Biosynthetic Studies on Ansatrienin A. Formation of the Cyclohexanecarboxylic Acid Moiety

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Abstract: The formation of the cyclohexanecarboxylic acid moiety in the biosynthesis of ansatrienin (mycotrienin) has been studied. ^{13}C- and ^{2}H-labeled samples of shikimic acid were used to probe the stereochemistry of processing the cyclohexane ring of shikimic acid and to establish the fate of all the precursor hydrogens in this transformation. A sample of [2-^{13}C]shikimic acid was fed to *Streptomyces collinus* Tü 1892, and ^{13}C in the resulting ansatrienin was found to reside exclusively at C-36. The l-cyclohexenecarboxylic acid accompanying the cyclohexanecarboxylic acid in the hydrolysis of the biosynthetic sample of ansatrienin carried the ^{13}C label not at C-2 but at C-6. Samples of [2-^{2}H]-, [3-^{2}H]-, [4-^{2}H]-, [2,5-$^{2}H_2$]-, [2,3,4,5-$^{2}H_4$]-, and [6-$^{2}H_1$]shikimic acid were fed to *S. collinus*. Deuterium from C-2, C-3, C-4, and C-5 was effectively incorporated and occupied the 36*R* (axial), 35*R* (equatorial), 34*E* (equatorial), and 33*R* (axial) positions, respectively, in the resulting ansatrienin A. However, absolutely no deuterium from C-6 of shikimic acid was incorporated. Potential intermediates specifically labeled with ^{13}C and ^{2}H were used to further delineate the pathway. Combined, the results from these studies define the pathway by which shikimic acid is converted into cyclohexanecarboxylic acid. 1,4-Conjugate elimination of the hydroxy group at C-3 and a proton from C-6 of shikimic acid gives rise to a cross-conjugated dihydroxy diene, which undergoes reduction of the double bond conjugated to the carbonyl group. Another 1,4-elimination involving the C-4 hydroxy group and the proton at C-1 gives a 5-hydroxy 1,3-diene. Reduction to 5-hydroxycyclohex-1-enecarboxylic acid proceeds either directly or via the Δ^2 isomer. Another reduction gives the hydroxy acid, which undergoes dehydration involving a nonacidic proton. Isomerization of the double bond into conjugation and a final reduction completes the sequence.

The ansatrienins[1] or mycotrienins[2] represent a small group of novel ansamycin antibiotics that have been isolated from *Streptomyces collinus*[3] and *Streptomyces rishiriensis*.[4] They show pronounced activity against fungi and yeasts but little antibacterial activity. Ansatrienin (**1**) is quite unique among the ansamycin antibiotics in that it contains a cyclohexanecarboxylic acid moiety which is linked via a D-alanine residue to the macrocyclic lactam ring, a feature shared only with the related trienomycins.[5] An unsubstituted cyclohexane ring at the terminus of a carbon chain is also found in asukamycin[6] and in ω-cyclohexyl fatty acids isolated from thermophilic and related bacteria.[7-9] In both those cases, cyclohexanecarboxylic acid serves as the starter unit for the formation of a polyketide[10]/fatty acid chain.[11,12] The unusual structural feature of an unsubstituted cyclohexanecarboxylic acid moiety in ansatrienin is interesting from a biosynthetic point of view and prompted us to carry out studies on its mode of formation. In previous work,[13] we had found that shikimic acid (**2**) is a precursor of the cyclohexanecarboxylic acid moiety, providing all seven of its carbon atoms. The terminal step in the conversion of shikimic acid into cyclohexanecarboxylic acid was believed to be a reduction of 1-cyclohexenecarboxylic acid as suggested by feeding experiments and by the detection of a new ansatrienin containing a 1-cyclohexene instead of the cyclohexane moiety. It was also found that the formation of the cyclohexylcarbonyl-D-alanine residue of ansatrienin involves the stepwise attachment of first alanine and then the acid moiety to the macrocycle rather than a preassembly of both units. Origin from shikimic acid has also been demonstrated for the cyclohexane ring of ω-cyclohexylundecanoic acid[11,12] and, indirectly, for the cyclohexane ring and the attached carbon in asukamycin.[10a]

In the present paper we describe our results on the mode of transformation of shikimic acid into the cyclohexanecarboxylic acid moiety of ansatrienin A in *S. collinus*. We have found that

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shikimic acid is converted into cyclohexanecarboxylic acid through a series of dehydrations and double bond reductions. Some of the results have been communicated in preliminary form.[14]

Results

Goals and Experimental Approach. The first two goals of this project were to determine whether shikimic acid is processed stereospecifically with respect to the two sides of the ring in the conversion into cyclohexanecarboxylic acid and to trace the fate of the carbon-bound hydrogens of shikimic acid. In order to initiate these studies, we synthesized the appropriately ^{13}C- and ^{2}H-labeled shikimic acid samples. Next, potential intermediates specifically labeled with ^{13}C or ^{2}H were synthesized and used to delineate the pathway further.

The resulting ansatrienin samples were analyzed by ^{13}C or ^{2}H NMR spectroscopy and by subsequent acid hydrolysis and GC-MS analysis of the resultant cyclohexanecarboxylic acid. It became evident very quickly that both methods of analysis were necessary to obtain unambiguous results, because the enzyme attaching the cyclohexylcarbonyl moiety to the antibiotic backbone is apparently not very specific. It was therefore necessary to establish not only that the precursor was incorporated into an ansatrienin-type compound but also that it had been reduced to the level of cyclohexanecarboxylic acid.

Synthesis of Labeled Shikimates. A number of syntheses of specifically ^{2}H- or ^{13}C-labeled shikimic acids have been reported in the literature,[15-19] but not all of these are very efficient and some result in scrambling of the isotopic label. In addition, some positions of the shikimate molecule had not been labeled before. We therefore developed and describe here procedures for the synthesis of [2-^{13}C]-, [2-^{2}H]-, [4-^{2}H]-, [2,5-$^{2}H_2$]-, [6-$^{2}H_1$]-, and [2,3,4,5-$^{2}H_4$]shikimic acids. Most of them are based on modifications of known methods, but some involve procedures developed in our laboratory.

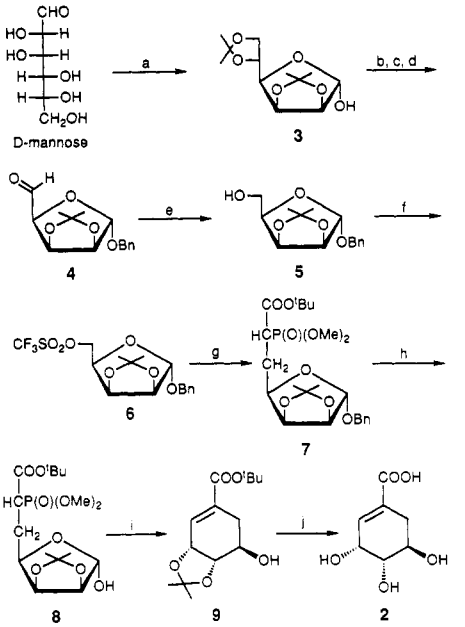
Fleet *et al.*[20] have reported a synthesis of optically pure (-)-shikimic acid (**2**), the natural enantiomer, from D-mannose in 39% yield (Scheme I). That procedure attracted our attention because it not only gave an excellent yield but also permitted easy access to isotopic labeling at various positions of shikimic acid. We adopted their procedure to synthesize several specifically labeled shikimic acids in optically pure form by replacing a starting material or a reagent by a labeled one. The only modification of the original procedure was the debenzylation step (**7** → **8**). We removed the benzyl protecting group of **7** by treatment with $HCOONH_4$ and Pd/C in 95% methanol instead of using Pd/C and hydrogen gas just for practical convenience.

By this approach, we recently synthesized [1,7-$^{13}C_2$]shikimic acid, using *tert*-butyl [1,2-$^{13}C_2$]dimethoxyphosphonoacetate prepared in good yield from [1,2-$^{13}C_2$]acetate.[21] [2-^{13}C]Shikimic acid was prepared from D-[1-^{13}C]mannose as a starting material instead of nonlabeled D-mannose (Scheme II). This labeled compound has not been reported before.

An enzymatic synthesis of [2-^{2}H]shikimic acid has been reported by Haslam and co-workers.[15] In their procedure, C-2 was the incorporation site, but the C-4 position was also enriched with deuterium to some extent. Above all, their procedure needed a long reaction time, and the preparation of enzymes was necessary for the synthesis. We prepared the same compound by oxidation of **3** followed by a $NaBD_4$ reduction to give [1-^{2}H]**3**, which then served as the precursor for the synthesis of [2-^{2}H]shikimic acid (Scheme II). Similarly, the reduction of **4** with $NaBD_4$ gave [5-^{2}H]**5**, which was further converted to [6-$^{2}H_1$]shikimic acid (Scheme II). NMR analysis of the product indicated that it was a 7:3 mixture of (6*R*)- and (6*S*)-(-)-[6-$^{2}H_1$]shikimic acid, pointing to considerable asymmetric induction in the reduction of **4**. (6*R*)-[6-$^{2}H_1$]Shikimic acid had been previously prepared by Ganem and co-workers[18] in racemic form, and Birch and co-workers[19] prepared both the 6*R* and 6*S* isomers of racemic [6-$^{2}H_1$]shikimic acid and resolved them into the pure enantiomers.

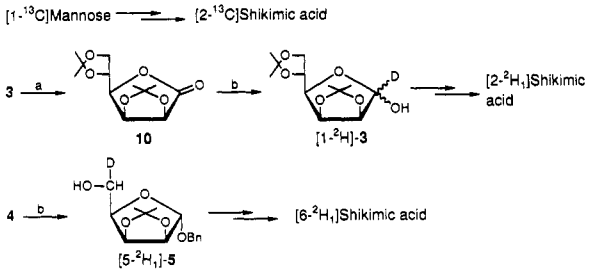
A previous approach[16,17] for the synthesis of [3-^{2}H]shikimic acid was to prepare 3-dehydroshikimic acid in a protected or unprotected form and to reduce it subsequently with sodium borodeuteride. Platinum oxide oxidation of (-)-shikimic acid followed by sodium borodeuteride reduction produced an almost equal mixture of [3-^{2}H]shikimic acid and [3-^{2}H]-3-epishikimic acid.[16] A tedious operation to separate them makes this procedure rather impractical. Zamir and Luthe[17] prepared a 3-dehydroshikimate ester with protecting groups at C-4 and C-5. Reduction of the latter with $NaBD_4$ produced the unwanted stereochemistry at C-3. Therefore it was necessary to invert the stereochemistry at C-3 before removing the protecting groups.

Scheme I. Synthesis of (-)-D-Shikimic Acid[a] by Fleet *et al.*[20]



[a] (a) Acetone, H^+. (b) BnCl, NaH. (c) HCl, aqueous MeOH. (d) $NaIO_4$. (e) $NaBH_4$. (f) $(CF_3SO_2)_2O$, pyridine. (g) *t*-BuOC(O)-$CH_2P(O)(OMe)_2$, NaH. (h) H_2, Pd/C. (i) NaH. (j) 60% aqueous CF_3COOH.

Scheme II. Synthesis of Various Labeled Shikimic Acids[a] by Modification of the Procedure of Fleet *et al.*[20]



[a] (a) DMSO, Ac_2O. (b) $NaBD_4$.

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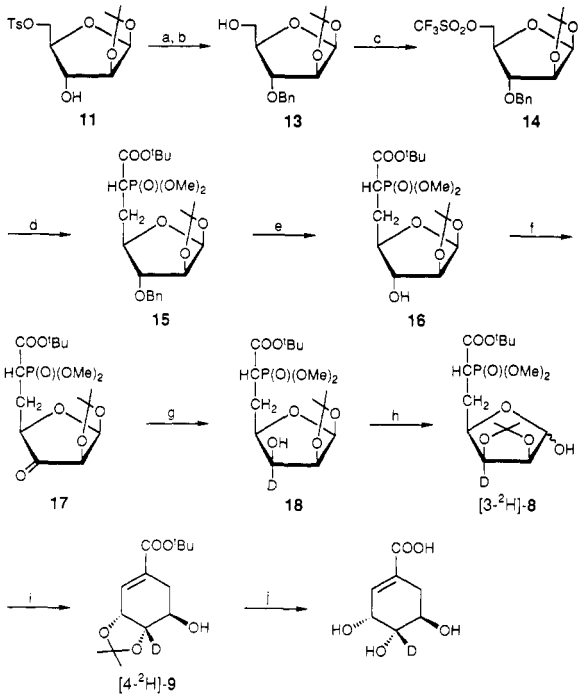
Scheme III. Synthesis of (–)-[4-^{2}H]Shikimic Acid[a]

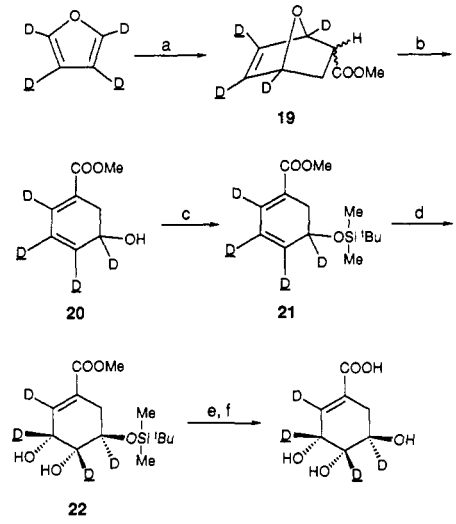


[a] (a) BnBr, NaH. (b) 4% Na(Hg). (c) $(CF_3SO_2)_2O$, pyridine. (d) *t*-BuOC(O)CH_2P(O)$(OMe)_2$, NaH. (e) $HCOONH_4$, Pd/C, MeOH. (f) P_2O_5, DMSO, DMF, 70 °C. (g) $NaBD_4$. (h) Acetone, Dowex-50W (H^+). (i) NaH. (j) 60% aqueous CF_3COOH.

This fact and the low yield are drawbacks of this procedure. We nevertheless followed their synthesis exactly to produce [3-^{2}H]-shikimic acid in about 1% yield based on the starting material, unlabeled shikimic acid. However, based on our experience with Fleet's synthesis, a far better route would be to generate D-[2-^{2}H]mannose by molybdate-catalyzed isomerization[22] of readily available D-[1-^{2}H]glucose, which can then be converted efficiently to [3-^{2}H]shikimic acid by the Fleet route.

The only previous synthesis of [4-^{2}H]shikimic acid was also reported by Zamir and Luthe.[17] Shikimate ester, with protecting groups at C-3 and C-5, was oxidized and reduced with $NaBD_4$ to introduce deuterium at the C-4 position. The hydroxyl function at C-4 was quite resistant to oxidation, and only 12% combined yield for the oxidation–reduction steps was reported. The overall yield was not specified in their report, but their procedure was reproduced in our laboratory in less than 1% yield. None of the other published syntheses of shikimic acid allowed easy introduction of deuterium at C-4. Therefore we decided to develop our own procedure to achieve that (Scheme III). Our first target was **16**, in which the hydroxyl group at C-3 has a stereochemistry opposite that of Fleet's intermediate **8**. Inversion of stereochemistry and deuterium incorporation at C-3 could be achieved by successive oxidation of **16** and reduction with $NaBD_4$. D-Arabinose was conveniently converted into its furanose form in a sequence of four reactions[23,24] to get **11**. Benzylation of **11** followed by treatment with 4% sodium amalgam afforded **13** in 90% yield. The alcohol **13** was esterified with $(CF_3SO_2)_2O$ in the presence of pyridine to give the trifluoromethanesulfonate **14** in quantitative yield. Alkylation of **14** with the sodium salt of *tert*-butyl dimethoxyphosphorylacetate in DMF in the presence of 15-crown-5 afforded a diastereomeric mixture of phosphonates (**15**) in 67% yield. The benzyl protecting group was removed by treatment of **15** with $HCOONH_4$ and Pd/C in 95% methanol in quantitative yield. The hydroxyl group of **16** was oxidized by

Scheme IV. Synthesis of (±)-[2,5-$^2H_2$]- and (±)-[2,3,4,5-$^2H_4$]Shikimic Acid[a] by Modification of the Procedure of Campbell *et al.*[25]



[a] (a) Methyl acrylate, ZnI_2. (b) $LiN(SiMe_3)_2$. (c) *tert*-Butyldimethylsilyl chloride, pyridine. (d) OsO_4, *N*-morpholine *N*-oxide. (e) *n*-Bu_4NF. (f) OH^-, H^+.

heating with P_2O_5 and DMSO in DMF at 70 °C for 1.5 h, at which time the OH stretching peak had disappeared completely and a new carbonyl peak appeared in the IR spectrum. The ketone **17** was then reduced with $NaBD_4$ to generate alcohol **18** (71% yield from **16**). The isopropylidene protecting group could be rearranged by treatment of **18** in acetone with acidic resin at room temperature for 3 h to give [3-^{2}H]**8** in 88% yield. Conversion of the latter to [4-^{2}H]shikimic acid then followed the procedure reported by Fleet *et al.*[20]

The preparation of shikimic acid specifically labeled with deuterium at C-5 has not been reported before and is difficult to achieve by known synthetic routes. As an alternative, we prepared (±)-[2,3,4,5-$^2H_4$]- and [2,5-$^2H_2$]shikimic acid by a modification of the synthesis reported by Campbell *et al.*[25] (Scheme IV). Commercial furan-d_4 and [2,5-$^2H_2$]furan, which could be readily prepared by treatment of furan with 2 equiv of butyl lithium followed by careful quenching with deuterium oxide, were employed as starting materials. Conversion of the Diels–Alder adduct of furan and methyl acrylate to dienol **20** through $LiN(SiMe_3)_2$-catalyzed ring-opening, followed by treatment with *tert*-butyldimethylsilyl chloride afforded **21**. Cyclohexadiene **21** was treated with a catalytic amount of OsO_4 and *N*-morpholine *N*-oxide in pyridine to give **22**. The synthesis of deuterated shikimic acid was then completed by removal of the protecting groups.

Stereochemistry of Shikimate Processing. In order to determine whether the asymmetry of the cyclohexane ring of shikimic acid is preserved in the product, i.e., whether shikimic acid is converted into cyclohexanecarboxylic acid stereospecifically, (–)-[2-^{13}C]shikimic acid was fed to cultures of *S. collinus*. ^{13}C NMR analysis of the resulting ansatrienin A showed a single enhanced signal indicating about 4.8% ^{13}C enrichment at either C-32, C-36, or C-17.[26] To determine if [2-^{13}C]shikimic acid labeled only C-32 or C-36 in the cyclohexanecarboxylic acid moiety, the ^{13}C-labeled ansatrienin A was hydrolyzed, and the cyclohexanecarboxylic acid was esterified and reduced to cyclohexylcarbinol. The latter was then esterified with (*S*)-(+)-mandelic acid (Scheme V).[27] The ^{13}C NMR spectrum of the corresponding unlabeled

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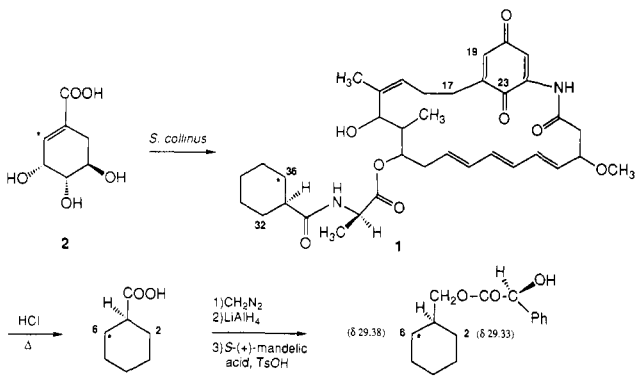
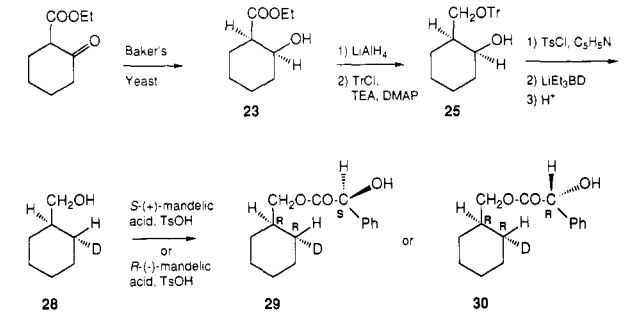
Scheme V. Stereochemical Fate of (–)-[2-^{13}C]Shikmic Acid (**2**) in the Conversion to the Cyclohexylcarbonyl Moiety of Ansatrienin A (**1**)



Scheme VI. Synthesis of (1*R*,2*R*)-[2-^{2}H]Cyclohexylcarbinol (*R*)-(–)- and (*S*)-(+)-Mandelate Esters



compound showed two signals at 29.38 and 29.33 ppm for C-2/C-6 of the cyclohexane ring; in the derivative obtained from the labeled ansatrienin A, only the signal at 29.38 ppm was enhanced. Hence, shikimic acid is indeed processed stereospecifically in the conversion to cyclohexanecarboxylic acid.

The absolute stereochemistry of this process was determined by synthesizing an authentic sample of (1*R*,2*R*)-[2-^{2}H]cyclohexylcarbinol by the route shown in Scheme VI. Fermenting baker's yeast reduction of 2-carbethoxycyclohexanone gave ethyl (1*R*,2*S*)-2-hydroxycyclohexanecarboxylate[28] (**23**), which was reduced to the diol **24** with $LiAlH_4$. Deuterium was then introduced by stereospecific displacement of the secondary alcohol function, as its tosylate, with $LiEt_3B^2H$. Aliquots of the (1*R*,2*R*)-[2-^{2}H]cyclohexylcarbinol (**28**) were esterified with (*S*)-(+)- and (*R*)-(–)-mandelic acid, and the broad-band {^{1}H,^{2}H} decoupled ^{13}C NMR spectra of the samples were recorded (Figure 1). Each spectrum showed for the C-2/C-6 pair of carbons one unshifted signal and one deuterium-shifted (0.34 ppm upfield ^{2}H isotope shift) signal. The chemical shift difference between the unshifted and the shifted signal was 0.39 ppm ($\Delta^2H + \Delta\delta_{C\text{-}2/C\text{-}6}$) in the (*S*)-(+)-mandelate derivative **29** and 0.29 ppm ($\Delta^2H - \Delta\delta_{C\text{-}2/C\text{-}6}$) in the (*R*)-(–)-mandelate ester **30**, indicating that in the (*S*)-(+)-mandelate ester the lower field (29.38 ppm) signal originates from C-6 (the pro-*S* carbon) and the higher field signal (29.33 ppm) from C-2 (pro-*R* carbon). The assignment is most easily deduced from the signal pattern of a 2:1 mixture of the (*R*)- (–) and the (*S*)-(+) esters (Figure 1). It was thus concluded that the label of ansatrienin A biosynthesized from [2-^{13}C]shikimic acid resided exclusively at C-6, i.e., the processing of [2-^{13}C]shikimic acid was stereospecific to give ansatrienin A with *S* configuration at C-1 of the cyclohexane ring.

While this finding shows that, in a formal sense, the double bond of shikimic acid is reduced on the 1*re* face, the following result reveals that this is the consequence of a more complex

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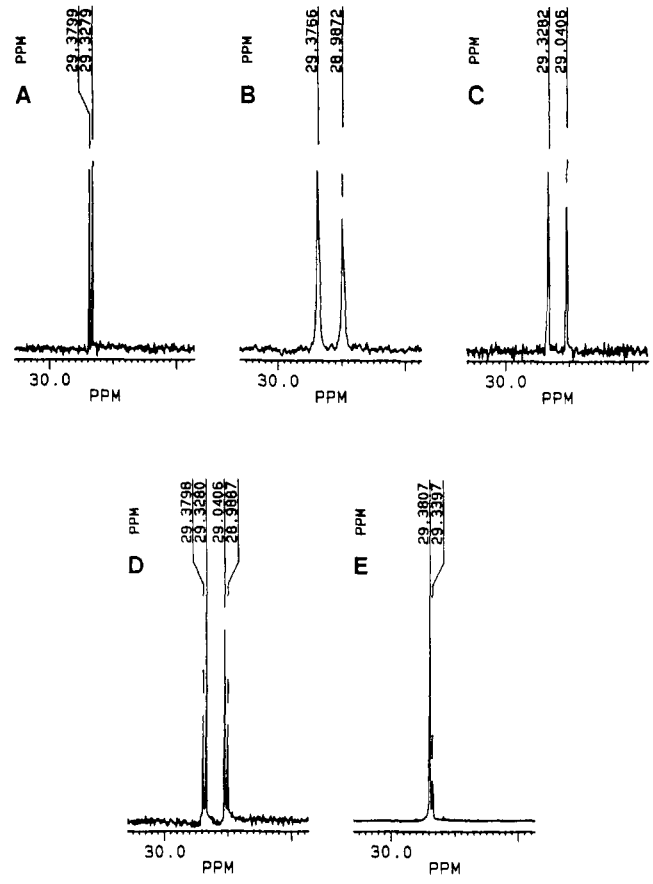


Figure 1. ^{13}C NMR spectra of cyclohexylcarbinyl mandelate, signals for C-2 and C-6 of the cyclohexane ring: (A) unlabeled cyclohexylcarbinyl (*S*)-(+)-mandelate; (B) (1*R*,2*R*)-[2-^{2}H]cyclohexylcarbinyl (*S*)-(+)-mandelate; (C) (1*R*,2*R*)-[2-^{2}H]cyclohexylcarbinyl (*R*)-(–)-mandelate; (D) 1:2 mixture of samples B + C; (E) [^{13}C]cyclohexylcarbinyl (*S*)-(+)-mandelate from **1** biosynthesized from [2-^{13}C]shikimic acid. Spectra A and E are {^{1}H} broad-band decoupled; B, C, and D are {^{1}H,^{2}H} broad-band decoupled.

series of reactions. We had observed earlier[13] that ansatrienin A is accompanied in the fermentation by a small amount of the Δ^1-cyclohexene analog (ansatrienin A_4). The ^{13}C NMR spectrum of the cyclohexanecarboxylic acid sample from the hydrolysis of ansatrienin A biosynthesized from [2-^{13}C]shikimic acid displayed the signals of a second minor component, 1-cyclohexenecarboxylic acid. By spectral subtraction, the spectrum of the minor component alone was generated (Figure 2). Comparison with the natural abundance spectrum shows unequivocally that the cyclohex-1-enecarboxylic acid carries the ^{13}C label not in the olefinic carbon, C-2, but rather in the methylene carbon, C-6. Thus, the double bond migrates in the ring during the transformation of shikimic acid into cyclohexanecarboxylic acid (Scheme VII).

Fate of Shikimate Hydrogens. The ^{2}H NMR spectrum of ansatrienin A biosynthesized from (–)-[2-^{2}H]shikimic acid showed a signal at δ 1.41. We knew from the previous experiment that C-2 of shikimic acid labels C-36 of ansatrienin A and that this carbon resonates at δ 29.38. The stereochemical assignment was made on the basis of the ^{1}H–^{13}C heteronuclear correlation (^{1}H–^{13}C COSY) spectrum of nonlabeled ansatrienin A. The ^{1}H–^{13}C COSY shows two cross peaks for C-36, indicating the correlation with the attached two hydrogens. The upfield signal (δ 1.38) corresponds to the axial (36*R*) hydrogen and the lower field signal (δ 1.80) represents the equatorial (36*S*) hydrogen. Therefore, the observation of a ^{2}H NMR signal at δ 1.41 for the ansatrienin A obtained by feeding [2-^{2}H]shikimic acid shows that the deuterium from C-2 of shikimic acid occupies the 36*R* (axial)

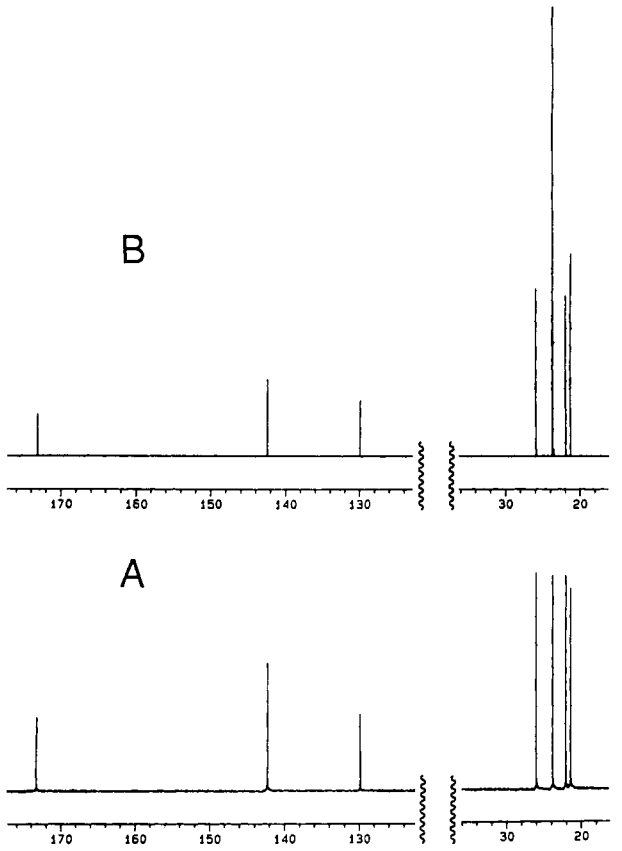
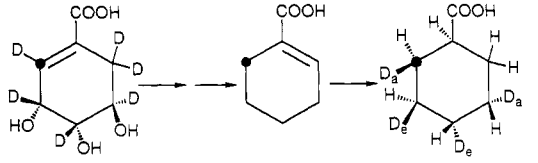


Figure 2. ^{13}C NMR spectra of natural abundance cyclohex-1-enecarboxylic acid (A) and the cyclohex-1-enecarboxylic acid obtained by hydrolysis of **1** biosynthesized from [2-^{13}C]shikimic acid (B).

Scheme VII. Summary of the Labeling Pattern of the 1-Cyclohexene- and Cyclohexanecarboxylic Acid Moieties of Ansatrienin A after Feeding Labeled Shikimic Acid Samples



position in the cyclohexanecarboxylic acid moiety of the resulting ansatrienin A (Scheme VII).

The fate of the C-6 hydrogens of shikimic acid was probed by feeding the sample of (–)-[6-$^2H_1$]shikimic acid (98% ^2H, 6*R*:6*S* = 7:3). The resulting ansatrienin contained no detectable (<0.1% enrichment) amount of deuterium. This negative result was confirmed by feeding a mixture of (–)-[2-^{13}C]shikimic acid and (–)-[6-$^2H_1$]shikimic acid to *S. collinus*. The NMR spectra of the resulting ansatrienin A showed ^{13}C enrichment at C-36 but no detectable ^2H enrichment. Hence, the two hydrogens at C-6 of shikimate are both replaced by hydrogens from other sources during the conversion into cyclohexanecarboxylic acid.

The remaining three ring hydrogens of shikimate are all retained during the incorporation into ansatrienin, as was revealed by experiments with (–)-[3-^2H]-, (–)-[4-^2H]- and (±)-[2,5-$^2H_2$]shikimic acids. The ^2H NMR spectra of ansatrienin A obtained by feeding [3-^2H]- and [4-^2H]shikimic acid showed signals at δ 1.74 and 1.66, respectively (Table I). Two atoms of deuterium from [2,5-$^2H_2$]shikimic acid were incorporated, giving ansatrienin A which displayed two ^2H NMR signals at δ 1.21 and 1.41 (Table I). The signal at δ 1.21 must originate from deuterium at C-5 of shikimic acid, because in the earlier experiment ansatrienin A from [2-^2H]shikimic acid had shown a signal at δ 1.41. Therefore, deuterium from C-3, C-4, and C-5 of shikimic acid occupied the

Table I. ^2H NMR Analysis of **1** Obtained from Feeding Experiments with ^2H-Labeled Shikimic Acids

	product (ansatrienin A)	
precursor	^2H NMR signal (ppm)	labeled hydrogen
(–)-[2-^2H]shikimic acid	1.41	pro-36*R*
(–)-[3-^2H]shikimic acid	1.74	pro-35*R*
(–)-[4-^2H]shikimic acid	1.66	34*E*
(±)-[2,5-$^2H_2$]shikimic acid	1.22, 1.42	pro-33*R*, pro-36*R*
(±)-[2,3,4,5-$^2H_4$]shikimic acid	1.21, 1.39, (1.66), 1.73	pro-33*R*, 34*E*, pro-35*R*, pro-36*R*
(–)-(6*R*,*S*)-[6-$^2H_1$]shikimic acid	no incorporation	

35*R* (equatorial), 34*E* (equatorial), and 33*R* (axial) positions, respectively, in the resulting cyclohexanecaboxylic acid moiety of ansatrienin A (Scheme VII).

Finally, (±)-[2,3,4,5-$^2H_4$]shikimate was fed to *S. collinus*. ^2H NMR analysis of the ansatrienin A from this experiment showed the labeling pattern expected on the basis of the results of the experiments with the corresponding mono- and dideuterated shikimic acids, as shown in Table I, except that the signal at 1.66 ppm was partially obscured by the signal at 1.73 ppm. To confirm the retention of all four deuterium atoms, the ansatrienin sample obtained from the feeding experiment with [2,3,4,5-$^2H_4$]shikimic acid was hydrolyzed and the acid fraction was derivatized to the methyl ester and analyzed by GC-MS (Table II). Significant enhancement of the $(M + 4)^+$ peak for both methyl cyclohexanecarboxylate and methyl 1-cyclohexenecarboxylate established that all four deuterium atoms of the [$^2H_4$]shikimic acid were indeed retained. The result of the mass spectral analysis also rules out the possibility of intermolecular transfers of any of these hydrogens during the biosynthesis.

Synthesis of Labeled Intermediates. As elaborated in the Discussion section, the results from the above deuterated shikimic acid feeding experiments considerably limit the number of reaction sequences which can be written for the transformation of shikimic acid to cyclohexanecarboxylic acid. However, they are not sufficient to define a single pathway for this transformation. In order to narrow the choices further, we synthesized and evaluated a series of potential intermediates (Figure 3). The synthetic strategies are described roughly in the order in which the compounds are presented in the next section. In most instances, known syntheses were modified to allow introduction of a stable isotope into the molecule.

Cyclohex-1- and -2-enecarboxylic acids have been synthesized by Wheeler[29] and Whitham,[30] respectively. In our syntheses, we utilized sodium [^{13}C]cyanide to introduce a ^{13}C label into the carboxyl carbon; otherwise, we followed their methods to prepare [7-^{13}C]cyclohex-1- and -2-enecarboxylic acids **31** and **32**.

Cyclohex-1-enecarboxylic acid (**31**) was additionally prepared carrying deuterium at the olefinic carbon C-2. The α protons of cyclohexanone were repeatedly exchanged with deuterium by treatment with deuterium oxide and potassium carbonate until cyclohexanone contained 91% of the calculated amount of deuterium.[31] The deuterated ketone was then converted under Shapiro conditions[32] (via the tosylhydrazone) to [2,6,6-$^2H_3$]**31** in 49% yield.

Four isomeric cyclohexadienecarboxylic acids were synthesized. Birch reduction of [7-^{13}C]benzoic acid[33] gave [7-^{13}C]cyclohexa-2,5-dienecarboxylic acid (**33**), which was then treated with KOH

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(30) Davies, S. G.; Whitham, G. H. *J. Chem. Soc., Perkin Trans. I* **1976**, 2279.
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Table II. Mass Spectral Analysis of Methyl Cyclohexanecarboxylate and Methyl 1-Cyclohexenecarboxylate Obtained by Hydrolysis of **1** from the Feeding Experiment with (±)-[2,3,4,5-^{2}H]Shikimic Acid

m/z	methyl cyclohexanecarboxylate from unlabeled ansatrienin (relative intensity)	methyl cyclohexanecarboxylate from labeled ansatrienin[a] (relative intensity)	m/z	methyl 1-cyclohexenecarboxylate from unlabeled ansatrienin (relative intensity)	methyl 1-cyclohexenecarboxylate from labeled ansatrienin[a] (relative intensity)
142	100	100	140	100	100
143	9.8	9.2	141	8.6	13
144	0.8	0.6	142	0.8	1.0
145	0	1.3	143	0	1.0
146	0	7.2	144	0	5.3

[a] Biosynthesized from (±)-[2,3,4,5-^{2}H$_4$]shikimic acid.

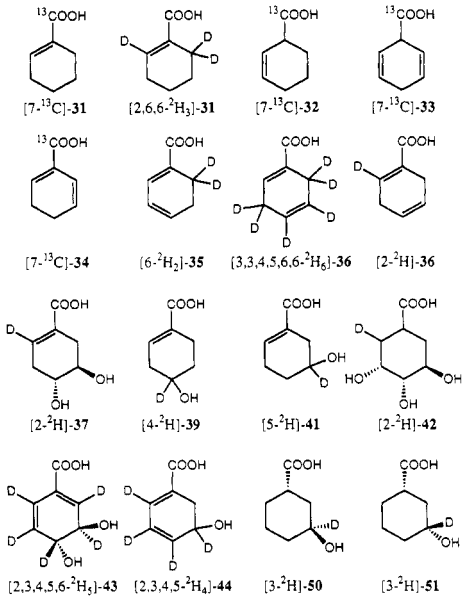

Figure 3. Structures of ^{13}C- and ^{2}H-labeled potential intermediates.

to isomerize the double bond, giving [7-^{13}C]cyclohexa-1,5-dienecarboxylic acid (**34**) in 82% yield.

Blanc has synthesized cyclohexa-1,3-dienecarboxylic acid from acrylic acid and 1-acetoxy-1,3-butadiene under Diels–Alder conditions, albeit in very low yields.[34] We followed this procedure using [2,3,3-^{2}H$_3$]acrylic acid to prepare [6,6-^{2}H$_2$]cyclohexa-1,3-dienecarboxylic acid (**35**) in 6% overall yield.

Grewe and Hinrichs have reported the synthesis of cyclohexa-1,4-dienecarboxylic acid by the Diels–Alder cycloaddition of 1,3-butadiene and propiolic acid.[35] Following their scheme, we used commercially available 1,3-butadiene-d_6 to prepare [3,3,4,5,6,6-^{2}H$_6$]cyclohexa-1,4-dienecarboxylic acid (**36**) in 88% yield. We also prepared [3-^{2}H]propiolic acid to be used in the above synthesis of [2-^{2}H]**36**. Initial attempts to exchange the acetylenic hydrogen of propiolic acid under equilibrium conditions with D_2SO_4 resulted in low yields. Similarly, treatment of propiolic acid and methyl propiolate with various bases (LDA followed by D_2O quench, NaOH/D_2O, K_2CO_3/D_2O) gave little or no product. We successfully prepared [3-^{2}H]propiolic acid from the monopotassium salt of acetylenedicarboxylic acid by modification of the Ingold synthesis.[36] Gentle heating in a large excess of D_2O gave the potassium salt of [^{2}H]propiolate. Acidification with D_2SO_4 gave [3-^{2}H]propiolic acid in 84% yield. Combination with 1,3-butadiene gave [2-^{2}H]**36**.

We continued with the Grewe synthesis[35] to prepare *trans*-4,5-dihydroxy[2-^{2}H]cyclohex-1-enecarboxylic acid (**37**) from [2-^{2}H]**36**. Antidihydroxylation of the diene methyl ester with H_2O_2 and formic acid followed by saponification gave [2-^{2}H]**37**.

Both 4- and 5-hydroxycyclohex-1-enecarboxylic acids, labeled at the secondary alcohol carbon with deuterium, could easily be made through extentions of syntheses developed by Danishefsky *et al.*[37] and Webster and Silverstein.[38] In the Danishefsky synthesis,[37] 1-methoxy-3-(trimethylsilyl)oxy-1,3-butadiene and methyl acrylate under Diels–Alder conditions gave an inseparable 1:1 mixture of methyl 4-oxocyclohex-1- and -2-enecarboxylates. Protection of the ketone under acidic conditions forced the migration of the double bond to the Δ^1 position. Deprotection of the ketal resulted in some slight migration of the double bond (9:1 mixture). Reduction of the mixed Δ^1- and Δ^2 keto esters with $NaBD_4$ resulted in the selective reduction of the saturated ketone to methyl 4-hydroxy[4-^{2}H]cyclohex-1-enecarboxylate (**38**), which could be easily isolated. Saponification with KOH gave the free acid [4-^{2}H]**39**. Webster and Silverstein[38] have made 5-oxocyclohex-1-enecarboxylic acid in a one-pot sequence. *m*-Anisic acid was reduced under Birch conditions to give 3-methoxycyclohexa-2,5-dienecarboxylic acid. Isomerization with KOH followed by acid hydrolysis of the vinylic ether gave the oxo acid in 60% overall yield. After esterification with diazomethane, treatment with $NaBD_4$ followed by saponification gave 5-hydroxy[5-^{2}H]cyclohex-1-enecarboxylic acid (**41**).

We exactly followed the Ikota and Ganem[39] synthesis of *trans*-3,4-dihydroxycyclohexa-1,5-diene (**43**) from benzoic acid except that we started with benzoic acid-d_5. The allylic bromination of [2,3,4,5,8-^{2}H$_5$]-8-bromo-7-oxo-6-oxabicyclo[3.2.1.]oct-2-ene with NBS resulted in partial loss of deuterium from C-4. Continuation of the synthesis gave [2,3,4,5,6-^{2}H$_5$]**43** which was only 76% deuterated at C-4.

Incorporation of Potential Intermediates. Based on our earlier observation of the natural occurrence of the Δ^1-cyclohexene analog ansatrienin A$_4$, we fed [7-^{13}C]cyclohex-1-enecarboxylic acid (**31**) to see if it is reduced to the level of cyclohexanecarboxylic acid. ^{13}C NMR analysis of the ansatrienin A revealed enrichment at δ 176.8 (C-30). A second enhanced resonance was observed at δ 167.6 which does not correlate to any carbons of ansatrienin A. Instead, this resonance was attributed to the presence of the small amount of the unsaturated ansatrienin A$_4$. This assignment was strengthened by ^{13}C NMR examination of cyclohex-1-enecarbonyl-D-alanine benzyl ester (**45**), synthetically prepared through the coupling of cyclohex-1-enecarboxylic acid and D-alanine benzyl ester toluenesulfonate.[40] The carbonyl carbon of the cyclohexenecarboxylate moiety in **45** resonates at δ 167.8. These conclusions were confirmed by GC-MS analysis of the acid hydrolysis products of the ansatrienin from this feeding experiment. Both cyclohexanecarboxylic acid and cyclohex-1-enecarboxylic acid were enriched at 29 and 90%, respectively. Similarly, [7-^{13}C]cyclohex-2-enecarboxylic acid (**32**) highly enriched C-30 of ansatrienin A.

The feeding of [2,6,6-^{2}H$_3$]**31** to *S. collinus* was used to probe the mechanistic outcome of the final reduction of cyclohex-1-enecarboxylic acid to cyclohexanecarboxylic acid. The deuterium resonances in the resulting ansatrienin A appeared at δ 1.42 and 1.82 in approximately a 1:2 ratio (Table III), indicating that the

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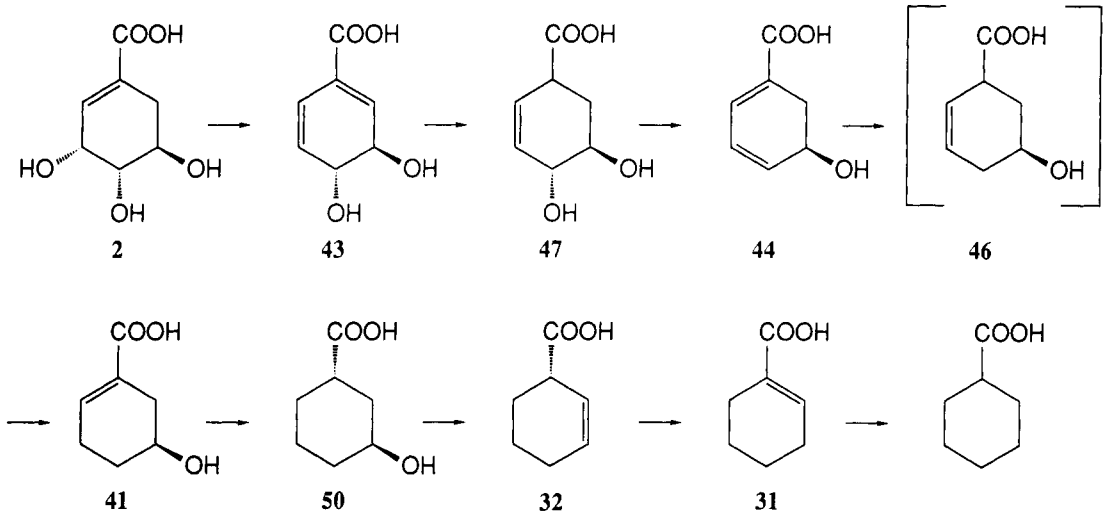
(39) Ikota, N.; Ganem, B. *J. Am. Chem. Soc.* **1978**, *100*, 351 and references therein.
(40) Shields, J. E.; McGregor, W. H.; Carpenter, F. H. *J. Org. Chem.* **1961**, *26*, 1491.

Table III. ^2H NMR Analysis of **1** Obtained from Feeding Experiments with ^2H-Labeled Potential Precursors

precursor	product (ansatrienin A)	
	^2H NMR signal (ppm)	labeled hydrogen
[2,6,6-$^2H_3$]cyclohex-1-enecarboxylic acid (**31**)	1.42, 1.82[a]	pro-36*R*, pro-36*S*, pro-32*R*
[6,6-$^2H_2$]cyclohexa-1,3-dienecarboxylic acid (**35**)	no incorporation[b]	
[3,3,4,5,6,6-$^2H_6$]cyclohexa-1,4-dienecarboxylic acid (**36**)	no incorporation[b]	
[2-^2H]cyclohexa-1,4-dienecarboxylic acid (**36**)	no incorporation[b]	
trans-4,5-dihydroxy[2-^2H]cyclohex-1-enecarboxylic acid (**37**)	no incorporation	
4-hydroxy[4-^2H]cyclohex-1-enecarboxylic acid (**39**)	1.20[c]	34*Z*
5-hydroxy[5-^2H]cyclohex-1-enecarboxylic acid (**41**)	1.19	pro-33*R*
[2-^2H]dihydroshikimic acid (**42**)	no incorporation	
trans-3,4-dihydroxy[2,3,4,5,6-$^2H_5$]cyclohexa-1,5-dienecarboxylic acid (**43**)	1.21, 1.39, (1.66), 1.73	pro-33*R*, 34*E*, pro-35*R*, pro-36*R*
5-hydroxy[2,3,4,5-$^2H_4$]cyclohexa-1,3-dienecarboxylic acid (**44**)	1.21, 1.39, (1.66), 1.73	pro-33*R*, 34*E*, pro-35*R*, pro-36*R*
trans-3-hydroxy[3-^2H]cyclohexanecarboxylic acid (**50**)	1.23	pro-33*R*
cis-3-hydroxy[3-^2H]cyclohexanecarboxylic acid (**51**)	1.23	pro-33*R*

[a] Ratio of δ 1.42:1.82 is approximately 1:2. [b] The 1,3- and 1,4-dienes were converted into cyclohex-3-enecarboxylic acid and benzoic acid which were attached to the antibiotic backbone. [c] A second deuterium signal at δ 3.95 originates from C-4 of 4-hydroxycyclohexanecarboxylic acid.

Scheme VIII. Proposed Conversion of Shikimic Acid to Cyclohexanecarboxylic Acid in *S. collinus*



C-2 deuterium of [2,6,6-$^2H_3$]**31** occupied the 32*R* (equatorial) position in the resulting cyclohexanecarboxylic acid moiety of ansatrienin A. It was shown earlier that [2-^{13}C]shikimic acid was processed stereospecifically to give ansatrienin A with *S* configuration at C-1 of the cyclohexane ring. Thus, the double bond reduction proceeds via the *anti* addition of hydrogen. Two additional deuterium resonances at δ 6.89 and 2.23 in a 1:2 ratio were attributed to the presence of the unsaturated cyclohex-1-enecarboxylic acid moiety.

Various cyclohexadienecarboxylic acids were tested as possible precursors. Of these, [7-^{13}C]cyclohexa-1,5-dienecarboxylic acid (**34**) was efficiently incorporated and fully reduced to the cyclohexanecarboxylic acid moiety. In contrast, [3,3,4,5,6,6-$^2H_6$]cyclohexa-1,4-dienecarboxylic acid (**36**) was incorporated as 3-cyclohexenecarboxylic acid, but it was not reduced to the cyclohexane oxidation level. The 2,5- and 1,3-double bond isomers (**33, 35**) suffered mixed fates. They were incorporated as such and partly reduced to the cyclohexane ring level. Additionally, these diene isomers were aromatized to benzoic acid, but neither seems to be on the biosynthetic pathway. Benzoic acid was excluded as an intermediate by a feeding experiment with [7-^{13}C]benzoic acid. Interestingly, the benzoic acid was attached intact to the backbone of the antibiotic, but it was not reduced at all.

We next tested 4-hydroxy[4-^2H]cyclohex-1-enecarboxylic acid (**39**) and 5-hydroxy[5-^2H]cyclohex-1-enecarboxylic acid (**41**). Two ^2H NMR signals at δ 1.20 and 3.95 were present in a 7:1 ratio in ansatrienin isolated from the experiment with [4-^2H]**39**. GC-MS analysis of the acid hydrolysis products showed that both cyclohexanecarboxylic acid and 4-hydroxycyclohexanecarboxylic acid were present and enriched, suggesting that the deuterium signals at δ 1.20 and 3.95 originate from C-4 of cyclohexanecarboxylic acid and 4-hydroxycyclohexanecarboxylic acid, respectively. The deuterium resonance of cyclohexanecarboxylic acid was not at the expected frequency of δ 1.66, the position labeled by [4-^2H]shikimic acid. Instead, the deuterium occupies the 34*Z* (axial) position. Hence, this compound cannot be an intermediate. In contrast, [5-^2H]**41** was very efficiently incorporated and reduced. The deuterium resonance in the resulting ansatrienin A appeared at δ 1.19 (Table III), corresponding to the chemical shift of the deuterium incorporated from C-5 of shikimic acid, and is clearly different from the position of the deuterium incorporated from C-3 of shikimic acid (δ 1.74, Table I). This crucial result establishes that **41** is an intermediate on the pathway and that the hydroxy group remaining in this compound originates from C-5 of shikimic acid.

Turning our attention to the beginning of the pathway, we found that [2-^2H]dihydroshikimic acid (**42**) was not incorporated, indicating that reduction of the double bond is not the first step in the transformation. This finding, coupled with the specific incorporation of [5-^2H]**41** and the results with the deuterated shikimic acids, indicates that the hydroxy group at C-3 of shikimic acid must be the first functionality to be eliminated and that the reaction must be a 1,4-conjugate elimination involving one of the hydrogens from C-6 (Scheme VIII). We tested this idea by feeding (±)-*trans*-3,4-dihydroxy[2,3,4,5,6-$^2H_5$]cyclohexa-1,5-dienecarboxylic acid (**43**). ^2H NMR (Table III) and GC-MS analysis data for the ansatrienin from this feeding experiment were virtually identical with those for ansatrienin obtained from (±)-[2,3,4,5-$^2H_4$]shikimic acid, indicating that **43** is an intermediate. As expected, the deuterium from C-2 of [2,3,4,5,6-

$^2H_5$]**43** (corresponding to C-6 of shikimic acid) was lost in the transformation to cyclohexanecarboxylic acid.

The C-4 hydroxy group of shikimic acid must be the second one to be eliminated. The feeding of 5-hydroxy[2,3,4,5-$^2H_4$]cyclohexa-1,3-dienecarboxylic acid (**44**) resulted in deuterium resonances in ansatrienin A which appeared at the same positions as those in ansatrienin derived from (±)-[2,3,4,5-$^2H_4$]shikimic acid. As with [2,3,4,5,6-$^2H_5$]**43**, the pattern of incorporation of [2,3,4,5-$^2H_4$]**44** into ansatrienin is fully consistent with it being an intermediate on the pathway. Thus, formation of 5-hydroxycyclohex-1-enecarboxylic acid must involve the reduction of **44**, either through the direct reduction of the Δ^3 double bond or through a two-step process involving the 1,4-conjugate reduction to 5-hydroxycyclohex-2-enecarboxylic acid (**46**) followed by isomerization.

The formation of **44** must involve reduction and dehydration of **43**. To avoid aromatization, a reduction of the diene must precede dehydration; this could occur either in a 1,4-fashion to give the Δ^1 monoene or in a 1,2-fashion to give the Δ^2 monoene. This was probed by feeding *trans*-4,5-dihydroxy[2-^{2}H]cyclohex-1-enecarboxylic acid (**37**). Analyses by ^{2}H NMR and GC-MS indicated no incorporation into the cyclohexanecarboxylic acid moiety of **1**. Hence, the transformation must proceed via *trans*-4,5-dihydroxycyclohex-2-enecarboxylic acid (**47**), which then undergoes a 1,4-conjugate elimination of water.

Refinement of the Pathway. We are also studying the pathway from shikimic acid to cyclohexanecarboxylic acid in *Alicyclobacillus acidocaldarius*, a producer of ω-cyclohexyl fatty acids. In this work we have isolated (1*S*,3*S*)-3-hydroxycyclohexanecarboxylic acid accumulated by a blocked mutant which is auxotrophic for cyclohexanecarboxylic acid.[12] This finding suggests that 5-hydroxycyclohex-1-enecarboxylic acid (**41**) is not dehydrated to the cross-conjugated diene **34** prior to double bond reduction to 2-cyclohexenecarboxylic acid (**32**), as proposed. Rather, the double bond of **41** may first be reduced to give the hydroxy acid, which was accumulated in the *Alicyclobacillus* mutant, before dehydration to **32**.

We thus synthesized 3-hydroxy[3-^{2}H]cyclohexanecarboxylic acid. Hydrogenation of [5-^{2}H]**40** followed by chromatography to resolve the diastereotopic mixture and saponification of the reduced methyl esters gave (±)-*trans*- and (±)-*cis*-3-hydroxy[3-^{2}H]cyclohexanecarboxylic acids (**50**, **51**). Both diastereomers were then individually administered to *S. collinus*.

The deuterium resonance in the resulting ansatrienin A from the feeding experiment with [3-^{2}H]**50** appeared at δ 1.23 (Table III), corresponding to the chemical shift of the deuterium incorporated from C-5 of shikimic acid. The GC-MS of the acid hydrolysis products of **1** from this feeding experiment indicated that cyclohexanecarboxylic acid was enriched at 11%. The ansatrienin A resulting from the diastereomer [3-^{2}H]**51** was likewise enriched with deuterium at δ 1.23. However, the cyclohexanecarboxylic acid from the hydrolysis of this sample of **1** was only enriched at 2%.

Discussion

The foregoing experiments show that shikimic acid is processed stereospecifically in the conversion to the cyclohexanecarboxylic acid moiety of ansatrienin A, i.e., the two sides of the six-membered ring remain distinct throughout the process. C-2 of shikimate labels the pro-*S* carbon, C-36 in ansatrienin A or C-6 in cyclohexanecarboxylic acid, and H-2 of shikimate gives rise to the axial hydrogen at that carbon (pro-36*R* hydrogen). A similar conclusion had been drawn by Furukawa *et al.*[41] on the biosynthesis of the cyclohexane ring in ω-cyclohexylundecanoic acid. They fed D-[6,6-$^2H_2$]glucose to *Curtobacterium pusilum* and degraded the resulting fatty acid to cyclohexanecarboxylic acid, which was further derivatized to introduce an auxiliary chiral center at C-7. The axial hydrogen at C-6 was the primary site of labeling with 66% deuterium enrichment, consistent with our result. The two hydrogens at C-2 each contained 5% deuterium and the remaining positions less than 2%. They interpreted this result to indicate reduction of the carbon–carbon double bond of shikimic acid by *syn* addition of hydrogen on the *re–re* face. Implicit in this interpretation is the assumption that the primary site of labeling in shikimic acid is at C-2. This assumption is questionable, as the hydrogens at C-6 of shikimate should also be labeled by D-[6,6-$^2H_2$]glucose (via phosphoenol [3-$^2H_2$]pyruvate), and, as our data suggest, their conclusion may only be fortuitously correct. The process involves a *re–re* reduction of the double bond of shikimate only in a formal sense; mechanistically the transformation is far more complex, as evidenced by the facts that the intermediate cyclohex-1-enecarboxylic acid is labeled at C-6, not the olefinic carbon C-2, and that both hydrogens from C-6 of shikimate are eliminated in the transformation.

The conversion of shikimate into the cyclohexanecarboxylic acid moiety of ansatrienin is a reductive process in which the double bond and all three hydroxyl groups are eliminated. The removal of the hydroxyl functions of shikimate must involve a series of alternate dehydrations and double bond reductions, arranged such that the ring system never becomes aromatic. The loss of both labeled hydrogens from C-6 of shikimate, and only those two hydrogens, indicates that two dehydrations must involve proton loss from C-6. Since 1,2-dihydroshikimic acid is not incorporated into ansatrienin, the reduction of the Δ^1 double bond of shikimate is not the first step, and the reaction sequence must be initiated by a dehydration. The retention of deuterium from both C-3 and C-5 of shikimic acid implies that the first dehydration must involve the hydroxyl group at C-3 or C-5, not that at C-4. The retention of deuterium at C-4 indicates that any dehydration step involving loss of a hydrogen from C-4, if it takes place at all, can only occur after the hydroxyl group at C-4 has been removed and replaced by another, unlabeled hydrogen.

The incorporation of 5-hydroxy[5-^{2}H]cyclohex-1-enecarboxylic acid (**41**) into ansatrienin A demonstrated that the hydroxy group in this compound originates from C-5 of shikimic acid. The deuterium resonance corresponded to the chemical shift of the deuterium incorporated from C-5 of shikimic acid and not that of deuterium incorporated from C-3 of shikimic acid. Therefore, the C-3 hydroxy group of shikimic acid must be the first one eliminated, through a 1,4-conjugate dehydration involving one of the hydrogens at C-6. This was confirmed by the successful incorporation of *trans*-3,4-dihydroxy[2,3,4,5,6-$^2H_5$]cyclohexa-1,5-dienecarboxylic acid (**43**). The dehydration may be facilitated by activation (phosphorylation or protonation) of the departing hydroxy group. This conjugate eliminaton is analogous to the conversion of 5-enolpyruvyl shikimate 3-phosphate to chorismate, which involves the removal of the pro-6*S* hydrogen and loss of phosphate with overall *anti* stereochemistry.[42] In fact, in *Klebsiella pneumoniae* (formerly *Aerobacter aerogenes*), chorismic acid undergoes cleavage of its enolpyruvyl side chain to produce (3*R*,4*R*)-**43**.[39] Hence, the formation of cyclohexanecarboxylic acid may branch off from the normal shikimate pathway at chorismic acid, at shikimic acid, or at any stage between the two.

5-Hydroxy[2,3,4,5-$^2H_4$]cyclohexa-1,3-dienecarboxylic acid (**44**) was also efficiently incorporated into **1**. Its formation must involve a 1,4-conjugate dehydration of *trans*-4,5-dihydroxycyclohex-2-enecarboxylic acid (**47**), as the corresponding Δ^1 isomer **37** was neither incorporated nor reduced. The dehydration of **47** involves the loss of the C-1 proton and the C-4 hydroxy group. Once

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again, the reaction may be facilitated by activation of the hydroxy group being eliminated.

The conversion of the monohydroxy 1,3-diene **44** to the monohydroxy 1-monoene **41** involves the overall reduction of the Δ^3 double bond. This conversion may occur by the direct reduction of the Δ^3 double bond, or, alternatively, **44** may undergo a 1,4-conjugate reduction to the corresponding Δ^2 isomer **46**, followed by isomerization of the double bond into conjugation with the carboxyl group to give **41**. The currently available data do not differentiate between these two possibilities.

The cross-conjugated diene **34** was the only diene which was efficiently reduced and incorporated into the cyclohexanecarboxylic acid moiety of **1**, whereas the other dienes (**33**, **35**, and **36**) were only partially reduced and/or aromatized to benzoic acid. Presumably, the conversion of **41** to **34** would involve the elimination of the C-5 hydroxy group and an allylic hydrogen at C-6. The eliminated proton would have to be the remaining hydrogen derived from C-6 of shikimic acid, since both C-6 hydrogens of shikimate are eliminated in the conversion to cyclohexanecarboxylic acid and one is lost in the initial dehydration to **43**.

The reduction of the diene **34** to the saturated cyclohexanecarboxylic acid via the monoene **32** was also observed at the CoA ester level in cell-free extracts of *S. collinus*.[43] In addition, the coenzyme A esters of **31** and **32** demonstrated isomerization of the double bond from the Δ^2 to the Δ^1 position and the double bond reduction to cyclohexanecarbonyl–CoA. However, the thioester of 3-cyclohexenecarboxylic acid was not a substrate. Later it was shown that the purified enoyl–CoA reductase which catalyzes the conversion of 1-cyclohexenecarbonyl–CoA into cyclohexanecarbonyl–CoA also was capable of catalyzing the reduction of **34** to **32**.[44] Consequently, we initially proposed that the conversion of **41** to **32** proceeds through the diene **34**.[45]

This segment of the pathway, however, was put into question by the *A. acidocaldarius* mutant experiments in which (1*S*,3*S*)-**50** rather than **34** was accumulated by a mutant auxotrophic for cyclohexanecarboxylic acid.[12] The synthetic diastereomers (±)-[3-^{2}H]**50** and (±)-[3-^{2}H]**51** were both reduced and incorporated into **1**. The deuterium resonance in both samples of **1** corresponded to the frequency of the deuterium incorporated from C-5 of shikimate. However, **50** was converted much more efficiently than **51**. Together with the configuration of the material accumulated by the *A. acidocaldarius* mutant, this suggests that **50** is the natural pathway intermediate. The isomer **51** may be an unnatural substrate which, as it is structurally related to **50**, also undergoes dehydration, albeit at a much slower rate. In any case, this result suggests that **41** is first reduced to **50** before dehydration to **32**. The elimination must involve the C-3 hydroxy group and a rather nonacidic hydrogen from C-2. As with the earlier proposed dehydration of **41**, the eliminated hydrogen must have originated from C-6 of shikimate.

The diene **34** apparently is not a pathway intermediate, yet when exogenously added it is shunted into the pathway via the monoene **32** through the nonspecific reduction by 1-cyclohexenecarbonyl–CoA reductase. This revision represents an important lesson exemplifying the caution required in deducing metabolic pathways solely from feeding, and even enzymatic, experiments.

The stereochemistry of the final double bond reduction was probed by feeding [2,6,6-$^2H_3$]**31**; the reaction was found to proceed by an *anti* addition of hydrogen. This result is in agreement with those of Reynolds *et al.* who showed at the enzymatic level that the reduction of 1-cyclohexenecarbonyl–CoA occurs by the addition of the pro-4*S* hydrogen of NADPH to the *si* face at C-2 of the cyclohexene ring and addition of a solvent proton at C-1.[46]

From the results presented here, we have been able to establish the sequence of reactions comprising this unique diversion of shikimic acid to cyclohexanecarboxylic acid. Most of the reactions probably do not occur on the free acid but rather on the coenzyme A thioester, as has been demonstrated at the enzymatic level for the transformations of **31**, **32**, and **34**.[43,46] Exactly at what stage in the pathway thioester formation takes place, however, is not clear yet, nor is the exact point of departure of this metabolic route from the common shikimate pathway known.

In our studies on the same pathway in *A. acidocaldarius*, we have been able to deduce the steric course of all but one of these transformations. Combined knowledge of the stereochemical fate of the carbon-bound hydrogens of shikimate in *S. collinus* with stereochemical information determined in *A. acidocaldarius* was crucial to the ultimate delineation of the stereochemistry of formation of cyclohexanecarboxylic acid. These results are presented in the following paper in this issue.[12]

Experimental Section

General Procedures. The ^{1}H, ^{13}C, and ^{2}H 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) relative to the TMS scale by reference to the solvent signal. Coupling constants (J) are given in hertz (Hz). {^{2}H} Broad-band decoupling was accomplished with a Bruker BSU-3 X-nucleus decoupler, using a PTS-250 synthesizer operating at 46.0724 MHz and tied to the spectrometer dwell clock as the frequency source. {^{2}H} Decoupler power was typically 2 W forward and <0.1 W reflected. GC-MS was carried out on a Hewlett–Packard 5790A gas chromatograph with a 5970A mass selective detector. Melting points were determined on a Mel-Temp Laboratory Device and are uncorrected. IR spectra were recorded with a Mattson Polaris FT-IR, only the major absorptions being cited. A New Brunswick G25 rotary shaker was used for fermentation. Analytical TLC was executed on precoated silica gel 60F-254 plates. Compounds on the plates were visualized under UV light or by spraying with a *p*-anisaldehyde solution (3.7 mL of *p*-anisaldehyde, 135 mL of ethanol, 5 mL of concentrated H_2SO_4, 1.5 mL of glacial acetic acid), phosphomolybdic acid (Aldrich), or $KMnO_4$ solution (1.0 g of $KMnO_4$, 100 mL of 1 N NaOH) and heating at 120 °C. Mobilities are quoted relative to the solvent front (R_f). Column chromatography[47] was performed on 230–400 mesh silica gel from Aldrich.

Materials. *S. collinus* Tü 1892 was obtained from Professor Axel Zeeck, Göttingen, and Professor Hans Zähner, Tübingen. All chemicals were of reagent grade and were used without further purification unless otherwise noted. Reaction solvents were purified by distillation from appropriate drying agents: THF, ether (Na/benzophenone), CH_2Cl_2 (P_2O_5), DMF (CaH_2). Other solvents were purified as described in ref 48. Ingredients for fermentations were purchased from Difco and Sigma. The following companies supplied stable-isotope-labeled compounds (atom % stable isotope): Na^{13}CN (99%), [7-^{13}C]benzoic acid (99%), [2,3,3-$^2H_3$]acrylic acid (98%), 1,3-butadiene-d_6 (98%), Cambridge Isotope Laboratories; benzoic acid-d_5 (99%), MSD Isotope; $NaBD_4$ (98%), furan-d_4 (99%), D_2O (99%), Aldrich; D-[1-^{13}C]mannose (99%), Los Alamos Stable Isotope Resourse.

Fermentation. *S. collinus* strain Tü 1892 was maintained on agar slants of the following medium: yeast extract, 0.4 g; malt extract, 1.0 g; glucose, 0.4 g; agar, 2.0 g; distilled water, 100 mL; pH 7.4. A part of the spore suspension was transferred under sterile conditions to a 500-mL Erlenmeyer flask containing 100 mL of seed medium and incubated for 2 days on a rotary shaker at 28 °C and 300 rpm. Of the seed culture, 10 mL was used to inoculate each 100 mL of production medium in 500-mL Erlenmeyer flasks, which were again grown for 72 h at 28 °C with rotary shaking at 300 rpm. Both the seed and production medium contained full fat soybean meal, 2.0 g; mannitol, 2.0 g; tap water, 100 mL; pH 7.3. All media were sterilized for 20 min at 121 °C in a steam autoclave.

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(44) Reynolds, K. A.; Wang, P.; Fox, K. M.; Speedie, M. K.; Lam, Y.; Floss, H. G. *J. Bacteriol.* **1992**, *174*, 3850.
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Feeding Experiments with Labeled Precursors. Feeding experiments were carried out in the normal production media. In general, single doses of precursor were administered to the fermentation after 24 h of growth, and the cultures were harvested 48 h later. Precursors were added as sterile solutions in the amounts indicated per culture volume: (–)-[2-^{13}C]shikimic acid, 12 mg/200 mL; (–)-[2-^{2}H]shikimic acid, 112 mg/700 mL; (–)-[3-^{2}H]shikimic acid, 14 mg/200 mL; (–)-[4-^{2}H]shikimic acid, 24 mg/200 mL; [2,5-$^{2}H_2$]shikimic acid, 18 mg/200 mL; [2,3,4,5-$^{2}H_4$]shikimic acid, 80 mg/500 mL; (–)-(6*R*,*S*)-[6-$^{2}H_1$]shikimic acid, 100 mg/2 L; [7-^{13}C]cyclohex-1-enecarboxylic acid, 14 mg/200 mL; [2,6,6-$^{2}H_3$]cyclohex-1-enecarboxylic acid, 18 mg/200 mL; [7-^{13}C]cyclohex-2-enecarboxylic acid, 37 mg/500 mL; [7-^{13}C]cyclohexa-2,5-dienecarboxylic acid, 12 mg/200 mL; [7-^{13}C]cyclohexa-1,5-dienecarboxylic acid, 10 mg/300 mL; [6-$^{2}H_2$]cyclohexa-1,3-dienecarboxylic acid, 3 mg/100 mL; [3,3,4,5,6,6-$^{2}H_6$]cyclohexa-1,4-dienecarboxylic acid, 72 mg/2.4 L; *trans*-4,5-dihydroxy[2-^{2}H]cyclohex-1-enecarboxylic acid, 31 mg/400 mL; 4-hydroxy[4-^{2}H]cyclohex-1-enecarboxylic acid, 16 mg/600 mL; 5-hydroxy[5-^{2}H]cyclohex-1-enecarboxylic acid, 18 mg/600 mL; [2-^{2}H]dihydroshikimic acid, 4 mg/100 mL; *trans*-3,4-dihydroxy[2,3,4,5,6-$^{2}H_5$]cyclohexa-1,5-dienecarboxylic acid, 15 mg/300 mL; 5-hydroxy[2,3,4,5-$^{2}H_4$]cyclohexa-1,3-dienecarboxylic acid, 12 mg/300 mL; *trans*-3-hydroxy[3-^{2}H]cyclohexanecarboxylic acid, 16 mg/300 mL; *cis*-3-hydroxy[3-^{2}H]cyclohexanecarboxylic acid, 15 mg/300 mL.

Isolation of Ansatrienin A. The cultures were filtered through Celite, and the mycelia were collected and suspended in acetone. The suspension was sonicated (Virsonic Model 16-850) for 5 min at maximum power setting and filtered, and the process was repeated once more. The combined filtrates were concentrated to remove acetone. The crude product was suspended in water and extracted with EtOAc. The extract was dried over Na_2SO_4, concentrated, and the crude ansatrienin was precipitated with petroleum ether with cooling to 0 °C overnight. The precitate was collected, washed with petroleum ether, and dissolved in a small volume of EtOAc. It was shaken briefly with a few milliliters of a saturated aqueous solution of $FeCl_3$ to oxidize ansatrienin B to ansatrienin A. The EtOAc layer was separated, concentrated, and purified by column chromatography (silica gel, hexane–EtOAc 2:1). The yield of **1** after purification averaged 60 mg/L.

Degradation of Ansatrienin A. One milligram of ansatrienin A in 2 mL of 10 N HCl was heated to 90 °C for 6 h. The reaction mixture was extracted with Et_2O (3 × 2 mL); the combined extract was concentrated, and cyclohexanecarboxylic acid was isolated by TLC on silica gel (*n*-PrOH–H_2O 7:3; or Et_2O). For GC-MS analysis, the undiluted acid was converted to the methyl ester by treatment with diazomethane in ether.

[2-^{13}C]Shikimic Acid (99% ^{13}C). This compound was prepared from D-[1-^{13}C]mannose as described:[20] ^{1}H NMR (CD_3OD) δ 2.18 (m, 1H), 2.69 (m, 1H), 3.66 (m, 1H), 3.97 (m, 1H), 4.36 (m, 1H), 6.79 (dm, *J* = 16.3 Hz, 1H); ^{13}C NMR (CD_3OD) δ 138.78 (enriched).

2,3:5,6-Di-*O*-isopropylidene-D-mannono-γ-lactone (10).[49] A solution of 2,3:5,6-di-*O*-isopropylidene-α-D-mannofuranose[20] (12.7 g, 48.8 mmol) in anhydrous DMSO (1 L) containing acetic anhydride (135 mL) was stirred at room temperature for 20 h. Air was bubbled through vigorously while the reaction mixture was warmed to 60 °C to remove dimethyl sulfide. Water (400 mL) was added, and the resulting mixture was extracted with ether (4 × 400 mL). The combined ether layers were washed with water (4 × 400 mL), concentrated under reduced pressure, and lyophilized to give about 9.5 g of crude product. The original aqueous layer was extracted with ether (400 mL), and the ether layer was concentrated and lyophilized to obtain an additional 1.5 g of crude product. The combined crude product (94% pure by GC analysis) was recrystallized from ligroin (500 mL) to give 10.4 g (81 %) of lactone **10**: mp 116 °C (lit.[49] mp 126 °C); ^{1}H NMR ($CDCl_3$) δ 1.35 (s, 3H), 1.38 (s, 3H), 1.43 (s, 3H), 1.44 (s, 3H), 4.02 (dd, *J* = 9.3, 3.9 Hz, 1H), 4.11 (dd, *J* = 9.3, 5.7 Hz, 1H), 4.32–4.43 (m, 2H) 4.78–4.85 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 25.06, 25.85, 26.71, 26.90, 66.40, 72.59, 75.82, 75.90, 78.16, 109.83, 114.41, 173.36; GC-MS *m*/*z* 243 $(M - CH_3)^+$.

2,3:5,6-Di-*O*-isopropylidene-D-[1-^{2}H]mannofuranose ([1-^{2}H]3). A mixture of **10** (20.9 g, 81 mmol) and $NaBD_4$ (3.4 g, 81 mmol) in 9:1 MeOH:H_2O (490 mL) was stirred for 2.5 h at room temperature. The reaction mixture was then concentrated to a syrup and extracted with ether (3 × 200 mL). The combined ether extracts were dried ($MgSO_4$) and concentrated to give 11.1 g (52%) of a diastereomeric mixture (α/β = 9:1) of 2,3:5,6-di-*O*-isopropylidene-D-[1-^{2}H]mannofuranose. This was used for the next reaction without further purification.

α-Anomer of [1-^{2}H]**3**: ^{1}H NMR ($CDCl_3$) δ 1.30 (s, 3H), 1.35 (s, 3H), 1.42 (s, 3H), 1.43 (s, 3H), 3.95–4.13 (m, 2H), 4.15 (dd, *J* = 7.0, 3.7 Hz, 1H), 4.37 (m, 1H), 4.58 (d, *J* = 5.9 Hz, 1H), 4.77 (dd, *J* = 5.9, 3.6 Hz, 1H); GC-MS *m*/*z* 246 $(M - CH_3)^+$.

[2-^{2}H]Shikimic Acid (98% ^{2}H). This compound was prepared from [1-^{2}H]**3** as described:[20] ^{1}H NMR (CD_3OD) δ 2.18 (ddd, *J* = 18.2, 5.7, 1.5 Hz, 1H), 2.69 (ddd, *J* = 18.2, 5.0, 1.8 Hz, 1H), 3.66 (dd, *J* = 7.5, 4.2 Hz, 1H), 3.98 (dt, *J* = 7.5, 5.4 Hz, 1H), 4.36 (m, 1H); ^{2}H NMR (CH_3OH) δ 6.77.

Benzyl 2,3-*O*-Isopropylidene-α-D-[5-^{2}H]lyxofuranoside ([5-^{2}H]5). This compound was prepared from **4** as described[20] using $NaBD_4$ (98 atom % ^{2}H) instead of $NaBH_4$ (43% yield from **3**): ^{1}H NMR ($CDCl_3$) δ 1.31 (s, 3H), 1.47 (s, 3H), 2.20 (br s, 1H), 3.94 (dd, *J* = 18.3, 5.4 Hz, 1H), 4.12 (t, *J* = 4.4 Hz, 1H), 4.50 (dd, *J* = 11.8 Hz, 1H), 4.67 (d, *J* = 5.9 Hz, 1H), 4.69 (d, *J* = 11.8 Hz, 1H), 4.80 (dd, *J* = 5.9, 3.8 Hz, 1H), 5.14 (s, 1H), 7.25–7.40 (m, 5H).

[6-$^{2}H_1$]Shikimic Acid (6*R*:6*S* = 7:3, 98% ^{2}H) . This compound was prepared from [5-^{2}H]**5** as described:[20] ^{1}H NMR (CD_3OD) δ 2.02 (m, 0.3H), 2.53 (m, 0.7H), 3.53 (m, 1H), 3.85 (m, 1H), 4.22 (m, 1H), 6.64 (m, 1H); ^{2}H NMR (CH_3OH) δ 2.02 (0.7D, 6*R*), 2.54 (0.3D, 6*S*).

1,2-*O*-Isopropylidene-3-*O*-benzyl-5-*O*-toluenesulfonyl-D-arabinofuranose (12). To a stirred solution of 1,2-*O*-isopropylidene-5-*O*-toluenesulfonyl-D-arabinofuranose (**11**)[23,24] (2.53 g, 7.35 mmol) in dry DMF (25 mL) were added benzyl bromide (2.5 mL, 21 mmol) and sodium hydride (80% in oil, 265 mg, 8.9 mmol) in several portions over a period of 10 min. After 15 min at room temperature, the reaction was quenched by addition of water (35 mL), and the resulting mixture was extracted with ether (2 × 180 mL, 3 × 125 mL). The combined extracts were dried ($MgSO_4$) and concentrated. Flash chromatography (hexane–EtOAc 3:1) of the residue afforded **12** (3.05 g, 96%) as a syrup: R_f 0.49 (hexane–EtOAc 2:1); ^{1}H NMR ($CDCl_3$) δ 1.25 (s, 3H), 1.34 (s, 3H), 2.41 (s, 3H), 3.94 (d, *J* = 1.8 Hz, 1H), 4.11 (d, *J* = 6.4 Hz, 2H), 4.24 (dt, *J* = 6.4, 1.8 Hz, 1H), 4.52 (s, 2H), 4.57 (d, *J* = 3.7 Hz, 1H), 5.84 (d, *J* = 3.8 Hz, 1H), 7.23–7.40 (m, 7H), 7.74 (d, *J* = 8.2 Hz, 2H); GC-MS *m*/*z* 329 $(M - CH_3)^+$.

1,2-*O*-Isopropylidene-3-*O*-benzyl-D-arabinofuranose (13). Four percent of sodium amalgam, prepared from 0.29 g (13 mmol) of sodium and 7.23 g of mercury,[50] was placed in a 100-mL round-bottom flask. A solution of **12** (652 mg, 1.50 mmol) in 85% aqueous MeOH (37 mL) was added slowly, and the mixture was stirred for 3 h, by which time all the sodium amalgam had disappeared. The solution was then neutralized by addition of excess dry ice and decanted. The mercury was washed with 10 mL of MeOH. The combined solutions were concentrated to ca. 5 mL, and 25 mL of water was added. The aqueous solution was then extracted with $CHCl_3$ (4 × 20 mL), and the extract was dried ($MgSO_4$), concentrated, and purified by column chromatography (hexane–EtOAc 1:1) to give **13** (396 mg, 94%) as a white solid: R_f 0.51 (hexane–EtOAc 2:1); mp 68–70 °C; ^{1}H NMR ($CDCl_3$) δ 1.33 (s, 3H), 1.51 (s, 3H), 1.94 (s, 1H, OH), 3.71 (d, *J* = 5.5 Hz, 2H), 3.96 (d, *J* = 3.4 Hz, 1H), 4.18 (m, 1H), 4.54 (d, *J* = 11.7 Hz, 1H), 4.63 (d, *J* = 11.7 Hz, 1H), 4.56 (d, *J* = 4.1 Hz, 1H), 5.89 (d, *J* = 4.1 Hz, 1H), 7.32 (m, 5H); GC-MS *m*/*z* 265 $(M - CH_3)^+$.

***tert*-Butyl (3-*O*-Benzyl-5,6-dideoxy-6-dimethoxyphosphoro-1,2-*O*-isopropylidene-D-*arabino*-heptofuranos)uronate (15).** A solution of **13** (615 mg, 2.20 mmol) in dry CH_2Cl_2 (7 mL) containing pyridine (0.40 mL, 4.8 mmol) was cooled to –30 °C and stirred while triflic anhydride (0.50 mL, 2.9 mmol) was added during 15 min. After 15 min at –30 °C, methanol (0.4 mL) was added to quench the reaction, and the resulting mixture was diluted with CH_2Cl_2 (10 mL). It was then washed successively with ice–water (5 mL) and cold aqueous NaH_2PO_4 (1 M, 5 mL), dried (Na_2SO_4), and concentrated to give 908 mg of crude triflate (**14**) (R_f 0.88, hexane–EtOAc 1:1). This was used for the next reaction without further purification.

Sodium hydride (100 mg, 3.3 mmol) was suspended in dry DMF (9 mL), and the mixture was cooled to 0 °C. A solution of *tert*-butyl dimethoxyphosphonoacetate (820 mg, 3.66 mmol) in dry DMF (3.5 mL) was added dropwise to the stirred mixture during 20 min. After the addition was complete, the cold bath was removed and the mixture was stirred for 1 h to give a clear solution. A solution of the triflate **14** (908 mg) in dry DMF (4 mL) was then added, followed by 15-crown-5 (3 drops). After being stirred for 13 h at room temperature, the reaction mixture was cooled to 0 °C, quenched with cold aqueous 1 N NaH_2PO_4 (25 mL), and extracted with $CHCl_3$ (1 × 25 mL, 2 × 15 mL). The combined extracts were washed with cold water (15 mL), dried (Na_2SO_4), concentrated, and purified by column chromatography (hexane–EtOAc 1:1). This provided 718 mg (67% from **13**) of the diastereomeric mixture

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(50) Holleman, A. F. *Org. Synth.* **1927**, *7*, 88.

of oily **15**: R_f 0.50 (EtOAc); ^{1}H NMR ($CDCl_3$) δ 1.28 (s, 3H), 1.39 (s, 3H), 1.44 (s, 9H), 1.46 (s, 9H), 1.49 (s, 3H), 1.52 (s, 3H), 2.08–2.45 (m, 2H), 3.05 (m, 1H), 3.31 (s, 1H), 3.72–3.78 (m, 6H), 4.11 (m, 1H), 4.54–4.62 (m, 3H), 5.82 (d, J = 4.8 Hz, 1H), 5.87 (d, J = 4.8 Hz, 1H), 7.24–7.33 (m, 5H); GC-MS m/z (relative intensity) 471 [$(M - CH_3)^+$, 10], 429 [$(M - O^tBu)^+$, 100].

***tert*-Butyl (5,6-Dideoxy-6-dimethoxyphosphoryl-1,2-*O*-isopropylidene-D-*arabino*-heptofuranos)uronate (16).** To a solution of **15** (1.57 g, 3.23 mmol) in 95% MeOH (50 mL) were added $HCOONH_4$ (904 mg, 14.4 mmol) and 10% Pd/C (1.04 g). After the solution was stirred for 25 min at 50 °C, Pd/C was filtered off and water (15 mL) was added to the methanol solution. The resulting mixture was concentrated to about 15 mL and extracted with $CHCl_3$ (4 × 30 mL). The combined $CHCl_3$ layer was dried (Na_2SO_4) and concentrated to give 1.34 g of a diastereomeric mixture of **16** which solidified on standing. It was used for the next reaction without further purification.

16 (mixture of diastereomers): mp 97–117 °C; IR (neat) 3390, 3000, 1730, 1420, 1375, 1250, 1155, 1065, 1030, 755 cm^{-1}; GC-MS m/z (relative intensity) 381 [$(M - CH_3)^+$, 18], 323 [$(M - O^tBu)^+$, 100].

16a: R_f 0.56 (hexane–EtOAc 4:1); ^{1}H NMR ($CDCl_3$) δ 1.28 (s, 3H), 1.46 (s, 9H), 1.52 (s, 3H), 2.10–2.30 (m, 2H), 3.29 (ddd, J = 23.5, 10.8, 4.0 Hz, 1H), 3.76 (d, J = 10.9 Hz, 3H), 3.77 (d, J = 10.9 Hz, 3H), 3.99 (dd, J = 9.8, 4.7 Hz, 1H), 4.12 (s, 1H), 4.51 (d, J = 3.8 Hz, 1H), 5.88 (d, J = 3.9 Hz, 1H).

16b: R_f 0.45 (hexane–EtOAc 4:1); ^{1}H NMR ($CDCl_3$) δ 1.28 (s, 3H), 1.45 (s, 9H), 1.52 (s, 3H), 2.15–2.45 (m, 2H), 3.13 (dt, J = 23.5, 5.8 Hz, 1H), 3.75 (d, J = 10.9 Hz, 3H), 3.76 (d, J = 10.9 Hz, 3H), 3.99 (m, 1H), 4.08 (s, 1H), 4.51 (d, J = 3.8 Hz, 1H), 5.85 (d, J = 3.9 Hz, 1H).

Oxidation–Reduction of 16. To a solution of **16** (540 mg, 1.36 mmol) in dry DMF (4 mL) was added dry DMSO (0.38 mL) and P_2O_5 (300 mg). After being heated at 70 °C for 3 h, the reaction mixture was allowed to cool to room temperature. Water (40 mL) was then added, and the mixture was extracted with $CHCl_3$ (4 × 40 mL). The combined $CHCl_3$ layers were washed with water (40 mL), and the latter was reextracted with $CHCl_3$ (40 mL). All the $CHCl_3$ layers were combined, dried (Na_2SO_4), and concentrated to give crude **17** (590 mg, R_f 0.58, hexane–EtOAc 4:1), perhaps still containing traces of solvents. The crude **17** (590 mg) in 90% MeOH (15 mL) was cooled to 0 °C, and $NaBD_4$ (250 mg) was added over a 5-min period. After 1 h at 0 °C, the reaction mixture was diluted with water (12 mL), concentrated to about 10 mL, and extracted with $CHCl_3$ (3 × 30 mL). The combined $CHCl_3$ layers were dried (Na_2SO_4) and concentrated to give 500 mg of a white solid. The latter was dissolved in hot EtOAc (2 mL) and treated with hexane (6 mL). A white solid separated which consisted of only **18a** (298 mg, mp 159–161 °C, R_f 0.50, EtOAc–acetone 4:1). Concentration of the mother liquor followed by column chromatography (EtOAc) afforded a 1:2 mixture of diastereomers **18a** and **18b** (87 mg). The diastereomer **18b** (R_f 0.40) did not solidify on standing. The total yield of **18** was 386 mg (71% from **16**).

18a: R_f 0.50 (EtOAc–acetone 4:1); mp 159–160 °C; ^{1}H NMR ($CDCl_3$) δ 1.32 (s, 3H), 1.43 (s, 9H), 1.58 (s, 3H), 2.20 (m, 2H), 3.31 (m, 1H), 3.73 (d, J = 10.9 Hz, 3H), 3.75 (d, J = 10.9 Hz, 3H), 3.95 (t, J = 7.0 Hz, 1H), 4.58 (d, J = 4.0 Hz, 1H), 5.69 (d, J = 4.2 Hz, 1H); GC-MS m/z (relative intensity) 382 [$(M - CH_3)^+$, 30], 324 [$(M - O^tBu)^+$, 100].

18b: R_f 0.40 (EtOAc–acetone 4:1); liquid; ^{1}H NMR ($CDCl_3$) δ 1.34 (s, 3H), 1.43 (s, 9H), 1.55 (s, 3H), 2.15–2.43 (m, 2H), 3.16 (m, 1H), 3.74 (d, J = 10.9 Hz, 3H), 3.77 (d, J = 10.9 Hz, 3H), 4.01 (dd, J = 8.3, 5.9 Hz, 1H), 4.58 (d, J = 4.0 Hz, 1H), 5.66 (d, J = 4.1 Hz, 1H).

***tert*-Butyl (5,6-Dideoxy-6-dimethoxyphosphoryl-2,3-*O*-isopropylidene-α-D-[3-^{2}H]*lyxo*-heptofuranos)uronate ([3-^{2}H]8).** A mixture of **18** (910 mg, 2.29 mmol) and Dowex-50W (H^+) (2.4 g, washed with acetone 3 times and dried under vacuum for 5 min) in dry acetone (20 mL) was stirred for 4 h at room temperature. It was then filtered, and the resin was washed with acetone (4 × 5 mL). The combined acetone layers were concentrated, and the residue was purified by column chromatography (EtOAc) to give 800 mg (88%) of a diastereomeric mixture of [3-^{2}H]**8** as a syrup (R_f 0.65, 0.58 EtOAc–acetone 4:1). It was identical with material synthesized by Fleet's route.[20]

[3-^{2}H]**8a**: R_f 0.65 (EtOAc–acetone 4:1); ^{1}H NMR ($CDCl_3$) δ 1.28 (s, 3H), 1.42 (s, 3H), 1.45 (s, 9H), 2.17–2.27 (m, 2H), 3.20 (ddd, J = 22.9, 10.3, 4.5 Hz, 1H), 3.75 (d, J = 10.9 Hz, 3H), 3.77 (d, J = 10.9 Hz, 3H), 4.11 (dd, J = 8.5, 4.6 Hz, 1H), 4.55 (s, 1H), 5.30 (s, 1H); GC-MS m/z 382 $(M - CH_3)^+$.

[3-^{2}H]-**8b**: R_f 0.58 (EtOAc–acetone 4:1); ^{1}H NMR ($CDCl_3$) δ 1.29 (s, 3H), 1.43 (s, 3H), 1.46 (s, 9H), 2.10–2.44 (m, 2H), 3.07 (ddd, J = 22.7, 9.5, 5.1 Hz, 1H), 3.74 (d, J = 10.9 Hz, 3H), 3.76 (d, J = 10.9 Hz, 3H), 4.21 (t, J = 7.0 Hz, 1H), 4.57 (s, 1H), 5.30 (s, 1H).

[4-^{2}H]Shikimic Acid (98% ^{2}H). This compound was prepared from [3-^{2}H]**8** as described:[20] ^{1}H NMR (CD_3OD) δ 2.18 (ddt, J = 18.2, 5.8, 1.7 Hz, 1H), 2.69 (ddt, J = 18.2, 5.0, 1.9 Hz, 1H), 3.97 (t, J = 5.3 Hz, 1H), 4.35 (m, 1H), 6.78 (dt, J = 3.8, 1.8 Hz, 1H); ^{2}H NMR (CH_3OH) δ 3.60.

Methyl 5-Hydroxy[2,3,4,5-^{2}H$_4$]cyclohexa-1,3-dienecarboxylate ([2,3,4,5-^{2}H$_4$]20). This compound was prepared from furan-d_4 according to Brion:[51] GC-MS m/z (relative intensity) 158 (M^+, 19), 143 [$(M - CH_3)^+$, 16], 127 [$(M - OCH_3)^+$, 15], 99 [$(M - CO_2CH_3)^+$, 100].

Methyl 5-((*tert*-Butyldimethylsilyl)oxy)[2,3,4,5-^{2}H$_4$]cyclohexa-1,3-dienecarboxylate ([2,3,4,5-^{2}H$_4$]21). To a stirred solution of [2,3,4,5-^{2}H$_4$]**20** (363 mg) in dry DMF (3.2 mL) was added *tert*-butyldimethylsilyl chloride (393 mg) and imidazole (358 mg). After 1 h at room temperature, the reaction mixture was taken up in ether (150 mL), washed with aqueous $NaHCO_3$ (2 × 20 mL) and water (3 × 20 mL) successively, dried ($MgSO_4$), and concentrated. Flash chromatography of the crude product afforded [2,3,4,5-^{2}H$_4$]**21** in 93% yield (587 mg): ^{13}C NMR ($CDCl_3$) δ -4.78, 17.98, 25.55, 31.22, 51.42, 64.61 (t), 122.98 (t), 126.93, 131.20 (t), 134.83 (t), 167.30; ^{2}H NMR ($CHCl_3$) δ 4.47 (1D), 6.09 (2D), 7.02 (1D); GC-MS m/z (relative intensity) 272 (M^+, 0.1), 257 [$(M - CH_3)^+$, 0.3], 241 [$(M - OCH_3)^+$, 0.6, 215 [$(M - {}^tBu)^+$, 12], 75 (100).

Methyl (3*R*,4*S*,5*R*)-3,4-Dihydroxy-5-((*tert*-butyldimethylsilyl)-oxy)[2,3,4,5-^{2}H$_4$]cyclohexenecarboxylate ([2,3,4,5-^{2}H$_4$]22). To a solution of [2,3,4,5-^{2}H$_4$]**21** (1.12 g, 4.15 mmol) in aqueous acetone (water 1.5 mL, acetone 3 mL) was added *N*-morpholine *N*-oxide (486 mg, 4.15 mmol) and OsO_4 solution (0.6 mL of 0.02 N solution in *t*-BuOH). After the solution was stirred for 20 h at room temperature, 1 N HCl (1.5 mL) was added, followed by $Na_2S_2O_3$ (100 mg) and Florisil (1.0 g). The resulting mixture was stirred for an additional 1 h. It was filtered over a Celite pad, and the Celite was washed with acetone (4 × 100 mL). The combined acetone layers were concentrated to about 3 mL, and the residue was extracted with ether (7 × 30 mL). The combined ether extracts were dried ($MgSO_4$), concentrated, and purified by column chromatography (hexane–EtOAc 2:1), to yield 867 mg (70%): mp 69–72 °C; ^{13}C NMR ($CDCl_3$) δ -4.91, -4.61, 17.89, 25.67, 31.22, 51.87, 65.45 (t), 67.74 (t), 71.32 (t), 129.79, 136.01 (t), 166.96; ^{2}H NMR ($CHCl_3$) δ 3.64, 4.04, 4.44, 6.82.

[2,3,4,5-^{2}H$_4$]Shikimic acid (98% ^{2}H). This compound was prepared from [2,3,4,5-^{2}H$_4$]**22** as described:[52] ^{13}C NMR (D_2O) δ 33.22, 68.21 (t), 68.04 (t), 73.50 (t), 132.60, 139.90 (t), 172.93; ^{2}H NMR (H_2O) δ 3.71, 4.00, 4.38, 6.79.

[2,5-^{2}H$_2$]Furan. To dry THF (100 mL) was added 10.0 M BuLi in hexane (44 mL) at -30 °C. Furan (14.5 mL, 0.2 mol) was then added to the BuLi solution at -30 °C, and the resulting solution was stirred at -15 °C for 4 h. The reaction was quenched by addition of D_2O (30 mL) over a 20-min period. Careful distillation by slowly warming the mixture afforded [2,5-^{2}H$_2$]furan (containing a significant amount of THF), which was immediately used for the next reaction without further purification.

[2,5-^{2}H$_2$]Shikimic Acid (75% ^{2}H). This compound was prepared from [2,5-^{2}H$_2$]furan as described earlier: ^{1}H NMR (CD_3OD) δ 2.17 (d, J = 18.5 Hz, 1H), 2.68 (d, J = 18.1 Hz, 1H), 3.66 (m, 1H), 3.98 (dt, J = 8.0, 5.5 Hz, 0.3H), 4.35 (m, 1H), 6.79 (m, 0.3H); ^{2}H NMR (CH_3OH) δ 3.93, 6.77.

Ethyl (1*R*,2*S*)-2-hydroxycyclohexanecarboxylate (23):[28] $[\alpha]^{20}_D$ = +25.8 (c = 0.6 in $CHCl_3$) [lit.[28] $[\alpha]_D$ = +24.25 (c = 1.45 in $CHCl_3$)]; ^{1}H NMR ($CDCl_3$) δ 1.27 (t, 3H, J = 7.1 Hz), 1.25–1.59 (m, 3H), 1.60–1.80 (m, 3H), 1.80–1.98 (m, 2H), 2.48 (ddd, J = 11.2, 3.6, 1.3 Hz, 1H), 3.22 (dd, J = 3.6, 1.3 Hz, 1H), 4.17 (q, J = 7.1 Hz, 2H), 4.24 (s, 1H, OH).

(1*S*,2*S*)-2-Hydroxymethylcyclohexan-1-ol (24). To a suspension of $LiAlH_4$ (1.44 g) in dry THF (60 mL) was added **23** (7.35 g, 42.7 mmol) in THF (15 mL) with ice cooling over a period of 10 min. The reaction mixture was then heated to reflux for 3 h. After that, 10 mL of 3% aqueous NaOH solution was added slowly to the cooled reaction mixture. The mixture was then stirred at room temperature for 20 min and filtered through a Celite pad. The Celite was washed with EtOAc (100 mL), and the combined organic layers were dried (Na_2SO_4), concentrated, and purified by column chromatography to give 4.41 g (79.3%) of **24**: R_f 0.32 ($CHCl_3$–acetone 7:3); ^{1}H NMR ($CDCl_3$) δ 1.21–1.84 (m, 9H), 3.33 (s, 2H, OH), 3.66–3.79 (m, 2H), 4.14 (m, 1H).

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(1*S*,2*S*)-2-((Triphenylmethoxy)methyl)cyclohexan-1-ol (25). To a stirred solution of **24** (2.40 g, 18.5 mmol) in CH_2Cl_2 (50 mL) were added triphenylmethyl chloride (5.65 g, 20.2 mmol), TEA (4.4 mL, 31 mmol), and DMAP (90 mg, 0.74 mmol). After being stirred overnight at room temperature, the reaction mixture was poured into ice–water (40 mL), and the resulting mixture was extracted with CH_2Cl_2 (3 × 40 mL). The combined organic layer was successively washed with saturated NH_4Cl solution (30 mL) and water (30 mL), dried (Na_2SO_4), and evaporated to dryness. Recrystallization of the crude solid from ethanol afforded 4.60 g (67%) of **25**: R_f 0.43 (hexane–EtOAc 8:2).

(1*S*,2*S*)-1-(Toluenesulfonyl)oxy-2-((triphenylmethoxy)methyl)cyclohexane (26). To a stirred solution of **25** (2.0 g, 5.4 mmol) in dry pyridine (8 mL) was added *p*-toluenesulfonyl chloride (1.05 g, 5.6 mmol). After 24 h, 0.50 g of additional *p*-toluenesulfonyl chloride was added, and stirring was continued at room temperature. After a total of 48 h, the reaction mixture was poured into water (50 mL) and extracted with ether (3 × 30 mL). The combined ether layers were dried (Na_2SO_4) and evaporated to dryness. Recrystallization of the crude solid from CH_2Cl_2–pentane afforded 1.59 g (56%) of **26**.

(1*R*,2*R*)-1-((Triphenylmethoxy)methyl)[2-^{2}H]cyclohexane (27). To 540 mg (1.03 mmol) of **26** in a 50-mL round-bottom flask was added under nitrogen 17.2 mL of $LiEt_3B^2H$ (1.0 M in THF). The reaction mixture was stirred overnight at room temperature, quenched with water (10 mL), and extracted with CH_2Cl_2 (2 × 50 mL). The combined organic layers were dried (Na_2SO_4) and evaporated. The crude product containing ((triphenylmethoxy)methyl)cyclohexene (R_f 0.30, hexane) as a contaminant was purified by column chromatography (pentane–cyclohexane–Et_3N 50:50:0.1) to give 186 mg (49%) of **27**: R_f 0.38 (hexane); ^{1}H NMR ($CDCl_3$) δ 1.08–1.48 (m, 5H), 1.75–2.08 (m, 6H), 3.27 (d, J = 6.4 Hz, 2H), 7.26 (t, J = 7.2 Hz, 3H), 7.34 (t, J = 7.2 Hz, 6H), 7.83 (d, J = 7.5 Hz, 6H); GC-MS m/z (relative intensity) 357 (M^+, 7), 243 (Tr^+, 100).

(1*R*,2*R*)-1-Hydroxymethyl[2-^{2}H]cyclohexane (28). To a solution of **27** (186 mg, 0.52 mmol) in THF (3.5 mL) was added 5 drops of concentrated HCl, and the mixture was stirred at room temperature. After 2 days, the reaction mixture was neutralized with solid $NaHCO_3$, filtered, and concentrated. Column chromatography of the crude product afforded 50 mg (83%) of **28**: ^{1}H NMR ($CDCl_3$) δ 0.85–1.75 (m, 9H), 1.90–2.10 (m, 1H), 3.30 (d, J = 6.4 Hz, 2H); GC-MS m/z 97 $(M - H_2O)^+$.

(*R*)-Mandelate Ester of (1*R*,2*R*)-1-Hydroxymethyl[2-^{2}H]cyclohexane (30). A mixture of **28** (50 mg, 0.44 mmol), (*R*)-mandelic acid (67 mg, 0.44 mmol), and *p*-toluenesulfonic acid (8 mg) in benzene (8 mL) was refluxed for 5 h. The reaction mixture was poured into 1 M Na_2CO_3, and the organic layer was separated. The aqueous layer was then extracted with ether (3 × 10 mL), and the combined organic layer was washed with brine (10 mL), dried ($MgSO_4$), and evaporated. Column chromatography of the crude product gave the (*R*)-mandelate ester **30**: ^{1}H NMR (C_6D_6) δ 0.55–0.72 (m, 2H), 1.31–1.42 (m, 2H), 1.42–1.50 (m, 3H), 1.90–2.10 (m, 3H), 3.76 (dd, J = 7.5, 5.8 Hz, 2H), 3.83 (m, 1H), 5.17 (d, J = 5.8 Hz, 1H), 7.06–7.35 (m, 3H), 7.50 (m, 2H); GC-MS m/z (relative intensity) 249 (M^+, 2), 107 (100).

(*S*)-Mandelate ester **29**: ^{1}H NMR (C_6D_6) δ 0.55–0.72 (m, 2H), 1.31–1.42 (m, 2H), 1.42–1.50 (m, 3H), 1.90–2.10 (m, 3H), 3.71 (dd, J = 6.3, 2.9 Hz, 2H), 3.80 (d, J = 5.2 Hz, 1H), 5.11 (d, J = 5.8 Hz, 1H), 7.06–7.35 (m, 3H), 7.50 (m, 2H).

[7-^{13}C]Cyclohex-1-enecarboxylic Acid ([7-^{13}C]31). This compound was prepared from cyclohexanone and sodium [^{13}C]cyanide:[29] ^{1}H NMR ($CDCl_3$) δ 1.64 (m, 4H), 2.24 (m, 4H), 7.14 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 21.3, 22.0, 23.7, 25.9, 129.8 (d, J = 70.4 Hz), 142.4, 173.0 (enhanced signal with small satellites symmetrically arranged, J = 70.4 Hz); GC-MS m/z (relative intensity) 127 (M^+, 33), 109 (31), 81 (100).

[2,6,6-$^2H_3$]Cyclohex-1-enecarboxylic Acid ([2,6,6-$^2H_3$]31). To a hot solution of *p*-tolylhydrazine hydrochloride (3.62 g, 22.8 mmol) in CH_3OD (8 mL) and D_2O (5 mL) was added [2,2,6,6-$^2H_4$]cyclohexanone[31] (2.0 g, 19.6 mmol). The tosylhydrazone immediately precipitated out of solution. The slurry was cooled to 10 °C for 12 h. The solids were collected by suction filtration, rinsed with 60% CH_3OD in D_2O (6 mL), and dried under vacuum at 50 °C for 12 h to give the tetradeuterated tosylhydrazone (4.63 g, 88% yield).

To a solution of the tosylhydrazone (750 mg, 2.78 mmol) in dry tetramethylethylenediamine (8 mL), cooled to −55 °C, was slowly added 1.6 M *n*-butyllithium in hexane (24 mL). The mixture was kept at −78 °C for 1 h and then at room temperature for 12 h. The orange slurry was then cooled to −78 °C and excess carbon dioxide gas was bubbled into the reaction mixture. The mixture was then acidified to pH 3 with 1 N HCl and extracted with ether. The combined ether layers were extracted with a 10% sodium bicarbonate solution. The aqueous layers were cooled and acidified to pH 3 with 3 N HCl and then extracted with ether. The combined ether layers were washed with brine, dried ($MgSO_4$), and concentrated in vacuo. Cromatography (silica gel, hexane–EtOAc 1:1) gave [2,6,6-$^2H_3$]**31** (200 mg, 1.55 mmol, 56%): ^{1}H NMR ($CDCl_3$) δ 1.52–1.67 (m, 4H), 2.19 (m, 1H), 2.33 (m, 1H), 7.05 (m, 0.04H); ^{2}H NMR ($CHCl_3$) δ 2.20 (2D), 7.10 (1D); GC-MS m/z (relative intensity) 129 ($C_7H_7D_3O_2$, 100), 128 ($C_7H_8D_2O_2$, 23), 127 ($C_7H_9DO_2$, 3).

[7-^{13}C]Cyclohex-2-enecarboxylic Acid ([7-^{13}C]32). This compound was prepared from 3-bromocyclohexene and sodium[^{13}C]cyanide:[30] ^{1}H NMR ($CDCl_3$) δ 1.5–2.0 (m, 6H), 3.05 (m, 1H), 5.76 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 20.60, 24.5, 25.0, 40.8 (d, J = 55.0 Hz), 123.6, 130.0, 181.0 (enhanced signal with small satellites symmetrically arranged, J = 55.0 Hz); GC-MS m/z (relative intensity) 127 (M^+, 7), 126 (1), 109 (21), 108 (3), 81 (100).

[7-^{13}C]Cyclohexa-2,5-dienecarboxylic Acid ([7-^{13}C]33). This compound was prepared from [7-^{13}C]benzoic acid under Birch conditions:[33] ^{1}H NMR ($CDCl_3$) δ 2.65 (m, 3H), 5.7–6.0 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 25.8, 41.5 (d, J = 55.8 Hz), 121.6, 126.8, 178.6 (enhanced signal with small satellites symmetrically arranged, J = 55.8 Hz).

[7-^{13}C]Cyclohexa-1,5-dienecarboxylic Acid ([7-^{13}C]34). A solution of [7-^{13}C]**33** (100 mg) and hydroquinone (8.5 mg) in 1 mL of 1 N KOH was heated to 60 °C for 2.5 h, cooled, acidified to pH 3 with 3N HCl, and extracted with ether. The combined ether extracts were washed with brine, dried ($MgSO_4$), and concentrated *invacuo*. Chromatography (silica gel, ether) removed the hydroquinone and gave 82 mg of [7-^{13}C]**34**: ^{13}C NMR ($CDCl_3$) δ 20.9, 23.0, 121.4, 127.3, 129.5 (d, J = 73.2 Hz), 139.5, 170.7 (enhanced signal with small satellites symmetrically arranged, J = 73.2 Hz).

[6-$^2H_2$]Cyclohexa-1,3-dienecarboxylic Acid ([6-$^2H_2$]35). This compound was prepared from 1-acetoxy-1,3-butadiene (622 mg, 5.6 mmol) and [2,3,3-$^2H_3$]acrylic acid (500 mg, 6.7 mmol) according to Blanc[34] to give [6-$^2H_2$]**35** (5mg, 6%): ^{1}H NMR ($CDCl_3$) δ 2.28 (m, 1H), 2.45 (m, 1H), 6.08 (m, 1H), 6.19 (m, 1H), 7.10 (d, 1H); ^{2}H NMR ($CHCl_3$) δ 2.4.

[3,3,4,5,6,6-$^2H_6$]Cyclohexa-1,4-dienecarboxylic Acid ([3,3,4,5,6,6-$^2H_6$]36). This compound was prepared from propiolic acid and 1,3-butadiene-d_6 as described:[35] ^{1}H NMR ($CDCl_3$) δ 7.05 (s); ^{2}H NMR (acetone) δ 2.72 (2D), 2.77 (2D), 5.76 (2D).

[3-^{2}H]Propiolic Acid.[36] A solution of potassium hydrogen acetylenedicarboxylate (5.0 g, 32.86 mmol) in D_2O (11 mL) was refluxed under nitrogen for 2 h. The cooled reaction mixture was acidified with concentrated D_2SO_4 (1mL) in D_2O (2.5 mL), extracted with ether, dried (Na_2SO_4), and evaporated. The residue was then distilled (bp 85–90 °C/120 mmHg) to give [3-^{2}H]propiolic acid (91% deuterated) as a clear colorless oil: ^{13}C NMR (acetone-d_6) δ 75.0, 75.8, 154; GC-MS m/z (relative intensity) 71 (C_3HDO_2, 100), 70 ($C_3H_2O_2$, 10).

[2-^{2}H]Cyclohexa-1,4-dienecarboxylic Acid ([2-^{2}H]36). This compound was prepared from [3-^{2}H]propiolic acid and 1,3-butadiene as described:[35] ^{1}H NMR ($CDCl_3$) δ 2.9 (m, 4H), 5.72 (m, 2H), 7.05 (m, 0.05H); ^{2}H NMR (acetone) δ 6.8.

***trans*-4,5-Dihydroxy[2-^{2}H]cyclohex-1-enecarboxylic Acid ([2-^{2}H]37).** To an ice-cooled solution of [2-^{2}H]**36** (1.5 g, 12 mmol) in ether (5 mL) was added excess diazomethane in ether. Chromatography (hexane–EtOAc 4:1) gave methyl [2-^{2}H]cyclohexa-1,4-dienecarboxylate.

To a stirred solution of methyl [2-^{2}H]cyclohexa-1,4-dienecarboxylate in 96% formic acid (7.5 mL) was slowly added 31% hydrogen peroxide (1.5 mL). The temperature of the reaction mixture was kept below 40 °C by cooling. After the addition was complete, the reaction was stirred at 40 °C for 4 h. The reaction mixture was concentrated, and the light yellow residue was taken up in water (5 mL) and heated on a steam bath for 3 h. After removal of the solvent, the light brown residue was distilled under vacuum (Kugelrohr, oven temperature 170 °C) to give a yellow oil which crystallized upon standing. The product was recrystallized from hot ether to give methyl *trans*-4,5-dihydroxy[2-^{2}H]cyclohex-1-enecarboxylate (400 mg). An additional 200 mg of product was obtained from concentration of the mother liquor: mp 95–96 °C (lit.[35] mp 97 °C).

Methyl *trans*-4,5-dihydroxy[2-^{2}H]cyclohex-1-enecarboxylate (100 mg, 0.58 mmol) was saponified with KOH as described earlier to give solid [2-^{2}H]**37** (68 mg): ^{1}H NMR (CD_3OD) δ 2.20 (m, 2H), 2.65 (m, 2H), 3.7 (m, 2H), 6.82 (m, 0.07H); ^{2}H NMR (CH_3OH) δ 6.8.

Methyl 4-hydroxy[4-^{2}H]cyclohex-1-enecarboxylate ([4-^{2}H]38). A solution of methyl 4-oxocyclohex-1-enecarboxylate ethylene ketal[37] (1.0 g, 5.1 mmol) was dissolved in a mixture of 5 mL of 1 N HCl and 5 mL of THF at 0 °C. The solution was stirred at room temperature for 13 h, whereupon it was diluted with water and extracted with ether. The

extract was dried (Na_2SO_4) and concentrated. Flash chromatography (hexane–EtOAc 4:1) gave 557 mg of a 9:1 mixture of methyl 4-oxo-1-cyclohexenecarboxylate and methyl 4-oxo-2-cyclohexenecarboxylate.

The mixture of keto esters was dissolved in MeOH (4 mL) and added slowly to an ice-cooled solution of $NaBD_4$ (162 mg) in MeOH (5 mL). After the addition was complete, the solution was stirred at room temperature for 2.5 h, and then the reaction was quenched with 1 N HCl. The reaction mixture was concentrated, the residue was diluted with water and extracted with ether, and the extract was dried (Na_2SO_4) and evaporated. Chromatography (hexane–EtOAc 3:2) gave [4-^{2}H]**38** (400 mg, 71%): ^{1}H NMR ($CDCl_3$) δ 1.62 (m, 1H), 1.67 (m, 1H), 1.87 (m, 1H), 2.17 (m, 1H), 2.30 (m, 1H), 2.5 (m, 1H), 3.65, (m, 0.15H), 3.70 (s, 3H), 6.85 (m, 1H).

4-Hydroxy[4-^{2}H]cyclohex-1-enecarboxylic Acid ([4-^{2}H]39). To a solution of [4-^{2}H]**38** (400 mg) in water (60 mL) were added four KOH pellets. The mixture was stirred at room temperature for 24 h and then carefully acidified to pH 4 with 1 N HCl. The aqueous solution was continuously extracted with ether for 72 h. The ether extract was dried (Na_2SO_4) and concentrated to give [4-^{2}H]**39** (220 mg): ^{2}H NMR (DMF) δ 3.82 (s).

Methyl 5-Hydroxy[5-^{2}H]cyclohex-1-enecarboxylate ([5-^{2}H]40). Methyl 5-oxocyclohex-1-enecarboxylate[38] (1.0 g, 6.49 mmol) was reduced with $NaBD_4$ as descibed above to give [5-^{2}H]**40** (717 mg): ^{1}H NMR ($CDCl_3$) δ 1.55 (m, 1H), 1.75 (m, 1H), 2.15 (m, 1H), 2.30 (m, 1H), 2.37 (m, 1H), 2.55 (m, 1H), 3.67 (s, 3H), 6.92 (m, 1H).

5-Hydroxy[5-^{2}H]cyclohex-1-enecarboxylic Acid ([5-^{2}H]41). [5-^{2}H]**40** (400 mg) was saponified with KOH as described above to give [5-^{2}H]**41** (310 mg): ^{1}H NMR δ ($CDCl_3$) 1.54 (m, 1H), 1.78 (m, 1H), 2.11–2.28 (m, 2H), 2.37 (m, 1H), 2.56 (m, 1H), 3.91 (m, 0.21H), 6.91 (m, 1H); ^{2}H NMR δ ($CHCl_3$) 3.86 (s).

[2-^{2}H]Dihydroshikimic Acid ([2-^{2}H]42). To a solution of [2-^{2}H]shikimic acid (27 mg) in MeOH (10 mL) was added 10% palladium on activated carbon (ca. 20 mg). The reaction mixture was shaken under an atmosphere of hydrogen for 20 h. The catalyst was then removed by filtration over Celite, and the filtrate was concentrated to give [2-^{2}H]**42** (6 mg): GC-MS m/z 159 $(M - H_2O)^+$.

(±)-*trans*-3,4-Dihydroxy[2,3,4,5,6-$^2H_5$]cyclohexa-1,5-dienecarboxylic Acid ([2,3,4,5,6-$^2H_5$]43). This compound was prepared from benzoic acid-d_5 as described[39] to give 68% [2,3,4,5,6-$^2H_5$]**43**, 22% [2,3,5,6-$^2H_4$]**43**, and 8% [2,4,5,6-$^2H_4$]- and 2% [2,5,6-$^2H_3$]-3-hydroxybenzoic acid: ^{2}H NMR (acetone) 4.34 (0.9D), 4.43 (1D), 5.92 (1D), 6.22 (1D), 6.85 (1D); EIMS m/z (relative intensity) 161 ($C_7H_3D_5O_4$, M_a^+, 41), 160 ($C_7H_4D_4O_4$, M_b^+, 13), 142 [$C_7H_2D_4O_3$, $(M_a - HOD)^+$, 100], 141 [$C_7H_3D_3O_3$, $(M_b - HOD)^+$, 33].

5-Hydroxy[2,3,4,5-$^2H_4$]cyclohexa-1,3-dienecarboxylic Acid ([2,3,4,5-$^2H_4$]44). To a solution of [2,3,4,5-$^2H_4$]**20** (82 mg, 0.52 mmol) in THF (2 mL) and water (0.3 mL) was added 1 N HCl (0.6 mL). The mixture was stirred at 0–5 °C for 3.5 h and then concentrated. The residue was taken up in water and washed with ether to remove unreacted [2,3,4,5-$^2H_4$]**20**. The aqueous layer was cooled, acidified to pH 3 with 3 N HCl, and extracted with ether. The combined ether layers were dried (Na_2SO_4) and concentrated to give [2,3,4,5-$^2H_4$]**44** (48 mg, 64%) as a white crystalline solid: ^{2}H NMR ($CDCl_3$) δ 4.4 (1D), 6.27 (2D), 7.20 (1D); GC-MS m/z (relative intensity) 126 [$(M - H_2O)^+$, 66], 109 ($C_7HD_4O^+$, 100), 81, ($C_6HD_4^+$, 100).

1-Cyclohexenecarbonyl-D-alanine Benzyl Ester (45). D-Alanine benzyl ester toluenesulfonate[40] (421 mg, 1.2 mmol) was coupled with 1-cyclohexenecarboxylic acid (126 mg, 1.0 mmol) using TEA (121 mg, 1.2 mmol) and DCC (240 mg, 1.2 mmol) in CH_2Cl_2 (10 mL). The resulting solution was stirred at −10 °C for 18 h. The white precipitate was filtered, and the filtrate was successively washed with aqueous $NaHCO_3$ and dilute HCl, dried ($MgSO_4$), and evaporated. Flash chromatography (hexane–EtOAc 4:1) afforded **45** (180 mg, 63%) as a clear oil: ^{1}H-NMR ($CDCl_3$) δ 1.43 (d, J = 6.9 Hz, 3H), 1.5–2.3 (m, 8H), 4.7 (q, 1H), 5.2 (m, 2H), 6.3 (s, 1H), 6.7 (m, 1H), 7.34 (m, 5H); GC-MS m/z (relative intensity) 287 (M^+, 0.8), 152 [$(M - CO_2Bz)^+$, 29], 109 ($C_6H_9CO^+$, 100), 91 (Bz^+, 27).

Methyl *trans*- and *cis*-3-Hydroxy[3-^{2}H]cyclohexanecarboxylate ([3-^{2}H]48, [3-^{2}H]49). A solution of [5-^{2}H]**40** (0.8 g) in EtOH (30 mL) was hydrogenated as described earlier. Chromatography (hexane–EtOAc 3:1) gave [3-^{2}H]**48** (222 mg) and [3-^{2}H]**49** (294 mg).

[3-^{2}H]**48**: R_f 0.41 (hexane–EtOAc 1:1); ^{1}H NMR ($CDCl_3$) 1.45–1.85 (m, 8H), 2.74 (tt, J = 11.0, 3.5 Hz, 1H), 3.63 (s, 3H), 4.02 (m, 0.1H); ^{2}H NMR ($CHCl_3$) δ 4.01 (s).

[3-^{2}H]**49**: R_f 0.49 (hexane–EtOAc 1:1); ^{1}H NMR ($CDCl_3$) 1.15–1.45 (m, 4H), 1.78–1.96 (m, 3H), 2.16 (dm, 1H), 2.34 (tt, J = 12.0, 3.5 Hz, 1H), 3.58 (m, 0.1H), 3.65 (s, 3H); ^{2}H NMR ($CHCl_3$) δ 3.51 (s).

***trans*-3-Hydroxy[3-^{2}H]cyclohexanecarboxylic Acid ([3-^{2}H]50).** To a solution of [3-^{2}H]**48** (222 mg) in water (15 mL) were added three NaOH pellets. The solution was stirred at room temperature for 24 h. The resulting solution was washed with ether (10 mL), and the aqueous layer was acidified with 6 N HCl. After the solution was saturated with NaCl, the liberated acid was extracted with ether and dried ($MgSO_4$). Removal of the solvent left [3-^{2}H]**50** (183 mg): ^{1}H NMR (acetone-d_6) δ 1.41–1.61 (m, 4H), 1.64–1.85 (m, 4H), 2.74 (tt, J = 12.1, 3.5 Hz, 1H), 3.97 (m, 0.1H); ^{2}H NMR (acetone) δ 3.93.

***cis*-3-Hydroxy[3-^{2}H]cyclohexanecarboxylic Acid ([3-^{2}H]51).** [3-^{2}H]**49** (294 mg) was saponified as described above to give [3-^{2}H]**51** (224 mg): ^{1}H NMR (acetone-d_6) δ 1.07–1.41 (m, 4H), 1.73–1.93 (m, 3H), 2.15 (dm, J = 12.4 Hz, 1H), 2.32 (tt, J = 12.1, 3.5 Hz, 1H), 3.47 (m, 0.1H); ^{2}H NMR (acetone) δ 3.47.

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