BIOSYNTHESIS OF ANSATRIENIN BY *Streptomyces collinus*: CELL-FREE TRANSFORMATIONS OF CYCLOHEXENE- AND CYCLOHEXADIENECARBOXYLIC ACIDS

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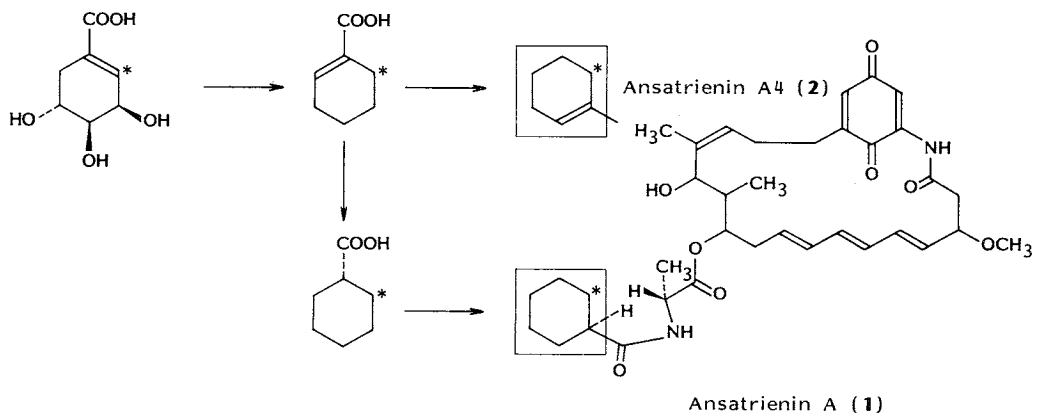
Cell-free extracts of *Streptomyces collinus* were tested with various cyclohexene- and cyclohexadienecarboxylic acids in order to determine the latter stages of the conversion of shikimic acid to cyclohexanecarboxylic acid. It was demonstrated that the final three steps of this process involve reduction of the α,β-double bond of 1(6),2-cyclohexadienylcarbonyl CoA, an isomerization of the double bond of the resulting 2-cyclohexenylcarbonyl CoA to afford 1-cyclohexenylcarbonyl CoA, and a subsequent reduction of the newly formed α,β-double bond. Both of the reduction steps were shown to require NADPH as a cofactor.

Ansatrienin A (mycotrienin I) (**1**) is an antibiotic that has been isolated from *Streptomyces collinus* Tü 1892[1] and *Streptomyces rhishiriensis*[2] and shown to exhibit pronounced activity against fungi and yeasts. The structure of this antibiotic is unusual in that it contains a cyclohexanecarboxamide moiety.

This moiety has been shown by feeding studies to be derived from shikimic acid.[3] Furthermore, the detection of an ansatrienin congener that contains a 1-cyclohexenecarboxamide moiety suggests that the pathway from shikimic acid leads through a 1-cyclohexenecarboxylic acid intermediate.[3] Experiments with specifically isotope labeled shikimic acid have shown that the conversion to this 1-cyclohexenecarboxylic acid is accompanied by migration of the original double bond in the ring, and that the final reduction of the double bond takes place with addition of hydrogen to the *re* face of C-1 (Fig. 1).[4~6] More recently a conversion of 1-cyclohexenecarboxylic acid activated as a coenzyme A thioester, to the corresponding

Fig. 1. Incorporation of isotopically labeled shikimic acid into ansatrienin A (**1**) and ansatrienin A4 (**2**).



thioester of cyclohexanecarboxylic acid in a partially purified cell-free system from *S. collinus* was observed.[7] The stereochemistry of this transformation was shown to be an addition of the *pro-4S* hydrogen of NADPH to the *si* face of C-2 of the cyclohexene ring and addition of solvent hydrogen at C-1 in an *anti* fashion.

The individual steps leading from shikimic acid to the 1-cyclohexenecarboxylic acid intermediate, however, have not been defined. Preliminary *in vivo* experiments revealed that both 1,4- and 2,5-cyclohexadienecarboxylic acid were incorporated at similar levels into ansatrienin.[3] At best only one of these compounds is likely to be the correct biosynthetic intermediate. Reported here are the results of *in vitro* studies with cyclohexene- and cyclohexadienecarboxylic acids that have helped clarify the late stages of the pathway, prior to the 1-cyclohexenecarboxylic acid intermediate.

Experimental

General Methods

^{13}C and ^{1}H NMR spectra were recorded on a General Electric QE300 FT NMR spectrometer. Chemical shifts were determined relative to TMS as an internal standard or were converted to the TMS scale by reference to the $CHCl_3$ resonance at $\delta_H = 7.26$ ppm and δ_C at 77.0 ppm as internal standards. GC-MS Analyses was conducted on a Hewlett-Packard 5970/5790A series gas chromatograph-mass selective detector that was equipped with a HP1 methyl silicone gum (0.33-μM film thickness) capillary column.

Barium carbonate (99 atom% ^{13}C) and sodium cyanide (99 atom% ^{13}C) were obtained from Sigma Chemical Company and Merck Sharp and Dohme Isotopes, respectively. 1-Cyclohexenecarboxylic acid, 3-cyclohexenecarboxylic acid and 1,4-cyclohexadienecarboxylic acid were obtained from Alfa Products and 3-bromocyclohexene from American Tokyo Kasei, Inc. All other chemicals and biochemicals were obtained from either Aldrich or Sigma Chemical Company. *S. collinus* cells were ruptured at 700 kg/cm^2 using a 40 ml pressure cell from American Institute Company and a Carver Laboratory Press. Protein concentrations were measured according to the method of BRADFORD.[8] Spectrophotometric enzyme assays were conducted on either Unicam SP 1800 or Gilford 2600 spectrophotometers.

Preparation of 2-[7-^{13}C]Cyclohexenecarboxylic Acid

This compound was prepared using a modification of a previously described synthesis.[9] A solution of anhydrous 1-methyl-2-pyrrolidinone (10 ml) (dried by azeotropic distillation with benzene) containing 12.3 mmol (2.0 g) of 3-bromocyclohexene was treated with 25 mmol (1.25 g) of anhydrous sodium cyanide (99 atom% ^{13}C) under nitrogen at room temperature for 90 minutes. The mixture was poured into water (50 ml) and extracted with diethyl ether. The ethereal extracts were combined, dried over magnesium sulfate and evaporated to yield the crude nitrile (1.16 g, 88%). Dry hydrogen chloride was bubbled vigoroulsy for 3 hours into a solution of the nitrile in methanol (50 ml) under reflux conditions. The reaction mixture was cooled, poured into cold water and extracted with ether. The ethereal extracts were combined, dried over magnesium sulfate and evaporated to afford the methyl 2-[7-^{13}C]cyclohexenecarboxylate. A solution of this ester in dioxane (27 ml) was treated with molecular sieves for 16 hours. The dioxane solution was decanted and the sieves were washed with additional dioxane (18 ml). Water (10 ml) and 2.3 mmol (400 mg) of *p*-toluenesulfonic acid were added to the dioxane solution and the mixture heated for 16 hours. The mixture was cooled, poured into water and extracted with ether. The ethereal extracts were combined, washed with brine, extracted with aqueous potassium carbonate (10% w/v) and water. The aqueous layers were acidified to pH 2 with hydrochloric acid (6 N), extracted with ether, dried over magnesium sulfate and evaporated to afford a clear oil. Contaminating dioxane was removed at room temperature under vacuum yielding the 2-cyclohexenecarboxylic acid (460 mg, 27% overall yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.5 (1H, br s, CO_2H), 5.5 (2H, m, 2-CH, 3-CH), 3.14 (1H, m, 1-CH), and 1.55~1.91 (6H, m); MS *m/z* 127 (M^+, 8), 109 (28), 81 (100), 80 (81), 79 (62).

Preparation of 1-[7-^{13}C]Cyclohexenecarboxylic Acid

To a solution of *p*-toluenesulfonylhydrazide in 60% methanol (30 ml) was added 12 mmol (1.18 g) of

cyclohexanone. The solution was immediately cooled and a precipitate formed. The solids were filtered, washed with methanol and dried to afford the tosylhydrazone (2.86 g, 89%). MP 136~142°C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.84 (2H, d, J=8.4 Hz), 7.31 (2H, d, J=8.4 Hz), 2.43 (3H, s), 2.21 (4H, m), and 1.61 (6H, m). A solution containing 3.1 mmol (831 mg) of this tosylhydrazone in freshly distilled *N,N,N',N'*-tetramethylethylenediamine (9 ml) was slowly cooled under nitrogen to −78°C. The resulting solid was treated with 15 mmol of *n*-butyl lithium (9.7 ml of a 1.6 M solution in hexanes) and allowed to warm to room temperature during which time a bright orange color formed. The reaction mixture was then cooled (−70°C), treated with carbon dioxide generated from 12 mmol (2.5 g) of barium carbonate (99 atom% in ^{13}C) and allowed to warm to room temperature overnight. The resulting slurry was poured into 1 N hydrochloric acid and extracted into ether. The ethereal extracts were combined, washed with 1 N sodium carbonate and water. The aqueous layers were combined, acidified to pH 2 with 6 N hydrochloric acid, extracted into ether and evaporated to afford the crude 1-[7-^{13}C]cyclohexenecarboxylic acid (99 atom% ^{13}C) (202 mg, 46% overall yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.33 (1H, br s, CO_2H), 7.14 (1H, m, 2-CH), 2.24 (4H, m, 3-CH_2, 6-CH_2), and 1.64 (4H, m, 4-CH_2, 5-CH_2); ^{13}C NMR (75 MHz, $CHCl_3$) δ 172.8 (C-7, enriched), 141.99, 129.23, 25.42, 23.16, 21.41, and 20.77; MS *m/z* 127 (M^+, 33), 109 (31), 81 (100), 80 (67), 79 (54).

Preparation of 1,3-Cyclohexadienecarboxylic Acid

A solution of 8.9 mmol (1 g) of 1-acetoxy-1,3-butadiene, 17.2 mmol (1.72 g) of ethyl acrylate, and 28 μmol (3 mg) of hydroquinone in benzene (2 ml) was placed in a Parr vessel, sealed and heated at 142°C for 16 hours. The reaction mixture was cooled, vented and poured into water (15 ml) and the benzene was removed by azeotropic distillation. The resulting aqueous solution was treated with 6 N hydrochloric acid (40 ml) for 4 hours under reflux conditions. The solution was cooled, filtered, extracted with ether and evaporated to afford a light brown oil containing the crude product (223 mg, 20% overall yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.13 (1H, m, 2-CH), 6.1~6.2 (2H, m, 3-CH, 4-CH), 2.29~2.46 (4H, m, 5-CH_2, 6-CH_2); MS *m/z* 124 (M^+, 36), 123 (33), 105 (7), 79 (100), 78 (21), 77 (69).

Preparation of 1(6),2-Cyclohexadienecarboxylic Acid

To a solution of 2.46 mmol (300 mg) of benzoic acid in methanol (1.2 ml) and liquid ammonia (15 ml) at −78°C was added 12.8 mmol (294 mg) of sodium metal. After all the sodium had dissolved the reaction mixture was allowed to warm to room temperature, 5.5 mmol (295 mg) of ammonium chloride was added and the excess ammonia was removed under a stream of nitrogen. The resulting solid was dissolved in water, acidified to pH 2 with 6 N hydrochloric acid and extracted into ether. The ethereal extracts were combined, dried over magnesium sulfate and evaporated to afford a colorless oil of 2,5-cyclohexadienecarboxylic acid (183 mg, 60%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.91 (2H, m), 5.86 (2H, m), 3.8 (1H, m), 2.72 (2H, m). A solution of 1.2 mmol (150 mg) of this acid in water (5 ml) and 28 μmol (3.0 mg) of hydroquinone was stirred under nitrogen for 5 minutes at which point a 20% w/w potassium hydroxide solution (5 ml) was added and the mixture heated under reflux conditions for 2 hours. The solution was cooled (20°C), acidified to pH 2 with 6 N hydrochloric acid and extracted with ether. The ethereal extracts were combined, dried over magnesium sulfate and evaporated to afford the crude 1(6),2-cyclohexadienecarboxylic acid as a pale yellow oil (149 mg, 49% overall yield). This acid was subsequently purified by flash column chromatography on silica gel with diethyl ether - hexane (1 : 1) as eluant. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.09 (1H, m, 6-CH), 6.36 (1H, m, 2-CH), 5.94 (1H, m, 3-CH), 2.35 (2H, m, 5-CH_2), 2.20 (2H, m, 4-CH_2); MS *m/z* 124 (M^+, 37), 123 (33), 105 (13), 81 (18), 80 (17), 79 (100), 78 (19), 77 (68).

Preparation of Coenzyme A Thioesters

The coenzyme A thioester of 1(6),2-cyclohexadienecarboxylic acid was prepared by the mixed anhydride method.[10] All of the remaining carboxylic acids were converted to the corresponding coenzyme A thioesters using 1,1'-carbonyldiimidazole according to literature procedures.[11] In both cases the coupling reaction was terminated by acidification to pH 4 and removal of the tetrahydrofuran by rotary evaporation. The aqueous solution was then extracted with ether, readjusted to pH 7 and used without further purification.

Culture conditions

S. collinus strain Tü 1892[1] was grown (30°C) and stored (4°C) on agar slants composed of the

following medium: yeast extract 0.4 g, malt extract 1.0 g, glucose 0.4 g, agar 2.0 g, tap water 100 ml, pH 7.4. Spores from these slants were used to inoculate 100 ml of seed medium in a 500-ml Erlenmeyer flask which was subsequently incubated for 48 hours at 28°C and 300 rpm. Of the seed culture, 10 ml was used to inoculate 100 ml of production medium in an Erlenmeyer flasks which was grown for a further 24 hours at 28°C and 300 rpm. Both the seed and the production medium contained soy bean meal, full fat 2.0 g, mannitol 2.0 g, tap water 100 ml, pH 7.3. The cells were harvested by centrifugation (20 minutes, $10,000 \times g$) at 4°C, resuspended and stirred in buffer A, containing potassium phosphate (50 mM), dithiothreitol (2.5 mM), pepstatin A (10 μM), ethylenediaminetetraacetic acid (1 mM) and glycerol (10% v/v) at pH 7.3, and were again collected by centrifugation. The washing procedure in buffer A was repeated once more and the wet cell paste (typically 18 g/100 ml of production media) was frozen (−70°C). The cells could be stored in excess of one year without any appreciable loss of activity.

S. collinus Cell-free Extract

In a typical cell-free conversion study 30 g of the frozen *S. collinus* cells were thawed, placed in 60 ml of buffer B containing potassium phosphate (50 mM), dithiothreitol (2.5 mM), ethylenediaminetetraacetic acid (1 mM), pepstatin A (10 μM), phenylmethylsulfonyl fluoride (1.4 mM), and glycerol (10%) at pH 7.4, and immediately ruptured using a French Press. The cell debris was removed by centrifugation ($25,000 \times g$ for 30 minutes) to yield a cell-free extract typically containing a total of 80 mg of protein. This extract was loaded onto a 1 × 15 cm DEAE cellulose column. Bound protein was subsequently eluted using buffer C (containing potassium phosphate (50 mM), ethylenediaminetetraacetic acid (1 mM), dithiothreitol (2.5 mM) and glycerol (10%) at pH 7.4) containing potassium chloride (1 M). The majority of the protein was collected in approximately 30 ml of buffer and was used directly for the cell-free incubations. All of these operations were carried out at 4°C.

Enzyme Assay

A solution containing 200 μl of the cell-free extract and 20 μl of either 10.5 mM NADPH (β-nicotinamide adenine dinucleotide phosphate, reduced form) or NADH (β-nicotinamide adenine dinucleotide, reduced form) was made up to 1.0 ml with buffer C and warmed to 30°C. Reaction was initiated by addition of 10 μl of a substrate solution (10 mM) and enzyme activity was monitored in a 1 cm path length cell by following the decrease in absorbance at 340 nm corresponding to oxidation of the nicotinamide cofactor.

Cell-free Incubations

The protein solution (30 ml) obtained from the DEAE cellulose column was incubated at 25°C with 6.5 mmol (5 mg) of NADPH and 5.6 mmol (5 mg) of the substrate to be studied. Samples (5 ml) were removed at specified time points and reaction was halted by adjustment to pH 12 with sodium hydroxide (20% w/v).

GC-MS Detection of the Products of Cell-free Incubations

Samples removed from cell-free incubations were hydrolyzed at pH 12 and room temperature for 30 minutes, were adjusted to pH 1 with 6 N hydrochloric acid and extracted with hexane. The hexane extracts were combined, dried over magnesium sulfate and evaporated. The residue was methylated with an ethereal diazomethane solution and was carefully evaporated to an approximate volume of 100 μl. A sample (1 μl) of this solution was analyzed by GC-MS with the mass spectrometer operating in either the scanning or selective ion monitoring mode.

Results and Discussion

Conversion of Cyclohexenecarboxylic Acids

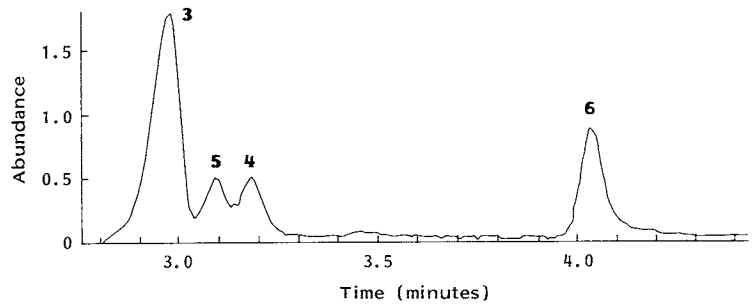
1-[7-^{13}C]Cyclohexenecarboxylic acid was prepared readily in two steps from cyclohexanone using barium [^{13}C]carbonate. The cell-free extract of *S. collinus* demonstrated no enzymatic activity with this acid with ether NADPH or NADH as reductant. When the coenzyme A thioester was prepared and tested using NADPH, the UV assay revealed that 30 g of wet cells afforded an enzyme activity corresponding

to approximately 100 mU (where a unit is defined as the amount of eznyme oxidizing 1 μmol of NADPH per minute). No enzymatic conversion could be effected with NADH as the cofactor. Indeed, in all cases where a cell-free conversion was observed the cofactor was NADPH and it was only the coenzyme A thioester, never the free acid, that served as substrate. In order to substantiate the results of the spectrophotometric assay, an incubation of 1-[7-^{13}C]cyclohexenylcarbonyl CoA (**7**) with the cell-free extract was conducted and samples were removed at various time points. Base hydrolysis both halted the enzyme incubation and hydrolyzed any thioesters present. The free acids were extracted at an acidic pH, methylated and examined by GC-MS under conditions where methyl 1-cyclohexenecarboxylate (**6**) and methyl cyclohexanecarboxylate (**3**) were well separated (see Fig. 2). The GC-MS traces revealed that as the incubation progressed the intensity of the peak for **6** decreased while a peak for **3** (methyl cyclohexanecarboxylate), containing ^{13}C in the carboxyl group, increased. These results indicate that under the conditions of the incubation greater than 50% conversion of 1-cyclohexenylcarbonyl CoA (**7**) to cyclohexanecarbonyl CoA (**8**) could be effected in less than 1 hour. It was clearly demonstrated that this conversion was dependent upon both the substrate being activated as the coenzyme A thioester and the presence of NADPH. This result strengthens the proposal that a 1-cyclohexenecarboxylic acid derivative is an intermediate on the pathway from shikimic acid to cyclohexanecarboxylic acid and would suggest that, at the very least, the late stages of this conversion occur with the carboxylic acids activated as their corresponding coenzyme A thioesters.

It should be noted that enoyl CoA reductases are ubiquitous in nature.[12] The possible caveat of these conclusions, therefore, is that an enoyl CoA reductase with a broad substrate specificity yet not involved in this pathway is giving rise to the observed enzyme activity. This explanation is precluded, however, by the demonstration of the high substrate specificity for 1-cyclohexene-1-carboxylic acid of the purified enzyme (K. A. REYNOLDS, P. WANG, K. M. FOX, Y. LAM & H. G. FLOSS: unpublished results).

2-[7-^{13}C]Cyclohexene-1-carboxylic acid was prepared by a nucleophilic displacement of bromide from 3-bromocyclohexene with potassium [^{13}C]cyanide. Hydrolysis of the nitrile afforded the free acid which was then converted to its coenzyme A thioester. A spectrophotometric assay of a cell-free extract of *S. collinus* with this compound and NADPH revealed enzymatic activity at a similar level to that observed for 1-cyclohexenylcarbonyl CoA. GC-MS analysis of the hydrolyzed products of an incubation revealed that as the incubation time increased, the peak from methyl 2-[7-^{13}C]cyclohexenecarboxylate (**4**)

Fig. 2. Gas chromatographic separation of methyl cyclohexanecarboxylate (**3**), methyl 2-cyclohexenecarboxylate (**4**), methyl 3-cyclohexenecarboxylate (**5**) and methyl 1-cyclohexenecarboxylate (**6**).



Gas chromatographic conditions: Column; HP1 Methyl Silicone Gum (0.33 μm particle size), column temperature; 60°C for 2 minutes and then an increase of 10°C/minute to 200°C.

Fig. 3. Plot of the relative percentages of methyl cyclohexanecarboxylate (**3**, □), methyl 2-cyclohexenecarboxylate (**4**, ■) and methyl 1-cyclohexenecarboxylate (**6**, ●) obtained from analyses of various time points of an incubation of 2-cyclohexenylcarbonyl CoA (**9**) with a *Streptomyces collinus* cell-free extract.

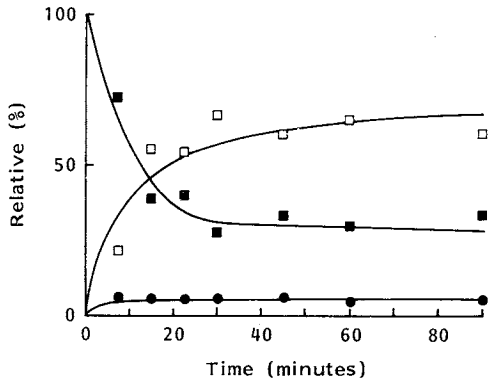
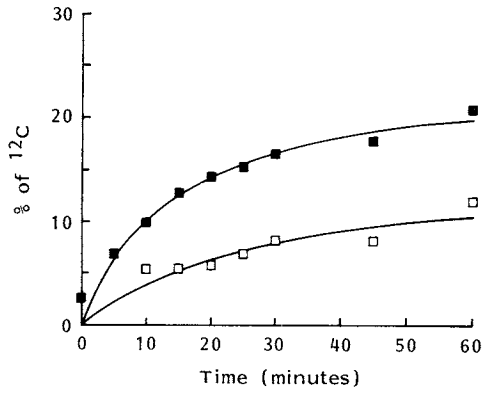


Fig. 4. Plot of the percentage of ^{12}C methyl cyclohexanecarboxylate (**3**, □) and 1-cyclohexenecarboxylate (**6**, ●) against time (obtained from analyses of an incubation of 2-cyclohexenylcarbonyl CoA and 1-[7-^{13}C]cyclohexenylcarbonyl CoA in a 1:1 ratio with a *Streptomyces collinus* cell-free extract).



decreased in intensity while the methyl [7-^{13}C]cyclohexanecarboxylate peak (**3**) increased in intensity. It was also noted from the GC-MS analyses, however, that for each incubation time point, with the exception of the sample examined at 0 minute, there was a small quantity of methyl 1-cyclohexenecarboxylate (**6**) present, which also carried a ^{13}C in the carboxyl group (**4** and **6** have distinctively different fragmentation patterns and, as shown in Fig. 2, are readily separable under the chromatographic conditions utilized). A coinjection of samples from the cell-free incubation with an authentic sample of **6** confirmed the identification. The percentage of **3**, **4**, and **6** at time points throughout the incubation was calculated by integration of each peak in the total ion chromatogram and is plotted in Fig. 3. The ratios of **4** and **6** indicate that while 50% of 2-cyclohexenylcarbonyl CoA (**9**) was reduced to cyclohexanecarbonyl CoA (**8**) the quantity of 1-cyclohexenylcarbonyl CoA (**7**) never exceeded 5%. Control experiments clearly showed that no double bond isomerization occurred in the work up procedure.

It is conceivable that a low level of double bond isomerization to afford **7** is occurring while the majority of **9** is converted to **8** by direct reduction of the β,γ-double bond. Alternatively, this analysis could be interpreted as indicative of a two step process where **9** is enzymatically converted, first to **7** and then to **8** (Fig. 5) and in which a steady state concentration of the intermediate **7** is quickly attained. In an attempt to distinguish between these two possibilities a *S. collinus* cell-free incubation with equimolar quantities of unlabeled 2-cyclohexenylcarbonyl CoA (**9**) and 1-[7-^{13}C]cyclohexenylcarbonyl CoA (**7**) was conducted. Samples were removed from the incubation at determined time points, hydrolyzed and examined as their methyl esters by GC-MS. MS analyses were run first in the scan mode for determination of the relative quantities of **3**, **4**, and **6** and then in the selected ion monitoring mode (for the 140, 141, 142, 143 ions) for accurate determination of the $^{13}C/^{12}C$ ratios of **3**, **4**, **6**. The percentage of ^{12}C for **3** and **6** is plotted against time in Fig. 4. It was observed that as the incubation time increased the ^{12}C content of the methyl 1-cyclohexenecarboxylate (**6**) increased. This can be attributed to the loss of ^{13}C labeled **7** by reduction and the formation of unlabeled **7** by isomerization of **9**. This result is consistent with the steady state concentration proposed for **7** because its quantity as determined by integration of the total ion chromatogram remained essentially unchanged although the percentage of ^{12}C material continually

increased. It was also observed that the increase of percentage of ^{12}C occurred faster for the methyl 1-cyclohexenecarboxylate (**6**) than for the methyl cyclohexanecarboxylate (**3**). This two is consistent with an isomerization of the double bond of **9** prior to reduction. It is noted that the methyl 2-cyclohexenecarboxylate (**4**) remained devoid of ^{13}C label throughout the incubation. This result indicates that under the conditions of the experiment the isomerization was essentially irreversible. A reexamination of the results obtained from incubation of 1-cyclohexenylcarbonyl CoA (**7**) with NADPH and the *S. collinus* cell-free extract revealed that there was no detectable formation of **9**, concurring with this conclusion. When the two-substrate experiment was repeated with 1-[7-^{13}C]cyclohexenecarbonyl CoA (**7**) and nonlabeled 2-cyclohexenylcarbonyl CoA (**9**) in a 1 : 9 ratio, only [7-^{13}C]cyclohexanecarbonyl CoA (**8**) was produced. Although some direct reduction of the double bond of **9** cannot be ruled out, these results would indicate that the majority of the conversion follows the pathway of isomerization to **7** prior to reduction to **8** (Figure 4).

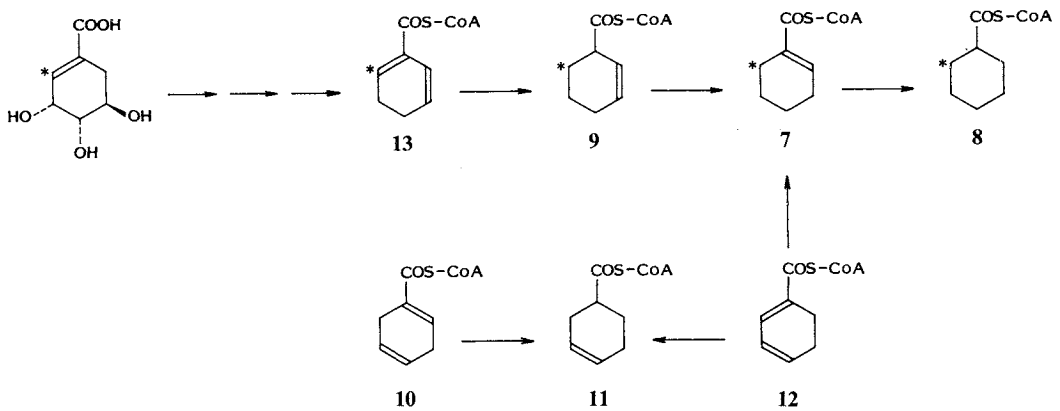
3-Cyclohexenylcarbonyl CoA was prepared and tested with the *S. collinus* cell-free extract. In contrast to the other isomers no conversion of this compound was observed thereby indicating that it is not on the pathway from shikimic acid to cyclohexanecarboxylic acid.

Conversion of Cyclohexadienecarboxylic Acids

The clean conversion of 2-cyclohexenylcarbonyl CoA (**9**), through 1-cyclohexenylcarbonyl CoA to afford cyclohexylcarbonyl CoA (**8**) indicates that **9** is an intermediate on the pathway from shikimic acid and raises the question of its immediate biosynthetic precursor.

Previous whole cell incorporation experiments had implicated either 2,5 or 1,4-cyclohexadienecarboxylic acid as possible intermediates.[3] Consequently, 2,5-cyclohexadienylcarbonyl CoA was prepared from the free acid (obtained from a Birch reduction of benzoic acid) and tested with the *S. collinus* cell-free extract. No enzymatic activity could be detected using the spectrophotometric assay nor could any reduction products be detected by GC-MS analyses. 1,4-Cyclohexadienylcarbonyl CoA (**10**) was also prepared from the corresponding free acid and in this case a UV spectrophotometric assay revealed activity of this thioester with the *S. collinus* cell-free extract. This activity was approximately 60% of that observed for **7**. A GC-MS analysis of the incubation products, however, revealed only methyl 1,4-cyclohexadienecarboxylate and methyl 3-cyclohexenecarboxylate (**4**) (as shown in Fig. 2 all methyl cyclohexenecarboxylates are separa-

Fig. 5. Cell-free transformations of cyclohexene- and cyclohexadienecarboxylic acids by *Streptomyces collinus*.



ble under the chromatographic conditions utilized and the identification of **4** in this experiment was confirmed by a series of coinjections). Clearly the α,β-double bond of the substrate **10** was reduced affording 3-cyclohexenylcarbonyl CoA (**11**) (Fig. 5) which was itself not transformed further by the cell-free extract. These results indicated that neither of these two acids is the biosynthetic precursor to **9**, and suggested that other isomers of dihydrobenzoic acid should be tested.

1,3-Cyclohexadienecarboxylic acid was prepared by acid treatment of the Diels Alder adduct of 1-acetoxy-1,3-butadiene and ethyl acrylate. The coenzyme A thioester of this acid was prepared and tested with the *S. collinus* cell-free extract and NADPH. A spectrophotometric assay revealed activity at a level of 25% of that observed with **7** and a GC-MS analysis of the hydrolyzed products of an incubation was carried out. The harsh work-up conditions did not allow for the observation of methyl 1,3-cyclohexadienecarboxylate. However, the appearance of methyl cyclohexenecarboxylates could be monitored and observation of these compounds indicated that 1) the α,β-double bond of 1,3-cyclohexadienylcarbonyl CoA (**12**) was reduced to afford 3-cyclohexenylcarbonyl CoA (**11**), 2) the γ,δ-double bond of **12** was also reduced, thus affording 1-cyclohexenylcarbonyl CoA (**7**) which was further reduced to cyclohexanecarbonyl CoA (**8**) (Fig. 5), and 3) no detectable amounts of 2-cyclohexenylcarbonyl (**9**) were formed in the process. Formation of **9** could result presumably *via* a 1,4-reduction of **12**. Precedence for such a reduction are the 2,4-dienoyl CoA reductases found in both eucaryotes and procaryotes[13~15] but the absence of any detectable levels of **9** in this experiment would argue against this possibility. This observation coupled with the relatively low level of activity with **12** and the relatively high levels of unreactive **3** formed would argue that despite some reduction to **8**, 1,3-cyclohexadienylcarbonyl CoA is not on the pathway from shikimic acid.

Finally 1(6),2-cyclohexadienecarboxylic acid was prepared *via* a base catalyzed isomerization of 2,5-cyclohexadienecarboxylic acid and converted to the coenzyme A thioester (**13**). A UV spectrophotometric assay with the thioester, NADPH and the *S. collinus* cell-free extract proved positive with approximately 100% of the activity observed with **7**. Subsequently an incubation was conducted and samples were removed, hydrolyzed and analyzed by GC-MS. The harsh work up conditions again meant that the starting material could not be observed. The analysis revealed, however, that cyclohexylcarbonyl CoA was being formed through the intermediate 1-cyclohexenylcarbonyl CoA. This transformation could occur simply *via* a reduction of the β,γ-double bond or by a reduction of the α,β-unsaturated double bond to afford 2-cyclohexenylcarbonyl CoA, (**9**) (which then undergoes further transformation as described above) (Fig. 5). The presence of small quantities of **9** at time points during the incubation would appear to support the latter pathway. Furthermore, **13** was the most active isomer of cyclohexadienylcarbonyl CoA with a cell-free extract of *S. collinus*, and it was the only one to afford cyclohexylcarbonyl CoA (**8**) cleanly. These results indicate that 1(6),2-cyclohexadienylcarbonyl CoA is the most likely precursor of **9**, and an intermediate on the pathway to cyclohexanecarboxylic acid.

It would appear from these studies that the last three steps of the pathway from shikimic acid to cyclohexanecarboxylic acid are a reduction of the α,β-double bond of 1(6),2-cyclohexadienylcarbonyl CoA, an isomerization of the double bond of the resulting 2-cyclohexenylcarbonyl CoA to afford 1-cyclohexenylcarbonyl CoA and a final reduction step (Fig. 5). Both of the reduction steps apparently require NADPH as a cofactor and occur with the carboxylic acids activated as their coenzyme A thioesters. It is worthy of note that this pathway would account for the double bond "migration" observed in the whole cell incorporation studies with isotopically labeled shikimic acid.[4~6]

The previous experiments[3] in which an incorporation of radiolabeled 2,5-cyclohexadiene- and 1,4-cyclohexadienecarboxylic acids into ansatrienin was observed appear to contrast with these conclusions. However, we have since demonstrated that these compounds are incorporated into the antibiotic either directly without reduction or with only partial reduction, for instance, to 3-cyclohexenecarboxylic acid. Furthermore whole cell studies with labeled 1(6),2-cyclohexadienecarboxylic acid fully support the intermediacy of this compound in ansatrienin biosynthesis (E. KENNEDY, K. A. REYNOLDS, H. CHO, B. E. MOORE, U. MOCEK, J. M. BEALE & H. G. FLOSS: unpublished results). These results are consistent with the variance observed in the moiety that is attached to the ansatrienin side chain[16] and points to the importance of distinguishing between a direct attachment to the ansa moiety and a conversion to cyclohexanecarboxylic acid prior to attachment.

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