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THE BIOSYNTHETIC ORIGINS OF REBECCAMYCIN[1]

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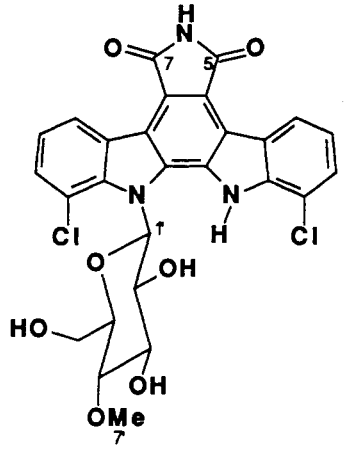
ABSTRACT.—Experimental evidence is presented to demonstrate that the antitumor-antibiotic rebeccamycin is biosynthesized by *Saccharothrix aerocolonigenes* from one unit of glucose, one of methionine, and two of tryptophan. Evidence is presented that suggests that the α-amino group of neither tryptophan unit provides the nitrogen of the phthalimide system.

Rebeccamycin [**1**] is an antitumor secondary metabolite isolated from cultures of *Saccharothrix aerocolonigenes* (1). The structure was obtained using a combination of spectroscopic approaches (2) and X-ray analysis (2) and by synthesis (3). It consists of a novel halogenated indolocarbazole chromophore to which is attached a 4-*O*-methylglucose via an *N*-glycosidic linkage. Other natural products containing a similar chromophore include staurosporine, isolated from a *Streptomyces* (4), and the arcyriaflavins isolated from the slime mold *Arcyria denudata* (5). Rebeccamycin was found to have significant activity against P388 leukemia, L1210 leukemia, and B-16 melanoma implanted in mice and inhibited the growth of human lung adenocarcinoma cells (1). Reports indicate that rebeccamycin produces single-strand breaks in DNA (1), and in a modified form it is currently being considered for clinical evaluation as a potential treatment for cancer in humans.

Rebeccamycin, together with staurosporine and the arcyriaflavins, represents a unique chemotype that until now has not been investigated biosynthetically. Knowledge of the biosynthesis of rebeccamycin would be useful to exploit fermentation possibilities leading to novel products and would also provide routes to isotopically labeled drugs for further investigation. Biogenetic analysis suggests that the indolocarbazole moiety is a tryptophan metabolite. In this respect, it should be noted that the some-



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what related antibiotic violacein was recently shown to be derived from two tryptophan units (6). Furthermore, the chromophore could be derived from two identical tryptophan metabolites with the phthalimide nitrogen originating from a different source, or there could be an intermediate in which the phthalimide nitrogen is derived from the tryptophan that provides either the left or the right portion of the compound. The most obvious precursors for the remainder of the molecule are glucose and methionine.

RESULTS AND DISCUSSION

Initial experiments were designed both to identify biosynthetic precursors and to optimize the extent of incorporation of these into rebeccamycin. Using the procedure described in the Experimental section, a series of *S. aerocolonigenes* cultures were prepared and ^{14}C-labeled precursors were added to these at various times. Rebeccamycin was isolated following 7 days incubation by extracting the mycelial cake with THF, and the concentrated extract was further purified by hplc (see Experimental section). The results summarized in Table 1, which represent the incorporation observed under optimal conditions, demonstrate that D-glucose, L-methionine, and L-tryptophan are efficiently incorporated. In order to determine the labeling pattern the following experiments were performed.

TABLE 1. Carbon Isotope Incorporation into Rebeccamycin.

	Labeled Precursor[a]					
	Glucose[b]		Methionine[b]		Tryptophan[c]	
	[U-^{14}C]	(1-^{13}C)	[^{14}Me]	(^{13}Me)	[3-^{14}C]	(2-^{13}C)
% Incorporation	1.58	na[d]	4.24	na	9.77	na
Positions Enriched . . .	na	1′	na	7′	na	5, 7
Enrichment Factor . . .	na	5.2	na	44	na	3.4, 4.2

[a]For each pair of carbon-labeled precursors a comparable feeding regimen was used.
[b]Added to a 3-day-old culture.
[c]Added to a 2-day-old culture.
[d]na, not appropriate.

This feeding approach was repeated using D-[1-^{13}C]glucose, DL-[methyl-^{13}C] methionine, or DL-[2-^{13}C]tryptophan. The resulting antibiotic was purified using the vacuum flash chromatography (7,8) technique described rather than by hplc. The labeling pattern was deduced using ^{13}C-nmr analysis, and the significantly enriched carbons are given in Table 1. These results confirm those obtained using radiolabeled precursors and demonstrate incorporation without extensive scrambling.

To determine the source of the phthalimide nitrogen the following experiment was performed. A defined medium for rebeccamycin production was prepared using $(^{15}NH_4)_2SO_4$ as the sole nitrogen source. After 3 days incubation, [2-^{13}C] tryptophan was added to the medium. Following a further 4 days incubation, the antibiotic was extracted, purified, and analyzed using proton-decoupled ^{13}C nmr. The spectrum shows signals for C-5 and C-7 as both singlets and doublets (Figure 1). The doublets A and B of Figure 1 correspond to C-5 and C-7, respectively, which are enriched 3- to 4-fold and result from incorporated ^{13}C label being adjacent to ^{15}N. The minor singlets a and b from C-5 and C-7, respectively, are from ^{13}C which is not coupled to ^{15}N. This pattern is consistent with ^{13}C and ^{15}N enrichment in the same molecule; thus, the majority of molecules that are ^{13}C-enriched at position 5 also contain ^{15}N at position 6, the phthalimide system, and similarly for C-7. Because the only source of ^{13}C-enrichment

is tryptophan and the only source of ^{15}N is the $(^{15}NH_4)_2SO_4$, and both are simultaneously incorporated into the same molecule, this excludes the possibility that the α-amino group of the added tryptophan is incorporated into rebeccamycin. These results do not exclude the possibility that the α-amino group of added tryptophan is exchanged with ^{15}N from the medium. Incorporation of the resulting doubly labeled tryptophan would give the results observed. For this to occur, the exchange process would have to be rapid in order to compete with the incorporation of tryptophan, which is added during idiophase at a time of maximal rebeccamycin biosynthesis and efficient tryptophan utilization. Because it is unlikely that all incorporated tryptophan will exchange the α-amino group with the nitrogen pool in the medium, incorporation of an amount of ^{14}N should occur. If the phthalimide nitrogen is from tryptophan, then it would be predicted that one of the enriched adjacent carbons be more frequently attached to ^{14}N. Depending upon the extent of this, it may be expected that peaks a and b of Figure 1 would show different intensities, which they do not. This argument assumes there is no symmetrical intermediate.

Thus, rebeccamycin is derived from two molecules of tryptophan, one of glucose and one of methionine. Glucose and methionine are probably incorporated via UDP-glucose and *S*-adenosyl methionine, respectively. Tryptophan is probably incorporated

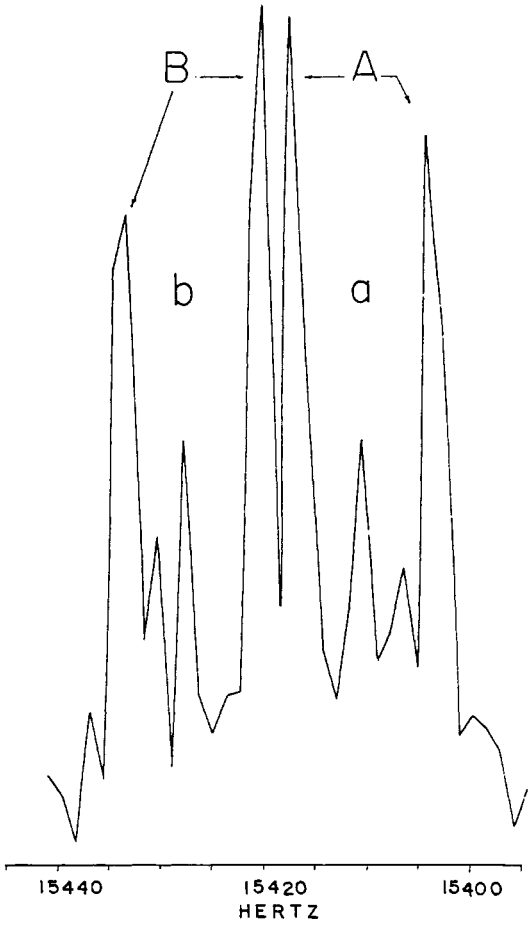

FIGURE 1. Expanded nmr spectrum showing the signals for C-5 and C-7. The pattern is explained in the text.

following deamination to yield indolepyruvic acid. In this respect it is worth noting that the microbial metabolism of L-tryptophan and its 7-chloro derivative to indolecarboxylic acid, via the indolepyruvyl and acetyl derivatives, has been reported (9). In addition it is interesting to note that indolepyruvic acid has been shown to be an intermediate between tryptophan and indolmycin (10). It seems a reasonable speculation that derivatives such as these could be involved in rebeccamycin biosynthesis. Further experiments on the enzymes involved in rebeccamycin biosynthesis are underway.

EXPERIMENTAL

CULTURE OF *S. AEROCOLONIGENES*.—*S. aerocolonigenes* (ATCC 39243) was grown in a medium containing cerelose 3%, Pharmamedia 1%, Nutrisoy 1%, and $CaCO_3$ 0.3%, at 28° and 250 rpm on a gyrorotary shaker. After 2 days, 3-ml aliquots were transferred to a 500-ml Erlenmeyer flask containing 100 ml of a rebeccamycin-production medium prepared using soluble starch 1%, $(NH_4)_2SO_4$ 0.25%, K_2HPO_4 0.2%, $MgCl_2$ 0.2%, and $CaCO_3$ 0.2%. This was also incubated at 28° on a rotary shaker at 250 rpm.

REBECCAMYCIN ISOLATION.—Approximately equal volumes of dicalyte and whole fermentation broth were mixed and stirred for 30 min. This was filtered using a scintered glass filter, and the pellet was washed with H_2O. The dicalyte-mycelial cake was placed in a beaker together with approximately two volumes of THF and stirred for 60 min. This was filtered through Whatman no. 1 filter paper, and the yellow filtrate containing rebeccamycin was concentrated. Rebeccamycin with purified further using either an hplc approach (in the case of radioactive experiments) or using vacuum flash chromatography (for experiments involving stable isotopes).

Hplc purification was achieved using a Waters hplc system with a C_{18} column and 0.1 M ammonium acetate-MeOH-MeCN (4:3:3) as solvent. Rebeccamycin was detected at 313 nm using a uv detector.

Vacuum flash chromatography (7,8) was used to purify stable isotope-labeled rebeccamycin. The THF extract from these experiments was mixed with 1 g Si gel H, Merck 10-40, and solvent removed to adsorb the rebeccamycin onto the Si gel. This was placed on the top of a short column of Si gel comprising 5 g Si gel in a 15-ml M porosity Kontes scintered glass funnel. This was successively developed using 50-ml aliquots of increasing concentration of EtOAc in hexane, rebeccamycin eluting as a sharp band with 60% EtOAc.

ISOTOPICALLY-LABELED PRECURSORS.—D-[U-^{14}C] Glucose (10 mCi/mmol), L-[side chain-3-^{14}C] tryptophan (51.8 mCi/mmol), and L-[methyl-^{14}C]methionine (40 mCi/mmol) were obtained from New England Nuclear, Boston, and DL-tryptophan-2-^{13}C (99.7 atom % ^{13}C), DL-[methyl-^{13}C] methionine (90 atom % ^{13}C), D-[1-^{13}C] glucose (99.5 atom % ^{13}C), and $(^{15}NH_4)_2SO_4$ (99 atom % ^{15}N) were from MSD Isotopes, Canada. Precursors were added to cultures as filtered-sterilized solutions at the times indicated. Radioactivity was determined using liquid scintillation counting employing aquasol cocktail and a Beckman counter. Samples for nmr analysis were dissolved in DMSO-d_6, and spectra were obtained on a Bruker WM-360 spectrometer.

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