ANTIBIOTIC A-16316-C, A NEW WATER-SOLUBLE BASIC ANTIBIOTIC

ATSUSHI TAMURA, RYUJI FURUTA and HIROTADA KOTANI

Research and Development Division, Dainippon Pharmaceutical Co., Ltd., Suita, Osaka, Japan

(Received for publication March 28, 1974)

A new water-soluble basic antibiotic named antibiotic A-16316-C was isolated together with antibiotic A-396-I and hygromycin B from a streptomyces strain identified as *Streptoverticillium eurocidicus*. The properties of the antibiotic A-16316-C were similar to those of destomycin B. But, it was found that the antibiotic A-16316-C was not identical with destomycin B on the basis of NMR analysis. On acidic degradation antibiotic A-16316-C gave N, N'-dimethyl-2-deoxystreptamine, destomic acid and D-mannose. The gross structure for antibiotic A-16316-C was deduced from chemical reactions and spectral data.

During the course of screening for new antibiotics from streptomyces in our laboratory, a streptomyces designated as A-16316 was found to produce three water-soluble antibiotics named antibiotic A-16316-A, B and C. Antibiotic A-16316-A and B were identified with antibiotic A-396-I[1,2] and hygromycin B[3], respectively. Antibiotic A-16316-C was similar to destomycin B[4], but not identical. The structure of destomycin B has not been elucidated hitherto. In this paper, the production, isolation and the gross structure of A-16316-C are described.

Production and Isolation of A-16316-A, B and C

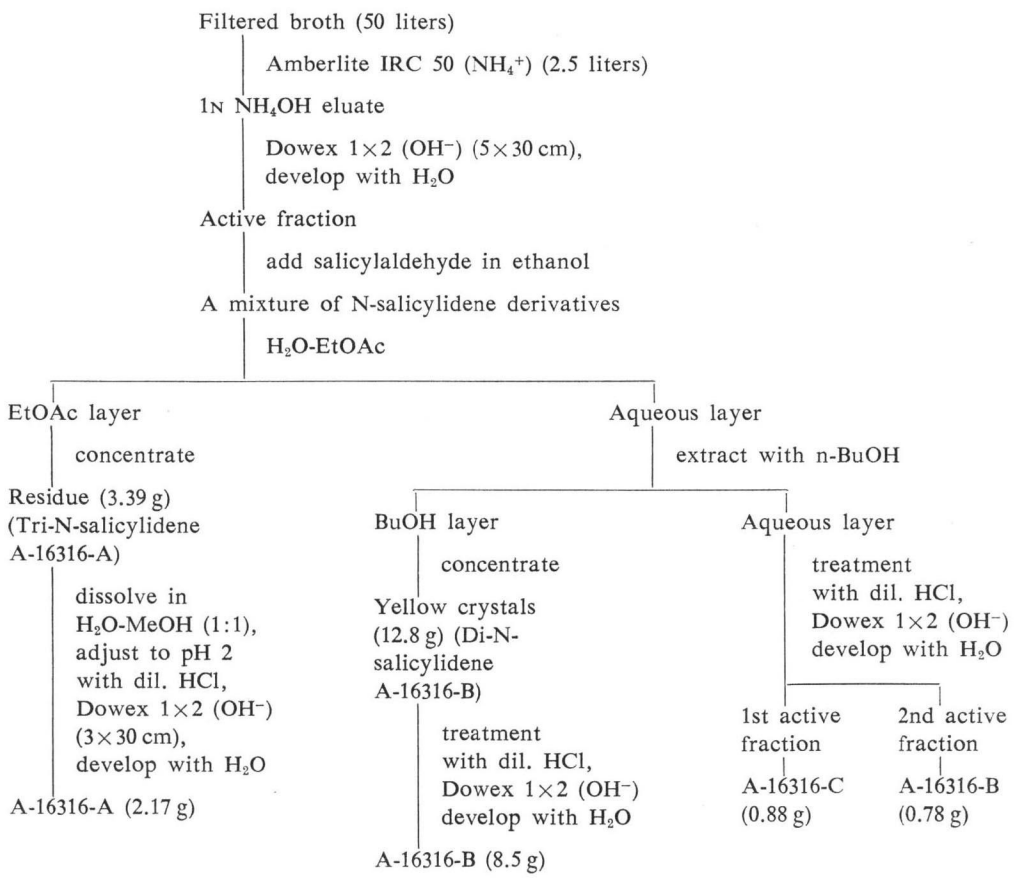
Strain A-16316 was isolated from a soil sample collected at Yasuura, Hiroshima Prefecture, Japan. According to taxonomic studies by ISP method[5] and comparative experiments using the type cultures *Streptoverticillium eurocidicus* A-396 (FERM-P No. 501), *Streptomyces eurocidicus* KCC S-0029 and *Streptomyces rimofaciens* ATCC 21066, this organism was proved to be identical with *Streptoverticillium eurocidicus*[6] (*Streptomyces eurocidicus*[7]) and designated as *Streptoverticillium eurocidicus* A-16316.

The culture of strain A-16316 was maintained on yeast-malt agar slant, and the following media were suitable for growth. Vegetative medium (%): maltose 1, glycerol 1, meat extract 0.5, Polypeptone 0.5, yeast extract 0.3, pH 7. Fermentation medium (%): soluble starch 3, defatted soybean meal 1, glucose 2, meat extract 0.5, Polypeptone 0.5, NaCl 0.3, $CaCO_3$ 0.3, soybean oil 0.2, silicon 0.06, pH 7.

A 100-liter fermentor containing 50 liters of the fermentation medium was inoculated with 3.5 liters of vegetative culture and incubated aerobically (1 liter air/liter/min.) under stirring (200 r.p.m.) at 30°C. The production of antibiotics was followed by the paper disc method using *Staphylococcus aureus* Terajima and *Escherichia coli* K-12 as test organisms. The maximum antibiotic activity was obtained after 94 hours of fermentation.

Antibiotic A-16316 substances were isolated from the culture filtrate (see Chart 1) by absorption on ion-exchange resin (Amberlite IRC 50) and *via* SCHIFF base formation. Further

Chart 1. Isolation procedure for A-16316-A, B and C



purification was achieved by chromatography on ion-exchange resin (Dowex 1×2). Pure A-16316-A 2.17 g, B 9.28 g and C 0.88 g were obtained from 50 liters of cultured broth.

Physico-Chemical Properties of A-16316-A, B and C

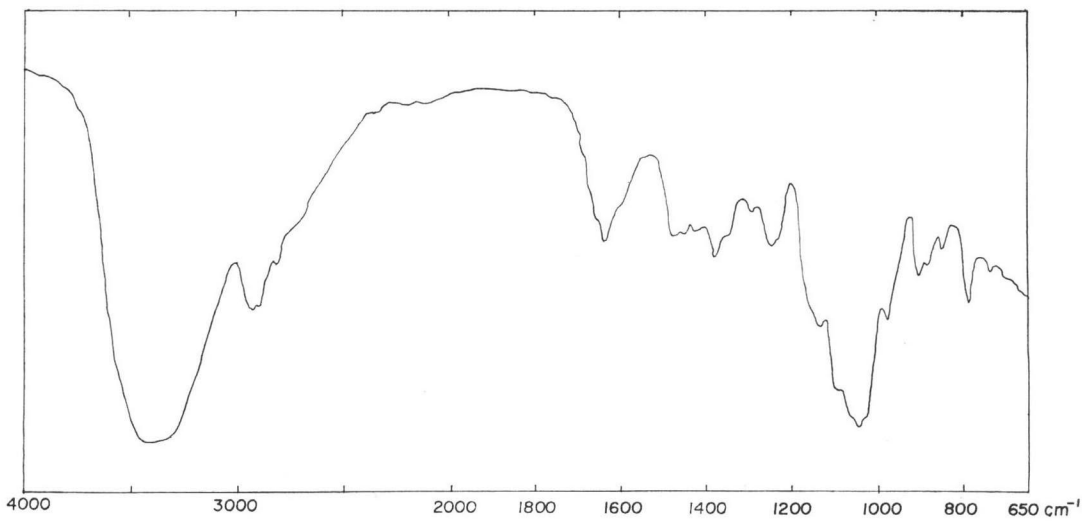
On the basis of physico-chemical properties and acid degradation products, A-16316-A, white powder $C_{19}H_{35}N_3O_{13}$, m.p. 185~195°C (dec.) $[\alpha]_D$+18° (*c* 1.0, H_2O), and A-16316-B, white powder $C_{20}H_{35}N_3O_{13}$, m.p. 170~185°C (dec.) $[\alpha]_D$ +19° (*c* 1.0, H_2O) were identified with antibiotic A-396-I[1] and hygromycin B[3], respectively. On the other hand, A-16316-C was suggested to be similar to destomycin B[4] by the following facts: (1) As shown in Table 1, its physico-chemical properties are similar to destomycin B. (2) A-16316-C and an authentic sample of destomycin B gave the same Rf value in TLC on silica gel G (0.24; $CHCl_3$-MeOH-4% NH_4OH, 12:1:1, upper layer). Its IR spectrum taken in KBr disk (Fig. 1) was similar on that of destomycin B[4]. (3) The activity of A-16316-C against *Mycobacterium* 607 is weaker than that of hygromycin B as shown in Table 2, while destomycin B is about one-tenth as active against *Mycobacterium* 607 as destomycin A[4].

However, the NMR spectrum of A-16316-C (Fig. 2) was not identical with that of an authetic sample of destomycin B. Namely, an axial proton of the methylene group in N, N'-dimethyl-2-deoxystreptamine part at δ 0.98 ppm in A-16316-C was observed as such in desto-

Table 1. Comparison of physico-chemical properties of A-16316-C and destomycin B

	A-16316-C	Destomycin B[4]
Free base	white powder	white powder
M.P. (°C)	175~185 (dec.)	140~200 (dec.)
$[\alpha]_D$ (*c* 1, H_2O)	+7.5°	+6°
UV max. (H_2O)	end	end
Mol. formula	$C_{21}H_{39}N_3O_{13}\cdot\frac{1}{2}H_2O$	$C_{21}H_{41}N_3O_{14}$ ($C_{21}H_{39}N_3O_{13}\cdot H_2O$)
Analysis:		
Calcd. C (%)	45.81	45.07
H (%)	7.42	7.39
N (%)	7.63	7.51
Found C (%)	46.12	45.34
H (%)	7.53	7.37
N (%)	7.13	7.69
Color reaction:		
ninhydrin	+	+
anthrone	+	+
SAKAGUCHI	−	−
Solubility:		
soluble	water, MeOH	water, MeOH
insoluble	EtOH, acetone, EtOAc, ether, benzene, hexane, $CHCl_3$ *etc.*	EtOH, acetone, EtOAc, ether, benzene, $CHCl_3$ *etc.*

Fig. 1. IR spectrum of A-16316-C (KBr)

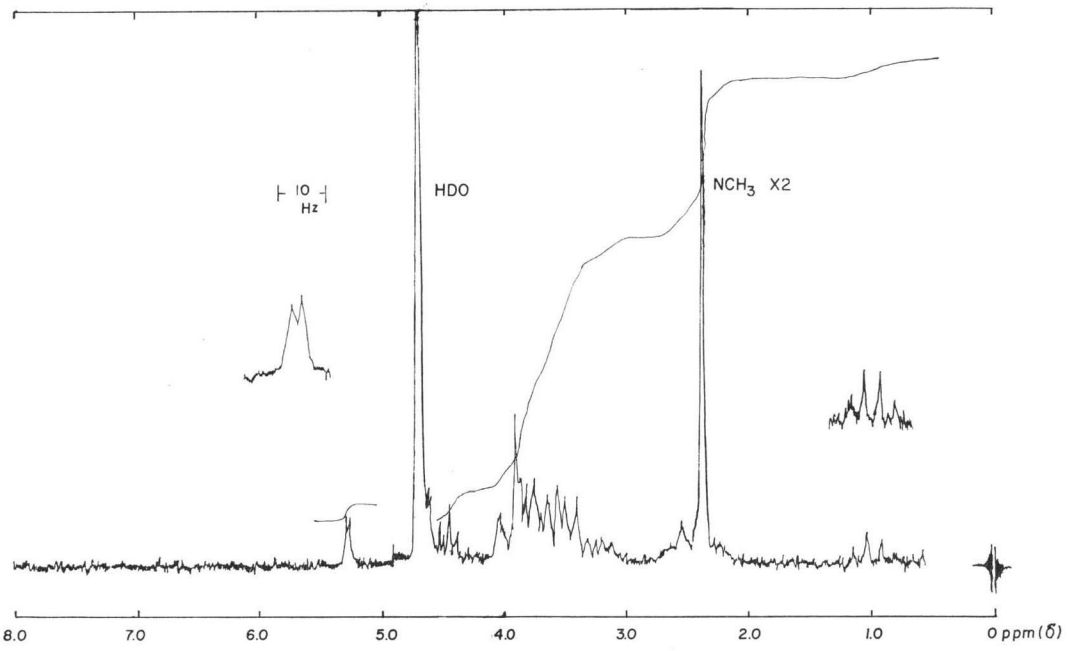


mycin B at δ 1.03 ppm. A signal attributable to two N-methyl groups of A-16316-C and that of destomycin B appeared at δ 2.39 and 2.44, respectively. Furthermore, the spectral pattern at δ 3.4~3.7 ppm corresponding to methine and methylene protons of A-16316-C was distinct from that of destomycin B.

Table 2. Antibacterial activities of A-16316-A, B and C assayed by the paper disc method (Paper disc: Thin "Toyo", 8 mm)

Antibiotic (mcg/ml)	Diameter of inhibition zone (mm)					
	A-16316-A		A-16316-B		A-16316-C	
	1,000	500	1,000	500	1,000	500
Staphylococcus aureus Terajima	17.7	14.8	18.3	15.4	19.6	16.0
Escherichia coli K-12	22.4	19.7	22.5	20.5	20.9	19.7
Mycobacterium smegmatis 607	34.6	30.0	36.0	26.0	14.5	10.0

Fig. 2. NMR spectrum of A-16316-C (D_2O, 100 Mc)



Acid Degradation Products and Gross Structure of A-16316-C

Acid degradation of A-16316-A, B and C was accomplished under conditions similar to those used for hygromycin B[8].

A-16316-C (**I**, 237 mg) was hydrolyzed with 1 N HCl at 100°C for 10 minutes to give a basic glycoside (**II**, 146 mg) and a polyhydroxy amino acid (**III**, 72 mg). These two products were isolated by means of column chromatography on Dowex 1×2 (OH^-, 2×24 cm). The fraction eluted with water was concentrated to dryness to give a white powder of basic glycoside (**II**) m.p. 124~126°C, $[\alpha]_D$ −32.1° (*c* 1.0, H_2O). Anal. Calcd. for $C_{14}H_{28}N_2O_8 \cdot \frac{1}{2}H_2O$: C 46.52, H 8.10, N 7.75 (%). Found: C 46.62, H 8.08, N 7.85 (%). After the elution of **II**, the column of Dowex 1×2 was successively eluted with 1 N acetic acid. From this fraction a polyhydroxy amino acid (**III**), colorless needles, m.p. 207~209°C (dec.), $[\alpha]_D$ +5.5° (*c* 0.97, H_2O), was obtained and identified with destomic acid by the direct comparison with an authentic sample prepared from destomycin A[9], *i.e.* TLC, IR and mixture melting point determinations.

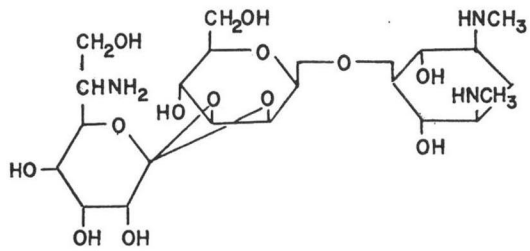
On treatment with 0.5 N H_2SO_4 at 100°C for 2 hours, the basic glycoside (**II**, 146 mg) gave a

hydrolysate from which two products, an aminocyclitol (**IV**) and D-mannose (**V**), were isolated by means of column chromatography on Dowex 1×2 (OH^-, 2×24 cm). From the fractions eluted with water, an aminocyclitol (**IV**, 69 mg) was obtained as colorless needles, m.p. 176~178°C (ethanol), $[\alpha]_D$ 0° (*c* 1.0, H_2O). Anal. Calcd. for $C_8H_{18}N_2O_3$: C 50.51, H 9.54, N 14.72 (%). Found: C 50.26, H 9.53, N 14.60 (%). Its IR spectrum lacked the bending vibration of an NH_2 group and **IV** did dot afford an N-salicylidene derivative. Its NMR spectrum taken in D_2O revealed the presence of a methylene group and two N-methyl groups appeared at δ 2.30 ppm. Acetylation of **IV** with acetic anhydride-pyridine gave a pentaacetate, colorless needles, m.p. 250~252°C ($CHCl_3$-ether). Anal. Calcd. for $C_{18}H_{28}N_2O_8$: C 53.98, H 7.06, N 7.00, mol. wt. 400. Found: C 53.94, H 6.73, N 6.77 (%), mol. wt. 400 (mass spectrum, M^+), whose NMR spectrum showed the signals of five acetyl and two N-methyl protons. The IR spectrum of the pentaacetate exhibited acetoxyl and acetamido carbonyl bands at 1730 and 1640 cm^{-1}, respectively. From these facts the aminocyclitol was considered to be N, N'-dimethyl-2-deoxystreptamine[10] and this structure was confirmed by direct comparison with the authentic sample in all respects.

The other product was isolated from subsequent 1N acetic acid elution of the Dowex 1×2 column, and identified as D-mannose by TLC (Rf 0.25 on silica gel; EtOAc-iso-PrOH-H_2O, 64:24:12) and mixed melting point determination of its methylphenylhydrazone, m.p. 174~176°C, $[\alpha]_D$ +8.6° (*c* 0.3, MeOH).

The degradation studies described above indicated that A-16316-C (**I**) consists of destomic acid, D-mannose and N, N'-dimethyl-2-deoxystreptamine and belongs to the destomycin group. The β-glycoside linkage between D-mannose and N, N'-dimethyl-2-deoxystreptamine was deduced from the NMR spectrum as shown in Fig. 2. Thus the anomeric proton of A-16316-C appeared at δ 5.35 ppm as doublet (J=2 Hz), quite typical for β-glycosides[2,8]. We have no definite

Fig. 3. Gross structure of A-16316-C



evidence for the mode of linkage of these three structural units in **I**, but the structure shown in Fig. 3 may be tentatively assigned to A-16316-C from a consideration of the biogenetic relationship to other fermentation components, hygromycin B and A-396-I.

Discussion

The destomycin group has been studied by several groups of workers in recent years. *Streptomyces rimofaciens* isolated by KONDO *et al.*[4] produced destomycin A as a major antibiotic and destomycin B as a minor. While *Streptoverticillium eurocidicus* isolated by SHOJI *et al.*[1] produced A-396-I and A-396-II (hygromycin B) in equal ratio, *Streptomyces eurocidicus* isolated by INOUYE *et al.*[11] produced SS-56 C and SS-56 D (A-396-I) in a ratio 1:2. *Streptoverticillium*

eurocidicus isolated in our laboratory producted A-16316-A (A-396-I), A-16316-B (hygromycin B) and A-16316-C in a ratio of 2:9:1.

The bioinactive substance SS-56 A containing D-mannose as a component was obtained from the culture broth of *Streptomyces eurocidicus* by INOUYE *et al.*[11], and they also suggested that an antibiotic containing β-D-mannose instead of β-D-talose of the destomycin group could be found in nature. N-Methyl-2-deoxystreptamine (hyosamine) has been found to be a component of hygromycin B[8] and destomycin A[12]. The isolation of N, N′-dimethyl-streptamine (actinamine) from the hydrolysate of actinospectacin has been reported by WILEY[13]. N, N′-Dimethyl-2-deoxystreptamine (N-methylhyosamine) has been synthesized by CLAES and VANDERHAEGHE[10], but this aminocyclitol is isolated here for the first time as a component of antibiotics. Therefore, it is verified newly from the biosynthetic point of view that A-16316-C belonging to the destomycin group contains D-mannose and N, N′-dimethyl-2-deoxystreptamine.

Acknowledgements

The authors express deep thanks to Dr. T. NIIDA, Meiji Seika Kaisha, Ltd. for the gift of destomycin B, to Phamaceutical Division of Meiji Seika Kaisha, Ltd. for the supply of destomycin A, to Dr. CLAES of Catholic University of Leuven (Belgium) for the gift of N, N′-dimethyl-2-deoxystreptamine, and Dr. A. SEINO of Kaken Chemical Co., Ltd. for the supply of the type culture (KCC S-0029). We are also very thankful to Director Dr. H. TAKAMATSU and Dr. Y. YOSHIMURA, Research Laboratories, Dainippon Phamaceutical Co., Ltd for their encouragement throughout this work. We are pleased to acknowledge the considerable assistance of Dr. S. NARUTO of our Research Laboratories. Thanks are also due to the members of Analytical Center of our Research Laboratories for microanalysis and spectroscopic measurements, to Mr. K. ISODA of our company (Hiroshima Branch) for the supply of the soil sample, and to Mr. O. TABUCHI of our laboratory for his cooperation during this work.

References

1) SHOJI, J.; S. KOZUKI, M. MAYAMA, Y. KAWAMURA & K. MATSUMOTO: Isolation of a new water-soluble basic antibiotic A-396-I. J. Antibiotics 23: 291~294, 1970
2) SHOJI, J. & Y. NAKAGAWA: Structural feature of antibiotic A-396-I. J. Antibiotics 23: 569~571, 1970
3) NEUSS, N.; K. F. KOCH, B. B. MOLLOY, W. DAY, L. L. HUCKSTEP, P. E. DORMAN & J. D. ROBERTS: Structure of hygromycin B, an antibiotic from *Streptomyces hygroscopicus*: The use of CMR spectra in structure determination. I. Helv. Chim. Acta 53: 2314~2319, 1970
4) KONDO, S.; M. SEZAKI, M. KOIKE, M. SHIMURA, E. AKITA, K. SATOH & T. HARA: Destomycins A and B, two new antibiotics produced by a streptomyces. J. Antibiotics, Ser. A 18: 38~42, 1965
5) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Inter. J. System. Bact. 16: 313~340, 1966
6) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type strains of *Streptomyces*. V. Additional description. *Streptoverticillium eurocidicus*. Inter. J. System. Bact. 22: 293, 1972
7) OKAMI, Y.; R. UTAHARA, S. NAKAMURA & H. UMEZAWA: On streptomyces producing a new antifungal substance mediocidin and antifungal substances of fungicidin-rimocidin-chromin group, eurocidin group and trichomycin-ascosin-candicidin group. J. Antibiotics, Ser. A 7: 98~103, 1954
8) WILEY, P. F.; M. V. SIGAL & O. E. WEAVER: Degradation products of hygromycin B. J. Org. Chem. 27: 2793~2796, 1962
9) KONDO, S.; E. AKITA & M. SEZAKI: A new polyhydroxy amino acid, destomic acid, the hydrolysis product of destomycin A. J. Antibiotics, Ser. A 19: 137~138, 1966
10) CLAES, P. & E. VANDERHAEGHE: Preparation of octa-N-methyl- and tetra-N-methylkanamycin. Bull. Soc. Chim. Belges 78: 561~569, 1969
11) INOUYE, S.; T. SHOMURA, H. WATANABE, K. TOTSUGAWA & T. NIIDA: Isolation and gross structure of a new antibiotic SS-56 C and related compounds. J. Antibiotics 26: 374~385, 1973
12) KONDO, S.; M. SEZAKI, M. KOIKE & E. AKITA: Destomycin A. The acid hydrolysis and the partial structure. J. Antibiotics, Ser. A 18: 192~194, 1965
13) WILEY, P. F.: The chemistry of actinospectacin. I. Actinamine. J. Amer. Chem. Soc. 84: 1514~1515, 1962