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Tetrahedron Letters 45 (2004) 29–33
Tetrahedron Letters

Brevetoxin B5, a new brevetoxin analog isolated from cockle *Austrovenus stutchburyi* in New Zealand, the marker for monitoring shellfish neurotoxicity

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Received 30 September 2003; revised 22 October 2003; accepted 23 October 2003

Abstract—A new brevetoxin analog, brevetoxin B5 (BTX-B5) was isolated together with BTX-B1 and PbTx-3 from the New Zealand cockle *Austrovenus stutchburyi* harvested at an outbreak of neurotoxic shellfish poisoning early in 1993. The structure was elucidated by comparison of its spectral data (NMR and CAD FAB MS/MS) with those of BTX-B1 and PbTx-2, and confirmed by synthesis from PbTx-2 with SeO_2 and H_2O_2. It was detected in the toxic greenshell mussel *Perna canaliculus* and Pacific oyster *Crassostrea gigas* collected at the same time.
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Human food poisoning caused by ingestion of shellfish exposed to the red tide of the dinoflagellate *Gymnodinium* (G.) *breve* is known as neurotoxic shellfish poisoning (NSP) because of the characteristic neurological symptoms in patients.[1,2] Due to the extreme difficulty of brevetoxin isolation from shellfish, only a few studies of these compounds have been carried out. An outbreak of NSP intoxication following ingestion of bivalves between December 1992 and March 1993 in New Zealand gave us the opportunity to initiate the chemical elucidation of the toxins involved in this incident.[3] During our study of this shellfish poisoning episode, we had isolated brevetoxins, PbTx-2, and PbTx-3 from oyster *Crassostrea* (C.) *gigas*, and the brevetoxin analog brevetoxin B1 (BTX-B1) and PbTx-3 from cockles, collected at Tiki Road, Coromandel Peninsula, and Whangarei in the North Island of New Zealand in January 1993, respectively (Fig. 1).[4–6] Recently four new brevetoxin analogs, named brevetoxin B5–B8 were detected in the above cockles. In this paper, we report the isolation, chemical structure and preparation of BTX-B5, in which the PbTx-2 skeleton is modified by oxidation of the aldehyde terminus to carboxylic acid. Detection of BTX-B5 in the toxic shellfish was also examined.

Cockles (160 kg) from Whangarei were shucked, lyophilized, and ground, then extracted twice with 80% MeOH under reflux. The extract was partitioned between CH_2Cl_2 and H_2O. The organic part was further partitioned between *n*-hexane and 80% MeOH. The methanolic part (109.2 g) was chromatographed on SiO_2 with CH_2Cl_2–MeOH–H_2O (95:5:0, 65:15:2, 65:20:3, and 65:45:10) to get fr-1 to fr-4, respectively. Fr-1, 2, and 3 showed potent neurotoxicity in mouse assay.

Fr-2 and 3 gave two active fractions, 70% and 80% MeOH eluates on ODS-A_{60} (YMC-Gel). Successive chromatography of fr-5 on Sephadex LH-20, and finally YMC ODS-A_{324} gave BTX-B1 (12 mg), as reported previously.[4]

Further purification of fr-1 by successive chromatography on Sephadex LH-20 with MeOH, SepPak C_{18} with 80% MeOH, Puresil C_{18} (Millipore) with 85% MeOH, and finally LiChroCART RP-18 (Merck) with 80%

Keywords: brevetoxin; BTX-B5; New Zealand; NSP.
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doi:10.1016/j.tetlet.2003.10.124

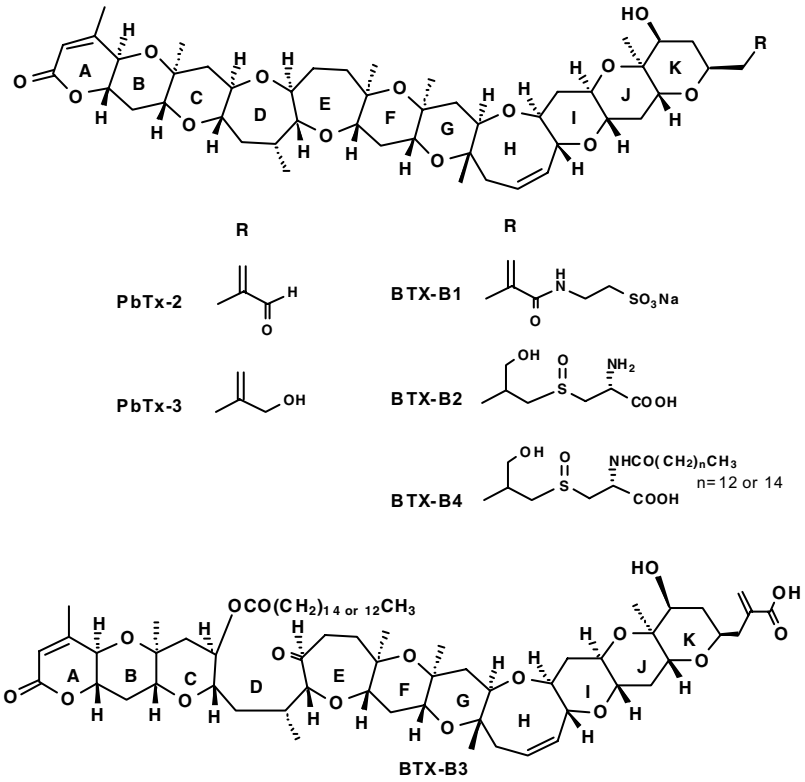

Figure 1. The structures of brevetoxins (PbTxs) and the analogs of PbTx-2 (BTX-Bs).

MeOH afforded PbTx-3 (ca. 2.4 mg) as in the previous report.[6,7]

Brevetoxin B5 (BTX-B5) (ca. 500 μg) from fr-6 was purified then by successive chromatography on Sephadex LH-20 with MeOH, Puresil C_{18} (×2) with 80% MeOH, and finally LiChroCART RP-18 with 80% MeOH. The toxic eluate was monitored by mouse bioassays. Full details of the isolation procedure for BTX-B5 will be published elsewhere.

The toxin BTX-B5 thus obtained is a colorless amorphous solid: FABMS m/z negative, 909 $(M-H)^-$; positive, 933 $(M+Na)^+$. HR-FBMS, m/z 933.4645 $(M+Na)^+$ (calcd for $C_{50}H_{70}O_{15}$, 933.4642). The IR absorptions at λ_{max} (KBr) 3446, 1735, 1652, 1609, 1230, 1211, and 865 cm^{-1} suggested the presence of hydroxyl and both conjugated carboxylate and carboxyl functions in the molecule. This compound has its absorption maximum near the end UV λ_{max} (MeOH) 205 nm (ε 27,300) due to the conjugated carboxylate and carboxylic acid chromophores, like PbTx-2 and BTX-B1.[4,8]

The 1D proton NMR (CD_3OD) spectrum of BTX-B5 resembled that of PbTx-2 but lacked the signal due to aldehyde, and is virtually identical with that of BTX-B1 except for the absence of signals due to the taurine moiety in the side chain (Fig. 1). ^1H–^1H COSY measurement revealed good agreement in the connectivities, chemical shifts, and coupling constants of protons from H2 to H40 between BTX-B5 and BTX-B1 (**2**). Hence, BTX-B5 has the same polycyclic ether part, including the stereochemistry, as that of BTX-B1. Connectivity of C40–C41–C50 was confirmed by allyl coupling observed between H2-40 and H2-50 and HMBC correlation from H2-50 to C40. Thus, the functional group at C42 in the side chain of BTX-B5 is –COOH (Fig. 2).

BTX-B5 (MeOH) showed a negative maximum (Δε −5.78, ene–lactone ππ*) at 227 nm and a positive one (Δε +6.88, ene–lactone nπ*) at 257 nm in the CD spectrum; they are similar to those observed for BTX-B1 and PbTx-2.[4,8] NOEs between H-35 and H2-40 indicated that the side chain is in β-orientation. These results show that BTX-B5 has the same absolute configuration as PbTx-2.

The proposed structure was well supported by collisionally activated dissociation, negative ion FAB MS/MS experiments carried out on the $(M-H)^-$ ion (m/z 909) of BTX-B5. Bond cleavage between C37-C36 and C39-O was evidenced by ion 111 (Fig. 3). Other prominent ions were generated by characteristic bond cleavage at ether rings, as established in BTX-B3,[9] and were consistent with the proposed structure.

BTX-B5 was transformed in 100% yield from PbTx-2 by oxidation of its aldehyde function with SeO_2 and 30% H_2O_2 in *t*-BuOH at room temperature.[10] All these data allowed us to assign the structure of BTX-B5 to be **1**. Details of the preparation procedure for BTX-B5 from PbTx-2 will be published elsewhere.

High levels of BTX-B5 with PbTx-3 were found in the New Zealand toxic shellfish, cockle, greenshell mussel *Perna* (P.) *canaliculus* and Pacific oyster, which had been

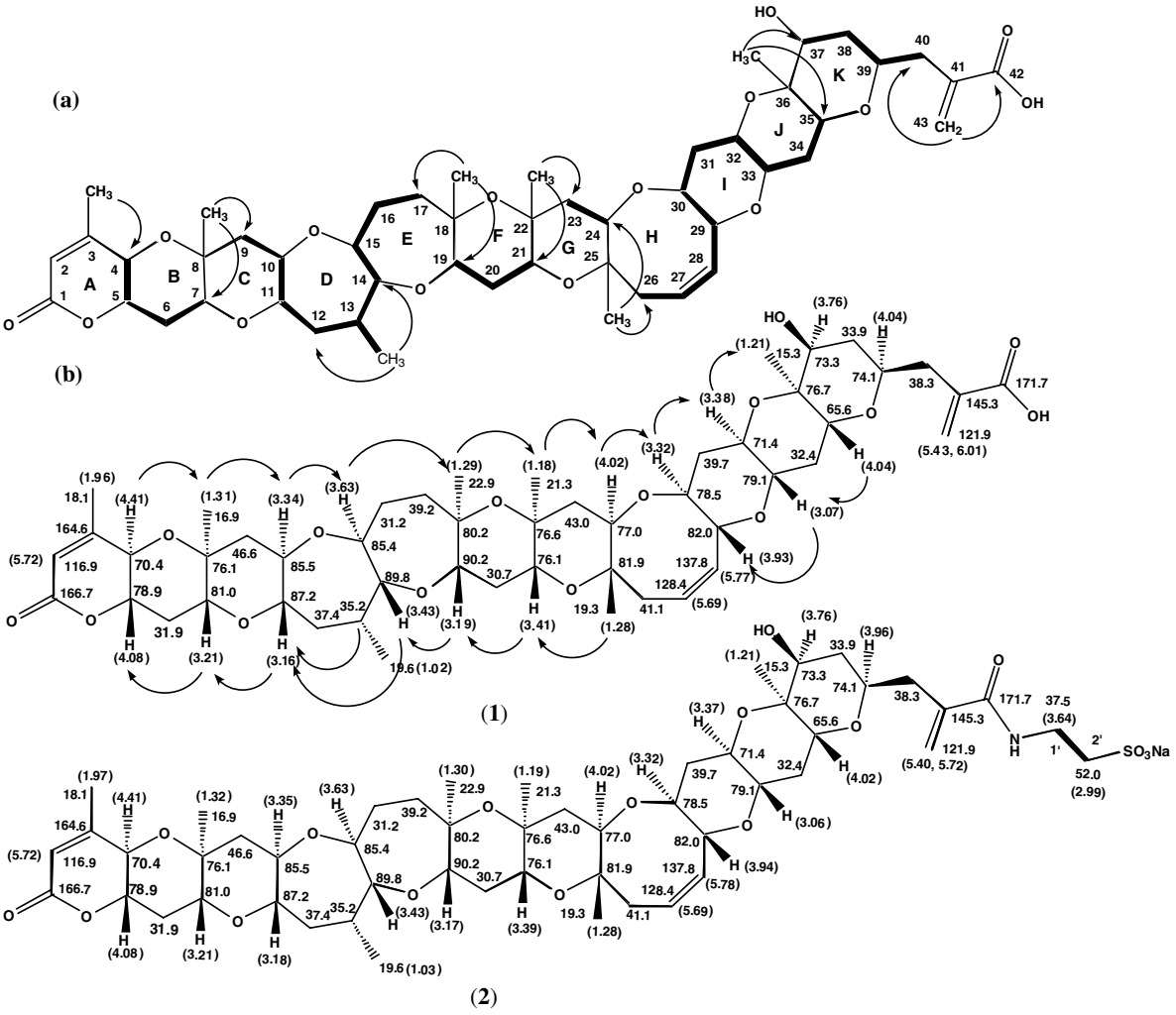

Figure 2. NMR techniques used for structural elucidation of BTX-B5 (**1**) (a) and its carbon and proton assignments (b), and the carbon and proton chemical shifts of BTX-B1 (**2**). (a) Heavy lines and arrows indicate the partial structures assigned by ^{1}H–^{1}H COSY and HMBC, respectively. (b) The attached numbers denote ^{13}C NMR (^{1}H NMR) chemical shifts, ppm in CD_3OD and arrows indicate NOE's, measured by NOE difference experiments, around ether linkage.

harvested at an outbreak of NSP early in 1993, while high and little levels of BTX-B1 in the first and the last two, respectively, by liquid chromatography–tandem mass spectrometry (Fig. 4).[11]

The minimum lethal dose of BTX-B5 thus isolated is ca. 0.3–0.5 mg/kg (ip) in mice. Immediately after injection, the animals exhibited neurological symptoms very similar to those caused by brevetoxins.[12]

Interestingly, although it is well known that several brevetoxins, such as PbTx-1,[13] PbTx-2,[8] and PbTx-3,[7] are produced in the approximate ratio of 1/7/2 by the dinoflagellate *G. breve*, the potent ichthyotoxins PbTx-1 and PbTx-2 were not detected in the cockles at significant levels, but the less ichthyotoxic or lethal PbTx-3, BTX-B1, and BTX-B5 were found in ca. 5/25/1 ratio.[6] BTX-B2, B3, and B4 had been isolated from greenshell mussels from New Zealand (Fig. 1).[9,14,15] Very recently significant level of PbTx-3 and low of BTX-B1 were detected in the same greenshell mussels.[6] Based on these data, it is shown that PbTx-3, BTX-B5, and BTX-B1 are responsible for the NSP-associated toxicity of cockles, while PbTx-3 and BTX-B2, B3, and B4 of the greenshell mussels and PbTx-3 and BTX-B5 of Pacific oyster. Thus, BTX-B5 and PbTx-3 could be a good marker for monitoring shellfish toxicity after *G. breve* algal bloom. BTX-B2, B3, and B4 were absent in the cockles, suggesting that brevetoxin metabolism is species specific. It was reported that brevetoxins, especially PbTx-1 and PbTx-2 are unstable in both acidic and basic conditions.[16] It is newly found that the aldehyde terminus of PbTx-2 is readily converted into the corresponding carboxylic acid by selective oxidation under mild condition. It is consumed that PbTx-2 is metabolized to BTX-B5 in all three shellfish and that a significant amount of BTX-B5 is converted to BTX-B1 by an internal cockle enzyme(s), whereas a little by mussel and Pacific oyster enzymes, for detoxification. Because of its similarity to the metabolic pathway of aldehyde in the human body, our suggested pathway for detoxication of brevetoxin in the cockle may have implications for the treatment of human poisoning

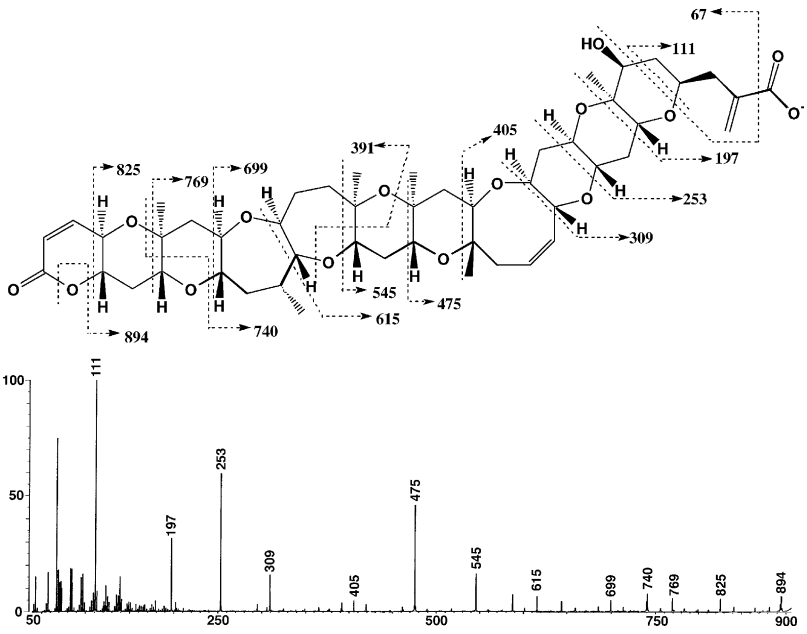

Figure 3. Negative FAB CAD MS/MS spectrum of BTX-B5 with a molecular ion at m/z 909.5 as a precursor.

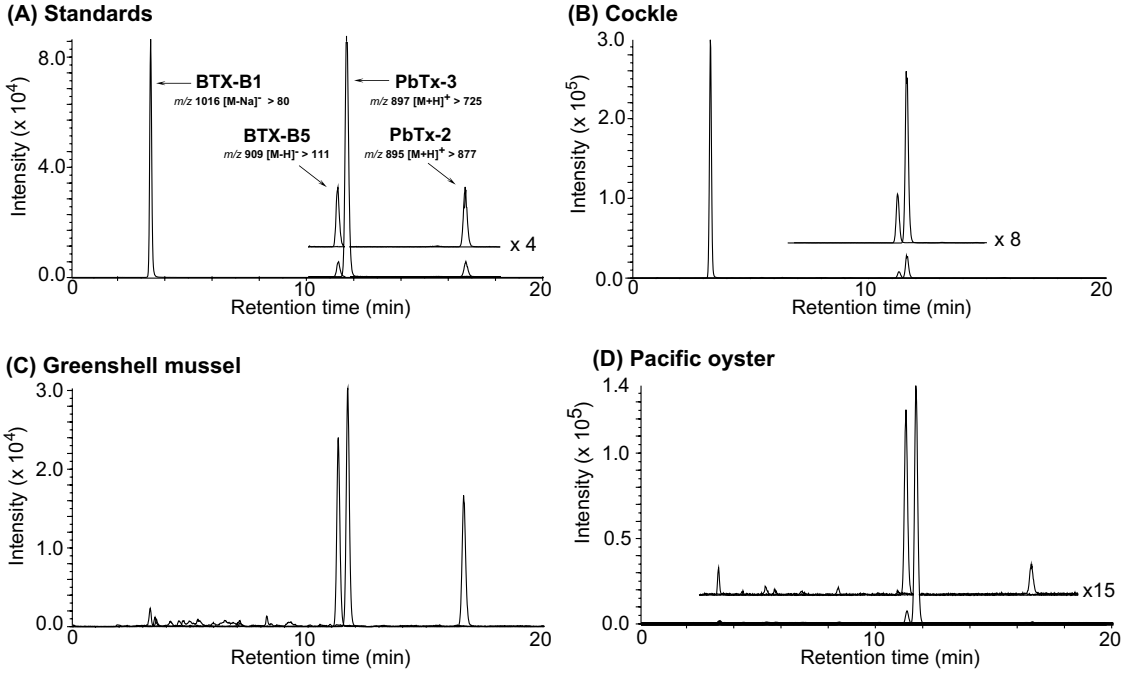

Figure 4. Selected reaction monitoring (SRM) liquid chromatography–tandem mass spectrometry chromatograms of standards (50 ng/mL) (A) and 80% MeOH fractions from 80% MeOH extracts of the toxic cockles (B), greenshell mussels (C) and Pacific oysters (D) (g/mL). Precursor–product ion combinations and polarity used in SRM detection are shown. HPLC conditions: column, Cadenza CD-C18 (3 mm × 150 mm, 3 μm); mobile phase, a gradient of 0.1% formic acid–acetonitrile (60–80% acetonitrile) for 20 min; flow rate, 0.2 mL/min; 20 μL injection. A cone voltage was set at 51, 96, 81, and 55 V, and collision-induced dissociation carried out with a collision energy of 130, 40, 50, and 20 eV for BTX-B1, BTX-B5, PbTx-3, and PbTx-2, respectively. Argon was used as the collision gas.

cases. The mechanism and enzyme(s) involved remain to be elucidated in detail. Highly sensitive analytical method using liquid chromatography coupled with tandem mass spectrometry technique for brevetoxins and their analogs could be important for shellfish toxin monitoring and metabolic study.[6,11]

Acknowledgements

The authors wish to thank Prof. Yuzuru Shimizu, Department of Pharmacognosy and Environmental Health Sciences, The University of Rhode Island, for providing PbTx-2.

References and Notes

1. McFarren, E. F.; Tanabe, H.; Silva, F. J.; Wilson, W. B.; Campbell, J. E.; Lewis, K. H. *Toxicon* **1965**, *3*, 111–123.
2. Morris, P. D.; Campbell, D. S.; Taylor, T. J.; Freeman, J. I. *Am. J. Pub. Health* **1991**, *81*, 471–474.
3. Bates, M.; Baker, M.; Wilson, N.; Lane, L.; Handford, S. *The Royal Society of New Zealand Miscellaneous Series* **1993**, *24*, 35–40.
4. Ishida, H.; Nozawa, A.; Totoribe, K.; Muramatsu, N.; Nukaya, H.; Kosuge, T.; Tsuji, K.; Yamaguchi, K.; Yasumoto, T.; Kaspar, H.; Barkett, N.; Kosuge, T. *Tetrahedron Lett.* **1995**, *36*, 725–728.
5. Ishida, H.; Muramatsu, N.; Nukaya, H.; Tsuji, K. *Toxicon* **1996**, *34*, 1050–1053.
6. Nozawa, A.; Tsuji, K.; Ishida, H. *Toxicon* **2003**, *42*, 91–103.
7. Chou, H. N.; Shimizu, Y. *Tetrahedron Lett.* **1982**, *23*, 5521–5524.
8. Lin, Y. Y.; Risk, M.; Ray, S. M.; Engen, D. V.; Clardy, J.; Golik, J.; James, J. C.; Nakanishi, K. *J. Am. Chem. Soc.* **1981**, *103*, 6773–6775.
9. Murata, K.; Satake, M.; Naoki, H.; Kaspar, H.; Yasumoto, T. *Tetrahedron* **1998**, *54*, 735–742.
10. Smith, C. W.; Holm, R. T. *J. Org. Chem.* **1957**, *22*, 728–746.
11. Ishida, H.; Nozawa, A.; Nukaya H.; Tsuji, K. *Toxicon*, in preparation.
12. Baden, M. G. *FASEB J.* **1989**, *3*, 1807–1817.
13. Shimizu, Y.; Chou, H. N.; Bando, H.; Duyne, G. V.; Clardy, J. C. *J. Am. Chem. Soc.* **1986**, *108*, 514–515.
14. Morohashi, A.; Satake, M.; Murata, K.; Naoki, H.; Kaspar, H. F.; Yasumoto, T. *Tetrahedron Lett.* **1995**, *36*, 8995–8998.
15. Morohashi, A.; Satake, M.; Naoki, H.; Kaspar, H. F.; Oshima, Y.; Yasumoto, T. *Nat. Toxins* **1999**, *7*, 45–48.
16. Hua, Y.; Cole, R. B. *Chem. Res. Toxicol.* **1999**, *12*, 1268–1277.