Biosynthesis of Ansatrienin: Stereochemical Course of the Final Reduction Step Leading to the Cyclohexanecarboxylic Acid Moiety

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Biosynthetic studies of the cyclohexanecarboxylic acid moiety of ansatrienin A (mycotrienin I) (**1**) have revealed that it is derived from shikimic acid via 1-cyclohexenecarboxylic acid.[1] The cyclohexanecarboxylic acid moiety of **1** was found to have exclusively *S* configuration at the C-1 of the cyclohexane ring upon incorporation of [2-^{13}C]shikimic acid (this carbon, normally possessing a plane of symmetry, becomes chiral by virtue of the ^{13}C label). Furthermore, it was observed that the 1-cyclohexenecarboxylic acid analogue (ansatrienin A4) accompanying **1** from this experiment carried at ^{13}C label at C-6 rather than C-2, thereby indicating that the double bond migrates in the ring (Figure 1).[2] The final step in this pathway is apparently a reduction involving addition of hydrogen at the *re* face of C-1 of cyclohexenecarboxylic acid. We report here a detailed stereochemical analysis of this reduction.

An NADPH-specific enoyl-CoA reductase which converted 1-cyclohexenylcarbonyl-CoA (**2**) to cyclohexylcarbonyl-CoA (**3**) was partially purified from *Streptomyces collinus*. The products of an incubation of **2** with this enzyme in ^2H_2O were hydrolyzed, extracted at acidic pH into ether, treated with diazomethane, and examined by GC-MS. The methyl cyclohexanecarboxylate obtained was shown to be 77% monodeuterated at the α-carbon (C-1).[3] Analogous results have been obtained for enoyl-CoA reductases involved in fatty acid synthesis.[4]

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.[5] Reduction of **2** by the 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 product. If the reduction of **2** is anti, this deuterium will be located at C-2 of **3** in a cis-1,2 relationship to the carbonyl-CoA functionality (this relationship will be trans for a syn addition). In order to differentiate between these by deuterium NMR, the resonances needed to be assigned. Accordingly, treatment of cyclohexanecarboxaldehyde with D_2O under basic conditions followed by oxidation afforded [1-^{2}H]-cyclohexanecarboxylic acid (**4**). Analysis of the ^{2}H NMR spectrum of the *p*-phenylphenacyl ester of **4** revealed a single signal at 2.51 ppm. (1*S*)-[2(*R*)-^{2}H]- and (1*R*)-[2(*S*)-^{2}H]cyclohexanecarboxylic acid (**5**) were prepared by catalytic hydrogenation of the Diels-Alder adduct of 1,3-butadiene and [3-^{2}H]-propiolic acid (Figure 2). Analysis of the *p*-phenylphenacyl ester of **5** by ^{2}H NMR revealed a single signal at 1.54 ppm (the catalytic nature of the hydrogenation affords two stereoisomers, both with a cis-1,2 relationship between the deuterium and the esterified carboxylate group).[3b] Catalytic hydrogenation with deuterium gas of the adduct of propiolic acid and 1,3-butadiene afforded two

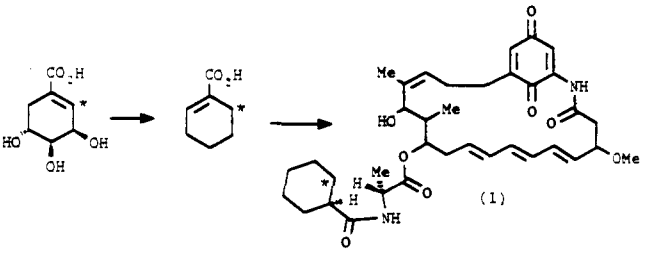

Figure 1. Stereochemical fate of D-(-)-[2-^{13}C]shikimic acid in the conversion to the cyclohexylcarbonyl moiety of ansatrienin (**1**).

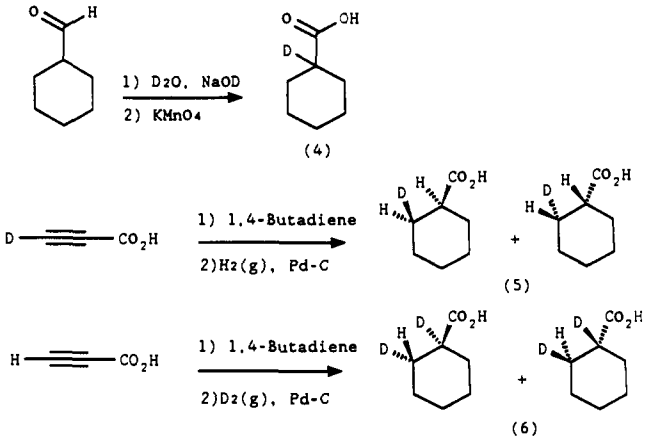

Figure 2. Synthesis of α- and β-deuterated cyclohexanecarboxylic acid.

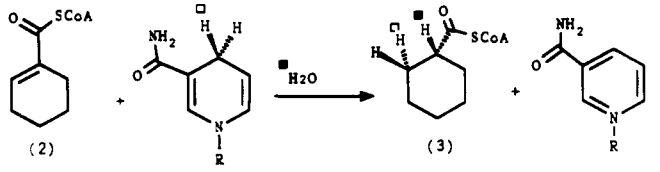

Figure 3. Stereochemical course of the conversion of 1-cyclohexenylcarbonyl-CoA (**2**) to cyclohexylcarbonyl-CoA (**3**) by an enoyl-CoA reductase from *S. collinus*.

stereoisomers, [2(*S*)-^{2}H,1(*S*)-^{2}H]- and [2(*R*)-^{2}H,1(*R*)-^{2}H]-cyclohexanecarboxylic acid (**6**). The *p*-phenylphenacyl derivative of this afforded two major signals in the ^{2}H NMR spectrum, at 2.49 ppm (deuterium at C-1) and 2.01 ppm (deuterium located at C-2 in a trans-1,2 relationship to the esterified carboxylate group). Analysis by ^{2}H NMR of the *p*-phenylphenacyl derivative of deuterated **3**, obtained from an incubation of **2** with the partially purified protein and [4(*S*)-^{2}H]NADPH, revealed a signal peak at 1.56 ppm, indicating a cis-1,2 relationship between the deuterium and the esterified carboxylate group and therefore anti addition of hydrogen at the α-carbon. This analysis does not allow direct distinction between (1*S*)-[2(*R*)-^{2}H]- or (1*R*)-[2(*S*)-^{2}H)]-cyclohexylcarbonyl-CoA. However, with the caveat that this enzyme is the same as that studied in the whole cell system,[1,2] the stereochemistry at the α-carbon of this product should be *R*, leading us to the conclusion that it is (1*R*)-[2(*S*)-^{2}H]cyclohexanecarboxylic acid.[6]

In summary, we propose that an enoyl-CoA reductase from *S. collinus* converts **2** to **3** by transfer of hydrogen from the *pro*-4*S* position of NADPH to the *si* face of the β-carbon of **2** and anti incorporation of hydrogen from solvent at the α-carbon (Figure 3). It is interesting to note that this follows a stereochemical pattern that has emerged for enoyl-CoA reductases involved in fatty acid synthesis; 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.[4,7]

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(2) Floss, H. G.; Casati, R.; Cho, H.; Beale, J. M. *Pure Appl. Chem.* **1989**, *61*, 485-488.
(3) (a) A McLafferty rearrangement of methyl cyclohexanecarboxylate with a subsequent cleavage of the α,β-carbon bond yields protonated methyl acrylate (m/z 77) which contains only one of the β-carbons. This fragment will contain the same level of deuterium content as the molecular ion in the case of α-deuteration (as observed here) and half the level in the case of β-deuteration). (b) Analysis by GC-MS of synthetically prepared α- and β-deuterated methyl cyclohexanecarboxylates strengthened this interpretation.
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(6) The assignment at the α-carbon is changed to *R* from *S* due to the fact that the deuterium label in this experiment was delivered to C-2 of **2** while the ^{13}C label in the whole cell study was located at C-6.

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It is worthwhile to note that these enoyl-CoA reductases typically incorporate solvent at the α-carbon in a syn fashion,[4,7,8] with the only exceptions (i.e., anti addition) being found in yeast[9] and *Escherichia coli*.[10] The enoyl-CoA reductase which catalyzes the final step in the formation of the cyclohexanecarboxylic acid now represents a third exception although clearly it has a very different metabolic role to these enzymes.

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Chaotropic Salt Effects in a Hydrophobically Accelerated Diels-Alder Reaction

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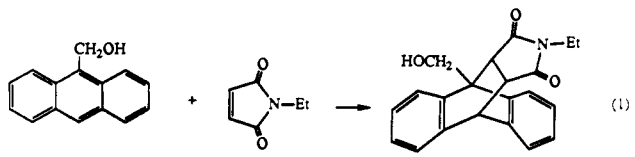
We have described the remarkable acceleration of some Diels-Alder reactions seen in water solution[1,2] and, as well, the changes in product selectivity.[2,3] Grieco has applied these water effects on selectivities to a number of cases of synthetic interest.[4] Although acids,[5] including hydrogen bonders,[6] can accelerate some Diels-Alder reactions, we offered several arguments that in our cases the hydrophobic effect is of principal importance. First of all, in reaction 1 the rate is slower in methanol than in less polar solvents, because an intracomplex hydrogen bond between the reactants is broken in the polar methanol.[1] However, despite this



(1)

the reaction is greatly accelerated in water, as the data in Table I show. Furthermore, recently Schneider has shown[7] that a Diels-Alder reaction rate as a function of solvent follows a "solvophobicity" parameter related to hydrocarbon solubility, not a polarity parameter. Hydrophobic packing of the two reactants in reaction 1, for instance, is the explanation that is consistent with both of these findings.

Some special salt effects on the reaction rates[1,2] and selectivities[2,3] of reaction 1 and other Diels-Alder reactions also argued for a hydrophobic packing effect in water solvent (similar salt effects are consistent with hydrophobic packing in the transition state for the benzoin condensation in water[8]). Reaction 1 in water is faster when LiCl is added, but slower when guanidinium chloride is added (Table I). LiCl is a "salting out" salt that increases the hydrophobic effect, by electrostriction of water that decreases the solubility of hydrocarbons and thus promotes their association.[9] Guanidinium chloride is a common denaturant of proteins and nucleic acids; such substances are sometimes called "chaotropic" agents.[9] They decrease the association of hydrocarbon residues in water and act as "salting in" materials that increase the water solubility of hydrocarbons such as butane or benzene.[9,10] Although it is usually thought that this occurs because materials like guanidinium ion or urea break up water structure, overcoming the electrostrictive effect of the chloride ion, we have recently shown that a different mechanism is responsible for the effects of such "salting in" denaturants.[11]

Table I. Second-Order Rate Constants for the Addition of *N*-Ethylmaleimide to Anthracene-9-carbinol (Reaction 1) in Various Media at 45 °C

solvent	$k_2 \times 10^3$, M^{-1} s^{-1}	k_{rel}
2,2,4-trimethylpentane[a]	8.0 ± 0.7	0.035
methanol[a]	3.4 ± 0.3	0.015
water[a]	226 ± 7	1.000
water[b]	230 ± 2	1.000
water + LiCl (4.86 M)[c]	560 ± 54	2.5
water + LiCl (4.0 M)[b]	498 ± 28	2.2
water + GnCl (4.86 M)[c]	72 ± 10	0.32
water + GnCl (2.0 M)[b]	129 ± 6	0.56
water + $LiClO_4$ (4.0 M)[b]	157 ± 3	0.68
water + $GnClO_4$ (2.0 M)[b]	86 ± 4	0.37

[a] Reference 1. [b] This work. All data are the average of at least three runs, in most cases of five runs. Reactions were carried to at least 7 half-lives. [c] Reference 2 and Ph.D. Thesis, D. Rideout, Columbia University, 1982.

The correlation between rate effects in the Diels-Alder reaction and salting out/in properties of the materials added might be a coincidence. Symons has pointed out the changes in water properties that occur when different types of ions are added.[12] In particular, he proposes that small coordinating cations bind the unshared electron pairs of water and lead to an excess of hydrogens available to hydrogen bond, while small coordinating anions bind to water protons and decrease its ability to hydrogen bond other substances. Our data on LiCl vs guanidinium chloride could be explained if Li^+ speeds reaction 1 and Cl^- slows it (but not as much) by the Symons effect and if hydrogen bonding by solvent is the principal modifier of the reaction rates.[13]

We have now tested this hypothesis. The hydrogen-bonding explanation would require that $LiClO_4$ speed the reaction even more than LiCl does; the salting-in explanation goes in the other direction,[8] contrasting the electrostrictive Cl^- with the charge-dispersed ClO_4^-. We find that $LiClO_4$ indeed slows reaction 1 (Table I), consistent only with the hydrophobic explanation. As an additional check, guanidinium perchlorate has an even larger salting-in effect than does guanidinium chloride;[14] we find that it slows reaction 1 even more (Table I). If the hydrogen-bonding explanation were operative, the order between these two salts should have been the reverse of that we observe.

These data confirm the idea that hydrophobic packing effects contribute to the Diels-Alder reaction in water, just as they do in the benzoin condensation. It seems likely that many organic

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