UDC 615.779.931-011

6. Tomoharu Okuda, Makoto Suzuki, Yoshiyuki Egawa, and Kokichi Ashino: Studies on Streptomyces Antibiotic, Cycloheximide. II. Naramycin-B, an Isomer of Cycloheximide.

(Tokyo Research Laboratory, Tanabe Seiyaku Co., Ltd.)*

In the preceding paper,[1] the authors reported that a Streptomyces (*Streptomyces naraensis novo sp.*) produces two antifungal antibiotics, named Naramycin-A and -B, and that Naramycin-A is identical with cycloheximide (Actidione) reported by Leach, *et al.*[2]

In the present paper the authors would like to report on the isolation and nature of the second component, Naramycin-B, which was found to be a stereoisomer of cycloheximide.

Crude Naramycin-B was isolated from the mother liquor left after removal of Naramycin-A. By means of alumina chromatography and repeated recrystallizations, Naramycin-B came as colorless thin plates, melting at 109~110°.

It is a dextrorotatory neutral substance of the formula $C_{15}H_{23}O_4N$, showing λ_{max}^{MeOH} at 292.5 mμ (log ε 1.49) and a shoulder at 232 mμ. Infrared spectrum of Naramycin-B in Nujol is different from that of Naramycin-A, especially in ν_{OH} region (Fig. 1).

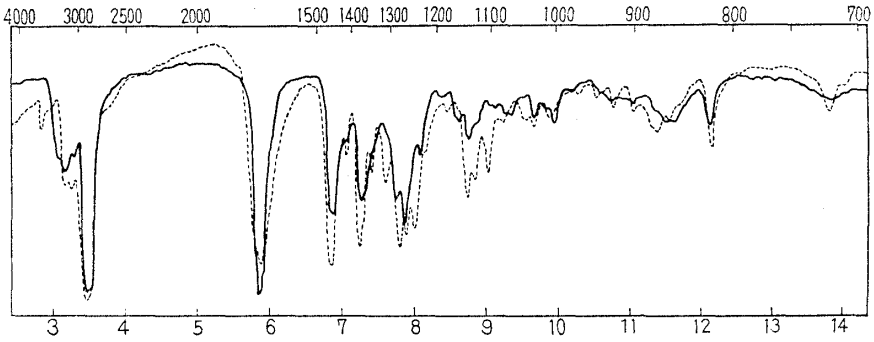
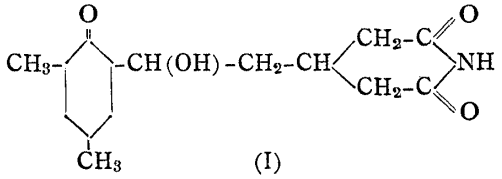


Fig. 1. Infrared Spectra of Naramycin-A and -B (Nujol mull)
..... Naramycin A
——— Naramycin B

Naramycin-B is active against microörganisms sensitive to cycloheximide but its activity seems to be less than that of Naramycin-A with few exceptions (Table I). From the result of cup-assay of the two Naramycins, Naramycin-B was found to have only 32% activity of Naramycin-A against *Saccharomyces sake*. Neither synergistic nor antagonistic action is found between the two antibiotics. LD_{50} of Naramycin-B is 70 mg./kg. for mice and 6 mg./kg. for rats when intraperitoneally administered.

Naramycin-B gives *cis-d*-dimethylcyclohexanone by alkaline degradation and gives anhydrocycloheximide by dehydration with phosphorus pentoxide or catalytic amounts of BF_3-ether complex, and gives dehydrocycloheximide by chromium trioxide oxidation. These products agreed well with those derived from cycloheximide and Naramycin-A.

From these experimental results Naramycin-B was proved to be one of the stereoisomers of cycloheximide due to any of the four asymmetric carbon atoms in the formula (I).



(I)

* 731 Daita 1-Chome, Setagaya-ku, Tokyo (奥田朝晴, 鈴木真言, 顕川吉之, 芦野孝吉).

1) Part I: This Bulletin, **6**, 711(1958).

2) B. E. Leach, J. H. Ford, A. J. Whiffen: J. Am. Chem. Soc., **69**, 474 (1947).

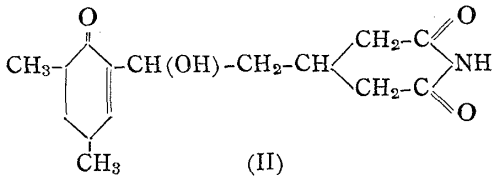
TABLE I. Antimicrobial Activity of Naramycin-A and -B by Agar-Streak Dilution Method

Test organism*		Minimum inhibitory concn. (γ/cc.) Naramycin-A	Naramycin-B
Saccharomyces sake		0.5	2.0
Saccharomyces cerevisiae		0.2	0.5
Saccharomyces formosensis		0.2	0.5
Torula rubra		1.0	5.0
Torula utilis		2.0	5.0
Torula candida		20.0	>100.0
Zygosaccharomyces soya		100.0	100.0
Zygosaccharomyces salsus		2.0	10.0
Hansenula Wil-7		0.5	2.0
Candida albicans		>100.0	>100.0
Candida krusei		2.0	5.0
Trichophyton asteroides		>100.0	>100.0
Aspergillus niger		>100.0	>100.0
Aspergillus oryzae		>100.0	>100.0
Mucor spinescens		>100.0	>100.0
Penicillium chrysogenum		>100.0	>100.0
Penicillium citrinum		20.0	50.0
(Test medium: Sabouraud's agar (27°, 48 hrs.)			
Piricularia oryzae		2.5	6.0
Botrytis cinerea		10.0	20.0
Mycosphaerella pinodes		1.0	10.0
Glomerella cingulate		5.0	10.0
Gibberella Fujikuroi		20.0	20.0
Ophiobolus miyabeanus		5.0	10.0
Gloeosporium Kaki		5.0	5.0
Alternaria Kikuchiana		5.0	1.0
Xanthomonas citri		>100.0	>100.0
*Sclerotinia Mali***	120 hrs.	0.5	0.25
	168 hrs.	2.0	0.5
(Test medium: Potato-sucrose-agar (27°, 48 hrs.)			

* The test organisms were kindly supplied by the National Institute of Health, Tokyo, the Institute of Applied Microbiology, University of Tokyo, the National Institute of Agricultural Sciences, the Fermentation Research Institute, Agency of Industrial Science and Technology, and Government Agricultural Experiment Station. The authors express their deep gratitude.

** Examined at NIKKEN Chemicals Co. Ltd.

Paul and Tchelitcheff[3] synthesized the three isomers of Actidione (they called them α-, β-, and γ-Isoactidione) by catalytic hydrogenation of Inactone (II), which is found as a by-product of Actidione in the fermentation broth of *Streptomyces griseus*. However, Naramycin-B does not seem to agree with any of them.



(II)

Hamilton, *et al.*[4] and Lemin, *et al.*[5] reported the application of an isomer of cycloheximide in the greenhouse test to control some plant diseases, but nothing was described regarding the origin and chemical properties of this isomer.

3) R. Paul, S. Tchelitcheff: Bull. soc. chim. France, **1955**, 1316.
4) J. M. Hamilton, M. Szkolnik, E. Sondheimer: Science, **123**, 1175 (1956).
5) A. J. Lemin, G. A. Boyack, W. C. Haskett, A. Steinhards, G. Swank: Abstr. Papers 132nd Meeting of the American Chemical Society, 24A (1957).

The authors express their deep gratitude to Prof. S. Umezawa of Keio-Gijuku University for his kind guidances, and to Dr. J. Hata and Dr. S. Yamada, the former and the present director of this Laboratory, for their helpful encouragement. The authors thank NIKKEN Chemicals Co. Ltd. for the supply of Naramycins, Mr. T. Yoda and Mrs. F. Hisamichi for elementary analyses, and Mr. K. Kodera for infrared spectral data.

Experimental

(All m.p.s are not corrected)

Isolation of Naramycin-B—The fermentation broth of *Streptomyces naraensis* was concentrated to a syrup as illustrated in Chart 1 (p.714 in the preceding paper). By treating the concentrate with equal amount of isoamyl acetate, Naramycin-A crystallized out and Naramycin-B was isolated from the residual mother liquor.

After removal of Naramycin-A 200 cc. of the mother liquor (188,000 U/cc.*) was concentrated *in vacuo* and the residual brownish syrup was extracted with benzene. After separating from insoluble oily substance, the benzene solution was poured into a column of activated alumina (H-form). The column was developed with benzene containing 3% of MeOH until the eluate showed no activity. Active fractions were collected and concentrated *in vacuo* to a brownish syrup, to which a large quantity of ether was added and the mixture was kept in a refrigerator overnight. From the ethereal solution, 50 g. of crude Naramycin-B (450U/mg.) crystallized out; m.p. 75~80°.

Purification of Naramycin-B—Crude Naramycin-B was dissolved in benzene and adsorbed on a column of activated alumina (H-form). The alumina column was washed with benzene to remove remaining Naramycin-A. After the main portion of Naramycin-A eluted, benzene was displaced with benzene containing 3% of MeOH, by which the main portion of Naramycin-B was eluted.** Active fractions were collected, evaporated *in vacuo*, and solidified with ether-hexane mixture (1:1) with cooling.

By repeated recrystallizations of this crude substance from AcOEt, m.p. of Naramycin-B was raised to 90~95°. At this stage the sample was recrystallized from water, m.p. of Naramycin-B rose gradually, and finally Naramycin-B came as colorless plates, m.p. 109~110°; $[\alpha]_D^9$ +48.8° (c=1, H_2O); $[\alpha]_D^{12.5}$ +50.2° (c=2, MeOH). Clear depression in m.p. (mixed m.p. 101.5~104°) was observed on admixture with Naramycin-A. *Anal.* Calcd. for $C_{15}H_{23}O_4N$; C, 64.03; H, 8.24; N, 4.98; mol. wt., 281.34. Found: C, 64.30; H, 7.80; N, 4.90; mol. wt. (Micro-Rast), 281.8.

Naramycin-B Acetate—To a solution of 200 mg. of Naramycin-B dissolved in 1 cc. of pyridine, 1 cc. of Ac_2O was added with cooling. After standing overnight at room temperature, a volatile product was removed *in vacuo* and the residue crystallized upon standing. Two recrystallizations from 99% *iso*-PrOH gave colorless prisms, m.p. 150.5~152°; $[\alpha]_D^{12.5}$ +62.15°(c=2, MeOH). Clear depression in m.p. (mixed m.p. 128~130°) was observed on admixture with Naramycin-A acetate (m.p. 147~147.5°). *Anal.* Calcd. for $C_{17}H_{25}O_5N$: C, 63.14; H, 7.79; N, 4.32. Found: C, 62.76; H, 7.61; N, 4.40.

Naramycin-B Oxime—A solution of 100 mg. of Naramycin-B in 0.2 cc. of MeOH was added to a solution of 140 mg. of $NH_2OH \cdot HCl$ and 240 mg. of anhyd. AcONa in 0.66 cc. of water. Upon standing overnight at room temperature 80 mg. of white crystals was obtained. Two recrystallizations from 50% MeOH gave colorless prisms, m.p. 142~144°. *Anal.* Calcd. for $C_{15}H_{24}O_4N_2 \cdot H_2O$; C, 57.32; H, 8.28; N, 8.91. Found: C, 57.44; H, 7.99; N, 9.04.

Naramycin-B Semicarbazone—A solution of 50 mg. of Naramycin-B in 0.25 cc. of MeOH was added to a solution of 50 mg. of semicarbazide hydrochloride and 65 mg. of anhyd. AcONa in 0.7 cc. of water. Upon standing overnight at room temperature 38 mg. of white crystals, m.p. 161~163°, was obtained; Recrystallization from 30% MeOH raised the m.p. to 168~169.5°. *Anal.* Calcd. for $C_{16}H_{26}O_4N_4 \cdot {}^1/_2H_2O$; C, 55.20; H, 7.78; N, 16.15. Found: C, 55.11; H, 7.39; N, 15.68.

Isolation of *cis-d*-2,4-Dimethylcyclohexanone by Alkaline Degradation of Naramycin-B—A solution of 300 mg. of Naramycin-B dissolved in 6 cc. of 20% NaOH solution was distilled until about one-half the original volume remained. The distillate was saturated with NaCl and extracted with ether. The extract was dried over Na_2SO_4 and the ether was removed. The residue was distilled, b.p. 175~176°; yield, 110 mg. $[\alpha]_D^{16}$ +10.84° (c=5, MeOH). The semicarbazone was prepared and purified by recrystallization from 50% EtOH, m.p. 213~214°(decomp.)(corr.).

These products agreed with *cis-d*-2,4-dimethylcyclohexanone and its semicarbazone which were obtained by the degradation of Actidione as reported by Kornfeld, *et al.*[6]

* For the assay of Naramycins, purified Naramycin-A (m.p. 116~116.5°) was used as a standard.

** It seemed that Naramycin-B forms a molecular complex with Naramycin-A and it was difficult to separate them completely by alumina chromatography alone. Thus, recrystallization from a polar solvent was necessary.

6) E. C. Kornfeld, R. G. Jones, T.V. Parke: J. Am. Chem. Soc., **71**, 150 (1949).

Oxidation of Naramycin-B—Naramycin-B was oxidized with CrO_3 in glacial AcOH in the same way as reported by Kornfeld, *et al.*[6] Oxidized product thus obtained was recrystallized from 50% EtOH; m.p. 171.5~172.5°. No depression in m.p. on admixture with Dehydroactidione (m.p. 172~173° and m.p. 174~175°, respectively derived from Naramycin-A and Actidione).

Dehydration of Naramycin-B—1) Dehydration with P_2O_5: Naramycin-B was dehydrated with P_2O_5 in dehyd. benzene in the same way as described by Kornfeld, *et al.*[6] Dehydrated product; m.p. 131.5~132.5°.

2) Dehydration with BF_3-ether complex: To a solution of 150 mg. of Naramycin-B dissolved in 1.5 cc. of dehyd. benzene, 0.075 cc. of BF_3-ether complex (47.3%) and 0.1 cc. of glacial AcOH were added. After standing for 5 hrs. at room temperature, the mixture was poured into ice water and extracted with benzene. The benzene extract was dried over Na_2SO_4 and benzene was removed. The residue was recrystallized from EtOH and 62 mg. of a crude product, m.p. 131~133°, was obtained (yield, 44%). The substance was recrystallized twice from EtOH; m.p. 132.5~133°; $[\alpha]_D^{11}$ −12.6° (c=1.33, MeOH). No m.p. depression was observed on admixture of both products with authentic specimens. Moreover, both of these dehydrated products showed no depression in m.p. on admixture with Anhydroactidione derived from Naramycin-A.

Summary

The second component, Naramycin-B, was isolated from the fermentation broth of *Streptomyces naraensis novo sp.* This antibiotic was also active against microörganisms sensitive to cycloheximide. The physical and chemical properties of Naramycin-B indicated that this antibiotic was one of the stereoisomers of cycloheximide.

(Received July 5, 1958)