Pyrrolo[1,4]benzodiazepine Antibiotics. Biosynthesis of the Antitumor Antibiotic Sibiromycin by *Streptosporangium sibiricum*†

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ABSTRACT: The biosynthesis of the antitumor antibiotic sibiromycin by *Streptosporangium sibiricum* requires the construction of four units: the amino sugar from glucose; the anthranilate ring from DL-tryptophan probably via kynurenine; the aromatic methyl group from methionine; the propylidene proline from L-tyrosine with the loss of two aromatic carbons and addition of a C-1 from methionine. Retention of tritium from DL-[5-^{3}H]tryptophan in sibiromycin suggests an NIH shift during hydroxylation of an intermediate.

Sibiromycin, an antitumor antibiotic produced by *Streptosporangium sibiricum* (Gause et al., 1969; Brazhnikov et al., 1972; Mesentev et al., 1974), is one of the pyrrolo[1,4]benzodiazepine antibiotics. The other antibiotics in this group are anthramycin (Leimgruber et al., 1965), 11-demethyltomaymycin (Kariyone et al., 1971), and the neothramycins A and B (Miyamoto et al., 1977).[1] The potent biological properties of sibiromycin (Brazhnikova et al., 1970; Shorin & Rossolima, 1970) are attributed to the fact that this antibiotic, like anthramycin and tomaymycin, binds covalently to DNA and thereby inhibits its template activity (Hurley et al., 1977a). A review on the comparative aspects, including the mode of action, of anthramycin, tomaymycin, and sibiromycin has recently been published (Hurley, 1977).

These antibiotics are biogenetically related (Hurley et al., 1975, 1976a; Hurley, 1977); in each case the anthranilate moiety of these antibiotics is derived from tryptophan probably via the kynurenine pathway (Hurley et al., 1976b; Hurley & Gairola, 1979). For anthramycin (Hurley et al., 1975) the acrylamide proline moiety is derived from tyrosine (seven carbons) and methionine (one carbon) (see labeling pattern shown in Scheme I). The ethylidene proline moiety of tomaymycin is derived similarly with one less carbon atom from methionine (Scheme I). The present results firmly establish the labeling pattern of tyrosine, methionine, and tryptophan in sibiromycin. A preliminary account of this work has appeared (Hurley et al., 1977b).

Experimental Procedures

All experimental details of this paper are given under supplementary material (see paragraph at the end of this paper regarding supplementary material).

Results and Discussion

The biosynthetic experiments were carried out by using the sibiromycin producing organism *S. sibiricum* ATTC 29053 which was grown in shake culture on a complex media. Sibiromycin production follows the common trophophase–idiophase relationship, starting at about 24 h after inoculation of the flask and maximizing at 100 μg/mL at 54 h.[2] Maximum incorporation of precursors into sibiromycin occurred when the radiotagged molecules were added at 44 h and harvested between 1 and 6 h later. Further incubation after this time resulted in a marked reduction in the incorporation into sibiromycin.[2]

Sibiromycin was harvested from the culture broth by using the procedure described by Gause et al. (1969). This method requires the extraction of the combined media and mycelium using a methylene dichloride–oleic acid mixture, followed by reextraction of the organic phase with a citric acid buffer at pH 4. After adjustment of the pH of the aqueous phase to 7.8, the sibiromycin is finally partitioned into chloroform. This procedure yields a product which is predominantly sibiromycin.

The efficiency of incorporation of potential precursors into sibiromycin was evaluated as described before for anthramycin and tomaymycin (Hurley et al., 1975, 1976a). The validity of these figures was checked for all substrates showing significant incorporation into sibiromycin by conversion of the amorphous sibiromycin to anhydrosibiromycin which was recrystallized from 1-butanol to constant specific activity (results not shown). The incorporation of expected precursors of sibiromycin into this antibiotic by *S. sibiricum* are summarized in Table I. As expected, based upon our previous biosynthetic studies on anthramycin and tomaymycin, the methyl group of methionine, L-Dopa, L-tyrosine, and the aromatic ring of tryptophan were all efficiently incorporated into sibiromycin. D-[6-^{14}C]Glucose was also incorporated presumably into the sibirosaminide moiety of sibiromycin.

On the basis of our previously reported biosynthetic studies on anthramycin and tomaymycin, the anticipated labeling pattern for L-[1-^{13}C]tyrosine, L-[*methyl*-^{13}C]methionine, and DL-[5-^{3}H]tryptophan in the aglycon moiety of sibiromycin should be as shown in Scheme II. This scheme predicts the following points. (1) L-Tyrosine should exclusively label the PPA[3] moiety of sibiromycin, and, furthermore, the only carbon atom enriched with carbon-13 in sibiromycin from a biosynthetic experiment with L-[1-^{13}C]tyrosine should be carbon atom 11. (2) L-[*methyl*-^{14}C]Methionine will label both the anthranilic acid as well as the PPA moiety of sibiromycin. The methyl groups transferred should reside exclusively at either carbon atoms 14 or 15 of the aglycon unit of sibiromycin and

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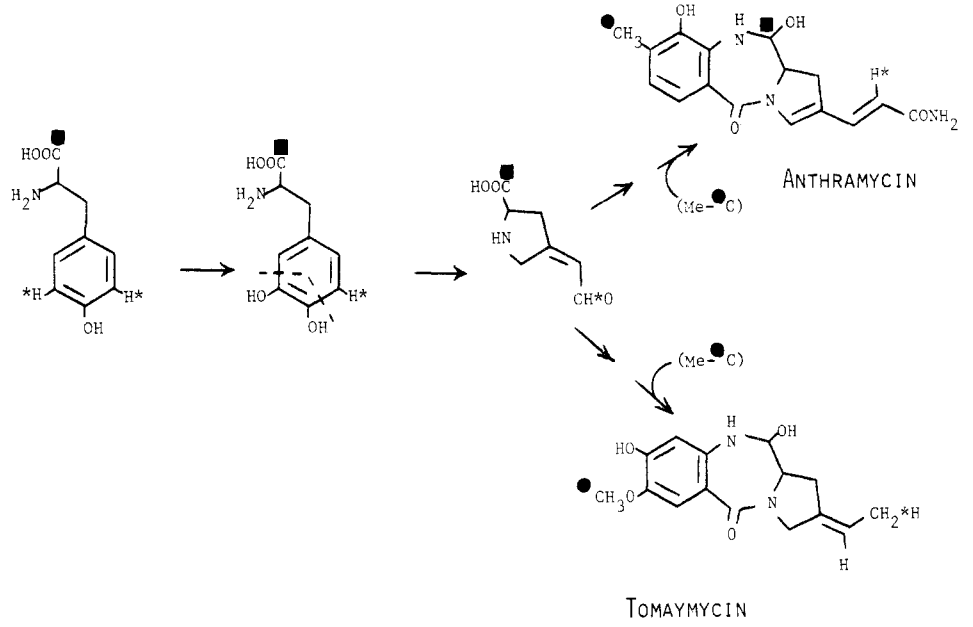
[1] It has recently been reported (Miyamoto et al., 1978) that the proline unit of the neothramycins is derived from an intact L-proline molecule rather than tyrosine.

[2] Time course production of sibiromycin and incorporation of L-[1-^{14}C]tyrosine into sibiromycin as well as the short-term time course incorporation of L-[1-^{14}C]tyrosine, L-[*methyl*-^{14}C]methionine, and DL-[7a-^{14}C]tryptophan into sibiromycin are given in Supplementary Material (see paragraph at end of this paper regarding supplementary material).

[3] Abbreviations used: AS, anhydrosibiromycin; AS-one, anhydrosibiromycinone; DAS-one, 7,9-diacetylanhydrosibiromycinone; DMAS-one, 7,9-dimethoxyanhydrosibiromycinone; DMAA, 3,5-dimethoxy-4-methylanthranilate; PPA, propylproline aldehyde; TLC, thin-layer chromatography; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

0006-2960/79/0418-4225$01.00/0

Scheme I: Established Labeling Pattern of Tyrosine and Methionine in Anthramycin and Tomaymycin



Scheme II: Proposed Labeling Pattern of L-[1-^{14}C]Tyrosine, L-[*methyl*-^{13}C]Methionine, and DL-[5-^{3}H]Tryptophan in Sibiromycin

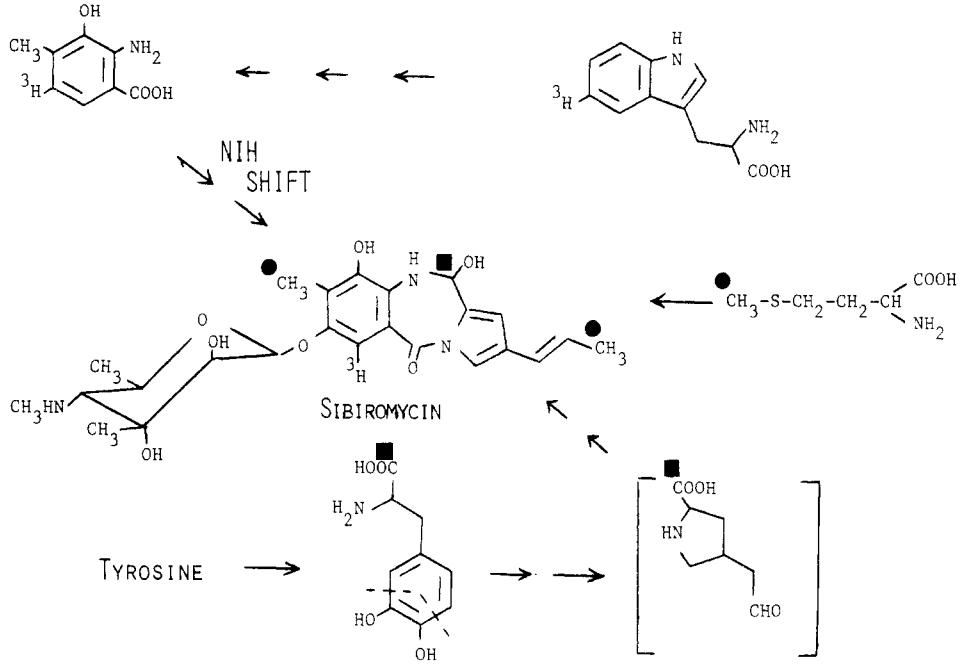


Table I: Incorporation of Labeled Substrates into Sibiromycin by *S. sibircum*

expt		quantity fed (μmol)	radioact. fed (dpm)	radioact. in BuOH extract (dpm)	% radioact. of BuOH extract in sibiromycin as detd by scanning	% incorpn into sibiromycin
1	D-[6-^{14}C]glucose	0.4	3.08×10^7	2.06×10^6	32	2.1
2	L-[1-^{14}C]dihydroxyphenylalanine	0.01	5.0×10^6	1.12×10^6	82	18.4
3	L-[*methyl*-^{14}C]methionine	3.2	6.2×10^7	1.52×10^7	100	24.5
4	DL-[7a-^{14}C]tryptophan	7.1	1.17×10^7	2.13×10^6	42	7.7
5	DL-[1-^{14}C]tyrosine	0.2	7.93×10^6	1.52×10^6	80	15.3
6	L-[U-^{14}C]tyrosine	0.03	6.70×10^6	6.54×10^5	67	6.5
7	L-[1-^{14}C]tyrosine	0.05	7.5×10^6	2.61×10^6	80	27.9

furthermore should be transferred intact, i.e., as a CH_3 unit. (3) DL-Tryptophan will be found solely in the anthranilate moiety of sibiromycin. Assuming that the tritium at position 5 of tryptophan undergoes an NIH shift (Daly et al., 1972) upon hydroxylation of some intermediate between tryptophan and sibiromycin, then this tritium atom will be retained in the antibiotic and should now reside at carbon atom 6 of sibiromycin. A series of experiments utilizing both radioactive

Table II: Enrichments of Carbons in DAS-one from Feeding Experiments with L-[*methyl*-^{13}C]Methionine and L-[1-^{13}C]Tyrosine

chemical shift[a]	multiplicity[b]	carbon atom[c]	rel enrichment[d] from L-[*methyl*-^{13}C]Met	rel enrichment[d] from L-[1-^{13}C]Tyr
11.17	q	14	3.1	0.8
18.49	q	15	3.0	0.8
20.72	q	17, 19	1.0	1.0
120.76	d	na[e]	1.0	1.0
121.16	s	na	1.5	0.8
121.75	d	na	0.8	0.7
122.54	d	na	1.0	1.0
123.52	d	na	0.8	0.9
128.58	d	na	0.8	0.8
128.89	s	na	0.7	0.8
131.60	s	na	0.7	0.9
137.32	s	na	0.8	0.9
146.79	d	11	0.9	3.9
148.32	s	7 or 9	0.8	0.8
149.20	s	7 or 9	0.9	0.9
160.53	s	5	0.7	0.8
168.66	s	16 or 18	1.0	0.6
169.00	s	18 or 16	1.1	0.9

[a] Chemical shifts are given relative to Me_4Si. [b] This multiplicity arises from one-bond ^{13}C proton coupling. [c] Numbering for DAS-one is shown in Scheme III. [d] Calculated by measuring peak heights in the spectrum of enriched DAS-one relative to the height of the acetyl methyl groups of DAS-one and then dividing these relative heights by the relative heights of the same peak (calculated in the same way) in the natural abundance spectrum. [e] na, not assigned.

and stable isotope techniques were carried out to test these predictions.

Chemical degradation of biosynthetically labeled species of sibiromycin was carried out by modification of a procedure described by Mesentsev et al. (1974) which is shown in Scheme III. Sibiromycin was chemically degraded through AS and AS-one to DMAS-one, which was then recrystallized and further checked for radiochemical purity by TLC. The intermediates AS and AS-one were not evaluated for specific incorporation of precursors due to lack of sufficient carrier material. DMAS-one was degraded by alkaline conditions to yield the anthranilate fragment as DMAA and the remaining part of the molecule as PPA. PPA was isolated and recrystallized to determine the relative contribution of tryptophan to this moiety. The DMAA isolated from the biosynthetic experiment with DL-[7a-^{14}C,5-^{3}H]tryptophan was diluted with cold authentic DMAA and recrystallized to a constant $^{3}H/^{14}C$ ratio.

The partial carbon NMR assignments for DAS-one are shown in Table II. Assignments for carbon atoms 5, 7, 9, 11, 14, 15, 16, 17, 18, and 19 were straightforward and made on the basis of multiplicity and specific neighboring groups (Stothers, 1972). The remaining carbon resonance signal assignments were not made due to both the complexity of the problem and also the fact that these atoms were neither expected nor found in practice to be enriched with carbon-13.

Specific Precursor Role of Tyrosine in Sibiromycin. L-[1-^{13}C]Tyrosine (95% ^{13}C) was added to 30 flasks of *S. sibiricum* just prior to the onset of antibiotic production (see Table III). After 24 h the sibiromycin was extracted from the culture and chemically converted to DAS-one (see Scheme III). Comparison of the ^{13}C NMR spectrum of the biosynthetically enriched species with the spectrum of the nonisotopically labeled species clearly showed that only carbon atom 11 of DAS-one was enriched in the isotopically labeled species (see Table II). This result therefore demonstrates that tyrosine

Table III: Conditions and Results of Stable Isotope Feeding Experiments with Deuterated and Carbon-13 Enriched Substrates

precursor fed[a]	feeding conditions[d]	sibiromycin yield[b]	DAS-one yield	isotopic anal. of DAS-one[c]
L-[1-^{13}C]-tyrosine (95% ^{13}C)	1	70 mg	5 mg	D_0 = 95.1% D_1 = 4.9% D_2 = 0.0%
L-[*methyl*-^{13}C]-methionine (90% ^{13}C)	5	40 mg[e]	7 mg	D_0 = 95.4% D_1 = 4.6% D_2 = 0.0%
L-[*methyl*-$^{2}H_3$]-methionine (95% ^{2}H)	15	60 mg	8 mg	D_0 = 79.9% D_1 = 2.1% D_2 = 0.0% D_3 = 12.0% D_6 = 8.1%

[a] Precursors were added at about 32 h of growth, and the sibiromycin was harvested 24 h later. [b] This value was based upon measurement of absorption at 310 nm (E = 21 800) of the sibiromycin dissolved and diluted out in methanol. [c] D_0, D_1, D_2, D_3, and D_6 represent the parent ions at *m/e* 366, 367, 368, 369, and 372, respectively. [d] Number of milligrams added per flask to 30 flasks. Each 500-mL baffled flask contained 100 mL of media. [e] Because of the low yield of sibiromycin, 70 mg of unlabeled sibiromycin was added before chemical conversion to DAS-one.

Table IV: Recrystallization of DAS-one Produced from Feeding Experiments with L-[1-^{14}C,2- or 6-^{3}H]Tyrosine, L-[U-^{14}C,2- or 6-^{3}H]Tyrosine, and L-[*methyl*-^{14}C,*methyl*-$^{3}H_3$]Methionine

expt	precursor	$^{3}H/^{14}C$ ratio of precursor	DAS-one $^{3}H/^{14}C$ ratio of recrystn: first	second	third	% final ^{3}H retention[a]
1	L-[1-^{14}C,2- or 6-^{3}H]tyrosine	6.50	2.24	2.31	2.20	34.6
2	L-[U-^{14}C,2- or 6-^{3}H]tyrosine	7.40	3.28	3.23	3.10	45.1
3	L-[*methyl*-^{14}C,*methyl*-$^{3}H_3$]-methionine	2.58	2.65	2.43	2.70	100.5

[a] Tritium retention = ($^{3}H/^{14}C$ of product)/($^{3}H/^{14}C$ of precursor) × 100.

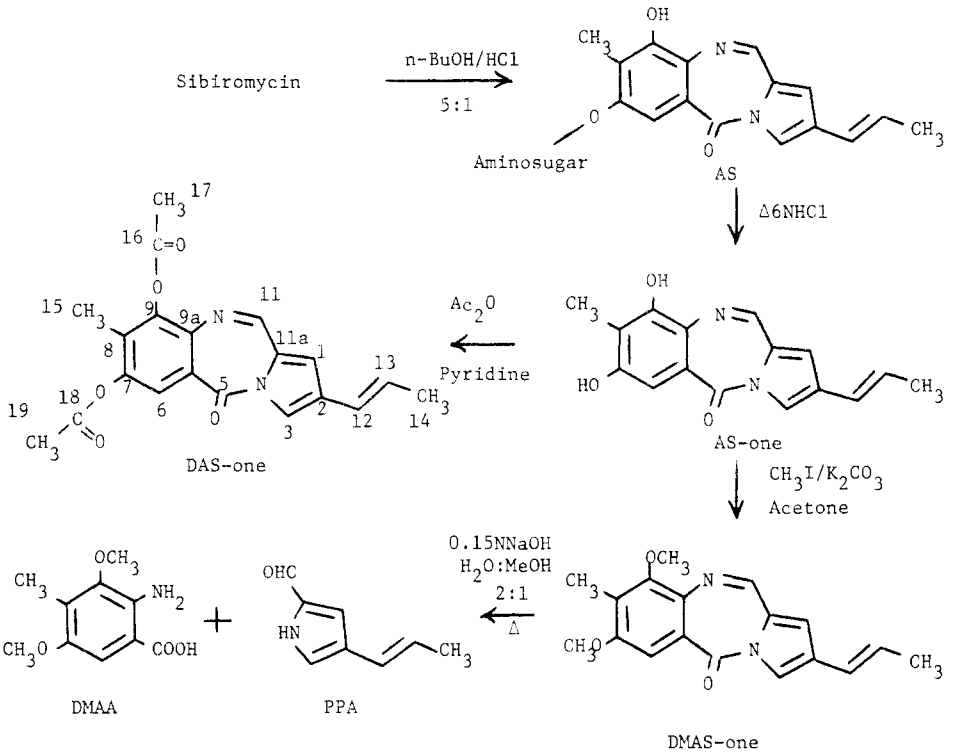
is incorporated into the aglycon moiety of sibiromycin in an analogous manner to that previously demonstrated for anthramycin and tomaymycin.

In order to determine how many carbon atoms of L-tyrosine were actually incorporated into sibiromycin, we compared the incorporation of L-[U-^{14}C]tyrosine with that of L-[1-^{14}C]-tyrosine using L-[2- or 6-^{3}H]tyrosine as a reference label. The data (experiments 1 and 2) obtained are presented in Table IV. The 45.1% retention of tritium in DAS-one (experiment 2, Table IV) is indicative of a transfer of seven of the nine carbon atoms of tyrosine to the DAS-one. Incorporation of six or seven or eight carbon atoms of tyrosine into DAS-one would be represented by tritium retentions of 38.9, 44.9, and 51.9%, respectively.[4]

Specific Precursor Role of Methionine in the Aglycon Part of Sibiromycin. The specific carbon atoms of DMAA and PPA that are labeled from methionine were determined by carbon NMR. L-[*methyl*-^{13}C]Methionine (90% ^{13}C) was fed to 30 flasks of *S. sibiricum* as described for the L-[1-^{13}C]-tyrosine experiment, and DAS-one was prepared from the isolated sibiromycin (see Table III). Analysis of the ^{13}C NMR

[4] Taking into account the 34.6% retention of tritium from L-[2- or 6-^{3}H]tyrosine during its conversion to DAS-one, then the retention of 6, 7, or 8 carbon atoms of L-tyrosine in DAS-one will be 9/6 × 34.6 = 51.9, 9/7 × 34.6 = 44.9, and 9/8 × 34.6 = 38.9, respectively.

Scheme III: Chemical Degradation of Sibiromycin [after Mesentsev et al. (1974)]



spectrum of DAS-one (Table II) showed that [*methyl*-^{13}C]methionine enriched the aromatic methyl group and the terminal propylidene methyl group of DAS-one. The C-1 pool origin of the terminal propylidene methyl group of sibiromycin is in line with both our previous studies on anthramycin (Hurley et al., 1975) and tomaymycin (Hurley et al., 1976a) and those of the Upjohn group on lincomycins A and B (Witz et al., 1971; Rolls et al., 1976) and suggests that the C_2- and C_3-proline units are derived from a common intermediate in which divergence takes place at the point at which either a proton or a methyl group is transferred to the branch point compound (Hurley et al., 1979).

In order to verify that both methionine-derived methyl groups in DAS-one were transferred as intact methyl units, we carried out feeding experiments with L-[*methyl*-^{14}C,-*methyl*-$^{3}H_3$]methionine and L-[*methyl*-$^{2}H_3$]methionine. The 100% retention of tritium (Table IV) in DAS-one is in accord with this assumption; however, to avoid any ambiguity due to an isotope effect, we conducted an experiment with deuterated methionine in which the DAS-one was analyzed by mass spectrometry. In this experiment, in addition to the nondeuterated species, two other predominant ions occurred corresponding to D_3 and D_6 (see Table III). This confirms that both methyl groups are transferred intact to the aglycon moiety of sibiromycin.

Specific Precursor Role of Tryptophan in Sibiromycin. The results in Table V show that DL-[7a-^{14}C]tryptophan labeled almost exclusively the DMAA moiety of sibiromycin. This unit of sibiromycin is derived from tryptophan via the kynurenine pathway (Hurley & Gairola, 1979), and the origin of this moiety is therefore analogous to that of variously substituted anthranilates found in anthramycin (Hurley et al., 1975), tomaymycin (Hurley et al., 1976a), and actinomycin D (Herbert, 1974; Salzmann et al., 1969).

The availability of DL-[5-^{3}H]tryptophan has allowed us to conveniently determine whether an NIH shift of the tritium originally located at the 5 position of tryptophan occurred during its conversion to sibiromycin. This experiment was

Table V: Feeding Experiment with L-[7a-^{14}C,5-^{3}H]Tryptophan

	rel sp act. of sibiromycin and degradn products	$^{3}H/^{14}C$ ratio	tritium retention (%)
[7a-^{14}C,5-^{3}H]-tryptophan[a]		12.7	100
sibiromycin[b]	100[c]	10.7	84.3
DMAS-one	89.0	9.63	76.0
DMAA			
first crystn	86.3	12.0	94.5
second crystn	87.5	11.7	92.3
PPA	0.0		

[a] DL-[7a-^{14}C]Tryptophan (1.40×10^7 dpm; sp act. 3.5 mCi/mmol) and DL-[5-^{3}H]tryptophan (1.78×10^8 dpm; sp act. 1 Ci/mmol) were fed to 24 100-mL cultures of *S. sibiricum*. [b] The sibiromycin isolated from this experiment (68.5 mg) had a specific activity of 9.10×10^3 dpm/μM. [c] Based upon carbon-14.

carried out using DL-[7a-^{14}C]tryptophan as a reference label and chemical degradation of the sibiromycin to DMAA. The tritium retention in DMAA (93%) (Table V) is a direct measure of the magnitude of the NIH shift, and since there is only one aromatic proton in DMAA, the tritium is presumably located exclusively at this position. An indication of the step at which hydroxylation of the aromatic ring occurs, resulting in an NIH shift, can be assessed from the following facts. In a feeding experiment with [1-^{14}C]-4-methyl-3-hydroxyanthranilic acid (Hurley & Gairola, 1979), we found a 14.1% incorporation into sibiromycin. An almost complete retention of tritium from DL-[5-^{3}H]tryptophan takes place in sibiromycin at C-6. The empirical rule that enzymatic hydroxylation para to a highly activating group such as $-NH_2$ results in virtually complete loss of isotopic hydrogen from the product (Daly et al., 1972) leads us to suspect that the hydroxylation may take place on a compound possessing a less activating para substituent such as an amide.

In summary, this study clearly demonstrates that the biogenetic building blocks for the aglycon moiety of sibiro-

mycin are tyrosine, tryptophan, and methionine. The determined biosynthetic labeling pattern of these three amino acids in the aglycon moiety of sibiromycin is shown in Scheme II. The paper immediately following this one supplies more information on the conversion of tyrosine to the C_2- and C_3-proline units of the pyrrolo[1,4]benzodiazepine antibiotics (Hurley et al., 1979). Our continuing studies are directed toward determining more about the biosynthetic pathways to these unique antibiotics and also their function, if any, to the producer organisms.

Acknowledgments

We are indebted to Dr. Gause at the Moscow Institute for New Antibiotics for supplying us with a sample of sibiromycin via Dr. H. Mahler at Indiana University. The excellent technical assistance of S. Estes is also acknowledged. Finally, we thank L. West for typing this manuscript.

Supplementary Material Available

Experimental procedures including fermentations, chromatography, labeled and nonlabeled compounds, isotope analysis, general techniques, and isolation, quantitative determination, and chemical degradation of sibiromycin and two figures showing the time course production of sibiromycin and incorporation of L-[1-^{14}C]tyrosine into sibiromycin as well as the short-term time course incorporation of L-[1-^{14}C]tyrosine, L-[*methyl*-^{14}C]methionine, and DL-[7a-^{14}C]tryptophan into sibiromycin (7 pages). Ordering information is given on any current masthead page.

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