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Studies of Aminosugars. XVII. Production of 3-Amino-3-deoxy-D-glucose by *Bacillus* Species[1]

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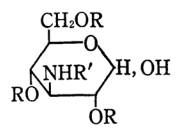
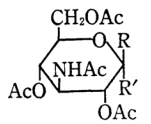
3-Amino-3-deoxy-D-glucose was isolated from a fermentation broth of a *Bacillus* species designated as *Bacillus aminoglucosidicus*. This demonstrated for the first time the presence of a monosaccharide aminodeoxypyranose in nature. The aminosugar was isolated in a high yield by using column chromatography with cation exchange resin. The identity of the product with 3-amino-3-deoxy-D-glucose was completely established by comparing the natural product and its derivatives with synthetic 3-amino-3-deoxy-D-glucose and its corresponding derivatives in elemental analyses, infrared spectra and mixed melting point determination.

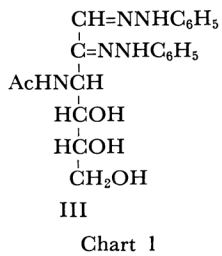
Many new aminosugars have recently been found as the constituents of useful antibiotics,[2] nucleotides of bacteria and lipo- and muco-polysaccharides[3] which occur in microbial cell walls and other cell tissues. The findings of these aminosugars, as well as the discovery of muramic acid in bacterial cell walls and the relation of muramic acid to the mechanism of antibacterial action of penicillin and other antibiotics have deepened the interest in this field of biologically significant compounds. These aminosugars usually occur as a component of polysaccharides or low molecular weight methabolic products, but have never been found in a form of monosaccharide in nature. In the work reported here the aminosugar produced by a *Bacillus* species was shown to be monosaccharide of 3-amino-3-deoxy-D-glucose (I) (abbreviated as 3AG).



I: R=R′=H
II: R=H; R′=Ac



IV: R=OAc; R′=H
V: R=H; R′=OCH$_3$



III

Chart 1

Synthetic derivatives[4] of 3AG have long been known. However, the occurence of this aminosugar in nature was not revealed until it was found[5] as a constituent of the antibiotic kanamycin[6] produced by a *Streptomyces* species. Since then, new syntheses[7,8] of the aminosugar and an improvement[9] of the method of Peat and Wiggins were reported.

The bacterium which produces the 3AG was found during screening for antibiotics exhibiting antibacterial activity against *Micrococcus pyogenes* var. *aureus* 209P; it was found that this aminosugar completely inhibited the growth of the above-mentioned bacterium at a dilution of 125 mcg/ml.

The bacterium is a Gram-positive *bacillus* species with peritrichous flagella, and the new strain was designated as *Bacillus aminoglucosidicus*. The studies on the cultural characteristics of this

1) This article constitutes Part XXXIII of a series entitled "Studies of Antibiotics and Related Substances," primarily by Sumio Umezawa. A part of this paper was presented at the 20 th Annual Meeting of the Chemical Society of Japan, Tokyo, April, 1967.

2) For review, see: "Chemistry of the Amino Sugars Derived from Antibiotic Substances" by J. D. Dutcher, in Advances in Carbohydrate Chem., **18**, pp. 259—308 (1963).

3) N. Sharon, "Distribution of Amino Sugars in Microorganisms, Plants and Invertebrates," in The Amino Sugars, Vol. IIA, Distribution and Biological Role, ed. by E. A. Balazs and R. W. Jeanloz, Academic Press, New York (1965).

4) S. Peat and L. F. Wiggins, *J. Chem. Soc.*, **1938** 1810.

5) a) M. J. Cron, O. B. Fardig, D. L. Johnson, H. Schmitz, D. F. Whitehead, I. R. Hooper and R. U. Lemieux, *J. Am. Chem. Soc.*, **80**, 2342 (1958): b) M. J. Cron, D. L. Evans, F. M. Palermiti, D. F. Whitehead, I. R. Hooper, P. Chu and R. U. Lemieux, *ibid.*, **80**, 4741 (1958).

6) H. Umezawa, K. Maeda, M. Ueda, K. Yagishita, S. Kondo, Y. Okami, R. Utahara, Y. Osato, K. Nitta and T. Takeuchi, *J. Antibiotics*, **10A**, 181 (1957).

7) R. D. Guthrie, *Proc. Chem. Soc.*, **1960**, 387.

8) H. H. Baer, *J. Am. Chem. Soc.*, **83**, 1882 (1961).

9) Y. Ito, S. Koto and S. Umezawa, This Bulletin, **35**, 1618 (1962).

organism and its fermentation conditions are presented in a separate paper.[10] This paper is concerned with isolation, purification and identification of the aminosugar.

The fermentation medium consists of glucose 1.0, soybean meal 1.5 and sodium chloride 0.3%. After removal of bacterial cells, the clear broth was passed over a column of Amberlite IRC-50, followed by elution with dilute aqueous ammonia. The resulting powder was further chromatographed on Dowex 50W×8 by eluting with dilute hydrochloric acid to give crystalline hydrochloride, which was recrystallized from absolute ethanol. Treatment of an aqueous solution of the hydrochloride with Amberlite IRA-410 afforded the free base of 3AG.

That the aminosugar is 3AG was demonstrated by the following evidences: The aminosugar gave a elemental constitution for a monoamino-monodeoxy-hexose, but did not agree with D-glucosamine which does not exhibit any antibacterial activity. The aminosugar showed mutarotation. Tollens, Fehling, Molisch, Elson-Morgan and ninhydrin reactions are positive. *N*-Acetyl derivative (II) gave a phenylosazone (III), which still possesses an acetamido group, showing that the amino group is linked to a carbon atom other than C-2. The melting point (decomp.) of fully acetylated derivative (IV) differed very markedly from 6-amino-6-deoxy-D-glucose obtained by hydrolysis of kanamycin. The molecular rotation and melting point of the free aminosugar and its all derivatives were in closest agreement with the reported values of those of 3-amino-3-deoxy-D-glucose. Confirmation was achieved by comparing with synthetic 3AG and its derivatives in the determination of mixed melting points, IR and NMR spectra: especially, the fully acetylated derivative of methyl glycoside (V) of the aminosugar showed no melting point depression on admixture with an authentic specimen of synthetic methyl 3-amino-3-deoxy-D-glucoside tetraacetate which melts without decomposition.

It is noteworthy that the strain of *Bacillus aminoglucosidicus* gave a high yield of 3AG; for example, a broth obtained by tank fermentation contained the aminosugar in a concentration of 2.6 g/*l*.

It can readily be imagined that the 3AG is formed by enzymatical transformation of nutrient glucose, and the presence of the aminosugar in large amounts in the broth may be due to the inability of the organism to utilize the aminosugar for the synthesis of some glycan containing this aminosugar. In any case, the presence of so large amount of free aminosugar in broth is unusual. Studies on the biosynthesis of the aminosugar is going on.

10) S. Umezawa, K. Umino, S. Shibahara and S. Omoto, *J. Antibiotics*, **20A** (1967), in press.

Experimental

General. Thin layer chromatography (TLC) was conducted on a silica gel (Daiichi Pure Chemicals Co.); the prepared plate was activated at 110°C and stored in a desiccator. Silica gel column chromatography was carried out using a silica gel (Kanto Chemical Co.) freshly activated at 110°C. Paper chromatography was run by the descending technique on Toyo filter paper No. 50 and the substance was detected by ninhydrin spray (0.25% in pyridine).

The synthetic derivatives of 3AG mentioned below, except fully acetylated methyl glycoside, were all synthesized according to the method of Baer.[8] The IR spectra of the derivatives of the natural 3AG were found to be identical with those of the corresponding synthetic derivatives throughout the whole range respectively.

Isolation of 3-Amino-3-deoxy-D-glucose from Fermentation Broth. The broth (2.0 *l*) was centrifuged to remove bacterial cells. The clear broth was chromatographed on a column (4.5×41 cm) of Amberlite IRC-50 (NH_4^+) and, after washing with water, elution was carried out with 1.0% aqueous ammonia (1800 m*l*). Ninhydrin-positive fractions (560 m*l*) were collected and evaporated in vacuum to give a yellowish powder, 6.8 g (yield by antibacterial assay 74%).*1 An aqueous solution of the crude product (2.5 g in 5 m*l* of water) was placed on a column of Dowex 50W×8 (H^+) (40 m*l*), and, after washing with water, the aminosugar was eluted with 0.5 N hydrochloric acid. Fractions which showed a single, ninhydrin-positive spot on a paper chromatogram were collected, the solvent system used for the paper chromatography being *n*-butanol - pyridine - water - acetic acid (6 : 4 : 3 : 1). Evaporation of the combined fractions in vacuum afforded 1.96 g of very hygroscopic crystalline hydrochloride of the aminosugar. Recrystallization from ethanol again gave hygroscopic colorless crystals of 3-amino-3-deoxy-D-glucose hydrochloride, mp 115—120°C (decomp.),[11] $[\alpha]_D^{20}$ +33° (*c* 0.91 in water, 1 hr).[12]

Found: C, 33.80; H, 6.78%. Calcd for $C_6H_{14}NO_5$-Cl: C, 33.42; H, 6.54%.

An aqueous solution of the hydrochloride (1.96 g in 13 m*l* of water) was neutralized by mixing with 10 m*l* of Amberlite IRA-410 (OH^-), and after removal of the resin by filtration, the solution was evaporated in vacuum to afford 1.60 g of colorless powder of 3-amino-3-deoxy-D-glucose (I), which was further purified by dissolving the product in a small quantity of water with subsequent addition of ethanol; mp 140—143°C (decomp.), $[\alpha]_D^{16}$ +39 → +19° (*c* 0.91 in water, 24 hr).[13]

Found: C, 40.54; H, 7.35; N, 7.40%. Calcd for $C_6H_{13}NO_5$: C, 40.22; H, 7.31; N, 7.82%.

The aminosugar gave positive results in the following

*1 By the cylinder-plate method, using a standard curve; the test organism used was *Micrococcus pyogenes* var. *aureus* 209P.

11) Reported mp 102—110.5°C (decomp.). Y. Ito, Dr. Thesis, p. 105, Keio University, 1963.

12) Reported $[\alpha]_D^{20}$ +47 (5 min) → +43° (6 hr, *c* 1 in water). Ref. 8.

13) Mp and optical rotation of 3AG obtained from the hydrolyzate of kanamycin were reported: mp 128°C (decomp.), $[\alpha]_D^{14}$ +19° (*c* 1.0 in water, 4 hr). K. Maeda, "Streptomyces Products Inhibiting Mycobacteria," Wiley (1963), p. 69.

tests: Tollens, Fehling, Molisch, Elson-Morgan and ninhydrin. IR spectra of the product and an authentic specimen of synthetic 3AG prepared by the method of Baer[8] were found to be identical throughout the whole range of wavelengths. No depression in melting point was observed on admixture with the authentic specimen (mp 140°C (decomp.)).

***N*-Acetyl Derivative (II).** To a solution of the above-mentioned free base of the aminosugar (40 mg) in methanol (2 m*l*) and water (0.6 m*l*), acetic anhydride (0.15 m*l*) was added under shaking and the mixture was allowed to stand at about 30°C for 24 hr. A small quantity of Amberlite IR-120 (H^+) was added to the reaction mixture to remove the unchanged aminosugar. After filtration, the solution was evaporated in vacuum to give a glassy solid, which was crystallized from isopropanol; fine needles, 20 mg, mp 198—202°C (decomp.), $[\alpha]_D^{21}$ +35→+41° (*c* 0.73 in water, 24 hr).[14]

Found: C, 43.51; H, 6.87; N, 6.32%; mol wt, 210. Calcd for $C_8H_{15}NO_6$ (221.2): C, 43.43; H, 6.84; N, 6.33%.

No depression in melting point was observed on admixture with the authentic specimen of synthetic 3-acetamido-3-deoxy-D-glucose[8] (mp 199—202°C (decomp.)).

***N*-Acetyl Phenylosazone (III).** To a solution of the free base of the aminosugar (284 mg) in water (1.42 m*l*) was added a solution of phenylhydrazine (0.57 m*l*) in acetic acid (0.57 m*l*) and water (1.42 m*l*) and the mixture was heated on a steam bath for 30 min. The mixture was cooled to room temperature and the deposit was collected, giving 170.2 mg of the substance. Crystallization from isopropanol gave brilliant yellow needles, mp 210—214°C (decomp.), $[\alpha]_D^{20}$ −180° (*c* 0.6 in ethanol). IR spectrum (KBr disk) showed absorptions at 3360 (broad, OH, NH), 1625 (amide I), 1600 (phenyl), 1575 (amide II) and 750 cm^{-1} (phenyl).

Found: C, 60.27; H, 6.75; N, 17.20%. Calcd for $C_{20}H_{25}N_5O_4$: C, 60.13; H, 6.31; N, 17.53%.

A specimen prepared from synthetic 3AG showed mp 214—216°C (decomp.) and $[\alpha]_D^{20}$ −183° (*c* 0.6 in ethanol). No depression in melting point was observed on admixture with the synthetic specimen.

Fully Acetylated Derivative. A mixture of the free base of the aminosugar (80 mg), acetic anhydride (12 m*l*) and pyridine (12 m*l*) was allowed to stand at about 27°C for 96 hr. The mixture was occasionally shaken. The mixture was evaporated in vacuum and the residue was dried by coevaporation with benzene to afford a crystalline solid, 156 mg. TLC of the product using a solvent system, methyl ethyl ketone (MEK) - benzene (3 : 2) and 50% sulfuric acid for the coloration showed two adjoining spots, suggesting the presence of α- and β-anomer. The product was chromatographed on a column of silica gel, using a solvent system MEK-benzene (1 : 1); early fractions were collected, evaporated and the residue was crystallized from isopropanol to give colorless needles of 3-amino-3-deoxy-β-D-glucose pentaacetate (IV), 40 mg, mp 206—208°C (decomp.), $[\alpha]_D^{20}$ +7.0° (c 1.0 in chloroform).[15]

Found: C, 49.17; H, 6.14; N, 3.73%. Calcd for $C_{16}H_{23}NO_{10}$: C, 49.35; H, 5.95; N, 3.60%.

The later fractions in the above-mentioned chromatography may contain the α-anomer. However, attempts to obtain it in a pure crystalline form was unsuccessful, giving an amorphous powder which showed a far higher dextro-rotation and an IR spectrum bearing a close resemblance to the β-anomer described above.

Fully Acetylated Methyl Glycoside. A solution of the free base of aminosugar (622 mg) in 5% methanolic hydrogen chloride (62 g) was refluxed on a steam bath for 36 hr. The mixture was evaporated in vacuum to dryness. The residue (0.73 g) was mixed with silica gel (3.0 g) and chromatographed on a column of silica gel (20.8 g); elution was carried out with MEK-ethanol (2 : 1). Fractions which showed a single spot on TLC by a solvent system MEK-ethanol (3 : 1) were combined and evaporated to dryness, giving a methyl glycoside, 247 mg.

A mixture of the crude product (245 mg), acetic anhydride (12 m*l*) and sodium acetate (600 mg) was heated on a steam bath for 2 hr and evaporated in vacuum to dryness. The residue was treated with chloroform and, after removal of sodium acetate by filtration, the chloroform solution was evaporated to dryness. The residue was mixed with silica gel (1.2 g) in a small quantity of benzene-MEK (2 : 1) and chromatographed on a column of silica gel (10 g), using the same solvent system for elution. The main fractions were combined and evaporated to give a solid, 160 mg. Recrystallization from ethanol-acetone gave colorless needles of methyl 3-amino-3-deoxy-α-D-glucoside tetraacetate (V), 60 mg; mp 174°C, $[\alpha]_D^{25}$ +99.3° (*c* 1.0 in chloroform).[16]

Found: C, 49.46; H, 6.13; N, 3.97%. Calcd for $C_{15}H_{23}NO_9$: C, 49.86; H, 6.42; N, 3.88%.

The product was identical in a mixed melting point comparison with the authentic specimen (mp 174—175°C, $[\alpha]_D^{24}$ +105.2° (*c* 0.8 in chloroform)) prepared according to Peat and Wiggins.[4]

We are indebted to Bristol Laboratories of Syracuse, N. Y., U. S. A. and Institute of Microbial Chemistry, Tokyo, for financial support, to Kawasaki-Factory of Meiji Seika Co. for technical support, to Mr. Saburo Nakada for microanalysis and to Mr. Shinkiti Koto for helpful discussions.

14) Reported mp 204—205°C (decomp.) and $[\alpha]_D^{20}$ +18.6 (2 min) → +52.7° (*c* 2.5 in water, 8 hr). Ref. 8.

15) Mp and optical rotation of pentaacetate of kanosamine (3AG) obtained from kanamycin were reported: mp 206—207°C, $[\alpha]_D^{25}$ +8.1° (*c* 0.8 in chloroform). Ref. 5a.

16) Reported mp 178°C and 172.5—173°C and $[\alpha]_D^{20}$ +101.9° (*c*1.0 in chloroform) and $[\alpha]_D$ +105.5° (*c* 0.5 in chloroform). See Ref. 4 and 5b.