On the Structure and Mode of Action of the Antibiotic Ristocetin A

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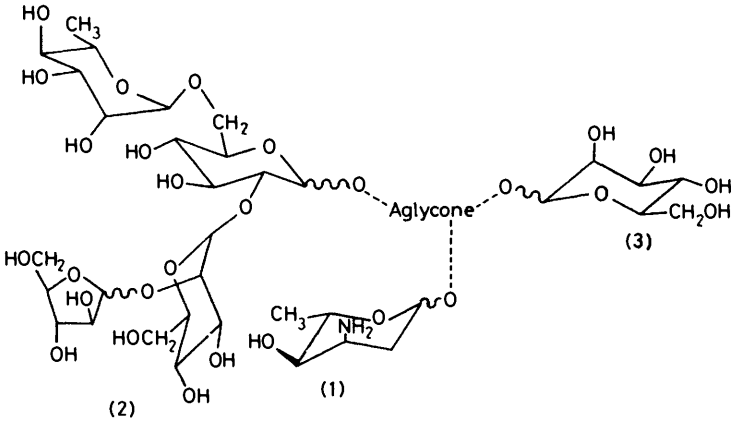
The antibiotics ristocetin A and ristomycin A are shown to be identical. It is concluded that ristocetin A is constituted from six sugars and an aglycone portion based on (i) a bis-ether containing three benzenoid rings, (ii) a fragment which on hydrolysis affords a 2',4,6-trihydroxybiphenyl-2,3'-diyldiglycinate derivative, (iii) a previously isolated fragment incorporating a biphenyl ether, and (iv) at least one (and perhaps two) moles of potential glycine from alkaline hydrolysis. An approximate molecular-weight determination (2 063 ± 5 Daltons) of the antibiotic by californium plasma desorption mass spectrometry, ^{13}C, and ^{1}H n.m.r. spectra, and functional group determinations, are consistent with the incorporation of the above structural units in a tetracyclic aglycone structure. A cell-wall component analogue, acetyl-D-alanyl-D-alanine, forms with ristocetin A a complex whose dissociation [in $(CD_3)_2SO$ solution at 40 °C] is slow on the ^{1}H n.m.r. time scale.

RISTOCETIN [1] and ristomycin [2] belong to the vancomycin group of glycopeptide antibiotics. Ristocetin is elaborated by *Nocardia lurida* in two forms, A and B, which differ in their sugar content.[3] Both forms were used in the past to combat infections by Gram-positive bacteria,[4] but more recently have been used in studies of blood platelet aggregation.[5] Ristomycin is produced by *Proactinomyces fructiferi*, also in A and B forms [6] differing in sugar content. The antibiotic is reported [7] to be manufactured for clinical use on a large scale in the U.S.S.R.

Evidence pointing to the identity, or near identity, of ristocetin A and ristomycin A, and of ristocetin B and ristomycin B, has previously been noted.[7,8] We have found that ristocetin A is indistinguishable from ristomycin A using an h.p.l.c. system which can readily separate it from ristocetin B. Furthermore, we have obtained well-resolved 270 MHz proton n.m.r. spectra of the A forms of the two antibiotics and found them to be the same (allowing for slight variations accountable for by differences in temperature and concentration). We therefore conclude that ristocetin A and ristomycin A have identical structures. This conclusion has been reached independently at the Institute for New Antibiotics, Moscow.[9]

The identity of ristocetin A and ristomycin A simplifies the structure elucidation of ristocetin A since the structures of the sugar portions of ristomycin A are known. Acid hydrolysis of ristomycin A affords the amino-sugar ristosamine (1),[10] and oligosaccharide units from which the presence of the tetrasaccharide (2) in ristomycin A can be deduced.[11] The structures of ristosamine, the disaccharide ristobiose, and the trisaccharide ristotriose have recently been confirmed by synthesis.[12-14] A further molar equivalent of mannose (3) is found in ristomycin A as a monosaccharide unit.[15]

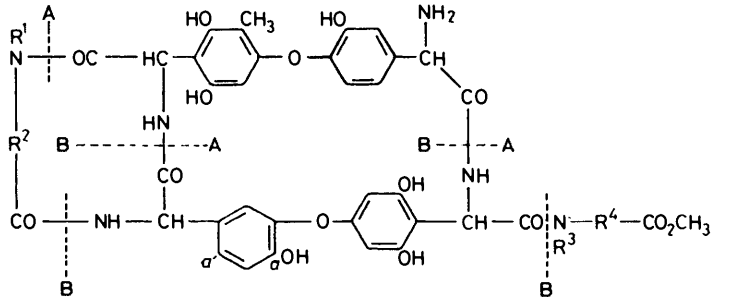
Independent evidence to support the presence of the same tetrasaccharide unit in ristocetin A has been obtained in the present work. Ristocetin A was subjected to mild acid hydrolysis and the liberated mixture of oligosaccharides permethylated using dimethyl sulphoxide anion as base, in conjunction with methyl iodide. The permethylated products were examined by ammonia chemical ionisation mass spectrometry.[16] The mass spectra contained high abundance and characteristic ions at *m/e* 472; 614 and 646; and 774 and 806. These *m/e* values are consistent with the occurrence of $(M + NH_4)^+$ ions associated with permethylated mannosylglucose (*m/e* 472), rhamnosyl(mannosyl)glucose (*m/e* 646) and rhamnosyl(arabinosylmannosyl)glucose (*m/e* 806); and further peaks (*m/e* 614 and 774) due to loss of MeOH from the $(M + NH_4)^+$ ions of the tri- and tetra-saccharide units. These assignments were confirmed by experiments which employed deuteriopermethylation with CD_3I. In these experiments, the previously cited peaks shifted to *m/e* 496; 641 and 676; and 807 and 842; the mass shifts establish the presence of the required 8, 10, and 12 hydroxyl-groups in the di-, tri-, and tetra-saccharide units prior to permethylation.



Much less is known about the structure of the aglycone

and, in the light of the identity of ristocetin A and ristomycin A, there appear to be inconsistencies in earlier conclusions. Russian workers [17] have proposed the partial structure (4) for the aglycone.

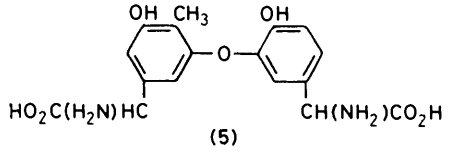
In particular, acid hydrolysis of either ristomycin A



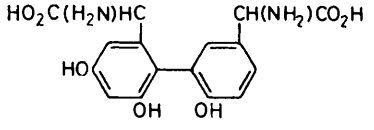
(4)

or aglycoristomycin A was reported to produce ristomycinic acid, $C_{17}H_{18}N_2O_8$, corresponding to the bis-amino-acid formed by hydrolytic cleavage of the molecule at the points marked A. A second bis-amino-acid available from acid hydrolysis was actinoidinic acid, $C_{16}H_{16}N_2O_8$, corresponding to the bis-amino-acid formed by hydrolytic cleavage of the molecule at the points marked B. It has been more recently suggested that actinoidinic acid is more likely to be the structure in which the phenolic hydroxy at *a* is relocated at *a'*.[18,19]

In contrast, Tarbell *et al.*[20,21a] have reported the isolation of (5) following acid hydrolysis of ristocetin A, and established its substitution pattern by independent synthesis of an oxidative degradation product of (5).[21a] They also obtained a second unusual amino-acid, to which they were unable to assign a structure. However, from mass spectra of derivatives,[20] it was believed to be a lower homologue ($C_{16}H_{16}N_2O_7$) of 5. We subsequently provide evidence that this amino acid should be formulated as 2',4,6-trihydroxybiphenyl-2,3'-diyldiglycine (6).* The latter is also a component of vancomycin.[22]



(5)

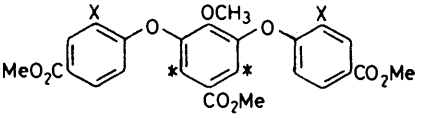


(6)

Our n.m.r. and mass spectral studies establish that ristocetin A (ristomycin A) contains only one aromatic methyl group (see later). Therefore, if the Russian and American workers have been working with homogeneous and identical samples of antibiotics, then either the structure of ristomycinic acid [17] or of (5) must be in error. Both structural proposals appear to be based on firm evidence and we cannot, at this stage, suggest how they should come to be different.

However, our own studies on ristocetin A (having the same n.m.r. spectrum as a sample of ristomycin A, provided by Dr. F. Sztaricskai) are entirely consistent with structure (5) but have provided no evidence to support the structure of ristomycinic acid which is incorporated in (4) (see later).[21b]

The work of Tarbell *et al.*[21a] has furnished a further aromatic fragment, (7), following hydrolysis and oxidation of methylated products from ristocetin A and finally, methylation of acidic products. A similar fragment, (8), was obtained by degradation of vancomycin.



(7) X = H
(8) X = Cl

The summarised literature data would, on the basis of the evidence presented so far, be consistent with the presence of at least 9 benzene rings in the antibiotic [actinoidinic acid from the lower central portion of (4), plus the units from (5), (6), and (7)]. We therefore obtained ^{13}C and ^1H n.m.r. spectra of ristocetin A, the latter at 360 MHz to give optimum separation of signals, in order to establish the approximate numbers of

TABLE 1

^{13}C Magnetic resonance spectrum of ristocetin A in D_2O or $(CD_3)_2SO$ solution [a]

Chemical shift range [b]	Approximate number of carbons	Assignment
180—165	6—7 (7)	*C*=O
160—140	10 ± 1 (11 ± 1)	aromatic *C*–O
140—110	25 ± 2 (28 ± 2)	aromatic *C*
110—90	11 ± 1 (13 ± 1)	high field aromatic *C*; 6 sugar anomeric *C*
90—63	20 ± 2 (24 ± 2)	sugar *C*–O/*C*–N
63—45	14 ± 2 (15 ± 3)	amino acid α-*CH*; $-OCH_3$
30—5	4—5 (5)	aromatic CH_3; rhamnose CH_3; ristosamine CH_3 and CH_2

[a] Spectra recorded at 67.8 Hz. [b] δ Relative to internal sodium 3-(trimethylsilyl)propanesulphonate.

* Since the preparation of this manuscript the same conclusion has been published by Harris and his co-workers.[21b]

chemically distinct carbons and hydrogens. The data are given in Tables 1 and 2.

TABLE 2

^{1}H Magnetic resonance spectrum of ristocetin A in $(CD_3)_2SO$/7% D_2O [a]

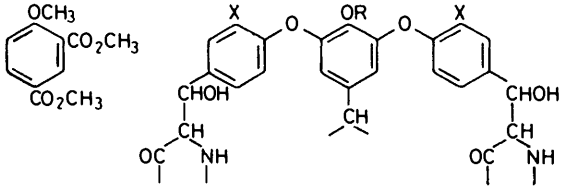
Chemical shift [b]	No. of protons	Assignment
1.11; 1.26	3; 3	Secondary methyl groups of rhamnose and ristosamine
2.00	3	aromatic methyl
2.0 and 2.2	2	CH_2 of ristosamine
2.8—4.1	31 ± 3 [c]	Sugar protons on carbons bearing one oxygen or one nitrogen; CH_3O– at 3.74
4.2—6.0	17	6 anomeric protons of sugars; α-protons of amino acid groups; high field aromatics
6—8	18	aromatic protons

[a] Spectrum recorded at 360 MHz. [b] δ Relative to internal sodium 3-(trimethylsilyl)propanesulphonate. [c] Estimated from relative areas of the sugar methyl peaks and the unresolved multiplet δ 2.8 to 4.1 in the 270 MHz spectrum of the antibiotic in $(CD_3)_2SO$–CD_3CO_2D (3 : 1) solution.

In Table 1, assignments of the various groups of resonances are based on those reported for suitable model compounds.[23] Signals in the range 90—160 must account for all the aromatic carbons as well as 6 sugar anomeric carbons; the spectra in D_2O and $(CD_3)_2SO$ then indicate 40 ± 4 and 46 ± 4 aromatic carbons, respectively. These data are clearly inconsistent with the presence of 9 benzene rings in ristocetin (54 aromatic carbons), but rather suggest the presence of only 7 benzene rings (42 aromatic carbons). We therefore conclude that one of the dibenzenoid fragments [(6), or that (actinoidinic acid) incorporated into the lower portion of (4)] is not present in ristocetin. The available evidence points to the fact that actinoidinic acid was incorrectly identified [as incorporated in (4)], and is, in fact, (6). Consistent with this conclusion are (i) the reported [18] resistance of actinoidinic acid to catalytic hydrogenolysis and, most importantly, (ii) the report [19] that vancomycin, ristocetin, ristomycin, and actinoidin all share, in actinoidinic acid, a common hydrolysis product; vancomycin, whose structure is now established from an *X*-ray study,[24] is known not to contain the unit from (4), but does incorporate (6). It is noteworthy that in proposing what appears to be an incorrect structure for actinoidinic acid, Lomakina *et al.*[19] excluded a biphenyl structure on the basis of its u.v. spectrum alone, and do not appear to have considered the possibility that the two rings may be almost orthogonal (as in vancomycin [24]). Additionally, their molecular weight appeared to rely solely on elemental analysis.

Our proposal that all benzene rings of ristocetin are accounted for by the units (5), (6), and (7) is also supported by the 360 MHz proton n.m.r. spectrum (Table 2). As in vancomycin,[25] two aromatic protons of ristocetin [those attached to the carbons marked with an asterisk in (7)] are likely to resonate in the range δ 5—6, due to (i) the high electron density in the substituted pyrogallol ring and (ii) shielding effects of the adjacent aromatic rings. The presence of a further 18 aromatic protons (Table 2) is in precise agreement with the presence of (5), (6), and (7) in ristocetin.

The product $C_{16}H_{16}N_2O_7$, isolated by Tarbell *et al.*[20] but not identified by them, is established in the present work to be (6). Ristocetin A was hydrolysed (6M-HCl) and two ninhydrin-staining and u.v.-absorbing (at 280 nm) fractions isolated after chromatography on a cation-exchange column. These two fractions were eluted at pH 3.25 and 4.25; the former fraction was esterified (MeOH–HCl) and acetylated (Ac_2O–MeOH). The product gave a molecular ion at *m/e* 460, corresponding to the formation of a di-*N*-acetyl dimethyl ester derivative. Treatment of this product with an ethereal solution of diazomethane resulted in an increase in molecular weight of 42 m.u., corresponding to the presence of three phenolic OH-groups. Although the hydrolysis product corresponding to this fraction could not be crystallised (perhaps due to the fact that it is likely to be a mixture of diastereoisomers), the proton n.m.r. spectrum was consistent with (6). Most convincingly, an identical fraction was produced on hydrolysis (and chromatographic separation of products) of vancomycin; this fraction showed spectroscopic (u.v., n.m.r., and mass) and chromatographic (ion-exchange, t.l.c., paper electrophoresis) properties which establish its identity with the product $C_{16}H_{16}N_2O_7$ isolated from ristocetin. Since (6) is rigorously established as being incorporated in the vancomycin structure,[24] then the same unit is also present in ristocetin. Finally, we note that Tarbell *et al.*[21] isolated dimethyl 4-methoxyisophthalate (9) from oxidative experiments on ristocetin A; the isolation of (9) is in accord with the presence of the biphenyl unit (6) in ristocetin.



(9)

(10) X = Cl, R = disaccharide
(11) X = H

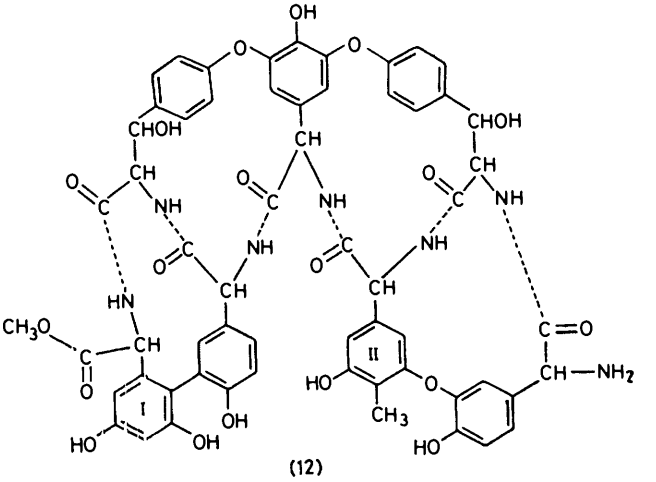
In the case of vancomycin, the degradation product (8) was extended to the structural unit (10) by establishing that an analogue of the retro-aldol reaction gave rise formally to 2 moles of glycine (experimentally, 1.2 moles) upon base-catalysed hydrolysis.[26] Similar experiments were, therefore, carried out on ristocetin A. Ristocetin A was hydrolysed under alkaline conditions, and the hydrolysate acidified and treated with dansyl chloride; t.l.c. established the presence of dansylglycine in the product. Quantitative determination by amino-acid analysis showed the presence of 0.8 mole glycine (per 2 300 g ristocetin). It therefore appears likely that ristocetin incorporates one or two potential glycine units, which may be incorporated [see (11)] as in vancomycin.

The structural similarities between vancomycin and

ristocetin suggest that the two antibiotics might inhibit cell-wall synthesis in bacteria in a closely similar way. Indeed, before the structural similarities were apparent, it had been shown that both antibiotics form complexes with mucopeptide precursors from various bacteria, and will specifically interact with acetyl-D-alanyl-D-alanine.[8,27] The complexation of vancomycin to acetyl-D-alanyl-D-alanine may also be studied by proton n.m.r. spectroscopy,[28,29] and a similar study of the ristocetin A/acetyl-D-alanyl-D-alanine system was, therefore, undertaken. When Ac-D-Ala-D-Ala (*ca.* 0.5 mol equivalents) is added to a solution of ristocetin A in $(CD_3)_2SO$ at 40 °C, only signals due to bound Ac-D-Ala-D-Ala are seen at 1.82 (*N*-acetyl), 0.83 and 0.40 (Ala-CH_3 resonances). In the absence of ristocetin A, under the same conditions these resonances are observed at 1.84, 1.29, and 1.20, establishing that one of the Ala-CH_3 resonances is in a strongly shielded environment in the antibiotic/peptide complex, probably due to its proximity to the face of a benzene ring (*cf.* the behaviour of the vancomycin/Ac-D-Ala-D-Ala complex [28]). In addition, separate signals can now be seen for the approximately equimolar proportion of bound and free ristocetin A. The assignment of two of the signals due to bound and free ristocetin A have been checked by varying the mole fraction of added Ac-D-Ala-D-Ala, and are aromatic CH_3 (bound, 2.06; free, 2.00) and secondary methyl of a sugar (bound 1.02; free 1.11). Upon increasing the temperature of the solution to 80 °C, the above pairs of resonances collapse to broad singlets. In addition, the CH_3-doublet signals of the bound Ac-D-Ala-D-Ala broaden continuously up to 100 °C, as the dissociation rate of the complex increases. This rate is calculated to be 60 s^{-1} at 80 °C.

An incidental finding from variable-temperature proton n.m.r. studies on ristocetin A was made in deuterium oxide solution. In such spectra, recorded at 90 °C, the three C-methyl resonances of the antibiotic appear at δ 2.16, 1.43, and 1.07; in the course of cooling to 24 °C, the doublet methyl resonance at 1.07 first broadens and finally splits into two broad resonances of approximately equal intensity centred at 0.98 and 1.28 p.p.m. These data show that in aqueous solution, a secondary methyl group of a sugar residue is undergoing relatively slow exchange between two almost equally populated sites. By taking data from spectra in which the line-width of this methyl resonance is determined by the exchange rate rather than the relaxation time, the unimolecular rate constant for the exchange process is calculated to be *ca.* 40 s^{-1} at 24 °C and 500 s^{-1} at 60 °C. The splitting of the methyl resonances does not occur in $(CD_3)_2SO$ solution at the corresponding temperatures.

The n.m.r. evidence for formation of a complex between ristocetin A and Ac-D-Ala-D-Ala, in which the *C*-terminal methyl resonance of the dipeptide is strongly shielded (*cf.* vancomycin [28,29]), is further evidence for closely analogous binding of the two antibiotics to the dipeptide. It appears likely therefore that the almost invariant parts [(6) and (7) (or possibly (11)] of these two antibiotics are joined in a like manner. If (5) is then incorporated into such a structure through the formation of secondary amide bonds, then the peptide nucleus must be tetracyclic. This follows since the peptide nucleus is known to contain only one free primary amine group and also one methyl ester group (CO_2CH_3).[18] One possible scheme of connectivity is shown in (12), where the putative secondary amide bonds are indicated by dotted lines. The structure of ristocetin A would then be completed by attachment of the three sugar units (1)—(3) to three of the eight hydroxys of the peptide nucleus.



(12)

Although the positioning of the H_2N–, $-CO_2CH_3$, and secondary amide bonds is relatively arbitrary (except insofar as they must be stereochemically feasible, and are chosen to give a high degree of similarity to the vancomycin structure [24]), further evidence for such a type of tetracyclic structure has been sought and found.

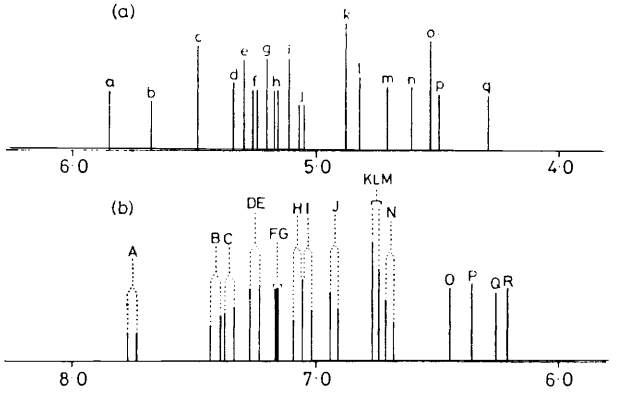
(i) In such a structure, ristocetin A would have a formula $C_{95}H_{110}N_8O_{44}$. In the two reported ^{13}C spectra (Table 1), the number of carbons are 90—92 (±8) and 103 (±9). The number of carbonyl carbons (7) is consistent with the proposal.

(ii) A structure of type (12) (as the glycoside) contains 110 protons, of which 31 should be replaceable by ^2H in the presence of D_2O, leaving 79 carbon-bound protons. The number of protons observed in the 360 MHz spectrum is 77 ± 3 (Table 2). Moreover, a detailed analysis of the 4.0—6.0 and 6.0—8.0 p.p.m. regions (Figure) provides further support. In the former region (Figure), by analogy to vancomycin,[25] we should find signals due to 2 C*H*OH protons (of β-hydroxytyrosine units), 6 anomeric protons of the sugar, 7 α-C*H* protons of the amino-acid residues, and the two high-field aromatic protons [see asterisks in (7)]. This is precisely the total number (17) found (Table 2). Although it was not possible to assign all the peaks in this region, the following assignments have been made: (a) α-C*H* of amino-acid residues: b, g, j, n, p, and q are decoupled on irradiating N*H* resonances at 7.7, 7.2, 7.4, 8.7, 9.2, and 7.1 p.p.m. respectively; all sharpen when D_2O is used to

exchange NH for ND; j in addition is coupled to h. (b) C*H*OH proton of β-hydroxytyrosine: h is coupled to α*CH* j. (c) anomeric protons: f, l; the former can be decoupled from a proton resonating at δ 3.6, and the latter from the ristosamine H-2 obscured by the aromatic methyl resonance at δ 2.0.

The protons on the 6 aromatic rings which will resonate in the 6.0—8.0 p.p.m. region should give rise to 12 signals which are split by *ortho*-coupling and 6 which are not. The observed splittings are consistent with this requirement; moreover, the observed chemical shift values are in accordance with expectations based on the reported shifts in vancomycin [25] and in a derivative of the biphenyl ether (5).[20] Particularly noteworthy are the 4 relatively high-field signals (O, P, Q, R) associated with protons *ortho* or *para* to two *meta*-oriented, electron-donating substituents of rings I and II in (12).

(iii) The proposed tetracyclic structure corresponds to a molecular weight of ristocetin A of 2066 Daltons. The



Line diagrams of the (a) δ 4.0—6.0 and (b) 6.0—8.0 regions of the ^{1}H n.m.r. spectrum of ristocetin A in $(CD_3)_2SO$ containing 7% D_2O at 50 °C

californium plasma desorption mass spectrum of ristocetin A (kindly determined by Professor R. Macfarlane) affords a peak in the positive ion spectrum at *m/e* 2 086 ± 5 Daltons. On the basis of the normal expectation [30] that this peak would correspond to a $(M + Na)^+$ species, then the molecular weight of the antibiotic is 2 063 ± 5 Daltons.

EXPERIMENTAL

^{1}H N.m.r. spectra were obtained with a Varian XL100 and Bruker 270 MHz and 360 MHz spectrometers operating in the Fourier transform mode. Electron impact mass spectra were obtained at 70 eV with an A.E.I. MS 902 instrument, and the ammonia chemical ionisation spectra were recorded with an A.E.I. MS 30 mass spectrometer.

Analytical t.l.c. was carried out on Merck plates coated with silica GF 254 and visualised under u.v. light and iodine vapour.

A Unicord 2000 fraction collector system was used for the ion-exchange chromatography. Paper electrophoresis was performed at pH 6.5 for 45 min at 3kV in pyridine–acetic acid buffer, with toluene as coolant. U.v. spectra were obtained on a Unicam SP 800 spectrophotometer. Amino-acid analysis was carried out on a Locarte analyser.

Base-catalysed Hydrolysis of Ristocetin.—Ristocetin A (11.3 mg) was dissolved in sodium hydroxide solution (4M; 1.5 ml), the vessel was sealed, and heated at 110 °C for 16 h. The reaction mixture was allowed to cool and was incubated with a solution of dansyl chloride (5 mg) in acetone (2 ml) at 37 °C overnight.

The reaction mixture was evaporated to dryness and the residue was extracted with chloroform containing 10% methanol. The extract was spotted on a polyamide plate (15 cm × 15 cm) and developed in two dimensions, using the following solvent systems: (*a*) 1.5% formic acid, (*b*) toluene–acetic acid (9 : 1), and (*c*) butyl acetate–methanol–acetic acid (20 : 1 : 1). Solvents (b) and (c) were run in the same direction, perpendicular to solvent (a). By comparison with dansylglycine as standard, the dansylated alkaline hydrolysate indicated the presence of dansylglycine. Dansylglycine appears as a green fluorescent spot under ultraviolet light.

Quantitative Analysis of Glycine.—A hydrolysis similar to that mentioned above was performed on an accurately weighed amount of ristocetin A. The hydrolysate was neutralised with concentrated hydrochloric acid. An amino-acid analysis of the hydrolysate showed the presence of 0.82 mole of glycine per 2300 gm of ristocetin A.

Strong Acid-catalysed Hydrolysis of Ristocetin.—A solution of ristocetin A (200 mg) in hydrochloric acid (6M; 200 ml) was heated under reflux under a nitrogen atmosphere at 105 °C for 12 h. The hydrolysate was freeze-dried, redissolved in a minimum volume of sodium citrate solution (2M; pH 1.5) and was applied to a cation exchange (Dowex 50X8, 200—400 mesh, H^+ resin) column (1.5 cm × 40 cm). The column was eluted with sodium citrate buffer (2M; pH 3.25) and the elution monitored at 280 nm. An ultraviolet absorbing component was eluted.

The eluate was freeze-dried and redissolved in a minimum volume of water and was acidified to pH 2.2 with concentrated hydrochloric acid. The solution was desalted by passage through a cation-exchange column of Zeo Carb ZK 225 (H^+ form) resin. The sodium citrate buffer was removed by eluting the column with water. The ultraviolet-absorbing (280 nm) component was collected by elution with ammonia solution (2M). The eluate was freeze-dried and weighed (12 mg).

Analytical t.l.c. developed in absolute ethanol–water (7 : 3) shows a u.v. absorbing spot (R_F 0.15) which gave positive reactions with ninhydrin and diazotised sulphanilic acid.

An ultraviolet spectrum shows a $\lambda_{max.}$ at 285 nm in acid media. A bathochromic shift of 18 nm to 303 nm occurs in alkaline media. Paper electrophoresis showed that at pH 6.5 the net charge was zero.

The 100 MHz ^{1}H n.m.r. spectrum in D_2O showed the presence of signals corresponding to the presence of aromatic protons (δ 6.5 to 7.4) and two methine protons (δ 4.42 and 4.80). The spectrum was poorly resolved.

Esterification (MeOH–5% HCl, overnight) and *N*-acetylation (MeOH–Ac_2O, 2 : 1, overnight) of the compound followed by electron impact studies gave a mass spectrum which showed *m/e* 460 (M^+), 442 ($M^+ - H_2O$), 428 (M^+ — MeOH), 417 ($M^+ - CH_3CO$), 401 ($M^+ - CO_2Me$), and 359 ($M^+ - CO_2Me - CH_2CO$).

A methanolic solution of the above derivatised compound was treated with an excess of diazomethane in ether for 5 h. A mass spectral study of the methylated compound showed

the following fragmentation pattern: *m/e* 502 (M^+), 470 ($M^+ - CH_3OH$), 459 ($M^+ - CH_3CO$), 443 ($M^+ - CO_2Me$), and 401 ($M^+ - CO_2Me - CH_2CO$).

Strong Acid-catalysed Hydrolysis of Vancomycin.—Vancomycin was hydrolysed under strong acid conditions (6M-HCl, 105 °C, 17 h) and the hydrolysate passed through the same cation-exchange (Dowex 50 × 8) column used in the above experiment. An ultraviolet-absorbing (280 nm) fraction was eluted at pH 3.25 (sodium citrate buffer) at about the same time the ristocetin fraction had eluted in the above experiment, allowing for a slight variation due to flow rate and temperature. The fraction was then desalted by passage through the Zeo Carb ZK 225 resin.

The vancomycin fraction had identical spectroscopic and chromatographic properties to the fraction isolated from the ristocein hydrolysate. The ultraviolet spectrum showed a $\lambda_{max.}$ of 285 nm in acid, and 303 nm in base. T.l.c. shows an R_F of 0.15 (ethanol–water 7 : 3 system). The ^1H n.m.r. spectrum recorded for a D_2O solution shows signals as above (representing aromatic and methine protons). Paper electrophoresis (pH 6.5, 3 kV, 45 min) shows it to have net zero charge (with reference to glycine).

The esterified (MeOH–5% HCl), *N*-acetylated (MeOH–Ac_2O) fraction gave the following ions on electron impact: *m/e* 460, 442, 428, 417, 401, and 359. The subsequently methylated (CH_2N_2) product gave *m/e* 502, 470, 459, 443, and 401.

Permethylation of Hydrolysed Ristocetin A.—Ristocetin A (11.3 mg) was dissolved in water (0.6 ml) and hydrochloric acid (4M; 0.13 ml) added. The reaction vessel was placed in a water bath (100 °C) for 90 s. The reaction mixture was subsequently divided into two equal volumes and freeze-dried.

One half was dissolved in dry Me_2SO (1 ml) and treated with sodium methylsulphonyl methide in Me_2SO [from complete reaction of sodium hydride (200 mg) with Me_2SO (4 ml)] till a drop of the solution gave a red colour with triphenylmethane. After 2 min, methyl iodide (2 ml) was added and after a further 5 min the reaction was quenched with water, chloroform (2 ml) was added, and the organic phase washed thoroughly with water.

The other half of the ristocetin hydrolysate was treated in a similar manner, where methyl iodide was replaced by [$^2H_3$]methyl iodide.

The permethylated and perdeuteriomethylated fractions were subsequently subjected to ammonia chemical ionisation mass spectral studies.

We thank Lundbeck and Co. (Copenhagen) for generous gifts of ristocetin A, and for h.p.l.c. comparison of ristocetin A and ristomycin A; Dr. J. Chapman (Kratos–AEI) for the ammonia chemical ionisation spectra, and Professor R. D. McFarlane (Texas A and M University) for the californium plasma desorption mass spectra; Professor R. Bognar and Dr. F. Sztaricskai (University of Debrecen, Hungary) for a sample of ristomycin A; and Professor G. F. Gause (Institute of New Antibiotics, Moscow), for an exchange of information. J. R. K. thanks the University of Sydney for the award of Eleanor Sophia Wood Travelling Fellowship. We are also grateful to the Universities of Copenhagen and Groningen for time on Bruker 270 MHz and 360 MHz n.m.r. spectrometers and Dr. C. A. Auffret for performing the amino acid analysis.

[8/451 *Received*, 13*th March*, 1978]

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