Structure Studies on Vancomycin

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Vancomycin is a clinically effective antibiotic. Investigations which have resulted in the elucidation of some of its structural features are described.

Vancomycin is an antibiotic which was isolated by McCormick and co-workers.[1] Laboratory[2] and clinical[2,3] studies demonstrated its activity and showed it to be particularly effective against refractory staphylococcal infections.

Reports[1,4] of its chemical properties revealed that vancomycin has an estimated molecular weight of 3300 based on sedimentation data. Titration showed the presence of carboxyl, amino, and phenolic groups. Hydrolyses produced 2 moles of aspartic acid and 2 moles of glucose/mole. Removal of the glucose led to isolation of an aglucovancomycin[5] which retains about three-fourths of the biological activity of vancomycin.

The chelating properties of vancomycin were utilized to develop a useful purification method[6] through the formation of a copper complex. Material purified in this manner was referred to as vancomycin c.r. (copper regenerated). All earlier significant reactions were repeated with this purer material with essentially identical results. Repeated copper precipitation and regeneration gave a sample[7] for which a tentative formula of $C_{118}H_{185}Cl_4N_{21}O_{56}$ can be calculated.

In addition to aglucovancomycin, vancomycin may be converted by mild acid hydrolysis to a crystalline degradation product (CDP-I) which is in turn converted to a second crystalline degradation product (CDP-II).

CDP-I had first been obtained as a result of stability studies[8] on vancomycin at pH 4.2 for 30 days. Examination by X-ray diffraction indicated a molecular weight[9] of 1840 for this product. A tentative formula of $C_{83}H_{185}Cl_2N_{10}O_{32-33}$ calculated from this weight and the analytical data is of interest for comparison with the antibiotic itself.

CDP-I is converted by 0.6 N HCl to CDP-II, a crystalline monohydrochloride which retains the two organic chlorines present in CDP-I. Based on analyses for both ionic and organic chlorines the molecular weight of CDP-II would be about 1350 and a tentative formula is $C_{60}H_{65}Cl_2N_7O_{22} \cdot HCl$.

The changes involved in the formation of the degradation products of vancomycin seem to involve one or more amino groups in the molecule. Vancomycin and CDP-II both form hydrochlorides while the intermediate CDP-I does not. Also, aglucovancomycin no longer shows basic amino groups.

Examination of an acid hydrolysate by the Moore and Stein method[10] showed the expected 2 moles of aspartic acid/mole and 1 mole of an unknown amino acid. An additional small peak indicated the presence of glycine.

After isolation of some of the unknown amino acid, its infrared spectrum and paper chromatographic behavior were suggestive of leucine. The spectrum of the hydrochloride salt showed it to be a secondary amine. These data and the empirical formula based on the elemental analysis suggested N-methylleucine. This was confirmed when oxidation with chloramine-T gave isovaleraldehyde and methylamine hydrochloride. The conclusion that the isomer obtained was actually N-methyl-D-leucine was based on the negative optical rotation[11] of −8.9. Under the conditions of the experiment, some racemization would be expected.

Prolonged acid hydrolysis of vancomycin yielded a monocarboxylic acid with a pK_a of 7.1. This will be referred to as the vancomycin acid.

The infrared spectrum of a barium salt of the vancomycin acid clearly showed carboxylate bands at 6.42 and 7.1 μ and a ketone band at 5.85 μ. That it was a γ-keto acid was established by a sodium borohydride reduction of the ketone carbonyl to give a product which showed the characteristic band (5.6 μ) for a five-membered lactone. Analytical results on a cyclic unsaturated toluidide and on the acid itself indicated a C_7 acid.

A positive iodoform test on the acid was misleading since, although there are exceptions,[12] it was felt that this definitely indicated a methyl ketone. Comparison of its infrared spectrum with those of some methyllevulinic acids and isolation of methylsuccinic acid from an oxidation reaction suggested a 4-keto acid with a 3-methyl substituent. N.m.r. showed that the acid was 3-methyl-4-ketohexanoic acid. Final chemical proof was achieved by preparation of an authentic sample and comparison of a number of derivatives.

The location of all the chlorines and the nature of four of the phenolic groups in the vancomycin molecule were learned from a study of the nitric acid oxidation of aglucovancomycin. Four products were obtained. On the basis of the excellent yield of I, 77% based on the presence of four chlorines in the molecule, it seems rea-

(1) M. H. McCormick, W. M. Stark, G. E. Pittenger, R. C. Pittenger, and J. M. McGuire, Antibiotics Annual 1955–1956, Medical Encyclopedia, Inc., New York, N. Y., 1956, p. 606.

(2) R. S. Griffith and F. B. Peck, Jr., *ibid.*, p. 619.

(3) W. L. Wilson, *Antibiot. Med. Clin. Therapy*, **6**, 167 (1959).

(4) H. M. Higgins, W. H. Harrison, G. M. Wild, H. R. Bungay, and M. H. McCormick, Antibiotics Annual 1957–1958, Medical Encyclopedia, Inc., New York, N. Y., 1958, p. 906.

(5) F. W. Kavanagh, unpublished studies, these laboratories.

(6) E. O. Davisson, unpublished studies, these laboratories.

(7) This sample, supplied by H. M. Higgins, showed C, 51.01; H, 5.68; Cl, 1.71; N, 8.88; O, 27.13 for a total analysis of 100.41%. There was no residue as had been present in previous analyses.

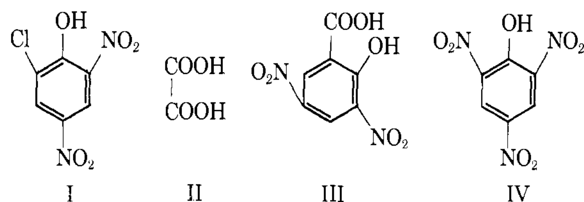
(8) Unpublished studies by S. W. Arnett, these laboratories.

(9) A private communication from Dr. Nelson J. Leonard suggests that his weight may be too high.

(10) See D. H. Sparkman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958), for a description of the method.

(11) E. H. Frieden, M. S. Dunn, and C. D. Coryell, *J. Phys. Chem.*, **47**, 10 (1943), give $[\alpha]^{25}_D$ −18.9° (c 0.611).

(12) V. I. Esafov and N. M. Stafeeva [*Zh. Analit. Khim.*, **6**, 195 (1951); *Chem. Abstr.*, **45**, 8401 (1951)] report positive tests with α,β-unsaturated ketones which can be cleaved to acetaldehyde or to methyl ketones.



sonable to assume that all of the chlorines are present in the form of an *o*-chlorophenol.

The 2-chloro-4,6-dinitrophenol could arise from either an attachment *ortho* or *para* to the phenol. When initial basic permanganate oxidation of vancomycin was unsuccessful, it was thought that prior introduction of a nitro group would stabilize the phenol during the subsequent oxidation. Utilization of this procedure ultimately led to isolation of both 3-chloro-4-hydroxybenzoic acid and the expected 3-chloro-4-hydroxy-5-nitrobenzoic acid. Thus it was established that the chlorophenol is attached to the rest of the molecule through at least a $-CH_2-$ and in the position *para* to the phenol. The 2-chloro-4,6-dinitrophenol could be formed by *ortho* nitration, oxidation of the $-CH_2-$ to COOH, decarboxylation, and nitration *para* to the phenolic group.

The formation of the dinitrosalicylic acid indicates the presence of an unsubstituted phenol attached *ortho* to the hydroxyl. The picric acid could come from an unsubstituted phenol with a *para* attachment but also could arise from the *o*-phenol by the nitration–decarboxylation–nitration route mentioned above. The amount of both of these derivatives was very small and no further assumptions could be made. In hopes of further elucidating the nature of the phenolic groups, aglucovancomycin was treated with chlorine and was then subjected to nitric acid oxidation. Chloranil was the only new product identified.

Vancomycin reacts rapidly and very extensively with periodate consuming 15.7 moles of periodate/mole of the antibiotic. The oxidation of aglucovancomycin, although a little slower, required essentially the same amount of periodate per mole. The two crystalline degradation products required 7.5 moles/mole of CDP-I and 8.6 moles/mole of CDP-II.

Further knowledge of some of the structural features of vancomycin was gained by the preparation of some derivatives. Alkylation studies did not lead to compounds of definite structure but, after treatment of vancomycin with a large excess of methyl iodide, the presence of N-methylleucine could no longer be shown chromatographically. When this methylated material was treated with 10 equiv. of acetic anhydride, a reaction that previously had completely destroyed the antibiotic activity of vancomycin, the product resulting had about half the activity of the starting material or about one-fourth that of the original vancomycin. Thus, preventing the acylation of the N-methylleucine appears to be a factor in retention of some activity.

Vancomycin was treated with diethylaminopropylene oxide and the extent of reaction was determined by nitrogen analysis. This indicated the introduction of 10 nitrogens/molecular weight of about 4500 based on the chlorine content. The antibiotic activity was almost entirely destroyed and the infrared spectrum no longer showed the presence of phenolic groups. This suggests a maximum of ten such groups. The nitric acid oxidations established that four of these are present as chlorophenols and that there is at least one unsubstituted phenol.

The data in Table I summarize present knowledge of the vancomycin molecule.

TABLE I

Component	No.	No. of C atoms
Glucose	2	12
Aspartic acid	2	8
N-Methylleucine	1	7
HO–⟨ ⟩–CH_2– (Cl)	4	28
OH ⟨ ⟩–CH_2–	1	7
$C_2H_5COCH(CH_3)CH_2COOH$	1	7

The 3-methyl-4-ketohexanoic acid is included as such although we have no evidence to indicate whether it is present in the molecule or arises during the acid treatment, perhaps from some unusual sugar.

It can be seen that the fragments which compose almost half of the molecule are now known. The nature of most of the nitrogen-containing portions of the molecule remains obscure at this time.

The information gained in this investigation suggests that vancomycin may be made up of repeating units, perhaps linked through the glucose and the amino acids. This theory would help explain the fact that there are two successively smaller degradation products, but each still shows the presence of *o*-chlorophenol and N-methyl-D-leucine. Also, the yield of CDP-I is 93% of the initial weight of vancomycin even though, from the molecular weight and the chlorine content, CDP-I appears to be slightly more than half of the vancomycin molecule. This would indicate that vancomycin contains two CDP-I units. The apparent discrepancy between the proposed carbon content of vancomycin and CDP-I and this theory may well indicate that the X-ray molecular weight[9] for CDP-I is too high.

Experimental

Aglucovancomycin.—Aglucovancomycin was prepared according to the method of Kavanagh.[5] Ten grams of crystalline vancomycin base was dissolved in 75 ml. of boiling water and 12.5 ml. of 4 *N* HCl was added. Boiling was continued for 2 min., and the mixture was then cooled to 55° and was filtered through a sintered-glass funnel. The damp cake was dissolved in 50 ml. of boiling water and 15 ml. of 4 *N* HCl was added to precipitate the aglucovancomycin. After filtration, washing with 12 ml. of 1 *N* HCl, and air drying, the colorless product weighed 8.8 g.

Crystalline Degradation Product I (CDP-I).—Vancomycin c.r. (2 g.) was placed in 30 ml. of water. The pH, which was 7.7, was adjusted to 4.2 by addition of concentrated HCl. This produced complete solution. The solution was heated for 40 hr. on a steam bath at an internal temperature of 60–70°. The heavy amber crystals present at the end of that time were isolated by filtration and weighed 1.8 g. The filtrate showed pH 4.6.

Anal. Found: C, 54.42; H, 5.32; Cl, 3.71; Cl^-, none; N, 7.34; O, 29.11.

X-Ray examination of the crystals confirmed that they were identical with those originally obtained in stability studies and indicated a molecular weight of 1840. Assay showed no antibiotic activity. Paper chromatography of an acid hydrolysate showed the presence of glucose. The infrared spectrum retained the characteristic vancomycin bands at 8.5–9 μ.

Extending the time of reaction to 64 hr. increased the yield only slightly, to 93% of the starting weight, but decreasing the time to 16 hr. reduced the yield to only 50% of the starting weight.

Crystalline Degradation Product II (CDP-II).—CDP-I (22 g., 0.12 mole) was placed in 380 ml. of 0.6 *N* HCl and, with hand stirring, was heated at the boiling point for 2 min. The mixture then set up solid so further boiling was impossible. After cooling to about 40°, the mixture was filtered and the product, after washing with 50 ml. of 0.5 *N* HCl and air drying, weighed 15 g. Titration showed pK_a (66% DMF) = 5.9, 8.0, 11.5, and 12.7. The infrared spectrum of this, and other samples also, showed COOH at 5.81 and amide bands at 6.1 and 6.63 μ. Assay showed no antibiotic activity. Chromatography indicated the presence of glucose but the absence of the infrared bands at 8.5–9 μ was similar to aglucovancomycin.

Anal. Found: C, 53.06; H, 4.87; Cl (total), 7.80; Cl^-, 2.60; N, 7.73; O, 26.02.

A 0.5-g. sample of the hydrochloride was dissolved in 250 ml. of 30% methanol to give a slightly turbid solution. When this was added to a column containing 25 ml. of IR-45 resin, much of the material came out of solution. The 250 ml. of solvent was allowed to run through the column and was then taken to dryness to give 0.15 g. of a colorless solid. X-Ray diffraction showed this free base to be crystalline, but it gave a poor pattern.

Anal. Found: C, 53.81; H, 5.59; Cl (total), 5.35; Cl^-, 0.00; N, 7.73; O, 28.43.

Amino Acids.—After overnight refluxing of 10 mg. of vancomycin, CDP-I, or CDP-II in 30 ml. of 6 *N* HCl, the mixture was filtered to remove some insoluble brown material. Paper chromatography, followed by ninhydrin spray, showed a brown immobile spot, aspartic acid, and a spot near where leucine would appear.

N-Methyl-D-leucine.—A 2-g. sample of aglucovancomycin was placed in 40 ml. of 1:1 sulfuric acid and the mixture was refluxed overnight. It was poured over ice and filtered to remove charred material. The filtrate was neutralized with barium hydroxide, filtered, and the filtrate was taken to dryness under reduced pressure. The cream-colored residue was extracted with boiling ethanol to give 50 mg. of a colorless solid which crystallized out of the ethanol on cooling. The material sublimed at 238–242°, showed $[\alpha]^{25}D$ −8.9° (*c* 1, water), and titrated well for an amino acid (pK_a in 66% DMF = 4.25 and 10.33). The infrared spectrum suggested leucine. It did not give a ninhydrin test after 2 min. of boiling.[13]

Paper chromatography showed that this material corresponded to the leucine-like spot mentioned above. A small sample was converted to a hydrochloride and infrared analysis clearly showed it to be a secondary amine.

Anal. Calcd. for $C_7H_{15}NO_2$: C, 57.90; H, 10.42; N, 9.65. Found: C, 57.21; H, 10.27; N, 9.79.

A later run gave a quantitative yield of crude N-methyl-D-leucine based on 1 mole/mole of vancomycin.

Titration of the material (640 mg.) insoluble in the ethanol indicated that it too contained an amino acid and paper chromatography showed it to be aspartic acid. It is probably present as the barium salt. In some later acid hydrolytic studies[14] ion-exchange and cellulose chromatography enabled the isolation of some of the aspartic acid which was shown to be the L-isomer.

Oxidation of N-Methyl-D-leucine.—A mixture of 145 mg. (1 mmole) of the amino acid and 280 mg. of chloramine-T in 14.5 ml. of water was allowed to stand for 3 hr. A little acetic acid was added and distillation was carried out using a bath at 110–120°. The vapors were passed into a flask containing 50 ml. of 0.5 *N* HCl in a bath at 80–90° and finally into a flask containing 25 ml. of 2 *N* HCl and 0.25 g. of 2,4-dinitrophenylhydrazine. Filtration of the resulting dinitrophenylhydrazone gave 30 mg., m.p. 113–115°. Crystallizations from ethanol and then from petroleum ether (b.p. 60–71°) raised the melting point to 117–119°.

Anal. Calcd. for $C_{11}H_{14}N_4O_4$: C, 49.62; H, 5.30. Found: C, 49.84; H, 5.51.

A mixture melting point with an authentic sample of isovaleraldehyde 2,4-dinitrophenylhydrazone (m.p. 116–118°) was not depressed. The ultraviolet and infrared spectra were also identical with those of the authentic sample.

The HCl solution was removed from the second flask and was concentrated to dryness. The residue melted at 225–230° after preliminary softening. This compares well with methylamine hydrochloride (226–228°).

Acid Hydrolysis of Vancomycin.—Hydrolysis of 48 g. of vancomycin in 960 ml. of concentrated HCl was carried out by refluxing gently for 48 hr. The mixture was filtered and a total of 1.7 g. of a yellow oil was isolated by continuous $CHCl_3$ extraction of the filtrate and of the brown insoluble solid. Separation by 2.5% KOH extraction gave 0.93 g. of an acid and 0.265 g. of a neutral material shown by infrared to be a lactone.

A Hickman tube was used for purification of 80 mg. of the acid. The bulb was kept in an oil bath at 60–80° and under a reduced pressure of 0.12 mm. for 3 hr. A colorless oil, later yellowing slightly, was collected and weighed about 50 mg. The estimated boiling point at atmospheric pressure was about 275–300°. The infrared spectrum was that of a carboxylic acid with water indicated by bands at 2.87 and 3–3.3 μ. Titration showed a pK_a (66% DMF) of 7.1 and gave an apparent molecular weight of 150.

Anal. Calcd. for $C_7H_{12}O_3 \cdot 0.5H_2O$: C, 54.88; H, 8.55; O, 36.56. Found: C, 54.96; H, 8.38; O, 36.53.

The water was eliminated by redistillation from a 100° bath and at a pressure of 0.6 mm. Titrations then indicated a molecular weight of 140 and 144 (calcd., 144.17).

Anal. Calcd. for $C_7H_{12}O_3$: C, 58.31; H, 8.39; O, 33.29. Found: C, 58.80; H, 8.61; O, 32.74.

Sodium Borohydride Reduction of the Vancomycin Acid. To 20 mg. of the acid in 1 ml. of reagent methanol was added, portionwise, a large excess (20 mg.) of sodium borohydride in 1 ml. of reagent methanol. The solution was allowed to stand for 2 hr. and was then adjusted to pH 5 with 5% HCl. After concentrating to about 0.5 ml., the solution was extracted with ether and the extracts were dried ($MgSO_4$). Removal of the ether gave about 7 mg. of colorless solid. A band in the infrared spectrum at 5.64 μ indicated a five-membered lactone.

***p*-Toluidide of Vancomycin Acid.**—A mixture of 0.225 g. of the acid and 0.8 g. of *p*-toluidine was heated in a 190° bath for 30 min. Purification from dilute ethanol gave 50 mg. of product melting at 100–101°. The infrared spectrum indicated an amide but the expected NH bands at 2.9 and 3.0 μ were absent. The ultraviolet spectrum showed that the derivative was cyclic and unsaturated.

Anal. Calcd. for $C_{14}H_{17}NO$: C, 78.10; H, 7.96; N, 6.51. Found: C, 78.08; H, 7.87; N, 6.55.

Iodoform Reaction on Vancomycin Acid.—From 100 mg. of the acid there was obtained 60 mg. of iodoform melting at 121°.

Sodium Hypobromite Oxidation of Vancomycin Acid.—A hypobromite oxidation of 500 mg. of the vancomycin acid was carried out by a procedure patterned after those described for substituted levulinic acids.[15] From the ether extracts of the acidified reaction mixture there was obtained 210 mg. of an oil which partially crystallized. Two crystallizations from benzene–petroleum ether (b.p. 60–71°) gave 110 mg. melting at 99–102°. A mixture melting point with authentic methylsuccinic acid, m.p. 110–112°, was 104–106°. The infrared spectra of the two were identical. Titration (66% DMF) showed pK_a = 6.3 and 8.6 and an apparent molecular weight of 137 (calcd., 132). A sublimed sample (m.p. 100–102°) was analyzed.

Anal. Calcd. for $C_5H_8O_4$: C, 45.45; H, 6.10. Found: C, 45.74; H, 6.45.

3-Methyl-4-ketohexanoic Acid.—Ozonolysis of 6 g. (0.048 mole) of α-allyl diethyl ketone, prepared by allylation of diethyl ketone using sodamide in liquid ammonia, was carried out according to the procedure of Whyte and Cope.[16] The yield of product was 1.7 g. (25%) of material which boiled at 92° (0.3 mm.), $n^{25}D$ 1.4383.

The *p*-phenylazophenacyl ester was prepared and was shown to be identical with the derivative of the vancomycin acid by mixture melting point, infrared spectrum, and X-ray crystallography. A sample from petroleum ether (b.p. 60–71°) melted at 85–85.5°.

Anal. Calcd. for $C_{21}H_{22}N_2O_4$: C, 68.83; H, 6.05; N, 7.65. Found: C, 69.05; H, 6.34; N, 7.52.

(13) P. A. Plattner and U. Nager [*Helv. Chim. Acta*, **31**, 2203 (1948)] obtained ninhydrin color with N-methylamino acids on paper but not in test tube reactions.

(14) R. B. Morin, unpublished studies, these laboratories.

(15) See R. Adams, E. F. Rogers, and F. J. Sprules, *J. Am. Chem. Soc.*, **61**, 2819 (1939); C. Djerassi, O. Halpern, D. I. Wilkinson, and E. L. Eisenbraun, *Tetrahedron*, **4**, 369 (1958).

(16) D. E. Whyte and A. C. Cope, *J. Am. Chem. Soc.*, **65**, 1999 (1943).

The semicarbazone melted at 153–154° (Whyte and Cope give 150–151.5°) and by mixture melting point, infrared, and X-ray crystallography was shown to be identical with the semicarbazone prepared from the vancomycin acid. This authentic acid was also found to give a positive iodoform test.

Vancomycin Acid from Crystalline Degradation Products.—Hydrolysis of 4 g. of CDP-I in 100 ml. of concentrated HCl in the same manner as above gave 175 mg. of crude product which showed a pK_a of 7.1 and its infrared spectrum was identical with that of the previously obtained acid.

Application of the hydrolysis procedure to 2 g. of CDP-II in 50 ml. of concentrated HCl gave 50 mg. of darker, more viscous material. The infrared spectrum showed it to be a mixture of acid and lactone but with a band at 6.34 μ not previously observed. Titration also indicated greater impurity by a higher apparent molecular weight.

Nitric Acid Oxidation of Aglucovancomycin.—To 25 ml. of nitric acid and 75 ml. of water was added, portionwise, 5 g. of aglucovancomycin. The resulting solution was heated on a steam bath for 19 hr. There were crystals present. Chilling and filtration gave 1.08 g. (77%) of yellow product which melted at 104–107° after some preliminary softening. An analytical sample from dilute ethanol melted at 109–110°. Identification as 2-chloro-4,6-dinitrophenol (m.p. 109°) was completed on the basis of analyses, infrared, and ultraviolet spectra and a mixture melting point with an authentic sample.

Anal. Calcd. for $C_6H_3ClN_2O_5$: C, 32.97; H, 1.38; Cl, 16.22; N, 12.82. Found: C, 33.36; H, 1.64; Cl, 16.23; N, 12.66.

The pH of the reaction mixture was adjusted to 7.5 to yield a bright yellow solid which was separated into ether-soluble and ether-insoluble fractions. The soluble material was, in turn, separated into two fractions—one insoluble (A) and one soluble (B) in benzene. (A) weighed 27 mg. and melted at 187° with evolution of much gas. After standing overnight, the material melted at 100–102°. These melting points corresponded to those of oxalic acid (189°) and its dihydrate (101°). The identification as oxalic acid was confirmed by its infrared spectrum and titration. (B) was isolated by concentration of the benzene and addition of petroleum ether (b.p. 60–71°). It weighed 20 mg. and melted at 153–155° but, after drying for analysis, it showed m.p. 168–170°. It was identified as 3,5-dinitrosalicylic acid by infrared and ultraviolet spectra, mixture melting point, and analysis.

Anal. Calcd. for $C_7H_4N_2O_7$: C, 36.85; H, 1.74; N, 12.28. Found: C, 36.94; H, 2.11; N, 12.44.

When concentrated nitric acid was used, the crystallization mother liquors of (B) yielded 6 mg. of yellow plates melting at 113–115°. Infrared analysis confirmed that this was picric acid.

Nitric Acid Oxidation of CDP-I.—When the same procedure as above was employed with CDP-I, a 42% yield of 2-chloro-4,6-dinitrophenol was obtained. The lower yield was due in part to the need for recrystallization to eliminate some higher melting material.

Nitric Acid Oxidation of CDP-II.—Treatment of CDP-II with dilute nitric acid (1:3) as above produced a 65% yield of 2-chloro-4,6-dinitrophenol.

Chlorination and Nitric Acid Oxidation of Aglucovancomycin.—To a solution of 12 g. (0.3 mole) of NaOH in 100 ml. of water was added 5 g. of aglucovancomycin. After bubbling in 7.1 g. (0.1 mole) of chlorine, the mixture was allowed to stand 5 hr. at room temperature. It was neutralized with nitric acid and concentrated to dryness under reduced pressure. There was then added 75 ml. of water and 25 ml. of nitric acid, and the mixture was warmed on a steam bath for 24 hr. Some insoluble material was isolated by filtration and was sublimed at 200° at atmospheric pressure to give 50 mg. of bright yellow material. Sublimation and decomposition made the melting point difficult to determine, but it appeared to be about 260°. The analyses and infrared spectrum confirmed that this was chloranil.

Anal. Calcd. for $C_6Cl_4O_2$: C, 29.31; Cl, 57.68. Found: C, 30.30; Cl, 56.72.

2-Chloro-4,6-dinitrophenol and oxalic acid were again isolated from the reaction mixture.

Nitration and Permanganate Oxidation of Aglucovancomycin. A. Isolation of 3-Chloro-4-hydroxybenzoic Acid.—Ten grams of aglucovancomycin was added portionwise to 100 ml. of dilute (1:4) nitric acid cooled in an ice bath. The mixture was then stirred at room temperature for 4 hr. It was neutralized with cooling, by addition of 50% NaOH solution, the volume was adjusted to 300 ml. by addition of water, and 6 g. of NaOH was added. To this was added, portionwise, with stirring, 17 g. of potassium permanganate, and the mixture was refluxed for 19 hr. Sulfur dioxide was bubbled through until the color became pale yellow. The solids were separated by filtration, and the filtrate was extracted with ether. After drying and removal of the ether, the residual oily solid was dissolved in 24 ml. of benzene and 2 ml. of ether and was placed on a column containing 10 g. of silica gel. The eluates were collected in 30-ml. portions. Four portions of benzene and two of benzene–10% ether gave little or nothing. The next five benzene–10% ether fractions gave, after washing the slightly gummy residue with 5 ml. of 1:1 benzene–petroleum ether (b.p. 60–71°), 100 mg. of cream-colored solid melting at 162–165°. Two crystallizations from benzene raised the melting point to 163–165°. The infrared spectrum agreed with that of an authentic sample of 3-chloro-4-hydroxybenzoic acid.

Anal. Calcd. for $C_7H_5ClO_3$: C, 48.72; H, 2.92; Cl, 20.54. Found: C, 48.42; H, 3.50; Cl, 19.69.

B. Isolation of 3-Chloro-4-hydroxy-5-nitrobenzoic Acid.—A run half the size of that above gave 300 mg. of yellow solid from the ether extracts. This was dissolved in 60 ml. of benzene and 3 ml. of ether and was placed on a silica gel column. Elution with 120 ml. of benzene and 120 ml. of benzene–5% ether gave fractions weighing a total of 60 mg. and all melting in the range 214–217°. Purification from benzene gave m.p. 215–216°. The infrared spectrum and mixture melting point with an authentic sample confirmed that this was 3-chloro-4-hydroxy-5-nitrobenzoic acid.

Anal. Calcd. for $C_7H_4ClNO_5$: C, 38.56; H, 1.85; Cl, 16.29; N, 6.44. Found: C, 38.47; H, 2.11; Cl, 16.14; N, 6.67.

Periodate Oxidation. A. Vancomycin.—Complete solution of 300 mg. (0.1 mmole) of vancomycin in 30 ml. of water was obtained by adjusting to pH 5 with a few drops of dilute sulfuric acid. There was then added 30 ml. of sodium periodate solution (about 0.1 *N*) and then water to a volume of 100 ml. Titration of an aliquot with standard sodium arsenite solution indicated that reaction was about 90% complete in 90 min. The final titration, after 23 hr., showed 15.7 moles of periodate consumed/mole of vancomycin. Two runs using copper purified vancomycin required 16.3 and 15.8 moles/mole.

A preparative reaction was filtered after 48 hr. and was adjusted to pH 7. Using the same method as in the oxidation of N-methyl-D-leucine, there was obtained 70 mg. of the 2,4-dinitrophenylhydrazone of acetaldehyde melting at 142–143°.

B. Aglucovancomycin.—A solution was prepared from 300 mg. of aglucovancomycin, 10 ml. of purified dioxane, 20 ml. of about 0.1 *N* sodium periodate, and water to 100 ml. Titrations showed that the reaction was 50% finished in 5 min., 75% finished in 2 hr. The final titration at the end of 48 hr. indicated consumption of 15.76 moles of periodate/mole of aglucovancomycin. The results were essentially identical (15.2 moles/mole) when a highly purified sample of aglucovancomycin was used.

C. Crystalline Degradation Product I.—The reaction mixture consisted of 300 mg. (0.163 mmole) of CDP-I, 40 ml. of water, a few drops of dilute sulfuric acid to give pH 5, 15 ml. of dioxane, 20 ml. of about 0.1 *N* periodate solution, and water to 100 ml. Titrations after 1.5 hr. and after 24 hr. were practically identical and indicated consumption of 7.5 moles/mole.

D. Crystalline Degradation Product II.—A suspension of 300 mg. (0.23 mmole) of CDP-II in water showed a pH of 2.5. Solution was achieved by adjusting to pH 6 with 5% NaOH solution. Addition of 5% sulfuric acid to pH 5 produced a slight turbidity. The oxidation was carried out as above and after 24 hr. showed that the consumption of periodate was 8.6 moles/mole of CDP-II. On a weight basis the consumption of periodate was 25% greater than for the equal amount of vancomycin.

Methylation of Vancomycin.—To a solution of 16 g. (5 mmoles) of vancomycin in 75 ml. of dimethylacetamide and 5 ml. of water were added 40 g. (0.28 mole) of methyl iodide, and the mixture was allowed to stand for 3 days. After neutralization with 5% sodium hydroxide, the product was precipitated by pouring the solution into 600 ml. of acetone. The rather gummy material was filtered, air-dried, and ground to give 16 g. of a gray powder. This showed decreased antibiotic activity, 525 units/mg. compared to 825 units/mg. for the starting vancomycin. An amino acid hydrolysis and chromatogram of this material showed almost a complete absence of the N-methylleucine spot.

Acetylation of Methylated Vancomycin.—Solution of 1.7 g. (about 0.5 mmole) of methylated vancomycin in 20 ml. of di-

methylacetamide became complete after addition of 0.5 g. (5 mmoles) of acetic anhydride. The mixture was allowed to stand for 3 days and was poured into 400 ml. of ether. Filtration and air drying gave 1.68 g. of yellow material which assayed 220 units/mg. A sample of vancomycin treated in the same fashion was devoid of antibiotic activity.

Reaction of Vancomycin with Diethylaminopropylene Oxide.—A solution of 1.6 g. (0.5 mmole) of vancomycin c.r. was prepared in 25 ml. of water acidified to pH 2 with 2 drops of concentrated sulfuric acid. The addition of 1.29 g. (10 mmoles) of diethylaminopropylene oxide raised the pH to about 9 but solution remained complete. After 20 hr., some barium carbonate was added; the mixture was filtered, and the filtrate was taken to dryness. The residue was triturated with acetone to help remove excess epoxide. After air drying, the product weighed 1.6 g. The antibiotic activity was almost entirely destroyed. A sample was retriturated with acetone prior to analysis.

Anal. Found: Cl, 3.15; N, 9.04.

The infrared spectrum no longer indicated the presence of any phenolic groups.

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The Chemistry of Cephalosporin Antibiotics. III. Acylation of Cephalosporadesates

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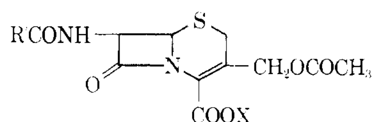
Deacetylated cephalosporins were prepared by enzymatic cleavage of the acetoxyl group. Although many usual methods of acylation failed, aroylation of the cephalosporadesic acids was possible in basic aqueous acetone. The biological activities of several derivatives are reported.

As indicated in other reports, 7-acylamidocephalosporanic acids prepared from 7-aminocephalosporanic acid and acylating agents have shown improved antibiotic activity when compared with naturally produced cephalosporin-C (I), where the 7-acyl function is an aminoadipyl group.[1] Cephalosporins have several points where modification of substituents might produce improved biological activity: one is the acetoxyl substituting the 3-methyl group.[2] This paper reports the chemistry involved in attempted variation of the acetoxyl and biological activity of some new derivatives. Cephalosporins in which the 7-acylamido group is either the 2-thiopheneacetamido group (II)[3] or the phenylmercaptoacetamido group (III) served as starting materials for this investigation.

Several routes to new 3-acyloxymethyl derivatives were explored. Nucleophilic displacement of the acetoxyl group of cephalosporin C has been reported.[4] Attempts to replace the acetoxyl group by nucleophilic displacement with an acid salt, in this case sodium butyrate, in water solution yielded no new product other than those of hydrolysis. Bioautographs of paper chromatograms of the product showed no new biologically active materials of expected mobility. This result is not surprising since aliphatic acid salts do not differ greatly in nucleophilicity.



I, R′ = $HO_2CCH(NH_2)(CH_2)_3$; X = H
II, R′ = $C_4H_3S—2—CH_2$; X = Na
III, R′ = $C_6H_5SCH_2$; X = Na

Another approach to the desired products would be to remove the acetoxyl group of a cephalosporin and acylate the resultant hydroxymethyl group. Abraham[1b] has shown that desacetylcephalosporins can be prepared by acid hydrolysis, but the concomitant hydrolysis of the β-lactam ring disqualified this method as one of practical value. Jensen, *et al.*,[5] have demonstrated that enzymatic hydrolysis of an acetoxyl can be performed with the orange peel enzyme, citrus acetylesterase. Using this technique desacetylcephalosporins were prepared in good yield. The latter have been assigned the trivial name of cephalosporadesic acids for convenience.

The anticipated simple acylation of the hydroxymethyl group was not realized in practice. One barrier to acylation was the easy formation of cephalosporadesolactones. The lactone was quite readily formed in acid solution although it was possible to isolate the free acid in an analytically pure state. It was found that acylation with an acidic reagent such as acetic anhydride is also precluded by the more facile lactone formation; in the case of 7-phenylmercaptoacetamidocephalosporadesic acid, treatment of the acid with acetic anhydride is a good method for the preparation of the corresponding lactone. Ketene at room temperature, likewise, did not acylate the hydroxyl group; lactone and cephalosporadesic acid were the only materials isolated.

(1) (a) R. R. Chauvette, E. H. Flynn, B. G. Jackson, E. R. Lavagnino, R. B. Morin, R. A. Mueller, R. P. Pioch, R. W. Roeske, C. W. Ryan, J. L. Spencer, and E. Van Heyningen, *J. Am. Chem. Soc.*, **84**, 3401 (1962); (b) B. Loder, G. G. F. Newton, and E. P. Abraham, *Biochem. J.*, **79**, 408 (1961).

(2) For the convention adopted for naming and numbering the cephalosporin nucleus, see R. B. Morin, B. G. Jackson, E. H. Flynn, and R. W. Roeske, *J. Am. Chem. Soc.*, **84**, 3400 (1962).

(3) Keflin® (cephalothin, Lilly).

(4) C. W. Hale, G. G. F. Newton, and E. P. Abraham, *Biochem. J.*, **79**, 403 (1961).

(5) E. F. Jensen, R. Jang, and L. R. MacDonnell, *Arch. Biochem.*, **15**, 415 (1947).