pyridazine 2-oxide (Ic), 3,4-dimethyl (Id) and 3-methylpyridazine 1-oxide (Ie) was examined and it was proved that Ia, Ib, Ic and Ie afforded γ-nitropyridazine N-oxides (IIa～IIe), whereas Id in which γ-position was substituted gave α-nitro N-oxide (IId). All of these nitro derivatives were derived to aminopyridazines (IIIa～IIIe) by catalytic reduction over Raney-nickel in methanol or over palladium-charcoal in dilute hydrochloric acid. Ultraviolet spectra of several aminopyridazines are also given.

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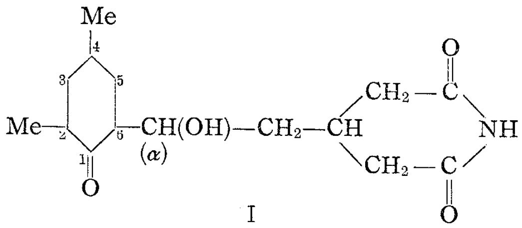
125. Tomoharu Okuda, Makoto Suzuki, Tamotsu Furumai, and Hiroko Takahashi : Studies on Streptomyces Antibiotic, Cycloheximide. XVIII.[1] Isomerization Study of Cycloheximides and Thermal Degradation of Naramycin-B. Chemical Support of the Proposed Absolute Configuration of Cycloheximides.[*1]

(*Tokyo Research Laboratory, Tanabe Seiyaku Co., Ltd.*[*2])

As evident from the plane structure (I) of cycloheximide which was elucidated by Kornfeld, *et al.*,[2] cycloheximide has four asymmetric centers in the molecule and, consequently, sixteen stereoisomers are possible to exist theoretically. Among these stereoisomers, three of them named as isocycloheximide,[3] Naramycin-B[4] and *α-epi*-isocycloheximide[*3, 5] were the main isomers found in a fermentation beer of a streptomyces or obtained by isomerizing cycloheximide or by synthesis.



I

Studies on the elucidation of the absolute configuration of cycloheximide and its isomers began with Eisenbraun, *et al.*[6] who conformed the absolute configuration of (−)-2,4-dimethylcyclohexanone, an alkaline degradation product of cycloheximides, belonging to (2R:4R)-series. Thus, it was made evident that the absolute configuration of C-4 of cycloheximide and other isomers belongs to (S)-series. Lemin and Ford,[3] who successfully derived cycloheximide into isocycloheximide, suggested that a more

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[*1] Preliminary accounts were published as a Communication to the Editor in this Bulletin, **10**, 639 (1962).

[*2] Toda-machi, Kitaadachi-gun, Saitama-ken (奥田朝晴, 鈴木真言, 古米 保, 高橋裕子).

[*3] A compound referred to as A_2 in J. Antibiotics, **14A**, 158 (1961).

1) Part XVII. T. Okuda, M. Suzuki : This Bulletin, **9**, 1014 (1961).

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

3) A.J. Lemin, J.H. Ford : J. Org. Chem., **25**, 344 (1960); U.S. Patents : No. 2,903,457 and 2,903,458 (1961).

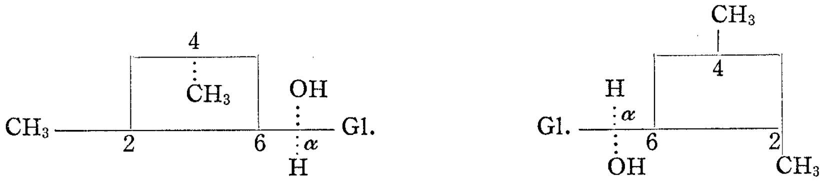
4) Part II. T. Okuda, M. Suzuki, Y. Egawa, K. Ashino : This Bulletin **7**, 27 (1959).

5) Part XV. M. Suzuki, Y. Egawa, T. Okuda : This Bulletin, **11**, 582 (1963).

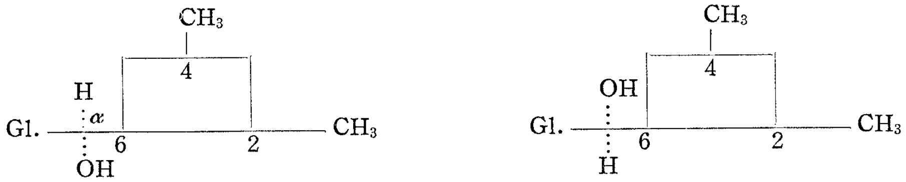
6) E.J. Eisenbraun, J. Osiecki, C. Djerassi : J. Am. Chem. Soc., **80**, 1261 (1958).

stable isomer isocycloheximide would have 2,6-*cis* configuration, while less stable one, cycloheximide, would have 2,6-*trans* configuration, and that the isomerism between both isomers would be ascribed to the C-6 position. In 1962, by thermal degradation of cycloheximide, Lawes⁷⁾ obtained (+)-2,4-*trans*-dimethylcyclohexanone of (2S:4R)-series which was readily isomerized to (2R:4R)-*cis* isomer by alkaline treatment and he deduced that cycloheximide should possess 2,4-*trans* relationship in the molecule and finally proposed to cycloheximide (2S:4S:6R)-configuration (ring structure of Formula (3)). Recently Schaeffer and Jain⁸⁾ assumed cycloheximide to have 2,4-*trans* configuration, based on the stereochemical study on anhydrocycloheximide, a dehydrated product of cycloheximide.

Independently from the above American groups, the present authors have been engaged in elucidating the absolute configuration of cycloheximides and proposed the absolute configurations of four cycloheximides as depicted in Fig. 1 in the preceding paper¹⁾. The elucidation was made principally based on optical rotatory dispersion analysis and other physicochemical methods. The absolute configurations proposed by the authors were not always the same as those of American groups. Therefore, the presentation of chemical proofs to support the authors' proposition was inevitably needed.*⁴ The present paper concerns a thermal degradation study and isomerization study of cycloheximides carried out along the above requirements. The paper also deals with the separation of cycloheximides by diatomeous earth (Celite) chromatography and discernment of isomers by infrared spectra, which facilitated the study remarkably.



cycloheximide (2) Naramycin-B (3)



isocycloheximide (4) α-*epi*-isocycloheximide (5)

Fig. 1. The Absolute Configuration of Cycloheximides

Thermal Degradation Study of Cycloheximides

After the procedure of Lawes⁷⁾ thermal degradations of cycloheximide and its stereoisomers were carried out and the liberated ketones were purified by redistillation and derived into semicarbazones. As a result of optical rotatory dispersion determination of the produced ketones and mixed melting point determination of their semicarbazones, it was found as illustrated in the Table I that Naramycin-B gave (2S:4R)-2,4-dimethylcyclohexanone of 2,4-*trans* relation which was identical with that derived

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*⁴ According to the recent correspondence from Dr. F. Johnson of the Dow Chemicals Co., nuclear magnetic resonance studies on cycloheximide and its stereoisomers gave the results which supported the authors' views. (c.f. Tetrahedron, 1173 (1962).

7) B. C. Lawes: J. Am. Chem. Soc., **84**, 239 (1962).

8) H. J. Schaeffer, V. K. Jain: J. Pharm. Sci., **50**, 1048 (1961).

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from cycloheximide, whereas isocycloheximide did (2R:4R)-2,4-dimethylcyclohexanone of *cis*-relation which was the same as the ketone obtained by an alkaline degradation of cycloheximide and other isomers. Anhydrocycloheximide has not undergone pyrolysis under the similar condition.

Table I. Thermal Degradation of Cycloheximides

Starting isomer	Ketone liberated				Semicarbazone	
	b.p. (°C)	Yield (%)	$[\alpha]_D$ [a]	ORD [b] (extr., mμ)	m.p. (°C)	$[\alpha]_D$ [c]
Cycloheximide	78～79/25 mm. 177～178	71	+59.2°	+771° (312.5) (positive)[d]	171～172	+151.0°
Naramycin-B	77～78/24 mm.	47	+58.2°	+684° (312.5) (positive)	170～171	+148.4°
Isocycloheximide	73～74/20 mm.	46	+11.3°	−245° (300.0) (negative)	208	+ 42.6°
Isocycloheximide (Alkaline degradation)	76～79/25 mm. 176～177	68	+10.8°[e]	−278° (297.5) (negative)	205	+ 40.6°

a) c=5.0, MeOH *b*) c=0.1, MeOH *c*) c=1.0, DMF *d*) Sign of Cotton Effect
e) c.f. J. Osiecki: Dissertation Abstr. **21**, No. 5, Mic. 60～3824 (1960)

Isomerization Study of Cycloheximides

Lemin and Ford[3)] found that cycloheximide isomerized into isocycloheximide by ageing the former for as long as 18 months in a solution or by treating the former with acid deactivated alumina. But the isomerization took place not only in such a neutral or acidic environment but also in basic conditions. As shown by the curve (d) in Fig. 2, when the benzene solution of cycloheximide was heated gently in the presence of sodium methoxide, the antimicrobial activity shown by the solution reduced rapidly to about one-third of the initial activity and then gradually. Reduction of the activity was found due to the isomerization of cycloheximide, since isocycloheximide was recovered from the reaction mixture reached at the level of one-third of the initial activity. Similar phenomena were observed when alkaline hydroxide or alkaline salts such as potassium hydroxide, carbonate, cyanate and phosphate were used as a catalyst instead of sodium methoxide, not only in benzene but also in other solvents like alcohol or tetrahydrofuran, some of the experiments being illustrated in Fig. 2. Among the basic catalysts tested, sodium methoxide and potassium carbonate were found most adequate when used in an amount of 10 to 20% of cycloheximide.

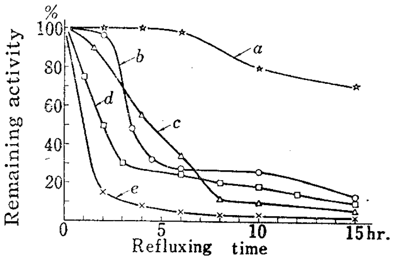

Fig. 2. Isomerization of Cycloheximide

Experimental Conditions: The solution of 1.0 g. of cycloheximide in benzene (20 cc.) was added with 0.2 g. of basic catalyst and refluxed gently. Aliquot of the solution was sampled at times and measured of its remaining activity against *S. cerevisiae*.

a, K_2HPO_4 (☆) *b*, K_2CO_3 (○) *c*, KCN (△)
d, NaOMe (□) *e*, KOH (×)

In the course of isomerization of cycloheximide using less amounts of catalyst, when the heating of the solution was discontinued at earlier stage, Naramycin-B was recovered from the resultant mixture of isomers by means of partition chromatography over Celite Analytical Filter-Aid.

Further isomerization studies under the like conditions were carried out on Naramycin-B, isocycloheximide and *α-epi*-isocycloheximide and it was found as summarized in the Table II, that, among three isomers, Naramycin-B turned into isocycloheximide as in the case of cycloheximide, while other two isomers were recovered intact.

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Table II. Isomerization of Cycloheximides[a)]

Result obtained by gently refluxing the benzene solution containing 5% cycloheximides and 1% MeONa for 4 hr.

Starting isomer	Recovered substance	Recovered yield (%)	Result
Cycloheximide	isocycloheximide	48	isomerized
〃 [b)]	Naramycin-B	33	〃
	isocycloheximide	8	〃
Naramycin-B	〃	61	〃
Isocycloheximide	〃	70	not isomerized
α-Epi-isocycloheximide	*α-epi*-isocycloheximide	65	〃
Anhydrocycloheximide	anhydrocycloheximide	68	〃
Deoxycyloheximide[c)]	deoxycyloheximide	65	〃

a) In the Table were shown the results carried out on anhydrocycloheximide and deoxycycloheximide for the sake of comparison.

b) In this case, 0.5% of MeONa was used and heating was discontinued 2 hr. later.

c) The compound VII in the Part V (This Bulletin, **7**, 666 (1959)).

Separation of Cycloheximides by Means of Diatomeous Earth (Celite) Partition Chromatography

Separation of cycloheximide isomers was carried out by modifying the procedure described by Eble, *et al.*,[9)] who succeeded in the separation of streptovitacins, cycloheximide and isocycloheximide by gradient partition chromatography on Celite 545 column using solvent systems composed of various ratio of ethyl acetate, cyclohexane and McIlvaine's pH 5.0 buffer.

In the present experiments, the solvent system employed was the same as used by Eble, *et al.* for the separation of isocycloheximide from cycloheximide, that is, comprising one part of ethyl acetate, nine parts of cyclohexane and one part of McIlvaine's buffer. Celite 545 partition chromatography made it possible as reported by Eble, *et al.* to isolate isocycloheximide from cycloheximide and Naramycin-B but was inadequate to isolate Naramycin-B from cycloheximide. Naramycin-B was successfully separated by employing finer diatomeous earth (Celite Analytical Filter-Aid) instead of Celite 545. As illustrated in the Fig. 3 which summarized the result run with Naramycin-B rich sample, isocycloheximide and Naramycin-B were recovered from pool No. 1 (fraction 5 to 10) and pool No. 2 (fraction 11 to 39) respectively, and cycloheximide was eluted following to Naramycin-B fraction. Naramycin-B thus recovered was freed from small amounts of contaminating cycloheximide by recrystallization from isopropyl acetate to afford a sample which was purer than that reported previously[4,10)] and showed the following properties.

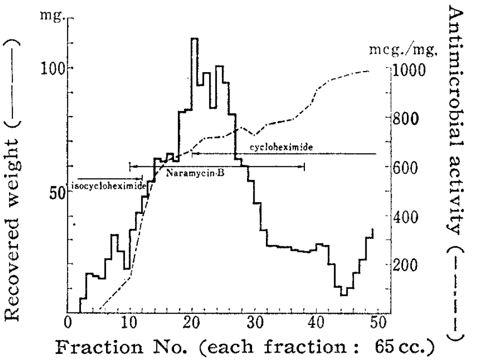

Fig. 3. Celite Analytical Filter-Aid Partition Chromatography of Cycloheximides

Result obtained by partition chromatography of 2.0 g. of Naramycin-B rich sample on 60 g. of celite analytical filter-aid, solvent system being AcOEt-cyclohexane-McIlvain's pH 5.0 phosphate buffer (1:9:1).

Naramycin-B: Colorless thin prisms, m.p. 112~113°, $[\alpha]_D^{21.8}$ +55.8° (c=5, MeOH), $[\alpha]_{317.5}^{23.2}$ +998 (peak) (c=0.1, MeOH, Xe), bioassay[*5] 350 mcg./mg.

[*5] Cycloheximide standard sample was used as a standard and *S. cerevisiae* was used as a test organism.

9) T.E. Eble, M.E. Bergy, R.R. Herr, T.A. Fox: Antibiotics & Chemotherapy, **10**, 479 (1960).

10) Part XIV. T. Okuda, M. Suzuki, Y. Egawa, K. Kotera: Yakugaku Kenkyu, **33**, 530 (1961).

Its acetate : Colorless prisms, m.p. 155.5°, $[\alpha]_D^{21.8}$ +74.5° (c=2, MeOH), $[\alpha]_{312.5}^{21.5}$ +1327° (peak) (c=0.1, MeOH, Xe).

It is to be added that, when cycloheximides contain anhydrocycloheximide as a contaminant, anhydrocycloheximide was found to precede isocycloheximide, and that there was found no evidence of dehydration or isomerization during the chromatography.

As evident from the infrared spectra in nujol mulls (Fig. 4) shown by cycloheximides in pure state, frequencies and intensities of the absorption bands existing in 1100~1000 cm^{-1} region were of great use to discern each of cycloheximides and to detect the contaminating isomer(s).

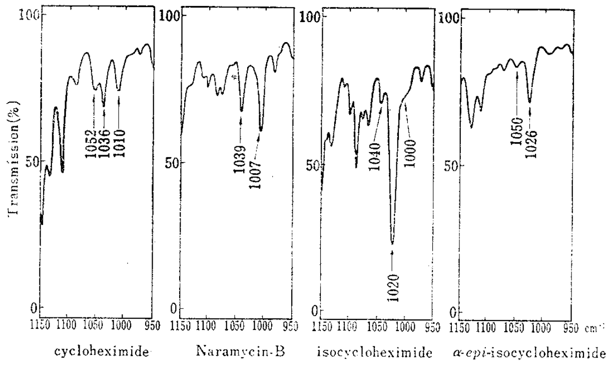

Fig. 4. Infrared Spectra of Cycloheximides (1150~950 cm^{-1} region in nujol mulls)

Reconsideration of the Absolute Configuration of Cycloheximides Proposed by the Authors

As described in the preface of this paper, the absolute configuration of cycloheximides proposed by the present authors were mainly elucidated as a result of the physicochemical analysis including optical rotatory dispersion, infrared etc., and presentation of some chemical supports appeared desirable. Now allow the authors to consider again on the absolute configurations based upon the several findings described in the present paper.

First of all, it became evident that the absolute configuration of α-asymmetric center existing in cycloheximide, Naramycin-B and isocycloheximide is the same, because the former two isomers were isomerized into isocycloheximide and because it is difficult to suppose that C-α situating far from activating centers might sustain isomerization. The identity of the configuration of α-center of these three isomers was deduced according to the Freudenberg's rule of shift by comparing $[\alpha]_D$ values shown by these isomers and their acetates respectively. The present chemical evidence does support the rightness of adopting this rule and consequently consideration made on α-center of α-*epi*-isocycloheximide is thought to be correct. Moreover, as to the spatial relationship between α-hydroxy and carbonyl groups which had much connection with the absolute configuration of α- and 6-assymmetric centers and was reflexed upon the infrared spectra of the compound, the infrared characteristic shown by α-*epi*-isocycloheximide was explained without any inconsistence along with the way made on other three isomers as described previously.$^{5)}$

Second, it is clear that Naramycin-B does have the configuration of 2,4-*trans* relation as proposed, because Naramycin-B gave (2S:4R)-*trans*-2,4-dimethylcyclohexanone on the thermal degradation.

According to the authors' proposition, isocycloheximide (4) and α-*epi*-isocycloheximide (5) had the most stable configuration of 2,4,6-(*cis, cis*) relation. The present finding that both of these isomers were recovered intact in the isomerization study does uphold the configuration proposed by the authors. Moreover, the assignment of stable configuration to these isomers is also supported by the fact that these isomers were

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obtained synthetically by condensing glutarimide-β-acetaldehyde with (2R:4R)-2,4-dimethylcyclohexanone as described already in the Part XV.5)

Finally, the correctness of the assignment of structure (2) and (3) to cycloheximide and Naramycin-B respectively is also reconsidered from the finding resulted from isomerization studies carried out on these isomers. The isomerization studies showed that Naramycin-B has the more stable configuration than cycloheximide, because Naramycin-B was found to exist in the course of isomerization of cycloheximide into isocycloheximide. On the other hand, among the structures having the 2,4-*trans* relation, structure (3) is considered more stable than structure (2) owing to the influence of "2-alkyl ketone effect." Therefore, the authors' assignment of structure (3) to Naramycin-B is consistent with the above chemical finding.

Thus, the above-mentioned chemical findings supported all of the structures of the cycloheximides proposed by the present authors as the result of physicochemical studies of the compounds, based upon the premise that all of the isomers had the cyclohexanone ring of chair conformation.

Experimental

Thermal Degradations of Cycloheximides: Cycloheximide——Five grams of cycloheximide (m.p. 116∼117°) was heated in an oil bath kept at 210∼230° under reduced pressure (20∼30 mm. Hg) and the volatile substance (b.p$_{25}$ 77∼79°) was collected, dissolved in Et$_2$O, washed with H$_2$O, dried with anhyd. Na$_2$SO$_4$ and concentrated. The resultant was redistilled under atmospheric pressure and 3.55 g. of an oil of positive Cotton effect was obtained, b.p. 177∼178°; yield, 71%. This oil was identical with (2S:4R)-2,4-dimethylcyclohexanone reported by Lawes and gave the same semicarbazone as Lawes'. As noticed by Lawes, small amounts of glutarimide-β-acetaldehyde was accompanied by the ketone during distillation after pyrolysis.

Naramycin-B——By the similar treatment, 1 g. of Naramycin-B (m.p. 111∼112°) was decomposed in an oil bath kept at 210∼220° under 20 mm. Hg pressure. The volatile substance was treated as above and redistilled to give 215 mg. of an oil (b.p$_{24}$ 77∼78°) of positive Cotton effect (yield, 47%). This oil and its semicarbazone were found identical with the above-mentioned (2S:4R)-2,4-dimethylcyclohexanone and its semicarbazone respectively.

Isocycloheximide——One gram of isocycloheximide (m.p. 99∼100°) was decomposed at 210∼230° under 20∼30 mm. Hg pressure and the volatile substance was treated as above and redistilled to give 210 mg. of an oil (b.p$_{20}$ 73∼74°) of negative Cotton effect (yield, 46%). This substance and its semicarbazone were identified with (2R:4R)-2,4-dimethylcyclohexanone and its semicarbazone which were obtained from cycloheximide by an alkaline degradation as reported by Kornfeld, *et al.*2)

Anhydrocycloheximide——This substance did not give any volatile substance by heating under 20∼30 mm. Hg pressure in an oil bath kept at the temperature as high as 250∼260°.

Isomerization of Cycloheximide into Isocycloheximide——The solution of 5 g. of cycloheximide in anhyd. benzene (100 cc.) was added with 1 g. of MeONa and gently refluxed in a water bath kept at about 80°, during which the solution turned to brown (cf. curve (d) in Fig. 2). After 4 hr., the solution was cooled and washed twice with 25 cc. of H$_2$O and separated from benzene layer. The aqueous solution was extracted with CHCl$_3$ and the extract was combined with the above benzene solution, dried over anhyd. Na$_2$SO$_4$, concentrated *in vacuo* to give the syrup, which gave 2.4 g. of crude isocycloheximide on treatment with AcOEt-Et$_2$O, yield, 48%; m.p. 76∼81°; $[\alpha]_D^{25}$ +22.0° (c=2, MeOH); bioassay,[*5] 350 mcg./mg.

The crude isocycloheximide thus obtained was chromatographed on Celite 545 according to the procedure of Eble, *et al.*9) and isocycloheximide recovered was recrystallized from AcOEt-Et$_2$O to give pure sample of isocycloheximide, m.p. 99∼100°; $[\alpha]_D^{20}$ +37° (c=5, MeOH); bioassay, 20 mcg./mg.[*6]

Isomerization of Cycloheximide into Naramycin-B——The solution of 5 g. of cycloheximide dissolved in 100 cc. of benzene was added with 0.5 g. of MeONa and gently refluxed in a water bath kept at 80°. After 2 hr., the solution was treated as above to give 3.75 g. of crystal, recovery yield, 65%; m.p. 90∼94°; $[\alpha]_D^{24.5}$ +9.5° (c=5, MeOH); bioassay, 760 mcg./mg. This crystal was found to compose of Naramycin-B, isocycloheximide and cycloheximide with the aid of IR spectroscopy.

[*6] Authors found that the purer isocycloheximide becomes, the less its antimicrobial activity becomes. Therefore, it seems that the purest sample of isocycloheximide, if obtained, would show no activity at all.

Two grams of the above crystal was chromatographed according to the procedure of Eble, *et al.*, using 60 g. of Celite Analytical Filter-Aid in place of Celite 545. The result was as shown in Fig. 3. Crystal (0.162 g.; yield, 8.1%) recovered from fraction No. 6 to 9 in Fig. 3 and crystal (0.666 g.; yield, 33.3%) recovered from fraction No. 11 to 39 were found to compose mainly of isocycloheximide and Naramycin-B respectively with aid of IR, ORD determinations.

Among Naramycin-B fraction, fractions from No. 13 to 17 gave 0.28 g. of crude Naramycin-B of m.p. 105∼107°, which gave on recrystallization from iso-PrOAc 0.21 g. of Naramycin-B, m.p. 111∼112°; $[\alpha]_D^{23}$ +52.3°(c=5, MeOH); bioassay, 350 mcg./mg.

Isomerization of Naramycin-B into Isocycloheximide——Isomerization of 0.5 g. of Naramycine-B (m.p. 106∼107°, $[\alpha]_D^{20}$ +40.9° (c=2, MeOH)) was carried out in 25 cc. of benzene in the presence of 0.1 g. of MeONa, and 320 mg. of isomerization product (m.p. 95∼96°, $[\alpha]_D^{20}$ +32°(c=5, MeOH)) was recovered from the solution (yield, 61%). The product was identified with isocycloheximide by means of IR and mixed melting point determinations.

Isomerization Study on Isocycloheximide——Isomerization was tried on 0.5 g. of isocycloheximide (m.p. 91∼93°, $[\alpha]_D^{20}$ +29.5°(c=5, MeOH)) according to the similar procedure described above, and 0.35 g. of crystal (m.p. 97∼98°) was recovered from benzene solution (recovery yield, 70%). This crystal was recrystallized from iso-PrOAc to give 0.255 g. of pure sample, m.p. 98∼99°; $[\alpha]_D^{20}$ +34.5°(c=5, MeOH), which was made sure of isocycloheximide by IR, ORD and mixed melting point determinations.

Isomerization Study on *α-epi*-Isocycloheximide——Isomerization of 100 mg. of *α-epi*-isocycloheximide (m.p. 105∼106°) was carried out in 5 cc. of benzene using 20 mg. of MeONa as a catalyst, and 64.5 mg. of crystal was recovered from benzene solution, m.p. 92∼96°; recovery yield, 64.5%. On recrystallization from iso-PrOAc, this crystal gave 52 mg. of pure sample, m.p. 103∼105°, which was identified with *α-epi*-isocycloheximide with the aid of IR, ORD and mixed melting point determinations.

Purification of Naramycin-B——Naramycin-B rich sample (18.5 g.) (m.p. 93∼97°; $[\alpha]_D^{21}$ +11.0°(c=5, MeOH); bioassay, 740 mcg./mg.) was chromatographed on Celite Analytical Filter-Aid column and 5.3 g. of crude Naramycin-B was recovered which was recrystallized from 10 cc. of iso-PrOAc to give 3.8 g. of a crystal of m.p. 108∼110°; $[\alpha]_D^{20}$ +45.7°(c=5, MeOH); bioassay, 450 mcg./mg., which was further recrystallized from 50 cc. of iso-PrOAc to afford 1.4 g. of pure sample of Naramycin-B having physicochemical properties shown in p. 733.

The authors express their gratitude to Dr. B. C. Lawes of Mead Johnson Research Center, Drs. F. McLafferty, F. Johnson and N. Starkovsky of Eastern Research Laboratory of the Dow Chemical Co., and Dr. A. J. Lemin of Upjohn Co. for their informative discussions and suggestions on the matter. The authors appreciate Emeritus Professor S. Sugasawa of Tokyo University and Dr. K. Abe, the Superintendent of this Laboratory, for their interest and encouragements. Thanks are also due to the members of the Analysis Room of this Laboratory for analysis.

Summary

Thermal degradation and isomerization studies on cycloheximide and its stereoisomers were carried out, and the results obtained did support chemically the absolute configuration of cycloheximides proposed previously.

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