Botryllamides E−H, Four New Tyrosine Derivatives from the Ascidian *Botrylloides tyreum*

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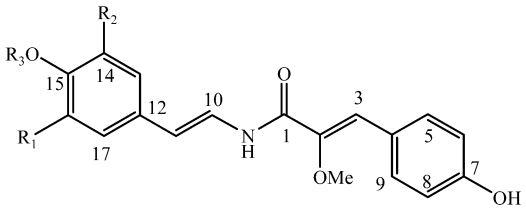
A specimen of the ascidian *Botrylloides tyreum* from Palau contained the known metabolites botryllamides A (**5**) and C (**7**) together with four new tyrosine derivatives, botryllamides E−H (**1**−**4**), the structures of which were elucidated by interpretation of spectroscopic data. The botryllamides exhibited weak cytotoxicity against the HCT-116 cell line.

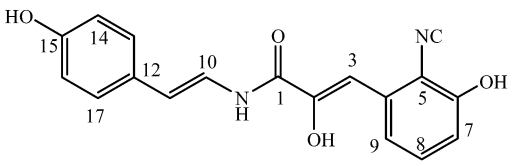
Ascidians (tunicates) are prolific producers of amino acid-derived secondary metabolites, many of which have been reported to possess cytotoxicity.[1−4] In 1995, McDonald et al. reported the isolation of four bromotyrosine derivatives, botryllamides A−D (**5**−**8**), from the styelid ascidian *Botryllus* sp. from Siquijor Island in the Philippines and from *Botryllus schlosseri* from the Great Barrier Reef, Australia.[5] In this paper, we report the structural elucidation of botryllamides E−H (**1**−**4**), which were isolated, together with the known metabolites botryllamides A (**1**) and C (**3**), from a specimen of *Botrylloides tyreum* from Palau.

The encrusting ascidian *B. tyreum* was collected by hand using scuba (−1 m) from a temporary floating bridge between Koror and Babeldaob, Republic of Palau.[6] The ethyl acetate-soluble material from a methanolic extract showed moderate activity against the HCT-116 cell line and was subjected to column chromatography on silica gel to obtain three active fractions, two of which consisted predominantly of botryllamide A (**5**). Further fractionation of the third active fraction by HPLC using normal- and reversed-phase supports gave botryllamides A (**5**), C (**7**), E (**1**), F (**2**), and G (**3**). Botryllamide H (**4**) precipitated from a more polar but inactive fraction. The ^{1}H NMR spectra of the fractions were devoid of a singlet at ca. 6.14 ppm, which is found in the spectra of botrylamides B (**6**) and D (**8**).

Botryllamide E (**1**) was obtained as a colorless gum. A molecular formula of $C_{19}H_{19}NO_4$, which requires 11 degrees of unsaturation, was deduced from high-resolution mass measurement of the $[M + Na]^+$ ion at *m/z* 348.1205 (Δ −0.1 mmu). The IR spectrum contained a very broad band centered at 3350 cm^{-1} (phenol) and bands at 3260 and 1635 cm^{-1} assigned to an amide group. The ^{13}C NMR spectrum contained only 15 signals, of which four at δ 114.9, 116.3, 127.4, and 132.5 were each assigned to two carbons on two 1,4-disubstituted aromatic rings. The HMBC data suggested that one of the aromatic rings was a *p*-methoxyphenyl group, while the other was a *p*-hydroxyphenyl moiety. The remaining three degrees of unsaturation were assigned to a carbonyl group at δ 163.7 and two olefinic groups that gave rise to signals at 115.7, 121.7, 122.2, and 147.0. Comparison of the ^{1}H and ^{13}C NMR spectra with those of botryllamides A (**5**) and C (**7**) indicated that botryllamide E (**1**) is a nonbrominated analogue of **5** and **7**, an assignment that was confirmed by analysis of the HMBC data (Table 1).



1 $R_1 = R_2 = H, R_3 = Me$
2 $R_1 = R_2 = R_3 = H$
3 $R_1 = R_2 = Br, R_3 = H$
5 $R_1 = R_2 = Br, R_3 = Me$
7 $R_1 = H, R_2 = Br, R_3 = Me$



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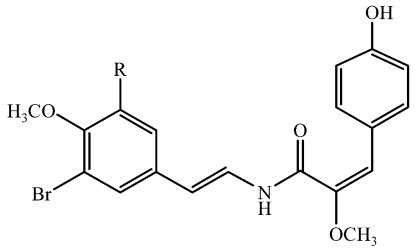

6 R = Br
8 R = H

Botryllamide F (**2**) was isolated in about 90% purity as a colorless oil. The molecular formula, $C_{18}H_{17}NO_4$, which was deduced from high-resolution mass measurement of the $[M + Na]^+$ ion at *m/z* 334.1062 (Δ +0.7 mmu), contained one methylene unit less than that of botryllamide E (**1**). The IR spectrum contained a very strong band at 3305 cm^{-1} (phenol). The ^{1}H and ^{13}C NMR spectra contained a single methoxyl signal that gave rise to signals at δ 3.56 and 59.7, respectively. The HMBC spectrum showed correlations between both the methoxyl signal and H-3 (δ 6.82) to C-2 (δ 147.0), which requires the methoxyl group to be at C-2 and infers the presence of two phenolic groups. Analysis of the NMR data confirmed this assignment.

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Table 1. ^{1}H and ^{13}C NMR Data (MeOH-d_4) for Botryllamides E−G (**1**−**3**)

	1				**2**			**3**		
carbon	δ_C	δ_H	mult., J (Hz)	HMBC	δ_C	δ_H	mult., J (Hz)	δ_C	δ_H	mult., J (Hz)
1	163.7				163.6			164.0		
2	147.0				147.0			146.8		
3	122.2	6.81	s	C-1, C-2, C-5	122.1	6.82	s	122.6	6.81	s
4	125.7				125.7			125.6		
5	132.5	7.46	d, 8.5	C-3, C-6, C-7	132.4	7.48	d, 8.5	132.5	7.62	d, 8.5
6	116.3	6.67	d, 8.5	C-4, C-5, C-6	116.3	6.69	d, 8.5	116.3	6.82	d, 8.5
7	159.1				159.1			159.2		
8	116.3	6.67	d, 8.5	C-4, C-5, C-6	116.3	6.69	d, 8.5	116.3	6.82	d, 8.5
9	132.5	7.46	d, 8.5	C-3, C-6, C-7	132.4	7.48	d, 8.5	132.5	7.62	d, 8.5
10	121.7	7.30	d, 14.5	C-1, C-11, C-12	121.1	7.25	d, 14.5	123.9	7.45	d, 14.5
11	115.7	6.33	d, 14.5	C-10, C-13	116.1	6.30	d, 14.5	112.4	6.34	d, 14.5
12	130.1				129.0			130.1		
13	127.4	7.16	d, 8.5	C-14, C-15, C-17	127.5	7.09	d, 8.5	129.8	7.52	s
14	114.9	6.73	d, 8.5	C-12, C-15, C-16	116.2	6.61	d, 8.5	112.7		
15	159.7				157.2			150.7		
16	114.9	6.73	d, 8.5	C-12, C-14, C-15	116.2	6.61	d, 8.5	112.7		
17	127.4	7.16	d, 8.5	C-13, C-15, C-16	127.5	7.09	d, 8.5	129.8	7.52	s
OMe-2	59.8	3.54	3H, s	C-2	59.7	3.56	3H, s	59.8	3.69	3H, s
OMe-15	55.7	3.66	3H, s	C-15						

Table 2. ^{1}H and ^{13}C NMR Data (DMSO-d_6) for Botryllamide H (**4**)

carbon	δ_C	δ_H	mult., J (Hz)	HMBC
1	162.6			
2	147.9			
3	102.3	7.57	s	C-1, C-4, C-5, C-9
4	122.2			
5	138.3			
6	153.5			
7	112.0	7.13	d, 7	C-5, C-6, C-9
8	127.8	7.43	t, 7	C-4, C-6
9	111.9	7.56	d, 7	C-5, C-7
10	120.8	7.43	dd, 15, 11	
11	114.3	6.52	d, 15	C-10, C-13, C-17
12	127.4			
13	126.7	7.25	d, 8.5	C-11, C-15, C-17
14	115.9	6.73	d, 8.5	C-12, C-15, C-16
15	156.4			
16	115.9	6.73	d, 8.5	C-12, C-14, C-15
17	126.7	7.25	d, 8.5	C-11, C-13, C-15
18	161.4			
OH-2		11.85	br	C-1, C-3, C-4
OH-6		10.00	s	C-5, C-6, C-7
OH-15		9.43	s	C-14, C-15, C-16
NH		11.23	d, 11	C-1, C-11 (weak)

Botryllamide G (**3**) was isolated as a colorless gum. The molecular formula, $C_{18}H_{15}Br_2NO_4$, which was deduced from the high-resolution mass measurement of the $[M + Na]^+$ ion at m/z 489.9272 (Δ +1.1 mmu), indicated two bromine atoms in place of two hydrogen atoms in botryllamide F (**2**). The IR spectrum again contained a very broad band at 3285 cm^{-1} (phenol). The position of the bromine atoms was elucidated by comparison of the NMR data with those of botryllamide A (**5**). The data differed from those of **5** in that the expected NMR signals due to the Me-15 group were missing.

Botryllamide H (**4**) was obtained as a white precipitate that was essentially insoluble in all common solvents except DMSO. The molecular formula, $C_{18}H_{14}N_2O_4$, was deduced from the high-resolution mass measurement of the $[M + H]^+$ ion at m/z 323.1032 (Δ +0.1 mmu). The IR spectrum contained a broad band at 3300 (phenol) and bands at 2130, 1630 cm^{-1} assigned to isonitrile and amide groups. Both the ^{1}H and ^{13}C NMR spectra contained signals that were assigned to a *p*-hydroxyphenyl ring (Table 2, C-12 to C-17). The H-13/17 signals showed a key HMBC correlation to C-11, and H-11 was correlated to C-13/17. The H-11 signal at δ 6.52 (d, 1 H, J = 15 Hz) was coupled to the H-10 signal at 7.43 (dd, 1 H, J = 15, 11 Hz),[7] which was in turn coupled to the amide NH signal at 11.23 (d, 1 H, J = 11 Hz). The left-hand portion of botryllamide H (**4**) was therefore identical to that found in botryllamide F (**2**). The amide NH signal and the H-3 signal at δ 7.57 (s, 1 H) showed HMBC correlations to C-1, while the broad OH-2 signal showed correlations to C-1, C-3, and C-4, indicating the presence of an enolized α-ketoamide adjacent to a second aromatic ring. The substitution pattern around the second aromatic ring was quite unexpected. In the HMBC experiment, H-3 is correlated to C-1, C-4, C-5, and C-9, while OH-6 correlated to C-5, C-6, and C-7. This requires the final substituent to be between the phenol and the enolized α-ketoamide substituents. This assignment was confirmed by the observation in the ^{1}H NMR spectrum of three mutually coupled signals at δ 7.13 (1H, d, J = 7 Hz, H-7), 7.43 (1H, t, J = 7 Hz, H-8), and 7.56 (1H, d, J = 7 Hz, H-9).[7] The remaining signal in the ^{13}C NMR spectrum was at δ 161.4, which can only be assigned to an isonitrile group, although the chemical shift was not exactly as expected, presumably due to the adjacent phenol group.

The C-10, C-11 double bond stereochemistry of compounds **1**−**4** was determined as "trans" on the basis of the coupling constants. NOE difference experiments confirmed the trans relationship between H-3 and the methyl ether for compounds **1**−**3**. It was also further confirmed through the ^{1}H and ^{13}C NMR chemical shift values of H-3, C-3, and C-2 of compounds **1**−**4** compared to similar compounds reported in the literature.[5]

The botryllamides were shown to be weak inhibitors of the human colon tumor (HCT-116) cell line (**5**, IC_{50} = 33 μM; **7**, IC_{50} = 28 μM; **1**, IC_{50} = 30 μM; **2**, IC_{50} = 85 μM (90%); **3**, IC_{50} = 110 μM). The activity was sufficient to allow bioassay-directed fractionation but did not meet the requirements for further biological evaluation.

Experimental Section

General Experimental Procedures. IR spectra were measured on a Perkin-Elmer 1600 FTIR spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 400 MHz spectrometer, and all 2D experiments were performed on a Varian Inova 300 MHz NMR spectrometer. HRMS data were obtained on a MALDI-FTMS mass spectrometer at the Scripps Research Institute. UV spectra were recorded on

a Beckman-Coulter DU 640 spectrophotometer. All solvents were redistilled prior to use.

Animal Material. The ascidian *Botrylloides tyreum* Herdman, 1886 (Collection # NCI-0394) was collected by hand using scuba (−1 m) from a temporary floating bridge between Koror and Babeldaob, Republic of Palau, in 2000 and was immediately frozen. The ascidian was identified by Shirley Parker-Nance (University of Port Elizabeth, South Africa). Voucher specimens are available on request.

Extraction and Purification. The ascidian (350 g wet wt) was cut into thin slices, lyophilized, and extracted with MeOH (4 × 400 mL). The extracts were concentrated and partitioned between EtOAc and H_2O to obtain organic (2.75 g) and aqueous (4.72 g) extracts. Only the organic extract inhibited the growth of the HCT-116 cell line (IC_{50} = 30 μg/mL). The organic extract was first subjected to column chromatography on silica gel using a stepwise gradient from hexane to EtOAc as eluant to obtain 13 fractions, of which three adjacent fractions showed HCT-116 inhibition (IC_{50} = ca. 6 μg/mL). The first of these fractions (300 mg) consisted of almost pure botryllamide A (**5**), while the second (342 mg) contained approximately 80% botryllamide A (**5**): these fractions were not purified further. A portion (190 mg) of the third active fraction (448 mg) was fractionated by HPLC on silica using 40% EtOAc in hexane as eluant to obtain botryllamides A (**5**, 24 mg), F (**2**, 8 mg), and G (**3**, 6.5 mg) and a mixed fraction that was further purified by HPLC on a reversed-phase C_{18} column to obtain botryllamides C (**7**, 34 mg) and E (**1**, 19 mg). A white solid was precipitated out from the more polar fraction. It was further purified by HPLC on a reversed-phase C_{18} column to afford botryllamide H (**4**, 4 mg).

HCT-116 Assay. The HCT-116 cells were plated in 96-well plates and incubated overnight at 37 °C in 5% CO_2/air. Compounds were added to the plate and serially diluted. Then the plate was incubated for a further 72 h. Cell viability was then assessed at the end of this period through the use of a CellTiter 96 AQ_{ueous} nonradioactive cell proliferation assay (Promega). Inhibition concentration (IC_{50}) values are interpreted from the bioreduction of MTS/PMS by living cells into a formazan product (proportional to the number of living cells in each well), which was then determined using a Molecular Devices Emax microplate reader that measured the amount of 490 nm absorbance in each well, and the IC_{50} value was calculated by a SOFTMax analysis program. Etoposide (Sigma) and DMSO (solvent) were used as positive and negative controls, respectively.

Botryllamide E (1): colorless gum; UV (MeOH) 207 nm (ϵ 14 997), 222 nm (ϵ 12 537), 339 nm (ϵ 29 804); IR (AgCl) ν_{max} 3350 (br), 3260 (br), 1635, 1515 cm^{-1}; ^1H NMR (300 MHz, CD_3OD), see Table 1; ^{13}C NMR (100 MHz, CD_3OD), see Table 1; ESIMS (+ve) *m/z* 673 $[2M + Na]^+$, 348 $[M + Na]^+$, 326 $[M + H]^+$, (−ve) *m/z* 324 $[M - H]^-$; HRMALDIMS $[M + Na]^+$ *m/z* 348.1205 (calcd for $C_{19}H_{19}NO_4Na$, 348.1206).

Botryllamide F (2): colorless oil, ~90% purity; UV (MeOH) 208 nm (ϵ 13 780), 220 nm (ϵ 11 998), 316 nm (ϵ 14 258); IR (AgCl) ν_{max} 3305 (br), 1600, 1505 cm^{-1}; ^1H NMR (300 MHz, CD_3OD), see Table 1; ^{13}C NMR (100 MHz, CD_3OD), see Table 1; ESIMS (+ve) *m/z* 645 $[2M + Na]^+$, (−ve) *m/z* 310 $[M - H]^-$; HRMALDIMS $[M + Na]^+$ *m/z* 334.1062 (calcd for $C_{18}H_{17}NO_4Na$, 334.1066).

Botryllamide G (3): colorless gum; UV (MeOH) 208 nm (ϵ 20 228), 332 nm (ϵ 22 152); IR (AgCl) ν_{max} 3285 (br), 1645, 1600, 1510 cm^{-1}; ^1H NMR (300 MHz, CD_3OD), see Table 1; ^{13}C NMR (100 MHz, CD_3OD), see Table 1; ESIMS (+ve) *m/z* 468 $[M + H]^+$; HRMALDIMS $[M + Na]^+$ *m/z* 489.9272 (calcd for $C_{18}H_{15}{}^{79}Br_2NO_4Na$, 489.9266).

Botryllamide H (4): white powder; UV (MeOH) 210 nm (ϵ 17 627), 249 nm (ϵ 12 568), 314 nm (ϵ 7787), 344 nm (ϵ 7964); IR (AgCl) ν_{max} 3300 (br), 2130, 1630, 1505 cm^{-1}; ^1H NMR (300 MHz, DMSO), see Table 2; ^{13}C NMR (100 MHz, DMSO), see Table 2; ESIMS (+ve) *m/z* 323 $[M + H]^+$; HRMALDIMS $[M + H]^+$ *m/z* 323.1032 (calcd for $C_{18}H_{15}N_2O_4$, 323.1032).

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References and Notes

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(6) The true origin of the ascidian is not known because the bridge had been towed from the Philippines. The ascidian was not found elsewhere in Palau.
(7) The coupling constants of the two overlapping signals at δ 7.43 (H-8 and H-10) could be determined by dropwise addition of acetone-d_6. The *meta*-coupling between H-7 and H-9 was observed in the DMSO-d_6/acetone-d_6 spectrum.

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