CALPHOSTINS, NOVEL AND SPECIFIC INHIBITORS OF PROTEIN KINASE C

II. CHEMICAL STRUCTURES

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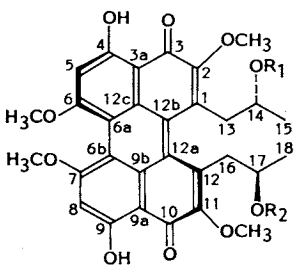
The structures of new antitumor antibiotics, calphostins (UCN-1028) A, B, C, D and I, which are specific and potent inhibitors of protein kinase C, were determined by spectral and chemical studies. All of the antibiotics have a 3,10-perylenequinone skeleton and calphostin D was revealed to be an antipode of isophleichrome.

Antitumor antibiotics, calphostin (UCN-1028) complex has been isolated from the fermentation broth of *Cladosporium cladosporioides* and shows strong inhibitory activity against protein kinase C. They consist of five major components, calphostins A[1], B, C, D and I. The isolation and biological properties of these compounds were reported in the preceding paper[2]. We wish to describe the structural elucidation of calphostins in this paper.

The Structure of Calphostin D (1)

The molecular formula was determined as $C_{30}H_{30}O_{10}$ by MS and elemental analysis. The IR spectrum



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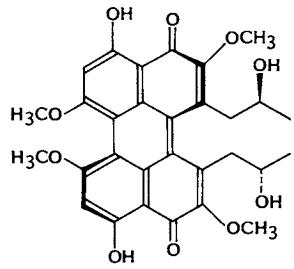

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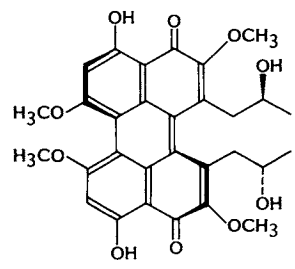

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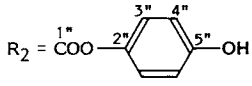
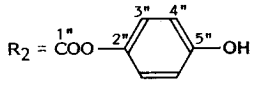
7 $R_1 =$  $R_2 =$ 

8 $R_1 =$  $R_2 =$ 

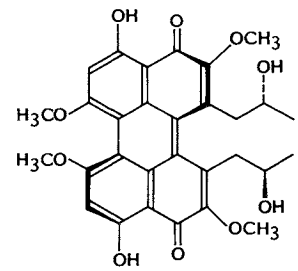


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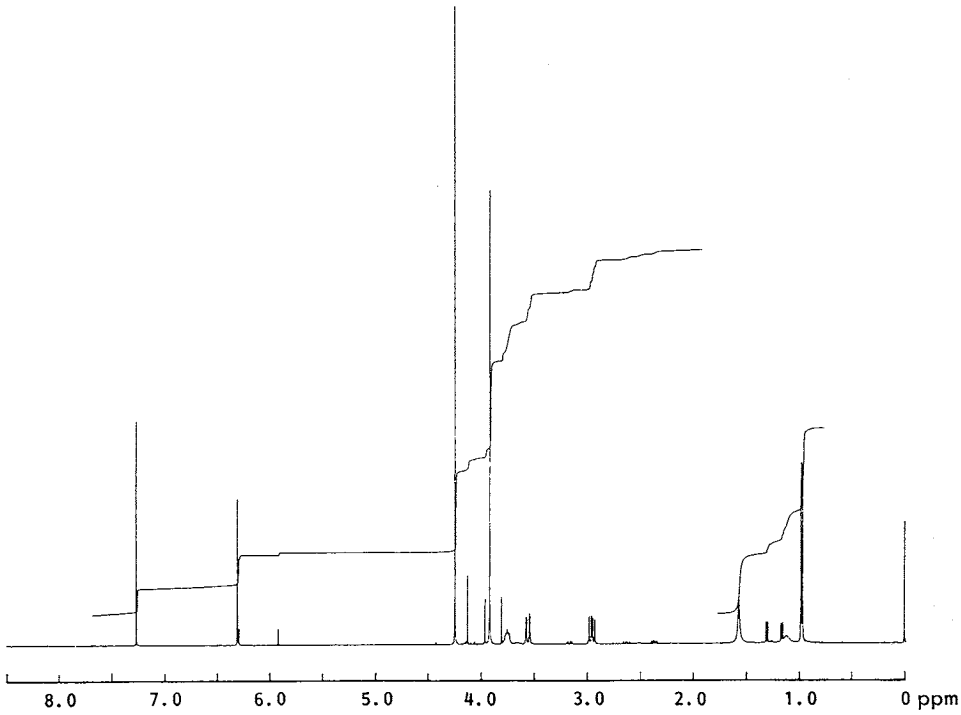
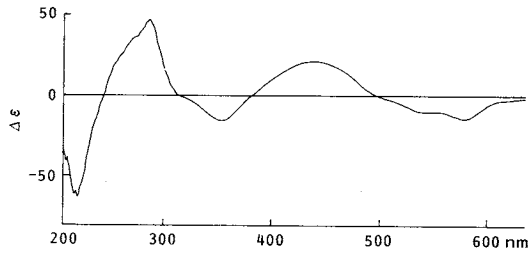
Fig. 1. ^{1}H NMR spectrum of calphostin D in $CDDl_3$ (400 MHz).



revealed the absorption band at 3000 (aromatic CH), 1600 (perylenequinone) and 1150 (alcoholic CO) cm^{-1} and the UV spectrum showed the absorption maximum at 226 (ε 44,800), 269 (29,500), 474 (23,500), 539 (sh, 11,900) and 582 (12,000) nm. The ^{1}H NMR spectrum, shown in Fig. 1 and Table 1, is superimposable with that of isophleichrome (**2**)[3] which is thermally isomerized product of phleichrome (**3**)[3,4], phytotoxin isolated from fungi. The CD spectrum (Fig. 2) of calphostin D was however opposite in sign to isophleichrome and equal with phleichrome. Phleichrome has two chiral centers in side chain and a dissymmetrical chromophore of 3,10-perylenequinone ring which affect the CD spectrum in sign. The axial chirality of the phleichrome and isophleichrome[3] was determined by comparing with that of cercosporin which had been established by X-ray analysis[5,6]. From these findings calphostin D (**1**) was determined to be the antipode of isophleichrome[3]. Thermal isomerization of calphostin D (**1**) on refluxing in toluene gave isocalphostin D (**4**), and the physico-chemical data except CD spectrum is completely identical with those of phleichrome. ^{13}C NMR spectral data is shown in Table 2 and those assignment was performed by selective proton decoupled and long range selective proton decoupled experiments.

Fig. 2. CD spectrum of calphostin D (MeOH).



The Structures of Calphostins A (**5**) and B (**6**)

The molecular formula of calphostins A (**5**) and B (**6**) were determined as $C_{44}H_{38}O_{12}$ and $C_{37}H_{34}O_{11}$, respectively by MS and elemental analysis. The ^{1}H and ^{13}C NMR spectral data are shown in Tables 1

Table 1. ^{1}H NMR spectral data of calphostins (400 MHz).

Proton	Chemical shifts (ppm) (multiplicity, coupling constants in J=Hz)				
	1 ($CDCl_3$)	**5** ($CDCl_3$)	**6** ($CDCl_3$)	**7** (CD_3OD)	**8** (CD_3OD)
2-OCH_3	4.24 (s)	4.33 (s)	4.31 (s)	4.19 (s)	4.19 (s)
4-OH	15.83 (s)	15.84 (s)	15.90 (s)		
5-H	6.30 (s)	6.22 (s)	6.30 (s)	6.31 (s)	6.35 (s)
6-OCH_3	3.91 (s)	3.88 (s)	3.87 (s)	3.82 (s)	3.88 (s)
7-OCH_3	3.91 (s)	3.88 (s)	3.88 (s)	3.64 (s)	3.65 (s)
8-H	6.30 (s)	6.22 (s)	6.30 (s)	6.30 (s)	6.29 (s)
9-OH	15.83 (s)	15.84 (s)	15.78 (s)		
11-OCH_3	4.24 (s)	4.33 (s)	4.26 (s)	4.18 (s)	4.19 (s)
13-H_a	2.95 (dd, 8.4, 13.2)	3.18 (dd, 10.0, 13.4)	3.22 (dd, 9.2, 13.4)	3.11 (dd, 10.1, 13.5)	3.08 (dd, 10.1, 13.5)
13-H_b	3.55 (dd, 3.2, 13.2)	3.60 (dd, 2.0, 13.4)	3.63 (dd, 2.1, 13.4)	3.60 (m)	3.60 (dd, 2.0, 13.5)
14-H	3.75 (m)	5.03 (m)	5.02 (m)	4.70 (m)	4.65 (m)
15-H_3	0.97 (d, 6.2)	1.29 (d, 6.3)	1.28 (d, 6.3)	1.22 (d, 6.3)	1.20 (d, 6.3)
16-H_a	2.95 (dd, 8.4, 13.2)	3.18 (dd, 10.0, 13.4)	2.90 (dd, 8.3, 13.2)	3.00 (dd, 9.8, 13.5)	3.00 (dd, 9.7, 13.5)
16-H_b	3.55 (dd, 3.2, 13.2)	3.60 (dd, 2.0, 13.4)	3.58 (dd, 3.2, 13.2)	3.60 (m)	3.60 (dd, 2.0, 13.5)
17-H	3.75 (m)	5.03 (m)	3.72 (m)	5.00 (m)	5.00 (m)
18-H_3	0.97 (d, 6.2)	1.29 (d, 6.3)	0.95 (d, 6.3)	1.10 (d, 6.3)	1.10 (d, 6.3)
3′-H		6.84 (dd, 1.3, 7.8)	6.89 (dd, 1.3, 7.9)	6.74 (dd, 1.3, 8.1)	
4′-H		6.90 (t, 7.8)	6.94 (t, 7.9)	6.86 (t, 8.1)	
5′-H		7.22 (tt, 1.3, 7.8)	7.25 (tt, 1.3, 7.9)	7.22 (tt, 1.3, 8.1)	
3″-H				6.36 (d, 8.9)	6.36 (d, 8.8)
4″-H				5.87 (d, 8.9)	5.87 (d, 8.8)
3‴-H					6.59 (d, 8.7)
4‴-H					6.20 (d, 8.7)

and 2, respectively. The ^{1}H NMR spectra of calphostin A (**5**) exhibited the presence of C_2 symmetry axis in its structure, and the down field shift of 14-H and 17-H compared with that of **1** and the appearance of the signals assigned to benzoate, indicate that calphostin A (**5**) is 14,17-di-*O*-benzoyl derivative of calphostin D (**1**). The NMR spectra of calphostin B (**6**) showed the existence of one benzoyl group and the down field shift of 14-H signal indicates that **6** is 14-monobenzoyl derivative of calphostin D (**1**).

The alcoholysis of both **5** and **6** with sodium methoxide in methanol gave calphostin D (**1**), and benzoylation of **1** gave **5** and **6**, which also assisted their structures including their stereochemistry.

The Structure of Calphostin C (7)

The molecular formula, $C_{44}H_{38}O_{14}$, of calphostin C (**7**) was determined by MS and elemental analysis. The IR spectrum revealed the absorption band at 3000 (aromatic CH), 1750 (carbonate), 1710 (ester) cm^{-1}, and the UV spectrum resembles that of calphostin A (**5**). From the comparison of the ^{1}H and ^{13}C NMR spectra of calphostins C (**7**) and B (**6**) it was ascertained that **7** has the additional signals arising from one carbonate carbon (δ_c 156.0) and one *para* substituted benzene ring of which protons were observed at rather high field (δ_H 5.87 and 6.36), which suggested the existence of *p*-hydroquinone. From these findings the structure of calphostin C was confirmed as **7**. ^{1}H and ^{13}C NMR spectra of the degradation products of **7** obtained by alcoholysis with CD_3ONa-CD_3OD in NMR tube, showed the signals of *p*-hydroquinone in addition to the signals of benzoic acid and calphostin D (**1**) (Figs. 3 and 4). The physico-chemical properties of the last compound was completely identical with those of authentic sample.

The Structure of Calphostin I (8)

Calphostin I (**8**) showed the molecular ion at *m/z* 806 in electron impact (EI)-MS. The molecular

Table 2. ^{13}C NMR spectral data of calphostins.

Carbon	Chemical shift (ppm)				
	1 (CD_3OD)	**5** ($CDCl_3$)	**6** ($CDCl_3$)	**7** (CD_3OD)	**8** (CD_3OD)
1	130.2	125.6	125.4	128.7	128.7
2	152.8	151.5	151.4	153.1	153.0
3	175.3	172.5	172.2	173.9	173.4
3a	107.1	106.3	106.2	107.3	107.4
4	178.1	177.9	178.6	179.4	179.6
5	101.8	101.2	101.4	102.4	102.2
6	168.6	166.2	166.5	168.5	168.6
6a	118.2	116.6	117.1	118.6	118.7
6b	118.2	116.6	117.4	118.4	118.7
7	168.6	166.2	166.8	168.4	168.6
8	101.8	101.2	101.4	101.9	101.9
9	178.1	177.9	178.9	179.1	179.6
9a	107.1	106.3	106.4	107.3	107.3
9b	127.5	127.4	127.5	126.8	126.8
10	175.3	172.5	171.7	173.8	173.5
11	152.8	151.5	151.2	153.0	153.0
12	130.2	125.6	125.7	128.9	128.9
12a	139.0	134.6	134.6	135.5	135.6
12b	139.0	134.6	136.4	136.3	136.4
12c	127.5	127.4	127.5	127.3	127.2
13	43.8	39.1	39.1	40.3	40.5
14	69.7	72.3	72.3	73.6	72.8
15	24.1	21.1	21.2	21.3	21.3
16	43.8	39.1	42.4	40.3	40.3
17	69.7	72.3	69.1	77.5	77.5
18	24.1	21.1	23.7	20.9	20.9
2-OCH_3	61.5	61.2	61.2	61.7	61.7
6-OCH_3	57.3	55.1	56.0	57.0	57.0
7-OCH_3	57.3	55.1	56.2	57.0	57.1
11-OCH_3	61.5	61.2	61.2	61.7	61.7
1′		164.6	164.6	165.9	
2′		129.1	129.2	130.1	
3′		128.4	128.4	129.3	
4′		127.5	127.5	128.8	
5′		132.0	132.0	133.6	
1″				156.0	156.0
2″				144.5	144.5
3″				122.1	122.1
4″				116.1	116.1
5″				154.1	154.1
1‴					166.0
2‴					120.9
3‴					131.4
4‴					115.4
5‴					162.7

The assingment of the carbons having the close chemical shift may be exchangeable.

formula was determined as $C_{44}H_{38}O_{15}$ from the MS and ^{13}C NMR spectral data. ^1H NMR spectrum showed a similar pattern with that of calphostin C (**7**), but it exhibited the protons assigned to *p*-hydroxybenzoate (δ_H 6.20 and 6.59) instead of benzoate of **7**, confirming the structure of calphostin I as **8**. The ^{13}C NMR spectral data (Table 2) also supported this structure.

Fig. 3. ^{1}H NMR spectrum of calphostin C methanolysis products in CD_3OD (400 MHz).

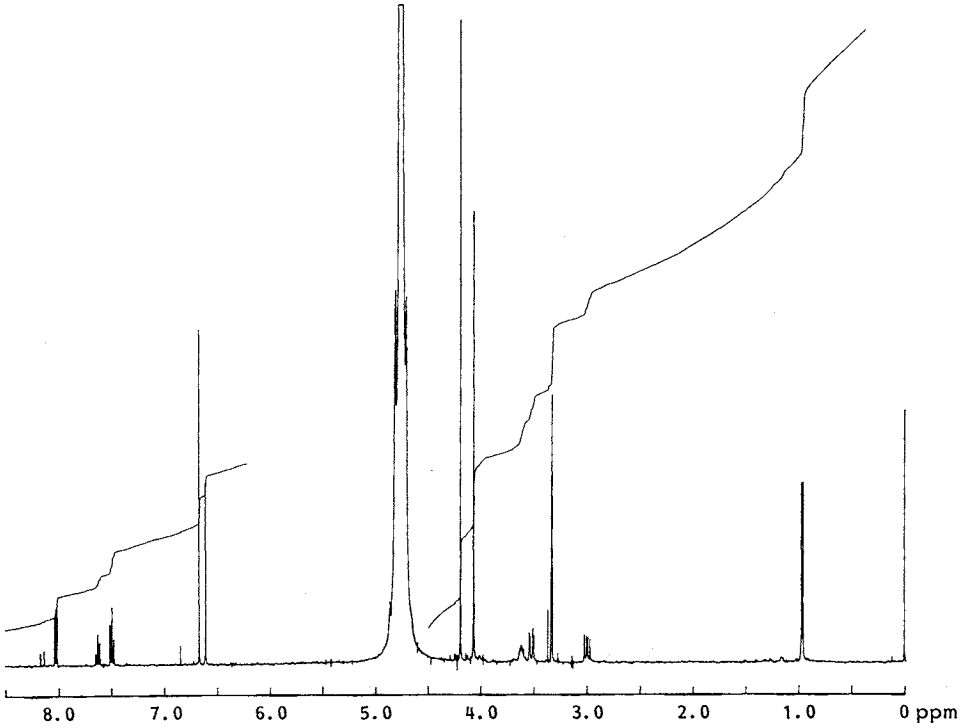
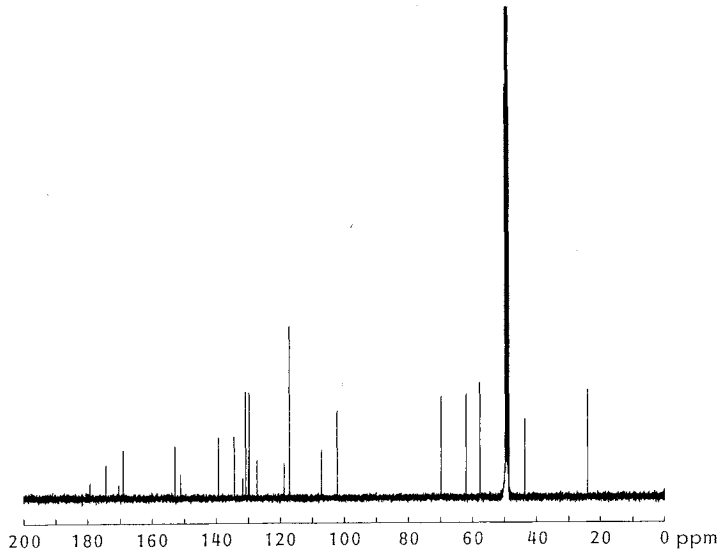


Fig. 4. ^{13}C NMR spectrum of calphostin C methanolysis products in CD_3OD (100 MHz).



Experimental

^{1}H and ^{13}C NMR spectra were measured with Bruker AM-400 and Jeol FX-100 spectrometer. MS were obtained with Hitachi M-80B spectrometer. IR spectra were recorded on Shimadzu IR-27G spectrometer. UV spectra were taken on Hitachi 200-20 spectrophotometer. CD spectra were obtained with Jasco J-500 spectropolarimeter.

Physico-chemical Properties

Calphostin D: EI-MS *m/z* 550 (M^+); UV λ_{max}^{MeOH} nm (ε) 226 (44,800), 269 (29,500), 474 (23,500), 539 (sh, 11,900), 582 (12,000); IR ($CHCl_3$) cm^{-1} 3000, 1600, 1440, 1270, 1150; ^1H NMR ($CDCl_3$) Table 1; ^{13}C NMR (CD_3OD) Table 2; CD (MeOH) nm ($\Delta\varepsilon$) 223 (−62.8), 287 (+46.5), 356 (−15.9), 441 (+21.2), 541 (−10.2), 582 (−14.3).

Anal Calcd for $C_{30}H_{30}O_{10}$: C 65.45, H 5.49.
Found: C 65.57, H 5.41.

Calphostin A: EI-MS *m/z* 758 (M^+); UV λ_{max}^{MeOH} nm (ε) 225 (56,100), 269 (25,300), 476 (20,400), 543 (sh, 10,200), 586 (9,880); IR ($CHCl_3$) cm^{-1} 3020, 1710, 1450, 1270, 830; ^1H NMR ($CDCl_3$) Table 1; ^{13}C NMR ($CDCl_3$) Table 2; CD (MeOH) nm ($\Delta\varepsilon$) 227 (−86.6), 284 (+45.1), 355 (−15.0) 444 (+20.1), 580 (−17.3).

Anal Calcd for $C_{44}H_{38}O_{12}\cdot 2H_2O$: C 66.49, H 5.33.
Found: C 65.15, H 5.47.

Calphostin B: EI-MS *m/z* 654 (M^+); UV λ_{max}^{MeOH} nm (ε) 225 (49,500), 269 (26,600), 476 (21,800), 543 (sh, 11,000), 585 (10,900); IR ($CHCl_3$) cm^{-1} 3000, 1710, 1600, 1450, 1270; ^1H NMR ($CDCl_3$) Table 1; ^{13}C NMR ($CDCl_3$) Tabel 2; CD (MeOH) nm ($\Delta\varepsilon$) 226 (−68.1), 284 (+42.8), 354 (−13.9), 443 (+21.9), 580 (−13.0).

Anal Calcd for $C_{37}H_{34}O_{11}\cdot H_2O$: C 66.07, H 5.39.
Found: C 66.51, H 5.43.

Calphostin C: EI-MS *m/z* 790 (M^+); UV λ_{max}^{MeOH} nm (ε) 223 (58,200), 270 (29,400), 476 (22,900), 543 (sh, 11,600), 586 (11,200); IR ($CHCl_3$) cm^{-1} 3000, 1750, 1710, 1605, 1500, 1270, 830, 700; ^1H NMR (CD_3OD) Table 1; ^{13}C NMR (CD_3OD) Table 2; CD (MeOH) nm ($\Delta\varepsilon$) 228 (−67.0), 287 (+53.1), 358 (−14.6), 446 (+24.1), 582 (−13.6).

Anal Calcd for $C_{44}H_{38}O_{14}$: C 66.83, H 4.84.
Found: C 66.56, H 4.74.

Calphostin I: EI-MS *m/z* 806 (M^+); UV λ_{max}^{MeOH} nm 217, 258, 348, 475, 540 (sh), 582; IR ($CHCl_3$) cm^{-1} 3350, 3000, 1750, 1700, 1600, 1505, 1450, 1270, 990, 830; ^1H NMR (CD_3OD) Table 1; ^{13}C NMR (CD_3OD) Table 2.

Isomerization of Calphostin D (1)

A solution of calphostin D (50 mg) in toluene (10 ml) was refluxed for 5 hours. Evaporation of the solvent gave a red residue of the equilibrium mixture of starting material and isomerized product. Chromatography of the residue on silica gel with $CHCl_3$-MeOH (100:1) gave isocalphostin D (19 mg): EI-MS *m/z* 550 (M^+); UV λ_{max}^{MeOH} nm (ε) 226 (44,200), 270 (29,300), 538 (sh, 11,100), 581 (11,300); IR (KBr) cm^{-1} 3470, 1600, 1445, 1395, 1270, 1235, 1205, 1150; ^1H NMR ($CDCl_3$) δ 0.53 (3H×2, dd, J=6.1 Hz), 2.95 (1H×2, dd, J=6.4 and 12.9 Hz), 3.41 (1H×2, m), 3.60 (1H×2, dd, J=6.6 and 12.9 Hz), 4.06 (3H×2, s), 4.20 (3H×2, s), 6.58 (1H×2, s), 15.79 (1H×2, s); CD (MeOH) nm ($\Delta\varepsilon$) 226 (+50.4), 288 (−42.4), 356 (+12.6), 443 (−20.8), 540 (+7.7), 578 (+11.7).

Anal Calcd for $C_{30}H_{30}O_{10}\cdot H_2O$: C 63.37, H 5.67.
Found: C 63.54, H 5.85.

Preparation of Calphostins A (5) and B (6) from Calphostin D (1)

To a solution of calphostin D (50 mg) in methylene chloride (1 ml) benzoic anhydride (100 mg), pyridine (0.1 ml) and dimethylaminopyridine (1 mg) was added, and the solution was stirred for 5 hours at room temperature. Methanol (0.2 ml) was added to the reaction mixture and stand for 1 hour at room temperature. The reaction mixture was extracted with ethyl acetate, and dried over anhydrous magnesium sulfate. The solution was evaporated and the residue was chromatographed on silica gel using $CHCl_3$-MeOH (160:1) as eluent. The first elution gave calphostin A (44 mg), and the following elution gave calphostin B (23 mg).

Alcoholysis of Calphostin C

Calphostin C (10 mg) was dissolved in CD_3ONa-CD_3OD prepared from a small amount of sodium metal and deuteriomethanol (0.5 ml), and kept for 24 hours at room temperature. After neutralization with

DCl, ^{1}H and ^{13}C NMR spectra were measured.

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