Article

Structure Determination of New Algal Toxins using NMR Methods

Alistair L. Wilkins*,[a] and Christopher O. Miles[b,c]

[a]Chemistry Department, Waikato University (e-mail: a.wilkins@waikato.ac.nz)

[b]AgResearch Limited, Ruakura

[c]National Veterinary Institute, Oslo, Norway

Introduction

Shellfish are considered a delicacy by many consumers. In NZ, as in many overseas countries, there is a now thriving shellfish industry servicing both domestic and international markets. Periodically shellfish accumulate harmful levels of a variety of algal toxins, including domoic acid, yessotoxins, pectenotoxins and brevetoxins. When this occurs, regulatory authorities may impose harvesting closures which have a consequential economic impact on both farmers and staff employed to harvest and market shellfish products.

Quantification of algal toxins in sea water or shellfish tissue is dependent on the prior identification and structure determination of the target toxin, and the subsequent availability of high purity reference material. Determination of the structure of an algal toxin, prior to its toxicity being established and its level regulated, requires the consideration of spectral data derived from a variety of techniques. UV spectroscopy can facilitate the identification of chromophoric groups, while IR data assist in identifying the presence of functional groups. However, these techniques rarely define the precise location or the stereochemical orientation of groups such as hydroxyl, acetoxyl, or sulfate.

Recent developments in mass spectrometry (MS), especially improvements in the design and sensitivity of electrospray ion trap and time-of-flight (TOF) spectrometers and the interfacing of liquid chromatography (LC) columns to MS systems, have dramatically improved the ability of scientists to detect and monitor the level of potentially harmful algal toxins in shellfish. LC-MS^n techniques can be used to define both the molecular weight of a target toxin, and characterize its fragmentation pattern. A particularly common approach is to determine the masses of a series of fragment ions generated by the progressive cracking of daughter ions generated by fragmentation of a parent ion. Ion trap and triple quad mass spectrometers are well suited to this approach, and while useful MS^n, for n = 2, 3, 4, *etc*., can be generated and used to define similarities or differences in structures of related algal toxins, mass spectrometry alone is rarely able to define the stereochemistry of chiral carbons, or in many cases the specific site of attachment of a functional group.

Thus, while a comparison of MS^n data (Fig. 1) determined for yessotoxin (YTX) and a new homoyessotoxin analogue **2** (Chart 1) showed that an additional 14 units were associated with ring B–C region of the new analogue **2**, it did not differentiate between, *e.g.* the replacement of a proton by a methyl group, the presence of a seven- rather than a six-membered ring, or replacement of a CH_2 group by a C=O group.[1]

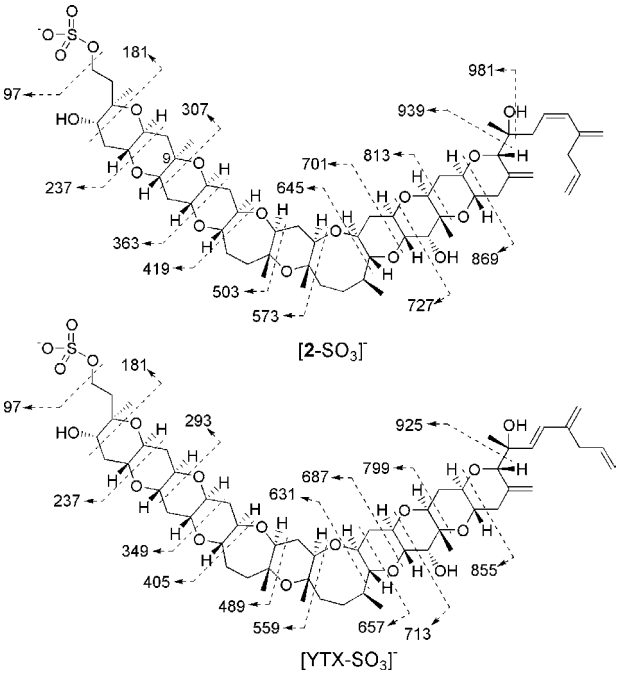

Fig. 1. MS^3 fragmentations observed for YTX and homoyessotoxin analogue **2** - see ref. 1.

Notwithstanding the power of modern MS techniques, NMR spectroscopy remains the method of choice for defining both atom connectivities and the three-dimensional stereochemistry of a molecule. The quantity of material required for the successful structure determination of algal toxins with molecular weights in the range 750–1250 Daltons has progressively decreased from more than 5 mg a decade or two ago, to less than 100 µg, even when using only moderate-field (400–500 MHz) instruments fitted with ambient temperature gradient shielded 5 mm probes. The sensitivity of state-of-the-art 750–900 MHz instruments fitted with cryogenically cooled microprobes is even better.

Raw power (highest possible field combined with the best hardware design) is always advantageous. However, careful attention to parameter setting, especially in two dimensional experiments, particularly the number of increments, repetition rate, number of scans per increment, and choice of transform conditions, can lead to the acquisition of data from a 400–500 MHz system, that approaches or sometimes even exceeds the quality and signal-to-noise of spectral data obtained from a less appropriately set up higher-field instrument, other than for factors related directly to field, *e.g.* spectral resolution. In our experience, there is considerable merit in optimizing the signal-to-noise ratio of spectral data that can be obtained in overnight or weekend experiments using known reference toxins, *e.g.* PTX-2 and yessotoxin, prior to embarking on

the more demanding task of defining the three dimensional structure and deriving a complete assignment of the ^{1}H and ^{13}C NMR resonances of a new algal toxin analogue.

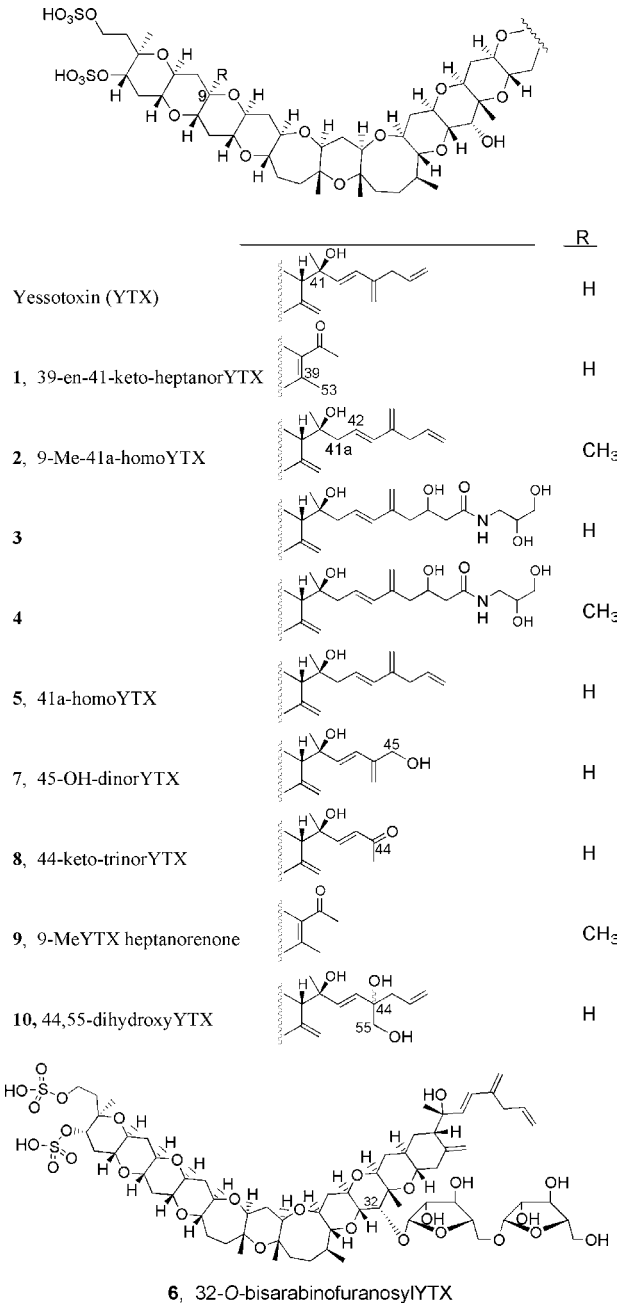

Chart 1. Structures of yessotoxins shown in their sulfonic acid forms.

Prior to a commitment being made in our laboratories in *ca.* 1996 to pursue the isolation and structural determination of sub-milligram quantities of new algal toxins, potentially harmful substances isolated from extracts of NZ shellfish have, with the notable exception of the determination of the absolute stereochemistry of gymnodimine,[2] been predominantly undertaken in Japanese or Canadian laboratories. Working collaboratively with other NZ and overseas scientists, we have reported the isolation and structure elucidation of numerous new yessotoxins, pectenotoxins, and several other algal toxins.[1,3-10] Recently, these studies have been aided greatly by access to a 600 MHz spectrometer equipped with microprobe and cryoprobe hardware, as part of a collaborative research agreement between the Chemistry Department of Oslo University, The National Veterinary Institute (NVI), Oslo, AgResearch at Ruakura (Miles) and a secondment agreement between the NVI and The University of Waikato (Wilkins).

^{1}H NMR Spectra

Despite the wide range of NMR experiments now available, a core group of 6–8 one- and two-dimensional NMR experiments frequently affords sufficient spectral data to define the structure of a new algal toxin. A standard approach is to firstly determine the ^{1}H NMR spectrum of the target compound. Invariably there is a substantial degree of overlap amongst methylene and methine proton signals in algal toxins with molecular weights in the range 800–1300 Daltons, as is apparent in the ^{1}H NMR spectrum (Fig. 2) of 42,43,44,45,46,47,55-heptanor-39-en-41-oxoyessotoxin (**1**) which was recently isolated from extracts of cultured *Protoceratium reticulatum*.[3] When solvent (or HOD) lines are excessive relative to target compound signals, they can be suppressed using single- or double-presaturation techniques that saturate the more slowly relaxing (long T_1) solvent (or HOD) lines, while not attenuating more rapidly relaxing target compound signals. Alternatively, this can be achieved using the WATERGATE technique. Modern spectrometer software allows these and other advanced NMR experiments to be set up and recalled by less experienced users as essentially *black box* experiments, other than for frequency setting and, if required, saturation power level adjustment.

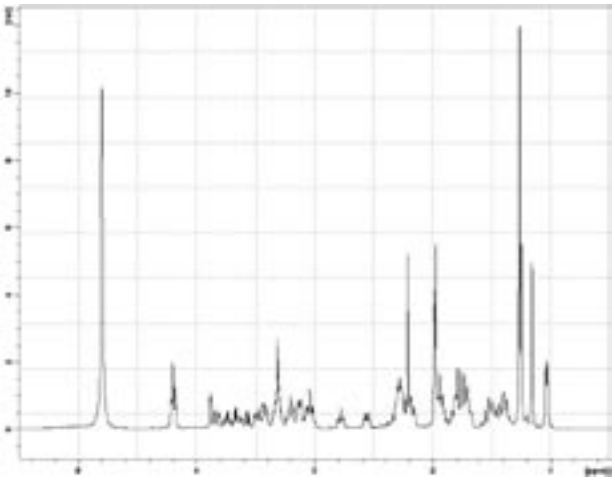

Fig. 2. ^{1}H NMR spectrum of 42,43,44,45,46,47,55-heptanor-39-ene-41-oxoyessotoxin (**1**).

COSY and TOCSY Spectra

Thereafter, proton chemical shifts can be correlated in two dimensional correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) experiments, or variants of these experiments, including double quantum filtered COSY, long range COSY, or COSY with solvent line presaturation. COSY data typically identify short range connectivities (^{2}J and ^{3}J couplings) whereas the spin-locked TOCSY experiment, also known as the HOHAHA experiment (Homonuclear Hartmann-Hahn Spectroscopy), can be optimized to detect short, medium, or long range correlations. Generally, in TOCSY experiments, a spin locked correlation (or mixing) time of the

order 15 msec affords a COSY-like spectrum whereas a mixing time of 150 msec enables connectivities for protons within 5–6 bonds of each other to be defined in vertical or horizontal columns appearing in the two dimensional spectra. The TOCSY spectrum of **1** is shown in Fig. 3 and detailed analyses of the multitude of correlations observed in the spectra of such medium to high molecular weight algal toxins is a time consuming, but rewarding task.

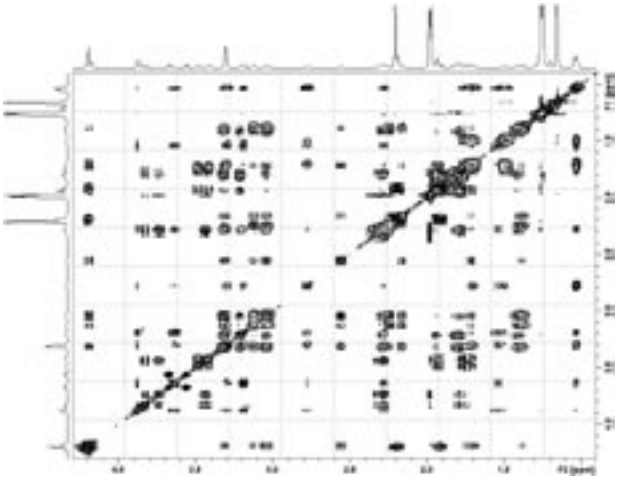

Fig. 3. TOCSY spectrum of **1**.

1D SELTOCSY

A powerful variant of the TOCSY experiment is the 1D-SELTOCSY (selective excitation TOCSY) experiment, a one dimensional selective excitation version of the more time-consuming two-dimensional experiment. Where knowledge of the correlations arising from only a limited number of protons is required, as is often the case for a substance known to differ from a known compound only in a single region of the structure, it may well be the method of choice. Advantages of the 1D-SELTOCSY experiment, compared to a conventional TOCSY experiment, include greater proton signal resolution (thereby allowing for coupling constant determination and the interpretation of vicinal couplings in six-membered rings using the Karplus equation), excitation only of a narrow region of the original ^{1}H NMR (typically 5–30 Hz), and a reduction in spectrometer time where only limited spectral data are required to define the location and stereochemical disposition of a particular group. Like TOCSY experiments, 1D-SELTOCSY experiments can be optimized for the detection of either short range (COSY-like) or long range correlations.

Selective excitation effectively eliminates solvent and impurity signals lines from these spectra. Despite ones best efforts at purification, detectable levels of phthalate and various tap grease components, surfactants and solvent stabilizers, are periodically encountered in precious samples that one is reluctant to subject to further cycles of clean-up and purification (particularly for samples that show a tendency to degrade during purification) pending initial establishment of the compound's structure. Provided target signals are not concealed by impurity signals, selective excitation techniques eliminate these impurity signals from consideration when setting up acquisition parameters such as receiver gain, and more importantly from plots of the resulting spectra.

Proton-Carbon Correlations

HMBC and HSQC Spectra

Having established short- and long-range proton connectivities, ^{1}J and longer range ^{2}J and ^{3}J proton–carbon correlations can be defined in gradient-selected heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond connectivity (HMBC) experiments, respectively, or variants of these experiments. Using gradient-selected inverse ^{1}H detection techniques, HMBC and HSQC spectra can now be obtained more readily than is the case for a conventional ^{1}H-decoupled ^{13}C NMR spectrum. Figs. 4 and 5 show the HSQC and HMBC spectra of **1**.

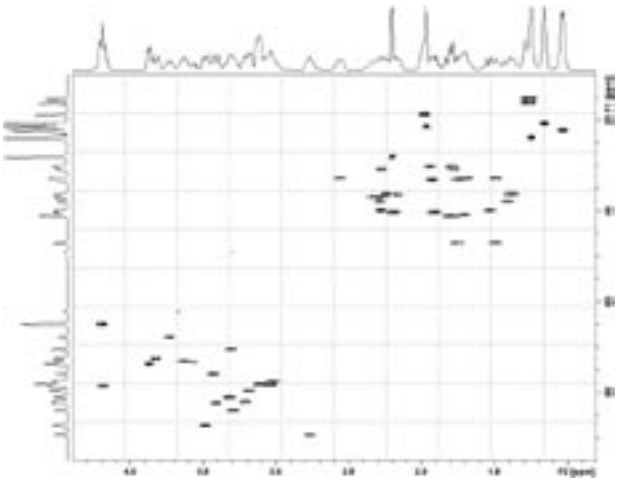

Fig. 4. HSQC spectrum of **1**.

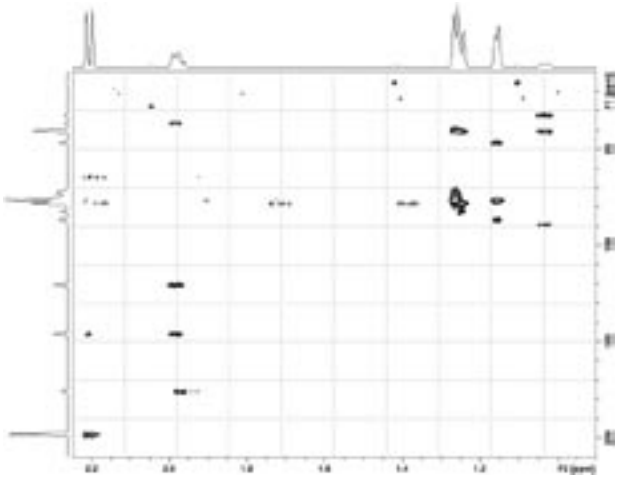

Fig. 5. Partial HMBC spectrum (CH_3 region) of **1**.

The resolution in the ^{1}H NMR axis of slices taken from the phase-sensitive HSQC spectrum of **1** was such that axial or equatorial orientation of methylene and methine protons in 6-membered ring systems could be defined. Typically, large ^{3}J axial-axial and ^{2}J vicinal couplings are resolved, whereas smaller ^{3}J axial-equatorial and ^{3}J equatorial-equatorial couplings are not resolved. Thus, the resonances of the axially and equatorially oriented $C(37)H_2$-protons can be readily distinguished (Fig. 6).

While ^{1}H-detected HSQC and HMBC experiments indirectly identify ^{13}C shifts with a precision of order 0.5–0.8 ppm, it is preferable to determine ^{13}C shifts directly with a precision of 0.1 ppm, and to define carbon types (C, CH, CH_2 or CH_3) using the distortionless enhancement by polarization transfer using a 135 degree selection pulse

(DEPT135) sequence. Frequently the acquisition of a ^{13}C spectrum requires 2–3 times more spectrometer time than is required to obtain good quality ^{1}H-detected HSQC and HMBC spectra.

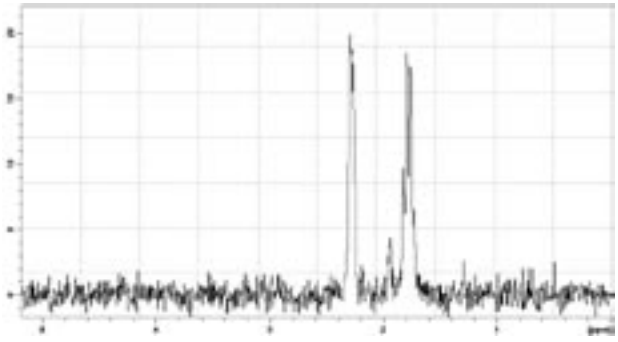

Fig. 6. HSQC slice showing the -C(37)H_2- methylene proton signals of **1**.

NOESY or ROESY Spectra

Careful consideration of a combination of ^{1}H, ^{13}C, DEPT135, COSY, TOCSY, HSQC and HMBC spectral data almost invariably enables a complete assignment of the ^{1}H and ^{13}C NMR assignments of new pectenotoxins, yessotoxins or other algal toxins to be derived, especially when considered alongside NMR data for known reference compounds. These data do not, however, allow for definition of the stereochemical relationship between specific functional groups. Data from nuclear Overhauser effect spectroscopy (NOESY) or the rotating frame variant ROESY, or from 1D-SELNOESY or 1D-SELROESY variants of these experiments, or the classic NOE-DIFFERENCE technique, can be utilized for this purpose. The spatial disposition of protons in ketone **1** was defined by correlations observed in its ROESY spectrum (Fig. 7).

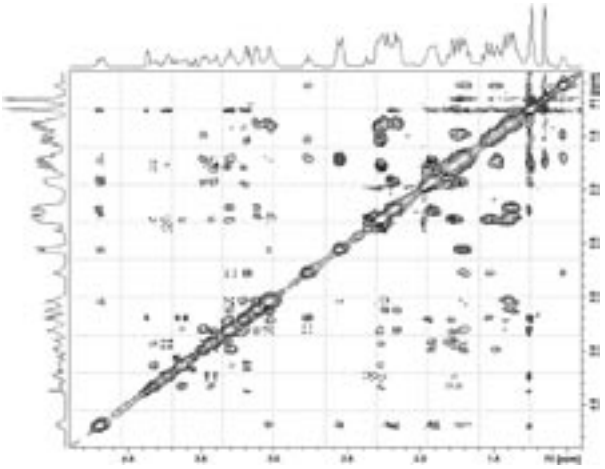

Fig. 7. ROESY spectrum of **1**.

We have used the NMR techniques described here to identify a number of new algal toxins, including a series of new yessotoxins, pectenotoxins and some gymnodimine and okadaic acid analogues. Some of the recently identified[1,3,4] yessotoxins are depicted by **1-10** (Chart 1) and we have also reported evidence for numerous other minor yessotoxins.[5] We anticipate that LC-NMR-MS^3 data will facilitate the identification of some of the minor components when considered alongside NMR spectral data for known YTX analogues. Arrangements are in place for LC-NMR investigations of the multitude of minor yessotoxins to be conducted collaboratively with our Norwegian colleagues.

Recently identified pectenotoxins include[6] **11-14** depicted in Chart 2 and a series of predominantly 37-*O*-acyl fatty acid esters of PTX-2 seco acid **15**.[7] Spectral data for PTX-11 **(11)** were determined collaboratively with Canadian workers while the structures of PTX-12 **(12)**, and the location of the acyl group in a series of PTX-2 seco acids esters, were determined collaboratively with Norwegian associates. Complete assignments of the ^{1}H and ^{13}C NMR assignments of PTX-2 SA **(15)** and 7-*epi*-PTX-2 SA **(16)** have also been achieved,[8] as has the structure elucidation of a number of other algal toxins including an okadaic acid *cis*-diol ester **(17)**[9] and gymnodimines B **(18)** and C **(19)**.[10]

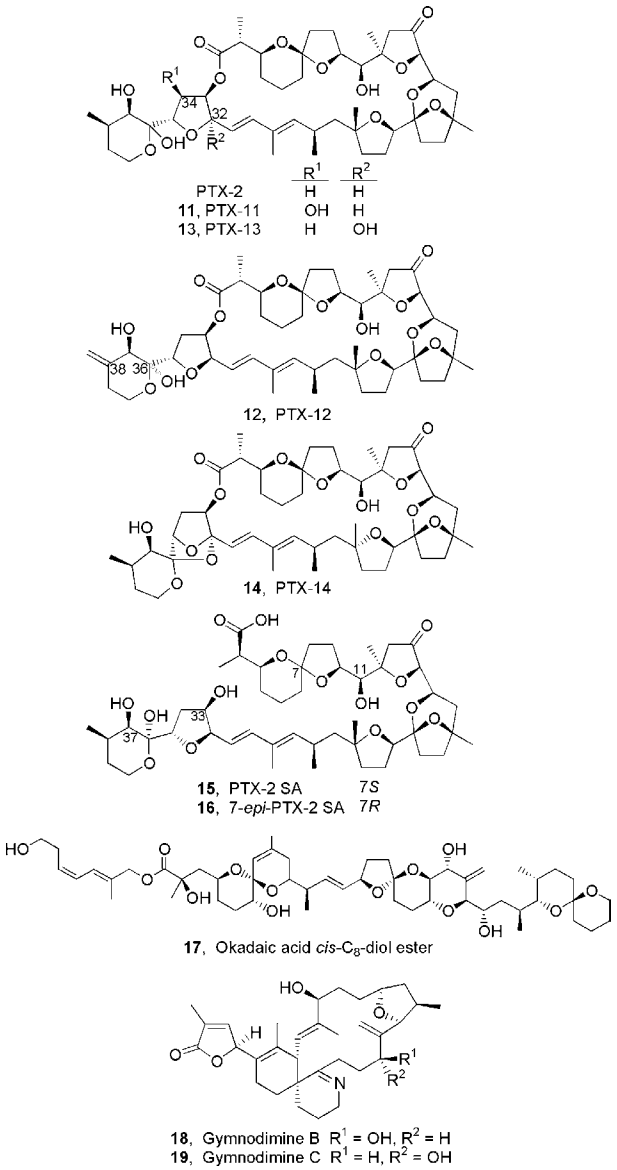

Chart 2. Structures of the pectenotoxins, okadaic acid derivatives, and gymnodimines.

Conclusion

The structure determination of sub-milligram quantities of newly isolation algal toxins using one- and two-dimensional NMR techniques, notably COSY, TOCSY, NOESY, ROESY, HSQC, HMBC, 1D-SELTOCSY, SELNOESY and SELROESY experiments, is now well es-

tablished. NMR analyses, in combination with MS^n data, have proved to be the cornerstone techniques for defining the structures of new algal toxins. It is likely that this will continue to be the case for the foreseeable future, since no other spectroscopic technique with the exception of X-ray crystallography (suitable crystals are rarely available) offers the same degree of insight into structural and stereochemical issues as does NMR spectroscopy.

Acknowledgements

We thank our NZ collaborators at AgResearch Ruakura (Hamilton) and the Cawthron (Nelson), those at the National Veterinary Institute and the University in Oslo, and the Institute for Marine Biosciences, NRC, (Halifax, Canada), the Marine Institute (Galway, Ireland), and the Tohoku National Fisheries Research Institute (Japan). We also thank the NZFRS&T (contract number CAWX0301), the IIOF (contract number C10X0406), the Norwegian Research Council (grant 139593/140), and the BIOTOX project (part funded by the EC (6^{th} Framework Programme contract 514074) for financial support.

References

1. Miles, C.O.; Wilkins, A.L.; Jensen, D.J.; Cooney, J.M.; Quilliam, M.A.; Aasen, J.; MacKenzie, A.L. *Chem. Res. Toxicol.* **2004**, *17*, 1414–1422.

2. Stewart, M.; Blunt, J.W.; Munro, M.H.G.; Robinson, W.T.; Hannah, D.J. *Tetrahedron Lett*. **1997**, *38*, 4889–4890.

3. Miles, C.O.; Wilkins, A.L.; Hawkes, A.D.; Selwood, A.; Jensen, D.J.; Aasen, J.; Munday, R.; Samdal, I.A.; Briggs, L.R.; Beuzenberg, V.; MacKenzie, A.L. *Toxicon* **2004**, *44*, 325–336.

4. Finch, S.C.; Wilkins, A.L.; Hawkes, A.D.; Jensen, D.J.; MacKenzie, A.L.; Beuzenberg, V.; Quilliam, M.A.; Olseng, C.D.; Samdal, I.A.; Aasen, J.; Selwood, A.I.; Cooney, J.M.; Sandvik, M.; Miles, C.O. *Toxicon* **2005**, *46*, 160–170. Miles, C.O.; Wilkins, A.L.; Hawkes, A.D.; Selwood, A.I.; Jensen, D.J.; Cooney, J.M.; Beuzenberg, V.; MacKenzie, A.L. *Toxicon* **2006**, *47*, 229–240. Miles, C.O.; Wilkins, A.L.; Hawkes, A.D.; Selwood, A.I.; Jensen, D.J.; Munday, R.; Cooney, J.M.; Beuzenberg, V. *Toxicon* **2005**, *45*, 61–71. Miles, C.O.; Wilkins, A.L.; Selwood, A.I.; Hawkes, A.D.; Jensen, D.J.; Cooney, J.M.; Beuzenberg, V.; MacKenzie, A.L. *Toxicon* **2006**, *47*, 510–516.

5. Miles, C.O.; Samdal, I.A.; Aasen, J.A.G.; Jensen, D.J.; Quilliam, M.A.; Petersen, D.; Briggs, L.M.; Wilkins, A.L.; Rise, F.; Cooney, J.M.; MacKenzie, A.L. *Harmful Algae* **2005**, *4*, 1075–1091.

6. Miles, C.O.; Wilkins, A.L.; Hawkes, A.D.; Jensen, D.J.; Selwood, A.I.; Beuzenberg, V.; Mackenzie, A.L.; Cooney, J.M.; Holland, P.T. *Toxicon* **2006**, *47*, in press. Miles, C.O.; Wilkins, A.L.; Samdal, I.A.; Sandvik, M.; Petersen, D.; Quilliam, M.A.; Naustvoll, L.J.; Rundberget, T.; Torgersen, T.; Hovgaard, P.; Jensen, D.J.; Cooney, J.M. *Chem. Res. Toxicol*. **2004**, *17*, 1423–1433. Suzuki, T.; Walter, J.A.; LeBlanc, P.; MacKinnon, S.; Miles, C.O.; Wilkins, A.L.; Munday, R.; Beuzenberg, V.; MacKenzie, A.L.; Jensen, D.J.; Cooney, J.M.; Quilliam, M.A. *Chem. Res. Toxicol*. **2006**, *19*, 310–318.

7. Wilkins, A.L.; Rehmann, N.; Torgersen, T.; Rundberget, T.; Keogh, M.; Petersen, D.; Hess, P.; Rise, F.; Miles, C.O. *J. Ag. Food Chem*. **2006**, *54*, in press.

8. Miles, C.O.; Wilkins, A.L.; Munday, R.; Dines, M.H.; Hawkes, A.D.; Briggs, L.R.; Sandvik, M.; Jensen, D.J.; Cooney, J.M.; Holland, P.T.; Quilliam, M.A.; MacKenzie, A.L.; Beuzenberg, V.; Towers, N.R. *Toxicon* **2004**, *43*, 1–9. Miles, C.O.; Wilkins, A.L.; Munday, J.S.; Munday, R.; Hawkes, A.D.; Jensen, D.J.; Cooney, J.M.; Beuzenberg, V. *J. Ag. Food Chem*. **2006**, *54*, 1530–1534.

9. Miles, C.O.; Wilkins, A.L.; Hawkes, A.D.; Jensen, D.J.; Cooney, J.M.; Larsen, K.; Petersen, D.; Rise, F.; Beuzenberg, V.; MacKenzie, A.L. *Toxicon* **2006**, *47*, in press.

10. Miles, C.O.; Wilkins, A.L.; Stirling, D.J.; MacKenzie, A.L. *J. Ag. Food Chem*. **2000**, *48*, 1373–1376, 2003, 51, 4838–4840.