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An NMR Study of the Structure of the Antibiotic Ristocetin A. The Negative Nuclear Overhauser Effect in Structure Elucidation

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Abstract: An analysis of the ^{1}H NMR spectrum of ristocetin A in dimethyl sulfoxide solution is reported. With the aid of negative nuclear Overhauser effects, and analogies between a part of the structure of the anitbiotic and a part of that of vancomycin, it has been possible to propose a structure for ristocetin A aglycone. The study leads to proposals for the absolute stereochemistry at eight of the nine asymmetric centers. Some details of the attachment of the six sugars of ristocetin A (previously known to be attached as two monosaccharides—ristosamine and mannose—and as a tetrasaccharide via glucose) are deduced.

Introduction

Recent work has established that the antibiotic ristocetin A (identical with ristomycin A[1]) is constituted from six sugars[2-4] (ristosamine, mannose (2 mol), glucose, arabinose, and rhamnose), and aromatic fragments **1**,[5] **2**,[1,6] and **3**.[5,7] The production of glycine upon alkaline hydrolysis of ristocetin A, in conjection with evidence from ^{13}C and ^{1}H NMR data, and a californium plasma desorption mass spectral determination of the molecular weight (to within 5 daltons) lead to the conclusion[1] that (like vancomycin) ristocetin A contains two "potential glycine" units, **4** and **4a**. The ^{1}H NMR data further establish the presence of six NH groups of secondary amide bonds.[1] Since ristocetin A is known to contain two free amino groups (one in the amino sugar ristosamine) and a methyl ester function,[8] the molecular weight and NMR data are satisfied by connection of the units **1** to **4** by secondary amide bonds. The six sugars are reported to be attached to the aglycone as a tetrasaccharide (comprising rhamnose, arabinose, mannose, and glucose) and two monosaccharides (ristosamine and mannose).[9] The parts of a total aglycone structure **5** which

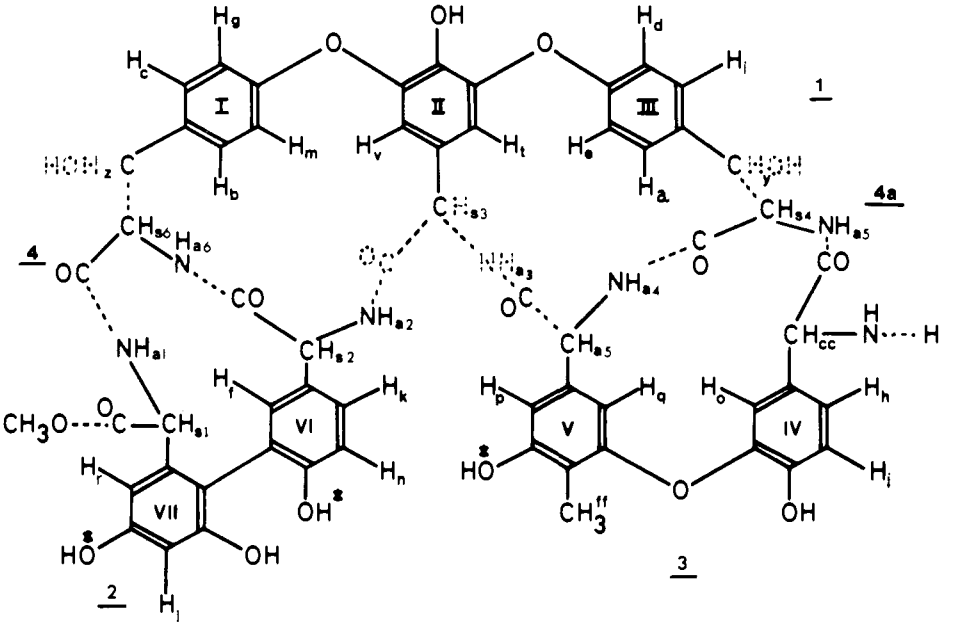

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Table I. NMR Parameters of Ristocetin A at 70 °C (270 MHz)

chemical shift, δ	intensity (multiplicity)	J, Hz	letter code	protons decoupled (J) on irradiation
9.26	1 (d)	5	a_1	s_1
8.58	1 (d)	6.5	a_2	s_2
7.86	1 (d)	8.0	a	e (i sharpened)
7.66	1 (d)	8.2	a_3	s_3
7.55	1 (d)	8.2	b	m (8), c (2)
7.41	1 (d)	~8	c	g (8), b (2)
7.41	1 (d)	~10	a_4	s_5
7.29	1 (d)	~8	d	i
7.26	1 (d)	~2	f	k
7.25	1 (d)	~8	e	a
7.21	1 (dd)	~8, ~2	g	c (8), m (2)
7.21	1 (d)	~9	a_5	s_4
7.20	1 (d)	~12	a_6	s_6
7.18	1 (dd)	~8, ~2	h	j (8), o (2)
7.12	1 (dd)	~8, ~2	i	d (8), a sharpened
7.03	1 (d)	~8	j	h
6.88	1 (dd)	8, ~2	m	b (8), g (2)
6.85	1 (d)	2	l	r
6.84	1 (dd)	8, ~2	k	n (8), f (2)
6.77	1 (d)	8	n	k
6.59	1 (brs)		o	h
6.45	1 (brs)		p	
6.42	1 (brs)		q	
6.32	1 (d)	2	r	l
5.85	1 (brs)		t	v; s_3
5.65	1 (d)	8.2	s_3	a_3
5.49	1 (s)		u	
5.38	1 (brs)		v	t
5.33	1 (s)		w	
5.28	1 (d)	7.8	x	[δ 3.70]
5.25	1 (d)	10	s_5	a_4
5.19	1 (d)	~5	y	s_4
5.17	1 (s)		z	
5.09	1 (dd)	9.5	s_4	a_5 (9), y (5)
4.92	1 (s)		aa	
4.88	1 (brs)		bb	ee_2
4.83	1 (brs)		cc	
4.73	1 (d)	6.5	s_2	a_2
4.57	1 (s)		dd	
4.55	1 (d)	5	s_1	a_1
4.38	1 (d)	12	s_6	a_6, z
3.9–2.8			sugar *CH* and *OH*: $COOCH_3$	
2.21	1 (d)	15	ee_1	ee_2
2.01	1		ee_2	ee_1; bb
2.01	3 (s)		ff	
1.25	3 (d)	~5	gg	[δ 3.46]
1.10	3 (d)	6	hh	[δ 3.31]

have been inferred from spectroscopic evidence[1] are indicated by dotted lines. We outline in the present paper reasons for the particular connectivity scheme of **5** and almost complete structural details for ristocetin A.

Code for Anomeric Protons

sugar	code	sugar	code
glucose	x	$mannose_2$	aa
ristosamine	bb	rhamnose	dd
$mannose_1$	w	arabinose	**u**

It has previously been established[10] that ristocetin A binds to acetyl-D-Ala-D-Ala, which is a model for a component involved in a key step of cell wall biosynthesis.[11] Moreover, we have already shown[1] that acetyl-D-Ala-D-Ala forms a strong complex with ristocetin A in Me_2SO-d_6 solution. This complex is in slow exchange with its free components on the NMR time scale (calculated free-energy barrier for dissociation ~18 kcal mol^{-1}). What follows is a systematic study of ristocetin A and (in the following paper) of the complex by ^1H NMR at 270 and 360 MHz.

Experimental Section

Solutions (0.5 mL) of ristocetin A (supplied by Abbott, Chicago, or Lundbeck, Copenhagen) in tubes of 5-mm o.d. were examined at concentrations around 12 mM in Me_2SO-d_6 and in Me_2SO-d_6 containing added D_2O (~7%) or added CF_3COOD (up to ca. 40 mM). Spectra were recorded at several temperatures ranging from 23 to 70 °C.

The spectra were obtained using Bruker WH270 or 360 spectrometers equipped with Fourier transform facilities. In a typical experiment, 100 scans were accumulated over a spectral width of 3000 Hz using 8K memory points; 0.4-Hz exponential line broadening was introduced on transformation. Double irradiations for decoupling experiments were performed by continuous irradiation at a power attenuation of 20 dB. The NOEs were measured either from these spectra or from spectra recorded using a gated irradiation pulse sequence, by comparison of peak heights of several spectra obtained under closely similar conditions.

Studies of Ristocetin A in Me_2SO Solution

Examination of spectra taken at a variety of temperatures between 24 and 70 °C with or without added trifluoroacetic-d_1 acid or D_2O, with the help of decoupling experiments at 50 and 70 °C enables us to list the protons in the chemical shift ranges δ 10–4.2 and 2.8–0 (Table I). Most protons in the δ 3–4 region are unassigned. The 270-MHz ^1H NMR spectrum of ristocetin A (12 mM in Me_2SO-d_6 at 70 °C) is reproduced in Figure 1; expansions and assignments of the δ 4.0–6.0 and 6.2–8.0 ppm regions are given in Figures 2 and 3, respectively.

Assignment of the Signals

A. Phenolic OH Protons. Addition of 0.3 molar equiv of trifluoroacetic-d_1 acid (TFA-d_1) causes the broad phenolic protons in the δ 9–10 region to sharpen; four phenolic OH protons are discerned at δ values of 9.73, 9.69, 9.57, and 9.39 ppm. These resonances are removed on addition of D_2O to the Me_2SO-d_6 solution, and lose intensity upon irradiation of the HOD peak.

B. Amine NH_2 Protons. Addition of 0.7 molar equiv of TFA-d_1 causes a three- to four-proton singlet to appear at 8.51 ppm, and also causes similar increase in integrated proton intensity at about 7.6 ppm. These resonances cannot be detected at higher pH owing to intermediate exchange rates. These signals correspond to two $-^+NH_3$ resonances.

C. Aliphatic OH Protons. A large number of aliphatic OH resonances are present in the region 6–3 ppm. These are distinguished from other resonances in the same region by broadening and upfield shifts with increasing temperature, and by pH-dependent line width. No attempt has been made to give detailed assignments of the aliphatic OH protons.

D. Protons of Secondary Amide Groups (–CON*H*–). There are unambiguously six secondary amides in ristocetin A, resonances which (1) disappear upon D_2O exchange but are slow to exchange on the NMR time scale and (2) are coupled to αCH protons in the 6–4-ppm region. The secondary amide NH protons are coded a_1, a_2, . . . , a_6 in Table I.

E. Aromatic Protons. There are 20 aromatic protons in ristocetin A. Of these, 18 resonances (coded a, b, c, . . . r) occur in the region 8.0–6.3 ppm and two at higher field (coded t and v). The aromatic protons are identified by their characteristic chemical shifts, their mututal couplings with standard values of $J_{ortho} \sim 8$ and $J_{meta} \sim 2$ Hz, and lack of exchange on addition of D_2O. The two high-field aromatic protons are distinguished from anomeric protons, α-CH, and C*H*OH protons in being mutually coupled but lacking a coupling to an amide NH. The chemical shifts of the protons t and v are consistent with values already reported for vancomycin[12] (which contains

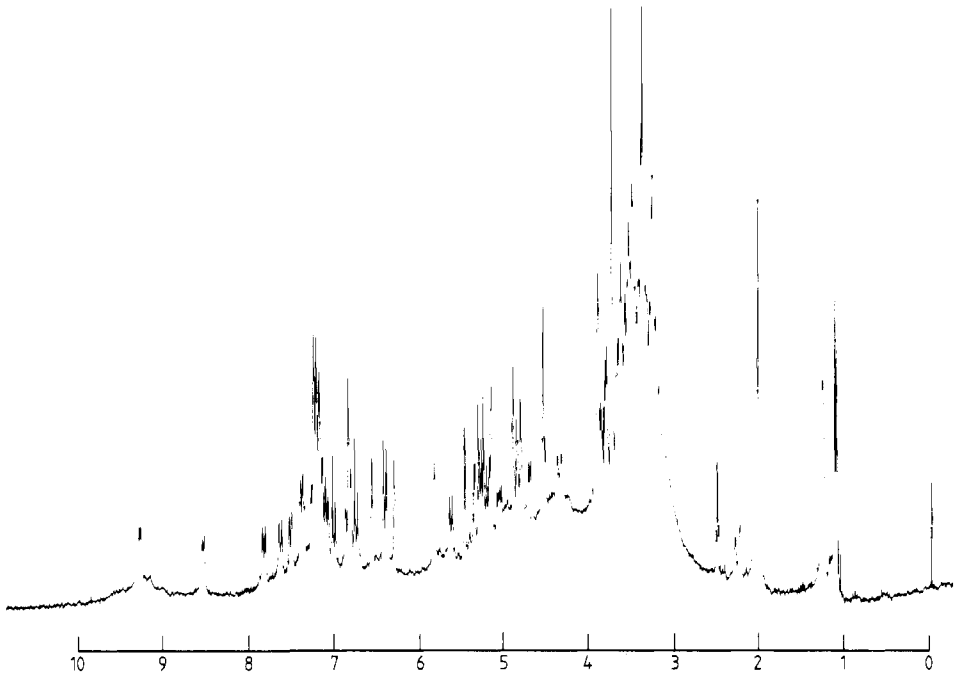


Figure 1. 270-MHz ^{1}H NMR spectrum of ristocetin A (12 mM in Me_2SO-d_6 at 70 °C).

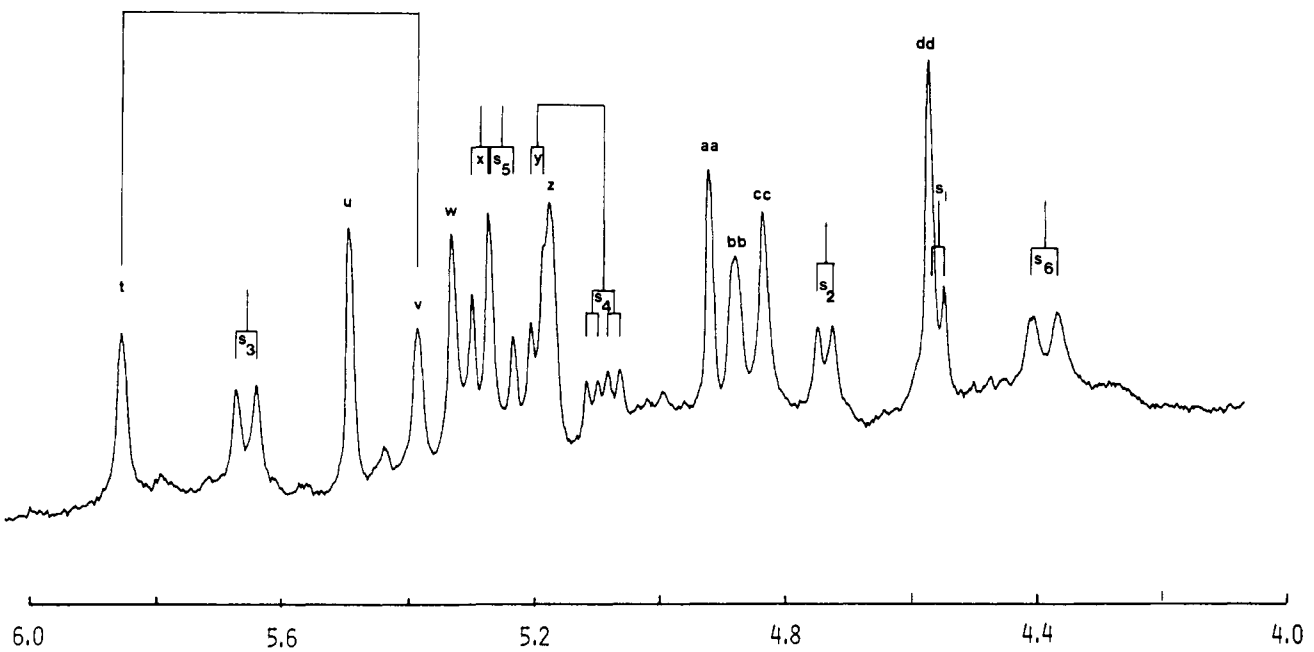


Figure 2. Expansion and assignment of the δ 4.0–6.0 ppm region in the 270-MHz ^{1}H NMR spectrum of ristocetin A (12 mM in Me_2SO-d_6 at 70 °C).

a three-ring fragment similar to **1**). The high-field chemical shifts of t and v establish that these protons lie under rings I and III, i.e., the planes of these rings are approximately perpendicular to the plane of ring II as in vancomycin.[12,13]

F. The αC*H*NH_2 Proton. Only a single resonance (coded cc) due to a carbon-bound proton in the expected chemical shift range 6–4 ppm shows a strong shift dependence on the acidity of the solvent. For example, upon addition of 1 molar equiv of TFA to ristocetin A in Me_2SO-d_6 at 30 °C, a resonance (cc) initially appearing at 4.87 ppm shifts downfield to 5.47 ppm.

G. ArCH(OR)CH– Protons. The α-CH resonance s_4 is a doublet of doublets, being not only coupled to an NH proton (a_5, 7.21 ppm, J = 9 Hz) but also to resonance y (5.19 ppm, J = 5 Hz). These data define the first -CH(OR)CHNH- group. Irradiation of the αCH resonance s_6 results in a perceptible sharpening of the broad singlet z (5.17 ppm), although the s_6/z mutual coupling is small (~1 Hz); thus the assignment of the second -CH(OR)CHNH- group is established. Further coupling of y and/or z to an aliphatic OH was demonstrated in solutions of suitable pH by irradiation of the superimposed doublet and broad singlet OH protons resonating at ~5.98 ppm. These data demonstrate two -CH(OR)CHNH- groups in ristocetin A, wherein at least one group R = H.

H. Sugar Protons. Most of the sugar CH resonances occur in the region 2.5–4 ppm, but the six anomeric protons resonate

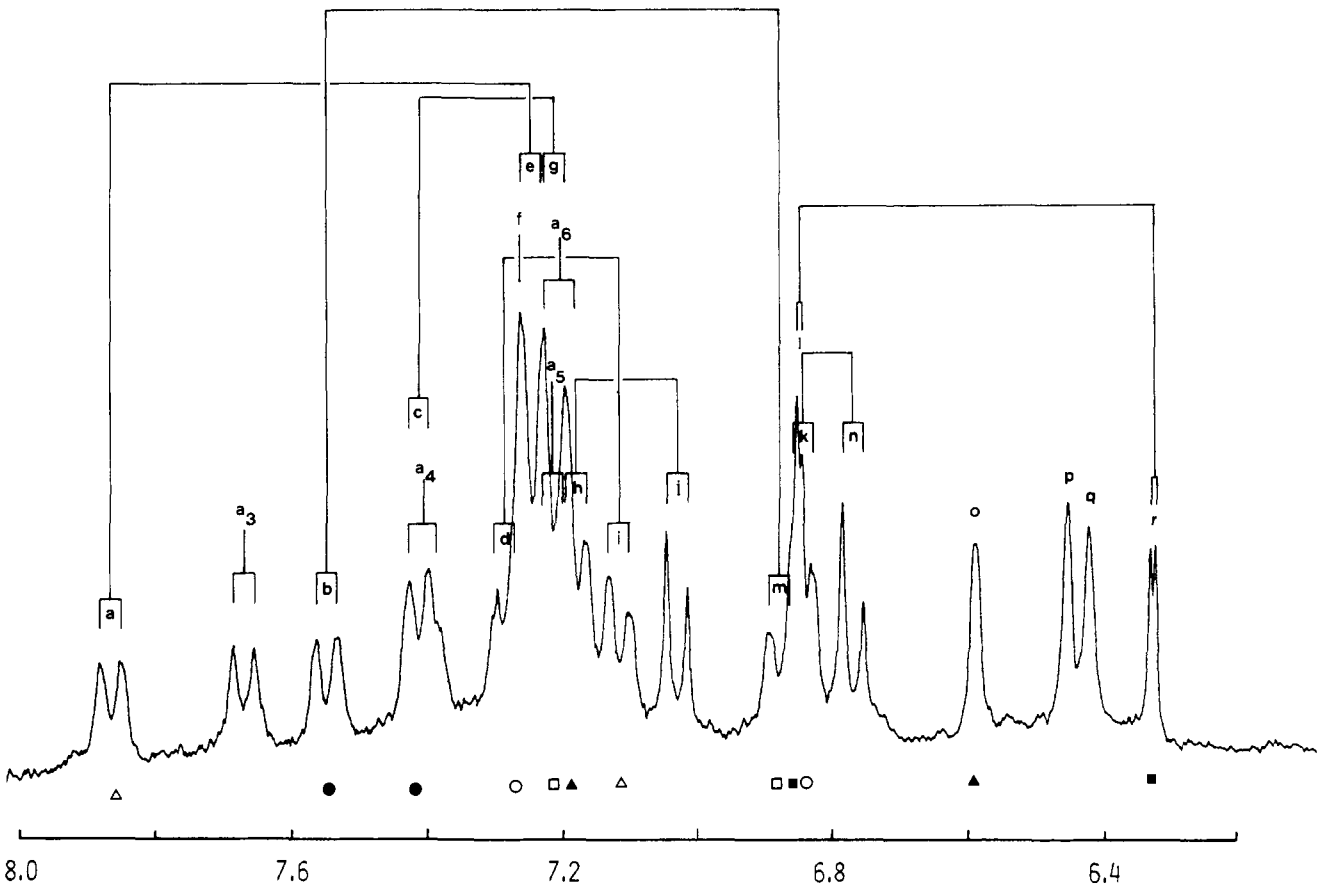


Figure 3. Expansion and assignment of the δ 6.2–8.0 ppm region in the 270-MHz ^{1}H NMR spectrum of ristocetin A (12 mM in Me_2SO-d_6 at 70 °C). Resolvable mutual couplings are indicated by straight lines; meta-coupled pairs of resonances are indicated by matching symbols below the spectrum.

in the 4–6-ppm region. The anomeric protons will be nonexchangeable and coupled, if at all, only to protons in the 2.5–4-ppm region (excepting the anomeric proton of the 2-deoxy sugar ristosamine). Of the six thus far unassigned resonances in the 4–6-ppm region, there is only a single doublet (x, 5.28 ppm); this is decoupled upon irradiation at 3.70 ppm. Of the remaining five (u, w, aa, bb, and dd), one resonance (bb) exhibits coupling to a one-proton resonance (ee_2) at 2.01 ppm; ee_2 is geminally coupled (J = 15 Hz) to ee_1 (2.21 ppm). Resonance bb is thus assigned to the ristosamine anomeric proton (see **6**).

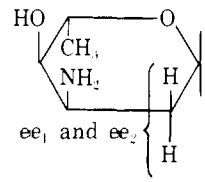
Resonances u, w, aa, and dd are all singlets, and none has been successfully decoupled from a resonance in the 2.5–4-ppm region. These anomeric protons do not therefore manifest large (e.g., diaxial) couplings with C-2 protons; the stereochemical information available from these observations is discussed subsequently.

The doublet methyl resonances at 1.25 (gg) and 1.10 ppm (hh) are coupled to protons at 3.46 and 3.31 ppm in the "sugar envelope", consistent with their being those of the 6-methyl groups of ristosamine and rhamnose. The protons of the aromatic methyl group of **3** resonate at 2.01 ppm (ff).

Specific Assignment of the Ristocetin A Spectrum in Me_2SO-d_6

The assignments considered so far, and many more to be detailed subsequently, are based on (1) decoupling experiments, (2) nuclear Overhauser effects (NOEs), and (3) studies of the Ac-D-Ala-D-Ala/ristocetin A complex (to be considered in more detail in the following paper[14]).

Aromatic Protons. These are grouped on the basis of decoupling experiments. The meta-coupled pairs are r + l; f + k; b + c; h + o; a + i, g + m; and t + v. From the coupling patterns, the assignments that follow for rings I–III are given in **7–9.**



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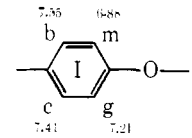

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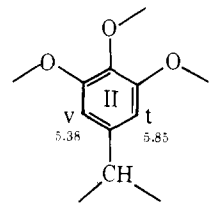

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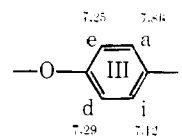

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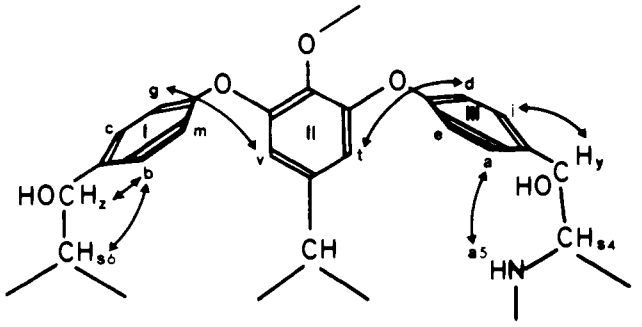
It would normally be expected that both the protons ortho to the CH group would be at lower field than those ortho to oxygen. Whereas this is the case for ring I (see **7** and below), it is not the case for ring III (**9**) since a and i are meta coupled. Confirmatory evidence for the above assignments, and further information, is available from the observation of nuclear Overhauser effects (NOEs) (Table II). These NOEs, and others detailed subsequently, are observed during the course of normal spin decoupling experiments; they are observed at a variety of temperatures. Since (1) ristocetin A has a relatively high molecular weight, and therefore tumbles in solution relatively slowly, and (2) our spectra have been obtained at high field

strengths (270- and 360-MHz spectra), these NOEs are negative in sign.[12] That is, when two protons are sufficiently close in space that one contributes to dipolar relaxation of the other, irradiation of one causes a decrease in the intensity of the resonance of the other. These observations allow crucial deductions to be made as to the molecular geometry of ristocetin A.

The NOE data are interpreted in terms of close proximity of the protons where the letter codes are joined by double-headed arrows in **10.**



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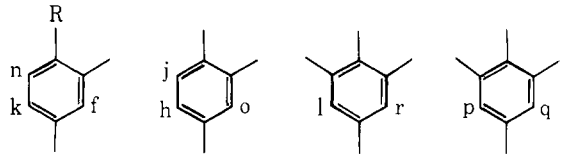
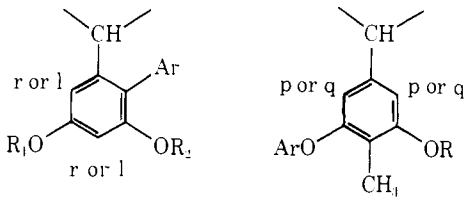
In addition, two 1,2,4-trisubstituted rings (**11** and **12**) and two 1,2,3,5-tetrasubstituted rings (**13** and **14**) can be characterized. Both p and q are broad resonances since the meta coupling cannot be resolved, whereas the meta coupling is readily resolved for the resonances l and r. This observation



11 **12** **13** **14**

suggests that the aromatic methyl group is present on the same ring as p and q, and advances the assignments to those shown in **15** and **16.**



15 **16**

These restrictions have been confirmed by NOE experiments performed on the Ac-D-Ala-D-Ala complex of ristocetin A.[14] The four phenolic protons which are evident in the spectrum of the complex were irradiated in turn. Resonances corresponding to r and l showed negative NOEs on irradiation of a single phenolic resonance. Therefore r and l must both be ortho to the same phenolic group, as in **15** (R_1 = H). Thus the oxygen substituent between r and l does not carry a sugar residue and is indeed a phenolic OH group. In sequential experiments, irradiation of two other phenolic protons led to negative NOEs for resonances correspong to n and p. Hence both n and p are ortho to phenolic groups. It follows that in **16,** R = H and that the proton ortho to the OH group is p, and that the group R in **11** is OH. In **15,** R_2 may be a sugar residue (as is established subsequently).

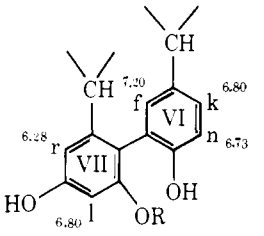
We have established that the attachment of **11** to **13** constitutes the biphenyl system (rather than **12** to **13**) by comparing the observed chemical shifts with those found in the same biphenyl unit when incorporated in vancomycin.[12] The

Table II. NOE Data Relevant to Assignments in Fragments 7-9

protons irrad	intensity reduced (%)	protons irrad	intensity reduced (%)
$d + a_6$	t (15%), v	b	z (12%)
$g + a_5$	a (30%)	x + y + s_5 + z	b (15%), i
a	$(a_5 + g)^a$	v	$(g + h)^a$
y + z	i (20%)	t	d (20%)
s_5 + x + y	i (20–50%)	s_6	b (20%), z (10%)

[a] Overlapping resonances.

relevant data are given in **17–19;** the ristocetin A data (**17** and **18**) refer to a temperature of 40 °C and the vancomycin data



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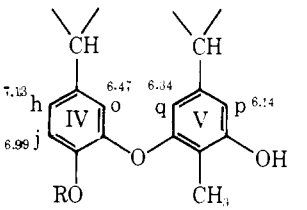

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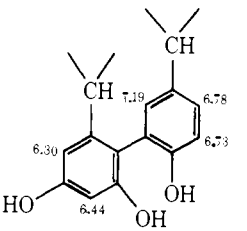

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to a temperature of 70 °C. There is a close correspondence of chemical shifts in the biphenyl units of ristocetin A (**17**) and vancomycin (**19**) with the exception of resonance l. This one major different is not unexpected, since it will be shown that the oxygen atom ortho to both l and the inter-ring bond of the biphenyl system carries a sugar in ristocetin A, but carries a hydrogen atom in vancomycin.[13]

Ring Connectivity. Assignment of αCH/NH Pairs and CHOH Groups

Nuclear Overhauser effects, which aid the proton assignments and structure elucidation, are listed in Table III. In developing the connectivity arguments which follow from NOEs, we will refer to the assigned aromatic ring systems of **10, 17,** and **18** by the Roman numerals I–VII indicated in these structures. On the basis of strong NOEs, we conclude that the following proton pairs are in close proximity: (a ↔ a_5) (a_3 ↔ t) (p ↔ s_5) (a_4 ↔ q) (a_5 ↔ q) (s_6 ↔ f) (s_6 ↔ s_2) (f ↔ s_2) (b ↔ z) (i ↔ y).

The β-hydroxytyrosine units [-CH(OH)CH(NH)-] are associated with the resonances y, s_4, and a_5 and z, s_6, and a_6. The former set can be placed on ring III by the proximity of a and a_5, the latter set on ring I by the proximity of b, s_6, and z (see **10**). Irradiation of t causes s_3 to sharpen slightly, placing s_3 on ring II. The NOE s_2 → f places s_2 on rings VI or VII, and p → s_5 places s_5 on ring V (or, less probably, on ring IV).

The mutual NOEs of s_2 and s_6 (and f) indicate that ring I is connected to the ring bearing s_2 (i.e., ring VI or VII). An NOE (c + f + a_6) → s_1, observed in the spectrum of the peptide-antibiotic complex,[14] shows that s_1 is also in this region of the molecule. It follows that the NOE (e + f) → s_1 must be due to the proximity of f and s_1 (since e is in ring III). We

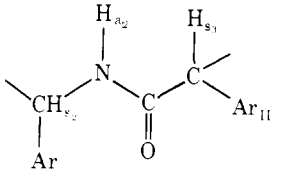
Table III. NOEs for Ristocetin A in Me_2SO-d_6 Solution under Various Conditions[a]

proton irrad	int reduced (%)	proton irrad	int reduced (%)
a	h (20%); a_5 + g	e + f + g + (a_5 + a_6)	s_2 (~20%), cc (13%)
a_3	t (20%)	a_5 + h (+g)	a
b	z (>12%)	s_6	b (15%)
c + a_4	q (20%), o (12%)	s_5	t, p (40%)
d + a_6	t (~15%)	b + c + a_5 + a_6	q (23%)
e + f	t (13%), s_2 (25%)		z (~10%), s_2 (14%)
	s_1 (~20%)	k + l	w (30%)
g + a_5	a (30%)	e + g + h (+f)	s_2 (20%)
k + l + m	w (25%), v (10%)	t	a_3
x + y + s_5	i (>20%)	p	s_5 (>20%)
s_2	s_6 (40%), f (30%)	a_2	s_3 (25%)
s_6	s_2 (60%), f (25%)		
	b (20%), z (10%)		
aa + bb + cc	h (15%)		

[a] The relevant spectra were recorded at a variety of temperatures between 24 and 70 °C. As a consequence, near coincidences of resonances recorded in Table I may be removed and others introduced.

conclude that the coupled pair of resonances s_1/a_1 must be the other α-CH/NH pair of rings VI and VII.

The NOE $a_2 \rightarrow s_3$ points to a link, through an amide bond (see **20**), of the coupled a_2/s_2 system to ring II.



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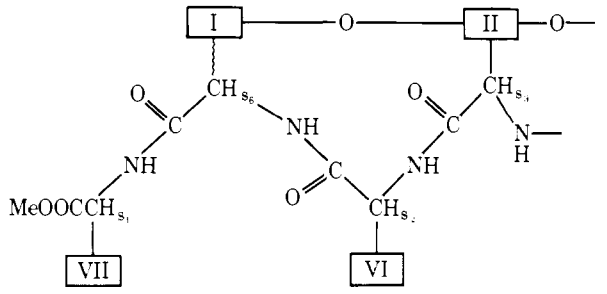
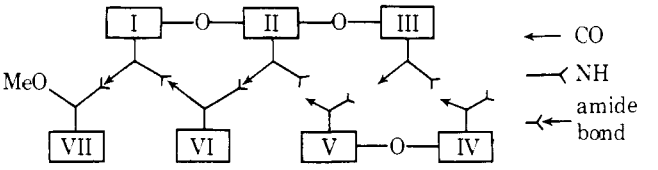
The carbonyl group bonded to the α carbon which also bears s_2 must provide the link to the ring I NH. This arrangement is evidenced by the proximity of s_2 and s_6 (Table III), bearing in mind also that the coupled z/s_6 system has previously been shown to be attached to ring I. Thus, the carbonyl group of ring I remains to be attached to a_1 (see **5**). On the basis of these arguments and model building it follows that the carbonyl group attached to the same carbon as s_1 cannot be involved in an amide bond; it must therefore be part of the methyl ester function[8] of ristocetin A (see **5**). If the NOE $s_2 \rightarrow$ f is taken to most probably indicate the attachment of the carbon bearing s_2 to ring VI, then we arrive at the connectivity scheme shown in **21.** This last point in the argument is dubious, since the s_2



21

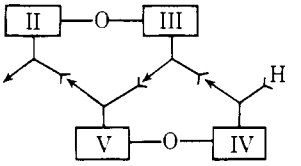
→ f NOE is shown by models to be consistent with attachment of the s_2-bearing carbon to either rings VI or VII. However, it will be seen subsequently that **21** provides a striking analogy to part of the vancomycin structure,[13] and further that there is indeed a close correspondence between the vancomycin[12] and ristocetin A chemical shifts and coupling constants for this part of the structure (see later).

We have now assigned the αCH-NH pairs for six rings: I, II, III, VI, VII, and one of IV and V. The seventh ring must bear the nuclear NH_2 group of ristocetin A. Although a number of NOEs have been observed for the region of the molecule around rings III, IV, and V, there are not sufficient data to distinguish the connectivity without considering all the possible models. This is most easily done diagrammatically; to illustrate, the scheme of connectivity for rings I, II, VI, and VII is presented in **22.**



22

We are fortunately able to limit the possibilities, since it has already been established that rings II and III bear αCH-NH groups where the NH is part of a secondary amide. We therefore consider only the structures where either ring IV or V bears the $-NH_2$ group. There are six such structures, three each for ring IV or V bearing the primary amine. Those for ring IV bearing the amine are illustrated in **23–25;** the others are obtained by interchanging IV and V.



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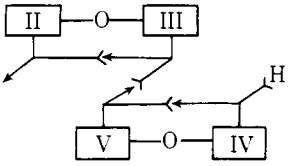

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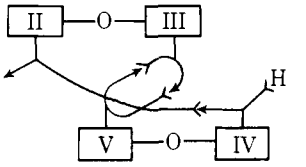


25

Structure **24** contains two rings neither of which can be constructed using CPK models. Structure **25** is in fact a diketopiperazine, which would require a fairly rigid six-membered ring. The relevant $J_{\alpha,NH}$ coupling constants are 10 and 9.3 Hz, characteristic of an almost exactly trans-coplanar arrangement of the αCH-H groups, and too large to be accommodated by a cis structure. Thus **23** is the only acceptable scheme of connectivity.

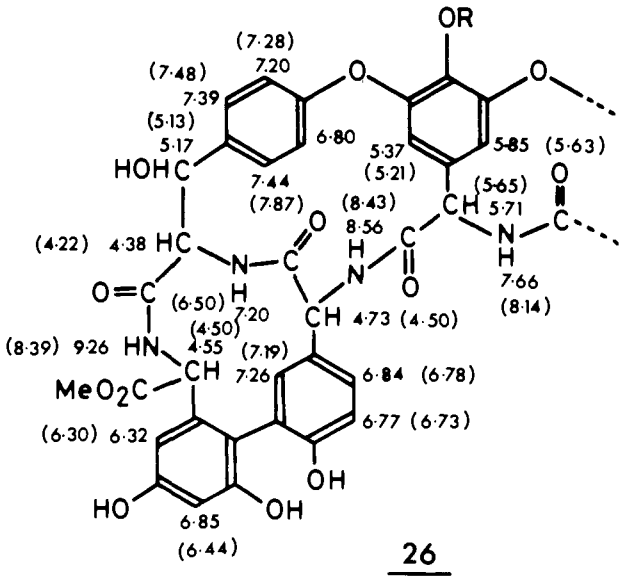
Location of, and Stereochemistry Associated with, Rings IV and V

The connectivity scheme which emerges from a combination of **22** and **23** (ambiguous only in the possible reverse location

Table IV. Comparison of Vancomycin and Ristocetin A Chemical Shifts and Coupling Constants (Me_2SO-d_6 Solution at 70 °C)

ring no.	anti-biotic	chemical shift values, δ							coupling constants, Hz	
		ring protons				βCHOH	αCH	NH	$J_{\alpha,\beta}$	$J_{\alpha,NH}$
I	R	7.20	7.39	(7.44)	(6.80)	5.17	4.38	7.20	~0	12
	V	7.28	7.48	(7.87)	(Cl)	5.13	4.22	6.50	~0	12
II	R		5.34	5.85			5.65	7.66		8.2
	V		5.21	5.63			5.71	8.04		8
VI	R	7.26	6.84	6.77			4.73	8.56		6.5
	V	7.19	6.78	6.73			4.50	8.43		5
VII	R		6.32	6.85			4.55	9.26		5
	V		6.30	6.44			4.50	8.39		5

of rings IV and V) shows that the connectivity of rings I, II, VI, and VII is precisely that found in vancomycin.[13] Since both antibiotics bind strongly to peptides terminating in D-Ala-D-Ala,[10] it is clearly likely that the system associated with rings I, II, VI, and VII provides a common part of a binding site for such peptides. This proposal would be in accord with the proposal for the binding site of vancomycin.[13] Such common parts of a binding site would further be expected to be the same in stereochemical detail. For the two antibiotics a comparison (Table IV and **26** (data for vancomycin in parentheses)) of the



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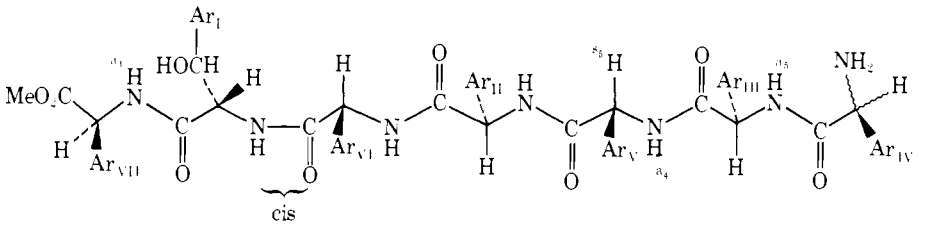
chemical shifts and coupling constants for the common parts (where they are likely to be remote from different structural features) shows striking agreement between the two sets of data. With the exception of proton 1, for the 14 carbon-bound protons which are compared, the chemical shifts are in all cases in agreement to 0.23 ppm or less, with an average deviation of only 0.10 ppm. We conclude that these parts of the two antibiotics are identically constituted (with the exceptions of the chlorine atom in ring I of vancomycin and the conversion of the ring VII CO_2H group in vancomycin to COOMe in ristocetin A), especially in the light of the near identity of the four pairs of $J_{\alpha,NH}$ values (Table IV). Thus, we conclude that the absolute stereochemistries at the amino acid α-CH groups of rings I, II, VI, and VII are *S*, *R*, *R*, and *S*, respectively, and that the absolute stereochemistry at the center carrying the aliphatic hydroxyl group is *R*. Particularly convincing in relation to these conclusions is the "nest" of mutual NOEs involving s_2, s_6, and f; the analogous protons are found in close proximity in vancomycin.[13]

With the structure of the molecule in the regions of rings I, II, VI and VII defined, we are now in a position to consider the relative locations of rings IV and V. The NOEs for the region of the molecule constituted from rings IV and V have already been listed in Table III. However, some of the ambiguities can now be removed. Thus a_5, already known to be near ring III (NOE a → a_5), must be responsible for the NOE (b + c + a_5 + a_6) → q, a_4 for the NOE (c + a_4) → q, and cc for the NOE (aa + bb + cc) → h. The simplified list of relevant NOEs is a ↔ a_5, a_3 → t, a_5 → q, p → s_5, cc → h. Also, in the spectra of the antibiotic-peptide complex,[14] we find NOEs corresponding to (o + p + q) → s_5. The NOEs p → s_5 and cc → h provide strong evidence for the structure given in **5**, i.e., with the free NH_2 group on ring IV.

There is also clear evidence that the absolute stereochemistry at the asymmetric centers of the second β-hydroxytyrosine is the same as that occurring in vancomycin.[13] The NOEs observed in ristocetin A establish that, as in vancomycin, z is on the "front" face of the molecule (the side that binds Ac-D-Ala-D-Ala[14]), whereas y is on the opposite face (see **10**). Moreover, $J_{\alpha,\beta}$ (the y/s_4 coupling constant) is similar in vancomycin (4 Hz) and ristocetin A (5 Hz), as is also $J_{\alpha,NH}$ (the s_4/a_5 coupling constant), which is 9 Hz in vancomycin and 9.3 Hz in ristocetin A. We conclude that the absolute configuration at the carbon bonded to s_4 is *R*, and that at the carbon bonded to y is *R*.

It is now possible to deduce the absolute stereochemistry at the carbon atom bearing s_5 (**5**). Since $J_{\alpha,NH}$ for a_4/s_5 is 10 Hz, the dihedral angle between the protons of the αCH–NH bond must be close to 180°. If we assume an *R* configuration at the carbon bearing s_5, then for a trans amide bond between rings V and III (i.e., amide bond involving a_4) the conformation of the antibiotic involves a peptide backbone in an extended conformation (**27**), except for the cis-amide bond indicated (which is known from the X-ray structure of vancomycin[13]).

Evidence to be presented subsequently[14] establishes that the NH protons a_1 and a_5 form hydrogen bonds to carbonyl groups of bound Ac-D-Ala-D-Ala. The peptide backbone of the anti-



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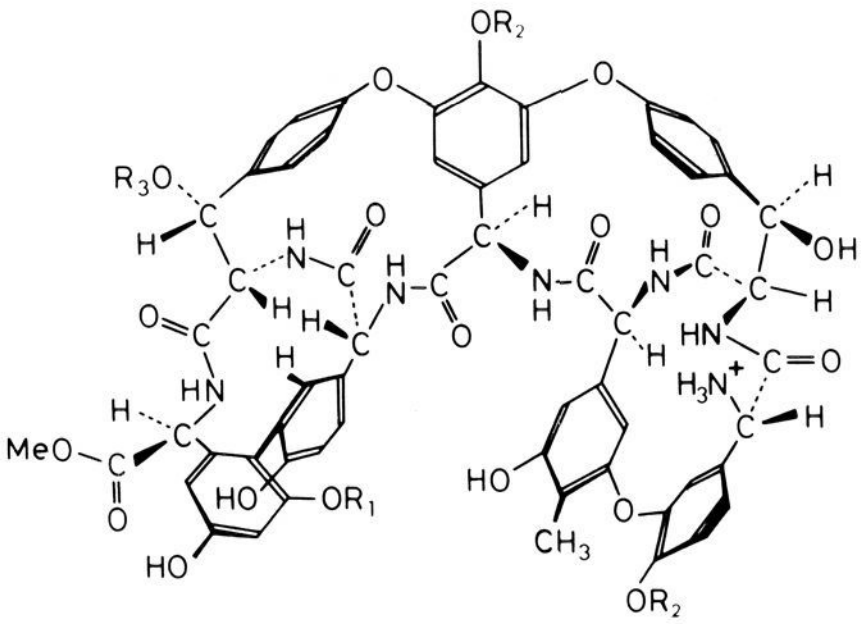
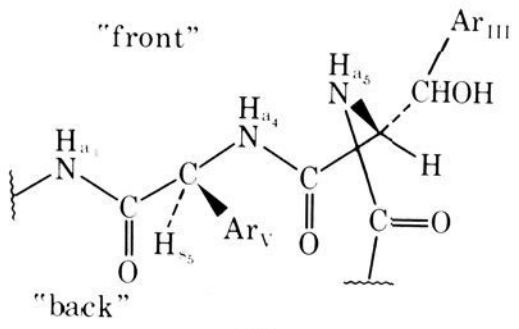


Figure 4. Proposed structure of the aglycone of ristocetin A ($R_1 = R_2 = R_3 = H$).

Figure 5. CPK model of ristocetin A aglycone, showing amide NH groups on the "front" face of the molecule. These NH groups are indicated by "hooked" hydrogen atoms, and from left to right are a_1, a_3, a_4, and a_5. The remaining two NH protons (a_2 and a_6) are hidden on the "back" face of the molecule and are completely exposed to solvent.

biotic does not have the flexibility to make this possible in this model (**27**) while keeping the angle between s_5 and a_4 near to 180° ($J_{\alpha,NH}$ = 10 Hz observed in the spectrum of the antibiotic–peptide complex).[14]

The alternative arrangement has the *S* configuration at the carbon bearing s_5. The $J_{\alpha,NH}$ coupling constant (10 Hz) now requires the ring V NH to be on the "front" face of the molecule (**28,** which shows the major modification caused to the



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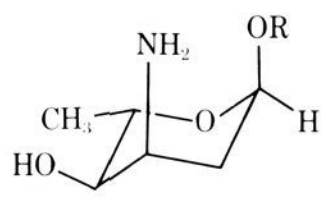
right-hand portion of **27**). The resulting peptide backbone is bent so that a_1 and a_5 approach each other more closely, enabling a binding model to be readily constructed.[14]

We conclude that the configuration at the carbon bearing s_5 is *S*. This conclusion is consistent with the NOEs p → s_5, a_4 → q, a_5 → q, and cc → h.

Thus, the most likely structure for ristocetin aglycone is **5** with the stereochemistry as previously detailed in this paper. The absolute configuration at the ring IV α-CH remains to be determined, although titration data support the *S* configuration;[15] we note that, if it is *S* as shown in Figure 4, the free NH_2 of this residue would approach close to the NH a_5 and provide a Coulombic contribution ($-CO_2^-\cdots{}^+NH_3-$) to the binding of Ac-D-Ala-D-Ala as its carboxylate. A CPK model of the proposed structure, given in Figure 5, shows that a_3, a_4, and a_5 are in a pocket on the "front" face and so should not be completely accessible to solvent. In contrast, a_1, although on the front face, should be more accessible (Figure 5), and a_6 and a_2, on the "back" face, more accessible still. These expectations are in accord with the measured temperature coefficients of the amide NH proton resonances, which are a_3 (−3), a_4 (−0.5), a_5 (−1.5); a_1 (−3), a_6 (−5.5), a_2 (−6 × 10^{-3} ppm/K in Me_2SO-d_6 solution).

Assignment of the Sugar Anomeric Protons and Related Structural Conclusions

The partial assignment of the six sugar anomeric protons is given immediately below **5**. The ristosamine anomeric proton bb is assigned unambiguously since ristosamine is the only 2-deoxy sugar in ristocetin A, and therefore can be decoupled from one of the high-field geminally coupled pair ee_1 and ee_2. Since bb resonates as a broad singlet, the ristosamine anomeric proton must be equatorial. On the basis of the known stereochemistry of ristosamine,[16] this may be accommodated in an α anomer **29** or a β anomer **30.** Since **30** should show a strong preference to exist in the alternative chair form (with three substituents equatorial and only one axial), we conclude that *ristosamine is present as the* α *anomer* **29.**



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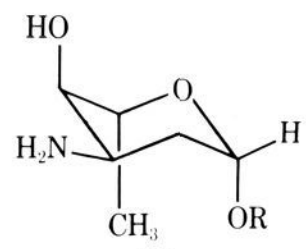

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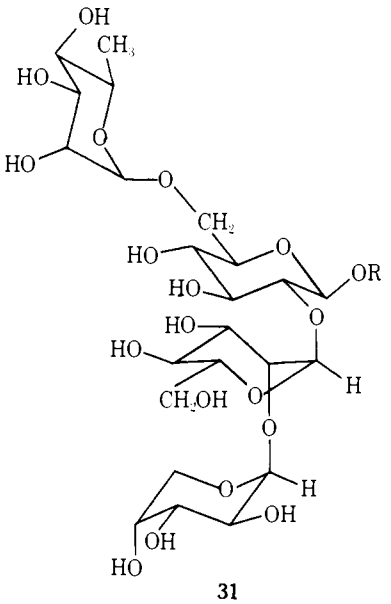
We have recently prepared a pseudoaglycone from ristocetin A by treatment with MeOH–HCl;[17] this product has lost five sugars but retains ristosamine. Ristosamine is therefore directly bonded to the aglycone. The resistance to hydrolytic removal of ristosamine by mild acid treatment, and the observation that it may be removed on treatment with base,[18] suggests that it is not linked via a phenolic OH but perhaps via one of the two aliphatic hydroxyl groups of **5**. This conclusion would be consistent with the earlier deduction[9] that a tetrasaccharide and mannose are separately bound to the aglycone, and yet allow for the observation of four phenolic OH resonances (rather than the three permitted if ristosamine were bound via a phenol) in the present work.

Of the six anomeric protons, only one (x) is split by a large coupling constant (7.8 Hz). The anomeric stereochemistry of a trisaccharide unit has been established by the determination of the structure of *O*-α-L-rhamnopyranosyl(1→6)-*O*-[α-D-mannopyranosyl(1→2)]-D-glucose;[3] this structure has been confirmed by synthesis.[19] The α stereochemistries at the anomeric carbons of L-rhamnose and D-mannose preclude large $J_{1,2}$ coupling constants for probable conformations of either of these sugars. The fourth sugar of the tetrasaccharide is attached at the 2 position of mannose as an *O*-β-D-arabinopyranosyl glycoside,[20] again excluding a large $J_{1,2}$ coupling constant for either chair conformation of arabinose, e.g., **31.** The same exclusion applies for the remaining D-mannose monosaccharide unit in a probable conformation of either an α or β anomer, due to its C-2 stereochemistry (axial substituent). Therefore, x is due to the anomeric proton of D-glucose, and we conclude that the tetrasaccharide unit is bound as its



31

β anomer at glucose. The present conclusion with regard to the anomeric stereochemistry at D-glucose is in agreement with those published earlier,[9] although the abstract does not provide the evidence for these conclusions. Resonance w is reduced in intensity by ~30% on irradiation of the coincident resonances k, l. This negative NOE is most likely due to the close proximity of the anomeric proton w and an aromatic ring proton ortho to the phenolic oxygen involved in the glycosidic bond. One (l) of the two irradiated protons (k, l) is indeed ortho to two phenolic oxygens. Since one of these oxygens has already been established to be present as a free OH group, then the remaining phenolic oxygen of ring VII must carry the sugar whose anomeric proton is w. On the basis of the above arguments that only the tetrasaccharide and an additional mannose are glycosidically bound via phenolic oxygens, then w is the anomeric proton of the mannose monosaccharide unit (mannose$_1$). The relative sharpness of resonance w is consistent with either an α- or β-glycoside of mannose$_1$; other workers[9] have stated that the sugar is attached to the aglycone as a β-glycoside, although the evidence for this conclusion is not available in the abstract.

Specific assignments of the anomeric protons of mannose$_2$, arabinose, and rhamnose (aa, dd, and u) are not made. However, their occurrence as singlets supports the presence of the chair conformations for rhamnose and mannose given in **31.** Since the phenolic oxygens indicated by asterisks in **5** are established to exist as free OH groups in ristocetin A, then the tetrasaccharide unit must be attached to the phenolic oxygen of ring II or ring IV. The chemical-shift analogies for H-1 and H-2 of glucose in vancomycin (5.35 and 3.62 ppm) and ristocetin A (5.25 and 3.66 ppm) are consistent with the attachment of the tetrasaccharide to ring II.

Conclusions

The structure of the ristocetin A aglycone which is proposed on the basis of the present work is given in Figure 4. The stereochemistry at the α carbon of ring IV remains in doubt.

In ristocetin A, mannose is attached to ring VII (Figure 4, R_1 = mannosyl). The tetrasaccharide **31** is bonded via the phenolic oxygen of ring II or ring IV, i.e., one of the R_2 groups in Figure 4 represents the tetrasaccharide. The point of attachment of ristosamine, as the α anomer **29,** remains in doubt but it is not bound via a phenolic oxygen. The available evidence would be satisfied, for example, by R_3 = ristosaminyl (Figure 4.)

A significant proportion of the structural and stereochemical information given in Figure 4 is based on the application of the nuclear Overhauser effect. However, it should be emphasized that the NOE should be applied with care since a single misassigned resonance can give rise to erroneous structural conclusions. In the present work, the possibility of error has been greatly reduced by the correspondence between a portion of the structure of ristocetin A (Figure 4) and that of vancomycin, which has been established by X-ray methods.[13]

Note Added in Proof. The remaining details of the structure of ristocetin A have now been established (Williams, D. H.; Rajananda, V.; Bojesen, G.; Williamson, M. *J. Chem. Soc., Chem. Commun.*, **1979,** 906).

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