32. *Structural Studies of Dextrans. Part I. A Dextran Containing α-1,3-Glucosidic Linkages.*

By I. J. Goldstein and W. J. Whelan.

A dextran (NRRL 1355-S) produced by a strain of *Leuconostoc mesenteroides* has been fragmented by acetolysis and separated into glucose (21%), isomaltose (2%), nigerose (20%), and 3-α-isomaltosylglucose (2%). The yield of nigerose was much greater than could be obtained by aqueous acid hydrolysis of the dextran. The structure of the trisaccharide proves that α-1,3- and α-1,6-bonds occur together in the same dextran molecule. The nature of the fragments obtained from the periodate-oxidized dextran indicated that few of the 1,3-linkages in the polysaccharide are contiguous.

The dextrans are a group of polyglucoses synthesized extracellularly by various micro-organisms, of which the most important is *Leuconostoc mesenteroides*. Different strains of this organism produce dextrans of different structures, as judged mainly by periodate oxidation studies.[1] Such studies, coupled with optical-rotational behaviour in cuprammonium solution,[2] indicate that the types of glucosidic linkage present may range from almost exclusive preponderance of 1,6-bonds to cases in which 1,2-, 1,3-, 1,4-, and 1,6-links are all present in substantial proportions. The very high optical rotations of the dextrans suggest also that the linkages have the α-configuration. Methylation studies have also detected all four types of bond in various dextrans. One of the best ways of identifying both the position and the configuration of a glucosidic linkage is by the isolation from the dextran of a characterizable oligosaccharide, but the literature contains exceedingly few instances of such studies. The isolation of isomaltose (6-*O*-α-D-glucopyranosyl-D-glucose) and its higher homologues [3,4] proved that the α-1,6-linkage is present and can occur in sequences in dextrans but there is only one case on record of the isolation and examination of a disaccharide other than isomaltose. This was by Barker *et al.*[4] who partly hydrolysed a dextran from *Betacoccus arabinosaceous* with acid and obtained isomaltose and a disaccharide whose behaviour on paper chromatography and electrophoresis indicated that it was a 1,3-linked diglucose. The presence of 1,3-links in several different dextrans was also detected by methylation analysis.[5]

The present investigation was designed to show the value of fragmentation by acetolysis as applied to dextrans and our preliminary account of this work [6] recorded the first isolation and full characterization of any di- and tri-saccharide other than isomaltose

[1] Jeanes, Haynes, Wilham, Rankin, Melvin, Austin, Cluskey, Fisher, Tsuchiya, and Rist, *J. Amer. Chem. Soc.*, 1954, **76**, 5041.
[2] Scott, Hellmann, and Senti, *J. Amer. Chem. Soc.*, 1957, **79**, 1178.
[3] Jeanes, Wilham, Jones, Tsuchiya, and Rist, *J. Amer. Chem. Soc.*, 1953, **75**, 5911; Turvey and Whelan, *Biochem. J.*, 1957, **67**, 49.
[4] Barker, Bourne, Bruce, Neely, and Stacey, *J.*, 1953, 2395.
[5] (*a*) Dimler, Jones, Schaefer, and Van Cleve, Amer. Chem. Soc. Meeting, 1956, Abs. 129, 2D; (*b*) Van Cleve, Schaefer, and Rist, *J. Amer. Chem. Soc.*, 1956, **78**, 4435; (*c*) Jones and Wilkie, *Canad. J. Biochem. Physiol.*, 1958, **37**, 377.
[6] Whelan, *Bull. Soc. Chim. biol.*, 1960, **42**, 1569.

and isomaltotriose from a dextran. More recently Aso and his co-workers [7] have confirmed the value of acetolysis and have isolated nigerose and kojibiose from other dextrans. Another dextran has yielded maltose.[8]

The dextran now chosen was kindly supplied by Dr. Allene Jeanes, and was isolated from a culture medium of a strain of *Leuconostoc mesenteroides* grown on sucrose and designated by her as dextran NNRL B-1355 fraction S. Periodate oxidation had indicated that 57% of the bonds were of the 1,6-type, 35% of the 1,3-type, and 8% of the 1,4-type.[1] In a trial experiment the dextran was hydrolysed with dilute sulphuric acid to degrees of conversion into glucose ranging from 20 to 51%. Paper chromatography showed the presence of glucose, isomaltose, higher saccharides, and a trace of a sugar migrating with nigerose (3-*O*-α-D-glucopyranosyl-D-glucose). This was not a satisfactory method of fragmenting the dextran and recourse was had to partial acetolysis. Experience in the fragmentation of yeast mannan had suggested that this might prove a method of preferentially preserving non-1,6-bonds. Peat, Whelan, and Edwards [9] hydrolysed this polysaccharide with acid and found the fragments to consist almost entirely of a series of α-1,6-linked mannose oligosaccharides. However, by partial acetolysis of a yeast mannan, Gorin and Perlin [10] obtained α-1,2-linked di- and tri-mannose in yields of 10% and 25%, respectively. Jones and Nicholson [11] also reported a preferential cleavage of 1,6-bonds during acetolysis of yeast mannan. In a trial experiment a paper chromatogram of dextran degraded by acetolysis showed the expected result, that the " nigerose " component was now present in a much greater quantity than was formed by aqueous acid hydrolysis. Correspondingly, the amount of " isomaltose " was considerably reduced. Accordingly, a larger amount of dextran was acetolysed, deacetylated, and fractionated on charcoal–Celite. The major products were glucose, isomaltose, nigerose, and a trisaccharide (see Table). The yield of nigerose was 20·4% of the dextran.

Properties of sugars obtained from fragmented dextran.

		Sugar		β-Acetate
	Wt. (g.) *	M. p.	$[\alpha]_D$ in H_2O	M. p.	$[\alpha]_D$ in $CHCl_3$
Glucose D	1·57	145°	+53°	135°	+4°
A	—	146	+53	135	+4
Isomaltose D	0·135	—	+121	143	+99
A	—	—	+122	143·5—144·5	+97
Nigerose D	1·464	—	+136	152—154	+83
A	—	—	+135	151—152	+84
3-α-Isomaltosylglucose D	0·13	—	+150	119—120·5	+117

D = from dextran, A = authentic material. * From 6·8 g. of dextran.

The trisaccharide component was identified as such by the fact that its capacity to yield glucose on total acid hydrolysis diminished by one-third after it had been reduced with sodium borohydride.[12] Partial acid hydrolysis of the trisaccharide gave disaccharide products which paper chromatography indicated were isomaltose and nigerose, whereas the only reducing disaccharide so identified in a partial hydrolysate of the reduced trisaccharide was isomaltose. On this evidence and because of the rigid identification of isomaltose and nigerose as the major disaccharide products from the dextran, the trisaccharide was assigned the structure *O*-α-D-glucopyranosyl-(1 → 6)-α-D-glucopyranosyl-(1 → 3)-D-glucose. Confirmatory evidence for this structure, and the enzymic synthesis of the trisaccharide from nigerose are reported in the following paper.

Structure of the Dextran.—The only monosaccharide detected in the partly fragmented dextran was D-glucose, confirming abundant earlier evidence that the dextrans are

[7] Matsuda, Watanabe, Fujimoto, and Aso, *Nature*, 1961, **191**, 278.
[8] Abdullah, Goldstein, and Whelan, unpublished work.
[9] Peat, Whelan, and Edwards, *J.*, 1961, 29.
[10] Gorin and Perlin, *Canad. J. Chem.*, 1956, **34**, 1796.
[11] Jones and Nicholson, *J.*, 1958, 27.
[12] Peat, Whelan, and Roberts, *J.*, 1956, 2258.

entirely constructed of glucose, although the mode of biosynthesis of dextran from sucrose suggests that one unit of fructose may be present in each polymer molecule.[13] Such a small amount of fructose could, however, escape detection.

The two disaccharides, isomaltose and nigerose, were isolated in amounts which eliminate any possibility that they were artefacts of the acetolysis prodecure. No information is available on the possibility that recombination reactions occur during acetolysis, corresponding to the reversion encountered during aqueous acid hydrolysis of a polysaccharide and acid treatment of monosaccharides. The effect of the acetolysis on glucose was tested and by paper chromatography there were found only traces of sugars migrating in the disaccharide region. Also, when isomaltose was treated in this way, nigerose was not formed. It is evident, therefore, that the dextran contains both 1,6- and 1,3-glucosidic linkages. This had already been indicated by periodate oxidation,[1] and the present work, in confirming the earlier work, now proves these linkages to be of the α-type. The present work goes further in proving that these linkages occur together in the same molecule, as shown by the isolation of the trisaccharide representing a fusion of isomaltose with nigerose. By periodate oxidation it was not possible to distinguish between an inter- and an intra-molecular mixture of 1,6- and 1,3-bonds. By partial hydrolysis Barker *et al.*[4] detected a trisaccharide which seemed to contain both 1,6- and 1,3-linkages but they did not examine it further.

Five other substances were detected on partial acetolysis of the dextran. These were all polyglucoses, three being disaccharides, the fourth a trisaccharide, and the fifth a tetrasaccharide. According to Jeanes *et al.*[1] periodate oxidation indicated 8% of 1,4-like bonds in this dextran. The oxidation cannot, however, distinguish 1,4- from 1,2-bonds. Scott, Hellmann, and Senti [2] devised a means of making this distinction but were unable to apply it to this particular dextran. If we assume the dextran to contain only α-glucosidic bonds, the evidence of periodate oxidation suggests that the dextran fragments should contain maltose or kojibiose, or both. Although the evidence of identification was only that of paper chromatography, ionophoresis, and differential staining, it seemed fairly certain that two of the disaccharides were maltose and kojibiose. The yields were, however, so low as to raise doubts whether these compounds were true structural fragments or artefacts of acetolysis. In the absence of information on the extent of recombination during acetolysis, no conclusion can be drawn.

The third disaccharide seemed to be laminaribiose (β-1,3-glucobiose). Again the yield was only a few milligrams and one may speculate here on the possibility that the glucosidic bond of nigerose underwent inversion of configuration in acetic anhydride–sulphuric acid, similar to that brought about by titanium tetrachloride in its reversible conversion of gentiobiose (β-1,6-link) into isomaltose (α-1,6-link).[14]

The trisaccharide fraction was partly hydrolysed with acid and gave rise to five disaccharide components, as judged by paper chromatography. These were the disaccharides already mentioned, namely, isomaltose, nigerose, maltose, kojibiose, and laminaribiose, the last three in traces. The complexity of the material and its relatively small amount precluded further investigation.

Finally, a tetrasaccharide component was obtained which seemed to contain only 1,6- and 1,3-linkages, since it gave rise on partial hydrolysis to glucose, nigerose, isomaltose, and a trisaccharide having the R_F value of 3-α-isomaltosylglucose. The relative simplicity of this mixture of degradation products suggested that the tetrasaccharide was a true structural fragment, although there was no proof that it was a single substance. There are twelve tetrasaccharides which can be made up of various combinations of α-1,6- and α-1,3-glucosidic linkages.

Nigerotriose was not detected in the fragments from the dextran, suggesting that the 1,3-linkages do not occur in sequence with each other. To test this conclusion the dextran

[13] Edelman, *Adv. Enzymol.*, 1956, **17**, 189.
[14] Lindberg, *Acta Chem. Scand.*, 1949, **3**, 1355.

was oxidized with periodate, reduced with borohydride, and then treated with cold dilute mineral acid, as in the procedure of Smith and his co-workers,[15] whereby the oxidized polysaccharide is preferentially split at the sites of oxidation. Glycerol, arising from non-reducing end groups and 1,6-linked glucose units, was obtained and characterized, and a non-reducing substance, shown by chromatography and periodate oxidation to be a 1-*O*-glucopyranosylglycerol, presumably the α-anomer. This is the expected product from a 3-substituted glucose residue joined in 1,6-combination to the next glucose residue. Two other substances were found. One appeared to be a mixture, giving glucose, glycerol, and erythritol on hydrolysis, but its R_F value made it unlikely that it was a derivative of a disaccharide. The other substance, present only in traces, could have been a disaccharide derivative. It is concluded that at least the majority of the 1,3-glucosidic bonds of the dextran are isolated from each other. There is also evidence from methylation analysis that the 3-substituted glucose units in this particular dextran are not substituted at position 6.[5a]

Experimental

General Methods.—Paper-chromatographic separations were made in ethyl acetate–pyridine–water (10 : 4 : 3, by volume). The reagents used to locate the sugars were (1) silver nitrate–sodium hydroxide,[16] (2) aniline–diphenylamine,[17] and (3) alkaline triphenyltetrazolium chloride.[18] Unless otherwise stated, the first of these spray reagents was used. R_g refers to the movement of a sugar relative to glucose. Paper electrophoresis was carried out in 12·5mM-sodium borate buffer pH 10·0 at 10 v/cm. Sugars were separated on Whatman No. 3MM paper and preparative paper-chromatographic operations were carried out on this paper, with a loading of sugar up to 2 mg. per cm. of starting line, or on Whatman No. 17 paper where 10 mg. of sugar per cm. could be applied. Evaporations of sugar solutions were conducted under reduced pressure at 45°; sugar syrups obtained from paper or charcoal columns were dissolved in 80% aqueous methanol and filtered, the solutions were evaporated, and the residues dissolved in water and treated with Biodeminrolit ion-exchange resin (Permutit Co., Ltd.) in the carbonate form.[19] Concentrations of oligosaccharide solutions were measured by acid hydrolysis to glucose,[20] unless otherwise stated. The time of hydrolysis was increased to 6 hr. when the molecule contained a 1,6-linkage (cf. Turvey *et al.*[3]). Partial hydrolysis of oligosaccharides was achieved in 0·33N-sulphuric acid at 100° (30 min.). Fractions from charcoal columns were tested qualitatively for carbohydrate content with phenol–sulphuric acid,[21] and when the presence of sugar was indicated the optical rotation was measured in a 4 dm. tube.

Acetolysis of Dextran.—To the dextran (8·0 g.; moisture content 15%) was added dropwise a mixture (32 ml.) of acetic anhydride and concentrated sulphuric acid (100 : 9, v/v) with cooling in an ice bath. The mixture was stored at 35° for 32 hr. with occasional shaking until the dextran had dissolved. The dark-brown solution was poured into iced water (200 ml.) and set aside overnight. The acetates were extracted with chloroform (3 × 100 ml.), and the combined extracts were washed with sodium hydrogen carbonate solution and water and then dried (Na_2SO_4). Evaporation of the chloroform was followed by addition of absolute methanol and evaporation. The acetates were de-esterified by the addition of sodium (50 mg.) to their solution in methanol. After being stored overnight, the solutions were neutralized with acetic acid and evaporated. The products were only partly soluble in water, so the deacetylation was repeated to give material completely soluble in water. This was treated with Biodeminrolit resin and evaporated to a syrup (6·0 g.), paper-chromatographic fractionation of which showed spots corresponding in R_g value to glucose, nigerose (R_g 0·74), maltose (R_g 0·65), isomaltose (R_g 0·56), isomaltotriose (R_g 0·32), and a sugar of R_g 0·43, among others.

Charcoal–Celite Fractionation of Degraded Dextran.—A charcoal–Celite column (5 × 90 cm.) was prepared, containing 650 g. of adsorbent (1 : 1, by wt.). The oligosaccharides, in 50 ml.

[15] Goldstein, Hay, Lewis, and Smith, Amer. Chem. Soc. Meeting, 1959, Abs. 134, 25D.
[16] Trevelyan, Procter, and Harrison, *Nature*, 1950, **166**, 444.
[17] Buchan and Savage, *Analyst*, 1952, **77**, 401.
[18] Wallenfels, *Naturwiss.*, 1950, **37**, 491.
[19] Woolf, *Nature*, 1953, **171**, 841.
[20] Pirt and Whelan, *J. Sci. Food Agric.*, 1951, **2**, 224.
[21] Dubois, Gilles, Hamilton, Rebers, and Smith, *Analyt. Chem.*, 1956, **28**, 350.

of water, were adsorbed and the column was eluted with water (2 l.) to remove glucose, fractions of about 125 ml. being collected. The glucose (fractions 1—17) was worked up to give 1·57 g. of crystals (from aqueous ethanol), m. p. 145°, $[\alpha]_D^{20}$ +53° at equilibrium (*c* 1·1 in H_2O). The derived β-penta-acetate had m. p. 135°, $[\alpha]_D^{18}$ +4° (*c* 1 in $CHCl_3$).

Elution of the column with 6% aqueous ethanol (*i.e.*, 6% of EtOH) gave further fractions (31—37) from which a syrup (0·20 g.) was obtained containing sugars migrating with glucose, isomaltose (R_g 0·54), nigerose (R_g 0·72), maltose (R_g 0·69), and kojibiose (R_g 0·62). The spot having R_g 0·69 gave the characteristic blue colour of 1,4-linked oligosaccharides with spray 2, while that having R_g 0·62 did not react with spray 3. On paper electrophoresis there were found substances with the M_G values of glucose (M_G 1·00), isomaltose and nigerose (M_G 0·59), maltose (M_G 0·42), and kojibiose (M_G 0·34). The isomaltose component was removed by chromatography on thick paper to give chromatographically pure material (0·135 g.), $[\alpha]_D^{18}$ +121° (*c* 2·7 in H_2O), which yielded a β-octa-acetate, m. p. and mixed m. p. 143°, $[\alpha]_D^{20}$ +99° (*c* 1 in $CHCl_3$).

Fractions 38—39 yielded a syrup (0·20 g.) containing substances migrating on paper with nigerose and kojibiose from which were separated nigerose (0·15 g.) and a sugar (9 mg.) not reacting with spray 3. Fractions 40—44 (0·487 g.), 45—49 (0·231 g.), and 50—57 (0·496 g.) contained the bulk of the nigerose. The first two batches contained traces of isomaltose, removed by paper chromatography. The nigerose had $[\alpha]_D^{18}$ +131° (*c* 1·1 in H_2O; concentration estimated by weight) or +136° (concentration estimated by reducing-sugar determination). The derived β-octa-acetate had m. p. and mixed m. p. 152—154°, $[\alpha]_D$ +83° (*c* 1·3 in $CHCl_3$), after two recrystallizations from ethanol and seeding with an authentic specimen. An additional amount of nigerose (0·10 g.) was obtained from fractions 58—79 (0·15 g.) by paper chromatography, the other sugar component apparently being isomaltose.

After fraction 75 had been collected the eluant was changed to 12% aqueous ethanol. Fractions 93—112 yielded a syrup (0·32 g.) which on fractionation on thick filter paper was separated into sugars having R_g values 0·38 (0·20 g.) and 0·74 (relative to nigerose, R_g 0·63). A little isomaltose was also present and persisted in the sugar having R_g 0·38. Further purification on thick paper gave chromatographically pure material (R_g 0·38) weighing 0·13 g. and having $[\alpha]_D^{20}$ +150° (*c* 1 in H_2O). The sugar was apparently a trisaccharide since paper chromatography of a partial acid hydrolysate showed sugars migrating with isomaltose, nigerose, and glucose. On complete hydrolysis with acid, only glucose was found. Reduction of the trisaccharide (20 mg.) with sodium borohydride (30 mg.) for 24 hr. followed by total acid hydrolysis gave 63% of the proportion of glucose obtained from the unreduced sugar, proving the compound to be a trisaccharide. Partial acid hydrolysis of the reduced trisaccharide and subsequent paper-chromatographic fractionation showed the presence of glucose and isomaltose; a reducing disaccharide corresponding in R_g value to nigerose was no longer present. The trisaccharide (20 mg.) was acetylated with sodium acetate–acetic anhydride, yielding a crystalline *acetate* when the solution was poured into water. This substance was separated and a syrup was extracted with chloroform from the filtrate, which crystallized on being seeded. The combined products were recrystallized and had m. p. 119—120·5°, $[\alpha]_D^{21}$ +117° (*c* 0·25 in $CHCl_3$) (Found: C, 49·8; H, 5·8. $C_{40}H_{54}O_{27}$ requires C, 49·7; H, 5·6%).

The component of fractions 93—112, R_g 0·74, crystallized spontaneously on being evaporated. It had the same R_g value as laminaribiose [0·69 in butanol–acetic acid–water (4 : 1 : 5, by vol.); nigerose had R_g 0·59], and a partial acid hydrolysate contained only glucose and unchanged component.

The charcoal column was eluted with 15% ethanol from fraction 116 onwards and with 18% ethanol at fraction 132. Fractions 152—200 yielded a syrup (0·470 g.) containing a trisaccharide having an R_g value (0·39) slightly greater than that of the trisaccharide already isolated (R_g 0·38), and a tetrasaccharide of R_g 0·23. The tetrasaccharide was twice purified by fractionation on thick filter paper, weighing 191 mg. after the final purification. This material had $[\alpha]_D^{20}$ +165° (*c* 1 in H_2O). A partial acid hydrolysate contained sugars migrating on paper with glucose, nigerose, and isomaltose. A trisaccharide (R_g 0·35) and unchanged material were also present.

The trisaccharide portion of fractions 152—200 was partly hydrolysed with acid; the mixture contained sugars migrating with glucose, laminaribiose, nigerose, kojibiose, and isomaltose.

The final material to be eluted from the column after fraction 200 was contained in 2 l. of

eluate and weighed 0·750 g. It consisted of higher oligosaccharides with traces of nigerose, isomaltose, trisaccharide, and tetrasaccharide and was not examined further.

Fragmentation of Periodate-oxidized Dextran.—The dextran (340 mg., dry wt.) dissolved in water (20 ml.) on being stored for several hours at room temperature. The solution was cooled to 3°, mixed with 0·1M-sodium metaperiodate (50 ml.), and diluted to 100 ml. with water. The flask was stored at 3° for 6 days, along with a dextran-free periodate solution. After this time a portion (3 ml.) of each solution was added to a mixture of water (20 ml.), 10% aqueous potassium iodide (3 ml.), and 3N-sulphuric acid (2 ml.), and the liberated iodine was titrated with 0·05N-sodium thiosulphate. The consumption of periodate by the dextran amounted to 1·50 moles per glucose residue.

Iodate and periodate were removed by stirring the dextran digest with barium carbonate. The precipitate was removed on a centrifuge and washed with water. The combined supernatant liquid and digest were added with stirring to 2·5% aqueous potassium borohydride (20 ml.) and stored overnight. N-Sulphuric acid was added to pH 7, and then 17 ml. of acid were added to the 160 ml. of solution, to bring the acid concentration to 0·1N. After the solution had been kept for 17 hr., the acid was removed by stirring in barium carbonate, and the precipitate was removed in a centrifuge. The supernatant solution was treated with Biodeminrolit resin and evaporated to a syrup containing boric acid. The acid was removed by addition of a few ml. of 0·5% methanolic hydrogen chloride, followed by distillation with repeated additions of methanol. The syrup was dissolved in water (75 ml.), again treated with ion-exchange resin, and evaporated to a syrup (0·20 g.) which on fractionation in butanol–acetic acid–water (4 : 1 : 5, by vol.) revealed non-reducing substances having the R_g value of glycerol (2·16) and the expected R_g value of 1-*O*-α-glucosylglycerol (1·0). A substance having R_g 1·37 and a trace of material having R_g 0·62 were present. These were resolved by paper-sheet chromatography into glycerol (23 mg.), glucosylglycerol (95 mg.), and substance of R_g 1·37 (35 mg.). The glycerol was identified as the crystalline tri-*p*-nitrobenzoate, m. p. and mixed m. p. 188°.

The glucosylglycerol (22·2 mg. in 2·5 ml.; concentration determined by acid hydrolysis) was mixed with 0·1M-sodium metaperiodate (5 ml.), diluted to 10 ml. with water, and kept overnight at room temperature. The consumption of periodate was then found, as above, to be 2·96 mol. (calc. for 1-*O*-glucosylglycerol, 3 mol.). Formic acid was determined by mixing a portion (2 ml.) of the digest with ethanediol (0·5 ml.) and, after 30 min., adding 10% potassium iodate (2 ml.) and 3N-sulphuric acid (2 ml.). Iodine was titrated with 10mN-thiosulphate. The yield of formic acid was 0·90 mol. (calc., 1 mol.). Formaldehyde was estimated with chromotropic acid,[22] 0·92 mol. being found (calc., 1 mol.).

The substance having R_g 1·37 was hydrolysed in 1·5N-sulphuric acid for 6 hr. at 100° and fractionated on paper. There were found substances migrating with glucose, glycerol, erythitol (trace), and original material (trace).

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THE LISTER INSTITUTE OF PREVENTIVE MEDICINE, LONDON, S.W.1.
[Present address (I. J. G.): DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF BUFFALO, BUFFALO, N.Y., U.S.A.]
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[22] Parrish and Whelan, *Stärke*, 1961, **13**, 231.