Pyrrolo[1,4]benzodiazepine Antibiotics. Biosynthetic Conversion of Tyrosine to the C_2- and C_3-Proline Moieties of Anthramycin, Tomaymycin, and Sibiromycin†

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ABSTRACT: This paper describes biosynthetic labeling experiments on the conversion of tyrosine to the C_2- and C_3-proline units of anthramycin, tomaymycin, and sibiromycin. The biosynthetic fate of all of the aromatic and side-chain hydrogens has been determined in each antibiotic by using dual tagged ($^3H/^{14}C$) and ^2H-labeled tyrosine molecules. In addition, experiments using [^{15}N]tyrosine and the tritiated D and L isomers of tyrosine have shed some light on the biochemical reactions which take place at the α position of tyrosine. On the basis of results of all these experiments, a biosynthetic scheme has been proposed to rationalize the apparent inconsistencies which occur between the results for the three antibiotics. This scheme proposes that a common main pathway involving proximal extradiol cleavage of Dopa and condensation to form the pyrrolo ring leads ultimately to a C-7 branch point compound. Parallel pathways from this central branch point compound lead by well-known biochemical transformations to the C_2- and C_3-proline units of anthramycin, tomaymycin, and sibiromycin. The reactions in these parallel pathways are suggested to be "cosmetic or after events".

Anthramycin, sibiromycin, and tomaymycin[1] are structurally and biosynthetically related antitumor antibiotics produced by various actinomycetes (Scheme I). The potent biological properties of these antibiotics, which we named as a group the "pyrrolo[1,4]benzodiazepine antibiotics", can be attributed to the effects of these compounds on nucleic acid biosynthesis (Hurley et al., 1977; Horwitz & Grollman, 1968; Gause & Dudnik, 1972; Nishioka et al., 1972).

Biosynthetic studies establishing the biosynthetic building blocks for anthramycin (Hurley et al., 1975), tomaymycin (Hurley et al., 1976), and sibiromycin (Hurley et al., 1979) have been published. These studies have demonstrated that the biosynthetic origins of the anthranilate and C_2- or C_3-proline units of these antibiotics are closely related as shown in Scheme I. The anthranilate moieties of these antibiotics are derived from tryptophan via the kynurenine pathway (Hurley & Gairola, 1979). Each antibiotic differs in the aromatic substitution pattern, insertion of the substituents appearing to occur at the kynurenate stage. The C_2- and C_3-proline units of these antibiotics are derived from catabolism of tyrosine. In the case of the C_3-proline units of anthramycin and sibiromycin, the additional carbon absent in the C_2-proline unit of tomaymycin is derived from methionine. The labeling patterns illustrated in Scheme I for anthramycin, sibiromycin, and tomaymycin have been demonstrated by radioactive and stable isotope techniques (Hurley et al., 1975, 1976, 1979).

One of the prime objectives of our biosynthetic studies on these antibiotics is to establish a general pathway for the unusual biosynthetic conversion of tyrosine through Dopa to the C_2- and C_3-proline moieties of these antibiotics. Specifically, we would like to determine at which intermediate the common pathway diverges to give rise to either the C_2- or C_3-proline unit and which subsequent modifications can be thought of as being cosmetic in character.

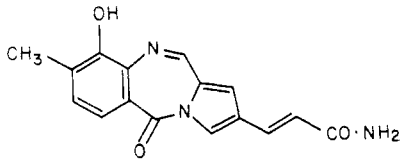
This paper describes the results of double-labeling experiments (^3H and ^{14}C) using various specifically labeled tyrosine molecules and also experiments utilizing ^2H- and ^{15}N-labeled substrates. These results, which have been obtained with all three antibiotics, are interpreted in terms of possible intermediates in the biosynthetic pathways to the C_2- and C_3-proline units. A preliminary account of some of this work has appeared (Lasswell et al., 1978).

Experimental Procedures

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

Results and Discussion

The optimum conditions for biosynthetic incorporation of tyrosine into anthramycin (Hurley et al., 1975), sibiromycin (Hurley et al., 1979), and tomaymycin (Hurley et al., 1976) have been described previously. Final tritium to carbon-14 ratios in anthramycin and tomaymycin were determined after recrystallization with cold carrier material (Hurley et al., 1975, 1976). Sibiromycin was converted to DAS-one (for structure see preceding paper) before recrystallization to a constant tritium to carbon-14 ratio (Hurley et al., 1979). Where exchangeable tritium was likely to be present in tyrosine samples, this was evaluated by either repeated addition and removal of water or recrystallization to a constant tritium to carbon-14 ratio. Anthramycin was chemically converted to DAA in certain cases (Hurley et al., unpublished experiments).



DAA

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[1] Abbreviations used: AME, anthramycin 11-methyl ether; TME, tomaymycin 11-methyl ether; Dopa, 3,4-dihydroxyphenylalanine; cyclo-Dopa, 6,7-dihydroxycyclophenylalanine; DAS-one, 7,9-diacetylanhydrosibiromycinone; DAA, 1,11a-didehydroanhydroanthramycin; F_3AcOH, trifluoroacetic acid; PPA, propylproline aldehyde.

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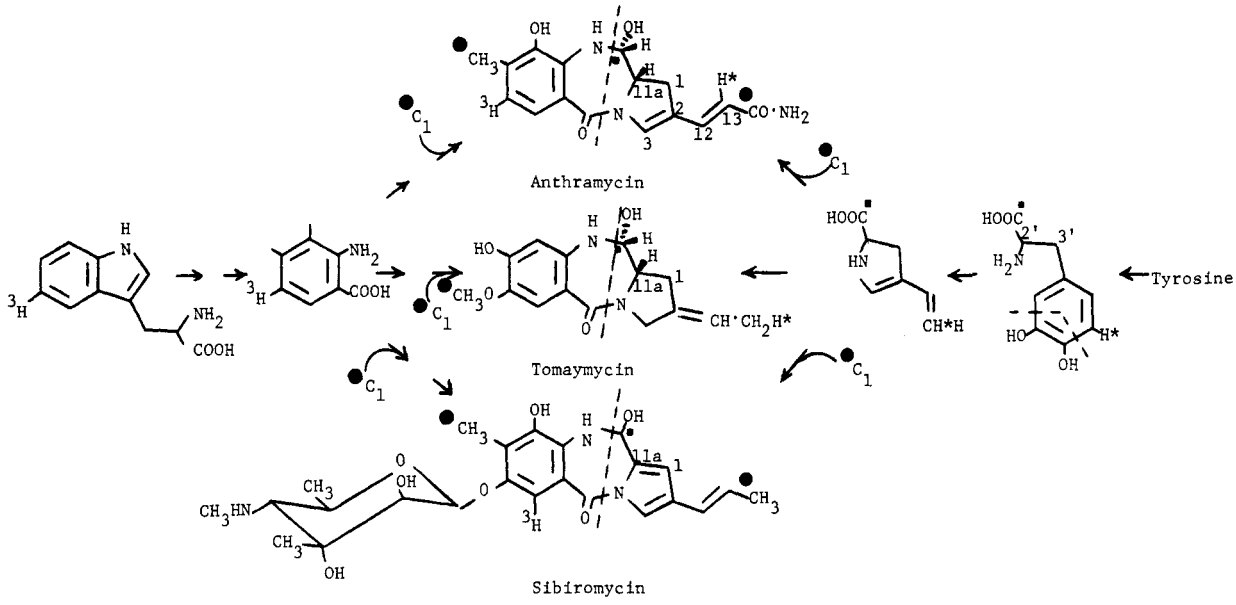
Scheme I: Structures of Anthramycin, Tomaymycin, and Sibiromycin and Their Demonstrated Biosynthetic Labeling Patterns from Tyrosine, Methionine, and Tryptophan



Sibiromycin was converted to PPA for mass spectral analysis of ^{15}N labeling (for structure see the preceding paper).

The double-labeled biosynthetic experiments were designed to answer specific questions. Each question will be considered in turn and the results rationalized in a suggested biosynthetic scheme leading to these antibiotics.

What routes via aromatic ring cleavage and cyclization to form the five-membered ring are possible? During the conversion of tyrosine through Dopa to the C_2- and C_3-proline moieties of the pyrrolo[1,4]benzodiazepine antibiotics, cleavage of the aromatic ring must take place. This in turn will be followed by condensation with the 2 or 6 position of tyrosine to form the pyrrolo ring. The alternative pathways for ring cleavage and condensation are shown in Scheme II. Biosynthetic pathways leading to structures of the type I, III, or V in Scheme II would necessarily require elimination of both tritium atoms from L-[3- or 5-^{3}H]tyrosine, whereas pathways leading to II, IV, or VI only require loss of one of the two tritium atoms from this substrate. The results in Table I (experiments 1–3) exclude pathways leading to I, III, or V as possible alternatives in the cases of anthramycin and tomaymycin. However, the almost complete loss of tritium from DAS-one would be in agreement with any of the pathways in Scheme II.[2] The position of the retained tritium from L-[3- or 5-^{3}H]tyrosine in anthramycin (Hurley et al., 1975) and tomaymycin (Hurley et al., 1976) was as expected at C-13 of these antibiotics. In view of the fact that the meta cleavage pathway is implicated not only for anthramycin and tomaymycin but also in the case of the lincomycin antibiotics (Rolls et al., 1976), which contain similar C_3- and C_2-proline units to the pyrrolo[1,4]benzodiazepine antibiotics, we suspect that the almost complete loss of tritium from L-[3- or 5-^{3}H]tyrosine in sibiromycin is due to a later biosynthetic transformation. A plausible hypothesis for this loss, peculiar to sibiromycin, is presented later.

Does ring cleavage of the aromatic ring take place before or after cyclization to form the five-membered ring? Just as L-[3- or 5-^{3}H]tyrosine can be used to differentiate between alternative methods for ring cleavage of Dopa, species of L-[2- or 6-^{3}H]tyrosine can be utilized to shed some information on the stage at which cyclization to form the five-membered ring of anthramycin, tomaymycin, and sibiromycin takes place. The intermediacy of cyclo-Dopa would require the loss of at least 50% of the tritium from L-[2- or 6-^{3}H]tyrosine in these antibiotics, whereas a pathway in which ring cleavage occurs prior to formation of the five-membered ring would not require loss of either of the tritium atoms from L-[2- or 6-^{3}H]tyrosine in these antibiotics. The results in Table I (experiments 5–7) show considerable variations in retention of tritium from one antibiotic to another. The 78% retention of tritium in tomaymycin would appear to rule out cyclo-Dopa as a plausible intermediate in this case. However, the 52 and 33% retentions in the cases of anthramycin and DAS-one are in agreement with either pathway.

Information on the labeling pattern of these species of tyrosine in anthramycin and sibiromycin was obtained by feeding the corresponding deuterated tyrosine molecules. L-[2- or 6-$^2H_2$]Tyrosine (97% $^2H_2$) was fed to anthramycin-producing cultures (20 mg/flask) and sibiromycin-producing cultures (20 mg/flask) by using conditions described previously (Hurley et al., 1975, 1979). The anthramycin isolated was crystallized as AME and subjected to mass spectra and ^{1}H NMR analysis. Mass spectral analysis of AME showed only species of D_0 and D_1 in the ratio of 48.15 to 51.85 (Table II), confirming that one deuterium atom was lost during the conversion of L-[2- and 6-$^2H_2$]tyrosine to anthramycin. The ^{1}H NMR analysis of the same sample showed a clear reduction of the doublet due to substitution of deuterium for the proton at C-12 of AME (Figure 1A,B). Significantly, no reduction in the intensity of the proton signal at C-3 was found. The sibiromycin isolated from a feeding experiment in which 20 mg/flask of the deuterated tyrosine was added was converted to DAS-one (Hurley et al., 1979). Mass spectral analysis on this sample showed only species of D_0 and D_1 in the ratio of 72.5 to 27.5 (Table II). The ^{1}H NMR analysis revealed that the remaining deuterium atom was located at C-12 of DAS-one (Figure 1C,D), as was also the case for AME.

These results, considered together with the experiment utilizing L-[2- or 6-^{3}H]tyrosine, lead to the conclusion that the

[2] We had previously reported a tritium retention of 17% in sibiromycin from an experiment with L-[1-^{14}C,3- or 5-^{3}H]tyrosine (Hurley, 1977). This result is correct; however, upon conversion of sibiromycin to DAS-one, further loss of tritium occurs such that only a 3.9% tritium retention is found in DAS-one.

Table I: Results of Cocrystallization of AME, TME, DAS-one, and DAA from Feeding Experiments with Various Double-Labeled Tyrosine Molecules

expt no.	species of tyrosine	product	$^3H/^{14}C$ ratio of tyrosine	$^3H/^{14}C$ ratio of crystallization			final tritium retention (%)
				first	second	third	
1	L-[1′-^{14}C,3- or 5-^{3}H]	AME	9.17	4.74	4.68	4.68	51
2	L-[1′-^{14}C,3- or 5-^{3}H]	TME	6.45	3.25	3.16	3.07	48.9
3	L-[1′-^{14}C,3- or 5-^{3}H]	DAS-one	8.91	0.32	0.26	0.47	3.9
4	L-[1′-^{14}C,3- or 5-^{3}H]	DAA	11.20 (5.79)[a]	5.53	5.69	*b*	98.4
5	L-[1′-^{14}C,2- or 6-^{3}H]	AME	8.16	4.31	4.31	4.25	52.6
6	L-[1′-^{14}C,2- or 6-^{3}H]	TME	8.75	7.22	6.83	6.82	78.0
7	L-[1′-^{14}C,2- or 6-^{3}H]	DAS-one	8.33	2.77	2.74	2.65	32.7
8	L-[1′-^{14}C,2- or 6-^{3}H]	DAA	18.38 (8.86)[a]	8.05	8.45	*b*	95.4
9	DL-[1′-^{14}C,3′-*RS*-(3′-^{3}H)]	AME	3.19	2.97	3.06	3.06	95.0
10	DL-[1′-^{14}C,3′-*RS*-(3′-^{3}H)]	TME	3.18	3.18	3.31	*b*	102.0
11	DL-[1′-^{14}C,3′-*RS*-(3′-^{3}H)]	DAS-one	4.87	2.84	3.00	3.07	61.0
12	DL-[1′-^{14}C,3′-*RS*-(3′-^{3}H)]	DAA	12.70 (9.97)[a]	4.76	4.92	5.44	54.5
13	DL-[1′-^{14}C,3′-*R*-(3′-^{3}H)]	AME	11.04	10.81	9.03	9.12	87.4
14	DL-[1′-^{14}C,3′-*R*-(3′-^{3}H)]	TME	4.86	4.29	4.49	*b*	90.3
15	DL-[1′-^{14}C,3′-*R*-(3′-^{3}H)]	DAS-one	9.70	9.68	9.37	9.40	97.8
16	DL-[1′-^{14}C,3′-*R*-(3′-^{3}H)]	DAA	11.04 (9.12)[a]	6.51	5.10	4.63	50.6
17	DL-[1′-^{14}C,3′-*S*-(3′-^{3}H)]	AME	9.85	10.03	8.12	7.81	80.9
18	DL-[1′-^{14}C,3′-*S*-(3′-^{3}H)]	TME	3.94	3.59	3.75	*b*	92.4
19	DL-[1′-^{14}C,3′-*S*-(3′-^{3}H)]	DAS-one	6.92	2.29	2.36	*b*	33.7
20	DL-[1′-^{14}C,3′-*S*-(3′-^{3}H)]	DAA	9.85 (7.81)[a]	4.04	3.48	3.43	44.2
21	L-[1′-^{14}C,Ala-2′,3′-^{3}H]	AME	3.30	1.64	1.64	*b*	49.7
22	L-[1′-^{14}C,Ala-2′,3′-^{3}H]	TME	3.30	1.47	1.69	*b*	47.9
23	L-[1′-^{14}C,Ala-2′,3′-^{3}H]	DAS-one	9.12	1.71	1.62	*b*	19.3
24	DL-[1′-^{14}C],L-[2- or 6-^{3}H]	AME	18.04	16.77	18.57	17.70	100.5[c]
25	DL-[1′-^{14}C],D-[2- or 6-^{3}H]	AME	11.86	0.75	0.84	0.54	5.8[c]
26	DL-[1′-^{14}C],L-[2- or 6-^{3}H]	DAS-one	5.82	2.89	2.92	3.34	53.8[c]
27	DL-[1′-^{14}C],D-[2- or 6-^{3}H]	DAS-one	6.17	0.55	0.63	0.48	9.0[c]

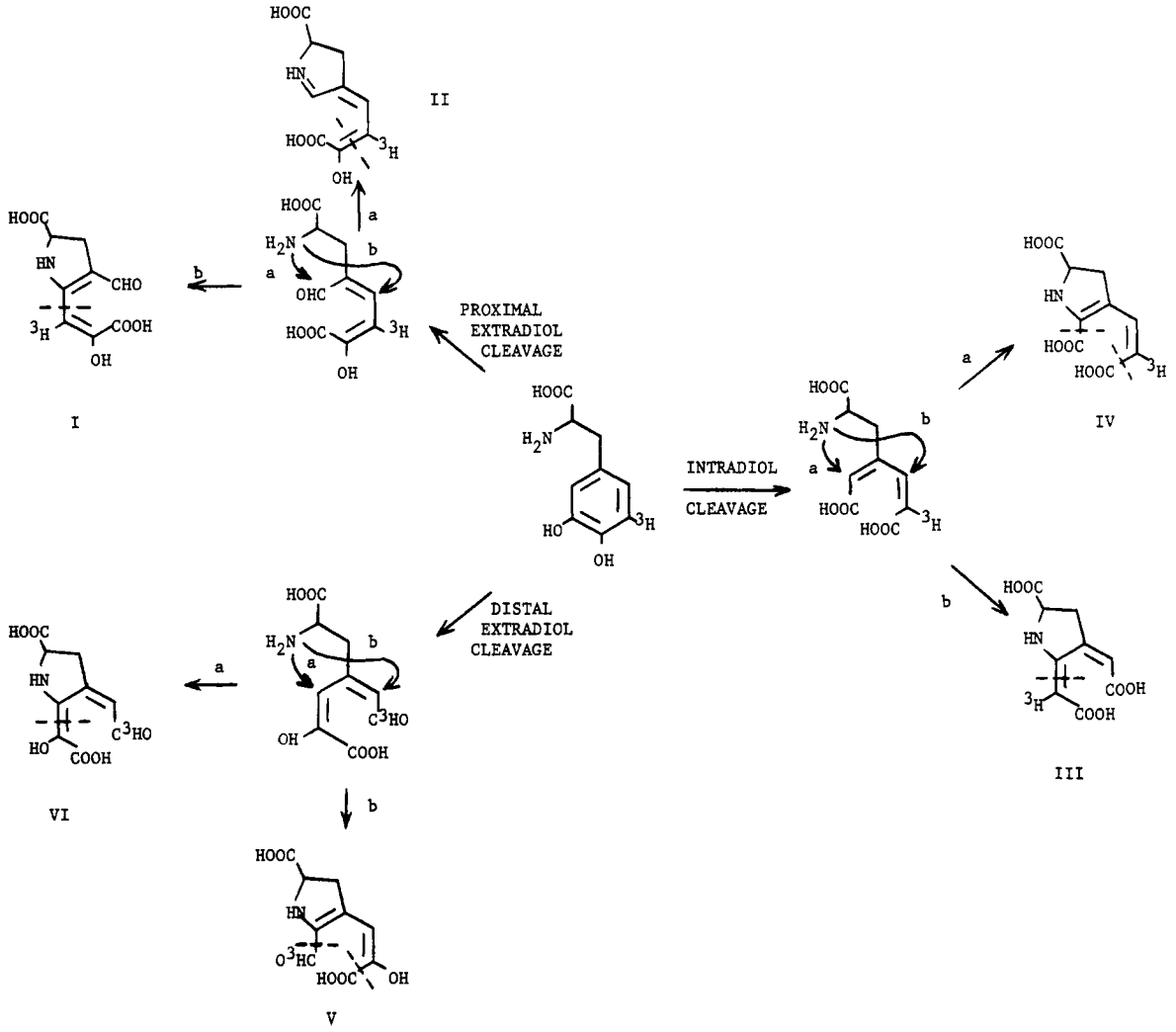
[a] Final $^3H/^{14}C$ ratio of AME after two crystallizations and before conversion to DAA. [b] Insufficient material for a third recrystallization. [c] Taking into account the retention of only approximately 50 and 32.7% of the tritium during the conversion of L-[1′-^{14}C,2- or 6-^{3}H]tyrosine to anthramycin and DAS-one, respectively (experiments 5 and 7, Table I), the experiments with DL-[1′-^{14}C] and L-[2- or 6-^{3}H] and DL-[1′-^{14}C] and D-[2- or 6-^{3}H]tyrosine have relative retentions of (100.5 × 100)/52.6 = 191.1% and 5.8 × 100/52.6 = 11.0% (for AME) and 53.8 × 100/32.7 = 165% and 9.0 × 100/32.7 = 27.5% (for DAS-one), i.e., the L isomer is incorporated at least 17 and 6 times more efficiently than the D isomer of tyrosine in AME and sibiromycin, respectively.[6]

Table II: Conditions and Results of Radioactive and Stable Isotope Feeding Experiments with ^{14}C-, ^{2}H-, and ^{15}N-Labeled Tyrosine Molecules Using *Streptomyces refuineus* and *Streptosporangium sibiricum*

species of tyrosine fed	feeding conditions	isotopic analysis of products		calcd enrichments (%)	
		mass spectroscopy[a] (%)	sp act. (μCi/mmol)	based upon mass spectroscopy	based upon ^{14}C dilution values[g]
L-[2- and 6-$^2H_2$] (97% $^2H_2$)	20 mg/flask added to 20 50-mL cultures at 6 h and harvested at 14 h	AME[b] D_0 = 48.1 D_1 = 51.9 D_2 = 0.0	na[f]	51.9 (^{2}H)	na
L-[2- and 6-$^2H_2$] (97% $^2H_2$)	20 mg/flask added to 20 100-mL cultures at 34 h and harvested at 54 h	DAS-one[c] D_0 = 72.5 D_1 = 27.5 D_2 = 0.0	na	27.5 (^{2}H)	na
L-[3′,3′-$^2H_2$] (98% $^2H_2$)	10 mg/flask added to 20 flasks at 32 h and harvested at 40 h	DAS-one[c] D_0 = 89.2 D_1 = 10.8 D_2 = 0.0	na	10.8	na
DL-[1-^{14}C,3- and 5-$^2H_2$,^{15}N] (sp act. 10.50 μCi/mmol; >95% $^2H_2$; 99% ^{15}N)	18 mg/flask added to 20 50-mL cultures at 6 h and harvested at 15 h	DAA[d] D_0 = 72.0 D_1 = 21.8 $D_1{}^{15}N_1$ = 6.2 $D_2{}^{15}N_1$ or $D_1{}^{15}N_2$ = 0.0	3.30	28.0 (^{2}H) 6.2 (^{15}N)	31.5
L-[1-^{14}C,^{15}N] (sp act. 19.11 μCi/mmol; 99% ^{15}N)	10 mg/flask added to 20 100-mL cultures at 32 h and harvested at 46 h	PPA[e] D_0 = 91.6 D_1 = 8.4 D_2 = 0.0	5.47	8.4 (^{15}N)	28.7

[a] Corrected for natural isotopic abundances after Biemann (1962). [b] D_0, D_1, and D_2 represent parent ions at *m/e* 296, 297, and 298. [c] D_0, D_1, and D_2 represent parent ions at *m/e* 366, 367, and 368. [d] D_0, D_1, $D_1{}^{15}N_1$, and $D_2{}^{15}N_1$ or $D_1{}^{15}N_2$ represent parent ions at *m/e* 295, 296, 297, and 298. [e] D_0, D_1, and D_2 represent parent ions at *m/e* 135, 136, and 137. [f] na, not applicable. [g] Dilution = (specific activity of tyrosine)/(specific activity of product).

Scheme II: Alternative Pathways for the Conversion of L-[3- or 5-^{3}H]Tyrosine into the C_2- and C_3-Proline Moieties of Anthramycin, Tomaymycin, and Sibiromycin Involving Various Cleavage Pathways



67% loss of tritium in DAS-one is due to complete loss of the tritium that will reside at C-6 of Dopa and a partial loss of tritium (17%) that ultimately labeled C-12 of DAS-one.

Stable isotope analysis of tomaymycin samples labeled from L-[2- and 6-^{2}H]tyrosine is lacking, due to the low antibiotic-producing ability of the available culture. Our double-labeling experiment with L-[1′-^{14}C,2- or 6-^{3}H]tyrosine in which we found a retention of 78% of tritium is, however, only compatible with a pathway involving ring cleavage of Dopa prior to formation of a five-membered ring.

What is the biosynthetic fate of the side-chain hydrogens of tyrosine in anthramycin, tomaymycin, and sibiromycin? On the basis of our previous biosynthetic results with the pyrrolo[1,4]benzodiazepine antibiotics, the 2′ and 3′ side-chain hydrogens of tyrosine should reside at carbon atoms 1 and 11a of these antibiotics, respectively (see Scheme I).

Conclusive evidence for the biosynthetic origin of C-1 of anthramycin as C-3′ of tyrosine was obtained, when it was found that species of AME biosynthetically labeled from DL-[1′-^{14}C,3′-*RS*-(3′-^{3}H)]tyrosine and the two stereospecifically labeled substrates lose approximately 50% of their radioactivity (experiments 12, 16, and 20, Table I) when they were converted to DAA by using F_3AcOH (Hurley et al., unpublished experiments). Control experiments in which L-[1′-^{14}C,2- or 6-^{3}H]tyrosine and L-[1′-^{14}C,3- or 5-^{3}H]tyrosine were administered to the anthramycin-producing culture and these samples were subsequently chemically converted to DAA showed, as expected, no further loss of tritium during chemical conversion of AME to DAA (experiments 4 and 8, Table I).

Inspection of the appropriate carbon atoms in tyrosine (side chain) and the three antibiotics (carbon atoms 11a and 1) reveals that, whereas in anthramycin and tomaymycin the oxidation state of the corresponding carbon atoms are similar, in sibiromycin an additional double bond exists. Furthermore, the stereochemistry at C-2′ of tyrosine is the same as that at C-11a of anthramycin and tomaymycin. These facts would therefore suggest that, whereas in anthramycin and tomaymycin all three side-chain hydrogens might be retained, in sibiromycin the 2′-hydrogen and one of the two diastereotopic 3′-hydrogens must be lost. Double-labeled species of tyrosine labeled with tritium either at the 3′-carbon [DL-[1′-^{14}C,3′-*RS*-(3′-^{3}H)], DL-[1′-^{14}C,3′-*R*-(3′-^{3}H)], and DL-[1′-^{14}C,3′-*S*-(3′-^{3}H)]] or at both the 2′- and 3′-carbons [L-[1′-^{14}C,-Ala-2′,3′-^{3}H]] were fed to all three antibiotic-producing strains, the antibiotics were isolated, and the tritium to carbon-14 ratios were used to determine the fate of these hydrogens in anthramycin, tomaymycin, and sibiromycin.

The results of feeding experiments with tyrosine nonstereospecifically labeled at C-3′ and the two largely stereospecifically labeled tyrosine molecules [DL-[3′-*S*-(3′-^{3}H)] and DL-[3′-*R*-(3′-^{3}H)]] with the anthramycin- and tomaymycin-producing cultures (Table I, experiments 9, 13, and 17 and 10, 14, and 18) as expected failed to show any significant loss of tritium. Therefore, any intermediate requiring loss of either

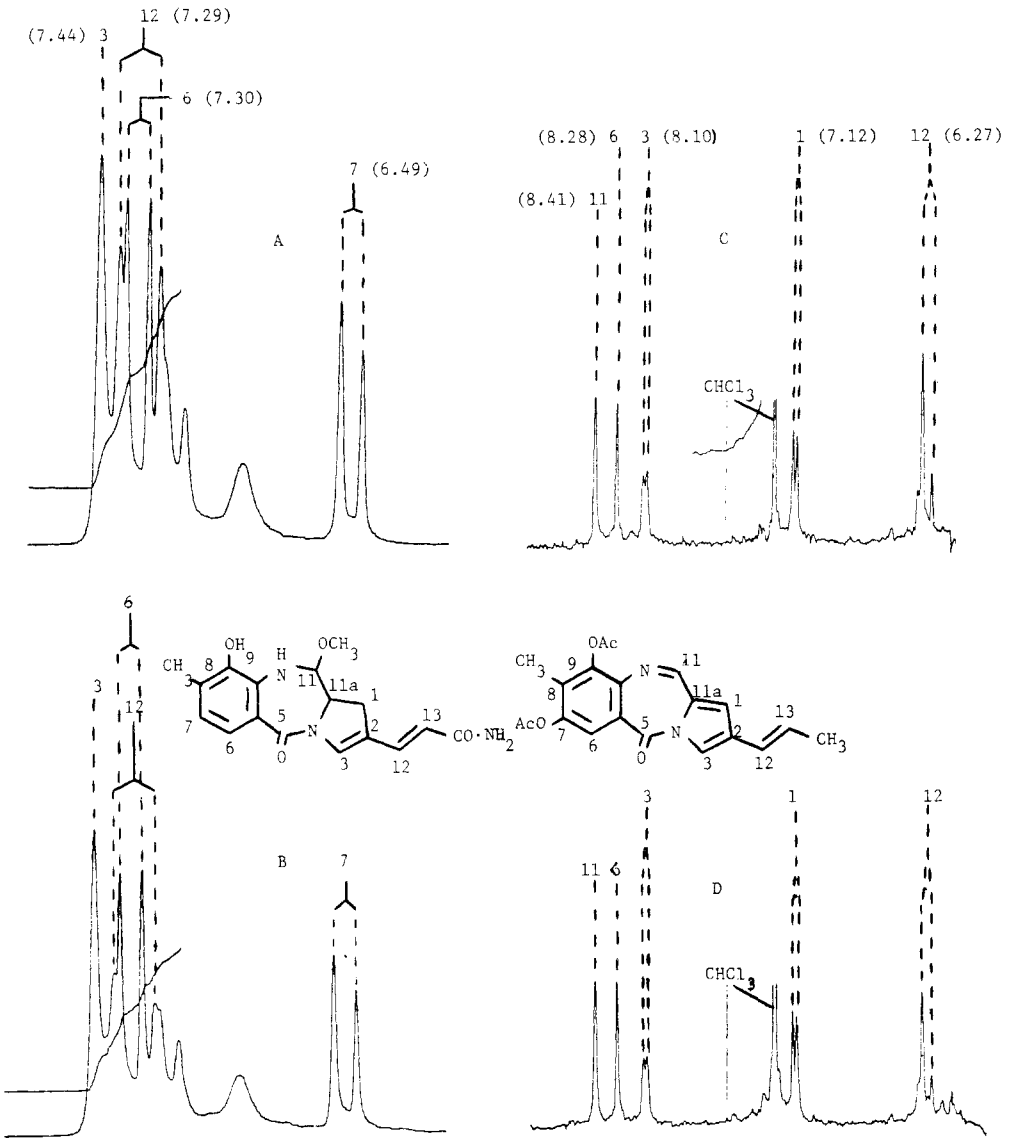


FIGURE 1: ^{1}H NMR spectra of nonisotopically labeled AME (A), AME labeled from L-[2- and 6-$^2H_2$]tyrosine (B) in [*methyl*-$^2H_6$]Me_2SO, and nonisotopically labeled DAS-one (C) and DAS-one labeled from L-[2- and 6-$^2H_2$]tyrosine (D) in C^2HCl_3.

one of these diastereotopic hydrogens can be eliminated from consideration. The equivalent experiments utilizing the same specifically labeled substrates but with the sibiromycin-producing organism showed that appreciable loss of tritium occurred from the DL-[1′-^{14}C,3′-*RS*-(3′-^{3}H)]- and DL-[1′-^{14}C,3′-*S*-(3′-^{3}H)]tyrosine in DAS-one (Table I, experiments 11 and 19). Very little loss occurred from DL-[1′-^{14}C,3′-*R*-(3′-^{3}H)]tyrosine (Table I, experiment 15), in accord with the stereospecific loss of the 3′-(*S*)-hydrogen from tyrosine. The predominant loss of the 3′-(*S*)-hydrogen from tyrosine is analogous to that found in secrurinine, mycelianamide, cryptoschiculine A and the ammonium-lyase reaction (Parry, 1978). The loss of one of the two diastereotopic 3′-hydrogens from tyrosine during its conversion to sibiromycin is further substantiated by an experiment in which L-[3′,3′-$^2H_2$]tyrosine was fed to a culture of *Streptosporangium sibiricum*. The isolated DAS-one showed only species of D_0 and D_1 in the ratio of 89.2 to 10.8 (Table II). Our results with DAS-one isolated from feeding experiments with DL-[1′-^{14}C,3′-*RS*-(3′-^{3}H)]- and DL-[1′-^{14}C,3′-*S*-(3′-^{3}H)]tyrosine (Table I, experiments 11 and 19) show tritium retentions (61 and 34%, respectively) which are higher than that theoretically expected (3′-*RS*, 50%; and 3′-*S*, 15%).[3] The excess tritium retentions for both of these species were reproducible from one experiment to another. As yet no firm basis for this excess tritium retention is known; however, it is possible that some competing reaction, unrelated to sibiromycin biosynthesis, could be selectively enriching the 3′-*S*-(3′-^{3}H) species of tyrosine relative to the 3′-*R*-(3′-^{3}H) species and (1′-^{14}C) species of tyrosine. Such an explanation has been suggested by Johns et al. (1975) to explain the unexpected results found in gliotoxin after feeding DL-[3′,-3′-$^2H_2$]phenylalanine to the fungus *Trichoderma viride*. In this case it was found that gliotoxin derived from [3′,3′-$^2H_2$]phenylalanine is mainly monodeuteriated but also contains some dideuterio species. Hydrogen loss is not therefore obligatory in gliotoxin biosynthesis, but some competing reaction selectively removes the 3′-*pro*-*R*-deuterium atom with retention of configuration.

The fate of the hydrogen at C-2′ of tyrosine in the three antibiotics was determined by using L-[1′-^{14}C,Ala-2′- or 3′-^{3}H]tyrosine since the tritium labeling pattern in this amino

[3] Each of the stereospecifically labeled species is only about 85% stereochemically pure (Parry, 1975).

acid is known (Kirby et al., 1975). First, the tritium is distributed equally between position 2′ and 3′ of tyrosine. Second, the tritium at C-3′ of tyrosine is predominantly in the *pro-S* position (41.5 ± 0.5%) relative to 8.5 ± 0.5% in the *pro-R* position. Since we had demonstrated using the C-3′ labeled species of tyrosine that complete retention of this tritium is found in anthramycin and tomaymycin, then any loss of tritium in these antibiotics from feeding experiments using L-[Ala-2′- or -3′-^{3}H]tyrosine would necessarily have to be from the C-2′ position. The results in Table I (experiments 21 and 22) show an approximate 50% retention of tritium in both anthramycin and tomaymycin; following that complete loss of the tritium from C-2′ of tyrosine occurs during its conversion of these antibiotics.

The result of the feeding experiment with L-[1′-^{14}C,Ala-2′- or -3′-^{3}H]tyrosine with the sibiromycin-producing organism revealed a retention of 19.3% of tritium in DAS-one (Table I, experiment 23). This is again larger than the expected 8.5% based upon the relative distribution of tritium in this species of tyrosine. However, this agrees with our finding in experiments 11 and 19 shown in Table I. As expected, the result with L-[Ala-2′- or -3′-^{3}H]tyrosine predicts the loss of the 3′-(*R*)-hydrogen and the 2′-hydrogen of tyrosine.

Is the nitrogen of tyrosine retained in anthramycin and sibiromycin? The complete loss of the 2′-hydrogen of tyrosine in the pyrrolo[1,4]benzodiazepine antibiotics could be associated with a rapidly equilibrating transaminase washing out the tritium from this position. In order to test this, we fed DL- or L-[^{15}N]tyrosine to the anthramycin- and sibiromycin-producing cultures together with DL- or L-[1′-^{14}C]tyrosine to act as an internal reference label to measure the dilution of tyrosine in these antibiotics. In the case of the anthramycin experiment, as an additional measure of the *intact* incorporation of the amino group with the carbon skeleton of tyrosine, a species containing deuterium (DL-[1′-^{14}C,3- or 5-$^2H_2$,^{15}N]tyrosine labeled intramolecularly with ^{15}N was prepared.[4] The results of experiments using the anthramycin and sibiromycin experiment are shown in Table II. The ^{14}C data indicated *theoretical* enrichments of 31.5 and 28.7% for ^{15}N in anthramycin and sibiromycin *provided the ^{15}N was completely retained*. The deuterium data in the case of anthramycin, measured as DAA, its chemical degradation product, showed an enrichment of 28.0%, i.e., in good agreement with the carbon-14 calculated theoretical enrichment. The mass spectral data on the ^{15}N in DAA and PAA, however, showed only 6.2 and 8.4% enrichments,[5] i.e., 22 and 29% of the comparative enrichments calculated from ^{14}C or ^{2}H data. These experiments therefore reveal that only a partial retention of ^{15}N is found in either case and therefore argue strongly against transamination being entirely responsible for the complete loss of the 2′-hydrogen of tyrosine in anthramycin.

Is L- *or* D-*tyrosine the precursor of the C_2- and C_3-proline units of anthramycin and sibiromycin*? An alternative explanation for the complete loss of the 2′-hydrogen of tyrosine could be a rapid racemization in which L- or D-tyrosine would serve equally well as precursors for these antibiotics. Experiments using L-[2- or 6-^{3}H]tyrosine and D-[2- or 6-^{3}H]-tyrosine, with DL-[1′-^{14}C]tyrosine as a reference label, have been utilized to answer this question. The results (experiments 24–27,[6] Table I) show that in both cases examined (anthramycin and sibiromycin) L-tyrosine serves almost exclusively as the precursor of these antibiotics, thus eliminating racemization as a means to explain the complete loss of the 2′-hydrogen of tyrosine in these cases. It appears, therefore, that the complete loss of the 2′-hydrogen, at least in the case of anthramycin and sibiromycin, is due to reaction in the biosynthetic pathway unrelated to either transamination or racemization of tyrosine.

Formulation of Parallel Biosynthetic Pathways to the C_2- and C_3-Proline Units of the Anthramycin, Tomaymycin, Sibiromycin, and Lincomycin Antibiotics. The overall objective of this detailed examination of the conversion of tyrosine into the C_2- and C_3-proline units of anthramycin, tomaymycin, and sibiromycin was to consolidate the biosynthetic information into a biosynthetic scheme that would accommodate some if not all of these apparent inconsistencies. The biosynthetic pathway shown in Scheme III was proposed, based upon the following assumptions. (1) Proximal extradiol cleavage of Dopa is involved as the common ring cleavage reaction. Neither the distal extradiol cleavage nor the ortho cleavage (pathways b and c in Scheme II) can be completely eliminated from consideration at this time; however, these were considered to be less likely than the proximal extradiol cleavage.[7] (2) Cyclo-Dopa is unlikely to be a common intermediate in the pathway. (3) Divergence of the pathways occurs at the step at which addition of either a CH_3^+ group or a H^+ to a common C_2-proline unit takes place. (4) Formation of the ethylidene methyl group of tomaymycin and the conjugated acrylamide side chain of anthramycin and the unsaturation in the side chain and pyrrolo ring of sibiromycin are cosmetic after events which occur subsequent to the main pathway.

The important features of the main and branch pathways leading to the C_2- and C_3-proline units of the pyrrolo[1,4]-benzodiazepine antibiotics and the lincomycins shown in Scheme III are as follows. (I) Following 2,3-extradiol cleavage of the aromatic ring of Dopa, a condensation reaction to form a Schiff base between the α-amino group and the aldehydic group takes place. (II) The conjugated enol (I) then undergoes enolization to yield the α-keto acid (II) which itself then loses two carbon atoms (carbon atoms 4 and 5 of Dopa) in a stepwise manner to form the diene (III). (III) The diene (III) is considered to be the branch point compound for which a 1,4 addition of H–X results in divergent pathways dependent upon the nature of X^+. If X^+ is H^+, then the pathway leads to the C_2-proline moieties of tomaymycin and lincomycin B, whereas if X^+ is S^+–CH_3, then the pathway leads to the C_3-proline moieties of anthramycin, sibiromycin, and lincomycin A. (IV) Subsequent modification of the first intermediates past the branch point leads to the propylproline unit

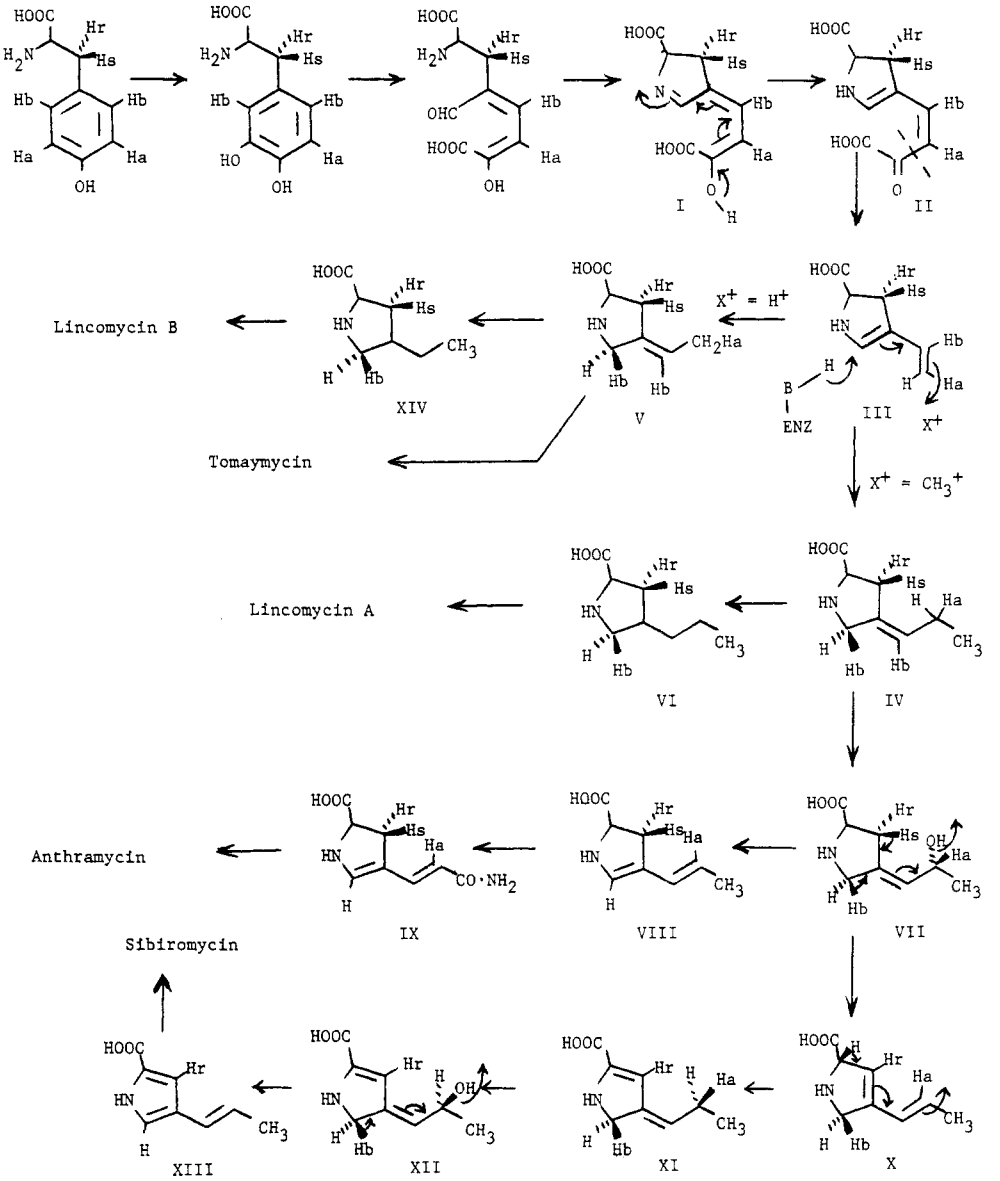
[4] This would not be useful with the sibiromycin experiment since both of these deuterium atoms are lost during biosynthesis of this antibiotic (see Results and Discussion).

[5] It could be argued that the ^{15}N could be equilibrating with the nitrogen pool and subsequently could be transferred back to the same or other tyrosine molecules. However, the complete absence of the $D_1{}^{15}N_2$ species of DAA (Table II) makes this possibility much less likely than the partial retention of ^{15}N from tyrosine in anthramycin.

[6] The company supplier of D-[2- or 6-^{3}H]tyrosine reports that the optical purity was about 97%, and therefore our results reported here are conservative estimates of the much more efficient utilization of L- than D-tyrosine.

[7] Our rationale for this conclusion was based upon the following observations. (a) When substituted catechol derivatives are used as substrates for metapyrocatechase, they are cleaved exclusively at this proximal site by the action of this enzyme (Nozaki et al., 1970); (b) mechanistically, the formation of a Schiff base (I in Scheme V) and its subsequent conversion to the diene (III in Scheme V) are attractive; (c) the presence of a similar yellow compound derived from tyrosine in the acid extract of the broths of all three antibiotic fermentations with spectral properties similar to products of extradiol ring fission of aromatic compounds is supportive evidence for a pathway involving a metapyrocatechase (Hurley, unpublished experiments).

Scheme III: Proposed Parallel Biosynthetic Pathways for the Conversion of Tyrosine into the C_2- and C_3-Proline Moieties of Anthramycin, Tomaymycin, Sibiromycin, and Lincomycins A and B



of lincomycin A (IV → VI), the acrylamideproline unit of anthramycin (IV → IX), the propylideneproline unit of sibiromycin (IV → XIII), and the ethylproline unit of lincomycin B (V → XIV). The ethylideneproline unit of tomaymycin is formed directly from the branch point compound (III → V). Where modifications are required, these would be considered as "cosmetic or after events" which occur subsequent to the main pathway. The cosmetic modifications leading to the lincomycins are straightforward and do not require further comment. For the branch pathways leading to the C_3-proline units of anthramycin and sibiromycin, hydroxylation at the allylic carbon[8] in a stereospecific manner leads to VII. This compound can then undergo a 1,4-conjugate elimination of phosphoric acid in two analogous but different ways (VII → VIII and VII → X).[9] These reactions would result in the stereospecific loss of hydrogen that was originally at C-2 or -6 of tyrosine (anthramycin pathway) or the 3′-*S* position of tyrosine (sibiromycin pathway). The conversion of VIII to IX requires oxidation and amination to produce the acrylamideproline moiety of anthramycin. In the case of the sibiromycin branch, X undergoes an allylic rearrangement to produce XI which is then hydroxylated at the allylic carbon thereby eliminating in a stereospecific manner the hydrogen that was originally located at C-3 or -5 of tyrosine. The product of this reaction, XII, is then able to undergo a second stereospecific 1,4-conjugate elimination of phosphoric acid which leads to loss of the hydrogen originally located at C-2 or C-6 of tyrosine and concomitantly the formation of the desired propylidene side chain of sibiromycin.

A consideration of the details in Scheme III allows us to explain the apparent inconsistencies between results from different antibiotic fermentations.

For example, the 1,4-conjugate elimination reactions (VII → VIII and XII → XIII) peculiar to the anthramycin and sibiromycin pathways explain the loss of the tritium originally located at C-6 of Dopa, which is at least partially retained in tomaymycin.

[8] Allylic hydroxylations carried out by microorganisms have been previously demonstrated (LeMahieu et al., 1970; Tabenkin et al., 1969; Robertson et al., 1978).

[9] Although stereochemical details for these compounds are as yet lacking, a similar postulated mechanism in which a 1,4-conjugate trans elimination involving a two-stage X-group mechanism (Cornforth, 1968) can be found in the chorismate synthetase reaction (Floss et al., 1972; Hill & Newkome, 1969) and might be a model for our suggested examples.

Furthermore, the complete loss of tritium from L-[3- or 5-^{3}H]tyrosine peculiar to the sibiromycin biosynthesis is linked indirectly to the introduction of the extra degree of unsaturation found solely in sibiromycin (11a–1 bond), which requires an additional postulated allylic hydroxylation at C-13 (XI → XII).

Although the proposed biosynthetic pathway shown in Scheme III is probably not the only one capable of explaining our results, we feel its development and existence is an important step in our program to establish a general biosynthetic pathway to these interesting antibiotics.

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Supplementary Material Available

Experimental procedures including fermentations, labeled and nonlabeled compounds, isotope analysis, general techniques, isotope composition, and isolation and purification of AME, TME, DAS-one, DAA, and PPA (3 pages). Ordering information is given on any current masthead page.

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