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Relationship of *Bugula neritina* (Bryozoa) antineoplastic constituents to the yellow sponge *Lissodendoryx isodictyalis*[a,b]

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Abstract - Lissodendoryx isodictyalis, a yellow marine animal of the Demospongiae class, was found to yield a series of closely related substances very active against the murine P388 lymphocytic leukemia. Separation studies guided by bioassay led to the isolation and characterization of the strongly antineoplastic (P388 leukemia) bryostatins 4-6 (1a-c) and 8 (1d) accompanied by new bryostatins A and B. Yields of the bryostatins from 108 kg of wet Gulf of California L. isodictyalis ranged from 3.2 mg of bryostatin 4 to 900 μg and 700 μg respectively of bryostatins A and B. The actual source of bryostatins 4-6 and 8 appeared to be from intrusion of the Bryozoan Bugula neritina. But bryostatins A (a deoxybryostatin 5) and B (a deoxybryostatin 4) have not hitherto been detected in B. neritina and may represent metabolic transformations performed by the sponge.

Marine Porifera have proved to be an exceptionally productive source of new substances with physiological activity (ref. 3). And starting, nearly 20 years ago, with initiation (ref. 4) of research directed at discovery of potentially useful marine animal antineoplastic constituents, we have been evaluating a broad selection of sponges from far-reaching geographical locations. As part of the program, specimens of the Gulf of California yellow sponge Lissodendoryx isodictyalis were collected in early 1972. Later a 2-propanol extract reached the U.S. National Cancer Institute's (NCI) confirmed active level by leading to 45% life extension (at 12.5 mg/kg) when used to treat the murine P388 lymphocytic leukemia (PS system). The antineoplastic activity displayed by the initial Mexican collection and recollections (from 1976-82) was surprising. Six collections (1968-74) of this yellow (interior) and very fibrous sponge from the coast of Florida and the Caribbean did not give PS active extracts (ref. 5).

Due to very limited quantities of Gulf of California L. isodictyalis available from recollections obtained in several periods from 1976-1978 combined with the substantial challenges involved in isolating what appeared to be a series of complex antitumor constituents, present in only trace amounts, all efforts to solve this problem until 1982 were unrewarding. During a 1976 expedition we explored some 1,500 miles of the eastern Gulf of California and Pacific Ocean coasts of Mexico and only found the yellow sponge in one location: the original collection site in Bahia de Kino, Sonora. The sponge was originally found under an abandoned dredge (damaged by a storm) and grew from a few kg. when first sampled in 1972 to considerably more than 100 kg. when a 108 kg. recollection was made in early 1981. In the intervening period, almost that amount had been removed for preliminary studies. In that habitat L. isodictyalis obviously flourished and this may have been due to a symbiotic relationship with Bugula neritina (Bryozoa) and its potent cell growth inhibitory constituents described in the sequel.

With the 1981 recollection and application of some of the most powerful isolation techniques (ref. 6) we developed to date for such challenging problems the active antineoplastic components were isolated as summarized in Scheme 1. Important features of the isolation now follow. After collection, the yellow sponge was stored in 2-propanol at ambient temperature and the solution phase became the first extract. The animal was next extracted with methanol-methylene chloride (1:1). The methylene chloride soluble fractions from each extract were subjected to a solvent partition sequence by dissolution in 9:1 methanol-water and successively partitioning the methylene chloride components between (9:1 → 4:1) hexane →

[a] Refer to ref. 1

[b] See ref. 2

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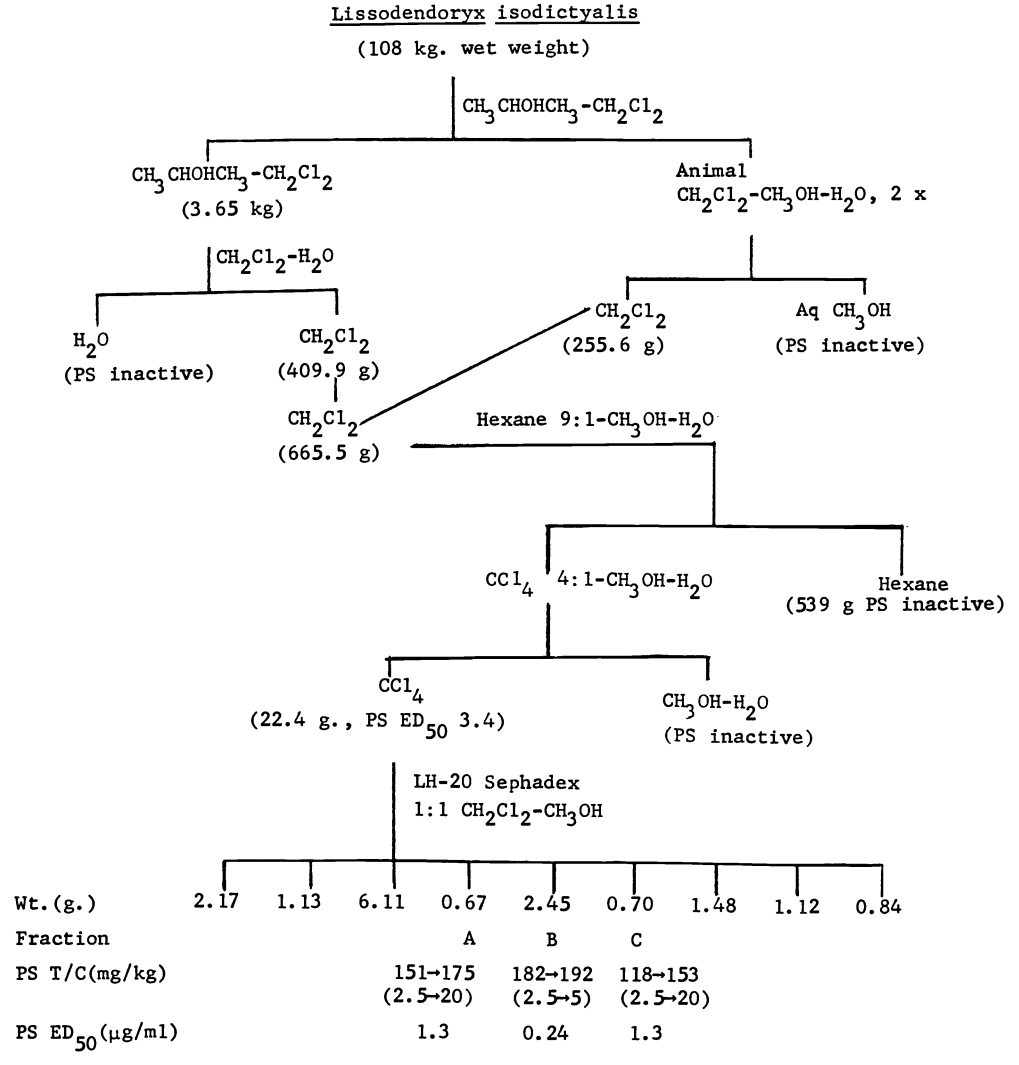
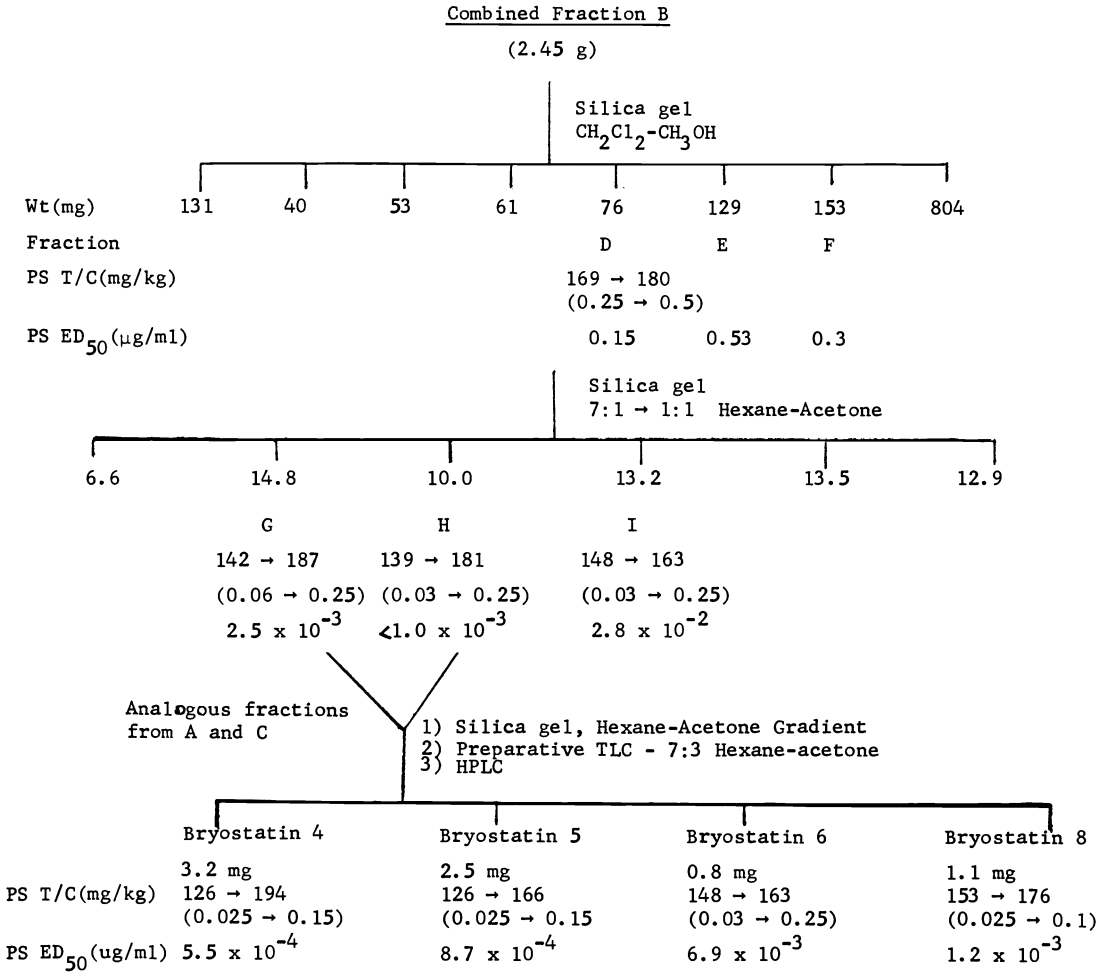
carbon tetrachloride. Each of these and subsequent separation steps were followed by bioassay (PS). Partition chromatography of the carbon tetrachloride fraction in 1:1 methylene chloride-methanol on Sephadex LH-20 constituted the next important step. The resulting active fractions A-C were individually chromatographed on silica gel using methylene chloride → methanol gradients. The next active fractions (D-F) were further separated using a hexane-acetone gradient. Enriched fractions G-I were purified to single antineoplastic agents by a series of preparative thin layer (7:3 n-hexane:acetone mobile phase) and high performance liquid chromatographic (Partisil M9 ODS-2, C-18 reversed phase methanol-water gradient, 1:1 → 9:1) procedures to yield six exceptionally potent antineoplastic substances. The method just summarized represents a considerable simplification resulting from a good number of less successful earlier attempts at reaching the pure antitumor components.

The reddish-purple color exhibited by side-products resulting from treating the antineoplastic active compounds on silica gel thin layer plates with an anisaldehyde-acetic acid-sulfuric acid mixture suggested the presence of bryostatins (ref. 1,6-8). From this clue four of the six antineoplastic (see Scheme 1 for biological data) constituents were identified (ref. 9) as bryostatins 4-6 (1a-c) and 8 (1d) obtained in respectively 3.0×10^{-6}, 2.0×10^{-6}, 7.4×10^{-7} and 1×10^{-6}% yields. The remaining two active compounds seemed to be new bryostatins bearing one oxygen atom less then those discovered to date. With this unusual oxygen content they appeared to be enzymatic transformation products and were not assigned a numerical position in the sequence. Instead they were designated bryostatins A and B (8.3×10^{-7} and 6.48×10^{-7}% yields). Due to the micro quantities (900 μg of A and 700 μg of B) isolated, the principal structural information was derived from solution phase-SIMS (ref. 10) spectra and high resolution ^{1}H-NMR. Unfortunately we were unable to obtain enough of A and B for a ^{13}C-NMR analysis that would have led to more definite structural assignments.

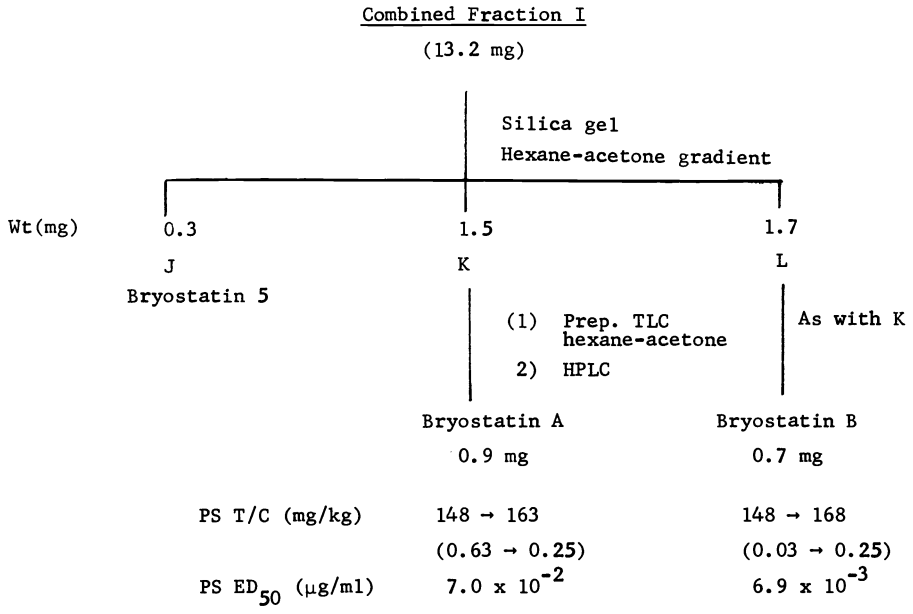
Separation Scheme: Part 1

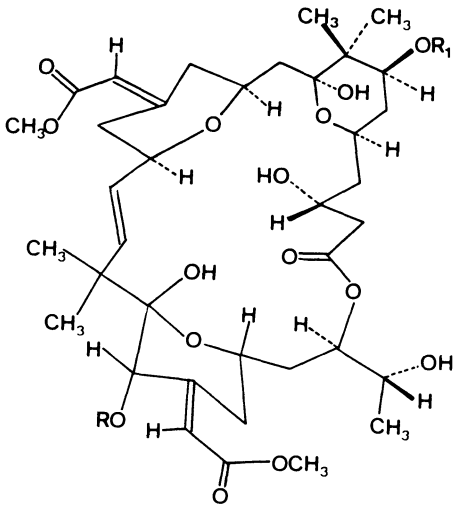


Separation Scheme: Part 2



Separation Scheme: Part 3





1a, R = $COCH_2CH_2CH_3$, R_1=$COCH_2CH(CH_3)_2$, Bryostatin 4

1b, R = $COCH_3$, R_1=$COCH_2CH(CH_3)_2$, Bryostatin 5

1c, R = $COCH_3$, R_1=$COCH_2CH_2CH_3$, Bryostatin 6

1d, R = R_1=$COCH_2CH_2CH_3$, Bryostatin 8

Interpretation of the ^{1}H-NMR spectra of bryostatins A and B indicated a close relationship in that order to bryostatins 5 (1b) and 4 (1a). That conclusion was further supported by results of the SP-SIMS (ref. 10) spectral data. The molecular formula selected for bryostatin A was $C_{44}H_{66}O_{16}$ corresponding to one less oxygen than that of bryostatin 5 (1b). The loss of acetate and valerate-type fragments observed in the SP-SIMS spectrum of A pointed to a possible bryostatin 5 origin. By the same means bryostatin B was assumed to be a deoxybryostatin 4.

When the presence of bryostatins in L. isodictyalis was recognized we had already discovered the first member of this potentially important class of new antineoplastic substances in a northern California collection of the marine Bryozoan Bugula neritina (ref. 11). Meanwhile, in a parallel study of the same animal from the Gulfs of California (e.g., Bahia de Kino) and Mexico, we isolated new units, bryostatins 4-8, of the series (ref. 6-8). Hence, we carefully examined museum specimens of the sponge and found Bugula neritina intrusions to the extent of some 2-5% by weight. The 1981 recollection that had undergone an even more complete exterior cleaning prior to extraction contained an interior amount closer to 2%. Doubtlessly, the bryostatins obtained from the yellow sponge extracts were derived from B. neritina. And the yields (cf., Table I) of bryostatins 4-6 were as would be expected from a few percent by weight of the Bryozoan. But on the assumption of even up to 5% invasion by B. neritina the yield of bryostatin 8 was considerably higher (~500%) than would be expected. As expected, a 2-propanol extract of B. neritina removed from the sponge led to 54% (at 6.2 mg/kg) life extension in the PS system.

Table I contains a summary of bryostatin yields from Bahia de Kino, Sonora and Gulf of Mexico B. neritina compared with that obtained from intrusions of this animal into the yellow sponge, the Bahia de Kino Tunicate Aplidium californicum (ref. 1) and the Florida

Table 1. Occurrence of Bryostatins in Four Marine Animals from the Gulfs of California and Mexico

Marine Organism	Amount (wet wt kg)	Bryostatins (mg) 4	5	6	7	8	A	B	Location/Date
Bugula neritina	12	12.3	3.0	2.8	0.6	0.5	---	---	Gulf of California Mexico, 1982
	50	44.5	14.1	61.9	31.2	1.5	---	---	Gulf of Mexico, Florida, 1982 (ref.8)
Lissodendoryx isodictyalis	33.7	1.0	1.2	---	---	0.5	---	---	Gulf of California, 1978
	108	3.2	2.5	0.8	---	1.1	0.9	0.7	Gulf of California, 1981
Aplidium californicum	34	0.9	0.5	---	---	---	---	---	Gulf of California, 1978 (ref. 1)
Amathia convoluta	245	7.6	3.1	6.3	---	4.2	---	---	Gulf of Mexico, 1981 Florida (ref. 6)

Bryozoan Amathia convoluta (ref. 6). The observations that bryostatin 7 was missing, bryostatin 8 was obtained in much larger quantity than would be expected and the discovery of new members A and B suggests that some concentration and/or enzymatic transformation of these antineoplastic constituents occurs in the sponge. Indeed the robust development of L. isodictyalis over the near decade of study suggests a very fruitful symbiotic relationship with B. neritina. Such adventures by this exceptionally resourceful and adaptable Bryozoan bearing highly active antineoplastic constituents continues to demand caution when evaluating new marine animal candidates for detailed biological and chemical examination.

GENERAL METHODS

All solvents used for chromatography were redistilled. The Sephadex LH-20 (25-100 μ) used for gel permeation and partition chromatographic procedures was supplied by Pharmacia Fine Chemicals AB, Uppsala, Sweden. Gilson FC-220 race track and FC-80 micro-fractionators with Gilson HM UV-visible Holochrome detectors were employed for chromatographic fractionation. Column chromatographic techniques (dry method) utilized columns of 70-230 mesh silica gel obtained from E. Merck (Darmstadt). A Partisil M9 10/50 ODS-2 (C-18 reversed phase) column (9.4 mm ID x 500 mm) was used for HPLC. Both the HPLC column and analytical layer plates (KC-18) were manufactured by Whatman, Inc., Clifton, New Jersey. The silica gel GF Uniplates for thin layer chromatography (TLC) were obtained from Analtech, Inc., Newark, Delaware. The thin layer chromatographic plates were viewed with UV light or developed with an anisaldehyde-acetic acid spray followed by heating at approximately 150° for 10-15 min.

Melting points (uncorrected) were observed with a Kofler-type melting point apparatus. Ultraviolet spectra were recorded using a Hewlett-Packard 8450A UV/VIS spectrophotometer. Optical rotation and infrared spectral data were recorded with a Perkin-Elmer 241 polarimeter and a Nicolet MX-1 FT infrared instrument. Mass spectra were obtained with a MAT 312 spectrometer equipped for solution phase SIMS (ref. 10). Nuclear magnetic resonance investigations were performed using a Bruker WH-400 instrument with deuteriochloroform as solvent and tetramethylsilane as internal standard.

ANIMAL COLLECTION AND PRELIMINARY EXPERIMENTS

In March 1972 about 1 kg. of Lissodendoryx isodictyalis Carter (Porifera phylum, Demospongiae class, and Poecilosclerida order, Myxillidae family) was collected in the Gulf of California (Bahia de Kino, Sonora, Mexico). The previously mentioned damaged and apparently abandoned dredge in the northeast section of the estuary was the source of this and all later recollections. Professional taxonomic studies were contributed by the Smithsonian Institution and museum samples are maintained in the ASU-CRI. The grey (yellow interior) fibrous sponge was preserved in 2-propanol. Evaporation of solvent gave an extract that reached a confirmed level ($\geqq$ T/C 120) of activity against the National Cancer Institute's murine P388 lymphocytic leukemia (PS system) and showed T/C 145 at 12.5 mg/kg. Recollections of the sponge were made in March 1976 and January 1977 for preliminary separation experiments.

In May 1978, 33.7 kg. (wet wt.) of L. isodictyalis was collected and preserved in 2-propanol. Removal of solvent from the aqueous 2-propanol extract gave a 0.89 kg. residue. A portion of the residue (113 g) was partitioned between methylene chloride-water and the chlorocarbon solution was concentrated to a fraction that was successively partitioned between 9:1 methanol:water-hexane, 4:1 methanol:water-carbon tetrachloride and 3:2 methanol:water-methylene chloride. By this technique (ref. 12) the antineoplastic activity was concentrated in the carbon tetrachloride fraction (1.5 g) and showed PS T/C 172 at 30 mg/kg and in the PS cell line ED_{50} 0.12 μg/ml. The challenging problem of isolating the antineoplastic components was partially solved (cf. Table I) using the 1978 recollection and completed starting from a March 1981 recollection amounting to 108 kg (wet wt.) of the Bahia de Kino sponge. The separation procedures employed with the 1981 recollection were found most efficient and will now be described in practical detail.

ANIMAL EXTRACTION

All of the aqueous 9:1 2-propanol-methylene chloride extract from the 108 kg (wet wt.) (1981) recollection preserving solution was concentrated and partitioned between methylene chloride and water. Residual animal was extracted with 1:1 methylene chloride-methanol (ref. 13). Enough water was added to produce two phases and the methylene chloride layer was separated and concentrated. Extraction of the sponge was repeated by adding sufficient methanol to the methanol-water-animal mixture to form a single phase. After adding water to separate the methylene chloride this phase was collected and concentrated.

SOLVENT PARTITION METHODS

A solution of the total methylene chloride extract (665.5 g) from the 1981 recollection in 9:1 methanol-water was extracted with hexane. Dilution of the methanol-water phase to 4:1 methanol-water was followed by extraction with carbon tetrachloride. The hexane (539 g), carbon tetrachloride (22.4 g), and methanol-water (56.2 g) fractions were concentrated and aliquots submitted for bioassay. Antineoplastic activity (see Separation Scheme Part 1) was found concentrated in the carbon tetrachloride fraction.

When the sponge extract antineoplastic constituents were found to be bryostatins, all prior recollections were examined for presence of the bryozoan, Bugula neritina. Inspection revealed internal invasion of 2-5% corresponding to Bugula neritina. The 1981 recollection contained approximately 2% B. neritina.

ISOLATION OF BRYOSTATINS 4–6, 8, A AND B

The following series of experiments began with the 22.4 g active carbon tetrachloride fraction (Separation Scheme Part 1) from the 1981 recollection. A solution of this fraction in 2:3 methylene chloride-methanol was chromatographed on a column of Sephadex LH-20 (10 x 120 cm). Combination of similar fractions led to the A-C active series entered in Separation Scheme Part 1. The principal in vivo active fraction (B, 2.45 g) was chromatographed on silica gel (3 x 120 cm column) using a gradient of methylene chloride to 4:1 methylene chloride-methanol. Fraction D (76 mg) was separated (Separation Scheme Part 2) by a dry column chromatographic procedure on silica gel (1 x 60 cm column) using gradient elution from 7:1 → 1:1 n-hexane-acetone. Detailed separation of resulting active fractions G-I by further silica gel column chromatography in gradients ranging from n-hexane-acetone (7:1 → 1:1) in 5 ml fractions followed by preparative TLC in 7:3 n-hexane-acetone and HPLC on a C-18 reversed phase column (a flow rate of 1.0 ml/min.) with a gradient from 1:1 → 9:1 methanol-water gave pure bryostatin 4 (1a, 3.2 mg). In these same experiments a total of 2.5 mg of bryostatin 5 (1b) and 1.1 mg of bryostatin 8 (1d) was obtained. The more polar fraction, I (13.2 mg, see Separation Scheme Part 3) was subjected to the same isolation sequence and by this means bryostatin A (0.9 mg), bryostatin B (0.7 mg) and bryostatin 6 (1c, 0.8 mg) were isolated.

Bryostatin A

An analytical specimen was obtained from methylene chloride-methanol as a colorless powder: TLC Rf 0.31 (n-hexane-acetone 7:3) and Rf 0.46 (n-hexane-ethyl acetate 1:1) on silica gel plates; mp 142-145°C; and SP-SIMS mass spec m/z (M^+ 850 for $C_{44}H_{66}O_{16}$) 873 $[M+Na]^+$, 855 $[M+Na-18]^+$, 845 $[M+Na-28]^+$, 829 $[M+Na-44]^+$, 827 $[M+Na-46]^+$, 815 $[M+Na-58]^+$, 813 $[M+Na-60]^+$, 797 $[M+Na-76]^+$, 785 $[M+Na-88]^+$, 783 $[M+Na-90]^+$, 769 $[M+Na-104]^+$; $[\alpha]_D^{27}$ +74.85° (CH_3OH, c = 0.04); UV $\lambda_{max}^{CH_3OH}$ 228 nm (ε36,400); IR ν_{max}^{KBr} 3465, 2980-2950, 1740, 1720, 1658-1640, 1440, 1380, 1365, 1285, 1240, 1160, 1100, 1090, 1075, 1050, 1000 and 870 cm^{-1}.

Bryostatin B

Bryostatin B was obtained pure as a colorless powder from methylene chloride-methanol: TLC Rf 0.35 (n-hexane-acetone 7:3) and Rf 0.51 (n-hexane-ethyl acetate 1:1) on silica gel plates; mp 137-140°C; and SP-SIMS mass spec m/z (M^+ 878 for $C_{46}H_{70}O_{16}$) 901 $[M+Na]^+$, 883 $[M+Na-18]^+$, 873 $[M+Na-28]^+$, 857 $[M+Na-44]^+$, 855 $[M+Na-46]^+$, 843 $[M+Na-58]^+$, 813 $[M+Na-88]^+$, 811 $[M+Na-90]^+$, 799 $[M+Na-102]^+$, and 797 $[M+Na-104]^+$; $[\alpha]_D^{27}$ + 71.30 (CH_3OH, c = 0.035); UV $\lambda_{max}^{CH_3OH}$ 227 nm (ε36,000); IR ν_{max}^{KBr} 3470, 2980-2945, 1740, 1725, 1660-1640, 1435, 1380, 1370, 1288, 1240, 1160, 1100, 1095, 1075, 1050, 1000 and 870 cm^{-1}.

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