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Studies on Amino-hexoses. XIII. The Synthesis of Methyl 4′,6′-*O*-Ethylidene-β-pseudocellobiouronoside Methyl Ester, and Its Degradation with Hydrazine

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A derivative of pseudocellobiouronic acid, methyl 4′,6′-*O*-ethylidene-β-pseudocellobiouronoside methyl ester was prepared and subjected to the treatment with hydrated or anhydrous hydrazine. Both of hydrated and anhydrous hydrazine have been found to cause the glycosidic cleavage, the extent of which has been estimated by measuring the amount of 4′,6′-*O*-ethylidene-D-glucose formed by a gas-liquid partition chromatography. The results obtained suggest that the polysaccharides which contain 1 → 4 glycosidic bonds to the uronic acids are degraded by the hydrazine treatment, conceivably due to β-elimination reaction.

The hydrazine treatment has been found efficient for removing the *N*-acetyl group of *N*-acetylamino-sugars such as methyl *N*-acetyl-α- and β-D-glucosaminide and methyl *N*-acetyl-4,6-*O*-ethylidene-α-D-glucosaminide with the exceptions of methyl *N*-acetyl-3-*O*-methyl-α- and β-D-glucosaminide.[1,2] It has been also noted that no or little, if any, glycosidic cleavage occurs in these instances.[1,2] These observations seem to show that de-*N*-acetylation with hydrazine is a promissing method for the structural studies of aminopolysaccharides, if a method such as nitrous deamination, periodate oxidation or acid hydrolysis is going to be applied to the de-*N*-acetylated amino glycans. However, a considerable degradation of the molecules which may give the confusing results has been noted when mucopolysaccharides were subjected to the hydrazinolysis.[3-5] Yosizawa and Sato observed that the blood group A specific substance, which is thought to contain no uronic acid, was degraded extensively[5] by the hydrazine treatments, while little glycosidic cleavage occurred in soluble starch and glycogen.[6] They also found that the decomposition of uronic acid component was scarce whereas some hexosamines were degraded in the hydrazinolysis of sodium chondroitin sulfate A and of sodium hyaluronate.[7] On these findings was based their degradation mechanism by which some of the hexosaminidic linkages were cleaved deaminatively to give 2,5-anhydrohexoses and/or pentoses.[7]

As it was noted that hydrazine hydrate caused little glycosidic cleavage of methyl glucosaminide,[1,2] some other interpretations for the degradation may be possible. Glycosidic linkages of uronic substances have been shown to be cleaved with aqueous alkali[8] and with neutral buffer,[9] being accompanied by the formation of 4,5-unsaturated uronic acid. This mechanism was thoroughly demonstrated in the reactions of methyl galacturonoside methyl ester and methyl digalacturonoside methyl ester with sodium methoxide in dry methanol.[10] Both anhydrous and hydrated hydrazine may possibly have similar influences on the uronic acid parts of polysaccharides. The present authors have synthesized the title compound as a model of uronoglycan[11,12] and examined the effect on it of hydrazine. Methyl β-cellobioside (I) was treated with paraldehyde in the presence of concentrated sulfuric acid to form methyl 4′,6′-*O*-ethylidene-β-cellobioside (II)[13] which was converted into methyl 4′,6′-*O*-ethylidene-β-pseudocellobiouronoside methyl ester (III) by the catalytic oxidation followed by esterification with diazomethane.

The hydrazine treatment of III caused degradation and the paper chromatogram of the reaction mixture showed clearly the existence of 4,6-*O*-ethylidene-D-glucose (IV),[14] together with several un-

1) M. Fujinaga and Y. Matsushima, This Bulletin, **37**, 468 (1964).
2) M. Fujinaga and Y. Matsushima, *ibid.*, **39**, 185 (1966).
3) Y. Matsushima and N. Fujii, *ibid.*, **30**, 48 (1957).
4) M. L. Wolfrom and B. O. Juliano, *J. Am. Chem. Soc.*, **82**, 2588 (1960).
5) Z. Yosizawa and T. Sato, *J. Biochem.*, **51**, 233 (1962).
6) Z. Yosizawa and T. Sato, *Tohoku J. Exp. Med.*, **76**, 100 (1962).
7) Z. Yosizawa and T. Sato, *J. Biochem.*, **51**, 155 (1962).
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13) A. Klemer, *Chem. Ber.*, **89**, 2583 (1956).
14) B. Herferich and H. Appel, *Ber.*, **64**, 1841 (1931).

identified substances. For the quantitative determination of IV in the reaction mixture, gas-liquid partition chromatography was used. As shown by Yosizawa and Sato,[15] extensive degradation will occur when a sugar with a free reducing group is heated with hydrazine. Accordingly, IV must have been degraded further, giving unidentified substances. In order to know the extent of the degradation with hydrazine of III, the values obtained in the gas-liquid chromatography were corrected for the further degradation of IV. For this purpose, the synthetic IV was treated with hydrazine in the identical conditions.

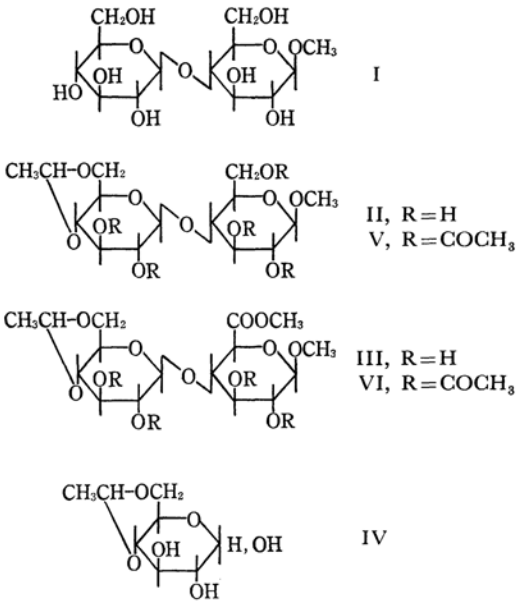

Fig. 1.

Experimental

Methyl 4′,6′-*O*-Ethylidene-β-cellobioside (II). In a three necked flask equipped with a condenser with a calcium chloride tube were placed methyl β-cellobioside (I, 10 g) and paraldehyde (200 m*l*). A few drops of concentrated sulfuric acid were stirred into this suspension and the mixture was heated to 50°C, being kept at this temperature for an hour. The precipitates obtained after the addition of chloroform (200 m*l*) were collected by filteration, washed twice with 50 m*l* of chloroform and then dissolved in cold water (100 m*l*). The aqueous solution thus obtained was treated with Dowex 1 X 4 (OH^- form) and with charcoal, filtered and evaporated *in vacuo* to a small volume (about 30 m*l*). Ethanol (30 m*l*) was added and the mixture was kept in a refrigerator. A thin layer chromatography on silica gel using a solvent mixture of *n*-butanol : methanol : water=3 : 1 : 1 showed that this preparation was homogeneous. Yield, 8.5 g (79%). A further recrystallization from water gave the pure substance which decomposed at 243—245°C and had $[\alpha]_D^{19}$ −40.6° (*c* 2.044, water).

Found: C, 47.11; H, 6.71%. Calcd for $C_{15}H_{26}O_{11}$: C, 47.11; H, 6.85%.

Klemer[13] reported for this compound, mp 279—281°C and no optical rotation value.

Methyl 4′,6′-*O*-Ethylidene-2,3,6; 2′,3′-penta-*O*-acetyl-β-cellobioside (V). Methyl 4′,6′-*O*-ethylidene-β-cellobioside (II, 1 g) was acetylated with acetic anhydride (10 m*l*) and anhydrous pyridine (20 m*l*) under mechanical shaking at room temperature for 2 days. The reaction mixture was then poured into ice-water and the precipitates formed were filtered, washed with water and recrystallized from ethanol. Yield, 1.3 g (84%). Mp 170—172°C; $[\alpha]_D^{20}$ −43.2° (*c* 2.716, chloroform). Found: C, 50.51; H, 5.97%. Calcd for $C_{25}H_{36}O_{16}$: C, 50.67; H, 6.12%.

Klemer[13] reported for this compound, mp 170—172°C and $[\alpha]_D$ −45° (*c* 1.0, chloroform).

Methyl 4′,6′-*O*-Ethylidene-β-pseudocellobiouronoside Methyl Ester (III). In a three necked flask, freshly reduced Adam's catalyst (1 g) was suspended in a solution of II (3.8 g) in 100 m*l* of water. While oxygen was introduced with vigorous stirring at 80°C, the pH of the solution was maintained at 7—8 by adding sodium bicarbonate (total, 0.9 g). The oxidation reaction was traced by a thin layer chromatography on silica gel using a moving phase of *n*-butanol: methanol: water =3: 1: 1. After a spot corresponding to II was disappeared (about 20 hr) the catalyst was filtered off and the almost colorless solution was freeze-dried. The residue was extracted with hot methanol, and after cooling, the methanol solution was passed through a cooled Dowex 50 X 4 column (H^+ form, 2×10 cm). When ethereal diazomethane was added to the methanol effluent, gas-evolution was observed together with the decoloration of the reagent. Evaporation of the solvent gave the residue which was taken up in ethyl acetate. Some insoluble materials were filtered off and the filtrate was again evaporated *in vacuo*. Recrystallization of the residue thus obtained from ethyl acetate-ether gave the substance which sintered at 100—102°C and had $[\alpha]_D^{17}$ −46.2° (*c* 1.882, water). A thin layer chromatogram with a solvent system of *n*-butanol: acetic acid: water =3: 1: 1 showed the homogeniety of the product.

Found: C, 46.64; H, 6.40%. Calcd for $C_{16}H_{26}O_{12}$: C, 46.83; H, 6.39%.

The infrared spectrum of III shows an absorption band at 1750 cm^{-1} characteristic to an ester carbonyl group.

Methyl 4′,6′-*O*-Ethylidene-2,3; 2′,3′-tetra-*O*-acetyl-β-pseudocellobiouronoside Methyl Ester (VI). Methyl 4′,6′-*O*-ethylidene-β-pseudocellobiouronoside methyl ester (III, 0.1 g) was acetylated as described in the preparation of V. Recrystallization from 50 vol% aqueous ethanol gave the fine needles which melted at 174—175°C and had $[\alpha]_D^{15}$ −63.2° (*c* 1.314, chloroform).

Found: C, 49.71; H, 5.98%. Calcd for $C_{24}H_{34}O_{16}$: C, 49.83; H, 5.92%.

The Hydrazine Treatments of Methyl 4′,6′-*O*-Ethylidene-β-pseudocellobiouronoside Methyl Ester (III) and of 4,6-*O*-Ethylidene-D-glucose (IV). Hydrazine (0.5 m*l*) and III (about 50 mg) were kept in a sealed tube at 100°C for 20 hr. Excess hydrazine was removed by an exhaustive evaporation *in vacuo*. On a paper chromatogram using a solvent system of ethyl

15) Z. Yosizawa and T. Sato, *Tohoku J. Exp. Med.*, **77**, 213 (1962).

acetate: acetic acid: water=4: 2: 1, 4,6-*O*-ethylidene-D-glucose (IV) was detected with an ammononiacal silver nitrate reagent. The gas-liquid chromatography also showed the existence of IV in the reaction mixture.

The gas chromatographic analyses were carried out as follow: the reaction products of the hydrazine treatments were dissolved in an appropriate volume of water and an aliquot of the solution was passed through a column of Dowex 50 X 4 (H^+ form) cooled with ice-water. Evaporation of a water effluent gave a syrup which was dissolved in dry pyridine containing a known amount of methyl β-D-glucoside as an internal standard substance and was trimethylsilylated with hexamethyldisilasan and trimethylsilylchloride. Thus an amount of IV released from III by the hydrazine treatment was quantitatively estimated on a 7.5 ft column of 15% polyethylene glycol succinate polyester on chromosorb W in which the following conditions were employed; flow rates of nitrogen, hydrogen and air were 75 ml/min, 60 ml/min and 0.9 l/min, respectively; oven temperature, 170°C and detector bath temperature, 180°C.

The retention times of 4,6-*O*-ethylidene-α-D-glucose, of its β-anomer and of methyl β-D-glucopyranoside were about 12 min, 17 min and 20 min, respectively.

On the other hand, IV (50 mg) was heated with hydrazine (0.5 ml) at 100°C for 20 hr and the reaction mixture was analyzed as described above and the amount of IV survived through the hydrazine treatment was determined. These results are shown in Table 1.

Table 1. The hydrazine treatments of methyl 4′,6′-*O*-ethylidene-β-pseudocellobiouronoside methyl ester (III) and of 4,6-*O*-ethylidene-D-glucose (IV)

Reagent	Starting compound	4, 6-*O*-Ethylidene-D-glucose* formed and/or survived
Hydrazine hydrate	III, 8.9 mg	1.76 mg (40%)
Anhydrous hydrazine	III, 28.0 mg	1.70 mg (12%)
Hydrazine hydrate	IV, 4.0 mg	1.80 mg (45%)
Anhydrous hydrazine	IV, 4.0 mg	2.37 mg (68%)

* Determined by the gas-liquid partition chromatography (see Text).

Discussion

The melting point of II reported by Klemer[13] is different from the present datum and no optical rotation value to be compared has been available. However, methyl 4′,6′-*O*-ethylidene-β-cellobioside (II) obtained here must be identical with that prepared by Klemer, since the physical constants of pentacetate (V) of II are in good agreement with his data. The location of an ethylidene group in II has been elucidated conclusively by the above author, so that a primary hydroxyl group to be oxidized catalytically is in a reducing end glucose moiety.

The hydrochloric acid hydrolysate of III gives the three spots corresponding to D-glucose, D-glucuronic acid and D-glucuronolactone on the one dimensional paper chromatograms with solvent systems, ethyl acetate : pyridine : water = 10 : 4 : 3 and *n*-butanol : acetic acid : water = 3 : 3 : 1. The infrared spectrum of III reveals the existence of an ester carbonyl group. These findings and the mode of the synthesis establish the structure of III. Owing to hygroscopic nature of III, no sharp melting point is observed, but the tetracetate (VI) of III is a fine crystalline compound and can well be characterized.

As is shown in Table 1, the amount of the eliminated 4,6-*O*-ethylidene-D-glucose (IV) were found, in the reaction mixtures of the hydrazine treatments, to be 40 and 12% in molar ratio to the starting compound, using hydrated and anyhdrous hydrazine, respectively. These figures infer the extents of glycosidic cleavages which may amount to 89 and 18% respectively when the corrections are made for the further degradation of 4,6-*O*-ethylidene-D-glucose (IV). In polysaccharides these glycosidic cleavages may degrade the molecules into dialysable substances. In fact, the preliminary studies showed that complete degradations were observed when pectin and alginic acid were similarly treated with anhydrous or hydrated hydrazine. On the similar treatment of I or II, which has no uronic acid, no glycosidic bond is cleaved, a fact which agrees with the observations in the case of soluble starch and glycogen.[6] These results lead the present authors to the conclusion that the hydrazine treatment causes the cleavages of the 1→4 glycosidic linkages of glycans having uronic acid as an aglycon group. Wolfrom and Juliano's observation[4] that the yield of the non-dialysable substances was much increased when carboxyl-reduced chondroitin sulfate A was subjected to hydrazinolysis is also compatible with the present results.

The reaction mechanism remains unknown yet, but a β-elimination by hydrazine seems most likely, as suggested by Yosizawa and Sato.[5,7] Several authors[9,10,16,17] have used the periodate-2-thiobarbituric acid test[18,19] for verifying a chemical or enzymic β-elimination reaction in which a prechromogen, 4,5-unsaturated uronic acid is formed. Thiobarbituric acid is known to be condensed with β-formylpyruvic acid and with malondialdehyde to give the red pigments with λ_{max} at 549 mμ and 532 mμ, respectively. Indeed, III treated with aqueous alkali gave the pigment with λ_{max} at 549 mμ in this test, suggesting that 4,5-unsaturated uronic acid was produced. Pectin, sodium alginate and III, treated with hydrazine, gave the pigments with λ_{max} at 532 mμ, similar to that obtained in the reaction of

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17) R. D. Edstrom and H. J. Phaff, *J. Biol. Chem.*, **239**, 2403 (1964).
18) A. Weissbach and J. Hurwitz, *ibid.*, **234**, 705 (1959).
19) G. B. Paerels and J. Schut, *Biochem. J.*, **96**, 787 (1965).

2-deoxy-D-glucose with periodate-thiobarbituric acid. It is not known why the pigments with λ_{max} at 549 mμ were not obtained but those with λ_{max} at 532 mμ. It may be possible that resultant β-formylpyruvic acid was decarboxylated to form malondialdehyde, or that 4,5-unsaturated uronic acid was decarboxylated to give malondialdehyde after the periodate oxidation.

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