Structure of a Polysaccharide from *Cassia tora* Seeds. I

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A new water-soluble complex polysaccharide consisting of D-galactose, D-glucose, D-mannose, and D-xylose in the molar ratio of 2:2:7:1 has been isolated from the defatted seeds of *Cassia tora* Linn. The methylated polysaccharide yielded 2,3,4,6-tetramethyl-D-glucose, 2,3,4-trimethyl-D-xylose, 2,3,4,6-tetramethyl-D-galactose, 2,3,4-trimethyl-D-glucose, 2,3,6-trimethyl-D-glucose, 2,3,6-trimethyl-D-mannose, 2,3-dimethyl-D-mannose, and 2,3-dimethyl-D-glucose in the approximate molar ratio of 1:4:8:1:1:20:8:5. The polysaccharide seems to be highly branched and probably contains α-linked D-galactopyranose and D-xylopyranose as end residues attached to 1,4- and 1,4,6-linked β-D-mannopyranose and glucopyranose units of the main chain as derived from the structural studies of other related polysaccharides. Graded hydrolysis indicated that D-xylose was liberated first, followed by D-galactose, D-glucose, and ultimately D-mannose.

Cassia tora Linn. (N.O. Leguminosae), an annual fetid found throughout India, is highly medicinal (Chopra *et al.*, 1956; Kirtikar and Basu, 1933) and a number of reports have appeared in the literature (Harborne and Sherratt, 1961; Narayan and Rangaswami, 1956; Pant and Singh, 1969; Patel and Patel, 1957; Rangaswami, 1963; Sharma and Gupta, 1965; Shibata *et al.*, 1969; Tiwari and Gupta, 1955; Wilkinson *et al.*, 1969). However, the seeds of the plant have not been studied for their mucilaginous content, therefore, the present investigations have been carried out to find out the nutritive value of the seeds of *C. tora*.

The purified polysaccharide, $[\alpha]^{27}D$ +34.5° (1 *N* NaOH), was extracted from the defatted seeds of *Cassia tora* and purified by the usual method (Khanna and Gupta, 1967; Rizvi *et al.*, 1971) until ash content was minimum (0.56%). Its homogeneity was checked by fractional precipitation followed by hydrolysis and quantitative estimation of the component sugars in each fraction, zone electrophoresis and acetylation, followed by deacetylation.

Acid hydrolysis followed by paper chromatographic examination and quantitative estimation showed the presence of D-galactopyranose, D-glucopyranose, D-mannopyranose, and D-xylopyranose in the molar ratio of 2:2:7:1 in the polysaccharide. Graded hydrolysis (Agrawal *et al.*, 1972; Rizvi *et al.*, 1971) with 0.05 *N* sulfuric acid followed by chromatographic identification revealed that D-xylopyranose units were first liberated, followed by D-galactopyranose, D-glucopyranose, and ultimately D-mannopyranose. As D-xylopyranose and D-galactopyranose units are liberated much earlier than D-glucopyranose and D-mannopyranose units, these are most probably attached to the main chain by α-linkages and predominantly form the nonreducing end groups. Attachment of D-galactopyranose units by α-linkages is quite in agreement with the results reported so far from the polysaccharides of Leguminosae and other families (Bishop *et al.*, 1962; Harvei and Wickstrom, 1964; Kapoor and Mukherjee, 1969; Khanna and Gupta, 1967; Morimoto *et al.*, 1962; Mukherjee *et al.*, 1961; Suba Rao and Rao, 1965; Tookey *et al.*, 1962). Probability of some D-glucopyranose units present as end groups cannot be ruled out.

The polysaccharide was methylated in two steps, first by the method of Parikh *et al.* (1958), giving a partly methylated product: $[\alpha]^{27}D$ +24.4° (chloroform); nmr (OMe) 39.84; and then by Purdie's method (Purdie and Irvine, 1903), which yielded a completely methylated polysaccharide: $[\alpha]^{27}D$ +20.2° (chloroform), nmr (OMe) 47.52. Methanolysis, followed by acid hydrolysis of the methylated product, yielded 2,3,4,6-tetra-*O*-methyl-D-glucopyranose, 2,3,4-tri-*O*-methyl-D-xylopyranose, 2,3,4,6-tetra-*O*-methyl-D-galactopyranose, 2,3,4-tri-*O*-methyl-D-glucopyranose, 2,3,6-tri-*O*-methyl-D-mannopyranose, 2,3-di-*O*-methyl-D-mannopyranose, and 2,3-di-*O*-methyl-D-glucopyranose in the approximate molar ratio of 1:4:8:1:1:20:8:5. These methyl sugars were separated on Whatman No. 3MM on a preparative scale and characterized by their specific optical rotations, melting point, mixed melting point, preparation of crystalline derivatives, and chromatography. The molar percentage of the end group calculated from the methylation studies was found to be 27.08.

From the above studies it is evident that the polysaccharide contains D-xylopyranose, D-galactopyranose, and some D-glucopyranose units as nonreducing residues which produced the respective methyl derivatives 2,3,4-tri-*O*-methyl-D-xylose, 2,3,4,6-tetra-*O*-methyl-D-galactose, and 2,3,4,6-tetra-*O*-methyl-D-glucose. The former product also shows that the D-xylose units are in pyranose form, while 2,3-di-*O*-methyl-D-mannose and 2,3-di-*O*-methyl-D-glucose show that the points of branching on D-mannopyranose and D-glucopyranose units are at the 1, 4, and 6 positions. A large total proportion of 2,3,6-tri-*O*-methyl-D-glucose (1 mol), 2,3,6-tri-*O*-methyl-D-mannose (20 mol), 2,3-di-*O*-methyl-D-mannose (8 mol), and 2,3-di-*O*-methyl-D-glucose (5 mol) is quite conclusive in showing that the backbone of the polysaccharide consists of 1→4 linked D-mannopyranose and D-glucopyranose units. The single unit branches of D-xylopyranose and D-galactopyranose and 1→6 linked two-unit branches of D-glucopyranose are attached to the C_6 of D-mannopyranose and D-glucopyranose units. The existence of 1→6 linked two-unit branches of D-glucopyranose was derived from the isolation of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3,4-tri-*O*-methyl-D-glucose in equal proportion (1:1), though in small amounts.

Determination of terminal groups by the periodate oxidation method (Khanna and Gupta, 1967; Rizvi *et al.*, 1971) corresponds to the liberation of 0.1862 mol of formic acid per 100 g of the polysaccharide. On the basis of methylation studies, the simplest repeating unit of the polysaccharide is supposed to consist of 48 units of pyranose sugar moieties, out of which 4 mol of D-xylopyranose, 8 mol of D-galactopyranose, and 1 mol of D-glucopyranose form terminal groups. For such a repeating unit the molar percentage of terminal residues was found to be 29.70, as determined by periodate oxidation, which is in close agreement to that revealed by methylation (27.08%). The consumption of periodate has been determined to be 63.2 mol per repeating unit of the polysaccharide. Examination of oxidized polysaccharide revealed that D-xylopyranose

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and D-galacotopyranose units took 36 hr, whereas D-glucopyranose and D-mannopyranose units required 208 hr for the complete oxidation. The considerable difference in the rates of oxidation of the component sugars is probably due to the steric effect resulting from the highly branched structure of the polysaccharide in which D-mannopyranose and D-glucopyranose units form all the branching points. Present knowledge, however, shows that this phenomenon is most probably due to the cyclic acetal formation (Cerezo, 1965). Resistance of some D-mannopyranose units to the periodate oxidation has already been observed in several cases of galactomannans (Andrews *et al.*, 1952; Khanna and Gupta, 1967; Rizvi *et al.*, 1971), although they do not contain 1→3 linked sugar residues. Infrared spectral studies (Barker *et al.*, 1956) also indicated the existence of α- and β-linkages in the polysaccharide by recording the absorption bands at 880, 820, and 785 cm^{-1}.

EXPERIMENTAL SECTION

All the specific rotations are equilibrium values and all melting points are uncorrected. Chromatographic separations were carried out at room temperature (25–35°) by the descending technique unless stated otherwise using the organic phase of any one of the following solvent systems: (A) *n*-butanol–ethanol–water (i) (5:1:4), (ii) (4:1:5); (B) benzene–acetic acid–methanol (1:1:3); (C) *n*-butanol–acetic acid–water (i) (5:1:4), (ii) (4:1:5); (D) *n*-butanol–isopropyl alcohol–water (11:6:3); (E) *n*-butanol–ethanol–water–ammonia (40:10:49:1); and (F) butanone–water–ammonia (100:50:3).

Isolation and Purification of the Polysaccharide. The defatted, dried, and crushed seeds (480 g) of *Cassia tora* were extracted with 1% acetic acid in cold by mechanical stirring. The procedure was repeated several times until no precipitate was obtained on addition of the excess of 95% ethanol to the extracts. The combined extracts were filtered, and to the clear solution was added nearly 2 vol of 95% ethanol with vigorous constant stirring, whereupon the crude polysaccharide was obtained as white fibers (45.6 g).

The crude polysaccharide was dissolved in 2.5 l. of 1% acetic acid by mechanical stirring, filtered, and treated with 250 ml of ethanol, whereupon a small amount of resinous precipitate obtained was filtered out. To the filtrate 4.7 l. of ethanol were further added, slowly with constant stirring. The precipitated polysaccharide was filtered and the process was repeated thrice, which finally yielded a white fibrous mucilage (32.5 g) with a minimum ash content of 0.56%; $[\alpha]^{27}D$ +34.5° (*c*, 0.8 in 1 *N* NaOH). It was slowly soluble in water, giving a turbid viscous solution which was used after filtration to determine the optical activity of the polysaccharide. The amount of polysaccharide in the solution was determined colorimetrically using phenol–sulfuric acid reagent (Dubois *et al.*, 1956; Marier and Boulet, 1959). An insoluble copper complex was obtained with Fehling's solution, but it was not reduced even on prolonged boiling.

Homogeneity of the Polysaccharide. *Fractional Precipitation.* The purified polysaccharide (5 g) was suspended in 600 ml of water and poured into 300 ml of ethanol with continuous stirring. The precipitate was filtered (Sample I). The filtrate was treated with another 300 ml of ethanol and the precipitate (Sample II) was filtered out. The filtrate was further diluted with 600 ml of ethanol to precipitate the rest of the polysaccharide (Sample III). The three samples and the original polysaccharide were separately analyzed (Hirst and Jones, 1949), which gave approximately the same ratio (2:2:7:1) between D-galactose, D-glucose, D-mannose, and D-xylose in each case.

Zone Electrophoresis. 70.5 mg of 0.174% solution of the polysaccharide were applied on a strip (15 × 45 cm) as a thin band at the anodic end and then separated by zone electrophoresis in borate buffer (pH 9.3) at 260 V and 18 mA for 6 hr. The strip was cut lengthwise into 30 equal segments, 1 cm in breadth from the anodic end down to cathodic end and numbered consecutively. The segments were eluted separately with 10 ml of distilled water and filtered through a plug of glass wool. A 5-ml portion of each extract was treated with 1 ml of 8.5% phenol and 15 ml of sulfuric acid. The absorbance of the color so produced was measured in a Klett–Summerson photoelectric colorimeter using filter No. 50. A blank was also run similarly. A plot of corrected absorbance *vs.* segment no. gave only one sharp peak (Figure 1). The high percentage of recovery (98.2%) from segments nos. 17 and 18 indicated the homogeneous character of the polysaccharide.

Acetylation. The polysaccharide (1 g) was acetylated with 5 g of anhydrous sodium acetate and 10 ml of acetic anhydride. The acetyl derivative recovered showed $[\alpha]^{27}D$ +7.5° (*c*, 1.2 in chloroform); nmr (acetyl) 46.01. Deacetylation of the acetate (Cerezo, 1965) produced the original polysaccharide: $[\alpha]^{27}D$ +33.8° (*c*, 0.5 in 1 *N* NaOH).

Hydrolysis of the Polysaccharide. The polysaccharide (3 g) was hydrolyzed with 75 ml of 2 *N* sulfuric acid on a water bath for 15 hr, neutralized ($BaCO_3$), filtered, and concentrated under reduced pressure. Paper chromatography in solvents A, C(ii), and D, while thin-layer chromatography of the hydrolyzate in C(i) revealed four spots in each case corresponding to the mobilities of D-galactose, D-glucose, D-mannose, and D-xylose.

Elution of the syrup (1.8 g) from a cellulose column with solvent D gave four fractions. Fraction I (70 mg) gave crystalline α-D-xylopyranose (crystallization from 85% aqueous methanol): melting point and mixed melting point 151°; $[\alpha]^{30}D$ +22.3° (*c*, 0.8 in water). It formed D-xylose benzylphenylhydrazone, mp 98–99° (crystallized from 95% ethanol) [lit. (Cheronis and Entrikin, 1963) mp 99°], and di-*O*-benzylidene-D-xylose-dimethyl acetal (crystallized from methanol), mp 207–210° [lit. (Breddy and Jones, 1945; Wise and Ratiff, 1947) mp 211–212°]. Fraction II (300 mg) gave α-D-mannopyranose (crystallized from aqueous methanol): melting point and mixed melting point 131°; $[\alpha]^{30}D$ +13.9° (*c*, 1.2 in water). It formed phenylhydrazone, mp 196° (without recrystallization) [lit. (Isbell and Frush, 1962) mp 199–200°], and D-

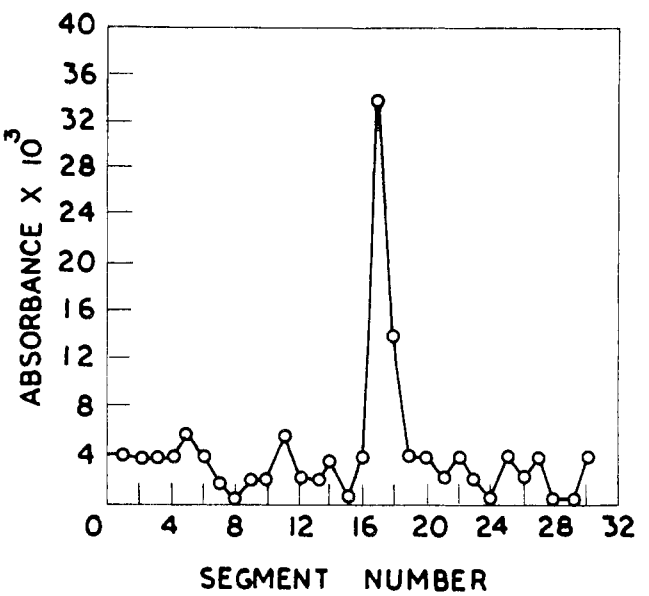

Figure 1. Homogeneity curve of polysaccharide from results of zone electrophoresis.

mannose-*p*-*N*-glycosyl aminobenzoic acid, mp 179° (recrystallized from ethanol) [lit. (Ellis, 1966), mp 182°]. Fraction III (105 mg) gave β-D-glucopyranose, melting point and mixed melting point 148° (recrystallized from absolute ethanol); $[\alpha]^{30}D$ +53.1° (*c*, 0.6 in water), and formed D-glucose-*p*-nitrophenylhydrazone, mp 185° (recrystallized from 95% ethanol) [lit. (Cheronis and Entrikin, 1963) mp 189–190°] and phenyl-D-glucosazone, mp 205° (recrystallized from aqueous ethanol) [lit. (Cheronis and Entrikin, 1963) mp 209–210°]. Fraction IV (100 mg) was identified as α-D-galactopyranose, melting point and mixed melting point 164° (recrystallized from aqueous methanol); $[\alpha]^{30}D$ +79.6° (*c*, 0.6 in water) and formed phenylhydrazone, mp 153° (crystallized from methanol) [lit. (Mester and Messmer, 1963) mp 159–160°] and *N*-*p*-nitrophenyl-D-galactosylamine, mp 215–216° (recrystallized from methanol) [lit. (Misaki and Smith, 1962) mp 219°].

Quantitative Estimation of the Component Sugars. The hydrolyzate was separated on Whatman No. 1 in solvent D and estimated by the periodate oxidation method (Hirst and Jones, 1949), which gave the molar proportion of 2:2:7:1, among D-galactose, D-glucose, D-mannose, and D-xylose.

Spectrophotometric Determination of Polysaccharide. Five-milliliter portions in triplicate of the solutions containing 5 to 100 μg/ml of the polysaccharide were treated with 1 ml of 8.5% aqueous phenol and 15 ml of sulfuric acid. The absorbances of the color produced were measured in a Klett–Summerson photoelectric colorimeter using filter No. 50. The standard graph was prepared by plotting the corrected absorbance against the amount ot polysaccharide in γ/ml of the solutions. The absorbance of the color obeyed Beer's law for solutions containing 5 to 70 γ per ml of the polysaccharide.

Graded Hydrolysis. Graded hydrolysis of the polysaccharide (100 mg) was carried out with 0.05 *N* sulfuric acid (50 ml) on a water bath for 9 hours. The hydrolyzate was taken out after 5, 10, 15, 20, 25, 30, 60, 90, 120, 150, 180, 240, 300, 420 and 540 minutes and examined chromatographically in solvent D. The results showed that D-xylose was first liberated followed by D-galactose, D-glucose and ultimately D-mannose along with the liberation of the oligosaccharides.

Periodate Oxidation. *Liberation of Formic Acid* (Brown *et al.*, 1948). The polysaccharide (300 mg) was dissolved in 50 ml of water and 3 g of potassium chloride, and 25 ml of 0.25 *M* sodium metaperiodate were added to it. The total volume was raised to 100 ml with water and the oxidation was carried out at room temperature in the dark. The final results (60 hr: 2.90 ml of N/103.8 ml of NaOH was used for a 5-ml aliquot) corresponded to the liberation of 0.1862 mol of formic acid per 540.5 and 537 g of the polysaccharide, respectively.

Three-milliliter portions of the reaction mixture after 36 and 60 hr were separately acidified with 5 ml of 2 *N* H_2SO_4 and then 2 ml of 10% KI were added to it. The liberated iodine was titrated immediately with 1 *N* sodium thiosulfate without indicator (starch) until the solution became colorless. The solutions were concentrated and hydrolyzed with 2 ml of 2 *N* H_2SO_4 and the hydrolyzate was obtained as usual. Chromatographic examination of the hydrolyzate in solvents A and D showed the presence of D-glucose and D-mannose.

Uptake of Periodate (Hough and Powell, 1960). To the polysaccharide (300 mg) dissolved in 25 ml of water was added 25 ml of 0.25 *M* sodium metaperiodate and the total volume was raised to 100 ml with water. The oxidation was carried out as usual and the final titer value at 208 hr (a 5-ml aliquot required 9.90 ml of N/40 ml of sodium thiosulfate solution) corresponds to the consumption of 0.825 mol of periodate per 100 g of the polysaccharide.

Periodate-oxidized solutions (5 ml each), taken out at 120 and 208 hr, were hydrolyzed as in the case of liberation of formic acid. Paper chromatographic examination showed a faint spot of D-glucose in the hydrolyzate of 120 hr, while no spot was obtained in the case of 208 hr.

Methylation of the Polysaccharide. The polysaccharide (8 g) was methylated in two steps, first according to the method of Parikh *et al.* (1958) with dimethyl sulfate and sodium hydroxide, giving a partly methyl derivative, $[\alpha]^{27}D$ +24.4° (chloroform); nmr (OMe) 39.84, and then by Purdie's method (Purdie and Irvine, 1903) using methyl iodide and silver oxide which yielded a brown-colored completely methylated derivative (5.6 g); $[\alpha]^{27}D$ +20.2° (*c*, 0.44 in chloroform); nmr (OMe) 47.52.

Hydrolysis of Methylated Polysaccharide and Identification of Methylated Sugars. The methylated polysaccharide was hydrolyzed according to the method of Bouveng *et al.* (1962). Chromatographic separation of the hydrolyzate in solvents A(i) and F gave as many as eight spots corresponding to the R_G (G = 2,3,4,6-tetra-*O*-methyl-D-glucose) values of 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,4-tri-*O*-methyl-D-xylose, 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-mannose, 2,3-di-*O*-methyl-D-mannose, and 2,3-di-*O*-methyl-D-glucose.

Quantitative Examination of Methylated Sugars. The methylated polysaccharide (60.4 mg) was hydrolyzed in 20 ml of 4% methanolic hydrogen chloride (Hirst *et al.*, 1949) and 15.2 mg of D-glucose were added. The dried residue was recovered as usual and hydrolyzed with 12 ml of 1.6 *N* H_2SO_4 for 10 hr. The hydrolyzate was neutralized ($BaCO_3$) and concentrated, and the component sugars were estimated (Hirst *et al.*, 1949) after chromatographic separation in solvent F using 1 ml of 0.1 *N* iodine with 1 ml of a solution containing 0.2 *M* sodium bicarbonate and 0.2 *M* sodium carbonate mixture. After 2.5 hr, the reaction mixtures were acidified with 2 *N* H_2SO_4, and 2 ml of 15% KI solution was added to it. The liberated iodine was titrated against 0.01 *N* solution of sodium thiosulfate. Found (results expressed as ml of 0.01 *N* iodine consumed) for 2,3,4,6-tetra-*O*-methyl-D-glucose: 0.24, 0.26, 0.22; for 2,3,4-tri-*O*-methyl-D-xylose: 0.94, 1.02, 0.86; for 2,3,4,6-tetra-*O*-methyl-D-galactose: 1.94, 2.00, 1.74; for 2,3,4-*O*-methyl-D-glucose: 0.26, 0.28, 0.20; for 2,3,6-tri-*O*-methyl-D-glucose: 0.23, 0.22, 0.26; for 2,3,6-tri-*O*-methyl-D-mannose: 4.76, 5.02, 4.40; for 2,3-di-*O*-methyl-D-mannose: 1.90, 2.04, 1.78; for 2,3-di-*O*-methyl-D-glucose: 1.18, 1.28, 1.08; and for D-glucose: 3.26, 3.52, and 3.10. These data gave the molar ratio of 1:4:8:1:1:20:8:5 among the respective methyl sugars and recovery was calculated on the basis of 100% recovery of D-glucose.

Qualitative Separation of Methylated Sugars. The methylated polysaccharide (5 g) was hydrolyzed with 45 ml of methanolic hydrogen chloride as described earlier and the methyl sugars were separated by paper chromatography on Whatman No. 3MM on a preparative scale.

Fraction I. The sugar was obtained as solid (75 mg), R_G 1.00 in solvents E and F (Found: OMe, 51.71; calculated for tetramethyl hexose: OMe, 52.54). It was recrystallized from petroleum ether (40–60°), mp 92–93°; $[\alpha]^{32}D$ +85.1° (*c*, 0.5 in water) [lit. (Irvine and Oldham, 1921) records for 2,3,4,6-tetra-*O*-methyl-α-D-glucopyranose mp 96°] and $[\alpha]D$ +92.2 → +83.8° (water). The sugar (45 mg) dissolved in 2 ml of ethanol on treatment with aniline (40 mg) yielded 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-glucopyranosylamine (42 mg), mp 135–136° after recrystallization from an ether–petroleum ether mixture; $[\alpha]^{32}D$ +224.5° (*c*, 0.3 in acetone) [lit. (Irvine and Moodie, 1908) records mp 132–134° and $[\alpha]^{20}D$ +229.5° (acetone)].

Fraction II. The sugar was obtained as solid (380 mg), R_G 0.94 and 0.92 in solvents E and F, respectively [Found: (OMe) 46.97; calculated for trimethylpentose: (OMe)

48.44]. It was recrystallized from petroleum ether (60–80°), mp 91°; $[\alpha]^{32}D$ +19.7° (*c*, 1.2 in water). The literature (Phelps and Purves, 1929) records mp 91–92° and $[\alpha]^{20}D$ +64.5 → +17.7° (water) for 2,3,4-tri-*O*-methyl-α-D-xylopyranose. A mixture of sugar (160 mg), redistilled aniline (90 mg), and absolute ethanol (2 ml) was refluxed, which yielded the colorless crystals (120 mg) of the anilide of 2,3,4-tri-*O*-methyl-D-xylopyranose after crystallization from ether; mp 96–97° and $[\alpha]^{32}D$ +45.3° (*c*, 0.8 in ethanol). The literature (Laidlaw and Percival, 1949) records mp 98–100° and $[\alpha]^{19}D$ −84 → +47° (ethanol) for 2,3,4-tri-*O*-methyl-*N*-phenyl-D-xylopyranosylamine.

Fraction III. The sugar was isolated as solid, R_G 0.88 and 0.81 in solvents E and F, respectively [found: (OMe) 51.32; calculated for tetramethylhexose: (OMe) 52.54]. It was recrystallized from petroleum ether (60–80°), mp 72–73°; $[\alpha]^{32}D$ +120.3° (*c*, 2.6 in water). The literature (Charlton *et al.*, 1927) records for 2,3,4,6-tetra-*O*-methyl-α-D-galactopyranose, mp 71–73° and $[\alpha]D$ +149.4 → 116.9° (water). The sugar (245 mg) dissolved in absolute ethanol (4 ml) was refluxed with 225 mg of redistilled aniline, which finally yielded the needles of 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-α-D-galactopyranosylamine (160 mg) having mp 192–193° and $[\alpha]^{32}D$ +42.2° (*c*, 1.1 in acetone). The literature (Whistler *et al.*, 1965) values are mp 192° and $[\alpha]D$ −83 → +41° (acetone).

Fraction IV. The fraction yielded a syrup, R_G 0.85 and 0.72 in solvents A and F, respectively [found: (OMe) 40.82; calculated for trimethylhexose: (OMe) 41.89]. It showed $[\alpha]^{32}D$ +76.3° (*c*, 0.4 in acetone), whereas the literature (Van Cleve *et al.*, 1956) value for 2,3,4-tri-*O*-methyl-D-glucopyranose is $[\alpha]^{25}D$ +71.9° (acetone). The sugar (40 mg) dissolved in ethanol (2 ml) was refluxed with 45 mg of *p*-nitroaniline and 1 drop of glacial acetic acid. The crystals of *p*-nitroanilide derivative of 2,3,4-tri-*O*-methyl-D-glucopyranose (46 mg) were obtained on recrystallization from ethanol, mp 221–222° and $[\alpha]^{32}D$ −244.5° (*c*, 0.3 in pyridine). The literature (Van Cleve *et al.*, 1956) records mp 224–225° and $[\alpha]^{25}D$ −251° (pyridine) for 2,3,4-tri-*O*-methyl-*N*-*p*-nitrophenyl-D-glucopyranosylamine.

Fraction V. The fraction yielded solid (80 mg), R_G 0.81 and 0.66 in solvents E and F, respectively [found: (OMe) 41.08; calculated for trimethylhexose: (OMe) 41.89]. The sugar was recrystallized from ether, mp 119° and $[\alpha]^{32}D$ +68.3° (*c*, 0.5 in water). The literature (Irvine and Hirst, 1922) records mp 122–123° and $[\alpha]D$ +90.2 → +70.5° (water) for 2,3,6-tri-*O*-methyl-α-D-glucopyranose. The sugar (50 mg) dissolved in pyridine (2 mg) was refluxed with *p*-nitrobenzoylchloride (200 mg) at 60–75°. After leaving overnight, it was treated dropwise with a saturated solution of $NaHCO_3$ until no effervescence occurred. Water (8 ml) was added to the reaction mixture and then extracted with chloroform (3 × 30 ml portions). The solvent from the combined extracts was evaporated to 1 ml *in vacuo* and the β anomer of 1,4-di-*O*-*p*-nitrobenzoate of 2,3,6-tri-*O*-methyl-D-glucopyranose (65 mg) was crystallized from petroleum ether (40–60°), mp 188°; $[\alpha]^{32}D$ +31.4° (*c*, 0.5 in chloroform). The literature (Rebers and Smith, 1954) records mp 189–190° and $[\alpha]^{23}D$ −33° (chloroform).

Fraction VI. The sugar was obtained as syrup (1400 mg), R_G 0.80 and 0.54 in solvents E and F, respectively [found: (OMe) 41.32; calculated for trimethylhexose: (OMe) 41.89]. It showed $[\alpha]^{32}D$ −8.7° (*c*, 2.6 in water) while the literature (Hirst and Jones, 1948) records $[\alpha]^{20}D$ −10° (water) for 2,3,6-tri-*O*-methyl-D-mannopyranose. The sugar (500 mg) was oxidized with bromine water and the product was crystallized from ether (280 mg), mp 80–81°. The literature (Andrews *et al.*, 1952) records mp 81° for 2,3,6-tri-*O*-methyl-D-mannono-γ-lactone. The lactone (180 mg) was boiled with phenylhydrazine (110 mg) under reflux which yielded 2,3,6-tri-*O*-methyl-D-mannonic acid phenylhydrazide (170 mg), mp 128-129° after crystallization from ethanol, $[\alpha]^{32}D$ −18.9° (*c*, 0.8 in water). The literature (Andrews *et al.*, 1952) records mp 131° and $[\alpha]^{19}D$ −20° (water) for 2,3,6-tri-*O*-methyl-D-mannonic acid phenylhydrazide.

Fraction VII. The fraction yielded a syrup (565 mg), R_G 0.53 and 0.33 in solvents E and F, respectively [found: (OMe) 29.44; calculated for dimethylhexose: (OMe) 29.81]. The sugar showed $[\alpha]^{32}D$ −16.8° (*c*, 1.8 in water), whereas the literature (Robertson, 1934) value is $[\alpha]D$ −15.8° (water) for 2,3-di-*O*-methyl-D-mannopyranose. The syrup (250 mg) dissolved in pyridine (10 ml) was refluxed with *p*-nitrobenzyl chloride (700 mg). The crude product was extracted with 2% H_2SO_4, washed with water, dissolved in chloroform, and recrystallized from ether, which yielded the crystals of 1,4,6-tri-*O*-*p*-nitrobenzoate of 2,3-di-*O*-methyl-D-mannopyranose (350 mg), mp 191–192° and $[\alpha]^{32}D$ +63.8° (*c*, 1.2 in chloroform). The literature (Smith and Montgomery, 1959) values are mp 194° and $[\alpha]D$ +65° (chloroform).

Fraction VIII. The sugar was obtained as solid (325 mg), R_G 0.57 and 0.25 in solvents E and F, respectively [found: (OMe) 29.53: calculated for dimethylhexose: (OMe) 29.81]. It was recrystallized from ethyl acetate, mp 116° and $[\alpha]^{32}D$ +50.9° (*c*, 0.8 in acetone). The literature (Irvine and Scott, 1913) records mp 108–110° and $[\alpha]^{20}D$ +6.5 → +50.9° (acetone) for 2,3-di-*O*-methyl-D-glucopyranose. The sugar (200 mg) dissolved in pyridine (8 ml) was mixed with *p*-phenylazobenzoyl chloride (1.7 g) at 0°. The reaction mixture was allowed to stand at 0° (2 days), followed by 25–30° (2 days), and again 0° (3 days). The crude product was purified over a small column of alumina and the chloroform eluate was concentrated, redissolved in chloroform, and precipitated with ethanol, mp 185° [lit. (Mertzweiller *et al.*, 1943) records mp 187–190°].

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Synthetic Precursors of Flavor Compounds with a Thiol Group

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Flavoring agents possessing a thiol group are usually difficult to apply in foodstuffs due to their volatility and instability. A search was made for a suitable protection of the thiol group to yield a precursor which releases the flavoring material during heating. The *O-tert*-alkyl thiocarbonates, particularly the *O-tert*-butyl thiocarbonate, appeared to be satisfactory. These esters hydrolyze in an aqueous medium at elevated temperatures to give the thiol of interest. The thiocarbonates of nine flavoring thiols were prepared and investigated.

Thiols are usually very powerful flavoring agents and are therefore generally used in concentrations less than 1 ppm. However, their application is limited as they are relatively volatile and highly reactive. They are, for example, easily oxidized to disulfides, and elimination of hydrogen sulfide may also occur. As a result of these two factors, thiol flavoring compounds rapidly disappear from food products during storage and heat treatment.

An illustrative example of a thiol flavoring compound is 1-methylthio-ethanethiol, which was isolated from beef broth in this laboratory (Brinkman *et al.*, 1972). When added to a foodstuff this compound is only perceived immediately after its addition, and then usually too strongly, since it is very volatile and unstable in an aqueous medium. Natural precursors of 1-methylthio-ethanethiol were found to be alanine, methionine, and cysteine (Schutte and Koenders, 1972). However, yields of formation of the flavoring compound from these precursors are very low and many other aromas are formed.

The addition of a nonvolatile derivative of the flavoring substance which slowly decomposes during heating, thus releasing the compound of interest in reasonable yields, would overcome the above drawbacks. It was therefore decided to investigate the slow formation of thiols from derivatives having a heat-labile thiol-protecting group. The protecting group increases the molecular weight and consequently decreases the volatility of the material so that the derivative should have little flavoring properties itself. The derivative should be sufficiently stable to be prepared, purified, and stored in a foodstuff, and finally, it should decompose during heating of the product to give the desired thiol.

In the present investigation we prepared some esters of thiols that are known to be important aroma compounds. The release of thiols during heating of the esters in an aqueous medium was conceived to take place as represented below.

$$R'-S-\overset{\overset{X}{\|}}{C}-R'' + H_2O \xrightarrow{\Delta} R'-SH + HO-\overset{\overset{X}{\|}}{C}-R'' \qquad (1)$$

$X = O, S$

The efficiency of release of the thiols from the precursors was determined by analytical methods. The thiol investigated primarily was 1-methylthio-ethanethiol [$R' = CH_3SCH(CH_3)$]. Later the study was extended to other flavoring substances with thiol groups. As examples of this important class, 1-butanethiol ($R' = C_4H_9$) and 2-butanethiol [$R' = CH(CH_3)C_2H_5$] were selected. Heterocyclic thiols, particularly tetrahydrofuran-, dihydrofuran-, and furanthiols, have been found to contribute to roasted meat odor (van den Ouweland and Peer, 1968); therefore this type of compound was also investigated. Finally, furfurylmercaptan, an important flavor compound contributing to the aroma of coffee (Staudinger and Reichstein, 1928) was

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