THE STRUCTURE OF HIKIZIMYCIN

PART I. IDENTIFICATION OF 3-AMINO-3-DEOXY-D-GLUCOSE AND CYTOSINE AS STRUCTURAL COMPONENTS*

B. C. DAS, J. DEFAYE**, AND K. UCHIDA
Institut de Chimie des Substances Naturelles, CNRS, 91–Gif-sur-Yvette (France)
(Received May 27th, 1971; accepted for publication, August 4th, 1971)

ABSTRACT

The molecular formula for hikizimycin, a new antibiotic isolated from *Streptomyces* A-5, is now established as $C_{21}H_{37}N_5O_{14}$. Evidence in support of the occurrence of 3-amino-3-deoxy-D-glucose and cytosine residues in the antibiotic molecule is presented. Methanolysis of *N,N'*-diacetylhikizimycin gave mainly two components. Fragment *A* was characterized as methyl 3-amino-3-deoxy-D-glucopyranoside by its conversion into the known methyl 3-acetamido-2,4,6-tri-*O*-acetyl-3-deoxy-α-D-glucopyranoside. Fragment *B*, on catalytic hydrogenation followed by acid hydrolysis, gave tetrahydropyrimidin-2-one, characterized as its picrate, thus establishing the presence of a cytosine residue in hikizimycin.

INTRODUCTION

Hikizimycin is a new antibiotic isolated from the fermentation broth of *Streptomyces* A-5, an organism obtained from a sample of soil collected at the side of the Hikizi river in Japan. The characteristics of the strain producing the antibiotic, the fermentation process, the isolation procedure, and some of the physical and biological properties of the antibiotic have already been described[1]. We now report on the molecular formula for hikizimycin, and present proof of the occurrence of 3-amino-3-deoxy-D-glucose (kanosamine) and cytosine as component parts of the molecule.

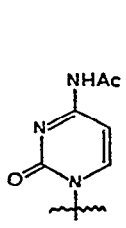
The presence of cytosine was previously suspected[1] from u.v. data that resemble those of cytidine[2]. The 60-MHz n.m.r. spectrum in deuterium oxide shows two doublets (J 8 Hz) at δ 6.3 and 7.95 p.p.m., indicative of a pyrimidine ring, and a doublet at 5.67 p.p.m. (J 8 Hz) indicative of an anomeric proton.

RESULTS AND DISCUSSION

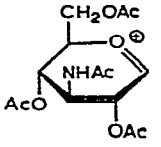
The n.m.r. spectrum of the peracetylated derivative of hikizimycin exhibits signals in the range 1.80–2.40 p.p.m. (39 protons), corresponding to 13 acetyl groups.

*This work was supported in part by a grant from the Ligue Nationale Française contre le Cancer.
**Present address; Centre de Recherches sur les Macromolécules Végétales, CNRS, Domaine Universitaire, Cedex 53, 38–Grenoble, France.

The mass spectrum of peracetylated hikizimycin exhibits a molecular-ion peak at *m/e* 1129, corresponding to the formula $C_{47}H_{63}N_5O_{27}$. A peak at *m/e* 977 is consistent with the loss of an *N*-acetylcytosine residue (**1**) from the molecular ion. The presence of this residue is also indicated by an intense peak at *m/e* 154 $(1+2H)^3$. The most intense peak (*m/e* 330) in the mass spectrum is associated (high-resolution data) with a fragment of composition $C_{14}H_{20}NO_8$, and is possibly the ion **2** which could have originated from a glycosidically linked tetraacetylhexosamine residue.

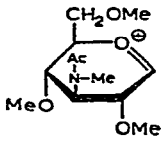


1



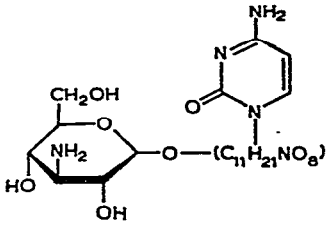
m/e 330

2



m/e 260

3



4

Peracetylated hikizimycin, on treatment with sodium methoxide at room temperature (conditions for *O*-deacetylation), gave a derivative (I) the n.m.r. spectrum of which indicates the presence of two acetyl groups (2.12 p.p.m.), presumably *N*-acetyl groups. The u.v. spectrum of I was identical with that of hikizimycin itself, indicating that the cytosine 4-*N*-acetyl group had been removed during deacetylation. This conclusion was supported by the observation that 4-*N*-acetyl-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)cytosine, (peracetylated cytidine) could be completely converted into cytidine under the above *O*-deacetylation conditions. Thus, of the 13 acetyl groups present in peracetylated hikizimycin, three are *N*-acetyl and ten are *O*-acetyl.

Treatment of *N,N*-diacetylhikizimycin (I) with methyl iodide and sodium hydride in *N,N*-dimethylformamide[4] gave a permethylated derivative (II) the spectrum of which showed a molecular-ion peak at *m/e* 863, corresponding to the formula $C_{39}H_{69}N_5O_{16}$, as determined by accurate mass-measurement. The per(deuteriomethylated) derivative gave a molecular-ion peak at *m/e* 905. The difference in molecular weight of 42 mass units indicates the incorporation of fourteen methyl groups into compound II, and is consistent with the methylation of ten hydroxyl groups and two acetamido groups, together with dimethylation of the primary amino group of the cytosine moiety.

It may therefore be concluded that hikizimycin contains ten hydroxyl and three primary amino groups, and that the molecular formula is $C_{21}H_{37}N_5O_{14}$ and not $C_{13}H_{29}N_3O_{10}$* as previously proposed[1].

The mass spectrum of the permethylated derivative II exhibited its most intense peak at *m/e* 260, corresponding to the oxonium ion[5] **3** derived from an *N,O*-methylated, *N*-acetylated glycoside of a hexosamine.

*Note added in proof: This formula was corrected in the publication[1] since the submission of the present manuscript.

Methanolysis of *N,N'*-diacetylhikizimycin (I) cleaves it mainly into two fragments, namely a ninhydrin-positive product (*A*) and a non-reducing, periodate-oxidizable compound (*B*) that absorbed u.v. light. The separation of these two compounds was achieved by using a cation-exchange resin.

Fragment *A* was eluted with 0.25M hydrochloric acid. Further purification by preparative, paper chromatography gave a hygroscopic product having analytical data for $C_7H_{16}ClNO_5$, corresponding to the methyl glycoside of a hexosamine hydrochloride. The n.m.r. spectrum exhibited two doublets in the region characteristic of anomeric protons, with a ratio H-*eq* to H-*ax* of 3:1.

Acetylation of fragment *A* with acetic anhydride–pyridine gave a crystalline derivative (*A'*) ($C_{15}H_{23}NO_9$), the n.m.r. and mass spectra of which indicate it to be an *N*-acetyl-tri-*O*-acetyl derivative of a methyl aminodeoxyhexoside.

O-Deacetylation of *A'* with sodium methoxide furnished the hygroscopic methyl glycoside (*A''*) of an *N*-acetylated hexosamine. The n.m.r. spectrum of *A''* showed only one doublet (δ 4.72, $J_{1,2}$ 3.5 Hz; H-1 *eq*) for anomeric protons, suggesting that only one anomer was present. In contrast to the behaviour of various methyl acetamidodeoxyglycosides[6], this compound failed to give a dithioacetal, and it did not consume sodium metaperiodate during 24 h. These data suggest that compound *A''* is a methyl 3-acetamido-3-deoxyaldohexopyranoside.

The specific rotation of *A'* is closely similar to that of methyl 3-acetamido-2,4,6-tri-*O*-acetyl-3-deoxy-α-D-glucopyranoside[7]. The identity of compound *A'* with the latter was firmly established by direct comparison (i.r., mass, and n.m.r. spectra and mixed melting point determination) with an authentic sample (kindly provided by Professor R. U. Lemieux). Thus, 3-amino-3-deoxy-D-glucose is shown to be a component of hikizimycin.

When fragment *B* was hydrogenated in the presence of Adams' catalyst, and the product hydrolysed with acid, two principal components (*B'* and *B''*) were formed (paper chromatography), and these were separated by ion-exchange chromatography. *B'* was further purified by preparative, paper chromatography, and identified as tetrahydropyrimidin-2-one by comparison of its n.m.r. and mass spectra with those of an authentic sample prepared[8] from cytidine by hydrogenation and acidic hydrolysis. Tetrahydropyrimidin-2-one, which is amorphous, was further characterized as the crystalline picrate. Thus, the presence of a cytosine residue in the antibiotic hikizimycin is established, and the partial structure may be represented as **4**.

Work on the structure of the second component *B''*, together with the complete structural elucidation of hikizimycin, is in progress. It is noteworthy that hikizimycin is the first example of an antibiotic having both 3-amino-3-deoxy-D-glucose and cytosine as its constituents.

EXPERIMENTAL

General methods. — Melting points were determined on a Leitz microstage and are corrected. Optical rotations were measured with a Roussel and Jouan "Quick

Polarimeter". N.m.r. spectra were recorded with a Varian A-60 spectrometer (interr tetramethylsilane) by Mme. L. Alais. Mass spectra were recorded with an A.E MS-9 spectrometer. Thin-layer chromatography (t.l.c.) was performed on Kieselg (DC–Fertigplatten F 254, Merck), followed by detection with sulphuric acid (10% or on microcrystalline cellulose (DC–Fertigfolien F 1440 LS 254, Schleicher Schüll), with u.v. detection. Paper chromatography was carried out on Whatm No. 1 paper by the descending method with 4:1:2 butyl alcohol–acetic acid–water the mobile phase, and Whatman No. 3 paper was used for preparative chromatograph

Peracetylated hikizimycin. — Hikizimycin hydrobromide[1] (350 mg) was su pended in a mixture of acetic anhydride (15 ml) and pyridine (10 ml) and stirred f 2 days at room temperature. The resulting, coloured solution was poured into ic water and extracted 5 times with chloroform. The combined extracts were wash successively with cold, saturated, aqueous potassium hydrogen sulphate, saturate aqueous sodium hydrogen carbonate, and water. The dried (Na_2SO_4) extract w evaporated do dryness to give a white solid (374 mg) which was applied in benze to a column of silica gel (Davison, 100–200 mesh, 70 g) and eluted with benzei The first 35 ml of effluent contained nothing, but the next 60 ml gave a solid (326 m Attempts to crystallize the product from chloroform–ether gave the peracetylat derivative of hikizimycin as a precipitate (77 mg) showing a single spot on t.l (Kieselgel), R_F 0.90 (45:55 methanol–benzene) and R_F 0.07 (45:55 acetone–benzen m.p. 167–172°; $[\alpha]_D^{25}$ +31.8° (*c* 0.5, chloroform); ν_{max}($CHCl_3$) 1748 (strong, ester) a 1675 cm^{-1} (amide); n.m.r. data (chloroform-*d*): δ 7.48 and 7.96 (doublets, $J_{5,6} = J$ 7.8 Hz, C-5 and C-6 protons of cytosine), 1.80–2.40 (39 protons, 13 OAc), 6.05 a 6.38 (doublets, $J \sim 9$ Hz, anomeric protons). The mass spectrum is partly record in the discussion: M^+ *m/e* 1129 ($C_{47}H_{63}N_5O_{27}$), and principal peaks at *m/e* 9 330, and 154.

N,N'-*Diacetylhikizimycin* (I). — To a solution of the above peracetylat derivative (4.10 g) in anhydrous methanol (40 ml) was added 0.20M methano sodium methoxide (15 ml). After stirring for 20 min at room temperature, the sol tion was neutralized with Amberlite IRC-50 (H^+) resin and then passed through column of the same resin. The column was washed with water, and the combin effluents and washings gave, after evaporation below 50°, a white residue (2.10 Attempts to crystallize the product from methanol afforded a white precipit (173 mg) of the title compound, m.p. 244–253°, $[\alpha]_D^{25}$ −15.7° (*c* 0.5, water). Pap chromatography (butyl alcohol–acetic acid–water, 4:1:2.5) showed one spot (ni hydrin, periodate–benzidine reagents, R_F 0.12); *cf.* hikizimycin hydrobromic R_F 0.05; ν_{max}(Nujol) 3000–3500 broad (OH), and 1655 cm^{-1} (amide); n.m.r. da (deuterium oxide): δ 6.17 and 7.82 (doublets, *J* 7.8 Hz, C-5 and C-6 protons of cyt sine), 4.70 and 5.65 (2 anomeric protons), 2.12 and 2.13 (3-proton singlets 2 NHAc)

Anal. Calc. for $C_{25}H_{41}N_5O_{16}.2H_2O$: C, 42.67; H, 6.40; N, 9.95. Foun C, 42.41; H, 6.95; N, 9.58.

Methylation of N,N'-*diacetylhikizimycin* (I). — A solution of the title compou (1 mg) in *N,N*-dimethylformamide (0.5 ml) was treated with sodium hydride (50

oil dispersion, 10 mg), and methyl iodide (0.3 ml) was added with magnetic stirring. After 45 min, water was added and the mixture was extracted with chloroform. The extract was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness. The crude, permethylated *N,N'*-diacetylhikizimycin was then submitted to mass-spectrometric analysis (M^+ *m/e* 863, $C_{39}H_{69}N_5O_{16}$). The perdeuteriomethylated derivative was prepared in the same way with trideuteriomethyl iodide.

Methanolysis of N,N'-*diacetylhikizimycin.* — A solution of the title compound (2.47 g) in anhydrous methanol (300 ml), previously saturated with hydrogen chloride at room temperature, was refluxed for 9 h. The solution was evaporated to dryness below 45°, and the remaining hydrogen chloride was removed by repeated evaporation of anhydrous methanol from the residue. Paper chromatography of the resulting, amorphous powder showed one ninhydrin-positive spot (R_F 0.20), one weakly ninhydrin-positive spot (R_F 0.25), and one u.v.-absorbing, periodate–benzidine-positive spot having R_F 0.06. This mixture (2.48 g), dissolved in the minimal amount of water, was applied to a column of Dowex-50W (X8, 100–200 mesh, H^+) resin (50 ml). The column was washed with water (100 ml) and eluted with hydrochloric acid of progressively increasing concentration. Fractions (50 ml) were collected, neutralized with Amberlite IR-45(OH^-) resin, and evaporated to dryness below 45°. The aqueous eluate gave a hygroscopic solid (375 mg) which, on paper chromatography, showed one weakly ninhydrin-positive spot having R_F 0.25. Elution with 0.25M hydrochloric acid gave (fractions 4–10) a hygroscopic, chromatographically homogeneous solid (420 mg), R_F 0.20 on paper chromatography, that was ninhydrin- and silver nitrate-positive and weakly periodate-positive (fragment *A*).

Elution with M hydrochloric acid gave (fractions 7–14) a solid (1.58 g) that was chromatographically homogeneous (R_F 0.06), u.v.-absorbing, periodate–benzidine and silver nitrate-positive, and weakly ninhydrin-positive (fragment *B*).

The ninhydrin-positive substance eluted with 0.25M acid (420 mg) was chromatographed on 3 sheets of Whatman No. 3 paper (butyl alcohol–acetic acid–water, 5:1:4). The principal product, located by means of guide strips, was eluted with water. Evaporation of the solution gave methyl 3-amino-3-deoxy-D-glucopyranoside hydrochloride as a white, hygroscopic powder (300 mg); n.m.r. data (methanol-d_4): δ 4.78 (doublet, $J_{1,2}$ 3.5 Hz, H-1 *eq*), 4.31 (doublet, $J_{1,2}$ 7 Hz, H-1 *ax*), 3.58 (singlet, eq OMe), 3.48 (singlet, ax OMe).

Anal. Calc. for $C_7H_{16}ClNO_5$: C, 36.60; H, 6.97; Cl, 15.40. Found: C, 36.20; H, 7.35; Cl, 15.96.

*Methyl 3-acetamido-2,4,6-tri-*O*-acetyl-3-deoxy-α-*D*-glucopyranoside* (*A'*). — 3-Amino-3-deoxy-D-glucose hydrochloride (260 mg) dissolved at 0° in a mixture of acetic anhydride (5 ml) and pyridine (5 ml) was stirred at room temperature for 20 h. The resulting, coloured solution was poured into ice–water which was then extracted with chloroform. The usual working up of the chloroform extract gave a crude product (280 mg) which, after decolorisation with charcoal (Norit) in methanol, was crystallized from benzene as needles (160 mg). A second recrystallization from benzene gave the analytical sample (109 mg, 22%), m.p. 171–175°, $[\alpha]_D^{20}$ +102.7° (*c* 0.5, chloroform);

ν_{max}(Nujol) 1740 (ester) and 1650 cm^{-1} (amide). N.m.r. data (benzene-d_6): δ 4.79 (doublet, $J_{1,2}$ 2.0 Hz, anomeric proton), 3.04 (singlet, OMe), 1.63, 1.67, 1.74, 1.78 (singlets, acetyl groups); this spectrum is identical with that of an authentic sample. Mass spectrum: *m/e* 361 (M^+), 330 ($M^+ - OMe$), 301 ($M^+ - AcOH$); this spectrum is also identical with that of the authentic sample .

Anal. Calc. for $C_{15}H_{23}NO_9$: C, 49.86; H, 6.42; N, 3.88. Found: C, 49.88; H, 6.20; N, 3.99.

Methyl 3-acetamido-3-deoxy-α-D-glucopyranoside (A″). — The peracetylated methyl hexosaminide *A′* (500 mg) was *O*-deacetylated as described for peracetylhikizimycin. The usual work up afforded a hygroscopic powder (310 mg) which was purified by preparative, paper chromatography. The silver nitrate-positive area (R_F 0.58), located by means of guide strips, was extracted with water; three other minor bands were not investigated. The aqueous eluate was evaporated and the residue was freed from ethanol-insoluble impurities to give *A″* as a hygroscopic powder (210 mg), $[\alpha]_D^{20}$ +136.6° (*c* 0.5, water). N.m.r. data (D_2O): δ 4.72 (doublet, $J_{1,2}$ 3.5 Hz, H-1*eq*); 3.45 (singlet, ax OMe), 2.00 (singlet, NHAc). Mass spectrum: *m/e* 204 ($M^+ - OMe$, corresponding to molecular formula $C_9H_{17}NO_6$). The extremely hygroscopic nature of the compound procluded satisfactory microanalysis.

A solution of *A″* (4.70 mg) in 0.02M aqueous sodium metaperiodate (5 ml) was kept in the dark at room temperature for 24 h. Saturated, aqueous sodium hydrogen carbonate was added, followed by aqueous potassium iodate (20%, 4 ml), and the mixture was kept for 15 min in the dark. Back titration with 5mM sodium arsenite showed that no periodate had been taken up, whereas methyl α-D-glucopyranoside, used as a reference sample, consumed 2.1 mol. of periodate after 3 h.

Isolation of the pyrimidine moiety; tetrahydropyrimidin-2-one. — Fragment *B* (500 mg), obtained after methanolysis of *N,N*-diacetylhikizimycin, was dissolved in water (20 ml) and added to a suspension of Adams' catalyst (50 mg) in water (10 ml) previously saturated with hydrogen. 2.8 Mol. of hydrogen were absorbed on vigorous shaking for 24 h in the presence of hydrogen and at room temperature and pressure. The n.m.r. spectrum of the product obtained after the usual work-up showed the disappearance of signals due to ethylenic protons. The product, without further purification, was dissolved in hydrochloric acid (1.5M, 15 ml), and the solution was refluxed for 1.5 h. After neutralization with Amberlite IR-45(OH^-), the solution was concentrated under diminished pressure. Paper chromatography of the concentrate showed a sugar-like component at R_F 0.12 (positive reactions with periodate–benzidine, alkaline silver nitrate, and aniline hydrogen phthalate) and a spot at R_F 0.43, detected with u.v. light (faint absorption) and alkaline silver nitrate (faint black). The mixture was applied to a column of Dowex-50W (X8, 100–200) resin (50 ml) and eluted successively with 0.1, 0.25, and M hydrochloric acid; 50-ml fractions were collected. The sugar-like component (338 mg) was eluted with 0.1M hydrochloric acid, and a component (62 mg) giving a positive reaction with silver nitrate was eluted with M hydrochloric acid. Further purification by preparative, paper chromatography gave an amorphous solid (50 mg), after elution with water of the zone having R_F 0.43,

which was identified as tetrahydropyrimidin-2-one by comparison (R_F, i.r., n.m.r., and mass-spectral data) with authentic material prepared as described below.

Cytidine (300 mg) dissolved in water (30 ml) was hydrogenated over platinum oxide (50 mg). Hydrogen uptake (2.9 mol.) occurred during 24 h. The solution, freed from catalyst by filtration through Celite, was evaporated to dryness. A solution of the residue in hydrochloric acid (1.5M, 30 ml) was refluxed for 1.5 h, and then evaporated to dryness. The residue, dissolved in the minimal amount of water, was applied to a column of Dowex-50W(H^+) resin (30 ml). The column was washed with water (~100 ml) and then eluted with M hydrochloric acid. Tetrahydropyrimidin-2-one (95 mg) was obtained as an amorphous, white powder after removal of hydrochloric acid in the eluate with Amberlite IR-45(OH^-) resin.

Tetrahydropyrimidin-2-one (50 mg) was dissolved in the minimal amount of water and added to a 2% solution of picric acid in methanol–water (1:1, 10 ml). The yellow crystals which separated immediately gave, on recrystallization from water, the picrate, (37 mg), m.p. 190–191°.

Anal. Calc. for $C_{10}H_{11}N_5O_8$: C, 36.48; H, 3.37; N, 21.27. Found: C, 36.22; H, 3.43; N, 21.38.

ACKNOWLEDGMENTS

We sincerely thank the Sanraku Ocean Research Laboratory, Japan, for providing hikizimycin, and Professor E. Lederer for his interest in this work.

REFERENCES

1 K. UCHIDA, T. ICHIKAWA, Y. SHIMAUCHI, T. ISHIKURA, AND A. OZAKI, *J. Antibiot., Ser. A*, 24 (1971) 259.
2 J. J. FOX AND D. SHUGAR, *Biochim. Biophys. Acta*, 9 (1952) 372.
3 K. BIEMANN AND J. A. MCCLOSKEY, *J. Amer. Chem. Soc.*, 84 (1962) 2005.
4 J. S. BRIMACOMBE, B. D. JONES, M. STACEY, AND J. J. WILLARD, *Carbohyd. Res.*, 2 (1966) 167.
5 A. ADAM, J. F. PETIT, J. WIETZERBIN-FALSZPAN, P. SINAY, D. W. THOMAS, AND E. LEDERER, *FEBS Letters*, 4 (1969) 87.
6 D. C. DEJONGH AND S. HANESSIAN, *J. Amer. Chem. Soc.*, 87 (1965) 3744.
7 S. PEAT AND L. F. WIGGINS, *J. Chem. Soc.*, 140 (1938) 1810.
8 H. IWASAKI, *Yakugaku Zasshi*, 82 (1962) 1358.