perature, the stirred reaction mixture was treated dropwise with 170 mg (0.833 mmol) of **12** dissolved in 8 mL of tetrahydrofuran. After an additional hour, phenylselenyl chloride (0.20 g, 1.093 mmol) in 5 mL of tetrahydrofuran was introduced in dropwise fashion. Stirring was maintained at −78 °C for 3 h prior to warming to room temperature. Following the addition of saturated ammonium chloride solution, the product was extracted into dichloromethane and the combined organic layers were washed with water, dried, and evaporated. The product was purified by preparative layer chromatography on silica gel (hexane–ether, 95:5). There were obtained 188 mg (88.3%) of the α-phenylseleno ketone and 49 mg of unreacted **12**.

A solution of 85% *m*-chloroperbenzoic acid (121 mg, 0.7 mmol) in dichloromethane (18 mL) was added to a solution of the α-phenylseleno ketone (187.5 mg) in 10 mL of the same solvent at −78 °C under nitrogen. The reaction mixture was stirred at −78 °C for 2 h, allowed to warm to −5 °C, and treated with 0.34 mL of triethylamine. After 1.5 h, room temperature was attained and a yellow solution was observed. This solution was transferred into 30 mL of hexane and heated at the reflux temperature for 2.5 h. Dichloromethane (100 mL) was added and this solution was washed with saturated sodium bicarbonate and brine solutions. The organic phase was dried and evaporated, and the crude product was purified by TLC on silica gel. There was obtained 95 mg of **13**: IR (neat, $cm^{-1}$) 2960, 2870, 1700, 1590, 1448; $^1H$ NMR ($CDCl_3$, δ) 7.31 (d, $J$ = 6 Hz, 1 H), 5.94 (d, $J$ = 6 Hz, 1 h), 4.93 (m, 1 H), 1.70 (d, $J$ = 2 Hz, 3 H), 1.63 (br m, 6 H), 1.10 (s, 3 H), 0.97 (s, 3 H); *m/e* calcd 202.1358, obsd 202.1361.

Anal. Calcd for $C_{14}H_{18}O$: C, 83.12; H, 8.97. Found: C, 82.90; H, 8.98.

**1,3,4,7-Tetramethyltricyclo[6.3.0.0$^{4,8}$]undec-2-en-5-one (14).** Lithium dimethylcuprate was prepared by adding slightly more than 2 equiv of methyllithium to 438 mg of cuprous iodide slurried in 1 mL of anhydrous ether. Subsequent to cooling to −20 °C, a solution of **13** (90 mg) of 4 mL of anhydrous ether was added dropwise and the mixture was stirred for 1 h at −20 °C and for 2 h at 0 °C. The usual workup afforded 122 mg of crude product which was used directly for the next step. For **14**: IR (neat, $cm^{-1}$) 2950, 2880, 1735, 1450, 845; $^1H$ NMR ($CDCl_3$, δ) 5.03 (br s, $W_{1/2}$ = 3 Hz, 1 H), 2.75 (q, $J$ = 17 Hz, 1 H), 2.27 (q, $J$ = 17 Hz, 1 H), 1.55 (d, $J$= 7 Hz, 3 H), 1.40 (br m, 7 H), 1.18 (s, 3 H), 1.10 (s, 3 H), 0.87 (d, $J$ = 7 H , 3 H); *m/e* calcd 218.1671, obsd 218.1674.

**(±)-Isocomene (1).** A mixture of unpurified **14** (122 mg), potassium carbonate (252 mg), hydrazine hydrate (0.23 mL), and triethylene glycol (2 mL) was heated at reflux for 1.5 h. A small distillation head was placed atop the flask in place of the condenser and the pot temperature was increased to 200 °C. After 1.5 h, the collected distillate was taken up in ether and washed with 10% hydrochloric acid. The pot residue was heated at 250 °C for 3 h, cooled, diluted with water, and extracted with ether. The combined ether phases were washed with 10% hydrochloric acid, water, and brine prior to drying and concentration. Dissolution in pentane followed by passage through a short silica gel column and VPC purification (2 ft × 0.25 in. 5% SE-30 on Chromosorb W, 100 °C) gave 70 mg (77%) of isocomene whose IR and $^1H$ NMR spectra were identical with those of the natural product. On standing, the colorless oil slowly crystallized: mp 59–62 °C; *m/e* calcd 204.1878, obsd 204.1882.

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# Stereochemical Studies of Isoprenoid Biosynthesis. Biosynthesis of Pentalenolactone from [U-$^{13}C_6$]Glucose and [6-$^2H_2$]Glucose

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*Contribution from the Department of Chemistry, Brown University, Providence, Rhode Island 02912.* *Received September 12, 1980*

**Abstract:** The biosynthesis of pentalenolactone (**1**) from mevalonate has been established by feeding [U-$^{13}C_6$]glucose to cultures of *Streptomyces* UC5319. The uniformly labeled glucose serves as an in vivo precursor of [1,2-$^{13}C_2$]acetyl-CoA. The $^{13}C$ NMR spectrum of the derived pentalenic acid (**5**) and pentalenolactone methyl esters show a pattern of enhancements and couplings consistent with a biosynthetic pathway involving cyclization of farnesyl pyrophosphate to humulene followed by further cyclization to a tricyclic intermediate pentalenene (**7**) and subsequent oxidative cleavage and rearrangment. The results of feeding [6-$^2$H]glucose, a potential precursor of [2-$^2H_2$]acetyl-CoA, were less clear-cut with label derived from both D-3′ and D-5 of mevalonate appearing in the methyl esters of **1**, **5**, and **6**. A stereochemical analysis establishes that the cyclization of farnesyl pyrophosphate involves electrophilic attack on the *si* face of the 10,11 double bond and suggests that humulene formation and cyclization may take place at the same active site.

The sesquiterpene *Streptomyces* antibiotic pentalenolactone (**1**) was isolated in 1970 by an Upjohn group as part of a screening program for antimetabolites having potential antitumor activity.[2] The same substance was independently isolated by Takeuchi et al. while screening for substances inhibitory against nucleic acid synthesis in bacterial cells.[3] The acidic lipophilic antibiotic proved to be identical with PA 132, first isolated by researchers at Chas. Pfizer and shown to be active against a variety of microorganisms including gram-negative and gram-positive bacteria, pathogenic and saprophytic fungi, and protozoan species.[4] Most recently pentalenolactone was also shown to be identical with arenaemycin E and found to block glycolysis in target organisms by selective inhibition of glyceraldehyde-3-phosphate dehydrogenase.[5]

The structure and absolute configuration of pentalenolactone were firmly established by X-ray diffracton analysis of the bromohydrin derived from tetrahydropentalenolactone[2] and corre-

(1) Fellow of the Alfred P. Sloan Foundation, 1978–1982. National Institutes of Health Research Career Development Award, 1978–1983.

(2) D. G. Martin, G. Slomp, S. Mizsak, D. J. Duchamp, C. G. Chidester, *Tetrahedron Lett.*, 4901 (1970); D. J. Duchamp and C. G. Chidester, *Acta Crystallogr., Sect. B* **B28**, 173 (1972).

(3) S. Takeuchi, Y. Ogawa, and H. Yonehara, *Tetrahedron Lett.*, 2737 (1969).

(4) B. K. Koe, B. A. Sobin, and W. D. Celmer, *Antibiot. Annu.*, 672 (1957).

(5) S. Hartmann, J. Neeff, U. Heer, and D. Mieke, *FEBS Lett.*, **93** 339 (1978); W. Keller-Schierlein, J. Lemke, R. Nyfeler, and H. Zaehner, *Arch. Microbiol.*, **84**, 301 (1972).

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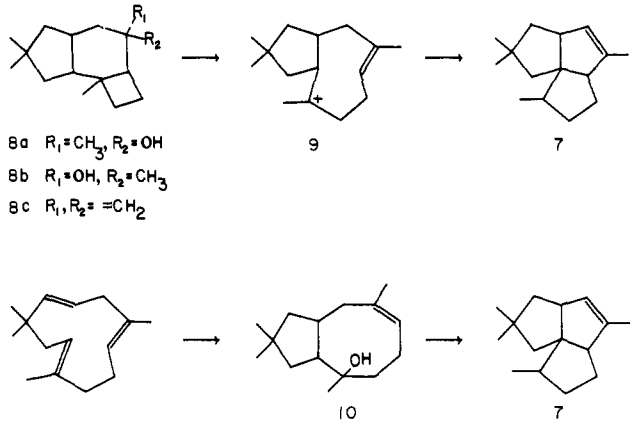
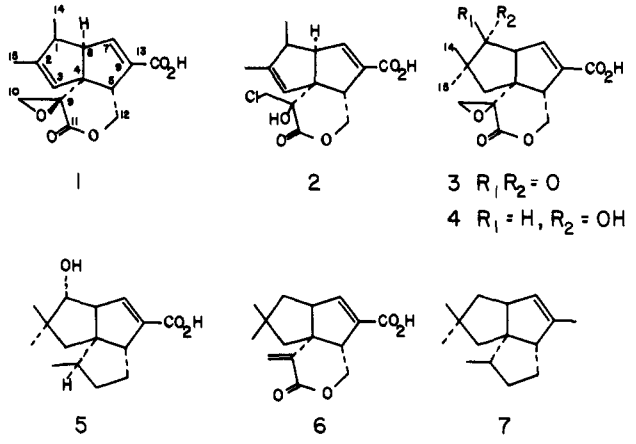
Scheme I




lation with NMR and IR spectroscopic data.[2,3] In the last 2 years two total syntheses of **1** have been reported.[6,7] In the meantime




screening of extracts of pentalenolactone-producing cultures has led to the isolation, as the corresponding methyl esters, of a series of structurally related metabolites, including the chlorohydrin (**2**) (AA-57, arenaemycin C),[5,8] pentalenolactones G (**3**)[9] and H (**4**),[10] and the tricyclic sesquiterpene pentalenic acid (**5**).[10] We have reported the isolation of pentalenolactone E (**6**)[11] while Seto has recently isolated the parent hydrocarbon, pentalenene (**7**).[12] Each of these substances is a potential intermediate or shunt metabolite of the biosynthesis of pentalenolactone itself. In parallel to the above investigations have been the extensive studies of Matsumoto and Shirahama and their collaborators on biogenetically modeled cyclizations of humulene and derived protoilludyl cations. Of particular interest to us was the finding that treatment of compounds **8a–c** in refluxing formic acid gave a 28% yield of pentalenene, presumably by way of the bicyclic cation **9**. **7** could also be synthesized by formolysis of **10**, obtained by $Hg(OAc)_2$ or $Hg(NO_3)_2$ cyclization of humulene and hydrogenolysis of the resultant 3,7- or 3,6-epoxy products (Scheme I).

## Results

Our initial biosynthetic investigations focussed on attempts to incorporate several potential isoprenoid precursors, including

Table I. $^{13}C$ NMR of Labeled Metabolites

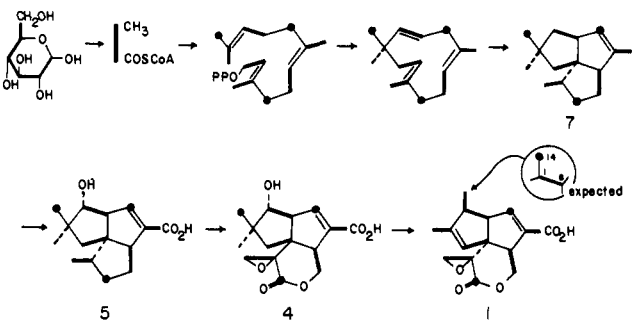
| | pentalenic acid methyl ester (5-Me)[a] | | pentalenolactone methyl ester (1-Me)[b] | |
|---|---|---|---|---|
| carbon | $\delta_C$[c] | $J_{CC}$,[d] Hz | $\delta_C$[c] | $J_{CC}$,[d] Hz |
| 1 | 85.9 (d)[e] | 37 | 44.5 (d) | 36 (d), 36 (t)[f] |
| 8 | 67.5 (d) | 37 | 56.7 (d) | 36 |
| 14 | 27.1 (q) | s | 15.5 (q) | 35 |
| 2 | 43.1 (s) | 35 | 147.9 (s) | 44 |
| 15 | 21.6 (q) | 36 | 14.6 (q) | 46 |
| 3 | 45.3 (t)[g] | 33 | 122.3 (d) | 43 |
| 4 | 60.4 (s) | 35 | 59.2 (s) | 44 |
| 9 | 44.6 (d) | 35 | 59.1 (s) | 32 |
| 10 | 17.0 (q) | 35 | 47.1 (t) | 33 |
| 11 | 32.8 (t)[g] | s | 169.4 (s) | s |
| 12 | 28.6 (t) | 33 | 67.7 (t) | 34 |
| 5 | 57.8 (d) | 35 | 51.0 (d) | 37 |
| 6 | 138.2 (s) | 76 | 133.4 (s) | 75 |
| 13 | 165.7 (s) | 74 | 164.3 (s) | 76 |
| 7 | 144.4 (d) | s | 146.1 (d) | s |
| 16 | 51.3 (q) | s | 51.8 (q) | s |

[a] Bruker HX-270, 67.86 MHz; spectral width 15 150 Hz, 8K data points, quadrature detection, 40° pulse, 4.0-s pulse delay, 16 480 transients, 13.1 mg in 0.5 mL of $CDCl_3$. [b] Bruker WP-60, 15.08 MHz; spectral width 4000 Hz, 4K data points, quadrature detection, 40° pulse, 4.0-s pulse delay, 44 288 transients, 18.5 mg in 0.5 mL of $CDCl_3$. The observed signal enhancements, calculated by comparing the normalized total area of each set of signals with that of the natural abundance methyl ester, ranged from 1.5–2.5% (average enrichment 1.9 ± 0.3%) in reasonable agreement with the value calculated from the measured $^{14}C$ activity, assuming two [$^{13}C$]acetates per glucose. [c] $Me_4Si$ = 0.00 ppm. [d] Observed coupling (±1 Hz) for satellite doublets for $^{13}C$-enriched sample. [e] Multiplicity in SFORD spectrum: s = singlet, d = doublet, t = triplet, q = quartet. [f] Area t/d ≃ 2. [g] Assignments of ref 10 reversed. New assignment confirmed by simultaneous broad-band proton, homonuclear $^{13}C$ decoupling on a Bruker WM 250 at 62.83 MHz. Irradiation of C-4 caused the 33-Hz doublet at 45.3 ppm to collapse to a singlet, while the signal at 32.8 Hz was unaffected.

[2-$^{14}C$]acetate, [2-$^{14}C$] mevalonate, [2-$^{14}C$,5-$^{3}H_2$]mevalonate, and benzyl [2-$^{14}C$]mevalonate. In spite of the use of a variety of feeding regimens and two different strains of *Streptomyces*,[14] our efforts were frustrated by the refusal of the organism to incorporate any of these labeled substrates. Although cyclized isoprenoids are themselves rare among bacterial metabolites[15] and failure of bacterial cultures to take up exogeneous mevalonate is apparently not uncommon,[16] we surmised that we were faced with overcoming permeability or compartmentation difficulties. As a tactic for overcoming such obstacles we therefore turned to the use of uniformly $^{13}C$-labeled [U-$^{13}C_6$]glucose.[17] Such a precursor has a number of attactive features, including the ability to penetrate the cell wall and act as an in vivo precursor of [1,2-$^{13}C_2$]-acetyl-CoA, an intermediate which has been a particularly powerful tool for the study of isoprenoid and polyketide biosynthetic pathways.[19]

(6) S. Danishefsky, M. Hirama, K. Gombatz, T. Harayama, E. Berman, and P. Schuda, *J. Am. Chem. Soc.*, **100**, 6536 (1978); **101**, 7020 (1979).
(7) W. H. Parsons, R. H. Schlessinger, and M. L. Quesada, *J. Am. Chem. Soc.*, **102**, 889 (1980).
(8) S. Aizawa, H. Akutsu, T. Satomi, S. Kawabata, and K. Sasaki, *J. Antibiot.*, **31**, 729 (1978).
(9) H. Seto, T. Sasaki, H. Yonehara, and J. Uzawa, *Tetrahedron Lett.*, 923 (1978).
(10) H. Seto, T. Sasaki, J. Uzawa, S. Takeuchi, and H. Yonehara, *Tetrahedron Lett.*, 4411 (1978).
(11) D. E. Cane and T. Rossi, *Tetrahedron Lett.*, 2973 (1979).
(12) H. Seto and H. Yonehara, *J. Antibiot.*, **33**, 92 (1980).
(13) Y. Ohfune, H. Shirahama, and T. Mastumoto, *Tetrahedron Lett.*, 2869 (1976); S. Misumi, T. Ohtsuka, Y. Ohfune, K. Susita, H. Shirahama, and T. Mastumoto, *ibid.*, 31 (1979).
(14) Strains of *S. roseogriseus*, ATCC 12414, failed to produce pentalenolactone by using a variety of media. Satisfactory production was achieved by using *Streptomyces* UC5319 obtained from the Upjohn Co.
(15) M. N. Gerber, *Phytochem.*, **11**, 385 (1972).
(16) Cf. F. W. Hemming In "Biochemistry of Lipids", Vol. 4, Biochemistry Series One, T. W. Goodwin, Ed., University Park Press, Baltimore, 1974, pp 50, 57. Exogeneous acetate and mevalonate act as efficient precursors of both triterpenes and phytosterols but fail to label the monoterpene glucosides loganin and foliamenthin in the higher plant *Menyanthes trifoliata*: Cf. D. Arigoni, *Pure Appl. Chem.*, **17**, 331 (168).
(17) Although [U-$^{13}C_6$]glucose has recently been used by White in an investigation of thiamine biosynthesis in which the distribution of label was examined by mass spectrometery,[18] the present study is the first use of this substrate in connection with multiple-label $^{13}C$ NMR. Very recently, in collaboration with Professor Steven Gould of the University of Connecticut, School of Pharmacy, we have used [U-$^{13}C_6$]glucose to study shikimate metabolism in the biosynthesis of the antibiotic streptonigrin. The results of this study will be reported separately.
(18) R. H. White, *Biochemistry*, **17**, 3833 (1978).

Scheme II



Preliminary incorporation experiments using [6-$^{14}$C]glucose did indeed confirm that satisfactory enrichments could be achieved with this precursor although several attempts at using replacement cultures and defined media resulted in reduced metabolite production without a sufficiently compensating increase in isotope enrichment. For the actual biosynthetic experiment, in order to minimize extraneous couplings due to excess intramolecular labeling,[20] the precursor [U-$^{13}C_6$]glucose (1.35 g, 85 atom % $^{13}$C) was mixed with unlabeled glucose (1.35 g) along with $1.74 \times 10^8$ dpm of [6-$^{14}$C]glucose as internal standard and fed to 2.4 L of *Streptomyces* UC5319 which had been grown in a modified medium for 30 h at 28 °C. After an additional 42 h the resulting pentalenolactone was isolated and rigorously purified as the methyl ester by PLC to separate it from pentalenolactone E. A total of 18.5 mg of **1**-Me was obtained whose specific activity ($2.78 \times 10^6$ dpm/mmol) corresponded to a calculated $^{13}$C enrichment of 2.2% per labeled site. Further purification of the extracts by high-pressure LC on μ-Bondapak CN led to isolation of 13.6 mg of pentalenic acid methyl ester (4.0% $^{13}$C/labeled site).

The $^{13}$C NMR spectrum of the biosynthetically labeled pentalenic acid methyl ester is completely consistent with a mevalonoid biosynthetic pathway, illustrated in Scheme II. As summarized in Table I, carbons 7, 11, and 14 each give rise to enhanced singlets, as predicted for carbons derived from C-2 of mevalonate which loses its paired acetate atom from C-1 of mevalonate in the formation of isopentenyl pyrophosphate.[19] The remaining carbon atoms of the sesquiterpene skeleton appear as six pairs of enhanced and coupled doublets. The $^{13}$C NMR assignments are those of Seto et al.[10] except that the assignments for C-3 and C-11 have been interchanged based on the observed coupling of 33 Hz between C-3 and 4 in the biosynthetically labeled sample. Because of the importance of these latter assignments to the interpretation of the biosynthetic results, $^{13}$C–$^{13}$C homonuclear decoupling of C-3 and 4 was used to confirm directly the assigned coupling. Well-precedented chemical shift parameters[21] were used to assign the upfield methyl resonance at 21.6 ppm to C-15, cis to the 1α-hydroxyl function, with the signal at 27.1 ppm being assigned to C-14.

Complete $^{13}$C NMR assignments for pentalenolactone methyl ester have been reported by Takeuchi.[22] We have independently confirmed these assignments by single-freuqency off-resonance decoupling and correlation with the $^1$H NMR spectrum by the method of Birdsall.[23] These assignments are further corroborated by the observed $^{13}$C–$^{13}$C couplings listed in Table I.

Pentalenolactone most probably is formed from pentalenic acid by oxidative cleavage of the 11,12 bond of ring C and Wagner–

Table II. Calculated Enrichments: Incorporation of D-[6,6-$^2$H,6-$^{14}$C]Glucose[a]

| substrate | % inc | dpm/mmol | % enrichment/ labeled site[b] |
|---|---|---|---|
| 5-Me | 0.12 | $7.51 \times 10^5$ | 6.5 |
| 1-Me | 0.05 | $7.51 \times 10^5$ | 6.5 |
| 6-Me | 0.04 | $4.87 \times 10^5$ | 4.2 |

[a] Specific activity $1.27 \times 10^6$ dpm/mmol. [b] (Specific activity of substrate/$1.27 \times 10^6$)/9; assumes one labeled acetyl-CoA per glucose and nine acetates per sesquiterpene.

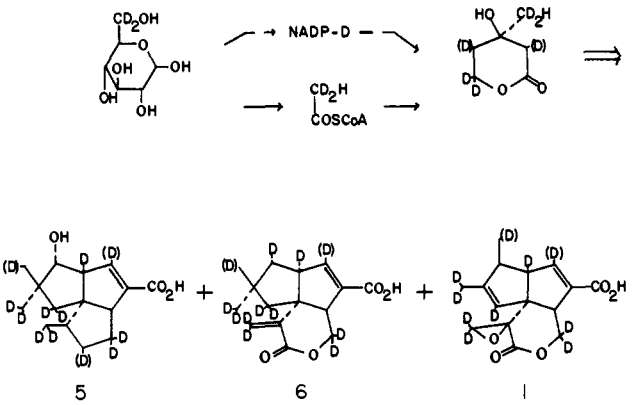


Figure 1. Apparent sites of deuterium labeling.

Meerwein rearrangment of the β-C-14 methyl carbon from C-2 to C-1. The $^{13}$C NMR spectrum of the biosynthetically labeled pentalenolactone methyl ester is consistent with such a proposal while exhibiting one unexpected feature. Carbons 7 and 11 each give rise to enhanced singlets, as expected, while the signals for C-6 and -13, C-5 and -12, C-9 and -10, and C-3 and -4 again appear as enhanced and coupled doublets. The paired doublets corresponding to C-2 and C-15 suggest that the allylic methyl of pentalenolactone is derived from C-15 (α) of pentalenic acid and therefore ultimately from C-3′ of mevalonate. On the other hand, C-14, which in pentalenic acid appears as a singlet, is now coupled to C-1 which appears as a triplet, $J$ = 36 Hz, and a doublet, J = 36 Hz, each centered on the enhanced natural abundance singlet. Carbon-1 therefore is coupled not only to C-8, as expected, but also to C-14, which has presumably migrated from C-2 at some stage. The triplet corresponds to those species in which C-14, C-1, and C-8 are all labeled while the doublet arises from molecules in which only the C-1, C-8 or C-1, and C-14 pairs are labeled. Although we have no really satisfactory explanation for the extra coupling, it is evident that the 14-methyl carbon of pentalenolactone (and presumably of pentalenic acid as well) must be derived from the same glucose molecule as the pair of carbons to which it has become attached, since no other extraneous couplings are evident in the remainder of the spectrum. While the addition of unlabeled glucose to the precursor can prevent two acetates from different glucose precursors from being labeled in the same molecule of product, there is no way a priori to prevent two acetates born from the same glucose from finding each other if there is little or no dilution by an endogeneous pool of triose phosphates at the site of glycolysis and mevalonate synthesis. Although there have been several independent reports of multienzyme glycolytic complexes and compartmentalization effects,[24] the precise origin of the observed extra couplings remains speculative. An attempt to demonstrate multiple labeling in pentalenic acid itself by refeeding $^{13}$C-labeled **5** and its methyl ester was unsuccessful due to failure of the organism to incorporate added pentalenate.

Having successfully used [U-$^{13}C_6$]glucose as a precursor of [1,2-$^{13}C_2$]acetyl-CoA, we also explored the use of [6,6-$^2H_2$] glucose

(19) M. Tanabe and K. Suzuki, *J. Chem. Soc., Chem. Commun.*, 445 (1974); A. G. McInnes, D. G. Smith, J. A. Walter, L. C. Vining, and J. L. C. Wright, *ibid.*, 282 (1974); H. Seto, T. Sato, and H. Yonehara, *J. Am. Chem. Soc.*, **95**, 8461 (1973).

(20) Cf. T. J. Simpson and J. S. E. Holker, *Phytochem.*, **16**, 229 (1977).

(21) (a) J. B. Stothers and C. T. Tan, *Can. J. Chem.*, **52**, 308 (1974); M. Christl, H. J. Reich, and J. D. Roberts, *J. Am. Chem. Soc.*, **93**, 3465 (1971). (b) G. C. Levy and G. L. Nelson, "Carbon-13 Nuclear Magnetic Resonance for Organic Chemists", Wiley-Interscience, New York, 1972.

(22) S. Takeuchi, J. Uzawa, H. Seto, and H. Yonehara, *Tetrahedron Lett.* 2943 (1977).

(23) B. Birdsall, N. J. M. Birdsall, and J. Feeney, *Chem. Commun.*, 316 (1972).

(24) J. Mowbray and V. Moses, *Eur. J. Biochem.*, **66**, 25 (1976); D. M. Gorringe and V. Moses, *Biochem. Soc. Trans.*, **6**, 167 (1978); F. R. Opperdoes and P. Borst, *FEBS Lett.*, **80**, 360 (1977).

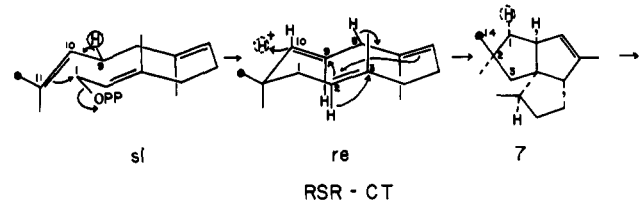
Table III. $^2H$ NMR Spectra of Labeled Metabolites[a]

| 5-Me[b] | | | 1-Me[c] | | | 6-Me[d] | | |
|---|---|---|---|---|---|---|---|---|
| $\delta_D$ | assignt | $\delta_H$[10] | $\delta_D$ | assignt | $\delta_H$[3] | $\delta_D$ | assignt | $\delta_H$ |
| 0.99 | D-10, -14, -15 | 0.93, 0.95 | (1.06)[e] | D-14 | 1.05 | 1.01 | D-14, -15 | 1.06 |
| 1.43 | D-3a, -12a | 1.40 | 1.66 | D-15 | 1.65 | 1.71 | D-3b | 1.74 |
| 1.97 | D-3b, -12b | 1.98, 2.0 | 2.82[f] | D-10a, -1 | 2.60, 2.81 | 1.90 | D-1$\alpha$ | 1.89 |
| 2.72 | D-8 | 2.73 | 3.27[f] | D-10b, -5 | 3.10 | 2.13 | D-3a | 2.16 |
| (6.87)[e] | D-7 | 6.83 | 3.43[f] | D-8 | 3.35 | 3.29 | D-5,[g] -8 | 3.29 |
| | | | 4.69 | D-12 | 4.55 | 4.30 | D-12 | 4.31 |
| | | | 5.15 | D-3 | 5.05 | 5.60 | D-10a | 5.57 |
| | | | (6.87)[e] | D-7 | 6.65 | 5.93 | D-10b | 5.91 |
| | | | | | | (6.90)[e] | D-7 | 6.85 |

[a] Bruker HX 270, 41.44 MHz; 4K data points, quadrature detection, 90° pulse. [b] Spectral width 2000 Hz, 11 173 transients, 15.5 mg in 0.5 mL of $CHCl_3$. [c] Spectral width 1000 Hz, 1.0-s pulse delay, 35 000 transients, 7.5 mg in 0.5 mL of $CHCl_3$. [d] Spectral width 1000 Hz, 55 000 transients, 9.6 mg in 0.5 mL of $CHCl_3$. [e] Very weak signal, <10% of next strongest signal. [f] Overlapping signals. Preferred assignment: D-10a, -10b, and -8, respectively. [g] Preferred assignment: D-5.

as an in vivo precursor of [2,2-$^2H_2$]acetyl-CoA. Such an intermediate would be expected to label C-2, -3′, and -4 of mevalonic acid resulting in deuterium enrichment at the corresponding positions of derived metabolites. [6,6-$^2H_2$]Glucose was chosen in preference to the [1-$^2H$]isomer since the latter can serve as an in vivo source of [4-$^2H$]NADPH by the action of glucose-6-phosphate dehydrogenase. In the event, feeding of 3.0 g of [6,6-$^2H_2$]glucose containing $4.0 \times 10^7$ dpm of [6-$^{14}C$]glucose as internal standard to 2.4 L of *Streptomyces* UC5319 culture did give rise to deuterated samples of pentalenic acid (15.5 mg), pentalenolactone (8.3 mg), and pentalenolactone E (9.6 mg), isolated and purified as their respective methyl esters. The calculated enrichments, based on the $^{14}C$ specific activities of each sample, are indicated in Table II. The corresponding 41.44-MHz $^2H$ NMR spectra, however, were much less clear-cut than anticipated.[25] Although unambiguous interpretations of several parts of the spectra were rendered difficult by limited signal resolution, two features of the spectra were immediately apparent (Figure 1). (1) While positions derived from C-3′ of mevalonate are labeled as expected, it appears that little or no deuterium from C-2 or C-4 of mevalonate is present in any of the derived metabolites. For example, while D-15 of pentalenolactone, derived from C-3′ of mevalonate, gives rise to a strong signal at 1.66 ppm, the intensity of the corresponding signal for D-14 at 1.06 ppm is at most 5% of that for D-15. Similarly, whereas D-10a and -b of pentalenolactone E are evident at 5.60 and 5.93 ppm, the corresponding signal for D-7 is extremely weak as it is also in both **1**-Me and **5**-Me. In the spectrum of pentalenic acid, no signal is apparent at 3.0 ppm corresponding to D-5, a position derived from C-4 of mevalonate. Although successful incorporations of acetate-$d_3$ into both polyketides[26] and isoprenoids[27] have been reported, unexpected loss of deuterium, possibly due to combinations of isotope and exchange effects, are not without precedent.[28] Nonetheless, the absence of signficant labeling at positions derived from C-2 and C-4 of mevalonate severely limits the usefulness of [6,6-$^2H_2$] glucose as an isoprenoid precursor and hinders the routine interpretation of derived spectra. (2) All of the biosynthetically deuterated samples are deuterium labeled at positions derived from C-5 of mevalonate. For example D-3 and D-12 of pentalenolactone give rise to strong signals (5.15 and 4.69 ppm, respectively) and as do the analogous positions of pentalenolactone E (1.71, 2.08, and 4.21 ppm). The corresponding protons ultimately come from the NADPH pool. Although unanticipated, this result is readily explained by Shulman's $^{13}C$ NMR experiments which establish that in both yeast and *E. coli* C-1 and C-6 of hexose substrates are rapidly equilibrated compared to the overall glycolytic flux.[29] Such a scrambling mechanism is apparently operating in *Streptomyces* UC5319, again restricting the general utility of [6,6-$^2H_2$]glucose as a mevalonoid precursor.

Scheme III




In spite of the above-described ambiguities, one feature of these $^2H$ NMR spectra was of particular interest. In the spectrum of pentalenolactone E, a signal at 1.85 ppm can be assigned to D-1. A corresponding signal for D-1 of pentalenic acid, however, which would be expected to appear at 3.42 ppm, is clearly absent, suggesting that the deuteron which has been replaced occupied the 1$\alpha$-position, assuming that oxidation has taken place with retention of configuration, as usually observed.[30] The D-1$\alpha$ assignment for pentalenolactone E is in fact supported by analysis of the corresponding 270-MHz proton NMR spectrum, in which the C-1 protons appear as the AB portion of an ABX system, centered at $\delta_A$ 1.89 and $\delta_B$ 1.44, $J_{AB}$ = 12.8 Hz, $J_{AX}$ = 9.2 Hz, and $J_{BX}$ = 6.2 Hz. On the basis of the observed coupling constants the downfield signal can be assigned to H-1$\alpha$, cis to H-8.[31] For obvious reasons, it is impossible to deduce the origin of D-1$\alpha$ in pentalenolactone E unambiguously. Although the corresponding carbon is clearly derived from C-4 of mevalonate, the absence of significant label from D-4 of mevalonate at other positions such as C-5 in **1**, **5**, and **6** does suggest that D-1$\alpha$ may well be derived from D-5 of mevalonate. The stereochemical significance of this possibility is discussed in more detail below.

## Discussion

From the above results pentalenolactone appears to be biogenetically related to the important class of humulene-derived dimethylcyclopentane sesquiterpenes which includes fomannosin,[32] illudins,[33] marasmic acid,[34] hirsutic acid,[35] the coriolins,[36] and

(25) For a review of the use of $^2H$ NMR in biosynthetic studies see M. J. Garson and J. Staunton, *Chem. Soc. Rev.*, **8**, 539 (1979).

(26) Y. Sato, T. Oda, and H. Saito, *Tetrahedron Lett.*, 2695 (1976); M. J. Garson, R. A. Hill, and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 624, 921 (1977).

(27) A. P. W. Bradshaw and J. R. Hanson, *J. Chem. Soc., Chem. Commun.*, 924 (1979).

(28) U. Sankawa, H. Shimada, T. Sato, T. Kinoshita, and K. Yamasaki, *Tetrahedron Lett.*, 483 (1977); U. Sankawa, H. Shimada, and K. Yamasaki, *ibid.*, 3375 (1978).

(29) K. Ugurbil, T. R. Brown, J. A. den Hollander, P. Glynn, and R. G. Shulman, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3742 (1978); J. A. den Hollander, T. R. Brown, K. Ugurbil, and R. G. Shulman, *ibid.*, **76**, 6096 (1979). For an example of labeling of the NADPH pool by acetate-$d_3$ see R. H. White, *Biochemistry*, **19**, 9 (1980).

(30) O. Hayaishi, "Oxygenases"; Academic Press, New York, 1962.

(31) Cf. L. M. Jackman and S. Sternhell, "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry", 2d ed., Pergamon Press, New York, 1969, pp 286–298 and reference cited. S. Sternhell, *Q. Rev., Chem. Soc.* **23**, 236 (1969). The assignment is also consistent with data from the following model compounds: **1**-Me, $J(\text{cis})_{1,8}$ = 9.0 Hz[3]; 4-Me, $J(\text{trans})_{1,8}$ = 5.3 Hz;[10] 1-*epi*-**4**-Me, $J(\text{cis})_{1,8}$ = 7.3 Hz;[10] **5**-Me, $J(\text{trans})_{1,8}$ = 5.5 Hz.[10]

(32) D. E. Cane and R. B. Nachbar, *J. Am. Chem. Soc.*, **100**, 3208 (1978).

(33) J. R. Hanson, T. Marten, and R. Nyfeler, *J. Chem. Soc., Perkin Trans. 1*, 876 (1976).

(34) J. J. Dugan, P. de Mayo, M. Nisbet, J. R. Robinson, and M. Anchel, *J. Am. Chem. Soc.*, **88**, 2838 (1966).

botrydial.[27,37] In connection with our own work on the biosynthesis of fomannosin we recently presented a detailed stereochemical analysis of the farnesyl pyrophosphate cyclizations leading to this family of sesquiterpenes and noted a possible correlation between the stereochemistry of these cyclizations and the absolute configuration of the eventual sesquiterpene metabolites.[32] Subsequently this analysis has been extended by Shirahama to a detailed consideration of the conformations of the postulated humulene intermediates.[38] By applying a similar analysis to the biosynthesis of pentalenolactone, it can be seen that the 4*R*, 5*S*, 8*S*, 9*R* configuration of pentalenene[39] is correlated with the *RSR*-CT conformation of humulene.[40] Furthermore since our $^{13}C$ NMR results have established that the C-14 ($\beta$) methyl of pentalenic acid is derived from C-2 of mevalonate, the corresponding *pro-S*-methyl at C-11 of humulene must also be derived from C-2 of mevalonate. Cyclization of the farnesyl pyrophosphate precursor must therefore have occurred exclusively by attack on the *si* face of the distal double bond by the developing allylic cation. It will be noted that this is the same stereochemical course as that previously deduced for fomannosin and illudin biosynthesis and that once again the stereochemistry of the farnesyl pyrophosphate cyclization appears to be correlated with the absolute configuration of the ultimate humulene cyclization product.[32] One obvious interpretation of this correlation is the possibility that humulene may be both formed and further cyclized by the same enzyme at a single active site. Further insight into this question comes from analysis of the $^2H$ NMR of biosynthetically deuterated pentalenolactone E. As noted above the signal at 1.85 ppm assigned to D-1$\alpha$ may well be derived via D-5 of mevalonate. For cyclization of *RSR*-CT humulene, protonation (deuteration) can only occur on the 10-*re* face of the 9,10 double bond, resulting in a deuterium atom at D-1$\alpha$ of pentalenene, as observed. In principle this deuterium could come from either attack of $D^+$ on the trans-9,10 double bond of humulene or by 9,10 $D^-$ migration within an intially formed 9-humulyl cation. Although the present experiment cannot distinguish between these two possibilities, we favor the former mechanism, since our previous $^2H$ NMR investigations of the biosynthesis of fomannosin, itself formed from *RSR*-CT humulene, clearly ruled out a quantitative transfer of hydride but were insufficiently sensitive to detect as little as 10% internal return of a proton.[41] Either mechanism, however, would be consistent only with formation and cyclization of humulene at the same active site.

Although further experimentation at the cell-free level, currently in progress, will be necessary to substantiate these conclusions, a tentative model for humulene formation and cyclization can be proposed. According to this hypothesis, farnesyl pyrophosphate, folded in a *proto-RSR*-CT conformation, undergoes ionization to an initial allylic cation–pyrophosphate anion pair.[42] Attack on the *si* face of the distal double bond generates a 9-humulyl cation which can be quenched by a basic group on the enzyme. The resulting conjugate acid may undergo varying degrees of proton exchange with the medium before redonating a proton to C-10 of humulene, thereby initiating cyclization and formation of the dimethylcyclopentane ring. The resultant 3-cation, it will be noted, can in principle be stabilzed by the paired pyrophosphate ion, by either covalent bonding or simple ion pairing. 2,3-Hydride migration generates the 2-cation, again stabilized by the pyrophosphate ion, which attacks the 6,7 double bond. The resultant 7-cation is then quenched by removal of H-8 by the original enzyme base. In this model the pyrophosphate ion is able to stabilize positive charge at either C-1, -2, or -3, while the enzyme base is responsible for deprotonation–reprotonation of C-8, -9, and -10 (Figure 2). Similar models can, in fact, be constructed for the formation of protoilludanes and other dimethylcyclopentane sesquiterpenes. As to the possibility that the pyrophosphate ion does not simply depart following initial ionization, we note the recent report by Croteau that an enzyme from Sage catalyzes the conversion of geranyl pyrophosphate to bornyl *pyrophosphate*.[43] Whether participation of pyrophosphate in the overall cyclization process is in fact a general phenomenon must await the results of future study. At the moment, however, a detailed stereochemical picture of the biosynthesis of an important class of sesquiterpenes has already emerged.

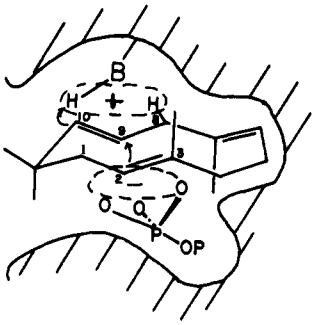



**Figure 2.** Proposed model for humulene formation and cyclization at a single active site.

## Experimental Section

**Instrumentation.** Proton and $^{13}C$ NMR spectra were obtained on Bruker WP 60 (60 and 15.08 MHz) and HX 270 (67.86 MHz) FT NMR spectrometers. Homonuclear $^{13}C$–$^{13}C$ decoupling experiments were carried out on a Bruker WM 250 NMR spectrometer at 62.83 MHz. Deuterium NMR spectra were recorded at 41.44 MHz by using the Bruker HX 270. NMR spectra are reported as parts per million downfield of $Me_4Si$ ($\delta$ 0). Multiplicities are as follows: s = singlet, d = doublet, t = triplet, b = broad, and m = multiplet. Infrared spectra were recorded on a Perkin-Elmer Model 257 grating spectrophotometer. High-pressure LC separations were carried out by using a Waters Model ALC 201 liquid chromatography unit. Radioactivity measurements were performed with a Packard 3330 liquid scintillation counter using 10-mL toluene solutions containing 7.20 g of Bu-PBD and 0.45 g of PBBO/L of toluene. Fermentations were carried out in a New Brunswick G25 gyrotory shaker.

**Materials.** D-[6-$^{14}C$]Glucose was purchased from New England Nuclear. D-[U-$^{13}C_6$]Glucose was obtained from the Stable Isotopes Resource, Los Alamos, NM. D-[6,6-$^2H_2$]Glucose was purchased from Stohler Isotopes.

Agar slants of *Streptomcyes* UC5319 were obtained from the Upjohn Co. and used to prepare an inoculum grown on a sterilized medium consisting of 2.5 g of Pharmamedia and 2.5 g of Bacto-Dextrose in 100 mL of distilled water in a 500-mL Delong flask shaken at 300 rpm and 28 °C for 3–4 days. For long-term storage this inoculum was pipetted in 0.5-mL lots into sterile 2.0-mL plastic vials which were sealed and stored in liquid nitrogen in an Orion ET-34 Dewar.

**Incorporation of D-[U-$^{13}C_6$]Glucose.** A vegetative inoculum of *Streptomyces* UC5319 was prepared by adding the contents of a rapidly thawed vial to 100 mL of the above-described inoculation medium and incubating at 300 rpm and 28 °C for 72 h. A production medium, consisting of 2.0 g of Black Strap molasses, 20.0 g of corn starch, 10.0 g of corn glutten meal, 5.0 g of calcium carbonate, 2.0 g of sodium chloride, and 1.125 g of Bacto-Dextrose/L distilled water, adjusted to pH 7.2 with 10% sodium hydroxide, was distributed in 24 500-mL DeLong flasks (100 mL/flask) fitted with Morton closures and autoclaved at 120 °C for 20 min. (For ordinary fermentations the amount of glucose was increased to 2.25 g/L.) Each flask was inoculated with 1.0 mL of vegetative culture and incubated at 28 °C and 300 rpm for 30 h, at which point a mixture of 1.35 g of D-[U-$^{13}C_6$]glucose (84 atom % excess), 1.35

(35) T. C. Feline, G. Mellows, R. B Jones, and L. Phillips, *J. Chem. Soc., Chem. Commun.*, 63 (1974).
(36) M. Tanabe, K. Suzuki, and W. C. Jankowski, *Tetrahedron Lett.*, 2271 (1974).
(37) J. R. Hanson and R. Nyfeler, *J. Chem. Soc., Chem. Commun.*, 72 (1976); A. P. W. Bradshaw, J. R. Hanson, and M. Siverns, *ibid.*, 819 (1977).
(38) H. Shirahama, E. Osawa, and T. Matsumoto, *J. Am. Chem. Soc.*, **102**, 3208 (1980); *Tetrahedron Lett.*, 1987 (1978).
(39) This absolute configuration has not been determined directly but is based on the known configuration of pentalenolactone itself.[2]
(40) The designation *RSR* refers to the chiralities of the three double bonds, $\Delta^{2,3}$, $\Delta^{6,7}$, and $\Delta^{9,10}$, respectively. C and T indicate crossed and parallel arrangements of the $\Delta^{9,10}$–$\Delta^{2,3}$ and $\Delta^{2,3}$–$\Delta^{6,7}$ double-bond pairs, respectively. Cf. J. K. Sutherland, *Tetrahedron*, **30**, 1651 (1974).
(41) D. E. Cane and R. B. Nachbar, *Tetrahedron Lett.*, **21**, 437 (1980).
(42) For a discussion of the role of such ion-pair intermediates in allylic pyrophosphate metabolism see: D. E. Cane, *Tetrahedron*, **36**, 1109 (1980); D. E. Cane and R. Iyengar, *J. Am. Chem. Soc.*, **101**, 3385 (1979); D. E. Cane, R. Iyengar, and M.-S. Shiao, *ibid.*, in press.
(43) R. Croteau and F. Karp, *Arch. Biochem. Biophys.*, **184**, 77 (1977).

g of D-glucose, and [6-$^{14}$C]glucose ($1.74 \times 10^8$ dpm) in 24.0 mL of distilled water was dispensed equally via a Millex disposable sterile filtration unit to the 24 flasks. After incubation for an additional 42 h, the culture was filtered through Celite. The pH of the filtrate was adjusted to 2.4 with 50% $H_2SO_4$, and the solution was extracted three times with 500 mL of chloroform. After drying of the extracts over sodium sulfate and evaporation of the solvent, the resulting brown oil (0.34 g) was dissolved in 25 mL of dry ether and filtered. An ethereal solution of distilled benzyl amine was added to the filtrate until no further precipitation was apparent. The mixture was allowed to stand for 15 min at room temperature, the ethereal supernatant was drawn off, and the amorphous benzylammonium salts were washed several times with dry ether. The acids were then regenerated by dissolving the salts in chloroform, shaking with concentrated phosphate buffer, pH 2.75, and then extracting the aqueous phase with two additional portions of chloroform. The combined organic extracts were washed once with saturated sodium chloride, dried over sodium sulfate, and evaporated to give 0.20 g of oil. This mixture of acids was dissolved in 1:1 tetrahydrofuran–ether and methylated at 0 °C for 5 min with diazomethane, freshly generated from *N*-nitrosomethylurea and potassium hydroxide in ether at 0 °C. The reaction was quenched by addition of a small amount of etheral acetic acid, and the solution was washed three times with saturated sodium bicarbonate and once with saturated sodium chloride, dried over sodium sulfate, and evaporated to yield 0.16 g of oil.

Initial separation of this oil into its components was effected by PLC on silica gel (buffered to pH 7, 2:1 benzene–ethyl acetate). Two major zones were eluted, a less polar, $R_f$ 0.65, containing pentalenolactone methyl ester (0.054 g), and a more polar $R_f$ 0.07–0.46, containing pentalenic acid methyl ester, (0.062 g). The less polar fraction was further enriched by a second PLC (2:1 benzene–ethyl acetate) to give two fractions, $R_f$ 0.65 (0.045 g) and $R_f$ 0.55 (0.009 g). The latter was combined with the more polar fraction from the previous separation while the former was subjected to purification on four 20 × 20 cm analytical silica gel plates (19:1 benzene–ethyl acetate, five developments) to yield 18.2 mg of pure pentalenolactone methyl ester[2,3] and 4.6 mg of pentalenolactone E methyl ester.[11]

Labeled pentalenic acid methyl ester was obtained by further purification of the 0.070 mg from the more polar fraction by PLC (2:1 benzene–ethyl acetate), excising the band $R_f$ 0.38–0.44, to give 27.1 mg of oil which was resubmitted to PLC (20:1 benzene–ethyl acetate, four developments) to yield 15.3 mg of **5**-Me. Final purification was effected by high-pressure LC using a 3.9 mm id × 30 cm column of μ-Bondapak CN, eluted with 8:1 hexanes–chloroform (2.5 mL/min, $k' = 4$), yielding finally 13.1 mg of pentalenic acid methyl ester.[10]

**Incorporation of D-[6-$^2H_2$]Glucose.** A solution of 3.0 g of D[-6-$^2H_2$]-glucose and $4.0 \times 10^7$ dpm [6-$^{14}$C]glucose was administered to 2.4 L of a 30-h fermentative culture of *Steptomyces* UC5319 incubated as described above in 24 Delong flasks at 27 °C. After an additional 42 h at 27 °C and 300 rpm, the cultures were extracted and the crude mixture of methyl esters (0.187 g), was isolated as before. Separation into the individual components was achieved by flash column chromatography on 0.040–0.063 mm Merck silica gel 60 (5:1 benzene–ethyl acetate), thick-layer PLC (3:1 benzene–ethyl acetate), and thin-layer PLC (20:1 benzene–ethyl acetate, four developments). Pentalenic acid methyl ester was further purified by high-pressure LC on μ-Bondapak CN, as above: pentalenolactone methyl ester, 8.3 mg; pentalenolactone E methyl ester, 9.6 mg; pentalenic acid methyl ester, 15.5 mg. **6**-Me: $^1$H NMR (270 MHz, $CDCl_3$) δ 1.06 (s, 3 H), 1.07 (s, 3 H) (H-14,15), 1.44 (dd, $J$ = 6.2, 12.8 Hz, 1 H, H-1β), 1.74 (d, $J$ = 13.9 Hz, 1 H, H-3b), 1.89 (dd, $J$ = 9.2, 12.8 Hz, 1 H, H-1α), 2.16 (d, $J$ = 13.9 Hz, 1 H, H-3a), 3.29 (m, 2 H, H-5,8), 3.76 (s, 3 H, H-16), 4.31 (m, 2 H, H-12), 5.57 (s, 1 H, H-10a), 5.91 (s, 1 H, H-10b), 6.85 (bs, 1 H, H-7). Irradiation of the signal at δ 1.89 caused the peaks at δ 1.44 to dollapse to a doublet and changed the shape of the multiplet at δ 3.29.

**Acknowledgment.** This work was supported by NIH Grant GM22172. The [U-$^{13}C_6$]glucose was provided by the National Stable Isotopes Resource, Los Alamos Scientific Laboratory, supported by the USDOE and the NIH (Grant RR-00962). The Bruker HX270 facility at Yale University was supported by NIH Grant 1-PO7-RR00798 from the Division of Research Resources. Homonuclear $^{13}C$–$^{13}C$ decoupling experiments were carried out on the Bruker WM 250 by Mr. Mark Mattingly of Bruker Instruments, Inc., Billerica, Ma. Generous gifts of crude pentalenolactone and strains of *Streptomyces* UC5319 were obtained from Dr. David G. Martin and Dr. L. J. Hanka of the Upjohn Co. A sample of pentalenolactone and a strain of *S. roseogriseus*, ATCC 12414, was also kindly provided by Dr. Walter D. Celmer of Chas. Pfizer, Inc.

# *Communications to the Editor*

## Evidence for the Isomerization of 1-Methylsilene to Dimethylsilylene

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*Received September 15, 1980*

Among the most convenient methods employed to generate simple silaethylenes (silenes) is the thermal fragmentation of appropriately substituted alkylsilacyclobutanes.[1] For example, the formation of 1,1-dimethylsilene and ethylene from the gas-phase pyrolysis of 1,1-dimethylsilacyclobutane has been firmly established by direct detection (mass spectrometry[2] and matrix isolation)[3]and kinetic[4] and chemical trapping studies.[5] Nevertheless, it is surprising that similar attempts to characterize silenes bearing a hydrogen on silicon by using chemical trapping and matrix-isloation techniques have met with only limited success.[6] We now wish to report evidence that such a silene, 1-methylsilaethylene, rapidly isomerizes to dimethylsilylene in the gas phase.

Low-pressure pyrolysis[7] of 1-methylsilacyclobutane[8] at 625 °C

(1) L. E. Gusel'nikov and N. S. Nametkin, *Chem. Rev.* **79**, 529 (1979).
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(7) All pyrolyses were carried out at pressures of 1–5 torr employing a seasoned hot zone consisting of a 10 mm i.d. x 30 cm quartz tube. Residence time in the hot zone was on the order of tenths of a second and was controlled by $^1/_{32}$-in. constriction placed at the end of the hot zone. Yields were determined relative to the $C_2H_4$ product or with hexane as an inert internal standard.
(8) D. Seyferth, R. Damrauer, S. B. Andrews, and S. S. Washburne, *J. Am. Chem. Soc.*, **93**, 3709 (1971).
(9) All compounds reported were fully characterized by their IR, NMR, and mass spectra.

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