Biosynthesis of the Cyclohexanecarboxylic Acid Starter Unit of ω-Cyclohexyl Fatty Acids in *Alicyclobacillus acidocaldarius*§

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Abstract: The formation of the cyclohexanecarboxylic acid starter unit of ω-cyclohexyl fatty acids from shikimic acid in *Alicyclobacillus acidocaldarius* (formerly *Bacillus acidocaldarius*) has been studied. Feeding experiments with ^{13}C- and ^{2}H-labeled samples of shikimic acid and potential intermediates in the wild type and two blocked mutants demonstrated that the formation of cyclohexanecarboxylic acid in this organism follows the same pathway as in *Streptomyces collinus* (*J. Am. Chem. Soc.*, preceding paper in this issue). (1*S*,3*S*)-3-Hydroxycyclohexanecarboxylic acid (**20**) is accumulated by blocked mutant 2, indicating that 5-hydroxycyclohex-1-enecarboxylic acid (**6**) is first reduced to the hydroxy acid **20** and then dehydrated to 2-cyclohexenecarboxylic acid (**8**). A sample of [6-$^{2}H_1$]shikimic acid was fed to blocked mutant 2. Deuterium occupied the *pro*-2*S* position in the resultant **20**, which was then lost when **20** was refed to blocked mutant 10. Thus, the dehydration of **20** involves the removal of the nonacidic *pro*-2*S* proton and the C-3 hydroxyl group in an *anti* fashion. The stereochemistries of other transformations along the pathway are also discussed.

The fatty acid pattern of the thermoacidophilic bacterium *Alicyclobacillus acidocaldarius* (formerly *Bacillus acidocaldarius*) is rather unusual in that the main components (70–90%) are ω-cyclohexyl fatty acids.[1] These fatty acids are suggested to have special physiological importance for the cells at high temperature and low pH.[2] Their dense packing properties provide a unique membrane structure which may play a role in enabling the organism to flourish under such extreme conditions. Consequently, mutants of *A. acidocaldarius* that are deficient in ω-cyclohexyl fatty acid biosynthesis grow poorly at high temperatures and low pH.[3] Model membranes consisting of lipids containing ω-cyclohexyl fatty acids are relatively dense even beyond the phase transition temperature.[4]

ω-Cyclohexyl fatty acids have also been isolated from the thermoacidophilic *A. acidoterrestis*[5] and the mesophilic *Curtobacterium pusillum*.[6] When these organisms are grown at pH 4, the percentage of ω-cyclohexyl fatty acids increases as the growth temperature is raised.[6,7]

This physiological adaptation may also be operative in the thermoacidophile *A. cycloheptanicus*, where ω-cycloheptyl fatty acids occur in high percentage,[8] and in thermoacidophilic archaebacteria containing, especially at high growth temperatures, tetraterpenoic tetraethers with disubstituted cyclopentane rings.[9] In our opinion, the occurrence of different alicycles in the lipids of thermoacidophiles is an example of evolutionary convergence. Recently, the three ω-alicyclic fatty acid containing *Bacillus* species have been placed in the new genus *Alicyclobacillus*.[10] They form a distinct phylogenetic group as supported by 16*S* rRNA sequence data.

Cyclohexanecarboxylic acid derivatives are also encountered in two examples of secondary metabolites in *Streptomyces*. Firstly, cyclohexanecarboxylic acid is found attached via a D-alanine unit to the macrocycle of the ansamycin antibiotic ansatrienin A from *Streptomyces collinus*[11] and to the related trienomycins.[12] Secondly, a singly substituted cyclohexane ring is found in asukamycin, produced by *S. nodosus*, at the terminus of a short polyene chain.[13]

Several groups have determined the shikimic acid origin of the cyclohexanecarboxylic acid starter unit in ω-cyclohexyl fatty acid synthesis.[14–16] In parallel to the present work, the metabolic steps for the conversion of shikimic acid to cyclohexanecarboxylic acid in *S. collinus* have been established.[17] The pathway (Scheme I) involves a series of dehydrations and double bond reductions interspersed in such a way that no intermediate is ever aromatic. 1,4-Conjugate elimination of the hydroxy group at C-3 and a

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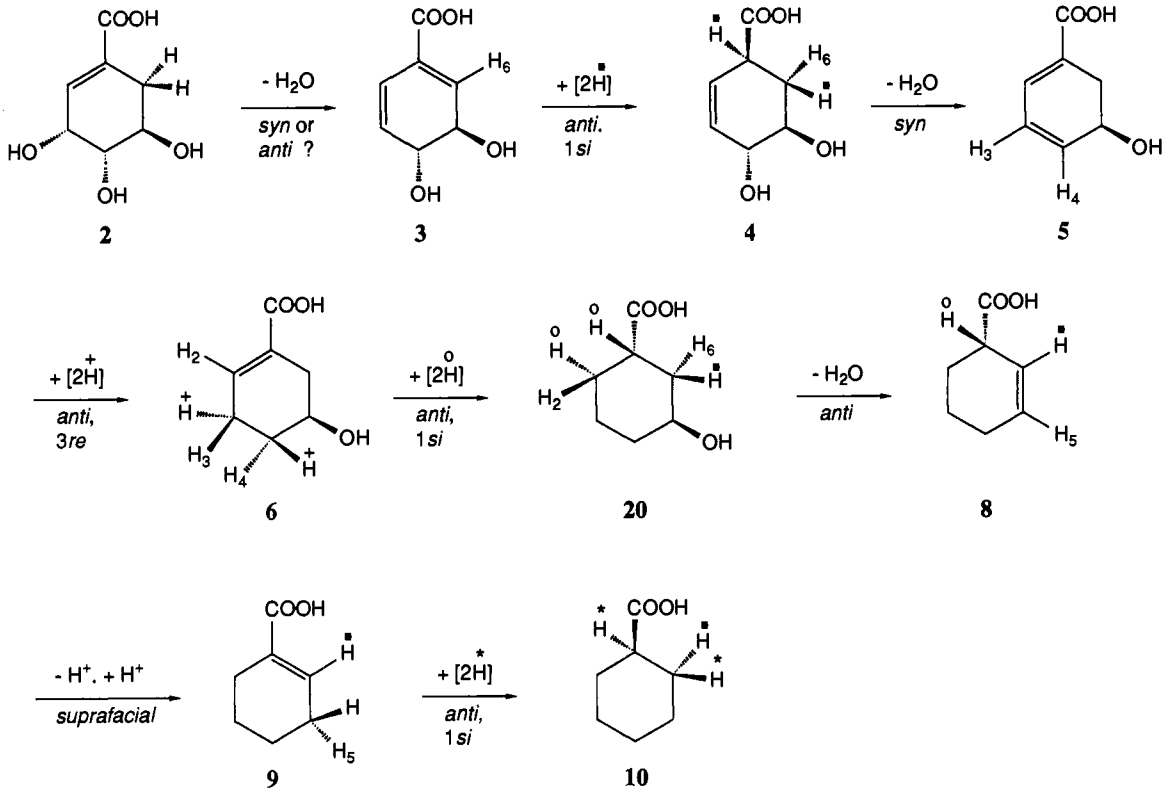
Scheme I. Proposed Biosynthetic Pathway of Cyclohexanecarboxylic Acid Formation in *A. acidocaldarius* and *S. collinus*[a]



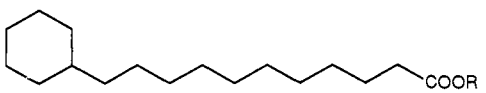
[a] The stereochemical fate of the carbon-bound protons of shikimic acid is depicted in the scheme. Each proton is denoted with a numerical subscript referring to its carbon of origin in shikimic acid.

proton from C-6 of shikimic acid gives rise to a cross-conjugated dihydroxy diene, which undergoes reduction of the double bond conjugated to the carbonyl group. Another 1,4-elimination involving the C-4 hydroxy group and the proton at C-1 gives a 5-hydroxy 1,3-diene. Reduction to 5-hydroxycyclohex-1-enecarboxylic acid proceeds either directly or via the Δ^2 isomer. Another reduction gives the hydroxy acid, which undergoes dehydration involving a nonactivated proton. Isomerization of the double bond into conjugation and a final reduction completes the sequence. At least some of the reactions take place at the level of the corresponding thioesters.

In the work reported here, feeding experiments with the wild-type and two mutants of *A. acidocaldarius* have shown that the pathway from shikimic acid to cyclohexanecarboxylic acid in this organism is very similar or identical to that found in *S. collinus* (Scheme I). Additional pathway information was obtained through analysis of the accumulation products of blocked mutants. This provided a vehicle to probe the stereochemistry of various conversions.

Results

Confirmation of the Shikimate Pathway Origin. In *A. acidocaldarius*, ω-cyclohexylundecanoic acid (**1a**) is the most abundant of the ω-cyclohexyl fatty acids. In our feeding



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a: R=H
b: R=Me

experiments with labeled precursors, the entire mixture of fatty acids from the hydrolysis of the fatty acid lipids was methylated with excess diazomethane in ether and analyzed by GC-MS. Incorporation into methyl ω-cyclohexylundecanoate (**1b**) was calculated[18] from measurements of the isotope content of M^+ (m/z 282) and of the cyclohexane fragment (m/z 83).

First, [2-^{13}C]shikimic acid[19] (**2**) was administered to *A. acidocaldarius* to measure its incorporation into ω-cyclohexyl fatty acids and to confirm earlier reports showing that the cyclohexane ring is derived from shikimic acid. GC-MS analysis of the fatty acid mixture from the feeding of [2-^{13}C]**2** (3 mg/100 mL culture) showed that the $(M + 1)^+$ peak of **1b** was enhanced 4.0%. The label was localized in the cyclohexane ring of **1b** as the ion at m/z 84 was similarly enhanced. Likewise, the homolog methyl ω-cyclohexyltridecanoate was enriched.

5-Hydroxy[5-^{2}H]cyclohex-1-enecarboxylic acid (**6**), which was efficiently reduced to the level of cyclohexanecarboxylic acid in *S. collinus*,[17] was also fed to determine whether this compound is a common intermediate in both organisms. Again, the ions at m/z 283 $(M + 1)^+$ and 84 were significantly enhanced (19.0%). The higher enrichment, as compared with that from [2-^{13}C]**2**, suggested that **6** is a more advanced precursor to cyclohexanecarboxylic acid.

Complementation of Mutants with Potential Precursors. Further experiments were carried out with two mutants of *A. acidocaldarius* that require cyclohexanecarboxylic acid for synthesis of ω-cyclohexyl fatty acids and hence growth.[3] Cross-feeding experiments established that the genetic block of mutant 10 precedes that of mutant 2 in the biosynthetic pathway. Ethyl acetate extracts of the spent media of mutant 2 grown on limiting branched-chain fatty acid precursors supported the growth of mutant 10, yet the converse was not true.

Several intermediates which occur late in the cyclohexanecarboxylic acid pathway established for *S. collinus*[17] supported the growth of mutant 10 (Table I). GC-MS analysis of the fatty acids from mutant 10 supplemented with cyclohex-2-enecarboxylic acid (**8**), cyclohex-1-enecarboxylic acid (**9**), and cyclo-

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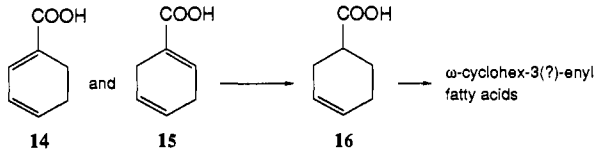
(19) Labeled and nonlabeled precursors had been previously synthesized. Experimental details (including isotopic enrichment) are given in ref 17.

Table I. Complementation of *A. acidocaldarius* Mutants by Potential Precursors of Cyclohexanecarboxylic Acid

precursor	growth[a] mutant 2	growth[a] mutant 10	cyclic fatty acids produced
shikimic acid (**2**)[3]	–	–	
trans-3,4-dihydroxycyclohexa-1,5-dienecarboxylic acid (**3**)		–	
5-hydroxycyclohexa-1,3-dienecarboxylic acid (**5**)	–	–	
5-hydroxycyclohex-1-enecarboxylic acid (**6**)	–		
5-hydroxycyclohex-1-enecarboxylic acid (**6**)		+	ω-cyclohexyl fatty acids
cyclohexa-1,5-dienecarboxylic acid (**7**)	+	+	ω-cyclohexyl fatty acids
cyclohex-2-enecarboxylic acid (**8**)		+	ω-cyclohexyl fatty acids
cyclohex-1-enecarboxylic acid (**9**)		+	ω-cyclohexyl fatty acids
cyclohexanecarboxylic acid (**10**)		+	ω-cyclohexyl fatty acids
trans-4,5-dihydroxycyclohex-1-enecarboxylic acid (**11**)	–	–	
4-hydroxycyclohex-1-enecarboxylic acid (**12**)	–	–	
cyclohexa-2,5-dienecarboxylic acid (**13**)	–	–	
cyclohexa-1,3-dienecarboxylic acid (**14**)	+	+	ω-cyclohex-3(?)-enyl fatty acids
cyclohexa-1,4-dienecarboxylic acid (**15**)	+	+	ω-cyclohex-3(?)-enyl fatty acids
cyclohex-3-enecarboxylic acid (**16**)	+	+	ω-cyclohex-3(?)-enyl fatty acids
5-ketocyclohex-1-enecarboxylic acid (**17**)	–		
5-ketocyclohex-1-enecarboxylic acid (**17**)		+	ω-cyclohexyl fatty acids

[a] (+) Signs indicate growth, and (–) signs indicate no growth.

Scheme II. Conversion of **14**, **15**, and **16** to ω-Cyclohex-3(?)-enyl Fatty Acids in *A. acidocaldarius*



hexanecarboxylic acid (**10**) indicated that ω-cyclohexyl fatty acids were abundantly produced in addition to the normally occurring branched-chain fatty acids. Additionally, both mutants produced cyclohexyl fatty acids and consequently sustained growth when supplemented with cyclohexa-1,5-dienecarboxylic acid (**7**). In contrast, the early intermediates shikimic acid (**2**) and 5-hydroxycyclohexa-1,3-dienecarboxylic acid (**5**) did not support the growth of either mutant. Intermediate **6** suffered a mixed fate as it did not support the growth of mutant 2, yet mutant 10 grew and produced cyclic fatty acids. Interestingly, 5-ketocyclohex-1-enecarboxylic acid (**17**), a compound not on the proposed pathway in *S. collinus*, behaved exactly like **6** as it supported the growth of only mutant 10.

trans-4,5-Dihydroxycyclohex-1-enecarboxylic acid (**11**), 4-hydroxycyclohex-1-enecarboxylic acid (**12**), and cyclohexa-2,5-dienecarboxylic acid (**13**), which are not expected to be on the pathway, did not support the growth of the mutants. However, cyclohexa-1,3-dienecarboxylic acid (**14**), cyclohexa-1,4-dienecarboxylic acid (**15**), and cyclohex-3-enecarboxylic acid (**16**), again not on the proposed pathway, supported the growth of both mutants. No cyclohexyl fatty acids were detected by GC-MS. Instead, cyclohexenyl fatty acids were produced, evident by characteristic MS peaks at m/z 280 (M^+) and 81 ($C_6H_9^+$). Presumably, compound **16**, formed by reduction of only the Δ^1 double bond of **14** and **15**, is incorporated as such, giving rise to unsaturated ω-cyclohex-3-enyl fatty acids (Scheme II).

Incorporation of Potential Intermediates. The above results were confirmed by feeding labeled versions of **7**, **8**, and **10** to blocked mutant 10 (Table II). The resulting ω-cyclohexyl fatty acids from these intermediates were highly enriched, yet the specific incorporations were not 100% as we would expect for cyclic fatty acids produced only from the added labeled precursors. Nonlabeled cyclic fatty acids in the mutants can arise from at least two sources. (1) There already exist cyclic precursors and cyclic fatty acids in the mutant seed culture which is used to inoculate the production culture supplemented with the labeled precursor. (2) The mutants can revert back to the wild type, although the reversion rate is low.[3]

Label from [7-^{13}C]**8** and [7-^{13}C]**10** enriched **1** to the extent of 88.1% and 97.7%, respectively. Additionally, the methylene-

Table II. Isotope Distribution in Methyl ω-Cyclohexaneundecanoate (**1b**) Enriched from Cyclohexanecarboxylic Acid Precursors Fed to *A. acidocaldarius* Mutant 10

precursor	% specific incorporation[a] (±0.5%)
5-hydroxy[5-^{2}H]cyclohex-1-enecarboxylic acid (**6**)[b]	48.2
[2,3,4,5,6-$^2H_5$]cyclohexa-1,5-dienecarboxylic acid (**7**)	84.0
[7-^{13}C]cyclohex-2-enecarboxylic acid (**8**)	88.1
[7-^{13}C]cyclohexanecarboxylic acid (**10**)	97.7
trans-3-hydroxy[3-^{2}H]cyclohexanecarboxylic acid (**20**)[c]	87.3
cis-3-hydroxy[3-^{2}H]cyclohexanecarboxylic acid (**21**)[c]	39.7

[a] Defined as (% enrichment of product)/(% enrichment of precursor) × 100. [b] Incompletely deuterated at C-5 (79%). [c] Incompletely deuterated at C-3 (90%).

Table III. Isotope Distribution in Methyl ω-Cyclohexaneundecanoate (**1b**) Enriched from Cyclohexanecarboxylic Acid Precursors Fed to Wild-Type *A. acidocaldarius*

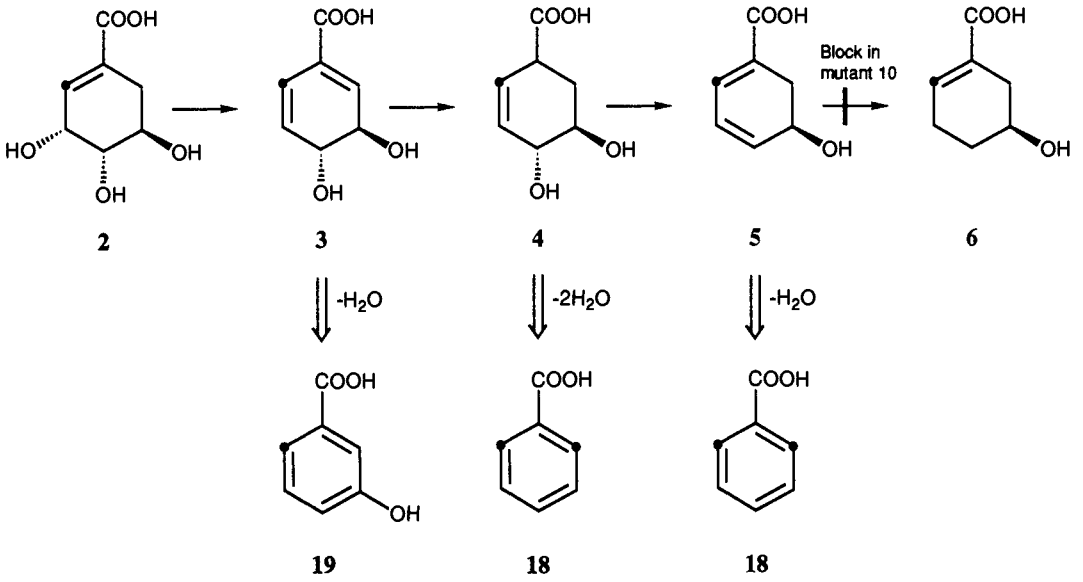
precursor	% specific incorporation[a] (±0.5%)
[2-^{13}C]shikimic acid	4.0
[2-^{2}H]shikimic acid	1.5
[2,5-$^2H_2$]shikimic acid[b]	2.8[c]
[6-$^2H_1$]shikimic acid	0.4
trans-3,4-dihydroxy[2,3,4,5,6-$^2H_5$]cyclohexa-1,5-dienecarboxylic acid (**3**)[d]	12.2[e]
5-hydroxy[5-^{2}H]cyclohex-1-enecarboxylic acid (**6**)[f]	19.0

[a] Defined as (% enrichment of product)/(% enrichment of precursor) × 100. [b] Incompletely deuterated at C-2 and C-5 (70%). [c] No dideuterio species was detected. [d] Incompletely deuterated at C-4 (76%). [e] The % enrichment of the product can be broken down as follows: 1.5% monodeuterio species, 2.6% dideuterio species, 7.1% trideuterio species, and 1.0% tetradeuterio species. [f] Incompletely deuterated at C-5 (79%).

cyclohexane fragment (m/z 97), but not the cyclohexane fragment (m/z 83), was enhanced in both samples. Thus, the ^{13}C label is positioned at C-11 of **1**. Secondly, [2,3,4,5,6-$^2H_5$]**7** enriched the $(M+5)^+$ peak of **1** to 84.0% and shifted the cyclohexyl fragment 5 mass units to m/z 88, indicating that all five deuterium atoms are retained. 5-Hydroxy[5-^{2}H]cyclohex-1-enecarboxylic acid (**6**), which was administered as the racemate and incompletely deuterated (79%), enriched **1** to only 38.1% (48.2% specific incorporation) in mutant 10. The natural and/or unnatural isomer may undergo oxidation–reduction at C-5 via the corresponding ketone (**17**), resulting in the loss of deuterium. This corroborates the interpretation of the finding with **17**.

Loss of deuterium from C-5 was also detected in feeding experiments with ^{2}H-labeled shikimic acids to the wild type (Table III). [2-^{2}H]Shikimic acid enriched the resultant cyclic fatty acid 1.5%, whereas [2,5-$^2H_2$]shikimic acid, incompletely deuterated

Scheme III. Proposed Formation of Benzoic Acid (**18**) and 3-Hydroxybenzoic Acid (**19**) in *A. acidocaldarius* Blocked Mutant 10[a]



[a] The ^{13}C-label from [2-^{13}C]shikimic acid is denoted as a dot and is incorporated into C-2/6 of **18** (1.7%) and C-6 of **19** (5.7%).

at C-2 and C-5 (70%), only singly enriched **1** to 2.8%. No dideuterio species was detected, indicating that much of the deuterium at C-5 is lost. Additionally, [6-$^2H_1$]shikimic acid (98% ^2H, 6*R*:6*S* = 7:3) was fed to the wild type to probe the fate of the C-6 hydrogens of shikimic acid. The resulting cyclohexyl fatty acids contained no detectable (0.4 ± 0.5% enrichment) amount of deuterium. Thus, the two C-6 hydrogens of shikimic acid are replaced during the conversion to cyclohexanecarboxylic acid, as had been found in the cyclohexanecarboxylic acid pathway in *S. collinus*.[17]

trans-3,4-Dihydroxy[2,3,4,5,6-$^2H_5$]cyclohexa-1,5-diene (**3**), corresponding to the first intermediate in the proposed pathway, was efficiently incorporated into **1** (12.2% specific incorporation). The occurrence of the tetradeuterio species $(M + 4)^+$ of **1** (1.0%) indicates that **3** is incorporated intact with the expected loss of deuterium from C-2 (equivalent to C-6 of shikimic acid). However, the major isotope satellites of the molecular ion are the $(M + 2)^+$ and $(M + 3)^+$ peaks at 2.6% and 7.1%, respectively. Keeping in mind that **3** is incompletely deuterated at C-4 (76%),[17] this finding is again in agreement with the partial loss of deuterium from C-3 (equal to C-5 of **2** and **6**) by oxidation–reduction.

Identification of Products Accumulated in Blocked Mutants. Mutant 10 is blocked in the conversion of **5** to **6**, evident from the mutant growth experiments in which **6** supported the growth of the mutant while **5** did not. We thus expected **5** and/or benzoic acid, which could result from the dehydration of **5**, to be accumulated. The spent medium from five 100-mL cultures of mutant 10 was extracted with ethyl acetate, and the extract evaporated to give 12.4 mg of a brown solid residue. Two metabolites (**18**, **19**) were detected in this material. Both were UV active at 254 nm, yet only the more polar **19** was visualized when sprayed with a $KMnO_4$ solution on TLC plates. This led us to believe that **18** was benzoic acid, and comparisons with authentic benzoic acid by chromatography, MS, and NMR confirmed this assumption. Presumably **5** was dehydrated nonenzymatically to **18** in the warm, acidic culture medium prior to isolation. We observed a similar decomposition of synthetic **5** to benzoic acid on storage at 0 °C.

Compound **19** was first suspected to be 5-hydroxycyclohexa-1,3-dienecarboxylic acid (**5**); however, **19** is inert to acidic conditions and is not dehydrated to **18**. Seven carbons, all at low field, are visible in the ^{13}C NMR spectrum of **19**; thus the molecule does not have a 2-fold axis of symmetry. Resonances at δ 167.57 and 158.31 are indicative of a carboxyl carbon and a phenolic carbon, respectively. Additionally, four aromatic protons are present in the ^1H NMR spectrum. Compound **19** was identified by comparison with authentic material (chromatography, NMR) as 3-hydroxybenzoic acid. Conceivably **19** may arise by the air oxidation of **5** during workup. More likely, however, **19** is formed through the nonenzymatic dehydration of **3**, which is accumulated in addition to **5** due to either product inhibition of **5** or pathway overflow. Nonenzymatic decomposition of synthetic **3** to 3-hydroxybenzoic acid has been observed.[17] Intermediate **4**, if also accumulated, would likely undergo decomposition involving two dehydrations in the warm, acidic culture media to **18** and not **19** (Scheme III).

The origin of **18** and **19** was further probed by administering [2-^{13}C]shikimic acid to blocked mutant 10. From this experiment, 3-hydroxybenzoic acid enriched 5.7% at C-6 (δ 121.65) was obtained, while benzoic acid was only enriched 1.7% at C-2/6 (δ 130.40). The higher enrichment in **19** further suggests that **19** originates from an intermediate which is nearer the beginning of the pathway than the precursor of **18**. Also, the hydroxy group remaining in **19** must originate from C-5 of shikimic acid as C-2 of shikimic acid labeled C-6 of **19** (Scheme III).

Growth experiments with mutant 2 suggested that this mutant is blocked in the originally proposed conversion of **6** to **7**.[20] However, we failed to detect **6** or **17** (the oxidized form of **6**) in our analysis of the organic components of the spent culture fluid from this mutant. Instead, two structurally related metabolites (**20**, **21**) were observed, each of MW 144. Esterification with excess diazomethane allowed for the isolation of **20b** and **21b** by silica gel flash chromatography. The ^1H and ^{13}C NMR spectra of both isomers indicated the presence of a secondary alcohol plus a methyl ester and the absence of double bonds. In addition, (±)-[5-^2H]**6** was entirely metabolized in this blocked mutant and enriched **20** and **21** with one deuterium atom each. The ^2H NMR resonance at δ 3.86 for **20** and δ 3.48 for **21** is characteristic of a methine proton on a carbon bearing an alcohol function. These observations led us to propose that **20a** and **21a** are *trans*- and *cis*-3-hydroxycyclohexanecarboxylic acid, respectively. These assignments were confirmed through comparisons with synthetically prepared (±)-**20** and (±)-**21**. The natural and synthetic **20** and **21** were identical chromatographically (TLC, GC) and spectroscopically (MS, ^1H and ^{13}C NMR). This finding indicates that **6** is not dehydrated to **7** before reduction to **8** as was first

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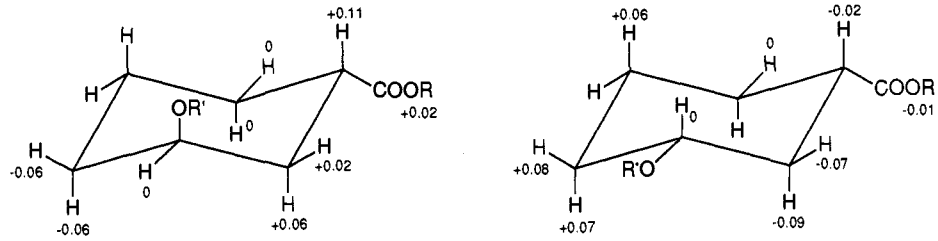
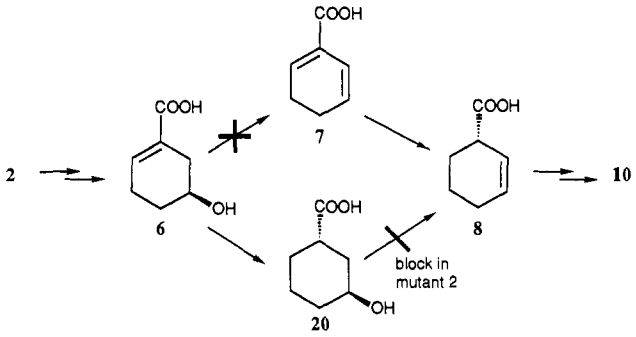


a: R = R' = H
b: R = Me, R' = H
c: R = Me, R' = MTPA

Figure 1. $\Delta\delta_{S-R}$ values obtained for the MTPA esters of methyl (1*S*,3*S*)-3-hydroxycyclohexanecarboxylate (**20c**) and methyl (1*S*,3*R*)-3-hydroxycyclohexanecarboxylate (**21c**). $\Delta\delta$ values are expressed in ppm.

Scheme IV. Sequence of Reduction and Dehydration Steps in the Conversion of **6** into **8**

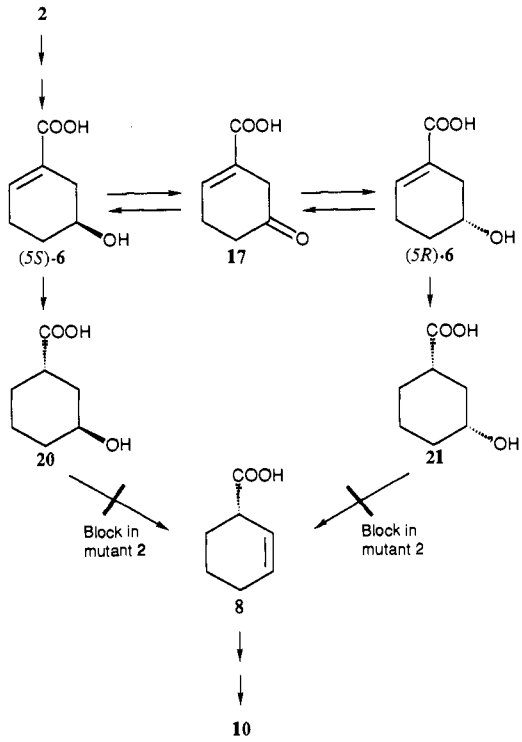


proposed.[20] Rather, **6** is first reduced to **20** and/or **21** and then dehydrated to **8** (Scheme IV).

The formation of the diastereomers **20** and **21** can be rationalized in a number of ways. For example, (5*S*)-**6** could be reduced nonstereospecifically to give two epimers at C-1. Alternatively, (5*S*)-**6** could be oxidized to **17** and reduced to (5*R*)-**6**, and then both enantiomers of **6** could be reduced either stereospecifically or nonstereospecifically. These scenarios were differentiated through analysis of the absolute configurations of **20** and **21**. This was done using Mosher's method, which has been employed recently to elucidate the stereochemistry of several secondary alcohol natural products.[21] Compounds **20b** and **21b** were converted separately to both the (*S*)- and (*R*)-methoxy-(trifluoromethyl)phenylacetate (MTPA) esters. The NMR assignments of the protons were established by 1D-decoupling experiments, and the $\Delta\delta$ ($\delta_S - \delta_R$) values (Figure 1) were found to be compatible only with the (1*S*,3*S*) and (1*S*,3*R*) configurations for **20** and **21**, respectively.[22] Since the stereochemistry at C-1 is fixed in both **20** and **21**, the possibility of nonstereospecific double bond reduction of **6** is eliminated. Rather, the results lend support to the oxidation–reduction branch in the pathway in which (5*S*)-**6** is oxidized to **17** and reduced either back to (5*S*)-**6** or forward to (5*R*)-**6**. Both enantiomers of **6** are then reduced stereospecifically to **20** and **21** (Scheme V).

The oxidation–reduction pathway was further probed by measurements of the ratio of **20**:**21** accumulated in feeding experiments with **6** and **17** in mutant 2. The ratio of **20**:**21** was determined by integrating the methine ^{1}H NMR signals at C-3 of the mixture of **20a** (δ 3.87) and **21a** (δ 3.47). Loading of the

Scheme V. Proposed Oxidation–Reduction Branch in the Cyclohexanecarboxylic Acid Pathway of *A. acidocaldarius* as Evident in Mutant 2



redox pathway with **17** shifted the ratio of **20**:**21** from 1.79:1 to 1.55:1. Furthermore, racemic **6** enhanced the production of **21** (1.42:1). Interestingly, from the feeding of (±)-[5-^{2}H]**6**, **20** was 9 times more enriched in deuterium than **21**, suggesting that the rate of oxidation of (5*R*)-**6** is faster than that of (5*S*)-**6** and/or that (5*S*)-**6** is reduced faster to **20** than (5*R*)-**6** to **21**, leaving (5*R*)-**6** around longer to be oxidized. These results further indicate that **20** is on the direct pathway from shikimic acid to cyclohexanecarboxylic acid, whereas **21** is formed by the redox branch in the pathway. This makes sense as the configuration at C-3 of **21** is opposite that of C-5 in shikimic acid, and formation of **21** therefore must involve the overall inversion of stereochemistry at this center.

We next fed (±)-[3-^{2}H]**20** and -**21** (each 90% deuterated) separately to mutant 10 to test whether both diastereomers are indeed metabolized to **10**. (±)-[3-^{2}H]**20** was very efficiently converted into ω-cyclohexyl fatty acids. The fatty acid extract contained approximately 50% cyclic fatty acids and 50% branched chain fatty acids. The resultant **1** was highly enriched (78.6%; 87.3% specific incorporation), indicating that **20** is converted to **10** efficiently and without loss of deuterium from C-3. Thus, **20** is not oxidized to the corresponding ketone as was **6**. In contrast, (±)-[3-^{2}H]**21** was poorly converted into cyclic fatty acids as the

(21) Ohtani, I.; Kusumi, T.; Kashman, Y.; Kakisawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 4092.

(22) In the marine triterpene sipholenol A, the $\Delta\delta$ values of the protons on the A and B rings of the MTPA derivatives are irregularly arranged, and thus the data could not be used to determine the absolute configuration.[21] The authors suggest that the MTPA group takes on a conformation that is greatly different from the ideal one, as molecular models indicated that the *O*-MTPA group takes on an axial-like orientation and is sterically compressed by two axial protons. However, in (1*S*,3*S*)-**20c**, where the *O*-MPTA group is axial along with protons at C-1 and C-5, the $\Delta\delta$ values are regularly arranged and can be used to determine the absolute configuration of **20**.

amount of ω-cyclohexyl fatty acids was approximately 5% of the total fatty acid content. The resultant **1** was only moderately enriched (35.8%; 39.7% specific incorporation). Although **21** is converted to **10**, the transformation is clearly not as efficient as that of **20**.

These results suggest that **21** is formed in significant concentrations only in mutant 2 due to the product inhibition of **20** and/or pathway overflow. The concentration of (5*S*)-**6** is elevated as **20** is accumulated. Consequently, levels of **17** are increased. The rate of ketone reduction of **17** back to (5*S*)-6 may be faster than the reduction to (5*R*)-**6** in the wild type, yet as concentrations of (5*S*)-**6** increase in the mutant, more (5*R*)-**6** is formed. The double bond reduction of (5*R*)-**6** would provide **21**, which is then also accumulated. This oxidation–reduction branch in the pathway does not appear to be very active in the wild type. We would expect to observe **21** accumulation in the spent medium as it is inefficiently converted to **8**; however, **21** was not detected in the wild type.

The mode of dehydration of **20** to **8** was probed by feeding 30.0 mg of [6-$^2H_1$]shikimic acid to a 1-L culture of blocked mutant 2. The resultant **20** and **21** were isolated as the methyl esters **20b** (16.6 mg) and **21b** (6.6 mg). Aliquots were converted to their (*R*)-MTPA esters as the ^1H NMR spectra of **20c** and **21c** are better resolved and had already been assigned. The ^2H NMR spectra of **20c** and **21c** enriched from [6-$^2H_1$]**2** each contained a single resonance, at δ 1.75 for **20c** and at δ 1.61 for **21c**. In both cases, the deuterium resides in the *pro-S* position at C-2. (2*S*)-[2-^2H]**20b** and -**21b** were saponified with 1.1 equiv of NaOH[17] to (2*S*)-[2-^2H]**20a** and -**21a**, which were refed to blocked mutant 10. ω-Cyclohexyl fatty acids were produced when the mutant was supplemented with (2*S*)-[2-^2H]**20a**, yet no deuterium was detectable in the product. This is consistent with the fact that in the wild type, (6*R*,*S*)-[6-$^2H_1$]shikimic acid did not enrich **1**. The loss of deuterium suggests that the dehydration of **20** to **8** procedes through an *anti* elimination of water involving the *pro-S* hydrogen at C-2. In contrast, if the dehydration of **21** to **8** also proceeds through an *anti* elimination of water, then the *pro-S* deuterium at C-2 of **21** would be retained. However, **21** is very inefficiently converted into cyclic fatty acids, and no appreciable deuterium content was observed in the mass spectrum of the methyl ester of **1** isolated from mutant 10. If deuterium was retained in the process, the low conversion of **21** may have obscured its detection. Alternatively, **21** may be dehydrated with *syn* stereochemistry involving the loss of the deuterium.

Discussion

The conversion of shikimic acid to cyclohexanecarboxylic acid in *A. acidocaldarius* follows the same pathway (Scheme I) as in *S. collinus*.[17] The only difference is the oxidative branch, which involves oxidation and subsequent reduction of **6** via **17**. Intermediates **2**, **3**, and **6** labeled with deuterium at C-5 partially lost the label in the *A. acidocaldarius* pathway, whereas in *S. collinus*, the C-5 proton of shikimic acid was retained entirely. The oxidative branch is accentuated in *A. acidocaldarius* blocked mutant 2. The accumulation of **20** in essence "turns on" the oxidative branch due to pathway overflow, and consequently diastereomer **21** is produced.

The elucidation of the pathway from shikimic acid to cyclohexanecarboxylic acid in both *A. acidocaldarius* and *S. collinus* was done in parallel, and the results from the two systems are complementary. Particularly important was the revision of the pathway based on the results with *Alicyclobacillus* blocked mutant 2. This revision represents an important lesson exemplifying the caution required in deducing metabolic pathways solely from feeding and even from enzymatic experiments. Both compounds **6** and **7** were efficiently reduced *in vivo* to the level of cyclohexanecarboxylic acid and incorporated as such into ω-cyclohexyl fatty acids (*A. acidocaldarius*) and ansatrienin A (*S. collinus*). In addition, the reduction of the diene **7** to the saturated cyclohexane **10** via the monoene **8**, at the level of the CoA esters, was observed in cell-free extracts of *S. collinus*.[23] Later it was shown that the purified enoyl reductase which catalyzes the conversion of 1-cyclohexenecarbonyl–CoA into cyclohexanecarbonyl–CoA also is capable of reducing **7** to **8**.[24] As a consequence, we initially proposed that **6** is dehydrated through the elimination of an allylic hydrogen from C-6 to give **7**, which then is reduced to **8**.[20]

Fortuitously, in *A. acidocaldarius* mutant 2 the conversion of **6** to **8** is blocked, and we were able to isolate the saturated hydroxy acids **20** and **21** as accumulated products. Consequently, we had to consider **20** and **21** as pathway intermediates. Synthetic (±)-[3-^2H]**20** was then shown to be efficiently converted to the level of cyclohexanecarboxylic acid in both organisms, yet synthetic (±)-[3-^2H]**21** was not. Thus, the unsaturated hydroxy acid **6** is not dehydrated to the diene **7** but instead undergoes double bond reduction to **20**. Both pathways cannot operate simultaneously, at least not in *A. acidocaldarius*, as the dehydration of **6** to **7** would bypass the mutant block and cyclic fatty acids would be produced. Thus, the cross-conjugated diene **7** is not an intermediate on the pathway but rather is shunted into the pathway via the monoene **8** through the nonspecific reduction by 1-cyclohexenecarbonyl–CoA reductase when added exogenously to the fermentation.

The steric course of the 1,2-elimination of **20** to **8** was probed by adding (–)-(6*R*,*S*)-[6-$^2H_1$]shikimic acid to a culture of blocked mutant 2 and determining which C-2 hydrogen of the accumulated **20** is enriched. It should be recalled that no deuterium was detectable in **1** when [6-$^2H_1$]shikimic acid was administered to the wild type. The loss of both labeled hydrogens indicates that two dehydrations must proceed with proton loss from C-6. The first dehydration involves a 1,4-conjugate elimination of the C-3 hydroxy group and one of the C-6 hydrogens of shikimic acid in the conversion to the dihydroxydiene **3**. The conversion of the hydroxy acid **20** to the Δ^2 monoene **8** must involve the loss of the other original C-6 hydrogen from shikimate. The resulting **20** from the feeding of [6-$^2H_1$]**2** contained a single atom of deuterium in the *pro*-2*S* position, indicating that this proton and the C-3 hydroxy group (equivalent to the C-5 hydroxy group in shikimate) must be eliminated in an *anti* fashion. This was confirmed by refeeding (2*S*)-[2-^2H]**20** to blocked mutant 10 and observing formation of **1** containing no deuterium.

The dehydration of **20** is a very unusual reaction. The *pro*-2*S* hydrogen which is eliminated is not acidic, and the hydroxyl group is a poor leaving group. This contrasts with the majority of biological dehydration and similar elimination reactions which involve the removal of a proton α to a carbonyl or some other activating group and a leaving group β to the carbonyl. The dehydration involves an *anti* 1,2-elimination which may be facilitated by the protonation or phosphorylation of the hydroxy group. The fact that the stereochemistry of the reaction is consistent with a concerted, ionic mechanism may be only fortuitous. The elimination may alternatively proceed through a carbocation intermediate or via a radical mechanism.

The dehydration of **20** is directly analogous to the conversion of 4-hydroxybutyryl–CoA to vinylacetyl–CoA in *Clostridium aminobutyricum* by 4-hydroxybutyryl–CoA dehydratase.[25] Willadsen and Buckel speculate that this conversion proceeds via a radical intermediate. The dehydrations of several 2-hydroxy CoA esters (i.e., lactyl–CoA, 2-hydroxybutyryl–CoA, and 2-hydroxyglutaryl–CoA) also involve the loss of an unactivated hydrogen β to the carbonyl function. However, it has been shown that these 1,2-dehydrations proceed in a *syn* fashion.[26,27] Thus, a concerted, ionic elimination is unlikely. Hofmeister and Buckel

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(25) Willadsen, P.; Buckel, W. *FEMS Microbiol. Lett.* **1990**, *70*, 187.
(26) Buckel. W. *Eur. J. Biochem.* **1980**. *106*, 439.
(27) Hofmeister. A. E. M.; Buckel, W. *Eur. J. Biochem.* **1992**, *206*, 547.

postulate that the overall dehydration of lactyl–CoA by (*R*)-lactyl–CoA dehydratase from *Cl. propionicum* proceeds in two steps: an S_N2 substitution of the hydroxyl group by a hydride ion followed by reoxidation to acryloyl–CoA.[27] In contrast, Kuchta and Abeles have proposed a radical mechanism for this reaction.[28]

In the next step, the Δ^2 monoene **8** undergoes isomerization to its Δ^1 isomer **9**. The stereochemistry at C-1 of **8** can be predicted to be *S* as the conversion of **20** to **8** presumably does not involve this stereocenter. Combining this knowledge of the stereochemistry at C-1 of **8** with our prior observation in *S. collinus* that the C-5 proton of shikimic acid occupies the *pro*-3*3R* (axial) position in ansatrienin,[17] we can deduce that the isomerization of **8** to **9** proceeds suprafacially. This is in agreement with the results of Reynolds et al., who have recently shown that the conversion of **8** to **9** in *S. collinus* proceeds via a suprafacial 1,3-allylic hydrogen shift.[29]

The double bond reduction of 1-cyclohexenecarboxylic acid (**9**) in *S. collinus* was shown to occur in an *anti* fashion.[17] This result again is in agreement with Reynolds et al., who have shown that the reduction of 1-cyclohexenecarbonyl–CoA occurs by the addition of the *pro*-4*S* hydrogen of NADPH to the *si* face at C-2 of the cyclohexene ring and the addition of a solvent proton at C-1.[30] The same stereochemical outcome was observed in the *Alicyclobacillus* pathway for the reductions of (5*S*)- and (5*R*)-**6** to (1*S*,3*S*)-**20** and (1*S*,3*R*)-**21**, respectively. Hydrogen addition to the *si* face of both C-1 and C-2 of the cyclohexene ring of **6** in an *anti* fashion will give the observed *S* stereochemistry at C-1 of **20**. The fate of the C-2 hydrogen of shikimate is also established during this reduction. It was observed in *S. collinus* that this hydrogen ends up occupying the *pro*-3*6R* (axial) position of ansatrienin,[17] which is in agreement with this model. Thus, it seems plausible that the enoyl reductases responsible for the three Δ^1 double bond reductions in the pathway all operate by the same mechanism and with the same stereochemistry. For that matter, a single reductase may be responsible for all three Δ^1 double bond reductions.

If so, the stereochemistry at C-1 of intermediate **4** created by the reduction of **3** can be predicted to be *S*. The C-2 hydrogen of **3**, originating from C-6 of **2**, would then occupy the *pro*-6*S* position in **4** as a result of the *anti* addition of hydrogen. That this is the case follows from the observation that the *pro*-2*S* position of **20** was enriched with deuterium from [6-$^2H_1$]shikimic acid. As the stereochemistry at C-2 of **20** is initially created by the reduction of **3** to **4**, the C-2 hydrogen of **3** must occupy the *pro*-6*S* position in **4**. It should be noted that **4** is the only compound on the pathway which has not been synthesized and tested directly for conversion into **1** or the cyclohexanecarboxylic acid moiety of ansatrienin A. However, all the indirect evidence[17] leaves little doubt that **4** or its CoA ester must be a pathway intermediate.

The next step in the pathway is a *syn* 1,4-conjugate dehydration involving the loss of the C-4 hydroxy group and the C-1 proton of **4** to give the monohydroxy 1,3-diene **5**. Once again, to facilitate the dehydration the C-4 hydroxy group may first be activated. However, in this case the proton being eliminated is reasonably acidic, particularly if the reaction takes place on the CoA thioester rather than on the free acid, and an ionic process thus seems quite feasible.[31] The observed *syn* stereochemistry of this elimination is consistent with a concerted mechanism. Both model studies[32] and theoretical investigations[33] favor a *syn* over an *anti* stereochemistry for concerted 1,4-conjugate eliminations of this type. This elimination contrasts with the conversion of 5-enolpyruvyl shikimate 3-phosphate to chorismate, which involves removal of the nonacidic *pro*-6*R* hydrogen and loss of phosphate with overall *anti* stereochemistry.[34] The chorismate synthesis is analogous to the 1,4-conjugate elimination of water from shikimic acid to the dihydroxydiene **3**, which we propose as the first step in the pathway to cyclohexanecarboxylic acid. However, we do not know which C-6 hydrogen of **2** is eliminated, whether the C-3 hydroxy group is activated, or even whether the branchpoint is **2** or, perhaps, chorismic acid. If the latter is the case, **3** would be generated by loss of the enolpyruvyl side chain of chorismic acid. In fact, this transformation of chorismate to **3** has precedence in *Klebsiella pneumoniae*.[35]

The conversion of the 1,3-diene **5** to the 1-monoene **6** involves the overall reduction of the Δ^3 double bond. This reduction may plausibly proceed in one of two ways. It may entail the direct reduction of the Δ^3 double bond by hydrogen addition to the *re* face at C-3 and the *si* face at C-4 of **5** in an *anti* fashion. This stereochemistry is deduced from the [3-^{2}H]- and [4-^{2}H]shikimic acid feeding experiments with *S. collinus*.[17] Deuterium from C-3 and C-4 of shikimate was effectively incorporated and occupied the *pro*-3*5R* (equitorial) and 3*4E* (equitorial) positions, respectively, in an *anti* orientation in the resulting ansatrienin. Alternatively, **5** may undergo a 1,4-conjugate double bond reduction to the corresponding Δ^2 monoene, followed by isomerization of the double bond into conjugation with the carboxyl group to **6**. Both pathways have precedent in linoleic acid degradation.[36] Bovine liver 2,4-dienoyl–CoA reductase catalyzes the reduction of 2-*trans*,4-*cis*-decadienoyl–CoA to 3-*trans*-decenoyl–CoA in a 1,4-addition of hydrogen across the diene system. This metabolite is in turn isomerized to 2-*trans*-decenoyl–CoA. In contrast, the same enzyme from *Escherichia coli* catalyzes a 1,2-addition of hydrogen to the Δ^4 double bond, giving 2-*trans*-decenoyl–CoA as the direct reduction product of 2-*trans*,4-*cis*-decadienoyl–CoA. Both reductases have been purified to homogeneity and use NADPH as an electron donor.

The results presented here define in considerable detail the mode of conversion of shikimic acid to cyclohexanecarboxylic acid, a diversion of the shikimate pathway of aromatic amino acid biosynthesis leading to a highly reduced hydroaromatic compound. We have been able to establish the sequence of reactions comprising this unique pathway and to deduce the steric course of all but one of these transformations. Most of the pathway reactions probably do not occur on the free acids but on their coenzyme A thioesters, as has been demonstrated at the enzymatic level for the transformations of **7**, **8**, and **9** in the *S. collinus* system.[23,30] Exactly at what stage in the pathway thioester formation takes place, however, is not clear yet, nor is the exact point of departure of this metabolic route from the common shikimate pathway known.

As has been stressed repeatedly, the pathway for cyclohexanecarboxylic acid formation in *A. acidocaldarius* is similar or identical to that in *S. collinus*. That suggests the possibility of a common evolutionary origin of the pathways in the two organisms. This and the mechanistic enzymology of the pathway will be the subject of future investigations.

Experimental Section

General Procedures. The ^{1}H, ^{2}H, and ^{13}C NMR spectra were obtained on an IBM AF-300 spectrometer operating at a field strength of 7.1 T. 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.) and a VG 7070H double focusing

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mass spectrometer (Manchester, U.K.). The isotopic distribution in methyl ω-cyclohexylundecanoate was detected by selective ion monitoring (SIM) and analyzed according to Biemann.[18] Analytical TLC was executed on precoated silica gel 60F-254 plates. Compounds on the plates were visualized under UV light 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. *Alicyclobacillus acidocaldarius* was obtained from the American Type Culture Collection, Rockville, MD (ATCC 27009). *A. acidocaldarius* blocked mutants 2 and 10 had been previously isolated and characterized.[3] All chemicals were of reagent grade and were used without further purification. Ingredients for fermentations were purchased from Difco and Sigma. $^{13}CO_2$ (99 atom % ^{13}C) for the synthesis of [7-^{13}C]**10** was provided by Isotech Inc. All other labeled precursors had been previously synthesized and are described in detail in ref 17.

Fermentation. *A. acidocaldarius* and mutants thereof were grown for 24 h at 50 °C and pH 4 on a sporulation medium[1] (medium N) containing 50 mM cyclohexanecarboxylic acid. Cultures were grown in 500-mL baffled Erlenmeyer flasks containing 100 mL of media on a rotary shaker at 150 rpm. 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 medium N minus cyclohexanecarboxylic acid. Prior to inoculation, the cells were washed in a sterile salt solution (58.5 mM NaCl, 4.1 mM $MgSO_4$) to remove excess cyclohexanecarboxylic acid. At this time, mutants were tested for revertants by growth on medium N and nongrowth on medium N without yeast extract and cyclohexanecarboxylic acid on 4% agar plates incubated at 50 °C for 3 days. 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 water, 5% $NaHCO_3$ in water, or ethanol to the following cultures in the amounts indicated per culture volume. Wild type: (–)-[2-^{13}C]shikimic acid, 1.0 mg/100 mL; (–)-[2-^{2}H]shikimic acid, 1.4 mg/100 mL; [2,5-$^{2}H_2$]shikimic acid, 2.1 mg/100 mL; (–)-(6*R*,*S*)-[6-$^{2}H_1$]shikimic acid, 1.7 mg/100 mL; *trans*-3,4-dihydroxy[2,3,4,5,6-$^{2}H_5$]cyclohexa-1,5-dienecarboxylic acid, 2.0 mg/100 mL; 5-hydroxy[5-^{2}H]cyclohex-1-enecarboxylic acid, 1.5 mg/100 mL. Mutant 2: (–)-(6*R*,*S*)-[6-$^{2}H_1$]shikimic acid, 30.0 mg/L; 5-hydroxy[5-^{2}H]cyclohex-1-enecarboxylic acid, 10.0 mg/100 mL; 5-ketocyclohex-1-enecarboxylic acid, 3.3 mg/100 mL. Mutant 10: *trans*-3,4-dihydroxy[2,3,4,5,6-$^{2}H_5$]cyclohexa-1,5-dienecarboxylic acid, 0.7 mg/100 mL; 5-hydroxy[5-^{2}H]cyclohex-1-enecarboxylic acid, 1.5 mg/100 mL; [2,3,4,5,6-$^{2}H_5$]cyclohexa-1,5-dienecarboxylic acid, 2.0 mg/100 mL; [7-^{13}C]cyclohex-2-enecarboxylic acid, 3.5 mg/100 mL; [7-^{13}C]cyclohexanecarboxylic acid, 1.0 mg/100 mL; *trans*-3-hydroxy[3-^{2}H]cyclohexanecarboxylic acid, 2.0 mg/100 mL; *cis*-3-hydroxy[3-^{2}H]cyclohexanecarboxylic acid, 2.0 mg/100 mL.

Mutant Growth Experiments. For these experiments, cyclohexanecarboxylic acid and yeast extract were omitted from medium N, and the glucose concentration was raised to 4 g/L. The cells were washed with the above salt solution before inoculation and tested for revertants. In general, 10–20 mol of the precursor was added at the time of inoculation to a 100-mL culture which was harvested 24 h later.

Isolation of Fatty Acids. The 100-mL cultures were centrifuged at 14 900*g* for 15 min. The wet cells were suspended in 4 mL of 2.5% KOH in 1:1 MeOH:H_2O, sealed in a 10-mL capped vial, and heated in an oven at 100 °C overnight. The basic solution was then extracted with 3 × 10 mL of hexane, acidified with 6 M HCl, and reextracted with 3 × 10 mL of hexane to give on average 3 mg of a mixture of fatty acids. The dried hexane extract was esterified with excess diazomethane in ether and analyzed by GC-MS.

Synthesis of [7-^{13}C]Cyclohexanecarboxylic Acid (10).[37] Cyclohexyl bromide (1.5 g, 9.2 mmol) was slowly added over 30 min to a stirred mixture of magnesium shavings (224 mg, 9.2 mmol) and anhydrous THF (5 mL) under nitrogen at 50 °C. One drop of allyl iodide was added to help initiate the reaction. After 1 h, THF (2 mL) was added and the flask was fitted with a nitrogen bubbler and cooled to –70 °C. After 10 min, the nitrogen line was disconnected and $^{13}CO_2$ (99 atom % ^{13}C, 206 mL, 9.2 mmol) was added via a 250-mL gas syringe. The solution was stirred for 2 h at 25 °C and then cooled to 0 °C, and 15 mL of 6 M H_2SO_4 was added. The solution was extracted with $CHCl_3$ (3 × 10 mL). The organic layers were combined and extracted with 10% KOH (3 × 20 mL), and the aqueous solution was acidified with concentrated HCl. The resultant precipitate was extracted into $CHCl_3$ (3 × 20 mL), and the extract dried ($MgSO_4$) and concentrated to give **10** (431 mg, 36%): ^{13}C NMR δ ($CDCl_3$) 25.3 (d, $^{3}J_{C3,5-C7}$ = 4.2 Hz, C-3,5), 25.7 (s, C-4), 28.8 (s, C-2,6), 42.9 (d, $^{1}J_{C1-C7}$ = 54.7 Hz, C-1), 182.3 (enhanced signal with small satellites symmetrically arranged, $^{1}J_{C1-C7}$ = 54.7 Hz, C-7).

Culture Conditions and Isolation of Benzoic Acid (18) and 3-Hydroxybenzoic Acid (19) from Blocked Mutant 10. Mutant 10 was grown in 0.5 L of medium N without yeast extract and supplemented with 200 mg of leucine, 100 mg of isoleucine, and 100 mg of valine per liter. After 24 h, the cells were removed by centrifugation at 14900*g* for 15 min. The spent medium was acidified with 1 N HCl to pH 2, extracted with 3 × 200 mL of EtOAc, dried over $MgSO_4$, and concentrated under reduced pressure to give a 1:1 mixture of **18** and **19** (12.4 mg). An aliquot was treated with excess diazomethane in ether for GC-MS analysis.

18: R_f 0.82 (hexane–EtOAc 1:1); ^{13}C NMR (acetone-d_6) δ 129.30 (C-3,5), 130.39 (C-2,6), 131.39 (C-1), 133.74 (C-4), 167.61 (C-7); GC-MS (methyl ester) *m/z* (relative intensity) 136 (M^+, 34), 105 [$(M - OCH_3)^+$, 100], 77 [$(M - CO_2CH_3)^+$, 73].

19: R_f 0.62 (hexane–EtOAc 1:1); ^{13}C NMR (acetone-d_6) δ 117.01 (C-2), 120.77 (C-4), 121.63 (C-6), 130.39 (C-5), 132.79 (C-1), 158.31 (C-3), 167.57 (C-7).

Culture Conditions and Isolation of (1*S*,3*S*)- and (1*S*,3*R*)-3-Hydroxycyclohexanecarboxylic Acids (20, 21) from Blocked Mutant 2. The culture conditions for mutant 2 and the subsequent workup were similar to those described for mutant 10 above. From a 1.4-L culture, 143 mg of a yellow oil was collected. The accumulated products (**20a** and **21a**) were treated with excess diazomethane in ether until GC-MS analysis indicated complete esterification. The diastereomers were separated on a silica gel flash column (hexane–EtOAc 3:1) to yield 23.7 mg of **20b**, 7.5 mg of **21b**, and 20.9 mg of a mixture of **20b** and **21b**.

20b: R_f 0.49 (hexane–EtOAc 1:1) ^{1}H NMR ($CDCl_3$) δ 1.45–1.85 (m, 8H), 2.74 (tt, *J* = 11.0, 3.5 Hz, 1H), 3.63 (s, 3H), 4.02 (m, 1H).

21b: R_f 0.41 (hexane–EtOAc 1:1) ^{1}H NMR ($CDCl_3$) δ 1.15–1.45 (m, 4H), 1.78–1.96 (m, 3H), 2.16 (dm, 1H), 2.34 (tt, *J* = 12.0, 3.5 Hz, 1H), 3.58 (tt, *J* = 11.0, 4.4 Hz, 1H), 3.65 (s, 3H).

MTPA Esters of Methyl (1*S*,3*S*)-3-Hydroxycyclohexanecarboxylate (20c). A solution of **20b** (3.8 mg, 24.1 μmol) in 0.5 mL of CH_2Cl_2 was treated with (*R*)-(–)-MTPA chloride (9.1 μL, 48.2 μmol), pyridine (100 μL), and a catalytic amount of DMAP. The mixture was shaken and allowed to stand overnight at room temperature. TLC analysis indicated that esterification was complete. The reaction mixture was filtered and purified by preparative TLC (hexane–EtOAc 3:1, R_f 0.74) affording the (*S*)-MPTA ester **20c** (5.7 mg, 63%): ^{1}H NMR ($CDCl_3$) δ 1.44 (m, H-6_{ax}), 1.55 (m, H-$4_{ax,eq}$), 1.81 (ddd, *J* = –14.2, 11.0, 2.8 Hz, H-2_{ax}), 1.89 (m, H-6_{eq}), 2.09 (dm, *J* = –14.2 Hz, H-2_{eq}), 2.57 (tt, *J* = 11.0, 3.9 Hz, H-1), 3.53 (s, OCH_3), 3.66 (s, $COOCH_3$), 5.38 (m, H-3), 7.35–7.53 (m, 5H). Using a similar procedure, 3.3 mg (20.9 μmol) of **20b** was converted with (*S*)-(+)-MTPA chloride to 5.3 mg (68%) of the (*R*)-MTPA ester **20c** (R_f 0.47): ^{1}H NMR ($CDCl_3$) δ 1.44 (m, H-6_{ax}), 1.61 (m, H-$4_{ax,eq}$), 1.75 (ddd, *J* = –14.4, 11.0, 2.8 Hz, H-2_{ax}), 1.89 (m, H-6_{eq}), 2.07 (dm, *J* = –14.4 Hz, H-2_{eq}), 2.46 (tt, *J* = 11.0, 3.6 Hz, H-1), 3.53 (s, OCH_3), 3.64 (s, $COOCH_3$), 5.38 (m, H-3), 7.35–7.53 (m, 5H).

MTPA Esters of Methyl (1*S*,3*R*)-3-Hydroxycyclohexanecarboxylate (21c). Using the above procedure, 1.9 mg (12.0 μmol) of **21b** was esterified with (*R*)-(–)-MTPA chloride to yield 3.2 mg (71%) of the (*S*)-MTPA ester **21c** (R_f 0.42): ^{1}H NMR ($CDCl_3$) δ 1.39 (m, H-4_{ax}), 1.52 (ddd, *J* = –12.2, 12.1, 11.0 Hz, H-2_{ax}), 1.90 (m, H-5_{ax}), 1.93 (dm, *J* = –11.3 Hz, H-6_{eq}), 2.07 (m, H-4_{eq}), 2.24 (dm, *J* = –12.2 Hz, H-2_{eq}), 2.42 (tt, *J* = 12.1, 3.6 Hz, H-1), 3.53 (s, OCH_3), 3.65 (s, $COOCH_3$), 4.97 (tt, *J* = 11.0, 4.4 Hz, H-3), 7.35–7.53 (m, 5H). Similarly, 4.3 mg (27.2 μmol) of **21b** was converted with (*S*)-(+)-MTPA chloride to 7.6 mg (75%) of the (*R*)-MTPA ester **21c** (R_f 0.63): ^{1}H NMR ($CDCl_3$) δ 1.32 (m, H-4_{ax}), 1.61 (ddd, *J* = –14.1, 11.8, 10.8 Hz, H-2_{ax}), 1.84 (m, H-5_{ax}), 1.93 (m, H-6_{eq}), 1.99 (m, H-4_{eq}), 2.31 (dm, *J* = –14.1 Hz, H-2_{eq}), 2.44 (tt, *J* = 11.8, 3.5 Hz, H-1), 3.53 (s, OCH_3), 3.66 (s, $COOCH_3$), 4.97 (tt, *J* = 10.8, 4.4 Hz, H-3), 7.35–7.53 (m, 5H).

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