STRUCTURAL INVESTIGATION OF THE ANTIBIOTIC RISTOMYCIN A

SYNTHESIS OF RISTOBIOSE AND RISTOTRIOSE

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Proof is given by synthesis confirming the structure of ristobiose as 2-O-α-D-mannopyranosyl-D-glucose (**IV**) and ristotriose as O-α-L-rhamnopyranosyl (1→6)-O-[α-D-mannopyranosyl (1→2)]-D-glucose (**X**) which are obtained from ristomycin A upon mild acid hydrolysis. Both oligosaccharides, **IV** and **X**, have been detected for the first time as the components of an antibiotic.

The antibiotic ristomycin[1] produced by *Proactinomyces fructiferi* var. *ristomycini*,[2] and presumably identical with the antibiotic ristocetin,[3] belongs to the vancomycin group of antibiotics. As shown by our investigations, ristomycin consists of the ristomycin A aglycone,[4,5] whose unusual peptide structure has not been completely elucidated as yet, connected separately to one molecule of D-mannose, one molecule of ristosamine,[6] and a branched heterotetrasaccharide side-chain.[7] The structure of ristosamine (2,3,6-trideoxy-3-amino-L-*ribo*-hexopyranose) has recently been substantiated by synthesis[8] and this result has been confirmed by LEE and his co-workers.[9]

We now report the synthesis of ristobiose and ristotriose resulting from the mild acid hydrolysis of the tetrasaccharide side chain of ristomycin A.

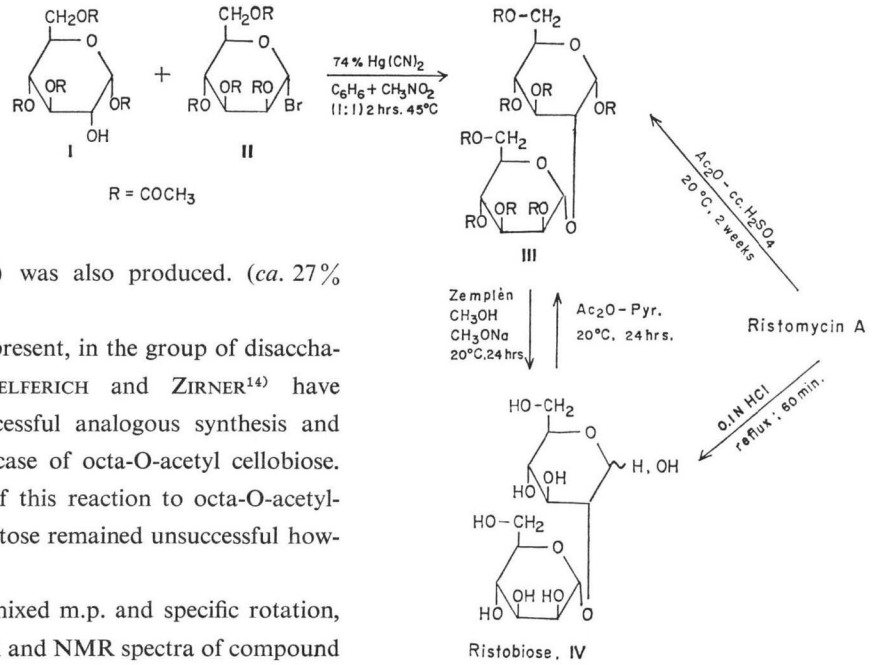
In previous studies on the sugar linkage, using permethylation followed by hydrolysis, ristobiose and ristotriose were shown to be 2-O-α-D-mannopyranosyl-D-glucose and O-α-L-rhamnopyranosyl (1→6)-O-[α-D-mannopyranosyl (1→2)]-D-glucose, respectively.[7]

In the present work, using the modified HELFERICH method,[10] 1,3,4,6-tetra-O-acetyl-α-D-glucose (**I**) and α-acetobromo-D-mannose (**II**) were allowed to react in benzene - nitromethane (1: 1), to obtain octa-O-acetyl-α-ristobiose (**III**) in 74% yield (Fig. 1). Octa-O-acetyl-2-O-α-D-mannopyranosyl-β-D-glucose has recently also been synthesized by DICK *et al.*[11] under different reaction conditions.

On the basis of the m.p., specific rotation and IR spectrum, compound **III** was identical in every respect with the acetylated derivative of ristobiose (**IV**)[7] prepared from ristomycin A by mild acid hydrolysis and acetylation. The specific rotation, as well as the paper chromatographic mobility of the compound obtained after deacetylation of **III** by ZEMPLÉN's method[11a] were in good agreement with the data of **IV** isolated from the 0.1 N hydrochloric acid hydrolyzate of the antibiotic (Table 1).

For the synthesis of ristotriose (Fig. 2), hexa-O-acetyl-α-rutinosyl halide[12,13] (**V**: R=CH_3CO: X = Cl or Br) was used as the starting material, from which hexa-O-acetyl-β-rutinose (**VII**), containing a free hydroxyl group at C-2, was obtained by the method of HELFERICH and ZIRNER.[14] As a result of the nucleophilic attack of acetate ion on compound **V** (prepared in both ways) in aqueous acetic acid, hepta-O-acetyl-α-rutinose (**VI**) was formed as the main product in 56% yield. The hexa-O-acetyl-

Fig. 1. Structure proof of ristobiose by synthesis.

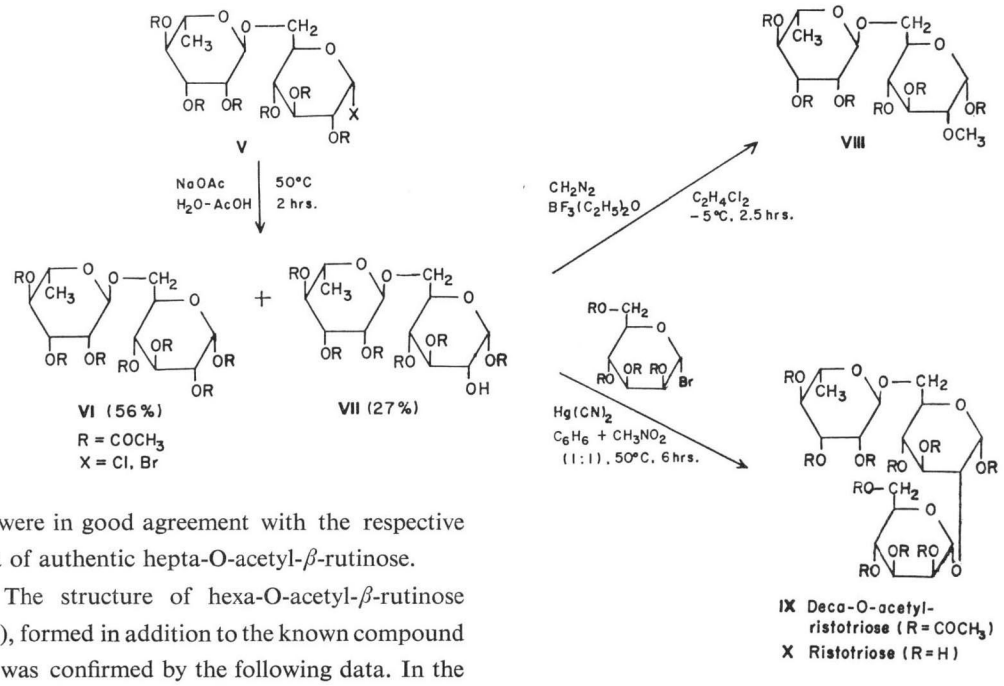


derivative (**VII**) was also produced. (*ca.* 27% yield).

Up to the present, in the group of disaccharides, only HELFERICH and ZIRNER[14] have achieved a successful analogous synthesis and this was in the case of octa-O-acetyl cellobiose. The extension of this reaction to octa-O-acetyl-lactose and maltose remained unsuccessful however.

The m.p., mixed m.p. and specific rotation, as well as the IR and NMR spectra of compound

Fig. 2. Synthesis of ristotriose



VI, were in good agreement with the respective data of authentic hepta-O-acetyl-β-rutinose.

The structure of hexa-O-acetyl-β-rutinose (**VII**), formed in addition to the known compound **VI**, was confirmed by the following data. In the 100 MHz NMR spectrum, the signal of the hydroxyl proton appears at δ=2.69 ppm, which can be confirmed by deuteration. At δ=4.20 ppm a distinct multiplet of one proton intensity can be found. On the basis of its chemical shift (appearing about 0.5~1.0 ppm higher than the signals of the methine protons of the -CH-OAc system), this multiplet can unequivocally be assigned to the H-2 proton.

Table 1. Paper chromatographic comparison of synthetic ristobiose and ristotriose with authentic samples isolated from ristomycin A

Origin		R_G*			
		1	2	3	4
Ristobiose	Synthetic sample	0.37	0.39	0.80	0.46
	Sample isolated from ristomycin A	0.37	0.40	0.81	0.45
Ristotriose	Synthetic sample	0.21	0.29	0.68	0.23
	Sample isolated from ristomycin A	0.22	0.29	0.68	0.23

* The term R_G means the chromatographic mobility of the compounds referred to D-glucose.
Paper: Schleicher-Schüll 2043b Mgl
Solvent systems: 1. Ethyl acetate - acetic acid - 1-butanol - H_2O (7: 3: 3: 3)
2. Ethyl acetate - acetic acid - H_2O (44: 20: 10)
3. 1-Butanol - pyridine - H_2O (6: 4: 3)
4. *t*-Amylalcohol - formic acid - methanol - H_2O (7.5: 0.5: 2:2)
The spots were detected by spraying with a 2% solution of aniline hydrogenphthalate in 1-butanol and drying at 105°C for 10 minutes.

Irradiation at this frequency caused the collapse of a doublet at δ=4.82 ppm, which can only be assigned to H-1, since H-3 should give a doublet of doublets. In addition, on the basis of its chemical shift, H-1 is, in all probability, in axial position. However, the resonance of H-1 is superimposed upon other signals, thus $J_{1,2}\approx 7\sim 8$ Hz can be established only as an approximate value. On the basis of these results, it is most likely that the anomeric configuration at the reducing end of compound **VII** is β.

The presence of the free OH group at C-2 in the molecule of **VII** was confirmed by the NMR spectrum of 2-O-methyl-hexa-O-acetyl-β-rutinose (**VIII**), obtained on methylation with diazomethane-boron trifluoride etherate. In the case of **VIII**, the signal of the OCH_3 group at C-2 appeared at δ= 3.50 ppm, being in good agreement with the chemical shifts of the methyl groups in several 2-O-methyl-D-glucose derivatives.[15~17] The nature of **VII** was unequivocally proved by thin-layer and gas chromatographic examinations of the 2-O-methyl-D-glucose obtained with 0.5 N sulfuric acid hydrolysis of **VIII** (6 hours, 120°C) followed by reduction with $NaBH_4$ and acetylation (see Experimental).

The mannosylation[10] of **VII** gave deca-O-acetyl-ristotriose (**IX**) which, on the basis of its physical properties, proved to be identical with the deca-O-acetyl derivative of ristotriose[7] originating from ristomycin A.

The mass spectra of the isolated and synthetic samples of **III** and **IX** proved to be identical within experimental error. The observed peaks of highest mass in both cases correspond to M-AcOH ions. Besides these peaks, due to successive losses of acetic acid and/or ketene molecules from this ion, each spectrum exhibits an intense peak at *m/e* 331 resulting from the cleavage of a glycosidic O-C bond. The appearance of this ion, as well as its fragments, is a common feature of hexopyranose pentaacetates.[18]

The saponification of **IX** in absolute methanol gave ristotriose **X**. The R_G values of **X** derived from the antibiotic and those of the synthetic product are compared in Table 1.

These results not only support the recently published[7] 2-O-α-D-mannopyranosyl-D-glucopyranose and O-α-L-rhamnopyranosyl-(1→6)-O-[α-D-mannopyranosyl (1→2)]-D-glucopyranose structure for ristobiose and ristotriose ,respectively, but also elucidate, in part, the structure of the hetero-tetrasaccharide side chain[7] in ristomycin A.

Both oligosaccharides have now been first observed in nature as the structural components of an antibiotic.

Experimental

Melting points were obtained with a KOFLER-BOETIUS apparatus and, in part, measured in a capillary tube, and are uncorrected. Optical rotations were measured with a Bendix NPL automatic polarimeter 143 D and a Polamat A (Zeiss) automatic photoelectric polarimeter. IR spectra were recorded in KBr pellets on a Perkin-Elmer 700 and an UNICAM SP 200 G instruments. NMR spectra were obtained on a Jeol MH-100 instrument for solutions in $CDCl_3$ with Me_4Si as the internal standard and mass spectra were taken on an AEI Ms-900 spectrometer. For gas chromatographic examinations (GLC), a Hewlett-Packard 5830 A instrument was used. Thin-layer chromatography (TLC) was performed on Kieselgel G (Merck) using the following solvent systems: (A) abs. benzene - methanol 95: 5, (B) abs. benzene - ethyl acetate 1: 1, (C) toluene - ethyl acetate - 96% ethanol 10: 5: 5, (D) benzene - acetone 1: 4+1% cc. NH_4OH, and (E) abs. benzene - methanol 9: 1. Spots were detected by spraying with 50% ethanolic sulfuric acid and heating at 120°C.

Octa-O-acetyl-α-ristobiose (III)

1,3,4,6-Tetra-O-acetyl-α-D-glucopyranose (1.04 g) and $Hg(CN)_2$ (0.83 g) were dissolved in 1: 1 benzene - nitromethane (60 ml) and the mixture was concentrated at atmospheric pressure to about 20 ml. After cooling to 40°C, 1.35 g (0.0033 moles) of α-acetobromo-D-mannose was added and the mixture was stirred in the absence of moisture for 2 hours. The mixture was concentrated to dryness *in vacuo*, the residue was dissolved in chloroform (100 ml), filtered and the filtrate was successively washed with 5% KI solution (3 × 40 ml) and water (3 × 20 ml). The resulting solution was dried over Na_2SO_4 and concentrated. The syrupy residue was purified by column chromatography on Kieselgel G (80 g) with 95: 5 benzene - methanol as the eluant to give 1.48 g (74%) of **III**. It was crystallized from a mixture of ethanol (5 ml) and water (4 ml), mp 68~69.5°C (KOFLER), $[\alpha]_D^{20}$ + 81.40° (*c* 0.64, $CHCl_3$). The physical properties as well as the IR spectrum and thin-layer chromatographic mobility of **III** were in good agreement with the corresponding values of the sample of **III** obtained upon 0.1 N hydrochloric acid hydrolysis of ristomycin A followed by acetylation in 1: 1 pyridine - acetic anhydride at 20°C [mp 67~69°C (KOFLER), $[\alpha]_D^{20}$ + 83.33° (*c* 0.60 CH_3OH), mixed mp 68~69°C (KOFLER)].

TLC: (A)R_f = 0.85; (B)R_f = 0.76.

Ristobiose (IV)

III (400 mg) was dissolved in abs. methanol (10 ml) and saponified with 0.1 N $NaOCH_3$ (0.5 ml). After 16 hours, compound **IV** spontaneously crystallized. It was filtered and washed with a small amount of methanol to give pure **IV** (212 mg; 98%), mp 157~158°C, $[\alpha]_D^{20}$ + 100° (*c* 0.56, H_2O), Lit.[11] $[\alpha]_{456}^{25}$ + 75.5° (*c* 1.0, H_2O). The paper chromatographic R_G values of the synthetic **IV** and ristobiose of natural origin in solvent systems 1~4 were completely identical (see Table 1).

Hepta-O-acetyl-β-rutinose (VI) and hexa-O-acetyl-β-rutinose (VII)

Acetochloro-rutinose (2.4 g) was dissolved in 99.5% acetic acid (24 ml) and treated with a solution of sodium acetate trihydrate (9.6 g) in water (9.6 ml) with stirring and the reaction mixture was heated to 50°C. After twenty minutes, it was treated again with the same amount of sodium acetate solution described above, and stirring was continued for additional 1.5 hours. The mixture was diluted with chloroform (250 ml) and washed to neutrality with water (6 × 50 ml).

The organic layer was dried over Na_2SO_4, filtered and concentrated. Crystallization of the residue from ethanol (50 ml) gave **VI** (1.40 g; 56%), mp 169~170°C (KOFLER), $[\alpha]_D^{20}$ −37.75° (*c* 0.98, pyridine). According to IR and NMR spectroscopic examinations, compound **VI** was found to be identical with authentic hepta-O-acetyl-β-rutinose,[12] mixed mp 170~171°C (KOFLER).

Compound **VII** was separated from the residue obtained after the concentration of the mother liquor of **VI** with chromatography on a Kieselgel G column (60 g; solvent system, benzene - methanol 95: 5); yield 627 mg (27%). It was crystallized from ethanol, mp 117~118°C (KOFLER), $[\alpha]_D^{20}$ + 15.56° (*c* 0.38 pyridine).

Anal. Calcd. for $C_{24}H_{34}O_{16}$ (578.51): C, 49.82: H, 5.92%
Found: C, 50.02: H, 5.99%

TLC: (A) **VI** $R_f = 0.44$; (A) **VII** $R_f = 0.26$

Hexa-O-acetyl-2-O-methyl-β-rutinose (**VIII**)

The solution of **VII** (100 mg) in dichloromethane (2 ml) was treated with 20 μl of boron trifluoride etherate, followed by the addition of a dichloromethane solution of diazomethane (20 ml) — prepared from 1 g of nitrosomethyl urea — over a period of 1.5 hours at −5°C. After standing for one hour at −5°C, the solid precipitate was filtered, and the filtrate was washed to neutral with water (3 × 30 ml), dried over Na_2SO_4 and concentrated to afford 82 mg of syrupy **VIII**.

TLC: (A) $R_f = 0.51$; NMR: $\delta_{OCH_3} = 3.50$ ppm.

Fifty mg of **VIII** was saponified with 0.05 ml of 0.1 N $NaOCH_3$ (24 hours 20°C) in abs. methanol (2 ml). The reaction mixture was neutralized with acetic acid, concentrated, and the residue was hydrolyzed with 0.5 M sulfuric acid (2.0 ml) at 100°C for 6 hours. The hydrolyzate was diluted with water (20 ml), neutralized by heating with barium carbonate, filtered, and evaporated to dryness. On the basis of TLC examination in solvent systems (C) and (D), one of the spots proved to be 2-O-methyl-D-glucose, (C) $R_f = 0.22$; (D) $R_f = 0.20$.

GLC examination: Ten mg of the hydrolyzate was reduced with $NaBH_4$ (20 mg) in 2 ml of water in the dark for two hours. After neutralization with acetic acid, the reaction mixture was concentrated, the residue was dried over P_2O_5 and then acetylated with 1: 1 pyridine - acetic anhydride (2 ml) at 100°C for 20 minutes. The solution was concentrated *in vacuo* to about 0.5 ml and allowed to stand in ice-water (10 ml) for one hour. The aqueous layer was extracted with chloroform (2 × 20 ml), the combined organic solution was successively washed to neutral with 1 M sulfuric acid (5 × 20 ml) and water (3 × 20 ml), dried over Na_2SO_4 and concentrated. The residue was dissolved in 0.2 ml of chloroform and the solution was injected into the gas chromatograph.

Experimental conditions: 6 ft stainless steel column: 10% OV-1, 80~100 mesh WHP, temperature 150~220/5°C/min. Detector: F.I.D. 300°C.

Inj. temp.: 250°C.

R_T: 10.34 min (penta-O-acetyl-L-rhamnitol)

R_T: 12.11 min (2-O-methyl-penta-O-acetyl-glucitol)

Deca-O-acetyl-ristotriose (**IX**)

Compound **IX** was prepared from **VII** (326 mg) and α-acetobromo-D-mannose (254 mg) using the method described above for **III**. A syrupy product was obtained, which was purified by short-column chromatography on Kieselgel G (30 g) with 9: 1 benzene - methanol as the eluant. Recrystallization from 2: 3 water - ethanol mixture gave pure **IX** (333 mg; 65%), mp 87~88°C (KOFLER), $[\alpha]_D^{20}$ + 12.5° (*c* 1.0, CH_3OH).

Anal. Calcd. for $C_{38}H_{52}O_{25}$ (908.79): C, 50.29: H, 5.00 %
Found: C, 50.55: H, 5.59 %

The physical properties, IR spectrum and chromatographic mobility of **IX** were in good agreement with the values of the acetylated derivative of authentic ristotriose, [mp 88~91°C, $[\alpha]_D^{20}$ + 34.60° (*c* 0.64, CH_3OH), mixed mp 87~88°C] obtained by the acetylation (1: 1 pyridine - acetic anhydride 20°C, 24 hours) of ristotriose[7] derived from the antibiotic.

TLC: (B) $R_f = 0.52$; (E) $R_f = 0.75$.

Ristotriose (**X**)

Saponification of **IX** was performed as described above for **IV**. Compound **X** was obtained in 97% yields as a hygroscopic white amorphous powder, mp 180~182°C (decomp.), $[\alpha]_D^{20}$ + 40.6° (*c* 0.44, H_2O). The paper chromatographic R_G values of the synthetic **X** and ristotriose derived from the antibiotic were completely identical (see Table 1).

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