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Pyrrolo(1,4)benzodiazepine Antitumor Antibiotics: Biosynthetic Studies on the Conversion of Tryptophan to the Anthranilic Acid Moieties of Sibiromycin and Tomaymycin

LAURENCE H. HURLEY* AND CHANDRACHURANAND GAIROLA†

Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Kentucky, Lexington, Kentucky 40506

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Biosynthetic intermediates between tryptophan and the anthranilate moieties of tomaymycin and sibiromycin have been suggested, based upon a combination of feeding experiments with either carbon-14-labeled substrates or competition experiments between radiolabeled tryptophan and unlabeled intermediates. In the case of sibiromycin and tomaymycin, substitution of the aromatic ring most likely takes place at the kynurenine stage. Feeding experiments with the anthramycin culture were inconclusive, most likely because of the cell impermeability.

Anthramycin, tomaymycin, and sibiromycin are structurally related antitumor antibiotics produced by various actinomycetes (1). Previous studies in this laboratory have shown that these compounds are biogenetically related (2, 4; L. H. Hurley, W. L. Lasswell, R. K. Malhotra, and N. V. Das, submitted for publication). Studies with both stable and radioisotopes have demonstrated that the C_3-proline moieties (8-carbon unit) of anthramycin (4) and sibiromycin (Hurley et al., submitted for publication) are derived from tyrosine (7-carbon unit) and methionine (1-carbon unit), whereas the C_2-proline moiety (7-carbon unit) of tomaymycin (2) is derived from tyrosine (7-carbon unit), without an extra C-1 unit from methionine (Fig. 1). Tryptophan is the precursor of the anthranilate units of all three antibiotics (2-4; Hurley et al., submitted for publication). This communication describes the results of experiments designed to determine at which stage the substituents are introduced into the aromatic ring.

MATERIALS AND METHODS

Growth, maintenance, and conditions for production of anthramycin (4), tomaymycin (2), and sibiromycin by *Streptomyces refuineus* NRRL 3143, *Streptomyces achromogenes* ATCC 21353, and *Streptosporangium sibiricum* ATCC 29053 (Hurley et al., submitted for publication), respectively, were as previously described.

Labeled substrates were added to the fermentation broths at times and for incubation periods previously determined to give maximum incorporation into each antibiotic (2, 4; Hurley et al., submitted for publication).

† Present address: Tobacco and Health Institute, University of Kentucky, Lexington, KY 40506.

Competition experiments between tryptophan and possible intermediates between this amino acid and sibiromycin were carried out by transferring 10-ml aliquots of culture broths from a 500-ml shaken flask containing 100 ml of a 30-h-old culture to 125-ml shaken flasks. These flasks were than incubated for an additional 6 h after the addition of DL-[7a-^{14}C]tryptophan and the suspected intermediate. At the end of the incubation period, the beers were extracted with 10 ml of a mixture of methylene chloride containing 8% (vol/vol) of oleic acid. The phases were separated by centrifugation, and the organic phase was extracted twice with 4 ml of a citric acid (0.1 M)-disodium phosphate (0.2 M) buffer (pH 4.0). The pooled extracts were then adjusted to pH 7.8 and extracted three times with 4 ml of $CHCl_3$. The combined extracts were dried over anhydrous sodium sulfate and concentrated under vacuum at 35°C. The residue was redissolved in 3 ml of methanol, and a 0.1-ml aliquot was counted to determine the total radioactivity in the sibiromycin. Another aliquot was chromatographed on a thin-layer chromatographic plate (5 by 20 cm; coated with silica gel F254; Merck) in a chromatography system (ethanol-chloroform, 1:4) together with sibiromycin as a reference (R_f = 0.3). In all cases the sibiromycin was >90% radiochemically pure. The amount of sibiromycin was determined by measurement of the absorption at 310 nm (E = 21,800), and the specific activity (disintegrations per minute per micromole) was then calculated.

Cellular uptake of radiolabeled compounds was determined by taking 0.2-ml samples of culture at various times, rapidly separating the mycelium through a 0.45-nm membrane filter (Millipore), and washing with 2 ml of cold saline solution. The filter paper and attached mycelium were then prepared for radioactivity determination as described previously (2).

DL-[7a-^{14}C]tyrptophan (3.5 mCi/mmol) was obtained from International Chemical and Nuclear; [COOH-^{14}C]anthranilic acid (48 mCi/mmol) came from Research Products International; and 3-hydroxy-

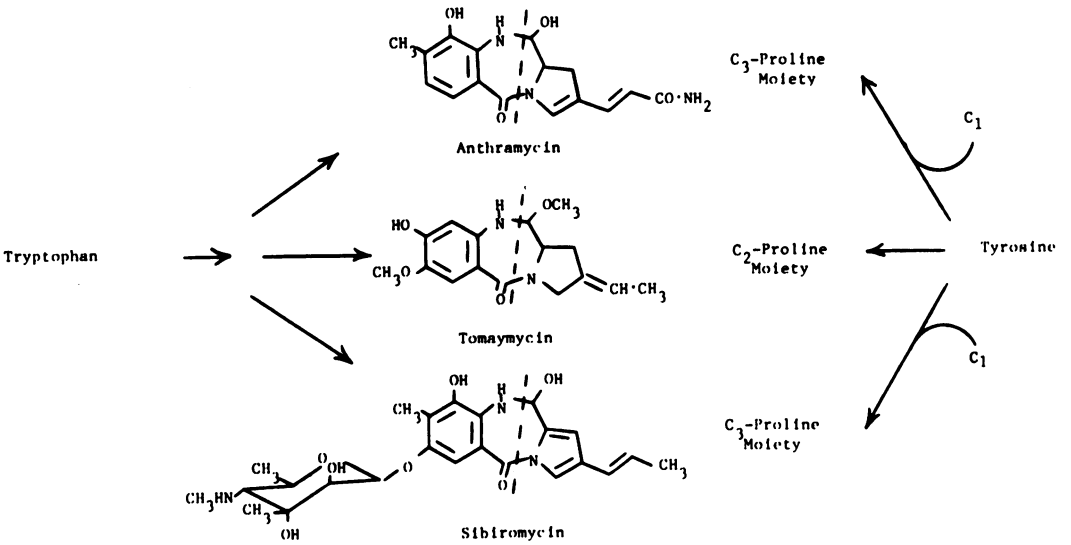

FIG. 1. *Structures and biosynthetic origin of the pyrrolo(1,4)benzodiazepine antitumor antibiotics.*

4-methyl-[2-^{14}C]anthranilic acid (0.063 mCi/mmol) was prepared from [9a-^{14}C]anthramycin, which had been prepared biosynthetically from DL-[7a-^{14}C]tryptophan as described previously (4). 3-Hydroxy-4-methylkynurenine, 3-hydroxykynurenine, and 3-hydroxy-4-methylanthranilic acid were generous gifts from K. L. Perlman (University of Wisconsin), R. K. Gholson (Oklahoma State University), and U. Hornemann (Purdue University), respectively. All other chemicals were obtained from commercial sources.

RESULTS

The results of the feeding experiments with radiolabeled anthranilic acid, 3-hydroxy-4-methylanthranilic acid, and tryptophan are shown in Table 1. Whereas anthranilic acid was efficiently incorporated into tomaymycin, this was almost certainly via tryptophan, since the incorporation into tomaymycin was reduced by greater than 80% when cold tryptophan was added in a parallel feeding experiment. Anthranilic acid and 3-hydroxy-4-methylanthranilic acid failed to be significantly taken up by the anthramycin-producing culture, and therefore their precursor role could not be properly assessed. Anthranilic acid was, however, effectively taken up by the sibiromycin culture (almost 20% of the fed [COOH-^{14}C]anthranilic acid was taken up by the cells in 3 h) but was not detectably incorporated into sibiromycin. On the other hand, 3-hydroxy-4-methylanthranilic acid was well incorporated into sibiromycin.

Since kynurenine, 3-hydroxykynurenine, 3-hydroxy-4-methylkynurenine, and 3-hydroxyanthranilic acid were not readily available in radiolabeled form, experiments were carried out with unlabeled anthranilic acid or kynurenine derivatives in conjunction with DL-[7a-^{14}C]tryptophan to determine which of these compounds are biosynthetic intermediates between tryptophan and sibiromycin. The experiments were designed in such a way that the effect of increasing amounts of the unlabeled compounds on the specific activity of sibiromycin was assessed (Fig. 2). Of the compounds evaluated, kynurenine, 3-hydroxykynurenine, 3-hydroxy-4-methylkynurenine, and 3-hydroxy-4-methylanthranilic acid very effectively reduced the specific activity of sibiromycin. The lesser effect of anthranilic acid on the specific activity of sibiromycin is presumably due to its conversion into tryptophan and subsequent dilution of the radiolabeled trypto-

TABLE 1. *Incorporation of labeled substrates into anthramycin, tomaymycin, and sibiromycin*

Compound fed	Percent incorporation into:		
	Anthramycin	Tomaymycin	Sibiromycin
[COOH-^{14}C]anthranilic acid	<0.1[a]	10.4[b]	<0.1
[COOH-^{14}C]anthranilic acid + DL-tryptophan	NE[c]	1.9[b]	NE[c]
3-Hydroxy-4-methyl-[2-^{14}C]anthranilic acid	<0.1[a]	NE[c]	14.1
DL-[7a-^{14}C]tryptophan	18.0	4.0	8.1

[a] Radiolabeled substrates failed to be taken up significantly by the *S. refuineus* cells in these experiments.

[b] Parallel feeding experiments in which a 100-ml culture of *S. achromogenes* was divided into halves prior to the addition of [COOH-^{14}C]anthranilic acid (4.42×10^6 dpm) to each flask and DL-tryptophan (16.5 μmol) to one of the two flasks.

[c] NE, Not examined.

phan. Significantly 3-hydroxyanthranilic acid has no appreciable effect on the specific activity of sibiromycin.

DISCUSSION

In addition to the pyrrolo(1,4)benzodiazepine antibiotics, actinomycin D also contains an anthranilic acid moiety derived from tryptophan. Perlman et al. (5) have suggested that the derivatization of the actinomine moiety of actinomycin takes place at the kynurenine stage, based upon competition experiments and the data obtained with washed cells and a cell-free extract which was able to convert 3-hydroxy-4-methylkynurenine to 3-hydroxy-4-methylanthranilic acid. Our results reported here suggest that the 5-sibirosaminide 3-hydroxy-4-methylanthranilic acid moiety of sibiromycin is derived in an analogous manner. Based upon the results with sibiromycin and actinomycin D, it is also possible that the 3-hydroxy-4-methylanthranilic acid moiety of anthramycin is derived in a similar fashion, although efforts to demonstrate this have failed, most likely due to impermeability of the cells to these compounds.

Since anthranilic acid is not an immediate precursor of tomaymycin but is incorporated via tryptophan, hydroxylation of the aromatic ring again may take place at the kynurenine level. Based upon previous results with DL-[5-^{3}H, 7a-^{14}C]tryptophan, in which it was found that the incorporation of this substrate into tomaymycin occurred with a 14% retention of tritium, and a knowledge of N.I.H. shift rules, we have suggested that the main pathway involves hydroxylation at C-8 prior to hydroxylation at C-7 (3). Figure 3 represents a postulated general scheme for the biosynthesis of anthranilic acid moieties found in these antibiotics.

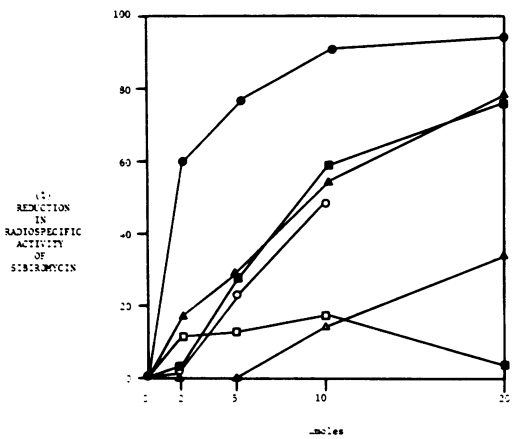

FIG. 2. *Effect of various compounds on the specific activity of sibiromycin labeled with* DL-*[7a-*^{14}*C]tryptophan.* DL-*[7a-*^{14}*C]tryptophan (2.5 x 10*^{6} *dpm) was added to 100-ml aliquots of a 30-h culture of S. sibiricum. Symbols: 3-hydroxy-4-methylanthranilic acid (●); kynurenine (■); 3-hydroxykynurenine (▲); 3-hydroxy-4-methylkynurenine (○); 3-hydroxyanthranilic acid (□); anthranilic acid (△).*

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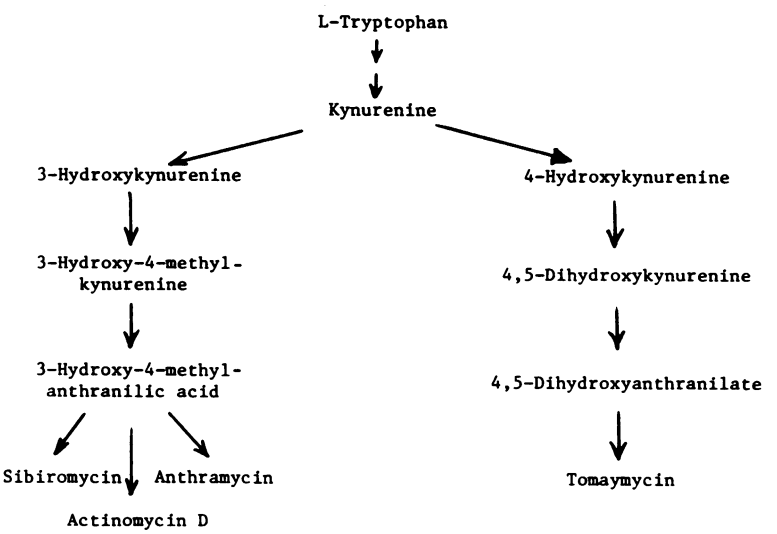

FIG. 3. *Proposed general pathway for the conversion of tryptophan to the anthranilate moieties of various antibiotics.*

We are grateful to U. Hornemann, R. K. Gholson, and K. L. Perlman for samples of 3-hydroxy-4-methylanthranilic acid, 3-hydroxykynurenine, and 3-hydroxy-4-methylkynurenine, respectively.

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