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AN IMPROVED SOURCE OF BRYOSTATIN 10, *BUGULA NERITINA* FROM THE GULF OF AOMORI, JAPAN[1]

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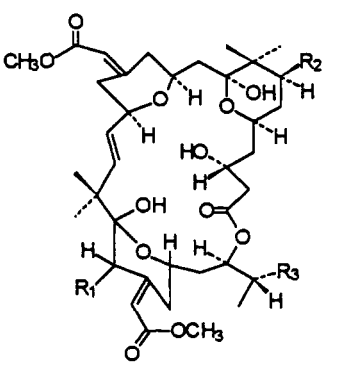
ABSTRACT.—Bryostatins have been isolated from diverse Japanese coastal specimens of *Bugula neritina* guided by inhibitory activity against fertilized sea urchin egg cell division. *B. neritina* from the Gulf of Aomori, Japan, has been found to contain bryostatin 10 [**1b**] in high yield (10^{-3}%) for this class of compounds. The ^{1}H- and ^{13}C-nmr signals of bryostatin 10 [**1b**] were reassigned by 2D nmr techniques. The conformation of bryostatin 10 [**1b**] in solution was revealed by nmr studies. This compound also exhibited activity in a steroidogenesis assay by increasing the production of adrenocortical hormones nearly twofold.

The marine Bryozoan *Bugula neritina* (L.) (Bugulidae) has maintained its position as a unique source of the important bryostatin series of macrocyclic lactones (2). In 1968 we began (3) a long-term study of *B. neritina* antineoplastic constituents that led to the discovery of bryostatin 1 [**1a**] in 1981 (4). During that 13-year period, we explored the bryostatin constituents of *B. neritina* collected in the North American Gulfs of California and Mexico and in the Eastern Pacific Ocean (California). The latter area proved to be a good source of bryostatins



1a R_1=  , R_2=$OCOCH_3$, R_3=OH, Bryostatin 1
1b R_1=H, R_2=$OCOC(CH_3)_3$, R_3=OH, Bryostatin 10
1c R_1=H, R_2=$OCOC(CH_3)_3$, R_3=$OCOCH_3$, Bryostatin 10 26-acetate
1d R_1=H, R_2=$OCOC(CH_3)_3$, R_3=$OCOCH_2CH_3$, Bryostatin 10 26-propionate
1e R_1=H, R_2=$OCOC(CH_3)_3$, R_3=$OCO(CH_2)_2CH_3$, Bryostatin 10 26-*n*-butyrate

[1]Series No. 342 (Arizona State University, Cancer Research Institute) on antineoplastic agents. Refer to Bai *et al.* (1) for the preceding contribution.

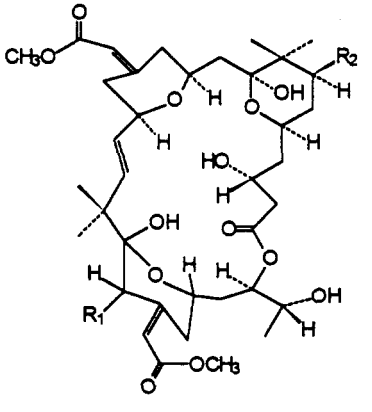
1–3 (2) and the Gulf specimens yielded bryostatins 4–15 (5–7) plus bryostatins A and B (8). When the geographical diversity of the *B. neritina* collections was

extended to the Gulf of Sagami, Japan, in 1982, we again isolated bryostatins 4–6, 8 and 9 (2, 9–11). Given the close bryostatin content relationship of the North American and Asian specimens, we predicted (6) that bryostatin 10 [**1b**] would eventually be found in Gulf of Sagami samples of *B. neritina*. Employing an April 1990 recollection from that general location, we have now isolated bryostatin 10 [**1b**]. Furthermore, 1988 collections of this moss animal in the Japanese Gulfs of Ohzuchi and Aomori led to detection of bryostatin 10 in the former and the latter proved to be a considerably improved [10^{-3} vs. 10^{-7}% yield, (6)] source of this potentially important bryostatin [**1b**].

Each Gulf (Japan) collection of *B. neritina* was extracted with CH_2Cl_2 and the extract was subjected to Sephadex LH-20 cc followed by reversed-phase flash cc. The isolation procedure was guided by the fertilized sea urchin egg cell division assay (12,13) instead of our usual P-388 lymphocytic leukemia cell line procedure (14). Final purification of the Gulf of Aomori, Asamushi, sample by reversed-phase hplc afforded bryostatins 4 [**2a**], 5 [**2b**], 6 [**2c**], 9 [**2d**], and 10 [**1b**]. An analogous isolation procedure was employed with *B. neritina* specimens collected in the Gulfs of Ohzuchi and Sagami.

The bryostatins obtained from these three geographically different collections of *B. neritina* and their yields are summarized in Table 1, along with the results of our 1982 collection in the Gulf of Sagami, Misaki. The most prominent feature observed was a considerably improved yield (10^{-3}%) of bryostatin 10 from *B. neritina* collected in the Gulf of Aomori, northern Japan. The earlier (6) yield of bryostatin 10 was 7×10^{-7}% from *B. neritina* collected in the Gulf of Mexico, Florida. The yields of bryostatins from the 1982 (Misaki) and 1990 (Ninomiya) collections from the Gulf of Sagami compared well and the major component was again bryostatin 4 [**2a**].

Bryostatin 10 [**1b**] was identical with an authentic sample (6). The increased supply combined with recent advances in 2D nmr techniques allowed us to make some revisions of prior nmr assignments. Detailed analysis of the 2D nmr spectral data for bryostatin 10 [**1b**] led to precise assignments for sets of closely spaced ^{13}C-nmr signals (C-2 and C-10, C-4 and C-20, C-22 and C-24, C-31 and C-35, and C-36 and C-37). The original ^{13}C-nmr assignments of carbons C-2/C-10 and C-22/C-24 were reversed (7). The revised chemical shifts are presented in italics in



2a R_1=$OCO(CH_2)_2CH_3$, R_2=$OCOC(CH_3)_3$, Bryostatin 4
2b R_1=$OCOCH_3$, R_2=$OCOC(CH_3)_3$, Bryostatin 5
2c R_1=$OCOCH_3$, R_2=$OCO(CH_2)_2CH_3$, Bryostatin 6
2d R_1=$OCO(CH_2)_2CH_3$, R_2=$OCOCH_3$, Bryostatin 9

TABLE 1. Bryostatins from Japanese Specimens of *Bugula neritina*.

Collection	Wet wt (kg)	Bryostatin (mg)					
		4	5	6	8	9	10
Gulf of Aomori ..... Asamushi 1988 (April)	1.5	0.6	1.5	trace		trace	15
Gulf of Ohzuchi ..... Ohzuchi 1988 (April)	ca. 0.2	[a]	[a]	[a]	[a]	[a]	trace
Gulf of Sagami ..... Ninomiya 1990 (April)	1.6	0.6	0.2	trace	0.2	trace	0.1
Gulf of Sagami ..... Misaki 1982 (May, July)	5	2.8	0.8	1.0	0.1	0.2	

[a]Detected by analytical hplc.

Table 2. Reassignments of the resonances for C-22 and C-24 were performed as follows. First, two pairs of geminal protons (δ 1.76/3.86 and δ 1.78/1.90) at C-22 and C-24 were assigned from the ^{1}H-^{1}H COSY correlations with 22a/22b and 24a/24b. Second, the ^{13}C-^{1}H couplings due to C-22 and C-24 were observed using the cross-peaks at δ_H 1.76/δ_C 35.72, δ_H 3.86/δ_C 35.72, δ_H 1.78/δ_C 36.12, and δ_H 1.90/δ_C 36.12 in the HMQC and ^{1}H-^{13}C COSY spectra. Finally, long-range couplings were observed due to H-22/C-21 in the HMBC spectrum and the ^{1}H-^{1}H coupling due to carbons C-24/C-25 in the ^{1}H-^{1}H COSY spectrum. In a similar manner, the C-2 and C-10 signals were reassigned as shown in Table 2. The 2D nmr experiments also allowed unambiguous assignments of ^{1}H-nmr signals. Because the ^{13}C-nmr signals due to C-4 and C-20 were overlapping, two pairs of geminal protons (δ 1.54/1.97 and δ 2.14/2.35) at C-4 and C-20 were difficult to assign. The HMBC spectrum showed a cross-peak from H-20 (δ 1.14)/C-19 and revealed correlations with H-20 (δ 2.35)/C-19 and H-20 (δ 2.35)/C-21. Thus, the two pairs of methylene protons were reaassigned. More importantly, an intense proton signal (9H, 3×CH_3) correlated with C-1′ (δ 178.13, s) and C-2′ (δ 39.05, s) in the HMBC spectrum, and this provided further support for the pivalate group at C-7 (7).

In our earlier (8) nmr study of bryostatin 1 [**1a**], temperature-dependent coupling patterns were observed in the ^{1}H-nmr spectrum. Bryostatin 10 [**1b**] also displayed such temperature dependence and chemical shifts in the ^{1}H-nmr spectrum (Figure 1). The most obvious feature was the upfield shift of the C-3 hydroxyl proton at higher temperature presumably due to intramolecular hydrogen bonding of the C-3 hydroxyl group. The X-ray crystal structure of bryostatin 1 [**1a**] indicated that the C-3 hydroxyl group extended into the macrocyclic lactone cavity and was hydrogen bonded (4). The hydrogen bonds, and in turn the conformation, would be affected by temperature changes. The temperature-dependent chemical shifts or coupling patterns were observed for H-2, H-5, H-7, H-30, and H-34. The spatial orientation of the two *gem*-dimethyl groups attached to C-8 and C-18 was determined from the NOESY experiment. The H-28 α-methyl protons (δ 0.94, s) showed strong nOe associations with both H-6b(α) and H-7α. A nOe was also observed between the H-29 β-methyl protons (δ 1.03 s) and H-6a (β). The *gem*-dimethyl group

TABLE 2. ^{1}H- and ^{13}C-Nmr (400 MHz) Chemical Shifts (δ ppm) for Bryostatin 10 [**1b**] in $CDCl_3$.

Position	^{13}C	^{1}H	*J* (Hz)	HMBC (^{1}H)
1	172.64 s	—		H_2-2, H-25
2a	42.05 t	2.44 d	(11.7)	
2b		2.54 dd	(12.2, 11.7)	
3	68.14 d	4.12 m		H_2-2, H-5
OH-3		4.03 s[a]		
4	39.78 t	1.54 d	(14.7)	H-7
		1.97 m		
5	65.64 d	4.23 d	(12.2)	H-6a
6	33.19 t	1.43 d		
		1.71 br s		
7	72.59 d	5.10 ddd	(11.9, 7.3, 4.4)	H-6a
8	41.26 s	—		H-7, H-10a, H_3-28, H_3-29
9	101.79 s	—		H-7, H_2-10, H-12a, H_3-28, H_3-29
OH-9		4.81 s[a]		
10	42.16 t	1.62 br s		
		2.08 m		
11	71.34 t	3.89 m		H-10b, H-12a
12	44.19 t	2.06 m		H-10a, H-14a, H-30
		2.18 d	(13.2)	
13	157.04 s	—		H-10b, H-14a
14	36.56 t	1.88 m		H-12b, H-30
		3.66 m		
15	78.95 d	4.11 m		H-17, H-30
16	130.43 d	5.36 dd	(8.3, 15.6)	
17	137.91 d	5.81 d	(15.6)	H-32, H-33
18	44.75 s	—		H-17, H-20a, H-32, H-33
19	100.93 s	—		H-20a, H-32, H-33
OH-19		5.91 s[a]		
20a	39.78 t	2.14 m		
20b		2.35 d	(14.2)	
21	157.04 s	—		H-22b
22a	35.72 t	1.76 d	(11.7)	H-20a, H-34
22b		3.86 m		
23	64.61 d	3.98 m		H-22a, H-24a, H-25, H-34
24a	36.12 t	1.70 m		
24b		1.95 m		
25	73.83 d	5.08 dd	(14.2, 3.9)	H_3-27, H_3-28, H_3-29
26	70.23 d	3.75 m		H_3-27
27	19.68 q	1.21 d	(6.8)	
OH-27		4.79 d[a]	(5.7)	
28	21.07 q	0.94 s		H-7, H_3-29
29	17.06 q	1.03 s		H-7, H_3-28
30	114.19 d	5.68 br s		H-10b, H-12a
31	167.01 s	—		H-36
32	20.39 q	1.03 s		H-17
33	24.45 q	1.08 s		H-17
34	115.73 d	5.69 br s		H-20a
35	166.81 s	—		H-34, H-37
36	51.08 q	3.71 s		
37	50.84 q	3.67 s		
1′	178.13 s	—		H-7
2′	39.05 s	—		H-3′, H-4′, H-5′
3′	27.16 q	1.19 s		
4′	27.16 q	1.19 s		
5′	27.16 q	1.19 s		

[a]The signals were recorded in DMSO-d_6 and disappeared after D_2O exchange.

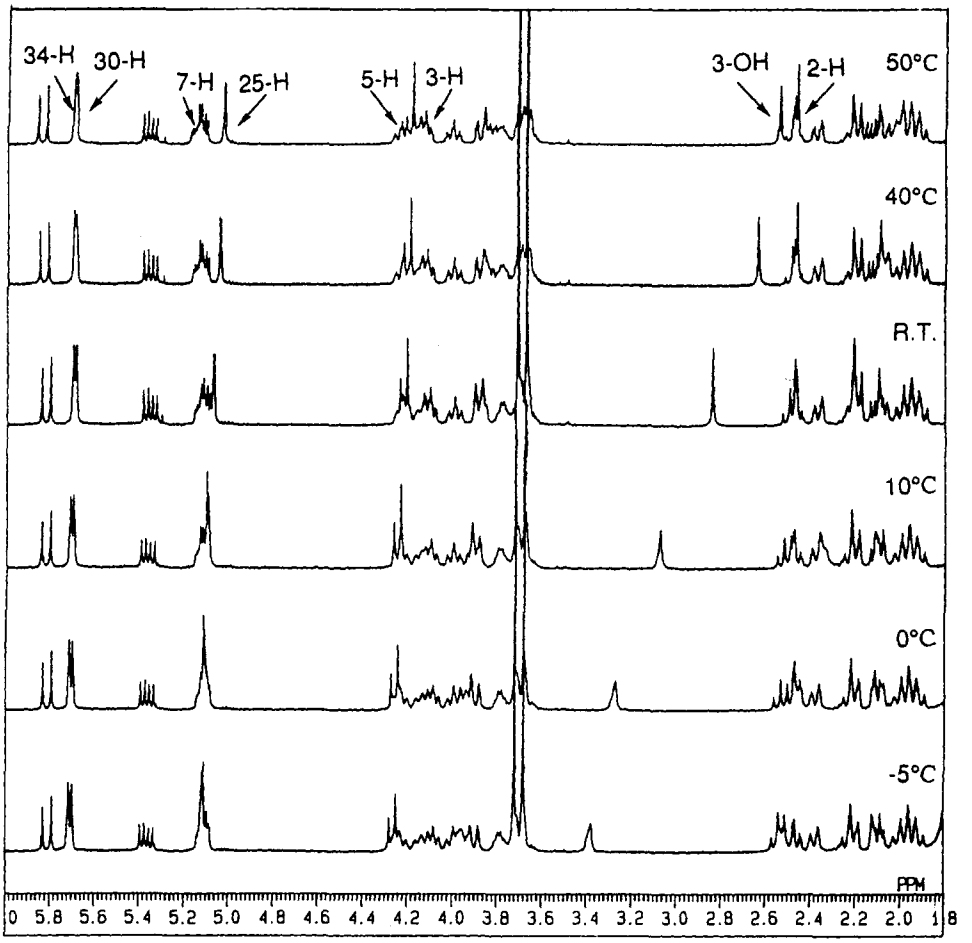


FIGURE 1. ^{1}H-Nmr spectra of bryostatin 10 [**1b**] at different temperatures.

Me-32/Me-33 on C-18 was identified by the NOESY cross-peak involving Me-33/H-17, Me-32/H-16, Me-32/19-OH (β), and Me-32/20b (β). These results again arose from the spin-lattice relaxation time (T_1) values (15), which are closely related with molecular mobility, for bryostatin 10 [**1b**] carbon atoms (Figure 2). For example, the T_1 values for the β-methyl groups (C-29, 466 msec; C-32, 259 msec) were smaller than those of the α-methyl groups (C-28, 648 msec; C-33, 452 msec). But the steric hindrance (molecular model) of the β-methyl groups was observed to be larger than that of the α-methyl groups.

The bryostatins are excellent examples of the biomedical potential of marine organism constituents and some pertinent aspects have been reviewed to 1991 (2). Subsequently, further evidence for the potential clinical use of bryostatin 1 against human leukemias (16–19) and lymphomas (20) as well as the very beneficial radioprotective (21–23) and hematopoietic growth factor effects of this protein kinase C modulator (21) have been described. Importantly, the first human phase I clinical trials results have been summarized (24,25) and phase II clinical trials are already in progress. With the replenished supply of bryostatin 10 [**1b**], some new biological facets of the bryostatins were explored.

Bryostatin 10 [**1b**] showed an ED_{50} value of 0.16 μg/ml in the fertilized sea urchin egg assay (12,13) and an ED_{50} value of 0.0018 μg/ml against the P-388

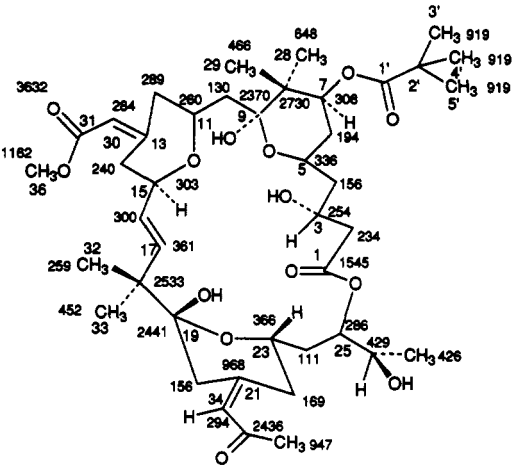
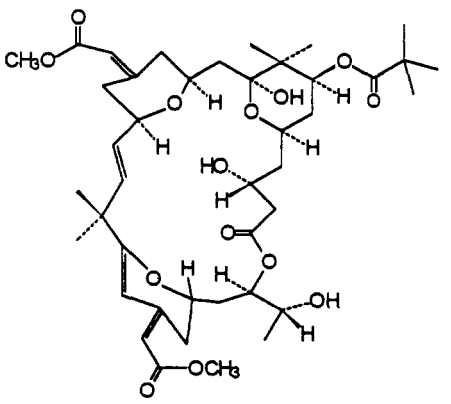


FIGURE 2. T_1-values (msec) for bryostatin 10 [**1b**].

cell line consistent with our previous result (6). Bryostatin 10 [**1b**] derivatives 26-acetate [**1c**], 26-propionate [**1d**], and 26-*n*-butyrate [**1e**] were prepared and found to show diminished activity as expected (26) against the P-388 leukemia line with ED_{50} values (μg/ml) of, respectively, **1c** (0.44), **1d** (0.52), and **1e** (0.32). But the $\Delta^{19,20}$-olefin derivative [**3**] we prepared earlier (6) showed an ED_{50} value of 3×10^{-4} μg/ml. Among the derivatives, only the $\Delta^{19,20}$-olefin [**3**] showed good activity (ED_{50} value of 0.3 μg/ml) in the fertilized sea urchin egg assay. Olefin **3** showed 100% inhibition of fertilization of sea urchin eggs at a dose of 1.0 μg/ml, while the ester derivatives exhibited 80% inhibition at this dose level. The 26-propionate [**1d**] also inhibited the movement of sea urchin sperm at a dose of 1.0 μg/ml.



3 $\Delta^{19,20}$ Bryostatin 10

More significantly, when employing bryostatin 10 [**1b**] with bovine adrenocortical cells (using an incubation period of 1 h at 37° and then measuring the steroidogenic response in the presence of ACTH) macrocyclic lactone **1b** increased by 1.8 times the ACTH-induced steroidogenesis. In the absence of ACTH, bryostatin 10 [**1b**] again stimulated steroidogenesis, but this effect was not observed with ester derivatives **1c–e** (27,28) in keeping with our pharmacophore proposal (26).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The ^{1}H- and ^{13}C-nmr spectra were recorded using $CDCl_3$ solutions with a JEOL JNM-EX400 spectrometer. The residual $CHCl_3$ resonances at δ_H 7.26 and δ_C 77.0 were used as internal references for ^{1}H- and ^{13}C-nmr spectra, respectively. The one-bond ^{13}C-^{1}H shift correlation experiment was carried out with the standard JEOL HMQC and JEOL ^{1}H-^{13}C COSY pulse sequence using delays optimized for J_{CH} of 140 Hz. The two- and three-bond correlation experiment was performed with the standard JEOL HMBC and JEOL COLOC pulse sequences using delays optimized for $^nJ_{CH}$ of 8 Hz. Homonuclear ^{1}H nOes were obtained by NOESY experiments using 500 msec mixing periods. Eims spectra were obtained using a JEOL JMS-AX505H spectrometer operating at 70 eV. A Sephadex LH-20 column (Pharmacia, 30 mm

i.d.×350 mm) and three solvent systems [A, CH_2Cl_2-MeOH (1:1); B, hexane-CH_2Cl_2-MeOH (4:5:1); and C, hexane-EtOAc-MeOH (4:5:1)] were employed for gel permeation and partition chromatography. Chromatorex ODS (200–300 mesh size; 28 mm i.d.×230 mm) columns were used for reversed-phase flash chromatographic separations. Prep. hplc was accomplished with the Hitachi Packed column Inersil PREP-ODS 5 μm (20 mm i.d.×250 mm), uv detection at 230 nm, and a flow rate of 9.0 ml/min.

ANIMAL MATERIAL.—All *Bugula neritina* specimens were deposited in the Graduate School of Human Informatics, Nagoya University, Furhocho, Chidane-ku, Nagoya, under the following voucher numbers: KM-43 (Ninomiya), KM-61 (Ohzuchi), and KM-62 (Asamushi).

EXTRACTION AND ISOLATION.—*Bugula neritina* collected (April, 1988) in the Gulf of Aomori (1.5 kg wet wt), Asamushi, Aomori-ken, Japan, was extracted with CH_2Cl_2. The CH_2Cl_2 extract (36.5 g) was directly subjected to Sephadex LH-20 cc with solvent system B followed by reversed-phase flash cc using a gradient of 1:1 H_2O to 100% CH_3CN. The active fractions eluted with 85–90% CH_3CN were combined and purified by reversed-phase hplc (eluent 15:85 H_2O-MeOH followed by 1:4 H_2O-CH_3CN) to afford bryostatin 10 (**1b**, 15 mg) and bryostatins 4 (**2a**, 0.6 mg), 5 (**2b**, 1.5 mg), 6 (**2c**, trace), and 9 (**2d**, trace).

In the same manner, the CH_2Cl_2 extract (3.5 g) of the specimens collected (April, 1988) in the Gulf of Ohzuchi (ca. 0.2 kg wet wt), Ohzuchi, Iwate-ken, Japan, was separated with the same isolation procedure described above, and yielded a major active component, which was identified as bryostatin 10 (**1b**, trace).

The MeOH extract of the specimens (collected April, 1990) from the Gulf of Sagami (1.6 kg wet wt), Ninomiya, Kanagawa-ken, Japan, was partitioned between MeOH-H_2O (9:1) and hexane. Next, the aqueous MeOH phase was diluted to MeOH-H_2O (7:3) and extracted with EtOAc. The EtOAc-soluble fraction (8.5 g) was chromatographed on Sephadex LH-20 using solvent systems A, B, and C. The active (sea urchin) fractions (ED_{50} values of 0.2–0.5 μg/ml) were combined and purified by reversed-phase hplc (elution with H_2O-MeOH, 3:2, to 100% MeOH) to afford bryostatins 4 (**2a**, 0.6 mg), 5 (**2b**, 0.2 mg), 6 (**2c**, trace), 8 (**2d**, 0.2 mg), 9 (**2d**, trace), and 10 (**1b**, 0.1 mg).

Bryostatin 10 [**1b**] was found to be identical (tlc, hplc, ^{1}H-nmr data) to an authentic sample (6). The other bryostatins exhibited the same chromatographic behavior (hplc and tlc) and ^{1}H-nmr spectra as authentic samples (5, 9–11).

NMR EXPERIMENTS WITH BRYOSTATIN 10 [**1b**].—The ^{1}H-, ^{13}C-, and HMBC nmr data are summarized in Table 2. Sample concentration was 15 mg of bryostatin 10 in 0.6 ml of $CDCl_3$. The following ^{1}H-^{1}H COSY correlations were found: (H/H) 2/3, 3/4a, 4a/4b (gem), 4a/5 (w), 4b/5, 5/6a, 5/6b, 6a/6b (gem), 6a/7, 6b/7, 10a/10b (gem), 10b/11, 11/12a, 11/12b, 12a/12b (gem), 14a/15, 14b/15 (w), 15/16, 16/17, 22a/22b (gem), 23/24a, 23/24b (w), 25/26, 26/27, 12a/30 (a), 14a/30 (b), 20b/34 (c), 22a/34 (d), 22a/34 (e) (these cross-peaks were considered as *W*-couplings); NOESY (H/H): 2a(α)/3-OH, 2a(α)/24b(α), 3-OH/5(α), 3-OH/6b(α), 4b(α)/6b(α), 4b(α)/7(α), 5(α)/6b(α), 6a(β)/7 (very weak), 6b(α)/7, 6a(β)/29(β), 6b/28(α), 7/28(α), 11(α)/12a(β) (w), 11(α)/12b(α), 14a(α)/15(α) (w), 15/16, 16/32(β), 17/33(α), 19-OH/32(β), 20b(α)/33(α), 22a(α)/24b(α), 22a(α)/34 (very weak), 22b(β)/24a(β), 23(β)/24a(β) (weak), 24a(β)/26(β), 25(α)/27(α).

ESTERIFICATION OF BRYOSTATIN 10 [**1b**].—A mixture of the appropriate acid anhydride (0.05 ml)-pyridine (0.01 ml) at room temperature (4 h) was used for esterifications of bryostatin 10 (**1b**, 0.2 mg). Upon treatment with ice-H_2O, each mixture was extracted with CH_2Cl_2. The CH_2Cl_2 solution was washed with dilute HCl and H_2O, dried, and the solvent evaporated. Each crude product was purified by prep. tlc on a Si gel plate with hexane-Me_2CO (7:3) as eluent. Thus, the 26-acetate [**1c**], 26-propionate [**1d**], and 26-*n*-butyrate [**1e**] were obtained in yields of 0.15, 0.11, and 0.07 mg, respectively. The esters were characterized by the following data:

26-Acetate [**1c**]: ms (sp-sims) using NaI in sulfolane (29,30), mol wt 850 for $C_{44}H_{66}O_{16}$, tlc (Si gel) R_f 0.55 (hexane-Me_2CO, 7:3); 26-propionate [**1d**]: ms (sp-sims), *m/z* 887 $[M+Na]^+$ and 869 $[M+Na-18]^+$, mol wt 864 for $C_{45}H_{68}O_{16}$, tlc (Si gel) R_f 0.58 (hexane-Me_2CO, 7:3); 26-butyrate [**1e**]: ms (sp-sims) *m/z* 901 $[M+Na]^+$ and 883 $[M+Na-18]^+$, mol wt 878 for $C_{46}H_{70}O_{16}$, tlc (Si gel) R_f 0.61 (hexane-Me_2CO, 7:3).

STEROIDOGENESIS ASSAY.—Primary cultured bovine adrenocortical cells were used in this assay (31). The amount of hormone was determined fluorometrically using cortisol as a standard (32). Results were expressed as picomoles of cortisol production per 10^5 cells/h (pmol/10^5 cells/h). The increase in the ACTH-induced steroidogenesis by bryostatin 10 [**1b**] was found to be about 1.8 times (1141 pmol/10^5 cells/h) the amount of steroidogenesis when stimulating only with 10 pM ACTH (635 pmol/10^5 cells/h).

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