ISOLATION OF NEOCOPIAMYCIN A FROM *STREPTOMYCES HYGROSCOPICUS* VAR. *CRYSTALLOGENES*, THE COPIAMYCIN SOURCE

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Fermentation of *Streptomyces hygroscopicus* var. *crystallogenes*, the copiamycin source, yielded several minor components with antifungal activity.

One of these minor components, neocopiamycin A, was isolated and characterized. The structure of neocopiamycin A was determined as *N*-demethylcopiamycin on the basis of spectroscopic evidence. The antibiotic was found to be more active against Gram-positive bacteria and fungi but less toxic than copiamycin.

Recently *Streptomyces hygroscopicus* var. *crystallogenes*, the source of copiamycin[1,2,3], was found to produce several minor components in addition to copiamycin, a macrocyclic lactone antifungal antibiotic[4]. One of these minor components was isolated and designated as neocopiamycin A. Copiamycin was found to possess novel synergistic activity with chlorinated imidazole antimycotics such as clotrimazole, miconazole, econazole and especially ketoconazole[5]. Neocopiamycin A proved to be more active against a number of fungi, and more synergistic with the above antimycotics, but less toxic than copiamycin. This paper describes the production, isolation, physicochemical and biological characteristics and skeletal structure of neocopiamycin A.

Fermentation

Spores of *Streptomyces hygroscopicus* var. *crystallogenes* IFM 1136 (ATCC 19040) grown on glucose - asparagine agar (Krainsky) were inoculated into 500 ml Sakaguchi flasks each containing 100 ml of a medium composed of 2% soy bean meal, 2% starch, 0.5% Ebios (dried yeast), 0.0007% $MnCl_2 \cdot 4H_2O$, 0.0007% $CuSO_4 \cdot 5H_2O$, 0.003% $ZnSO_4 \cdot 7H_2O$, 0.35% $CaCO_3$ and Silicone TSA 0.003%. The

Table 1. Physico-chemical properties of neocopiamycin A.

Nature	Neutral, colorless powder, hygroscopic	
Mp	134~137°C (dec)	
$[\alpha]_D^{25}$	+23° (*c* 1.0, MeOH)	
UV λ_{max}^{MeOH}	205 nm (log ε 4.2)[a]	
Elementary analysis (%)	Found:	C 58.59, H 8.81, N 3.69
	Calcd for $C_{53}H_{93}N_3O_{17} \cdot 2H_2O$:	C 58.94, H 8.99, N 3.89
MW	1,043	
FD-MS	1,044 (MH^+)	
FAB-MS	1,044 (MH^+)	
SIMS	1,044 (MH^+)	
Solubility	Soluble in pyridine, dimethyl sulfoxide, MeOH, EtOH, BuOH	
	Insoluble in acetone, $CHCl_3$, EtOAc, benzene, ether, *n*-hexane, water	
IR (KBr)	See Fig. 2	

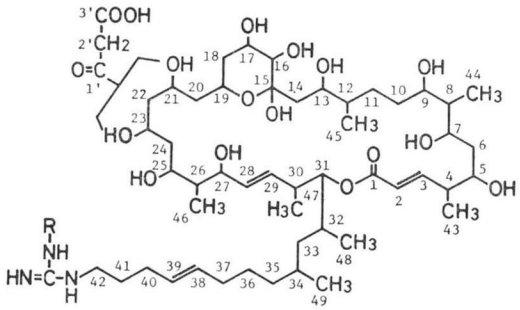
[a] Hitachi double wavelength double beam spectrophotometer model 557.

flasks were incubated on a reciprocal shaker at 125 strokes per minute, at 27°C for 48 hours. One liter of the culture broth was transferred into a 200-liter fermentor containing 100 liters of the medium as described above. The fermentation was carried out under aeration of 50 liters/minute, agitation at 250 rpm, inner pressure of 0.5 kg/cm^2 and at 27°C. Time course of antibiotic production was followed by paper disc method using *Candida albicans* IFM 40009 as a test organism. The production of the antibiotics reached maximum after 72 hours of fermentation.

Isolation and Purification

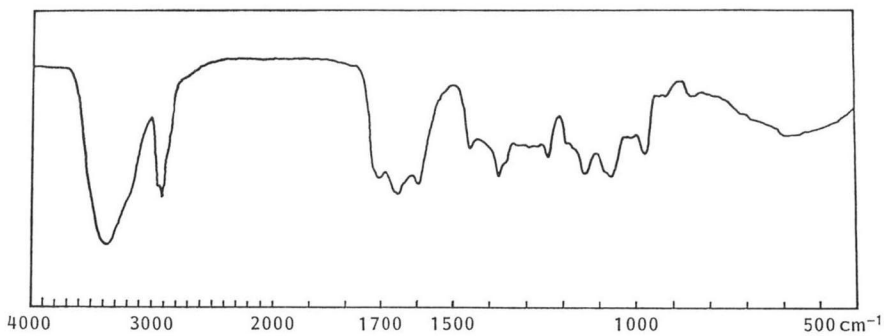
Copiamycin group antibiotics mainly accumulated in the mycelia and were scarcely found in the culture filtrate. Thus, the mycelial cake was collected by continuous centrifugation and washed with water. The collected wet cake, 7.8 kg, was extracted twice with 3 and 1.5 volumes of methanol successively. The solvent extracts were combined and concentrated to one tenth of original volume under reduced pressure. An equal volume of water was added to the concentrate and the mixture was extracted twice with an equal amount of 1-butanol. The extract was evaporated under reduced pressure to give a brown solid (250 g). The solid was dissolved in a small amount of methanol and chromatographed on a silica gel column (Mallincrodt Co., U.S.A.) and developed with chloroform, followed by a solvent mixture of chloroform - methanol (4: 1). Copiamycins were not found in these eluates but were found in those which were eluted with chloroform - methanol (3: 2) and (1: 1). The active eluates were combined and evaporated *in vacuo* to give a slightly yellowish powder (43 g). A methanolic solution of the powder, allowed to stand at room temperature, gave a crystalline precipitate which yielded colorless prisms (23 g) by recrystallization from aqueous methanol. The crystalline materials thus obtained were shown to be a complex composed of one major component (copiamycin, Rf 0.25) and several minor components including neocopiamycin A (Rf 0.35) by TLC analysis (silica gel 60 F_{254}, Merck; solvent system: 2-butanol - H_2O, 4 : 1; detection: iodine or sulfuric acid). Isolation of neocopiamycin A from the copiamycin complex was achieved by preparative TLC on Merck silica gel 60 F_{254} (20 cm × 20 cm, 0.5 mm thick). The developing solvent mixture was, 2-butanol - water

Fig. 1. Structures of neocopiamycin A and copiamycin.



Neocopiamycin A (**I**) R=H
Copiamycin (**II**) R=CH_3

Fig. 2. IR spectrum of neocopiamycin A.



(4: 1). The neocopiamycin A containing materials, scrapped out from the TLC plates under UV light, were combined and eluted with ethanol. The solvent was evaporated under vacuum resulting in a white powder. From 1 g of the crystalline copiamycin complex, 35 mg of pure neocopiamycin A was obtained. Attempts to crystallize neocopiamycin A have so far been unsuccessful.

Physico-chemical Properties and the Structure

Neocopiamycin A was obtained as a neutral, hygroscopic, colorless powder, soluble in dimethyl sulfoxide, pyridine and lower alcohols, but insoluble in other organic solvents and water.

The antibiotic showed a positive reaction to iodine on silica gel TLC plate. The physico-chemical properties of neocopiamycin A are summarized in Table 1 and its skeletal structure is illustrated in Fig. 1 together with that of copiamycin (**II**). Field desorption (FD), fast atom bombardment (FAB) and secondary ion mass spectrometric (SIMS) analyses of neocopiamycin A gave the protonated molecular ion peak, MH^+, at m/z 1,044 which is smaller by 14 mass units than that of copiamycin, whose skeletal structure has been elucidated already[4,6,7,8]. The molecular formula of neocopiamycin A was postulated to be $C_{53}H_{93}N_3O_{17}$ (MW 1,043) from the above mass spectrometric analyses, elementary analysis and the number of peaks in the ^{13}C NMR spectrum. Its ultraviolet (UV) absorption maximum at 205 nm ($\log \varepsilon$ 4.2) suggested the presence of the same chromophore as in **II** (α,β-unsaturated carboxylic ester group).

Table 2. ^{13}C NMR data for neocopiamycin A in CD_3OD.

Signal No.	Chemical shift (ppm)	Multiplicity	Assignment[a]	Signal No.	Chemical shift (ppm)	Multiplicity	Assignment[a]
1	10.57	q		27	45.41	d	
2	11.39	q		28	46.19	t	2′
3	14.54	q		29	65.75	d	
4	15.18	q		30	65.84	d	
5	16.66	q	43	31	69.04	d	
6	17.72	q	47	32	69.76	d	
7	20.77	q		33	71.19	d	[b]
8	27.67	t	40	34	72.06	d	
9	29.87	t		35	72.56	d	
10	30.62	t+t*		36	74.66	d	
11	30.73	d*		37	75.29	d	
12	32.92	d		38	75.94	d	
13	33.39	t		39	77.09	d	16
14	33.78	t		40	80.32	d	31
15	37.18	t		41	99.78	s	15
16	39.62	t+d*		42	123.29	d	2
17	40.61	d		43	129.95	d	39
18	41.31	t	42	44	132.95	d	38
19	41.62	t		45	134.79	d	28
20	41.94	t		46	134.82	d	29
21	42.01	t		47	152.59	d	3
22	42.34	t		48	158.78	s	[c]
23	43.18	t		49	168.28	s	1
24	43.40	d	4	50	171.61	s	1′
25	44.69	d		51	174.30	s	3′
26	44.80	t					

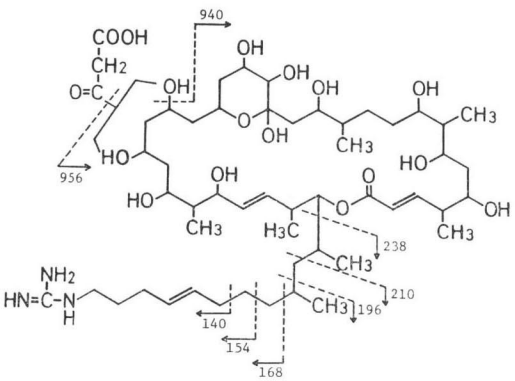
[a] Assignments were made by ^1H-^{13}C selective decoupling experiments.
[b] The carbon bearing malonyl hemiester.
[c] Guanido carbon.
* Insensitive nuclei enhanced by polarization transfer technique was used.

Table 3. Assigned ^{1}H NMR signals of neocopiamycin A in CD_3OD.

Signal No.	Chemical shift (ppm)	Multiplicity and coupling constant (J in Hz)	Assignment[a]
1	6.92	1H, dd, $J_{3,2}$=15.9 Hz, $J_{3,4}$=9.1 Hz	H-3
2	5.87	1H, d, $J_{2,3}$=15.9	H-2
3	5.55	1H, dd, $J_{29,28}$=15.8, $J_{29,30}$=7.3	H-29
4	5.49	1H, m, $J_{38,39}$=15.2	H-38
5	5.46	1H, m, $J_{28,29}$=15.8	H-28
6	5.42	1H, m, $J_{39,38}$=15.2	H-39
7	5.21	1H, m	[b]
8	3.89	1H, m	H-27
9	3.85	1H, m	H-17
10	3.74	1H, m	H-5
11	3.37	1H, d, $J_{16,17}$=9.4	H-16
12	3.24	2H, s	H_2-2′
13	3.17	2H, t, $J_{42,41}$=7.2	H_2-42
14	2.54	1H, m, $J_{30,29}$=7.4, $J_{30,31}$=6.9, $J_{30,47}$=6.8	H-30
15	2.48	1H, m	H-4
16	2.07	2H, m	H_2-40
17	1.99	2H, m	H_2-37
18	1.91	1H, m	H-32
19	1.65	2H, m, $J_{41,42}$=7.2	H_2-41
20	1.12	3H, d, $J_{43,4}$=6.9	H_3-43
21	0.99	3H, d, $J_{47,30}$=6.7	H_3-47
22	0.90	3H, d	H_3-48
23	0.78	3H, d, J=6.9	

[a] Assignments of respective signals were made by decoupling experiments.
[b] The methine group bearing malonyl hemiester.

Fig. 3. Speculative fragmentations in the positive FAB mass spectrum of neocopiamycin A.



The IR spectrum (Fig. 2) of neocopiamycin A was quite similar to that of **II**. The ^{13}C NMR (67.5 MHz) and ^{1}H NMR (270 MHz) spectral data of neocopiamycin A are listed in Tables 2 and 3, respectively.

Comparison of the NMR data of neocopiamycin A with those of **II**[4] revealed that signals corresponding to the *N*-methyl group found in **II**, H 2.83 ppm and C 28.35 ppm, are absent from the spectra of neocopiamycin A.

This led to the conclusion that the structural difference between these two antibiotics lies only in the guanidine moieties, in which a methyl group is present in **II**, but not in neocopiamycin A. The conclusion was also supported by the comparison of the FAB mass spectra of both antibiotics. Accompanied by the MH^+ ion peaks (m/z 1,044 and 1,058), a series of the fragment ion peaks were found as follows; m/z 1,026 (MH^+-H_2O), 956 ($MH-HOOCCH_2CO)^+$, 940 ($MH-HOOCCH_2COO)^+$, 434, 346, 334, 306, 280, 268, 238, 210, 196, 168, 154 and 140 in neocopiamycin A: m/z 1,040, 970, 954, 448, 360, 348, 320, 294, 282, 252, 224, 210, 182, 168 and 154 in **II**. All these fragment ion peaks in the spectrum of neocopiamycin A shift to positions 14 mass units lower. This evidence supports the conclusion above. Some of the fragment ions observed in the spectrum of neocopiamycin A are presumed to be produced by cleavage at the

Table 4. Antibacterial spectra of neocopiamycin A and copiamycin.

Test organism	MIC (μg/ml)	
	Neocopiamycin A	Copiamycin
Bacillus subtilis PCI 219	12.5	>100.0
Micrococcus luteus IFM 2066	6.25	100.0
Staphylococcus aureus 209P	12.5	>100.0
S. albus IFM 2013	12.5	>100.0
S. citreus IFM 2025	12.5	>100.0
Streptococcus faecalis IFM 2001	100.0	>100.0
Corynebacterium diphteriae IFM 2056	3.13	50.0
Escherichia coli	>100.0	>100.0
Klebsiella pneumoniae IFM 3008	25.0	>100.0
Proteus vulgaris IFM 3014	>100.0	>100.0
Pseudomonas aeruginosa IFM 3011	>100.0	>100.0
Salmonella typhimurium IFM 3023	>100.0	>100.0
Serratia marcescens IFM 3027	>100.0	>100.0
Mycobacterium sp. 607	>100.0	>100.0
Nocardia asteroides IFM 0042	25.0	>100.0
N. brasiliensis IFM 0082	25.0	>100.0
Streptomyces paraguayensis IFM 1148	50.0	>100.0

Table 5. Antifungal spectra of neocopiamycin A and copiamycin (1).

Test organism	MIC (μg/ml)	
	Neocopiamycin A	Copiamycin
Candida albicans IFM 40001	1.56	12.5
C. albicans IFM 40002	3.13	25.0
C. albicans IFM 40003	3.13	25.0
C. albicans IFM 40004	6.25	100.0
C. albicans IFM 40005	1.56	25.0
C. albicans IFM 40007	3.13	12.5
C. albicans IFM 40008	1.56	12.5
C. albicans IFM 40009 (7N)	6.25	100.0
C. guilliermondii IFM 40017	3.13	100.0
C. tropicalis IFM 40018	3.13	100.0
C. krusei IFM 40019	3.13	100.0
C. parapsilosis IFM 40020	3.13	100.0
C. stellatoidea IFM 40021	3.13	100.0
C. utilis IFM 40099	12.5	>100.0
Cryptococcus neoformans IFM 40037	<0.78	1.56
C. neoformans IFM 40038	<0.78	1.56
C. neoformans IFM 40047	1.56	6.25
Geotrichum candidum IFM 40068	1.56	1.56
Torulopsis glabrata IFM 40065	6.25	100.0
Trichosporon cutaneum IFM 40066	6.25	100.0
Saccharomyces cerevisiae sake IFM 40025	12.5	>100.0
Sporothrix schenckii IFM 40751 (yeast phase)	3.13	100.0

positions of the molecule shown in Fig. 3. Although no chemical degradation experiment was attempted, the skeletal structure of neocopiamycin A was elucidated as **I** on the basis of the spectroscopic evidence and the analytical data.

Table 6. Antifungal spectra of neocopiamycin A and copiamycin (2).

Test organism	MIC (μg/ml)	
	Neocopiamycin A	Copiamycin
Aspergillus flavus 23	6.25	100.0
A. fumigatus 25	25.0	>100.0
A. nidulans 21	6.25	100.0
A. niger 22	6.25	>100.0
A. oryzae IFM 40607	6.25	>100.0
A. versicolor 26	12.5	>100.0
Penicillium expansum IFM 40619	3.13	100.0
Epidermophyton floccosum IFM 40747	0.78	0.39
Microsporum canis	1.56	3.13
M. gypseum IFM 40727	1.56	6.25
Trichophyton mentagrophytes IFM 40737	1.56	3.13
T. mentagrophytes Kamiyama	1.56	3.13
T. rubrum IFM 40732	0.39	3.13
Sporothrix schenckii IFM 40750 (hyphal phase)	6.25	50.0
Fonsecaea pedrosoi IFM 40756	3.13	100.0
Histoplasma capsulatum IFM 40752 (hyphal phase)	0.78	0.78

Biological Activity

Antimicrobial Activity

The minimum inhibitory concentration (MIC) of neocopiamycin A and copiamycin against bacteria, yeasts and fungi were determined by a serial two-fold agar dilution method. The medium used was Mueller-Hinton agar for bacteria and Sabouraud dextrose agar for yeasts and fungi. The antibiotics were dissolved in a small amount of ethanol and diluted with sterilized water to give final agar media containing less than one percent of the solvent, which was not a harmful concentration against the organisms tested. The MICs were determined after incubation for 24 hours or 4 days at 37°C for bacteria and 24 hours to 7 days at 37°C for yeasts and fungi, depending on the test strain.

Antimicrobial spectra of neocopiamycin A and copiamycin are given in Tables 4, 5 and 6. The tables show that neocopiamycin A is active against a wide range of yeasts, fungi and Gram-positive bacteria but inactive against Gram-negative bacteria and that it has much higher antimicrobial activity than copiamycin. Furthermore, it should be pointed out that the antibiotic inhibits the growth of both filamentous and yeast-like fungi at concentrations below 10 μg/ml. The growth of *Trichomonas vaginalis* was inhibited at the concentration of 1.56 μg/ml in thioglycolated medium with 0.5% dextrin and 15% bovine serum.

Toxicity

Acute toxicity of neocopiamycin A was determined with *dd*Y mice weighing 20 to 21 g. Neocopiamycin A was finely pulverized and suspended in 10% ethanol solution for intravenous, intraperitoneal and oral administrations. Neocopiamycin A was relatively nontoxic as follows: All mice tolerated for 14 days without any toxic signs 30 mg/kg of the antibiotic by intravenous injection and more than 1,000 mg/ml by intraperitoneal and oral administrations.

Discussion

When copiamycin was first isolated, the antibiotic was found to be sparingly soluble or insoluble in

most organic solvents and water, fungistatic rather than fungicidal, and no detailed studies on its chemotherapeutic efficacy were conducted. During the cource of studies on the mode of action of copiamycin and its novel synergism with imidazole antimycotics, crude preparations of copiamycin were found to contain a minor component which is more active than copiamycin. Considerable difficulties have been encountered in obtaining the minor component in a pure state. The component was finally isolated and designated as neocopiamycin A. The new antibiotic seems to be promising as antifungal chemotherapeutic because it is more soluble in organic solvents, more active against a variety of microorganisms, but less toxic than copiamycin. The antibiotic also exhibits strong synergism with imidazole antimycotics as dose copiamycin. Further *in vitro* and *in vivo* evaluation of the antibiotic is now being carried out in our laboratories.

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