CARBAZOMYCINS C, D, E AND F, MINOR COMPONENTS OF THE CARBAZOMYCIN COMPLEX

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Carbazomycins C (III), D (IV), E (V) and F (VI), the minor components of the carbazomycin complex, were isolated from the cultured broth of *Streptoverticillium ehimense* together with carbazomycins A (II) and B (I). Among them, III and IV were shown to be new substances and their structures were elucidated as 4-hydroxy-3,6-dimethoxy-1,2-dimethylcarbazole and 3,4,6-trimethoxy-1,2-dimethylcarbazole, respectively, by spectroscopic and chemical means. The other components, V and VI, were found to contain an aldehyde function and were identified as carbazomycinal and 6-methoxycarbazomycinal, respectively. The antimicrobial activity of III and IV are also reported.

Carbazomycins A and B are the first antibiotics containing a carbazole nucleus and inhibit mainly the growth of phytopathogenic fungi. Their isolation[1], structure elucidation[2,3] and biosynthesis[4] have been reported previously and the producing microorganism has also been assigned as *Streptoverticillium ehimense* H 1051-MY 10[4].

During the course of biosynthetic studies[4] of the main component, carbazomycin B (I), we found that DL-[3-^{14}C]tryptophan was incorporated into I with the high efficiency of 15%. We also observed that carbazomycins gave characteristic strong colors on silica gel TLC by heating after spraying with 10% sulfuric acid. By making use of these two points, we tried to obtain biosynthetic analogs or precursors of I from the cultured broth of the producing microorganism.

First, we added DL-[3-^{14}C]tryptophan to the culture at 48 hours after inoculation and the cultivation was continued for an additional 48 hours. Then, we examined by TLC the acetone extract of the mycelial cake and the ethyl acetate extract of the broth filtrate. The radioautogram obtained from the above TLC is shown in Fig. 1, where we named the spots different from I and

Fig. 1. Radioautogram obtained from the silica gel TLC of the EtOAc extract of the broth filtrate and Me_2CO extract of the mycelial cake.

The plate was developed with *n*-hexane - EtOAc (3: 1). The letters on the sides are the names of carbazomycins.

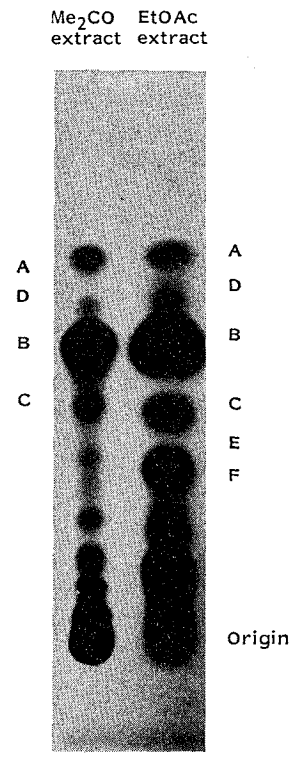
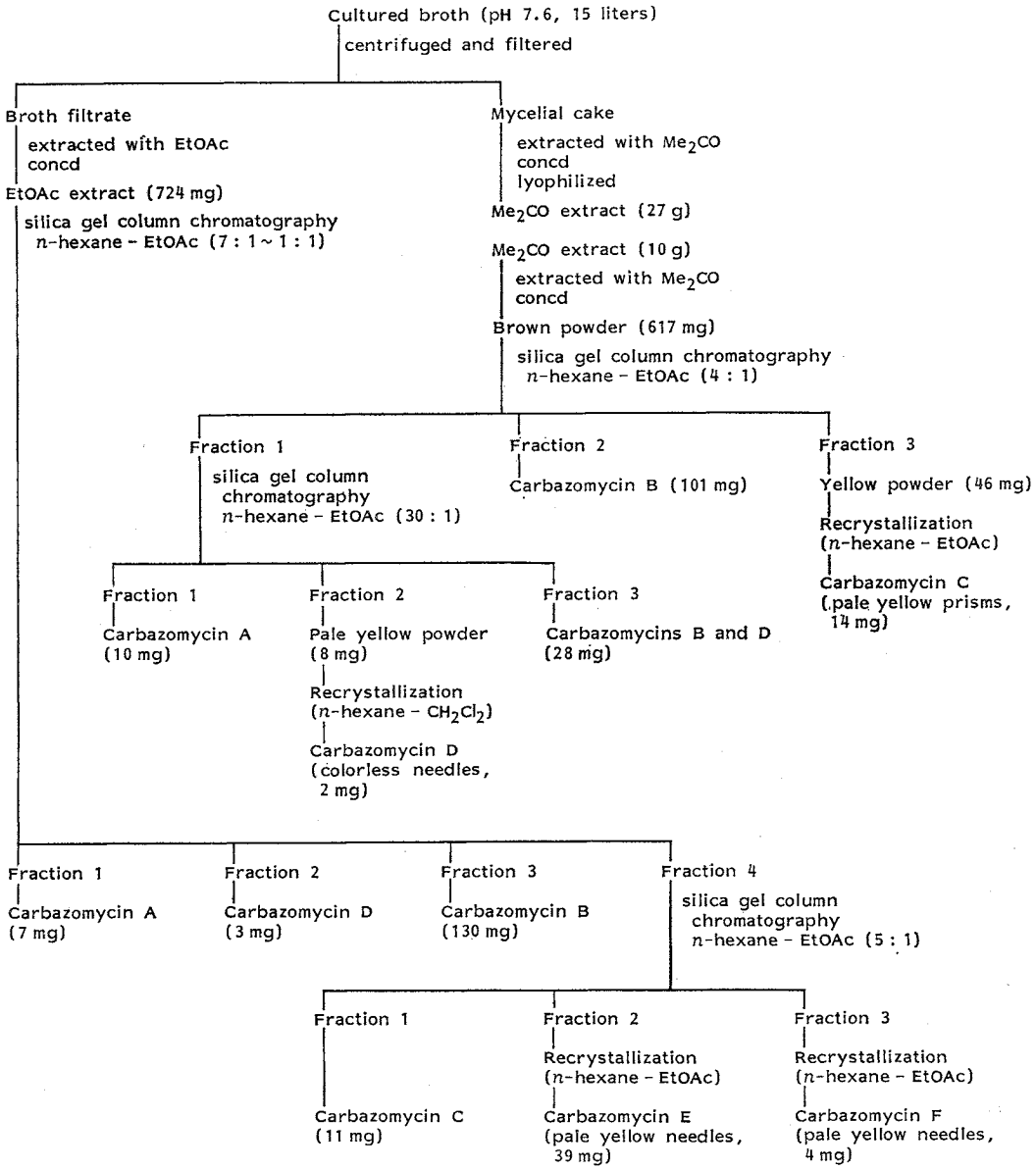


Fig. 2. Isolation and purification procedures for carbazomycins.



carbazomycin A (II) as carbazomycins C, D, E and F. Among them, carbazomycins C (III) and D (IV) proved to be new components, while carbazomycins E (V) and F (VI) were found to be identical with carbazomycinal and 6-methoxycarbazomycinal, respectively, isolated from a strain of *Streptoverticillium* sp. and reported by MARUMO *et al.*[5]. The isolation, purification, physico-chemical and biological properties, and structure elucidation of the new components are described in this paper.

Fermentation and Isolation

Streptoverticillium ehimense was cultured in the same production medium as reported previously[1] for 7 days at 27°C in 500-ml Erlenmeyer flasks on a rotary shaker (amplitude 7 cm, 180 rpm).

Table 1. Rf Values of carbazomycins on silica gel TLC.

Solvent system	A	B	C	D	E	F
n-Hexane - EtOAc (1: 1)	0.92	0.82	0.76	0.88	0.60	0.48
Toluene - acetone (9: 1)	0.70	0.54	0.42	0.59	0.37	0.31
Benzene - chloroform (4: 1)	0.49	0.34	0.26	0.36	0.14	0.11
n-Hexane - ether (2: 1)	0.60	0.42	0.28	0.46	0.20	0.15
Benzene - *n*-hexane - acetone (20: 5: 3)	0.74	0.59	0.48	0.65	0.38	0.33
Benzene	0.36	0.26	0.14	0.28	0.13	0.10

Table 2. Physico-chemical properties of carbazomycins C and D.

	C (III)	D (IV)
Appearance	Pale yellow prisms	Colorless needles
MP (°C)	198.0~198.5	129.5~130.0
Molecular formula	$C_{16}H_{17}NO_3$	$C_{17}H_{19}NO_3$
MS *m/z* (M^+)	271	285
HR-MS *m/z* (M^+)		
Found:	271.1191	285.1374
Theory:	271.1209	285.1366
UV λ_{max}^{MeOH} nm (ε)	227 (24,900), 245 (24,000), 260 (sh, 12,500), 287 (sh, 7,600), 295 (12,200), 341 (3,600), 354 (4,200)	229 (30,500), 247 (27,800), 255 (sh, 19,300), 291 (sh, 10,300), 300 (17,000), 340 (4,300), 356 (4,500)
IR ν_{max}^{KBr} cm^{-1}	3400, 3290, 2900, 1630, 1590, 1500, 1480, 1460, 1400, 1320, 1295, 1210, 1140, 1110, 1040, 1025, 1000, 880, 760	3330, 2920, 1630, 1580, 1500, 1480, 1460, 1390, 1340, 1290, 1210, 1140, 1050, 1030, 1015, 975, 770

HR-MS: High resolution mass spectra.

The cultured broth was centrifuged and filtered. The broth filtrate was extracted with ethyl acetate and the mycelial cake was extracted with acetone. Carbazomycins in each extract were separately purified by repeated column chromatography on silica gel eluted with a solvent composed of *n*-hexane and ethyl acetate. The isolation and purification procedures for the carbazomycins are shown in Fig. 2.

The Rf values of carbazomycins on silica gel TLC plates developed with several solvent systems are listed in Table 1. Carbazomycins C and D gave a brownish purple color by heating after spraying with 10% sulfuric acid on a TLC plate, while carbazomycins E and F gave yellowish brown and brown colors, respectively.

Physico-chemical Properties and Structure Elucidation

The physico-chemical properties of **III** and **IV** are summarized in Table 2. The UV absorption spectra of **III** and **IV** were very similar to each other and also to that of **I** suggesting the existence of a carbazole nucleus in **III** and **IV**. The molecular formulae of **III** and **IV** were established by high resolution mass spectrometry as $C_{16}H_{17}NO_3$ and $C_{17}H_{19}NO_3$, respectively.

The ^1H NMR spectra of **III** and **IV** together with that of **I** are shown in Table 3. The proton signals of **III** suggested the existence of two aromatic methyl groups (δ 2.33 and 2.36) and two methoxyl groups (δ 3.74 and 3.84), one hydroxyl group (δ 8.06, exchangeable with D_2O) and three aromatic protons (δ 6.91, 7.31 and 7.77) together with a carbazole-NH group (δ 9.76, exchangeable with D_2O) in **III**. The proton signals of **IV** were very similar to those of **III** and indicated the existence of two

Table 3. ^{1}H NMR data of carbazomycins C and D compared with those of carbazomycin B.

Protons	Carbazomycin B (I) R_1=CH_3, R_2=OH, R_3=H	Carbazomycin C (III) R_1=CH_3, R_2=OH, R_3=OCH_3	Carbazomycin D (IV) R_1=CH_3, R_2=OCH_3, R_3=OCH_3
5-H	8.23 (br d, J=6.8 Hz)	7.77 (d, J=2.4 Hz)	7.71 (d, J=2.4 Hz)
6-R_3	7.03~7.48 (m)	3.84 (s)	3.87 (s)
7-H		6.91 (dd, J=8.8, 2.4 Hz)	6.97 (dd, J=8.8, 2.4 Hz)
8-H		7.31 (d, J=8.8 Hz)	7.36 (d, J=8.8 Hz)
1-R_1	2.39 (s)	2.36 (s)	2.40 (s)
2-CH_3	2.35 (s)	2.33 (s)	2.32 (s)
3-OCH_3	3.75 (s)	3.74 (s)	3.83 (s)
4-R_2	8.11 (s)	8.06 (s)	4.06 (s)
NH	9.98 (br s)	9.76 (br s)	9.98 (br s)

Spectra were taken with a Jeol FX-100 spectrometer at 99.55 MHz in Me_2CO-d_6 (δ in ppm), using TMS as an internal standard.

Table 4. ^{13}C NMR data of carbazomycins C (III) and D (IV) compared with those of carbazomycins B (I) and A (II) (δ in ppm).

Carbon No.	Multiplicity	I	II	III	IV
C-1	s	109.7 (109.3)	(113.5)	109.8	114.5
C-2	s	127.8 (127.0)	(128.7)	127.9	129.0
C-3	s	139.3 (138.5)	(144.4)	139.0[b]	144.5
C-4	s	143.6 (142.0)	(145.9)	143.6	146.6
C-4a	s	110.6 (109.3)	(114.4)	110.8	115.0
C-4b	s	124.3 (123.3)	(122.8)	124.7	123.8
C-5	d	123.1 (122.7)	(122.5)	106.2	105.9
C-6	s (d)[a]	119.2 (119.5)	(119.4)	154.0	154.4
C-7	d	124.8 (124.7)	(125.0)	113.9	114.5
C-8	d	111.0 (110.0)	(110.3)	111.5	112.0
C-8a	s	140.7 (139.3)	(139.4)	135.6	135.9
C-9a	s	137.9 (136.8)	(136.4)	138.9[b]	138.7
C-10 (1-CH_3)	q	13.3 (13.1)	(13.6)	13.4	13.7
C-11 (2-CH_3)	q	12.8 (12.7)	(12.6)	12.8	12.7
C-12 (3-OCH_3)	q	61.3 (61.4)	(61.1)	61.3	61.0
C-13 (4-OCH_3)	q	—	(60.5)	—	60.6
C-14 (6-OCH_3)	q	—	—	56.0	56.0

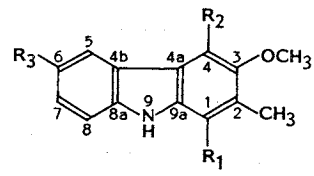
Spectra were taken with a Jeol FX-100 spectrometer at 25.00 MHz using TMS as an internal standard and Me_2CO-d_6 as solvent. Values in parentheses were taken in $CDCl_3$. Parameters are as follows: Spectral width 5 KHz, pulse width 14 μseconds (45°), repetition time 1.8 seconds.

[a] Multiplicity for I and II. [b] Signals could be interchanged.

aromatic methyl groups (δ 2.32 and 2.40), three aromatic protons (δ 6.97, 7.36 and 7.71) and a carbazole-NH group (δ 9.98), but they showed the presence of three methoxyl groups (δ 3.83, 3.87 and 4.06) and the absence of a hydroxyl group. These data and the molecular formula difference between **III** ($C_{16}H_{17}NO_3$) and **IV** ($C_{17}H_{19}NO_3$) indicated the replacement of the hydroxyl group in **III** by a methoxyl group in **IV**. Thus, *O*-methylation of **III** with dimethyl sulfate and potassium carbonate in boiling acetone was carried out to yield **IV**, which confirmed that **IV** is the monomethyl ether of **III**.

The proton signals for H-4 and H-5 of carbazole are generally observed at lower field than the other aromatic protons because of anisotropic effect of the opposite aromatic ring[6]. Accordingly, the broad doublet signal at δ 8.23 was assigned to H-5 in I[2,3]. In the case of **III**, the aromatic proton signal in the lowest field was a *meta*-coupled doublet at δ 7.77 and this was attributed to H-5. The

Fig. 3. Structures of carbazomycins.



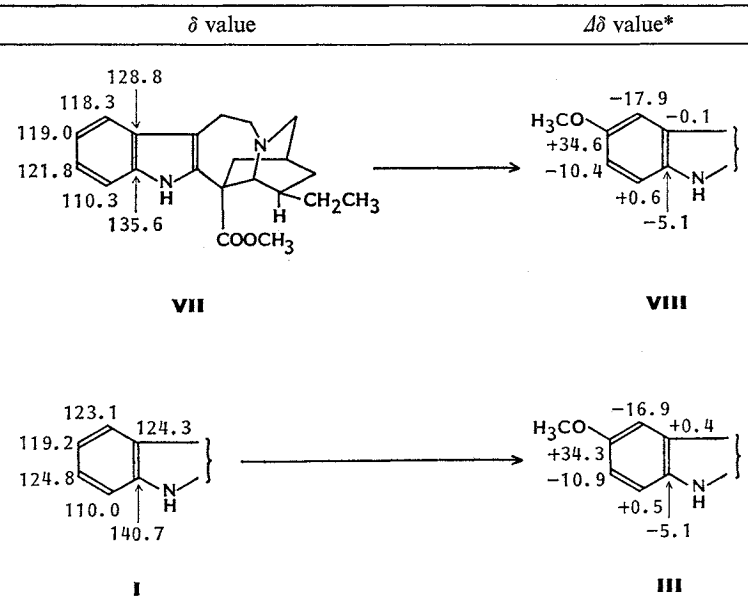
Carbazomycin B (**I**)	$R_1 = CH_3$	$R_2 = OH$	$R_3 = H$
Carbazomycin A (**II**)	$R_1 = CH_3$	$R_2 = OCH_3$	$R_3 = H$
Carbazomycin C (**III**)	$R_1 = CH_3$	$R_2 = OH$	$R_3 = OCH_3$
Carbazomycin D (**IV**)	$R_1 = CH_3$	$R_2 = OCH_3$	$R_3 = OCH_3$
Carbazomycin E (**V**) (Carbazomycinal)	$R_1 = CHO$	$R_2 = OH$	$R_3 = H$
Carbazomycin F (**VI**) (6-Methoxycarbazomycinal)	$R_1 = CHO$	$R_2 = OH$	$R_3 = OCH_3$

upfield shift of H-5 by 0.46 ppm on going from **I** to **III** can easily be interpreted by the introduction of a methoxyl group at the *ortho* position[6,7]. Consequently, among the other two aromatic proton signals, the *ortho*, *meta*-coupled double doublet at δ 6.91 should be assigned to H-7 and the other *ortho*-coupled doublet at δ 7.31 should be due to H-8. These findings suggested that one aromatic ring of a carbazole nucleus of **III** might have one methoxyl group at C-6 and the other aromatic ring should have four other substituents, two methyl, one methoxyl and one hydroxyl groups. Among them, the hydroxyl group should be at C-4 in **III** because, on going from **III** to **IV**, the new methoxyl group suffered anisotropic deshielding by the opposite aromatic ring and appeared at significantly lower field (δ 4.06) than the other two methoxyl signals (δ 3.74 and 3.84 in **III**; δ 3.83 and 3.87 in **IV**).

The ^{13}C NMR data of **III** and **IV** together with those of **I** are shown in Table 4. The assignments were made by the substitution-induced shift trend[8] and by comparison with the data of **I** and **II** whose assignments were made by chemical and spectral methods[2,3] and finally established during the biosynthetic experiments[4].

As seen in Table 4, the carbon chemical shifts of **III** are almost identical with those of **I** except for the chemical shifts due to C-5, C-6, C-7 and C-8a, the methoxylated carbon and its *ortho*- and *para*-carbons, the chemical shifts of which were strongly affected by the introduction of a methoxyl group at C-6[8]. The methoxyl signal at δ 56.0 of **III** and **IV** could be assigned to 6-OCH_3 (C-14) because it is at an isolated position and shows an usual methoxyl value, while the other methoxyl signals appeared at lower field than usual (δ 61.3 in **III**; δ 60.6 and 61.0 in **IV**) and should be located in the *ortho*-disubstituted positions[9,10]. The presence of a methoxyl group at C-6 in **III** was also supported by the ^{13}C NMR data of **III** compared with those of **I**. Chemical shift changes of the carbons of the aromatic ring on going from **I** to **III** caused by introduction of a methoxyl group at C-6 were observed as shown in Fig. 4 and these values are in good accordance with the calculated values from the substitution-induced shift trend[8]. Furthermore, these changes from **I** to **III** agreed well with the chemical shift changes from coronaridine (**VII**) to voacangine (10-methoxycoronaridine) (**VIII**)[11], members of *Iboga* alkaloids, as shown in Fig. 4. On the other hand, the *O*-methylation of 4-OH in **III** to give **IV** caused chemical shift changes of the fully substituted aromatic ring carbons and this substitution-induced shift trend was in good accordance with the trend from **I** to **II** which was confirmed

Fig. 4. Substitution-induced shifts of aromatic carbons in I and III compared with those in VII and VIII.



* $\Delta\delta = \delta_{R-OCH_3} - \delta_{R-H}$.

by comparison with that of model compounds[4].

From the above results, the structures of III and IV have been elucidated as 4-hydroxy-3,6-dimethoxy-1,2-dimethylcarbazole and 3,4,6-trimethoxy-1,2-dimethylcarbazole, respectively, as shown in Fig. 3.

The structures of carbazomycins E (V) and F (VI) have been determined by MARUMO *et al.*[5] as shown in Fig. 3. We attempted chemical correlation of V to I and VI to III. Thus, reduction of V with $LiAlD_4$ and $AlCl_3$ in boiling ether afforded dideuterated carbazomycin B (I') having a CHD_2 group at C-1 (MS: M^+ *m/z* 243). In ^1H NMR spectrum of I', the intensity of the proton signal due to this methyl group at δ 2.39 was greatly reduced and could not be observed practically. The ^{13}C NMR spectrum of I' was identical with that of I except for the carbon signal of CHD_2 at C-1 which virtually disappeared[12], and only one methyl signal due to a methyl group at C-2 was observed at δ 12.8. The reduction of VI in the same way as V afforded dideuterated carbazomycin C (III') (MS: M^+ *m/z* 273). This compound gave an identical ^{13}C NMR spectrum to III except for the methyl signal at C-1 (δ 13.4) which also essentially disappeared.

Antimicrobial Activity

Antimicrobial spectra of carbazomycins C and D together with that of carbazomycin B are shown in Table 5. These minor components showed very weak inhibition against several kinds of fungi as shown.

Experimental

General

Melting points were measured on a Yanaco hot plate apparatus and uncorrected. UV spectra were measured on a Hitachi model 124 spectrophotometer. IR spectra were recorded on a Shimadzu

Table 5. Antimicrobial spectra of carbazomycins B, C and D.

Test organisms	MIC (μg/ml)		
	B	C	D
Micrococcus flavus FDA 16	25	50	>100
M. lysodeikticus IFO 333	50	>100	>100
M. luteus PCI 1001	25	>100	>100
Staphylococcus aureus FDA 209P	25	50	>100
S. aureus Smith	25	100	>100
Bacillus anthracis	25	25	>100
B. subtilis PCI 219	50	100	>100
Escherichia coli K-12	>100	>100	>100
Klebsiella pneumoniae	>100	>100	>100
Serratia marcescens	>100	>100	>100
Candida albicans YU-1200	>100	>100	>100
Saccharomyces cerevisiae	>100	>100	>100
Alternaria kikuchiana	ND	12.5	>100
Aspergillus niger F-16	>100	>100	>100
Colletotrichum gloeosporioides	12.5	100	>100
C. lagenarium	50	50	>100
Gloeosporium laeticolor	50	50	>100
Glomerella cingulata	25	100	>100
G. cingulata No. 3	3.1	25	>100
Trichophyton asteroides 429	6.3	25	100
T. mentagrophytes 833	6.3	100	100

Agar dilution method on glucose - nutrient agar for bacteria and yeasts, and on potato - sucrose agar for fungi. Incubated at 33°C for 24 hours for bacteria and yeasts, and 26°C for 48 hours for fungi.
ND: Not detected.

IR 408 spectrophotometer. ^{1}H and ^{13}C NMR spectra were measured with a Jeol JNM-FX 100 spectrometer at 99.55 MHz and 25.00 MHz, respectively. Mass spectrometry was carried out using a Shimadzu 7000S and a Jeol JMS-DX 300 (JMA 3100 data system) mass spectrometer by the direct inlet method. For column chromatography, Merck Kieselgel 60, Art 7734, was used.

O-Methylation of **III** to **IV**

To a solution of **III** (3.2 mg) in dry $(CH_3)_2CO$ (3 ml) was added anhydrous K_2CO_3 (300 mg) and $(CH_3)_2SO_4$ (0.2 ml) and the mixture was stirred under reflux for 1 hour. After usual work-up, the product was extracted with CH_2Cl_2 and purified by preparative TLC developed with *n*-hexane - EtOAc (3: 1) to give an almost colorless powder (2 mg). Recrystallization from *n*-hexane - CH_2Cl_2 afforded 0.7 mg of colorless needles; mp 129.0°C. This compound obtained here was identified with **IV** by TLC and mixed mp and by comparison of MS and IR (KBr) spectra.

Reduction of **V** with $LiAlD_4$ and $AlCl_3$

To a solution of $AlCl_3$ (335 mg) in dry ether (5 ml) was added $LiAlD_4$ (32 mg) and then **V** (16.4 mg), and the mixture was refluxed under stirring for 3 hours. After usual work-up, the product was purified by preparative TLC developed with *n*-hexane - EtOAc (3: 1) to give 8.5 mg of pale yellow powder. Recrystallization from *n*-hexane and EtOAc yielded 4 mg of dideuterated carbazomycin B (**I'**), pale yellow prisms: MP 137°C; MS M^+ *m/z* 243.

Reduction of **VI** with $LiAlD_4$ and $AlCl_3$

VI (8 mg) was reduced with $LiAlD_4$ (32 mg) and $AlCl_3$ (203 mg) in boiling dry ether (5 ml) as above and purified by preparative TLC to give 2.2 mg of pale yellow powder. Recrystallization from *n*-hexane and EtOAc afforded pale yellow prisms of dideuterated carbazomycin C (**III'**), 1 mg: MP 198~199°C; MS M^+ *m/z* 273.

Acknowledgment

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