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MECHANISTIC STUDIES OF A Δ^1,Δ^2-CYCLOHEXENYLCARBONYL COA ISOMERASE CATALYZING THE PENULTIMATE STEP IN THE BIOSYNTHESIS OF THE CYCLOHEXANECARBOXYLIC ACID MOIETY OF ANSATRIENIN A

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ABSTRACT.—Mechanistic studies of the conversion of 2-cyclohexenylcarbonyl CoA to 1-cyclohexenylcarbonyl CoA in a cell-free extract of *Streptomyces collinus* are shown to be consistent with a stereochemical preference for the 1*S* isomer of 2-cyclohexenylcarbonyl CoA and a mechanism of a 1,3-allylic suprafacial shift of a hydrogen, catalyzed by a single base.

The cyclohexanecarboxylic acid moiety of the antibiotic ansatrienin A [**1**], produced by *Streptomyces collinus*, has been shown to be derived from shikimic acid [**2**] (1,2). Studies have revealed that the final two steps in this pathway proceed from 2-cyclohexenylcarbonyl CoA [**3**] with an isomerization of the double bond to form 1-cyclohexenylcarbonyl CoA [**4**], and a reduction of this newly formed α,β-double bond to afford cyclohexylcarbonyl CoA [**5**] (3). The enzyme catalyzing this final reduction step has recently been purified and characterized (4). The stereochemical course of the reduction of **4** has previously been investigated and shown to involve the transfer of a hydrogen from the 4-*proS* position of NADPH to the si face of the β-carbon and an anti addition of hydrogen from solvent at the α-carbon (Figure 1) (1,5). The stereochemical and mechanistic features of the isomerization of **3** to give **4**, on the other hand, have not been investigated. In accord with all other similar enzyme catalyzed 1,3-allylic rearrangements that have been studied, it would be predicted that the mechanism of isomerization would proceed via a suprafacial shift of a hydrogen catalyzed by a single base (6–9). Furthermore, **3** has a chiral center at the α-carbon, and it would seem likely that the enzyme catalyzing the isomerization would exhibit a preference for either the 1*S* or 1*R* isomer. This preference would reflect the isomer most likely formed in the pathway from **2** to **5**. We report herein results of experiments consistent with the mechanistic predictions and with a preference on the part of the isomerase for the 1*S* isomer of **3**.

RESULTS AND DISCUSSION

A crude cell-free extract of *S. collinus* containing both Δ^1,Δ^2-cyclohexenylcarbonyl CoA isomerase and 1-cyclohexenylcarbonyl CoA reductase activities and therefore capable of converting **3** to **5** was used in the following experiments. An aliquot of the products of an incubation of [1-$^2H_1$]-2-cyclohexenylcarbonyl CoA (69 atom % in deuterium) [**3**] with this cell-free extract was base-hydrolyzed, extracted at acidic pH into Et_2O, treated with CH_2N_2 and analyzed by gc-ms. The methyl 1-cyclohexenecarboxylate observed was shown to be 27% monodeuterated. This reflects a 39% retention of deuterium label on the substrate and is consistent with a single base catalyzing the 1,3-allylic rearrangement (Figure 2). In order to confirm that this deuterium was located at C-3 of the cyclohexane ring, another aliquot of the reaction products of this incubation was base-hydrolyzed and extracted at acidic pH with Et_2O. The carboxylic acids so isolated were converted to their *p*-phenylphenacyl derivatives, purified, and analyzed by deuterium nmr. Two major signals were observed at 3.31 ppm and 1.81 ppm and two minor signals at 2.33 ppm and 1.33 ppm (integrations of these peaks were 1.0:0.155:0.054:0.040, respectively).

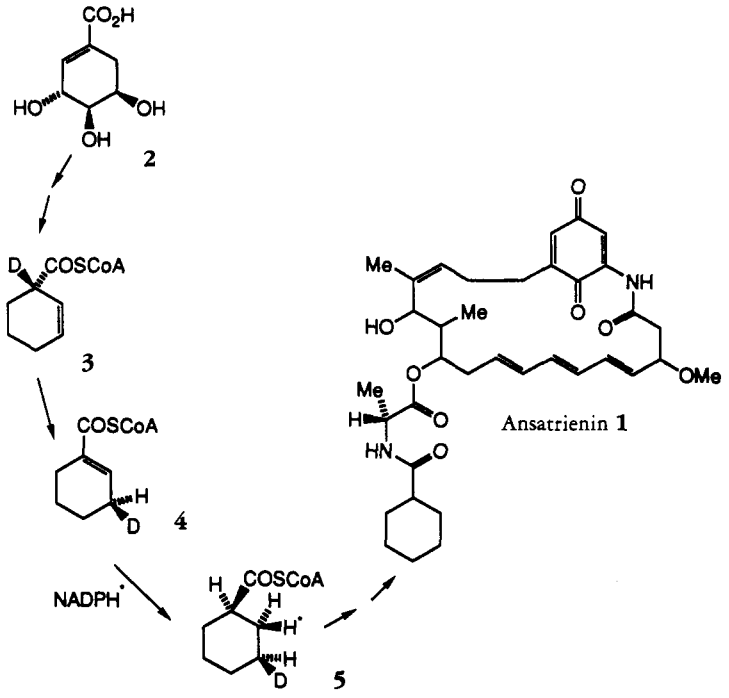

FIGURE 1. Conversion of 2-cyclohexenylcarbonyl CoA [**3**] to cyclohexylcarbonyl CoA [**5**], the final two steps in the biosynthesis of the cyclohexanecarboxylic acid moiety of ansatrienin [**1**] from shikimic acid [**2**].

The signal at 1.81 ppm reflects deuterium located at C-3 of the cyclohexane ring in a *cis*-1,3 relationship to the esterified carboxylate (Figure 2i) (from the ^{1}H nmr of the *p*-phenylphenacyl derivative of cyclohexanecarboxylic acid which was unambiguously assigned using a 2D COSY spectrum). This relationship means, by virtue of the stereochemical course of reduction catalyzed by 1-cyclohexenylcarbonyl CoA reductase, that deuterium was located in the 3*S* position of **4**. This would lead to the conclusion that the substrate of the isomerase must have 1*S* configuration, assuming that the enzyme mechanism is one of an intramolecular suprafacial shift of a hydrogen (Figure 2i). Unreacted **3** gives rise to the peak at 3.31 ppm, while the peak at 2.33 ppm arises from a small quantity of the intermediate of [3-^{2}H$_1$]-1-cyclohexenylcarbonyl CoA [**4**] (Figure 2). The gc-ms analyses of the cell-free incubation products confirmed the presence of **4**. Finally, the smallest signal at 1.33 ppm arises from deuterium located at C-3 of the cyclohexane ring in a *trans*-1,3 relationship to the esterified carboxylate, consistent with the reaction of (1*R*)-[2-^{2}H$_1$]-2-cyclohexenylcarbonyl CoA with the isomerase and reductase in the manner shown in Figure 2ii. Therefore, while the enzyme has a preference for the 1*S* isomer, it apparently can react at a slower rate with the 1*R* isomer. This too was confirmed by the gc-ms analyses of the cell-free incubation products, which revealed that under the conditions of the experiment greater than 50% of the racemic **3** reacted. This result is similar to the partial regiospecific promiscuity that has been observed between two carbons in the reprotonation step of Δ^5-3-ketosteroid isomerases [in this case the catalytic group has been shown to be a freely rotation carboxylate of an aspartate residue (10,11)].

It was also demonstrated, using a coupled enzyme assay with purified 1-cyclohexenylcarbonyl CoA reductase and NADPH (following the oxidation of NADPH

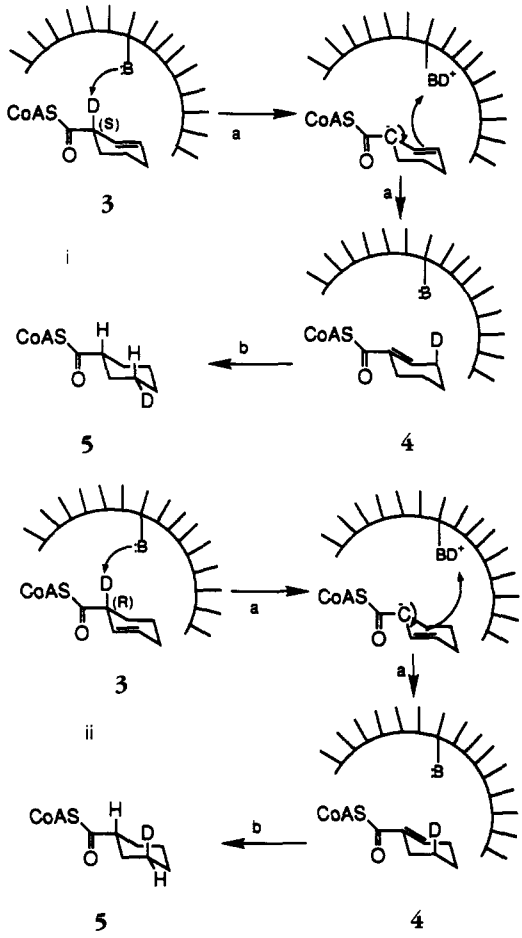

FIGURE 2. Conversion of (1*S*)- and (1*R*)-[2^2H_1]-2-cyclohexenylcarbonyl CoA to deuterated cyclohexylcarbonyl CoA by Δ^1,Δ^2-cyclohexenylcarbonyl CoA isomerase and 1-cyclohexenylcarbonyl CoA reductase. a. Δ^1,Δ^2-Cyclohexenylcarbonyl CoA isomerase. b. 1-Cyclohexenylcarbonyl CoA reductase.

spectrophotometrically at 340 nm), that the Δ^1,Δ^2-cyclohexenylcarbonyl CoA activity in the cell-free activity can be separated from the reductase activity by partial purification through DEAE cellulose and phenyl Sepharose.

In summary, these results are consistent with the presence of a Δ^1,Δ^2-cyclohexenylcarbonyl CoA isomerase that has a sterochemical preference for (1*S*)-2-cyclohexenylcarbonyl CoA and that catalyzes, using a single base, a 1,3-allylic suprafacial shift of a hydrogen. Enoyl CoA isomerases that play a role in both the biosynthesis and β oxidation of unsaturated fatty acids have previously been studied (6–9, 12–16). As such, these enzymes are involved in primary metabolism and catalyze reactions with acyclic substrates that have no chiral center at the α carbon. The mechanistic studies described here have been conducted on an enoyl CoA isomerase, which is unusual because it is the first such enzyme to be implicated in a secondary metabolic process and because it catalyzes a reaction with a cyclic substrate that has a chiral center at the α carbon where it exhibits a preference for the *S* isomer.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—3-Bromocyclohexene was obtained from American Tokyo Kasei and general biochemicals from Sigma. Nmr spectra were obtained on either a General Electric QE 300 MHz or Bruker 500 MHz FT nuclear magnetic resonance spectrometer. Gc-ms analyses were conducted on a Hewlett-Packard 5970-5970A series gas-chromatograph-mass spectrometer equipped with a HP1 methyl silicone gum (0.33-μM film thickness) capillary column.

PREPARATION OF [1-$^2H_1$]-2-CYCLOHEXENYLCARBONYL CoA [**3**].—A solution of anhydrous 1-methyl-2-pyrrolidinone (10 ml) (dried by azeotropic distillation with C_6H_6) containing 12.5 ml (2.0 g) of 3-bromocyclohexene was treated with 25 ml (1.25 g) of anhydrous NaCN under N_2 at room temperature for 90 min. The mixture was poured into H_2O (10 ml) and extracted with Et_2O. The Et_2O extracts were combined, dried over $MgSO_4$, and evaporated to yield the crude nitrile. The nitrile was dissolved in THF and treated at room temperature for 2 h with 5 ml of D_2O (98 atom % in deuterium) and NaOH (50 mg). The solution was extracted with Et_2O, and the Et_2O extracts were dried ($MgSO_4$) and evaporated to afford the deuterated nitrile. Dry HCl was bubbled vigorously for 3 h into a solution of the nitrile in MeOH (50 ml) under reflux conditions. The reaction mixture was cooled, poured into H_2O (100 ml), and extracted with Et_2O. The Et_2O extracts were combined, dried, and evaporated to yield methyl [1-$^2H_1$]-2-cyclohexenecarboxylate. This was then treated at room temperature for 1 h with 50 ml of a solution containing pig liver esterase (100 units), glycine (3.58 g), and tris(hydroxymethyl)aminomethane (790 mg) at pH 8.3. The reaction mixture was acidified and extracted with Et_2O. The Et_2O extracts were combined, washed with brine, and extracted with aqueous K_2CO_3 (10 % w/v) and H_2O. The Et_2O extracts were again combined and dried to afford a clear oil of [1-$^2H_2$]-2-cyclohexenecarboxylic acid (96 atom % in deuterium): ^{1}H nmr (300 MHz, $CDCl_3$) δ 9.5 (1H, br s, CO_2H), 5.5 (2H, m, H-2, H-3), 1.55–1.91 (6H, m); ms *m/z* $[M]^+$ 127 (9), 126 (0.4), 82 (100), 81 (73), 80 (56). This acid was converted to its corresponding coenzyme A thioester as previously described (3,4) and stored at −20° in a 10 mM aqueous solution (partial exchange of the deuterium was observed to occur under these storage conditions).

PREPARATION OF A CELL-FREE EXTRACT OF *S. COLLINUS*.—*S. collinus* (Tü 1862) cells were collected after a 24-h period of incubation with 3 liters of fermentation broth and ruptured by passage through a French press as previously described (3,4).

ISOLATION AND ANALYSIS OF CELL-FREE EXTRACT INCUBATION PRODUCTS BY NMR.—The cell-free extract of *S. collinus* was centrifuged (25,000×*g* for 30 min) and the supernatant stirred with 10 g of DEAE-cellulose (4°, 20 min) and filtered. The DEAE cellulose was then treated with 50 ml of a solution containing potassium phosphate (50 mM), KCl (500 mm), EDTA (1 mM), dithiothreitol (2.5 mM), and glycerol (10% v/v) at pH 7.3 for 30 min and filtered. The filtrate was treated with NADPH (75 mg) and [1-$^2H_1$]-2-cyclohexenylcarbonyl CoA [**3**] (50 mg) at 30° for 90 min and subsequently adjusted to pH 1. The protein precipitate was removed by centrifugation (5,000×*g* for 15 min), and a 1 ml aliquot of the solution was removed and analyzed by gc-ms as previously described (4). The remaining solution was adjusted to pH 11 and stirred with cyclohexanecarboxylic acid (50 mg) for 15 min, acidified (pH 1), and extracted with Et_2O (3×50 ml). The Et_2O extracts were dried over $MgSO_4$ and evaporated. The residue was dissolved in H_2O (2 ml) at pH 11, and the resulting solution was then adjusted to pH 7.5 and heated with 2-bromo-4-phenylacetophenone (134 mg) in THF under reflux conditions for 18 h. The resulting *p*-phenylphenacyl derivative of cyclohexanecarboxylic acid (82 mg, 82% yield based on quantity of cyclohexanecarboxylic acid carrier added) was purified by flash cc on Si gel using CH_2Cl_2-hexane (40:60) as eluent (derivatives of cyclohexenecarboxylic acids were not separated by this technique and were therefore present as minor impurities). ^{1}H nmr (300 MHz, $CDCl_3$) δ 7.4–8.0 (9H, m, Ph-Ph), 5.35 (2H, s, OCH_2), 2.51 (1H, m, H-1), 2.02 (2H, m, H_{eq}-2, H_{eq}-6), 1.81 (2H, m, H_{eq}-3), H_{eq}-5), 1.54 (3H, m, H_{ax}-2, H_{ax}-6, H_{eq}-4), 1.34 (3H, m, H_{ax}-3, H_{ax}-5, H_{ax}-4).

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