CHARACTERISATION OF SUCROSE LACTATE AND OTHER OLIGOSACCHARIDES FOUND IN THE CLADOPHORALES

ELIZABETH PERCIVAL AND MARGARET YOUNG
Department of Chemistry, Royal Holloway College, Englefield Green, Surrey (Great Britain)
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ABSTRACT

Oligosaccharides not previously recorded in the Cladophorales (green algae) have been separated from the 80% ethanolic extract and characterised. 4-*O*-Lactyl-β-D-fructofuranosyl α-D-glucopyranoside (sucrose lactate) was found only in *Cladophora laetevirens* and a fresh-water *Rhizoclonium* species. Panose, maltotriose, maltotetraose, and 6-*O*-D-glucosylmaltotriose appear to be common to all the species examined[1].

INTRODUCTION

In a systematic survey[1] of the carbohydrates of nine different species of green algae belonging to the Cladophorales, an unknown ketose-containing oligosaccharide was observed in *Cladophora laetevirens* and in a fresh-water species of *Rhizoclonium*, but was absent from all the other species examined. It was extracted from the alga, together with glucose, fructose, sucrose, and several reducing oligosaccharides, with 80% ethanol. The present paper describes the separation and characterisation of this ketose and also the four major reducing oligosaccharides.

RESULTS AND DISCUSSION

Ketose-containing oligosaccharide **1**. This oligosaccharide was obtained as a chromatographically homogeneous substance from the 80% ethanolic extract of the alga. It has a paper-chromatographic mobility lower than that of sucrose in most solvents, but in complex mixtures it tends to be obscured by the sucrose streak; this may explain why **1** has not been found previously. Preparative paper chromatography (butyl alcohol–ethanol–water) completely separated **1** from sucrose but not from a reducing disaccharide (tentatively identified as maltose). A second separation (isopropyl alcohol–butanone–*N*,*N*-dimethylformamide) was necessary to obtain chromatographically homogeneous material. Oligosaccharide **1** is neutral and immobile on ionophoresis in a neutral buffer. It gives a positive test for ketose and reduces silver nitrate slowly. On acid hydrolysis, the sole products appeared to be equimolar proportions of glucose and fructose. These results are consistent with a

glucosylfructose. Chromatographic sprays[3] have been developed to distinguish these disaccharides (Table I).

TABLE I

PAPER-CHROMATOGRAPHIC BEHAVIOUR OF GLUCOSYLFRUCTOSES

	$R_{Sucrose}$ (*Solvent B*)	*Colour reaction*	
		Diphenylamine–urea	*Diphenylamine–p-anisidine*
Leucrose (5-*O*-α-D-Glc*p*-D-Fru)	0.64	Cream	Brown
Turanose (3-*O*-α-D-Glc*p*-D-Fru)	1.02	Purplish brown	Reddish brown
Cellobiulose (4-*O*-β-D-Glc*p*-D-Fru)	0.75		Reddish brown
Maltulose (4-*O*-α-D-Glc*p*-D-Fru)	0.78	Brownish-grey	Reddish brown
1-*O*-β-D-Glucosylfructose	0.56	Brownish-grey	Reddish brown
Isomaltulose (6-*O*-α-D-Glc*p*-D-Fru)	0.66	Brownish-grey	Reddish brown
Sucrose	1.0	Slate-grey	Greenish yellow
Oligosaccharide **1**	0.63	State-grey	Greenish yellow

It can be seen (Table I) that oligosaccharide **1** gave the same colour reactions as sucrose, but had a different mobility. Moreover, where the mobilities were similar, as in leucrose and isomaltulose, the colour reaction was different.

G.l.c. of the *O*-trimethylsilyl (TMS) derivative of oligosaccharide **1** gave a single peak having a retention time only slightly lower than that of sucrose. On g.l.c. of a mixture, oligosaccharide **1** could only be detected as a small shoulder or broadening of the sucrose peak.

After methylation and methanolysis of **1**, methyl 2,3,4,6-tetra-*O*-methylglucosides, methyl 1,3,6-tri-*O*-methylfructosides, and traces of methyl 1,3,4,6-tetra-*O*-methylfructosides were detected by g.l.c. It should be noted that methyl 1,3,4-, 1,4,6-, and 1,3,6-tri-*O*-methylfructosides have similar retention times on column *1*, but are readily distinguished on columns *2* and *3* (Table II).

G.l.c. of methanolysates of methylated maltulose and methylated oligosaccharide **1**, gave identical results, although **1** differed from maltulose in its paper-chromatographic mobility and colour reactions.

It appeared from these results that **1** is sucrose substituted at position 4, and possible substitutents were then investigated. After treatment of **1** with alkali, paper chromatography showed the presence of sucrose and a monocarboxylic acid of low molecular weight which could not be distinguished readily from oxalic, glyceric, pyruvic, or lactic acids. A pure sample of this acid was obtained, after removal of phenolic substances by ionophoresis, from an ether extract of an acid hydrolysate of **1**. With 4-hydroxybiphenyl and concentrated sulphuric acid[4], the acid gave a deep-violet colour (λ_{max} 560 nm), as did α-hydroxybutyric, pyruvic, and lactic acids. With 2-hydroxybiphenyl in concentrated sulphuric acid[4], a blue fluorescent solution was obtained (λ_{max} 322 nm), identical with that given by lactic acid and reduced pyruvic acid but not by pyruvic acid itself.

On electrophoresis over a range of pH values[5], the acid had a mobility and colour reaction identical with that of lactic acid, and the retention time of its methyl ester on g.l.c. was the same as that of methyl lactate. Oligosaccharide **1** is therefore sucrose esterified at C-4 with lactic acid. It is present in the two algae to the extent of *ca.* 1% of the dry weight and is clearly a significant metabolite. It is of interest that both species in which sucrose lactate has been found grow in situations where the amount of available oxygen may fluctuate violently. *C. laetevirens* is found in shallow pools which often empty at low tide and also become very warm in sunny conditions. The *Rhizoclonium* sp. grows on surfaces which can vary over a short period between being completely submerged and completely exposed.

The ability to form a neutral lactic ester might enable the plant to reduce pyruvic acid more extensively than would otherwise be possible, at times when oxidation through the cytochrome system is diminished.

Reducing oligosaccharides. The four major reducing oligosaccharides (**2**–**5**) were isolated in the same way as oligosaccharide **1**. Paper chromatography with solvents *A* and *E* was necessary to remove contaminating inositols and sugar alcohols. Paper chromatography then showed (Table III) that the oligosaccharides had the mobilities of maltotriose, maltotetraose, panose, and 6-*O*-D-glucosylmaltotriose, respectively.

On hydrolysis, each oligosaccharide gave only glucose; on partial hydrolysis, maltose could also be detected. In addition, in the partial hydrolysates from oligosaccharides **3** and **5**, trace quantities of maltotriose could be seen. Oligosaccharide **2** had a d.p. of 3. The R_{Maltose} values of maltose and oligosaccharides **2** and **3** indicate that they belong to the (1→4)-linked D-glucose homologous series, since a plot made of $R_{\text{Maltose}} = \log[(1/R_F) - 1]$ values against d.p. gave a straight line[8].

After methylation of oligosaccharide **2** followed by hydrolysis and glucosidation, g.l.c. examination showed the presence of methyl 2,3,4,6-tetra-*O*-methylglucosides, and methyl 2,3,6-tri-*O*-methylglucosides (Table II) in the molar ratio of 1:2, confirming the identity of oligosaccharide **2** as maltotriose.

Oligosaccharides found in the 80% aqueous ethanol are unlikely to be degradation products or artefacts formed during the extraction process. On several occasions, freezing, grinding, and extraction of the weed was carried out within 30 min of collection, with no resulting change in the quantities of oligosaccharides present.

Duncan and Manners[6] investigated the oligosaccharides formed *in vitro* from maltose by a *Cladophora rupestris* trans-α-D-glucosidase preparation. The products, in order of yield, were panose, maltotriose, maltotetraose, and 6-*O*-D-glucosylmaltotriose. It now appears that these same compounds occur naturally in members of the Cladophorales. At a low concentration (0.04%) of maltose, the sugar was completely hydrolysed[6] *in vitro* by the crude trans-α-D-glucosidase. Only when concentrations were greater than 7% did oligosaccharide formation take place. The oligosaccharides now reported occur in cell fluid having a low concentration of sugar, *i.e.* <0.04% of maltose. However, in the living cell, the hydrolytic activity and trans-β-D-glycosylase activity may be completely separated, so that these results are not necessarily comparable with those of the previous authors.

EXPERIMENTAL

Evaporations were done at 40° under reduced pressure. Paper chromatography was carried out on Whatman No. 1 and 3M papers with the following solvent systems (v/v) (*A*) butyl alcohol–ethanol–water (40:11:19), (*B*) butyl alcohol saturated with water, (*C*) butyl alcohol–pyridine–water (6:4:3), (*D*) isopropyl alcohol–butanone–*N*,*N*-dimethylformamide–water[2] (50:25:5:20), (*E*) butyl alcohol–pyridine–water–benzene (5:3:3:1), (*F*) butyl alcohol–acetic acid–water (4:1:5), (*G*) ethyl acetate–pyridine–water (10:4:3). Reducing oligosaccharides and sugars were located by (*a*) aniline oxalate; the ketose-containing oligosaccharides by (*b*) urea hydrochloride[9] and (*c*) the *p*-anisidine reagent[9]; and both reducing and non-reducing carbohydrates with (*d*) silver nitrate–sodium hydroxide[10]. Acids were detected with (*e*) silver nitrate or phenol–sodium hydroxide[11]; (*f*) potassium iodide–starch[12]; and (*g*) ferric chloride–ferricyanide[13]. All sugars and their derivatives were characterised by comparison with authentic materials. R_S-values refer to the distance migrated relative to sucrose. Carbohydrates were assayed by the phenol–sulphuric acid method[14]. Paper electrophoresis was conducted on Whatman No. 3MM paper at 3000 volts and 30 mamp for 50 min, using acetic acid–pyridine buffer at pH 6.7. G.l.c. (Pye–Argon gas chromatograph) of mixtures of *O*-methyl sugars was carried out on columns of (*1*) 15% by weight of poly(butane-1,4-diol succinate), (*2*) 10% polyphenyl ether [*m*-bis(*m*-phenoxyphenoxybenzene)], (3) 10% poly(ethylene glycol adipate) on HMDS-treated Celite (80–100 mesh) at 175°. TMS derivatives of carbohydrates were run on (*4*) 3% SE 30 and (*5*) 7.5% Apiezon K on HMDS-treated Chromosorb W (80–100 mesh) at 175° for monosaccharides and 208° for disaccharides. Acids, as their methyl esters, were analysed on (*6*) 15% di(ethylene glycol succinate) (DEGS) on HDMS-treated Chromosorb W (80–100 mesh) at 112°. Retention times (*T*) of methylated sugars are given relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside, and of the TMS ethers of the disaccharides relative to sucrose.

Isolation of the oligosaccharides. — Freshly collected, damp weed was frozen in liquid nitrogen, ground to a powder, and exhaustively extracted with 80% aqueous ethanol. The combined extracts were concentrated to a dark-green syrup. Ethanol was added with stirring, and the mixture was set aside for 20 h. The precipitated solid was removed, and the filtrate, after deionisation, was concentrated to a syrup. Paper-chromatographic examination (solvent *A*, spray *b*) of this syrup revealed the presence of a number of ketose-containing oligosaccharides[1], including one having R_S 0.78; spray *a* showed, in addition, some reducing oligosaccharides. The oligosaccharide with R_S 0.78 and the four major reducing oligosaccharides were separated on a preparative scale by development for 5–7 days on a number of 3M papers with solvent *A*. Paper-chromatographic examination of the ketose-containing material R_S 0.78 (solvent *D*, spray *d*) showed the presence of two substances having R_S 0.86 and 0.98, respectively. The material with R_S 0.98 (oligosaccharide **1**) was non-reducing and gave a positive test for ketose, and that with R_S 0.86, which was reducing to aniline oxalate, had the same mobility as maltose in solvents *A*, *E*, *F*, and *G* and gave only glucose on

hydrolysis. A second separation on 3M papers (solvent *D*) resulted in the isolation of chromatographically homogeneous oligosaccharide **1**.

Examination of oligosaccharide **1**. — The compound was a white, powdery solid (5 mg) with R_S 0.80 (solvent *C*) and 0.63 (solvent *B*), and had zero mobility on ionophoresis at pH 6.7. Hydrolysis of an aliquot with 2% oxalic acid for 1 h at 80° was followed by neutralisation with calcium carbonate and paper-chromatographic examination. A portion of the hydrolysate was taken to dryness, the residue was converted into the TMS derivatives, and these were subjected to g.l.c. in the usual manner.

Oligosaccharide **1** was converted into the TMS derivative by treatment with the silylating reagents[1] for 1 h at 20°. The product was analysed by g.l.c.

Methylation of oligosaccharide **1**. — An aliquot of **1** (4 mg) was dissolved in dry methanol (1 ml), methyl iodide (1 ml) and silver oxide (500 mg) were added, and the mixture was refluxed for 8 h. The cooled mixture was filtered, the silver salts were extracted with hot chloroform, and the combined filtrate and washings were concentrated to a syrup which was then methylated twice with the omission of methanol. The methylated disaccharide glycoside was hydrolysed in methanol and 40% oxalic acid for 1 h at 60° and then in 2% aqueous oxalic acid[15] for 2 h at 80°. The mixture, after neutralisation ($CaCO_3$), was filtered and evaporated to dryness. The methylated sugars were converted into their respective glycosides with dry methanol and Amberlite IR-120 (H^+) resin[16], and then analysed by g.l.c. (Table II).

TABLE II

RELATIVE RETENTION TIMES (*T*) OF METHYLGLYCOSIDES IN G.L.C.

	Column		
	1	2	3
2,3,4,6-Tetra-*O*-methylglucose	1.00, 1.43	1.00, 1.36	1.00, 1.36
2,3,6-Tri-*O*-methylglucose	3.21, 4.21	1.75, 2.3	3.55, 2.36, 4.68
1,3,4,6-Tetra-*O*-methylfructose	0.98, 1.19	1.01, 1.22	—
1,3,6-Tri-*O*-methylfructose	3.6, 3.0, 2.75, 2.5, 3.2	2.2, 1.70, 2.00	4.00, 3.53, 2.72, 3.21, 1.70
1,3,4-Tri-*O*-methylfructose	2.3, 3.4, 3.9, 1.8	1.36, 1.79, 2.5	5.2, 3.9, 0.9
1,4,6-Tri-*O*-methylfructose	1.9, 2.1, 3.3	0.73, 2.0, 1.6, 1.45, 2.2	0.9, 2.5, 3.72, 4.6
Products from oligosaccharide **1** and maltulose	1.00, 1.43, 2.70, 1.9, 3.2, 3.6	1.0, 1.36, 1.7, 2.0, 2.2	1.0, 1.36, 2.72, 1.70, 3.21, 3.53, 4.2

Hydrolysis and separation of lactic acid from **1**. — A portion of **1** was hydrolysed with 90% formic acid for 6 h at 100° in a sealed tube in an atmosphere of carbon dioxide. The cooled solution was diluted with 6 vol. of water and heated for 2 h at 100°. The cooled solution was extracted with ether, and the ether-soluble material

was examined by paper chromatography in solvents (*A*) and (*C*) with sprays (*e*), (*f*), and (*g*), and by electrophoresis in pyridine–acetic acid buffer at pH 6.7 and over a range[5] of pH from 4.0 to 9.2 (spray *g*).

An aliquot of the pure acid was obtained from the ether-soluble material by ionophoretic separation at pH 6.7, this was converted into the methyl ester by refluxing with methanolic hydrogen chloride, and the product was analysed by g.l.c.

Reducing oligosaccharides. — Compounds with R_S 0.44 (**2**), 0.19 (**3**), 0.33 (**4**), and 0.18 (**5**), respectively, in solvent *A* were re-separated on 3M paper chromatograms in solvent *E*. Separate aliquots of each were hydrolysed with M sulphuric acid for 3 h at 100°, and with 0.125M sulphuric acid for 1 h at 80°. The solutions were worked up in the usual way, and the derived syrups were analysed by paper chromatography (Table III). The presence of D-glucose was confirmed with D-glucose oxidase[17]. The d.p. of a separate aliquot of **2** was determined[18].

TABLE III

PAPER-CHROMATOGRAPHIC MOBILITIES OF OLIGOSACCHARIDES **2–5**

Oligosaccharide	$R_{Maltose}$, *solvent*			
	A	*E*[6]	*F*[6]	*G*[7]
Maltose	1.0	1.0	1.0	1.0
2 (maltotriose)	0.52	0.59	0.62	0.60
3 (maltotetraose)	0.27	0.35	0.33	0.36
4 (panose)	0.45	0.44	0.48	0.47
5 (6-*O*-D-glucosylmaltotriose)	0.24	0.28	0.25	0.28

Methylation of oligosaccharide **2**. — A portion (0.4 mg) of oligosaccharide was methylated as for oligosaccharide **1**, except that the methylation was repeated four times. It was then methanolysed, and the methylated glycosides were analysed by g.l.c. (Table II).

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