The Structure of an Amino-Sugar from the Antibiotic Vancomycin

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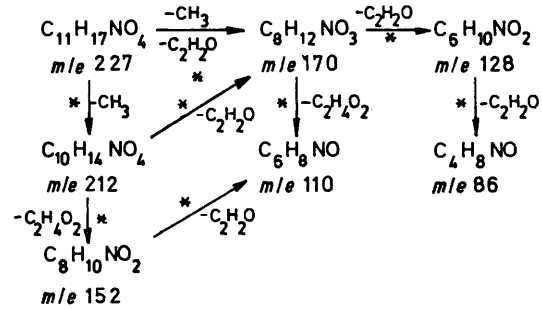
Mild acid hydrolysis of the antibiotic vancomycin, previously known to liberate glucose, has been shown to liberate a second sugar formulated as 3-amino-2,3,6-trideoxy-3-*C*-methyl-*lyxo*-hexopyranose. On vigorous treatment with acid, under conditions previously shown to give vancomycin acid (3-methyl-4-oxohexanoic acid) from vancomycin, the novel amino-sugar affords vancomycin acid, thus establishing the origin of this acidic degradation product.

VANCOMYCIN is an antibiotic first isolated in 1956 from an actinomycete, *streptomyces orientalis* by McCormick *et al.*[1] Its structure has been studied by various groups,[1-7] but on the basis of a probable molecular weight of about 1500—1600, substantial parts of the antibiotic have not been isolated as identifiable fragments. We now report the identification of a novel amino-sugar, 3-amino-2,3,6-trideoxy-3-*C*-methyl-*lyxo*-hexopyranose, obtained upon mild acid hydrolysis of vancomycin.

It has previously been reported[3,4] that on treatment of vancomycin with boiling 0·6N-hydrochloric acid for 2 min a white compound, named aglucovancomycin, is precipitated. Glucose has been identified as a compound which is liberated in this transformation.[3,4] However, the ^{1}H n.m.r. spectrum of the solution which remains after precipitation of aglucovancomycin indicates that a second component is liberated from vancomycin in this reaction. Neutralisation of the solution with potassium hydroxide, and subsequent evaporation to dryness, affords a mixture which contains *ca.* 75% potassium chloride, roughly equimolar quantities of glucose and the unknown, and traces of aglucovancomycin.

The unknown was isolated (as an oil) from the other components of the mixture by ion-exchange chromatography [Amberlite IR 120 (H^+)]. It did not give a satisfactory mass spectrum and therefore, since there was evidence (on the basis of its ^{1}H n.m.r. spectrum) for the presence of OH and/or NH_2 groups, the preparation of a more volatile and stable derivative was attempted. Treatment with acetic anhydride–pyridine afforded a triacetyl derivative (as established from the ^{1}H n.m.r. spectrum). The high resolution mass spectrum of this derivative was obtained with the aid of an on-line computer, and showed the highest mass ions at *m/e* 212 ($C_{10}H_{14}NO_4$) and 227 ($C_{11}H_{17}NO_4$). With the aid of 'metastable peaks' the fragmentation map in Scheme 1 was established; the transition *m/e* 227 ⟶ 170 probably corresponds to two successive metastable transitions occurring in the same field-free region.



SCHEME 1

When the acetylation experiment was repeated with [$^2H_6$]acetic anhydride, the mass spectral peaks at *m/e* 227 and 212 were replaced by peaks at *m/e* 233 and 218. Therefore, *m/e* 227 does not correspond to the molecular ion of a triacetyl derivative, but at least part of one acetyl group has been lost in the formation of *m/e* 227. Since the molecular ion of a compound containing one nitrogen atom will contain an odd number of hydrogen atoms, *m/e* 227 must have been formed by the loss of an even-electron species (*e.g.* acetic acid), and not a radical, from the molecular ion. Since field-ionisation mass spectra have proved especially useful in obtaining molecular ions from model sugars where none were obtained from electron impact spectra,[8] a field-ionisation mass spectrum of the triacetyl derivative of the unknown was

[1] M. H. McCormick, W. M. Stark, G. E. Pittenger, R. C. Pittenger, and J. M. McGuire, 'Antibiotics Annual,' 1955—1956, Medical Encyclopaedia Inc., New York, 1956, p. 606.
[2] H. M. Higgins, W. H. Harrison, G. M. Wild, H. R. Bungay, and M. H. McCormick, 'Antibiotics Annual,' 1957—1958, Medical Encyclopaedia Inc., New York, 1958, p. 906.
[3] E. J. Marshall, *J. Medicin. Chem.*, 1965, **8**, 18.
[4] C. R. Johnson, Ph.D. Thesis, University of Illinois, 1962.
[5] N. N. Lomakina, I. A. Spiridonova, R. Bognar, M. Puskas, and F. Sztaricskai, *Antibiotiki*, 1968, **13**, 975 (*Chem. Abs.*, 1969, **70**, 34,350).
[6] N. N. Lomakina, L. I. Murav'eva, A. S. Mezentsev, M. S. Yurina, and F. Sztaricskai, *Antibiotiki*, 1969, **14**, 594 (*Chem. Abs.*, 1969, **71**, 124,870).
[7] N. N. Loumakina, L. I. Murev'eva, and M. S. Yurina, *Antibiotiki*, 1970, **15**, 21 (*Chem. Abs.*, 1970, **72**, 107,083).
[8] H. D. Beckey, *Angew. Chem. Internat. Edn.*, 1969, **8**, 623.

obtained. This spectrum contained the highest mass ion at *m/e* 288, and a ' metastable peak ' establishing the loss of acetic acid from this species to give a daughter ion at *m/e* 228. Field-ionisation mass spectra frequently give $M^+ + 1$ rather than M^+ ions (owing to ion-molecule reactions at the blade tip or wire where ionisation occurs),[8] and therefore the molecular weight to be inferred for a compound containing one nitrogen atom is 287, *i.e.* *m/e* 227 in the electron-impact mass spectrum does indeed arise *via* loss of acetic acid from a triacetyl derivative of the formula $C_{13}H_{21}NO_6$. This conclusion is supported by the presence of an $M + 1$ peak at *m/e* 297 in the field-ionisation spectrum of the derivative prepared with [$^2H_6$]acetic anhydride, *i.e.* a [$^2H_9$]triacetyl derivative results. Therefore the molecular formula of the unknown is $C_7H_{15}NO_3$.

Details of the ^1H n.m.r. spectrum of the unknown in deuterium oxide solution are given in the Table. The

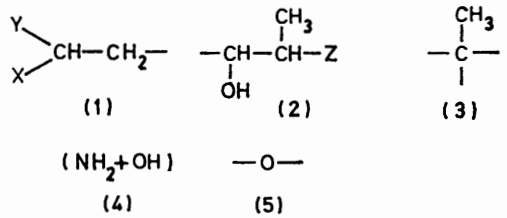
TABLE

^1H N.m.r. spectrum of the unknown *

Integrated intensity	δ (p.p.m.	Multiplicity	*J* Values in Hz (β-anomer)
1	5·01 (5·41)	q	2·6, 9·7
2	*ca.* 1·90	AB of ABX	2·6, 9·7, 12·6
1	3·46	d	0·7
1	4·00 (4·36)	pair of q	0·7, 6·5
3	1·23 (1·20)	d	6·5
3	1·48 (1·62)	s	

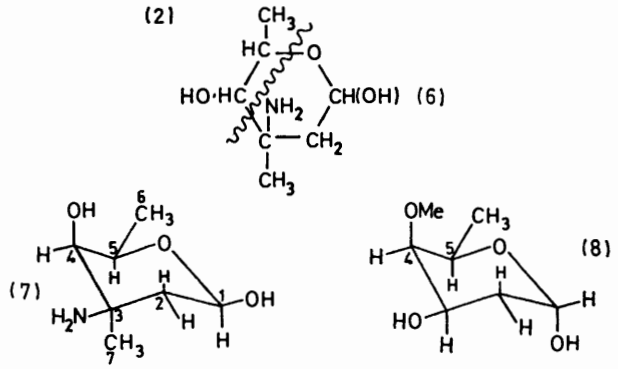
* δ Values for the β-anomer are given first, and for the α-anomer are given in parentheses; chemical shifts are measured relative to internal sodium 2,2-dimethyl-2-silapentane-5-sulphonate at pD 8·5.

spectrum could be interpreted in terms of a major and a minor isomer, now known to correspond to β- and α-anomers of a sugar. Since the spectrum contains signals due to 11 protons, 4 protons are relatively acidic and removed by exchange with the solvent. The data are consistent with the formation of a triacetate if the active hydrogen atoms are those from two hydroxy-groups and one primary amino-group. Spin-decoupling experiments established that the low-field proton (δ 5·01 p.p.m.) is coupled unequally to the two high-field protons (δ *ca.* 1·90 p.p.m.), which are themselves sufficiently non-equivalent to allow a geminal coupling of 12·6 Hz between them to be discerned. These three protons form an isolated ABX system (1).



The chemical shift (δ 3·46 p.p.m.) of the one-proton doublet split by *J* 0·7 Hz does not unambiguously indicate whether this proton is attached to the same carbon atom as an OH or NH_2 group, but we temporarily assume that it is attached to the same carbon atom as an OH group, for reasons to be given subsequently; the further couplings, multiplicities, and intensities allow the unit (2) to be inferred.[1] The singlet methyl resonance at δ 1·48 p.p.m. accounts for a sixth carbon atom, which, judging from its chemical shift, must be attached to the seventh carbon atom, which carries no protons (3). The required molecular formula is completed by the necessary amino- and hydroxy-groups (4) and an ether oxygen atom (5). The double-bond equivalent of the compound must be accounted for by a ring, since its i.r. spectrum establishes the absence of carbonyl absorption.

The lack of further coupling of the CH_2 group of system (1) is accommodated by attachment of system (3) (plus the amino-group) to (1) to give system (6), and the whole n.m.r. spectrum is satisfied by attachment of system (2) as shown. Thus the unknown is formulated



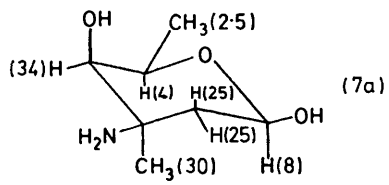
as a 2,3,6-trideoxy-3-amino-sugar, and evidence is now presented to support the tentative stereochemistry (7) (although it is emphasised that the absolute configuration shown is arbitrarily chosen). The very small vicinal H-4,H-5 coupling (0·7 Hz) indicates that the electronegative substituents attached to C-4 and C-5 are in *trans*-coplanar configurations with respect to the protons attached to C-5 and C-4, respectively.[9,10] Thus, the heterocyclic oxygen atom must be *trans*-coplanar with respect to an equatorial hydrogen atom at C-4, and the C-4 hydroxy-group axially orientated with respect to a *trans*-coplanar hydrogen atom attached to C-5, so that the electronegativity effects can act optimally in reducing the magnitude of the vicinal coupling constant. A stereochemistry which is analogous at C-4 and C-5 to the one proposed here is found in α-D-chromose A [(8) olivomose], where $J_{4,5}$ is reported [11] as 1 Hz.

Relative to the positions of the methyl resonances in the n.m.r. spectra of the β-anomer, in the α-anomer the $C(6)H_3$ resonance appears at higher field by 0·03 p.p.m., and while the $C(7)H_3$ resonance appears at lower field by 0·14 p.p.m. (Table). Thus the effect of inversion of configuration at C-1 (β ⟶ α) is to shield C-6 but to

[9] N. S. Bhacca and D. H. Williams, *J. Amer. Chem. Soc.*, 1964, **86**, 2742.
[10] H. Booth, *Tetrahedron Letters*, 1965, 411.
[11] M. Miyamoto, Y. Kawamatsu, M. Shinohara, Y. Asahi, Y. Nakadaira, H. Kakisawa, K. Natanishi, and N. S. Bhacca, *Tetrahedron Letters*, 1963, 693.

deshield C-7, and we therefore conclude that these methyl groups are not (even approximately) symmetrically oriented with respect to C-1, *i.e.* the methyl group at C-7 is axial. The stereochemistry (7) is further supported by the relatively large downfield shift (0·36 p.p.m.; Table) of the H-5 signal upon inversion at the anomeric carbon (β ⟶ α); the large downfield shift is in accord with the 1,3-diaxial interaction which is introduced between H-5 and OH.

The gross structure is further substantiated by measurement of chemical shift changes in the spectrum of the unknown on changing the pD of a D_2O solution from 8·6 to 10·5 by the controlled addition of 0·2N-NaOD in deuterium oxide solution. The observed upfield shifts (in Hz) of the various proton resonances are summarised in structure (7a).

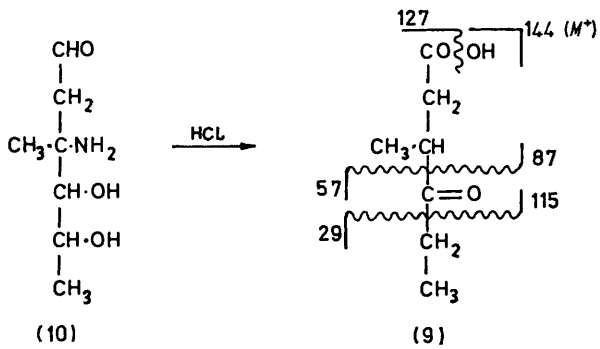


(7a)

We make the reasonable assumption that these upfield shifts are primarily due to deprotonation of the amino-group as the pH (pD) is raised. It can be seen that only those protons which have been placed in the immediate vicinity of the amino-group undergo large shifts. In particular, the equatorial position of the NH_2 group is supported by the equal upfield shifts observed for the protons attached to C-2.

Strong support for the carbon skeleton which is deduced from the molecular formula and n.m.r. data is forthcoming from the previous isolation of vancomycin acid [3-methyl-4-oxohexanoic acid (9)] [3] following acid hydrolysis of vancomycin. It appeared probable to us that compound (9) is in fact a product of elimination and internal oxidation–reduction from the amino-sugar (7), shown in its acyclic form (10).

To test this possibility, the mixture of sugars obtained from mild acid hydrolysis of vancomycin was subjected to the same vigorous acid hydrolysis [3] that afforded vancomycin acid (9) from vancomycin. The acidic



(10) (9)

material isolated was identified by its highly characteristic mass spectrum as 3-methyl-4-oxohexanoic acid [see (9)]. Since this acid cannot arise from glucose, it must arise from the amino-sugar (7). It is noteworthy that our n.m.r. spectrum of vancomycin establishes that a structural unit closely resembling (9) is certainly not present in the original antibiotic (*e.g.* the n.m.r. spectrum of vancomycin does not show signals due to a $\cdot CH_2 \cdot CH_3$ unit). Thus the postulated conversion (10) ⟶ (9) is in accord with the n.m.r. spectrum. In addition, the identification of the amino-sugar accounts for the previously recorded liberation of acetaldehyde from the antibiotic on treatment with periodate.[3,4]

Although the amino-sugar did not give a structurally useful mass spectrum prior to formation of a derivative, the spectrum obtained contained a large peak at *m/e* 108, and further peaks at integral multiples of *m/e* 108 (*m/e* 216, 324, and 432). An element listing of these peaks indicated the compositions $(C_7H_8O)_n$, which in retrospect appear to arise *via* loss of one molecule of ammonia and two of water from the amino-sugar, followed by partial polymerisation of the resulting C_7H_8O unit within the mass spectrometer.

It should be noted that the Russian workers [5] have described the isolation (but not the identification) of a deoxyamino-sugar from ristomycin, and report that this same sugar is also obtained by hydrolysis of vancomycin. They believe their sugar to have the molecular formula $C_{12}H_{25}N_2O_5$.

EXPERIMENTAL

Mass spectra were obtained on A.E.I. MS9 and MS902 instruments operating at accelerating voltages of 8 kV and electron beam energies of 70 eV for electron-impact spectra. Field-ionisation spectra were obtained using the standard A.E.I. source on an MS9 instrument. N.m.r. spectra were obtained on Varian HA100 and XL100 instruments.

Isolation of the ' Unknown ' Sugar.—Vancomycin (5·0 g) was dissolved in water (37·5 ml) and heated to the b.p. 4N-Hydrochloric acid (6·25 ml) was added and boiling was continued for 2 min. The brown solution was cooled to 55 °C and the amorphous precipitate (impure aglucovancomycin) was filtered off (Whatman no. 1 filter paper). 4N-Hydrochloric acid (6·27 ml) was added to the filtrate to make the solution 1N in HCl and further precipitated material was filtered off. N-Potassium hydroxide was added until the pH of the solution was 2·3; further precipitation occurred and this precipitate was also isolated as before. The pH of the filtrate was again raised slowly, but no precipitation of unchanged vancomycin occurred between pH 7 and 9. Finally the pH was adjusted to neutrality and the solution was evaporated to dryness with a rotary evaporator.

The mixture (294 mg) of sugars and potassium chloride was taken up in water (20 ml) and put on a column of Amberlite IR 120 resin (H^+ form; 2 cm diam., 11 cm height) which had been previously washed with 55% aqueous ethanol (500 ml). The column was then successively developed with water (300 ml), 1·8N-hydrochloric acid–ethyl alcohol (9 : 11; 520 ml), N-hydrochloric acid (130 ml), and 6N-hydrochloric acid (130 ml) (flow rate *ca.* 3 ml min^{-1}; fractions *ca.* 100 ml). The eluates were evaporated to dryness under vacuum, the temperature of the solutions being

maintained below 40 °C. The residues were analysed by t.l.c. on Merck silica plates (previously soaked in N-sodium acetate solution and dried) developed with acetone–water (85 : 15). The first fractions (420 ml) contained glucose (40 mg). The following fractions (300 ml) contained the ' unknown ' sugar (26 mg), and further fractions (220 ml) a mixture (10 mg) of the ' unknown ' sugar with potassium chloride. The last fractions contained only potassium chloride.

The ' unknown ' sugar isolated in this manner was an oil, which gave a white solid on trituration with acetone. Microanalyses of this product were not satisfactory, as it was highly hygroscopic and contained traces of inorganic material.

Triacetate of the ' Unknown ' Sugar.—The mixture (310 mg) of sugars and potassium chloride just described was shaken with acetic anhydride–pyridine (1 : 1) for 2 h at room temperature. The insoluble portion (235 mg) of the mixture was then isolated and dried; t.l.c. showed that it contained no organic material. The solution was kept at room temperature for a further 24 h and then evaporated under reduced pressure (temp. 38 °C). The residue (161 mg) was applied to two preparative thin-layer plates [20 × 20 cm; silica gel previously washed (×3) with acetone] in acetone (1 ml). The plates were developed with benzene–methanol (9 : 1), and the bands were located by developing a thin strip by means of a hot wire. The acetylated ' unknown ' sugar was isolated as a yellow oil (59 mg) by extraction with acetone. Analytical t.l.c. of this material showed only one spot; its ^{1}H n.m.r. spectrum ($CDCl_3$) contained bands at δ 5·88 [1H, C(1)H], 5·80br (1H, N–H) 4·91 [1H, C(4)H], 4·02 [1H, C(5)H], 2·42 [2H, C(2)H_2], 2·18 (3H, OAc), 2·09 (3H, OAc), 1·64 (3H) and 1·87 (3H) [OAc, C(7)H_3], and 1·18 [3H, C(6)H_3]. Crystallisation from benzene–ether gave a white solid, m.p. 70—71°.

Microanalysis of this product gave figures in agreement with the mass spectrometric data (Found: C, 54·1; H, 7·5; N, 4·8. $C_{13}H_{21}NO_6$ requires C, 54·4; H, 7·3; N, 4·9%).

[$^2H_9$]*Triacetate of the ' Unknown ' Sugar.*—This derivative was prepared as just described from the mixture (75 mg), [$^2H_6$]acetic anhydride (2·4 ml), and pyridine (3 ml). The derivative (15 mg) gave an $M^+ + 1$ peak in its field-ionisation mass spectrum at *m/e* 297, and in its electron-impact mass spectrum gave the highest mass ion at *m/e* 233 ($M^+ - CD_3{\cdot}CO_2H$).

Vancomycin Acid from the Mixture of Sugars.—The mixture (40 mg) of sugars and potassium chloride was heated under reflux with concentrated hydrochloric acid (1 ml) for 48 h, cooled, and extracted with chloroform (5 × 2 ml). The combined extracts were then extracted with aqueous 2·5% potassium hydroxide (4 ml). The alkaline solution was made acidic with 12N-hydrochloric acid and then extracted with chloroform (3 × 2 ml). The combined chloroform extracts were evaporated to give a residual brown oil (1 mg) which gave the same mass spectrum as a sample of vancomycin acid (3-methyl-4-oxohexanoic acid) obtained from vancomycin by the method of Marshall.[3] The mass spectrum contained the following characteristic ions: *m/e* 144 (8%, M^+), 127 (2, $M^+ - OH$), 115 (21, $M^+ - C_2H_5$), 87 (13, $M^+ - CO{\cdot}C_2H_5$), 57 (100, $C_2H_5{\cdot}CO^+$), and 29 (40, $C_2H_5^+$).

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