Biosynthesis of Puromycin in \textit{Streptomyces alboniger}

ENZYMATIC METHYLATION OF \textit{O-DEMETHYLPUROMYCIN}*

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SUMMARY

An enzyme has been found in soluble extracts of a puromycin-producing culture of \textit{Streptomyces alboniger} which catalyzes the transfer of the methyl group from \textit{S-adenosyl-L-methionine-methyl-14C} to \textit{O-demethylpuromycin}. Properties of this system are presented. The product has been characterized as puromycin, and the labeled methyl group has been shown to be located solely in the \textit{O-methyl-L-tyrosine} moiety. A convenient synthesis of \textit{O-demethylpuromycin} is described.

Puromycin, the antibiotic and antitumor agent excreted by \textit{Streptomyces alboniger}, has the structure shown in Diagram 1 (2). On the assumption that the addition of methyl groups to the tyrosine and adenine moieties might be terminal enzymatic steps in the biosynthesis of this compound, we decided to study the methylation of possible precursors of puromycin. Evidence is presented in the present communication for the enzymatic conversion of \textit{O-demethylpuromycin} and \textit{S-adenosyl-L-methionine} to puromycin as shown in Diagram 1.

EXPERIMENTAL PROCEDURE

\textit{Materials}

\textit{S-Adenosyl-L-methionine-methyl-14C} was obtained from New England Nuclear; puromycin \textit{HCl}, puromycin aminonucleoside, Woodward's reagent \textit{K}, and protamine sulfate from Nutritional Biochemicals; \textit{O,N-dicarbobenzoxy-L-tyrosine} from Miles Laboratories, Elkhart, Indiana; 10\% palladium charcoal from Matheson Coleman and Bell; silica gel (100 to 200 mesh) from Davison Chemical Company, Baltimore; sucrose from Mann; bovine serum albumin from Armour; \textit{S-adenosyl-L-methionine}, \textit{L-tyrosine ethyl ester}, \textit{L-tyrosine amide}, and \textit{L-epinephrine bitartrate} from Sigma; \textit{N⁶,N⁸-dimethyladenine} and \textit{N⁴-methyladenine} from Cyclo; \textit{L-tyrosine} from Calbiochem. Catechol (Matheson Company, East Rutherford, New Jersey) was recrystallized from toluene. \textit{O-Methyl-\textit{L-tyrosine HCl}} and \textit{3-amino-3-deoxy-D-ribose-HCl} were generous gifts from Lederle Laboratories (Division of American Cyanamid Company, Pearl River, New York). Precast thin layer cellulose and silica gel plastic sheets and glass plates were purchased from Brinkmann Instruments, Inc., Westbury, New York. All other reagents were analytical grade commercial chemicals.

\textit{Methods}

\textit{Preparation of \textit{O-Demethylpuromycin}}

A modification of the procedure of Baker, Joseph, and Williams (3) was used. The aminonucleoside was coupled with \textit{O,N-dicarbobenzoxy-L-tyrosine} in the presence of Woodward's reagent \textit{K} (4), followed by hydrogenolysis of the carbobenzoxy groups. A solution of triethylamine (0.2 ml; 0.34 mmole) in dimethylformamide was added over a 1-min period to a magnetically stirred suspension of \textit{O,N-dicarbobenzoxy-L-tyrosine} (0.153 g, 0.34 mmole) in 1.6 ml of dimethylformamide. Stirring was continued until the Woodward's reagent had completely dissolved (25 min). Then, 3 ml of a solution of puromycin aminonucleoside (0.10 g, 0.34 mmole) and Woodward's reagent \textit{K} (0.086 g, 0.34 mmole) in 1.6 ml of dimethylformamide. Stirring was continued until the Woodward's reagent had completely dissolved (25 min). Then, 3 ml of a solution of puromycin aminonucleoside (0.10 g, 0.34 mmole) and Woodward's reagent \textit{K} (0.086 g, 0.34 mmole) in dimethylformamide were added, and stirring was continued for an additional 18 hours. The solvent was removed in a vacuum, and the sticky residue was triturated with 15 ml of water. The granular precipitate formed after standing for 3 hours at 4° was collected by filtration, washed several times with water, and dried in a vacuum overnight (yield, 0.213 g, 86\%). After this product was heated to boiling in 5 ml of absolute ethanol and cooled, the residue was collected by filtration (m.p. 184-187°).

The above residue (0.173 g) was dissolved in 5 ml of Methyl

\textit{1 Katchalski and Sela (5) reported a melting point of 117° for \textit{O,N-dicarbobenzoxy-L-tyrosine}. However, samples obtained from Cyclo or Miles-Yeda, or those prepared by us according to the procedure of Katchalski and Sela, had a lower melting point (97-98°).}
Cellosolve, at 60–70°, 0.04 g of 10% palladium charcoal was added, and the suspension was placed in a water bath kept at 60–70°. A slow stream of hydrogen was passed through the solution until the evolution of carbon dioxide, as tested with barium hydroxide solution on the exit gasses, was complete (14 hours). The catalyst was removed by filtration, the solvent removed in a vacuum, and the glassy residue dried overnight in a vacuum (yield, 0.105 g).

The crude O-demethylpuromycin was further purified by column chromatography on silica gel. The sample in 0.1 ml of methanol was absorbed on a column of silica gel (1 x 10 cm) in chloroform and eluted with 3% methanol in chloroform. The bulk of product, as measured by absorption at 275 μm, appeared between 130 to 410 ml of effluent. This material was concentrated to dryness in a vacuum and the chromatography was repeated. Final purification to remove traces of yellow impurity was achieved by preparative paper electrophoresis in pyridine-acetic acid-water (2: 1: 20) (pH 5.2) followed by elution with ethanol. The ultraviolet absorption spectra of this product are shown in Fig. 1. Absorption maxima and approximate molar extinction coefficients were as follows: λ_{max} ethanol 275 μm (ε, 15,000); λ_{max} 0.1 N HCl 265 μm (ε, 14,400); λ_{max} 0.1 N NaOH 277 μm (ε, 14,300), 245 μm (ε, 9,700).

**Enzyme Assay**

All reagents for enzyme analyses were dissolved in glass-distilled water.

The general procedure used for estimation of methylase activity was as follows. S-Adenosyl-L-methionine-methyl-^{14}C (40 to 55 μC per μmole), O-demethylpuromycin, and enzyme were incubated in 0.05 M sodium phosphate buffer (pH 7.4) at 38° in a final volume of 20 μl in test tubes (10 x 75 mm.) Concentrations and times are indicated in the text. Control samples were mixtures incubated either without enzyme or O-demethylpuromycin. Reactions were terminated by the addition of 20 μl of ethanol-acetic acid (9:1), and protein was removed by centrifugation in a clinical centrifuge. One-half of each supernatant was carefully removed with a micropipette and spotted on precontd cellulose layers on plastic sheets. The sheets were developed in saturated ammonium sulfate-μ sodium acetate-isopropyl alcohol (80:18:2) (6) with puromycin as marker. After drying, the sheets were again developed in the same solvent. This procedure served to separate the unreacted S-adenosyl-L-methionine and its decomposition products from puromycin, which remained at the origin. In some cases, the development was again repeated with fresh solvent in order to lower the background radioactivity. The puromycin markers were located under ultraviolet light, and the areas corresponding to puromycin were cut out (approximately 1.5-cm squares). Each piece was placed in a scintillation vial, 3 ml of ethanol and 10 ml of Spectrafluor (Nuclear-Chicago) (1:25 dilution in toluene) were added, and the radioactivity was determined in a Nuclear-Chicago.
Growth of S. alboniger Cells

A puromycin-producing mutant strain of S. alboniger (ATCC 12462) was grown at 28° with constant agitation on a New Brunswick gyratory shaker in the following medium: 6% corn steep liquor and 2% corn starch, pH 6.4. To 500 ml of inoculated medium in two-liter shake flasks with added Dow-Corning Antifoam, 2 ml of 20% CaCO₂ were added after 12 and 28 hours of growth. After 72 hours of growth, the cells were harvested by centrifugation at 4°, washed 5 times with 0.15 M KCl, and stored at -65°.

Enzyme Purification

For determinations of enzyme activity, the assay mixtures, containing 0.02 to 0.05 mM S-adenosyl-L-methionine and 1 mM O-demethylpuromycin, were incubated for 20 to 60 min. All purification procedures were carried out at 0-4°.

Preparation of Cell-free Extract—Cell-free extracts were prepared by sonic disintegration of a 10% (wt/v) suspension of cells in 0.1 M phosphate buffer (pH 7.5, Na+) in an ultrasonic disintegrator (Measuring and Scientific Equipment, Ltd., London, 60 watt) for 20 min. The cell suspension was cooled by circulating ice water during the sonic disruption. Unbroken cells and particulate matter were removed by centrifugation at 18,000 x g for 90 min, and the supernatant fluid was dialyzed overnight against 1 mM EDTA (pH 7.5) (crude extract, 2.4 mg of protein per ml; specific activity, 0.06 to 0.07).

Ammonium Sulfate Fractionation—The crude extract was brought to 45% saturation by the gradual addition of solid ammonium sulfate (0.277 g per ml). The precipitate was removed by centrifugation, and the concentration of ammonium sulfate was raised to 70% saturation by the further addition of salt (0.171 g per ml). The precipitate was collected by centrifugation, dissolved in a small volume of glass-distilled water, and dialyzed against 6 to 8 changes of 1 mM EDTA (pH 7.5) (45 to 70% ammonium sulfate fraction, 24 mg of protein per ml; specific activity, 0.28).

Protamine Sulfate Treatment—Nucleic acids were removed from the above fraction by the addition of a 2% solution of protamine sulfate (pH 8.0) until precipitation was complete (0.29 ml per ml of enzyme solution). The supernatant was collected by centrifugation with no loss of activity (protamine supernatant, 16 mg of protein per ml; specific activity, 0.41).

Sucrose Density Gradient Centrifugation—Further purification of the enzyme was achieved by sucrose gradient centrifugation of the protamine supernatant by the procedure of Martin and Ames (7) (Fig. 2). The enzyme activity appeared in a single sharp peak, which corresponded in position to that of bovine serum albumin run under identical conditions. The molecular weight of the methylase was therefore assumed to be about 68,000. An approximate 30-fold increase in specific activity over the crude extract was found in the peak enzyme fractions (Fractions 14 and 15, 0.62 mg per ml; specific activity, 1.95).

All enzyme preparations could be stored at -65° for several months without loss of activity.

Specificity Studies

Methylation of L-tyrosine (0.7 mM) was tested as described under “Enzyme Assay” with crude extract (12 mg of protein) and 0.018 mM S-adenosyl-L-methionine (26,300 cpm); incubation was carried out for 60 min. O-Methyl-L-tyrosine, the expected product, was found to remain at the origin upon chromatography in ammonium sulfate-sodium acetate-isopropyl alcohol.

The other phenolic compounds were tested as follows. The reaction systems consisted of S-adenosyl-L-methionine (0.11 mM, 170,000 cpm), 1 mM concentration of phenol in place of O-demethylpuromycin, and sucrose gradient peak fraction (3.1 mg of protein) incubated in a final volume of 20 µl for 30 min. After incubation, the incorporation of radioactivity into each phenolic compound was determined by subjecting the incubation mixtures to the following treatments: (a) L-epinephrine, extraction with toluene-isooamyl alcohol as described by Axelrod (9); (b) catechol and l-tyrosine ethyl ester, addition of 0.5 ml of 0.1 M phosphate (pH 9.0, Na⁺) to each reaction mixture, followed by extraction with 2 ml of chloroform (1 ml of the organic phase was counted by liquid scintillation); (c) L-tyrosine amide, the same procedure as (b), except that extraction was performed with 2 ml of benzene.

It had been established by ultraviolet absorption measurements that significant amounts (at least 25%) of the non-methylated substrates were extracted into the respective organic solvents under these conditions. It was assumed that if methylation of the phenolic hydroxyl group occurred, the solubilities in the organic phase would be the same or greater. Methylation of O-demethylpuromycin could also be assayed by this procedure by addition of phosphate buffer (pH 9) followed by chloroform extraction. Controls without enzyme were included for each compound tested. Under these conditions, no radioactivity was transferred into chloroform from a control of S-adenosyl-L-methionine and enzyme.

Other Methods

Protein was determined by the micro Lowry procedure (10) with bovine serum albumin as standard. High voltage paper electrophoretic separations were run in a Pherograph (Brinkmann Instruments). Radioactivity on paper chromatograms and thin layer sheets was located with either a Packard model 7200 or Baird-Atomic model SC525 radiochromatogram scanner.

RESULTS

Properties of Enzyme—Preliminary experiments showed that the labeled methyl group of S-adenosyl-L-methionine-14C was transferred to O-demethylpuromycin when incubated with dialyzed crude extract from S. alboniger at pH 7.4 and 38°. As shown in Table 1, very little radioactivity was incorporated in the absence of either extract or O-demethylpuromycin, and enzyme activity was destroyed upon boiling. In Experiment 3, the 45 to 70% ammonium sulfate fraction produced a quantitative transfer of labeled methyl groups to product.
No metal or other cofactor requirement could be shown. Added puromycin (1 mM), puromycinaminonucleoside (1 mM), β-mercaptoethanol (10 mM), or dithiothreitol (5 mM) had no effect on incorporation of radioactivity into O-demethylpuromycin.

Enzyme activity as a function of time and protein concentration are shown in Figs. 3 and 4. Formation of product was linear with respect to both parameters when no more than 10% of the S-adenosyl-L-methionine was used. A broad pH optimum was found over the range pH 7 to 9 with a maximum at pH 8 (Fig. 5).

The effects of varying concentrations of S-adenosyl-L-methionine and O-demethylpuromycin on enzyme activity are shown in Figs. 6 and 7, respectively. From plots of v versus v/[S], apparent Kₐ values of 0.01 mM for S-adenosyl-L-methionine and 0.21 mM for O-demethylpuromycin were obtained (Figs. 6 and 7, inset). No inhibition was observed with S-adenosyl-L-methionine concentrations as high as 3 mM.

Specificity—A high degree of specificity was found for this methylase with respect to the source of the phenolic hydroxyl group. No demonstrable radioactivity was incorporated from S-adenosyl-L-methionine-methyl-14C into the following compounds: L-tyrosine, L-tyrosine ethyl ester, L-tyrosine amide, catechol, or L-epinephrine. The incorporation of radioactivity from S-adenosyl-L-methionine into O-demethylpuromycin (1 mM) under the conditions used for testing methylation of L-tyrosine was 1,510 cpm (0.02 mmole) and under those used for testing the other phenols, 13,500 cpm (0.17 mmole).
FIG. 5. pH optimum of methylase. The reaction mixtures contained 1.0 mM O-demethylpuromycin, 0.025 mM S-adenosyl-L-methionine (32,600 cpm), and protamine supernatant fraction (16 μg of protein). Incubation was for 20 min. Incubated controls without enzyme were included for each pH value.

Isolation and Characterization of Radioactive Product

The results discussed above clearly showed that the transfer of the labeled methyl group to O-demethylpuromycin was an enzyme-catalyzed reaction with a high degree of specificity. The identity of this reaction product with puromycin and confirmation of the location of the radioactive methyl group in the O-methyl-L-tyrosine moiety were established as described below.

Isolation—A large scale preparation of radioactive product was obtained as follows: S-adenosyl-L-methionine (0.106 μmole, 537,000 cpm), electrophoretically purified O-demethylpuromycin (0.116 μmole), sucrose gradient peak fraction (82 μg of protein), and 5 μmoles of phosphate buffer (pH 7.4) were incubated in a final volume of 0.105 ml for 2 hours at 38°. After termination of the reaction by addition of 0.105 ml of ethanol-acetic acid, the product was separated from unreacted S-adenosyl-L-methionine on a thin layer cellulose plate, as described under "Enzyme Assay." The radioactive product was eluted from the cellulose with 85% ethanol and taken to dryness in a vacuum. The residue was dissolved in 2 ml of 0.01 N NaOH and exhaustively extracted with chloroform. Most of the radioactivity (64,000 cpm) was extracted in this latter step, indicating that the product had no free phenolic hydroxy group. The amount of radioactivity in the chloroform-soluble product corresponded to a 12% conversion of added S-adenosyl-L-methionine.

Electrophoresis and Chromatography—Upon paper electrophoresis in 0.1 M K2PO4 (pH 12.2), the radioactive product and puromycin remained at the origin, whereas O-demethylpuromycin moved toward the anode, thus confirming the absence of a...
free phenolic hydroxyl group in the product. Electrophoresis of the radioactive product in pyridine-acetic acid-water (pH 5.2) (Fig. 8) showed that the major radioactive component had a mobility identical with puromycin. A small amount of puromycin marker and radioactivity remained at the origin. Thin layer chromatography in four different solvent systems (Table II) and paper chromatography in n-butyl alcohol-water (86:14) (Fig. 9A) further confirmed that the major radioactive component was identical with puromycin.

The identity of the other radioactive component (or components) (e.g. radioactivity near the solvent front in Fig. 9A) was not established; however, this product was not formed in the absence of O-demethylpuromycin.

Ethanolysis of Radioactive Product—In the presence of absolute ethanol saturated with HCl, puromycin is split into its constituent units: N²,N⁴-dimethyladenine, 3-amino-3-deoxy-β-ribose, and O-methyl-L-tyrosine, the latter moiety being simultaneously esterified (2). These compounds could be separated from each other, and also from N²,N⁴-dimethyladenine and adenine, by paper chromatography in n-butyl alcohol-water (86:14). Results of paper chromatography of the radioactive product after alcoholysis in ethanolic HCl are shown in Fig. 9B. Radioactivity was associated only with the spot corresponding to O-methyl-L-tyrosine ethyl ester. No radioactivity was found in the N²,N⁴-dimethyl-adenine moiety nor in the area where 3-amino ribose was found. The latter compound had a mobility less than half that of free O-methyl-L-tyrosine.

The above observation was further confirmed by hydrolyzing the ethanolic HCl product in 6 N HCl to convert the O-methyl-L-tyrosine ethyl ester to the free acid. Paper chromatography of this product in the above solvent showed that most of the radioactivity corresponded to O-methyl L-tyrosine and a small amount of unhydrolyzed ethyl ester (Fig. 9C).

Permanganate Oxidation of Radioactive Product—it had been previously shown that alkaline permanganate oxidation of puromycin yielded p-methoxybenzoic acid (2). Therefore, radioactive product was treated with permanganate to confirm that the label was solely present in the O-methoxy group.

For this experiment, radioactive product was prepared essentially as described above with 0.00834 μmole of S-adenosyl L-methionine-methyl-¹⁴C (approximately 370,000 cpm), 0.38

### Table II

**Thin layer chromatography of radioactive product and puromycin**

Ascending chromatography was performed in the following solvent systems with precoated plastic sheets. System A: cellulose layer, N²,N⁴-dimethylformamide-isopropyl alcohol-ammonia (22:65:10) (13); System B: cellulose layer, n-butyl alcohol-acetic acid-water (5:1:4) (upper phase); System C: polyethyleneimine cellulose layer, 0.8 × NaCl for 10 min followed by 1 M NaCl for 30 min without intermediate drying (14) (before spotting the compounds, the sheet was washed with water and dried); System D: silica gel layer; l-amyl alcohol-formic acid-water (3:2:1) (8).

<table>
<thead>
<tr>
<th>Compound</th>
<th>System</th>
<th>Rf</th>
<th>Rf</th>
<th>Rf</th>
<th>Rf</th>
</tr>
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<tr>
<td>Puromycin</td>
<td></td>
<td>0.83</td>
<td>0.81</td>
<td>0.48</td>
<td>0.0</td>
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<tr>
<td>Radioactive product</td>
<td></td>
<td>0.84</td>
<td>0.81</td>
<td>0.47</td>
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### Table III

**Thin layer chromatography of permanganate oxidation product**

The following solvent systems were used with precoated plastic sheets: System A: silica gel layer, benzene-acetic acid-dioxane (90:4:26) (16); System B: silica gel layer, benzene-methanol (95:5) (16); System C: cellulose layer, benzene-acetic acid-water (125:72:3) (17). Nonradioactive spots were visualized by spraying with a 0.04% aqueous solution of bromophenol blue.

<table>
<thead>
<tr>
<th>Compound</th>
<th>System</th>
<th>Rf</th>
<th>Rf</th>
<th>Rf</th>
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<tbody>
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<td>p-Methoxybenzoic acid</td>
<td></td>
<td>0.54</td>
<td>0.37</td>
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<tr>
<td>Oxidation products of radioactive material</td>
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<td>0.45</td>
<td>0.57</td>
<td>0.09</td>
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<tr>
<td>Puromycin</td>
<td></td>
<td>0.45</td>
<td>0.37</td>
<td>0.90</td>
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Fig. 9. Paper chromatography of radioactive product and its derivatives after acid ethanolysis and hydrolysis. Descending chromatography for 17 hours at room temperature on Whatman No. 1 paper in n-butyl alcohol-water (86:14) in chamber saturated with NH₃ (15). Purine bases were visualized under ultraviolet light, and tyrosine derivatives by spraying with ninhydrin. A, chloroform-soluble product. Radioactivity was determined by cutting the radiochromatogram strip into 1- or 2-cm pieces and counting by liquid scintillation as described under "Methods." B, product after alcoholysis in ethanolic HCl. The radioactive product used in this experiment was from a preparation similar to that described in the text by using dialyzed crude extract and a 6-hour incubation period. Chloroform-soluble product was heated for 2 hours at 80-82° in 0.1 ml of ethanolic saturated with dry HCl and flash evaporated to dryness before chromatography. All marker compounds were the corresponding hydrochlorides. C, product of B after further heating for 1½ hours in 2 N HCl at 100° and flash evaporation.


μ mole of O-demethylpuromycin, and dialyzed crude extract (350 μg of protein) incubated in a final volume of 0.48 ml for 14 hours. Before isolation of the product, 2.4 mg of cold puromycin was added. Radioactive product was eluted from the cellulose with water. A total of 32,000 cpm was found in the chloroform extract.

To a portion of this product (10,800 cpm) in 0.15 ml of 3.3 % NaOH, an additional 1.8 mg of cold puromycin were added, followed by an excess of saturated KMnO₄ solution. After standing for 3 hours at room temperature, excess permanganate was destroyed by addition of sodium sulfite and concentrated HCl, and the solution was exhaustively extracted with ethyl acetate. In the ethyl acetate extract, 90 % of the radioactivity was recovered. Thin layer chromatography in three different solvent systems showed that the oxidation products of puromycin and of the radioactive material were identical with p-methoxybenzoic acid (Table III).

**DISCUSSION**

**Comparison with Known Methyl Transferases—**The O-demethylpuromycin methylase of *S. alboniger* appears to be quite distinct from other known methylases. Catechol-O-methyl transferase (9) can methylate a large number of catechol derivatives but not monophenolic compounds, and in addition has an absolute requirement for a divalent cation. A general stimulation of RNA methylases from *Escherichia coli* by Mg²⁺ has also been reported (18, 19), whereas the DNA methylase does not (20). Nevertheless, it is of interest that the Kₘ values reported for these four different enzymes agree very closely for both S-adenosyl-L-methionine and the various methyl acceptors.

**Time Sequence of Methylation in Antibiotic Biosynthesis—**There is some evidence from studies on antibiotic biosynthesis in other streptomycetes which suggests that methylation may generally occur after formation of the basic carbon skeleton. Thus, Miller et al. (21) found that the addition of L-ethionine to cultures of *Streptomyces rimosus* caused the accumulation of four different demethyl precursors of tetracycline. One of these precursors, 4′-demethylaminou-4′-amino-5a,6-anhydrotetracycline, was methylated to 5a,6-anhydrotetracycline by S-adenosyl-L-methionine in the presence of cell-free extracts of *Streptomyces aureofaciens*. Also, two probable precursors of novobiocin, O-demethylnovobiocin and O-demethyledecarbamylnovobiocin, have been found to accumulate during the fermentation of *Streptomyces niveus* (22).

From our present studies, it appears that O-methylation of the tyrosine moiety may be the terminal step in puromycin biosynthesis. It remains to be established at which point in the biosynthesis of this antibiotic the methyl groups are added to the amino group at position 6 of the adenine moiety.

**Acknowledgment—**Thanks are due to William B. Lawson for advice on the synthesis of O-demethylpuromycin.

**REFERENCES**

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Biosynthesis of Puromycin in *Streptomyces alboniger*: ENZYMATIC METHYLATION OF O-DEMETHYLPUROMYCIN
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