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Identification of indolepyruvic acid as an intermediate of rebeccamycin biosynthesis

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SUMMARY

Experimental evidence is presented to demonstrate that indolepyruvic acid is an intermediate in the rebeccamycin biosynthetic pathway. [3-^{14}C]Indolepyruvic acid was prepared and efficiently incorporated (8%) into rebeccamycin by *Saccharothrix aerocolonigenes*.

INTRODUCTION

Rebeccamycin (Fig. 1) is an antitumor agent isolated from cultures of *Saccharothrix aerocolonigenes* [2]. Its structure, consisting of a novel halogenated indolocarbazole chromophore N-glycosylated with 4-*O*-methylglucose, was elucidated using a combination of spectroscopic methods [16] and by total synthesis [8] and confirmed by X-ray analysis [16]. Other natural products containing a similar chromophore include staurosporine [5], k-252a, b, c, d [10,15,26], UCN-01 and UCN-02 [24,25], and arcyriaflavins [22]. The biosynthesis of this type of chromophore has recently been investigated. We demonstrated that rebeccamycin is derived from one unit of glucose, one of methionine, and two of tryptophan [18]. The biosynthesis of a related compound, staurosporine was reported by Meksuriyen and Cordell [13,14], who showed that the aglycone moiety of staurosporine was derived from two units of tryptophan. In this communication, we present evidence that indolepyruvic acid (IPA) is a precursor for rebeccamycin biosynthesis.

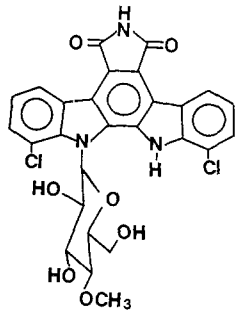

Fig. 1. The structure of rebeccamycin.

MATERIALS AND METHODS

Microorganism. The rebeccamycin-producing culture, *S. aerocolonigenes*, had been deposited with the American Type Culture Collection with the accession number ATCC 39243.

Media and culture conditions. Strain ATCC 39243 was grown on slants of yeast-malt extract agar. This medium consisted of 4 g glucose, 4 g yeast extract, 10 g malt extract, 1.5 g $CaCO_3$ and 15 g agar in 1 liter of distilled water (pH 7.6 before sterilization). To prepare an inoculum for the shake flask culture, surface growth from a 5-day-old culture was transferred to a 500-ml Erlenmeyer flask containing 100 ml of medium consisting of 30 g glucose, 10 g soy flour, 10 g cottonseed embryo meal and 3 g $CaCO_3$ in 1 l of distilled water (pH 7.5 before sterilization). This vegetative culture was incubated at 28 °C and 250 rpm on a rotary shaker. After 48 h, 3-ml aliquots were transferred to a 500-ml Erlenmeyer flask containing 100 ml of a rebeccamycin-production medium prepared using 10 g soluble starch, 2.5 g L-threonine, 1 g $MgCl_2$, 2 g KH_2PO_4 and 2 g $CaCO_3$ in 1 l of distilled water (pH 7.0 before sterilization). The production culture was incubated at 28 °C and 250 rpm for 6 days to obtain the titer of 25–30 μg/ml.

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Preparation of [3-^{14}C]IPA. [3-^{14}C]IPA was prepared using a modified version of the procedure published by Badenoch-Jones et al. [1]. One μmol of [3-^{14}C] tryptophan (50 μCi, New England Nuclear) was dissolved in 0.2 mM potassium phosphate buffer (pH 7.6, 10 ml) containing 500 U catalase (from dog liver, 900–1000 U/mg, Sigma Chemical Co.). Two units of L-amino acid oxidase (from *Crotalus atrox* venom, 1–3 U/mg, Sigma Chemical Co.) were added and the mixture was incubated at 37 °C for 1 h. The mixture was acidified to pH 3.0 and extracted 3 times with an equal volume of ethylacetate. The ethylacetate phase was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure at a temperature less than 30 °C. The dried extract, containing [3-^{14}C]IPA, was stored at − 80 °C until use.

Extraction and analytical methods. The fermentation extract was processed by stirring approximately equal volumes of dicalite and whole fermentation broth for 30 min. This was filtered through a sintered glass filter and the pellet was washed with distilled water. The dicalite mycelial cake was placed in a beaker together with approximately 2 volumes of tetrahydrofuran and stirred for 60 min. This was filtered through Whatman no. 1 filter paper, and the yellow filtrate containing rebeccamycin was concentrated. The analysis of rebeccamycin in fermentation extracts was performed by HPLC using a C-18 reversed-phase column (μ-Bondapak, 3.9 × 300 mm, Waters Associates). The solvent system was ammonium acetate (0.1 M)/methanol/acetonitrile (4 : 3 : 3, v/v/v) at a flow rate of 1.5 ml/min with the detector wavelength set at 313 nm. For determining the amount of radioactivity incorporated into rebeccamycin, the concentrated extract was injected into HPLC and the fraction containing rebeccamycin was collected and counted for radioactivity using Aquasol cocktail and a Beckman scintillation counter.

RESULTS

Inhibition of [3-^{14}C]tryptophan incorporation into rebeccamycin by indole metabolites

The aglycone of rebeccamycin is derived from two molecules of tryptophan [18]. The formation of aglycone from tryptophan is probably the initial step of rebeccamycin biosynthesis. We were interested in determining which other indole metabolites could be incoporated into rebeccamycin since this may lead to the identification of intermediates between tryptophan and rebeccamycin. Preliminary experiments were based on the assumption that if the exogenously added indole metabolite is a pathway intermediate, it would compete with [3-^{14}C]tryptophan incorporation into, and thus reduce the specific activity of rebeccamycin. Five indole metabolites were tested, with IPA exhibiting the highest dilution of [3-^{14}C]tryptophan incorporation into rebeccamycin (Table 1). IPA inhibited the incorporation of [3-^{14}C]tryptophan into rebeccamycin by 79%, followed by indoleacetaldehyde (36%), indoelacetamide (19%) and indoleacetic acid (4%). Tryptamine did not show any inhibition effect.

Incorporation of [3-^{14}C]IPA into rebeccamycin

[3-^{14}C]IPA was prepared as described in MATERIALS AND METHODS. The purified [3-^{14}C]IPA was analyzed by reversed-phase HPLC and showed one single peak with both the same retention time (9.7 min) and UV spectrum as the authentic IPA standard (Fig. 2). The conversion yield of [^{14}C]tryptophan to [^{14}C]IPA was 97%.

The efficiency of incorporation of [3-^{14}]tryptophan, [3-^{14}C]IPA and [2-^{14}C]tryptamine was compared (Table 2). The ^{14}C-metabolites (1 μCi) were added to the cultures at 48 h. In this experiment, 8.9% of added [3-^{14}C]tryptophan was incorporated into rebeccamycin, compared with 8.3% of added [3-^{14}C]IPA. [2-^{14}C]Tryptamine was not incorporated into rebeccamycin.

TABLE 1

Inhibition of [3-^{14}C]tryptophan incorporation into rebeccamycin by indole metabolites

Indole metabolite	Rebeccamycin production (μg/ml)	Specific activity of rebeccamycin (dpm/mg)	% Inhibition
None (Control)	27.2 ± 0.7	84360 ± 1146	–
IPA	28.1 ± 1.4	17546 ± 1912	79.2 ± 2.3
Indoleacetaldehyde	25.8 ± 0.7	54159 ± 2062	35.8 ± 2.4
Indoleacetamide	27.4 ± 0.9	68669 ± 1119	18.6 ± 1.3
Indoleacetic acid	26.6 ± 1.0	80732 ± 1024	4.3 ± 1.2
Tryptamine	30.5 ± 1.3	84933 ± 1065	0

Indole metalobites and [3-^{14}C]tryptophan (1 μCi) were added to the cultures of *S. aerocolonigenes* (100 ml) at 48 h. The final concentration of the indole metabolites in the culture was 0.05 mM.

TABLE 2

Incorporation of [3-^{14}C]IPA into rebeccamycin

^{14}C-Precursor	Rebeccamycin production (μg/ml)	Total radioactivity in rebeccamycin (dpm)	Specific activity of rebeccamycin (dpm/mg)	% Incorporation
[3-^{14}C]Tryptophan	27.9 ± 1.2	1.98 ± 0.13 × 10^5	70968 ± 1495	8.9 ± 0.6
[3-^{14}C]IPA	26.3 ± 1.7	1.85 ± 0.18 × 10^5	70342 ± 2075	8.3 ± 0.8
[2-^{14}C]Tryptamine	27.7 ± 0.8	0	0	0

Radiolabeled precursors (1 μCi) were added to the cultures of *S. aerocolonigenes* (100 ml) at 48 h.

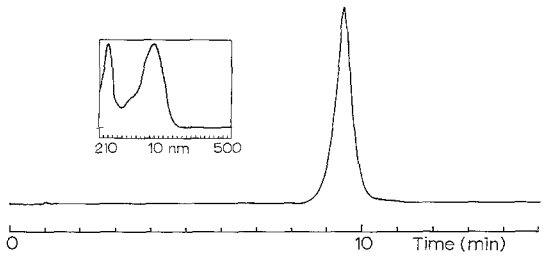

Fig. 2. HPLC chromatogram of [3-^{14}C]IPA. Approximately 5 μg of [3-^{14}C]IPA (prepared by the procedures described in MATERIALS AND METHODS) was injected onto a C-18 reversed-phase column (μ-Bondapak, 3.9 × 300 mm, Waters Associates) and eluted with the solvent system of ethanol: water (3 : 7, v/v) containing 0.12% acetic acid, at a flow rate of 1 ml/min and the detector wavelength set at 294 nm.

Inhibition of incorporation of [3-^{14}C]IPA into rebeccamycin by tryptophan

The inhibition of the incorporation of [3-^{14}C]IPA into rebeccamycin by tryptophan was examined (Table 3). At 0.05 mM added tryptophan concentration, 58% inhibition of [3-^{14}C]IPA into rebeccamycin was observed.

TABLE 3

Inhibition of [3-^{14}C]IPA incorporation into rebeccamycin by tryptophan

Tryptohan (mM)	Rebeccamycin production (μg/ml)	Specific activity of rebeccamycin (dpm/mg)	% Inhibition
0	28.7 ± 0.7	76615 ± 2150	–
0.05	26.5 ± 1.2	31948 ± 1466	58.3 ± 1.9
0.25	27.4 ± 1.7	11186 ± 1050	85.4 ± 1.4
0.50	24.2 ± 0.6	11722 ± 1236	84.7 ± 1.6

[3-^{14}C]IPA (1 μCi) and various amounts of tryptyphan were added to the cultures of *S. aerocolonigenes* (100 ml) at 48 h.

Note that at the same concentration of 0.05 mM, IPA exhibited a significantly greater effect (79%) on [3-^{14}C]tryptophan incorporation into rebeccamycin (Table 1). The inhibition of [3-^{14}C]IPA into rebeccamycin was greatest (85%) when added together with 0.25 mM tryptophan.

DISCUSSION

IPA is an unstable compound and it is easily broken down to form indoleacetic acid, indoleacetamide and indoleacetaldehyde but not tryptophan [1,9]. From Table 1, the inhibition of the incorporation of [3-^{14}C]tryptophan into rebeccamycin by these breakdown products was much less than IPA, although they still may be considered potential biosynthetic intermediates. Even with its instability, we demonstrated that [3-^{14}C]IPA was incorporated into rebeccamycin as efficiently as [3-^{14}C]tryptophan. Based on the data from the inhibition of incorporation of [3-^{14}C]IPA into rebeccamycin by tryptophan (Table 3) and the specific activity of rebeccamycin produced by [3-^{14}C]IPA (Table 2), we conclude that IPA is an intermediate of rebeccamycin pathway.

The presence of IPA as an intermediate between tryptophan and antibiotic is not without precedent. IPA had been shown to be an intermediate between tryptophan and indolmycin in *Streptomyces griseus* [7,21]. In indolmycin biosynthesis, tryptophan is transaminated to IPA which is subsequently methylated to form 3-methyl-IPA. Aromatic amino acid transaminases have been reported for a number of microorganisms such as *Escherichia coli* [3,19], *Rhizobium leguminosarium* [6] and a rhizobacterial isolate from *Festuca octoflora* [4], *Agrobacterium tumefaciens* [23], *Clostridium sporogens* [17] and *Pseudomonas* species [11,12,20]. We are in the process of identifying the transaminase activity in the cell free extract of *S. aerocolonigenes*.

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