Application of Carbon-13 Magnetic Resonance to Isoprenoid Biosynthesis. II. Ovalicin and the Use of Doubly Labeled Mevalonate

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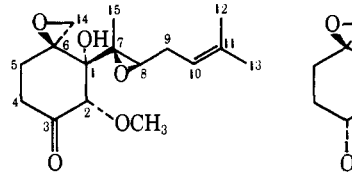
Abstract: The biosynthesis of the antibiotic ovalicin (**1**) has been studied by carbon magnetic resonance techniques. Feeding of [4-^{13}C]mevalonate and [3,4-^{13}C]mevalonate to *P. ovalis* resulted in labeling of **1**. Analysis of the ^{13}C NMR spectrum of labeled **1** supported a biosynthetic pathway involving the intermediacy of β-bergamotene (**8**). The use of doubly labeled mevalonate is shown to be complementary to incorporation of [1,2-^{13}C]acetate.

The sesquiterpene-methyl ether ovalicin[1] (**1**) isolated from culture filtrates of the fungus *Pseudorotium ovalis* Stolk shows antibiotic as well as immunosuppressive and antitumor activity. The structure and stereochemistry of this substance have been determined by a Sandoz group using a combination of chemical and x-ray crystallographic techniques and these workers also recognized the identity of ovalicin with the lettuce seed germination stimulant graphinone,[2] a metabolite of a *Graphium* sp. fungus. Ovalicin is closely related to fumagillin (**2**),[3] an antibiotic metabolite of *Aspergillus fumigatus* with antiparasitic and carcinolytic properties. The monoterpene carquejol acetate (**3**),[4] isolated from the essential oil of *Baccharis genistelloides* Pers., also belongs to this unusual structural class of metabolites. Ovalicin (**1**) and its relative fumagillin (**2**) possess a rare sesquiterpene carbon skeleton and may be formally considered as derivatives of *o*-menthane. The biosynthetic problem presented by these substances involves the rigorous distinction among a number of plausible explanations which may be advanced a priori to account for their biological formation or biogenesis. In order to make this distinction one must consider the biogenetic relationship of each carbon atom in ovalicin to a corresponding atom in mevalonate.



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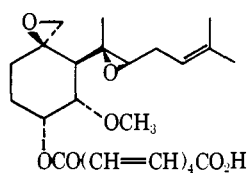

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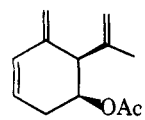

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In Scheme I is illustrated a biosynthetic route in which three units of mevalonate (**4**) are converted, via farnesyl pyrophosphate (**5**), to a bisabolenelike cation (**6**), formally belonging to the *p*-menthane class. Six conceivable simple modes of rearrangement exist for the conversion of **6** to the *o*-menthane framework of ovalicin. Each ovalicin structure has been numbered so as to indicate the mevalonoid origin of its individual carbon atoms. As is evident, each hypothetical rearrangement results in a unique numbering pattern for the derived ovalicin. Experimental determination of these numbering patterns and correlation to a specific rearrangement pathway is complicated by degeneracies between pairs of rearrangements as to the origin of specific carbon atoms. For example, (1) pathway a, in which there is a 1,3 migration of the eight-carbon side chain, and pathway b, involving a 1,3 migration of the ring methyl, both result in labeling of the carbonyl carbon, the hydroxyl-bearing carbon, and the monosubstituted olefinic carbon of ovalicin by carbon 4 of mevalonate. (2) In pathways a and c identical carbon atoms become labeled by C-3 mevalonate. (3) In pathways d and e, C-5 of mevalonate is the precursor of identical carbon atoms in each resulting ovalicin molecule.

Scheme I

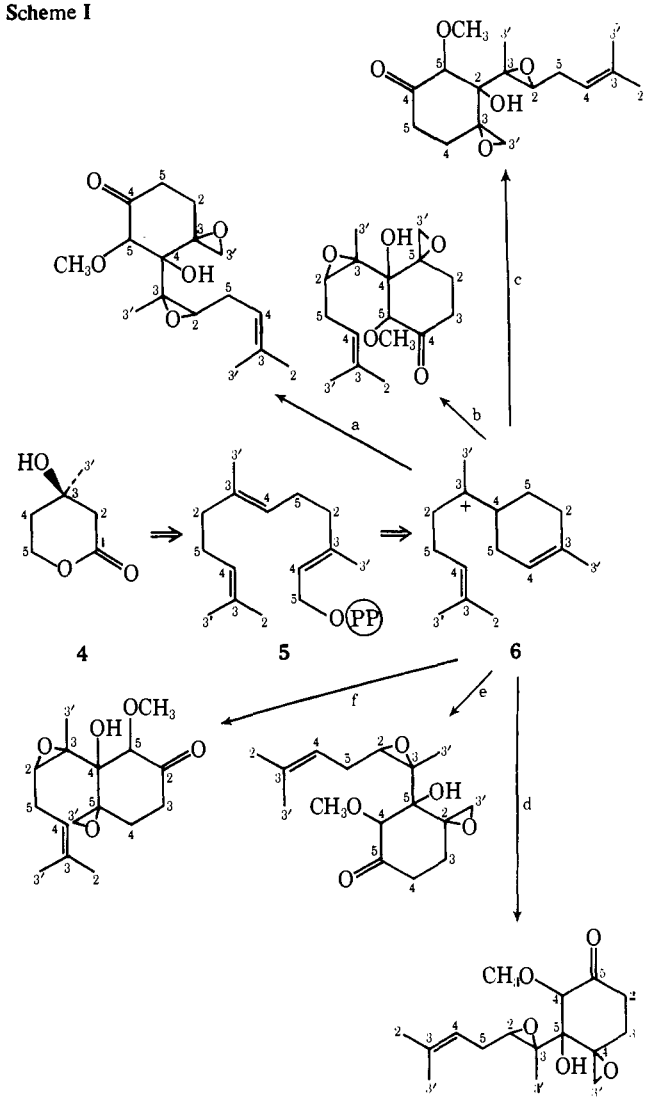

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Table I. ^{13}C NMR Spectrum of Ovalicin[a-c]

C-3	206.2 s[d,e]	C-8	56.5 d
C-11	135.0 s[f]	C-14	51.0 t
C-10	117.8 d[f]	C-4	36.4 t
C-2	85.9 d	C-5	30.0 t
C-1	78.2 s[g]	C-9	26.8 t
C-6	60.2 s[g]	C-13	25.4 q
C-7	60.0 s	C-12	17.7 q
C-16	58.9 q	C-15	14.1 q

[a] Varian XL-100-15; spectral width 6000 Hz, acquisition time 0.6666 sec, pulse delay 4.00 s, pulse width 20 μs, 256 transients, 0.85 M solution in $CDCl_3$, 12-mm sample tube. [b] ^{13}C NMR spectrum of **1a**: 6711 transients. [c] ^{13}C NMR spectrum of **1b**: 11 685 transients. [d] TMS = 0.00 ppm. [e] Multiplicity in off-resonance decoupled spectrum: s = singlet, d = doublet, t = triplet, q = quartet. [f] Doublet, J = 74 Hz in spectrum of **1b**. [g] Doublet, J = 53 Hz in spectrum of 1b.

The experimental goal must be to determine as efficiently and as unambiguously as possible the actual "numbering pattern" of natural ovalicin thereby demonstrating which of the conceivable structural rearrangements is operative.

A preliminary study of fumagillin biosynthesis has been reported by Birch[5] who showed that the terminal isopropylidene residue of fumagillin derived from labeling experiments with [2-^{14}C]mevalonate carried one-third of the incorporated radioactivity. While supporting the proposed mevalonoid origin of fumagillin, this experiment did not distinguish among any of the postulated rearrangements leading to formation of the requisite *o*-menthane skeleton. We have applied the techniques of carbon-13 magnetic resonance to the solution of this problem, using both singly and doubly labeled precursors, and report our results below.[6]

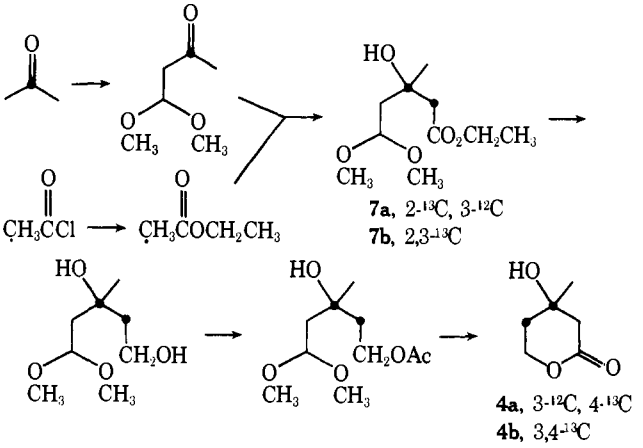
Results and Discussion

The natural abundance proton-noise-decoupled ^{13}C NMR spectrum of ovalicin shows 16 signals corresponding to the 15 carbon atoms of the sesquiterpene framework as well as the single carbon of the methyl ether (Table I). In the off-resonance decoupled spectrum (decoupling centered ca. 700 Hz upfield of TMS) five peaks appeared as singlets. Those at 206.2, 135.0, and 78.2 ppm are assigned to C-3, C-11, and C-1, respectively, on the basis of chemical shift. The two remaining singlets at 60.2 and 60.0 ppm are assigned to the quaternary epoxide carbons C-6 and C-7.[7] The off-resonance decoupled spectrum shows three doublets at 117.8 ppm (C-10), 85.9 ppm (C-2), and 56.5 ppm (C-8). Assignments are based on known chemical shifts and on the observed enhancement and decoupling of the signal at 85.9 ppm by irradiation of the C-2 proton at δ 4.24.

The four triplets in the off-resonance decoupled spectrum are assigned to C-14 (51.0 ppm), C-4 (36.4 ppm), C-5 (30.0 ppm), and C-9 (26.8 ppm). The latter carbon should experience γ shielding[8] from both C-15 and C-12. Differentiation of C-4 and C-5 was based on comparison with known chemical shift data. The four methyl carbons appear as quartets at 58.9 (C-16), 25.4 (C-13), 17.7 (C-12), and 14.1 (C-15) ppm. Irradiation of the methoxyl protons at δ 3.58 causes the signal at 58.9 ppm to collapse to a singlet. Similarly irradiation at δ 1.38 decouples the highest field signal at 14.1 ppm. Irradiation at δ 1.67 and 1.76 caused the signals at both 25.4 and 17.7 to collapse essentially to singlets but does not allow further distinction between the two carbons. The two allylic methyl groups have been assigned by comparison with known chemical shift values; in geraniol the analogous methyl resonances appear at 25.6 and 17.6 ppm.[9] The cis methyl at C-12 is shifted upfield by a γ effect.

A number of methods are available for the synthesis of labeled mevalonates.[10] Many of these involve a Reformatsky reaction between acetoacetaldehyde dimethyl acetal and ethyl bromoacetate. Recently Rathke has demonstrated the feasibility of quantitatively generating ester enolates by the use of lithium salts of hindered amines.[11] This procedure appeared to be ideally suited to the synthesis of ^{13}C-labeled mevalonates and was adopted for the synthesis of the labeled mevalonates **4a** and **4b**, shown in Scheme II.

Scheme II



Commercial [2-^{13}C]acetyl chloride was converted to ethyl acetate by treatment with absolute ethanol in the presence of anhydrous triethylamine. This ethyl acetate was then metalated with lithium diisopropylamide in tetrahydrofuran and the resulting lithium enolate was treated with acetoacetaldehyde dimethyl acetal to yield **7**. In the ^1H NMR spectrum of **7a**, the signal at δ 2.50 corresponding to the protons at C-2 is reduced in intensity. The ^{13}C-^1H satellite (J = 128 Hz) integrates for >85% ^{13}C. Conversion of **7** to [4-^{13}C]mevalonolactone (**4a**) was accomplished by successive reduction with lithium aluminum hydride, acetylation, and performic acid oxidation (48% yield based on acetyl chloride). Treatment of the ester acetate with an excess of ozone in ethyl acetate[12] also led to formation of **4a** but in less satisfactory yield.

The above sequence was adapted to the synthesis of [3,4-$^{13}C_2$]mevalonate (**4b**). [2-^{13}C]Acetoacetaldehyde dimethyl acetal was prepared by treatment of [2-^{13}C]acetone with sodium methoxide and methyl formate followed by methanolic hydrogen chloride.[13] Reaction with [2-^{13}C]2-lithio ethyl acetate gave **7b**. The ^1H NMR spectrum of **7b** showed a doublet of doublets ($J_{^{13}CH}$ = 128 Hz, $J_{^{13}CCH}$ = 4 Hz) centered at δ 2.50 as well as a low-intensity doublet ($J_{^{13}CCH}$ = 4 Hz) at δ 2.50. Conversion of **7b** to [3,4-$^{13}C_2$]mevalonate (**4b**) was carried out as above.

In order to determine both percent incorporations and isotopic enrichments from mevalonate, a series of experiments with [2-^{14}C]mevalonate was carried out (Table II). In each experiment an increasing amount of mevalonate (from 0.01 to 100 mg) was administered to cultures of *P. ovalis*. The total activity of each sample of mevalonate was kept constant. The ovalicin produced in each case was isolated and purified and the ^{14}C content was measured. The amount of substrate fed did not significantly affect either percent incorporation or yield of ovalicin. The specific incorporation depended on the amount of mevalonate administered in each experiment.

Ten 500-ml DeLong flasks, each containing 100 ml of nutrient broth, were innoculated with *P. ovalis* and shaken aerobically at 30 °C for 7 days. Sodium [4-^{13}C]mevalonate (0.55 mmol, 90% enriched) was added and incubation was continued for an additional 7 days. The culture filtrates were then extracted with methylene chloride and the extracts purified by PLC on silica gel. Recrystallization gave 10.5 mg of labeled ovalicin (**1a**). The proton-decoupled ^{13}C

Table II.[a] Incorporation of (3*RS*)-[2-^{14}C]Mevalonate

Mevalonate,[b,c] mmol	Incorp,[d] %	1 mmol	Enrich,[e] %
0.0001	2.5	0.013	0.008
0.15	2.2	0.023	2.1
0.85	1.2	0.017	9

[a] Two 100-ml shake cultures, *P. ovalis*. [b] Fed as the sodium salt. [c] 3.67×10^6 dpm. [d] Based on (3*R*)-mevalonate. [e] Enrichments based on 3 moles of mevalonate per mole of ovalicin.

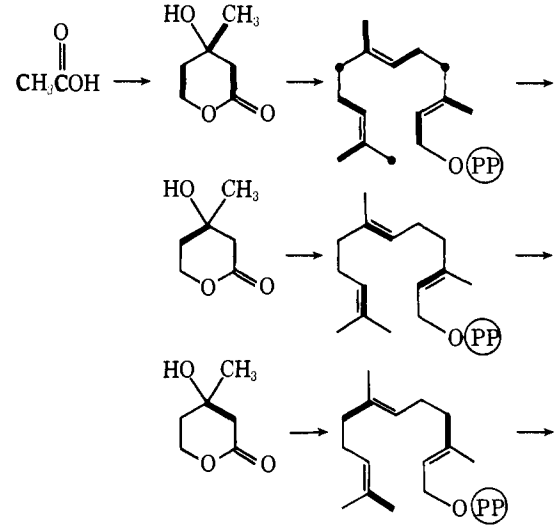
NMR spectrum of **1a** showed an approximate threefold peak enhancement for the signals assigned to C-1, C-3, and C-10 of ovalicin. The magnitude of the peak enhancements corresponded to ca. 3% ^{13}C enrichment at each of the respective carbons.[14]

A sample of [3,4-$^{13}C_2$]mevalonolactone [0.38 mmol, 81% $^{13}C_2$] was mixed with an equal weight of unlabeled mevalonolactone and, after saponification, the sodium salt was administered as above to 1 l. of 7-day-old *P. ovalis* culture. After an additional 7 days at 30 °C, the cultures were worked up in the usual manner yielding, after recrystallization, 24 mg of ovalicin (**1b**). The proton decoupled ^{13}C NMR displayed the following characteristics: a pair of doublets (J = 74 Hz) centered at 135.5 and 118.1 ppm; a second pair of doublets (J = 53 Hz) centered at 78.7 and 60.6 ppm; and enhanced singlets at 206.7 and 60.4 ppm. The magnitude of the peak enhancements corresponded to ca. 1.5–2% enrichment at each labeled position.

The observed enrichment of carbons 1, 3, and 10 of ovalicin by [4-^{13}C]mevalonate demonstrates the intermediacy of mevalonate in the biosynthesis of ovalicin. Pathways c through f are clearly excluded by the ^{13}C NMR data; only pathways a or b are consistent with the observed labeling pattern.

The use of substrates doubly labeled with stable isotopes is ideally suited to the study of molecular rearrangements. In the field of biosynthesis the classic studies of Bloch[15] and Cornforth[16] of methyl migrations during lanosterol biosynthesis relied on mass spectrometric analysis of samples of labeled acetic acid obtained by degradation. More recently, the combination of double labeling techniques and ^{13}C NMR has utilized vicinal carbon-carbon couplings as a probe for both bond-making and bond-scission processes. For example in Battersby's elegant studies of the rearrangement leading to the formation of type III porphyrins, two labeled carbons initially separated by two intervening atoms become adjacent as a result of an intramolecular rearrangement, giving rise to an observed carbon-carbon coupling in the ^{13}C NMR spectrum.[17] Alternatively, the use of [1,2-$^{13}C_2$]acetate, developed by both Seto and Tanabe[18] and McInnes[19] to study terpenoid and polyketide biosynthesis, results in pairs of doublets arising from vicinal carbons which originate from a common molecule of acetate. Any process which breaks a bond between paired acetate carbons will result in ^{13}C NMR signals which are enhanced but unsplit. The use of [1,2-^{13}C]acetate leads to formation of mevalonate containing three groups of paired carbon atoms (Scheme III): C-1 and -2, C-3 and -3′, and C-4 and -5. Since C-1 is subsequently lost as carbon dioxide in the formation as isopentenyl pyrophosphate, all carbons derived from C-2 of mevalonate give rise to singlets. As long as the C-3,3′ pair or the C-4,5 pair remains intact the corresponding signals in any derived metabolite appear as coupled doublets. If at any time either of these bonds is broken the resulting spectra exhibit enhanced singlets. While extremely powerful, this method cannot be used to detect directly the breaking of bonds derived from either the 2,3 or the 3,4 bond of mevalonate. The latter two processes may be observed by using mevalonate doubly labeled at the appropriate sites. The use of labeled mevalonate has of course the further advantage of providing direct evidence for the intermediacy of this key substance in the biosynthetic sequence. Incorporation of [3,4-^{13}C]- or [2,3-^{13}C]mevalonate is therefore complementary to the use of [1,2-^{13}C]acetate.

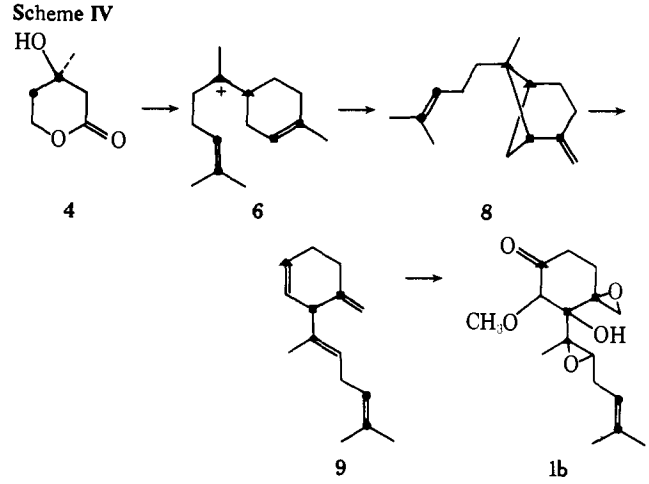
Scheme III



The magnitude of the enrichments observed from incorporation of [4-^{13}C]mevalonate into ovalicin indicates that each molecule of labeled mevalonate has become diluted with ca. 32 molecules of endogenous mevalonate during the biosynthesis of ovalicin. Therefore, for a given unit of mevalonate, the chances that its neighbor in farnesyl pyrophosphate will be labeled are only 1 in 33. Dilution of labeled mevalonate with exogenous unlabeled mevalonate prior to feeding further decreases the probability of finding adjacent labeled mevalonates.

The observed spectrum of ovalicin derived from incorporation of [3,4-^{13}C]mevalonate is consistent only with pathway a (Scheme IV). Carbons 10 and 11 of ovalicin are derived from carbons 4 and 3, respectively, of mevalonate. In 90% of the molecules in which C-10 is labeled, C-11 is also labeled and the enriched carbon appears as a doublet with J = 74 Hz.[20] In the remaining 10% of those molecules in which C-10 is labeled, C-11 is unlabeled and the enriched carbon gives rise to a singlet superimposed on the natural abundance signal. The magnitude of the enhancement of the natural abundance signal is equal to one-ninth of the total intensity of the satellite doublet. Looking at C-11, one observes the expected doublet J = 74 Hz, centered on the enhanced natural abundance singlet.

Scheme IV



Carbons 1 and 6 of ovalicin, derived from C-4 and C-3, respectively, of a common molecule of mevalonate, give rise to the observed pair of doublets, J = 53 Hz.[20] By contrast, carbons 1 and 7, although adjacent, are derived from distinct mevalonate units and the statistical chance that both will be labeled within the same molecule is essentially zero. Therefore the two resonances corresponding to these carbons are not coupled and the carbon at C-7, as well as its former neighbor, now at C-3 of ovalicin, gives rise to an enhanced singlet.[21,22]

Conclusion

The observed pattern of enhancements and couplings is completely consistent with pathway a for the generation of the *o*-menthane skeleton of ovalicin, and excludes all other simple possibilities.

A plausible intermediate consistent with pathway a is the bicyclo[3.1.1]sesquiterpene, β-bergamotene (**8**).[5,23,24] Oxidative ring opening of **8** might generate the tetraene **9** which in turn would undergo appropriate oxidation to yield, ultimately, ovalicin. While the two epoxides as well as the hydroxyl function are most likely derived from molecular oxygen, it is conceivable that either the carbonyl or the methoxyl oxygen originates from water.

Recently, Tanabe[25] has also examined the biosynthesis of ovalicin by the use of [1,2-^{13}C]acetate. His results indicate, inter alia, that carbons 14 and 6 of ovalicin (carbons 13 and 1 using Tanabe's numbering) must originate from the same molecule of acetate. This is clearly inconsistent with pathway b and supports pathway a.

The above experiments have illustrated the utility of doubly C-13 labeled mevalonate in isoprenoid biosynthetic studies while suggesting a number of plausible intermediates in the biosynthesis of ovalicin and fumagillin. Further work designed to test some of these predictions is in progress in our laboratories.

Experimental Section

Instrumentation. Carbon magnetic resonance spectra were obtained with a Varian XL-100-15 pulsed Fourier Transform system operating at 25.16 MHz. The center of the deuteriochloroform triplet was assigned a value of 77.11 ppm. Proton NMR were recorded on a Varian A60A. ^{1}H NMR shifts are expressed in parts per million downfield from internal tetramethylsilane (δ = 0). Infrared spectra were taken using a Perkin-Elmer Model 257 grating spectrophotometer. Measurements of carbon-14 activity were obtained using a Packard 3330 Liquid Scintillation counter to measure 10-ml toluene solutions containing 7.20 g of Bu-PBD and 0.45 g of PBBO per liter of toluene. Fungal fermentations were carried out in a New Brunswick Scientific G25 Gyrotory Incubator Shaker.

Materials. Acetone [2-^{13}C, 90 atom % C-13] and acetyl chloride [2-^{13}C, 90 atom % C-13] were purchased from Merck and Co. DL-[2-^{14}C]Mevalonic acid lactone (17.5 mCi/mmol) was obtained from Amersham/Searle. Strains of *P. ovalis* were a gift of Sandoz, A.G. Basel, Switzerland.

n-Butyllithium (ca. 2 *M* hexane solution, Alfa Inorganics) was standardized by double titration just prior to use. Acetoacetaldehyde dimethyl acetal was purchased from Aldrich. Diisopropylamine and triethylamine were distilled from calcium hydride. Methyl formate was dried over potassium carbonate and then distilled from phosphorus pentoxide. Tetrahydrofuran was freshly distilled from lithium aluminum hydride. Ethanol was Gold Shield absolute alcohol. Pyridine was distilled from barium oxide.

[2-^{13}C]Acetoacetaldehyde Dimethyl Acetal.[13] A mixture of anhydrous methyl formate (0.62 g, 10.3 mmol) and 0.50 g of [2-^{13}C]acetone (90 atom % C-13) in 2.0 ml of anhydrous ether was added dropwise to a stirred suspension of 0.47 g (8.6 mmol) of sodium methoxide in 5.0 ml of anhydrous ether under nitrogen. The reaction mixture was refluxed for 1 hr then cooled, and the ether was carefully evaporated at aspirator pressure without stirring. Methanol (2.06 ml, 1.65 g, 51.5 mmol) was added to the solid residue and the suspension was stirred for 3 min before addition of 1.37 ml (17.4 mmol) of 12.7 *M* methanolic hydrogen chloride. After an additional 2 hr at room temperature the pH was adjusted to 8 by addition of saturated methanolic potassium hydroxide, ether was added, and the mixture was filtered. The filtrate was concentrated on a rotary evaporator and the residue was redissolved in ether and once again filtered and evaporated. Care was taken to avoid loss of product by prolonged evaporation. The flask containing the residue was connected to a short-path condenser and immersed in liquid nitrogen while the system was evacuated to 24 μ. The receiver was cooled in liquid nitrogen and the residue was allowed to warm to room temperature. After 45 min the distillate was dissolved in ether and the solution was dried over anhydrous sodium sulfate and a spatula-full of Linde 4 Å molecular sieves (to remove any residual methanol). Filtration and careful evaporation yielded 0.48 g (42%) of [2-C^{13}]acetoacetaldehyde dimethyl acetal: ^{1}H NMR ($CDCl_3$) δ 2.18 (d, J_{CCH} = 6 Hz on s, CH_3, 3 H), 2.73 (d × d, J_{CCH} = 5 Hz, J_{HH} = 5.5 Hz, CH_2, 2H), 3.37 (s, OCH_3, 6 H), 4.80 (t × d, J_{HH} = 5.5 Hz, J_{CCCH} = 1.5 Hz, CH, 1 H); ir ν_{max} ($CHCl_3$) 1673 (^{13}C=O), shoulder at 1718 cm^{-1} (C=O).

Ethyl [2-^{13}C]-3-Hydroxy-3-methyl-5,5-dimethoxypentanoate (7a).[10] [2-^{13}C] Acetyl chloride (90 atom % C-13) (0.50 g, 6.37 mmol) in 5.0 ml of anhydrous ether was added dropwise to a stirred solution of 0.707 g (7.0 mmol) of anhydrous triethylamine and 0.293 g (6.37 mmol) of absolute ethanol in 5.0 ml of ether at −78 °C under nitrogen. After 15 min at −78 °C, the mixture was stirred overnight at 4 °C. The reaction mixture was transferred via syringe to a sintered glass filter funnel and filtered under positive nitrogen pressure into a pressure-equalizing addition funnel which was attached to a 100-ml three-neck flask equipped with a rubber septum and a three-way stopcock, fitted with a nitrogen balloon. The reaction mixture was rinsed with four additional 3-ml portions of ether which in turn were filtered into the addition funnel.

The reaction flask was charged with 20 ml of dry tetrahydrofuran containing 0.68 g (6.70 mmol) of diisopropylamine and the solution was cooled to −78 °C before addition of 3.04 ml (6.37 mmol) of 2.10 *M* *n*-butyllithium.[11] After 10 min at −78 °C the solution was cooled to −90 °C and the ether solution of [2-^{13}C]ethyl acetate was added dropwise. The reaction mixture was stirred an additional 15 min at −90 °C, then warmed to −78 °C and 0.80 g (6.06 mmol) of acetoacetaldehyde dimethyl acetal in 1.5 ml of tetrahydrofuran was added. After 25 min 10 ml of saturated aqueous ammonium chloride was added and the mixture was allowed to warm to room temperature. The tetrahydrofuran was evaporated and the residual mixture was extracted with ether. The ether extracts were shaken with saturated sodium chloride, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The oily residue was distilled in a bulb-to-bulb apparatus to yield 0.968 g (70%) of **7a** (bp 70 °C (0.01 mm)): ^{1}H NMR (**7**, $CDCl_3$) δ 1.26 (t, J = 7 Hz, CH_2CH_3) and 1.30 (s, CH_3) (total 6 H), 1.90 (d, J = 5.5 Hz, CH_2CH, 2 H), 2.50 (s, CH_2CO_2, 2 H), 3.33 (s, OCH_3, 6 H), 3.90 (s, OH, 1 H), 4.15 (q, J = 7 Hz, CH_2CH_3, 2 H), 4.63 (t, J = 5.5 Hz, $CH(OCH_3)_2$, 1 H); ^{1}H NMR (**7a**, $CDCl_3$) δ 1.26 (t, J = 7 Hz, CH_2CH_3) and 1.30 (d, J_{CCCH} = 3 Hz, CH_3) (total 6 H), 1.88 (d × d, J_{HH} = 5.5 Hz, J_{CCCH} = 3 Hz, CH_2CH, 2 H), 2.50 (d, J_{CH} = 128 Hz, $^{13}CH_2CO_2$, 1.7 H and s, CH_2CO_2, 0.3 H), 3.31 (s, OCH_3, 6 H), 3.88 (s, OH, 1 H), 4.16 (q, J = 7 Hz, CH_2CH_3, 2 H), 4.64 (t, J = 5.5 Hz, $CH(OCH_3)_2$, 1 H); ir ν_{max} ($CHCl_3$) 3500 (OH), 1725 cm^{-1} (C=O).

Ethyl [2,3-^{13}C]3-Hydroxy-3-methyl-5,5-dimethoxypentanoate (7b).[10] [2-^{13}C]Ethyl acetate (4.05 mmol) prepared as described above was metalated with 4.05 mmol of lithium diisopropylamide and the resulting enolate was reacted with 0.45 g (3.38 mmol) of [2-^{13}C]acetoacetaldehyde dimethylacetal. After work-up in the usual manner the crude product was purified by PLC (3:1 methylene chloride–ether, silica gel, pH 7). The zone of R_f 0.24–0.36 was removed and the product which was eluted was bulb-to-bulb distilled to yield 0.518 g (70%) of **7b** (bp 75 °C (0.01 mm): ^{1}H NMR (**7b**, $CDCl_3$) δ 1.27 (t, J = 7 Hz, CH_2CH_3 superimposed on m, CH_3), (total 6 H), 1.92 (m, CH_2CH, 2H), 2.52 (d × d, J_{CH} = 128 Hz, J_{CCH} = 4 Hz, $^{13}CH_2CO_2$, 1.75 H and d, J_{CCH} = 4 Hz, $^{13}CCH_2CO_2$, 0.25 H), 3.33 (s, OCH_3, 6 H), 3.9 (bs, OH, 1 H), 4.18 (q, J = 7 Hz, CH_2CH_3, 2 H), 4.63 (bt, J = 5.5 Hz, $CH(OCH_3)_2$, 1 H).

[2-^{13}C]3-Methyl-5,5-dimethoxypentane-1,3-diol and [2,3-

^{13}C]**Diol.**[10] A solution of 0.580 g (2.63 mmol) of **7a** in 2 ml of tetrahydrofuran was added to a stirred suspension of 0.20 g (5.26 mmol) of lithium aluminum hydride in 10 ml of tetrahydrofuran under nitrogen. After 1 hr at room temperature the reaction mixture was refluxed for 1 hr and then cooled, whereupon 0.2 ml of water, 0.2 ml of 15% sodium hydroxide, and 0.6 ml of water were added in succession. The granular precipitate was removed by filtration and the filtrate was concentrated under reduced pressure to yield 0.463 g (99%) of diol: ^{1}H NMR (unlabeled diol, $CDCl_3$) δ 1.27 (s, CH_3, 3 H), 1.78 (m, CH_2CH_2OH) and 1.82 (d, J = 5.5 Hz, CH_2CH) (total 4 H), 3.37 (s, OCH_3, 6 H), 3.45–4.0 (m, CH_2OH, OH, 4 H), 4.68 (t, J = 5.5 Hz, $CH(OCH_3)_2$, 1 H); ^{1}H NMR ([2-^{13}C]diol, $CDCl_3$) δ 1.28 (d, J_{CCCH} = 3.5 Hz, CH_3, 3 H), 1.75 (d × d × d, J_{CH} = 125 Hz, J_{HH} = 4.5 Hz, J_{HH} = 6.5 Hz, $^{13}CH_2CH_2OH$) and 1.85 (m, CH_2CH) (total 4 H), 3.37 (s, OCH_3, 6 H), 3.5–4.0 (m, CH_2OH, OH, 4 H), 4.68 (t, J = 5.5 Hz, $CH(OCH_3)_2$, 1 H); ir ν_{max} ($CHCl_3$) 3400 cm^{-1} (OH).

In the doubly labeled series 0.489 g (2.22 mmol) of ethyl [2,3-^{13}C]3-hydroxy-3-methyl-5,5-dimethoxypentanoate (**7b**) was converted to 0.394 g of [2,3-^{13}C]diol: ^{1}H NMR ($CDCl_3$) δ 1.22 (m, CH_3, 3 H), 1.72 (d × m, J_{CH} = 125 Hz, CH_2CH_2OH, 1.8 H), 1.8 (m, CH_2, 2.2 H), 3.39 (s, OCH_3, 6 H), 3.8 (m, CH_2OH, OH, 4 H), 4.68 (bt, J = 5.5 Hz, $CH(OCH_3)_2$, 1 H).

[2-^{13}C]-3-Methyl-5,5-dimethoxypentane-1,3-diol-1-acetate and [2,3-^{13}C]Acetate.[10] [2-^{13}C]Diol (0.463 g, 2.60 mmol) was stirred overnight at 4 °C under nitrogen in a mixture of 10 ml of dry pyridine and 10 ml of acetic anhydride. The reaction mixture was concentrated at 0.05–1.0 mm (bath temperature 30 °C) and the residue was distilled in a bulb-to-bulb apparatus to yield 0.515 g (90%) of acetate (bp 105 °C (0.02 mm)): ^{1}H NMR (unlabeled acetate, $CDCl_3$) δ 1.24 (s, CH_3, 3 H), 1.82 (d, J = 5.5 Hz, CH_2CH) and 1.84 (t, J = 7 Hz, CH_2CH_2O) (total 4 H), 2.03 (s, CH_3CO, 3 H), 3.36 (bs, OCH_3, OH, 7 H), 4.23 (t, J = 7 Hz, CH_2O, 2 H), 4.67 (t, J = 5.5 Hz, $CH(OCH_3)_2$, 1 H); ^{1}H NMR ([2-^{13}C]acetate, $CDCl_3$) δ 1.23 (d, J_{CCCH} = 3.5 Hz on s, CH_3, 3 H), 1.82 (d × d, J_{CCCH} = 2 Hz, J_{HH} = 5.5 Hz, CH_2CH, 2 H), 1.84 (d × t, J_{CH} = 125.5 Hz, J_{HH} = 7 Hz, $^{13}CH_2CH_2O$, 1.8 H and t, $^{12}CH_2CH_2O$, 0.2 H), 2.03 (s, CH_3CO, 3 H), 3.36 (s, OCH_3, OH, 7 H), 4.22 (t × d, J_{HH} = 7 Hz, J_{CCCH} = 2 Hz, CH_2O, 2 H), 4.66 (t, J = 5.5 Hz, $CH(OCH_3)_2$, 1 H); ir ν_{max} ($CHCl_3$) 3500 (OH), 1730 cm^{-1} (C=O).

In the doubly labeled series, 0.394 g (2.21 mmol) of [2,3-^{13}C]diol was converted to 0.455 g (94%) of the corresponding acetate: ^{1}H NMR ($CDCl_3$) δ 1.23 (m, CH_3, 3 H), 1.82 (m, CH_2, 2 H), 1.83 (d × d × t, J_{CH} = 125 Hz, J_{CCH} = 3.5 Hz, J_{HH} = 7 Hz, CH_2CH_2O, 2 H), 2.03 (s, CH_3CO, 3 H), 3.37 (s, OCH_3, OH, 7 H), 4.23 (m, CH_2O, 2 H), 4.68 (bt, J = 5.5 Hz, $CH(OCH_3)_2$, 1 H).

[4-^{13}C]-Mevalonolactone (4a).[10] [2-^{13}C]3-Methyl-5,5-dimethoxypentane-1,3-diol-1-acetate was refluxed for 1 hr in a mixture of 1.2 ml of formic acid, 0.6 ml of 30% hydrogen peroxide, and 1.2 ml of 1% aqueous sulfuric acid. The reaction mixture was cooled to room temperature and 60 mg of anhydrous potassium carbonate was added. Following evaporation of the solvent the residue was dissolved in chloroform and filtered. The concentrated filtrate was distilled in a bulb-to-bulb apparatus (110 °C (0.01 mm) to yield 0.218 g (78%) of oily [4-^{13}C]mevalonolactone. Recrystallization of 0.180 g of lactone from ether–acetone at −78 °C gave 0.088 g of [4-^{13}C]mevalonolactone (**4a**): mp ca. 25 °C; ^{1}H NMR (**4**, $CDCl_3$) δ 1.36 (s, CH_3, 3 H), 1.92 (m, CH_2CH_2O, 2 H), 2.56 (m, CH_2CO, 2 H), 3.6 (m, OH, 1 H), 4.4 (m, CH_2O, 2 H); ^{1}H NMR (**4a**, $CDCl_3$) δ 1.34 (d, J_{CCCH} = 4.5 Hz on s, CH_3, 3 H), 1.92 (d × d × d, J_{CH} = 127 Hz, J_{HH} = 7 Hz, J_{HH} = 4 Hz, $^{13}CH_2CH_2O$, 1.9 H on d × d, J_{HH} = 7 Hz, J_{HH} = 5 Hz, CH_2CH_2O, 0.1 H), 2.54 (m, CH_2CO, 2 H), 4.16 (s, OH) and 4.4 (m, CH_2O) (total 3 H); ir ν_{max} ($CHCl_3$) 3600, 3500 (OH), 1735 cm^{-1} (C=O).

In the doubly labeled series, 0.455 g (3.50 mmol) of [2,3-^{13}C]-3-methyl-5,5-dimethoxypentane-1,3-diol-1-acetate was converted to 0.245 g of **4b.** Recrystallization from ether–acetone at −78 °C gave 0.103 g of [3,4-^{13}C]mevalonolactone (**4b**): mp ca. 26 °C; ^{1}H NMR ($CDCl_3$) δ 1.35 (d × d, J_{CCCH} = 4 Hz, J_{CCH} = 4 Hz, on d, J_{CCH} = 4 Hz, CH_3, 3 H), 1.9 (d × m, J_{CH} = 129 Hz, $^{13}CH_2CH_2O$, 1.7 H on m, 0.3 H), 2.58 (m, CH_2CO, 2 H), 3.69 (bs, OH, 1 H), 4.45 (m, CH_2CO, 2 H), 3.69 (bs, OH, 1 H), 4.45 (m, CH_2O, 2 H).

Incorporation of Labeled Mevalonate. *Pseudorotium ovalis* Stolk (S 3484 Sandoz) obtained as a lyophilized preparation was rehydrated and grown out on malt-agar slants at 27 °C for 19 days. Slants were stored at 4 °C.

A nutrient solution[1] consisting of 20.0 g of dextrose, 2.0 g of malt extract (Difco), 2.0 g of Bacto-yeast extract (Difco), 2.0 g of Bacto-peptone (Difco), 2.0 g of magnesium sulfate heptahydrate, and 2.0 g of potassium dihydrogen phosphate per 1 l. demineralized water was distributed in 500-ml DeLong flasks (100 ml per flask) fitted with Morton closures and the contents autoclaved at 120 °C for 20 min.

Each flask was inoculated with a 5-mm plug of mycelium of *P. ovalis* and then incubated at 30 °C and 220 rpm in an incubator-shaker. After 7 days when production of ovalicin had begun, labeled mevalonate, as an aqueous solution of the sodium salt, was introduced via a Swinnex-13 Millipore Filter unit. Incubation was continued an additional 7 days after which the culture was filtered with suction and the filtrate extracted with three half-volumes of either methylene chloride or ethylene dichloride. The solvent was dried over magnesium sulfate and evaporated. The residue was purified by PLC on pH 7 buffered silica gel (3:1 methylene chloride–ether, R_f 0.5). The ovalicin which was isolated was then recrystallized from ether–pentane.

Incorporation of [4-^{13}C]Mevalonate. [4-^{13}C]Mevalonolactone (0.072 g, 0.55 mmol) was dissolved in 0.28 ml (0.56 mmol) of 2 N sodium hydroxide. After 5 min the solution was diluted to 8 ml and added under sterile conditions to eight 100-ml cultures of *P. ovalis*. After 7 days, the culture filtrate was extracted as described above to yield 0.095 g of crude extract. Purification by PLC gave 0.018 g which was recrystallized to give 0.0105 g of ovalicin.

[3,4-^{13}C]Mevalonate. [3,4-^{13}C]Mevalonolactone (0.052 g, 0.40 mmol) and 0.052 g (0.40 mmol) of unlabeled mevalonolactone were dissolved in 0.40 ml (0.80 mmol) of 2 N sodium hydroxide. The solution was diluted to 10.0 ml and added to ten 100-ml cultures of *P. ovalis* as described above. Extraction in the usual manner gave 0.218 g of crude extract from which 0.029 g of ovalicin was isolated. Recrystallization gave 0.023 g of ovalicin.[26]

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Low-Resolution Microwave Spectroscopy. III. Three Conformers in Ethyl Trifluoroacetate, Ethyl Chloroformate, and Ethyl Cyanoformate[1]

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Abstract: Low-resolution microwave spectra of ethyl trifluoroacetate display three a-type band series characterized by $B + C$ values of 1842 ± 2, 1947 ± 2, and 1904 ± 5 MHz associated with three rotameric forms designated extended, compact, and intermediate, respectively. Ethyl chloroformate and ethyl cyanoformate also display band spectra of three conformations with $B + C$ values of 2819 ± 3 MHz (2762 ± 3 MHz, ^{37}Cl), 3061 ± 3 MHz (2989 ± 3 MHz, ^{37}Cl), and 2954 ± 5 MHz (2885 ± 17 MHz, ^{37}Cl) for the chloroformate and 2752 ± 2, 2952 ± 5, and 2870 ± 2 MHz for the cyanoformate. For each compound the rotational constants of the extended species are consistent with a cis-trans [τ(O=COC) = 0°, τ(COCC) = 180°] heavy-atom planar structure, the compact species with a cis-gauche [τ(O=COC) = 0°, τ(COCC) ~ 90°] structure, and the intermediate species with a gauche-gauche [τ(O=COC) ~ 45°, τ(COCC) ~ 240°] structure. The cis-trans and cis-gauche structures have been previously identified in ethyl formate. For all three ethyl esters, the temperature dependence of the band intensities indicates that the extended and compact forms have nearly equal energy and the intermediate species have 1–2 kcal/mol higher energy. Characteristics of the band shape of the intermediate forms suggest that they have unusually flexible configurations.

Studies of the structures of methyl esters have consistently demonstrated the presence of a single conformer while studies of ethyl esters have frequently indicated the presence of more than one conformer.[2] The cis-heavy-atom-planar configuration of Figure 1a is the observed conformation for all methyl esters which have been unambiguously characterized. The cis-planar conformation of methyl chloroformate was determined by electron diffraction.[3] That work and a dipole moment study,[4] which showed a strong temperature dependence of the dipole moment, indicate a large amplitude of torsional motion about the carbonyl carbon to the ether oxygen bond. Methyl formate[5-11] and acetate[9-11] were demonstrated to have the cis-planar configuration. No conclusive evidence for other rotational isomers has been reported for either of these molecules in the gas phase. Similar results were obtained by infrared and microwave spectroscopic studies of methyl chloroformate,[12-15] methyl cyanoformate,[11,16] methyl fluoroformate[11,16] (here the similarity of the atomic masses of fluorine and oxygen prevented an unambiguous deduction of molecular structure), methyl propiolate,[11,16] and methyl trifluoroacetate.[17]

Spectroscopically distinct rotational isomers about both the C–O and O–ethyl bonds are possible for ethyl formates and acetates. Two rotational isomers of ethyl formate have been assigned by microwave spectroscopy[18] to the cis-trans and cis-gauche configurations of Figures 1b and 1c. They differ in energy by 186 ± 60 cal/mol, the planar form being more stable. The trans-trans structure (Figure 1d) was not observed. Small populations of other rotational isomers were not ruled out and models such as Figure 1e involving nonplanar configurations about the C–O bond were not considered. Rotational isomerism in ethyl chloroformate,[19] ethyl cyanoformate,[19] and ethyl fluoroformate[20] have been demonstrated in recent infrared studies. The number and nature of the isomers present could not be unambiguously determined and the evidence for rotational isomerism in ethyl chloroformate was not conclusive. Dipole moment measurements on ethyl chloroformate were interpreted in terms of a trans-trans structure[4] (Figure 1d).

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