Comparison of Two Unusual Enoyl-CoA Reductases in Streptomyces collinus

Kevin A. Reynolds

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COMPARISON OF TWO UNUSUAL ENOYL-CoA REDUCTASES IN *STREPTOMYCES COLLINUS*[1]

KEVIN A. REYNOLDS

Department of Biomedicinal Chemistry, School of Pharmacy, The University of Maryland at Baltimore, Baltimore, Maryland 21201

ABSTRACT.—Ansatrienin [**1**], an antifungal antibiotic isolated from *Streptomyces collinus*, is unusual in that it contains a cyclohexanecarboxylic acid moiety that is derived from shikimic acid. An enoyl CoA reductase catalyzing the conversion of 1-cyclohexenylcarbonyl CoA to cyclohexylcarbonyl CoA reductase was purified to homogeneity, and it is suggested that this enzyme catalyzes the final step in the pathway from shikimic acid to cyclohexanecarboxylic acid. An enzyme catalyzing the conversion of crotonoyl CoA to butyryl CoA was also purified to homogeneity. The stereochemical course of the reactions catalyzed by these two enoyl CoA reductases, together with their physical properties, is discussed in the light of an evolutionary relationship.

Evidence of a Δ^2,Δ^3-enoyl-CoA isomerase catalyzing the conversion of 2-cyclohexenylcarbonyl CoA to 1-cyclohexenylcarbonyl CoA has been obtained. Results of mechanistic studies with this enzyme, which is also thought to be involved in the pathway to cyclohexanecarboxylic acid, are shown to be consistent with a preference for the 1*S* isomer of the substrate and a mechanism of a 1,3-suprafacial shift of a hydrogen catalyzed by a single base at the active site.

Shikimic acid is made by plants and microorganisms and used to synthesize the aromatic amino acids phenylalanine, tyrosine, and tryptophan (1). It is also used as a precursor to *p*-aminobenzoic acid and *p*-hydroxybenzoic acid, essential aromatic compounds in the formation of folic acid; ubiquinone; and a wide variety of secondary metabolites. All of these aromatic compounds are derived at or beyond the branch point intermediate in the pathway, chorismate. There are a few diversions from the pathway which occur before this point, one of the more unusual of which is reduction of shikimic acid to cyclohexanecarboxylic acid.

This pathway is unusual because clearly shikimic acid is typically a precursor to aromatic compounds, yet here it produces a fully reduced-six membered ring. Furthermore, the occurrence of the pathway is rare; therefore this structural unit is rarely seen. Only three antibiotics produced by streptomycetes contain cyclohexanecarboxylic acid or derivatives thereof in their structures. In the ansamycin antibiotic ansatrienin [**1**] and the related trienomycins it is located as a side chain attached to the macrocycle via a D-alanine moiety (2,3). In asukamycin, cyclohexanecarboxylic acid forms the starter unit of a short polyene chain (4). The only other occurrences of this structural moiety are the ω-cyclohexyl fatty acids of the thermophilic/acidophilic bacteria (5,6).

In the case of ansatrienin produced by *Streptomyces collinus* Tü 1892, it has been shown (7) using feeding studies that the moiety is derived from shikimic acid. Furthermore, the detection of an ansatrienin congener that contains a 1-cyclohexenecarboxylic acid (ansatrienin A4 [**2**]) suggests that the pathway leads through a 1-cyclohexenecarboxylic acid intermediate. Experiments (8,9) with D-(−)-[2-^{13}C]shikimic acid have shown that the conversion to this cyclohexenecarboxylic acid moiety is accompanied by an apparent migration of the original double bond in the ring, and that the final reduction of the double bond occurs with addition of hydrogen to the *si* face of C-1 (Scheme 1).

Our laboratory has been studying the late stages of this intriguing conversion of

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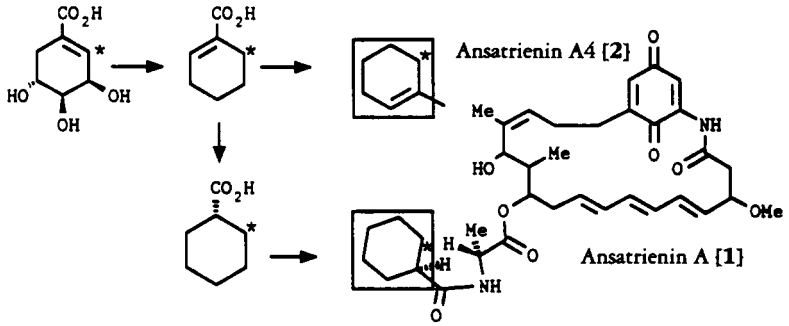
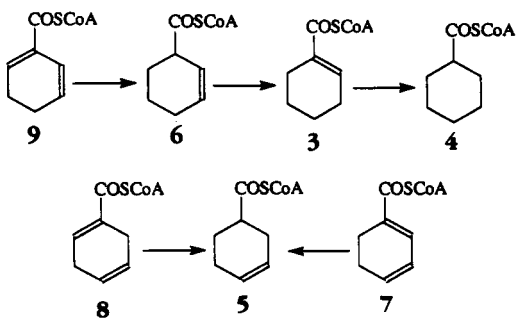


SCHEME 1. Stereochemical fate of D-(−)-[2-^{13}C]shikimic Acid in the conversion to the cyclohexylcarbonyl moiety of ansatrienin [**1**].

shikimic acid to cyclohexanecarboxylic acid at the level of purified and partially purified purified enzymes.

CELL-FREE TRANSFORMATIONS OF CYCLOHEXENE- AND CYCLOHEXADIENE-CARBOXYLIC ACIDS.—A uv spectrophotometric assay revealed that a cell-free extract of *S. collinus*, taken after 24 h in a fermentation medium, was able to convert the coenzyme-A-activated thioester **3** of 1-cyclohexenecarboxylic acid to the corresponding thioester **4** of cyclohexanecarboxylic acid (Scheme 2) (10). This conversion required NADPH as the cofactor. In order to substantiate the results of the spectrophotometric assay, the products of the cell-free incubation were hydrolyzed and analyzed as their methyl ester by gc-ms, which revealed clearly that the α,β double bond was clearly being reduced. No such activity could be observed with 3-cyclohexenylcarbonyl CoA [**5**]. However, a spectrophotometric assay of a cell-free extract of *S. collinus* with 2-cyclohexenylcarbonyl CoA [**6**] and NADPH revealed activity at a similar level to that observed with 1-cyclohexenylcarbonyl CoA. Analysis by gc-ms of this incubation revealed that not only was the majority of the product being converted to **4** but that small quantities of **3** were also present throughout the incubation (Figure 1). This could be attributed to a low level of isomerization of **6** while the majority is reduced directly to **4**, or it could be interpreted as indicative of a two-step process where **6** is converted first to **3** and then to **4**, and in which a steady state concentration of intermediate **3** is quickly attained (Scheme 2). In an attempt to distinguish between these possibilities, a cell-free extract of *S. collinus* was incubated with equimolar quantities of unlabeled 2-cyclohexenylcarbonyl CoA [**6**] and 1-[7-^{13}C]cyclohexenylcarbonyl CoA [**3**]. Once again



SCHEME 2. Cell-free transformations of cyclohexene- and cyclohexadienecarboxylic acids by *Streptomyces collinus*.

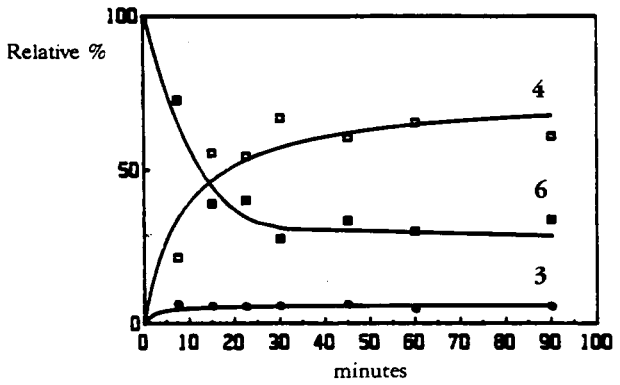


FIGURE 1. Plot of the relative percentages of cyclohexylcarbonyl CoA [**4**] and 1-cyclohexenylcarbonyl CoA [**3**], obtained from analyses of various time points of an incubation of 2-cyclohexenylcarbonyl CoA [**6**] with a *Streptomyces collinus* cell-free extract.

gc-ms analysis of the incubation showed that the relative quantity of **3** remained essentially unchanged throughout. However, when the precentage of ^{12}C at C-7 in **3** and **4** was calculated and plotted, it was observed that it increased for **3** and did so at a faster rate than for **4** (Figure 2). This result is consistent with the sequential two-step process with a steady state concentration of **3** in which the ^{13}C-labeled substrate is reduced to **4** and replaced by unlabeled substrate formed by the isomerization of **6**.

This result naturally raises the question of the immediate biosynthetic precursor to **6**. Previous in vivo experiments (7) had already indicated that cyclohexadienecarboxylic acids were possible intermediates in this pathway. Accordingly, 1,3-cyclohexadienylcarbonyl CoA [**7**], 1,4-cyclohexadienylcarbonyl CoA [**8**], and 1(6),2-cyclohexadienylcarbonyl CoA [**9**] were prepared and tested with a cell-free extract of *S. collinus* and NADPH. Analysis by gc-ms revealed that both **7** and **8** were converted primarily to 3-cyclohexenylcarbonyl CoA [**5**] which did not undergo further transformation, while **9** was converted cleanly through **6** and **3** to give **4** (Scheme 2). It is reasonable to suggest

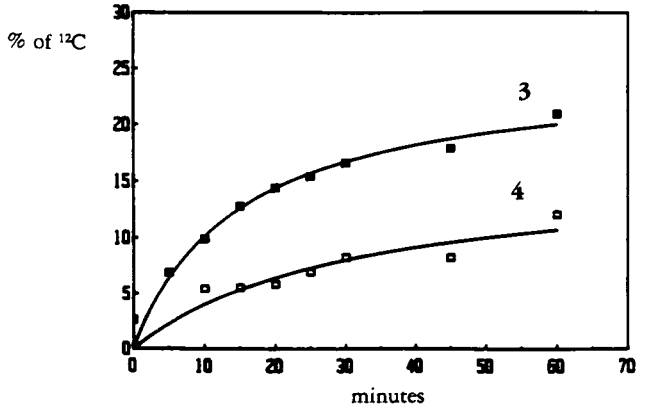
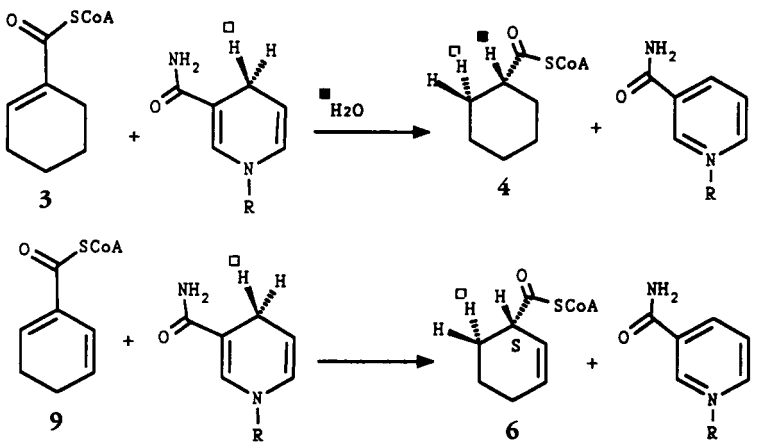


FIGURE 2. Plot of the percentage of ^{12}C for cyclohexylcarbonyl CoA [**4**] and 1-cyclohexenylcarbonyl CoA [**3**] against time, obtained from analyses of an incubation of 2-cyclohexenylcarbonyl CoA and 1-[7-^{13}C]cyclohexenylcarbonyl CoA in a 1:1 ratio with a *Streptomyces collinus* cell-free extract.

that the last steps of the pathway from shikimic acid to cyclohexanecarboxylic acid follow this sequence of events.

STEREOCHEMICAL COURSE OF THE REACTION CATALYZED BY 1-CYCLOHEXENYLCARBONYL CoA REDUCTASE.—Elucidation of the stereochemical course of the reduction of the α,β double bond during the conversion of **3** to **4** was conducted in the following manner (11). The enzyme catalyzing this reduction was partially purified from a cell-free extract of *S. collinus* using DEAE-cellulose and phenyl-Sepharose. The enzymatic products of an incubation of **3**, NADPH, and this partially purified enzyme in ^2H_2O, were hydrolyzed, extracted, and examined by gc-ms as their methyl esters. The methyl cyclohexylcarboxylate observed was clearly labeled 77% at the α-carbon with deuterium, based on examination of the fragmentation pattern (11). This indicated that it is H_2O that provides the hydrogen that the in vivo experiments (7–9) demonstrated was added to the *si* face of the α carbon of **3**. The caveat to this conclusion is that the reaction studied in the in vivo and in vitro experiments is one and the same.

In order to determine the stereochemical course of the hydrogen incorporation at the β carbon, [4(*R*)-^{2}H]NADPH and [4(*S*)-^{2}H]NADPH were synthesized (12). Reduction of 1-cyclohexenylcarbonyl CoA by partially purified enzyme in the presence of [4(*R*)-^{2}H]NADPH afforded nonlabeled product whereas [4(*S*)-^{2}H]NADPH led, under optimal conditions, to 88% monodeuterated cyclohexylcarbonyl CoA. Clearly it is the hydrogen from the *pro*-4*S* position of NADPH that is transferred in the reduction. If the addition of solvent hydrogen at the α carbon is anti then it would be predicted that the NADPH derived hydrogen would be located in a cis-1,2 relationship to the carbonyl CoA functionality. Conversely this relationship would be trans for a syn addition. In order to differentiate between these, the deuterated **4** obtained from the incubation of **3** with [4*S*-^{2}H]NADPH and the partially purified enzyme was isolated and converted to its *p*-phenylphenacyl derivative and analyzed by ^{2}H nmr. A single resonance was observed at 1.56 ppm, which is attributed to deuterium located in a cis-1,2 relationship to the esterified carboxylate (11). Therefore the addition of solvent hydrogen to the *si* face of the α carbon of **3** occurs in an anti fashion, indicating that the transfer of a hydrogen from the *pro*-4*S* position of NADPH is to the *si* face of the β carbon (Scheme 3).



SCHEME 3. Stereochemical course of conversion of 1-cyclohexenylcarbonyl CoA [**3**] to cyclohexylcarbonyl CoA [**4**] and predicted stereochemical course of conversion of 1(6),2-cyclohexadienylcarbonyl CoA [**9**] to 2-cyclohexenylcarbonyl CoA [**6**] by a 1-cyclohexenylcarbonyl CoA reductase from *Streptomyces collinus*.

These results can be compared with the stereochemical course of reduction that has been elucidated for a variety of enoyl thioester reductases (13–18) (Table 1). In doing so it is noticeable that they conform to a stereochemical pattern that has emerged for enoyl thioester reductases; the nucleotide specificity, either *pro*-4*R* or *pro*-4*S*, determines the stereospecificity of hydrogen incorporation at the β carbon of the fatty acid, *pro*-3*R* or *pro*-3*S*, respectively. No discernible pattern has been observed for the course of solvent addition at the hydrogen (i.e., either syn or anti addition).

TABLE 1. Stereochemistry of the Reaction Catalyzed by Various Enoyl Reductases.[a]

Enzyme source	Stereopecificity of NAD(P)H	Attack of hydrogen		Type of addition
		C-3	C-2	
Brevibacterium ammoniagenes	*pro*-4*S*	*si*	*re*	syn
Yeast	*pro*-4*S*	*si*	*si*	anti
Rat Liver	*pro*-4*R*	*re*	*si*	syn
Escherichia coli (chain elongation)	*pro*-4*R*	*re*	*re*	anti
E. coli (de novo synthesis)	*pro*-4*S*	*si*	*re*	syn
Streptomyces collinus (1-cyclohexenylcarbonyl CoA)	*pro*-4*S*	*si*	*si*	anti
S. collinus (crotonoyl CoA)	*pro*-4*S*	*re*	?	?

[a]The hydride of the reduced pyridine nucleotide attacks the C-3 position and the proton of the medium attacks at the C-2 position.

PURIFICATION AND CHARACTERIZATION OF 1-CYCLOHEXENYLCARBONYL CoA REDUCTASE.—The enzyme catalyzing the conversion of **3** to **4** was purified 6200-fold by six chromatographic steps to homogeneity (Table 2) (19). It was demonstrated that NADPH is the sole electron donor for this process (no measurable activity was observed with NADH, and neither FAD or FMN appeared to have an effect when added to an enzyme assay). The Km values for NADPH and 1-cyclohexenylcarbonyl CoA were determined to be 1.5 and 25 μM, respectively. This enzyme is a dimer with subunits of identical M_r (36,000), is most active at 30°, and has a pH optimum of 7.5. The enzyme is inhibited at high concentrations of NADPH and by both divalent cations ($MgCl_2$, $MnCl_2$, $ZnCl_2$, and $CaCl_2$) and thiol group inhibitors (*N*-ethylmaleamide and *p*-chloromercuribenzoate) in the absence of either NADPH or 1-cyclohexenylcarbonyl CoA.

TABLE 2. Purification of 1-Cyclohexenylcarbonyl CoA Reductase from *Streptomyces collinus*.[a]

Step	Protein (mg)	Total activity (mU)[b]	Specific activity (mU/mg)	Purification factor	Activity recovery
Cell-free	8232	4540	0.55	—	—
DEAE cellulose	3060	5630	1.8	3.3	100%
Phenyl-sepharose	702	4400	6.2	11	97%
Sephadex G-100	346	4800	14	25	100%
Green agarose	27	2200	83	150	48%
Mono-Q	1.2	970	808	1470	21%
Phenyl-superose	0.37	1200	3400	6200	26%

[a]Weight of wet *S. collinus* cells = 1280 g.
[b]1 Unit = Oxidation of 1 μmol of NADPH in 1 min with 1-cyclohexenylcarbonyl CoA.

The substrate specificity of this enzyme proved to be interesting. It was shown that not only is the enzyme able to reduce the α,β double bond of 1-cyclohexenylcarbonyl CoA but also the α,β double bonds of a variety of cyclohexadienylcarbonyl CoA compounds (Table 3). It is interesting to note that of these, 1(6),2-cyclohexadienylcarbonyl CoA [**9**] is reduced most readily and affords **6** as a product (Scheme 2). This suggests that the last two reductions in this pathway may be catalyzed by the same enzyme. The following two high confidence internal amino acid sequences were generated for this enzyme: Asn-Ile-Val-Asp-Leu-Gly-Pro-His-His-Leu-Asp-Arg and Ile-Val-Ala-Leu-Ser-Ser-Tyr-Gly-Ser-Val-Arg. No significant homology (20) of these peptides with proteins in the SwissProt data base was observed using FastA (21). However, it was noticed that the first peptide contained the sequence Val-Asp-Leu, which has been observed to be a sequence motif strongly conserved among enzymes of the shikimate pathway (dehydroquinase, shikimate synthase, dehydroquinate synthase, anthranilate synthase) (22). It has been suggested that this motif may play a role in the binding of one of the hydroxyl groups (23). While clearly little significance can be placed on the observation of this motif it does raise the question of whether 1-cyclohexenylcarbonyl CoA reductase can bind a compound such as 5-hydroxy-1-cyclohexenylcarbonyl CoA and reduce the double bond. Accordingly, this compound was obtained in racemic form as the free acid from Dr. H.G. Floss (University of Washington), converted to its corresponding coenzyme A thioester, and observed to react at approximately 50% of the rate of 1-cyclohexenylcarbonyl CoA with the enzyme (Table 3). This preliminary result raises interesting questions with regard to the evolutionary origin of this enzyme and to the number of reductive steps in the formation of the cyclohexanecarboxylic acid that can be catalyzed by this enzyme.

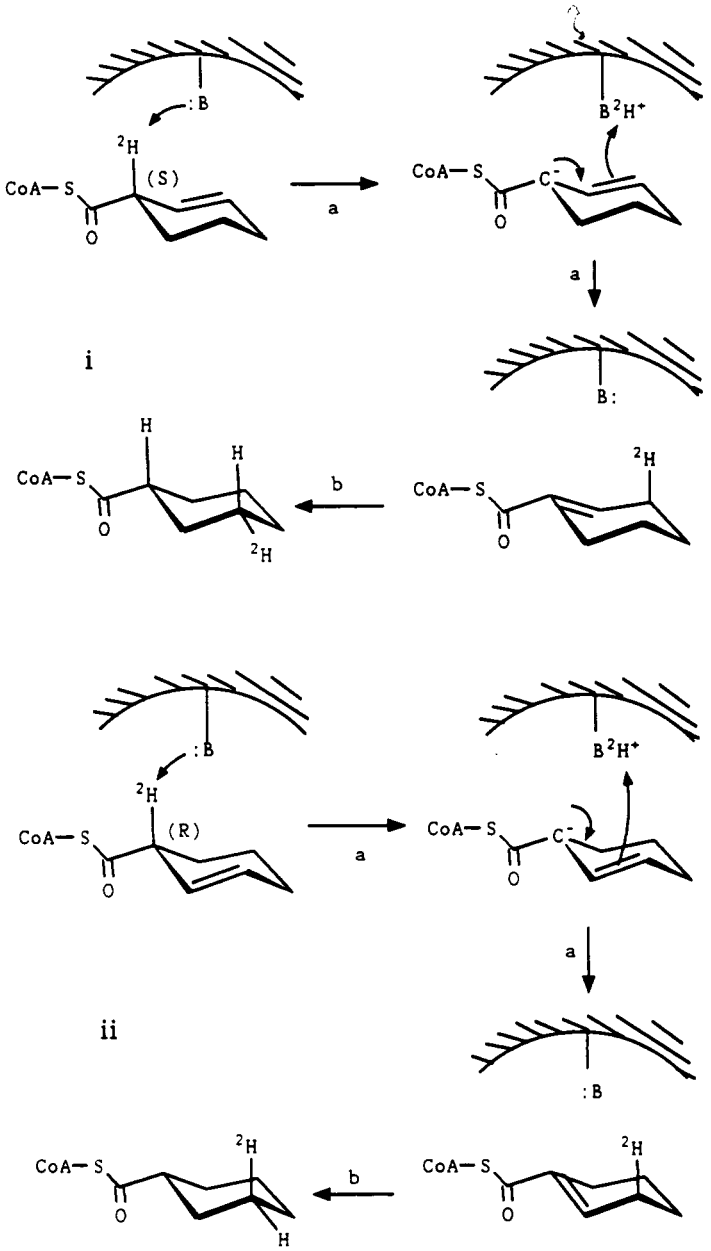
TABLE 3. Substrate Specificity of 1-Cyclohexenylcarbonyl CoA Reductase.

Substrate (100 μM)	Relative Activity
1-Cyclohexenylcarbonyl CoA [**3**]	100
2-Cyclohexenylcarbonyl CoA [**6**]	0
3-Cyclohexenylcarbonyl CoA	0
1(6),2-Cyclohexadienylcarbonyl CoA [**9**]	100
1,3-Cyclohexadienylcarbonyl CoA	28
1,4-Cyclohexadienylcarbonyl CoA	13
Crotonoyl CoA	0
5-Hydroxy-1-cyclohexenylcarbonyl CoA	50
Benzoyl CoA	0

MECHANISTIC STUDIES OF THE Δ^1,Δ^2-CYCLOHEXENYLCARBONYL COA REDUCTASE CATALYZING THE CONVERSION OF **6** TO **3**.—It is reasonable to assume that if 1-cyclohexenylcarbonyl CoA reductase reduces the α,β double bond of both **9** and **3** that it would do so in the same stereochemical fashion (that is, the enzyme would bind the both substrates in the active site in the same orientation). If this were the case, the stereochemical center at the α carbon of the **6** that is generated from **9** should be *S* (Scheme 3). Furthermore, in accord with 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 (24–27). A set of experiments were conducted which provided results consistent with these predictions (28).

It was demonstrated that a cell-free extract of *S. collinus* is able to convert 2-[1-$^2H_1$]

cyclohexenylcarbonyl CoA to cyclohexylcarbonyl CoA, via a double bond isomerization and reduction, with 39% retention of deuterium label. This is consistent with a single base catalyzing the 1,3-allylic rearrangement (Scheme 4). In order to confirm that this deuterium was located at C-3 of the cyclohexane ring, the deuterated cyclohexylcarbonyl CoA generated from the 2-[1-$^2H_1$]cyclohexenylcarbonyl CoA was isolated and analyzed as its *p*-phenylphenacyl derivatives by ^2H nmr. Two signals associated with



SCHEME 4. 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.

the derivatized cyclohexanecarboxylic acid were observed (1.81 ppm and 1.33 ppm in the ratio 4:1). The major 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 (24). This result is consistent with a base-catalyzed 1,3-allylic suprafacial shift of the deuterium from (1*S*)-2-[2-$^2H_1$]cyclohexenylcarbonyl CoA (Scheme 4i). The minor 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 (24), consistent with the reaction of (1*R*)-[2-$^2H_1$]-2-cyclohexenylcarbonyl CoA with the Δ^1,Δ^2-isomerase in the manner shown in Scheme 4ii. Therefore, while the enzyme has a clear preference for the 1*S* isomer, it apparently can react at a slower rate with the 1*R* isomer. Further support for this came from 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 **6** reacted. This result is similar to the partial regiospecific promiscuity that has been observed (29,30) 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 rotating carboxylate of an aspartate residue).

These results are clearly consistent with the presence of a Δ^1,Δ^2-cyclohexenylcarbonyl CoA isomerase that has a stereochemical preference for (1*S*)-2-cyclohexenylcarbonyl CoA and that catalyzes, using a single base, a 1,3-allylic suprafacial shift of a hydrogen. It is interesting to observe an enoyl CoA isomerase implicated in the biosynthesis of the cyclohexanecarboxylic acid moiety of an antibiotic. The only previously studied enoyl CoA isomerases are those that play a role in the primary metabolic processes of biosynthesis and β-oxidation of fatty acids (25, 26, 28, 31–35).

PURIFICATION AND CHARACTERIZATION OF A SHORT CARBON CHAIN LENGTH SPECIFIC TRANS-2-ENOYL-CoA REDUCTASE.—Enoyl thioester reductases which have been observed in both eukaryotes and prokaryotes can be divided into two classes: Enoyl ACP (acyl carrier proteins) reductases that are involved in fatty acid biosynthesis (14,15) and *trans*-2-enoyl CoA (coenzyme A) reductases that are typically involved in fatty acid elongation (36–41). 1-Cyclohexenylcarbonyl CoA reductase bears many resemblances to the latter of these two classes (19). There have been no reports of the isolation of a *trans*-2-enoyl CoA reductase from a streptomycete, and it is clear that it would be of interest to isolate one such enzyme from the same source as the 1-cyclohexenylcarbonyl CoA reductase. A comparison of the two enzymes might then reveal a relationship between enzymes involved in ansatrienin antibiotic biosynthesis (a secondary metabolic process) and fatty acid metabolism (a primary metabolic process). Such comparisons have attracted considerable interest over recent years (42–44).

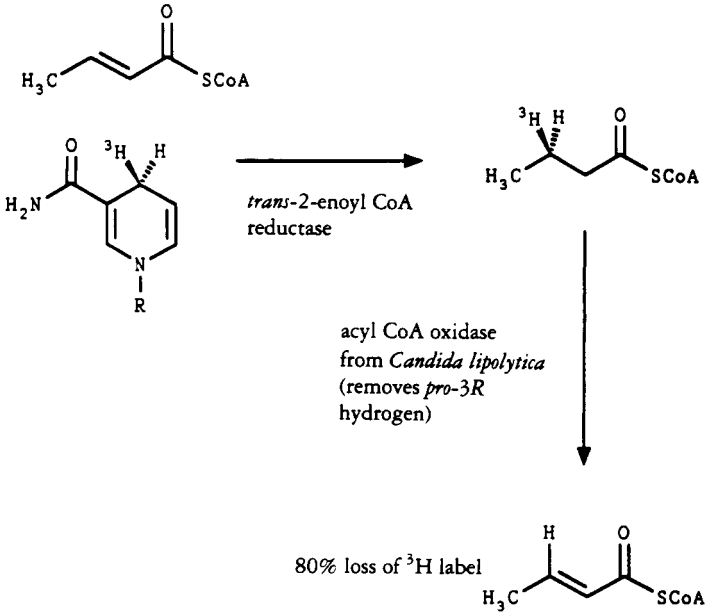
Accordingly, a *trans*-2-enoyl CoA reductase catalyzing the conversion of crotonoyl CoA to butyryl CoA was purified to homogeneity from *S. collinus* (Table 4). This enzyme, like 1-cyclohexenylcarbonyl CoA reductase, specifically requires NADPH as the sole electron donor (no measurable activity was observed with NADH, and neither FAD or FMN appeared to have an effect when added to the enzyme assay). The K_m values for NADPH and crotonoyl CoA were determined to be 15 μM and 18 μM, respectively. This enzyme is also inhibited similarly at high concentrations of NADPH and by both divalent cations ($MgCl_2$, $MnCl_2$, $ZnCl_2$, and $CaCl_2$) and thiol group inhibitors (*N*-ethylmaleamide and *p*-chloromercuribenzoate) in the absence of either NADPH or 1-cyclohexenylcarbonyl CoA reductase. This enzyme is also a homodimer, although the subunits have a larger M_r (46,000). Neither enzyme is able to convert the substrate when it is activated as its *N*-acetylcysteamine thioester, although this has been observed in the case of enoyl ACP reductases (15). A pH optimum of 6.5 was observed in contrast to the 7.5 seen for 1-cyclohexenylcarbonyl CoA. The following high confidence *N*-terminal sequence was obtained: Thr-Val-Lys-Asp-Ile-Leu-Asp-Ala-Ile-Gln-Ser-Lys-Asp-Ala-Thr-Ser-Ala-Asp-Phe-Ala.

TABLE 4. Purification of Crotonoyl-CoA Reductase from *Streptomyces collinus*.

Chromatographic Step	Volume (ml)	Protein (mg)	Total activity (mU)[a]	Specific activity (mU/mg)	Purification (fold)	Activity Recovery (%)
Cell-free extract	550					
DEAE cellulose	250	1415	1131	0.80		
Phenyl-sepharose	400	772	755	0.98	1.3	67
Sephadex G100	66	137	1123	8.20	8.4	99
DEAE-cellulose	19.5	54	819	15.2	19	72
Mono-Q	11.5	2.7	783	290	362	69
Phenyl-superose	6.5	0.41	552	1346	1683	49
Hydroxyl apatite	22	0.17	346	2036	2545	31

[a]1 U = oxidation of 1 μmol of NADPH in 1 min with crotonoyl-CoA.

In order to determine the stereochemical course of reduction of crotonoyl CoA by this enzyme, [4(*R*)-^{3}H]NADPH and [4(*S*)-^{3}H]NADPH were synthesized (12). The butyryl CoA obtained from an incubation of crotonoyl CoA, [4(*S*)-^{3}H]NADPH, and this *trans*-2-enoyl CoA reductase was purified by hplc with a reversed-phase C_{18} column and was found to contain a ^{3}H label (Scheme 5). Conversely, when the experiment was repeated under the same conditions with [4*R*-^{3}H]NADPH, the butyryl CoA obtained did not contain a label, thereby demonstrating that the reduction proceeds with transfer of hydrogen from the *pro*-4*S* position of NADPH. In order to determine on which face of the crotonoyl CoA this addition takes place, use was made of a commercially available acyl CoA dehydrogenase from *Candida lypolitica*. This enzyme converts acyl CoA compounds to their corresponding enoyl CoA compounds with abstraction of the *pro*-3*R* and *pro*-2*R* hydrogens (45). When the tritiated butyryl CoA generated from the experiment with [4(*S*)-^{3}H]NADPH was combined with [1-^{14}C]butyryl CoA and this acyl CoA dehydrogenase, it was observed that the conversion to crotonoyl CoA took place with 80% loss of label (Scheme 5). This implies that the label was located in the



SCHEME 5. Stereochemical course of the reduction of crotonoyl CoA by a *trans*-2-enoyl CoA reductase from *Streptomyces collinus*.

pro-3*R* position of butyryl CoA, and that therefore the addition of the hydrogen from the *pro*-4*S* position of NADPH is to the *re* face of crotonoyl CoA. This represents the first exception to the general pattern for enoyl thioester reductases where transfer is either from the *pro*-4*R* position of NADPH to the *re* face or from the *pro*-4*S* face to the *si* face of the β carbon of the fatty acid (Table 1).

The substrate specificity of this *trans*-2-enoyl CoA reductase raises some very interesting questions concerning its possible physiological role. Previously studied *trans*-2-enoyl-CoA reductases (36–41) are typically thought to play a role in a fatty acid elongation system. In accord with such a role they typically exhibit either a substrate preference for longer carbon chain lengths, or an ability to react with substrates of broad ranging carbon chain lengths. This trans-2-enoyl-CoA from *S. collinus* was shown to react with crotonoyl CoA (carbon chain length of four), and it seems unlikely therefore that its role is that of fatty acid elongation. There have been, to the best of our knowledge, two previous examples of short-carbon-chain-length-specific *trans*-2-enoyl-CoA reductases that have been isolated and characterized. The first example is a crotonoyl-CoA reductase activity associated with bovine mammary fatty acid synthetase of mol wt 530,000 (46). This activity provides butyryl-CoA, rather than acetyl-CoA, as a decidedly better primer unit for the fatty acid synthetase. The second example is a short-carbon-chain-length-specific *trans*-2-enoyl-CoA reductase from mitochondria of *Euglena gracilis* reacting preferentially with crotonoyl-CoA (38). This enzyme has been implicated in playing a role in a malonyl-CoA-independent fatty acid synthesis from acetyl CoA. In this system fatty acids are synthesized by the reverse of β-oxidation, except that the reduction of *trans*-2-enoyl-CoA to acyl-CoA is catalyzed by a *trans*-2-enoyl-CoA reductase instead of an acyl-CoA dehydrogenase. In order for long carbon chain fatty acid biosynthesis to occur in this manner by *E. gracilis*, *trans*-2-enoyl-CoA reductases operating on larger carbon chain-length substrates would be required, and these have been observed (47). Preliminary results have revealed a second NADPH-dependent *trans*-2-enoyl CoA reductase, specific for hexenoyl-CoA, in *S. collinus*. This enzyme is separable from both the crotonoyl-CoA specific *trans*-2-enoyl CoA reductase and 1-cyclohexenylcarbonyl-CoA reductase activities. This would suggest that hexanoyl CoA could be formed via a malonyl-CoA-independent fatty acid synthetic system in *S. collinus*. To date there has been no report of such a fatty acid synthetic system within streptomycetes. If hexanoyl CoA is formed in this manner in *S. collinus* it may be converted by a similar process to longer carbon chain fatty acids, used as a primer unit for the more commonly observed malonyl-CoA-dependent fatty acid synthetic process, or serve in a hitherto unknown function.

CONCLUSION.—Two unusual enoyl CoA reductases have been isolated from *S. collinus*, one likely involved in the biosynthesis of the cyclohexanecarboxylic acid moiety of the antibiotic ansatrienin [**1**] and the other thought to be involved in a novel fatty acid metabolism. Some of the characteristics of these enzymes are the same, yet some clear differences also exist, most noticeably the stereochemical course of addition of hydrogen at the β carbon. Work is underway to clone and sequence these enzymes to determine what, if any evolutionary relationships exist between them.

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