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Botryllamides A-D, New Brominated Tyrosine Derivatives from Styelid Ascidians of the Genus *Botryllus*

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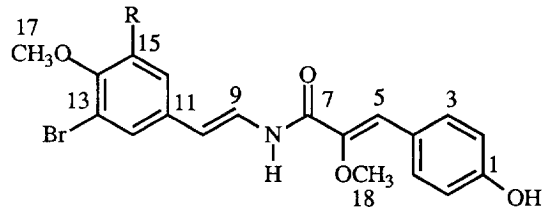
Abstract: Four new bromotyrosine derivatives, botryllamides A-D (**1-4**) were isolated from the styelid ascidian *Botryllus* sp. from Siquijor Is., Philippines, and from *Botryllus schlosseri* from the Great Barrier Reef, Australia. Their structures were deduced from 1D and 2D NMR spectral data.

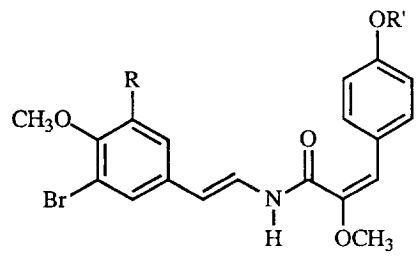
Chemical studies of ascidians have concentrated predominantly on species from the order Aplousobranchia. The main types of compounds isolated from these species have been peptides and other alkaloids.[1,2] Studies of ascidians from the other two orders (Phlebobranchia and Stolidobranchia) although rare, have shown them to be a source of alkaloids too. Only a small number of chemical studies of ascidians from the family Styelidae (order Stolidobranchia) have been reported. Among these studies, the isolation of cytotoxic α-carbolines (the grossularines) from *Dendrodoa grossularia*[3] is notable. Another example is the isolation of eusynstyelamide, a peptide alkaloid from *Eusynstyela misakiensis* which was recently reported.[4]

In this paper we describe the independent isolation and structural determination of the same series of mildly cytotoxic brominated tyrosine derivatives from two geographically distinct regions. The compounds were isolated from the brilliantly colored Styelid ascidian *Botryllus schlosseri* (Pallas, 1766), from Little Trunk Reef (Great Barrier Reef), Australia, and from a *Botryllus* sp. from Siquijor Is., Philippines. These tyrosine derivatives, botryllamides A-D (**1-4**), exhibit striking structural similarities to the tunichromes.[5-7]

The encrusting Styelid ascidian belonging to the genus *Botryllus*[8] was kept frozen until extracted. Initial extraction and separation on reversed phase silica gel yielded a single component (botryllamide D(**4**)). A recollection, after extraction and isolation on reversed phase silica gel yielded **1-4**. *Botryllus schlosseri*[9] from Little Trunk Reef, Australia was freeze dried and extracted. Separation of the crude extract on silica gel by HPLC also yielded **1-4**.



1: R = Br
3: R = H



2: R = Br, R' = H
4: R = H, R' = H
5: R = H, R' = Me

The formula for botryllamide A (**1**) was established as $C_{19}H_{17}Br_2NO_4$ by high resolution mass spectrometry. Two methoxy groups were evident from the ^1H and ^{13}C NMR spectra in $CDCl_3$ (3.68, 3.87; 59.5, 60.7 ppm). The presence of two symmetrically substituted aromatic rings was suggested by the presence of only 15 signals in the ^{13}C NMR spectrum; a *para*-substituted benzene ring was indicated by the 2H doublets in the ^1H NMR spectrum at δ 6.78 and 7.29 and by the resonances at 115.8 and 132.0 ppm (each 2C) in the ^{13}C NMR spectrum, while a 1,3,4,5 substituted benzene ring was suggested by the degenerate ^{13}C signals at 129.4 (2H signal at δ 7.48) and 118.3 ppm (Table 1). HMBC[10,11] correlation between δ 3.87 and 152.5 places the methoxy substituent of the 1,3,4,5 substituted ring, while HMBC correlations between δ 7.48 and ^{13}C signals at 152.5 (C14), 135.0 (C11), and 118.3 (C13 & C15) ppm established the remaining substituents as bromine. The presence of a *trans*-enamide group was indicated by the 14.6 Hz coupling between the signals at δ 6.08 and 7.53 and by coupling (J = 11.1 Hz) of the signal at δ 7.53 to an NH signal at δ 8.45. The NH signal strongly correlated with the signal for a (conjugated) carbonyl carbon (162.0 ppm) in the HMBC spectrum. HMBC correlations linked the olefinic protons (δ 6.08 and 7.53) to the tetrasubstituted benzene ring (correlations to 135.0 ppm). The *trans*-enamide system was thus part of a 3,5-dibromo-tyramine methyl ether residue. An enamide is found in molecules such as the tunichromes,[5-7,12] clionamide,[13,14] and the celenamides[15] which offered excellent models. The remaining ^{13}C NMR signals (122.3, 145.3 ppm) were attributed to a trisubstituted polarized double bond. The proton attached to the carbon resonating at 122.3 gave a signal at δ 7.13 suggesting it was the β proton on an α,β-unsaturated carbonyl system. HMBC correlation between the methoxy protons (δ 3.68) and the sp^2 carbon signal at 145.3 ppm indicated the polarized double bond to be an enol methyl ether function. These data led to the proposed structure for botryllamide A (**1**), an amide consisting of a methylated dibromotyramine residue linked to a modified tyrosine unit. The nature of the tyrosine residue was supported by the mass spectral fragment at *m/z* 177 $[C_{10}H_9O_3]^{\cdot+}$.

High resolution mass spectrometry established that botryllamide B (**2**) was isomeric with botryllamide A (**1**). The only significant differences in the NMR data for the two isomers were the shifts associated with the enol methyl ether. The NMR signals at position 5 in **1** (^{13}C; 122.3 ppm,

Table 1. ^{1}H and ^{13}C NMR Assignments for Botryllamides A-D (**1-4**).

	Botryllamide A (1)		**Botryllamide B (2)**	
Atom	δ ^{13}C†	δ ^{1}H†	δ ^{13}C‡	δ ^{1}H‡
1	156.9	OH, 5.85 (br s)	157.2	OH, 5.75 (br s)
2	115.8	6.88 (d, 8.4, 2H)	115.4	6.78 (d, 8.7, 2H)
3	132.0	7.59 (d, 8.4, 2H)	131.3	7.29 (d, 8.7, 2H)
4	125.2	-	126.4	-
5	122.3	7.13 (s, 1H)	109.9	6.14 (s, 1H)
6	145.3	-	148.2	-
7	162.0	-	162.0	-
8	-	8.45 (d, 11.1, 1H)	-	8.23 (d, 11.6, 1H)
9	124.0	7.53 (dd, 11.1, 14.6, 1H)	125.9	7.46 (dd, 11.6, 14.6, 1H)
10	110.7	6.08 (d, 14.6, 1H)	110.5	5.95 (d, 14.6, 1H)
11	135.0	-	137.3	-
12	129.4	7.48 (s, 1H)	130.1	7.42 (s, 1H)
13	118.3	-	118.8	-
14	152.5	-	152.8	-
15	118.3	-	118.8	-
16	129.4	7.48 (s, 1H)	130.1	7.42 (s, 1H)
17	60.7	3.87 (s, 3H)	60.9	3.85 (s, 3H)
18	59.5	3.68 (s, 3H)	56.1	3.78 (s, 3H)

	Botryllamide C (3)		**Botryllamide D (4)**		
Atom	δ ^{13}C†	δ ^{1}H†	δ ^{13}C§	δ ^{1}H§	δ ^{1}H‡
1	158.9	OH, 5.95 (br s)	156.0	OH, 9.38 (br s)	OH, 5.75 (br s)
2	116.4	6.86 (d, 8.6, 2H)	114.9	6.64 (d, 8.5, 2H)	6.74 (d, 8.6, 2H)
3	132.5	7.60 (d, 8.6, 2H)	129.4	7.11 (d, 8.5, 2H)	7.26 (d, 8.6, 2H)
4	125.8	-	124.9	-	-
5	121.3	7.12 (s, 1H)	105.9	5.93 (s, 1H)	6.13 (s, 1H)
6	147.8	-	148.4	-	-
7	162.4	-	161.7	-	-
8	-	8.37 (d, 11.4, 1H)	-	10.40 (d, 10.0, 1H)	8.22 (d, 11.7, 1H)
9	123.8	7.49 (dd, 11.4, 14.6, 1H)	122.5	7.34 (m, 1H)	7.41 (dd, 14.6, 11.7, 1H)
10	113.5	6.14 (d, 14.6, 1H)	111.8	6.21 (d, 14.8, 1H)	6.04 (d, 14.6, 1H)
11	132.5	-	130.6	-	-
12	130.7	7.55 (d, 2.0, 1H)	129.5	7.55 (d, 2.0, 1H)	7.49 (d, 2.1, 1H)
13	112.3	-	111.0	-	-
14	155.0	-	153.9	-	-
15	112.3	6.85 (d, 8.4 1H)	112.9	7.01 (d, 8.7, 1H)	6.81 (d, 8.6, 1H)
16	126.4	7.27 (dd, 8.4, 2.0, 1H)	125.7	7.34 (m, 1H)	7.23 (dd, 8.6, 2.1, 1H)
17	56.6	3.89 (s, 3H)	56.2	3.82 (s, 3H)	3.87 (s, 3H)
18	59.7	3.68 (s, 3H)	55.6	3.66 (s, 3H)	3.78 (s, 3H)

† $CDCl_3$; ^{13}C 75.5 MHz; ^{1}H 300 MHz ‡ Acetone-d_6 ; ^{13}C 75.5 MHz; ^{1}H 300 MHz

§ DMSO-d_6 ; ^{13}C 125 MHz; ^{1}H 500 MHz

^{1}H; δ 7.13) differed significantly from those for **2** (^{13}C; 109.9 ppm, ^{1}H; δ 6.14). An nOe difference experiment confirmed the *cis* relationship between H5 (δ 6.14) and the methyl ether (δ 3.78) for **2**. Botryllamides A and B were thus geometric isomers about the enol ether function.

Botryllamides C and D (**3** and **4**) were similarly confirmed to be geometric isomers about an enol ether function. The EI mass spectra for both **3** and **4** showed parent ion doublets at *m/z* 403/405 (1:1), indicating the presence of only one bromine. The molecular formula for **3** and **4** were established as $C_{19}H_{18}BrNO_4$ by high resolution mass spectrometry. The EI mass spectrum of **4** contained fragment ion doublets at *m/z* 212/214 and 226/228 corresponding to $[C_9H_9OBr]^{\bullet+}$ and $[C_9H_{10}ONBr]^{\bullet+}$ respectively (Fig. 1). This indicated the methylated dibromotyramine moiety of **1** and **2** was replaced by a methylated bromotyramine in **3** and **4**. Proton NMR couplings confirmed the nature of the brominated rings in **3** and **4**. For example, **4** showed a one proton doublet (δ 7.01, J = 8.7 Hz) coupled to a doublet of doublets at δ 7.34 (J = 8.7, 2.0 Hz), which was in turn coupled to a fine doublet at δ 7.55, J = 2.0 Hz). This defined the substitution pattern of a trisubstituted aromatic ring. The identity of the other portion of the amide **4** was indicated by the nitrogen bearing *m/z* 193 fragment $[C_{10}H_{11}O_3N]^{\bullet+}$ together with the non-nitrogen bearing *m/z* 177 fragment $[C_{10}H_9O_3]^{\bullet+}$ fragment (Fig. 1).

Analysis of the ^{1}H and ^{13}C NMR data for botryllamide D (**4**, Table 1) together with ^{1}H-^{1}H COSY,[16] TOCSY,[17,18] ROESY,[19-22] HMQC,[23,24] and HMBC[10] spectra in DMSO-d_6 enabled full assignment of all resonances. Strong ROESY cross peaks (Fig. 2) between H5 and H18 indicated they were *cis* and enabled the geometry of the enol ether to be established as identical with that of botryllamide B (**2**). The geometry of the enol ether for botryllamide C (**3**) is then the same as in botryllamide A (**1**). Methylation of **4** with diazomethane yielded 1-O-methylbotryllamide D (**5**).

Botryllamide D (**4**) showed marginal cytotoxicity, after 72 hour exposure, against the human colon cancer cell line HCT116 (IC_{50} = 17 μg/mL) but was inactive *in vivo*.

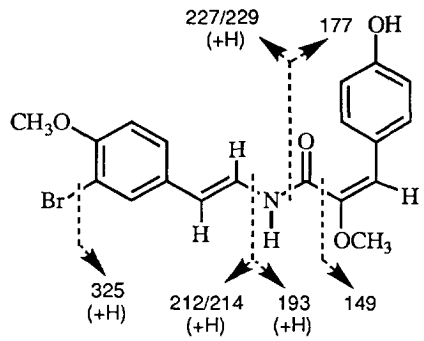


Fig. 1. Major EI fragments for botryllamide D (**4**).

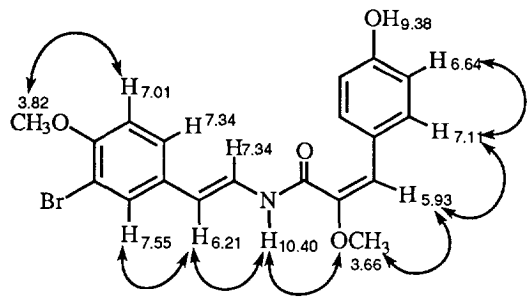


Fig. 2. Elucidative ROESY correlations for botryllamide D (**4**).

EXPERIMENTAL SECTION

At Utah, mass spectral data was acquired on a Finnigan MAT 95 spectrometer. ^{1}H NMR spectra were measured at 500 MHz and ^{13}C NMR spectra at 125 MHz on a Varian Unity 500 spectrometer. HPLC separations were carried out on a Rainin Dynamax-Microsorb C18 column (4.6 x 250 mm).

In Australia, UV spectra in ethanol were measured on a Varian 634 UV spectrophotometer, and IR spectra with chloroform solutions on a Perkin-Elmer 297 FTIR spectrometer. Mass spectral data was supplied by the Australian National University mass spectrometer unit. Proton NMR spectra were measured at 300 MHz and ^{13}C NMR spectra at 75.5 MHz on a Bruker AM-300 spectrometer. HPLC separations were carried out with a Waters 4500A solvent delivery system connected to a Waters U6K injector and a Waters R401 differential refractometer. HPLC columns were from Techsil (250 x 8 mm, filled with Techsil 5 mm silica). All solvents used were either freshly distilled or of analytical reagent grade.

Collection and Extraction of the Ascidian

The encrusting Styelid ascidian belonging to the genus *Botryllus*[8] was collected by SCUBA (-10 m) in April 1991 near Siquijor Island, Philippines and kept frozen until extracted. The frozen specimen (10 g wet weight) was repeatedly extracted with methanol to give a yellow extract which after solvent removal gave a yellow residue (1.01 g). A recollection of the ascidian (69 g) was similarly extracted.

Botryllus schlosseri[9] was collected from Little Trunk Reef, Australia, by SCUBA (-10 m) in June 1992 and freeze dried prior to being extracted. The dried animals (14.3 g) were extracted exhaustively with acetone/dichloromethane to yield an orange/brown solid (1.04 g) after removal of the solvent.

Isolation of Botryllamides A-D

The initial extract from the Philippine collection after reversed phase chromatography on C18 silica gel with 70% methanol/water afforded a single component, botryllamide D (**4**, 7.1 mg). The extract from the recollection, after reversed phase chromatography on C18 silica gel with stepped gradient elution afforded botryllamide A (**1**, 120 mg) eluted with 80% methanol/water, botryllamide B (**2**, 15 mg) eluted with 70% methanol/water, botryllamide C (**3**, 55 mg) eluted with 70% methanol/water, and botryllamide D (**4**, 31 mg) eluted with 60-70% methanol/water.

The extract from the Australian collection was rapidly chromatographed on silica using a gradient from petroleum ether to acetone. The botryllamides (135 mg) were eluted with petroleum ether/acetone (1:1). HPLC separation of the botryllamide fraction on silica gel with ethyl acetate/petroleum ether (4:6) yielded four fractions: botryllamide A (**1**, 34 mg), botryllamide B (**2**, 13 mg), botryllamide C (**3**, 26 mg) and botryllamide D (**4**, 8 mg).

Botryllamide A (1). White needles from chloroform, m.p. 169-171 °C; UV (EtOH) 205, 220, 225 sh, 325 nm (ε 10900 10300, 9900, 15900); IR ($CHCl_3$) 3410, 3018, 2927, 2855, 1735, 1650, 1607, 1513, 1496, 1468, 1423, 1265, 1171, 1087, 909 cm^{-1}; ^1H NMR data: See Table 1; ^{13}C NMR data: See Table 1; High resolution EI mass measurement 480.9526, $C_{19}H_{17}{}^{79}Br_2NO_4$ requires 480.9524; EIMS, *m/z* (relative intensity) 484.9 (3%), 482.9 (6), 480.9 (3), 177 (17), 149 (20), 134 (100), 106 (25), 78 (15), 77(13).

Botryllamide B (2). Colorless gum; UV (EtOH) 206, 214, 230 sh, 328 nm (ε 11800, 9600, 9400, 14100); IR ($CHCl_3$) 3409, 3018, 1700, 1650, 1607, 1513, 1468, 1423, 1250, 1171, 1087, 1001, 949 cm^{-1}; ^1H NMR data: See Table 1; ^{13}C NMR data: See Table 1; High resolution mass EI measurement 480.9526, $C_{19}H_{17}{}^{79}Br_2NO_4$ 480.9524; EIMS, *m/z* (relative intensity) 484.9 (4%), 482.9 (8), 480.9 (4), 177 (21), 149 (21), 134 (100), 106 (19), 78 (10), 77 (9).

Botryllamide C (3). White needles from chloroform, m.p. 173-175 °C; UV (EtOH) 204, 227 sh, 329 nm (ε 13200, 10600, 14100); IR ($CHCl_3$) 3409, 3018, 2927, 2855, 1730, 1693, 1653, 1607, 1513, 1486, 1463, 1441, 1259, 1171, 1089, 1054, 1019 cm^{-1}; ^1H NMR data: See Table 1; ^{13}C NMR data: See Table 1; HMBC correlations (optimized to observe J = 8 Hz): H3/C1, C3', C5, C2; H5/C3, C4; H10/C9, C11, C12, C16; H12/C10, C14, C16; H15/C13, C11; H16/C14, C10; 17-CH3/C14; 18-CH3/C6; High resolution EI mass measurement 403.0418, $C_{19}H_{18}{}^{79}BrNO_4$ requires 403.0419; EIMS, *m/z* (relative intensity) 405 (7%), 403 (6), 229 (7), 227 (7), 177 (11), 149 (16), 78 (13), 77 (15).

Botryllamide D (4). Colorless gum; UV (EtOH) 203, 224 sh, 326 nm (ε 11100, 9100, 11700); IR ($CHCl_3$) 3413, 3018, 2935, 1710, 1650, 1607, 1513, 1466, 1425, 1248, 1168, 1087, 1001, 949 cm^{-1}; ^1H NMR data: See Table 1; ^{13}C NMR data: See Table 1; HMBC correlations (optimized to observe J = 8 Hz): H2/C1, C4; H3/C1, C2, C5; H5/C3, C6, C7; H8/C7, C9, C10; H9/C7, C10, C11; H10/C9, C12, C16; H12/C10, C13, C14, C16; H15/C11, C13, C14; H16/C10, C11, C14; 17-CH3/C14; 18-CH3/C6; High resolution mass measurement 403.0418, $C_{19}H_{18}{}^{79}BrNO_4$ requires 403.0419; EIMS, *m/z* (relative intensity) 405 (8%), 403 (7), 229 (8), 227 (7), 177 (12), 149 (18), 135 (9), 134 (100), 106 (21), 78 (13), 77 (16).

1-O-Methylbotryllamide D (5). ^1H-NMR (DMSO-d_6): δ 3.62 (3H, s, 18-Me); 3.79 (3H, s, 1-OMe); 3.83 (3H, s, 17-Me); 6.44 (1H, d, J = 14.7 Hz, H10); 6.64 (1H, s, H5); 6.99 (2H, d, J = 8.8 Hz, H2); 7.04 (1H, d, J = 8.6 Hz, H15); 7.36 (1H, dd, J = 8.6, 2.1 Hz, H16); 7.41 (1H, dd, J = 14.7, 9.6 Hz, H9); 7.56 (1H, d, J = 2.1 Hz, H12); 7.70 (2H, d, J = 8.8 Hz, H3); 10.53 (1H, s, 1H, J = 9.6 Hz, H8).

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REFERENCES AND NOTES

1. Ireland, C. M.; Roll, D. M.; Molinski, T. F.; McKee, T. C.; Zabriskie, T. M.; Swersey, J. C. Uniqueness of the Marine Chemical Environment: Categories of Marine Natural Products from Invertebrates. In *Biomedical Importance of Marine Organisms*; Fautin, D. G. Ed.; California Academy of Sciences: San Francisco, 1988; Vol. 13; pp. 41-56.
2. Ireland, C. M.; Molinski, T. F.; Roll, D. M.; Zabriskie, T. M.; McKee, T. C.; Swersey, J. C.; Foster, M. P. Natural Product Peptides from Marine Organisms. In *Bioorganic Marine Chemistry*; Scheuer, P. J. Ed.; Springer-Verlag: Berlin, 1989; Vol. 3; pp. 1-46.
3. Moquin-Pattey, C.; Guyot, M. *Tetrahedron* **1989**, 45, 3445.
4. Swersey, J. C.; Ireland, C. M.; Cornell, L. M.; Peterson, R. W. *J. Nat. Prod.* **1994**, 57, 842-845.
5. Bruening, R. C.; Oltz, E. M.; Furukawa, J.; Nakanishi, K. *J. Am. Chem. Soc.* **1985**, 107, 5298-5300.
6. Bruening, R. C.; Oltz, E. M.; Furukawa, J.; Nakanishi, K.; Kustin, K. *J. Nat. Prod.* **1986**, 49, 193-204.
7. Oltz, E. M.; Bruening, R. C.; Smith, M. J.; Kustin, K.; Nakanishi, K. *J. Am. Chem. Soc.* **1988**, 110, 6162-6172.
8. The ascidian was identified by Dr. Françoise Monniot, Muséum National d'Histoire Naturelle, Paris, France.
9. The ascidian was identified by Mr. Robert McCauley, Australian Institute of Marine Science, Townsville 4811, Australia.
10. Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, 108, 2093-2094.
11. The HMBC experiment was optimized to observe $^{n}J_{CH}$ couplings of 8 Hz.
12. Kim, D.; Li, Y.; Horenstein, B. A.; Nakanishi, K. *Tetrahedron Lett.* **1990**, 31, 7119-7122.
13. Anderson, R. J. *Tetrahedron Lett.* **1978**, 29, 2541-2544.
14. Anderson, R. J.; Stonard, R. J. *Can. J. Chem.* **1979**, 57, 2325-2328.
15. Stonard, R. J.; Anderson, R. J. *Can. J. Chem.* **1980**, 58, 2121-2126.

16. Martin, G. E.; Zektzer, A. S. *Two-Dimensional NMR Methods for Establishing Molecular Connectivity: A Chemist's Guide to Experiment Selection, Performance, and Interpretation*; VCH Publishers, Inc.: New York, 1988; pp. 58-157.
17. Braunschweiler, L.; Ernst, R. R. *J. Magn. Reson.* **1983**, 53, 521-528.
18. The TOCSY experiment was run with a mixing time of 50 ms.
19. Bothner-By, A. A.; Stephens, R. L.; Lee, J.-M.; Warren, C. D.; Jeanloz, R. W. *J. Am. Chem. Soc.* **1984**, 106, 811-813.
20. Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, 63, 207-213.
21. Kessler, H.; Griesinger, C.; Kressebaum, R.; Wagner, K.; Ernst, R. R. *J. Am. Chem. Soc.* **1987**, 109, 607-609.
22. The ROESY experiment was run with a mixing time of 400 ms.
23. Summers, M. F.; Marzilli, L. G.; Bax, A. *J. Am. Chem. Soc.* **1986**, 108, 4285.
24. The HMQC experiment was optimized to observe $^1J_{CH}$ couplings of 140 Hz.

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