Antineoplastic Agents. 340. Isolation and Structural Elucidation of Bryostatins 16−18[1]

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Separation of two trace cancer cell growth inhibitory (P388 leukemia) fractions from about 1000 kg of wet Gulf of Mexico *Bugula neritina* (Bryozoa) has led to the isolation of bryostatins 16−18 (**2**−**4**). A combination of HRFABMS and high-field (400 MHz) ^{1}H- and ^{13}C-NMR spectral analyses were employed to assign the structures. The three new 20-desoxybryostatins 16 (**2**), 17 (**3**), and 18 (**4**) showed significant growth inhibitory activity (P388 ED_{50}, **2**, 9.3×10^{-3} μg/mL, **3**, 1.9×10^{-2} μg/mL, and **4**, 3.3×10^{-3} μg/mL) against murine P388 lymphocytic leukemia.

The marine bryozoan *Bugula neritina* L. (family Bugulidae) has proved to be an exciting and exceptionally useful source of new anticancer drugs of the bryostatin class.[2] From our initial observations[3] in 1968 that *B. neritina* offered promise of containing very potent antineoplastic constituents, we proceeded (1968−1981) to isolate and determine the structures of bryostatins 1−15 and bryostatins A and B.[2,4−10] After the isolation and structural determination of bryostatin 1 (**1**) in 1981, preclinical development was undertaken and the first phase I human clinical trials began in early 1991. Promising initial clinical results have been reported.[11−14] Currently, the U.S. National Cancer Institute has entered bryostatin 1 into phase II clinical trials.

Because of the encouraging clinical progress of bryostatin 1 and the considerable utility in employing the bryostatins as biochemical probes,[15−22] we have continued to study structural modifications[23,24] and isolate new members[10] of this important series.[2,4−10,25] Since Gulf of Mexico (Florida) collections of *B. neritina*[4] have been found to afford the greatest variety of new bryostatins, we further explored the trace components of a 1986 recollection (1000 kg, wet wt) employing assays against the murine P388 lymphocytic leukemia cell line to guide the separation.

From a complex fraction containing bryostatins 4−8,[4,5,6] bryostatin 10,[8] and bryostatin 14,[10] we have succeeded in isolating the new trace cancer cell growth inhibitory constituents designated bryostatins 16 (**2**), 17 (**3**), and 18 (**4**) (Chart 1). Successive column chromatographic separations using Sephadex LH-20 and silica gel followed by high speed countercurrent distribution[26] led to two penultimate fractions. The first of these was separated by HPLC on silica gel and found to contain bryostatin 16 (**2**) accompanied by bryostatins 4−8 and bryostatin 17. Separation of the second fraction was achieved by normal-phase silica gel semipreparative HPLC to give bryostatin 18 accompanied by bryostatins 4−8 and 10.

Bryostatins 16 (**2**) and 17 (**3**) proved to be stereoisomers. Mass spectra of the two isomers led to the same molecular formula. Structural elucidation of the two isomers was based primarily on careful analysis of the high-field (400 MHz) 2D NMR spectra. From the ^{1}H-NMR data, significant differences were exhibited by the signals corresponding to H-20, H-22a, and H-22b (see Table 1). The dramatic downfield shift of the H-20 proton signal from δ 5.50 in bryostatin 16 to δ 6.78 in bryostatin 17 was attributed to proximity of a deshielding carbonyl oxygen. The H-22a proton of bryostatin 16 was found at δ 3.57 compared to the H-22a chemical shift at δ 2.48 in bryostatin 17. Otherwise, the remaining ^{1}H-NMR signals of bryostatins 16 and 17 were very similar. When the ^{13}C-NMR spectra of the two compounds were compared, the chemical shift for the C-20 of bryostatin 16 was δ 101.55 *vs*. 97.14 for bryostatin 17 and for C-22 was δ 32.82 *vs*. 37.10, respectively (Table 2). On the basis of ^{1}H-NMR spectral data, the bryostatin 16 (**2**) C-21, 34 double bond was assigned the same geometry as in bryostatin 1 and the opposite geometry for bryostatin 17 (**3**). Apparently, the C-35 carbonyl group of bryostatin 17 resides closer to the C-20 proton than in the case of bryostatin 16. An overall interpretation of the ^{1}H - and ^{13}C-NMR data (Tables 1 and 2) combined with the NOE and HRFAB mass spectral data showed that bryostatin 16 was the $\Delta^{19,20}$-derivative of bryostatin 10 (**5**)[8] and bryostatin 17 was the $\Delta^{19,20}$-derivative of bryostatin 18 (see below). While it is conceivable that both bryostatin 16 and 17 were formed from bryostatins 10 and 18, respectively, during the isolation procedures, this is unlikely in view of the usual good stability of the bryostatin C-19 hydroxyl group.

The HRFABMS data from bryostatin 18 (**4**) indicated the molecular formula $C_{42}H_{64}O_{15}$. The low resolution FABMS indicated ready loss of water by the appearance of ions at *m/z* 815 $[M + Li]^+$(100) and 797 $[M + Li - H_2O]^+$(42). The molecular formula of bryostatin 18 corresponded to that of bryostatin 10 (**5**). Thus, the detailed structure of bryostatin 18 was determined by direct comparison of its NMR spectra with those of bryostatin 10.[8] While both the ^{1}H- and ^{13}C-NMR spectra were very similar, some differences were obvious. In the ^{1}H-NMR spectrum of bryostatin 18, the two H-20 proton signals were at δ 3.79 and 2.10 and the two from H-22 were at δ 2.23 and 2.02. By comparison, the H-20 protons of bryostatin 10 appear at δ 2.37 and 2.20 and signals for the two H-22 protons at δ 4.00 and 1.95. In addition, the ^{13}C-chemical shifts for the C-20

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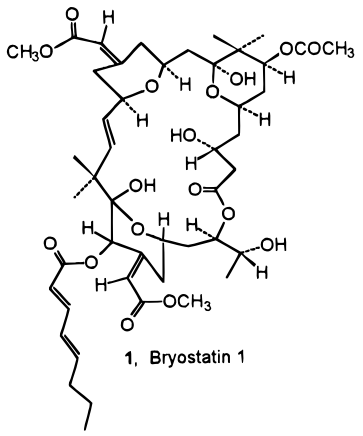
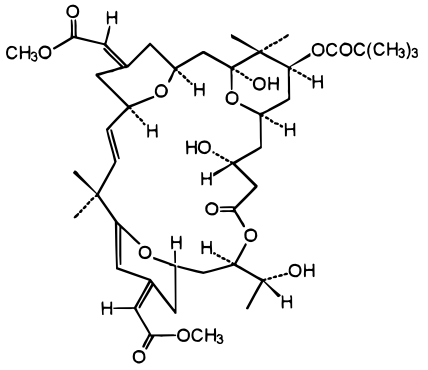
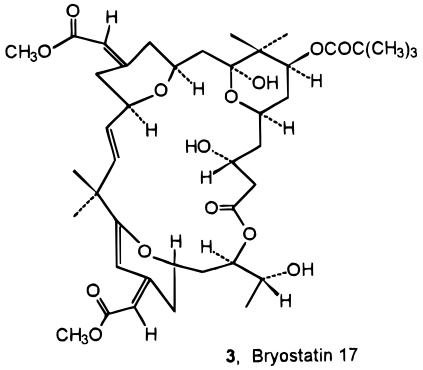
Chart 1



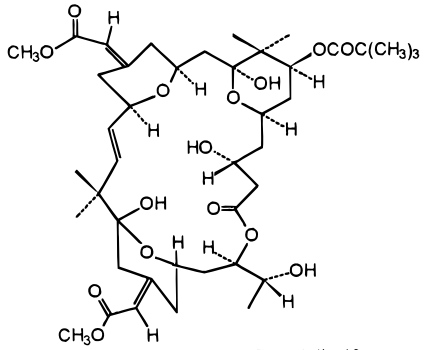
1, Bryostatin 1



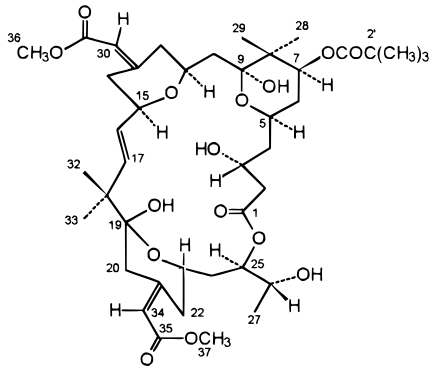
2, Bryostatin 16



3, Bryostatin 17



4, Bryostatin 18



5, Bryostatin 10

and C-22 carbons of bryostatin 18 (**4**) appeared at δ 33.70 and 43.13 while the C-20 and C-22 carbon atoms of bryostatin 10 were assigned, respectively, δ 40.39 and 36.63 (Tables 1 and 2). Therefore, bryostatin 18 was assigned the structure **4** corresponding to the C-21 → C-34 geometrical isomer of bryostatin 10.

The discovery of bryostatins 16−18 proved to be useful for a variety of reasons. Foremost among these was the opportunity to further assess limits of the structural pharmacophore proposed for the bryostatin effects on protein kinase C.[27] Loss of the C-19 hydroxyl group led to a 100-fold decrease in PKCα activity by bryostatins 16 and 17 compared with bryostatin 10, while the geometry of the isomers (*cf.* bryostatins 16 and 17) made little difference. Furthermore, it permitted experimental evaluation of the role of the C-9 hydroxyl group in the phorbol ester pharmacophore.[27−29] In contrast to the availability of derivatives at the other oxygens, elimination of the C-9 oxygen has so far been synthetically inaccessible in phorbol and interpretation has been further clouded by potential hydrogen bonding between the C-9 hydroxyl and the carbonyl of the phorbol 13-esters. Modeling indicates that the C-9 hydroxyl of phorbol is homologous to the C-19 hydroxyl of the bryostatins.[28] Binding of bryostatins 16 and 17 to protein kinase C α, assayed under our usual conditions in the presence of phosphatidylserine/Triton X-100,[30] yielded K_i values of 118 ± 2 nM (n = 3) and 188 ± 7 nM (n = 3), respectively. The latter value is in good agreement with our previous bryostatin findings.[31] For comparison, bryostatin 10 yielded a K_i of 1.56 ± 0.16 nM (n = 3) and bryostatin 18 yielded a K_i of 4.82 ± 0.06 nM (n = 3); the value for bryostatin 10 is in good agreement with previous measurements.[27−32] We conclude that (a) the configuration of the C-21 → C-34 double bond has a rather modest influence on binding affinity, with slightly higher affinity for the bryostatin 1 configuration, and (b) the C-19 hydroxyl contributes

Table 1. ^{1}H-NMR (400 MHz) Assignments (δ, ppm) for Bryostatins 16−18 in Deuterioacetonitrile Solution with Tetramethylsilane as Reference

position	bryostatin 16	bryostatin 17	bryostatin 18
2a	2.47 *	2.61 brd (16)	2.50 brs
2b	2.47 *	2.38 dd (6, 16)	2.50 brs
3	4.16 m	4.18 m	4.04 m
4a	1.64 m	1.52 brdd (11,12)	1.80 *
4b	1.64 m	1.47 brdd (12,12)	1.70 *
5	4.12 m	3.70 brt (12)	4.20 brt (12)
6a	1.67 m	1.64 brdd (5, 12)	1.64 *
6b	1.40 dt (12, 12)	1.40 dt (12, 12)	1.45 dt (12, 12)
7	5.10 dd (5, 12)	4.89 dd (5, 12)	5.07 dd (5, 12)
10a	1.86 *	2.25 dd (2, 15)	1.99 *
10b	1.78 dd (3, 15)	1.75 dd (11, 15)	1.73 *
11	3.78 *	3.95 brt (11)	3.92 *
12a	2.23 *	2.26 brd (15)	2.17 *
12b	2.18 *	2.17 brdd (11, 15)	2.17 *
14a	3.68 *	3.70 brd (12)	3.57 brd (12)
14b	1.89 *	2.02 brt (12)	1.91 *
15	3.98 brdd (7.3, 12)	4.00 brdd (8, 12)	4.10 *
16	5.41 dd (7.3, 16)	5.54 dd (8, 16)	5.31 dd (8, 16)
17	5.92 d (16)	6.12 d (16)	5.87 d (16)
20a	5.50 brs	6.78 brs	3.79 d (12)
20b			2.10 *
22a	3.57 brd (17)	2.48 brdd (11, 15)	2.23 *
22b	2.25 brdd (11, 17)	2.32 brd (15)	2.02 *
23	3.82 brt (11)	3.87 brt (11)	3.96 *
24a	1.98 brt (11)	1.96 *	1.95 *
24b	1.88 m	1.85 m	1.62 m
25	5.20 brd (11)	5.35 brd (11)	5.12 *
26	3.76 *	3.75 dq (6.7, 6.7)	3.75 *
27	1.10 d (6.6)	1.10 d (6.7)	1.07 d (6.7)
28	0.97 s	0.98 s	1.02 s
29	0.89 s	0.87 s	1.01 s
30	5.75 brs	5.72 brs	5.76 brs
32	1.21 s	1.21 s	1.09 s
33	1.19 s	1.29 s	0.89 s
34	5.47 brs	5.25 brs	5.73 brs
36	3.66 s	3.65 s	3.66 s
37	3.61 s	3.62 s	3.63 s
OR			
3′−5′	1.16 s	1.16 s	1.16 s
19OH			4.74 d (3)

*Couplings for these signals were obscured.

Table 2. ^{13}C NMR Assignments (δ, ppm at 100 MHz) for Bryostatins 10, and 16−18 in Deuterioacetonitrile Solution with Tetramethylsilane as Internal Standard

	bryostatin 10	bryostatin 16	bryostatin 17	bryostatin 18
C-1	172.34	172.80	174.85	172.37
2	42.72	42.92	42.73	42.73
3	69.82	67.41	65.77	69.81
4	40.93	42.54	42.64	40.95
5	66.70	65.97	69.01	66.70
6	34.02	34.31	34.00	34.02
7	73.15	73.48	75.09	73.20
8	42.24	41.86	42.11	42.26
9	102.05	102.50	101.71	102.75
10	42.72	43.04	37.08	42.73
11	73.42	74.42	75.97	73.44
12	44.70	45.29	43.75	44.71
13	158.76	158.95	157.68	158.89
14	37.32	36.48	37.10	37.36
15	80.53	79.94	81.27	80.51
16	130.91	128.20	127.45	130.94
17	139.77	139.49	140.70	139.85
18	45.56	42.07	43.75	45.68
19	102.73	151.91	149.80	101.98
20	40.39	101.55	97.14	33.70
21	158.53	168.22	167.97	158.81
22	36.63	32.82	37.10	43.13
23	65.76	73.48	74.37	66.17
24	36.11	36.48	36.26	36.03
25	73.42	73.48	73.35	73.25
26	69.07	69.02	69.02	69.08
27	19.08	19.14	19.36	19.18
28	20.46	21.29	21.37	20.55
29	17.34	17.23	15.28	17.35
30	114.53	114.82	115.50	114.52
31	167.70	167.78	167.61	167.73
32	21.32	27.72	27.93	21.33
33	24.71	23.45	22.64	24.75
34	116.23	108.90	108.89	116.26
35	167.47	170.02	170.23	167.67
36	51.60	51.59	51.58	51.61
37	51.40	51.21	51.29	51.40
OR				
1′	178.49	178.51	178.42	178.54
2′	39.64	39.65	39.67	39.67
3′−5′	27.45	27.47	27.46	27.46

a factor of 100 to the binding affinity, consistent with its forming an element of the pharmacophore. A further conclusion is that not all elements of the pharmacophore contribute equally to binding. Thus, for example, epimerization of the C-26 hydroxyl of bryostatin 4 (which is homologous to the C-20 hydroxyl of phorbol) caused complete loss of binding affinity under comparable binding conditions.[32]

Another important aspect of the biological evaluations of bryostatins 16−18 concerned effects against the P388 lymphocytic leukemia and a minipanel of the U. S. National Cancer Institute's human cancer cell lines.[33] The GI_{50} (μg/mL) for bryostatin 16 ranged from 0.019 to 2.2, for bryostatin 17, 0.16 to 3.2, and for bryostatin 18, 0.42 to 3.7. In each case the greatest activity was displayed against the lung (NCI-H460) cell line. Specifically, bryostatin 16 resulted in inhibitory activity against ovarian (OVCAR-3: GI_{50} 1.9 μg/mL), CNS (SF-295: GI_{50} 1.4 μg/mL), renal (A498: GI_{50} 1.9 μg/mL), lung (NCI-H460: GI_{50} 0.019 μg/mL), colon (KM20L2: GI_{50} 2.2 μg/mL), and melanoma (SK-MEL-5: GI_{50} 1.2 μg/mL). Bryostatins 17 and 18 were evaluated against the same minipanel with very similar results.

Experimental Section

General Experimental Procedures. Solvents used for column chromatography were redistilled. Sephadex LH-20 was obtained from Pharmacia Fine Chemicals AB. All ^{1}H- and ^{13}C-NMR data were obtained using a Bruker AM 400 spectrometer. Optical rotations were measured using a Perkin-Elmer Model 241 polarimeter. The IR spectra were recorded using a Nicolet MX-1 FT-IR spectrometer. UV detection at 254 nm was used for HPLC. For other general experimental procedures refer to ref 10.

Animal Material. An approximate 1000 kg (wet wt) amount of *B. neritina* L. was collected in May 1986 in the Northern Gulf of Mexico (Florida) and preserved in 2-propanol. A voucher specimen of *B. neritina* is maintained at the Arizona State University Cancer Research Institute. For animal taxonomy and earlier recollection data, see ref 4.

Isolation. The biomass was reduced to a murine P388 lymphocytic leukemia (PS) active fraction weighing 906 g as previously described.[10] This fraction was further separated as summarized for isolation of bryostatin 14.[10] The two PS active and complex fractions from the high speed countercurrent distribution step were separated as follows.

The fraction containing bryostatins 16 and 17 was separated by reversed-phase semipreparative HPLC using an RP C-8 column (10 mm × 25 cm) with 4:1 CH_3OH-H_2O as eluting solvent (1.0 mL/min). Bryostatin 16 (4.3 mg) was eluted at 25.7 min and bryostatin 17

(3.4 mg) at 18.0 min. Separation of the fraction containing bryostatin 18 was accomplished by normal-phase silica gel semipreparative HPLC (10.0 mm × 25 cm column) using hexane−2-propanol (9:1) as eluant. Bryostatin 18 (6.7 mg) eluted at 26 min. The known bryostatins 4−8 and bryostatin 10 were also isolated from this fraction.

Bryostatin 16: $[\alpha]_D = +84°$ ($c = 0.43$, CH_3OH); LRFABMS (3-NBA/glycerol matrix) m/z (rel int), 813 $[M + Na]^+$ (10), 790 $[M]^+$ (30), 773 $[M - OH]^+$ (80), 741 $[773 - CH_3OH]^+$ (100); HRFABMS ($C_{42}H_{62}O_{14}Na$, calcd 813.4037) found 813.4038; NMR data, see Tables 1 and 2.

Bryostatin 17: $[\alpha]_D = +231°$ ($c = 0.34$, CH_3OH); LRFABMS (3-NBA/glycerol matrix) m/z (rel int), 813 $[M + Na]^+$ (4), 790 $[M]^+$ (29), 773 $[M - OH]^+$ (100), 741 $[773 - CH_3OH]^+$ (78); LRFABMS (3-NBA/LiCl matrix) m/z (rel int) 797 $[M + Li]^+$ (100); HRFABMS ($C_{42}H_{62}O_{14}$-Li, calcd 797.4277) found 797.4301; NMR data, see Tables 1 and 2.

Bryostatin 18: $[\alpha]_D = +136°$ ($c = 0.67$, CH_3OH); IR (film) ν_{max} 3465, 3370 (OH), 1740 (COOR), 1650 (C═C), 1610 (conjugated C═C), 1380 (CH_3), 1155 (COOR), 860 (CH_3); LRFABMS (3-NBA/LiCl matrix) m/z (rel int), 815 $[M + Li]^+$ (100), 797 $[M + Li - H_2O]^+$ (42); HRFABMS ($C_{42}H_{64}O_{15}Li$ calcd 815.4405), found 815.4432; NMR data, see Tables 1 and 2.

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