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Isolation and Structure of a New Brevetoxin Analog, Brevetoxin B2, from Greenshell Mussels from New Zealand

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Abstract: A new brevetoxin analog, brevetoxin B2 (BTXB2), was isolated from greenshell mussels, *Perna canaliculus*, collected at the time of the neurotoxic shellfish poisoning incident in New Zealand. The structure was elucidated based on NMR, CAD FAB MS/MS, and chemical degradation experiments.
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INTRODUCTION

Food poisoning due to ingestion of bivalve shellfish that have been exposed to blooms of the toxic dinoflagellate, *Gymnodinium breve*, is termed neurotoxic shellfish poisoning (NSP) because of the prevailing neurological symptoms in patients.[1,2] Although precise knowledge on toxin structures in shellfish is essential for developing proper determination methods and for toxicological evaluation, little chemical study has been conducted previously, presumably due to the notion that those toxins were the same brevetoxins as were in *G. breve*.[3] In early 1993 a large scale incident of neurotoxic shellfish poisoning (NSP) occurred for the first time in New Zealand.[4] Over 280 people had symptoms after consumption of shellfish. Concurrent blooms of *G. breve* were also observed. The incident prompted us to initiate chemical studies on the toxins in the shellfish. Previously we elucidated the structure of a new brevetoxin analog, brevetoxin B3 (BTXB3).[5] Now we report the structure of another new analog designated brevetoxin B2 (BTXB2, **1**). BTXB2 differed from brevetoxin B (BTXB, **2**) in the structure of the side-chain (Fig. 1).

RESULTS

Isolation

Excised hepatopancreas of greenshell mussels, *Perna canaliculus*, collected in January 1993 at Coromandel Peninsula, North Island of New Zealand, were extracted with acetone. After solvent removal the residue was partitioned first between water and EtOAc to obtain BTXB3 and an additional unidentified toxin in the organic phase. BTXB2 retained in the aqueous layer was then extracted twice with BuOH. After removal of

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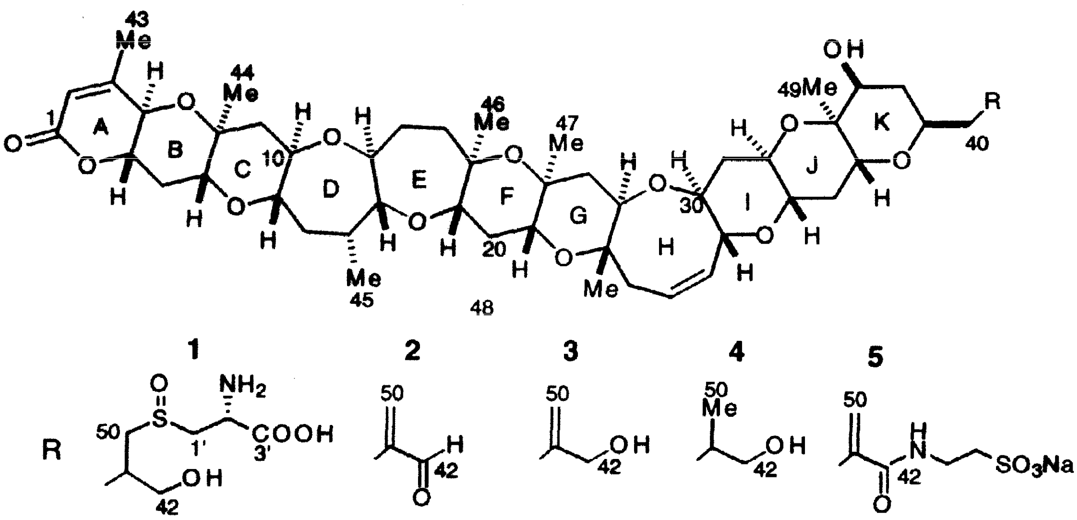

Fig. 1 Structures of the brevetoxins. Carbon numbers of **1** were given based on those of **2**.

BuOH by evaporation, the residue was purified by successive chromatography on gel permeation and reversed phase columns. The toxic eluates were monitored by UV absorption at 210 nm and mouse bioassays. BTXB2 was a major toxin in the polar fraction and accounted for one third of total mouse lethality.

Structure

From 30 kg of hepatopancreas 29.4 mg of BTXB2 was obtained as colorless amorphous solid; HR-FABMS, *m/z* 1034.5171 $[M+H]^+$ (cald. for $C_{53}H_{80}O_{17}NS$, 1034.5147); no UV maxima above 210 nm; $[\alpha]_D^{25}$ +19° (*c* 0.58, $MeCN/H_2O$ 1:1); CD λext ($MeCN/H_2O$ 1:1, 256 nm $\Delta\varepsilon$ +1.98; 227 nm $\Delta\varepsilon$ -1.97); positive to ninhydrin test. The result of the ninhydrin test and the IR spectrum (1610 cm^{-1}) suggested that an α-amino group existed in the molecule. The presence of a sulfur atom in the molecule was confirmed by energy dispersive X-ray analysis (solid pellet). BTXB2 oxidized HI to liberate I_2 and produced by *m*-chloroperbenzoic acid (MCPBA) oxidation[6] a sulfone ($[M+H]^+$, *m/z* 1050). Thus, the sulfur atom should exist as sulfoxide.

The 1D proton NMR spectrum resembled that of BTXB (**2**) but lacked signals due to aldehyde and exo-methylene in the side-chain of **2**. ^1H-^1H COSY, HSQC, and HMBC measurements revealed good agreements between **1** and **2** in the connectivities, chemical shifts and coupling constants of protons from H-2 to H-39. Hence, **1** and **2** had the same backbone structure including the stereochemistry (Table 1). On ^{13}C NMR and ^1H-^1H COSY spectra of **1**, signals arising from the poly-cyclic-ether part were clearly observed, while those of the side-chain were split and broadened. Changing temperature or radiowave frequency did not improve the signal shape, ruling out the possible conformational perturbation observed in ciguatoxin.[7] Therefore, **1** seemed to be a diastereomeric mixture having two chiral centers at C41 and sulfoxide. In spite of the broadened and split signals, the terminal unit was deduced to be cysteine sulfoxide, based on the typical chemical shifts of proton and carbon signals: α-amino methine, ^1H 4.05 and 4.07 ppm and ^{13}C 49.9 and 50.9 ppm; β-methylene, ^1H 3.04-3.35 ppm and ^{13}C 51.2 and 51.4 ppm. The cysteine sulfoxide unit was also supported by cross peaks

Table 1. NMR Chemical Shifts (δ) of **1** and **2**.

	BTXB2 (1)			BTXB (2)[c]			BTXB2 (1)			BTXB (2)[c]	
Pos.	^{13}C	^1H		^1H		Pos.	^{13}C	^1H		^1H	
1	165.2					28	134.6	5.69		5.69	
2	114.3	5.69		5.69		29	79.0	3.97		3.97	
3	162.7					30	75.6	3.49		3.49	
4	67.7	4.37		4.37		31	36.1	1.45	2.09	1.45	2.09
5	76.1	4.03		4.03		32	68.4	3.39		3.39	
6	28.9	1.80	2.14	1.80	2.14	33	76.1	3.09		3.01	
7	78.2	3.16		3.16		34	29.2	1.48	1.91	1.38	1.70
8	74.4					35	62.7	3.85		3.91	
9	43.5	1.41	2.04	1.41	2.04	36	74.4				
10	82.7	3.37		3.37		37	70.3	3.73		3.73	
11	84.5	3.09		3.09		38	31.9	1.57	2.18	1.57	2.18
12	34.2	1.48	1.69	1.48	1.69	39	69.2-70.2[a]	3.98		3.84	
13	32.2	1.88		1.88		40	33.2-33.5[a]	1.32-2.44[a]		2.21	3.13
14	86.9	3.49	2.55	3.49	2.55	41	32.6	2.06			
15	83.4	3.58		3.58		42	61.3-63.7[a]	3.48-3.59[a]		9.36	
16	28.2	1.49	2.04	1.49	2.04	43	16.2	1.87		1.87	
17	36.9	1.60	1.69	1.60	1.69	44	14.7	1.20		1.20	
18	78.1					45	17.5	0.92		0.92	
19	87.1	3.12		3.12		46	20.3	1.19		1.19	
20	27.6	1.59	1.69	1.59	1.69	47	18.7	1.07		1.07	
21	73.2	3.35		3.35		48	17.2	1.18		1.60	
22	73.9					49	13.1	1.13		1.09	
23	39.9	1.39	1.76	1.39	1.76	50	53.1-55.0[a]	2.82-3.07[a]		6.13	6.37
24	74.6	3.93		3.93		1'	51.2, 51.4[b]	3.04, 3.35[b]			
25	79.9					2'	49.9, 50.9[b]	4.05, 4.07[b]			
26	38.1	2.21	2.43	2.21	2.43	3'	170.5, 170.8[b]				
27	126.7	5.68		5.68							

NMR chemical shifts in CD_3CN/D_2O (1:1), at 400 MHz. CHD_2CN as δ 1.91.

[a] Chemical shifts were not determined precisely because of split signals.

[b] Pairs of signals were observed.

[c] ^{13}C NMR spectrum was not measured because of the poor solubility in this solvent.

from both H_2-1' and H-2' to C-3' carboxyl carbon on the HMBC spectrum. Attempts to separate diastereomers by HPLC after acetylating the amino and hydroxyl groups or by methylating the carboxyl group were unsuccessful. However, both methylation of the terminal carboxyl and acetylation of the amino group gave rise to sharpened cross peaks on COSY spectra, thereby allowing us to elucidate connectivity from H-39 to H_2-42. The chemical shifts of H_2-50 (about 3.1 ppm) and C-50 (55 ppm) well matched those of a sulfoxide-bearing methylene. NOEs between H-35 and H_2-40 indicated that the side-chain is in β-orientation, as is in **2**. The slight difference of chemical shifts around rings J and K from those in **2** can be accounted for by the modification in the side-chain.

The planar structure of **1** was further tested by collisionally activated dissociation (CAD) in negative FAB MS/MS experiments carried out on $[M-H]^-$ ion (*m/z* 1032) of **1**. The validity of this methodology was well established in previous structural studies on yessotoxin,[8] maitotoxin,[9] and brevetoxin B3.[5] Prominent ions (*m/z* 87, 135 and 897) generated by bond cleavages at both sides of sulfoxide as well as other fragment ions were consistent with the proposed structure (Fig. 2).

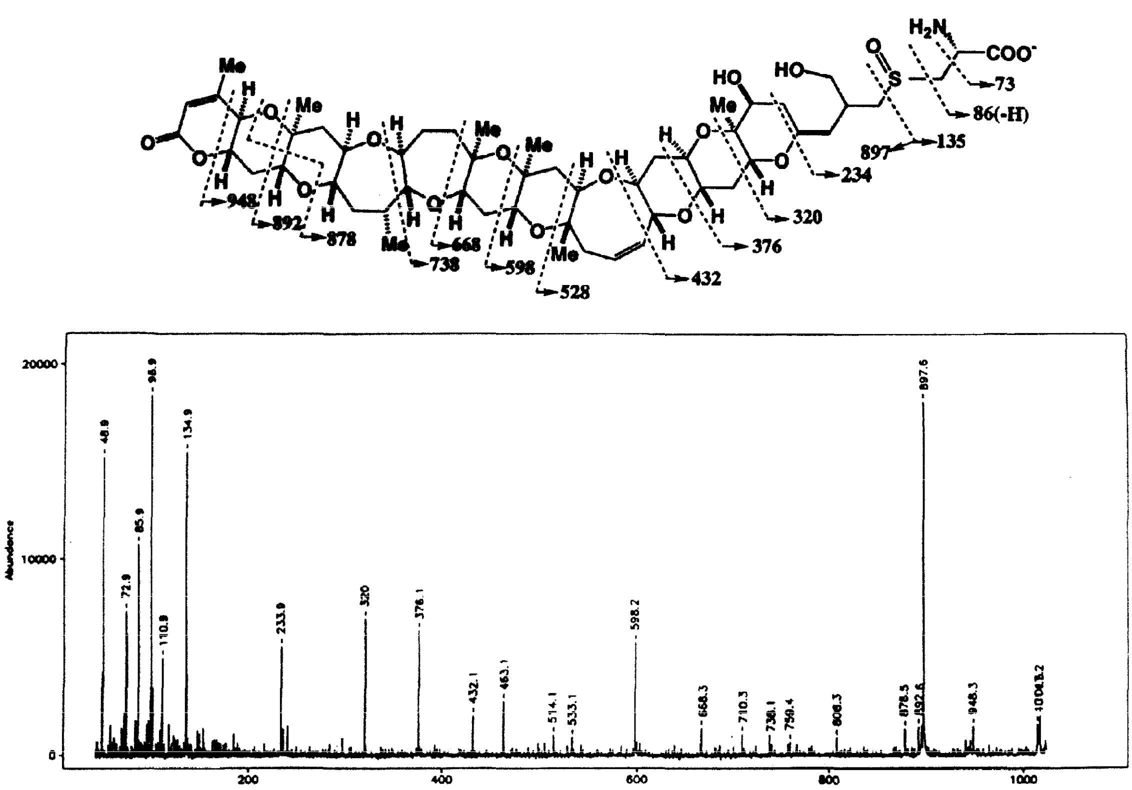

Fig. 2. Negative CAD MS/MS of **1** with a molecular ion at *m/z* 1032 as a precursor.

BTXB2 has never been reported from *G. breve*.[4] Nor was it found in other bivalves collected in New Zealand. Most likely **1** was a specific metabolite of greenshell mussels and produced from **2** by 1,4 addition of cysteine followed by oxidation of the sulfide to sulfoxide and reduction of the aldehyde to alcohol. Alternatively, **1** could be produced from 42-dihydrobrevetoxin B (acronym PbTx-3, **3**), another major toxin of for by racemization during reduction. On the NMR spectra of *dl*-*S*-methyl-L- cysteine sulfoxide prepared by oxidation of *S*-methyl-L-cysteine, all proton and carbon signals were split into a 1:1 ratio, confirming the stereochemical effect of the sulfoxide function. Thus, all the results unanimously pointed to the structure **1**.

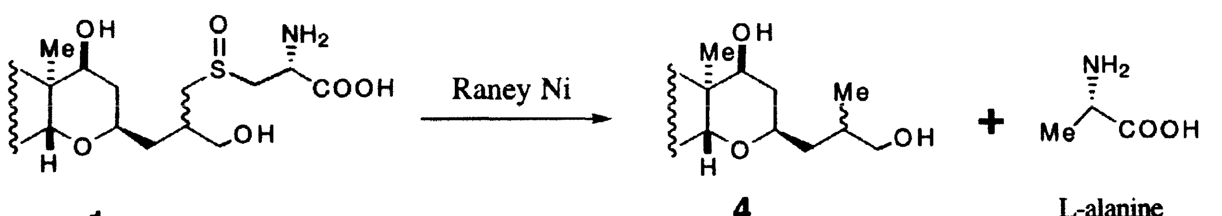

Fig. 4. Reductive degradation scheme of **1**.

Biological properties

The minimum lethal dose of **1** to mice (ddY, male, i.p.) was estimated to be 306 µg/kg. Mice showed convulsion, paralysis of limbs, and dyspnea and died within 1 hr. Unlike the brevetoxins, **1** did not kill freshwater fish *Tanichthys albonubes* at 0.1 ppm. Cytotoxicity to Neuroblastoma cells through Na-channel