

Characterisation of  $\beta$ -Hydroxytyrosine Units in Ristocetin A

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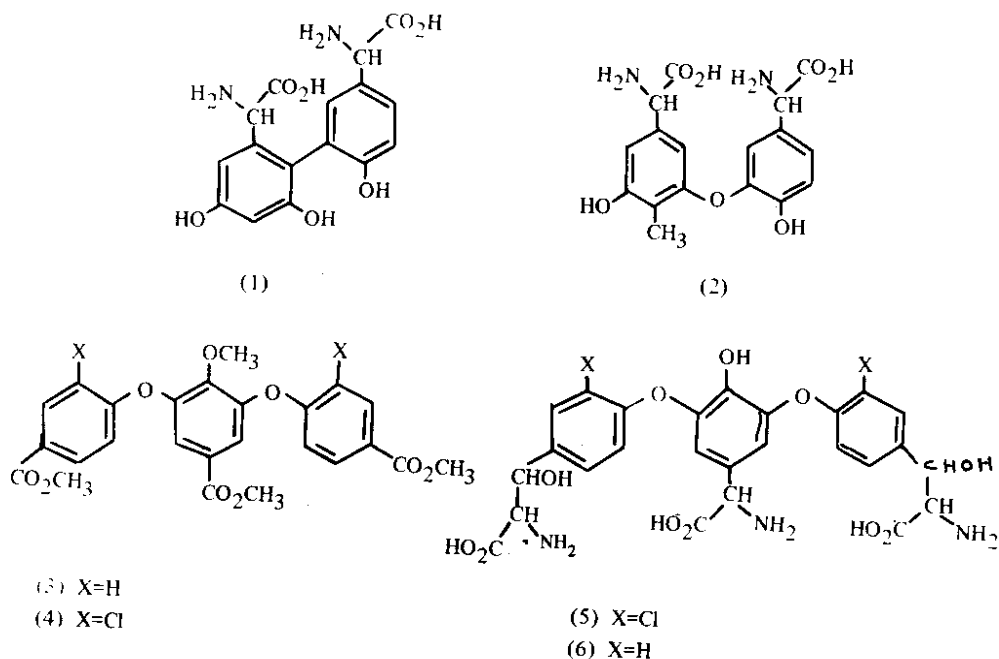
**Summary:** Ristocetin A is shown to contain a fragment formally derived by phenol oxidative coupling of two  $\beta$ -hydroxytyrosine units to a *p*-hydroxyphenylglycine.

Ristocetin A,<sup>1</sup> a glycopeptide antibiotic elaborated by *Nocardia lurida*, belongs to the vancomycin class of antibiotics.<sup>2</sup> It functions by interfering with the synthesis of the bacterial cell wall.<sup>3</sup>

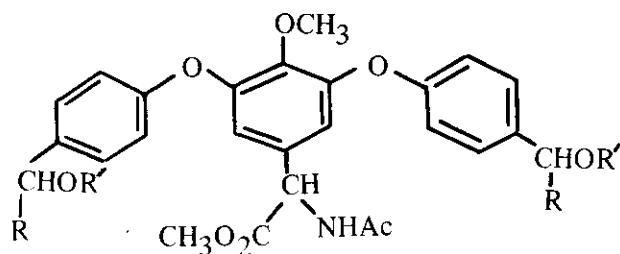
Ristocetin A has recently been shown to be identical to ristomycin A,<sup>4</sup> the antibiotics are composed of a central and biologically active polypeptide aglycone and a peripheral carbohydrate moiety. The nature and linkages of the sugars of the carbohydrate moiety have been fully characterised.<sup>5-11</sup> Structural work on the aglycone<sup>4,12-14</sup> has shown the presence of two unusual aromatic bis-amino acids (1) and (2). From oxidative degradation experiments, Harris and co-workers<sup>13</sup> have obtained, after methylation, the fragment (3). A similar dichloro-derivative (4) was obtained from the oxidative degradation of (5) in vancomycin. Here we present evidence for the presence of the bis-ether (6) in ristocetin A.

A  $\psi$ -aglycone of ristocetin A was prepared by me-

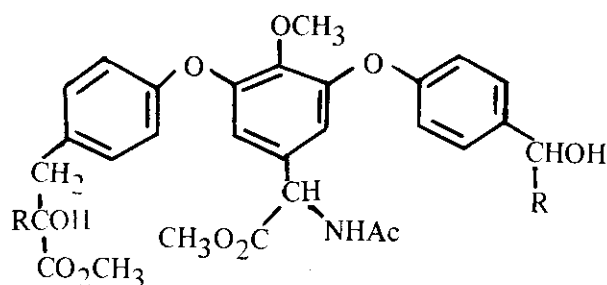
thanolysis (5% HCl in MeOH; reflux 1hr.) of the antibiotic; this product was shown by <sup>1</sup>H NMR (270 and 360 MHz) to contain ristosamine<sup>6</sup> as the only sugar. This  $\psi$ -aglycone was protected [acetylation (Ac<sub>2</sub>O/MeOH) and methylation (CH<sub>2</sub>N<sub>2</sub>)] and then subjected to reductive alkaline hydrolysis (NaBH<sub>4</sub>/NaOH). The hydrolysate was derivatised (5% HCl in MeOH, and then Ac<sub>2</sub>O/MeOH) and the products separated by preparative t.l.c. (silica GF<sub>254</sub>, developed with 10% MeOH/CHCl<sub>3</sub>). One fraction obtained (R<sub>f</sub> 0.3) corresponded to the diol 7 (Found M<sup>+</sup>, 481.1810; C<sub>26</sub>H<sub>27</sub>NO<sub>8</sub> requires 481.1737). As in vancomycin,<sup>15,16</sup> this can arise by retro-aldol cleavages of the  $\beta$ -hydroxytyrosine units (6) to generate initially a bisaldehyde, which is then reduced *in situ* to a diol by NaBH<sub>4</sub>. The occurrence of this sequence of events was established by carrying out the hydrolysis with NaBD<sub>4</sub>, when (8) (M<sup>+</sup> at m/e 483) was produced. As expected, acetylation (Ac<sub>2</sub>O in pyridine) of (7) gave (9) (M<sup>+</sup> 565).



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- (7), R=R'=H  
 (8), R=D, R'=H  
 (9), R=H, R'=OAc



- (10) R=R'=H  
 (11) R=R'=D  
 (12) R=H, R'=D

A second fraction obtained from the reductive basic hydrolysis is formulated as (10) (Found  $M^+$  553.1972;  $C_{29}H_{31}NO_{10}$  requires 553.1948). Such a product can arise by base-catalysed elimination of water from a  $\beta$ -hydroxytyrosine group (6), and hydrolysis of an enamine (formally formed during hydrolysis) to an  $\alpha$ -keto-acid;  $NaBH_4$  reduction of such a product would lead to (10). In reactions carried out in the presence of  $NaBD_4$ , compounds showing  $M^+$  in similar proportions at  $m/e$  554 and 555 were produced. The latter is the anticipated product (11), whereas  $m/e$  554 may arise due to partial exchange of deuterium adjacent to a carbonyl group (12).

The presence of two  $\beta$ -hydroxytyrosine units in ristocetin A is not only further supported by the production of glycine upon base hydrolysis,<sup>4</sup> but also from  $^{13}C$  and  $^1H$  spectra of the  $\psi$ -aglycone. In the  $^{13}C$  spectrum of this compound there are 13 resonances in the 40-80 ppm region, as required by 7 amino acid  $\alpha$ -carbons of (1),(2) and (6) the 2 hydroxylated  $sp^3$  carbons of (6), 3  $sp^3$  carbons of ristosamine attached to one electronegative atom,<sup>6</sup> and one  $CH_3$  carbon of a methyl ester.<sup>17</sup> The  $^1H$  spectrum (270 and 360 MHz) shows two  $\alpha$  CH-NH systems where the  $\alpha$  CH-resonances are further coupled ( $J \approx 1$ Hz in one case,  $J=5.5$  Hz in the other) to protons resonating at 5.12 and 5.21 ppm ( $d_6$ -DMSO solution).

## Experimental

$^1H$  NMR spectra were obtained with Bruker 270 MHz and 360 MHz spectrometers operating at 80°C and in the Fourier transform mode.  $^{13}C$  NMR spectra were obtained with a varian XL-100 spectrometer and

recorded with proton noise decoupling. Mass spectra were recorded at 70 eV by direct insertion with an A.E.I. MS 902 spectrometer. High resolution mass measurements were made on an A.E.I. MS30, on-line to a DS50S data acquisition system

Analytical t.l.c. was carried out on Merck plates coated with silica GF<sub>254</sub>, and preparative t.l.c. on 20 x 20 cm plates coated in our laboratory with Merck silica GF<sub>254</sub>. The abbreviation BWA refers to  $\eta$ -butanol-water-glacial acetic acid (3:1:1). Compounds were recovered from preparative t.l.c. plates by elution of the silica with acetone, except where stated otherwise.

A Uvicord 8300 fraction collector system was used for the gel-filtration chromatography. HPLC was performed on a Waters Associates machine using reverse phase columns.

### Preparation of the $\psi$ -aglycone

Ristocetin A (300mg) was heated under reflux in methanolic hydrogen chloride (5%, 15ml) for 1 hour. The methanolysate was evaporated to dryness and the residue redissolved in water (10 ml). A solution of potassium carbonate (IN) was added dropwise until the crude  $\psi$ -aglycone precipitated (pH7.5). The precipitate was removed by centrifugation, redissolved in a minimum volume of acetic acid (6%) and applied to a gel-filtration (Sephadex G-25) column (1cm x 120 cm). The column was eluted with acetic acid (6%, 18ml/hr) and the elution monitored at 280nm. An u.v.-absorbing component was eluted after 7hr. 40min.

The eluate was freeze-dried to give the  $\psi$ -aglycone (80mg). The material was found to be pure by t.l.c. (BWA,  $R_f$  5.5) and by hplc (Bondapak C<sub>18</sub> column, eluted with 0.05M  $KH_2PO_4/CH_3CN$ , 3:1, 2ml/min).

*Protection of the  $\psi$ -Aglycone*

$\psi$ -aglycone (50mg) was acetylated by suspension in methanol-acetic anhydride (2:1, 9ml) overnight at room temperature. The mixture was evaporated to dryness and the purity of the di-N-acetyl- $\psi$ -aglycone checked by t.l.c. (BWA,  $R_f$  0.85).

The acetylated  $\psi$ -aglycone was dissolved in methanol (80ml) and treated with excess ethereal diazomethane for 6 hours.

The resulting protected (penta-O-methyl-di-N-acetyl)- $\psi$ -aglycone (25mg) was purified by t.l.c. (developed with 10% methanol in chloroform,  $R_f$  0.3), and eluted from the silica with 25% methanol in chloroform.

*Reductive ( $\text{NaBH}_4$ ) Alkaline Hydrolysis*

The protected aglycone (25 mg) was suspended in sodium hydroxide solution (20%, 25ml,  $\text{N}_2$ -saturated) containing sodium borohydride (720mg). The mixture was heated under reflux under a nitrogen atmosphere for 24 hours.

The cooled mixture was neutralised with dilute hydrochloric acid (3N) and evaporated to dryness. The residual dry solid was suspended in a 5% solution of hydrogen chloride in methanol and the mixture stirred overnight at room temperature.

The solvent was then removed and the residue redissolved in methanolic potassium carbonate-acetic anhydride (2:1).

The mixture was evaporated to dryness and the residue extracted with acetone. A u.v.-absorbing component ( $< 2\text{mg}$ ) obtained by preparative t.l.c. (developed in 10% methanol in chloroform,  $R_f$  0.3) was the diol (7),  $m/e$  481 ( $\text{M}^+$ ), 463 ( $\text{M}^+ - \text{H}_2\text{O}$ ) 449 ( $\text{M}^+ - \text{CH}_3\text{OH}$ ), 438 ( $\text{M}^+ - \text{CH}_3\text{CO}$ ), 422 ( $\text{M}^+ - \text{CO}_2\text{CH}_3$ ), 380 ( $\text{M}^+ - \text{CO}_2\text{CH}_3 - \text{CH}_2\text{CO}$ ). The above product was treated with acetic anhydride - pyridine (1:1, 2ml) overnight. The reaction mixture was evaporated to dryness and the product identified as (9) [(the triacetate of (7))] by its mass spectrum ( $\text{M}^+$  565).

Another fraction obtained from the same preparative t.l.c. was identified as the hydroxy-ester (10),  $m/e$  553 ( $\text{M}^+$ ), 536 ( $\text{M}^+ - \text{OH}$ ), 521 ( $\text{M}^+ - \text{CH}_3\text{OH}$ ), 510 ( $\text{M}^+ - \text{CH}_3\text{CO}$ ), 494 ( $\text{M}^+ - \text{CO}_2\text{CH}_3$ ), 452 ( $\text{M}^+ - \text{CO}_2\text{CH}_3 - \text{CH}_2\text{CO}$ ).

*Reductive ( $\text{NaBD}_4$ ) Alkaline Hydrolysis*

The above experiment was repeated using sodium borodeuteride instead of sodium borohydride. One of the u.v. absorbing fractions obtained by t.l.c. was identified as (8) by its mass spectrum ( $\text{M}^+$  483).

Another component from the t.l.c. was found to be a 1:1 mixture of the mono- and di-deuterated com-

pounds (11) and (12) ( $\text{M}^+$  554, 555 respectively).

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