cussions of this research. Prof. Westheimer is in entire agreement with the use of the stereochemical outcome at phosphorus as a criterion for free metaphosphate and with the conclusion from the experiments reported here, i.e., that the Conant-Swan reaction does not produce free metaphosphate but that the reaction must proceed with preassociation between the incipient metaphosphate and the alcoholic nucleophile. Support for this work was provided to Prof. F. H. Westheimer through NSF Grant 3CHE-7922045.

Biosynthesis of the Hypocholesterolemic Agent Mevinolin by *Aspergillus terreus*. Determination of the Origin of Carbon, Hydrogen, and Oxygen Atoms by ^{13}C NMR and Mass Spectrometry

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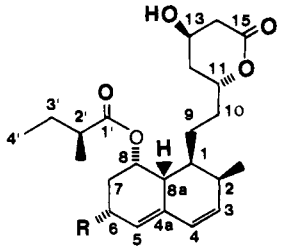
Abstract: The ^1H and ^{13}C NMR spectra of mevinolin (**1**), an inhibitor of hydroxymethylglutaryl-coenzyme A reductase, were fully assigned by using selective homonuclear and heteronuclear decoupling and two-dimensional DEPT heteronuclear shift correlation. Sodium [1-^{13}C]-, [2-^{13}C]-, [1,2-$^{13}C_2$]-, [1-^{13}C,$^{18}O_2$]-, [1-^{13}C,$^2H_3$]-, and [2-^{13}C,$^2H_3$]acetate as well as $^{18}O_2$ and [*methyl*-^{13}C]methionine were incorporated into mevinolin (**1**) by cultures of *Aspergillus terreus* ATCC 20542. Double quantum coherence (2D INADEQUATE) NMR spectra of **1** derived from sodium [1,2-^{13}C]acetate independently confirmed the ^{13}C NMR assignment and provided the location of intact carbon-carbon bonds from acetate. Mevinolin (**1**) is formed from two polyketide chains (4-carbon and 18-carbon) of acetate units coupled in head to tail fashion, with each chain bearing a methionine-derived methyl group. Material obtained from ^2H and ^{18}O incorporations was analyzed by mass spectrometry and by ^{13}C NMR detection of α and β isotope shifts. The results show that oxygen atoms on the main chain are introduced by aerobic oxidation of a deoxygenated precursor. Several mechanisms, including a biological Diels-Alder reaction, are proposed to account for formation of bicyclic ring systems in mevinolin (**1**), compactin (**2**), and related metabolites.

Mevinolin (**1**)[1,2a] and compactin (**2**)[2b,c] are potent inhibitors of cholesterol biosynthesis in humans and possess potential in treatment of atherosclerosis and coronary heart disease.[3] These



1 R=CH_3

2 R=H

fungal metabolites and their corresponding 4a,5-dihydro derivatives (**3** and **4**)[4] block isoprenoid formation because of competitive inhibition of the key enzyme in the pathway, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase: EC 1.1.1.34), by the corresponding lactone-opened forms (e.g., **5** and **6**).[5] The resulting decrease in availability of mevalonate caused by these compounds not only lowers mammalian sterol levels[3,5a,6] but also interferes with production of ubiquinone,[7] sea urchin dolichol,[8] insect juvenile hormones,[9] plant sterols and pigments,[10] and fungal gibberellins.[11] The importance of mevinolin (**1**) and compactin (**2**) as biochemical tools[12] and their unusual structures

(1) Mevinolin (**1**) from *Aspergillus terreus* and monacolin K from *Monascus ruber* (Endo, A. *J. Antibiot.* **1979**, *32*, 852-854. Endo, A. *Ibid.* **1980**, *33*, 334-336) appear to be the same compound; compactin (**2**) is also known as ML-236B.

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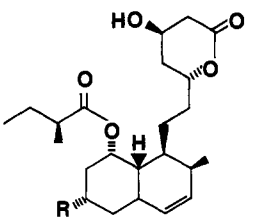
have inspired total[13] and partial[14,15] syntheses of both compounds as well as structural modifications by chemical[16] and microbiological[17] approaches. A number of structural relatives have also been isolated from various fungi. Mevinolin (**1**) co-occurs with dihydromevinolin (**3**) and the hydroxy acid **5** in *Aspergillus terreus*[2a,4a] and with monacolin J (**7**) in *Monascus ruber*.[18] Compactin (**2**) is found together with dihydrocompactin (**4**),[4b] ML-236A (**8**), ML-236C (**9**),[2c] and compound **10**[18] in *Penicillium citrinum*. Other naturally occurring analogues which possess the truncated three-carbon side chain include diplodiatoxin (**11**) from *Diplodia maydis*[19] and antibiotic LL-N313 ξ (versiol) (**12**) from *Sporormia affinis* and *Aspergillus versicolor*.[20] The common structural features of these compounds suggest that they may be formed by relatively similar and widespread biosynthetic pathways.

In addition to its inherent interest and challenge, an understanding of how Nature manufactures such materials affords hints for their chemical synthesis and is essential for rational control of their biological production. In this paper we expand our preliminary work[21] on the biosynthesis of mevinolin (**1**) by *Aspergillus terreus* ATCC 20542 and show it proceeds from acetate via a polyketide pathway. Some more modern NMR techniques (e.g., double quantum coherence, heteronuclear polarization transfer, isotope shift methods) are used in conjunction with mass spectrometry to determine the origin of the carbon, hydrogen, and oxygen atoms in **1** and to examine possible mechanisms for its formation.

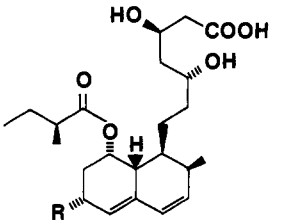
Experimental Section

General Remarks. Commercial reagents and solvents were analytical grade, or were purified by standard procedures[22] prior to use. Isotopically labeled oxygen gas, water, and sodium acetates were purchased from Cambridge Isotope Laboratories (Woburn, MA) except for sodium [$^2H_3$,^{13}C]acetate samples which were prepared by literature procedure[23] from the corresponding ^{13}C-labeled compounds. Isotopic content of all sodium acetate precursors was determined by conversion to the phenacyl ester[24] followed by mass spectrometric and/or ^{13}C NMR analysis. The isotopic purities were [1-^{13}C]acetate, 90% ^{13}C; [2-^{13}C]acetate, 97% ^{13}C; [1,2-$^{13}C_2$]acetate, 97% $^{13}C_2$; [1-^{13}C,$^{18}O_2$]acetate, 90% ^{13}C, 18% $^{18}O_1$, 81% $^{18}O_2$; [1-^{13}C,$^2H_3$]acetate, 90% ^{13}C, 98% ^2H; [2-^{13}C,$^2H_3$]acetate, 97% ^{13}C, 90% ^2H. Difco culture media were employed for fermentations unless otherwise stated.



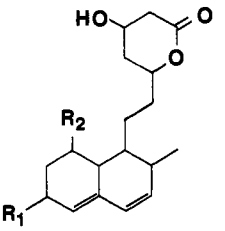
3 R = CH_3

4 R = H



5 R = CH_3

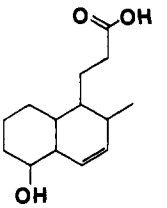
6 R = H



7 R_1 = CH_3, R_2 = OH

8 R_1 = H, R_2 = OH

9 R_1 = R_2 = H



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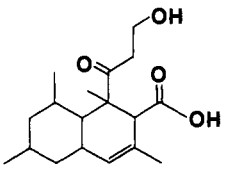

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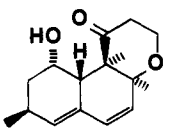

12

Fermentation of *Aspergillus terreus* and Incorporation of Labeled Sodium Acetate. *Aspergillus terreus* ATCC 20542 (American Type Culture Collection, Rockville, MD) was grown as described[2a] with the following modifications. Twelve-day-old agar slants (per liter: 20 g of malt extract, 20 g of D-glucose, 3 g of peptone, 20 g of agar) were used to inoculate 500 mL of sterile medium A[2a] (one 10-mL slant/flask). These were incubated on a rotary shaker (180 rpm) for 48 h at 26 °C. This inoculum (100 mL) was then partitioned across six similar flasks of medium B[2a] which were incubated in similar fashion for 48 h. Each flask was then injected with 1.0 mL of aqueous labeled sodium acetate solution (1.0 g/18 mL) every 24 h for 3 days. After a further 24 h, the combined fermentation mixtures were acidified to pH 4.0 with HCl, homogenized with ethyl acetate (600 mL) in a Waring blender, and filtered through multiple layers of cheesecloth. The mycelium was extracted again with ethyl acetate (600 mL), and the combined organic layers were dried (Na_2SO_4), concentrated in vacuo, and heated 30 min in refluxing benzene (200 mL). Concentration in vacuo and column chromatography on silica gel (50 g) with CH_2Cl_2/ethyl acetate (gradient elution: 9/1 to 6/4) afforded crude **1** which was further purified by preparative thin layer chromatography on Merck silica gel (5% methanol/ethyl ether or 10% methanol/chloroform) and recrystallization (ethyl acetate) to typically give 30 mg of pure labeled mevinolin (**1**). Its spectral (^1H NMR, ^{13}C NMR, IR, MS), chromatographic, and physical properties were compared to those of an authentic unlabeled sample donated by Merck, Sharp and Dohme Research Laboratories. Unlabeled material prepared by the above method had the following properties: mp 160–161 °C (lit.[2a] 174.5

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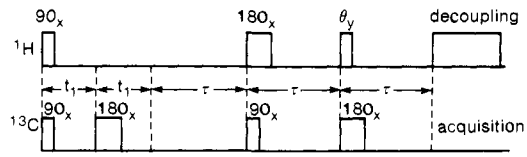

Figure 1. Pulse sequence for selective two-dimensional DEPT heteronuclear shift correlation. The t_1 value is incremented, τ is set to $1/(2J_{CH})$, and θ is set to 30°, 90°, and 150° to acquire spectra as described in the Experimental Section.

°C);[25] IR ($CHCl_3$ cast) 3200, 1725, 1280 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 0.1 M, 25 °C) δ 0.89 (3 H, t, J = 8, H-4′), 0.91 (3 H, d, J = 7, 2-Me), 1.08 (3 H, d, J = 8, 6-Me), 1.12 (3 H, d, J = 8, 2′-Me), 1.30 (1 H, m, H_a-10), 1.35-1.40 (3 H, m, H-9, H_a-3′), 1.5-1.75 (3 H, m, H_b-3′, H-1, H_a-12), 1.8-2.0 (4 H, m; H_b-10, H_b-12, H-7), 2.16 (1 H, br s, OH), 2.26 (1 H, dd, J = 12, 3, H-8a), 2.3-2.4 (2 H, m, H-2, H-2′), 2.45 (1 H, m; H-6), 2.62 (1 H, m, J = 18, 4, 2, H_a-14), 2.74 (1 H, dd, J = 18, 5, H_b-14), 4.38 (1 H, m, J = 4, H-13), 4.62 (1 H, m, H-11), 5.40 (1 H, q, J = 3, H-8), 5.54 (1 H, m, H-5), 5.78 (1 H, dd, J = 10, 6, H-3), 6.00 (1 H, d, J = 10, H-4).

Incorporation of [^{18}O]Oxygen Gas into Mevinolin (1) by *A. terreus*. Cultures of *A. terreus* were first grown 48 h on medium A as described above. A 100-mL portion was then transferred to 1.0 L of sterile medium B in a closed system fermentor (see supplementary material)[26] which was sealed under normal atmosphere. A modified Optima aquarium pump (Rolf Hagen Inc., Montreal), which had been sealed in glass, was used to circulate the atmosphere at 4.0 L/min through a coarse glass frit (65 mm) which supported the fermentation medium. The temperature of the circulating medium B was maintained at 26 °C by a water jacket connected to a Colora thermostated bath. Moisture was condensed from the exiting air by a water-cooled condenser coil, and CO_2 was removed by passing the air stream over 1 L of freshly prepared 3 N KOH. During the first 24 h, depleted oxygen was replaced by unlabeled oxygen gas via a pressure-equalizing water buret (2.0 L); [^{18}O]oxygen gas (50% isotopic purity) was then added for the next 72 h, and this was followed by normal $^{16}O_2$ for another 24 h. Oxygen consumption averaged about 25 mL of O_2 per hour per 100 mL of fermentation medium. Extraction of the mixture and purification as described above for sodium acetate experiments gave 7.0 mg of pure ^{18}O-labeled mevinolin (**1**). In other experiments the circulating fermentor was replaced by modified 500-mL Erlenmeyer flasks, each containing 100 mL of sterile medium B. These were connected in series to the system and incubated on a rotary shaker (320 rpm) at 26 °C with oxygen addition as described above. In a typical unlabeled experiment 23 mg of pure mevinolin was obtained from 1.0 L of fermentation medium B.

^{13}C NMR of Mevinolin (1). All spectra were obtained at 100.6 MHz on a Bruker WH400 spectrometer in the Fourier transform mode on solutions of mevinolin (**1**) in $CDCl_3$ (0.025-0.10 M) (5-mm tubes) at ambient temperature with Me_4Si internal standard. Normal broad band proton-decoupled spectra were obtained by using a 32K data block, 500-1000 scans, 25 000-Hz sweep width, 0.65-s acquisition time, and 45° pulse angle. For analysis of ^{18}O-labeled samples, signals for carbons bearing oxygen were subsequently expanded by using a 32K data block, 3700-6000 scans, 2000-Hz sweep width, 8.2-s acquisition time, and 45° pulse angle. These spectra were repeated with the spin-echo Fourier transform[27] (SEFT) pulse sequence $(90°-\tau-180°-\tau-\text{acquisition}-T)_N$ where τ is set to $1/(2J_{cc})$, T is the delay between sequences (3-10 s), and N is the number of accumulations (600). The two-bond coupling constant, J_{cc}, was estimated from the normal ^{13}C NMR spectrum, and the resulting value of τ was generally about 0.111 s. Analysis of mevinolin (**1**) derived from sodium [1-^{13}C,$^2H_3$]acetate for deuterium-induced β-isotope shifts employed conditions essentially identical with those used to detect ^{18}O isotope shifts. Examination of samples obtained from sodium [2-^{13}C,$^2H_3$]acetate incorporations for α-isotope shifts required a triple resonance probe with broad band ^1H and ^2H decoupling and a ^{19}F lock. These spectra were measured by using solutions of **1** (9-23 mg) in $CDCl_3$ (5-mm tubes) at ambient temperature with Me_4Si and C_6F_6 (5%) as internal standard and lock, respectively. Typical conditions were as follows: 64K data block, 6000 scans, 29 400-Hz sweep width, 1.11-s acquisition time, and 40° pulse angle. Two-dimensional INADEQUATE experiments on mevinolin (**1**) (0.1 M in $CDCl_3$, 5-mm tubes) derived from sodium [1,2-$^{13}C_2$]acetate followed literature procedures[28] optimized for $^1J_{cc}$ = 42 Hz and accumulated (128 scans) with 5-s recycle delay time and using spectral widths of ±3520 Hz (70 ppm) by 3496 Hz with a data block of (148 × 1 K). Selective two-dimensional DEPT heteronuclear shift correlation[29] spectra of unlabled **1** were measured on a Bruker WH200 spectrometer using the reported pulse sequence (Figure 1). The τ value ($1/(2J_{CH})$) was set to 0.0029 s (average $^1J_{CH}$ = 174 Hz), the recycle delay was 1.8 s, and the incremented ($1/(4$ ^{1}H sweep width)) time was initially 5 μs. Frequency ranges of 2000 Hz for ^{13}C and 3000 Hz for ^{1}H were covered in a typical experiment. The methine (CH) plot was available directly from the free induction decays (FID) with θ = 90°. The methylene (CH_2) and methyl (CH_3) plots were obtained by manipulation of FID (θ = 150°) - FID (θ = 30°) and FID (θ = 30°) + FID (θ = 150°) - FID (θ = 90°), respectively, as described previously.[29]

Mass Spectrometric Determination of $^{18}O_2$ Incorporation. The high-resolution electron impact (EI) (70 eV) mass spectrum of mevinolin (**1**) was determined on a Kratos AEI MS50 instrument: m/z (relative intensity) 404 (1.74, M^+), 302 (7.87, $M^+ - C_5H_{10}O_2$), 284 (7.37, $M^+ - C_5H_{10}O_2 - H_2O$), 269 (3.06, $C_{18}H_{21}O_2$), 251 (1.75, $C_{18}H_{19}O$), 224 (13.1, $C_{17}H_{20}$), 198 (66.3, $C_{15}H_{18}$), 159 (100, $C_{12}H_{15}$); exact mass calcd for $C_{28}H_{24}O_{28}$: 404.2566. Found 404.2567. Chemical ionization (CI) (isobutane) mass spectra on this sample were obtained on an AEI MS12 instrument: m/e (relative intensity) 405 (100, MH^+), 387 (7.1, $MH^+ - H_2O$), 302 (58.9, $M^+ - C_5H_{10}O_2$), 284 (25.8, $M^+ - C_5H_{10}O_2 - H_2O$). Mevinolin (**1**) derived from a fermentation with $^{18}O_2$ was analyzed under the same conditions as the unlabeled sample in both EI and CI modes. The regions for the peaks listed above were normalized separately to spectra of unlabeled compounds to obtain the ^{18}O content of each fragment. To determine the labeling pattern in the side chain, 1.08 mg of [^{18}O]mevinolin (**1**) in dry ether (1.0 mL) was treated with 1 M methyllithium in ether (0.5 mL) at 0 °C. This mixture was stirred 30 min under Ar atmosphere, quenched by careful dropwise addition of cold water (0.5 mL), extracted with ether (2 × 0.5 mL), and dried by filtration of the organic extracts through anhydrous Na_2SO_4. The resulting solution was injected onto an OV225 column (3% on Chromsorb W, 80-100 mesh, $^1/_8$ in. × 10 ft) mounted on a Varian Aerograph 1400 gas chromatograph attached to an AEI MS12 mass spectrometer. A temperature program of 5 min at 40 °C followed by increase at 10 °C/min to 150 °C eluted 2,3-dimethyl-2-pentanol (**13**) at 9.0 min after injection: mass spectrum, m/z (relative intensity) 101 (8.5, $M^+ - CH_3$), 98 (1.8), 83 (5.4), 59 (100). The identity of this sample and the total absence of ^{18}O labeling was confirmed by comparison to the gas chromatographic and mass spectral properties of 2,3-dimethyl-2-pentanol (**13**) prepared on a large scale by reaction of methyllithium with methyl 2-methylbutanoate.

Results and Discussion

Assignment of ^{1}H and ^{13}C NMR Spectra. Partial assignments of the ^{1}H NMR spectra of mevinolin (**1**) and dihydromevinolin (**3**) had been reported[2a,4a] prior to our initial work.[21] Since complete determination of NMR spectral positions is a prerequisite for stable isotope incorporation studies,[30] the 400-MHz proton spectrum of **1** was first fully assigned by using chemical shifts and selective homonuclear decoupling experiments. Our assignments (see Experimental Section) are generally in accord with data reported earlier[2a] except for H-8a, whose resonance position at δ 2.26 in the presence work is in agreement with that of the corresponding proton in 6α-hydroxycompactin.[17b]

Subsequent examination of both broad band proton-decoupled and gated ^{1}H-decoupled spin-echo Fourier transform[27] ^{13}C NMR spectra of mevinolin (**1**) did not permit complete unambiguous resonance identification, even with selective heteronuclear (^{1}H) decoupling experiments. The similarity of a number of carbon

(25) The melting point is uncorrected and was not changed by admixture with authentic mevinolin (**1**) obtained from Merck, Sharp and Dohme.

(26) We gratefully acknowledge Professor G. Maurice Gaucher (Chemistry Department, University of Calgary) for advice on fermentor design and Dr. R. Murray Ratcliffe (Chembiomed Ltd., Edmonton) for suggestions on the air pumping system.

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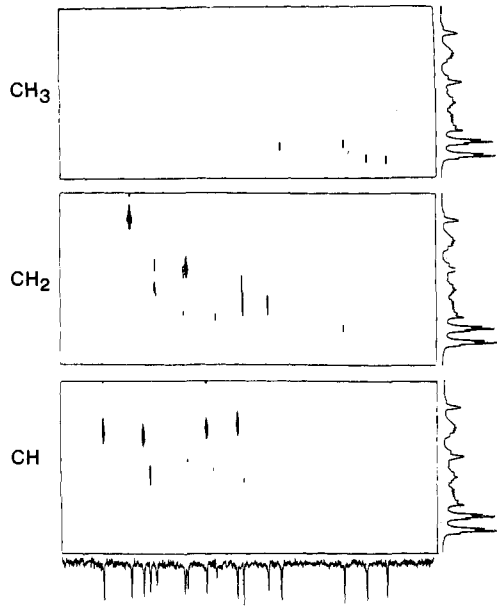

Figure 2. Selective 2D heteronuclear shift correlation maps for CH, CH_2, and CH_3 groups of unlabeled mevinolin (**1**). In each case the ^1H chemical shift (δ 0–2.5) is on the vertical axis and the carbon chemical shift (δ 10–50) is on the horizontal axis, with the groups appearing as contour plots. Normal ^1H and inverted ^{13}C NMR spectra are shown to the right and below, respectively. Some low-intensity "breakthrough" can be seen (e.g., the methyl signal in the bottom right corner of the CH_2 plot).

signals in multiplicity and ^{13}C NMR chemical shift was unfortunately reflected by ^1H NMR overlap of their attached hydrogen resonances. Two new NMR methods[31] overcame this difficulty. The first of these is selective two-dimensional heteronuclear shift correlation spectroscopy[29] which combines the distortionless enhanced polarization transfer (DEPT) pulse sequence[32] and a proton–carbon shift correlation experiment.[33] Application of this technique (Figure 1) to unlabeled mevinolin (**1**) produced separate maps for its CH, CH_2, and CH_3 groups (Figure 2) in which the chemical shift dispersion available from the carbon spectrum (horizontal axis) allowed resolution of heavily overlapped hydrogen resonances (vertical axis). This "spectroscopy of groups" simplifies assignment, identifies the multiplicity of each carbon resonance, and often permits clear observation of AB systems in the CH_2 plot which are normally buried under mounds of other proton resonances.

The second method of carbon signal assignment employed double quantum coherence NMR[28,34] in the form of the two-dimensional INADEQUATE experiment on a sample of **1** derived from a fermentation (see below) with sodium [1,2-$^{13}C_2$]acetate (97% $^{13}C_2$). In unlabeled molecules (1.1% ^{13}C per carbon) this NMR technique affords the carbon connectivity pattern by observation of natural abundance carbon–carbon coupling, but large quantities (0.5–1.0 g) of sample are often required for spectral analysis. However, application of this experiment to the multiply labeled mevinolin (**1**) routinely gave good spectra (Figure 3) on less than 50 mg (~0.1 mmol) of sample and permitted complete ^{13}C NMR assignment[35] because of a 200-fold increase in the relative intensity of coupled carbon signals (from 0.01% to about 2% for each adjacently coupled pair of carbon atoms). This approach to spectral assignment requires no knowledge of the

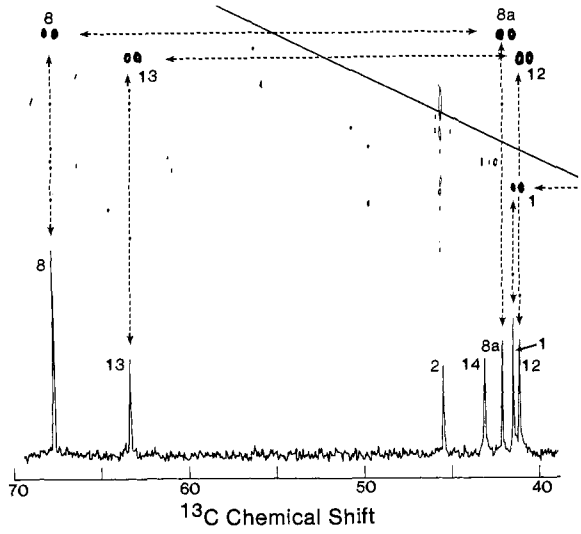

Figure 3. Overlay of a normal ^1H-decoupled ^{13}C NMR spectrum (50 MHz) of unlabeled mevinolin (**1**) on a 2D INADEQUATE spectrum of (**1**) derived from sodium [1,2-$^{13}C_2$]acetate.

Table I. ^{13}C NMR Data of Mevinolin (**1**) Derived from Sodium [1-^{13}C]-, [2-^{13}C]-, and [1-^{13}C,$^2H_3$]Acetate[a]

carbon	δ	^{13}C enhancement[b] 1-^{13}C	2-^{13}C	[1-^{13}C, $^2H_3$]acetate incorp ^2H: ^1H[c]	$\Delta\delta$[d]
1	36.7		2.4	24:76	
2	30.7	4.1			0.25, −0.17, −0.082[e]
2-Me	13.9		2.3	76:8:8:8[e]	
3	133.1		3.1	0:100	
4	128.4	3.3			
4a	131.6		4.4		
5	129.6	3.0			
6	27.5		5.2	0:100	
6-Me	22.9				
7	32.8	3.2			
8	68.0		1.4	45:55	
8a	37.4	4.6			+0.013
9	24.3	4.1			−0.096
10	33.0		2.2	80:20	
11	75.7	4.6			−0.06
12	36.2		2.4	50:50	
13	62.6	7.7			−0.12
14	38.7		3.4	50:50	
15	170.7	2.8			+0.04
1′	176.9	2.4			+0.018
2′	41.6		2.9	75:25	
2′-Me	16.3				
3′	26.8	4.4			−0.21, −0.14, −0.071[e]
4′	11.7		2.9	76:8:8:8[e]	

[a] For spectral conditions see Experimental Section. [b] Ratio of carbon signal intensities for enriched and natural abundance sample measured under identical conditions. [c] Ratio of carbon peak areas for β-isotope shifted signals. [d] β-Isotope shifts due to ^2H. [e] CD_3:CHD_2:CH_2D:CH_3.

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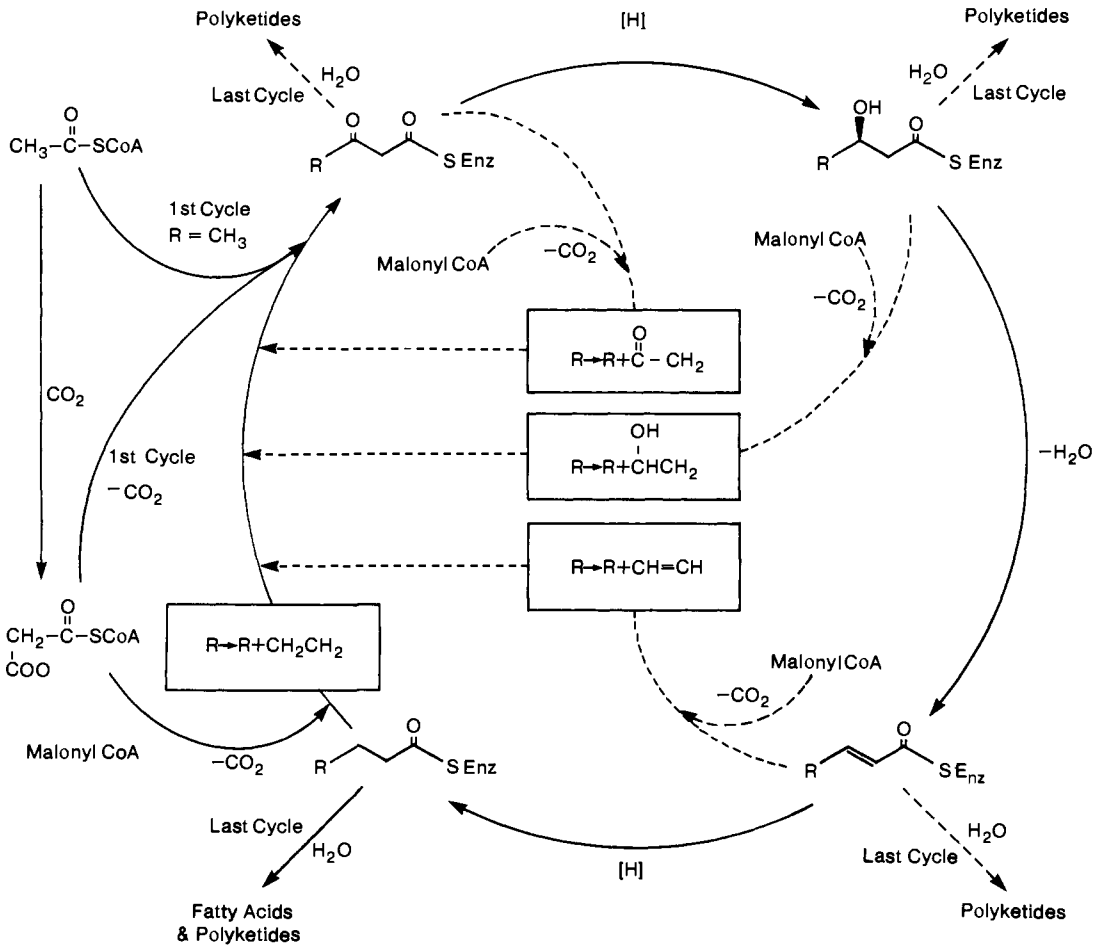
Scheme I. Relationship of Fatty Acid and Polyketide Biosynthesis[a]



[a] Solid lines in outer circle represent stages of fatty acid formation initiated in the first cycle by condensation of malonate and acetate and resulting in addition of a C_2H_4 unit ($R \rightarrow R + CH_2CH_2$) with each complete cycle. Dashed lines represent polyketide formation by addition of the next malonate before reduction is complete which causes incorporation of keto ($R \rightarrow R + COCH_2$), hydroxyl ($R \rightarrow R + CHOHCH_2$), or olefinic ($R \rightarrow R + CH=CH$) functionality on the chain.

biosynthetic distribution of label since it depends exclusively on increasing the level of ^{13}C in the molecule. Nevertheless, intact biosynthetic units can still be identified (see below). The complete carbon assignments obtained by selective 2D shift correlation and by double quantum coherence spectroscopy are given in Table I.

Incorporation of ^{13}C-Labeled Precursors. To define the biosynthetic origin of the carbon skeleton of mevinolin (**1**), we incorporated in separate experiments sodium [1-^{13}C]acetate, [2-^{13}C]acetate, and [1,2-^{13}C]acetate, as well as [*methyl*-^{13}C]-methionine, into fully grown shake cultures of *Aspergillus terreus*. The acetate precursors typically gave 2- to 7-fold peak enhancements for each labeled carbon in the ^{13}C NMR spectra (Table I), whereas the methionine enriched only the methyls at C-6 and C-2′ to afford remarkable 29-fold and 45-fold peak enhancements, respectively. Incorporation rates were confirmed by mass spectral comparison of the molecular ion regions of labeled and unlabeled samples. The pulse feeding technique which was used momentarily flooded the fermentation system with labeled precursor and led to multiple incorporations of [^{13}C]acetate within a single molecule of mevinolin (**1**). This allowed ^{13}C NMR observation of two-bond $^{13}C-^{13}C$ coupling between carbons of neighboring biosynthetic units and helped confirm the spectral assignments through selective homonuclear (^{13}C) decoupling experiments. Application of the two-dimensional INADEQUATE pulse sequence to **1** derived from sodium [1,2-$^{13}C_2$]acetate not only yielded the complete ^{13}C NMR assigment (see above) but also picked out the carbon–carbon bonds of acetate which remained intact during biosynthesis.[35] This combination of double quantum coherence spectroscopy[28,34] and incorporation of multiply labeled ^{13}C precursors in biosynthetic studies was reported in 1982[36] and has rapidly proved to be a very productive approach.[21,37] Even though it is possible to observe $^{13}C-^{13}C$ coupling at low levels (e.g., natural abundance) and thereby determine the complete carbon connectivity pattern, intact biosynthetic units can still be easily identified because there is a much higher level of coupled signals between carbons comprising them if multiply ^{13}C-labeled precursors are used.

The results (Table I) show that the main portion of mevinolin (**1**) consists of a polyketide chain of nine intact acetate units with a methionine-derived methyl group at C-6 (Figure 4). Interestingly, the α-methylbutyryl side chain is constructed in an analogous fashion. Somewhat unexpectedly, [U-^{14}C]-L-isoleucine, which has the correct configuration for the methyl group of the side chain, was not incorporated to any significant extent despite its well-known degradation to α-methylbutyryl-coenzyme A.[38] The origin of the branching methyl groups from L-methionine is typical of fungal metabolism, whereas Actinomycetes generally utilize propionate to provide the extra carbon.[39] According to the Birch–Collie hypothesis, the head-to-tail arrangement of

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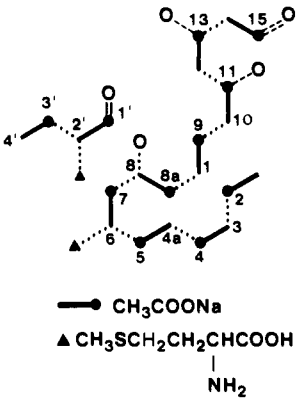

Figure 4. Biosynthetic distribution of acetate units and methionine-derived carbons in mevinolin (**1**).

acetate units in the main chains of polyketides results from a series of condensation–reduction steps similar to fatty acid formation.[40]

The genesis of functionality and the mode of cyclization remain central questions in biosynthetic studies on polyketides. In principle, keto, hydroxyl, or olefinic functionality could arise at appropriate points by condensation of the next acetate unit (as malonyl-coenzyme A) to the enzyme-bound growing chain before the normal fatty acid sequence of reduction–dehydration–reduction is complete (Scheme I). In this scenario, a highly reactive C-18 linear functionalized polyketide would cyclize either while it is still attached to enzyme which manufactured it or shortly after it is released. A few further modifications like oxidation at C-8 followed by addition of the ester side chain and/or methylation at C-6 would give mevinolin (**1**). Alternatively, every functionality required for cyclization as well as those remaining in **1** could be introduced by separate enzymes reoxidizing a partially or completely saturated fatty acid (e.g., stearic acid). Attempts to incorporate long fatty acids intact usually fail because of rapid β-oxidation and cleavage to acetate,[41] but recently hexanoate has been specifically utilized in the side chain of averufin.[42] In contrast, considerable evidence supports the "incomplete reduction pathway" for biosynthesis of aromatic compounds[40b,43] and for at least part of the route to polyether antibiotics[44] and macrolides.[44b,45] In order to help distinguish between the two major pathways and to determine possible intermediate oxidation states at various points along the chain, a series of oxygen and deuterium labeling experiments were done.

Incorporation of Sodium [1-^{13}C,$^{18}O_2$]Acetate and $^{18}O_2$. Since the appearance of intact carbon–oxygen bonds derived from acetate would confirm the incomplete reduction hypothesis, we administered doubly labeled sodium [1-^{13}C,$^{18}O_2$]acetate to cultures of *A. terreus* and analyzed the resulting mevinolin (**1**) by ^{13}C NMR. Detection of upfield shifts induced in the carbon spectrum by isotopic substituion with ^{18}O is a proven method for biosynthetic and mechanistic studies[43,46] which can detect unbroken bonds if doubly labeled precursors are used.[46a] Both normal and SEFT ^{13}C NMR spectra[27,43] showed extensive labeling of the doubly bonded oxygen at C-1′ (isotope shift 0.038 ppm). Even though ^{13}C incorporation was high (2- to 7-fold peak enhancement in ^{13}C NMR), the amount of oxygen-18 at other sites derived from C-1 of acetate, such as C-11, C-13, and C-15, was less than 5% of the carbon labeling. Initial experiments indicated that very small amounts of oxygen label might be present at these positions, but removal of interfering two-bond ^{13}C–^{13}C couplings by SEFT techniques failed to reveal consistently detectable quantities of ^{18}O (except at C-1′). This implies either solvent (H_2O) exchange at carbonyls during growth of the C-18 polyketide or late-stage aerobic oxidations of a deoxygenated chain after its release from the enzyme responsible for assembly from nine acetate units.

The latter hypothesis was tested by fermentation of *A. terreus* in a closed-system fermentor (see supplementary material)[26] wherein depleted oxygen was replaced by $^{18}O_2$. The quantity (7 mg) of [^{18}O]mevinolin (**1**) obtained proved to be too small for reliable ^{13}C NMR detection of isotope shifts, and it was therefore analyzed by mass spectrometry. High-resolution electron impact (EI) and normal chemical ionization (CI) mass spectra clearly showed that although most molecules bore only one or two oxygen-18 atoms, some contained up to four. The first EI fragmentation of unlabeled **1** produced a peak at m/z 302 which corresponds to the loss of α-methylbutyrate ($-C_5H_{10}O_2$). With [^{18}O]mevinolin (**1**) this cleavage of the side chain resulted in loss of 37% of the oxygen-18 present in the molecular ion. Although the exact structure of subsequent fragments has not been rigorously proven, it is clear from exact mass data and multiple labels in the ions that further fragmentations of the main mevinolin nucleus (with side chain absent) removed 20 ± 3% of the ^{18}O label as each remaining oxygen was lost. CI (isobutane) mass spectra confirm these observations. To check which oxygens in the α-methylbutyrate side chain were labeled by $^{18}O_2$, the mevinolin (**1**) was treated with excess methyllithium in ether to produce 2,3-dimethyl-2-pentanol (**13**), which was analyzed by gas chromatography/mass spectrometry. No oxygen-18 was detected in this compound, thereby showing that the label (37% of the total) resides exclusively in the singly bonded oxygen at C-8. This is completely consistent with labeling of only the doubly bonded oxygen at C-1′ by sodium [1-^{13}C,$^{18}O_2$]acetate and with the origin of C-8 from a methyl carbon of acetate.

The situation is quite different for the main chain formed from nine acetate units. Extensive utilization of $^{18}O_2$ at every oxygenated site and lack of substantial acetate oxygen incorporation appears to require late-stage aerobic oxidation of a deoxygenated precursor. Moreover, the unexpected appearance of label from $^{18}O_2$ in *both* oxygens at the carboxyl terminus (C-15) of **1** suggests either overreduction followed by reoxidation or cleavage of a longer chain precursor. It might be argued that the failure to incorporate aerobic oxygen at the α-methylbutyrate carbonyl and the inability to utilize exogenous acetate oxygen on the main chain could be due to exchange processes in combination with compartmentalization of the two biosyntheses at different cellular locations. Alternatively, acetate could be regenerated from a fatty acid by β-oxidation.[47] However, conversion of aerobic oxygen-18 to

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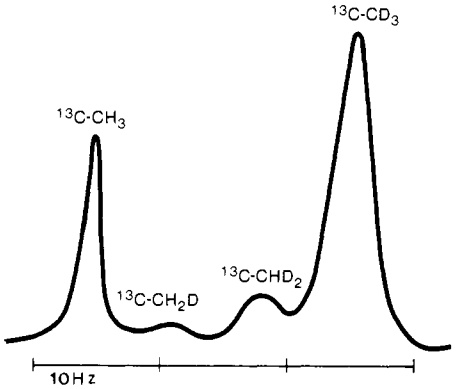


Figure 5. 100-MHz ^{13}C NMR spectrum of C-2 of mevinolin (**1**) derived from sodium [1-^{13}C,$^{2}H_3$]acetate showing β-isotope shifts.

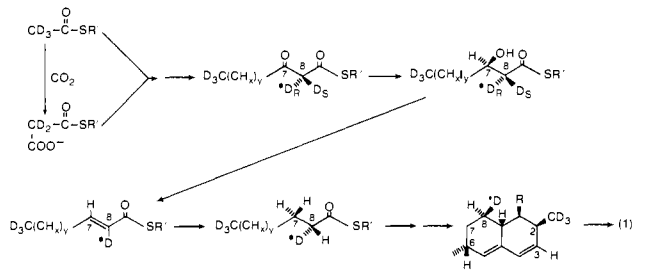
[^{18}O]water cannot account for the relatively large amount of incorporation from mixed $^{16}O/^{18}O$ atmosphere (total amount less than 0.5 mol) in unlabeled aqueous media (ca. 55 mol). At the inception of mevinolin (**1**) production, cell growth is essentially complete, glucose and acetate concentrations are low, and lipid amounts are large.[48] The high aeration requirement for production of **1** implies an oxidative process to form this highly reduced compound. This probably occurs by elaboration of a deoxygenated (but perhaps functionalized) chain. Compartmentalized generation of [^{18}O]acetate by aerobic oxidation of some acetate-derived intermediate appears unlikely in view of the relatively uniform carbon incorporation in both chains of mevinolin (**1**) and the failure to utilize aerobic oxygen at the C-1′ carbonyl. In order to confirm this and to obtain additional information on biosynthetic mechanisms, the source of hydrogens in **1** was examined.

Incorporation of Deuterated Precursors. Since the regiochemical and stereochemical distribution of acetate-derived hydrogens during fatty acid biosynthesis is known,[45b,49] knowledge of the fate of these atoms during mevinolin (**1**) formation would provide valuable information about possible intermediates. Of the many methods developed to detect labeled hydrogens by magnetic resonance,[30a,50] the observation of deuterium-induced β-isotope shifts in ^{13}C NMR is among the simplest and most useful.[46k,l,51] Incorporation of sodium [1-^{13}C,$^{2}H_3$]acetate into **1** and application of this technique indicated high deuterium retention at all expected sites except C-3 and C-6 (Table I). The presence of species bearing three deuteriums at C-4′ and at the C-2 methyl (Figure 5) identifies these carbons as starter units which come directly from acetate without the intermediacy of malonyl-coenzyme A.[45b,52,49a] Presence of relatively large amounts of CD_3 species at the latter methyl group demonstrates that regeneration of acetate by β-oxidation of an intermediate fatty acid is *not* a major pathway for the main chain biosynthesis. If that were the case, most of the acetate would have lost deuterium during the construction–degradation sequence.[47,49] Apparently the major route involves aerobic oxidation of a longer deoxygenated chain. The presence of only a single deuterium (within experimental error) at the other sites supports the intermediacy of a fat-like molecule.

Since β-isotope shifts may be upfield, downfield, or possibly zero,[51f] there was a small chance that C-3 and C-6 bore deuterium on the basis of these experiments. However, incorporation of sodium [2-^{13}C,$^{2}H_3$]acetate into mevinolin (**1**) and analysis of the ^{13}C NMR spectrum with simultaneous ^{1}H and ^{2}H decoupling (^{19}F lock)[30a,50d] confirmed the lack of significant amounts of deuterium at these two positions because of the absence of observable α-isotope shifts.

Stereochemistry at deuterium-labeled positions can provide valuable hints about biosynthesis of reduced polyketides.[45b] The co-occurrence of monacolin J (**7**) and mevinolin (**1**) in *Monascus ruber*[18] and the isolation of ML-236A (**8**) and ML-236C (**9**) with compactin (**2**) in *Penicillium citrinum*[2c] suggest that addition of the side chain proceeds by hydroxylation of a methylene at C-8 followed by esterification. If we make the reasonable assumption that hydroxylation occurs with the usual retention of configuration,[53] the deuterium at C-8 would have occupied the pro-*R* position in the growing polyketide chain after complete reduction of an adjacent carbonyl to the methylene group which becomes C-7 (Scheme II). This is the same stereochemistry that has been elegantly demonstrated for fatty acid synthesis in *Penicillium brefeldianum*.[45b] The exact meaning of deuterium retention at C-2′ and C-1 and of its loss at C-6 and C-3 remains unclear because the stereochemistry of additional bond-forming reactions at those positions is unknown. Configurations of the single deuteriums at methylene groups like C-14, C-12, and C-10 are uncertain but are currently being investigated to determine whether they correspond to that at C-8.

Scheme II. Possible Mechanism for Stereospecific Deuterium Incorporation at C-8 of Mevinolin (1)



Conclusions

The present work illustrates the power of modern NMR techniques in combination with multiple isotope labeling to assign spectra and detect cleavage or integrity of bonds during biosynthesis. In particular, application of 2D DEPT heteronuclear shift correlation to unlabeled mevinolin (**1**) and 2D INADEQUATE to samples derived from doubly labeled [$^{13}C_2$]acetate facilitated a difficult assignment of ^{13}C NMR spectra. The latter method also showed that this fungal metabolite consists of two polyketide chains (4-carbon and 18-carbon) with intact acetate units coupled in head-to-tail fashion (Figure 5). Two remaining carbons are supplied by the methyl group of L-methionine. Location of intact C–H and C–O bonds derived from acetate by observation of ^{18}O- and ^{2}H-induced isotope shifts in normal and SEFT ^{13}C NMR spectra demonstrated that enzymes very similar to those of fatty acid biosynthesis are involved in mevinolin (**1**) formation. CI and EI mass spectrometric analyses of **1** obtained by fermentation of *A. terreus* under $^{18}O_2$ atmosphere suggested that all oxygens on the main polyketide chain are introduced primarily by late-stage

(47) Compartmentalization is known to occur in mammalian β-oxidation of fatty acids: Bremer, J.; Osmundsen, H. In "Fatty Acid Metabolism and its Regulation"; Numa, S., Ed.; Elsevier: New York, 1984; pp 113–154. See also: Kindl, H., *Ibid.* pp 187–191.

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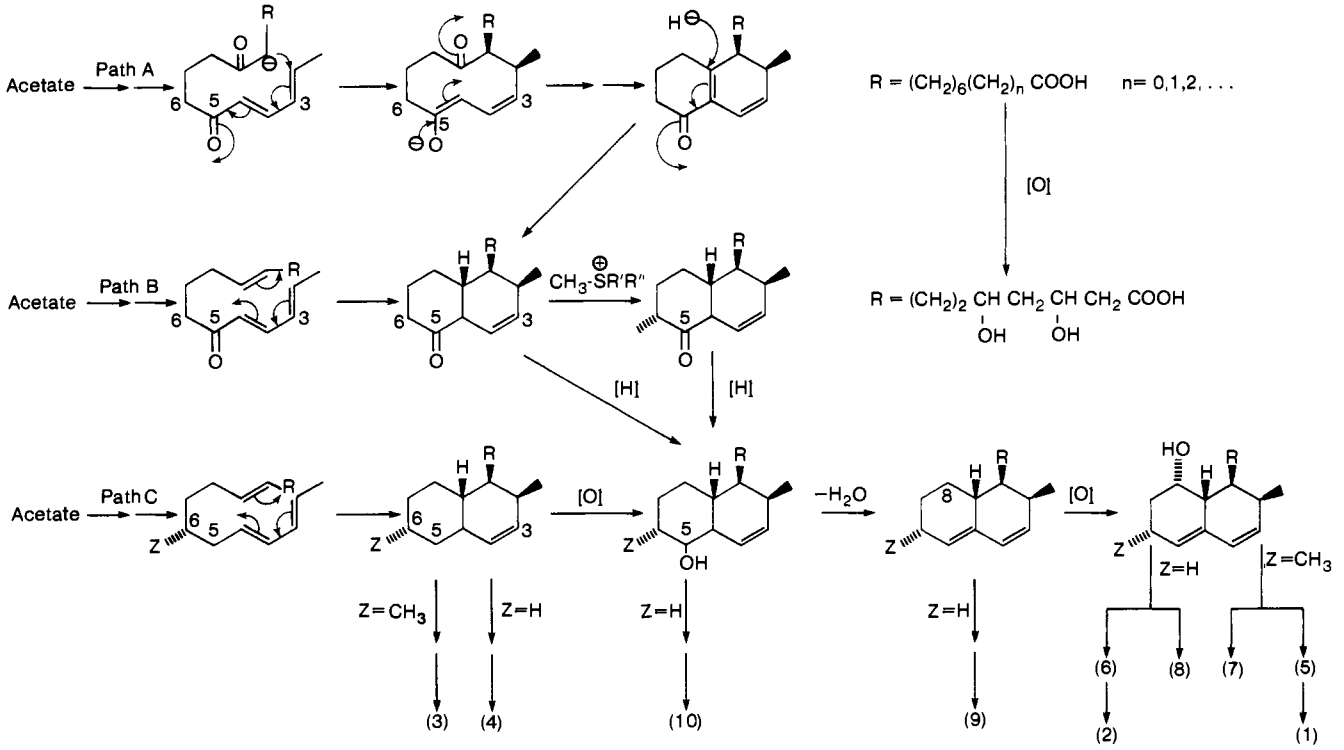
Scheme III. Possible Mechanisms for Formation of Mevinolin (**1**), Compactin (**2**), and Related Metabolites (3 → 10)



oxidation of a longer deoxygenated precursor.

Although the exact structures of the key intermediates between acetate and mevinolin (**1**) are still unknown, the present study permits construction of plausible working hypotheses. Condensation of acetate units (via malonate) with incomplete reduction (Scheme I) could lead to long carbon chain ($\geq C_{18}$) in one of a variety of oxidation states, as shown in Scheme III. Path A relies on intramolecular aldol and Michael condensations which are well precedented in biosynthesis of aromatic compounds[40] but require retention of acetate oxygen near the starter end of the polyketide chain. With some exceptions like brefeldin A,[45b] such chains tend to be most highly reduced at this end.[46a] Paths B and C make use of a biological Diels–Alder reaction to generate the correct ring stereochemistry in a single step. Such ring-forming processes have been suggested for a number of reduced polyketide metabolites, including cytochalasins,[54] nargenicin and related systems,[46h] and endiandric acids,[55] but to the best of our knowledge no enzyme-catalyzed Diels–Alder reaction has yet been demonstrated. Paths A and B could explain the loss of acetate hydrogen at C-3 and C-6 through enolizations of the keto group at C-5 which are employed to close the rings and introduce the methyl. Path C does not obviously encourage the acetate hydrogen loss at C-3, but it is very attractive because it can lead directly to dihydromevinolin (**3**) or dihydrocompactin (**4**) from intermediates on the route to **1** or **2**. Aerobic hydroxylation at C-5 (path C) or reduction of a carbonyl at that position (paths A and B) would give an alcohol whose side chain could be oxidatively shortened to three carbons to form **10**. Oxidative modification of the *R* group to a seven-carbon dihydroxy carboxylic acid moiety would generate the other co-occurring metabolites (**5–9**) as shown in Scheme III. In contrast to *Penicillium* species which produce main-chain unmethylated metabolites (Z = H), *Aspergillus* and *Monascus* fungi add a C_1 unit at C-6.[18] The timing of this is unknown, but may occur during polyketide assembly.[40] Determining the actual sequence of events in these biosyntheses as well as those of other reduced cyclic polyketides[56] will continue to challenge biologists and chemists. Work is in progress to elucidate the biochemical details of the formation of mevinolin (**1**), compactin (**2**), and related metabolites.

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Registry No. 1, 75330-75-5; O_2, 7782-44-7; acetic acid, 64-19-7; L-methionine, 63-68-3.

Supplementary Material Available: Description of apparatus for closed system fermentations under ^{18}O atmosphere (1 page). Ordering information is given on any current masthead page.

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