Studies on the Biosynthesis of the Antibiotic Reductiomycin in *Streptomyces xanthochromogenus*

Hyeongjin Cho,[†,‡,∥] John M. Beale,[†,‡,⊥] Cynthia Graff,[†] Ursula Mocek,[†] Akira Nakagawa,[§,#] Satoshi Ōmura,[§] and Heinz G. Floss*[,†,‡,√]

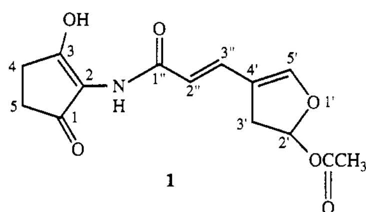
Contribution from the Departments of Chemistry, University of Washington, Seattle, Washington 98195 and The Ohio State University, Columbus, Ohio 43210, and School of Pharmaceutical Sciences, The Kitasato University and Kitasato Institute, Minato-ku, Tokyo 108, Japan

Received June 16, 1993[⊗]

Abstract: The biosynthesis of the antibiotic reductiomycin (**1**) in *Streptomyces xanthochromogenus* was investigated by feeding experiments with radioactive and stable isotope-labeled precursors. NMR and mass spectroscopic analyses of the labeled **1** samples revealed that the acetoxy group comes from acetate, the 2-amino-3-hydroxycyclopent-2-enone moiety arises by a novel intramolecular cyclization of 5-aminolevulinic acid (ALA), and the dihydrofuranylacrylic moiety is formed by aromatic ring cleavage of a symmetrical product of the shikimate pathway. Both 4-hydroxy-[7-^{13}C]benzoic acid and 4-hydroxy-[7-^{13}C]benzaldehyde label **1** very efficiently, and deuterium from various positions in these precursors is incorporated into the predicted positions in the dihydrofuranylacrylic acid moiety of **1**. The results are interpreted in terms of a dioxygenase mechanism for the ring cleavage reaction and pyridoxal phosphate catalysis for the ALA cyclization.

Introduction

Reductiomycin (**1**), an antibiotic produced in culture by several strains of *Streptomyces*, possesses antitumor activity[1] as well as weak activity against Gram-positive bacteria and fungi.[2] The



1

original isolation of reductiomycin was reported by Tamura *et al.*,[3] who purified the compound from *Streptomyces griseorubiginosus*. In the initially reported structure, based on X-ray crystallography[4] and spectroscopic studies,[5] the nitrogen and 1′-oxygen atoms were reversed relative to structure **1**. After isolation of reductiomycin from two other bacteria, *Streptomyces xanthochromogenus*[1] and *Streptomyces orientalis*,[5] the structure was revised, on the basis of chemical and spectroscopic data, to that given for **1**. Any remaining ambiguity was eliminated by a total synthesis of racemic reductiomycin.[6] While there is now no doubt about the correctness of structure **1**, the absolute configuration of reductiomycin remains in question.

Reductiomycin consists of two unique structural units, the biosyntheses of which have attracted our attention for some time. One is a 2-amino-3-hydroxycyclopent-2-enone moiety (C_5N unit), which is also found in the antibiotics asukamycin,[7,8] manumycin,[9] moenomycin,[10,11] senecarcin A,[12] virustomycin A,[13] bafilomycin B_1,[14] and L-155,175.[15] The other is an unusual acetoxydihydrofuran unit bearing an acrylic acid side chain. Working with *S. xanthochromogenus* AM-6201, we have elucidated the biosynthetic origins of both of these units. Some of our results have been published in preliminary form.[16] In the present paper we provide a complete account of this work.

Results

NMR Assignments. Unequivocal assignments for all of the proton and ^{13}C NMR signals were made for **1**. Initial proton assignments were determined by analysis of the coupling patterns, multiplicities, and chemical shift parameters. These were very straightforward for this relatively simple system and led readily to a complete ^{13}C assignment from a ^{1}H/^{13}C heteronuclear correlation experiment.[17] The amide proton signal was readily identified by its relative broadness, and the enolic -OH proton was detected as a high frequency (ca. δ 13.0 ppm) singlet. The

† University of Washington.
‡ The Ohio State University.
§ The Kitasato University and Kitasato Institute.
∥ Present address: Department of Chemistry, Inha University, Inchon 402-751, Korea.
⊥ Present address: Division of Medicinal Chemistry, College of Pharmacy, University of Texas, Austin, TX 78712.
\# Present address: Department of Biosciences, Teikyo University, Utsunomiya 320, Japan.
√ To whom reprint requests should be addressed at Department of Chemistry BG-10, University of Washington, Seattle, WA 98195.
⊗ Abstract published in *Advance ACS Abstracts*, December 1, 1993.

(1) Konda, Y.; Onda, K.; Hinotozawa, K.; Ōmura, S. *J. Antibiot.* **1981**, *34*, 1222.
(2) Shimizu, K.; Tamura, G. *J. Antibiot.* **1981**, *34*, 649.
(3) Hirayama, N.; Shimizu, K.; Shirahata, K.; Ueno, K.; Tamura, G. *Agric. Biol. Chem.* **1980**, *44*, 2083.
(4) Shimizu, K.; Tamura, G. *J. Antibiot.* **1981**, *34*, 654.
(5) Shizuri, Y.; Ojika, M.; Yamada, K. *Tetrahedron Lett.* **1981**, *22*, 4291.
(6) Ojika, M.; Niwa, H.; Shizuri, Y.; Yamada, K. *J. Chem. Soc., Chem. Commun.* **1982**, 628.
(7) Kakinuma, K.; Ikekawa, N.; Nakagawa, A.; Ōmura, S. *J. Am. Chem. Soc.* **1979**, *101*, 3402.
(8) Ōmura, S.; Kitao, C.; Tanaka, H.; Oiwa, R.; Takahashi, Y.; Nakagawa, A.; Shimada, M.; Iwai, Y. *J. Antibiot.* **1976**, *29*, 876.
(9) Schröder, K.; Zeeck, A. *Tetrahedron Lett.* **1973**, 4995.
(10) Welzel, P.; Witteler, F. J.; Muller, D.; Riemer, W. *Angew. Chem.* **1981**, *93*, 130.
(11) Tschesche, R.; Lenoir, D.; Weidenmuller, H. *Tetrahedron Lett.* **1969**, 141.
(12) Nakano, H.; Yoshia, M.; Shirahata, K.; Ishii, S.; Arai, Y.; Morimota, M.; Tomita, F. *J. Antibiot.* **1982**, *35*, 760.
(13) Ōmura, S.; Imamura, N.; Hinotozawa, K.; Otoguro, K.; Lukacs, G.; Faghih, R.; Tolmann, R.; Arison, R. H.; Smith, J. L. *J. Antibiot.* **1983**, *36*, 1783.
(14) Werner, G.; Hagenmaier, H.; Drautz, H.; Baumgartner, A.; Zähner, H. *J. Antibiot.* **1984**, *37*, 110.
(15) Goetz, M. A.; McCormick, P. A.; Monaghan, R. L.; Ostlind, D. A.; Hensens, O. D.; Liesch, J. M; Albers-Schönberg, G. *J. Antibiot.* **1985**, *38*, 161.
(16) Beale, J. M.; Lee, J. P.; Nakagawa, A.; Ōmura, S.; Floss, H. G. *J. Am. Chem. Soc.* **1986**, *108*, 331.
(17) Bax, A.; Morris, G. *J. Magn. Reson.* **1981**, *42*, 501.

0002-7863/93/1515-12296$04.00/0 © 1993 American Chemical Society

Table I. ^1H NMR Signal Assignments for Reductiomycin Measured in $CDCl_3$

chemical shift (ppm)	assignment	J_{HH} (Hz)
2.14 (s, 3 H)	CH_3CO	
2.46–2.63 (m, 4 H)	4-CH_2, 5-CH_2	
2.68 (dm, 1 H)	H-3′	16.1
3.03 (dddd, 1 H)	H-3′	16.1, 7.5, 2.2, 1.0
5.81 (dd, 1 H)	H-2″	15.0, 0.7
6.71 (dd, 1 H)	H-2′	7.5, 2.4
6.84 (dt, 1 H)	H-5′	1.6, 0.6
7.47 (d, 1 H)	H-3″	15.0
7.76 (s, 1 H)	NH	
13.71 (s, 1 H)	enolic OH	

^{13}C signals of C-1, C-2, and C-3, representing carbon atoms in tautomeric exchange, were observable as sharp lines if very dry solvent was used for NMR analysis. The presence of moisture caused these signals to broaden and to become difficult to observe. The proton signals for H-2′ and H-3′ were readily assignable by their couplings, which fit the Karplus relationship well. Tables I and II, respectively, show the complete proton and ^{13}C NMR assignments for **1**.

Biosynthesis of the 2-Amino-3-hydroxycyclopent-2-enone (C_5N) Moiety. Our working hypothesis for the biosynthesis of the C_5N unit centered around 5-aminolevulinic acid (**2**). This compound, which typically undergoes intermolecular condensations in the biosynthesis of porphyrin- and corrin-type compounds,[18] could, via a novel intramolecular cyclization, give rise to the entire C_5N unit. The chemistry of this cyclization can be rationalized as a pyridoxal phosphate mediated reaction, in which an α-carbanion is generated adjacent to the amino group of **2**. Attack of the carbanion on the carboxyl group would, after elimination of water, give rise to the C_5N unit (Figure 1). Our earlier experiments with asukamycin strongly supported this hypothesis.[19] They also suggested that ALA in *S. asukaensis is* formed via the Shemin pathway rather than via the C_5 pathway.[20] Consistent with a similar biosynthesis of **2** in *S. xanthochromogenus* from glycine and succinyl coenzyme-A, during which the carboxyl group of glycine is lost, [2-^{14}C]glycine but not [1-^{14}C]glycine was incorporated into **1** [specific incorporation 20.9% vs 0.8%, respectively (Table III)]. [1(4)-^{14}C]Succinate and [5-^{14}C]aminolevulinic acid were incorporated into **1** significantly, but less efficiently than glycine (2.3% and 1.8%) (Table III). The coupling pattern observed in **1** after feeding [1,2-$^{13}C_2$]acetate (Table II) also supports the precursor role of succinate. **1** shows high enrichment of carbons 1, 3, 4, and 5 of the C_5N unit and coupling of C-1 to C-5 and C-3 to C-4. The origin of the 2-amino-3-hydroxycyclopent-2-enone unit from **2** was unequivocally proven by feeding [4,5-$^{13}C_2$]aminolevulinic acid and observing the expected enrichments and couplings in the ^{13}C NMR spectrum of the resulting

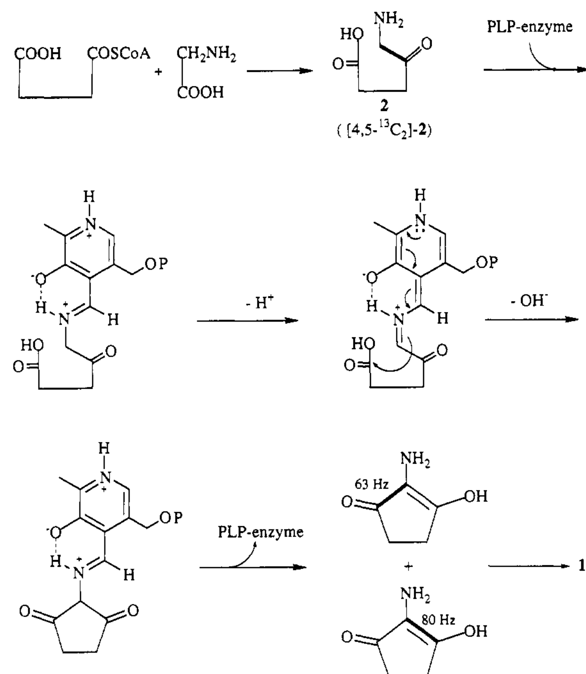


Figure 1. Labeling pattern of the C_5N unit of **1** from [4,5-$^{13}C_2$]-**2** and proposed mechanism for the intramolecular cyclization of **2**.

1. C-2 is twice as heavily enriched as C-1 and C-3; in half of the labeled molecules it is coupled to C-1, and in the other half to C-3, as expected for a system in tautomeric equilibrium (Figure 1, Table II).

Origin of the Acetoxydihydrofuran Unit. Inspection of the dihydrofuranylacrylic acid moiety of **1** suggests the possibility that the entire nine-carbon assembly, including the acetoxy group, could be derived by a ring cleavage of phenylalanine or tyrosine, or a derivative thereof, followed by a Baeyer–Villiger oxidation. In accord with this assumption, both [1,6-^{14}C]shikimic acid and L-[U-^{14}C]phenylalanine were incorporated (1.0% and 4.3%, respectively)(Table III). [1-^{14}C]Tyrosine, however, was not incorporated at all (0.02%), and analysis of the enrichment and coupling patterns of **1** labeled from [1,2-$^{13}C_2$]acetate (Table II) indicated that the acetoxy group is derived intact and with high efficiency from a molecule of acetate. These data clearly did not agree with the original assumption. In order to obtain additional information on the source of the remaining seven carbon atoms of the dihydrofuranylacrylic acid moiety, we took advantage of the efficient incorporation of glycerol (13–15%) and analyzed the labeling and ^{13}C–^{13}C coupling pattern of **1** derived from [U-$^{13}C_3$]glycerol. This precursor has, in our hands, proved to be

Table II. ^{13}C NMR Chemical Shift Assignments of **1**, ^{13}C-Labeling and ^{13}C–^{13}C Coupling Patterns in **1** Biosynthesized from ^{13}C-Labeled Precursors

		^{13}C-enrichment (%) ($^nJ_{CC}$, Hz, n = 1 or 2)			
chemical shift (ppm)	assignment	[U-$^{13}C_3$]glycerol	[1,2-$^{13}C_2$]acetate	[4,5-$^{13}C_2$]-**2**[a]	4-hydroxy-[7-^{13}C]benzoic acid
20.92	*CH*$_3$CO	4.35 (60)	11.50 (60)		
25.52	C-4	4.35 (45)	15.60 (45)		
32.14	C-5	4.35 (40)	15.60 (40)		
34.19	C-3′	6.70 (41)	3.10		
98.45	C-2′	4.70 (41)	3.10		
114.68	C-4′	6.30 (78,64,41)	2.20		
115.09	C-2	3.70	3.20	3.70 (62.6, 80)	
115.35	C-2″	3.50 (68,73.5)	1.80		
135.48	C-3″	5.90 (64,73.5,11)	2.10		
150.52	C-5′	6.70 (78,11)	2.10		64
165.71	C-1″	4.20 (68)	2.40		
169.53	CH_3*CO*	3.80 (60)	11.50 (60)		
173.90	C-3	4.70 (45)	12.50 (45)	1.80 (80)	
197.56	C-1	4.00 (40)	11.50 (40)	1.80 (62.6)	

[a] Signals for which no figures are given showed no significant enrichment.

Table III. Incorporation of Radioactive Precursors Into **1**

precursor	specific activity of precursor (dpm/μmol, ×10³)	amount of precursor fed (μmol)	specific activity of isolated **1** (dpm/μmol)	specific incorporation (%)
[1-^{14}C]glycine	1.10 × 10³	1600	9	0.80
[2-^{14}C]glycine	2.75 × 10³	1600	574	20.9
[1(4)-^{14}C]succinate	3.74 × 10³	1200	88	2.3
5-Amino-[5-^{14}C]levulinate hydrochloride	2.77 × 10³	1200	77	3.4
[1,6-^{14}C]shikimate	2.81 × 10³	1140	29	1.0
L-[U-^{14}C]phenylalanine	1.16 × 10³	600	491	4.3
L-[1′-^{14}C]tyrosine	3.67 × 10³	1200	0.64	0.018
sodium [2-^{14}C]acetate	3.66 × 10³	1000	763	21.4
[1(3)-^{14}C]glycerol	5.95 × 10³	1520	770	13.2
[2-^{14}C]glycerol	6.21 × 10³	1520	924	14.8

extremely useful due to its typically efficient incorporation and the readily recognizable, highly diagnostic coupling patterns it produces. Intact incorporations of glycerol are identifiable in NMR spectra as (ideally) AMX or ABX-type spin systems.[21] If a carbon–carbon bond is cleaved during biosynthesis, subsequent to metabolic dilution, a singly-coupled (AB or AX) pattern results. Obviously, if both carbon–carbon bonds are cleaved, all biochemical coupling is lost and potentially enriched (but not coupled) carbon atoms are detected. If the ^{13}C NMR spectrum of the compound is not congested, simple first-order analysis of coupling satellites, looking for the $^{2}J_{CC}$ systems, characteristic of the AMX or ABX patterns will serve to identify doubly-coupled species.

The broad-band ^{1}H-decoupled ^{13}C NMR spectrum of **1** labeled from [U-$^{13}C_3$]glycerol indicated extensive enrichment and coupling throughout the molecule (Table II). For quantitative purposes, the absolute ^{13}C enrichment of the methyl carbon atom of the 2′-acetoxy group was determined by integration of the methyl proton signal and its ^{13}C satellites in the ^{1}H NMR spectrum; the integral of the corresponding ^{13}C signal in the ^{13}C NMR spectrum was then used as the reference against which enrichments at other carbon atoms were calculated. As one would expect from the known metabolic fate of glycerol, pairs of carbon atoms (singly-coupled species) were derived intact from glycerol; these were the same ones that originated from intact acetate units, i.e., the acetoxy group as well as C-1/C-5 and C-3/C-4 in the succinate-derived portion of the C_5N unit. One additional singly-coupled pair of carbon atoms was observed for C-2′/C-3′ (Table II). A doubly-coupled assembly of three carbon atoms, indicating intact incorporation of a glycerol unit, was identified at C-3″/C-4′/C-5′, as evidenced by an 11 Hz coupling between C-3″ and C-5′ (Table II). However, the C-4′ signal showed a pattern of at least eight lines, instead of the expected four predicted for a doubly-coupled carbon. This finding implied the presence of another coupled two- or three-carbon system involving C-3′, exhibiting a coupling constant coincidental with $^{1}J_{2',3'}$ such that the satellites associated with C-3′ would be a doublet. A 2D INADEQUATE[22] experiment clearly revealed that C-3′ was indeed coupled to both C-2′ and C-4′ with a coincident coupling constant of 41 Hz. The contour projection through the INADEQUATE spectrum at δ 114.7 (C-4′) showed an eight-line pattern, which would be expected for a superimposition of two arrays of three coupled carbon atoms. The two possible arrangements of an intact three-carbon unit involving C-3′ and C-4′, i.e. C-3′/C-4′/C-5′ and C-3′/C-4′/C-3″, were distinguished by spectral simulation with the Bruker PANIC routine, using the known δ and J_{CC} values as input. There was correspondence of the observed pattern to that calculated for the superimposition of a C-3″/C-4′/C-5′ and a C-3′/C-4′/C-5′ coupling pattern, and a very poor match for the other alternative, C-3″/C-4′/C-5′ plus C-3″/C-4′/C-3′. An additional doubly-coupled three-carbon assembly was identified at C-1″/C-2″/C-3″ in the acrylic acid side chain. Therefore, [U-^{13}C]glycerol gives rise to two species of **1**, one bearing the labeling pattern A and the other pattern B (Figure 2).

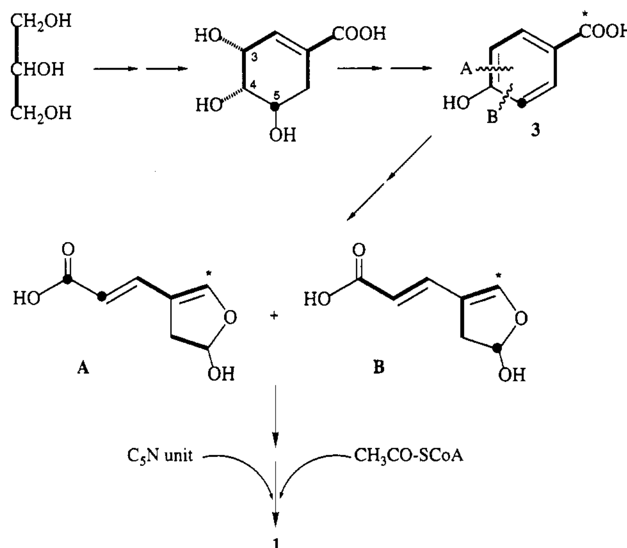


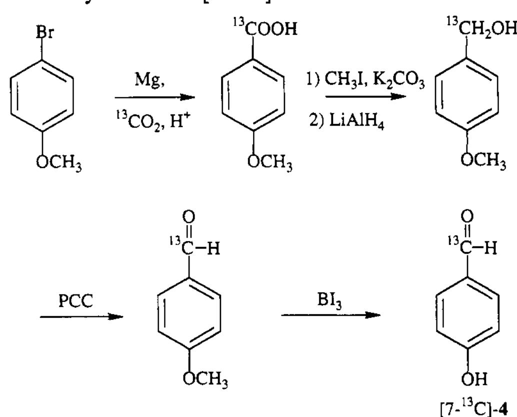
Figure 2. Labeling and ^{13}C–^{13}C coupling pattern in the dihydrofuranylacrylic acid moiety of **1** derived from [U-$^{13}C_3$]glycerol.

These results pointed to the shikimate pathway as the biosynthetic source of the dihydrofuranylacrylic acid unit of **1**. On the basis of the known biochemistry of this pathway,[23] [U-$^{13}C_3$]-glycerol will label shikimic acid as shown in Figure 2. Coupling pattern B implies ring cleavage between C-4 and C-5 of shikimate or one of its metabolites, whereas coupling pattern A reflects bond cleavage between C-3 and C-4. The presence of the two patterns in equal quantities indicates that the precursor to the dihydrofuranylacrylic acid moiety must be a symmetrical compound containing all seven carbon atoms of shikimate. 4-Hydroxybenzoic acid (**3**) (Figure 2), was considered a likely candidate to be such a precursor, containing all carbon atoms and the proper oxygen atoms to furnish the entire moiety. This notion was tested by synthesizing 4-hydroxy-[7-^{13}C]benzoic acid[24] and feeding it to *S. xanthochromogenus*. A very high enrichment (64%) solely in C-5′ of the resulting **1** confirmed that indeed 4-hydroxybenzoic acid or a closely related metabolite must be the substrate for the ring cleavage reaction leading to the dihydrofuran moiety of **1**.

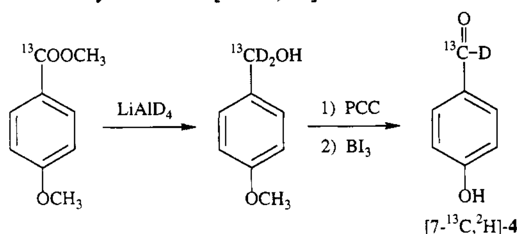
The efficient incorporation of 4-hydroxy-[7-^{13}C]benzoic acid into **1** clearly demonstrates that this compound is a likely pathway intermediate. Since the carboxyl carbon of **3** becomes a (masked)

(18) (a) Leeper, F. *Nat. Prod. Rep.* **1985**, *2*, 19. (b) Battersby, A. R. *Acc. Chem. Res.* **1986**, *19*, 147. (c) Scott, A. I. *Pure Appl. Chem.* **1986**, *58*, 753.
(19) Nakagawa, A.; Wu, T. S.; Keller, P. J.; Lee, J. P.; Ōmura, S.; Floss, H. G. *J. Chem. Soc., Chem. Commun.* **1985**, 519.
(20) Beale, S. I. *Plant Physiol.* **1990**, *93*, 1273.
(21) Beale, J. M.; Cottrell, C. E.; Keller, P. J.; Floss, H. G. *J. Magn. Reson.* **1987**, *72*, 574.
(22) Bax, A.; Freeman, R.; Frenkiel, T. A. *J. Am. Chem. Soc.* **1981**, *103*, 2002.
(23) Haslam, E. *The Shikimate Pathway*; Wiley: New York, 1974; pp 4–21.
(24) Ott, D. G. *Synthesis with Stable Isotopes of Carbon, Nitrogen, and Oxygen*; Wiley: New York, 1981; p76.

Scheme I. Synthesis of [7-^{13}C]-**4**



Scheme II. Synthesis of [7-^{13}C,^{2}H]-**4**



aldehyde function in **1**, it seemed possible that 4-hydroxybenzaldehyde (**4**) might be an even more proximate precursor of **1**. This idea was tested by a feeding experiment with 4-hydroxy-[7-^{13}C]benzaldehyde, prepared as shown in Scheme I, which gave a 35% enrichment at C-5′ of the resulting **1**. This result clearly indicates very efficient incorporation of **4** into **1**. However, the enrichment is lower than in the previous experiment with [7-^{13}C]-**3**, and since many microorganisms can oxidize aldehydes to carboxylic acids,[25] the outcome of this experiment does not necessarily reveal whether 4-hydroxybenzaldehyde is a true intermediate in the biosynthetic pathway, or whether its incorporation results from oxidation to 4-hydroxybenzoic acid and incorporation of the latter. In order to determine whether 4-hydroxybenzaldehyde occupies a later position on the biosynthetic pathway than 4-hydroxybenzoic acid, [7-^{13}C,7-^{2}H]-**4** was synthesized (Scheme II) and fed to *S. xanthochromogenus*. The amount of precursor fed was substantially lower than in the [7-^{13}C]-**4** experiment. The resulting sample of **1** showed a 4% enrichment of ^{13}C at C-5′, and about 75% of the ^{13}C carried a directly attached deuterium (Figure 3). Hence the majority of the **4** incorporated had retained the original aldehyde hydrogen, i.e., cannot have been incorporated via **3**. About 25% of the deuterium had been exchanged for protons from other sources, producing reductiomycin labeled with ^{13}C but not ^{2}H. The relatively low incorporation compared with the 4-hydroxy-[7-^{13}C]benzaldehyde feeding probably is the result of biological variations between individual fermentations, combined with the fact that a smaller amount of precursor was fed. These results establish that 4-hydroxybenzaldehyde can be incorporated into **1** independently, i.e., not via 4-hydroxybenzoic acid, and they suggest that the ring cleavage reaction may occur at the aldehyde stage, rather than at that of the acid.

The above results allow the formulation of a hypothetical pathway from shikimic acid to the dihydrofuranylacrylic acid moiety of **1**, shown in Figure 4, although the order in which some of the reactions take place may be different. To further examine the validity of this pathway we carried out experiments probing the fate of various hydrogens in the transformation. For ease of

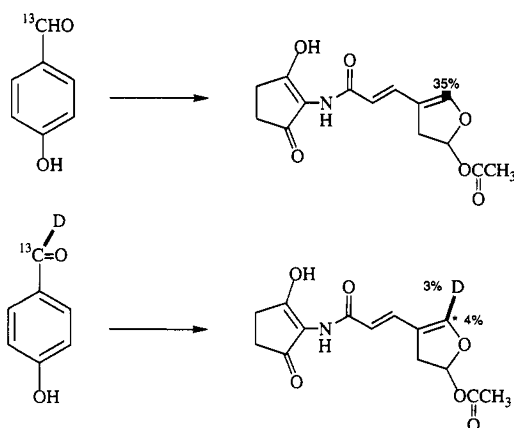


Figure 3. Incorporation of [7-^{13}C]-**4** and [7-^{13}C,^{2}H]-**4** into **1**.

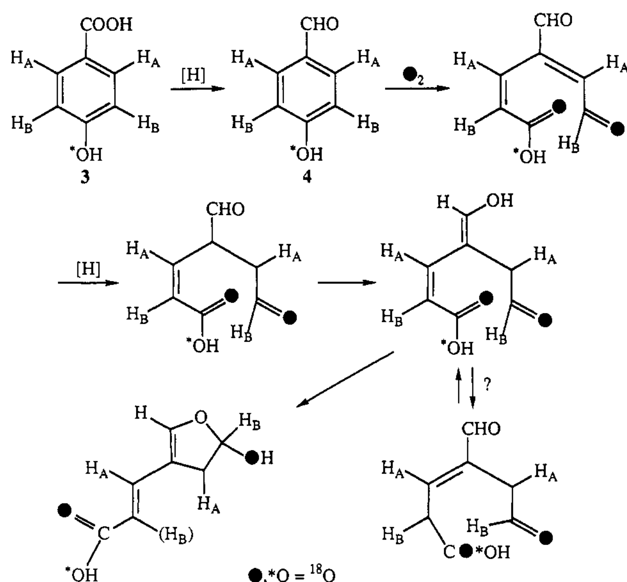
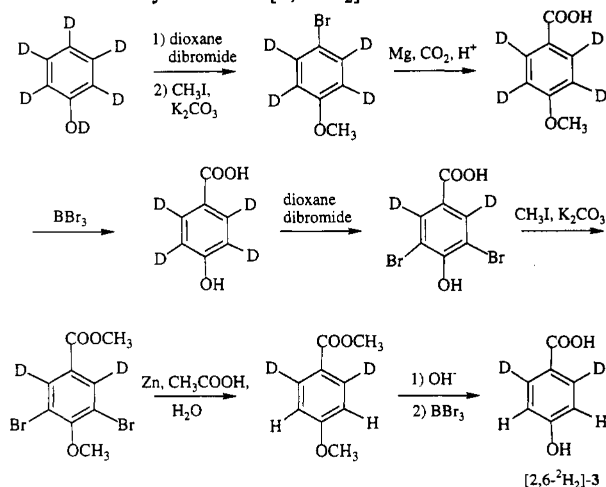


Figure 4. Hypothetical pathway and mechanism for the formation of the dihydrofuranylacrylic acid moiety of **1**, and expected isotope distribution in **1** based on this pathway.

Scheme III. Synthesis of [2,6-$^2H_2$]-**3**



synthesis, and because it gave better incorporations, we chose **3** rather than **4** as the labeled precursor. According to the proposed pathway (Figure 2), **3** labeled with deuterium at C-2 and/or C-6 should label the hydrogens at C-3′ and C-3″ in **1**. Hence, [2,6-$^2H_2$]-**3** was synthesized as shown in Scheme III and fed to *S. xanthochromogenus*. The resulting **1** was analyzed by ^{1}H and ^{2}H NMR spectroscopy. The ^{2}H NMR spectrum showed two

(25) Kieslich, K. *Microbial Transformations of Nonsteroidal Cyclic Compounds*; Thieme: Stuttgart, 1976; p 124.

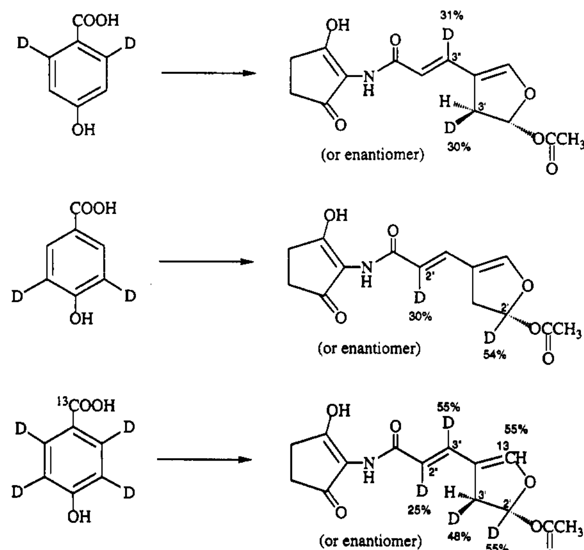


Figure 5. Observed labeling patterns in 1 after feeding [2,6-$^2H_2$]-, [3,5-$^2H_2$]-, and [7-^{13}C,2,3,5,6-$^2H_4$]-3.

Table IV. Mass Spectral Analyses of 1 Obtained from Deuterated and/or ^{13}C-Labeled Precursors

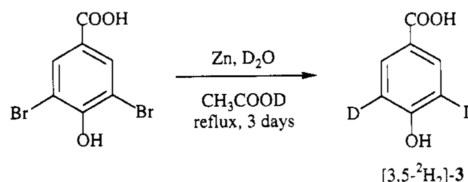
	observed					calculated[a]		
m/z	STD[b]	R26[c]	R35[d]	RD4C[e]	R7C[f]	R26[c]	R35[d]	RD4C[e,g]
293[h]	76	55	37	30	61	54 (45)	33 (14)	32 (14)
294	24	15	23	9	34	17 (32)	27 (32)	9 (22)
295		30	31	3	5	29 (18)	30 (28)	3 (6)
296			8	7		(5)	9 (10)	4(2)
297				25				23 (17)
298				17				22 (26)
299				7				7 (8)
230				3				2 (2)
233[i]	79	54	36	30	63	57 (49)	37 (16)	32 (15)
234	18	20	26	12	30	13 (32)	27 (43)	7 (22)
235	3	21	30	3	6	24 (16)	30 (34)	2 (5)
236		5	7	12	1	6 (3)	7 (7)	7 (1)
237			1	21				22 (9)
238				14				20 (28)
239				5				7 (6)
240				1				2 (1)

[a] Relative abundances are calculated on the basis of the NMR analysis results shown in Figures 3 and 5, assuming no intermolecular transfer of deuterium atoms. The numbers in parentheses are relative intensity values expected if one of the deuterium atoms had been transferred between molecules. [b] Unlabeled 1. [c] 1 biosynthesized from [2,6-$^2H_2$]-3. [d] 1 biosynthesized from [3,5-$^2H_2$]-3. [e] 1 biosynthesized from [7-^{13}C,2,3,5,6-$^2H_4$]-3. [f] 1 biosynthesized from [7-^{13}C]-4. [g] The labeled precursor was 98% ^{2}H enriched and 99% ^{13}C enriched; this was considered in the calculation. [h] m/z = 293 is the molecular ion peak, relative abundance of m/z 293 to m/z 233 is 40:100. [i] m/z = 233 is the M – AcOH peak.

signals of almost equal intensity at δ 2.68 and δ 7.50 ppm which correspond to H-3′ and H-3″, respectively (Figure 5), consistent with the prediction (Figure 4). Integration of the ^{1}H NMR spectrum showed that both sites were labeled to the extent of 30% with deuterium. Moreover, on the basis of coupling constant and Karplus[26] considerations, we were able to determine that the deuterium at C-3′ had been introduced *anti* to the acetoxy group. The mass spectrum of the sample demonstrated an isotope distribution in the molecular ion cluster (Table IV) that exactly matched the pattern expected on the basis of the NMR results, assuming that no intermolecular transfer of deuterium had occurred. In addition, the mass spectral data also support the conclusion that the deuterium at C-3′ is located *anti* to the acetoxy group. Elimination of HOAc from 1 to give m/z 233 presumably involves a McLafferty rearrangement, implying that the *syn* hydrogen must be removed from C-3′, and this fragment ion is still predominantly dideuterated (Table IV).

(26) Karplus, M. J. Am. Chem. Soc. 1963, 85, 2870.

Scheme IV. Synthesis of [3,5-$^2H_2$]-3



In a complementary experiment, 4-hydroxy-[3,5-$^2H_2$]benzoic acid, prepared as shown in Scheme IV, was fed. The ^{2}H NMR spectrum of 1 biosynthesized from this precursor showed two signals, at δ 5.81 and δ 6.71, corresponding to H-2″ and H-2′. Integration of the ^{2}H NMR spectrum gave an intensity ratio of 0.54:1.00 for these two signals, which were found, by proton integration, to represent enrichments of 30% at H-2″ and 54% at H-2′ (Figure 5). The mass spectral analysis matched the NMR results, again assuming no intermolecular transfer of deuterium during biosynthesis (Table IV).

A comparison of these two experiments clearly shows that not all four ring hydrogens of 3 are incorporated to the same extent. However, in the absence of any reference label which would allow one to correct for biological variation between the two fermentations, it was not entirely clear which hydrogen or hydrogens were partly removed during biosynthesis. We therefore carried out an additional experiment with [7-^{13}C,2,3,5,6-$^2H_4$]-3 in order to relate the incorporation of each deuterated hydrogen to that of the ^{13}C. In addition, this experiment allowed us to probe whether any of the hydrogens incorporated from 3 into 1 had undergone an intermolecular transfer between different precursor molecules during the biosynthesis. [7-^{13}C,2,3,5,6-$^2H_4$]-3 was synthesized as shown in the first part of Scheme III, but using $^{13}CO_2$ in step C. The analysis of 1 obtained from this feeding experiment provided results consistent with the data from the dideuterated 4-hydroxybenzoic acids (Figure 5). The ^{13}C enrichment at C-5′ was determined by integration of the C-5′ proton satellites to be 55%. Almost identical deuterium enrichments of 48–55% were observed at H-3″, H-3′, and H-2′, whereas H-2″ was only enriched to the extent of 25%. Less incorporation of deuterium at H-2″ than H-2′ is consistent with the experimental result from the [3,5-$^2H_2$]-3 feeding. The analysis indicates that, of the four ring hydrogen atoms of 3, three are completely retained in the conversion to 1 and one, appearing at C-2″, is only retained to the extent of about one-half.

The ^{1}H NMR spectrum provides evidence against the possibility of intermolecular transfer of the deuterium at C-2″ or C-3″. Figure 6 shows all possible labeling patterns at C-2″ and C-3″, and the proton NMR signal patterns that would correspond to these species. If either the deuterium atom ending up at C-2″ or C-3″ had been transferred to the same position in a different molecule during the biosynthesis, species **B** and **C** would be produced in addition to species **A** and **D**. This possibility was ruled out by the observation of only species **A** and **B** (see arrow, Figure 6), but not **C**, in the experimental ^{1}H NMR spectrum of 1 derived from the tetradeuterated precursor. The only alternative mode of formation of species **B** is by exchange of the deuterium atom labeling C-2″ with unlabeled hydrogens from other sources, e.g., solvent, at some point in the biosynthesis. The mass spectral analysis of 1 labeled from [7-^{13}C,2,3,5,6-$^2H_4$]-3 gave the results summarized in Table IV. The relative abundances of the M^+, $(M + 1)^+$, $(M + 4)^+$, $(M + 5)^+$, and $(M + 6)^+$ ions match reasonably well those predicted for the conversion of the precursor into 1 without any intermolecular transfer of deuterium. Also given in Table IV are the calculated isotope distributions for the M^+ and $(M - AcOH)^+$ ions assuming intermolecular transfer of one of the deuterium atoms to the same position in a different

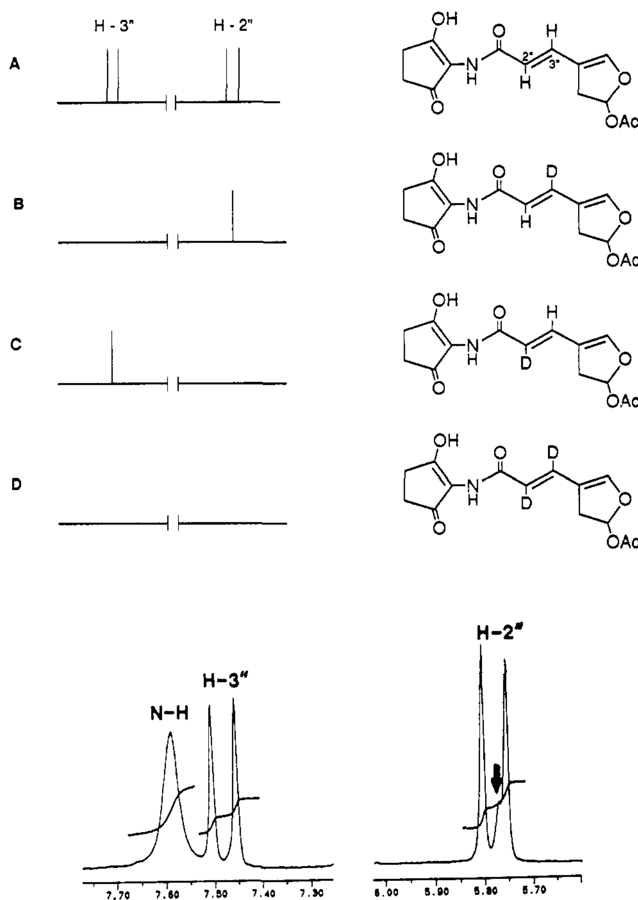


Figure 6. (a) Predicted ^{1}H NMR spectral patterns for H-2″ and H-3″ of **1** in four possible labeling patterns and (b) observed ^{1}H NMR spectrum corresponding to H-2″ and H-3″ of **1** obtained from a feeding experiment with [7-^{13}C,2,3,5,6-^{2}H$_4$]-**3**.

molecule. The significant differences between the observed pattern and that predicted for such transfer argues against intermolecular transfer of deuterium during **1** biosynthesis.

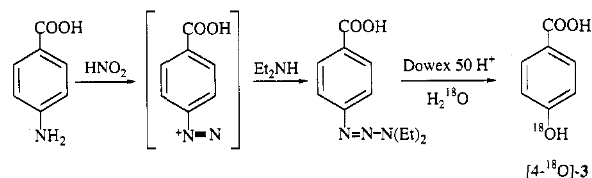
Biosynthesis of the dihydrofuranylacrylic acid moiety from **3** or **4** obviously involves some type of oxidative aromatic ring cleavage reaction. On the basis of the precedent of the well-known ortho and meta cleavage reactions of catechols,[27] one might suspect that the ring cleavage of **3** or **4** also proceeds by a dioxygenase mechanism, as suggested in Figure 4. Such a mechanism would predict the incorporation of two atoms of ^{18}O from the same molecule of $^{18}O_2$ gas into the cleavage product, with resultant ^{18}O labeling of **1** in the ester bridge oxygen at C-2′ and, to a lesser extent, in the amide carbonyl oxygen. On the other hand, the aromatic ring cleavage of genetisic aldehyde in the biosynthesis of patulin appears to involve a monooxygenase mechanism.[28] An analogous mechanism for the cleavage of **3** or **4** would place only one atom of ^{18}O from $^{18}O_2$ into the molecule, presumably at the ester bridge oxygen at C-2′. To obtain information on the origins of the oxygen atoms in **1**, a fermentation of *S. xanthochromogenus* was carried out in a closed system[29] under an atmosphere of $^{18}O_2$ gas (98 atom % ^{18}O). After 30 h, the resulting reductiomycin (18 mg) was isolated, purified, and analyzed by mass spectrometry (Table V). A substantial (25%) enhancement of the $(M + 2)^+$ peak (m/z = 295), but almost no (<2%) increase in the $(M + 4)^+$ peak, indicates predominant incorporation of a single atom of ^{18}O. Since the intensity of the

Table V. Mass Spectral Analysis of **1** Obtained from Fermentation of *S. Xanthochromogenus* in an $^{18}O_2$-Containing Atmosphere[a]

m/z	233[b]	234	235	236	237	293[c]	294	295	296	297
standard[d]	100	24	2.9	3.2	0.7	100	15	2.7		1.1
labeled[e]	100	17	5.4	1.0	1.0	100	20	28	6.2	2.5

[a] Relative abundance of m/z = 293 to m/z = 233 is 40:100. [b] m/z = 233 is the M − AcOH peak. [c] m/z = 293 is the molecular ion peak. [d] Nonlabeled **1**. [e] **1** from fermentation in ^{18}O-containing atmosphere.

Scheme V. Synthesis of [4-^{18}O]-**3**



$(M - AcOH + 2)^+$ peak (m/z = 253) was not enhanced significantly (<3%) the ^{18}O must reside in the acetoxy group. Since the acetyl group is known to derive from acetate, presumably through acetyl-CoA, the oxygen atom connecting C-2′ with the acetyl group is thus indicated as the site of labeling, with 25% ^{18}O enrichment. ^{13}C NMR analysis of the same sample gave results consistent with the mass spectral data. The appearance of upfield isotope shifts[30] on the C-2′ (δ 98.55, $\Delta\delta$ 0.028 ppm) and acetoxy carbonyl (δ 169.46, $\Delta\delta$ 0.034 ppm) carbon signals indicated that the C-2′ oxygen was enriched with ^{18}O. No isotope shift was observed for the C-1″ carbon, implying no significant ^{18}O enrichment at the amide oxygen.

The failure to detect ^{18}O incorporation at C-1″ may mean that the ring cleavage proceeds by a monooxygenase mechanism; alternatively, however, it is possible that a biochemical process leads at some stage in the biosynthesis to washout of ^{18}O from the carbon giving rise to C-1″. Since half of the ^{18}O originally present at that position will be lost during activation of the carboxyl group for amide formation, only an enrichment of 12.5% would be expected at C-1″ anyway; an additional exchange process could easily reduce this below the ^{13}C NMR detection limits to the low levels (1.4–2.5%) possibly present in the mass spectra. We therefore carried out control experiments in which [4-^{18}O]-**3**, synthesized as shown in Scheme V, was fed alone or in a mixture with [7-^{13}C]-**3**. The phenolic oxygen, as shown in Figure 4, should also label C-1″; yet no significant (< 0.2%) enrichment was detected in the molecular ion or the M − AcOH fragment, although co-administered [7-^{13}C]-**3** was efficiently incorporated (data not shown). Thus, it seems likely that ^{18}O present at the carbon giving rise to C-1″ is indeed washed out extensively during the biosynthesis, and the $^{18}O_2$ experiment is inconclusive as to a distinction between a dioxygenase and a monooxygenase mechanism.

Discussion

The results presented here demonstrate the origin of reductiomycin from one molecule each of 5-aminolevulinic acid, *p*-hydroxybenzoic acid or *p*-hydroxybenzaldehyde, and acetate. Formation of the 2-aminocyclopentenolone moiety of **1** represents an entirely new pathway of metabolism of **2**, which so far had only been known to undergo the intermolecular condensation reaction to give porphobilinogen, the universal precursor of porphyrins and corrins.[18] Its intramolecular cyclization to the C_5N unit of **1** and other antibiotics, e.g., asukamycin,[19] is most plausibly formulated via pyridoxal phosphate catalysis, as shown in Figure 1. Further evidence for or against this proposed mechanism will have to come from experiments at the enzymatic level. Some efforts have been made toward detection of an enzyme catalyzing this reaction in cell-free extracts of *S. xanthochro-*

(27) Hayaishi, O.; Nozaki, M.; Abbott, M. T. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1975; Vol. 12, pp 119–189.
(28) Iijima, H.; Noguchi, H.; Ebizuka, Y.; Sankawa, U.; Seto, H. *Chem. Pharm. Bull.* **1983**, *31*, 362.
(29) McIntyre, C. R.; Scott, F. E.; Simpson, T. J.; Trimble, L. A.; Vederas, J. C. *Tetrahedron* **1989**, *45*, 2307.
(30) Vederas, J. C. *J. Am. Chem. Soc.* **1980**, *102*, 374.

mogenus,[31] but the extreme instability of unacylated 2-amino-3-hydroxycyclopent-2-enone has so far frustrated these attempts.

The origin of the dihydrofuranylacrylic acid moiety of **1** from a symmetrical intermediate of the shikimate pathway was discovered by detailed analysis of the $^{13}C-^{13}C$ coupling patterns in **1** derived from [U-$^{13}C_3$]glycerol, a highly diagnostic general precursor of *Streptomyces* metabolites. The data also leave little doubt that **3** is one such symmetrical intermediate on the pathway. In Nature, **3** can be formed by two routes, one involving aromatization of chorismic acid, which operates mainly in microorganisms,[32] and another, operating in higher plants, which proceeds via phenylalanine and/or tyrosine and involves side chain degradation of their deamination products, the corresponding cinnamic acids.[33] The coupling pattern in **1** from [U-$^{13}C_3$]glycerol is only compatible with the direct pathway from chorismic acid, since it indicates that all seven carbon atoms of shikimic acid, including the carboxyl group, are retained. The relatively substantial incorporation of ^{14}C from L-[U-^{14}C]phenylalanine (Table III) must therefore be due to metabolic breakdown of this compound, presumably to acetate which is efficiently incorporated into **1**.

The data also demonstrate that 4-hydroxybenzaldehyde can be converted directly, without oxidation to **3**, into **1**, and that this conversion occurs quite efficiently. It thus seems plausible that reduction of the carboxyl group of **3** to the aldehyde is the first step in the conversion to the dihydrofuranylacrylic acid moiety of **1**, followed by the oxidative ring cleavage, as proposed in Figure 4. However, one cannot dismiss the possibility that **4** is not a true intermediate in the biosynthesis, but is converted into **1** when added to the fermentation, and that **3** is the natural substrate for the ring-cleavage reaction followed by reduction of the carboxyl group of a later stage. The fact that **4** was always incorporated less efficiently than **3**, although clouded by the fact that incorporation rates generally varied greatly between individual fermentations, argues for this alternative. Further work will be necessary to clarify this aspect of the biosynthetic pathway.

The formation of the dihydrofuranylacrylic acid moiety of **1** adds another example to the list of natural products that arise by cleavage of an aromatic ring, which also includes the betacyanins,[34] patulin,[35] and the anthramycin family of antibiotics[36]. With some exceptions,[28] the majority of these aromatic ring-cleavage reactions proceed by a dioxygenase mechanism.[27] However, an attempt to support such a mechanism for **1** by an $^{18}O_2$ experiment gave inconclusive results. As the control experiment with [4-^{18}O]-**3** showed, one of the ^{18}O atoms would be introduced by a dioxygenase into a position, the carbon giving rise to C-1″ of **1**, from which virtually all ^{18}O is washed out by an unknown biochemical process, possibly a futile cycle of repeated activation of the carboxyl group and hydrolysis. Thus the question of the oxidative ring cleavage mechanism remains open.

The steps proposed in Figure 4 for the conversion of **3** into **1** require, after the ring cleavage, reduction of one double bond and *cis-trans* isomerization of the other. The fate of the four ring hydrogens of **3** is entirely consistent with, and thus supports, the mechanistic pathway shown in Figure 4. Each deuterium label appears in the product in the position predicted by the mechanism. The absolute configuration of the isotopically chiral center at C-3′ generated by incorporation of [2,6-$^{2}H_2$]-**3** is determined in the double bond reduction step, the relative configuration with respect to C-2′ in the closure of the dihydrofuran ring by attack of the enol oxygen at C-5′ on the carbonyl oxygen at C-2′. No mechanistic significance can at present be attached to the finding that deuterium at C-2′ is *anti* to the acetoxy group.

Of considerable interest is the mechanism of the *cis–trans* isomerization of the C-2″/C-3″ double bond, particularly in view of the observation that only about one-half of the theoretical amount of deuterium from [2,6-$^{2}H_2$]-**3** is incorporated at C-2″. The partial loss of deuterium from what may simply be due to nonenzymatic exchange of an intermediate, for example as shown in Figure 4. In that case the actual isomerization may proceed without protonation/deprotonation at C-2″ as seems to be the case in the malyeyl-fumarylpyruvate isomerase reaction.[37] If the partial deuterium loss at C-2″ is an inherent part of the isomerization mechanism, this reaction would presumably involve mandatory protonation/deprotonation at that carbon. To account for partial loss deuterium either the protonation or deprotonation, or both, must be nonstereospecific. Alternatively, the isomerization must involve intermolecular hydrogen recycling accompanied by partial exchange, as has been demonstrated for a *cis–trans* isomerization in the biosynthesis of ergot alkaloids.[38] Since both the NMR and mass spectral data exclude any significant intermolecular deuterium transfer, particularly of the deuterium at C-2″, the latter mechanism can be excluded. The proton NMR data on **1** derived from [7-^{13}C,2,3,5,6-$^{2}H_4$]-**3**, i.e., the pattern shown in Figure 6, rather point to a partial exchange of the deuterium at C-2″ along the pathway. Obviously, further insight into this intriguing mechanism can only be gained once the enzyme catalyzing the reaction is at hand.

In summary, the results presented here provide considerable evidence for the pathway and mechanisms shown in Figures 1 and 4 for the biosynthesis of reductiomycin.

Experimental Section

Materials. General synthetic reagents were obtained from Aldrich Chemical Company. Radioactive precursors were purchased from the following sources: [1-^{14}C]- and [2-^{14}C]glycine, (each 56 mCi/mmol), [1(4)-^{14}C]succinic acid (118 mCi/mmol), L-[1-^{14}C]tyrosine (53.7 mCi/mmol), and L-[U-^{14}C]phenylalanine (513 mCi/mmol) from Amersham; [1,6-^{14}C]-shikimic acid (19.7 mCi/mmol) and amino-[5-^{14}C]levulinic acid (49.0 mCi/mmol) from New England Nuclear; [1(3)-^{14}C]glycerol (50 mCi/mmol) from ICN. Stable isotope-labeled compounds were obtained from the following suppliers: [4,5-$^{13}C_2$]Aminolevulinic acid (90% ^{13}C) from Cambridge Isotopes; sodium [1,2-$^{13}C_2$]acetate (99% ^{13}C) from British Oxygen Company, Ltd.; acetic acid-*d* (98% ^{2}H), deuterium oxide (99.9% ^{2}H), $H_2{}^{18}O$ (97.2% ^{18}O), $^{18}O_2$ gas (98% ^{18}O) and [$^{2}H_6$]phenol (98% ^{2}H) from Aldrich Chemical Company; and $^{13}CO_2$ gas (99% ^{13}C) from Mound Laboratories. 4-Hydroxy-[7-^{13}C]benzoic acid (99% ^{13}C) was synthesized from *p*-bromoanisole by reaction of the Grignard derivative with $^{13}CO_2$[24] followed by deprotection with BBr_3. [U-^{13}C]-Glycerol was synthesized from $K^{13}CN$ and [1,2-$^{13}C_2$]acetic acid (both 99%), provided by the Los Alamos Stable Isotope Resource, via diethyl malonate and diethyl-2-acetoxymalonate.[39]

General Methods. Melting points were determined on a Mel-Temp Laboratory apparatus and are uncorrected. Thin-layer chromatography was carried out on precoated silica gel plates (EM) obtained from Brinkmann. Infrared spectra were recorded on a Mattson FT-IR instrument. Mass spectra were recorded in the EI mode on Kratos MS-55 or Profile mass spectrometers by direct probe introduction. Isotopic enrichments were calculated from mass spectral data as described by Biemann.[40] Routine GC–MS identification of synthetic intermediates was carried out on a Hewlett-Packard 5790A gas chromatograph with a HP-5970A mass selective detector.

NMR Spectroscopy. ^{1}H NMR, ^{2}H NMR, and ^{13}C NMR spectra were acquired in $CDCl_3$ ($CHCl_3$ in the case of ^{2}H NMR) at 307 K on either Bruker WM-300 or IBM AF-300 instruments, operating at field strengths of 7.1 T. Chemical shifts for both ^{1}H and ^{13}C spectra are

(31) Shibuya, M.; Floss, H. G. Unpublished work.
(32) Lawrence J.; Cox, G. B.; Gibson, F. *J. Bacteriol.* **1974**, *118*, 41.
(33) Gross, G. G. In *Biosynthesis and Biodegradation of Wood Components*; Higuchi, T., Ed.; Academic Press: Boca Raton, FL, 1985; p 229.
(34) Fischer, N.; Dreiding, A. *Helv. Chim. Acta* **1972**, *55*, 6491.
(35) Scott, A. I.; Beadling, L. *Bioorg. Chem.* **1974**, *3*, 281.
(36) Hurley, L. H. *Acc. Chem. Res.* **1980**, *13*, 263.
(37) Seltzers, S. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed., Academic Press: New York, 1972; Vol. 6, pp 381–406.
(38) Floss, H. G.; Tcheng-Lin, M.; Chang, C.-j.; Naidoo, B.; Blair, G. E.; Abou-Chaar, C. I.; Cassady, M. J. *J. Am. Chem. Soc.* **1974**, *96*, 1898.
(39) Ott, C. G. *Synthesis with Stable Isotopes of Carbon, Nitrogen and Oxygen*; Wiley: New York, 1981; pp 33–35; 37. Murray, A. W.; Williams, D. L. *Organic Synthesis with Isotopes*; Interscience: New York, 1958; pp 931–932.
(40) Biemann, K. *Mass Spectrometry, Organic Chemical Applications*; McGraw-Hill: New York, NY, 1962;, pp 224–227.

referenced to their respective internal solvent resonances. Data are reported as follows: Chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broadened, m = multiplet), coupling constants (Hertz), assignment. Proton and ^{13}C NMR assignments of **1** are based on multiplicity analysis, chemical shift theory, and data from $^{1}H/^{13}C$ heteronuclear correlation experiments. A continuous-wave deuterium decoupler as employed for analysis of samples containing $^{13}C-^{2}H$ bonds.

Fermentation. *S. xanthochromogenus* AM-6201[1] was grown on slants of yeast–malt extract agar (Difco) which were incubated at 28 °C and stored in sealed, screw-capped tubes at 4 °C until used. For the production of **1**, the organism was cultivated according to a two-stage (seed/production) fermentation protocol in a medium consisting of (both stages identical) 2% glucose, 2% soybean meal, and 0.3% NaCl in double deionized water. The pH of the medium was adjusted to 7.0 prior to autoclaving. Cultures were grown in 100 mL volumes of medium contained in 500-mL baffled, foam-plugged Erlenmeyer flasks at 28 °C and 300 rpm agitation on a New Brunswick Scientific G-25 gyrotory incubator shaker (2 in. throw). The seed stage flask was inoculated with the contents of a single slant per 100 mL of medium, propagated for 48 h, and used to provide a 10% inoculum into each production stage flask. Incubation conditions were identical in both stages. Labeled precursors were typically added after 24 h of production stage growth, and fermentations were harvested 48 h later.

Isolation and Purification of 1. Mycelia were separated from the broth by filtration through a bed of Celite. The broth was acidified to pH 4.5 and extracted with ethyl acetate (3 × equal volumes). The mycelial mass was treated with acetone to remove cell-associated product. The acetone layer was filtered and combined with the ethyl acetate extracts, and the combined organic extract was dried with anhydrous sodium sulfate. Following evaporation of solvent on a rotary evaporator at 40 °C, **1** was purified from the residue by preparative layer chromatography (silica gel GF_{254}, $CHCl_3$–acetone 9:2). The band containing **1** (R_f 0.45) was scraped from the TLC plate and eluted with ethyl acetate to yield, after evaporation of solvent, a yellow solid. The solid was washed with hexane to remove lipid material and recrystallized from methanol. The yield of **1** was typically 150–250 mg/L after purification.

Feeding Experiments with Labeled Precursors. Feeding experiments were carried out in the normal production medium. In general, precursors were administered to the fermentation at a time at which production of **1** was just detectable in the medium by TLC (24 h into stage 2). Feeding experiments were conducted with single-dose protocols, and labeled compounds were added as filtration-sterilized aqueous solutions. Radioactive precursors were diluted with unlabeled carrier material and added to the fermentation after sterile filtration. Radioactivity in the starting materials and isolated antibiotic (after crystallization to constant specific activity) was determined with a Beckman LS-7000 liquid scintillation counter using Aquasol-2 LSC cocktail (New England Nuclear).

Feeding experiments with stable isotope-labeled precursors were carried out by the same methods. Precursors were added in the amounts indicated as follows: sodium [1,2-$^{13}C_2$]acetate, 9.6 mmol/L; [U-$^{13}C_3$]glycerol, 6.3 mmol/L; [4,5-$^{13}C_2$]-**2**, 1.5 mmol/L; [7-^{13}C]-**3**, 4.0 mmol/L; [3,5-$^{2}H_2$]-**3**, 3.8 mmol/L; [2,6-$^{2}H_2$]-**3**, 3.6 mmol/L; [7-^{13}C,2,3,5,6-$^{2}H_4$]-**3**, 3.6 mmol/L; [7-^{13}C]-**4**, 3.6 mmol/L; [7-^{13}C, 7-^{2}H]-**4**, 1.6 mmol/L; $^{18}O_2$, 1 L per 100 mL culture.

The incubation with $^{18}O_2$ gas was conducted in a closed system[29] containing the gas. A 500 mL baffled Erlenmeyer flask containing a normally grown 24-h-old production culture was connected to the system, a modified Optima Aquarium pump was employed to circulate the atmosphere, and a 5 N aqueous KOH solution was used to trap expired carbon dioxide. A one-liter bottle of $^{18}O_2$ gas was connected to the system, with a slightly positive pressure being maintained by a water reservoir to insure diffusion into the culture. After 30 h of fermentation under $^{18}O_2$, **1** was isolated, purified (18 mg), and analyzed by mass spectrometry.

Synthesis of Labeled Precursors. 4-Hydroxy-[3,5-$^{2}H_2$]benzoic Acid ([3,5-$^{2}H_2$]-3). 3,5-Dibromo-4-hydroxybenzoic acid (7.39 g, 25 mmol) was added to a suspension of zinc dust (9.8 g, 0.15 mol) in CH_3COOD (13.5 mL)/D_2O (48 mL) and the reaction mixture was heated at reflux for 3 days. After cooling to room temperature, it was extracted with diethyl ether (4 × 50 mL). The combined organic layers were dried ($MgSO_4$) and concentrated to give 4-hydroxy-[3,5-$^{2}H_2$]benzoic acid (3.14 g, 22.4 mmol, 90%) as a white solid: mp 202–205 °C; ^{1}H NMR (acetone-d_6, 300 MHz) δ 7.93 (s, 2 H).

4-Bromo-[2,3,5,6-$^{2}H_4$]phenol. To an ice-cold solution of phenol-d_6 (2.04 g, 20.4 mmol) in dry diethyl ether (3 mL) was added dioxane dibromide over a period of 3 h, until the reaction had come to 95% completion as determined by GC–MS. The reaction was then quenched by addition of water (5 mL). The organic layer was separated and the aqueous layer was extracted with diethyl ether (4 × 20 mL). The combined organic extracts were dried ($MgSO_4$) and concentrated to give crude 4-bromo-[2,3,5,6-$^{2}H_4$]phenol (2.7 g), 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: R_f 0.51 (hexane–EtOAc 2:1); GC–MS (*m*/*z*, rel intensity) 178 (96), 176 (100), 97 (32), 69 (97), 41 (39).

4-Bromo-[2,3,5,6-$^{2}H_4$]anisole. To a solution of the product obtained above in dry acetone (20 mL) was added K_2CO_3 (3.2 g, 23.2 mmol) and CH_3I (3 mL, 48.2 mmol), and the mixture was gently refluxed for 12 h. The resulting mixture was filtered, and the remaining solid was washed with dry acetone (5 mL). Concentration of the organic layer afforded crude 4-bromo-[2,3,5,6-$^{2}H_4$]anisole (3.4 g): R_f 0.66 (hexane–EtOAc 3:1).

4-Methoxy-[2,3,5,6-$^{2}H_4$]benzoic Acid. A two-neck flask fitted with a condenser and a rubber septum was charged with magnesium turnings (545 mg, 22.4 mmol). The bromoanisole obtained above (3.37 g, 17.7 mmol) was dissolved in dry THF (10 mL) and 3 mL of this solution was added to the flask containing the magnesium. The mixture was heated to initiate the reaction. When the reaction had started, the remaining solution was added over a 5-min period. After the reaction had subsided, the reaction mixture was heated at reflux for 30 min. After the mixture was cooled to room temperature, carbon dioxide was bubbled into the Grignard solution for 10 min and then 2 N HCl was added to the reaction mixture until no more precipitate was formed. The mixture was extracted with diethyl ether (9 × 20 mL) and the combined extracts were dried ($MgSO_4$) and concentrated to give 2.27 g of the product: R_f 0.24 (hexane–EtOAc 2:1).

4-Hydroxy-[2,3,5,6-$^{2}H_4$]benzoic Acid. To the material obtained in the previous step (2.27 g) in dry CH_2Cl_2 (40 mL) was added 20 mL of a 1 M solution of BBr_3 in CH_2Cl_2, and the mixture was stirred under a N_2 atmosphere. After 8 h at room temperature, the reaction was quenched by addition of ice–water (5mL). The mixture was then extracted with diethyl ether (5 × 50 mL), dried ($MgSO_4$), and concentrated to obtain crude [2,3,5,6-$^{2}H_4$]-**3** (1.52 g). A small sample was purified by recrystallization from water: mp 204–207 °C.

3,5-Dibromo-4-hydroxy-[2,6-$^{2}H_2$]benzoic Acid. Dioxane dibromide was added over a 2-h period to an ice-cold solution of the crude deuterated **3** (1.52 g) obtained above in dioxane–diethyl ether (30 mL, 1:1) until the reaction was complete. Reaction progress was monitored by treating aliquots of the reaction mixture with a solution of diazomethane followed by GC–MS analysis. The final reaction mixture was extracted with diethyl ether (5 × 20 mL), and the extract was dried ($MgSO_4$) and concentrated to give a yellow solid.

Methyl 3,5-Dibromo-4-methoxy-[2,6-$^{2}H_2$]benzoate. To a solution of the product from the previous reaction in dry acetone (50 mL) was added CH_3I (7 mL, 112.4 mmol) and K_2CO_3 (7 g, 50.6 mmol), and the mixture was heated at reflux overnight. The resulting crude methyl [2,6-$^{2}H_2$]-3,5-dibromo-4-methoxybenzoate, containing some tribromide and other impurities was purified by column chromatography on silica gel. The yield was 2.20 g (33% overall from phenol-d_6): R_f 0.51 (hexane–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 4-Methoxy-[2,6-$^{2}H_2$]benzoate. The compound obtained above (1.95 g) was added to a suspension of zinc dust (2.30 g) in dilute aqueous acetic acid (20% by volume) and the mixture was heated at reflux for 3 days. Extraction of the reaction mixture with diethyl ether (3 × 30 mL), followed by concentration, afforded 1.04 g of the product: R_f 0.68 (hexane–EtOAc 2:1); ^{1}H NMR (acetone-d_6, 300 MHz) δ 3.82 (s, 3 H), 3.86 (s, 3 H), 7.94 (s, 2 H).

4-Hydroxy-[2,6-$^{2}H_2$]benzoic Acid ([2,6-$^{2}H_2$]-3). The compound obtained above (1.04 g) was heated at reflux for 1 h with 10% aqueous NaOH (5 mL). After cooling to room temperature, the reaction mixture was acidified to pH 1 with 2 N HCl and extracted with diethyl ether (9 × 15 mL). The extract was dried ($MgSO_4$) and concentrated to obtain crude product (722 mg), mp 177–178 °C. The material was dissolved in dry CH_2Cl_2 (10 mL) and boron tribromide in CH_2Cl_2 (6 mL of a 1 M solution) was added to the stirred solution. The mixture was stirred for 8 h at room temperature and the reaction was then quenched by addition of ice water (10 mL). After separation of the CH_2Cl_2 layer, the aqueous layer was extracted with diethyl ether (8 × 20 mL). The combined organic layers were dried and concentrated to give [2,6-$^{2}H_2$]-**3** (630 mg, 4.5 mmol, 75% from methyl 3,5-dibromo-4-methoxybenzoate) which was

further purified by recrystallization from water: R_f 0.61 (hexane–EtOAc 40:20); mp 203–206 °C; ^{1}H NMR (acetone-d_6, 300 MHz) δ 6.92 (s).

4-Hydroxy-[7-^{13}C,2,3,5,6-^{2}H$_4$]benzoic Acid ([7-^{13}C,2,3,5,6-^{2}H$_4$]-3). The procedure used to prepare [2,3,5,6-^{2}H$_4$]-3 was repeated, except that $^{13}CO_2$ was employed instead of nonlabeled CO_2.

Methyl 4-Methoxy-[7-^{13}C]benzoate. This compound was prepared from *p*-bromoanisole in the same way as described earlier for 4-methoxy-[2,3,5,6-^{2}H$_4$]benzoic acid, except that $^{13}CO_2$ was used instead of nonlabeled CO_2. The resulting methoxy-[7-^{13}C]benzoic acid (2.45 g, 16.0 mmol), CH_3I (6 mL, 96.4 mmol), and K_2CO_3 (2.5 g, 18.1 mmol) in acetone (30 mL) were refluxed for 24 h. The mixture was then filtered and the filtrate concentrated to give a quantitative yield of the title compound (2.65 g, 16 mmol).

4-Methoxy-[7-^{13}C]benzyl Alcohol. The crude product obtained above (2.65 g, 16 mmol) in THF (20 mL) was added slowly to a suspension of $LiAlH_4$ (1.0 g, 26 mmol) in THF (40 mL), and the mixture was heated at reflux for 5 h. After the mixture was cooled to room temperature, water (5 mL) was added slowly, and the resulting mixture was heated at reflux for 15 min. It was then filtered and the cake suspended in THF (80 mL) and heated again for 10 min. The mixture was filtered again and the filtrate was concentrated. The resulting oil was taken up in diethyl ether (200 mL), dried ($MgSO_4$), and concentrated to give 2.10 g of the product: R_f 0.59 (hexane–EtOAc 4:1).

[7-^{13}C]Anisaldehyde. To a stirred solution of the alcohol obtained above (2.1 g, 15.1 mmol) in dry CH_2Cl_2 (100 mL) was added pyridinium chlorochromate (3.5 g, 16.2 mmol) and powdered molecular sieves (8 g). After 2 h at room temperature, the reaction mixture was filtered and the solid was washed with CH_2Cl_2 (2 × 40 mL). The combined CH_2Cl_2 layers were concentrated and purified by column chromatography to give *p*-anisaldehyde in 89% overall yield from 4-methoxybenzoic acid.

4-Hydroxy-[7-^{13}C]benzaldehyde ([7-^{13}C]-4). To a stirred solution of *p*-[7-^{13}C]anisaldehyde (1.23 g, 8.91 mmol) in dry CH_2Cl_2 (80 mL), BI_3 (3.5 g, 8.9 mmol) was added rapidly. The resulting mixture was stirred vigorously for 2 min and the reaction was quenched by addition of water (10 mL). After separation of the CH_2Cl_2 layer, the aqueous layer was extracted with diethyl ether (4 × 50 mL). The combined organic extracts were concentrated and purified by column chromatography to obtain [7-^{13}C]-4 (643 mg, 5.2 mmol, 58%): mp 114–115 °C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 6.91 (d, 2 H, J = 8.5 Hz), 7.75 (dd, 2 H, J = 8.5 Hz, 4.7 Hz), 9.78 (d, 1 H, $^1J_{CH}$ = 172.9 Hz); ^{13}C NMR (DMSO-d_6, 300 MHz) δ 190.01 (enriched).

4-Hydroxy-[7-^{13}C,7-^{2}H]benzaldehyde ([7-^{13}C,7-^{2}H]-4). The procedure used to prepare [7-^{13}C]-4 was repeated except that $LiAl^2H_4$ was employed instead of $LiAlH_4$ in the reduction step. [7-^{13}C,7-^{2}H]-4: mp 115–116 °C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 6.92 (d, 2 H, J = 8.5 Hz), 7.75 (dd, 2 H, J = 8.5 Hz, 4.6 Hz), 10.52 (s, br, 1 H); ^{13}C NMR (DMSO-d_6, 300 MHz) δ 190.17, 190.52, 190.87 (enriched).

4-Hydroxy-[4-^{18}O]benzoic Acid[41] ([4-^{18}O]-3). A solution of 1-(4′-carboxyphenyl)-3,3-diethyltriazene[42] (224 mg, 1.02 mmol) in hot acetonitrile was added dropwise to a boiling mixture of Dowex 50H$^+$ cation exchange resin (975 mg) and $H_2{}^{18}O$ (2 mL). The mixture was refluxed for 40 min, when TLC indicated that the reaction was complete. The reaction mixture was freeze-dried in a closed system and the residue dissolved in 12 N NaOH (10 mL). The solution was filtered and extracted with ether, and the ether extract was discarded. The aqueous phase was acidified with HCl and extracted with ether, and the extract was dried ($MgSO_4$) and evaporated to dryness. Preparative TLC of the residue (solvent: EtOAc) gave of [4-^{18}O]-3 (55.7 mg, 0.45 mmol, 40%). A second preparation gave a yield of 80%: EI-MS m/z 140 ([M + 2]$^+$, 72.1), 138 (M$^+$, 25.1), 123 ([M + 2 – OH]$^+$), 100), 121 ([M – OH]$^+$, 35.8), 95 ([M + 2 – COOH]$^+$, 28.6), 93 ([M – COOH]$^+$, 10.6); ^{18}O-enrichment 74%; ^{1}H NMR (methanol-d_4, 300 MHz) δ 7.87 (d, J = 8.7, 2 H), 6.80 (d, J = 8.7, 2 H); ^{13}C NMR (methanol-d_4, 75.4 MHz) δ 160.55, 153.86, 123.50, 113.21, 106.52.

Acknowledgment. We thank the National Institutes of Health for a research grant (AI 20264 to H.G.F.) and a postdoctoral fellowship (GM 10207 to J.M.B.). The services of the Los Alamos Stable Isotope Resource, supported by NIH grant RR 02231 and USDOE/OHER, are also gratefully acknowledged, as is helpful advice by Prof. J. C. Vederas, Edmonton, on the fermentation under $^{18}O_2$ gas and loan of the apparatus. We also appreciate useful comments by one of the reviewers of this paper.

(41) Satyamurthy, N.; Barrio, J. R.; Bida, G. T.; Phelps, M. E. *Tetrahedron Lett.* **1990**, *31*, 4409.

(42) Ku, H.; Barrio, J. R. *J. Org. Chem.* **1981**, *46*, 5239.