Structural Studies on the Antibiotic Vancomycin; the Nature of the Aromatic Rings

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It is shown that the antibiotic vancomycin contains five benzene rings. In a three-ring unit, connected through ether linkages, two sugars (glucose and vancosamine) are sequentially attached to a central pyrogallol system. A two-ring unit is connected as a biphenyl system, incorporating three phenolic OH groups. It is concluded that vancomycin contains carbamoyl and carboxy-groups. The identified units, plus the previously isolated aspartic acid and *N*-terminal *N*-methyl-leucine, appear to account for all, or almost all the carbon skeleton of vancomycin if reasonable assumptions are made regarding interconnection of the units through amide bonds.

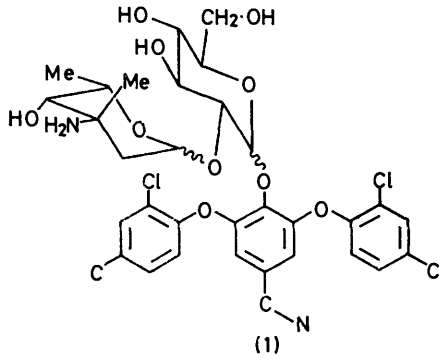
VANCOMYCIN is an antibiotic first isolated in 1956 from an actinomycete, *Streptomyces orientalis*.[1] Interest in its structure has been aroused recently by the discovery that it complexes selectively with peptides having the *C*-terminal sequence D-Ala-D-Ala.[2] This binding is probably the basis of the antibiotic activity of vancomycin,[3] since mucopeptides terminating in the sequence

[1] M. H. McCormick, W. M. Stark, G. E. Pittenger, R. C. Pittenger, and G. M. McGuire, in 'Antibiotics Annual, 1955—56,' Medical Encyclopedia Inc., New York, 1956, p. 606.

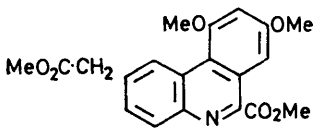
[2] H. R. Perkins, *Biochem. J.*, 1969, **111**, 195.

[3] M. Nieto and H. R. Perkins, *Biochem. J.*, 1971, **123**, 789.

D-Ala-D-Ala are precursors involved in all wall biosynthesis. In the present paper, we give details of evidence which defines a partial structure (1) for vancomycin,[4] and also report the isolation of the phenanthridine (2) as a degradation product of vancomycin, believed to arise from a portion of the antibiotic represented by the partial structure (3).



(1)



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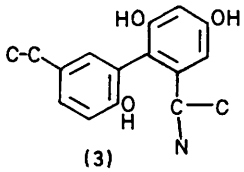
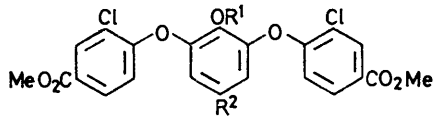
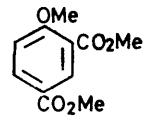


(3)

Linkage of the Sugars.—The possibility that the two sugars (glucose [5] and vancosamine [6,7]) of vancomycin were linked together was investigated by two sets of partial permethylation experiments. In the first method, vancomycin was permethylated (dimethyl sulphoxide anion, followed by an excess of methyl iodide) and the product hydrolysed with methanolic hydrogen chloride. A portion of the partially methylated glucose was then further methylated with C^2H_3I–$^-CH_2$·SOMe. The mass spectrum of the product established that it was a 2-*O*-[$^2H_3$]methyl derivative of permethylglucose.[8] In the second method, the vancomycin was permethylated and the sugars were cleaved with methanolic hydrogen chloride as above, but the resulting partially methylated glucose was then hydrolysed with aqueous hydrochloric acid and subsequently reduced with sodium borohydride and acetylated with acetic anhydride in pyridine to give 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylhexitol, identified from its characteristic mass spectrum.[9] These experiments show that in vancomycin there is a glycosidic bond to position 2 of the glucose. Since tests for reducing sugars have proved negative on both vancomycin [10] and aglucovancomycin (the product obtained from vancomycin on removal of the two sugars by acid hydrolysis [5–7]), the amino-sugar vancosamine must be linked glycosidically to C-2 of the glucose and this disaccharide is linked glucosidically to the remainder of the antibiotic at C-1 [as in (1)].* These conclusions are in accord with the suggestion of Johnson *et al.*[7] These authors also indicated the possibility that a labile substituent might be attached to C-6 of the glucose. However, in our studies no sign of a 2,6-di-*O*-[$^2H_3$]methyl derivative of permethyl glucose was found when the first method described was applied, nor was any tetra-acetyldimethylhexitol found when the second method was applied. We conclude that the 6-hydroxy-group of glucose is free in vancomycin.

Nature of the Chlorine-containing Aromatic Rings.—Earlier degradative studies on vancomycin led to the isolation of chloro-phenols in which the chlorine was substituted *ortho* to the phenolic hydroxy-group.[5] Attempts to isolate larger fragments of the antibiotic incorporating these chlorine-substituted benzene rings, through the use of acidic hydrolysis, were unsuccessful when the hydrolysis was carried out either on the vancomycin or aglucovancomycin, or on methylated derivatives prepared by use of a variety of reagents (CH_2N_2, Me_2SO_4–K_2CO_3, MeI–K_2CO_3, MeI–$^-CH_2$·SOMe). We therefore protected the phenolic groups of aglucovancomycin (MeI–K_2CO_3–MeOH) and attempted selective cleavage of the methylated product through oxidation with permanganate in aqueous ammonia. The products were expected to be carboxylic acids and these were therefore methylated with diazomethane, or [$^2H_2$]diazomethane,[11] prior to separation *via* preparative t.l.c. This procedure afforded five pure products; the structures of three of these [(A)—(C)] have been determined as (4)—(6) from spectroscopic and microanalytical





(6)

(4) R^1 = Me , R^2 = CO_2Me
(5) R^1 = Me , R^2 = CO·NH_2
(7) R^1 = $C(^2H)_3$, R^2 = CO_2Me

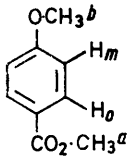
data. The structures of the remaining two are still in some doubt, but one of them must be closely related to (4) and (5), and its identification is unlikely to add much to what is known already. Likewise, the other compound must be closely related to a biphenyl derivative

* Free vancosamine contains the six-membered ring shown in (1).[6,7] Evidence that this is also present in vancomycin (rather than a furanoid ring) comes from the observation (J. P. Brown, personal communication) that the chemical shifts of the vancosamine methyl groups in vancomycin are close to the shifts shown by the β-anomer of the free sugar. These observations also suggest that the vancosamine is present in the β-configuration in vancomycin.

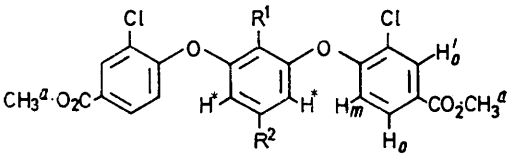
[4] Preliminary report, P. J. Roberts, O. Kennard, K. A. Smith, and D. H. Williams, *J.C.S. Chem. Comm.*, 1973, 773.
[5] F. J. Marshall, *J. Medicin. Chem.*, 1965, **8**, 18.
[6] W. D. Weringa, D. H. Williams, J. Feeney, J. P. Brown, and R. W. King, *J.C.S. Perkin I*, 1972, 443.
[7] A. W. Johnson, R. M. Smith, and R. D. Guthrie, *J.C.S. Perkin I*, 1972, 2153.
[8] N. K. Kochetkov and O. S. Chizov, *Biochim. Biophys. Acta*, 1964, **83**, 134.
[9] H. Bjorndal, C. G. Hellergvist, B. Lindberg, and S. Svensson, *Angew. Chem. Internat. Edn.*, 1970, **9**, 610.
[10] H. M. Higgins, W. H. Harrison, G. M. Wild, H. R. Bungay, and M. H. McCormick, in 'Antibiotics Annual, 1957—58,' Medical Encyclopedia Inc., New York, 1958, p. 906.
[11] S. M. Hecht and J. M. Kozarich, *Tetrahedron Letters*, 1972, 1501.

isolated from alkaline hydrolysis studies to be described subsequently and therefore will not be given further consideration.

Product (*A*). The mass spectrum shows molecular ions at *m/e* 534, 536, and 538 in the ratio 9 : 6 : 1, establishing the presence of two chlorine atoms in the molecule. The ^{1}H n.m.r. spectrum contains resonances corresponding to two equivalent 1,2,4-trisubstituted benzene rings; these must be the two chlorine-substituted rings, whose substitution pattern is already known from the isolation of 3-chloro-4-hydroxybenzoic acid by Marshall.[5] The spectrum also shows the presence of two other equivalent aromatic protons (δ 7·56) and four methoxy-groups, two of which are equivalent. The possibility of ester links between the aromatic rings is ruled out by the i.r. spectrum, and by the mass spectrum which shows none of the fragments expected from such systems. In the light of the molecular formula, $C_{25}H_{20}Cl_2O_9$, determined by microanalysis, the only possible structures are (4) and an alternative in which the positions of OR^1 and R^2 are reversed. These possibilities can be differentiated through the use of the shift reagent Eu(fod)$_3$. Our experience with shift reagents led us to expect that Eu(fod)$_3$ would complex more strongly at a methyl ester than at an aromatic ether. This supposition was confirmed by examination of the shifted spectrum of methyl *p*-methoxybenzoate (8) in which, at low molar ratios of Eu(fod)$_3$ to substrate, the shift ratios, relative to CH_3^a (=1), are as follows: $H_o : H_m : CH_3^b = 0{\cdot}90 : 0{\cdot}17 : 0{\cdot}06$.



(8)



(9) R^1 = O·CH$_3$, R^2 = $CO_2CH_3^b$

(10) R^1 = $CO_2CH_3^b$, R^2 = O·CH$_3$

(11) R^1 = O·C(^{2}H)$_3$, R^2 = CO_2CH_3

Since the effect of shift reagent falls of rapidly with distance, the three rings of product (A) can be treated independently, provided that the shift reagent complexes mainly at the ester groups. This supposition is supported by the good agreement between the shift ratios observed for the chlorinated rings of product (A) [(9) or (10)] in comparison with (8), *viz.*: $CH_3^a : H_o' : H_o : H_m = 1{\cdot}0 : 0{\cdot}85 : 0{\cdot}94 : 0{\cdot}22$. The shift ratio CH_3^b : H* is 1·0 : 1·07, thus establishing (9) as the structure of product (A).

Product (*B*). The spectra of this product showed it to be a methyl methoxy-methoxycarbonyl-benzoate. It was identified as dimethyl 4-methoxyisophthalate (6) from its m.p. and by direct comparison with synthetic material.[12] It seems likely that this product has the same origin as 4,6-dinitrosalicyclic acid, isolated from a nitric acid oxidation of vancomycin by Marshall.[5]

Product (*C*). The mass spectrum shows molecular ions at *m/e* 519, 521, and 523 (relative abundance 9 : 6 : 1), demonstrating the presence of two chlorine atoms. The ^{1}H n.m.r. spectrum is similar to that of product (A), but shows one less methoxy-group and an additional broad two-proton signal, which is removed on shaking with deuterium oxide containing a trace of acid. The i.r. spectrum confirms that it is an amide. In the light of the structure of product (A), product (C) can be assigned the primary amide structure (5).

It is possible, though unlikely, that the nitrogen in the amide product (C) arises from the ammonia used in the oxidation. This possibility was excluded by carrying out an oxidation in potassium carbonate solution; (C) was isolated in almost the same yield as before.

To test whether the primary amide group of (5) was originally present in vancomycin, an oxidation was carried out on an acid hydrolysate of vancomycin. The hydrolysis was carried out under vigorous conditions (concentrated hydrochloric acid, reflux, 48 h), such that survival of any amides is very unlikely.[13] The hydrolysate was acetylated to protect amino-groups and then methylated, oxidised, and methylated as before. Product (C) was again isolated (although in rather lower yield than from aglucovancomycin), indicating that the primary amide group of (5) is formed during the oxidation. The oxidation of amines to amides by permanganate is well known and can involve C–C cleavage.[14]

The isolation of (A) and (C) from the oxidation of methylated aglucovancomycin, although oxidation of methylated vancomycin gives only methyl 3-chloro-4-methoxybenzoate,[15] suggests that the sugars in vancomycin are linked to the central ring of the three-ring system. Vancomycin was therefore methylated as above, and then treated with acid to remove the sugars; the methylation was then repeated with [^{2}H$_3$]methyl iodide. Oxidation and methylation of the product in the usual way gave (11), confirming that the sugars are linked to the central ring. These experiments lead to assignment of the partial structure (1) to vancomycin.

Nature of the Remaining Aromatic Rings.—The above oxidation experiments provide evidence for only one type of phenolic ring in vancomycin, *i.e.* that giving rise to (6). However, there is much evidence that points to the presence of several phenolic groups in vancomycin. For example, Nieto and Perkins [16] report pK_a values of 2·9, 7·2, 8·6, 9·6, 10·5, and 11·7 on titration of vancomycin in water. The last four were assigned to phenols on the basis of spectrophotometric titration results. Lomakina *et al.*[17] obtained similar results but associated the pK_a value of 8·5 with an amino-group. On the basis of reasonable models, the pK_a of the amino-group of vancosamine is likely to lie between 8·0 and 9·6, and

[12] See also A. W. Johnson and R. M. Smith, *J. Antibiotics*, 1972, **25**, 292.

[13] R. L. Hill, *Adv. Protein Chem.*, 1965, **20**, 37.

[14] L. J. Chinn, 'Selection of Oxidants in Organic Synthesis,' Dekker, New York, 1970, p. 142.

[15] Unpublished work by the present authors.

[16] M. Nieto and H. R. Perkins, *Biochem. J.*, 1971, **123**, 773.

[17] N. N. Lomakina, L. I. Murav'eva, and M. S. Yurina, *Antibiotiki*, 1970, **15**, 21 (*Chem. Abs.*, 1970, **72**, 107,083t).

that of the *N*-terminal amino-acid *N*-methyl-leucine, known to be present in vancomycin,[5] to be near the range 7·6—8·0. Thus, we assign the pK_a values 8·6 and 7·2 to the basic nitrogen functionalities of vancomycin, that at 2·9 to a carboxylic acid, and the remaining three to phenols (9·6, 10·5, and 11·7). The presence of three free phenolic groups in vancomycin was established through methylation of aglucovancomycin with diazomethane; the methylated product showed signals for five methoxy-groups in the ^1H n.m.r. spectrum. These are derived through methylation of one carboxy-group, of three phenolic hydroxy-groups which are also free in vancomycin, and of a further phenolic hydroxy-group which is freed during the conversion of vancomycin into aglucovancomycin.

The two phenolic hydroxy-groups which have hitherto remained unidentified have now been located as a result of alkaline hydrolysis experiments. Vancomycin was hydrolysed with 4N-sodium hydroxide under reflux. The hydrolysate was acidified and ethyl-acetate-soluble products were extracted. In addition to the products of acidic hydrolysis (*N*-methyl-leucine and aspartic acid), the extracts afforded 1·2 mol of glycine per 1560 g of vancomycin [slightly less than the quantity (1·5 mol) reported by Johnson [18]]. When the ethyl acetate-soluble fraction was treated with diazomethane, two other major products were isolated, one of which was methyl 3-chloro-4-methoxybenzoic acid. The formula of the other, product (D), was established as $C_{20}H_{19}NO_6$ by high resolution mass spectrometry. Its ^1H n.m.r. spectrum (Table 1) confirms that the molecule contains 19 protons and decoupling experiments show that H_a is coupled to H_d, and H_c to H_e. Treatment of (D) with sodium [$^2H_3$]methoxide in [$^2H_4$]methanol gave a [$^2H_8$]-derivative, showing the presence of one CO_2Me and one $CH_2{\cdot}CO_2Me$ group. The aromatic region of the ^1H n.m.r. spectrum (Table 1) shows the presence of two

TABLE 1

^1H N.m.r. spectrum of product (D)

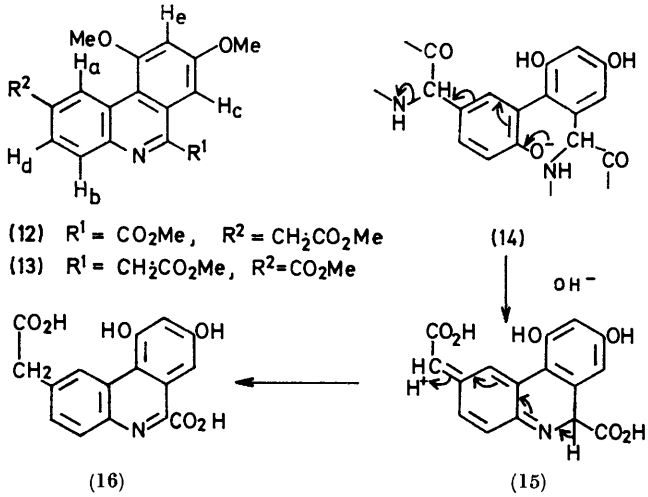
Proton(s)	δ $CDCl_3$	δ C_6D_6	J/Hz	Intensity	Relative shift with $Eu(fod)_3$
H_a	9·34	9·45	2	1	15
H_b	8·22	8·36	8	1	100
H_c	7·66	7·82	2	1	25
H_d	7·63	7·41	8,2	1	21
H_e	6·96	6·68	2	1	7
Me [a] *	4·12	3·71 †		3	23
Me [b]	4·12	3·50 †		3	4
Me [c]	3·98	3·35 †		3	7
CH_2 *	3·89	3·62		2	21
Me [d] *	3·71	3·33 †		3	14

* Exchange by sodium [$^2H_3$]methoxide in [$^2H_4$]methanol.
† These assignments are arbitrary and may be interchanged.

aromatic rings which are 1,2,3,5-tetrasubstituted and 1,2,4-trisubstituted.

‡ In support of the partial structure (14), hydrolysis experiments have led to the isolation of a product having the molecular weight required for a peracetylated dimethyl ester derived from (14) (R. M. Smith, J. Elix, and A. W. Johnson, unpublished work), and to a product having the molecular weight required for a di-*N*-acetyl tri-*O*-methyl dimethyl ester derived from (14) (G. A. Smith and D. H. Williams, unpublished work).

The ^1H n.m.r. spectrum of aglucovancomycin in [$^2H_6$]dimethyl sulphoxide containing *ca.* 10% of [^2H]-trifluoroacetic acid and 10% of deuterium oxide (to exchange all acidic protons) contains signals due to 13 ± 1 aromatic hydrogens. Product (D) and structure (1) together therefore account for all the aromatic rings of vancomycin. It follows that (6) must be derived by degradation of the 1,2,4-trisubstituted ring of (D). Moreover, the above ^1H n.m.r. spectrum of aglucovancomycin shows clearly the two *meta*-coupled protons analogous to H_c and H_e (Table 1), and in particular the higher field signal of this pair is removed by exchange with deuterium when the solution is maintained at 85° for several minutes. We conclude that the replaceable hydrogen atom lies between two phenolic hydroxy-groups in aglucovancomycin. The above information leads to (12) as a structure of (D) (supported by its u.v. spectrum).



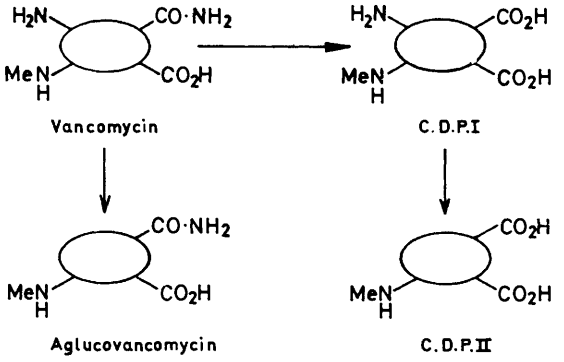
The shifts induced by europium chelates in the spectra of heterocycles related to pyridine have been studied by several workers,[19-21] and the results are largely consistent with a dominant pseudo-contact shift with association of the metal near to the heterocyclic nitrogen atom. Thus, the relatively large shift of H_b places it *peri* or α to the nitrogen atom, and the coupling constant data only permit the former possibility. The much smaller shift of the CH_2 group excludes (13) as a possibility.

Since C-9 of (12) carries a hydroxy-group in vancomycin [see (6)], this group must have been replaced by nitrogen during the alkaline hydrolysis of vancomycin. Such a process would be readily understandable [(14) ⟶ (16)] if vancomycin contained phenylglycine units of the type believed to occur in the related antibiotic ristomycin.[22] ‡

[18] C. R. Johnson, Thesis, University of Illinois, 1962.
[19] J. K. M. Sanders and D. H. Williams, *J. Amer. Chem. Soc.*, 1971, **93**, 641.
[20] R. L. Atkins, D. W. Moore, and R. A. Henry, *J. Org. Chem.*, 1973, **38**, 400.
[21] T. Heigl and G. K. Mucklow, *Tetrahedron Letters*, 1973, 649.
[22] N. N. Lomakina, V. A. Zenkova, P. Bognar, F. Starichkai, Yu. N. Sheinker, and K. F. Turchin, *Antibiotiki*, 1968, **13**, 675.

Relationship between Vancomycin, Aglucovancomycin, C.D.P.I, and C.D.P.II.—C.D.P.I is formed by keeping vancomycin at pH 4·2 for 40 h at 60—70°, and upon treatment with 0·6N-hydrochloric acid C.D.P.I is converted into C.D.P.II (by removal of both sugars, a process analogous to the conversion of vancomycin into aglucovancomycin).[5] Marshall [5] reported that C.D.P.I does not form a hydrochloride and that aglucovancomycin does not show basic amino-groups. However, our analytical results establish that we obtain aglucovancomycin as a monohydrochloride, although C.D.P.I (which seems to be related to vancomycin only through the loss of 1 mol of ammonia [18]) as isolated contains no chloride ions. The latter observation does not prove that C.D.P.I contains no basic groups, and it appeared probable that it exists as a neutral zwitterion, with two protonated basic groups (MeLeu and vancosamine) and two carboxylate anions (one more than vancomycin). In accord with this supposition, vancomycin (free base) and C.D.P.I both form diacetates upon treatment with acetic anhydride in methanol; this reaction is specific for amino-groups.[23]

Treatment of aglucovancomycin with methanolic hydrogen chloride gave a mixture of two products. ^{1}H N.m.r. spectroscopy suggested that a mixture of mono- and di-methyl esters was formed. Under the same conditions, C.D.P.II gave a dimethyl ester. We interpret the results as summarised in the Scheme. Partial methanolysis of the primary amide probably accounts for the formation of some diester from aglucovancomycin.



SCHEME

These conclusions are confirmed by paper electrophoresis studies of numerous derivatives, summarised in Table 2; acetylaglucovancomycin and acetyl-C.D.P.II were prepared *via* mild acidic hydrolysis of diacetylvancomycin and diacetyl-C.D.P.I, respectively. Mobility is expected to be predominantly determined by charge as the various compounds are of similar size,[24] and the influence of differential adsorption on the paper support is assumed to be small. The results indicate, in accord with the Scheme, that as we pass from the top to the bottom of the Table the net charges at pH 6·5 are +1, −1, 0, −1, 0, −2, −1, and −2.

[23] D. W. Thomas, B. C. Das, S. D. Gero, and E. Lederer, *Biochem. Biophys. Res. Comm.*, 1968, **32**, 519.

Conclusions.—Vancomycin contains five benzene rings [(1) and (3)], incorporating three free phenolic hydroxy-groups in the unit (3) and two sugars in the unit (1). The molecule additionally contains carbamoyl and carboxy-groups, as well as previously detected [5] aspartic acid and *N*-terminal *N*-methyl-leucine. Vancomycin and its degradation products and their derivatives tenaciously retain solvents such as diethyl ether, methanol, and water, as evidenced from ^{1}H n.m.r. spectra of rigorously dried products. It is therefore possible that our analysis of dried C.D.P.I ($C_{64}H_{74}Cl_2N_8O_{26}$) [4] may reflect the retention of at least one or two molecules of water. However, if this analytical figure is used as a working basis, then vancomycin would correspond to $C_{64}H_{75}Cl_2N_9O_{25}$. It is noteworthy that interconnection of the above-mentioned units through amide bonds on the rather arbitrary assumption that all the benzene rings are incorporated as phenylglycine units would lead to a formula $C_{64}H_{71}Cl_2N_9O_{22}$ for vancomycin. Since the nitrogen atoms which have not been isolated in degradation products are non-basic and probably incorporated as amide linkages, it appears that all, or almost all the carbon skeleton of vancomycin has been identified, and the task that remains is one of determining the nature of the interconnecting functionalities.

TABLE 2

Electrophoresis of vancomycin derivatives *

Compound	Net charge −2	−1	0	+1
Vancomycin				×
Diacetylvancomycin		×		
Aglucovancomycin			×	
Acetylaglucovancomycin		×		
C.D.P.I			×	
Diacetyl–C.D.P.I	×			
C.D.P.II		×		
Acetyl–C.D.P.II	×			

Origin ↑ Neutral marker ↑

* pH 6·5, 3 kV, 55 min.

EXPERIMENTAL

N.m.r. spectra were determined on a Varian HA-100 spectrometer operating at normal probe temperature. Coupling constants (J) are quoted in Hz and chemical shifts, unless otherwise stated, are relative to internal Me_4Si. Mass spectra were recorded at 70 eV by direct insertion with an A.E.I. MS12 spectrometer. High resolution mass measurements were made on an A.E.I. MS902 spectrometer at 10,000 resolving power using perfluorokerosene as reference, on-line to a PDP8 (DS10) data acquisition system.

Analytical t.l.c. was carried out on Merck plates coated with silica GF_{254}, and preparative t.l.c. on 20 × 20 cm plates coated in our laboratory with Merck silica GF_{254}. The abbreviation BPA refers to butanol–propanol–aqueous 2N-ammonia (2 : 5 : 3). Compounds were recovered from

[24] C. J. O. R. Morris and P. Morris, ' Separation Methods in Biochemistry,' Pitman, London, 1963, p. 621.

preparative t.l.c. plates by elution of the silica with acetone.

Permethylation of Vancomycin.—Vancomycin (200 mg; dried under vacuum over P_2O_5) in Me_2SO (4 ml; dried as above) was treated with sodium methylsulphonylmethanide in Me_2SO [from complete reaction of sodium hydride (200 mg) with Me_2SO (4 ml)] till a drop of the solution gave a red colour with triphenylmethane. Methyl iodide (8 ml) was then added and after 45 min water (10 ml) and chloroform (10 ml) were added. The chloroform layer was separated, washed with aqueous 2N-sodium thiosulphate (10 ml) and water (4 × 10 ml), dried (Na_2SO_4), and evaporated to give the product (179 mg).

Methanolysis of Permethylated Vancomycin.—Methanol (15 ml; dried with magnesium and distilled) was cooled in ice and acetyl chloride (1·2 ml) added dropwise. The solution was added to the permethylated vancomycin in a tube cooled in ice, and nitrogen was bubbled through the solution for 30 min. The tube was sealed and maintained at 105° for 24 h. The methanol was evaporated off, chloroform (3 ml) and charcoal (500 mg) were added, and the solution was filtered. Preparative t.l.c. (ethyl acetate) gave material (16 mg; R_F 0·1—0·6) which was utilised directly in the following reaction.

Permethylation of Methanolysed Permethylated Vancomycin.—Two-thirds of the above methanolysis product in dry Me_2SO (0·2 ml) was treated successively with sodium methylsulphonylmethanide in Me_2SO (0·5 ml; prepared as above) and [$^2H_3$]methyl iodide (0·3 ml). After 45 min, water (1 ml) and chloroform (1 ml) were added, the water was decanted, and the chloroform layer was washed with water (4 × 1 ml) and evaporated. The mass spectrum of the product showed it to be a 2-*O*-[$^2H_3$]methyl derivative of permethylglucose.[8]

Reduction and Acetylation of Methanolysed Permethylated Vancomycin.—The remaining methanolysis product was treated with 4N-hydrochloric acid (1 ml) for 6 h in a bath at 80—90° to hydrolyse the methyl glycosides. The solution was evaporated and water (2·5 ml) and sodium borohydride (27 mg) were added. After 3 h, Amberlite IR120(H^+) cation exchanger (50 mg) was added. When hydrogen evolution ceased, the solution was decanted from the ion exchanger and freeze-dried. Acetic anhydride (1 ml) and pyridine (1 ml) were added and the mixture was left at room temperature for 16 h, then evaporated to dryness. The mass spectrum of the product showed it to be 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylhexitol.

Methylation of Aglucovancomycin.—Anhydrous potassium carbonate (1·2 g) was added to a suspension of aglucovancomycin (0·70 g)[5] in methanol (60 ml) and methyl iodide (15 ml). The mixture was heated under reflux for 6 h. The solvents were evaporated off and the solid residue was suspended in water (10 ml), filtered off, washed with water (2 × 5 ml), and dried under vacuum (P_2O_5). The product (0·73 g) had $\nu_{max.}$ 1735 and 1690—1640 cm^{-1} and $\lambda_{max.}$ (95% EtOH) 281 nm (ε 8600, assuming *M*, 1300), unchanged by addition of aqueous sodium hydroxide.

Permanganate Oxidation of Methylated Aglucovancomycin.—Methylated aglucovancomycin (400 mg), prepared as above, was suspended in water (20 ml) and a solution of potassium permanganate (2·4 g) in water (45 ml) and 2N-ammonia (7 ml) was added. The mixture was stirred in a bath at 70—75° until no permanganate was detected by spotting on filter paper (4 h). The mixture was filtered (Hyflo), acidified (HCl), and extracted with ethyl acetate (3 × 40 ml). The extract was dried ($MgSO_4$) and evaporated. The product (220 mg) was dissolved in methanol (5 ml), cooled in ice, and treated with an excess of ethereal diazomethane. After 10 min the solution was evaporated and the products separated by preparative t.l.c. (petroleum–acetone, 5 : 1) to give three fractions [(I)—(III)]. Preparative t.l.c. of fraction (I) (dichloromethane) gave the products (A) and (B).

Product (A) was further purified by preparative t.l.c. (petroleum–benzene, 2 : 1) to give white crystals, recrystallised from petroleum–benzene (yield 20 mg) and identified as *methyl 3,5-bis-(2-chloro-4-methoxycarbonylphenoxy)-4-methoxybenzoate*, m.p. 112—114°, R_F 0·4 ($CHCl_3$); $\nu_{max.}$ ($CHCl_3$) 1720 cm^{-1}; $\lambda_{max.}$ (95% EtOH) 255 (ε 31,000) and 290sh nm (3800); δ ($CDCl_3$) 8·16 (2H, d, *J* 2), 7·88 (2H, q, *J* 8 and 2), 7·56 (2H, s), 6·84 (2H, d, *J* 8), 3·90 (6H, s), 3·86 (3H, s), and 3·85 (3H, s); *m/e* 538 (5%), 536 (69), 534 (100), 507 (1), 506 (1), 505 (6), 504 (3), 503 (12), 502 (1), and 501 (5), *m** 475 (Found: C, 56·1; H, 3·7. $C_{25}H_{20}Cl_2O_9$ requires C, 56·1; H, 3·7%).

Product (B) was obtained as white crystals (3·5 mg), m.p. and mixed m.p. with synthetic dimethyl 4-methoxyisophthalate (see below) 95—96·5° (from methanol). The n.m.r. and mass spectra, and the t.l.c. behaviour (four solvents) of product (B) were also identical with those of the synthetic ester.

Preparative t.l.c. of fraction (III) (benzene) gave product (C) as white crystals (10·1 mg), m.p. 175—177° (from benzene), of *3,5-bis-(2-chloro-4-methoxycarbonylphenoxy)-4-methoxybenzamide*, R_F 0·1 ($CHCl_3$); $\nu_{max.}$ ($CHCl_3$) 1720 and 1675 cm^{-1}; $\lambda_{max.}$ (95% EtOH) 255 nm (ε 34,000) and 290sh nm (3900); δ ($CDCl_3$) 8·15 (2H, d, *J* 2), 7·9 (2H, q, *J* 8 and 2), 7·35 (2H, s), 6·8 (2H, d, *J* 8), 6·0br (2H, s, removed by D_2O–trifluoroacetic acid), 3·9 (6H, s), and 3·85 (3H, s); *m/e* 523 (11%), 522 (17), 521 (60), 520 (26), 519 (100), 505 (5), 504 (2), 503 (8), 492 (2), 491 (2), 490 (9), 489 (4), and 488 (16) (Found: C, 55·2; H, 3·7; N, 2·8. $C_{24}H_{19}Cl_2NO_8$ requires C, 55·4; H, 3·7; N, 2·7%).

Synthesis of Dimethyl 4-Methoxyisophthalate.—*p*-Cresol (14·5 g) was subjected to the Reimer–Tiemann reaction to give 2-hydroxy-5-methylbenzaldehyde (1·5 g), m.p. 52—54° (from ethanol) (lit.,[25] 55°). The aldehyde (0·3 g) was methylated *via* a standard procedure (MeI–K_2CO_3–MeOH) and the crude product oxidised with sodium dichromate in sulphuric acid to give 4-methoxyisophthalic acid (0·15 g), m.p. 260—261° (from acetone–petroleum) (lit.,[26] 261°). This was methylated with diazomethane to give dimethyl 4-methoxyisophthalate (0·12 g), m.p. 95—96·5° (from methanol) (lit.,[27] 94°).

Oxidation of Methylated Aglucovancomycin in Sodium Hydrogen Carbonate Solution.—Methylated aglucovancomycin (100 mg), prepared as above, was oxidised as before with potassium permanganate (0·6 g) in water (16 ml) and aqueous M-sodium hydrogen carbonate (2 ml). The products were separated as before to give (A) (5·0 mg) (C) (1·9 mg).

Oxidation of Hydrolysed Vancomycin.—Vancomycin (300 mg) in concentrated hydrochloric acid (12 ml) was heated under reflux for 48 h, and the solution was evaporated to dryness. The solid residue was dissolved in 0·5N-acetic acid and run through a 6 × 1 cm column of Amberlite

[25] N. V. Sidgwick and E. N. Allott, *J. Chem. Soc.*, 1923, **123**, 2828.

[26] C. Shall, *Ber.*, 1879, **12**, 828.

[27] L. S. Fosdick and D. E. Faucher, *J. Amer. Chem. Soc.*, 1941, **63**, 1277.

CG400 anion exchanger. The solution was evaporated, methanol (10 ml) and acetic anhydride (3 ml) were added, and after 20 h the solution was again evaporated. Potassium carbonate (0·3 g), methanol (20 ml), and methyl iodide (6 ml), were then added and the mixture was heated under reflux for 16 h. The mixture was then evaporated and the residue triturated with water (2 × 2 ml) and dried (P_2O_5).

The resulting acetylated and methylated product (60 mg) was oxidised in the same way as methylated aglucovancomycin (above) with potassium permanganate (300 mg) in water (9 ml) and 2N-ammonia (1·2 ml). The products were methylated and separated in the usual way to give (A) (2·0 mg) and (C) (0·5 mg).

Attachment of the Sugars to the Aromatic Rings.—Vancomycin (300 mg) was methylated *via* the usual procedure with methyl iodide (5 ml), methanol (20 ml), and potassium carbonate (0·5 g). A suspension of the product (250 mg) in 0·6N-hydrochloric acid was heated under reflux for 2 min, cooled, and filtered, and the solid was washed with water (2 ml) and dried (P_2O_5). The product was heated under reflux with methanol (5 ml), [$^2H_3$]methyl iodide (2·0 g), and potassium carbonate (0·2 g) for 17 h. The mixture was then evaporated to dryness and the residue triturated with water (3 × 1 ml) and dried (P_2O_5).

The product (100 mg) was oxidised in the same way as methylated aglucovancomycin with potassium permanganate (0·60 g) in water (16 ml) and 2N-ammonia (2 ml). The products were methylated and separated in the usual way to give product (A) (6·1 mg), *m/e* 541 (13%), 540 (17), 539 (66), 538 (30), 537 (100), 510 (3), 509 (4), 508 (16), 507 (8), and 506 (27); n.m.r. spectrum identical with that of undeuteriated product (A) except for the absence of the singlet at δ 3·86. Product (B) (1·0 mg) was also isolated; its n.m.r. and mass spectra were identical with those of synthetic material.

Methylation of Aglucovancomycin with Diazomethane.—Aglucovancomycin (50 mg) was suspended in methanol (10 ml) and water was added slowly to give a clear solution. A freshly distilled solution of diazomethane in ether was added and the addition was continued during 2 h to maintain an excess of the reagent. The solution, which showed one diffuse spot (R_F 0·3) on t.l.c. (10% methanol–chloroform), was evaporated and the residue dried at 80° *in vacuo*. The ^1H n.m.r. spectrum [$(CD_3)_2SO$; 90°] showed three OMe resonances at δ 4·06, 3·85, and 3·76 and two incompletely resolved OMe resonances at δ 3·67—3·69. The presence of isomers was indicated by two resonances assigned to the *N*-terminal NMe at δ 2·30 and 2·32 (shifted to δ 2·9 on acetylation).

Alkaline Hydrolysis of Vancomycin.—Vancomycin (25 mg) was hydrolysed with aqueous 4N-sodium hydroxide (5 ml) under the conditions used by Johnson.[18] The solution was acidified (HCl) and extracted with ethyl acetate (3 × 5 ml). The aqueous fraction was evaporated and then converted into *N*-trifluoracetyl n-butyl esters [28] and examined by g.l.c. Three peaks were observed and identified as derivatives of glycine, *N*-methyl-leucine, and aspartic acid by comparison with synthetic materials. The yield of glycine was 1·2 mol from 1560 g of vancomycin. The ethyl acetate fraction was evaporated, the product was dissolved in methanol (2 ml), and an excess of diazomethane was added. After 16 h the solution was evaporated. T.l.c. ($CHCl_3$) showed two major spots.

In a series of experiments, vancomycin (50 mg) in aqueous 4N-sodium hydroxide (10 ml) was heated under reflux, under nitrogen, in glass apparatus for 1, 11, 20, 48, and 60 h. The reactions were worked up as above to give yields of ethyl acetate-soluble material of 5·0, 4·5, 6·0, 10·1, and 5·7 mg, respectively. T.l.c. ($CHCl_3$) of these products, after methylation with diazomethane as above, showed the same two major spots as in the initial experiment, except for the product of 1 h hydrolysis which showed only base-line material.

Vancomycin (500 mg) in aqueous 4N-sodium hydroxide (100 ml) was heated under reflux, under nitrogen, in glass apparatus, for 48 h. The reaction was worked up as above, and the methylated products were separated by preparative t.l.c. (petrol–acetone, 4 : 1) to give products (E), white crystals (2·8 mg), and (D). Product (E), after recrystallisation from petroleum–benzene was identical (mass spectrum, n.m.r., t.l.c., m.p. and mixed m.p.) with synthetic methyl 3-chloro-4-methoxybenzoate. Product (D) was further purified by preparative t.l.c. (dichloromethane) to give a white solid (3·1 mg), $\nu_{max.}$ ($CHCl_3$) 1730 and 1615 cm^{-1}; $\lambda_{max.}$ (95% EtOH) 253 (ε 26,000), 278sh (19,000), 318sh (4400), and 376 (2400); *m/e* 369 (100%, $C_{20}H_{19}NO_6$) and 311 ($C_{18}H_{17}NO_4$), *m** 262; for ^1H n.m.r. spectrum see Table 1.

Reaction of Product (D) with [$^2H_4$]Methanol–Sodium [$^2H_3$]Methoxide.—Sodium (1·4 mg) was dissolved in [$^2H_4$]-methanol (0·6 ml). A portion (30 μl) of this solution was added to a solution of product (D) (0·3 mg) in [$^2H_4$]methanol (0·6 ml). After 1 h a drop of [$^2H_4$]methanol acidified with acetyl chloride was added, and the mixture was evaporated. The product was identical with starting material [t.l.c. ($CHCl_3$ and petroleum–acetone, 4 : 1)] and had *m/e* 377 (100%) and 317 (95), *m** 267. Repeated treatment of the product with sodium [$^2H_3$]methoxide under the same conditions for 6 h produced no further change in the mass spectrum.

Acetylation of Vancomycin.—Amberlite CG400 resin (200 mesh; Cl^-) (5 g) was washed in a Buchner funnel with aqueous 3M-sodium acetate (250 ml) and then water (100 ml). The resultant acetate form of the resin was suspended in aqueous 0·5N-acetic acid and poured into a 1 cm diam. column (resultant length of 6 cm). Vancomycin (100 mg) in aqueous 0·5N-acetic acid (1 ml) was put on the column and eluted with aqueous 0·5N-acetic acid. U.v.-absorbing material (eluted between 6 and 12 ml) was dried under vacuum (P_2O_5 and NaOH). The product (91 mg) was suspended in methanol (4 ml) and acetic anhydride (1 ml) was added. All the solid dissolved in about 2 h. After 4 h the solvents were evaporated off and the residue was dried under vacuum (P_2O_5 and NaOH). The product (96 mg) had R_F 0·55 (BPA); $\lambda_{max.}$ (MeOH) 278 nm (ε 7500, assuming *M* 1600); $\lambda_{max.}$ (MeOH–NaOH) 303 nm (ε 8500); $\nu_{max.}$ (Nujol) 3500—2900 and 1730—1600 cm^{-1}. The n.m.r. spectrum [$(CD_3)_2SO$; external Me_4Si reference] was like that of vancomycin but showed the NMe peak shifted to δ 3·0 and additional peaks at δ 1·9 and 2·3 (NAc) and a large peak at δ 2·1 (probably acetic acid).

Acetylation of C.D.P.I.—C.D.P.I (65 mg) was suspended in methanol (10 ml) and acetic anhydride (2·5 ml) was added. All the solid had dissolved after 2 h. After 4 h the solvents were evaporated off and the residue was dried under vacuum (P_2O_5 and NaOH). The product (68 mg)

[28] W. M. Lamkin and C. W. Gehrke, *Analyt. Chem.*, 1965, **37**, 383; C. W. Gehrke, R. W. Zumwalt, and L. L. Wall, *J. Chromatog.*, 1968, **37**, 398.

had R_F 0·45 (BPA); $\lambda_{max.}$ (MeOH) 298 nm (ε 6800, assuming *M* 1600); $\lambda_{max.}$ (MeOH–NaOH) 303 nm (ε 9600). Its n.m.r. spectrum showed the same new peaks as that of acetylated vancomycin.

Reaction of Aglucovancomycin with Methanolic Hydrogen Chloride.—Aglucovancomycin (50 mg) was suspended in methanol (10 ml; dried by distillation from magnesium methoxide) and methanolic 1·4N-hydrogen chloride (1·4 ml) was added. The aglucovancomycin immediately dissolved. After 24 h the solvent was evaporated off. T.l.c. (BPA) of the product showed two spots, R_F 0·65 and 0·75, in addition to starting material.

Reactions carried out for shorter periods gave as the main product the material of R_F 0·65, whose n.m.r. spectrum [$(CD_3)_2SO$; external Me_4Si reference] showed a new methoxy-peak at δ 4·0, and a small peak at δ 3·75, associated with the product of R_F 0·75. After further treatment of the product with methanolic hydrogen chloride under the same conditions for 48 h, t.l.c. showed only a trace of starting material, and after further treatment for 7 days t.l.c. showed one major spot, R_F 0·75.

Reaction of C.D.P.II with Methanolic Hydrogen Chloride.—C.D.P.II (50 mg) was dissolved in methanol (3 ml; dried as above) and methanolic N-hydrogen chloride (0·3 ml) was added. A white precipitate gradually formed. After 40 h the precipitate was filtered off, washed with methanol (0·2 ml), and dried under vacuum (P_2O_5). The product (25 mg) had R_F 0·75$_5$(BPA), $\nu_{max.}$ (Nujol) 1735 and 1680—1640 cm^{-1}. The n.m.r. spectrum [$(CD_3)_2SO$; external Me_4Si reference] was like that of C.D.P.II, with the exception of additional peaks at δ 3·75 and 4·0.

Acetylaglucovancomycin from Acetylated Vancomycin.—Acetylated vancomycin (50 mg) in water (0·4 ml) was heated under reflux, 4N-hydrochloric acid (0·07 ml) was added, and heating was continued for 2 min. The mixture was left to cool, then filtered, and the precipitate was washed with 0·5N-hydrochloric acid (0·5 ml) and dried under vacuum (P_2O_5). The product (30 mg) had R_F 0·6 (BPA); $\lambda_{max.}$ (MeOH) 279 nm (ε 5200, assuming *M* 1260); $\lambda_{max.}$ (MeOH–NaOH) 299 (ε 12,000); $\nu_{max.}$ 3500—2800 and 1710—1630 cm^{-1}; the n.m.r. spectrum [$(CD_3)_2SO$; external Me_4Si reference] was like that of aglucovancomycin but lacked the peak at δ 2·5 and had additional peaks at δ 2·0 (NAc) and 2·8 (NMe).

Acetyl-C.D.P.II from Acetylated C.D.P.I.—Acetylated C.D.P.I (40 mg) was heated under reflux in 0·6N-hydrochloric acid (4 ml) for 2 min. The solution was cooled and filtered, and the precipitate washed with 0·6N-hydrochloric acid (0·5 ml) and dried under vacuum (P_2O_5). The product (25 mg) had R_F 0·55 (BPA); $\lambda_{max.}$ (MeOH) 279 nm (ε 5900, assuming *M* 1260); $\lambda_{max.}$ (MeOH–NaOH) 298 nm (ε 12,700); the n.m.r. spectrum [$(CD_3)_2SO$; external Me_4Si reference] was like that of C.D.P.II but lacked the peak at δ 2·5 and had additional peaks at δ 2·0 (NAc) and 2·8 (NMe).

Electrophoresis of Vancomycin and its Derivatives.—Electrophoresis was carried out on strips of Whatman 3MM paper hung in a tank of toluene coolant. The buffer was made up of water (6·3 l), pyridine (0·7 l), and acetic acid (20 ml), adjusted to pH 6·5 by addition of more acetic acid (*ca.* 5 ml). Samples were applied as solutions in this buffer. A spot of leucine was placed at both edges of each paper strip. Electrophoresis was carried out for 55 min at 3 kV. The paper was dried in an oven at 60—70° for 30 min and then sprayed with ninhydrin along the edges to detect the leucine, and with diazotised sulphanilic acid over the remaining area to detect vancomycin derivatives. The distance (cm) from the centres of the spots to the origin (positive towards the cathode) were as follows: run 1, leucine +3·6, vancomycin +7·5 (with minor, rather streaky spots at about +5·5 and +3·3), aglucovancomycin +2·6; run 2, leucine +3·5, C.D.P.I +4·8, C.D.P.II −1·6, acetylvancomycin −2·5; run 3, leucine +3·6, acetyl-C.D.P.II −7·5, acetylaglucovancomycin −2·0.

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