methyl appeared at δ 2.153 as a sharp singlet in the ^{1}H NMR spectrum.

26-Oxobryostatin 10 (4b). The oxidation of bryostatin 10 (**2a**) (3.0 mg; with chromium trioxide, 1.2 mg) in pyridine (1.2 mL) and purification of product was executed as summarized for obtaining 26-oxobryostatin 4 (**4a**). Recrystallization from methylene chloride–methanol gave pure plates (1.8 mg, 60% yield) of 26-oxobryostatin 10 (**4b**): mp 163–165 °C; MS (SP-SIMS), mol wt 860 for $C_{42}H_{62}O_{15}$, from m/z 829 ([M + Na]$^+$), 817 ([M + Na − 18]$^+$), 771 ([M + Na − 58]$^+$), 739 ([M + Na − 90]$^+$), 727 ([M + Na − 102]$^+$); $[\alpha]^{27}_D$ + 95.2° (c 0.035, CH_3OH); UV $\lambda_{max}^{CH_3OH}$ 227 (ϵ 36 050) nm; IR (KBr) 3450, 2980–2938, 1745, 1724, 1650, 1435, 1370, 1360, 1285, 1240, 1165, 1100, 1085, 1045, 1025, 1000, 870 cm^{-1}. The high-resolution proton NMR spectrum and interpretation has been summarized in Table IV.

13,30-Epoxybryostatin 4 (4c). To a solution of bryostatin 4 (**1b**, 3.2 mg) in methylene chloride (0.5 mL) was added 1.5 mg of *m*-chloroperbenzoic acid, and the mixture was allowed to remain at room temperature for 48 h. Upon dilution with ice–water and extraction with methylene chloride the solution was washed with aqueous sodium bisulfite and potassium iodide followed by water. After removal of the solvent, the crude product (3.1 mg) was purified by the same HPLC method described above for obtaining acetate **1c**. Recrystallization from methylene chloride–methanol afforded 2.0 mg (62.5% yield) of the 13,30-epoxide **4c** as plates melting at 189–192 °C: MS (SP-SIMS), mol wt 910 for $C_{46}H_{70}O_{18}$, from m/z 933 ([M + Na]$^+$), 915 ([M + Na − 18]$^+$), 901 ([M + Na − 32]$^+$), 875 ([M + Na − 58]$^+$), 861 ([M + Na − 72]$^+$), 845 ([M + Na − 88]$^+$), and 831 ([M + Na − 102]$^+$); $[\alpha]^{27}_D$ + 56.75 (c 0.04, CH_3OH); UV $\lambda_{max}^{CH_3OH}$ 227 (ϵ 35 800) 227 nm; IR (KBr) 3450, 2985–2440, 1740, 1725, 1660–1640, 1440, 1380, 1360, 1290, 1240, 1160, 1100, 1080, 1060, 1045, 1000, and 870 cm^{-1}. The proton NMR data is given in Table III.

13,30-Epoxybryostatin 10 (4d). The epoxidation of bryostatin 10 (**2a**, 3.0 mg) was pursued and epoxide **4d** isolated in 63% yield (1.9 mg) as summarized above in the parallel experiment employing bryostatin 4. After recrystallization from methylene chloride–methanol the 13,30-epoxide **4d** was obtained as plates melting at 184–186 °C; MS (SP-SIMS), mol wt 824 for $C_{42}H_{64}O_{16}$, from m/z 847 ([M + Na]$^+$), 829 ([M + Na − 18]$^+$), 815 ([M + Na − 32]$^+$), 789 ([M + Na − 58]$^+$), 757 ([M + Na − 90]$^+$), and 745 ([M + Na − 102]$^+$); $[\alpha]^{27}_D$ +88.5° (c 0.035, CH_3OH); UV λ_{max} 226 (ϵ 36 000) nm; IR (KBr) 3460, 2980–2940, 1740, 1720, 1650, 1435, 1380, 1358, 1283, 1235, 1165, 1100, 1080, 1030, 1000, and 872 cm^{-1}. A summary of the proton NMR spectrum with assignments is given in Table III.

Acknowledgment. The very necessary financial assistance was provided by Eleanor W. Libby, the Waddell Foundation (Donald Ware), the Fannie E. Rippel Foundation, the Flinn Foundation, Mary Dell Pritzlaff, the Olin Foundation, the Robert B. Dalton Endowment Fund, the Upjohn Company, and the Division of Cancer Treatment, NCI, National Institutes of Health DHHS (Grants CA-16049-07 and 09), awarded by the National Cancer Institute. Other helpful assistance was contributed by the Smithsonian Institution Oceanographic Sorting Center, the National Science Foundation Regional Facility at the University of South Carolina (Grant CHE 82-07445), Drs. H. Cohen, D. L. Doubek, P. D. Ellis, G. Hendler, L. W. Knapp, P. Lohavanijaya, M. I. Suffness, and J. M. Schmidt, and the Mexican Government, Departamento de Pesquerias (Dr. Edith Polanco Jaime and Rogelio Castañares Ferrer).

Registry No. **1b**, 91523-82-9; **1c**, 97850-08-3; **1d**, 108268-84-4; **2a**, 102580-65-4; **2b**, 108268-90-2; **2c**, 108268-85-5; **2d**, 102580-63-2; **4a**, 108268-86-6; **4b**, 108268-87-7; **4c**, 108268-88-8; **4d**, 108268-89-9; *m*-bromobenzoyl chloride, 1711-09-7.

Isolation and Structure of Bryostatins 12 and 13[1a]

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Bryostatins 12 (**4**, 3.7 mg) and 13 (**5**, 0.7 mg) were isolated from ~1000 kg of the marine bryozoan *Bugula neritina* (Linnaeus) found in the Eastern Pacific Ocean (California). The new bryostatins led to strong cell growth inhibitory (PS cell line ED_{50} 0.014 and 0.0054 μg/mL) and antineoplastic activity (for bryostatin 12, a 47–68% increase in life extension at 30–50 μg/kg) against the marine P388 lymphocytic leukemia (PS system). In addition to the previously known constituents (bryostatins 1–3) of *Bugula neritina* from this ocean area the presence of bryostatins 8 (13.2 mg) and 9 (16.4 mg) was also established. A detailed series of ^{1}H–^{1}H COSY, 2D-*J*-resolved, ^{1}H–^{13}C 2D-shift correlated, and ^{1}H–[^{1}H] NOE difference ^{1}H NMR experiments combined with ^{13}C NMR and SP-SIMS studies were employed to elucidate the structures of bryostatins 12 and 13.

Chemical and biological evaluation of Bryozoan biosynthetic products appears to offer exceptional scientific and medical potential. The seven species[2,3] (of 4000+) of these fascinating marine animals so far examined have afforded marine alkaloids[4] and macrocyclic lactones[2,5] as diverse as the remarkable β-lactam chartelline A[3] from *Chartella papyracea* and bryostatin 1 (**1a**)[6] from *Bugula neritina*. Bryostatin 1 (**1a**) has been found to provide a 96% increase in life-span against the U.S. National Cancer Institute's P388 lymphocytic leukemia (PS system) at 70 μg/kg with activity starting at very low (<10 μg/kg) dose levels. Presently bryostatin 1 is undergoing preclinical

(1) (a) Paper 126 of the series Antineoplastic Agents. For 125, refer to: Singh, S. B.; Pettit, G. R.; Schmidt, J. M., submitted for publication in *Synth. Commun.* (b) Polyscience, Inc., Paul Valley Industrial Park, Warrington, PA 18976. (c) The Upjohn Co., Kalamazoo, MI 49001.

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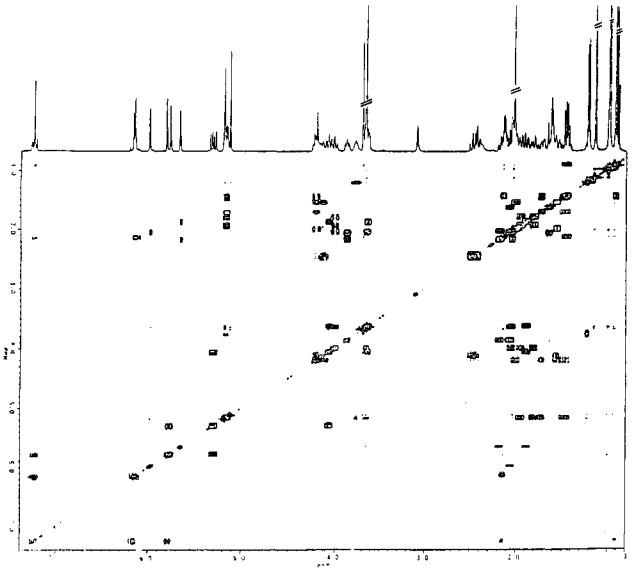


Figure 1. The ^1H–^1H COSY spectrum of bryostatin 1 (**1a**). The spectrum at the top of the diagram is the fully coupled ^1H NMR spectrum recorded by using 37.6 mmol/L at 313 K.

development and total syntheses are under way.[7]

We discovered bryostatins 1–3[8] in the Eastern Pacific Ocean collections of *Bugula neritina*, bryostatins 4–7 and 9–11[2,5,9] in Gulf of California, Mexico, and Sagami, Japan, specimens of this cosmopolitan, fouling bryozoan, bryostatin 8 in *Amathia convoluta* (Gulf of Mexico bryozoan),[2] and bryostatins A and B[10] in the yellow sponge *Lissondendoryx isodictyalis* invaded by *Bugula n.* All of the bryostatins have exhibited inhibition of the PS leukemia similar to that shown by bryostatin 1 (**1a**). While the bryostatins like the avermectins[11] do not appear to have antibacterial properties[12] typical of the macrolide antibiotics,[13] they do display other very interesting biological activities ranging from inhibition of RNA synthesis[14] to strong protein kinase C binding,[15] stimulation of protein phosphorylation, and activation of intact human polymorphonuclear leukocytes.[16]

In order to isolate and structurally identify the remaining bryostatins we originally detected[17] in microgram or less quantities and obtain enough bryostatin 1 for homonuclear correlation (COSY), two-dimensional carbon–proton chemical shift correlations (^1H–^{13}C 2D), 2D-*J*-resolved experiments, proton–proton differential nuclear Overhauser enhancement (NOEDS, or ^1H-[^1H] NOE) experiments, and additional biological evaluation, a 4000-L recollection of *Bugula neritina* (~1000 kg damp weight)

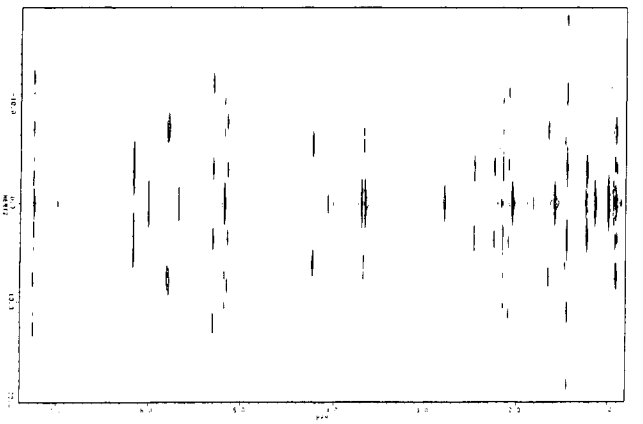
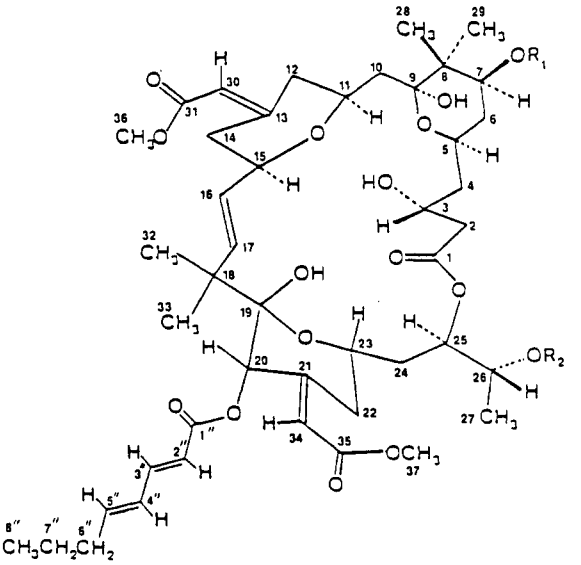


Figure 2. The 2D-*J*-resolved spectrum of bryostatin 1 (**1a**) at 4.4 mmol/L and 297 K.

from the Eastern Pacific Ocean (California) was extracted with 2-propanol and the product subjected to careful separation[9,10] guided by bioassay employing the PS leukemia. The initial large-scale separation procedure was based on our earlier techniques[6,9,10] except for introduction of a process-scale high-pressure liquid chromatography (HPLC) step following partition of the first methylene chloride fraction between methanol–water (9:1) and hexane. By this means the methanol–water fraction (1.8 kg) was separated into 26 major fractions. Bryostatin 1 (**1a**) was detected in fractions 8–12 and bryostatin 2 (**1b**) in 13–15. Similar fractions were combined and subjected to a series of chromatographic separations.[9,10] The bryostatin-enriched fractions yielded as major components bryostatin 1 (**1a**, 630 mg) and bryostatin 2 (**1b**, 588 mg).



1 a, R_1 = $COCH_3$, R_2=H, bryostatin 1

b, R_1=R_2=H, bryostatin 2

c, R_1=R_2=$COCH_2CH_2CH_3$, bryostatin 2 dibutyrate

The residual fractions from separation of bryostatins 1 and 2 were further fractionated by reversed-phase HPLC (C-18, methanol–water gradient). In this manner bryostatins 3[8] (1.6 mg), 8[2] (13.2 mg), and 9[18] (16.4 mg) were obtained. The latter two bryostatins had not previously been located in the California *Bugula neritina*. More importantly, two new substances designated bryostatins 12 (**3**, 3.7 mg) and

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Table I. Bryostatin ^{1}H NMR (400-MHz) Chemical Shift Assignments Relative to Tetramethylsilane in Deuteriochloroform Solution

	bryostatin 1 (c 4.4 mmol/L)			bryostatin 12 (c 12.8 mmol/L)		
H	δ	mult	$J_{H,H}$, Hz	δ	mult	$J_{H,H}$, Hz
2	2.46	dd	6.7, 1.3	2.46	m	
3	4.20	m		4.11	d	3
4a	2.02	m		2.01	m	
4b	1.58	d	8	1.56	d	8
5	4.23	d	12.1	4.23	d	12
6a	1.48	ddd	12.7, 12.7, 12.7	1.45	m	
6b	1.74	ddd	13.5, 1.5	1.72	m	
7	5.15	dd	12.1, 4.9	5.15	dd	12.2, 4.9
10a	2.07	m		2.06	m	
10b	2.23	d	7.5	2.16	m	
11	3.83	m		3.85	m	
12a	2.07	m		2.07	dd	15.5, 7.2
12b	3.68	dd	16.5, 0.7	3.67	dd	15, 0.7
14a	1.88	m		1.88	m	
14b	3.67	dd	13.3, 1.5	3.66	dd	11.2, 1.5
15	4.07	dt	8.5, 2	4.07	dt	9.6, 2.2
16	5.30	dd	15.8, 8.5	5.30	dd	15.6, 8.3
17	5.79	d	15.8	5.78	d	15.6
20	5.18	s		5.18	s	
22a	2.08	dd	15, 7.4	2.08	m	
22b	1.66	d	15	1.66	d	15
23	4.01	m		4.01	m	
24a	1.82	dd	11.5, 11.8	1.81	dd	11.3, 11.3
24b	1.97	dd	11.8, 11.8	1.95	dd	11.8, 11.8
25	5.17	dddd	17.6, 12.1, 3.0, 3.0	5.17	dddd	17.6, 11.8, 2.9, 2.9
26	3.78	m		3.78	m	
27	1.22	d	6.5	1.23	d	6.5
28[a]	0.94	s		0.94	s	
29[a]	1.00	s		1.00	s	
30	5.66	dd	1.3, 1.3	5.68	dd	2.2, 2.2
32[b]	1.15	s		1.15	s	
33[b]	1.00	s		1.00	s	
34	6.00	d	1.6	6.00	d	1.8
36	3.69	s		3.70	s	
37	3.66	s		3.66	s	
2′	2.05	s		2.28	t	7.5
3′				1.64	sextet	7.2
4′				0.94	t	7.3
2″	5.80	d	15.5	5.80	d	15.4
3″	7.25	m		7.25	m	
4″	6.16	dd	8.5, 2.4	6.16	dd	8.5, 2.0
5″	6.16	dd	4.8, 1.5	6.16	dd	4.9, 1.5
6″	2.15	dq	6.8, 3.0	2.14	dq	6.0, 2.5
7″	1.45	sextet	6.5	1.45	sextet	7.5
8″	0.92	t	7.6	0.92	t	7.3

[a] Chemical shift values are interchangeable. [b] Chemical shift values are interchangeable.

13 (**5**, 0.7 mg) were discovered, and their structural elucidation is recorded in the sequel. Bryostatin 12 (NSC 606294) exhibited activity against the PS leukemia cell line at ED_{50} 0.014 μg/mL and gave a 47–68% life extension at 30–50 μg/kg doses, while bryostatin 13 (NSC 606740) led to ED_{50} 0.0054 μg/mL.

In order to unambiguously complete the proton and carbon NMR assignments for the bryostatin series and undertake the structure of bryostatins 12 and 13 detailed high-resolution (400-MHz) NMR experiments were initially focused on bryostatin 1 (**1a**) where the X-ray crystal structure was well established.[6] A series of 2D NMR experiments allowed definitive assignments (Table I) for all the bryostatin 1 protons. The COSY experiment[19] uncovered proton–proton couplings critical to the chemical shift assignments (Figure 1). Multiplicities and coupling constants for the numerous multiplets in the spectrum were ascertained where possible from the 2D-*J*-resolved experiment[20] (Figure 2, Table I). Assignment of the ^{13}C NMR (proton decoupled) spectrum of bryostatin 1 (**2**) was achieved by using the ^{1}H–^{13}C 2D technique[21] (Figure 3). These experiments uncovered proton signals that were not detected by using earlier NMR techniques.[8] For example, the two one-proton multiplets at δ 3.64 and 3.65 (H-14a and H-12a, respectively) were previously obscured by the large methoxyl signals (δ 3.66 and 3.69 in the usual ^{1}H NMR spectrum). In the COSY spectrum (Figure 1) significant spin-couplings between protons in the region bearing the methoxyl groups (δ 3.66–3.69) with methylene hydrogens at δ 2.05 (H-12b) and 1.88 (H-14b) and the olefinic proton at δ 5.66 (H-30) were observed, pointing to the presence of two one-proton signals hidden by the methoxyl resonances. Further examination of this region in the 2D-*J*-resolved and ^{1}H–^{13}C 2D spectra provided additional confirmation.

During the ^{1}H NMR study, concentration and temperature dependent splitting patterns were noted for several multiplets in the spectrum, and most striking was the

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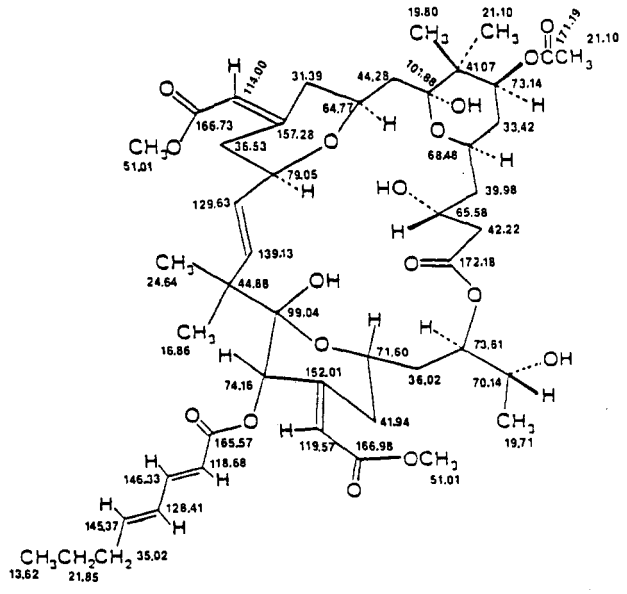

2

Bryostatin 1

(with ^{13}C-NMR)

methylene region at δ 2.46 (H-2). When the ^{1}H NMR spectrum was determined at a concentration of 34 mg/mL (37.6 mmol/L), the δ 2.46 region appeared as a triplet and a doublet of doublets (Figure 4). In the 2D-*J*-resolved experiment (Figure 5 at 17.7 mmol/L) this area showed four signals, while the peak integral in the normal ^{1}H NMR spectrum indicated only two hydrogens. To test the possibility that this discrepancy might be due to conformational changes, the spectra were redetermined at several concentrations and temperatures. At lower concentration (4 mg/mL, 4.4 mmol/L), the region in question collapsed to a single two-proton doublet of doublets, thereby simplifying the 2D-*J*-resolved spectrum. Similar results were observed at higher temperatures (Figures 4 and 5). A detailed conformational study of bryostatin 1 in solution is presently in progress.

With the NMR behavior of bryostatin 1 better understood, the stage was set for more readily solving the structures of bryostatins 12 and 13. Preliminary examination of the 400-MHz ^{1}H NMR spectrum of bryostatin 12 revealed a series of signals characteristic of the bryopyran ring system of bryostatins 1–9. Noteworthy were the proton signals attributable to the octadienoate ester side chain at C-20 of bryostatins 1 and 2. The C-20 carbon resonance at δ 74.15 and the H-20 proton signal at δ 5.18 (sharp singlet) further substantiated this assumption. The C-7 acetyl protons of bryostatin 1 normally at δ 2.05 were absent. Since the ^{13}C NMR spectra of bryostatins 12 and 1 both showed five signals in the region δ 165–174 corresponding to ester carbonyls, the C-7 carbon appeared part of a new ester. The C-7 carbon resonance at δ 72.47 and the H-7 proton at δ 5.15 supported this conclusion.

Application of solution phase secondary ion mass spectrometry (SP-SIMS)[22] to the bryostatin 12 structure problem proved decisive. High-resolution mass measurements were obtained by using potassium iodide in 2-hydroxyethyl disulfide and the K_6I_5 ion proved convenient for peak matching procedures. Bryostatin 12 exhibited a molecular ion complex $[M + K]^+$ at m/z 971 (for $C_{49}H_{72}O_{17}$) and a prominent fragment ion at m/z 897 ($[M + K - 74]^+$), indicating loss of a butyryl group along with three hydrogen atoms. Comparison with the closely related bryostatin 2 (**1b**, $C_{45}H_{66}O_{16}$)[17] suggested that they differed only by presence of a butyrate ester. Assignment of the remaining ^{1}H (Table I) and ^{13}C NMR (4) signals showed the ester to be derived from *n*-butyric acid. To provide chemical evidence for this hypothesis, bryostatin 2 (**1b**) was treated with excess *n*-butyric anhydride in pyridine, and the resulting dibutyrate ester (**1c**) was subjected to mild acid hydrolysis with 6 N hydrochloric acid in ethanol at room temperature. One of the monobutyrate ester products (20–30% yields) was identical with bryostatin 12 (**3**).

The synthetic conversion of bryostatin 2 to 12 (**1b** → **3**) was employed to obtain sufficient material for detailed 400-MHz NMR experiments necessary to finally locating (C-7 vs. C-26 or C-3) the butyrate ester. A ^{1}H–[^{1}H] NOE

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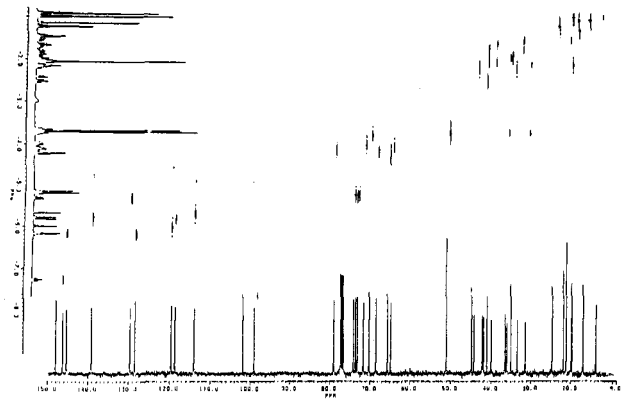


Figure 3. The ^{1}H–^{13}C shift correlation spectrum of bryostatin 1 (**1a**) at 400.14/100.62 MHz and a concentration of 110.6 mmol/L at 303 K.

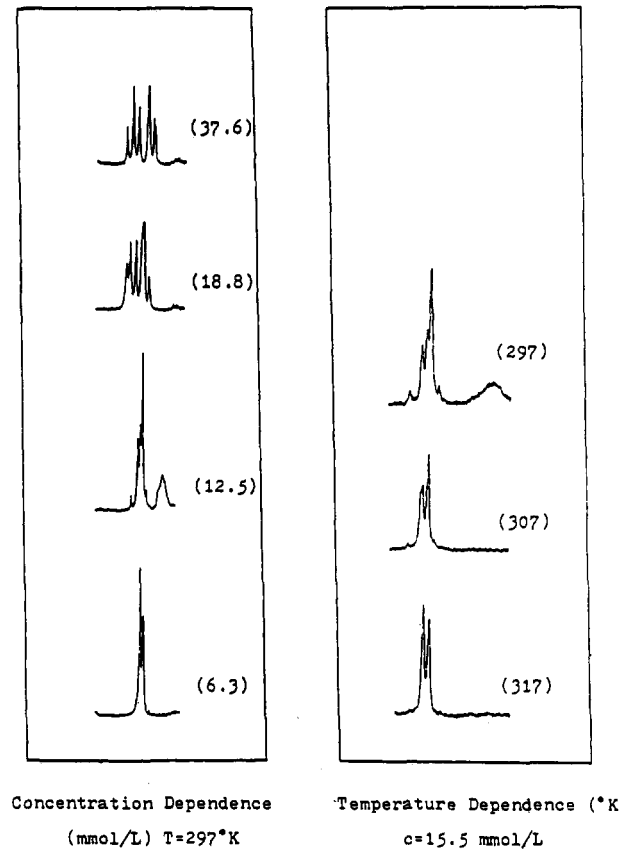
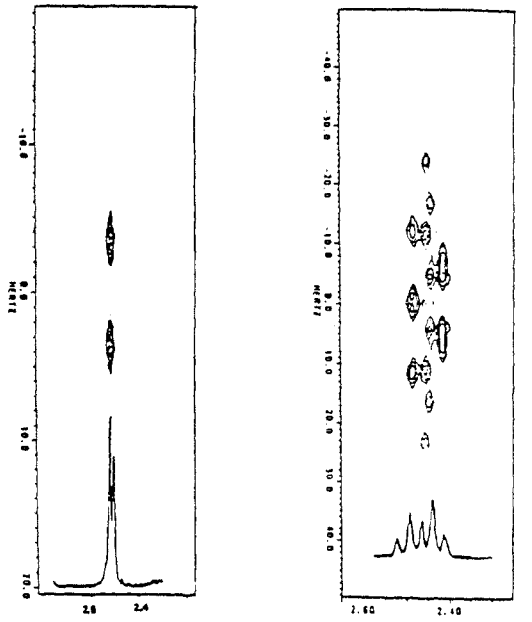


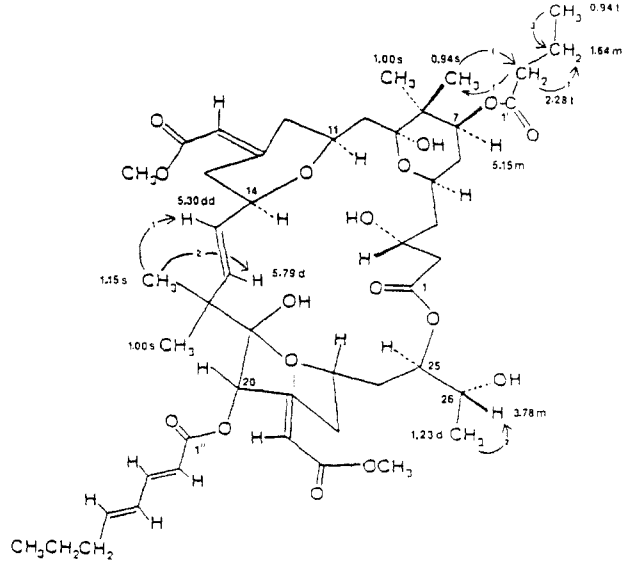
Figure 4. Concentration and temperature effects on the H-2 multiplet (δ 2.46) of bryostatin 1 (**1a**).



4.4 mmol/L 17.7 mmol/L

Figure 5. Concentration dependent 2D-*J*-resolved spectrum of the H-2 multiplet (δ 2.46) of bryostatin 1 (**1a**).

difference[23] study clearly allowed placement of the butyrate ester at C-7 (**3**). Irradiation of the methyl singlet at



3

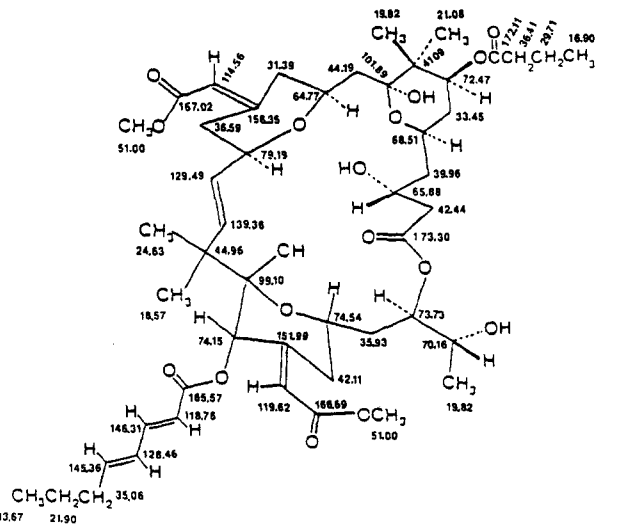
Bryostatin 12

(with partial NOEDS)

δ 0.94 (H-28) resulted in enhancement of the butyrate methylene triplet [δ 2.28 (H-2′)]. When the methyl doublet at δ 1.23 (H-27) was irradiated, it affected the adjacent H-26 signal at δ 3.75 and nicely allowed rejection of ester substitution at C-26. The methyl singlet at δ 1.15 upon irradiation enhanced the olefinic proton signals at δ 5.30 and 5.78 (H-16 and H-17, respectively), indicating placement of this methyl group at C-32, rather than at C-28 as tentatively assigned earlier.[17] In addition to the NOEDS analysis, COSY and 2D-*J*-resolved experiments allowed complete chemical shift assignments for bryostatin 12.

The high-resolution SP-SIMS spectrum of bryostatin 13 (**5**) obtained in the presence of potassium iodide dis-

(23) Hall, L. D.; Sanders, J. K. M. *J. Am. Chem. Soc.* **1980**, *102*, 5703.

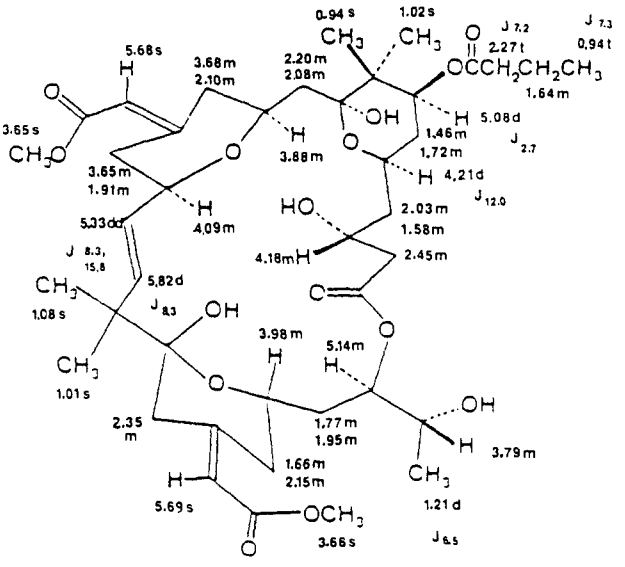


4

Bryostatin 12

(with ^{13}C-NMR)

played *m/z* 833 ($[M+K]^+$, corresponding to molecular formula $C_{41}H_{62}O_{15}$. The 400-MHz ^{1}H NMR spectrum revealed a series of signals characteristic of the bryostatins. A key difference was absence of the octadienoate ester at C-20 and the downfield signal at δ 5.18–5.19, corresponding to H-20 in bryostatins 1 and 12. A new two-proton signal



5

Bryostatin 13

(with ^{1}H-NMR)

at δ 2.35 suggested that C-20 was a methylene carbon, reminiscent of the 20-deoxybryostatins 10 and 11 isolated from the Gulfs of California and Mexico specimens of *Bugula neritina*.[5] Interestingly, when the 20-deoxybryostatins 10, 11, and 13 were chromatographed on silica gel thin-layer plates and developed with an anisaldehyde–acetic acid–sulfuric acid spray reagent, they displayed a bright pink coloration, whereas the other bryostatins with oxygen at C-20 gave the usual darker purple-red color. Presence of a butyrate ester substituent was supported by a downfield two-proton methylene triplet at δ 2.27 (H-2′), a multiplet at δ 1.64 (H-3′), and a methyl triplet at δ 0.94 (H-4′). Confirmation was obtained from the low-resolution SP-SIMS spectrum. When measured

by employing 2-hydroxyethyl disulfide, bryostatin 13 exhibited m/z 817 ($[M + Na]^+$ and 745 ($[M + Na - 72]^+$) for loss of a butyl group plus hydrogen and m/z 727 ($[M + Na - 90]^+$) for loss of a butyrate ester and three hydrogen atoms. Location of the butyl ester was settled by ^1H NMR. With elimination of the C-3 position due to presence of the usual C-3 proton signal at δ 4.18, attention was directed to C-26 and C-7. When the multiplet at δ 3.79 (C-26H) was irradiated in a simple proton-decoupling experiment, the adjacent methyl doublet (C-27) collapsed to a singlet, thereby eliminating esterification at C-26. The more downfield proton signal at δ 5.08, due to the adjacent butyl ester, was assigned to the C-7 hydrogen. Since detailed analysis of the two-dimensional NMR spectra was not feasible due to the 0.7 mg (7×10^{-8}% yield) originally isolated, all other ^1H NMR assignments for bryostatin 13 (**5**) were deduced from the definitive studies of bryostatins 1 and 12.

Bugula neritina in the Gulfs of California, Mexico, and Sagami contain the biosynthetic capability and/or the necessary exogenous microorganism(s) to produce or concentrate bryostatins with simple aliphatic ester substituents, particularly butyl, at the C-7 and C-20 positions. In contrast, results of the present investigation confirm that Eastern Pacific Ocean *Bugula neritina* is almost devoid of that capability and instead is best at producing or concentrating the C-20-octadienoate ester-type bryostatins. Discovery of the 20-deoxybryostatin 13 (**5**) in 7×10^{-8}% yield (even less than the 7 to 2×10^{-7}% recovery of the corresponding bryostatins[5] 10 and 11 from the various Gulf collections of *Bugula neritina*) suggests that the 20-deoxybryostatins will generally be quite rare in this bryozoan. The unequivocal structures assigned bryostatins 1–13 combined with their remarkable biological properties now provides the necessary foundation for molecular modeling and extended structure–activity investigations.

Experimental Section

General Procedures. Solvents employed for chromatography were redistilled. Sephadex LH-20 (25–100 μm) used for gel permeation and partition chromatography was obtained from Pharmacia Fine Chemicals, AB, Uppsala, Sweden. Other ambient-pressure column chromatographic separations were performed with silica gel 60 (70–230 μm) or Lobar size B Silica 60 (40–63 μm) columns supplied by E. Merck (Darmstadt). Gilson UV monitor Model HM and Gilson microfractionators were used for collecting fractions. An HPLC column (10 mm i.d. × 500, Supelco, Inc., Bellefonte, PA) packed with Techsil-10M-C-18 reversed-phase adsorbent (Phenomenex) prepared by HPLC Technology Ltd., Cheshire, U.K., was used for final purification procedures. The HPLC column was interfaced with an Axxiom 710 controller and with two Altex Model 110A pumps. Preparative-layer silica gel plates (F254; 0.5- and 0.25-mm layers) and silica gel GF uniplates for analytical thin-layer chromatography (TLC) were manufactured respectively by E. Merck and Analtech, Inc., Newark, DE. The thin-layer chromatograms were viewed by short wavelength UV light and/or developed by anisaldehyde–acetic acid–sulfuric acid (1:97:2) spray reagent followed by heating at approximately 150 °C for 5–10 min.

Ultraviolet spectra were obtained by using a Hewlett-Packard 8450 UV–vis spectrophotometer equipped with a HP-7225A plotter. Optical rotations were measured with a Perkin-Elmer 241 variable wavelength polarimeter. Infrared spectra were recorded by using a Nicolet FTIR Model MX-1 instrument. All SP-SIMS[22] mass spectra were obtained by using a V.G. Analytical MM ZAB-2F mass spectrometer.[1c] For high-resolution mass measurements, the sample was dissolved in 2-hydroxyethyl disulfide containing potassium iodide.

The NMR spectra were measured on a Bruker AM-400 narrow bore spectrometer, with an ASPECT 3000 computer and pulse programmer, operating at 400.13 and 100.62 MHz for ^1H and ^{13}C NMR respectively. Spectra were obtained in deuteriochloroform solution by using a Bruker 5-mm ^1H–^{13}C dual switchable probehead (quadrature detection and 8-transient image-suppression phase cycling). The 2D spectra were acquired with quadrature detection in the f_2 dimension, sine-bell multiplication in f_1 and f_2, and zero-filling in f_1. The 90° pulse lengths for ^1H and ^{13}C were 10 and 7.5 μs. Specific experiments were as follows: ^1H–[^1H] NOE, size 16K, sweep width 4700 Hz, 3712 transients each on-resonance and off-resonance, recovery delay 3.5 s, decoupling time 0.5 s, decoupler offset cycled;[23] **^1H–^1H COSY** sequence, Δ –90°–t_1 –90°–t_2, with phase cycling,[24] Δ 0.4 s, acquisition time (t_2) 360.45 ms, spectral width in f_1 and f_2 2840.9 Hz, size 2K, 8 transients and 512 increments in f; **^1H–^1H 2D-*J*-resolved** sequence, Δ –90°–t, –180°–t,–t_2, Δ 0.5 s, sweep width in f_1 and f_2 3067.5 and 48 Hz, respectively, size 4K, acquisition time (t_2) 667.65 ms, 160 transients and 128 increments; **^1H–^{13}C heteronuclear shift correlated spectrum** sequence,[20b] Δ –90° (^1H)–$t_1/2$–180° (^{13}C)–$t_1/2$–Δ_1–90° (^1H, ^{13}C)–Δ_2–t_2, Δ 0.5 s, Δ_1 3.3 ms, Δ_2 17 ms, size 4K, spectral width in f_1 1285.7 Hz, width in f_2 3599.7 Hz, 256 transients, 256 increments.

***Bugula neritina* (Linnaeus).** In 1980 approximately 500 kg damp weight of the marine sea mat *Bugula neritina* (Bryozoan phylum) was collected near Monterey, CA, and preserved in 2-propanol. In 1981 4000 L corresponding to ~1000 kg damp weight of *Bugula neritina* was recollected from the same California site and stored in 2-propanol for 30 months.

Animal Extraction: Procedure A. From the 1980 collection, the 2-propanol solution (577 L) was removed and concentrated to 8 L. The concentrate was partitioned (5×) between methylene chloride and water, in portions. The marine animal was further extracted with methylene chloride–methanol (1:1) as described before.[9] The combined methylene chloride extracts, 877 g, were partitioned between hexane (4×) and methanol–water (9:1) in portions giving 430 g of hexane extract. Water was added to the aqueous methanol to give 4:1 methanol–water, and this solution was finally partitioned with carbon tetrachloride (9×), giving 214 g of concentrate. The 214 g of extract was divided among five glass columns (Glenco, 10 × 112 cm) prepared with Sephadex LH-20 in methylene chloride–methanol (1:1). The bioassay guided chromatographic separation yielded 122.8 g of PS in vivo active material. This fraction was again divided among five columns for chromatography on LH-20 equilibrated with hexane–toluene–methanol (3:1:1).[25] Test tube fractions, 19 mL ea, were combined according to simularities based on TLC (5% methanol in methylene chloride). Bryostatin 1 (**1a**) was found in test tubes 353–375 out of 780 and crystallized from solution upon evaporation at ambient temperature. Crystalline bryostatin 1 (120 mg) was recrystallized from methylene chloride–methanol as described before.[6] Bryostatin 2 (**1b**) was also isolated.[17]

Procedure B. For the 1981 recollection the 2-propanol was removed from the 4000 L of *Bugula neritina* and the semidry animal (~1000 kg) was reextracted with 2-propanol (91%) for 2 weeks. The combined 2-propanol extracts were concentrated to 190 L, placed in a 1000-L stainless steel vessel, and partitioned (5×) between 180-L volumes each of distilled water and methylene chloride. After concentration the methylene chloride extract (16.4 kg) was partitioned between hexane (4 × 100 L) and methanol–water (9:1). The hexane phase was separated, and the methanol–water portion was evaporated to dryness to afford 1.8 kg of solid. The 1.8 kg residue was chromatographed on a series of 15 × 305 cm stainless steel HPLC columns prepared from 61 kg of Davisil 633 grade silica gel (200–400 mesh) in methylene chloride. Elution was begun with methylene chloride followed by an increasing methanol gradient. The flow rate was approximately 68 L/h. Fractions measuring 20 L were collected and combined on the basis of TLC (4–7% methanol in methylene chloride), giving 26 major fractions. Bryostatin 1 (**1a**) was detected by TLC in fractions 8–12 and bryostatin 2 (**1b**) in fractions 13–15.

Isolation of Bryostatins 1 (1a) and 2 (1b). From procedure B bryostatin 1 (**1a**) fractions (8–12, 167 g total) were combined

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and chromatographed in methanol on Sephadex LH-20. Typically, a 20–30-g portion in methanol was subjected to steric exclusion chromatography on a 7.5 × 120 cm column prepared from Sephadex LH-20 (1 kg) and methanol. Progress of the chromatogram was followed by TLC using methylene chloride–methanol (95:5) as the mobile phase. The fraction (26.4 g) containing bryostatin 1 was chromatographed in 2–10-g amounts on columns of silica gel 60 in methylene chloride. Elution was conducted with methylene chloride followed by an increasing gradient of methanol. The bryostatin-containing fraction (2.2 g total) was rechromatographed at 5 psi by using a Lobar B silica gel 60 column with a hexane–acetone gradient to afford 630 mg of bryostatin 1 (**1a**). When bryostatin 2 containing fractions 13–15 (38 g) were subjected to the same chromatographic procedures used to isolated bryostatin 1 a total of 588 mg of pure bryostatin 2 (**1b**) was obtained.

Isolation of Bryostatins 12 and 13. Analytical TLC of adjacent fractions from the preceding purification of bryostatins 1 and 2, using 7:3 hexane–acetone and 3:2 ethyl acetate–hexane, revealed the possible presence of other bryostatin-type constituents in relatively minor amounts. Such fractions were individually chromatographed by employing RP-18 reversed-phase HPLC. In each case, elution was begun with methanol–water (1:1) at a flow rate of 2.0 mL/min with a gradient to methanol. By this means pure specimens of amorphous bryostatin 3 (1.6 mg), bryostatin 8 (13.2 mg),[2] bryostatin 9 (16.4 mg),[18] bryostatin 12 (3.7 mg), and bryostatin 13 (0.7 mg) were isolated. Known bryostatins 1, 2, 3, 8, and 9 were identified by direct comparison (principally by 400-MHz NMR and SP-SIMS molecular weight determinations) with authentic samples and by co-TLC in several solvent systems.

Bryostatin 12 (4): $C_{49}H_{72}O_{17}$; TLC R_f 0.56 (CH_2Cl_2–CH_3OH, 95:5); MS (SP-SIMS), m/z 971 ($[M + K]^+$), 957 ($[M + K - CH_3 + H]^+$), 897 ($[M + K - COCH_2CH_2CH_3 - 3H]^+$), and 883 ($[M + K - OCOCH_2CH_2CH_3 - H]^+$); $[\alpha]^{27}_D$ +39° (c 0.108, CH_3OH); UV (CH_3OH) λ_{max} 231 and 263 nm (log ϵ 4.46, 4.47); IR (thin film on NaCl) ν_{max} 3470, 3346, 2964–2949, 1734, 1717, 1660–1640, 1440, 1380, 1365, 1270, 1250, 1220, 1164, 1100, 1070, 1055, 1000, and 860 cm^{-1}. The ^1H NMR and ^{13}C NMR data have been entered in Table I and structure **4**, respectively.

Bryostatin 13 (5): $C_{41}H_{62}O_{15}$; TLC R_f 0.51 (CH_2Cl_2–CH_3OH, 95.5); MS (SP-SIMS), m/z 833 ($[M + K]^+$); low-resolution MS, m/z 817 ($[M + Na]^+$), 745 ($[M + Na - COCH_2CH_2CH_3 - H]^+$), and 727 ($[M + Na - OCOCH_2CH_2CH_3 - 3H]^+$); UV ($CH_3OH$) λ_{max} 228 nm (log ϵ 3.96); IR (thin film) ν_{max} 3475, 3359, 2926, 1734, 1717, 1685, 1653, 1606, 1436, 1380, 1153, 1096, and 1077 cm^{-1}. The ^1H NMR data has been recorded on structure **5**.

Conversion of Bryostatin 2 (1a) to Bryostatin 12 (4). A 20-mg sample of bryostatin 2 (**1a**) was treated with pyridine (0.25 mL and butyric acid anhydride (0.5 mL). The solution was allowed to stand for 44 h at room temperature (under nitrogen) and concentrated to dryness. Preparative TLC using 7:3 hexane–acetone led to 12 mg of bryostatin 2 dibutyrate (**1c**, $C_{53}H_{78}O_{18}$); TLC R_f 0.80 (CH_2Cl_2–MeOH, 95:5); MS (SP-SIMS), m/z 1041 ($[M + K]^+$).

To a solution of bryostatin 2 dibutyrate (**1c**, 12 mg) in ethanol (1 mL) was added 6.0 N hydrochloric acid (0.25 mL). After removal of the ethanol the residue was partitioned between methylene chloride and water. The chlorocarbon phase was dried over anhydrous sodium sulfate, the solvent evaporated and the residue purified by preparative TLC using 95:5 methylene chloride–methanol as the mobile phase to afford 2.5 mg of bryostatin 12 (**4**) identical (by comparison TLC and ^1H NMR, ^{13}C NMR, IR, UV, $[\alpha]_D$, and SP-SIMS spectral properties) and the natural product (**4**). Recovered starting material **1c** was resubmitted to the same hydrolysis procedure to improve the yield of bryostatin 12.

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Registry No. **1a**, 83314-01-6; **1b**, 87745-28-6; **1c**, 108268-91-3; **4**, 107021-10-3; **5**, 107021-11-4; bryostatin 3, 87370-86-3; bryostatin 8, 102580-64-3; bryostatin 9, 102604-78-4; butyric acid, 107-92-6.

Potent Prostacyclin Analogues Based on the Bicyclo[4.2.0]octane Ring System[1]

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The novel and biologically active prostacyclin mimetics **2** and **29** were prepared in a sequence based on the regioselective opening of epoxide **7** with a lithium acetylide in the presence of boron trifluoride etherate. The regioselectivity of epoxide opening was consistent with a mechanism involving coordination in the transition state of the epoxide and endo acetal oxygens with the Lewis acid boron. Diastereomers **10** and **11** were separated through formation of their dicobalt hexacarbonyl complexes **12** and **13**, followed by chromatography and oxidative cleavage. The individual racemic diastereomers **10** and **11** were then resolved into the four enantiomeric keto diols **23–26** through a four-step sequence. The side-chain alcohol (*S*)-**9a** was obtained through reduction of acetylenic ketone **27** with (*S*)-*B*-isopinocampheyl-9-borabicyclo[3.3.1]nonane, and its absolute stereochemistry was determined by its transformation to (*S*)-hexahydromandelic acid. The absolute stereochemical assignments for keto diols **23–26** were made on the basis of CD spectroscopy, single-crystal X-ray structural determination of **23**, and the transformation of (*S*)-**9a** into **23** and **26**. Prostacyclin mimetics **2** and **29**, which were obtained through Wittig olefination of **23** and **26**, had ca. 10 times the potency of PGE_1 in inhibiting the ADP-induced aggregation of human platelets.

Our previous research on prostacyclin mimetics resulted in the preparation of the bicyclo[3.2.0]heptane **1**, which was a potent inhibitor of ADP-induced aggregation of human platelets.[2] In seeking to extend this finding to the

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