Butirosin, a New Aminoglycosidic Antibiotic Complex: Isolation and Characterization

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Butirosin, a new water-soluble aminoglycosidic antibiotic complex, active against gram-positive and gram-negative bacteria, has been isolated from fermentation filtrates of Bacillus circulans by ion exchange. The complex has been resolved into isomeric A and B components, C$_{38}$H$_{41}$N$_{5}$O$_{12}$, by means of Dowex 1 $\times$ 1 or Dowex 1 $\times$ 2 chromatography. Properties of the free bases and the N,N',N'',N'''-tetraacetyl derivatives of butirosin A and B are given.

ISOATION AND PURIFICATION

Because of the mucoid nature of the culture filtrates, butirosin is isolated by batch adsorption at pH 6 to 7 onto a cation-exchange resin, IRC-50 (NH$_4^+$). The resin adsorbate is packed into a column and washed with 0.1 M ammonium hydroxide. The eluate is concentrated and reabsorbed on a column of IRC-50 (NH$_4^+$); the resin column is washed with three equivalents of 0.1 M ammonium hydroxide to remove impurities, and the antibiotic complex is eluted with 1 M ammonium hydroxide. After concentration of the eluate in vacuo, the aqueous concentrate is (i) lyophilized to give the free base which may then be treated with excess hydrochloric or sulfuric acid to give the respective salts upon acetone precipitation, or (ii) adjusted to pH 6.2 with dilute sulfuric acid and carbon-treated to give the sulfate of butirosin.

The antibiotic complex can be separated into butirosin A and B by means of ion-exchange chromatography. A solution of the base is percolated through a column of Dowex 1 $\times$ 1 or Dowex 1 $\times$ 2 in the borate form. Development of the resin column with water gives butirosin A free of butirosin B. Subsequent elution of the resin with increasing concentrations of aqueous boric acid up to 5% gives butirosin B free of A. The separated bases are then isolated by treatment with IRC-50 (NH$_4^+$), and the remaining traces of boric acid are removed with Amberlite XE-243, a boron-specific anion-exchange resin.

CHARACTERIZATION

The amorphous sulfate of butirosin exhibits no sharp melting point, decomposing at ca. 225°C. It is very soluble in water and relatively insoluble in the common organic solvents, including the lower alcohols; the free base exhibits the same solubility characteristics as the sulfate except for its increased solubility in the lower alcohols (7.5 mg/ml of methanol at room temperature). It gives positive ninhydrin and orcinol tests but negative Elson-Morgan, biuret, and Sakaguchi tests. It analyzes for C$_{38}$H$_{41}$N$_{5}$O$_{12}$·2H$_2$SO$_4$·2H$_2$O, exhibits a rotation of [a]$_{D}$ + 29° (2% in water), has pK'a values in water of 5.5, 7.2, 8.5, 9.4, and exhibits no maximum in water in the ultraviolet in the 220- to 320-nm region. The infrared spectrum is typical of aminoglycosidic antibiotics except for an amide band at 1,650 to 1,652 cm$^{-1}$.

The base or sulfate can be differentiated from most aminoglycosidic antibiotics by using paper chromatography with the following systems: (i) methanol-water-acetic acid-25% aqueous sodium chloride (600:212:75:20.8, v/v) using Whatman no. 1 paper buffered at pH 3, $R_f$ 0.21; (ii) methanol-5% aqueous sodium chloride (2:1, v/v)
using Whatman no. 1 paper buffered at pH 3, 3; (ii) ethanol-water-acetic acid-25% aqueous sodium chloride (250:500:38:7.5, v/v), Rf 0.71.

Butirosin base readily forms a N', N', N''-tetraacetyl (N-Ac) derivative by treatment with acetic anhydride in methanol. Paper chromatography of the above N-Ac derivative, using 1-butanol-pyridine-5% boric acid (6:4:3), gives two zones: Rf 0.34 to 0.38 (N-Ac butirosin A) and Rf 0.17 to 0.20 (N-Ac butirosin B). The zones are detected by using a modification of the method of Pan and Dutcher (5), chlorine gas being used instead of sodium hypochlorite. Quantitative estimation of the two zones according to the method of Majumdar and Majumdar (4) gives the relative proportion of N-Ac butirosin A and B and thus provides a means of estimating the ratio of A and B in various butirosin preparations. On the average, the various lots obtained by the IRC-50 isolation method mentioned above were found to consist of 80 to 85% A and 15 to 20% B.

As mentioned previously, butirosin can be separated into the components A and B by means of Dowex 1 × 1 or Dowex 1 × 2 chromatography. The properties of purified A and B are given in Table 1, and the biological properties are described by Howells et al. (3) and Heifetz et al. (2).

On a preparative scale, pure N-Ac butirosin A was obtained free from the B component by partition chromatography of the N-Ac derivatives using 1-butanol-1% aqueous boric acid-pyridine (10:10:3) as solvent system on a Celite column. Pure N-Ac butirosin B was obtained by similar chromatography of the crude N-Ac derivative prepared from the Dowex 1 × 2-resolved butirosin B base, using 1-butanol-water-pyridine (10:10:3) as solvent system. The properties of the purified N-Ac derivatives are given in Table 2.

The structures of butirosin A and B (Fig. 1) were elucidated by Woo et al. (6–8) and found to be isomeric, differing only in a pentose moiety. Butirosin A is N', [S]-(−)-4-amino-2-hydroxybutyryl]-4-O-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-5-O-β-D-xylouranosyl-2-deoxystreptamine, also known in the literature (6) as N'(−)-4-amino-2-hydroxybutyryl]-4-O-(2,6-diamino-2,6-dideoxy-D-glucopyranosyl)-5-O-D-xylouranosyl-2-deoxystreptamine; butirosin B is

### Table 2. Properties of N', N', N''-tetraacetyl (N-Ac) butirosin A and B

<table>
<thead>
<tr>
<th>Properties</th>
<th>N-Ac butirosin A</th>
<th>N-Ac butirosin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance</td>
<td>White amorphous solid</td>
<td>White amorphous solid</td>
</tr>
<tr>
<td>Molecular formula</td>
<td>C21H41N5O12</td>
<td>C21H41N5O12</td>
</tr>
<tr>
<td>Rotation</td>
<td>+26° (1.46% in water)</td>
<td>+33° (1.5% in water)</td>
</tr>
<tr>
<td>Melting point</td>
<td>163 – 196°C (2.11% in water)</td>
<td>168 – 186°C (1.34% in water)</td>
</tr>
<tr>
<td>pK'a in water</td>
<td>No titratable groups</td>
<td>No titratable groups</td>
</tr>
<tr>
<td>UV spectrum</td>
<td>End absorption at 220 nm</td>
<td>End absorption at 220 nm</td>
</tr>
</tbody>
</table>

*a* Ultraviolet.
the corresponding β-D-ribofuranosyl isomer. They are, therefore, different from all other known antibiotics.

**EXPERIMENTAL**

**Assay.** Chemical fractionation samples were assayed routinely by a paper-disc agar-diffusion method using *Escherichia coli* P-D 04863 as the test organism (3).

**Isolation of the butirosin complex from harvested beers.** The unfiltered harvested beer (305 gal [1,155 liters]; pH 6.8) was treated with 48 liters of Amberlite IRC-50 (NH₄⁺), and the mixture was stirred for 1 hr. The resin adsorbate was allowed to settle, and water washed free of suspended solids by decantation. The washed resin was charged into a 6-inch (15.2-cm) glass column already containing 10.5 liters of fresh IRC-50 (NH₄⁺) as a bottom layer. The packed column was washed with 0.1 M NH₄OH to remove impurities, and the butirosin was eluted with 100 gal (378.5 liters) of 1 M NH₄OH. The eluate was concentrated to 22 liters, adjusted to pH 7 with dilute sulfuric acid, and percolated through a column of 275 ml of IRC-50 (NH₄⁺). The resin adsorbate was then layered on top of 0.82 liter of fresh IRC-50 (NH₄⁺) in a 2-inch (5-cm) glass column. The resin column was washed with 49 liters of 0.1 M NH₄OH, and the butirosin was eluted with 4 liters of 1 M NH₄OH. The eluate was concentrated in vacuo, filtered, and lyophilized to give butirosin base, [α]⁺₆ + 26° (1.1% in water).

**Analysis:** C₇₂H₇₄N₂O₁₂·2H₂SO₄·2H₂O; calculated: C, 45.40; H, 7.44; N, 12.61. Found: C, 45.41; H, 7.26; N, 12.83.

A portion (150 mg) of the above butirosin base was dissolved in 2 ml of water and 0.4 ml of 5 N H₂SO₄. The solution was centrifuged, and the supernatant fluid was added to 10 ml of acetone. The resulting oily precipitate was dissolved in 3 ml of water and reprecipitated by addition of 11 ml of acetone. The white solid was filtered off, washed with acetone, and dried to give the sulfate of butirosin.

**Analysis:** C₇₂H₇₄N₂O₁₂·2H₂SO₄·2H₂O; calculated; C, 32.02; H, 6.27; N, 8.89. Found: C, 32.32; H, 6.20; N, 8.64.

In another isolation experiment of butirosin, the concentrated 1 M NH₄OH eluate from the second IRC-50 (NH₄⁺) column, obtained from 600 gal of beer, was adjusted to pH 6.2 with dilute H₂SO₄ and treated with 0.81 kg of water-washed carbon (Darco G-60) and 400 g of diatomaceous earth (Celite #545). The mixture was stirred for 1 hr and filtered, and the filter cake was washed with 80 liter of water. The filtrate and wash were concentrated, filtered, and lyophilized to yield the sulfate of butirosin; [α]⁺₆ + 29° (2% in H₂O), pKa in water, 5.5, 7.2, 8.5, 9.4.

**Analysis:** C₇₂H₇₄N₂O₁₂·2H₂SO₄·2H₂O; calculated: C, 32.02; H, 6.27; N, 8.89; S, 8.14. Found: C, 31.85; H, 6.28; N, 8.83; S, 8.01.

**Determination of the ratio of butirosin A and B in various preparations.** The Majumdar process (4) for the quantitative determination of the components of neomycin was employed with several modifications.

(i) **Acetylation.** Butirosin base preparations were acetylated by treatment of 50 mg of the base with 1.4 ml of methanol and 0.8 ml of acetic anhydride. The solutions were allowed to stand at room temperature for 5 hr and then evaporated to dryness in vacuo.

(ii) **Chromatography.** The dried acetylated products were dissolved in water at concentrations of 15, 10, 7.5, and 5 mg/ml, and 5 μl of each solution was spotted on Whatman no. 1 paper. The chromatograms were developed descendingly for 24 hr at 25 to 30 C in n-butyl alcohol-pyridine-5% aqueous boric acid (6:4:3).

(iii) **Quantitative estimation.** The developed chromatograms, run in pairs for each sample, were dried in air for 1 hr and then exposed to chlorine gas in a desiccator for 3 min. The desiccator was then evacuated to remove most of the chlorine, and the chromatograms were dipped in anhydrous ethanol and air-dried. The spots corresponding to A and B components were located on one of the chromatogram pairs by the starch-KI-HCl reagent (4); the amount of the butirosin N-Ac in each spot was determined on the second chromatogram by elution of the material from the paper with water, development of the color with the starch-KI-HCl reagent, and measurement of the absorbance at 570 nm. Standard curves of pure N-Ac-butilrosin A and B were used as reference.

**Chromatographic separation of butirosin A and B on Dowex 1 × 2.** The procedure for the separation of butirosin A and B by means of Dowex 1 is exactly the same whether the × 1 or × 2 resin is used. The method using Dowex 1 × 2 was as follows. The 3 liters of resin (Cl⁻, 50 to 100 mesh) in a 2-inch (5-cm) column was converted to the hydroxide form with 16 liters of 2 NaOH, washed with ca. 9.5 liters of water, treated with 17.5 liters of 5% boric acid, and washed with several holdups of water. Butirosin base (6.01 g) in 15 ml of water was added to the prepared resin column. After percolation of the sample, the column was washed with 6.42 liters of water at a flow rate of approximately 420 ml/hr. The column was then developed with 4 liters of 1%
boric acid, 4 liters of 2% boric acid, and finally with several holdups of 5% boric acid. Portions of eluate fractions were lyophilized, and the residues were analyzed as the N-acetyl derivative by paper chromatography for butirosin A and B content. Most of the butirosin A (ca. 4.5 g) free of B was found in fractions in the initial effluent volume of 1.5 to 4.95 liters. Most of the butirosin B (0.6 g) was found in the effluent volume of 2.64 to 4 liters after application of 5% boric acid to the column.

The butirosin A and B, present in the effluent volumes from the Dowex 1 columns, were purified by means of IRC-50 (NH₄⁺), XE-243 (free base form), and sometimes Dowex 1 × 16 resin columns. Examples of the final purification steps are given below.

An 0.6 liter portion of an initial effluent volume from one of the above Dowex 1 column containing ca. 0.85 g of butirosin A was percolated through a column of 11 ml of IRC-50 (NH₄⁺); the column was washed with water and then 40 ml of 0.1 M ammonium hydroxide. The butirosin A was eluted with two 80-ml portions of 1 M ammonium hydroxide. The first 80 ml of eluate was lyophilized. Assay for boron, using carmine red (1), indicated that the product contained 0.1% boron. The solid (0.49 g), dissolved in 5 ml of water, was percolated through a column of 4 ml of Amberlite XE-243 (free base form), and the resin was washed with 8 ml of water. The water percolate and wash were combined and percolated through 6 ml of Dowex 1 × 16 (OH⁻). The resin column was washed with 15 ml of water. Lyophilization of the effluent and water wash gave butirosin A base, 0.43 g.

Analysis: C₂₁H₄₁N₅O₁₂; calculated: C, 45.40; H, 7.44; N, 12.61. Found: C, 44.94; H, 7.05; N, 12.23.

The butirosin B (0.8 g) present in effluent volume 0.5 to 4.6 liters (5% boric acid as eluant) from the Dowex 1 column was purified by slowly percolating the aqueous solution through 5 ml of IRC-50 (NH₄⁺). The column was washed with water and then eluted with 90 ml of 1 M ammonium hydroxide. Concentration of the eluate and subsequent lyophilization gave butirosin B containing 0.3% boron (1). The powder (0.62 g) was dissolved in water, and the solution was percolated slowly through 4 ml of XE-243 resin (free base form). The column was washed with 10 ml of water. Lyophilization of the eluate and wash gave butirosin B (0.6 g), containing ca. 0.02% boron.

Analysis: C₁₉H₂₁N₅O₁₂·2H₂O; calculated: C, 42.63; H, 7.67; N, 11.84. Found: C, 42.79; H, 7.53; N, 11.45.

Preparation of N-Ac butirosin A and B. Butirosin base (16.5 g) was dissolved in 350 ml of acetic anhydride and 900 ml of dry methanol, and the solution was left at room temperature for 48 hr in a stoppered flask. The reaction mixture was then added to 7 liters of diethyl ether, and the resulting white precipitate was isolated (18 g). The product, dissolved in 50 ml of the top phase of 1-butanol-1% aqueous boric acid-pyridine (10:10:3), was partitioned on a column of 1,850 g of Celite no. 545 wetted with 925 ml of the bottom phase of the above solvent mixture. The column was developed with the top phase of the latter system; 20-ml cuts were taken. Those fractions (no. 562 to 640) containing the pure N-Ac butirosin A according to paper chromatography (1-butanol-pyridine-5% boric acid [6:4:3]) were combined and concentrated in vacuo. The aqueous concentrate (100 ml) was percolated successively through two columns of 100 ml of XE-243 resin (28 ml/hr) to remove the boric acid and then lyophilized to give N-Ac butirosin A; 2.5 g.

Analysis: C₂₉H₄₉N₅O₁₆·H₂O; calculated: C, 46.96; H, 6.93; N, 9.44; O, 36.67. Found: C, 47.08; H, 6.97; N, 9.43; O, 37.39.

The above partition column yielded fractions containing pure N-Ac butirosin B, but the amount was so little in this particular experiment that it was not isolated. To obtain pure N-Ac butirosin B, the base isolated from Dowex 1 × 2 (0.9 g) was acetylated with acetic anhydride in methanol and then partitioned as above but using the solvent system 1-butanol-water-pyridine (10:10:3) (1.05 g of product on 100 g of Celite no. 545). The column cut containing the desired material was concentrated in vacuo and transferred to water. The latter solution was percolated through a mixed bed resin (MB-3) to remove traces of ash, and the percolate was lyophilized. Yield: 0.44 g.

Analysis: C₂₉H₄₉N₅O₁₆·H₂O; calculated: C, 46.96; H, 6.93; N, 9.44. Found: C, 46.75; H, 7.00; N, 9.30.

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LITERATURE CITED