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Cho, Hyeongjin, Ph.D.
The Ohio State University, 1990
STUDIES IN ANTIBIOTICS BIOSYNTHESIS
PART 1: ANSATRIENIN
PART 2: REDUCTIOMYCIN

DISSERTATION

Presented in Partial Fulfillment of
the Requirements for the Degree of Doctor
of Philosophy in the Graduate School of
The Ohio State University

By

Hyeongjin Cho, M.S.

* * * * *

The Ohio State University
1990

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To my parents and my wife
ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

DMF N,N-dimethylformamide
DMSO dimethyl sulfoxide
FT-IR Fourier transform - infrared
GC-MS gas chromatography - mass spectrometry
H-C HETCOR proton, carbon heteronuclear correlation spectroscopy
J coupling constant
NMR nuclear magnetic resonance
PCC pyridinium chlorochromate
THF tetrahydrofuran
TLC thin layer chromatography
UV ultraviolet
PART 1: ANSATRIENIN
CHAPTER I
INTRODUCTION

The ansatrienins (mycotrienins) represent a new type of benzoquinoid ansamycin antibiotics containing a 21-membered polyene macrolide. Since the first ansatrienin was isolated from Streptomyces sp. ACC 1293, in 1965 as triene MM₈¹, several other macrocyclic lactams which appear to be identical with ansatrienin B have been reported²,³. Recently, the ansatrienins were also isolated from Streptomyces collinus⁴ and Streptomyces rishirienis⁵. The two main products are ansatrienin A (=mycotrienin I) and ansatrienin B (=mycotrienin II) interconvertible via a redox reaction. The structures of ansatrienin A and ansatrienin B were established independently by the groups of Zeeck⁶ and Otake⁷,⁸ largely on the basis of NMR spectroscopy. A minor difference between the structures published by the two groups regarding the stereochemistry of the alanine moiety has been resolved in favor of the D configuration reported by the Japanese group⁹. Several minor components have also been reported. Otake et al.¹⁰ isolated mycotrienol I and II in which the cyclohexanecarbonyl-alanine ester group at C-11 in
Figure 1. Structures of ansatrienin
ansatrienin A and B is replaced by a hydroxyl function. The 22-0-methyl ether of mycotrienine II and 19-deoxymycotrienin II (=trienomycin A) were also reported by the same group\textsuperscript{11}. The congeners ansatrienin A\textsubscript{2} and A\textsubscript{3}\textsuperscript{12} carry a 2- and a 3-methylbutyryl moiety respectively in place of the cyclohexanecarbonyl group and ansatrienin A\textsubscript{4}\textsuperscript{13} contains a cyclohexene moiety instead of a cyclohexane ring.

The ansatrienins show pronounced activity against fungi and yeasts but little antibacterial activity\textsuperscript{5}. The trienomycins\textsuperscript{11,14-19}, also isolated from a \textit{Streptomyces} species, have all the structural features of ansatrienin B but lack the 19-hydroxyl group. However, the trienomycins have no antimicrobial activity against bacteria, fungi and yeasts, but do show cytocidal and antitumor activities stronger than the ansatrienins\textsuperscript{18}. The only structural difference between the trienomycins and the ansatrienins is the 19-oxygen atom; therefore it seems reasonable to believe that this feature must determine the differences in biological activity.

The ansamycin antibiotics represent a class of natural products in which an aromatic nucleus is combined with a branching aliphatic side chain\textsuperscript{20}. The aromatic moiety can be either benzenic or naphthalenic and the
length of the aliphatic ansa chain varies mostly from 15 to 23 carbons. The structural differences result in considerable differences in biological properties. However, in every case, one of the aliphatic-aromatic junctions is an amide bond.

Recent studies on the biosynthesis of ansatrienin A, a metabolite of *Streptomyces collinus* \(^\text{13}\) and *Streptomyces rishiriensis* \(^\text{8,10}\), established the building blocks of ansatrienin as shown in Figure 2. The work of Otake et al. \(^\text{8,10}\) demonstrated that carbon atoms 1-16 of ansatrienin, representing the ansa bridge, are derived from two propionate and six acetate units by a polyketide-type biosynthesis. As with most ansamycin antibiotics, the aliphatic chain is biosynthesized through the condensation, in an appropriate order, of 2-methylmalonyl and malonyl-CoA units. The methoxy group at C-3 is derived from methionine.

The seven carbon unit carrying a nitrogen (m-C\(_7\)N unit) in the chromophore is not derived from the acetate/propionate pathway \(^\text{21,22}\). The m-C\(_7\)N unit, which is also found in a variety of other natural products, consists of a six-membered carbocycle plus one carbon atom and a nitrogen attached meta to each other on the ring.
Figure 2. Biosynthetic origin of ansatrienin (mycotrienin)
origin of the \( m-C_{7}N \) unit has been traced to the shikimate pathway. The labeling pattern of the \( m-C_{7}N \) unit in ansatrienin A biosynthesized from \([U-^{13}C]glycerol\) supports this notion\(^{22}\). The formation of the \( m-C_{7}N \) unit is considered to branch off early in the shikimate pathway because shikimic acid itself is not incorporated into the \( m-C_{7}N \) unit but served as an efficient precursor for the cyclohexanecarboxylic acid residue\(^{23}\). The \( m-C_{7}N \) unit was found to originate from 3-amino-5-hydroxybenzoic acid which is derived via the shikimate pathway\(^{13}\). Very little is actually known about how 3-amino-5-hydroxybenzoic acid is formed by the shikimate pathway, but the branch point should be at a very early stage, possibly the very first reaction. Recently Floss and coworkers\(^{24}\) proposed a pathway to 3-amino-5-hydroxybenzoic acid which envisions 4-amino-3,4-dideoxy-D-arabinohexulonic acid 7-phosphate as the key intermediate.

Wu et al.\(^{13}\) suggested that D-alanine is a more immediate precursor of the alanine moiety of ansatrienin than the L isomer. Feeding experiments with \(^{15}N\)-labeled D- and L-alanine showed that feeding D-[\(^{15}N\)]alanine resulted in more than twice the enrichment in the cyclohexanecarbonyl-alanine moiety than did feeding of the L-isomer. This is consistent with the established D
configuration of the alanine moiety of ansatrienin\textsuperscript{8,9}. Further studies have shown that the cyclohexanecarbonylalanine side chain was formed by stepwise attachment of first alanine and then the acid moiety to the macrocycle rather than preassembly of cyclohexylcarbonylalanine. The latter, doubly labeled with \textsuperscript{13}C and \textsuperscript{15}N in the amide linkage, was incorporated only after hydrolysis not as an intact unit\textsuperscript{13}.

The cyclohexanecarboxylic acid moiety in ansatrienin originates from the shikimate pathway but the exact mode of formation of the fully reduced hydroaromatic ring system is not well understood. Cyclohexane rings are also found in another antibiotic, asukamycin, isolated from \textit{Streptomyces nodosus} var. \textit{asukaensis}\textsuperscript{25,26} and in the \(\omega\)-cyclohexyl fatty acids of thermophilic and other bacteria\textsuperscript{27-30}, e.g., \(\omega\)-cyclohexylundecanoic acid. In ansatrienin the cyclohexane ring is present as a cyclohexanecarboxylic acid which is linked via a D-alanine residue to the macrocyclic lactam ring, whereas in asukamycin, as in the \(\omega\)-cyclohexyl fatty acids, the cyclohexane ring is attached to the end of a hydrocarbon chain. It seemed likely that in both the latter cases the cyclohexane ring also originates from cyclohexanecarboxylic acid which, in the form of its CoA ester, serves as the starter unit for
a polyketide chain. The experimental evidence supports this notion, both in the case of the \(\omega\)-cyclohexyl fatty acids and in that of asukamycin. For example, feeding experiments with \(^{13}\text{C}\)-labeled acetate and malonate clearly established the polyketide origin of the two olefinic hydrocarbon chains in asukamycin\(^3\). Cyclohexane[carboxy-\(^{14}\text{C}\)]carboxylic acid was incorporated into both asukamycin\(^3\) and ansatrienin\(^2\); degradation of labeled ansatrienin showed that essentially all the label was confined to the cyclohexanecarbonyl moiety. Further studies showed that the cyclohexane rings in both asukamycin and ansatrienin are derived via the shikimate pathway and not by reduction of a benzene ring\(^2,\(^3\). Benzoic acid was not a precursor of the cyclohexanecarbonyl moiety but the partially reduced systems 2,5-dihydro- and 1,4-dihydrobenzoic acid, and cyclohexanecarboxylic acid itself were incorporated. Degradation of ansatrienin derived from \([U-^{14}\text{C}]\)shikimic acid showed that all seven carbon atoms of the precursor are incorporated to give rise to the seven carbon atoms of cyclohexanecarboxylic acid. Consistent with this finding was the inefficient incorporation of 2,5-dihydrophenylalanine\(^1\).

The biosynthesis of the cyclohexanecarboxylic acid raises the question of the stereochemistry of processing
Figure 3. Formation of the N-(cyclohexylcarbonyl)alanine moiety of ansatrienin (mycotrienin) from shikimic acid.
of intermediates in the pathway from shikimic acid, i.e.,
the cryptic stereochemistry of formation of the symmetri-
cal cyclohexane ring. Casati et al. synthesized samples
of D-(-)-[2-\(^{13}\)C]shikimic acid and fed this material to
Streptomyces collinus. \(^{13}\)C-NMR analysis of the resulting
ansatrienin A showed a single enhanced signal indicating
about 4.8\% \(^{13}\)C enrichment at C-32 or C-36. To determine
the absolute stereochemistry of this process, i.e.,
whether (-)-[2-\(^{13}\)C]shikimic acid stereospecifically
labeled C-32 or C-36 in the cyclohexanecarboxylic acid
moiety, they degraded the \(^{13}\)C-labeled ansatrienin A to
cyclohexylmethanol and derivatized it with (S)-(+)-
mandelic acid to give 3 (Figure 4). The \(^{13}\)C-NMR spectrum
of the corresponding unlabeled compound showed two signals
at 29.33 ppm and 29.38 ppm for C-2 and C-6 of the
cyclohexane ring; in the derivative obtained from the
labeled ansatrienin A only the signal at 29.38 ppm was
enhanced. This experiment confirmed that shikimic acid
was processed stereospecifically in the conversion to
cyclohexanecarboxylic acid. An unambiguous assignment of
the \(^{13}\)C NMR signals at 29.33 ppm and 29.38 ppm was made by
synthesizing an authentic sample of (1R,2R)-[2-\(^{2}\)H]cyclo-
hexylmethanol. Analysis of the (R)- and (S)-mandelate
esters of this sample revealed that in the (S)-mandelate
derivative the higher field signal (29.33 ppm) originates
Figure 4. Stereochemical fate of D-(-)-[2-\textsuperscript{13}C]\textit{shikimic} acid in the conversion to the cyclohexylcarbonyl moiety of ansatrienin.
from C-2 (the pro-R carbon) and the lower field signal (29.38 ppm) from C-6 (the pro-S carbon). Based on these results Casati et al. concluded that C-2 of shikimic acid gives rise to C-6 of the cyclohexanecarboxylic acid moiety of ansatrienin A.

A similar conclusion had been drawn by Furukawa et al. on the biosynthesis of the cyclohexane ring in 11-cyclohexylundecanoic acid. They fed D-[6-2H]glucose to Curtobacterium pusillum and degraded the resulting fatty acid to cyclohexanecarboxylic acid. This was further derivatized by reaction with phenyllithium to give cyclohexylphenyl ketone, followed by asymmetric reduction with (-)-N-methylephedrin/N-ethylaniline/LiAlH₄ to give (S)-α-cyclohexylbenzyl alcohol. NMR analysis of the latter revealed that the axial hydrogen at C-6 was the primary site of labeling with 66% deuterium enrichment. The two hydrogens at C-2 each contained 5% deuterium and the remaining positions less the 2% each (Figure 5). 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.
Figure 5. Stereochemical analysis of cyclohexane ring formation in \( \omega \)-cyclohexylundecanoic acid biosynthesis.
Their finding agrees with the result of Casati et al. in that C-2 of shikimic acid gives rise to C-6 of the cyclohexanecarboxylic acid moiety of ansatrienin A. However, their interpretation that this represents a direct double bond reduction of shikimic acid on the Re face is contradicted by the observation of Floss and coworkers that the small amount of 1-cyclohexenecarboxylic acid accompanying the cyclohexanecarboxylic acid in the hydrolysis of the biosynthetic sample of ansatrienin A carried the $^{13}$C label not at C-2 but at C-6, i.e., the double bond migrates in the ring. This notion is also supported by the observation that (6R,S)-(-)-[6-$^2$H$_1$]-shikimic acid gave no detectable incorporation into ansatrienin A, indicating that there had been loss of both methylene hydrogens. Therefore, the reaction sequence in the formation of ansatrienin A is considered to be more complex than the direct reduction of the carbon-carbon double bond of shikimic acid as proposed by Furukawa et al. in the 11-cyclohexylundecanoic acid case.

The objective of the research presented in this thesis was to further examine the conversion of shikimic acid to cyclohexanecarboxylic acid in ansatrienin. One way to achieve that is to follow the fate of all hydrogen atoms on the ring of shikimic acid. Therefore, we decided
to synthesize samples of shikimic acids specifically labeled with deuterium at various positions and to feed this material to *Streptomyces collinus*. Syntheses of (-)-[2-^2H]−, (-)-[3-^2H]−, (-)-[4-^2H]−, (±)-[2,5-^2H₂], and (±)-[2,3,4,5-^2H₄]shikimic acid are described as well as the results of feeding experiments with those precursors. Analysis of the resulting ansatrienin would show which hydrogens are retained and would reveal the stereochemical result of processing of shikimic acid and intermediates in the pathway. With these results in hand the choices for the biosynthetic pathway from shikimic acid to cyclohexanecarboxylic are reduced to a limited number of possible routes.
A. Synthesis of Labeled Precursors

1. Synthesis of (-)-[2-²H]shikimic acid

Recently, Fleet et al.34 reported the preparation of the natural isomer of shikimic acid from D-mannose in 39% yield. Their scheme for the enantiospecific synthesis of shikimic acid is shown in Figure 6. Acetonation of D-mannose gave 2,3:5,6-di-O-isopropylidene-α-D-mannofuranose which, in turn, can be converted to benzyl 2,3-O-isopropylidene-α-D-lyxofuranoside without isolation of any intermediates. The lyxo alcohol(8) was esterified with trifluoromethanesulfonic anhydride to give the trifluoromethanesulfonate(9). Alkylation of the sulfonate(9) with the sodium salt of t-butyl trimethoxyphosphorylacetate followed by hydrogenolysis of the benzyl protecting group afforded the phosphonate(11). The latter was converted to methyl 3,4-O-isopropylideneshikimate(12) by an intramolecular Wadsworth-Emmons olefination. The synthesis of (-)-shikimic acid was completed by removal of both protecting groups with 60% aq. CF₃COOH.
Figure 6. Scheme for the synthesis of (-)-D-shikimic acid by Fleet et al.

a) acetone, H⁺  b) BzCl, NaH  c) HCl, aq. MeOH

d) NaIO₄  e) NaBH₄  f) (CF₃SO₂)₂O, py.

g) t-BuOC(O)CHP(O)(OMe)₂, NaN  h) H₂, Pd/C

i) NaN  j) 60% aq. CF₃COOH
The procedure of Fleet et al. attracted our attention because it not only gives excellent yield but also permits easy access to isotope labeling at various positions of shikimic acid. We adopted their procedure to synthesize shikimic acid in optically pure form. The only modification of the original procedure was the debenzylation step (10 → 11). We removed the benzyl protecting group of 10 by treatment with HCOONH₄ and Pd/C instead of using Pd/C and hydrogen gas, just for practical convenience. Oxidation of 2,3:5,6-di-O-isopropylidene-α-D-mannofuranose(4) with DMSO and acetic anhydride afforded lactone(13) in 81% yield (Figure 7). Reduction of the lactone with NaBD₄ gave the anomeric mixture (α/β = 9:1) of [1-²H]-2,3:5,6-di-O-isopropylidene-D-mannofuranose(14) in 52% yield. The latter was converted to [2-²H]shikimic acid by the procedure of Fleet et al. as described above.

![Figure 7. Scheme for introduction of deuterium at C-2 of 4](image)

- a) DMSO, Ac₂O
- b) NaBD₄
Figure 8. $^1$H-NMR spectrum of [2-$^2$H]shikimic acid
2. Synthesis of (-)-[3-2H]shikimic acid

Zamir and Luthe\textsuperscript{35} reported a procedure for the synthesis of shikimic acid labeled with deuterium at C-3. The detailed synthetic steps are shown in Figure 9. Their approach consists of generating the protected 3-keto species\textsuperscript{(18)}. Reduction of the latter with NaBD\textsubscript{4} introduces deuterium at C-3 position but this operation produces the unwanted stereochemistry. Therefore it was necessary to invert the stereochemistry at C-3 before removing the protecting groups. We followed their procedure in exactly the same way to obtain samples of [3-2H]shikimic acid albeit in low yield.

3. Synthesis of (-)-[4-2H]shikimic acid

The only previous synthesis of [4-2H]shikimic acid was also reported by Zamir and Luthe\textsuperscript{35}. They used nonlabeled (-)-shikimic acid as a starting material. Shikimate ester, with protecting groups at C-3 and C-5, was oxidized and reduced with NaBD\textsubscript{4} to introduce deuterium at the C-4 position. The hydroxyl function at C-4 is resistant to oxidation and gives only 12\% combined yield for the oxidation-reduction steps. The overall yield was not specified in their report but their procedure was reproduced in our laboratory in less than 1\% yield. Starting from 10g of (-)-shikimic acid we were not able to
Figure 9. Scheme for the synthesis of (-)-[3-\(^2\)H]-shikimic acid by Zamir and Luthe
a) MeOH, H\(^+\)  b) HC(O\(\text{Me}\))\(_3\), TsOH
  c) t-butyldimethylsilyl chloride, imid.
  d) MeOH/H\(_2\)O/HCl=100:1:0.1  e) PDC
  f) Ac\(_2\)O, DMAP  g) NaBD\(_4\)  h) MsCl, Et\(_3\)N
  i) KOAc, MeOH  j) MeOH/H\(_2\)O/AcOH=30:10:1
  k) Ac\(_2\)O, DMAP  l) n-Bu\(_4\)NF  m) OH\(^-\); H\(^+\)
Figure 10. $^1$H-NMR spectrum of [3-$^2$H]shikimic acid
produce enough material for a feeding experiment. Furthermore, none of the many reported syntheses of non-labeled shikimic acid lend themselves to easy introduction of deuterium at the C-4 position. We therefore decided to develop our own procedure modeled after Fleet's synthesis\textsuperscript{34} (Figure 12). Our first target was 31 in which the hydroxyl group at C-3 has a stereochemistry opposite to Fleet's intermediate 11. This compound is advantageous in that the inversion of stereochemistry and deuterium incorporation at C-3 can be achieved by successive oxidation and reduction with NaBD\textsubscript{4}. D-Arabinose can be conveniently converted into its furanose form in four sequential reactions\textsuperscript{36,37} to get 26 (Figure 11). Benzylation of 26 followed by treatment with 4% sodium amalgam afforded 28 in 90% yield. The alcohol(28) was esterified with (CF\textsubscript{3}SO\textsubscript{2})\textsubscript{2}O in the presence of pyridine to give the trifluoromethanesulfonate(29) in quantitative yield. Alkylation of 29 with the sodium salt of t-butyl dimethoxyphosphorylacetate in DMF in the presence of 15-crown-5 afforded a diastereomeric mixture of phosphonates (30) in 72% yield. The benzyl protecting group was removed by treatment of 30 with HCOONH\textsubscript{4} and Pd/C in quantitative yield. The hydroxyl group of 31 was oxidized by heating with 1.5 equivalents of P\textsubscript{2}O\textsubscript{5} and 3 equivalents of DMSO in DMF at 70°C for 1.5h, at which time the OH
Figure 11. Scheme for the conversion of D-arabinose to 1,2-O-isopropylidene-5-O-toluenesulfonyl-D-arabinofuranose (26)
a) EtSH, HCl  b) TsCl, py.  c) CdCO₃, HgCl₂, ag. acetone  d) acetone, H₂SO₄, CuSO₄
Figure 12. Scheme for the synthesis of (−)-[4-2H]-shikimic acid

a) BzBr, NaH  b) 4% Na(Hg)  c) (CF$_3$SO$_2$)$_2$O, pyridine  d) t-BuOC(O)CHP(O)(OMe)$_2$, NaH  
e) HCOONH$_4$, Pd/C, MeOH  f) P$_2$O$_5$, DMSO, DMF, 70 C  g) NaBD$_4$, h) acetone, Dowex-50W(H$^+$)  
i) NaH  j) 60% aq. CF$_3$COOH
Figure 13. $^1$H-NMR spectrum of [4-\textsuperscript{2}H]shikimic acid
stretching peak in the IR spectrum had disappeared completely and a new carbonyl peak had appeared. The ketone (32) was then reduced with NaBD$_4$ to generate alcohol 33 (71% yield from 31). The isopropylidene protecting group could be rearranged by treatment of 33 in acetone with acidic resin at room temperature for 3h to give [3-$^2$H]-11 in 88% yield. Conversion of the latter to shikimic acid could then be effected by the procedure reported by Fleet et al.$^{35}$

4. Synthesis of (±)-[2,5-$^2$H$_2$]shikimic acid

Shikimic acid deuterated at C-2 and C-5 was prepared in its racemic form by a modification of the procedure of Campbell et al.$^{38}$ (Figure 14). These workers employed the Diels-Alder adduct of furan and methyl acrylate as a starting material to obtain (±)-methyl shikimate. According to their scheme, C-2 and C-5 of furan give rise to the C-2 and C-5 positions of the resulting shikimic acid. Therefore, [2,5-$^2$H$_2$]furan should be a precursor of [2,5-$^2$H$_2$]shikimic acid. [2,5-$^2$H$_2$]Furan was obtained by treatment of furan with 2 equivalents of butyl lithium followed by quenching with D$_2$O. Base-catalyzed ring opening$^{39}$ of the Diels-Alder adduct$^{39}$, obtained by warming the mixture of deuterated furan and methyl acrylate in the presence of zinc iodide, afforded dienol 35. Catalytic
Figure 14. Scheme for the synthesis of $(\pm)$-[2,5-\(^2\)H\(_2\)] and $(\pm)$-[2,3,4,5-\(^2\)H\(_4\)] shikimic acid by modification of the procedure by Campbell et al.

- a) methyl acrylate, ZnI\(_2\)
- b) LiN(SiMe\(_3\))\(_2\)
- c) t-butyldimethylsilyl chloride, py.
- d) OsO\(_4\), N-morpholine-N-oxide
- e) n-BuNF
- f) OH\(^-\); H\(^+\)
Figure 15. $^1$H-NMR spectrum of [2,5-$^2$H$_2$]shikimic acid
Figure 16. $^2$H-NMR spectrum of [2,3,4,5-²H₄]shikimic acid
osmylation (OsO₄, N-morpholine N-oxide) of the t-butyl-dimethylsilyl ether of the dienol(35) produced diol 37 with complete regio- and stereochemistry. The synthesis of deuterated shikimic acid was then completed by desilylation and alkaline hydrolysis.

5. Synthesis of (±)-[2,3,4,5-²H₄]shikimic acid

The same procedure as for [2,5-²H₂]shikimic acid was repeated except that commercially available furan-d₄ was employed as starting material (Figure 14).

B. Fermentation

The biosynthesis of ansatrienin A was studied in feeding experiments using shake cultures of Streptomyces collinus Tü 1892. Optimal conditions for the fermentation of Streptomyces collinus Tü 1892 and for the feeding of shikimic acid had been established earlier by Dr. Rosangela Casati and these were used for the present feeding experiments. Both the seed and the production medium contained full fat soy been meal, 2.0g; mannitol, 2.0g; tap water, 100mL; pH 7.3. Samples of labeled shikimic acid were fed to 24h old production cultures which were harvested 48h later. The mixture of ansatrienins as extracted was treated with aqueous FeCl₃ solution to convert ansatrienin B to ansatrienin A. After
column chromatography about 30mg of ansatrienin A was obtained from 1L of production culture.


The 2H-NMR spectrum of ansatrienin A biosynthesized from [2-2H]shikimic acid showed a signal at δ1.41 (Figure 19). It is already known that C-2 of shikimic acid labels ansatrienin A exclusively at C-36, and the 13C-NMR spectrum of the corresponding unlabeled ansatrienin A showed the signal for C-36 at δ29.36. Unambiguous assignments of the 13C-NMR spectrum were made by the groups of Otake and Floss. The stereochemical assignment was made based on the H-C HETCOR spectrum of nonlabeled ansatrienin A (Figure 18). The H-C HETCOR shows two cross peaks for C-36 indicating the correlation between C-36 and two hydrogens attached to it. The upfield signal (δ1.31-1.47) corresponds to the axial(36R) hydrogen and the lower field signal (δ1.73-1.92) represents the equatorial(36S) hydrogen. Therefore the observation of a 2H-NMR signal at δ1.41 for ansatrienin A obtained by feeding [2-2H]shikimic acid shows that the deuterium from C-2 of shikimic acid occupies the 36R(axial) position in the cyclohexanecarboxylic acid moiety of the resulting ansatrienin A. This result is consistent with the report by Furukawa et al.33
Figure 17. Labeling pattern of the cyclohexanecarboxylic acid moiety in ansatrienin from shikimic acid

Table 1. $^2$H-NMR Analysis of ansatrienin A samples obtained from feeding experiments with $^2$H-labeled shikimic acids

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$^2$H-NMR signal (ppm)</th>
<th>Labeled hydrogen(s) in product</th>
</tr>
</thead>
<tbody>
<tr>
<td>[2-$^2$H]-1</td>
<td>1.41</td>
<td>pro-36R</td>
</tr>
<tr>
<td>[3-$^2$H]-1</td>
<td>1.74</td>
<td>pro-35R</td>
</tr>
<tr>
<td>[4-$^2$H]-1</td>
<td>1.66</td>
<td>34E</td>
</tr>
<tr>
<td>[2,5-$^2$H$_2$]-1</td>
<td>1.21, 1.42</td>
<td>pro-33R, pro-36R</td>
</tr>
<tr>
<td>[2,3,4,5-$^2$H$_4$]-1</td>
<td>1.21, 1.39, (1.66), 1.73</td>
<td>pro-33R, 34E, pro-35R, pro-36R</td>
</tr>
<tr>
<td>(6R,S)-[6-$^2$H$_2$]-1</td>
<td>No incorporation</td>
<td></td>
</tr>
</tbody>
</table>
Figure 18. H-C HETCOR of ansatrienin A
on the biosynthesis of \( \omega \)-cyclohexylundecanoic acid in *Curtobacterium pusillum*. They found that D-[6-\( ^2 \)H]glucose labeled the axial hydrogen at C-6 of the resulting fatty acid to the extent of 66\% while other positions carried less than 5\% deuterium. Assuming that the pathways in the two organisms are the same, our result provides substantiation for their otherwise unfounded assumption that the high level of deuterium enrichment at C-2 of \( \omega \)-cyclohexyl-undecanoic acid originated from C-2 of shikimic acid.

Deuterium from both C-3 and C-4 of shikimic acid was effectively incorporated and occupied the 35R (equatorial) and 34E(trans, equatorial) positions, respectively, in the resulting ansatrienin A (Figure 17, Table 1). The \( ^2 \)H-NMR spectra of ansatrienin A obtained by feeding [3-\( ^2 \)H]- and [4-\( ^2 \)H]shikimic acid showed signals at 61.75 and 61.66, respectively (Figure 20 and 21).

Two atoms of deuterium from [2,5-\( ^2 \)H\(_2\)]shikimic acid were incorporated giving ansatrienin A which displayed two \( ^2 \)H-NMR signals at 61.21 and 61.41 (Figure 22). The signal at 61.21 must originate from deuterium at C-5 of shikimic acid because in the earlier experiment (see above) ansatrienin A from [2-\( ^2 \)H]shikimic acid had shown a signal at 61.41.
Ansatrienin A from the feeding experiment with $[2,3,4,5-^{2}\text{H}_4]$shikimic acid showed the labeling pattern expected based on the results of the experiments with the appropriate mono or dideuterated shikimic acids as shown in Table 1 except that the signal at 1.66 ppm is obscured by the signal at 1.73 ppm (Figure 23). To confirm the retention of all four deuterium atoms the ansatrienin A sample obtained from the feeding experiment with $[2,3,4,5-^{2}\text{H}_4]$shikimic acid was degraded to cyclohexanecarboxylic acid. This was then derivatized to its methyl ester and analyzed by GC-MS (Table 2). Significant enhancement of

<table>
<thead>
<tr>
<th>degradation product</th>
<th>Methyl cyclohexane-carboxylate</th>
<th>Methyl cyclohex-1-ene-carboxylate</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/z</td>
<td>142 143 144 145 146</td>
<td>140 141 142 143 144</td>
</tr>
<tr>
<td>Nonlabeled 1</td>
<td>100 9.8 0.8 0 0</td>
<td>100 8.6 0.8 0 0</td>
</tr>
<tr>
<td>Labeled 1 a)</td>
<td>100 9.2 0.6 1.3 7.2</td>
<td>100 12.9 1.0 1.0 5.3</td>
</tr>
</tbody>
</table>

a) Ansatrienin A obtained from feeding experiments with $(\pm)$-$[2,3,4,5-^{2}\text{H}_4]$shikimic acid
the (M+4)$^+$ peak for both methyl cyclohexanecarboxylate and methyl cyclohex-1-enecarboxylate established that all four deuterium atoms of [2,3,4,5-$^{2}$H$_4$]shikimic acid were retained and the signal expected at 1.66 ppm is indeed obscured by the neighboring signal in the $^{2}$H-NMR spectrum (Figure 23). The result of the mass spectral analysis also rules out the possibility of intermolecular transfer of deuterium in the biosynthetic pathway.
Figure 19. $^2$H-NMR spectrum of ansa'trienin A from feeding experiment with (−)-[2-$^2$H]shikimic acid
Figure 20. $^2$H-NMR spectrum of ansatrienin A from feeding experiment with (−)-[3-$^2$H]shikimic acid
Figure 21. $^2$H-NMR spectrum of ansatrienin A from feeding experiment with (-)-[4-$^2$H]shikimic acid.
Figure 22. $^2$H-NMR spectrum of ansatrienin A from feeding experiment with (±)-[2,5-$^2$H$_2$]shikimic acid
Figure 23. $^2$H-NMR spectrum of ansatrienin A from feeding experiment with ($\pm$)-[2,3,4,5-$^2$H$_4$]shikimic acid
A. Discussion on the synthesis of labeled precursors

An enzymatic preparation of [2-²H]shikimic acid has previously been reported by Haslam and coworkers⁴¹. According to their procedure C-2 was the major incorporation site, but the C-4 position was also enriched with deuterium to some extent. Above all their procedure needs a long reaction time and the preparation of enzymes involved in the synthesis. Our procedure, on the other hand, afforded shikimic acid with deuterium labeling exclusively at C-2 position in good yield. The previous approach⁴²,⁴³ for [3-²H]shikimic acid synthesis was to prepare 3-dehydroshikimic acid as a protected or an 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 [3-²H]shikimic acid and [3-²H]-3-epishikimic acid⁴². A tedious operation to separate them makes this procedure less valuable for practical purposes. Zamir and Luthe³⁶ prepared a 3-dehydroshikimate ester with protecting groups at C-4 and C-5. Reduction of the latter with
NaBD4 produced the unwanted stereochemistry at C-3. Therefore it was necessary to invert the stereochemistry at C-3 before removing the protecting groups. This fact and the low yield are drawbacks of this procedure, but we followed their procedure in exactly the same way to obtain samples of [3-^2H]shikimic acid. The only previous synthesis of [4-^2H]shikimic acid was also reported by Zamir and Luthe^36. Shikimate ester, with protecting groups at C-3 and C-5, was oxidized and reduced with NaBD4 to introduce a deuterium at the C-4 position. As described earlier, the hydroxyl function at C-4 is resistant to oxidation and gives only 12% combined yield for the oxidation-reduction steps. The overall yield was too low (<1%) to obtain enough material for a feeding experiment. Our procedure, on the other hand, produced [4-^2H]shikimic acid in 30% overall yield starting from 26, which can be conveniently prepared from D-arabinose in 4 sequential reactions^37. Many of the reactions can be carried out without purification of intermediates and this fact, together with the excellent yield, is an advantage of our procedure. The preparation of shikimic acid specifically labeled with deuterium at C-5 is not unprecedented. Many trials to oxidize the OH group at C-5 of shikimic acid were unsuccessful and, therefore, the strategy of successive oxidation-reduction as in the case
of [3-\textsuperscript{2}H]shikimic acid could not be implemented in this case. We synthesized [2,5-\textsuperscript{2}H\textsubscript{2}]shikimic acid from readily available [2,5-\textsuperscript{2}H\textsubscript{2}]furan by a modification of the procedure of Campbell et al.\textsuperscript{38} in good yield, and this provided an access to shikimic acid specifically labeled with deuterium at C-5 position. The synthesis of [2,3,4,5-\textsuperscript{2}H\textsubscript{4}]shikimic acid by the same procedure allowed deuterium labeling at four positions at the same time.

B. Discussion on results of the feeding experiments

As described earlier the cyclohexanecarboxylic acid moiety in ansatrienin A is formed by reduction of shikimic acid. In the process, the double bond and all three hydroxyl functions of shikimic acid are removed. Presumably, the removal of the hydroxyl groups must involve a series of alternate dehydrations and double bond reductions, arranged such that the ring system never becomes aromatic. The retention of deuterium at 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 C-4. The retention of deuterium at C-4 implies that any dehydration step involving loss of a hydrogen from C-4, if at all, can only occur after the hydroxyl group at C-4 has been removed and replaced by another, unlabeled hydrogen. These boundary conditions play an important role in delineating the
pathway for the transformation of shikimic acid into cyclohexanecarboxylic acid, as do the additional limitations described earlier, i.e., the fact that no deuterium from C-6 of shikimic acid was incorporated and the fact that the small amount of 1-cyclohexenecarboxylic acid accompanying the cyclohexanecarboxylic acid in the hydrolysis of the sample of ansatrienin A derived from [2-13C]shikimic acid carried the 13C label not at C-2 but at C-6.

Another important observation was made by Dr. Eileen Kennedy in this laboratory43. Racemic 5-hydroxy-[5-2H]-cyclohex-1-ene carboxylic acid was efficiently incorporated into ansatrienin A to give a fully reduced cyclohexanecarboxylic acid residue. The resulting ansatrienin A showed a 2H-NMR resonance at 61.21, the same position labeled by deuterium from C-5 of shikimic acid and different from the position labeled by [3-2H]shikimic acid (61.75). This result strongly suggests that a) 5-hydroxycyclohex-1-ene carboxylic acid is an intermediate in the transformation of shikimic acid to cyclohexanecarboxylic acid and b) that the remaining hydroxy group in this intermediate originates from C-5, not C-3, of shikimic acid.
If the first dehydration eliminates either the hydroxyl group at C-3 or C-5 of shikimic acid and it does not involve the hydrogen at C-4, there are two possible dehydration products of shikimic acid, 39 and 42, which could be intermediates on the biosynthetic pathway. Diols 39 and 42 can be converted independently into 40 and 43, most probably by the pathways shown in Figure 24. The route shown on the right side in Figure 24 satisfies all the boundary conditions described above, but the pathway on the left would produce a cyclohexanecarboxylic acid moiety retaining one of the two deuterium atoms from C-6 of shikimic acid. As mentioned before, Kennedy’s result provides another piece of evidence favoring loss of the hydroxyl group from C-3 as the first step. According to the two possible pathways, 5-hydroxy-[5-2H]-cyclohex-1-ene carboxylic acid can be derived either from [3-2H]-shikimic acid (via 39) or [5-2H]shikimic acid (via 42). The 2H-NMR spectrum of ansatrienin A derived from [5-2H]-5-hydroxycyclohex-1-ene carboxylic acid matches that expected for ansatrienin A biosynthesized from shikimic acid deuterated at C-5, not at C-3. This observation clearly rules out the intermediacy of 39 in the biosynthetic pathway. The finally proposed pathway is shown in Figure 25.
Figure 24. Two possible pathways from shikimic acid to cyclohexanecarboxylic acid moiety in ansatrienin
Figure 25. Proposed biosynthetic pathway from shikimic acid to cyclohexanecarboxylic acid
In addition to the facts discussed above the proposal considers the findings$^{44}$ that neither 1,2-dihydroshikimic acid nor chorismic acid are incorporated into ansatrienin A, and it assumes that only double bonds conjugated to the carbonyl groups are reduced, but that double bonds $\beta,\gamma$ to carbonyl groups can be isomerized into the $\alpha,\beta$ position. Both these assumptions are biochemically well preceded, e.g., in enzymes like enoyl-CoA reductase and in vinyl-CoA : crotonyl-CoA isomerase$^{45}$. Also consistent with these assumptions and with the proposed pathway, an enzyme catalyzing the reduction of 1-cyclohexenecarbonyl-CoA to cyclohexanecarbonyl-CoA has been purified about 1000 fold from $S$. collinus$^{44}$, and the conversion of cyclohex-2-ene-carboxyl-CoA to cyclohex-1-ene-carboxyl-CoA in a cell-free extract of the same organism has been observed$^{44}$.

The results reported in this thesis have been crucial in delineating the sequence of steps leading from shikimic acid to cyclohexanecarboxylic acid in $S$. collinus. They provide additional information which will be useful in the future in establishing the steric course of the various reactions in this sequence.
A. Materials and General Methods

1. Materials

All chemicals and solvents for synthetic reactions were reagent grade and were used without further purification unless otherwise noted. Reaction solvents were purified by distillation from appropriate drying agents; THF (Na/benzophenone), CH$_2$Cl$_2$ (P$_2$O$_5$), ether (Na/benzophenone), DMF (CaH$_2$). Other solvents were purified as described in reference$^{46}$. *Streptomyces collinus* Tü 1892 was a gift from Professor H. Zähner, Universität Tübingen, Institut für Biologie II, Lehrstuhl Mikrobiologie I, Tübingen, West Germany. Full fat soy bean meal for medium preparation was also obtained from Dr. Zähner. Other materials for medium preparation were purchased from Difco.

2. General methods

Melting points were determined on a Mel-Temp Laboratory device and are uncorrected. IR spectra were obtained on a Mattson Polaris FT-IR with absorptions being
cited. The $^1$H, $^{13}$C, and $^2$H NMR spectra were obtained on IBM AF-300 spectrometer. Chemical shifts are given in parts per million (ppm) relative to (CH$_3$)$_4$Si as internal standard or adjusted to the (CH$_3$)$_4$Si scale by reference to the solvent signal. Coupling constants (J) are given in Hertz(Hz). Splitting patterns are designated: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Routine GC/MS identification of synthetic intermediates was performed on a Hewlett-Packard 5790A gas chromatograph with 5970A mass selective detector. Analytical TLC was executed on precoated silica gel 60F-254. Compounds on the plates were visualized by spraying a p-anisaldehyde solution (3.7mL p-anisaldehyde, 135mL ethanol, 5mL conc. H$_2$SO$_4$, 1.5mL glacial acetic acid), phosphomolybdic acid (Aldrich), or KMnO$_4$ solution (10g KMnO$_4$ in 1L of 1N 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. A New Brunswick rotary shaker was used for fermentations.

B. Syntheses of Labeled Precursors

1. Synthesis of [2-$^2$H]shikimic acid

2,3:5,6-Di-O-isopropylidene-D-mannonic-$\gamma$-lactone (13)

A solution of 2,3:5,6-di-O-isopropylidene-$\alpha$-D-mannofuranose (12.7g, 48.8mmol) in anhydrous DMSO (1L)
containing acetic anhydride (135mL) was stirred at room temperature for 20h. Air was bubbled vigorously through the solution while warming the reaction mixture to 60°C to remove dimethylsulfide. Water (0.4L) was added to the reaction mixture and the resulting mixture was extracted with ether (4 * 400mL). The combined ether layer was washed with water (4 * 400mL), concentrated under reduced pressure, and lyophilized to give ca. 9.5g of crude product. The original aqueous layer was extracted with ether (400mL) and the ether layer was concentrated and lyophilized to give ca. 1.5g of crude product. The combined crude product which was 94% pure by GC analysis was recrystallized from ligroin (500mL) to produce 10.4g (81%) of lactone (13): mp 116°C; \textsuperscript{1}H-NMR (CDCl\textsubscript{3}, 300MHz) \(\delta\) 1.35 (s,3H), 1.38 (s,3H), 1.43 (s,3H), 1.44 (s,3H), 4.02 (dd,1H, \(J=9.3Hz, 3.9Hz\)), 4.11 (dd,1H, \(J=9.3Hz, 5.7Hz\)), 4.32-4.43 (m,2H) 4.78-4.85 (m,2H); \textsuperscript{13}C-NMR (CDCl\textsubscript{3}, 75.5MHz) \(\delta\) 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, rel intensity) 243(M\textsuperscript{+}−CH\textsubscript{3},73), 185(10), 157(10), 137 (9), 115(11), 101(100), 97(12), 85(30), 83(18), 73(29), 72 (23), 69(15), 68(15), 59(41), 57(11), 55(25)
A mixture of 13 (20.9 g, 81.0 mmol) and NaBD₄ (3.4 g, 81 mmol) in 90% aq. MeOH (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 ether layers were dried (MgSO₄) and concentrated to give 11.1 g (52%) of the diastereomeric mixture (α/β = 9:1) of [1-²H]-2,3:5,6-di-O-isopropylidene-D-mannofuranose. It was used for the next reaction without further purification.

α anomer of 14: ¹H-NMR (CDCl₃, 300 MHz) 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, 1H, J=7.0 Hz, 3.7 Hz), 3.37 (q, 1H, J=6.6 Hz), 4.58 (d, 1H, J=5.9 Hz), 4.77 (d, 1H, J=5.9 Hz, 3.6 Hz); GC-MS (m/z, rel intensity) 246 (M⁺-CH₃, 38), 188 (11), 141 (7), 130 (11), 128 (12), 101 (96), 99 (10), 98 (10), 86 (16), 85 (21), 81 (15), 74 (11), 73 (30), 72 (36), 71 (9), 70 (10), 69 (15), 68 (19), 61 (13), 60 (14), 59 (100), 58 (10), 57 (11), 55 (31)

β anomer of 14: ¹H-NMR (CDCl₃, 300 MHz) δ 1.30 (s, 3H), 1.37 (s, 3H), 1.43 (s, 3H), 3.92–4.00 (m, 2H), 4.08 (dd, 1H, J=8.6 Hz, 6.5 Hz, 2.1 Hz), 4.38 (q, 1H, J=6.6 Hz), 4.45–4.51 (m, 1H), 4.58–4.65 (m, 2H), 4.75–4.85 (m, 1H), 7.22–7.37 (m, 5H); GC-MS (m/z, rel intensity) 246 (M⁺-CH₃, 38), 188 (11), 141 (7), 130 (11), 128 (12), 101 (96), 99 (10), 98 (10), 86 (16), 85 (21), 81 (15), 74 (11), 73 (30), 72 (36), 71 (9), 70 (10), 69 (15), 68 (19), 61 (13), 60 (14), 59 (100), 58 (10), 57 (11), 55 (31)
intensity) 336(M⁺-CH₃, 74), 144(12), 141(11), 101(43), 98(12), 92(16), 91(100), 85(12), 72(13), 69(16), 65(17), 60(15), 59(17)

β anomer of 5: R_f 0.34 (hexane-EtOAc 3:1)

[1⁻²H]Benzyl 2,3-O-isopropylidene-α-D-mannofuranoside (6):

³¹H-NMR (CDCl₃, 300MHz) δ 1.27 (s, 3H), 1.41 (s, 3H), 4.40 (dd, 1H, J=4.3Hz, 1.0Hz), 4.50 (d, 1H, J=11.6Hz), 4.62-4.71 (m, 2H), 5.07 (dd, 1H, J=5.7Hz, 4.3Hz), 7.25-7.38 (m, 5H), 9.65 (d, 1H, J=1.0Hz); GC-MS (m/z, rel intensity) 264(M⁺-CH₃, 1), 130(27), 113(17), 92(8), 91(100), 74(9)

[1⁻²H]Benzyl 2,3-O-isopropylidene-α-D-lyxofuranoside (7):

³¹H-NMR (CDCl₃, 300MHz) δ 1.29 (s, 3H), 1.45 (s, 3H), 3.86-4.03 (m, 2H), 4.10 (q, 1H, J=4.5Hz), 4.48 (d, 1H, J=11.9Hz), 4.64-4.69 (m, 2H), 4.77-4.80 (m, 1H), 7.23-7.35 (m, 5H); GC-MS (m/z, rel intensity) 266(M⁺-CH₃, 4), 132(11), 113(8), 91(100), 86(11), 85(23), 59(18), 43(23)

[1⁻²H]-t-Butyl(Benzyl 5,6-dideoxy-6-dimethoxyphosphoryl-2,3-O-isopropylidene-α-D-lyxo-heptofuranosid)uronate (10):

³¹H-NMR (CDCl₃, 300MHz) δ 1.27 (s, 3H), 1.42(s) & 1.43(s) & 1.46 (s) (12H), 2.10-2.50 (m, 2H), 2.97-3.35 (m, 1H), 3.95-4.06 (m, 1H), 4.40 (d, 1H, J=11.8Hz), 4.58-4.65 (m, 3H), 7.17-7.35 (m, 5H)
[1-$^2$H]-t-Butyl(5,6-Dideoxy-6-dimethyloxyphosphoryl-2,3-O-isopropylidene-α-D-lyxo-heptofuranos)uronate (11)$^{34}$: $^1$H-NMR (CDCl$_3$, 300MHz) δ1.29 (s,3H), 1.43 (s,3H), 1.46 (s, 9H), 2.00-2.40 (m,2H), 3.00-3.30 (m,1H), 3.77 (d,3H, J=10.8Hz), 3.78 (d,3H,J=10.8Hz), 4.08-4.27 (m,1H), 4.45-4.60 (m,1H), 4.61-4.68 (m,1H)

[2-$^2$H]Shikimic acid$^{34}$: $^1$H-NMR (CD$_3$OD, 300MHz) δ2.18 (ddd,1H, J=18.2Hz, 5.7Hz, 1.5Hz), 2.69 (ddd,1H,J=18.2Hz, 5.0Hz, 1.8Hz), 3.66 (dd,1H,J=7.5Hz, 4.2Hz), 3.98 (dt,1H, J=7.5Hz, 5.4Hz), 4.34-4.38 (m,1H); $^2$H-NMR (CH$_3$OH, 46.1MHz) δ6.77

2. Synthesis of [3-$^2$H]shikimic acid$^{35}$

[3-$^2$H]Shikimic acid: $^1$H-NMR (CD$_3$OD, 300MHz) δ2.16 (ddd,1H, J=17.9Hz, 8.0Hz, 2.4Hz), 2.81 (dd,1H,J=17.9Hz, 5.5Hz), 3.54 (d,1H,J=9.0Hz), 3.90 (ddd,1H,J=9.0Hz, 8.0Hz, 5.5Hz), 6.46-6.48 (m,1H); $^2$H-NMR (CH$_3$OH, 46.1MHz) δ4.27

3. Synthesis of [4-$^2$H]shikimic acid

1,2-O-Isopropylidene-3-O-benzyl-5-O-toluenesulfonyl-D-arabinofuranose (27)

To a stirred solution of 1,2-O-isopropylidene-5-O-toluenesulfonyl-D-arabinofuranose (26) (2.53g, 7.35mmol) in dry DMF(25mL) was added benzyl bromide (2.5mL, 21mmol)
and sodium hydride (80% in oil, 265mg, 8.9mmol) in several portions over a period of 10 minutes. After 15 minutes at room temperature, the reaction was quenched by addition of water (35mL) and the resulting mixture was extracted with ether (2 * 180mL, 3 * 125mL). The combined extracts were dried (MgSO₄), and concentrated. Flash chromatography (hexane-EtOAc 3:1) of the residue afforded 27 (3.05g, 96%) as a syrup. 27: R_f 0.49 (hexane-EtOAc 2:1); ¹H-NMR (CDCl₃, 300MHz) δ1.25 (s,3H), 1.34 (s,3H), 2.41 (s,3H), 3.94 (d,1H, J=1.8Hz), 4.11 (d,2H, J=6.4Hz), 4.24 (dt,1H, J=6.4Hz, 1.8Hz), 4.52 (s,2H), 4.57 (d,1H, J=3.7Hz), 5.84 (d,1H, J=3.8Hz), 7.23-7.40 (m,7H), 7.74 (d,2H, J=8.2Hz); GC-MS (m/z, rel intensity) 329(m⁺-CH₃,74), 215(17), 172(25), 159(33), 155(56), 139(69), 129(30), 101(21), 97(33), 91(78), 73(29), 65(24), 59(100)

1,2-O-Isopropylidene-3-O-benzyl-D-arabinofuranose (28).

Four percent of sodium amalgam was prepared using 0.29g (13mmol) of sodium and 7.23g of mercury as described elsewhere⁴⁸ and it was placed in a 100mL round bottom flask. A solution of 1,2-O-isopropylidene-3-O-benzyl-5-O-toluenesulfonyl-D-arabinofuranose (27) (652mg, 1.50mmol) in 85% aq. MeOH (37mL) was added slowly and the mixture was stirred for 3h at 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 10mL of methanol. The combined solution was concentrated to ca. 5mL and 25mL of water was added to the residue. It was then extracted with CHCl₃ (4 * 20mL), and the extract was dried (MgSO₄), concentrated, and chromatographed on a silica gel column (hexane-EtOAc 1:1) to give 28 (396mg, 94%) as a white solid. 28: Rₚ 0.51 (hexane-EtOAc 2:1); mp 68-70°C; ¹H-NMR (CDCl₃, 300MHz) δ1.33 (s,3H), 1.51 (s,3H), 1.94 (s,1H, OH), 3.71 (d,2H, J=5.5Hz), 3.96 (d,1H,J=3.4Hz), 4.18 (m, 1H), 4.54 (d,1H, J=11.7Hz), 4.63 (d,1H,J=11.7Hz), 4.56 (d,1H, J=4.1Hz), 5.89 (d,1H,J=4.1Hz), 7.32 (m,5H); GC-MS (m/z, rel intensity) 265(M⁺-CH₃,49), 249(20), 92(38), 91(100)

t-Butyl (3-benzyl-5,6-dideoxy-6-dimethoxyphosphoryl-1,2-O-isopropylidene-D-arabino-heptofuranos)uronate (30).

A solution of 1,2-O-isopropylidene-3-O-benzyl-D-arabinofuranose (28) (615mg, 2.20mmol) in dry CH₂Cl₂ (7mL) containing pyridine (0.40mL, 4.8mmol) was cooled to -30°C and stirred while triflic anhydride (0.50mL, 2.9mmol) was added during 15 minutes. After 15 minutes at -30°C, 0.4mL of methanol was added to quench the reaction and the resulting mixture was diluted with 10mL of CH₂Cl₂. It was then washed successively with ice-water (5mL) and cold aq. NaH₂PO₄ (1M, 5mL), dried (Na₂SO₄), and concentrated to give
908mg of crude triflate (29) (Rf 0.88, hexane-EtOAc 1:1). This was used for the next reaction without further purification. Sodium hydride (100mg, 3.3mmol) was suspended in dry DMF (9mL) and the mixture was cooled to 0°C. A solution of t-butyl dimethylphosphonium acetate (820mg, 3.66mmol) in dry DMF (3.5mL) was added dropwise to the stirred mixture during 20 minutes. After the addition was completed the cold bath was removed and the mixture was stirred for 1h to give a clear solution. A solution of triflate 29 (908mg) in dry DMF (4mL) was then added, followed by 15-crown-5 (3 drops). After stirring for 13h at room temperature the reaction mixture was cooled to 0°C, quenched with cold aq. 1N NaH₂PO₄ (25mL) and extracted with CHCl₃ (1 * 25mL, 2 * 15mL). The combined extracts were washed with cold water (15mL), dried (Na₂SO₄), concentrated, and chromatographed on a column of silica gel (hexane-EtOAc 1:1). This provided 718mg (67% from 28) of a diastereomeric mixture of oily 30: Rf 0.50 (EtOAc); ¹H-NMR (CDCl₃, 300MHz) δ 1.28(s) & 1.39(s) (3H), 1.44(s) & 1.46(s) (9H), 1.49(s) & 1.52(s) (3H), 2.08-2.45 (m,2H), 2.99-3.12 (m) & 3.24-3.38(s) (1H), 3.72-3.78 (m, 6H), 4.06-4.16 (m,1H), 4.54-4.62 (m,3H), 5.82(d, J=4.8Hz) & 5.87(d, J=4.8Hz) (1H), 7.24-7.33 (m,5H); GC-MS (m/z, rel intensity) 471(M⁺-CH₃, 0.3), 429(M⁺-t-BuO,3),
313(5), 307(5), 301(8), 293(6), 266(6), 265(7), 211(34), 193(14), 91(100)

t-Butyl (5,6-dideoxy-6-dimethoxyphosphoryl-1,2-O-isopropylidene-D-arabino-heptofuranose)uronate (31).

To a solution of 30 (1.57g, 3.23mmol) in 95% MeOH (50mL) was added HCOONH₄ (904mg, 14.4mmol) and 10% Pd/C (1.04g). After stirring for 25 minutes at 50°C, the catalyst was filtered off and water (15mL) was added to the methanol solution. The resulting mixture was concentrated to about 15mL and extracted with CHCl₃ (4 * 30mL). The combined CHCl₃ layers were dried (Na₂SO₄) and concentratred to give 1.34g of a diastereomeric mixture of 31 (Rf 0.56 & 0.45, hexane-EtOAc 4:1) which solidified on standing. It was used for the next reaction without further purification.

31(mixture of diastereomers): mp 97-117°C; IR (neat) 3390, 3000, 1730, 1420, 1375, 1250, 1155, 1065, 1030, 755cm⁻¹; GC-MS (m/z, rel intensity) 381(M⁺-CH₃,6), 325(44), 323(M⁺-t-BuO,33), 307(57), 293(22), 283(24), 265(34), 247(15), 235(17), 223(22), 211(78), 193(100), 168(46), 165(16), 163(11), 151(19), 150(12), 137(34), 133(11), 110(18), 109 (26), 105(11), 100(79), 85(20), 79(16), 59(44), 57(52), 55(22)
31a: R\textsubscript{f} 0.56 (hexane-EtOAc 4:1); \textsuperscript{1}H-NMR (CDCl\textsubscript{3}, 300MHz)
\begin{align*}
\delta & 1.28 (s, 3H), 1.46 (s, 9H), 1.52 (s, 3H), 2.10-2.30 (m, 2H), \\
& 3.29 (ddd, 1H, J=23.5Hz, 10.8Hz, 4.0Hz), 3.76 (d, 3H, J=10.9Hz), 3.77 (d, 3H, J=10.9Hz), 3.99 (dd, 1H, J=9.8Hz, 4.7Hz), 4.12 (s, 1H), 4.51 (d, 1H, J=3.8Hz), 5.88 (d, 1H, J=3.9Hz);
\end{align*}

31b: R\textsubscript{f} 0.45 (hexane-EtOAc 4:1); \textsuperscript{1}H-NMR (CDCl\textsubscript{3}, 300MHz)
\begin{align*}
\delta & 1.28 (s, 3H), 1.45 (s, 9H), 1.52 (s, 3H), 2.15-2.45 (m, 2H), \\
& 3.13 (dt, 1H, J=23.5Hz, 5.8Hz), 3.75 (d, 3H, J=10.9Hz), 3.76 (d, 3H, J=10.9Hz), 3.95-4.03 (m, 1H), 4.08 (s, 1H), 4.51 (d, 1H, J=3.8Hz), 5.85 (d, 1H, J=3.9Hz)
\end{align*}

**Oxidation-reduction of 31**

To a solution of 31 (540mg, 1.36mmol) in dry DMF (4mL) was added dry DMSO (0.38mL) and P\textsubscript{2}O\textsubscript{5} (300mg). After heating at 70°C for 3h the reaction mixture was allowed to cool to room temperature. Water (40mL) was then added to the mixture and the latter was extracted with CHCl\textsubscript{3} (4 * 40mL). The combined CHCl\textsubscript{3} layers were washed with water (40mL) and the latter was reextracted with CHCl\textsubscript{3} (40mL). All the CHCl\textsubscript{3} layer was combined, dried (Na\textsubscript{2}SO\textsubscript{4}), and concentrated to give crude 32 (590mg, R\textsubscript{f}=0.58, hexane-EtOAc 4:1), which may still contain trace amounts of solvents. Crude 32 (590mg) in 90% MeOH (15mL) was cooled to 0°C and NaBH\textsubscript{4} or NaBD\textsubscript{4} (250mg) was added over a 5 minute period. After 1h
at 0°C the reaction mixture was diluted with water (12mL). It was then concentrated to about 10mL, and extracted with CHCl₃ (3 * 30mL). The combined CHCl₃ layers were dried (Na₂SO₄), and concentrated to give 500mg of white solid. The latter was dissolved in hot EtOAc (2mL) and triturated with hexane (6mL). The white solid was separated; it contained only one of the two diastereomers (298mg, mp 159-161°C, Rₙ 0.50, EtOAc:acetone 4:1). Concentration of the mother liquor followed by column chromatography (EtOAc) afforded a mixture of diastereomers (87mg, Rₙ 0.50 / Rₙ 0.40 = 1/2, EtOAc:acetone=4:1). The diastereomer of Rₙ 0.40 did not solidify on standing. Total yield was 385mg (71% from 31).

33a: Rₙ 0.50 (EtOAc-acetone 4:1); mp 159-160°C; ¹H-NMR (CDCl₃, 300MHz) δ1.34 (s,3H), 1.45 (s,9H), 1.60 (s,3H), 2.22 (q,2H,J=8.0Hz), 3.24-3.38 (m,1H), 3.75 (d,3H, J=10.9Hz), 3.77 (d,3H,J=10.9Hz), 3.98 (q,1H,J=7.5Hz), 4.23 (t,1H,J=6.2Hz), 4.59 (dd,1H,J=6.0Hz, 4.2Hz), 5.70 (d,1H, J=4.1Hz); ¹³C-NMR (CDCl₃, 75.5MHz) δ26.17, 26.20, 26.87, 26.93, 27.83, 40.88, 42.61, 53.16, 53.20, 53.24, 70.81, 79.00, 79.18, 79.23, 81.96, 104.58, 113.63, 167.50, 167.57; GC-MS (m/z, rel intensity) 381(M⁺-CH₃,0.7), 325(23), 323(M⁺-t-BuO,12), 293(36), 283(23), 265(68), 235(20), 211(69), 192(100), 168(39), 137(22), 109(23), 100(25), 99(26), 59(48), 57(59), 55(26)
33b: $R_f$ 0.40 (EtOAc-acetone 4:1); liquid; $^1$H-NMR $\delta$1.36 (s,3H), 1.45 (s,9H), 1.57 (s,3H), 2.17-2.45 (m,2H), 3.09-3.24 (m,1H), 3.77 (d,3H, $J$=10.9Hz), 3.78 (d,3H, $J$=10.9Hz), 4.02 (ddd,1H, $J$=8.1Hz, 6.5Hz, 6.0Hz), 4.18 (t,1H, $J$=6.0Hz), 4.60 (dd,1H, $J$=6.0Hz, 4.1Hz), 5.67 (d,1H, $J$=4.1Hz); $^{13}$C-NMR (CDCl$_3$, 300MHz) $\delta$26.58, 26.64, 27.29, 27.35, 27.77, 41.76, 43.49, 53.16, 53.20, 53.24, 70.28, 79.84, 79.94, 81.84, 104.58, 114.15, 167.79, 167.86

[3-2H]-33a: $^1$H-NMR (CDCl$_3$, 300MHz) $\delta$1.32 (s,3H), 1.43 (s,9H), 1.58 (s,3H), 2.20 (q,2H, $J$=8.0Hz), 3.24-3.38 (m,1H), 3.73 (d,3H, $J$=10.9Hz), 3.75 (d,3H, $J$=10.9Hz), 3.95 (t,1H, $J$=7.0Hz), 4.58 (d,1H, $J$=4.0Hz), 5.69 (d,1H, $J$=4.2Hz); GC-MS (m/z, rel intensity) 382($M^+$-15,1.5), 350(3), 326(38), 324($M^+$-t-BuO,5), 308(13), 295(12), 294(41), 284(32), 266(62), 211(73), 193(100), 168(38), 137(36), 109(25), 100(56), 59(46), 57(56)

[3-2H]-33b: $^1$H-NMR (CDCl$_3$, 300MHz) $\delta$1.34 (s,3H), 1.43 (s,9H), 1.55 (s,3H), 2.15-2.43 (m,2H), 3.09-3.24 (m,1H), 3.74 (d,3H, $J$=10.9Hz), 3.77 (d,3H, $J$=10.9Hz), 4.01 (dd,1H, $J$=8.3Hz, 5.9Hz), 4.58 (d,1H, $J$=4.0Hz), 5.66 (d,1H, $J$=4.1Hz)
[3-\textsuperscript{2}H]-t-Butyl (5,6-dideoxy-6-dimethoxyphosphoryl-2,3-O-isopropylidene-\alpha-D-lyxo-heptofuranos)uronate ([3-\textsuperscript{2}H]-11).

A mixture of 33 (910 mg, 2.29 mmol) and DOWEX-50W(H\textsuperscript{+}) (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 purified by chromatographed (EtOAc) to give 800 mg (88%) of [3-\textsuperscript{2}H]-11 as a syrup. It was identical with the material synthesized by the procedure of Fleet et al\textsuperscript{34}. 

R\textsubscript{f} 0.65, 0.58 (EtOAc:acetone=4:1).

11a: R\textsubscript{f} 0.65 (EtOAc-acetone 4:1); \textsuperscript{1}H-NMR (CDCl\textsubscript{3}, 300 MHz) 61.29 (s, 3H), 1.43(s, 3H), 1.45 (s, 9H), 2.14-2.28 (m, 2H), 3.21 (dd, 1H, J=22.9 Hz, 10.3 Hz, 4.5 Hz), 3.76 (d, 3H, J=10.9 Hz), 3.78 (d, 3H, J=10.9 Hz), 4.12 (5tet, 1H, J=4.1 Hz), 4.57 (d, 1H, J=5.8 Hz), 4.64 (dd, 1H, J=5.8 Hz, 3.5 Hz), 5.30 (s, 1H); GC-MS (m/z, rel intensity) 381 (M\textsuperscript{+}-CH\textsubscript{3}, 10), 323 (M\textsuperscript{+}-t-BuO, 31), 265 (30), 253 (81), 247 (19), 236 (27), 235 (50), 211 (70), 207 (42), 203 (37), 193 (100), 168 (40), 137 (55), 109 (35), 59 (42), 57 (79), 55 (31)

11b: R\textsubscript{f} 0.58 (EtOAc-acetone = 4:1); \textsuperscript{1}H-NMR (CDCl\textsubscript{3}, 300 MHz) 61.29 (s, 3H), 1.42 (s, 3H), 1.45 (s, 9H), 2.14-2.38 (m, 2H), 3.07(dd, 1H, J=22.7 Hz, 9.5 Hz, 5.1 Hz), 3.75 (d, 3H, J=10.9 Hz), 3.77 (d, 3H, J=10.9 Hz), 4.20 (m, 1H), 4.57 (d, 1H, J=6.0 Hz), 4.64 (dd, 1H, J=5.7 Hz, 3.6 Hz), 5.30 (s, 1H); GC-MS same as 7a.
[3-2H]-11a: Rf 0.58 (EtOAc-acetone = 4:1); ¹H-NMR (CDCl₃, 300MHz) δ1.28 (s,3H), 1.42 (s,3H), 1.45 (s,9H), 2.17-2.27 (m,2H), 3.20 (ddd,1H, J=22.9Hz, 10.3Hz, 4.5Hz), 3.75 (d,3H, J=10.9Hz), 3.77 (d,3H, J=10.9Hz), 4.11 (dd,1H, J=8.5Hz, 4.6Hz), 4.55 (s,1H), 5.30 (s,1H); GC-MS (m/z, rel intensity) 382(M⁺-15,1.5), 350(3), 326(38), 324(5), 308 (13), 295(12), 294(41), 284(32), 266(62), 252(15), 248 (12), 236(16), 224(10), 211(73), 208(12), 193(100), 168 (38), 165(16), 151(15), 150(13), 137(36), 110(16), 109 (25), 100(56), 85(18), 79(17), 59(46), 57(56), 55(20)

[3-2H]-11b: Rf 0.58 (EtOAc-acetone = 4:1); ¹H-NMR (CDCl₃, 300MHz) δ1.29 (s,3H), 1.43 (s,3H), 1.46 (s,9H), 2.10-2.44 (m,2H), 3.07 (ddd,1H, J=22.7Hz, 9.5Hz, 5.1Hz), 3.74 (d,3H, J=10.9Hz), 3.76 (d,3H, J=10.9Hz), 4.21 (t,1H, J=7.0Hz), 4.57 (s,1H), 5.30 (s,1H)

[4-2H]-t-Butyl (3R,4S,5R)-3,4,5-trihydroxy-3,4-0-isopropylidene-cyclohex-1-enecarboxylate ([4-2H]-12): ³H-NMR (CDCl₃, 300MHz) δ1.38 (s,3H), 1.44 (s,3H), 1.47 (s,9H), 2.15 (ddt,1H, J=17.4Hz, 8.8Hz, 2.0Hz), 2.76 (dd,1H, J=17.3Hz, 4.7Hz), 3.84 (dd,1H, J=8.8Hz, 4.7Hz), 4.70 (d,1H, J=3.4Hz), 6.81 (t,1H, J=2.9Hz); GC-MS (m/z, rel intensity) 256(M⁺-CH₃,45), 215(5), 198(9), 140(55), 139(10), 96(20), 59(19), 57(100)
[4-²H]Shikimic acid\textsuperscript{34}: \textsuperscript{1}H-NMR (CD\textsubscript{3}OD, 300MHz) \textdelta 2.18 (ddt, 1H, J=18.2Hz, 5.8Hz, 1.7Hz), 2.69 (ddt,1H,J=18.2Hz, 5.0Hz, 1.9Hz), 3.97 (t,1H,J=5.3Hz), 4.33-4.37 (m,1H), 6.78 (dt, 1H,J=3.8Hz, 1.8Hz); \textsuperscript{2}H-NMR (CD\textsubscript{3}OH, 46.1MHz) \textdelta 3.60

4. (±)-[2,5-²H\textsubscript{2}]Shikimic acid

[2,5-²H\textsubscript{2}]Furan

To dry THF(100mL) was added 10.0M BuLi in hexane (44mL) at -30°C. Furan (14.5mL, 0.2mole) was then added to the BuLi solution at -30°C and the resulting solutions was stirred at -15°C for 4h. It was quenched by addition of D\textsubscript{2}O(30mL) over a 20min period. Careful distillation by slowly warming the mixture afforded [2,5-²H\textsubscript{2}]furan which was about 75% deuterated. The deuterated furan, containing a significant amount of THF, was immediately used for the next reaction without further purification.

[1,4-²H\textsubscript{2}]Exo-2-methoxycarbonyl-7-oxabicyclo[2,2,1]hept-5-ene ([1,4-²H\textsubscript{2}]-34a)\textsuperscript{39}: R\textsubscript{f} 0.25(hexane-EtOAc 6:1); \textsuperscript{1}H-NMR (CDCl\textsubscript{3}, 300MHz) \textdelta 1.59 (dd,1H,J=11.7Hz, 8.7Hz), 2.18 (dd, 1H,J=11.7Hz, 3.7Hz), 2.46 (dd,1H,J=8.6Hz, 3.8Hz), 3.76 (s, 3H), 6.41 (d,2H,J= 5.9Hz)
[\textit{1,4-}^{2\text{H}_2}]\textit{Endo-2-methoxycarbonyl-7-oxabicyclo[2,2,1]hept-5-ene} ([\textit{1,4-}^{2\text{H}_2}]\textit{34b})^{39}: R_f 0.34 (hexane-EtOAc 6:1); \textit{^1H-NMR} (CDCl_3, 300MHz) \delta 1.56 (dd, 1H, J=11.4Hz, 3.7Hz), 2.09 (dd, 1H, J=11.3Hz, 9.5Hz), 3.09Hz (dd, 1H, J=9.8Hz, 3.7Hz), 3.62 (s, 3H), 6.20 (d, 1H, J=5.8Hz), 6.34 (d, 1H, J=5.9Hz)

\[2,5-^{2\text{H}_2}\]\textit{1-Methoxycarbonyl-5-hydroxycyclohexa-1,3-diene} ([\textit{2,5-}^{2\text{H}_2}]\textit{35})^{39}: \textit{^1H-NMR} (CDCl_3, 300MHz) \delta 1.58 (br.s, 1H), 2.61 (d, 1H, J=18.8Hz), 2.90 (d, 1H, J=18.8Hz), 3.75 (s, 3H), 6.17-6.27 (m, 2H)

\[2,5-^{2\text{H}_2}\]\textit{Shikimic acid}^{38}: \textit{^1H-NMR} (CD_3OD, 300MHz) \delta 2.17 (d, 1H, J=18.5Hz), 2.68 (d, 1H, J=18.1Hz), 3.63-3.69 (m, 1H), 3.98 (dt, 0.3H, J=8.0Hz, 5.5Hz), 4.33-4.38 (m, 1H), 6.75-6.82 (m, 0.3H); \textit{^2H-NMR} (CH_3OH, 46.1MHz) \delta 3.93, 6.77

5. (±)-[\textit{2,3,4,5-}^{2\text{H}_2}]\textit{Shikimic acid}

\[1,4,5,6-^{2\text{H}_4}]\textit{Exo-2-methoxycarbonyl-7-oxabicyclo[2,2,1]-hept-5-ene} ([\textit{1,4,5,6-}^{2\text{H}_4}]\textit{34a})^{39}: R_f 0.25 (hexane-EtOAc 6:1); \textit{^13C-NMR} (CDCl_3, 75.5MHz) \delta 28.73, 42.41, 51.73, 77.43(t), 80.28(t), 133.96(t), 136.34(t), 173.85; \textit{^2H-NMR} (CHCl_3, 46.1MHz) \delta 5.03, 5.15, 6.36, 6.39; \textit{GC-MS} (m/z, rel intensity) 143(M^+-CH_3, 0.01), 127(M^+-OCH_3, 1.3), 85(9), 72(100), 55(80)
[1,4,5,6-2H₄]endo-2-Methoxycarbonyl-7-oxabicyclo[2,2,1]-hept-5-ene ([1,4,5,6-2H₄]-34b)³⁹: R₇ 0.34 (hexane-EtOAc 6:1); ¹³C-NMR (CDCl₃, 75.5MHz) δ28.30, 42.42, 51.39, 78.09(t), 78.51(t), 131.96(t), 136.38(t), 172.35; ²H-NMR (CHCl₃, 46.1MHz) δ4.99, 5.13, 6.23, 6.45; GC-MS (m/z, rel intensity) 127(M⁺-OCH₃,0.3), 85(13), 72(69,55(100)

[2,3,4,5-2H₄]-1-Methoxycarbonyl-5-hydroxycyclohexa-1,3-diene ([2,3,4,5-2H₄]-35)³⁹: GC-MS (m/z, rel intensity) 158(M⁺,19), 143 (M⁺-CH₃,16), 127(M⁺-OCH₃,15), 126(15), 99(M⁺-CO₂CH₃,100), 81(45)

[2,3,4,5-2H₂]-1-Methoxycarbonyl-5-t-butyldimethylsilyloxy-cyclohexa-1,3-diene ([2,3,4,5-2H₂]-36)³⁹: ¹³C-NMR (CDCl₃,75.5MHz) 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; ²H-NMR (CHCl₃, 46.1MHz) δ4.47 (¹²H), 6.09 (²²H), 7.02 (¹²H); GC-MS (m/z, rel intensity) 272(M⁺,0.1), 257(M⁺-CH₃,0.3), 241(M⁺-OCH₃, 0.6), 215(M⁺-t-Bu,12), 109(81), 89(17), 81(20), 75(100), 73(25)

[2,3,4,5-2H₄]Methyl (3R,4S,5R)-3,4-dihydroxy-5-t-butyldimethylsilyloxy-cyclohex-1-ene-carboxylate ([2,3,4,5-2H₄]-37)³⁸: mp 69-72°C; ¹³C-NMR (CDCl₃, 75.5MHz) 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$_3$, 46.1MHz) δ3.64, 4.04, 4.44, 6.82

[2,3,4,5-^2$H_4$]Shikimic acid$^{38}$: $^{13}$C-NMR (D$_2$O, 75.5MHz) δ33.22, 68.21 (t), 68.04 (t), 73.50 (t), 132.60, 139.90 (t), 172.93; $^2$H-NMR (H$_2$O, 46.1MHz) δ3.71, 4.00, 4.38, 6.79

C. Fermentation

*Streptomyces collinus* strain Tu 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 prepared from well-sporulated slants was transferred under sterile conditions to a 500 mL Erlenmeyer flask containing 100 mL of seed medium and incubated for 2 days on a New Brunswick rotary shaker at 28°C and 300 rpm. Of the seed culture, 10mL each was used to inoculate 100 mL of production medium in 500mL Erlenmeyer flasks, which was again grown for 72h at 28°C with rotary shaking at 300 rpm. Both the seed and production medium contained full fat soy bean meal, 2.0 g; mannitol, 2.0 g; tap water, 100 mL; pH 7.3. Labeled precursors were added after 24h growth in the production medium, and the cultures were harvested 24h later. All media were sterilized for 20 min at 121°C in a steam autoclave.
D. Isolation and Purification of Ansatrienin A

The cultures were filtered through Celite; the mycelia were collected and suspended in acetone. The suspension was sonicated for 5 min at maximum power setting and filtered, and the process was repeated once more. The combined filtrates were concentrated to remove acetone, and the crude ansatrienin was precipitated with petroleum ether with cooling to 0°C overnight. The precipitate was collected, washed with petroleum ether, and dissolved in a small volume of EtOAc. It was shaken briefly with a few mL of a saturated aqueous solution of FeCl₃ to oxidize ansatrienin B to ansatrienin A. The EtOAc layer was separated, concentrated, and purified by column chromatography (hexane-EtOAc 2:1)

E. Degradation of Ansatrienin A

One mg of ansatrienin A in 2mL 10N HCl was heated to 90°C for 6h. The reaction mixture was extracted with Et₂O (3 * 2mL); the combined extract was concentrated to about 1mL and it was used for GC-MS analysis without further purification.
PART 2: REDUCTIOMYCIN
CHAPTER I
INTRODUCTION

Reductioycin, an antibiotic which shows pronounced activity against tumors, Gram-positive bacteria, fungi, and certain yeasts, was first isolated from *Streptomyces reeseorubiginosus* in 1980 by Tamura et al. Based on X-ray crystallographic and spectroscopic studies they reported a structure for reductioycin in which the positions of the nitrogen and 1' oxygen atom are reversed relative to those shown in Figure 26. At almost the same time two other groups isolated reductioycin independently from *Streptomyces xanithochromogenus* and *Streptomyces orientalis*. They derived the structure of reductioycin as shown in Figure 26 largely on the basis of chemical and spectroscopic data. The structural ambiguity was resolved by Yamada et al. by total synthesis of racemic reductioycin. The finally established structure is shown in Figure 26 but the absolute configuration is still unknown.

Reductioycin consists of two unique structural units. One is a 2-amino-3-hydroxycyclopent-2-enone moiety
(C₅N unit) which is also found in the antibiotics asukamycin²⁵,²⁶, manumycin⁵⁵, moeromycin⁵⁶,⁵⁷, virustomycin A⁵⁸, senecarcin A⁵⁹, bafilomycin B₁⁶⁰, and antibiotic L-155,17⁵⁶. The other is an unusual acetoxydihydrofuran carrying an acrylic acid side chain.

In manumycin and asukamycin⁶², the 2-amino-3-hydroxycyclopent-2-enone moiety is known to arise by an intramolecular cyclization of 5-aminolevulinic acid, which in turn is formed from glycine and succinic acid (Figure 28). In a series of feeding experiments with [2-¹³C,¹⁵N]glycin, 5-amino-[5-¹⁴C]levulinic acid, and other labeled precursors it was established that succinyl-CoA combines with glycine with decarboxylation to form 5-amino-levulinic acid, which then undergoes an
Figure 27. Mechanism for the formation of the C₅N unit in asukamycin from 5-aminolevulinic acid proposed by Floss and coworkers.
intramolecular cyclization. Floss and coworkers\textsuperscript{62} proposed a mechanism for this reaction, invoking pyridoxal phosphate (PLP) catalysis to generate a carbanion at C-5 which attacks the carboxyl carbon. This is shown in Figure 27.

Feeding experiments with labeled precursors established the same biosynthetic origin of the 2-amino-3-hydroxycyclopent-2-enone (C\textsubscript{5}N unit) moiety of reductiomyacin\textsuperscript{63}. [2-\textsuperscript{14}C]Glycine, but not [1-\textsuperscript{14}C]glycine, was efficiently incorporated into reductiomyacin. As in the case of asukamycin, [1-\textsuperscript{14}C]succinic acid and [5-\textsuperscript{14}C]-5-aminolevulinic acid were incorporated significantly but less efficiently than [2-\textsuperscript{14}C]glycine. Unequivocal proof for the direct origin of the 2-amino-3-hydroxycyclopent-2-enone moiety from an intact molecule of 5-aminolevulinic acid was obtained by a feeding experiment with [4,5-\textsuperscript{13}C\textsubscript{2}]-5-aminolevulinic acid. The \textsuperscript{13}C-NMR spectrum of the resulting sample of reductiomyacin showed labeling exclusively at C-1, C-2, and C-3 of the C\textsubscript{5}N unit. C-2 was twice as heavily enriched as C-1; in half of the labeled molecules C-2 was coupled to C-1 and in the other half to C-3. As in the case of asukamycin, cyclization of 5-aminolevulinic acid is expected to involve pyridoxal phosphate-generated carbanion $\alpha$ to the nitrogen.
The remaining nine carbon atoms of reductiomycin were first thought to arise from tyrosine, e.g., by ring cleavage of tyrosine or a derivative thereof followed by a Baeyer-Villiger oxidation\textsuperscript{63}. However, this notion was quickly dismissed when it was found that $[1^{-14}\text{C}]$tyrosine was not incorporated and that $[1,2^{-13}\text{C}_2]$acetate was incorporated efficiently and intact into the acetoxy group. The origin of the remaining seven carbon atoms making up the dihydrofuranylacrylic acid moiety was probed in a feeding experiment with $[\text{U}^{-13}\text{C}]$glycerol. The complex spectral pattern of the resulting reductiomycin was resolved as a superimposition of two distinct coupling patterns. Half the molecules (B in Figure 28) contained two intact molecules of glycerol and a single enriched but not coupled carbon and the other half (A in Figure 28) contained one C$_3$ segment, one pair of coupled carbons, and two single enriched, but not coupled, carbon atoms. These coupling patterns led Floss and coworkers\textsuperscript{63} to propose the shikimate pathway as the source of the dihydrofuranylacrylic acid moiety of reductiomycin. Coupling pattern A implies ring cleavage between C-3 and C-4 of shikimate. The additional presence of coupling pattern B reflects cleavage between C-4 and C-5, indicating that the substrate must be a symmetrical
Figure 28. Biosynthesis of reductiomyacin proposed by Beale et al.
compound formed from shikimic acid. Floss and coworkers considered 4-hydroxybenzoate as a plausible candidate\(^6^3\). To test this notion, they fed 4-hydroxy-(carboxy-\(^{13}\)C)-benzoic acid to *Streptomyces xanthochromogenus*. A 64% enrichment exclusively in the expected C-5' position confirmed that 4-hydroxybenzoate or a closely related product must be the substrate for the ring cleavage reaction leading to the formation of the dihydrofuranyl-acrylic acid moiety of reductiomyacin. The proposed pathway of reductiomyacin biosynthesis is shown in Figure 28.

The objective of the research presented in this thesis was to examine further the biosynthetic pathway leading to the dihydrofuranyl-acrylic acid moiety of reductiomyacin. The three aspects addressed were: (i) To test the pathway proposed by Beale et al.; (ii) To examine if 4-hydroxybenzaldehyde is involved in the conversion and, if this is the case, at which stage the ring cleavage occurs; (iii) To probe the mode of ring cleavage. Firstly, we intended to synthesize samples of [\(^{2}\)H\(_2\)]\(_{2}\), [\(^{3}\)H\(_2\)]\(_{2}\), and [\(^{2}\)H\(_4\),\(^{7}\)C]-4-hydroxybenzoic acid and to feed these materials to *Streptomyces xanthochromogenus*. The labeling pattern of the resulting reductiomyacin would confirm or disprove the proposed
pathway and provide valuable information to probe the pathway in more detail. Secondly, we intended to synthesize samples of [7-\textsuperscript{13}C]- and [7-\textsuperscript{13}C,7-\textsuperscript{2}H]-4-hydroxybenzaldehyde. Feeding experiments with these materials would reveal at which stage the ring cleavage occurs. Thirdly, we planned to try fermentation in an \textsuperscript{18}O\textsubscript{2} atmosphere to establish the origin of the oxygen atoms in reductiomyycin. With the result from this experiment we wished to obtain information on the mode of ring cleavage which is involved in the conversion of 4-hydroxybenzoic acid to the dihydrofuranylacrylic acid moiety of reductiomycin.
CHAPTER II
RESULTS

A. Syntheses of Labeled Precursors

1. Synthesis of \([3,5-^{2}\text{H}_2]\)-4-hydroxybenzoic acid

Reduction of 3,5-dibromo-4-hydroxybenzoic acid with zinc dust in refluxing CH$_3$COOD/D$_2$O afforded \([3,5-^{2}\text{H}_2]\)-4-hydroxybenzoic acid in 90\% yield and >99\% deuterium enrichment.

2. Synthesis of \([2,6-^{2}\text{H}_2]\)-4-hydroxybenzoic acid

Preparation of the title compound involved the synthesis of 3,5-dibromo-4-hydroxybenzoic acid carrying deuterium at C-2 and C-6 and subsequent debromination by the same procedure employed above.

![Synthesis of \([3,5-^{2}\text{H}_2]\)-4-hydroxybenzoic acid](image.png)

Figure 29. Synthesis of \([3,5-^{2}\text{H}_2]\)-4-hydroxybenzoic acid
Figure 30. Synthesis of [2,6-2H2]-4-hydroxybenzoic acid

a) dioxane dibromide  
b) CH₃I, K₂CO₃  
c) Mg; CO₂; H⁺  
d) BBr₃  
e) dioxane dibromide  
f) CH₃I, K₂CO₃  
g) Zn, CH₃COOH, H₂O  
h) OH⁻  
i) BBr₃
[2,6-2H2]-3,5-Dibromo-4-hydroxybenzoic acid was obtained from phenol-d6 in 5 reaction steps without purification of any intermediates. Phenol-d6 was converted to 4-bromophenol-d4 by treatment with dioxane dibromide. Dioxane dibromide was used to brominate the para position predominantly. Treatment of 4-bromophenol-d4 with CH3I/K2CO3 in acetone gave anisol-d4. The Grignard reagent prepared from the latter was quenched with CO2 to give [2,3,5,6-2H4]-4-methoxybenzoic acid. Removal of the methyl protecting group with BBr3 gave [2,3,5,6-2H4]-4-hydroxybenzoic acid. Bromination of the latter with dioxane dibromide followed by exhaustive methylation (CH3I, K2CO3) afforded methyl [2,6-2H2]-3,5-dibromo-4-methoxybenzoate. This was purified by column chromatography and the yield was 33% based on phenol-d6. Reduction of 61 with zinc dust in CH3COOH/H2O gave methyl [2,6-2H2]-4-methoxybenzoate. Saponification followed by removal of the methyl protecting group afforded [2,6-2H2]-4-hydroxybenzoic acid in 75% yield from 61 or 25% yield from phenol-d6.

3. Synthesis of [2,3,5,6-2H4,7-13C]-4-hydroxybenzoic acid

Substitution of 13CO2 for CO2 in the preparation of [2,3,5,6-2H4]-4-hydroxybenzoic acid afforded [2,3,5,6-2H4,
Figure 31. Synthesis of [7-\(^{13}\)C]-4-hydroxybenzaldehyde
a) Mg; \(^{13}\)CO\(_2\); H\(^+\) b) CH\(_3\)I, K\(_2\)CO\(_3\)
c) LiAlH\(_4\) d) PCC e) B\(_3\)I

Figure 32. Synthesis of [7-\(^2\)H,7-\(^{13}\)C]-4-hydroxybenzaldehyde
a) LiAlD\(_4\) b) PCC c) B\(_3\)I
7-$^{13}$C]-4-hydroxybenzoic acid. Purification of the latter by recrystallization from water completed the synthesis without isolation or purification of any intermediates.

4. Synthesis of [7-$^{13}$C]-4-hydroxybenzaldehyde

Carboxylation of the Grignard reagent prepared from 4-bromoanisol with $^{13}$CO$_2$ gas afforded [7-$^{13}$C]-4-methoxybenzoic acid. The methyl ester of the latter was reduced with LiAlH$_4$ to [7-$^{13}$C]-4-methoxybenzyl alcohol which was then oxidized with PCC to obtain [7-$^{13}$C]-4-methoxybenzaldehyde. Removal of the methyl protecting group by treatment with boron triiodide afforded [7-$^{13}$C]-4-hydroxybenzaldehyde.

5. Synthesis of [7-$^{13}$C,7-$^2$H]-4-hydroxybenzaldehyde

Reduction of methyl [7-$^{13}$C]-4-methoxybenzoate with LiAlD$_4$ instead of LiAlH$_4$ gave [7-$^{13}$C,7-$^2$H$_2$]-4-methoxybenzyl alcohol. Oxidation and deprotection of the alcohol as above afforded [7-$^{13}$C,7-$^2$H]-4-hydroxybenzaldehyde.

B. Fermentation

The biosynthesis of reductiomyacin was studied using shake cultures of Streptomyces xanthochromogenus (AM 6201). Culture conditions developed in our group were used for the feeding experiments. Five milliliters of 72h
old cultures of *S. xanthochromogenus* AM 6201 grown in 100mL of seed medium (glucose, 1.0g; peptone, 2.0g; yeast extract, 0.5g; CaCO₃, 0.4g; distilled water, 100mL; pH 7.0) were transferred into production medium (glucose, 2.0g; soybean meal, 2.0g; NaCl, 0.3g; distilled water, 100mL; pH 7.0) in 500mL baffled flasks. Labeled precursors were added in one portion to 24h old production cultures which were harvested 48h later. The average yield of purified reductiomycin was 250mg per liter of production culture.

C. Feeding Experiments

1. Feeding of [2,6-²H₂]-4-hydroxybenzoic acid

   According to the pathway of reductiomycin biosynthesis proposed by Floss and coworkers⁶, ⁴-hydroxybenzoic acid labeled with deuterium at the C-2 and C-6 position should label the C-3' and C-3'' positions in the resulting reductiomycin. To test this notion [2,6-²H₂]-4-hydroxybenzoic acid was fed to *Streptomyces xanthochromogenus* and the resulting reductiomycin was analyzed by ¹H- and ²H-NMR spectroscopy. Deuterium from [2,6-²H₂]-4-hydroxybenzoic acid was incorporated giving reductiomycin with two ²H-NMR signals of almost equal intensity at 2.68 ppm and 7.50 ppm. These two signals correspond to H-3' and H-3'', respectively, (Figure 33); this result is consistent with that expected from the
Figure 33. Results of feeding experiments with [3,5-\textsuperscript{2}H\textsubscript{2}], [2,6-\textsuperscript{2}H\textsubscript{2}], and [2,3,5,6-\textsuperscript{2}H\textsubscript{4},7-\textsuperscript{13}C]-4-hydroxybenzoic acid
pathway shown in Figure 28. The $^1$H-NMR spectrum showed that both positions are 30% enriched with deuterium.

The relative stereochemistry of the deuterated center at C-3' in reductiomycin biosynthesized from [2,6-$^2$H$_2$]-4-hydroxybenzoic acid and the chiral center at C-2' was deduced from the relationship between the coupling constants and the molecular geometry of reductiomycin. A molecular model shows that the conformation of the dihydrofuran ring of reductiomycin is fairly rigid and the dihedral angle between H$_c$-C$_3$, and H$_2'$-C$_2'$, is close to 90° and that between H$_t$-C$_3$, and H$_2'$-C$_2'$, is close to 0° (figure 34). The $^1$H-NMR of nonlabeled reductiomycin showed that the hydrogen atom at C-2' couples with the hydrogen atoms at C-3' (δ2.68 and δ3.03) with coupling constants of 2.4Hz and 7.5Hz. The Karplus equation$^{64}$, which relates dihedral angle and the vicinal coupling constant, led us to assign the coupling constant between H$_c$ and H$_2'$ to 2.4Hz and the other to 7.5Hz. Based on this interpretation, the $^1$H-NMR signal at δ2.68 was assigned to H$_t$ and that at δ3.03 to H$_c$. Reductiomycin derived from [2,6-$^2$H$_2$]-4-hydroxybenzoic acid showed a $^2$H-NMR resonance at 2.68 ppm but not at 3.03 ppm. This result established the anti relationship of the deuterium at C-3' to the acetoxy group.
Figure 34. The relationship between the coupling constants and the molecular geometry of reductiomycin
In the mass spectrum of the sample, the observed pattern of isotope distribution in the molecular ion peak exactly matches with the pattern expected on the basis of the results described above assuming no intermolecular transfer of deuterium. The calculated pattern is shown in Table 3. Intermolecular transfer of deuterium atoms does not seem to occur because the observed pattern is considerably different from that expected if one of the deuterium atoms were transferred between molecules on the biosynthetic pathway.

2. Feeding of [3,5-$^{2}$H$_{2}$]-4-hydroxybenzoic acid

The $^{2}$H-NMR spectrum of reductiomyacin biosynthesized from [3,5-$^{2}$H$_{2}$]-4-hydroxybenzoic acid showed two signals at the expected positions, 65.83 and 66.73. Integration of these two peaks corresponding to H-2" and H-2′, respectively, gave an intensity ratio of 0.54:1.00. The $^{1}$H-NMR spectrum showed deuterium enrichments of 30% at H-2" and 54% at H-2′. These results, together with those obtained from the feeding experiment with [2,6-$^{2}$H$_{2}$]-4-hydroxybenzoic acid, provides evidence supporting the pathway proposed by Floss and coworkers$^{63}$.

The mass spectral analysis gave almost the same result. The observed isotope distribution of both
molecular ion and M-AcOH peak matches very well with the pattern calculated on the basis of the result obtained from the NMR study. Also assumed in this case is that deuterium atoms did not transfer to another molecule of reductioycin precursor on the biosynthetic pathway. This is a reasonable assumption because the observed isotope distribution pattern is considerably different from the pattern expected if intermolecular transfer of deuterium atoms had occurred (Table 3). The question of intermolecular transfer of deuterium atoms and the unequal incorporation of deuterium into reductioycin was studied further with tetradeuterated 4-hydroxybenzoic acid.

3. Feeding of [2,3,5,6-²H₄,7-¹³C]-4-hydroxybenzoic acid

Reductioycin from the feeding experiment with [2,3,5,6-²H₂,7-¹³C]-4-hydroxybenzoic acid showed the deuterium labeling pattern expected from the results of the feeding experiments with dideuterated hydroxybenzoic acids. The ¹³C enrichment at C-5' was determined to be 55% by comparing the integral of the ¹³C-coupled 5'-H peak with that of the uncoupled peak. The deuterium labeling pattern is summarized in Figure 33. Almost identical deuterium enrichments of about 55% were observed at H-3", H-3', and H-2' position but H-2" was only 25% enriched. Less incorporation of deuterium at H-2" compared to H-2'
is consistent with the result obtained from the feeding experiment with the \([3,5-^{2}H_{2}]\)-4-hydroxybenzoic acid. Of the ring hydrogens of tetradeuterated 4-hydroxybenzoic acid three were completely retained in the conversion to reductiomyacin and the one appearing at C-2" of reductiomyacin was only about half retained.

The \(^1\text{H}-\text{NMR}\) spectrum also provides evidence against the possibility of intermolecular transfer of the deuterium atom at C-2". Figure 36 shows all possible labeling patterns at C-2" and C-3" and the \(^1\text{H}-\text{NMR}\) spectra corresponding to those species. If the deuterium atom ending up at C-2" position had been transferred intermolecularly on the biosynthetic pathway, the species B and C would be produced in addition to A and D. This possibility was ruled out by the observation of only A and B, but not C, in the \(^1\text{H}-\text{NMR}\) spectrum of reductiomyacin obtained from the feeding experiment with [2,3,5,6-\(^2\text{H}_4\),7-\(^{13}\text{C}\)]-4-hydroxybenzoic acid. The only alternative way of producing species B is exchange of the deuterium atom at C-2" with solvent protons.

Mass spectral analysis of reductiomyacin from [2,3,5,6-\(^2\text{H}_4\),7-\(^{13}\text{C}\)]-4-hydroxybenzoic acid gave the results as summarized in Table 3. The relative abundance of the
Figure 35. (a) Expected $^1$H-NMR spectral patterns of H-2" and H-3" of reductiomyacin in four possible labeling states
(b) Observed $^1$H-NMR spectrum corresponding to H-2" and H-3" of reductiomyacin obtained from feeding experiment with [2,3,5,6-2H$_4$, 7-13C]-4-hydroxybenzoic acid
Table 3. Results of the mass spectral analysis of reductiomyacin samples obtained from feeding experiments with labeled precursors

<table>
<thead>
<tr>
<th>m/z</th>
<th>STD</th>
<th>R26</th>
<th>R35</th>
<th>RD4C</th>
<th>R7C</th>
<th>R26</th>
<th>R35</th>
<th>RD4C&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>293&lt;sup&gt;3&lt;/sup&gt;</td>
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<td>55</td>
<td>37</td>
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<td>54 (45)</td>
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<td>34</td>
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<td>27 (32)</td>
<td>9 (22)</td>
</tr>
<tr>
<td>295</td>
<td>30</td>
<td>8</td>
<td>7</td>
<td>5</td>
<td>29 (18)</td>
<td>(5)</td>
<td>9 (10)</td>
<td>4 (2)</td>
</tr>
<tr>
<td>296</td>
<td>25</td>
<td>17</td>
<td>30</td>
<td>3</td>
<td>29 (18)</td>
<td>30 (28)</td>
<td>3 (6)</td>
<td></td>
</tr>
<tr>
<td>297</td>
<td>3</td>
<td>17</td>
<td>21</td>
<td>3</td>
<td>29 (18)</td>
<td>30 (34)</td>
<td>2 (5)</td>
<td></td>
</tr>
<tr>
<td>298</td>
<td>1</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>6 (3)</td>
<td>7 (7)</td>
<td>7 (6)</td>
<td></td>
</tr>
<tr>
<td>299</td>
<td>14</td>
<td>21</td>
<td>12</td>
<td>6</td>
<td>24 (16)</td>
<td>30 (34)</td>
<td>2 (5)</td>
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<tr>
<td>300</td>
<td>23</td>
<td>17</td>
<td>12</td>
<td>3</td>
<td>13 (32)</td>
<td>27 (43)</td>
<td>7 (22)</td>
<td></td>
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<td>24 (16)</td>
<td>30 (34)</td>
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</tr>
<tr>
<td>302</td>
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<td>7</td>
<td>12</td>
<td>1</td>
<td>6 (3)</td>
<td>7 (7)</td>
<td>7 (6)</td>
<td></td>
</tr>
</tbody>
</table>

(Numbers in the table are relative intensities recalculated as percentages)

R26 : Reductiomyacin biosynthesized from [2,6-<sup>2</sup>H<sub>2</sub>]-4-hydroxybenzoic acid
R35 : Reductiomyacin biosynthesized from [3,5-<sup>2</sup>H<sub>2</sub>]-4-hydroxybenzoic acid
RD4C : Reductiomyacin biosynthesized from [2,3,5,6-<sup>13</sup>C<sub>4</sub>]-4-hydroxybenzoic acid
R7C : Reductiomyacin biosynthesized from [7-<sup>13</sup>C]-4-hydroxybenzaldehyde

1) Relative abundances are calculated based on the NMR analysis results shown in Figure 8 and 10 assuming no intermolecular transfer of deuterium atoms. The numbers in parenthesis are relative intensities values expected when one of the deuterium atoms has transferred between molecules.
2) The labeled precursor was 98% 2H-enriched and 99% <sup>13</sup>C-enriched and this was considered in the calculation.
3) m/z=293 is molecular ion peak, relative abundance of m/z 293 : m/z 233 is 40:100.
4) m/z=233 is M-AcOH peak.
M^+ / M^+1 / M^+4 / M^+5 / M^+6 peaks matches reasonably well with that predicted for the conversion of [2,3,5,6-2H4,7-13C]-4-hydroxybenzoic acid into reductiomycin without any intermolecular transfer of deuterium between molecules to give the observed enrichments; i.e., 25% at H-2", and 55% each at the C-5', H-3", H-3', and H-2' positions. Also shown in Table 3 are the calculated isotope distributions of the M^+ and (M-AcOH)^+ peaks assuming intermolecular transfer of deuterium. Significant discrepancy of those values from the observed spectral pattern provides additional evidence against intermolecular transfer of deuterium.

4. Feeding of [7-13C]-4-hydroxybenzaldehyde

The intermediacy of 4-hydroxybenzaldehyde in the biosynthetic pathway of reductiomycin was tested by feeding [7-13C]-4-hydroxybenzaldehyde to Streptomyces xanthonochromogenus. A 35% 13C enrichment at C-5' of the resulting reductiomycin established that 4-hydroxybenzaldehyde can serve as a precursor of reductiomycin. This observation does not tell whether 4-hydroxybenzaldehyde is a true intermediate on the pathway or whether its incorporation only reflects an artifactual oxidation to 4-hydroxybenzoic acid and not the normal biosynthetic pathway.
Figure 36. Incorporation of [7-\(^{13}\)C]- and [7-\(^2\)H,7-\(^{13}\)C]-hydroxybenzaldehyde into reductioycin by cultures of \textit{S. xanthochromogenus}
5. Feeding of \([7^{-13}C,7^{-2}H}]-4\text{-hydroxybenzaldehyde}

\([7^{-13}C,7^{-2}H}]-4\text{-Hydroxybenzaldehyde was fed to Streptomyces xanthochromogenus to determine if 4-hydroxybenzaldehyde is a true intermediate which occupies a stage later than 4-hydroxybenzoic acid in the biosynthetic pathway to reductiomycin. Deuterium and }^{13}C \text{ from } [7^{-13}C, 7^{-2}H] \text{-4-hydroxybenzaldehyde would be incorporated into reductiomycin if 4-hydroxybenzaldehyde were a later intermediate but only incorporation of }^{13}C \text{ would be observed if it were incorporated only via the acid, because oxidation of } [7^{-13}C,7^{-2}H}]-4\text{-hydroxybenzaldehyde to the carboxylic acid would wash out the deuterium label from the aldehyde.}

Reductiomycin biosynthesized from \([7^{-13}C,7^{-2}H}]-4\text{-hydroxybenzaldehyde showed }4\% ^{13}C\text{-enrichment and }3\% ^{2}H\text{-enrichment at the C-5'} \text{ position. }^{13}C\text{-NMR analysis revealed that }75\% \text{ of the }^{13}C \text{ was incorporated intact with the original deuterium attached; }25\% \text{ of the deuterium had exchanged with solvent protons producing reductiomycin labeled with }^{13}C, \text{ but not with }^{2}H. \text{ The relatively low enrichment in this experiment compared to the earlier experiment with } [7^{-13}C]-4\text{-hydroxybenzaldehyde is a result of biological variation between cultures, and to some}
extent also reflects the fact that a smaller amount of precursor was fed. The result from this feeding experiment with [7-\textsuperscript{13}C,7-\textsuperscript{2}H]-4-hydroxybenzaldehyde establishes 4-hydroxybenzaldehyde as a later intermediate in the pathway, past 4-hydroxybenzoic acid, and suggests that the ring cleavage occurs at the stage of 4-hydroxybenzaldehyde, rather than the acid.

D. Fermentation in an \textsuperscript{18}O\textsubscript{2}-Containing Atmosphere

To obtain information on the origin of the oxygen atoms in reductiomyacin, a fermentation was carried out in a closed system containing \textsuperscript{18}O\textsubscript{2} gas. A 500mL baffled flask containing a normally grown 24h old production culture of \textit{Streptomyces xanthochromogenus} was connected to the system shown in Figure 37. A modified Optima aquarium pump was employed to circulate the atmosphere and 5N aqueous KOH solution was used to trap expired carbon dioxide. A one liter bottle of \textsuperscript{18}O\textsubscript{2} gas was connected to the system so that labeled oxygen can diffuse into the system and a slightly positive pressure was maintained inside the system using a water reservoir. After 30h fermentation under \textsuperscript{18}O\textsubscript{2} atmosphere reductiomyacin was isolated and purified, and the resulting material (18mg) was analyzed by mass spectrometry (Table 4). Significant enhancement of the (M+2)\textsuperscript{+} peak (m/z=295), but not the
Figure 37. Diagram of the system for fermentation in \(^{18}O\)-containing atmosphere
(M+4)$^+$ peak, indicated incorporation of a single atom of $^{18}O$. Absence of enhancement of the (M-AcOH+2)$^+$ peak (m/z=235) established the acetoxy function as the $^{18}O$ incorporation site. Of the two oxygen atoms of the acetoxy group, the carbonyl oxygen is unlikely to be the $^{18}O$ incorporation site because the acetyl group in reductiomycin is known to arise from acetate, presumably via acetyl-CoA. The bridge oxygen atom connecting the acetyl group to C-2' is thus suggested as the labeled position with 25% $^{18}O$ enrichment.

Table 4. Result of mass spectral analysis of reductiomycin obtained from fermentation of S. xanthochromogenus in $^{18}O$-containing atmosphere

<table>
<thead>
<tr>
<th>m/z</th>
<th>233$^1$</th>
<th>234</th>
<th>235</th>
<th>236</th>
<th>237</th>
<th>293$^2$</th>
<th>294</th>
<th>295</th>
<th>296</th>
<th>297</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std.</td>
<td>100</td>
<td>24</td>
<td>2.9</td>
<td>3.2</td>
<td>0.7</td>
<td>100</td>
<td>15</td>
<td>2.7</td>
<td>-</td>
<td>1.1</td>
</tr>
<tr>
<td>Labeled</td>
<td>100</td>
<td>17</td>
<td>5.4</td>
<td>1.0</td>
<td>1.0</td>
<td>100</td>
<td>20</td>
<td>28</td>
<td>6.2</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Std.: Nonlabeled reductiomycin  
Labeled: Reductiomycin from fermentation in $^{18}O$-containing atmosphere

1) m/z 233 is M-AcOH peak  
2) m/z 293 is molecular ion peak  
3) Relative abundance of m/z=293 : m/z=233 is 40:100
$^{13}$C-NMR analysis of the reductiomycin sample obtained from fermentation in an $^{18}$O atmosphere gave the results confirming that of the mass spectral analysis. Detection of upfield shifts induced in the carbon spectrum by isotope substitution with $^{18}$O is a proven method for biosynthetic and mechanistic studies which can detect the carbon atom(s) directly connected to $^{18}$O atom. We applied the same principle to $^{18}$O-enriched reductiomycin to identify the incorporation site. Examination of the resonances assigned to C-2'(δ98.55) and to the carbonyl carbon (δ169.46) of the acetyl group revealed upfield-shifted peaks (0.028 and 0.034 ppm respectively, Figure 37), indicating $^{18}$O-enrichment at the bridge oxygen atom connecting the acetyl group to C-2'. No upfield-shifted peak was observed for C-1''(δ165.87) and this result implies no $^{18}$O-enrichment at the amide oxygen.
Figure 38. $^{13}$C-NMR spectrum of reductiomyin from fermentation in an $^{18}$O-containing atmosphere.
Figure 37. $^{13}$C-NMR spectrum of reductiomyacin from fermentation in an $^{18}$O-containing atmosphere (continued)
Figure 37. $^{13}$C-NMR spectrum of reductiomycin from fermentation in an 18O-containing atmosphere (continued)
As was described in the Results section, H-2 and H-6 / H-3 and H-5 of 4-hydroxybenzoic acid labeled H-3' and H-3" / H-2" and H-2' of reductiomycin. These results are consistent with those expected for the pathway of reductiomycin formation proposed by Beale et al.⁶³ which envisions that the dihydrofuranylacrylic acid moiety of reductiomycin is formed via ring cleavage between C-3 and C-4 of 4-hydroxybenzoic acid or a closely related compound. It is noteworthy that the deuterium enrichment at H-2" was only about half that of the enrichments at H-3', H-3", and H-2'. Feeding experiments with 4-hydroxybenzaldehydes labeled with $^{13}$C and $^2$H at the aldehyde group established the fact that the ring cleavage occurs at the stage of 4-hydroxybenzaldehyde, not the acid. The results described above provide evidence supporting the earlier part of the pathway proposed by Beale et al.⁶³ However, their assumption on the mechanism of the ring cleavage reaction had to be dismissed when the fermentation in an $^{18}$O atmosphere was carried out. The idea implicit in the pathway proposed by Beale et al. on the
mode of the ring cleavage reaction of 4-hydroxybenzaldehyde was that the ring cleavage occurs by addition of an intact oxygen molecule to a double bond between C-3 and C-4 of 4-hydroxybenzaldehyde in a dioxygenase reaction, followed by a retro [2+2]cycloaddition type ring cleavage. This mode of aromatic ring cleavage has been encountered often in microorganisms. However, this idea was disproved because $^{18}O$ was incorporated only at the acetoxy function in reductiomycin and the amide oxygen was not enriched with $^{18}O$ at all. The site of $^{18}O$ incorporation at 4-hydroxybenzaldehyde is obviously C-3, but the incorporation of deuterium from $[3,5-^{2}H_{2}]-4$-hydroxybenzaldehyde at C-2' of reductiomycin rules out the intermediacy of 3,4-dihydroxybenzaldehyde. In view of the above considerations we propose a mechanism for the ring cleavage of 4-hydroxybenzaldehyde in reductiomycin biosynthesis as shown in Figure 39. In the proposed mechanism, invoking a flavin-dependent monooxygenase, the intermediate 73 is attacked by a molecule of water rather than aromatizing as in the 4-hydroxybenzoate hydroxylase reaction. This mechanism accommodates well the result of the $^{18}O$ incorporation experiment and the deuterium incorporation patterns from feeding experiments with deuterated 4-hydroxybenzoic acids, except for the partial loss of the deuterium at C-2'' of reductiomycin. Part of the deuterium
Figure 39. Proposed mechanism for the conversion of 4-hydroxybenzaldehyde to acetoxydihydrofuranylacrylic acid moiety in reductiomyein.
atoms at C-2" of reductiomycin are thought to be exchanged with solvent protons during cis-trans isomerization of the double bond between C-2" and C-3" of reductiomycin. It was also concluded that intermolecular transfer of deuterium atoms has not occurred during the conversion of 4-hydroxybenzoic acid to the dihydrofuranylacrylic acid moiety of reductiomycin. This observation provides an important boundary condition on the mode of cis-trans isomerization. Of the two most common mechanisms A and B in Figure 40, A produces reductiomycin with complete retention of \( ^2\)H at C-2". On the other hand, via mechanism B, reductiomycin will either lose all deuterium labeling at C-2" or intermolecular transfer of deuterium should be observed. Assuming addition-elimination of a nucleophile as a necessary steps in the double bond isomerization, mechanism C can be proposed to accommodate the boundary conditions described above. In the proposed mechanism addition of a nucleophile to the double bond of the substrate is accompanied by the addition of a proton from a solvent water molecule on either face of the double bond. Rotation around the newly generated single bond followed by abstraction of a proton or a deuteron generates the product with trans double bond configuration. Intermolecular recycling of deuterium is diluted below the detection limit by the exchange of the
Mechanism A: Complete retention of deuterium

Mechanism B: Complete loss of deuterium
Intermolecular transfer of deuterium

Mechanism C: Partial loss of deuterium
Negligible intermolecular transfer of deuterium

Figure 40. Possible mechanisms for cis-trans double bond isomerization
Figure 41. Modified mechanism for the biosynthesis of reductiomyacin
water molecules in the enzyme active site with those outside the active site. The major reservation about this mechanism is the fact that either proton addition or proton abstraction, or both, must be nonstereospecific, i.e., not mediated by a functional group of the enzyme. Finally, a modified mechanism for reductiomyacin biosynthesis is shown in Figure 41.
CHAPTER IV
EXPERIMENTAL

A. Materials and General Methods

1. Materials

All chemicals and solvents for synthetic reactions were of reagent grade and were used without further purification unless otherwise noted. Reaction solvents were purified by distillation from appropriate drying agents; THF (Na/benzophenone), CH₂Cl₂ (P₂O₅), ether (Na/benzophenone), DMF (CaH₂). Other solvents were purified as described in reference 46. Materials for fermentation medium preparation were purchased from Difco. Oxygen-18 gas and ¹³CO₂ gas were purchased from Cambridge Isotope Lab. and ISOTEC Inc., respectively.

2. General Methods

Melting points were determined on a Mel-Temp Laboratory device and are uncorrected. IR spectra were recorded on a Mattson Polaris FT-IR with absorptions being cited. The ¹H, ¹³C, and ²H NMR spectra were obtained on an IBM AF-300 spectrometer. Broadband proton decoupling was employed for ¹³C-NMR if not otherwise noted. Chemical
shifts are given in parts per million (ppm) relative to (CH$_3$)$_4$Si as internal standard or adjusted to the (CH$_3$)$_4$Si scale by reference to the solvent signal. Coupling constants (J) are given in Hertz (Hz). Splitting patterns are designated: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Mass spectra were recorded on a DS-55 mass spectrometer. Routine GC/MS identification of synthetic intermediates was performed on a Hewlett-Packard 5790A gas chromatograph with 5970A mass selective detector. Analytical TLC was carried out on precoated silica gel 60F-254. Compounds on the plates were visualized under UV light or by spraying with a p-anisaldehyde solution (3.7mL p-anisaldehyde, 135mL C$_2$H$_5$OH, 5mL conc.H$_2$SO$_4$, 1.5mL glacial acetic acid), phosphomolybdic acid (Aldrich), or KMnO$_4$ solution (10g, KMnO$_4$ in 1L of 1N NaOH), and heating at 120°C. Mobilities are quoted relative to the solvent front($R_f$). Column chromatography was performed on 230-400 mesh silica gel from Aldrich.

B. Synthesis of Labeled Precursors

1. Synthesis of [3,5-$^2$H$_2$]-4-hydroxybenzoic acid (54)

3,5-Dibromo-4-hydroxybenzoic acid (7.39g, 25mmol) was added to a suspension of zinc dust (9.8g, 0.15mol) in CH$_3$COOD (13.5mL)/D$_2$O (48mL) and the reaction mixture was
heated to reflux for 3 days. After cooling to room
temperature it was extracted with ether (4 * 50mL). The
combined ether layer was dried(MgSO₄) and concentrated to
give 3.14g(90%) of [3,5-²H₂]-4-hydroxybenzoic acid as a
white solid: mp. 202-205°C; ¹H-NMR (acetone-d₆,300MHz)
δ7.93(s,2H)

2. Synthesis of [2,6-²H₂]-4-hydroxybenzoic acid (64)
4-Bromophenol-d₄ (56)

To an ice-cold solution of phenol-d₆ (98% ²H, 2.04g,
20.4mmol) in dry ether(3mL) was added dioxane dibromide
over 3h. Progress of the reaction was checked
occasionally by GC-MS. The reaction was quenched after
95% completion by addition of water (5mL). The organic
layer was separated and the water layer was extracted with
ether (4 * 20mL). The combined organic layer was dried
(MgSO₄) and concentrated to give 2.7g of crude 4-
bromophenol-d₄ which contained approximately 6% of the
ortho isomer and 6% dibromo compound as well as 5% of
unreacted starting material. The crude product was used
for the next reaction without further purification.
56: Rₖ 0.51 (hexane-EtOAc 2:1); GC-MS (m/z, rel intensity)
178(96), 176(100), 97(32), 69(97), 41(39)
[2,3,5,6-$^2$H$_4$]-4-Bromoanisol (57)

To a solution of the 4-bromophenol-$d_4$ obtained above in dry acetone (20mL) was added K$_2$CO$_3$ (3.2g) and CH$_3$I (3mL) and the mixture was gently refluxed for 12h. The resulting mixture was filtered and the remaining solid was washed with dry acetone (5mL). Concentration of the organic layer afforded 3.40g of crude 57: $R_f$ 0.66 (hexane-EtOAc 3:1)

[2,3,5,6-$^2$H$_4$]-4-Methoxybenzoic acid (58)

A two neck-flask fitted with a condenser and a rubber septum was charged with magnesium (545mg, 22.4mmol). 4-Bromoanisol-$d_4$ (3.37g, 17.7mmol) obtained above was dissolved in dry THF (10mL) and 3mL of this solution was introduced into the flask containing magnesium. The mixture was heated to initiate the reaction. When the reaction had started the remaining solution of 4-bromoanisol-$d_4$ was added over a 5 minute period. After the reaction had subsided the reaction mixture was heated to reflux for 30 min. After cooling to room temperature carbon dioxide was bubbled into the Grignard solution for 10 minutes and then 2N HCl solution was added to the reaction mixture until no more precipitate formed. The mixture was extracted with ether (9 * 20mL) and the
extract was dried (MgSO₄), and concentrated to give 2.27g of 58: Rf 0.24 (hexane-EtOAc 2:1)

[2,3,5,6-²H₄]-4-Hydroxybenzoic acid (59)

To the 4-methoxybenzoic acid (2.27g) obtained above in dry CH₂Cl₂ (40mL) was added 20mL of a 1M solution of BBr₃ in CH₂Cl₂, and the mixture was stirred under an N₂ atmosphere. After 8h at room temperature the reaction was quenched by addition of ca.5mL of ice-water. The mixture was then extracted with ether (5 * 50mL), dried (MgSO₄), and concentrated to obtain 1.52g of crude 4-hydroxybenzoic acid-d₄. The crude product can be purified by recrystallization from water. 59: mp. 204-207°C

[2,6-²H₂]-3,5-Dibromo-4-hydroxybenzoic acid (60)

To an ice-cold solution of the crude 59 (1.52g) obtained above in 30mL of dioxane-ether (1:1) mixture was added dioxane dibromide over a 2h period. Aliquots of the reaction mixture were taken, treated with a solution of diazomethane to be checked by GC-MS analysis. The reaction was quenched after 95% completion by addition of water (15mL). The resulting mixture was extracted with ether (5 * 20mL), dried (MgSO₄), and concentrated to give a yellow solid.
Methyl [2,6-\(^{2}\text{H}_2\)]-3,5-dibromo-4-methoxybenzoate (61)

To a solution of the [2,6-\(^{2}\text{H}_2\)]-3,5-dibromobenzoic acid obtained in the previous reaction in dry acetone (50mL) was added CH\(_3\)I (7mL) and K\(_2\)CO\(_3\) (7g), and the mixture was refluxed overnight. The resulting crude methyl [2,6-\(^{2}\text{H}_2\)]-3,5-dibromo-4-methoxybenzoate containing tribromide and other impurities was readily purified by column chromatography. The overall yield was 2.20g or 33% from phenol-d\(_6\). 61: R\(_f\) 0.51 (hexane-\text{EtOAc} 4:1); mp. 91-93°C; GC-MS (m/z, rel intensity) 328(49), 326(99), 324(50), 297(50), 295(100), 293(52)

Methyl [2,6-\(^{2}\text{H}_2\)]-4-methoxybenzoate (62)

Methyl [2,6-\(^{2}\text{H}_2\)]-3,5-dibromo-4-methoxybenzoate (1.95g, 6.0mmol) obtained above was added to a suspension of zinc dust (2.30g) in CH\(_3\)COOD (3mL)/D\(_2\)O (11mL) and the mixture was refluxed for 3 days. Extraction of the reaction mixture with ether (3 * 30mL) followed by concentration afforded 1.04g of 62: R\(_f\) 0.68 (hexane-\text{EtOAc} 2:1); \(^1\text{H}\)-NMR (acetone-d\(_6\), 300MHz) \(\delta\) 3.86 (s,3H), 3.82 (s,3H), 7.94(s,2H)

[2,6-\(^{2}\text{H}_2\)]-4-Methoxybenzoic acid (63)

Methyl [2,6-\(^{2}\text{H}_2\)]-4-methoxybenzoate (1.04g) obtained above was added to 10% aqueous NaOH solution (15mL) and
refluxed for 1h. After cooling to room temperature the reaction mixture was acidified to pH 1 by addition of 2N HCl. It was then extracted with ether (9 * 15mL), dried (MgSO₄), and concentrated to obtain 722mg of [2,6-²H₂]-4-methoxybenzoic acid: mp. 177-178°C

[2,6-²H₂]-4-Hydroxybenzoic acid (64)

The crude [2,6-²H₂]-4-methoxybenzoic acid (722mg) obtained above was dissolved in dry CH₂Cl₂ (10mL). Boron tribromide in CH₂Cl₂ (6mL of a 1M solution) was then added to the stirred solution. The mixture was stirred 8h at room temperature and the reaction was quenched by addition of ice-water (10mL). After separation of the CH₂Cl₂ layer, the aqueous layer was extracted with ether (8 * 20mL). The combined organic layers were dried, and concentrated to give 630mg (75% from 61) of [2,6-²H₂]-4-hydroxybenzoic acid. This can be further purified by recrystallization from water. 64: Rf 0.61 (hexane-EtOAc 40:20:1); mp. 203-206°C; ¹H-NMR (acetone-d₆, 300MHz) 66.92 (s)

3. Synthesis of [2,3,5,6-²H₄,7-¹³C]-4-hydroxybenzoic acid (65)

The procedure used to prepare [2,3,5,6-²H₄]-4-hydroxybenzoic acid was repeated except that ¹³CO₂ (99% ¹³C) was employed instead of nonlabeled CO₂.
4. Synthesis of $[7^{-13}\text{C}]$-4-hydroxybenzaldehyde (70)

**Methyl $[7^{-13}\text{C}]$-4-methoxybenzoate (67)**

$[7^{-13}\text{C}]$-4-methoxybenzoic acid was prepared from 4-bromoanisol in the same way as described earlier for $[2,3,5,6^{-2}\text{H}_4]$-4-methoxybenzoic acid except that $^{13}\text{CO}_2$ was used instead of $^{12}\text{CO}_2$. A mixture of $[7^{-13}\text{C}]$-4-methoxybenzoic acid (2.45g, 16.0mmol), CH$_3$I (6mL), and K$_2$CO$_3$ (2.5g) in acetone (30mL) was refluxed for 24h. The mixture was then filtered and the filtrate was concentrated to give a quantitative yield of 67 (2.65g).

**[7-$^{13}\text{C}$]-4-Methoxybenzyl alcohol (68)**

The crude methyl $[7^{-13}\text{C}]$-4-methoxybenzoate (2.65g, 16.0mmol) in THF (20mL) was added slowly to a suspension of LiAlH$_4$ (1.0, 26mmol) in THF (40mL) and the mixture was refluxed for 5h. After cooling to room temperature water (5mL) was added slowly and the resulting mixture was refluxed for 15 min. It was filtered and the cake was suspended in THF (80mL) and refluxed for 10 min. This was filtered again and the organic filtrates were combined and concentrated. The resulting liquid was taken up in ether (200mL), dried (MgSO$_4$), and concentrated to give 2.10g of $[7^{-13}\text{C}]$-4-methoxybenzyl alcohol: $R_f$ 0.59(hexane-EtOAc 4:1)
[7-13C]Anisaldehyde (69)

To a stirred solution of the alcohol (2.10g, 15.1mmol) obtained above in dry CH2Cl2 (100mL) was added PCC (3.5g, 16.2mmol) and powdered molecular sieve (8g). After 2h at room temperature the reaction mixture was filtered and the solid was washed with CH2Cl2 (2 * 40mL). The combined CH2Cl2 layers were concentrated and purified by column chromatography to give [7-13C]-anisaldehyde in 89% yield from 66.

[7-13C]-4-hydroxybenzaldehyde (70)

To a stirred solution of [7-13C]anisaldehyde (1.23g, 8.91mmol) in dry CH2Cl2 (80mL) was added B13 (3.5g) quickly. The resulting mixture was stirred vigorously for 2 minutes and the reaction quenched by addition of water (10mL). After separation of the CH2Cl2 layer the aqueous layer was extracted with ether (4 * 50mL). The combined organic layers were concentrated and purified by column chromatography to obtain 643mg (58%) of 70: mp. 114-115°C; 1H-NMR (DMSO-d6, 300MHz) 66.91 (d, 2H, J=8.5Hz), 7.75 (dd, 2H, J=8.5Hz, 4.7Hz), 9.78 (d, 1H, J=172.9Hz); 13C-NMR (DMSO-d6, 300MHz) 6190.01
5. Synthesis of [7-\textsuperscript{13}C, 7-\textsuperscript{2}H]-4-hydroxybenzaldehyde (72)

The procedure used to prepare [7-\textsuperscript{13}C]-4-hydroxybenzaldehyde was repeated except that LiAlD\textsubscript{4} was employed instead of LiAlH\textsubscript{4} in the reduction step. 72: mp. 115-116\textdegree C; \textsuperscript{1}H-NMR (DMSO-d\textsubscript{6}, 300MHz) \delta 6.92 (2H,d, J=8.5Hz), 7.75 (2H,dd, J=8.5Hz, 4.6Hz), 10.52 (1H, br.s); \textsuperscript{13}C-NMR (DMSO-d\textsubscript{6}, 300MHz) \delta 190.17, 190.52, 190.87

C. Fermentation

\textit{Streptomyces xanithochromogenus} AM 6201 was maintained on yeast-malt extract agar (Difco) and sporulated agar slants were used to inoculate liquid media. Seed medium containing 1.0g of glucose, 2.0g of peptone, 5.0g of yeast extract, and 4.0g of CaCO\textsubscript{3} in 100mL of distilled water was adjusted to pH 7.0 with 1N NaOH solution and autoclaved at 121\textdegree C for 20 min in a 500mL baffled flask. This medium was then inoculated with a loopful of spores from a sporulated agar slant and incubated for 3 days on a rotary shaker at 28\textdegree C and 300rpm. Of the seed culture, 5mL was used to inoculate 100mL of sterilized production medium in a 500mL baffled flask. The production medium consisted of 2.0g of glucose, 2.0g of Japanese soybean meal, and 0.3g of NaCl in 100mL of distilled water, adjusted to pH 7.0 with 1N NaOH solution. Inoculated production culture were
again grown for 72h at 28°C with rotary shaking at 300rpm. Reductiomycin was then isolated as described later.

D. Precursor Incorporation

Labeled precursors (3.6mmol per liter of production culture, 1.6mmol/L in case of [7-2H,7-13C]-4-hydroxybenzaldehyde) were dissolved in minimum amounts of distilled water and adjusted to pH 7.0 with solid NaHCO₃. They are added to 24h old production cultures through a Millex disposable sterile filtration unit. The labeled reductiomycin was harvested 48h later.

E. Isolation and Purification of Reductiomycin

The cultures (100mL) were shaken with 5mL of 1N HCl and 100mL of EtOAc for 1 min and sonicated for 10 min at maximum power setting. The EtOAc layer was separated and the same process was repeated once more without addition of HCl. The combined EtOAc extracts were concentrated, and the resulting crude oily product was triturated with petroleum ether. Crystallization of reductiomycin was induced by addition of a small amount of MeOH. The methanolic mother liquor was removed from the solid reductiomycin using a small pipet. The crude reductiomycin was then purified by recrystallization from MeOH. About 25mg of pure reductiomycin was usually obtained from
100mL of production culture. In general further purification was not necessary but repeated recrystallization gave better purity.

F. Biosynthesis of Reductiomycin in the presence of $^{18}\text{O}$Oxygen Gas

A 24h old production culture of *S. xanthochromogenus* was connected to the closed system shown in Figure 37. The oxygen reservoir contained 1L of 98% $^{18}\text{O}$-enriched oxygen gas. The system utilizes a pump to circulate air and the air in the fermentation flask was kept in contact with the circulating air through a sponge plug. A modified Optima aquarium pump (Rolf Hagen Inc., Montreal, Canada), which had been sealed in glass, was used to circulate the gas at 4L/min. Expired CO$_2$ was removed by passing the air stream over 1.5L of freshly prepared 5N KOH. Concentrated H$_2$SO$_4$ was employed to remove excess moisture from the air stream. An adequate level of moisture was supplied to the air stream by passing it over water. Slightly positive pressure was maintained in the system by a pressure-equalizing buret (2L) containing 1N CuSO$_4$ solution. The average oxygen consumption rate was approximately 40mL per hour per 100mL of fermentation medium. Extraction of the mixture and purification as
described above for other feeding experiments gave 20 mg of pure reductiomyacin.
APENDIX A

Assignment of $^1$H- and $^{13}$C-NMR signals of reductiomyacin
Table 5. Assignment of $^1$H-NMR signals of reductiomyacin

<table>
<thead>
<tr>
<th>Chemical shift (ppm)</th>
<th>Assignment</th>
<th>Coupling constants (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.14 (s,3H)</td>
<td>CH$_3$C(O)</td>
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</tr>
<tr>
<td>2.46-2.63 (m,4H)</td>
<td>4-H$_2$, 5-H$_2$</td>
<td>-</td>
</tr>
<tr>
<td>2.68 (dm,1H)</td>
<td>3'</td>
<td>16.1</td>
</tr>
<tr>
<td>3.03 (dddd,1H)</td>
<td>3'</td>
<td>16.1, 7.5, 2.2, 1.0</td>
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<tr>
<td>5.81 (dd,1H)</td>
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<td>15.0, 0.7</td>
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<tr>
<td>6.71 (dd,1H)</td>
<td>2'</td>
<td>7.5, 2.4</td>
</tr>
<tr>
<td>6.84 (dt,1H)</td>
<td>5'</td>
<td>1.6, 0.6</td>
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<tr>
<td>7.47 (d,1H)</td>
<td>3''</td>
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<tr>
<td>7.76 (s,1H)</td>
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<tr>
<td>13.71 (s,1H)</td>
<td>OH</td>
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</table>

Table 6. Assignment of $^{13}$C-NMR signals of reductiomyacin

<table>
<thead>
<tr>
<th>Chemical shift (ppm)</th>
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<tr>
<td>20.92</td>
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<td>CH$_3$C(O)</td>
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APENDIX B

NMR spectra of reductiomyacin samples obtained from feeding experiments with labeled precursors
Figure 42. $^1$H-NMR spectrum of nonlabeled reductiomyacin in CDC$_3$
Figure 42. $^1$H-NMR spectrum of nonlabeled reductiomycin in CDCl$_3$ (continued)
Figure 43. $^1$H-NMR spectrum of reductiomyacin from feeding experiment with [2,6-$^2$H$_2$]-4-hydroxybenzoic acid in CDCl$_3$
Figure 43. $^1$H-NMR spectrum of reductiomyacin from feeding experiment with $[2,6-^{2}H_2]$-4-hydroxybenzoic acid in CDCl$_3$ (continued)
Figure 43. $^1$H-NMR spectrum of reductiomyacin from feeding experiment with [2,6-2H$_2$]-4-hydroxybenzoic acid in CDCl$_3$ (continued)
Figure 44. $^2$H-NMR spectrum of reductiomyacin from feeding experiment with [2.6-$^2$H$_2$]-4-hydroxybenzoic acid in CDCl$_3$
Figure 45. $^1$H-NMR spectrum of reducimycin from feeding experiment with [3,5-²H₂]-4-hydroxybenzoic acid in CDCl₃.
Figure 45. $^1$H-NMR spectrum of reductiomyacin from feeding experiment with [3,5-$^2$H$_2$]-4-hydroxybenzoic acid in CDCl$_3$ (continued)
Figure 45. $^1$H-NMR spectrum of reductiomyacin from feeding experiment with [3,5-$^2$H$_2$]-4-hydroxybenzoic acid in CDCl$_3$ (continued)
Figure 46. $^2$H-NMR spectrum of reductiomyacin from feeding experiment with $[3,5-^2\text{H}_2]$-4-hydroxybenzoic acid in CDCl$_3$
Figure 47. $^1$H-NMR spectrum of reductiomyacin from feeding experiment with $[2,3,5,6-^{2}$H$_4,7-^{13}$C]-4-hydroxybenzoic acid in CDCl$_3$
Figure 47. $^1$H-NMR spectrum of reductomycin from feeding experiment with [2,3,5,6-$^2$H$_2$,7-$^{13}$C]-4-hydroxybenzoic acid in CDCl$_3$ (continued)
Figure 47. $^1$H-NMR spectrum of reductiomyacin from feeding experiment with [2,3,5,6-$^2$H$_2$,7-$^{13}$C]-4-hydroxybenzoic acid in CDCl$_3$ (continued)
Figure 48. $^2$H-NMR spectrum of reductomycin from feeding experiment with [2,3,5,6-$^2$H$_2$,7-$^{13}$C]-4-hydroxybenzoic acid in CDCl$_3$
Figure 49. $^{13}$C-NMR spectrum of reductiomyein from feeding experiment with [2, 3, 5, 6-2H$_2$, 7-$^{13}$C]-4-hydroxybenzoic acid in CDCl$_3$.
Figure 50. $^{13}$C-NMR spectrum of reductiomyacin from feeding experiment with [7-$^{13}$C]-4-hydroxybenzaldehyde in CDCl$_3$. 
Figure 51. $^1$H-NMR spectrum of reductionycin from feeding experiment with [7-$^{13}$C]-4-hydroxybenzaldehyde in CDCl$_3$
Figure 51. $^1$H-NMR spectrum of reductiomyacin from feeding experiment with [7-$^{13}$C]-4-hydroxybenzaldehyde in CDCl$_3$ (continued)
Figure 52. $^{13}$C-NMR spectrum of reductionmycin from feeding experiment with [7-$^2$H, 7-$^{13}$C]-4-hydroxybenz-aldehyde
Figure 53. $^1$H-NMR spectrum of reductiomycin from feeding experiment with [7-$^2$H,7-$^{13}$C]-4-hydroxybenzaldehyde in CDCl$_3$ (continued)
A: broadband proton decoupled, deuterium coupled spectrum
B: broadband proton deuterium decoupled spectrum
REFERENCES


33. In the original paper of Furukawa et al. C-6 in this thesis appears as C-2 and vice versa. We changed their numbering according to the systematic IUPAC numbering system, and this system is used all through this thesis.


43. Kennedy, E.; unpublished results.

44. Reynold, K.; unpublished results.


