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RELATIONSHIP OF STRUCTURE TO FUNGITOXICITY OF CYCLOHEXIMIDE AND RELATED GLUTARIMIDE DERIVATIVES

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Abstract—The effect on biological activity of various group substitutions and stereochemical changes in the *l*-cycloheximide molecule was studied. Compounds were assayed for toxicity to cells of *Saccharomyces pastorianus* and to a cell-free protein-synthesizing system from this organism.

Substitution of the imide hydrogen with a methyl group, esterification of the hydroxyl group with acetate, or conversion of the ketone to an oxime, semicarbazone, or hydroxyl group resulted in greatly diminished or complete loss of activity. Stereoisomers which differed from *l*-cycloheximide in configuration of the trisubstituted cyclohexanone ring were low in activity. An isomer with the same cyclohexanone ring configuration as *l*-cycloheximide, but with a different orientation of the methyl group at the 4-position, was half as active as *l*-cycloheximide. A cyclic ketone was not an absolute requirement for biological activity of glutarimide antibiotics because streptimidone which contains an aliphatic ketone was about 30 per cent as active as *l*-cycloheximide.

The principal steric requirement in the cyclic ketone moiety appears to be proper orientation of the carbonyl group in respect to the remainder of the molecule. Substitution data suggest that the keto, hydroxyl, and imide nitrogen groups may be involved in a 3-point attachment of the glutarimide antibiotics to the substrate.

Acetoxycycloheximide and streptovitacin-A were less toxic to cells but more toxic to the cell-free protein-synthesizing system than was cycloheximide. No glutarimide derivatives were active against whole cells or a cell-free protein-synthesizing system of the cycloheximide-resistant yeast, *S. fragilis*.

l-CYCLOHEXIMIDE is a naturally occurring glutarimide derivative with high antifungal activity. It is the best-known member of a series of glutarimide derivatives isolated from various streptomycetes.

An extensive investigation of the mechanism of action of *l*-cycloheximide in cells of *Saccharomyces pastorianus* has indicated that toxicity results from inhibition of protein synthesis.[1] Specifically the antibiotic inhibits transfer of amino acids from soluble RNA to ribosomes and their polymerization into protein.[2, 3] It has also been demonstrated that this aspect of protein synthesis in a mammalian cell-free system[4] and in a cell-free system from *S. elipsoideus*[5] is inhibited by cycloheximide.

Although cycloheximide inhibits both DNA and protein synthesis in intact yeast[1,6] and mammalian cells[4, 7–9] it does not inhibit incorporation of deoxynucleoside monophosphates into DNA in mammalian cell-free systems.[9] This suggests that DNA synthesis in intact cells may not be directly inhibited by cycloheximide, but only indirectly affected because protein synthesis is inhibited.[1, 9]

Cell-free protein-synthesizing systems from the cycloheximide-resistant yeast *S. fragilis* are resistant to cycloheximide, whereas those from the cycloheximide-sensitive yeast *S. pastorianus* are highly sensitive to the antibiotic.[3] This evidence supports the hypothesis that *l*-cycloheximide is a specific inhibitor of protein synthesis in sensitive cells.

Although considerable attention has been devoted to the mechanism of action of *l*-cycloheximide, the relationship of chemical structure to biological activity of such glutarimide antibiotics has not been studied in much detail. Introduction of a hydroxy or acetoxy group into the cycloheximide molecule at position 4 of the cyclohexanone ring, as in the case of acetoxycycloheximide and streptovitacin-A, greatly increases the antitumor activity.[10] Increased activity has also been reported for streptovitacin-A in other mammalian cell systems[11] and for actoxycycloheximide in a mammalian cell-free system, incorporating amino acids into protein.[4, 12] Conversion of the ketone in *l*-cycloheximide to an oxime or semicarbazone[13] causes a marked loss in activity. Esterification of the hydroxyl group of acetoxycycloheximide with acetate, propionate, butyrate, or benzoate does not lower antitumor activity.[10] On the other hand, the acetate derivative of *l*-cycloheximide has been reported to have no antifungal activity against cells of *S. pastorianus*.[13, 14] Rearrangement of the asymmetric centers in the cyclohexanone ring, as in the stereoisomers naramycin-B[15] and isocycloheximide,[16] results in a 70 per cent loss of antifungal activity. However, a cyclic ketone is not an absolute requirement for antifungal activity of glutarimide derivatives. This is apparent from the case of streptimidone, a biologically active compound which contains an aliphatic rather than a cyclic ketone.[17]

In the present study, several glutarimide derivatives were compared to *l*-cycloheximide as inhibitors of growth of cells of *S. pastorianus* and as inhibitors of protein synthesis in a cell-free system. Data from these tests were used to assess the influence on biological activity of various structural deviations from the *l*-cycloheximide molecule.

METHODS

Testing in vivo. The test organisms used for toxicity measurements were *S. pastorianus* Hansen, *S. fragilis* Jorgensen (ATCC 8635), and *Neurospora crassa*. The basal medium and cultural procedures for growing *S. pastorianus* and *S. fragilis* were those of Coursen and Sisler.[13] The basal medium, a glucose-mineral salt solution (pH 6·4) containing vitamins and trace elements, was modified by the addition of 2·5 g yeast extract/liter. Cell suspensions were prepared from cultures (12–18 hr old) grown at 30° on a reciprocating shaker. Cells were separated from the culture medium by centrifugation and washed twice with distilled water; suspensions were standardized to an optical density of 1·0 at 450 mμ (0·65 mg dry weight/ml) in water. Test tubes containing 9·4 ml basal medium (minus yeast extract), 0·5 ml standardized cell suspension and 0·1 ml methanol containing the appropriate concentration of toxicant were placed on a reciprocating shaker and incubated at 30° until an optical density of 0·60–0·75 was reached in the control tubes. At this time all the tubes were read for absorbancy. ED_{50} values (50 per cent inhibitory doses) were determined from curves plotted on log-probability paper.

Certain compounds were also tested against *N. crassa* for toxicity, Chemically defined agar growth medium[18] was inoculated with small mycelial plugs and the cultures

incubated for 5–7 days at 27°. Spores were harvested with physiological saline, washed three times with saline, and standardized to a concentration of 6×10^7 spores/ml. Test tubes containing 9·4 ml growth medium, 0·5 ml spores, and 0·1 ml methanol containing appropriate concentrations of toxicant were incubated on a shaker at 30° for 5 hr. One ml formaldehyde was pipetted into each test tube to stop further spore germination. Germinated and non-germinated spores were counted, and ED_{50} values were calculated as described above.

Testing in vitro. A cell-free system from *S. pastorianus* and *S. fragilis* capable of incorporating ^{14}C-phenylalanine into acid-precipitable protein was prepared as previously described.[2,3] Each milliliter of reaction mixtures consisted of 0·05 M Tris-HCl (pH 7·5), 0·008 M magnesium acetate, 0·005 M KCl, 0·001 M ATP, 0·005 M phosphoenolpyruvate (PEP), 0·0002 M GTP, 75 μg pyruvic kinase (ATP pyruvic phosphotransferase, EC 2·7·1·40), 0·5 mg soluble RNA, 0·25 μc ^{14}C-L-phenylalanine (specific activity 360 μc/μmole), 1·0 mg ribosomal and supernatant protein and appropriate concentrations of toxicant. The pH of the solutions of ATP, PEP, GTP and ^{14}C-L-phenylalanine was adjusted to 7·0 prior to addition of the reaction mixture. The reaction was stopped by the addition of 1·2 volumes of cold 10% trichloroacetic acid containing 0·01 M phenylalanine. Samples were placed in an ice bath for 10 min and then centrifuged. The protein precipitate was washed and prepared for counting as previously described.[2] ED_{50} values were determined in the same manner as ED_{50} values *in vivo*.

MATERIALS

Glutarimide derivatives. Source or method of preparation. *l*-Cycloheximide,* melting point (m.p.) 114–115°, isolated from *Streptomyces griseus.*[19, 20] *Cycloheximide oxime,** m.p. 203–204°, reaction of cycloheximide with hydroxylamine hydrochloride.[20] *Cycloheximide acetate,** m.p. 148–149°, reaction of cycloheximide with acetatic anhydride in dry pyridine.[20] *Cycloheximide semicarbazone,** m.p. 182–183°, reaction of cycloheximide with semicarbazide hydrochloride.[20] *ψdl-Cycloheximide-I acetate*, m.p. 140°, total synthesis.† *dl-Cycloheximide*, m.p. 139–140°, total organic synthesis of racemic cycloheximide starting with 2,4-dimethylphenol and 3-glutarimidylacetyl chloride.[21] *α-Dihydrocycloheximide*, m.p. 163°, reduction of cycloheximide with diphenyltin dihydride.[22] *β-Dihydrocycloheximide*, m.p. 164°, reduction of cycloheximide with diphenyltin dihydride.[22] *N-Methyl cycloheximide acetate*, m.p. 140–141°, methylation of cycloheximide acetate with CH_3I/K_2CO_3 in acetone.[22] *N-Methyl cycloheximide*, prepared fron N-methyl cycloheximide acetate by an esterase preparation from maize.[23] The compound was identified by nuclear magnetic resonance (n.m.r.) spectroscopy, infred spectrometry, thin-layer chromatography, and by conversion of the product (N-methyl cycloheximide) to N-methyl cycloheximide acetate with acetic anhydride in dry pyridine. *d-Isocycloheximide*, m.p. 101–103°, a stereoisomer of cycloheximide, isolated from *S. griseus*. Also formed by the isomerization of cycloheximide with acid-deactivated alumina.[16] *d-Naramycin-B*,‡ sample 1, m.p. 112–113°, a stereoisomer of cycloheximide isolated from *Streptomyces naraensens novo.*[15,24,25] *d-Naramycin-B*, sample 2, rhodium-catalyzed hydrogenation of inactone to a mixture

* A gift from the Upjohn Co., Kalamazoo, Mich.

† N. A. Starkousky and F. Johnson, unpublished observations.

‡ A gift from Dr. T. Okuda, Tanabe Seiyaku Co. Ltd., Tokyo, Japan.

of 32% *l*-cycloheximide and 68% *d*-naramycin-B.[22] *dl-α-Epi-isocycloheximide*, m.p. 153°, total organic synthesis starting with *cis*-2,4-dimethylcyclohexanone and 3-glutarimidylacetylchloride.[26] *Actiphenol*,* m.p. 200–201°, isolated from a cycloheximide producing streptomycete;[27] Total synthesis.[28] *Inactone*,† ,mp. 116°, isolated from *S. griseus*.[29] *Streptovitacin-A*,‡ m.p. 156–159°, isolated from *S. griseus*.[30, 31] *Acetoxycycloheximide*§ (E–73), m.p. 140°, isolated from *Streptomyces albulus*.[32, 33] *Streptimidone*,¶ m.p. 72°, isolated from *Streptomyces rimosus forma paramomycicus*.[34, 35] *Streptimidone acetate*,¶ m.p. 94–96°, acetylation of streptimidone.[36] *Anhydrocycloheximide*, m.p. 135–136°, reaction of cycloheximide and phophorus pentoxide in benzene.[37]

Purity of glutarimide derivatives. Compounds which showed toxicity were examined for *l*-cycloheximide contamination by either n.m.r. analysis[38] or by silica gel chromatography. *d*-Naramycin-B, *d*-isocycloheximide, and *dl-α*-epi-isocycloheximide were analyzed by the n.m.r. technique which will detect contamination of 5% or greater. The remaining compounds were chromatographed on glass plates coated with 0·5 mm of silica gel G (Brinkmann Instruments Inc.). The plates were developed in a solvent of ethyl acetate: methylene chloride (5:1) and compounds were detected by spraying with a solution of 1% iodine in 95% ethanol. Five-mg samples of the antibiotics were spotted on the plates. L-Cycloheximide contamination of 1–2% could be detected in these tests.

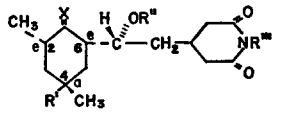
Chemicals. Potassium ATP, pyruvic kinase, and PEP were purchased from Calbiochem Co. Yeast-soluble RNA was purchased from General Biochemical Co. ^{14}C-L-Phenylalanine was purchased from New England Nuclear Corp. The culture of *S. fragilis* (ATCC 8635) was obtained from the American Type Culture Collection.

RESULTS AND DISCUSSION

The structure of L-cycloheximide and that of related glutarimide derivatives investigated for biological activity are shown in Figs. 1–4. Structures of compounds possessing the same steric arrangement as *l*-cycloheximide, but differing in substituents at certain positions, are shown in Fig. 1. Acetoxycycloheximide and streptovitacin-A are included in this group, although it is not certain that the orientation of the group at the 4- and 6-positions of the cyclohexanone ring are the same as that in *l*-cycloheximide.[22] Glutarimide derivatives which differ from *l*-cycloheximide only in respect to the steric configuration or orientation of the methyl groups of the 2,4,6-trisubstituted cyclohexanone ring are shown in Fig. 2. Structures of the third group of miscellaneous glutarimide derivatives are shown in Fig. 3. Inactone is a conformational mixture of the two species shown. Asymmetric centers in the cyclic ketone occur at the 2- and 4-positions, but the 6-position is planar. In actiphenol, the cyclohexanone ring is replaced by a substituted phenol. Since there are no asymmetric centers the ring is completely planar. Streptimidone is a glutarimide antibiotic in which the ketone is aliphatic rather than cyclic as in *l*-cycloheximide. The enantiomer of *l*-cycloheximide, *d*-cycloheximide, is shown in Fig. 4.

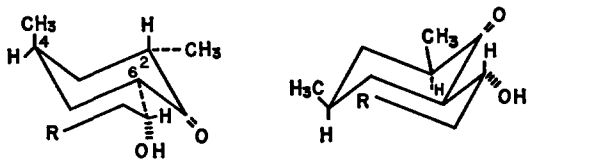
* A gift from Prof. V. Prelog, Eidg., Technische Hochschule, Zurich, Switzerland.
† A gift from Dr. R. Paul, Rhone Poulenc, Paris, France.
‡ A gift from the Upjohn Co., Kalamazoo, Mich. U.S.A.
§ A gift from Chas. Pfizer and Co., Maywood, N.J., U.S.A.
¶ A gift from Park-Davis and Co., Detroit,Mich., U.S.A.

Effectiveness of glutarimide antibiotics as inhibitors of growth of *S. pastorianus*, *in vivo* was compared with their effectiveness as inhibitors of cell-free protein synthesis *in vitro*. Results of tests of compounds with the same steric configuration as *l*-cycloheximide (Fig. 1) but with different substituents at certain positions are shown in Table 1.

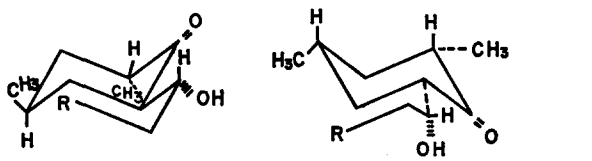


Cycloheximide (X = O, R', R'', R''' = H)
Cycloheximide oxime (X = NOH, R', R'', R''' = H)
Cycloheximide semicarbazone
(X = N-NHC(=O)NH$_2$, R', R'', R''' = H)
ψ cycloheximide-I acetate
(X = O-C(=O)-CH$_3$, R', R''' = H)
αdihydrocycloheximide (X = OH-e, R', R'', R''' = H)
β dihydrocycloheximide (X = OH-a, R', R'', R''' = H)
Acetoxycycloheximide
(X = O, R' = O-C(=O)-CH$_3$, R'', R''' = H)
Streptovitacin-A (X = O, R' = OH, R'', R''' = H)
Cycloheximide acetate
(X = O, R'' = -C(=O)-CH$_3$, R', R''' = H)
N-methylcycloheximide (X = O, R', R'' = H, R''' = CH$_3$)
N-methylcycloheximide acetate
(X = O, R' = H, R'' = -C(=O)-CH$_3$, R''' = CH$_3$)

FIG. 1. Derivatives of *l*-cycloheximide (oxygen at R'' position is ketone in ψ cycloheximide-I acetate).



1- CYCLOHEXIMIDE (2e,4a,6e) d-ISOCYCLOHEXIMIDE(2e,4e,6e)



d-NARAMYCIN B (2a,4e,6e) 1-α epi ISOCYCLOHEXIMIDE(2e,4e,6e)

FIG. 2. Conformational structures of *l*-cycloheximide and three of its stereoisomers (R = glutarimide).

l-Cycloheximide was more toxic to whole cells than to the cell-free amino-acid-incorporating system. No compound in this group was as effective *in vivo* as *l*-cycloheximide, but the compounds acetocycycloheximide and streptovitacin-A, with an acetyl or hydroxyl substituent at the 4-position of the cyclohexanone ring, were superior to cycloheximide *in vitro*. In contrast to cycloheximide, these two compounds were far less effective *in vivo* than *in vitro*. There is a striking contrast between the

ratio ED_{50} *in vivo*/ED_{50} *in vitro* for *l*-cycloheximide and that of acetoxycycloheximide and streptovitacin-A. The ratio for *l*-cycloheximide is about 0·1 while the ratios for acetoxycycloheximide and streptovitacin-A are about 10 and 100 respectively. This suggests that permeability restrictions may limit toxicity of acetoxycycloheximide and streptovitacin-A to intact yeast cells. Unfortunately, the stereochemistry of trisubstituted cyclohexanone ring in acetoxycycloheximide and streptovitacin-A has not been completely elucidated so it is not possible to conclude that the difference in activity between these derivatives and that of *l*-cycloheximides results solely from substitution at the 4-position.

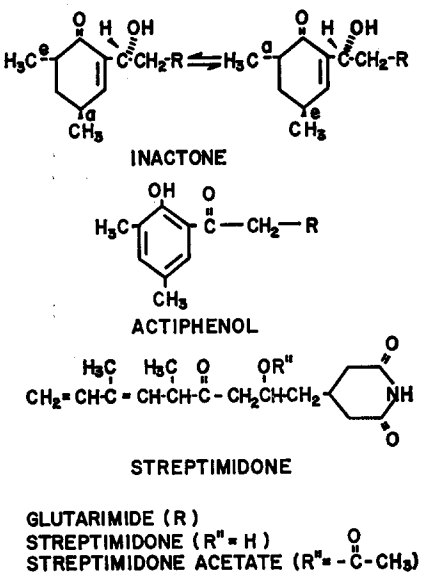


FIG. 3. Miscellaneous glutarimide derivatives.

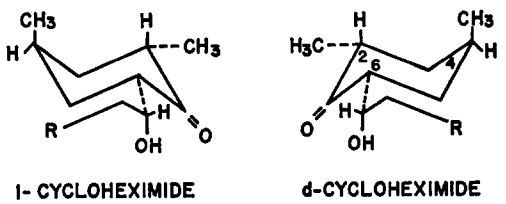


FIG. 4. Conformational structures of *l*-cycloheximide and *d*-cycloheximide (R = glutarimide).

The superiority of *l*-cycloheximide over acetoxycycloheximde as an inhibitor of yeast growth has also been observed by Rao and Cullen.[32] Low activity against germination of conidia of *N. crassa* is also characteristic of acetoxycycloheximide and streptovitac-A. The two antibiotics are only about 10% and 1%, respectively, as effective as *l*-cycloheximide. In mammalian systems, however, acetoxyxycloheximide and streptovitacin-A are more effective both *in vivo* and *in vitro* than *l*-cycloheximide.[4, 10–12]

In regard to modification of functional groups of the *l*-cycloheximide molecule, esterification of the hydroxyl group with acetic acid or methylation of the imide nitrogen leads to complete loss of effectiveness. Rao[10] reported that esterification of the hydroxyl group of acetoxycycloheximide with acetic, propionic, butyric, or benzoic acid did not change the antitumor activity. It is likely, however, that acetoxycycloheximide released by esterases present in the animal tissue was responsible for the observed activity. Tissues of a number of higher plants[14, 23] fungal cells, and pancreatic

TABLE 1. RELATIVE EFFECTIVENESS OF GLUTARIMIDE DERIVATIVES OF THE L-CYCLOHEXIMIDE CONFIGURATION AS INHIBITORS OF GROWTH OF *Saccharomyces pastorianus* (*in vivo*) AND AS INHIBITORS OF CELL FREE PROTEIN SYNTHESIS (*in vitro*)

Compounds	*In vitro* ED_{50} (μg/ml)	Effectiveness* (%)	*In vivo* ED_{50} (μg/ml)	Effectiveness* (%)
l-Cycloheximide	0·36	100	0·028	100
Cycloheximide oxime	>50		12·5	0·2
Cycloheximide semicarbazone	>50		1·4	2
Cycloheximide acetate	>50		>250	
ψ-Cycloheximide-I acetate	>50		>250	
Streptovitacin-A	0·15	240	13·0	0·2
Acetoxycycloheximide	0·044	818	0·50	6
α-Dihydrocycloheximide	4·5	8	3·5	0·8
β-Dihydrocycloheximide	>50		52·0	0·05
N-methyl cycloheximide†			>100	
N-methyl cycloheximide acetate	>50		>250	

* Effectiveness relative to that of *l*-cycloheximide.
† N-methyl cycloheximide not tested *in vitro* or against *S. fragilis*.

lipase preparations[23] release cycloheximide from cycloheximide acetate. *l*-Cycloheximide acetate is not toxic to cells of *S. pastorianus*, even at 1000 ppm or greater,[14] but neither whole cells nor cell-free extract of this organism are active in releasing *l*-cycloheximide from the acetate ester.[23]

Substitution of the ketone carbonyl group with an oxime or semicarbazone or reduction to an axially or equatorially oriented hydroxyl group markedly reduces or destroys biological activity. When the keto group is replaced with an axially oriented hydroxyl group, toxicity is eliminated, but when it is replaced with an equatorially oriented hydroxyl group, toxicity is reduced to about 8% of that of *l*-cycloheximide *in vitro*. The semicarbazone derivative showed some toxicity *in vivo* but none *in vitro*. This phenomenon can be explained, however, on the basis of the release of cycloheximide through a pH-dependent hydrolysis of the semicarbazone.[23] At pH 3·5 the semicarbazone is about 60% as toxic to cells as cycloheximide, but it is only about 2% as effective at pH 6·5. Lack of toxicity of this derivative in the *in-vitro* system at pH 7·5 is attributed to the fact that little or no hydrolysis occurs at this pH. A similar mechanism of cycloheximide release probably accounts for toxicity of the oxime derivatives *in vivo*.

Toxicity data for glutarimide antibiotics which differ in configuration from *l*-cycloheximide (Figs. 2–4) are shown in Table 2. The effectiveness of *dl*-cycloheximide relative to that of *l*-cycloheximide was 46% *in vitro* and 52% *in vivo*. Since the racemic mixture was 50% *l*-cylohexiimide it is evident that *d*-cycloheximide possesses little or no toxicity. The isomer, *l*-α-epi-isocycloheximide differs from *l*-cycloheximide only

TABLE 2. RELATIVE EFFECTIVENESS OF STEREOISOMERS OF CYCLOHEXIMIDE AND RELATED COMPOUNDS AS INHIBITORS OF GROWTH OF *Saccharomyces pastorianus* (*in vivo*) AND AS INHIBITORS OF CELL FREE PROTEIN SYNTHESIS (*in vitro*)

Compounds	*In vitro* ED_{50} (μg/ml)	Effectiveness* (%)	*In vivo* ED_{50} (μg/ml)	Effectiveness (%)	*l*-cycloheximide contamination %
l-Cycloheximide	0·36	100	0·028	100	
dl-Cycloheximide	0·78	46	0·054	52	50
d-Naramycin-B, Sample 1	1·05	34	0·11	26	25–33
d-Naramycin-B, Sample 2 (reduction product of inactone)	1·25	29	0·95	30	32
d-Isocycloheximide	2·6	14	0·21	13	<5
dl-α-Epi-isocycloheximide†			0·12	24	<5
Inactone	7·5	5	0·36	8	unknown
Actiphenol	>50		>250		
Streptimidone	1·4	26	0·084	33	<1
Streptimidone acetate	>50		>250		
Anhydrocycloheximide†			>100		
3-Glutarimidylacetic acid	>50		>250		
3-Glutarimidylacetaldehyde	>50		>250		
Trans-2,4-dimethyl-cyclohexanone	>50		>250		

* Effective as compared to cycloheximide.

† *dl*-α-Epi-isocylcoheximide and anhydrocycloheximide not tested *in vitro* or against *S. fragilis*.

in respect to the orientation of the methyl group at the 4-position of the cyclohexanone ring. The sample of α-epi-isocycloheximide tested was a racemic mixture. Since *d*-cycloheximide is apparently inactive it is probable that *d*-α-epi-isocycloheximide is also inactive. α-Epi-isocycloheximide was 24% or 48% as effective as *l*-cycloheximide, dependent on whether calculations were based on the activity of the *dl* mixture or on the amount of *l*-isomer present. This indicated that orientation of the methyl group at the 4-position does not have a marked effect on biological activity.

The two isomers, *d*-naramycin-B and *d*-isocycloheximide, differ from *l*-cycloheximide not only in arrangement of asymmetric centers of the cyclohexanone ring but also in absolute configuration of the ring (Fig. 2). The conformational structure of the rings in these two isomers belong to the same mirror-image series as the ring in *d*-cycloheximide. Orientation of the side chain, however, is the same as that in *l*-cycloheximide. *d*-Isocycloheximide and *l*-α-epi-isocycloheximide have the same orientation of groups around asymmetric centers of the cyclohexanone ring, but they differ in ring configuration. Sample 1 of *d*-naramycin-B was 34% as effective *in vitro* and 26% as effective *in vivo* as cycloheximide. However, n.m.r. measurements showed a 25–33% contamination with cycloheximide. Thus *d*-naramycin-B accounted for little if any of the toxicity of the sample. The problem was investigated further in the reduction

product of inactone (sample 2 of *d*-naramycin-B). The product contained 32% *l*-cycloheximide and 68% *d*-naramycin-B. Toxicity corresponded closely to that expected from the *l*-cycloheximide present. Toxicity of *d*-cycloheximide is apparently 13–14% of that of *l*-cycloheximide. No *l*-cycloheximide was found in the sample by n.m.r. measurements which will detect contamination of more than 5%. If it is assumed that that 5% or less cycloheximide was present, toxicity of *d* isocyclohexmide would still be greater than 8% of that of *l*-cycloheximide. It is apparent from these observations that configurational differences in the trisubstituted cyclohexanone ring can markedly affect biological activity of glutarimide antibiotics.

The ketone ring in inactone is unsaturated between carbons 5 and 6, and therefore it contains only two asymmetric centers. Whether inactone is toxic at all is uncertain, since the possibility of cycloheximide contamination could not be eliminated. At most, its toxicity is only 5–8% of that of *l*-cycloheximide. Replacement of the cyclohexanone with the corresponding phenol, as in actiphenol. leads to complete loss of toxicity. Dehydration of the *l*-cycloheximide molecule resulting in loss of the side-chain hydroxyl group and unsaturation betweeen carbon-6 of the cyclohexanone ring and the α-carbon of the side chain produces the completely inactive compound, anhydrocycloheximide. Recently Lee and Wilkie[39] reported that anhydrocycloheximide was as active as cycloheximide against *Saccharomyces cerevisiae*. Data presented in this paper, however, indicate that anhydrocycloheximide is not fungitoxic. The reason for the lack of agreement between the results presented here and those of Wilkie and Lee is unknown. Possibly, rehydration of anhydrocycloheximide occurred under the conconditions of their experiments but not under ours. The fact that rehydration of anhydrocycloheximide does occur under certain conditions has been demonstrated by Garrett and Notari.[40] However, *l*-cycloheximide is undoubtedly responsible for any toxicity arising from anhydrocycloheximide because it is immediately toxic to cells of *S. pastorianus*, whereas anhydrocycloheximide is without toxic effects even after 7 hr.

Although the absolute configuration of the cyclohexanone ring appears to have a marked effect on toxicity of glutarimide antibiotics, a cyclic ketone is not an absolute requirement for activity. This is apparent from the case of streptimidone in which the ketone is aliphatic, but toxicity is still 26–33% of that of *l*-cycloheximide. Esterification of the hydroxyl group of streptimidone with acetate destroyed toxicity, as was the case with *l*-cycloheximide.

The principal steric requirement in the ketone portion of the glutarimide antibiotics appears to be the proper orientation of the carbonyl group in relation to the hydroxyethylglutarimide portion of the molecule. The best orientation for toxicity apparently occurs in *l*-cycloheximide, but the orientation in streptimidone is better than that of isocycloheximide or naramycin-B. The structure of streptimidone differs from that of *l*-cycloheximide only in the moiety attached to the carbonyl carbon opposite the hydroxyl group. It is possible that a variety of substituents in this position would yield effective antibiotics. The structural requirements in the remainder of the molecule seem to be highly specific and probably cannot be modified without loss of toxicity.

In addition to the compounds already discussed, 3-glutarimidylacetic acid, 3-glutarimidylacetaldehyde, and trans-2,4-dimethylcyclohexanone were tested for toxicity and found to be inactive.

All compounds described here, unless otherwise specified, were tested against whole cells and a cell-free amino acid-incorporating system from the cycloheximide-resistant yeast, *S. fragilis*, but none was found to be active at 250 ppm *in vivo* or 50 ppm *in vitro*.

Cycloheximide inhibits the step in protein synthesis involving transfer of amino acids from soluble RNA to ribosomes and their subsequent polymerization into protein.[3,4] However, the exact mechanism of action within this step is unknown,* and therefore little can be said concerning the relationship between structure of glutarimide antibiotics and the basis of their biochemical action. Proper orientation of the ketone carbonyl, hydroxyl, and imide groups of the antibiotic molecules is probably critical for attachment to the site of action. Substitution data suggest that these groups may be involved in a 3-point attachment of glutarimide antibiotics to the substrate. Attachment to the site must also involve weak linkages such as hydrogen bonds and Van der Waals forces, because toxicity is readily reversible in either whole-cell[13] or in cell-free systems.[4] Furthermore, very specific structural requirements in the protein-synthesizing system are apparently necessary for attachment of these toxicants, because none was effective in systems from the resistant yeast, *S. fragilis*. It is possible that metabolic activation or detoxication accounts for susceptibility or resistance, but no evidence at present would indicate that this is so.

* See References 41–43 for a further discussion of the mechanism of action of cycloheximide.

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