Structural Studies on the Antibiotic Vancomycin: Evidence for the Presence of Modified Phenylglycine and β-Hydroxytyrosine Units

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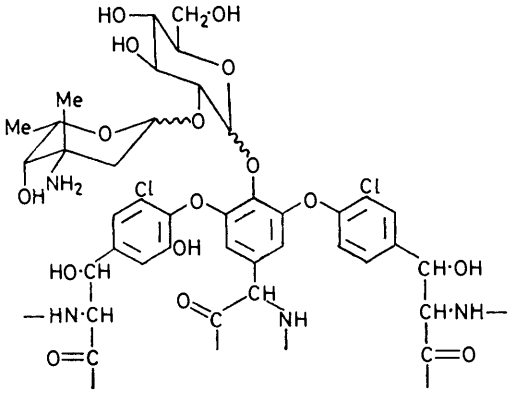
Chemical and spectroscopic evidence is presented to support the presence of three oxygenated phenylglycine units in the molecule of the antibiotic vancomycin. The glycine (2 mol. equiv.) reported as a product of alkaline hydrolysis of vancomycin is believed to arise from the fission of two chloro-β-hydroxytyrosine units. The ^{1}H n.m.r. spectrum of a crystalline degradation product, hexa-*O*-methyl-*N*-acetyl-CDPII, is in accord with the presence of the above units, as is the ^{13}C n.m.r. spectrum (sp^3 carbon atoms) of CDPII itself. The presence of two hydroxy-groups in hexa-*O*-methyl-*N*-acetyl-CDPII is established through the preparation of a bisdinitrobenzoate.

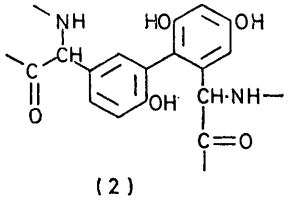
VANCOMYCIN is an antibiotic which complexes selectively with peptides having the *C*-terminal sequence D-Ala-D-Ala.[1] It is believed that this binding is probably the basis of the antibiotic activity,[2] since mucopeptides terminating in the sequence D-Ala-D-Ala are precursors involved in cell wall biosynthesis. However, it will not be possible to propose a detailed mechanism for the action of vancomycin until the structure of the antibiotic is known. The elucidation of its structure has proved to be a remarkably intractable problem, although recent studies have led to the identification of a novel sugar vancosamine,[3,4] and established the presence and substitution pattern of five benzene rings in vancomycin.[5] We now provide evidence that the units (1)—(3), together with one molecule of ammonia [6] and one molecule of aspartic acid,[6,7] account for all the structural units

[1] H. R. Perkins, *Biochem. J.*, 1969, **111**, 195.
[2] M. Nieto and H. R. Perkins, *Biochem. J.*, 1971, **123**, 789.
[3] W. D. Wringa, D. H. Williams, J. Feeney, J. P. Brown, and R. W. King, *J.C.S. Perkin I*, 1972, 443.
[4] A. W. Johnson, R. M. Smith, and R. D. Guthrie, *J.C.S. Perkin I*, 1972, 2153.
[5] K. A. Smith, D. H. Williams, and G. A. Smith, *J.C.S. Perkin I*, 1974, 2369.
[6] C. R. Johnson, Thesis, University of Illinois, 1962.
[7] F. J. Marshall, *J. Medicin. Chem.*, 1965, **8**, 18.

present in vancomycin, and that connection of these units solely through peptide bonds will provide the complete structure, with only D-*N*-methyl-leucine (3) [6,7] present as a free *N*-terminal amino-acid residue (the amino-group of vancosamine is also free).[5]



(1)



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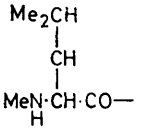
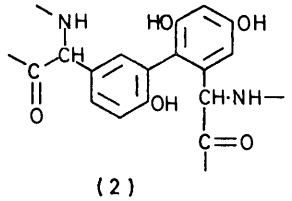


(3)

The Biphenyl System.—We felt that, if the suggested mechanism [5] for the formation of the previously isolated phenanthridine (4) were correct, methylation of the free phenols prior to base-catalysed hydrolysis might prevent the proposed cyclisation [which involves an anion (5)] and allow isolation of a biphenyl di(amino-acid).



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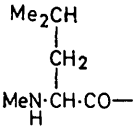
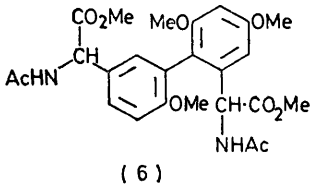
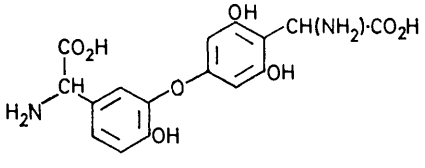


(3)

Aglucovancomycin [7] was methylated with diazomethane, and the product was hydrolysed with sodium hydroxide (in the presence of sodium borohydride—see later). The aqueous phase from the work-up was esterified (MeOH–HCl) and acetylated, and a single u.v. absorbing product isolated *via* preparative t.l.c. This component was identified, in the light of the structural information available from previous work (see, for example, (4)],[5] and its ^{1}H n.m.r. and mass spectra, as the di-*N*-acetyl dimethyl ester of a biphenyl di(amino-acid) (6). In particular, an exact mass measurement on the molecular ion was in accord with the molecular formula $C_{25}H_{30}N_2O_9$.



(6)



(7)

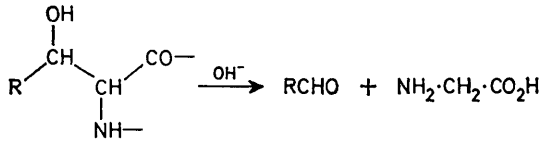
The isolation of phenylglycines from natural sources is unusual. Two previously reported examples are the isolation of *m*-hydroxy- and 3,5-dihydroxy-phenylglycines from latex [8] and actinoidinic acid.[9] It has been suggested that the amino-acids B and C found in hydrolysates of actinoidin, ristomycin, ristocetin, and vancomycin (all related antibiotics) are optical isomers of the same compound, actinoidinic acid, for which structure (7) has been suggested.[9] In the light of the present work, it appears likely that actinoidinic acid should be correctly formulated as the compound derived by deactylation and demethylation of (6). The Russian work [9] relied heavily on ^{1}H n.m.r. evidence, but no mass spectrometric evidence was supplied to support the proposed molecular weight.

The Origin of Glycine.—The studies of Johnson [6] revealed that basic hydrolysis of vancomycin or CDPII [7] gave *ca.* 2 mol. equiv. of glycine, although this substance was not produced in significant amounts from acidic hydrolyses. No satisfactory explanation of this observation has been given. We considered the possibility that vancomycin might contain two β-substituted serine units (8), which might undergo a cleavage reminiscent of the retro-aldol reaction in the presence of base. There is precedent for this type of reaction: a substituted serine unit in the desulphurised product from the antibiotic verticillin A fragments to yield glycine under basic conditions.[10]

Basic hydrolyses did not give any ketones or aldehydes, but in view of the severe reaction conditions necessary to bring about hydrolysis, this was not too surprising. The hydrolysis was therefore performed in the presence of sodium borohydride, to reduce *in situ* any carbonyl compounds produced *via* fragmentation. Aglucovancomycin [7] was hydrolysed ($NaOH–NaBH_4–H_2O–EtO·CH_2·CH_2·OH$), and chromatography of the crude material afforded mainly 3-chloro-4-hydroxybenzyl alcohol, identified by comparison with synthetic material. 3-Chloro-4-hydroxybenzaldehyde was also isolated from this reaction.

[8] P. Miller and H. R. Schutte, *Z. Naturforsch.*, 1968, **23b**, 659.
[9] N. N. Lomakina, M. S. Yurina, Yu. N. Scheinker, and K. F. Turchin, *Antibiotiki*, 1972, **17**, 488.
[10] H. Minato, M. Matsumato, and T. Katayama, *Chem. Comm.*, 1971, 44.

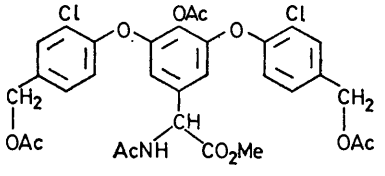
There is no chemical or unambiguous spectroscopic evidence for the presence of any ketone or aldehyde groups in vancomycin, although the i.r. spectrum does not rule out this possibility. In particular, the ^1H n.m.r. spectra of derivatives of aglucovancomycin and



(8)

CDPII (see later) establish the absence of aldehyde protons. Thus the aldehyde group must be produced by the action of alkali. Moreover, the 3-chloro-4-hydroxybenzyl alcohol must be produced by reduction of the aldehyde, since when the hydrolysis was repeated in the presence of sodium borodeuteride, 3-chloro-4-hydroxy-[$^2H_1$]benzyl alcohol was produced; 3-chloro-4-hydroxybenzaldehyde isolated in the same experiment did not contain any deuterium. These experiments provide strong evidence that the 2 mol. equiv. of glycine may arise *via* basic hydrolysis of two units of (8) where R is a 3-chloro-4-oxygenated benzene ring, especially since earlier work [5] has established the presence of two 3-chloro-4-aryloxybenzene units in vancomycin, both of which carry oxidisable carbon atoms at C-1 [see (1)].

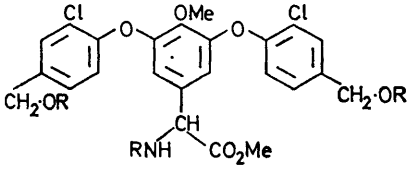
The above reaction carried out in the presence of sodium borohydride also afforded a more polar product, which was isolated *via* esterification [CH_3OH–CD_3OH (1 : 1)–HCl] and acetylation (Ac_2O–C_5H_5N). The isotope pattern of the molecular ion of this compound established the presence of one methyl ester group and two chlorine atoms, the *m/e* value for the molecular ion containing two ^{35}Cl atoms and one OCH_3 group being 661. This suggests that the product has the structure (9). The whole skeleton of (9) (including the location of the nitrogen atom) has been previously established [5] with the exception of the carbonyl carbon atom attached to the pyrogallol ring. Further experiments were carried out to provide additional evidence for this third phenylglycine unit in vancomycin.



(9)

Aglucovancomycin was methylated (CH_2N_2); half of the product was hydrolysed with sodium hydroxide and a large excess of sodium borohydride (relative to the quantity used in earlier experiments) and the other half similarly treated with sodium hydroxide and a large excess of borodeuteride. In this way it was hoped to avoid aldehyde production and to prevent oxidation of the pyrogallol ring. The crude product was esterified (MeOH–HCl) and the less polar products were purified *via* chromatography. This procedure produced, as major product, a compound affording a base peak in its mass spectrum at *m/e* 448; the isotope pattern (*m/e* 448/450/452 ratio 9 : 6 : 1) established the presence of two chlorine atoms. The presence of an ion at *m/e* 507, possibly a molecular ion, suggested the loss of a methoxycarbonyl group (59 mass units) in the production of *m/e* 448. These findings are in accord with the molecular weight of the expected product (10), and its anticipated fragmentation by loss of a benzylic methoxycarbonyl group to give a stable iminium ion ($ArCH{=}\overset{+}{N}H_2$, *m/e* 448); the elemental composition of *m/e* 448 was established as $C_{22}H_{20}Cl_2NO_5$ by high resolution measurement. The presence of three acylatable sites in structure (10) was established by acetylation, which led to a product (11) giving a molecular ion at *m/e* 633 [507 + (3 × 42)]. Moreover, in the reduction by borodeuteride, this product incorporated two deuterium atoms, as required by our earlier proposals.

The experiments already described suggest that the partial structure of vancomycin can be extended to (1), which incorporates a trioxygenated phenylglycine and two chloro-β-hydroxytyrosine units. If (1)—(3), together with aspartic acid and ammonia, are connected through the formation of amide bonds, with the proviso that vancomycin contains one each of the functions NH_2, MeNH, $CONH_2$, and CO_2H,[5] then its molecular formula would be $C_{66}H_{75}Cl_2N_9O_{24}$ and that of the degradation product CDPI would be $C_{66}H_{74}Cl_2N_8O_{25}$. This formula for CDPI is in close agreement with the formula ($C_{64}H_{74}Cl_2N_8O_{26}$) calculated from microanalysis of carefully dried CDPI.[11] The molecular weight of CDPI determined in an *X*-ray study is 1 420 ± 30,[11] to be compared with 1 448 calculated from combination of the five units which have now been deduced chemically.



(10) R = H
(11) R = Ac

The proposals with regard to the numbers and types of protons in vancomycin, and the presence of two hydroxy-groups, have been checked by ^1H n.m.r. Vancomycin, CDPI, CDPII, and aglucovancomycin give extremely poorly resolved ^1H n.m.r. spectra [even in $(CD_3)_2SO$ at 90 °C], partly owing to intermolecular association. We therefore aimed to prepare a derivative in which all ionisable groups would be protected, in the hope that this might be soluble in organic solvents and amenable to the purification techniques conventionally

[11] P. J. Roberts, O. Kennard, K. A. Smith, and D. H. Williams, *J.C.S. Chem. Comm.*, 1973, 773.

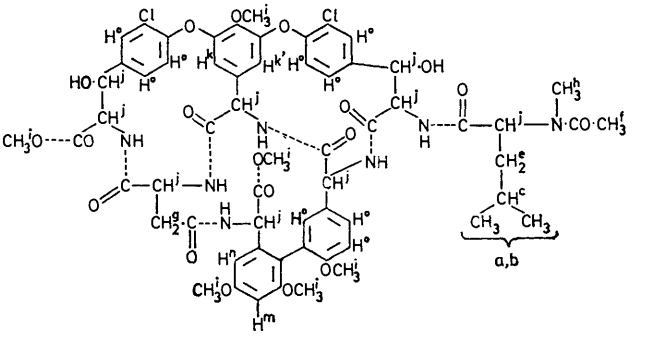
applied to relatively non-polar compounds (rather than gel filtration, which has hitherto been applied to purify vancomycin and its derivatives [12,13]). The carboxy and phenolic groups of the aglycone CDPII were methylated with diazomethane, and the terminal *N*-methylleucine unit was acetylated with acetic anhydride in methanol. In chromatography of the product on silica a small fraction gave two spots (relative intensities *ca.* 5 : 1) which were barely separable; the majority of the product remained at the base-line and appeared, on the basis of its behaviour in various solvents, to be polymeric. The mobile fraction crystallised from methanol to give hexa-*O*-methyl-*N*-acetyl-CDPII. Our evidence requires a formula $C_{61}H_{65}Cl_2N_7O_{19}$ for this derivative and microanalytical data (see Experimental section) are in accord with this. As with all other materials containing the five benzene rings of vancomycin that we have examined, this derivative did not give a molecular ion or any useful fragments in its electron impact mass spectrum. Neither did it give a mass spectrum by field desorption or chemical ionisation techniques.

The ^{1}H n.m.r. spectrum of hexa-*O*-methyl-*N*-acetyl-CDPII (Table) was obtained at 85 °C, in $(CD_3)_2SO$ containing 20% $CD_3{\cdot}CO_2D$, so that all acidic hydrogen atoms were exchanged.

^{1}H N.m.r. data of hexa-*O*-methyl-*N*-acetyl-CDPII

δ	No. of protons	Multiplicity	*J*/Hz	Assignment
0.92	3	d	7	a, b
0.95	3	d	7	
1.5—1.9	3	m		c, e
2.12	3	s		f
2.3 and 3.1	2	m		g
2.95	3	s		h
3.54	3	s		
3.70	3	s		
3.72	3	s		
3.78	3	s		
3.87	3	s		
4.06	3	s		
4.30	1	br		
4.64	1	s		
4.69	2.7	br		j
4.82		t	2.5	
4.97	1	br		
5.22	3	br		
5.81	1	br, s		
5.87	0.5	*ca.* q	2.5 and 1	k, k'
6.00	0.5	*ca.* q	2.5 and 1	
6.38	1	d	2.0	m
6.78	1	d	2.0	n
6.9—7.7	8	m	8	o
7.86	1	d	2.5	

The units known to be present in hexa-*O*-methyl-*N*-acetyl-CDPII are shown in structure(12), which illustrates one possible scheme for interconnecting these units through amide bonds and locating the two methyl ester functions. Many of the amide bond connections (indicated by dotted lines) are made in an arbitrary manner, although some tentative restrictions on the connectivity scheme can be made on the basis of evidence to be presented subsequently. It is evident that connection must be made through six amide bonds, leaving 8 hydrogen atoms replaceable by deuterium (6 NH, 2 OH), and an expectation that signals for 57 protons will appear in the ^{1}H n.m.r. spectrum. To the nearest whole number this is the value (56.7) obtained by integration. Assignments are given in the Table.



(12)

The sharpness of the signals associated with many of the protons (especially f, h, i, m, and n) supports the belief that hexa-*O*-methyl-*N*-acetyl-CDPII is a pure compound. However, one proton gives rise to two clear half-proton resonances (δ 5.87 and 6.00; Table). These, with a further one-proton resonance at 5.81, together necessarily represent 2 of the 13 known aromatic protons in the molecule, but occur at relatively high field. Such chemical shifts are close to those anticipated for two protons (k) of a pyrogallol ring [see (12)], which are unusually shielded by the aromatic rings of the chlorotyrosine units (which are expected to lie in planes roughly perpendicular to the plane of the pyrogallol ring). Such an arrangement might decrease non-bonded interactions within the system of three aromatic rings *via* diphenyl ether links; the proposed arrangement is that believed to occur in thyroxine and its analogues [14] where the proton corresponding to k is also unusually shielded (δ *ca.* 6). The splitting of one of the k proton resonances could then be due to one of the chlorotyrosine aromatic rings populating approximately equally the two positions obtained by a rotation of *ca.* 180° round the $O{-}C_6H_3Cl{-}CH(OH)$ axis. An alternative possibility is that the asymmetric centre adjacent to the pyrogallol ring has been partially inverted during the acid-catalysed conversion of vancomycin into CDPII. This acid-catalysed equilibration would then have to be relatively slow, since the splitting of a k proton resonance is not observed in aglucovancomycin (prepared from vancomycin by the action of 0.6N-HCl for 2 min) but only in CDPII and its derivatives (CDPII may be prepared from vancomycin by heating at pH 4.2 for 2 days, followed by treatment of the resulting CDPI with 0.6N-HCl for 2 min). Further support for the assignment of the two half-proton resonances at δ 5.87 and 6.00 to one of the k protons is provided by the appearance of each of these resonances as a distinct quartet (*J* 1.5 and 2.5 Hz) in

[12] M. Nieto and H. R. Perkins, *Biochem. J.*, 1971, **123**, 773.
[13] G. K. Best, N. H. Best, and N. H. Durham, ' Antimicrobial Agents and Chemotherapy,' American Society for Microbiology, Bethesda, Maryland, 1968, p. 115.
[14] P. A. Lehman and E. C. Jorgensen, *Tetrahedron*, 1965, **21**, 363.

expanded spectra obtained with resolution enhancement; the smaller coupling is removed on irradiation of the broad three-proton singlet centred at 5.22. These data are in accord with $J_{k,k'} = 2.5$ and $J_{j,k} = 1.5$ Hz, where the j proton referred to here is that α to the pyrogallol ring (δ 5.22). The mutual coupling of protons m and n is also established by decoupling experiments; further it has been shown [5] that in the free phenol, proton m can be replaced by deuterium in acidic media.

The occurrence of six amide NH groups in CDPII [see (12)] is supported by ^{1}H n.m.r. data for vancomycin itself, obtained in $(CD_3)_2SO$ solution at 90 °C. A one-proton resonance at δ 8.9 broadens rapidly as the temperature approaches 90 °C and a further 7 replaceable NH resonances are estimated to occur in the 7.95—8.5 region (by comparison with the integration of the methyl peak of the *N*-methyl-leucine). Thus, vancomycin is estimated to contain 8 amide NH groups, consistent with the conversion $CONH_2 \longrightarrow CO_2H$ in passing from vancomycin to CDPII (*via* CDPI).[7]

A second pure compound suitable for ^{1}H n.m.r. studies has been prepared *via* methylation (CH_2N_2) and acetylation (Ac_2O–MeOH) of aglucovancomycin. Penta-*O*-methyl-*N*-acetylaglucovancomycin was isolated *via* preparative t.l.c. in poor yields (2.5%). Its ^{1}H n.m.r. spectrum, recorded under conditions similar to those used for the CDPII analogue, was very similar to that of hexa-*O*-methyl-*N*-acetyl-CDPII but lacked the methoxy-resonance at δ 3.54, in accord with the change $CONH_2 \longrightarrow CO_2H$ on passing from aglucovancomycin to CDPII.[5] In this penta-*O*-methyl derivative, the protons analogous to k and k′ in (12) give broad singlets at 5.70 and 5.77 (1 H and 1 H) and the half-proton resonances observed earlier (Table) are not evident.

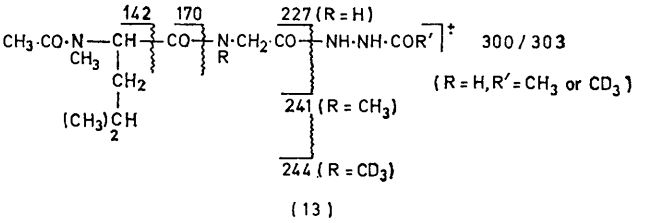
Two further consequences of the presence of β-hydroxytyrosine units in (12) have been tested experimentally. First, the proposed presence of vicinal proton signals in the 'j' region of the spectrum (4.06—5.22; Table) allows for the possibility of mutual coupling of j protons. In fact, irradiation of the resonance centred at 4.82 resulted in a sharpening of the broad resonance at 4.97 into a two-line pattern with a splitting of 3 Hz; in view of the separation of the signal of one of the k protons into two half-proton resonances, the residual splitting of 3 Hz may well be due to the postulated existence of two forms of (12). Secondly, it should be possible to diesterify (12); this was achieved by prolonged reaction with dinitrobenzoyl chloride in pyridine. The product was isolated *via* preparative t.l.c. and shown to be a bisdinitrobenzoate from its ^{1}H n.m.r. spectrum, which contained sharp resonances due to six protons in the δ 9.0—9.3 range. Signals for two protons which occurred in the 4.3—5.3 range in the spectrum of (12) were shifted downfield to 6.48 [1 H; broad singlet ($W_{\frac{1}{2}}$ *ca.* 4 Hz)] and 6.46 and 6.34 [two half-proton broad singlets ($W_{\frac{1}{2}}$ *ca.* 4 Hz)]; these signals are assigned to C*H*·OH.

[15] A. C. Chibnall and M. W. Rees, *Biochem. J.*, 1958, **68**, 105.
[16] H. R. Morris, D. H. Williams, and R. P. Ambler, *Biochem. J.*, 1971, **125**, 189.

Although many of the amide links indicated by dotted lines in (12) are made in an arbitrary manner, there is evidence (varying from firm to extremely tentative) to support some of the linkages and to limit the number of possibilities. The earlier evidence [6] for *N*-terminal *N*-methyl-leucine is confirmed by the monoacetylation of CDPII with acetic anhydride in methanol, which is accompanied by a downfield shift of the *N*-methyl signal from δ 2.3 to 2.95 (Table). A carboxy-terminal amino-acid was not detected by the hydrazinolysis method,[6] and it thus appears unlikely that the aspartic acid residue is *C*-terminal; if the terminal carboxy-group were a part of one of the modified tyrosine or phenylglycine units then it would remain undetected upon hydrazinolysis, since these unusual amino-acids are destroyed by normal hydrolytic procedures.

It is not clear from the original work whether the hydrazinolysis experiments were carried out on both vancomycin and CDPII. We have also failed to find evidence for *C*-terminal aspartic acid *via* hydrazinolysis of CDPII, which suggests that aspartic acid in CDPII is fully involved in amide linkages and that the conversion of vancomycin into CDPII does not involve the modification of an asparagine to an aspartic acid residue with a free carboxy-group ($CONH_2 \longrightarrow CO_2H$). Further support for this conclusion was derived *via* reduction of CDPII dimethyl ester with lithium borohydride.[15] The i.r. spectrum of the product showed no absorption above 1 700 cm^{-1} and its ^{1}H n.m.r. spectrum showed no methoxy-resonances, indicating that reduction of both ester groups had occurred. The hydrolysate of this reduction product, as a mixture of *N*-trifluoroacetyl n-butyl ester derivatives, was examined by g.l.c. Comparison with a standard sample of *N*-trifluoracetylaspartic acid di-n-butyl ester showed the yield of aspartic acid to be 80% of that obtained by hydrolysis of vancomycin.

Evidence to support the attachment of the terminal *N*-methyl-leucine to a potential glycine unit [see (12)] has been obtained by mass spectrometric analysis of the products of mild hydrazinolysis of (12). The products were acetylated with 1 : 1 $(CH_3CO)_2O$–$(CD_3CO)_2O$ in methanol, and then examined by mass spectrometry (i) prior to permethylation, (ii) after permethylation with CH_3I, and (iii) after permethylation with CD_3I. On the



(13)

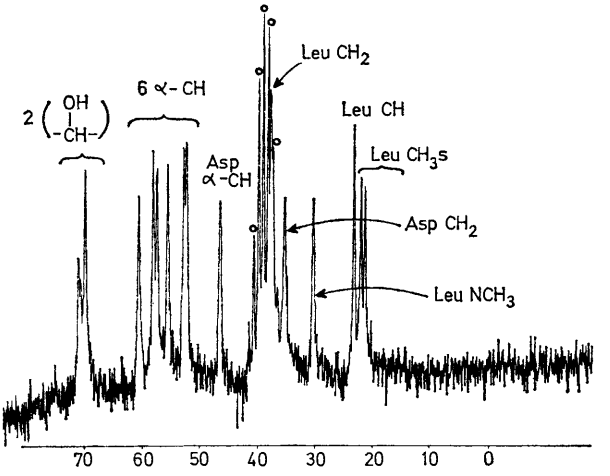
basis of the strategy developed for the sequencing of proteins by mass spectrometry,[16,17] abundant sequence ions at the *m/e* values indicated in (13) support the presence of the *N*-acetylhydrazide of *N*-acetyl-*N*-methyl-

[17] H. R. Morris, D. H. Williams, G. Midwinter, and B. S. Hartley, *Biochem. J.*, 1974, **141**, 701.

leucylglycine (13) in the products of mild hydrazinolysis.

There is no compelling evidence for the location of the two ester groups in hexa-*O*-methyl-*N*-acetyl-CDPII, and their location in (12) is arbitrary except for the following evidence. Nieto and Perkins [12] have observed that the electromeric titration of a carboxy-group in vancomycin is accompanied by a progressive change in the u.v. extinction coefficient at 294 nm. The 294 nm band is appropriate for the biphenyl system, and the carboxy-group of vancomycin must therefore be near the biphenyl system (the c. d. spectra of vancomycin in acid and neutral solution are very similar, and therefore the change in the u.v. spectrum is unlikely to be due to a conformational change).[12]

A final test that the four structural units incorporated in (12) account for the total skeleton of CDPII is available from the ^{13}C n.m.r. spectrum of CDPII itself. Since



^{13}C N.m.r. spectrum of CDPII in $(CD_3)_2SO$ at 80 °C (sp^3 carbon atoms only); chemical shifts in p.p.m. form internal Me_4Si; the five intense signals arising from solvent are marked by open circles (○)

CDPII forms aggregates in relatively concentrated solutions, satisfactory ^{13}C spectra are obtained only with difficulty [*e.g.* at 80 °C in $(CD_3)_2SO$]. The spectrum obtained under the quoted conditions [with CDPII (300 mg) in $(CD_3)_2SO$ (3 ml) and accumulating 100 000 transients] contains satisfactorily resolved resonances which are of structural utility only for the sp^3 carbon atoms (Figure). In accord with the units shown in (12), the spectrum shows seven resonances associated with the α-carbon atoms of the proposed seven amino-acid residues, an *N*-methyl carbon resonance, five resonances due to the remaining sp^3 carbon atoms which are not directly attached to electronegative atoms, and resonances due to two carbon atoms at relatively low field assigned to Ar*C*(OH). One of these low-field resonances is split into two signals; this phenomenon seems likely to be associated with the same structural feature that gives rise to the two half-proton resonances discussed previously (Table).

It appears likely that all the structural units present in vancomycin have been identified. The ambiguities that remain for the amide bond connections appear unlikely to be readily resolved by chemical methods owing to the sensitivity of the molecule to strong acid or base. Subsequent work should probably be aimed at the preparation of a suitable derivative for *X*-ray analysis; *X*-ray analysis of crystalline hexa-*O*-methyl-*N*-acetyl-CDPII has not yet led to a structural solution.

EXPERIMENTAL

Vancomycin was kindly provided by Eli Lilly and Co. Ltd., U.K. Proton and ^{13}C magnetic resonance spectra were obtained with a Varian XL100 spectrometer, operating in the Fourier transform mode; the ^{13}C spectrum was recorded with proton noise decoupling. Mass spectra were obtained with an A.E.I. MS 902 instrument at 70 eV, samples being introduced *via* a direct insertion lock.

Alkaline Hydrolysis of Methylated Aglucovancomycin in the Presence of a Large Excess of Sodium Borohydride.—Aglucovancomycin (120 mg) in aqueous methanol was treated with an excess of diazomethane in ether for 3 h. The solution was evaporated to dryness and one half of the residue hydrolysed with 50% sodium hydroxide (2 ml), in the presence of sodium borohydride (200 mg), under reflux for 24 h. The remainder of the residue was similarly treated in the presence of sodium borodeuteride. Each product was worked up in the following manner.

The cooled mixture was washed with ether and the aqueous phase acidified with concentrated hydrochloric acid and evaporated to dryness. The residual dry solid was suspended in a 3% solution of hydrogen chloride in methanol and left overnight at room temperature. The solvent was then removed and the solid residue dried and extracted with ethyl acetate containing methanol (10%). The solid remaining was acetylated by suspension in acetic anhydride–pyridine (1 : 1) overnight; the mixture was evaporated to dryness and the residue extracted with ethyl acetate–methanol. The single u.v. absorbing spot obtained by t.l.c. on silica gel (developed with 10% methanol in chloroform) afforded an oil (5 mg), which was further purified by multiple-run t.l.c. to give *dimethyl* NN′-*diacetyl*-2′,4,6-*trimethoxybiphenyl*-2,3′-*diyldiglycinate* (6) as a gum (2 mg), *m/e* 502 (90%, M^+), 470, (95, $M^+ - CH_3OH$), 459 (100 $M^+ - CH_3CO$), 443 (60, $M^+ - CO_2CH_3$), and 401 (90, $M^+ - CO_2CH_3 - CH_2CO$); no deuterium incorporation with $NaBD_4$ (Found: M^+, 502.1979. $C_{25}H_{30}N_2O_9$ requires *M*, 502.1950). Evidence for the presence of stereoisomers of (6) was available from the ^1H n.m.r. spectrum: in $(CD_3)_2SO$ *N*-acetyl resonances were present at δ 1.80 (3 H, s) and 1.90 (s) and 1.91 (s) (total 3 H); in $(CD_3)_2SO$ containing 20% $CD_3{\cdot}CO_2D$ the spectrum showed five methoxy-resonances at 3.52 (s) and 3.53 (s) (total 3 H), 3.61 (3 H, s), 3.64 (6 H s,), and 3.82 (3 H, s); two amino-acid α-CH signals at 5.07 (s) and 5.08 (s) (total 1 H) and 5.42 (1 H, s); and signals for five aromatic protons at 6.6 (2 H, s, assigned to accidentally equivalent *meta*-oriented protons of the trisubstituted benzene ring), 6.98 (1 H, d, *J* 9 Hz), 7.10br (1 H, s), and 7.35br (1 H, d, *J* 9 Hz).

Alkaline Hydrolysis of Aglucovancomycin in the Presence of a Restricted Quantity of Sodium Borohydride.—Aglucovancomycin (50 mg) was heated under reflux with sodium hydroxide solution (50%; 1 ml) and 2-ethoxyethanol (1 ml) containing sodium borohydride (10 mg) for 3 h. The solution was cooled and diluted with water and the solvent was removed by multiple extraction with ether. The resulting aqueous phase was acidified with dilute hydrochloric acid and extracted with ether. The extract was dried and evaporated and the residue chromatographed on silica plates,

developed with 10% methanol in chloroform. The main iodine-staining spot was eluted and shown to be 3-chloro-4-hydroxybenzyl alcohol by mass spectral and ^{1}H n.m.r. comparison with authentic material. A less polar, strongly u.v. absorbing spot was also eluted and shown to be 3-chloro-4-hydroxybenzaldehyde by comparison with authentic material.

The acidified aqueous phase was evaporated to dryness *in vacuo* and was esterified with 1 : 1 methanol–[$^2H_4$]-methanol containing 3% hydrogen chloride. After 3 h at room temperature the suspension was evaporated to dryness and the residue suspended in pyridine and treated with an excess of acetic anhydride at 50 °C for 2 h. The reagents were removed by evaporation under reduced pressure and the residual solid was extracted with chloroform to give an oil, from which the major constituent was separated *via* preparative t.l.c. on silica plates (developed with chloroform). The mass spectrum of this constituent identified it, in the light of earlier structural studies,[5] as the bis-ether (9) [M^+ showing the expected isotope pattern for 2 Cl and 1 OCH_3 or OCD_3: 661 (100%), 662 (40), 663 (65), 664 (125), 665 (50), 666 (70), 667 (20), and 668 (10)].

The experiment was repeated, with sodium borodeuteride instead of borohydride, and the corresponding materials were isolated from the ethereal extract. The 3-chloro-4-hydroxybenzaldehyde was unchanged, but the corresponding alcohol contained one deuterium atom per molecule (M^+ 159/161).

Alkaline Hydrolysis of Methylated Aglucovancomycin in the Presence of a Large Excess of Sodium Borohydride: Isolation of the Bis-ethers (10) *and* (11).—The ethyl acetate–methanol extract referred to in the first experiment of the Experimental section was evaporated to dryness to give an oil (30 mg), which was subjected to preparative t.l.c. on silica, the plates being developed with chloroform containing methanol (10%). The developed plates showed six u.v. active bands, and the most polar band (R_F *ca.* 0.3) gave a compound whose mass spectrum showed *m/e* 507 (M^+, 5%), 506 (M^+ −H, 3), 490 (M^+ −OH, 7), 476 (M^+ −OMe, 3), 462 (M^+ −CO_2H, 10), 448 (M^+ −CO_2Me, 100), 432 (8), and 418 (15). An exact mass measurement on the base peak (M^+ −CO_2CH_3) established the ion composition $C_{22}H_{20}Cl_2NO_5$ (Found: 448.0733. Required: 448.0717), corresponding to the molecular formula $C_{24}H_{23}Cl_2NO_7$. All the above peaks (*m*) in in the mass spectrum were accompanied by peaks at *m* + 2 and *m* + 4 in the ratio [m : (m + 2) : (m + 4)] 9 : 6 : 1, establishing the presence of 2 chlorine atoms.

The above product (10) was treated with acetic anhydride–pyridine (1 : 1) for 1 h at 80 °C and the reaction mixture was evaporated to dryness. The product was identified as (11) [the triacetate of (10)] by its mass spectrum (M^+ 633).

When the above experiment was repeated with $NaBD_4$, and the most polar u.v.-absorbing band was isolated as above and examined directly by mass spectrometry, the base peak of the product occurred at *m/e* 450.

Hexa-O-methyl-N-acetyl-CDPII.—CDPII (500 mg) was dissolved in methanol (100 ml) and a few ml of water were added to clarify the solution. The solution was treated with an excess of diazomethane in ether, and further diazomethane was added during 2 h to maintain an excess. The ether was then removed by evaporation and acetic anhydride (25% by volume) added to the remaining methanolic solution, which was then left overnight at room temperature. It was then evaporated to dryness to give a solid, which on t.l.c. (silica; developed with 7.5% MeOH in $CHCl_3$) showed much base-line material (polymer) and two less-polar products in the ratio 5 : 1 (R_F 0.40 and 0.42, respectively). These could not be separated by multiple-run preparative t.l.c. (15 runs with 2% MeOH–$CHCl_3$), and the mixture was therefore isolated as a white solid (140 mg). Two crystallisations from methanol gave *hexa-O-methyl-N-acetyl-CDPII* (50 mg) as well-defined rhombic crystals, m.p. 308—310° (t.l.c. of the melt showed only a base-line spot, establishing complete reaction of the crystalline product in the melting process) (Found: C, 57.5; H, 5.2; Cl, 5.2; N, 7.6. $C_{61}H_{65}Cl_2N_7O_{19}$ requires C, 57.65; H, 5.15; Cl, 5.6; N, 7.7%). Hexa-*O*-methyl-*N*-acetyl-CDPII did not give a useful mass spectrum, presumably owing to thermal decomposition on the probe.

Penta-O-methyl-N-acetylaglucovancomycin.—Aglucovancomycin (500 mg) was dissolved in aqueous methanol (100 ml) and treated with an excess of diazomethane in ether for 30 min. The ether and excess of diazomethane were removed under reduced pressure and the residual solution was treated with acetic anhydride (25 ml) at room temperature overnight. The solution was evaporated at reduced pressure and the residual gum shown to consist mainly of base-line material upon t.l.c. The major non-polar constituent was isolated by preparative t.l.c. on silica (plates developed with 10% methanol in chloroform) to give a white solid (20 mg). The solid was dissolved in methanol–ethyl acetate (50 : 50) and the solution concentrated at low pressure to give a white precipitate (12 mg) which was homogeneous by t.l.c. (R_F 0.5 in 10% methanol–chloroform).

Hexa-O-methyl-N-acetyl-CDPII Bis-(3,5-dinitrobenzoate).—Hexa-*O*-acetyl-*N*-methyl-CDPII (15 mg) was dissolved in pyridine (10 ml) and stirred with 3,5-dinitrobenzoyl chloride (1 g) at room temperature for 2 days. The mixture was poured into dilute hydrochloric acid and extracted twice with chloroform. The extracts were washed with dilute hydrochloric acid and an excess of saturated sodium hydrogen carbonate solution (twice), dried, and evaporated to give a white solid (20 mg). The product was purified by preparative t.l.c. on silica gel (chloroform) to give a pale yellow solid (14 mg). This was taken up in the minimum volume of chloroform and precipitated with methanol to give a pale yellow solid (12 mg) which was homogeneous by t.l.c.; for ^{1}H n.m.r. spectrum, see Discussion section.

Reduction of CDPII Dimethyl Ester with Lithium Borohydride.—CDPII (200 mg) in dry methanol (12 ml) and methanolic N-hydrogen chloride (1.2 ml) was kept at room temperature. A white precipitate gradually formed and after 40 h the precipitate (100 mg) was filtered off, washed with methanol (0.8 ml), and dried under vacuum (P_2O_5). The ^{1}H n.m.r. spectrum [$(CD_3)_2SO$; external Me_4Si reference] was like that of CDPII, with additional peaks at δ 3.75 and 4.00. This product (100 mg) was suspended in tetrahydrofuran (2.6 ml; dried over calcium hydride), lithium borohydride (17 mg) was added, and the mixture was heated under reflux for 6 h. Methanolic hydrogen chloride was then added and the solution evaporated. The product was heated under reflux with 6N-hydrochloric acid for 24 h, and the isolated hydrolysate dissolved in methanol (10 ml) containing 1.2 equiv. of HCl and stirred for 1 h. The methanol was evaporated off, n-butanol (20 ml) containing 1.2 equiv. of HCl was added, and the solution was stirred in a 90 °C bath for 3 h. The n-butanol was evaporated off, dichloromethane (10 ml) and trifluoroacetic anhydride (1.0 ml) were added, and the solution stirred for 30 min and evaporated. G.l.c. of the volatile products (on a column of 10% diethylene glycol

succinate on 60—80 Chromosorb W at 140 °C) showed a ratio of *N*-trifluoroacetyl n-butyl ester derivatives of aspartic acid and *N*-methyl-leucine which was reduced by only 10% relative to vancomycin hydrolysates. Comparisons (by g.l.c.) with standard solutions of the aspartic acid derivative indicated the yield of aspartic acid to be 1 mol per 1 600 g of CDPII dimethyl ester (following lithium borohydride reduction), as compared with 1 mol from 1 580 g of vancomycin.

*Mild Hydrazinolysis of Hexa-*O*-methyl-*N*-acetyl-CDPII.*—Hexa-*O*-methyl-*N*-acetyl-CDPII (5 mg) was heated in hydrazine–water (1 : 1; 2 ml) for 15 min at 75 °C. The solution was then diluted with water and the reagents removed under vacuum with warming. Portions of the residue were acetylated,[16] or acetylated [16] and permethylated [18] according to the published procedures, prior to examination by mass spectrometry.

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[18] H. R. Morris, *F.E.B.S. Letters*, 1972, **22**, 257.