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Bradley S. Moore, and Heinz G. Floss

J. Nat. Prod., **1994**, 57 (3), 382-386• DOI:
10.1021/np50105a008 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 57, No. 3, pp. 382-386, March 1994

BIOSYNTHETIC STUDIES ON THE ORIGIN OF THE CYCLOHEXANECARBOXYLIC ACID MOIETY OF ANSATRIENIN A AND ω-CYCLOHEXYL FATTY ACIDS

BRADLEY S. MOORE and HEINZ G. FLOSS*

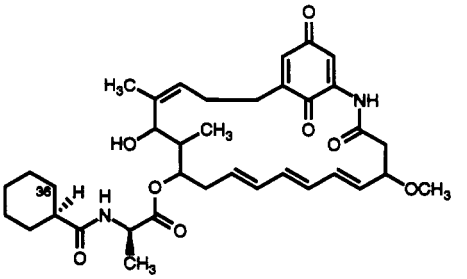
Department of Chemistry, University of Washington, Seattle, WA 98195

ABSTRACT.—Feeding experiments with [2,6,10,10-$^2H_4$]chorismate in *Streptomyces collinus* (ansatrienin A) and *Alicyclobacillus acidocaldarius* (ω-cyclohexyl fatty acids), and inhibitor experiments with glyphosate in the latter organism, have shown that the biosynthesis of cyclohexanecarboxylic acid branches off from the shikimate pathway at a point prior to enolpyruvylshikimate 3-phosphate, either at shikimate or shikimate 3-phosphate.

The metabolic pathway leading to the formation of cyclohexanecarboxylic acid [**10**] in *Streptomyces collinus* (1) and *Alicyclobacillus acidocaldarius* (2) has recently been elucidated in considerable detail. Cyclohexanecarboxylic acid is formed from shikimic acid [**3**] through a series of dehydrations and double-bond reductions in which no intermediate is ever aromatic. In *S. collinus*, the cyclohexanecarboxylic acid moiety is attached via a D-alanine to the macrocyle of the ansamycin antibiotic ansatrienin A [**1**] (3,4), whereas in *A. acidocaldarius*, **10**, probably as its CoA thio ester, serves as the starter unit for the formation of ω-cyclohexyl fatty acids (2,5). The most abundant of the ω-cyclohexyl fatty acids is ω-cyclohexylundecanoic acid [**2**].

One of the unsolved questions in the proposed biosynthetic pathway of **10** (1,2) is the point of divergence from the common shikimic acid pathway of aromatic biosynthesis (6). Either **3** or shikimate 3-phosphate [**4**] may undergo a 1,4-elimination of H_2O or phosphoric acid, respectively, to give (3*R*,4*R*)-3,4-dihydroxycyclohexa-1,5-dienecarboxylic acid [**7**], the first pathway intermediate (Scheme 1). This elimination is analogous to the conversion of 5-enolpyruvylshikimate 3-phosphate [**5**] to chorismate [**6**], which involves the removal of the *pro*-6*R* hydrogen and phosphate (7,8). Alternatively, the enolpyruvyl side chain of **6** may be hydrolyzed to yield **7** (Scheme 1). In fact, this transformation of chorismate has precedence in *Klebsiella pneumoniae* (9).

To probe whether **6** is the starting point for **10** biosynthesis, we performed feeding experiments with [^{2}H]-**6** in both organisms and glyphosate inhibition studies in *A. acidocaldarius*. From these experiments, it is evident that chorismate is not an intermediate in cyclohexanecarboxylic acid formation.



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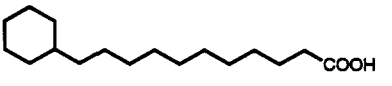

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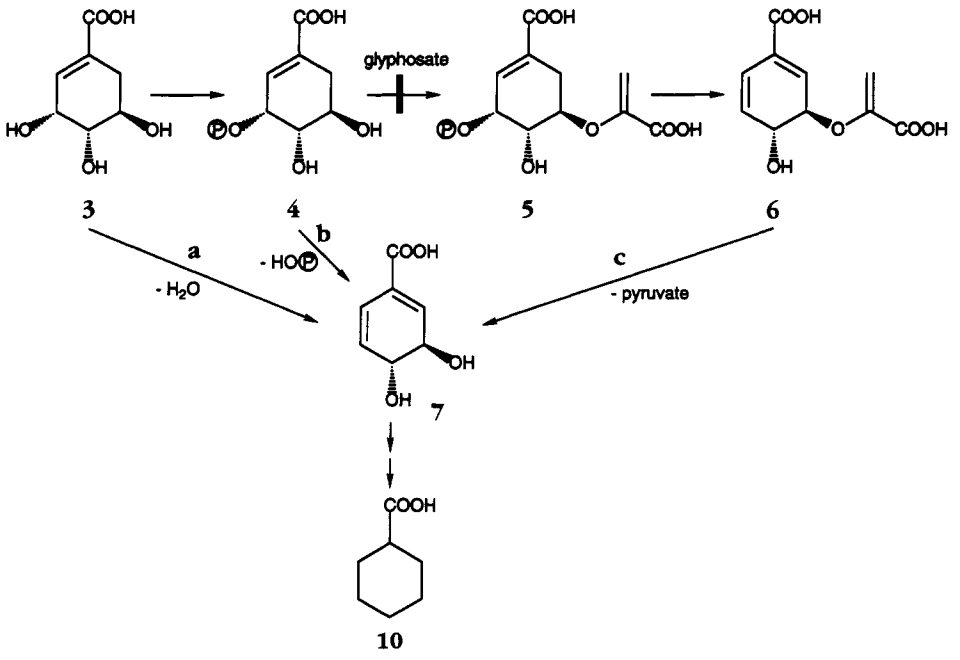
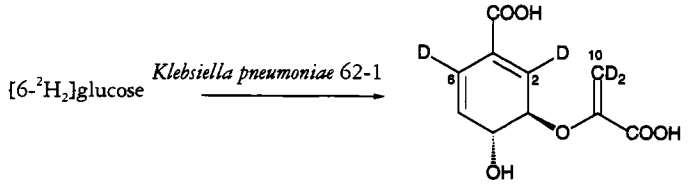


SCHEME 1. Possible Modes of Formation of **7** in the Biosynthesis of **10**: (a) 1,4-Elimination of Water from **3**; (b) 1,4-Elimination of Hydrogen Phosphate from **4**; (c) Loss of the Pyruvyl Side Chain of **6**.

RESULTS AND DISCUSSION

[2,6,10,10-$^2H_4$]Chorismic acid was prepared in vivo from D-[6-$^2H_2$]glucose with *K. pneumoniae* 62-1 (Scheme 2), which is deficient in the shikimic acid pathway enzyme chorismate mutase (10). The deuterium enrichment in **6** was determined by ^1H and ^2H nmr as 42, 67, 34, and 35% at positions 2, 6, 10a, and 10b, respectively. A minor amount of deuterium was also detectable at C-5 (6%). The hydrogen at C-6 is labeled with deuterium via [4-$^2H_2$]erythrose 4-phosphate, whereas the hydrogens at C-2 and C-10 are labeled via phosphoenol-[3-$^2H_2$]pyruvate. The small amount of deuterium at C-5 probably results from the reduction of dehydroshikimate to shikimate by NADPH which acquired deuterium at C-4 during the metabolism of D-[6-$^2H_2$]glucose.

[2,6,10,10-$^2H_4$]Chorismic acid was then fed to *A. acidocaldarius*, and the resultant fatty acid mixture was esterified with CH_2N_2 and examined by gc-ms. Methyl ω-cyclohexylundecanoate (**2**) was singly enriched to the extent of 13.1%. The pathway (1,2) predicts that the deuterium at C-2 of **6** (equivalent to C-6 of **3**) will be lost in the conversion to **10**, but that the deuterium at C-6 will be retained resulting in incorporation of a single atom of deuterium. A minor amount of doubly-labeled **2** (0.9%) was also detected, reflective of the additional presence of deuterium at C-5. Obviously, the



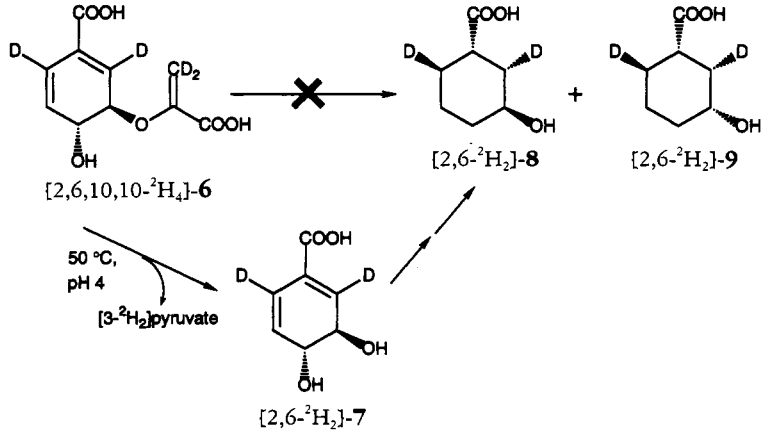
SCHEME 2. Formation of [2,6,10,10-$^2H_4$]-**6** from D-[6-$^2H_2$]Glucose by *K. pneumoniae* 62-1.

deuterium atoms at C-10 of **6** would be lost when the pyruvyl side-chain is eliminated as [3-$^2H_2$]pyruvate.

The labeling orientation was then explored in a second feeding experiment with *A. acidocaldarius* blocked mutant 2, which is auxotrophic for **10** (11). Mutant 2 accumulates (1*S*,3*S*)-3-hydroxycyclohexanecarboxylic acid [**8**] in its spent medium as it is unable to convert **8** to (1*S*)-2-cyclohexenecarboxylic acid (2). The diastereomer (1*S*,3*R*)-3-hydroxycyclohexanecarboxylic acid [**9**], which results from a redox side pathway, is additionally accumulated. [2,6,10,10-$^2H_4$]Chorismic acid (13.0 mg) was fed to a 500 ml culture of blocked mutant 2, and the resultant **8** and **9** were isolated as their methyl esters. The ^{2}H-nmr spectra of both **8** and **9** contained two deuterium resonances each. Aliquots of the **8** and **9** samples were converted to the (*R*)-MTPA esters as their ^{1}H-nmr spectra are better resolved and have already been assigned (2). In both cases, the H-2_{ax} and H-6_{eq} hydrogens were labeled with deuterium. In an earlier experiment when [6-$^2H_1$]shikimic acid was fed to this mutant (2), the H-2_{ax} hydrogen was enriched with deuterium. As C-6 of shikimate corresponds to C-2 of chorismate, the deuterium atoms at C-2 and C-6 of **6** label the H-2_{ax} and H-6_{eq} hydrogens, respectively, of **8** and **9**, which is in agreement with the pathway model.

The chorismate feeding experiment was next repeated in the *S. collinus* system. [2,6,10,10-$^2H_4$]Chorismic acid (12.5 mg) was fed to a 300 ml culture, and the resultant **1** was isolated and analyzed for deuterium enrichment at the *pro*-3*6R* position in **1** (δ 1.41). This hydrogen was labeled with deuterium from [2-$^2H_1$]-**3**, whereas both deuterium atoms at C-6 of **3** are lost in the course of the conversion to **10** (1). However, no deuterium was detectable by ^{2}H nmr at this or any other position of **1**. Ansatrienin from this feeding experiment was then hydrolyzed, and the liberated **10** was esterified. Analysis of the methyl ester by gc-ms again failed to detect any incorporation of deuterium. Thus, it appears that **6** is either poorly taken up by the cells or is not converted into **10** in *S. collinus*.

The thermal decomposition of **6** at 100° in 0.05 M HCl has been studied (9,12) and found to produce mainly 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, phenylpyruvate, and, coincidentally, **7**, the first proposed intermediate in the cyclohexanecarboxylic acid pathway. The bacterium *A. acidocaldarius* is a thermoacidophile grown at 50° and pH 4, and under these conditions, **6** is probably converted non-enzymatically to **7**, which in turn is used to biosynthesize **10** (Scheme 3). In contrast, *S. collinus* is grown at 28° and pH 7.2; conditions under which **6** is not converted to **7** non-enzymatically.



SCHEME 3. Conversion of [2,6,10,10-$^2H_4$]-**6** by *A. acidocaldarius* Mutant 2 into [2,6-$^2H_2$]-**8** and -**9** through the Decomposition Product [2,6-$^2H_2$]-**7**.

In order to determine whether **6** is an intermediate in the *A. acidocaldarius* cyclohexanecarboxylic acid pathway, chorismate biosynthesis was blocked by the addition of *N*-(phosphonomethyl)-glycine (the herbicide glyphosate), an inhibitor of 5-enolpyruvylshikimic acid 3-phosphate synthase (13,14). Glyphosate at a concentration of 100 mg per 100 ml culture was found to inhibit growth of *A. acidocaldarius* in medium N (2) without cyclohexanecarboxylic acid and yeast extract and supplemented with branched-chain amino acids. However, growth in the presence of glyphosate (15) was sustained by the addition of the three aromatic amino acids, *p*-hydroxybenzoic acid, *p*-aminobenzoic acid, and yeast extract (20% of the normal amount indicated for this medium), although cell growth was slower than normal. If the yeast extract was omitted, even if the concentrations of the aromatic compounds were raised fivefold, cell growth was inhibited. It thus appears that an additional aromatic metabolite in the yeast extract is essential for cell growth.

The fatty acid pattern of *A. acidocaldarius* grown in the presence of glyphosate indicated that ω-cyclohexyl fatty acids were produced in amounts comparable to those in the control without glyphosate relative to the naturally occurring branched-chain fatty acids. As cyclic fatty acids were produced in the presence of glyphosate, the starter unit **10** for their formation must be derived from a shikimate pathway intermediate prior to **5**, and thus **6**.

In conclusion, the results argue against chorismic acid being an intermediate in cyclohexanecarboxylic acid biosynthesis. They point to either shikimic acid or shikimic acid 3-phosphate as the branch point at which the biosynthesis of **10** departs from the normal shikimate pathway. The glyphosate inhibition studies do not reveal whether **4** is still on the pathway to **10**; as with the other two dehydrations in the biosynthesis of **10**, we do not know whether water is eliminated directly or if the hydroxyl groups are first activated, possibly as their phosphate esters, before elimination.

EXPERIMENTAL

GENERAL EXPERIMENTAL 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, UK).

MATERIALS.—*Alicyclobacillus acidocaldarius* wild-type and blocked mutant 2 were obtained from Professor Karl Poralla, Tübingen, and *S. collinus* Tü 1892 was obtained from Professor Axel Zeeck, Göttingen, and Professor Hans Zähner, Tübingen. *Klebsiella pneumoniae* strain 62-1 (ATCC 25306) was purchased from the American Type Culture Collection. Ingredients for fermentations were from Difco and Sigma. D-[6,6-$^{2}H_2$]Glucose (98 atom % D) was obtained from Cambridge Isotope Laboratories.

[2,6,10,10-$^{2}H_4$]CHORISMIC ACID [**6**].—*Klebsiella pneumoniae* (formerly *Aerobacter aerogenes*) strain 62-1 was grown as described by Gibson (10). The accumulation medium contained no unlabeled glucose. Instead, D-[6-$^{2}H_2$]glucose (3.6 g), 50% less glucose than prescribed, in H_2O (10 ml), was administered by sterile filtration to four 100 ml cultures (2.5 ml/flask) at the time of inoculation. Isolation of **6** as described gave 101.5 mg of [2,6,10,10-$^{2}H_4$]-**6**: ^{1}H nmr (Me_2CO-d_6) δ 4.71 (1.00 H, dd, J=11.0 and 2.5 Hz, H-4),[1] 4.93 (0.68 H, d, J=2.7 Hz, H-10a), 5.02 (0.96 H, d, J=11.0 Hz, H-3), 5.48 (0.67 H, d, J=2.6 Hz, H-10b), 6.03 (0.90 H, m, H-5), 6.32 (0.33 H, dd, J=9.9 and 2.0 Hz, H-6), 6.88 (0.56 H, d, J=2.7 Hz, H-2). ^{2}H

[1]This proton signal at δ 4.71 has the largest relative intensity, and has been assigned a value of 1.00 H. The other proton signal intensities are relative to this value.

[2]This deuterium signal at δ 6.31 has the largest relative intensity. As the relative intensity of the corresponding proton signal is 0.33 H, its deuterium relative intensity is assigned a value of 0.67D (1.00–0.33). All the other deuterium signal intensities are relative to this value. The deuterium relative intensity values correspond to the deuterium incorporation.

nmr (Me_2CO) δ 4.93 (0.34 D, H-10a), 5.50 (0.35 D, H-10b), 6.05 (0.06 D, H-5), 6.31 (0.67 D, H-6),[2] 6.87 (0.42 D, H-2).

A. ACIDOCALDARIUS FEEDING EXPERIMENT WITH [2,6,10,10-$^2H_4$]-**6**.—Cells from a 24-h-old 100 ml culture of *A. acidocaldarius* were washed in a sterile salt solution and resuspended in 100 ml medium N without **10** (2). [2,6,10,10-$^2H_4$]-**6** (3.1 mg) in H_2O (1 ml) was introduced by sterile filtration during inoculation. After 24 h, the fatty acids were extracted, esterified with CH_2N_2, and analyzed by gc-ms as previously described (2). Methyl ω-cyclohexylundecanoate: gc-ms *m/z* 284 ([M+2]$^+$, 7), 283 ([M+1]$^+$, 37), 282 ([M]$^+$, 100).

A. ACIDOCALDARIUS BLOCKED MUTANT 2 FEEDING EXPERIMENT.—Mutant 2 was grown in 0.5 liter of medium N without yeast extract and **10** but supplemented with leucine, isoleucine, and valine (2). At the time of inoculation, [2,6,10,10-$^2H_4$]-**6** (13 mg) in H_2O (5 ml) was introduced by sterile filtration. After 24 h, **8** (14.5 mg) and **9** (4.0 mg) were isolated from the cultures as their methyl esters (2). Compound **8** (methyl ester at C-7): ^2H nmr (Me_2CO) δ 1.64, 1.70; ($CHCl_3$) δ 1.78. Compound **9** (methyl ester at C-7): ^2H nmr (Me_2CO) δ 1.22, 1.77; ($CHCl_3$) δ 1.40, 1.86. Aliquots of the methyl esters of **8** and **9** were converted with (*S*)-(+)-MTPA chloride to their (*R*)-Mosher esters as previously described (2). Compound **8** [(*R*)-Mosher ester at C-3, methyl ester at C-7]: ^2H nmr ($CHCl_3$) δ 1.75 (H-2_{ax}), 1.86 (H-6_{eq}). Compound **9** [(*R*)-Mosher ester at C-3, methyl ester at C-7]: ^2H nmr ($CHCl_3$) δ 1.61 (H-2_{ax}), 1.95 (H-6_{eq}).

S. COLLINUS FEEDING EXPERIMENT.—*S. collinus* was grown in 300 ml of the normal production medium (1). After incubation for 24 h, [2,6,10,10-$^2H_4$]-**6** (12.5 mg) in H_2O (3 ml) was added by sterile filtration. After an additional 48 h, the culture was harvested and ansatrienin A (13.2 mg) was isolated as previously described (1).

A. ACIDOCALDARIUS GLYPHOSATE INHIBITION EXPERIMENT.—Washed cells of *A. acidocaldarius* were resuspended in 100 ml of medium N without yeast extract and **10**. Glyphosate (100 mg), L-leucine (200 mg), L-isoleucine (100 mg), L-valine (100 mg), L-phenylalanine (5 mg), L-tyrosine (5 mg), L-tryptophan (5 mg), *p*-aminobenzoic acid (0.15 mg), *p*-hydroxybenzoic acid (0.15 mg) and yeast extract (20 mg; the normal medium N contains 100 mg) were added to the medium before sterilization. After 48 h, the fatty acids were extracted, esterified and analyzed by gc-ms (2).

ACKNOWLEDGMENTS

We are indebted to Professor Karl Poralla, Universität Tübingen, for providing cultures of *A. acidocaldarius* wild-type and blocked mutant 2, and Professor Axel Zeeck, Universität Göttingen, and Professor Hans Zähner, Universität Tübingen, for providing the culture of *S. collinus* Tü 1892. Financial support by the National Institutes of Health through Research Grant AI 20264 is greatly appreciated. B.S.M. is the recipient of a National Research Service Award in Biotechnology (Training Grant GM 08437) from the NIH.

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Received 5 October 1993