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ANTINEOPLASTIC AGENTS, 116. AN EVALUATION OF THE MARINE ASCIDIAN *APLIDIUM CALIFORNICUM*[1]

GEORGE R. PETTIT, JOHN E. LEET, CHERRY L. HERALD, YOSHIAKI KAMANO, and DENNIS L. DOUBEK

Cancer Research Institute and Department of Chemistry, Arizona State University, Tempe, Arizona 85287

ABSTRACT.—The antineoplastic constituents of the marine ascidian *Aplidium californicum* were found to be bryostatins 4 and 5. Bioassay-directed isolation procedures using the National Cancer Institute's P-388 lymphocytic leukemia system led to the bryostatins. The probable symbiotic relationship between *A. californicum* and the bryozoan *Bugula neritina* was discussed.

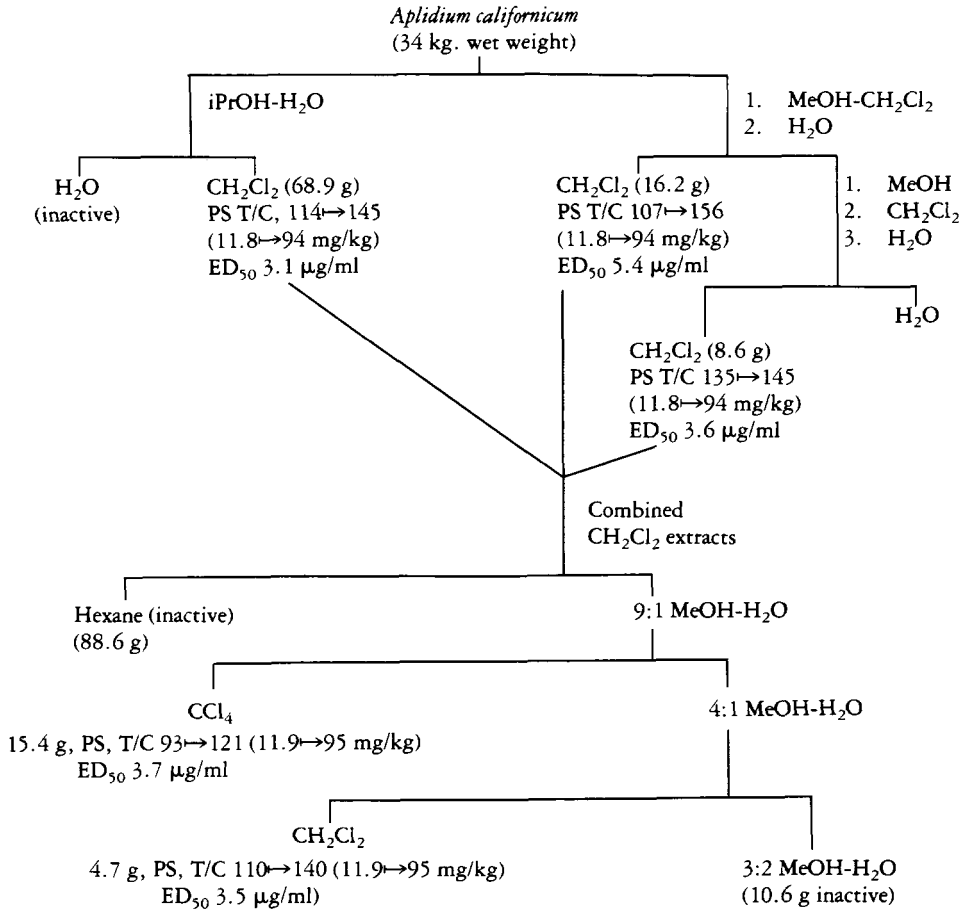
Among the marine invertebrates, tunicates of the subphylum Urochordata most clearly resemble our Vertebrata subphylum. They have a dorsal tubular nerve cord that terminates in an enlarged anterior end forming a single brain. Members of the class Ascidiacea (sea squirts) originate from fertilized eggs (2), representing a quite interesting group of organisms for biological and chemical studies. Indeed, during our initial (1965-1968) worldwide evaluation of marine animals as sources of new anticancer substances, we uncovered the first tunicates (e.g., *Styela plicata*) with such constituents (3). On this Institute's 1976 expedition to the coast of Honduras, we located a very promising [NCI confirmed active, T/C 173 (9.37 mg/kg)] tunicate of the *Tridemnum* genus that was later found independently by A.J. Weinheimer and colleagues to yield potent antineoplastic cyclic depsipeptides (the didemnins).[2] More recently, three other tunicates have provided a series of antimicrobial and/or cell growth inhibitory substances (6-10).

In early 1973, we collected the Gulf of California ascidian *Aplidium californicum* Ritter and Forsyth for antineoplastic evaluation. *A. californicum* is found as yellow-to-orange pulpy masses (sea pork) growing on the submerged portions of docks and other hard substrates exposed to strong tidal currents. Extracts of this animal reached the confirmed active stage during 1975 in the U.S. National Cancer Institute's murine P-388 lymphocytic leukemia (PS system), affording a 68% increase in life span at a dose of 60 mg/kg. Four re-collections of the tunicate made from 1976-1982 gave quite variable biological results ranging from loss of activity to the 1978 sample with good results (approximately that of the original specimens). While this investigation was in progress, Howard and co-workers (11) found San Francisco Bay (California) specimens of *A. californicum* to yield prenylhydroquinone (38% life extension against the PS system at 3.1 mg/kg) and the related chromenol. Both substances had been isolated previously from a terrestrial plant and were found to be anticarcinogenic. Now we will summarize our conclusions reached employing the 1978 re-collection of *A. californicum*.

Extracts from the 1978 re-collection gave a 45% life extension increase against the PS leukemia and were considered sufficiently active to complete the study of *A. californicum*. A combination of results from the previous investigation with some of the best experimental techniques (13) we had developed to that date for solving such challenging problems led to the solution as outlined in Separation Scheme Parts 1-3 and discussed in the Experimental section. The thin-layer chromatogram of fraction T uncovered potential antineoplastic components that responded to ultraviolet light and gave a reddish-purple color upon development with anisaldehyde-HOAc-H_2SO_4. We recognized such behavior as reminiscent of the bryostatins (14), and this was confirmed by

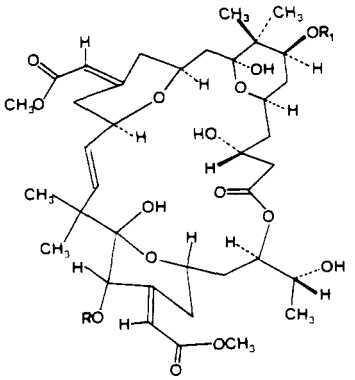
[1]For the preceding contribution, see Pettit and Nelson (1).

[2]The structures of didemnins A-C have been proposed: Rinehart *et al.* (4,5).



Separation Scheme Part I

isolation of the strongly PS-active bryostatins 4 (**1a**) and 5 (**1b**). Both constituents appeared to account for the principal antitumor activity and were identical with authentic specimens previously isolated (14,15) in our laboratory from the marine Bryozoan *Bugula neritina*. Against the PS leukemia bryostatin 4 provided 62% life extension at very low doses (46↦92 μg/kg), and similarly, bryostatin 5 gave 45↦88% life extension at 46↦185 μg/kg.



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)

Combined CCl_4-CH_2Cl_2 Fractions
(20.1 g)

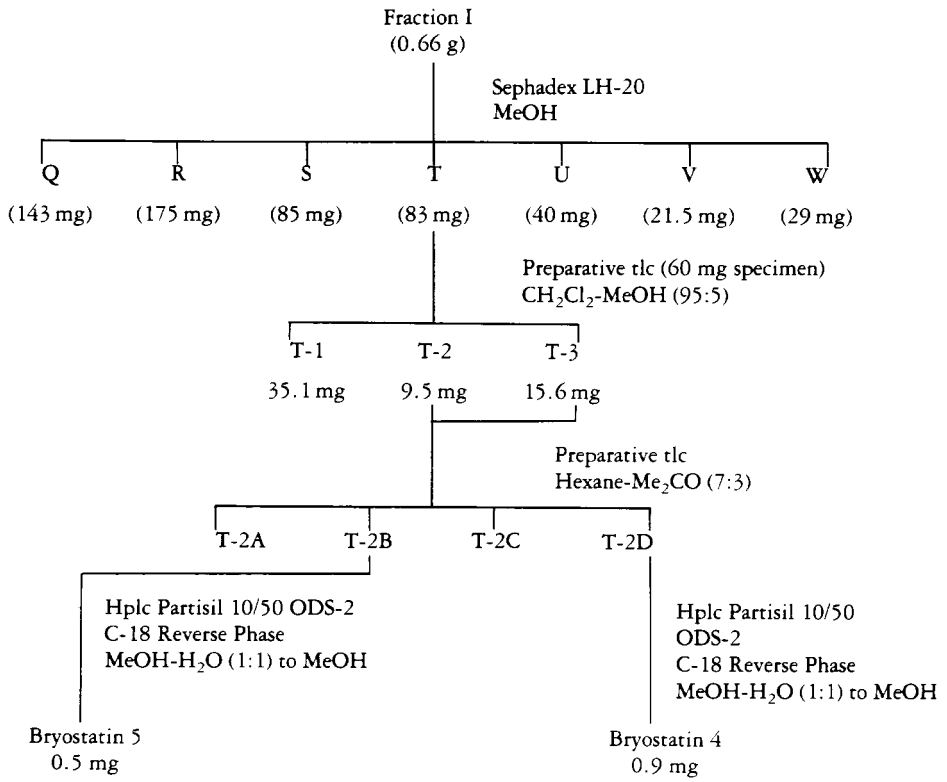
Sephadex LH-20
CH_2Cl_2-MeOH (1:1)

	A	B	C	D	E	F	G
Wt (g)	(6.2)	(3.0)	(2.2)	(1.2)	(1.2)	(0.5)	(0.8)
PS ED_{50}	33	6.2	10	34	30	2.4	29
T/C	102-107	125-163	100-102	93-102	93-100	100-102	98-109
(mg/kg)	(3.1-25)	(3.1-25)	(3.1-25)	(3.1-25)	(3.1-25)	(3.1-25)	(3.1-25)

Sephadex LH-20
Hexane-$CHCl_3$-MeOH (10:10:1)

	H	I	J	K	L	M	N	O	P
Wt (g)	(0.53)	(0.66)	(0.52)	(0.24)	(0.26)	(0.17)	(0.11)	(0.10)	(0.42)
PS ED_{50}	100	6.5	14.5	25	23.5	26	20	23	0.07
T/C	95-104	152-177	110-129	104-121	104-127				118-toxic
(mg/kg)	(4.2-34)	(4-16)	(4.7-38)	(4.1-33)	(4.5-36)				(4.4-35)

Separation Scheme Part 2



Separation Scheme Part 3

Near the time bryostatins 4 and 5 were discovered in material from *A. californicum*, we found these very interesting substances in extracts of the marine Bryozoan *Amathia convoluta* invaded by *B. neritina* (13). A careful examination of the museum specimens preserved from the *A. californicum* re-collections revealed that all contained small quantities of interstitial *B. neritina* undetected during initial cleaning and processing of the original animal collection. Because ascidians continue to secrete an external connective tissue from living cells passing out through the epidermis, it is not clear whether the *B. neritina* invaded *Aplidium* or was encased in the growing skin (a tunic or test). The anthropod *Polycheria osborni* invades *A. californicum* by burrrowing into the tests (2), but any invasion route by *B. neritina* would probably involve passage with the feeding water from pharynx to atrium. Whatever the mechanism, for defensive purposes *Aplidium* may enjoy its relationship with *Bugula*. Indeed, the luxuriant growth of *A. californicum* at the Bahia Kino, Sonora, collection site suggests a symbiotic relationship with *B. neritina*.

As emphasized when summarizing the *A. convoluta* research, *B. neritina* contains very potent antineoplastic constituents with the capacity to intrude upon or otherwise become associated with certain other marine organisms (13). Therefore, the possibility of *Bugula* contamination should be considered prior to selecting a new marine animal for detailed chemical study directed at isolation of possible antineoplastic constituents.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Summaries of these general methods appear in introductions to the experimental sections of our prior contributions (13-15) concerned with the isolation and structural elucidation of bryostatins 4 and 5.

ANIMAL COLLECTION AND INITIAL SEPARATION.—The initial collection (March 12, 1973) of *A. californicum* (Chordata phylum, Urochordata subphylum, Ascidiacea class, Enterogona order, Aplousobranchia suborder, and Polyclinidae family) (12) was made by one of us (GRP) assisted by M.J. Pettit, Dr. C.G. Zubrod, and K. Zubrod in an estuary about 2 mi southeast of Bahia Kino, Sonora, Mexico. Re-collections were obtained from the same location in March 1976, January 1977, May 1978, and January 1982, assisted at various times by Drs. P. Brown, D. Doubek, P. Lohavanijaya, and R. Ode and by G.C. Bryan, G.R. Pettit, III, M.S. Pettit, L.D. Vanell, and L. Wakeford. In each case, the animal was preserved in iPrOH, and the 34 kg (wet wt) 1978 re-collection was subjected to extraction and separation guided by bioassay (PS system) as summarized in Scheme 1. Details of the approach to extraction and elimination of inactive material appear in Pettit *et al.* (13).

The taxonomic study of *A. californicum* was completed by Dr. Ivan Goodbody, and we very much appreciate this contribution to the research. Authentic specimens of this tunicate and *B. neritina* are also maintained in our Institute.

ISOLATION OF BRYOSTATINS 4 AND 5.—The CCl_4 and CH_2Cl_2 fractions (see Scheme 1) were combined (20.1 g) and chromatographed on a column of Sephadex LH-20 (4.8×234 cm), using CH_2Cl_2-MeOH (1:1) as eluting solvent. Fractions measuring 12 ml were collected at the rate of 60 ml/h. Examination by tlc and combination of similar fractions provided seven sets (A-G) for biological (PS) evaluation. The principal active fraction (B, 3.0 g, PS T/C 163 at 25 mg/kg, ED_{50} 6.2 μg/ml) was further separated by partition chromatography (3.5×115 cm column) on Sephadex LH-20 using hexane-$CHCl_3$-MeOH (10:10:1) as eluent (Scheme 2). Fractions of 13 ml each were collected and consolidated on the basis of tlc properties. In this manner, fractions H-P were obtained. The principal in vivo active fraction (I, 0.66 g, PS T/C 177 at 16.8 mg/kg) was further fractionated in MeOH on a column of Sephadex LH-20 (3×150 cm). Combination of like fractions provided the series Q-W. A thin-layer chromatogram of fraction T (60 mg), in particular, revealed a uv light responsive component that gave a reddish-purple color reminiscent of the bryostatins (13-15) upon development with anisaldehyde-HOAc-H_2SO_4 reagent. Fraction T was subjected to preparative tlc using CH_2Cl_2-MeOH (95:5). The uv active zones (T-2, 9.5 mg and T-3, 15.6 mg) were collected, recombined, and subjected to preparative tlc using hexane-Me_2CO (7:3) as the developing solvent. After developing the plate (50 mm Whatman PK5F Silica Gel) three consecutive times, the resulting well-separated uv active bands (T-2-B, 1 mg and T-2-D, 1 mg) were collected. Analytical tlc using hexane-Me_2CO (7:3) still revealed the presence of impurities in T-2-B and T-2-D. A solution of fraction T-2-D in MeOH was chromatographed using hplc on a C-18 reverse phase column. Elution was begun with MeOH-H_2O (1:1) at a flow rate of 2.0 ml/min with a gradient to MeOH. In this manner, 0.9 mg of bryo-

statin 4 was obtained. Fraction T-2-B was purified using identical hplc procedures to afford bryostatin 5 (0.5 mg). Bryostatins 4 and 5 were identified by spectral methods [principally sp-sims (16,17) ms (13-15), and by co-tlc with authentic samples using two different solvent systems (Scheme 3)].

BRYOSTATIN 4.—$C_{46}H_{70}O_{17}$; ms (sp-sims) using 0.15 M NaI in sulfolane: *m/z* 917 $[M+Na]^+$, 901 $[M+Na-OH+H]^+$, 859 $[M+Na\text{-}COOCH_3+H]^+$, 844 $[M+Na-COCH_2CH_2CH_3\text{-}2H]^+$, 830 $[M+Na-OCOCH_2CH_2CH_3]^+$, 813 $[M+Na-OCOCH_2CH(CH_3)_2-3H]^+$; Rf 0.22 (hexane-$Me_2CO$, 7:3), 0.46 (EtOAc-hexane, 6:4 v/v).

BRYOSTATIN 5.—$C_{44}H_{66}O_{17}$; ms (sp-sims) using 0.15 M NaI in sulfolane: *m/z* 889 $[M+Na]^+$, 844 $[M+Na-COCH_3-2H]^+$, 830 $[M+Na-OCOCH_3]^+$, 785 $[M+Na-OCOCH_2CH(CH_3)_2-3H]^+$; Rf 0.14 (hexane-$Me_2CO$, 7:3), 0.38 (EtOAc-hexane, 6:4 v/v).

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