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(36) Based on the known tendency of 6β-amidopenams to undergo oxidation on sulfur syn to the neighboring amide group, the configuration of sulfinyl oxygen was expected to be *S* (β) [see R. D. G. Cooper, L. D. Hatfield, and D. O. Spry, *Acc. Chem. Res.*, **6**, 32 (1973), and references cited therein]. This was confirmed by examination of sulfoxide bond chemical shift perturbations and aromatic solvent induced shifts. In the *S* configuration (β) the sulfoxide bond of penicillin sulfoxide is known to exert deshielding effects on H-3 and 2β-CH_3–, and shielding effects on H-5 and 2α-CH_3, whereas in the *R* configuration (α) all protons are deshielded except H-3, which is shielded. The observed sulfoxide perturbation shifts (Δ_{SO}) for **50** where $\Delta_{SO} = \delta_{sulfide} - \delta_{sulfoxide}$ are H-3, −0.32; H-5, +0.56. This is in good agreement with Δ_{SO} for penicillin *S*-oxides in general, especially in comparison to the nonmethoxylated *S*-oxide derivative of **50**: Δ_{SO} H-3, −0.25; H-5, +0.56. See P. V. DeMarco and R. Nagarajan in ref 30, pp 349–353 and references cited therein.
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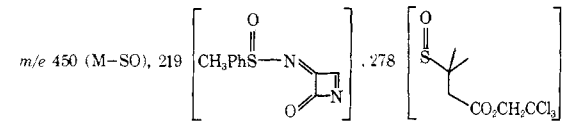
(40) See ref 7c,d. We thank Dr. W. A. Slusarchyk for providing this authentic sample.
(41) Mass spectral fragments of **55** are assigned as follows:



(42) The extinction coefficients reported for this compound in our preliminary communication were incorrect.

Streptonigrin Biosynthesis. 3. Determination of the Primary Precursors to the 4-Phenylpicolinic Acid Portion[1]

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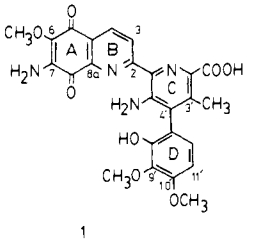
Abstract: The 4-phenylpicolinic acid portion of streptonigrin (**1**), a metabolite of *Streptomyces flocculus* with an unusual heterocyclic structure and striking anticancer activity, has been shown to be derived from tryptophan (**4**). All four methyls are derived from methionine, and C-3 of serine is likewise incorporated, although indirectly after apparently being degraded and entering the C-1 pool. From the serine findings and the results of specially timed feedings of [$^{13}CH_3$]-L-methionine, it was revealed that C-methylation occurs at a significantly earlier stage of the fermentation than does O-methylation; the dynamics of the pathway could be observed directly using ^{13}C NMR spectroscopy. The serine results also showed that no de novo biosynthesis of **4** was occurring, at the time of the incorporation experiments. A rational hypothesis for the formation of **1** is presented.

Introduction

Streptonigrin (**1**), a potent anticancer antibiotic, was first isolated from *Streptomyces flocculus* (ATCC 13852),[2] and subsequently from other actinomycetes, e.g., *S. albus* var. *bruneomycini*,[3] *S. echinatus*, and *S. rufochromogenes* 662.[4] The structure of **1** was originally deduced from chemical degradations,[5] and was confirmed by X-ray crystallography.[6] A partial assignment of the ^{13}C NMR spectrum has been reported.[7]



1

Streptonigrin is active against a variety of Gram-positive and Gram-negative bacteria, and has pronounced activity against numerous experimental tumors.[8,9] In the late 1960s testing progressed to a phase III clinical trial for the treatment of lymphoproliferative diseases; the antibiotic appeared to be as effective as chlorambucil but exhibited a somewhat greater degree of toxicity,[10] and interest in streptonigrin declined thereafter in the U.S. However, promising results from France[11] and the Soviet Union,[12] where streptonigrin has been used in combination therapies, have indicated a need to reassess the potential of this antibiotic.

The lethality of streptonigrin is apparently due to a redox process, involving the quinone portion of the molecule, that leads to uncoupling of oxidative phosphorylation and depletion of cellular ATP.[13,14] As a secondary effect, superoxide produced in the redox reaction reacts with its dismutation product, hydrogen peroxide, to yield hydroxyl radicals that cause single-strand breaks in DNA.[13]

A few simple derivatives of streptonigrin have been prepared by methylation of the carboxylic acid or modification of the quinone portion,[15,16] and a minimum structure required for activity has been suggested.[17] Although the synthesis of streptonigrin has not yet been reported, a number of analogues have been prepared.[18]

Our interest has focused on the biosynthesis of **1**.[1] The molecular structure, composed of a quinoline ring joined to a 4-phenylpyridine, is quite unlike that of any other known natural product.[2] While the quinoline quinone portion seemed reminiscent of the tryptophan-derived ommochromes (e.g., xanthomatin (**2**)),[19] pigments produced by various insects, the origin of the fully substituted pyridine ring seemed far less obvious. As a working hypothesis (shown in Figure 1) we considered that streptonigrin might be derived from the

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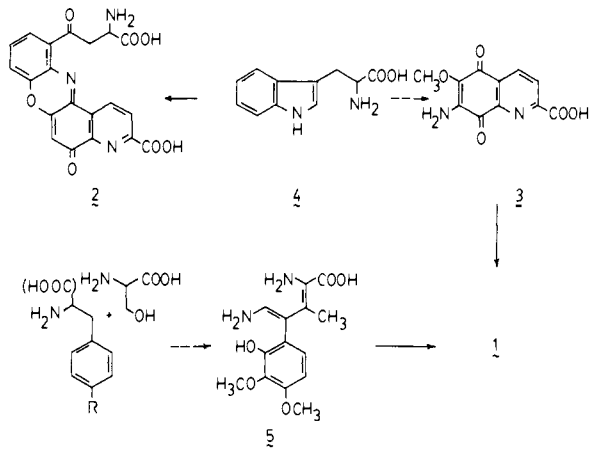

Figure 1.

equivalent of two biogenetic units, one (**3**) proposed to come from tryptophan (**4**) and the other (**5**) from phenylalanine or tyrosine and serine. All four methyls would be derived from methionine.

In this communication we report results which reveal a previously unknown pathway of tryptophan metabolism leading to formation of the 4-phenylpicolinic acid moiety (C and D rings), rather than the quinoline quinone (A and B rings).

Results and Discussion

The biosynthesis of streptonigrin was studied in shake cultures of *S. flocculus* which were grown in a complex medium based on soy flour and distillers' solubles[20] inoculated with spores or growing mycelium.[21] Based on time-course studies, sterile addition of labeled precursors 48 h after inoculation and harvest of the cultures 48 h later were chosen as standard conditions for feeding experiments. Streptonigrin was isolated by a series of acid–base extractions (see Experimental Section). After spectrophotometric determination of the amount of streptonigrin produced (UV max 380 nm), authentic streptonigrin (50–100 mg) was added when only radioactive labels were involved; in all cases the isolated **1** was recrystallized to constant specific activity.

[$^{14}CH_3$]Methionine, Phenylalanine, and Tyrosine. A series of preliminary feedings was carried out to test our initial hypotheses. The results of these and some later feedings are given in Table I. The specific incorporation of [$^{14}CH_3$]-L-methionine was confirmed by Kuhn–Roth oxidation and Schmidt degradation of the resulting acetic acid. Twenty-two percent of the radioactivity present in **1** was found in the *p*-bromophenacyl ester of the acetic acid, and essentially all of this was in the methyl group[22] (see Figure 2 for the degradations and Table II for the distribution of radioactivity).

Table I. Results of Feeding Experiments with Radioactive Precursors

precursor added	radioactivity fed, dpm $\times 10^{-7}$	mg **1** produced	% incorporation	% radioactivity remaining in broth
[$^{14}CH_3$]-L-Met	2.20	3.0	3.7	n.d.
[β-^{14}C]-DL-Trp	4.40	7.0	7.48	8.0
	3.90	5.1	5.48	1.0
[3-^{14}C]-DL-Ser	2.20	2.57	1.07	n.d.
	5.90	2.59	0.32	n.d.
[3-^{14}C]-DL-Phe	8.80	2.82	0.005	20.2
[3-^{14}C]-DL-Tyr	5.50	0.39	0.005	19.2
	2.75	2.02	0.005	12.2
[U-^{14}C]shikimic acid	2.09	2.75	0.005	70
[^{14}COOH]anthranilic acid	5.10	5.69	0.005	4.9
[$^{13}CH_3$, $^{14}CH_3$]-L-Met:				
single pulse[c]	2.59[a]	31.25[b]	1.44	n.d.
double pulse[c]	0.82[a]	51.2	2.74	n.d.
[β-^{13}C, β-^{14}C]-DL-Trp	2.07[a]	75.6	2.08	n.d.

[a] Fed to eight 500-mL fermentations. [b] Three of the eight 500-mL fermentations were contaminated by bacterial growth and were discarded. [c] See text for explanation.

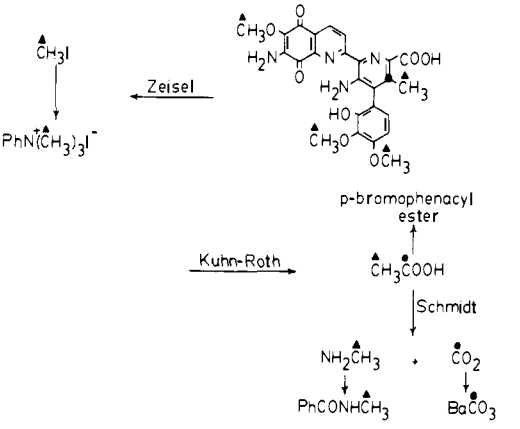

Figure 2.

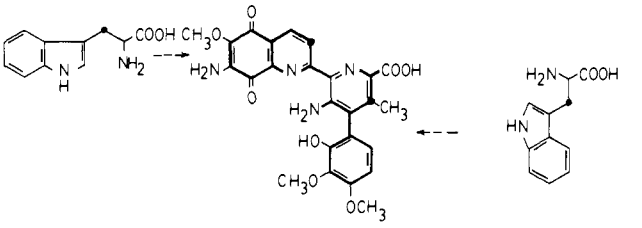

Figure 3.

Our expectations that phenylalanine or tyrosine might have been involved in the formation of the C/D rings of **1** were quickly eliminated. However, the positive incorporation of [β-^{14}C]-DL-tryptophan appeared to lend support to our proposal for the origin of the quinoline moiety, while the magnitude of the incorporation led us to expand the hypothesis to view streptonigrin as a highly modified dimer of **4**, as shown in Figure 3.

[β-^{14}C]- and [β-^{13}C]Tryptophan. Based on Figure 3, [β-^{14}C]-**4** should have labeled C-3 and C-3′ of **1**; the latter position was accessible via Kuhn–Roth oxidation and Schmidt degradation (Figure 2). The results, given in Table II, showed that *all* the activity was found at C-3′ and gave us the first indications for the biosynthesis of a major fragment of the molecule. Although the published ^{13}C NMR assignments[7] of the pyridine carbon atoms were tenuous, the specific labeling of C-3′ by the β carbon of **4** provided an opportunity to evaluate the published assignments. [β-^{13}C]-DL-Tryptophan was synthesized[23] from [^{13}C]formaldehyde (90% enriched) in 45% overall yield. The proton noise decoupled ^{13}C NMR spectrum of the tryptophan taken in 10% NaOH showed a single enriched signal at 31.1 ppm.[24] This material was fed to eight 500-mL shake cultures of *S. flocculus* at a concentration of 0.1 mM, which had pre-

Table II. Results of Chemical Degradations of Radioactive Streptonigrin

precursor added	% of streptonigrin activity in phenacyl ester	$BaCO_3$	$PhCONHCH_3$	$PhN^+Me_3I^-$
[$^{14}CH_3$]-L-Met	21.6	3.7	20.4	
[β-^{14}C]-DL-Trp	101	65.5	0	
[3-^{14}C]-DL-Ser	3.4			96
[3-^{14}C]-DL-Ser		2.0	5.1	81

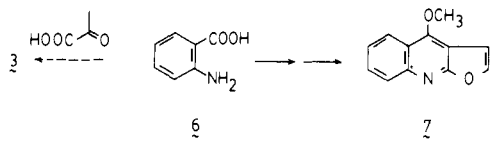

Figure 4.

viously been determined to give the optimum balance between low dilution and acceptable streptonigrin production. In this experiment 76 mg of labeled **1** was produced, and the purified material was analyzed by ^{13}C NMR spectroscopy.

Although pyridine-d_5 had previously been used as the NMR solvent, this was unsatisfactory for our present purposes owing to the relatively poor solubility of streptonigrin and the presence of nine solvent signals overlapping the aromatic region of the ^{13}C NMR spectrum of **1**. Me_2SO-d_6 proved to be a superior solvent and all 25 streptonigrin signals were clearly resolved.[25] Only the signal at 136.1 ppm in the ^{13}C NMR spectrum of the enriched sample was enhanced (eightfold when normalized relative to the C-8 signal at 180.2 ppm). This corresponded to the 137.8-ppm resonance in pyridine-d_5, which had been assigned to C-4′,[7] but which now clearly is due to C-3′.

The specificity of the incorporation of **4** had been confirmed, but an alternative biogenesis for the origin of the quinoline equivalent (**3**) was now needed. Since only the side chain of **4** had been eliminated from consideration, it was still possible that **3** was formed from anthranilic acid (**6**) (possibly derived from **4**[26]) and a three-carbon unit, such as pyruvic acid, as shown in Figure 4, which would be analogous to the formation of the quinoline ring system of dictamnine (**7**).[27] To test this hypothesis, [U-^{14}C]shikimic acid was fed, but no incorporation was obtained. However, when the fermentation was worked up, greater than 70% of the radioactivity fed had remained in the aqueous phase after streptonigrin has been removed by extraction, indicating a possible lack of a mechanism for active transport of shikimic acid into the cell.[28]

When [^{14}COOH]anthranilic acid was fed, again no incorporation was obtained. In this case, only 5% of the radioactivity had remained in the original broth, clearly indicating cellular uptake. The high degree of anthranilic acid uptake suggested an active metabolism, the simplest explanation being incorporation into tryptophan, although in this case the labeled carboxyl group would have been lost. However, as described in the next section, there was apparently no de novo biosynthesis of tryptophan during the feeding experiments.

While shikimic acid may or may not be able to enter the cells, the lack of incorporation of carboxyl-labeled anthranilic acid and the lack of de novo tryptophan biosynthesis (next section) decrease the significance of the nonincorporation of shikimic acid relative to streptonigrin biosynthesis. Additional efforts to unravel the origin of the quinoline portion will be reported in future communications.

[3-^{14}C]Serine Incorporation. Serine plays a key role in several primary metabolic pathways[29] and could have been incorporated into streptonigrin at C-3′ (if serving as precursor of the tryptophan side chain), at the four methyls (if involved via the C-1 pool), and/or at C-2 (if converted to pyruvic acid by serine dehydrase). C-3′ and the *C*-methyl were again obtained by Kuhn–Roth oxidation. The three *O*-methyls were obtained by a Zeisel reaction and subsequent trapping of the methyl iodide produced with dimethylaniline (Figure 2). With the first serine-labeled sample only 3.4% of the activity of **1** was found in the acetic acid phenacyl ester, and there was too little activity available for Schmidt degradation. However, using another portion of the labeled **1** in the Zeisel reaction, 96% of the activity was found in the methoxyl groups. With **1** obtained from a second serine feeding all the acetic acid was degraded to yield 5.1% of the activity in the benzamide and only 2% in the barium carbonate, while 81.3% of the activity was found in the trimethylanilinium iodide. These results are given in Table II.

Table III. Incorporation of [$^{13}CH_3$]-L-Methionine at Different Stages of the Fermentation

carbon atom	chem shift, ppm from Me_4Si[a]	single pulse S/11′[b]	double pulse S/11′[b]
3′-CH_3	17.8	1.9	0.8
6-OCH_3	56.1	0.8	2.4
9′-OCH_3	60.1	1.3	2.2
10′-OCH_3	60.5	1.3	2.3
11′	105.1		

[a] Spectra were run in pyridine-d_5. [b] Peak intensities of the enriched methyl signals were normalized to signals in an unenriched spectrum by using the C-11′ signal as an internal standard in each spectrum.

Clearly, serine was serving exclusively as a methyl source, although the label distribution was strikingly different from that obtained with methionine. It was reasoned that, if C-methylation were occurring at an early stage in the biosynthesis and O-methylations at late stages, C-methylation could have been (nearly) completed by the time the serine had been metabolized and its β carbon had entered the methionine pool. In any event, the data clearly indicated the lack of de novo tryptophan biosynthesis in the fermentation at the *stage* the feeding experiments are conducted, since no incorporation at C-3′ of **1** was found.

[$^{13}CH_3$]-L-Methionine Incorporations. In order to acquire additional data to support our hypothesis of different timings for C- vs. O-methylations, two feeding experiments were carried out with [$^{13}CH_3$]-L-methionine. In the first, a single pulse of labeled methionine (0.12 mM) was fed to cultures of *S. flocculus* at the normal feeding point. In the second experiment, in which twice as much methionine was used, half was added at the normal feeding point and the second half was added 24 h later. Both experiments were worked up 48 h after the initial addition of methionine. The results are summarized in Table III, which gives the pertinent data for the four methyls; signal intensities were normalized relative to the signal at 105.1 ppm (in pyridine) assigned to C-11′,[7] which is one of the few other hydrogen-bearing carbon atoms in **1**. While in the single-pulse experiment the *C*-methyl was enriched nearly twice as much as the *O*-methyls, this relationship was reversed in the double-pulse feeding with the *O*-methyl signals now being enhanced three times as much as the *C*-methyl signal. Since the first pulse was administered at the same stage of the fermentation process in both feedings, the change in relative enrichment was due to the second pulse, and the results are consistent with O-methylation occurring at a significantly later stage of the fermentation than C-methylation. Thus, with the use of differential timings for introduction of the precursor we were able directly to observe the dynamics of the streptonigrin pathway using ^{13}C NMR spectroscopy.

Conclusions

Although our original hypothesis for the primary precursors required extensive revision, our biogenetic cleavage of **1** into fragments *equivalent* to **3** and **5** proved correct and the results so far described led us to propose the partial pathway shown in Figure 5. According to this biosynthesis, **4** is methylated to β-methyltryptophan (**8**) and condenses with an acid (**3**) or a precursor thereof. Formation of the pyridine ring then results from the intramolecular attack on an amide carbonyl by the nucleophilic α position of the indole, followed by aromatization to a β-carboline and eventual cleavage of the heterocyclic ring. This proposal provides a number of points for experimental verification, the results of which will be reported in a subsequent communication.

Table IV. Results of the Kuhn-Roth Oxidations of **1**

precursor	streptonigrin: mg oxidized	streptonigrin: sp act, dpm/mmol	phenacyl acetate: yield, mg	phenacyl acetate: sp act	phenacyl acetate: % act of **1**
[$^{14}CH_3$]-L-Met	102.33	3.85×10^5	25.68	8.32×10^4	21.62
[β-^{14}C]-DL-Trp	99.40	4.36×10^5	21.25	4.41×10^5	101
[3-^{14}C]-DL-Ser	102.57	4.45×10^5	29.40	1.50×10^4	3.37

Experimental Section

General. Melting points were determined in a Hoover capillary melting point apparatus. ^{1}H NMR spectra were taken on a Hitachi Perkin-Elmer R-24 spectrometer, while ^{13}C NMR spectra were taken on a Bruker WP-60 spectrometer at 15.08 MHz. All ^{13}C NMR were proton noise decoupled (pnd) and were obtained at 30 °C in 10-mm tubes containing a cylindrical 0.50-mL capacity insert (Wilmad Glass Co.). 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 using EI. All radioactive measurements were carried out in a Packard Tri-Carb 3375 liquid scintillation counter in toluene scintillation solution (0.5% PPO), or dioxane scintillation solution (10% naphthalene and 0.5% PPO), or Bray's solution purchased from New England Nuclear and ICN. Microsamples were weighed on a Cahn Model 4400 electrobalance. All measurements were done in duplicate to a ±4% standard deviation. Counting efficiencies were determined by spiking with [^{14}C]- or [^{3}H]-*n*-hexadecane standards purchased from Amersham/Searle. Barium [^{14}C]carbonate was counted as a suspension in Cab-O-Sil (Cabot Corp., Boston, Mass.), 4% w/v in toluene scintillation solution and spiked with barium [^{14}C]carbonate (1.20×10^3 dpm/mg). Radioactive samples of trimethylanilinium iodide were counted in Bray's solution in the presence of sodium borohydride, which prevented the discoloration of the samples due to the formation of iodine. 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 except for phenylalanine, which was obtained from Schwarz/Mann. ^{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. Starks catalyst (tri-*n*-caprylmethylammonium chloride) was purchased from General Mills Chemical Co., Minneapolis, Minn. All chemicals were of reagent grade, and all solvents were distilled prior to use.

Culture Conditions. *S. flocculus* was maintained on agar slants composed of 1% malt extract, 0.4% yeast extract, 0.4% glucose, and 2% agar. Seed cultures were prepared by inoculating 100 mL of medium, containing 1% glucose, 1.5% soybean meal, 0.2% distillers' solubles, 0.5% $K_2HPO_4 \cdot 3H_2O$, 0.2% NaCl, and 0.2% $CaCO_3$, with spores from an agar slant. The cultures, contained in 500-mL Erlenmeyer flasks, were incubated at 27–29 °C on a New Brunswick Model VS gyrotary shaker at 220 rpm for 48 h. Production medium of the same composition as that used to prepare seed cultures was inoculated 5% v/v with vegetative inoculum from seed cultures and incubated for 3.5–4 days. For ^{14}C feedings 250 mL of medium in 1-L Erlenmeyer flasks was used, while for each ^{13}C feeding eight 2-L Erlenmeyer flasks each containing 500 mL of medium were used. Precursors were added in a sterile manner through disposable Millipore filters at the first detection of streptonigrin production, approximately 36 h after inoculation with seed culture. The fermentations were continued for an additional 2–2.5 days.

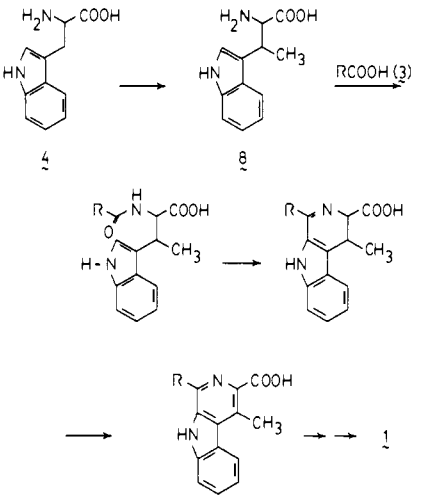

Figure 5.

Isolation. The cultures were centrifuged in an International Equipment Co. size 2 Model K centrifuge at ca. 4500 rpm for 20 min to remove the mycelia and the solid components of the broths. The supernatant was adjusted to pH 5.0 with 1 N HCl, then suction filtered through a pad of Celite to remove fine debris and particles. The filtrate was extracted three times with chloroform. Upon evaporation of the combined chloroform extracts, a brownish residue was obtained, which was then redissolved in pH 7.50 sodium phosphate buffer (0.05 M). After washing with petroleum ether (bp 30–60 °C) twice and then with isopropyl ether twice, the aqueous solution was adjusted to pH 5.0 and extracted with chloroform. The combined chloroform extracts were dried over Na_2SO_4 and concentrated to dryness in vacuo, and the chemical purity of the residue was checked by TLC in *n*-butyl alcohol-acetic acid-water (4:1:1) (R_f 0.36). The streptonigrin isolated was quantitated by its absorbance in ethyl acetate at 380 nm using a Beer's law plot (slope = 0.0380) and aliquots were taken for radioactivity analysis. Authentic streptonigrin (50–100 mg) was then added and recrystallized to constant specific radioactivity.

Kuhn-Roth Oxidation of Streptonigrin (1). Kuhn-Roth oxidations were performed according to the procedure of Wissenberger,[30] and the derived acetic acid was converted to its *p*-bromophenacyl ester using Starks catalyst.[31] The acetic acid was titrated with lithium hydroxide, concentrated in vacuo to dryness, taken up in 1 mL of water, and mixed vigorously with a solution of Stark's catalyst (4% w/v) and an excess of freshly recrystallized *p*-bromophenacyl bromide

Table V. Results of the Schmidt Degradations of Acetic Acid Derived from **1**

precursor	streptonigrin: mg	streptonigrin: sp act	barium carbonate: mg	barium carbonate: sp act	barium carbonate: % of **1**	*N*-methylbenzamide: mg	*N*-methylbenzamide: sp act	*N*-methylbenzamide: % of **1**
[$^{14}CH_3$]-L-Met	53.21	4.56×10^5	15.1	1.67×10^4	3.66	8.51	8.31×10^4	20.4
[β-^{14}C]-DL-Trp	53.32	5.54×10^6	9.6	3.61×10^6	65.5	4.9	0	0
[3-^{14}C]-DL-Ser	49.47	8.60×10^5	14.0	1.72×10^4	1.99	10.53	4.4×10^4	5.1

Table VI. Zeisel Degradation of Serine-Labeled **1**

feeding	streptonigrin: mg	streptonigrin: sp act	trimethylanilinium iodide: mg	trimethylanilinium iodide: yield, %	trimethylanilinium iodide: sp act	trimethylanilinium iodide: % of **1**[a]
first	24.0	1.28×10^6	27.0	72	4.07×10^5	96
second	31.2	2.66×10^5	29.0	60	7.20×10^4	81.3

[a] Calculated by multiplying the specific activity times three to account for the three methoxyl groups found in **1**.

(120 mg, 0.43 mmol) in 10 mL of benzene. After stirring overnight, the mixture was percolated through a column of silicic acid (1 g, dry-packed) which was washed with diethyl ether and then with ethyl acetate. The combined effluents were concentrated in vacuo and the phenacyl ester was purified by PLC developed with benzene–chloroform (1:1). Finally, the ester was sublimed (75 °C, 0.5 mm) and recrystallized from aqueous ethanol. The data for these degradations are given in Table IV.

Degradation of Acetic Acid Obtained from the Kuhn–Roth Oxidation of 1. Schmidt degradations were carried out by the procedure of Britt.[32] The methylamine was then converted to *N*-methylbenzamide.[33] The residue of the Schmidt degradation was neutralized with 20% NaOH and distilled into 10 mL of cold 2 N HCl. Additional water (20 mL) was added to the distilling flask and also distilled into the acid. After the distillate was concentrated in vacuo to dryness, the benzamide was prepared using standard Schotten–Baumann conditions and purified by sublimation (65 °C, 0.01 mm). Data for these degradations are given in Table V.

Hydriodic Acid Cleavage of Streptonigrin. The Zeisel reaction was performed according to the procedure of Clutterbuck and Raistrick,[34] and the trimethylanilinium iodide was recrystallized from absolute ethanol. Data for these degradations are given in Table VI; the percent activities given are for three methoxyl equivalents.

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