DNACINS, NEW ANTIBIOTICS

II. ISOLATION AND CHARACTERIZATION

MASAYUKI MUROI, SEIICHI TANIDA, MITSUKO ASAI and TOYOKAZU KISHI

Microbiological Research Laboratories, Central Research Division,
Takeda Chemical Industries, Ltd.,
17–85, Jusohonmachi 2-chome, Yodogawa-ku, Osaka 532, Japan

(Received for publication August 28, 1980)

Dnacins A_1 and B_1, new basic antibiotics with strong and broad antibacterial activities, were isolated as dark red or reddish brown needles from the culture broth of *Nocardia* sp. No. C-14482. The characteristic absorption maxima at 213 nm, 281 or 283 nm and 496 nm in the UV and visible range and other physicochemical properties indicated that dnacins A_1 and B_1 are novel antibiotics which belong to the group having aminobenzoquinone moieties.

In the course of our screening program for new antibiotics that inhibit DNA synthesis[1] two new antibiotics, dnacins A_1 and B_1,* have been isolated from the fermentation broth of *Nocardia* sp. No. C-14482.[2] Dnacins A_1 and B_1 showed strong and broad antibacterial activities against Gram-positive and negative bacteria including *Pseudomonas aeruginosa*. This paper deals with their isolation and characterization.

Isolation

An Hfr strain of *Escherichia coli*[1] and *Proteus mirabilis* were used as the assay organism for thin-layer chromatography (TLC) to monitor the isolation of dnacins A_1 and B_1 from the culture broth of *Nocardia* sp. No. C-14482.

Since both antibiotics have basic properties and relatively soluble in polar organic solvents and water, they were isolated by the usual methods for such products from the culture filtrate as shown in the Chart 1.

Firstly, antibiotic dnacin was adsorbed on a column of nonionic adsorption resin such as Diaion HP-10 and eluted with aqueous methanol or acetone. The eluate was concentrated to remove the organic solvent and the resulting aqueous solution was extracted with polar organic solvents such as *i*-butanol or *n*-butanol at pH 8.0. Dnacin was mainly extracted into the above organic solvent but the aqueous layer (F-II) still contained about one-third of the total activity. The organic layer (F-I) was reextracted with dilute acid and the aqueous layer was concentrated to remove organic solvent. The aqueous concentrate was applied to a column of Diaion HP-10 and antibiotic was eluted with aqueous methanol.

The eluate was concentrated to 1/50 volume and repeatedly extracted with chloroform at pH 8.0. The activity was transferred into aqueous layer from chloroform with 0.02 N HCl and the wine-colored aqueous solution was repeatedly back extracted with chloroform at pH 8.0. The chloroform extracts were combined and concentrated to deposit reddish brown crystalline B_1, which was recrystallized from acetone-hexane to give pure dnacin B_1.

* Dnacins A_1 and B_1 were formerly named antibiotics C-14482 A_1 and B_1.

Chart 1a. Isolation procedure for dnacins A_1 and B_1 (1).

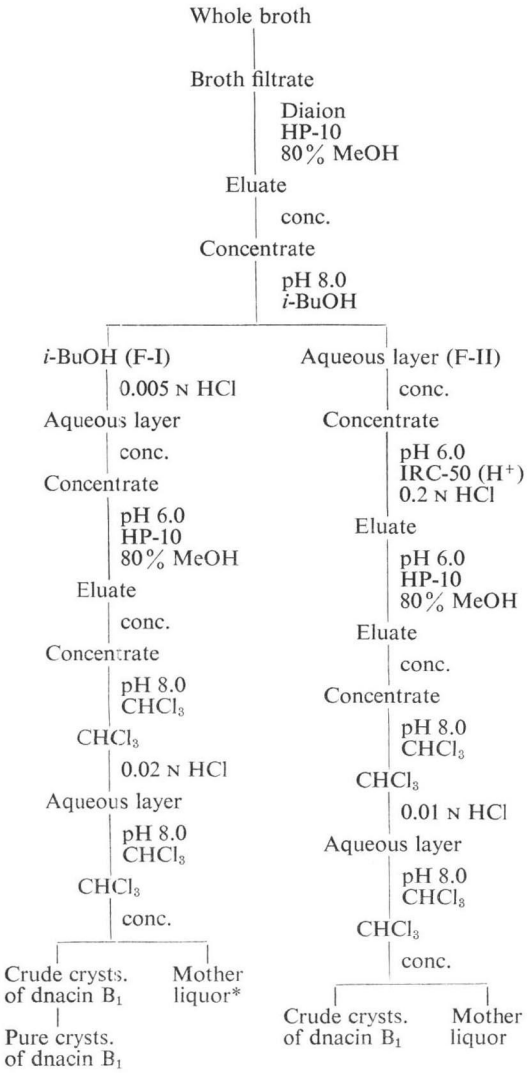
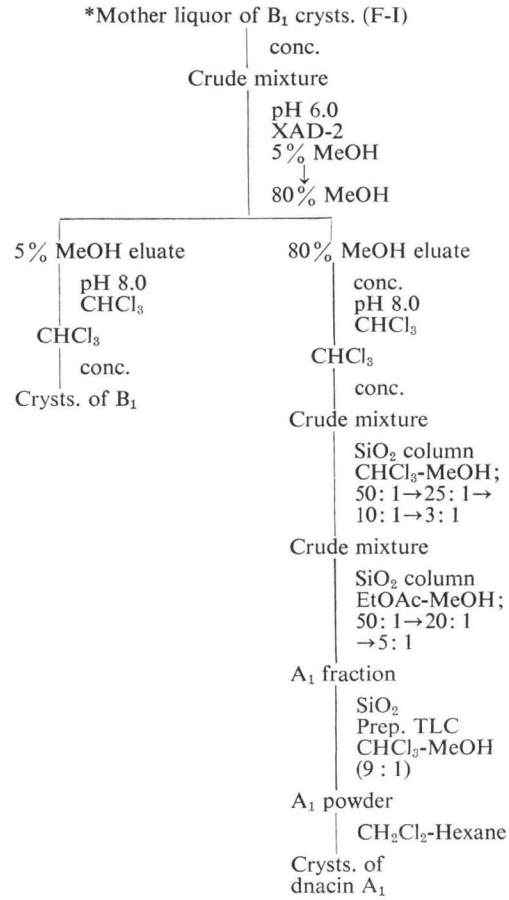


Chart 1b. Isolation procedure for dnacins A_1 and B_1 (2).



The mother liquor of crude B_1 was evaporated to dryness to give a solid which consisted of many components and was applied to a column of Amberlite XAD-2 in 5% aqueous methanol of pH 6.0. The column was first developed with 5% aqueous methanol and then with 80% aqueous methanol. From the first effluent was recovered crystalline B_1 after extraction with chloroform at pH 8.0 and concentration of the extract.

The eluate with 80% aqueous methanol was concentrated to remove methanol, extracted with chloroform and the extract was again evaporated to give a mixture consisting of dnacin A_1 and other components.

The mixture thus obtained was chromatographed on a column of silica gel with chloroform - methanol as the solvent system. Dnacin A_1 was eluted with chloroform - methanol (25 : 1), while the other minor components were later eluted with chloroform and increasing amounts of methanol.

Fractions containing dnacin A_1 were concentrated to dryness and the residue was again chromatographed on a column of silica gel with ethyl acetate-methanol as the eluant. Dnacin A_1 fraction thus obtained was further purified by preparative TLC on silica gel with chloroform - methanol (9 : 1) as the solvent system to afford a reddish brown amorphous powder of dnacin A_1 which was crystallized from dichloromethane or chloroform-hexane as reddish brown needles.

The aqueous layer (F-II), which still contained active substances after extraction of the concentrate of the eluate from the first Diaion HP-10 column, was purified by use of Amberlite IRC-50 and thereafter by a similar manner to F-I as shown in the chart to give further crops of dnacins A_1 and B_1.

Physicochemical Properties

Dnacins A_1 and B_1 were obtained as dark red or reddish brown needles from dichloromethane or chloroform-hexane and from acetone-hexane. Pure dnacin A_1 is readily soluble in methanol and dimethylsulfoxide; soluble in chloroform and dichloromethane; slightly soluble in ethanol, *n*-butanol, ethyl acetate and water; insoluble in hexane and petroleum ether. Likewise, dnacin B_1 is readily soluble in methanol and dimethylsulfoxide; soluble in ethanol; slightly soluble in ethyl acetate, chloroform, diethyl ether, and water; insoluble in hexane and petroleum ether. The physicochemical properties of dnacins A_1 and B_1 are presented in Table 1.

Both antibiotics gave positive reactions with DRAGENDORFF's and $KMnO_4$ reagents, whereas they showed negative reactions with ninhydrin, EHRLICH and SAKAGUCHI's reagents. The UV-visible spectra of dnacins A_1 and B_1 were almost superimposable and had λ_{max}^{MeOH} ($E_{1cm}^{1\%}$) 213 nm (550), 281 nm (222) and 496 nm (52.7), and 213 nm (592), 283 nm (227) and 496 nm (50.1), respectively, suggesting that they belong to the aminobenzoquinone group.[3]

The absorption maxima in visible spectra showed no remarkable shifts in alkaline conditions. Both antibiotics also showed similar absorption bands in the IR spectra (Figs. 2 and 3) and characteristic

Table 1. Physicochemical properties of dnacins A_1 and B_1.

	A_1	B_1
Appearance	Dark red or reddish brown needles	Dark red or reddish brown needles
m.p.	>300°C (dec.)	>300°C (dec.)
$[\alpha]_D$ (EtOH)	+150°	—
Elemental analysis	C; 59.10 H; 5.70 N; 17.14	C; 55.61 H; 6.31 N; 13.64
Molecular weight (V.P.O.)	460 (EtOAc)	400 (EtOH)
UV-Visible spectrum λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	213 (550) 281 (222) 496 (52.7)	213 (592) 283 (227) 496 (50.1)
IR spectrum ν_{max}^{KBr} cm^{-1}	3,430, 3,175, 2,940, 2,890, 2,850, 1,680, 1,650, 1,625, 1,600, 1,455, 1,390, 1,345, 1,250, 1,170, 1,140, 1,110, 1,075, 1,025, 995, 935, 910, 825	3,580, 3,420, 3,175, 2,950, 2,900, 2,840, 1,685, 1,650, 1,610, 1,455, 1,395, 1,345, 1,330, 1,250, 1,230, 1,175, 1,110, 1,075, 1,025, 1,000, 965, 940, 915, 855, 825, 780, 760

Fig. 1. UV-visible spectrum of dnacin B_1.

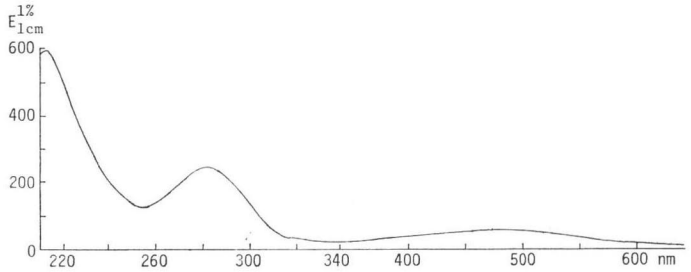


Fig. 2. IR spectrum of dnacin A_1 (KBr).

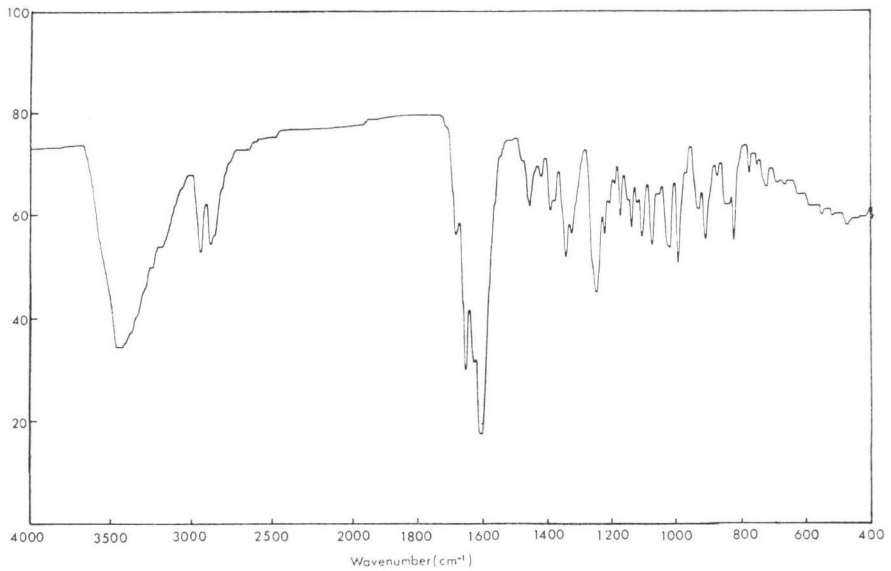


Fig. 3. IR spectrum of dnacin B_1 (KBr).

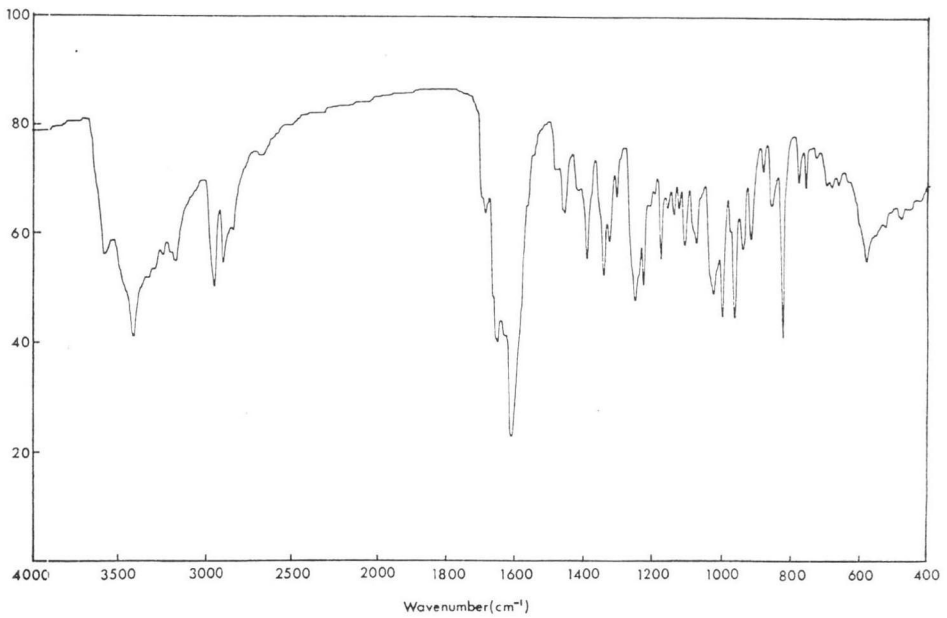


Fig. 4. ^{1}H-NMR spectrum of dnacin A_1 ($CDCl_3$).

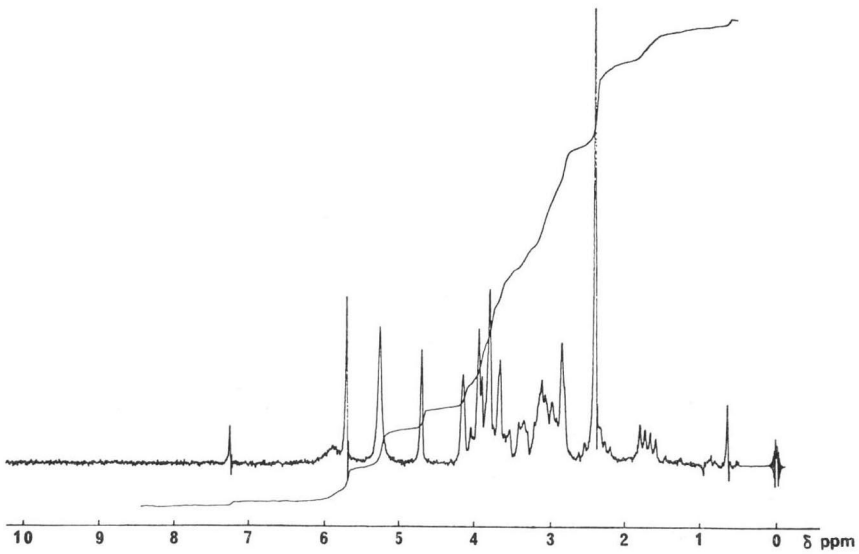
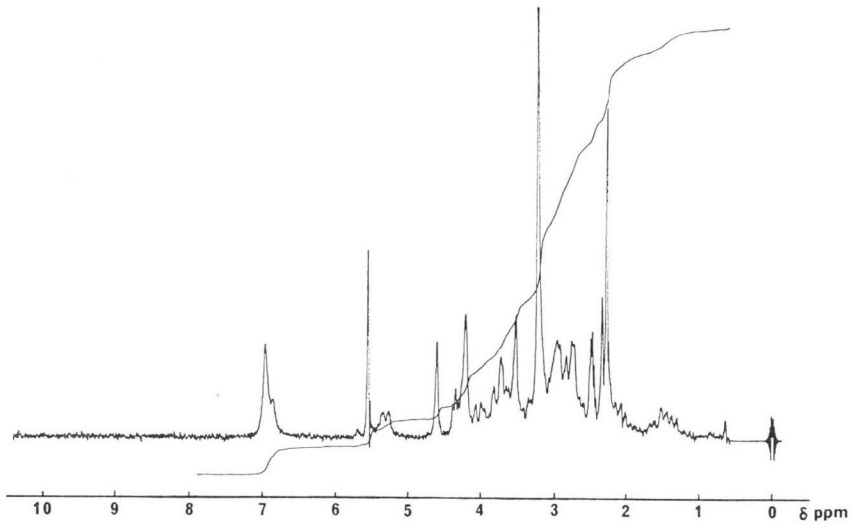


Fig. 5. ^{1}H-NMR spectrum of dnacin B_1 (d_6-DMSO).

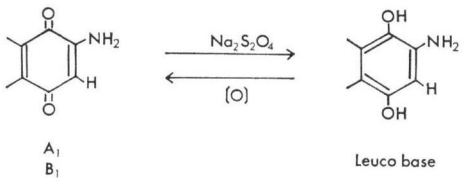


absorptions at 1650, 1625 and 1610 or 1600 cm^{-1} together with the UV-visible spectra indicated the presence of aminobenzoquinone moieties.

The presence of quinonoid ring protons at δ 5.70 in dnacin A_1 and at δ 5.52 in dnacin B_1 and the signals of NH_2 attached to the benzoquinone nuclei at δ 5.27 in dnacin A_1 and at δ 6.92 in dnacin B_1 in their ^{1}H-NMR spectra (Figs. 4 and 5) supported the conclusion that both dnacins A_1 and B_1 are 2-aminobenzoquinone derivatives and the C-3 position of the quinone ring are unsubstituted.

Both antibiotics gave their leucobases by reduction with reducing agents such as $Na_2S_2O_4$ and ascorbic acid and the leucobases were readily reoxidized by air to restore original red colors.

Fig. 6.



A_1
B_1

Leuco base

Table 2. TLC Rf values of dnacins A_1 and B_1.

Solvent system	Support	Rf	
		A_1	B_1
$CHCl_3$ - MeOH (9 : 1)	SiO_2	0.52	0.37
EtOAc - MeOH (1 : 1)		0.52	0.31
EtOAc - MeOH (10 : 1)		0.38	0.07
n-PrOH - H_2O (7 : 3)	Cellulose	0.78	0.49

Table 3. Comparison of dnacins A_1 and B_1 with known antibiotics.

	Dnacin A_1	Dnacin B_1	Mitomycin C	Porfiromycin	Streptonigrin
Producing organism	*Nocardia* sp. No. C-14482	*Nocardia* sp. No. C-14482	*Streptomyces caespitosus*	*Streptomyces ardus*	*Streptomyces flocculus*
M.P.	>300°C (dec.)	>300°C (dec.)	>360°C	204~206°C (dec.)	275°C (dec.)
Anal. Found C	59.10	55.61	53.84	55.23	58.66
H	5.70	6.31	5.14	5.92	4.93
N	17.14	13.64	14.49	16.19	11.01
M.W.	460	400	334	348	506
UV-visible spectrum	213 (550)	213 (592)	216 (742)	216 (665)	245 (760)
	281 (222)	283 (227)	360 (742)	358 (638)	390 (344)
λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	496 (52.7)	496 (50.1)	560 (0.06)	550 (6.5)	

Dnacins A_1 and B_1 did not give any significant ion peaks in the E.I. mass spectra and the accurate molecular weights were not determined. However, molecular weights of dnacins A_1 and B_1 estimated by vapor pressure osmometry (V.P.O.) were 460 and 400, respectively.

Dnacin A_1 was stable in weakly acidic solutions and relatively unstable in alkaline solutions, while dnacin B_1 was slightly unstable even in weakly acidic solutions and very unstable in alkaline solutions. Both components are observed as red or reddish pink spots on silica gel and cellulose TLC plates and their Rf values are summarized in Table 2.

Discussion

Dnacins A_1 and B_1 are dark red or reddish brown antibiotics with broad antibacterial activities,[2] but they are differentiated from anthracycline antibiotics such as cinerubine A[4] because of the presence of aminobenzoquinone nuclei in dnacins. Although mitomycin C,[5] porfiromycin[6] and streptonigrin[7] contain the aminobenzoquinone moieties and show similar values to dnacins in elemental analyses and have broad antibacterial activities, the presence of a quinone ring proton in dnacins and the UV-visible spectra differentiate dnacins from the known aminobenzoquinonoid antibiotics. Therefore, dnacins A_1 and B_1 are concluded to be new antibiotics. As described in the isolation section, the culture broth of *Nocardia* sp. No. C-14482 produced other minor components besides A_1 and B_1, and studies on their isolation and on structural elucidation are now in progress.

Experimental

Melting points were determined with a Mettler FP-5 instrument. UV and visible spectra were re-

corded with a Hitachi EPS-3T spectrometer. IR spectra were recorded with a Hitachi 285 grating infrared spectrophotometer. ^{1}H-NMR spectra were obtained with Varian EM-390 instrument with tetramethylsilane as the internal reference. Chemical shifts are reported on the δ scale. Optical rotations were measured with a Perkin-Elmer model 141 polarimeter.

Thin-layer chromatography (TLC) for monitoring the isolation of the antibiotics was performed on silica gel (Tokyo Kasei Co.) unless otherwise noted. Dnacins A_1 and B_1 are abbreviated as A_1 and B_1.

Isolation of dnacins A_1 and B_1

The culture broth (5,250 liters, combined broth of four 2,000 liters tank fermentations) of *Nocardia* sp. No. C-14482 (N-1020)[2] was adjusted to pH 5.0 with d.H_2SO_4 and filtered with Hyflo-Supercel (Johns-Manville Products, U.S.A.) as the filter aid to yield 6,020 liters of filtrate. The filtrate was applied to a column (560 liters) of Diaion HP-10 (Mitsubishi Chemicals, Japan), which was washed with 1,680 liters of water and eluted with 2,100 liters of 80% aqueous methanol. The eluate was concentrated under reduced pressure at pH 4.5~5.0 to remove methanol and the resulting aqueous solution (310 liters) was extracted three times with one-third of *i*-butanol. The combined *i*-butanol extracts contained almost two-thirds of the total activity, and were transferred twice with 0.005 N HCl into the aqueous layers. The combined aqueous extracts (390 liters) were adjusted to pH 4.5 and concentrated to remove the organic solvent. The resulting aqueous concentrate was passed through a column (100 liters) of Diaion HP-10, which was washed with water and eluted with 80% aqueous methanol (395 liters).

The eluate was again concentrated to a small volume (8 liters) under reduced pressure at low temperature after adjustment to pH 4.5. The concentrate was extracted five times with one-third of chloroform and the combined extract was concentrated to a half volume (4 liters) and transferred twice with 0.02 N HCl (2 liters) into the aqueous layers. The aqueous solution was reextracted five times with chloroform at pH 8.0 and the combined organic extract was again concentrated to give crude reddish brown crystals of B_1 (4.26 g). The mother liquor residue left a mixture of A_1, B_1 and other minor components which were detected by TLC. Crude crystals of B_1 thus obtained were dissolved in methanol, evaporated to dryness and recrystallized from acetone-hexane to give pure crystals of B_1 (1.58 g).

The mother liquor of crude B_1 crystals was evaporated to dryness to afford a dark brown solid, which was dissolved in 5% aqueous methanol at pH 6.0 and applied to a column (1 liter) of Amberlite XAD-2 (Rohm & Haas Co., U.S.A.). The column was developed successively with 3 liters of aqueous methanol and 80% aqueous methanol. The first eluate was extracted five times with chloroform (1 liter) at pH 8.0 and the combined and dried extract was concentrated to give reddish brown crystals (1.1 g) of B_1, while the eluate with 80% aqueous methanol was concentrated, extracted with chloroform at pH 8.0 and evaporated to afford a mixture (11.075 g) of A_1 and other components.

The latter fraction was chromatographed on a column of silica gel (275 g, Merck, Germany) and elution was carried out successively with 2.4 liters of chloroform - methanol (50 : 1), 2.4 liters of chloroform - methanol (25 : 1), 1.8 liters of chloroform - methanol (3 : 1) and 1.8 liters of chloroform - methanol (1 : 1). A_1 was eluted with chloroform - methanol (25 : 1) and other components eluted later.

A_1 fraction was concentrated to afford a crude powder of A_1 (2.94 g) which was again applied to a column of silica gel (145 g, Merck). The column was developed successively with 1 liter of ethyl acetate - methanol (50 : 1), 1.6 liters of ethyl acetate - methanol (20 : 1) and 1.5 liters of ethyl acetate - methanol (5 : 1), where 20-ml fractions were collected. Fractions containing A_1 were combined and evaporated to afford a partially purified powder of A_1 (1.059 g), which was subjected to preparative thin - layer chromatography (Merck HF_{254}) with chloroform - methanol (9 : 1) as the solvent system. A essentially pure dark red powder of A_1 (491 mg) thus obtained was crystallized from dichloromethane - hexane to give reddish brown needles of dnacin A_1 (160 mg).

The aqueous layer (F-II) in the extraction with *i*-butanol still contained smaller amounts of A_1, B_1 and other components and was concentrated at pH 4.5 to remove the remaining organic solvent. The resulting solution (169 liters) was passed through a column (240 liters) of Amberlite IRC-50 (H^+), which was washed with water (720 liters) and then eluted with 0.2 N HCl (610 liters).

The eluate was adjusted to pH 6.0 with d.NaOH solution and applied to a column (100 liters)

of Diaion HP-10, which was washed with water (305 liters) and then eluted with 80% aqueous methanol (380 liters). The eluate was adjusted to pH 4.5, concentrated to a small volume (8 liters) and extracted with chloroform (2.6 liters × 5) at pH 8.0. The antibiotics were transferred from the combined chloroform extract concentrated to a half volume (4 liters) into the aqueous layer by shaking with 0.01 N HCl (2 liters × 2).

The aqueous layer was reextracted five times with chloroform (2 liters) at pH 8.0 and the combined and dried extract was evaporated to give reddish brown crystals of B_1 (2.0 g). The mother liquor residue (4.06 g) containing A_1, B_1, and other components was purified as in the case of the F-I fraction.

Acknowledgement

We are grateful to Drs. E. OHMURA and M. YONEDA for their encouragement throughout this work. Thanks are also due to Mr. K. KOYAMA and the members of fermentation, purification and physicochemical analysis for their cooperative work.

References

1) TANIDA, S.; T. HASEGAWA & M. YONEDA: Use of Hfr strain of *E. coli* for prescreening for antitumor antibiotics. Agr. Biol. Chem., in preparation.
2) TANIDA, S.; T. HASEGAWA, M. MUROI, & E. HIGASHIDE: Dnacins, new antibiotics. I. Producing organism, fermentation, and antimicrobial activity. J. Antibiotics 33: 1443~1448, 1980
3) a) MOORE, H. W. & K. FOLKERS: Structure of rhodoquinone. J. Am. Chem. Soc. 88: 567~570, 1966
b) POWLS, R. & F. W. HEMMING: The properties and significance of rhodoquinone-9 in autotrophic and etiolated cultures of *Euglena gracilis* var. *bacillaris*. Phytochemistry 5: 1235~1247, 1966
c) SASAKI, K.; K. L. RINEHART, Jr., G. SLOMP, M. F. GROSTIC & E. C. OLSON: Geldanamycin. I. Structure assignment. J. Am. Chem. Soc. 92: 7591~7593, 1970
4) ETTLINGER, L.; E. GÄUMAN, R. HÜTTER, W. KELLER-SCHIERLEIN, F. KRADOLFER, L. NEIPP, V. PRELOG, P. REUSSER & H. ZÄHNER: Stoffwechselprodukte von Actinomyceten. XVI. Cinerubin. Chem. Ber. 92: 1867~1879, 1959
5) a) WAKAKI, S.; H. MARUMO, K. TOMIOKA, G. SHIMIZU, E. KATO, H. KAMADA, S. KUDO & Y. FUJIMOTO: Isolation of new fractions of antitumor mitomycin. Antibiot. & Chemoth. 8: 228~240, 1958
b) STEVENS, C. L.; K. G. TAYLER, M. E. MUNK, W. S. MARSHALL, K. NOLL, G. D. SHAH & K. UZU: Chemistry and structure of mitomycin C. J. Med. Chem. 8: 1~10, 1965
6) a) HERR, R. R.; M. E. BERGY, T. E. EBLE & H. K. JANKE: Porfiromycin, a new antibiotic. II. Isolation and characterization. Antimicr. Agents Annual 1960: 23~26, 1961
b) WEEB, J. S.; D. B. COSULICH, J. H. MOWAT, J. B. PATRICK, R. W. BROSCHARD, W. E. MEYER, R. P. WILLIAMS, C. F. WOLF, W. FULMOR, C. PIDACKS & J. E. LANCASTER: The structures of mitomycins A, B and C and porfiromycin-part I. J. Am. Chem. Soc. 84: 3185~3187, 1962
7) RAO, K. V. & W. P. CULLEN: Streptonigrin, an antitumor substance. Antibiotics Annual 1959/1960: 950~953, 1960