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# BIOCHEMISTRY OF THE USTILAGINALES

## IX. THE β-D-CELLOBIOSIDE UNITS OF THE USTILAGIC ACIDS[1]

By R. U. Lemieux, J. A. Thorn,[2] and H. F. Bauer[3]

ABSTRACT

The two D-glucose residues in the two main components of the ustilagic acid mixture are shown to be present as β-D-cellobiopyranoside units.

The glucoustilic acid mixture (6) was completely methylated by standard procedures for methylation and the product was subjected to methanolysis followed by hydrolysis to yield a mixture of methyl derivatives of D-glucose and the ustilic acids (7). The presence in the hydrolyzate of only tri-*O*- and tetra-*O*-methyl derivatives of D-glucose was established by paper partition chromatography. Semimicro amounts of the two fractions were obtained by preparative paper partition chromatography (13). Since the fractions crystallized readily and completely, there could be no doubt that each fraction contained only one component. The compounds were characterized as 2,3,6-tri-*O*-methyl-D-glucose and 2,3,4,6-tetra-*O*-methyl-D-glucose by the preparation of suitable derivatives. Quantitative estimations showed the glucose derivatives to be present, within experimental error, in equimolar amounts. The mixture of partially methylated ustilic acids isolated from the hydrolyzate possessed the composition expected for a mixture of mono-*O*-methylustilic acid A and di-*O*-methylustilic acid B. Therefore, both the ustilagic acids A and B possessed disaccharide units which were derived either from D-cellobiose or D-maltose.

The A and B components of the glucoustilic acid mixture must be present in the same proportion as are the ustilic acids A and B in the ustilic acid mixture. The ustilic acid mixture has been shown (7) to contain about 70% ustilic acid A and about 30% ustilic acid B. The molar rotations for the mixtures listed in Table I were calculated on this basis. Thus, the possible presence in these mixtures of a small amount of impurities (8) was disregarded.

Acetylation of methyl glucoustilate mixture yielded a crystalline mixture of the acetyl derivatives with molar rotation −25,100 in chloroform. Several recrystallizations of this substance gave pure methyl octa-*O*-acetylglucoustilate A with molar rotation −25,200 in chloroform. Since the lactol carbon atoms of glucose derivatives make a strong contribution to molar rotation (about ±25,000) (4), these rotations show conclusively that the corresponding anomeric centers in the glucoustilic acids A and B are of the same configuration.

The molar rotations of acetylated glycosides of same configuration derived from a sugar and optically inactive alcohols are well-known to be remarkably constant in value. On this basis, a comparison of the molar rotation of the

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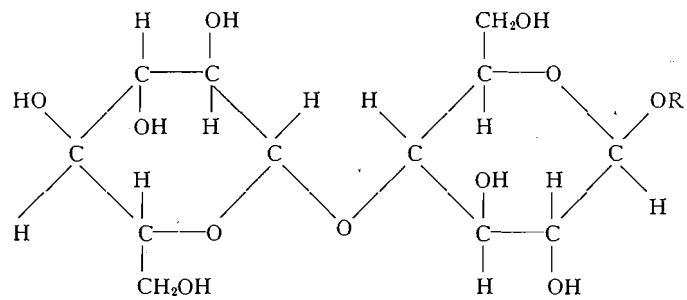
TABLE I
THE MOLAR ROTATIONS OF GLUCOUSTILIC ACID DERIVATIVES AND RELATED SUBSTANCES

| Substance | $[M]_D^{23-26}$ (chloroform) | References |
|---|---|---|
| Di-*O*-acetyl methyl ustilate A | +200° | |
| Tri-*O*-acetyl methyl ustilate B | +4600 | |
| Acetylated methyl ustilate mixture | +2000 | |
| Methyl octa-*O*-acetyl glucoustilate A | −25,200 | |
| Acetylated methyl glucoustilate mixture | −25,100 | |
| Octa-*O*-acetyl-β-D-cellobiose | −9900 | 4 |
| Octa-*O*-acetyl-α-D-cellobiose | +27,800 | 4 |
| Octa-*O*-acetyl-β-D-maltose | +42,500 | 4 |
| Octa-*O*-acetyl-α-D-maltose | +83,000 | 4 |

acetylated methyl glucoustilate mixture with the molar rotations of the α- and β-octa-*O*-acetyl derivatives of D-cellobiose and D-maltose (see Table I) would lead to the conclusion that the contribution to the rotation made by the optically active methyl ustilate portions would be −50,000 or greater (numerically) should the acetylated methyl glucoustilates possess α-D-cellobiose, β-D-maltose, or α-D-maltose residues. Since such a strong levorotation is exceedingly unlikely for residues derived from the weakly dextrorotatory acetylated methyl ustilates (see Table I), the optical rotatory data strongly suggested that the glucoustilic acids are β-D-cellobiosides. Chemical evidence was obtained which substantiated these conclusions. Acetolysis of the methyl glucoustilate mixture yielded octa-*O*-acetyl-α-D-cellobiose (1, p. 124). Therefore, the glucoustilic acids are definitely cellobiosides. It is well established (14) that hepta-*O*-acetyl-β-D-cellobiopyranosides usually can be isomerized to the α-anomers. Treatment of the levorotatory methyl octa-*O*-acetylglucoustilate A, $[M]_D$ −25,200, with boron trifluoride in chloroform solution (9) gave a dextrorotatory isomer, $[M]_D$ +24,200. These molar rotations are in excellent agreement with the values expected for anomeric cellobiopyranosides on the basis of Hudson's rules of isorotation (4). Therefore, the reaction must have effected a *beta* to *alpha* isomerization. Since the glucosidic union which joins the glucose residues in acetylated cellobiosides is known to be highly resistant to anomerization (10), the reaction must have involved the cellobiosyl to ustilic acid bond. Therefore, the glucoustilic acids must be β-D-cellobiopyranosides and their structures can be represented by the formulas I(*a*) and I(*b*).



I(*a*). Glucoustilic acid A, R = $C_{16}H_{31}O_3$.
I(*b*). Glucoustilic acid B, R = $C_{16}H_{31}O_4$.

Although cellobiose can be considered to be a repeating unit of such substances as cellulose and lichenin, it appears that the ustilagic acids comprise the first *true* cellobiosides of biological origin to be characterized.

## EXPERIMENTAL

### *Methylation of Glucoustilic Acid Mixture*

Ustilagic acid (6), 25 gm., was methylated with dimethyl sulphate, 90 ml., and 30% sodium hydroxide, 210 ml., in the usual way. The acidified reaction mixture was extracted with ether to yield 22.4 gm. of a partially methylated derivative. After two further methylations using methyl iodide, 100 ml., and silver oxide, 75 gm., the yield was 19.4 gm. of partially crystalline material which contained 36.5% methoxyl groups. The methoxyl group content calculated for a mixture of 70% nona-*O*-methylglucoustilate A and 30% deca-*O*-methylglucoustilate B is 38.5%.

### *Quantitative Estimation of Tri-O- and Tetra-O-methyl-D-glucoses*

The fully methylated glucoustilic acid, 100 mgm., was heated for six hours in a sealed tube at 100°C. with 2 ml. of 5% methanolic hydrogen chloride. The solution was evaporated to dryness under reduced pressure and the residue was heated at 100°C. with 2 ml. of 10% hydrochloric acid for three hours. The hydrochloric acid was destroyed by the addition of an excess of an ethereal solution of diazomethane. The ether was removed by evaporation and the aqueous residue was filtered. The filter was washed with water to yield 25 ml. of filtrate.

Inspection of the solution by paper partition chromatography with butanol–ethanol–water (3) as developing phase showed only two reducing components detectable by the aniline phthalate reagent. The $R_f$ values indicated the presence of a tri- and a tetra-*O*-methyl-D-glucose.

A portion of the solution, 150 cmm., was developed on a sheet of Whatman 1 paper in the usual way as a 20 cm. streak across the paper. After the resultant bands were located by use of spots developed simultaneously, the zones were cut out and the methylglucoses eluted with water (2). The amounts of tri- and tetra-*O*-methyl-D-glucoses were determined by use of the anthrone reaction (2, 12). The blank was made up from the eluate of a strip of filter paper of equal area taken from the same chromatogram but which contained no reducing sugar. The transmission readings were compared to standard curves prepared from pure samples of 2,3,6-tri-*O*-methyl and 2,3,4,6-tetra-*O*-methyl-D-glucose. The ratio of tri- to tetra-*O*-methylglucose was 0.91. The yields were about 70% of theory.

### *Hydrolysis of Fully Methylated Glucoustilic Acid Mixture*

The methylated glucoustilic acid mixture, 5 gm., was heated under reflux with 50 ml. of 5% methanolic hydrogen chloride for 17 hr. The solution was added to 100 ml. of water and extracted several times with chloroform. After it was dried with sodium sulphate, the solution was evaporated to an oil. A solution of the material in 25 ml. of ethanol and 10 ml. of *N* potassium hydroxide was refluxed for half an hour. After neutralization with hydrochloric acid,

the solution was evaporated to near dryness. The residue was stirred with sodium sulphate to yield a solid which was extracted with chloroform. Evaporation of the chloroform gave 1.7 gm. of the methylated glucoses. The residue from the extraction was dissolved in hot water, the solution was made strongly acid with hydrochloric acid and extracted with chloroform. Evaporation gave 1.75 gm. of a crystalline mixture of methylated ustilic acids, m.p. 41–55°C. After several recrystallizations from acetone the material melted at 56–70°C. Calc. for a mixture containing 60% mono-*O*-methylustilic acid A and 40% di-*O*-methylustilic acid B: neutral equiv., 314; $OCH_3$, 13.8%. Found: neutral equiv., 313; $OCH_3$, 13.8%.

The methylated glucose fraction, 400 mgm., was hydrolyzed by heating at 100°C. with 8 ml. of 10% hydrochloric acid for three hours. The solution was neutralized with diazomethane and evaporated to about a 2 ml. volume. The two methylated glucose components were separated by paper chromatography on a preparative scale (13) and characterized as follows.

*2,3,4,6-Tetra-O-methyl-α-D-glucose*

The tetra-*O*-methyl-D-glucose fraction crystallized at once on seeding with an authentic sample of 2,3,4,6-tetra-*O*-methyl-D-glucose. After two recrystallizations from petroleum ether, the material, $[\alpha]_D$ $+102 \longrightarrow +84°$ (*c*, 1 in water), melted at 95.5°C. (with some melting from 81°C.). These constants are in agreement with the published values (5) for 2,3,4,6-tetra-*O*-methyl-α D-glucose. The anilide derivative (15) melted at 137–139°C. and did not depress the melting point of an authentic sample of the same melting point.

*2,3,6-Tri-O-methyl-D-glucose*

The tri-*O*-methyl-D-glucose fraction crystallized on standing several days. A 10 mgm. sample was heated for three minutes on the steam bath with 0.5 ml. of pyridine and 20 mgm. of *p*-nitrobenzoyl chloride. Water was added, the sirup was triturated with sodium bicarbonate solution, washed with water, and dried *in vacuo*. A chloroform extract was treated with charcoal, filtered, and evaporated to yield a crystalline residue which was recrystallized from 0.8 ml. of methanol. The substance possessed the specific rotation and did not depress the melting point of 1,4-di-*O*-*p*-nitrobenzoyl-2,3,6-tri-*O*-methyl-D-glucose, m.p. 190°C., $[\alpha]_D$ $-32$ (*c*, 0.2 in chloroform).

*Acetylated Methyl Glucoustilate Mixture*

A solution of glucoustilic acid in methanol was treated with an excess of diazomethane. Addition of petroleum ether precipitated the methyl glucoustilate mixture in quantitative yield. The material was heated at 100°C. for three hours with sodium acetate and acetic anhydride. The acetyl derivative crystallized on shaking the acetylation mixture with cold water. The yield of crude material, m.p. 94–95.5°C., $[\alpha]_D$ $-25.6°$ (*c*, 4.3 in chloroform), was quantitative. Calc. for a mixture containing 70% methyl octa-*O*-acetylglucoustilate A and 30% methyl nona-*O*-acetylglucoustilate B: methoxyl, 3.17; acetyl, 36.5%. Found: methoxyl, 3.21; acetyl, 35.2%.

*Methyl Octa-O-acetylglucoustilate A*

The mixture of acetylated methyl glucoustilates was recrystallized once from benzene – petroleum ether, three times from isopropanol, and finally three times from isopropanol – petroleum ether. The yield was 7 gm. of a substance which had reached constant physical properties, m.p. 108–109.5°C., $[\alpha]_D$ −27° (*c*, 2.3 in chloroform). Calc. for $C_{45}H_{70}O_{22}$: C, 56.12; H, 7.32%. Found: C, 56.10; H, 7.35%. Methanolysis of a sample and alkaline hydrolysis of the methyl ustilate fraction thus formed (7) to yield ustilic acid gave a substance, m.p. 110–111°C., after one crystallization from ethanol. This melting point was unchanged by admixture with ustilic acid A of the same melting point. When the melting of ustilic acid A is observed with a heating stage microscope, a transformation in crystal form is observed at 102–103°C.

*Methyl Di-O-acetylustilate A*

Pure methyl ustilate A (7) was acetylated with acetic anhydride and sodium acetate in the usual manner. The crystalline product was recrystallized from ethanol. The substance melted at 26–27°C. and $[\alpha]_D$ +0.6 (*c*, 4.3 in chloroform). Calc. for $C_{17}H_{32}O_4$ $(COCH_3)_2$: acetyl, 22.3%. Found: acetyl, 23.2%.

*Methyl Tri-O-acetylustilate B*

Acetylation of pure methyl ustilate B (7) as described above gave a sirup which resisted crystallization. The material, $[\alpha]_D$ +10.5° (*c*, 4 in chloroform), for analysis was distilled at a bath temperature of 170–180°C. at $10^{-3}$ mm. Calc. for $C_{17}H_{31}O_5(COCH_3)_3$: acetyl, 29.0%. Found: acetyl, 29.3%.

*Acetylated Methyl Ustilate Mixture*

Acetylation of purified methyl ustilate mixture (7) as described above gave a sirupy product, $[\alpha]_D$ +4.9° (*c*, 1.9 in chloroform). Calc. for a mixture containing 70% methyl di-*O*-acetylustilate A and 30% methyl tri-*O*-acetylustilate B: acetyl, 24.3%. Found: acetyl, 23.8%.

*Octa-O-acetyl-α-D-cellobiose*

The methyl glucoustilate mixture, 0.50 gm., was cooled with 1.4 ml. acetic anhydride in an ice–salt mixture. Concentrated sulphuric acid, 0.28 ml., was added to 0.60 ml. acetic anhydride cooled in the ice–salt mixture. The mixtures were combined (1, p. 124) and shaken at room temperature until solution was complete. The next day crystallization was initiated by scratching and after 2.5 days reaction time the crystals were collected, washed with ether, and recrystallized from ethanol. The yield was 20 mgm., m.p. 220–222°C. Recrystallization from ethanol raised the melting point to 226.5–228°C. and $[\alpha]_D$ +42° (*c*, 0.7 in chloroform). The substance did not depress the melting point of an authentic sample of octa-*O*-acetyl-α-D-cellobiose prepared by acetolysis of cellulose (1).

*Anomerization of Methyl Octa-O-acetylustilate A*

Methyl octa-*O*-acetylglucoustilate A, $[\alpha]_D$ −27° (chloroform), 2.90 gm., was dissolved in 50 ml. of pure chloroform and the solution was saturated with boron trifluoride. After 48 hr. at room temperature, the mixture was shaken

with aqueous bicarbonate solution. The chloroform was washed with water, dried, and evaporated to a solid which was recrystallized from ethanol–petroleum ether mixture. The substance, 1.30 gm., $[\alpha]_D$ +8° (chloroform) was shown to be heterogeneous by chromatography on a 5:1 Magnesol-Celite column (11). The material, 1.00 gm., was treated again under the above conditions for anomerization except that the reaction time was 24 hr. The product was recrystallized from ethanol–petroleum ether mixture to yield 0.70 gm. of material, $[\alpha]_D$ +22.6° (chloroform). The substance, 100 mgm., in 2 ml. of benzene was added to the top of 140 × 35 mm. (in diam.) column of 5 parts Magnesol to 1 part Celite (11), and the chromatogram was developed with 1500 ml. of 100: 1 benzene to ethanol mixture. A band was located on the extruded column by streaking with 1% potassium permanganate in 2.5 *N* sodium hydroxide which extended from 3 mm. to 25 mm. from the top of the column. Elution with warm acetone and crystallization from ethanol gave 50 mgm. of material, m.p. 103–105°C., $[\alpha]_D$ +25.2° (*c*, 1.5 in chloroform). Calc. for $C_{29}H_{46}O_{14}(COCH_3)_8$: C, 56.12; H, 7.32; acetyl, 35.8%. Found: C, 56.22; H, 7.36; acetyl, 36.1%.

## ACKNOWLEDGMENTS

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