Skeletal Structure of Neocopiamycin B from *Streptomyces hygroscopicus* var. *crystallogenes*

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Streptomyces hygroscopicus var. *crystallogenes*, IFM 1136 (FERM BP-576), produces antibiotic copiamycin (niphithricin, **2**, Fig. 1)[1~4] along with minor macrocyclic lactone antibiotics, neocopiamycin A (**3**), demalonylcopiamycin (**4**), demalonylmethylcopiamycin (**5**) and guanidolide A, possessing a guanidino alkenyl side chain.[5~7] Copiamycin had been found to possess synergistic activity with chlorinated imidazole antimycotics such as clotrimazole, miconazole, econazole and especially ketokoazole.[8] ARAI *et al.* had reported that the strain produces a new macrocyclic lactone antibiotic, neocopiamycin B, when cobalt (II) chloride is added into the original medium.[9] Neocopiamycin B proved to be more active against some fungi than copiamycin (**2**) and/or neocopiamycin A (**3**) and low toxicity for mice (LD_{50}: >25 mg/kg, iv).[9] In this paper, we describe the skeletal structure of neocopiamycin B.

Materials and Methods

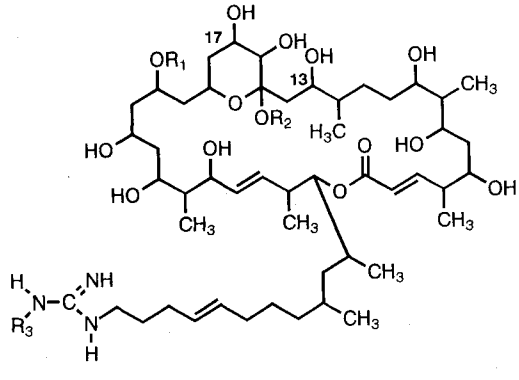
Instruments

FAB-MS spectrum was obtained with a Jeol DX303 mass spectrometer. MALDI-TOF MS spectrum was obtained with a PerSeptive Biosystems Voyager-DE STR with nitrogen laser (337 nm). Instrument operation was performed in delayed extraction reflector mode. The solid matrix used for MALDI was α-cyano-4-hydroxycinnamic acid. The milli-mass spectrum with MALDI-TOF MS was calibrated using angiotensin I and des-Arg(1)-bradykinin as the internal standard: Number of data points was 50000 and the data were collected from 500 to 1550 mass range with 128 laser shots. Time interval between subsequent data points was 0.5 nsec. Accelerating voltage is 20 kV and grid voltage is 14.7 kV. NMR spectra were recorded on a Jeol JNM-α-500 (500 MHz for ^1H and 125 MHz for ^{13}C) spectrometer at 50°C. As ^1H NMR chemical shift reference, the center signal of CD_3OD at δ 3.30 and DMSO-d_6 at δ 2.49, and as ^{13}C NMR reference, the methyl signal of CD_3OD at δ 49.0 were used. ^1H NMR spectrum of **1** was assigned with COSY and HOHAHA (59 msec) spectra. ^1H and ^{13}C NMR spectra of **2** were assigned with ^1H-^1H COSY, HOHAHA (35, 61, and 109 msec), *J*-resolved, HSQC, and ^{13}C-^1H COSY spectra.

Purification of Neocopiamycin B (1) and Copiamycin (2)

Crude neocopiamycin B (10 mg)[9] was purified by preparative TLC (silica gel, 2-BuOH - H_2O - 28% ammonia (8 : 2 : 1)) to give pure **1** (2.5 mg). Purified copiamycin with preparative TLC (silica gel, 2-BuOH - H_2O

Fig. 1. Structures of copiamycin (**2**) and its derivatives (**3**~**5**).



Copiamycin (**2**)
R_1 = $COCH_2COOH$, R_2 = H, R_3 = CH_3

Neocopiamycin A (**3**)
R_1 = $COCH_2COOH$, R_2 = R_3 = H

Demalonylcopiamycin (**4**)
R_1 = R_2 = H, R_3 = CH_3

Demalonylmethylcopiamycin (**5**)
R_1 = H, R_2 = R_3 = CH_3

Table 1. ^{13}C and ^{1}H NMR data of neocopiamycin B (**1**) and copiamycin (**2**) in CD_3OD.

C	1 δ_C	2 δ_C	1 δ_H	2 δ_H
1	168.3	168.3	———	———
2	123.2	123.2	5.87 (d, J = 15.5)	5.86 (d, J = 15.5)
3	152.5	152.5	6.95 (dd, J = 8.5, 15.5)	6.93 (dd, J = 8.5, 15.5)
4	43.4	43.5	2.46 (m)	2.46 (m)
5	75.4	75.3	3.76	3.76 (td, J = 5, 10)
6	39.4	39.5	~ 1.50, ~ 1.75	~ 1.51, ~ 1.75
7	74.8	(75.0)*	3.72	~ 3.77
8	45.1	44.5	1.50 (m)	~ 1.55
9	72.6	(72.5)	3.75 (m)	~ 3.88
10	45.2	(44.7)		(~ 1.75)
11	37.0	(33.4)		(~ 1.58, ~ 1.65)
12	42.9	(44.5)		(~ 1.53)
13	33.4	(72.3)		~ 3.88
14	39.6	41.9		~ 1.67, ~ 1.78
15	102.6	99.8	———	———
16	76.1	77.3	3.75 – 3.9	3.35 (d, J = 9)
17	67.6	(69.8)	3.75 – 3.9	~ 3.87
18	42.8	41.2	~ 1.62, ~ 1.98	~ 1.29, ~ 1.90
19	65.8	65.9	4.00 (m)	4.09
20	42.1	42.1	~ 1.75, ~ 1.85	~ 1.67, ~ 1.78
21	71.4	71.1	5.29 (septet-like)	5.22 (septet-like)
22	40.9	40.6	~ 1.75, ~ 1.85	1.63 – 1.81
23	65.2	(65.8)	3.95 (m)	~ 3.88
24	43.0	43.1	~ 1.4, ~ 1.6	~ 1.39, ~ 1.60
25	69.3	(69.3)	4.18 (td, J = 5, 10)	4.13 (ddd, J = 2, 4.5, 10)
26	45.3	(45.4)	1.50 (m)	~ 1.50
27	76.1	(76.0)	3.88 (t, J = 8)	~ 3.88
28	134.5	134.7†	5.46	5.47 (dd, J = 8, 15.5)
29	134.9	134.7†	5.55 (dd, J = 8, 15.5)	5.53 (dd, J = 8, 15.5)
30	39.6	39.7	2.54 (m)	2.52 (m)
31	80.9	80.4	4.73 (t, J = 6)	4.75 (dd, J = 5, 7)
32	33.3	33.0	1.90 (m)	~ 1.92
33	42.3	(42.4)	~ 1.30	(~ 0.90, ~ 1.32)
34	30.6	(30.8)	~ 1.60	(~ 1.58)
35	37.1	37.3		~ 1.09, ~ 1.28
36	27.5	27.6		~ 1.28, ~ 1.37
37	33.7	33.7	~ 1.98 (m)	~ 1.97
38	132.9	132.8	5.48 (dd-like, J = 6, 15)	5.48
39	130.0	129.9	5.43 (dt, J = 6, 15)	5.42 (td, J = 6, 15.5)
40	30.3	30.6	2.08 (td, J = 6, 7)	2.07 (td, J = 7, 7)
41	29.9	29.9	~ 1.65 (m)	~ 1.65
42	42.0	42.0	3.17 (t, J = 7)	3.17 (t, J = 7)
43	16.5	16.5	1.12 (d, J = 6.5)	1.12 (d, J = 7)
44	10.6	(10.6)	0.88 (d, J = 7)	0.89 (d, J = 7)
45	14.7	(14.7)	0.88 (d, J = 8)	(0.88, d, J = 7)
46	11.5	11.4	0.79 (d, J = 7)	0.79 (d, J = 7)
47	17.8	17.7	0.99 (d, J = 7)	0.99 (d, J = 7)
48	15.3	(15.1)	0.87 (d, J = 7)	(0.88, d, J = 7)
49	20.8	(20.7)	0.92 (d, J = 7)	(0.87, d, J = 7)
50	158.4	158.4	———	———
51	28.3	28.3	2.83 (s)	2.84 (s)
1'	171.5	171.6	———	———
2'	——— (a)	——— (a)	——— (a)	——— (a)
3'	——— (b)	174.0	———	———

* Signals show in parentheses are tentative assignments. † Appeared at δ 134.66 (correlated with the signal at δ 5.55) and 134.69 (correlated with signal at δ 5.46). (a): The signal(s) did not appear because of easily exchange of H_2-2′ with deuterium at the measurement temperature (50°C), [**1**: δ 3.06 (2H, br s), **2**: δ 3.05 (2H, br s) in DMSO-d_6)]. (b): The signal was not observed because of a limitation in the quantity of the available sample.

(4:1)) was recrystallized from MeOH-H_2O.

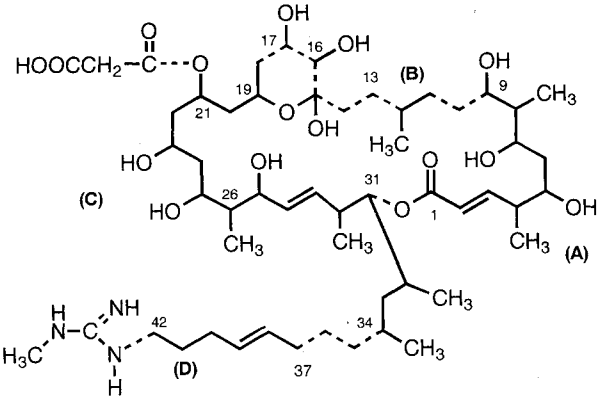
Neocopiamycin B (1)

Neocopiamycin B (**1**) was obtained as a colorless powder. MALDI-TOF MS: *m/z* 1042.6802 $(M+H)^+$, $\Delta+1.1$ milli-mass unit (mmu) for $C_{54}H_{96}N_3O_{16}$, 1024.6681, $\Delta-0.4$ mmu for $C_{54}H_{94}N_3O_{15}$, 956.6801, $\Delta+1.3$ mmu for $C_{51}H_{94}N_3O_{13}$, 938.6700, $\Delta+1.9$ mmu for $C_{51}H_{92}N_3O_{12}$, 920.6546, $\Delta-2.9$ mmu for $C_{51}H_{90}N_3O_{11}$. FAB-MS: *m/z* 1042 $(M+H)^+$, 1024, 938, 448, 360, 348, 320, 294, 282, 266, 252, 224, 210, 182, 168, 154, 100, 87, 73, 57. ^1H NMR (DMSO-d_6): δ 0.68 (3H, d, $J=7$ Hz, H_3-46), 0.72 (3H, d, $J=7$ Hz, H_3-49), 0.76 (3H, d, $J=6.5$ Hz, H_3-48), 0.80 (3H, d, $J=7$ Hz, H_3-45), 0.85 (3H, d, $J=7$ Hz, H_3-44), ~0.86 (H-33), 0.87 (3H, d, $J=6.5$ Hz, H_3-43), 1.06 (3H, d, $J=7$ Hz, H_3-47), ~1.22, ~1.30 (H-33), ~1.32 (H-34), ~1.42 (H-26), ~1.51 (H-12), ~1.52 (H_2-41), ~1.53 (H-8), ~1.62, ~1.81 (H_2-20 and/or H_2-22), ~1.77 (H-32), ~1.93 (H_2-37), 1.98 (2H, H_2-40), 2.46 (1H, br, H-4), 2.54 (1H, br, H-30), 2.70 (3H, s, H_3-51), 2.95 (1H, d, $J=14$ Hz, H-16), 3.05 (3H, br, H-17 and H_2-42), ~3.50, ~1.27, ~3.80 (HOC*H*$CH_{(2)}$C*H*OH), ~3.52, ~1.33, ~3.72 (HOC*H*$CH_{(2)}$C*H*OH), ~3.58 (H-19), ~3.79 (H-27), 3.98 (1H, br d, $J=10$ Hz, H-25), 4.60 (1H, dd, $J=5$ and 8 Hz, H-31), 5.04 (1H, m, H-20), 5.33~5.43 (3H, m, H-28, -38, and -39), 5.48 (1H, dd, $J=7$ and 15 Hz, H-29), 5.89 (1H, d, $J=16$ Hz, H-2), 6.85 (1H, dd, $J=9$ and 16 Hz, H-3).

Results and Discussion

Crude neocopiamycin B[9] was purified by preparative TLC to give pure neocopiamycin B (**1**). Previously the molecular formula of **1** was reported as $C_{53}H_{91}O_{17}N_3 \cdot 4H_2O$ by its FAB-MS and elemental analysis.[9] But, matrix-assisted laser desorption/ionization time-of-flight mass spectrum (MALDI-TOF MS) of **1** gave a MW, *m/z* 1042.6802 $(M+H)^+$, which fits the molecular formula, $C_{54}H_{95}O_{16}N_3$. The mass spectrum also showed the fragment ions at *m/z* 1024.6681 $(MH-H_2O)^+$, 956.6801 $[MH-(malonyl-H)]^+$, 938.6700 $(M-O\text{-malonyl})^+$, and 920.6546 $(938-H_2O)$ indicating that the compound is a malonyl ester. The other significant ion was not observed in the MALDI-TOF MS. The ^1H and ^{13}C NMR spectra of **1** (Table 1) showed the presence of the alkenyl side chain and macrocyclic moieties consist with eight methyl groups (one is substituted with a nitrogen atom; δ_C 28.3), six olefinic carbons, one sp^2 carbon

Fig. 2. Partial structures (A~D) of neocopiamycin B (**1**).



bearing to nitrogen atoms (δ_C 158.4), one carbonyl carbon (δ_C 168.3), one hemiketal carbon (δ_C 102.6) and 34 methine or methylene carbons in which 11 carbons are substituted with an oxygen function (δ_C 65.2~80.9). The spin-spin network founded with the ^1H-^1H COSY spectrum (in CD_3OD) showed four partial structures, A~D (Fig. 2). By the comparison of chemical shifts and coupling patterns of **1** with those of copiamycin (**2**), three partial structures A, C, and D could be assigned as C1~C9, C18~C34, and C37~C42, respectively. The 21-H signal of copiamycin and its derivatives shows a significant coupling pattern (septet-like signal). The 21-H signal of **1** appeared at δ 5.29 (septet-like signal) indicating that the malonyl group links to the C21 position (the signal of desmalonyl compounds, **4** and **5**, appears at δ 4.04 and 4.05, respectively, and that of malonylated compounds, **2** and **3**, appears at δ 5.22 and 5.21, respectively).[5~7] These partial structures were supported further by the ^{13}C NMR spectra: On the comparison of ^{13}C NMR spectra of **1** and **2**, the chemical shifts of the assignable signals for the partial structures of **1** resembled to those of **2** (Table 1). On the other hand, the FAB-MS of **1** gave the fragment ions at *m/z* 100, 154, 168, 182, 210, 224 and 254 derived from cleavages on the guanidino alkenyl side chain (Fig. 3) as well as that of copiamycin.[5] Thus, the compound **1** has the same alkenyl side chain as **2**, **4** and **5**. These above data suggested that neocopiamycin B may be 13-deoxy or 16-deoxy or 17-deoxy copiamycin. The 13-deoxy or 17-deoxy structure for **1** was suggested by the chemical shifts of C15 of **1** (δ 102.6)

Fig. 3. Fragmentations in the positive FAB-MS of neocopiamycin B (**1**).

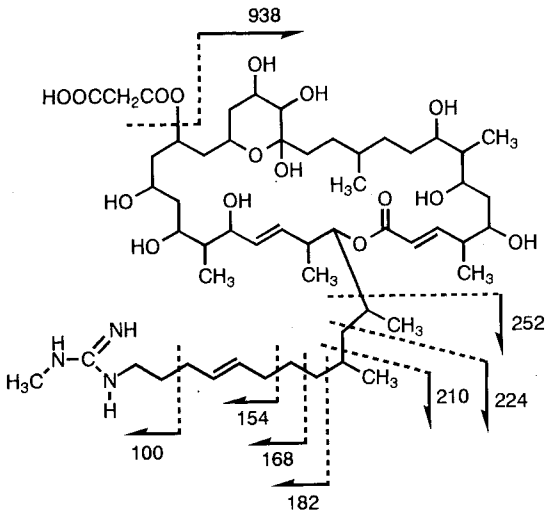
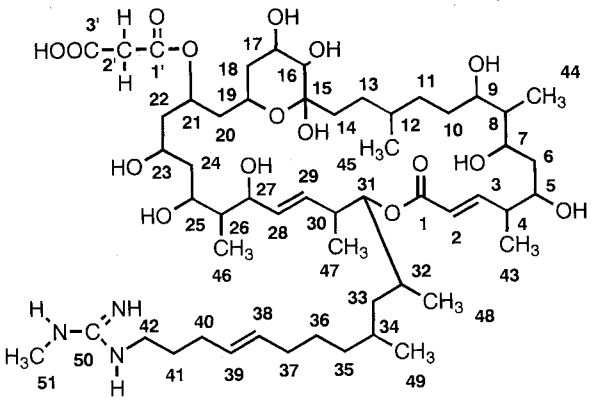


Fig. 4. Structure of neocopiamycin B (**1**).



as following. The signals of **2**~**4** appeared more upfield (about 3 ppm, δ 99.3~99.8) than that of **1**. The upfield shift could be explain by the *γ-trans* effect of a hydroxy group (C13-OH or C17-OH).[10] Furthermore, if compound **1** is 16-deoxycopiamycin, the C15 signal must be observed at near δ 90 with a release from β effect of C16-OH (downfield shift of 8~10 ppm).[10] Thus, the 16-deoxy structure was eliminated. The discrimination between the remained two structures was established by the ^{1}H NMR spectrum of **1** measured in DMSO-d_6. In the solvent, the spectrum was observed as broad signals, but hydroxy methine proton signals appeared in relatively wide range (δ 2.95~4.00). The H-16 signal was appeared at δ 2.95 as a doublet (J = 14 Hz) correlated to the signals at δ 3.05 (H-17 and H_2-42) in the ^{1}H-^{1}H COSY spectrum indicating the presence of the C16-OH and C17-OH groups. From the above all data, the skeletal structure of neocopiamycin B was assigned to 13-deoxycopiamycin (**1**, Fig. 4).

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