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Gymnocin-A, a cytotoxic polyether from the notorious red tide dinoflagellate, *Gymnodinium mikimotoi*

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Abstract—A new cytotoxic polyether, gymnocin-A, was isolated from the notorious red tide dinoflagellate, *Gymnodinium mikimotoi*, and its structure consisting of 14 contiguous ether rings and a 2-methyl-2-butenal side-chain was determined by NMR and CID MS/MS experiments. © 2002 Elsevier Science Ltd. All rights reserved.

Worldwide blooms of unicellular microalgae, or red tides, pose serious threats to aquaculture and marine ecosystems. The noxious effects of some species are so devastating that involvement of toxins in the phenomena is easily perceived. However, obstacles due to the difficulty of culturing the organisms and the low production of the toxins have hampered chemical studies. Hitherto, only two toxin groups have been elucidated as the causative agents: the brevetoxins and prymnesins produced, respectively, by *Gymnodinium breve* and *Prymnesium parvum*.[1,2] In many other species, occurrence of toxic agents was difficult to prove by conventional fish assays and the mechanism of fish kills was left to speculative hypoxia or mechanical gill damages. The dinoflagellate, *Gymnodinium mikimotoi*, is a representative species that causes devastating damages worldwide and the mechanism of the toxic effect yet remains unknown.[3,4] Using a cytotoxicity assay instead of an elusive fish toxicity assay used in the past and by improving the extraction and purification conditions, we succeeded in isolating a new toxin designated gymnocin-A (**1**, Fig. 1). Working on **1** and its derivatives by NMR and FAB MS/MS spectrometry, we elucidated its absolute structure consisting of 14 contiguous ether rings and a 2-methyl-2-butenal side-chain. The present result sheds new light on the structural diversity of red tide toxins.

The dinoflagellate, *G. mikimotoi*, isolated at Kushimoto Bay, Japan, was cultured in 3 L Fernbach flasks with 2 L of seawater media enriched with T1 nutrients for 28 days at 25°C. Cells harvested by centrifugation were

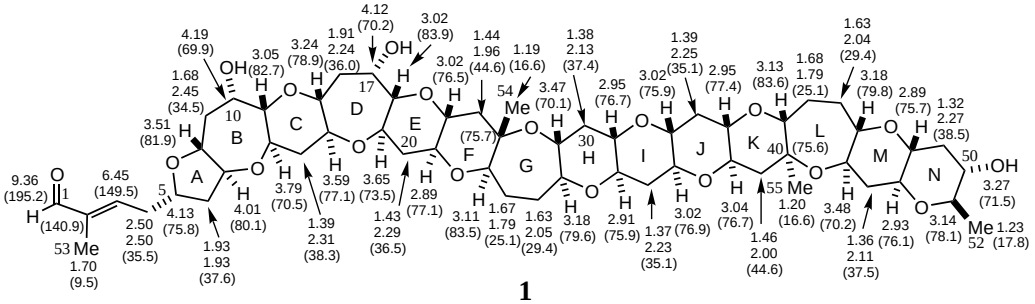

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Figure 1. Structure and NMR assignments of gymnocin-A (**1**). ^{1}H and ^{13}C (in parentheses) NMR chemical shifts of **1** were those in $CDCl_3$.

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extracted once with acetone/hexane (1:9) and thrice with 80% PrOH. The toxic residue extracted with 80% PrOH was partitioned between hexane and 80% MeOH, and the residue in the methanolic phase in 40% MeOH and $CHCl_3$. The toxins in the $CHCl_3$ phase were chromatographed on DEAE cellulose with $CHCl_3$ and MeOH. The toxins eluted with MeOH were dissolved in 80% PrOH and chromatographed on a JAIGEL W 251 column (2×50 cm, Nihon Bunseki Kogyo) with the same solvent. Finally, **1** was purified on a Develosil RPAQUEOUS column (4.6×150 mm, Nomura Chemicals) with 29% PrOH. Throughout the purification, elution of **1** was monitored with a diode array detector at 225 nm and by a cytotoxic assay.

From 1440 L cultures, 1.5 mg of the major compound (**1**) was obtained as a colorless amorphous solid: $[\alpha]_D^{20}$ +12.5 (*c* 0.11, $CHCl_3$); UV absorption maxima: 236 nm (ε 16 000); negative to a ninhydrin test; cytotoxicity against P388 at 1.3 μg/ml. The HR-FAB MS and NMR experiments indicated the molecular formula $C_{55}H_{80}O_{18}$ ($[M+Na]^+$ 1051.5244, Δ +0.2 mmu) for **1**. When ESI MS was measured by injecting an aqueous PrOH solution of **1**, dimeric ions frequently appeared at *m*/*z* 2061 $[2M+H]^+$ and 2082 $[2M+Na]^+$, obscuring the true molecular weight. In the FAB MS, however, a protonated ion $[M+H]^+$ was observed at *m*/*z* 1029 and the shift of the ion to *m*/*z* 1035 $[M+Li]^+$ by addition of Li^+ confirmed the molecular weight. Structural elucidation was performed mainly by NMR experiments.[5] The NMR spectra indicated that **1** has a cyclic polyether structure analogous with brevetoxins (BTX), yessotoxins (YTX), ciguatoxins (CTX) and maitotoxin (MTX).[6] The ^1H NMR, ^{13}C NMR, DEPT and HSQC spectra showed that **1** contains 3 singlet and 1 doublet methyls, 17 aliphatic methylenes, 29 oxymethines, 2 quaternary oxycarbons, 1 olefinic methine, 1 quaternary olefinic carbon and an aldehyde. Thus, one of the three singlet methyls resided on an olefinic carbon and the other two on ring junctions. The 2-methyl-2-butenal side-chain was determined by long range couplings from Me53 to C1, C2, and C3 on HMBC spectra, ^1H and ^{13}C NMR chemical shifts (Fig. 1), an IR absorption band at 1687 cm^{-1} and the UV maxima. Detailed analysis of ^1H–^1H COSY and TOCSY spectra in $CDCl_3$ and pyridine-d_5 led to elucidation of three partial structures from H1 to H_2-23, from H25 to H_2-39, and from H41 to H_3-52 which were interrupted by two quaternary oxycarbons bearing angular methyls. Assembling the partial structures was accomplished by HMBC experiments. Observed cross-peaks from Me54 to C23, C24 and C25, and Me55 to C39, C40 and C41 allowed the carbon skeleton to be traced from C1 to C55. The molecular formula and NMR analysis indicated that 14 ether rings and 3 hydroxy protons existed in the molecule of **1**. After acetylation of **1**, signals of H10, H17 and H50 shifted down field to 5.23, 5.15 and 4.45 ppm in $CDCl_3$, respectively. Thus, hydroxy groups were positioned at C10, C17 and C50 and the rest of oxycarbons formed ether linkages. Observed NOEs, H5/H8, H7/H12, H11/H15, H14/H19, H21/H25, Me54/H29, H28/H32, H37/H41, Me55/H45, H44/H48, and H47/H51, confirmed the positions of the ether linkages except for the rings E, I and J. Close chemical shifts of H18 and H22, H31 and H37, and H34 and H38 hampered assignment of NOE correlations, H18/H22, H31/H35 and H34/H38. The rings E, I and J were deduced from the signal shapes and chemical shifts of H_2-20, H_2-33 and H_2-36, which were typical for methylene protons in a tetrahydropyran ring. Thus, the planar structure of **1** was determined.

The planar structure was supported by FAB collision induced dissociation (CID) MS/MS experiments, in

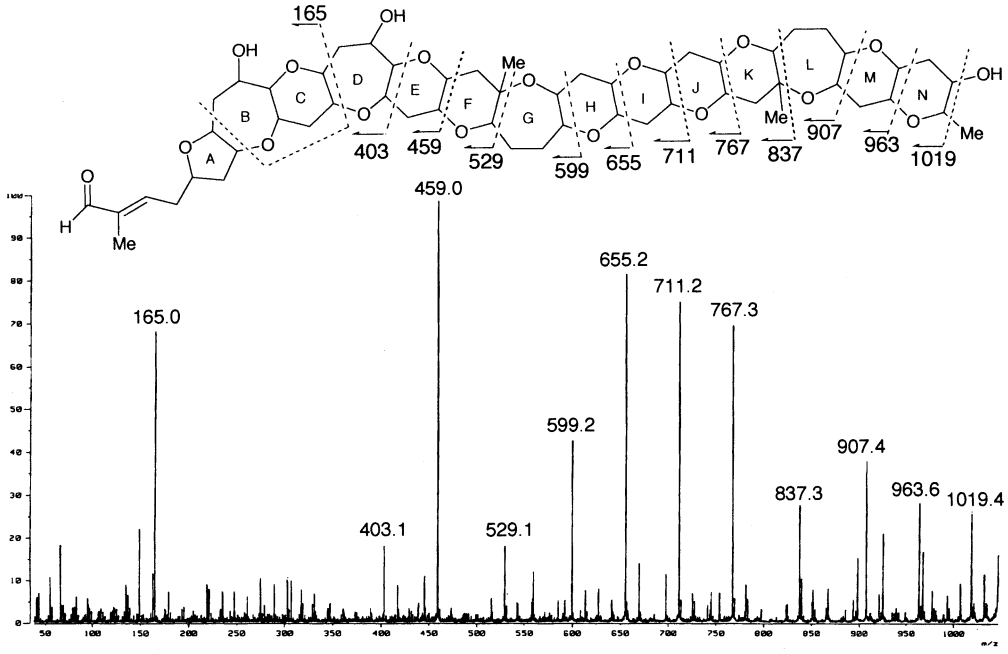

Figure 2. Positive ion FAB CID MS/MS spectrum and fragmentation patterns of **1**.

which a precursor ion at m/z 1051 $[M+Na]^+$ produced prominent product ions typical for ladderlike polyethers, YTX, MTX, and CTX.[7,8] Product ions derived from bond cleavages of rings E–N were clearly observed (Fig. 2), and the ions at m/z 599, 655, 711, 767, and 837 enabled confirmation of the contiguous six-membered rings H–K, where NMR signals severely overlapped. Product ions due to similar ring fissions of rings A–D were not observed, suggesting that the sodium ion was not set on the terminal aldehyde but on the vicinal oxygen atoms at C10-OH and the ether oxygen on the ring C or on the C17-OH and the oxygen on ring E. The location of the positive ion was further confirmed by MS/MS experiments on deuterium exchanged **1** using the precursor ion at m/z 1054 $[M-3H+3D+Na]^+$. The product ions from m/z 400 to 1200 produced by ring fissions were 2 mass units larger than those of intact **1**. The prominent ion at m/z 165 in the spectrum of intact **1** shifted to m/z 166 supporting the involvement of either C10-OH or C17-OH in localizing the positive charge. Thus, all the prominent ions in the spectra were in support of the structure deduced from the NMR data.

The NOE correlations and proton coupling constants revealed that all ether rings were fused in a *trans–cisoid* manner like in BTX and CTX. A small coupling constant (1 Hz) between H10 and H11, and NOEs, H8/H9β, H8/H11, H9α/H10, H9β/H10, H9β/H11, and H10/H11, indicated a pseudo axial disposition for 10-OH on the oxepane ring B. Similarly, a pseudo axial orientation was deduced for 17-OH from a small coupling constant between H17 and H18 and from NOEs, H15/H16β, H16α/H17, H16β/H18, and H17/H18. A large coupling constant (9 Hz) between H50 and H51 and NOE correlations from Me52 to H50 and H51 indicated equatorial orientations for both 50-OH and Me52. Based on NOE correlations from H1 to H3 and Me53 to H_2-4, the geometry of the C2–C3 double bond was determined to be *E*. All of these data allowed us to assign the relative stereostructure of **1**.

The absolute configuration of **1** was elucidated by the modified Mosher method.[9] A small amount (200 μg) of **1** was reacted with (*R*)- or (*S*)-MTPA chloride (1 mg) in pyridine with existence of TEA and DMAP at 20°C for 3 h. The reaction mixture was partitioned between $CHCl_3$ and H_2O. The pure (*S*)-MTPA (**2**) and (*R*)-MTPA (**3**) esters were obtained from the organic fraction. The position of esterification with MTPA at C50 in **2** and **3** was deduced by the proton chemical shift of H50 in $CDCl_3$ and by the HR-FAB MS.[10] By analyzing the COSY and TOCSY spectra of **2** and **3**, $\Delta\delta$ ($\delta_S-\delta_R$) values of the protons were obtained and are indicated in the partial structure in Fig. 3. The signs of $\Delta\delta$ values were symmetrically distributed around C50: those for protons Me52 and H51 were positive, while those for protons H_2-49 and H48 were negative. From these results, the absolute configuration at C50 in **1** was determined to be *S*. As the relative stereostructures of other asymmetric centers were established as discussed previously, the structure and the absolute configuration of gymnocin-A were successfully determined as shown in **1**.

Gymnocin-A is characterized by 14 contiguous and saturated ether rings and a 2-methyl-2-butenal side-chain. Two repeating ring systems 6/6/7/6/6 construct rings E–I and J–N. The number of contiguous ether rings (14) is the same as that of C-CTX-1,[11] but exceeds those of other polyethers hitherto known: BTX-B (10), CTX (12), and MTX (10). The complemental use of FAB MS/MS by choosing the sodiated molecular ion was highly effective to confirm the complex structure. The positive ion sets on vicinal oxygen atoms locating in proximity (2.8 Å between C10-OH and the ether oxygen) but not on oxygen atoms disposed *anti* (ether oxygens in rings and C50-OH and the ether oxygen in ring N). This observation will be useful in structural studies of polyethers by FAB MS/MS. Despite the structural similarities, **1** differs from BTX-B in being cytotoxic but only weakly toxic to fish. When a mixture of the gymnocins was tested on a freshwater fish, *Tanichthys albonubes*, the toxicity was 250 times less potent than that of 42-dihydroBTX-B.[12] The discrepancy between the observed massive fish kills in the field and the weak toxicity in the laboratory assay may arise from the extremely low solubility of the gymnocins to water that prevents them from reaching the fish gills. In the red tide events, *G. mikimotoi* cells were observed to stuff the fish gills, thereby enabling direct contact of the gymnocins to the gills. A similar mechanism may apply to many other red tide species that kill fish in the field but appear to be nontoxic when extracts are tested by conventional fish assays. The use of aqueous PrOH for extraction and purification was the key element for our success. The limited solubility of the gymnocins to commonly used extracts such as pure or aqueous MeOH and MeCN explains why previous attempts by others failed to detect gymnocins. Finally, the intriguing structure of **1** is a new addition to the fascinating synthetic targets of polyether compounds.[13]

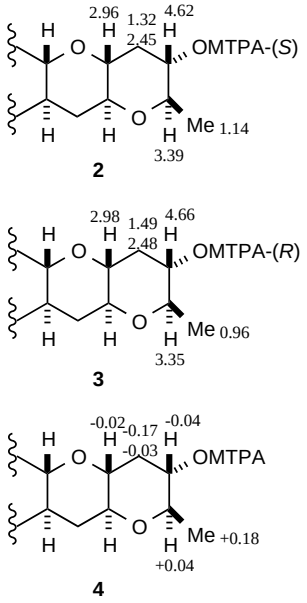

Figure 3. Partial structures of MTPA esters of **1** with the $\Delta\delta$ ($\delta_S-\delta_R$) values in ppm.

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