· Original article ·

Studies on antineoplastic constituents from marine bryozoan *Bugula neritina* inhabiting South China Sea: isolation and structural elucidation of a novel macrolide

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[**ABSTRACT**] **Objective**: To investigate the antineoplastic constituents from marine bryozoan *Bugula neritina* in South China Sea. **Methods**: Antineoplastic compounds were screened by bioassay-guided method with partitive extraction and multiple column chromatographies (Sephadex LH-20, ODS and preparative HPLC). Structures of active compounds were determined by high-resolution 2D NMR (600 MHz) and ESI-MS techniques. **Results**: A new antineoplastic macrolide bryostatin 19(**1**) was isolated from this animal. Five known antineoplastic bryostatins: bryostatin 4(**2**), bryostatin 5(**3**), bryostatin 6(**4**), bryostatin 18(**5**) and bryostatin 10(**6**), were for the first time obtained from this bryozoan in South China Sea. Bryostatin 19(**1**) shows strong inhibition activity against U937 cell line with ED_{50} value being 3.2 nmol/L *in vitro*. **Conclusion**: The animal resource of *Bugula neritina* is abundant in South China Sea. It contains potential antineoplastic new macrolide of bryostatin series and will be developed as potentially useful source of anticancer agents in the future.

[**KEY WORDS**] *Bugula neritina*; antineoplastic drugs; bryostatins

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The marine bryozoan *Bugula neritina* is a common fouling organism. Its potential medicinal value was not discovered until 1968 when Pettit *et al*[1] found that certain Bryozoa members contained potential antineoplastic constituents. After a long-term study on antineoplastic constituents from this animal, an encouraging progress was achieved in 1981 by the isolation and structure elucidation of bryostatin 1[2]. From then on, Pettit's group sharply focused their research efforts on uncovering the marine animal constituents for potential use in cancer chemotherapy. Their 15-year endeavor led to the isolation of bryostatin 2-18 and neristatin A and B from *Bugula neritina*[3,4].

In our initial studies, we found *Bugula neritina* on the south coast of China was very rich. The author initiated an extensive investigation of antineoplastic constituents from this animal collected off Dapen Bay in South China Sea. In the present work, a new macrolide named bryostatin 19 together with 5 known bryostatins were isolated from this animal in the course of a large-scale isolation of bryostatins. We now wish to further contribute to this emerging subject by describing the structure of bryostatin 19(**1**).

1 MATERIALS AND METHODS

1.1 Animal material About 100 kg (dry weight) of specimens were collected off Nanwan Bay (Guangxi province) in March 1997 at a depth of 0-1.5 m. Taxonomic identification was performed by professor LI Chuan-Yan (Third Institute of Oceanography, National Bureau of Oceanography, China). A voucher specimen of the animal in this study was maintained in our labrary.

1.2 General experimental procedures Optical rotation was measured on a Perkin-Elmer MC-241 polarimeter. Melting points were determined on a ZMD-H melting point apparatus and were uncorrected. UV was carried out on a Varian Cary 300

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Bio instrument. Infrared spectra were obtained on a SIMADUZO infrared spectrophotometer in KBr pellets. The NMR experiments were conducted with a Bruker Am-600 instrument ($CDCl_3$ as solvent); chemical shift values were in ppm(δ) with TMS as internal standard. The ESI-MS was acquired with a Micromass Quattro mass spectrum instrument.

Sephadex LH-20(25-100 μm) for gel permeation and partition chromatography was obtained from Pharmacia Fine Chemicals, AB, Uppsala, Sweden. Reversed phase flash column chromatographic separations were carried out by ODS (200-300 mesh), performed by FUJI Chem. Other ambient-pressure column chromatographic separations were performed with silica gel 60(70-230 μm, or 200-300 mesh) supplied by Qingdao Ocean Chem-factory. Medium pressure liquid chromatography(MPLC) was run on a Lobar column packed with RP-C_{18} silica(40-63 μm). TLC analyses were performed on Merk TLC plates. Component positions were visualized 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. The preparative HPLC separation was performed on a Whatman Partisil-10 M-9-ODS-2(C_{18}) HPLC column(9.4 mm×500 mm) with 80% MeOH in H_2O. The HPLC instrument was equipped with a Waters UV PDA 996 detector(set for 228 nm from the range 200-400 nm), a Waters 510 pump, and a Millennium 2000 data station. All solvents were AR grade.

1.3 Extraction and purification Fresh animals (100 kg, dry weight after extraction) were extracted with 95% EtOH(4×500 L) at room temperature with each extraction taking one week. The extract solution was filtered and concentrated in vacuum to get aqueous extracts(2.0 kg), which were suspended into 9 : 1 methanol-water solution, and extracted with *n*-hexane(10 L) for 5 times. The *n*-hexane phase was separated and concentrated in vacuum to get 560 g *n*-hexane extracts(no activivity, HL-60). The methanol-water phase was adjusted to be a concentration of 4 : 1 and partitioned with carbon tetrachloride(10 L) for 5 times. The carbon tetrachloride phase was removed and concentrated in vacuum to get an active portion(60 g, IC_{50} = 0.7 μg/ml, HL-60).

A bryostatin-containing fraction 2BH-10(2.7 g, IC_{50} = 0.024 μg/ml, HL-60) was obtained from the silica gel flash column chromatography with SiO_2(200-300 mesh) using CH_2Cl_2→MeOH-CH_2Cl_2 (1 : 9) as eluting solvent. 2BH-10 was subjected to chromatography on a column of Sephadex LH-20 (3.8 cm×110 cm), using CH_2Cl_2-MeOH(1 : 1) as eluting solvent. Fractions(12 ml) were collected at the rate of 60 ml/h. This procedure caused a rapid concentration in fraction A(1.7 g, IC_{50} = 0.003 μg/ml, HL-60) and B(1.1 g, IC_{50} = 0.008 μg/ml, HL-60) of the major active compounds. Further gel permeation-partition type chromatographic separation employing Sephadex LH-20 with *n*-hexane-CH_2Cl_2-MeOH(4 : 5 : 1) and (10 : 10 : 1) as eluting solvent led to the more pure and more active bryostatin-containing fractions of C(0.7 g, IC_{50} = 0.001 μg/ml, HL-60) and D(0.5 g, IC_{50} = 8×10^{-4} μg/ml, HL-60). These 2 fractions still contained some kinds of green pigments. Therefore, C and D were combined for further separation. By intensive ODS flash column chromatographic method with solvent gradient of 50%→80% MeOH, a major bryostatin-containing fraction E(0.8 g, IC_{50} = 2.4×10^{-4} μg/ml, HL-60) was selected for further purification. The following silica gel column chromatography using *n*-hexane-acetone(5 : 1 : 1 : 1) as eluting solvent led to 2 fractions F(0.4 g) and G (0.2 g). F was subjected to HPLC using a Partisil C_{18} reverse phase column with 83% MeOH as eluting solvent. In this manner, compounds **1**(5 mg, 5×10^{-7}% of dry specimen weight), **2**(10 mg), **3** (65 mg) and **5**(50 mg) were obtained. G was still challenging mixtures that need further purification before HPLC separations. As a result of Lobar MPLC method with solvent gradient of 50%→80% MeOH, G1(5 mg), G2(12 mg) and G3(34 mg) were obtained. G1 was subjected to preparative HPLC using a Partisil C_{18} reverse phase column with 75% MeOH as eluting solvent, com-

pound **4**(2.1 mg) were obtained. G2 and G3 was subjected to preparative HPLC using a Partisil C_{18} reverse phase column with 85% MeOH as eluting solvent, compound **6**(5.8 mg) were obtained.

2 RESULTS AND DISCUSSION

2.1 Structural elucidation

2.1.1 *Bryostatin* 19(1) Bryostatin 19(1) was obtained as an amorphous powder, m. p. 140-142 C, $[\alpha]_D^{25}$ + 28°(*c* 0.1, MeOH). The ESI mass spectrum of **1**(*m/z* 902[M+Na+H]$^+$, 918[M+K+H]$^+$) and its ultimate analysis indicated the formula of $C_{45}H_{66}O_{17}$. The UV absorption maximum at 228 nm was indicative of the presence of an α,β-unsaturated ester moiety. The ^{1}H and ^{13}C NMR chemical shifts of **1** were listed in Tab 1.

Tab 1 ^{1}HNMR(600 MHz) and ^{13}CNMR(150 MHz)data[a] for bryostatin 19(1) in $CDCl_3$[b]

Position	^{1}HNMR	^{13}CNMR	Position	^{1}HNMR	^{13}CNMR
1	/	172.51	21	/	166.98
2[a]	2.53 t(12.2)	41.93	22	4.47 d(8.9)	81.34
2[b]	2.47 dd(12.2,2.6)		23	3.76 ddd(11.6,9.2,2.4)	69.06
3	4.06 m	68.32	24[a]	2.37 m	32.95
3-OH	4.34 brd		24[b]	1.88 m	
4[a]	2.00 t(12.5)	39.38	25	5.06 ddd(9.2,5.3,3)	72.87
4[b]	1.60 brd(15)		26	3.86 m(overlapped)	69.57
5	4.22 brt(11.6)	65.77	27	1.23 d(6.6)	19.19
6ax	1.44 q(12.0)	33.11	28	1.036 s	17.04
6eq	1.72 m		29	0.95 s	21.03
7	5.13 dd(11.9,4.6)	72.27	30	5.17 s	114.61
8	/	41.28	31	/	166.76
9	/	101.80	32	1.15 s	21.21
10[a]	2.10 dd(14.8,7.9)	42.12	33	1.042 s	24.49
10[b]	1.67 d(15.2)		34	5.85 s	114.30
11	3.86 brt(6.3)	71.25	35	/	171.90
12ax	2.19 t(12.5)	44.01	36	3.71 s	51.10
12eq	2.09 d(14.5)		1'	/	177.96
13	/	156.23	2'	/	39.04
14ax	1.90 m(overlapped)	36.22	3'	1.20 s	27.16
14eq	3.69 brd(14)		4'	1.20 s	27.16
15	4.15ddd(11.3,8.5,2.7)	78.17	5'	1.20 s	27.16
16	5.43 dd(15.8,8.3)	132.58	1''	/	172.11
17	5.72 d(15.5)	136.37	2''	2.43 t(7.6)	36.13
18	/	45.24	3''	1.70 m(overlapped)	19.19
19	/	101.57	4''	0.98 t(7.6)	13.74
20	5.85(overlapped)	68.61			

[a]: All the signals were assigned by DQF-COSY, TOCSY, HMQC and NOESY spectra; [b]: *J* values(in parentheses) were reported in Hz

^{1}HNMR spectrum revealed the presence of 2 olifinic protons at δ 5.43(dd, J = 15.8, 8.3 Hz) and δ 5.72(d, J = 15.5 Hz), which were unambiguously assigned to H-16 and H-17 respectively. The DQF-COSY experiments gave straightforward connectivities from C-17 to C-14ax. In the same manner, several spin systems could also be found, such as H-2-3-4-5-6-7 and H-10-11-12, by the combination of the TOCSY experiments. The above date indicated **1** consisted of the same pryopran ring with other bryostatins. Nevertheless, **1** was characterized by the presence of only one methoxyl group(δ 3.71, 3 H; 51.1, single cross peak in the corresponding HMQC spectrum) as distinguished from other bryostatins except bryostatin 3 showing 2 methoxyl groups[5]. The correlation peak between δ 3.71(H-36) and 3.69(H-14eq) in the NOESY spectrum indicated that the methoxyl group located on the C-11-C-15 pyran ring. In addition, the major structural difference between **1** and other bryostatins had to mostly involve the C-19-C-23 pyran ring. In the IR spectrum of **1**, the new infrared absorption

band at 1 795 cm^{-1} indicated the presence of a 5 or 6 membered lactone carbonyl group. The coupling signals in the DQF-COSY and TOCSY spectra led to the identification of spin systems of H-17, H-16, H-15, H-14ax as well as H-12ax, H-11, H-10a and H-2, H-3, H-4, H-5, H-6, H-7. This assignment indicated that **1** shared the same fragments C-2-3-4-5-6-7, C10-11-12 and C14-15-16-17 with the other known bryostatins. Obviously, the proton doublet at δ 1.23 (3 H, d, J = 6.6 Hz) was unambiguously assigned to H-27. In the DQF-COSY spectrum, the C-27 methyl protons were correlated to an oxygenated methine (δ 3.86, 26-H), which was in turn coupled to an oxygenated methine at C-25 (δ 5.06, 25-H). This oxygenated proton was in turn correlated to a methylene at C-24 (δ 1.88, 2.37), which coupled to another oxygenated methine at C-23 (δ 3.76, 23-H). H-23 coupled to an oxygenated methine at C-22 (δ 4.47, 22-H), which showed allylic coupling to an olifinic proton at C-34 (δ 5.85, 34-H). Therefore, the spin system including H-27, H-26, H-25, H-24, H-23, H-22 and H-34 was also identified in the DQF-COSY and TOCSY spectra. Furthermore, the proton signal at δ 4.47 (1 H, d, J = 8.9 Hz, H-22) showed the cross-peak with the corresponding carbon signal at δ 81.34 (C-22) in the HMQC spectrum, which suggested an oxygen-containing group attached at C-22 directly. The above data indicated that **1** and bryostatin 3[5] shared the same skeleton structure. The ^{1}H and ^{13}C NMR chemical shifts including H-1-H-36 and C-1-C-36 were almost the same, which confirmed the same skeleton structure of these 2 bryostatins. The difference between the 2 bryostatins was mainly due to the side chains at C-7 and C-20. A pivalate was identified from the strong singlet at δ 1.20 with 9 protons in the ^{1}HNMR spectrum of **1**. The chemical shift of H-7 at δ 5.13 suggested the pivalate group attached at C-7, which was confirmed by the strong cross peak of δ 1.20/5.13 (H3'-5'/H7) in the NOESY spectrum of **1**. In the upfield of ^{1}HNMR, the signal at δ 0.98 (3 H, t, J = 7.6 Hz) was unambiguously assigned to H-4″. The cross peaks between H-4″, H-3″ and between H-3″, H-2″ at δ 0.98/1.70 and 1.70/ 2.43 were readily observed in the DQF-COSY spectrum of **1**, which suggested another side chain was butyrate ester. The proton signal in the downfield at δ 5.85 (1 H, s, H-20) suggested the butyrate ester side chain attached at C-20, which was confirmed by the cross peak between δ 1.70 (2 H, m, H-3″) and 5.85 (1 H, s, H-20) in the NOESY spectrum of **1**.

2.1.2 *Relative stereochemistry of bryostatin* 19(**1**)

2.1.2.1 C-14-C-17 fragment The E geometry of the $\Delta^{16,17}$ double bond was derived from the large vicinal coupling constant of 15.8 Hz between H-16 and H-17. H-15 was suggested to be axial orientation mainly based on the observation of vicinal constants of 8.5 and 11.3 Hz between H-15 and H-16, H-15 and H-14ax, respectively, which was confirmed by the NOESY spectrum (cross peak H-17 and H-15). Interpretation of the DQF-COSY spectrum revealed H-4eq resonated at downfield δ 3.69, whereas H-14ax resonated at upfield δ 1.90. The high nonequivalence of the methylene at C-14 was diagnostic in confirming the configuration of the α, β-unsaturated methyl ester attached at C-13. The chemical shift of equatorial proton at C-14 strongly downfields to δ 3.69 owing to 2 reasons. One is H-14eq lays outside the shielding cone of the unsaturated ester, whereas H-14ax lays inside the shielding cone of the double bond. Another may be the effect of the carbonyl group at C-31 to H-14eq or the NOE effect between the methoxyl group at the unsaturated methyl ester and H-14ax.

2.1.2.2 C-2-C-7 fragment A large coupling constant (11.6 Hz) between H-5 and H-6ax and a NOESY correlation between H-5 and H-6eq indicated that H-5 was in an axial arrangement. In the same manner, H-7 was suggested to be axial orientation by the observation of a NOESY correlation between H-7 and H-5. There was no NOESY correlation between H-3 and H-5, which suggested the opposite arrangement of these 2 protons and confirmed the orientation of the hydroxyl group at C-3.

2.1.2.3 C-22-C-27 fragment Coupling constants of the H-23 (J = 11.6, 9.2, 2.4) were consistent with the axial orientation of H-23, whereas the $J_{22\text{-}23}$ value of 8.9 Hz was in agreement with trans-rela-

tionship between H-22 and H-23. No NOESY correlation was found between H-23 and H-25, which suggested the opposite arrangement of these 2 protons. The orientation of H-25 was also confirmed by the observation of a NOESY correlation between H-25 and H-17.

2.1.2.4 Four geminal methyl groups at C-8 and C-18 In the upfield of ^{1}HNMR, 4 singlets were suggested to be the signals of the geminal methyl groups (C-28, C-29 and C-32, C-33). The unambiguous assignment of these geminal methyl groups was achieved by NOESY spectrum. In the NOESY spectrum of 1, the cross peaks of δ 1.15/5.43 (H-16) and δ 1.042/5.72 (H-17) were consistent with the assignments of the C-32 methyl at δ 1.15 and the C-33 methyl at δ 1.042. In the same manner, the singlet at δ 1.036 was assigned to the C-28 methyl according to the cross peaks between δ 1.036/1.44 (H-6β), δ 1.036/2.19 (H-10ax) and δ 1.036/1.20 (9-H, pivilate), whereas the singlet at δ 0.95 was assigned to the C-29 methyl by the observation of the cross peak of δ 0.95/5.13 (H-7), δ 0.95/1.72 (H-6eq) and δ 0.95/2.09 (H-10eq).

2.1.2.5 The unique orientation of H-20 In the ^{1}HNMR of 1, the signals of H-20 and H-34 were overlapped at δ 5.85, which made it difficult to determine the relationship of some cross peaks in the NOESY spectrum. This problem was settled by intensive interpretation of the NOESY cross peak. The axial H-20 (δ 4.47) was correlated with δ 5.85 (H-20 or H-34) in the NOESY spectrum of 1, which indicated that H-20/H-34 or H-20/H-22 had NOE efforts. The configuration of H-22 had been settled by the large coupling constant (J = 8.9 Hz) with the adjacent axial proton (H-23). It seemed to be impossible for H-22 and H-34 to have NOE correlation, owing to the long distance between these 2 protons. Therefore, the cross peak at δ 4.47/5.85 was assigned to be the NOE correlation between H-22 and H-20, which indicated the axial orientation of H-20 (Fig 1). The configuration of H-20 was also confirmed by the NOE correlations between H-20 and H-33 as well as H-34 and H-32 at δ 5.85/1.042 and δ 5.85/1.15, respectively. The axial orientation of H-20 was unique in the bryostatin series. All NOESY correlations were on full agreement with the assigned stereochemistry and 3D solution structure. The structure of **1** was established and named bryostatin 19.

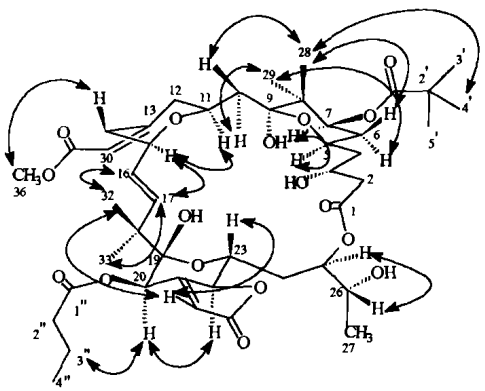

Fig 1 Summary of NOESY correlations for bryostatin 19(1)

t_m 800 ms, $CDCl_3$ (Geminal and some vicinal correlations were omitted for clarity)

2.1.3 *Bryostatin* 4(**2**) A white amorphous powder, m. p. 198-200 °C; $[\alpha]_D^{25}$ +91.8° (c 0.1, MeOH); UV (MeOH) γ_{max} (log ε) 228.7 (4.32) nm; ESI-MS m/z 918 $[M+Na+H]^+$, 1814 $[2M+Na+3H]^+$; IR (KBr, cm^{-1}) ν_{max} 3 500 (OH), 2 980-2 945, 1 740 (COOR), 1 660 (C=C), 1 440, 1 390, 1 370 (CH_3), 1 290, 1 260, 1 160-1 170 (COOR), 1 100, 1 080, 1 060, 1 000, 860 (CH_3) and 800. ^{1}HNMR (600 MHz, $CDCl_3$) and ^{13}CNMR (150 MHz, $CDCl_3$), see ref 6.

2.1.4 *Bryostatin* 5(**3**) A white amorphous powder, m. p. 168-172 °C; $[\alpha]_D^{25}$ +110.4° (c 0.1, MeOH); UV (MeOH) γ_{max} (log ε) 228.7 (4.24) nm. ESI-MS m/z 890 $[M+Na+H]^+$, 1 757 $[2M+Na+2H]^+$; IR (KBr, cm^{-1}) ν_{max} 3 500-3 400 (OH), 2 980-2 935, 1 740 (COOR), 1 660 (C=C), 1 440, 1 385, 1 370 (CH_3), 1 295, 1 260, 1 240, 1 160-1 165 (COOR), 1 100, 1 080, 1 060, 1 000, 860 (CH3) and 805; ^{1}HNMR (600 MHz, $CDCl_3$) and ^{13}CNMR (150 MHz, $CDCl_3$), see ref 6.

2.1.5 *Bryostatin* 6(**4**) A white amorphous powder, m. p. 173-176 °C; $[\alpha]_D^{25}$ +41.2° (c 0.05, MeOH); UV (MeOH) γ_{max} (log ε) 228.7 (4.28) nm; ESI-MS m/z 876 $[M+Na+H]^+$, 1 729 $[2M+Na+2H]^+$; IR (KBr, cm-1) ν_{max} 3 500-3 400 (OH), 2 980-2 935, 1 740 (COOR), 1 675 (C=C), 1 450, 1 390, 1 300,

1 260，1 240，1 170(COOR)，1 100，1 080，1 060，1 000，860(CH_3) and 820；^{1}HNMR(600 MHz，$CDCl_3$) and ^{13}CNMR(150 MHz，$CDCl_3$)，see ref 6.

2.1.6 *Bryostatin* 18(**5**) A white amorphous powder，m. p. 156-159 ℃；$[\alpha]_D^{25}$ + 133.5°(*c* 0.3，MeOH). UV(MeOH) γ_{max}(logε) 228.7(4.52) nm；ESI-MS *m/z* 832[M+Na+H]$^+$，1 641[2M+Na+2H]$^+$；IR(KBr，cm-1) ν_{max} 3 500(OH)，2 980-2 935，1 740(COOR)，1 660(C=C)，1 620(conjugated C=C)，1 440，1 380，1 290，1 240，1 150(COOR)，1 100，1 060，860(CH3). ^{1}HNMR(600 MHz，$CDCl_3$) and ^{13}CNMR(150 MHz，$CDCl_3$)，see ref 7.

2.1.7 *Bryostatin* 10(**6**) A white amorphous powder，m. p. 160-164 ℃，$[\alpha]_D^{25}$ + 96.4o(c 0.1，MeOH)；UV(MeOH) γ_{max}(logε) 228.7(4.68) nm；ESI-MS *m/z* 832[M+Na+H]$^+$，1 641[2M+Na+2H]$^+$；IR(KBr，cm-1) ν_{max} 3 450(OH)，2 980-2 935，1 735(COOR)，1 660(C=C)，1 620(conjugated C=C)，1 440，1 380，1 290，1 240，1 150(COOR)，1 100，1 060，860(CH_3). ^{1}HNMR(600 MHz，$CDCl_3$) and ^{13}CNMR(150 MHz，$CDCl_3$)，see ref 7.

2.2 Results of cytotoxicities The cytotoxicities of **1** against U937 and K562 leukemia cell lines were carried out by MTT(Microculture Tetrazolium) assay. As a result，bryostatin 19(**1**) showed strong cytotoxic activities against U937 leukemia cell line at an ED_{50} value of 3.2 nmol/L；bryostatin 19(**1**) also exhibited prominent inhibitory activities against K562 cell line at an ED_{50} value of 20.1 nmol/L.

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中国南海总合草苔虫抗癌活性成分研究：一个新的大环内酯的分离鉴定

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[**摘要**] **目的**：研究中国南海总合草苔虫中的抗癌活性成分。**方法**：通过分级萃取和多种硅胶柱层析(Sephadex LH-20 凝胶过滤、ODS 反相层析和 HPLC)追踪分离抗癌活性成分；利用电喷雾离子质谱(ESI-MS)和高分辨(600 MHz)核磁共振二维谱技术鉴定化合物的结构。**结果**：分离得到一个新的具有显著抗肿瘤活性的大环内酯 bryostatin 19(1)。同时还首次自中国海域的该种苔虫中分离鉴定了 5 个 bryostatins 类大环内酯抗癌活性化合物：bryostatin 4，5，6，10，18。Bryostatin 19 显示很强的体外抗癌活性，对 U937 细胞的 ED_{50} 值为 3.2 nmol/L。**结论**：我国南海总合草苔虫资源丰富，并且含有新结构的抗癌活性草苔虫内酯。本研究结果为今后的新药研制与开发提供了参考。

[**关键词**] 总合草苔虫；抗肿瘤药；草苔虫素
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