PREPARATION AND PURIFICATION OF [^{14}C] LABELED N-DEMETHYL LINCOMYCIN AND PROPYLPROLINE FROM [^{14}C] TYROSINE

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SUMMARY

Propylproline and N-demethyl-lincomycin, two intermediates in lincomycin biosynthesis, were prepared in radiolabeled form. [^{14}C]-labeled N-demethyl-lincomycin was synthesized by a resting cell suspension of a lincomycin negative mutant of Streptomyces lincolnensis that was fed methylthiolincosaminide and uniformly labeled [^{14}C]-tyrosine. [^{14}C]-N-demethyl-lincomycin was isolated from the culture medium by ion exchange chromatography and reverse phase-ion pair high pressure liquid chromatography. [^{14}C]-propylproline was generated by the base hydrolysis of [^{14}C]-N-demethyl-lincomycin and isolated by anion exchange chromatography. The products had radiochemical purities greater than 95% and specific activities of 5.9 mCi/mmole.

Key Words: [^{14}C]N-demethyl-lincomycin, [^{14}C]propylproline, Streptomyces lincolnensis, high pressure liquid chromatography.

INTRODUCTION

Previous biosynthetic studies have suggested that the last two steps of lincomycin biosynthesis involve the coupling of propylproline (PPL) and methyl-thiolincosaminide (MTL) to produce N-demethyl-lincomycin (N-deMe-L) followed by the N-methylation of N-deMe-L to produce lincomycin A (L_A) (1). One way to examine these putative enzyme activities is to follow the incorporation of radioactively labeled substrate into the product. This report describes the preparation of two [^{14}C]-labeled lincomycin intermediates, PPL and N-deMe-L, from commercially available [^{14}C]-tyrosine for use in the study of lincomycin biosynthesis.

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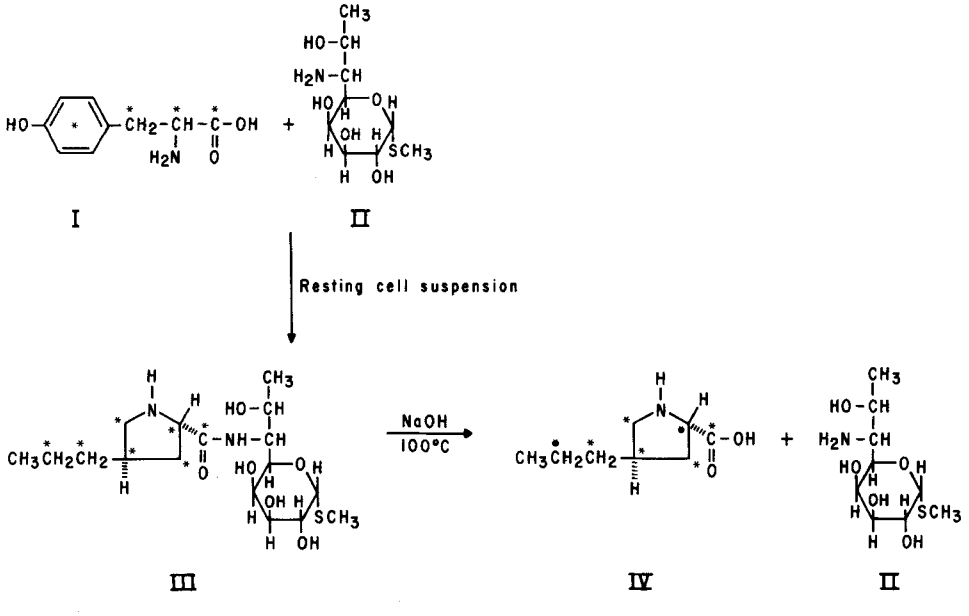
MATERIALS

L-[^{14}C-(U)]-tyrosine (493 mCi/mmole) was purchased from New England Nuclear. Propylproline, N-demethyl-lincomycin and methylthiolincosaminide were obtained from The Upjohn Company. High pressure liquid chromatography (HPLC) solvents were purchased from Burdick and Jackson Laboratories. Milli Q-UF (Millipore) reagent water was used throughout the experiments. All other chemicals were purchased commercially and used without further purification. Thin layer chromatography was performed with silica gel GF, 250 microns (Analtech). HPLC was carried out on a Varian 5010 liquid chromatograph equipped with a variable wavelength detector (No. 1840, ISCO). Radioactivity was measured with a Packard Tri-Carb liquid scintillation counter.

SYNTHETIC PATHWAY



I: L-tyrosine II: methylthiolincosamide III: N-demethyl-lincomycin IV: propylproline

* indicates position of [^{14}C] label

EXPERIMENTAL

Bacterial Strain

A non-sporulating lincomycin negative mutant was found to accumulate neither PPL nor MTL during the fermentation. Thin layer chromatography of the culture medium did not indicate either compound with the following conditions, silica gel GF developed with MeOH:$CHCl_3$:NH_4OH (35:61:4) and visualized using an iodine vapor. When MTL was added to the fermentation to a final concentration of 1 mg/ml, substantial amounts of N-deMe-L accumulated in the medium as well as minor amounts of L_A and L_B. This appears to indicate that lesions exist in both the MTL biosynthetic pathway and in the final N-methylation step.

Preparation of ^{14}C-N-demethyl-lincomycin

The fermentation conditions were as described previously (3). After a total of 4 days of incubation in the fermentation medium, the flask was cooled on ice and the cells harvested by centrifugation at 1000 xg for 5 minutes. The mycelial layer was removed and washed three times by centrifugation with cold 10mM potassium phosphate buffer, pH 7.0. The washed mycelial pellet was resuspended to the original volume in 50mM potassium phosphate buffer, pH 7.0 containing 100mM glucose, 5mM $MgCl_2$ and 10mM K_2SO_4. The spin volume of the suspension was 25% when centrifuged for 15 minutes at 1000 xg.

Twenty ml of the cell suspension was added to a 125 ml flask that contained 1.3 ml of a MTL solution (15 mg/ml) and 10 µCi of L-[^{14}C-(U)]-tyrosine (S.A. = 493 mCi/mmole) in 100 µl of 2% ethanol in H_2O. The flask was incubated at 28°C in a gyratory water bath shaker (400 rpm, G76, New Brunswick Scientific). The flask was removed after one hour and cooled in an ice bath to stop the reaction.

The contents of the flask were centrifuged for 5 minutes at 1000 xg. The supernatant was saved. Ten ml of water was used to wash the mycelial pellet by centrifugation. This supernatant was combined with the previous supernatant to give a total volume of 27 ml. The solution was made basic by the addition of 540 µl of 58% NH_4OH and the mixture allowed to stand for 15 minutes in an ice bath.

The small amount of precipitate that formed was removed by centrifugation (5 minutes, 1000 xg). The solution was loaded onto an anion exchange column of AG 1-X2, OH- form, 200-400 mesh, 1.5 ml bed volume (Bio-Rad) that had been equilibrated with 0.5% (w/v) NH_3. The eluant and 5 ml of a 0.5% (w/v) NH_3 rinse of the column were collected. The combined solution (32 ml) was loaded by gravity feed onto a SEP-PAK C_{18} cartridge (Waters) that had been previously treated with three washes: 10 ml of CH_3CN, 10 ml of H_2O and 5 ml of 0.5% (w/v) NH_3. An additional 5 ml of 0.5% (w/v) NH_3 was used to rinse the column after loading. The [^{14}C]-N-deMe-L was eluted with 5 ml of a H_2O:CH_3CN mixture (3:1). This solution was taken to dryness with N_2 and the residue redissolved in the HPLC mobile phase. The [^{14}C]-N-deMe-L was finally purified by reverse phase-ion pair chromatography (Figure 1). N-deMe-L was observed to have a k' of 11 in this system. Thirty drop fractions were collected from the column and examined for [^{14}C]-N-deMe-L by

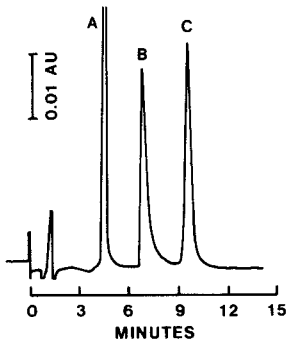


Figure 1: Reverse phase-ion pair HPLC separation of lincomycin. Peaks: A=lincomycin B, B=N-demethyl-lincomycin A, C=lincomycin A. Column: Zorbax C-8 (Dupont), 4.6x150 mm. Mobile phase: 10 mM K-PO_4, pH 7.0:CH_3CN (8:2) plus 5 mM Na 1-octanesulfonate. Flow rate: 2 ml/min. UV detection at 200 nm.

counting 3 µl of each fraction in 5 ml of toluene:Triton X-100 (2:1) solution containing 5.5 grams of Permablend III (Packard). Appropriate fractions were combined and taken to dryness with a stream of N_2. In order to remove the ion pair agent, the residue was taken up in 1.0 ml of water and acidified with 2N HCl. The sample was loaded onto a cation exchange column of AG 50W-X4, H^+ form, 200-400 mesh, 1.0 ml bed volume (Bio-Rad). The column was washed with 10 ml of

H_2O and then eluted with 10 ml of 0.5% NH_3. The first two ml of the ammonia elution were discarded and the next 8 ml collected in a single vial. This solution was taken to dryness and redissolved in H_2O. Determination of radiochemical purity of [^{14}C]-N-deMe-L was carried out using the same HPLC conditions with a 50 µl sample loop and counting the radioactivity in the collected fractions.

[^{14}C]-N-demethyl-lincomycin (0.5 µC) that had a radiochemical purity of greater than 95% was obtained by this method. Incorporation of the radiolabel from [^{14}C]-tyrosine into N-deMe-L was 10%. The isolation yield of the [^{14}C]-N-deMe-L was 50%.

Preparation of ^{14}C-Propylproline

[^{14}C]-PPL was prepared by base hydrolysis of [^{14}C]-N-deMe-L. 0.4 µCi of [^{14}C]-N-deMe-L were dissolved in 800 µl of H_2O and added to a one ml Reacti-Vial (Pierce) with 120 µl of 50% NaOH and a spine vane. The vial was capped tightly and heated to 100°C in an oil bath for 3 hours with stirring. The entire reaction mixture was loaded onto an anion exchange column AG 1-X2, OH- form, 200-400 mesh, 1.0 ml bed volume (Bio-Rad) equilibrated with 0.01N NaOH. The column was washed with 10 ml of H_2O. [^{14}C]-PPL was eluted with 0.1N HCl and 1 ml fractions were collected. PPL eluted in fractions 7 and 8. The radiochemical purity of PPL was determined by thin layer chromatography on silica gel GF developed with MeOH-$CHCl_3$:NH_4OH (35:61:4); PPL was visualized using an iodine vapor. R_f values of 0.36 and 0.69 were observed for PPL and N-deMe-L, respectively. Radioactivity was determined by scraping zones of the TLC plates into scintillation vials and counting the vials after addition of scintillation cocktail. Quantitation of propylproline was determined by derivatization with phenylisothiocyanate using a Waters PICO-TAG system.

This method produced 0.36 µCi of [^{14}C]-propylproline with a radiochemical purity greater than 95%. The yield was 90% and the specific activity was determined to be 5.9 mCi/mmole.

DISCUSSION

Tyrosine has been shown to be a precursor of lincomycin by examining the incorporation of radioactive and stable isotopes into lincomycin (2,3). Specifically the label is incorporated into the propylhygric acid (PHA) moiety of L_A. Previous studies have shown that seven of the nine carbons of tyrosine are incorporated into PHA (2). The gamma carbon of the propyl chain as well as the N-methyl group are derived from methionine (4). Consequently all but two of the carbons in PHA are labeled when starting from uniformly labeled tyrosine. Therefore, it was expected that feeding this strain [^{14}C(U)]-tyrosine in the presence of MTL would produce [^{14}C] labeled N-deMe-L. The short incubation time reported here, one hour under nongrowing conditions, reduces the total incorporation of [^{14}C]-label from tyrosine into N-deMe-L but enhances the specific activity. Since this mutant exhibits little MTL biosynthetic ability and since substantial amounts of exogenous MTL were added (0.9 mg/ml), little if any [^{14}C]-label is expected in the MTL moiety of [^{14}C]-N-deMe-L. Therefore, it is assumed that the specific activity of the [^{14}C]-N-deMe-L is the same as the [^{14}C]-PPL. This assumption is further supported by the 90% radioactive yield during the base hydrolysis of [^{14}C]-N-deMe-L to produce [^{14}C]-PPL.

The isolation of [^{14}C]-N-deMe-L is straightforward. The use of reverse phase-ion pair HPLC is important to isolate N-deMe-L from two other similar products, lincomycin A and B. The chromatographic conditions reported give good separation of all three compounds (Figure 1). The inclusion of the ion pair agent dramatically increases the k' of N-deMe-L from 5 to 11 with little effect on either L_A or L_B.

Base hydrolysis of [^{14}C]-N-demethyl-lincomycin to produce radiolabeled [^{14}C]-propylproline proved to be a relatively easy and high yielding procedure.

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