NEW ANTIBIOTICS, CARBAZOMYCINS A AND B

I. FERMENTATION, EXTRACTION, PURIFICATION AND PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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An unidentified *Streptomyces*, tentatively designated as Strain H 1051-MY 10, was proved to produce viomycin and two new antibiotics. The new antibiotics were extracted from the cultured mycelia with acetone and transferred to ethyl acetate after acetone was removed *in vacuo*. The extracted antibiotics were separated into two components by alumina column chromatography and named carbazomycins A and B, because both antibiotics were proved to contain a carbazole nucleus. The molecular formulae of carbazomycins A and B were determined to be $C_{16}H_{17}NO_2$ and $C_{15}H_{15}NO_2$, respectively. Further, carbazomycin B was methylated with diazomethane to give carbazomycin A. Carbazomycins inhibited the growth of phytophathogenic fungi and further showed weak antibacterial and antiyeast activities.

In the course of our screening of cholesterol esterase produced by microbes, the primary screening was performed by examining silica gel thin-layer chromatogram of the incubation product of the culture filtrate and cholesterol linoleate[1]. Cholesterol linoleate or resulting cholesterol on the thin-layer was detected by heating after spraying with 40% sulfuric acid. Strain H 1051-MY 10 was shown to produce two substances different from cholesterol linoleate or cholesterol by the above test. These two substances developed a blue to green color on thin-layer by the test and showed very weak inhibition against *Bacillus subtilis* by bioautogram. Thus, these substances were purified using *B. subtilis* as a test microbe. The active substances were separated into two components by alumina column chromatography and the first eluted component was designated as A and the later as B. The ultraviolet absorption spectra of A and B are related to that of carbazole derivatives[2,3]. Further, the ultraviolet absorption spectra of zinc dust distillation products of B were closely related to that of carbazole[2]. B was converted into A by methylation with diazomethane. Thus, A and B were proved to contain a carbazole nucleus and were named carbazomycins A and B, respectively.

Strain H 1051-MY 10, isolated from a soil sample collected at Ni-imi City, Okayama Prefecture, was also shown to produce viomycin simultaneously.

Fermentation, extraction, purification and physico-chemical and biological properties of carbazomycins A and B are described in this paper.

Fermentation

Strain H 1051-MY 10 was cultured in shaking flasks each containing 100 ml of an inoculation medium composed of 1.0% maltose and 0.2% yeast extract (pH 7.0) and incubation was at 27°C for 24 hours on a reciprocal shaker (amplitude 7 cm, 160 strokes per minute). This inoculum (2%) was used to inoculate 500 ml Erlenmeyer flasks each containing 100 ml of a production medium composed of 1.5% soluble starch, 1.0% glucose, 2.0% soy bean meal, 0.5% Ebios (dried yeast, distributed by

Tanabe Pharmaceutical Co. Ltd.), 0.25% NaCl and 0.3% $CaCO_3$ (pH 7.6 before sterilization). The culture was grown at 27°C for 91 hours on a rotary shaker (amplitude 7 cm, 145 strokes per minute).

Extraction and Purification

Carbazomycins were extracted from the mycelial cake with acetone and transferred into ethyl acetate after acetone was removed *in vacuo*, as summarized in Chart 1. Carbazomycins were purified by alumina column chromatography to yield carbazomycins A and B as shown in Chart 2. Carbazomycin A was crystallized from *n*-hexane and ethyl acetate to give pale yellow needles, m.p. 51.0~52.5°C. Carbazomycin B showing dimorphism was crystallized from *n*-hexane and benzene to give pale yellow prisms, m.p. 137.5~138.0°C, and from *n*-hexane and ethyl acetate to give pale yellow prisms, m.p. 158.5~160.0°C.

A basic water-soluble antibiotic in the broth filtrate was recovered by absorption on Amberlite IRC-50 (Na^+-type) followed by elution with 0.5 N hydrochloric acid. The antibiotic was precipitated as the reineckate from the eluate after concentrated *in vacuo*. The reineckate was dissolved in water and an aqueous solution of pyridine hydrochloride was added to precipitate pyridine reineckate. Pyridine reineckate was removed by filtration and the antibiotic hydrochloride was recovered from the filtrate by lyophilization. The ultraviolet absorption spectra of the antibiotic in acidic and alkaline aqueous solutions were closely related with that of viomycin group antibiotics and identity with viomycin was confirmed by silica-gel thin-layer chromatography[4,5].

Chart 1. Extraction of carbazomycins A and B.

Culture broth (pH, 7.6)
↓ filtered
Mycelial cake — Broth filtrate (8,500 ml)
↓ extracted with acetone
Extract (1,960 ml) — Mycelial cake
↓ concentrated *in vacuo* to 840 ml and extracted with AcOEt at pH 8.0
AcOEt layer (1,270 ml) — H_2O layer (1,090 ml, total activity 20%)
↓ washed with H_2O (250 ml) at pH 2.0
AcOEt layer (1,270 ml)
↓ evaporated to dryness *in vacuo*
Viscous mixture of antibiotics

Chart 2. Purification of carbazomycins A and B

Viscous mixture of antibiotics (709 mg, total activity 100%)
↓ dissolved in 2 ml of AcOEt
Alumina column (Aluminiumoxid 90, Merck, 54×2.2 cm, diam.)
↓ developed with 150 ml of AcOEt - *n*-hexane (1 : 19) and following with 850 ml of AcOEt - *n*-hexane (1 : 9) the eluate was collected in 12 ml fractions

Fraction No. 15~22	Fraction No. 37~73
↓ evaporated to dryness *in vacuo*	↓ evaporated to dryness *in vacuo*
Purified carbazomycin A (9.0 mg)	Purified carbazomycin B (233.5 mg, total activity 75%)
↓ recrystallized from AcOEt - *n*-hexane	↓ recrystallized from benzene - *n*-hexane
Pale yellowish needles (4.3 mg)	Pale yellowish prisms (155.5 mg)

Physico-chemical Properties

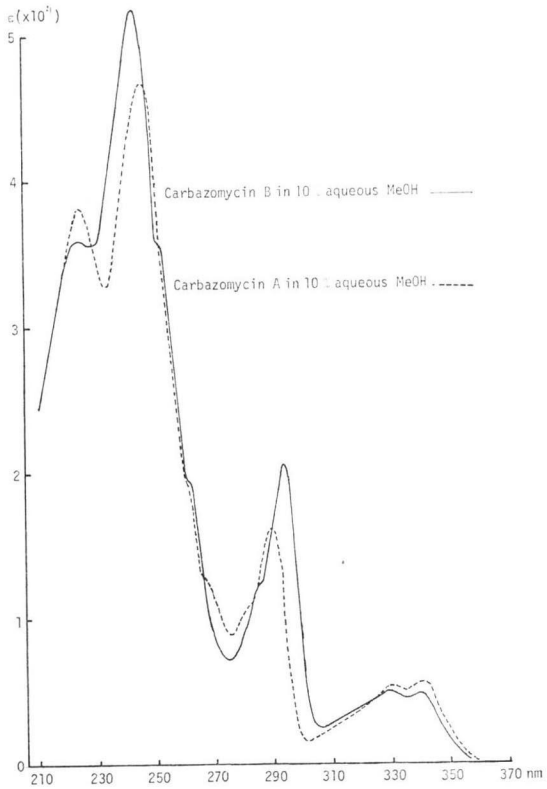
The main constituent of carbazomycins was carbazomycin B and the molecular formula was determined to be $C_{15}H_{15}NO_2$ by mass spectroscopy and elementary analysis. The mass spectrum of carbazomycin B gave a molecular ion peak at *m/e* 241.

Analysis Calculated for $C_{15}H_{15}NO_2$: C, 74.66; H, 6.27; N, 5.81.
Found: C, 74.57; H, 6.34; N, 5.68.

The formula, $C_{16}H_{17}NO_2$ for carbazomycin A was supported by a moelcular ion peak at *m/e* 255 and chemical conversion of carbazomycin B into carbazomycin A by methylation as described later.

Carbazomycins A and B gave no optical rotation in methanol. Ultraviolet absorption spectra of carbazomycins A and B are shown in Fig. 1. Carbazomycin A: $\lambda_{max.}^{10\%\,aq.MeOH}$ nm (ε); 223 (36,000), 242 (51,000), 293 (20,700), 327 (4,700) and 340 (4,600). Carbazomycin B: $\lambda_{max.}^{10\%\,aq.MeOH}$ nm (ε); 224 (38,000), 245 (46,800), 289 (16,100), 330 (5,300) and 340 (5,800).

Fig. 1. UV spectra of carbazomycins A and B.



Infrared absorption spectra of carbazomycins A and B are shown in Fig. 2 and Fig. 3, respectively.

Chromatographic behavior of both carbazomycins on silica gel thin-layer plates is shown in Table 1.

Carbazomycins A and B are soluble in lower alcohols, acetone, ethyl acetate, chloroform and benzene, but not in water. Though carbazomycin A is soluble in carbon tetrachloride, carbazomycin B is slightly soluble in the same solvent.

Carbazomycin A reacts positively with dichlorodicyanobenzoquinone[6] (bluish green) and 40% H_2SO_4 (blue) but gives negative ferric chloride, 2,4-dinitrophenylhydrazine and nin-

Table 1. Silica gel thin-layer chromatography behaviors of carbazomycins A and B.

Solvent system	Rf value	
	Carbazomycin A	Carbazomycin B
n-Hexane - AcOEt (4: 1)	0.63	0.39
Benzene	0.34	0.20
Benzene - *n*-hexane - acetone (20: 5: 3)	0.68	0.50
Toluene - acetone (9: 1)	0.65	0.47
Benzene - $CHCl_3$ (4: 1)	0.44	0.27
Benzene - cyclohexane (1: 1)	0.15	0.06
n-Hexane - ether (2: 1)	0.64	0.35

SPOTFILM, fluorescent, Silica Gel f (Tokyo Kasei) was used.

Fig. 2. IR spectrum of carbazomycin A (KBr).

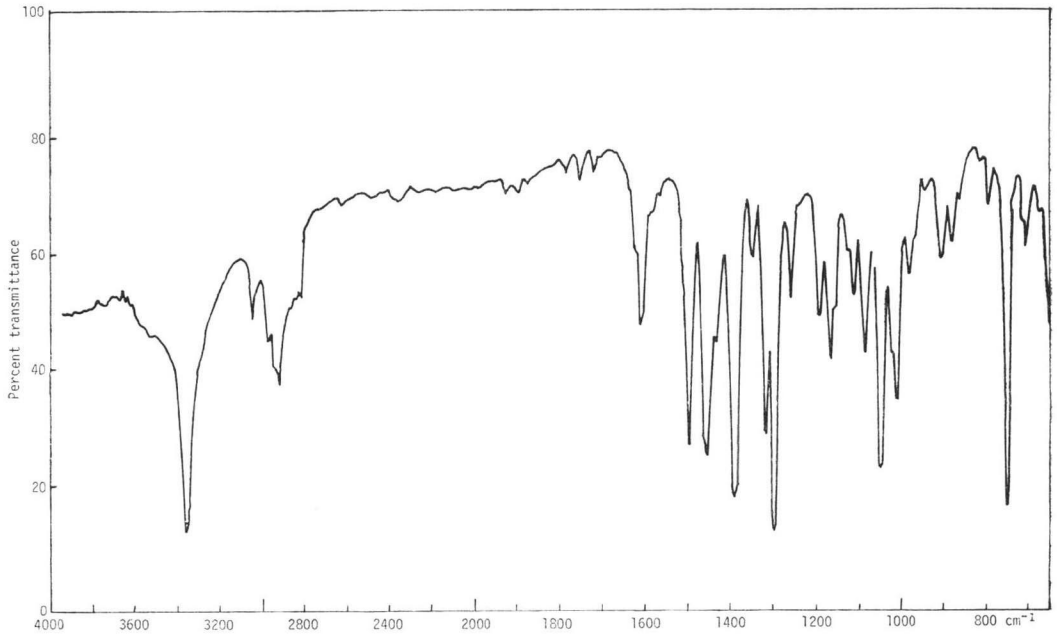
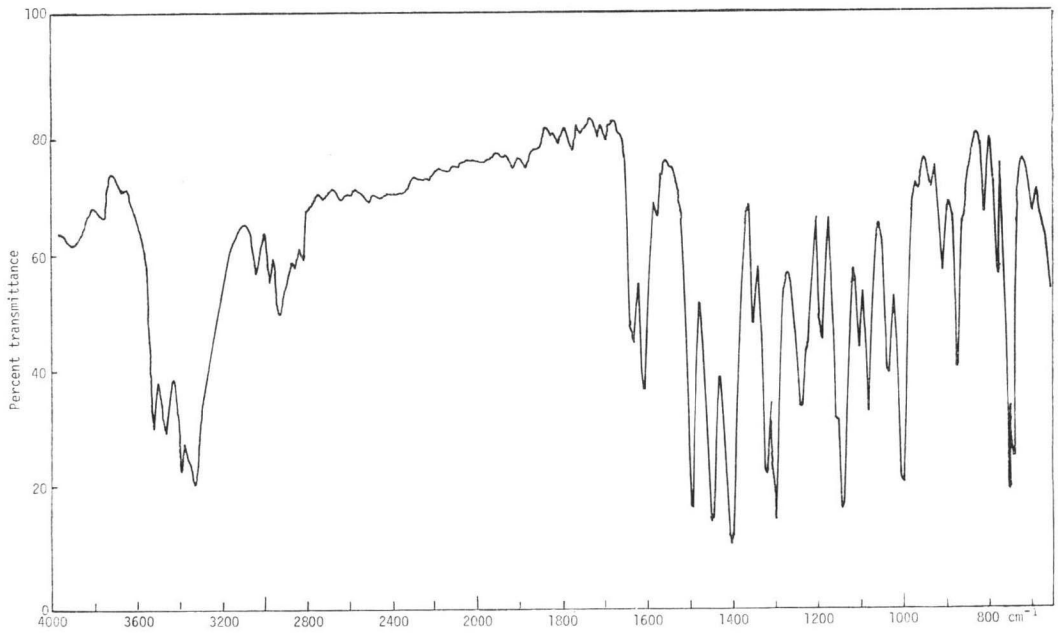


Fig. 3. IR spectrum of carbazomycin B (KBr)*.



* Recrystallzed from benzen - *n*-hexane (m.p. 137.5~138.0°C).

hydrin tests. Carbazomycin B gives positive dichlorodicyanobenzoquinone (green), 40% H_2SO_4 (bluish green) and ferric chloride tests but negative 2,4-dinitrophenylhydrazine and ninhydrin tests.

Carbazomycins A and B were stable in an aqueous solution (pH 2~9) when heated at 100°C for 5 minutes, but a solution of carbazomycin B was gradually oxidized to develop reddish brown color

in the air.

^{1}H-NMR spectra of carbazomycins A and B were taken in $CDCl_3$ at 100 MHz. Carbazomycin A gave a double doublet for one aromatic proton (1H) at δ 8.25, a broad singlet for NH proton (1H) at δ 7.89, multiplets for aromatic protons (3H) at δ 7.13~7.42, two singlets for aromatic methoxyl groups (2×3H) at δ 4.13 and 3.92 and two closely overlapped singlets for aromatic methyl groups (2×3H) at δ 2.40. The ^{1}H-NMR spectrum of carbazomycin B showed a double doublet for one aromatic proton (1H) at δ 8.31, a broad singlet for NH proton (1H) at δ 7.71, multiplets for aromatic protons (3H) at δ 7.14~7.38, a singlet for a phenolic OH proton (1H) at δ 6.21, a singlet for one aromatic methoxyl group (3H) at δ 3.80 and two singlets for two aromatic methyl groups (2×3H) at δ 2.36 and 2.28.

Zinc Dust Distillation of Carbazomycin B

Carbazomycin B (50 mg) was treated with zinc dust (500 mg) at 400°C for 30 minutes and the resulting mixture was extracted with ethyl acetate. The extract was evaporated *in vacuo* and partially purified on a silica gel thin-layer plate developed with *n*-hexane - AcOEt (4: 1). The partially purified

Table 2. Antimicrobial spectra of carbazomycins A and B.

Test organisms	MIC (mcg/ml)	
	Carbazomycin A	Carbazomycin B
Micrococcus flavus FDA 16	200	25
Micrococcus lysodeikticus IFO 333	>200	100
Staphylococcus aureus FDA 209P	>200	50
Staphylococcus aureus Smith	12.5	25
Sarcina lutea PCI 1001	>200	25
Bacillus anthracis	>200	12.5
Bacillus subtilis PCI 219	>200	25
Corynebacterium bovis	>200	50
Mycobacterium phlei	>200	100
Mycobacterium smegmatis ATCC 607	>200	100
Escherichia coli B	>200	100
Escherichia coli K-12	>200	>200
Escherichia coil K-12 ML 1629	>200	>200
Escherichia coli NIHJ	>200	200
Proteus vulgaris OX 19	>200	>200
Salmonella typhosa T-63	>200	>200
Shigella sonnei 191-66	>200	>200
Klebsiella pneumoniae	>200	100
Pseudomonas aeruginosa Ishii 14	>200	>200
Serratia marcescens	>200	>200
Candida albicans 3147	>200	100
Candida albicans Yu-1200	>200	100
Candida krusei NI 7492	>200	200
Candida pseudotropicalis NI 7494	>200	100
Candida tropicalis NI 7495	>200	50
Saccharomyces cerevisiae	>200	100

Agar dilution method on glucose nutrient agar.
Incubated for 15 hours at 37°C.

product showed four peaks, designated as Peaks I, II, III and IV, by gas chromatography on 1.5% SE-30 column (100 cm) at column temperature 170°C under 1.2 kg/cm² of nitrogen gas. The retention times of Peaks I, II, III and IV were 4.2, 5.0, 5.8 and 6.6 minutes, respectively. Peak I showed a molecular ion peak at *m/e* 167 by the GC-mass spectrum and the same retention time with carbazole (M.W. 167) by the gas chromatography. Ultraviolet absorption spectra of Peak III showing a molecular ion peak at *m/e* 181 by the GC-mass spectrum and Peak I were essentially identical with that of carbazole; $\lambda_{max.}^{MeOH}$ nm (log ε) 236 (4.3), 255 (4.2), 295 (4.2), 323 (3.6) and 337 (3.5)[2].

Conversion of Carbazomycin B into Carbazomycin A

Carbazomycin B (81 mg) dissolved in ether (5 ml) was methylated with diazomethane in ether at room temperature for 17 hours. The reaction mixture was purified on a silica gel column (53×2.2 cm, diam.) developed with *n*-hexane - AcOEt (20: 1) and the eluate was fractionated in 15-ml fractions. Fractions No. 14~19 were evaporated and recrystallized from *n*-hexane and ethyl acetate to give pale yellow needles (11 mg), m.p. 50~52°C. Identity of methylated carbazomycin B with carbazomycin A was confirmed by m.p. admixture and by comparison of infrared absorption spectra of both compounds.

Biological Properties

Carbazomycin B inhibited the growth of some kinds of phytophathogenic fungi and showed rather weak antibacterial and anti-yeast activities as shown in Tables 2 and 3. On the other hand, carbazomycin A showed very weak inhibition against a few kinds of fungi and bacteria.

No toxic symptom was observed when carbazomycin B was administered intraperitoneally at a dose of 5 mg/mouse.

Table 3. Antimicrobial spectra of carbazomycins A and B.

Test organisms	MIC (mcg/ml)	
	Carbazomycin A	Carbazomycin B
Alternaria kikuchiana	>200	12.5
Aspergillus niger F-16	>200	200
Colletotrichum gloeosprioides	>200	12.5
Colletotrichum lagenarium	>200	100
Elsinoë fawcetti	>200	3.1
Gloeosporium laeticolor	>200	100
Glomerella cingulata	>200	100
Glomerella cingulata No. 3	>200	3.1
Helminthosporium oryzae	>200	6.3
Pyricularia oryzae	25	12.5
Trichophyton asteroides 429	12.5	12.5
Trichophyton mentagrophytes 833	12.5	12.5

Agar dilution method on potato sucrose agar.
Incubated for 40 hours at 27°C.

Discussion

The existence of a carbazole nucleus in carbazomycins A and B was strongly suggested by their ultraviolet absorption spectra and positive dichlorodicyanobenzoquinone test. Peak I, a zinc dust distillation product of carbazomycin B, was proved to be carbazole by the ultraviolet absorption spec-

trum, the gas chromatogram and the GC-mass spectrum. Consequently, Peak III showing the molecular ion peak at *m/e* 181 was postulated to be one of methylcarbazoles. Up to the present time, no antibiotic related to carbazole is reported and carbazomycins A and B are first antibiotics containing a carbazole nucleus.

Carbazomycin B, $C_{15}H_{15}NO_2$, was shown to have one phenolic hydroxyl, one methoxyl and two methyl groups by the ^{1}H-NMR spectrum. Thus, the structure of carbazomycin B can be considered to be hydroxy-methoxy-dimethylcarbazole. Furthermore, the structure of carbazomycin A, $C_{16}H_{17}NO_2$ should be dimethoxy-dimethylcarbazole.

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