Carbon-13 Nuclear Magnetic Resonance Study of the Biosynthesis of Cycloheximide. Stereospecific Incorporation of Malonate into the Glutarimide Ring[1]

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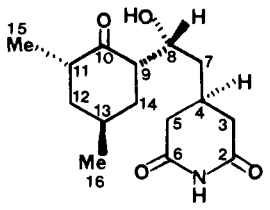
Abstract: The biosynthesis of the antibiotic cycloheximide (**1**) has been studied utilizing sodium [1-^{13}C]acetate, [2-^{13}C]acetate, and [1,2-^{13}C]acetate and sodium [^{13}C]bicarbonate. The results differ from those previously reported in that the incorporation of acetate and malonate units into the cycloheximide ring is shown to be in stereospecific. The two stereoheterotropic ligands represented by the C(2)–C(3) and C(5)–C(6) carbons are shown to be derived from malonate and acetate, respectively.

Cycloheximide (**1**), an antibiotic which has some commercial



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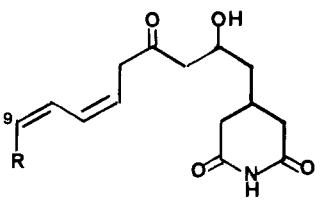


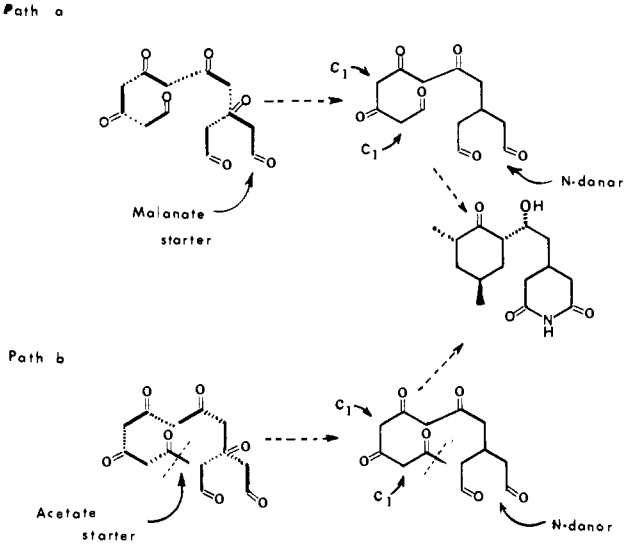
2 R=H
3 R=Me

value as an antifungal agent[2] and is commonly used as a biochemical tool for the inhibition of protein synthesis,[3] was first isolated[4] from a streptomycin producing strain of *Streptomyces griseus*. The structure and stereochemistry of the antibiotic were provided largely from a series of elegant studies by Johnson and co-workers[5] with confirmation forthcoming from a recent X-ray crystallographic structure determination.[6]

The glutarmide ring is a characteristic feature of a small group of related fungal metabolites which include stereoisomers of **1** in naramycin B[8] and isocycloheximide[9] and the open-chain analogues containing additional carbons in streptimidone (**2**)[10] and 9-methylstreptimidone (**3**).[11]

The close structural relationships between the glutarimide antibiotics which suggest a common biosynthetic origin have been confirmed by ^{14}C-tracer studies which have established that **1**, **2**, and **3** originate via the polyacetate pathway. Early studies in this field with [1-^{14}C]- and [2-^{14}C]acetate have shown a pattern of incorporation in accord with either of two possible pathways which are summarized in Scheme I. Evidence for the derivation of the two methyl groups from the C(1) pool rather than from mixed condensation of acetate and propionate units was provided by the appropriate [^{14}C]-labeling experiments.[12]

A distinction between these two postulated pathways presented in Scheme I in favor of pathway a was made by Vanek and



co-workers from a series of interlocking experiments. The principal evidence was derived from the incorporation of [1,3-^{14}C]malonate and [^{14}C]carbonate. In the case of the latter precursor, degradation of the labeled cycloheximide derived from this experiment indicated that the majority of the label resided in the carbonyl(s) of the glutarimide ring.[13] Supporting evidence for pathway a was provided by the finding that the mode of incorporation of [1,3-^{14}C]malonate was in keeping with a labeling pattern in which intact incorporation of one malonate unit occurs in the glutarimide ring of cycloheximide.[14]

The derivation of the carbons of the glutarimide ring in **1** from separate units of acetate and malonate has possible stereochemical implications in that the C(2)–C(3) carbons (*pro-R*) and the C(5)–C(6) carbons (*pro-S*) are diastereotopic. The results of ^{14}C-tracer studies were reported to indicate that malonate was incorporated preferentially into the C(5)–C(6) carbons over that of the C(2)–C(3) carbons by a ratio of 2.2:1.[15] A similar

(1) (a) Taken in part from the MA Thesis of D.M., Duke University, Aug 1980. (b) Presented by P.W.J. at the SE-SW Regional American Chemical Society Meeting, Dec 10–13, 1980, New Orleans, Abstr. ORG 384.
(2) Wiffin, A. J.; Bohonas, N.; Emerson, R. L. *J. Bacterio.* **1946**, *52*, 610.
(3) Ford, J. H.; Klomparens, W.; Hamner, C. L. *Plant Dis. Rep.* **1958**, *42*, 680.
(4) Garrasco, L.; Jiminez, A.; Vazquez, D. *Eur. J. Biochem.* **1976**, *64*, 2. Emmerich, B.; Hoffman, H.; Erben, V.; Rastetter, J. *Biochim. Biophys. Acta* **1976**, *447*, 460.
(5) Johnson, F.; Starkovsky, N. A. *Tetrahedron Lett.* **1962**, 1173. Johnson, F.; Starkovsky, N. A.; Carlson, A. A. *J. Am. Chem. Soc.* **1965**, *87*, 3442.
(6) Sayers, J.; Schindler, D.; Sundaralingam, M. *J. Am. Chem. Soc.* **1977**, *99*, 4308.
(7) For a comprehensive review of the chemistry of these compounds, see: Johnson, F. *Fortschr. Chem. Org. Naturst.* **1971**, *29*, 1401.
(8) Okuda, T.; Suzuki, M.; Egawa, Y.; Ashino, K. *Chem. Pharm. Bull.* **1958**, *6*, 328.
(9) Lemin, A. J.; Ford, J. H. *J. Org. Chem.* **1960**, *25*, 344.
(10) Frohardt, R. P.; Dion, H. W.; Jakubowski, Z. L.; Ryder, A.; French, J. I.; Bartz, Q. R. *J. Am. Chem. Soc.* **1959**, *81*, 5500.
(11) Allen, M. S.; Becker, A. M.; Rickards, R. W. *Aust. J. Chem.* **1976**, *29*, 673.
(12) Kharatyan, S.; Puza, M.; Spizek; Dolezilova, L.; Vanek, Z. *Chem. Ind. (London)* **1963**, 1038.
(13) Vanek, Z.; Puza, M.; Cudlin, J.; Dolezilova, L. *Biochem. Biophys. Res. Commun.* **1964**, *17*, 532.
(14) Vanek, Z.; Puza, M.; Cudlin, J.; Vandracek, M.; Rickards, R. W. *Folia Microbiol. (Prague)* **1969**, *14*, 388.
(15) Cudlin, J.; Puza, M.; Vandracek, M.; Vanek, Z.; Rickards, R. W. *Folia Microbiol. (Prague)* **1967**, *12*, 376.

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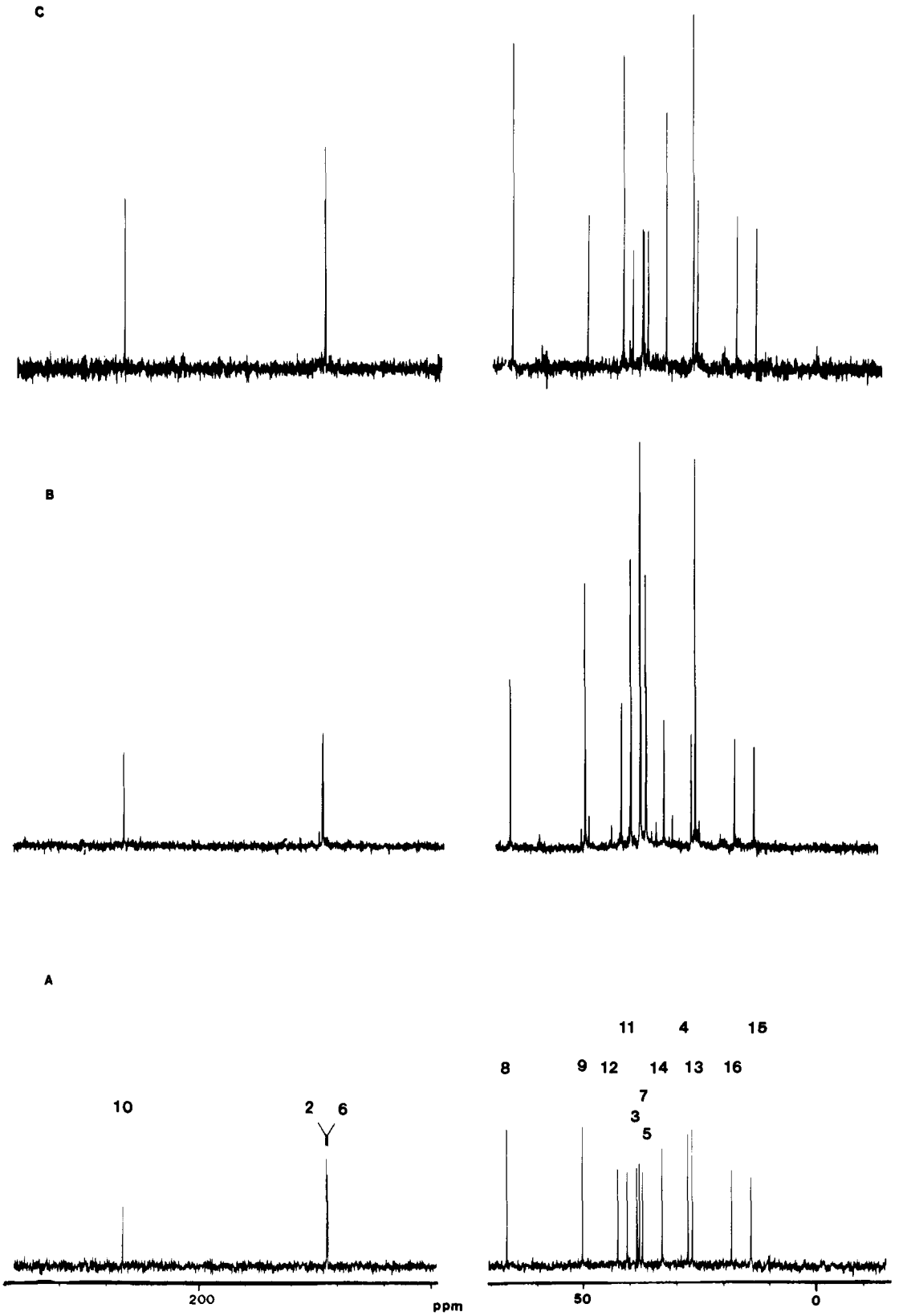


Figure 1. Proton noise decoupled FT ^{13}C NMR spectra of cycloheximide at 63.54 MHz: (A) natural abundance sample (560 transients, 3.0-s pulse delay); (B) isotopically enriched from biosynthetic incorporation of $^{13}CH_3CO_2Na$ (560 transients, 3.0-s delay); (C) isotopically enriched from biosynthetic incorporation of $CH_3{}^{13}CO_2Na$ (560 transients, 3.0-s pulse delay).

nonstereospecific incorporation of malonate into the glutarimide ring of streptimidone was subsequently reported.[16]

This lack of stereospecificity was in our view somewhat surprising, and it appeared that this aspect of the biosynthesis of cycloheximide might be particularly amenable to further study employing ^{13}C labeling in combination with ^{13}C NMR spectroscopy.

(16) Cudlin, J.; Puza, M.; Vanek, Z.; Rickards, R. W. *Folia Microbiol. (Prague)* **1969**, *14*, 406.

Results and Discussion

Chemical Shift Assignments. The proton noise-decoupled 63.5-MHz ^{13}C spectrum of cycloheximide is shown in Figure 1.

Table I. Carbon Chemical Shifts[a] of Cycloheximide and Related Compounds

carbon no.	compds						
	1	4[f]	5	6	7	8	14[e]
2	172.0 (s)	172.4	172.0[b] (s)	171.9 (s)	173.4 (s)	172.2 (s)	170.1 (s)
3	38.5 (t)	38.6	38.3[c] (t)	37.5 (t)	38.5[d] (t)	38.5 (t)	36.2 (s)
4	27.6 (d)	26.8	27.3 (d)	30.3 (d)	27.3 (d)	26.9 (d)	29.0 (d)
5	37.2 (t)	36.9	36.9[c] (t)	37.5 (t)	37.2[c] (t)	37.4 (t)	40.3 (t)
6	171.8 (s)	172.5	171.8[b] (s)	171.9 (s)	173.4 (s)	172.2 (s)	173.0 (s)
7	38.0 (t)	38.3	38.9 (t)	36.3 (t)	39.6 (t)	36.5 (t)	32.6 (t)
8	66.5 (d)	68.8	69.5 (d)	131.8 (d)	73.1[d] (d)	71.3[d] (d)	83.0 (d)
9	50.2 (d)	55.1	49.3 (d)	139.3 (s)	31.0 (d)	30.1 (d)	42.6 (d)
10	216.4 (s)	217.4	212.3 (s)	203.3 (s)	76.5[d] (d)	72.5[d] (d)	70.7 (d)
11	40.5 (d)	39.2	40.8 (d)	44.1 (d)	40.5 (d)	40.2 (d)	31.8 (d)
12	42.6 (t)	45.0	42.7 (t)	40.7 (t)	33.2 (t)	33.9 (t)	33.9 (t)
13	26.8 (d)	31.4	26.8 (d)	30.3 (d)	26.7 (d)	26.8 (d)	27.6 (d)
14	33.1 (t)	44.8	36.4 (t)	32.6 (t)	23.6 (t)	28.3 (t)	27.9 (t)
15	14.2 (q)	14.0	14.2 (q)	15.8 (q)	18.0 (q)	18.1 (q)	18.4 (q)
16	18.4 (q)	21.0	18.1 (q)	21.2 (q)	18.4 (q)	18.3 (q)	18.6 (q)

[a] In parts per million from internal Me_4Si for solution in $CDCl_3$: s = singlet, d = doublet, t = triplet, and q = quartet in the single-frequency $^{13}C\{^1H\}$ off-resonance spectrum. [b-d] Reversible assignments. [e] Shifts reported for a $(CD_3)_2CO$ solution. [f] Sample contaminated with ~15% of compound 1; assignments tentative since no off-resonance studies could be performed.

All 15 carbons of the antibiotic give separate signals at different chemical shifts. The chemical shifts of the glutarimide ring carbons are of special interest. The diastereotopic methylene carbons at C(3) and C(5) give rise to well-separated signals at δ 37.2 and δ 38.5, and, while the carbonyl signals have very similar chemical shifts at δ 171.8 and 172.0, they are, nevertheless clearly resolved at 63.5 MHz.

The ability to discriminate the carbon signals of the glutarimide ring was a very important result in that it meant it would be possible to detect the differential utilization of various ^{13}C-labeled substrates into the two stereoheterotopic—CH_2CO—groups of the glutarimide ring by ^{13}C NMR spectroscopy.

Assignment of all 15 carbon resonances in the spectrum of cycloheximide could be accomplished through the application of chemical shift theory, off-resonance $^{13}C\{^1H\}$ decoupling, and single-frequency $^{13}C\{^1H\}$ and through spectral comparisons with related compounds.

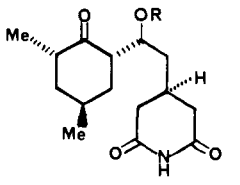
Using simple chemical shift criteria, we could assign six of the carbon resonances in the spectrum of cycloheximide readily. The two signals at highest field at 14.2 and 18.4 ppm are assigned to the equatorial methyl carbon at C(15) and to the axial methyl at C(16), respectively. This assignment is in agreement with the shift of the signal at 18.4 to 21.0 ppm upon converting cycloheximide (**1**) to isocycloheximide (**4**). The latter transformation has been shown by Johnson and co-workers[5] to involve configurational change at C(9) and C(11) and the accompanying conformational change of the cyclohexane ring as shown in **4a**.

The C(8) signal in **1** is assigned to the resonance at 66.5 ppm and is supported by a 3.0-ppm downfield shift of the signal upon conversion of cycloheximide to the *O*-acetate **5**. The latter transformation is also accompanied by an upfield shift of the cyclohexanone carbonyl signal from 216.4 to 212.3 ppm. Both the low-field position of the carbonyl resonance in **1** and its accompanying upfield shift of the signal associated with the conversion to the *O*-acetate **5** (with the resulting disruption of the intramolecular hydrogen bond) are in accord with a solution conformation of cycloheximide in which the C(8) hydroxyl is intramolecularly hydrogen bonded to the C(9) carbonyl oxygen as portrayed in structure **1a**.[17]

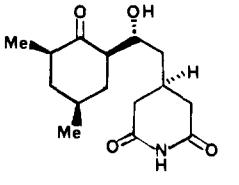
Finally, the two imide carbonyl resonances appear at 171.8 and 172.0 ppm in cycloheximide and at very similar shifts in both *O*-acetylcycloheximide and isocycloheximide (see Table I).

The off-resonance spectrum of **1** was consistent with the foregoing assignments and in addition allowed the identification of four methine carbons at 50.5, 40.5, 26.8, and 27.6 ppm and five methylene resonances at 42.6, 38.5, 38.0, 37.2, and 33.1 ppm.

Designation of the two low-field methine carbon signals as originating from C(9) and C(11), respectively, was made largely on the basis of comparison of the changes associated with the conversion of cycloheximide to the known transformation products **5**, **6**, **7**, and **8** (see Table I). The occurrence of the C(9) resonance



1 R = H
5 R = Ac



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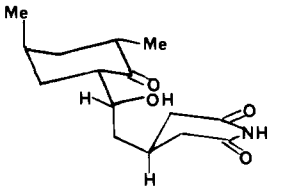
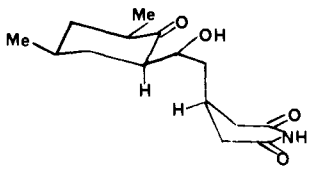
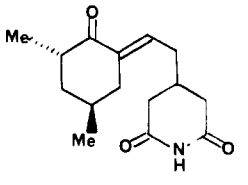


1a



4a



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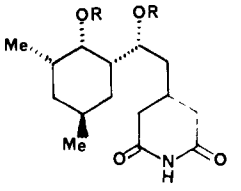


7 R = H
8 R = Ac

downfield of C(11) is accounted for by the deshielding of the former by the β-hydroxyl at C(8). The assignment of the C(9) signal is in agreement with the slight upfield shift (0.9 ppm) of the resonance when cycloheximide is converted to the *O*-acetate **5**.[18]

The two remaining methine resonances at 26.8 and 27.6 ppm proved more difficult to assign. None of the transformation products **4**–**8** showed any marked differential effects on the shifts of these carbons which aided in their assignment. Ultimately a

(17) The evidence for the conformation of cycloheximide in chloroform solution existing as **1a** is supported by IR spectral studies in which the existence of an intramolecularly H-bonded >C=O···H—O– species exists over the concentration range 1.0×10^{-2} M to 1.0×10^{-3} M. The conformation in solution differs from the two conformations which have been shown to coexist in the crystalline state by a recent X-ray study.[6]

(18) Reich, H. J.; Jautaulai, M.; Hesse, M. T.; Weigeri, F. J.; Roberts, J. D. *J. Am. Chem. Soc.* **1969**, *91*, 7445.

Table II. Time-Based Studies for Incorporation of Radio Labeled [2-^{14}C]Acetate in Cycloheximide

time of introduction of [2-^{14}C]-acetate, h	amount, μCi	specific activity of cycloheximide, μCi/mg	% incorporation
48	6.25	0.056	0.89
72	6.25	0.029	0.46
96	6.25	0.088	1.4

single-frequency $^{13}C\{^1H\}$ decoupling experiment was used to assign the C(13) resonance at 26.8 ppm. Homonuclear $\{^1H\}\{^1H\}$ decoupling in the 250-MHz ^1H NMR spectrum of cycloheximide was used to locate the H(13) signal at δ 2.22 by monitoring the collapse of the C(16) methyl doublet at δ 1.22 to a singlet by stepping the decoupling field through the δ 1.5–2.5 region of the spectrum. Observation of a carbon singlet at 26.8 ppm in the ^{13}C spectrum while decoupling at the H(13) frequency in the proton region served to confirm the assignment of this signal as originating from the carbon at position 13. The signal at 27.6 ppm is accordingly assigned to C(4).

The five signals remaining belong to the methylene carbons at C(3), C(5), C(7), C(12), and C(14). Since the signals at 37.2 and 38.5 ppm in the spectrum of cycloheximide appear at comparable shifts (see Table I) in dihydrocycloheximide (**7**), its *O*,*O*-diacetate (**8**), and *O*-acetylcycloheximide (**5**), these resonances could be restricted to the diastereotopic carbons at C(3) and C(5). These assignments were confirmed by single-frequency $^{13}C\{^1H\}$ decoupling of the H(3) and H(5) equatorial protons[19] at 2.78 ppm which resulted in considerable simplification of the coupled carbon signals at 37.2 and 38.5 ppm.

The substitution pattern on the cyclohexane ring of cycloheximide suggests from simple additivity effects that the C(12) resonance should occur in the region of 44 ppm. The only methylene carbon resonance in this region is the most highly deshielded of the methylene carbons at 42.6 ppm. Assignment of this signal to C(12) is supported by its 9.4-ppm upfield shift in the spectrum of dihydrocycloheximide **7** as a consequence of the introduction of a β-axial hydroxyl group.

The remaining two signals at 38.0 and 33.1 ppm which belong to the C(7) and C(14) methylenes could be differentiated on a similar basis. A comparison of the shifts in the spectra of cycloheximide and dihydrocycloheximide indicated that the C(14) signal in **1** could be assigned to the peak at 33.1 ppm since it was shifted 9.5-ppm upfield in the spectrum of **7** in accord with the comparable effects noted previously for the shifts of the C(12) signal in these two compounds. Curiously, the expected differentiation of the C(7) and C(14) signals in **1** which we anticipated would be clearly reflected by a comparison of the spectra of **1** with that of anhydrocycloheximide **6** was not realized. The conversion was associated with a 1.7-ppm upfield shift of the signal at 38.0 ppm while the peak at 33.1 ppm remained essentially unaffected. In retrospect, the absence of any significant change in the C(14) signal may be attributed to offsetting factors resulting from the loss of a γ-anti hydroxyl shielding of C(14) in a conformation such as **9** being replaced by the shielding of the "cis effect" in the enone **6**.

All of the foregoing assignments are supported by the subsequent biosynthetic studies with both C(1)- and C(2)-labeled acetate incorporations into cycloheximide which lead to internally consistent results with the only ambiquities being the individual assignments of the diastereotopic methylenes at C(3) and C(5) and the diastereotopic carbonyls at C(2) and C(6).

(19) The complete assignment of ^1H chemical shifts for all the hydrogens in cycloheximide has been accomplished from high-field proton studies at 600 MHz: McWilliams, D. M.A. Thesis, Duke University, 1980.

(20) Our work was greatly helped by the gift of a modified strain of *S. griseus* UC 2001 which consistently gave 0.7 mg/mL of cycloheximide in shake cultures. We are very much indebted to Dr. B. W. Churchill, Upjohn Co., Kalamazoo, MI, for a gift of this organism and for details of its culture.

Table III. Labeling of Cycloheximide by ^{13}C-Enriched Substrates

position	% enriched by $^{13}CH_3COOH$	% enriched by $CH_3{}^{13}COOH$	% enriched by $^{13}CO_2$
2	1.90	1.70	1.49
3	3.61		
4		2.24	
5	3.01		
6		2.43	
7	2.67		
8		2.08	
9	2.63		
10		2.54	
11	2.96		
12		2.20	
13	2.95		
14		2.08	
15			
16			

[a] The enrichments were calculated by the method of Wright et al.[21] For each resonance in each spectrum $R = I_\delta/I_{18.2}$ was calculated where I_δ is the integrated intensity of the resonance and $I_{18.2}$ is the integrated intensity of the C(16) methyl resonance (unlabeled by any of the precursors used in this study) at 18.2 ppm. For $R_{\text{natural abundance}}$ the average R of four natural abundance spectra ran under identical instrument conditions was used. R for the $^{13}CO_2$-labeled cycloheximide was the average of two spectra. Then $r = R_{\text{labeled}}/R_{\text{natural abundance}}$ was calculated. Resonances with $r > 1$ are labeled. Enrichment at each site was found by multiplying r of the labeled carbons by the factor necessary to normalize the average r of unlabeled carbons to 1.11%.

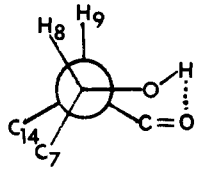
Biosynthetic Studies. Culture conditions required in order to induce *Streptomyces griseus* to produce adequate quantities of cycloheximide for the biosynthetic studies proved to be critical and details may be found in the Experimental Section.

After optimization of cycloheximide production, an examination of uptake and incorporation of acetate was undertaken with time-based studies by using sodium [2-^{14}C]acetate. A summary of these studies presented in Table II shows that maximum incorporation is found when acetate is introduced after 96 h. An explanation for the incorporation results is not obvious, particularly since cycloheximide production is found to increase linearly from 0 to 100 h after which it levels off, and therefore incorporation of acetate over the 96–120-h time period would be expected to be less than if it was introduced at an early stage of the growth cycle.

It is possible that the maximum incorporation observed at 96 h may occur as a result of the endogenous acetate in the medium being exhausted in which case the labeled acetate introduced at this stage is likely to undergo incorporation without significant dilution by unlabeled material prior to uptake by the cells. As a result of this study, all labeled test precursors were introduced 96 h after the medium was innoculated.

Incorporation of Singly Labeled Acetates. For confirmation of the acetate labeling pattern in cycloheximide observed by Vanek and co-workers[12] using ^{14}C-labeled acetates, *S. griseus* was cultured in separate experiments in the presence of [1-^{13}C]- or [2-^{13}C]acetate (90% isotopic purity). The distribution of the label was ascertained by comparing the ^{13}C NMR spectra of the labeled and natural abundance spectra recorded under identical conditions (see Figure 1). The results of ^{13}C enrichment at each carbon were conveniently calculated[21] by using the intensities of the C(16) methyl resonance as an internal standard since in view of its biosynthetic origin via (*S*)-adenosylmethionine, it is not labeled by acetate or any of the other precursors used in this study. These data, which are summarized in Table III, clearly show that the labeling pattern is consistent with that derived by Vanek et al.[13] as illustrated in structure **10**. Six carbons originate from the methyl carbon of acetate, C(3), C(5), C(7), C(9), C(11), and

(21) Wright, J. S. C.; Vining, L. C.; McInnes, A. G.; Smith, D. G.; Walter, J. A. *Can. J. Biochem.* **1977**, *55*, 678.



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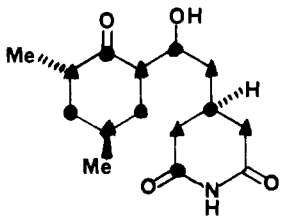

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C(13), and six from the carbonyl carbon of acetate, C(4), C(6) (or C(2)), C(8), C(10), C(12) and C(14). These results also served to confirm the chemical shift assignments of C(7) which is derived from the methyl carbon of acetate and C(14) which is derived from the carbonyl carbon.

An important finding was the observation that both [1-^{13}C]- and [2-^{13}C]acetate caused an enrichment of the downfield C(2) (or C(6)) carbonyl at 172.0 ppm. The magnitude of the enrichment was somewhat lower than the other carbons labeled in the same experiment but was shown to occur reproducibly. This result suggested that introduction of label at C(2) (or C(6)) was occurring via the degradation of the labeled acetates to CO_2 by *S. griseus* and subsequent reincorporation though carboxylation of acetate to malonate. Two further experiments served to confirm this viewpoint.

In the first of these, *S. griseus* was cultured in the presence of $Na_2{}^{13}CO_3$ and the cycloheximide produced was examined by ^{13}C NMR. The spectrum of the sample showed a small but significant enrichment of 1.49% of the downfield imide carbonyl at 172.0 ppm. No other carbons in this sample showed any enrichment above natural abundance.

If the incorporation of malonate had been nonstereospecific and incorporation of label had occurred into both carbonyl groups of the glutarimide ring in the same ratio as claimed by the Czech workers, the calculate percentage enrichment for the other carbonyl would have been 1.27%. While it is likely that such enrichment if it occurred would have been detected, nevertheless it is approaching a value which raises questions regarding its reliability.

Incorporation of [1,2-^{13}C]Acetate. Fortunately the stereospecificity of the separate incorporation of acetate and malonate into the glutarimide ring could be clearly established by the mode of labeling resulting from the incorporation of [1,2-^{13}C]acetate into cycloheximide.

Examination of the ^{13}C spectrum (Figure 2) of the enriched sample derived from [1,2-^{13}C]acetate shows 12 of the 15 carbon signals as triplets[22] (with overlap occurring for C(3), C(5), and C(7) signals between 37.0 and 38.0 ppm) while the C(15) and C(16) methyls together with *one of the imide carbonyl signals appear as singlets*. The key feature of the spectrum which establishes the stereospecific incorporation of acetate into the glutarimide ring of cycloheximide can be most clearly seen in the expansion of the C(2) and C(6) carbon signals in Figure 3.

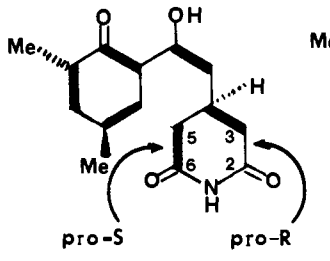
The single line at 172.0 ppm is due to an enriched but uncoupled imide carbonyl resonance, indicating, clearly, that this carbon is not derived from acetate. The fact that it is enriched is consistent with the results from the incorporation of singly labeled acetate and serves to corroborate that endogenous acetate is rapidly degraded to CO_2 by *S. griseus* and then reutilized by the organism for the carboxylation of acetyl coenzyme A to malonate.

Table IV. ^{13}C-^{13}C Coupling Constants for [1,2-^{13}C]Acetate Derived Cycloheximide

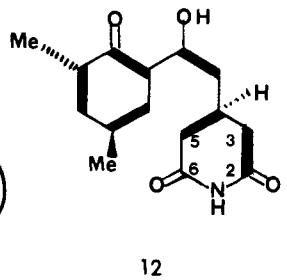
carbon	δ(Me$_4$Si)	coupled carbons	J_{C-C}, Hz
2	172.0		
3	38.5	C(3)-C(4)	33.4
4	27.4	C(3)-C(4)	33.5
5	37.2	C(5)-C(6)	44.3
6	171.8	C(5)-C(6)	44.2
7	38.0	C(7)-C(8)	39.4
8	66.5	C(7)-C(8)	38.8
9	50.2	C(9)-C(10)	37.4
10	216.7	C(9)-C(10)	37.5
11	40.4	C(11)-C(12)	29.6
12	42.6	C(11)-C(12)	29.5
13	26.7	C(13)-C(14)	33.4
14	33.1	C(13)-C(14)	33.5
15	14.2		
16	18.4		

The second imide carbonyl signal appears as a triplet centered at 171.8 ppm in accord with its derivation from intact incorporation of a [1,2-^{13}C]acetate unit into this carbon and the adjacent methylene group. The spectrum of cycloheximide derived from [1,2-^{13}C]acetate also provided the J_{CC} values which are summarized in Table IV. Important information which is derived from this table is that not only does it confirm the ^{13}C chemical shift assignments but also it allows the assignment of the methylene signal at 37.2 ppm (J = 49.3 Hz) and the upfield imide carbonyl signal at 172.4 ppm (J = 49.2 Hz) to be identified as originating from adjacent carbons, i.e., the C(2)–C(3) pair or, alternatively, the C(5)–C(6) pair.

Assignment of the Stereochemistries of Incorporation of Malonate and Acetate into the Glutarimide Ring. While the stereospecificity of incorporation of malonate and acetate into the carbons of the glutarimide ring of cycloheximide is established by the foregoing studies, it remained to correlate each of these separate biogenetic building blocks with the origin of the diastereotopic $-CH_2C{=}O$ groups of the glutarimide ring. The incorporation of [1,2-^{13}C]acetate as discussed in the previous section could lead to one of the two labeling patterns expressed in structures **11** and **12**.



11



12

The C(2)–C(3) carbons and the C(5)–C(6) carbons may be considered as two stereoheterotopic ligands emanating from C(4) and are assigned the stereochemical descriptors *pro-R* and *pro-S*, respectively. In structure **11**, the C(5) and C(6) carbons of the *pro-S* ligand are portrayed as being derived from the intact incorporation of [1,2-^{13}C]acetate, whereas in **12** it is the C(2) and C(3) carbons of the *pro-R* ligand which are indicated to originate from [1,2-^{13}C]acetate.

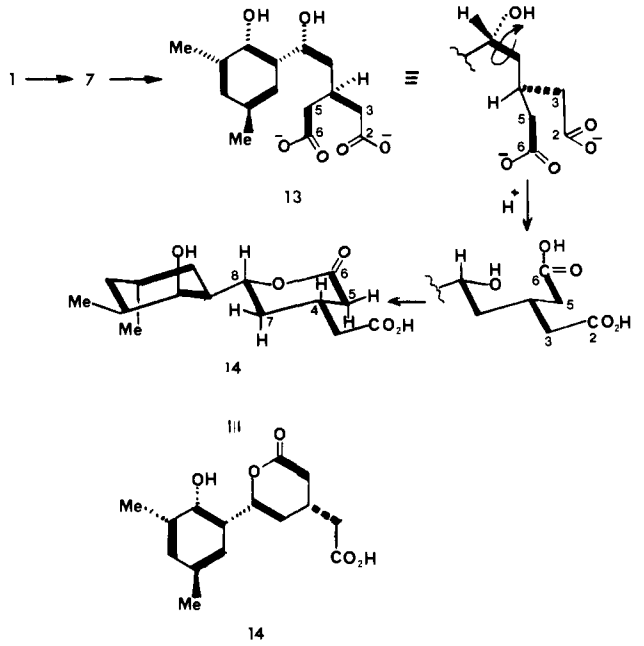
The carbonyl groups of the glutarimide ring of **1** may be differentiated chemically by utilizing the known conversion of cycloheximide to the lactone–acid **14** via the intermediate dihydrocycloheximide **7**. With the assumption that the carbonyls of the lactone and carboxylic acid in the ^{13}C NMR of **14** can be assigned, this transformation is capable of providing a means of determining whether the cycloheximide derived from [1,2-^{13}C]-

(22) The appearance of these signals as triplets occur as a result of the natural abundance ^{13}C singlets being flanked on either side by the peaks of the doublet from the ^{13}C–^{13}C coupled signal arising from the incorporation of doubly labeled acetate.

(23) The degradation of acetate to CO_2 by *S. griseus* is in accord with earlier studies in which it was found that both [2-^{14}C]propionic acid and [1,4-^{14}C]succinic acid were degraded to CO_2 by *S. noursei* prior to incorporation into cycloheximide.[13]

(24) This statement is true for all carbons except the C(4)–C(5) and C(13)–C(14) pairs where the similarity of the magnitude of $J_{C,C}$ is such that they could not be differentiated on this criteria.

(25) The spectra in Figures 2 and 3 show additional satellites for each of the carbon signals appearing as doublets. These satellites originate from various individually labeled cycloheximide molecules in which two intact $^{13}CH_3{}^{13}CO_2H$ units have been incorporated at adjacent sites. The presence of the satellite peaks was practically eliminated in the spectrum of cycloheximide in samples of the metabolite in which the concentration of $^{13}CH_3{}^{13}CO_2H$ administered to the culture medium was reduced from 1 to 0.5 mg/mL (see Experimental Section).



acetate is labeled as in **11** or **12**.

Since hydrolysis–lactonization of dihydrocycloheximide gives a single lactone product, lactonization has been reasonably assumed to occur, but not proven, between the C(8) hydroxyl and the carboxyl of the (*pro-S*)-CH_2CO_2H ligand in the diacid intermediate **13** to afford the γ-lactone in which the cyclohexyl and acetic acid residues are in a cis 1,3-diequatorial relationship. It should be noted that cyclization of the C(8) hydroxyl and the carboxyl of the (*pro-R*)-CH_2CO_2H group in **13** would afford a γ-lactone with a trans 1,3-eq–ax relationship of the substituents.

The ultimate assignment of stereospecificity of acetate incorporation into the glutarimide ring rests upon the structure of the lactone–acid. Therefore it was imperative to establish the stereochemistry of this compound beyond reasonable doubt.

Fortunately a study of 600-MHz proton NMR spectrum of the lactone–acid demonstrated that its structure is indeed correctly represented as **14**. The chemical shifts of all of the protons on the γ-lactone ring of **14** were uniquely defined and interrelated by a series of $\{^1H\}\{^1H\}$ decoupling studies leading to the chemical shifts and coupling constants summarized in Table V. The most critical parameters in terms of defining the stereochemistry of **14** are the large ^3J coupling constants for protons on adjacent carbons at positions 4, 7, and 8 (cycloheximide numbering). The magnetitude of these vicinal couplings which range from 10.0 to 11.3 Hz are only in accord with trans-diaxial arrangements of the hydrogens as required by structure **14**.

The PND ^{13}C spectrum of the lactone–acid at 63.5 MHz was assigned as shown in Table I with the aid of $^{13}C\{^1H\}$ off-resonance and the ^{13}C–^{13}C coupling constants in the PND ^{13}C spectrum of the ^{13}C-enriched lactone–acid obtained from the [1,2-^{13}C]acetate derived cycloheximide. In the spectrum of the ^{13}C-enriched lactone–acid the carbonyl signal at 171.1 ppm had the typical triplet appearance characteristic of the natural abundance singlet and an overlapping doublet due to coupling with an adjacent carbon-13, whereas the carbonyl carbon at 173.7 ppm occurred as a singlet.

The similarity of the two carbonyl shifts in **14** made it impossible to assign them. Fortunately, a solution to this dilemma was provided by the knowledge that the carbonyl resonance of carboxylic acids are shifted downfield when they are converted to their carboxylate salts.[26]

To effect the conversion of the carboxylic acid to its anion in the presence of the lactone group in **14** required a nonnucleophilic base. Model studies showed that 2,6-lutidine fulfilled this requirement in that the carbonyl of cyclohexane carboxylic acid underwent a downfield shift in the presence of an excess of this base while the carbonyl shift of valerolactone remained essentially unaffected under the same conditions.

(26) Hagen, R.; Roberts, J. D. *J. Am. Chem. Soc.* **1969**, *91*, 4504.

Table V. ^1H Chemical Shifts and Coupling Constants for the Lactone Ring of 14 Obtained at 600 MHz

					J, Hz		
	4 ax δ 2.16	5 ax δ 2.72	5 eq δ 2.42	7 ax δ 1.36	7 eq δ 2.33	8 ax δ 4.29	9 ax δ 2.42
4 ax		10.0	5.9	10.9	4.0		
5 ax			17.2				
5 eq					1.7		
7 ax					13.3	11.3	
7 eq						2.8	
8 ax							8.1
9 ax							

When a solution of the lactone–acid **14** in D_2O–dioxane was run in the presence of a ~3 M excess of 2,6-lutidine the upfield partner of the two carbonyls at 171.3 ppm remained unaffected while the peak at 173.4 ppm was shifted to 175.0 ppm allowing the latter to be assigned to the carboxyl group.

Consistent with these assignments are the T_1 values obtained for the two carbonyl groups. The longer T_1 (13.0 s) obtained for the carboxyl carbon in comparison to T_1 of the lactone carbon (9.4 s) is agreement with the different environments of these two functional groups in the lactone–acid.

The assignment of the carbonyl frequencies in **14** demonstrate that the labeling pattern of the lactone–acid is as shown in Scheme II in which the lactone carbonyl and its adjacent methylene are derived from the intact incorporation of [1,2-^{13}C]acetate. This establishes that the *pro-R* C(2)–C(3) carbons in the glutarimide ring of cycloheximide are derived from malonate with acetate providing the *pro-S* C(5)–C(6) carbons of the ring.[27]

These results differ from those obtained by Vanek et al. in that not only is the incorporation of malonate and acetate units into the glutarimide ring in the current study stereospecific but also the stereospecific utilization of *malonate* for the *pro-R* C(2)–C(3) carbons contrasts with the nonspecific incorporation reported by the Czech workers in which *acetate* was favored over malonate by a 2.2:1 ratio in providing these carbons. Since the earlier biosynthetic studies reporting this latter result were obtained using *S. noursei*, it is possible that the markedly differing results may be accounted for by the operation of different pathways for cycloheximide biosynthesis in *S. griseus* and *S. noursei*.[28]

Experimental Section

General Methods. IR spectra were recorded on a Perkin-Elmer 621 instrument, and NMR spectra were recorded on JEOL MH-100, FX60, and Bruker WM 250 instruments. The 600-MHz ^1H spectra were obtained at the Carnegie-Mellon high-field NMR Facility. All chemical shifts are reported by using Me_4Si as an internal standard. The ^{14}C-labeled acetate was obtained from New England Nuclear, Inc., whereas all ^{13}C-labeled compounds were purchased from Merck, Sharpe and Dohme, Canada, Ltd. Preparative layer chromatography was carried out an 0.5-mm plates of Merck Silica Gel H. Radioactivity measurements were accomplished with a Beckman LS 150 scintillation counter by using Research Products International 3a70 cocktail. Cycloheximide (mp 111–113 °C) was obtained from Aldrich Chemical Co.

Culture Conditions. *Streptomyces griseus* UC 2001 (Upjohn Co.) was maintained in sub-cultures on CMI agar prepared by the method of Hopwood.[29] These subcultures were used for the innoculation of the seed

(27) The chemical shift correlations obtained by the conversion of the [1,2-^{13}C]acetate enriched cycloheximide to the lactone–acid provided the final information necessary to identify the chemical shifts of diastereotopic pairs of carbons C(2)–C(3) and C(5)–C(6) in cycloheximide provided in Table I.

(28) Following the completion of this work we became aware that the biosynthesis of cycloheximide in *Streptomyces naraensis* employing ^{13}C NMR is one of several topics discussed in a lecture entitled "Application of ^{13}C–^{13}C and ^{13}C–^2H Couplings to Biosynthetic Studies": Shimada, H.; Sato, T.; Kinoshita, T.; Ebizuka, Y.; Akiyama, T.; Noguchi, H.; Iitaka, Y.; Sankawa, U.; Yamasaki, K. "Proceedings of the 21st National Products Conference, Hakkaido University, Department of Agriculture, Sapporo 1978; **1979**, *Chem. Abstr. 90*, 68567.

(29) Hopwood, D. A. *Bacteriol. Rev.* **1967**, *31*, 373.

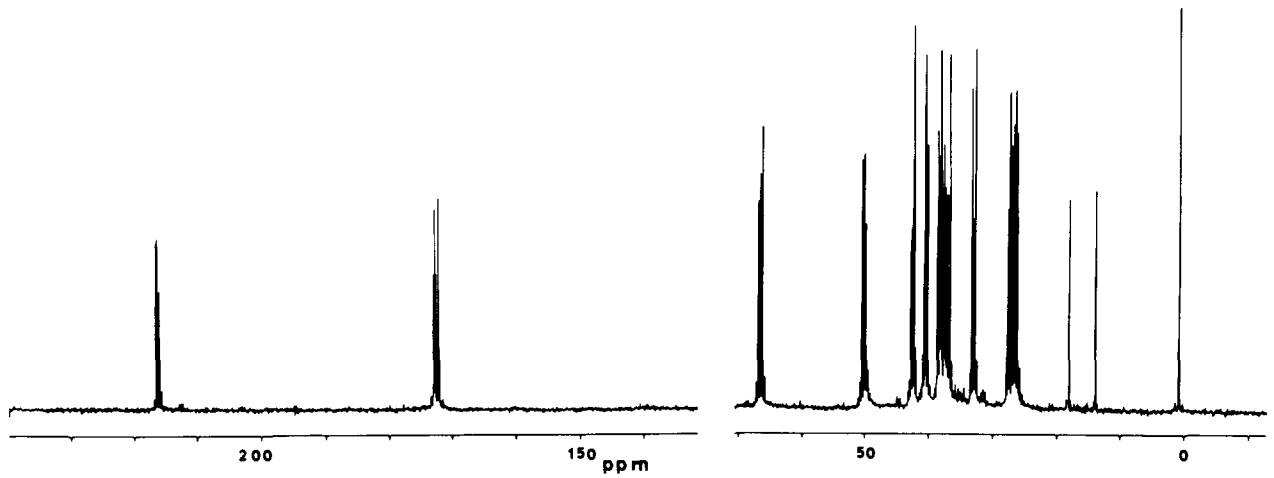


Figure 2. Proton-noise-decoupled FT ^{13}C NMR spectrum of cycloheximide isotopically enriched from the biosynthetic incorporation of $^{13}CH_3{}^{13}CO_2Na$ (15991 transients, 3.0-s pulse delay).

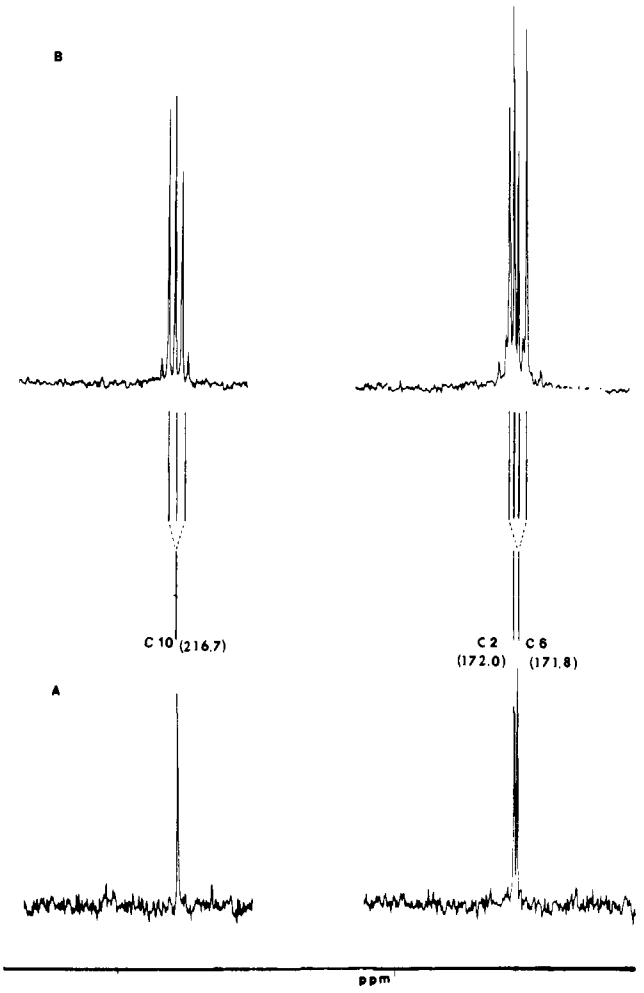


Figure 3. (A) Carbonyl region of PND ^{13}C spectrum of cycloheximide from Figure 1A. (B) Carbonyl region of PND spectrum of isotopically enriched cycloheximide from the biosynthetic incorporation of $^{13}CH_3{}^{13}CO_2Na$.

medium. Typically, refrigerated agar slants of *S. griseus* were allowed to come to room temperature during a 2-day period. A block of spore-covered agar was cut from this slant and placed in a 500 mL Erlenmeyer flask containing 100 mL of innoculation medium prepared according to Churchill[30] (see below). The cultures were incubated at 28 °C for 48 h on a New Brunswick rotary shaker at 240–250 rpm. Production cultures were innoculated with approximately 5 mL of the vegetative inoculum from the seed medium by using 100 mL of the F-3 fermentation medium specified by Churchill[30] contained in stippled wide-mouth Erlenmeyer flasks. On the basis of time-course studies with [2-^{14}C]acetate, addition of ^{13}C precursors to the medium was effected after 92 h and the cultures were harvested after 120 h. The precursors were added to the medium as solutions in sterile water through a Millipore filter.

Isolation and Purification of Cycloheximide. The isolation procedure was based upon that reported by Churchill.[31] Filtration of the fermentation medium, first through a filter pad made of gauze and then through a celite pad, gave a clear aqueous filtrate. Acidification of the aqueous filtrate (400 mL) with 10% HCl followed by immediate extraction with CH_2Cl_2 (3 × 250 mL) and concentration of the CH_2Cl_2 extract afforded a residue which by TLC on silica gel (Merck) in the following solvent systems showed that it was essentially pure cycloheximide: 6% MeOH:THF (R_f = 0.60); 2% MeOH:EtOAc (R_f = 0.40), and EtOAc (R_f = 0.34). A molybdic acid reagent was used for detecting the cycloheximide. Crystalline cycloheximide, mp 112–113 °C (EtOAc), could be obtained from the material by either preparative layer chromatography on silica gel H in ethyl acetate or by low-pressure liquid chromatography on Lichrosorb (40 μm) in ethyl acetate at 30 psi with flow rates at 0.75 mL/min. Typical yields of cycloheximide obtained were in the range 0.5–0.7 mg/mL of medium.

Innoculation Medium. The medium was prepared as described by Churchill[29] as follows. Difco beef extract (10 g), Difco Bacto Peptone (5 g), glucose (10 g), and NaCl (5.0 g) were dissolved in water (1 L) and sterilized at 121 °C for 20 min.

Incorporation of [2-^{14}C]Acetic Acid into Cycloheximide. Three culture flasks, each containing 100 mL of fermentation medium, were innoculated with a seed culture of *S. griseus*. Sodium [2-^{14}C]acetate (6.25 μCi) was added separately to each of the growing cultures at the time intervals of 48, 72, and 96 h from the time of innoculation. After 120 h each of the fermentations were worked up separately and 100 mg of pure cycloheximide was added to the extract from each fermentation. The cycloheximide was chromatographed and purified to constant activity by several recrystallizations. The results, which are summarized in Table II, showed maximal incorporation of label when acetate was administered at 96 h.

Incorporation of ^{13}C-Enriched Acetates into Cycloheximide. The following experimental conditions were used for the incorporation of ^{13}C-labeled acetates into cycloheximide.

Solutions of 50 mg (90 atom %) of the ^{13}C-labeled acetate in 100 μL of sterile water were added by using sterile techniques to each of the cultures of *S. griseus* growing on 100 mL of the fermentation medium at the 96-h period following innoculation. After 120 h the cultures were harvested and the cycloheximide was isolated as described above. In one experiment with [1,2-^{13}C]acetate 200 μL of solution containing 100 mg

(30) Churchill, B. W., private communication.

(31) Churchill, B. W. U.S. Patent, 2885326, 1959.

(32) **Note Added in Proof.** Following the submission of this manuscript an article describing the incorporation of [1,2,3-^{13}C]malonate into cycloheximide in *S. naraensis* has appeared [Shimada, H.; Noguchi, H.; Yoichi, I.; Sankawa, U. *Heterocycles* **1981**, *15*, 1141]. The results reported show the stereospecific incorporation of malonate into the pro-R unit of the glutarimide ring in agreement reported with the independent findings of the ^{13}C-labeled acetate studies presented in our work. This paper, in conjunction with our studies, must now place the results of the earlier studies[16] on the biosynthesis of cycloheximide in *S. noursei* into serious doubt.

of 90 atom % ^{13}C was administered to each of three flasks containing 100 mL of growing cultures. The ^{13}C NMR spectrum which is shown in Figure 2 was derived from this experiment.

Incorporation of $^{13}CO_2$ into Cycloheximide. The general procedure followed that described for the administration of the ^{13}C-labeled acetates. At the 96-h period 1 mL of a solution containing 150 mg of $Na_2{}^{13}CO_3$ was administered to each of three growing cultures of *S. griseus*. The fermentation was terminated at 120 h and the ^{13}C-enriched cycloheximide isolated as described above.

T_1's and Effect of the Addition of 2,6-Lutidine on the ^{13}C-Carbonyl Shifts of 14. The ^{13}C spectra of cyclohexane carboxylic acid and valerolactone were obtained in D_2O–dioxane and gave ^{13}C-carbonyl shifts at 179.3 and 179.6 ppm, respectively. Addition of an excess (~3 M) of 2,6-lutidine to each of these solutions gave the following changes in carbonyl shifts: cyclohexanecarboxylic acid, ^{13}CO = 179.69 ($\Delta\delta$ = +1.39 ppm); valerolactone, ^{13}C = 179.32 ppm ($\Delta\delta$ = −0.27 ppm). The analogous experiment with the lactone–acid **16** gave the following results: ^{13}CO shifts in D_2O–dioxane: C(2) carboxylic acid 174.6 ppm and C(6) lactone 173.6 ppm. With the addition of 3 M excess of 2.6-lutidine the following changes were observed: C(2), 176.6 ppm ($\Delta\delta$ = +2.0 ppm); C(6), 171.1 ppm ($\Delta\delta$ = 0.1 ppm).

The T_1 values obtained by the inversion-recovery process with a 180–τ–90 sequence for **14** in acetone-d_6 were as follows: C(2) carboxylic acid, 13.0 s; C(6) lactone, 9.4 s.

Acknowledgment. We are indebted to the Biomedical Research Support Grant, Duke University, for partial support of this research. Dr. Bruce Churchill, The Upjohn Co., Kalamazoo, MI, provided the selected strain of *S. griseus* used in this study as well as much valuable advice. Proton NMR spectra at 600 MHz were obtained through the courtesy of the NIH High Field NMR Facility at Carnegie-Mellon (Grant No. RR 00292). The ^{13}C NMR of isocycloheximide reported in Table I was made possible through a generous gift of the compound from Dr. A. J. Lemin, The Upjohn Co., Kalamazoo, MI.

Catalytic Mechanisms of Acyl Transfer Reactions in Dipolar Aprotic Media. 2. Electrophilic Activation of the Carbonyl Group by Quaternary Alkylammonium and Imidazolium Functions[1]

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Contribution from the Department of Chemistry, Boston College, Chestnut Hill, Massachusetts 02167. *Received January 30, 1981*

Abstract: Crown ether solvated potassium acetate in dipolar aprotic media readily converts *p*-nitrophenyl *o*-toluate to the corresponding acetyltoluyl mixed anhydride. The reaction proceeds at room temperature in quantitative yield. The rate of acyl transfer is 0.0118 M^{-1} s^{-1} in acetonitrile, 0.0223 M^{-1} s^{-1} in dimethyl sulfoxide, and 0.0898 M^{-1} s^{-1} in dimethylformamide, exhibiting strictly bimolecular kinetics up to the solubility limit of the desolvated nucleophile. The reaction shows absolute dependence on dipolar aprotic media; addition of hydroxylic solvents results in strong inhibition. Formation of the mixed anhydride is efficiently catalyzed by imidazolium and quaternary ammonium neighboring groups located in close proximity to the scissile carbonyl oxygen. The electrophilic catalysis results in 1000-fold rate enhancements. The acceleratory participation of the cationic neighboring groups is consistent with stabilization of the developing negative charge at the reaction center in the course of formation of the tetrahedral intermediate. The catalytic rates are one order of magnitude faster than the aminolytic cleavage of *p*-nitrophenyl acetate by benzamidine in nonprotic media. They also exceed the rate of hydrolysis of *p*-nitrophenyl acetate by hydroxide ion in water. The mechanism of the reaction is being investigated in reference to the catalytic hydrolyses by proteolytic and lipolytic enzymes which have no serine residues at the active site.

Introduction

X-ray crystallographic studies of the past several years have clearly demonstrated that the active sites of proteolytic as well as lipolytic enzymes contain hydrophobic regions.[2,3] The fact that rather low concentrations of aqueous portions are found at the catalytic centers may indicate that the medium of enzyme-catalyzed nucleophilic reactions of carboxylic acid derivatives is aprotic in nature. The mechanistic significance and possible catalytic advantage of transferring these hydrolytic reactions into nonpolar media have remained essentially unexplored.[2]

In contrast to the abundance of bioorganic model studies of hydrolytic reactions in aqueous solutions[4] and more recent work involving micellar systems,[5] relatively few attempts have been made to elucidate the mechanism of catalytic acyl transfer reactions in nonpolar or dipolar aprotic media.[6] The limited data presently available focuses almost entirely on aminolysis of esters,[2,6-9] clearly indicating that there are substantial changes both in the reactivities of the nucleophiles[9] and in the manner by which the substituents influence the reaction rates in nonprotic vs. aqueous solutions. It appears, for example, that the rate-limiting step of the reaction between *p*-nitrophenyl acetate and pyrrolidine in acetonitrile involves the collapse rather than the formation of the tetrahedral intermediate.[6a] Analogous results were obtained in imidazole and piperidine aminolyses of *p*-nitrophenyl esters in toluene and benzene.[6b,7] These conclusions are further supported by the observation that aminolytic reactions in nonprotic media are more sensitive to substituent effects on the leaving group than on the

(1) Presented in part at the Fith IUPAC Conference on Physical Organic Chemistry, Santa Cruz, CA, Aug 17-22, 1980.
(2) Bender, M. L. In "Bioorganic Chemistry", Van Tamelen, E. E., Ed.; Academic Press: New York, 1978; Vol. I, pp 19-57.
(3) Verheij, H. M.; Volwerk, J. J.; Jansen, E. H. J. M.; Puyk, W. C.; Dijkstra, B. S.; Drenth, J.; de Haas, G. J. *Biochemistry* **1980**, *19*, 743-750.
(4) (a) Bender, M. L. "Mechanisms of Homogeneous Catalysis from Protons to Proteins"; Wiley-Interscience: New York, 1971; pp 95-193. (b) Jencks, W. P. "Catalysis in Chemistry and Enzymology"; McGraw Hill: New York, 1969; pp 463-554.
(5) Menger, F. M. In "Bioorganic Chemistry", Van Tamelen, E. E., Ed.; Academic Press: New York, 1977; Vol. III, pp 137-152.
(6) (a) Menger, F. M.; Smith, J. H. *Tetrahedron Lett.* **1970**, *48*, 4163-4168. (b) Menger, F. M.; Vitale, A. C. *J. Am. Chem. Soc.* **1973**, *95*, 4931-4034.
(7) Rivetti, F.; Tonellato, V. *J. Chem. Soc., Perkin Trans. 2*, **1977**, 1176-1179.
(8) Parker, A. J. *Chem. Rev.* **1969**, *69*, 1-32.
(9) Menger, F. M. *J. Am. Chem. Soc.* **1966**, *88*, 3081-3084.

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