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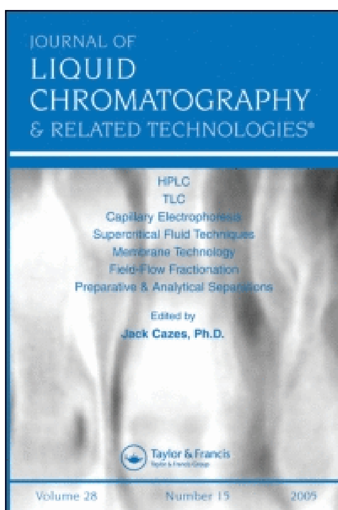

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SEPARATION OF THE BRYOSTATIN DERIVATIVES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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SEPARATION OF THE BRYOSTATIN DERIVATIVES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The Separation of bryostatin derivatives (26-acetate, 26-metabromobenzoate, 26-ketone, and 13→30 epoxide) by high performance liquid chromatography (HPLC) using normal phase and reverse phase systems was accomplished.

Using *n*-hexane/EtOAc/MeOH/H_2O (26:5:1:0.01) as mobile phase for normal phase HPLC and acetonitrile (CH_3CN)/H_2O system for reverse phase HPLC provided good separation of bryostatin derivatives. In addition, contributions of functional group for eluate order were discussed.

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INTRODUCTION

The macloride bryostatins were isolated mainly from the marine bryozoan *Bugula neritina*. Now, the bryostatins have been promised as the new anti-cancer agent etc., since they were found to be an activator of protein kinase C, an antineoplastic and antitumor promoter and an immunomodeling agent.[1] Also, we have found that bryostatin **10** inhibits the infection of HIV cells.[2]

For analysis of bryostatins, previously we have reported a fast and selective HPLC separation[3] and a systematic thin-layer chromatographic separation,[4] respectively. The present paper describes a useful result for the separation of derivatives of bryostatins (Figure 1), in addition to the analysis of reaction products by acid hydrolysis of bryostatins, **6** and **7**, and sodium borohydride ($NaBH_4$) reduction of bryostatin **4** 26-ketone. In Figure 1, structures of bryostatins and their derivatives were illustrated.

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EXPERIMENTAL

Chemicals and Reagents

All solvents and reagents were purchased from commercial sources in the best available grade of purity.

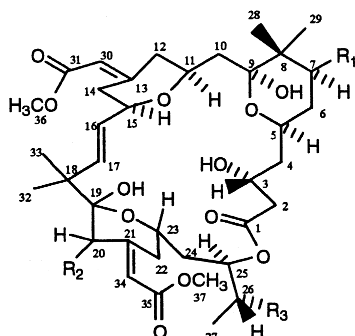
Biological Material

Bryostatins, **4,**[5] **5,**[6] **6,**[6] **7,**[6] and **10**[7,8] were employed the authentic samples, which were isolated from the marine bryozoan *Bugula neritina*.

The 26-acetates (26Ac) of bryostatins, **4,**[5] **5,**[6] **6,**[6] **7,**[6] and **10,**[7,8] the 26-metabromobenzoates (26MBB) of bryostatins, **4**[5] and **10,**[7] the 26-ketones (26K) of bryostatins, **4**[5] and **10,**[7] and the 13→30 epoxides (13Ep) of bryostatins, **4**[5] and **10,**[7] used the synthetic samples from the corresponding bryostatins. The acid hydrolysis mixtures of bryostatins, **6**[6] and **7**[6], employed the stored samples, which were already reported. Also, the $NaBH_4$ reduction of bryostatin **4** 26-ketone to bryostatin **4** and its 26-hydroxy isomer was briefly reported.[9] The detailed separation of reaction mixture into bryostatin **4** and its 26-hydroxy isomer is described in this paper.

Instruments and Chromatographic Conditions

Reverse phase HPLC solvents were delivered by two Gilson Model 302 pumps, which were controlled by an Apple IIe programmer through a Rheodyne



Bryostatins[1]	R_1(C-7)	R_2(C-20)	R_3(C-26)	other
B4	$-OCOC(CH_3)_3$	$-OCO(CH_2)_2CH_3$	-OH	
B4-26Ac	"	"	$-OCOCH_3$	
B4-26MBB	"	"	$-OCOC_6H_5Br$	
B4-26K	"	"	ketone	
B4-13Ep	"	"	-OH	13→30epoxy
B5	"	$-OCOCH_3$	-OH	
B5-26Ac	"	"	$-OCOCH_3$	
B6	$-OCO(CH_2)2CH_3$	$-OCOCH_3$	-OH	
B6-26Ac	"	"	$-OCOCH_3$	
$B6H_1(B7H_1)$	-OH	"	-OH	
$B6H_2$	$-OCO(CH_2)_2CH_3$	-OH	-OH	
B7	$-OCOCH_3$	$-OCOCH_3$	-OH	
B7-26Ac	"	"	$-OCOCH_3$	
B10	$-OCOC(CH_3)_3$	-H	-OH	
B10-26Ac	"	"	$-OCOCH_3$	
B10-26MBB	"	"	$-OCOC_6H_5Br$	
B10-26K	"	"	ketone	
B10-13Ep	"	"	-OH	13→30epoxy

Figure 1. Structures of bryostatins and their derivatives. 1)B**4** (bryostatin **4**), B**4**-26Ac (bryostatin **4** 26-acetate), B**4**-26MBB (bryostatin **4** 26-meta-bromobenzoate), B**4**-26K (bryostatin **4** 26-ketone), B**4**-13Ep (bryostatin **4** 13→30-epoxide), B**5** (bryostatin **5)**, B**5**-26Ac (bryostatin **5** 26-acetate), B**6** (bryostatin **6**), B**6**-26Ac (bryostatin **6** 26-acetate), B**6**H_1(B**7**H_1)(bryostatin **6** or **7** 7-hydroxide), B**6**H_2 (bryostatin **6** 20-hydroxide), B**7** (bryostatin **7**), B**7**-26Ac (bryostatin **7** 26-acetate), B**10** (bryostatin **10**), B10-26Ac (bryostatin **10** 26-acetate), B**10**-26MBB (bryostatin **10** 26-meta-bromobenzoate), B**10**-26K (bryostatin **10** 26-ketone), B**10**-13Ep (bryostatin **10** 13→30-epoxide).

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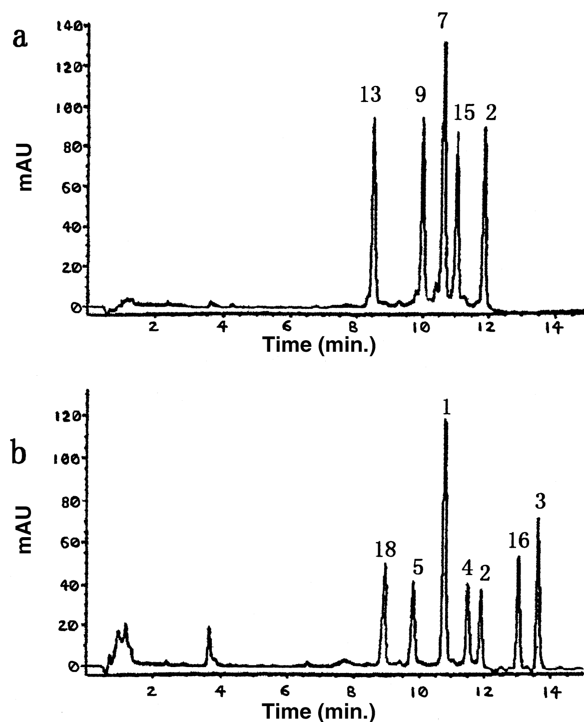

Figure 2. HPL Chromatogram of the bryostatin derivatives. a: chromatogram of the 26-acetate of bryostatin **4**(2), **5**(7), **6**(9), **7**(13) and **10**(15). b: chromatogram of the bryostatin **4**(1) and its derivatives, 26Ac(2), 26MBB(3), 26K(4), 13Ep(5) with bryostatin **10** derivatives, 26MBB(16) and 13Ep(18). HPLC conditions, column: RP-18 (3µm, 100×4.6mm I.D.), mobile phase: CH_3CN/H_2O (1:1) to CH_3CN in 15min, flow rate: 1mL/min, wave length: 230nm.

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7161 injector and 0.5 µm in-line precolumn filter (Rainin). For solvent degassing, helium was used. Samples (approx. 0.1mg/mL) were dissolved in methanol (RP separation) and the injection size was 10 µL. Reverse phase separations were performed with a RP-8 3 µm Ultremex column (100×4.6 mm I.D.; Phenomenex, Rancho Palos Verde CO.) using a linear gradient of 1:1 aqueous acetonitrile (CH_3CN) to pure CH_3CN in 15 min at a flow rate of 1 mL/min.

Normal phase HPLC was performed by the followed apparatus and conditions. Apparatus: Water Associates Model 6000, column: Partisil 5 silica (Phenomenex), Length of 30 mm and I.D. of 3.9 mm, solvent: *n*-hexane/EtOAc/MeOH/H_2O (26:5:1:0.01), flow rate: 0.3 mL/min for isolation and 0.7 mL/min for calculation of yields, recorder speed: 1/15 inch/min for

analysis. For injection sample, 50 μg of reaction mixture was dissolved in 50 μL of MeOH and the 2-3 μL of sample solution was injected. Solvents were degassed by helium.

For thin-layer chromatography (TLC), UNIPLATE from ANALTECH Inc., was used. Detection of compounds was conducted by use of UV-light (UV GL-25, Mineralight Lamp, Upland, CA. USA) and by spraying of the anisaldehyde reagent. As a solvent, *n*-hexane/acetone (7:3) was employed.

RESULTS AND DISCUSSION

HPLC of the Bryostatin Derivatives

A completed separation of the 26-acetates of bryostatins, **4**, **5**, **6**, **7**, and **10** was achieved on a short analytical column in less than 15 minutes, using linear gradient elution with CH_3CN-H_2O, as in the same case of natural bryostatins.[3] Chromatography was shown in Figure 2a. The technique was effective for separation of other bryostatin derivatives.

Figure 2b showed another excellent separation for the mixture of the bryostatin **4** and their derivatives, together with the 13→30 epoxide and 26-metabromobenzoate of bryostatin **10**.

In Table 1, retention times and relative retention times of the derivatives of bryostatins, **4**, **5**, **6**, **7** and **10**, in addition to those of the corresponding original bryostatins, were indicated. The retention times were found to be correlated with the difference of structures of the bryostatin derivatives. This point will be discussed in a later section.

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Application of HPLC to Acid Hydrolysis of Bryostatins, 6 and 7

Figure 3 (a-c) showed the chromatogram of acid hydrolysis of bryostatin **7**. After 24hr from starting as shown in Figure 3a, a new peak 10 (RT 4.30) appeared, besides a peak of bryostatin **7** (peak 12). This peak 10 was going up in order to the progress of reaction, as shown in Figure 3b (after 64 hr). After 96 hr, another new peak X (RT 2.50) was observed (Figure 3c). On the other hand, acid hydrolysis of bryostatin **6** provided two peaks, 10 (RT 4.30) and 11 (RT 6.80), respectively, as shown in Figure 3d. In this case, a peak 10 (B**7**H_1) was assigned to the 20-monoacetate of bryostatin **6** (B**6**H_1), which corresponded to a peak 10 (B**7**H_1) from bryostatin **7**. Also, a peak 11 from bryostatin **6** was determined to be 7-monobutyrate (B**6**H_2).

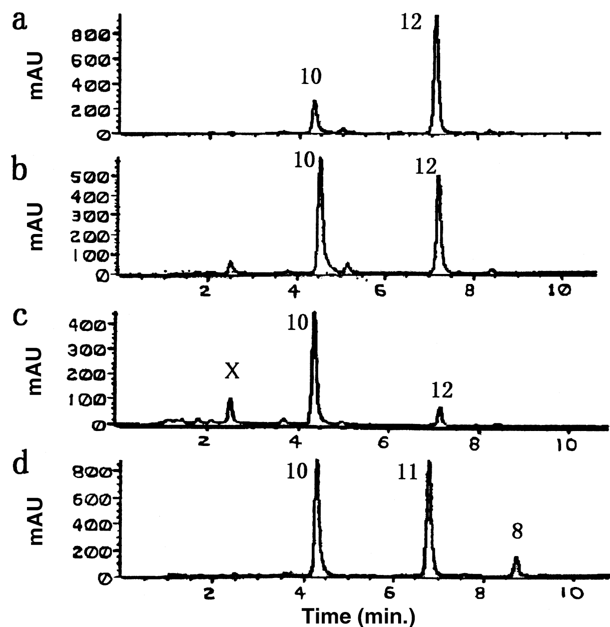

Figure 3. HPLC chromatogram of acid hydrolysis of bryostatin **7** (a-c) and bryostatin **6** (d). a: after 24 hrs from starting, b: after 64 hrs from starting, c: after 96 hrs from starting, d: after 96 hrs from starting. Peaks: 12(B**7**), 10 (B**7**H_1), X (unknown), 8 (B**6**), and 11 (B**6**H_2). HPLC conditions were same as Figure 2.

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Retention Times and Their Contribution for Functional Groups

In Table 1, the retention times on the reverse phase column and their relative retention times in comparison with retention time of the 26-hydroxy-7,20-diacetates (B**7**) were presented. Also, the relative retention times at position C-26, C-20 and C-7, respectively, were estimated.

From this data, it was found that the relative retention times of both bryostatin **4** derivatives and bryostatin **10** derivatives were close, notwithstanding each functional group was different at position C-20. The difference of between the 7,20-diacetate (B**6**H_2) and 26-hydroxy-7,20-diacetates (B**7**) was slight. The big difference appeared between the 7-hydroxide and the 7-pivarate. For the retention time, the contribution of the functional groups on the reversed phase column may be in the following sequential order: 20-OH < 7-OH < 26-OH < 20-OAc < 7-OAc < 13→30 epoxy < 26-ketone < 26-OAc < 26-$OCOC_6H_5Br$ < 20-$OCO(CH_2)_2CH_3$ < 7-$OCO(CH_2)_2CH_3$ < 7-$OCOC(CH_3)_3$. Thus, the contribution of position in bryostatin structure was found to be in the following sequential order : C-20<C-7<C-26.

Table 1

Retention Times and Relative Retention Times

No.	**Bryostatins**	**RT**[1]	**Relative RT**[2]			
			for B7	**at R_3(C-26)**	**at R_2(C-20)**	**at R_1(C-7)**
1	**B4**	10.77	1.49	1.00	1.14	
2	**B4**-26AC	12.10	1.70	1.12		
3	**B4**-26MBB	13.71	1.93	1.27		
4	**B4**-26K	11.70	1.65	1.09		
5	**B4**-Ep	9.92	1.40	0.82		
6	**B5**	9.44	1.33	1.00	1.00	1.33(2.20)
7	**B5**-26Ac	10.63	1.49	1.12		
8	**B6**	8.78	1.24	1.00	1.29	1.24(2.04)
9	**B6**-26AC	10.03	1.41	1.14		
10	**B6**H_1(**B7**H_1)	4.30	0.60			0.61(1.00)
11	**B6**H_2	6.80	0.96		1.00	
12	**B7**	7.09	1.00	1.00		1.00(1.65)
13	**B7**-26Ac	8.54	1.20	1.20		
14	**B10**	9.85	1.36	1.00	1.05	
15	**B10**-26Ac	11.08	1.56	1.13		
16	**B10**-26MBB	13.14	1.85	1.33		
17	**B10**-26K	10.64	1.49	1.08		
18	**B10**-Ep	8.83	1.25	0.88		

[1] RT: retention times. [2] Relative RT: relative retention times.

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Separation of Bryostatin 4 and Its 26-Hydroxy Isomer

In Figure 4, both $NaBH_4$ reductive of bryostatin **4** 26-ketone and CrO_3 oxidation of 26-hydroxy isomer were illustrated. The $NaBH_4$ (50 μg) reduction of bryostatin **4** 26-ketone (50 μg) in MeOH (10 drops) at room temperature (26°C) for 4 hr gave the crude product after extraction with CH_2Cl_2, washing with water, and dried with nitrogen gas. The crude product afforded two spots on TLC with *n*-hexane/acetone (7:3) by two times development, as shown in Figure 4. One of spots, the front spot A, was identical with that of bryostatin **4** (26-αOH). The rear spot B was a new product and assumed to be 26-hydroxy isomer (26-βOH).

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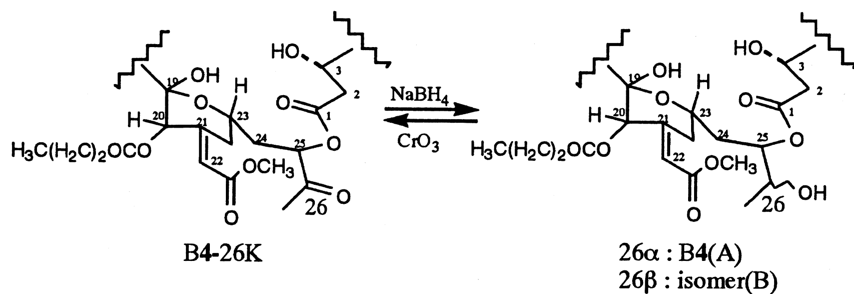

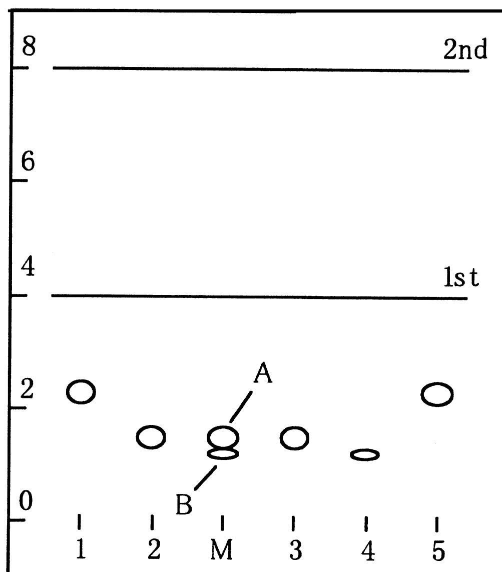

Figure 4. TL Chromatogram of $NaBH_4$ reduction of bryostatin **4** 26-ketone to 26-hydroxy isomers. Samples: 1 (B**4**-26K), 2 (B**4**), M (reduction mixture of $NaBH_4$ reduction of B**4**-26K), 3 (isolated A; B**4**), 4 (isolated B; B**4**-26-βOH), and 5 (CrO_3 oxidation of B; B**4**-26K). TLC conditions, plate: SiO_2, solvent : *n*-hexane/acetone (7:3), method: two times development.

Separation of the products, A and B by reverse phase HPLC failed, while separation by normal phase HPLC with Partisil 5 silica (Phenomenex) using *n*-hexane/EtOAc/MeOH/H_2O (26:5:1:0.01) provided satisfactory results, as shown in Figure 5. Therefore, isolation of products, A and B, was examined by use of normal phase HPLC condition, which was described in the experimental section.

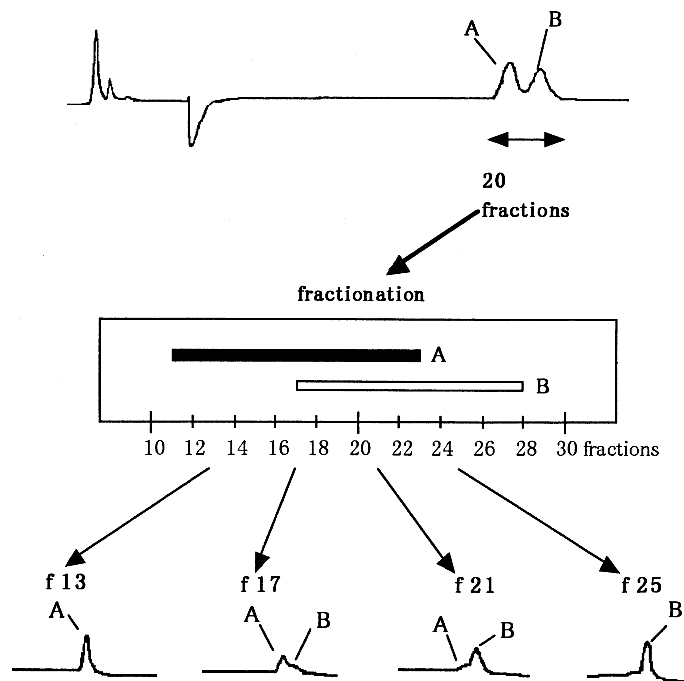

Figure 5. Separation of $NaBH_4$ reduction mixture of bryostatin **4** 26-ketone. HPLC conditions, mobile phase: *n*-hexane/EtOAc/MeOH/H_2O(26:5:1:0.01), flow rate: 0.3 mL/min.

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By signals of UV detector, 20 fractions of Fr. 10 to 29 with each 20 drops was collected. A product A (bryostatin **4**) was eluted until Fr. 11 to Fr. 23, while a product B (bryostatin **4** 26-hydroxy isomer) from Fr.17 to Fr.28, respectively. The overlapping 7 fractions (Fr. 17-23) of products, A and B, were rechromatographed by the same HPLC conditions. Each pure fraction was combined. Thus, the yields of products, A and B, were 9.5 µg and 6.0 µg, respectively. The product A was identical to bryostatin **4** (26-αOH) by comparison with the authentic specimen. Identification of the product B to the 26-β-hydroxy isomer of bryostatin **4** was established by CrO_3 oxidation of the product B. Figure 4 showed the result on TLC.

CONCLUSION

For separation of the bryostatin derivatives as shown in Figure 1, the use of HPLC by reverse phase system with CH_3CN-H_2O mixture afforded an effec-

tive result as the same as that of previously reported natural bryostatins.[3] On the other hand, for separation of bryostatin **4** and its 26β-hydroxy isomer, the careful chromatographic technique by normal phase system with *n*-hexane/EtOAc/MeOH/H_2O (26:5:1:0.01) was required.

As an application, separations of acid hydrolysis mixture of both bryostatin **6** and bryostatin **7** were established. Interestingly, the retention time contributions for the functional groups in bryostatin structure were recognized to be in the following order : C-20<C-7<C-26.

In the functional groups, the contributions were found to be in the following sequential order: epoxy < ketone < hydroxy < acetate <meta-bromobenzoate < butyrate < pivalate.

It is hoped that the proposed chromatographic method may serve for the analysis of other bryostatins and related compounds.

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