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Note

Assay of methylthiolincosaminide in fermentations by high-performance liquid chromatography with fluorescence detection

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Lincomycin[1], a clinically important antibiotic produced by *Streptomyces lincolnensis*, consists of a unique aminooctose moiety, α-methylthiolincosaminide (MTL), attached via an amide linkage to a propylhygric acid unit. Biosynthetically, it has been proposed that MTL is coupled to propylproline to form N-demethyllincomycin which is then N-methylated to become lincomycin (Fig. 1)[1]. The existence of MTL and its precursors in the pathway was inferred from labeling studies using α-D-[$^{13}C_6$]glucose. Our intention in developing an assay for MTL was to demonstrate the presence of MTL in fermentation beers of *S. lincolnensis* and to provide a quick, sensitive and specific method to aid our studies of lincomycin biosynthesis.

Since MTL does not possess a chromophore for detection and since fermentation broths contain a rich abundance of primary and secondary metabolites, unconsumed media and cellular debris, derivatization of MTL with a fluorescent tag and high-performance liquid chromatography (HPLC) separation of the derivatized MTL from the other broth constituents was employed. In the method described below, we used 4-chloro-7-nitrobenzofurazan[2] as the derivatizing agent based on a number of considerations discussed in the text.

EXPERIMENTAL

Fermentation

The lincomycin producing culture, *Streptomyces lincolnensis* UC® 5124 was grown on Hickey and Tresner agar (BBL, Cockeysville, MD, U.S.A.) and stored above liquid nitrogen as agar plugs. The seed flasks (100 ml medium in 500-ml wide mouth Erlenmeyer flasks) were inoculated with four agar plugs of the stock culture and incubated at 28°C for $2\frac{1}{2}$ days on a rotary shaker. The seed medium consisted of yeastolac (Vico Products, Decatur, IL, U.S.A.; 10 g/l), NZ-amine B (Sheffield Chemical, Norwich, NY, U.S.A.; 5 g/l) and glucose monohydrate (cerelose; 20 g/l). Presterilization pH of the medium was adjusted to 7.2. The fermentation flasks (100 ml medium in 500-ml wide mouth Erlenmeyer flasks) were inoculated with 5 ml of the

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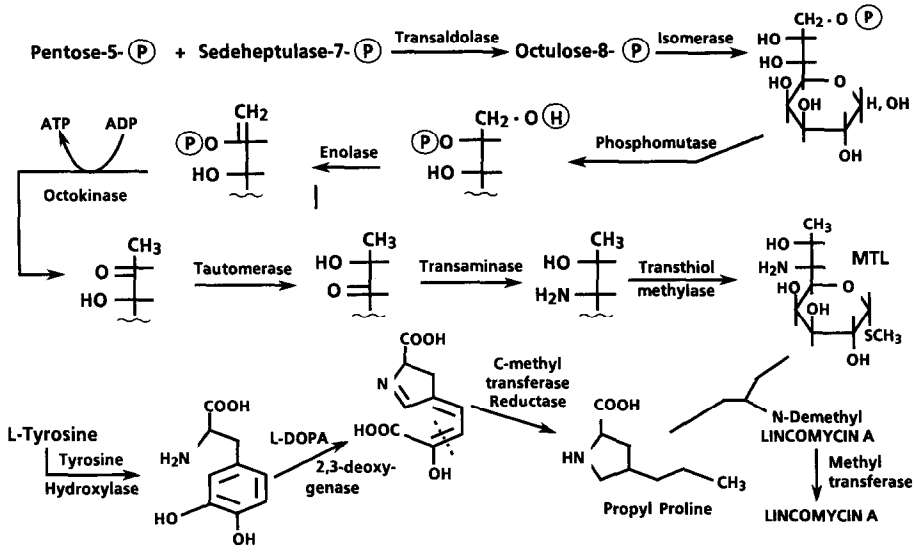

Fig. 1. Proposed biosynthetic pathway to lincomycin A.

seed culture and incubated at 28°C on a rotary shaker for 4 days. Fermentation beers were then pooled and kept frozen until use. The production medium F-13 consisted of glucose monohydrate (15 g/l), black strap molasses (Pacific Molasses, San Francisco, CA, U.S.A.; 20 g/l), hi-starch (Lauhoff Grain, Danville, IL, U.S.A.; 40 g/l), cotton seed hydrolysate (Pharmamedia, Traders Oil Mill, Fort Worth, TX, U.S.A.; 25 g/l), calcium carbonate (8 g/l), potassium sulfate (2 g/l) and antifoam (Ucon LB625, Union Carbide; 3 drops per 100 ml). Presterilization pH was adjusted to 7.2.

Derivatization

Whole beer was diluted 1:1 with water and samples were sonicated (Fisher sonicator, small tip, setting of 25 for 5 min) in centrifuge tubes on ice to break open cells. The pH was adjusted to 8.5 with 10 μl 1 *M* sodium hydroxide per ml beer. The sonicated mixture (400 μl) was placed in an autosampler vial and 100 μl of 1 *M* sodium hydrogencarbonate and 500 μl of a 30 mg 4-chloro-7-nitrobenzofurazan per ml methanol stock solution were added. Sodium hydroxide and sodium hydrogencarbonate were obtained from Mallinckrodt while 4-chloro-7-nitrobenzofurazan was purchased from Aldrich. Vials were sealed and heated in a water bath at 60°C for 4–6 h. Vials were shaken periodically to mix. Samples were then cooled on ice and filtered prior to injection. For testing the linearity and recovery from beer, the final reaction volume was made to contain 0, 2, 4, 10, 20 and 40 μg MTL per ml by adding appropriate amounts of a 0.2 mg MTL per ml stock solution to the water used to dilute the whole beer prior to sonication. (MTL was obtained by cleavage of lincomycin with base[1].) To determine the efficiency of derivatization, water was substituted for beer. The precision was determined by derivatizing nine replicates of a beer sample spiked with MTL to yield a concentration of 10 μg MTL per ml in the final reaction volume.

Chromatography

HPLC was performed using a Varian 8055 autosampler that filled a 10-μl loop on a Varian 5500 HPLC system. Fluorescent detection was performed by an EM Science Model F1000 fluorescence detector set at an excitation frequency of 420 nm and an emission frequency of 525 nm. The signal was integrated by a Hewlett-Packard 3392A integrator. An Alltech Econosphere cartridge column with a guard cartridge was utilized to provide the separation (C_{18}, 5 μm, 250 × 4.6 mm I.D.). Gradient elution was used employing a linear ramp from 10 to 33% methanol and 90 to 67% 2.2 m*M* NaH_2PO_4, pH 6.9, in 5 min followed by a linear gradient from this point to a 50:50 mixture at 20 min. A wash with 100% methanol for 10 min followed by an equilibration for 8 min at starting conditions was employed to recycle the column prior to the next injection.

RESULTS AND DISCUSSION

Assay of samples as complex as a fermentation beer requires great selectivity in the method of identification of the compound of interest. Fluorescence derivatization offers this selectivity as well as high sensitivity. The choice of the derivatization reagent must also consider the complexity of the sample and be as specific as possible to the compound of interest. The MTL amino group lends itself to derivatization with many fluorescent tags. 4-Chloro-7-nitrobenzofuran was selected as the reagent of choice since it produces highly fluorescent products only on reaction with amino groups resulting in less background fluorescence and a smaller hydrolysis product peak. A final 4-chloro-7-nitrobenzofuran concentration of 15 mg/ml in the reaction vial was determined to give the most effective derivatization of added MTL. Since the reaction produces hydrochloric acid as a by-product, a concentration of 0.1 *M* sodium hydrogencarbonate was necessary to maintain the slightly alkaline conditions necessary for derivatization.

The reaction was complete after 4 h at 60°C with no change up to 6 h. Variation in the observed peaks occurred with the fermentation organism, media used and date of sampling. However, the MTL peak is still resolved from any large interferents observed thus far. The HPLC conditions may have to be modified for individual fermentations. Buffer concentration and pH are used to modify the chromatogram in the reversed-phase mode. Gradient elution provides the separation with good peak shape. Fig. 2A shows a chromatogram of the derivatization of 10 μg/ml of MTL in water. Fig. 2B is an elution of a derivatized beer sample spiked with 10 μg MTL per ml. Fig. 2C is a blank beer sample. Fig. 3 is the HPLC trace for another lincomycin producing strain that has a larger amount of MTL. Many small peaks are seen to elute in the vicinity of the MTL derivative in the beer sample. This puts the lower limit on the detection of MTL in fermentation beers at 1 μg/ml.

A linear relationship ($r^2 = 0.9999$) was obtained for the assay of 0–40 μg MTL per ml in aqueous solution. The linear regression equation was $y = 1.60 \cdot 10^6 x - 3.58 \cdot 10^4$. The precision of the assay was determined for beer with added MTL. Nine samples spiked with MTL to give a final concentration of 10 μg/ml were assayed. A value of 8.44 ± 0.14 μg/ml relative standard deviation (R.S.D.) was obtained. The accuracy was tested by adding MTL to beer samples in the concentration range 0–40 μg/ml. The recovery was 73 ± 1.7% (R.S.D.). Regression analysis showed a linear

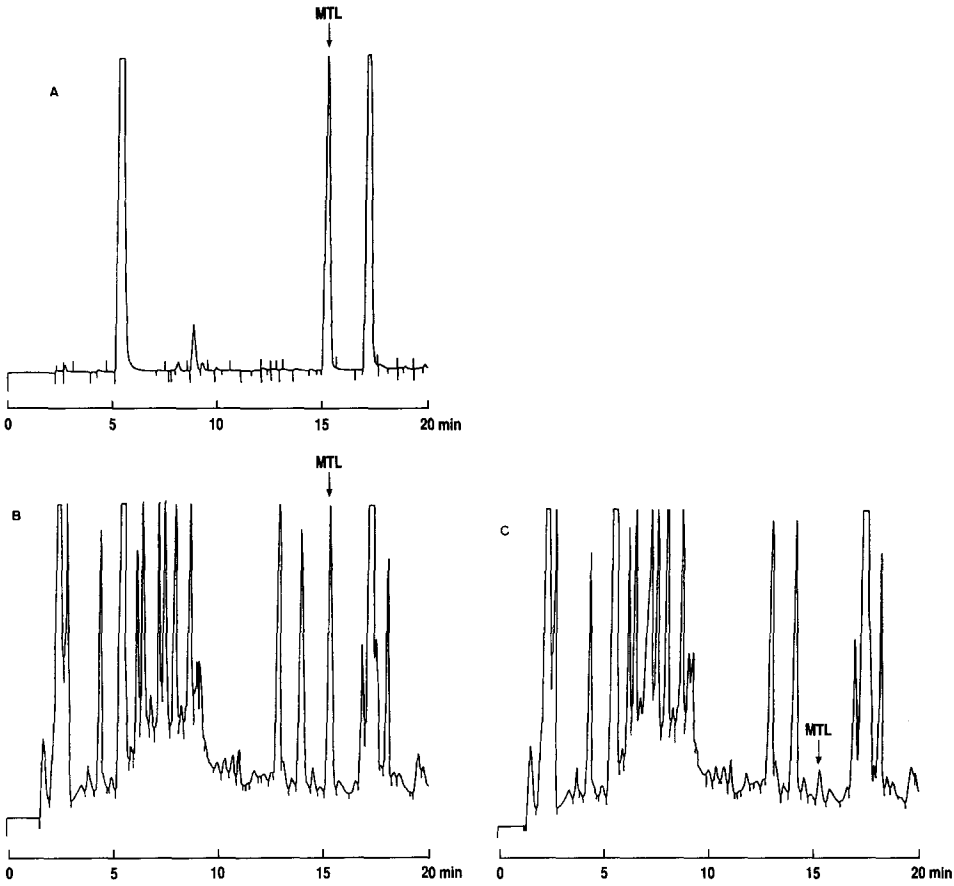

Fig. 2. (A) HPLC trace of an MTL sample in water after derivatization. Gradient elution from 10 to 33% A and 90 to 67% B in 5 min followed by a gradient to A–B (50:50) in 15 min. Solvent A, methanol; solvent B, 2.2 m*M* NaH_2PO_4, pH 6.9. (B) HPLC trace of a beer sample with added MTL after derivatization. Elution conditions as in (A). (C) HPLC trace of a derivatized beer sample. Elution conditions as in (A).

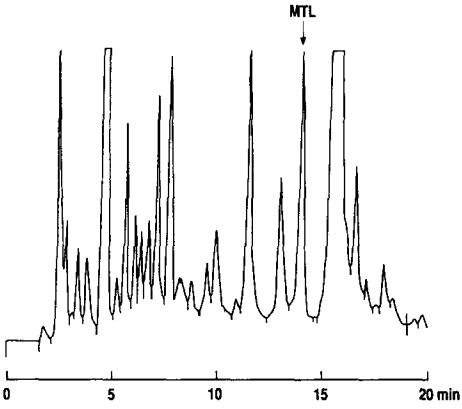

Fig. 3. HPLC trace of derivatized beer sample from another lincomycin producing strain. Elution conditions as in Fig. 2A.

relationship ($r^2 = 0.999$) between added and recovered MTL: $y = 0.742x - 0.74$. Since the recovery is not 100%, a standard curve generated in the fermentation beer must be used to determine the MTL concentration in a beer sample. A standard curve for MTL added to whole beer is linear ($r^2 = 0.999$) with an equation of: $y = 1.18 \cdot 10^6 x + 1.07 \cdot 10^6$. The y-intercept reflects the amount of MTL in an unspiked fermentation and is equivalent to 0.8 μg MTL per ml in the derivatization mixture or 4 μg/ml in the fermentation broth.

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