DC-86-M, A NOVEL ANTITUMOR ANTIBIOTIC

II. STRUCTURE DETERMINATION AND BIOLOGICAL ACTIVITIES

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A novel antibiotic, DC-86-M was isolated from the culture broth of *Streptomyces luteogriseus* DO-86. The antibiotic has the molecular formula of $C_{17}H_{14}N_2O_5$ and belongs to the phenazine antibiotics. Its structure has been elucidated by mass and NMR spectra. It is active against Gram-positive and Gram-negative bacteria and experimental murine sarcoma 180.

In the course of our screening program for novel antitumor antibiotics, a streptomycete strain DO-86 identified as *Streptomyces luteogriseus* DO-86[1] was found to produce a novel antitumor antibiotic DC-86-M. DC-86-M was active against Gram-positive and Gram-negative bacteria and murine tumors.

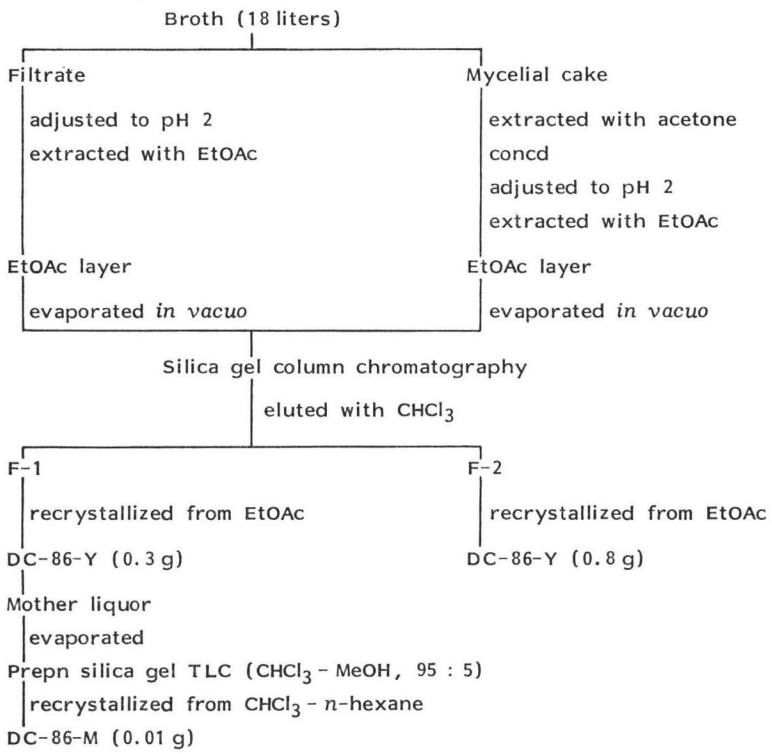
In this paper, the isolation and characterization, the structure elucidation, the synthesis, and the biological activities are described.

Isolation and Purification

Activity against *Bacillus subtilis* and thin-layer chromatography were used to monitor DC-86-M and DC-86-Y during isolation from the culture broth. The whole broth (18 liters) was adjusted to pH 2.0 with concentrated H_2SO_4, and 15 liters of acetone and 20 liters of ethyl acetate were added and mixed vigorously. After the mixture was filtered with aid of Celite pad, the upper layer was separated and washed with water.

Evaporation of the extract afforded a brown syrup, which was applied to a silica gel column, and chromatographed with chloroform. The active fraction 1 containing DC-86-M (**2**) and DC-86-Y (**1**) was eluted first and then the fraction 2 containing DC-86-Y (**1**) was eluted. The active fraction was concentrated to dryness to leave a yellow solid which was recrystallized from ethyl acetate to give 300 mg DC-86-Y as yellow needles. The mother liquor was concentrated and purified on silica gel TLC using chloroform - methanol (95: 5) as a developing solvent. The major component was extracted with chloroform. The extract was concentrated, and crystallized from chloroform - *n*-hexane to give 10 mg of DC-86-M (**2**) as yellow needles. The second fraction was concentrated, and recrystallized from ethyl acetate to give 800 mg of DC-86-Y (**1**) as yellow needles. The isolation and purification procedures are summarized in Fig. 1.

Fig. 1. Isolation and purification of DC-86.



Physico-chemical Properties and Structure Elucidation

The molecular formula of DC-86-Y (**1**) was determined as $C_{15}H_{12}N_2O_3$ by EI-MS and elementary analysis. ^{1}H NMR spectrum of DC-86-Y (**1**) (in $CDCl_3$) showed the signals at 1.82 (3H, d, J=6.3 Hz), 5.85 (1H, q, J=6.3 Hz), 7.9~8.3 (4H, aromatic protons), 8.53 (1H, dd, J=1.5, 8.8 Hz), 8.99 (1H, dd, J=1.5, 7.3 Hz), and 14.5 ppm (1H, br s, COOH).

The UV absorption maxima of DC-86-Y were observed at 253 and 367 nm in methanol, which resembled closely a typical phenazine type spectrum[2]. These results suggested that DC-86-Y might be the phenazine having two substituents at 1 and 6 positions or 1 and 9 positions, but the exact positions of the two substituents could not be determined by the NMR spectrum. The structure of saphenamycin, a member of the phenazine group of antibiotics, was determined by X-ray crystallography, and has the same substituents except 2-hydroxy-6-methylbenzoyl group[3]. Alkaline hydrolysis of saphenamycin, a sample of which was kindly provided by Dr. H. UMEZAWA, led to a chromophore, whose ^{1}H NMR spectrum (in $CDCl_3$) matched exactly that of DC-86-Y.

The molecular formula of DC-86-M (**2**) was determined as $C_{17}H_{14}N_2O_5$ by EI-MS and elementary analysis. The UV absorption maxima of DC-86-M were closely related to that of DC-86-Y.

^{1}H NMR spectrum of DC-86-M (**2**) (in $CDCl_3$) showed the signals at 1.82 (3H, d, J=6.8 Hz), 4.30 (2H, d, J=0.7 Hz), 7.35 (1H, q, J=6.8 Hz), 7.9~8.3 (4H, aromatic protons), 8.57 (1H, dd, J=1.7, 8.8 Hz), 9.00 (1H, dd, J=1.7, 7.1 Hz) and 15.49 (1H, br, COOH).

These spectral data indicate that DC-86-M is an ester compound of DC-86-Y with glycolic acid. Other physico-chemical properties are summarized in Table 1.

Table 1. Physico-chemical properties of DC-86.

	DC-86-Y (1)	DC-86-M (2)
Appearance	Yellow needles	Yellow needles
MP (°C)	223~225	185~187
Molecular weight	268	326
Molecular formula	$C_{15}H_{12}N_2O_3$	$C_{17}H_{14}N_2O_5$
Specific rotation $[\alpha]_D^{20}$	+55.8°	−43.8°
	(*c* 1.0, DMSO)	(*c* 0.5, $CHCl_3$)
IR ν_{max}^{KBr} cm^{-1}	3410, 1728, 1700	3450, 1743, 1715
UV λ_{max}^{MeOH} nm (ε)	253 (84,000), 367 (16,000)	251 (79,000), 363 (14,000)

Fig. 2. Synthesis of DC-86 derivatives.

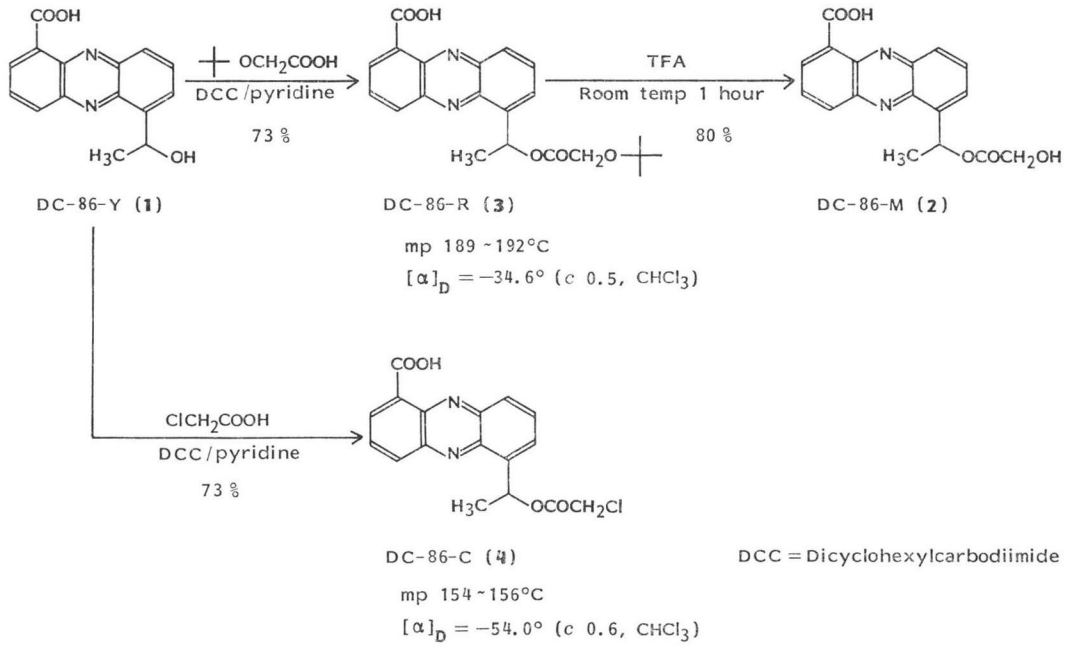


Table 2. Antimicrobial activity of DC-86 (MIC, μg/ml).

Test organism	DC-86-Y (1)	DC-86-M (2)	DC-86-R (3)	DC-86-C (4)
Bacillus subtilis	>100	0.1	0.7	0.01
Staphylococcus aureus	>100	0.3	3	0.2
Enterococcus faecalis	>100	0.3	3	0.4
Escherichia coli	>100	100	>100	>100
Klebsiella pneumoniae	>100	100	>100	>100
Shigella sonnei	>100	10	>100	10
Salmonella typhosa	>100	10	>100	10
Proteus vulgaris	>100	1	100	2
Pseudomonas maltophilia	>100	100	>100	>100
P. fluorescens	—	>25	>25	>25
Aeromonas salmonicida	—	0.1	1.56	<0.1
Pasteurella piscicida	—	<0.1	>25	0.78
Vibrio anguillarum	—	0.2	>25	1.56
Candida albicans	>100	>100	>100	>100

—: Not tested.

Synthesis of DC-86-M

Since the active component DC-86-M was obtained only in small amounts, we attempted synthesis of DC-86-M (**2**) from DC-86-Y (**1**) to prove the structure of DC-86-M (**2**) and to obtain larger amounts for biological experiments. The esterification of DC-86-Y (**1**) with *tert*-butoxyacetic acid[4] in the presence of dicyclohexylcarbodiimide and pyridine gave a product (DC-86-R, **3**) which was cleaved by trifluoroacetic acid (TFA) to give DC-86-M with mp, spectral, and chromatographic properties identical with those described for the natural product. We also synthesized the DC-86-C (**4**) by condensation of DC-86-Y with monochloroacetic acid. Synthetic procedures are described in the section of Experimental and are summarized in Fig. 2.

Table 3. Antitumor activity of DC-86-M (**2**) against sarcoma 180 (sc-ip).

Dose (mg/kg)		T/C
DC-86-M[a]	40	Toxic
	20	0.36
	10	0.82
	5	0.97
Mitomycin C	6	0.28

[a] LD_{50} <25 mg/kg (ip).

Biological Activities

The *in vitro* activities of four compounds against various microorganisms are shown in Table 2. DC-86-Y (**1**) is inactive, but the other three compounds showed strong activities against Gram-positive bacteria and weak activities against Gram-negative bacteria. The LD_{50} value of DC-86-M (**2**) was below 25 mg/kg when administered intraperitoneally.

Table 3 shows the effect of DC-86-M (**2**) on murine sarcoma 180. DC-86-R (**3**) and DC-86-C (**4**) have no effect against murine sarcoma 180. However, it is of interest that DC-86-C showed strong antibacterial activity and further modification of DC-86-Y is in progress.

Experimental

General

The spectroscopic data were measured by the following instruments. IR spectra; Shimadzu IR-27G. Mass spectra; Jeol JMS-01SG-2. NMR spectra; Jeol FX-100. Optical rotations; Perkin-Elmer 141 polarimeter. UV spectra; Hitachi Model 200-20 spectrophotometer.

DC-86-R

To a solution of DC-86-Y (**1**) (1.0 g) and *tert*-butoxyacetic acid (0.8 g) in pyridine (30 ml) was added dicyclohexylcarbodiimide (1.2 g), and the solution was stirred for 9 hours at room temp. The reaction mixture was poured into ice-water, adjusted to pH 4.5 with 6 N HCl, and extracted with EtOAc. The extracts were washed with H_2O, dried over Na_2SO_4 and concd to about 50 ml, the precipitates were filtered off, and the filtrate was concd to dryness. The residue was chromatographed on silica gel (250 ml) using $CHCl_3$ as eluant. The first fraction gave DC-86-R (**3**) as a yellow solid, which was recrystallized from EtOAc - *n*-hexane to give yellow needles (1.0 g), mp 189~192°C, $[\alpha]_D^{25}$ −34.6° (*c* 0.5, $CHCl_3$), MS *m/z* 382 (M^+), ^1H NMR ($CDCl_3$) δ 1.26 (9H, s), 1.80 (3H, d, J=6.6 Hz), 4.17 (2H, s), 7.28 (1H, q, J=6.6 Hz), UV λ_{max}^{MeOH} 252, 364 nm.

Anal Calcd for $C_{21}H_{22}N_2O_5$: C 65.96, H 5.80, N 7.32.
Found: C 65.74, H 5.90, N 7.32.

Second fraction gave DC-86-Y (**1**) (0.3 g) as a yellow solid.

DC-86-M

A solution of DC-86-R (**3**) (900 mg) in TFA (20 ml) was stirred for 1 hour at room temp. After removal of solvent at 25°C, the residue was chromatographed on silica gel (200 ml) using $CHCl_3$ - MeOH as eluant. Fraction of $CHCl_3$ - MeOH (99: 1) gave DC-86-M as a yellow solid, which was

recrystallized from $CHCl_3$ - *n*-hexane to give yellow needles (610 mg). The physico-chemical properties of synthetic DC-86-M (**2**) were identical to those of natural DC-86-M.

DC-86-C

To a solution of DC-86-Y (**1**) (200 mg) and monochloroacetic acid (140 mg) in pyridine (5 ml) was added dicyclohexylcarbodiimide (260 mg), and the solution was stirred for 1 hour at room temp. The reaction mixture was poured into ice-water (100 ml) and adjusted to pH 5.0 with 6 N HCl, and extracted with EtOAc. The extracts were washed with H_2O, dried over Na_2SO_4, and evaporated to about 15 ml, the precipitates were removed and the filtrate was concd to dryness. The residue was chromatographed on silica gel (200 ml) using $CHCl_3$ as eluant. The fraction of $CHCl_3$ gave DC-86-C (**4**) as a yellow solid, which was recrystallized from benzene - *n*-hexane to give yellow crystals (190 mg), mp 154~156°C, $[\alpha]_D^{25}$ −54.0° (*c* 0.6, $CHCl_3$), MS *m/z* 346 (M^+), 344, UV λ_{max}^{MeOH} 254, 364 nm, ^1H NMR ($CDCl_3$) δ 1.83 (3H, d, J=6.6 Hz), 4.12 (2H, s), 7.30 (1H, q, J=6.6 Hz).

Anal Calcd for $C_{17}H_{13}N_2O_4Cl$: C 59.23, H 3.80, N 8.12.
Found: C 59.44, H 3.78, N 8.22.

Acknowledgments

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