BIOSYNTHESIS OF TRITIUM, DEUTERIUM AND CARBON-13 LABELED CYCLOHEXIMIDE

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SUMMARY

Tritium, deuterium, and carbon-13 labeled cycloheximide were prepared through fermentation of *Streptomyces griseus* in the presence of S-methyl labeled L-methionine. Cycloheximide-^{3}H of specific activity 4.96 mCi/mg (1.40 Ci/mmol) was obtained in 25% overall radiochemical yield. Nuclear magnetic resonance and mass spectral analyses of the deuterium and carbon-13 labeled products showed that isotope incorporation occurred virtually exclusively in the two methyl groups in cycloheximide, with negligible (<5%) incorporation in the rest of the molecule. Furthermore, deuterated species contained either three or six deuterium atoms, indicating that the methyl groups were transferred intact from L-methionine. Also, the two methyl groups in cycloheximide were of equal isotope enrichment. The overall enrichment achieved in the methyl groups was 55% and 45%, respectively, in the deuterium and carbon-13 labeled compounds.

Key Words: Cycloheximide; biosynthesis; tritium, deuterium, carbon-13

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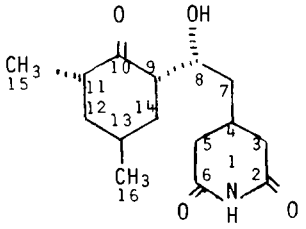
INTRODUCTION

Cycloheximide *(*1*), an antibiotic first isolated by Whiffen, Bohonos, and Emerson (1, 2) from *Streptomyces griseus*, possesses antitumor (3, 4, 5) and amebicidal (6) activities. It is also a potent rodent repellent (7) and has agricultural utilities as an abscission agent and fungicide (8-12). It is currently under development as a drug candidate for treatment of dermatologic diseases. Synthesis of cycloheximide was undertaken to meet the need for radioactive drug for conducting absorption and metabolism studies in test animals and man.

DISCUSSION AND RESULTS

Because of anticipated topical applications, radioactive drug of high specific activity was needed. The total synthesis of cycloheximide was reported by Johnson *et al.* in 1966 (13). Unfortunately, the chemistry is not readily applicable to the preparation of high activity tritium labeled material. We therefore turned to biosynthesis from radioactive precursors.



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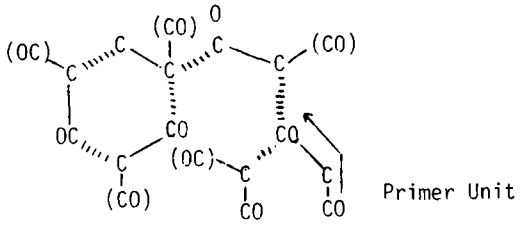

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The biogenesis of cycloheximide was studied by Vaněk *et al.* (14-16). The carbon skeleton of the compound results from condensation of five three-carbon units of malonate, initiated by a sixth "primer" malonate unit, as shown in structure *2*. One carbon atom, shown in parentheses, is lost through

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* Also known as naramycin A and Acti-Dione® (The Upjohn Company).

decarboxylation from each unit except the primer unit. The malonyl building blocks are in turn derived from two-carbon acetate units. Fermentation of *S. griseus* in the presence of sodium acetate-^{3}H, therefore should produce cycloheximide bearing tritium labels at the odd-numbered carbon atoms. However, the mode of condensation would suggest that at least two-thirds of the tritium in sodium acetate-^{3}H would be lost through dilution by the fermentation medium. This coupled with the expected low incorporation (2-8%) of acetate, as reported in the literature (16) when sodium acetate-^{14}C was used as substrate, raised doubts as to whether the required specific activity of 1 mCi/mg in cycloheximide could be achieved. Moreover, most of the tritium labels in such a product would be located at potentially exchangeable positions *alpha* to existing and latent carbonyl groups and the carboximide function, an undesirable situation when the compound is used for metabolism studies.

The two methyl groups in cycloheximide can be derived from L-methionine (*4*) through trans-methylation (14). Cycloheximide-15,16-^{14}C was produced with 14-17% incorporation (16) during fermentation in the presence of L-methionine (*4*) labeled with carbon-14 in the S-methyl group. By analogy, it appeared that by using L-methionine, labeled with tritium in the S-methyl group, as the precursor, cycloheximide could be obtained with specific tritium labels in the methyl groups. The superior incorporation efficiency, specificity, and the location of tritium at chemically stable positions in the product made L-methionine-S-Me-^{3}H the potential radioisotope label source of choice for preparing high specific activity cycloheximide with non-labile tritium labels.

A low radioactivity trial fermentation was carried out to determine tritium incorporation efficiency and whether the methyl group was transferred from L-methionine to cycloheximide as an intact group or through some transient species of higher oxidative state, to be subsequently reduced back to the methyl group. The latter point was of concern because such transformations would release tritium as volatile tritiated water, which in a multi-curie

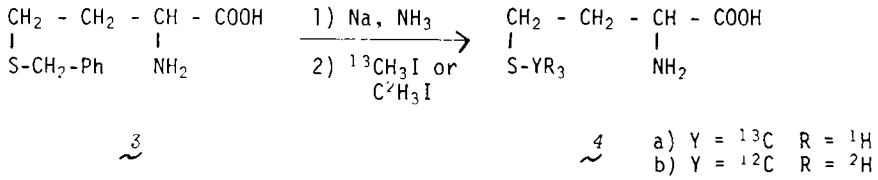
experiment would constitute a health hazard. To minimize such hazards, the fermentation was carried out in an environmentally contained apparatus under a regulated stream of sterile air which, after exit from the fermentation chambers, was led through a series of cold traps and drying agents to remove any escaping moisture. The labeled methionine was added in increments to the fermentation medium during the period of presumed maximum production of cycloheximide in order to effect optimum incorporation, since it was expected that methionine would probably also be dissipated through biotransformations other than those directed towards the synthesis of the product. From 4.45 mCi of tritiated methionine of 201 mCi/mmol, 0.796 mCi of cycloheximide of 2.33 mCi/mmol was produced, which represented a 17.8% radiochemical yield. Only 0.03% of radioactivity used was found in the substantial amounts of trapped water and/or other volatile products condensable at -70° C, suggesting the intact methyl group as the predominant if not sole mode of transfer.

Similar results were obtained in a biosynthesis carried out with a much larger amount of radioactivity. Fermentation in the presence of 2.64 Ci of methionine, nominally 77 Ci/mmol, yielded 661 mCi of cycloheximide with a specific activity of 4.96 mCi/mg or 1.40 Ci/mmol, which represented a 25% radiochemical yield. It is of interest to note that exposure to multi-curie amounts of radioactivity had no apparent deleterious effect on *S. griseus*. The product, however, known to be unstable in both acidic and basic media (13), proved highly susceptible to radiolytic decomposition as well. In the crystalline state, high specific activity cycloheximide declined in purity at a rate of 1% per day at -15° C. The material was purified by column chromatography on silica gel and stored at -15° C in ethyl acetate at a concentration of ~5 mCi/ml to maintain the radiochemical stability of labeled cycloheximide.

Carbon-14 labeled methyl group of L-methionine was reported (14) to be incorporated into the two methyl groups of cycloheximide exclusively and equally. By analogy, the tritium labels in our cycloheximide were therefore expected to be also localized evenly at carbon atoms number 15 and 16. To

validate this assumption and determine isotope distribution, particularly with respect to whether there was any significant randomization of labels elsewhere in the cycloheximide molecule, we prepared deuterium and carbon-13 labeled cycloheximide through fermentation of *S. griseus* in the presence of L-methionine labeled with deuterium and carbon-13 in the methyl group. The exclusivity of the label positions, or lack of it, in tritiated cycloheximide, is of interest because of its anticipated use in metabolism studies.

The labeled L-methionines *4a* and *4b* were prepared from S-benzyl-L-homocysteine (*3*) and deuterium (99% enrichment) and carbon-13 (90% enrichment) labeled methyl iodide according to known procedures (17). In order to maximize utilization of labeled precursor while minimizing the effects of isotope



3 *4* a) Y = ^{13}C R = ^{1}H
b) Y = ^{12}C R = ^{2}H

dilution* inherent during the course of the fermentation, relatively massive amounts of deuterium and carbon-13 labeled L-methionine (750-1000 mg), in comparison to tritiated L-methionine (~5 mg), were fed to the culture in numerous increments over a prolonged period of time. Although, as noted earlier, exposure to radiation apparently had no effect on the fermentation, prolonged presence of high concentrations of L-methionine exerted an inhibitory effect on culture growth and/or cycloheximide production, resulting in low product titers (40-50% of normal). Nevertheless, adequate amounts of deuterium and carbon-13 labeled products were obtained. These materials were subjected

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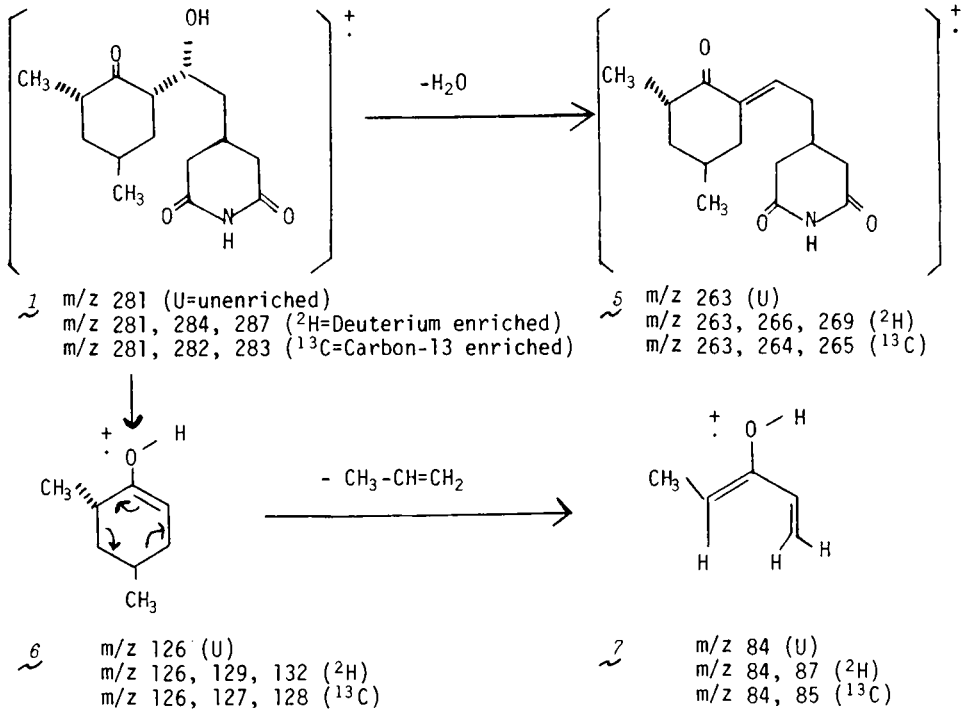
* Comparison of specific activities of L-methionine-^{3}H and cycloheximide-^{3}H shows a one-hundred-and-ten fold isotopic dilution during fermentation.

to proton and carbon-13 nuclear magnetic resonance, as well as mass spectral analyses. The NMR spectra clearly showed exclusive isotope enrichment (overall 55% deuterium, 45% carbon-13) of equal distribution between the two methyl groups. The mass spectral fragmentation pattern of interest are shown in Scheme 1. Loss of the elements of water from cycloheximide afforded the ionized species *5*, and a McLafferty rearrangement followed by a retro Diels-Alder type fragmentation of *1* led to ions *6* and *7*. All the listed labeled and unlabeled ions, with the exception of the molecular ions which were rather weak, were present in good abundance and readily measured in the mass spectra. The relative intensities for m/z 263-271, attributable to *5*, are tabulated in Table 1, along with the calculated distribution for the various labeled and unlabeled forms of *5*. The spectrum of deuterated cycloheximide showed that virtually all the labeled molecules were either trideuterated or hexa-deuterated, with all other deuterated species combined, e.g., d_1, d_2, d_4, d_5, etc., accounting for less than 5% of the total mixture. The spectrum of the carbon-13 labeled compound showed presence of less than 2% of materials containing more than two carbon-13 atoms per molecule. These spectral data showed conclusively that there was negligible random incorporation of labels during fermentation, the methyl groups were transferred intact from methionine to cyclohemixide, and the products were specifically labeled in the methyl groups.

Scheme 1. Fragmentation of Cycloheximide



EXPERIMENTAL

Radioactivity determinations were carried out by means of external standard method with a Packard Tri-Carb liquid scintillation spectrometer, Model 2425. Diotol (Burdick-Jackson) was used as the scintillation solvent. Thin-layer chromatographic (TLC) analyses were done on 2.5 x 10 cm glass plates precoated with a 250 μm layer of silica gel GF (Analtech). Developed zones were visualized by exposure to iodine vapor. Radioactive zones were detected by scanning on a Vanguard Model 880 Autoscanner equipped with Model 885 glass plate scanner, and were quantified by eluting the sectioned plate with MeOH and counting aliquots of the eluates. Infrared (IR) spectra were obtained with a Digilab Model 14D Fourier transform spectrometer. Nuclear magnetic resonance (NMR) spectral analyses were carried out with a Varian

Table 1. Ion Intensity and Isotope Distribution

Compound	Relative Ion Intensity* (% of Total 5)**									
	m/z 263	264	265	266	267	268	269	270	271	272
Unenriched	1997	370	45	14	0	0	0	0	0	0
Deuterium Enriched	4440 (23.3)	926 (1.2)	348 (0.6)	6522 (34.0)	1376 (1.8)	617 (1.4)	7372 (37.7)	1503 (0)	208 (0)	0
Carbon-13 Enriched	2678 (38.5)	3101 (37.9)	2044 (22.0)	433 (1.6)	77 (0)	0	0	0	0	0

*Relative intensities are based on the most intense ion in each spectrum, which is assigned the intensity of 9999.

**The distribution of various labeled species of 5 were calculated after correcting for contributions to relative intensities by natural isotopic abundances, as determined from the spectrum of unlabeled cycloheximide.

CFT-20 spectrometer for carbon-13 and a Varian XL-100 for proton. Mass spectral analyses were done with a Varian MAT CH-5 DF spectrometer. Melting points were determined in capillary tubes and were uncorrected. Microanalyses were obtained for the elements listed where indicated, and the results, except as noted, were within ±0.4% of theory.

L-Methionine S-Methyl-^{13}C and ^{2}H (*4a* and *4b*)

S-Benzyl-L-homocysteine (Sigma Chemical Co.), 2.93 g, 13.0 mmoles, was dissolved in 100 ml of distilled liquid ammonia in a predried 250 ml 3-necked round bottom flask fitted with a silicon rubber septum and a dry ice-acetone condenser. To the clear solution was added small chunks of sodium metal until an intense blue color persisted. The dry ice-acetone cooling bath (-72° C) was replaced with a dry ice-acetonitrile bath (-50° C), and methyl-^{13}C iodide (Stohler Chemical Co., 90% ^{13}C-enrichment), 1.86 g, 13.0 mmoles, was added dropwise with stirring from a syringe. The colorless mixture, which gave a negative sodium nitroprusside test (a violet color signified a positive test), was allowed to evaporate at room temperature overnight. The residual solids were dissolved in 120 ml of water, and the solution (pH ∿11) was titrated with concentrated hydriodic acid to pH ∿5. The mixture was filtered through a pad of celite to remove gummy materials, and the clear filtrate was concentrated at 40° C under vacuum to ∿15 ml. The mixture was heated to reflux to dissolve precipitates, 300 ml of hot ethanol was added, and the mixture was kept at 0° C overnight. The resulting crystals were filtered, washed with absolute ethanol, and dried, 1.707 g (88% yield) of L-methionine S-methyl-^{13}C (*4a*), m.p. 278-9° C (dec.); homogeneous and identical to a standard sample of L-methionine by TLC (10:1:3 butanol:acetic acid:water, ninhydrin spray, Rf 0.39); IR spectrum conformed with that of a reference sample of L-methionine; mass spectrum: m/z 150; proton NMR in $^{2}H_2O$:3.9δ (t, 1H, J = 7 Hz, -C<u>H</u>-COOH), 2.6-2.8δ (m, 2H, S-C<u>H</u>$_2$), 2.05-2.35δ (m, 2H, C<u>H</u>$_2$-CH), 2.17δ (s, <1H, S-^{12}C<u>H</u>$_3$), 1.46 and 2.87δ (d, >2H, $J_{H-^{13}C}$ = 141 Hz, S-^{13}C<u>H</u>$_3$), methyl carbon on sulfur ∿85% deuterated; *anal.*- C(-0.97%), H, N.

Similarly, from 2.80 g, 12.4 mmoles, of S-benzyl-L-homocysteine and 1.8 g, 12.4 mmoles of methyl-^{2}H iodide (Stohler Chemical Co., 99% ^{2}H-enrichment), there was obtained 1.304 g (69% yield) of L-methionine S-methyl-^{2}H (*4b*), mp.p. 275-7° C (Dec.); identical to a reference sample of L-methionine by IR and TLC (Rf 0.39 in 10:1:3 butanol:acetic acid:water); proton NMR in ^2H_2O: 3.9δ (t, 1H, J = 7 Hz, -CH-COOH), 2.67δ (t, 2H, J = 7 Hz, S-CH_2), 2.05-2.35δ (m, 2H,-CH_2-CH), S-CH_3 not detectible, indicating virtually total ^{2}H-enrichment; *anal.*-C, H, ^{2}H, N; mass spectrum: m/z 152.

Fermentation

Streptomyces griseus was the organism used in these studies. The composition of the agar slant medium was the same as described previously (18). Spores from the agar slant cultures were used as the inoculum for a seed medium of the following composition: Carbohydrate, 10 g; yeast, 10 g; cottonseed flour, 5 g; NaCl, 4 g; $CaCO_3$, 4 g; polyalkylene glycol, 0.1 ml; and tap water to 1000 ml. The inoculated medium (100 ml in a 500-ml Erlenmeyer flask) was incubated at 28° C for 3 days on a reciprocating shaker. The fermentation medium was composed of the following: Carbohydrate, 80 g; yeast, 2.5 g; $(NH_4)_2SO_4$, 5 g; $CaCO_3$, 8 g; NaCl, 4 g; KH_2PO_4, 0.2 g; soybean flour, 14 g; polyalkylene glycol, 0.1 ml; and tap water 1000 ml. This medium (50 ml in a wide-mouthed 500-ml Erylenmeyer flask) was inoculated with 5% of the seed medium. The flasks were incubated at 25° C on a rotary shaker (300 rpm) in a Psycro Therm controlled environment incubator shaker (New Brunswick Scientific Co., Inc.) for 7 days. All media were sterilized by autoclaving at 121° C at 15 lbs/in^2 pressure for 30 min.

Where radioactive precursors were used, the cotton and gauze covers on the flasks were replaced with sterile rubber stoppers fitted with air inlet and outlet tubes, and an injection port topped with a silicone rubber septum. The two flasks were connected in series with sterile rubber tubings. Filtered sterile air was pumped through the two flasks at 15 ml/min and led through two cold traps (dry ice-acetone bath) and finally two 5 cm x 60 cm drying

towers filled with a mixture of calcium chloride and Dririte indicator (calcium sulfate) granules, prior to venting into a hood. The cold trap baths were periodically replenished with dry ice as needed.

Isolation

Typically, the combined contents from two fermentation flasks, diluted with an equal volume of water, were mixed with 6-8 g of filteraid FW-40 and adjusted to pH 2.5-3.0 with ~2 ml of 6N H_2SO_4. The mixture was filtered through a wet pad of 10 g of FW-40, and the cake was washed twice with 50 ml of H_2O. The combined filtrate and washings were extracted twice with 160-190 ml of CH_2Cl_2. Overly vigorous mixing should be avoided to minimize emulsification. The combined extracts were washed with 90 ml each of H_2O and brine, and dried over $MgSO_4$.

The filtered CH_2Cl_2 solution was concentrated at 25° C and 25 torr pressure, and the residual foam was dissolved in 0.2 ml of warm amyl acetate. The clear mixture was cooled to room temperature, seeded with cycloheximide, and allowed to crystallize in the refrigerator overnight. The supernate was removed with an eye-dropper, and the crystals lining the flask walls were rinsed with 0.3-0.5 ml portions of ice-cold 1:1 and 1:2 amyl acetate:hexane in that order, and finally broken up under hexane and filtered. Yields of 64-89 mg of cycloheximide were obtained.

Cycloheximide Methyl-^{3}H

The L-methionine S-methyl-^{3}H, supplied by New England Nuclear Corp. as a 10 mCi/ml solution in 7:3 EtOH:H_2O, nominally 77 Ci/mmol, was added to the medium in two portions at 48 and 72 hours after the start of fermentation. In each instance 150 ml of the solution, nominally 1.5 Ci, i.e., 2.91 mg of methionine, was concentrated at 35° C and 25 torr pressure. The residue was dissolved in 5 ml of H_2O, sterilized by filtration (Nalgene filter, 0.20 μm), and the filter was washed with 5 ml of water. The combined filtrate and washings were weighed and assayed for radioactivity (first batch: 8.60 g,

1.32 Ci; second batch: 8.20 g, 1.32 Ci). Half of the solution was added to each of two fermentation flasks, each containing 50 ml of fermentation medium. After 7 days, the contents of the two flasks were combined and worked up as described above. There was obtained 89 mg of crystalline product with a specific activity of 4.96 mCi/mg, 98.8% radiochemically pure by TLC (95:5 v/v $CHCl_3$:MeOH, Rf 0.35, same as a standard sample of cycloheximide). The residue from the mother liquor was mixed with 150 mg of non-radioactive cycloheximide and crystallized from 0.2 ml of amyl acetate to give 118 mg of crystals, sp. act. 1.18 mCi/mg, 97.8% radiochemically pure by TLC. The residue from the second mother liquor was chromatographed on a column of 50 g of silica gel packed in and eluted with 95:5 v/v $CHCl_3$:MeOH. The eluate was collected in 7 ml fractions at 2 min per fraction. The combined fractions 33-45 afforded 90.3 mCi of tritiated cycloheximide, 97.8% radiochemically pure by TLC, as a solution in EtOAc at a concentration of 6.0 mCi/ml. The total yield of radioactive cycloheximide was 661 mCi, or 25% of the 2.64 Ci of tritium labeled L-methionine used.

After 6 days in the crystalline state at -15° C, the 4.96 mCi/mg material declined in radiochemical purity from 98.8% to 92.0% as determined by TLC. The material, 430 mCi, was therefore purified by column (24 mm ID) chromatography (60 g of silica gel, 97:3 v/v $CHCl_3$:MeOH) to give 396 mCi of product, 99.7% radiochemically pure by TLC, which was stored as a solution in EtOAc at -15° C, at a concentration of 4.78 mCi/ml.

Cycloheximide Methyl-^{2}H

A sterile solution of 1.0 g of L-methionine S-methyl-^{2}H (99% enrichment) in 56 ml of H_2O was added in 7 portions to four fermentation flasks each containing 50 ml of fermentation medium. The additions spanned 72 hours, beginning on the second day of the 7-day fermentation. The oily crude product, 172 mg, was chromatographed on a 60 g column (24 mm ID) of silica gel packed in and eluted with 97:3 v/v $CHCl_3$:MeOH to afford 87 mg of deuterated cycloheximide,

after crystallization from benzene-hexane, m.p. 113-114.5° C; homogeneous and identical to a reference sample of cycloheximide by TLC (95:5 v/v $CHCl_3$:MeOH, Rf 0.39); mass spectral data shown in Scheme 1 and Table 1; proton NMR in C^2HCl_3 (TMS): 0.99δ (d, $\lesssim$1.5H, J = 7 HZ 16-CH_3), 1.24δ (d, $\lesssim$1.5H, J = 7 Hz, 15-CH_3), ∿55% deuterated in both methyl groups; *anal.*-C, H, N.

Cycloheximide Methyl-^{13}C

A sterile solution of 750 mg of L-methionine S-methyl-^{13}C (90% enrichment) in 56 ml of H_2O was added in 7 portions to four fermentation flasks each containing 50 ml of fermentation medium. The additions spanned 72 hours, beginning on the second day of the 7-day fermentation. The crude product, 127 mg of oil, was chromatographed on a 60 g column of silica gel packed in and eluted with 97:3 v/v $CHCl_3$:MeOH to give 91 mg of colorless oil, which afforded 64 mg of crystals from benzene-hexane, m.p. 114-115° C; single component identical to a reference sample of cycloheximide by TLC (95:5 v/v $CHCl_3$:MeOH, Rf 0.39); mass spectral data shown in Scheme 1 and Table 1; carbon-13 NMR in C^2HCl_3 (TMS): enrichment at 18.88 ppm (16-$^{13}CH_3$) and 14.17 ppm (15-$^{13}CH_3$); proton NMR in C^2HCl_3 (TMS): 0.99δ (d, $\lesssim$1.5H, J = 7 Hz, 16-$^{12}CH_3$), 0.36 and 1.62δ (d, $\lesssim$1.5H, J_{H-H} = 7 Hz, $J_{H-^{13}C}$ = 126 Hz, 16-$^{13}CH_3$), 1.25δ (d, $\lesssim$1.5H, J_{H-H} = 7 Hz, 15-$^{12}CH_3$), 0.62 and 1.88δ (d, $\lesssim$1.5H, J_{H-H} = 7 Hz, $J_{H-^{13}C}$ = 126 Hz, 15-$^{13}CH_3$), ∿45% carbon-13 labeled in both methyl groups; *anal.*-C, H, N.

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