Spiroimine shellfish poisoning (SSP) and the spirolide family of shellfish toxins: Isolation, structure, biological activity and synthesis

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Spirolides are metabolites of the dinoflagellates *Alexandrium ostenfeldii* and *Alexandrium peruvianum.* Spirolides (A–D) were isolated from the digestive glands of contaminated mussels (*Mytilus edulis*), scallops (*Placopecten magellanicus*) and toxic plankton from the east coast of Nova Scotia in Canada. Fourteen members of the spirolide family of marine biotoxins have since been identified from around the world. The relative stereochemistry of these compounds was established *via* molecular modelling and NMR studies. Related marine toxins containing a spiroimine unit have been classified as fast-acting toxins and the spirolides also target muscarinic and nicotinic acetylcholine receptors and are weak activators of L-type transmembrane Ca^{2+} channels. The spirolides are macrocycles that are comprised of two parts: a *bis*-spiroacetal moiety and an unusual 6,7-bicyclic spiroimine.

1 Introduction: microalgae, harmful algal blooms and shellfish poisoning

1.1 Impact of microalgae on the environment

Microalgae play an important role in the ecological balance of marine ecosystems. Oceans contain over 5,000 known species of planktonic microscopic algae. The phytoplankton form the base of the marine food chain (crucial food for filter-feeding bivalve shellfish, such as oysters, mussels, scallops and clams) and produce ~50% of the oxygen we inhale by photosynthesis. Phytoplankton are divided into eight main phylogenetic groups: Chlorophyta (green algae), Chrysophyta (golden algae), Phaeophyta (brown algae), Dinophyta (dinoflagellata), Euglenophyta (most characteristic genus is *Euglena*), Rhodophyta (red algae), Diatoms (yellow or brown unicellular organisms) and Cyanobacteria (blue-green algae).[1]

An increase in the phytoplankton population may become a cause of death in other organisms. A high concentration of microalgae can lead to visible patches near the surface of the water commonly called "red or brown tides" due to the presence of pigments in the phytoplankton. In the case of toxic microalgae, this phenomenon is specifically called a "Harmful Algal Bloom" (HAB) that is often responsible for the death of fish, birds and mammals. The associated toxins may also accumulate in tissues and organs of shellfish without causing any apparent toxicity to the molluscs, but human consumption of a sufficient amount of contaminated seafood can result in mild to severe illness and in rare cases, death. For example, mussels filter approximately 20 litres of water per hour, and during an algal bloom the water contains several million algae per litre, thus accumulation of toxins in the shellfish can reach potentially high toxic levels.

Among 500 known species, about 300 species of phytoplankton are known to produce dense visible patches near the surface of the ocean. Only 85 species have the capacity to produce potent toxins that can affect the health of humans and/or marine fauna.[2,3] Flagellated species of microalgae account for 90% of harmful algal blooms and in this group approximately 75% are dinoflagellates.

1.2 Harmful algal blooms

Harmful algal blooms are a natural phenomena that have been recorded throughout history, with the first written reference in

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the Bible (1000 BC) "…all the waters that were in the river were turned to blood. And the fish that were in the river died; and the river stank, and the Egyptian could not drink the water of the river" (Exodus 7 : 20–21). However, over the past few decades, such ecological events appear to have increased in frequency, intensity and geographic distribution, causing significant economic and public health impacts. HABs are an international health problem being responsible for more than 60,000 intoxications per year with an overall mortality of approximately 1.5%.[1] The apparent global increase in the occurrence of HABs has been accompanied by enhanced efforts to identify factors controlling the population and toxic dynamics of HABs.

Various explanations have been proposed for the apparent increase in the incidence of HABs. Rigorous monitoring of these events and the development of new techniques to identify harmful toxins has led to an increase in the reporting of HABs. Secondly, human activities including shellfish and fish farming have both directly and indirectly contributed to the increased incidence of HABs and their associated paralytic, diarrhetic and amnesic shellfish poisoning. Worldwide transfer of shellfish and ship ballast can also directly act as a medium to transfer toxin producing species by moving non-indigenous species from one side of the world to the other. An increase in coastal HABs is currently considered as one of the first biological signs of global warming of ocean waters. Eutrophication due to an increase of the human population near coastal waters results in pollution (loading of untreated domestic, agricultural and industrial waste) leading to climate variations on a local and global scale.[4] The real consequence of the increase in HABs is not yet determined but the effects on public health and the resultant economic impacts are now showing signs of a truly global epidemic. Consequently, global climate change studies need to consider that algal bloom events and increased population around coastal waters could become a major problem for human and marine health and for shellfish production.

1.3 Shellfish poisoning

Most harmful algal toxins are heat stable meaning that cooking contaminated seafood does not remove their toxicity. Six seafood poisoning syndromes have been identified[5,6] (usually the poisoning syndrome is named based on the related human symptoms after ingestion of the toxic seafood) (Fig. 1):

—*Paralytic shellfish poisoning (PSP)*[7] occurs due to the consumption of shellfish contaminated by heterocyclic guanidine toxins as represented by the main compound saxitoxin (**1**). Saxitoxin (**1**) was isolated in butter clams (*Saxidomus giganteus*) in 1957 by Schantz.[8] These toxins cause almost 2,000 cases of human poisonings per year worldwide with a 15% mortality rate. PSP symptoms are purely neurological. In mild cases, PSP induces tingling, numbness, and burning of the perioral region, ataxia, giddiness, drowsiness, fever, rash, and staggering. In extreme cases, this poisoning leads to muscular paralysis and pronounced respiratory difficulty. PSP is also implicated in the deaths of birds and humpback whales. In July 2002, the death of two fishermen was reported after consumption of ribbed mussels in the Patagonia Chilean fjords. The post mortem analysis showed PSP toxins including saxitoxin (**1**) and goniotoxin in the tissues and blood of the victims.[9]

—*Neurotoxic shellfish poisoning (NSP)* results after the consumption of molluscs contaminated by the cyclic polyether brevetoxins A (**2**) or B (**3**) and their derivatives. The primary producer is the dinoflagellate *Gymnodinium breve* which often forms red tides along the Florida coast, the Gulf of Mexico, the Caribbean Sea and the coastal waters of New Zealand.[10,11] The principal characteristic symptoms of NSP are gastrointestinal (diarrhoea accompanied by progressive paresthesias) and neurological (vertigo, general muscle weakness, headache). However, no fatal human intoxication has been reported; the toxicological importance of the brevetoxins is largely due to massive fish death.

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Margaret A. Brimble

Margaret Brimble completed an MSc (1st Class Hons) in Chemistry at The University of Auckland, New Zealand. She was awarded a UK Commonwealth Scholarship to undertake her PhD studies at Southampton University. She currently holds the Chair of Organic and Medicinal Chemistry at the University of Auckland where her research program focuses on the synthesis of spiroacetal-containing natural products, pyranonaphthoquinone antibiotics, alkaloids and peptidomimetics for the treatment of neurodegenerative disorders, the synthesis of fish antifreeze neoglycopeptides and neoglycopeptides as vaccine components. She was awarded the 2010 Royal Society of Chemistry Natural Products Chemistry Award.

—*Ciguatera fish poisoning (CFP)* results after the consumption of coral reef fish contaminated by the ladder-like polyether toxin ciguatoxin (**4**) and analogues. These toxins are primarily produced by the dinoflagellate *Gambierdiscus toxicus*.[12] CFP is estimated to affect over 50,000 people annually and on rare occasions can be fatal by ingestion of coral reef fish that have become toxic due to accumulation of such compounds. CFP produces gastrointestinal, neurological, and cardiovascular symptoms. Generally, diarrhoea, vomiting, and abdominal pain occur initially, followed by neurological dysfunction including reversal of temperature sensation (cold things feel hot), muscular aches, dizziness, anxiety, sweating, and low heart rate and blood pressure. This poisoning is well known from the coral reef areas of the Caribbean, Australia and especially French Polynesia.

—*Diarrhetic shellfish poisoning (DSP)* is a milder seafood intoxication attributed to a class of acidic polyether toxins. The main causative agent is okadaic acid (**5**) that was first isolated from the marine sponges *Halichondria okadai* and

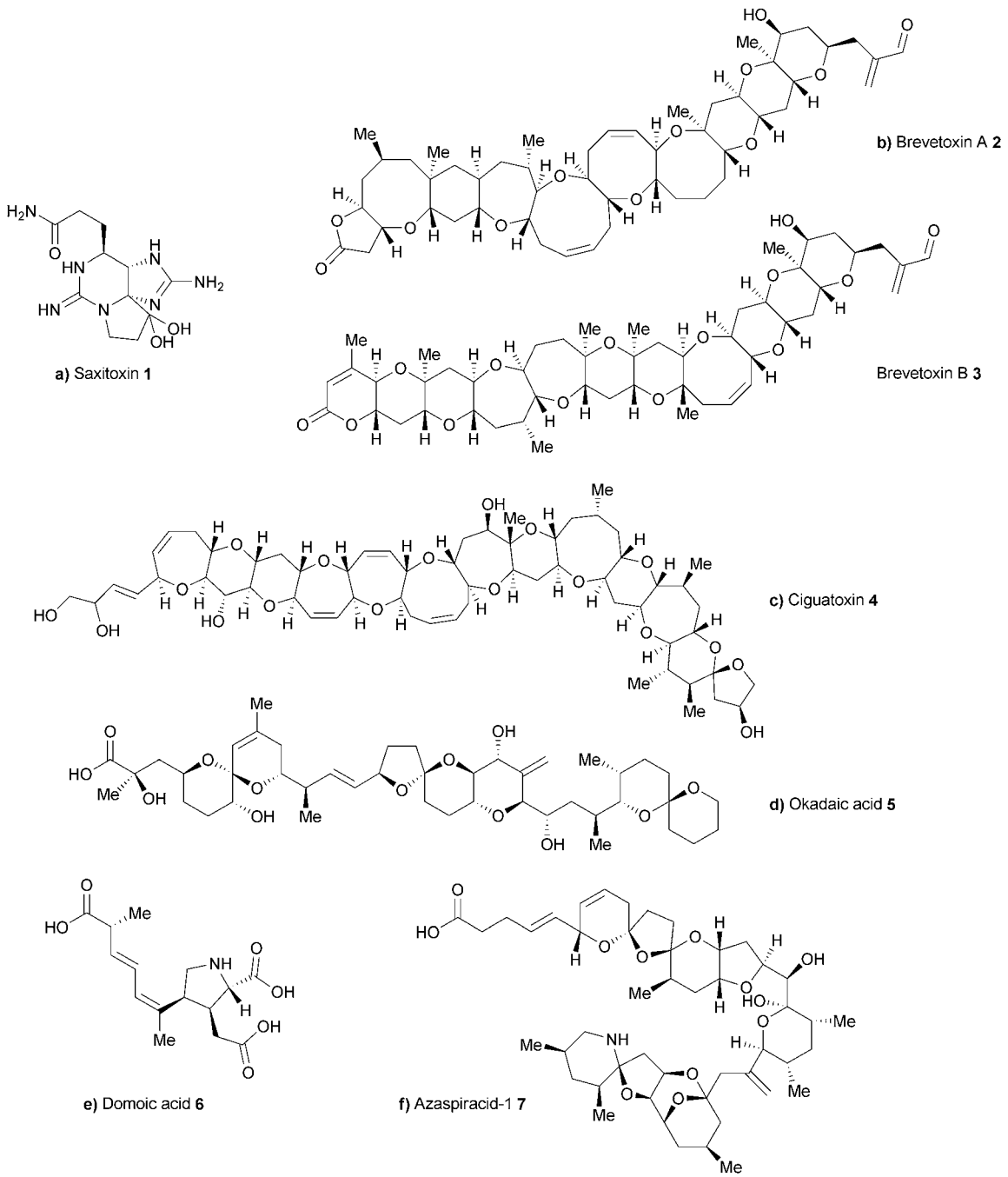


Fig. 1 (a) Saxitoxin (**1**): PSP, hydrophilic, purine derivatives. (b) Brevetoxins A (**2**) and B (**3**): NSP, lipophilic, *trans*-fused structure. (c) Ciguatoxin (**4**): CFP, lipophilic, *trans*-fused polyether. (d) Okadaic acid (**5**): DSP, lipophilic, linear and macrocyclic non-nitrogenated polyether. (e) Domoic acid (**6**): ASP, hydrophilic, amino-acid like structure. (f) Azaspiracid (**7**): AZP, lipophilic, nitrogenated polyether.

H. malanodocia.[13] Okadaic acid was later found to be produced by two species of toxic dinoflagellates *Dinophysis* and *Prorocentrum* spp.[14] The illness, which is not fatal, is characterized by diarrhoea, nausea, vomiting and abdominal cramps. This phenomenon was first documented in 1976 in Japan where it caused major problems for the scallop industry.

—*Amnesic shellfish poisoning (ASP)* occurs after the consumption of shellfish contaminated with domoic acid (**6**) and its congeners.[15] It is the only shellfish intoxication caused by a diatom (*Pseudo-nitzschia* spp.) and was first observed in 1987 when 105 people became ill and 3 people died after consuming contaminated blue mussels from Prince Edward Island, Canada. ASP is characterised by initial gastrointestinal symptoms such as nausea, vomiting, abdominal cramps, and diarrhoea. In severe cases, neurological symptoms also appear including dizziness, hallucinations, short-term memory loss seizures, respiratory difficulty and coma.

—*Azaspiracid poisoning (AZP)* is the latest poisoning syndrome and the causative toxin is azaspiracid (**7**). In November 1995, at least eight people in the Netherlands became ill after eating mussels (*Mytilus edulis*) cultivated at Killary Harbour, Ireland. AZP is a gastrointestinal illness and the symptoms resemble those of DSP but the concentration of these related toxins are very low. In addition, a slowly progressing paralysis was observed in the mouse bioassay, which was different from the usual DSP toxicity. Subsequently, azaspiracid (**7**) was identified and the new toxic syndrome was called azaspiracid poisoning (AZP).[16] In laboratory experiments, azaspiracid can induce widespread organ damage in animals; hence it is probably more dangerous than previously known classes of shellfish toxins.[17] Recently, one strain of a new species of dinoflagellate, *Azadinium spinosum*, has been reported as one of the organisms that produces azaspiracid.[18]

—*Spiroimine shellfish poisoning (SSP)*. The spirolide family of toxins are closely related to other fast-acting toxins, the gymnodimines and the pinnatoxins (see section 2.1) that are not currently classified under the categories above. Their inclusion in a new class of shellfish poisoning by marine biotoxins containing a spiroimine moiety is therefore proposed.

1.4 Methods for detecting marine toxins

New techniques have been established to identify the presence and quantify the levels of toxins in shellfish tissues to protect seafood consumers. Scientific observations of HABs and toxic mussels were reported as early as 1937, and soon after many countries implemented regulations on the level of toxins developing a mouse bioassay to monitor the occurrence of algal toxins.[19] To date, the mouse bioassay is the most commonly used method for detecting marine toxins. However this assay is costly, time consuming, and only detects relatively high toxin levels. Quicker techniques to detect toxins at a lower level need to be established. To this end, various methods of detection have been developed including a fluorometric assay,[20] high pressure liquid chromatography (HPLC) often coupled with fluorescence detection or with ion-pair chromatography,[21–23] LC-MS techniques for the detection of ASP, PSP and lipophilic toxins[24–26] and also enzyme immunoassay techniques.[27] More specific techniques have recently been developed for the detection of particular toxic dinoflagellates and toxins, such as a rapid method for detection of *A. ostenfeldii* spp. using a DNA biosensor[28] and also a non-radioactive quantitative method for detection of both gymnodimine A and 13-desmethyl spirolide C (see section 2 for structures) in shellfish using an inhibition fluorescence polarisation assay. This method is based on the fact that gymnodimine and spirolide toxins target muscular and neuronal nicotinic acethylcholine receptor (nAChR) subtypes with high affinity (see section 3.2).[29,30]

Each country has determined their own legal regulations for determination of toxins in shellfish prior to distribution and sale. In the European Union, HPLC-MS techniques are the official methods. The Canadian Food Inspection Agency (CFIA) used the mouse bioassay to detect the presence of saxitoxin (**1**) and derivatives known to cause PSP until 2005, when it was superseded by the HPLC-MS method developed by Lawrence.[31]

2 A new family of marine toxins, the spirolides

2.1 Marine toxins containing a spiroimine unit, SSP

Several toxins have been discovered that contain a spiroimine unit.[32] The syntheses of these specific toxins including procentrolide A (**8**), spiroprorocentrimine (**9**), pinnatoxins A–C (**10–12**), pteriatoxins A–C (**13–15**) and gymnodimine (**16**) (Fig. 2) and others have been described in a 2007 review.[33]

For example, pinnatoxin A (**10**) was isolated from the extract of digestive glands of clams (*Pinna muricata*) from sub-tropical waters around Okinawa and was linked to several major poisoning events in Japan and China.[34] Gymnodimine (**16**) was isolated from oysters collected at Foveaux Strait in the South Island of New Zealand.[35]

2.2 Structure and assignment of the relative stereochemistry of the members of the spirolide family

In 1995 during routine monitoring of polar lipophilic toxic compound in mollusc extracts, an unusual toxicity was found during regular mouse bioassay testing of scallop (*Placopecten magellanicus*) and mussel (*Mytilus edulis*) viscera harvested from certain aquaculture sites along the south-eastern coast of Nova Scotia in Canada. Hu and co-workers,[36] after chromatographic purification of methanolic extracts of frozen digestive glands of shellfish, isolated four pure novel compounds, spirolides A–D (**17–20**) (Fig. 3).

Two of these toxins were isolated in sufficient quantity to enable structural elucidation. HR-LSIMS (High Resolution Liquid Secondary Ion Mass Spectrometry) determined the molecular formula to be $C_{42}H_{63}NO_7$ and $C_{43}H_{65}NO_7$ respectively, for spirolides B (**18**) and D (**20**). IR spectra and ^{13}C analysis clearly established that the molecule contained a γ-lactone, a vinyl group and a double bond between a carbon and either a carbonyl or imine functionality. The TOCSY, HMBC and COSY spectra were obtained for spirolide D (**20**) allowing a partial structure elucidation of this new toxin: a γ-lactone ring attached through C5 to the six-membered ring, establishment of an olefin as the C5–C6 bond and also the *E* configuration of the double bond between C8 and C9 due to a strong nOe correlation between H8 and H10. The C29 quaternary centre was revealed by HMBC data showing the

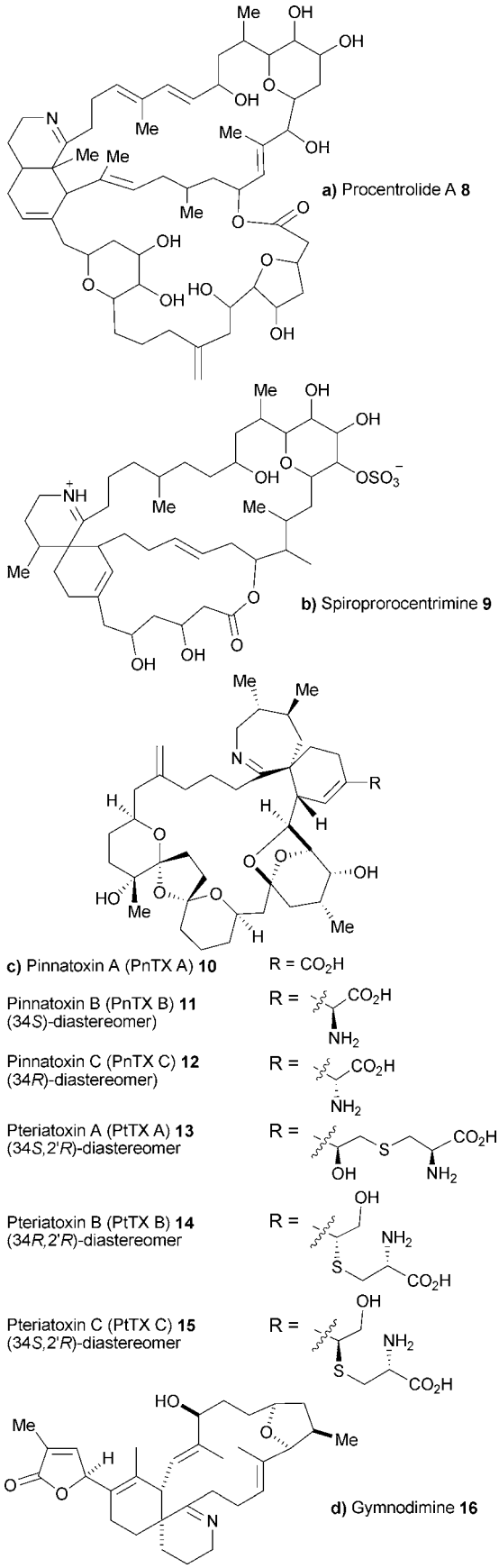
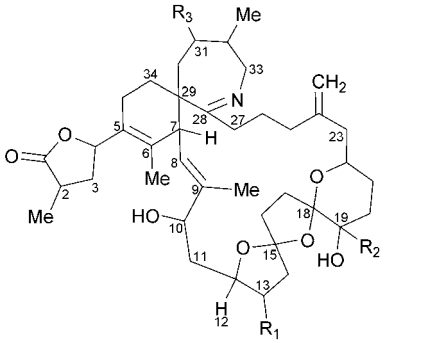
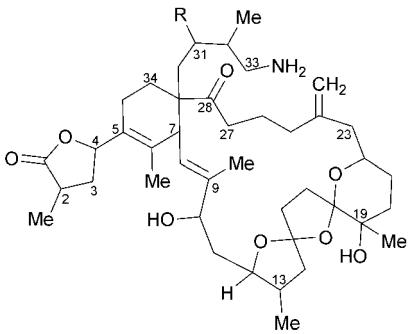


Fig. 2 Marine toxins containing a spiroimine unit.



a) spirolide A **17** $\Delta^{2,3}$; R_1 = Me; R_2 = Me; R_3 = H
spirolide B **18** R_1 = Me; R_2 = Me; R_3 = H
spirolide C **19** $\Delta^{2,3}$; R_1 = Me; R_2 = Me; R_3 = Me
spirolide D **20** R_1 = Me; R_2 = Me; R_3 = Me
13-desMe spirolide C **21** $\Delta^{2,3}$; R_1 = H; R_2 = Me; R_3 = Me
13-desMe spirolide D **22** R_1 = H; R_2 = Me; R_3 = Me



b) spirolide E **23** $\Delta^{2,3}$; R = H
spirolide F **24** R = H

Fig. 3 Structures of spirolides A–D (**17–20**), 13-desmethyl spirolide C (**21**), 13-desmethyl spirolide D (**22**) and keto-amine derivatives E (**23**) and F (**24**).

relation between C7, C29, H7 and H34. The position of the single nitrogen was assigned in the seven-membered ring due to a characteristic chemical shift of the C28 imine carbon and the HMBC correlations between C28 and the downfield H33. The quaternary centre C29 is linked to both the six and seven-membered rings thus proving the presence of the spiro-linked imine. The partial structure of the lower part of the molecule containing a *bis*-spiroacetal was confirmed by recording ^{13}C NMR in CD_3OH and then in CD_3OD and observation of HMBC correlations, such as the oxygen bearing C15 and C18 quaternary centre linking the two tetrahydrofurans and the tetrahydrofuran and tetrahydropyran rings and the presence of a tertiary hydroxyl group at C19. A third exchangeable proton was identified at C27 as a result of iminium ion tautomerization. Spirolide B (**18**) lacks a methyl group compared to spirolide D (**20**). MS/MS spectra showed that this methyl group was located on the imine-containing ring.

In 1996, the same research group reported the isolation of two further spirolides E (**23**) and F (**24**) from shellfish extracts.[37] The final HPLC step showed the presence of two peaks eluting later than the known spirolides A–D (**17–20**). NMR data (^1H, ^{13}C, ^1H TOCSY and DQF-COSY) of spirolide F (**24**) revealed a close

resemblance to spirolide B (**18**) but a difference appeared around C28. The resonance for position 33 was shifted upfield as a result of the presence of a primary amine instead of an imine. Moreover, the resonance for H27 was shifted downfield which is in accordance with hydrolysis of the imine to a ketone functionality. In addition, the HRMS of compound **24** established the molecular formula $C_{42}H_{65}NO_8$ corresponding to the addition of water to spirolide B (**18**). The absence of the imine moiety in spirolide F (**24**) was also confirmed by the dramatic difference in the MS/MS fragmentation pathway of compounds **18** and **24**. The IR data also confirmed the presence of an amine and a ketone, establishing spirolide F (**24**) as a keto-amine derivative of spirolide B (**18**). For spirolide E (**23**), the molecular formula was determined to be $C_{42}H_{64}NO_8$ by HRMS which suggested this new compound to be a keto-amine derivative of spirolide A (**17**). These conclusions were confirmed by the acid-catalysed hydrolysis of spirolides A (**17**) and B (**18**) giving compounds with identical spectroscopic data to spirolides E (**23**) and F (**24**), respectively.

In 2001, the structural elucidation of spirolides A (**17**) and C (**19**) was reported without assigning stereochemistry. High resolution LSIMS established the molecular formula of spirolide A (**17**) as $C_{42}H_{61}NO_7$ which contains two protons less than the known spirolide B (**18**). Additional NMR data (^1H, ^{13}C, DEPT, HSQC, TOCSY, COSY and HMBC of selected protons), IR, tandem mass spectrometry (MS/MS) and further spectral comparison with the known data for spirolide B (**18**), confirmed the close resemblance of these two compounds. However, spirolide A (**17**) contained an additional double bond between C2 and C3 conjugated to the carbonyl group in the γ-lactone ring. Using the same analytical methods with comparison to data previously obtained for spirolide D (**20**), the molecular formula of spirolide C (**19**) was established to be $C_{43}H_{63}NO_7$ with the extra methyl group being assigned to position 31 on the cyclic imine system and the extra double bond positioned between C2 and C3 in the γ-lactone unit.

In addition, the structure of another new congener, 13-desmethyl spirolide C (**21**) (Fig. 3) was reported. The molecular formula of compound **21** was attributed to be $C_{42}H_{61}NO_7$ showing that this new derivative was an isomer of spirolide A (**17**). NMR data demonstrated that part of the structure is identical to spirolide C (**19**) with the only difference occurring at C13. TOCSY data and COSY correlations clearly indicated that H12 is adjacent to two methylene groups and the absence of a methyl signal from C13 confirmed the notable difference between the MS/MS spectra of compounds **17**, **19** and **21**.[38] Moreover, LC-MS and MS/MS analysis of phytoplankton extracts also identified 13-desmethyl spirolide D (**22**), another desmethyl derivative of spirolide D, but no structural elucidation was carried out for this new congener.

Sleno *et al.*[39,40] identified important collision-induced dissociation (CID) products of 13-desmethyl spirolide C (**21**) and their probable mechanism of formation. FTICR experiments confirmed the accurate mass for 13-desmethyl spirolide C ($[M + H]^+$ *m/z* 692.45192). Various fragmentation pathways were proposed using MS/MS data. One of the major fragmentation pathways is summarized in Fig. 4: initially the six-membered unsaturated ring undergoes an intramolecular retro-Diels–Alder reaction that leads to the formation of intermediate **25** containing the dienophilic end of the molecule with the cyclic imine untouched. This intermediate then undergoes fragmentation by a six-centered concerted loss of a water molecule, giving a fragment ion at $[M + H]^+$ *m/z* 444 due to the highly conjugated species **26** that contains the untouched spiroimine and the *bis*-spiroacetal moiety. The major fragmentation pathway for ion **26** leads to the formation of a conjugated 7-membered ring spiroimine species **27** ($[M + H]^+$ *m/z* 164) *via* a four-centered reaction (common in charge-remote fragmentation). Fragment **27** is characteristic of the C/D class of the spirolides, containing vicinal dimethyl groups on the seven membered imine ring.

The spirolides A–D (**17–20**) and 13-desmethyl spirolide C (**21**) all contain a 6,7-bicyclic imine moiety with the same atom-to-atom

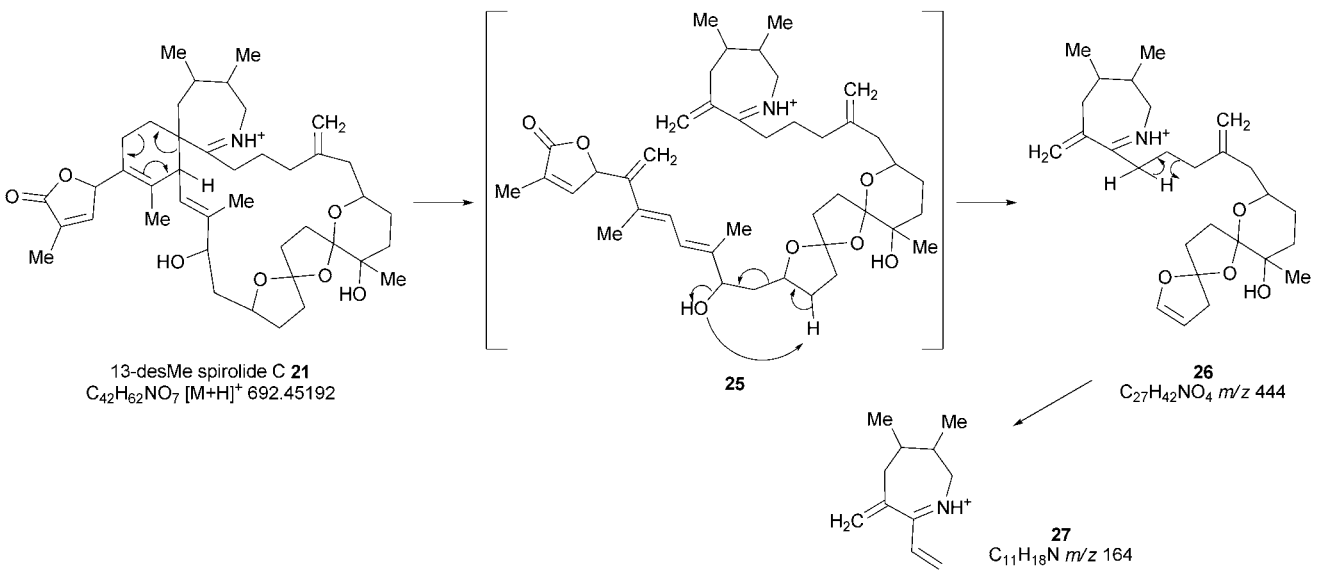


Fig. 4 One of the possible fragmentation pathways for 13-desmethyl spirolide C (**21**) as proposed by Sleno *et al.*[39] The arrows depict a possible mechanism for the fragmentation reaction.

connectivity as that found in the pinnatoxins (**10–12**) and the pteriatoxins (**13–15**) (Fig. 2(c)) and bear some similarity to gymnodimine (**16**) (Fig. 2(d)). The relative stereochemistry of 13-desmethyl spirolide C (**21**) and spirolides B (**18**) and D (**20**) (Fig. 5) was established from NMR data (series of NOESY and ROESY spectra) combined with modelling studies using Tripos Sybyl molecular modelling software with the ConGen routine and the HyperChem software using the XMConGen routine.[41] As compound **21** was obtained in a larger quantity, a list of 47 unambiguous restraints was drawn up from observed ^{1}H–^{1}H NOESY and ROESY cross peaks and measurements of interproton distances which was sufficient to yield a unique configuration using both molecular modelling programs. The optimum configuration established the following relative chirality 7*S**, 10*S**, 12*S**, 15*R**, 18*R**, 19*R**, 22*S**, 29*R**, 31*S**, 32*R**. The *anti* relation between the two protons at C31 and C32 and their assignment to one specific enantiomer was confirmed by the predicted dihedral angle H31–C31–C32–H32 (163°) which was consistent with the large measured value for $^{3}J_{(31,32)}$ = 15.9 Hz. Also all H···H distances in the optimum configuration were in agreement with the collected nOe data. However, the configuration at C4 was uncertain due to the rotation of the γ-lactone ring at the C4–C5 bond, leading to four possible isomers with a distribution of 67 : 50 : 59 : 51 indicating complete and dense coverage of configuration space. The relative stereochemistry of spirolides B (**18**) and D (**20**) was determined using the same modelling procedure but due to the smaller quantity of material, the NOESY and ROESY spectra were determined with a single mixing time. Only the partial stereochemistry could be determined that was in accordance with that obtained for **21**. The relative configuration of the spirolides agrees with the stereochemistry of pinnatoxins A (**10**) and D in the overlapping region.

Ten years after the first detection of the spirolides, two novel spirolide derivatives were isolated and characterised from the culture of two clonal strains of *A. ostenfeldii* isolated from Limfjorden in Denmark. The LC-MS profile of these extracts revealed the presence of the known 13-desmethyl spirolide C (**21**) and two unknown components suspected to be spirolide C/D derivatives, due to the presence of a fragment ion at $[M + H]^+$ *m*/*z* 164 in the MS/MS spectra. HMRS established the molecular formula to be $C_{41}H_{59}NO_7$ and $C_{42}H_{61}NO_7$ for the two new compounds, called 13,19-didesmethyl spirolide C (**28**) and spirolide G (**29**), respectively (Fig. 6). The partial structure of 13,19-desmethyl spirolide C (**28**) was established by comparing IR and NMR data with the data already reported for 13-desmethyl spirolide C. The magnitude of the chemical shift for C15 and C18 confirmed the presence of the 5,5,6-*bis*-spiroacetal ring system and the fragment ions of the parent compound **28** were in agreement with the absence of a methyl group at C19. COSY,

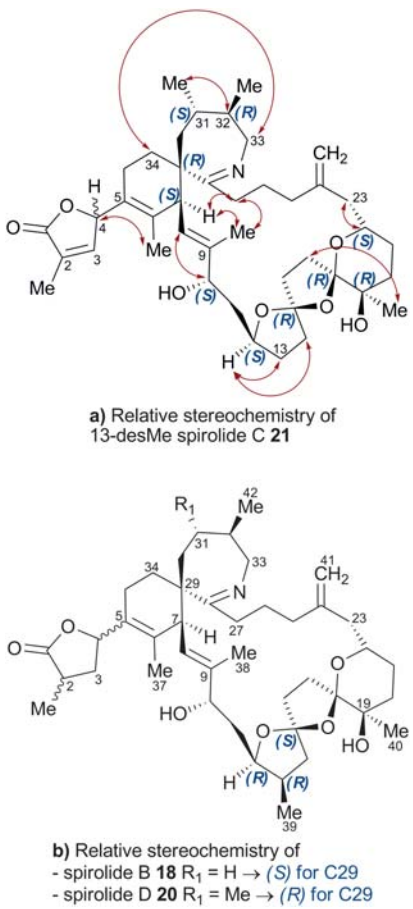


Fig. 5 (a) Relative stereochemistry of 13-desmethyl spirolide C (**21**). The chirality is indicated in blue for each stereocentre and the red double arrows represent some of the strong nOe correlations. (b) Relative stereochemistry of spirolides B (**18**) and D (**20**). The chirality of specific stereocentres indicated in blue show a trivial chirality reversal owing to priority changes.

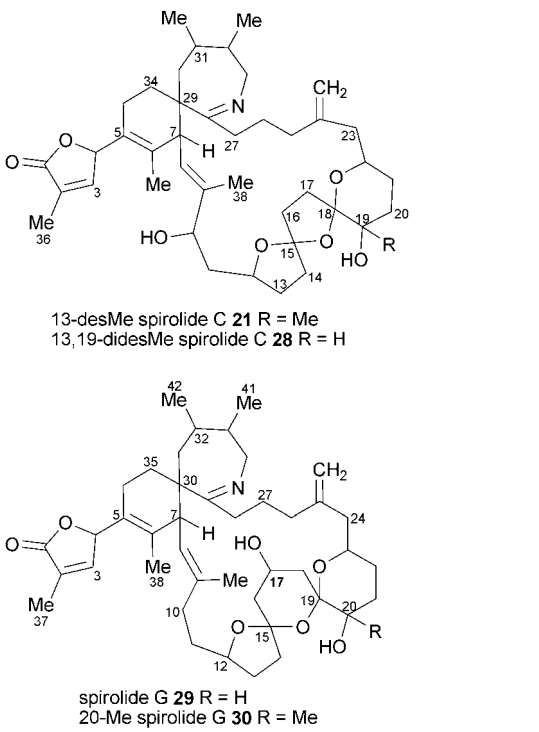
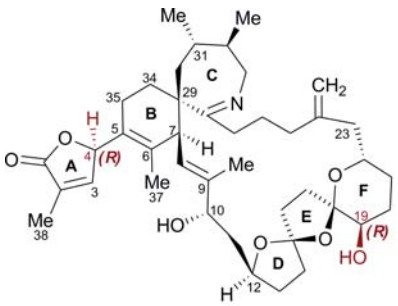


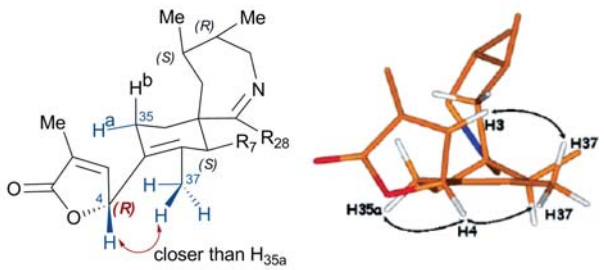
Fig. 6 Structure of 13,19-desmethyl spirolide C (**28**) in comparison with previously described 13-desmethyl spirolide C (**21**) (top). Structure of spirolide G (**29**) and 20-methyl spirolide G (**30**) (bottom).

TOCSY and HMBC correlations confirmed that compounds **28** and **29** have the same structure for the upper part of the molecule (6,7-spiroimine and γ-lactone). However, ^{13}C DEPT and HSQC NMR data for compound **29**, supported by the difference in HRMS analysis compared to compound **28**, confirmed the presence of an extra methylene carbon leading to a change in the atom numbering. HMBCs between H13, H14, H16 and C15 and between H18 and C19 of spirolide G (**29**) allowed assignment of the resonance at 109.7 ppm characteristic of a spiroacetal carbon at a 5,6 ring junction to be the quaternary C15 and the resonance at 101.2 ppm characteristic of a spiroacetal carbon at a 6,6 ring junction to be C19. COSY and TOCSY spectra and mass spectral fragmentation data also confirmed the presence of a novel 5,6,6-*bis*-spiroacetal ring system on the lower part of spirolide G (**29**). Moreover, mass spectral fragmentation data and ^{13}C NMR spectra in CD_3OH and CD_3OD indicated the presence of a secondary alcohol at positions C17 and C20 due to the upfield shift of the methine carbons C17 and C20.[42]

Subsequently, analysis of blue mussel (*Mytilus edulis*) digestive glands and algal biomass samples collected during a bloom at Sognefjord, Skjer in Norway allowed the structure elucidation of a new analogue of spirolide G (**29**), which contained an additional methyl group at C20 indicated by MS/MS fragmentation, NMR data and comparison with all previous data for the known spirolides. The accurate mass of compound **30** was determined to be $C_{43}H_{64}NO_7$.[43]



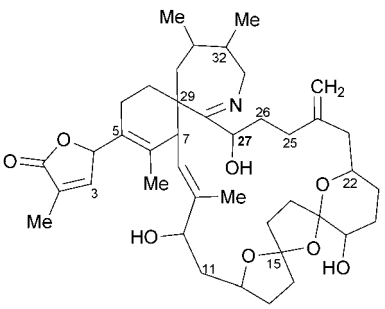
a) 13,19-didesMe spirolide C **28**



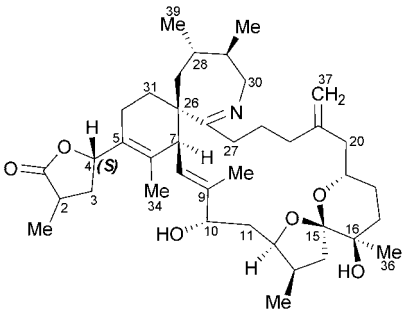
b) configuration at C4

Fig. 7 (a) Full relative stereochemistry for 13,19-didesmethyl spirolide C (**28**). In red is represented the stereochemistry of the two newly-determined stereocentres C4 and C19. (b) (Left) Spatial configuration at C4 and orientation of the unsaturated lactone with respect to the cyclohexene ring. The groups in blue lie on the same side of the molecule. Red arrows show the strongest ROESY cross peak. (Right) *gauche*$^-$ conformer of 4*R**. Black arrows indicate possible ROEs, reproduced from Ciminiello *et al.*[44]

Eight years after the report of the partial stereochemical assignment for 13-desmethyl spirolide C (**21**), the full relative stereochemical assignment of 13,19-didesmethyl spirolide C was reported by Ciminiello *et al.*[44] using a combination of NMR and molecular modelling. Using the previously reported relative stereochemistry for 13-desmethyl spirolide C and knowing that the ^1H and ^{13}C chemical shifts of these two spirolide derivatives overlapped except the missing methyl group at C19 in **21**, the determination of the relative stereochemistry at C4 and C19 was elucidated (Fig. 7(a)). A *gauche*-like orientation between H4 and both C6 and C35 was deduced from the small carbon–proton vicinal coupling constant observed, and by comparison of the dihedral angle α (H4–C4–C5–C6) obtained for all the possible conformers using molecular modelling, thus leading to only two possible conformers. Further study of the z-TOCSY and ROESY correlations showed that H3 is spatially closer to Me37; H4 is closer to Me37 than H35a; and H4, H35a and Me37, all showing ROESY correlations, were positioned on the same side of the molecule. These conclusions led to the assignment of the *R** configuration at C4 (Fig. 7(b)). Using NMR data and MM conformers of the possible molecular models, the six-membered ring of the *bis*-spiroacetal moiety was established to adopt a *chair* conformation with H19 in an axial position thus establishing an *R** configuration for C19. Also three major conformers of 13,19-didesmethyl spirolide C **28** appeared to be in equilibrium in solution. The two six-membered rings **F** and **B** adopted only one conformation, respectively, a *chair* and "*pseudo-chair*" respectively. Only one conformation was observed for the seven-membered imine ring **C**. The methyl at C38 was assumed to adopt an "*endo*" position in ring **A**. In contrast, rings **D** and **E**



27-hydroxy-13,19-desMe spirolide C **31**



spirolide H **32** $\Delta^{2,3}$
spirolide I **33**

Fig. 8 Basic structure of 27-hydroxy-13,19-desmethyl spirolide C (**31**), spirolide H (**32**) and spirolide I (**33**).

showed a rapid equilibrium in solution between two conformers. C9 and C10 adopted an *anti*-periplanar and a *gauche*-like orientation, respectively, due to the two possible configurations of ring **D**.

In 2007, investigation of a large culture of an Adriatic *A. ostenfeldii* strain allowed the isolation and structure elucidation of another congener of the spirolide family, 27-hydroxy-13,19-desmethyl spirolide C (**31**) (Fig. 8) with a molecular formula of $C_{41}H_{59}NO_8$. The mass spectral fragmentation pattern was similar to 13,19-didesmethyl spirolide C (**28**) with a difference of 16 mass units for all the peaks and an additional loss of water occurred from the protonated molecular ion in the MS/MS spectrum thereby indicating the presence of an additional hydroxyl group. 1D and 2D NMR analysis revealed a notable change in the chemical shift in the ^{1}H and ^{13}C spectra at positions 25, 26 and 27 compared to compound **28** and also the presence of an hydroxymethine functionality at C27 instead of the common methylene group described in all the spirolides isolated previously.[45]

To date spirolides H (**32**) and I (**33**) (Fig. 8) are the two most recent reported compounds of the spirolide family, containing an unusual 5,6-spiroacetal ring system compared to all previously isolated spirolides that contained a 5,5,6- or 5,6,6-*bis*-spiroacetal. High resolution mass spectroscopy established $C_{40}H_{60}NO_6$ and $C_{40}H_{62}NO_6$ as a molecular formula for spirolide H and I, respectively. DEPT, HSQC, COSY, TOCSY NMR and HMBC correlations supported the difference in molecular formula between compounds **32** and **33**, due to the presence of a double bond between C2 and C3 in the γ-lactone ring in compound **32**. The MS/MS spectra of these two compounds exhibited a $[M + H]^+$ *m/z* 164 fragment which proved the presence of the bicyclic spiroimine containing two vicinal methyl groups identical to previously isolated spirolides; this observation was confirmed by NMR data. The presence of a 5,6-spiroacetal was established by observation of the characteristic resonance of the C15 quaternary centre at 112.9 ppm, the absence of carbon signals between 112 and 119 ppm, showing no additional linkages, and analysis of the HMBC connectivities. The full relative configuration of spirolide H (**32**), as represented in Fig. 8, was established from ROESY data. The same configuration as that for 13-desmethyl spirolide C (**21**) was assigned for the stereocentres in the 6,7-bicyclic spiroimine unit, the C10 secondary hydroxyl group and the stereocentres in the spiroacetal moiety. z-TOCSY and ROESY correlations (H4 to H3 and Me34 only, H3 to H4 and Me34 only) established the favoured *S** configuration at C4 which is opposite to the relative configuration found in 13,19-didesmethyl spirolide C (**28**). Finally the MS/MS fragmentation pattern for compound **32** was similar to the one described previously for 13-desmethyl spirolide C (**21**).[46]

2.3 Detection of spirolides around the world

In order to obtain more information about the presence of spirolides in European coastal water after the first detection of *A. ostenfeldii* cells in Denmark,[47] samples from the Scottish east coast and the North Atlantic were collected in 2000 and analyzed by LC-MS. The spirolides were detected in 75% of the samples collected in a zone of approximately 45,000 km^2 containing *A. tamarense* and *A. ostenfeldii*. A variation in the spirolide profile from different sampling areas was observed. Spirolide C (**19**) was the major component for all sample sites. Two minor compounds corresponding to spirolide A (**17**) and/or 13-desmethyl spirolide C (**21**) were found in samples from different coastal areas. Smaller amounts of unknown spirolides, in variable quantities, were also observed.[48,49]

In November 2003, in the northwestern Adriatic Sea along the Emilia-Romagna coast in Italy, a high concentration of toxic *A. ostenfeldii*, with a maximum of 15,600 cell L^{-1}, was detected during routine monitoring for toxic phytoplankton in farmed shellfish. Analysis of a culture of this organism concluded that Adriatic strains of *A. ostenfeldii* produced spirolides but not PSP toxins. Comparison with authentic samples proved the presence of 13-desmethyl spirolide C (**23**), 13,19-didesmethyl spirolide C (**28**) and 27-hydroxy-13,19-didesmethyl spirolide C (**31**) as well as minor unknown compounds showing fragmentation patterns similar to that observed for the spirolides.[45,50] Moreover, lipophilic extracts of toxic mussels (*Mytilus galloprovincialis*), collected during the same period and subjected to semi-quantitave analysis by LC-MS for various common marine toxins contained the same spirolides as the ones reported in the culture of *A. ostenfeldii* cells. For the first time the spirolides represented the main compounds responsible for toxicity in the lipophilic mussel extract as opposed to the pectenotoxin family of toxins usually found in shellfish in the Adriatic Sea.[51,52]

In 2005, analysis of extracts of mussels, clams and razor clams collected in various shellfish farms in the area of Galicia in northwestern Spain showed the presence of 13-desmethyl spirolide C (**21**) at low levels using LC-MS.[53]

As part of a regular French shellfish monitoring program carried out by the Phytoplankton and Phycotoxin Monitoring Network (REPHY), spirolides were detected for the first time in April 2005, together with the usual DSP toxins. Spirolide A (**17**), the major component, and 13-desmethyl spirolide C (**21**) were detected in oysters and mussels collected from the Arcachon Bay, Atlantic coast. A smaller amount of spirolide A (**17**), as well as 13-desmethyl spirolide C (**21**) and 13-desmethyl spirolide D (**22**) were also observed in south Britanny. The maximum concentration of spirolides, in association with the occurrence of *A. ostenfeldii* in French coastal water was close to 60 μg kg^{-1} of shellfish tissue. Due to the presence of the spirolides and lack of an European regulatory limit for this family of toxins, the French health authority declared the shellfish to be unfit for consumption for 10 weeks after their detection.[54,55]

Culture of vegetative cells of *A. peruvianum* and *A. ostenfeldii* isolated from the southwest and northeast coast of Ireland revealed the production 13-desmethyl spirolide C (**21**), spirolides C (**19**) and D (**20**) but the absence of saxitoxin derivatives (PSP toxins).[56]

Detection of spirolide toxins seems to have increased around coastal European waters, however, after their first detection in Canada these toxins have also been found in the eastern coastal waters of the USA. A five year program called ECOHAB (ECOlogy of Harmful Algal Blooms) was initiated to address several fundamental issues regarding *Alexandrium* blooms in the Gulf of Maine. This study showed that *A. ostenfeldii* co-occurs with *A. fundyense*, the main dinoflagellate monitored in this program. LC-MS analysis established the presence of spirolides in 36 to 70% of field samples in the large area between Cape

Elizabeth and the Bay of Fundy. Spirolide A (**17**) was the major component of the samples followed by a smaller amount of spirolide B (**18**) and two unknown structural isomers. Culture of *A. ostenfeldii* isolated from the same area showed a totally different spirolide composition compared to the field sample, for example spirolides C (**19**), D (**20**) and 13-desmethyl spirolide C (**21**) were only detected in the culture sample.[57]

The latest new occurrence of spirolides in shellfish in the southern hemisphere was reported at Coquimbo Bay in northern Chile. HPLC-MS/MS analysis of the lipophilic extract of two bivalves: razor clam (*Mesodesma donacium*) and clam (*Mulinia edulis*) revealed the presence of 13-desmethyl spirolide C (**21**) in both species present above the 6.78 μg kg^{-1} limit of detection. Moreover, in some samples, spirolides and azaspiracids were detected in the same shellfish extract.[58]

2.4 Dinoflagellate origin of the spirolides

Dinoflagellates have an extraordinary ability to produce red tide and in particular, paralytic shellfish poisoning. Numerous dinoflagellate blooms have been reported in diverse regions of the world such as the South Atlantic coast, the coasts of Chile, Venezuela, Peru, Spain, Japan, Norway, the coastal waters of the Romanian Black sea and in the United States, where many outbreaks have occurred along the New England coast, the Gulf coast of Florida and off Alaska.[59] Neurotoxins produced by these organisms may be 50 times as potent as the paralytic poison curare.[60] The three genera of dinoflagellates, *Alexandrium*, *Gymnodinium* and *Pyrodinium*, are known to produce secondary toxic metabolites. These toxins are in many cases fascinating polyketide compounds with unique and complex chemical structures. The exact relationship between dinoflagellates and the structure of the related toxins has not yet been proven. However, primitive dinoflagellates (*Dinophysoid*, *Prorocentroid*, *Gonyaulacoid*, *Alexandrium* spp.) produce only linear or macrocyclic polyether compounds.[61] Elucidation of the exact biosynthetic route for the formation of these secondary metabolites could possibly lead to viable synthetic routes and a potential opportunity to genetically engineer analogues of interesting shellfish toxins. However, even if some metabolites display specific activities such as antifungal activity[62] or target the mammalian central nervous system (see section 3.2) their actual role in the natural environment is not yet explained.

The genus *Alexandrium* is one of the most intensively studied genera of free living planktonic dinoflagellates and it contains more neurotoxin-producing members than any other algal genus. *Alexandrium* blooms tend to be located in two typical areas: firstly, in salt ponds, bays and lagoons and secondly, in coastal waters or large estuaries such as the Saint Lawrence estuary in Canada and the Gulf of Maine in the US.[63] *Alexandrium* population development (sometimes leading to blooms) is rarely mono-specific and *Alexandrium* spp. are often found with other species of the genus and also other morphologically similar dinoflagellates. Several conclusions have been drawn, however: blooms are not long lasting and seem to be restricted in both space and time by oceanographic conditions (temperature, light availability, turbulence) and possibly biological interactions (life cycle transition, predation, pathogenicity).[7]

More specifically, *A. ostenfeldii* is an armoured, planktonic dinoflagellate and a photosynthetic species with radiating chloroplasts. *A. ostenfeldii* has often been mistaken for *A. tamarase* and/or *A. peruvianum* due to similar morphology. In general, populations of *A. ostenfeldii* tend not to dominate the dinoflagellate community and often co-occur with other *Alexandrium* spp. This species is best known for its capacity to produce PSP neurotoxins. Hansen *et al.*[64] reported a study on the taxonomy and the toxicity of *A. ostenfeldii*. This species was first believed to be a cold-water estuarine species found in low numbers and confined to Greenland and the Western European coast including Iceland, the Faroe Islands, United Kingdom (strains WW516 and WW517), Denmark (strains K0287 and K0324), Norway[65–67] and East Asiatic Russia.[68] Recently however, *A. ostenfeldii* has been isolated from Alexandria Harbour, Egypt and around the west coast of the Pacific Ocean of the USA (strains HT140-E4 and AOCB03)[57,69] demonstrating that this species is widely distributed around the world. Moreover, *A. ostenfeldii* and/or *A. peruvianum* have been reported in coastal waters off the central and Northern Baltic Sea,[70] in the Mediterranean sea around Spain,[71] in Irish coastal waters,[56] in Malaysian waters[72] and in sediment collected between Taharoa and Kaitaia, on the west coast of the North Island of New Zealand.[73,74]

In Nova Scotia, during spring and early summer, high spirolide levels in shellfish have been found on an annually recurring basis. Golden-pigmented spherical cells (commonly called "golden balls"(GB)) were also observed in the viscera of scallops and mussels. In order to determine the origin of the spirolides, samples of water were analyzed during May through July from 1994 to 1998 at Graves Shoal, Indian Point, Snake Island and Ship Harbour, Nova Scotia.[75–77] Plankton sized between 26–44 μm and 44–56 μm had higher concentrations of spirolides which was correlated with the abundance of *Alexandrium* spp. (predominantly *A. ostenfeldii* and *A. tamarense*) and GB cells being 42 μm in diameter. Nevertheless, no spirolides were evident upon LC-MS analysis of cultured strains of *A. tamarense* isolated from water samples containing a high concentration of spirolides. In contrast these species yielded only PSP toxins. Around the same period in Denmark, spirolides and PSP toxins were observed in one culture of *A. ostenfeldii*.[78] Isolation of GB and motile dinoflagellate cells from the 21–56 μm size fractions, by micropipette techniques and cytometry, showed the presence of spirolides. As is the case for many other phycotoxins such as brevetoxins and the PSP toxins, the spirolide family exhibits a high degree of molecular diversity within cultured strains and between geographical populations. During the peak of toxicity in June 1996, the total yield of spirolides from the 26–44 μm fraction was more than twice as high at Graves Shoal than at Ship Harbour and this was in relation to a higher amount of *Alexandrium* cells. The spirolide profile in water samples from Graves Shoal was identical over time showing a dominance of spirolides B (**18**) and D (**20**) and the observation of a new isomer 13-desmethyl spirolide D (**22**) but an annual variation in the abundance of spirolides A (**17**) and 13-desmethyl spirolide C (**21**) was also observed. At Ship Harbour, the phytoplankton was rich in spirolides A (**17**), B (**18**) and 13-desmethyl spirolide C (**21**) with spirolides C (**19**) and D (**20**) often present as minor constituents. On the other hand, LC-MS and MS/MS analysis of an axenic

unialgae isolate of *A. ostenfeldii* from a dinoflagellate bloom population at Ship Harbour proved the presence of spirolides C (**19**), D (**20**), 13-desmethyl C (**21**) and 13-desmethyl D (**22**).[79] Moreover, spirolides have also been detected in cultures of *A. ostenfeldii* and *A. peruvianum* from Irish water,[56] Spain and the Baltic Sea.[70]

In the Gulf of St. Lawrence, Canada, *Alexandrium* spp. has been monitored on a weekly basis since 1989. Until 1994 only *A. tamarense* had been detected but following a microscopic examination of the morphology, *A. ostenfeldii* (strain AOSH1) was also identified.[80] Cells have also been isolated from the south-eastern coast of Nova Scotia that have proven to produce PSP toxins and spirolides.[75] However, toxin analysis of the New Zealand strain (CAWD16) showed the presence of PSP toxins such as saxitoxins and goniotoxins but, in contrast to the Canadian species, the spirolides were not detected. Since 1997 in the northern Baltic Sea, strong bioluminescent blooms of *A. ostenfeldii* have been observed as a recurring phenomenon in late summer. At this time, the temperature of the surface water rose to around 20 °C which appears to be optimal for cell growth. In the bloom of 2002, the spirolides were detected in mussel tissue and algal biomass by LC-MS/MS analysis in co-occurrence with PSP toxins.[43] In August 2004, the phytoplankton biomass was almost mono-specific with the highest cell concentration (2×10^6 cells L^{-1}) of *A. ostenfeldii* ever reported for this species. Water surface samples and cultures of strains of *A. ostenfeldii* were collected at the time of this strong bioluminescence and showed the presence of PSP toxins but LC/MS-MS analysis surprisingly did not prove the presence of any spirolide in the isolated strains.[70]

These studies concluded that the dinoflagellates *A. ostenfeldii* and *A. peruvianum* are the most likely primary causative organisms of spirolide production, although the spirolide profile analyzed from a culture of a single cell isolate of *A. ostenfeldii* differed significantly from the one from phytoplankton harvested from the same site.

Spirolides E (**23**) and F (**24**) were not found in phytoplankton samples or even in unialgal cultures of *A. ostenfeldii*. These two keto-amine derivates have only been observed in shellfish extracts, indicating that they are likely formed through chemical or enzymatic hydrolysis in the shellfish.

A description of the spatio-temporal distribution of these polyketide-derived metabolites of *A. ostenfeldii* has been reported by Cembella *et al.*[75] This study showed that spirolides and PSP toxins in plankton and blooms often occur at the same time. However PSP toxins were present at Graves Shoal earlier than spirolides. While there is some temporal overlap of these two toxins, they are produced by different *Alexandrium* spp. By the end of June an increase in diatoms such as *Chaetoceros laciniosus* and *Skeletonema costatum* and the absence of *Alexandrium* spp. in the water column coincided with the disappearance of spirolide toxins. In contrast, diatom cells at Ship Harbour continued to dominate the plankton composition in the presence of a considerable amount of *Alexandrium* spp. cells and detectable levels of spirolides.

Physical data from the water column indicated that the appearance of spirolides is dependent on the temperature and salinity resulting from seasonal warming, and increased freshwater run-off in late spring. Further studies were carried out on the effect of environmental conditions on growth and toxin production by various strains of *A. ostenfeldii*, collected from Ship Harbour. Growth rate and cell concentration in batch cultures were measured by *in vivo* fluorescence to determine the effects of light, salinity and added inorganic nitrogen.[3] Maximum cell concentration and optimum exponential growth were observed for one specific irradiation level (100 μmol m^{-2} s^{-1}) and for the highest nitrate concentration (880 μmol L^{-1}). The observation of *A. ostenfeldii* growth under various light levels demonstrated that this species prefers lower light levels in comparison to other planktonic dinoflagellates. This light level corresponds to the typical one found at 3 to 5 metres beneath the surface in late spring at Ship Harbour. *A. ostenfeldii* can tolerate and utilise much higher levels of inorganic nitrogen than that typically available in the environment. Growth rates appear largely unaffected by salinity, although in lower salinity conditions the maximum cell concentration reached was significantly smaller. The toxin profile remained constant through the culture life cycle excluding environmental changes. The dominant component of the profile was 13-desmethyl spirolide C (**21**) followed by spirolide C (**19**), 13-desmethyl spirolide D (**22**) and some unknown spirolide derivatives as determined by LC-MS analysis. Spirolide concentration was higher at 100 μmol m^{-2} s^{-1} light intensity and higher salinity but no difference was observed for the total spirolide cell quota. In the case of inorganic nitrogen addition, spirolide concentration per cell displayed an apparent inverse relationship. At low nitrogen addition (20 μmol L^{-1}), the lowest growth rate of *A. ostenfeldii* was observed out with a higher proportion of extra-cellular spirolides, thus showing that nutrient stress affects the leakage or excretion of these compounds. A recent study shows the first evidence that external environmental factors can influence the toxin profile of *A. ostenfeldii* dinoflagellate.[81]

3 Toxicity and biological activity of the spirolides

3.1 Fast-acting toxins and the spiroimine moiety as the pharmacophore

The first discovered spirolides showed characteristic symptoms and a highly potent response in the mouse bioassay (LD_{100} 250 μg kg^{-1} *via* intra-peritoneal (i.p.) injection). Death of mice occurred within 7 min after separate i.p. injections of 5 μg of pure spirolides A–D (**17–20**) dissolved in 1 mL of 1% Tween 80.[36] For various dose groups of 13-desmethyl spirolide C (**21**) (75, 260 and 2,000 μg kg^{-1}), adult mice died within 8, 4 and 2 min respectively. In rats, administration of 2,000 μg kg^{-1} of 13-desmethyl spirolide C led to the death of the animal within 2 min. All spirolides A–D, 13-desmethyl spirolide C and 20-methylspirolide G (**30**) showed similar high toxicity in mice, with i.p. LD_{50} values between 6.9 to 99 μg kg^{-1}. The spirolides were therefore considered as fast-acting toxins. The general symptoms observed in mice after a low dose (75 μg kg^{-1}) i.p. administration of 13-desmethyl spirolide C (**21**), included the following: decreased activity and exploratory behaviour, decreased coordination, piloerection, marked abduction, moderate exophthalmia with increased lacrimation, marked abdominal muscle spasms, respiratory distress with mouth breathing developing into gasping, tremor in the hind end limbs

when moving, hyperextension of the back and arching of the tail to the point of touching the nose, contraction of front legs, respiratory arrest, convulsions and tremors involving the whole body and urination immediately preceding death within a total of 8 min.[82] The toxicity of spirolides was determined using a crude extract of a culture of *A. ostenfeldii* (strain AOSH1) containing more than 90% 13-desmethyl spirolide C (**21**) on a molar basis with other spirolide derivates. Assessment of the i.p. effect of this extract in mice revealed an LD_{50} toxicity of approximately 40 μg kg^{-1}. It has also been demonstrated that if a mouse survives past 20 min even though demonstrating characteristic symptoms after i.p. injection, it will fully and quickly recover, confirming the determination of spirolides as all or nothing fast-acting toxins.

Intragastric toxicity of the spirolide family was also studied knowing that the opening of the imine ring does not occur in stomach acid conditions for the two different classes of spirolides C/D and A/B, regardless of the presence of vicinal dimethyl groups in the seven membered imine ring. Intragastric administration of 50 μg of spirolide D (**20**) in three mice and 40 μg of spirolide A (**17**) in two mice resulted in the death of all three within 4 to 8 min for spirolide D (**20**) and the death of only one out of two in 19 min for spirolide A (**17**).[83] This result suggests that the spirolides containing vicinal dimethyl groups on the imine ring are more stable to acid or enzymatic hydrolysis and might be more potent. Moreover, oral administration of the minimal lethal dose of an extract of toxic *A. ostenfeldii* culture, containing mainly 13-desmethyl spirolide C (**21**), resulted in death of mice within 15 to 18 min. Assessment of the oral toxicity for this extract revealed an LD_{50} toxicity of 1 mg kg^{-1}, thus showing that spirolides are 25 times less potent when administered orally rather than intraperitoneally in mice.

Spirolides E (**23**) and F (**24**), the keto-amine hydrolysis derivatives of spirolides A (**17**) and B (**18**), were inactive in the mouse bioassay. Substantial studies demonstrated that the secondary amine product, obtained from the reduction of the imine in spirolide A (**17**) with sodium borohydride, also failed to elicit any toxic effect after administration to mice even at four times the equivalent dose used for spirolides A–D (**17–20**).[37] These results allowed the establishment of the spiroimine moiety as the pharmacophore in the spirolide family. This was confirmed by the fact that other marine toxins containing a similar cyclic imine group, such as gymnodimine (**16**) and the pinnatoxins (**10–12**), showed the same toxicity in the mouse bioassay *via* i.p. injection. This is also true for spiroprorocentrimine (**9**) that contains a 6,6-spiroimine unit but not a *bis*-spiroacetal moiety as found in the spirolides.

Although the pharmacophore of the spirolide family is the spiroimine unit, structural changes in other parts of the molecule also influenced the toxicity. For example, the loss of the methyl group at C19 results in a 5-fold loss of toxicity for 13,19-didesmethyl spirolide C (**28**) compared to 13-desmethyl spirolide C (**21**).[42] Spirolides containing vicinal dimethyl groups on the imine ring and also a methyl at C19 are highly toxic in mice *via* i.p. administration. Moreover, an i.p. injection of 2,000 μg kg^{-1} of spirolide H (**32**), containing a spiroacetal unit instead of a *bis*-spiroacetal ring system, did not induce death of the animal but only transient hunching and lethargy.[46]

3.2 Weak L-type calcium activators and antagonists of nicotinic and muscarinic acetylcholine receptors

To determine the biological activity of the spirolides, Perovic *et al.*[84] investigated the effect of supernatants from various dinoflagellate clones, isolated from algal blooms, on the intracellular free calcium concentration in PC12 cells (a model system for neuronal differentiation), on rat primary neuronal cells and on free calcium levels in guinea-pig cortical synaptosomes. However, the presence of spirolides or other shellfish toxins have not been proven in this study. A strain of *A. ostenfeldii* (BAHME-141) was not toxic to the PC12 cells and only induced a non-significant minimal increase in the intracellular free calcium concentration in both the PC12 cells and the synaptosomes over time. However, this species of dinoflagellate was toxic for primary neurons (TC_{50} 1.64×10^{-3} cells μL^{-1}) and a significant increase of the calcium influx in the synaptosomes was observed but only after addition of 40 mM of potassium chloride.

In vitro assays established that spirolides do not affect the NMDA receptor, the AMPA receptor and the kainate receptor. The spirolide toxins do not inhibit serine/threonine phosphoprotein phosphatases (enzymes that account for different neuronal functions including learning and memory). They also do not activate or block voltage-dependant sodium channels but are, however, weak activators (1.7 μmol dm^{-3}) of L-type calcium channels.[36] These channels are high voltage activated calcium channels found in excitable cells with permeability to calcium ions. They are responsible for excitation-contraction coupling of skeletal, smooth and cardiac muscle and also for hormone secretion in endocrine cells. Activation of these channels allows the entry of calcium ions into cells resulting in muscular contraction, upregulation of gene expression or release of hormones or neurotransmitters depending on the cell type.

The pharmacological effect of the spirolides was studied by administration of a lethal dose of toxic *A. ostenfeldii* (AOSH1) extract to mice in the presence of therapeutic doses of various drugs, leading to differences in the time to death compared to the control. The raw extract caused death in approximately 5 min. The time to death was accelerated in the presence of atropine and gallamine drugs, which are both antagonists of the muscarinic acetylcholine (mACh) receptors, whereas the time to death was extended in the presence of physostigmine and methacholine therapeutics considered as mACh agonists. Spirolides therefore demonstrated a strong affinity for the muscarinic acetylcholine receptors and appeared to mimic the action of mACh antagonists.[83] Histological examination and use of biomarkers allowed a better understanding of the toxicity of the spirolides especially for 13-desmethyl spirolide C (**21**) that was isolated in sufficient quantity from the laboratory culture of *A. ostenfeldii*. In treated mice, histological evaluation using specific stainings of various tissues and organs revealed distinct morphological differences in the brain only in comparison to control mice. Important damage was visible, the severity of the lesion being dose dependant. The hippocampus and the brain stem appeared to be the most affected areas and no transcription alterations were observed. Surprisingly, in rats, however, no clear morphological differences in brain tissues between the treated animals and the controls were observed but major transcriptional alterations were seen, by

analysis of biomarkers, for the HSP-72 (early injury markers of heat shock protein 72 KDa involved in the regulation of the apoptosis signal kinase 1) and c-jun (gene and protein that form the activator protein 1). mACh receptors 1, 4 and 5 were up-regulated by gene expression which confirmed the fact that the spirolides are antagonists of these receptors as previously described. Moreover, nicotinic acetylcholine receptor subtypes α2 and β4 also resulted in up-regulation. In short, the spirolides are fast-acting neurotoxins affecting exclusively the central nervous system (particularly the brain stem and the hippocampus) and also have an effect on astrocytes, endothelial cells and neurons.[82,85]

Bourne and co-workers[86] have very recently published an important contribution to the understanding of the biological activity of 13-desmethyl spirolide C (**21**). Binding assays, voltage-clamp recordings and complementary kinetic and equilibrium binding assays were performed on gymnodimine A (**16**) and on 13-desmethyl spirolide C (**21**) from *A. ostenfeldii*. Their binding to AChBP exhibited picomolar affinities governed by diffusion limited association and slow dissociation that account for apparent irreversibility over a short time frame. Binding and voltage-clamp recording of both toxins on muscle-type and neuronal nicotinic acetylcholine receptors revealed sub-nanomolar affinities and potent antagonism of nAChR. Moreover, the crystal structure of the A-AChBP (*Aplysia californica* ACh-Binding Protein) complex with 13-desmethyl spirolide C (**21**) was solved at 2.4/2.5 Å and showed the orientation and the conformation adopted in the binding pocket and interactions with neighbouring amino-acid residues. Multiple binding interactions involving the 5,5,6-*bis*-spiroacetal unit, 7-membered cyclic imine, cyclohexane ring and terminal γ-butyrolactone ring proved the importance of these chemical functionalities and played a role in the remarkable surface complementarity of the ligand in the binding pocket. Also, the hydrogen bonding of the protonated nitrogen of the imine unit appears key to the high affinity and the slow dissociation of these marine toxins. This study proved that 13-desmethyl spirolide C (**21**) is the most potent general non-peptidic nicotinic antagonist providing a new perspective for the detection of spiroimine toxins in shellfish and the design of novel drugs.

3.3 Metabolism of the spirolides in biological systems

To determine if the spirolides are metabolised in the shellfish biological system, Christian *et al.*[87] incubated cell-free enzyme extracts of non-toxic bivalve shellfish with the methanolic extract of a toxic strain (KO287) of *A. ostenfeldii*. LC-MS analysis of both the extract of *A. ostenfeldii* and the *in vitro* incubated shellfish tissue showed the presence of the same spirolides 13-desmethyl spirolide C (**21**), 13,19-didesmethyl spirolide C (**28**) and spirolide G (**29**) in identical percentages. This study established that spirolides C/D survived enzymatic hydrolysis conditions in shellfish and were not converted to the biologically inactive spirolides E (**23**) and F (**24**). As this class of spirolides is also not hydrolysed when incubated under human acid stomach conditions, it can therefore be toxic to humans.

However, during monitoring of mussel samples in Norway collected after the disappearance of toxic bloom of *A. ostenfeldii*, the mouse bioassay displayed an unusually long death time. LC-MS analysis of the mussel extracts showed the presence of fatty acid esters of the main 20-methyl spirolide G (**30**) present during the previous toxic episode. Various chain length fatty acid methyl esters were attached to the readily available secondary hydroxyl group at C17 in this spirolide. This conclusion was confirmed by the synthesis of the palmitoyl derivative of 20-methyl spirolide G after acylation of 20-methyl spirolide G with palmitic acid. The acylation of this spirolide is due to metabolism in the shellfish and action of enzymes such as acyl transferases.[88]

4 Biosynthesis of 13-desmethyl spirolide C

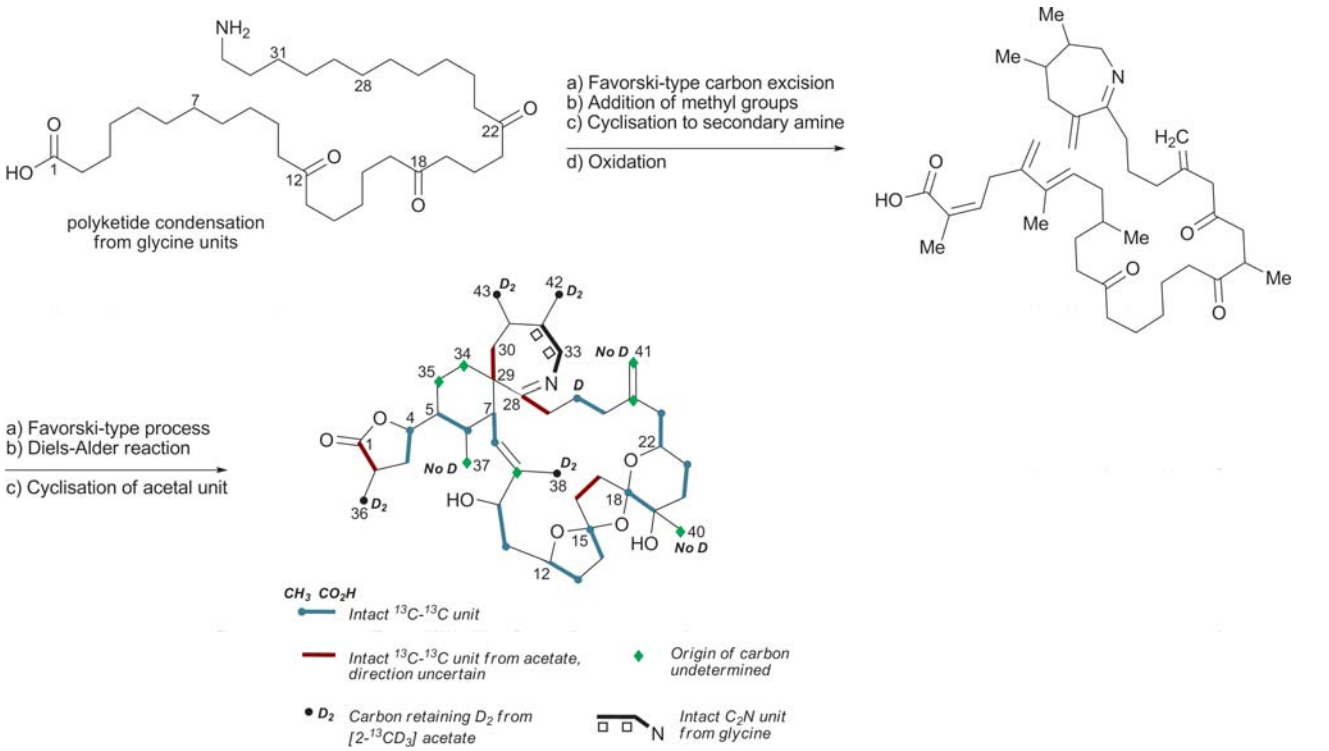
Knowing that for DSP toxins the polyketide biogenetic pathway has been proven by stable isotope labeling studies, MacKinnon *et al.*[89] reported the biosynthesis of 13-desmethyl spirolide C (**21**) by supplementing a culture of toxic *A. ostenfeldii* with the stable isotope-labeled precursors [1,2-$^{13}C_2$]acetate, [1-^{13}C]acetate, [2-$^{13}CD_3$]acetate and [1,2-$^{13}C_2$,^{15}N]glycine and measuring the incorporation pattern. Most of the carbons of this spirolide were polyketide derived and glycine was incorporated into the cyclic imine moiety (Scheme 1). The proposed biosynthesis suggested that glycine is a starter unit for polyketide condensation followed by Favorski-type rearrangement, addition of methyl groups from acetate, cyclisation to a secondary amine, oxidation to form an imine in the presence of several double bonds and finally undergoing a series of enzyme-induced reductive cyclisations to obtain the final ring system of the spirolides. This polyketide pathway for spirolide biogenesis was supported by the fact that at least four recognized PKS genes have been identified from expressed sequence tags generated from a normalized cDNA library of the same strain of toxic *A. ostenfeldii*.

5 Synthetic approaches to the spirolides

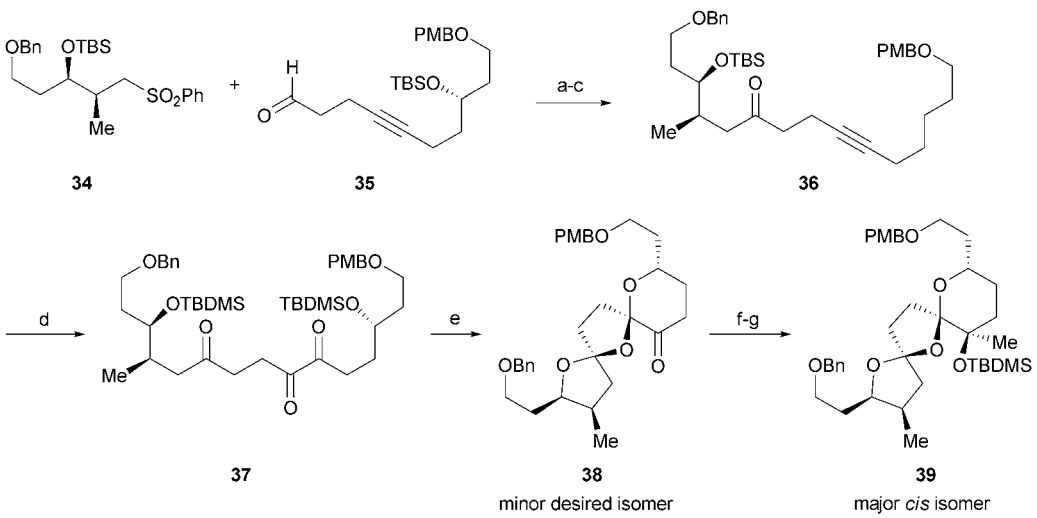
The structure of the spirolides can be divided into two parts: a *bis*-spiroacetal moiety and an unusual 6,7-bicyclic spiroimine. To date the total synthesis of the spirolides has not yet been reported, but the syntheses of the 5,5,6-*bis*-spiroacetal moiety of spirolide B (**18**) and D (**19**) have been reported by Ishihara *et al.*[90] and the Brimble group.[91,92]

In the Ishihara group synthesis (Scheme 2), the carbon skeleton of the *bis*-spiroacetal was obtained by coupling sulfone **34** with aldehyde **35** followed by oxidation and desulfurization. The resultant alkyne **36** was then oxidized in presence of ruthenium tetraoxide to give triketone **37**. The key step of this study was the one-step *bis*-spiroacetalization of this triketone using HF-pyridine which proceed smoothly to give a mixture of 4 : 1 in favour of the undesired isomer. This mixture of isomers was methylated *via* stereoselective axial addition of methyl lithium to the ketone and further silylation of the free alcohol resulted in isomerisation of the 5,5-spirocentre to give the desired compound **39** as the major *cis bis*-spiroacetal.

The Brimble group approach provides rapid access to the fully functionalized C10 to C23 *bis*-spiroacetal fragment of spirolides B (**18**) and D (**20**) (Scheme 3). Aldehyde **40** was synthesized in 5 steps in 44% overall yield from monoprotected 1,3-propanediol in which the key step was a Brown enantioselective crotylboration to establish the desired *R* configuration for both

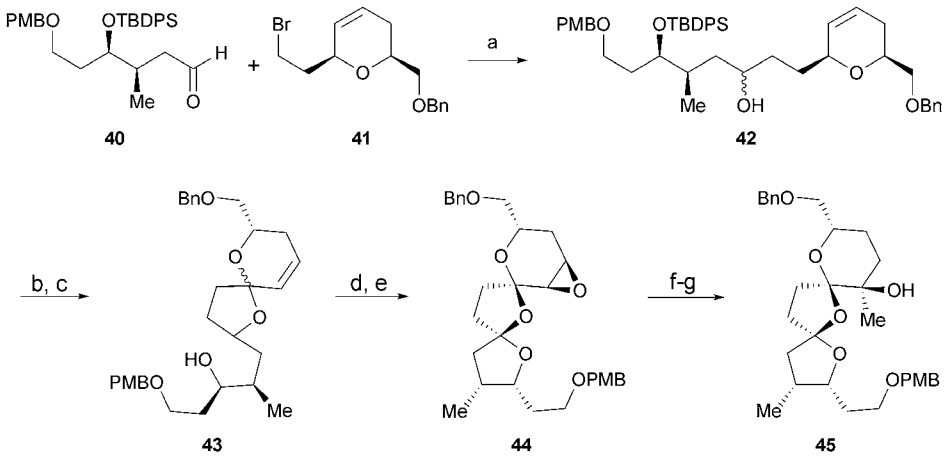


Scheme 1 Proposed biosynthesis of **21** and structure of 13-desmethyl spirolide C (**21**), showing incorporation of stable isotope labels from [1,2-$^{13}C_2$]acetate, [1-^{13}C]acetate, [2-$^{13}CD_3$]acetate and [2,3-$^{13}C_2$,^{15}N]glycine reproduced from MacKinnon *et al.*[89]



Scheme 2 Synthesis of the 5,5,6-*bis*-spiroacetal unit of spirolide B (**18**) by Ishihara group. *Reagents and conditions*: a) *n*-BuLi, THF, −78 °C, 30 min then **34**; b) DMP, $NaHCO_3$, CH_2Cl_2, rt, 40 min; c) SmI_2, MeOH, THF, 0 °C, 40 min, 86% (3 steps); d) $RuO_2 \cdot H_2O$, $NaIO_4$, CCl_4/MeCN/H_2O (1 : 1 : 5), rt, 10 h, 74%; e) HF·py, MeCN, rt, 8.5 h, 85%; f) MeLi, THF, −78 °C, 30 min, 99%; g) TBDMSOTf, 2,6-lutidine, CH_2Cl_2, 0 °C to rt, 1 h, 53%.

stereocentres. The 1,3-*cis* (*cis*/*trans* > 4 : 1) dihydropyran fragment **41** was obtained in 3 steps from enantiomerically pure *O*-benzyl protected (*R*)-(+)-glycidol making use of a remarkable silyl-modified Prins cyclisation catalysed by indium trichloride. Aldehyde **40** and bromide **41** were coupled using a Barbier reaction to afford the fully assembled linear carbon skeleton. The 5,6-spiroacetal **43** was synthesised *via* an iterative radical oxidative cyclisation[93] using $PhI(OAc)_2$ in 86% yield. Deprotection of the silyl ether followed by a second radical oxidative cyclisation afforded an equal mixture of isomers of the *bis*-spiroacetal. Further equilibration of this mixture to the most thermodynamically favored isomer due to the presence of *meta*-chlorobenzoic acid and water in the *m*-CPBA and further stereoselective epoxidation gave the β-epoxide **44** as a single diastereoisomer. Regioselective reductive opening of the epoxide **44** and oxidation of the resulting alcohol afforded a ketone that



Scheme 3 Synthesis of the 5,5,6-*bis*-spiroacetal unit of spirolide B (**18**) and D (**20**) by the Brimble group. *Reagents and conditions*: a) Mg, $Br(CH_2)_2Br$, I_2, Et_2O, rt, 88%; b) $PhI(OAc)_2$, I_2, hν, cyclohexane, rt, 86%; c) TBAF, DMF, 80 °C, 82%; d) $PhI(OAc)_2$, I_2, hν, cyclohexane, rt, 81%; e) *m*-CPBA, CH_2Cl_2, 0 °C to rt, 63%; f) DIBAL-H, hexane, 0 °C, 54%; f) DMP, CH_2Cl_2, rt, 88%; g) MeMgBr, Et_2O, −78 °C, 86%.

was finally stereoselectively methylated with methylmagnesium bromide to afford the thermodynamically favoured *trans bis*-spiroacetal **45**. Incorporation of this moiety into the larger macrocyclic system of spirolides is expected to induce re-equilibration to the desired *cis* stereochemistry.

The spiroimine unit of the spirolides has not yet been synthesised and appears to be a synthetic challenge. Studies on the unusual 6,7-spiroimine moiety of the spirolides are in progress in the Zakarian[94] and Brimble groups.[95–99] For synthesis of related marine toxins containing a spiroimine unit see the 2007 review.[33]

The successful synthesis of the 6,7-spiroimine unit of the spirolides would be a major advance for the chemical and pharmacological evaluation of the toxicity of these and related compounds. Better knowledge of spiroimine formation and stability is expected to facilitate a viable pathway for the total synthesis of the spirolides. Importantly, as the spiroimine moiety is the putative pharmacophore for the observed toxicity, its synthesis will enable extended biological testing to determine the exact mode of action of this compound class.

6 Conclusions

Due to the possible adverse impact on the shellfish consumer, HABs are monitored all over the world and various methods have been established to determine the presence of toxins in shellfish extracts. These studies have also led to the discovery of new families of marine toxins. Further studies on the development of detection methods for marine biotoxins are required to allow the simultaneous detection of known and the discovery of unknown toxins. Many problems remain unsolved making the microalgae and their toxins an attractive area of study. To date, the causes of HAB occurrence and the role of the toxic secondary metabolites in the natural environment are not yet well understood.

The spirolides were discovered for the first time along the east coast of Nova Scotia in Canada in 1995. They are metabolites of the dinoflagellate *A. ostenfeldii* and *A. peruvianum* and are sometimes found in the presence of other toxins such as PSP toxins. To date fourteen members of the spirolide family have been isolated all over the world. The relative stereochemistry of 13,19-didesmethyl spirolide C (**28**) and spirolide H (**32**) has been established by a combination of molecular modelling and NMR studies, and bears a close resemblance to the absolute stereochemistry established for pinnatoxins. The structure of the spirolides can be divided into two parts; the upper part containing the spiroimine moiety believed to be the pharmacophore, and the lower unit containing a spiroacetal or *bis*-spiroacetal unit. The spirolides are considered as fast-acting neurotoxins due to their effect in the mouse bioassay. Biological studies have shown that the spirolides are antagonists of the nicotinic and muscarinic acetylcholine receptors and also weak activators of L-type calcium channels, rendering these compounds possible candidates for the treatment of cardiovascular disorders.

In conclusion, the spirolide family of marine biotoxins discovered 15 years ago still represents an active area of research in both the chemical and biological sciences. To date, the total synthesis of spirolides remains an unmet synthetic challenge and even the putative pharmacophore of the spirolides has not yet been synthesised. Moreover, the low natural abundance of spirolides currently hampers efforts towards pharmacological evaluation, detection assay, development and possible medicinal applications.

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