ISOLATION AND IDENTIFICATION OF 3-PROPYLIDENE-Δ^1-PYRROLINE-5-CARBOXYLIC ACID, A BIOSYNTHETIC PRECURSOR OF LINCOMYCIN

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An accumulated lincomycin intermediate in UC 8292, a lincomycin nonproducing strain of *Streptomyces lincolnensis*, has been isolated and purified by employing an assay system based on complementation of UC 11066, another lincomycin nonproducing strain of *S. lincolnensis*. The structure of the purified intermediate is shown to be 3-propylidene-Δ^1-pyrroline-5-carboxylic acid, or 1, 2, 3, 6-tetradehydro-propylproline by mass spectrometry and NMR spectroscopic studies. Based on the structure of this newly found intermediate, a biosynthetic pathway for propylproline is proposed as tyrosine→L-3-hydroxytyrosine (Dopa)→→→→3-propylidene-Δ^1-pyrroline-5-carboxylic acid→3-propyl-Δ^2-pyrroline-5-carboxylic acid→propylproline.

It has been shown previously that *Streptomyces lincolnensis* strain UC 8292 (originally designated NTG-3), a lincomycin nonproducer, is incapable of synthesizing lincomycin cosynthetic factor (LCF), a deazariboflavin[1,2]. Lacking this deazariboflavin, UC 8292 cannot synthesize propylproline and consequently lincomycin. In this paper, we report that a previously unidentified lincomycin intermediate in UC 8292 has been isolated and purified by employing an assay system based on complementation of *S. lincolnensis* strain UC 11066 (originally designated IIc-2-3-3)[3], another lincomycin nonproducer.

Materials and Methods

Bacterial Strains

Two mutants of *S. lincolnensis* blocked in the biosynthesis of lincomycin were used in this study, UC 8292, a riboflavin auxotroph which does not produce LCF necessary for the biosynthesis of lincomycin[1,2], and UC 11066, a mutant which does not produce propylproline, an intermediate in the lincomycin biosynthetic pathway. The latter strain was obtained from a derivative of *S. lincolnensis* strain UC 5124 by transposon mutagenesis with the *Streptomyces fradiae* transposon Tn*4560*[3].

Fermentation Media and Conditions

The media and fermentation conditions for shake flask fermentations were described previously[2]. Twenty liter fermentations were carried out in the same media at 29°C with an agitation speed of 700 rpm and an aeration rate of 20 liters per minute. Both flasks and tanks were harvested at 4 days.

Assay for Lincomycin Biosynthetic Intermediates (LBI)

In an effort to find a nonproducing strain of *S. lincolnensis* capable of complementing UC 8292, several other strains, including UC 11066, were tested in cross-feeding experiments. Fermentations of all strains were carried out in 500 ml shake flasks and supernatants and mycelia mixed in all combinations at 96 hours. Mixed test flasks were incubated for an additional 24 hours and assayed for lincomycin activity. Addition of UC 8292 supernatant to mycelia of UC 11066 also resulted in lincomycin production indicating that the genetic lesion in UC 11066 precedes the block in UC 8292. Based on these results, *S. lincolnensis*

strain UC 11066 was selected for use in an assay for accumulated LBI in UC 8292 fermentations. This assay procedure is very similar to that used to detect LCF[1,2]. Fractions to be assayed were lyophilized, resuspended in 1 ml of sterilized water and added into frozen UC 11066 assay flasks. The flasks were incubated for 18 hours on a rotary shaker at 28°C and assayed for lincomycin using a *Micrococcus luteus* disc-plate agar assay[4].

Spectroscopic Methods

NMR spectra were recorded on a Bruker AM-300 spectrometer operated at 300 MHz and 75 MHz for the proton and carbon experiments, respectively. Spectra were run in deuterated water and reported as ppm relative to tetramethylsilane. Two dimensional ^{1}H-^{1}H correlation (COSY) and ^{1}H-^{13}C correlation spectra were obtained by the standard sequences. FAB-MS were obtained on a MAT CH-5 spectrometer operated in the positive ion mode.

Isolation of LBI

For the isolation of LBI produced by UC 8292 in 20 liters tank fermentations, a series of chromatography procedures were performed. Briefly, the clear beer was adjusted to pH 3 with HCl and passed over a 3 liters column of Dowex 50WX2(Na^+) cation exchange resin. The column was washed with water and 0.5 M NH_4OH. Fractions containing LBI were determined by drying 10 ml of every 20th fraction (25 ml each) and adding the residue to UC 11066 fermentations as described above. LBI containing fractions were pooled and passed over a 500 ml column of Dowex 1X4 (Cl^-). The resin was washed with water and eluted with 0.1 M HOAc. Five ml aliquots from each fraction were assayed in the UC 11066 fermentation as before. The LBI containing fractions were pooled and passed over an Diaion HP-20 column. After washing with water, a step-gradient of MeOH - H_2O was used to elute the column. LBI containing fractions (1 ml aliqout per fraction was assayed) were pooled, concentrated *in vacuo* and loaded to a 250 ml DEAE Sephadex A-25 column. Elution of the DEAE column was with a step-gradient of NaCl in water. A 0.5 ml aliquot from each fraction was assayed. Fractions with LBI were combined and loaded to another HP-20 column for desalting. The column was eluted with a slow gradient of H_2O - MeOH. The LBI containing fractions were pooled and dried for structure analysis.

TLC Analyses

TLC analyses were performed on 250 μm silica plates (Analtech, Ann Arbor, MI) developed with a *n*-BuOH - Acetone - HOAc - H_2O (35 : 35 : 10 : 20) system. To visualize compounds eluted from columns, TLC plates were sprayed with ninhydrin reagent and heated on a hot plate for 10 minutes.

Results and Discussion

Isolation of LBI

The isolation procedures for LBI from fermentation broths of UC 8292 were detailed in the Materials and Methods section. Briefly, LBI was purified by a series of chromatographies over Dowex 50X2, Dowex 1X4, HP-20, DEAE Sephadex A-25 and a second HP-20 columns. The levels of LBI in column fractions were determined by the amounts of lincomycin produced when dried aliquots of fractions were added to fermentations of UC 8292. The concentration of LBI was progressively higher after each chromatography as evidenced by the fact that increasingly smaller amount of sample were needed to produce approximately the same level of lincomycin in the UC 11066 fermentation assay. The purity of column fractions was assessed by TLC analysis and NMR spectroscopy. During early purification procedures, phenylalanine was identified as the major component in fractions active in the UC 11066 assay. However, after the 1st HP-20 chromatography, fractions eluting between 30～50% MeOH were shown to contain a number of components in low levels along with phenylalanine. After DEAE chromatography, the major components were found to be phenylalanine and a new compound which has a lower Rf value than that of phenylalanine.

This new compound gives a yellow color after reacting with the ninhydrin reagent instead of the pink color observed for phenylalanine. Significantly, the amount of this new compound, when added to fermentations of UC 11066, was found to correlate with the amount of lincomycin produced. The addition of phenylalanine under the same conditions did not result in lincomycin production. The second HP-20 chromatography finally removed the majority of phenylalanine from the above mentioned mixture.

Structure Elucidation of LBI

The molecular formula of LBI was deduced as $C_8H_{11}NO_2$ by high resolution FAB-mass spectrometry ($(M+H)^+$, found 154.0869, theory 154.0868). The one-dimensional ^1H and ^{13}C NMR spectra results are listed in Table 1. The 2D ^1H-^1H COSY spectrum revealed that protons 6, 7 and 8 are coupled in sequence, while protons 4 and 5 are coupled to each other. Furthermore, the COSY spectrum also indicated that protons 6 and 4 are long-range coupled to each other. This long-range coupling also was indicated in the one-dimensional spectrum where peaks due to H-4 and H-6 showed coupling constants in the neighborhood of 1.5 Hz. Based on chemical shift considerations, structure 1 can be deduced.

The NMR data also suggests that LBI contains an imine (C-2) and a carboxylic acid group (C-9). This is consistent with the yellow color observed after with the ninhydrin staining and the amphoteric character of LBI exhibited during isolation. Thus, every element of the molecular formula has been accounted for. Furthermore, from the molecular formula, it was deduced that LBI has four unsaturation equivalents and therefore is a monocyclic compound. That the imino group can be placed between C_3 and C_5 to form a pyrroline ring was indicated by the chemical shift values of C-3, H-5 and C-5. Finally, the carboxyl group was placed at C-5 as it has the only and the last available valency. The structure of LBI is, therefore, elucidated as 3-propylidene-Δ^1-pyrroline-5-carboxylic acid, or 1, 2, 3, 6-tetradehydro-propylproline (**I**).

Biosynthesis of Lincomycin

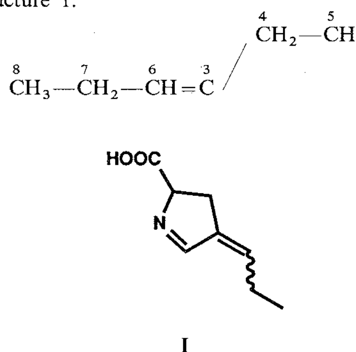
In a previous study[5], propylproline biosynthesis was shown to go through the 2,3-extradiol cleavage pathway of L-3-hydroxy-tyrosine (L-Dopa). A series of reactions (Scheme 1A) leading to propylproline and ethylproline from L-Dopa were suggested by BRAHME *et al.* In view of our findings, Scheme 1A should be modified. After the formation of the enamine, methylation or protonation of the enamine forms an α,β-unsaturated imine, (**I**) (see Scheme 1B). Scheme 1B is still consistent with all the labeling experiments published to date.

Knowing that **I** is the substrate of a reductase which requires LCF as a cofactor allows us to speculate

Table 1. ^1H and ^{13}C NMR data of **I**.

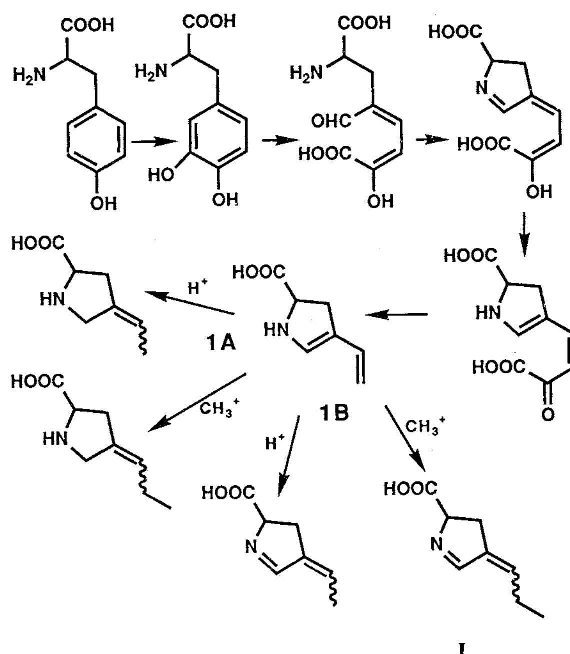
	^1H Chemical shift (multiplicity)		^{13}C Chemical shift (multiplicity)	
1	—		—	
2	8.7	(S)	157	(D)
3	—		136	(S)
4	2.95, 2.30	(DD)	29	(T)
5	5.20	(DD)	66	(D)
6	7.10	(T)	128	(D)
7	2.35	(Quintet)	25	(T)
8	1.05	(T)	12	(Q)
9	—		172	(S)

Structure 1.

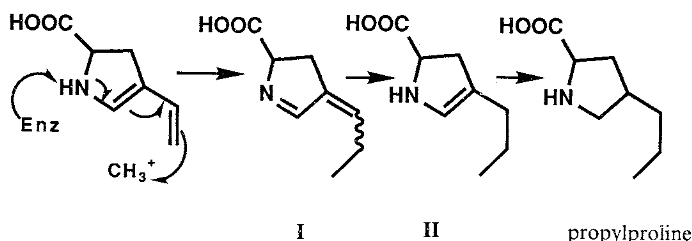


Scheme 1. Biosynthesis of ethyl- and propyl-proline precursors.

1A: Proposed by BRAHME *et al.*[1] 1B: Modified pathway based on this work.



Scheme 2. Proposed biosynthetic pathway of propylproline.



on the biosynthetic steps leading to propylproline. LCF was previously reported[6] as a cofactor in the enzymatic reduction of the chlortetracycline precursor to chlortetracycline. The two electron reduction was carried out *via* the 1,4 addition of hydrides to the α,β-unsaturated ketone. In our case, the hydrides may be added to the α,β-unsaturated imine *via* 1,4 addition. Thus, we propose that the enamine (**II**) (3-propyl-Δ^2-pyrroline-5-carboxylic acid) is the immediate precursor of propylproline (Scheme 2).

In conclusion, we have isolated 3-propylidene-Δ^1-pyrroline-5-carboxylic acid and identified it as a lincomycin intermediate accumulating in UC 8292 fermentations. Based on the structure of **I**, a modified propylproline biosynthetic pathway is proposed as Tyr→L-Dopa→→3-propylidene-Δ^1-pyrroline-5-carboxylic acid→3-propyl-Δ^2-pyrroline-5-carboxylic acid→propylproline→lincomycin.

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