701. *Studies of* Aspergillus niger. *Part VII.** *The Enzymic Synthesis of* 3-O-β-D-*Glucopyranosyl*-D-*xylose.*

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A disaccharide produced by the action of a cell-free extract of *A. niger* (152) on a cellobiose–xylose mixture has been characterised as 3-*O*-β-D-glucopyranosyl-D-xylose.

We have reported [1] that when a cell-free extract of *A. niger* (152) is incubated with a cellobiose–D-xylose mixture two unknown components (X, Y), with the mobilities of disaccharides, are produced in addition to those formed on incubation of the cell-free extract and cellobiose alone. We now report the isolation and characterisation of one of these components, disaccharide X.

After fractionation on a charcoal column [2] and thereafter on a Grycksbo filter-paper column, chromatographically pure X was isolated from the oligosaccharide mixture. The other unidentified component Y was produced only in minute amount. When separated by ionophoresis as its *N*-benzylglycosylammonium ion, X had the mobility expected [3] of a glucosylxylose. On the assumption that oxidation with hypoiodite was quantitative,[4] the

* Part VI, *J.*, 1957, 2448.

[1] Barker, Bourne, Hewitt, and Stacey, *J.*, 1955, 3734.
[2] Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677.
[3] Barker, Bourne, Grant, and Stacey, *Nature*, 1956, **177**, 1125.
[4] Hirst, Hough, and Jones, *J.*, 1949, 928.

molecular weight of disaccharide X was 281 (a glucosylxylose requires 312). The elementary analysis of X was in good agreement with a molecular formula $C_{11}H_{20}O_{10},H_2O$—the presence of water was confirmed by infrared analysis.

Acid hydrolysis of disaccharide X gave only glucose and xylose. The sequence of sugar units was determined by reduction of X to its alcohol and subsequent acid hydrolysis. Only glucose and xylitol could be detected in the hydrolysate, indicating that X was a glucosylxylose. The low optical rotation ($[\alpha]_D$ −0·6°) and the infrared spectrum [5] of X both indicated that the glycosidic linkage was β. The fact that both X and its osazone were attacked by emulsin was further confirmation of this structural feature.

Since X formed a disaccharide osazone (rather than being broken down into a mixture of monosaccharide osazones) the glycosidic linkage was not 1 : 2. Disaccharide X reacted rapidly with methanolic hydrogen chloride at room temperature. This indicated methyl furanoside formation and hence that the 4-hydroxyl group of the reducing sugar unit was free. The methyl furanoside of disaccharide X was isolated and methylated with sodium hydroxide–methyl sulphate and thereafter with the Purdie reagents. The methoxyl content (53·2%) was in good agreement with the value of 52·9% expected for a hepta-*O*-methyl derivative of a glucosylxylose. Hydrolysis of the methyl ether gave 2 : 3 : 4 : 6-tetra-*O*-methylglucose and a di-*O*-methylxylose. The latter (which could be differentiated chromatographically from both 3 : 4- and 2 : 4-di-*O*-methylxylose) was reduced to the corresponding xylitol derivative. When oxidised with sodium periodate the di-*O*-methylxylitol consumed almost 1 mol. of periodate and produced no formaldehyde or formic acid. The only di-*O*-methylxylitol (other than 1 : 4) which would give these values on oxidation is the 2 : 5-di-*O*-methyl derivative. This would only have been produced if the original disaccharide X was 3-*O*-β-D-glucopyranosyl-D-xylose, a structure which is consistent with the observation that X, when subjected to filter-paper ionophoresis in borate buffer, pH 10, had the same mobility as laminaribiose and gentiobiose, and moved much faster than cellobiose and sophorose.

The alcohol of disaccharide X appears to be overoxidised with periodate. Although the alcohol would be expected to consume 4 mols. of periodate and liberate 1 mol. of formic acid and 2 mols. of formaldehyde, a rapid uptake of 3 mols. of periodate followed by a slow uptake of another 4 mols. of periodate, making 7 mols. in all, was observed. After this time, 3·6 mols. of formic acid and 3·5 mols. of formaldehyde were liberated. This over-oxidation may be due to the formation of a substituted malondialdehyde structure, which would be expected with a 1 : 3-linked glucosylxylitol, but the phenomenon is very sensitive to reaction conditions and no attempt has been made to employ these results in the determination of the structure of disaccharide X.

Experimental

Enzymic Synthesis of Disaccharide X.—Cellobiose (15 g.) and D-xylose (15 g.) were dissolved in a cell-free extract [1] (50 c.c.) of *Aspergillus niger* (152) (*ca.* 9 g.) and incubated at 30° for 48 hr. The pH of the solution was then adjusted to 7 with sodium hydroxide and enzyme action arrested by boiling for 20 min. Paper-chromatography in the organic phase of butanol–ethanol–water–ammonia (40 : 10 : 49 : 1) revealed the presence of components having R_F values identical with those of xylose, glucose, laminaribiose, and cellobiose together with two unknown disaccharides X and Y (trace). After fractionation on a charcoal column [2] [gradient elution with aqueous ethanol (0 ⟶ 20%)], the monosaccharide fraction and a fraction containing cellobiose and a trace of gentiobiose were discarded. Attempts to fractionate the disaccharide mixture of X, Y, cellobiose, and laminaribiose on a cellulose and on a charcoal column in the presence of borate [6] were unsuccessful. Fractionation was finally achieved on a 3391 Grycksbo chromatographic filter-paper column with the organic phase of a butanol (40), ethanol (10), and water (50) mixture. Those fractions containing disaccharide X only were combined, concentrated, and dried to a white powder (1·40 g.), $[\alpha]_D^{19}$ −0·6° (*c* 0·35 in H_2O).

[5] Barker, Bourne, Stacey, and Whiffen, *J.*, 1954, 171.
[6] Barker, Bourne, and Theander, *J.*, 1955, 4276.

Characterisation of Disaccharide X.—(i) The *compound* (Found: C, 40·0; H, 6·9. $C_{11}H_{20}O_{10}$ requires C, 42·2; H, 6·45. $C_{11}H_{20}O_{10},H_2O$ requires C, 40·0; H, 6·7%), had infrared absorption at 1640 ± 5 cm.$^{-1}$ due to water. In the range 700—1100 cm.$^{-1}$ absorption peaks were at 1021, 936, 895, 758, and 719 cm.$^{-1}$.

(ii) *Paper chromatography and ionophoresis.* Paper chromatography of the free sugar and of its benzylamine derivative [7] in the first solvent mixture described above showed that disaccharide X was chromatographically pure and moved with an R_F between those of laminaribiose and glucose. When submitted to paper ionophoresis [8] in borate buffer, pH 10, the mobility of disaccharide X was approx. the same as those of laminaribiose and gentiobiose. When separated as its *N*-benzylglycosylammonium ion in an electrolyte of sodium hydroxide–formic acid (pH 1·8),[3] the mobility (*M*) was 0·80.

(iii) *Oxidation with hypoiodite.* A series of solutions containing glucose, xylose, and disaccharide X was oxidised with iodine in alkaline solution (Hirst, Hough, and Jones [4]). Glucose and xylose were oxidised stoicheiometrically. The molecular weight of disaccharide X, calculated on the assumption that the oxidation was stoicheiometric, was 281.

(iv) *Formation of osazone.* Disaccharide X (25 mg. in 0·4 c.c.), with phenylhydrazine (50 mg.) and 50% acetic acid (0·05 c.c.) at 100° for 2 hr., gave yellow crystals after cooling which after recrystallisation from ethanol had m. p. 213—215°. On circular paper chromatography in toluene–ethanol–water (270 : 30 : 1), the osazone had an R_F approx. the same as that of cellobiosazone and very different from those of glucosazone and xylosazone.

The osazone (5 mg.), dissolved in dioxan (0·2 c.c.), was treated with almond emulsion (5 mg.). Paper chromatography showed that the only free sugar liberated was glucose. Electrophoresis [3] in sodium formate–formic acid (pH 1·8) showed that while the mobility of the disaccharide X osazone was approx. the same as that of cellobiosazone, the product of almond emulsin action had the mobility of a monosaccharide osazone.

(v) *Hydrolysis.* Disaccharide X (2 mg.) was heated with 1·5N-sulphuric acid at 100° for 2 hr. The solution was neutralised with barium carbonate and filtered. Disaccharide X (10 mg.) was incubated with almond emulsin (10 mg. in 0·2 c.c. of water) at 30° for 2 days. Paper chromatography of both the acidic and the enzymic hydrolysate showed only the presence of glucose and xylose.

(vi) *Reduction.* Disaccharide X (25 mg.) in water (12 c.c.) was reduced with sodium borohydride (25 mg.) at room temperature for 2 hr. Amberlite IR-120 (H^+) (1 g.) was added and the mixture shaken for 15 min., then filtered and evaporated to dryness *in vacuo*. Dry methanol (3 × 15 c.c.) was added and the whole evaporated to dryness *in vacuo*. The residue was dissolved in water and freeze-dried.

Part of the disaccharide alcohol (2 mg.) was heated with 2N-sulphuric acid at 100° for 2 hr. Paper chromatography of the neutral hydrolysate and selective spraying with aniline hydrogen phthalate and buffered bromocresol-purple revealed the presence of components having R_F values identical with those of glucose and xylitol. The same sprays indicated that the disaccharide alcohol moved as a single component.

The disaccharide alcohol (15·8 mg.) was oxidised with 0·025M-sodium periodate (50 c.c.) at 18°. Some mannitol (11·2 mg.) was similarly treated. Results were:

Mols. of periodate consumed per:	mol. of disaccharide alcohol	2·9	6·0	—	6·5	6·8
	mol. of mannitol	4·8	—	5·0	5·0	5·0
Time (hr.)		0·5	23·25	27	47·5	124

After 124 hr., 3·6 mols. of formic acid and 3·6 mols. of formaldehyde were produced per mol. of disaccharide alcohol.

(vii) *Conversion into its methyl furanoside.* Disaccharide X (900 mg.) was left in 4% methanolic hydrogen chloride (40 c.c.) at room temperature for 1·5 hr. before being neutralised with silver carbonate (8 g.). After filtration, the solution was concentrated *in vacuo* and then freeze-dried to a white powder (692 mg.) (Found: OMe, 10·1. Methyl glucosylxyloside requires OMe, 9·5%).

(viii) *Methylation of the methyl furanoside.* The methyl furanoside (686 mg.) was suspended in dioxan (20 c.c.) and methylated with dimethyl sulphate (35 c.c.) and 30% aqueous sodium hydroxide (70 c.c.), added in ten equal portions at 10 min. intervals. The product (618 mg.) was then methylated three times with the Purdie reagent. The resulting syrup (462 mg.)

[7] Bayly and Bourne, *Nature*, 1953, **171**, 385.
[8] Foster, *J.*, 1953, 982.

(Found: OMe, 52·2. Hepta-*O*-methylglucosylxylose requires OMe, 52·9%) showed neglible infrared absorption in the O–H stretching region (3400—3700 cm.$^{-1}$).

(viii) *Hydrolysis of methylated disaccharide X.* The methyl ether (440 mg.) was heated with 0·5N-sulphuric acid (15 c.c.) at 100° for 2 hr., and the solution neutralised with barium carbonate, filtered, and evaporated to a syrup (305 mg.). Paper chromatography of the syrup showed that it contained only two components, one with an R_F identical with that of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose and the other with an R_F somewhat less than those of 3 : 4- and 2 : 4-di-*O*-methylxylose.

The syrup was fractionated by Bell's method [9] to give 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose (171 mg.) and the new di-*O*-methylxylose {63 mg.; $[\alpha]_D^{19}$ +11·5° (*c* 0·31 in $CHCl_3$)}. The latter was reduced to the alcohol with sodium borohydride (50 mg.) as described for disaccharide X. On periodate oxidation the di-*O*-methylpentitol consumed 0·5 (0·25 hr.), 0·9 (3 hr.), and 0·9 mol. (25 hr.) of periodate. No formic acid or formaldehyde was produced.

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[9] Bell, *J.*, 1944, 473.