Biosynthesis of the Phenazine Antibiotics, the Saphenamycins and Esmeraldins, in *Streptomyces antibioticus*

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The biosynthesis of the phenazine antibiotics, the esmeraldins and the related saphenamycins, was studied by feeding radioactive and stable isotope-labeled precursors to *Streptomyces antibioticus*, strain Tü 2706. After purification, the labeled antibiotics were degraded to the diastereomeric esmeraldic acid dimethyl esters **3** and **4** and racemic saphenic acid methyl ester (**5**), respectively. The ^{1}H- and ^{13}C-NMR spectra of these were assigned by single and multiple bond correlation experiments. Although ^{14}C- and ^{13}C-labeled shikimic acid was not incorporated, ^{13}C-NMR analysis of the samples biosynthesized from [U-$^{13}C_3$]-, [1,3-$^{13}C_2$]-, and [2-^{13}C]glycerol provided evidence that these phenazine antibiotics are derived from the shikimic acid pathway and display the same head-to-tail coupling pattern of monomer units seen in other phenazine antibiotics. The extra methyl group attached to the carbon corresponding to the shikimate carboxyl group originates from C-2 of acetate. Radiolabeled saphenic acid was incorporated into the diphenazine ring system of the esmeraldins. Mechanistic aspects of the formation of the phenazine and diphenazine ring systems are discussed.

Esmeraldin A (**1b**) and B (**1c**), intensely green metabolites isolated from *Streptomyces antibioticus*, strain Tü 2706, belong to a class of antibiotics containing the phenazine ring system.[1] In addition to these diphenazines, the organism also produces simpler phenazines such as saphenic acid (**2a**),[2] the saphenyl esters (**2b**),[3] and saphenamycin (**2c**), as well as the parent compound, phenazine itself.[4] Several of these compounds possess antibacterial activity against Gram-positive and Gram-negative bacteria or antitumor activity against murine tumor cells. Although there are nearly 75 naturally occurring phenazines,[5–8] all of which are produced by bacteria, unsubstituted phenazine and the novel diphenazines were isolated from this organism for the first time.[4]

In the work reported here, the biosynthesis of the phenazine and diphenazine systems of the saphenamycins and esmeraldins A and B in *S. antibioticus* was investigated to determine whether these antibiotics are assembled from shikimic acid pathway intermediates in the same way as the phenazines produced by *Pseudomonas*[9–13] and other *Streptomyces*[14,15] species and what the origin of the extra methyl group(s) in these compounds is.

Results

Fermentation. *S. antibioticus* Tü 2706 was grown in baffled 500-mL shake flasks containing 100 mL of a soybean–mannitol medium[16] using a two-stage fermentation process. Maximum production of the saphenyl derivatives occurred between 72 and 108 h after inoculation, and that of the esmeraldins between 96 and 144 h (cf. ref 17). The organism tended to degenerate fairly easily, and frequent selections were necessary to maintain acceptable yields. At the end of the fermentation the metabolites were extracted from the separated mycelia, defatted by partitioning between hexane and saturated $NaHCO_3$ solution, and separated by chromatography on silica gel into a yellow fraction of saphenic acid derivatives and a green esmeraldin fraction. Since each of these fractions was a very complex mixture of related compounds, each fraction was hydrolyzed with NaOH, and the resulting saphenic and esmeraldic acids were esterified with diazomethane in ether. Chromatographic purification gave saphenic acid methyl ester (**5**)(8 mg/L) from the yellow fraction and an approximately 1:1 mixture of two blue compounds, esmeraldic acid dimethyl esters **3** and **4** (15 mg/L), from the green fraction. The latter could be resolved by preparative HPLC on a polar amino–cyano column.

Characterization and NMR Assignments of 3–5. Compounds **3** and **4** showed almost identical ^{1}H- and very similar ^{13}C-NMR spectra, suggesting that they are diastereomers. The proton resonances of **3–5** were assigned based on COSY[18] spectra, slightly revising earlier assignments by Keller-Schierlein and co-workers[1] for a mixture

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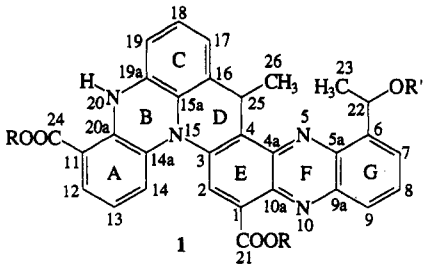
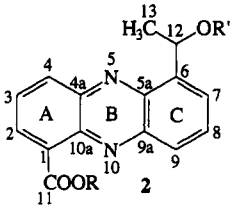


1a: Esmeraldic acid, R=R'=H
1b: Esmeraldin A, R=H, R'= a: 12-Methyltridecanoyl
b: Tetradecanoyl
c: 12-Methyltetradecanoyl
d: 14-Methylpentadecanoyl
e: Hexadecanoyl
f: 14-Methylhexadecanoyl
g: 15-Methylhexadecanoyl
h: Heptadecanoyl
i: Unassigned C-18 Acyl
j: 16-Methylheptadecanoyl
1c: Esmeraldin B, R=H, R'=2-Hydroxy-6-methylbenzoyl
3,4: Esmeraldic acid dimethyl ester, R=CH_3, R'=H



2a: Saphenic acid, R=R'=H
2b: Saphenyl esters, R=H, R' as in **1b**
2c: Saphenamycin, R=H, R' as in **1c**
5: Saphenic acid methyl ester, R=CH_3, R'=H

Figure 1. Phenazines produced by *S. antibioticus* Tü 2706 and their derivatives.

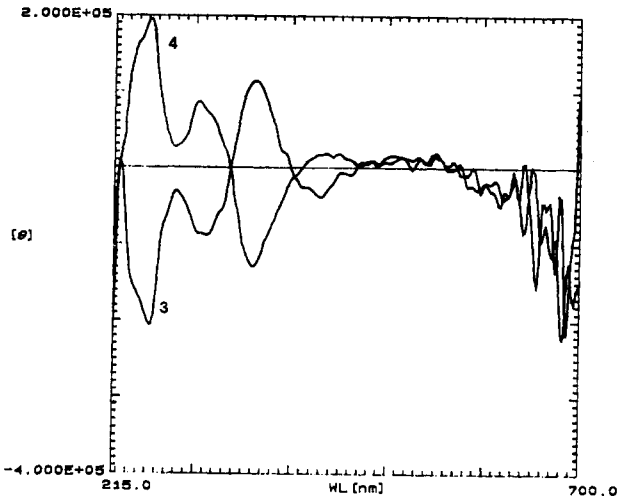


Figure 2. CD spectra of compounds **3** and **4**.

of **3** and **4** and those for **5**. On the basis of these proton assignments, HMQC,[19] HMBC,[20] HETCOR,[21] and COLOC[22] spectra gave the assignments for the ^{13}C resonances of all three compounds. The data for **4** and **5** are listed in Table III. A few remaining ambiguities, e.g., distinction of C-4a and C-10a in **5**, were resolved by analysis of ^{13}C-labeled samples from the biosynthetic experiments described below.

The relationship of **3** and **4** was probed further by examining their CD spectra. The spectra are almost, but not quite, mirror images of each other (Figure 2), indicating that they must have opposite configurations at the chiral center dominating the spectra. The high molar ellipticities (Table I), approaching those of helical compounds,[23] suggest that this stereocenter is due to atropisomerism resulting from the angular annellation of four six-membered rings giving rise to steric interference between H-2 and H-14. Derivatization of **3** and **4** with (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride[24] and ^1H-NMR analysis of the resulting Mosher esters revealed that each compound again represented a mixture of at least two diastereomers. Each spectrum displayed two sets of signals for the methoxy protons of the Mosher acid and for the two *C*-methyl groups of the esmeraldic acid moiety. The larger chemical shift difference for the C-23 relative to the C-26 methyl protons suggests that the two isomers in each sample are epimeric at C-22; whether the configurations at C-25 are constant or also vary cannot be deduced with certainty from these data. Methyl saphenate (**5**) was also converted to its Mosher ester derivative; the spectra of this derivative, too, showed two sets of signals for the C-13 and the Mosher acid methoxy protons in ratios of 3:2 and 2:1 in two different experiments. Hence, **5** is obtained as at least a partial racemate. Attempts were made to demonstrate epimerization at C-22 with incorporation of deuterium from solvent or interconversion of **3** and **4** under the conditions of the alkaline hydrolysis. However, refluxing a mixture of **1b** and **1c** or a pure sample of **3** with NaOD in CH_3OD followed by methylation of the resulting acids showed no incorporation of deuterium into **3** or **4** and no conversion of **3** into **4**. Hence, the esmeraldins most likely occur in Nature as mixtures of diastereomers, as had already been suspected by Keller-Schierlein *et al.*[1] This finding does not, however, rule out the possibility that epimerization at C-12 of **2** and at C-22 of **1** is an artefact of the degradation leading to **5** or **3** and **4**, respectively.

Table I. Molar Ellipticities for Compounds 3 and 4

3		4	
wavelength (nm)	$[\Theta] \times 10^4$	wavelength (nm)	$[\Theta] \times 10^4$
254	−20.37	254	+19.80
314	−8.65	304	+8.89
362	+11.66	362	−12.73
430	−3.61	436	+2.19

Biosynthetic Experiments. ^{13}C-Labeled precursors were administered aseptically to 10–20 100-mL shake cultures, usually in two portions at about 85 and 99 h after inoculation; the cultures were harvested about 60 h after the first addition. These experiments were usually preceded by a feeding experiment with the same precursor carrying a ^{14}C-label to check on its general incorporation. The resulting esmeraldins and saphenyl esters were then isolated and converted into **3**–**5**, which were analyzed by ^{13}C-NMR spectroscopy.

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Table II. ^{13}C–^{13}C Couplings Observed in 3–5 Biosynthesized from [U-$^{13}C_3$]Glycerol

coupled carbon atoms in 3 and 4	coupling constants (Hz)	coupled carbon atoms in 5	coupling constants (Hz)
C-2, C-3	60.7	C-2, C-3, C-4	51.9 and 59.4
C-6, C-22	46.8	C-6, C-12	48.9
C-8, C-9	46.6	C-8, C-9	50.2
C-16, C-25	41.2		
C-17, C-18, C-19	50.5 and 54.5		

An initial feeding of [U-^{14}C]shikimic acid gave very little incorporation of label into the esmeraldins. This suggests that either shikimic acid is not a precursor or it does not penetrate to the site of antibiotic synthesis. Isolation and acid hydrolysis of the cellular protein also showed little incorporation of radioactivity (0.2%) into the total amino acid fraction isolated on a Dowex 50 column, supporting the notion of poor cellular uptake of shikimic acid by this organism. In an attempt to overcome this problem, a larger quantity of [1,7-$^{13}C_2$]shikimic acid[25] was fed. However, no significant enrichment of ^{13}C and no coupling for the signal pairs C-1/C-21, C-6/C-22, C-11/C-24, or C-16/C-25 was evident in the spectra of the resulting samples of **3** and **4**.

Evidence for an origin of **1** and **2** from the shikimate pathway did, however, come from feeding experiments with [U-$^{13}C_3$]glycerol. The experiment yielded 9.1 mg of **3**, 16.5 mg of **4**, and 9.2 mg of **5**. The proton decoupled ^{13}C-NMR spectra of **3** and **4** were very complex, due to the overlap of many resonances arising from the aromatic carbons. In spite of this, it was immediately apparent from the presence of satellites indicating coupled spin systems that intact units of glycerol had been incorporated into the esmeraldins, e.g., the satellites at C-21 (76.2 Hz) and C-24 (68.5 Hz) suggest coupling to their adjacent carbons, C-1 and C-11, respectively. Most ^{13}C-^{13}C connectivities were deduced the from 2D-INADEQUATE NMR[26] spectra of **3** and **4**. The presence of two-carbon connectivities between C-6/C-22, C-16/C-25, C-2/C-3, and C-8/C-9 was established. The data also revealed incorporation of an intact unit of glycerol into C-17/C-18/C-19. The NMR spectra of **5** biosynthesized from [U-$^{13}C_3$]-glycerol were analyzed in the same manner. In addition to two-carbon units (C-6/C-12 and C-8/C-9), one intact glycerol moiety was incorporated (C-2/C-3/C-4). The data are summarized in Table II.

Since many of the expected couplings could not be identified due to extensive signal overlaps, we further delineated the mode of incorporation of glycerol by feeding [1,3-$^{13}C_2$]glycerol and [2-^{13}C]glycerol. From the fermentations we obtained samples of **3** and **4** which were analyzed by proton decoupled inverse gated ^{13}C-NMR spectroscopy. The average enrichment of labeled carbons in **4** was 0.6%. The results, which are listed in Table III, are entirely consistent with formation of the esmeraldin and saphenamycin ring systems and their directly attached carbons from an intermediate of the shikimate pathway, as illustrated in Figure 3.[27] In the process all seven carbon atoms of the shikimate moiety are retained, as evidenced by the couplings of C-21, C-22, C-24, and C-25 to their adjacent ring carbons in the [U-$^{13}C_3$]glycerol experiment, ruling out a pathway via phenylpropanoid intermediates. The intact incorporation of a glycerol unit into C-17/C-18/C-19 reveals the orientation of the two shikimate-equivalent monomer units in the dimerization to the phenazine system.

The shikimate pathway accounts for all carbons of **1a** and **2a** except the *C*-methyl groups. These could be provided either by the methyl group of methionine or C-2 of acetate. The fact that they are efficiently labeled by [1,3-$^{13}C_2$]glycerol does not distinguish between the two possibilities, because C-1 of *syn*-glycerol gives rise to the methyl group of methionine (via serine) and to C-2 of acetate (via pyruvate). Transfer of the methyl group of methionine to a carbonyl carbon is mechanistically not very plausible, and a feeding experiment with [*methyl*-^{13}C]methionine gave no detectable enrichment in the *C*-methyl groups of **3** and **4** (data not shown). In contrast, feeding of sodium [2-^{13}C]acetate to 20 cultures and sodium [1-^{13}C]acetate to 10 cultures gave **3**–**5**, which by proton-decoupled inverse gated ^{13}C-NMR were clearly shown to be specifically enriched by C-2 but not by C-1 of acetate. The label from C-2 of acetate appeared in C-23 and C-26 of **3** and **4** and in C-13 of **5** (Table III), leaving little doubt that C-2 of acetate is indeed the source of the *C*-methyl groups.

In a preliminary experiment the nature of the monomeric precursor of the diphenazine system of the esmeraldins, i.e., the biosynthetic relationship of the saphenamycins and the esmeraldins, was probed. A sample of **2a** tritiated in the *C*-methyl group was prepared by feeding 2-tritiated acetic acid (8.58 mCi, 1.0 Ci/mmol) to *S. antibioticus*. Labeled **4** and **5** were obtained from the fermentation and purified to constant specific radioactivity (87 and 91 mCi/mmol, respectively).[29] The sample of **5** was then hydrolyzed to **2a** (97 mCi/mmol), which was fed to a new culture. The isolated **4** and **5** from this fermentation were again purified to give constant specific radioactivities of 1.06 and 0.93 mCi/mmol, respectively. These preliminary results suggest that **2a** is indeed a precursor of the esmeraldins, although the experiment does not reveal whether it is incorporated into both halves of **1b** and **1c** or only into the "northwestern" portion of the molecule. The close correspondence of the specific radioactivities of the two compounds, if it is not fortuitous, could be interpreted in favor of the latter option, as could the fact that unlike **2a**, the free esmeraldic acid **1a** has not been detected in the fermentation.

Discussion

The results presented here demonstrate that the saphenamycins and esmeraldins are biosynthesized via the shikimate pathway, as has been demonstrated for other phenazines in various organisms.[7,9–15] The failure of added

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(27) It is noteworthy that little ^{13}C from [1,3-$^{13}C_2$]glycerol appears in any of the carbons corresponding to C-5 of shikimic acid, i. e., in C-4a, C-9a, C-14a, and C-19a of **4** and in C-4a and C-9a of **5**. This carbon is derived from C-1 of erythrose 4-phosphate, which in turn arises from C-3 of hexose 6-phosphates. It has been observed in other work[28] that glycerol labels the "top" and "bottom" halves of hexose phosphates differentially, surprisingly giving much higher enrichment in the "bottom" than in the "top" half.

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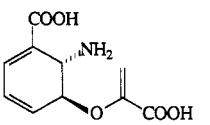
(29) The sample of tritiated acetate fed was actually sodium (*R*)-[2-$^2H_1$,^3H]acetate, and an attempt was made to determine the overall steric course of *C*-methyl group formation. However, Kuhn–Roth oxidation of **4** gave acetic acid with extensive loss of tritium and, concomitantly, complete racemization.

Table III. ^{13}C-Enrichments in 4 and 5 Biosynthesized from [1,3-$^{13}C_2$]- and [2-^{13}C]Glycerol and [2-^{13}C]Acetate

4 carbon	relative ^{13}C-enrichment[a] from			5 carbon	relative ^{13}C-enrichment[a] from		
(δ, ppm)	[1,3-$^{13}C_2$]glycerol	[2-^{13}C]glycerol	[2-^{13}C]acetate	(δ, ppm)	[1,3-$^{13}C_2$]glycerol	[2-^{13}C]glycerol	[2-^{13}C]acetate
C-1 (129.5)	0.1	1.0		C-1 (131.3)	0	1.0	
C-2 (125.0)	0.6	0		C-2 (132.1)	0.6	0.2	
C-3 (137.0)	0.1	0.9		C-3 (129.2)	0.2	1.1	
C-4 (126.3)	0–0.6[b]	0		C-4 (133.1)	0.6	0.3	
C-4a (139.6)	0	0.1		C-4a (140.7)	0.1	0.1	
C-5a (140.9)	0.4	0		C-5a (141.5)	0.9	0.4	
C-6 (142.2)	0	1.1		C-6 (142.6)	0	1.0	
C-7 (127.1)	0.4	0		C-7 (127.2)	0.5	0.2	
C-8 (128.9)	0	0.6		C-8 (130.7)	0.1	1.1	
C-9 (129.4)	0.6	0		C-9 (129.5)	0.4	0.1	
C-9a (142.0)	0	0		C-9a (144.0)	0.4	0.5	
C-10a (139.0)	0.8	0.1		C-10a (140.5)	0.8	0.1	
C-11 (111.6)	0–0.3[b]	0–1.4[b]		C-11 (166.9)	0.8	0.1	
C-12 (126.3)	0–0.6[b]	0		C-12 (68.6)	0.6	0.1	
C-13 (119.2)	0	0.8		C-13 (23.4)	0.6	0.1	1.5
C-14 (120.5)	0.4	0		C-14 (52.6)	0	0	0
C-14a (129.7)	0.1	0.2					
C-15a (126.8)	0.8	0					
C-16 (129.0)	0	0.9					
C-17 (120.8)	0.4	0					
C-18 (125.8)	0.1	0.7					
C-19 (111.5)	0–0.3[b]	0–1.4[b]					
C-19a (133.5)	0.2	0.2					
C-20a (142.2)	0.7	0					
C-21 (166.6)	0.4	0					
C-22 (69.0)	1.0	0					
C-23 (24.0)	0.7	0	0.9				
C-24 (168.1)	0.5	0					
C-25 (31.3)	0.6	0					
C-26 (23.4)	0.7	0	1.0				
C-27 (52.7)	0	0	0				
C-28 (52.0)	0	0.1					

[a] Relative to the abundance at C-27 (**4**) and C-14 (**5**) = 1.1% (0% enrichment). [b] The relative ^{13}C-enrichments reported as a range are a result of coincident ^{13}C signals.

labeled shikimic acid to label **3–5** is most likely due to impermeability of the cells of this organism to shikimate.[30] However, the alternative possibility that the biosynthesis of these phenazines branches off from the main pathway at a point prior to shikimic acid cannot be entirely dismissed. Although not all of the expected ^{13}C–^{13}C couplings were detected in the samples of **3** and **4** derived from [U-$^{13}C_3$]glycerol, the patterns observed are sufficient to demonstrate that the assembly of two shikimate-equivalent units to each of the phenazine moieties of **1** involves the same pairing scheme demonstrated for the formation of the phenazines of *Pseudomonas* species[9,10,12] (Figure 3), i.e., each nitrogen forms a bridge between C-6 of one and C-5 of the other shikimate-equivalent unit. Based on indirect evidence it is generally assumed, but has not been proven, that chorismate is an intermediate in phenazine formation and that the actual species undergoing dimerization is an intermediate in anthranilate biosynthesis, probably compound **6**.[33] On the other hand, recent work[34] on the biosynthesis of the mC_7N units encountered in various antibiotics from Actinomycetes, e.g., rifamycin, has uncovered a new branch of the shikimate pathway leading to 3-dehydro-5-deoxy-5-aminoshikimic acid (**7**), which is then aromatized to 3-amino-5-hydroxybenzoic acid, the mC_7N unit precursor. It should be pointed out that **7** could produce the same pairing pattern in phenazines by a mechanistically more plausible route involving reduction to 5-deoxy-5-aminoshikimic acid, followed by dehydration and dimerization. Since this route does not involve shikimic acid as an intermediate it would provide a logical alternative explanation for the lack of incorporation of this compound into **1** and **2**. Although the weight of the evidence from the extensive work on phenazine formation in *Pseudomonas* favors the intermediacy of **6**, the possibility that phenazines in Streptomycetes may be formed via **7** should at least be explored.



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The saphenamycins and esmeraldins differ from other natural phenazines by the presence of the extra *C*-methyl groups. These were found to arise from C-2 of acetate, as in a number of other examples, e.g., the antibiotics myxovirescin,[35] elaiomycin,[36] and virginiamycin M_1[37] and brevetoxin B.[38] The process presumably involves condensation of a monothioester of the phenazine precursor, probably phenazine-1,6-dicarboxylic acid, with malonyl-CoA or acetyl-CoA followed by thioester hydrolysis and

(30) We have encountered several other instances of impermeability of microbial cells to shikimic acid, e.g., in *S. nodosus* ssp. asukaensis (producer of asukamycin)[31] and in *Claviceps sp.*, strain SD58 (producer of clavine alkaloids).[32]

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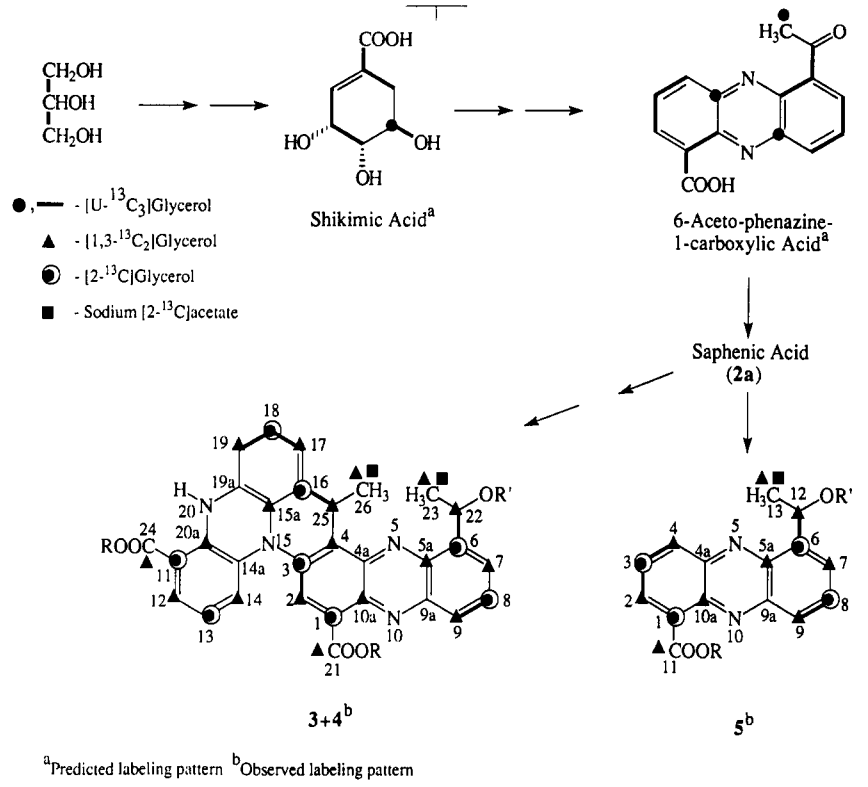

Figure 3. Labeling pattern of **3–5** from [^{13}C]glycerol and [^{13}C]acetate.

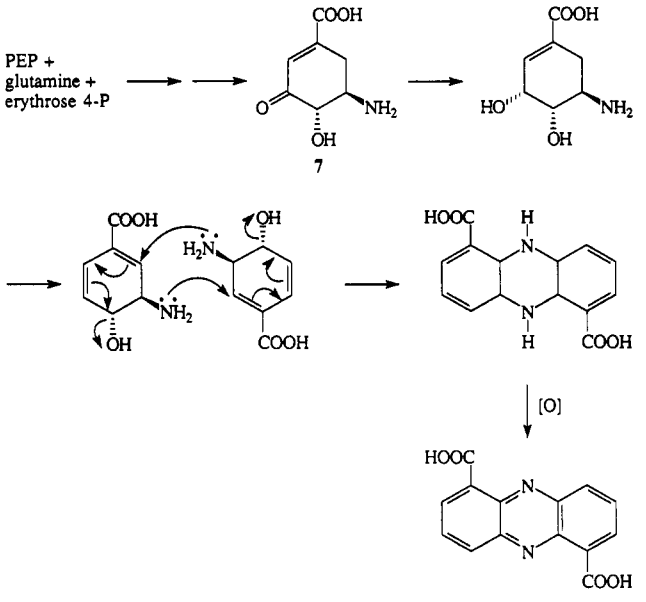

Figure 4. Alternate hypothetical mechanism for phenazine formation in streptomycetes.

β-decarboxylation. Reduction of the resulting methyl ketone then gives **2a**.

The biosynthesis of the saphenamycins and esmeraldins poses some interesting stereochemical questions. It appears that **1** indeed exists in Nature as a mixture of diastereoisomers, as had originally been suggested by Keller-Schierlein *et al.*[1] The available evidence strongly suggests that the ring system of **1** has three, not two, stereogenic centers, those at C-22 and C-25 and an additional one due to the two helical senses of the nonplanar arrangement of rings A, B, D, and E. The latter dominates the chirooptical properties. Derivatives **3** and **4** have opposite configurations at this center, but each is again a mixture of epimers at C-22, as shown by the NMR analysis of their Mosher esters. This analysis also suggests that only one epimer at C-25 is present, but this point is not strictly proven by these data because the two epimers at C-25 could have degenerate NMR spectra. However, that **3** and **4** must indeed each have only one configuration at C-25 is strongly indicated by the fact that their CD spectra are not exact mirror images of each other, but show small differences, and that they can be separated on an achiral chromatography column. This can only be explained by **3** and **4** having the same configuration at C-25.

The epimerization at C-12 of **2** and C-22 of **1** observed in their derivatives **3–5** may take place naturally in the fermentation or as an artefact during the degradation or both. It seems rather unlikely that racemic **2a** is formed by reduction of the precursor methyl ketone, since this would require that either one or two enzymes generate the two enantiomers in equal amounts. More likely the reduction produces one configuration and epimerization occurs subsequently. A plausible mechanism via a dihydrophenazine, a vinylogous carbinolamine which can reversibly eliminate water to give an achiral species, is shown in Figure 5A. The same achiral species would also be a plausible precursor for the ring A, B, and C segment in the dimerization to give the diphenazine system of **1**. One possible mechanism is shown in Figure 5B. The order in which the C-4/C-25 and the C-3/N-15 bonds are formed is dictated by the fact that both **3** and **4** have the same configuration at C-25. This requires that the C-4/C-25 bond must be formed first and under stereocontrol by the enzyme; the subsequent formation of the C-3/N-15 bond may not be under enzyme control. The reverse order of

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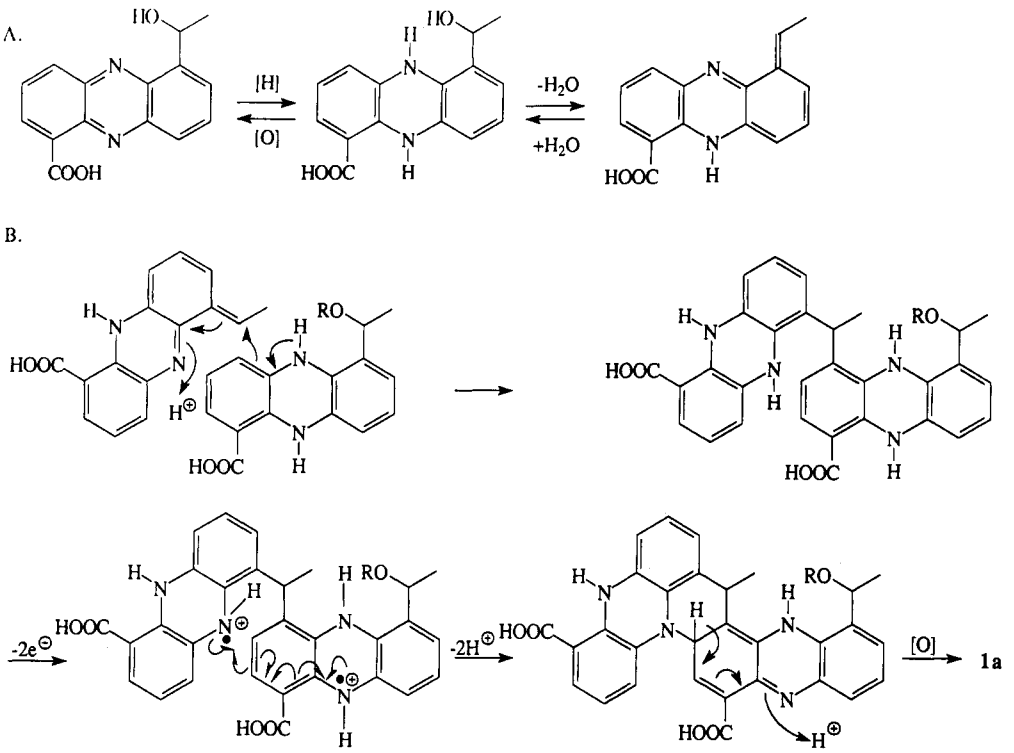

Figure 5. Hypothetical mechanism for the dimerization of saphenic acid derivatives to esmeraldins.

bond formation would of necessity lead to a correlation between the configurations at C-25 and at the helical stereocenter, i.e., **3** and **4** would have to be enantiomers, not diastereomers.

Exploration of the validity of some of these ideas will be the subject of future investigations.

Experimental Section

Materials. All organic solvents were reagent or HPLC grade purchased from E. M. Science, Mallinckrodt, or J. T. Baker Chemical Co. All aqueous solutions used in fermentation media and reactions were made up in deionized Milli-Q (Millipore) filtered water. All commercially available reagents were purchased from Aldrich or Sigma Chemical Co. and were usually of the highest purity available. Silica gel was purchased from E. M. Science Co. or J. T. Baker Co.

Radioactive precursors were obtained from the following sources: [U-^{14}C]Shikimic acid (21.9 mCi/mmole) from Dupont NEN Research Products; [1,3-^{14}C]glycerol (50 mCi/mmol) from ICN. Sodium (*R*)-[2-$^{2}H_1$,^{3}H]acetate (1 Ci/mmol) was synthesized at the National Tritium Labeling Facility, Berkeley, CA following a published procedure.[39] Stable isotope-labeled precursors were purchased from the following sources: Sodium [2-^{13}C]acetate, sodium [1-^{13}C]acetate, K^{13}CN, [1-^{13}C]acetic acid, and [2-^{13}C]-glycerol from Cambridge Isotope Laboratories. D-[1,7-$^{13}C_2$]Shikimic acid[25] and [U-$^{13}C_3$]glycerol[28] were samples previously synthesized in this laboratory, [1,3-$^{13}C_2$]glycerol was synthesized[40] from K^{13}CN and [1-^{13}C]acetic acid. All ^{13}C-compounds were 99 atom % ^{13}C enriched.

NMR Spectroscopy. ^{1}H- and ^{13}C-NMR spectra were acquired in $CDCl_3$ on an IBM AF-300 FT-NMR spectrometer operating at a field strength of 7.1 T. Samples were prepared in 5-mm tubes and analyzed at 298 K. Spectra were internally referenced to the solvent resonance. 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. The proton decoupled, inverse-gated ^{13}C-NMR spectra were obtained by setting the delays at 5 s. The incorporations were calculated by comparing the integration of individual signals with those of an unlabeled standard sample which was run under the same conditions.

Fermentation Conditions. *Streptomyces antibioticus* strain Tü 2706 was grown on plates of yeast–malt extract agar (yeast extract, 4%; malt extract, 10%; glucose, 4%; agar, 20%; pH adjusted to 7.3 before addition of 20% agar). The media were autoclaved (121°C, 19 psi, 15 min), poured into petri dishes, and left to cool. After inoculation and incubation at 28 °C for 6–10 days, the plates were stored at 4 °C. The cultures were also stored at −20 °C or −80 °C as frozen vegetative mycelia (FVM) in seed medium containing 20% glycerol. The seed and production medium consisted of 2% soybean meal (full-fat) and 5% mannitol in deionized water (pH 7.5). The seed cultures (100 mL) were grown in 500-mL baffled Erlenmeyer flasks by inoculating with an agar plug of spore culture or a FVM (5 mL/flask) and incubated for 48–72 h (28 °C, 150 rpm with a throw of 50 mm). The production cultures were inoculated from the seed cultures (5 mL/flask) and incubated for 144 h under the same conditions.

Product Isolation, Derivatization, and Purification. After centrifugation of the fermentation broth, the supernatant was filtered through cheesecloth under vacuum. The mycelial pellet was suspended in methanol/methylene chloride (2:1) and stirred overnight. This mixture was centrifuged and the green supernatant again filtered. This extraction procedure was repeated four times. The combined organic solutions were concentrated to a green oil which was taken up in saturated $NaHCO_3$ solution and extracted with *n*-hexane. After acidifying the mixture to pH 1 with 2 N HCl, the aqueous solution was extracted three times with methylene chloride. The solvent was removed *in vacuo* and the resulting residue dissolved in a minimum amount of methylene chloride. The sample was applied to a silica gel column of 40 and 60-μm silica gel packed in a 2:1 ratio on top of each other. The yellow metabolite was eluted with methylene chloride, followed by methylene chloride/ethyl acetate (step gradient from 9:1 to 1:1) and then adding 5% methanol to the last solvent mixture. The green fractions were eluted by addition of 0.5% of formic acid to the latter, followed

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by methanol containing 0.5% formic acid. The appropriate fractions were combined and concentrated *in vacuo*.

The yellow and green sample were each dissolved in methylene chloride and 2 N methanolic NaOH solution added. The mixtures were heated at reflux for 5 h under a nitrogen atmosphere. After cooling to room temperature, the mixtures were acidified to pH 1 with 2 N HCl and extracted three times with methylene chloride and the extracts were dried over magnesium sulfate, filtered, and evaporated to dryness. The residues were then converted to the corresponding methyl esters with diazomethane in ether. The color of the samples turned from green to blue and yellow to golden brown, respectively, upon addition of diazomethane.

The blue sample was applied to a silica gel flash column (60-μm) and fractionally eluted using the same solvents as described above. It was then further purified by HPLC on a semipreparative Partisil 10 PAC column (10 μm, 9.6 × 250 mm) which was eluted with methylene chloride/ethyl acetate (9:1) at flow rates of 2.0–3.0 mL/min and monitored at 365 nm. Two compounds with retention times of 12 (**3**) and 14.5 min (**4**) were separated. The fractions containing **3** and **4** were pooled separately, dried over magnesium sulfate, filtered, and taken to dryness by rotary evaporation.

The yellow sample was applied to a preparative silica gel TLC plate (Merck K 60 μm, F254 nm, 2 mm), which was developed in methylene chloride/ethyl acetate/methanol (90:10:2). The major yellow band at R_f 0.40 was eluted with methylene chloride/acetone (1:1). The eluate was dried over magnesium sulfate, filtered, and concentrated *in vacuo*. This chromatography procedure was repeated once or twice, affording pure saphenic acid methyl ester.

Esmeraldic acid dimethyl ester (3): ^{1}H NMR ($CDCl_3$) δ 1.44 (3 H, d, 6.9, H-26), 1.84 (3 H, d, 6.5, H-23), 3.93 (3 H, s, H-28), 4.03 (3 H, s, H-27), 5.15 (1 H, q, 7.0, H-25), 5.74 (1 H, q, 6.5, H-22), 6.52 (1 H, dd, 7.9, 1.1, H-19), 6.76 (1 H, dd, 7.9, 7.6, H-13), 6.86 (1 H, dd, 7.2, 1.0, H-17), 6.96 (1 H, dd, 7.9, 7.2, H-18), 7.18 (1 H, d, 7.6, H-14), 7.60 (1 H, dd, 7.6, 0.9, H-12), 7.71 (1 H, m, H-7), 7.72 (1 H, m, H-8), 8.16 (1 H, dd, 8.3, 1.7, H-9), 8.43 (1 H, s, H-2), 9.51 (1 H, s, H-20).

Esmeraldic acid dimethyl ester (4): ^{1}H NMR ($CDCl_3$) δ 1.42 (3H, d, 6.9, H-26), 1.82 (3H, d, 6.5, H-23), 3.93 (3H, s, H-28), 4.03 (3H, s, H-27), 5.15 (1H, q, 7.0, H-25), 5.76 (1H, q, 6.5, H-22), 6.51 (1H, dd, 7.9, 1.1, H-19), 6.75 (1H, dd, 7.9, 7.6, H-13), 6.85 (1H, dd, 7.2, 1.0, H-17), 6.95 (1H, dd, 7.9, 7.2, H-18), 7.17 (1H, d, 7.6, H-14), 7.59 (1H, dd, 7.9, 0.9, H-12), 7.71 (1H, m, H-7), 7.72 (1H, m, H-8), 8.14 (1H, dd, 8.3, 1.7, H-9), 8.41 (1H, s, H-2), 9.50 (1H, s, H-20).

Saphenic acid methyl ester (5): ^{1}H NMR ($CDCl_3$) δ 1.76 (3H, d, 6.6, H-13), 4.08 (3H, s, H-14), 5.68 (1H, q, 6.4, H-12), 7.75 (1H, dd, 7.3, 2.0, H-7), 7.79 (1H, dd, 7.3, 7.3, H-8), 7.82 (1H, dd, 8.5, 8.5, H-3), 8.19 (1H, dd, 7.3, 2.0, H-9), 8.22 (1H, dd, 8.5, 1.5, H-2), 8.32 (1H, dd, 8.5, 1.5, H-4).

22-[(2′*S*)-2′-Methoxy-2′-(trifluoromethyl)phenylacetoxy]-esmeraldic acid dimethyl ester (Mosher ester of 3): 1.42 (3H, d, 7.1, H-26), 1.45 (3H, d, 7.0, H-26), 1.86 (3H, d, 6.4, H-23), 1.92 (3H, d, 6.6, H-23), 3.47 (3H, d, 0.9, OMe of Mosher ester), 3.65 (3H, d, 1.1, OMe of Mosher ester).

22-[(2′*S*)-2′-Methoxy-2′-(trifluoromethyl)phenylacetoxy]-esmeraldic acid dimethyl ester (Mosher ester of 4): 1.40 (3H, d, 6.5, H-26), 1.84 (3H, d, 6.4, H-23), 1.89 (3H, d, 6.4, H-23), 3.53 (3H, d, 1.1, OMe of Mosher ester), 3.62 (3H, d, 0.8, OMe of Mosher ester), 3.92 (3H, s, H-27).

Preparation of [13-^{3}H,^{2}H$_1$]saphenic acid:[29] [13-^{3}H,^{2}H$_1$]-Saphenic acid methyl ester was obtained from a feeding experiment with sodium (*R*)-[2-^{2}H$_1$,^{3}H] acetate. The sample was dissolved in methylene chloride and methanolic NaOH solution (2 M, 1 mL) added. The mixture was heated at reflux under argon for 1 h. Water (5 mL) was added and the solution acidified to pH 1 using 1 M HCl. After extraction with methylene chloride (3 times), the organic layers were combined and dried *in vacuo*. The crude sample was applied to a preparative TLC plate, which was developed in methylene chloride/ethyl acetate/methanol (90:8:2). The yellow band at R_f 0.29 was eluted with methylene chloride/acetone (1:1) and the eluate dried over magnesium sulfate, filtered, and evaporated *in vacuo*. The sample was purified to constant specific activity (97.5 μCi/mmol).

Feeding Experiments. The ^{14}C-labeled precursors, [U-^{14}C]-shikimic acid (5.1 mg, 29.4 μmol, 1.7 μCi, 0.06 μCi/μmol) and [1,3-^{14}C]glycerol (0.026 μmol, 1.3 μCi, 50 μCi/μmol) were each added to one production culture between 80–84 h after inoculation. The cultures were harvested 60–64 h later and the products isolated as described above. The incorporation of ^{14}C-label was determined by analysis of the crude green fraction obtained from the silica gel flash column on a semipreparative HPLC column. The sample was dissolved in methylene chloride, an aliquot injected onto a Partisil 10 PAC column, with flow rates of 2–3 mL/min, and eluted with methylene chloride/ethyl acetate/formic acid (1:1:0.001). Fractions were collected and counted in a Beckman Model 7500 liquid scintillation counter.

The tritiated precursor, [13-^{3}H,^{2}H$_1$]saphenic acid (0.87 μmol, 0.085 μCi, 97.5 μCi/mmol) was dissolved in ethanol (150 μL) and administered to one production culture at 90 h after inoculation. The culture was harvested in two equal portions 24 and 48 h later and **3**–**5** were prepared and isolated as described above. Esmeraldic acid dimethyl ester (**4**) and saphenic acid methyl ester (**5**) were purified to constant specific activities of 1.06 and 0.93 μCi/mmol, respectively.

The feeding experiments with ^{13}C-labeled precursors were carried out in the following manner: The substrates (0.23–1 g) were dissolved in water (20 mL) and added to 10–20 production cultures, either in one addition at 96 h after incubation or two additions at 85 and 99 h. The cells were harvested 45 h later. Esmeraldic acid dimethyl esters and saphenic acid methyl ester were obtained as described above. The amounts of products obtained were 10–45 mg for **3**, 9–44 mg for **4** and 9–26 mg for **5**.

Acknowledgment. We are greatly indebted to Professor Hans Zähner, Tübingen, Germany, for suggesting these studies and for providing the culture of *Streptomyces antibioticus* strain Tü 2706 as well as reference samples. We also acknowledge with gratitude the supply of ^{13}C-labeled compounds by the Los Alamos Stable Isotope Resource, supported by NIH grant RR 02231 and USDOE/OHER, and the financial support provided by the NIH through research grant AI 20264.