268. *Confirmatory Evidence for the Structure of Carrobiose.*

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Partial methanolysis of a polysaccharide extracted from the red alga *Furcellaria fastigiata* afforded carrobiose dimethyl acetal, which was then fully methylated. Mild acid hydrolysis of the product yielded a strongly reducing hexa-*O*-methyl ether of carrobiose, which was then reduced to the corresponding glycitol. Acetolysis of the glycitol, followed by *O*-deacetylation of the products, yielded 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,5-di-*O*-methyl-DL-galactitol. These and other results confirm that carrobiose is 3,6-anhydro-4-*O*-β-D-galactopyranosyl-D-galactose.

Carrobiose (3,6-anhydro-4-*O*-β-D-galactopyranosyl-D-galactose) has been isolated from a polysaccharide extracted from the red alga *Furcellaria fastigiata* (Huds.) Lamour.[1] The disaccharide was identified by comparison of its crystalline derivatives with specimens previously isolated from κ-carrageenan by O'Neill.[2] However, since O'Neill's specimens had been identified by periodate oxidation alone,[2] confirmatory evidence for the structure of these preparations [1,2] and of carrobiose was sought by the method of methylation.

Syrupy carrobiose dimethyl acetal was prepared by partial methanolysis [3,4] of the polysaccharide from *Furcellaria fastigiata*,[1] and was purified by conversion into its crystalline hexa-*O*-acetate, followed by *O*-deacetylation. To confirm its identity, a portion

[1] Painter, *Canad. J. Chem.*, 1960, **38**, 112.
[2] O'Neill, *J. Amer. Chem. Soc.*, 1955, **77**, 6324.
[3] Araki and Hirase, *Bull. Chem. Soc. Japan*, 1956, **29**, 770.
[4] Clingman and Nunn, *J.*, 1959, 493.

was heated in dilute oxalic acid to remove the dimethyl acetal grouping;[3,4] reduction of the product with aqueous sodium borohydride afforded crystalline carrobi-itol, identical with the specimen described earlier.[1]

The carrobiose dimethyl acetal was methylated, first with methyl sulphate and aqueous sodium hydroxide, and then with methyl iodide and silver oxide. Hydrolysis of the resulting hexamethyl ether with hot dilute oxalic acid[3,4] yielded a strongly reducing hexa-*O*-methyl ether of carrobiose, which was then converted into the corresponding glycitol by reduction with sodium borohydride.

The carrobi-itol hexa-*O*-methyl ether was acetolysed under conditions designed[5] to cleave not only the galactosidic linkage but also the hydrofuranol ring in the 3,6-anhydro-D-galactitol moiety. The products, after *O*-deacetylation with methanolic sodium methoxide, were separated by paper chromatography into two major components. One was 2,3,4,6-tetra-*O*-methyl-D-galactose. The other, a crystalline di-*O*-methylhexitol, yielded a crystalline tetra-*O*-acetate, and afforded crystalline DL-galactitol after demethylation with hydrobromic acid.[6] Its m. p. was different from that of authentic 2,4-di-*O*-methyl-D-galactitol, kindly provided by Dr. C. T. Bishop,[7] and unlike Dr. Bishop's compound, it was optically inactive. Upon periodate oxidation, the compound rapidly consumed 1·0 mol. of oxidant, without the liberation of formic acid or formaldehyde. Periodate oxidation yielded approximately 2 mol. of an optically inactive mono-*O*-methyltriose as the only detectable product; this compound must have been 2-*O*-methyl-DL-glyceraldehyde, since it was converted by borohydride reduction into 2-*O*-methylglycerol, identified by comparison (of the di-*O*-*p*-nitrobenzoate) with an authentic specimen synthesised by the method of Hibbert and his co-workers.[8]

The di-*O*-methylhexitol was therefore identified as 2,5-di-*O*-methyl-DL-galactitol. Since it must have originated from the reducing 3,6-anhydro-D-galactose part of carrobiose,[2] the non-reducing D-galactose residue must have been attached to position 4 of this portion.

Samples of the carrobiose dimethyl acetal isolated in this work, and of the carrobi-itol isolated earlier,[1] were subjected to periodate oxidation. Carrobiose dimethyl acetal consumed 1·95 mol. of oxidant, and liberated 0·97 mol. of formic acid, but did not liberate formaldehyde. Carrobi-itol consumed 2·96 mol. of periodate, and liberated 0·99 mol. each of formic acid and formaldehyde.

As pointed out by O'Neill,[2] the numerically low specific optical rotations of carrobiose derivatives indicate that the D-galactopyranosidic linkage is in the β-configuration. This, together with the evidence provided by O'Neill and by the present work, establishes that carrobiose, as isolated from κ-carrageenan and the polysaccharide from *Furcellaria fastigiata*, is 3,6-anhydro-4-*O*-β-D-galactopyranosyl-D-galactose. The same disaccharide has also been isolated by Araki and Hirase[3] from a polysaccharide extracted from *Chondrus ocellatus*, and by Clingman and Nunn[4] from a polysaccharide extracted from *Hypnea specifera*.

Experimental

General Methods.—Qualitative paper chromatography was carried out on Whatman No. 1 filter paper, but large sheets (57 × 46 cm.) of Whatman No. 3MM paper were used for preparative work. The following solvent systems were used: (*a*) butan-1-ol–pyridine–water (6 : 4 : 3 v/v), (*b*) butan-1-ol–ethanol–water (3 : 1 : 1 v/v), or (*c*) butan-2-one–ethanol–water (85 : 4 : 11 v/v). Sugar derivatives were located by spraying chromatograms with (*i*) silver nitrate in acetone, followed by sodium hydroxide in ethanol,[9] (*ii*) *p*-anisidine hydrochloride in

[5] Cottrell and Percival, *J.*, 1942, 749.
[6] Hough, Jones, and Wadman, *J.*, 1950, 1702.
[7] Bishop, *Canad. J. Chem.*, 1960, **38**, 1636.
[8] Hill, Whelen, and Hibbert, *J. Amer. Chem. Soc.*, 1928, **50**, 2235.
[9] Trevelyan, Procter, and Harrison, *Nature*, 1950, **166**, 444.

butan-1-ol,[6] or (*iii*) 0·01M-sodium metaperiodate, followed by aqueous starch–potassium iodide solution.[10] Chromatographic mobilities (R_F) are calculated relative to the solvent front.

Solvents were removed at 30° by distillation under diminished pressure. Melting points were determined with a Kofler block. Optical rotations were measured at 20—22°. The polysaccharide from *Furcellaria fastigiata* was isolated as described under " Method (*b*) " in an earlier paper.[1]

Periodate oxidation of carrobiose dimethyl acetal and carrobi-itol was carried out with 12-mg. samples of substrate in 0·01M-sodium metaperiodate (50 ml.) in the dark at 22°. For the measurement of periodate consumed, portions (5 ml.) of the mixture were added to a mixture of phosphate buffer (pH 7·0; 5 ml.) and aqueous potassium iodide (30% w/v; 1 ml.), and the liberated iodine was titrated with 0·01M-sodium thiosulphate. Samples (5 ml.) of the mixture, treated 10 min. previously with ethane-1,2-diol (1 ml.), were analysed for formic acid by titration with 0·01N-sodium hydroxide (Methyl Red as indicator). For the estimation of formaldehyde, samples (1 ml.) were added to aqueous sodium sulphite (0·52% w/v; 1 ml.), and the resultant solution was analysed by the chromotropic acid method.[11]

Partial Methanolysis of the Polysaccharide.—The polysaccharide (potassium salt; 20 g.) was shaken with methanolic hydrogen chloride (0·5% w/w) and anhydrous silica gel (50 mesh; 20 g.) at room temperature for 24 hr., and the mixture was then boiled under reflux for 4¼ hr., cooled, neutralised with excess of lead carbonate, and filtered. On concentration to 50 ml., the filtrate formed optically inactive crystals (yield, 2·5 g.), m. p. 215—220° [Found: ash, 57·9; ester sulphate (as SO_4 after acid-hydrolysis [1]), 63·8; OMe, 20·4. Calc. for KCH_3SO_4: ash (as K_2SO_4), 58·0; sulphate, 64·0; OMe, 20·7%].

The supernatant solution was diluted with water (500 ml.), and residual ionic material was removed by passage through a column (20 × 4 cm.) of a mixture (1 : 1 v/v) of Amberlite IR-120 (H^+ form) and Amberlite IR-45 (OH^- form) ion-exchange resins. The effluent and washings were concentrated, and the syrup (11·0 g.) examined on chromatograms. In solvent (*b*), it was resolved into four major components (*A*, *B*, *C*, and *D*), which had R_F = 0·64, 0·45, 0·33, and 0·16, respectively. All four components were readily detected with the periodate spray (*iii*), but *A* and *C* reacted only feebly with the silver nitrate spray (*i*). Components *A* and *C* could be detected selectively with the *p*-anisidine spray (*ii*) when the treated chromatograms were heated at 110° for 15 min. (cf. Clingman and Nunn [4]). Components *A* and *B* migrated at the same rates as 3,6-anhydro-D-galactose dimethyl acetal and methyl α-D-galactopyranoside, respectively. These observations suggested that component *C* was carrobiose dimethyl acetal. Chromatography of the mixture in solvent (*a*) showed that a small amount of a fifth component, having a mobility slightly lower than that of *C*, was also present; this component was probably methyl carrobioside (cf. Araki and Hirase [3]).

Carrobiose Dimethyl Acetal.—The crude methanolysate (5·0 g.) was separated on Whatman No. 3MM paper (20 sheets) with solvent (*b*). The bands containing component *C* were cut out and extracted with methanol; evaporation of the extracts yielded a syrup (2·4 g.), which failed to crystallise. It was therefore acetylated at 80° for 1 hr. with acetic anhydride (20 ml.) and pyridine (30 ml.). After crystallisation by solution in ethanol and dilution with water (to turbidity) the product (2·7 g.) had m. p. 154·5°, $[\alpha]_D$ −8·5° (*c* 1·0 in benzene) [Found: C, 50·3; H, 6·1; Ac, 42·7; OMe, 10·2%; *M* (Rast), 624. Calc. for $C_{12}H_{14}O_9Ac_6(OMe)_2$: C, 50·2; H, 6·2; Ac, 41·5; OMe, 10·0%; *M*, 622].

The crystals (2·6 g.) were dissolved in methanol (50 ml.), and methanolic sodium methoxide (10 ml.; from 50 mg. of sodium) was added. The mixture was kept at 4° for 24 hr., and then treated with Amberlite IR-120 (H^+ form) resin (10 g.), filtered, and concentrated to yield a syrup (1·6 g.), $[\alpha]_D$ +17° (*c* 1·0 in water) [Found: OMe, 16·9. Calc. for $C_{12}H_{20}O_9(OMe)_2$: OMe, 16·8%]. A portion (100 mg.) was converted into crystalline carrobi-itol, m. p. and mixed [1] m. p. 173°, as described by Clingman and Nunn.[4]

Hexa-O-methylcarrobiose Dimethyl Acetal.—Carrobiose dimethyl acetal (1·4 g.) was dissolved in aqueous sodium hydroxide (30% w/w; 20 ml.), and dimethyl sulphate (10 ml.) was added slowly with stirring and ice-cooling. Five further additions of aqueous sodium hydroxide (10 ml.) and dimethyl sulphate (10 ml.) were made to the continuously stirred mixture at daily intervals for the next 5 days. The mixture was then heated at 80° for 2 hr., cooled, and concentrated to 50 ml. It was extracted 5 times with chloroform (50 ml.); the chloroform

[10] Metzenberg and Mitchell, *J. Amer. Chem. Soc.*, 1954, **76**, 4187.
[11] Frisell, Meech, and Mackenzie, *J. Biol. Chem.*, 1954, **207**, 709.

extracts were combined, washed, dried (Na_2SO_4), and concentrated to a syrup (1·7 g.). After three further methylations with methyl iodide (10 ml.) and silver oxide (2 g.), the product (1·4 g.) showed no absorption in the infrared spectrum corresponding to free hydroxyl groups [Found: OMe, 53·5. Calc. for $C_{12}H_{14}O_3(OMe)_8$: OMe, 54·6%]. On chromatograms developed in solvent (*c*), sprayed with the *p*-anisidine reagent (*ii*), and heated at 110° for 15 min., the product appeared as a single spot, having R_F 0·88.

Hexa-O-methylcarrobi-itol.—Hexa-*O*-methylcarrobiose dimethyl acetal (1·4 g.) was heated in 0·01N-oxalic acid (100 ml.) at 100° for 3 hr., and the solution was then cooled, neutralised with excess of calcium carbonate, and filtered. Sodium borohydride (0·5 g.) was added to the filtrate, and the solution was kept at room temperature for 24 hr. It was then brought to pH 7 with N-sulphuric acid, and extracted with chloroform (200 ml.) in a continuous extraction apparatus. The chloroform extract was dried (Na_2SO_4), filtered, and concentrated to yield a syrup (1·1 g.).

Acetolysis of Hexa-O-methylcarrobi-itol.—Hexa-*O*-methylcarrobi-itol (1·1 g.) was dissolved in acetic anhydride (18 ml.), and concentrated sulphuric acid (3 ml.) was added. The mixture was kept, with occasional stirring, at 37° for 54 hr.,[5] and then poured into ice-cold aqueous sodium acetate (1% w/v; 1 l.) with vigorous stirring. The resultant solution was extracted six times with chloroform (300 ml.); the combined extracts were dried ($CaSO_4$), filtered, and concentrated to yield a syrup (0·8 g.). This, in methanol (20 ml.), was treated with methanolic sodium methoxide (5 ml.; from 25 mg. of sodium), and the mixture kept at 4° for 12 hr. Amberlite IR-120 (H^+ form) resin (5 g.) was added, and the mixture was filtered and concentrated, to yield a syrup (0·5 g.). On chromatograms developed in solvent (*c*), one component reacted with the *p*-anisidine reagent (*ii*), and travelled at same rate as 2,3,4,6-tetra-*O*-methyl-D-galactose. However, a second major component (R_F = 0·22) was readily detected when the chromatograms were sprayed with the periodate reagent (*iii*); this component also reacted feebly with the silver nitrate reagent (*i*).

The deacetylated acetolysate was separated with solvent (*c*) on two sheets of Whatman No. 3MM paper. The bands corresponding to the two major components were extracted with methanol, and yielded *ca.* 150 mg. of each as a syrup.

2,3,4,6-*Tetra*-O-*methy*-D-*galactose.*—The component that reacted with the *p*-anisidine reagent (*ii*) had $[\alpha]_D$ +114° (*c* 1·0 in water) and was indistinguishable on chromatograms developed in solvent (*a*), (*b*), or (*c*) from authentic 2,3,4,6-tetra-*O*-methyl-D-galactose. It crystallised slowly and had m. p. and mixed m. p. 70—72°. The derived *N*-phenylglycosylamine had m. p. and mixed m. p. 189—190°.

2,5-*Di*-O-*methyl*-DL-*galactitol.*—The component (150 mg.) that did not react with the *p*-anisidine spray (*ii*) was purified by being heated under reflux for 10 hr. with aqueous barium hydroxide (100 ml.; saturated at 23°), cooled, and filtered. Barium was precipitated by carbon dioxide, and the filtrate heated at 80° for 15 min. to decompose barium hydrogen carbonate and again filtered. Concentration yielded a pale gold syrup (110 mg.), which crystallised (ethanol–ether) to give an optically inactive *compound*, m. p. 183·5° [Found: C, 45·4; H, 8·7; OMe, 29·6. $C_6H_{12}O_4(OMe)_2$ requires C, 45·7; H, 8·6; OMe, 29·5%].

A portion (20 mg.) of this compound was acetylated with acetic anhydride (2 ml.) and pyridine (3 ml.). The *product*, which crystallised from ether–light petroleum, was optically inactive and had m. p. 88·5° [Found: C, 51·0; H, 7·1; OMe, 16·0; Ac, 45·8%; *M* (Rast), 398. $C_6H_8O_4(OMe)_2Ac_4$ requires C, 51·0; H, 6·9; OMe, 16·4; Ac, 45·5%; *M*, 378].

Another portion (20 mg.) of the crystals was demethylated [6] with hydrobromic acid (48% w/w; 1 ml.) at 100° for 15 min. The product (15 mg.), was shown by chromatography and mixed m. p. to be mainly DL-galactitol, m. p. 188°.

A third portion (30 mg.) was dissolved in 0·01M-sodium metaperiodate (100 ml.), and the solution kept at room temperature for 2 hr. Samples (two of 5 ml., and one of 1 ml.) were then withdrawn for the estimation of periodate uptake, formic acid, and formaldehyde, respectively. The remainder of the solution was extracted continuously with chloroform (250 ml.). The extract was dried (Na_2SO_4), filtered, and concentrated to yield a syrup (23 mg.) [Found: reducing Equiv. by iodometric titration,[12] 58. Calc. for $C_3H_7O_2{\cdot}CHO$: Equiv., 52]. The syrup (20 mg.) was treated with sodium borohydride (100 mg.) in water (10 ml.). The mixture was kept at room temperature for 24 hr., then acidified with N-sulphuric acid (5 ml.), and

[12] Ingles and Israel, *J.*, 1948, 810.

extracted continuously with chloroform. Evaporation of the extract yielded 2-*O*-methylglycerol (18 mg.), characterised by conversion into its di-*O*-*p*-nitrobenzoate,[8] which, after recrystallisation from benzene–ethanol, had m. p. 159·5—160·5°, undepressed on admixture with a specimen synthesised by the method of Hibbert and co-workers.[8]

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