***trans*-anti-*trans*-4,5-[5α-(3-Oxobutyl)-4-oxo-1,4,5,6-tetrahydrobenzo]-7aβ-methylhydrindan-1-spiro-2′-(1′β-5′-oxotetrahydrofuran) (47).** A mixture of mercuric trifluoroacetate (53 mg, 0.124 mmol), the lactone **46** (30 mg, 0.08 mmol), and 2 mL of methylene chloride was stirred for 1 h at room temperature. The reaction mixture was diluted with 10 mL of water and extracted with chloroform (20 mL × 3). The combined extracts were washed with saturated aqueous NaCl solution and dried ($MgSO_4$). The residue upon workup was chromatographed on silica gel (1 g) with methylene chloride–chloroform (1:4 v/v) to give the diketone **47** (28 mg, 98%) as a colorless oil: IR ($CHCl_3$) 1760 (C=O), 1705 (C=O), 1665 (C=O) cm^{-1}; ^1H NMR (90 MHz, $CDCl_3$) δ 1.06 (3 H, s, CH_3), 2.15 (3 H, s, O=CCH_3), 6.00 (1 H, dd, J = 10.5 and 2.9 Hz, COCH=C*H*), 6.82 (1 H, d, J = 10.5 Hz, COC*H*=CH); mass spectrum, m/z 344 (M^+); exact mass calcd for $C_{21}H_{28}O_4$ 344.1987 (M^+), found 344.2012 (M^+).

19-Norcanrenone (3). A mixture of the diketone **47** (39 mg, 0.113 mmol), 0.4 mL of concentrated hydrochloric acid, 0.1 mL of water, and 4 mL of acetic acid was stirred for 90 h at room temperatue, diluted with 20 mL of water, and extracted with chloroform (20 mL × 3). The combined extracts were washed successively with saturated aqueous $NaHCO_3$ and NaCl solutions and dried ($MgSO_4$). The residue upon workup was chromatographed on silica gel (1 g) with methylene chloride–chloroform (1:1 v/v) to give 19-norcanrenone (**3**) (20.1 mg, 54%) as colorless prisms (from methylene chloride–*n*-hexane): mp 192–193 °C (lit.[2] 191–192 °C); IR ($CHCl_3$) 1765 (C=O), 1655 (C=O) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.88 (1 H, dd, J = 6.0 and 14.0 Hz), 1.03 (3 H, s), 1.10–1.20 (1 H, m), 1.26–1.43 (5 H, m), 1.52–1.65 (2 H, m), 1.85–1.96 (3 H, m), 2.22 (1 H, t, J = 12.5 Hz), 2.28–2.41 (4 H, m), 2.46–2.67 (3 H, m), 5.80 (1 H, s), 6.16 (1 H, d, J = 10.1 Hz), 6.22 (1 H, dd, J = 10.1 and 3.0 Hz); mass spectrum, m/z 326 (M^+); exact mass calcd for $C_{21}H_{26}O_3$ 326.1882 (M^+), found 326.1883 (M^+).

Acknowledgment. We thank Professor W. S. Johnson and Dr. C. Newton at Stanford University for a generous gift of authentic samples of 19-norcanrenone and 19-norspironolactone, which were used throughout this work. We also thank K. Mushiake, K. Koike, M. Inada, and H. Nagai of this Institute, Tohoku University, for microanalyses, spectral measurements, and preparation of the manuscript.

Biosynthesis and Full NMR Assignment of Fungichromin, a Polyene Antibiotic from *Streptomyces cellulosae*

Hiroshi Noguchi, Paul H. Harrison, Kunizo Arai, Thomas T. Nakashima, Laird A. Trimble, and John C. Vederas*

Contribution from the Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2. *Received August 10, 1987*

Abstract: The biosynthesis and NMR signal assignments of the antifungal polyene antibiotic fungichromin (**1**) were determined. NMR analysis after administration of sodium [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]acetates, [1-^{13}C]propionate, and [1-^{13}C]- and [3-^{13}C]octanoates to cultures of *Streptomyces cellulosae* ATCC 12625 showed that **1** is derived from one propionate unit, 12 acetate units, and one intact octanoate unit, condensed in the head-to-tail fashion typical of polyketide biogenesis. The results with octanoate are the first case of incorporation of a fatty acid with more than four carbon atoms as a unit into a polyketide without significant degradation. Incorporation of ethyl [CD_3]oleate gave **1** labeled specifically at C-6′, thereby demonstrating that this unit is produced from oleate. Separate incorporations of sodium [1-^{13}C,$^{18}O_2$]acetate, [1-^{13}C,$^{18}O_2$]propionate, and [1-^{13}C,$^{18}O_2$]octanoate as well as $^{18}O_2$ into **1** followed by ^{13}C NMR analysis indicated retention of intact carbon–oxygen bonds at all expected sites. Diethyl [2-^{13}C]malonate was incorporated into **1** as acetate. Efficient incorporation of a mixture of diethyl [2-^{13}C]- and [1,3-$^{13}C_2$]malonates gave **1**, the 2D INADEQUATE spectrum of which gave "interunit" carbon–carbon connectivities complementary to the "intraunit" connectivities obtained from incorporation of sodium [1,2-$^{13}C_2$]acetate. This technique allowed full assignment of the ^{13}C NMR spectrum of **1** for the positions derived from acetate with small amounts of compound.

Fungichromin (**1**) and filipin III (**2**) belong to the class of macrocyclic polyene antibiotics, a group of over 200 compounds, produced primarily by *Streptomyces* species, that possess antifungal and antiprotozoal activity.[1,2] Structurally, all of these compounds contain a chain of three to eight conjugated double bonds within a macrocyclic lactone ring that also has a corresponding saturated fragment adorned with hydroxyl groups.[1a] Despite their toxicity[1b,3] and the development of other classes of antifungal antibiotics,[4] the polyenes (especially amphotericin B and nystatin) remain the best treatment for many fungal infections in humans.[1b,4a] The antihypercholesterolemic,[3a,5] antitumor,[6] and antiviral[7] activities of such steroid-binding[8] polyenes have also

(1) For reviews, see: (a) Omura, S.; Tanaka, H. In *Macrolide Antibiotics–Chemistry, Biology and Practice*; Omura, S., Ed.; Academic: New York, 1984; pp 351–404. (b) Schaffner, C. P. *Ibid.* pp 457–507. (c) Kotler-Brajtburg, J.; Medoff, G.; Kobayashi, G. S.; Boggs, S.; Schlessinger, D.; Pandey, R. C.; Rinehart, K. L., Jr. *Antimicrob. Agents Chemother.* **1979**, *15*, 716–722.

(2) Fungichromin (**1**) from *S. cellulosae* is identical with lagosin from *S. roseoluteus* and with cogomycin from *S. fradiae*: (a) Pandey, R. C.; Guenther, E. C.; Aszalos, A. A. *J. Antibiot.* **1982**, *35*, 988–996. (b) Tytell, A. A.; McCarthy, F. J.; Fisher, W. P.; Bolhofer, W. A.; Charney, J. *Antibiot. Annu.* **1955**, *716*–718. (c) Shirling, E. B.; Gottlieb, D. *Int. J. Syst. Bacteriol.* **1968**, *18*, 279–392. (d) Berry, M. P.; Whitting, M. C. *J. Chem. Soc.* **1964**, 862–863. (e) Pozsgay, V.; Tamas, J.; Czira, G.; Wirthlin, T.; Levai, A. *J. Antibiot.* **1976**, *29*, 472–476.

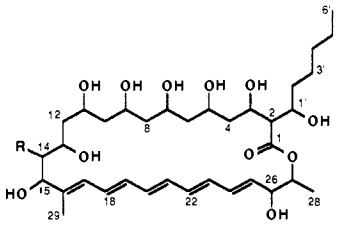
(3) (a) Nilsson-Ehle, I.; Yoshikawa, T. T.; Edwards, J. E.; Schotz, M. C.; Guze, L. B. *J. Infect. Dis.* **1977**, *135*, 414–422. (b) Nilsson-Ehle, I. *Acta Pathol. Microbiol. Scand., Sect. B: Microbiol.* **1977**, suppl. *259*, 61–66. (c) Witzke, N. M. W. Ph.D. Thesis, Rutgers, **1981**; *Diss. Abstr. Int. B* **1981**, *42*, 191.

(4) (a) Lefler, E.; Brummer, E.; Perlman, A. M.; Stevens, D. A. *Antimicrob. Agents Chemother.* **1985**, *27*, 363–366. (b) Hopfer, R. L.; Fainstein, V. *Clin. Lab. Annu.* **1984**, *3*, 425–438.

(5) (a) Gordon, H. W.; Schaffner, C. P. *Proc. Natl. Acad. Sci. U.S.A.* **1968**, *60*, 1201–1208. (b) Schaffner, C. P.; Gordon, H. W. *Proc. Natl. Acad. Sci. U.S.A.* **1968**, *61*, 36–41.

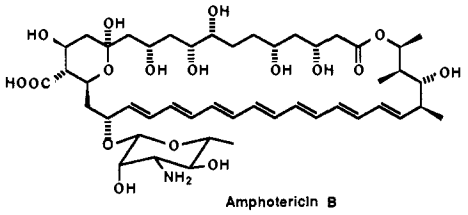
(6) (a) Akiyama, S.; Hidaka, K.; Komiyama, S.; Kuwano, M. *Cancer Res.* **1979**, *39*, 5150–5154. (b) Laurent, G.; Dewerie-Vanhouche, J.; Machin, D.; Hildebrand, J. *Cancer Res.* **1980**, *40*, 939–942. (c) Vertut-Croquin, A.; Brajtburg, J.; Medoff, G. *Cancer Res.* **1986**, *46*, 6054–6058 and references therein. (d) Zhen, Y. S.; Reardon, M. A.; Weber, G. *Biochem. Biophys. Res. Commun.* **1986**, *140*, 434–439.

0002-7863/88/1510-2938$01.50/0



1 R = OH Fungichromin

2 R = H Filipin III



Amphotericin B

attracted considerable interest. Very recently, the total chemical synthesis of amphotericin B and associated degradation studies were reported.[9]

Biosynthetically, the macrocyclic ring of the polyenes derives from acetate and propionate, as shown by labeling studies with radioactive precursors.[10] Birch and co-workers found good incorporations of ^{14}C-labeled acetate and propionate into nystatin aglycon.[11] Perlman and Semar used [2-^{14}C]acetate to prepare radiolabeled amphotericin B for clinical studies,[12] and other analogous results have been reported for this drug[13] as well as for lucensomycin,[14] candicidin,[15] fungimycin,[16] and levorin.[17] Inhibition of polyene production by cerulenin[10b] and failure to incorporate [1-^{14}C]mevalonic acid[11] or [*methyl*-^{14}C]-methionine[11,12,13a] were interpreted as evidence for a polyketide biogenesis.[10b] However, the arrangement of biosynthetic units and possible intermediate oxidation states remained speculative because of the difficulty in determining the sites of labeling by degradation of these antibiotics. No NMR studies of macrolide polyene antibiotic biosynthesis using stable isotope techniques appear to have been published, with the exception of our preliminary report on fungichromin (**1**).[18] Current interest in polyketide formation[19,20] and our belief that biosynthetic isotopic enrichment could be a valuable tool for NMR assignment and structure elucidation prompted the present investigation of fungichromin biosynthesis by *Streptomyces cellulosae* ATCC 12625.

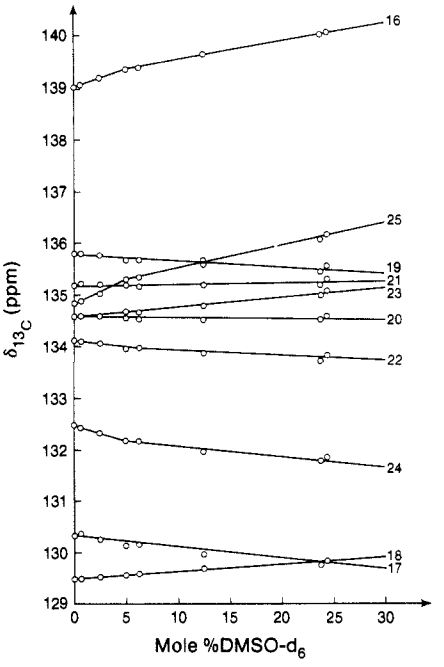


Figure 1. ^{13}C NMR chemical shifts of the "polyene" region (C-16–C-25) of fungichromin (**1**) as a function of DMSO concentration.

Results

Assignment of ^{1}H and ^{13}C NMR Spectra. In order to obtain sufficient **1** in solution for 2D NMR experiments, varying amounts of DMSO-d_6 in methanol-d_4 were used as solvent. Optimum solubility is obtained when DMSO is added before methanol, which precludes the use of a premixed standard solvent. Systematic variation of solvent composition showed that the ^{13}C NMR chemical shifts of some of the polyene carbons cross over or coalesce at certain concentrations of DMSO (Figure 1). Although chemical shifts of other (non-polyene) carbons do change, no crossover of those signals occurs with change in mole fraction of DMSO. The mole fraction values given were obtained by comparison of the integrals of the carbon signals of the two solvents in spectra measured under identical conditions. However, the fractions do not represent exact compositions because of differences in the relaxation rates of the solvents.

Initial NMR experiments using ^{1}H and ^{13}C chemical shifts, ^{1}H homonuclear decoupling, proton COSY, and ^{1}H,^{13}C heteronuclear shift correlation allowed assignment of a number of proton and carbon-13 signals of fungichromin (**1**) as follows.[2,21-23] The H-28 signal appears as a unique methyl doublet; the C-28 signal

(7) (a) Schneider, M. A. *Mol. Genet., Mikrobiol. Virusol.* **1984**, *5*, 41–46. (b) Malewicz, B.; Momsen, M.; Jenkin, H. M.; Borowski, E. *Antimicrob. Agents Chemother.* **1984**, *25*, 772–774.

(8) For a review, see: Norman, A. W.; Spielvogel, A. M.; Wong, R. G. *Adv. Lipid Res.* **1976**, *14*, 127–170.

(9) (a) Nicolaou, K. C.; Daines, R. A.; Chakraborty, T. K.; Ogawa, Y. *J. Am. Chem. Soc.* **1987**, *109*, 2821–2822 and references therein. (b) Nicolaou, K. C.; Chakraborty, T. K.; Daines, R. A.; Ogawa, Y. *J. Chem. Soc., Chem. Commun.* **1987**, 686–689.

(10) For reviews see: (a) Martin, J.-F. *Annu. Rev. Microbiol.* **1977**, *31*, 13–38. (b) Martin, J.-F. In *Biochemistry and Genetic Regulation of Commercially Important Antibiotics*; Vining, L. C., Ed.; Addison-Wesley: Reading, 1983; pp 207–229. (c) Martin, J.-F. In *Macrolide Antibiotics–Chemistry, Biology and Practice*; Omura, S., Ed.; Academic: New York, 1984; pp 405–424.

(11) Birch, A. J.; Holzapfel, C. W.; Rickards, R. W.; Djerassi, C.; Suzuki, M.; Westley, J.; Dutcher, J. D.; Thomas, R. *Tetrahedron Lett.* **1964**, 1485–1490.

(12) Perlman, D.; Semar, J. B. *Biotechnol. Bioeng.* **1965**, *7*, 133–137.

(13) (a) Linke, H. A. B.; Mechlinski, W.; Schaffner, C. P. *J. Antibiot.* **1974**, *27*, 155–160. (b) Monji, N. Ph.D. Thesis, Rutgers, 1975; *Diss. Abstr. Int. B* **1975**, *36*, 2195–2196. (c) Liu, Y. T. Ph.D. Thesis, Rutgers, 1981; *Diss. Abstr. Int. B* **1982**, *42*, 3577.

(14) Manwaring, D. G.; Rickards, R. W.; Gaudiano, G.; Nicolella, V. *J. Antibiot.* **1969**, *22*, 545–550.

(15) (a) Liu, C. M.; McDaniel, L. E.; Schaffner, C. P. *J. Antibiot.* **1972**, *25*, 116–121. (b) Martin, J.-F.; Liras, P. *J. Antibiot.* **1976**, *29*, 1306–1309.

(16) Liu, C. M.; McDaniel, L. E.; Schaffner, C. P. *J. Antibiot.* **1972**, *25*, 187–188.

(17) Belousova, I. I.; Lishenevskaya, E. B.; Elgart, R. E. *Antibiotiki (Moscow)* **1971**, *16*, 684–687.

(18) Harrison, P. H.; Noguchi, H.; Vederas, J. C. *J. Am. Chem. Soc.* **1986**, *108*, 3833–3834.

(19) For reviews, see: (a) Simpson, T. J. *Nat. Prod. Rep.* **1985**, *2*, 321–347. (b) Vederas, J. C. *Ibid.* **1987**, *4*, 277–377. (c) Simpson, T. J. *Chem. Soc. Rev.* **1987**, *16*, 123–160.

(20) For discussions of the mechanism of polyketide formation, see: (a) Moore, R. N.; Bigam, G.; Chan, J. K.; Hogg, A. M.; Nakashima, T. T.; Vederas, J. C. *J. Am. Chem. Soc.* **1985**, *107*, 3694–3701. (b) Yue, S.; Duncan, J. S.; Yamamoto, Y.; Hutchinson, C. R. *J. Am. Chem. Soc.* **1987**, *109*, 1253–1255. (c) Cane, D. E.; Yang, C. C. *J. Am. Chem. Soc.* **1987**, *109*, 1255–1257.

(21) For recent descriptions of 2D NMR, see: (a) Derome, A. E. *Modern NMR Techniques for Chemical Research*; Pergamon: Oxford, 1987. (b) Sanders, J. K. M.; Hunter, B. K. *Modern NMR Spectroscopy*; Oxford University: Oxford, 1987.

(22) For some previous NMR studies on polyene antibiotics, see: (a) Brown, J. M.; Sidebottom, P. J. *Tetrahedron* **1981**, *37*, 1421–1428. (b) Tweit, R. C.; Pandey, R. C.; Rinehart, K. L., Jr. *J. Antibiot.* **1982**, *35*, 997–1012. (c) Radics, L.; Incze, M.; Dornberger, K.; Thrum, H. *Tetrahedron* **1982**, *38*, 183–189. (d) Sowinski, P.; Pawlak, J. K.; Borowski, E.; Iwashita, T. *J. Antibiot.* **1985**, *38*, 175–180. (e) Aszalos, A.; Bax, A.; Burlinson, N.; Roller, P.; McNeal, C. *J. Antibiot.* **1985**, *38*, 1699–1713.

(23) We are indebted to Dr. Charles Rodger (Bruker Canada) and to Dr. David Bundle (NRC, Ottawa) for time to accumulate spectra on 500-MHz instruments.

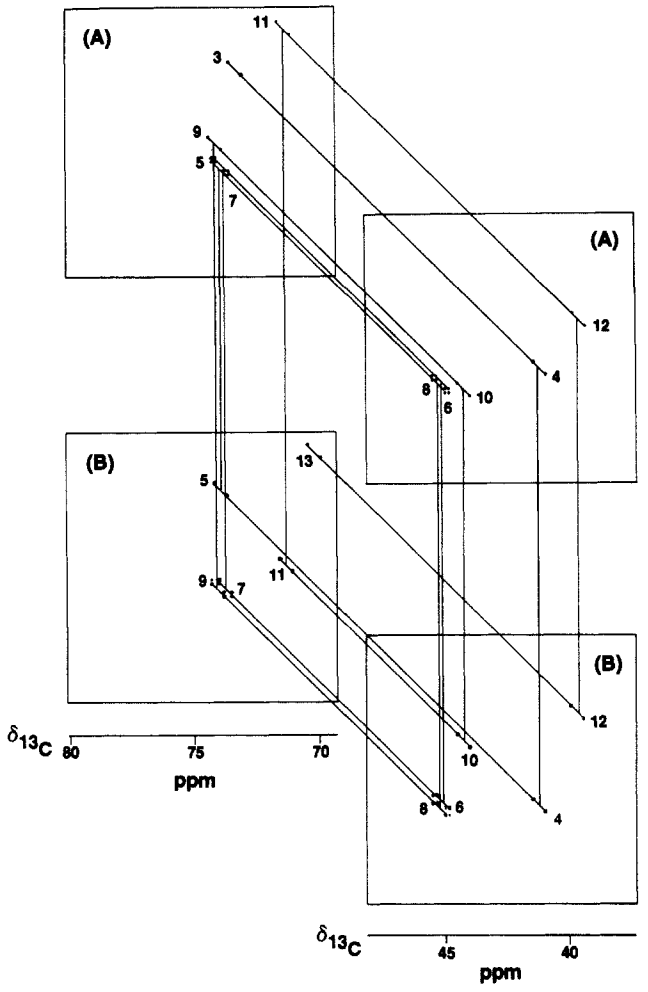


Figure 2. Tilted 2D INADEQUATE spectra of the "hydroxyl" (80–70 ppm) and "methylene" (50–35 ppm) regions of **1** enriched by incorporation of (A) sodium [1,2-$^{13}C_2$]acetate; (B) a 1:1 mixture of diethyl [2-^{13}C]- and [1,3-$^{13}C_2$]malonates.

was therefore assigned by ^{1}H,^{13}C correlation. Decoupling of H-28 assigned H-27 and hence C-27. Upon decoupling H-26, the signals at H-25 and H-27 partially collapse. Thus, C-26 and C-25 could be assigned by correlation. From H-25, the COSY spectrum gave H-24 and hence C-24. Also from the COSY spectrum, H-17 could be assigned since it couples to only one other proton in the polyene region, H-18. The H-18 couples to H-19, and assignments for C-17, C-18, and C-19 followed from ^{1}H,^{13}C correlation. Similar reasoning from such experiments allowed assignment of C-16 (by attached proton test[24] as the only quaternary polyene carbon), H-29/C-29, H-15/C-15, H-14/C-14, H-13/C-13, H-2/C-2, H-6′/C-6′, and C-1. Assignments for C-2′ to C-5′ agree with those of Pandey and co-workers,[2] and are based on extensive studies of ^{13}C chemical shifts for compounds with long alkyl chains. However, a large number of resonances in the polyhydroxyl and polyene regions could only be grouped according to type because of extensive overlap in both the ^{1}H and ^{13}C NMR spectra.

To solve this problem, 2D INADEQUATE spectra[21,25] were obtained on samples of fungichromin (**1**) biosynthetically derived (see below) from sodium [1,2-$^{13}C_2$]acetate and from a 1:1 mixture of diethyl [2-^{13}C]- and [1,3-$^{13}C_2$]malonates.[26] The former sample (A) shows only *intraunit* couplings (i.e., coupled carbons are within the same acetate unit) whereas the latter sample (B) displays only *interunit* couplings (i.e., coupled carbons are in adjacent acetate units). Portions of these spectra for the "northern hemisphere" (C-3 to C-12) of **1** are shown in Figure 2 for both the "intraunit" sample A and the "interunit" sample B. Looking first at the spectra of A, each set of signals in the two boxes on top is a section expanded from the full spectrum for clarity, with the empty region between $\delta_{^{13}C}$ ~48 and 69 ppm removed. The chemical shift of C-3 was independently determined by ^{1}H,^{13}C correlation to H-3, which was in turn assigned by decoupling of H-2 (see above). From the INADEQUATE spectra of A, C-4 is easily assigned. Correlations are also obvious between the other pairs of signals (e.g., C-11 to C-12), but since none of these signals could be independently identified by other techniques, absolute assignments cannot be made solely from this spectrum due to lack of coupling between carbons in adjoining acetate units. In sample B, however, all couplings are *interunit* and arise from fortuitous biosynthetic linking of a [1-^{13}C] and a [2-^{13}C] unit head to tail to form a new ^{13}C–^{13}C bond. Its INADEQUATE spectrum therefore contains only complementary connectivities to those in the upper spectrum. Thus starting at the known position C-3 in the upper spectrum, the sloping line shows the intraunit correlation to C-4. Dropping vertically to the chemical shift of C-4 in the lower "interunit" spectrum and then following the diagonal gives the interunit correlation to C-5. Ascending back at the chemical shift of C-5 reveals the intraunit correlation to C-6. Continuing in similar fashion, the connectivity pattern eventually terminates at the signal for C-13, which was independently assigned above. When the

Table I. ^{13}C and ^{1}H Chemical Shifts and Isotopic Incorporations for Fungichromin (**1**)

carbon[a]	^{13}C δ^a	^{1}H δ^b (mult, J)[c]	enhancement(s)[d]	precursor(s)
1	172.98		2.9	*e*
16	138.55			
19	135.36	6.38 (ddm, 14.4, 11.4)	2.7	*f*
21	134.81	6.42 (dd, 12, 12)	2.4	*f*
25	134.28	6.12 (dd, 14.3, 5.0)	2.9	*f*
23	134.21	6.44 (dd, 12, 12)	2.5	*f*
20	134.13	6.43 (dd, 12, 12)	2.2, 8	*g, k*
22	133.66	6.32 (m)	2.5, 8	*g, k*
24	131.97	6.48 (dm, 14.3)	2.6, 8	*g, k*
17	129.91	6.08 (dd, 11.2, 2.0)	2.5	*f*
18	129.06	6.55 (dd, 14.1, 11.2)	2.2, 8	*g, k*
15	80.43	3.86 (d, 9)	5.6	*h*
14	78.31	3.55 (dd, 9, 1.5)	2.8, 9	*g, k*
27	75.25	4.79 (q, 7, d, 7)	2.5	*f*
9	74.20	4.02 (~dd, ~6, ~6)	2.5	*f*
5	74.08	4.02 (m)	2.6	*f*
7	73.92	4.14 (~m, ~6)	2.7	*f*
3	73.41	4.05 (m)	2.3	*f*
26	73.25	4.07 (dd, 6, 6)	2.7, 9	*g, k*
1′	72.59	3.70 (m)	2.5	*i*
11	71.45	3.95 (m)	2.3	*f*
13	70.34	3.30 (d, 11)	2.5	*f*
2	60.35	2.55 (dd, 9, 7)		
8	45.33	1.33 (m), 1.49 (m)	2.9, 9	*g, k*
6	45.17	1.38 (m), 1.47 (m)	2.8, 9	*g, k*
10	44.34	1.51 (m)	2.5, 9	*g, k*
4	41.38	1.52 (m)	2.9, 9	*g, k*
12	39.58	1.39 (m), 1.52 (m)	2.5, 9	*g, k*
2′	36.22	1.35 (m), 1.52 (m)		
4′	32.88	1.30 (m)		
3′	26.01	1.40 (m), 1.53 (m)		
5′	23.65	1.33 (m)		
28	17.96	1.16 (d, 6)	3.0, 9	*g, k*
6′	14.38	0.88 (t, 7)	100 ± 20	*j*
29	11.74	1.78 (s)	50 ± 10	*j*

[a] 100.6-MHz ^{13}C NMR spectrum in methanol-d_4 with solvent reference at 49.00 ppm. [b] 400- or 500-MHz ^{1}H NMR spectrum in 25 mol % DMSO-d_6/methanol-d_4 (see text). [c] Multiplicities (mult) and coupling constants (J, in hertz) determined from the normal ^{1}H spectrum, the 2D-COSY spectrum, or slice through the ^{13}C,^{1}H heteronuclear shift correlation. [d] Ratio of carbon signal intensities for enriched and natural abundance samples measured under identical conditions. [e] Sodium [1-^{13}C]octanoate. [f] Sodium [1-^{13}C]acetate. [g] Sodium [2-^{13}C]acetate. [h] Sodium [1-^{13}C]propionate. [i] Sodium [3-^{13}C]octanoate (**3a**). [j] Ethyl [CD_3]oleate. [k] Diethyl [2-^{13}C]malonate.

(24) (a) Brown, D. W.; Nakashima, T. T.; Rabenstein, D. L. *J. Magn. Reson.* **1981**, *45*, 302–314. (b) Rabenstein, D. L.; Nakashima, T. T. *Anal. Chem.* **1979**, *51*, 1465A–1474A.

(25) Mareci, T. H.; Freeman, R. *J. Magn. Reson.* **1982**, *48*, 158–163.

(26) Diethyl malonate is hydrolyzed and decarboxylated before incorporation as a C_2 unit in the growing polyketide chain.[19,20]

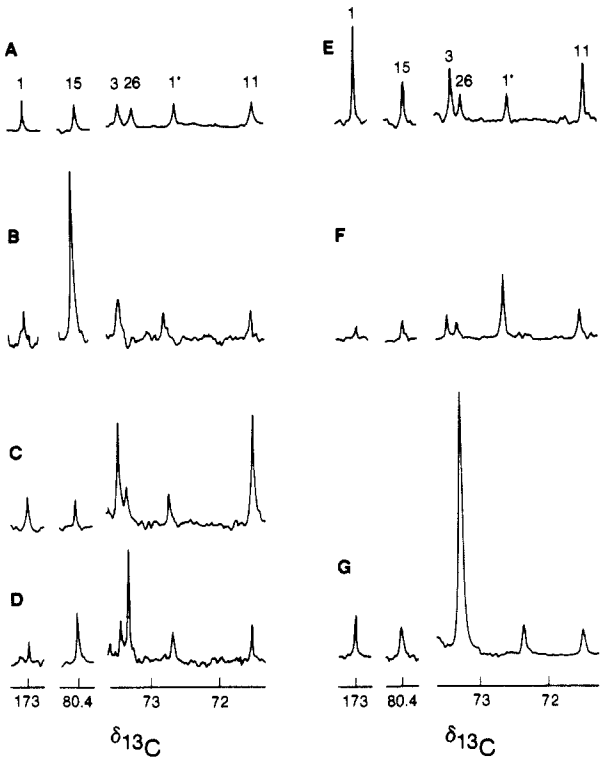


Figure 3. Partial ^{13}C NMR spectra of fungichromin (**1**): (A) natural abundance, and enriched by incorporation of (B) sodium [1-^{13}C]-propionate, (C) sodium [1-^{13}C]acetate, (D) sodium [2-^{13}C]acetate, (E) sodium [1-^{13}C]octanoate, (F) sodium [3-^{13}C]octanoate, and (G) diethyl [2-^{13}C]malonate.

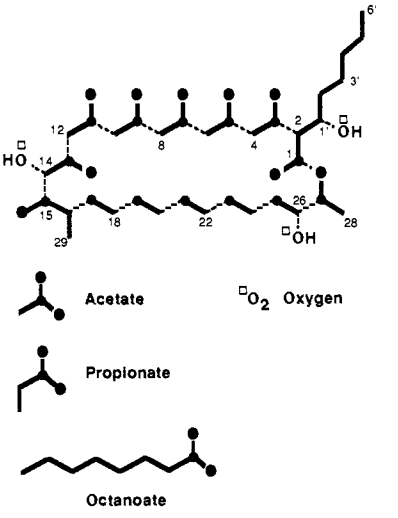
full spectrum is examined, correlation is observed between C-13 and C-14, as well as between C-27 and C-28. The connectivity stops at C-2, C-15, and C-16 since these sites are not enriched by acetate (see below).

The polyene carbons were also assigned by 2D INADEQUATE spectra of the two enriched fungichromin (**1**) samples. With all of the carbon atoms assigned, the exact proton chemical shifts within overlapping multiplets could be obtained from ^1H,^{13}C shift correlations. The complete carbon and proton assignment is given in Table I.

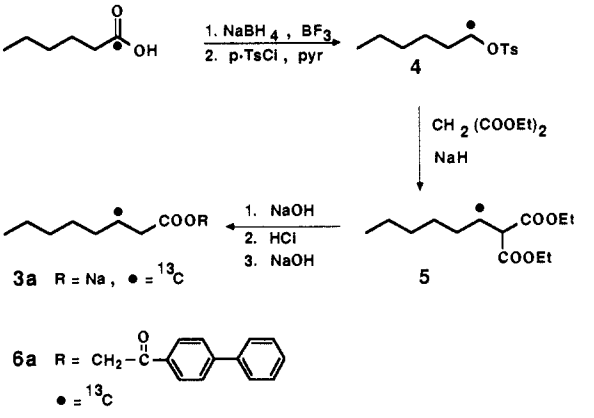
Origin of the Carbon Skeleton of 1. With the spectral assignment complete, the biosynthetic source of the carbon atoms in fungichromin (**1**) could be determined. Addition of sodium [1-^{13}C]acetate to growing cultures of *S. cellulosae* ATCC 12625 gives **1**, which is labeled (Figure 3c) at 12 alternating positions around the macrocyclic ring (see Table I for enrichments). A similar experiment with sodium [2-^{13}C]acetate enhances the ^{13}C NMR signals for 12 carbons adjacent to the first set (Figure 3d). Incorporation of sodium [1,2-$^{13}C_2$]acetate gives rise to 12 pairs of coupled signals in the ^{13}C NMR spectra of **1**, which were fully analyzed by the 2D INADEQUATE technique as described above (Figure 2). The results show that the carbon–carbon bond of each acetate unit is incorporated intact (Scheme I). Administration of diethyl [2-^{13}C]malonate or of the mixture of diethyl [2-^{13}C]- and [1,3-$^{13}C_2$]malonates gave very high incorporation rates (up to 10% per site) at the corresponding acetate-derived carbons (Figure 3g),[26] but no "starter effect"[19,27] could be detected. This suggests that rapid biological interconversion of malonate and acetate precedes incorporation. Incorporation of sodium [1-^{13}C]propionate affords **1** labeled specifically at C-15 (Figure 3b). None of these experiments gave significant ^{13}C enrichment at C-1, C-2, or in the C-1′–C-6′ side chain. Indeed, coupled satellites could not be observed around the ^{13}C NMR signals for these carbons in samples of **1** derived from [1,2-$^{13}C_2$]acetate, even though this approach is known to be very sensitive for detection

(27) Chandler, I. M.; Simpson, T. J. *J. Chem. Soc., Chem. Commun.* **1987**, 17–18.

Scheme I. Distribution of Biosynthetic Units in **1** Showing C–C and C–O Bonds Remaining Intact



Scheme II. Preparation of Sodium [3-^{13}C]Octanoate (**3a**)



of low incorporations.[28] However, in that experiment, small coupled signals are seen at the C-29 resonance due to conversion of acetate to propionate via succinyl-CoA and the citric acid cycle.[19]

In order to determine the origin of the eight-carbon fragment (C-1, C-2, and C-1′–C-6′), larger potential precursors were examined. Upon incorporation of sodium [1-^{13}C]octanoate into **1**, specific enrichment of C-1 occurs (Figure 3e). Only small (ca. 25%) enhancements of ^{13}C NMR signals for carbons derived from the carboxyl of acetate are apparent due to degradation of octanoate by β-oxidation.[29] In contrast, no incorporation of sodium [1-^{13}C]hexanoate could be detected.

Sodium [3-^{13}C]octanoate (**3a**) was prepared (Scheme II) to confirm utilization of the intact eight-carbon fragment. Diborane reduction[30] of [1-^{13}C]hexanoic acid affords [1-^{13}C]hexanol, which without isolation is converted to its tosylate **4**.[31] Displacement with the anion of diethyl malonate[32] gives diethyl[1-^{13}C]hexylmalonate (**5**).[33] Schwab and co-workers have recently reported an improved procedure for this step.[31] Hydrolysis and decarboxylation of **5** affords [3-^{13}C]octanoic acid, which is isolated as its sodium salt **3a**. For analysis of isotopic purity, a portion of

(28) (a) In one experiment very small satellites appeared to be present at C-6′ only. (b) Leete, E. *J. Nat. Prod.* **1982**, *45*, 197–205. (c) Hedges, S. H.; Herbert, R. B.; Wormwald, P. C. *J. Chem. Soc., Chem. Commun.* **1983**, 145–147.

(29) Kindl, H. In *Fatty Acid Metabolism and Its Regulation*; Numa, S., Ed.; Elsevier: Amsterdam, 1984; pp 181–204 and references therein.

(30) Westerhill, R. B.; Brown, H. C.; Subba Rao, B. C. *J. Org. Chem.* **1957**, *22*, 1135–1136.

(31) Schwab, J. M.; Habib, A.; Klassen, J. B. *J. Am. Chem. Soc.* **1986**, *108*, 5304–5308.

(32) Marshall, J. A.; Pike, M. T.; Caroll, R. D. *J. Org. Chem.* **1966**, *31*, 2933–2941.

(33) Anker, H. S. *Methods Enzymol.* **1957**, *4*, 779–809.

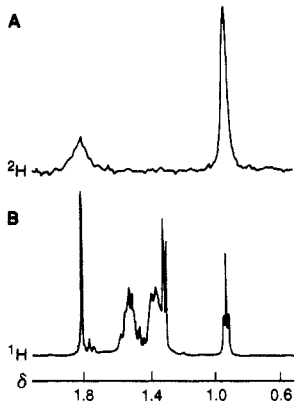


Figure 4. A: Partial ^{1}H-decoupled deuterium NMR spectrum of **1** enriched by ethyl [CD_3]oleate. B: Corresponding ^{1}H NMR spectrum of unlabeled **1**. The methyl hydrogen resonances for C-6′ and C-29 are at δ 0.9 and 1.8, respectively.

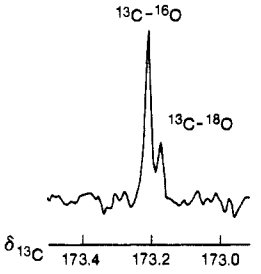


Figure 5. C-1 signal of (**1**) labeled with sodium [1-^{13}C,$^{18}O_2$]octanoate (**3b**).

3a was converted to its *p*-phenylphenacyl ester **6a** by using the method of Risley and Van Etten.[34] The mass spectrum of **6a** shows no detectable molecular ion from unenriched material (isotopic purity >99%).

Administration of the sodium [3-^{13}C]octanoate (**3a**) to *S. cellulosae* and ^{13}C NMR analysis of the resulting fungichromin (**1**) shows exclusive enrichment at C-1′ with no detectable enhancements at the sites derived from C-1 of acetate (Figure 3f). The results suggested that degradation of fats[29] present in the medium (e.g., Span 85)[35] probably accounts for octanoate formation under normal circumstances. To test this, *S. cellulosae* was grown on a medium in which Span 85 had been replaced with ethyl [CD_3]oleate[36] (10% isotopic purity). Deuterium NMR spectroscopy demonstrated that essentially all of the C-6′ methyl of fungichromin (**1**) originated from ethyl oleate (quantitative incorporation rate) (Figure 4), presumably by in vivo degradation of oleate to octanoate. About 50% of the propionate-derived methyl group (C-29) also came from the terminal methyl of the oleate, but deuterium could not be detected at any other sites, in accord with labeling experiments described below.

The Origin of Oxygen and Hydrogen Atoms in 1. To determine possible oxidation states during the assembly of fungichromin (**1**), the sources of its carbon–oxygen and carbon–hydrogen bonds were examined with doubly labeled precursors and isotope shifts induced in ^{13}C NMR spectra.[19]

Sodium [1-^{13}C,$^{18}O_2$]octanoate (**3b**) was readily prepared by reaction of heptyl iodide with potassium [^{13}C]cyanide, hydrolysis of the resulting [1-^{13}C]octanonitrile with potassium *tert*-butoxide in [^{18}O]water, and isolation as the sodium salt by the method of Cane and co-workers.[37] Mass spectrometric analysis of the

Table II. Oxygen-18 Isotopically Shifted Resonances in Fungichromin (**1**) from Labeled Precursors

carbon	^{13}C δ	^{18}O isotope shift, ppb	precursor
1	172.98	40	*a*
15	80.43	20	*b*
14	78.31	16	*c*
27	75.25	28	*d*
9	74.20	20	*d*
5	74.08	20	*d*
7	73.92	20	*d*
3	73.41	20	*d*
26	73.25	10	*c*
1′	72.59	12	*c*
11	71.45	20	*d*
13	70.34	20	*d*

[a] Sodium [1-^{13}C,$^{18}O_2$]octanoate (**3b**). [b] Sodium [1-^{13}C,$^{18}O_2$]-propionate. [c] $^{18}O_2$ gas. [d] Sodium [1-^{13}C,$^{18}O_2$]acetate.

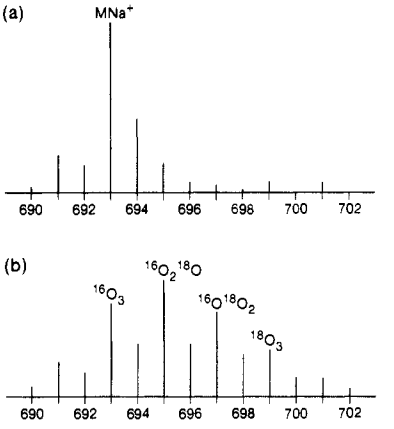


Figure 6. FAB-MS of **1** for the [M + Na]$^+$ region: (a) natural abundance, (b) enriched by $^{18}O_2$ gas.

derived[37a] *p*-phenylphenacyl ester **6b** shows isotopic purities of >99% ^{13}C and 92% ^{18}O per site. Incorporation of octanoate **3b** gives fungichromin (**1**) whose ^{13}C NMR spectrum displays only a single isotope shift at C-1 (Figure 5) of ca. 40 ppb (Table II). The magnitude of the shift demonstrates that one of the carbon–oxygen bonds of octanoate becomes the carbon–oxygen double bond[19,38] at C-1 of fungichromin.

An analogous experiment with sodium [1-^{13}C,$^{18}O_2$]acetate gives **1**, which has isotope shifts at all oxygen-bearing carbons derived from C-1 of acetate (Table II, Scheme I]. As expected, sodium [1-^{13}C,$^{18}O_2$]propionate incorporation shows that the carbon–oxygen bond at C-15 is the only one derived from this precursor. Incubation of *S. cellulosae* in a closed system[20a,39] containing $^{18}O_2$ gas (ca. 50% isotopic purity) labels the remaining three oxygens (at C-14, C-26, and C-1′) of fungichromin (**1**). Although isotope shifts are visible at these three positions, the natural broadness of the carbon resonances and the small sample size encouraged confirmation of this result by fast atom bombardment mass spectrometry (FAB-MS). The data show incorporation of 0–3, but not more, ^{18}O atoms per molecule (Figure 6) and best fit a statistical analysis for 35% ^{18}O per site over three sites.

Elucidation of the biosynthetic origin of the hydrogen atoms of **1** could provide valuable mechanistic information about polyketide assembly.[19,40] However, incorporation of sodium [2-^{13}C,$^2H_3$]acetate into **1** leads to material that contains no significant amount of deuterium despite good ^{13}C enrichment. Fermentation of *S. cellulosae* in a medium containing D_2O extensively labels fungichromin (**1**) throughout the molecule except at positions 6′, 5′, 4′, 3′, and 2′, as demonstrated by ^{2}H NMR and ^{1}H,^{2}H-de-

(34) Risley, J. M.; Van Etten, R. L. *J. Am. Chem.* Soc. **1980**, *102*, 4609–4614.

(35) (a) McCarthy, F. J.; Fisher, W. P.; Charney, J.; Tytell, A. A. *Antibiot. Annu.* **1955**, 719–723. (b) Span 85 is a mixture whose principal component is sorbitan trioleate: *The Merck Index*, 10th ed.; Merck and Co.: Rahway, NJ, 1983; no. 8578.

(36) A generous gift of [CD_3]oleic acid was obtained from Dr. A. P. Tulloch, Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Canada.

(37) (a) Cane, D. E.; Liang, T.-C.; Hasler, H. *J. Am. Chem. Soc.* **1981**, *103*, 5960–5962. (b) Cane, D. E.; Liang, T.-C.; Hasler, H. *J. Am. Chem. Soc.* **1983**, *104*, 7274–7281.

(38) Hansen, P. E. *Annu. Rep. NMR Spectrosc.* **1983**, *15*, 105–234.

(39) Vederas, J. C. In *Mycotoxins and Phycotoxins*; Steyn, P. S., Vleggaar, R., Eds.; Elsevier: Amsterdam, 1986; pp 97–108.

(40) Garson, M. J.; Staunton, J. *Chem. Soc. Rev.* **1979**, *8*, 539–561.

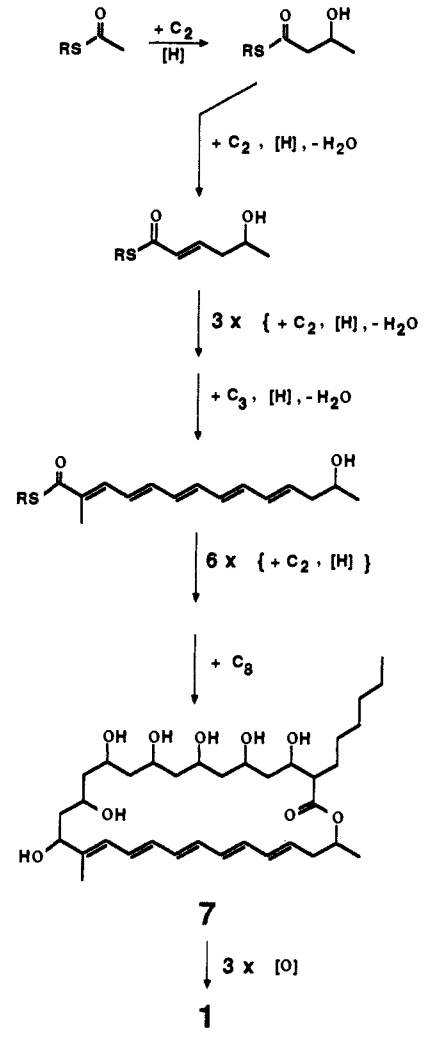
coupled ^{13}C NMR spectroscopy.[40] This result is expected since these positions originate from oleate via octanoate. *S. cellulosae* grown in media containing sodium [2-^{13}C,$^{2}H_3$]acetate in the absence of added fatty acid esters produces fatty acids containing no deuterium (by GC–mass spectrometry of the corresponding methyl esters).[41]

Discussion

The NMR assignment of polyene antibiotics is normally very difficult because of the presence of repeating identical structural fragments.[22] Resulting extensive overlap of both proton resonances and carbon-13 signals even limits the usefulness of 2D NMR experiments. In addition, the low solubility and tendency toward self-association of these compounds further hinders acquisition of such spectra. A potential solution to this problem involves biosynthetic enrichment with a multiply ^{13}C-labeled precursor followed by identification of coupled carbons with 2D INADEQUATE[25,42] or ^{13}C COSY[43] NMR techniques. This approach has become quite popular[19b] for other types of metabolites since its first applications.[44] No previous knowledge of the biogenetic path is necessary to obtain a partial carbon connectivity pattern; the only requirement is an educated guess as to a possible precursor (usually acetate or glucose). In recent studies on a tetramethylated porphyrinogen, Müller, Scott, and co-workers demonstrated that connectivity between two different biosynthetic units could also be detected by using properly labeled precursors and a 1D INADEQUATE experiment.[45] In the present study, two types of fungichromin samples were generated. The first sample, derived from sodium [1,2-$^{13}C_2$]acetate, consists primarily of molecules in which adjacent carbon-13 nuclei are part of the *same* biosynthetic unit. The other sample, obtained from a mixture of diethyl [1,3-$^{13}C_2$]- and [2-^{13}C]malonates, contains molecules in which neighboring ^{13}C atoms originate from *different* units. A 2D INADEQUATE spectrum could be obtained on 25 mg (0.037 mmol) of the "intraunit" sample despite an acetate incorporation rate of only 1–2% per site. The "interunit" material ostensibly has an incorporation rate of 5–9% per site, but the intensity of coupled signals is much stronger than expected on purely statistical grounds (0.25–0.81%) because pulse feeding of the labeled precursors momentarily floods the assembly enzyme(s) with ^{13}C substrate. As a result, a similar quantity of "interunit" sample suffices for the 2D INADEQUATE experiment. Together (Figure 2) the two spectra provide a complete carbon connectivity pattern and ^{13}C assignment for fungichromin (**1**) with the exception of the propionate- and octanoate-derived carbons. This methodology should be a very powerful tool for structure elucidation as well as for NMR assignment and biosynthetic studies. Completion of the ^{13}C NMR assignment permits facile identification of all proton resonances through use of ^{1}H,^{13}C chemical shift correlation.[21,22c,46]

Biosynthetic units in fungichromin (**1**) (Scheme I) have the C-1–C-2 (head-to-tail) connection typical of polyketide biogenesis.[19] A highly unusual feature is termination of the chain by an intact octanoate unit derived from oleate. Martin had proposed introduction of a preformed eight-carbon fragment during biogenesis of filipin (**2**).[10a] However, until our initial report on fungichromin,[18] the only example of intact incorporation of a carboxylic acid longer than four carbons into a microbial polyketide was the utilization of hexanoic acid as a starter unit for averufin biosynthesis.[47,48] Very recently Hutchinson, Cane, and co-workers

Scheme III. Probable Biosynthetic Sequence Leading to Formation of **1**[a]



[a] Construction of **7** may occur on a single polyketide synthase enzyme complex. See ref 20 for detailed descriptions of polyketide synthesis.

demonstrated that functionally and stereochemically correct six-carbon "diketides" derived from two propionate units can be incorporated intact into *Streptomyces* metabolites like tylactone and erythromycin A.[20,49] Their results provide convincing support for the current theory that considerable functionality on partially reduced polyketides appears as a result of an assembly process that resembles incomplete fatty acid biosynthesis.[19a,20] A unique feature of the present work is that octanoate is neither degraded to acetate nor synthesized from it to any major extent, as seen both from carbon- and deuterium-labeling studies. Presumably the β-oxidation enzymes usually involved in degradation of such precursors are fully occupied in processing oleate in the medium to both acetate and octanoate.[50] Failure of [1-^{13}C]hexanoate to significantly label any part of fungichromin (**1**) supports this idea, as does the quantitative incorporation rate of label from ethyl [CD_3]oleate into C-6′.

The oxygen-18 incorporation experiments identify all carbon–oxygen bonds remaining intact from acetate, propionate, and

(41) *S. cellulosae* grows well in the absence of added fatty acid esters but does not produce significant quantities of fungichromin (see also ref 35a).
(42) Turner, D. L. *J. Magn. Reson.* **1982**, *49*, 175–178.
(43) Bax, A.; Freeman, R.; Morris, G. *J. Magn. Reson.* **1981**, *42*, 164–168.
(44) (a) Mackenzie, N. E.; Baxter, R. L.; Scott, A. I.; Fagerness, P. E. *J. Chem. Soc., Chem. Commun.* **1982**, 145–147. (b) Bacher, A.; LeVan, Q.; Bühler, M.; Keller, P. J.; Eimicke, V.; Floss, H. G. *J. Am. Chem. Soc.* **1982**, *104*, 3754–3755. (c) Chan, J. K.; Moore, R. N.; Nakashima, T. T.; Vederas, J. C. *J. Am. Chem. Soc.* **1983**, *105*, 3334–3336.
(45) Müller, G.; Schmiedl, J.; Schneider, R.; Sedlmeier, R.; Wörner, G.; Scott, A. I.; Williams, H. J.; Santander, P. J.; Stolowich, N. J.; Fagerness, P. E.; Mackenzie, N. E.; Kriemler, H. P. *J. Am. Chem. Soc.* **1986**, *108*, 7875–7877.
(46) Bax, A.; Morris, G. *J. Magn. Reson.* **1981**, *42*, 501–505.
(47) Townsend, C. A.; Christensen, S. B.; Trautwein, K. *J. Am. Chem. Soc.* **1984**, *106*, 3868–3869.
(48) Octanoic acid and 5-oxooctanoic acid have been incorporated intact into eight-carbon alkaloids (e.g., coniine) in hemlock plants: (a) Leete, E. *Acc. Chem. Res.* **1971**, *4*, 100–107. (b) Leete, E.; Olson, J. O. *J. Am. Chem. Soc.* **1972**, *94*, 5472–5477.
(49) A "triketide" can also be incorporated into tylactone; see ref 20b.
(50) Oleate is probably catabolyzed in *S. cellulosae* by β-oxidation to *cis*-dodec-3-enoate, which isomerizes and adds water to form β-hydroxydodecanoate. This would then undergo further β-oxidation to the C-8 stage.[29] See also: Stumpf, P. K. *Annu. Rev. Biochem.* **1969**, *38*, 159–212.

octanoate. These results together with the modern polyketide hypothesis[19,20] suggest that assembly of **1** proceeds as shown in Scheme III. Addition of each fatty acid unit (via decarboxylation of its malonyl homologue) would give a β-keto acyl moiety bound to the assembly enzyme. In situ reduction to the β-hydroxy derivative would be followed either by dehydration to generate an olefinic bond or by direct addition of the next carbon fragment to leave a hydroxyl in the growing chain. Finally, addition of the octanoate unit and lactonization would produce **7** as the first complete polyene antibiotic. The proposal that the three aerobic oxidations at C-26, C-1′, and C-14 happen after assembly is supported by the cooccurrence of filipin III (**2**), filipin II (deoxyfilipin III), and filipin I (dideoxyfilipin III) in *S. cellulosae*.[1a,51] A closely related antibiotic from *Chainia multisclerotica*, chainin, possesses a potential six-carbon fragment in place of the octanoate unit of **1** and also lacks the hydroxyls at C-1′ and C-14.[1a,52]

In summary, the present work shows that fungichromin (**1**) is formed by a polyketide pathway from 12 intact acetate units, one propionate unit, and one octanoate unit. The latter is derived extremely efficiently from degradation of oleate rather than de novo synthesis from acetate. Intact carbon–oxygen bonds from the fatty acid precursors are present at all expected sites as shown in Scheme I. This study also illustrates a possible general approach to structure elucidation and NMR assignment of polyketide natural products. A combination of 2D INADEQUATE NMR spectra obtained on samples multiply enriched with carbon-13 within a biosynthetic unit with such spectra of samples having multiple enrichments only between different units allows extensive carbon connectivity determination on small amounts of material. Further applications of this technique and studies employing advanced intermediates in polyketide biosynthesis are currently under investigation.

Experimental Section

General Procedures.[20a] Labeled precursors (>98% isotopic purity) were purchased from Cambridge Isotope Laboratories (Woburn, MA) unless otherwise stated. Diethyl [1,3-$^{13}C_2$]malonate and ethyl [CD_3]-oleate were prepared by esterification of the corresponding acids by using the method of Boissonnas et al.[53] Commercial TLC plates were Merck 60F-254 (silica gel) or Merck RP-8F-254S (reverse phase). Silica gel for column chromatography was Merck type 60, 70–230 mesh. Medium-pressure liquid chromatography (MPLC) employed Merck Lobar RP-8 columns, size B. Gas chromatography (GC) was performed on a Hewlett-Packard 5890A gas chromatograph fitted with a 3% OV-101 on WHP-100-120 6 ft × $^1/_4$ in. column with helium as the carrier gas and a flame-ionization detector. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker WP-80, WH-200, AM-300, WM-360, WH-400, or AM-500[23] instruments with tetramethylsilane (TMS) as internal standard for ^{1}H spectra and solvent (referenced to TMS) as internal standard for ^{13}C spectra. Infrared (IR) spectra were determined on a Nicolet 7199 FT-IR spectrometer. Mass spectra (MS) were recorded at an ionizing voltage of 70 eV on an AEI MS-50 instrument for high-resolution electron-impact (EI) ionization. Fast atom bombardment mass spectra (FAB-MS) were recorded on an MS-9 instrument with glycerol–sulfolane matrix and Ar.

NMR Methods. Isotopic incorporations into fungichromin (**1**) were determined by comparison of the heights of peaks, due to labeled and unlabeled sites, in the ^{1}H-decoupled ^{13}C NMR spectrum, accumulated with a relaxation delay of 1.2 s. Unlabeled **1** gave peaks of reproducible height under these conditions. The ^{18}O isotope shifts were determined in the usual fashion[20a] by accumulation of the ^{1}H-decoupled ^{13}C NMR spectrum over a narrow window; the FID was then zero-filled once to give a resolution of ca. 0.1 Hz/point. The ^{2}H NMR spectra were accumulated at 61.42 MHz with an ^{19}F lock (C_6F_6) and ^{1}H broad-band decoupling.

The 2D INADEQUATE[25,42] spectra were recorded at 300 MHz. For the polyene region of **1**, a 64 × 1K data matrix was obtained, accumulating 1152 scans per t_1 value over a 625-Hz sweep width centered at δ 132.2. The relaxation delay was 1 s. The value of J_{C-C} selected was 56 Hz. The data were zero-filled to 512 words in F1, subjected to Fourier transformation using Gaussian data manipulation in the F1 dimension, and symmetrized for improved appearance. For the polyhydroxy and methylene region of **1**, a 128 × 2K data matrix was recorded, accumulating 1408 scans per t_1 value over a 3290-Hz sweep width centered at δ 54.60. The relaxation delay was 1 s. The value of J_{C-C} selected was 38 Hz. The data were zero-filled to 1K in F1, subjected to Fourier transformation using Gaussian data manipulation in F1, and symmetrized to improve appearance.

The 2D ^{1}H,^{13}C heteronuclear shift correlation[46] experiment of the polyene and polyhydroxy region of **1** was performed on a AM-500[23] instrument. A 317 × 2K data matrix was generated accumulating 400 scans per t_1 value over 10640-Hz sweep width centered at δ 102 (F2). The ^{1}H sweep width was 2050 Hz, centered at δ 5.00 (F1). The relaxation delay was 1 s, and the value of J_{C-H} selected was 131 Hz. The data were zero-filled to 4K in F2 and to 1K in F1 and subjected to Fourier transformation using Lorentzian data manipulation.

The 2D ^{1}H,^{13}C heteronuclear shift correlation experiment of the methylene and methyl region of **1** was performed on a WH-400 instrument. A 128 × 2K data matrix was generated accumulating 352 scans per t_1 value over a 5500-Hz sweep width centered at δ 41.5 (F2). The ^{1}H sweep width was 900 Hz centered at δ 1.86 (F1). The relaxation delay was 1 s, and J_{C-H} was selected at 130 Hz. The data were zero-filled to 4K in F2 and to 512 words in F1, and were subjected to Fourier transformation using Lorentzian data manipulation in F1.

The ^{1}H–^{1}H COSY[54] spectrum (410 × 2K) of the polyene region of **1** was obtained on an AM-500[23] instrument, accumulating 48 scans per t_1 value over a 500-Hz sweep width centered at δ 6.3. The relaxation delay was 1 s. The data were zero-filled to 1K in F1, subjected to Fourier transformation using Gaussian line shaping in F1, and symmetrized to improve appearance. In all cases, symmetrization did not affect the overall results obtained.

Fermentation of *S. cellulosae* and Isolation of Fungichromin (1). Freeze-dried specimens of *S. cellulosae* (ATCC 12625) were soaked in H_2O (1 mL) for 5 min and transferred to 10 slants, prepared from Bacto yeast malt extract agar (19 g) and H_2O (500 mL), which had been sterilized at 121 °C for 20 min. The slants were incubated at 25 °C for 7 days. The resulting mycelium was suspended in H_2O (2 mL), and the suspension was added to two Erlenmeyer flasks (500 mL), each containing liquid media (100 mL) prepared from bactopeptone (5 g), DIFCO yeast extract (2.5 g), NaCl (4 g), glucose (10 g), and Span 85 (Sigma, 10 mL), made up to 1 L with H_2O, buffered to pH 7.0 with $NaHCO_3$, and then autoclaved at 121 °C for 20 min. The preculture flasks were incubated in a fermenter at 26 °C and 165 rpm in the dark for 48 h. A 2-mL portion of the resulting suspension was then transferred to each of 10 flasks containing medium prepared as above (100 mL/flask); the flasks were then incubated under the same conditions. After 3–4 days, the contents became yellow, and isotopically labeled precursors (for labeled acetates, propionates, hexanoate, and octanoates, 500 mg in 10 mL of H_2O; for labeled diethyl malonates, 500 mg in 5 mL of EtOH and 5 mL of H_2O) were added aseptically in 4 portions at 24-h intervals. At 24 h after the last feeding, the mycelium (ca. 25 g fresh weight) was collected by vacuum filtration. The filtrate was extracted (hexane 66%/benzene 34%, 2 × 500 mL, and then EtOAc, 2 × 500 mL). The mycelium was gently boiled in hexane 66%/benzene 34% (500 mL, 30 min). The cooled mixture was filtered, and the filter cake was extracted with boiling EtOAc (500 mL, 10 min). The combined EtOAc extracts were concentrated in vacuo to afford ca. 1.3 g of yellow solid, which was taken up in MeOH and filtered. The filtrate was concentrated in vacuo. Column chromatography of the residue on Sephadex LH-60 (MeOH) afforded UV-active fractions, which were concentrated in vacuo. The residue was taken up in MeOH (5 mL), H_2O (2.7 mL) was added, and the thick precipitate was removed on a centrifuge. Medium-pressure liquid chromatography (reverse phase, MeOH/H_2O, 65:35, 1 mL/min, 6-mL fractions) of the supernatant (5 mL) afforded 20–40 mg of fungichromin (**1**) from fractions 35–40 after azeotropic removal of solvent in vacuo with EtOH. Small amounts of fillipin III (**2**) were also obtained: TLC R_f (SiO_2, $CHCl_3$/MeOH/EtOAc/H_2O, 22:22:45:11, lower phase) **1**, 0.31; **2**, 0.38; R_f (RP-8, MeOH/H_2O, 65:35) **1**, 0.35; **2**, 0.27; ^{1}H and ^{13}C NMR spectral data are given in Table I; FAB-MS (glycerol–sulfolane matrix), *m/e* 693 (MNa^+), 670 (M^+).

The procedure outlined above was followed for fermentation with D_2O except that H_2O in the culture flasks were replaced by D_2O 20%/H_2O 80%. The same approach was used for fermentation under $^{18}O_2$ atmosphere except that the flasks were grown in a closed atmosphere in which consumed oxygen was replenished with $^{18}O_2$ (50 atom % ^{18}O), as has been previously described.[20a,39] Experiments using ethyl oleate were similar

(51) Bergy, M. E.; Eble, T. E. *Biochemistry* **1968**, *7*, 653–659.
(52) Pandey, R. C.; Narasimhachari, N.; Rinehart, K. L., Jr.; Millington, D. S. *J. Am. Chem. Soc.* **1972**, *94*, 4306–4310.
(53) Boissonnas, R. A.; Guttman, S.; Jaquenoud, P. A.; Waller, J. P. *Helv. Chim. Acta* **1955**, *38*, 1491–1501.
(54) (a) Aue, W. P.; Bartholdi, E.; Ernst, R. R. *J. Chem. Phys.* **1976**, *64*, 2229–2246. (b) Nagayama, K.; Kumar, A.; Wuthrich, K.; Ernst, R. R. *J. Magn. Reson.* **1980**, *40*, 321–334.

to the general procedure except that Span 85 was omitted from the preculture medium and was replaced in the culture medium by unlabeled ethyl oleate (9 g/L of medium), ethyl [CD_3]oleate[36] (1 g/L), and ethyl [U-^{14}C]oleate (22.1 μCi/L).

[1-^{13}C]Hexyl *p*-Toluenesulfonate (4). The procedure of Brown and co-workers[30] was adapted. The [1-^{13}C]hexanoic acid (750 mg, 6.40 mmol) was dissolved in Et_2O (15 mL), and $NaBH_4$ (620 mg, 16.4 mmol) was added. The mixture was stirred, and BF_3·Et_2O (48% BF_3, 2 mL, 12.6 mmol) was added dropwise over 30 min. The mixture was stirred and monitored by GLC (3% OV-101 column) until starting material was no longer present (2 days). Water (50 mL) was added, and the mixture was extracted (CH_2Cl_2, 2 × 50 mL). The extracts were washed (5% Na_2CO_3, 100 mL) and dried. Pyridine (6 mL) and *p*-toluenesulfonyl chloride (1.5 g, 7.62 mmol) were added, and the mixture was stirred and monitored by GLC (as above). After 64 h, the mixture was extracted (saturated $CuSO_4$ (2 × 100 mL), 10% $NaHCO_3$ (100 mL), and H_2O (100 mL)). The organic phases were dried and concentrated in vacuo to give 1.44 g (87% based on hexanoic acid) of **4**. For unlabeled material:[31] IR ($CHCl_3$ cast) 3060, 3030, 2958, 2931, 2860, 1600, 1468, 1360, 1189, 1177, 1100, 929, 816, 665 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.79, 7.36 (AB, J = 8.0 Hz, 4 H, Ar *H*), 4.03 (t, J = 6.4 Hz, 2 H, CH_2OTs), 2.45 (s, 3 H, Ar CH_3), 1.64 (m, 2 H, CH_2CH_2OTs), 1.24 (br m, 6 H, 3 CH_2), 0.85 (t, J = 6.4 Hz, 3 H, CH_3); ^{13}C NMR (50 MHz, $CDCl_3$) δ 144.43, 133.01, 129.56, 127.55, 70.46, 30.78, 28.50, 24.72, 22.10, 21.27, 13.59; exact mass 256.1133 (256.1133 calcd for $C_{13}H_{20}SO_3$). Anal. ($C_{13}H_{20}SO_3$) C, H, S. For [^{13}C]hexyl tosylate **4**, NMR spectra were identical except for the following: ^{1}H NMR δ 4.03 (d, $^1J_{CH}$ = 130 Hz); ^{13}C NMR δ 70.46 (enriched), 30.78 (d, $^2J_{CC}$ = 4 Hz), 28.50 (d, $^1J_{CC}$ = 38 Hz).

Diethyl [1-^{13}C]Hexylmalonate (5). A modification of the method of Marshall et al.[32] was used. DMF (5 mL) was added to NaH (50% oil dispersion, 132 mg, 2.74 mmol), which had been washed with THF. Diethyl malonate (327 mg, 2.09 mmol) was added, and the mixture was stirred until effervescence ceased. A solution of **4** (446 mg, 1.74 mmol) in DMF (5 mL) was added, and the mixture was stirred at 70 °C for 16 h. Water (100 mL) was added, and the mixture was acidified with concentrated HCl and extracted ($CHCl_3$, 2 × 100 mL). The extracts were dried and concentrated in vacuo. Column chromatography ($CHCl_3$) afforded 226 mg (54%) of **5** as a clear oil. For the unlabeled analogue: IR ($CHCl_3$ cast) 2959, 2931, 2855, 1753, 1736, 1462, 1365, 1035 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.19 (q, J = 7.2 Hz, 4 H, 2 CH_2O), 3.31 (t, J = 7.6 Hz, 1 H, $CH(CO_2Et)_2$), 1.88 (m, 2 H, $CH_2CH(CO_2Et)_2$), 1.22–1.38 (m, 8 H, 4 CH_2), 1.27 (t, J = 7.2 Hz, 6 H, 2 CH_3CH_2O), 0.88 (t, J = 6.8 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.29, 60.88, 51.83, 31.28, 28.63, 28.51, 27.02, 22.56, 13.80, 13.69; exact mass 244.1676 (244.1675 calcd for $C_{13}H_{24}O_4$). Anal. ($C_{13}H_{24}O_4$) C, H. For **5**, NMR spectra were identical except for the following: ^{1}H NMR δ 3.31 (d, $^2J_{CH}$ = 4.6 Hz), 1.88 (d, $^1J_{CH}$ = 130 Hz); ^{13}C NMR δ 28.51 (enhanced).

Sodium [3-^{13}C]Octanoate (3a). A modification of the method of Anker[33] was used. Aqueous 10% NaOH (10 mL) was added to a solution of **5** (175 mg, 729 μmol) in dioxane (5 mL). The mixture was stirred for 24 h, acidified to pH 1 with concentrated HCl, and heated at reflux for 36 h. The mixture was cooled and extracted (Et_2O, 3 × 20 mL). The extracts were dried and concentrated in vacuo. The residue was adjusted to pH 8.5 with NaOH, and EtOH (30 mL) was added. Organic solvents were removed, and the aqueous solution was lyophilized to afford 120 mg (99%) of **3a** as a white powder. For the unlabeled analogue: ^{1}H NMR (360 MHz, D_2O) δ 2.08 (t, J = 7.5 Hz, 2 H, CH_2CO_2), 1.44 (m, 2 H, $CH_2CH_2CO_2$), 1.18 (br m, 8 H, 4 CH_2), 0.76 (t, J = 6.3 Hz, 3 H, CH_3); ^{13}C NMR (90 MHz, D_2O) δ 184.04, 38.30, 31.76, 29.53, 28.97, 26.51, 22.59, 13.95. For **3a**, the NMR spectra were identical except for the following: ^{1}H NMR δ 2.08 (d, $^2J_{CH}$ = 3.2 Hz), 1.44 (d, J obscure); ^{13}C NMR δ 26.51 (enhanced).

***p*-Phenylphenacyl [3-^{13}C]Octanoate (6a).** The method of Risley and Van Etten[34] was used. A mixture of *p*-phenylphenacyl bromide (33.4 mg, 134 μmol), **3a** (22.7 mg, 121 μmol), and anhydrous EtOH (1 mL) was heated at reflux for 16 h. The mixture was cooled, and H_2O (25 mL) was added. The mixture was extracted ($CHCl_3$, 2 × 25 mL), and the extracts were dried and concentrated in vacuo. Column chromatography (benzene) afforded 12.5 mg (30%) of virtually pure **6a**; an analytical sample was recrystallized (EtOH/H_2O): mp 64.5–65.5 °C; IR ($CHCl_3$ cast) 3050, 2950, 2923, 2845, 1750, 1699, 1171, 760 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.02, 7.73 (AB, J = 8 Hz, 4 H, disubstituted Ar *H*), 7.40–7.70 (m, 5 H, monosubstituted Ar *H*), 5.41 (s, 2 H, OCH_2CO), 2.53 (m, 2 H, CH_2CO_2), 2.06 (m, 7 Hz, 1 H, part of $^{13}CH_2CH_2CO_2$), 1.34 (m, 9 H, 4 CH_2 and part of $^{13}CH_2CH_2CO_2$), 0.92 (t, J = 7 Hz, 3 H, CH_3); exact mass 339.1915 (339.1915 calcd for $C_{21}H_{26}{}^{13}CO_3$), 338 not observed (<1% ^{12}C isotopomer).

Sodium [1-^{13}C,$^{18}O_2$]Octanoate (3b) and Its *p*-Phenylphenacyl Derivative (6b). Compound **3b** was prepared by the method used to prepare the propionate analogue[37] from K^{13}CN and heptyl iodide. Derivative **6b** was prepared by the method used to prepare the propionate analogue.[37] Thus, **3b** (7.11 mg, 41.5 μmol) afforded 2.45 mg (17%) of **6b**; spectra were the same as for **6a** except for the following: IR 1671 ($^{13}C{=}^{18}O$); ^{1}H NMR δ 5.37 (d, $^3J_{CH}$ = 4.5 Hz, 2 H, OCH_2CO), 2.50 (d, $^2J_{CH}$ = 7.3 Hz, t, J = 7.3 Hz, 2 H, CH_2CO_2), 1.72 (m, 2 H, $CH_2CH_2CO_2$); ^{13}C NMR (partial) δ 174.01 ($^{13}C{=}^{16}O_2$), 173.97 ($^{13}C^{18}O_2$), 173.98 ($^{13}C{=}^{18}O(^{16}O)$); MS (EI), m/e 338 ($^{12}C^{16}O_2$, 0%), 339 ($^{13}C^{16}O_2$, 1.9%), 340 ($^{12}C^{16}O^{18}O$, 0%), 341 ($^{13}C^{16}O^{18}O$, 26.2%), 342 ($^{13}C_2{}^{16}O^{18}O$, $^{12}C^{18}O_2$, 5.6%), 343 ($^{13}C^{18}O_2$, 53.8%), 344 ($^{13}C_2{}^{18}O_2$, 12.5%).

Acknowledgment. We thank Dr. Ramesh C. Pandey (Xechem Inc.) for a sample of authentic fungichromin and Dr. A. P. Tulloch (Plant Biotechnology Institute, National Research Council of Canada) for a gift of [CD_3]oleic acid. We are grateful to Dr. Charles Rodger (Bruker Canada) and Dr. David Bundle (National Research Council of Canada) for assistance in spectral acquisition. These studies were supported by the Natural Sciences and Engineering Research Council of Canada and by the Alberta Heritage Foundation for Medical Research.