Conclusions

(1) On the basis of potentiometric studies, we conclude that a single proton is displaced from the primary amine moiety of the diaminopropionamide portion of the bleomycin molecule on binding Ca(II) and Tb(III).

(2) The binding of the lanthanides occurs with a large entropy of complexation suggesting that both the metal ions and the antibiotic are desolvated on complex formation.

(3) The complexes of bleomycin with the lanthanides are relatively long lived. These results, taken together with our previous studies on the Zn(II) and Ga(III) complexes of these antibiotics, indicate that these metal complexes are kinetically stable. This suggests that the interpretation of Gd^{3+}-induced relaxation rate enhancements in terms of intermolecular distances may not be possible.

(4) The perturbations induced by Pr(III) in the ^1H spectrum of the antibiotic indicate that the valeric acid group, the dissaccharide, as well as the histidine, are in close proximity to the site of metal complexation. Further structural studies based on these Pr(III) results are in progress in our laboratories.

Structural Studies of Ristocetin A (Ristomycin A): Carbohydrate–Aglycone Linkages

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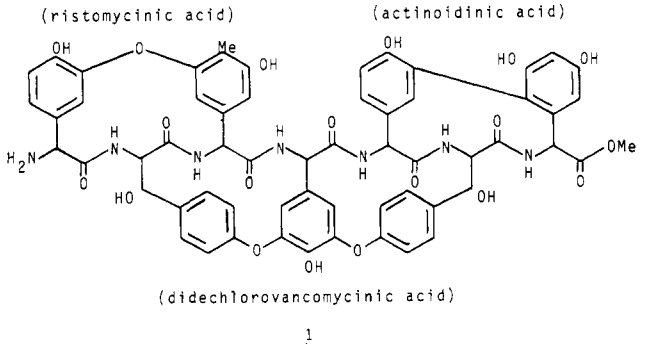
Abstract: The structure of the carbohydrate component of the antibiotic ristocetin A has been elucidated. The amino sugar L-ristosamine has been identified along with previously isolated D-glucose, D-mannose, D-arabinose, and L-rhamnose. Acid-catalyzed acetolysis of the antibiotic yielded acetylated ristotetrose along with mono-, di-, and trisaccharides derived from it. Base hydrolysis of O-methylated antibiotic in the presence of $NaBH_4$ has shown that the O-α-D-arabinofuranosyl-(1→2)-*O*-α-D-mannopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranosyl residue is attached to the phenolic hydroxyl group of didechlorovancomycinic acid and the L-ristosaminyl residue to one of the two alcoholic hydroxyl groups in that amino acid. Controlled acid hydrolysis of the antibiotic yielded a partial hydrolysis product which still contained ristosamine; ^{13}C NMR spectroscopy indicated an α linkage for that sugar. An additional mannose residue is attached to actinoidinic acid. The phenolic hydroxyl group to which it is attached was identified unambiguously by ^1H NMR measurements on a degradation product and by an independent synthesis of that compound; the anomeric configuration of mannose was not established. Further evidence is presented supporting the belief that ristocetin A and ristomycin A are identical. The structural data now available permit conclusions to be drawn concerning the mechanism of action of antibiotics in this class, particularly with regard to differences in structural specificity observed in the binding of small aliphatic peptides to vancomycin and ristocetin.

The glycopeptides ristocetin and ristomycin, produced by *Nocardia lurida*[1] and *Proactinomyces fructiferi* var. *Ristomycini*,[2] respectively, belong to the vancomycin group of antibiotics. Both antibiotics contain two biologically active components, A and B, of which the A form predominates.[3,4] Mounting evidence, both chemical and biological, indicates that the two antibiotics are identical. Studies in this laboratory and elsewhere have established that the peptide moieties of both ristocetin A[5,6] and ristomycin A[7] contain ristomycinic acid, actinoidinic acid, and didechlorovancomycinic acid. Structure **1** has been proposed for this peptide



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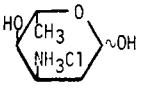
by analogy with the known structure of vancomycin;[5,6] N-terminal analysis of ristocetin A and ristomycin A supports this proposal.[8]

Studies of ristomycin A have shown that it contains 2 mol of D-mannose and 1 mol each of D-glucose, D-arabinose, L-rhamnose,[9,10] and the amino sugar L-ristosamine (**2**).[11] The



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structure of **2** was established by Sztaricskai via independent synthesis.[12–14] He has also inferred the presence of a tetra-

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(2) Brazhnikova, M. G.; Lomakina, N. N.; Sztaricskai, F.; Puskás, M.; Makleit, S.; Bognár, R. *Kem. Kozl.* **1967**, *27*, 143.
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(8) Harris, C. M.; Harris, T. M. *Tetrahedron Lett.* **1979**, 3905.
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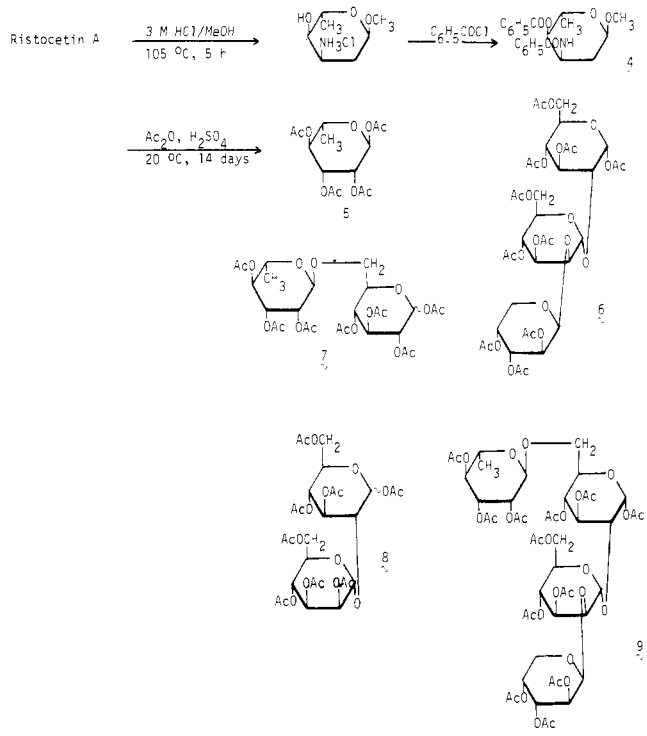
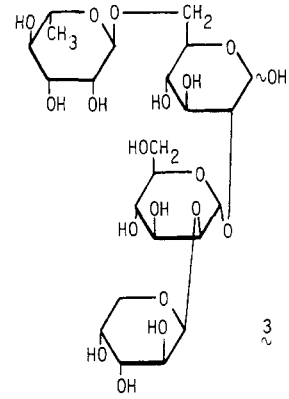
Scheme I



saccharide fragment.[15] Acetolysis of the antibiotic yielded acetate derivatives of the disaccharides rutinose and ristobiose and the trisaccharides ristriose and ristotriose. By comparison of these materials with natural and synthetic samples[16] and by ^{13}C NMR spectroscopy,[17] the fugitive tetrasaccharide was assigned the structure *O*-β-D-arabinopyranosyl-(1→2)-*O*-α-D-manno-pyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→6)]-D-gluco-pyranose (**3**). The same neutral sugars have been isolated from



ristocetin A,[18] and spectroscopic evidence has been put forth that ristosamine is also present.[6,19] Williams, employing chemical ionization mass spectroscopy of an oligosaccharide mixture resulting from partial hydrolysis of permethylated ristocetin A, has obtained molecular and fragment ions from di-, tri-, and tetrasaccharide products which are consistent with a rhamnosyl(arabinosylmannosyl)glucose unit being present.[6] We now report our studies of the carbohydrate portion of ristocetin A which confirm many of the earlier structural conclusions. Key findings include identification of the three sites of carbohydrate attachment to the aglycone, assignment of a β configuration for the D-glucosyl residue, and assignment of an α-furanosyl structure for the D-arabinosyl residue.

Table I. Anomeric Signals in the ^{13}C NMR Spectrum of Dodeca-*O*-acetyl-α-ristotetrose (**9**)

assignment	chemical shift, ppm	$^1J_{CH}$, Hz
α-D-glucosyl	88.3	173
β-D-arabinosyl	94.2	172
α-D-mannosyl	95.9	171
α-L-rhamnosyl	98.2	173

Results

Presence of Ristosamine. Ristocetin A was subjected to acid-catalyzed methanolysis in order to establish that ristosamine was, in fact, a constituent of the antibiotic. Methyl α-L-ristosaminide hydrochloride was isolated after ion-exchange chromatography; acylation with benzoyl chloride gave methyl di-*N*,*O*-benzoyl-α-L-ristosaminide (**4**) (Scheme I). Both ristosamine derivatives were identical with synthetic samples and with materials isolated from ristomycin A by Bognár et al.[11-14]

Isolation of Dodeca-*O*-acetyl-α-ristotetrose (9). Ristocetin A was treated with acetic anhydride, following the procedure previously employed for analysis of ristomycin A.[15] Chromatography of the acetolysis products revealed that degradation had not been as extensive as had been observed with ristomycin; the principal products were tetra-*O*-acetyl-α-L-rhamnose (**5**), deca-*O*-acetyl-α-ristriose (**6**), and a product which had not been observed in the ristomycin studies. Trace amounts of hepta-*O*-acetylrutinose (**7**) and octa-*O*-acetylristobiose (**8**) were detected by TLC.

The new compound has been shown to be dodeca-*O*-acetyl derivative **9** of the fugitive ristotetrose (**3**). Acid hydrolysis of **9** gave the four constituent monosaccharides. The ^{13}C NMR spectrum of **9** contained four signals for anomeric carbons (see Table I) which could be assigned by appropriate chemical shift comparisons with peracetates of α-ristriose (**6**), α-rutinose (**7**), and α-ristobiose (**8**).[17] With the method of Bock and Pedersen, configurational assignments of the anomeric centers in **9** have been made from $^1J_{CH}$ coupling constants.[20] The α assignments for mannose and rhamnose and the β assignment for arabinose (Table I) were identical with those previously deduced.[17] The configuration of the anomeric center in glucose was assigned as α on the basis of the coupling constant (173 Hz) and the chemical shift (88.3 ppm). Preferential cleavage during acetolysis to give rhamnose and ristriose is consistent with the previous observations that (1) loss of rhamnose and other 6-deoxy sugars is facile[21] and (2) 1→6 glycosidic linkages are ruptured more readily than 1→2 linkages.[22]

Of particular note in relation to results to be described subsequently is the fact that the ^{13}C NMR spectrum of **9** is fully consistent with D-arabinose being β-pyranosyl rather than α-furanosyl; the latter would have yielded a ^{13} NMR signal for the anomeric carbon in the region of 107 ppm in contrast to the observed signal at 94.2 ppm.

Methylation. The sites of attachment of sugars to the aglycone were determined by a procedure involving sequential methylation

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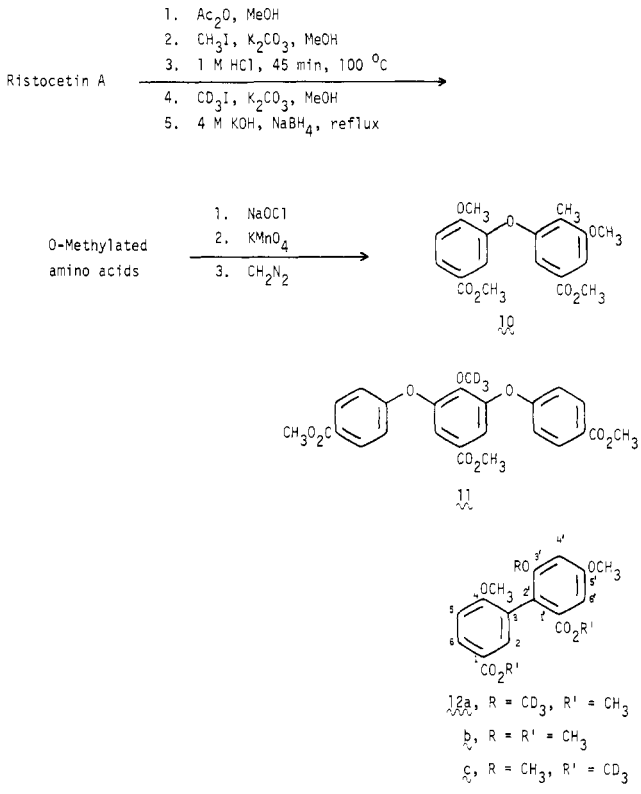
(21) (a) Compton, J. *J. Am. Chem. Soc.* **1938**, *60*, 1203: (b) Lindberg, B.; Lönngren, J.; Svensson, S. *Adv. Carbohydr. Chem. Biochem.* **1975**, *31*, 185.
(22) Guthrie, R. D.; McCarthy, J. F. *Adv. Carbohydr. Chem.* **1967**, *22*, 11.

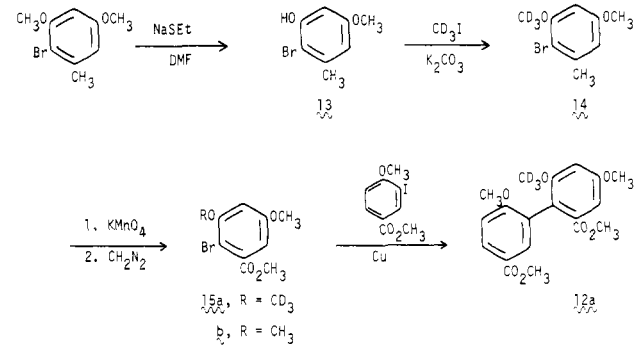
Scheme II



with CH_3I and CD_3I; similar techniques have been used previously in structural studies of carbohydrate linkages in vancomycin[23] and avoparcin.[19] Ristocetin A was methylated with CH_3I in the presence of K_2CO_3. Removal of the sugars by mild acid hydrolysis was followed by further methylation with CD_3I/K_2CO_3 (Scheme II). The aglycone was then hydrolyzed by using KOH and $NaBH_4$, and the resulting amino acids were degraded by oxidation with NaOCl followed by $KMnO_4$ to give substituted benzoic acids. The methyl esters, obtained by CH_2N_2 treatment, were separated chromatographically.

Diester **10**, derived from ristomycinic acid, contained only a trace of CD_3 in the *O*-methyl groups. Triester **11**, derived from didechlorovancomycinic acid, contained one CD_3 group, indicating that one carbohydrate linkage to the antibiotic occurred through the phenolic group on this amino acid. Diester **12a**, arising from actinoidinic acid, also contained one CD_3 group, indicating a second site of carbohydrate attachment. The site of this CD_3 group was determined spectroscopically. The five *O*-methyl signals in the ^1H NMR spectrum of undeuterated diester **12b** appear at δ 3.56, 3.70, 3.77, 3.86, and 3.88; the signal at δ 3.70 is missing in the spectrum of CD_3 analogue **12a**. The signals at δ 3.56 and 3.86 were assigned to the ester methoxyl groups by transesterification of **12b** with CD_3OH and CD_3ONa to give bis(trideuteriomethyl) ester **12c**. Assignments of the remaining three methoxyl signals in the spectrum of **12c** were made by using NOE measurements. Enhancements of individual aromatic signals were monitored during irradiation of each of the methoxyl signals. Peak heights were measured rather than peak areas because two of the aromatic signals (δ 6.94 and 7.04) were not fully resolved from each other at the 90-MHz observation frequency. The observed signal enhancements consequently reflect a combination of NOE and removal of weak long-range spin–spin coupling ($J < 0.3$ Hz) between methoxyl and aromatic protons. Interpretation of the results requires unambiguous assignments for the aromatic portion of the spectrum, but the aromatic signals can be reliably assigned on the basis of chemical shifts, multiplicities, and coupling constants. Irradiation of the δ 3.88 methoxyl group (4-OMe) caused

Scheme III



a 31% enhancement in the aromatic signal at δ 6.94 (5-H), irradiation of the δ 3.77 methoxyl (5′-OMe) caused a 27% enhancement of the signal at δ 7.04 (6′-H), and irradiation of the δ 3.70 methoxyl (3′-OMe) caused a 35% enhancement of the signal at δ 6.69 (4′-H). All other changes in peak heights were 10% or less and have been disregarded. On the basis of these enhancements, the deuterated compound derived from ristocetin A is assigned structure **12a**.

The assignment for the location of the CD_3 group in **12a** was confirmed by an independent synthesis (Scheme III). Treatment of 2-bromo-3,5-dimethoxytoluene with sodium ethanethiolate[24] brought about selective removal of the 3-*O*-methyl group to give 2-bromo-3-hydroxy-5-methoxytoluene (**13**). The structure of **13** was established by ^1H NMR. The aromatic protons at positions 4 and 6 were assigned to the signals at δ 6.48 and 6.43, respectively, on the basis of spin–spin coupling to the *C*-methyl group; the proton at position 6 exhibits a coupling constant of 0.65 Hz whereas the coupling constant for the 4 proton is substantially smaller such that splitting is not readily observed. The location of the hydroxyl group was assigned on the basis of changes in chemical shifts measured after addition of $Eu(fod)_3$; the OH, 4-H, 6-H, *C*-Me, and *O*-Me groups were shifted downfield 0.61, 0.19, 0.07, 0.04, and 0.03 ppm, respectively. Alkylation of **13** with CD_3I/K_2CO_3 gave **14**, which was identical with the starting material for the synthesis except the methoxyl signal at δ 3.82 was missing. Oxidation by hot $KMnO_4$ followed by esterification with CH_2N_2 gave ester **15a**. Undeuterated ester **15b** gives methoxyl signals at δ 3.81, 3.88, and 3.93 in the ^1H NMR spectrum; the signal at δ 3.88 is missing in the spectrum of **15a**. The structure of **15a** was supported by assignments of the three methoxyl signals in the spectrum of **15b** as the 5-*O*Me, 3-*O*Me, and ester respectively, on the basis of NOE measurements which showed substantial enhancement for the 6-H signal (δ 6.80) when the 5-*O*Me group was irradiated and for the 4-H signal (δ 6.58) when the 3-*O*Me group was irradiated. The final step in the synthesis involved coupling **15a** with methyl 3-iodo-4-methoxybenzoate in the presence of the Cu powder[25] to give **12a**, identical with the material derived from ristocetin. The cross-coupling reaction to give **12a** predominated over self-coupling processes when an excess of the iodo compound was employed and the reaction was held at 220 °C.

Base Hydrolysis. As a means of identifying the sugars attached to the two phenolic hydroxyl groups which had been shown above to be sites of glycosylation, hydrolysis of ristocetin A under alkaline conditions was investigated. Earlier work on ristomycin A had suggested that the tetrasaccharide, the second mannose, and ristosamine might each be attached directly to the peptide, requiring that one of these moieties be attached to the peptide at a site not detected by the methylation experiment.[9,10] As a consequence, base hydrolysis was carried out in the presence of $NaBH_4$ so that if one of the sugars should be linked to the peptide via the alcoholic hydroxyl group of one of the β-hydroxytyrosine residues in didechlorovancomycinic acid the sugar, when released by β elimination, would be reduced to the alditol rather than

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(24) Feutrill, G. I.; Mirrington, R. N. *Tetrahedron Lett.* **1970**, 1327.
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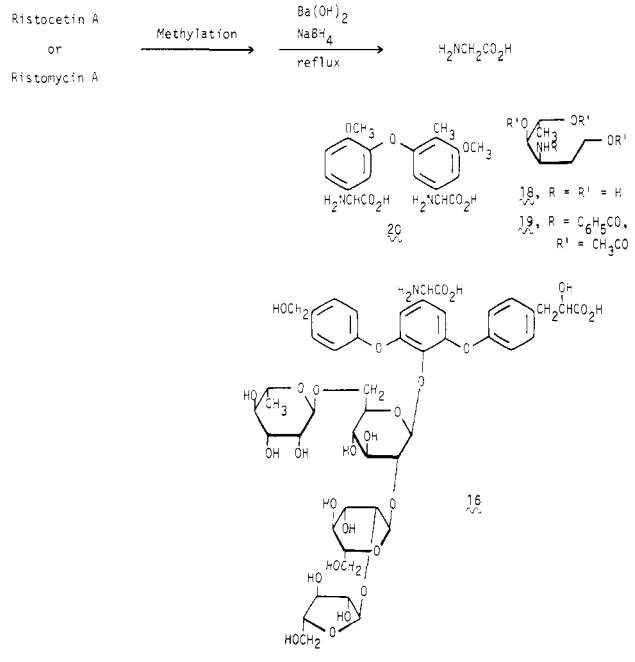
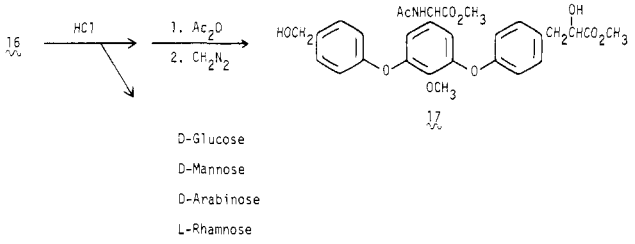
Scheme IV



Scheme V



undergoing alkaline degradation. Tanaka and Pigman have exploited base hydrolysis in the presence of $NaBH_4$ for structural identification of glycopeptides containing sugars linked to serine and threonine.[26] This process was recently used to demonstrate novel glycosidic linkages in the enzyme cutinase involving sugars linked to the alcoholic hydroxyl groups of β-hydroxytyrosine and β-hydroxyphenylalanine.[27]

Prior to the employment of the Tanaka and Pigman procedure, ristocetin A was treated with diazomethane to minimize oxidative degradation of unglycosylated phenols during alkaline treatment; it is probable that this procedure does not lead to complete derivatization of the four free phenolic hydroxyl groups. Hydrolysis was carried out for 48 h with a refluxing mixture of 0.2 M $Ba(OH)_2$ and 0.2 M $NaBH_4$ under N_2 (Scheme IV). Barium ion was removed from the hydrolysate by precipitation as $BaCO_3$ and $BaSO_4$. Chromatography on Sephadex G-25 gave compound **16** as the main product; the molecular weight of **16** is approximately half that of the parent antibiotic. The constitution of **16** was demonstrated by acid hydrolysis, which gave the four sugars and the amino acid (Scheme V). Acetylation and methylation of the latter gave **17**, for which the ^1H NMR and mass spectral data were identical with those of the compound prepared earlier by base–borohydride degradation of O-methylated aglycoristocetin[5] and aglycoristomycin.[7] Compound **17** is a partially degraded didechlorovancomycinic acid in which one of the β-hydroxytyrosine fragments has been transformed to a benzyl alcohol and the other to phenyllactate.

The ^{13}C NMR spectrum of **16** contained six signals in the 85–125-ppm region. A broad signal at 115.4 ppm is assigned to the two protonated carbons on the center aromatic ring. A somewhat more intense signal at 119.3 ppm, which was also broad, is assigned to the protonated carbons ortho to the oxy substituent on the two flanking aromatic rings. The four remaining signals appeared at 100.2, 101.8, 103.1, and 109.7 ppm and are assigned to the anomeric carbons of the four sugars. The assignments for the aromatic and carbohydrate signals were confirmed by single-frequency heteronuclear decoupling at various locations in the ^1H NMR spectrum. The strongly deshielded signal at 109.7 ppm is not consistent with the tetrasaccharide structure (**9**) which had been inferred from the acetolysis studies, irrespective of whether the unassigned anomeric configuration of the glucopyranosyl residue in **16** is α or β. However, the chemical shift is readily rationalized if one of the sugars is present in a furanose structure.[28] ^{13}C NMR values in the range 108–110 ppm are observed for the anomeric carbons of methyl pento- and hexofuranosides which have opposing configurations at C-1 and C-2. We conclude the D-arabinose is present in **16** as the α-furanoside. ^{13}C NMR signals at 82.0 and 84.4 ppm, which can be assigned to C-2 and C-4, respectively, of an α-D-arabinofuranosyl residue, support this conclusion. Furthermore, the furanosyl assignment for arabinose is consistent with the observation by Sztaricskai and co-workers that permethylation of ristomycin A yielded 2,3,5-tri-*O*-methylarabinose,[16] although that result had subsequently been questioned when acetolysis studies provided strong evidence for arabinose having a β-pyranoside structure in deca-*O*-acetylristriose (**6**).[17] A comparison of the ^3C NMR spectrum of **16** with that of the native antibiotic indicates that the α-arabinofuranoside structure rather than the β-pyranoside is present in the antibiotic. Apparently, ring expansion of arabinose to the thermodynamically more stable pyranose form occurs during acid-catalyzed acetolysis. Detailed studies of the ^{13}C NMR spectra of **16** and the antibiotic will be reported elsewhere.

The configuration of D-glucose in **16** has been assigned as β from ^1H NMR spectra recorded at 250 and 600 MHz. The spectra reveal a large vicinal coupling constant (J = 7.85 Hz) for one of the anomeric protons (δ 4.95), which is consistent with H-1 and H-2 being antiparallel in a $^4C_1$-β-(D)glucosyl residue.

Glycine, which is another product of the alkaline degradation of didechlorovancomycinic acid, was also present and was identified by TLC. A third product was identified as L-ristosaminol (**18**). The compound gave a positive reaction with ninhydrin; the ^1H NMR spectrum indicated the absence of aromatic structures but was suggestive of a carbohydrate. Benzoylation of the amino group of **18** by the Schotten–Baumann procedure and acetylation gave the *N*-benzoyl tri-*O*-acetyl derivative **19**. The structure of **19** was deduced spectroscopically and confirmed by reduction of L-ristosamine with $NaBH_4$ followed by benzoylation and acetylation. Had **2** been linked to a phenolic site, ristosaminol would not have been generated; the acetal linkage of methyl α-L-ristosaminide is not reduced by $Ba(OH)/NaBH_4$. Consequently, **2** must be linked to one of the β-hydroxytyrosyl alcoholic hydroxyl groups of didechlorovancomycinic acid. The amino sugar can be released from such a site under alkaline conditions by β elimination. It is noteworthy that **17** was the only compound isolated having the triaryl nucleus. Hydrolysis of aglycoristocetin and aglycoristomycin gave an additional product having both of the β-hydroxytyrosines coverted to benzyl alcohols (by processes of retroaldol cleavage followed by reduction). With the intact antibiotic, the glycosylated residue is protected against retroaldolization and undergoes elimination of the ristosamine residue instead, leading ultimately to the lactate side chain.

The hydrolysis also yielded partially O-methylated ristomycinic acid. Di-O-methylated ristomycinic acid (**20**) was obtained from base hydrolysis of ristocetin A which had been methylated more

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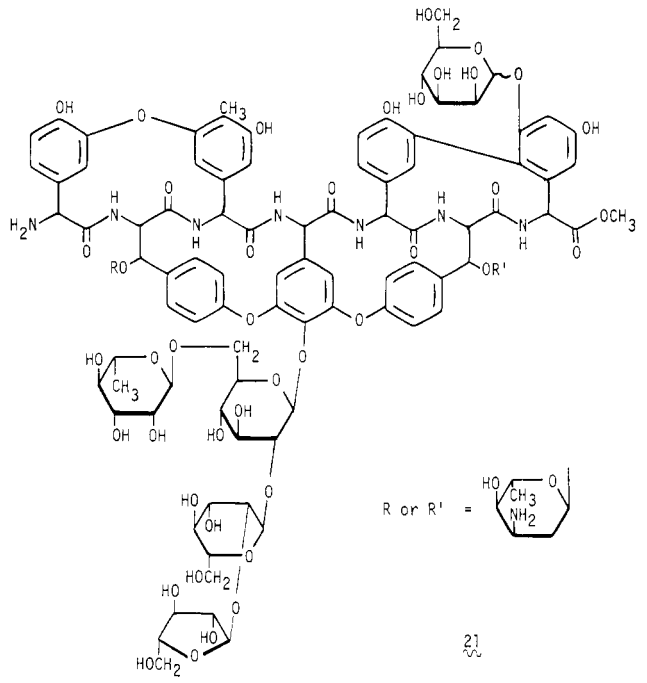
completely with MeI/K_2CO_3. The mannosylactinoidinic acid fragment was never isolated in sufficient quantity and purity to establish the anomeric configuration of mannose. The existence of multiple stereoisomeric forms and occurrence of degradation reactions[29] contributed to this failure.

Repetition of the alkaline cleavage reaction with O-methylated ristomycin A gave the same results, thus providing additional evidence that ristocetin A and ristomycin A are identical.

Acid Hydrolysis. Partial acid hydrolysis is potentially a useful tool for establishing the sites of glycosidic linkages to the aglycone and for simplifying the structure so that spectral measurements can be interpreted more readily. In the present case, the method is complicated by low yields of products retaining only a single sugar and by the complexity of the mixtures resulting from these reactions. Previous workers have employed this method in investigations of ristomycin A, obtaining evidence for glucose and mannose being attached to the aglycone at separate but unidentified sites.[9,10] We have explored this method with ristocetin A. After hydrolysis for 1 h in refluxing 1 M HCl, the products were fractionated by precipitation during stepwise neutralization. The aglycone precipitating at pH 6, although not completely homogeneous, was essentially free of neutral sugars but still contained ristosamine, as indicated by characteristic ^{13}C NMR signals for the 6-methyl group (18.0 ppm) and the 2-methylene group (30.4 ppm) and by further acid hydrolysis to give free ristosamine. The anomeric configuration was assigned as α on the basis of the chemical shift of the anomeric carbon (94.0 ppm). By comparison, the signal for C-1 of the methyl α-L-ristosaminide appears at 97.6 ppm and that of a ristosamine in the related antibiotic avoparcin[19] at 94.0 ppm. The latter is also assigned an α linkage.

Discussion

On the basis of the above experiments and other results obtained in this laboratory, structure **21** is proposed for ristocetin A. Although the peptide sequence is not fully established, steric



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constraints of the biphenyl and diphenyl ether linkages rule out most alternatives. The structure provides a basis for speculation on the mechanism of action of ristocetin and related antibiotics. The process of complexation with acyl-D-Ala-D-Ala peptides by antibiotics in this class undoubtedly involves primarily the C-terminal portion of the peptide chain. Actinoidinic acid and vancomycinic acid (or the dechloro analogues) appear to be invariant components of the antibiotics, based on studies now reported on vancomycin,[30] ristocetin[5] (ristomycin),[7] actinoidin,[31] avoparcin,[19] A35512B,[32] and teichomycin A_2,[33] whereas the remaining residues are highly variable, involving a pair of linked or separate aromatic amino acids or even, in the case of vancomycin, aliphatic amino acids. Actinoidinic acid is at the C-terminus of vancomycin and ristocetin[34] (and probably the other antibiotics as well). The variable amino acids are in the N-terminal portion of the peptides, at least in those examples where the question has been examined. Williams has made a specific proposal of a binding mechanism for vancomycin which involves hydrogen bonding between carbonyl and NH groups of amides utilizing actinoidinic acid and vancomycinic acid.[30] His proposal is based upon studies of the structure of a derivative of vancomycin, CDP-I, which was determined by X-ray diffraction and is supported by NMR studies of chemical shift changes occurring during the binding process.

Detailed studies of the effect of structural modification in the binding peptides by Nieto and Perkins[35] by using vancomycin and ristocetin B have revealed that while the two antibiotics are remarkably similar in their binding affinities some significant differences exist. In a series of peptides having the structure Ac_2-L-Lys-D-C(R_2)H-CO-NH-C(R_1)H-CO_2H, ristocetin B could accommodate increases in the size of R_1 better than vancomycin, but the converse was true for R_2. In fact, ristocetin B binds Ac_2-L-Lys-Gly-D-Ala better than it binds Ac_2-L-Lys-D-Ala-D-Ala. Ristocetin B lacks arabinose and one molecule of mannose; Lomakina, in studies of ristomycin A and B,[9,10] has presented evidence indicating that the differences in the two forms of the antibiotic involves only the tetrasaccharide and that the mannose attached to actinoidinic acid is still in place in the B form of the antibiotic. We conclude, therefore, that the peptide-binding properties of ristocetin A are very similar to those of B since the two additional sugars are distal to the binding site.[36]

We presume that the chiral centers near the C terminus of ristocetin have the same absolute configurations as those in the corresponding locations in vancomycin and that these portions of the two antibiotics have similar conformations. By inspection of models, it becomes possible to account for differences in binding affinities observed by Nieto and Perkins by using William's mechanistic proposal. The decreased restriction on the size of R_1 in ristocetin A arises because the vancomycin chlorine substituents are missing. The chlorine atom on the β-hydroxytyrosine residue closest to the C terminus of vancomycin is in close contact with the binding peptide as indicated by a large chemical shift change observed for the adjacent aromatic proton during the binding process. The increased restriction on the size of R_2 in ristocetin can be ascribed to steric effects of the mannosyl residue attached to actinoidinic acid. The model places R_2 close to this sugar.

A binding model which assigns no role to the amino acids near the N terminus is probably an oversimplification of a complex situation. Certainly, in ristocetin, the ristomycinic acid must play a significant part in the complexation process. Nieto and Perkins[35] observed that UV difference spectra for vancomycin which were generated by the binding process were clearly different from those for ristocetin B. Williams has found that the ^1H NMR chemical

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(36) In agreement with this, Nieto and Perkins[35] found that ristocetin A and B exhibited virtually identical ORD spectra upon binding Ac_2-L-Lys-D-Ala-D-Ala.

shift of the C-methyl group of ristomycinic acid is altered by peptide binding.[6] The absolute configurations of the chiral centers in ristomycinic acid (and the other amino acids, as well) must be known before this observation can be explained. Further work on the structures and the binding affinities of antibiotics in the vancomycin class will be necessary before a comprehensive view of the binding process can be evolved.

The role of the sugars in these antibiotics is not clear; they are not required for peptide binding or for antibiotic activity since aglycoristocetin is active in both respects. There is much diversity in the carbohydrate composition and structure in vancomycin-group antibiotics,[37] although the presence of an amino sugar appears to be a common factor. Actinoidin is similar to ristocetin in that it has three glycosylation sites (the phenolic and one alcoholic hydroxyl group of the triphenyl amino acid and a phenolic site on actinoidinic acid)[31d] whereas vancomycin contains only one (the phenolic hydroxyl of the triphenyl amino acid) and avoparcin[19] has several, including the phenolic group of its N-terminal amino acid but not of those on actinoidinic acid. The carbohydrates undoubtedly lead to subtle differences in binding specificities and may play a part in transport processes in vivo.[37b]

Experimental Section

Melting points were determined on a Kofler hot stage or in open capillaries and are uncorrected. ^1H and ^{13}C NMR spectra were recorded with a JEOL FX-90Q spectrometer. CH_3CN, CH_3COCH_3, and DSS were used as internal standards for spectra run in D_2O; Me_4Si was used in $CDCl_3$. IR spectra were recorded with a Unicam SP-200G or Perkin-Elmer 727 spectrometer employing KBr pellets. Optical rotations were measured with a Bendix NPL, a Zeiss Polamat A, or an Autopol III automatic spectropolarimeter. Open-column chromatography was carried out on Merck silica gel 60F-254 (TLC grade). TLC was performed on Merck silica gel 60F-254 plates unless otherwise indicated, using the following solvent systems: (A) C_6H_6/MeOH (94:6); (B) *n*-propanol/25% NH_4OH/H_2O (6:2:1); (C) $CHCl_3$/MeOH (95:5); (D) C_6H_6/MeOH (85:15); (E) EtOAc/HOAc/*n*-butanol/H_2O (7:3:3:3); (F) *n*-butanol/HOAc/H_2O (3:1:1). Low-resolution mass spectra were obtained on an LKB-9000A mass spectrometer (70 eV) with the direct inlet. High-resolution mass spectra were obtained at Florida State University.

Methyl α-L-Ristosaminide Hydrochloride. Ristocetin A (2.1 g), dried over P_2O_5 in vacuo at room temperature, was methanolized in dry 3 M methanolic HCl (21 mL) in a sealed ampule at 105 °C for 5 h. The reaction mxture was worked up as described previously[11] for isolation of this compound from ristomycin, using Dowex 50 (NH_4^+) resin for ion-exchange chromatography with 0.5 M NH_4OH as eluent, and yielded 76 mg (41%) of methyl α-L-ristosaminide hydrochloride, which was recrystallized from a small amount of ethanol; mp 165–167 °C, $[\alpha]^{20}_D$ −140° (*c* 0.28, H_2O). Literature data[11] for material obtained by methanolysis of ristomycin A: mp 168–170° C, $[\alpha]^{20}_D$ −123.8° (*c* 1, H_2O). Literature data[13,14] for synthetic material: mp 165–168 °C, $[\alpha]^{20}_D$ −160° (C 0.23, MeOH). TLC (solvent B, R_f 0.85) and IR spectra of the natural and synthetic samples were identical: ^{13}C NMR (D_2O) δ 17.9 (C-6), 31.4 (C-2), 49.8 (C-3), 55.8 (OMe), 64.5 (C-4 or C-5), 68.8 (C-5 or C-4), 97.5 (C-1, $^1J_{CH}$ = 172 Hz).

Methyl Di-*N*,*O*-benzoyl-α-L-ristosaminide (4). Methyl ristosaminide hydrochloride (75 mg) from above was treated in the conventional manner with benzoyl chloride (0.10 mL) in pyridine (1.2 mL) at ambient temperature for 24 h. The reaction mixture was poured into ice water (50 mL), and the precipitate was filtered off, washed with H_2O, and dried (121 mg). Purification by chromatography as silica gel (solvent A) gave 31 mg (19%) of the title compound, which was crystallized from 95% EtOH; mp 146–148 °C, $[\alpha]^{20}_D$ −175° (*c* 0.60, $CHCl_3$) [lit.[13,14] mp 150–151 °C, $[\alpha]^{20}_D$ −181° (*c* 0.66, $CHCl_3$)]. Isolated and authentic material gave the same IR spectrum and R_f (0.68) on TLC in solvent A.

Acetolysis of Ristocetin A. Ristocetin A (2.0 g, dried over P_2O_5) in vacuo (0.02 mmHg at room temperature) was suspended with cooling in a mixture of acetic anhydride (18 mL) and concentrated H_2SO_4 (0.2 mL); the mixture was cooled to 0 °C and then allowed to stand at room temperature. After several days, the antibiotic dissolved completely to give a dark brown solution. After 14 days, the solution was diluted with 10 mL of acetic acid and poured onto 500 g of ice. The yellow-green precipitate was filtered off, washed with H_2O to neutral pH, and dried at room temperature to give 2.2 g of product. The dried material was extracted with 3 × 50 mL of hot C_6H_6, and the combined extracts were filtered and evaporated in vacuo. The mixture of sugar acetates (180 mg) was placed on a column (21 × 2.4 cm) of silica gel G (~30 g) in solvent A; the column was developed with that solvent at a flow rate of 5 mL/h. The homogeneity of the fractions was assayed by TLC in solvent A. Spots were detected by spraying with 50% ethanolic H_2SO_4 and heating at 120 °C. Fraction A (2.7 mg), tetra-*O*-acetyl-α-L-rhamnose (**5**): ^1H NMR ($CDCl_3$) δ 1.23 (6 CH_3, d, *J* = 6 Hz), 2.00, 2.06, 2.16, 2.17 (4 CH_3CO, 4s), 3.93 (5 CH, dq, *J* = 9, 6 Hz), 5.0–5.45 (2 CH, 3 CH, 4 CH, m), 6.02 (1 CH, d, *J* = 2 Hz). Hepta-*O*-acetylrutinose (**7**), R_f 0.29, and octa-*O*-acetylristobiose (**8**), R_f 0.26 (fraction B), were present in trace amounts and were identified by TLC comparison (solvent A) with samples prepared from ristomycin A. Fraction C (34.4 mg), deca-*O*-acetylristriose (**6**): R_f 0.21; mp 84–87 °C; ^{13}C NMR identical to reported spectrum.[17] Compound **6** was isolated from ristomycin A; R_f 0.20, mp 81–84 °C. Fraction D (10 mg), dodeca-*O*-acetyl-α-ristotetrose (**9**); R_f 0.14, $[\alpha]^{20}_D$ +39.7° (*c* 0.35, MeOH). The ^1H NMR spectrum of **9** showed the 6-Me group of rhamnose at 1.21 ppm (*J* = 6.5 Hz). ^{13}C NMR ($CDCl_3$): δ 17.4 (C-6 of rhamnose), 20.6 (12 $COCH_3$), 61.9 (C-6 of mannose and arabinose), 66.2–75.2 (13–14 peaks), 88.3–98.2 (4 peaks, see Table I), 169.2–169.7 (12 $COCH_3$).

Differential Methylation of Ristocetin A. A mixture of the antibiotic (1.0 g), CH_3I (4 mL), K_2CO_3 (3.0 g), and MeOH (15 mL) was refluxed for 16 h, evaporated to dryness, dialyzed to remove salts, and lyophilized. UV absorption of the material at 280, 290, and 310 nm showed no change upon addition of base, indicating that methylation of the phenols had been complete. Acid hydrolysis (1 M HCl, 45 min, 100 °C) followed by lyophilization yielded 0.380 g of aglycone, which was remethylated as above but by using CD_3I (2 mL). The resulting methylated aglycone was hydrolyzed by using 4 M KOH (15 mL) and $NaBH_4$ (0.5 g) refluxed under N_2 for 24 h. The mixture, after cooling, was adjusted to pH 7.2 and treated with 4 mL of commercial NaOCl solution (Clorox) added dropwise. After 4 h, excess oxidant was destroyed with Na_2SO_3. Further oxidation with excess 5% $KMnO_4$ (16 h, 25 °C) followed by acidification, reduction of MnO_2 with Na_2SO_3, extraction (EtOAc/MeOH, 9:1), and esterification (CH_2N_2) led to a complex mixture containing mainly benzoate esters **10**, **11**, and **12a** (70.4 mg). Preliminary fractionation of the mixture on silica gel (elution with pentane/EtOAc, 9:1) followed by high-pressure liquid chromatography (high-pressure LC) (2 ft Waters μ-Porasil, pentane/EtOAc, 9:1) gave benzoates **11**, **10**, and **12a**, eluting in that order. Compound **11** was identical by TLC and ^1H NMR with previously prepared material[5] except that the methoxyl signal at 3.71 ppm was absent; mass spectrum (MS), *m/e* 469 (100), 438 (51). Compound **10** was identical with previously isolated material.[5] The mass spectrum of **10** indicated minor contamination by the mono-CD_3 analogue; MS, *m/e* 363 (10), 360 (100), 332 (3), 329 (29). Compound **12a** was identical with prevously isolated material[5] except that the methoxyl signal at 3.70 ppm in the ^1H NMR spectrum was only 0.1 of the intensity of the other methoxyl groups; MS, *m/e* 363 (100), 360 (8), 332 (76), 329 (0.7). A detailed discussion of the ^1H NMR spectrum of **12a** appears in the text.

Synthesis of Diester 12a. 2-Bromo-3,5-dimethoxytoluene (460 mg, 2 mmol) was added to a solution of sodium ethanethiolate (6 mmol) in 20 mL of DMF.[24] The mixture was stirred for 9 h at 65 °C, poured into dilute HCl, and extracted three times with Et_2O. The extracts were combined and extracted with 1 M KOH. The alkaline extract was acidified and reextracted with Et_2O. Evaporation gave an oil which was purified by high-pressure LC on silica gel (elution with $CHCl_3$) to yield 160 mg (37%) of 2-bromo-3-hydroxy-5-methoxytoluene (**13**): mp 68–70 °C (lit.[39] mp 68–70 °C); ^1H NMR ($CDCl_3$) δ 2.33 (C–Me, s), 3.72 (5 OMe, s), 5.70 (OH, br s), 6.39 (6 H, d, *J* = 3 Hz), 6.45 (4 H, d, *J* = 3 Hz); MS, *m/e* 218 (100), 216 (100). A high-resolution ^1H NMR spectrum of **13** showed 0.65-Hz coupling between H-6 and the C–Me group which was clearly resolved when the OMe group was decoupled by irradiation. The proton at position 4 was also coupled to the C–Me group but to a lesser extent; both the H-4 and H-6 signals were sharpened by irradiation of the C–Me group. Experiments with $Eu(fod)_3$ are described in the text. Compound **13** was remethylated in the usual fashion

(37) (a) For a compilation, see: Bardone, M. R.; Paternoster, M.; Coronelli, D. *J. Antibiot.* **1978**, *31*, 170. (b) We call the reader's attention to a communication[38] by Williams and co-workers which appeared after this manuscript was completed. Using ^1H NMR spectra obtained at 270 and 360 MHz in conjunction with the known structure of vancomycin, they have been able to define the structure of ristocetin A. Our chemical studies generally complement their spectroscopic findings.

(38) Williams, D. H.; Rajananda, V.; Bojesen, G.; Williamson, M. P. *J. Chem. Soc., Chem. Commun.* **1979**, 906.

(39) Cannon and co-workers (Cannon, J. R.; Cresp, T. M.; Metcalf, B. W.; Sargent, M. W.; Vinciguerra, G.; Elix, J. A. *J. Chem. Soc. C.* **1971**, 3495) have reported the preparation of compound **13** by using a different synthetic route. The melting point of our material is identical with that of theirs, but they found the aromatic protons to be isochronous (δ 6.47).

by using CD_3I (0.75 mL), K_2CO_3 (0.5 g), and acetone (5 mL) to give 2-bromo-3-(trideuteriomethoxy)-5-methoxytoluene (**14**) in quantitative yield: ^1H NMR ($CDCl_3$) δ 2.39 (C–Me, s), 3.78 (5 OMe, s), 6.33 (4 H, d, J = 3 Hz), 6.42 (6 H, d, J = 3 Hz); MS, m/e 235 (100), 233 (100). Oxidation of **14** (70 mg, 0.3 mmol) with hot $KMnO_4$[40] gave the corresponding benzoic acid which was esterified with CH_2N_2 to give methyl 2-bromo-3-(trideuteriomethoxy)-5-methoxybenzoate (**15a**, 20.8 mg, 25%): ^1H NMR ($CDCl_3$) δ 3.81 (5 OMe, s), 3.93 (ester OMe, s), 6.58 (4 H, d, J = 2.7 Hz), 6.80 (6 H, d, J = 2.7 Hz); MS, m/e 279 (100), 277 (100), 248 (50), 246 (50). Undeuterated compound **15b** gave the same ^1H NMR spectrum except that the 3-OMe group yielded a singlet at 3.88 ppm; NOE measurements are discussed in the text. A mixture of **15a** (20.8 mg, 75 μmol), ethyl 3-iodo-4-methoxybenzoate[41] (67 mg, 221 μmol), and finely powdered Cu (867 mg, pretreated with 0.02 M EDTA)[42] was heated at 220 °C for 30 min.[25] After the reaction mixture was cooled, it was extracted several times with CH_2Cl_2. The extracts were combined, washed with 0.02 M EDTA, dried, and evaporated to yield 74.7 mg of a mixture containing primarily starting iodo ester, the biaryl compound derived from its self-coupling and the desired cross-coupling product, diester **12a**. Preparative TLC (elution with pentane/EtOAc, 1:1) gave **12a** (7.6 mg, 28%) which was further purified by high-pressure LC (2 ft Waters μ-Porasil, pentane/EtOAc, 85:15) to give material identical by ^1H NMR with that isolated from ristocetin A; MS, m/e 363 (100), 360 (10), 332 (65), 329 (6.5).

Base Hydrolysis of *O*-Protected Ristocetin A and Ristomycin A. Partially *O*-methylated ristocetin A (1.0 g), obtained by treatment of the antibiotic with CH_2N_2 for 24 h in 70% acetone, was refluxed in 0.2 M $Ba(OH)_2$ (50 mL) containing $NaBH_4$ (0.38 g) for 48 h under N_2. The reaction mixture was cooled, filtered, treated with CO_2 and filtered to remove $BaCO_3$, treated with $(NH_4)_2SO_4$ and filtered to remove $BaSO_4$, and lyophilized. The residue was partitioned by gel filtration on a Sephadex G-25 (fine) column (42 × 2.4 cm) which was developed with deionized water at 0.5 mL/min and monitored at 254 nm with a Uviscan III and by TLC on silica gel (solvent B). Individual components were rechromatographed (flow rate 0.25 mL/min) to obtain further purification. The first constituents eluting from the column appeared to be products of incomplete hydrolysis. These were followed by a large UV-absorbing peak (170 mg) containing compound **16**: R_f 0.35 (solvent B); $[\alpha]^{20}_D$ +20.0° (c 0.50, H_2O); ^1H NMR (D_2O) (poorly resolved spectrum) δ 1.25 (rhamnose Me), 2.74 (CH_2CH), 3.42–4.11 (carbohydrate), 4.60 ($ArCH_2OH$), 5.30 (amino acid CH), 6.88–7.46 (aromatic protons); ^{13}C NMR (D_2O) δ 17.2 (rhamnose Me), 40.0 (CH_2CH), 56.5–84.1 (~18 lines, amino acid CH and sp^3 carbons bearing one oxygen substituent), 99.8–109.3 (4 lines, anomeric sugar carbons, see text), 115.0 (CH's of central ring), 118.9 (CH's ortho to oxy substituents on outer rings), 129.6, 131.3 (CH's meta to oxy substituents on outer rings), 134.5–137.8 (3 lines, alkyl-substituted aromatic carbons), 175.0, 180.6 (carbonyl groups). Subsequent fractions contained glycine (23 mg), which was identified by TLC comparison, R_f 0.41 (solvent B), with authentic material and L-ristosaminol (**18**, 65 mg, R_f 0.49, solvent B), which was identified by derivatization as described below. A compound tentatively identified as mono-*O*-methylristomycinic acid was also obtained along with uncharacterized substances believed to be derived from actinoidinic acid. A parallel experiment employing ristomycin A gave identical results. Repetition of the alkaline hydrolysis of ristocetin A, employing material that had been methylated more exhaustively (CH_3I/K_2CO_3 in refluxing acetone/H_2O), yielded di-*O*-methylated ristomycinic acid [^1H NMR (D_2O) δ 2.04 (C–Me, s), 3.82, 3.87 (OMe's, 2s), 6.51, 6.84, and 7.16 (aromatic protons, br signals)] as well as **16**, **18**, and glycine, but gave no characterizable product derived from actinoidinic acid.

Acid Hydrolysis of 16. Compound **16** (30 mg) was treated for 30 min with refluxing 1 M HCl (1.0 mL). The sugars in the reaction mixture were examined by TLC on Avicel F plates with solvent system E followed by visualization with a 2% solution of aniline/hydrogen phthalate in 1-butanol (105 °C, 10 min). The presence of D-glucose (R_G 1.00), D-mannose (R_G 1.06), D-arabinose (R_G 1.44), and L-rhamnose (R_G 2.34) was established by comparison with standards. The reaction mixture was diluted with H_2O (1 mL), adjusted to pH 8–9 with aqueous NaOH, and treated with Ac_2O and NaOH added alternately to maintain the pH of the solution. The mixture was acidified and extracted with EtOAc/MeOH (9:1). The extracts were dried ($MgSO_4$) and evaporated to dryness. The residue was taken up in MeOH (1 mL) and treated with excess CH_2N_2 for 12 h to give **17** after purification by TLC (solvent C). Compound **17** was identified by comparison with authentic material previously obtained by base hydrolysis of O-methylated aglycoristocetin[5] (and aglycoristomycin):[7] ^1H NMR ($CDCl_3$) δ 1.68 (OH, s), 2.00 (CH_3CO, s), 3.03 (CH_2CH, m), 3.70, 3.75, 3.78 (MeO's, 3s), 4.44 (CH_2CH, m), 4.67 ($ArCH_2OH$, s), 5.42 ($CHNH$, d, J = 8 Hz), 6.41 ($CHNH$, br d, J = 8 Hz), 6.77 (aromatic CH's of center ring, s), 6.90–7.33 (aromatic CH's of outer rings, 2 A_2B_2); MS, m/e 553 (44), 535 (15), 521 (42), 510 (72), 503 (65), 494 (50), 493 (53), 452 (100).

Preparation of 19 from L-Ristosaminol. Compound **18**, isolated as described above, was treated with benzoyl chloride for 30 min at 5 °C with the pH being maintained at 7–8 by addition of $NaHCO_3$. After an additional 30 min at room temperature, the solution was deionized by treatment with Dowex 1 × 8 (HCO_3^-) and 50W-X8 (H^+) resins and lyophilized. The residue was crystallized from MeOH/H_2O (1:1) and acylated with 2 mL of 1:1 Ac_2O/pyridine for 24 h at room temperature. The usual workup gave 27.5 mg of **19** which was further purified by TLC in solvent D to give crystals: mp 90–91 °C; R_f 0.40 (solvent D); ^1H NMR ($CDCl_3$) δ 1.32 (C–Me, d, J = 6 Hz), 1.87 (2 CH_2, m), 2.00, 2.04, 2.14 ($COCH_3$'s, 3s), 4.15 (1 CH_2, t, J = 6 Hz), 4.57 (3 CH, m), 5.12 (4 CH, 5 CH, m), 6.73 (NH, d, J = 9 Hz), 7.3–8.0 (aromatic protons, m); ^{13}C NMR ($CDCl_3$) δ 14.9 (6 Me), 20.8 (2 $COCH_3$), 21.0 ($COCH_3$), 30.1 (2 CH_2), 46.7 (3 CH), 61.0 (1 CH_2), 69.4 (4- or 5-CH), 76.1 (5- or 4-CH), 127.0, 128.6, 131.7, 134.1 (C_6H_5CO), 167.0 (CO), 170.5 (2 CO), 170.8 (CO); MS (high-resolution), m/e 319.1410 (1.2%, $C_{17}H_{21}NO_5$), 277.1319 (1.4% $C_{15}H_{19}NO_4$), 260.1290 (4.3%, $C_{15}H_{18}NO_3$), 220.0977 (24.8%, $C_{12}H_{14}NO_3$), 160.0719 (100%, $C_7H_{12}O_4$), no parent ion at m/e 379. An authentic sample of **18** was prepared from methyl α-L-ristosaminide hydrochloride (20 mg, derived from ristomycin A) by hydrolysis with 1 M HCl (1.0 mL) at 100 °C for 1 h, followed by neutralization with Dowex 1 × 8 (HCO_3^-) resin and treatment with $NaBH_4$ (8 mg) at 20 °C for 3.5 h. The resulting L-ristosaminol (**18**) was identical by TLC, R_f 0.49 (solvent B), with **18** prepared above. Benzoylation and acetylation as described above gave 9.5 mg of **19**, mp 91–92 °C (Kofler), R_f 0.40 (solvent D), which was identical in all respects with the above sample.

Partial Acid Hydrolysis of Ristocetin A. The antibiotic (1.0 g) was heated with 1 M HCl (25 mL) for 45 min at 100 °C and then chilled in an ice bath. The resulting precipitate (0.439 g) was removed by filtration. The filtrate was neutralized to pH 6.0 with $KHCO_3$ and allowed to stand at 5 °C for 18 h. The precipitate which formed (0.189 g) was isolated by filtration. The ^{13}C NMR spectrum of the second precipitate showed an aglycone still containing ristosamine, as evidenced by prominent signals at δ 18.0 (6 CH_3), 30.4 (2 CH_2), and 94.0 (1 CH). The presence of ristosamine was confirmed by further hydrolysis (4 h, 1 M HCl, 100 °C) to give the free amino sugar, which was identified by TLC comparison with authentic materials (Avicel F plates, solvent E) and by methanolysis to give methyl α-L-ristosaminide, which was identified by TLC comparison with authentic material (silica gel, solvents B, E, and F).

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