Structure of Bryostatin 20: A Symbiont-Produced Chemical Defense for Larvae of the Host Bryozoan, *Bugula neritina*†

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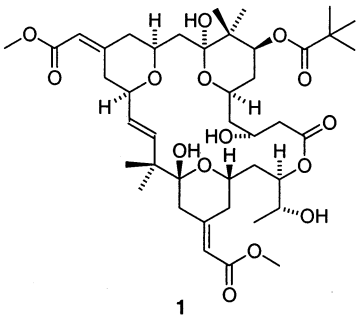
Larvae of the marine bryozoan *Bugula neritina* are defended against potential predators by high concentrations of bryostatins, which are produced by a bacterial symbiont of the bryozoan. From the larvae of *B. neritina*, three bryostatins, bryostatin 10 (**1**), the novel bryostatin 20 (**2**), and an as yet uncharacterized bryostatin, were isolated that were unpalatable to fish. These deterrent bryostatins represent the first example from the marine environment of a microbial symbiont producing an antipredator defense for its host. The structure of bryostatin 20 (**2**) was determined by spectral comparison with previously described bryostatins.

The production of defensive chemistry by microbial symbionts that protects their hosts against consumers or pathogens is common in terrestrial plants;[1] however, among marine organisms, there is limited empirical support for defensive symbiosis. Gil-Turnes and co-workers[2] provide rigorous data demonstrating that compounds produced by microbial symbionts can protect the host against pathogens, but no data have been previously reported showing that symbiont-produced chemistry defends marine hosts against consumers. Although many sessile marine invertebrates, such as sponges, bryozoans, and tunicates, harbor microbial symbionts and possess secondary metabolites with structural similarities to known microbial metabolites,[3] few studies have progressed sufficiently to even demonstrate symbiont production of these metabolites.[4] Lopanik and co-workers[5] recently provided a convincing demonstration that *Endobugula sertula*, a bacterial symbiont of the marine bryozoan *Bugula neritina*,[6,7] produces the bryostatin class of complex polyketides isolated from *B. neritina*[8−10] and that the bryostatins are concentrated on the bryozoan's larvae and protects them from predation by fish. From the larvae of *B. neritina*, we extracted a complex mixture of bryostatins, of which three, including bryostatin 10 (**1**) and the novel bryostatin 20 (**2**), were found to deter fish feeding. This paper describes a novel bryostatin acquisition methodology and the structural elucidation of bryostatin 20 (**2**).



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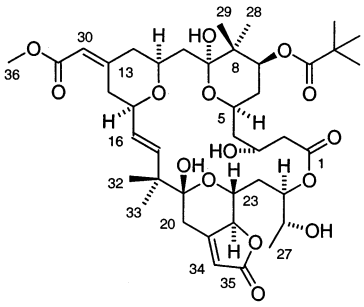

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Reproductive colonies of *B. neritina* collected in North Carolina were stimulated to release larvae by exposure to sunlight after being held in the dark overnight. Released larvae were collected with a pipet and concentrated on a sieve. Larvae on the sieves were briefly soaked in a 0.56 M potassium chloride solution, which caused the larvae to exude >90% of their bryostatin load.[5] The aqueous potassium chloride solution was then solvent extracted with dichloromethane and *n*-butanol. Facile separation of the bryostatins from this extract was accomplished by reverse-phase HPLC. Each purified bryostatin was tested at its natural volumetric concentration in a fish feeding bioassay,[5] which identified three deterrent bryostatins: bryostatin 10 (**1**), the novel bryostatin 20 (**2**), and an as yet uncharacterized bryostatin, at concentrations of 0.8, 0.6, and 0.2 mg·mL^{-1} of larvae, respectively.

Bryostatin 10 (**1**) was readily identified by comparison of its MS and NMR spectral data with appropriate literature values.[9,10] The molecular formula of bryostatin 20 (**2**) was established as $C_{41}H_{60}O_{15}$ by high-resolution electrospray ionization MS measurements of the sodium adduct $[M + Na]^+$, *m/z* 815.3826 (calcd 815.3830 for $C_{41}H_{60}O_{15}Na$, Δ −0.5 ppm). This formula was one carbon and four hydrogens less than the formula of **1**, and it required one additional unsaturation equivalent in **2**. Careful analysis of the ^1H and ^{13}C NMR data of **2** (Table 1) revealed it had many of the same structural features as bryostatin 10 (**1**). It was possible to assign a partial structure for **2** from C-1

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Table 1. ^{1}H and ^{13}C NMR Data for Bryostatin 20 (**2**) Obtained in CD_3CN

pos.	δ_H mult. (*J* in Hz)	δ_C[a]	pos.	δ_H mult. (*J* in Hz)	δ_C[a]
1		172.01	19		101.56
2	2.50 br s, 2H	41.62	20	2.67 d (13.3)	33.54
3	4.00 m	68.85		2.80 d (13.3)	
4	1.67 dt (3.5, 14.7)	39.85	21		168.91
	1.72 br d (14.7)		22	4.42 dd (1.0, 8.9)	82.30
5	4.18 tt (3.0, 11.8)	65.83	23	3.70 m	70.21
6	1.43 q (12.0)	33.00	24	1.87 m	32.54
	1.62 ddd (3.0, 4.9, 12.6)			2.26 td (2.8, 13.0)	
7	5.08 dd (5.3, 12.1)	72.28	25	5.11 ddd (3.0, 4.3, 12.6)	71.61
8		41.54	26	3.80 m	67.87
9		101.31	27	1.08 d, 3H (6.7)	18.00
10	1.72 br d (15.5)	41.67	28	0.88 s, 3H	20.38
	1.98 dd (7.1, 15.5)		29	1.00 s, 3H	16.42
11	3.91 m	72.55	30	5.75 br s	113.80
12	2.17[b] 2H	43.72	31		167.45
13		157.76	32	1.04 s, 3H	19.70
14	1.93[b]	36.30	33	1.11 s, 3H	23.23
	3.58 br d (13.8)		34	5.82 br s	114.16
15	4.10 m	79.37	35		172.91
16	5.36 dd (8.4, 16.1)	131.10	36	3.65 s, 3H	50.72
17	5.80 d (16.1)	138.01	1′		178.37
18		44.85	2′		38.74
			3′−5′	1.15 s, 9H	26.28

[a] Assignments made from HSQC and HMBC data. [b] Signal obscured.

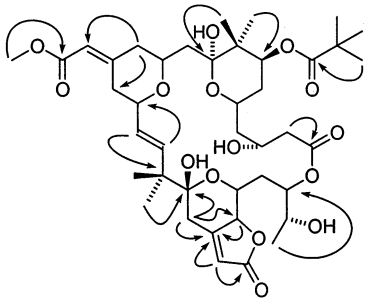

Figure 1. Selected HMBC correlations observed in **2**.

to C-18 that was identical to that portion of **1**. The only significant differences were observed in the vicinity of the C-19 to C-23 tetrahydropyran ring. In compound **2**, H-22 was shifted downfield to δ 4.42 and it showed an 8.9 Hz vicinal coupling to H-23 and a 1.0 Hz allylic coupling to H-34. An oxygen substituent on C-22 was evident from the low-field carbon resonance at δ 82.30. While bryostatin 10 (**1**) has methyl ester groups at C-31 and C-35, only one methyl ester moiety was present in **2**, and it was assigned to the C-31 carbonyl on the basis of HMBC correlation data (Figure 1). Bryostatin 20 (**2**) appeared to be a structural homologue of **1** in which the C-35 carbonyl was esterified to the oxygen on C-22, thus forming a buteneolide ring. An absorption band at 1779 cm^{-1} in the IR spectrum of **2** was consistent with this assignment. An unsaturated γ-lactone fused to the C-19 to C-23 tetrahydropyran ring is characteristic of several other bryostatins including bryostatin 3,[8] several bryostatin 3 congeners,[11,12] and bryostatin 19.[13] NMR data from this bicyclic region of **2** corresponded well with the data from these other bryostatins. HMBC correlations from H-20 to C-19, C-21, and C-22 and correlations from H-34 to C-21 and C-35 supported this assignment. The NMR data for **2**, which we obtained in CD_3CN, were carefully compared to the published data for bryostatins 3, 16, 17, and 18,[10] which also were recorded in CD_3CN. Close similarity in the NMR chemical shift values and coupling constants indicated that

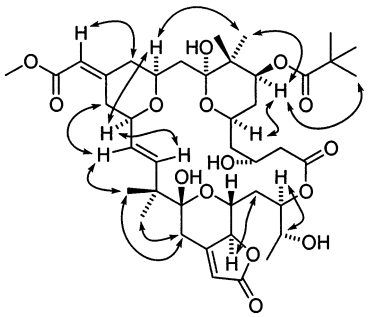

Figure 2. Selected NOE interactions observed in **2**.

the relative stereochemistry in **2** was the same as these other bryostatins. A comprehensive set of NOESY correlations observed with **2** (Figure 2) was fully consistent with the proposed stereochemistry. While several diagnostic ^{1}H resonances in the C-11 to C-15 portion of **2** were obscured (Table 1), NOE interactions between H-11 and H-15 were indicative of a diaxial orientation for these protons. The relative configuration at C-22 was assigned on the basis of NOE interactions between H-22 and the C-24 methylene protons and the 8.9 Hz coupling between H-22 and H-23, which was similar to the *trans* diaxial coupling of these protons observed in bryostatin 3.[11] This completed the structural characterization of bryostatin 20 (**2**).

The vast majority of reported symbioses between marine invertebrates and microorganisms involve nutritional enhancements critical to the fitness of the host.[14] Through a symbiont knock-out experiment, Lopanik and co-workers[5] showed that *E. sertula* likely does not contribute nutritionally to *B. neritina* because asymbiotic colonies grew and reproduced as well as symbiotic colonies. In contrast, larvae of asymbiotic colonies lacked bryostatins, whereas colonies hosting a natural abundance of *E. sertula* produced larvae with high bryostatin levels. Further, Lopanik et al. clearly demonstrated that bryostatins [e.g., bryostatins 10 (**1**) and 20 (**2**)] protect *B. neritina* larvae from predation by fishes. High larval concentrations of bryostatins also occur in California populations.[15]

The *B. neritina*−*E. sertula* association is the first example from the marine environment of a symbiont producing an antipredator defense for its host, one that appears to have evolved to protect the highly vulnerable larval stage. In conjunction with the increasing number of secondary metabolites isolated from marine invertebrates shown to have a microbial origin,[3,4,16,17] our results with *B. neritina* and studies by Fenical and co-workers[2,18] suggest that symbiont production of ecologically active secondary compounds may be widespread among diverse taxa of marine invertebrates.

While our basic research on the chemical ecology of *B. neritina* has yielded new insights into the understudied area of defensive symbioses in the marine environment, much of the recent research on the symbiosis between *B. neritina* and *E. sertula* has focused on using state-of-the-art genetic, molecular, and microbial manipulations to optimize bryostatin production for biomedical applications.[19] Our discovery of high bryostatin levels in larvae of *B. neritina*, however, suggests that current aquaculture methodologies developed for *B. neritina*[20] could be modified to substantially increase bryostatin yields. This increase would be accomplished not by the harvest of the adults but by perpetuating them for their daily production of bryostatin-rich larvae, which, if not captured, results in the loss of the majority of bryostatins produced over the lifetime of a colony.[21]

Experimental Section

General Experimental Procedures. The optical rotation was measured with a Perkin-Elmer 241 polarimeter, the UV spectrum was recorded on a Beckman DU 640 spectrophotometer, and the IR spectrum was obtained on NaCl disks in a Perkin-Elmer Spectrum 2000 FT-IR spectrometer. NMR spectra were obtained with a Varian INOVA NMR spectrometer at 500 MHz for ^{1}H and 125 Hz for ^{13}C using residual solvent peaks at δ_H 3.30 and at δ_C 49.00 ppm as chemical shift reference signals. High-resolution electrospray ionization (ESI) mass spectrometry data were acquired on a Thermo Finnigan TSQ Quantum mass spectrometer operating in the positive ion mode and using sodiated PEG as a reference. Bryostatins were purified using a Waters HPLC system consisting of two 510 pumps, a 717 Plus autosampler, and a 996 photodiode-array detector. A methanol−water gradient [80% methanol (0.6 mL/min) increasing to 100% methanol (0.75 mL/min) in 28 min)] over a Rainin Microsorb ODS column (4.6 × 250 mm) with 200−300 nm PDA detection was used to purify the individual bryostatins.

Animal and Collection Information. Reproductive colonies of *B. neritina* (Linneaus) were collected from 4 to 10 m depth along the Radio Island Jetty near Morehead, NC. Collected colonies were placed in containers of seawater and transported to the UNC Chapel Hill Institute of Marine Sciences (IMS). Fifteen to twenty individual colonies were woven into the strands of 0.5 m long lengths of 3-strand polypropylene line, which were then placed in a 5 m by 10 m by 0.75 m deep concrete block pond continuously supplied with fresh seawater. Vigorous aeration circulated seawater in the pond, and under these conditions, the colonies grew and reproduced. A voucher specimen is on deposit at IMS.

Extraction and Isolation. At 2-day intervals, the polypropylene lines with attached *B. neritina* were placed in clear, 12 L glass jars filled with seawater. The jars were positioned in full sunlight approximately 3 h after sunrise, which stimulated the reproductive colonies to begin releasing larvae within 10−15 min. After about 30 min, larvae aggregated at the rim of the jars were gently pipetted into a small sieve having a 100 μm NITEX mesh bottom. The sieve was partially submerged in a container of ice-chilled seawater to prevent larval settlement. After collecting the released larvae, the lines with the adults were placed back in the pond for subsequent larval collections. Extraction of bryostatins from the larvae was accomplished by draining the water from the sieve, leaving the spherical larvae (250−350 μm diameter) on the sieve mesh, and then placing the sieve into a 0.56 M potassium chloride (KCl) solution for 30 s. This brief KCl soaking caused the larvae to leach >90% of their bryostatins along with some pigments.[5] The KCl solution was then solvent extracted twice with DCM and then twice with *n*-butanol. The DCM and butanol partitions were combined and the solvents removed by rotary evaporation. Bryostatins were then purified in one step from the larval exudate using reverse-phase HPLC. A fish feeding bioassay, as described by Lopanik and co-workers,[5] identified three unpalatable compounds, corresponding to bryostatin 10 (**1**) (0.8 mg·mL^{-1} of larvae), bryostatin 20 (**2**) (0.6 mg·mL^{-1} of larvae), and a third, as yet unidentified, bryostatin (0.2 mg·mL^{-1} of larvae), with the total bryostatin content of the larvae ranging from 1 to 2% of their dry mass.

Bryostatin 20 (2): colorless oil; $[\alpha]^{24}_D$ +52.1° (*c* 0.04, MeOH); UV (MeOH) λ_{max} (log ϵ) 218 (4.13) nm; IR (neat, NaCl plate) ν_{max} 3453, 2925, 1779, 1728, 1285, 1154 cm^{-1}; ^{1}H NMR and ^{13}C NMR, see Table 1; HRESIMS *m/z* 815.3826 [M + Na]$^+$ (calcd for $C_{41}H_{60}O_{15}Na$, 815.3830).

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References and Notes

(1) Saikkonen, K.; Faeth, S. E.; Helander, M.; Sullivan, T. J. *Annu. Rev. Ecol. Syst.* **1998**, *29*, 319−343.
(2) Gil-Turnes, M. S.; Hay M. E.; Fenical, W. *Science* **1989**, *246*, 116−118.
(3) Kobayashi, J.; Ishibashi, M. *Chem. Rev.* **1993**, *93*, 1753−1769.
(4) Faulkner, D. J.; Harper, M. K.; Salomon, C. E.; Schmidt, E. W. *Mem. Queensland Mus.* **1999**, *44*, 167−173.
(5) Lopanik, N. B.; Lindquist, N.; Targett, N. *Oecologia* **2004**, *139*, 131−139.
(6) Haygood, M. G.; Davidson, S. K. In *New Developments in Marine Biotechnology*; Le Gal, Y., Halvorson, H. O., Eds.; Plenum Press: New York, 1998; pp 281−284.
(7) Davidson, S. K.; Allen, S. W.; Lim, G. E.; Anderson, C. M.; Haygood, M. G. *Appl. Environ. Microbiol.* **2001**, *67*, 4531−4537.
(8) Pettit, G. R.; Herald, C. L.; Kamano, Y. *J. Org. Chem.* **1983**, *48*, 5354−5356.
(9) Pettit, G.; Kamano, Y.; Herald, C. L. *J. Org. Chem.* **1987**, *52*, 2848−2854.
(10) (a) Pettit, G. R.; Herald, D. L.; Gao, F.; Sengupta, D.; Herald, C. L. *J. Org. Chem.* **1991**, *56*, 1337−1340. (b) Pettit, G. R.; Gao, F.; Blimberg, P. M.; Herald, C. L.; Kamano, Y.; Lewin, N. E.; Schmidt, J. M.; Chapuis, J.-C. *J. Nat. Prod.* **1996**, *59*, 286−289.
(11) Schaufelberger, D. E.; Chmurny, G. N.; Beutler, J. A.; Koleck, M. P.; Alvarado, A. B.; Schaufelberger, B. W.; Muschik, G. M. *J. Org. Chem.* **1991**, *56*, 2895−2900.
(12) Chmurny, G. N.; Koleck, M. P.; Hilton, B. D. *J. Org. Chem.* **1992**, *57*, 5260−5264.
(13) Lin, H.; Yi, Y.; Li W.; et al. *Zhongguo Haiyang Yaowu* **1998**, *17*, 1−3.
(14) Douglas, A. E. *Symbiotic Associations*; Oxford University Press: Oxford, UK, 1994.
(15) Lim, G.; Faulkner, D. J. Personal communication.
(16) Stierle, A. C.; Cardellina, J. H., II; Singleton, F. L. *Experientia* **1988**, *44*, 1021.
(17) Elyakov, G. B.; Kuznetsova, T.; Mikhailov, V. V.; Maltsev, I. I.; Voinov, V. G.; Fedoreyev, S. A. *Experientia* **1991**, *47*, 632−633.
(18) Gil-Turnes, M. S.; Fenical, W. *Biol. Bull.* **1992**, *182*, 105−108.
(19) Haygood, M. G.; Schmidt, E. W.; Davidson, S. K.; Faulkner, D. J. *J. Mol. Microbiol. Biotechnol.* **1999**, *1*, 33−43.
(20) Mendola D. In *Drugs from the Sea*; Fusetani, N., Ed.; Karger: Basel, 2000; pp 120−133.
(21) Lindquist, N.; Lopanik, N. B. U.S. patent application 20030233000, 2003.

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