Biosynthetic Studies of ω-Cycloheptyl Fatty Acids in *Alicyclobacillus cycloheptanicus*. Formation of Cycloheptanecarboxylic Acid from Phenylacetic Acid

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The formation of the structurally novel, mono-substituted cycloheptane ring in ω-cycloheptyl fatty acids in *Alicyclobacillus cycloheptanicus* (formerly *Bacillus cycloheptanicus*) has been examined. Feeding experiments with ^{13}C- and ^{2}H-labeled intermediates demonstrated that cycloheptanecarboxylic acid (**3**), probably as its CoA thioester, is the starter unit for ω-cycloheptyl fatty acid biosynthesis. Analysis of the resultant labeling pattern from a feeding experiment with [U-$^{13}C_6$]-glucose suggested a shikimate pathway origin of **3** via aromatic amino acids. [1,2-$^{13}C_2$]Phenylacetic acid (**6**) was efficiently metabolized into the **3**-derived moiety in a manner reminiscent of the seven-membered ring *Pseudomonas* metabolite thiotropocin. The fates of the aromatic and benzylic hydrogens of **6** were determined; these dictated various boundary conditions for the biosynthetic pathway from **6** to **3**. Taken together with the results from feeding experiments with postulated cycloheptenylcarboxylate biosynthetic intermediates, the data lead us to propose a pathway which involves an oxidative ring-expansion of **6** to a hydroxynorcaradiene intermediate followed by a series of double bond reductions and dehydrations to the saturated **3**.

Most bacilli predominantly possess branched chain fatty acids which are biosynthetically derived from branched chain amino acids.[1] Three thermoacidophilic bacilli alternatively produce ω-cycloalkyl fatty acids as their main fatty acid components.[2] As they form a distinct phylogenetic group, they have recently been reclassified into the new genus *Alicyclobacillus* based on this common phenotypic trait.[3] Comparative 16*S* rRNA sequence data for *Alicyclobacillus* are markedly different from those of the traditional *Bacillus* species, including the thermophiles *Bacillus stearothermophilus* and *Bacillus coagulans*. *Alicyclobacillus acidocaldarius*[4] and *Alicyclobacills acidoterrestris*[5] both contain 70−90% ω-cyclohexyl fatty acids, whereas *Alicyclobacillus cycloheptanicus*[6] possesses ω-cycloheptyl fatty acids. Considerable evidence[7−9] suggests that these compounds serve a membrane-stabilizing function and are essential to the growth of these organisms at high temperature and low pH.

Several groups have determined the shikimic acid origin of the cyclohexanecarboxylic acid starter unit in ω-cyclohexyl fatty acid synthesis.[10−12] We recently reported the complete sequence of metabolic steps for the conversion of shikimic acid to cyclohexanecarboxylic acid in *A. acidocaldarius*[13,14] and in the ansatrienin A producer *Streptomyces collinus*.[14,15] The pathway involves a series of dehydrations and double bond reductions interspersed in such a way that no intermediate is ever aromatic.

Unlike the occurrence of a cyclohexane ring in ω-cyclohexyl fatty acids of *A. acidocaldarius*, *A. acidoterristris*, *Curtobacterium pusillum*,[16] and *Achyranthes aspera*[17] and in several *Streptomyces* antibiotics,[18−25] the occurrence of a fully saturated, monosubstituted cycloheptane ring is unique to the ω-cycloheptyl fatty acids of *A. cycloheptanicus*. ω-Cycloheptylundecanoic (**1a**), -tridecanoic, and -α-hydroxyundecanoic acids comprise nearly

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(1) Kaneda, T. *Microbiol. Rev.* **1991**, *55*, 288.
(2) Deinhard, G.; Poralla, K. *Biospektrum* **1996**, *2*, 40.
(3) Wisotzkey, J. D.; Jurtshuk, P.; Fox, G. E.; Deinhard, G.; Poralla, K. *Int. J. Syst. Bacteriol.* **1992**, *42*, 263.
(4) Darland, G.; Brock, T. D. *J. Gen. Microbiol.* **1971**, *67*, 9.
(5) Deinhard, G.; Blanz, P.; Poralla, K.; Altan, E. *Syst. Appl. Microbiol.* **1987**, *10*, 47.
(6) (a) Deinhard, G.; Saar, J.; Krischke, W.; Poralla, K. *Syst. Appl. Microbiol.* **1987**, *10*, 68. (b) Allgaier, H.; Poralla, K.; Jung, G. *Liebigs. Ann. Chem.* **1985**, 378. (c) Poralla, K.; König, W. A. *FEMS Microbiol. Lett.* **1983**, *16*, 303.
(7) DeRosa, M.; Gambacorta, A.; Minale, L.; Bu`Lock, J. D. *J. Chem. Soc., Chem. Comm.* **1971**, 1334.
(8) Krischke, W.; Poralla, K. *Arch. Microbiol.* **1990**, *153*, 463.
(9) Mantsch, H. H.; Madec, C.; Lewis, R. N. A. H.; McElhaney, R. N. *Biochim. Biophys. Acta.* **1989**, *980*, 42. Kannenberg, E.; Blume, A.; Poralla, K. *FEBS Lett.* **1984**, *172*, 331.
(10) DeRosa, M.; Gambacorta, A.; Bu`Lock, J. D. *Phytochemistry* **1974**, *13*, 1793. DeRosa, M.; Gambacorta, A.; Minale, L.; Bu`Lock, J. D. *Biochem. J.* **1972**, *128*, 751.
(11) Oshima, M.; Ariga, T. *J. Biol. Chem.* **1975**, *250*, 6963.
(12) Furukawa, J.; Tsuyuki, T.; Morisaka, N.; Uemura, N.; Koiso, Y.; Umezawa, B.; Kawaguchi, A.; Iwasaki, S.; Okuda, S. *Chem. Pharm. Bull.* **1986**, *34*, 5176.
(13) Moore, B. S.; Poralla, K.; Floss, H. G. *J. Am. Chem. Soc.* **1993**, *115*, 5267.
(14) Moore, B. S.; Floss, H. G. *J. Nat. Prod.* **1994**, *57*, 382.
(15) Moore, B. S.; Cho, H.; Casati, R.; Kennedy, E.; Reynolds, K. A.; Mocek, U.; Beale, J. M.; Floss, H. G. *J. Am. Chem. Soc.***1993**, *115*, 5254.
(16) Suzuki, K.; Saito, K.; Kawaguchi, A.; Okuda, S.; Komagata, K. *J. Gen. Appl. Microbiol.* **1981**, *27*, 261.
(17) Misra, T. N.; Singh, R. S.; Pandey, H. S.; Prasad, C.; Singh, B. P. *Phytochemistry* **1993**, *33*, 221.
(18) Ansatrienins: Damberg, M.; Russ, P.; Zeeck, A. *Tetrahedron Lett.* **1982**, 59. Weber, W.; Zähner, H.; Damberg, M.; Russ, P.; Zeeck, A. *Zbl. Bakt. Hyg., I. Abt. Orig.* **1981**, *C2*, 122.
(19) Mycotrienins: Hosokawa, N.; Naganawa, H.; Hamada, M.; Takeuchi, T.; Ikeno, S.; Hori, M. *J. Antibiot.* **1996**, *49*, 425. Hiramoto, S.; Sugita, M.; Ando, C.; Sasaki, T.; Furihata, K.; Seto, H.; Otake, N. *J. Antibiot.* **1985**, *38*, 1103. Sugita, M.; Sasaki, T.; Furihata, K.; Seto, H.; Otake, N. *J. Antibiot.* **1982**, *35*, 1467. Sugita, M.; Natori, Y.; Sasaki, T.; Furihata, K.; Shimazu, A.; Seto, H.; Otake, N. *J. Antibiot.* **1982**, *35*, 1460.

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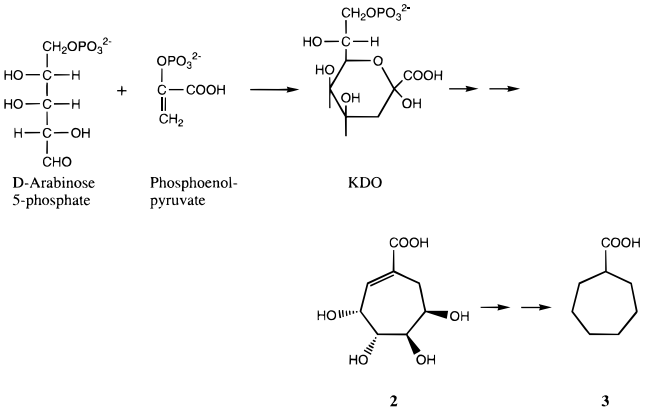
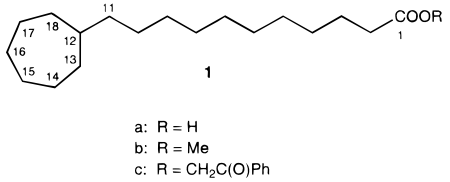
Scheme 1. Early Hypothesis for the Biosynthesis of Cycloheptanecarboxylic Acid via 3-Deoxy-D-*manno*-2-octulosonate 8-Phosphate (KDO) in *A. cycloheptanicus*



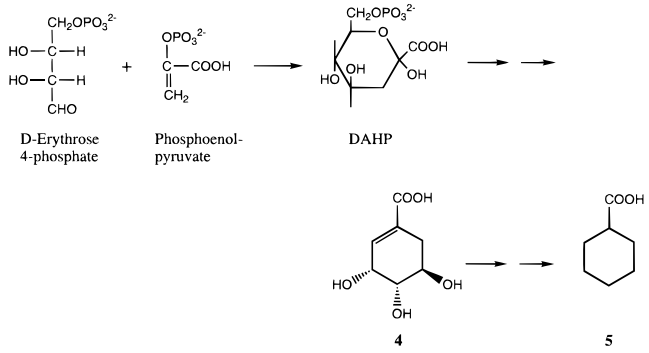
80% of the fatty acids of *A. cycloheptanicus*.[6a,26] Three additional ω-cycloheptyl fatty acids, ω-cycloheptylnonanoic, -decanoic and -α-hydroxytridecanoic acids, were recently identified in the minor fatty acid components.[26] The biologically novel structural feature of the saturated cycloheptane ring is interesting from a biosynthetic point of view and prompted us to carry out a study of its mode of formation.



a: R = H
b: R = Me
c: R = $CH_2C(O)Ph$

On the basis of the biosynthesis of the cyclohexanecarboxylic acid (**5**) starter unit of ω-cyclohexyl fatty acids, we initially contemplated an analogous pathway to the hypothetical starter unit cycloheptanecarboxylic acid (**3**) (Scheme 1). 3-Deoxy-D-*manno*-2-octulosonate 8-phosphate (KDO), an integral constituent of the lipopolysaccharide in most Gram-negative bacteria, is derived from the condensation of phosphoenolpyruvate (PEP) and

Scheme 2. Biosynthesis of Cyclohexanecarboxylic Acid in *A. acidocaldarius* and *S. collinus*.[13,15]



arabinose 5-phosphate,[27] and may be metabolized to the homoshikimate analog **2** (Scheme 1) in a fashion similar to the conversion of 3-deoxy-D-*arabino*-2-heptulosonate 7-phosphate (DAHP) into shikimic acid (**4**) (Scheme 2).[28] A series of dehydrations and double bond reductions of **2**, akin to those in the conversion of **4** to **5**,[13,15] would then yield **3**. In fact, although **3** (probably as its CoA thioester) is the starter unit of ω-cycloheptyl fatty acid synthesis in *A. cycloheptanicus*, it is rather derived via a ring expansion of phenylacetic acid.

Results

Isolation and Characterization of ω-Cycloheptyl Fatty Acids. Biosynthesized ω-cycloheptyl fatty acids were typically analyzed by a combination of GC−MS (as methyl esters such as **1b**) and NMR (as phenacyl esters such as **1c**). Derivatization of the fatty acid extract to the UV-absorbing phenacyl ester derivatives allowed for the isolation of **1c** by reverse phase HPLC[29] and subsequent analysis by NMR.[30]

The unequivocal ^{13}C-NMR signal assignment of **1c**, the most abundant ω-cycloheptyl fatty acid, was neccessary in order to conduct labeling experiments. The ring methylene carbon signals, which each integrate to two due to the symmetry-induced degeneracy of the ring, were initially assigned on the basis of the known ^{13}C assignments of cycloheptanecarboxylic acid.[31] The complete assignments were eventually determined through coupling analyses of ^{13}C-labeled samples of **1c** from various feeding experiments. These assignments will be discussed throughout the manuscript in conjunction with the appropriate feeding experiment. The ^{1}H signals were then assigned by correlation to their respective carbons via a heteronuclear mutliple quantum coherence (HMQC) experiment.

Acetate Feeding Experiments. Compound **1** may be biosynthesized from a functionalized straight chain fatty acid analogous to the formation of dictyotene from

(20) Trienomycins: Smith, A. B., III; Wood, J. L.; Wong, W.; Gould, A. E.; Rizzo, C. J.; Funayama, S.; Omura, S. *J. Am. Chem. Soc.* **1990**, *112*, 7425. Funayama, S.; Okada, K.; Iwasaki, K.; Komiyama, K.; Umezawa, I. *J. Antibiot.* **1985**, *38*, 1677. Funayama, S.; Okada, K.; Komiyama, K.; Umezawa, I. *J. Antibiot.* **1985**, *38*, 1107. Umezawa, I.; Funayama, S.; Okada, K.; Iwasaki, K.; Satoh, J.; Masuda, K.; Komiyama, K. *J. Antibiot.* **1985**, *38*, 699.

(21) Thiazinotrienomycins: Hosokawa, N.; Naganawa, H.; Iinuma, N.; Hamada, M.; Takeuchi, T.; Kanbe, T.; Hori, M. *J. Antibiot.* **1995**, *48*, 471.

(22) Asukamycin: Kakinuma, K.; Ikekama, N.; Nakagawa, A.; Omura, S. *J. Am. Chem. Soc.* **1979**, *101*, 3402.

(23) Phospholine: Ozasa, T.; Tanaka, K.; Sasamata, M.; Kaniwa, H.; Shimizu, M.; Matsumoto, H.; Iwanami, M. *J. Antibiot.* **1989**, *42*, 1339. Ozasa, T.; Suzuki, K.; Sasamata, M.; Tanaka, K.; Kobori, M.; Kadota, S.; Nagai, K.; Saito, T.; Watanabe, S.; Iwanami, M. *J. Antibiot.* **1989**, *42*, 1331.

(24) Phoslactomycins: Fushimi, S.; Nishikawa, S.; Shimazu, A.; Seto, H. *J. Antibiot.* **1989**, *42*, 1019. Fushimi, S.; Furihata, K.; Seto, H. *J. Antibiot.* **1989**, *42*, 1026.

(25) 2-Pyranone derivative I-i: Kohama, T.; Nakamura, T.; Kinoshita, T.; Kaneko, I.; Shiraishi, A. *J. Antibiot.* **1993**, *46*, 1512. Kohama, T.; Enokita, R.; Okazaki, T.; Miyaoka, H.; Torikata, A.; Inukai, M.; Kaneko, I.; Kagasaki, T.; Sakaida, Y.; Satoh, A.; Shiraishi, A. *J. Antibiot.* **1993**, *46*, 1503. Maeda, M.; Kodama, T.; Asami, S. *Jpn. Kokai* 304893 ('89), Dec 8, 1989.

(26) Moore, B. S.; Poralla, K.; Floss, H. G. *J. Nat. Prod.* **1995**, *58*, 590.

(27) Unger, F. M. *Adv. Carbohydr. Chem. Biochem.* **1981**, *38*, 323 and references therein.

(28) Haslam, E. *Shikimic Acid: Metabolim and Metabolites*; John Wiley & Sons, Chichester, 1993.

(29) Borch, R. F. *Anal. Chem.* **1975**, *47*, 2437.

(30) Aside from this method of derivatization being rapid and convenient, the presence of the phenacyl moiety permits (1) the direct quantitation of the molar ratios of the fatty acid derivatives based on HPLC peak areas[29] and (2) the determination of ^{13}C abundances in the biosynthesized **1c** by integration of the ^{13}C-NMR signals of the fatty acid carbons relative to that of the phenacyl ester methylene carbon.

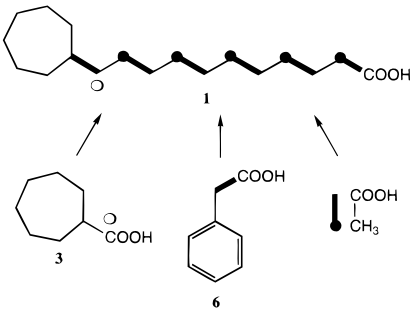
(31) *The Stadtler Standard Carbon-13 NMR Spectra*, Stadtler Research Laboratories, Division of Bio-Rad Laboratories Inc.: Philidelphia, 1980.

Table 1. ^{13}C Distribution in 1c Biosynthesized from Singly Labeled General Precursors

		relative ^{13}C abundances[a]					
carbon no.	δ_c (ppm[b])	[1-^{13}C]acetate	[2-^{13}C]acetate	[1-^{13}C]glucose	[2-^{13}C]glucose	[3-^{13}C]glucose	[6-^{13}C]glucose
1	173.2	13.1	0.9	nd[c]	0.4	nd	nd
2	33.9	0.7	18.1	2.3	1.0	1.0	1.5
3	24.9	28.0	2.6	1.2	2.1	0.9	0.6
4	29.1	nd	17.8	2.1	0.9	0.9	1.5
5	29.3	30.2	nd	1.3	1.5	1.0	0.8
6	29.5	nd	18.9	2.1	1.4	0.8	1.6
7	29.6	32.3	nd	1.4	1.8	0.9	0.8
8	29.7	nd	26.6	2.2	1.4	0.9	1.6
9	30.0	28.7	nd	1.2	1.6	0.9	0.7
10	27.4	0.7	17.4	2.1	0.7	1.4	1.3
11	38.3	1.0	1.3	1.1	4.4	1.0	0.6
12	39.3	0.9	1.2	3.4	0.6	0.6	1.9
13/18	34.7	1.8	2.5	4.7	4.6	1.5	4.0
14/17	26.6	1.7	2.6	4.7	2.9	4.1	3.7
15/16	28.6	2.1	2.1	2.3	3.1	5.8	1.1

[a] Relative to the abundance of the phenacyl ester methylene carbon (65.5 ppm) = 1.0. [b] Referenced to $CDCl_3$. [c] nd = not detected.

Scheme 3. Precursors of ω-Cycloheptylundecanoic Acid (1a)



arachidonic acid.[32] Conversely, cycloheptanecarboxylic acid may be the starter unit for fatty acid synthesis similar to ω-cyclohexyl fatty acid formation in which cyclohexanecarboxylic acid initiates chain elongation.[13] Feeding experiments with ^{13}C-labeled acetate differentiated between these two scenarios.

Sodium [1-^{13}C]- and [2-^{13}C]acetate both highly enriched (20−30%) two different sets of five chain carbons in **1c** (Table 1). Absolutely no label was detected in the ring carbons, thus eliminating the fatty acid cyclization hypothesis. The data rather indicate that five acetate units are added to some non-acetate-derived precursor which provides C-11 through C-18. The one remaining chain methylene carbon (38.3 ppm) not labeled by acetate, by difference, must be C-11.

The ^{13}C NMR chemical shift assignments of the chain carbons of **1c** were in part determined from **1c** biosynthesized from sodium [1,2-$^{13}C_2$]acetate (Scheme 3). Intact incorporation of acetate was evident from the presence of satellites in the 1D ^{13}C-NMR spectrum, and the presence of several C−C connectivities was confirmed by 2D INADEQUATE[33] NMR. The high acetate incorporation allowed assignment of carbons 1−4. A third pair involving carbons resonating at 30.0 and 27.4 ppm was also observed. However, the remaining two acetate-derived pairs of carbons involving signals at 29.3, 29.5, 29.6, and 29.7 were not detected due to near chemical shift equivalence of these carbons and consequent strong second order coupling.

Origin of the Cycloheptane Ring. Based on the results of the [^{13}C]acetate experiments with **1**, [8-^{13}C]**3** was next administered to *A. cycloheptanicus* (Scheme 3). [8-^{13}C]**3** was synthesized from cycloheptyl magnesium bromide and $^{13}CO_2$ in analogy to the procedure of Gilman.[34] Concentrations of [8-^{13}C]**3** greater than 1 mg per 100 mL culture inhibited the growth of *A. cycloheptanicus*. This inhibition may be due to **3** acting like a detergent. ^{13}C-NMR analysis of the resultant **1c** indicated ^{13}C-enrichment at 38.3 ppm (C-11) of 50% (Table 2). Due to the high incorporation, the signals of the carbons adjacent to C-11 were split into doublets roughly centered around the natural abundance singlet, thus providing further ^{13}C-chemical shift assignments. From the coupling constants, the two [2-^{13}C]acetate-derived carbons resonating at 27.4 ppm (AM doublet, ^1J = 34.9 Hz) and 29.7 (^3J = 2.1 Hz) must be C-10 and C-8, respectively. By difference, the remaining unassigned [2-^{13}C]acetate-derived carbon at 29.5 ppm should be C-6. In addition, two ring carbon signals at 39.3 ppm (AB doublet, ^1J = 34.9 Hz) and 26.6 ppm (^3J = 2.6 Hz) were assigned to C-12 and C-14/17, respectively.

[7-^{13}C]Cyclohexanecarboxylic acid[13] was also fed to *A. cycloheptanicus* to probe whether ω-cycloheptyl fatty acids are enriched and/or if ω-cyclohexyl fatty acids are formed.[35] ^{13}C-NMR analysis of the resultant **1c** showed no incorporation into this compound. However, ω-cyclohexyl-α-hydroxyundecanoate and ω-cyclohexylundecanoate were produced in amounts comparable to the corresponding ω-cycloheptyl fatty acids. Both were enriched 100% at C-11 (37.6 ppm). Phenacyl ω-cyclohexylundecanoate isolated from *A. acidocaldarius* was identical chromatographically (GC, HPLC) and spectroscopically (MS, NMR) to that formed in this experiment by *A. cycloheptanicus*.

The biosynthesis of **3** was next probed in feeding experiments with [1,7-$^{13}C_2$]shikimic acid[36] and L-[*methyl*-^{13}C]methionine. A shikimic acid-derived precursor conceivably may ring expand with the addition of a carbon from the one-carbon pool to a cycloheptyl intermediate, which, in turn, is converted into **3**. The arrangement of ^{13}C in positions 1 and 7 of shikimic acid allows for the distinction of biosynthetic pathways in which shikimic acid is incorporated intact with all seven carbons from a pathway in which it is incorporated via the aromatic

(32) Stratmann, K.; Boland, W.; Müller, D. G. *Tetrahedron* **1993**, *49*, 3755. *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 1246.

(33) Bax, A.; Freeman, R.; Kempsell, S. P. *J. Am. Chem. Soc.* **1980**, *102*, 4849−4851.

(34) Gilman, H.; Kirby, R. H. *Organic Syntheses*; Wiley: New York, 1941; Collect. Vol. I, p 361.

(35) In the ω-cyclohexyl fatty acid producer *A. acidocaldarius*, various cycloalkylcarboxylic acids, when exogenously added, were converted to the corresponding ω-cycloalkyl fatty acids.[8]

(36) Cho, H.; Heide, L.; Floss, H. G. *J. Labelled Compd. Radiopharm.* **1992**, *31*, 589.

Table 2. ^{13}C-NMR Analysis of 1c Obtained from Feeding Experiments with ^{13}C-Labeled Precursors.

precursor	amount (mg) fed per 1 L culture	% enrichment at C-11 (38.3 ppm) in **1c**
[8-^{13}C]cycloheptanecarboxylic acid (**3**)	10	50[a]
[1,2-$^{13}C_2$]phenylacetic acid (**6**)	20	20[b,c]
[1,2-$^{13}C_2$]phenylacetic acid (**6**)	100	27[b,c]
[8-^{13}C]cyclohepta-2,4,6-trienecarboxylic acid (**14**)	20	3[a]
[1-^{2}H,8^{13}C]cyclohepta-2,4,6-trienecarboxylic acid (**14**)	15	4[a]
[8-^{13}C]cyclohepta-1,4,6-trienecarboxylic acid (**15**)	17	8[a]
[3-^{2}H,8^{13}C]cyclohepta-1,4,6-trienecarboxylic acid (**15**)	24	7[a]
[8-^{13}C]cyclohepta-1,3-dienecarboxylic acid (**17**)	5.5	8[a]
[8-^{13}C]cyclohepta-2-enecarboxylic acid (**18**)	5	26[a]
[8-^{13}C]cyclohepta-2-enecarboxylic acid (**18**)	10	48[a]
[8-^{13}C]cyclohept-1-enecarboxylic acid (**19**)	10[d]	12[a]

[a] % Specific incorporation = $(A - B)/B$, where A = intergrated NMR signal of enriched carbon and B = intergrated NMR signal of the natural abundance carbon. [b] (doublet, $^1J_{C11-C12} = 34.6$ Hz). [c] % specific incorporation = C/B, where C = summation of the integrated NMR signals of the enriched carbon doublet. [d] 10 mg fed to 300 mL culture.

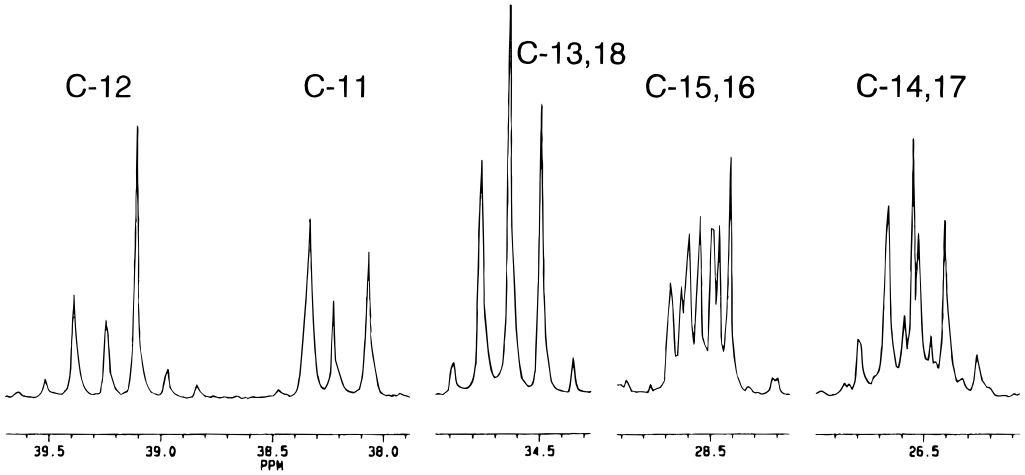


Figure 1. Partial 125.8 MHz ^{13}C-NMR spectrum in $CDCl_3$ of phenacyl ω-cycloheptylundecanoate (**1c**) enriched in a feeding experiment with [U-$^{13}C_6$]glucose. Individual resonances for the cycloheptanecarboxylic acid derived carbons are shown.

amino acids with loss of C-7. Analyses of the ^{13}C-NMR spectrum of **1c** and the GC−MS spectrum of **1b** from the experiment revealed no ^{13}C incorporation.[37] Secondly, no label was detected in the resultant **1c** from L-[*methyl*-^{13}C]methionine, even when *A. cycloheptanicus* was grown in a defined medium (medium B) in which the unlabeled methionine was entirely replaced with labeled methionine.

Glucose Feeding Experiments. Since none of the previous experiments had provided labeling of the cycloheptanecarboxylic acid-derived carbons (C-11 to C-18), [U-$^{13}C_3$]glycerol was fed as a more general precursor to probe the biosynthesis of the starter unit **3**. By the known metabolism of glycerol via glycolysis and the Krebs cycle, a general acetate labeling pattern should at least be observed from C-1 to C-10 in addition to any labeling in the cycloheptanecarboxylic acid-derived carbons C-11 to C-18. However, no incorporation of label from glycerol was observed, even when the feeding experiment was conducted in the defined medium in which the glucose was entirely replaced with labeled glycerol. Clearly, glycerol is not being assimilated or metabolized by *A. cycloheptanicus*.

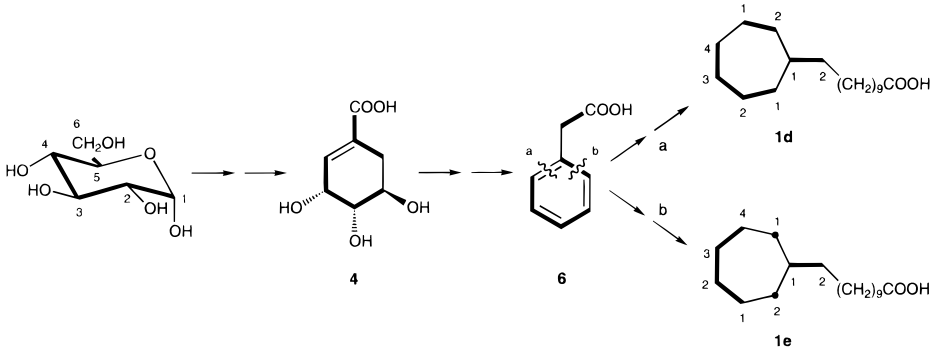
Alternatively, feeding experiments were performed with ^{13}C-labeled glucose, another general precursor which, if metabolized, will also produce metabolites via glycolysis and the Krebs cycle. In the normal sporulation medium (medium A), [U-$^{13}C_6$]glucose did not significantly enrich **1**. However, [U-$^{13}C_6$]glucose extensively labeled **1** when *A. cycloheptanicus* was fermented in the defined medium from which unlabeled glucose and L-alanine were excluded. A drawback of the feeding experiments in the defined medium is that considerably more branched chain fatty acids, and consequently less ω-cycloheptyl fatty acids, are produced.

Analysis of the 1D ^{13}C-NMR spectrum (Figure 1) revealed that each carbon is coupled to at least one of its neighboring carbons. The spectrum is rather complex due to overlapping multiplets and to the symmetry-induced degeneracy of the ring methylene carbons. Initial assignments of labeling patterns were made from the analysis of the coupling patterns and spectral simulation with the Bruker PANIC program. The ^{13}C-NMR signals for C-11 and C-12 appear as a pair of enhanced and coupled AB doublets ($J = 34.6$ Hz) approximately centered around the natural abundance singlet. For C-13/18, the AX-type doublet ($J = 34.2$ Hz) is less intense than the enhanced central resonance, indicative of an additional single enrichment. Small triplets are also apparent at C-12 and C-13/18 due to the high overall

(37) The nonincorporation of [1,7-$^{13}C_2$]shikimic acid turned out to be a false negative result, as was later demonstrated in a feeding experiment with [U-$^{13}C_6$]glucose. We have observed in some organisms that labeled-shikimic acid failed to be incorporated into shikimate pathway-derived metabolites, probably because of poor uptake into the cells. For example, ^{13}C-labeled shikimate was not incorporated into the cyclohexanecarboxylic acid moiety of asukamycin.[38] However, the expected shikimate type labeling pattern from [U-$^{13}C_3$]glycerol was observed.[38]

(38) Thiericke, R.; Zeeck, A.; Nakagawa, A.; Ōmura, S.; Herrold, R. E.; Wu, S. T. S.; Beale, J. M.; Floss, H. G. *J. Am. Chem. Soc.* **1990**, *112*, 3979.

Scheme 4. ^{13}C-Labeling and $^{13}C-^{13}C$ Coupling Patterns in ω-Cycloheptyl Fatty Acids Biosynthesized from [U-$^{13}C_6$]Glucose



incorporation of glucose (approximately 30%). The signals at C-14/17 and C-15/16 are much more complex due to overlapping first and second order couplings. First, both the C-14/17 and C-15/16 signals contain doublets of 34.8 and 33.4 Hz, respectively. The doublet at C-14/17 actually represents two independent two-bond couplings with C-15/16 and C-13/18; this became very apparent when the ^{13}C-NMR spectrum was run at lower field (75.1 MHz), as two doublets of types AB and AX of 32.9 and 34.6 Hz, respectively, were detected. The remaining lines of the signals for C-14/17 and C-15/16 are part of an ABX spin system. The originally degenerate carbons C-15 and C-16 have become nondegenerate and thus couple due to a nonsymmetrical alignment of the ^{13}C-enriched four-carbon unit within the cycloheptane ring.

Routine double quantum (DQ) and triple quantum (TQ) INADEQUATE experiments were attempted to help clarify this coupling system. However, low sample concentrations caused these experiments to be unsuccessful. To overcome the low sensitivity of the INADEQUATE experiment, an inverse-detected version of this experiment was developed. Details of this experiment have been published.[39]

The results from this successful experiment corroborated the interpretation of the 1D coupling pattern. In addition, the assignment of each carbon resonance was unambiguously determined. Five signals appeared at the DQ freqencies of the 10 chain carbons (C-1 through C-10) which were acetate-derived. Four additional DQ transitions were detected which correspond to the cycloheptanecarboxylic acid-derived portion; one of these comes from two degenerate signals on equivalent sides of the ring (C-15 and C-16). Additionally, two nonadjacent DQ signals are present, indicative of intact three-carbon units. The TQ spectrum yielded cross peaks corresponding to those for the nonadjacent DQ signals. The two overlapping three-carbon units (C-13,14,15 and C-14,15,-16) are part of an overall four-carbon unit.

In addition to the four-carbon unit from C-13 to C-16 (or likewise C-15 to C-18), there are two two-carbon units at C-11 to C-12 and C-17 to C-18 (or likewise C-13 to C-14) to complete the labeling of the ring (**1d**, Scheme 4). However, there are two signals which are not addressed in this pattern: the strong single enrichment at C-13/18 and the AB-type doublet between C-14/17 and C-15/16. There must therefore be a second labeling pattern present in C-13 through C-18. As there are only these two remaining NMR signals, the labeling pattern must be symmetrical. There may be an intact four-carbon unit extending from C-14 to C-17, but due to the symmetrical arrangement of the unit within the cycloheptane ring, it is impossible to distinguish, from coupling alone, between a four-carbon unit (**1e**) and two two-carbon units.

Singly ^{13}C-labeled glucose samples were also fed to probe the orientation of the various glucose-derived fragments (Table 1). [1-^{13}C]- and [6-^{13}C]glucose both labeled **1c** in roughly the same manner. Through glycolysis, carbons at positions 1 and 6 of glucose become equivalent. In addition to labeling the [2-^{13}C]acetate-derived carbons, carbons 12, 13/18, and 14/17 were enriched at 2.8, 1.6, and 1.8%, respectively. [2-^{13}C]-Glucose enriched the [1-^{13}C]acetate-derived carbons and carbons 11, 13/18, 14/17, and 15/16 at 3.4, 1.6, 0.7, and 0.7%. And lastly, [3-^{13}C]glucose labeled only carbons 14/17 and 15/16 at 1.4 and 2.2%, respectively.

The labeling patterns of the cycloheptanecarboxylic acid moiety are not consistent with the KDO model in which PEP and arabinose 5-phosphate condense (Scheme 1). In this scenario, intact three- and five-carbon units would label C-11 through C-13 and C-14 through C-18, respectively.[40] Rather, the labeling pattern is identical to that recently reported for the shikimate-derived thiotropocin, which was shown to be formed via phenylacetic acid (**6**).[41] "Oxidative ring expansion [of phenylacetic acid] would involve either of the ortho carbon atoms of the aromatic ring with an equal probability of either cleaving the bond between the two-carbon and four-carbon units [route a] or scission of the two-carbon unit itself [route b], resulting in the generation of a pair of carbon atoms without labeled neighbors [Scheme 4]." [41]

Feeding Experiments with Aromatic Precursors. To test whether the formation of the cycloheptane ring of **1** is similar to that of the tropolone ring of thiotropocin,[41] 20 mg of [1,2-$^{13}C_2$]**6** was administered to a 1 L culture of *A. cycloheptanicus*. The ^{13}C-NMR spectrum of the resultant **1c** showed two enhanced AB doublets for C-11 and C-12 (J = 34.6 Hz, 20% specific incorporation) (Table 2), indicating intact incorporation (Scheme 4), which is consistent with the pathway proposed by Cane and co-workers.[41] The phenylacetic acid presumably arises from L-phenylalanine (**7**), and the following experiments bear this out.

(39) Pratum, T.; Moore, B. S. *J. Mag. Res.* Series B, **1993**, *102*, 91.

(40) Depending upon the mode and degree of glucose metabolism, the arabinose 5-phosphate moiety may not appear solely as an intact five-carbon unit. In addition, the labeling pattern may appear as intact two- and three-carbon units corresponding to C-1 → C-2 and C-3 → C-5 of the pentose, respectively. Catabolism of glucose to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate during glycolysis would account for this.

(41) Cane, D. E.; Wu, Z.; Van Epp, J. E. *J. Am. Chem. Soc.* **1992**, *114*, 8479.

Table 3. Mass Spectral Analysis of 1b Obtained from Feeding Experiments with ^{2}H-Labeled Potential Precursors

	m/z (relative intensity)							
precursor	296	297	298	299	300	301	302	303
unlabeled control	100	21.4	2.7	0.3				
[$^2H_7$]phenylacetic acid (**6**)	100	35.5	9.4	17.4	51.6	74.2	45.2	13.9
L-[*ring*-$^2H_5$]phenylalanine (**7**)	100	34.0	10.0	6.4	52.0	64.0	20.8	3.9
D,L-[3,3-$^2H_2$]phenylalanine (**7**)	100	42.3	7.5	1.1	0.1			
(±)-[2-$^2H_1$]mandelic acid (**12**)	100	20.8	2.7	0.3				
[1-$^2H_1$,8-^{13}C]cyclohepta-2,4,6-trienecarboxylic acid (**14**)	100	25.0	3.8	0.4				
[3-$^2H_1$,8-^{13}C]cyclohepta-1,4,6-trienecarboxylic acid (**15**)	100	21.6	8.4	2.2				

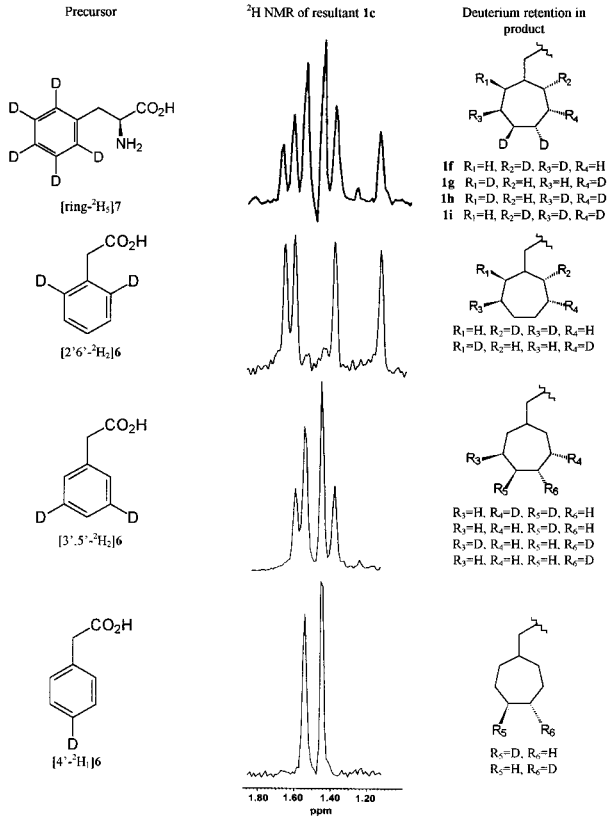
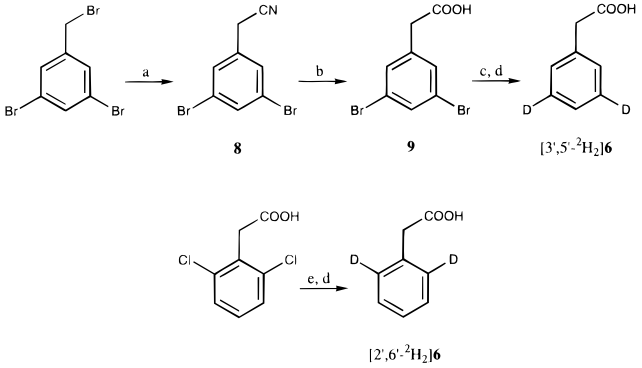


Figure 2. ^{2}H-NMR spectra of **1c** biosynthesized from L-[*ring*-$^2H_5$]**7**, [2′,6′-$^2H_2$]**6**, [3′,5′-$^2H_2$]**6**, and [4′-^{2}H]**6**.

The fate of the aromatic hydrogens of phenylacetic acid was followed in a feeding experiment with L-[*ring*-$^2H_5$]**7**. Phenylalanine should be converted to phenylacetic acid by successive transamination and α-oxidation. Ten percent of the aromatic deuterium atoms were lost in the conversion of L-[*ring*-$^2H_5$]**7** to deuterated **1**. Equal amounts of tetra- and pentadeuterio species (21.7% and 22.7%, respectively) (Table 3) were measured for the resultant **1b** by mass spectrometry. In addition, minor amounts of mono-, di-, and trideuterio species were detected (5%, 2%, and 2%, respectively), which may reflect reduction(s) of an unlabeled cycloheptanecarboxylic acid pathway intermediate by NAD(P)H that has acquired deuterium from the catabolism of L-[*ring*-$^2H_5$]-**7**. This rationale also explains the occurrence of a hexadeuterio species (3.5%), reflecting the reduction of an L-[*ring*-$^2H_5$]**7**-derived intermediate by labeled NAD(P)H.

The ^{2}H-NMR spectrum (Figure 2) of **1c** from this feeding experiment displayed six signals (Table 4). Each unique ring methylene hydrogen (six total) contained deuterium, and furthermore, each methylene hydrogen equatorial/axial pair (three total) was equally enriched. The ratio of enrichment at H-13/18, H-14/17, and H-15/16 was approximately 1:1.5:2. If no deuterium is lost during the metabolism and if there is no deuterium migration, this ratio should theoretically be 1:2:2. From the MS and NMR data, it thus appears that one-fourth of the deuterium from C-14/17 is lost. Therefore at least two tetradeuterio species (**1f** and **1g**) are required to account for the equal deuterium loss from either C-14/17 methylene hydrogen. In addition, two pentadeuterio species (**1h** and **1i**) are likewise needed to account for the equal distribution of deuterium at H-13/18$_{ax}$ and H-13/18$_{eq}$. Consequently, the four deuterated products are present in a 1:1:1:1 ratio. C-12 is epimeric in each pair of tetra- and pentadeuterated **1**. Thus, a biosynthetic pathway proceeding through a C_2 symmetrical intermediate would account for the equal distribution of deuterium between the axial and equatorial positions at each ring methylene carbon.

Scheme 5. [a] Synthesis of [3′,5′-$^2H_2$]- and [2′,6′-$^2H_2$]Phenylacetic Acids



[a] (a) NaCN, H_2O/EtOH; (b) KOH/H_2O, Δ; (c) (i) Zn, NaOD/D_2O, Δ, (ii) HCl; (d) (i) KOH/H_2O, Δ, (ii) HCl; (e) Raney Ni, NaOD/D_2O.

Phenylacetic acids with deuterium exclusively at the ortho-, meta-, or para-positions were synthesized and fed in order to corroborate the above interpretation and determine which aromatic hydrogen is partially lost during the biosynthetic transformation from **6** to **1**. 4′-Bromophenylacetic acid was dehalogenated with zinc powder in 30% NaOD/D_2O to give [4′-^{2}H]phenylacetic acid as previously described.[42] This approach was also utilized for the synthesis of [3′,5′-$^2H_2$]phenylacetic acid from 3′,5′-dibromophenylacetic acid (**9**), which in turn was prepared from 3′,5′-dibromobenzyl bromide via the cyanide **8** (Scheme 5). Dehalogenation of 2′,6′-dichlorophenylacetic acid to [2′,6′-$^2H_2$]phenylacetic acid was alternately achieved with Raney Ni−Al alloy in 30% NaOD/D_2O in a fashion similar to the dehalogenation of 2,6-dichlorophenol.[43] In each synthesis, deuterium (99%) was also detected at the benzylic position of the resultant phenylacetic acid; this deuterium was completely exchanged with hydrogen by refluxing in 30% KOH/H_2O.

(42) Langhals, H.; Fischer, H. *Chem. Ber.* **1978**, *111*, 543.
(43) Tashiro, M.; Fukata, G. *J. Org. Chem.* **1977**, *42*, 835.

Table 4. ^{2}H-NMR Analysis of 1c Obtained from Feeding Experiments with ^{2}H-Labeled Potential Precursors

	product (phenacyl ω-cycloheptylundecanoate)	
precursor	δ ^{2}H-NMR signal(s)[a] (relative abundance)	labeled hydrogen(s)
[2′,6′-$^2H_2$]phenylacetic acid (**6**)	1.64 (1), 1.58 (1), 1.36 (1), 1.11 (1)	H-13/18$_{eq}$, H-14/17$_{eq}$, H-14/17$_{ax}$, H-13/18$_{ax}$
[3′,5′-$^2H_2$]phenylacetic acid (**6**)	1.58 (1), 1.52 (1.6), 1.42 (1.7), 1.36 (1)	H-14/17$_{eq}$, H-15/16$_{eq}$, H-15/16$_{ax}$, H-14/17$_{ax}$
[4′-$^2H_1$]phenylacetic acid (**6**)	1.52 (1), 1.42 (1)	H-15/16$_{eq}$, H-15/16$_{ax}$
[2,2,4′-$^2H_3$]phenylacetic acid (**6**)	1.58 (1), 1.52 (1.8), 1.42 (1.8), 1.36 (1)	H-14/17$_{eq}$, H-15/16$_{eq}$, H-15/16$_{ax}$, H-14/17$_{ax}$
[$^2H_7$]phenylacetic acid (**6**)	1.64 (1), 1.58 (1.5), 1.52 (2), 1.42 (2.1), 1.35 (1.4), 1.11 (1)	H-13/18$_{eq}$, H-14/17$_{eq}$, H-15/16$_{eq}$,H-15/16$_{ax}$, H-14/17$_{ax}$, H-13/18$_{ax}$
L-[*ring*-$^2H_5$]phenylalanine (**7**)	1.64 (1), 1.58 (1.7), 1.52 (2.4), 1.42 (2.5), 1.36 (1.8), 1.11 (1)	H-13/18$_{eq}$, H-14/17$_{eq}$, H-15/16$_{eq}$, H-15/16$_{ax}$, H-14/17$_{ax}$, H-13/18$_{ax}$
D,L-[3,3-$^2H_2$]phenylalanine (**7**)	1.60 (1), 1.38 (1)	H-14/17$_{eq}$, H-14/17$_{ax}$
L-[2,3*S*-$^2H_2$]phenylalanine (**7**)	1.60 (1), 1.39 (1.2)	H-14/17$_{eq}$, H-14/17$_{ax}$
L-[3*R*-$^2H_1$]phenylalanine (**7**)	no incorporation	
(±)-[2-$^2H_1$]mandelic acid (**12**)	no incorporation	
[1-$^2H_1$,8-^{13}C]cyclohepta-2,4,6-trienecarboxylic acid (**14**)	1.38	H-14/17$_{ax}$
[3-$^2H_1$,8-^{13}C]cyclohepta-1,4,6-trienecarboxylic acid (**15**)	1.59 (1), 1.37 (1)	H-14/17$_{eq}$, H-14/17$_{ax}$

[a] $CHCl_3$ used as reference.

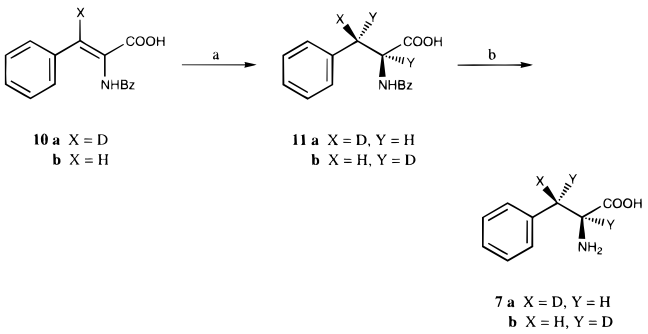
[2′,6′-$^2H_2$]Phenylacetic acid was fed to *A. cycloheptanicus*, and the resultant **1** was doubly enriched. The corresponding ^{2}H-NMR spectrum (Figure 2) indicated that both ortho hydrogens are fully retained, as it showed four peaks with identical intensities at H-13/18 and H-14/17 (Table 4). In contrast, **1** biosynthesized from [3′,5′-$^2H_2$]**6** was both singly and doubly enriched. The ratio of deuterium at the equatorial and axial positions of C-14/17 to that at C-15/16 was 1:1.7 (Figure 2). Therefore, approximately 50% of one of the meta-hydrogens of **6**, the one that is ultimately positioned at C-14/17 in **1**, is lost during the biosynthetic conversion from **6** to **1**. And thirdly, deuterium from [4′-$^2H_1$]**6** was incorporated equally at both H-15/16 positions of **1** as expected. The NMR data corroborate the above interpretation that the biosynthetic pathway must proceed through a symmetrical intermediate.

To determine whether the benzylic hydrogens are retained, a feeding experiment with D,L-[3-$^2H_2$]phenylalanine was performed. GC−MS analysis of the resultant **1b** revealed the presence of 17.3% monodeuterated species (Table 3). No dideuterio species was detected. The location of the deuterium atom was determined by ^{2}H-NMR of the corresponding **1c**. Two deuterium signals of equal intensity were observed at 1.36 ppm (H-14/17$_{ax}$) and 1.58 ppm (H-14/17$_{eq}$) (Table 4).

In order to ascertain the individual fate of the *pro*-3*S* and *pro*-3*R* hydrogens of **7**, L-[3*R*-^{2}H]**7** and L-[2,3*S*-$^2H_2$]**7** were prepared by adaptations of procedures in the literature (Scheme 6).[44] 2-Benzamido[3-^{2}H]cinnamic acid[44] ([3-^{2}H]**10**) was hydrogenated in the presence of the chiral catalyst [Rh((*R*)-prophos)(NBD)]ClO_4[45] to produce (2*S*,3*R*)-*N*-benzoyl[3*R*-^{2}H]phenylalanine ([3*R*-^{2}H]**11**) in 90% ee (Scheme 6). Alternatively, unlabeled **10** was hydrogenated in D_2 with the rhodium catalyst to (2*S*,3*S*)-N-benzoyl[2,3-$^2H_2$]phenylalanine ([2,3*S*-$^2H_2$]**11**). Deprotection yielded L-[3*R*-^{2}H]**7** and L-[2,3*S*-$^2H_2$]**7**, respectively.

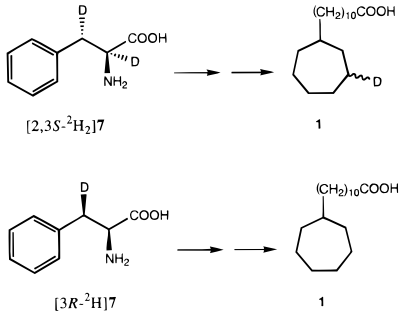
The resultant **1c** from the feeding experiment with L-[2,3*S*-$^2H_2$]**7** (Scheme 7) was singly enriched and contained an equal amount of deuterium at δ 1.39 and 1.60 in a regiospecific manner identical to that observed with **1c** from D,L-[3-$^2H_2$]**7**. In contrast, **1c** derived from L-[3*R*-^{2}H]**7** was not enriched with deuterium. Thus, the *pro*-3*S* hydrogen of phenylalanine is stereoselectively retained during the conversion to **1** at C-14/17.

Scheme 6. [a] Synthesis of L-[3*R*-$^2H_1$]- and L-[2,3*S*-$^2H_2$]Phenylalanines



[a] (a) Rh((*R*)-prophos)(NBD)ClO_4, D_2 (H_2), THF; (b) 6 N HCl, Δ.

Scheme 7. Conversion of L-[3*R*-$^2H_1$]- and L-[2,3*S*-$^2H_2$]7 by *A. cycloheptanicus* into 1

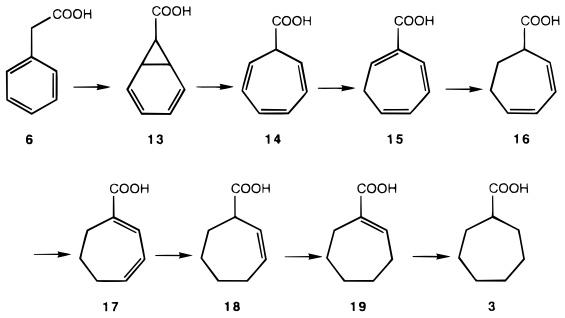


The incorporation of the deuterium from the benzylic position relative to that of the aromatic ring was assessed by feeding [2,2,4′-$^2H_3$]phenylacetic acid. In addition to the two ^{2}H-NMR signals present in the resultant **1c** at δ 1.42 and 1.52 in a 1:1 ratio, a second pair of equivalent singlets resided at δ 1.36 and 1.58, but in a 1:1.8 ratio relative to the first set of signals (Table 4). The relative incorporation of the deuterium from the benzylic position to that from the aromatic ring was 55%, indicating that approximately half of the *pro*-3*S* hydrogen of **7** is lost during the conversion.

Mandelic acid (**12**) was next tested as a possible intermediate as the ring expansion may proceed via an α-hydroxylation of phenylacetic acid. Introduction of deuterium at C-2 of methyl mandelate was achieved by the reduction of methyl benzoylformate with boron deuteride. Treatment with $NaBD_4$, unfortunately, resulted in the reduction of both carbonyl groups. However,

(44) Wightman, R. H.; Staunton, J.; Battersby, A. R.; Hanson, K. R. *J. Chem. Soc., Perkin Trans. 1* **1972**, 2355.
(45) Fryzuk, M. D.; Bosnich, B. *J. Am. Chem. Soc.* **1978**, *100*, 5491.

Scheme 8. Hypothetical Formation of Cycloheptanecarboxylic Acid (3) via Phenylacetic Acid (6)



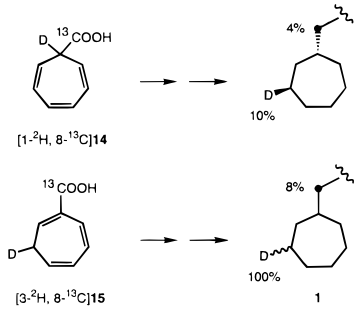
reduction with $Zn(BD_4)_2$ (formed from $NaBD_4$ and $ZnCl_2$)[46] in Et_2O cleanly gave methyl [2-^{2}H]mandelate in 80% yield. Base hydrolysis of the methyl ester completed the synthesis.

The resultant **1** from the feeding of [2-^{2}H]**12** was analyzed by ^{2}H-NMR and MS. The ^{2}H-NMR spectra failed to show any deuterium signals at H-14/17 nor at any other positions. In addition, the MS did not reveal any enhancement of the $(M + 1)^+$ peak. It thus appears that mandelic acid is not a precursor and that the rearrangement may proceed directly from phenylacetic acid.

Cycloheptatrienecarboxylic Acid Feeding Experiments. Ring expansion of phenylacetic acid in *A. cycloheptanicus* may proceed via a coenzyme-B_{12}-mediated or similar rearrangement to the norcaradiene **13** (Buchner's acid[47]), which is in equilibrium with cyclohepta-2,4,6-trienecarboxylic acid[48] (**14**). A suprafacial 1,3-allylic isomerization of the Δ^2-double bond of **14** catalyzed by a single enzyme base[49] to give cyclohepta-1,4,6-trienecarboxylic acid (**15**) would then rationalize the retention of the *pro*-3*S* hydrogen of phenylalanine. Subsequent double bond reductions and isomerizations through cyclohepta-1,3-trienecarboxylic acid (**17**) would yield the saturated **3**. To test whether such a pathway (Scheme 8) is operative, labeled **14** and **15** were synthesized and fed to *A. cycloheptanicus*.

Betz and Daub have reported the synthesis of **14** through methanolysis of cyclohepta-2,4,6-trienylcyanide (**20**) to the methyl ester **21** followed by saponification.[50] [8-^{13}C]**20** was prepared analogously to the procedure of Doering and Knox,[51] instead using tropylium tetrafluoroborate[52] and potassium [^{13}C]cyanide. We then followed the Betz and Daub procedure[50] to convert [8-^{13}C]**20** to [8-^{13}C]**14**, although we modified the methanolysis step (**20** → **21**). The cyano group was converted to the methyl ester by treatment of **20** with chlorotrimethylsilane in methanol for 48 h instead of saturating the methanol solution with HCl gas. [1-^{2}H,8-^{13}C]**14** was additionally prepared by the treatment of [8-^{13}C]**14** with 2 N NaOD in D_2O.[53]

Scheme 9.[a] Conversion of [1-^{2}H,8-^{13}C]14 and [3-$^2H_1$,8-^{13}C]15 by *A. cycloheptanicus* into 1



[a] The ^{13}C-label is denoted with a dot and is incorporated into C-11 of **1** with the respective percentages. The percentage deuterium retention is relative to that of ^{13}C. The implied absolute stereochemistry of the cycloheptane moiety is not known, but is shown to illustrate the relative stereochemistry.

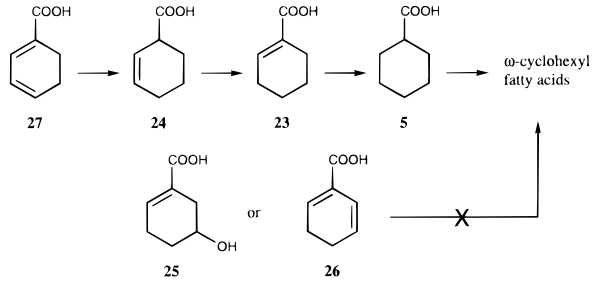
[8-^{13}C]**14** (20 mg) was fed to a 1 L culture of *A. cycloheptanicus*. The resultant **1c** was labeled with ^{13}C at C-11 (3%) (Table 2), indicating that **14** was completely reduced to the saturated cycloheptane. However, the percent incorporation is low, especially relative to that of **6** (20%). [8-^{13}C]**14** is not labile in the warm, acidic culture medium, as first thought, as it was detected unaltered in the spent culture medium after the 24 h fermentation. No other triene isomer was detected. A competition experiment with [1,2-$^{13}C_2$]**6** was conducted to probe whether the incorporation of **6** would diminish that of **14**. Ten milligrams of [8-^{13}C]**14** and 5 mg of [1,2-$^{13}C_2$]**6** were fed to a 500 mL culture of *A. cycloheptanicus*, and the resultant **1c** was singly enriched at C-11 (4.5%) and doubly enriched at C-11 and C-12 (10%).

The fate of the C-1 hydrogen of **14** was examined in a second feeding experiment with [1-^{2}H,8-^{13}C]**14** (Scheme 9). The resultant **1c** was enriched once again with ^{13}C at C-11 (4%). The ^{2}H-NMR spectrum indicated a single signal at 1.38 ppm (H-14/17_{ax}); no deuterium was evident at 1.60 ppm (H-14/17_{eq}). Recall that both methylene hydrogens at C-14/17 of **1c** biosynthesized from L-[2,3*S*-$^2H_2$]phenylalanine were equally enriched with deuterium, which is attributed to the pathway from phenylalanine proceeding through a symmetrical intermediate. Yet in this case, only one position is labeled, indicating that if **14** is a pathway intermediate, a C_2 symmetrical intermediate must occur before and not after **14**. The total incorporation of [1-^{2}H,8-^{13}C]**14** was measured by MS on the resultant **1b** and found to be 3.9% [$(M + 1)^+ = 3.5\%$ enrichment and $(M + 2)^+ = 0.4\%$ enrichment], while the relative deuterium incorporation was only 10% (Table 3), corresponding to a greater deuterium washout than that observed from the benzylic position in **7**. No deuterium was lost from the recovered [1-^{2}H,8-^{13}C]**14**.

We followed the Takahashi *et al.* syntheses of **15**[53] when preparing [8-^{13}C]**15** from [8-^{13}C]**20** and [3-$^2H_1$, 8-^{13}C]**15** from [8-^{13}C]**14**. In both feeding experiments, **15** was recovered from the spent culture medium unaltered and, in the second case, without loss of deuterium. As in the feeding experiment with **14**, no other triene isomer was detected by GC−MS. The resultant **1c** from the feeding of [8-^{13}C]**15** was more highly enriched at C-11 (8%) than that from [8-^{13}C]**14**, which may indicate that **15** is a more advanced precursor than **14**. A competition experiment with [1,2-$^{13}C_2$]**6** was also conducted. Ten milligrams of [8-^{13}C]**15** and 5 mg of [1,2-$^{13}C_2$]**6** were fed to 500 mL of culture, and the resultant **1c** was singly

(46) Gensler, W. J.; Johnson, F.; Sloan, D. B. *J. Am. Chem. Soc.* **1960**, *82*, 6074.
(47) Braren, W.; Buchner, E. *Ber. Dtsch. Chem. Ges.* **1901**, *34*, 982, and references therein; for a review, see Maier, G. *Angew. Chem.* **1967**, *79*, 446.
(48) Wehner, R.; Günther, H. *J. Am. Chem. Soc.* **1975**, *97*, 923.
(49) Hanson, K. R.; Rose, I. A. *Acc. Chem. Res.* **1975**, *8*, 1.
(50) Betz, W.; Daub, J. *Chem. Ber.* **1972**, *105*, 1778.
(51) Doering, W. von E.; Knox, L. H. *J. Am. Chem. Soc.* **1957**, *79*, 352.
(52) Conrow, K. *Org. Syntheses* **1963**, *43*, 101.
(53) Takahashi, K.; Yamamoto, H.; Nozoe, T. *Bull. Chem. Soc. Jpn.* **1970**, *43*, 200.

Scheme 10. Conversion of Cyclohexylcarboxylic Acid Derivatives by *A. cycloheptanicus* into ω-Cyclohexyl Fatty Acids



enriched at C-11 (8%) and doubly enriched at C-11 and C-12 (10%).

The fate of the C-3 hydrogens of **15** was examined in a feeding experiment with [3-$^2H_1$,8-^{13}C]**15** (Scheme 9). Once again, the resultant **1c** was enriched with ^{13}C at C-11 to the extent of 7%. The ^{2}H-NMR spectrum showed the expected two signals in equal proportions at 1.59 and 1.37 ppm corresponding to H-14/17$_{eq}$ and H-14/17$_{ax}$, respectively (Table 4). The relative deuterium incorporation was measured by MS on the corresponding **1b**. The $(M + 1)^+$ peak was not increased, whereas the $(M + 2)^+$ peak was enhanced 7.8%, indicating that the deuterium retention relative to ^{13}C is 100% (Table 3).

Feeding Experiments with Cycloheptyl Mimics. Intermediates late in the metabolic pathway from shikimic acid to cyclohexanecarboxylic acid[13,15] were administered to *A. cycloheptanicus* to determine whether these intermediates mimic their corresponding cycloheptyl counterparts and, as a consequence, are inadvertently converted into ω-cyclohexyl fatty acids. The resultant fatty acid methyl esters from these experiments were analyzed by GC−MS.

Cyclohex-1- and -2-enecarboxylic acids[54] (**23**, **24**) were both converted by *A. cycloheptanicus* into fully reduced ω-cyclohexyl fatty acids. The ratio of methyl ω-cyclohexylundecanoate to methyl ω-cycloheptylundecanoate in the two experiments was 1:3.5 and 1:2.5, respectively. These ratios roughly equate to relative incorporations of the cyclohexyl mimics, giving values of 22 and 29%, respectively. Conversely, 5-hydroxycyclohex-1-ene- and cyclohexa-1,5-dienecarboxylic acids (**25**, **26**) were not metabolized to ω-cyclohexyl fatty acids. Cyclohexa-1,3-dienecarboxylic acid (**27**), which is not an intermediate in the *A. acidocaldarius* cyclohexanecarboxylate pathway but was instead converted into ω-cyclohex-3-enyl fatty acids,[13] was also tested as a mimic. GC−MS analysis revealed that the fully reduced methyl ω-cyclohexylundecanoate was formed in addition to methyl ω-cycloheptylundecanoate in a 1:11 ratio (8% relative incorporation). On the basis of these results, it is suggested that the final steps in the formation of **3** may involve two double bond reductions of **17**, which is consistent with the proposed pathway outlined in Scheme 8, and similar to that proposed for the reduction of the mimic **27** (Scheme 10).

Cycloheptenylcarboxylic Acid Feeding Experiments. The corresponding cycloheptenylcarboxylic acids were synthesized with a ^{13}C-label at the carboxyl carbon and fed to *A. cycloheptanicus* to substantiate the above results with the cyclohexyl mimics. Direct dehydrogenation of the methyl ester of [8-^{13}C]**3** by selenoxide syn elimination[55] followed by ester hydrolysis yielded the α,β-unsaturated [8-^{13}C]**19**. The Δ^2-monoene [8-^{13}C]**18** was synthesized from 3-bromocycloheptene[56] and sodium [^{13}C]-cyanide based on the synthesis of **24**.[57] *anti*-Addition of bromine to [8-^{13}C]**18** followed by two base-induced dehydrobrominations of the crude stereoisomeric dibromides afforded a 3.6:1 mixture of the 1,3- and 1,6-regioisomeric dienes. Resultant chromatography and recrystallization yielded pure [8-^{13}C]**17**. ^{13}C-Label from each of the [8-^{13}C]-cycloheptenylcarboxylic acids was efficiently incorporated into the C-11 position of the resultant **1c** (Table 2), lending support for the conversion of **17** to **3** via **18** and **19**.

Discussion

Initial experiments on the biosynthesis of the unique ring structure in ω-cycloheptyl fatty acids have been presented. As in the case of the ω-cyclohexyl fatty acids from *A. acidocaldarius*,[13] the corresponding cycloheptanecarboxylic acid (probably as its CoA thioester) serves as the starter unit for ω-cycloheptyl fatty acid biosynthesis in *A. cycloheptanicus*. The [U-$^{13}C_6$]glucose feeding experiment demonstrated that all eight carbons of the cycloheptanecarboxylic acid moiety are derived from glucose. Analysis of the resultant labeling pattern suggested a shikimate pathway origin, but via aromatic amino acids rather than directly from the seven carbons of shikimate.

The labeling pattern of the cycloheptanecarboxylic acid moiety in **1** biosynthesized from [U-$^{13}C_6$]glucose was reminiscent of that recently published by Cane *et al.* for the seven-membered ring *Pseudomonas* metabolite thiotropocin.[41] A feeding experiment with [1,2-$^{13}C_2$]phenylacetic acid in *A. cycloheptanicus* demonstrated that the cycloheptanecarboxylic acid-derived moiety of **1** was also elaborated from phenylacetic acid. The fates of the aromatic and benzylic hydrogens of phenylacetic acid were then determined through feeding experiments with deuterated phenylalanine and phenylacetic acid. The results from these experiments dictate the following boundary conditions for the biosynthetic pathway from phenylacetic acid to cycloheptanecarboxylic acid:

(1) The biosynthetic pathway proceeds through a C_2 symmetrical intermediate; this would account for the equal distribution of deuterium between the equatorial and axial positions at each ring methylene carbon.

(2) Fifty percent of the aromatic meta-hydrogen that ultimately resides equally at H-14/17$_{ax}$ and H-14/17$_{eq}$ of **1** is lost during the biosynthetic transformation. Otherwise, all of the other aromatic hydrogens are retained.

(3) The *pro*-3*S* hydrogen of **7** is retained at 50% in the resultant **1** equally at H-14/17$_{ax}$ and H-14/17$_{eq}$, whereas the *pro*-3*R* hydrogen is entirely lost. The partial retention and migration of the *pro*-3*S* hydrogen is suggestive of a suprafacial 1,3-allylic isomerization.[49]

Evidence in support of the final stages in the sequence (**17** → **3**) comes from the experiments with the cyclohexyl mimics and the incorporations of **17**−**19**. In fact, incubations of *A. cycloheptanicus* cell-free extracts with the

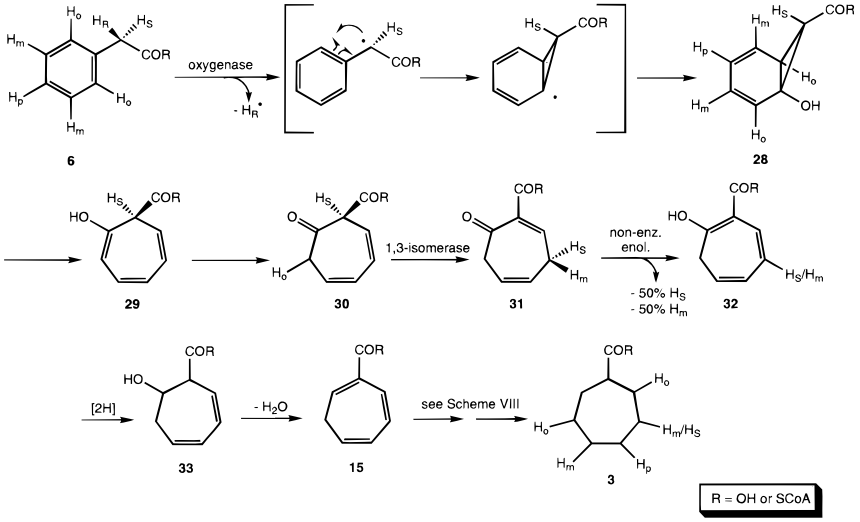
(54) All of the cyclohexyl intermediates were previously synthesized and are described in ref 15.

(55) Reich, H. J.; Renga, J. M.; Reich, I. L. *J. Am. Chem. Soc.* **1975**, *97*, 5434.

(56) Hatch, L. F.; Bachmann, G. *Chem. Ber.* **1964**, *97*, 132.

(57) Davies, S. G.; Whitman, G. H. *J. Chem. Soc., Perkin Trans. 1* **1976**, 2279.

Scheme 11. Proposed Oxidative Ring Expansion of Phenylacetic acid (6) by *A. cycloheptanicus*



coenzyme A thioesters of monoenes **18** and **19** with NADPH resulted in the formation of **3**.[58]

The hypothetical pathway outlined in Scheme 8 does not allow for the symmetrization of the labeling patterns nor for the observed partial losses of deuterium from **6** as dictated above. Likewise, various features from the feeding experiment with the hypothetical intermediate **14** raise questions as to whether it is an intermediates on the pathway from **6** to **3** or whether its incorporation represents nonphysiological channeling of added compounds onto the pathway. For instance, it is incorporated into **1** much less efficiently than **6**, and the fate and percent transfer of the deuterium did not match that from the corresponding position in precursors **6** and **7**.

For these reasons an alternate pathway is proposed which invokes an oxidative ring-expansion reaction which could be mediated by a cytochrome P_{450}-like enzyme[59] (Scheme 11). Enzymatic or nonenzymatic ring expansion of the hydroxynorcaradiene intermediate **28** followed by tautomerization would yield the ketodiene **30**. A suprafacial 1,3-allylic isomerization of the methine hydrogen (H_S), which originated from the *pro*-3*S* hydrogen in **7**, gives **31**. A subsequent nonenzymatic enolization to **32** would result in the observed 50% losses of hydrogen originating from both the aromatic meta (H_m) and pro-*S* benzylic (H_S) positions in **6**. Enol reduction followed by dehydration gives the triene **15**, which is then processed to the saturated **3** as illustrated in Scheme 8.

The pathway presented in Scheme 11 accounts for the observed losses of deuterium as described above but is limited by the fact that it does not advance through a symmetrical intermediate to allow for the observed scrambling of deuterium atoms from the feeding experiments with **6** and **7**. We have not been able to reconcile these observations to our satisfaction without jeopardizing the other pathway constraints. However, if the final reduction of the Δ^1-monoene **19** were to proceed without overall stereocontrol, then the resultant **3** would be epimeric at C-1, resulting in the observed "scrambling" of deuterium atoms. Cell-free experiments are in progress to probe the early stages in the pathway in order to define the complete sequence of reactions.

(58) Tornus, I.; Floss, H. G. Unpublished results.

(59) Ortiz de Montellano, P. R. Cytochrome P-450: Structure, Mechanism, and Biochemistry; Plenum Press, New York, 1986.

Experimental Section

General Procedures. The ^{1}H-, ^{2}H-, and ^{13}C-NMR spectra were recorded on an IBM AF-300 spectrometer operating at a field strength of 7.1 T. Additional ^{13}C-NMR measurements on D-[U-$^{13}C_6$]-glucose-derived **1c** were performed on a Bruker AM-500 spectrometer. Chemical shifts are given in parts per million (ppm) and are adjusted to the TMS scale by reference to the solvent signal. Coupling constants (*J*) are given in Hertz (Hz). GC−MS was carried out on a Kratos Profile mass spectrometer (Manchester, U.K.). Analytical TLC was executed on precoated silica gel 60F-254 plates. Compounds on the plates were visualized under UV light and/or by spraying with a $KMnO_4$ solution (1.0 g of $KMnO_4$, 100 mL of 1 N NaOH) and heating at 120 °C. Mobilities are quoted relative to the solvent front (R_f). Column chromatography was performed on 230−400 mesh silica gel from Aldrich.

Materials. *A. cycloheptanicus* (formerly *B. cycloheptanicus*)[3] was obtained from the Deutsche Sammlung für Mikroorganismen (DSM 4006) and *A. acidocaldarius* (formerly *B. acidocaldarius*)[3] from the American Type Culture Collection, Rockville, MD (ATCC 27009). All chemicals were of reagent grade and were used without further purification. Ingredients for fermentations were purchased from Difco and Sigma. The following companies supplied the stable-isotope labeled compounds (all ^{13}C or ^{2}H enrichments were at least 98 atom %): sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate, sodium [1,2-$^{13}C_2$]-acetate, [1-^{2}H]benzaldehyde, D_2, 30% NaOD in D_2O, D_2O, Cambridge Isotope Laboratories; D-[U-$^{13}C_6$]glucose, D-[1-^{13}C]-glucose, D-[2-^{13}C]-glucose, D-[3-^{13}C]glucose, D-[6-^{13}C]glucose, D-[1,2-$^{13}C_2$]glucose, L-[*methyl*-^{13}C]methionine, $^{13}CO_2$, K^{13}CN, [1,2-$^{13}C_2$]phenylacetic acid, L-[*ring*-$^2H_5$]phenylalanine, D,L-[3-$^2H_2$]phenylalanine, Isotec Inc; [U-$^{13}C_3$]glycerol, Los Alamos Stable Isotope Resource; [$^2H_7$]phenylacetic acid, C/D/N Isotopes Inc; $NaBD_4$, Aldrich.

Fermentation. A loopful of *A. cycloheptanicus* from a plate culture was inoculated into 100 mL of the sporulation medium (medium A) in a 500 mL baffled Erlenmeyer flask. The medium contained $(NH_4)_2SO_4$ (0.2 g), $CaCl_2 \cdot 2H_2O$ (0.25 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), KH_2PO_4 (3.0 g), glucose (3.0 g), yeast extract (3.0 g), and 1 mL of trace element solution per liter. The trace element solution consisted of $FeSO_4 \cdot 7H_2O$ (0.28 g), $MnCl_2 \cdot 4H_2O$ (1.25 g), and $ZnSO_4 \cdot 7H_2O$ (0.48 g) per liter. The pH of the sporulation medium was adjusted to 4.0 with 6 M H_2SO_4. The inoculated culture was incubated at 50 °C for 24 h on a rotary shaker at 150 rpm. The inoculum (5−10 mL) was then transferred to 100 mL of the same sporulation medium or to 100 mL of a defined medium (medium B) and incubated at 50 °C for 24 h with shaking at 150 rpm. Medium B consisted of the sporulation medium elements minus yeast extract, plus an additional 2 g of glucose and the following amino acids and vitamins per liter: L-methionine (50 mg), L-alanine (320 mg), L-isoleucine (280 mg), L-valine (160 mg),

L-leucine (180 mg), biotin (100 μg), niacin (2 mg), thiamine (150 μg), vitamin B_{12} (100 μg), and pantothenic acid (5 mg) per liter. The trace element solution for this medium (1 mL per liter) is $CaCl_2 \cdot 2H_2O$ (0.66 g), $ZnSO_4 \cdot 7H_2O$ (0.18 g), $CuSO_4 \cdot 5H_2O$ (0.16 g), $MnSO_4 \cdot H_2O$ (0.15 g), $CoCl_2 \cdot 6H_2O$ (0.18 g), H_3BO_3 (0.1 g), $Na_2MoO_4 \cdot 2H_2O$ (0.3 g), and $FeSO_4 \cdot H_2O$ (1.0 g) per liter. All media were sterilized for 20 min at 121 °C in a steam autoclave.

Feeding Experiments with Labeled Precursors. Feeding experiments were carried out in either medium A or B. In general, single doses of precursor were administered to the fermentation at the time of inoculation and the cultures were harvested 24 h later. Precursors were added as sterile solutions in either water or a 3% $NaHCO_3$ solution to the following media in the amounts indicated per culture volume. Medium A: sodium [1-^{13}C]acetate (450 mg/900 mL), sodium [2-^{13}C]acetate (250 mg/1 L), sodium [1,2-$^{13}C_2$]acetate (250 mg/1 L), [U-$^{13}C_3$]glycerol (100 mg/1 L), L-[*methyl*-^{13}C]methionine (100 mg/1 L), (−)-[1,7-$^{13}C_2$]shikimic acid (5 mg/100 mL; 100 mg/1L), [8-^{13}C]cycloheptanecarboxylic acid (10 mg/1 L), [7-^{13}C]-cyclohexanecarboxylic acid (20 mg/1 L), cyclohex-1-enecarboxylic acid (1.5 mg/100 mL), cyclohex-2-enecarboxylic acid (1.9 mg/100 mL), 5-hydroxycyclohex-1-enecarboxylic acid (1.2 mg/100 mL), cyclohexa-1,5-dienecarboxylic acid (1.4 mg/100 mL), cyclohexa-1,3-dienecarboxylic acid (1.4 mg/100 mL), [1,2-$^{13}C_2$]-phenylacetic acid (20 and 100 mg/1 L), [2′,6′-$^2H_2$]phenylacetic acid (100 mg/1 L), [3′,5′-$^2H_2$]phenylacetic acid (100 mg/1 L), [4′-^{2}H]phenylacetic acid (100 mg/1 L), [$^2H_7$]phenylacetic acid (100 mg/1 L), L-[*ring*-$^2H_5$]phenylalanine (100 mg/1 L), D,L-[3-$^2H_2$]phenylalanine (100 mg/1 L); L-[3*R*-$^2H_1$]phenylalanine (100 mg/1 L), L-[2,3*S*-$^2H_2$]phenylalanine (100 mg/1 L), (±)-[2-^{2}H]-mandelic acid (90 mg/900 mL), [8-^{13}C]cyclohepta-2,4,6-trienecarboxylic acid (20 mg/1 L), [1-^{2}H,8-^{13}C]cyclohepta-2,4,6-trienecarboxylic acid (15 mg/1 L), [8-^{13}C]cyclohepta-1,4,6-trienecarboxylic acid (17 mg/1 L), [3-$^2H_1$,8-^{13}C]cyclohepta-1,4,6-trienecarboxylic acid (24 mg/1 L), [8-^{13}C]cyclohepta-1,3-dienecarboxylic acid (5.5 mg/1 L), [8-^{13}C]cyclohept-2-enecarboxylic acid (5 and 10 mg/1 L), [8-^{13}C]cyclohept-1-enecarboxylic acid (10 mg/300 mL). Medium A without unlabeled glucose: D-[U-$^{13}C_6$]glucose (250 mg/500 mL). Medium B without unlabeled L-alanine and glucose: sodium [1,2-$^{13}C_2$]-acetate (100 mg/1 L), D-[1-^{13}C]glucose (50 mg/500 mL), D-[2-^{13}C]glucose (50 mg/500 mL), D-[3-^{13}C]glucose (100 mg/1 L), D-[6-^{13}C]glucose (100 mg/1 L), D-[1,2-$^{13}C_2$]glucose (100 mg/1 L), D-[U-$^{13}C_6$]glucose (200 mg/2 L), [U-$^{13}C_3$]glycerol (50 mg/500 mL). Medium B without unlabeled L-methionine: L-[methyl-^{13}C]-methionine (25 mg/500 mL).

Isolation of Phenacyl ω-Cycloheptyl Fatty Acids. The cultures were centrifuged at 14900*g* for 15 min. The wet cells were suspended in 10−20 mL of 2.5% KOH in 1:3 MeOH:H_2O, sealed in 2−4 10 mL capped vials and heated in an oven at 100 °C overnight. The basic solution was then extracted with 3 × 20 mL *n*-hexane and acidified with 6 M HCl, and the fatty acids were then extracted with 3 × 20 mL of 1:2 *n*-hexane:EtOAc. The extract was evaporated, and per 10 mg of residue, 12 mg phenacyl bromide and 10 mg triethylamine in 2 mL acetone were added and the mixture was stirred overnight at room temperature.[29] The fatty acid derivatives were purified by reverse-phase HPLC on a 10 × 300 mm C-18 (10 μm) column with 9:1 MeCN:H_2O (4 mL/min, 254 nm detection) to give phenacyl ω-cycloheptyl-α-hydroxyundecanoate (t_r 28 min) and phenacyl ω-cycloheptylundecanoate (t_r 56 min). The average amount of phenacyl ω-cycloheptylundecanoate varied according to the medium [medium A (6.0 mg/L), medium B without unlabeled glucose and L-alanine (1.0 mg/L)].

Phenacyl ω-Cycloheptylundecanoate (1c): R_f 0.90 (hexane:EtOAc 2:1). ^{1}H-NMR ($CDCl_3$) δ 1.08 (m, H-13/18$_{ax}$, 1H), 1.14 (m, H-11, 2H), 1.18−1.28 (m, H-4 to H-10, 14H), 1.32 (m, H-14/17$_{ax}$, 1H), 1.36 (m, H-12, 1H), 1.41 (m, H-15/16$_{ax}$, 1H), 1.49 (m, H-15/16$_{eq}$, 1H), 1.55 (m, H-14/17$_{eq}$, 1H), 1.62 (m, H-13/18$_{eq}$, 1H), 1.66 (m, H-3, 2H), 2.44 (t, J = 7.6 Hz, H-2, 2H), 5.31 (s, 2H), 7.47 (t, J = 7.6 Hz, 2H), 7.58 (tm, J = 7.3 Hz, 1H), 7.90 (dm, J = 7.8 Hz, 2H); ^{13}C-NMR ($CDCl_3$) δ 24.9 (C-3), 26.6 (C-14/17), 27.4 (C-10), 28.6 (C-15/16), 29.1 (C-4), 29.3 (C-5), 29.5 (C-6), 29.6 (C-7), 29.7 (C-8), 30.0 (C-9), 33.9 (C-2), 34.7 (C-13/18), 38.3 (C-11), 39.3 (C-12), 65.5 (C-1′), 127.8, 128.8, 133.8, 134.4, 173.2 (C-1), 192.4 (C-2′); GC−MS *m*/*z* (relative intensity) 400 (M^+, 0.1), 281 [(M − $CH_2C(O)Ph)^+$, 2], 265 [(M − $OCH_2C(O)Ph)^+$, 2], 105 ($PhCO^+$, 100), 97 ($C_7H_{13}^+$, 7).

Phenacyl ω-cycloheptyl-α-hydroxyundecanoate: R_f 0.64 (hexane:EtOAc 2:1); ^{13}C-NMR ($CDCl_3$) δ 24.8, 26.6, 27.4, 28.6, 29.4, 29.5, 29.6, 29.7, 30.0, 34.6, 34.7, 38.3, 39.3, 66.5, 70.7, 127.8, 129.0, 134.0, 134.1, 175.1, 191.4.

Isolation of Phenacyl ω-Cyclohexylundecanoate from *A. acidocaldarius*. The fermentation of *A. acidocaldarius* and isolation of the fatty acids was done as described in ref 13. The fatty acids were derivatized to their phenacyl esters and purified by C-18 HPLC as described above. Phenacyl ω-cyclohexylundecanoate (3 mg/1 L culture) eluted at 44 min: ^{13}C-NMR ($CDCl_3$) δ 24.9, 26.5, 26.8, 26.9, 29.1, 29.3, 29.5, 29.6, 29.7, 30.0, 33.5, 34.0, 37.6, 37.7, 65.8, 127.8, 128.8, 133.8, 134.4, 173.2, 192.3.

Synthesis of Labeled Precursors. [8-^{13}C]Cycloheptanecarboxylic acid ([8-^{13}C]3) was prepared from cycloheptyl bromide (1.5 g, 8.5 mmol) and $^{13}CO_2$ (206 mL, 9.2 mmol) to give [8-^{13}C]**3** (662 mg, 55%) in an analogous manner to the synthesis of [7-^{13}C]**5**:[13] ^{13}C-NMR δ ($CDCl_3$) 26.3 (d, $^3J_{C3,6-C8}$ = 4.2 Hz, C-3,6), 28.3 (s, C-4,5), 30.6 (s, C-2,7), 44.9 (d, $^1J_{C1-C8}$ = 54.0 Hz, C-1), 183.6 (enhanced signal with small satellites symmetrically arranged, $^1J_{C8-C1}$ = 54.0 Hz, C-8).

3′,5′-Dibromobenzyl cyanide (8) was prepared from 3′,5′-dibromobenzyl bromide (2 g, 6.1 mmol) and NaCN (377 mg, 7.7 mmol) in 80% yield analogous to the synthesis of benzyl cyanide:[60] EIMS *m*/*z* (relative intensity) 273 (M^+, 0.50), 275 [$(M + 2)^+$, 1.00], 277 [$(M + 4)^+$, 0.48]; High-resolution EIMS *m*/*z* 272.87897 (calcd for $C_8H_5Br_2N$, 272.87887); ^{1}H-NMR (acetone-d_6) δ 4.04 (s, H-2), 7.63−7.75; ^{13}C-NMR (acetone-d_6) δ 22.7 (C-2), 118.2 (C-1), 123.8 (C-4′), 131.1 (C-3′,5′), 134.0 (C-2′,6′), 136.9 (C-1′). Anal. Calcd for $C_8H_5Br_2N$: C, 35.03; H, 1.84; N, 5.11. Found: C, 34.90; H, 1.83; N, 5.04.

3′,5′-Dibromophenylacetic Acid (9). Compound **8** (1 g, 3.6 mmol) was hydrolyzed in KOH to give **9** in 90% yield:[60] EIMS *m*/*z* (relative intensity) 292 (M^+, 0.50), 294 [$(M + 2)^+$, 1.00], 296 [$(M + 4)^+$, 0.49]; high-resolution EIMS *m*/*z* 291.87324 (calcd for $C_8H_6Br_2O_2$, 291.87345); ^{1}H-NMR (acetone-d_6) δ 3.76 (s, H-2), 7.55−7.65. ^{13}C-NMR (acetone-d_6) δ 39.9 (C-2), 123.0 (C-4′), 132.5 (C-3′,5′), 132.8 (C-2′,6′), 140.4 (C-1′), 171.8 (C-1). Anal. Calcd. for $C_8H_6Br_2O_2$: C, 32.66; H, 2.06; O, 10.88. Found: C, 32.48; H, 1.97; O, 10.89.

[3′,5′-$^2H_2$]Phenylacetic Acid ([3′,5′-$^2H_2$]6). Compound **9** (755 mg, 2.57 mmol) was stirred for 2 h at 80 °C with Na_2CO_3 (286 mg, 2.70 mmol) in D_2O (2 mL). The D_2O was removed under vacuum at 100 °C, and the sodium salt was dissolved in 7.5% NaOD in D_2O (4 mL). Zinc dust (2 g, 30.6 mmol) was added in one portion, and the mixture stirred at reflux for 36 h. The reaction mixture was then cooled to room temperature, diluted with H_2O (40 mL), and acidified by dropwise addition of concentrated HCl. The phenylacetic acid was extracted into Et_2O (3 × 40 mL), dried ($MgSO_4$), and concentrated to give **6** (264 mg, 73% yield). The product was refluxed overnight in 30% KOH/H_2O to exchange the deuterated benzylic position with hydrogen. Workup as described above gave [3′,5′-$^2H_2$]**6** as a white solid (255 mg): mp 76−77 °C (lit.[61] mp 76.5 °C); high-resolution EIMS *m*/*z* 138.06502 (calcd for $C_8H_6D_2O_2$, 138.06498); ^{1}H-NMR (acetone-d_6) δ 3.62 (s, 2H, H-2), 7.24, 7.31 (m, 3H); ^{13}C-NMR (acetone-d_6) δ 40.9 (C-2), 127.3 (C-4′), 128.8 (t, J = 24.4 Hz, C-3′,5′), 130.0 (C-2′,6′), 135.7 (C-1′), 172.8 (C-1).

[2′,6′-$^2H_2$]Phenylacetic Acid ([2′,6′-$^2H_2$]6). 2′,6′-Dichlorophenylacetic acid (1.5 g, 7.3 mmol) was stirred with Na_2CO_3 (848 mg, 8.0 mmol) in D_2O at 80 °C for 3 h. The D_2O was removed, and the sodium salt was stirred with 30% NaOD in D_2O (5 mL) at 80 °C for 36 h. After heating, the D_2O was removed until a wet paste remained. D_2O (10 mL) was added to the mixture in an ice bath, and Ni−Al alloy (1.4 g) was added in one portion. After the Al dissolved and the evolution of D_2 ceased, the mixture was warmed to room temperature and stirred for 30 min. The reaction was worked up as

(60) Vogel, A. Textbook of Practical Organic Chemistry, 4th ed.; Longman: London, 1978.

(61) The Merck Index; Merck & Co., Inc.: Rahway, N.J., 1989.

described above to give [2′,6′-$^2H_2$]**6** (800 mg) in 80% yield: mp 74.5−76 °C; ^{1}H-NMR (acetone-d_6) δ 3.63 (s, 2H, H-2), 7.20−7.36 (m, 3H); ^{13}C-NMR (acetone-d_6) δ 41.1 (C-2), 127.5 (C-4′), 129.0 (C-3′,5′), 129.9 (t, J = 24.3 Hz, C-2′,6′), 135.6 (C-1′), 172.8 (C-1).

L-**[3*R*-^{2}H]Phenylalanine ([3*R*-^{2}H]7).** [3*R*-^{2}H]**11** was prepared by hydrogenation of [3-^{2}H]**10**[44] with [Rh((*R*)-prophos)(NBD)]ClO_4 as described.[45] Deprotection yielded [3*R*-^{2}H]**7**:[44] $[\alpha]^{25}_D = -26.5$ (c = 0.9 in H_2O), [lit.[62] $[\alpha]^{25}_D = -29.4$ (c = 1.0 in H_2O)]; ^{1}H-NMR (D_2O) δ 3.09 (d, J = 5.5 Hz, H-3*S*), 3.80 (d, J = 5.5 Hz, H-2), 7.14−7.29 (m, 5H); ^{13}C-NMR (D_2O) δ 36.4 (t, J_{C3-2H} = 19.4 Hz, C-3), 56.3 (C-2), 127.8, 129.3, 129.7, 135.6, 173.5 (C-1).

L-**[2,3*S*-^{2}H$_2$]Phenylalanine ([2,3*S*-^{2}H$_2$]7).** [2,3*S*-^{2}H$_2$]**11** was prepared by hydrogenation of **10** with D_2.[45] Deprotection yielded [2,3*S*-^{2}H$_2$]**7**:[44] $[\alpha]^{25}_D = -26.5$ (c = 1.0 in H_2O); ^{1}H-NMR (D_2O) δ 2.93 (s, H-3*R*), 7.11−7.24 (m, 5H); ^{13}C-NMR (D_2O) δ 36.4 (t, J_{C3-2H} = 19.7 Hz, C-3), 55.3 (t, J_{C2-2H} = 18.5 Hz, C-2), 127.9, 129.4, 129.6, 135.4, 173.0 (C-1).

Zinc Borodeuteride.[46] A mixture of $ZnCl_2$ (1.25 g, 9.2 mmol) and anhydrous Et_2O (50 mL) was refluxed for 2 h in a 100 mL flask. The mixture was cooled to room temperature, and the supernatant, a saturated solution of $ZnCl_2$ in Et_2O (33 mL, ca. 6 mmol), was slowly added to a stirred solution of $NaBD_4$ (0.5 g, 11.9 mmol) in 17 mL of Et_2O. This mixture was stirred for 2 d at room temperature. The solids were allowed to settle, and the solution of $Zn(BD_4)_2$ (approximately 238 mM in Et_2O) was removed and stored in a stoppered bottle at 0 °C.

Methyl [2-^{2}H]Mandelate. $Zn(BD_4)_2$ (2.6 mL of a 238 mM solution in Et_2O) was added dropwise to a stirred solution of methyl benzoylformate (200 mg, 1.22 mmol) in anhydrous Et_2O (5 mL) in an ice bath. After stirring for 1 h, the reaction was quenched by the addition of H_2O (10 mL) followed by 1 N HCl (2 mL). The Et_2O layer was decanted, and the aqueous solution was extracted again with Et_2O (2 × 10 mL). The Et_2O fractions were dried ($MgSO_4$) and evaporated to give an oil (163 mg, 0.98 mmol, 80% yield): R_f 0.46 (hexane:EtOAc 2:1); IR (neat) 3500, 1740 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 3.65 (br s, 1H), 3.74 (s, 3H), 5.17 (s, 0.01H), 7.32−7.42 (m, 5H); ^{2}H-NMR ($CHCl_3$) δ 5.16; ^{13}C-NMR ($CDCl_3$) δ 53.0, 72.5 (t, $J^{13}_{C-^2H}$ = 22.6 Hz, C-2), 126.6, 128.5, 128.6, 138.1, 174.1.

[2-^{2}H]Mandelic Acid ([2-^{2}H]12). To a solution of methyl [2-^{2}H]mandelate (100 mg, 0.6 mmol) in H_2O (5 mL) was added one KOH pellet. The mixture was stirred at room temperature for 1 h. The resulting solution was washed with Et_2O (3 × 10 mL), and the aqueous layer was acidified with 6 N HCl (approximately 0.5 mL). The liberated acid was extracted with Et_2O (5 × 10 mL), and the Et_2O fractions were combined, dried ($MgSO_4$), and concentrated to give [2-^{2}H]**12** (83.5 mg, 91%): ^{1}H-NMR (acetone-d_6) δ 5.20 (s, 0.02H), 7.29−7.51 (m, 5H); ^{2}H-NMR (acetone) δ 5.14; ^{13}C-NMR (CD_3OD) δ 73.7 (t, $J^{13}_{C-^2H}$ = 22.6 Hz, C-2), 127.8, 129.2, 129.4, 140.6, 176.1.

[8-^{13}C]Cyclohepta-2,4,6-trienyl Cyanide ([8-^{13}C]20) was prepared from K^{13}CN (742 mg, 11.2 mmol) and tropylium tetrafluoroborate[52] (2.00 g, 11.2 mmol) according to Doering and Knox[51] to give [8-^{13}C]**20** (1.237 g, 10.5 mmol, 94%): R_f 0.60 (hexane:EtOAc 5:1); ^{1}H-NMR ($CDCl_3$) δ 2.97 (m, H-1, 1H), 5.34 (m, H-2,7, 2H), 6.29 (dt, J = 8.9, 3.0 Hz, H-3,6, 2H), 6.70 (t, J = 3.2 Hz, H-4,5, 2H); ^{13}C-NMR ($CDCl_3$) δ 29.6 (d, J_{C1-C8} = 61.5 Hz, C-1), 115.9 (d, J = 2.9 Hz), 119.6 (enhanced signal with small satellites symmetrically arranged, J_{C8-C1} = 61.1 Hz, C-8), 127.1 (d, J = 5.5 Hz), 131.2 (s).

Methyl [8-^{13}C]Cyclohepta-2,4,6-trienecarboxylate ([8-^{13}C]21). [8-^{13}C]**20** (1.237 g, 10.5 mmol) was dissolved in anhydrous Et_2O (15 mL) and MeOH (20 mL). Chlorotrimethylsilane (5 mL, 39.4 mmol) was next added slowly to the above stirred solution in an ice bath. The solution was stirred at room temperature for 48 h, at which time it turned pinkish. Water (30 mL) was slowly added over 30 min. The solution was then extracted with Et_2O layers (4 × 40 mL), and the Et_2O layers were combined, dried ($MgSO_4$), and evaporated to give a dark oil (1.197 g). Flash chromatography (silica gel, hexane:EtOAc 6:1) gave pure [8-^{13}C]**21** as an oil (909 mg, 6.0 mmol) and a mixture of [8-^{13}C]**20** and -**21** (172 mg): R_f 0.70 (hexane:EtOAc 5:1); GC−MS m/z (relative intensity) 151 (M^+, 7), 119 (7), 91 (100). ^{1}H-NMR ($CDCl_3$) δ 2.52 (m, H-1, 1H), 3.76 (d, J = 3.9 Hz, COOCH_3, 3H), 5.40 (m, H-2,7, 2H), 6.23 (dm, J = 8.8 Hz, H-3,6, 2H), 6.62 (t, J = 3.1 Hz, H-4,5, 2H); ^{13}C-NMR ($CDCl_3$) δ 43.8 (d, J_{C1-C8} = 61.9 Hz, C-1), 52.1 (d, J = 3.9 Hz, COOCH_3, 3H), 116.9, 125.5 (d, J = 4.9 Hz), 130.8, 173.4 (enhanced signal with small satellites symmetrically arranged, J_{C8-C1} = 62.0 Hz, C-8).

[8-^{13}C]Cyclohepta-2,4,6-trienecarboxylic Acid ([8-^{13}C]-14). [8-^{13}C]**21** (909 mg, 6.0 mmol) was hydrolyzed as described by Betz and Daub[50] to give [8-^{13}C]**14** (680 mg, 5.0 mmol, 92% yield based on consumed **21**): GC−MS m/z (relative intensity) 137 (M^+, 15), 119 (12), 91 ($C_7H_7^+$, 100); ^{1}H-NMR ($CDCl_3$) δ 2.58 (m, H-1, 1H), 5.36 (m, H-2,7, 2H), 6.29 (dm, J = 8.8 Hz, H-3,6, 2H), 6.64 (t, J = 3.2 Hz, H-4,5, 2H), 11.27 (br s, COO*H*, 1H); ^{13}C-NMR ($CDCl_3$) δ 42.9 (d, J_{C1-C8} = 59.8 Hz, C-1), 113.3, 125.9 (d, J = 4.7 Hz), 130.7, 179.9 (enhanced signal with small satellites symmetrically arranged, J_{C8-C1} = 59.8 Hz, C-8).

[1-^{2}H,8-^{13}C]Cyclohepta-2,4,6-trienecarboxylic acid ([1-^{2}H,8-^{13}C]14) was prepared from [8-^{13}C]**14** (133 mg, 0.97 mmol) and 2 N NaOD in D_2O as described[53] to give [1-^{2}H,8-^{13}C]**14** (127 mg): GC−MS m/z (relative intensity) 138 (M^+, 14), 120 (12), 92 ($C_7H_6D^+$, 100); ^{1}H-NMR ($CDCl_3$) δ 2.58 (m, H-1, 0.01H), 5.35 (dd, J = 8.7, 4.0 Hz, H-2,7, 2H), 6.29 (ddd, J = 8.6, 3.4, 3.1 Hz, H-3,6, 2H), 6.64 (dd, J = 3.3, 3.1 Hz, H-4,5, 2H); ^{2}H-NMR ($CHCl_3$) δ 2.53 (br s, H-1); ^{13}C-NMR ($CDCl_3$) δ 42.5 (dt, J_{C1-C8} = 59.6 Hz, J_{C1-2H} = 20.6 Hz, C-1), 113.1, 125.9 (d, J = 4.8 Hz), 130.6, 179.8 (enhanced signal with small satellites symmetrically arranged, J_{C8-C1} = 59.7 Hz, C-8).

[8-^{13}C]Cyclohepta-1,4,6-trienyl Cyanide ([8-^{13}C]22). [8-^{13}C]**20** (614 mg, 4.80 mmol) was isomerized to [8-^{13}C]**22** (250 mg) as described:[53] R_f 0.60 (hexane:EtOAc 5:1); ^{1}H-NMR ($CDCl_3$) δ 2.37 (t, J = 7.0 Hz, H-3), 5.38 (dt, J = 9.3, 6.7 Hz, H-4), 6.03 (q, J = 7.3 Hz, H-2), 6.23 (dd, J = 9.3, 5.5 Hz, H-5), 6.55 (dd, J = 11.2, 3.3 Hz, H-7), 6.72 (dd, J = 11.2, 5.6 Hz, H-6); ^{13}C-NMR ($CDCl_3$) δ 27.8 (d, J = 6.1 Hz, C-3), 112.1 (d, J_{C1-C8} = 78.3 Hz, C-1), 118.2 (enhanced signal with small satellites symmetrically arranged, J_{C8-C1} = 78.3 Hz, C-8), 121.5 (s), 127.3 (d, J = 2.8 Hz), 127.5 (s), 133.8 (d, J = 2.0 Hz), 133.9 (d, J = 5.9 Hz).

[8-^{13}C]Cyclohepta-1,4,6-trienecarboxylic acid ([8-^{13}C]-15) was prepared from [8-^{13}C]**22** (250 mg, 1.82 mmol) to give [8-^{13}C]**15** (214 mg) as described:[53] GC−MS m/z (relative intensity) 137 (M^+, 18), 91 ($C_7H_7^+$, 100); ^{1}H-NMR ($CDCl_3$) δ 2.37 (t, J = 7.3 Hz, 2H, H-3), 5.41 (dt, J = 9.2, 6.7 Hz, 1H, H-4), 6.23 (dd, J = 9.3, 5.5 Hz, 1H, H-5), 6.59 (q, J = 7.0 Hz, 1H, H-2), 6.73 (dd, J =11.4, 5.5 Hz, 1H, H-6), 7.13 (br d, J = 11.5 Hz, 1H, H-7), 11.29 (br s, COO*H*); ^{13}C-NMR ($CDCl_3$) δ 27.8 (d, J = 5.0 Hz, C-3), 120.5 (s), 127.7 (d, J = 2.7 Hz), 127.8 (s), 129.4 (d, J_{C1-C8} = 70.8 Hz, C-1), 131.8 (d, J = 2.4 Hz), 132.2 (d, J = 4.9 Hz), 172.4 (enhanced signal with small satellites symmetrically arranged, J_{C8-C1} = 70.8 Hz, C-8).

[3-^{2}H$_1$,8-^{13}C]Cyclohepta-1,4,6-trienecarboxylic acid ([3-^{2}H$_1$,8-^{13}C]15) was prepared from [8-^{13}C]**14** (135 mg, 0.98 mmol) and 2 N NaOD in D_2O to give [3-^{2}H$_1$,8-^{13}C]**15** (125 mg):[53] GC−MS m/z (relative intensity) 138 (M^+, 14), 120 (11), 92 ($C_7H_6D^+$, 100). ^{1}H-NMR ($CDCl_3$) δ 2.32 (t, J = 7.1 Hz, 1H, H-3), 5.40 (dd, J = 9.2, 6.7 Hz, 1H, H-4), 6.23 (dd, J = 9.3, 5.5 Hz, 1H, H-5), 6.59 (t, J = 6.8 Hz, 1H, H-2), 6.73 (dd, J =11.4, 5.5 Hz, 1H, H-6), 7.13 (dd, J = 11.5, 1.2 Hz, 1H, H-7). ^{13}C-NMR ($CDCl_3$) δ 27.4 (dt, J_{C3-C8} = 5.0 Hz, J_{C3-2H} = 20.2 Hz, C-3), 120.4 (s), 127.7 (d, J = 2.3 Hz), 127.8 (s), 129.3 (d, J_{C1-C8} = 70.6 Hz, C-1), 131.7 (d, J = 2.7 Hz), 132.1 (d, J = 4.2 Hz), 172.5 (enhanced signal, C-8).

Methyl [8-^{13}C]cyclohept-1-enecarboxylate[63] was prepared from [8-^{13}C]**3** (50 mg, 0.35 mmol) in 63% yield in analogy to the synthesis of methyl cyclohex-1-enecarboxylate from **5**:[55] R_f 0.15 (Et_2O:hexane 1:9); ^{1}H-NMR ($CDCl_3$) δ 1.44−1.49 (m, 4H), 1.65−1.69 (m, 2H), 2.22 (dd, J = 11.1, 6.7 Hz, 2H), 2.44 (dd, J = 5.4, 5.3 Hz, 2H), 3.65 (s, 3H), 7.11 (t, J = 6.7 Hz, 1H); ^{13}C-NMR ($CDCl_3$) δ 25.7, 26.1, 27.3, 28.7, 31.9, 51.6, 136.4 (d, J_{C1-C8} = 59.2 Hz, C-1), 144.4, 168.5 (enhanced signal, C-8).

(62) The optical purity was confirmed by comparison of the product's optical rotation with that of authentic material from Sigma.

(63) Pawlak, J. L.; Berchtold, G. A. *J. Org. Chem.* **1988**, *53*, 4063.

[8-^{13}C]Cyclohept-1-enecarboxylic Acid ([8-^{13}C]19). To a solution of methyl [8-^{13}C]cyclohept-1-enecarboxylate (31 mg, 0.2 mmol) in MeOH (0.5 mL) at 0 °C was added a solution of lithium hydroxide monohydrate (21 mg, 0.5 mmol) in H_2O (0.5 mL). The mixture was stirred at 0 °C for 2 h and then the MeOH was removed *in vacuo*. The aqueous solution was diluted with H_2O (5 mL), extracted with Et_2O (2 × 10 mL), acidified to pH 1 with dilute HCl, and extracted with Et_2O (3 × 15 mL). The latter extracts were combined and dried (Na_2SO_4), and the solvent was removed *in vacuo* to give [8-^{13}C]**19** (26 mg, 92% yield) as a colorless oil: ^{1}H-NMR ($CDCl_3$) δ 1.48−1.53 (m, 4H), 1.69−1.79 (m, 2H), 2.30 (dd, J = 11.1, 6.7 Hz, 2H), 2.49 (dd, J = 5.5, 5.3 Hz, 2H), 7.33 (t, J = 6.7 Hz, 1H); ^{13}C-NMR ($CDCl_3$) δ 25.6, 26.1, 27.0, 29.0, 32.0, 135.9 (d, J_{C1-C8} = 59.1 Hz, C-1), 147.3, 173.5 (enhanced signal, C-8).

[8-^{13}C]Cyclohept-2-enecarboxylic Acid ([8-^{13}C]18). Freshly distilled 3-bromocycloheptene[56] (350 mg, 2 mmol) was added dropwise at room temperature to a stirred solution of sodium [^{13}C]cyanide (200 mg, 4 mmol) in 1-methyl-2-pyrrolidinone (1.5 mL). After stirring for 2.5 h, the mixture was diluted with Et_2O (40 mL) and extracted with H_2O (30 mL). The aqueous phase was extracted with Et_2O (2 × 40 mL), and the collected Et_2O extracts were washed with water (10 mL), dried ($MgSO_4$), and evaporated. Chlorotrimethylsilane (1.3 mL, 10 mmol) was added to the crude nitrile (brown oil, ^{13}C-NMR in benzene-d_6: δ_{CN} 120.7 ppm) in absolute Et_2O (3 mL) and anhydrous MeOH (4 mL) at 0 °C and stirred at room temperature for 4 d. The reaction mixture was poured into crushed ice (10 mL) and the product extracted with Et_2O (4 × 20 mL). Drying ($MgSO_4$) and evaporation of the solvent gave the crude methyl ester as a yellowish oil (175 mg) that was directly subjected to a solution of lithium hydroxide monohydrate (95 mg, 2.26 mmol) in 40% aqueous MeOH (2.5 mL). After stirring for 10 h at room temperature, the alkaline solution was washed with Et_2O (3 × 5 mL) and acidified to pH 2 with concentrated HCl. The solution was extracted with Et_2O (3 × 30 mL), the extract dried ($MgSO_4$), and the solvent removed in vacuum to yield [8-^{13}C]**18** as an oil (94 mg, 33% yield): ^{1}H-NMR ($CDCl_3$) δ 1.30−2.20 (m, 8H), 3.29 (m, 1H, H-1), 5.89 (m, 2H); ^{13}C-NMR ($CDCl_3$) δ 26.5, 28.5, 30.3 (d, J = 4.6 Hz), 30.4, 45.8 (d, J = 55 Hz, C-1), 129.6, 133.7 (d, J = 5.2 Hz), 181.6 (enhanced signal, C-8). Anal. Calcd. for $C_8H_{12}O_2$: C, 68.55; H, 8.63. Found: C, 68.12; H, 8.57 (for unlabeled **18**).

[8-^{13}C]Cyclohept-1,3-dienecarboxylic Acid ([8-^{13}C]17). Bromine (53 μL, 1.03 mmol) in chloroform (60 μL) was slowly added to a stirred solution of [8-^{13}C]**18** (141 mg, 1 mmol) in chloroform (0.5 mL mL) at −10 °C. After stirring at room temperature for 10 min, the solvent was removed under vacuum (1 Torr) to quantitatively furnish a 2.8:1 (*cis*:*trans*) mixture of diastereomeric 2,3-dibromo[8-^{13}C]cycloheptanecarboxylic acids as a yellow-brownish solid. The bromides were dissolved in THF (5 mL), treated at −78 °C with slow addition of potassium *tert*-butoxide (95%, 354 mg, 3 mmol), and stirred at −10 °C. After 1 h, water (10 mL) was added, and the aqueous phase was washed with pentane (20 mL) before adjusting to pH 2 (concentrated HCl). The collected organic layers from the Et_2O extraction (3 × 20 mL) were dried ($MgSO_4$) and concentrated to give 98 mg of a yellowish oil. Silica flash column chromatography (hexane:EtOAc 5:1) afforded a 3.6:1 mixture of [8-^{13}C]cyclohepta-1,3- and -1,6-dienecarboxylic acids (colorless crystals, 63 mg, 45%) according to ^{1}H-NMR (ratio of the olefinic protons). Recrystallization (30 mg in 1 mL hexane, −78 °C) afforded pure [8-^{13}C]**17** (5.5 mg): ^{1}H-NMR ($CDCl_3$) δ 1.86 (m, 2H, H-6), 2.42 (m, 2H, H-5), 2.63 (m, 2H, H-7), 5.93 (m, 1H, H-4), 6.21 (dt, J = 11.7, 4.9 Hz, 1H, H-3), 7.14 (t, J = 7.4, 1H, H-2); ^{13}C-NMR ($CDCl_3$) δ 24.5 (d, J = 2.9 Hz), 29.2 (d, J = 2.9 Hz), 33.0, 123.6 (d, J = 7.6 Hz), 123.9, 133.3 (d, J = 69.5 Hz, C-1), 136.3 (d, J = 2.0 Hz), 173.8 (enhanced signal, C-8). Anal. Calcd. for $C_8H_{10}O_2$: C, 69.55; H, 7.30. Found: C, 69.49; H, 7.11 (for unlabeled **17**).

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