Pergamon

Tetrahedron 57 (2001) 7507–7511

TETRAHEDRON

Three new bromotyrosine derivatives lethal to crab from the marine sponge, *Pseudoceratina purpurea*☆

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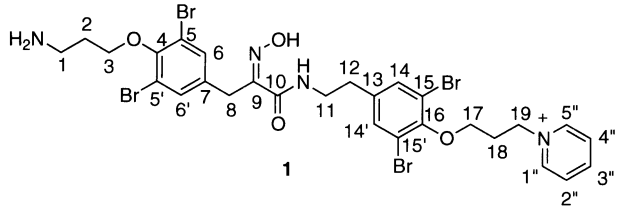
Received 4 June 2001; accepted 16 July 2001

Abstract—Three new bromotyrosine-derived metabolites, tokaradines A (**1**), B (**2**), and C (**3**), were isolated from the marine sponge *Pseudoceratina purpurea*. Their structures were determined by spectral methods. Tokaradines were lethal to the crab *Hemigrapsus sanguineus*.

1. Introduction

Marine sponges of the order Verongida are a rich source of bromotyrosine-derived compounds[2] which showed various biological activities such as antifouling,[3,4] cytotoxicity,[5–7] antibacterial,[6] and Na,K-ATPase inhibitory.[5] In our search for potential insecticides from Japanese marine invertebrates, we found that the hydrophilic extract of the marine sponge *Pseudoceratina purpurea* collected in southern Japan was lethal to the crab *Hemigrapsus sanguineus*. Bioassay-guided isolation afforded three new bromotyrosine derivatives, tokaradines† A (**1**), B (**2**) and C (**3**), along with the known purealidins A (**4**)[5] and C (**5**).[6] This paper describes the isolation, structural elucidation, and biological activity of these compounds.



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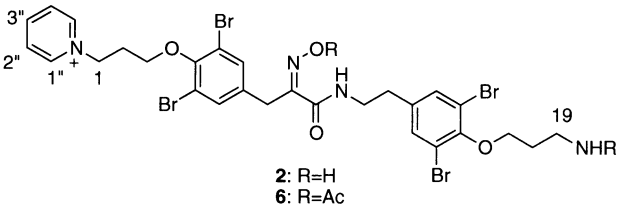

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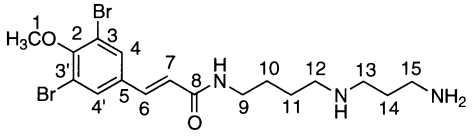

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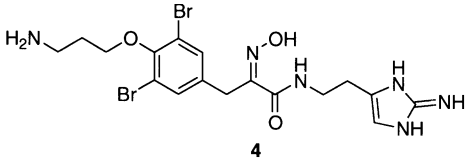

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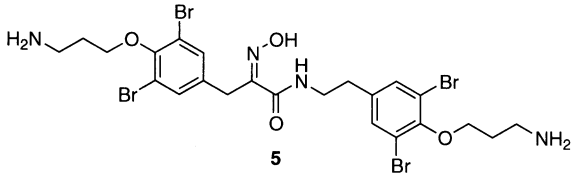

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2. Results and discussion

The MeOH extract of the frozen sponge (4.8 kg) was partitioned between Et_2O and H_2O. The aqueous layer was extracted with *n*-BuOH, and the *n*-BuOH layer was separated by ODS flash chromatography, gel filtration, silica

☆ Bioactive Marine Metabolites series, Part 109. For Part 108, see Ref. 1.

Keywords: amine; biologically active compounds; pyridinium salt; sponges.

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† Nakanoshima Island where the sponge was collected is the member of the Tokara Archipelago.

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PII: S0040-4020(01)00735-9

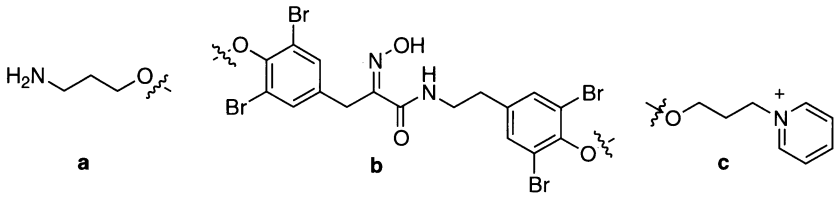

Figure 1. Partial structures of tokaradines A (**1**) and B (**2**).

gel column chromatography, and reversed phase HPLC to afford tokaradines A (**1**, 9.7 mg, 2.0×10^{-4}% based on wet weight), B (**2**, 12.2 mg, 2.5×10^{-4}%), and C (**3**, 6.3 mg, 1.3×10^{-4}%), along with the known purealidins A (**4**, 4.8 mg, 6.0×10^{-3}%), and C (**5**, 4.0 mg, 5.0×10^{-3}%).

Tokaradine A (**1**) exhibited a 1:4:6:4:1 ion cluster peak at *m/z* 803/805/807/809/811, indicating the presence of four bromine atoms. The molecular formula of $C_{28}H_{31}Br_4N_4O_4$ was determined on the basis of HR-FABMS and NMR data. The ^{1}H NMR spectrum (in CD_3OD containing TFA) was composed of nine methylenes, a pair of aromatic singlets, and protons assignable to an *N*-substituted pyridinium unit. Interpretation of the COSY spectrum classified the nine methylenes into four spin systems; two units of N–CH_2–CH_2–CH_2–O (H_2-1–H_2-3 and H_2-17–H_2-19), N–CH_2–CH_2 (H_2-11–H_2-12), and an isolated singlet methylene (H_2-8). Two sets of 4-alkyl-2,6-dibromophenol moiety (C-4–C-7 and C-13–C-16) were also implied from ^{1}H and ^{13}C chemical shifts as well as 2D NMR data. The carbon signals at δ 151.9 (C-9) and 165.5 (C-10) could be assigned to the amide–oxime conjugated system which was supported by the presence of two exchangeable proton signals at δ 8.13 and 12.09 in the ^{1}H NMR spectrum measured in DMSO-d_6. HMBC[8] cross peaks, H_2-8/C-6, C-6′, C-7, C-9, C-10, H_2-11/C-10, C-13, H_2-12/C-13, C-14, C-14′, H-14, H-14′/C-12 accommodated this amide–oxime system between two 4-alkyl-2,6-dibromophenol units, thus constructing partial structure **b**. Partial structure **c** was composed of an N–CH_2–CH_2–CH_2–O unit and an *N*-substituted pyridinium moiety, which were connected by an HMBC cross peak, H_2-19/C-1″. The partial structure **a** was the same C_3 unit as above, but terminating at the amino group in place of pyridinium group; the other end of this C_3 unit was linked to the oxygen atom of the 2,6-dibromophenol moiety. The chemical shift values of C-8 implied that the oxime was *Z* geometry[9] (Fig. 1).

An HMBC correlation, H-17/C-16 connected partial structures **b** and **c**. Although there was no cross peak which supported connectivity between C-3 and C-4, chemical shift values of H-3/C-3 and the molecular formula were consistent with the presence of an ether linkage between these carbons.

Tokaradine B (**2**) was isomeric to tokaradine A (**1**). Interpretation of 2D NMR spectra resulted in three partial structures found in **1**. Although crucial HMBC cross peaks were not observed among partial structures, tokaradine B (**2**) must have an alternative sequence of partial structures. This problem was overcome by FABMS data. Tokaradine A (**1**) gave two sets of a 1:2:1 triplet peak at *m/z* 411/413/415 and 347/349/351, while tokaradine B (**2**) gave those at *m/z* 426/428/430, and 409/411/413. These fragment ions could

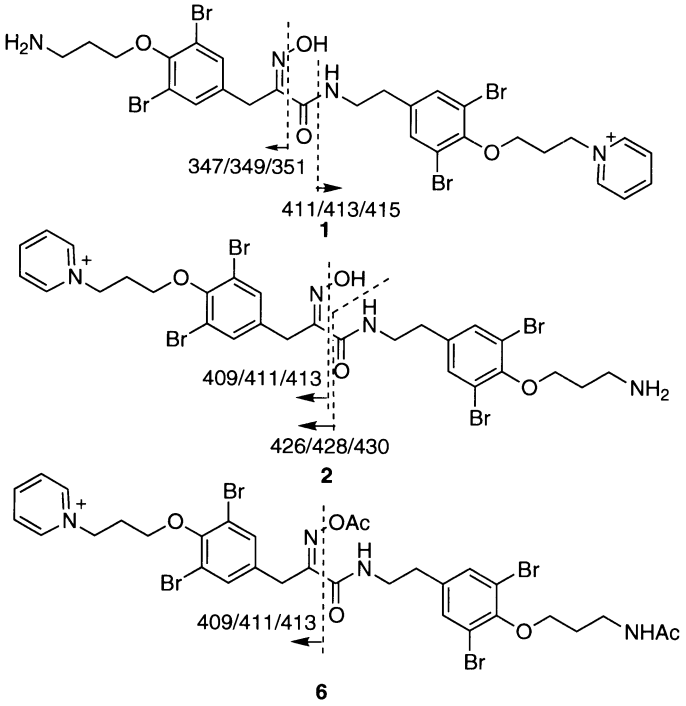

Figure 2. FABMS fragmentation of tokaradines A (**1**), B (**2**), and *N*,*O*-diacetyltokaradine B (**6**).

have arisen by cleavages as shown in Fig. 2, which was further supported by FABMS data of tokaradine B diacetate (**6**) exhibiting intense triplet ions at *m/z* 409/411/413 (Fig. 2).

Tokaradine C (**3**) had a molecular formula of $C_{17}H_{25}Br_2N_3O_2$ which was determined by HR-FABMS. The ^{1}H NMR spectrum of **3** indicated the existence of seven methylenes, one methoxy group, an α,β-unsaturated carbonyl group, and one 4-alkyl-2,6-dibromophenol moiety. UV absorption maxima at 229 and 279 nm were consistent with 6,8-dibromo-7-methoxycinnamoyl moiety,[10] which was supported by HMBC cross peaks, H4/C6, H6/C4, C5, H7/C5, H6, H7/C8, and H1/C2. Interpretation of the COSY spectrum and chemical shift values lead to two substructures, N–CH_2–CH_2–CH_2–N and N–CH_2–CH_2–CH_2–CH_2–N; HMBC cross peaks, H12/C13 and H13/C12 connected these substructures to complete a spermidine unit (C9–15). An HMBC cross peak, H9/C8 linked the spermidine unit to dibromomethoxycinnamoyl moiety via an amide bond to construct structure **3**.

Tokaradines A (**1**) and B (**2**) were lethal to the crab *H. sanguineus* at concentrations of 50 and 20 μg/g, respectively. Tokaradine C (**3**), purealidins A (**4**) and C (**5**) were less toxic (Table 4). Tokaradines A and B contain an *N*-substituted pyridinium unit, while the less toxic tokaradine C does not possess any strong cationic group. This may suggest that a cationic group is important for toxicity. Tokaradines A and B are the rare example of marine bromotyrosine-derived metabolites containing an *N*-substituted pyridinium group; another example is purpureamine C from the marine sponge *Psammaplysilla purpurea*.[11]

3. Experimental

3.1. General

NMR spectra were recorded on a JEOL A600 NMR spectrometer. ^{1}H and ^{13}C NMR chemical shifts were referenced to the solvent peaks: δ 3.30 and 49.0 for CD_3OH, δ 2.49 for DMSO-d_6. FAB mass spectra were measured on a JEOL JMX-SX102/SX102 tandem mass spectrometer using glycerol as a matrix. UV spectra were recorded on a Shimadzu UV-mini 1240 UV–VIS Spectrophotometer.

3.2. Animal material

Sponge samples were collected using SCUBA at a depth of 15–20 m off Nakanoshima Island in the Tokara Archipelago (129°51′0″N, 29°50′18″E). The specimens were frozen immediately and preserved at −20°C until extraction. The sponge was identified *P. purpurea* and deposited at the Institute for Systematics and Ecology, University of Amsterdam (ZMA16717).

3.3. Isolation

The frozen sponge (4.8 kg, wet weight) was extracted with MeOH (6 L×3), and the combined extracts were concentrated and partitioned between Et_2O (9 L×3) and H_2O (9 L). The aqueous phase was further extracted with *n*-BuOH (9 L×3); the *n*-BuOH phase which was lethal to crabs was separated by ODS flash chromatography with aqueous MeOH containing 0.05% TFA. The fraction eluted with 60% MeOH containing 0.1% TFA (10.7 g) was gel filtered on Sephadex LH-20 with MeOH–AcOH (99:1). The active fraction was further fractionated by ODS flash chromatography with aqueous MeCN containing 5% AcOH. The fraction eluted with MeCN–H_2O–AcOH (20:75:5) was separated on silica gel with $CHCl_3$–MeOH–AcOH (8:2:0–5:5:1). The fractions eluted with $CHCl_3$–MeOH–AcOH (6:4:1 and 5:5:1) were combined and separated by reversed phase HPLC (Cosmosil 5C_{18}-ARII using gradient elution of 20–40% MeCN containing 0.05% TFA) to furnish three crude compounds, which were finally purified on the same column with aqueous 30% MeCN containing 0.05% TFA to afford tokaradines A (**1**: 9.7 mg), B (**2**: 12.2 mg), and C (**3**: 6.3 mg).

Table 1. ^{1}H and ^{13}C NMR data for tokaradine A

Position	δ_H (mult., *J* in Hz)	δ_C	COSY	HMBC
1	3.28 (t, 7.7)	38.9	H-2	C-2, C-3
2	2.19 (tt, 5.7, 7.7)	29.0	H-1, H-3	C-1, C-3
3	4.08 (t, 5.7)	71.6	H-2	C-1, C-2
4		152.2		
5,5′		117.8		
6,6′	7.49 (s)	134.4		C-4, C-5, C-6, C-6′, C-8
7		137.9		
8	3.82 (s)	30.7		C-6, C-7, C-9, C-10
9		151.9		
10		165.5		
11	3.41 (t, 7.1)	41.5	H-12	C-10, C-12, C-13
12	2.74 (t, 7.1)	35.2	H-11	C-11, C-13, C-14
13		140.3		
14,14′	7.43 (s)	134.6		C-12, C-14, C-14′, C-15, C-16
15,15′		118.7		
16		152.4		
17	4.10 (t, 5.4)	70.8	H-18	C-18, C-19,C-16
18	2.60 (tt, 5.4, 6.9)	32.5	H-17, H-19	C-17, C-19
19	4.99 (t, 6.9)	60.9	H-18	C-17, C-18, C-1″
1″,5″	9.13 (d, 5.8)	146.4	H-2″	C-19, C-2″, C-5″
2″,4″	8.14 (dd, 5.8, 7.7)	129.5	H-1″, H-3″	C-1″, C-4″
3″	8.60 (t, 7.7)	147.1	H-2″	C-1″

Table 2. ^{1}H and ^{13}C NMR data for tokaradine B

Position	δ_H, (mult., J in Hz)	δ_C	COSY	HMBC
1	4.98, (t 7.1)	60.8	H-2	C-2, C-1″
2	2.60, (tt, 5.6,7.1)	32.5	H-1, H-3	
3	4.10, (t, 5.6)	70.9	H-2	
4		152.3		
5,5′		118.5		
6,6′	7.47, (s)	134.6		C-4, C-5, C-6, C-6′, C-8
7		138.0		
8	3.81, (s)	30.7		C-6, C-7, C-9, C-10
9		151.9		
10		165.4		
11	3.42, (t, 7.1)	41.5	H-12	C-10
12	2.74, (t, 7.1)	35.2	H-11	C-11, C-13, C-14
13		140.2		
14,14′	7.43, (s)	134.4		C-12, C-14, C-14′, C-15, C-16
15,15′		118.7		
16		152.3		
17	4.08, (t, 5.8)	71.6	H-18	
18	2.20, (tt, 5.8,7.7)	29.0	H-17, H-19	
19	3.29, (t, 7.7)	38.9	H-18	C-18
1″,5″	9.13, (d, 5.8)	146.4	H-2″	C-19, C-2″, C-5″
2″,4″	8.13, (dd, 5.8,7.7)	129.5	H-1″, H-3″	C-1″, C-4″
3″	8.59, (t, 7.7)	147.1	H-2″	C-1″

The fractions eluted with $CHCl_3$–MeOH–AcOH (8:2:0.1 and 7:3:0.5) from the silica gel column were combined to afford 1.25 g of crude material; a 20 mg portion was separated by reversed phase HPLC [Cosmosil $5C_{18}$-ARII (20×250 mm); 30% MeCN containing 0.05% TFA; 8.0 mL/min; UV detection at 220 nm] to yield purealidin C (**5**, 4.0 mg). From the polar fraction, purealidin A (**4**, 4.8 mg) was separated by reverse phased HPLC [Cosmosil $5C_{18}$-AR-II (20×250 mm); gradient elution of aqueous MeCN containing 0.05% TFA; 8.0 mL/min; UV detection at 220 nm].

3.3.1. Tokaradine A (1). Yellow amorphous solid; UV (MeOH) λ_{max} 216 nm (ϵ 21,800), 258 sh (4,000); ^{1}H and ^{13}C NMR data in CD_3OD containing TFA, see Table 1. ^{1}H NMR in DMSO-d_6; δ 12.09 (9-NOH), 9.17 (H1″, H5″), 8.60 (H3″), 8.17 (H2″, H4″), 8.13 (10-NH), 7.86 (1-NH), 7.47 (H6, H6′), 7.44 (H14, H14′), 4.88 (H19), 3.99 (H17), 3.97 (H3), 3.73 (H8), 3.33 (H11), 3.05 (H1), 2.71 (H12), 2.49 (H18), 2.05 (H2); FABMS (glycerol matrix) m/z 803/805/807/809/811 $[M]^+$, 645/647/649, 466/468/470, 411/413/415, 347/349/351; HR-FABMS (glycerol matrix) m/z 806.9037 $[M]^+$ (calc. $C_{28}H_{31}Br_4N_4O_4$, Δ 0.0 mmu).

3.3.2. Tokaradine B (2). Yellow amorphous solid; UV (MeOH) λ_{max} 214 nm (ϵ 20,600), 258 sh (3,800); ^{1}H and ^{13}C NMR data in CD_3OD containing TFA, see Table 2. ^{1}H NMR in DMSO-d_6; δ 12.07 (9-NOH), 9.18 (H1″, H5″), 8.60 (H3″), 8.17 (H2″, H4″), 8.12 (10-NH), 8.04 (19-NH), 7.47 (H14, H14′), 7.44 (H6, H6′), 4.88 (H1), 4.01 (H3), 3.97 (H17), 3.74 (H8), 3.34 (H11), 3.05 (H19), 2.73 (H12), 2.49 (H2), 2.07 (H18); FABMS m/z 803/805/807/809/811 $[M]^+$, 466/468/470, 426/428/430, 409/411/413; HR-FABMS m/z 806.9007 $[M]^+$ ($C_{28}H_{31}Br_4N_4O_4$, Δ −3.1 mmu).

3.3.3. Tokaradine C (3). Yellow amorphous solid; UV (MeOH) λ_{max} 229 nm (ϵ 12,200), 279 (9,900); ^{1}H and ^{13}C NMR data in CD_3OD containing TFA, see Table 3. ^{1}H NMR in DMSO-d_6; δ 8.18 (8-NH), 7.86 (H4, H4′), 7.32 (H6), 6.65 (H7), 3.80 (H1), 3.18 (H9), 2.96 (H13), 2.90 (H12), 2.86 (H15), 1.88 (H14), 1.59 (H11), 1.50

Table 3. ^{1}H and ^{13}C NMR data for tokaradine C

Position	δ_H, (mult., J in Hz)	δ_C	COSY	HMBC
1	3.87, (s)	61.2		C-2
2		156.3		
3,3′		119.5		
4,4′	7.78, (s)	132.9		C-2, C-3, C-4, C-4′, C-6
5		135.3		
6	7.38, (d, 15.8)	138.2	H-7	C-4, C-5, C-7, C-8
7	6.56, (d, 15.8)	123.8	H-6	C-5, C-6, C-8
8		168.0		
9	3.34, (t, 7.7)	39.5	H-10	C-8, C-10, C-11
10	1.65, (tt, 6.9,7.7)	27.5	H-9, H-11	C-9, C-11, C-12
11	1.75, (tt, 6.9,8.1)	24.4	H-10, H-12	C-9, C-10
12	3.06, (t, 8.1)	48.5	H-11	C-10
13	3.12, (t, 7.7)	45.7	H-14	C-12, C-14, C-15
14	2.07, (tt, 7.7,8.1)	25.3	H-13, H-15	C-13, C-15
15	3.05, (t, 8.1)	37.8	H-14	C-14

(H10); FABMS *m/z* 462/464/466 $[M+H]^+$, 317/319/321, 238/240; HR-FABMS *m/z* 464.0366 $[M+H]^+$ ($C_{17}H_{25}Br_2N_3O_2$, Δ −0.5 mmu).

3.4. Acetylation of 2

Tokaradine B (0.8 mg, in MeOH) was treated with Ac_2O (0.1 mL) for 1 h at rt to furnish *N,O*-diacetyltokaradine B (**6**).

3.4.1. *N,O*-Diacetyltokaradine B (6). ^{1}H NMR (DMSO-d_6): δ 9.16 (2H, d), 8.59 (1H, d), 8.16 (2H, dd), 8.11 (1H, br), 7.89 (1H, br), 7.44 (2H, s), 7.43 (2H, s), 4.87 (2H, t), 4.00 (2H, t), 3.89 (2H, t), 3.73 (2H, s), 3.33 (2H, t), 3.22 (2H, t), 2.72 (2H, t), 2.49 (2H, m), 2.49 (3H, s), 1.88 (2H, tt), 1.78 (3H, s); FABMS (pos.) *m/z* 887/889/891/893/895 $[M]^+$.

3.5. Bioassay

Individuals of the crab *H. sanguineus* were collected at a rocky beach on the Miura Peninsula and kept in an aquarium containing seawater until used for assay. Crabs of 1–5 g were used for the bioassay. The procedure of bioassay was performed essentially according to the method of Kem.[12,13] Samples were dissolved in MeOH/H_2O (1:1), and 5 μL aliquots were injected into a crab (3±2 g) by inserting a 26-gauge syringe needle at dorsal membranous junction between the cephalothorax and tail exoskeletons. For one dose, 5–10 crabs were used. After injection, each crab was kept in a plastic case (3×5×7 cm), which was allowed to stand at rt. An hour after injection, the number of dead crabs was counted (Table 4).

Table 4. Lethality of tokaradines to crabs

Compounds	LD_{99} (μg/g crab)
Tokaradine A	50
Tokaradine B	20
Tokaradine C	>100
Purealidin A	100
Purealidin C	>100
Quisqualate	0.5
Kinate	Non toxic[a]
NMDA	Non toxic[a]

[a] At 100 μg/mg crab.

Acknowledgements

We thank Professor P. J. Scheuer of the University of Hawaii for editorial comments. This work was partly supported by a Grant-in-Aid from the Ministry of Education, Science, Culture and Sports of Japan.

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