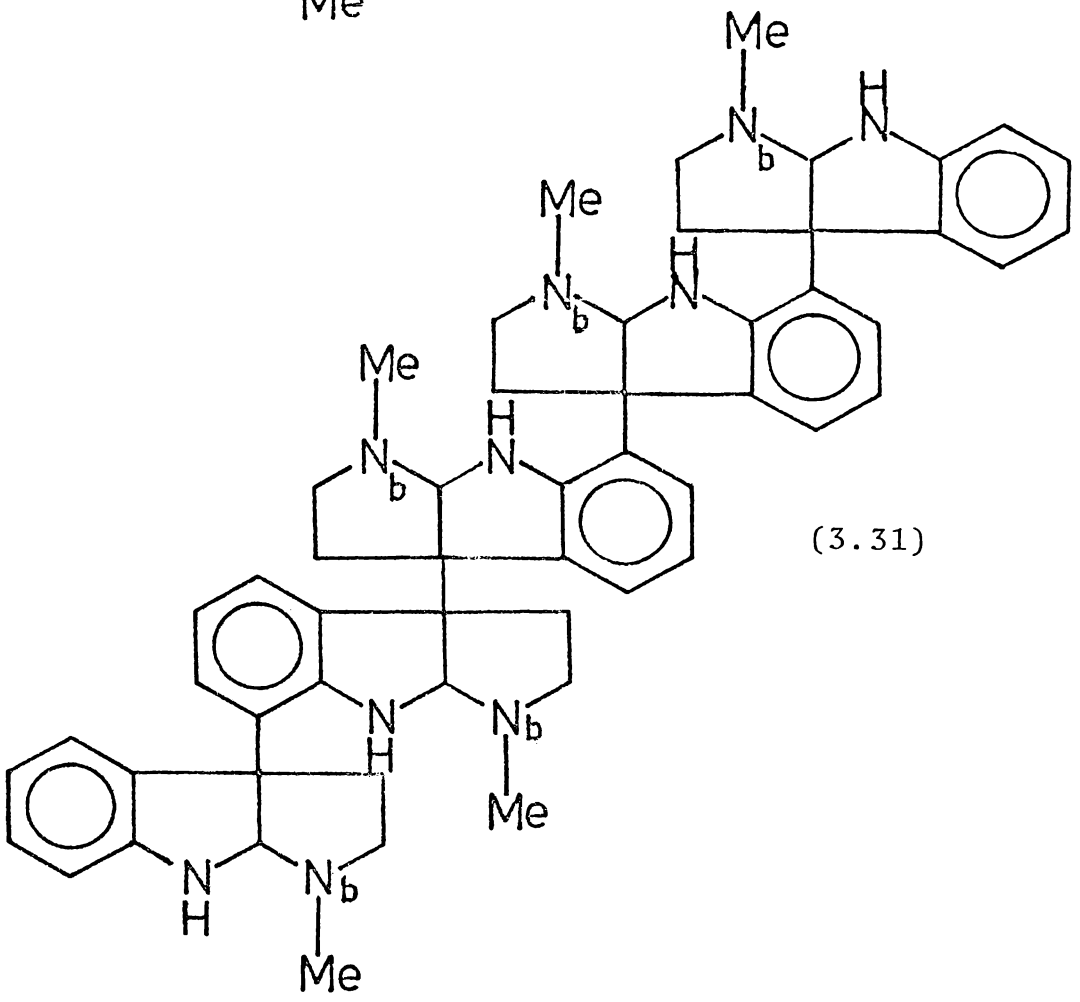
(ii) *At* 82.5 *ppm (sdm) and* 80.4 *ppm (d) (sdm-D)*, J 158.7 *Hz*.-In a list of secondary alcohols (Stothers, 1972g) the shieldings decrease from propan-2-ol (63.7 ppm) to 2,2,4,4-tetramethylpentan-3-ol (85.0 ppm) as the carbinyl groups become more sterically crowded with α-substitution; similarly with the carbinyl group in these sdms. On the one side the α-substitution was with an aromatic group (the OH on this carbon was *trans*) and on the other it was with a lactam carbonyl which was more sterically crowding in sdm than in sdm-D. In the 17β-hydroxy-steroids (testosterones) the carbinyl carbon chemical shifts are comparable (81.3 ppm) (Stothers, 1972m).

2-, 3- And 4-carbinyls in pyranoses (3.8) have coupling constants of 141—148 Hz (Bock and Pedersen, 1974). Because of the symmetry of the dioxopiperazine ring, the argument (see above), deduced from Haake, *et al.* (1964) for the influence of the electronegativity of the C-3 carbon upon the coupling constant of the methyl group, accounted for the coupling constant of the CH of this carbinyl group being 10 Hz larger.

(iii) *At* 90.4 *ppm (sdm) and* 90.3 *ppm (s) (sdm-D)*.-In the undecoupled spectrum of sdm-D this was a broad peak ($W_{\frac{1}{2}}$ *c.* 12 Hz), showing some multiplicity. It consisted of a quaternary carbon singlet with the upfield half of the split adjacent resonance partly superimposed upon it. The only carbon to which this singlet could be attributed was the remaining quaternary carbon C-10b (no appearance of nuclear Overhauser enhancement). This hydroxylated carbon was β to two nitrogens in a 1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole ring system. These two facts accounted for

(3.30)




(3.31)

the degree of deshielding. Applying the relationship between the shieldings of carbons α to methyl groups and the shieldings of carbons α to hydroxy-groups (Roberts, *et al.*, 1970), to the ring-junction quaternary C-9 of *trans*-9-methyldecalin (34.8 ppm, Dalling, *et al.*, 1973) (of comparable spatial arrangement but not ring size) gives *c.* 73 ppm. Hence the environment of C-10b was associated with special parameters which may appear in aspidophytine (3.3) (Yates, *et al.*, 1973). Here C-12 (57.3 ppm) is a quaternary bridgehead carbon to three rings of which only the indoline is strictly comparable with sdms. Though this carbon (in aspidophytine) bears a $CH_2CH_2N$ group instead of a methyl, yet applying the above relationship (Roberts, *et al.*, 1970) gives more than 90 ppm for the resonance of this carbon when hydroxylated.

(iv) *At* 95.3 *ppm (sdm) and* 96.0 *ppm (d)*, J 161.1 *Hz (sdm-D)*.-This resonance was attributed to the one remaining non-aromatic methine-*C* which occurred between two nitrogen atoms. For this reason it was at low field and was $^{14}N$-quadrupole broadened ($W_{\frac{1}{2}}$ *c.* 11 Hz) (Levy and Nelson, 1972a).

In aspidophytine (3.3) (Yates, *et al.*, 1973) the corresponding methine-*C*, which is adjacent to only one nitrogen and at a ring junction between an indoline and a cyclohexene, resonates at 72 ppm. This carbon could be deshielded, a few ppm, by the adjacent double bond in the cyclohexene ring. That the methine-*C* in the sdms was deshielded by 20 ppm more than that in aspidophytine was accounted for by the second adjacent nitrogen (a nitrogen deshields by *c.* 20 ppm, Levy and Nelson, 1972b) and by being β to a hydroxy-group (5—10 ppm, Stothers, 1972f).

In hodgkinsine (3.30) and psychotridine (3.31) which both possess the 1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole ring system as do the sdms, the methine-*C*s of the N-CH-N groups resonate between 82.2 and 83.1 ppm (Hart, *et al.*, 1974). β To each of these methines (resonating in this range) and influencing each is an adjacent hexahydropyrroloindole

ring system. An estimate of this influence, was obtained from the shieldings of a carbon which is in a side chain (at C-3a of the pyrrolo[2,3-*b*]pyrrole rings) and which is γ to the methine as, for example, in roquefortine (3.29) (Scott, *et al.*, 1976). The appropriate carbon here is an olefinic methine in a 1,1-dimethylprop-2-enyl group and it resonates at 143.4 ppm. This is 6 ppm shielded from that of the corresponding olefinic-methine carbons in neohexene (149.5 ppm, Johnson and Jankowski, 1972) and 3-methylbutene (148.4 ppm, Stothers, 1972d). Therefore, it is considered that each methine in hodgkinsine and psychotridine is similarly shielded since each one bears the same relationship to a 1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole ring system, hence this methine in 1-methyl-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole would be expected to resonate at *c.* 6 ppm downfield from those in hodgkinsine and psychotridine (i.e. *c.* at 89 ppm). In sdm and sdm-D this methine-*C* was β to a hydroxy-function which deshielded it by a further 5—10 ppm (Stothers, 1972f). In hodgkinsine and psychotridine the methines are adjacent to $N_b$-methyls which condition was comparable with that in the sdms where the methines were adjacent to $N_a$-methyls. Amido-carbonyl oxygens have strong shielding effects upon their adjacent *N*-methyl carbons (as observed under a,iii) but apparently not so upon adjacent *N*-methine carbons in rings as in 1,3-dimethyluracil (143.2 ppm) and 2-pyridone (141.6 ppm, Johnson and Jankowski, 1972) compared with that in bicyclo-[2,2,1]hepta-2,5-diene (143.3 ppm, Johnson and Jankowski, 1972).

Further, in roquefortine (3.29) the N-CH-N methine-*C* resonates at 78.3 ppm. In a series of olefins (*cis*-hex-2-ene, hept-2-ene, oct-2-ene and oct-1-ene, Johnson and Jankowski, 1972) the carbon γ to the double bond (C=C) resonates at the same (within 1 ppm) ppm as that of the corresponding alkanes (Stothers, 1972a). When a methyl group is substituted for the dimethylpropanyl group in 3,3-dimethylpentane the resonance of the carbon β (6.8 ppm, Stothers, 1972a) to the dimethyl-

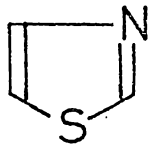
propanyl group is deshielded by *c.* 9 ppm (to 15.6 in propane, Stothers, 1972a). So it was calculated that the methine-*C* would resonate at *c.* 87 ppm (78 + 9) if the dimethylpropenyl group of roquefortine was replaced by a methyl group. Further, substitution with a hydroxy-group at the same position would deshield the methine even further (5—10 ppm, Stothers, 1972f). Then finally, the methyl group on the adjacent nitrogen in sdm would deshield the methine by another (*c.*) 9 ppm (α carbons in pyrrolidine, 47.1 ppm, and in *N*-methylpyrrolidine, 56.7 ppm, Levy and Nelson, 1972b,c).

Hence it is not surprising that this methine in sdm and sdm-D resonated so far downfield (95.3 and 96.0 ppm compared with 78.3 ppm in roquefortine).

This methine proton was between two nitrogen atoms, only one of which (*N*-methyl) had lone pair electrons; the other was a lactam nitrogen. The CH coupling constant for methylamine is *c.* 132 Hz (Stothers, 1972ℓ) or 137 Hz for diaminomethylenes (Levy and Nelson, 1972d), reflecting the nitrogen electronegativity, but in the sdms the proton was rigidly held in approximately the same plane (Dreiding model) as the lone pair of electrons on the indoline-*N*, hence the large value (Bock and Pedersen, 1974) of 161 Hz. The proximity of the equatorial H-1 to the lone pairs on the pyranoid-ring oxygen atom, in α-anomers of hexopyranoses (Bock and Pedersen, 1974), makes the coupling constant 7—8 Hz greater than that of the axial hydrogen atom.

(v) *Long-range spin-spin coupling.*-In the undecoupled spectrum of sdm-D there were three non-aromatic resonances which showed long-range splittings, two doublets of 3.7 and one of 2.4 Hz. These occurred on the *N*-methyl quartet (41.3 ppm), the pyrrolo[1,2-*a*]pyrazine quaternary carbon singlet (73.1 ppm) and a carbonyl (165 ppm) singlet (resp.). Each of these carbons was three bonds removed, through a nitrogen atom,

from the same methine proton (5a) which was itself between two nitrogen atoms. There were two opposing factors affecting the value of these long-range coupling constants. One factor was that $^{3}J_{HCNC}$ values in a planar five-membered ring are about 15.5 Hz, e.g. thiazole (3.32)



(3.32)

(Bojesen, *et al.*, 1971). Against this large value was the fact that in a Dreiding model these C-N-C-H groups have a dihedral angle of either $120^{o}$ or $60^{o}$ (approx.) which by Conroy's curve (Conroy, 1960) would reduce the coupling constant to about a quarter of the value expected in a planar molecule. There were two more C-N-C-H groups both influencing the same aromatic-*C* (141.0 ppm, C-6a). One group was CNCH and the other $CNCH_3$, but the resonance (141.0 ppm) was broad ($W_{\frac{1}{2}}$ *c.* 7 Hz) and unresolved. The 2-Me protons were in a $^{3}J_{CNCH_3}$ relationship to two carbons, C-3 (69.7 ppm) and C-1 (167.1 ppm), but both these resonances showed $^{14}N$-quadrupole broadening, hence in the former no splitting pattern was observed while in the latter there was only a suggestion of it.

In addition to the *N*-methyl being split by a methine proton there was also the methine carbon resonance (96 ppm) being split into an unsymmetrical poorly defined quartet by long-range coupling to the hydrogens of the *N*-methyl group (at 41.3 ppm): $^{3}J_{H_3CNC}$ *c.* 3.7 Hz.

There were two other carbon nuclei (the CHOH and the aromatic carbon 10a) which bore the same spacial relationship to the methine proton (5a) but the grouping was H-C-C-C and the splitting was not observable (i.e. it was less than 1 Hz). Similarly with the same groupings centred upon the proton nuclei at C-10 and C-11, the former could be expected to split the resonance of carbon C-10b and the latter those of carbons C-1, C-5a and C-10a, but likewise no splitting appeared.

(c) *The Aromatic Peaks and Their Long-Range Couplings*

Because of additivity it is interesting to note the average shieldings for the six aromatic carbons: in benzenes, 128.7 ppm (Stothers, 1972j); in tabersonine (3.5a) and four 1-methyl-2,16-dihydrovincadifformines (and derivatives) (3.5b—e) (indolines without aromatic substitution), 127.1±0.5 ppm (Lukacs, *et al.*, 1974); in six *Aspidosperma* alkaloids (3.10-3.13, 3.15, 3.16) (indolines with 6-methoxy-, 5,6-dimethoxy- or 6-methoxy-1-methyl substitution), 126.6±0.4 ppm (Wenkert, *et al.*, 1973b); in a series of eight 3-methoxymorphinanes (3.9a—h), six codeines (3.18a—f) and two thebaines (3.17a,b) (6-methoxy-1,2,3,4-tetrahydronaphthalenes with or without an ether-linked ring at position 5), 129.7±0.4 ppm (Terui, *et al.*, 1975a); in aspidophytine (3.3) and haplophytine (3.4) (6,7-dimethoxy-1-methylindolines), 134 and 135.7 ppm (resp.) (Yates, *et al.*, 1973); and for sdm, 134.1 and sdm-D, 134.6 ppm. It appears that a 1,2-dimethoxy-3-methylamino-grouping on an aromatic ring or a 1-methyl-6,7-dimethoxyindoline has an overall deshielding effect upon the aromatic-*C* resonances.

Stothers (1972j) and Levy and Nelson (1972f) together listed a number (29) of aryl-carbon shieldings for monosubstituted benzenes in which, in general, those compounds whose additivity showed shielding, had activating groups (Packer and Vaughan, 1958) except phenylcyanate, phenylcyanide, trifluoromethylbenzene and iodobenzene while those whose additivity showed deshielding had deactivating groups except phenolate ion, diphenyl and alkylbenzenes. That the additivity in sdm showed deshielding and, that deshielding may be indicative of deactivation, may account, in part, for the failure to achieve diazonium coupling at the aromatic hydrogen of sdm (Chaper 2).

On considering that in sdm-D the sole aromatic proton, adjacent to C-Cl, had a coupling constant of 166.0 Hz, i.e. 8.5 Hz greater than in

benzene, and that Tarpley and Goldstein (1971) working on *o*-dichlorobenzenes observed the coupling constant for the methine adjacent to a C-Cl as 166.5 Hz, it is suggested that in the aromatic ring, the chlorine atom has a strong influence on the coupling constant of an adjacent CH group. This influence extended further because (1) there was a well defined long-range coupling (at 119.1 ppm) of 3.7 Hz comparable with the 3.5 Hz recorded by Tarpley and Goldstein for the (Cl)$^{13}$CCH group, and (2) in sdm-D there was an unsplit aromatic-quaternary-carbon resonance (128.3 ppm) similarly comparable with Tarpley and Goldstein's record of a negligible coupling constant (0.02 Hz) for the $^{13}$CCH(CCl) group. Hence these three peaks (i)—(iii):

(i) *At* 118.8 *ppm (sdm) and* 119.1 *ppm (sdm-D).*-These were attributed to the carbon bearing the chlorine atom. This resonance was shielded by 9.6 ppm (from $C_6H_6$); the summation of the shielding (15.5 ppm, Stothers, 1972j) by an *o*-methoxy-group together with the deshielding (6.4 ppm, Stothers, 1972j) effect of the chlorine atom totaled 9.1 ppm. The closeness of the calculated result may be fortuitous. The same calculation does not apply in 1-chloro-2,4-dimethoxy-5-nitrobenzene (difference 5.8 ppm), 5-chloro-2,4-dimethoxyaniline (difference 5.8 ppm) and 2-chloro-4-methoxyanisole (difference 3.4 ppm) (Johnson and Jankowski, 1972).

(ii) *At* 121.0 *ppm (sdm) and* 121.8 *ppm (d),* J 166.0 *Hz (sdm-D).*-These were unambiguously attributed to the sole aromatic CH group (nuclear Overhauser enhancement). It was shielded by 7 ppm (from $C_6H_6$) by being *para* to a methoxy-group (8.9 ppm, Stothers, 1972j). In 2-(3,4-dimethoxyphenyl)ethylamine the corresponding carbons resonate at 120.7 ppm (Johnson and Jankowski, 1972).

(iii) *At* 126.9 *ppm (sdm) and* 128.3 *ppm (s) (sdm-D).*-These were assigned to the ring-junction carbon (C-10a). The small shielding (by 1.4 ppm) in sdm arose from reduced steric crowding by the secondary

hydroxy-group (at C-11) when the dioxopiperazine ring was strained by the -S-S- bridge. In a number of indolines with or without 6- and/or 7-methoxy-substitution (3.3, Yates, *et al.*, 1973; 3.5 a—e, Lukacs, *et al.*, 1974; 3.10, 3.11, Wenkert, *et al.*, 1973b) their C-3a carbons (corresponding to C-10a) resonate at 130 ppm or more (except in the indoline alkaloids haplophytine, (3.4, and vindoline and derivatives, 3.12, 3.13) but when there is substitution in the 5-position (MeO, 3.16 or Cl) then C-3a is shielded to *c.* 128 ppm.

(iv) *At* 140.8 *ppm (sdm) and* 141.0 *ppm (sdm-D).*-Of the three remaining aromatic resonances this one (in sdm-D) showed $^{14}N$-quadrupole broadening (Levy and Nelson, 1972a) ($W_{1/2}$ *c.* 7.3 Hz) and no resolution; therefore it was attributed to the ring-junction carbon (C-6a) bearing the NMe.

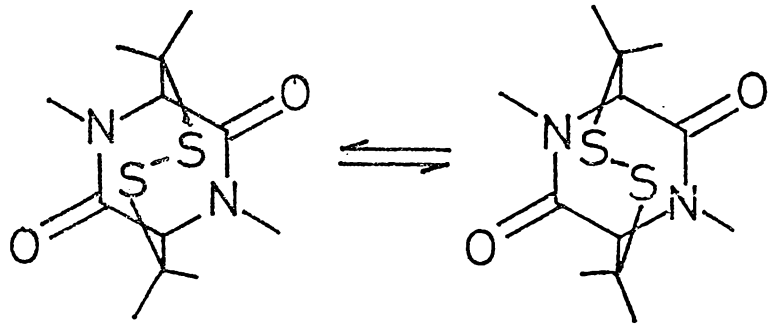
(v) *At* 145.8 *ppm (sdm) and* 146.1 *ppm (sdm-D).*-This resonance showed two quartets ($^3J_{COCH}$ 6.1 Hz) separated by ($^4J_{CCCCH}$) 2.4 Hz so it was attributed to the methoxy-carbon (C-7).

(vi) *At* 151.6 *ppm (sdm) and* 151.3 *ppm (sdm-D).*-Here similarly, this resonance showed to quartets ($^3J_{COCH}$ 7.3 Hz) separated by ($^3J_{CCCH}$) 3.7 Hz so it was assigned to the methoxy-carbon (C-8).

The two adjacent methoxy-groups had a mutual shielding effect (15.5 ppm, Stothers, 1972j) upon their α-ring carbon resonances. The adjacent (to the 7-OMe) *N*-methyl group had an additional shielding effect (15.6 ppm) on C-7. The CCl group on the other hand had a negligible (0.2 ppm) deshielding effect upon the resonance of C-8, hence C-7 was shielded more than C-8.

(d) *The Carbonyl Resonances of the Lactams*

(i) *At* 164.9 *ppm (sdm) and* 165.0 *ppm (sdm-D).*-Though showing $^{14}N$-quadrupole broadening ($W_{1/2}$ *c.* 7.3 Hz), this resonance was clearly split into a doublet ($^3J_{CNCH}$ 2.4 Hz) as discussed above. Hence this resonance was attributed to the 4-CO group in the sdms.



P-helical

(3.33a)

M-helical

(3.33b)

(ii) *At* 166.7 *ppm (sdm) and* 167.1 *ppm (sdm-D).*-This peak also showed $^{14}N$-quadrupole broadening ($W_{\frac{1}{2}}$ *c.* 8.5 Hz). It was unsymmetrically split (3.6 Hz) and the spectrum showed a suggestion of splitting into a quartet as would be expected from the protons of the methyl group on the adjacent nitrogen. The strain applied to this 1-CO by the -S-S- bridge in sdm did not have a profound effect (0.4 ppm) nor was there much change in orientation in a Dreiding model.

Deslauriers, *et al.* (1975) synthesized a number of symmetrical and unsymmetrical dioxopiperazines and reported the $^{13}C$ carbonyl shieldings to be between 167.2 and 170.2 ppm (dimethylsulphoxide-$d_6$). In the same solvent the dioxopiperazine from cystine shows two resonances at low temperature and only one above 40° (Ottnad, *et al.*, 1975). The main resonance is at 170.7 ppm while the minor one (for the M-helicity) is at 166.7 ppm. These two helicities (3.33a,b) arise from the two arrangements that the $-CH_2-S-S-CH_2-$ bridge can assume. In all the above dioxopiperazine rings the 3-C and the 6-C are both methines (or methine and methylene) which give the carbonyls a different environment from that in the sdms. Nevertheless the carbonyls of the sdms, of tryptophan-dehydrobutyrine dioxopiperazine (3.2) and the M-helical cystine dioxopiperazine resonated within a range (160.5-167.1 ppm) which is very little shielded from that of the above (synthesized) dioxopiperazine carbonyls. Furthermore, in a list of amides (Levy and Nelson, 1972h) the carbonyls of the sdms resonated at frequencies between (more or less) those of the deshielded formamides (163.4 ppm, except formamide itself, 165.5 ppm and *trans-N*-methylformamide, 166.7 ppm) and the shielded acetamides (169.1 ppm).

*Conclusions*

From studying the undecoupled spectrum of sdm-D it was possible to assign the $^{13}C$ peaks in the decoupled spectra of both sdm-D and sdm. The long-range coupling especially in the aromatic ring and that through

hetero-atoms was particularly helpful.

Had these complex molecules been of unknown structure then the elucidation of their structures from the $^{13}C$ n.m.r. spectra would have been difficult, e.g. the highly deshielded position of the diazamethine-*C* (95–96 ppm) was anomalous as explained above, since it resonated at the same position as the β-olefinic-*C* in vinyl acetate (Levy and Nelson, 1972e).

The relaxation of strain was clearly manifested in the differences between the spectra of the strained epidithiodioxopiperazine ring (sdm) and the opened -S-S- bridge (sdm-D), indicated by the dotted lines (Fig. CM3.1) between the two spectra. This is valuable in understanding the sdm molecule but such shifts in an unknown could not be readily recognized till a strained -S-S- bridge had been established.

# CHAPTER 4. SPORIDESMIN-E, ITS SYNTHESIS, AND COMPARISON OF ITS INFRA-RED AND $^{13}C$ NUCLEAR MAGNETIC RESONANCE SPECTRA WITH THOSE OF SPORIDESMIN

*Synthesis*

Rahman, *et al.* in 1969 isolated another sdm analogue from cultures of *Pithomyces chartarum*. This analogue had three sulphur atoms ($C_{18}H_{20}ClN_3O_6S_3.C_4H_{10}O$) instead of the two as in sdm: they named the compound sdm-E (1.10). They also synthesized it from sdm, by heating the latter for two min with sulphur (13 atom equivalents) and phosphorus pentasulphide (2 equivalents) in carbon disulphide (yield 36%). In 1970, Murdock and Angier converted acetylaranotin (1.2b) to the epitrithio-compound by simply warming (25°) acetylaranotin with elemental sulphur (1 atom equivalent) in pyridine. The synthetic method (A-B3) adopted in this work was the latter (which is new for the sdm series) with the same observation that Murdock and Angier made that the epitetrathio-compound (sdm-G) (1.17) also formed; the yield of crystals was 92% (A-B3iii). Column chromatography separated unreacted sdm and sdm-G from sdm-E, after which the sdm-E etherate crystallized freely. But, though carefully prepared and crystallized in subdued light, two-dimensional chromatography always yielded as many as three spots, two of them minor ones (sdm and sdm-G).

The study of this compound was taken up because it had been claimed (Brewer, *et al.*, 1968) that sdm-E was 10 times more toxic than sdm. It was known that sdm as prepared by the method of Ronaldson, *et al.* (1963) contained *c.* 5% of sdm-E. The experimental pathologists needed sdm-E in order to check that it was so toxic and for this purpose they required it as pure as possible similarly sdm for comparison was also required pure (A-A).

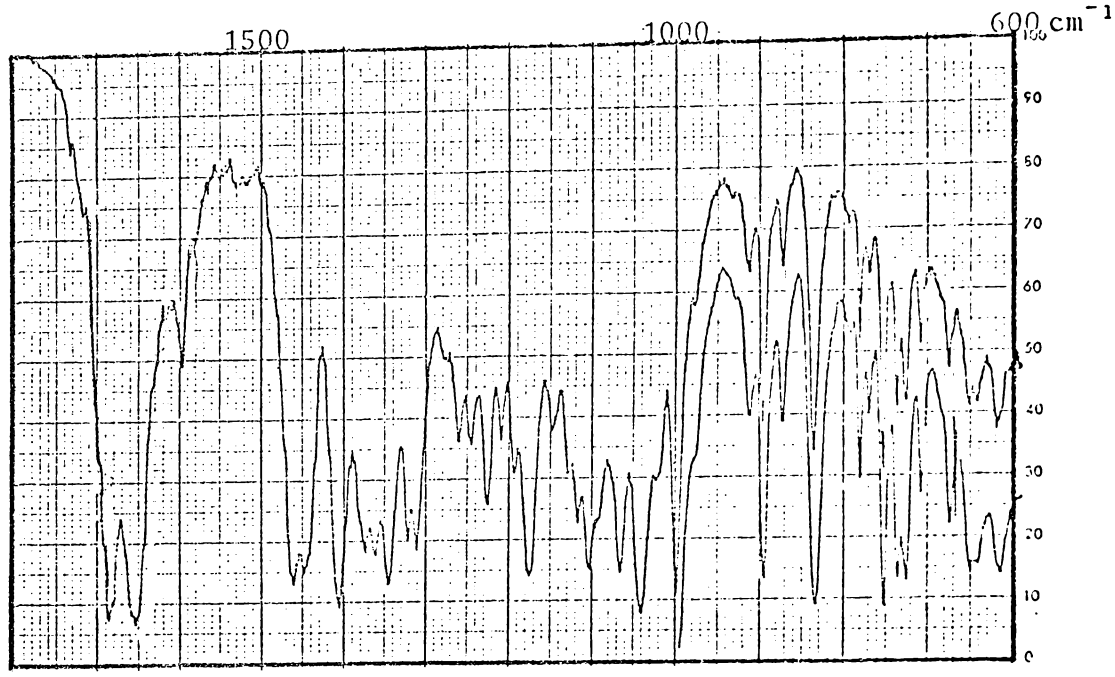



Fig. IR4.1. The i.r. spectrum of sporidesmin-E.EtOEt in KBr ground with ether (A-B3iii).

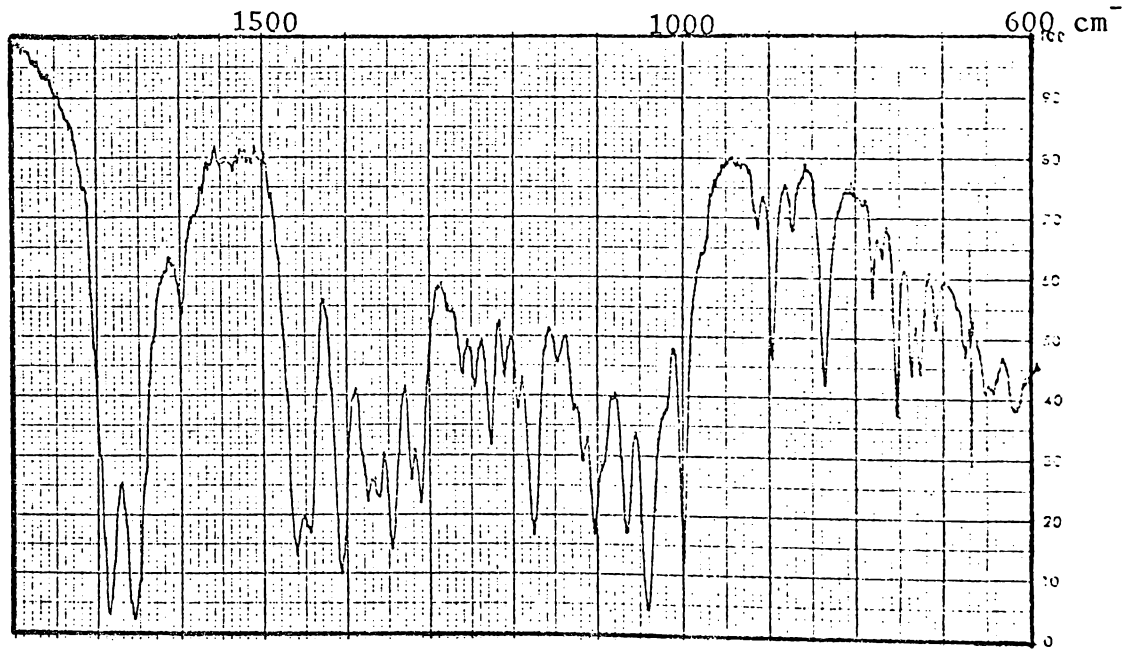



Fig. IR4.2. The i.r. spectrum of authentic sporidesmin-E.EtOEt received from A. Taylor, and prepared as for IR4.1.

*Properties*

From a chemical point of view this compound, sdm-E, was enigmatic as the following discussion demonstrates.

The parent peak for the mass spectrum of the crystals, sdm-E.EtOEt, showed the expected values 507 and 505 (A-B3iv) for the two chlorine isotopes. The mass spectra of both sdm and sdm-E were mainly the same (there were minor differences) except that sdm-E clearly showed the stepwise loss of each of the three sulphur atoms down to *m/e* 409 and a peak at *m/e* 256 equivalent to $S_8$. Sdm, on the other hand, showed only the loss of $S_2$ from the parent peak (*m/e* 473) to *m/e* 409 and no $S_8$ peak.

The optical rotation, $[\alpha]_D$ ($-166^o$, *c* 0.06 in $CHCl_3$), was comparable with that ($-132^o$, *c* 0.064 in $CHCl_3$) reported by Rahman, *et al.* (1969) and considerably different from that ($+6.9^o$, *c* 1.4 in $CHCl_3$) for sdm (Ronaldson, *et al.*, 1963). The melting point presented a problem: Rahman, *et al.* (1969) quoted sdm-E.EtOEt, m.p. $180^o$ (from ether) whereas it was found that sdm-E.EtOEt melted at 144.5–$148^o$. In case the ether could be driven off readily at the efflorescent point (125–$135^o$) sdm-E was maintained at that temperature (45 min) to allow it to recrystallize (A-B3iv): instead it liquified and decomposed. By dissolving sdm-E (as a gum) in a minimum of ethyl acetate and adding acetic acid, sdm-E free of solvent of crystallization was obtained and it melted at 181–182.5$^o$ (lit. 180–185$^o$) (A-B3iv). The melting point of this unsolvated sdm-E after storing in a refrigerator for three months was altered only slightly (179–182.5$^o$). The melting point of a mixture of this sample with unsolvated sdm showed no depression (178.5–182.5$^o$) so the melting point could not be relied on as an indicator of purity.

*Infra-red Spectra* (A-B3iii,iv)

The i.r. spectrum (KBr) of sdm-E.EtOEt (ground in EtOEt) (Fig. IR4.1) was identical with that of an authentic sample of sdm-E.EtOEt (also ground

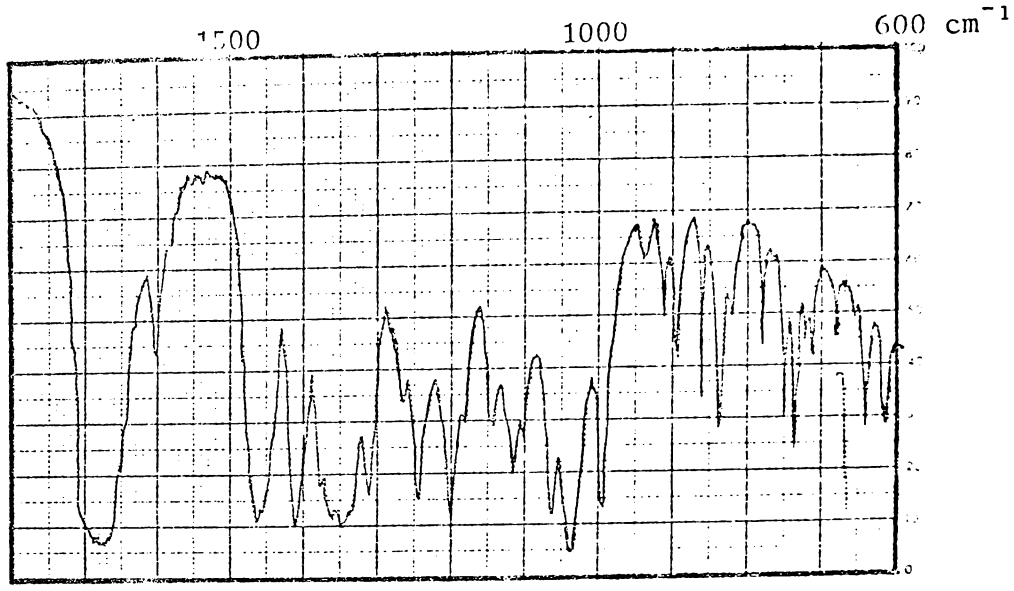



Fig. IR4.3. The i.r. spectrum of unsolvated sporidesmin in KBr ground with ether.

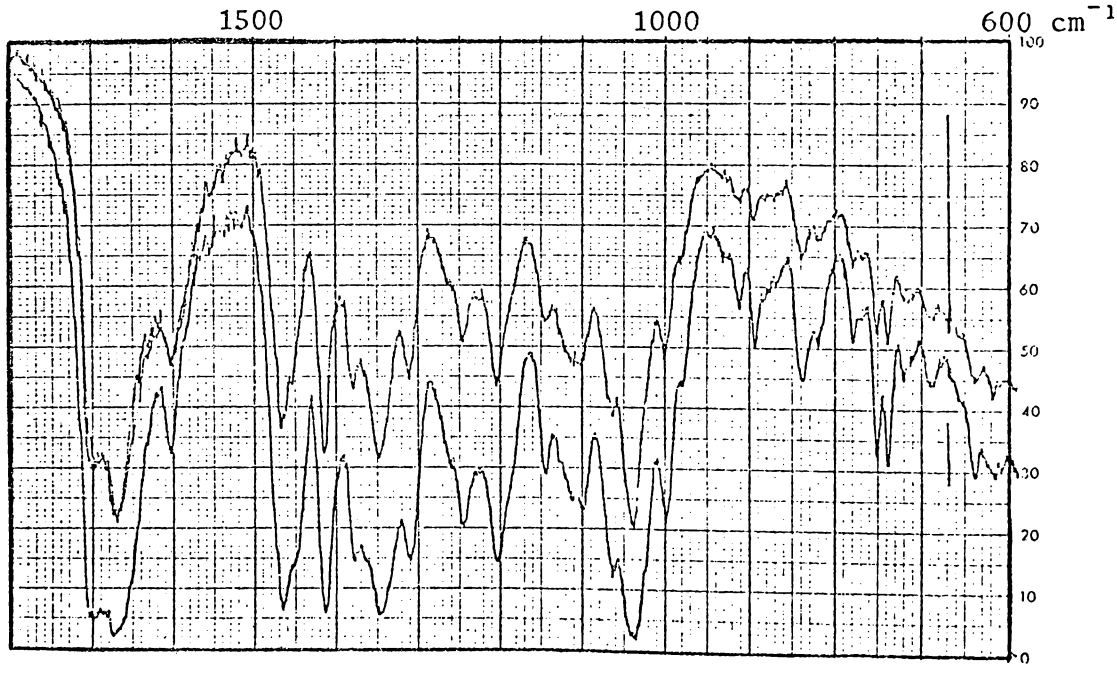



Fig. IR4.4. The i.r. spectrum of sporidesmin.$C_6H_6$ in KBr ground with methanol.

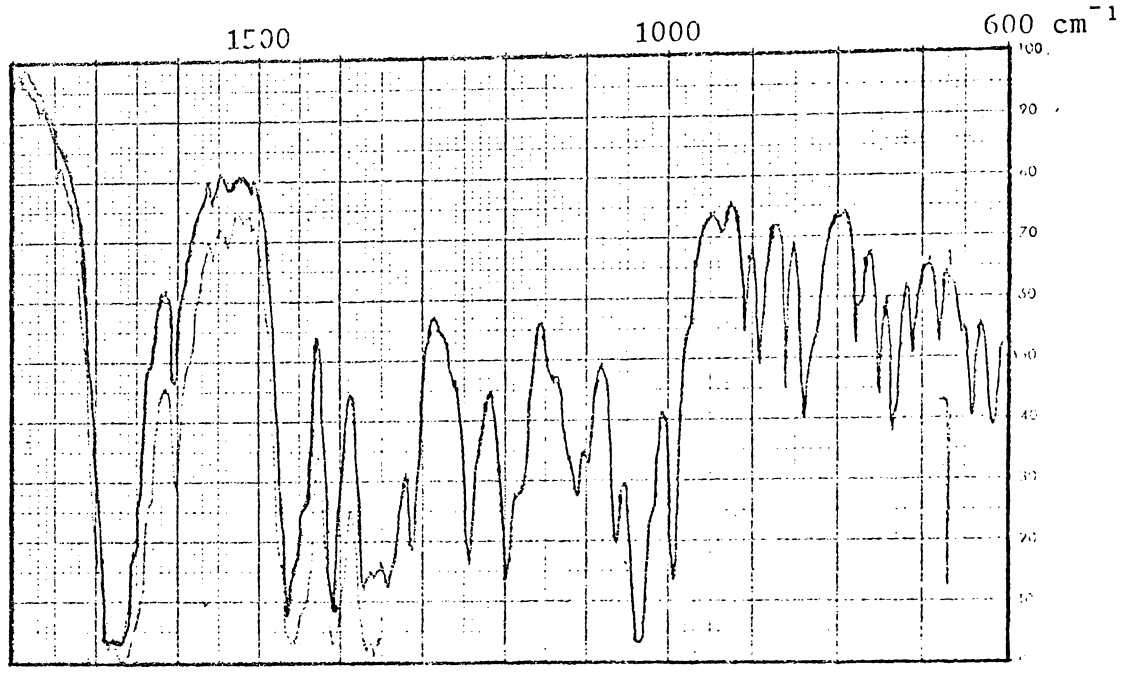



Fig. IR4.5. The i.r. spectrum of unsolvated sporidesmin-E in KBr ground with ether.

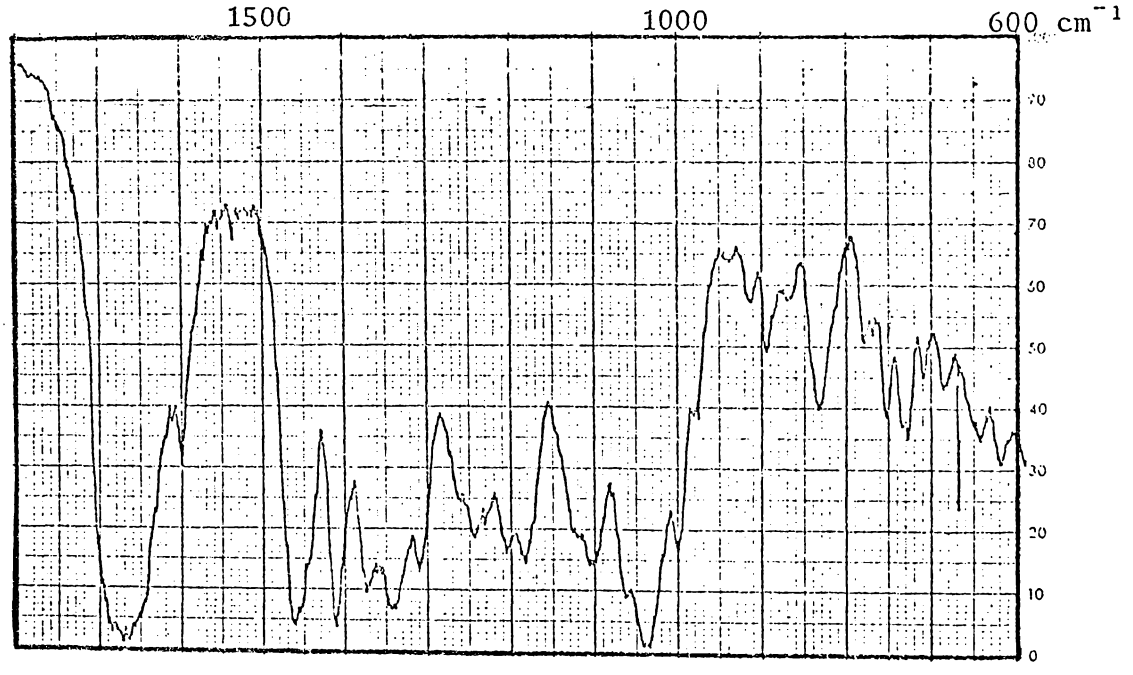



Fig. IR4.6. The i.r. spectrum of sporidesmin-E.EtOEt in KBr ground with methanol.

in EtOEt) (Fig. IR4.2), obtained from A. Taylor, of Safe and Taylor (1971). (Samples for i.r. spectroscopy were usually ground in MeOH.)

When unsolvated sdm was ground with potassium bromide in the presence of ether the spectrum (Fig. IR4.3) was different from that obtained when sdm was ground in the presence of methanol (Fig. IR4.4) (perhaps an etherate of sdm forms). Sdm ground in the presence of methanol did not form a methanolate seeing that unsolvated sdm was obtained from methanol-water. Furthermore, this (ex EtOEt) spectrum of sdm (Fig. IR4.3) was markedly different from that of sdm-E.EtOEt (Fig. IR4.1) prepared the same way. But the spectrum of unsolvated sdm-E ground in the presence of ether (Fig. IR4.5) was also markedly different from that obtained from the solvate, sdm-E.EtOEt, (Fig. IR4.1) and by contrast, little different from that for unsolvated sdm, ex ether (Fig. IR4.3). This suggests that the formation of solvated (EtOEt) sdm-E from unsolvated sdm-E was a slow reaction. The differences between the i.r. spectra of unsolvated sdm and sdm-E (both ex ether) (Figs IR4.2, IR4.4) were: the carbonyl shoulder in sdm (1700 $cm^{-1}$) was absent in sdm-E; the small peak (1380 $cm^{-1}$) in sdm was shifted (1375 $cm^{-1}$) and stronger; the peak at 1270 $cm^{-1}$ in sdm was absent in sdm-E; the medium peak at 1140 $cm^{-1}$ as almost absent in sdm-E; the weak peak at 820 $cm^{-1}$ was reduced to a shoulder in sdm-E; a weak peak at 770 $cm^{-1}$ in sdm-E was absent in sdm; the medium singlet at 737 $cm^{-1}$ was split into a medium doublet (735 and 730 $cm^{-1}$) in sdm-E; and the weak doublet at 723 and 714 $cm^{-1}$ in sdm appeared as a singlet in sdm-E at 710 $cm^{-1}$.

When sdm-E.EtOEt was ground in the presence of methanol (Fig. IR4.6), the spectrum was again only little altered from that of sdm (Fig. IR4.4). The following minor differences were observed: the strong shoulder at 1600 $cm^{-1}$ in sdm was a weak shoulder in sdm-E; the strong peak at 1350 $cm^{-1}$ in sdm was weaker (relative to that at 1410 $cm^{-1}$) in sdm-E and shifted to 1340 $cm^{-1}$; the strong peak at 1205 $cm^{-1}$ in sdm

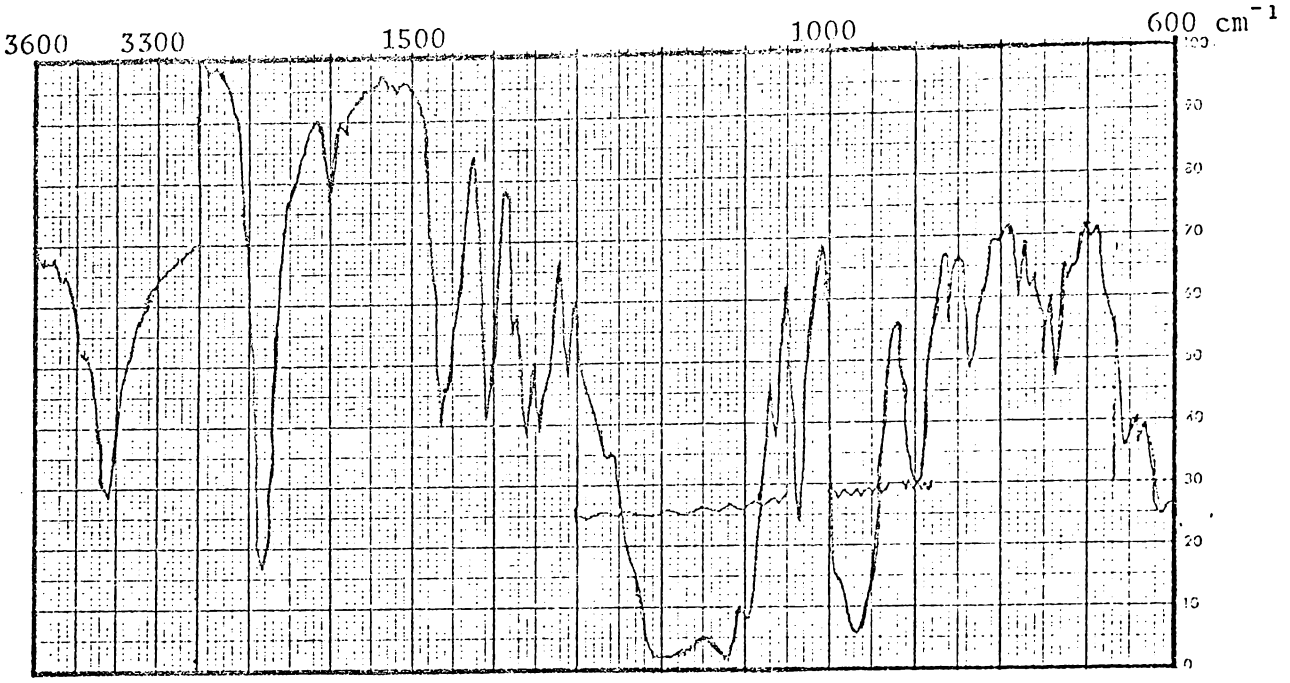



Fig. IR4.7. The i.r. spectrum of unsolvated sporidesmin ground in halocarbon oil.

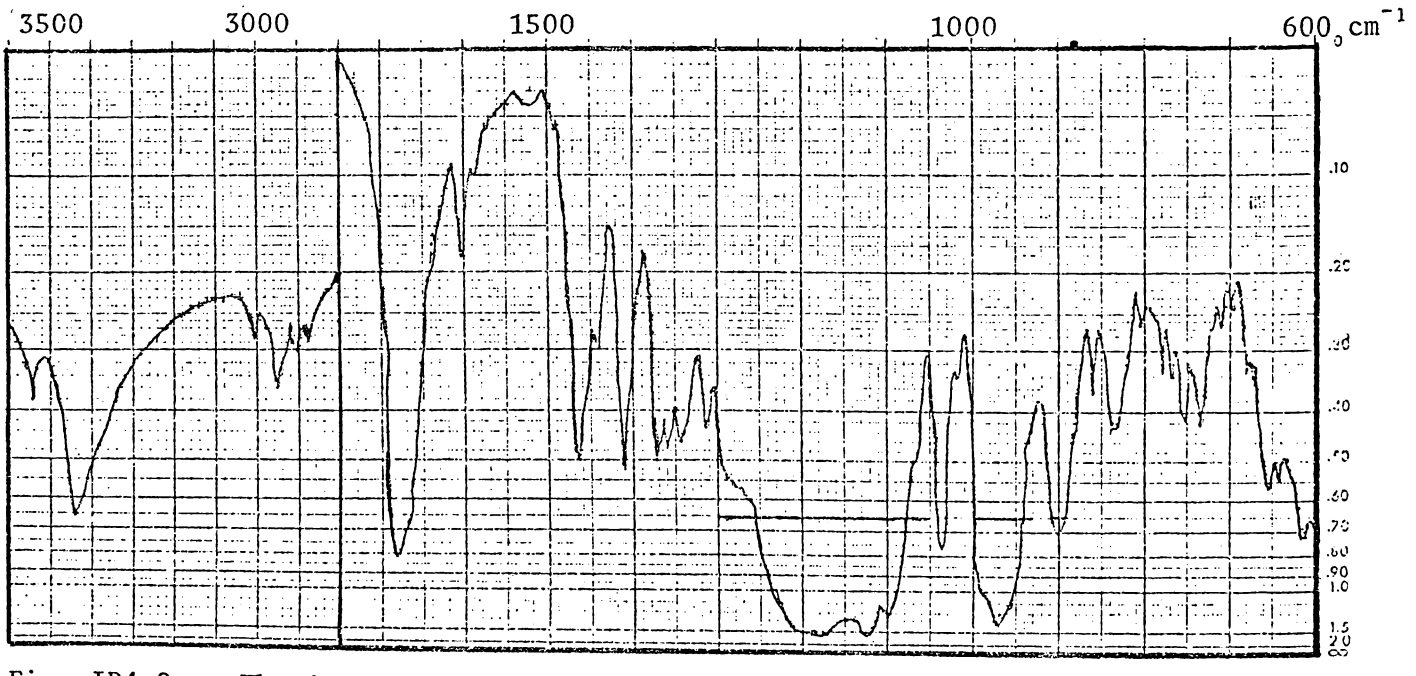



Fig. IR4.8. The i.r. spectrum of unsolvated sporidesmin-E ground in halocarbon oil.

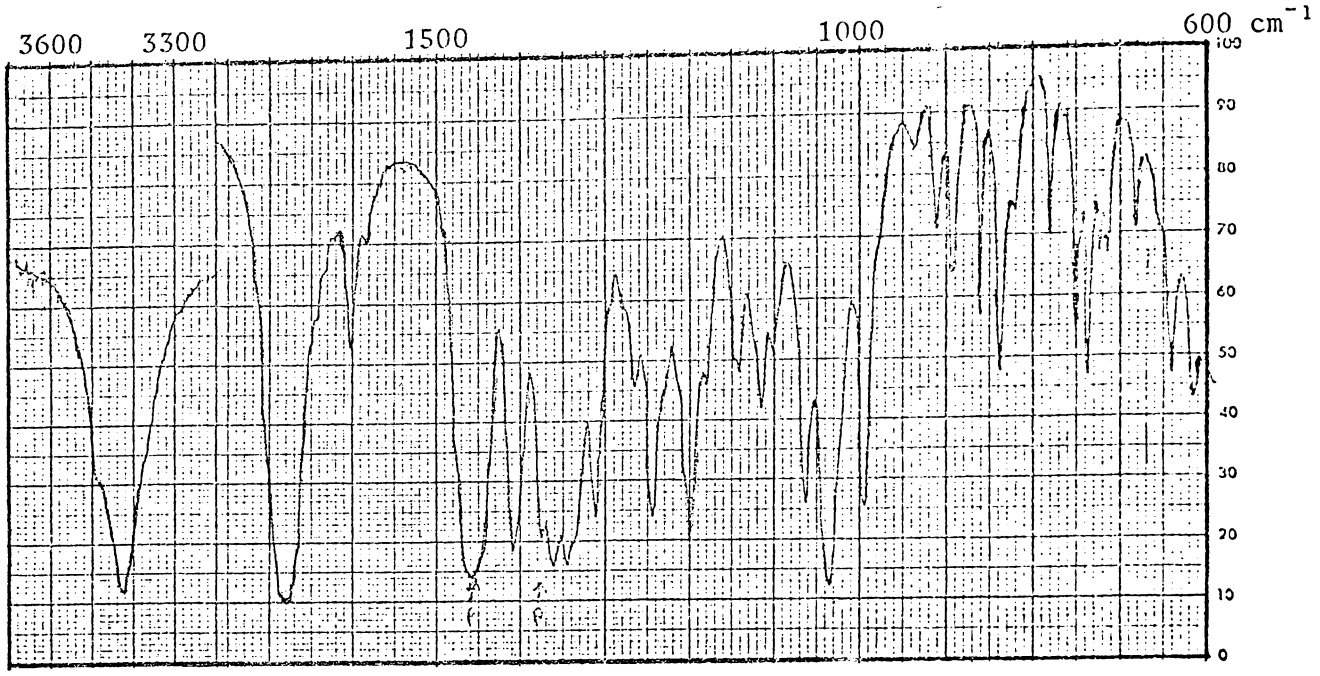



Fig. IR4.9. The i.r. spectrum of unsolvated sporidesmin ground in nujol.

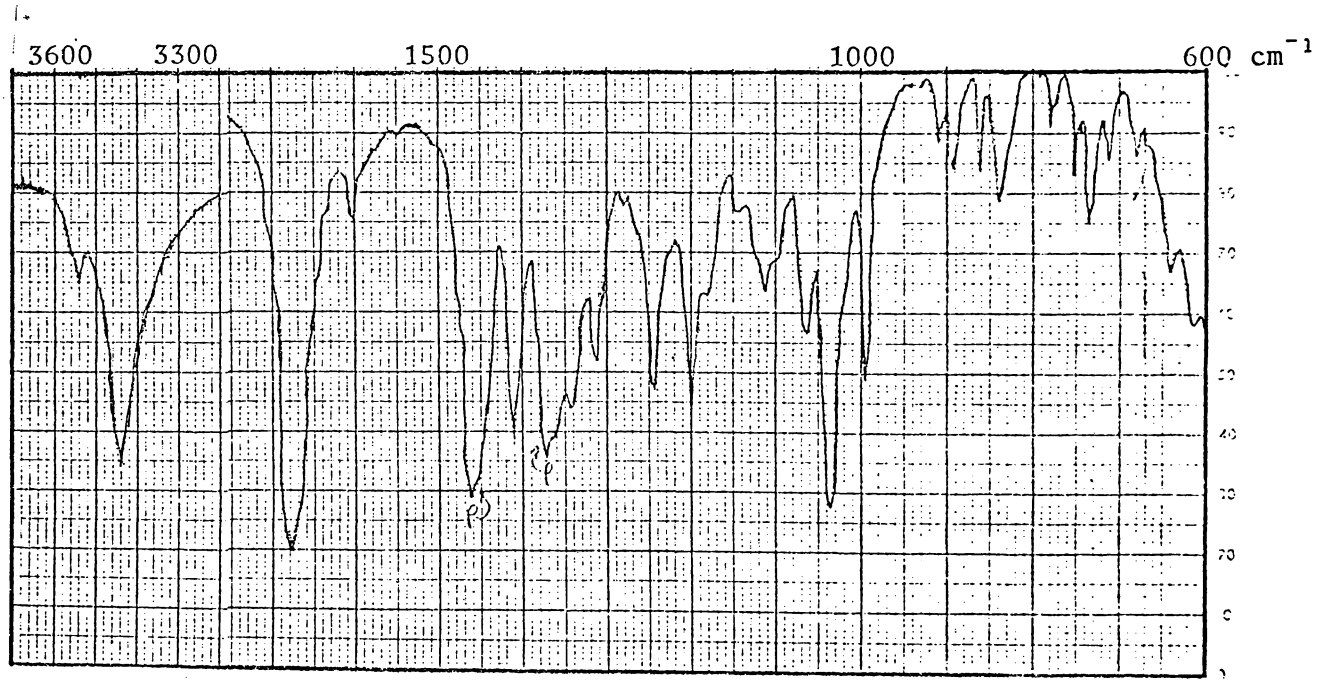


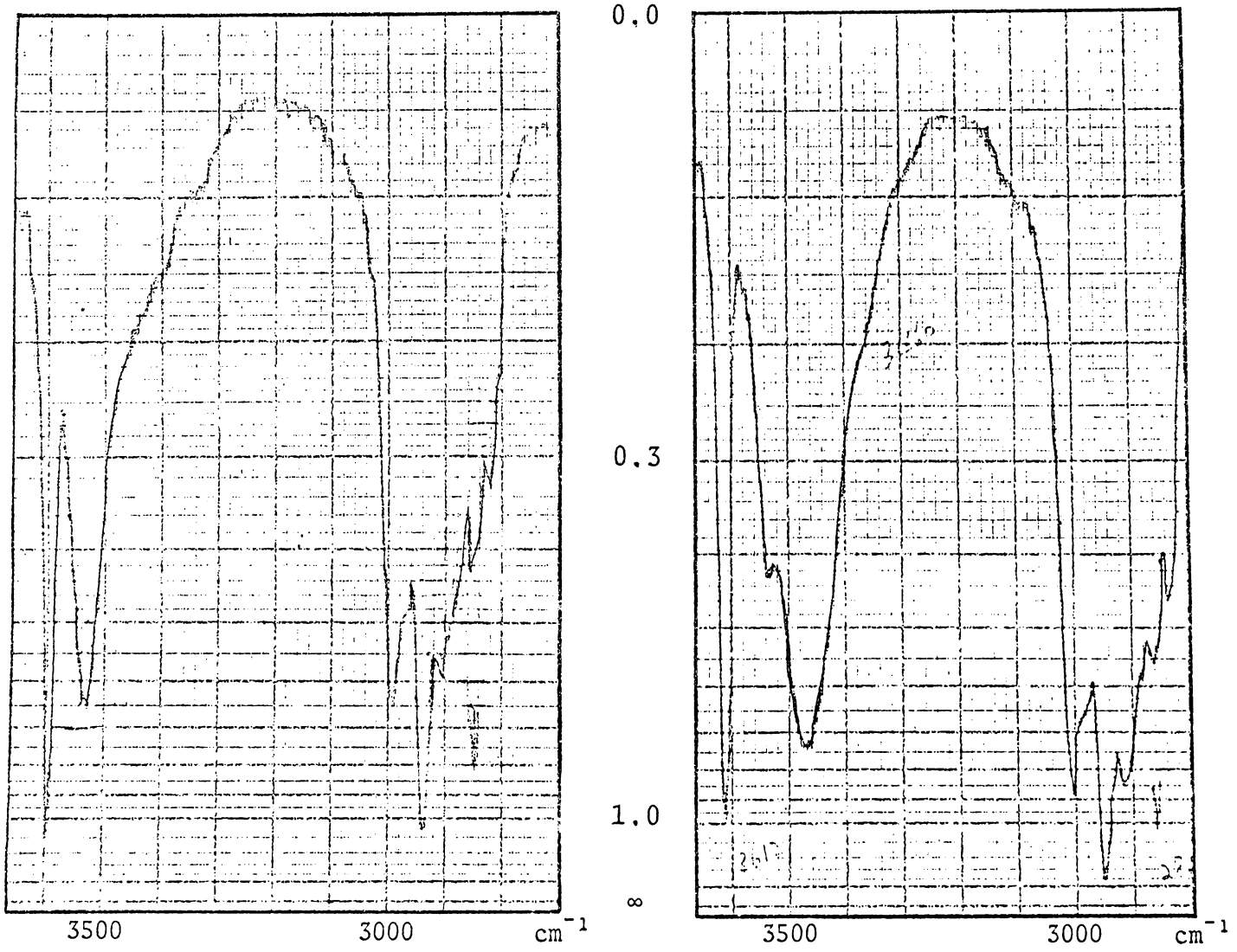

Fig. IR4.10. The i.r. spectrum of unsolvated sporidesmin-E ground in nujol.

was split into two almost equal peaks at 1205 and 1185 $cm^{-1}$ in sdm-E; the medium peak at 1145 $cm^{-1}$ in sdm was absent in sdm-E; a weak broad peak in sdm-E at 940 $cm^{-1}$ was absent in sdm; a weak peak in sdm at 820 $cm^{-1}$ was absent in sdm-E; a singlet medium peak at 740 $cm^{-1}$ in sdm appeared as two medium peaks at 735 and 728 $cm^{-1}$ in sdm-E while a weak broad peak in sdm at 720 $cm^{-1}$ appeared as a weak but sharp peak in sdm-E.

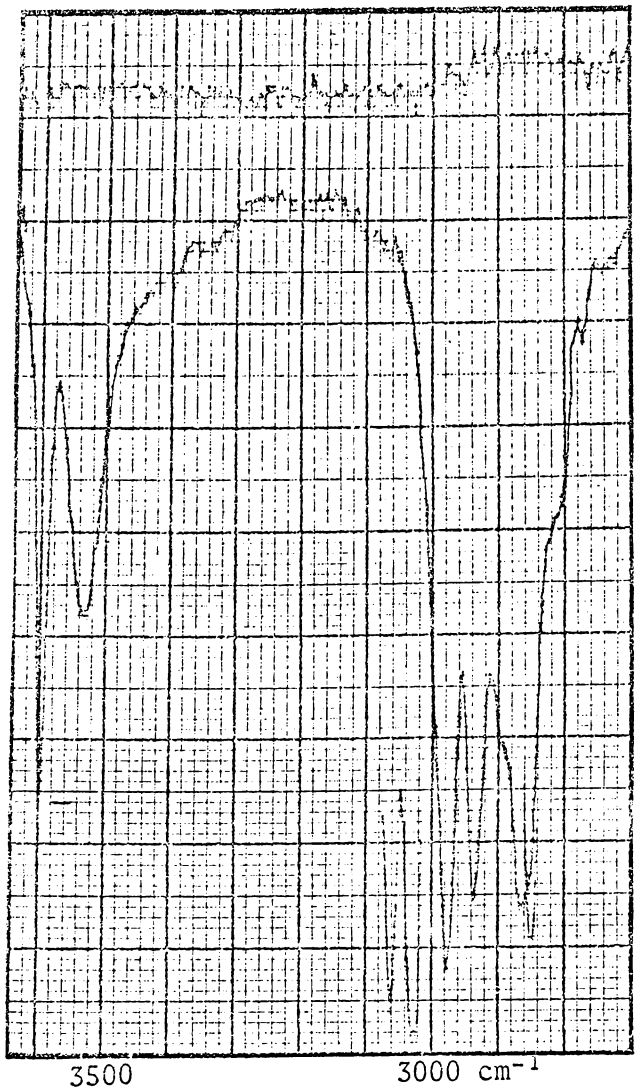
When unsolvated sdm and sdm-E were compared in halocarbon oil (Figs IR4.7, IR4.8), there were these differences: sdm-E showed a small OH stretching peak at 3540 $cm^{-1}$ which was a weak shoulder in sdm at 3490 $cm^{-1}$ on the strong peak at 3420 $cm^{-1}$; the $\Delta\nu_{\frac{1}{2}}^{a}$ for the C=O stretching peak (1680 $cm^{-1}$) in sdm-E was broader than that in sdm; a weak peak at 1440 $cm^{-1}$ in sdm-E was absent in sdm; the weak peak at 1380 $cm^{-1}$ in sdm was strong in sdm-E and shifted to 1370 $cm^{-1}$; the asymmetrical peak in sdm at 835 $cm^{-1}$ was almost an equal doublet at 840 and 830 $cm^{-1}$; the weak peak at 765 $cm^{-1}$ in sdm was somewhat stronger in sdm-E and the medium sharp peak at 750 $cm^{-1}$ was broader in sdm-E.

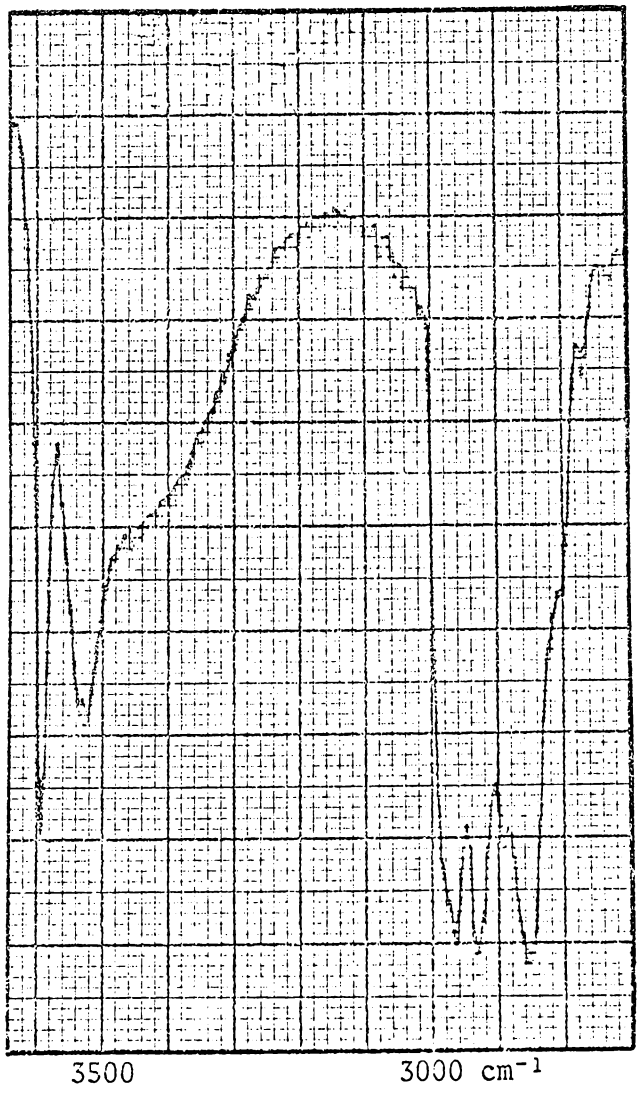
Some of these differences were also present in the nujol mulls (Figs IR4.9, IR4.10) of the two compounds but there were also other differences: the shoulder at 3490 $cm^{-1}$ in sdm was shifted to a weak sharp peak at 3540 $cm^{-1}$ in sdm-E; the weak peak in sdm at 1265 $cm^{-1}$ was absent in sdm-E; the medium peak (more or less a doublet) at 1150–1140 $cm^{-1}$ was very weak in sdm-E; the very weak (almost a shoulder) sharp peak at 800 $cm^{-1}$ was absent in sdm-E; the medium sharp singlet at 738 $cm^{-1}$ was almost a doublet in sdm-E at 735–730 $cm^{-1}$; while the weak well defined doublet at 723 and 715 in sdm was a sharp singlet at 712 $cm^{-1}$; and the two medium peaks at 640 and 615 $cm^{-1}$ were only weak ones in sdm-E.

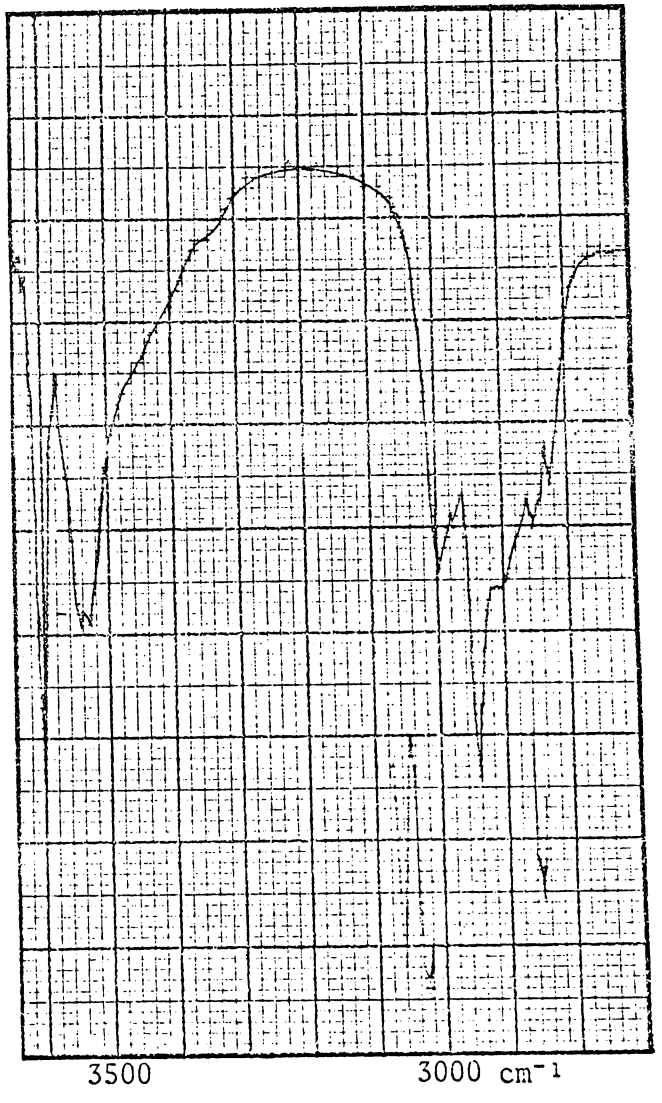
It is appropriate to find only minor i.r. differences between these two compounds since sdm-E is sdm with an extra sulphur atom in the -S-S- bridge.




Figs IR4.11 and IR4.12. The i.r. spectra of unsolvated sporidesmin-E and sporidesmin (resp.) (2 mM) in carbon tetrachloride (50 mm cell).




Figs IR4.13, IR4.14 and IR4.15. I.r. spectra of sporidesmin-E. Fig. IR4.13 is of etherate crystals (Experiment A-B3iii) in carbon tetrachloride (50 mm cell). Fig. IR4.14 is of etherate crystals (in $CHCl_3$-$CCl_4$ 1:19, 10 mm cell), crystallized after evaporating solution (AcMe) to a gum. Fig. IR4.15 is of unsolvated sporidesmin-E ($CCl_4$, 6 mM, 10 mm) after dissolving ($CHCl_3$) and evaporating to a film.

*Hydrogen bonding in sdm-E*

In Dreiding models of sdm and sdm-E the distance between the oxygen of the 1-CO and the hydrogen of the 11-OH was 22% greater in sdm-E. This difference was reflected in the hydrogen-bonding frequencies in the i.r. solution ($CCl_4$) spectra of the two compounds. [For the general discussion of the hydrogen bonding see Chapter 5, 'The infra-red solution spectra (4000–2700 $cm^{-1}$) of sporidesmin and its derivatives'.]

The spectrum of sdm-E unsolvated (Fig. IR4.11) (*c.* 2 mM in $CCl_4$, 5 cm cell) (B-E1) showed the following peaks:

1. $\nu$ 3700 $cm^{-1}$, $\varepsilon^a$ 24, $\Delta\nu_{\frac{1}{2}}^a$ 60 $cm^{-1}$,
2. 3590 " , 103, 32 " ,
3. 3530 " , 60, 100 " .

This spectrum was markedly different from that of sdm (Fig. IR4.12) but it had similarities to those of sdm-B (Fig. IR5.7) and 3,11a-dimercaptosecosdm (Fig. IR5.19). The differences between the two spectra (for sdm and sdm-E) indicated the configurational change effected by the incorporation of the third sulphur atom (S-13) between the two (3-S and S-12) in sdm. Not only did the 11-OH group move away (Plate 5.1) (going from sdm to sdm-E) from the 1-CO but also the hydroxy-group was less colinear with the lone pair electrons on the carbonyl oxygen.

The crystals from the synthesis (A-B3iii) were examined in a saturated solution (5 cm, $CCl_4$) (B-E3) and there was no sign of any shoulder corresponding to the peak of sdm at 3450 $cm^{-1}$ (Fig. IR4.13). But after the $^{13}C$ n.m.r. experiment (A-B3iii) where an acetone solution was evaporated to a resin to remove the ether of crystallization it was recrystallized several times from ether. Each time there was always a broad shoulder at 3450 $cm^{-1}$ (Fig. IR4.14) (B-E2). Similarly, when it was dissolved in chloroform and evaporated to a film there was the appearance of a shoulder (3450 $cm^{-1}$, $CHCl_3$ did not absorb at this frequency) (Fig. IR4.15):

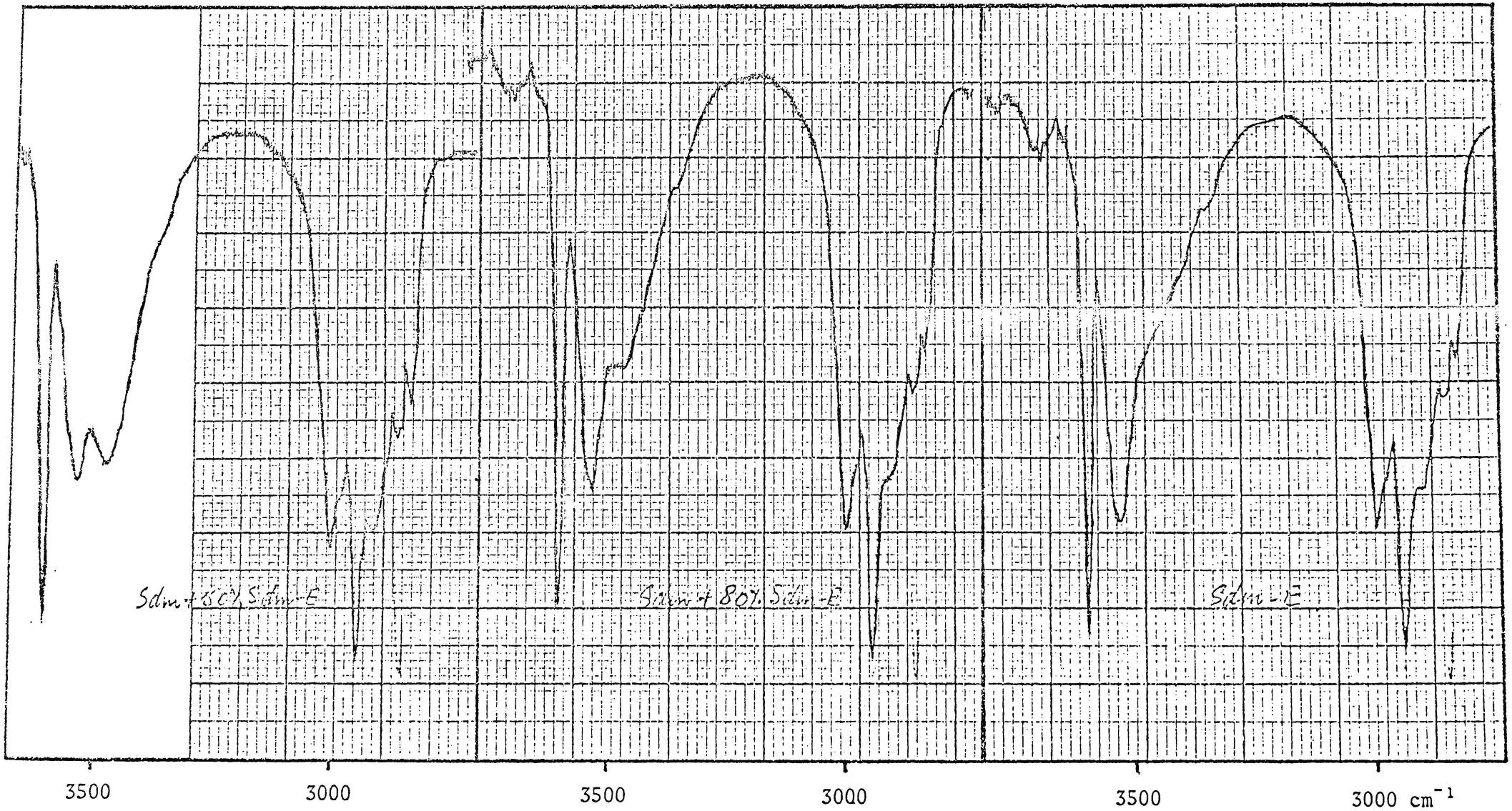



Fig. IR4.16. The i.r. spectra of mixtures of sporidesmin (50, 20, 0%) and sporidesmin-E (50, 80, 100%) (contaminated with sporidesmin).

the film dissolved readily in carbon tetrachloride.

In a series of spectra of mixtures of sdm and sdm-E (contaminated with some sdm, Fig. IR4.16), when there was *c.*25% of sdm the shoulder at 3450 $cm^{-1}$ was beginning to appear as a peak. After the $^{13}C$ n.m.r. experiment (referred to above) the shoulder corresponding to sdm was consistent with the estimated 15% as assessed on the relative intensities observed in the $^{13}C$ n.m.r. spectrum (Fig. CM4.1).

*C-H Stretching Region*

There was only a negligible difference (as mentioned in Chapter 5, 'The infra-red solution spectra (4000–2700 $cm^{-1}$) of sporidesmin and its derivatives') between the solution spectra of the C-H stretching region for sdm (Fig. IR4.12) and sdm-E (Fig. IR4.11). In the former there was a trough between the peaks at 2940 and 2900 $cm^{-1}$ while in the latter the peak at 2900 $cm^{-1}$ appeared as a shoulder on the 2940 $cm^{-1}$ peak.

*Instability of Sdm-E*

Whenever sdm-E was analysed by t.l.c. (A-B3ii) a spot corresponding to sdm was always present. In two dimensional t.l.c. that spot which separated from sdm, etc., and was in the position for sdm-E in the first direction, was always, in the second direction apparently resolved again into two spots: the minor one corresponding to where sdm would be, if it had been spotted onto the sdm-E spot at the end of chromatographing in the first direction. From this latter observation it was deduced that sdm-E broke down when it was evaporated to dryness on a silica gel G $F_{254}$ plate. To confirm this sdm-E was eluted after chromatographing in two directions (A-B3v) and rechromatographed; this resulted in three spots. Hence t.l.c. on silica gel G $F_{254}$ plates was not a satisfactory system to monitor the disappearance of sdm (in the synthesis of sdm-E) or to determine the purity of sdm-E. Although purification of some sort may have been achieved by column chromatography, in the process of dispersing the

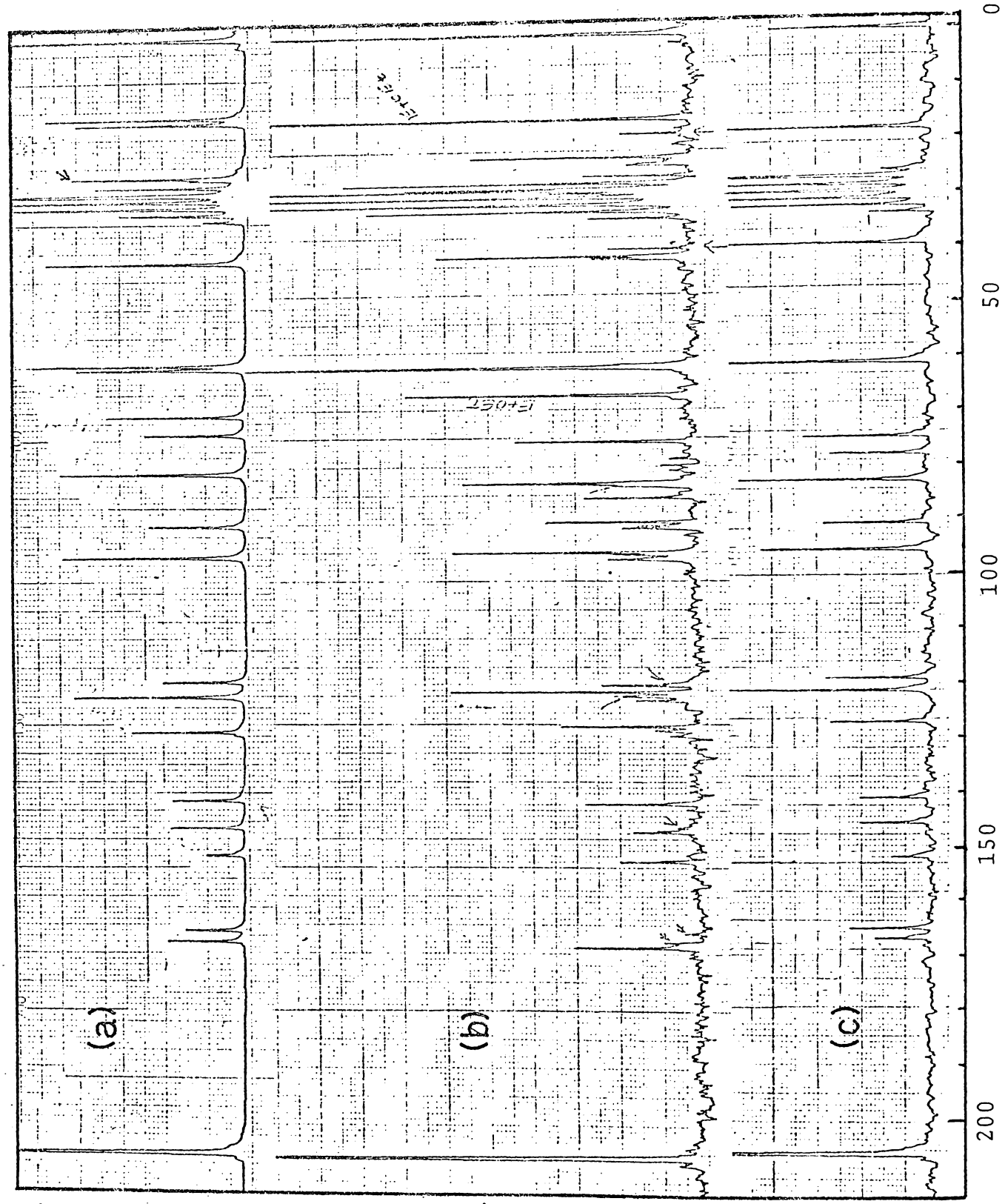



Fig. CM4.1. The decoupled $^{13}C$ nuclear magnetic resonance spectra of (a) sporidesmin-D, (b) sporidesmin-E (showing the peaks of residual EtOEt and the contaminating sporidesmin) and (c) sporidesmin, in acetone-$d_6$ (t.m.s.).

reaction residue on the silica gel G (A-B3i,ii) and evaporating the fractions dry for weighing, decomposition would occur and finally, after spotting on silica gel G plates t.l.c. monitoring would give only a false answer.

Sdm-E broke down not only on silica gel but also on evaporating to an expanded resin. The least handled crystals were those from experiment (A-B3iii) where the gum after evaporating the chloroform (work-up) solution (without column chromatography) spontaneously precipitated crystals upon dissolving in ether. In order to have the spectrum of the sample free of the $^{13}C$ n.m.r. peaks of ether and of the traces of pyridine, the above sdm-E.EtOEt crystals (A-B3iii) were dissolved in acetone (in subdued light) and evaporated to an expanded resin. When this expanded resin was dissolved again in deuteroacetone for $^{13}C$ n.m.r. analysis there was a precipitate of elemental sulphur.

$^{13}C$ *Nuclear Magnetic Resonance* (Fig. CM4.1)

Consistent with the appearance of sulphur in the solution (A-B3iii) the $^{13}C$ n.m.r. spectrum of sdm-E showed the complete spectrum of sdm. By comparing the intensities of the peaks for the residual sdm with the shifted peaks for the corresponding carbons in sdm-E (at 18.2 ppm, sdm, compared with those at 22.5, 23.6 and 23.9 ppm, sdm-E, and 39.2 ppm, sdm, with those at 40.5 and 41.0 ppm, sdm-E) it was calculated that 15% of the whole was sdm.

The sample of sdm-E from a $^{13}C$ n.m.r. experiment was recrystallized several times from ether (B-E2) but these sdm-E.EtOEt crystals gave the same $^{13}C$ n.m.r. spectrum with sdm still present and at about the same proportion.

Rahman, *et al.* (1969) showed from $^{1}H$ n.m.r. spectroscopy that in solution sdm-E existed in two conformers because of the duplication of peaks and suggested that they arose from the two conformations of the

-S-S-S- bridge (Fig. PM4.1). Here again in the $^{13}C$ n.m.r. of sdm-E there was evidence of conformers. The following observations on the $^{13}C$ n.m.r. spectrum of sdm-E have been made after making allowance for the presence of the sdm peaks.

Where the peaks for the two conformers were well resolved (and from those of sdm) the relative peak intensities suggested that at the temperature of observation (*c.* 30°) 28% was as the minor conformer. This figure was the average of the following percentages: at 22.5 ppm, 31%; at 40.5 ppm, 35%; at 89.1 ppm, 28%; at 94.7 ppm, 28%; and at 120.4 ppm, 21%.

The resonances of the four carbons in the dioxopiperazine ring i.e. the two carbonyl carbons (167.4 ppm) and the two carbons linked through the epitrithio-group (74.3, 84.7 ppm), all appeared as single peaks for the two conformers. That for the aromatic carbon which bore the methylated nitrogen (141.0 ppm) was not split either. In these cases the splitting might not be apparent because of the instrument's limit of sensitivity which was 1.2 Hz between any two data points.

For the aromatic carbon bearing the 8-OMe (151.6 ppm), the carbinyl carbon (81.9 ppm), the methoxy-carbons (60.9 ppm) and the aromatic carbon bearing the 7-OMe (146.2 ppm), the resonances for the minor conformer were shielded by 0.3 , 0.4, 0.5 and 0.9 ppm resp. While for the methyl-*C* on the indoline-*N* (40.5 ppm), the quaternary carbon bearing a hydroxy-group (89.1 ppm), its adjacent aromatic carbon (126.8 ppm), the methyl-*C* of the amido-*N*-methyl (28.2 ppm), the methyl-*C* of the *C*-methyl (22.5 ppm), the methine carbon between the two nitrogens (94.7 ppm), the aromatic methine-*C* (120.4 ppm) and the aromatic-*C* bearing the chlorine (119.2 ppm), the resonances for the minor conformer were deshielded by 0.5, 0.8, 0.8, 0.9, 1.3, 1.3, 1.7 and 2.1 ppm resp. In the former list of upfield resonances (for the minor conformer) the carbinyl-*C* had a

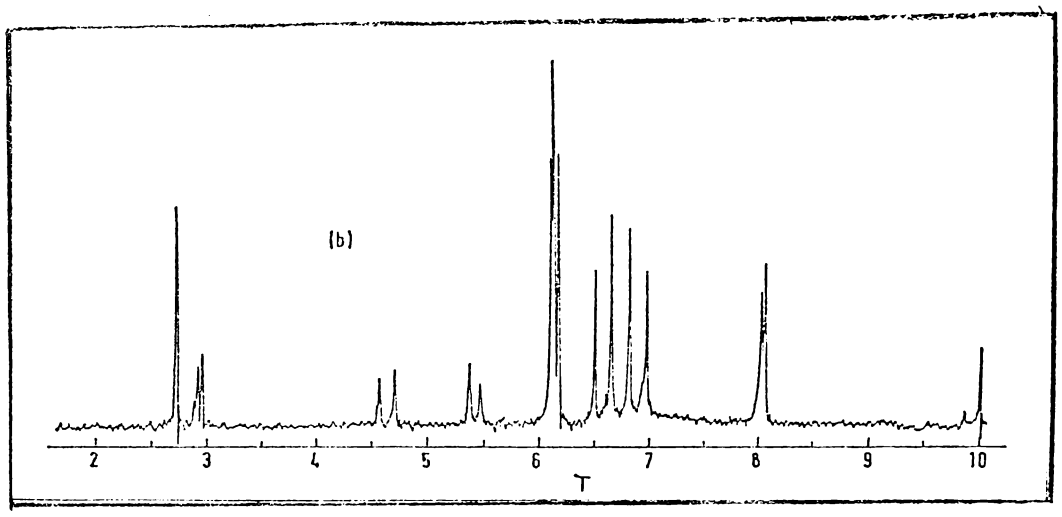



Fig. PM4.1. The $^{1}H$ nuclear magnetic resonance spectrum of sporidesmin-E at $0^{o}$. The chloroform-*d* solution of sporidesmin-E.EtOEt was cooled to $-40^{o}$ and the ether removed as the solution warmed to $0^{o}$. (Copied from Safe and Taylor, 1971.)

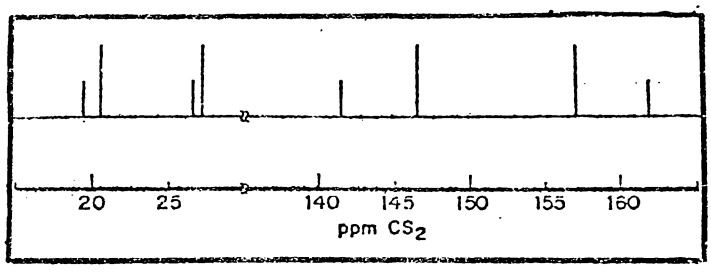



Fig. CM4.2. Schematic representation of the $^{13}C$ nuclear magnetic resonance spectrum of *N*-formylsarcosine. The more intense set of peaks represents the spectrum of the *trans*-conformer. (Copied from Dorman and Bovey, 1973.)

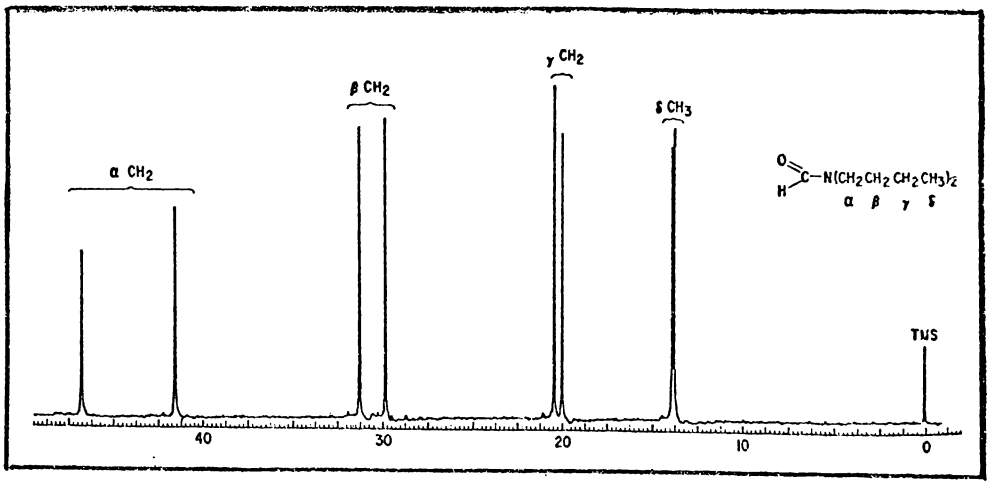



Fig. CM4.3. The $^{13}C$ nuclear magnetic resonance spectrum of *N*,*N*-di-n-butylformamide (aliphatic region shown). (Copied from Levy and Nelson, 1972.)

corresponding proton which also resonated upfield (0.10 ppm) in the minor conformer (Safe and Taylor, 1971). In the published $^{1}H$ n.m.r. spectrum of sdm-E ($CDCl_3$, $0^{o}$) (Safe and Taylor, 1971) (Fig. PM4.1) it was not possible to determine which proton peaks in the methoxy-group of resonances belonged to the minor conformer. The peak for the amido-*N*-methyl-*C* (in the $^{13}C$ spectrum) were obscured by those for the deutero-acetone, nevertheless that for the conformer appeared to be downfield while in the $^{1}H$ n.m.r. spectrum that for the corresponding protons was upfield (0.12 ppm). In the other cases where the carbon resonances were deshielded so were the corresponding proton resonances; the protons on the methyl on the indoline-*N* (0.17 ppm), *C*-methyl protons (0.05 ppm), the methine proton between the two nitrogens (0.12 ppm) and the aromatic proton (0.05 ppm from the displayed spectrum).

Dorman and Bovey (1973) displayed the $^{13}C$ n.m.r. spectrum of N-formylsarcosine (Fig. CM4.2) which demonstrated the *cis*- and *trans*-conformers arising from the partial rigidity of the amido-grouping. Walter and Maerten (1968) reported the $^{1}H$ n.m.r. spectrum of *N*-isopropylformamide and of *N*-isobutylformamide and showed the existence of two conformers for each ($\delta$ 4.01 and 3.50, and $\delta$ 3.05 and 3.00 resp. for the $\alpha$-protons). Then, Levy and Nelson (1972g) published the $^{13}C$ n.m.r. spectrum of *N,N*-di-n-butylformamide which showed (Fig. CM4.3) each of the two alkyl chains had a separate pattern, the one upfield demonstrating the carbonyl compression. In the *N,N*-di-n-butylformamide spectrum (Levy and Nelson, 1972g) the more removed that the carbon was from the amido-group the smaller was the $\Delta\delta$ value.

If the general case was that the carbons nearest the centre of conformational interchange showed the greatest shift, then in sdm-E it could be argued that the potential centre for conformational interchange was not the epitrithio-group. The carbon whose resonance showed the greatest $\Delta\delta$ value (2.1 ppm) was the aromatic CCl (its adjacent carbon (8-MeO*C*)

showed a minimal shift, 0.3 ppm) and it was, in the ring system, about the farthest removed from the epitrithio-group. But this deduction was not consistent with the absence of conformers in the $^{13}C$ n.m.r. spectra of sdm and sdm-D. Seeing that both sdm-D and sdm-E have been derived from sdm, they all had the same basic ring structure (apart from the sulphur atoms) and therefore, apart from the sulphurs, they all should have the same conformers.

For some of these (in sdm-E) conformer shifts, the sulphur atom (S-13) could be considered to modify the electric field of the carbonyls in the dioxopiperazine ring. When it (the S-13 atom) was one way it had a shielding effect upon the carbinyl-*C* (C-11) (0.4 ppm) (*anti* to the 4-CO) and a deshielding effect upon (in increasing order of the effect) the indoline-*N*-methyl-*C*, (0.5 ppm) the quaternary-carbinol-*C* (C-10b) (0.8 ppm) (*syn* to the 4-CO), 3-methyl-*C* (*anti* to the 1-CO) (1.3 ppm) and the carbon (C-5a) between the two nitrogens (1.3 ppm) (*syn* to the 4-CO, Dorman and Bovey, 1973).

The following is a discussion of the resonance positions observed for the major conformer, comparing them with those for sdm and sdm-D.

(i) *At* 22.5, 28.2 *and* 40.5 *ppm*.-These methyl-*C* peaks (3-methyl, 2-methyl and 6-methyl resp.) each resonated at positions between those for sdm and sdm-D. Each peak was 60% nearer that of sdm-D, indicative of the relaxation of the strain imposed by the -S-S- bridge in sdm. Not only did the bond lengths of the C-S and S-S bonds apply a strain to the dioxopiperazine ring but also the dihedral angle of the C-S-S-C group. This dihedral angle, $90^{o}$ when unrestrained (Rahman, *et al.*, 1970), was about $10\text{-}20^{o}$ (Safe and Taylor, 1971) in sdm. If a Dreiding model can be relied upon, the epitrithio-group applied an expanding strain (Plate 5.1) on the dioxopiperazine ring in contrast to the contracting strain in sdm, i.e. the C-S bonds converge in sdm, were more or less parallel in sdm-D and diverge in sdm-E (see under 'sdm-E', Chapter 5). So the

relaxation was not as complete as in sdm-D. The C-S-S-S dihedral angle was *c.* 60° (in sdm-E) which angle was considerably closer to the unrestrained dihedral angle (90°) for sulphur atoms, therefore it could be expected that the epitrithio-group in sdm-E would be a stable condition. Instead, as observed above, sdm-E showed considerable instability.

(ii) *At* 60.9 *ppm.*-This peak for the two methoxy-*C*s was at the same position as that in sdm.

(iii) *At* 74.3 *ppm.*-This peak and that at 84.7 ppm showed no nuclear Overhauser enhancement agreeing with their corresponding carbons bearing no protons. Although the methyl-*C* of the C-methyl resonated between that of sdm and sdm-D the carbon (C-3) here bearing that methyl, resonated at the same frequency as that in sdm. This might be attributed to the effect of the divergence of the C-S bond (mentioned above) i.e. in the relatively relaxed condition in sdm-D carbon-3 resonated at 69.7 ppm while either the convergent strain (sdm) or the divergent strain (sdm-E at C-3) deshielded to *c.* the same value (74.3 ppm).

The peak (84.7 ppm) for the carbon '*para*' (in the dioxopiperazine ring) (C-11a) to the above carbon (C-3) was subject to the same divergent strain but was also subject to the restraint of the fused pyrrolidine ring. Because of the fusing of the dioxopiperazine and pyrrolidine rings this bridgehead C-S bond had a rigid direction to which the orientation of the epitrithio-group was applying a modifying stress in the direction of the pyrrolidine ring (in respect of the sulphur end of the C-S bond) while the dioxopiperazine ring was being forced away. This strain at C-11a was reflected in the deshielding to 84.7 ppm from 73.1 ppm (in sdm-D).

In sdm and sdm-D the resonances for carbons-3 and -11a were 3.3 ppm apart but in the epitrithio-compound (sdm-E) they were 9.6 ppm apart. This downfield shift of 6.3 ppm indicated that the epitrithio-group had a profound effect compared with the epidithio-group (sdm) or the dimethyl-

thio-group (sdm-D).

(iv) *At* 81.9, 89.1 *and* 94.7 *ppm.*-These three peaks assigned to the carbinyl-*C*, the tertiary hydroxy-group-*C* and the diazamethine-*C* (resp.) were all in the pyrrolidinediol ring and each reflected the propagation of the above C-S bond divergence effect in different ways. They were all shielded in respect of the corresponding resonances in sdm (by 0.6, 1.3 and 0.6 ppm resp.) but only that for the carbinyl-*C* occurred between those for sdm and sdm-D (deshielded from that of the latter by 1.5 ppm). The tertiary hydroxy-group-*C* and the diazamethine-*C* lie opposite (in the pyrrolidinediol ring) the carbon (C-11a) bearing the divergent C-S bond: they were shielded by 1.2 ppm from corresponding values for the relatively relaxed molecule sdm-D.

(v) *The aromatic carbon peaks at* 119.2, 120.4, 126.8, 141.0, 146.2 *and* 151.6 *ppm.*-As before, these peaks were assigned to the chloro-carbon (C-9), the aromatic methine carbon (C-10), its adjacent ring-junction carbon (C-10a), the carbon (C-6a) bearing the methylated nitrogen and the carbons (C-7 and -8) bearing the 7- and 8-methoxy-groups respectively. The carbon bearing the methylated nitrogen resonated at the frequency of that for both sdm and sdm-D (within 0.2 ppm), the chloro-carbon and C-7 both resonated at the same frequency as in sdm-D, and both C-10a and C-8 resonated as in sdm while the remaining methine carbon resonated at less than in either sdm (0.6 ppm) or sdm-D (1.4 ppm).

Here the average (134.2 ppm) of these shieldings fell between those for sdm and sdm-D. That for the minor conformer was 134.8 ppm (sdm-D, 134.6 ppm). For the major conformer these differences were small.

(vi) *At* 167.4 *ppm.*-This was the peak for the carbonylcarbons. Although the two carbonyls were in quite different environments and although their resonances were well resolved in both sdm and sdm-D, in sdm-E they were coalesced and they occurred slightly downfield from the most deshielded (167.1 ppm, sdm-D) of the carbonyl resonances for sdm and sdm-D.

Of the resonances of the minor conformer which were not within the range of those of sdm and sdm-D, one was 1.2 ppm upfield (the chlorocarbon) from that for sdm-D while the rest were either within the range or within 0.7 ppm of it. Likewise, of the resonances of the major conformer which were not within this range one was deshielded from sdm-D by 7.0 ppm (C-11a) (which has been discussed) and another by 1.2 ppm (C-10b) and the rest fall within 0.7 ppm of the range. So on the whole, the resonances of the two sdm-E conformers occurred quite closely to those of the parent compound (sdm) and the *S,S'*-dimethyl compound (sdm-D).

*Conclusion*

Seeing that mixed melting point determinations on sdm-E (with sdm) did not show the expected depression and seeing that sdm and sdm-E melted within 3.5° of each other (sdm, 179°, Ronaldson, *et al.*, 1963; sdm-E, 181-182.5°, this work) a more definitive indication that an epitrithiocompound had been formed from sdm was needed: the molecular weight by mass spectrometry (505 = 473 + 32) provided this indication.

The following is a suggestion for obtaining sdm-E.EtOEt as pure as possible, since conventional techniques, the results from which have been described, caused decomposition to sdm from which sdm-E could not be subsequently separated by recrystallization.

The chloroform solution of the reaction product after the synthesis of sdm-E (A-B3iii) should be washed with dilute acid to remove the pyridine and then concentrated (at less than 45°) to a small volume (of $CHCl_3$). Ether would need to be added and evaporated repeatedly, taking care not to evaporate to a gum, till crystals appeared on cooling. It may be possible to recrystallize from ether.

The $^{13}C$ n.m.r. peaks of ether (17.1 and 67.4 ppm, Stothers, 1972h) and pyridine (124.1, 136.1, 150.4 ppm, Stothers, 1972k) do not interfere in the spectrum of sdm-E, therefore, the above preparation dissolved

directly in deuteroacetone should give a spectrum with a minimum of sdm.

The quantity of contamination of the sdm-E.EtOEt with sdm could possibly be determined by $^1$H n.m.r. spectroscopy; the 11-H peak for sdm (δ 4.58) occurred between those of the conformers of sdm-E (δ 4.62 and 4.52) (Fig. PM4.1); similarly, for the 2-$CH_3$ peak which occurred at δ 3.07 for sdm and δ 3.14 and 3.02 for sdm-E; for the 6-$CH_3$ and 3-$CH_3$ those for sdm (δ 3.30 and 2.03 resp.) were upfield from the peaks for sdm-E at δ 3.50 and 3.33, and δ 2.00 and 1.95 (resp.). Though most of the peaks for sdm are shifted only 0.03 to 0.05 ppm from the nearest conformer peaks of sdm-E, with careful spectrometric resolution, an assessment of purity could be made.

There was a suggestion from the observation of the hydrogen bonding spectra of unsolvated sdm-E that recrystallization through ethyl acetate-acetic acid may effect purification of sdm-E. But the yield at each crystallization was far from quantitative. It would not be easy to recover sdm-E from the acetic acid mother liquors.

Crystalline sdm-E.EtOEt (A-B3i) was checked on tissue culture and sheep by the Ruakura Experimental Pathology Section. The *in vitro* tissue culture results showed that sdm-E was no more toxic than sdm and similarly in sheep there was no significant differences in liver damage between the two compounds. By both methods they were equally toxic. Nevertheless, the sdm-E intoxicated animals were, on average, photosensitive in fewer days and there was a difference in the bladder lesions between the two groups (sdm and sdm-E treated).

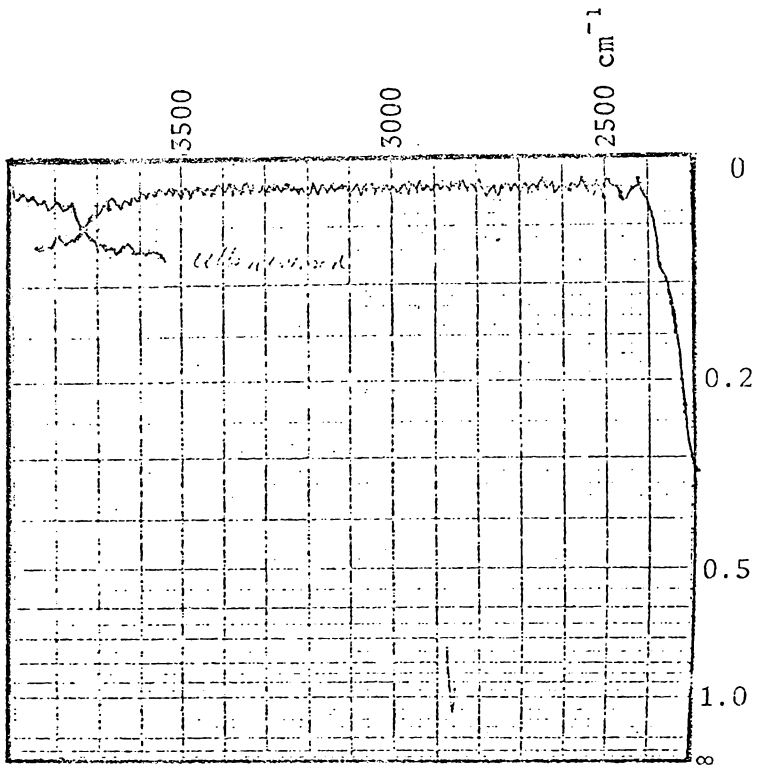



Fig. IR5.1. Spectrum of carbon tetrachloride recorded against carbon tetrachloride in the reference beam. The instrument was set out of 'balance'. Between 3800 and 3550 $cm^{-1}$ the sample and reference cells were reversed.

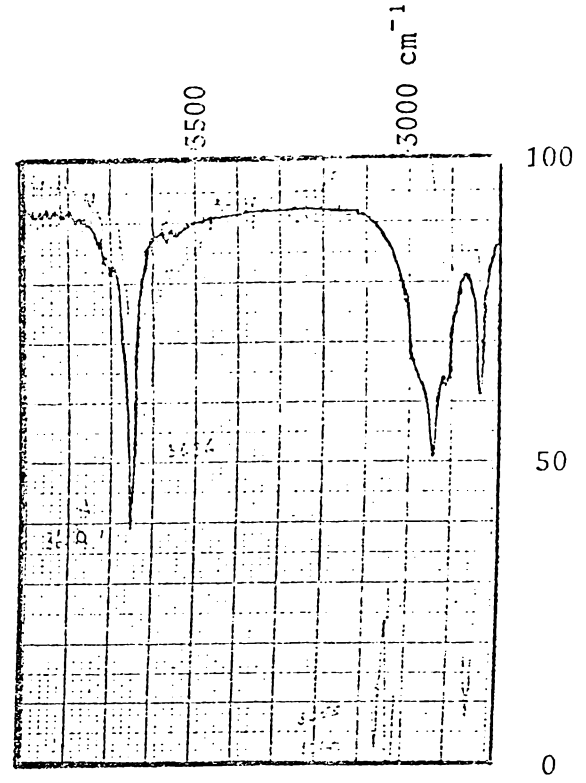



Fig. IR5.2. Spectrum of methanol (8 mM in 10 mm cell) against carbon tetrachloride.

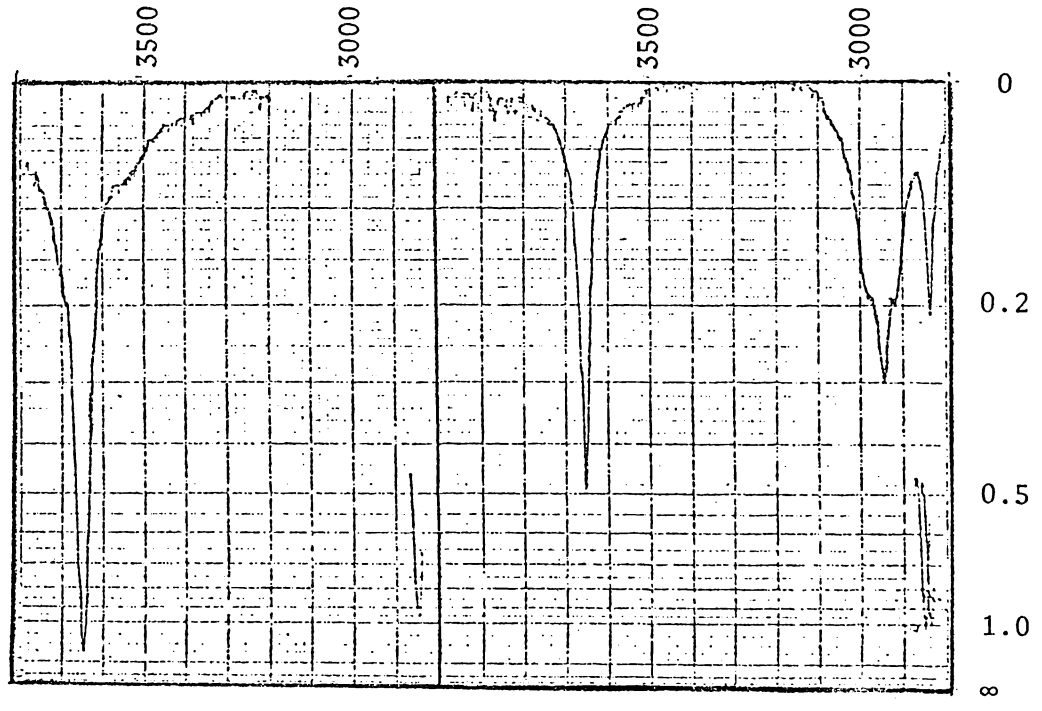



Fig. IR5.3A and 3B. Spectra of methanol (5.5 mM) in 50 mm and 20 mm cells respectively.

# CHAPTER 5. THE INFRA-RED SOLUTION SPECTRA (4000—2700 $cm^{-1}$) OF SPORIDESMIN AND ITS DERIVATIVES

This study was made as an extension to the comparison of the hydrogen-bonding spectra of sdm and sdm-E and because of the anomaly that the 11a-mercaptoseccosdms had no νSH peaks. For the discussion of the comparison of sdm with sdm-E see Chaper 4, 'Sporidesmin-E, its synthesis and comparison of its infra-red and $^{13}C$ nuclear magnetic resonance spectra with those of sporidesmin'.

The frequency range 4000 to 2700 $cm^{-1}$, covers the absorbances due to free and hydrogen-bonded O-H stretching (Tichy, 1965). To avoid solvent interaction with the solutes carbon tetrachloride was chosen for the solvent. Carbon tetrachloride has absorption bands (augmented by the 50 mm path length) in this frequency range but these, when balanced out did not absorb so greatly that a spectrum could not be obtained (Fig. IR5.1). Spectrum (Fig. IR5.1) was obtained with the instrument set in 'imbalance' (B-A1) to show up the areas where 'blanketing' (Jones and Sandorfy, 1956) would render spectra unreliable. Blanketing began at 2400 $cm^{-1}$, owing to the broad bands of atmospheric carbon dioxide (2349 $cm^{-1}$, Bellamy, 1958a), moisture (*c.* 2340 $cm^{-1}$, Sadtler, 1962), carbon tetrachloride (*c.* 2280 $cm^{-1}$, Jones and Sandorfy, 1956) and the end absorption of the silica cells.

*Methanol.*-To verify that the carbon tetrachloride used was satisfactory for these measurements and to calibrate the instrument (a 1965 Beckman IR8), solutions of methanol (*c.* 5.5 and 8 mM) (B-A2,3) (Figs IR5.2, 5.3) were examined. Only the free O-H stretching peak was observed even at the higher concentration. Compared with the position that the instrument recorded the 2850 $cm^{-1}$ peak (which was recorded on each spectrum) for the polystyrene film that for methanol was 3652-3654

$cm^{-1}$ which was 10 $cm^{-1}$ higher than published results, determined with correctly calibrated instruments (Bellamy and Pace, 1966, 3642 $cm^{-1}$; van der Maas and Lutz, 1974, 3644 $cm^{-1}$). The frequencies reported here were adjusted for this error.

The peak heights increased approximately linearly (within the capability of the instrument) as the path length was increased (from 10 to 50 mm) (e.g. 20 and 50 mm, Fig. IR5.3). At the longest path length (50 mm) the apparent molecular extinction coefficient ($\varepsilon^a$, Jones and Sandorfy, 1956) for methanol was *c.* 47 and the apparent width at half height ($\Delta\nu^a_{\frac{1}{2}}$) was 46 $cm^{-1}$. Van der Maas and Lutz (1974) with more sophisticated instruments, reported a $\Delta\nu^a_{\frac{1}{2}}$ of 21 for methanol.

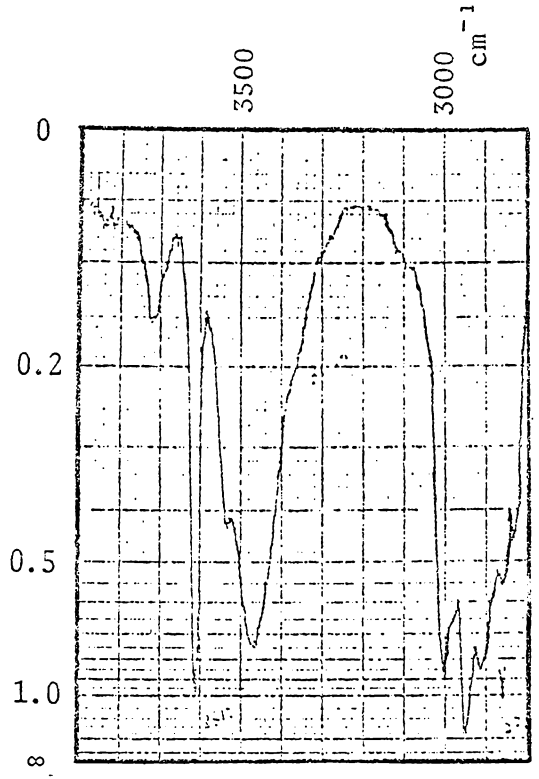



Fig. IR.5.4. Spectrum of sporidesmin in carbon tetrachloride (2 mM in 50 mm cell).

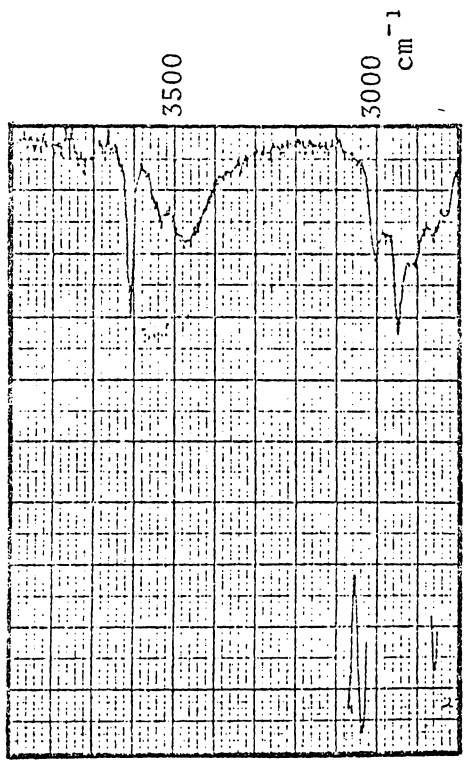



Fig. IR5.5. Spectrum of sporidesmin in tetrachloroethylene (saturated, in 10 mm cell).

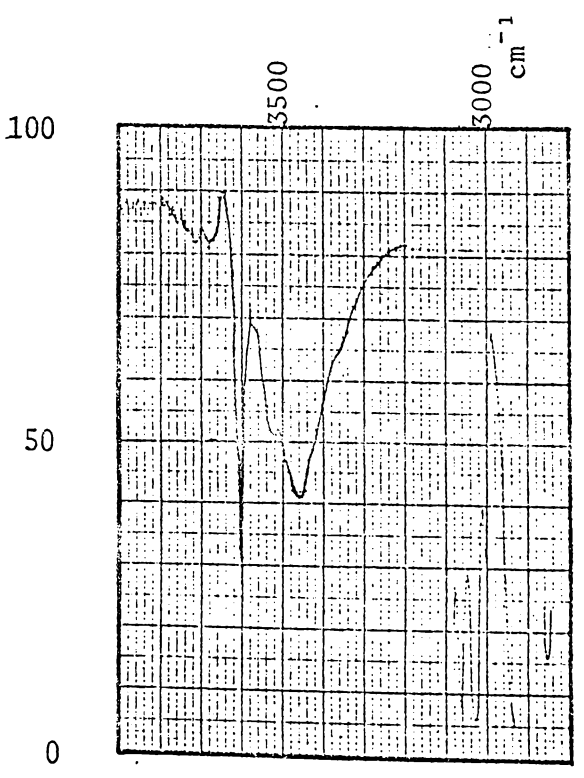
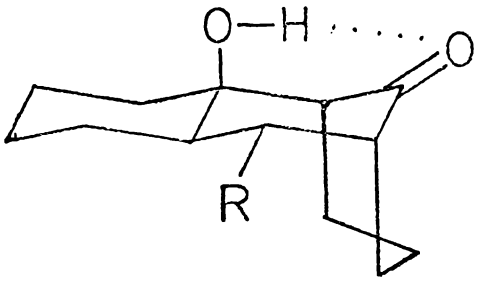


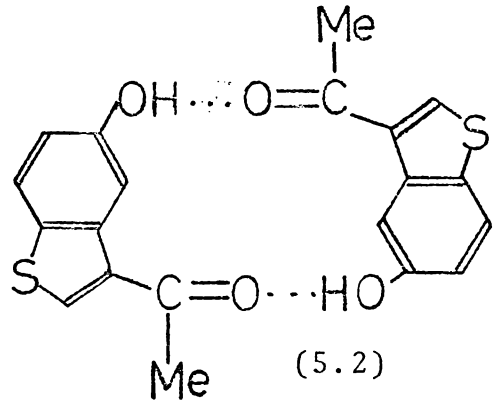

Fig. IR5.6. Spectrum of sporidesmin in 5% chloroform in carbon tetrachloride (6 mM in 10 mm cell).

## §1. *Sporidesmin* (1.1)

To avoid intermolecular hydrogen bonding absorptions which are not expected in dihydroxy-compounds in solutions of lower concentration than 5 mM (Kuhn, 1952) the concentrations of the sdm solutions were 5 mM or less. Two hydrogen-bonded dimers (involving carbonyl oxygen acceptors) are known at lower concentrations (1 mM solution of a derivative of 3-hydroxycyclohexanone (5.1), Hanousek, 1964, and 0.24 mM solution of 3-acetyl-5-hydroxybenzothiophene (5.2), Brown, *et al.*, 1963) but in both cases the band corresponding to the dimer was at less than 3400 $cm^{-1}$.




(5.1)




(5.2)

No OH stretching bands in the sdm compounds examined occurred as low as this. Unsolvated sdm and unsolvated sdm-E (B-B1, B-E1) were fairly insoluble ($CCl_4$); nearly saturated solutions, at room temperature were *c.* 2 mM.

The spectrum of sdm (*c.* 2 mM in a 50 mm cell, B-B1) (Fig. IR5.4) showed the following peaks:

1. ν3592 $cm^{-1}$, $\varepsilon^a$ 95, $\Delta\nu_{\frac{1}{2}}^a$ 26 $cm^{-1}$, 16 relative peak area.
2. 3520 " 39, * 3 " " "
3. 3450 " 70, 150 " , 113 " " "

The approximate (±10%) relative peak areas were obtained by cutting out the peaks (derived from 50 mm of 2 mM solutions for each compound) and weighing. *Peak No.2 above was a small definite peak on the side of peak No.3.

The relationship between the two hydroxy-groups in the sdms was comparable with that in 1,2-*trans*-cyclopentanediol which is the simplest case (Kuhn, 1952) and which shows only a free OH stretching band (at 3620 $cm^{-1}$). In the absorptions observed here the most intense peak was more than 25 $cm^{-1}$ less than this frequency.

In a Courtauld model of sdm (Plate 1.1) the hydrogen of the 11-OH was contiguous to the oxygen of the 1-CO so the broad peak at 3450 $cm^{-1}$ was assigned to this structure. The two oxygens (centre to centre in the model) were *c.* 2.5 Å apart hence the interatomic distance between the hydrogen and its hydrogen-bonded oxygen was *c.* 1.5 Å (Dreiding model 1.3 Å). Applying the Kubota, *et al.* (1966) formula $\Delta\nu = 412 - 143R$, where R is the internuclear distance in Å, gives 1.6 Å. $\Delta\nu$ Is calculated from 3627 $cm^{-1}$ for secondary alcohols (Oki, *et al.*, 1968). In the models the hydrogen of the 10b-OH was not apparently contiguous to any other atom. The narrow sharp peak (3592 $cm^{-1}$) was consistent with this environment.

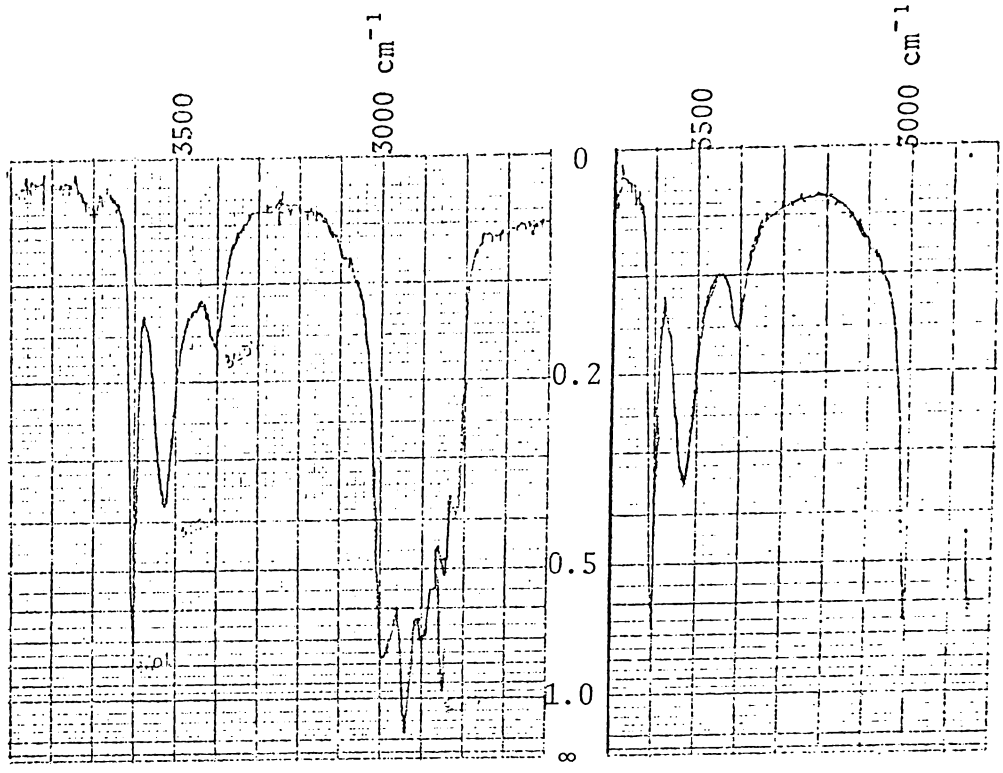
Among the hundreds of saturated aliphatic compounds listed by Tichy (1965) the greater number of the free νOH values occurred at or more than *c.* 3618 $cm^{-1}$. (This value is approximate; many free values occur below it and many π-bonded values occur above it.) Where an aromatic ring bears a hydroxy-group or the hydroxy-group is α or β to an aromatic ring then in most cases the 'free' νOH value occurs at or less than *c.* 3618 $cm^{-1}$ which shift in frequency suggests that in the latter there are OH•••π-bondings. From the work of Hanaya, *et al.* (1974) the $\Delta\nu_{\frac{1}{2}}^{a}$ for OH•••π-bonding (11—16 $cm^{-1}$) appeared to be less than for the free OH (15—25 $cm^{-1}$). The 10b-OH of sdm was α to an aromatic ring so it is suggested that the shifting to 2592 $cm^{-1}$ was the result of OH•••π-bonding.

Schleyer (1958) observed that the intensity of the non-bonded peak relative to the π-bonded one decreased as the number of carbons between

the functional groups decreased and that the strength of the hydrogen bond also decreased ($\Delta\nu$ decreased). Thus in going from γ-phenylpropanol to phenol the band for the free primary hydroxy-group (3638 $cm^{-1}$ in γ-phenylpropanol) became a shoulder in benzyl alcohol and disappeared in phenol; while the OH•••π-band, absent in γ-phenylpropanol, of comparable intensity with the free one in β-phenylethanol (3606 $cm^{-1}$), dominant in benzyl alcohol (3615 $cm^{-1}$) was alone in phenol (3614 $cm^{-1}$) (Huggins and Pimentel, 1956). Therefore, because the 10b-OH was adjacent to a benzene ring the absence of a band about or more than the free-tertiary-hydroxy-group frequency (3618 $cm^{-1}$ , Oki, *et al.*, 1968) in the sdm compounds was not unexpected.

The small peak (relative peak area 3) at 3520 $cm^{-1}$ was difficult to interpret. Joris, *et al.* (1968) pointed out that a small peak on the side of another might have apparent high intensity but when decomposed with a Curve Resolver this small peak represented only a small percentage of the recorded peak area. Hence the use of the relative peak area even though it was approximate compared with results using a Curve Resolver. This peak is further discussed under 'sporidesmin-B'.

*Other solvents.*-Because of the low solubility of sdm in carbon tetrachloride, only low intensity spectra were obtained with 10 mm cells. Till longer path length cells arrived another non-polar solvent was employed i.e. tetrachloroethylene (B-B2) (Fig. IR5.5). In this solvent the solubility and therefore the intensity for sdm was 20% less but the band frequencies were unaltered. For the same reason sdm was dissolved in chloroform (50 μℓ) and diluted (to 1 ml, $CCl_4$, 6 mM sdm) (Fig. IR5.6). This procedure produced an intensity 2.6 times as great as 10 mm of the saturated solution ($CCl_4$). The band frequencies were again unchanged but the intensity ratios ($\varepsilon^a_{3590}/\varepsilon^a_{3450}$) were 10% greater than that for the spectrum in carbon tetrachloride alone.




Figs IR5.7A, IR5.7B. Spectra of sporidesmin-B in carbon tetrachloride (A. 3.3 mM in 30 mm cell; B. 2 mM in 50 mm cell).

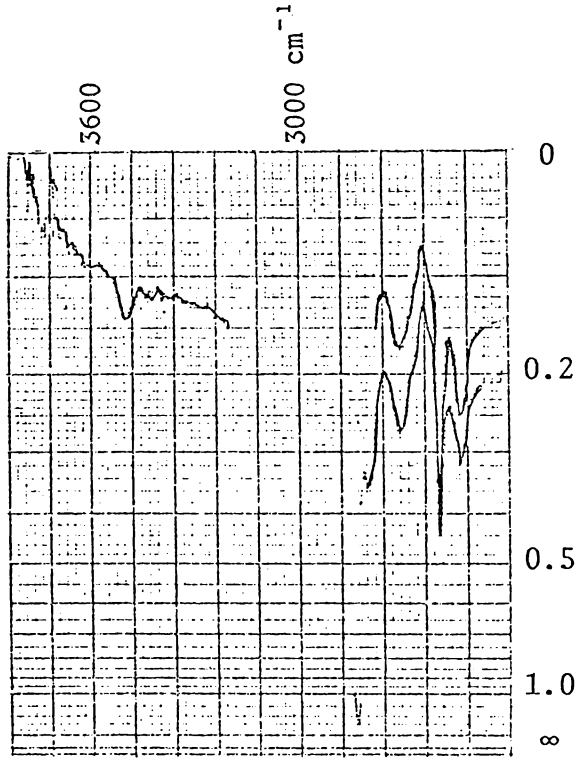



Fig. IR5.8. Spectrum of sporidesmin-B in carbon tetrachloride-deuterium oxide.

## §2. *Sporidesmin-B* (1.15)

The second epidithiodioxopiperazine toxin to be isolated from *Pithomyces chartarum* cultures (Ronaldson, *et al.*, 1963; Rahman and Taylor, 1967), sdm-B ($C_{18}H_{20}ClN_3O_5S_2$), had no secondary hydroxy-group, only the tertiary one. Without the secondary hydroxy-group absorptions, assignment of the peaks from sdm-B should be simplified.

Sdm-B dissolved more readily (in $CCl_4$, B-Cl) (Fig. IR5.7) than sdm and was made a 3.3 mM solution. The i.r. spectrum of this solution showed the following peaks in the *OH*-stretching region (30 mm cell):

1. $\nu$ 3592 $cm^{-1}$, $\varepsilon^a$ 69, $\Delta\nu^a_{\frac{1}{2}}$ 20 $cm^{-1}$, 12 relative peak area.
2. 3520 " , 35, 72 " , 50 " " "
3. 3394 " , 14, 70 " , 5 " " "

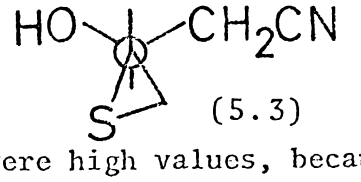
It was apparent that the main bands displayed by sdm-B (at 3592 and 3520 $cm^{-1}$) were also present in the spectrum of sdm. Whereas the peak at 3520 $cm^{-1}$ in sdm was small (rel. peak area 3) the corresponding peak in sdm-B was *c.* 16 times larger (rel. peak area 50) and at the same time the size of the peak at 3592 $cm^{-1}$ was smaller in sdm-B (*c.* 75% of that in sdm, by rel. peak area).

Apart from the $\pi$-bonding, discussed above, (and apart from the 11-*trans*-hydroxy-group in other sdm derivatives) the possible atoms to which the 10b-*OH*-hydrogen might hydrogen bond were 1. the amido-nitrogen, N-5, 2. the indoline nitrogen, N-6, and 3. the sulphur atom, S-12. There is no possibility of hydrogen bonding to an amido-nitrogen because an amido-nitrogen is planar, having no lone pair electrons (Robin, *et al.*, 1970). The indoline nitrogen in the sdm compounds was, if anything only very weakly basic; this nitrogen did not quaternize (A-E). Added to this, from Tichy's review (1965), it would seem that of the five-membered heterocycles only the thiacyclopentan-3-ol compounds showed intramolecular hydrogen bonding. In the Dreiding model, whether the C-S

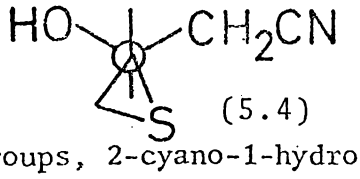
bonds were converging (epidithio-compounds), parallel (*S*-alkylated compounds) or diverging (epitrithio-compounds) there were minimal changes in the heterocyclic ring of the indoline moiety. Instead, upon modification of the -S-S- bridge of the epidithiodioxopiperazine ring (by *S*-alkylation, reduction to the dimercapto or incorporation of a third sulphur as in sdm-E, see later) the spectra were markedly altered: the absorptions of the 10b-OH were shifted to other frequencies (3553-3520 $cm^{-1}$). These changes will be shown to be consistent with the structure: 12-S···HO-10b. Some of these changes necessitated modification also in the OH···π-bonding which peak suffered reduction in size till it was absent in one compound (methyl 11a-mercaptosecosdm-3-*S*-acetate). So therefore, it is suggested that in sdm-B the intramolecular hydrogen bond was from the tertiary hydroxy-group, 10b-OH, to the sulphur atom, S-12, of the epidithio-group.

In the models the hydrogen atom was about 3.6 Å (Courtauld 3.4 Å and Dreiding 3.8 Å) from the sulphur atom and not far removed from the orbital of one of the latter's lone pair electrons. Applying the Kubota, *et al.* (1966) formula $\Delta\nu = 412 - 143R$ gave R = 2.2 Å. Although the Kubota formula may not strictly apply to these conditions the result did indicate that the electronic and atomic orientation in this part of the sdm and sdm-B molecules was such that hydrogen bonding was favoured.

Carlson, *et al.* (1970) described OH···S interaction in 1-cyano-2-hydroxy-3,4-epithiobutanes where $\Delta\nu$ was 138 and 123 $cm^{-1}$ (for the *threo* (5.3) and *erythreo* (5.4) isomers in $CCl_4$). In comparison with sdm-B




(5.3)




(5.4)

these were high values, because the two groups, 2-cyano-1-hydroxyethyl and cyclothiapropyl, could freely rotate to a position favouring intramolecular hydrogen bonding. In the sterically rigid sdm-B the $\Delta\nu$ was only 97 $cm^{-1}$, indicative of a weaker hydrogen bond.

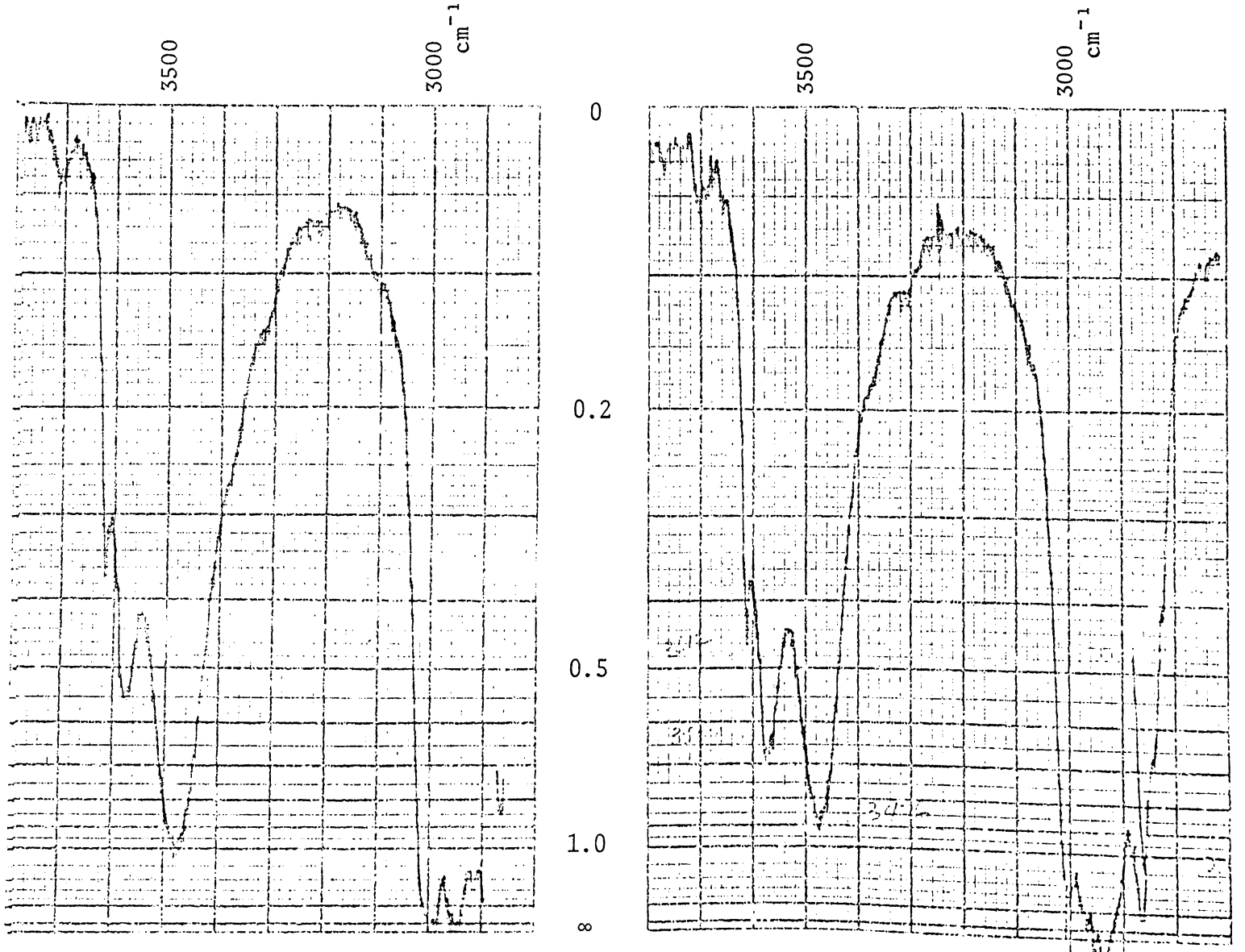
Where there are two OH stretching peaks (one free and the other hydrogen bonded) in the solution i.r. spectrum of a non-rigid monohydroxy-thio-compound their presence has been explained in terms of the possible conformations of the molecule (Luttringhaus, *et al.*, 1962). The free peak is proposed to arise when the conformation is in the form where the hydroxy-*H* cannot bond to any lone pair electrons and *vice versa* for the hydrogen-bonded peak. Thiacyclohexan-3-ol and thiacyclopentan-3-ol (Luttringhaus, *et al.*, 1962) both show this phenomenon but the above explanation does not explain the two peaks observed in the spectrum of thiacyclobutan-3-ol (thietan-3-ol, 3617 and 3601 $cm^{-1}$). This compound, according to the abstract (Chemical Abstracts, 1973) of the paper by Arbuzov, *et al.* (1971), has a saddle configuration with the 3-C substituent in a pseudoequatorial position. The rigidity in the conformation of thietan-3-ol is of such a kind that it allows only weak hydrogen bonding of the OH hydrogen to the S atom ($\Delta\nu$ 16). Thus, it is plausible to think that the hydrogen atom spends only a portion of its time within the range of the lone pair electrons of the sulphur atom hence the minor peak at 3601 $cm^{-1}$ and the major portion of its time outside the influence of the lone pair electrons hence the major sharp peak at 3617 $cm^{-1}$ (similar to a B rotamer, as defined by Joris, *et al.*, 1968). So with sdm-B assuming that there was OH•••π-interaction (3592 $cm^{-1}$) (as discussed above) the hydrogen of the tertiary-hydroxy-group shared its time between the influence of either the aromatic π-electrons or the lone pair electrons of the sulphur atom (S-12). The OH bond in sdm-B was (by the Courtauld model) more colinear (Tichy, 1965) with the sulphur lone-pair orbital than in thietan-3-ol, hence the greater $\Delta\nu$ value (*c.* 97 $cm^{-}$) for sdm-B.

The greater relative peak area (50 at 3520 $cm^{-1}$) in sdm-B than in sdm (rel. peak area 3) indicated that a greater percentage of hydroxy-hydrogens were participating in hydrogen bonding to the sulphur atom than

in sdm. Because there was no secondary hydroxy-group (11-OH) hydrogen bonding to the 1-CO, it is suggested that there was a local steric relaxation at the 11-methylene sufficient to allow a higher percentage of hydrogen bonding to the sulphur atom to occur in sdm-B. [$^{13}$C n.m.r. of sdm-B might reveal this effect if it could be determined in carbon tetrachloride (or $CS_2$) instead of the proton accepting solvent acetone.]

To obtain some information on the significance of the small broad peak (at 3394 $cm^{-1}$, sdm-B) the 3.3 mM solution was diluted to 2 mM and read in a 50 mm cell (B-C1) (Fig. IR5.7B). There was no change in the relative peak height or area thus establishing that it did not arise from dimerisation (Hanousek, 1964) or intermolecular hydrogen bonding. The peak remained after the sample was carefully dried (B-C2) so it was not due to hydrogen bonding to extraneous water. When the sample was deuterated (B-C3, 60°, 30 min) this peak was still present in the O-H stretching region (3394 $cm^{-1}$) (Fig. IR5.8) and not in the O-D stretching region (<2600 $cm^{-1}$). Thus it is suggested that it was an overtone from the carbonyl absorptions (1700—1650 $cm^{-1}$).

See end of next section (3) for the discussion of the effect of a proton acceptor solvent (EtOEt).




Figs IR5.9A and IR5.9B. Spectra of sporidesmin-D (A. sdm-D.EtOEt, 10 mM in 10 mm cell; B. sdm-D unsolvated, 2 mM in 50 mm cell) in carbon tetrachloride.

## §3. *Sporidesmin-D* (1.9)

In sdm and sdm-B the C-S-S-C bonds of the epidithio-group were rigidly held in position. In sdm-D the -S-S- bridge was opened and the two sulphur atoms methylated (Jamieson, *et al.*, 1969) (A-C1aiii). It was not possible to obtain crystals of sdm-D unsolvated; it occurred as the etherate.

Sdm-D ($C_{20}H_{26}ClN_3O_6S_2.C_4H_{10}O$), isolated from *Pithomyces chartarum* cultures by Jamieson, *et al.* (1969) was found to be relatively inactive, toxicologically (Taylor, 1971), which suggests that the toxicity of sdm and its toxic analogues lay in the epipolythio-group.

Although sdm-D.EtOEt still had the two hydroxy-groups it was infinitely more soluble ($CCl_4$) (B-D1) than either sdm or sdm-E. The i.r. spectrum of the 10 and 2 mM solutions (10 and 50 mm cells) of sdm-D showed negligible differences (between each other) in the following peaks (Fig. IR5.9).

1. $\nu$ 3592 $cm^{-1}$, $\varepsilon^a$ 40, $\Delta\nu_{\frac{1}{2}}^a$ shoulder, 4 relative peak area.
2. 3550 " , 59, " , 16 " " "
3. 3455 " , 110, *c.* 190 $cm^{-1}$, 146 " " "

Compared with sdm, sdm-B and sdm-E, the spectrum of sdm-D showed considerable differences. Though the frequency of the peak at 3592 $cm^{-1}$ was unchanged from the former, it was only a minor peak (rel. peak area 4). The $\Delta\nu$ (3618 - 3550 = 68 $cm^{-1}$, Oki, *et al.*, 1968) for the peak at 3550 $cm^{-1}$ indicated a weaker hydrogen bonding for this structure (OH•••-O=C, sdm-D) than in the former compounds ($\Delta\nu$ 98, 98). While the peak at 3455 $cm^{-1}$ showed its hydrogen bonding to be only fractionally weaker (than sdm, 3450 $cm^{-1}$) in sdm-D ($\Delta\nu$ = 3627 - 3455 = 172 $cm^{-1}$). Although O-H stretching absorption intensities or areas do not correlate quantitatively with structure (unlike the correlation between protons and proton peak intensities in n.m.r.), in a semiquantitative way peak areas

have some significance. Where a considerable percentage (*c.* 12 and 18%) of the O-H stretching appeared in the OH•••π-condition in sdm and sdm-B, in sdm-D only a small percentage (*c.* 2.4%) occurred as OH•••π-bonding. But what was a small peak (3520 $cm^{-1}$) in sdm (rel. peak area 3) was five times as large in sdm-D (rel. peak area 16) suggesting that the configuration of sdm-D enabled more of the hydroxy-hydrogens (10b-OH) to bond to the sulphur atom rather than to that aromatic π-electrons. The configuration of sdm-D had these features: 1. in the Dreiding model the 10b-OH•••S plane was *c.* 10% further removed from the hydrogen of the CH-11 than in sdm, so the 10b-OH•••S hydrogen bonding was less sterically crowded by the methine-*H* of the 11-carbinyl (whose OH was hydrogen bonded to the 1-CO, Δν 77 $cm^{-1}$) and 2. the methylthio-group was free to rotate till one pair of the sulphur's lone-pair electrons was more nearly colinear with the O-H bond. But the result of these combined effects was not as great as that observed above in sdm-B, where the relative peak (3520 $cm^{-1}$) area was *c.* 50, because in sdm-B there was no 11-OH•••O=C hydrogen bond to thrust the C-11 hydrogen in the way of the 10b-OH•••S hydrogen bond.

Though the bond length (the 1-CO oxygen moved away from the 11-OH hydrogen on opening the -S-S- bridge, Plate 5.1) of the 11-OH•••O=C hydrogen bond was slightly greater in sdm-D (*c.* 1.7 Å calc., compared with 1.6 Å for sdm) and therefore, that much weaker, the corresponding band (3455 $cm^{-1}$) was *c.* 30% larger (by rel. peak area) than the one in sdm.

*The effect of ether on the spectra of sdm-D and sdm-B.*-According to the work of Bellamy, *et al.* (1966) and Kuhn (1952) ether functioned as a hydrogen acceptor in hydrogen bonding. The former workers used 5% solutions (*c.* 480 mM) of ether in carbon tetrachloride, which was a large excess over the concentration of the solutes (2—10 mM).

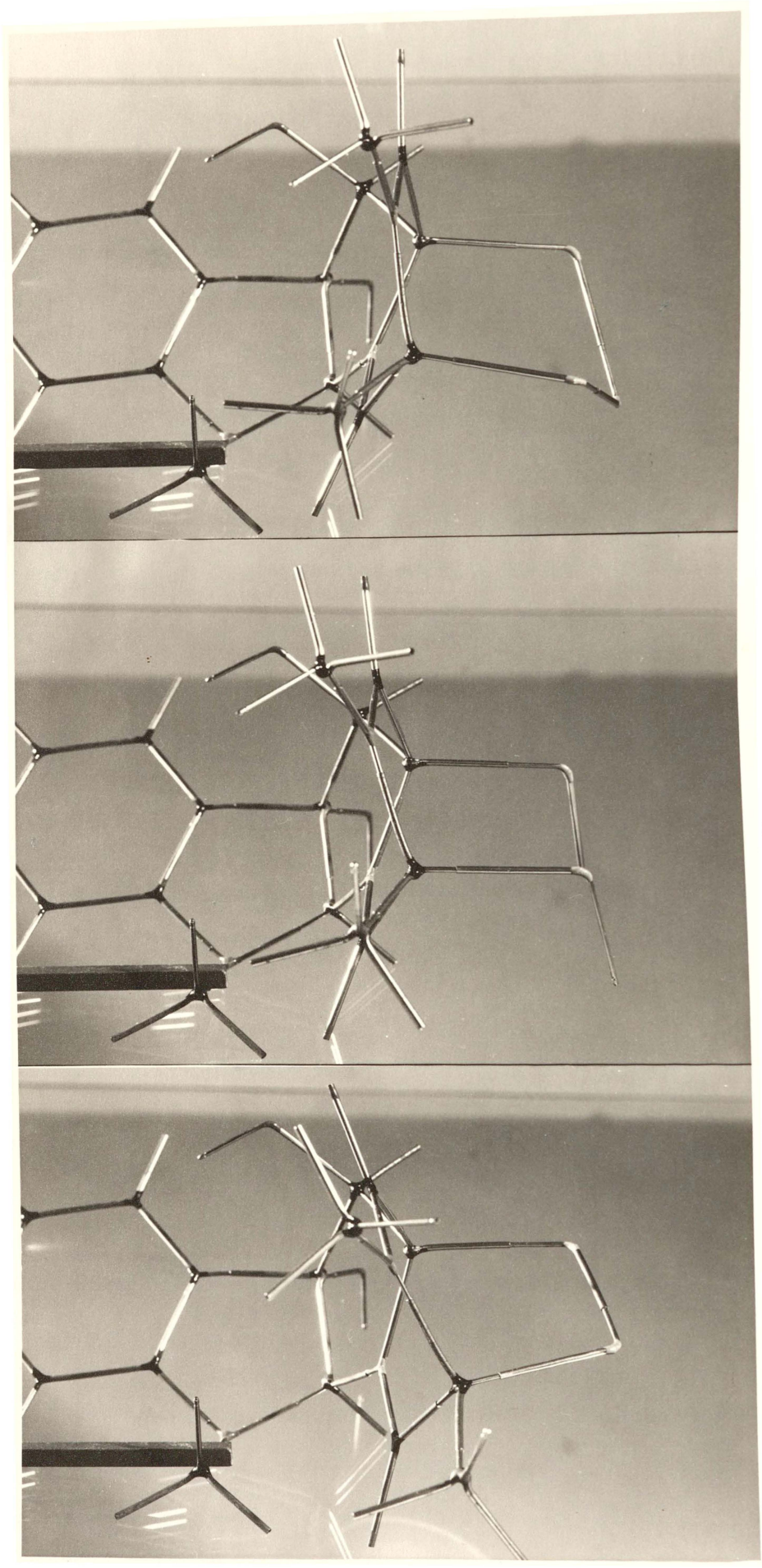



Plate 5.1. For caption see facing page.

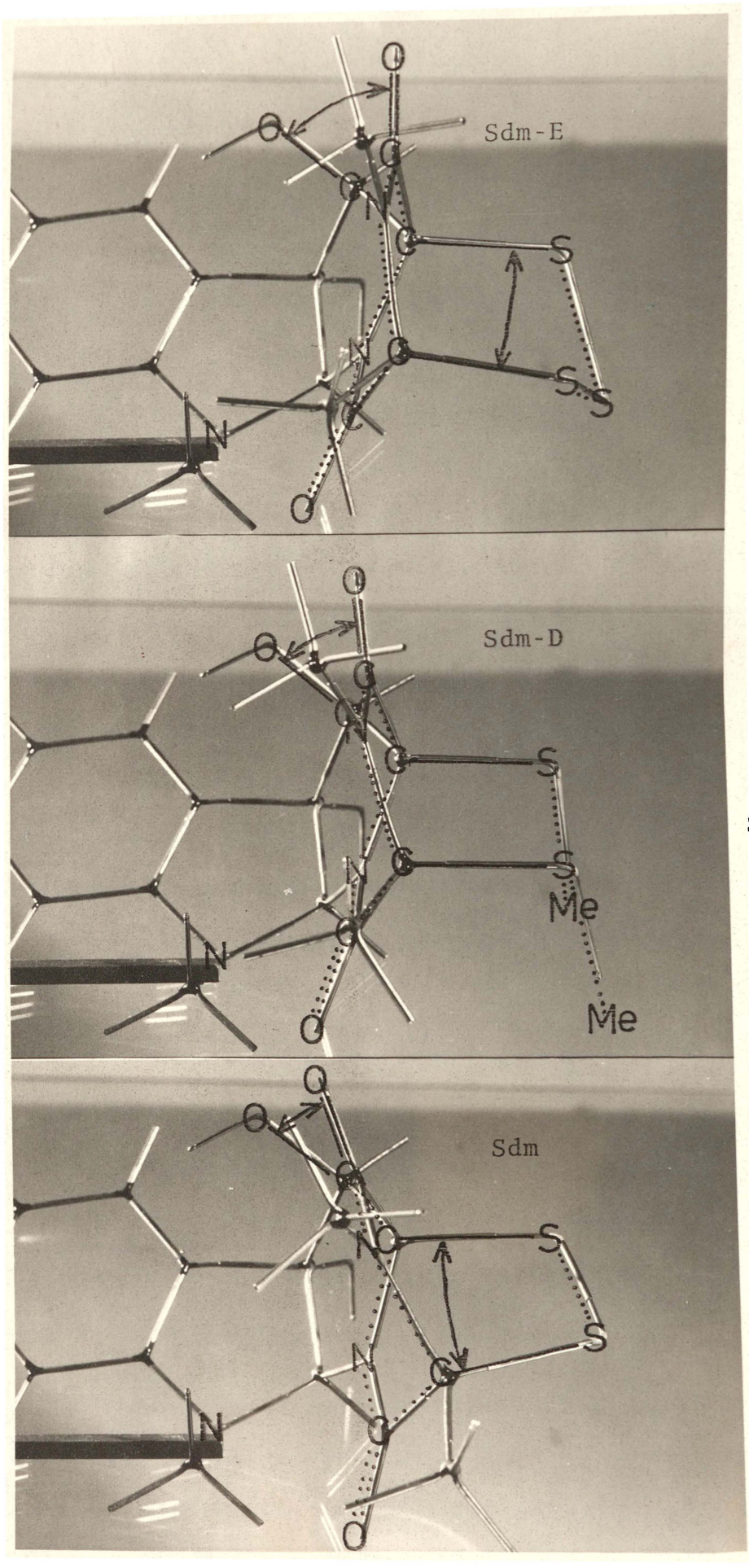
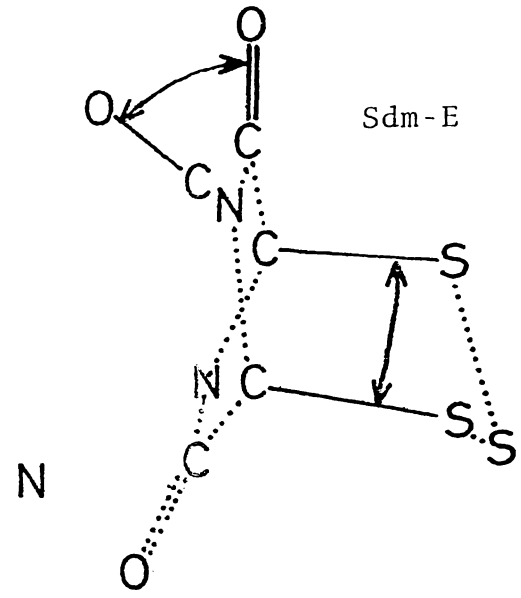
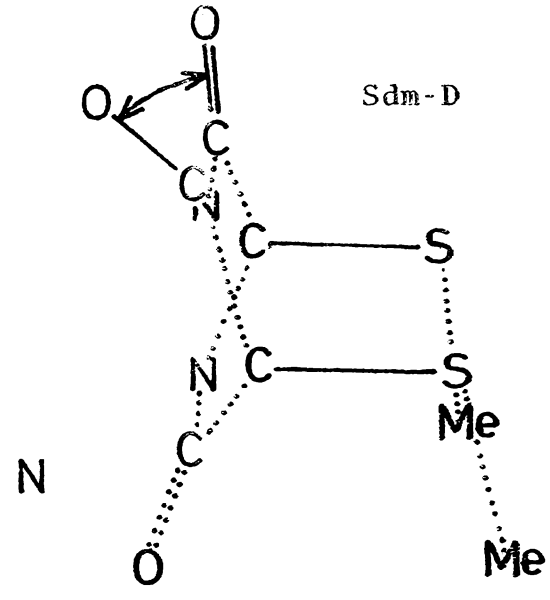



Plate 5.1. For caption see facing page.


Sdm-E


Sdm-D
Me
Me

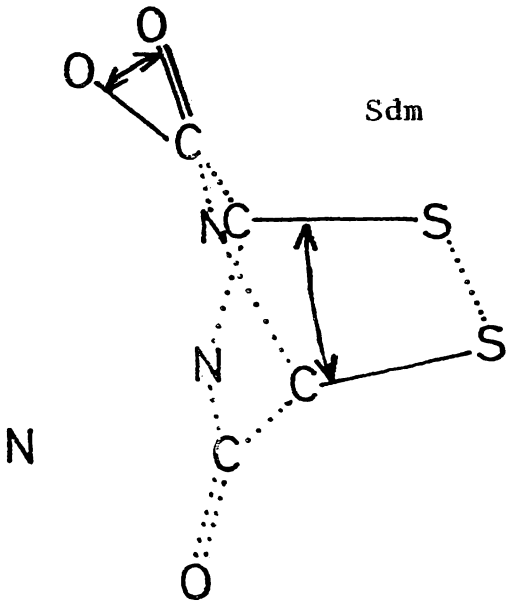

Sdm

Plate 5.1. Dreiding models showing the orientation of some bonds in three sporidesmins: sporidesmin-E (sdm-E) (epitrisulphide group on the right), sporidesmin-D (sdm-D) (methyl groups of the *S*-methyls not in the model but shown opposite) and sporidesmin (sdm). The arcs between the C-S bonds indicate their divergence, parallelness and convergence (resp.), while the arcs between the C-O bonds (11-OH, 1-C=O) indicate the respective changes resulting from the changes made to the orientation of the C-S bonds. (The hydrogen of the 11-OH in these models is not in the hydrogen-bonded position.) The greater the arc the greater the interatomic distance between the oxygens. The dotted lines (on the right) indicate S-S and S-Me bonds while the others indicate the dioxopiperazine rings. The positions of the third nitrogen are also indicated.

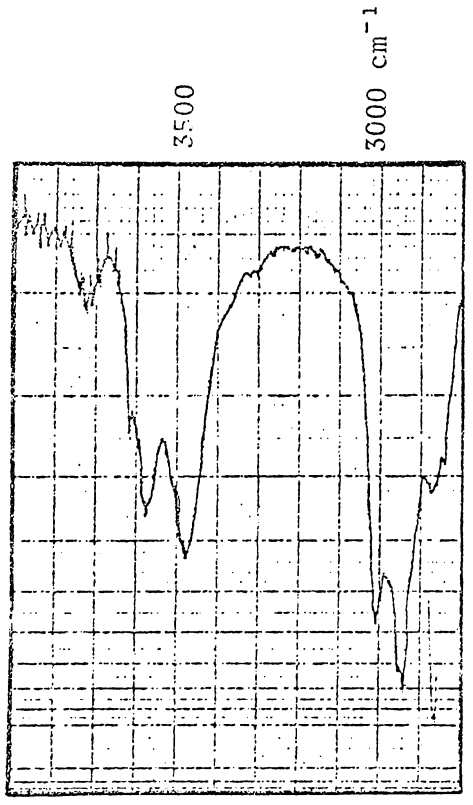



Fig. IR5.10. Spectrum of sporidesmin-D unsolvated, in carbon tetrachloride (2 mM in 20 mm cell).

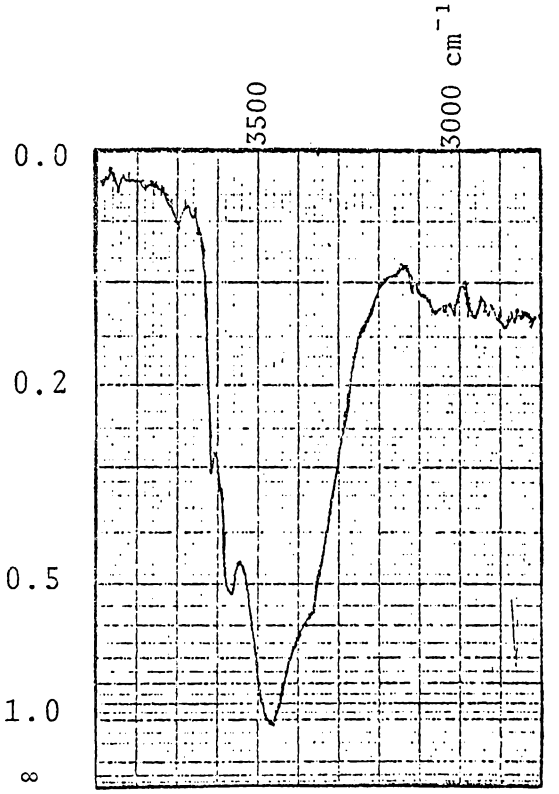



Fig. IR5.11. Spectrum of sporidesmin-D.EtOEt in 5% ether-carbon tetrachloride (*c.* 10 mM in 10 mm cell).

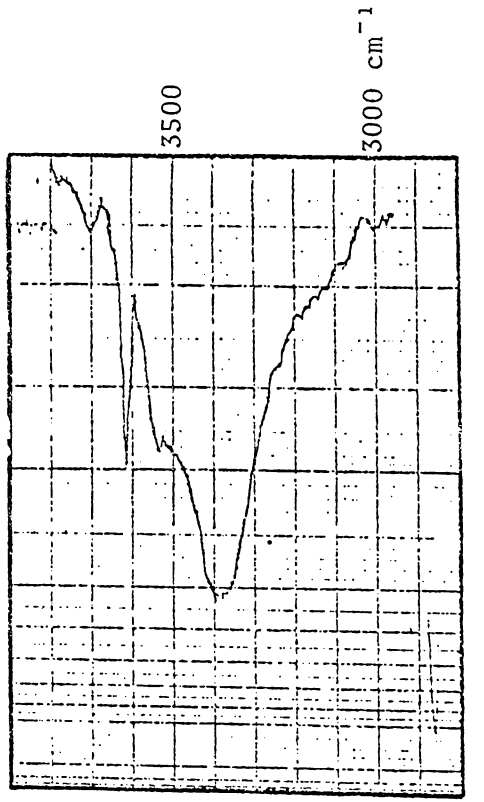



Fig. IR5.12. Spectrum of sporidesmin-B in 5% ether-carbon tetrachloride (5 mM in 20 mm cell).

For sdm-D.EtOEt (a 1:1 molar ratio) (Fig. IR5.9A) intermolecular hydrogen bonding to the ether molecule was not observed between the 2 and 10 mM solutions (the 10 mM solution had been evaporated before dilution to 2 mM). This observation was not surprising since intermolecular hydrogen bonding between the same kind of molecules begins at about 10 mM and increases with increasing concentration. After evaporating the solution to dryness and desiccating there was no change in any of the absorptions (Figs IR5.9B, 5.10).

But when the concentration of the ether (sdm-D) was raised (to *c.* 480 mM, a 48 molar excess) (B-D3) a shoulder appeared at *c.* 3360 $cm^{-1}$ (Fig. IR5.11). There was no alteration in the intensity of the peak at 3455 $cm^{-1}$. Both the peaks at 3592 and 3550 $cm^{-1}$ were reduced in area. Though the former was small in sdm-D, yet it was still present in the 5% ether solution. It was as though the proton accepting molecules of ether had incompletely competed with the sulphur atom and the π-electrons for hydrogen bonding with the 10b-OH and not at all with the 1-CO for the 11-OH hydrogens.

This observation was confirmed with sdm-B (in 5% EtOEt in $CCl_4$), where the spectrum (Fig. IR5.12) (B-C4) showed a large peak ($\varepsilon^a$ 52, $\Delta\nu^a_{\frac{1}{2}}$ 240 $cm^{-1}$) at 3460 $cm^{-1}$. Here again both the peaks (at 3592 and 3520 $cm^{-1}$) were reduced in area; the latter was only a shoulder ($\varepsilon^a$ 26). So the ether competed with both the sulphur atom and the π-electrons of the aromatic ring for the 10b-OH hydrogen.

## §4. *Sporidesmin-E* (1.10)

(See also Chapter 4, 'Sporidesmin-E, its synthesis, and comparison of its infra-red and $^{13}C$ nuclear magnetic resonance spectra with those of sporidesmin'.)

The spectrum of sdm-E (Fig. IR4.11) (*c.* 2 mM in $CCl_4$, 50 mm cell) (B-E1) showed the following peaks:

1. $\nu$ 3590 $cm^{-1}$, $\varepsilon^a$ 103, $\Delta\nu^a_{\frac{1}{2}}$ 32 $cm^{-1}$, 14.4 relative peak area.
2. 3530 " , 60, 100 " , 81 " " "

Although the incorporating of the third sulphur atom (S-13) between the two in sdm (3-S, S-12) was only a small change to the molecule (see Chapter 4), the alteration shown in the solution spectra (from sdm, Fig. IR5.4, to sdm-E) was considerable. This alteration was reasonably attributed to the influence of the S-13 sulphur atom. By the figure (Plate 5.1) it may be observed that in sdm the C-S bonds converged, in sdm-D they were almost parallel and in sdm-E they diverged. This divergence of the C-S bonds distorted the dioxopiperazine ring from the relaxed condition in sdm-D or the strained condition (in the opposite sense) in sdm. This distortion was propagated into the adjacent pyrrolidine ring so that the interatomic distance between the oxygen of the 1-CO and the hydrogen of the 11-OH (as seen in Plate 5.1) was increased from that in sdm. Intramolecular hydrogen bonds weaken with increase in internuclear distance and the weakening is indicated by smaller $\Delta\nu$ values (3627 - 3530 = 97 $cm^{-1}$). This state was reflected in the spectrum (Fig. IR4.11) where the peak which in sdm (Fig. IR5.4, 3450 $cm^{-1}$) indicated the strong hydrogen bonding ($\Delta\nu$ 177 $cm^{-1}$) of the 11-OH hydrogen to the oxygen of the 1-CO, was now in a position (3530 $cm^{-1}$) which was similar to that for the 10b-OH•••S in sdm-B (3520 $cm^{-1}$). So the spectrum showed only two peaks; the 10b-OH•••$\pi$-interaction at 3590 $cm^{-1}$ (as observed in previous spectra) and the broad one at 3530 $cm^{-1}$ consisting of the two intramolecular absorptions 11-OH•••OC (as discussed above) and

Table 5.1. Relative peak areas for sporidesmin and derivatives

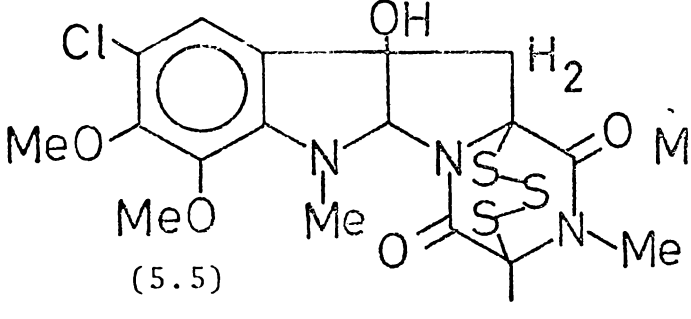
| Frequency | 3592-0 | 3553-43 | 3535-30 | 3520 | 3474-0 | 3455-0 $cm^{-1}$ | Total relative peak area |
|---|---|---|---|---|---|---|---|
| Sdm (1.1) | 16 | | | 3 | | 113 | 132 |
| Sdm-B (1.15) | 12 | | | 50 | | | 62 |
| Sdm-D (1.9) | 4 | 16 | | | | 146 | 166 |
| Sdm-E (1.10) | 14.4 | | 81 | | | | 95 |
| 3-SH-COOMe (1.13) | 1.6 | 10 | | | 147 | | 158 |
| 11a-SH-COOMe (1.14a) | | 17 | | | 107 | | 124 |
| di-SH (1.11) | *c.* 9 | | *c.* 4 | *c.* 54 | | | - |

10b-OH•••S (which was common to the previous spectra). In the spectrum (Fig. IR4.11) there is a suggestion that the broad peak at 3430 $cm^{-1}$ was split into a doublet.

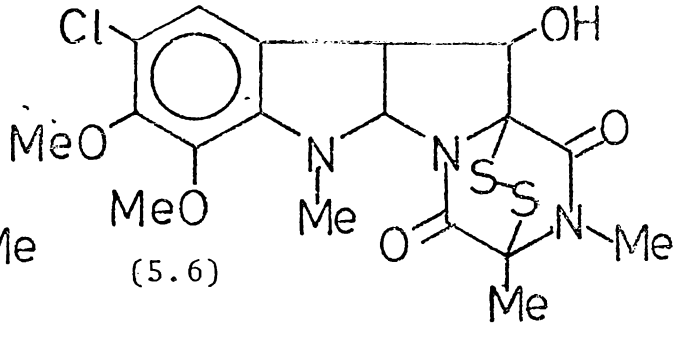
Applying the Kubota, *et al.* (1966) formula $\Delta\nu = 97\ cm^{-1} = 412 - 143R$ gave R = 2.3 Å whereas the measured value on a Dreiding model was 1.7 Å. This wide discrepancy pointed to the lack of colinearity of the O-H bond with the long pair electrons on the C=O oxygen.

It is noteworthy that the total relative peak area (for the dihydroxy-compounds, 124-166, Table 5.1) was least (95) for this compound. This is a function of the well-known observation that the lower the frequency of a peak the broader and intenser the peak.

To examine the individual contributions by the tertiary hydroxy- or the secondary hydroxy-hydrogens to the hydrogen-bonding peaks, it would be necessary to synthesize the epitrithio-derivatives of sdm-B (5.5) and 10b-deoxysdm (5.6). The synthesis of the latter compound from sdm is a




(5.5)




(5.6)

major undertaking, while that of the former is delayed by the lack of supply of sdm-B.

*Other solvents.*-As with sdm the problem of the low solubility ($CCl_4$) when only 10 mm cells were available, was partially overcome by dissolving the solvated compound in chloroform (50 µl) and making up to volume (1 ml) with carbon tetrachloride (e.g. B-E2) and using the same solvent mixture in the reference beam (in 10 mm cells). In this way (e.g. Fig. IR4.14) although the band frequencies were unaltered the peak heights were enhanced by *c.* 3 times since this solution was *c.* 5.3 mM.

(Hence the concentration of the nearly saturated solution of unsolvated sdm-E ($CCl_4$) was *c.* 2 mM, at room temperature.)

As with sdm-D.EtOEt, when a saturated solution of sdm-E.EtOEt (B-E3) was examined in carbon tetrachloride (Fig. IR4.13), although the molar ratio of ether to sdm-E was 1:1 there was no sign of intermolecular hydrogen bonding to the ether (at less than 3500 $cm^{-1}$).

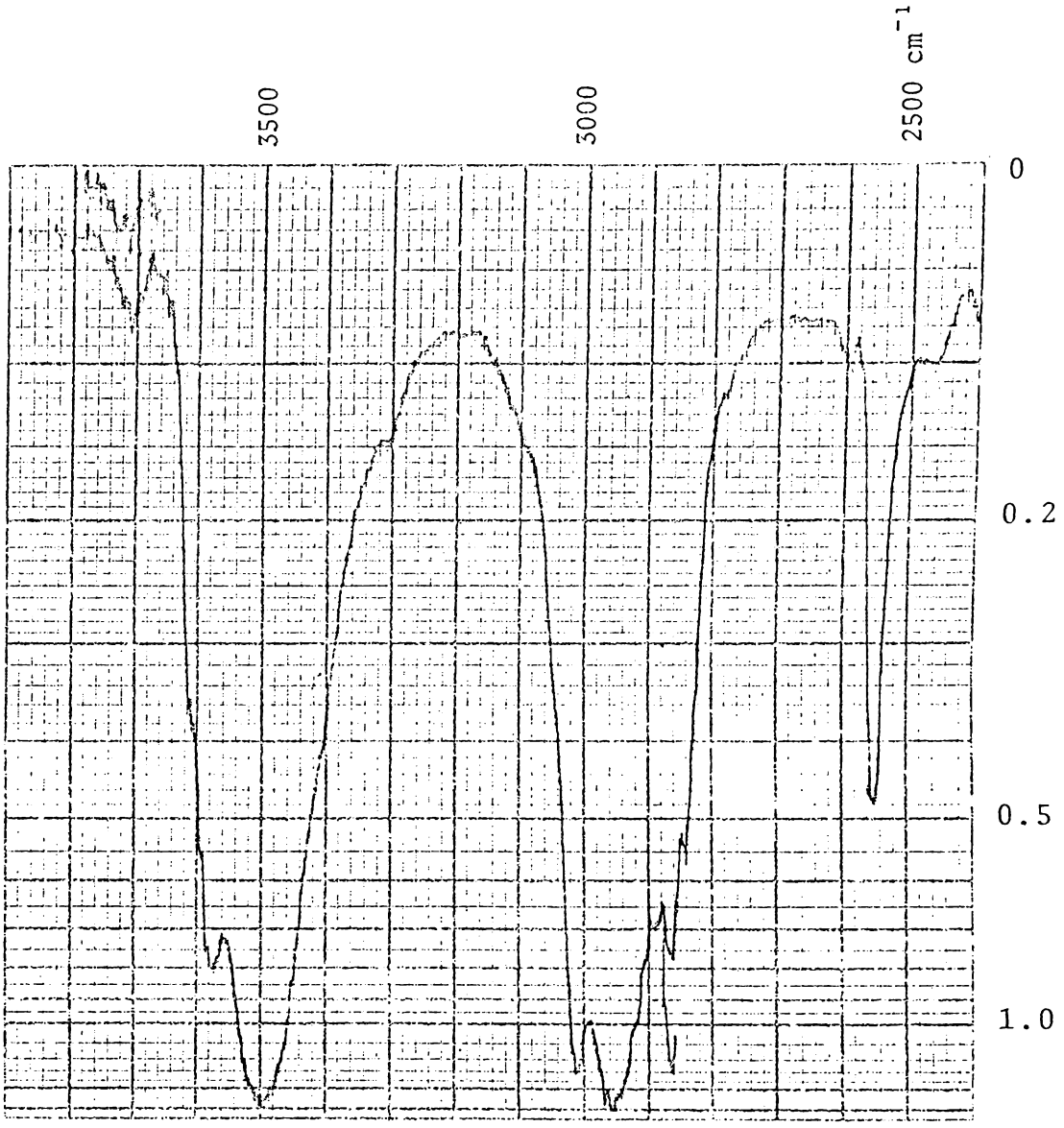



Fig. IR5.13. Spectrum of methyl 3-mercaptosecosporidesmin-11a-*S*-acetate in carbon tetrachloride (6 mM in 20 mm cell).

## §5. *Methyl 3-mercaptoecosporidesmin-11a-S-acetate* (1.13)

The high frequency i.r. spectrum ($CCl_4$) of this compound, synthesized (A-C5c) from sdm, showed these peaks (Fig. IR5.13); it was more freely soluble ($CCl_4$) (B-F1) than sdm, so a 6.3 mM solution was examined in a 20 mm cell:

1. $\nu$ 3592 $cm^{-1}$, $\varepsilon^a$ shoulder, $\Delta\nu_{\frac{1}{2}}^a$ - 1.6 relative peak area.
2. 3550 " , weak peak, - 9.6 " " "
3. 3474 " , 126, 230 $cm^{-1}$, 147 " " "
4. 2540 " , 32, 30 " , 16.6 " " "

(The relative peak areas recorded here have been adjusted from the values for the 20 mm of the 6.3 mM solution to those for 20 mm of a 5 mM solution).

As it might be expected the spectrum (Fig. IR5.13) of this compound between 3700 and 3200 $cm^{-1}$ was similar to that of sdm-D (Fig. IR5.9), except that the peak at 3592 $cm^{-1}$ has nearly disappeared, and that at 3550 $cm^{-1}$ was weaker and the third peak has shifted to a higher frequency (from 3455 to 3474 $cm^{-1}$).

The major peak (3474 $cm^{-1}$) was attributed (as in the previous compounds) to the 11-OH•••O=C interaction. The intramolecular hydrogen bonding was progressively weaker in going from sdm ($\Delta\nu$ 177 $cm^{-1}$), to sdm-D ($\Delta\nu$ 172 $cm^{-1}$) and to this compound ($\Delta\nu$ 153 $cm^{-1}$), but the relative peak areas for the latter two compounds were the same (146 and 147 resp.). Though, from the Kubota, *et al.* (1966) formula $\Delta\nu = 412 - 143R$, the internuclear distance between the hydrogen of the 11-OH and oxygen of the 1-C=O was 1.8 Å (0.2 Å greater than in sdm and consequently weaker) yet the percentage of hydrogens participating would be the same as for sdm (100%) since there were no other $\nu$OH bands attributable to these groups in the spectra of either compound. This progressive weakening of the hydrogen bonding was associated with the relaxation of the

strain applied to the epidithiodioxopiperazine ring in going from sdm to sdm-D (as mentioned above) and then to the steric effect of the methoxycarbonylmethylene on the environment of the S-12 sulphur atom. That the relative peak area for a hydroxy-group of one compound cannot be used to predict quantitatively the number of hydroxy-groups for another compound was exemplified here. For this compound and sdm-D the relative peak area for one 11-OH group was 147 and for the corresponding group in sdm and in methyl 11a-mercaptosecosdm-3-*S*-acetate the relative peak areas were 113 and 107 (resp.) (see Table 5.1); the former were 40% larger.

By the same token, for the corresponding hydroxy-groups (10b-OH) in this compound and sdm-D, the combined relative peak area (11) of the two absorbances at 3592 and 3550 $cm^{-1}$ was only about half of the area of those in sdm-D (20). It seems that the OH•••π-bonding was least in this compound compared with the previous ones so the S-12 sulphur atom must be in the most favourable position to compete against the aromatic π-electrons for the 10b-OH hydrogens compared with its position in the others. In the models the internuclear distances between the hydroxy-hydrogens and the sulphur atoms in sdm-D and this compound (1.13) were the same: the frequency of the absorbances (3550 $cm^{-1}$) were the same in the two compounds but the intensities were considerably different.

The fourth absorbance at *c.* 2540 $cm^{-1}$ was typical of an S-H stretching absorbance (Jones and Sandorfy, 1956). Among the possibilities of intramolecular SH hydrogen bonding (SH•••X) in this compound the following were the more likely, in increasing order of likelihood: an interaction between the thiol group and

1. either of the oxygens of the carbonyl groups (1-CO or 4-CO); the thiol group was about equidistant (3.3—3.5 Å) from either oxygen and could form either a 6- or 5-membered ring (resp.)
2. either of the oxygens of the 11a-*S*-acetate group; this group may

readily dispose to form a strong hydrogen bond, the ring would be 10 membered and

3. the S-12 sulphur atom; by the Dreiding model this sulphur atom was *c.* 1.4 Å from the thiol hydrogen, a 7-membered ring.

In the $^1$H n.m.r. spectrum (see Chapter 2) of this compound, long range coupling ($^4J$ *c.* 2 Hz) was observed between a split methyl peak and a single proton peak split into a quartet. Long range coupling requires coplanarity (Jochims, *et al.*, 1969): the H-S of the $HSCCH_3$ group must be coplanar with the $CCH_3$ group. For this to occur the S-H group must be either adjacent to the methyl group or opposite to it, i.e. hydrogen bonded to the S-12 sulphur atom. So this $^1$H n.m.r. observation ruled out the possibility of the 3-SH hydrogen bonding to either of the carbonyl oxygens (1-CO or 4-CO) or to either of the oxygens of the oxycarbonyl group of the 11a-*S*-acetate, for in none of these cases would the S-H bond be coplanar with the C-$CH_3$ bond. Further, from the work of Mori, *et al.* (1971) it would appear that α- and β-mercaptoalkanoates (e.g. derivatives of acetic to butyric acids) and β- and γ-mercaptoethoxyalkanes do not form intramolecular hydrogen bonds. An intramolecular hydrogen bond in these compounds would form 5- or 6-membered rings with either the carbonyl-*O* or the alkoxy-*O*. In these compounds the peaks they observed (*c.* 2583 and *c.* 2577 $cm^{-1}$) were comparable with those they observed in alkyl mercaptans (2586 and *c.*2576 $cm^{-1}$).

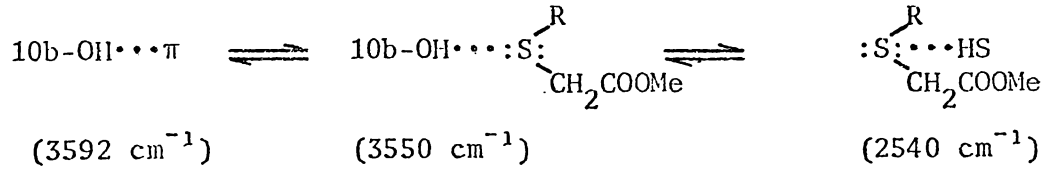
The frequency and intensity of the peak for this SH group (in methyl 3-mercaptosecosdm-11a-*S*-acetate) was comparable with that for the intramolecularly hydrogen bonded thiols (Mori, *et al.*, 1971) of *o*-mercaptobenzyl ether and thiosalicylic acid and esters (2560—2523 $cm^{-1}$, $\varepsilon^a$ 8—32). They attributed these low frequency bands to hydrogen bonding because they were absent in mercaptobenzenes and *p*-mercaptobenzoates.

In the S-H stretching region, the bands did not cover such a great

number of frequencies as was observed in the O-H stretching region. As yet not much data have been recorded for intramolecularly hydrogen-bonded thiol compounds especially the SH•••S type (Zuika and Bankovskii, 1973) so it is not possible to make structural deductions from the S-H stretching band frequencies and intensities. All that could be observed was that the well defined peak at 2540 $cm^{-1}$ was consistent with the SH•••S bond suggested from the $^1H$ n.m.r. work.

In the spectrum of methyl 3-mercaptosecosdm-11a-*S*-acetate (Fig. IR5.13) there was the suggestion of a peak at 2580 $cm^{-1}$ ($\varepsilon^a$ 2) which may be the absorbance for the free 3-SH group.

So it is suggested that one lone pair of electrons on the S-12 sulphur atom was hydrogen bonded to the 10b-OH (10b-OH•••S) and the lone pair on the other side hydrogen bonded to the 3-SH (3-SH•••S). They may or may not hydrogen bond simultaneously but there could be an overall equilibrium thus:



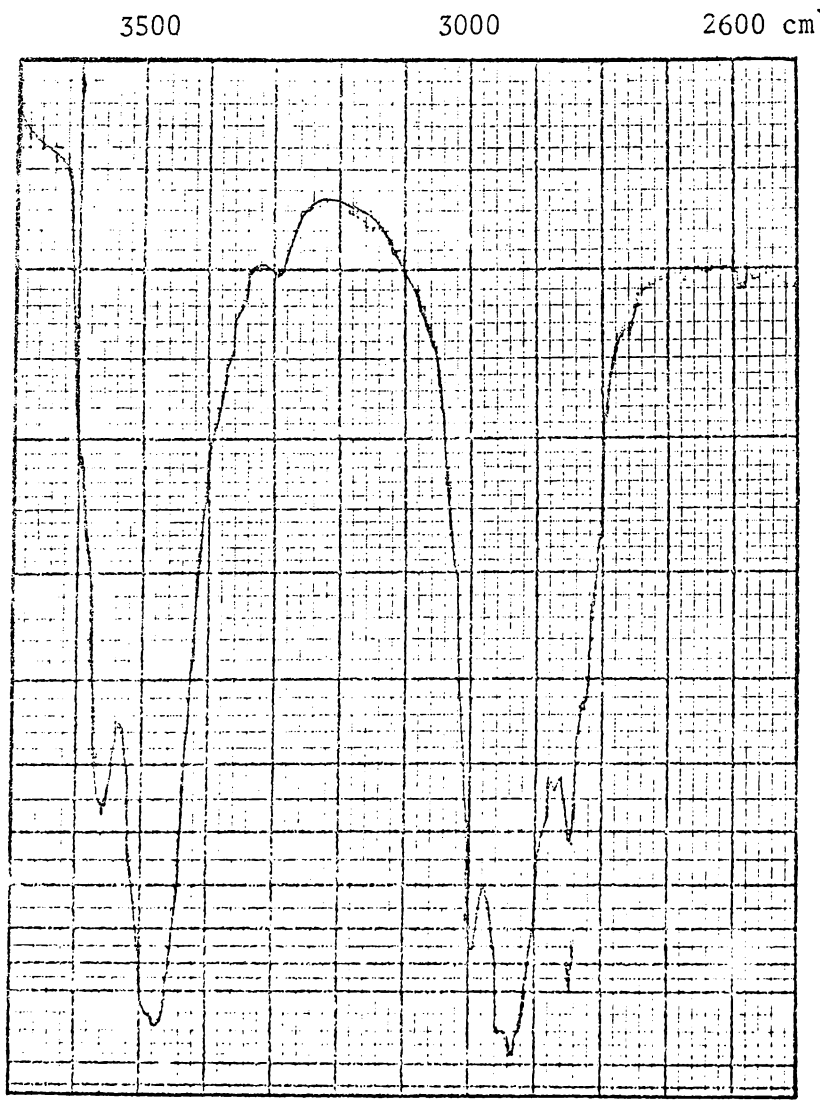



Fig. IR5.14A. Spectrum of methyl 11a-mercapto-secosporidesmin-3-*S*-acetate in carbon tetrachloride (1.8 mM in 50 mm cell).

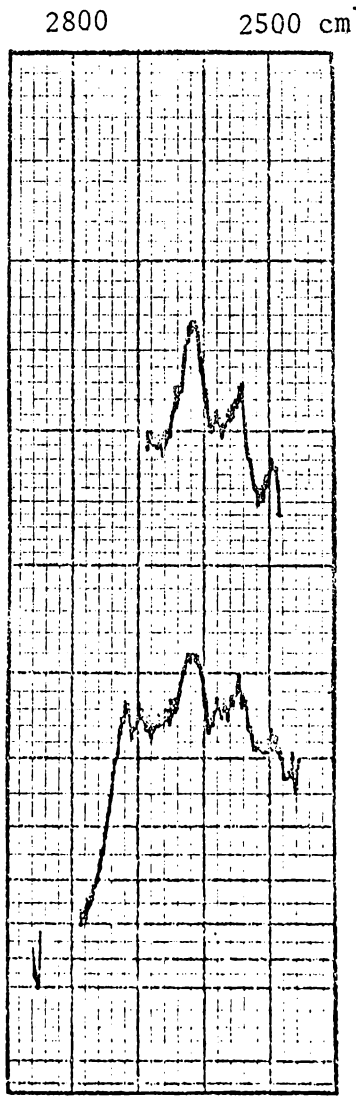



Fig. IR5.14B. Spectrum (2700 to 2500 cm$^{-1}$) of methyl 11a-mercaptosecosporidesmin-3-*S*-acetate in 2.5% chloroform-carbon tetrachloride (13.3 mM in 50 mm cell).

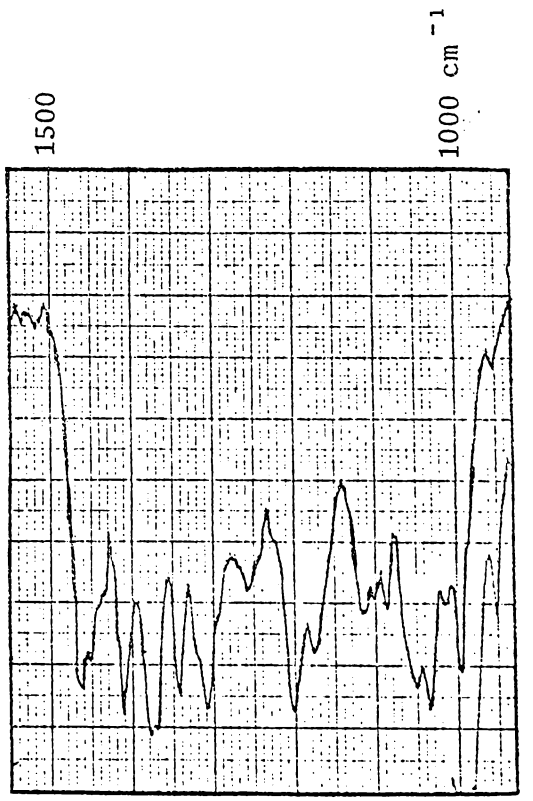



Fig. IR5.15. Spectrum (1500—1000 cm$^{-1}$) of methyl 11a-mercaptosecosporidesmin-3-*S*-acetate (KBr).

§6. *Methyl* 11a-*mercaptosecosporidesmin*-3-S-*acetate*.$H_2O$ (1.14a)

This isomer (of the previous compound) also synthesized from sdm (A-C5b) was poorly soluble in carbon tetrachloride. The unsolvated compound was about twice as soluble. Upon heating (56°) a suspension of the crystalline monohydrate (sufficient to make 5 ml of 1.8 mM solution in $CCl_4$) under reduced pressure (*c.* 400 mm Hg) the water and any residual methanol (from recrystallization) were removed as the crystals dissolved (B-G1). This solution (50 mm cell) gave the following peaks (Fig. IR5.14A):

1. ν 3553 $cm^{-1}$, $\varepsilon^a$ 60, $\Delta\nu^a_{\frac{1}{2}}$ - 17 relative peak area.
2. 3470 " , 129, 120 $cm^{-1}$, 107 " " "
3. 2510 " , 1.7, - a very weak peak.

The mercapto-structure of this compound was established from mass spectral data (see Chapter 2).

This spectrum (3650 — 3200 $cm^{-1}$) was similar to those of both sdm-D and methyl 3-mercaptosecosdm-11a-*S*-acetate, except that there was no peak for OH•••π-electron interaction. This was present in sdm-D, negligible in the 3-mercapto-compound and absent in this compound. The peak at 3470 $cm^{-1}$ was consistent with the corresponding peak in the 3-mercapto-compound (3474 $cm^{-1}$). As assigned previously this band (3470 $cm^{-1}$) was attributed to the 11-OH•••O=C interaction. Though the compound was a mercapto-compound any S-H stretching band (2600–2500 $cm^{-1}$) was non-existent.

It is suggested that hydrogen bonding of the 10b-OH hydrogen to the S-12 sulphur atom was so strongly favoured that 10b-OH•••π-interaction was inhibited. The 10b-OH then showed complete hydrogen bonding to the 11a-SH sulphur atom (3553 $cm^{-1}$). In these two isomers as described under 'sporidesmin-D' (above), the steric interference of the methine-*H* (C-11) with the 10b-OH•••S-12 interaction was negligible, owing to the

relaxation of the dioxopiperazine ring consequent upon the opening of the -S-S- bridge.

Since both the thiol in the 3-mercapto-compound and that in this compound were '*para*' to each other on a dioxopiperazine ring it would be expected that the two thiol groups would have comparable S-H stretching bands. Instead, in this compound, the i.r. spectrum (Fig. IR5.14A) would indicate that there was no SH group present. When a 13.3 mM solution ($CHCl_3$-$CCl_4$ 1:39) (B-G2) was examined, three peaks (Fig. IR5.14B) appeared between 2600 and 2500 $cm^{-1}$. [Above it was indicated that hydroxy-compounds begin intermolecularly hydrogen bonding with increasing concentration (at *c.* 10 mM in $CCl_4$) but thiol compounds tend not to be so active till the concentration is considerably greater (*c.* 163 mM benzenethiol in $CCl_4$, Josien, *et al.*, 1957a).] The strongest of these showed an apparent ε of 1.7. This value was less than that obtained by Mori, *et al.* (1971) for simple aliphatic (methyl to t-butyl) mercaptans ($\varepsilon^a$ 2.2–2.7). Bellamy (1958b) noted that SH peaks are sometimes difficult to detect but gave no examples. On the other hand, these peaks may not arise from S-H stretching vibrations. Josien, *et al.* (1957b) suggested that a weak peak at 2580–2570 $cm^{-1}$ in a series of *p*-halogenobenzenethiols or even as low as 2544 $cm^{-1}$ (*o*-bromobenzenethiol) arose from the sum of two other strong peaks in the 1500–1000 $cm^{-1}$ region. This weak peak at 2510 $cm^{-1}$ almost equalled the sum of the two at 1465 and 1040 $cm^{-1}$ in the KBr spectrum (Fig. IR5.15). Deuteration was not attempted because when the 10b-OH hydroxy-group was deuterated then its O-D stretching peak would cover the 2600–2500 $cm^{-1}$ band so that an unequivocal answer would not be obtained.

In case the S-H stretching band should be suppressed by hydrogen bonding to the 10b-OH, the methyl 11a-mercaptosecosdm-3-*S*-acetate di-(chloroacetate) was synthesized (B-G3) to remove the 10b-OH function. Methyl 11a-mercaptosecosdm-3-*S*-acetate was dissolved in pyridine and

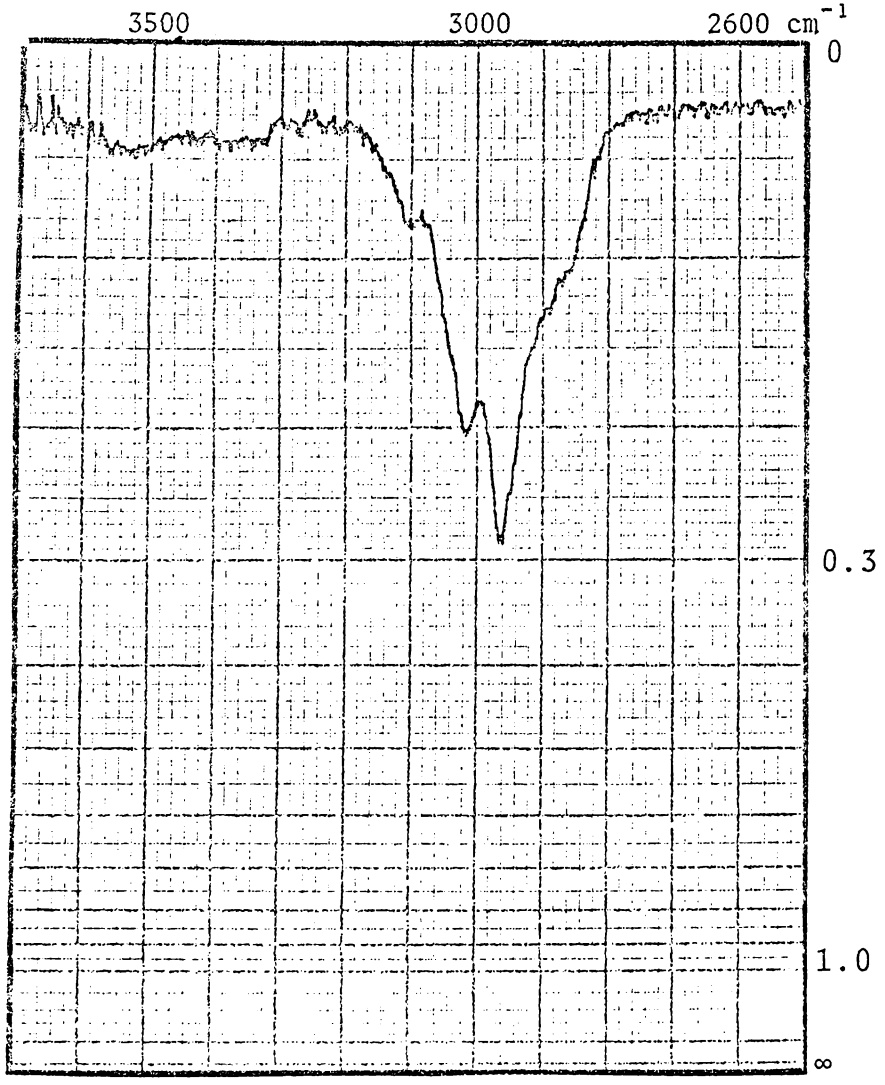



Fig. IR5.16. The i.r. spectrum (film) of methyl 11a-mercaptosecosporidesmin-3-*S*-acetate di(chloroacetate) from 3700 to 2500 $cm^{-1}$.

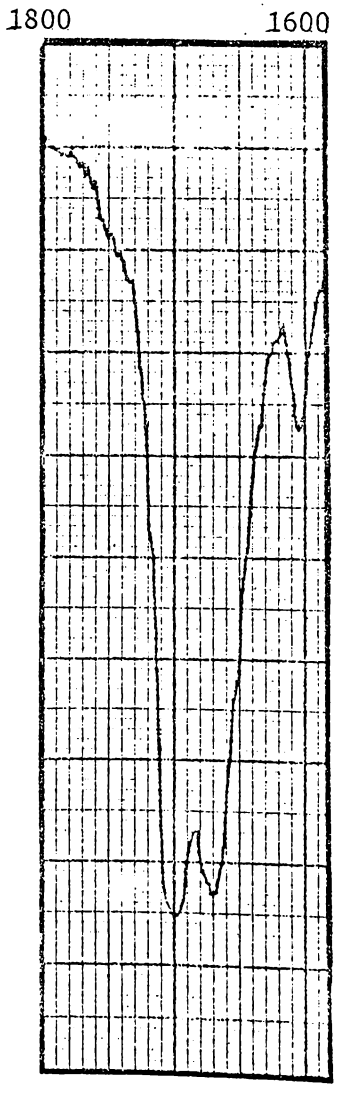



Fig. IR5.17. The νCO peaks of sporidesmin (film).

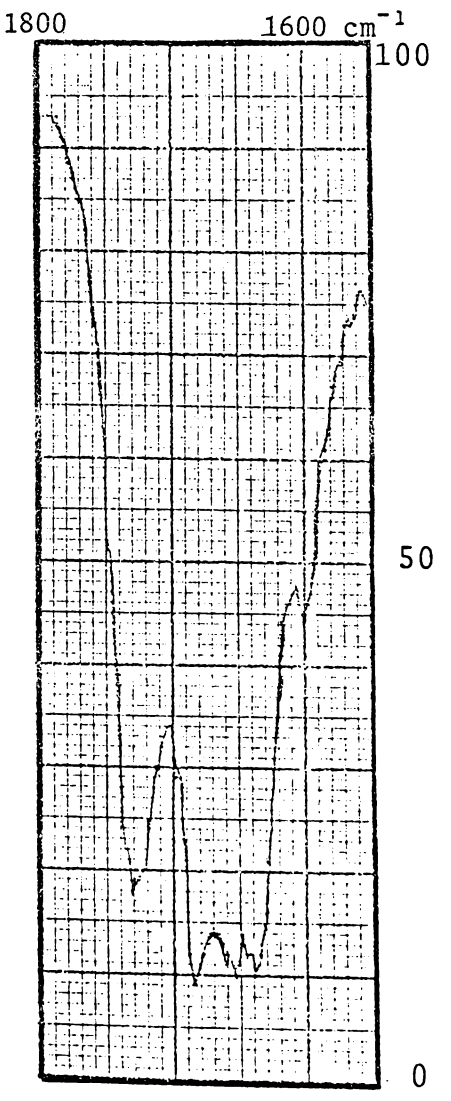
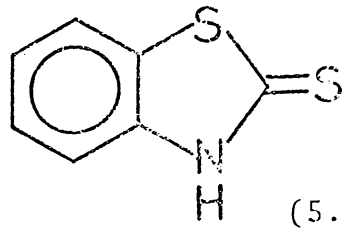



Fig. IR5.18. The νCO peaks of methyl 3-mercapto-secosporidesmin-11a-*S*-acetate (KBr).

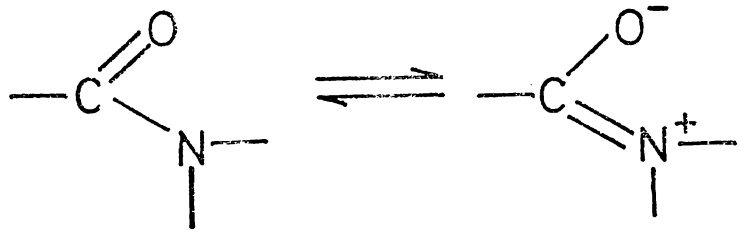
treated for a short time (45 min) with chloroacetic anhydride. The mass spectrum of the product after chromatography showed a stronger set (>4 times as intense) of peaks (3 chlorine-atom pattern) at $M^+$-$H_2S$ (*m/e* 665, 667, 669, 671) than the parent peaks (*m/e* 699, 701, 703, 705 $M^+$). This loss of the elements of $H_2S$ to give a fragment of greater intensity than the parent peaks was characteristic of the fragmentation of methyl 11a-mercaptosecosdm-3-*S*-acetate (see Chapter 2) and suggests that only *O*-acylation had occurred and no *S*-acylation. Further, supposing it had been acetylated on one oxygen and the sulphur, then there would have been an OH function remaining but in the i.r. spectrum (Fig. IR5.16 acetone film) there was no ν OH peak. Also this i.r. spectrum showed no bands in the SH stretching region. So suppression of the SH stretching peak was not attributable to the effect of the 10b-OH hydrogen bonding to the SH.

The difference between the environments of the 3-SH and the 11a-SH (in their respective compounds) was that the latter was adjacent to a nitrogen atom, a carbinol-*C* (C-11) in a pyrrolidine ring and a carbonyl group (1-CO) hydrogen bonded to the carbinol-*OH*, (11-OH) while the former was on a carbon bearing a methyl, an *N*-methyl group and a non-bonded carbonyl (4-CO). (The two C=O groups of the sdm absorbed (νCO) at different frequencies (Fig. IR5.17) indicative of the difference in environment.) It appears that the 11-OH•••O=C (1-CO) bond inhibited 3-SH•••O=C (1-CO) bonding in the 3-mercapto-compound. But in the 11a-SH isomer the 4-CO was otherwise non-bonded and so free to hydrogen bond to the hydrogen of the 11a-SH (6-membered ring).

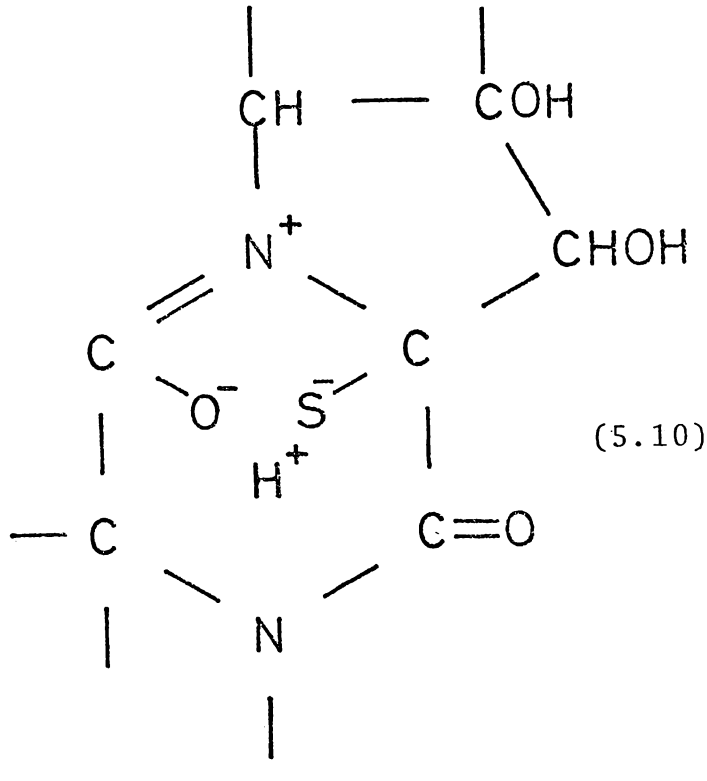
Within a group of keto-enol compounds where the equilibrium is very much in one direction; this direction is ascertained by the presence or absence of the appropriate stretching frequencies e.g. from the absence of any S-H stretching absorptions in the spectrum of mercaptobenzthiazole, it was given a thioketone structure (5.7) (Bellamy, 1958b). Again no



(5.7)



(5.8) (5.9)



(5.10)

O-H stretching peaks were detected in the spectra of either 2-hydroxy-1-nitrosonaphthalene (Amstutz, *et al.*, 1951) or 1-hydroxyanthraquinone (Flett, 1948). Bellamy and Pace (1969) suggested here that there are very large frequency shifts producing very broad and very weak OH bands. Other workers (Silverstein and Bassler, 1968; Bellamy, 1975c) indicated that resonance conditions explain broad shallow bands for tautomeric species. Silverstein and Bassler (1968) also pointed out that this resonance effect in β-diketones influences the C=O stretching peaks as well, i.e. the peaks shift to a lower frequency, are more intense and are broader. In the C=O stretching region on the i.r. spectrum (Fig. IR5.15 in KBr) of the 11a-mercapto-compound, the amido-carbonyl peak at 1650 $cm^{-1}$ was more intense and broader than the other amido-carbonyl peak (1680 $cm^{-1}$), whereas in the 3-mercapto-compound the C=O stretching peaks were of equal intensity (Fig. IR5.18 in KBr).

Although the dioxopiperazine ring is not a β-diketone and hence unable to present a resonating structure, amides can be represented in two valence-bond structures (5.8, 5.9) (Robin, *et al.*, 1970). Under the influence of the 11a-SH hydrogen and the rest of its environment the 4-CO and the pyrrolidine-*N*, it is suggested, favoured the dipolar structure (5.10), the $O^-$ of which withdrew the proton from the weaker (than an O-H bond) S-H bond (11a-SH). Thus the hydrogen was not residing on either the $O^-$ oxygen or the 11a-$S^-$ sulphur atom. This relationship is akin to hyperconjugation, so that there is no SH group *per se* to produce S-H stretching peaks. Altogether this no-bond hydrogen bridge completed a 6-membered ring; it seems that intramolecular hydrogen bonding from S-H to oxygen (SH•••OC) occurs in (at least) 6-membered rings rather than 5-membered rings (Zuika and Bankovskii, 1973; they cited thioglycollic acid but Mori, *et al.*, 1971, did not observe intramolecular hydrogen bonding in the ester).

So the S-H stretching peak was absent because the proton was participating in a no-bond hydrogen bridge. This phenomenon is similar to what was observed by Blinc and Hadzi (1958); they called it the tunnelling of the hydrogen. The overall hydrogen bonding around the S-12 sulphur atom was:

| | | |
|---|---|---|
| 10b-OH•••$S^-$ | $H^+$ | $O^-$-C=$\overset{+}{N}$ |
| 3543 $cm^{-1}$ | (no νSH peak) | 1650 $cm^{-1}$ |

An attempt was made to confirm this type of structure by examining the compound in the presence of a high concentration of an organic alkali, e.g. 25% diethylamine (B-G5). Since as little as a 1 mm path length was required to obtain no more than 50% transmission, insufficient of the mercapto-compound could be dissolved in the mixture to give a definitive νSH peak.

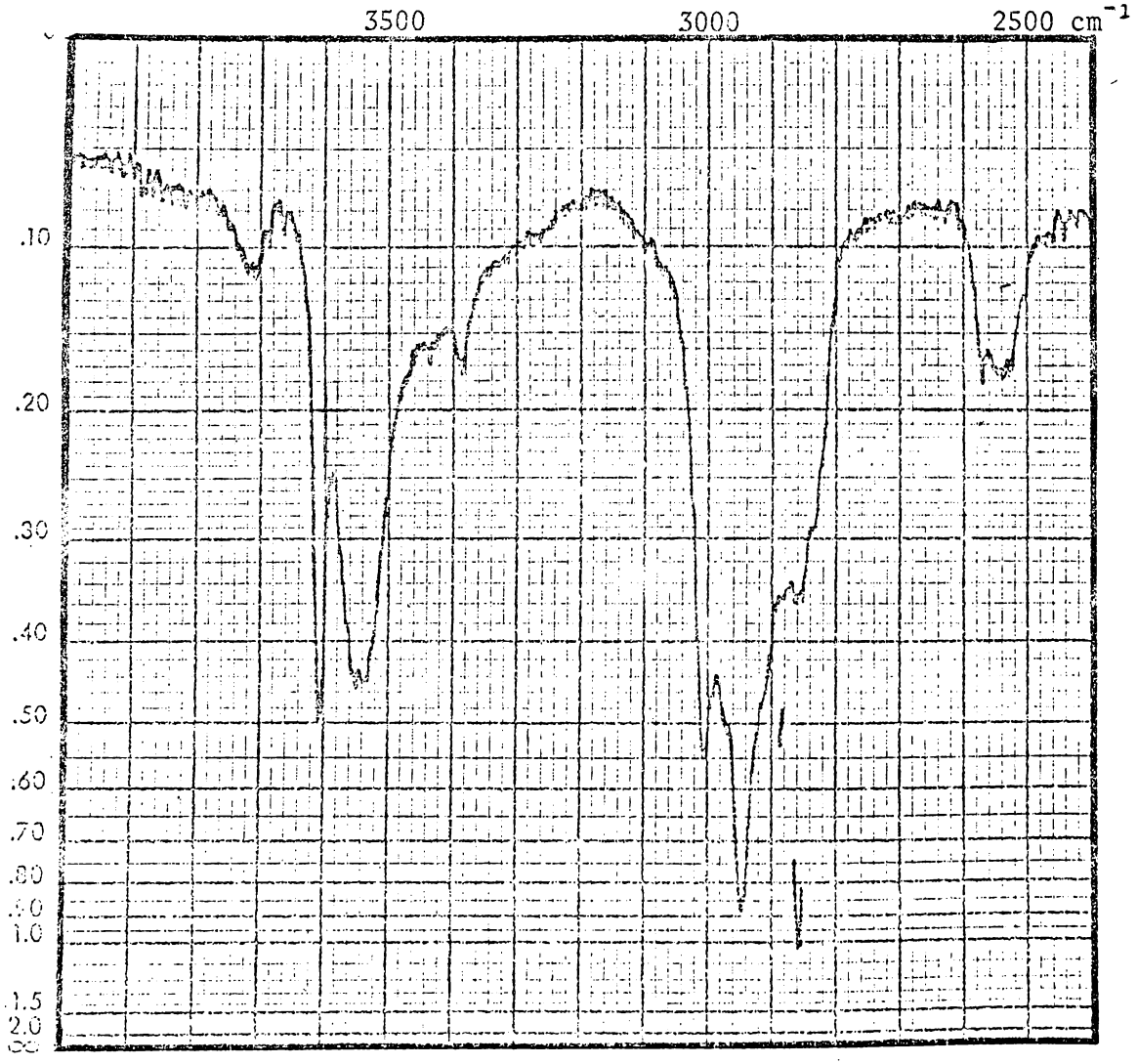



Fig. IR5.19. The i.r. spectrum of 3,11a-dimercaptosecosporidesmin in carbon tetrachloride (saturated solution).

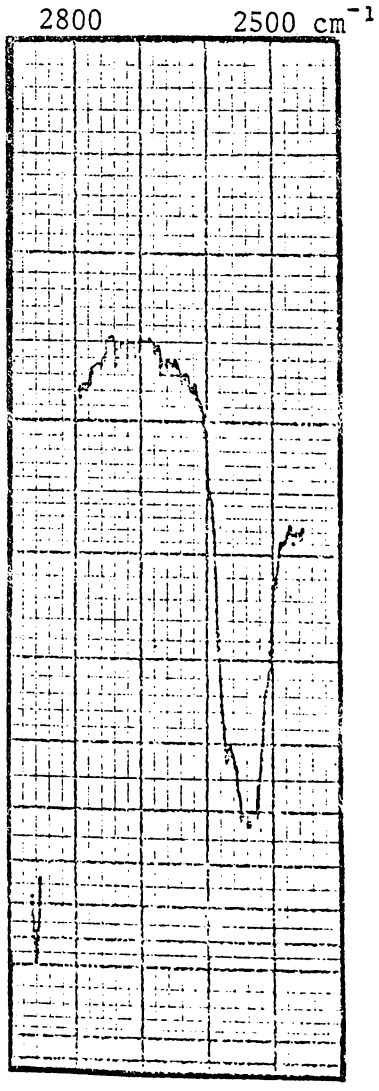



Fig. IR5.20. The νSH spectrum of 3,11a-dimercaptoseco-sporidesmin in 10% chloro-form-carbon tetrachloride.

## §7. 3,11a-*Dimercaptosecosporidesmin* (1.11)

This compound, synthesized from sdm (A-B1) was not sufficiently soluble ($CCl_4$) to make a 5 mM solution so the spectrum (Fig. IR5.19) (B-H1) was of a saturated solution (hence no $\varepsilon^a$ or rel. peak area assessment).

| | | | |
|---|---|---|---|
| 1. | $\nu$ 3590 $cm^{-1}$, | $\Delta\nu_{\frac{1}{2}}^a$ 26 $cm^{-1}$ | |
| 2. | 3535 " | | unresolved from peak 3. |
| 3. | 3520 " | 90 " | |
| 4. | 2564 " | | unresolved from peak 5. |
| 5. | 2520 " | 80 " | |

This spectrum (3700—3400 $cm^{-1}$) bore a close resemblance to that of sdm-E (Fig. IR4.11) except that in the latter the broad peak *c.* 3530 $cm^{-1}$ was by comparison less intense in respect of the peak at 3590 $cm^{-1}$ than in this compound. Furthermore in sdm-E this peak was sharper with only a suggestion of being a doublet, while in this compound it was quite clearly split into a doublet and thus broader. In a Dreiding model the internuclear distance between the hydrogen of the 10b-OH group and the S-12 sulphur atom did not seem to change in going from the structure of sdm-E to that of the opened ring, so it is suggested that the peak at 3535 $cm^{-1}$ (which was 5 $cm^{-1}$ more than in sdm-E) be attributed to the 10b-OH···S ($\Delta\nu$ *c.* 83 $cm^{-1}$) interaction, as deduced under 'Sporidesmin-B' (above). Since the strong sharp peak at 3590 $cm^{-1}$ was relatively less intense than in sdm-E, it is suggested that in this compound the $\pi$-electrons compete for the 10b-OH less effectively than they do in sdm-E. By slight contrast to sdm-E the peak at 3520 $cm^{-1}$ attributed to the 11-OH···O=C-1 interaction indicated that this interaction was slightly stronger ($\Delta\nu$ = 107 $cm^{-1}$) than in sdm-E (97 $cm^{-1}$).

In order that this dimercapto-compound and sdm-E should produce similar i.r. spectra (between 3700 and 3400 $cm^{-1}$) there must be similarity

in structure associated with the functional groups which distinguish the compounds. Chemically, the difference between the functional groups was great: sdm-E had an -S-S-S- bridge while the dimercapto-compound had two -SH groups in the place of this sulphur bridge. In sdm-E the spectrum reflected the conformational effect of the 'diverging' C-S bonds (discussed under 'Sporidesmin-E' above) so with this compound the intramolecular hydrogen-bonding interaction and the mutual polar repulsion between the two mercapto-groups produced a divergence of the C-S bonds similar to what was observed in sdm-E. The forces of mutual attraction between the two SH···S hydrogen bonds (11a-SH···S-3 $\rightleftharpoons$ 3-SH···S-11a) though weak so held the two hydrogens of the SH groups juxtaposed that polar repulsive forces (greater than the attractive H-bonding forces) thrusted the two SH groups apart. This thrusting apart resulted in divergence of the C-S bonds giving a similar conformation to that in sdm-E and consequently a similar spectrum. As a result of this, the 1-CO oxygen was rotated (on the 3,6 axis of the 2,5-dioxopiperazine ring) away from the 11-OH hydroxy-*H*, producing almost as weak a H-bond as in sdm-E: $\Delta\nu$ = 3627 - 3520 = 107 $cm^{-1}$, compared with 97 $cm^{-1}$ in sdm-E and 177 $cm^{-1}$ in sdm.

That the orientation in relation to the rest of the molecule, of the S-H bond of the 3-SH group was different from that in methyl 3-mercapto-secosdm-11a-*S*-acetate was indicated in the $^1$H n.m.r. spectrum (see Chapter 2). For the latter compound the 3-Me proton peak appeared as a doublet and the S-H proton peak as a quartet which indicated the orientation of the S-H bond (in the same plane as the $CCH_3$ bonds). But in the former there was no such splitting and therefore the S-H bond was not confined in the plane of the $CCH_3$ bonds. This observation is consistent with the orientation deduced here from hydrogen-bonding observations.

The two thiol groups were *cis* to each other at positions 3 and 6 on a chair ring (2,5-dioxopiperazine ring): they were so close together it

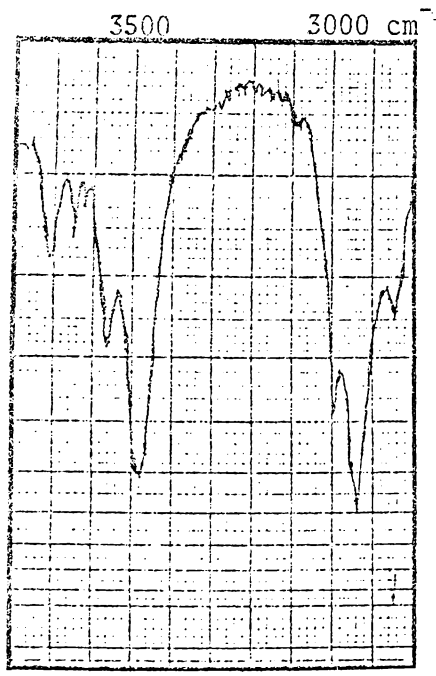



Fig. IR5.21. The i.r. spectrum of methyl 11a-mercaptoseco-sporidesmin-3-*S*-acetate.$H_2O$ in carbon tetrachloride showing the water peaks at *c.* 3710 and 3610 $cm^{-1}$.

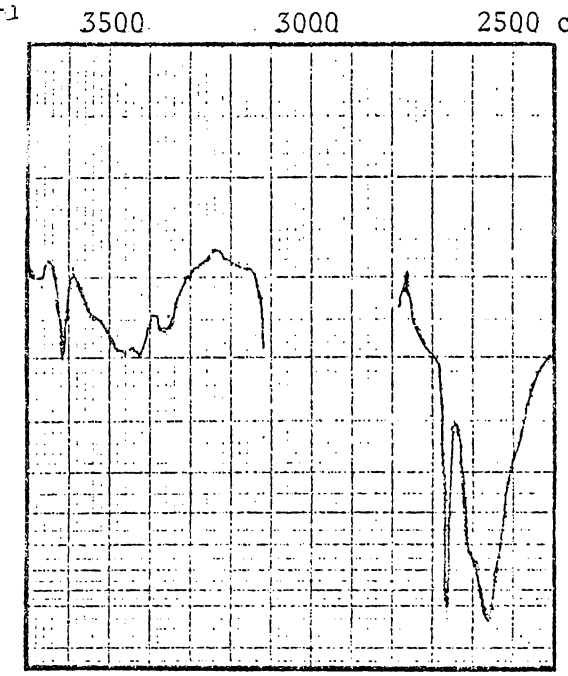



Fig. IR5.22. The i.r. spectrum of sporidesmin in carbon tetrachloride-deuterium oxide showing the unaffected peaks at 3390 and 3330 $cm^{-1}$.

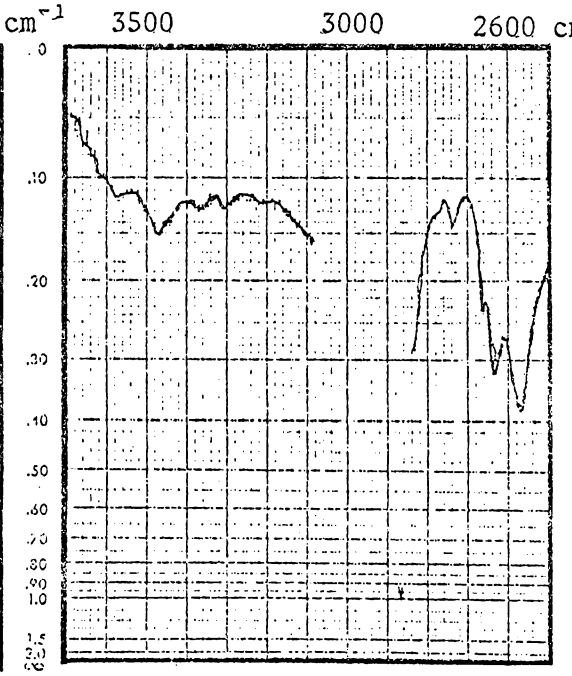



Fig. IR5.23. The i.r. spectrum of sporidesmin-D in carbon tetrachloride-deuterium oxide showing the unaffected peaks at 3340 and 3290 $cm^{-1}$.

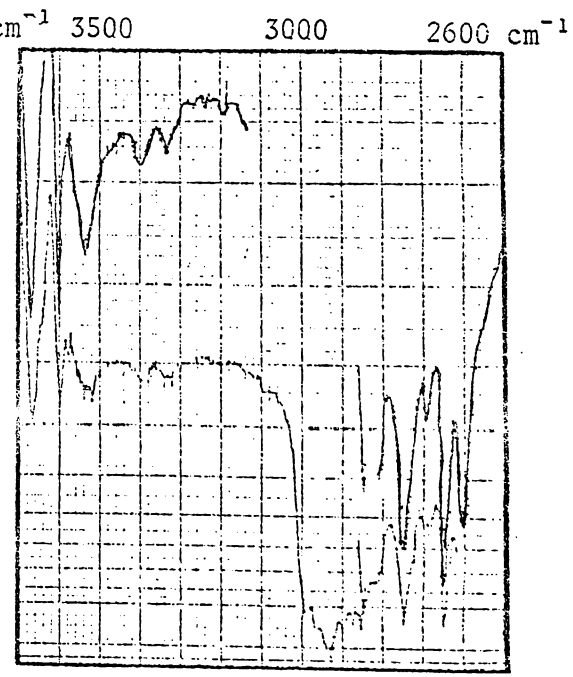



Fig. IR5.24. The i.r. spectrum of sporidesmin-E in carbon tetrachloride-deuterium oxide showing the unaffected peaks at 3390 and 3330 $cm^{-1}$.

is not surprising that ring closure occurred in the presence of dissolved oxygen between the two sulphurs when they were ionized (A-B1i,ii), C-S-S-C dihedral angle notwithstanding.

The two peaks in the S-H stretching region (2600—2500 $cm^{-1}$) (Figs IR5.19, 5.20) might indicate the presence of two mercapto-groups especially since, as shown above, the hydrogen of the 11a-SH was not in the same state as in methyl 11a-mercaptosecosdm-3-*S*-acetate (where the S-H stretching absorption was absent) so that it could have a much greater $\varepsilon^a$ value comparable with that of the 3-SH group in methyl 3-mercaptosecosdm-11a-*S*-acetate (Fig. IR5.13). Or the splitting might arise from the S-12 being sometimes hydrogen bonded to 10b-OH (3535 or 3520 $cm^{-1}$) and sometimes not (3590 $cm^{-1}$).

$$\underset{3590\ cm^{-1}}{\pi\cdots HO\text{-}10b} \rightleftharpoons \underset{3535\ cm^{-1}}{10b\text{-}OH\cdots :SH} \rightleftharpoons 11a\text{-}S:\cdots HS\text{-}3 \rightleftharpoons \underset{2564\ \text{and}\ 2520\ cm^{-1}}{3\text{-}S:\cdots HS\text{-}11a}$$

## §8. *Miscellaneous Observations*

*The extraneous peak at 3700 $cm^{-1}$*

When methyl 11a-mercaptosecosdm-3-*S*-acetate.$H_2O$ was examined (Fig. IR5.21) (B-G4) in the same way that the previous compounds were, there were extraneous peaks at *c.* 3700, 3645 and 3613 $cm^{-1}$. Saumagne and Josien (1958) showed the spectrum of water (in $CCl_4$) absorbing at 3708 and 3613 $cm^{-1}$. So in case two of these extraneous peaks might be attributable to water the solution of the methyl 11a-mercaptosecosdm-3-*S*-acetate was evaporated at 56° (reduced pressure) (B-G1). The result was (Fig. IR5.14A) where all three extraneous peaks had disappeared and where 3700 $cm^{-1}$ peak had been, there was now a trough indicating that the reference carbon tetrachloride contained traces of water. (The peak at 3645 $cm^{-1}$ was due to methanol from the freshly crystallized substance.) In the two spectra (Figs IR5.21, 5.14a) with or without water, the absorption frequencies were identical, indicating that although the carbon tetrachloride was wet the concentration of the water was too low to alter the spectra. In each of the other spectra there was a peak of variable intensity at *c.* 3700 $cm^{-1}$ which was attributed to water adsorbed on the solutes.

*Spectra after deuteration*

The result after adding deuterium oxide to sdm-B was discussed under the heading of 'Sporidesmin-B' above. Three of the other compounds (sdm, sdm-D, sdm-E) were also treated with deuterium oxide to check that the weak shoulders or peaks (3500—3200 $cm^{-1}$) in their spectra were not due to O-H stretching. Whereas for sdm-B there was only one peak which did not shift on deuteration, for each of the above compounds there were two weak peaks: sdm, well-defined weak peaks at 3390 and 3330 $cm^{-1}$ (Fig. IR5.22); sdm-D, weak peaks at 3340 and 3290 $cm^{-1}$ (Fig. IR5.23); and sdm-E, well-defined weak peaks at 3390 and 3330 $cm^{-1}$ (Fig. IR5.24).

Table 5.2. CH Stretching peaks of sporidesmin and derivatives ($CCl_4$ solution).

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Sdm | (1.1) | IR5.4 | 2995s | 2970sh* | | 2940vs | | 2900s | 2870sh | 2850m | 2825m |
| Sdm-B | (1.15) | IR5.7A | 3000sbr | | | 2940vs | | 2900s | | 2855m | 2825m |
| Sdm-D | (1.9) | IR5.9B | 2995s | | | 2940vs | 2930vs | 2900sh | 2860m | | 2830w sharp |
| Sdm-E | (1.10) | IR4.11 | 2990s | 2970sh* | | 2940vs | | 2900sh | | 2850m | 2820m |
| 3-SH-COOMe | (1.13) | IR5.13 | 3000s | | 2960w | 2940vs | | 2900sh | 2880 | 2850m | 2830w |
| 11a-SH-COOMe | (1.14a) | IR5.14A | 3000s | | 2960sh | 2945vs | | | 2870 | 2850m | 2830sh |
| Di-SH | (1.11) | IR5.19 | 2995s | 2970sh** | | 2940vs | | 2900wsh | 2870sh | 2850w | 2830sh |

* shoulder on peak at 2995—2990 $cm^{-1}$

** shoulder on peak at 2940 $cm^{-1}$

These four spectra gave sufficient evidence to conclude that the shoulders also observed in the spectra of methyl 3-mercaptosecosdm-11a-*S*-acetate (Fig. IR5.13, 3390 and 3300 $cm^{-1}$), methyl 11a-mercaptosecosdm-3-*S*-acetate (Fig. IR5.14a, 3390 and 3300 $cm^{-1}$), and 3,11a-dimercaptosecosdm (Fig. IR5.19, 3420 and 3380 $cm^{-1}$) were not associated with O-H stretching phenomena but to overtones from carbonyl absorptions.

*The CH stretching frequencies*

The C-H stretching peaks (3200—2700 $cm^{-1}$) for these compounds were very complex but the following observations might be made (Table 5.2).

Of the C-H functions in the sdm molecule and some derivatives (sdm-E, dimercaptosdm) there were 5 methyls (one C-Me, two O-Me, two N-Me) one aromatic methine and two aliphatic methines. The single aromatic methine did not appear (3100—3000 $cm^{-1}$) in any of the spectra and the aliphatic methine (CHOH, NCHN) bands were expected to be so weak that they would not be apparent (Jones and Sandorfy, 1956, Bellamy, 1975b). Hence the complex C-H stretching peak was mainly attributable to the five methyl groups.

In alkanes the methyl $\nu_{as}$ peak was strong and occurs at 2962 ± 10 $cm^{-1}$ (Bellamy, 1975a) but at this frequency in these compounds there were no strong peaks. Polar groups not directly attached to the methyl group could produce upward shifts of the νCH bands (Bellamy, 1975a). Pozefsky and Coggeshall (1951) reported increased frequencies (2976—2965 $cm^{-1}$) for methyl groups β to sulphur atoms and Gotoh and Takenaka (1961) reported $\nu_{as}$ 2986 $cm^{-1}$ for the β-methyl in propionic acid. Altogether there were three polar groups β to the 3-Me (-S-, -CO-, -NMe-) contributing to an increase in frequency so the peaks at 3000—2990 $cm^{-1}$ were ascribed to the asymmetrical stretching frequency of the 3-Me group. Compared with the narrowness of this peak in the other compounds that in sdm-B was broad. This broadness could be ascribed to its $-CH_2-$ stretch-

ing peak shifted for the same reasons that the 3-Me peak was (Kivelson, *et al.*, 1961).

The $\nu_s$ band was expected to be *c.* 90 $cm^{-1}$ less: in the spectra of sdm and sdm-B there were well marked peaks at 2900 $cm^{-1}$; in the spectra of sdm-D, sdm-E, 3-mercapto-compound and the dimercapto-compound there were shoulders at 2910—2900 $cm^{-1}$; while in the 11a-mercapto-compound the peak was eclipsed by the very strong broad peak at 2940 $cm^{-1}$.

The most intense peak in all the spectra (except sdm-D) was at 2940 $cm^{-1}$ which was too low to be that of the C-Me $\nu_{as}$. Henbest, *et al.* (1957) reported 2948 $cm^{-1}$ for the methoxy-stretching peak of veratrole. It is suggested that the intense peak at 2940 $cm^{-1}$ arose from the $\nu_{as}$ (Nolin and Jones, 1956) of the aromatic dimethoxy-group of sdm and its derivatives. The shift to lower frequency might be due to the influence of the two adjacent polar groups (9-Cl, NMe-6). The corresponding $\nu_s$ bands could be those at *c.* 2850 $cm^{-1}$ (Nolin and Jones, 1956). Henbest, *et al.* (1957) and Briggs, *et al.* (1957) both reported absorptions at 2832—3 $cm^{-1}$ for veratrole. In the spectra of the sdms there were well marked peaks at 2930—20 $cm^{-1}$ which might be attributable to either or both the aromatic methoxy-groups or the indoline-N methyl. In the spectra of the two mono-mercapto-compounds there were two small but well-defined peaks at 2960 and 2880—70 $cm^{-1}$. These two peaks were attributed to their methoxy-groups (of the methoxycarbonyl group) (Bellamy, 1968). The peak at 2960 $cm^{-1}$ was a doublet with that at 2940 $cm^{-1}$ and together they formed a broad peak.

The 2-Me group in sdms was on an amido-*N* hence (Braunholtz, *et al.*, 1958) there were no absorptions at 2805—2780 $cm^{-1}$.

Sdm-D had a very strong peak at 2930 $cm^{-1}$ (stronger than at 2940 $cm^{-1}$) and a weaker one at 2860 $cm^{-1}$ which were absent from all the other spectra. These were attributed to the methyls of the two S-methyls

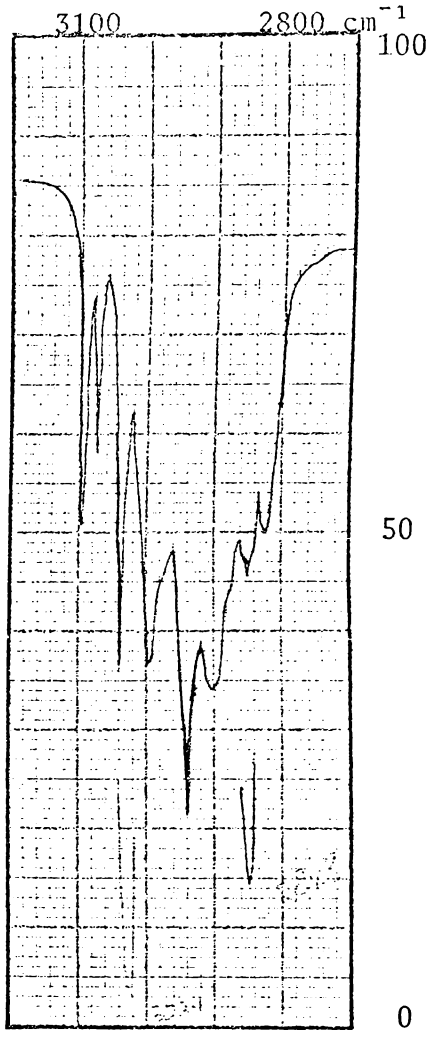



Fig. IR5.25. The νCH spectrum of sporidesmin.-$C_6H_6$ showing that the peaks of benzene (>3020 $cm^{-1}$) do not overlap those (<3020 $cm^{-1}$) of sporidesmin.

(Pozefsky and Coggeshall, 1951).

Comparing the C-H stretching regions of the spectra of the two mercapto-isomers revealed only minor differences between them. This was not surprising. While as would be expected, the spectrum for the dimercapto-compound differed from them both, by virtue of the absence of either of the carbomethoxy-peaks. Although there was no difference between sdm, sdm-E and dimercaptosdm except in the constitution of the sulphurs, there were quite marked differences among the spectra. There was a shoulder (2960 $cm^{-1}$) on the side of the strong peak (2940 $cm^{-1}$) in dimercaptosdm which was a trough in both sdm and sdm-E and where there was a well defined peak (2825 $cm^{-1}$, sdm, sdm-E) there was only a very weak shoulder in the dimercapto-compound. Further the peak in sdm at 2900 $cm^{-1}$ was a sharp shoulder in sdm-E and a smooth shoulder in dimercaptosdm.

Where the spectra were obtained for solvated molecules (Figs IR4.14, 5.9) the spectra of the C-H stretching region were masked by those of the solvents. The sdm benzene solvate (Fig. IR5.25) was an exception because the spectrum of benzene (3100—3000 $cm^{-1}$) occurred outside the C-H stretching region for sdm. In order to obtain the unsolvated spectrum of sdm-D it was dissolved in acetone and evaporated to dryness twice (B-D2) (Fig. IR5.10).

## §9. *Conclusion*

The solution spectra (as discussed above) of sdm and sdm-E were distinctive; either compound when sdm-E is synthesized from sdm may be identified by the νOH (3450 $cm^{-1}$ is absent in sdm-E) bands.

The study made the suggestion for the absence of a νSH peak in the spectrum of methyl 11a-mercaptosecosdm-3-*S*-acetate. The proton of the SH was participating in a no-bond hydrogen bridge between an $O^-$ and an $S^-$.

On the whole, for structural elucidation the νOH intense peaks indicated that there was at least one hydroxy-group in each compound and that it was hydrogen bonded (broad, intense and at <3580 $cm^{-1}$). In sdm, sdm-B, sdm-E and 3,11a-dimercaptosecosdm the well-defined sharp peak (*c.* 3590 $cm^{-1}$) indicated a hydroxy-group, α- to and π-bonded to an aromatic ring. The presence of a νSH peak showed the presence of a thiol group but the absence of a νSH peak did not prove the absence of a potential thiol group, as in methyl 11a-mercaptosecosdm-3-*S*-acetate.

But when the structures of these molecules were known then the solution spectra in the νOH region (the 11-OH•••OC-1 bonding peaks at 3530—3450 $cm^{-1}$) assumed importance in showing the conformational strain in the dioxopiperazine rings of the different compounds. This conformational strain was controlled by the spatial relationship of the two C-S bonds to each other, whether they were converging, almost parallel or diverging. For each of these 3 relationships of the bond pairs the strain in the dioxopiperazine ring and its adjacent pyrrolidine ring was different. When the bonds were converging (sdm) the 11-OH hydrogen was nearest the oxygen of the 1-CO so that that bond was the strongest and the band frequency lowest (3450 $cm^{-1}$). Where the bonds were *c.* parallel (sdm-D, methyl 3-mercaptosecosdm-11a-*S*-acetate, methyl 11a-mercaptosecosdm-3-*S*-acetate) the interatomic distance (H to O) was great-

er and consequently the frequency not so low (3455, 3470, 3474 $cm^{-1}$ resp.). Finally for the diverging bonds (sdm-E, 3,11a-dimercaptosecosdm) the interatomic distance was greatest and the frequency highest (3530, 3535 $cm^{-1}$ resp.). That the conformation of the dioxopiperazine ring was a boat was not altered but the distance between its 1 and 4 carbons changed. This interatomic distance was least in the converging condition in sdm and increased through the parallel (relaxed condition) to the diverging condition in sdm-E and 3,11a-dimercaptosecosdm. When more is known about the relationship between interatomic distance and hydrogen-bonding frequency shifts it might be possible to compute such a distance as that between carbons 1 and 4 in these dioxopiperazine rings.

Diagnostically, the i.r. solution spectrum (4000—2700 $cm^{-1}$) might be used to identify any one of the 7 compounds (§§ 1-7 above) (provided they were nearly pure) thus:

1a. The π-bonding peak (*c.* 3590 $cm^{-1}$) is well-defined and the most intense. 2.

b. The π-bonding peak is weak or absent. 5.

2a. A well-defined doublet (unequal) in the νSH region. 3,11a-dimercaptosecosdm.

b. No νSH peak. 3.

3a. Strong broad peak at 3450 $cm^{-1}$. sdm.

b. No peak at 3450 $cm^{-1}$, medium broad well-defined peak at 3530—3520 $cm^{-1}$. 4.

4a. Peak at 3520 $cm^{-1}$ a singlet, a weak peak at 3394 $cm^{-1}$ (not νOH, $D_2O$). sdm-B.

b. Peak at 3530 $cm^{-1}$ a doublet (*c.* 10 $cm^{-1}$ apart), peak at 3390 $cm^{-1}$ only a weak shoulder. sdm-E.

5a. νSH peak well-defined. methyl 3-mercaptosecosdm-11a-*S*-acetate.

b. νSH peak absent.

6a. Strongest νCH peak at *c*. 2930 $cm^{-1}$ ($S-CH_3$) adjacent to the 2945 $cm^{-1}$ ($Ar-O-CH_3$) peak, π-bonding peak weak. sdm-D.

b. Strongest νCH peak at 2940 $cm^{-1}$ , 2960 $cm^{-1}$ (COOMe) peak; adjacent to the π-bonding peak negligible.

methyl 11a-mercaptosecosdm-3-*S*-acetate.

# CHAPTER 6. EXPERIMENTAL

*General experimental conditions*

Thin layer chromatography (t.l.c.) on Eastman Chromagram sheets 6060 silica gel, with fluorescent indicator, was used to monitor reactions and column chromatography.

In mixtures of solvents the numbers indicate the volume ratios of each solvent. The following abbreviations for solvents have been used: carbon disulphide, $CS_2$; carbon tetrachloride, $CCl_4$; benzene, $C_6H_6$; chloroform, $CHCl_3$; ether (diethyl ether), EtOEt; ethyl acetate, EtOAc; acetone, AcMe; t-butyl alcohol (2-methylpropan-2-ol), $Bu^tOH$; ethanol, EtOH; methanol, MeOH; water, $H_2O$; pyridine, py; and acetic acid, AcOH. Only 'Analar' solvents were used. Any ether used was peroxide-free otherwise, in sdm work, dark blue and green products formed (substituted indigotin) resulting in drastic losses (A-C5aii). To check for peroxide, ether was shaken with sodium iodide solution then starch solution added. To check that a colourless negative was not the result of decomposed starch, a drop of hydrogen peroxide was added.

For most column chromatography (50 mm long by 25 mm wide or less) 'Merck' silica gel G was used under pressure (blow ball). These columns gave results which were comparable with those obtained by t.l.c. Where the sample was not soluble in the less polar solvent, used to make up the column, then the sample was dispersed on a small amount of the silica gel with a more polar solvent (usually AcMe) and dried. Care was taken that the silica gel, after the sample was dispersed on it, was free flowing and finely ground. Fraction sizes were measured by the distance that the free surface of the developing solvent travelled.

Melting points (Kofler block) are uncorrected. I.r. spectra were recorded for micro-potassium bromide pellets (because of the presence

of extraneous water, values above 3100 $cm^{-1}$ were neglected) and the u.v. spectra in ethanol (stated in absorbance units (A.U.)). Mass spectra were measured with a Varian MAT CH5 instrument (by Dr P. Holland). The $^{1}H$ n.m.r. spectra in deuterochloroform ($CDCl_3$) (unless stated otherwise) with tetramethylsilane (t.m.s.) as internal standard, were determined on a JEOL C-60HL spectrometer (some by S. Gumbley, some by Miss K.J. Ronaldson and some by Dr A.L. Wilkins) or a Varian T60 (by D. Calvert at the University of Auckland).

For the $^{13}C$ n.m.r. spectra these were recorded at the University of Auckland (D. Calvert) on a JEOL JNM-FX60 FT n.m.r. spectrometer, in deuteroacetone ($CD_3COCD_3$) solutions and t.m.s. for the internal standard. It was not possible to run the spectra in the usual n.m.r. solvent, $CDCl_3$, because at the high concentration needed for $^{13}C$ n.m.r., sdm crystallized out as the deuterochloroform solvate.

When the -S-S- bridge (3-S, S-12) in sdm was being opened by sodium borohydride there was always a sulphuretted smell but, nevertheless, the yield of modified sdm was satisfactory. The reaction mixture (from any experiment involving sdm) must be evaporated carefully under reduced pressure, as it turned brown if the water bath reached $50^{o}$ and, although the products after chromatography were satisfactory, the yield was reduced. Because of this heat instability of sdm and sdm products the latter could not be purified for analysis by sublimation. Ultimate analyses were performed by the Microchemical Laboratory, University of Otago.

The electrophoresis of the protein preparations (some by T. Stanbridge and some by B. Coe) were performed in a Gelman Instrument Co. instrument, using Sepraphore III strips, tris-barbital-HCl buffer, pH 8.3, Ponceau S staining and scanning.

Table 6.1. Reagents used in the synthesis of 3,11a-dimercaptosecosporidesmin (A-B).

| Expt | Sdm.$C_6H_6$ (μmol) | $NaBH_4$ (mg) | Conditions | Yield of di-SH cpd (νSH AU) | (Crystals %) |
|---|---|---|---|---|---|
| i. | 120 | 34 | Air not excluded | - | - |
| ii. | 4 | 14 | do. | - | - |
| iii. | 23 | 7 | Air excluded | 0.035 | - |
| iv. | 110 | 44 | do. | 0.35 | |
| v. | 200 | 88 | do. | | 46 |

A. *Syntheses*

A-A. *Sporidesmin free of sdm-E*

Sdm.$C_6H_6$ (637 mg) was chromatographed on silica gel G (14 g) with benzene. In the benzene-chloroform (9:1) fractions (60—110 cm) no sdm-E (by t.l.c.) was detected in the sdm (410 mg). The mixture of sdm and sdm-E remaining on the column was eluted with increasing concentrations of chloroform and with ether-acetone (4:1). The sdm was crystallized once by dissolving in acetone, evaporating twice (waterbath at less than 50°) to an expanded resin, dissolving in methanol, warming with charcoal if necessary, then crystallizing by adding drops of water. This procedure produced sdm, m.p. 179°, without solvent of crystallization (if recrystallization had to be repeated then it was usually necessary to repeat the charcoal step); $\nu_{max}$ (KBr ground in MeOH) see Figs IR2.1, IR2.2, IR2.3, IR4.4; (KBr ground in EtOEt) see Fig. IR4.3; (halocarbon) see Fig. IR4.7; (nujol) see Fig. IR4.9; (film from AcMe) see Fig. IR5.17; ($CCl_4$) see Fig. IR4.12 or IR5.4; ($CCl_2$:$CCl_2$) see Fig. IR5.5; ($CHCl_3$-$CCl_4$ 1:20) see Fig. IR5.6: $^{13}C$ n.m.r. see Fig. CM3.1: *m/e* 475, 473 ($M^+$), 409, 373: sdm.$C_6H_6$ $\lambda_{max}$ 220 (log ε 4.68), 250 (4.30), 300 (3.96): $\nu_{max}$ ($CCl_4$) see Fig. IR5.25; ($CCl_4$-$D_2O$) see Fig. IR5.22.

A-B. *Reactions at the sulphur atoms* (other than alkylation)

A-B1. 3,11a-*Dimercaptosecosporidesmin* (1.11)

i. Sdm.$C_6H_6$ (Table 6.1) in methanol was reacted with a cooled methanolic solution of sodium borohydride. The i.r. spectrum (after 0.5 h) showed no -SH stretching peaks (2600—2500 $cm^{-1}$).

ii. In this second experiment (Table 6.1) the i.r. spectrum of the crude reaction mixture (after 15 min) showed no -SH stretching peaks. The i.r. spectra of the chloroform extract of the residue, either before or after acidification did not show any -SH stretching peaks but only those of essentially unaltered sdm. The mass spectrum of the acidified extract showed *m/e* 475 and 473 ($M^+$) (*m/e* sdm 475, 473, $M^+$).

A-B1. Cont'd

iii. Sdm.$C_6H_6$ (Table 6.1) and sodium borohydride were cooled in a small (5 ml) flask and cold methanol added. As the suspension warmed it effervesced and dissolved. Before (10 min) effervescence subsided, chloroform (1 ml) was added (no ppte) and water to fill the flask completely. After shaking vigorously, the chloroform emulsion was filtered into a silica cell (u.v. 1 mm light path). The i.r. spectrum (against $CHCl_3$) of the solution showed no peak between 2700 and 2400 $cm^{-1}$.

The addition of conc. hydrochloric acid (20 µℓ) turned the aqueous phase turbid. Shaking with more chloroform (1 ml) resulted in both layers becoming clear. The i.r. spectrum of this extract ($CHCl_3$) showed a small (0.035 A.U.) broad ($W_{\frac{1}{2}}$ 7 $cm^{-1}$) peak (2535 $cm^{-1}$).

iv. The above experiment was repeated (Table 6.1) and to exclude air water was added before effervescence ceased. The chloroform extract after acidification (120 µℓ conc. HCl) gave a large (0.35 A.U.) i.r. peak of the same width ($W_{\frac{1}{2}}$ 7 $cm^{-1}$) at 2535 $cm^{-1}$ ($CHCl_3$ solution) as found previously. Crystals appeared on adding water (1 drop) to a methanol solution. They were soluble in ether, chloroform or acetone but t.l.c. ($C_6H_6$-EtOEt 7:3) gave a brownish tail from the origin to $R_{sdm}$ 0.5 and a small spot corresponding to sdm, while with methanol-benzene (1:19) there was a tail all the way from the origin to sdm.

v. Experiment iv. was repeated at 0.2 mmol level (Table 6.1). Crystals (52 mg) of 3,11a-*dimercaptosecosporidesmin* (1.11) were obtained from methanol-water, m.p. 170—172.5° (Found: *m/e* 477, 475 ($M^+$); C, 45.5; H, 4.7; N, 8.8. $C_{18}H_{22}{}^{37}ClN_3O_6S_2$ and $C_{18}H_{22}{}^{35}ClN_3O_6S_2$ require *M* 477, 475 resp.; C, 45.4; H, 4.7; N, 8.8%): $\nu_{max}$ (KBr) see Figs IR2.1, IR2.2, IR2.4; ($CCl_4$) see Fig. IR5.19; ($CHCl_3$-$CCl_4$) see Fig. IR5.20: δ ($CDCl_3$ sat. sol.) 7.10, 1 H, s, H-10; 5.30, 1 H, s, H-5a;

A-B1v. cont'd

4.64, 1 H, d *J* 2.9 Hz, H-11, collapsed to a singlet with $D_2O$ and with irradiation at 3.36; 3.86, 3 H, s, $CH_3O$; 3.81, 3 H, s, $CH_3O$; 3.8—2.0, 3 H, m, 11-OH and 2 SHs, disappeared with $D_2O$; 3.36, 3 H, s, $CH_3N$-6; 3.09, 3 H, s, $CH_3N$-2; 1.96, 3 H, s, $CH_3C$-3; 1.25, 1 H, brs, 10b-OH, disappeared with $D_2O$.

An attempt was made to separate the dimercaptosdm from the *N*-methoxycarbonylmethylpyridinium salt in the reaction mixture. The latter was dissolved in chloroform and extracted with alkali (*c.* pH 10). Then the alkaline solution was acidified and extracted ($CHCl_3$). But this extract contained nothing showing absorptions in the νSH region.

The 5,5'-dithiobis-(2-nitrobenzoic acid) test was positive; but although the sodium nitroprusside test was positive to dithiothreitol, it was negative to the dimercaptosecosdm.

vα. Alkylation of 3,11a-dimercaptosecosporidesmin

Dimercaptosecosdm (50 mg, 0.1 mmol), sodium iodide (20 mg) methyl chloroacetate (200 μl, 2 mmol), and pyridine (0.5 ml) were dissolved in methanol (1 ml). T.l.c. of the washed product (after 90 min) showed only a spot at the origin and at the position of sdm.

A-B2. *Copper derivatives of dimercaptosecosporidesmin*

i. Sdm.$C_6H_6$ (55 mg, 0.1 mmol) and cupric chloride dihydrate (17 mg, 0.1 mmol) were dissolved in methanol and cooled: cold sodium borohydride (39 mg) in methanol (2 ml) was added and the whole turned to a red brown precipitate. A control (without sdm) gave the same result: when the precipitate settled the supernatant liquid was colourless but with time the precipitate dissolved giving a green solution. The sample containing sdm turned brown. Nothing crystalline was recovered from the evaporation of solvents from the sdm reaction product. The major spot on t.l.c. (MeOH-$C_6H_6$ 1:4) was sdm.

A-B2 Cont'd

ii. 3,11a-Dimercaptosecosdm (4.2 mg, 8.9 μmol) was dissolved in methanol (0.3 ml). Cupric acetate ($H_2O$) (2 mg, 10 μmol) was dissolved in methanol (0.5 ml) and added slowly. One drop of the copper solution turned the sdm solution olive-green from the green-blue of the cupric acetate solution. The olive-green changed to amber overnight. A white cloudiness resulted from the addition of water. On evaporation no crystals formed but there was a strong acetic-acid smell: this gum was examined by i.r. spectroscopy, $\nu_{max}$ (KBr) 1655vsbr, 1465s, 1410vs, 1385s, 1345m, 1310w, 1040vs, 915vw, 890vw, 830vwbr, 750w, 740w, 620 vw $cm^{-1}$.

iii. The zinc derivative was formed as under ii with zinc salts.

A-B3. *Sporidesmin-E*

i. After Murdock and Angier (1970), sdm.$C_6H_6$ (116 mg, 0.21 mmol) and sulphur (6.8 mg, 0.21 mmol of S) were dissolved in pyridine (1.2 ml) the flask being wrapped in foil and warmed (32°) (water-bath 2 h). On t.l.c. (1st direction EtOEt-$C_6H_6$ 1:4 and 2nd $Bu^tOH$-petroleum spirit 40—60° 1:4) there was a weak sdm spot, a strong spot (sdm-E) and a third weak spot (sdm-G ?) with an $R_F$ similar to that of sdm-D.

To react the remaining sdm more reactants were added and excess (10%) sulphur; sdm.$C_6H_6$ (214 mg), sulphur (14.7 mg). T.l.c. (after 1.5 h) showed much sdm-G and still some sdm.

The acid washed product was chromatographed with benzene-ethyl acetate (9:1). Sdm-E was eluted in the first fraction (20 cm). By t.l.c. (2 dimensional as above) it contained sdm; further fractions contained sdm and sdm-G as well as the sdm-E.

The sdm-E.EtOEt crystals were obtained from ether and were used in animal feeding trials.

A-B3 Cont'd

ii. Sdm.$C_6H_6$ (663 mg, 1.2 mmol) and sulphur (38.2 mg, 1.2 mmol of S) were mixed with pyridine (1.5 ml). Not all the sulphur dissolved immediately. The flask, wrapped in foil, was maintained at 32° (2.5 h). The following process and chromatography were carried out in subdued light. The pyridine was evaporated under reduced pressure and the residue distributed onto silica gel G. Chromatography ($Bu^tOH$-petroleum spirit, 40—60° 1:19) yielded a gum (415 mg). The fractions were dissolved (EtOEt) for t.l.c. monitoring: sdm-E.EtOEt crystallized (316 mg) spontaneously. Each fraction on t.l.c. showed a fast moving trace (sdm) and a slow one (sdm-G), the latter two compounds increasing with fraction number. In two dimensional t.l.c., the sdm-E spot (from the first direction) in the second direction invariably had a shadow corresponding to sdm.

The above crystals (316 mg) were rechromatographed with benzene-ethyl acetate (49:1) to yield a gum 254 mg from which sdm-E.EtOEt (200 mg) was obtained.

iii. The above experiment was repeated with sdm.$C_6H_6$ (800 mg, 1.45 mmol), sulphur (46.4, 1.45 mmol of S) and pyridine (5 ml, 4 h). The pyridine solution was evaporated to a small volume, which (in $CHCl_3$) was acid washed and then evaporated to a fluffy gum. This gum dissolved readily in a small amount of ether and immediately precipitated crystals. These crystals (724 mg) had the same i.r. spectrum (KBr ex EtOEt, see Fig. IR4.1) as was obtained from those prepared under (i) and (ii). But when the KBr and sdm-E.EtOEt were ground in the presence of methanol instead of ether then the i.r. spectrum was essentially that of sdm except between 1240 and 1080, 890 and 800, and 780 and 700 $cm^{-1}$, see Fig. IR4.6; for solution spectra see (H-E) later.

For $^{13}C$ n.m.r. these crystals were dissolved in acetone and in the dark evaporated to a film to remove the ether of crystallization, then redissolved in acetone-$d_6$, see Fig. CM4.1.

A-B3 Cont'd

iv. *Unsolvated sporidesmin-E*

Recrystallized sdm-E.EtOEt melted 144.5—148° (180—185° Rahman, *et al.*, 1969), $[\alpha]_D$ -166° (*c* 0.060 in $CHCl_3$). A sample from these authors melted at 139—142°. Sdm-E.EtOEt effloresced at 125—135° and when the sample was held (45 min) at 135° it all melted with decomposition.

When ether-free sdm-E (by evaporating either $CHCl_3$ or AcMe solutions of the etherate) was dissolved in a minimum of ethyl acetate and acetic acid added sdm-E crystallized, m.p. 181—182.5°: $\nu_{max}$ (KBr ground in MeOH) see Figs IR2.1, IR2.2, IR2.3; (KBr ground in EtOEt) see Fig. IR4.5; (halocarbon oil) see Fig. IR4.8; (nujol) see Fig. IR4.10; ($CCl_4$) see Fig. IR4.11; ($CCl_4$-$D_2O$) see Fig. IR5.24: *m/e* 507, 505 ($M^+$), 473, 441, 409, 256 ($S_8$).
In three months (5°) the m.p. was 179—182.5; mixed m.p. of this sample of sdm-E with sdm was 178.5—182.5°.

v. *Stability of sporidesmin-E*

A sample (*c.* 20 µg) sdm-E was chromatographed in two directions on a t.l.c. plate (1st direction EtOEt-$C_6H_6$ 1:4 and 2nd $Bu^tOH$-petroleum spirit 40—60° 1:4) and the sdm-E spot eluted and spotted onto another plate; this latter chromatogram showed three spots.

The same three-spot phenomenon was observed ($C_6H_6$-$CHCl_3$ 7:13) when sdm-E.EtOEt and unsolvated sdm-E were spotted on the same plate: the amounts of the corresponding spots were about the same.

vi. [13-$^{35}$S]*Sporidesmin-E*

Sdm.$C_6H_6$ (338 µg, 0.6 µmol) and sulphur-35 (24 µg, 0.75 µmol of $^{35}S$) were dissolved in pyridine (100 µl), wrapped in foil and warmed (32°, 3 h). In subdued light the chloroform solution of the product was acid washed and evaporated to a small volume. Benzene was added

Table 6.2. Reagents used in the synthesis (A-C1) of sporidesmin-D.

| Expt | Sdm.$C_6H_6$ (μmol) | Pyridine (ml) | MeI (μmol) | MeOH (ml) | $NaBH_4$ (mg) | Product | Crude yield (%) |
|---|---|---|---|---|---|---|---|
| i. | 190 | 0.4 | 32 | 3 | 70 | - | - |
| ii. | 490 | 1 | 80 | 7 | 124 | Sdm | 6 |
| | | | | | | Sdm-D | 94 |
| iii. | 1000 | 2 | 160 | 12 | 221 | Sdm | 10 |
| | | | | | | Sdm-D | 87 |

A-B3vi. cont'd

twice and each time evaporated to a small volume. This was chromatographed (1 g $SiO_2$ gel G in 7 mm column) and developed ($C_6H_6$-$CHCl_3$ 4:1). The fractions (10 cm) were counted (by R. Fairclough, Ruakura Agricultural Research Centre) in a Nuclear Chicago β scintillator for radioactivity. Their relative activities were: fraction 1, 105; 2, 24; 3, 21; 4, 19; 5, 39; 6, 483; 7, 449; 8, 232; 9, 161; 10, 107; 11, 61; 12, 34; 13, 75 and 14, 72. Fraction 8 was used for the radioimmunoassay work.

A-B3a *Sporidesmin-E diacetate*

After Rahman, *et al.* (1969), sdm-E.EtOEt (56.5 mg, 0.10 mmol) was dissolved in acetic anhydride (100 µl, 1 mmol) and pyridine (400 µl) and held in the dark (3 d). The whole was evaporated to an expanded gum which could not be crystallized (3 y) (Found: C, 45.3; H, 4.2; Cl, 6.5; N, 7.0; S, 16.1. Calc. for $C_{22}H_{24}ClN_3O_8S_3$: C, 44.8; H, 4.1; Cl, 6.0; N, 7.1; S, 16.3%): $\nu_{max}$ (nujol) 1754, 1695sh, 1688 and 1597 $cm^{-1}$, (no sign of any AcOAc or py peaks): *m/e* 621, 589 ($M^+$), 557, 525, 493, 433, 391, 373 at 135°.

A-C. S *alkylation*

A-C1a *Sporidesmin-D* (sdm-D) (1.9) (after the method of Jamieson, *et al.*, 1969)

i. Sdm.$C_6H_6$ (Table 6.2) was dissolved in methanol, pyridine and methyl iodide. The solution turned yellow and then a precipitate formed which was dissolved in more methanol (0.8 ml). The sodium borohydride which had been dissolved in methanol with violent effervescence was added to the above solution. The i.r. spectrum (after 4 h) showed unchanged sdm. More sodium borohydride (42 mg) was added directly to the solution. A small sample dissolved in chloroform-water and washed with water gave an i.r. spectrum different from sdm. T.l.c. (EtOEt-$C_6H_6$ 3:22) showed unreacted sdm still present.

A-C1ai cont'd

The residue after washing was chromatographed to yield four products each in a small amount.

ii. The above experiment was repeated: sdm.$C_6H_6$ (Table 6.2) was dissolved in methanol, pyridine and methyl iodide and cooled (dry ice-AcMe). Sodium borohydride was dissolved in cooled (dry ice-AcMe) methanol and added to the sdm solution. Bubbles of hydrogen appeared only slowly. After allowing to warm (3 h), and evaporating off the methanol and methyl iodide (reduced pressure) and washing, the i.r. spectrum showed no sdm. Chromatography (EtOEt-$C_6H_6$ 3:22; 5 cm fractions) yielded sdm (14 mg) and a gum (240 mg) which crystallized spontaneously after evaporating ether from it.

iii. In this experiment (Table 6.2) the chloroform solution in work-up was washed with dil. hydrochloric acid and the residue chromatographed yielding sdm (60 mg) and sdm-D (464 mg) as a gum which crystallized (367 mg) spontaneously in the presence of ether. Sporidesmin-D etherate was obtained (EtOEt), m.p. the crystals dissolved in the ether of crystallization at *c.* 80°, boiled to an opaque gum which was clear and fluid at 110°, (lit. above 110—120°) (Found: *m/e* 505, 503 ($M^+$); C, 50.1; H, 6.3; Cl, 6.3; N, 7.2; S, 11.5. Calc. for $C_{20}H_{26}ClN_3O_6S_2$.EtOEt: *M* 505 ($^{37}Cl$), 503 ($^{35}Cl$); C, 49.9; H, 6.2; Cl, 6.1; N, 7.3; S, 11.1%): $\nu_{max}$ (KBr ground in MeOH) see Figs IR2.1, IR2.2, IR2.3; ($CCl_4$) see Fig. IR5.9A; (EtOEt-$CCl_4$ 1:19) see Fig. IR 5.11: sdm-D (unsolvated) $\nu_{max}$ ($CCl_4$) see Figs IR5.9B, IR5.10; (unsolvated) ($CCl_4$-$D_2O$) see Fig. IR5.23). δ 7.07, 1 H, s, ArH; 5.30, 1 H, s, NCHN; 4.63, 1 H, d *J* 3.5 Hz, C*H*OH collapsed to a singlet with $D_2O$; 3.90, 1 H, s, COH; 3.87, 3.80, 6 H, 2 s, $2CH_3OAr$; 3.45, 4 H, q *J* 6 Hz, $CH_2$ of EtOEt; 3.37, 3 H, s, $ArNCH_3$; 3.07, 3 H, s, $CONCH_3$; 2.97, 1 H, d *J* 3.5 Hz, CHO*H*, disappeared with $D_2O$; 2.40, 2.34, 6 H, 2 s, $2CH_3S$; 1.87, 3 H, s, $CCH_3$; 1.20, 6 H, t *J* 6 Hz,

A-C1aiii cont'd

$CH_3$ of EtOEt: *m/e* 457, 455, 410, 408, 242, 240.

For $^{13}C$ n.m.r. the sdm-D.EtOEt crystals were dissolved in acetone and evaporated twice (waterbath at 50°) to an expanded resin and then dissolved in acetone-$d_6$, see spectrum CM3.1.

A-C1b. [3,11a-$(SC^3H_3)_2$]*Sporidesmin-D*

A cooled (dry ice-AcMe) ampoule of [$^3H_3$]methyliodide (25 mCi) was broken and the contents dissolved out in 3 lots of methanol (total 750 µl). Sdm.$C_6H_6$ (41 µg, 0.08 µmol) was dissolved in this methanol with pyridine (2 µl) and then cooled. Several drops of cold (dry ice-AcMe) methanol solution of sodium borohydride (*c.* 30 µg) were added and bubbles appeared on warming. The residue after washing the chloroform solution was diluted with benzene, evaporated to a small volume, rediluted with benzene, and again concentrated to a small volume and applied to a silica gel G (1 g) column (57 mm long). After the dead volume (1.7 ml) 9 fractions were collected (2x25 mm, 4x50 mm, 3x100 mm movement of free solvent surface) in ether-benzene (1:9). To have a sufficient concentration of solute for t.l.c. the volume of fraction 5 was reduced to 1/10th.

A-C2. *Sporidesmin-D diacetate* (after the method of Jamieson, *et al.*, 1969)

Sdm-D.EtOEt (52 mg, 0.09 mmol) was dissolved in pyridine (160 µl) and acetic anhydride (40 µl, 0.4 mmol). After allowing to stand (9 d), the reaction mixture was evaporated to a gum, dissolved in ether, washed (dil. acid) dried and evaporated again to a gum which crystallized spontaneously, giving *m/e* 589 and 587 ($M^+$) for $C_{24}H_{30}{}^{35}ClN_3O_8S_2$ and $C_{24}H_{30}{}^{37}ClN_3O_8S_2$, resp.

A-C3. *Secosporidesmin-S,S'-diacetic acid*

[Sodium chloroacetate was prepared from chloroacetic acid (1.95 g), neutralized with sodium carbonate (1.06 g) and concentrated to a wet crystalline mass, $\nu_{max}$ (KBr) 1595vs ($COO^-$), 1415vs, 770 (C-Cl) $cm^{-1}$.]

i. Sdm.$C_6H_6$ (112 mg, 0.2 mmol), sodium chloroacetate (167 mg, wet, *c.* 1.4 mmol) and sodium iodide (8 mg) were dissolved in pyridine (300 µl), and methanol and cooled. Sodium borohydride (50 mg) was dissolved in cold (dry ice-AcMe) methanol and added. After 30 min the chloroform extract of the alkaline solution showed sdm (i.r.); acidification and chloroform or ether extraction yielded i.r. spectra without peaks at *c.* 1410 or 1460 $cm^{-1}$. This chloroform extract showed 2 spots on t.l.c. ($C_6H_6$-MeOH-AcOH 45:8:4). Since no fractions from chromatography ($C_6H_6$-AcOH 23:2 with increasing concentrations of MeOH) showed peaks at 1410 $cm^{-1}$ (for sdm or its derivatives) they were discarded.

ii. The above experiment was repeated using chloroacetic acid (96 mg). Sdm (73 mg) was recovered. As in preparation i. the extract ($CHCl_3$) after acidification gave the same i.r. spectrum and 2 spots on t.l.c. ($C_6H_6$-MeOH-AcOH).

iii. Sdm.$C_6H_6$ (56 mg, 0.1 mmol) (dissolved in AcMe and evaporated to an expanded resin) was dissolved in a sodium sulphide solution (48 mg, 0.2 mmol $Na_2S$, $9H_2O$ in 4.8 ml $H_2O$, pH >10.5). To this was added a solution of chloroacetic acid (18.9 mg, 0.2 mmol in 1.9 ml $H_2O$). Sdm was recovered unchanged from the alkaline solution and the extract ($CHCl_3$) of the acidified solution had an i.r. spectrum similar to those above, so the preparation was again discarded.

A-C4. *S-alkylation with ethylchloroacetate*

A-C4a. *Diethyl secosporidesmin-S,S'-diacetate* (1.12b)

i. Sdm.$C_6H_6$ (Table 2.1) was dissolved in pyridine, methanol and ethyl

A-C4ai. cont'd

chloroacetate. A solution of sodium borohydride dissolved in methanol was added to the above solution. After 5 h the i.r. spectrum of a test sample (washed, $CHCl_3$-$H_2O$) showed only sdm. T.l.c. of the final gum after work-up also showed only sdm which was recovered.

ii. Sdm.$C_6H_6$ (Table 2.1) was dissolved in the pyridine, methanol and ethyl chloroacetate. This time sodium iodide was added and the solution cooled. Sodium borohydride, dissolved in cooled methanol was added, then the reaction mixture was allowed to warm. Some sodium chloride crystallized out (cubic system). The i.r. spectrum (1500—600 $cm^{-1}$) at 10 min. of the crude reaction mixture was the same as that of the final product. When at 2 h the mixture was evaporated to a gum and washed ($CHCl_3$-$H_2O$) crystals appeared on drying, which it is suggested were *N*-ethoxycarbonylmethylpyridinium salt. This suggestion was made because the crystals were only slightly soluble in methanol, acetone, ethyl acetate or ether-benzene but they were soluble in water. Their i.r. spectrum was the same as that obtained for the crystals from ethyl chloroacetate and pyridine. Chromatographing the mixture of gum and crystals with ether-benzene (3:22) yielded a gum (40 mg t.l.c., EtOEt-$C_6H_6$ 1:4, $R_{sdm}$ 0.46).

iii. The above experiment (Table 2.1) was repeated. After 2 h reaction time, the methanol was evaporated and the residue acid-washed ($CHCl_3$) and chromatographed. Ether-benzene (1:9, 25 cm) removed sdm (1.2 mg), then (1:4, 40 cm) a gum (111 mg, $R_{sdm}$ 0.85 on t.l.c., EtOEt-$C_6H_6$ 7:13) of *diethyl secosporidesmin*-S,S'-*diacetate* which could not be crystallized (Found: *m/e* 647.1333 ($^{35}Cl$) and 649.1341 ($^{37}Cl$); C, 48.2; H, 5.0; Cl, 6.0; N, 6.5; S, 9.5. $C_{26}H_{34}{}^{35}ClN_3O_{10}S_2$ and $C_{26}H_{34}{}^{37}Cl$-$N_3O_{10}S_2$ require 647.1374 and 649.1345 resp.; C, 48.2; H, 5.3; Cl, 5.5; N, 6.5; S, 9.9%): $\lambda_{max}$ 217 nm (log ε 4.62), 252 (4.06), 303 (3.29):

A-C4aiii. cont'd

$\nu_{max}$ (KBr) see Figs IR2.1, IR2.2, IR2.3: δ 7.08, 1 H, s, H-10; 5.31, 1 H, s, H-5a; 4.64, 1 H, s, H-11; 4.21 and 4.27, 4 H, 2 *q*, *J* 7 Hz, $2OCH_2CH_3$; 3.79 and 3.86, 6 H, 2 s, $2CH_3O$; 3.68, 4 H, s, $2SCH_2COO$; 3.35, 3 H, s, 6-$CH_3$; 3.07, 3 H, s, 2-$CH_3$; 1.87, 3 H, s, 3-$CH_3$; 1.31 and 1.34, 6 H, 2 t, *J* 7 Hz, $2CH_2CH_3$.

A-C4b. *Ethyl* 11a-*mercaptosecosporidesmin*-3-S-*acetate* (1.14b)

iv. The above experiment (Table 2.1) was repeated at a 1 mmol level. Chromatography in benzene with increasing concentrations of ether yielded sdm, diethyl secosdm-*S,S'*-diacetate (243 mg) and a gum (118 mg, $R_{sdm}$ 0.46 on t.l.c. EtOEt-$C_6H_6$ 7:13) giving crystals of ethyl 11a-mercaptosecosporidesmin-3-*S*-acetate from ethanol, m.p. 135—145°: $\nu_{max}$ (KBr) see Figs IR2.1, IR2.2, IR2.4: *m/e* 645, 643 ($M^+$).

A-C5. S *alkylation with methyl chloroacetate*

A-C5a. *Dimethyl secosporidesmin*-S,S'-*diacetate* (1.12a)

[Methyl chloroacetate (after Cohen, 1926) was prepared from chloroacetic acid (10 g) by refluxing (5.5 h) with acidified ($H_2SO_4$) methanol (15 ml). After washing ($NaHCO_3$) the residue was distilled (131.5°) (131.5° Chemical Rubber, 1964).]

This dimethyl ester was prepared in a similar way to that for the diethyl ester:

i. Sdm.$C_6H_6$ (Table 2.2), sodium iodide, and methyl chloroacetate were dissolved in methanol and reacted with sodium borohydride solution in the cold. After 20 min the i.r. spectrum showed sdm unchanged so pyridine (1.2 ml) was added to the reaction mixture. After 30 min the i.r. spectrum had changed. Chromatography of the residue (after $CHCl_3$-$H_2O$) yielded sdm (60 mg), a trace of low $R_F$ material and *dimethyl secosporidesmin*-S,S'-*diacetate* (1.12) (420 mg) as a gum which could not be

A-C5ai cont'd

crystallized (Found: *m/e* 619.1017 and 621.0982. $C_{24}H_{30}{}^{35}ClN_3O_{10}S_2$ and $C_{24}H_{30}{}^{37}ClN_3O_{10}S_2$ require 619.1061 and 621.1032 resp.): $\lambda_{max}$ 216 nm (log ε 4.65), 251 (4.11), 302 (3.34): $\nu_{max}$ (KBr) see Figs IR2.1, IR2.2, IR2.3: δ 7.12, 1 H, s, H-10; 5.34, 1 H, s, H-5a; 4.65, 1 H, s, H-11; 4.00, 1 H, s, OH; 3.88, 3.83, 3.80 and 3.76, 12 H, 4 s, $2CH_3O$ and $2COOCH_3$; 3.74 and 3.70, 4 H, 2 s, $2SCH_2COO$; 3.36, 3 H, s, 6-$CH_3$; 3.09, 3 H, s, 2-$CH_3$; 1.90, 3 H, s, 3-$CH_3$.

ii. The above preparation was repeated (Table 2.2) with half quantities.

In chromatography the column showed no colours when loaded but blue colours (the substituted indigotin) appeared as it developed. It was suspected that the ether was contaminated with peroxide: the peroxide test (NaI dissolved in starch solution) was positive. As a consequence of the peroxide the yield was negligible.

iii. The above experiment (i) was repeated (Table 2.2).

Chromatography with ether-benzene (1:4) yielded sdm (80 mg) then dimethyl secosdm-*S,S'*-diacetate (110 mg) and another gum (120 mg); the fractions were accumulated according to the t.l.c. (EtOEt-$C_6H_6$ 2:3) result.

A-C5b. *Methyl* 11a-*mercaptosecosporidesmin*-3-S-*acetate* (1.14a)

iv. Experiment iii. above was repeated (Table 2.2). This time the low $R_F$ (t.l.c.) gum crystallized to yield *methyl* 11a-*mercaptoseco-sporidesmin*-3-S-*acetate* from methanol, m.p. 150—156° after repeated recrystallizations (Found: *m/e* 547.0828; C, 44.8; H, 4.5; Cl, 6.5; N, 7.6; S, 11.3. $C_{21}H_{26}{}^{35}ClN_3O_8S_2$ requires 547.0850 and $C_{21}H_{26}ClN_3O_8S_2,H_2O$ requires C, 44.6; H, 5.0; Cl, 6.3; N, 7.4; S, 11.3%: $\lambda_{max}$ 222 nm (log ε 4.62), 253 (4.21), 300 (3.65) almost a shoulder; $\nu_{max}$ (KBr) see Figs. IR2.1, IR2.2, IR2.4, IR5.15; ($CCl_4$) see Fig. IR5.21;

A-C5biv cont'd

Compound (1.14a) unsolvated $\nu_{max}$ ($CCl_4$) see Fig. IR5.14A;

ditto ($CHCl_3$-$CCl_4$ 1:39) see Fig. IR5.14B

(νSH): δ 7.07, 1 H, s, H-10; 5.35, 1 H, s, H-5a; 5.04, 1 H, s(b), OH; 4.64, 1 H, s(b), H-11; 3.78 and 3.83, 9 H, 2 s, $2CH_3O$ and $COOCH_3$; 3.75, 2 H, s, $SCH_2COO$; 3.37, 3 H, s, 6-$CH_3$; 3.00, 3 H, s, 2-$CH_3$; 2.02, 3 H, s, 3-$CH_3$; *m/e* 515, 513 ($M^+$-$H_2S$) (176 and 300% of $M^+$ resp.).

iva. *Alkylation of methyl* 11a-*mercaptosecosporidesmin*-3-S-*acetate*

Methyl 11a-mercaptosecosdm-3-*S*-acetate (10 mg) in methanol (2 ml) and pyridine (36 μl) with methyl chloroacetate (18 μl) and sodium iodide (27 mg) were heated 34°, 1 d). Examination by t.l.c. revealed no formation of dimethyl secosdm-*S*,*S*'-diacetate.

No new compounds (t.l.c.) were observed when methyl iodide (250 μl) was used in a similar experiment.

A-C5c. *Methyl* 3-*mercaptosecosporidesmin*-11a-S-*acetate* (1.13)

v. For quantities in this preparation see Table 2.2.

Chromatography of the product yielded sdm (32 mg in 20 cm EtOEt-$C_6H_6$ 1:9), a fraction (5 cm) consisting of a gum (51 mg, $R_{sdm}$ 0.61 and a trace of sdm, t.l.c. EtOEt-$C_6H_6$ 2:3), a large (477 mg) fraction (40 cm, 1:4) showing two spots on t.l.c. ($R_{sdm}$ 0.61 and 0.59) very close together, a fraction (60 cm) consisting of dimethyl secosdm-*S*,*S*'-diacetate ($R_{sdm}$ 0.59) and another fraction (60 cm 7:13) containing methyl 11a-mercaptosecosdm-3-*S*-acetate ($R_{sdm}$ 0.19) with traces of the preceding compound.

That gum (477 mg) showing the two substances, was rechromatographed (14 mm Vitreosil column) on silica gel G $F_{254}$ (5 g). The latter silica gel was used to facilitate the 'cutting' of the fractions (under the 254 nm lamp). The column was loaded using light petroleum (40—60°) and eluted with light petroleum containing increasing concentrations of ether.

A-C5cv cont'd

The major quenching zone did not begin to move till the concentration of ether reached 60%, to yield a single component fraction (240 mg, t.l.c. $R_F$ 0.83, 9:1) followed by another (155 mg) containing dimethyl secosdm-*S*,*S*'-diacetate as well, and finally a fraction (10 mg) which crystallized to methyl 11a-mercaptosecosdm-3-*S*-acetate.

The single component fraction (240 mg) was dissolved in acetone and diluted with ether. Repeated evaporation and addition of ether produced crystals of *methyl 3-mercaptosecosporidesmin*-11a-S-*acetate* (1.13). This commenced melting at 103°, resolidified at 114° and remelted at 151—166° (dec.) (Found: *m/e* 547.0795 ($^{35}$Cl) and 549.0893 ($^{37}$Cl). $C_{21}H_{26}{}^{35}ClN_3O_8S_2$ and $C_{21}H_{26}{}^{37}ClN_3O_8S_2$ require 547.0850 and 549.0822 resp.): $\lambda_{max}$ 221 nm (log ε 4.67), 254 (4.14), 305 (3.64); $\nu_{max}$ (KBr) see Figs IR2.1, IR2.2, IR2.4, IR5.18 (νCO); ($CCl_4$) see Fig. IR5.13: δ 7.07, 1 H, s, H-10; 5.30, 1 H, s, H-5a; 4.75, 1 H, s, OH; 4.65, 1 H, d, *J* 4 Hz, H-11; 3.98, 1 H, q, *J* 2 Hz, 3-SH; 3.78, 3.80 and 3.82, 9 H, 3 s, $2CH_3O$ and $COOCH_3$; 3.75, 2 H, s, $SCH_2COO$; 3.34, 3 H, s, 6-$CH_3$; 3.04, 3 H, s, 2-$CH_3$; 1.87, 3 H, d, *J* 2 Hz, 3-$CH_3$: *m/e* 513, 515 ($M^+$-$H_2S$) (51 and 57% of $M^+$ resp.).

vi. A 2 mmol preparation (Table 2.2)

Chromatography of the (dil.) acid-washed residue with ether-benzene (1:4) yielded sdm (250 mg), a mixture of 3 compounds (550 mg), dimethyl secosdm-*S*,*S*'-diacetate (150 mg), a mixture of 2 compounds (62 mg), and methyl 11a-mercaptosecosdm-3-*S*-acetate (210 mg).

Rechromatography of the mixture (550 mg) developed with light petroleum-ether yielded sdm (18 mg), methyl 3-mercaptosecosdm-11a-*S*-acetate (61 mg), and a mixture of this methyl compound and dimethyl secosdm-*S*,*S*'-diacetate (400 mg) which was chromatographed (ether-benzene) again. This yielded methyl 3-mercaptosecosdm-11a-*S*-acetate (23 mg) and dimethyl secosdm-*S*,*S*'-diacetate (360 mg).

A-C5cvi cont'd

An attempt was made at separating the 3-mercapto-compound from the diacetate by partition between sodium hydroxide solution (1.2M) and chloroform. The chloroform extract contained the same relative concentrations as the original mixture. The raffinate after acidification contained only a trace of the two compounds.

A-C5d. *Hydrolyzing dimethyl secosporidesmin-*S,S'*-diacetate with ion exchange resin*

After Davies and Thomas (1952), an ether solution of dimethyl secosdm-*S,S'*-diacetate (60 mg, 0.1 mmol) was evaporated to a film over the wall of a small flask. Ion exchange resin, IR 100 (H) (35 mg) was added with water (1.6 ml) and shaken (20 d). The resin was filtered out and the filtrate extracted ($CHCl_3$ then EtOEt). The residue (EtOEt extract) showed 2 broad peaks between 2700 and 2500 $cm^{-1}$ and another at 1720vs $cm^{-1}$.

A-C5e. *Trimethylsilylation of secosporidesmin-*S,S'*-diacetate*

After Safe and Taylor (1972), dimethyl secosdm-*S,S'*-diacetate (36 mg) was dissolved in pyridine (500 µl), hexamethyldisilazane (200 µl) and trimethylsilyl chloride (100 µl) and stood (3 d) at room temperature. Washing the solution ($CHCl_3$) with water removed a whitish material ($NH_4Cl$ ?). The noncrystalline residue was dimethyl *O,O'*-bis(trimethylsilyl)secosporidesmin-*S,S'*-diacetate: δ 6.82, 1 H, s, H-10; 5.38, 1 H, s, H-5a; 4.61, 1 H, s, H-11; 3.87, 3.80, 6 H, 2 s, $2CH_3OAr$; 3.75, 6 H, s, $2COOCH_3$; 3.37, 3 H, s, 6-$CH_3$; 3.09, 3 H, s, 2-$CH_3$; 1.85, 3 H, s, 3-$CH_3$; 0.05, 9 H, s, $Me_3Si$; -0.17, 9 H, s, $Me_3Si$. When the product was subjected to sublimation (0.001 mmHg) it was stable to 123° but turned brown at 130°; the sublimate was hairlike crystals which did not appear to grow with time (2 d). Both the sublimate and the original

A-C5c cont'd

material had the same i.r. spectra.

An attempt at chromatographing ($SiO_2$ gel, $C_6H_6$-EtOEt) the trimethylsilyl ether, either by column or t.l.c., resulted in hydrolysis to the ester.

A-C6. *Complexing the ester derivatives of sporidesmin to proteins and lysine.*

A-C6a. *To poly(*L-*lysine)* (p11)

i. *With diethyl secosporidesmin-*S,S'-*diacetate*

P11.HBr (Sigma) (see Table 2.3) was dissolved in water (4.2 ml) and ethanol (2.8 ml) added. Diethyl secosdm-*S,S'*-diacetate was dissolved in ethanol (0.8 ml) and water (1.2 ml) added which turned the solution cloudy. This cloudiness disappeared on addition of the p11 solution. The pH was corrected to *c.* 10 (paper) with sodium hydroxide (0.1M) and the solution allowed to stand overnight. The alcohol was evaporated from the neutralized solution and excess ester (by i.r. spectrum) extracted ($CHCl_3$, 7.6 mg). The dried residue of (ethoxy)-poly(secosporidesmin-*S,S'*-diacetyl)poly-(L-lysine) which showed the peaks of modified sdm (1470, 1410, 1380 $cm^{-1}$) but no ester peaks (1725 $cm^{-1}$), was forwarded for immunological studies... Preparation 1. (Table 2.5).

ii. *With methyl* 11a-*mercaptosecosporidesmin-*3-S-*acetate*

P11.HBr (Sigma, *M* 139,000, and Miles, *M* 70,000, Table 2.3) was dissolved in water and alcohol as above. Methyl 11a-mercaptosecosdm-3-*S*-acetate as a gum was dissolved in ethanol but crystallized out. The pH of the mixture was raised to 9 (paper) and shaken (2 d). The aqueous suspension after concentration was diluted ($H_2O$, 50 ml) and extracted ($CHCl_3$, 74 mg). The dried residue of poly-(11a-mercaptosecosporidesmin-3-*S*-acetyl)poly-(L-lysine) was again forwarded for immunological studies. .. .. .. .. Preparation 2. (Table 2.5).

A-C6a Cont'd

iii. *With dimethyl secosporidesmin-*S,S'*-diacetate*

P11.HBr (Miles, Table 2.3) was dissolved in water (29 ml). Dimethyl secosdm-*S,S'*-diacetate was dissolved in ethanol (12 ml) and transferred to the protein solution (EtOH, 7 ml). In 24 h the pH fell from 9 to 8 (paper). The aqueous suspension after concentration was extracted ($CHCl_3$, 30 mg) and dried for immunological studies.

.. .. .. .. .. .. .. Preparation 3. (Table 2.5). This complex, (methoxy)poly(secosporidesmin-*S,S'*-diacetyl)poly-(L-lysine), was found to be insoluble in water but soluble in aqueous methanol or aqueous ethanol.

iv. *Preparation iii. was repeated*

The aqueous suspension from this preparation (see Table 2.3) after evaporation of the alcohol was made up to 9.8 ml i.e. 1 ml ≡ 50 μmol of lysyls.

The percentage substitution was estimated in aliquots of this solution by the Boyd, *et al.* (1972) modification of the Moore and Stein (1948) method. The ninhydrin solution used was that prepared for the Amino Acid Autoanalyser.

The readings for the p11.HBr were:

0.588, 0.589, 0.562 Average: 0.579 A.U.

The readings for the substituted p11 were:

0.510, 0.518, 0.490 Average: 0.506 A.U.

The difference was 0.073, therefore, the percentage substitution was 0.073/0.579 = 12.6% substitution.

The aqueous concentrate from evaporating the above alcoholic solution under reduced pressure, was gently extracted (EtOEt, 5.6 mg, *m/e* 647 ($M^+$) i.e. unchanged diacetate) and dried for immunological studies.

.. .. .. .. .. .. .. Preparation 6. (Table 2.5).

A-C6aiv cont'd

The u.v. spectrum for this preparation (against an equal concentration of p11.HBr) showed bands at 217 (shoulder on end absorption), 251 and 302 nm equivalent to those for the diacetate.

v. *Attempt to saturate p11 with modified sporidesmin*

The reagents were dissolved as before (Table 2.3) and the pH adjusted to 9. After 4 d the preparation was analyzed by the same method as under iv. above and gave the following results in absorbance units:

Sdm complex: 0.439, 0.443, 0.374 Average: 0.419.

P11 control: 0.536, 0.531, 0.524 Average: 0.530. Therefore:

$$\frac{0.419}{0.530} \times 100 = 79.0\% \text{ unsubstituted.}$$

Replicate: Sdm complex: 0.489, 0.536, 0.503 Average: 0.509.

P11 control: 0.572, 0.555, 0.560 Average: 0.562.

Therefore $\frac{0.509}{0.562} \times 100 = 90.5\%$ unsubstituted.

Replicate: Sdm complex: 0.478, 0.451, 0.458 Average: 0.462.

P11 control: 0.522, 0.525, 0.598 Average: 0.548.

Therefore $\frac{0.462}{0.548} \times 100 = 84.5\%$ unsubstituted.

The average unsubstitution was 85% suggesting that only *c.* 15% of the ε -amino-groups of the p11 have been substituted.

The aqueous suspension was ether extracted (9.6 mg) and dried to yield (methoxy)poly(secosporidesmin-*S*,*S*'-diacetyl)poly-(L-lysine).

.. .. .. .. .. .. .. Preparation 7. (Table 2.5).

vi. *Second attempt to saturate p11 with modified sporidesmin* (Table 2.3)

As the pH dropped from 9 it was corrected and during this process a gel precipitated (22.8 mg). This gel was washed and dried for immunological studies, see viii. below.

A-C6a cont'd

vii. *Third attempt*

The reagents (Table 2.3) were mixed in the same way as previously and the pH manually maintained at 9 till constant. This again yielded an insoluble material (27.7 mg), cross-linked (ethoxy)poly(secosporidesmin-*S*,*S*'-diacetyl)poly-(L-lysine), which was collected by centrifuging (washed, EtOH-$H_2O$ 2:3). It was insoluble in the supernatant liquid (pH 2—10), methanol, chloroform, *N*,*N*-dimethylformamide or trifluoroacetic acid. When heated in the latter solvent it turned dark blue without dissolving. This preparation was combined with viii. below.

viii. *Fourth preparation, using a pH titrator*

P11.HBr (Table 2.3) and dimethyl secosdm-*S*,*S*'-diacetate were mixed with sufficient ethanol-water (2:3, *c*. 30 ml) for the titrator which maintained the pH at 10.5 (under $N_2$, with 0.33M NaOH). The insoluble material (67.3 mg), collected (5 h) as under vii. and washed gave *cross-linked poly(methoxy)poly(secosporidesmin-*S,S'*-diacetyl)poly-(L-lysine)* (Found: C, 46.9; H, 6.3; Cl, 3.7; N, 12.2; S, 5.4. If 62% of the ε-amino-groups were substituted it would require C, 51.2; H, 6.3; Cl, 3.7; N, 13.6; S, 6.6%): $\nu_{max}$ (KBr) 1465, 1410, 1380, 1350, 1305, 1100, 1035, 1000, 855 and 780 $cm^{-1}$. The remainder of this preparation together with those from vi. and vii. above was sent for immunological studies. .. .. .. .. .. Preparation 8. (Table 2.5).

The i.r. spectrum of the dried supernatant (from the gel) which did not precipitate white material upon evaporation under reduced pressure, showed no peaks (i.r.) characteristic of the dimethyl secosdm-*S*,*S*'-diacetate.

ix. *As a control for immunology, poly-(L-lysine) was acylated with methyl chloroacetate*

Methyl chloroacetate (24 μl, 0.27 mmol) in alcohol was added to

A-C6aix cont'd

poly(L-lysyls).HBr (105 mg, 0.5 mmol as lysyls) in water (15 ml) and the pH adjusted to *c.* 9. After 3 days the preparation, poly(chloroacetyl)-poly-(L-lysine), was dried for immunology.

A-C6b. *To lysine*

i. *Attempt at acylation of the copper chelate of lysine*

After Taniyama, *et al.* (1971) lysine (128 mg, m.p. 240—255° therefore dihydrochloride dihydrate, 0.5 mmol) was dissolved in water (200 µl) and adjusted to pH 9.5. Copper sulphate (bluish white, nearly anhydrous, 57.0 mg, *c.* 0.35 mmol) was dissolved in a minimum of water and added to the lysine solution producing a deep blue solution (pH 8.5, $\lambda_{max}$ 630 nm) which formed two phases with ethanol, instead of precipitating the copper chelate.

There was no reaction between this copper chelate of lysine and either dimethyl secosdm-*S*,*S*'-diacetate or ethyl acetate: there was still the ester peak at 1725 $cm^{-1}$. The synthesis was discarded.

ii. *Attempt at acylation at pH 7—8 without copper chelation*

After Okawa and Hase (1965), dimethyl secosdm-*S*,*S*'-diacetate (20 mg) in ethanol-water (2:3) was added to lysine.2HCl (17 mg) dissolved in ethanol-water (2:3, total 1.2 ml) and adjusted to pH 7.5—8 (paper). T.l.c. ($Bu^nOH$ saturated with 1.5M HCl) showed dimethyl secosdm-*S*,*S*'-diacetate ($R_F$ 0.9) and lysine at the origin with no other spot. Lysine was visualized with sodium β-naphthoquinone-4-sulphonate (1%) and sodium carbonate (5%) (Folin, 1922).

iii. *Attempt at acylation at pH* c. *9 without copper chelation*

Lysine.2HCl (8.3 mg) and dimethyl secosdm-*S*,*S*'-diacetate (10 mg) were dissolved together (EtOH-$H_2O$ 2:3) and maintained at pH 9 for 5 d. T.l.c. (EtOEt-$C_6H_6$ 3:7) showed only one mobile spot but t.l.c. ($CHCl_3$-

A-C6biii cont'd

MeOH-1M $NH_3$ 4:4:1) showed a second spot slower than the ester ($R_{ester}$ 0.65) which increased as the ester spot decreased. The lysine spot was visualized with Folin's (1922) reagent and by ninhydrin (Randerath, 1964a). The plates were also sprayed with bromocresol purple (Randerath, 1964b). None of the u.v. quenching spots appeared as spots upon spraying with either the ninhydrin, the bromocresol purple or the Folin's reagent.

A-C6c. *To bovine plasma albumin* (bpa)

Bpa (Table 2.6) was dissolved in water (5 ml) but turned cloudy when alcohol (2.3 ml) was added. The cloudiness disappeared upon raising the pH to 9. To this solution was added dimethyl secosdm-*S*,*S*'-diacetate in alcohol (2 ml) and water (3 ml) (just cloudy). Electrophoresis after one day's reaction showed 33% of the modified-sdm-treated protein to move at a slower rate ($R_{bpa}$ 0.77). After 2 d the acidified (pH 4) aqueous residue was extracted ($CHCl_3$, 16 mg). Chloroform tended to cause these aqueous solutions to become cloudy: the cloudiness redissolved in ethanol. The suspension, (methoxy)poly(secosporidesmin-*S*,*S*'-diacetyl)bovine plasma albumin, was evaporated to a small volume for immunological studies. .. .. .. Preparation 4. (Table 2.5).

A-C6d. *To rabbit serum albumin* (rsa)

i. *With dimethyl secosporidesmin*-S,S'-*diacetate*

To the dimethyl secosdm-*S*,*S*'-diacetate (Table 2.6) dissolved in ethanol (1 ml) and water (1.5 ml) was added rsa dissolved in water (3 ml, pH 9) and ethanol (2 ml). After 2 d the preparation was checked electrophoretically but the migration showed no difference from that of natural rsa. The aqueous residue was extracted with ether and the raffinate examined spectrophotometrically. This u.v. spectrum showed no peaks due to sdm. No substitution had occurred.

A-C6d cont'd

ii. *With methyl 3-mercaptosecosporidesmin-11a-S-acetate*

The methyl 3-mercaptosecosdm-11a-*S*-acetate (Table 2.6) dissolved in ethanol-water (2:3) was added to rsa in ethanol-water (2:3) making a slightly cloudy suspension (7.5 ml, pH 5.5). When correcting the pH there was a heavy precipitate (pH 6--6.25 *c.* the isoelectric point) which cleared (pH 8). Electrophoresis after 21 h showed two bands. The areas under the scanning curves indicated 57% had reacted. At 45 h there were again 2 bands: 75% had reacted. After 62 h 91% of the protein moved in the slower band.

Taking the whole sample up with water (6 ml) caused a copious precipitate which did not extract with ether (3.8 mg). The u.v. spectrum of the raffinate (in EtOH-$H_2O$ solution) showed peaks at 215 and 252 nm, characteristic of sdm. The suspension, poly-(3-mercaptosecosporidesmin-11a-*S*-acetyl)rabbit serum albumin was dried for immunological studies.

.. .. .. .. .. .. .. Preparation 5. (Table 2.5).

iii. *Preparation ii. repeated* (Table 2.6)

After 6 d, electrophoresis showed only one band moving at the same rate as that of natural rsa. Therefore, the experiment was unsuccessful.

iv. *With diethylsecosporidesmin-S,S'-diacetate*

The reaction mixture (Table 2.6) was maintained at pH 9—10. After 3 d, electrophoresis showed only one discrete band moving more slowly than rsa. The concentrated aqueous solution was extracted (EtOEt, 3.9 mg) and the raffinate showed the u.v. spectral pattern characteristic of sdms superimposed upon the end absorption of rsa. The dried suspension of (ethoxy)poly(secosporidesmin-*S*,*S*'-diacetyl)-rabbit serum albumin, was forwarded for immunological studies.

.. .. .. .. .. .. .. Preparation 9. (Table 2.5).

A-C6e. *To Ovalbumin*

i. To diethyl secosdm-*S,S'*-diacetate (Table 2.6) in ethanol, ovalbumin in water was added. A white curd separated immediately and did not redissolve when the pH was raised to 8.5. Excess water did not redissolve it either. In less than 1 h, it was carefully extracted (EtOEt, 87 mg). The i.r. spectrum of this extract was consistent with the original spectrum of the diacetate.

ii. An aqueous (15.5 ml) solution of ovalbumin (Table 2.6) was added to a film of diethyl secosdm-*S,S'*-diacetate, and the pH raised to 9.5. After stirring for 2 d, no impression seemed to be made on the film. When a sample was made 16% with ethanol (containing the diester), it was cloudy but the cloudiness was extractable with ether. Hence, alcohol (3 ml) was added to enhance the rate of solution of the ester film. This gave a slightly cloudy suspension. Next day electrophoresis showed no slow moving bands. Four days after adding the ethanol, more (1 ml) was added. Electrophoresis now showed a band remaining at the origin. After another 8 d of continual stirring the aqueous suspension was extracted (19 mg) with ether leaving a cloudy raffinate. The filtered raffinate showed the peaks of sdm superimposed on the end absorption in the u.v. The dried suspension of (ethoxy)poly(secosporidesmin-*S,S'*-diacetyl)ovalbumin was forwarded for immunological studies.

.. .. .. .. .. .. .. Preparation 10. (Table 2.5)

A-C6f. *To bovine thyroglobulin* (btg)

To bovine thyroglobulin (Sigma, Table 2.6) in water (0.7 ml), diethyl secosdm-*S,S'*-diacetate in ethanol (2.5 ml) and water (3.75 ml) was added and formed a cloudy suspension which remained cloudy after more water (3 ml) was added. On raising the pH to 9.5 (BDH paper) the cloudiness disappeared. In the electrophoretic pattern after 7 d the substituted thyroglobulin moved faster ($R_{btg}$ 1.2) than natural thyroglobulin

A-C6f cont'd

but tailed back to the origin.

The aqueous suspension (pH 5.5 without correction) containing the poly(ethoxy)poly(secosporidesmin-*S,S'*-diacetyl)bovine thyroglobulin, was extracted (1 mg) with ether then concentrated to 5 mg equivalent thyroglobulin/ml for injection into sheep.

In collaboration with R. Fairclough, 4 Perendale lambs were selected and each was injected with *c.* 1.25 mg equivalents of thyroglobulin blended with Freund's adjuvant. A 'booster' injection of the same kind was given after 2 months.

A-C7. *S-alkylation with the chloroacetate of a 'good leaving group' and one which promotes crystallization*

i. *Sodium picrate and chloroacetyl chloride*

Chloroacetyl chloride (191 mg, 1.7 mmol) was added to the dried sodium salt of picric acid (427 mg, 1.7 mmol), and warmed. The i.r. spectrum of the product showed no peaks around 1700 $cm^{-1}$ or more, nor a broad peak around 1150 $cm^{-1}$.

Only picric acid was recovered from picric acid plus chloroacetyl chloride and even when pyridine was added.

ii. p-*Nitrophenyl chloroacetate* (After Auwers, *et al.*, 1927)

To *p*-nitrophenol (354 mg, 2.55 mmol), chloroacetyl chloride (400 mg, 3.54 mmol) and ether (5 ml), pyridine (285 µℓ, 3.54 mmol) was slowly added. After refluxing (30 min) and filtering out the pyridinium hydrochloride the ether evaporated leaving crystals (68%). The brown crystals from benzene were sublimed (80—88° at 0.001 mmHg) m.p. 94° (lit. 94°). The i.r. spectrum (KBr from AcMe-EtOEt) of these crystals showed a doublet peak at 1770 $cm^{-1}$ (ester C=O) and 1140 $cm^{-1}$ (chloroacetic ester C-O-C).

A-C7 cont'd

iii. p-*Nitrophenol chloroacetate and sdm/sodium borohydride*

Sdm.$C_6H_6$ (55.3 mg, 0.1 mmol), *p*-nitrophenyl chloroacetate (66.6 mg, 0.3 mmol) sodium iodide (3.3 mg) were dissolved in pyridine (130 µl) and methanol, and cooled. Sodium borohydride (33 mg) in methanol (cold) was added. Chromatography ($C_6H_6$-EtOEt) of the product yielded 2 fractions, one (48 mg), *p*-nitrophenol (i.r.), and two (39 mg), dimethyl secosdm-*S*,*S*'-diacetate (i.r.).

A-C8. S,S'-*Homosporidesmin*

i. Sdm.$C_6H_6$ (25 mg, 50 µmol) was dissolved in methanol (1.5 ml), pyridine (0.1 ml) and methylene diiodide (320 mg, 1.2 mmol, washed with thiosulphate solution) and cooled. Sodium borohydride (14 mg) was dissolved in cooled (dry ice-AcMe) methanol (0.7 ml) and added. T.l.c. after 1 h showed a negligible amount of unreacted sdm. Chromatography of the washed product yielded sdm (1.8 mg), a gum (14.5 mg) and a third substance (2.6 mg).

ii. Sdm.$C_6H_6$ (109 mg, 0.2 mmol) was treated as above. Crystals formed in the cold, presumably $I^-$ $py^+$-$CH_2$-$py^+$ $I^-$. After 1 h the i.r. spectrum of the crude reaction mixture showed negligible sdm but a new pattern. So the residue from evaporating the methanol, was washed ($CHCl_3$-$H_2O$) and chromatographed ($C_6H_6$-EtOEt 22:3). The gum which was eluted between 20 and 40 cm of solvent (13 mm column) remained uncrystallized for 4 months during which time it decomposed to a deep orange colour. Chromatography yielded a pink zone (substituted isatin) and an orange zone (anhydrodethiosdm) followed by a colourless gum which was dissolved (with the gum under i.) in acetone. Repeated evaporation and addition of ether produced crystals, m.p. 238.5—240.5, of S,S'-*homosporidesmin* (Found: *m/e* 489, 487 ($M^+$); C, 46.8; H, 4.7; Cl, 7.5; N, 8.9; S, 12.9. $C_{19}H_{22}{}^{35}ClN_3O_6S_2$ requires *M* 487; C, 46.8; H, 4.5; Cl, 7.3; N, 8.6; S, 13.1%):

A-C8ii cont'd

δ 7.05, 1 H, s, H-10; 5.35, 1 H, s, H-5a; 4.48, 1 H, d, *J* 2Hz, H-11, collapsed to a singlet with $D_2O$; 4.02, 3.98, 2 H, 2 s, $SCH_2S$ (conformational isomers); 3.87, 3.83, 6 H, 2 s, $2CH_3OAr$; 3.55, 1 H, d, *J* 2 Hz, 11-OH, disappeared with $D_2O$; 3.40, 3 H, s, 6-$CH_3$; 3.18, 1 H, s, 10b-OH, disappeared with $D_2O$; 3.08, 3 H, s, 2-$CH_3$; 1.90, 3 H, s, 3-$CH_3$: $\nu_{max}$ (KBr) see Figs IR2.1, IR2.2, IR2.4, IR5.18.

iii. *A 1 mmol level of production*

Chromatography (EtOEt-$C_6H_6$ 3:22) yielded sdm (10 mg), the gum (homosdm, 390 mg), a substance (1:3) by t.l.c. (EtOEt-$C_6H_6$ 2:3) $R_{homosdm}$ 0.63 (62 mg) which turned brown on the t.l.c. plate and another (EtOEt-$C_6H_6$-MeOH 18:27:5) at $R_F$ 0.0 (40 mg). These latter were not identified.

A-C9. *Attempted preparation of* 12a-*phenyl*-S,S'-*homosporidesmin* (2.2)

Sdm-$C_6H_6$ (111 mg, 0.2 mmol) was dissolved in methanol, pyridine (550 μℓ) with sodium iodide and benzylidene dichloride (320 mg, 1.3 mmol), and cooled. Sodium borohydride (60 mg) was dissolved in cooled methanol and added. After allowing to warm (2 h) the evaporated residue in chloroform was washed with acid and bicarbonate. Chromatography (EtOEt-$C_6H_6$ 1:9) yielded 23 mg sdm (50 cm) and 2 mg (7:13) of gum. A further 11 mg was obtained with methanol (50 cm). Over 90 mg were unaccounted for. This synthesis was not pursued any further.

A-C10. *Attempted preparation of* S,S'-*dihomosporidesmin* (2.3)

i. To sdm.$C_6H_6$ (72 mg, 0.13 mmol) dissolved in pyridine, methanol and ethylene dibromide (229 mg, 2.3 mmol from ethylene and bromine, Gattermann, 1938) and cooled, was added sodium borohydride (34 mg). The residue after washing ($CHCl_3$-$H_2O$) was chromatographed. Sdm (11 mg) as a gum (19 mg) was recovered.

A-C10 cont'd

ii. The above was repeated with 100 mg sdm.$C_6H_6$.

The silica gel used in the chromatography gave an unsatisfactory answer: sdm (45 mg) and a long indefinite tail was obtained.

A-C11. *Attempted preparation of* 12a,12b-*di(methoxycarbonyl)*-S,S'-*di-homosporidesmin* (2.4)

Sdm.$C_6H_6$ (78 mg, 0.14 mmol) was dissolved in pyridine (0.3 ml) and methanol with sodium iodide (170 mg) and dimethyl 2,3-dibromo-succinate (from fumaric acid and bromine, after Rhinesmith, 1943, methylated with diazomethane, DeBoer and Backer, 1956), and cooled. Sodium borohydride (36 mg) in cooled methanol was added. After 18 h the product, dissolved in chloroform, was washed with sodium thiosulphate and water. Chromatography ($SiO_2$ gel, EtOEt-$C_6H_6$ 2:3) yielded no fractions containing sdm derivatives. In the search for the fractions the t.l.c. plates were sprayed with silver nitrate (5%) to indicate the position of sulphur containing compounds but none appeared.

A-D. O-*Acylation*

A-D1. *With monomethyl glutarate* (mmg)

A-D1a. *In the presence of trifluoroacetic anhydride* (tfaa)

*Sporidesmin* 11-*(methyl glutarate)* (2.5)

i. Tfaa (140 µl, 1 mmol) and mmg (59.4 mg, 0.4 mmol) were mixed (dry box). Sdm.$C_6H_6$ (108.5 mg, 0.2 mmol) was added and the solution immediately turned dark brown. After 40 min the excess tfaa was removed under reduced pressure and the residue washed ($NaHCO_3$ solution). The main product was anhydrodethiosdm (i.r.) (1.16).

ii. Tfaa was mixed (mol/mol as near as possible) with mmg and sealed in glass phials and held (3°) till needed. The mixture turned to a light amber colour but remained that colour (3°).

A-D1a cont'd

iiα. Sdm.$C_6H_6$ (54 mg, 0.1 mmol) was dissolved (not completely) in a few (3) drops of acetone and, in a dry box, the mixture (60 μℓ) of tfaa (0.24 mmol) and mmg (0.24 mmol) added. The reaction mixture turned brown slowly. After 30 min the mixture was washed ($NaHCO_3$). T.l.c. of the residue showed much unreacted sdm and another spot of higher $R_F$ than sdm. The mass spectrum of the crude residue showed a series of peaks 32 mass units apart.

iiβ. To sdm.$C_6H_6$ (53 mg, 0.1 mmol) in acetone was added (in a dry box) the mixture (130 μℓ) of tfaa (0.5 mmol) and mmg (0.5 mmol). After 20 min the mixture was washed ($NaHCO_3$ solution) and concentrated. Chromatography ($C_6H_6$-EtOEt 9:1) yielded unchanged sdm (33 mg) and a gum (16 mg) with an $R_{sdm}$ of 0.75 ($C_6H_6$-EtOEt 1:4).

iiγ. To sdm.$C_6H_6$ (111 mg, 0.2 mmol) in acetone (nearly all dissolved) was added the mixture (260 μℓ) of tfaa (1 mmol) and mmg (1 mmol). After 30 min the mixture (in $CHCl_3$) was washed ($NaHCO_3$, till alkaline) and evaporated to dryness immediately. Chromatography (as under iiβ) yielded sdm (70 mg) and a gum (50 mg), which yielded crystals of *sporidesmin 11-(methyl glutarate)* (2.5) from benzene, m.p. 167—169° (Found: *m/e* 603 and 601; S, 10.3. $C_{24}H_{28}{}^{37}ClN_3O_9S_2$ and $C_{24}H_{28}{}^{35}ClN_3O_9S_2$ require *m/e* 603 and 601 resp.; S, 10.7%): $\nu_{max}$ (KBr) 3300 (OH), 1750vs and 1740vs (ester C=O), 1710vs, 1665vs (sdm-COs), 1470s, 1410s, 1350s (sdm peaks), 1330s, 1200s (RCOOR), 1160vs (RCOOMe) $cm^{-1}$.

iii. To sdm.$C_6H_6$ (111 mg, 0.2 mmol) was added mmg (62 mg, 0.42 mmol) and acetone (200 μℓ) to dissolve the sdm completely. When tfaa (133 mg, 0.63 mmol) was added (in dry box) the mixture turned amber quickly. After 30 min the mixture (taken up in $CHCl_3$) was washed ($NaHCO_3$ solution). By diluting (repeatedly) a filtered (to remove anhydrodethiosdm, insoluble in AcMe) acetone solution with ether and evaporating (to reduce the concen-

A-D1aiii cont'd

tration of AcMe) crystals formed whose i.r. spectrum showed a strong peak at 1800 $cm^{-1}$ (trifluoroacetate). The whole reaction product was chromatographed (as under iiß.) to yield a yellow band (31 mg) and sdm (48 mg) but no fractions containing esters. When the yellow band was dissolved (AcMe) crystals of anhydrodethiosdm deposited.

iv. Tfaa (348 mg, 0.63 mmol) and mmg (200 mg, 1.37 mmol) were mixed and allowed to stand (warmed to 40° for 15 min) for 4 h. This mixture of anhydrides was added to sdm.$C_6H_6$ (348 mg, 0.63 mmol) dissolved in acetone (500 µℓ). The washed (as above) reaction mixture was chromatographed to yield sdm (300 mg) and ester (65 mg).

v. Tfaa (48 mg, 0.23 mmol) and mmg (28 mg, 0.19 mmol) were mixed and warmed (40° for 30 min). Sdm.$C_6H_6$ (53 mg, 0.096 mmol) was dissolved in acetone and evaporated to a fluff and then desiccated. To this was added the mixed anhydride which dissolved the fluff but not the film on the wall of the reaction vessel. After 15 min acetone (over 'drierite', a drop) was added which dissolved the film immediately. The washed reaction mixture upon chromatography yielded sdm (25 mg) and ester (6 mg).

vi. Tfaa (431 mg, 2.05 mmol) and mmg (243 mg, 1.7 mmol) were mixed and held (3°) for 60 days. Sdm.$C_6H_6$ (220 mg, 0.4 mmol) was dissolved in acetone and evaporated to a fluff and redissolved in acetone (120 µℓ). The mixed anhydride was added to this solution which turned amber with anhydrodethiosdm crystallizing out. The residue after washing (as above) was chromatographed and yielded sdm (100 mg) and a mixture (50 mg) containing some ester and anhydrodethiosdm.

A-D1b. *With carbodiimide*

After Sheehan, *et al.* (1965), sdm.$C_6H_6$ (55 mg, 0.1 mmol), mmg (18 mg, 0.12 mmol) and triethylamine (15 µℓ) were dissolved in methylene

A-D1b cont'd

dichloride (14 µℓ). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (21.6 mg, 0.11 mmol) was added and the mixture allowed to stand (1 d) at room temperature. The i.r. spectrum after washing was that of sdm. It was still the same after warming (40°, 1 h). After 7 months standing there were still no ester peaks in the i.r. spectrum of the acid washed product.

A-D2. *With* p-*toluene-sulphonyl chloride*

Sdm-$C_6H_6$ (56.5 mg, 0.1 mmol) was dissolved in pyridine (0.8 ml) and toluene-*p*-sulphonyl chloride (38 mg, 0.2 mmol), recrystallized according to Fieser and Fieser (1967), added. After 48 d (3°), there were no crystals but the solution had turned a light amber. Although t.l.c. suggested that *c.* 20% had reacted the i.r. spectrum showed unchanged sdm with a small peak at 1175 $cm^{-1}$.

A-D3. *With chloroacetyl chloride*

i. *With a mixture of chloroacetyl chloride and* N,N-*dimethylaniline*

Mixtures of dimethyl aniline and chloroacetyl chloride became a thick, black mass in an hour.

By the method of Hauser, *et al.* (1944) sdm.$C_6H_6$ (112 mg, 0.2 mmol) was added to a mixture of *N,N*-dimethylaniline (50 µℓ, 0.4 mmol) and chloroacetyl chloride (31 µℓ, 0.4 mmol). It was warmed in a bath (65°) for a short time to dissolve all the sdm. The i.r. spectrum (2 h) showed mainly sdm. There was a small faster moving spot on the t.l.c. plate (EtOEt-$C_6H_6$ 1:4). This spot was no larger after another 16 h reaction time. At this stage the mass was sticky but clear and deep green. On chromatography ether-benzene (3:97) eluted 14% of the product: the rest was sdm. The i.r. spectrum of this 14% fraction had peaks at 1765m, and 1135brs $cm^{-1}$, but no large peak in the C-Cl absorbing region.

A-D3 cont'd

ii. *With chloroacetyl chloride added to a solution of sporidesmin*

Sdm.$C_6H_6$ (111 mg, 0.2 mmol) was dissolved in *N,N*-dimethylaniline (50 µℓ, 0.4 mmol) and acetone (200 µℓ) and chloroacetyl chloride (31 µℓ, 0.4 mmol) added, with cooling. After 90 min it was evaporated under reduced pressure and partitioned between ether and water. The ether layer contained mainly sdm (i.r.).

iii. No. ii. repeated with the same sdm sample and same order of adding the reagents. The sdm fluff completely dissolved (very slowly at 45°) without adding any acetone. Before adding the chloroacetyl chloride the solution was frozen (Westheimer and Shookhoff, 1940). After warming and standing at room temperature (2 h), the washed sample showed only sdm (i.r.).

A-D4. *With chloroacetic anhydride*

i. *Synthesis of chloroacetic anhydride*

After Katyshkina and Kraft (1959) chloroacetic acid (11.95 g, 127 mmol), boiled to remove water, was added to chloroacetyl chloride (21.55 g, 192 mmol) and potassium chloride (100 mg); the whole mixture was gently refluxed till there was no appearance of chloroacetic acid peaks in the i.r. spectrum (less than 7 h) (the HCl gas was trapped). At about 7 h the refluxing mixture darkened. By next morning the whole mass had crystallized. Upon distilling the mixture on the teflon spinning band column excess chloroacetyl chloride was recovered but the residue in the still pot was a black solid even at 120°. This solid had no anhydride peaks in the i.r. spectrum.

ii. The above experiment was repeated. Instead of distilling the product it was filtered to remove as much of the chloroacetyl chloride as possible without hydrolyzing the anhydride. The product was not free of chloride but was satisfactory in the following experiment.

A-D3 cont'd

iii. *Chloroacetylation of sporidesmin after White* (Ronaldson, *et al.* 1963)

To sdm (residue from experiment A-D3iii. above) dissolved in pyridine (0.4 ml) was added the crude chloroacetic anhydride (300 mg). After 30 min there was a single spot ($R_{sdm}$ 1.4 EtOEt-$C_6H_6$ 3:7). Overnight the whole reaction mass set to a glass. T.l.c. (EtOEt-$C_6H_6$ 3:7) showed only material at the origin. The reaction time had been too long.

iv. *The above repeated*

To sdm.$C_6H_6$ (53 mg, 0.1 mmol) in pyridine (200 µl) chloroacetic anhydride (102 mg, <0.3 mmol) was added and the solution turned an amber colour. T.l.c. (10 min) showed no sdm but another spot $R_{sdm}$ 1.3 (EtOEt-$C_6H_6$ 3:7). Therefore at 20 min the reaction mass was dissolved in chloroform and washed with water. T.l.c. showed only one spot faster than sdm and the i.r. spectrum showed peaks at 1765s (*CO*OR), 1140bvs (*ROC*OCCl), 780m (CCl) $cm^{-1}$. Chromatography (ether-benzene 1:49) yielded 44 mg of an ester with *m/e* 629, 627, 625 ($M^+$).

v. *Complexing sporidesmin di(chloroacetate) to bovine plasma albumin*

As described by Marks (1967) bpa (200 mg) was acylated with *S*-acetylmercaptosuccinic anhydride (samsa) (31.8 mg). After separating the protein from unreacted samsa (sephadex column), the protein was treated with hydroxylamine.HCl (19 mg) sodium iodide (20 mg) and the sdm di(chloroacetate) (44 mg, prepared under iv.) in an automatic titrator to maintain the pH constant at 7.5. There was no change in pH with time (i.e. the titrator did not add any alkali). After 1 h iodoacetamide (15 mg) was added but the titrator did not respond. The aqueous phase was concentrated (in vacuo) and the extract (EtOEt 14 mg) was sdm (i.r.). When this raffinate was compared with natural bpa (u.v.) there were no sdm peaks.

A-E. *Attempt at quaternization with methyl iodide*

Sdm. $C_6H_6$ (11.5 mg, 20 μmol) was dissolved in methyl iodide (1.02 g, 7 mmol) in sealed tube. No changes were observed upon storing in the dark (2 d). Upon heating the tube in a boiling water bath (34 h) two phases appeared.

Upon treating the residue (from evaporation of the iodide) dissolved in dil. acid with a filtered solution of Reinecke salt there was an immediate formation of crystals. The quantity was negligible and the i.r. spectrum did not show peaks characteristic of sdm.

A-F. *Diazonium coupling*

A-F1. p-*Nitroaniline*

A-F1a. *Reaction of diazotized* p-*nitroaniline with veratrole*

i. *p*-Nitroaniline (140 mg, 1 mmol) was diazotized according to Bunnett and Hoey (1958) (in $H_2SO_4$ + AcOH). The reaction mixture turned pale yellow almost immediately upon diazotization and if shaken it crystallized to a solid mass. When veratrole (128 μℓ in AcOH) was added to the diazonium suspension (after 30 min) there was an immediate reaction to dark purple. After 15 min the reaction mixture was allowed to warm to room temperature, was poured into ice-water (2.5 g in 7.5 ml) and filtered. No precipitate was found on the filter; dark red material was expected (Kokkinos and Wizinger, 1971).

ii. The above reaction was performed with more acetic acid (12 ml instead of only 1.6 ml). The diazotization was satisfactory, yielding an off white to pale yellow solution almost immediately on mixing. The diazonium sulphate crystallized readily on the ice bath from the acetic acid solution; it dissolved quickly on warming (40°). When the veratrole (in 2 ml AcOH) was added, the mixture turned darker and finally a brown colour. The mixture (after 2 h at room temperature) was poured into ice-water (15 g in 45 ml) producing a yellow orange solution. When

A-Flaii cont'd

the pH was made 2.5 (pellets of NaOH) the solution turned cloudy. (A test sample of the solution, at this stage, turned red with β-naphthol, indicating that there was still free diazonium salt.)

At pH 3—3.5 the colour was extracted (EtOAc). As more caustic (NaOH) was added so more cloudiness formed which could be extracted (EtOAc) as an orange solution. At high pH (>7) the solution turned rusty red.

The material extracted (EtOAc) was examined by mass spectroscopy which showed peaks at *m/e* 259 and 380, (mono- and di-(*p*-nitrophenyl)veratrole but no peak at 287 (3,4-dimethoxy-4'-nitroazobenzene).

iii. *Diazotizing* p-*nitroaniline in conc. hydrochloric acid, after Nietzki* (1887)

*p*-Nitroaniline (690 mg, 5 mmol) in conc. hydrochloric acid (10 ml) and sodium nitrite (380 mg, 5.5 mmol) in water (10 ml). The *p*-nitroaniline was not freely soluble in the acid but dissolved immediately upon addition of the sodium nitrite solution to give a light amber solution. If veratrole (138 μℓ, 1 mmol) in methanol (1.5 ml) and water (1.8 ml) was added slowly at this stage the reaction turned red with only a trace of precipitate. When alkali (10M NaOH) was added (to pH 7) a dark brown frothy mass resulted which turned dark purple with veratrole (138 μℓ, 1 mmol in MeOH). But when the alkali was added more slowly there was a sudden change to a brown precipitate at *c.* pH 4.5, at which pH, addition of veratrole produced a brown suspension. This brown suspension became a dark purple on raising the pH. A similar series of colour changes were obtained when the veratrole was added to the diluted ($H_2O$) diazonium salt solution and the pH slowly raised to about 9.

The ethyl acetate extracts of the above preparations were combined and chromatographed ($CHCl_3$). When some (100 mg) of the leading and main fraction (500 mg out of a theoretical 1.5 g) was sublimed (107°, 0.001

A-F1aiii cont'd

mmHg) the following zones were obtained: 1. a colourless liquid, veratrole (i.r. spectrum); 2. crystals, *p*-nitroaniline (i.r. spectrum); 3. a yellow oil containing crystals; and 4. dark brown at the origin. The mass spectrum of the crystals in zone 3. showed *m/e* 259, 380, 501 and 622 (mono- and poly-(*p*-nitrophenyl)veratroles).

A-F1b. *Synthesis of* 3,4-*dimethoxy*-4'-*nitroazobenzene*

A-F1bi. *Synthesis of* 4-*hydroxy*-3-*methoxy*-4'-*nitroazobenzene from guaiacol*

*p*-Nitroaniline (135 mg, 1 mmol) suspended in water (3 ml) and conc. hydrochloric acid (0.26 ml) and sodium nitrite (76 mg, 1.1 mmol) in water (0.75 ml) were mixed (in an ice bath). After 30 min guaiacol (116 mg, 0.94 mmol) dissolved in methanol (1 ml) and water (1 ml), was added. There was an immediate reaction which (during 90 min) deposited a dark orange mass (140 mg crystals). The supernatant upon raising the pH to 5.5 turned purple which precipitated more orange material on acidification (same i.r. spectrum as the dark-orange mass before). From the combined orange masses, 4-*hydroxy*-3-*methoxy*-4'-*nitroazobenzene* was crystallized (MeOH), m.p. 170.5—173° (125—135°, Colombano and Leonardi, 1908) (Found: $M^+$ 273; C, 57.4; H, 4.2; N, 15.3. Calc. for $C_{13}H_{11}N_3O_4$: *M* 273; C, 57.1; H, 4.1; N, 15.4%.): $\nu_{max}$ (KBr) see Table 2.9.

ii. 3,4-*Dimethoxy*-4'-*nitroazobenzene*

4-Hydroxy-3-methoxy-4'-nitroazobenzene (83 mg, 0.3 mmol), suspended in ether, was methylated with diazomethane, generated (Aldrich, 1970) from *N*-methyl-*N*-nitro-*p*-toluenesulphonamide (Diazald ®), (848 mg, 4 mmol) in an Aldrich diazomethane kit. After 30 h, the ether was full of crystals. Filtration yielded 3,4-dimethoxy-4'-nitroazobenzene, m.p. 157.5—160° (158°, Kokkinos and Wizinger, 1971) (Found: *m/e* 287 ($M^+$).

A-F1bii cont'd

Calc. for $C_{14}H_{13}N_3O_4$: *M* 287.): $\nu_{max}$ (KBr) see Table 2.9.

A-F1c. p-*Nitrobenzenediazonium chloride coupling to sdm*

i. *p*-Nitroaniline (138 mg, 1 mmol) was diazotized according to Schwalbe (1905) in hydrochloric acid (6 ml, 5M) with sodium nitrite (75 mg, 1.1 mmol). To this Schwalbe solution was added sdm.$C_6H_6$ (54 mg, 0.1 mmol) in methanol (5 ml). After several hours there was a strong smell of nitrobenzene (Sandmeyer reaction) and bubbles of nitrogen. Next day there was a red amorphous precipitate, giving crystals (3 mg) ($CHCl_3$) whose mass spectrum showed well defined peaks at *m/e* 578 and 576 ($M^+$-18), and a weak one at *m/e* 594 ($M^+$). 10-(4-Nitrophenyl)sporidesmin, $C_{24}H_{23}{}^{35}ClN_4O_8S_2$, requires *M* 594.

ii. The above experiment (i) was repeated but the sdm was added in acetone solution. The mixture darkened slowly and dark red flocks precipitated. This experiment was no improvement on the one above.

The filtrate was a dark colour whose extract (EtOAc) showed a leading purple spot on t.l.c. ($CHCl_3$). The mass spectrum of the leading zone from chromatography ($CHCl_3$-$C_6H_6$ 1:4) showed small peaks at *m/e* 295 and 293 and major peaks at *m/e* 256 and 254. These were not the kind of products desired so the experiment was abandoned.

iii. Experiment (i) above was repeated but with the sdm dissolved in ethanol. The precipitate (72 mg, after 4 d, 5°) was chromatographed. In none of the fractions was there any suggestion (i.r.) of a sdm derivative. A deep violet fraction (3.2 mg, 8 μmol) was obtained, which yielded crystals of 5-*chloro*-2-*hydroxy*-6,7-*dimethoxy*-1-*methyl*-4'-*nitro*-*indole*-3-*azobenzene* from $CHCl_3$-MeOH, m.p. 246—247.5° (Found: *m/e* 390.073 ($M^+$); $C_{17}H_{15}{}^{35}ClN_4O_5$ requires *M* 390.073): $\nu_{max}$ (KBr) see Table 2.9; and (nujol) 3400wbr (OH), 1660m (CO) 1600s $cm^{-1}$.

A-F1ciii cont'd

A control experiment consisting of the above reagents and ethanol but no sdm produced bubbles of nitrogen for *c.* 1 h. The reaction with sdm continued producing bubbles till the point of filtration.

iv. In another repeat of experiment (i) above, the pH was raised to above 7 with drops of sodium hydroxide solution (10M). There was no violent evolution of gas. The solution was extracted ($CHCl_3$) then acidified and re-extracted. In the chromatographed products there was no sign (i.r.) of any sdm derivatives only a trace of the above, 5-chloro-2-hydroxy-6,7-dimethoxy-1-methyl-4'-nitroindole-3-azobenzene, in the extract of the acidified raffinate.

A-F2. 2,4-*Dinitroaniline*

A-F2I. *Diazotization in conc. hydrochloric acid*

A-F2Ia. *Coupling to veratrole*

i. 2,4-Dinitroaniline (366 mg, 2 mmol) and sodium nitrite (151 mg, 2.2 mmol) were ground together. Conc. hydrochloric acid (18.5°) was added and the mixture stirred (2 h). A pressure developed in the flask (the stopper blew off). When veratrole (255 µℓ, 2 mmol) was added an orange solution and a 'red tar' (157 mg) resulted. When the orange solution (filtered) was poured into water (100 ml) there was no precipitation. Continued extraction ($CHCl_3$ then EtOEt) leached out only a yellow material. When resorcinol was added to the raffinate, there was a copious red precipitate which showed that all the diazonium chloride had not reacted in the precipitation of the 'red tar'.

Recrystallization of the 'red tar' yielded dark red woolly crystals (16.4 mg) and a mother liquor smelling strongly of veratrole which was evaporated (70° 0.5 mmHg). T.l.c. of the crystals showed an orange spot with the same $R_F$ as 2-chloro-3',4'-dimethoxy-4-nitroazobenzene and a

A-F21ai cont'd

purple spot ($R_{orange}$ 0.26) while the mother liquor showed the same spots in reversed intensities.

Chromatography ($CHCl_3$-$C_6H_6$ 1:4) of the 'red tar' produced deep red orange crystals (54 mg) and two dark purple bands (5 mg ea.). The crystals were 2-*chloro*-4,5-*dimethoxy*-2',4'-*dinitroazobenzene* from acetone-methanol, m.p. 222—224° (Found: *m/e* 368, 366 ($M^+$): C, 46.3; H, 3.1; N, 15.1. $C_{14}H_{11}{}^{37}ClN_4O_6$ and $C_{14}H_{11}{}^{35}ClN_4O_6$ require *M* 368 and 366 resp.: C, 45.9; H, 3.0; N, 15.3%.): $\nu_{max}$ (KBr) see Table 2.9: δ ($CDCl_3$) 8.77, 1 H, d, *J* 2 Hz, H-3'; 8.48, 1 H, dd, *J* 10 and 2 Hz, H-5'; 7.88, 1 H, d, *J* 10 Hz, H-6'; 7.33, 1 H, s, H-6; 7.03, 1 H, s, H-3; 3.96, 3 H, s, $OCH_3$; 3.87, 3 H, s, $OCH_3$: *m/e* 199, *m** 108.3 (366 → 199 ($C_8H_8ClN_2O_2$) + 167).

ii. The above synthesis (i) was repeated but the veratrole was added in acetic acid (1 ml) dropwise. Reaction took place immediately but, even after adding more (2.5 ml) acetic acid there were still unreacted crystals of the diazonium chloride. There was no appearance of the 'red tar' (i). The reaction mixture was poured (after 30 min) into water (100 ml) but this did not result in the precipitation of any azo-compound.

iii. *Discharging the excess nitrous acid with urea*

2,4-Dinitroaniline (367 mg, 2 mmol) and sodium nitrite (145 mg, 2.1 mmol) were diazotized (ice bath) in conc. hydrochloric acid (4 ml) and acetic acid (3 ml). As much as 1 g of urea was needed. There was an immediate reaction upon addition of veratrole (255 μl, 2 mmol in 1 ml AcOH).

The very dark brown extract ($CHCl_3$) (547 mg) yielded (at 80°, 0.5 mmHg) a pale yellow oil (136 mg) giving yellow crystals of 2-*chloro*-3',-4'-*dimethoxy*-4-*nitrobiphenyl* from acetone, m.p. 126.5—127° (Found: *m/e*

A-F2Iaiii cont'd

293($M^+$): C, 56.9; H, 4.0; N, 4.6. $C_{14}H_{12}{}^{35}ClNO_4$ requires *M* 293; C, 57.3; H, 4.1; N, 4.8%): $\nu_{max}$ (KBr) see Table 2.9: δ ($CDCl_3$) 8.36, 1 H, d, *J* 2.5 Hz, H-3; 8.18, 1 H, dd, *J* 8 and 2.5 Hz, H-5; 7.53, 1 H, d, *J* 8 Hz, H-6; 7.02, 3 H, s, H-2', -5', -6'; 3.93, 6 H, s, $2OCH_3$.

iv. When the diazotization under (i) was carried out in conc. hydrochloric acid (4 ml, 2 h) and then acetic acid (4 ml) added, diazonium chloride crystals still (1 h) remained. Veratrole (255 μl) in acetic acid (750 μl) was added. The mixture was poured (next day) into water (100 ml) and extracted ($CHCl_3$) (394 mg). This residue when heated (80°) *in vacuo* (0.4 mmHg) for several days lost weight (166 mg, perhaps veratrole). Repeated extraction (0.2M NaOH) of the residue (in $CHCl_3$) gave a chloroform solution (176 mg).

Sublimation (0.04 mmHg, 200°) of the chloroform extract (40 mg) gave a mixture (8 mg) (m.p. 120—126°, AcMe-MeOH) of 2-chloro-3',4'-dimethoxy-4-nitrobiphenyl (i.r.) (*m/e* 295 and 293 ($M^+$)) and 2,2'-dichloro-4',5'-dimethoxy-4-nitrobiphenyl (*m/e* 331, 329 and 327 ($M^+$)) and an orange zone (9 mg) which was not 2-chloro-3',4'-dimethoxy-4-nitroazobenzene (i.r.).

A-F2Ib. *Synthesis of 2-chloro-3',4'-dimethoxy-4-nitroazobenzene*

i. *2-Chloro-4'-hydroxy-3'-methoxy-4-nitroazobenzene from guaiacol*

2,4-Dinitroaniline (365 mg, 2 mmol) and sodium nitrite (151 mg, 2.2 mmol) were ground together. Cold conc. hydrochloric acid (4 ml) and acetic acid (3 ml) were added and urea (*c.* 1 g, after 1 h) was added till there was no further evolution of carbon dioxide and no smell of nitrogen dioxide. Then guaiacol (247 mg, 2 mmol) in acetic acid (1 ml) was added and the reaction mixture left (overnight, at 5°). The red precipitate (500 mg) was filtered off and fine red needles of *2-chloro-4'-hydroxy-3'-methoxy-4-nitroazobenzene* were obtained from methanol, m.p.

A-F2Ibi cont'd

150.5—152° (Found: *m/e* 309 and 307 ($M^+$): C, 50.5; H, 3.4; Cl, 11.8; N, 13.2. $C_{13}H_{10}{}^{37}ClN_3O_4$ and $C_{13}H_{10}{}^{35}ClN_3O_4$ require *M* 309 and 307 resp.: C, 50.8; H, 3.3; Cl, 11.5; N, 13.7%): $\nu_{max}$ (KBr) see Table 2.9: δ ($CDCl_3$) 8.41, 1 H, d, *J* 2 Hz, H-3; 8.20, 1 H, dd, *J* 8.5 and 2 Hz, H-5; 7.8—7.5, 3 H, *6*, H-2', -5', -6'; 7.07, 1 H, d, *J* 8.5 Hz, H-6; 6.17, 1 H, s, OH; 4.00, 3 H, s, $OCH_3$.

ii. *Methylation of 2-chloro-4'-hydroxy-3'-methoxy-4-nitroazobenzene*

2-Chloro-4'-hydroxy-3'-methoxy-4-nitroazobenzene (104 mg, 0.34 mmol) was methylated in the same way as under (A-F1bii) above. The mixture after evaporating the solvent (EtOEt and MeOH) was dissolved (AcMe) and crystals of 2-*chloro*-3',4'-*dimethoxy*-4-*nitroazobenzene* obtained, m.p. 157—158° (Found: *m/e* 323 and 321 ($M^+$): C, 52.1; H, 3.9; Cl, 11.2; N, 12.9. $C_{14}H_{12}{}^{37}ClN_3O_4$ and $C_{14}H_{12}{}^{35}ClN_3O_4$ require *M* 323 and 321 resp.: C, 52.3; H, 3.8; Cl, 11.0, N, 13.1%): $\nu_{max}$ (KBr) see Table 2.9: δ ($CDCl_3$) 8.39, 1 H, d, *J* 2 Hz, H-3; 8.16, 1 H, dd, *J* 8.5 and 2 Hz, H-5; 7.8—7.5, 3 H, m, *5*, H-2', -5', -6'; 7.02, 1 H, d, *J* 8.5 Hz, H-6; 4.00 3 H, s, $OCH_3$; 3.98, 3 H, s, $OCH_3$.

A-F2Ic. *Coupling to sdm*

i. 2,4-Dinitroaniline (185 mg, 1 mmol) and sodium nitrite (74 mg, 1.07 mmol) were ground together. Cold (ice bath) conc. hydrochloric acid (4 ml) was added and stirred (2 h). All the dinitroaniline dissolved to give a slightly yellow suspension of fine needles of the diazonium chloride. The whole was diluted (to 10 ml) with conc. hydrochloric acid. To an aliquot (1.2 ml) of this suspension, sdm.$C_6H_6$ (55 mg, 0.1 mmol) in acetone (3 ml) was added. The mixture (after 15 min) was evaporated under reduced pressure (removing AcMe and excess HCl) and the chloroform solution of the residue washed (saturated $NaHCO_3$ and $H_2O$). T.l.c. ($Al_2O_3$ T, $C_6H_6$) showed dinitroaniline (at the origin), sdm

A-F2Ici cont'd

($R_F$ 0.06) a purple spot ($R_F$ 0.5) and a colourless spot (quenching, $R_F$ 0.6).

ii. Synthesis (i) above was repeated and the reaction mixture left ($5^o$) overnight. Purple crystals (m.p. 234.5—242.5$^o$) were filtered off (9.9 mg, 24 μmol). (The filtrate darkened while exposed to light and air. T.l.c. of an extract ($CHCl_3$) of the filtrate showed no spot corresponding to the crystals.) 2',5-*Dichloro*-2-*hydroxy*-6,7-*dimethoxy*-1-*methyl*-4'-*nitroindole*-3-*azobenzene* was obtained from chloroform-methanol, m.p. 244—245$^o$ (Found: *m/e* 428, 426, 424.032 ($M^+$). $C_{17}H_{14}{}^{37}Cl_2N_4O_5$, $C_{17}H_{14}{}^{35}Cl{}^{37}ClN_4O_5$ and $C_{17}H_{14}{}^{35}Cl_2N_4O_5$ require *M* 428, 426 and 424.032 resp.): $\nu_{max}$ (KBr) see Table 2.9; (nujol) 3400wbr (OH) 1665m (CO) 1595s $cm^{-1}$.

iii. Synthesis (ii) was repeated and the reaction mixture left ($5^o$, 2½ d) but the yield (11 μmol) was poor.

iv. Acetone and conc. hydrochloric acid on mixing turned pale yellow (1 h), then yellow (overnight), and finally to a deep orange with the smell of mesityl oxide (Schmidt, 1932). To avoid this side reaction sdm was dissolved in chloroform.

The above synthesis (ii) was repeated with 2,4-dinitroaniline (37 mg, 0.2 mmol), sodium nitrite (14.8 mg, 0.21 mmol) in cold conc. hydrochloric acid and sdm.$C_6H_6$ (108 mg, 0.2 mmol) in chloroform (2.5 ml). With vigorous shaking the chloroform phase turned pale purple but did not increase in colour density with time (t.l.c.). Only a trace of the purple compound was recovered.

v. *Sdm dissolved in conc. hydrochloric acid*

2,4-Dinitroaniline (10.4 mg, 57 μmol) was ground together with sodium nitrite (only 1 mg because of 'self-diazotization' effect) and cold conc. hydrochloric acid (0.5 ml) added (ice bath). Sdm.$C_6H_6$

A-F2Icv cont'd

(26.7 mg, 48 μmol) in conc. hydrochloric acid (1 ml) (turned green slowly) was added to the diazonium suspension. The reaction produced a grey-brown suspension which was extracted ($CHCl_3$). The i.r. spectrum showed the extract to be mainly sdm.

vi. The above experiment (v) was repeated using double the quantities. Upon the addition of the sdm there was no immediate reaction but the mixture turned brownish (1 h) and purplish brown (20 h, 5°). T.l.c. of the extract ($CHCl_3$) (40 mg) showed a black spot at the origin and a faint pink purplish spot. Chromatography ($C_6H_6$) yielded several spots (t.l.c.)

1. sulphur (t.l.c. and i.r. spectrum) (3 mg) ≡ to all the sulphur in the sdm.
2. a quenching spot (colourless) (4.6 mg)
3. a slower quenching spot (colourless) (0.8 mg)
4. a purple and quenching spot (0.5 mg)
5. a yellow spot (1.1 mg).

A-F2Id. *Reaction with potassium iodide*

2,4-Dinitroaniline (368 mg, 2 mmol) was diazotized with sodium nitrite (147 mg, 2.1 mmol) in conc. hydrochloric acid. After Hodgson and Walker (1933), this suspension was added to potassium iodide (420 mg, 2.5 mmol) solution (400 μl $H_2O$). The extract ($CHCl_3$) was washed (aq. $Na_2S_2O_3$, aq. $NaHCO_3$, $H_2O$) and the residue (408 mg, 72%) sublimed (80°, 0.3 mmHg). 2-Chloro-1-iodo-4-nitrobenzene was obtained from methanol, m.p. 96—98.5° (99°, Wallagh and Wibaut, 1936). Mass spectrum *m/e* 285 and 283 ($M^+$) 269, 267, 255, 253, 239, 237, 226, 224, 128, 127, 126, 112, 110, 88, 86, 84, 82, 80, 75. Calc. for $C_6H_3{}^{37}Cl$-$INO_2$ and $C_6H_3{}^{35}ClINO_2$: *M* 285 and 283 resp.

A-F2II. *Diazotization in sulphuric acid*

A-F2IIa. *Coupling to guaiacol*

i. 2,4-Dinitroaniline (367 mg, 2 mmol) was dissolved in acetic acid (2 ml) with warming but it crystallized immediately on cooling (ice bath). Sodium nitrite (152 mg, 2.2 mmol) was dissolved in conc. sulphuric acid (1 ml, 70°), cooled (ice bath) and added to the acetic acid suspension (after Hodgson and Walker, 1935; Gunstone and Tucker, 1952). There was darkening, leading to a brown suspension, containing dendritic plates.

Guaiacol (247 mg, 2 mmol), dissolved in acetic acid (1.5 ml), was added and it immediately reacted to give a deep red. Crystals appeared around the neck of the flask, hence an attempt was made to concentrate by evaporating the acetic acid under reduced pressure. This served only to enhance the destructive (dehydrating) action of the sulphuric acid and led to the production of tar (which appeared when poured into 70 ml $H_2O$).

ii. *Diazotization according to Saunders* (1949a)

Sodium nitrite (148 mg, 2.1 mmol) was dissolved in conc. sulphuric acid (1 ml, 70°). Finely ground 2,4-dinitroaniline (365 mg, 2 mmol) was added (dry) to the above solution (30—35°) to produce acicular crystals in a brown matrix. After Haginiwa, *et al.* (1958), guaiacol (249 mg, 2 mmol) in acetic acid (2.5 ml) was added to the above suspension (ice bath). The reaction mixture was poured into ice water (100 ml) and filtered. T.l.c. (polyamide with MeOH) showed the tarry precipitate to be a mixture of a red, brown, and 2 yellow spots of comparable intensity (360 nm) while the filtrate ($CHCl_3$ extract) showed another grey spot as well. The experiment was discarded.

A-F2III. *Diazotization in orthophosphoric acid* (s.g. 1.75)

A-F2IIIa. *Coupling to veratrole*

i. 2,4-Dinitroaniline (183 mg, 1 mmol) was ground and mixed with orthophosphoric acid (4 ml): it was not completely soluble. Sodium nitrite (79.5 mg, 1.15 mmol) was ground and added dry to the suspension (ice bath). Veratrole (128 μl, 1 mmol) (after 1 h) in orthophosphoric acid (1 ml) was added, resulting in an immediate reaction to give a deep red, which was extracted ($CHCl_3$, after 15 min, 24 mg, after evaporation of volatile material). Sublimation (gradient) of the crystalline residue yielded two substances: a. dark red crystals (proximal) and b. yellow crystals (distal to heat source).

ii. Experiment (i) was repeated: the reaction mixture (after 15 min) showed no unreacted veratrole (by adding a drop of the reaction mixture to water). Residual veratrole, from the pipette used to transfer it in acid solution to the reaction mixture, appeared as droplets when diluted with water. The reaction mixture was poured into ice water and extracted ($CHCl_3$) yielding crystals (26 mg) after any traces of veratrole had evaporated. Sublimation (gradient) yielded the same pair of substances as under (i) above.

a. Dark red crystals of 3',4'-*dimethoxy*-2,4-*dinitroazobenzene*, m.p. 152—152.5° (135°, Haginiwa, *et al.*, 1958), were obtained (Found: *m/e* 332.077 ($M^+$). $C_{14}H_{12}N_4O_6$ requires *M* 332.076.): $\nu_{max}$ (KBr) see Table 2.9.

b. Yellow crystals of 3',4'-dimethoxy-2,4-dinitrobiphenyl, m.p. 177—178°, were obtained (Found: *m/e* 304 ($M^+$). $C_{14}H_{12}N_2O_6$ requires *M* 304.)

A-F2IIIb. *Synthesis of* 3',4'-*dimethoxy*-2,4-*dinitroazobenzene*

i. *Synthesis of* 4'-*hydroxy*-3'-*methoxy*-2,4-*dinitroazobenzene*

2,4-Dinitroaniline (368 mg, 2 mmol) was ground and mixed (with warming) with orthophosphoric acid (8 ml). Sodium nitrite (166 mg, 2.4 mmol) was ground and added dry to the suspension of dinitroaniline in

A-FIIIbi cont'd

phosphoric acid (ice bath). A solution of guaiacol (252.8 mg, 2 mmol) in orthophosphoric acid (5 ml) was added (dropwise) (after 50 min). Upon pouring into ice-water (after 40 min) an amorphous solid floated. This was dissolved ($CHCl_3$) and filtered to yield a residue (530 mg). Crystals (344 mg, AcMe-MeOH), m.p. 174.5--176.5° (sublimed) of 4'-*hydroxy*-3'-*methoxy*-2,4-*dinitroazobenzene* were obtained (Found: *m/e* 318 ($M^+$); C, 49.3; H, 3.1; N, 17.3. $C_{13}H_{10}N_4O_6$ requires *M* 318; C, 49.1; H, 3.2; N, 17.6%): $\nu_{max}$ (KBr) see Table 2.9: δ ($CD_3COCD_3$) 8.85, 1 H, d, *J* 2 Hz, H-3; 8.63, 1 H, dd, *J* 9 and 2 Hz, H-5; 7.94, 1 H, d, *J* 9 Hz, H-6; 7.63, 1 H, dd, *J* 9 and 2 Hz, H-6'; 7.45, 1 H, d, *J* 2 Hz, H-2'; 7.03, 1 H, d, *J* 9 Hz, H-5'; 3.91, 3 H, s, $OCH_3$; 2.98, 1 H, s, OH.

ii. *Methylation of* 4'-*hydroxy*-3'-*methoxy*-2,4-*dinitroazobenzene*

4'-Hydroxy-3'-methoxy-2,4-dinitroazobenzene (19.6 mg, 60 μmol) was evaporated to a film (from AcMe) and dissolved in ether (some crystallized out). It was methylated in the same way as under (A-FIbii) above. Because of the formation of crystals the ethereal solution of diazomethane was poured off, the crystals redissolved in acetone and evaporated to a film, and the ethereal solution of diazomethane returned. The orange solution (AcMe) turned dark blue from alkali in the glass. This pH was corrected with some HCl vapour from conc. hydrochloric acid and a film again formed. After sublimation and chromatography, red crystals (AcMe-EtOEt) of 3',4'-*dimethoxy*-2,4-*dinitroazobenzene* (11.8 mg), m.p. 151—152°, were obtained. $\nu_{max}$ (KBr) 3100m, 2840w (OMe, $\nu_s$), 1590s (Ph conjugated with N=N), 1530vs ($NO_2$, $\nu_{as}$), 1505s (Ph), 1460m (Ph) 1340vs ($NO_2$, $\nu_s$), 1270vvs (Ph, 1,2,4-subst., in-plane bending, intensified by $NO_2$), 1245s (OMe, $\delta_{as}$), 1020m (OMe, $\delta_s$), 860m (C-N stret.), 835m (Ph, adjacent aromatic Hs), 620 ($NO_2$, CNO bending) $cm^{-1}$ (Nakanishi, 1964).

A-F2IIIc. *Coupling to sporidesmin*

2,4-Dinitroaniline (18.2 mg, 0.1 mmol) was diazotized as above in orthophosphoric acid to which sdm.$C_6H_6$ (55 mg, 0.1 mmol) in orthophosphoric acid (400 µl) was added. The pale green sdm solution in acid turned a deeper green. Upon extraction ($CHCl_3$) the sdm was almost quantitatively recovered (57.6 mg crude).

A-F2IV. *Diazotization in saturated trichloroacetic acid* (tca)

A-F2IVa. *Coupling to veratrole*

2,4-Dinitroaniline (184 mg, 1 mmol) was ground and dissolved in saturated tca (4 ml), with warming but the solution crystallized on cooling (ice bath). Sodium nitrite (72.6 mg, 1.05 mmol) was ground and added dry. Saturated tca was far less viscous than orthophosphoric acid. Veratrole (128 mg, 1 mmol), dissolved in saturated tca (freely soluble), was added (after 15 min), yielding a dark red-brown colour immediately. After 15 min the washed ($Na_2CO_3$) extract ($CHCl_3$) yielded 109 mg which was chromatographed. Red crystals of 3',4'-dimethoxy-2,4-dinitroazobenzene (78 mg, 0.23 mmol), m.p. 150--151, m.m.p. 150—151.5°, were obtained from acetone-ether.

A-F2IVb. *Coupling to anhydrodethiosporidesmin* (adsdm)

2,4-Dinitroaniline (18.2 mg, 0.1 mmol) was dissolved in saturated tca (400 µl) and sodium nitrite (8.6 mg, 0.11 mmol) was ground and added dry (ice bath). A few drops of a solution of adsdm (37.8 mg, 0.1 mmol) in saturated tca (500 µl) were added without reaction. After Binks and Ridd (1957), sodium hydroxide (10M) was added, dropwise, to the solution of adsdm (which became insoluble and then redissolved) till alkaline (paper) then the diazonium salt added, giving an immediate colour reaction. The colour was extracted ($CHCl_3$) giving crystals (23.4 mg, 0.05 mmol) from methanol, m.p. 258—259°, of 5-*chloro*-6,7-*dimethoxy*-8-

A-F21Vb cont'd

*methyl-2',4'-dinitropyrrolo[2,3-b]indole-2-azobenzene* (Found: C, 48.5; H, 3.2; N, 17.6. $C_{19}H_{15}ClN_6O_6.H_2O$ requires C, 47.9; H, 3.6; N, 17.6%): $\nu_{max}$ (KBr) see Table 2.9; (nujol) 3420wbr, 3300wbr (NH), 1650m, 1610s $cm^{-1}$: $\delta$ ($CDCl_3$) 12.4, 1 H, brs, NH, $D_2O$ exchange; 9.05, 1 H, d, 2 Hz, H-3'; 8.18, 1 H, dd, 9.5 and 2 Hz, H-5'; 8.03, 1 H, d, 9.5 Hz, H-6'; 7.30, 1 H, s, H-4; 6.65, 1 H, s, H-3; 4.27, 6 H, s, 2 $CH_3O$; 3.62, 3 H, s, $NCH_3$: *m/e* 460 and 458 ($M^+$).

A-F21Vc. *Coupling to sporidesmin*

2,4-Dinitroaniline (18.6 mg, 0.1 mmol) was ground and dissolved in saturated tca (400 µl). Sodium nitrite (7.1 mg, 0.105 mmol) was ground and added dry (ice bath). Sdm.$C_6H_6$ (54.6 mg, 0.1 mmol) in saturated tca (sdm turned green as quickly as in $H_3PO_4$) was added (after 2 h): a dark purple colour slowly developed, which was extracted ($CHCl_3$). Crystals (3.6 mg, 8 µmol) of *5-chloro-2-hydroxy-6,7-dimethoxy-1-methyl-2',4'-dinitroindole-3-azobenzene* appeared, m.p. 277.5--280.5°, in the residue; methanol separated the matrix from the crystals (Found: *m/e* 435.058 ($M^+$). $C_{17}H_{14}{}^{35}ClN_5O_7$ requires *M* 435.058): $\nu_{max}$ (KBr) 1680 $cm^{-1}$ and Table 2.9, and (nujol) 3400wbr, 1710m, 1620s, 1590s $cm^{-1}$.

B. *The hydrogen-bonding experiments for Chapters* 4 *and* 5.

B-A1. The carbon tetrachloride (Analar) used in the subsequent experiments was examined in 50 mm Hellma Infrasil cells between 4000 and 2400 $cm^{-1}$. The instrument was set out of 'balance'. See Fig. IR5.1. Between 3800 and 3550 $cm^{-1}$ a further reading was made by inserting the reference cell in the sample beam and the sample cell in the reference beam ('cells reversed').

For each of the subsequent observations (in Hellma Infrasil cells) the instrument was correctly 'balanced' and the position of the

B-A1. cont'd

2850 $cm^{-1}$ peak for polystyrene recorded.

B-A2. In 10, 20, 30 and 50 mm cells, 5.5 mM methanol (Analar) in carbon tetrachloride was examined. For the spectra of this solution in the 50 and 20 mm cells see Figs 5.3A and 5.3B (resp.).

B-A3. In a 10 mm cell 8 mM methanol in carbon tetrachloride was examined. See Fig. IR5.2.

B-B. *Sporidesmin* (1.1)

B-B1. Unsolvated sdm (A-A) (6.2 mg) was ground and dissolved ($CCl_4$, 6.5 ml) with warming (45°). This solution (*c.* 2 mM) after standing, was filtered and recorded in a 50 mm cell, see Fig. IR4.12 or IR5.4.

B-B2. A saturated solution (5 ml $CCl_2=CCl_2$ with warming) of unsolvated sdm (10 mg, <2 mM) was examined in a 10 mm cell, see Fig. IR5.5. About 8 mg of the sdm dissolved.

B-B3. Unsolvated sdm (2.4 mg) was dissolved in chloroform-carbon tetrachloride (85 µl 1:1) and made up to 850 µl ($CCl_4$, 6 mM). Spectrum (Fig. IR5.6) was recorded for a 10 mm cell using chloroform-carbon tetrachloride (1:19) in the reference cell.

B-B4. Sdm.$C_6H_6$ (4.4 mg) was dissolved in carbon tetrachloride (1.3 ml, *c.* 6 mM). For the spectrum (10 mm cell) of this solution see Fig. IR5.25.

B-B5. The above solution (B-B4) was treated with deuterium oxide ($D_2O$) was warmed (45°), see spectrum (Fig. IR5.22) of this solution, using carbon tetrachloride saturated with deuterium oxide for the reference beam.

B-C. *Sporidesmin-B* (1.15)

B-C1. Sdm-B (prepared by E.P. White) (4.6 mg) was dissolved ($CCl_4$, 3 ml, 3.3 mM) with warming. This solution was recorded (30 mm cell, see Fig. IR5.7A) and then diluted (to 5 ml, 2 mM) and reread (50 mm cell, see Fig. IR5.7B).

B-C2. The above solution was evaporated (45°, reduced pressure) to dryness to remove any water azeotropically, desiccated and recorded (3 ml).

B-C3. Sdm-B (2.3 mg) was dissolved in carbon tetrachloride (2 ml, 2.5 mM) and deuterium oxide added. After warming (60°, 30 min) the solution was recorded (50 mm cell) against carbon tetrachloride saturated with deuterium oxide, see spectrum (Fig. IR5.8).

B-C4. Sdm-B (4.6 mg) was dissolved in ether-carbon tetrachloride (1:19, 2 ml, 5 mM), see Fig. IR5.12 for the spectrum (20 mm cell) recorded against ether-carbon tetrachloride (1:19 in reference cell).

B-D. *Sporidesmin-D* (1.9)

B-D1. Sdm-D.EtOEt (A-C1aiii) (5.8 mg) was dissolved in carbon tetrachloride (1.1 ml, *c.* 10 mM) and observed in a 10 mm cell, see Fig. IR5.9A. The sdm-D dissolved immediately in the few drops of solvent ($CCl_4$) used to rinse the measuring cylinder. The same solution was evaporated (45°, reduced pressure) to remove the ether of crystallization and redissolved (5 ml, *c.* 2 mM), see Fig. IR5.9B (50 mm cell) for the spectrum.

B-D2. Sdm-D.EtOEt (2.8 mg) was dissolved in acetone and evaporated (this process repeated) to remove the ether. Carbon tetrachloride (2 ml, 2 mM) was added, see Fig. IR5.10 for the spectrum (20 mm cell).

B-D3. Sdm-D.EtOEt (5.8 mg) was dissolved in carbon tetrachloride (950 μℓ) and ether (50 μℓ) added (total volume 1 ml, *c.* 10 mM). The spectrum (Fig. IR5.11, 10 mm cell) was recorded against ether-carbon tetrachloride (1:19, in the reference beam).

B-D4. The solution from experiment B-D2 was saturated with deuterium oxide with warming (50°). The spectrum of this solution (50 mm cell) against carbon tetrachloride saturated with deuterium oxide was Fig. IR5.23.

B-E. *Sporidesmin-E* (1.10)

B-E1. Unsolvated sdm-E (A-B3iv) (6.5 mg) was ground and washed with carbon tetrachloride into a measuring cylinder (6.5 ml, *c.* 2 mM). The suspension dissolved readily on warming. Some crystals appeared on cooling. See Fig. IR4.11 for the spectrum (50 mm cell).

B-E2. The sdm-E which was recovered from the $^{13}C$ n.m.r. experiment (A-B3iii) was recrystallized from ether several times (for further $^{13}C$ n.m.r. analysis). This sdm-E.EtOEt (3.2 mg) was dissolved in chloroform (50 μℓ) and made up to 1 ml ($CCl_4$, *c.* 6 mM) and recorded (10 mm cell) against chloroform-carbon tetrachloride (1:19), see Fig. IR4.14.

B-E3. A saturated solution of sdm-E.EtOEt (A-B3iii) in carbon tetrachloride was examined in a 50 mm cell, see Fig. IR4.13.

B-E4. Unsolvated sdm-E (A-B3iv) (5.0 mg) was dissolved in carbon tetrachloride (5 ml, *c.* 2 mM) with warming (45°). The solution was saturated with deuterium oxide (45°, 30 min) and recorded (Fig. IR5.24) against carbon tetrachloride saturated with deuterium oxide.

B-E5. Unsolvated sdm-E (A-B3iv) (2.9 mg) was dissolved in chloroform and evaporated to a film. For the spectrum of this film ($CCl_4$, 6 mM, 10 mm cell) see Fig. IR4.15.

B-F. *Methyl 3-mercaptosecosporidesmin-11a*-S-*acetate* (1.13) (A-C5c)

B-F1. This compound (1.13) (7.1 mg) dissolved freely in carbon tetrachloride (2 ml, *c.* 6.3 mM) to give the spectrum, Fig. IR5.13 (20 mm cell).

B-G. *Methyl 11a-mercaptosecosporidesmin-3*-S-*acetate* (1.14a) (A-C5b)

B-G1. This compound (1.14a) (5.0 mg) was dissolved in carbon tetrachloride and evaporated dry. (50°, reduced pressure) to azeotrope the water of crystallization out and then redissolved (5 ml, *c.* 1.8 mM), see Fig. IR5.14A for the spectrum (50 mm cell).

B-G2. For a stronger spectrum in the νSH region this compound (37.5 mg) was dissolved in chloroform (125 μℓ) and made up to 5 ml ($CCl_4$, 13.3 mM), see Fig. IR5.14B (50 mm cell).

B-G3. *Methyl 11a-mercaptosecosporidesmin-3*-S-*acetate di(chloroacetate)*

Compound (1.14a) (16 mg) and chloroacetic anhydride (33 mg, 0.19 mmol) were dissolved in pyridine (65 μℓ). The crude film (after 45 min) showed no νOH peaks. Upon chromatography ($C_6H_6$-EtOEt) methyl 11a-mercaptosecosporidesmin-3-*S*-acetate di(chloroacetate) was obtained as a gum (Found: *m/e* small peaks at 703, 701, 699 ($M^+$) and intense at 671, 669, 667 (most intense), 665 (characteristic 3 chlorine peaks). $C_{25}H_{28}{}^{35}Cl_3N_3O_{10}S_2$ requires $M^+$ 699 and $M^+$- $H_2S$ 665.) Its i.r. spectrum as a film from acetone (Fig. IR5.16) showed no νOH or νSH peaks.

B-G4. Fig. IR5.21 was the spectrum (50 mm cell) of a saturated solution of crystals (solvated with $H_2O$) of compound (1.14a) in carbon tetrachloride.

B-G5. A solution of diethylamine in chloroform-carbon tetrachloride (1:2.5:6.5) was examined. Even in a 1 mm cell (silica) the transmission was only 50% (against $CHCl_3$-$CCl_4$ 2.5:6.5).

B-II. *3,11a-Dimercaptosecosporidesmin* (1.11) (A-B1)

B-III. Fig. IR5.19 was recorded (50 mm cell) for a saturated solution of compound (1.11). Carbon tetrachloride (5 ml) was warmed and shaken with 11.8 mg of the compound and then filtered.

B-H2. Compound (1.11) (11.8 mg) was dissolved in chloroform (500 µℓ) and made up to 5 ml ($CCl_4$) and recorded (50 mm cell) against chloroform-carbon tetrachloride (1:9). For the νSH region see Fig. IR5.20.

C. For the Experimental for crepidotine (C) see the end of Chapter 7.

Table 7.1. Ultra-violet spectra (White, pers. comm.)

Crepidotine.

In methanol.

| | | |
|---|---|---|
| 205 nm log ε | 4.45 | |
| 226 | 4.29 | Conjugated/aromatic |
| 238 | 4.28 | system |
| 332 | 4.23 | |

In acidified methanol.

| | | |
|---|---|---|
| 206 nm log ε | 4.45 | |
| 259 | 4.14 | |
| 377 | 4.33 | A nitrogen in a different ring from that of the tautomerizing one. |

In alkaline methanol.

| | | |
|---|---|---|
| 257 nm log ε | 4.39 | |
| 352 | 4.13 | |

Benzalacetone in methanol.

| | |
|---|---|
| 209, 221, 226 and 289 nm. | Phenyl conjugated to carbonyl. |

Benzalacetophenone in methanol.

| | |
|---|---|
| 206, 218, 226 and 305 nm. | Phenyl conjugated to carbonyl. |

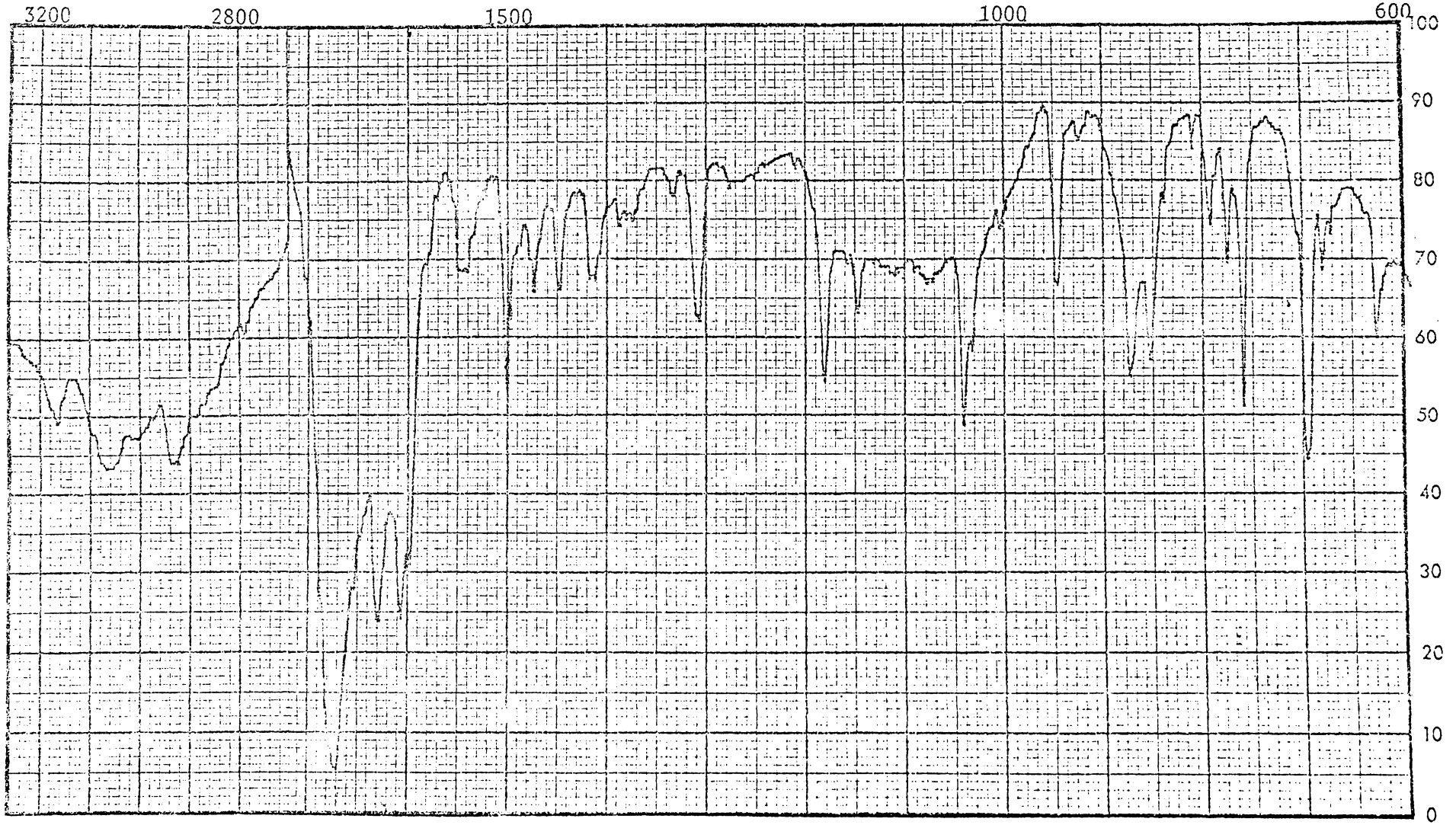



Fig. 7.1. The infra-red (KBr) spectrum of crepidotine.

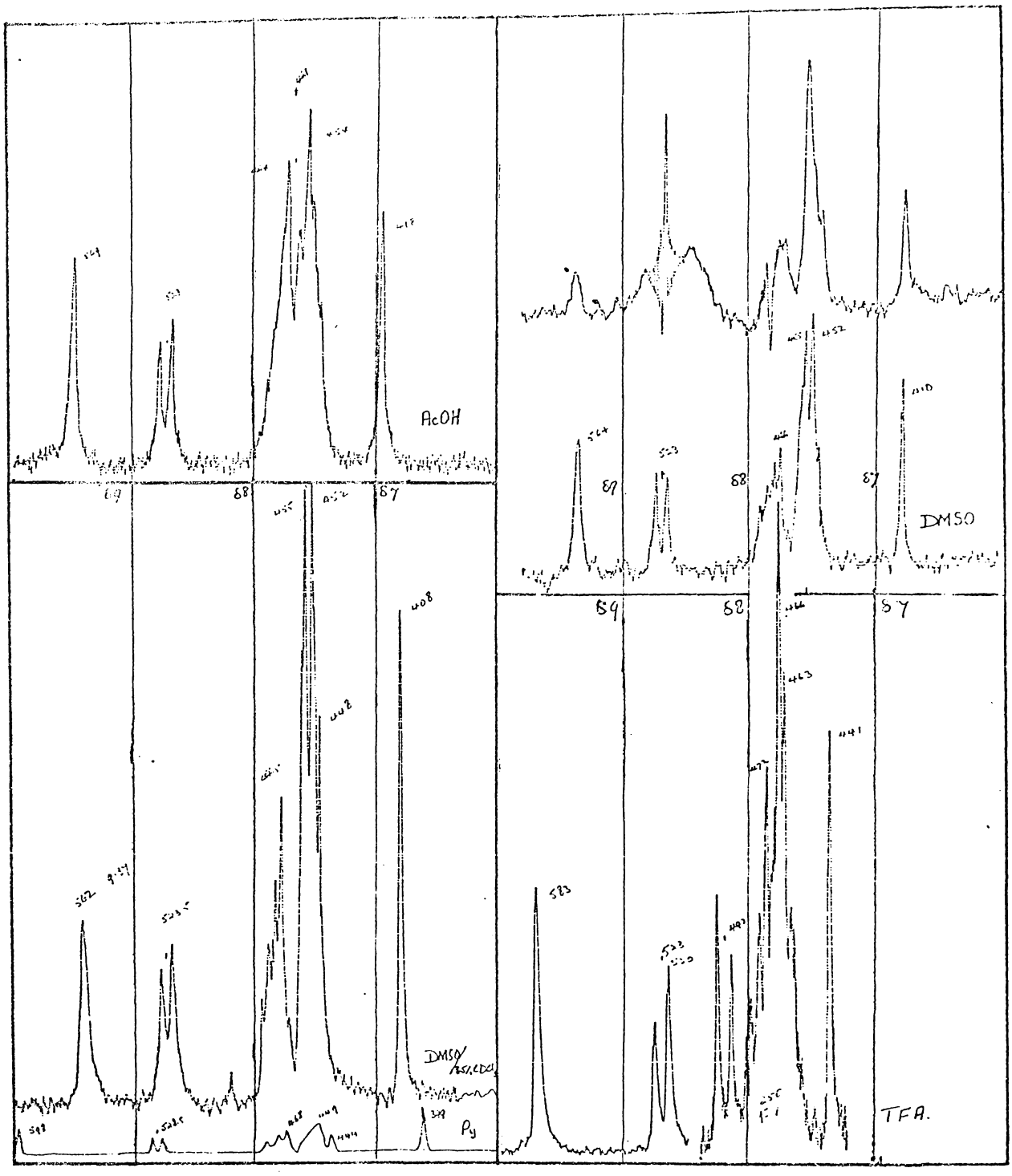



Fig. 7.2. The $^1$H n.m.r. spectra of crepidotine in acetic acid (AcOH, 85°), dimethyl sulphoxide (dmso-$d_6$, 85°), dimethyl sulphoxide-chloroform (dmso-$d_6$-$CDCl_3$, 38:5, 85°), pyridine (py-$d_5$, 30°) and trifluoroacetic acid (tfa-$d$, 65°).

Table 7.2. $^1$H Nuclear magnetic resonance spectral data for crepidotine and model compounds (δ).

| Compound | Solvent | Temp. | CH/NH, | $H_a$, | $H_b$, | $H_c$, | Benzo, | Phenyl, | CH-CO, | CH=C-CO, | *J*. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | s | d | d | m | m | s | | |
| Crepidotine | dmso-$d_6$ | 85° | 11.45, | 9.36, | 8.69, | 7.54, | | {7.9 —7.65, 7.65—7.35,} | 6.81, | | 6. |
| | AcOH | 85° | - | 9.47, | 8.70, | 7.67, | | 7.9 —7.4, | 6.93, | | 6. |
| | tfa-*d* | 65° | - | 9.73, | 8.73, | 8.20, | | 8.05—7.5, | 7.35, | | 6.6. |
| | tfa-*d* | 30° | - | 9.67, | 8.67, | 8.15, | | 7.95—7.55, | 7.32, | | 6.6. |
| | py-$d_5$ | 30° | | 9.97, | 8.80, | * | | {7.95—7.7, 7.7 —7.3,} | 6.65, | | |
| Isoquinoline | dmso-$d_6$ | 85° | | 9.42, | 8.63, | 8.25 .. | .. 7.5, | | | | 5.6. |
| | AcOH | 65° | | 9.58, | 8.58, | 8.4 .. | .. 7.7, | | | | 6.1. |
| | AcOH | 30° | | 9.69, | 8.65, | 8.28, | 8.5 —7.4, | | | | 6.1. |
| | tfa-*d* | 65° | | 9.63, | 8.7 .. | .. .. | .. 8.0, | | | | |
| | tfa-*d* | 30° | | 9.65, | 8.4 .. | .. .. | .. 8.0, | | | | |
| | py-$d_5$ | 85° | | 9.42, | 8.68, | 8.0 .. | .. 7.4, | | | | 5.6. |
| | neat | 85° | | 9.38, | 8.71, | 7.9 .. | .. 7.2, | | | | 6.0. |
| | | | | | | | | | d | d | |
| 2-Quinolone | dmso-$d_6$ | 85° | 11.45, | | | | 7.75—7.0, | | 6.51, | 7.88, | 9.5. |
| | AcOH | 65° | - | | | | 7.8 —7.1, | | 6.81, | 8.00, | 9.5. |
| | AcOH | 30° | - | | | | 7.8 —7.1, | | 6.82, | 8.00, | 9.5. |
| | tfa-*d* | 65° | - | | | | 8.2 —7.6, | | 7.37, | 8.70, | 9.0. |
| | tfa-*d* | 30° | - | | | | 8.2 —7.6, | | 7.42, | 8.75, | 9.0. |
| | py-$d_5$ | 85° | 8.55, | | | | 7.6 —6.9, | | 6.73, | 7.70, | 9.7. |
| | | | | | | | | | H-3, -6, m | H-4, -5, m | |
| 2-Pyridone | dmso-$d_6$ | 85° | 11.1, | | | | | | 6.4 —6.1, | 7.6 —7.3, | |
| | AcOH | 65° | - | | | | | | 6.9 —6.4, | 7.95—7.5, | |
| | AcOH | 30° | - | | | | | | 6.95—6.4, | 7.9 —7.55, | |
| | tfa-*d* | 65° | - | | | | | | 7.6 —7.2, | 8.5 —8.0. | |
| | tfa-*d* | 30° | - | | | | | | 7.6 —7.3, | 8.6 —8.1. | |

Proton $H_c$ is adjacent to proton $H_b$, while protons $H_a$ and $H_b$ are on either side of a nitrogen.

s = singlet; d = doublet; m = multiplet. .. .. Represents extent of multiplet.

* Resonance under phenyl envelope.

# CHAPTER 7 CREPIDOTINE

This fungal metabolite presented a challenge in deducing a structure. It came from a fungus, *Crepidotus* sp., which was discovered (White, pers. comm.) growing on a log in the local Claudelands Bush. When the fungus was discovered all that was available was collected. A year later, when the fungus was expected to be fruiting, it was found that there was no log left and no fungus growing at the site. Only 50 mg of the white crystalline alkaloid, crepidotine, m.p. 242—3° was obtained. Because of the shortness of supply of this compound and no hope of obtaining more of it, more or less non-degradative methods (mainly $^1$H n.m.r.) were applied to deduce its structure.

The following properties (White, pers. comm.) were already known: molecular formula $C_{14}H_{10}N_2O$, *m/e* 222.0790 ($M^+$), (calc. 222.0793), insoluble in common organic solvents, soluble in dil. acid and unchanged upon hot alkaline ethanolic hydrolysis. The u.v. spectrum, in neutral methanol, acidified methanol, and in alkaline methanol, was that of a highly conjugated aromatic system (Table 7.1). In the i.r. spectrum (Fig. 7.1) there were no peaks between 2300 and 1750 cm$^{-1}$.

## §1. *Rings and Double Bonds in the Structure*

Since crepidotine was so insoluble in the usual organic solvents e.g. acetone or chloroform, for the $^1$H n.m.r. study it was examined first in dimethylsulphoxide (dmso-$d_6$) (Fig. 7.2, Table 7.2) in which solvent it dissolved on heating (85°). The spectrum obtained had no resonances corresponding to protons in either a saturated environment or an exocyclic double bond (< δ 6.5 ppm). And since there was an absence of peaks between 2300 and 1750 cm$^{-1}$ in the i.r. spectrum (Fig. 7.1) there were no triple bonds or allene functions. Hence the molecule must be a conjugated or aromatic system which was substantiated by

Table 7.3. The mass spectral data for crepidotine (R. Hodges, pers. comm.)

*m/e* 222 ($M^+$); $C_{14}H_{10}N_2O^+$.

$C_{14}H_{10}N_2O^+ \rightarrow C_{13}H_9NO^+ + HCN$; $222^+ \rightarrow 195^+ + 27$; m* 171.3.

$C_{14}H_{10}N_2O^+ \rightarrow C_{13}H_{10}N_2^+ + CO$; $222^+ \rightarrow 194^+ + 28$; m* 169.5.

$C_{13}H_{10}N_2^+ \rightarrow C_{12}H_9N^+ + HCN$; $194^+ \rightarrow 167^+ + 27$; m* 143.7.

$C_{14}H_{10}N_2O^+ \rightarrow C_7H_5NO^+ + C_7H_5N$; $222^+ \rightarrow 119^+ + 103$; m* 63.8.

$C_7H_5NO^+ \rightarrow C_6H_5N^+ + CO$; $119^+ \rightarrow 91^+ + 28$; m* 69.6.

$C_{13}H_9N_2^+$ $193^+$.

$C_{12}H_8N^+$ $166^+$.

$C_{11}H_8$ $140^+$.

the u.v. spectrum (Table 7.1). Since the molecular formula, $C_{14}H_{10}N_2O$, was equivalent to $C_{16}H_{12}$ which in the saturated form was $C_{16}H_{34}$, its unsaturation index was 11. As there are no more than 8 conjugated double bonds for 16 carbons there must be at least 3 rings (or 7 double bonds and 4 rings, etc.).

*The phenyl group*

The aromatic envelope between δ 7.9 and 7.35 (Fig. 7.2) consisted of 6 protons, one (δ 7.54) of which, by decoupling the doublet at δ 8.69, belonged to a different system. The remaining 5 protons occurred in two groups (δ 7.9—7.65 and 7.65—7.35) of 2 and 3 protons (resp.) which arrangement was reminiscent of that of a benzoyl, PhCO (Emsley, *et al.*, 1966c) e.g. in benzamide, benzohydroxamic acid, bromoacetophenone and benzaldehyde (Pouchert and Campbell, 1974). The i.r. spectrum (Fig. 7.1) showed two strong peaks, at 750 and 690 $cm^{-1}$, attributable to a phenyl group. The mass spectrum (Table 7.3) showed the loss of a $C_7H_5N$ neutral fragment ($C_{14}H_{10}N_2O^+ \rightarrow C_7H_5NO^+ + C_7H_5N$; $222^+ \rightarrow 119^+ + 103$; *M** 63.8) which it is suggested was phenyl cyanide. Hence the phenyl group was adjacent to a nitrogen atom and was not on a carbonyl group ( no loss of *m/e* 105 in the mass spectrum).

*The basic carbon skeleton*

Because of the diversities of the 2- and 3-ring structures of the alkaloids set out by Raffauf (1970), it was considered necessary to look at every possible basic ring structure which could be applied to crepidotine. Compared with the ring systems in the alkaloids produced by higher plants the ring systems of alkaloids from fungi tend to be atypical. The ring systems of higher-plant compounds colour the thinking in deducing the structures of compounds derived from fungi, e.g. all the evidence indicated that another fungal alkaloid, lepistine, had a pyrrolizidine-type structure but X-ray crystallography showed the ring structure

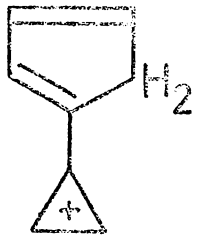
to be a perhydro-2,5-methano-5*H*-pyrido[3,2-*b*]-1,4-oxazine (C.A. name, 1975; Laing, *et al.*, 1975).

Since, in the phenyl group (shown above), there are 3 double bonds and 1 ring, there remains now a total of 7 units of double bonds and rings, i.e. at least 2 rings and up to 5 double bonds for the $C_8H_5N_2O$ or for this reasoning (each N ≡ CH and O is neglected) $C_{10}H_{7(8)}$.

In tricyclo $C_{10}H_8$ structures there would be 4 double bonds. For the 8 protons to be all downfield (n.m.r.) they must all be associated with the 4 double bonds. The only tricyclic structure which fulfils this requirement is the unlikely tricyclo[2,2,2,2]decatetraene. But the u.v. spectrum of this compound would not be that of a conjugated chromophore which the spectrum (Table 7.1) of crepidotine showed. (A tetracyclic compound of $C_{10}H_8$ would have fewer (3) double bonds for the 8 protons.)

*The fulvalene types.*-In the conjugated-double bond series where there are two odd-numbered rings linked together by a double bond (e.g. fulvalene) for $C_{10}H_8$ there are 4 possibilities: 5-(2,4-cyclopentadien-1-ylidene)-1,3-cyclopentadiene (fulvalene), 7-(2-cyclopropen-1-ylidene)-1,3,5-cycloheptatriene (triaheptafulvalene), 1-(2,4-cyclopentadien-1-ylidene)-2-(2-cyclopropen-1-ylidene)ethane (the vinologue of triapentafulvalene or of calicene) and 1,4-bis-(2-cyclopropen-1-ylidene)-2-butene (the divinologue of triafulvalene). The properties of these fulvalenes (except perhaps the vinologue of calicene, Kende, *et al.*, 1966) are those of the polyenes (Hess and Schaad, 1971) whose u.v. spectra would show absorbances at more than 332 nm (Scott, 1964). Fulvalene (obtained only in solution) and the 3 triafulvalene derivatives are unstable (Hess and Schaad, 1971).

Triapentafulvalenes are protonated in trifluoroacetic acid (Bergmann, 1968) to



but in the n.m.r. spectrum of crepidotine in this solvent (Table 7.2) there was no aliphatic proton resonance. The same observation applies to their vinologues, the 1-(2,4-cyclopentadien-1-ylidene)-2-(2-cyclopropen-1-ylidene)ethane. But for the triaheptafulvalene since both heptafulvene and triafulvene tend to be cationic (Bergmann, 1968) there is no such protonation.

On the basis of stability and u.v. spectrum (Table 7.1) it would seem that crepidotine does not have basically a fulvalene type of structure but this cannot be stated categorically. The phenyl group (of crepidotine) and the two nitrogen atoms could stabilize the molecule. Using Hess and Schaad's (1971) system of prediction 2 phenyl groups would be needed to stabilize fulvalene (*c.* -0.033β to *c.* 0.017β with 2 phenyl groups), cyclopropenylidene cycloheptatriene (*c.* -0.040β to *c.* 0.031β with 2 phenyl groups), biscyclopropenylidene-2-butene (*c.* -0.100β to *c.* 0.009β with 2 phenyl groups). Triafulvenes are stable when 2,3-disubstituted with phenyl groups (Bergmann, 1968), and in triapentafulvalenes when the triafulvene group is 2,3-disubstituted with diisopropylamino-groups (Yoshida, *et al.*, 1975). The contribution of the nitrogen atoms to stability is dependent upon their positions in the molecule: they sometimes stabilize and other times destabilize (Hess, *et al.*, 1975). Further, the two nitrogen atoms could effect a hypsochromic shift in the u.v. (e.g. 5-azaazulene with multiple absorptions between 420 and 680 nm compared with 6-dimethylamino-5,7-diazaazulene with absorptions ending at *c.* 500 nm; Muller-Westerhoff and

Hafner, 1967). The position and effect of the nitrogen atoms as indicated by $^1$H n.m.r. will be discussed later.

*Fused bicyclo systems.*-Theoretically, for the bicyclodecapentaenes, $C_{10}H_8$, there are 9 structures of the form bicyclo[x,y,z]decapentaene, where $x \geq y \geq z$ and where $x + y + z = 8$. (A spiro-condition would demand an allene group or a methylene and a triple bond.) If all these types existed, each type would be a conjugated double bond system, hence their protons would resonate downfield (at *c.* δ 5 or more).

The bicyclo[7,1,0]deca-1,3,5,7,9-pentaene type of compound has been reported only as the bicyclodecane (Moore and Ward, 1962). Bicyclo[7,1,0]deca-1,3,5,7,9-pentaene is a triafulvene in a cyclonona-tetraene ring. It is referred to by Toyota and Nakajima (1973) who infer that it is too unstable to exist.

Bicyclo[6,2,0]deca-2,4,6,9-tetraene readily isomerizes to *trans*-9,10-dihydronaphthalene at 70° (Masamune, *et al.*, 1967) so it is inferred that the 1,3,5,7,9-pentaene compound would be even less stable. Several attempts at its synthesis have failed (Elix, *et al.*, 1970). Cyclooctatetraene has been isolated and its protons resonate at δ 5.7 (Fritz and Sellmann, 1967; Meinwald and Tsuruta, 1970), indicating its lack of aromaticity (Hess and Schaad, 1971). The presence of the unsaturated cyclobutadiene ring might help to flatten the molecule (Rosowsky *et al.*, 1960): it would still not be aromatic. Three 9-t-butoxybicyclo[6,2,0]deca-1,3,5,7,9-pentaenes have been synthesized (Schroder and Rottele, 1968) where the 6 cyclooctatetraene protons resonate between δ 6.5 and 6.0, more olefinic than aromatic (though more aromatic than cyclooctatetraene δ 5.7), while the one cyclobutenyl proton resonating at δ 6.9 reflects a more aromatic state. These compounds are all coloured red and are air-sensitive.

Bicyclo-[5,2,1]-, -[4,2,2]- and -[3,3,2]-decapentaenes are all

(with -[6,2,0]- above) etheno-derivatives of cyclooctatetraene (-1,3-; -1,4- and -1,5- resp.) and hence they would lack planarity; their protons would resonate in the olefinic area. Each of these compounds has a double bond at both ring-junctions. Some 'anti-Bredt' compounds where $S \leqslant 8$ (Buchanan, 1974) have been prepared but these have only one ring-junction double bond. Therefore none of them (with two ring-junction double bonds) is likely to be found; each is an impossible structure (Prinzbach, pers. comm.): and thus none is likely to be the basic structure of crepidotine. (Bicyclo[5,2,1]decapentaene is the fulvene isomer of bicyclo[7,1,0]decapentaene, above.) Only a bicyclo[5,2,1]-decatrienone has been described (Press and Shechter, 1975). The nearest structure to bicyclo[4,2,2]decapentaene is bicyclo[4,2,2]deca-2,4,7,9-tetraene (Press and Shechter, 1975), which is ethylenic (by $^1$H n.m.r.) and stable. Again the nearest sturcture to bicyclo[3,3,2]decapentaene is 3-phenylbicyclo[3,3,2]deca-2,6,9-triene which is also stable and ethylenic (Schroder, 1965).

According to the Ring Indexes (Patterson, *et al.*, 1960) not even the saturated hydrocarbon bicyclo[6,1,1]decane has been obtained. The 8-ketone of its homologue bicyclo[5,1,1]nonane has been isolated (Gutsche and Smith, 1960). The hydroxypropellatriene, 7-hydroxybicyclo[4,3,1]-deca-2,4,8-triene (Willcott, *et al.*, 1971), is the nearest to bicyclo-[4,3,1]decapentaene. From a Courtauld model there would be no room for the proton, H-10, in such a planar aromatic pentaene compound. These two types (bicyclo-[6,1,1]- and -[4,3,1]-decapentaenes) are also 'anti-Bredt' compounds with two ring-junction double bonds. Nevertheless, Vogel and Roth (1964) proposed the structure bicyclo[4,4,1]undeca-1,3,5-7,9-pentaene for a $C_{11}H_{10}$ hydrocarbon i.e. a methano-bridge joining two ring-junction double bonds. The two methano-protons of this almost planar compound resonate at $\delta$ -0.5, while the 8 peripheral protons resonate in a multiplet centred at $\delta$ 7.2. This doubly 'anti-Bredt'

compound is not borderline for one double-bond ring junction (S = 9) but it is suggested it is for two (Buchanan, 1974). Though the peripheral protons resonate in the aromatic region the compound exhibits olefinic properties. A 10π electron seemingly aromatic system has been isolated as an anion (Radlick and Rosen, 1966), bicyclo[4,3,1]deca-2,4,6,-8,-tetraenyl ion, showing 2 protons resonating at δ 7.06, 5 at 6.02 and one each at -0.45 and -0.95. The anion was unstable, reverting to a tricyclic compound in the presence of water. In 1972 Ahlberg, *et al.*, protonated bicyclo[4,2,2]decatetraene and obtained the same carbon skeleton, bicyclo[4,3,1]deca-2,4,7-trienyl, as a cation. This bishomotropylium ion showed δ 8.03 (3-H and 4-H), 6.87 (2-H and 5-H), 6.85 (8-H) and 6.62 (7-H and 9-H) for the tropylium ring protons and δ 1.04 and 0.00 for the methano-bridge protons. In Vogel and Roth's compound and in both these ions the methano-protons are strongly shielded from the usual methylene position (*c.* δ 1.25 ppm) which state would be characteristic of H-10 in bicyclo[4,3,1]decapentaene if this impossible structure (Prinzbach, pers. comm.) could be formed. In crepidotine there is no such strongly shielded proton. From the literature it seems that methino- and etheno-bridged compounds in the decapentaene series are rare.

Bicyclo[6,1,1]decapentaene would be an analogue of cyclooctatetraene (therefore its protons would resonate in the olefinic region) where one double bond is replaced by a cyclobutadiene group. Though cyclooctatetraene as a 'tub' is unstrained and therefore stable, fitting a cyclobutadiene into it would accentuate the inherent strain of the latter by bending opposite *exo*-cyclic bonds out of the plane of the ring. Cyclobutadiene *per se* is so unstable that it has been detected only as a mass spectrometer peak during flash vacuum pyrolysis of photo-α-pyrone (Herndon, 1976). (Cyclobutadiene is stable as the phenyl derivative or as an iron carbonyl complex.) Hence bicyclo[6,1,1]decapentaene need not be considered as a basic skeleton for crepidotine: it is unstable

(Prinzbach, pers. comm.).

The aromatic hydrocarbons bicyclo[5,3,0]- and -[4,4,0]-decapentaene are well known as azulenes and naphthalenes.

*The phenylpyridone ring system*

Returning to the discussion of the $^1$H n.m.r., for a number (9) of benzoyl compounds (Varian, 1963) the aromatic multiplet is 0.8 to 1.0 ppm wide and the width of the trough between the groups is 0.3 to 0.5 ppm (measuring from furthest downfield peak of the *meta*-and *para*-resonances to the furthest upfield peak of the *ortho*-), while for crepidotine (Fig. 7.2) the corresponding values were 0.6 and 0.17 ppm. Where a phenyl group is β to a carbonyl of an α,β-unsaturated ketone then the aromatic multiplet is contracted to a single envelope of peaks (*c.* 0.5 ppm wide) e.g. *trans*-cinnamic acid (Varian, 1963), *trans*-cinnamaldehyde, α-methylcinnamaldehyde and β-phenylcinnamaldehyde (Pouchert and Campbell, 1974) and where the phenyl is α to the nitrogen in a hexacyclic lactam as in 3,5-diacetyl-4-methyl-6-phenyl-2-pyridone and 3-acetyl-5-cyano-4,6-diphenyl-2-pyridone (in dmso-$d_6$) the phenyl protons resonate in a singlet (at *c.* δ 7.4, Kato, *et al.*, 1975). But when the phenyl group of the β-phenyl-α,β-unsaturated ketone is adjacent to the hetero-atom in a ring as in flavone, 5-hydroxy-7-methoxyflavone and 5,7-dihydroxyflavone (Pouchert and Campbell, 1974) the aromatic multiplet (for the phenyl protons) is again divided into two groups. This time the aromatic envelope is 0.7—0.8 ppm wide (not as wide as for the benzoyl group, 0.8—1.0 ppm) and the trough between the two groups *c.* 0.2 ppm. For 5,6-dimethyl- (δ 7.9—7.3), 3,6-dimethyl- (δ 7.7—7.3), 6-ethyl-5-methyl-2-phenyl-4-pyrone (δ 7.9—7.3) and 3,5,6-trimethyl-2-phenyl-4-pyridone (δ 7.7—7.3) (Wittek, *et al.*, 1973); 6-methyl-2-phenyl- (δ 7.65—7.28) and 2,6-diphenyl-4-pyridone (δ 7.9—7.3) (Kashima, *et al.*, 1969); and 2,3-dihydro-2,6-diphenyl-4-pyridone (δ 7.7—7.2) (Kashima, *et al.*, 1970)

the width of the aromatic envelope is between 0.4 and 0.6 ppm, comparable with that of crepidotine (0.6 ppm). Similarly, where the phenyl is part of a -CPh=C-CO-NH- group as in 3-acetyl-5-cyano-*4*,6-diphenyl-2-pyridone the phenyl protons resonate as a multiplet (δ 7.45—7.90, Kato, *et al.*, 1975). [Neither Wittek (1973), Kashima (1969, 1970) nor Kato (1975) published their spectra so it is not known whether the aromatic multiplet for these 2-phenyl-4-pyridones or 6-phenyl-2-pyridones occurred in two groups or not.] Since the aromatic envelope for crepidotine showed similarities to that of flavones and 2-phenyl-4-pyridones and the elements of phenyl cyanide must occur together it is suggested that

$$\text{-NH-CPh=CH-CO-} \rightleftarrows \text{-N=CPh-CH=COH-}$$

formed part of a ring in crepidotine. Were this nitrogen an unsaturated one, e.g. CH=N-CPh=C, and the keto-group tautomerizing with the other nitrogen then the structure -N=CPh- could not form. This -N=CPh- has to form (by tautomerism) in order that the neutral fragment phenyl cyanide should result in mass spectrometry. Only two single bonds have to be ruptured to form phenyl cyanide from -N=CPh-, other wise two double bonds would have to be ruptured. The labile proton in the tautomerizing system resonated as a very broad ($W_{\frac{1}{2}}$ 30 Hz), shallow peak at δ 11.45 (dmso-$d_6$; Table 7.2).

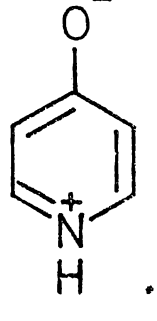
It is interesting to note in this context the similarity between the two resonances of the 2- or 6-phenyl groups on *N*-methylated 4- or 2-pyridones. Weber (1975) published the spectra for both 1-methyl-6-phenyl-2-pyridone and 1-methyl-2-phenyl-4-pyridone showing both the phenyl resonances as singlets (dmso-$d_6$-$D_2O$) at δ 7.5 and 7.55 (both *c.* 0.3 ppm wide).

That the oxygen of crepidotine existed as a carbonyl conjugated to a phenyl group was consistent with the strong peaks at 1670 and 1605 $cm^{-1}$ in its i.r. spectrum (Fig. 7.1). The i.r. spectrum of benzalacetone (nujol) shows strong peaks at 1670 and 1605 $cm^{-1}$ (White, pers. comm.), 2-phenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one (Ring Index nomenclature) at

1680 $cm^{-1}$ (Mendel, 1972) and 1-methyl-2-phenyl-4-pyridone at 1685 $cm^{-1}$ (Weber, 1975).

The singlet at δ 6.81 in the $^1H$ n.m.r. spectrum (dmso-$d_6$, Fig. 7.2, Table 7.2) was consistent with this structure. The following compounds, having the Ph-C=CH-C=O group in an unsaturated ring, are reported to have resonances at about this position: flavone (δ 6.71), 5-hydroxy-7-methoxy-flavone (δ 6.87, dmso-$d_6$-$CDCl_3$), 5,7-dihydroxyflavone (δ 6.74, dmso-$d_6$-$CDCl_3$), α-naphthoflavone (δ 6.69), β-naphthoflavone (δ 6.98) (Pouchert and Campbell, 1974), 5,6-dimethyl-2-phenyl-4-pyrone and 6-ethyl-5-methyl-2-phenyl-4-pyrone (δ 6.70 each, Wittek, *et al.*, 1973) and 2,6-diphenyl-4-pyridone (δ 6.75, Kashima, *et al.*, 1969). 2-Quinolone and 2-pyridone also have =CH-CO- groups and the positions where these protons resonate (in the same solvents as for crepidotine) are set out in Table 7.2. Matter, *et al.*, (1969a) obtained a set of values for predicting the resonance of an olefinic proton by the addition of a set of parameters which had been arrived at by examining (statistically) a number of model compounds. By applying these values to the resonance for H-3 it was found that the observed value was downfield by 1.13 ppm from the calculated value. This downfield difference was the same as that calculated by Matter, *et al.*, (1969b) for pyrone (1.17 ppm) which was a further suggestion that crepidotine had the -NH-CPh=CH-CO- structure.

Crepidotine dissolved in sodium hydroxide solution and was recovered unchanged on acidification (White, pers. comm.). This observation was consistent with this 4-pyridone formula since 2- and 4-pyridones can have a zwitterionic structure:



That pyridones can exist as these zwitterions, enables them to form

salts with either acids or alkalis (Smith, 1976).

*The pyrido-ring*

Returning to the original $^1$H n.m.r. spectrum (dmso-$d_6$, Fig. 7.2) there was an outstanding feature in the $^1$H n.m.r. spectrum of a well-defined sharp singlet at δ 9.36 and an equally sharp doublet at δ 8.69, *J* 6 Hz. Decoupling this doublet revealed the other half of the AB pattern to be centred at δ 7.54 under the phenyl multiplet. (There was no νCO peak at *c.* 1735 cm$^{-1}$ which might have indicated an aldehyde for the sharp singlet at δ 9.36.) Since the association of the oxygen and one nitrogen atom has been accounted for, this $^1$H n.m.r. pattern must be that of the resonances of the remaining hydrogens deshielded by an unsaturated nitrogen (i.e. the other nitrogen). Protons adjacent to aromatic-*N* atoms tend to be deshielded in comparison with those in benzene rings, e.g. δ 8.6 in pyridine (Pouchert and Campbell, 1974) as against δ 7.23 in benzene (Simons and Zanger, 1972). When protons are adjacent to an azo-group in an *N*-heteroaromatic ring they are further downfield than in pyridine (δ 9.21, pyridazine, Varian, 1963) but with crepidotine in view of the reasoning above, there could not be an azo-group, only an =N-NH- group where the adjacent protons do not resonate so far downfield (δ 7.63, pyrazole, Pouchert and Campbell, 1974). The protons in some NH groups resonate at *c.* δ 9.3 but the peak is broad, e.g. pyrrole (Varian Associates, 1963). But when the proton is adjacent to both an aromatic-*N* and a ring-junction carbon, as the H-1 proton of isoquinoline (δ 9.42, dmso-$d_6$, 85°) or the H-4 proton in 6-phenyl-5-aza-azulene (δ 9.40, Hafner, *et al.*, 1970), it resonates at a δ value comparable with that for crepidotine (δ 9.36, dmso-$d_6$, 85°).

Not only did the H-1 proton of isoquinoline resonate at a similar frequency to one in crepidotine but also did the H-3 proton in isoquinoline resonate (δ 8.63, dmso-$d_6$, 85°, Table 7.2) at a frequency similar

to another proton in crepidotine (δ 8.69, dmso-$d_6$, 85°, Table 7.2). (The upfield half of the AB quartet for isoquinoline was hidden under the benzo-proton resonances.) (Pyrimidine has a very similar resonance pattern with sharp peaks at δ 9.28, s, 1 H and 8.8 d, 2 H (Pouchert and Campbell, 1974) but both nitrogens are unsaturated.) These similar resonances for both isoquinoline and crepidotine suggest that there was this structure in crepidotine,

C=CH-N=CH-CH=

as a pyrido-ring. This partial structure is consistent with crepidotine soluble in dilute acid (White, pers. comm.)

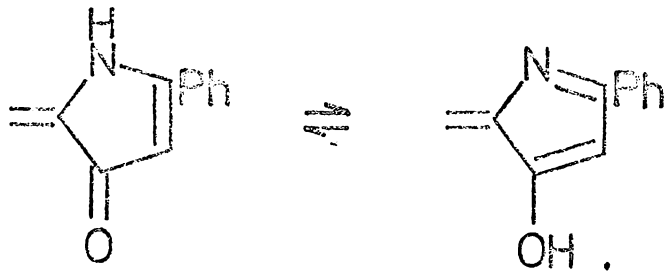
## §2. *The Total Structure*

Having suggested the immediate environments of the phenyl group, the oxygen, the 2 nitrogens and the 10 hydrogens, now they are to be considered in relation to the carbon skeletons discussed earlier.

### *As a fulvalene derivative*

In the fulvalene series, the ring double bonds of fulvalene itself will add tetracyanoethylene (Bergmann, 1968) hence these double bonds are olefinic so, were the 3-CH group replaced by a N atom, the adjacent protons would not resonate as far downfield (δ 9.4 and 8.7) as was observed for crepidotine. The 2 and 3 protons of 6,6-diphenyl-1,4-diazafulvene resonate at δ 8.28 (Rohr and Staab, 1965). When these resonances are compared with those of the fully aromatic compound, pyrazine, whose protons resonate at δ 8.64 (Pouchert and Campbell, 1974) the difference indicates that azafulvalenes would tend to be intermediate between being fully aromatic or fully olefinic. From the work of Watanabe, *et al.*, (1975) and Kobayashi, *et al.*, (1975) it appears that the red 2-azacyclopenta-2,4-dien-1-ylidene group is unstable, dimerizing readily to a pyrrole derivative.

The tautomeric half of the molecule would have the structure

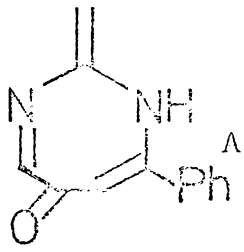


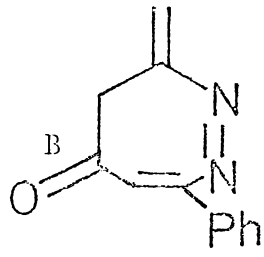
Metler, *et al.*, (1968) synthesized 5-benzylidene-1,2-diphenyl-2-pyrrolin-4-one which structure, apart from the N-phenyl, was basically the same as the left hand tautomer. Metler's compound was red, sensitive to both dilute acid and base, showed vinylic proton resonances (δ 5.71 or 6.27); these properties are well removed from those of crepidotine. Indigotin which is a highly coloured (indigo) fulvalene derivative, 2,2'-diaza-3,4:3',4'-dibenzo-5,5'-dihydroxyfulvalene, is stable. A substituted indigotin is the stable decomposition product from sporidesmin. The heterocyclic ring in indigotin is comparable with that of Metler's pyrrolinone above.

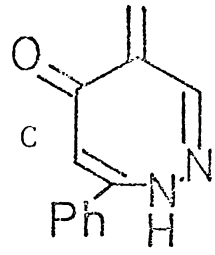
5-Methoxy-2,5-dimethyl-2-pyrrolin-4-one and 5-hydroxy-5-methoxymethyl-2-methyl-2-pyrrolin-4-one, comparable with Metler's pyrrolinone, were recently synthesized (Lightner and Low, 1975) in which the proton α to the CO resonated at δ 5.8 and the C=O stretching frequency was at 1700 $cm^{-1}$. These values are markedly different from those of crepidotine (δ 6.81 and 1670 $cm^{-1}$).

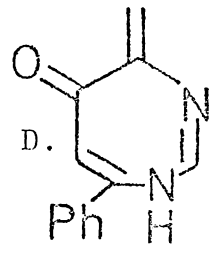
Among the triafulvalenes, were the 2-CH (in the cyclopropenylidene ring) replaced by a N then the adjacent proton would resonate further downfield than δ 9.36 (δ 10.0, 3-styryl-1-azirine, Isomura, *et. al.*, 1972). In the same ring if the phenyl group were on 3-C, it would help to stabilize the azirine ring but it would not be β to a carbonyl group. The cyclopropenylidene ring (without a nitrogen) would be unstable unless it had two phenyl groups on it. For the cyclohepta-

trienylidene moiety (of triaheptafulvalene) there could be these structures and their tautomers



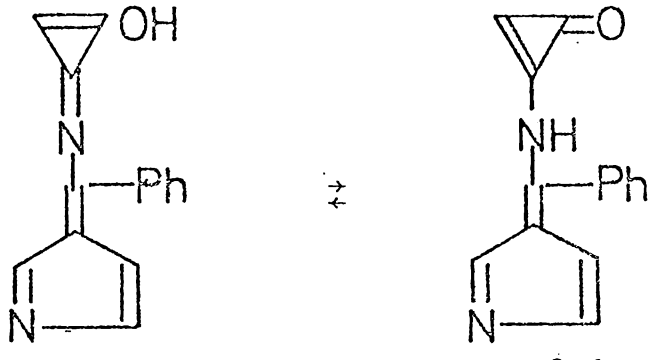




The nitrogens in structure B cannot tautomerize and structure B also has two aliphatic protons. In structure A the proton adjacent to the unsaturated nitrogen, would, because of the adjacent keto-enol group, not be deshielded to δ 9.36. Were the nitrogen adjacent to the carbonyl, so that this proton would be between an unsaturated nitrogen and an *exo*-cyclic double bond, it would not resonate further downfield than δ 8.5 (δ 8.13 isonitrosoacetophenone, Varian, 1963). For structures C and D, the proton adjacent to the unsaturated nitrogen would resonate *c.* δ 8.1 (indazole, Pouchert and Campbell, 1974) or *c.* δ 8.4 (benzimidazole, Pouchert and Campbell, 1974) respectively. In none of the structures A—D would there be a proton resonating as far downfield as δ 9.36.

Two vinologue structures were suggested above: the ethano-groups (=CH-CH=) would have olefinic protons. And this structure for a vinologue




cannot be entertained because when the elements of phenyl cyanide are removed (in mass spectrometry) the remainder does not form an ion of *m/e* 119 (Table 7.3).

On the basis of (a) the lack of colour (b) the stability (c) the carbonyl stretching absorption at 1670 $cm^{-1}$ and (d) the $^1H$ n.m.r. spectrum of crepidotine, it is considered that it is not a derivative of fulvalene or its isomers, all of which are strongly coloured.

*As a bicyclo-structure*

In the bicyclo-series outlined above, the two nitrogen atoms and the phenyl group have particular influences on their properties.

In 2-methoxyazocine (an 8-membered-heterocyclic tetraene) the proton adjacent to the unsaturated nitrogen resonates in the olefinic region (δ 6.54, Paquette, *et al.*, 1971 compared with δ 8.5 for isoquinoline, Simons and Zanger, 1972). Since in the fully unsaturated aza-ring systems larger than 7 members the protons resonate upfield from the aromatic region, it is unlikely that crepidotine is one of the isomers of either phenyldiazabicyclo[7,1,0]decatetraenone or phenyldiaza-1,x-ethenocyclooctatrienone (x = 2,3,4 or 5).

To contribute stability to the triafulvene group in bicyclo[7,1,0]-decatetraenes the phenyl group would need to be at position 10 (i.e. on the triafulvene group). Were this so then a nitrogen would have to be at either positions 1 or 9 (i.e. to form phenyl cyanide in mass spectrometry) in which position the nitrogen could not tautomerize as described above. For the same reason the cyclopropene group cannot be an azirine.

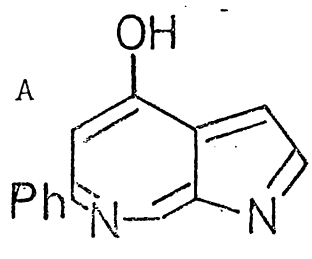
Crepidotine could not be a bridged (etheno) diazacyclooctatetraene (i.e. a diazabicyclo-[6,2,0]-, -[5,2,1]-, -[4,2,2]- or -[3,3,2]-decapentaene) because the above reasoning required both rings of the bicyclo-structure to be aromatic when protonated (tfa-*d*, Table 7.2). Protonation (on a heteroatom) would produce a completely conjugated system but cyclooctatetraenes tend to be non-planar (Person, *et al.*, 1952) and to have olefinic protons except when protonated. In this condition five of the protons are aromatic (δ 8.6), three olefinic (δ 6.6 and 5.2) and

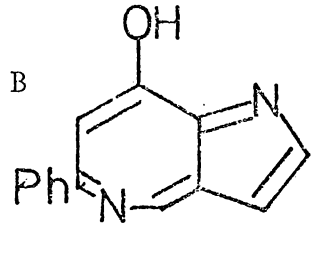
one strongly shielded (δ -0.6, Laszlo and Stang, 1971). The presence of nitrogen in isoquinoline deshields one proton (H-1) by 1.5 ppm compared with H-1 in naphthalene (centred at δ 7.68 ppm, Simons and Zanger, 1972). If an unsaturated nitrogen were in a cyclooctatetraene ring it might deshield an adjacent proton by 2 ppm (to *c.* δ 7.7) so that it would appear to be in an aromatic environment but it would take more deshielding than that to account for the resonances, δ 9.36 and 8.69, recorded for crepidotine (Table 7.2). As indicated above, the proton adjacent to the nitrogen in 2-methoxyazocine resonates at δ 6.54 (Paquette, *et al.*, 1971), more olefinic than aromatic.

It would appear that only the bicyclo-[4,4,0]- and -[5,3,0]-decapentaenes are stable aromatic types of compounds. The former, naphthalene, is colourless with neither half ionic; the latter, azulene, is highly coloured and dipolar (the 7-membered ring tends to be cationic while the 5-membered ring tends to be anionic). By contrast the other bicyclodecapentaenes, where they exist, have polyolefinic properties (not aromatic properties). The latter become aromatic only when protonated (to be a cation) or deprotonated (to be an anion). Crepidotine was aromatic (see $^1$H n.m.r.) without ionization. The same observation can be made for the diazabicyclodecatetraenones.

*As an azaazulene*

Given these partial structures: -NH-CPh=CH-CO and C=CH-N=CH-CH= (or C=CH-N= and -N=CH-CH=) the following phenylpyrroloazepinones or phenyldiazaazulenones and their tautomers were possibilities:



Azulenes are strongly blue and violet coloured (Gordon, 1952). 5-Aza-azulene is also violet coloured (Hafner and Kreuder, 1961) but a series (>14) of 2-oxocyclohepta[*b*]pyrroles and 2,8-dioxocyclohepta[*b*]pyrroles were yellow or orange (Nakao, *et al.*, 1965; Sato, *et al.*, 1973). Also several (>7) 1*H*-cyclohepta[1,2-*d*:3,4-*d'*]diimidazoles were yellow (Cariello, *et al.*, 1974a,b). Up to 1976 pyrroloazepinones had not been described but by comparison with the above compounds it would appear that such compounds would be yellow coloured or strongly absorbing in the near u.v. Crepidotine is colourless and has no intense absorptions at more than 332 nm (Table 7.1).

Structures A and B above could both show the AB pattern of crepidotine (δ 8.69, d, and 7.54, d) for protons H-2 and H-3. The singlet at δ 6.77 could be attributed to proton H-5 in structure A, and H-7 in B. Only if the azepino-N did not tautomerize (and it would, Bodor, *et al.*, 1970) could the proton adjacent to the N resonate at δ 9.36 (δ 9.40 in 6-phenyl-5-azaazulene, Hafner, *et al.*, 1970). Hence on the whole, crepidotine was unlikely to have a phenylpyrroloazepinone structure (or phenyldiazabicyclo[5,3,0]deca-1,3,5,7,9-pentaenol).

*As a naphthyridine*

The remaining bicyclo-system is the bicyclo[4,4,0]decapentaene, naphthalene. As discussed above the $^1$H n.m.r. spectrum suggests there are two moieties in the molecule: (a) the tautomeric system

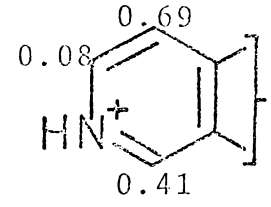
$$-NH-CPh=CH-CO- \rightleftarrows -N=CPh-CH=COH-$$

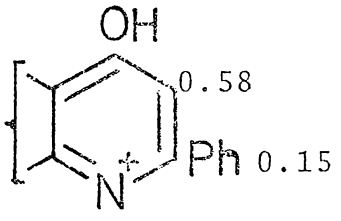
as in 2-phenyl-4-pyridone and (b) the unsaturated nitrogen environment

$$C-CH=N-CH=CH$$

as in isoquinoline. These two moieties together form a 2-phenyl-1,6- or -1,7-naphthyridin-4-one.

*The $^1H$ nuclear magnetic resonance in acids.*--The $^1H$ n.m.r. spectrum of crepidotine shifted downfield in acetic acid (Fig. 7.2, Table 7.2 at 85°) and even further in trifluoroacetic acid (tfa) (Fig. 7.2, Table 7.2). The extent of the downfield shift in tfa-*d* from that in dmso-$d_6$ (85°) (in ppm) is set out here:-

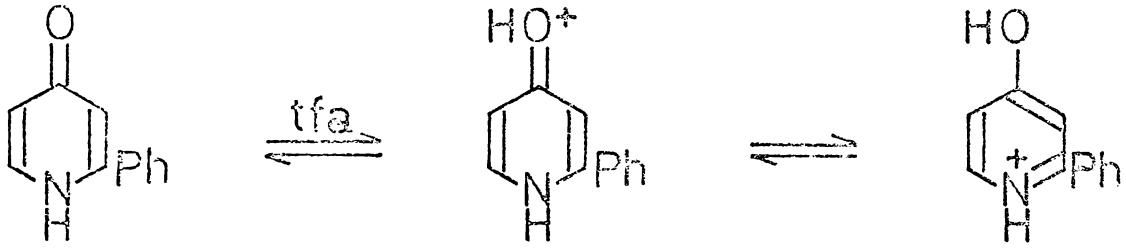



Consistent with the 4-pyridone ring becoming aromatic upon protonation in tfa-*d* the resonances for the protons of the phenyl group now appear as an almost single envelope i.e. the influence of the carbonyl group has been lost because the carbonyl group has become a phenolic-*OH*. The phenyl-proton multiplet for 6-nitro-2-phenyl-4-quinolone in tfa-*d* appears as a single envelope (δ 8.2—7.5, Pouchert and Campbell, 1974). The most intense peak for the single aromatic envelope for crepidotine in tfa-*d* was at δ 7.67 while that for 6-nitro-2-phenyl-4-quinolone (tfa-*d*) is at δ 7.81. Another comparison would be with the $^1H$ n.m.r. spectrum of the phenyl group in 2-phenylpyridine but this is complicated by the resonances for the unsymmetrically arranged pyridine protons. It appears that these phenyl protons resonate as a multiplet between δ 7.6 and 7.35 ($CDCl_3$, Pouchert and Campbell, 1974) with the most intense peak at δ 7.4.

Since the shift for the PhC=C*H* was not as great as that for the proton at δ 7.54 (dmso-$d_6$), it could be reasoned that the environment of the PhC=C*H* had not altered and that the observed shift was the result only of the $^1H$ n.m.r. spectrum being determined in tfa. When an aromatic proton is *ortho* to an OH and a phenyl group that proton is shielded (by *c.* 0.5 ppm for OH and *c.* -0.2 ppm for $C_6H_5$, Laszlo and Stang, 1971). Hence for the PhC=C*H* proton, were there no adjacent OH, a shift of *c.* 0.88 ppm could be considered to have taken place (δ tfa-*d* - dmso-$d_6$,

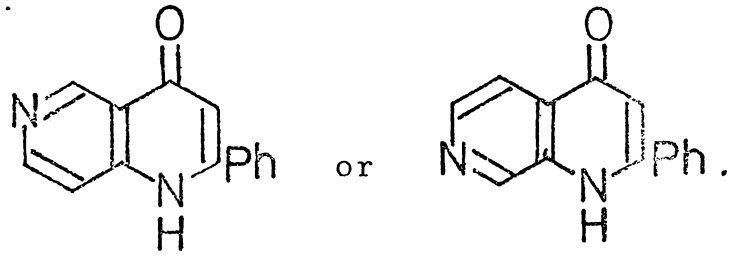
0.58 + 0.5 - 0.2 = 0.88 ppm). For 2-quinolone (and 2-pyridone) the resonance for proton H-3 shifts 0.86 ppm downfield in going from dmso-$d_6$ to tfa-$d$ (δ 6.51, dmso, to 7.37, tfa see Table 7.2). In the above cases it is suggested that in the strong acid, tfa, the molecules are protonated so that the condition of the ring changes from that of a 4-pyridone to that of a 4-hydroxypyridinium.




Since cyclohexa-2,4-dienone is a tautomer of phenol (Green, 1968) the shift of the H-2 proton resonance in going from 6,6-disubstituted cyclohexa-2,4-dienone to phenol would be comparable with that observed above. The H-2 proton in 6-acetoxy-6-methyl-2,4-cyclohexadienone resonates at δ 6.16 ($CDCl_3$, Botherner-By and Moser, 1968), that in 6-dichloromethyl-6-methyl-2,4-cyclohexadienone at δ 6.02 ($CCl_4$, Friedrich, 1968) and 6-allyl-6-methyl-2,4-cyclohexadienone at δ 5.92 (Hansen, *et al.*, 1968) compared with phenol at δ 7.3—6.65 (Simons and Zanger, 1972, or δ 6.7 in hydroquinone, Pouchert and Campbell, 1974).

This confirms the suggested structure and points to a 6-membered aromatic ring, which is capable of tautomerizing, as in 4-pyridone.

From the above observations two structures for crepidotine are proposed:




Both are consistent with the mass spectral data (see Table 7.3), u.v. (see later), and $^1H$ n.m.r. (dmso-$d_6$). Czuba and Wozniak (1975) examined

the $^1$H n.m.r. spectra of both 1,6- and 1,7-naphthyridin-4-ones in which the following shieldings (δ) were observed, and the difference from crepidotine shown:

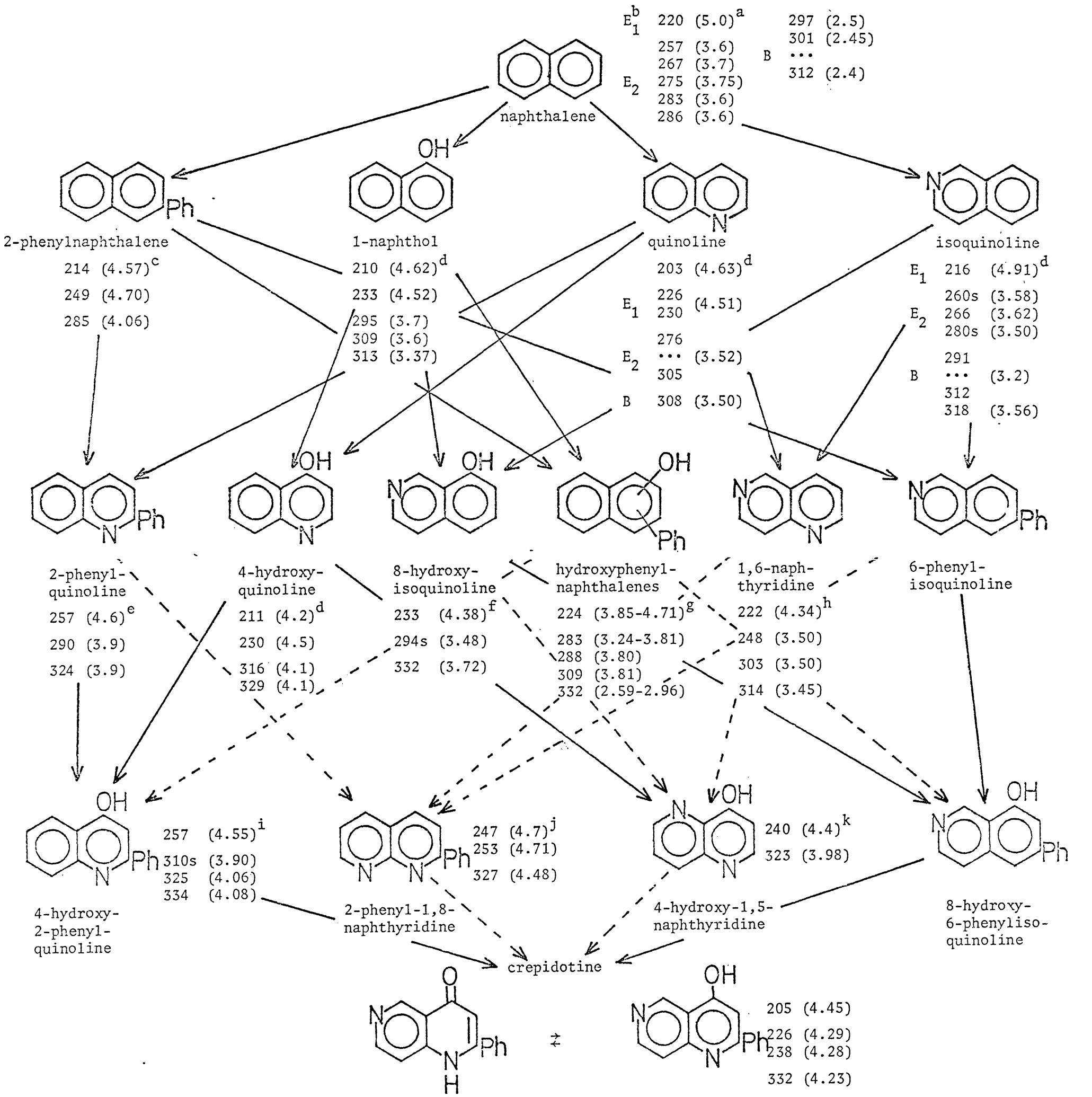
| Proton: | | $H_a$ | N | $H_b$ | $H_c$ | 2 | 3 | Total difference |
|---|---|---|---|---|---|---|---|---|
| 1,6-naphthyridin-4-one: | δ | 9.42 | - | 8.80 | 7.66 | 8.19 | 6.38 | |
| Difference: | | 0.06 | - | 0.11 | 0.12 | | | =0.29 |
| Crepidotine: | | 9.36 | - | 8.69 | 7.54 | Ph | 6.81 | |
| Difference: | | 0.05 | - | 0.06 | 0.55 | | | =0.66 |
| 1,7-naphthyridin-4-one: | | 9.31 | - | 8.63 | 8.09 | 8.25 | 6.37. | |

Considering only the resonances for the pyrido-protons, $H_a$, $H_b$ and $H_c$ which are the appropriate ones to compare, for $H_a$ and $H_b$ those for crepidotine lay between those for the 1,6- and 1,7-isomers. The resonance for the crepidotine proton $H_a$ was midway between those for the two model isomers while that for $H_b$ was 65% in favour of the 1,7-isomer. But the resonance for $H_c$ for crepidotine was upfield from that of the 1,6-isomer which also was upfield from that of the 1,7-isomer so at this point crepidotine strongly favoured the 1,6-naphthyridine structure. Further, the overall difference for these three protons again strongly favours the 1,6-isomer.

It is interesting to note that the sum of the 4 δ values for crepidotine was 32.40 ppm while those for the two isomers are 32.26 and 32.40 ppm (resp.) which tends to confirm the structure of crepidotine to be in the naphthyridine class.

The $^1$H n.m.r. spectra in the different solvents (see Fig. 7.2) showed the single proton peak at δ 6.81 (dmso-$d_6$) to be taller and sharper than the single proton peak at δ 9.36. When crepidotine was examined in tfa-*d* the hidden doublet that was at δ 7.54 in dmso-$d_6$ now appeared separately from the aromatic envelope and appeared taller and sharper than its corresponding downfield doublet. This observation

Table 7.4. Ultraviolet spectra, $\lambda_{max}$ (log ε), of naphthalene and crepidotine (preferred structure) with those of the intermediate combinations of the latter's substituents.




a. Friedel and Orchin, 1951.
b. Silverstein and Bassler, 1968.
c. Holloway, *et al.*, 1968.
d. DMS, 1966
e. Moszew, *et al.*, 1961.
f. Schenker, *et al.*, 1966.
g. Williams, *et al.*, 1968.
h. Albert and Armarego, 1963.
i. Goodwin, *et al.*, 1957.
j. Hawes and Wibberley, 1967.
k. Mason, 1975b.
s. = shoulder.

suggested that the resonances at δ 9.73 and 8.73 (tfa-*d*) were broadened by either unresolved long-range splitting or $^{14}N$-quadrupole broadening. Decoupling the doublet at δ 8.69 (dmso-$d_6$) caused only a deformation of the peak at δ 9.36, instead of a sharpening of it. Whether it was mutual unresolved long-range splitting or $^{14}N$-quadrupole broadening, this minor observation is consistent with the suggested structure: =CH-N=CH-CH=.

*The ultra-violet spectra.*-Scott (1964) pointed out that the introduction of a nitrogen atom into the ring system of naphthalene has a remarkably slight effect on the u.v. spectrum. In Table 7.4 is set out the u.v. spectra of naphthalene and crepidotine (suggested structure) with all the intermediate combinations of the substituents and their u.v. spectra where known or that of a near isomer. That for naphthalene reveals a fine structure (in three groups $E_1$-, $E_2$- and B-bands, Silverstein and Bassler, 1968) with many (*c.* 6) minor peaks between 257 and 312 nm and the reduction of intensity with increase in wavelength. Upon the introduction of one substituent (either phenyl or hydroxy) much of the fine structure is lost but the absorptions are still in three groups and the intensities at longer wavelengths are greater; also there are still absorptions between 250 and 300 nm: similarly with the introduction of the two nitrogen atoms, but the effect is not so marked. Upon the introduction of a second group there are mostly only three absorptions, all of which are intense (log ε >4.35) and absent between 250 and 290 nm, except those for phenylnaphthols. When there are three groups the u.v. spectrum shows a peak about 240—260 nm and another group at 310—335 nm (all of log ε >4) with a trough between. Finally crepidotine showed, as well as the peak at 205 nm, absorptions at 226 and 238 separated by a trough from the absorption at 332 nm, all of intensity greater than 4 (log ε). The u.v. spectrum of crepidotine was therefore consistent with

its being a highly conjugated and aromatic system. [4-Phenyl-2-quinolone has a u.v. spectrum of $\lambda_{max}$ (log ε) 226 (4.57), 278 (3.89), and 331 (3.79) nm (Iwai and Hiraoka, 1963), i.e. an absorption between 250 and 300 nm, absent in crepidotine, and the intensity at $\lambda_{max}$ 331 nm less than half of that in crepidotine.]

The u.v. spectra of crepidotine, benzalacetophenone and benzalacetone all have a common feature (see Table 7.1). (Both these compounds have olefinic protons.) In each of them there are two unresolved peaks of almost equal intensity occurring in the benzenoid region (Scott, 1964) between 218 and 238 nm, suggesting their extended conjugated systems, β-phenyl-α,β-unsaturated ketones.

Upon the addition of acid to crepidotine the peak at 205 nm was not affected (Table 7.1) but those at 226 and 238 nm became degenerate bathochromically to 259 nm, with reduced intensity (log ε *c.*4.14). This agreed with the pyridone ring or the extended conjugated system (above) becoming aromatic on protonation, so the molecule became like 3-phenylphenol in acid (250 nm, log ε 4.14, Kreiter, *et al.*, 1954). Further, the absorption at 332 nm shifted markedly to 377 nm with increase in intensity (log ε *c.* 4.33). This red shift also appeared when crepidotine became a pink solution in tfa-*d* (for $^1$H n.m.r.). Similarly upon raising the pH the peaks at 226 and 238 nm again became degenerate bathochromically to 257 nm with greater intensity (log ε *c.* 4.39) and that at 332 nm also shifted (bathochromically) to 352 nm (with almost equal intensity, see Table 7.1). 1-Naphthol shows similar shifts in alkali: 233 to 247 nm and 295 to 333 nm (DMS, 1966). This suggests that for crepidotine in contrast to 1,5-naphthyridin-4-one (Bailey, *et al.*, 1967) the keto-form (ethanol solution) did not absorb at the same wavelength as the anion. (Crepidotine was not sufficiently soluble in non polar solvents to determine the absorptions of the enol form.)

Table 7.5. νCO absorptions of tautomeric oxo-*N*-heteroaromatic compounds.

a. α-Carbonyl compounds from Mason's (1957a) table.

| Frequency ($cm^{-1}$) | Compound | Relation of CO to N | Classification |
|---|---|---|---|
| 1640 | 2-quinolone | 2-oxo-1-aza | the CO α to one N. |
| 1650 | 2-pyridone | do. | |
| 1653 | 1-isoquinolone | 1-oxo-2-aza | |
| 1658 | phthalazin-1-one | 1-oxo-2,3-diaza | the CO α to a N which is adjacent to another N. |
| 1660 | cinnolin-3-one | 3-oxo-1,2-diaza | |
| 1678, 1652 | pyridazin-3-one | do. | |
| 1690, 1642 | quinozalin-2-one | 2-oxo-1,4-diaza | the CO α to one N and β or *peri* to another. |
| 1692, 1650 | 5-hydroxy-1,4,-6-triazanaphthalene | 1-oxo-2,5,8-triaza | |
| 1693 | 1,7-naphthyridin-8-one | 1-oxo-2,8-diaza | |
| 1710, 1662 | pyrazin-2-one | 2-oxo-1,4-diaza | |
| 1710, 1670 | 4-hydroxy-1,3,5-triazanaphthalene | 1-oxo-2,4,5-triaza | the CO α to one N and γ to another. |
| 1716m, 1684 | pyrimidin-4-one | 1-oxo-2,4-diaza | |
| 1733m, 1647 | pyrimidin-2-one | 2-oxo-1,3-diaza | the CO α to two Ns. |

b. γ-Carbonyl compounds.

| Frequency ($cm^{-1}$) | Compound | | Relation of CO to N | Reference |
|---|---|---|---|---|
| 1624 | 1,5-naphthyridin-4-one | | 1-oxo-4,8-diaza | Mason (1957a) |
| 1625 | 4-hydroxy-1,5,8-triaza-naphthalene | | 1-oxo-4,5,-8-triaza | do. |
| 1638 | 4 quinolone | | 1-oxo-4-aza | do. |
| 1640 | 2,8-dimethyl-<br>2,6,8-trimethyl-<br>2,5,6,8-tetramethyl | 1,7-naphthyridin-4-one | 1-oxo-4,6-diaza | Achremowicz and Mlochowski (1973) |

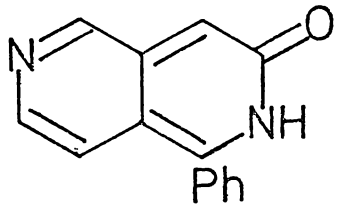
Neither 4-quinolone, 2-methyl-4-quinolone (EtOH, Ewing and Steck, 1946) nor 2-phenyl-4-quinolone (EtOH, Goodwin, *et al.*, 1957) shows a bathochromic shift in either dilute base or acid. But both 4-hydroxy-1,5-naphthyridine and 8-hydroxyquinoline show marked bathochromic shifts in aqueous acid (Bailey, *et al.*, 1967) which shifts, they suggested, were associated with the 4-OH and the 5-aza (in the former compound) rather than the 1-aza. Comparable model compounds for crepidotine are either 5-hydroxyisoquinoline (for a 1,7-naphthyridine-type structure) or 8-hydroxyisoquinoline (for a 1,6-naphthyridine-type structure). Both these compounds show bathochromic shifts in acid (the former 17 and 39 nm, Ewing and Steck, 1946; Nakanishi, *et al.*, 1961: the latter 11 and 47 nm, Schenker, *et al.*, 1966) comparable with that of crepidotine (27 and 45 nm, for the bands at 230 and 330 resp.).

## §3. *An Alternative Structure*

Although the i.r. absorptions at 1670vs and 1610s $cm^{-1}$ (KBr) for crepidotine appeared to support an -NH-CPh=CH-CO- structure, there are only absorptions at 1632s and 1608s $cm^{-1}$ (KBr) for 2-phenyl-4-quinolone (Staskun, 1966) whose structure is closely analogous to that suggested for crepidotine. Crepidotine had strong absorptions at 1630 and 1610 $cm^{-1}$ but that at 1670 $cm^{-1}$ was a great deal stronger. The frequencies of the $\nu C{=}O$ absorptions of 2-pyridones (Table 7.5) are nearer that of crepidotine than are those of 4-pyridones (see Table 7.5). Bajwa and Joullie (1972) observed that 2-quinolones absorb between 1640 and 1670 $cm^{-1}$ while 4-quinolones do between 1620 and 1650 $cm^{-1}$. For the 1-isoquinolone series the $\nu C{=}O$ peak occurs at 1653s $cm^{-1}$ for 1-isoquinolone; 1693s $cm^{-1}$ for 2,8-naphthyridin-1-one (Mason, 1957a); 1700, 1675, 1660, 1600 $cm^{-1}$ for 4-quinazolinone (Pouchert, 1970); and 1710, 1670 for 4-hydroxy-1,3,5-triazanaphthalene (Mason, 1957a): i.e., the more nitrogens in the aromatic molecule the higher the $\nu C{=}O$ frequency. By contrast in

the 4-quinolone series the νC=O absorbs between 1624 and 1640 $cm^{-1}$ for a number of compounds (Table 7.5) i.e. there is no apparent increase in the νC=O frequency with increase in the number of nitrogens in the aromatic molecule. This evidence suggests that crepidotine was not a derivative of 4-pyridone. The presence of a phenyl group conjugated to the carbonyl of 4-quinolones did not change the frequency of their carbonyl absorptions either (1638 $cm^{-1}$, 4-quinolone, Mason 1957a; 1637 $cm^{-1}$, 7-nitro-2-phenyl-4-quinolone; 1639 $cm^{-1}$, 7-fluorosulphonyl-2-phenyl-4-quinolone, Pouchert, 1970).

In order that the compound should be able to lose the neutral fragment, phenyl cyanide, in mass spectrometry and should have a 2-pyridone structure, a structure comparable with 1-phenyl-3-isoquinolone is suggested. This type of compound




has some phenolic properties (Bentley, *et al.*, 1952), has the H-4 proton resonating at *c.* δ 6.8 (δ 6.79 in 2-methyl-3-isoquinolone, Evans, *et al.*, 1967) and has the CO absorbing between 1650 and 1675 $cm^{-1}$ (3-isoquinolone, 1650 $cm^{-1}$, Baumgarten, *et al.*, 1961; 1-benzyltetramethoxy-3-isoquinolone, 1663 $cm^{-1}$, Elliott, 1972; 1-phenyl-1,4-dihydro-3-isoquinolone, 1670 $cm^{-1}$, Deak, *et al.*, 1973; cinnolin-3-one, 1672 $cm^{-1}$, Baumgarten, *et al.*, 1961). Although they have these properties, 3-isoquinolones tend to be unstable (2-methyl-3-isoquinolone), yellow (1-methyl-3-isoquinolone, Evans, *et al.*, 1967; $\lambda_{max}$ for 1-phenyl-3-isoquinolone 350 (3.76), 420 (3.34) nm, Jones, 1969) compounds. In spite of these νCO frequencies (1651—1672 $cm^{-1}$) being close to that of crepidotine, the νCO frequencies for 1,2,4-triphenyl- and 4-benzyl-1-phenyl-

3-isoquinolones are at 1620 $cm^{-1}$ (Deak and Hazai, 1973; Nakazawa, *et al.*, 1974). So it would appear, therefore, that 3-isoquinolones do not fall within the νCO range of 2-pyridones (Table 7.5). Further the phenyl group would not be β to the carbonyl in an α,β-unsaturated ketone system as is suggested by the u.v. spectrum (218—238 nm) when compared with those of benzalacetophenone and benzalacetone (see above).

From the shape of the phenyl envelope in the $^1H$ n.m.r. spectra of 4-phenylpyrimidine and 2,5-diphenyl-1,3,4-oxadiazole, phenyl derivatives of single heterocyclic rings, (δ 8.3—8.0 and 7.85—7.4 resp., Pouchert and Campbell, 1974) the phenyl group does not have to be constituted as a benzoyl or be β to the carbonyl in an α,β-unsaturated ketone ring system in order to resonate in two groups. Though the resonances of the phenyl groups of these two compounds are in two groups the *ortho*-protons resonate (δ 8.3—8.0) much further downfield than those of crepidotine (δ 7.9—7.65) and the width of the trough is greater (0.2 and 0.35 ppm resp.) than that for crepidotine. Against this occurrence of phenyl resonances in two groups is the observation (before) that the phenyl resonances of both 3,5-diacetyl-4-methyl-6-phenyl-2-pyridone and 3-acetyl-5-cyano-4,6-diphenyl-2-pyridone (dmso-$d_6$) occur as singlets (Kato, *et al.*, 1975). These structures are nearer to what crepidotine might be than the two former whose phenyl groups resonate in two groups.

It is possible that the second nitrogen (the 6- or 7-aza in 1-phenyl-2,6- or -2,7-naphthyridin-3-one) may cause a hypsochromic shift in the u.v. spectrum from *c.* 400 nm (for 3-isoquinolones) to that of crepidotine at 332 nm. This shift is comparable with that observed by Muller-Westerhoff and Hafner (1967) in the u.v. absorptions in going from 6-dimethylamino-5-azaazulene to 6-dimethylamino-5,7-diazaazulene, where there was a hypsochromic shift of *c.* 100 nm.

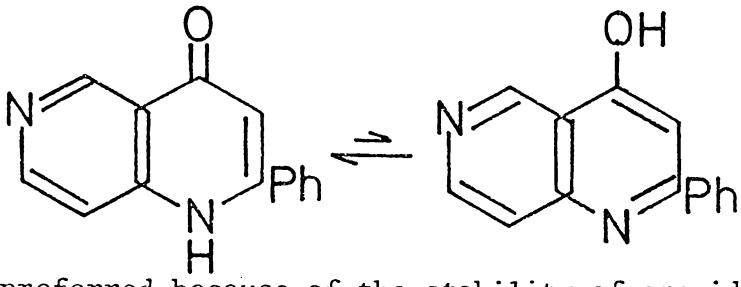
Bailey, *et al.*, (1967; see above) demonstrated for hydroxynaphthy-

ridines that their u.v. spectra were a function of the hydroxy-group in one ring and the nitrogen in the other. Nakanishi, *et al.*, (1961) worked with the u.v. spectra of 5-, 6-, 7- and 8-hydroxyisoquinolines and showed that there was significance in the position of the deepest trough in these compounds and their ions. For 5- and 8-hydroxyisoquinoline the deepest trough occurs *c.* 260 nm in methanol, 265—270 nm in acid and 275 and 290 nm (resp.) in alkali: while for 6-hydroxyisoquinoline they all occur at *c.* 270—275 nm and for 7-hydroxyisoquinoline at *c.* 295—310 nm. When crepidotine (275, 305 and 295 nm in methanol, acid and alkali resp.) was compared with the above values it was nearest to that of 7-hydroxyisoquinoline. Nevertheless, the spectra for 7-hydroxyisoquinoline have peaks (log ε 3.5 to 4.0) between 260 and 300 nm, which were absent for crepidotine.

As stated above, though 1-phenyl-3-isoquinolones may have νCO peaks at *c.* 1670 $cm^{-1}$ and their H-4 protons resonate at c. δ 6.81, their phenyl groups do not seem to resonate in two groups of multiplets neither do their u.v. spectra have marked similarities to those (neutral, acid, base) of crepidotine. On the other hand 2,6-naphthyridines are known in the plant kingdom: Harkiss (1971) discovered 4-methyl-2,6-naphthyridine in *Antirrhinum majus* L. In the literature there is a paucity of data (u.v., i.r., n.m.r. etc.) on 3-isoquinolones and their derivatives, by which an exhaustive comparison could be made between them as model compounds and crepidotine.

## §4. *Conclusion*

In conclusion, this structure for crepidotine is proposed




It is preferred because of the stability of crepidotine and because, apart from the νCO value, the weight of evidence above favours 2-phenyl-1,6-naphthyridin-4-one. With this proposed structure for crepidotine it is difficult to see how it could be degraded to elucidate the structure. Given a larger supply of crepidotine the application of lithium aluminium hydride reduction has been suggested but crepidotine is insoluble in either ether or tetrahydrofuran which are the usual solvents for this reaction. Were such a reaction successful the carbonyl might be reduced to a methylene so then this 1,4-dihydropyridine ring might open during oxidation. Further with the larger supply, the $^{13}C$ n.m.r. could be investigated. This would have to be done in tfa-*d*, in which solvent very few other compounds for comparison have been examined. On the whole the final structure may have to await an X-ray crystallographic examination.

## *Experimental for Chapter 7, Crepidotine*

C. *Spectra of crepidotine*

C-A. $^1$H *Nuclear magnetic resonance spectra*, see Fig. 7.2 and Table 7.2.

i. *Crepidotine*

The crystalline crepidotine (supplied by E.P. White) (*c.* 50 mg) was dissolved in deuterodimethylsulphoxide (dmso-$d_6$) at 85° and filtered in a hot air bath. This solution was transferred to the n.m.r. tube, while it was still hot and the spectrum recorded at 85°.

The same sample (of crepidotine) was recovered and redissolved in acetic acid (85°) then in deuterotrifluoroacetic acid (tfa-*d*) (65° and 30°) for the respective spectra. Crepidotine was freely soluble in tfa-*d*. The pyridine (py-$d_5$) spectrum was of a saturated solution (30°) by E.P. White (pers. comm.).

ii. *Model compounds*

In Table 7.2 is set out the $^1$H n.m.r. results for isoquinoline, 2-quinolone and 2-pyridone determined in dmso-$d_6$ (85°), acetic acid (65° and 30°), tfa-*d* (65° and 30°) and py-$d_5$ (85°, except 2-pyridone). The spectrum for neat isoquinoline was also determined.

C-B. *Ultra-violet spectra* (u.v.)

This was determined in methanol, neutral, acidified and alkaline, by E.P. White (pers. comm.).

C-C. *Infra-red spectrum* (i.r.)

This was recorded for a micropellet of potassium bromide (KBr), see Fig. 7.1.

C-D. *Mass spectrum*

The mass spectrum was determined by Prof. R. Hodges, Massey University.

# CHAPTER 8. CONCLUSION

1. Derivatives of sporidesmin were produced which would complex with proteins. When these materials were used as antigens in animals, antibodies were detected in their sera.

2. An antibody titre of 1:500 from treating sheep with the antigen, modified sporidesmin complexed to bovine thyroglobulin, strongly suggests that there is some hope of immunizing sheep against Facial Eczema.

3. Being able to chloroacetylate sdm supplies a route for complexing sdm to protein (provided the chlorine atom does not hydrolyze too readily) without altering the disulphide bridge which is the toxic centre of the molecule.

4. *p*-Nitrobenzene diazonium salt or derivatives of it (2-chloro- or 2-nitro-) are too active an environment for sdm. The sdm molecule and even the otherwise stable anhydrodethiosdm break down in the presence of the salt without coupling at the aromatic hydrogen. The breakdown involves an unusual elimination reaction.

5. Though sdm-E is only a little different from sdm (i.e. an epitrithio-bridge instead of an epidithio-one), it is labile. Its main decomposition product is sdm (plus some sulphur and sdm-G). A method of preparation, to obtain it as pure as possible, has been suggested. Its mixed melting point with sdm does not depress but the degree of contamination with sdm is indicated in the i.r. spectrum of the carbon tetrachloride solution. Sdm-E does not have the νOH band at 3450 $cm^{-1}$ which sdm has.

6. The carbon tetrachloride solution spectra between 3650 and 3300 $cm^{-1}$ of sdm and some derivatives serve to identify structural features in the molecules. Sdm-E and 3,11a-dimercaptosecosdm have similar spectra therefore their C-S bonds are similarly orientated. Sdm-B has only one hydroxy-group (10b-OH) but two strong νOH peaks. The high frequency one

of these, strong in sdm, sdm-E and 3,11a-dimercaptosecosdm, is nearly absent in sdm-D, methyl 3-mercaptosecosdm-11a-*S*-acetate and methyl 11a-mercaptosecosdm-3-*S*-acetate, although all these compounds have the same hydroxy-group (10b-OH) as sdm-B. This points to the radical change in the structure of the epidithiodioxopiperazine ring when the -S-S- bridge is opened.

7. A suggestion is made to explain the absence of any νSH band or of chemical evidence for a thiol group for methyl 11a-mercaptosecosdm-3-*S*-acetate when the mass spectral data indicated the presence of a potential thiol group. The elements of the thiol group are present but they with an adjacent amido-group are participating in a state which is akin to hyperconjugation.

8. Further information on the change in the dioxopiperazine ring (from opening the -S-S- bridge) was obtained from the $^{13}C$ n.m.r. spectra of sdm and sdm-D. The comparison of these spectra indicated the strain under which the dioxopiperazine ring existed in sdm.

9. Arising out of this work, three papers have been published. Xerox copies of these papers are in the Appendices of this work.
Appendix a. *The production of rabbit antibodies to sporidesmin,* Jonas and Ronaldson (1974) describes the results from using the antigen, modified sporidesmin complexed to poly-(L-lysine), whose synthesis is described in chapter 2.
Appendix b. *Sporidesmins.* XIV *Modifications to the opened -S-S- bridge of sporidesmin for coupling to proteins by transacylation,* Ronaldson (1975) describes the synthesis of the antigens used in the above publication.
Appendix c. *Sporidesmins.* XV *The $^{13}C$ nuclear magnetic resonance of sporidesmin and sporidesmin-D. The evidence in the spectra for strain imposed by an epidithio bridge,* Ronaldson (1976) gives the assignments of the peaks as described in chapter 3.

10. The structure of crepidotine was elucidated. Since there was only 50 mg of this fungal product available, non-destructive methods were used to arrive at the structure, 2-phenyl-1,6-naphthyridin-4-one. Of these methods, $^{1}H$ nuclear magnetic resonance spectroscopy was the most important. This spectrum ($^{1}H$ n.m.r.) of crepidotine was compared with the known or expected spectra of possible isomeric structures most of which were unknown. Infra-red and ultra-violet spectra of compounds of comparable structure (where known) were also considered. A metastable peak in the mass spectrum indicating the formation of the neutral fragment, phenyl cyanide, was important in showing that there was a phenyl group and that the elements of phenyl cyanide occurred together. Confirmation of the structure deduced awaits the synthesis of crepidotine.

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Appendix a.



# THE PRODUCTION OF RABBIT ANTIBODIES TO SPORIDESMIN

W. E. JONAS* and J. W. RONALDSON†

## INTRODUCTION

SPORIDESMIN, the causative agent of facial eczema, is a low molecular weight (473) hepatotoxin produced by the fungus *Pithomyces chartarum*. Being a small molecule, it is unlikely that sporidesmin would be capable, by itself, of inducing an antibody response in an animal. However, an antibody response can often be produced against a low molecular weight molecule, provided that the molecule is covalently coupled to a protein with a molecular weight of approximately 10 000 or greater.

The work reported in this paper is part of an investigation concerning the possibility of controlling facial eczema by vaccination. The results presented show that a sporidesmin-poly-L-lysine complex when injected into some rabbits resulted in an antibody response to sporidesmin. The sporidesmin-poly-L-lysine complex was prepared by breaking the disulphide bridge across the dioxopiperazine ring of sporidesmin, alkylating the sulphydryl groups and then reacting this complex with the ε-amino groups of poly-L-lysine.

## MATERIALS AND METHODS

### MATERIALS

Crystalline sporidesmin prepared as sporidesmin benzene solvate was used as the source of sporidesmin. Other substances used were: Poly-L-lysine hydrobromide Type I, molecular weight 139 000: DL-tryptophan (Sigma Chemical Company, St Louis, Mo.); keyhole limpet haemocyanin (Pacific Bio-Marine Supply Co., Venice, Calif.); bovine albumin powder (Armour Pharmaceutical Company, Illinois); rabbit albumin, crystallized (Mann Research Laboratories, N.Y.); serotonin-creatinine-phosphate (5-hydroxy - tryptamine - creatinine - phosphate) (BDH Ltd, England); 6-nitroveratraldehyde (K & K Laboratories Inc., Hollywood); sulphamethoxydiazine (Bayrena: Bayer Pharmaceutical Company, Germany); sulphadimethoxine (Madriben: Roche Products Ltd, England); sulphamethoxypyridazine (Midicel: Park Davis and Company, Sydney). 5,5'-dichloro-6,6',7,7' - tetramethoxy - *N,N'* dimethyl indigo, 5-chloro,6,7-dimethoxy *N*-methyl isatin, 5-chloro-6-hydroxy-7-methoxy *N*-methyl isatin, gliotoxin and berberine were obtained from Dr E. P. White (Ruakura Agricultural Research Centre). 5-chloroindole-2-carboxylic acid was synthesized by A. Erasmuson (Victoria University of Wellington). 5-chlorovanillin was synthesized by K. McNatty (Wallaceville Animal Research Centre).

### PREPARATION OF SPORIDESMIN COMPLEXES FOR IMMUNIZATION

*Sporidesmin-poly-L-lysine complex*: The method of coupling sporidesmin to poly-L-lysine and albumins will be described in detail elsewhere. In brief, sporidesmin was treated with sodium borohydride to open the disulphide bridge across the dioxopiperazine ring. The sulphydryl groups were then alkylated and the alkylated *S,S'*-seco sporidesmin diacetic acid methyl ester then reacted with poly-L-lysine. The preparation used in these experiments had approximately 28% of the lysine residues substituted with sporidesmin.

*Sporidesmin-poly-L-lysine-keyhole limpet haemocyanin complex*: The method described by Jaff *et al.* (1971) for coupling prostaglandin $PGF_{2\alpha}$ to succinylated bovine albumin was used for coupling sporidesmin-poly-L-lysine to keyhole limpet haemocyanin.

### PRODUCTION OF ANTISERA

Two rabbits received approximately 1.4 mg sporidesmin-poly-L-lysine (28% preparation) at multiple intradermal

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sites on 3 occasions, each course of injections being 2 weeks apart. Four rabbits each received approximately 1.4 mg of the sporidesmin-poly-L-lysine intramuscularly as above. One rabbit received 1.6 mg of the sporidesmin-poly-L-lysine-keyhole limpet haemocyanin on 5 occasions, each injection being 4 weeks apart. Four rabbits were injected intramuscularly with poly-L-lysine; 2 received 2 mg and 2 received 20 mg per injection for 3 injections, each 6 weeks apart. Four guinea-pigs were immunized on three occasions with rabbit albumin. All antigens were given in Freund's complete adjuvant.

### Preparation of Sporidesmin-Albumin Complexes for Serology

The sporidesmin-rabbit albumin complex was prepared as described above. The dried complex was taken up in distilled water and dialysed against a number of changes of distilled water for 2 days. The dialysate was centrifuged and the deposit was taken up in 0.15M pH 7.2 phosphate buffer.

A sporidesmin-bovine albumin complex was prepared as above, but was not dialysed prior to use.

All the sporidesmin-complex preparations were subjected to spectral analysis in a Shimadzu QV spectrophotometer. Some preparations were sieved through columns of coarse Sephadex G25 (Pharmacia Ltd).

### Coupling of the Sporidesmin-Rabbit Albumin Complex to Erythrocytes for the Indirect Haemagglutination Test

The *bis*-diazotized benzidine method described by Gordon *et al.* (1958) was used with slight modifications. The incubation period was reduced from 15 minutes to 12 minutes and the final concentration of erythrocytes reduced from 1% to 0.33%.

### Indirect Haemagglutination Test (IHA)

IHA tests were done in 50 × 8 mm tubes with 0.2 ml volumes of each reactant. All dilutions were made in 0.15M pH 7.2 phosphate buffer containing 0.5% of heat-inactivated normal guinea-pig serum. For the IHA inhibition tests, 0.2 ml volumes of the albumins or low molecular weight molecules were added to the antisera at least 30 min prior to adding the coupled erythrocytes. All IHA tests were left on the bench overnight and read the following day.

### Equilibrium Dialysis

To 3.8 ml of the serum under test was added 0.68 mg of sporidesmin benzene solvate suspended in 0.2 ml of saline. Two dialysis bags (Visking dialysis tubing, boiled and rinsed in a number of changes of distilled water) containing 2 ml of the sporidesmin-serum mixture were prepared from the 4 ml volume of sporidesmin-serum mixture. The bags were rinsed, dried and placed in a glass-stoppered tube with 4 ml of benzene. The tubes were rotated at room temperature for 24 h and the optical density of the benzene phase read at 300 nm.

## RESULTS

### Sporidesmin Complexes: Spectroscopic Analysis

*Sporidesmin-poly-L-lysine complex*: The absorption spectra of poly-L-lysine or treated poly-L-lysines are shown in Fig. 1.

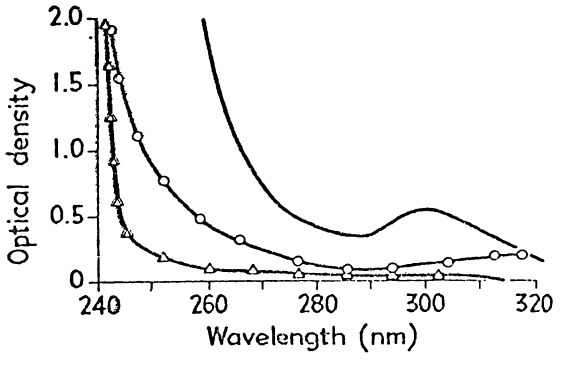



FIG. 1: *The absorption spectrum of the sporidesmin-poly-L-lysine complex* (—, *1 mg/ml*) *used for immunization compared with chloroacetylated poly-L-lysine* (o—o, *1 mg/ml*) *and untreated poly-L-lysine* (Δ—Δ, *10 mg/ml*).

The sporidesmin-poly-L-lysine complex shows end absorption in the vicinity of 260 nm and a peak at 300 nm. Chloroacetylated poly-L-lysine shows end absorption in the vicinity of 240 nm and a small peak at 320 nm. Poly-L-lysine, at 10 mg per ml, showed end absorption only in the vicinity of 240 nm. The absorption spectrum of sporidesmin-poly-L-lysine complex was unaltered even after sieving the material through a Sephadex G25 (coarse) column.

*Sporidesmin-albumin complexes*: Both the sporidesmin-bovine albumin and the sporidesmin-rabbit albumin complexes showed the characteristic sporidesmin peak at 300 nm (see sporidesmin-poly-L-lysine, Fig. 1). The sporidesmin-rabbit albumin complex was separated into a supernatant and a deposit fraction by dialysis and then centrifugation. The supernatant (water-soluble fraction) showed a characteristic protein absorption spectrum with a peak of 280 nm, but the deposit (water-insoluble fraction) gave an absorption spectrum with a peak at 300 nm similar to that shown in Fig. 1 for sporidesmin-poly-L-lysine.

*Sporidesmin-poly-L-lysine-keyhole limpet haemocyanin complex*: The absorption spectrum of the Sephadex G25 (coarse) exclusion peak of the sporidesmin-poly-L-lysine-keyhole limpet haemocyanin complex differed markedly from untreated haemocyanin. The spectrum of the complex did not show the 300 nm peak seen with sporidesmin-poly-L-lysine (Fig. 1).

## Coupling of Sporidesmin-Rabbit Albumin Complex to Erythrocytes by *bis*-Diazotized Benzidine

Initial experiments showed that the sporidesmin-rabbit albumin complex, prior to dialysis, could not be coupled to erythrocytes by *bis*-diazotized benzidine. After dialysis against distilled water and separation by centrifugation into a supernatant and deposit fraction, it was shown that the deposit fraction (in phosphate buffer) could be coupled to erythrocytes with *bis*-diazotized benzidine.

## Reaction of Erythrocytes Coupled to Sporidesmin-Rabbit Albumin with Various Antisera

The results in Table 1 give the titres of some of the antisera used when tested against erythrocytes or erythrocytes coupled to rabbit albumin or the sporidesmin-rabbit albumin complex. The antisera from the rabbits injected with sporidesmin - poly - L - lysine or sporidesmin-poly-L-lysine - keyhole limpet haemocyanin reacted with erythrocytes coupled to sporidesmin-rabbit albumin only. The antisera from the guinea-pigs injected with rabbit albumin reacted with both lots of coupled erythrocytes. The anti-poly-L-lysine did not react with any erythrocytes.

TABLE 1

Titre of Antisera (Reciprocal) from Rabbits Injected with Sporidesmin-poly-L-lysine complexes. Reaction with Rabbit Albumin or Sporidesmin-Rabbit Albumin Complex Coupled to Erythrocytes by *bis*-Diazotized Benzidine. Indirect Haemagglutination Test.

| *Serum from* | *Erythrocytes Coupled to* | | |
|---|---|---|---|
| | *Nothing* | *Rabbit Albumin* | *Sporidesmin-Rabbit Albumin* |
| Rabbits injected with: | | | |
| Nothing .... .... | 0* | 0 | 0 |
| Sporidesmin-poly-L-lysine | | | |
| Intradermally .... .... .... | 0 | 0 | 160 |
| Intramuscularly .... .... .... | 0 | 0 | 160 |
| Sporidesmin-poly-L-lysine-keyhole limpet haemocyanin .... | 0 | 0 | 20 |
| Poly-L-lysine .... .... .... .... | 0 | 0 | 0 |
| Guinea-pigs injected with: | | | |
| Rabbit albumin .... .... .... | 0 | > 2560 | > 2560 |

*Negative at 1:10 dilution.

TABLE 2

Indirect Haemagglutination Inhibition Test. Reaction of Antisera from the Rabbits Injected Intradermally with Sporidesmin-poly-L-lysine in the presence of Sporidesmin and/or Albumins

| *Inhibitor Used and Concentration* | *Titre (reciprocal) of Anti-serum with Sporidesmin-Rabbit Albumin Coupled Erythrocytes* |
|---|---|
| Nothing | 160 |
| Sporidesmin (saturated solution) | 10 |
| Rabbit albumin (1 mg/ml) | 160 |
| Sporidesmin plus rabbit albumin | 10 |
| Sporidesmin-rabbit albumin complex (approx. 1 mg/ml) | 20 |
| Bovine albumin (1 mg/ml) | 160 |
| Sporidesmin plus bovine albumin | 20 |
| Sporidesmin-bovine albumin complex (approx. 1 mg/ml) | 20 |

INHIBITION TESTS WITH SPORIDESMIN-RABBIT ALBUMIN COUPLED ERYTHROCYTES AND ANTISERA FROM RABBITS INJECTED WITH SPORIDESMIN COMPLEXES (INDIRECT HAEMAGGLUTINATION TESTS)

The results in Table 2 show the effect of sporidesmin, rabbit or bovine albumin, mixtures of sporidesmin and the albumin and the sporidesmin-albumin complexes on the ability of the antisera from the rabbits injected intradermally with sporidesmin-poly-L-lysine to agglutinate the sporidesmin-rabbit albumin coupled erythrocytes. It can be seen that the antibody activity was inhibited only when sporidesmin was present.

The results presented in Table 3 show the effect of various low molecular weight molecules on the same indirect haemagglutination system as described for Table 2. Only those molecules having a structure similar to the substituted indole nucleus of sporidesmin and 5-chlorovanillic acid had any inhibitory effect.

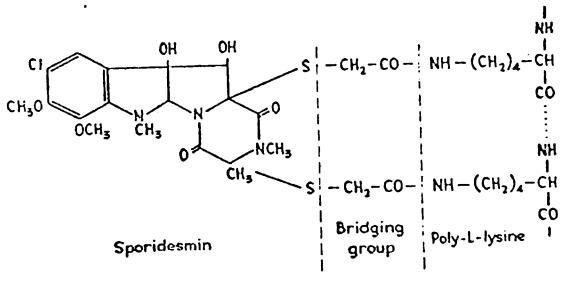
Similar results to those shown in Tables 2 and 3 were obtained with the antisera from the 4 rabbits injected intramuscularly with sporidesmin-poly-L-lysine and the rabbit injected with the sporidesmin-poly-L-lysine-keyhole limpet haemocyanin complex.

EQUILIBRIUM DIALYSIS EXPERIMENTS

Using a modified equilibrium dialysis system it was consistently found that the pooled antisera from the two rabbits injected intradermally with sporidesmin-poly-L-lysine bound more sporidesmin than did serum from normal rabbits or serum from a number of rabbits each injected with different antigens. Control experiments showed that an antiserum containing antibodies to bovine albumin had the same titre before and after a 24-hour period of dialysis against benzene.

## DISCUSSION

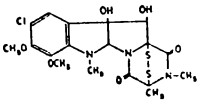
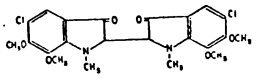
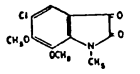
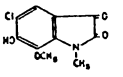
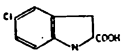
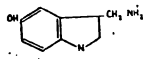
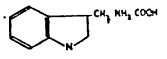
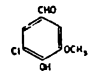
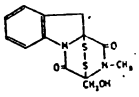
The method which has been used in experiments to couple sporidesmin to poly-L-lysine has relied on the opening of the disulphide bridge in the dioxopiperazine ring of the sporidesmin molecule. Thus, one or both of the sulphur atoms could be available for coupling with the poly-L-lysine or albumin (presumably through the ε-amino group of lysine residues). The sporidesmin-poly-L-lysine complex may have the following structure.




Poly-L-lysine, the high molecular weight carrier used in these experiments may by itself be non-antigenic (Maurer *et al.*, 1959). It has been shown, however, that penicillin-poly-L-lysine (Parker *et al.*, 1965) or 2,4-dinitrophenol-poly-L-lysine (Kantor *et al.*, 1963) complexes are capable of inducing an antibody response

TABLE 3

Indirect Haemagglutination Inhibition Test. Reaction of Antisera from the Rabbits Injected Intradermally with Sporidesmin-poly-L-lysine in the Presence of Low Molecular Weight Molecules Structurally Similar to Sporidesmin*

| *Inhibitor Used and Concentration* | *Titre (reciprocal) of Antisera with Sporidesmin-Rabbit Albumin Coupled Erythrocytes* | *Formula* |
|---|---|---|
| None | 160 | — |
| Sporidesmin (saturated solution) | 10 |  |
| 5,5'-dichloro-6,6'7,7'-tetra-methoxy-*N.N'* dimethyl indigo (saturated solution) | 0† |  |
| 5-chloro-6,7,-dimethoxy *N*-methyl isatin (saturated solution) | 0 |  |
| 5-chloro-6-hydroxy-7-methoxy *N*-methyl isatin (0.08 mg/ml) | 0 |  |
| 5-chloroindole-2-carboxylic acid (saturated solution) | 0 |  |
| 5-hydroxy-tryptamine (1 mg/ml) | 160 |  |
| Tryptophan (saturated solution) | 160 |  |
| 5-chlorovanillin (1 mg/ml) | 40 |  |
| Gliotoxin (1 mg/ml) | 160 |  |

*In addition to the compounds listed, some other molecules possessing methoxy ($OCH_3$) groups were tested. They were 6-nitro-veratraldehyde, berberine, sulphamethoxydiazine, sulphadimethoxine sulphamethoxypyridazine. None of these compounds inhibited the reaction.
†Negative at 1:10 dilution.

specific for the penicillin or 2,4-dinitrophenol molecules. The experiments of Parker *et al.* (1965) showed that the amount of hapten coupled to poly-L-lysine for the purposes of immunization was critical. Therefore, the weak antibody response exhibited by the rabbits to sporidesmin-poly-L-lysine may have been due to a suboptimal immunizing preparation of sporidesmin-poly-L-lysine.

A major practical problem in experiments with sporidesmin is its solubility in physiological solutions such as saline and phosphate buffers that are usually used in serological tests. Consequently, equilibrium dialysis — a valuable method in hapten immunology — is of limited use. A modified form of the method was used initially as a method of detecting antibodies to sporidesmin. Although benzene moved across the dialysis membrane into the serum and resulted in cloudiness, the titre of a control antibody (anti-bovine albumin), measured by an independent method, remained unchanged. Although the antisera from the rabbits injected with sporidesmin-poly-L-lysine always bound more sporidesmin

than normal serum or any other immune serum, the difference was very small. It is now appreciated that this small difference could be due to the low titre of antibodies produced and not a fault of the method used.

The sporidesmin-rabbit albumin complex was prepared for the indirect haemagglutination test with two factors in mind. First, rabbit albumin was used as it was considered unlikely that rabbits injected with sporidesmin-poly-L-lysine would produce antibodies that would cross-react with rabbit albumin. Secondly, as it was considered that sporidesmin would probably couple to the ε-amino group of the lysine in albumin (there are 60 to 70 lysines in albumin), the amount of sporidesmin coupled was limited to leave some lysine residues to react with the *bis*-diazotized benzidine to enable the sporidesmin-rabbit albumin complex to be coupled to an erythrocyte.

Despite these considerations, the indirect haemagglutination test used did not work in the initial experiments. Subsequently it was found that some factor in the undialysed sporidesmin-rabbit albumin complex prevented the complex from coupling to erythrocytes via the *bis*-diazotized benzidine.

Once a satisfactory indicator particle was produced, it was shown (Table 1) that the antisera from some of the rabbits injected with sporidesmin-poly-L-lysine immunizing antigens would agglutinate the particles. That the antisera acted specifically with sporidesmin was indicated by the following results:

(1) The anti-sporidesmin-poly-L-lysine or anti-sporidesmin-poly - L - lysine-keyhole limpet haemocyanin did not react with the erythrocytes or the rabbit albumin (Table 1).

(2) The reaction between the antisera and the indicator particle was only inhibited by sporidesmin (Table 2) or compounds that are structurally related to the indole end of the sporidesmin molecule (Table 3).

It also appears from the results in Table 3 that the chlorine radical may be important in the antigenic structure of sporidesmin because the 5-chloroindole-2-carboxylic acid and, to a lesser extent, the 5-chlorovanillin inhibited the anti-sporidesmin antibodies. Attempts were made to inhibit the reaction with poly-L-lysine, poly-L-lysine plus sporidesmin and sporidesmin-poly-L-lysine, but all these compounds, even in very low concentrations, caused "non-specific" agglutination of erythrocytes.

Although a number of other "potential" immunizing antigens have been prepared and injected into rabbits and guinea-pigs, only the three lots of antisera from the seven rabbits injected with sporidesmin-poly-L-lysine or sporidesmin-poly-L-lysine-keyhole limpet haemocyanin have so far contained antibodies to sporidesmin.

## SUMMARY

Rabbits were injected with a sporidesmin-poly-L-lysine complex. The complex was prepared by breaking the disulphide bridge in the dioxopiperazine ring of sporidesmin, alkylating the sulphydryl groups and then reacting this preparation with the ε-amino group of poly-L-lysine. Antibodies were detected by a modified equilibrium dialysis method and an indirect haemagglutination test utilizing an erythrocyte-*bis*-diazotized benzidine - rabbit albumin - sporidesmin complex. Indirect haemagglutination inhibition tests showed that the antibodies were inhibited by sporidesmin and indole derivatives structurally related to sporidesmin.

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Maurer, P. H.; Subrahmanyam, D.; Katchalski, E.; Blout, E. R. (1959): Antigenicity of polypeptides (poly alpha amino acids). *J. Immun., 83*: 193-7.

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Appendix b.

*Aust. J. Chem.*, 1975, 28, 2043–50

# Sporidesmins. XIV*
# Modifications to the Opened –S–S– Bridge of Sporidesmin for Coupling to Proteins by Transacylation

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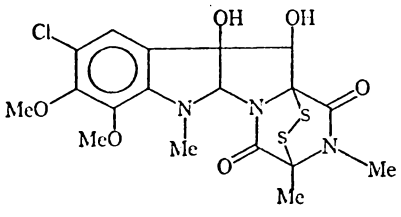
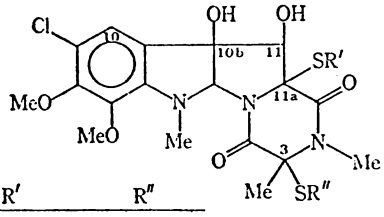
*Abstract*

Alkylation of the opened –S–S– bridge of sporidesmin formed diethyl secosporidesmin-*S*,*S*′-diacetate (3), dimethyl secosporidesmin-*S*,*S*′-diacetate (4), methyl 3-mercaptosecosporidesmin-11a-*S*-acetate (5) and methyl 11a-mercaptosecosporidesmin-3-*S*-acetate (6) which were condensed with ε-amino groups of poly(L-lysine), bovine plasma albumin and rabbit serum albumin. The thiol group in compound (6) could not be demonstrated by conventional tests but its presence was established by its mass spectrum.

Sporidesmin (sdm) (1) has been shown to be the toxin causing facial eczema,[1] an economically important disease in farm animals in New Zealand. For immunological studies on this disease,[2] it is essential to link the toxin to a large molecule. This paper describes the covalent linking of modified sdm to protein.



(1)



| | R′ | R″ |
|---|---|---|
| (2) | Me | Me |
| (3) | $CH_2COOEt$ | $CH_2COOEt$ |
| (4) | $CH_2COOMe$ | $CH_2COOMe$ |
| (5) | $CH_2COOMe$ | H |
| (6) | H | $CH_2COOMe$ |

## Syntheses

Taylor *et al.*[3] opened the –S–S– bridge of sdm in the presence of methyl iodide and formed *S*,*S*′-dimethylsecosporidesmin (sdm-D) (2). The same procedure was adopted but using methyl or ethyl chloroacetate and sodium iodide instead of methyl iodide. When an excess of 20 moles of ester to each mole of sdm was used the main

* Part XIII, *J. Chem. Soc., Perkin Trans. 1*, 1972, 472.

[1] Mortimer, P. H., and Collins, B. S., *Res. Vet. Sci.*, 1968, 9, 136.
[2] Jonas, W. E., and Ronaldson, J. W., *N. Z. Vet. J.*, 1974, 22, 111.
[3] Jamieson, W. D., Rahman, R., and Taylor, A., *J. Chem. Soc., C*, 1969, 1564.

product was the non-crystalline diethyl (or methyl) secosporidesmin-*S*,*S*′-diacetate (3) or (4). But when an excess of only 5 moles of ester to that of sdm was used, as well as the diacetate, crystalline methyl 3-mercaptosecosporidesmin-11a-*S*-acetate (5) and crystalline methyl 11a-mercaptosecosporidesmin-3-*S*-acetate (6) were obtained.

Poly(L-lysine) (pll), bovine plasma albumin (bpa) and rabbit serum albumin (rsa) were selected for complexing to the substituted sdm compounds because they possessed primary ε-amino groups which could be transacylated[4,5] by the esters described above. Solutions of the protein in water and the ester in alcohol were mixed so that the hapten* concentration was somewhat higher than that finally required.

## Experimental

The pll,HBr was supplied by Sigma (mol. wt > 70000 and 139000) and Miles (mol. wt 30000–70000) and the bpa and rsa by Dr Jonas, Wallaceville Animal Research Centre.

Thin-layer chromatography on Eastman Chromagram sheets 6060 silica gel, with fluoroscent indicator, was used to monitor column chromatography. Any ether used was peroxide-free otherwise dark blue products formed resulting in drastic losses.

Melting points (Kofler block) are uncorrected. I.r. spectra were measured in micro potassium bromide pellets (because of the presence of extraneous water, values above 3100 $cm^{-1}$ were neglected) and the u.v. spectra in ethanol. Mass spectra were measured by a Varian MAT CH5 instrument and p.m.r. spectra were recorded on a JEOL C-60HL spectrometer or a Varian T60 with $CDCl_3$ solutions.

When the -S-S- bridge was being opened by sodium borohydride there was always a sulphuretted smell but nevertheless the yield of modified sdm was satisfactory. The reaction mixture must be evaporated carefully, as it turns brown if the water bath reaches 50° and, although the products after chromatography are satisfactory, the yield is reduced.

**Table 1. Quantities of reactants in synthesis from sdm**

Solvent system for t.l.c. ether–benzene 2 : 3

| Expt | sdm (mmol) | $NaBH_4$ (mmol) | NaI (mmol) | $ClCH_2COOR$ R | (mmol) | Products | $R_{sdm}$ | Crude yield (%) |
|---|---|---|---|---|---|---|---|---|
| 1 | 0·2 | 2 | 4 | Et | 4 | (3) | 0·8 | 86 |
| 2 | 0·4 | 1·6 | 0·13 | Me | 2 | (4) | 0·53 | 70 |
| | | | | | | (6) | 0·13 | 20 |
| 3 | 2 | 8 | 0·66 | Me | 11·5 | (1) | 1·0 | 20 |
| | | | | | | (4) | 0·59 | 27 |
| | | | | | | (5) | 0·70 | 27 |
| | | | | | | (6) | 0·22 | 18 |

*Diethyl Secosporidesmin-S,S′-diacetate (3)*

Table 1 sets out the quantities of reagents used in synthesizing (3) by the following method. A cooled (dry ice/acetone) solution of sodium borohydride dissolved in methanol (3·8 ml) was added to a cooled solution of sdm benzene solvate, sodium iodide and ethyl chloroacetate in pyridine (0·55 ml) and methanol (0·8 ml). The mixture was allowed to warm slowly (2½ h) then evaporated under reduced pressure. The residual pyridine solution was acidified and the extract ($CHCl_3$) concentrated and filtered through silica gel G (4·5 g). Those fractions which were

* That portion of an antigenic molecule or antigenic complex, that determines its immunological specificity, but which fails by itself to elicit the formation of a detectable amount of antibody.

[4] Ansell, M. F., and Gigg, R. H., in 'Rodd's Chemistry of Carbon Compounds' (Ed. S. Coffey) Vol. 1c, p. 167 (Elsevier: Amsterdam 1965).

[5] Pinckard, R. N., Hawkins, D., and Farr, R. S., *Arthritis Rheum.*, 1970, 13, 361.

**Table 2. Vibrational spectra for compounds (1)–(6)**

| (1) | (2) | (3) | (4) | (5) | (6) | (1) | (2) | (3) | (4) | (5) | (6) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 3020sh | 1144m | 1152w | 1153w | 1160w | 1160w | |
| 3010m,sh | 3010s, 2990s [A] | 3000s | 3010m,sh | 3010m,sh | | 1114w | 1120sh | | | 1118m | 1115m |
| | | | | | | 1100m | 1100s | 1100m | 1105m | | 1100vw |
| 2960s | | 2960s | 2960s | 2955s | | | | | | | 1085m |
| | 2950s | | | | 2935s | 1065m | 1067w | 1060sh | 1060sh | 1060sh | |
| | 2880w,sh | | | | 2890w,sh | | | | | | 1050t |
| 2870m,sh | | 2870sh | 2870m | | | 1037vs | 1040vs | 1040vs | 1040vs | 1035vvs | 1037vs |
| | | | | 2865m | 2860w,sh | | | | | | 1015w |
| | | | | 2535m | | 1000s | 1009s | 1000s | 1000s | 999s | 995s |
| | | 1725vs | 1730vs | 1732s | 1720s | 978sh | 985m | | | | 985sh |
| 1700vs | | | | | | | 965sh | | 955vw | 960vw | 953w |
| | 1680vs | 1685vs | | 1685vs | 1680vs | 910w | 920w | 920w | 910w | 915vw | |
| 1675vs | | | 1675vs | | | 894m | 897w | 897w | 895w | 895m | 890w |
| | 1655vs | | | 1655vs | 1650vvs | | 897m | 860m | | | 850m |
| | | 1640vs | 1640vs | 1640vs | | | 855m,sh | 850m | 852w | 850m | 850m |
| 1603m | 1602m | 1600w | 1600w | 1600w | 1600vw | 840m | 843w | | | | |
| 1465vs | 1465vs | 1465vs | 1465vs | 1465vs | 1465s | 820w | 816m | 825w | 820w | 820m | |
| | | | | | 1452m | | | | | | 810w |
| | | | 1435m | | 1435w | | 785m | | | | |
| 1412vs | 1410vs | 1412vs | 1412vs | 1410vs | 1410s | 780w | 777m | 780m | 780w | 780m | 780w |
| 1380m | 1385sh | 1383vs | 1383vs | 1380vs | 1380vs | 752m | 753m | 752w | 753w | 753w | 750w |
| | 1375vs | | | | 1370vs | 738m | 738m | | | | |
| 1346vs | 1348s | 1348m | 1348m | 1348s | 1343s | | 730m | 730w | 730vw | 730w | |
| | 1323m | | | | | 720w | | | | | 723w |
| 1310m | 1305m | 1300vs | 1303vs | 1305s | 1310s | | 712w | 710w | 710w | 709vw | 707vw |
| 1260w,sh | 1258m | 1262m | 1260sh | 1258m | 1260w | 690w | 690m | 690w | | | 690w |
| 1246m | 1248m | | | | 1248sh | 680sh | 680m | 680w | 680w | 680w | 677w |
| | 1230w | | 1228w | | | | 650v | | | | |
| 1203s | 1200s | | 1200vs | 1200vs | 1203vs | 638w | 630w | 630vw | 630vw | | |
| | 1190sh | 1185vs | | | 1177m | 615vw | | | | | 623m |

[A] Unresolved peaks.

eluted in ether–benzene (1 : 4) and which on t.l.c. had an $R_{sdm}$ of 0·85 ($Et_2O$–$C_6H_6$ 7 : 13) were collected and yielded a white fluff (111 mg, 86%) (which has not crystallized in more than 7 months) of *diethyl secosporidesmin*-S,S′-*diacetate* (Found: *m/e* 647·1333 ($^{35}Cl$) and 649·1341 ($^{37}Cl$); C, 48·2; H, 5·0; Cl, 6·0; N, 6·5; S, 9·5. $C_{26}H_{34}{}^{35}ClN_3O_{10}S_2$ and $C_{26}H_{34}{}^{37}ClN_3O_{10}S_2$ require 647·1374 and 649·1345 resp.; C, 48·2; H, 5·3; Cl, 5·5; N, 6·5; S, 9·9%). $\lambda_{max}$ 217 nm (log ε 4·62), 252 (4·06), 303 (3·29). $\nu_{max}$ Table 2 (3). P.m.r. δ 1·31 and 1·34, t, *J* 7 Hz, $2CH_2CH_3$; 1·87, s, C3-$CH_3$; 3·07, s, N6-$CH_3$; 3·35, s, N2-$CH_3$; 3·68, s, $2SCH_2COO$; 3·79 and 3·86, s, $2CH_3O$; 4·21 and 4·27, q, *J* 7 Hz, $2OCH_2CH_3$; 4·64, s, H11; 5·31 s, H5a; 7·08, s, H10.

*Dimethyl Secosporidesmin*-S,S′-*diacetate* (*4*)

This was prepared similarly to (3) with modifications as in Table 1 (expt 2). *Dimethyl secosporidesmin*-S,S′-*diacetate* was obtained by chromatography ($Et_2O$–$C_6H_6$ 1 : 4) as a colourless gum (Found: *m/e* 619·1017 and 621·0982. $C_{24}H_{30}{}^{35}ClN_3O_{10}S_2$ and $C_{24}H_{30}{}^{37}ClN_3O_{10}S_2$ require 619·1061 and 621·1032 resp.). $\lambda_{max}$ 216 nm (log ε 4·65), 251 (4·11), 302 (3·34); $\nu_{max}$ Table 2 (4). P.m.r. δ 1·90, s, C3-$CH_3$; 3·09, s, N6-$CH_3$; 3·36, s, N2-$CH_3$; 3·70 and 3·74, s, $2SCH_2COO$; 3·76, 3·80, 3·83 and 3·88, s, $2CH_3O$ and $2COOCH_3$; 4·00, s, OH; 4·65, s, H11; 5·34, s, H5a; 7·12, s, H10.

*Methyl 11a-Mercaptosecosporidesmin-3*-S-*acetate* (*6*)

By further elution of the above silica gel with ether–benzene (2 : 3) another gum (54 mg) was obtained, $R_{sdm}$ 0·13 (Table 1), giving stellate clusters of *methyl 11a-mercaptosecosporidesmin-3*-S-*acetate* from methanol, m.p. 150–156° after repeated crystallizations (Found: *m/e* 547·0828; C, 44·8; H, 4·5; Cl, 6·5; N, 7·6; S, 11·3. $C_{21}H_{26}{}^{35}ClN_3O_8S_2$ requires 547·0850 and $C_{21}H_{26}ClN_3O_8S_2$,-$H_2O$ requires C, 44·6; H, 5·0; Cl, 6·3; N, 7·4; S, 11·3%). $\lambda_{max}$ 222 nm (log ε 4·62), 253 (4·21), 300 (3·65) almost a shoulder; $\nu_{max}$ Table 2 (6). P.m.r. δ 2·02, s, C3-$CH_3$; 3·00, s, N6-$CH_3$; 3·37, s, N2-$CH_3$; 3·75, s, $SCH_2COO$; 3·78 and 3·83, s, $2CH_3O$ and $COOCH_3$; 4·64, s(b), H11; 5·04, s(b), OH; 5·35, s, H5a; 7·07, s, H10. Mass spectrum ($M^+ - H_2S$) 513, 515 (300 and 176% of $M^+$ resp.).

*Methyl 3-Mercaptosecosporidesmin-11a*-S-*acetate* (*5*)

The quantities as set out in Table 1 (expt 3) were allowed to react (1¾ h) in pyridine (2·4 ml) and methanol (24 ml) and chromatographed (11 g silica gel G, $C_6H_6$–$Et_2O$); 90 ml (9 : 1) yielded (1), 100 ml (4 : 1) yielded (4) and (5) as a gum, 180 ml (4 : 1) yielded (4) and finally 400 ml (13 : 7) yielded (6). The gum was rechromatographed (5 g silica gel G) in ether–light petroleum (40–60°) and 80 ml (3 : 2) eluted a trace of (1) and 50 ml (4 : 1) removed a gum (400 mg). This was dissolved in acetone and ether added. Repeated evaporation and addition of ether produced crystals of *methyl 3-mercaptosecosporidesmin-11a*-S-*acetate* (*5*). This commenced melting at 103°, resolidified at 114° and remelted at 151–166° (dec.) (Found: *m/e* 547·0795 ($^{35}Cl$) and 549·0893 ($^{37}Cl$). $C_{21}H_{26}{}^{35}ClN_3O_8S_2$ and $C_{21}H_{26}{}^{37}ClN_3O_8S_2$ require 547·0850 and 549·0822 resp.). $\lambda_{max}$ 221 nm (log ε 4·67), 254 (4·14), 305 (3·64); $\nu_{max}$ Table 2 (5). P.m.r. δ 1·87, d, *J* 2, C3-$CH_3$; 2·14, s, ½ mole acetone of crystallization; 3·04, s, N6-$CH_3$; 3·34, s, N2-$CH_3$; 3·75, s, $SCH_2COO$; 3·78, 3·80 and 3·82, s, $2CH_3O$ and $COOCH_3$; 3·98, q, *J* 2 Hz, C3-SH; 4·65, d, *J* 4 Hz, H11; 4·75, s, OH; 5·30, s, H5a; 7·07, s, H10. Mass spectrum ($M^+ - H_2S$) 513, 515 (51 and 57% of $M^+$ resp.).

*Covalent Bonding to Protein*

The general method was to dissolve the protein in water (*c.* 30 mg/ml) and the ester in alcohol (*c.* 8 mg/ml) using the quantities set out in Table 3 and adjusting the final solution to pH 9. When the solvent was adjusted to 40% alcohol both the protein and ester remained in solution. Some difficulty was experienced with compound (6) (expt 2, Table 3) because of its low solubility; the reaction mixture was therefore continuously shaken but even so only about 1/3 of the ester

reacted. Experiments 4 and 5 (Table 3) were monitored electrophoretically,* and experiment 6 colorimetrically by ninhydrin.[6] Each of the above preparations after removing the alcohol was extracted with either chloroform or ether before evaporating to dryness. Experiment 7 (Table 3) was performed under nitrogen in a pH-Stat at pH 9 and the insoluble product examined by ultimate analysis.

Table 3. Quantities of reagents in complexing to proteins

| Expt | Protein | | | sdm derivatives | | Reaction | Unchanged sdm |
|---|---|---|---|---|---|---|---|
| | Name | Wt (mg) | Mol. wt | No. | Wt (mg) | time (days) | deriv. (mg) |
| 1 | pll,HBr | 22 | 139000 | (3) | 19·3 | 1 | 7·6 |
| 2 | pll,HBr | 144 | 139000 | (6) | 121 | 3 | 74 |
| 3 | pll,HBr | 148 | 30–70000 | (4) | 130 | 2 | 30 |
| 4 | bpa | 300 | | (4) | 26 | 2 | 16 |
| 5 | rsa | 56 | | (5) | 23 | 3 | 4 |
| 6 | pll,HBr | 103 | > 70000 | (4) | 30 | 4 | 6 |
| 7 | pll,HBr | 72 | > 70000 | (3) | 400 | 5 h | [A] |

[A] Insoluble precipitate, 94 mg, obtained.

*Results of Complexing Modified sdm to Protein*

Each complexed protein preparation (except that from expt 7, Table 3) was water-insoluble but soluble in aqueous alcohol. When the cloudy aqueous suspensions were extracted with chloroform a bulky precipitate resulted (denatured) but they were apparently unaffected by extraction with ether. In electrophoresis 1/3 of the product from experiment 4 (Table 3) moved as a single entity and at a slower rate than unsubstituted bpa. But in experiment 5, 60% appeared in the slow moving band after one day, 80% after two days and after three days there was less than 7% remaining in the rsa band. The u.v. spectrum of this aqueous suspension after ether extraction showed bands at 215 and 252 nm, characteristic of compound (5). The u.v. spectrum for preparation 6 (after extraction with ether) showed bands at 217 (shoulder on the end absorption of the pll), 251 and 302 nm equivalent to those for compound (4). The ninhydrin estimation on this preparation showed that 12·5% of the ε-amino groups had been substituted with modified sdm. It is suggested that the insoluble (in water, pH 1–9, alcohol, trifluoroacetic acid and dimethylformamide) material from experiment 7 (Table 3) is a cross-linked product (Found: C, 46·9; H, 6·3; Cl, 3·7; N, 12·2; S, 5·4. If 62% of the amino groups were substituted it would require C, 51·2; H, 6·3; Cl, 3·7; N, 13·6; S, 6·6%).

## Discussion

For compound (5), methyl 3-mercaptosecosporidesmin-11a-*S*-acetate, colour tests for thiols, 5,5′-dithiobis(2-nitrobenzoic acid) (dtnb),[7] and azide–iodine[8] were positive (the Grote test[9] was negative owing to the insolubility of the compound in the reagent). Its i.r. spectrum showed a peak at 2535 $cm^{-1}$, in the S–H stretching region. The p.m.r. spectrum indicated the position of this thiol group because the single proton quartet at $\delta$ 3·98 collapsed to a singlet when the 3-proton doublet at $\delta$ 1·89 was irradiated. The $J$ value of 2 Hz is low for the possible alternative of the

* Gelman Instrument Co., Sepraphore III, tris–barbital–HCl buffer, pH 8·3, Ponceau S staining and scanning.

[6] Slobodian, E., Mechanic, G., and Levy, M., *Science*, 1962, 135, 441.
[7] Beutler, E., Duron, O., and Kelly, B. M., *J. Lab. Clin. Med.*, 1963, 61, 882.
[8] Feigl, F., 'Spot Tests in Organic Analysis' p. 219 (Elsevier: Amsterdam 1966).
[9] Grote, I. W., *J. Biol. Chem.*, 1931, 93, 25.

methyl being split by a methine ($J$ 6–8 Hz) but is consistent with long-range coupling, e.g. $^4J(HSCCH_3)$. Jochims *et al.*[10] have observed long-range couplings of $^4J$ *c.* 2 in several deoxypyranoses. They point out that a coplanar arrangement of the two protons and of the connecting atoms was neccessary and that long-range coupling rapidly diminished at even slight deviations from coplanarity. In a Courtauld model of compound (5) coplanarity obtains when the S–H bond is more or less antiparallel to the $CH_3$–C bond, so hydrogen bonding to the adjacent carbonyl oxygen of the dioxopiperazine ring is structurally impossible; furthermore, this oxygen–hydrogen distance is too great. Consideration of the mass spectral results for the 3-SH compound suggests that, under the conditions of fragmentation, the SH group forms $H_2S$ at the expense of one of the protons of the adjacent methyl group, comparable with the synthesis of anhydrodethiosporidesmin from sdm diacetate.[11]

For the isomeric compound (6) dtnb, azide–iodine on paper and Grote tests were negative for SH (azide–iodine was positive in the test tube but the test is non-specific). When the i.r. of this compound was run normally there was no S–H stretching peak but when about 15 times as much compound was compressed into the micro-KBr pellet a weak absorption showed at 2520 $cm^{-1}$. The p.m.r. spectrum shows no single proton peak which could be assigned to an SH but it is suggested that a different environment has shifted such a peak upfield from *c.* $\delta$ 3·98 (as in the 3-SH compound) so that it is lost under the overlapping multiplet of methyls at $\delta$ 3·9–3·7. Conclusive proof of the presence of an SH group came from this mass spectral data that there is an $M^+ - 34$ peak and that it is about 6 times (in relation to the $M^+$ peak) as intense as that in the mass spectrum of compound (5). The great ease of formation of $H_2S$ in the mass spectrometer could arise from the close proximity of the thiol to the exchangeable hydrogens, hence the structure (6), methyl 11a-mercaptosecosporidesmin-3-*S*-acetate.

That the 11a-SH of compound (6) should be so chemically inert suggests steric hindrance and/or hydrogen bonding. From a Courtauld model the 10b-OH group hinders approach to this SH group so much that prolonged treatment with either iodoacetic ester or methyl iodide at 34° would not alkylate it. The 11a-SH bears a similar relationship to the dioxopiperazine ring as does the 3-SH (see above) so that hydrogen bonding to the adjacent carbonyl oxygen is unlikely, but structurally the hydrogen of the SH group is so close to the oxygen of the 10b-OH group that there could be hydrogen bonding to it.

In each of the p.m.r. spectra there are a set of overlapping peaks, more or less unresolved in the region of $\delta$ 3·9–3·7, which include the two aromatic methoxyls and (for the esters) the ester methoxyls and the methylenes occurring between the sulphur atoms and the carboxyl group. Compound (5) shows a split proton peak ($\delta$ 4·65, $J$ 4 Hz) but there is no sign of the other split peak; the latter is the peak of a hydroxyl and consequently broad and of indefinite position, perhaps hidden under the overlapping methyl peaks.

The aliphatic 3-methyl in sdm (1) is deshielded ($\delta$ 2·03)[12] by the strained condition of the epidithiadioxopiperazine ring system in which the dihedral angle

[10] Jochims, J. C., Otting, W., Seeliger, A., and Taigel, G., *Chem. Ber.*, 1969, **102**, 255.
[11] Hodges, R., Ronaldson, J. W., Shannon, J. S., Taylor, A., and White, E. P., *J. Chem. Soc.*, 1964, 26.
[12] Ronaldson, J. W., Taylor, A., White, E. P., and Abraham, R. J., *J. Chem. Soc.*, 1963, 3172.

C–S–S–C is forced to be 10–20° and the dioxopiperazine ring is constrained by the two sulphurs to be in the boat form.[13] When this strained condition is released as in sdm-D (2) and compounds (3), (4) and (5) the methyl peak shifts upfield to $\delta$ 1·90–1·86. But under the environment as suggested above for compound (6) there is deshielding in this compound comparable with that in sdm.

Perhaps it is this strained condition that accounts for the intensity of the band at 308 nm in the u.v. spectrum of sdm (1) (log $\varepsilon$ 3·96) for it is double that in compounds (2),[3] (3), (4), (5) and (6) where the sulphurs are alkylated.

In the i.r. spectra, the C–H stretching region shows two distinct patterns, that for compounds (1), (4), (5) and (6) (Table 2) and that for compounds (2) and (3). Only the esters (3), (4), (5) and (6) have peaks at 1720–1732 $cm^{-1}$; the latter two at half the intensity of the former two. The C=O stretching region (1600–1700 $cm^{-1}$) is similar in (3), (4) and (5) while (2) and (6) are similar to each other but not to the former; as expected sdm (1) is different from any. In the asymmetrical methyl (1465 $cm^{-1}$) C–H bending and the 'perturbed methylene' (1410 $cm^{-1}$) region, they all have the characteristic pattern, though methylenes are absent in sdm and sdm-D, but the symmetrical vibrations around 1380 $cm^{-1}$ in compounds (2), (3), (4), (5) and (6) show increased intensity for the extra S-methyls, C-methyls and carboxymethyls (as the case may be) over those in sdm (1). The molecule is so large in comparison with the size of the modifications in the sulphur area that it is not surprising that there are so many common i.r. peaks at 1340–1350, 1300–1310, 1240–1262, 1200, 1035–1040 (C–O–C function), 999–1000, 890–897, 780, 750–753 and 680 $cm^{-1}$ (Table 2), each one having a characteristic intensity.

When non-covalent binding (of compounds to proteins) takes place the process is rapid, e.g. tetracycline is bound to ribosomes in 8 min,[14] or with oestradiol added to rabbit uterine cytoplasm, 90% is bound in less than 30 min.[15] Hence the slowness of the reaction between the esters and protein suggests that, although some protein binding of the ester may have taken place the major reaction has been a transacylation resulting in a specific number (for the albumins) of lysine-ε-aminos (*c.* 10 in the case of expt 4 and 40 in expt 5, Table 3) being converted to amides and yielding a single molecular entity of acylated albumin (microheterogeneity[16] apart). This transacylation is comparable with that of lysine residues in serum albumin when the latter is treated *in vivo* and *in vitro* with acetylsalicylic acid.[5] That an immunological response has been elicited with preparation 1 (Table 3) is indicative of covalent bonding to the protein substrate.[2]

The ultimate analysis of the insoluble product from experiment 7 (Table 3), showed a carbon percentage (46·9%) which is lower than that in either of the lysyl (56·4%) or secosporidesmin-*S*,*S*′-bis(acetyl-ε-*N*-lysyl) (50·4%) moieties. But the percentage of chlorine and hydrogen agree with 62% of the amino groups of the pll being substituted with modified sdm, and the values for sulphur and nitrogen are about equally displaced. For values less than 62% substitution the calculated percentage of sulphur decreases and that of nitrogen increases.

[13] Rahman, R., Safe, S., and Taylor, A., *Quart. Rev., Chem. Soc.*, 1970, 24, 208; Safe, S., and Taylor, A., *J. Chem. Soc., C*, 1971, 1189.
[14] Last, J. A., *Biochim. Biophys. Acta*, 1969, 195, 506.
[15] Mester, J., Robertson, D. M., Feherty, P., and Kellie, A. E., *Biochem. J.*, 1970, 120, 831.
[16] Hansson, V., Larsen, J., and Reusch, E., *Steroids*, 1972, 20, 555.

Boyd *et al.*[17] and Slobodian *et al.*[6] used ninhydrin estimation to show that they had acetylated the ε-amino groups of proteins (including bsa). Similarly in this work, ninhydrin estimation shows that the observed electrophoretic changes are due to modified sdm being covalently bound to ε-amino groups of proteins (the percentage of α-amino groups being negligible).

Acknowledgment

Thanks are due to Dr P. T. Holland (Ruakura Ag. Res. Centre) for measurements of mass spectra.

Manuscript received 21 February 1975

[17] Boyd, H., Leach, S. J., and Milligan, B., *Int. J. Peptide Protein Res.*, 1972, 4, 117.

Appendix c.-

*Aust. J. Chem.*, 1976, 29, 2307–14

# Sporidesmins. XV*
# The $^{13}C$ Nuclear Magnetic Resonance Spectra of Sporidesmin and Sporidesmin-D. The Evidence in the Spectra for Strain Imposed by an Epidithio Bridge

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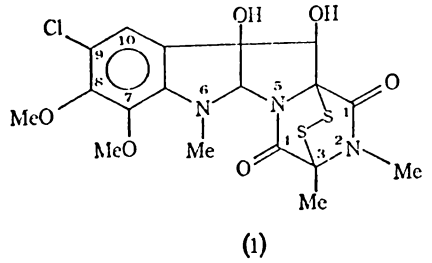
*Abstract*

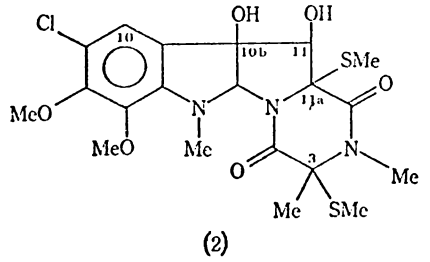
The proton-noise-decoupled $^{13}C$ n.m.r. spectra of sporidesmin and sporidesmin-D are presented. The detailed assignments were worked out from the undecoupled spectrum of sporidesmin-D. The 3-methyl resonance moved downfield (by 7·3 ppm) upon cleavage of the –S–S– bridge in sporidesmin while the peaks of the two quaternary carbons to which the sulphurs are bound moved upfield (by 4·7 ppm). Lactam *N*-methyl and indoline *N*-methyl proton resonance decoupling showed that their p.m.r. assignments should be reversed. The electronegativity of nitrogen, sulphur and carbonyl groups increased the coupling constants of the 3-$CH_3$ by 7 Hz and of the 11-CH by 10 Hz. The carbon of the lactam *N*-methyl was shielded by 14 ppm. There were six three-bond couplings, four HCN$^{13}C$ and two HCO$^{13}C$, evident but no non-aromatic HCC$^{13}C$ couplings.

## Introduction

Ronaldson[1] reported that when the –S–S– bridge across the dioxopiperazine ring in sporidesmin (sdm) (1) is opened and alkylated as in sdm-D (*S,S'*-dimethylsecosporidesmin) (2), the proton resonances of the 3-methyl group move upfield by 0·16 ppm (in $CDCl_3$ or 0·23 in $CD_3COCD_3$), indicating a relaxation of the strained condition in sdm. This paper sets out the proton-noise-decoupled $^{13}C$ n.m.r. spectra of both sdm and sdm-D with the undecoupled spectrum of the latter, and discusses the seemingly anomalous deshieldings which attended the carbon resonances in the above relaxation.




(1)




(2)

* Part XIV, *Aust. J. Chem.*, 1975, 28, 2043.

[1] Ronaldson, J. W., *Aust. J. Chem.*, 1975, 28, 2043.

Previous $^{13}C$ n.m.r. data for 6,7-dimethoxy-1-methylindolines[2] and for some dioxopiperazine rings[3,4] are available but no data are available on 3,3a-dihydroxy-1,2,3,3a,-8,8a-hexahydropyrrolo[2,3-*b*]indole ring systems nor on the dioxoepidithiopiperazine ring, so sdm and sdm-D are useful model compounds where both these latter systems are fused into one molecule. These systems are integral moieties of an important series of mycotoxins, so the data presented will be useful in structural elucidation of more mycotoxins in this series.

## Experimental

The spectra were recorded at the University of Auckland on a JEOL JNM-FX60 FT n.m.r. spectrometer with $CD_3COCD_3$ solutions and tetramethylsilane as internal standard. It was not possible to run the spectra in the usual n.m.r. solvent, $CDCl_3$, because at the high concentration needed for $^{13}C$ n.m.r. sdm crystallized out as the deuterochloroform solvate.

In Fig. 1 the spectral width for the two decoupled spectra was 4 kHz and for the undecoupled spectrum 2·5 kHz, which gives 1·221 Hz between any two data points, hence the coupling constants quoted in the text are multiples of this value and are ± 1·2 Hz or better. To correct for the difference in intensity between the two decoupled spectra, that for sdm-D in Fig. 1 was increased by a factor (1·18), determined by making the average height of the three least-shifted resonances (at 90·3, 119 and 141 ppm) in sdm-D equal to that of the same three in sdm.

Where a resonance shows long-range multiplicity and no one-bond carbon–hydrogen splitting, it is considered, in the first instance, as a singlet and $W_{h/2}$ is the width at half height.

The system used to number the carbon atoms is that adopted by Chemical Abstracts.

### *Sporidesmin Free from Analogues*

Sporidesmin, concentrated according to the scheme of Ronaldson and Fyvie,[5] has a small amount (sometimes as much as 5%) of naturally occurring sdm-D and sdm-E co-crystallized with it. To obtain sdm free from these contaminants, sdm benzene solvate (637 mg) was chromatographed ($CHCl_3$–$C_6H_6$ 1 : 9) on a column (24 mm diam.) of silica gel G (14 g). Those fractions (50 ml) which showed no sdm-D or sdm-E (t.l.c. $CHCl_3$–$C_6H_6$ 13 : 7) were collected (417 mg), and crystallized ($MeOH/H_2O$).

## Results and Discussion

Fig. 1 sets out the proton-noise-decoupled spectra of the two compounds and the undecoupled spectrum of sdm-D. The peaks fall naturally into four groups: (*a*) the methyl carbon peaks from $\delta$ 10 to 65, (*b*) the methine and quaternary carbon peaks from $\delta$ 65 to 100, (*c*) the aromatic peaks from $\delta$ 115 to 155 and, (*d*) the two lactam carbonyl peaks about $\delta$ 165.

The following discussion deals with the carbon-13 shieldings and $^1J$ spin–spin couplings. The discussion of the long-range coupling relevant to sections (*a*) and (*b*), is at the end of section (*b*).

### (*a*) *The Methyl Peaks*

(i) *At 14·5 and 15·6 ppm* (*q*), J *140 Hz* (*sdm-D*).—The two *S*-methyl resonances are more shielded than those in dimethyl sulphide ($\delta$ 19·5[6c]). This shielding is attributed

[2] Yates, P., MacLachlan, F. N., Rae, I. D., Rosenberger, M., Szabo, A. G., Willis, C. R., Cava, M. P., Behforouz, M., Lakshmikantham, M. V., and Zeiger, W., *J. Am. Chem. Soc.*, 1973, 95, 7842.
[3] Ottnad, M., Hartter, P., and Jung, G., *Hoppe-Seyler's Z. Physiol. Chem.*, 1975, 356, 1011.
[4] Deslauriers, R., Grzonka, Z., Schaumburg, K., Shiba, T., and Walter, R., *J. Am. Chem. Soc.*, 1975, 97, 5093.
[5] Ronaldson, J. W., and Fyvie, A. A., *Lab. Pract.*, 1973, 22, 734.
[6] Stothers, J. B., 'Carbon-13 N.M.R. Spectroscopy' (*a*) p. 139; (*b*) pp. 140–1; (*c*) p. 158; (*d*) p. 197; (*e*) p. 337; (*f*) p. 447 (Academic Press: New York 1972).

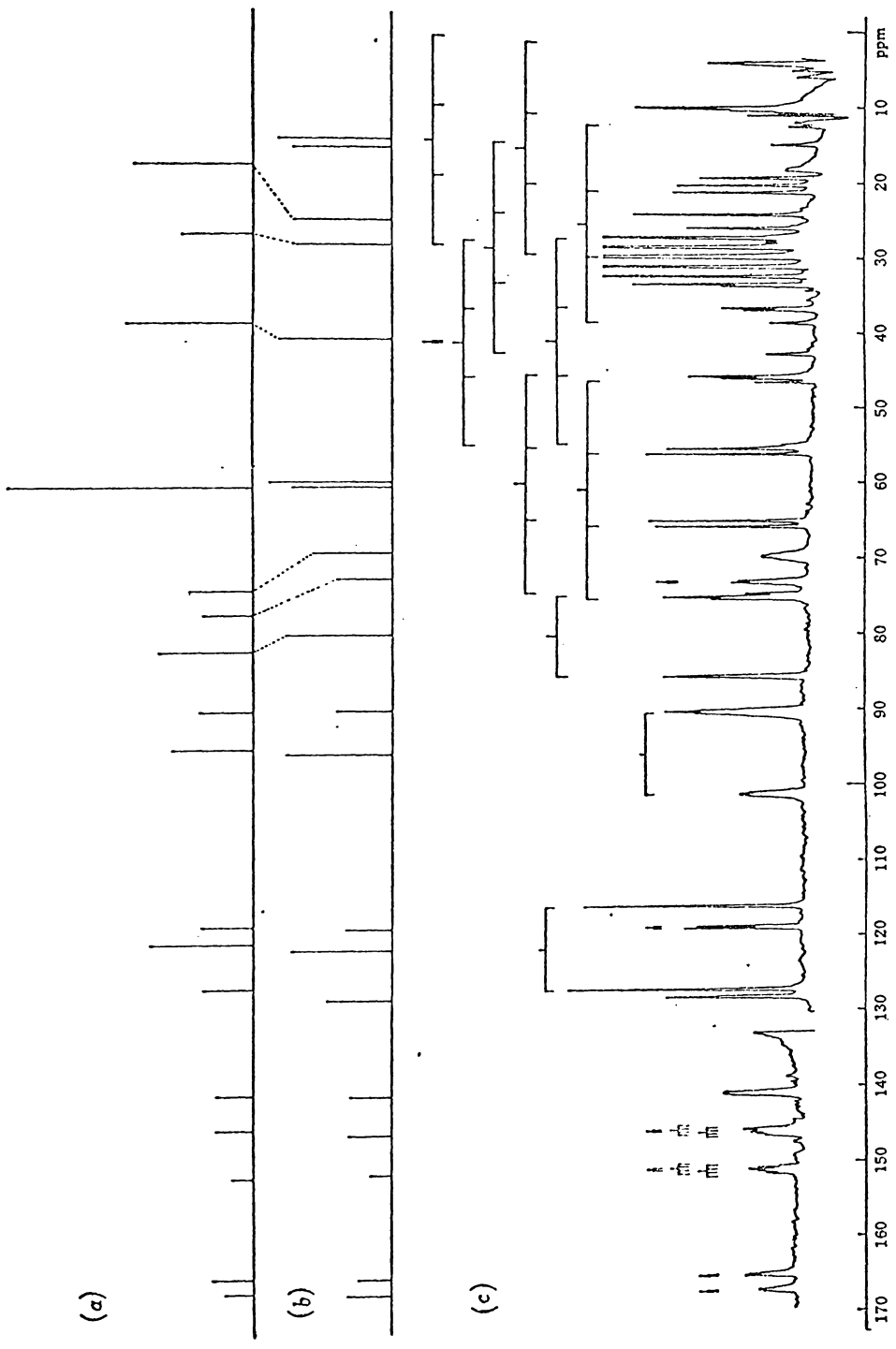



Fig. 1. (*a*) Decoupled spectrum of sdm; (*b*) decoupled spectrum of sdm-D; (*c*) undecoupled spectrum of sdm-D, showing the multiplicities. The peaks in (*c*) between 140 and 155 ppm have been doubled in intensity. The five truncated peaks in the (*c*) spectrum, between 27 and 33 ppm, are those of $CD_3COCD_3$.

to the proximity of the lactam carbonyl groups (see below). The proton peaks of the *S*-methyl are downfield (by 0·5 ppm) from that of the *C*-methyl,[7] while these *S*-methyl carbon peaks are upfield. The coupling constants in sdm-D are comparable with those in dimethyl sulphide (138 Hz).

(ii) *At 18·2 ppm (sdm) and 25·5 ppm (q), J 131·2 Hz (sdm-D).*—In sdm there was no ambiguity in assigning the peak at 18·2 ppm to the *C*-methyl; the 2-methyl peak of 1,2,4-trimethylpiperazine absorbs at 17·8 ppm.[8]

By irradiating the *C*-methyl protons at 1·79 ppm in the proton spectrum of sdm-D (acetone), the carbon peak that was at 18·2 ppm in sdm was observed to be downfield by 7·3 ppm. This deshielding of the carbon stands in contrast to what was observed for the *C*-methyl protons.[1,7] Dorman and Bovey[9] have observed the strong carbon-13 shielding effect of amide carbonyl oxygens on *N*-alkyl carbons and they conclude that a similar effect could occur at the *β*-carbon. In a Dreiding model of sdm-D the methyl carbon (*β* to the nitrogen) is not coplanar with the 1-carbonyl oxygen, hence the deshielding observed by releasing the strain upon the dioxopiperazine ring by opening the –S–S– bridge.

According to Haake *et al.*[10] increased electronegativity of the atom upon which a methyl resides increases the coupling constant of that methyl group. The coupling constant for the four methyl groups on the quaternary carbon of 2,2-dimethylpropane[6e] is 124 Hz but in sdm-D the value is 131 Hz, hence the sulphur, nitrogen and carbonyl adjacent to the C3 in the di(methylthio)dioxopiperazine ring confer electronegativity on this carbon atom, which accounts for the increased one-bond coupling constant of the methyl.

(iii) *At 27·3 ppm (sdm) and 28·8 ppm (q), J 140·4 Hz (sdm-D).*—This methyl resonance of the *N*-methyl lactam in sdm is strongly shielded compared with that of the 1-methyl (42·9 ppm) in 1,2,4-trimethylpiperazine.[8] This shielding is attributed to the strong carbon-13 shielding effect of the lactam carbonyl oxygen.[9] This shielding is less in sdm-D by 1·5 ppm because, when the strain of the –S–S– bridge is relaxed, the coplanarity of the lactam is lessened; in a model where a pyrrolidine ring is fused to a dioxopiperazine ring the lactam group tends to be less coplanar. In sdm-D the coupling constant is comparable with that of the *N*-methyl acetamides (138 Hz).[6e]

(iv) *At 39·2 ppm (sdm) and 41·3 ppm (q), J 137·9 Hz (sdm-D).*—Yates *et al.*[2] studied the carbon-13 spectrum of aspidophytine which contains a 6,7-dimethoxy-1-methylindoline moiety, and assigned the peak at 35·4 ppm to the *N*-methyl carbon. Similarly, Lukacs *et al.*,[11] studying 1-methyl-2,16-dihydrovincadifformines containing a 1-methylindoline moiety, found that the *N*-methyl carbon absorbed at 35–36 ppm. In both sdm and sdm-D the *N*-methyl carbon adsorbs at lower fields and in both compounds the methyl is sterically crowded by a carbonyl of the dioxopiperazine ring, producing a deshielding of 5·9 ppm in sdm-D. The strain of the –S–S– bridge in sdm tends to move the carbonyl oxygen away from the *N*-methyl, hence the absorption at higher field.

[7] Jamieson, W. D., Rahman, R., and Taylor, A., *J. Chem. Soc. C*, 1969, 1564.
[8] Ellis, G., and Jones, R. G., *J. Chem. Soc., Perkin Trans. 2*, 1972, 437.
[9] Dorman, D. E., and Bovey, F. A., *J. Org. Chem.*, 1973, 38, 1719.
[10] Haake, P., Miller, W. B., and Tyssee, D. A., *J. Am. Chem. Soc.*, 1964, 86, 3577.
[11] Lukacs, G., De Bellefon, M., Le Men-Olivier, L., Levy, J., and Le Men, J., *Tetrahedron Lett.*, 1974, 487.

By swamping the protons of the *N*-methyl group (in sdm-D) at $\delta$ 3·01 the carbon resonance at 28·8 ppm remains a singlet while that at 41·3 ppm is split (q). Conversely, when the protons of the *N*-methyl group at $\delta$ 3·3 are irradiated the peak at 41·3 ppm is unsplit while that at 28·8 ppm is split (q). These observations reverse the assignments which have been made for these *N*-methyl protons.[1]

The coupling constant (137·9 Hz) exceeds that reported[6e] for methylamine (133 Hz). The significantly greater value for this constant in sdm-D is attributed to enhancement of the electronegativity of the indoline nitrogen by the benzene ring.

(v) *At 60·9 ppm (sdm) and 60·3 and 61·0 ppm (q), J 144·9 Hz (sdm-D).*—Here there is no ambiguity in either compound, for the intensity of the single peak in sdm about equals the sum of the two in sdm-D and these occur within the range assigned to comparable aromatic methoxy groups in aspidophytine and haplophytine (55·8–61·1 ppm).[2]

Here the coupling constant (144·9 Hz) can be compared with that reported[6e] for methanol (142 Hz), the greater value in the former case may be attributed to the aromatic enhancement of electronegativity of the oxygen atoms.

(*b*) *The Peaks from Quaternary and Methine Carbons, and Long-Range Coupling*

(i) *At 74·5 ppm (sdm) and 69·7 (s) (sdm-D) and at 77·7 ppm (sdm) and 73·1 ppm (s) (sdm-D).*—The intensity ratio of these two peaks in sdm is comparable with the ratio of the two in sdm-D (Fig. 1) and so both peaks in going from sdm to sdm-D have shifted to higher field by a similar amount (4·8 and 4·6 ppm). Hence these two peaks are assigned to those quaternary carbons in the dioxopiperazine ring which are affected by the opening of the –S–S– bridge. Because of the symmetry of the di(methylthio)-dioxopiperazine ring system each of these carbon atoms is deshielded by a nitrogen atom, a sulphur atom and a carbonyl group. One (C 3) carries a methyl group, so it is assigned to the higher field while the other carbon (C 11a) is adjacent to an alcohol function in a pyrrolidinediol ring[6a,12] and is therefore deshielded (comparatively).

(ii) *At 82·5 ppm (sdm) and 80·4 ppm (d) (sdm-D), J 158·7 Hz.*—In a list of secondary alcohols[6b] the shieldings decrease from propan-2-ol (63·7 ppm) to 2,2,4,4-tetramethylpentan-3-ol (85·0 ppm) as the carbinyl group becomes more sterically crowded with $\alpha$-substitution; similarly with the carbinyl group in these sporidesmins. On the one side the $\alpha$-substitution is with an aromatic group (the OH on this carbon is *trans*) and on the other it is with a lactam carbonyl which is more sterically crowding in sdm than in sdm-D. In the 17$\beta$-hydroxy steroids (testosterones) the carbinyl carbon chemical shifts are comparable (81·3 ppm).[6f]

2-, 3- and 4-Carbinyls in pyranoses have coupling constants of 141–8 Hz.[13] Because of the symmetry of the dioxopiperazine ring, the argument (see above), deduced from Haake *et al.*,[10] for the influence of the electronegativity of the C 3 carbon upon the coupling constant of the methyl group, accounts for the coupling constant of the CH of this carbinyl group being 10 Hz larger.

(iii) *At 90·4 ppm (sdm) and 90·3 ppm (s) (sdm-D).*—In the undecoupled spectrum of sdm-D this is a broad peak ($W_{h/2}$ *c.* 12 Hz), showing some multiplicity. It consists of a quaternary carbon singlet partly hidden by the upfield half of the split adjacent

[12] Grover, S. H., and Stothers, J. B., *Can. J. Chem.*, 1974, 52, 870.
[13] Bock, K., and Pedersen, C., *J. Chem. Soc., Perkin Trans. 2*, 1974, 293.

resonance. The only carbon to which this singlet can be attributed is the quaternary carbon C 10b. This hydroxylated carbon is $\beta$ to two nitrogens in a 1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole ring system. These two facts account for the degree of deshielding. Applying the relationship between the shieldings of carbons $\alpha$ to methyl groups and the shieldings of carbons $\alpha$ to hydroxyl groups,[14] to the bridgehead quaternary C 9 of *trans*-9-methyldecalin[15] (of comparable spatial arrangement but not ring size) gives *c.* 73 ppm. Hence the environment of C 10b is associated with special parameters which may appear in aspidophytine.[2] Here C 12 (57·3 ppm) is a quaternary bridgehead carbon to three rings of which only the indoline is strictly comparable with sporidesmins. Though this carbon (in aspidophytine) bears a $CH_2CH_2N$ group instead of a methyl, yet applying the above relationship[14] gives more than 90 ppm for the resonance of this carbon when hydroxylated.

(iv) *At 95·3 ppm (sdm) and 96·0 ppm (d), J 161·1 Hz (sdm-D).*—This resonance is attributed to the one remaining non-aromatic methine which occurs between two nitrogen atoms. For this reason it is at low field and is $^{14}$N-quadrupole-broadened[16a] ($W_{h/2}$ *c.* 11 Hz).

In aspidophytine[2] the corresponding methine carbon, which is adjacent to only one nitrogen and at a bridgehead between an indoline and a cyclohexene, resonates at 72 ppm. This carbon could be shielded, a few ppm, by the adjacent double bond in the cyclohexene ring. That the methine in the sporidesmins is deshielded by more than 20 ppm than that in aspidophytine is accounted for by the second adjacent nitrogen; a nitrogen deshields by *c.* > 20 ppm.[16b]

Though this methine proton is between two nitrogen atoms, only one (*N*-methyl) has lone pair electrons; the other is a lactam nitrogen. The CH coupling constant for methylamine is *c.* 132 Hz[6c] or 137 Hz for diaminomethylenes,[16c] reflecting the nitrogen electronegativity, but in the sporidesmins the proton is rigidly held in approximately the same plane (Dreiding model) as the lone pair of electrons on the indoline nitrogen atom, hence the large value[13] of 161 Hz. The proximity of the equatorial H 1 to the lone pairs on the pyranoid-ring oxygen atom, in $\alpha$ anomers of hexopyranoses,[13] makes the coupling constant 7–8 Hz greater than that of the axial hydrogen atom.

(v) *Long-range spin–spin coupling.*—In the undecoupled spectrum of sdm-D there are three non-aromatic resonances which show long-range splittings, two doublets of 3·7 and one of 2·4 Hz. These occur on the *N*-methyl quartet, the pyrrolo[1,2-*a*]-pyrazine quaternary carbon singlet and a carbonyl (165 ppm) singlet (respectively). Each of these carbons is three bonds removed, through a nitrogen atom, from the same methine proton (5a) which is itself between two nitrogen atoms. There are two opposing factors affecting the value of these long-range coupling constants. One factor is that $^3J_{HCNC}$ values in a planar five-membered ring are about 15·5 Hz, e.g. thiazole.[17] Against this large value is the fact that in a Dreiding model these C–N–C–H groups have a dihedral angle of either 120° or 60° (approx.) which by Conroy's curve[18]

[14] Roberts, J. D., Weigert, F. J., Kroschwitz, J. I., and Reich, H. J., *J. Am. Chem. Soc.*, 1970, 92, 1338.
[15] Dalling, D. K., Grant, D. M., and Paul, E. G., *J. Am. Chem. Soc.*, 1973, 95, 3718.
[16] Levy, G. C., and Nelson, G. L., 'Carbon-13 Nuclear Magnetic Resonance for Organic Chemists' (*a*) p. 31; (*b*) p. 52; (*c*) p. 58 (Wiley–Interscience: New York 1972).
[17] Bojesen, I. N., Høg, J. H., Nielsen, J. T., Petersen, I. B., and Schaumburg, K., *Acta Chem. Scand.*, 1971, 25, 2739.
[18] Conroy, H., in 'Advances in Organic Chemistry: Methods and Results' (Eds R. A. Raphael, E. C. Taylor, and W. Wynberg) Vol. 2, p. 265 (Interscience: New York 1960).

would reduce the coupling constant to about a quarter of the value expected in a planar molecule. A fourth C–N–C–H group occurs in the molecule where the $^{13}C$ is aromatic, but the resonance is broad ($W_{h/2}$ *c.* 7 Hz) and unresolved.

In addition to the *N*-methyl being split by a methine proton there is also the methine carbon resonance being split into an unsymmetrical poorly defined quartet by long-range coupling to the hydrogens of the *N*-methyl group: $^3J_{MeNC}$ *c.* 3·7 Hz.

There are two other carbon nuclei (the CHOH and the aromatic carbon 10a) which bear the same special relationship to this (5a) methine proton but the grouping is H–C–C–C and the splitting does not appear (i.e. it is less than 1 Hz).

(*c*) *The Aromatic Peaks and Their Long-Range Couplings*

Because of additivity it is interesting to note the average shieldings for the six aromatic carbons: in benzene 128·7 ppm,[6d] in five 1-methyl-2,16-dihydrovincadiffor-mines[11] (indolines without aromatic substitution) 127·1 ± 0·5 ppm, in six *Aspidosperma* alkaloids[19] (indolines with 6-methoxy; 5,6-dimethoxy or 6-methoxy-1-methyl substi-tution) 126·6 ± 0·4 ppm, in a series of eight 3-methoxymorphinanes, six codeines and two thebaines[20] (6-methoxy-1,2,3,4-tetrahydronaphthalenes with or without an ether-linked ring at position 7) 129·7 ± 0·4 ppm, in two aspidophytines[2] (6,7-dimethoxy-1-methylindolines) 134 ppm and for sdm 134·1 and sdm-D 134·6 ppm. It appears that a 1,2-dimethoxy-3-*N*-methyl group on an aromatic ring has an overall deshielding effect.

On considering that in sdm-D the sole aromatic proton, adjacent to C–Cl, has a coupling constant of 166·0 Hz, i.e. 8·5 Hz greater than in benzene, and that Tarpley and Goldstein[21] working on *ortho* dichlorobenzenes observed the coupling constant for the methine adjacent to a C–Cl as 166·5 Hz, it is suggested that in the aromatic ring, the chlorine atom has a strong influence on the coupling constant of an adjacent CH group. This influence extends further because (A) there is a well defined long-range coupling (at 119·1 ppm) of 3·7 Hz comparable with the 3·5 Hz recorded by Tarpley and Goldstein for the (Cl)$^{13}$CCH group, and (B) in sdm-D there is an unsplit aromatic quaternary carbon resonance (128·3 ppm) similarly comparable with Tarpley and Goldstein's record of a negligible coupling constant (0·02 Hz) for the $^{13}$CCH(CCl) group. Hence these three peaks (i)–(iii):

(i) *At 118·8 ppm (sdm) and 119·1 ppm (sdm-D).*—These are attributed to the carbon bearing the chlorine atom. This resonance is shielded by 9·6 ppm (from $C_6H_6$); the summation of the shielding (15·5 ppm)[6d] by an *ortho* methoxy group together with the deshielding (6·4 ppm)[6d] effect of the chlorine atom totals 9·1 ppm.

(ii) *At 121·0 ppm (sdm) and 121·8 ppm (d), J 166·0 Hz (sdm-D).*— These are un-ambiguously attributed to the sole aromatic CH group. It is shielded by 7 ppm (from $C_6H_6$) by being *para* to a methoxy group (8·9 ppm[6d]).

(iii) *At 126·9 ppm (sdm) and 128·3 ppm (s) (sdm-D).*—These are assigned to the bridgehead carbon (C 10a). The small shielding (by 1·4 ppm) in sdm arises from reduced steric crowding by the secondary hydroxyl (at C 11) when the dioxopiperazine ring is strained by the –S–S– bridge. In a number of indolines with or without 6-

[19] Wenkert, E., Cochran, D. W., Hagaman, E. W., Schell, F. M., Neuss, N., Katner, A. S., Potier, P., Kan, C., Plat, M., Koch, M., Mehri, H., Poisson, J., Kunesch, N., and Rolland, Y., *J. Am. Chem. Soc.*, 1973, 95, 4990.

[20] Terui, Y., Tori, K., Maeda, S., and Sawa, Y. K., *Tetrahedron Lett.*, 1975, 2853.

[21] Tarpley, A. R., Jr, and Goldstein, J. H., *J. Mol. Spectrosc.*, 1971, 37, 432.

and/or 7-methoxy substitution[2,11,19] their C 3a (corresponding to C 10a) resonates at 130 ppm or more (except in the indoline alkaloid vindoline and derivatives)[19] but when there is substitution in the 5-position (MeO,[19] or Cl) then C 3a is shielded to *c.* 128 ppm.

(iv) *At 140·8 ppm (sdm) and 141·0 ppm (sdm-D).*—Of the three remaining aromatic resonances this one (in sdm-D) shows $^{14}$N quadrupole broadening[16a] ($W_{h/2}$ *c.* 7·3 Hz) and no resolution; therefore it is attributed to the bridgehead carbon (C 6a).

(v) *At 145·8 ppm (sdm) and 146·1 ppm (sdm-D).*—This resonance shows two quartets ($^3J_{COCH}$ 6·1 Hz) separated by ($^4J_{CCCCH}$) 2·4 Hz so it is attributed to the methoxy carbon (C 7).

(vi) *At 151·6 ppm (sdm) and 151·3 ppm (sdm-D).*—Here similarly, this resonance shows two quartets ($^3J_{COCH}$ 7·3 Hz) separated by ($^3J_{CCCH}$) 3·7 Hz so it is applied to the methoxy carbon (C 8).

The two adjacent methoxy groups have a mutual shielding effect (15·5 ppm[6d]) upon their $\alpha$ ring carbon resonances but the *N*-methyl group, adjacent to the 7-OMe, has an additional shielding effect (15·6 ppm[6d]) on C 7, while the CCl group has a negligible (0·2 ppm[6d]) deshielding effect upon the resonance of C 8, hence C 7 is shielded more than C 8.

(*d*) *The Carbonyl Resonances of the Lactams*

(i) *At 164·9 ppm (sdm) and 165·0 ppm (sdm-D).*—Though showing $^{14}$N quadrupole broadening ($W_{h/2}$ *c.* 7·3 Hz), this resonance is clearly split into a doublet ($^3J_{CNCH}$ 2·4 Hz) as discussed above. Hence this resonance is attributed to the 4-CO group in the sporidesmins.

(ii) *At 166·7 ppm (sdm) and 167·1 ppm (sdm-D).*—This peak also shows $^{14}$N quadrupole broadening ($W_{h/2}$ *c.* 8·5 Hz). It is unsymmetrically split (3·6 Hz) and the spectrum shows a suggestion of splitting into a quartet as would be expected from the protons of the methyl group on the adjacent nitrogen. The strain applied to this 1-CO by the –S–S– bridge in sdm does not have a profound effect (0·4 ppm) nor is there much change in orientation in a Dreiding model.

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