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Biosynthesis of Phenazine Antibiotics in *Streptomyces antibioticus*: Stereochemistry of Methyl Transfer from Carbon-2 of Acetate

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Abstract: Stable isotope labeling experiments have shown that the biosynthesis of the monomeric phenazines, the saphenyl esters, and their dimerization products, the esmeraldins, in *Streptomyces antibioticus* Tü 2706 proceeds from phenazine-1,6-dicarboxylic acid by chain extension with C-2 of acetate to 6-acetylphenazine-1-carboxylic acid, which is reduced to saphenic acid. The latter is incorporated into both halves of the esmeraldins, albeit differentially. By feeding of chiral acetate, degradation of the resulting saphenyl esters and esmeraldins, and configurational analysis of the acetic acid formed, the chain extension process was found to proceed with overall inversion of configuration at the methyl group. This suggests that the decarboxylation of a hypothetical intermediate β-keto acid proceeds in an inversion mode. This result is discussed with reference to analogous C-methylations of polyketide backbones by addition of C-2 of acetate.

Phenazines are a group of natural products which are quite common in many bacteria; most of its about 75 members are produced by *Pseudomonas* species, but a few are also found in Streptomycetes.[1,5] *Streptomyces antibioticus*, strain Tü 2706, produces a series of dimeric phenazine derivatives (Figure 1), the intensely green esmeraldins A (**1b**) and B (**1c**),[2] as well as simpler phenazines such as saphenic acid (**2a**) and its esters (**2b** and **2c**).[3] Several of the compounds showed antibacterial activity and anticancer activity against murine tumors.[2] Degradation and derivatization of the esmeraldins allowed their separation into a pair of diastereomers, esmeraldic acid dimethyl esters I and II (**3** and **4**), which are epimeric at N-15. NMR analysis of their Mosher's esters further revealed that each compound in turn was a mixture of epimers at C-22, but with a single configuration at C-25.[4]

As reported previously,[4] the biosynthesis of the esmeraldins involves the same head-to-tail dimerization of an unknown metabolite of the shikimic acid pathway that has been demonstrated for the phenazines elaborated by *Pseudomonas* sp.[5−10]

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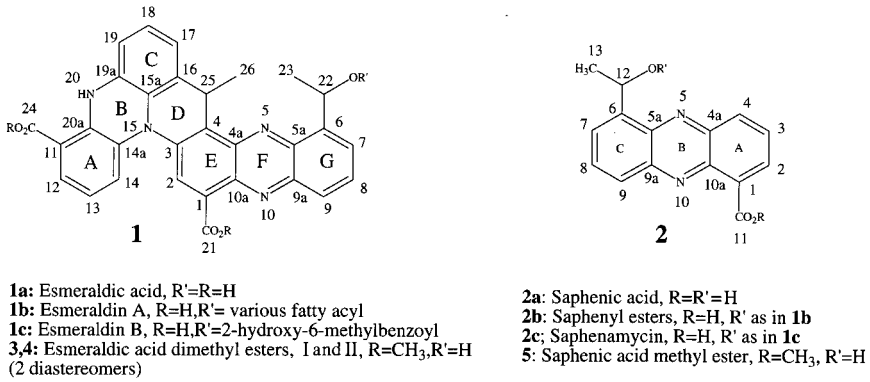


Figure 1. Structures of esmeraldins and saphenic acid derivatives from *Streptomyces antibioticus* Tü 2706.

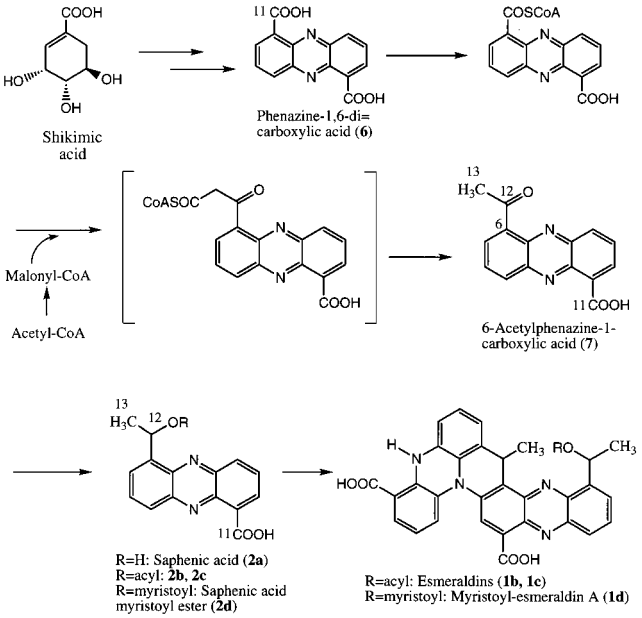
and by some other Streptomycetes.[11,12] The product, presumably phenazine-1,6-dicarboxylic acid (**6**),[13] is then further converted into saphenic acid **2a** or a derivative thereof by addition of a one-carbon unit, a methyl group derived from C-2 of acetate. Such one-carbon extensions of various structural backbones, particularly polyketides, have been encountered in the biosynthesis of a number of compounds, such as myxovirescin A_1,[14] virginiamycin,[15] myxopyronin A,[16] aurantinin,[17] pseudomonic acid,[18,19] oncorhyncolide,[20] cylindrophane,[1] and pulvomycin.[22] Preliminary evidence supports the notion that **2a** in turn is converted into **1b** and **1c** by dimerization and esterification.[4]

In the present paper we report experiments which provide further support for the proposed pathway by which the esmeraldin structures are assembled and which shed some light on the one-carbon extension process by determining its steric course at the acetate-derived methyl group.

Results

A plausible pathway by which C-2 of acetate can be converted into the methyl groups of the esmeraldin (**1**) and saphenamycin (**2**) type compounds is outlined in Scheme 1. Acetate as its coenzyme A thioester derivative is converted by acetyl-CoA carboxylase to malonyl-CoA, which then undergoes a decarboxylative Claisen condensation with the mono-CoA thioester of phenazine-1,6-dicarboxylic acid **6**. Hydrolysis of the resulting thioester and decarboxylation of the β-keto acid produces 6-acetylphenazine-1-carboxylic acid (**7**), which is then reduced to saphenic acid **2a**. Support for this pathway was ascertained

Scheme 1. Proposed Pathway for the Biosynthesis of the Dimeric Phenazines, the Esmeraldins, in *Streptomyces antibioticus* Tü 2706



by feeding stable isotope-labeled phenazine-1,6-dicarboxylic acid **6**, 6-acetylphenazine-1-carboxylic acid **7**, and saphenic acid **2a** to *Streptomyces antibioticus* Tü 2706 and demonstrating their specific incorporation into esmeraldins **1b**/**1c** and/or saphenyl esters **2b**/**2c**. Labeled **6** carrying 99% ^{13}C in one carboxyl group was synthesized from [7-^{13}C]benzoic acid and 2-chloro-3-nitrobenzoic acid by the method of Holliman and co-workers.[23] Feeding of this material (50 mg to ten 100 mL shake cultures) produced **1**, which was degraded[4] to esmeraldic acid dimethyl esters **3** and **4** (16.2% excess M + 1 and 2.2% excess M + 2 species by MS). These showed enhanced ^{13}C NMR signals for C-21, C-22, C-24, and C-25. A second feeding experiment with [*carboxy*-$^{13}C_1$]-**6** (20 mg to five 100 mL cultures) produced **2** isolated as **5** (15 mg) after base hydrolysis and esterification with diazomethane, which displayed enhanced ^{13}C NMR signals at C-11 and C-12 (0.5 and 0.7% enrichment), whereas MS analysis indicated the presence of 2.1% excess M + 1 species. The esmeraldins from the same experiment, isolated similarly as **3** (1.0 mg) and **4** (1.2 mg), showed 1.8% excess M + 1 species by MS. The amounts of sample and enrichments were too small to obtain meaningful ^{13}C NMR data.

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6-Acetylphenazine-1-carboxylic acid **7** bearing three deuterium atoms (98% ^{2}H) in its methyl group was synthesized by exchange of the unlabeled compound with D_2O and CD_3OD in the presence of NaOD; part of this material was further converted to saphenic acid **2a** bearing four deuterium atoms in the hydroxyethyl group by reduction with NaB^2H_4. 6-[13-$^2H_3$]-Acetylphenazine-1-carboxylic acid (17 mg) was fed to five 100 mL cultures and gave **1** isolated as esmeraldin dimethyl esters **3** and **4** (8 and 10 mg, respectively) carrying deuterium in both C-methyl groups, C-23 and C-26, as shown by deuterium NMR and ES-MS. Mass spectrometry indicated about 6% enrichment in each C-methyl group, and careful analysis of the deuterium NMR spectrum by line-fitting revealed, within the limits of accuracy, equal enrichment in the two methyl groups (**3**, intensity ratio C-26:C-23 1.0:0.988; **4**, ratio C-26:C-23 = 1.0:0.9). Similarly, [12,13-$^2H_4$]saphenic acid (50 mg) was fed to ten 100 mL cultures and gave esmeraldic acid dimethyl esters **3** and **4** (8 and 10 mg, respectively) showing a deuterium distribution by MS of 0.8% D_2, 4.2% D_3, 14.7% D_4, 0% D_5, 0.4% D_6, 1.4% D_7, and 2.0% D_8 species. Deuterium NMR showed an intensity ratio of the methyl to the methine signal of 3.94:1. Line-fitting analysis of the deuterium NMR spectrum of the labeled **4** from this experiment indicated that [12,13-$^2H_4$]-**2a** labels the two halves of **1** unequally (intensity ratio C-26:C-23 = 1.0:1.53 and C-25:C-22 = 1.0:1.85). Since the precursor was incorporated predominantly (70−80%) with retention of all four deuterium atoms, it is evident that (a) **2a** is incorporated directly, not by way of **7**, (b) it is incorporated preferentially into the "Eastern" half of **1** and (c) both methyl groups remain intact during the dimerization process.

The facts that esmeraldic acid **1a** has not been isolated from the esmeraldin fermentation and that esmeraldin A and B, **1b** and **1c**, carry the same acyl substituents as the saphenyl esters **2b** and **2c** suggested the possibility that the Eastern half of **1** may be derived more directly from the saphenyl esters than from **2a**. This could explain the unequal incorporation of [12,13-$^2H_4$]-**2a** into the two halves of **1**, although it would be more consistent with lower enrichment in the "Eastern" half than with the observed higher enrichment. To examine this point, we prepared the myristoyl ester of [12,13-$^2H_4$]-**2a**, [12,13-$^2H_4$]-**2d**, by acylation of [12,13-$^2H_4$]-**2a** with myristoyl chloride according to the procedure of Bahnmüller et al.[24] [12,13-$^2H_4$]-**2d** (8.5 mg) was fed to five 100 mL cultures and the resulting mixture of esmeraldins was analyzed by mass spectrometry. The analysis gave no evidence for the presence of myristoyl-esmeraldin A **1d** in the mixture. Deuterium NMR of the derived esmeraldic acid dimethyl esters, **3** and **4**, showed no detectable signal for deuterium at either C-22 or C-23. This experiment thus provided no indication for the direct incorporation of saphenyl esters into the esmeraldins.

The finding that the methyl groups of [12,13-$^2H_4$]-**2a** were incorporated intact into **1** made it possible to examine the steric course of the one-carbon chain extension from C-2 of acetate. To this end, sodium (*R*)-[2-$^2H_1$,^{3}H]acetate (8.58 mCi)[25] was administered to a 100 mL culture of *S. antibioticus* Tü 2706. The resulting **2**, isolated as **5** (0.24 μCi), was purified to constant specific radioactivity and degraded by ozonolysis and oxidation with $KMnO_4/K_2CO_3$ to carve out the hydroxyethyl group as sodium acetate. The purified sodium acetate (25% radiochemical yield) was subjected to configurational analysis by the malate synthase/fumarase assay,[26] which indicated a 45% enantiomeric excess of *S* configuration. To confirm this result, sodium (*S*)-[2-$^2H_1$,^{3}H]acetate (3.84 mCi) was fed to two 100 mL cultures of *S. antibioticus* in the same way and both **2**, isolated as **5** (1.36 μCi), and **1**, isolated as **3** (0.23 μCi) and **4** (0.16 μCi), were obtained. Degradation of **5** produced sodium acetate in 12.5% yield, which assayed for 18% ee *R* isomer in the configurational analysis.[27] Degradation of **3** from the same experiment gave sodium acetate (22% yield), which analyzed for 42% ee *R* configuration. Thus, the conversion of the methyl group of acetate into the C-methyl groups of **1** and **2** proceeds with predominant overall retention of configuration accompanied by a slightly more than 50% decrease in configurational purity.

Discussion

The results of the feeding experiments with the stable isotope-labeled compounds **6**, **7**, and **2a** provide considerable support for the pathway of esmeraldin assembly proposed in Scheme 1. The fact that deuterated **2a** labels the two halves of **1** differentially suggests that the dimerization reaction to produce the phenazine ring system must involve two different phenazines rather than dimerization of two identical molecules, such as **2a**. It is peculiar that such unsymmetrical labeling of **1** is not seen with deuterated **7** as precursor, but this may be a fortuitous result of the kinetics of the fermentation, i.e., the time courses of incorporation of isotope into the two halves may just accidentally cross over at the time of harvest. One could speculate that the two molecules undergoing dimerization to the esmeraldin ring system are saphenic acid, giving rise to the "Northwestern" half, and a saphenyl ester, giving rise to the "Eastern" half. However, the feeding experiment with saphenic acid myristoyl ester **2d** provides no support for such a process, although its negative outcome could be due to lack of cellular uptake of the precursor. Thus, although we cannot rule out the possibility that one of them is a saphenyl ester, the exact nature of the two molecules undergoing dimerization to **1** is not known at this time.

The experiments with chiral acetate shed some light on the process by which one of the carboxyl groups of **6** is extended to a two-carbon unit by addition of C-2 of acetate to give the C-methyl groups of **7**, **2**, and **1**. Scheme 2 elaborates on a plausible mechanism for this process, involving carboxylation of acetyl-CoA (**8**) to malonyl-CoA (**9**), decarboxylative Claisen condensation with the coenzyme A thioester of **6**, followed by thioester hydrolysis and decarboxylation. The reaction stereochemistry of several members of the three classes of enzymes presumed to act upon the methyl group in this sequence has been studied in other systems using the chiral methyl group methodology. Sedgwick and Cornforth[28] have shown that acetyl-CoA carboxylase replaces a hydrogen in the methyl group of acetyl-CoA by COOH with retention of configuration and a negligible deuterium isotope effect. Numerous enzymes catalyzing Claisen condensations have been shown without exception to operate with inversion of configuration at the methyl group.[26c] However, β-decarboxylases do not show such stereochemical consistency, as examples of both inversion and retention of configuration have been found.[29] In the acetyl-CoA

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(27) This sodium acetate sample was inadvertently exposed to base for a prolonged period of time, which probably caused substantial racemization.

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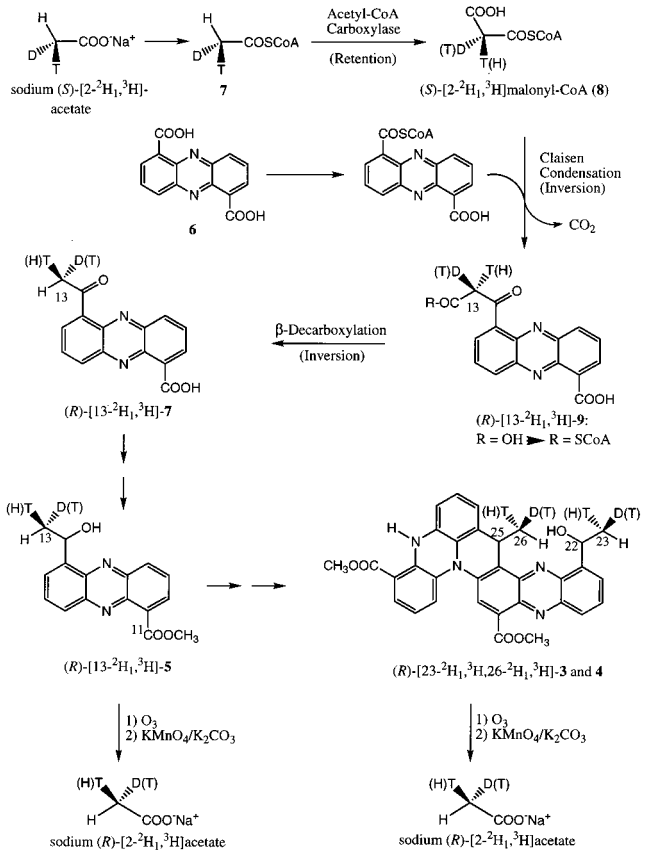
Scheme 2. Proposed Mechanism and Stereochemical Course of the Conversion of C-2 of Chiral (*S*)-Acetate into the Methyl Groups of the Saphenic Acid Derivatives and Esmeraldins in *Streptomyces antibioticus* Tü 2706[a]

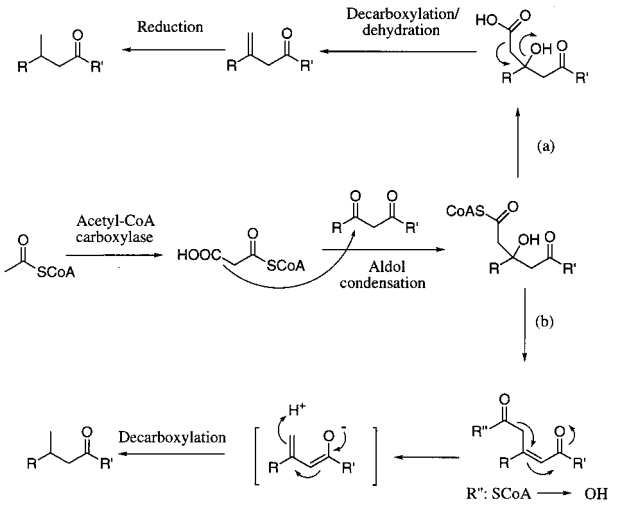


[a]Hydrogen isotopes in parentheses denote the expected results if a deuterium is abstracted by the acetyl-CoA carboxylase rather than a hydrogen.

carboxylase reaction the removal of H or D from the chiral methyl group without an isotope effect will produce two isotopomers of **9**, one containing H and T and the other D and T, in a 1:1 ratio. In the final decarboxylation reaction the latter will give a chiral methyl group and the former an achiral one; thus, the configurational purity of the final methyl group should be decreased to 50% of that of the starting material. The experimental values, 45 and 42% ee, are very close to this prediction. On the basis of the stereochemical precedence, a sample of (*S*)-acetate will give malonyl-CoA **9** of predominantly *S* configuration, which upon decarboxylative Claisen condensation will produce **10** of predominantly *R* configuration. To generate a chiral methyl group of predominantly *R* configuration in the final products, the observed overall stereochemical outcome from (*S*)-acetate as substrate, the β-decarboxylation of **10** must proceed with inversion of configuration (Scheme 2). The complementary result with (*R*)-acetate confirms this conclusion.

The mechanism of the one-carbon extension from C-2 of acetate in the formation of **1** and **2** may be unique and somewhat different from those of the analogous processes encountered in various polyketide biosyntheses.[14−22] Whereas the process described here must involve a decarboxylative Claisen condensation between two acyl derivatives, the one-carbon chain extensions of polyketides occur by connecting C-2 of acetate to a carbonyl group of the polyketide chain. They should therefore involve a mixed aldol reaction, which would be predicted to proceed with retention of configuration, since

Scheme 3. Hypothetical Mechanisms for the One-Carbon Extension of Polyketides from C-2 of Acetate



enzymatic aldol condensations proceed in a retention mode.[26c,29,30] Unlike in the phenazine case, the nature of the subsequent steps is uncertain and may in fact differ in different systems. Two mechanisms have been proposed in the literature (Scheme 3). For the introduction of the acetate-derived methyl groups attached to C-2 and C-14 of cylindrocyclophane D, Bobzin and Moore[21] propose a decarboxylation/dehydration of the initial aldol product to generate an *exo*-methylene group from C-2 of the acetate, which is then reduced to the methyl group (path a).[31] Experiments with deuterated acetate support this mechanism The overall stereochemical outcome in this case cannot be predicted and may not be mechanistically informative. An alternative mechanism has been proposed for the methylation, from C-2 of acetate, at C-7 and C-11 of the pulvomycin polyketide backbone.[22] Aldol condensation was postulated to be followed by dehydration to an α,β-unsaturated polyketide in which the stage is set for a vinylogous β-decarboxylation to give the methyl group (path b). On the basis of stereochemical precedence, such a mechanism would probably involve aldol condensation with retention and decarboxylation with inversion, resulting in overall retention of the methyl group configuration. To the best of our knowledge these reactions have not yet been examined for their stereochemical outcome.

Experimental Section

General Materials and Methods. ^{1}H, ^{2}H, and proton-decoupled ^{13}C NMR spectra were recorded on Brucker AF-300 (^{1}H 300 MHz) or WM-500 (^{1}H 500 MHz) spectrometers at 298 K. Chemical shifts in parts per million (ppm), using residual solvent signal as internal standard (^{1}H, ^{2}H: 7.24 (singlet) for $CDCl_3$, 3.30 (quintet) for $^2H_4$-methanol, 11.50 (singlet) for CF_3COOD; ^{13}C: 77.0 (t) for $CDCl_3$, 49.0 (septet) for methanol, 116.4 (quartet) and 164.6 (quartet) for CF_3COOD), are given on the δ scale. Coupling constants (J) are given in hertz (Hz). The proton-decoupled, inverse-gated ^{13}C NMR spectra were obtained by setting the delays at either 4 or 5 s. Mass spectral (MS) data were determined on a Micromass Ltd. Quattro II Tandem Quadrupole electrospray ionization mass spectrometer or on a Micromass 80SEQ Tandem Hybrid mass spectrometer for FAB-MS. Column chromatography was performed with use of Merck 60 μm silica gel. Preparative TLC was performed with use of Merck silica gel 60 F-254 plates (2 mm precoated). A Hewlett-Packard HP-5890A series II gas chromatograph (GC) with a capillary column coupled to a Hewlett-Packard 5971A mass selective detector was used for GC-MS. HPLC was carried

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(31) Cf.: O'Hagan, D. *Nat. Prod. Rep.* **1995**, *12*, 1.

Table 1

precursor	amount fed	culture vol	metabolite produced
[11-^{13}C]-**6** (1st feeding)	50 mg	1000 mL	1.2 mg of **3**; 1.1 mg of **4**
[11-^{13}C]-**6** (2nd feeding)	20 mg	500 mL	1.0 mg of **3**; 1.2 mg of **4**; 15 mg of **5**
[13-$^{2}H_3$]-**7**	17 mg	500 mL	8.0 mg of **3**; 10 mg of **4**
[12,13-$^{2}H_4$]-**2a**	50 mg	1000 mL	8.0 mg of **3**; 10 mg of **4**
[12,13-$^{2}H_4$]-**2d**	8.5 mg	500 mL	6.8 mg of **3**, 5.7 mg of **4**
(*R*)-[2-$^{2}H_1$,^{3}H]acetate	8.58 mCi	100 mL	0.24 μCi of **5** (97.5 μCi/mmol)
(*S*)-[2-$^{2}H_1$,^{3}H]acetate	3.84 mCi	200 mL	1.36 μCi of **5** (35 μCi/mmol)
			0.23 μCi of **3** (52.3 μCi/mmol)
			0.16 μCi of **4** (46.0 μCi/mmol)

out on a Beckman dual pump System Gold HPLC equipped with a Beckman UV detector module monitoring at 254 and 365 nm. Radioactivity was measured in Atomlight (DuPont) or Biosafe II (Research Products International Corp.) liquid scintillation fluid with a Beckman LS-1801 liquid scintillation counter with [^{3}H]- or [^{14}C]-*n*-hexadecane (Amersham) as internal standards. Melting points were determined on a Mel-Temp apparatus and are uncorrected. *Streptomyces antibioticus* Tü 2706 was a gift from Prof. Hans Zähner, University of Tübingen, Germany.

Feeding Experiments. Production cultures of *Streptomyces antibioticus* Tü 2706 were grown and their phenazine metabolites harvested as previously described.[4] After 4 days of growth of the production culture, appearance of a faint green color indicates the beginning of esmeraldin production. At that point (approximately 100 h after inoculation), half of the precursor (dissolved in water or dilute sodium bicarbonate) was administered by sterile filtration to the production flasks in equal quantities. Twelve to 16 h later, the second half of the precursor was administered to the production flasks in the same manner as the first. The cultures were harvested 6 days (approximately 150 h) after inoculation and were worked up as described,[4] producing the results shown in Table 1.

Purification of Phenazines from (*S*)-[2-$^{2}H_1$,^{3}H]Acetate Feeding. (*S*)-[2-$^{2}H_1$,^{3}H]Acetate (3.84 mCi) was pulse-fed to 200 mL of *S. antibioticus* fermentation medium as described above. The fermentation broth was harvested by centrifugation 6 days after inoculation. The supernatant was acidified to pH 2 with 2 N HCl and extracted with methylene chloride (3 × 100 mL) to give a yellow-brown oil after concentration. The cellular pellet was broken up in 100 mL of a mixture of CH_2Cl_2/MeOH (1:2) by stirring overnight. This mixture was filtered, and the extraction was repeated twice. The residue obtained after removing solvent from the combined filtrates was dissolved in 5% aqueous sodium bicarbonate (200 mL) and washed with 100 mL of *n*-hexane. After acidification to pH 1−2 with 2 N HCl, the aqueous solution was extracted with methylene chloride (3 × 50 mL). Solvent was removed in vacuo to give a green oily residue that was chromatographed on silica gel (*n*-hexane/acetone 1:1) to give yellow and green fractions. All yellow fractions (saphenyl metabolites **2**) were concentrated, combined with the residue from the supernatant extraction, and converted to methyl saphenate **5** by hydrolysis and esterification with diazomethane.[4] The crude **5** was chromatographed on silica gel (*n*-hexane/acetone 1:1) and the major yellow fraction was collected and evaporated to dryness (R_f = 0.35; 85 mg, 78 μCi/mmol). This residue was chromatographed on a preparativeTLC plate (CH_2Cl_2/EtOAc:MeOH 85:10.5) and the major yellow band at R_f = 0.45 was scraped off and eluted with CH_2Cl_2/acetone 1:1 (30 mg, 65 μCi/mmol). This sample was recrystallized from hexane/acetone yielding 16.5 mg of **5** (42 μCi/mmol), which was dissolved in CH_2Cl_2 and purified further by preparative TLC (hexane/acetone 1:1) to give, after elution with CH_2Cl_2/acetone, 14 mg of **5** (32 μCi/mmol). A final preparative TLC (CH_2Cl_2/EtOAc/MeOH 85:10:5) was carried out to demonstrate radiochemical purity. The yellow band at R_f = 0.4 yielded 12 mg of **5** (35 μCi/mmol); its ^{1}H and ^{13}C NMR data were in agreement with published values.[2,4] The green fractions from the initial column chromatography, containing the esmeraldins **1**, were hydrolyzed and esterified, using the same procedure as with the saphenyl metabolites. The resulting blue oil was chromatographed on silica gel (CH_2Cl_2/EtOAc 9:1) to give 6.2 mg of the diastereomers **3** and **4** (56 μCi/mmol). The two diastereomers were separated by HPLC on a semipreparative Partisil 10 PAC column (10 μm, 9.6 × 250 mm) with CH_2Cl_2/EtOAc 9:1 as the mobile phase at a flow rate of 2.5 mL/min to give 2.4 mg of **3** (52.3 μCi/mmol) and 1.9 mg of **4** (46.0 μCi/mmol).

Degradation of Saphenic Acid Derived from (*R*)-[2-$^{2}H_1$,^{3}H]-Acetate. The biosynthetically labeled saphenic acid methyl ester **5** (1.4 × 10^5 dpm)[4] was diluted with unlabeled material (6.8 mg, 0.02 mmol), dissolved in 10 mL of methanol, and cooled to 0 °C. Ozone was bubbled through the cold solution for 45 min, after which it was warmed to room temperature and purged with argon for 5 min. Solvent was removed in vacuo and the residue dissolved in 2 mL of water and stirred at 0 °C. A solution of potassium carbonate (69 mg, 0.5 mmol) and potassium permanganate (79 mg, 0.5 mmol) in 5 mL of water was added, and the resulting mixture warmed to room temperature with stirring. After 20 min, another 5 mL of the basic permanganate solution was added to the brown suspension and stirring was continued for an additional 40 min. This mixture was adjusted to a pH of 2 with 2 N sulfuric acid and volatiles were isolated by bulb-to-bulb lyophylization. To the collected volatiles, 0.3 mL of 50% sulfuric acid and 110 mg of mercuric sulfate were added, and the solution was heated to reflux for 1 h to oxidize any formic acid present. After the solution was cooled to room temperature, the pH was adjusted to 10 with 5 N NaOH and the solution was freeze-dried. The residue was taken up in 5 mL of water and adjusted to pH 2 with 50% sulfuric acid, and the volatiles were again isolated by bulb-to-bulb lyophilization. The collected volatiles were basified to pH 10 with 5 N NaOH and the solution was lyophilized to yield sodium acetate (3.5 × 10^4 dpm, 25% overall radiochemical yield). This procedure was repeated on the methyl saphenate (2.2 × 10^6 dpm) and the combined esmeraldic acid dimethyl esters (8.9 × 10^5 dpm) isolated from the (*S*)-[2-$^{2}H_1$,^{3}H]-acetate feeding experiment and yielded 3.8 × 10^5 (12.5%) and 1.95 × 10^5 dpm (22%) of sodium acetate, respectively. The chirality of the methyl groups in these acetate samples was analyzed by using the coupled malate synthase/fumarase assay developed by Cornforth, Arigoni, and their co-workers.[26]

[11-^{13}C]Phenazine-1,6-dicarboxylic Acid ([11-^{13}C]-6). [11-^{13}C]-**6** was prepared from [7-^{13}C]benzoic acid according to the procedure of Holliman and co-workers[23] for unlabeled **6**: mp >360 °C [lit. mp >290 °C]; ^{1}H NMR (CF_3COOD, 500 MHz) δ 9.54 (broad d, J = 6.84 Hz, 2H), 9.23 (d, J = 7.81 Hz, 2H), 8.76 (dd, J = 6.84, 7.81 Hz, 2H); ^{13}C NMR (CF_3COOD, 125 MHz, inverse gated) δ 170.53 (s, enriched), 144.39 (s, 2C), 140.06 (s, 4C), 138.47 (s, 2C), 134.01 (s, 2C), 124.30 (d, J = 69.82 Hz, 1C), 124.32 (s, 1C); ESI-MS (dimethyl ester) *m*/*z* 268 (0.3), 269 (M^+ + H for unlabeled **6**, 2.7), 270 (100), 271 (24.9), 272 (3.3), 273 (1.1).

2-[3-(1-Hydroxyethyl)anilino]-3-nitrobenzoic Acid (11). 2-Chloro-3-nitrobenzoic acid (3 g), 3.2 g of 3-(1-hydroxyethyl)aniline, 2.3 g of potassium carbonate, and 185 mg of copper powder in 30 mL of anhydrous ethanol were refluxed for 16 h under an argon atmosphere. After the solution was cooled to room temperature, ethanol was removed under reduced pressure and the residue taken up in 50 mL of water. Insoluble material was removed by filtration and washed thoroughly with water. The filtrate was acidified with 2 N HCl to afford a sticky brown-orange precipitate, from which the aqueous layer was decanted. The residue was taken up in chloroform, dried over $MgSO_4$, and concentrated to give 3.0 g of orange-red crystals of **11**: mp 145−147 °C; ^{1}H NMR (CD_3OD, 300 MHz) δ 8.28 (broad d, J = 8.31 Hz, 1 H), 8.00 (d, J = 7.81, 1H), 7.20 (t, J = 7.81, 1H), 7.02 (broad d, J = 7.81, 1H), 6.99 (t, J = 7.82, 1H), 6.90 (broad s, 1H), 6.80 (broad d, J =

7.81, 1H); ^{13}C NMR (CD_3OD, 75 MHz) δ 165.1, 148.9, 142.7, 141.8, 138.9, 136.3, 131.9, 130.2, 128.4, 121.9, 119.2, 118.6, 116.3, 70.5, 24.75; GC/MS (methyl ester) *m*/*z* 316 (M^+ 100), 317 (18), 223 (49), 195 (61).

Saphenic Acid (2a). Sodium ethoxide (8.5 g) in 20 mL of anhydrous ethanol was added to a solution of 1.5 g of **11** in 15 mL of ethanol. After the addition, 390 mg of sodium borohydride was stirred in and the reaction mixture was heated to reflux for 7 h under argon. Upon cooling the solution to room temperature, the resulting precipitate was dissolved by adding water, and the ethanol was removed in vacuo. Careful acidification of the aqueous residue with 5 N HCl and subsequent extraction with $CHCl_3$ (3 × 100 mL) produced, after drying and solvent removal, 1.36 g of crude product. Flash chromatography (CH_2Cl_2/EtOAc 9:1) yielded 903 mg of **2a**: mp 203−204 °C [lit. 202−204 °C]; ^{1}H and ^{13}C NMR data were in agreement with literature values.[3]

6-Acetylphenazine-1-carboxylic Acid (7). Saphenic acid (100 mg) and activated MnO_2 (700 mg) in 50 mL of dioxane were heated at reflux for 5 h. Upon cooling the solution, the insoluble material was filtered off and solvent was removed under reduced pressure. The residue was purified by chromatography ($CHCl_3$) to give 70 mg of **7**: mp 218−219 °C [lit. 219−221 °C]; ^{1}H and ^{13}C NMR data were in agreement with literature values.[3]

[13-$^2H_3$]Acetylphenazine-1-carboxylic Acid ([13-$^2H_3$]-7). Sodium deuterioxide (0.3 mL, 30% in deuterium oxide) was added to a solution of 30 mg of **7** in 2 mL of [$^2H_4$]methanol and 4 mL of deuterium oxide. The reaction mixture was stirred at room temperature overnight before acidifying with 1 N DCl in D_2O to pH ∼2. Methanol was removed by rotary evaporation and the precipitate recovered and dried, giving 17 mg of [13-$^2H_3$]-**7**: mp 216−219 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.01 (dd, J = 7.4, 1.9, 1H), 8.55 (dd, J = 8.7, 1.9, 1H), 8.41 (dd, J = 8.7, 1.2, 1H), 8.29 (dd, J = 6.8, 1.2, 1H), 8.07 (dd, J = 8.7, 7.4, 1H), 8.05 (dd, J = 8.7, 6.8, 1H), 3.10−3.00 (m, 0.3H); ^{2}H NMR ($CHCl_3$, 46.1 MHz) δ 3.05 (s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 200.6, 165.5, 143.6, 142.2, 140.5 140.2, 140.0, 138.6, 135.8, 133.0, 132.9, 132.1, 131.5, 125.9; ESI-MS (methyl ester) *m*/*z* 280 (2.2), 281 (M^+ + H for unlabeled **7**, 11.2), 282 (11.5), 283 (44.7), 284 (100), 285 (19.0).

[12,13-$^2H_4$]Saphenic Acid ([12,13-$^2H_4$]-2a). Sodium deuterioxide (0.3 mL, 30% in deuterium oxide) was added to a solution of 70 mg (0.26 mmol) of **7** in 2 mL of [$^2H_4$]methanol and 4 mL of deuterium oxide. After 5 h of stirring at room temperature, a half molar equivalent (5.3 mg, 0.13 mmol) of sodium borodeuteride (98% ^{2}H) was added and the reaction mixture was left overnight. Saturated ammonium chloride solution was used to destroy the excess reducing agent, and methanol was evaporated from the mixture. The remaining aqueous solution was acidified with 1 N HCl and extracted with chloroform (3 × 25 mL). The combined organic phase was dried ($MgSO_4$) and concentrated to give 50 mg of [12,13-$^2H_4$]-**2a**: mp 198−200 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.97 (dd, J = 7.0, 1.4, 1H), 8.50 (dd, J = 8.8, 1.4, 1H), 8.17 (dd, J = 7.9, 2.1, 1H), 8.03 (dd, J = 8.8, 7.0, 1H), 8.00−7.91 (m, 2H); ^{2}H NMR ($CHCl_3$, 46.1 MHz) δ 5.76 (s, 1D), 1.78 (s, 3D); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 165.8, 143.7, 142.2, 141.5, 140.2, 139.7, 137.4, 134.9, 133.2, 130.5, 127.8, 127.0, 124.7, 68.5−66.9 (m); ESI-MS *m*/*z* 269 (M^+ + H for unlabeled **2a**, 1.7), 270 (40.0), 271 (15.0), 272 (6.2), 273 (100), 274 (21.0).

[12,13-$^2H_4$]Saphenic Acid Myristoyl Ester (2d). 2d was prepared according to the procedure of Keller-Schierlein and co-workers for saphenic acid acylation:[24] ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.97 (dd, J = 7.1, 1.2, 1H), 8.55 (dd, J = 8.7, 1.2, 1H), 8.19 (m, 1H), 8.05−7.93 (m, 3H), 1.75−1.55 (m, 2H), 1.40−1.10 (m, 20H), 0.78 (t, J = 6.5 Hz, 3H); ^{2}H NMR ($CHCl_3$, 46.1 MHz) δ 7.05 (s, 1D), 1.56 (s, 3D); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 172.9, 165.9, 142.5, 142.1, 141.3, 139.9, 139.6, 137.5, 135.5, 132.9, 130.2, 127.2, 126.9, 124.8, 34.6, 33.6, 31.9, 31.6, 30.9, 29.6−29.1 (several overlapping peaks), 25.0, 24.7, 22.7, 22.6, 14.1.

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