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Tetrahedron Letters
Tetrahedron Letters 46 (2005) 3537–3540

Gymnocin-B with the largest contiguous polyether rings from the red tide dinoflagellate, *Karenia* (formerly *Gymnodinium*) *mikimotoi*

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Received 7 February 2005; revised 9 March 2005; accepted 11 March 2005
Available online 5 April 2005

Abstract—A new cytotoxic polyether, gymnocin-B, was isolated from the red tide dinoflagellate, *Karenia mikimotoi*. Its structure was characterized by 15 contiguous ether rings and a 2-methyl-2-butenal side chain. Gymnocin-B showed cytotoxicity against P388 cells.

Globally red tides due to blooms of phytoplankton cause massive fish kills in the field. Although involvement of toxins in the phenomena was easily perceived, only the brevetoxins produced by *Karenia brevis* (formerly *Gymnodinium breve*) and prymnesins produced by *Prymnesium parvum* have been elucidated so far.[1,2] The dinoflagellate *Karenia* (formerly *Gymnodinium*) *mikimotoi* is one of the most notorious red tide species that cause devastating damages to aquaculture and marine ecosystems worldwide and the mechanism of the toxic effect to fish as yet remains unknown.[3,4] Using a cytotoxicity assay instead of the elusive fish toxicity assay used in the past and improving extraction and purification conditions, we isolated new toxins named gymnocins. Recently we successfully determined the structure of gymnocin-A (**1**), which was characterized by 14 contiguous saturated ether rings and a 2-methyl-2-butenal side chain.[5] The total synthesis of **1** was already accomplished by Tsukano and Sasaki.[6] In our continued efforts to clarify other congeners, we successfully isolated a new toxin, gymnocin-B (**2**). Its NMR spectra indicated that **2** had the 2-methyl-2-butenal side chain as in **1**, but the arrangement and the number of the ether rings of **2** differed from those of **1**. In this letter we report the structure including the relative stereostructure of gymnocin-B (Fig. 1).

The dinoflagellate *K. mikimotoi* was collected at Kushimoto Bay, Wakayama, Japan, and cultured in 3 L Fernbach flasks each containing 2 L of seawater media enriched with T1 nutrients for 28 days at 25 °C. A total of 2800-L cultures of *K. mikimotoi* was carried out. The purification of **2** was accomplished by slightly modifying the method previously reported. Cells were harvested by centrifugation, defatted with acetone/hexane (1:9), and extracted with 80% PrOH three times. After evaporating PrOH, the residue was partitioned between hexane and 80% MeOH. The residue in the latter solution was redissolved in 40% MeOH and extracted with $CHCl_3$. The toxins obtained in the $CHCl_3$ phase were chromatographed on DEAE cellulose (3 × 13 cm) with $CHCl_3$, MeOH, and $CHCl_3$–AcOH (3:1) in this order. The $CHCl_3$ fraction was dissolved in 50% THF and chromatographed on a Develosil Lop ODS column (2 × 18 cm, Nomura Chemicals) with 50%, 60%, 80% THF in this order. The toxins in the first eluate were chromatographed on a JAIGEL W251 column (2 × 50 cm, Nihon Bunseki Kogyo) with 80% PrOH and then on Develosil ODS UG-5 (10 × 250 mm, Nomura Chemicals) with 50% THF. Finally, **2** was purified on a Develosil C30 UG-5 column (10 × 250 mm, Nomura Chemicals) with 35% PrOH. Throughout the purification, elution of **2** was monitored with a diode array detector at 230 nm and by a cytotoxic assay. Finally,

Keywords: Gymnocin-B; Polyether; Red tide dinoflagellate; *Karenia mikimotoi*.
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doi:10.1016/j.tetlet.2005.03.115

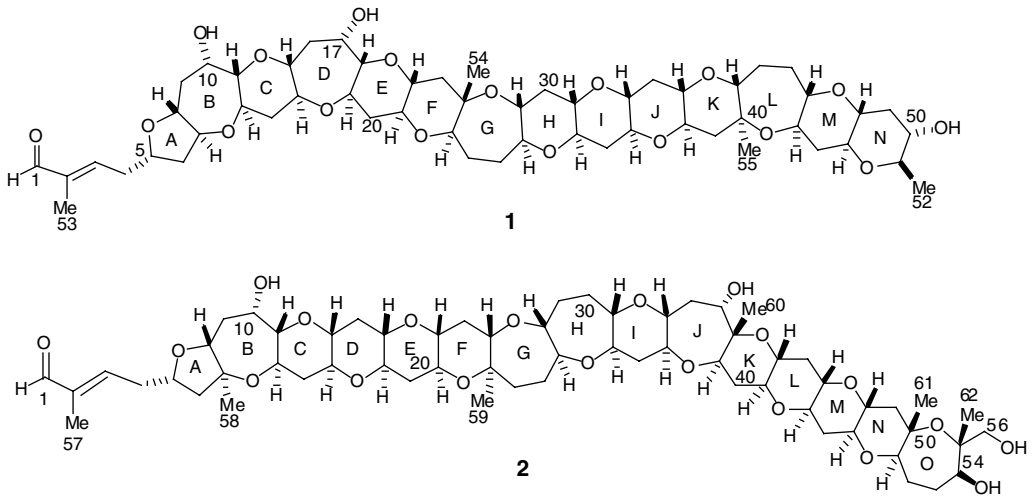

Figure 1. Structures of gymnocin-A (**1**) and gymnocin-B (**2**).

2.8 mg of the major compound (**2**) was obtained as a colorless amorphous solid.

The UV absorption maxima was observed at 230 nm (ε 17,300). The HR-FAB MS and NMR experiments indicated the molecular formula $C_{62}H_{92}O_{20}$ ($[M+H]^+$ 1157.6270, Δ +0.9 mmu) for **2**. In the NMR spectra of **2**, signals belonging to a 2-methyl-2-butenal side chain were observed analogous with **1**, but other signals belonging to a skeletal structure distinctly differed from those of **1**. The ^{1}H NMR, ^{13}C NMR, DEPT, and HSQC spectra showed that **2** contained 6 singlet methyls, 19 aliphatic methylenes, 1 oxymethylene, 28 oxymethines, 5 quaternary oxycarbons, 1 olefinic methine, 1 quaternary olefinic carbon, and 1 aldehyde. Thus, one of the singlet methyls resided on an olefinic carbon and the other five on ring junctions, interrupting the proton connectivities. The 2-methyl-2-butenal side chain was determined by long-range correlations from Me57 to C1, C2, and C3 on the HMBC spectra and the analogous ^{1}H and ^{13}C NMR chemical shifts (Table 1) with **1**. Detailed analysis of ^{1}H–^{1}H COSY and TOCSY spectra in $CDCl_3$ and pyridine-d_5 measured with Varian INOVA600 led to elucidation of 6 partial structures from H1 to H6, from H8 to H23, H25 to H37, H39 to H49, H51 to H54, and H_2-56. The partial structures interrupted by the five quaternary oxycarbons at the ring junctions were assembled by HMBC experiments. The observed cross-peaks from Me58 to C6/C7/C8, from Me59 to C23/C24/C25, from Me60 to C37/C38/C39, from Me61 to C49/C50/C51, and from Me62 to C54/C55/C56 made it possible to trace the carbon skeleton from C1 to C62. Hydroxy protons were observed at 2.25, 2.51, 1.59, and 2.10 ppm in $CDCl_3$, and those were coupled with H10, H37, H54, and H56, respectively. Thus the position of hydroxy groups was assigned at C10, C37, C54, and C56, and the rest of oxycarbons formed ether linkages. The observed NOEs, H5/H8, Me58/H12, H11/H15, H20/Me59, H23/H28, H27/H32, H31/H35, H34/H39, Me60/H42, H47/H51, and Me61/Me62 confirmed the positions of the ether linkages except for those on rings D, E, L, and M. Strong NOE correlations between protons δ_H 2.95 and 3.05 were observed. However, close chemical shifts of protons hampered attempts to distinguish NOE correlations H14/H18, H17/H21, H41/H45, and H44/H48. Therefore, rings D, E, L, and M were

Table 1. ^{1}H and ^{13}C chemical shifts of **2** in $CDCl_3$

Pos	^{1}H	^{1}H	^{13}C	Pos	^{1}H	^{1}H	^{13}C
1	9.37		195.1	34	3.49		78.3
2			140.9	35	3.23		78.7
3	6.46		149.4	36	1.79	2.39	35.8
4	2.51	2.56	35.7	37	3.75		74.7
5	4.13		74.9	38			79.7
6	1.63	2.23	47.1	39	3.82		74.0
7			81.6	40	1.58	2.09	32.3
8	3.50		82.2	41	2.93		77.9
9	1.63	2.39	30.4	42	3.24		69.2
10	4.22		68.1	43	1.34	2.14	35.4
11	3.18		82.4	44[a]	3.04		76.9
12	3.93		64.6	45[a]	3.05		76.7
13	1.45	2.28	36.5	46	1.43	2.29	35.2
14	2.93		78.4	47	2.97		77.7
15[a]	3.06		77.2	48	3.02		76.1
16	1.35	2.24	35.1	49	1.47	2.00	45.9
17[a]	3.06		77.3	50			77.0
18[a]	3.05		77.4	51	3.05		85.3
19	1.35	2.15	35.5	52	1.64	1.88	23.5
20	3.21		68.9	53	1.75	1.85	27.5
21	2.95		76.4	54	3.76		72.2
22	1.54	2.00	32.7	55			81.2
23	3.13		83.2	56	3.17	3.34	71.1
24			77.2	57	1.70		9.5
25	1.69	1.71	36.9	58	1.35		19.1
26	1.68	2.03	29.4	59	1.14		15.9
27	3.42		83.4	60	1.11		14.4
28	3.43		83.8	61	1.44		18.5
29	1.84	1.84	30.4	62	1.27		20.5
30	1.74	1.76	27.6	10-O*H*	2.25		
31	2.84		82.1	37-O*H*	2.51		
32	3.06		81.5	54-O*H*	1.59		
33	1.36	2.24	39.1	56-O*H*	2.10		

^{1}H internal reference was set at 7.21 ppm. ^{13}C internal reference was set at 77.0 ppm.

[a] ^{13}C chemical shifts were interchangeable.

confirmed from the unsaturation number, and coupling constants (d, d, d, $J = 9$–10 Hz) of axial protons and chemical shifts of H_2-16, H_2-19, H_2-43, and H_2-46, which were typical for methylene protons in a tetrahydropyran ring. The signal shapes of H_2-16, H_2-19, H_2-43, and H_2-46 and NOE correlations H_{ax}-16/H14/H18, H_{ax}-19/H17/H21, H_{ax}-43/H41/H45, and H_{ax}-46/44/H48 indicated a chair conformation of rings D, E, L, and M. The planar structure was further supported by FAB collision-induced dissociation (CID) MS/MS experiments, in which a precursor ion at *m/z* 1163.6 $[M+Li]^+$ produced prominent product ions typical for ladderlike polyethers, YTX, MTX, and CTX.[7,8] Interestingly, two sets of product ions were generated. The lithium ion was set on two nearby oxygen atoms: on 54-OH and 56-OH on ring O, and 10-OH and 11-ether-O of ring C. From the former lithiated (Li-adducted) ion, product ions from rings A to L were clearly observed. The ions at *m/z* 937, 881, 825, 769, and 713 confirmed the contiguous six-membered rings C–F, where NMR signals severely overlapped. Furthermore, the product ions at *m/z* 713, 629, 559, 503, 417, and 347 confirmed the ring arrangement G-K as 7/7/6/7/6, and the methyl on the junction between rings F and G, and J and K and the hydroxy on ring J. From the lithiated ion in which the charge was set on rings B and C, the observed product ions at *m/z* 1031, 961, 905, 849, 427, 371, and 315 were generated by cleavage of the six-membered rings. Thus, all the prominent ions in the spectra supported the planar structure deduced from the NMR data (Fig. 2).

The NOE correlations and proton coupling constants revealed that all ether rings were fused in a *trans-cisoid* manner as in BTX and CTX. A small coupling constant (1 Hz) between H10 and H11, and NOEs, H8/H9β, H8/H11, H9α/H10, H9β/H10, and H10/H11, indicated a pseudoaxial disposition for 10-OH on the oxepane ring B. Similarly, a pseudoaxial orientation was deduced for 37-OH from NOEs, H37/Me60, 37-OH/H39, H36α/H37, and H36β/Me60. An NOE correlation between H51 and H54 indicated β orientation for 54-OH. The geometry of the C2–C3 double bond was *E* based on NOE correlations from H1 to H3 and Me57 to H_2-4. The relative stereostructure of **2** was thus assigned. Esterification of 54-OH with MTPA was unsuccessful probably due to steric hindrance of ring O. Therefore, the absolute stereostructure of gymnocin-B is unelucidated. We are continuing studies for determining the absolute stereostructure.

Gymnocin-B has 15 saturated contiguous ether rings and a 2-methyl-2-butenal side chain. The number of contiguous ether rings (15) is the largest among the polyether compounds hitherto known.[5,9] Gymnocin-B showed cytotoxicity against mouse lymphoide P388 cells at 1.7 μg/mL. Although the structural backbones of **1** and **2** were quite different except for the terminal side chain, their cytotoxic potency was very close. The complex biosynthetic route to produce structurally and biologically diverse polyethers in dinoflagellates is intriguing. The use of FAB MS/MS by choosing the lithiated ion was highly effective to confirm the complex structure. Especially, the lithium ion was set on respective terminus, producing two sets of ions complemental to each other. Gymnocin-B possesses two contiguous oxepane rings in the middle of the structure and a terminal conjugated aldehyde analogous with BTXB. Despite the structural similarities, **2** differs from BTXB in being cytotoxic but only weakly toxic to fish. When a mixture of the gymnocins was tested on a fresh water fish, *Tanichthys albonubes*, the toxicity was 250 times less potent than that of 42-dihydroBTXB.[10] The discrepancy between the observed massive fish kills in the field and the weak toxicity in the laboratory assay may arise from the fact that the extremely low solubility of the gymnocins to water prevents them from reaching the fish gills. In the red tide events, *K. mikimotoi*, cells were observed

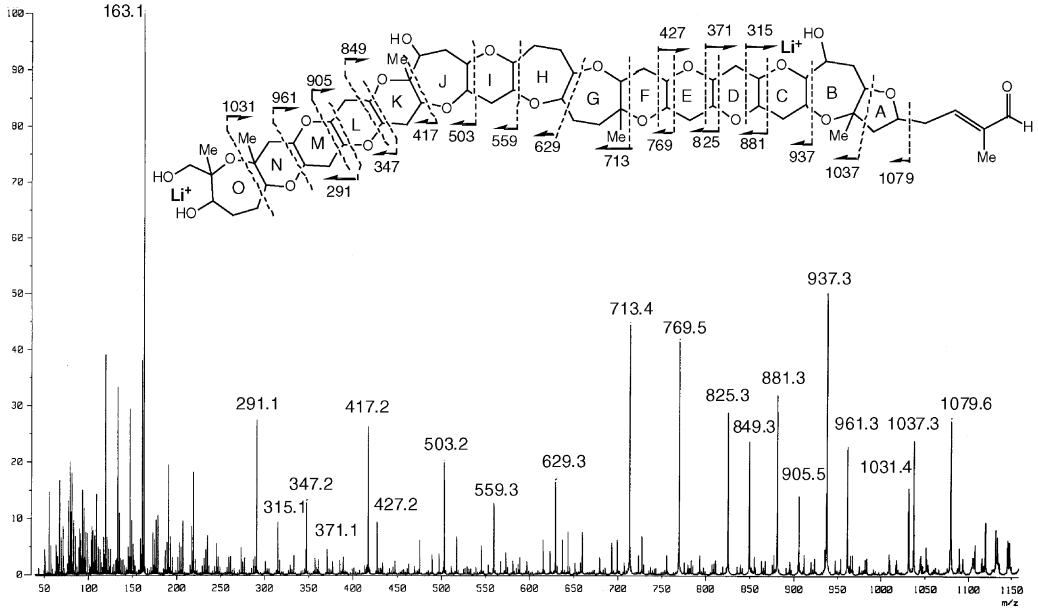

Figure 2. Positive ion FAB CID MS/MS spectrum and fragmentation patterns of **2**.

to stuff the fish gills, enabling thereby 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 recent progress of total synthesis of polyether compounds was outstanding.[11] The intriguing structure of **2** is a new addition to the fascinating synthetic targets.

Acknowledgements

We are grateful to Mr. T. Yano, Miss A. Konno, M. Shoji, and Miss M. Abe of Tohoku University for culturing *K. mikimotoi* and purification of gymnocins. This work was supported by grant-in-aid from the Ministry of Education, Science, Sports and Culture, Japan (No. 13760080) and CREST.

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