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ANTINEOPLASTIC AGENTS 100

THE MARINE BRYOZOAN *AMATHIA CONVOLUTA*[1]

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Abstract—An intensive investigation of the marine animal *Amathia convoluta* (Bryozoa phylum) for antineoplastic constituents has led to the isolation and structural determination of bryostatin 8 (2). A total of 100 kg of *Amathia convoluta* was required to obtain some 4.2 mg of bryostatin 8. Evaluation of bryostatin 8 against the murine P388 lymphocytic leukemia showed substantial inhibition of growth (PS, cell line ED_{50}, 1.3×10^{-3}, T/C 174 at 0.11 mg/kg). A major part of the very strong antineoplastic activity exhibited by fractions prepared from *Amathia convoluta* was accounted for by the isolation of bryostatins 4 (**1a**), 5 (**1c**) and 6 (**1b**). All three bryostatins were obtained in yields comparable to that of bryostatin 8 and appear derived from the closely related marine bryozoan *Bugula neritina*. The epiphytic-like relationship of *Bugula neritina* to *Amathia convoluta* is discussed.

From present fossil and geological dating evidence living organisms as highly developed as algae were already in oceans existing 3.6 billion years ago and some recent molecular evidence suggests an even longer 3.8 billion years. Biosynthetic evolutionary processes over such an incredibly long period should favor the development of very sophisticated chemical protective agents in marine organisms that will prove exceptionally useful with a variety of refractory human medical problems. Furthermore, terrestrial and marine invertebrates do not have a thymus system for immunological protection and must have developed other chemical mechanisms of intercellular control. In addition to phagocytosis the biosynthetic compounds employed in such control mechanisms should be especially important in the discovery of potentially useful cancer chemotherapeutic drugs. Less speculative is the fact that cancer is essentially unknown among terrestrial and marine invertebrates.[2]

Some of the preceding concepts led one of us (GRP) in 1957 to initiate a study of amphibian venoms of the steroidal bufadienolide[3] type as potential sources of new antineoplastic substances. Eventually we found some of the toad venom bufadienolides such as marinobufagin to significantly inhibit growth of the National Cancer Institute's KB cell line derived from a human nasopharynx carcinoma and lead to a curative response with the murine Ehrlich ascites system.[3d] But the therapeutic indices were unattractive for further development. And this effort was extended in 1965–66 to a broad and systematic program to evaluate marine invertebrates[4] and arthropods[5] as new sources of potentially useful anticancer drugs. From certain terrestrial arthropods we isolated the first such antineoplastic constituents and these studies are still in progress.[6]

By 1968 we were able to show conclusively that some 9–10% of marine invertebrates and vertebrates from exploratory collections would display a confirmed level of activity against the U.S. National Cancer Institute's (NCI) murine P388 lymphocytic leukemia (PS system) or Walker carcinosarcoma 256 in the rat.[2,4] Subsequently we have isolated and characterized the first marine animal antineoplastic and/or cell growth inhibitory constituents from a variety of these organisms. And these have been found to be as diverse as the terpene aplysistatin from the sea hare *Aplysia angasi* (Mollusca),[7] lanostane glycosides of the stichostatin 1[8] type from the sea cucumber *Stichopus chloronotus* (Echinodermata), macrocyclic lactones illustrated by bryostatin 1[9] from *Bugula neritina* (Bryozoa), unique cyclic peptides of the dolastatin 3[10] class from the shell-less mollusc *Dolabella auricularia*, to glycoproteins such as strongylostatin 1 obtained from the sea urchin *Strongylocentrotus drobachiensis*[11] (Echinodermata) and the sphyrnastatins[12] from the hammerhead shark, *Sphyrna lewini*. Most importantly, one of the dolastatins (1) has been found to afford a curative response (33%) at a dose of 11 μg/kg (T/C 240, T/C 139 at 1.37 μg/kg) against the NCI murine B16 melanoma and on that basis may be one of the most active (in terms of low dose) presently known antineoplastic agents. Similarly bryostatin 1 has given 31–68% life extension at 5–40 μg/kg against the NCI M531 murine ovary sarcoma and is presently undergoing further antineoplastic and other biological evaluation.

The discovery of each such interesting marine animal antineoplastic constituent has required an extensive series of biological and chemical studies where separation guided by the PS *in vitro* and/or *in vivo* systems have played a major role. However, by sharply focussing our research efforts on uncovering only marine animal constituents of potential use in cancer chemotherapy we have succeeded in uncovering a number of potentially useful substances of

completely unanticipated structural types. Any unlocking of nature's important secrets can be fraught with difficulties and this approach to completely new types of anticancer drugs is no exception. The following summary of our 15 year endeavors directed at isolation of antineoplastic constituents from the marine bryozoan *Amathia convoluta* provides an illustration.

A 1968 collection of *Amathia convoluta* from the Northeastern Gulf of Mexico was found to yield extracts that more than doubled the life span of animals bearing the PS lymphocytic leukemia. Indeed this was an exceptionally promising lead and was vigorously pursued until brought to fruition in 1983. While biologically and chemically fascinating, the physical appearance of *Amathia convoluta* is deceptively mundane. Perhaps this accounts in part for the absence of any prior chemical study and no biological investigation beyond distribution and taxonomy.[13,14] *A. convoluta* occurs in small shrub-like clusters from 1 to 3 cm tall, where each individual animal of the greyish-brown colony approximates 0.1 mm in size. In general *A. convoluta* resembles the bryozoan *Bugula neritina* L. and we have found both to occur together in certain areas of the Northeastern Gulf of Mexico. That observation becomes very important in the sequel.

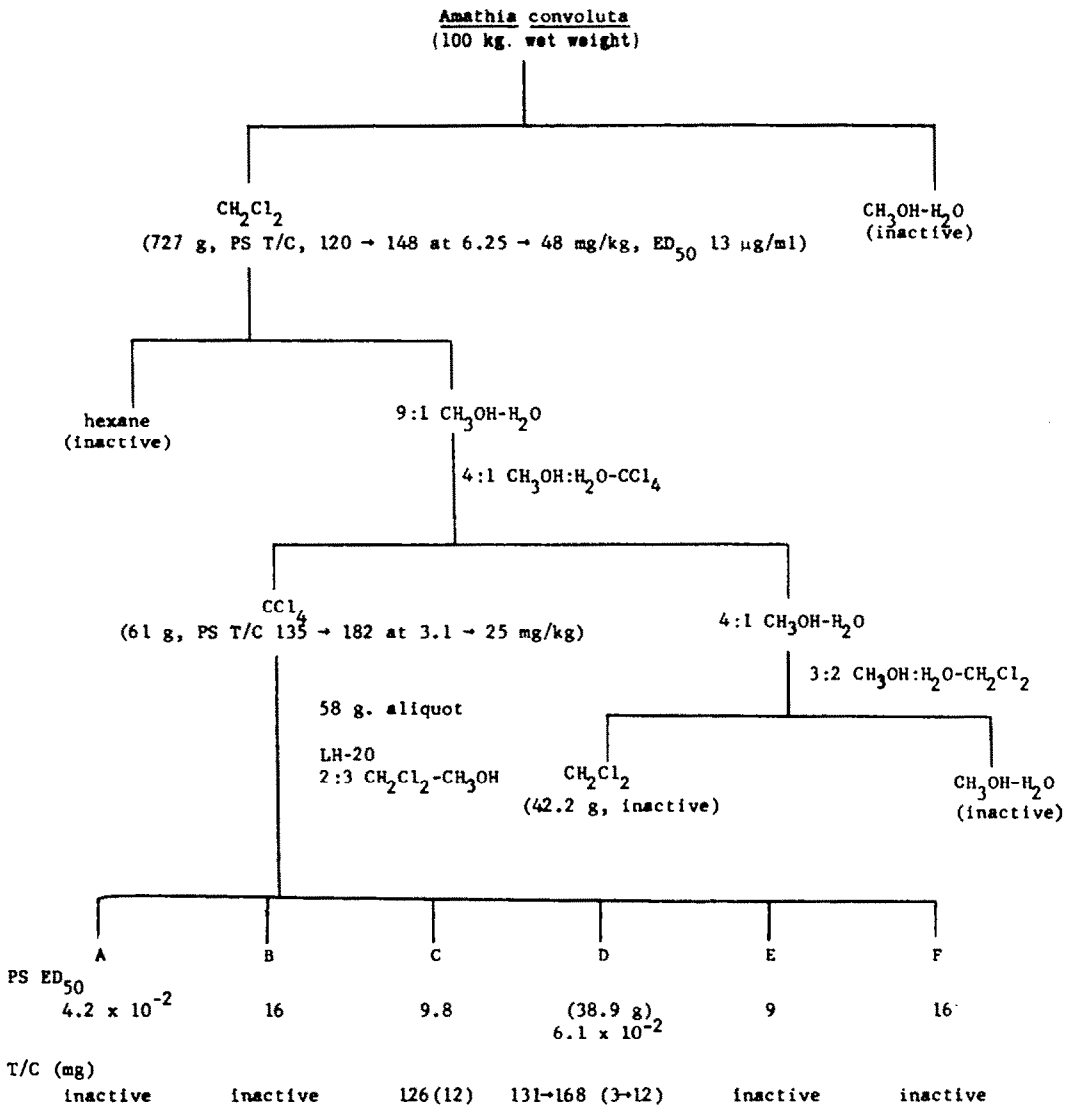
The chemical investigation of *A. convoluta* began in earnest in 1970 with recollections made in the spring and fall that totalled some 50 kg. Over the next 6 years various methods of initial extraction and separation of active constituents were attempted with these collections to no avail. In this period most of the improved extraction and initial separation techniques that we devised were applied to the *A. convoluta* objectives. Most encouraging progress was noted when the aqueous 2-propanol extract was partitioned between methylene chloride and water followed by successive partitioning of the methylene chloride fraction between (9:1 → 4:1 → 3:2) methanol–water and hexane → carbon tetrachloride → methylene chloride. By this means the PS *in vivo* activity was concentrated in the carbon tetrachloride fraction.

History of the above very useful technique began over 30 years ago with studies of methanol–water as upper layer and chloroform as lower layer for extraction of lipids from biological materials.[15] By 1959 such experiments were developed into a practical technique by Bligh and Dyer for extracting marine vertebrate lipids.[16] Later the 9:1 → 4:1 → 3:2 methanol–water with ligroin → carbon tetrachloride → chloroform variation was very productively used by Professor Kupchan (and colleagues) and by our group for the preliminary investigation of higher plants containing antineoplastic components. Because of safety considerations and side-reactions (from carbene, hydrogen chloride and phosgene formation), by 1975 we further improved the technique by replacing chloroform with methylene chloride.[17] And this substitution has proved to be even more useful in terms of concentrating biologically active constituents. The overall preliminary separation procedure for *A. convoluta* (and for other animals and plants under study in our Institute) was further improved when we developed a very simple and effective extraction procedure for animal and plant materials employing 1:1 methylene chloride–methanol.[18] After extraction the solvent mixture was diluted with sufficient water to cause phase separation and the methylene chloride fractions were collected. Sufficient methanol and methylene chloride was added to the aqueous methanol mixture to produce a homogeneous solvent. The extraction was allowed to proceed and the overall procedure repeated as necessary. By this method the great mass of inactive materials was removed and the active fraction reduced to a manageable weight.

The first two years spent exploring the active fractions from *A. convoluta* were devoted to evaluating separation methods that ranged from gel permeation (Sephadex G-10 and LH-20), partition (Avicel A microcrystalline cellulose), ion exchange (Amberlite CG-120), and silica gel column chromatography to chromatography on macroreticular resins such as XAD-4. Of these approaches Sephadex LH-20 seemed most promising for introduction at an early stage. By 1976 it was clear that the antineoplastic constituent(s) of *A. convoluta* must be present in only trace amounts and it was decided to recombine all fractions and begin again with the best technique developed to that time. Although the high level of antineoplastic activity was still apparent in the mixture obtained by recombination, it soon became apparent that the paucity of antineoplastic component(s) would continue to elude detection unless the quantities could be greatly increased. In 1978 approximately 118 kg (wet weight) was recollected followed by another 100 kg of the wet animal in early 1981. All of the experience gained from previous separation experiments was applied to the 1978 recollection and microgram amounts of the principal antineoplastic constituents were isolated over the next few years. Again the isolation procedures were further simplified, improved and applied to the 1981 recollection that formed the basis of this contribution.

The 1981 recollection (100 kg, wet weight) was initially reduced to a 61 g carbon tetrachloride PS active fraction as outlined in Separation Scheme Part 1. The gel permeation-partition type chromatographic separation employing Sephadex LH-20 with 2:3 methylene chloride–methanol as solvent caused a rapid concentration in fraction D of the major antineoplastic constituents. A useful further separation and concentration was achieved by repeating this step as shown in Part 2 of the Separation Scheme. Fraction H seemed to contain the most potent antineoplastic constituents and was further separated by careful fractionation on silica gel to yield active fraction M. The silica gel chromatographic separation with changes in solvent was expanded to produce active fraction N. The relatively small weight of N (37.5 mg) allowed partial separation by preparative layer chromatography to yield active fraction 0. Even the 18.2 mg fraction 0 from some 100 kg of animal was still a challenging mixture that required extensive reverse phase and normal phase HPLC procedures for final separation. By this means bryostatins 4 (**1a**) and 6 (**1b**) were isolated in 1.6 mg and 0.6 mg yields respectively.

Meanwhile the promising Sephadex LH-20 fractions G and I were combined as outlined in Separation Scheme Part 3 and subjected to the same type of fractionation as just summarized for obtaining bryostatins 4 and 6. However, the active fraction P

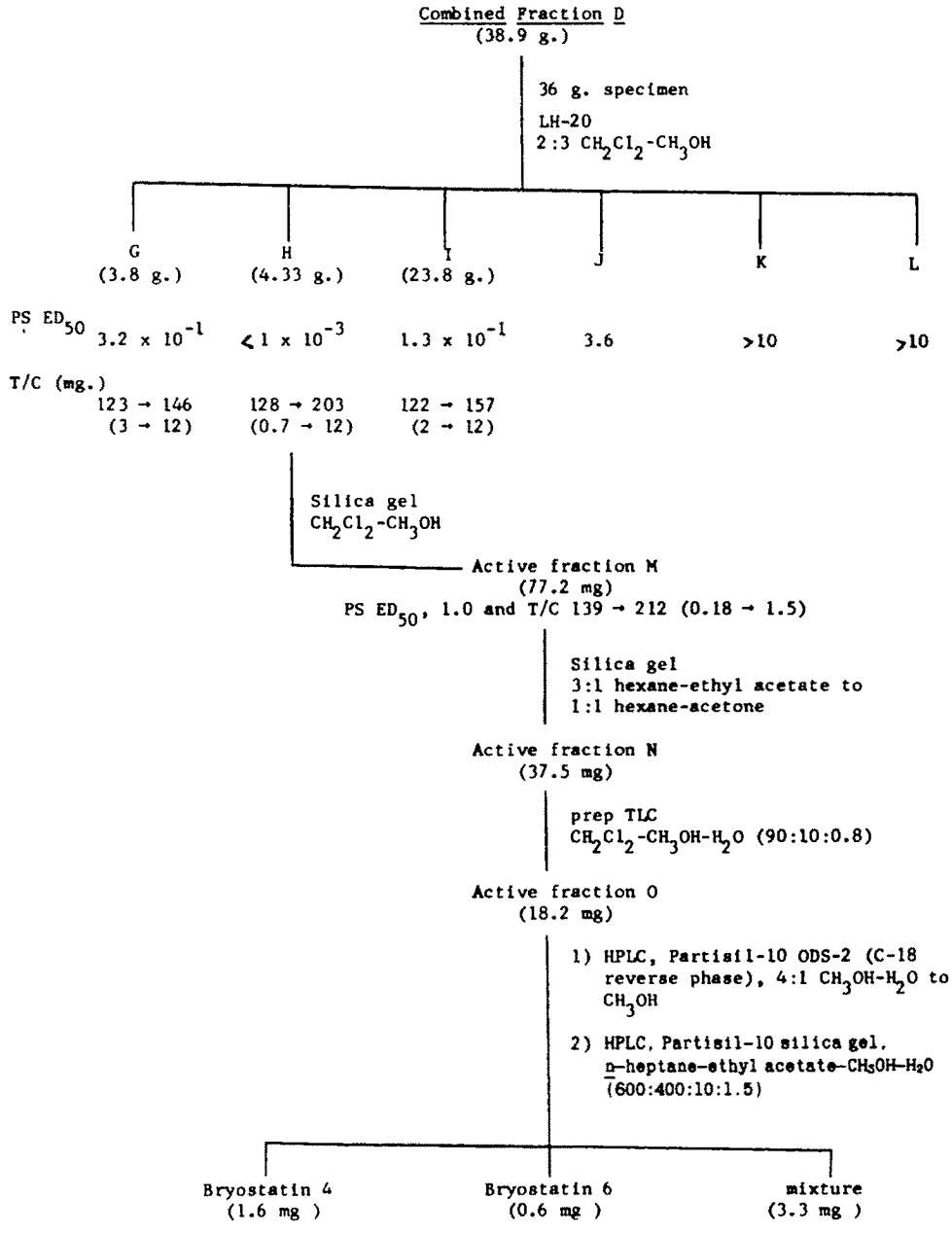


Separation Scheme Part 1

corresponding to fraction M of the preceding separation was considerably more complicated and required the introduction of a careful partition chromatographic step utilizing the LH-20 gel. The resulting PS *in vivo* active fraction Q was separable into the principal active antineoplastic constituent of *A. convoluta* utilizing the techniques developed for separating fractions M–O. Additional quantities of bryostatins 4 (**1a**, 6.0 mg) and 6 (**1b**, 5.7 mg) were isolated accompanied by bryostatin 5 (**1c**, 3.1 mg) and a new bryostatin designated 8 (**2**, 4.2 mg). The combined total yields of bryostatins 4 and 6 amounted to 7.6×10^{-6} and 6.3×10^{-6}%, bryostatin 5, 3.1×10^{-6}% and bryostatin 8, 4.2×10^{-6}%.

A parallel study of *Bugula neritina* antineoplastic components ongoing in our laboratory from about the same time in 1968 had by 1981 led to the discovery of bryostatins 1[9] and 2[19] (**1d, e**) and by the summer of 1982 structure **1d** by X-ray crystal structure determination for bryostatin 1.

Subsequently we were able to assign the structures indicated here for bryostatins 4 and 6.[20,21] Recognition of bryostatin 8 as a possible member of this unique series of macrocyclic lactones first arose from viewing the characteristic reddish-purple color produced upon heating a thin-layer chromatogram of the substance sprayed with anisaldehyde reagent. Detailed interpretation of the 400 MHz ^{1}H-NMR (Table 1) and ^{13}C-NMR spectra (Table 2) indicated that bryostatin 8 retained the basic bryopyran ring system and substitution pattern of bryostatin 1. The principal differences seemed to be at C-7 and C-20 where the acetate and (*E,E*)-octa-2,4-dienoate esters appeared to be absent. Application of the solution phase secondary ion mass spectrometric techniques to bryostatin 8 that we[22] developed for routine detection of molecular ions

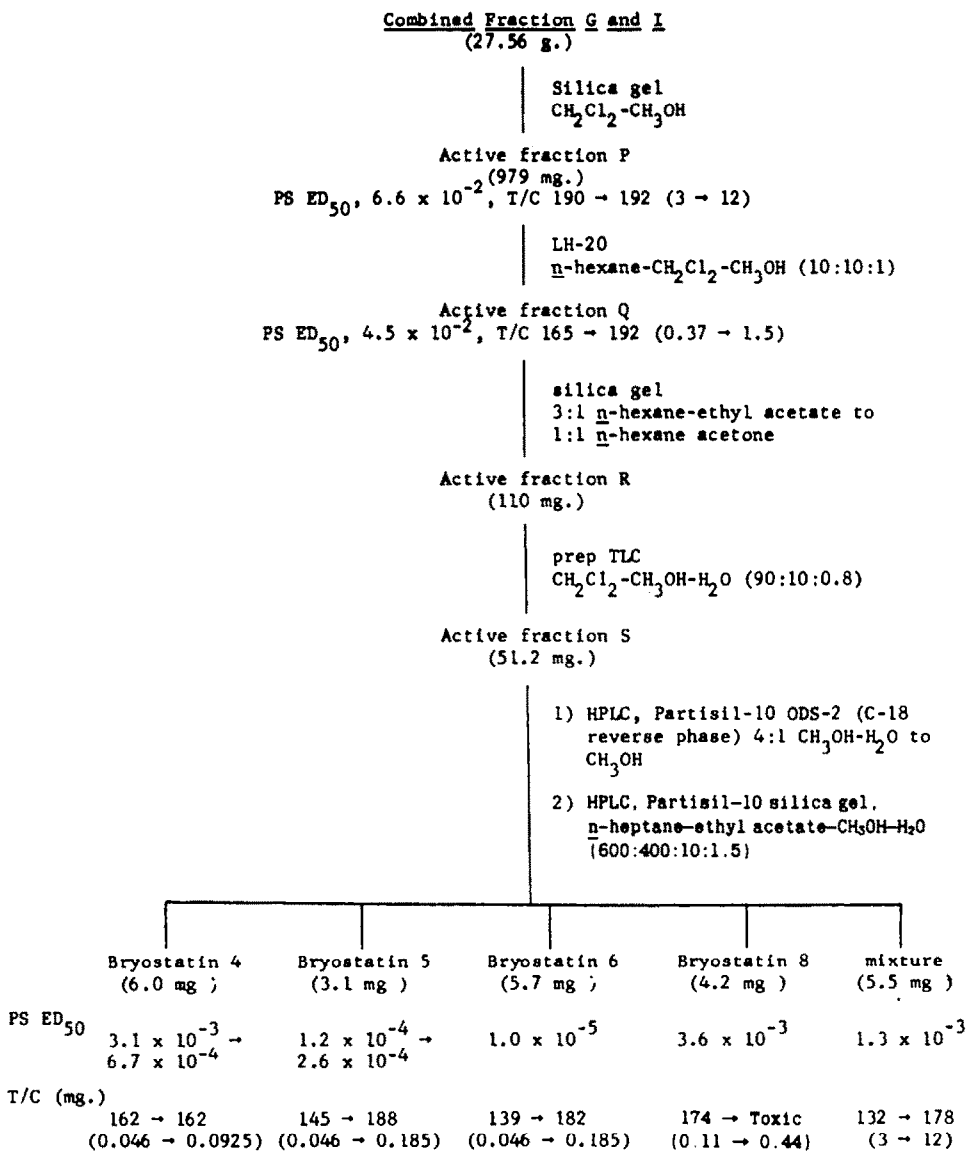


Separation Scheme Part 2

proved rewarding. A sulfolane solution containing sodium iodide gave a molecular ion complex at 903 $[M + Na]^+$ and an important fragment ion at 815 $[M + Na - 88]^+$. Elimination of only 88 mass units led to the assumption that bryostatin 8 was esterified at C-7 and C-20 with butyrate substituents.

As part of the structural elucidation of bryostatin 2 (**1e**)[19] it was found that bryostatin 1 could be selectively hydrolyzed using 1% hydrochloric acid in aqueous methanol. The reaction led to bryostatin 2 as major product and suggested that the steric hindrance around C-20 was responsible for this very helpful selectivity. When bryostatin 8 was allowed to react with 1% hydrochloric acid in aqueous methanol for 1 day at room temperature the major product was presumed to be C-20 butyrate ester **1f** accompanied by the isomeric **1g** on the basis of mass spectral and proton magnetic resonance studies. A solution phase secondary ion mass spectrum of the hydrolysis product exhibited a molecular ion complex at 833$[M + Na]^+$ and a quite revealing fragment ion at 745 $[M + Na - 88]^+$. Thus loss of one butyl ester by selective hydrolysis and the second by mass spectral promoted cleavage provided compelling support for the C-7, C-20 dibutyrate structure **2** for bryostatin 8. Non-branching of the butyl grup was ascertained by the NMR studies of bryostatins 4, 6 and 8.

When structures for the principal antineoplastic constituents of *A. convoluta* were established, a possible relationship between this animal and the related



Separation Scheme Part 3

Bugula neritina came under scrutiny. Voucher animal specimens from all prior collections along with 100 plus kilogram amounts of *A. convoluta* available from the 1981 and more recent (1982) recollections were closely examined. Amounts ranging from <0.2% to approx. 3% dry weight of *Bugula neritina* were found growing from the *A. convoluta* in a parasitic or epiphytic-like manner. The 1981 recollection described herein was found to have approx. 2.5% by weight of attached *Bugula neritina*. From our experience with the isolation and characterization of bryostatins 4–6 from *B. neritina* collected in the same general area as *A. convoluta*, the yields of these bryostatins seem to be two to four times greater than would be expected from *A. convoluta* containing some 2.5% of *B. neritina*. But this proportion was too close to safely conclude that they were produced by *A. convoluta*. On the other hand bryostatin 8 (2) was isolated from *A. convoluta* in a quantity 50 times greater than would be expected from the companion *B. neritina*. Thus, bryostatin 8 seems to be a genuine constituent of *A. convoluta* and/or both animals have a relationship where the bryostatins may be transferred and concentrated by the *A. convoluta*. Alternatively, if the real source of the bryostatins resides in a common food source such as a dinoflagellate, these relationships may be even more complex.

The preceding observations pose a number of interesting scientific questions and lead to some immediate practical considerations. The ability of *B. neritina* with its reservoir of exceptionally potent antineoplastic bryostatins to attach itself and/or invade other organisms prompts us to urge that new marine animal candidates for biological investigation be carefully inspected for such contamination. Otherwise the bryostatins represent a very significant advance

Table 1. ^{1}H NMR (400 MHz, deuteriochloroform solution)

Position	Bryostatin 8 (2) δ (ppm)	Multiplicity (J Hz)	Bryostatin 1 (1d) δ (ppm)	Multiplicity (J Hz)
2	2.450	m	2.45	m
3	4.166	m	4.19	m
4	1.64, 2.01	m,m	1.55, 1.95	m,m
5	4.202	m	4.1	m
6	1.46, 1.76	m,m	1.4, 1.5	m,m
7	5.164	m	5.15	m
10	1.70, 2.06	m,m	2.1~2.2	m
11	3.820	m	~3.95	m
12	2.10~2.19	m	2.1~2.2	m
14	1.83, 2.03	m,m	1.9, ~2.0	m,m
15	4.04	m	4.08	m
16	5.295	dd(8.45, 15.75)	5.300	dd(8.3, 15.9)
17	5.758	d(15.75)	5.758	d(15.9)
20	5.103	s	5.162	s
22	1.89, 2.02	m,m	~1.90	m
23	3.982	m	~3.65	m
24	1.80, 1.97	m,m	1.95	m
25	5.170	m	5.19	m
26	3.767	m	3.73	m
27	1.214	d(6.50)	1.226	d(6.3)
28[a]	1.135	s	1.132	s
29[a]	0.983	s	0.982	s
30	5.663	s	5.657	s
32[a]	0.983	s	0.982	s
33[a]	0.922	s	0.919	s
34	5.961	s	5.983	s
36	3.685	s	3.68	s
37	3.655	s	3.65	s
2'	2.27	m	2.051	s
3'	1.66	m		
4'	0.923	t(7.2)		
2''	2.27		5.796	d(15.3)
3''	1.66		7.261	m
4''	0.917	t(7.2)	6.157	m
5''			6.157	m
6''			~2.15	m
7''			1.42	m
8''			0.904	t(7.3)

[a]Assignments for these four groups may be interchanged

in our knowledge of structure requirements for anticancer activity and one or more of these important new substances may be developed for eventual clinical trial.

EXPERIMENTAL

General methods. Solvents used for chromatographic procedures were redistilled. The Sephadex LH-20 (25–100 μ) employed for gel permeation and partition chromatography was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Gilson FC-220 race track and F-80 micro-fractionators connected to Gilson HM UV-visible Holochrome detectors were used for chromatographic fractionation experiments. Column chromatographic procedures with silica gel utilized the 70–230 mesh or silica gel 60 prepacked columns supplied by E. Merck (Darmstadt). Both the Partisil M9 10/50 and Partisil M9 10/50 ODS-2 (C-18 reverse phase) columns (9.4 mm i.d. × 500 mm) were used for HPLC and obtained from Whatman, Inc., Clifton, New Jersey. Preparative layer plates were also obtained from Whatman, Inc. and the silica gel GF Uniplates for TLC were supplied by Analtech, Inc., Newark, Delaware. The TLC plates were viewed with UV light or developed with an anisaldehyde–AcOH–H_2SO_4 spray (heating at approx. 150° for 10 min).

M.p.s are uncorrected and were observed with a Kofler-type m.p. apparatus. UV spectra were recorded using a Hewlett–Packard 8450A UV/VIS spectrophotometer equipped with a HP7225A plotter. Optical rotation and IR spectral data were obtained using a Perkin–Elmer 241 polarimeter and a 299 IR spectrophotometer, respectively. Recently, IR spectra have been obtained with a Nicolet MX-1 FT instrument. All mass spectra (70 eV and FAB) were

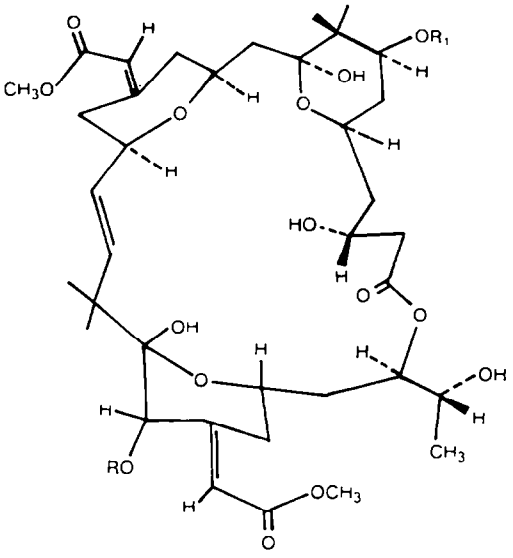
Table 2. ^{13}C NMR (deuteriochloroform solution)[a]

Position	Bryostatin 8 (2) δ (ppm)	Bryostatin 1 (1d) δ (ppm)
1	173.26	172.29
2	44.26	44.29
3	64.86	71.62
4	31.29	31.42
5	73.76	73.73
6	33.47	35.09
7	72.46	73.11
8	41.14	41.11
9	101.94	101.94
10	42.50	36.04
11	65.93	68.50
12	42.18	36.56
13	156.30	157.18
14	36.62	40.00
15	70.22	70.22
16	139.37	139.24
17	129.59	129.66
18	44.94	44.94
19	99.04	99.11
20	79.26	79.13
21	151.85	152.08
22	36.04	42.31
23	74.35	64.83
24	40.00	42.02
25	71.62	74.19
26	68.56	65.71
27	19.85	21.90
28, 29	16.93	16.90
30	114.64	114.15
31	167.02	167.06
32	24.63 or 21.12	21.15 or 19.79
33	21.12 or 24.63	19.79 or 21.15
34	119.71	119.65
35	166.70	166.80
36, 37	51.02	51.05
1'	172.09	171.15
2'	36.59	33.43
3'	18.59	
4'	13.68	
1''	171.96	165.63
2''	36.59	118.74
3''	18.29	146.39
4''	13.68	128.49
5''		145.42
6''		35.09
7''		24.66
8''		13.68

a, tentative assignments

recorded by Mr. D. Adams with a MAT 312 spectrometer. NMR experiments were conducted with Varian XL-100, Bruker WH-90 and Bruker WH-400 instruments. $CDCl_3$ was employed as solvent and TMS as internal standard.

Animal collection and preliminary experiments. In the spring of 1968 approx. 1 kg of *Amathia convoluta* (Bryozoa phylum, Gymnolaemata class, Ctenostomata order, Stolonifera suborder, Vesiculariidae family, and Vesicularioidea



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

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

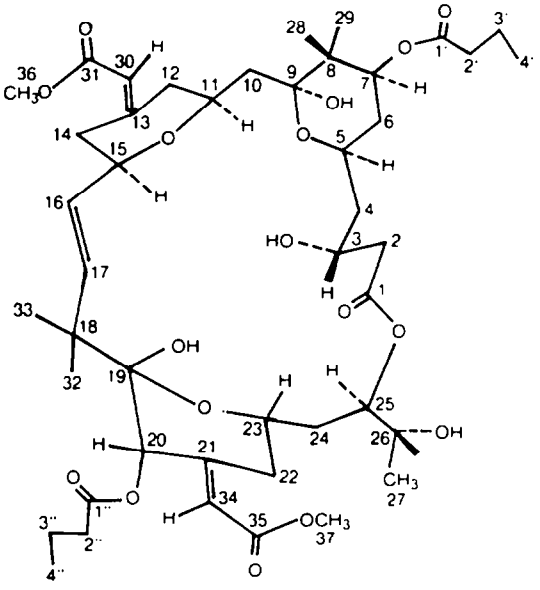
1c, R = $COCH_3$, R_1 = $COCH_2CH(CH_3)_2$, Bryostatin 5

1d, R = COCH = CHCH = $CHCH_2CH_2CH_3$, R_1 = $COCH_3$, Bryostatin 1

1e, R = COCH = CHCH = $CHCH_2CH_2CH_3$, R_1 = H, Bryostatin 2

1f, R = $COCH_2CH_2CH_3$, R_1 = H

1g, R = H, R_1 = $COCH_2CH_2CH_3$



2, Bryostatin 8

subfamily) was collected in the Gulf of Mexico (Alligator Harbor, Franklin County, Florida). The same general area of the Northeastern Gulf of Mexico was the source of all later recollections. Taxonomic identification was generously provided by the Smithsonian Institution.

The initial sample of *Amathia convoluta* was preserved in isopropyl alcohol. Removal of solvent gave an extract that reached a confirmed level of activity against the National Cancer Institute's murine P388 lymphocytic leukemia (PS system) with a response of T/C 200 at 400 mg/kg. A hot ethanol extract of the residual animal gave material that exhibited PS T/C 125 at 200 mg/kg.

In 1970 approx. 50 kg (wet wt) of *Amathia convoluta* was collected during the spring and fall. The 2-propanol extract from a 4.6 kg specimen of the fall collection was concentrated and the residue partitioned between hexane and water. The aqueous phase was partitioned between $CHCl_3$ and water. After separation of the water phase it was extracted with $CHCl_3$, lyophilized and the residue was extracted with EtOH. By this means the hexane extract provided 120 g, the $CHCl_3$ 35 g, the EtOH 138 g and the water extract 387 g. The hexane fraction led to a confirmed level of activity against the Walker carcinosarcoma 256 system (T/C 41 at 200 mg/kg) and the $CHCl_3$ fraction displayed PS T/C 140 at 100 mg/kg.

The detailed chemical study of *Amathia convoluta* begun in 1970 was initiated using the Walker carcinosarcoma 256 as bioassay. Eventually this tumor system was abandoned in favor of the PS leukemia for bioassay purposes. By 1976 it was decided to recombine all fractions arising from the 1970 collections and partition between $CHCl_3$ and water. The $CHCl_3$ phase was successively partitioned between 9:1 MeOH:water–hexane, 4:1 MeOH:water–CCl_4 and 3:2 MeOH:water–CH_2Cl_2.[17] By this means the antineoplastic activity was concentrated in the CCl_4 fraction (98.5 g) and showed PS T/C 169 at 200 mg/kg and in the PS cell line ED_{50} 2.8 μg/ml. The results were reassuring and indicated that about 6 years of separation procedures had not destroyed the original antineoplastic activity. But it was clear that a solution to the problem would require another and larger recollection.

In the fall of 1978, 118 kg (wet wt) of *Amathia convoluta* was collected and preserved in 2-propanol. The resulting aqueous 2-propanol extract was concentrated to a 2.5 kg residue. A soln of the 2.5 kg extract in 9:1 MeOH–water was subjected to the partition sequence just described for the 1976 recombination to yield hexane (157 g), CCl_4 (130 g), CH_2Cl_2 (79 g) and MeOH–water fractions. Again the antineoplastic activity was concentrated in the CCl_4 fraction which led to PS T/C 141 at 40 mg/kg and in the PS cell line ED_{50} 7.8 μg/ml. Furthermore, the CCl_4 fraction exhibited a reasonable level of activity against the NCI KB (human nasopharynx carcinoma) cell line (ED_{50} 2.4 μg/ml). The CH_2Cl_2 fraction showed similar *in vitro* activity with the PS ED_{50} at 8 μg/ml and the KB ED_{50} at 3.3 μg/ml. The overall problem of uncovering the antineoplastic constituents came close to solution using the 1978 recollection and finally yielded, starting with a spring 1981 recollection amounting to 100 kg (wet wt) and a minimum of 45 kg (dry wt) of *Amathia convoluta*. The following approach to the 1981 recollection entails the most convenient and successful experimental methods.

Animal extraction. The aqueous 2-propanol extract of the 1981 recollection was concentrated and partitioned between CH_2Cl_2 and water. The residual animal was extracted with 1:1 CH_2Cl_2–MeOH.[18] Sufficient water was added to create two phases and the CH_2Cl_2 layer was separated and concentrated. The extraction procedure was repeated by adding sufficient MeOH to the MeOH–water and animal mixture to form a single phase. Again, water was added to separate the CH_2Cl_2 which was collected and concentrated.

Solvent partition sequence. The combined CH_2Cl_2 extract from the 1981 recollection was dissolved in 9:1 MeOH–water and extracted with hexane. The MeOH–water phase was diluted to 4:1 MeOH–water and extracted with CCl_4. The MeOH–water phase was diluted to 3:2 and extracted with CH_2Cl_2. The resulting hexane (469 g), CCl_4 (61 g), CH_2Cl_2 (45 g) and MeOH–water (91 g) fractions were concentrated and aliquots submitted for bioassay. Antineoplastic activity (PS T/C 182 at 25 mg/kg, ED_{50} 10 μg/ml) again resided in the CCl_4 fraction.

After the principal antineoplastic constituents of *Amathia convoluta* were isolated in 1982 and early 1983 and found to be bryostatins, we very carefully investigated some of the recent recollections for presence of the closely related bryozoan, *Bugula neritina*. Close inspection uncovered contamination of less than 0.2% to some 3% corresponding to *Bugula neritina*. The contaminating species was found growing on the *Amathia convoluta* in an epiphytic manner. The 1981 recollection that forms the basis of all subsequent experiments contained approx. 2.5% *Bugula neritina*.

Isolation of the bryostatins. In a typical series of experiments the 61 g active CCl_4 fraction (Separation Scheme Part 1) in 2:3 CH_2Cl_2–MeOH was chromatographed on a column of Sephadex LH-20 (10 × 120 cm). The procedure was repeated until the total (61 g) CCl_4 fraction was separated by this gel permeation procedure. Combination of similar fractions provided the A–F series noted in Separation Scheme Part 1. The principal *in vivo* active fraction (D, 38.9 g) was further separated by another application of the Sephadex LH-20 procedure using 2:3 CH_2Cl_2–MeOH. With a 36 g amount of fraction D aliquots of 20 ml were collected. Recombination of fractions on the basis of TLC properties led to fractions G–L (Separation Scheme Part 2). Fraction H (4.33 g) was next separated by a dry column chromatographic procedure on silica gel using gradient elution from CH_2Cl_2 to CH_2Cl_2–MeOH mixtures. By means of 12 ml fractions a total of 480 ml was collected and of these a fraction noted as M on Separation Scheme Part 2 was found to give the highest level of antineoplastic activity. Extensive separation of fraction M by further silica gel column chromatography in gradients ranging from n-hexane–EtOAc (3:1 → 1:1) → n-hexane–acetone (5:1 → 1:1) mixtures in 7 ml fractions (280 ml total) afforded active fraction N (37.5 mg). By preparative TLC in CH_2Cl_2–MeOH–water (90:10:0.8) the main PS active fraction was isolated and found to weigh 18.2 mg (fraction O). Although fraction O contained a majority of the active constituents emanating from fraction H it was still quite complex and required extensive HPLC methods for separation. A soln of fraction O in 4:1 MeOH–water was chromatographed using HPLC techniques with a C-18 reverse phase column and a flow rate of 1.0 ml/min with a gradient to methanol. The fraction with most promising PS activity was further separated by HPLC using a silica gel column and EtOAc–heptane–MeOH–water (400:600:10:1.5) as mobile phase with a flow rate of 1.0 ml/min. By this means 1.6 mg of **1a** and 0.6 mg of **1b** was isolated.[23]

Separation of the gel permeation fractions G and I was even more challenging. Both fractions were combined and the 27.56 g combined fraction (Separation Scheme Part 3) was first separated as summarized for fractions H → M to yield PS active fraction P (979 mg). At this stage it was found best to utilize a partition chromatographic procedure employing Sephadex LH-20 and the solvent system n-hexane–CH_2Cl_2–MeOH (10:10:1). A 3 cm × 120 cm sized column was used and 12 ml fractions totalling 240 ml were collected. The resulting most active fraction (Q, 266 mg) was further fractionated as noted above for fraction M → N. The remaining separation steps were accomplished by the same general procedures employed with fraction O (Separation Scheme Part 2). In order of elution appearance from the HPLC silica gel, were obtained **1a** (6.0 mg), **1c** (3.1 mg), **1b** (5.7 mg) and **2** (4.2 mg). Bryostatins 4 and 6 were recently isolated from *Bugula neritina* in our laboratory and structural determinations have been completed.[20,21] Presumably these substances are derived from the association of *Amathia convoluta* with *Bugula neritina*. However, **2** appears to be a genuine constituent of *Amathia convoluta* and was characterized as follows.

Bryostatin 8 (**2**). Bryostatin 8 (**2**) was brought to analytical purity by rechromatography of the original 4.2 mg on a HPLC reverse phase C-18 column with MeOH–water (from 1:1 to 9:1) as eluent. The resulting 2.8 mg of bryostatin 8 was obtained as a colorless amorphous powder melting at 170–173°: $[\alpha]_D^{27}$ + 49.9 (*c*, 0.04, CH_3OH); uv λ_{max}^{MeOH} 226 (ε37,500); ir ν_{max}^{KBr} 3551, 3475, 3415, 2975–2950, 1742, 1721, 1640, 1616, 1449, 1380, 1245, 1225, 1165, 1095, 1075, 1050 and 870 cm^{-1}; MS: 903 $[M + Na]^+$ and 815 $[M + Na-88]^+$. The 400 MHz proton and carbon magnetic resonance data has been displayed in Tables 1 and 2 in comparison with **1d.**

Acid-catalyzed hydrolysis of bryostatin 8 (**2**) *to monobutyrate esters* **1f** *and* **1g.** An 0.8 mg specimen of **2** was hydrolyzed (24 hr at room temp) in 0.2 ml of 1% HCl in

MeOH. The products (0.5 mg, **1f** and **1g**) were isolated by HPLC reverse phase column (C-18) chromatography with MeOH–water (from 1:1 to 9:1). Results of the soln phase secondary ion mass and high resolution ^{1}H-NMR (400 MHz) spectral studies of the hydrolysis product have been recorded in the discussion section.

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