Streptonigrin Biosynthesis. 4. Details of the Tryptophan Metabolism[1]

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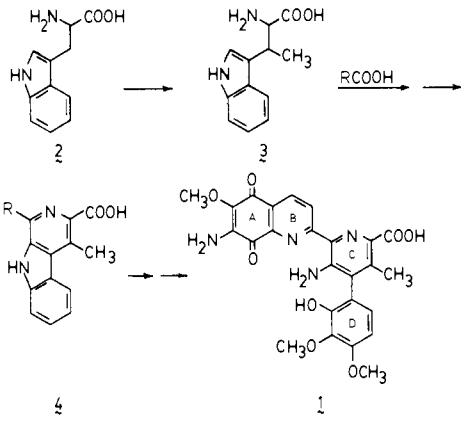
Abstract: Details of the metabolism of tryptophan leading to the biosynthesis of streptonigrin have been elucidated. These include identification of L-tryptophan as the natural precursor, the isolation of an early intermediate in the pathway, and the use of ^{15}N-^{13}C heteronuclear spin couplings to eludicate the N-C bond cleavage leading to the 4-phenylpicolinic acid portion (C-D rings). Additional feedings have apparently eliminated the possible involvement of all known pathways leading to quinoline rings; suggestions for the origin of this portion (A-B rings) are presented.

In the preceding paper[3] we reported an account of early studies on the biosynthesis of streptonigrin (**1**), a potent anticancer agent produced by *Streptomyces flocculus*.[4] These studies led to the formation of the partial biosynthetic pathway shown in Scheme I, in which tryptophan (**2**) serves as the primary precursor to the C and D rings of **1**. The present communication presents further evidence for this pathway and examines some of the details of the metabolism of tryptophan in the formation of **1**. A summary of the feedings to be discussed is given in Table I.

Scheme I



2 3 4 1

Results and Discussion

Intermediacy of β-Methyltryptophan. A critical aspect of Scheme I is the involvement of β-methyltryptophan (**3**), which provides a rational origin for the methyl group at C 3′ of **1**, as well as explains our findings[3] that the C-methylation occurs considerably earlier in the biosynthesis than the O-methylations.

The C-methylation of amino acids is quite rare and in the few cases so far known the amino acid was obtained from the hydrolysis of a polypeptide-type antibiotic.[5-7] Although hydrolysis of telomycin afforded β-methyltryptophan,[7] it has not previously been detected as a free natural product. We have now established the presence of **3** in cultures of *S. flocculus*, using an isotope dilution technique, and the intermediacy of **3** in the streptonigrin pathway.[1b]

Authentic samples of the 2*RS*,3*SR* isomer "A", **3a**, and the 2*RS*,3*RS* isomer "B", **3b**, were synthesized according to the procedure of Snyder and Matteson,[8] separation being effected by fractional crystallization of the acetamides. Isomeric purity was clearly evident from the ^1H NMR spectra of the acetamides, as well as those of the β-methyltryptophans. The signal from the amide N-H of the isomer A acetamide appeared at δ 8.10, whereas that from the isomer B acetamide appeared at δ 8.83. After hydrolysis to the amino acids, the signals due to the α and β hydrogens appeared at δ 3.45 and 4.05 and at 4.30 and 4.90 for **3a** and **3b**, respectively. LC analysis[9] on a reverse phase column also indicated that each isomer was free of the other.

To test for the production of **3**, [$^{14}CH_3$]-L-methionine (4.4 μCi) was added to a 50-mL fermentation of *S. flocculus* just at the start of streptonigrin production. Six hours later the broth was divided in two, one half receiving a small quantity (10 mg) of isomer A (**3a**) and the other receiving a small quantity (15 mg) of isomer B (**3b**). After cell disruption by sonication to release any endogenous labeled **3** and centrifugation to remove solids, each supernatant was purified by ethyl acetate extractions at pH 2 and 10, followed by neutralization to pH 7 and chromatography on Dowex 50W-X4 (H^+) ion exchange resin. The β-methyltryptophan was eluted from the column using increasing concentrations of ammonium hydroxide to yield relatively pure material that was free of radioactive methionine.[10] Preparative paper chromatography was used to further purify this material, half of which was then diluted with an additional 50 mg of the relevant isomer **3** and recrystallized repeatedly.[11] In this manner it was shown that *S. flocculus* had produced one of the enantiomers of the 2*RS*,3*SR* isomer A, with incorporation of 2% of the methionine fed.

The remaining half of the purified, labeled isomer A (6×10^4 dpm) was fed to a new 100-mL fermentation of *S. flocculus*, and the resulting labeled **1** (1.72 mg) was diluted with authentic streptonigrin (25 mg). Recrystallization to constant specific activity demonstrated that **3a** had been incorporated to the extent of 4%, thus demonstrating the intermediacy of **3a** in the biosynthesis of **1**.

Specificity of the Pathway for L-Tryptophan. In order to determine whether there was an enantiomeric preference[12] for L- or D-tryptophan in the streptonigrin pathway, doubly labeled tryptophan consisting of one enantiomer (L) labeled with ^{14}C mixed with racemic tryptophan labeled with ^3H was fed to cultures of *S. flocculus*. Only tritium in the benzene ring would be retained in streptonigrin, and fortunately [5-^{3}H]-DL-tryptophan was commercially available.

The proximity of C-5 of tryptophan to the positions that would be eventually hydroxylated necessitated a preliminary control feeding to rule out the possibility that the C-5 hydrogen was labilized during the biosynthesis of **1**. Thus, a mixture of [5-^{3}H]-DL-tryptophan and [β-^{14}C]-DL-tryptophan ($^3H/^{14}C$ = 19.39) was first fed. The ratio (18.26) found in the streptonigrin subsequently isolated indicated that essentially no loss

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Table I. Incorporation of Precursors into Streptonigrin

fed precursor	fed activity, dpm	fed mg[a]	product	product mg[a]	% incorp
[$^{14}CH_3$]-L-Met	9.78×10^6		β-methyltryp (**3**)		2
[^{14}C]-**3**	6×10^4	5	streptonigrin	1.72	4
[5-^{3}H]-DL-Trp + [β-^{14}C]-DL-Trp	2.27×10^8 / 1.17×10^7		streptonigrin	3.07	2.19[b]
[5-^{3}H]-DL-Trp + [β-^{14}C]-L-Trp	1.85×10^8 / 1.11×10^7		streptonigrin	2.40	2.78[b]
[β-^{14}C]-DL-Trp + [7a-^{14}C]-DL-Trp	3.28×10^7 / 3.19×10^7		streptonigrin	3.99	1.34
[β-^{14}C,2-^{13}C,1-^{15}N]-DL-Trp	2.80×10^7	58	streptonigrin	82	3.71

[a] Where applicable. [b] Refers to incorporation of ^{14}C.

of the tritium occurred (94% retained) in the formation of streptonigrin.

When [5-^{3}H]-DL-tryptophan mixed with [β-^{14}C]-L-tryptophan (^{3}H/^{14}C = 16.64) was fed, the ratio (8.29) in the streptonigrin obtained was exactly one-half that of the starting ratio, indicating that only the L enantiomer had been incorporated. The results of both feedings are summarized in Table II.[13]

[β-^{14}C, 7a-^{14}C]-DL-Tryptophan Feeding. It remained to identify the precursor(s) of the rest of the molecule, which in a formal sense would be equivalent to a quinoline quinone carboxylic acid **5**. There are five known pathways leading to the formation of quinoline rings, four of which involve tryptophan (Scheme II). The pathway leading to camptothecin (**6**)[14] was clearly not applicable owing to the significantly different substitution patterns. Formation from tryptophan via kynurenine (**7a**) or hydroxykynurenine (**7b**) without loss of the side chain, analogous to the formation of xanthommatin (**8**),[15] was eliminated when it was demonstrated that the side chain only labeled the pyridine ring.[3] The condensation of a three-carbon acid with anthranilic acid (**9**), derived directly from shikimic acid (**10**)[16] or via degradation of kynurenine,[17] appeared to be ruled out by the lack of incorporation of [^{14}COOH]anthranilic acid.[3] The remaining possibility, involving 3-hydroxyanthranilic acid (**11**), which is formed from **7b**[17] rather than by hydroxylation of **9**, was then tested.

Since labeled **11** was unavailable, it was decided to feed commercially available [7a-^{14}C]-DL-tryptophan (**2a**), which would be expected to label C-8a and C-8′ of **1**, although owing to the possibility of different pool sizes the percent label at each of these positions could not be predicted. Unfortunately, extensive chemical degradations would have been necessary to carve out either of these individual atoms. Therefore, [β-^{14}C]-DL-tryptophan (**2b**), which would exclusively label C-3′,[3] was included in the feeding. The possible distribution of labels is shown in Scheme III. Kuhn-Roth oxidation would afford acetic acid from C-3′ and the attached methyl group, and could be used as an indirect measure of labeling at C-8a and C-8′.

Scheme II

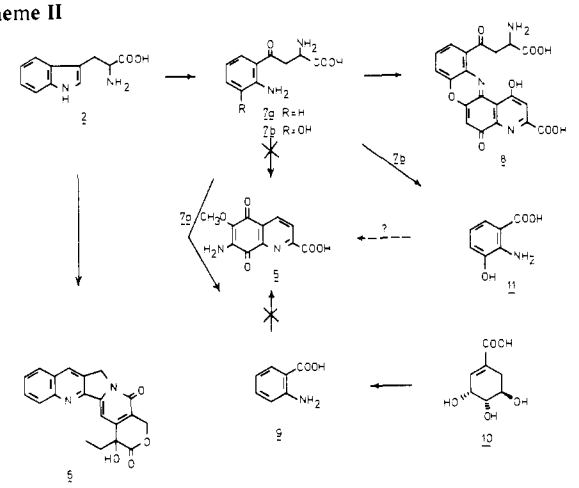


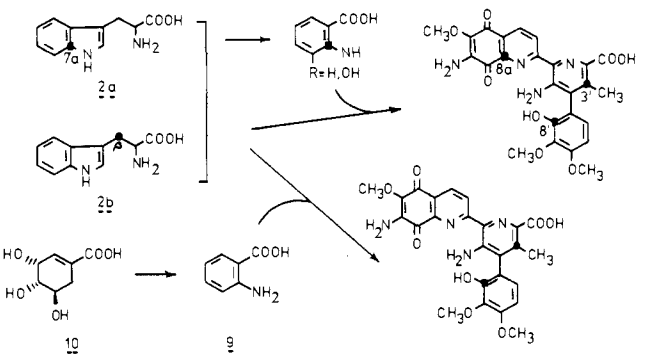
Table II. ^{3}H/^{14}C Ratios of the ^{3}H,^{14}C Doubly Labeled Tryptophan and Streptonigrin

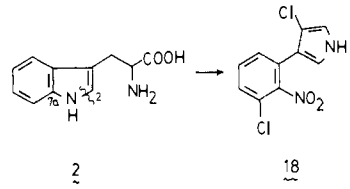
precursor mixture	precursor ^{3}H/^{14}C	streptonigrin ^{3}H/^{14}C	streptonigrin % ^{3}H remaining
[5-^{3}H,β-^{14}C]-DL-Trp	19.39	18.26	94
[5-^{3}H-DL-,β-^{14}C-L-]Trp	16.64	8.29	50

A 46:54 mixture of **2a** and **2b** was fed and the streptonigrin (3.1% incorporation) was isolated. As shown in Scheme IV, if tryptophan were only involved in formation of the C/D rings, the acetic acid would have contained 54% of the streptonigrin activity, but only 46% was found. However, when the labeled streptonigrin was oxidized with hypochlorite[18] to 2,3,6-pyridinetricarboxylic acid (**12**) and this decarboxylated to the diacid **14**, these compounds—measured as their permethyl esters **13** and **15**—contained only 4.8 and 1.6% of the activity,[19] respectively. Furthermore, streptonigric acid (**16**)[18] derived by basic peroxide oxidation of **1** contained 100% of the activity when measured as its tetramethyl derivative **17**.[18] Therefore, these results ruled out the direct involvement of tryptophan in formation of the A/B rings.

Cleavage of the Indole Ring. The pathway suggested in Scheme I, involving a β-carboline intermediate, required the cleavage of the original indole ring of tryptophan at a C–N bond. Cleavage of an intact indole in such a fashion has been previously reported only in the biosynthesis of pyrrolnitrin (**18**).[20] In this case it was demonstrated that the C_2–N bond of tryptophan was cleaved and the indole nitrogen was retained in the nitro group. If a similar cleavage occurred in the strep-

Scheme III





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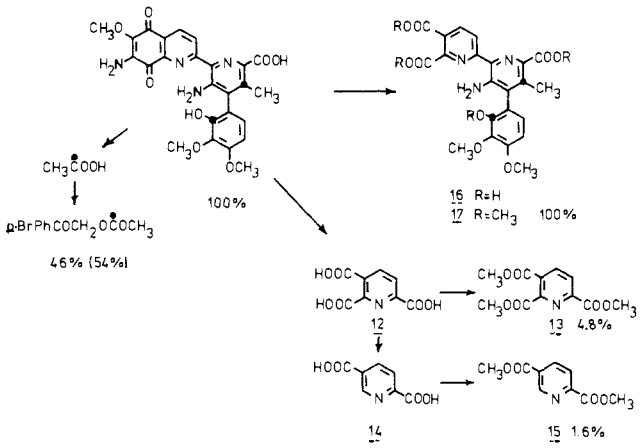
tonigrin pathway, it would necessitate exchange of the indole nitrogen for oxygen (C-8′ OH) and introduction of a new nitrogen at C-5′. This seemingly uneconomical possibility could be avoided by a C_{7a}–N bond cleavage and the extensive oxygenation pattern of the D ring of streptonigrin allows a number of feasible mechanisms for such a cleavage.

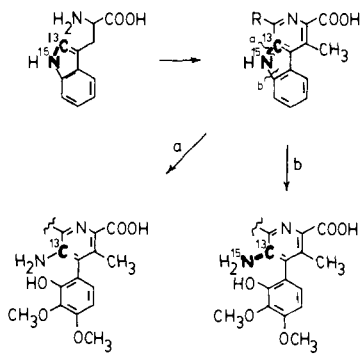
Simply stated, a distinction between C_2–N and C_{7a}–N cleavage amounted to a study of secondary nitrogen metabolism: was the indole nitrogen lost or retained? There are no long-lived radioactive isotopes of nitrogen and detection of ^{15}N by mass spectrometry is likely to be equivocal when there is more than one nitrogen present unless clearly defined fragmentations can be identified. While the use of ^{15}N NMR had recently been demonstrated in a study of the biosynthesis of penicillin,[21] we were unsure that the currently available technology would be of adequate sensitivity for our studies, considering our low streptonigrin production and the moderate levels of incorporation obtainable. Since the value of ^{13}C–^{13}C homonuclear spin couplings obtained by feeding doubly ^{13}C labeled precursors had been amply demonstrated,[22] and ^{2}H–^{13}C heteronuclear spin couplings had recently been reported,[23] it seemed that the fate of ^{15}N could be determined indirectly by ^{13}C NMR via heteronuclear spin coupling to an adjacent ^{13}C.[24]

[2-^{13}C,1-^{15}N]-DL-Tryptophan was synthesized[25] from [^{15}N]ammonium chloride (95.2% ^{15}N) and sodium [^{13}C]-formate (90% ^{13}C) and fed to *S. flocculus*. The possible outcomes are shown in Scheme V. If C_2–N cleavage occurred (pathway "a"), the ^{15}N would be lost and a simple enriched

Scheme IV



Scheme V



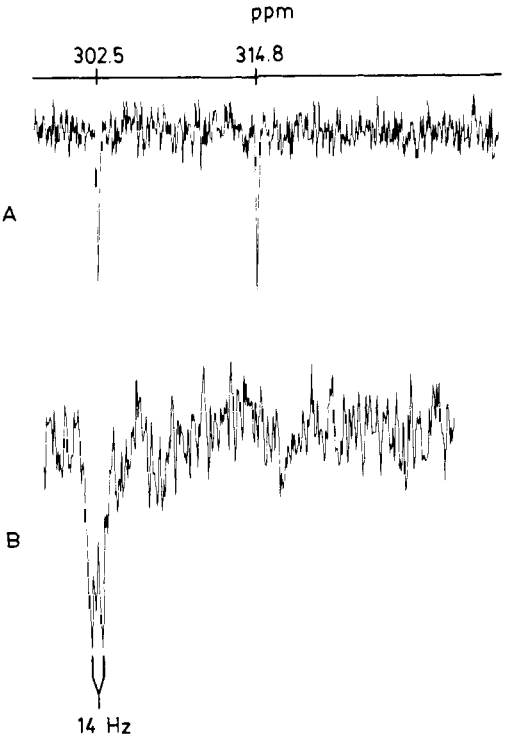


Figure 1. ^{15}N NMR spectra obtained on a Bruker HFX-10 spectrometer using a 38° (30 s) pulse width, 1.2-s repetition time, and 1200-Hz sweep width. Parts per million are relative to $H^{15}NO_3$. (A) The spectrum of streptonigrin at natural abundance (42 604 transients). (B) The spectrum of streptonigrin produced in the presence of [2-^{13}C,1-^{15}N]tryptophan (51 423 transients).

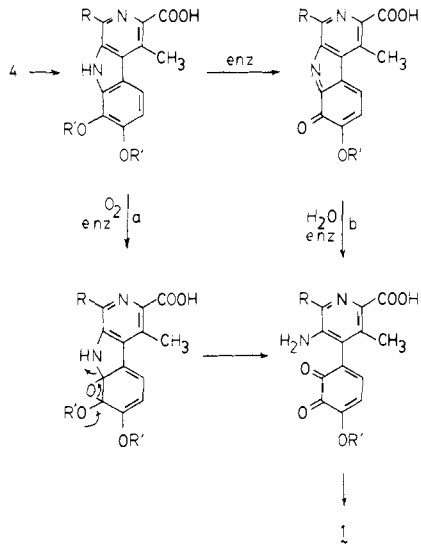
singlet at 145.6 ppm should be observed in the ^{13}C NMR spectrum. However, if C_{7a}–N cleavage occurred (pathway "b"), both isotopes would be incorporated, the C_2–N bond between them would remain intact, and the natural-abundance singlet at 145.6 ppm[1a] should be flanked by a doublet due to the enriching ^{13}C being split by the adjacent ^{15}N. The latter result was obtained; the coupled signals ($J_{^{13}C^{15}N}$ = 14.7 Hz) were even larger than the natural-abundance singlet. A comparison of the area of the natural-abundance singlet with that of the doublet indicated a 3.2-fold enrichment.

Having the doubly labeled sample of streptonigrin in hand, we investigated whether the coupled signal could be detected by ^{15}N NMR spectroscopy. A 300-mg sample of streptonigrin in 16 mL of tetrahydrofuran and 2 mL of acetone-d_6 exhibited signals for the amino groups only (Figure 1A). The signal for the C-5′ amino group appeared at 302.5 ppm and the signal for the C-7 amino group appeared at 314.8 ppm, upfield from external $H^{15}NO_3$, respectively. The spectrum (Figure 1B) obtained from the enriched streptonigrin (50 mg) in the same solvent system only showed the enriched signal: a doublet at 302.5 ppm ($J_{^{13}C^{15}N}$ = 14 Hz). It would appear that, if there are long relaxation times, as for aromatic nitrogens, ^{15}N NMR may be difficult to use for biosynthetic studies when only small samples are available.

Conclusions

Significant details of the pathway leading from tryptophan to the C/D rings of **1** have been clarified. L-Tryptophan is the specific precursor and, as predicted, is converted to β-methyltryptophan, which has now been identified for the first time as a free natural product. The unprecedented ring cleavage of a putative β-carboline intermediate affords the 4-phenylpicolinic acid grouping, with the C-5′ amine derived from the original indole nitrogen. While there are a number of possibilities for the mechanism of the cleavage, two attractive alternatives are presented in Scheme VI. These can potentially

Scheme VI



be distinguished using $^{18}O_2$, since in path "a" the C-8′ hydroxyl is derived from molecular oxygen, whereas in path "b" it is derived from water.

It would appear that all of the known pathways that were potentially relevant to the formation of the A/B rings have been eliminated. We anticipate that yet another new metabolism by *S. flocculus* will be revealed when we have determined the precursors of this portion of the antibiotic.

Experimental Section

General. Melting points were determined on a Hoover capillary melting point apparatus. ^{1}H NMR spectra were taken on a Hitachi Perkin-Elmer R-24 spectrometer. ^{13}C NMR spectra were proton noise decoupled and were taken on Bruker WP-60 and WP-90 spectrometers at 30 °C in 10-mm tubes containing a cylindrical 0.5-mL capacity insert (Wilmad Glass Co.). ^{15}N NMR spectra were taken on a Bruker HFX-10 spectrometer at 18.25 MHz using a 0.1 M $H^{15}NO_3$ solution as an external reference; the samples were dissolved in 16 mL of tetrahydrofuran and 2 mL of acetone-d_6. IR spectra were obtained with a Beckman Acculab 3 spectrometer, and UV spectra on a Cary 118 instrument. Mass spectra were measured on an AEI MS-902 instrument using EI. LC was conducted on a Waters analytical instrument using a MicroSil C_{18} column (30 cm × 5 mm) from Micronetics, Inc., Norcross, Ga.; the solvent was 8% aqueous acetonitrile buffered at pH 4.0 with 0.01 M NaOAc. All radioactive measurements were carried out on a Packard Tri-Carb 3375 liquid scintillation counter in toluene scintillation solution (0.5% PPO), dioxane scintillation solution (10% naphthalene and 0.5% PPO), or Bray's solution purchased from New England Nuclear or ICN. Microsamples were weighed on a Cahn Model 4400 electrobalance. All measurements were done in duplicate to a ±3% standard deviation. Counting efficiencies were determined by spiking with [^{14}C]- or [^{3}H]-*n*-hexadecane standards purchased from Amersham/Searle. Cells were disrupted using a Branson Sonifier cell disruptor 200 and centrifuged in a Sorvall refrigerated centrifuge. Analytical paper chromatograms were scanned for radioactivity with a Vanguard Autoscanner 800. Paper chromatograms were run on 1-in. wide Whatman No. 1 strips (analytical) and on 4-in. wide Whatman No. 3 MM strips (preparative). Thin layer (TLC) and preparative (PLC) chromatography were done using Merck silica gel PF-254 adsorbent. Evaporation in vacuo refers to solvent removal on a rotary evaporator at aspirator pressure and 25–35 °C.

^{14}C-Labeled compounds were obtained from New England Nuclear. [5-^{3}H]-DL-Tryptophan was obtained from CEA (France). ^{15}N- and ^{13}C-labeled compounds were obtained from Merck Sharp and Dohme. Strains of *S. flocculus* were generous gifts from Dr. J. DeZeeuw of Pfizer and Co., Inc., Groton, Conn. Streptonigrin was a generous gift from Dr. J. Douros of Drug Research and Development, Chemotherapy, National Cancer Institute. Soybean meal and distillers' solubles were kindly supplied by the Nutritional Science Department and the School of Agriculture at the University of Connecticut, respectively. All chemicals were of reagent grade, and all solvents were distilled prior to use.

Culture conditions and the procedure for the isolation of streptonigrin were the same as before.[3]

Synthesis of the β-Methyltryptophans.[8] 3-(Isopropylaminoethylidene)indole. The reaction of isopropylamine (31.72 g, 0.56 mol), indole (58.94 g, 0.50 mol), and acetaldehyde (22.85 g, 0.52 mol) according to the published procedure afforded 31.7 g (31%) of 3-(isopropylaminoethylidene)indole: mp 112–117 °C (lit. 110–117 °C); IR ($CHCl_3$) 3440, 2800–3000, and 1170 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.00 (d, 6 H, J = 6 Hz), 1.5 (d, 3 H, J = 8 Hz), 2.9 (m, 1 H), 4.3 (q, 1 H, J = 7 Hz), 7.0 (m, 4 H), 7.7 (dd, 1 H, J = 7, 3 Hz), and 8.52 (s, 1 H).

Dibenzyl Acetamidomalonate. Diethyl acetamidomalonate (38.0 g, 0.175 mol) was converted to dibenzyl acetamidomalonate (36.0 g, 60.5%) according to the literature procedure: mp 108–110 °C (lit. 110–113 °C); IR ($CHCl_3$) 3420, 1750, and 1680 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.86 (s, 3 H), 5.05 (s, 4 H), and 7.21 (s, 10 H).

Dibenzyl (3-Indolylethylidene)acetamidomalonate. A mixture of 3-(isopropylaminoethylidene)indole (9.4 g, 0.046 mol), dibenzyl acetamidomalonate (17 g, 0.050 mol), and a catalytic amount of sodium methoxide (15 mg) in 65 mL of toluene yielded 20.6 g (78%) of dibenzyl (3-indolylethylidene)acetamidomalonate: mp 160–162 °C (lit. 161–163 °C); IR ($CHCl_3$) 3400, 3000, 1740, and 1690 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.5 (d, 3 H, J = 8 Hz), 1.88 (s, 3 H), 4.02 (m, 1 H), 5.13 (s, 4 H), and 7.4 (m, 16 H).

Ammonium (3-Indolylethylidene)acetamidomalonate. Dibenzyl (3-indolylethylidene)acetamidomalonate (17.0 g, 0.035 mol) was reduced catalytically, yielding ammonium (3-indolylethylidene)-acetamidomalonate (9.33 g, 78.4%): mp 154–161 °C (lit. 160–165 °C); IR (Nujol) 3500, 2900–3000, and 1590–1650 cm^{-1}; ^{1}H NMR (D_2O) δ 1.18 (d, 3 H, J = 8 Hz), 2.1 (s, 3 H), 3.8 (d, 1 H, J = 8 Hz), and 7.0–7.7 (m, 5 H).

***N*-Acetyl-β-methyltryptophans.** Ammonium (3-indolylethylidene)acetamidomalonate (5.9 g, 17.8 mmol) was refluxed in 6 mL of water and 6 mL of pyridine under a nitrogen atmosphere for 5 h, during which time a crystalline mass (ammonium carbonate) deposited in the spiral reflux condenser. After the solution was diluted with 30 mL of water, it was acidified to pH 3 with dilute H_2SO_4. The solution was allowed to stand overnight at 0 °C, yielding the impure crystalline *N*-acetyl-β-methyltryptophan isomer A (2.6 g, 56%), which was recrystallized from dimethylformamide-water. The yield of purified isomer A was 1.4 g (30%): mp 207–210 °C (lit. 211–215 °C); IR ($CHCl_3$) 3400, 2800–3000, 1770, and 1580 cm^{-1}; ^{1}H NMR (Me_2SO-d_6) δ 1.34 (d, 3 H, J = 8 Hz), 1.87 (s, 3 H), 3.56 (m, 1 H), 4.67 (dd, 1 H, J = 3, 6 Hz), 7.0–7.7 (m, 5 H), 8.10 (d, 1 H, J = 9 Hz), and 10.9 (s, 1 H).

N-Acetyl-β-methyltryptophan isomer B, which was contained in the mother liquor of the isomer A crystallization, was isolated by first extracting with four 20-mL portions of ethyl acetate, then washing the organic phase with two 10-mL portions of water and two 20-mL portions of sodium bicarbonate. The combined aqueous solution was acidified to pH 3 with 4 N H_2SO_4 and extracted with three 20-mL aliquots of ethyl acetate. After the organic extract was washed with two 5-mL portions of water and dried over magnesium sulfate, the solution was concentrated in vacuo until a white solid was obtained. The material was dried under high vacuum to yield isomer B (1.69 g, 36%): mp 207–210 °C; IR ($CHCl_3$) 3550, 2980, 1710, and 1660 cm^{-1}; ^{1}H NMR (Me_2SO-d_6) δ 1.34 (d, 3 H, J = 8 Hz), 1.87 (s, 3 H), 3.45–3.68 (m, 1 H), 4.67 (q, 1 H, J = 3, 6 Hz), 7.0–7.7 (m, 5 H), 8.83 (d, 1 H, J = 9 Hz), and 10.9 (s, 1 H).

β-Methyltryptophan, 2*RS*,3*SR* Isomer A (3a). *N*-Acetyl-β-methyltryptophan isomer A (1.0 g, 3.6 mmol) was hydrolyzed to yield crude, crystalline β-methyltryptophan isomer A (0.454 g, 58%). This was recrystallized from 4 mL of water yielding 0.36 g (46%) of the purified compound: mp 240–247 °C dec (lit. 246–250 °C); IR (Nujol) 3400, 1615, and 1330 cm^{-1}; ^{1}H NMR (trifluoroacetic acid) δ 1.65 (d, 3 H, J = 9 Hz), 4.3 (m, 1 H), 4.9 (m, 1 H), 6.9–7.8 (m, 9 H).

β-Methyltryptophan, 2*RS*,3*RS* Isomer B (3b). *N*-Acetyl-β-methyltryptophan isomer B (1.95 g, 7.06 mmol) was hydrolyzed to yield impure product (0.85 g, 55.2%), which was recrystallized from 15 mL of water to afford 0.65 g (44%) of pure β-methyltryptophan isomer B: mp 241–247 °C dec (lit. 247–251 °C); IR (Nujol) 3400, 1580, and 1320 cm^{-1}; ^{1}H NMR (trifluoroacetic acid) δ 1.25 (d, 3 H, J = 9 Hz), 3.45 (m, 1 H), 4.05 (m, 1 H), and 6.20–7.3 (m, 9 H).

Incorporation of [^{14}CH$_3$]-L-Methionine into β-Methyltryptophan.

A 50-mL sterile fermentation broth was inoculated with spores of *S. flocculus* and incubated on a rotary shaker (220 rpm) at 27–29 °C. Following a 48-h growth period, 3-mL aliquots of the culture were used to inoculate another 50-mL broth and two 25-mL broths. These were also incubated with shaking at 27–29 °C for 3 days, at which time [$^{14}CH_3$]-L-methionine (9.78×10^6 dpm) was added to the 50-mL fermentation, and incubation was continued for 6 h. The [$^{14}CH_3$]-L-methionine-fed fermentation was removed and divided into two 25-mL portions. Authentic β-methyltryptophan isomer A (15 mg in H_2O) was added to one of these, while the authentic B isomer (10 mg in H_2O) was added to the other. One of the original 25-mL fermentations was removed at the same time and assayed for the production of streptonigrin. The second original 25-mL fermentation was assayed after an additional 30-h incubation period. These showed streptonigrin concentrations of 6 and 10 mg/L, respectively.

Each of the broths diluted with an authentic β-methyltryptophan isomer was treated in the following manner. Immediately after addition, the sample mixture was sonicated in order to disrupt the cells, and then centrifuged for 20 min at 24600*g*. The supernatant (pH 7.6) was brought to pH 2 with concentrated HCl, and, after centrifugation to remove some precipitate, was extracted with three 15-mL portions of ethyl acetate. Following adjustment to pH 10 with concentrated NH_4OH, the aqueous solution was again centrifuged, and then extracted with three 15-mL aliquots of ethyl acetate. The aqueous solution was brought to pH 7 with 10% NH_4OH and applied to a chromatographic column (18 mm × 15 cm) packed with activated Dowex-X4, 100–200 mesh (H^+ form) cation exchange resin. After addition of the sample to the resin bed the column was eluted with 2% NH_4OH, and ten 6-mL fractions were collected. This was followed by elution with 10% NH_4OH, and ten 6-mL fractions were again taken. The fractions were analyzed for radioactivity by scintillation counting, and the bulk of the β-methyltryptophan was determined by paper chromatography to be present in the late 2% NH_4OH fractions.

The fractions containing β-methyltryptophan were combined, reduced in volume by lyophilization to ca. 3 mL, and chromatographed preparatively on Whatman 3MM paper. After an overnight elution, a thin lengthwise strip was cut from the paper and sprayed with ninhydrin, in order to locate the β-methyltryptophan component by comparison of this strip with the R_f value of authentic β-methyltryptophan. A band corresponding to the identified position was then cut from the paper, sewed to a Whatman No. 1 paper leader at one end, and eluted with distilled water. A portion of the eluted material was counted for radioactivity and then tested for chemical purity on analytical paper chromatography. Radioactive purity was determined by scanning an analytical paper chromatogram, which indicated that the radioactivity was localized on a portion of the chromatogram corresponding to the R_f value of authentic β-methyltryptophan. Following a reduction in volume to ca. 10 mL, one-half of the sample was removed and diluted with 50 mg of the corresponding β-methyltryptophan isomer. The material was then recrystallized repeatedly from acetic acid–benzene, until constant specific activity was obtained. In this manner the **3a** sample remained constant at 3.76×10^5 dpm/mmol (loss of 17% of the initial specific activity) for the third through sixth recrystallizations, indicating approximately a 2% incorporation of methionine. After seven recrystallizations the **3b** sample was still losing activity (9.2×10^4 dpm/mmol, loss of 47% of the initial specific activity) but not enough material remained for further recrystallizations.

Incorporation of [^{14}C]-β-Methyltryptophan into Streptonigrin. Forty-eight hours after inoculation of a 100-mL broth with 6 mL of an *S. flocculus* seed fermentation the streptonigrin concentration was assayed as 5.6 mg/L. At this point the remaining half of the radioactive β-methyltryptophan isomer A (6×10^4 dpm), isolated previously via preparative paper chromatography, was added to the fermentation. After an additional 15-h incubation, workup for streptonigrin and UV assay showed the isolation of 1.72 mg of streptonigrin from the fermentation (17.2 mg/L). This was diluted with 25 mg of authentic streptonigrin and repeatedly recrystallized from THF–methanol to constant specific radioactivity (4.53×10^4 dpm/mmol), indicating 4% incorporation of **3a**.

Kuhn–Roth Oxidation of Streptonigrin (1). Kuhn–Roth oxidation was performed according to the procedure of Wissenberger,[26] and the derived acetic acid was converted to its *p*-bromophenacyl ester using dicyclohexyl 18-crown-6 ether.[27] Oxidation of 104.34 mg of streptonigrin (1.59×10^6 dpm/mmol) yielded 45 mg (86%) of the phenacyl ester, which was recrystallized from 95% ethanol to constant specific activity (7.28×10^5 dpm/mmol).

2,3,6-Pyridinetricarboxylic Acid (12) and Trimethyl Ester (13). Streptonigrin (250 mg, 0.5 mmol) was suspended in a small volume of distilled water (1.5 mL), and Clorox (2.5 mL) (5.25% sodium hypochlorite) was added in small portions. The suspension was kept in a water bath at a temperature below 50 °C and the reaction flask was occasionally shaken manually over a 3-h period. The streptonigrin slowly dissolved and the solution turned yellowish, whereupon a magnetic stirring bar was added and the mixture stirred for an additional 1–2 h. The reaction mixture eventually turned a clear yellow as a gummy reside occluded to the stirring bar. Approximately 1.65 g of sodium bisulfite (66.7% assayed as SO_2, 10.5 mmol) was then added slowly to destroy the excess hypochlorite, and the solution was acidified to ca. pH 1.0 with concentrated sulfuric acid while the solution was cooled to keep the temperature below 50 °C. After acidification, the solution was continuously extracted with ether (freshly distilled from lithium aluminum hydride) for at least 2 days. The ether extract was dried over anhydrous sodium sulfate and was immediately methylated.

Diazomethane generated from Diazald (2.15 g, 9.7 mmol) and potassium hydroxide (0.4 g) in 10 mL of 95% ethanol was slowly distilled into a cooled Erlenmeyer flask containing the dried ethereal solution of **12.** The flask was shaken manually to facilitate the mixing of the two solutions, and after 1 h a small amount of formic acid (88%) was added to destroy the excess diazomethane. The ethereal solution was then shaken with saturated sodium bicarbonate and washed with saturated brine. Finally, the solution was dried, filtered, and concentrated in vacuo, yielding a brownish-orange residue, which was purified by preparative TLC, using 10% methanol in chloroform. Elution of the UV band (R_f 0.71) containing the trimethyl ester with 15% methanol–chloroform afforded an orange oil, which was subjected to further purification by additional preparative TLC on silica gel in ether–dichloromethane (1:9). The UV band (R_f 0.33) containing the trimethyl ester was again eluted with 15% methanol–chloroform, yielding 23 mg (30% yield from streptonigrin) of **13** as an oil: ^{1}H NMR ($CDCl_3$) δ 3.98 (s, 3 H), 4.03 (s, 3 H), 4.04 (s, 3 H), 8.36 (s, 2 H); mass spectrum *m/e* (rel intensity) 253 (M^+, 3.1) and 163 (100); high-resolution mass spectrum 253.0579 for $C_{11}H_{11}NO_6$.

2,5-Pyridinedicarboxylic Acid (14). The dried ethereal solution containing 2,3,6-pyridinetricarboxylic acid (**12**) was concentrated in vacuo, and the dark brown residue was transferred to a sublimation tube. Upon heating at 150 °C (0.02 mm), **14** was obtained as white crystals in 10–15% yield: mp 256–258 °C dec (lit. 256–258 °C);[18] IR (KBr pellet) 3070–2300, 1700, and 1680 cm^{-1}; ^{1}H NMR (Me_2SO-d_6) δ 8.15 (d, 1 H, J = 8 Hz), 8.45 (dd, 1 H, J = 2, 8 Hz), 9.15 (d, 1 H, J = 2 Hz); mass spectrum *m/e* (rel intensity) 167 (M^+, 4.9) and 123 (100).

Dimethyl Ester of 2,5-Pyridinedicarboxylic Acid (15). The dimethyl ester of 2,5-pyridinedicarboxylic acid was prepared from 2,5-pyridinedicarboxylic acid (**14**) and diazomethane using the same procedure as that in the preparation of trimethyl ester **13.** The R_f value of **15** was 0.70 in 10% methanol–chloroform and 0.25 in 10% ether–dichloromethane. Upon removal of the solvent, 5 mg of **15** was obtained as white crystals (60–70% yield based on 2,5-pyridinedicarboxylic acid), which could be sublimed at 100 °C (0.2 mm) or recrystallized from methanol: mp 162 °C (lit. 162–163 °C);[18] IR ($CHCl_3$) 1715 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.99 (s, 3 H), 4.04 (s, 3 H), 8.25 (d, 1 H, J = 8 Hz), 8.52 (dd, 1 H, J = 1, 8 Hz), and 9.35 (d, 1 H, J = 2 Hz); mass spectrum *m/e* (rel intensity) 195 (M^+, 10.3) and 137 (100); high-resolution mass spectrum 195.0526 for $C_9H_9NO_4$.

The dimethyl ester could also be obtained by direct methylation of the unpurified dicarboxylic acid (**14**) as follows. The dried ethereal solution containing pyridinetricarboxylic acid (**13**) was concentrated to a small volume on a rotary evaporator, transferred to a 250-mL Erlenmeyer flask, and dried further with a stream of nitrogen. After the flask was placed in an oven at 130 °C for 30 min to effect the decarboxylation, the black residue was suspended in freshly distilled ether, and methylation with diazomethane was carried out as described previously.

Hypochlorite Oxidation of Labeled Streptonigrin. Streptonigrin (158 mg, 1.17×10^6 dpm/mmol) obtained from the [β-^{14}C,7a-^{14}C]-tryptophan feeding was oxidized with hypochlorite as described above. Half of the ethereal solution was directly methylated to yield labeled triester **13** (3 mg, 7.11×10^4 dpm/mmol) which had 4.8% of

the specific activity of **1**. The remaining half was decarboxylated in an oven and then methylated to yield the diester **15** (2.5 mg, 2.36 × 10^4 dpm/mmol) containing 1.6% of the specific activity of **1**.

Tetramethyl Streptonigrinate (17). Streptonigrin (69 mg, 0.14 mmol) in 10 mL of 5% sodium hydroxide was treated with 5 mL of 30% hydrogen peroxide and stirred for 3 h while the deep red-brown solution discolored to an orange-yellow solution. Sodium bisulfite (0.6 g, 66.7% SO_2, 3.82 mmol) was added to the cooled solution to destroy the excess hydrogen peroxide, and the solution was acidified to pH 1.5 with concentrated sulfuric acid, at which pH an orange compound started to precipitate out. The orange-yellow suspension was extracted with ethyl acetate, and upon evaporation of the dried solution an orange residue was obtained. This residue was further dried with a stream of nitrogen for 1–2 h. It was then dissolved in 50 mL of acetone (dried over anhydrous potassium carbonate), and anhydrous potassium carbonate (250 mg, 1.81 mmol) and dimethyl sulfate (230 mg, 1.83 mmol) were added. The mixture was stirred in the dark at room temperature for 4 days, and then filtered through a sintered-glass funnel. The solid residue that remained on the funnel was further washed with acetone and chloroform. Concentration in vacuo of the combined filtrates yielded the crude tetramethyl ester of streptonigric acid which was purified by preparative TLC in hexane–chloroform–methanol (60:34:6). The plate was developed at least three times to obtain good resolution; however, prolonged contact with silica gel or the solvent system or prolonged exposure to light resulted in some degradation of the compound. The yellow band (R_f 0.62 in 2.5% methanol in chloroform, or R_f 0.51 when run three times in hexane–chloroform–methanol (70:39:1)) corresponding to the tetramethyl ester was eluted with 10% methanol in chloroform to yield 32 mg (45% yield) of **17** as a golden-yellow residue which was recrystallized from chloroform–methanol: mp 169–169.5 °C (lit. 166–167 °C);[18] ^{1}H NMR ($CDCl_3$) δ 2.33 (s, 3 H), 3.75 (s, 3 H), 3.99 (s, 9 H), 4.00 (s, 6 H), 6.94 (s, 2 H), 8.42 (d, 1 H, J = 8.5 Hz), 9.05 (d, 1 H, J = 8.5 Hz); mass spectrum *m/e* (rel intensity) 525 (M^+, 27) and 434 (100); high-resolution mass spectrum 525.1749 for $C_{26}H_{27}N_3O_9$.

Tetramethyl Streptonigrinate (17) from Labeled Streptonigrin. Radioactive streptonigrin (70.5 mg, 3.0 × 10^6 dpm/mmol) from the [β-^{14}C,7a-^{14}C]-tryptophan feeding was oxidized with basic peroxide and methylated as described above to yield 32 mg (44%) of labeled **17**. This material was recrystallized to constant specific activity (3.0 × 10^6 dpm/mmol), which was the same as that of the starting material.

Acknowledgments. We are indebted to Dr. John DeZeeuw of Pfizer and Co., Inc., Groton, Conn., for strains of *S. floc-culus* and to Dr. John Douros of Drug Research and Development, Chemotherapy, NCI, for generous gifts of streptonigrin. Professor Edward Samulski and Dr. Thomas Leipert of the Department of Chemistry, University of Connecticut, are thanked for obtaining the ^{13}C NMR spectra; the Bruker WP-90 spectrometer used for this was purchased in part by a grant from the National Science Foundation (CHE 76-05739). Dr. James Stuart of the Department of Chemistry, University of Connecticut, is thanked for advice and help with the LC analysis of the β-methyltryptophans. This work was supported in part by grants from the University of Connecticut Research Foundation and the National Science Foundation (PCM 76-15197) to S.J.G. and Public Health Service Grant GM-11072 from the Division of General Medical Sciences to J.D.R.

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