[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, COLLEGE OF AGRICULTURE, UNIVERSITY OF NEBRASKA]

The Synthesis and the Enzymolysis of Methyl Glycosides of Malto-oligosaccharides[1]

BY JOHN H. PAZUR, JEAN M. MARSH AND TADAHIKO ANDO[2]

RECEIVED OCTOBER 16, 1958

The methyl α- and β-D-glycosides of malto-oligosaccharides were synthesized enzymatically from cyclohexaamylose and appropriate cosubstrates, methyl α-D-glucoside or methyl β-D-glucoside, by use of *macerans* amylase. The glycosides of maltose, maltotriose and maltotetraose have been isolated in pure form by chromatographic procedures. Methyl β-D-maltoside, methyl α-D-maltotrioside and methyl α-D-maltotetraoside have been obtained in crystalline form. Yeast maltase and fungal amyloglucosidase hydrolyzed the methyl maltosides to D-glucose and methyl D-glucoside. Crystalline salivary amylase hydrolyzed all of the α-D(1 → 4)-glucosidic bonds in the trioside and tetraoside but at varying rates and was without action on the bonds of the methyl maltosides.

Prior to the recent report of Peat, Whelan and Jones,[3] the synthesis of methyl α-D-maltoside and the methyl glycosides of higher molecular weight malto-oligosaccharides had not been achieved. Methyl heptaacetyl α-D-maltoside had been prepared earlier by Inouye, *et al.*,[4] by isomerization of the corresponding β-compound with the aid of antimony pentachloride catalyst. Deacetylation of the α-compound should yield the methyl α-D-maltoside. Although this procedure could be used for the synthesis of the methyl glycoside of maltose, it is not readily applicable to the synthesis of glycosides of higher molecular weight malto-oligosaccharides. Peat and associates[3] prepared the methyl α-D-glycosides of maltose, maltotriose and maltotetraose in amorphous but chromatographically pure form by an enzymatic procedure. Methyl α-D-glucoside and amylopectin incubated with D-enzyme[5] were converted into a series of non-reducing methyl malto-oligosaccharides and into a series of reducing malto-oligosaccharides. The separation of the complex mixture of compounds though eventually achieved was nevertheless quite tedious. In our laboratory the enzymatic synthesis of the methyl glycoside series of malto-oligosaccharides has been effected by use of the coupling and redistribution reactions of *macerans* amylase. This method possesses certain advantages as pointed out below and has resulted in the preparation of some of the glycosides in crystalline form. Information on the course of hydrolysis of the methyl glycosides by yeast maltase, fungal amyloglucosidase and salivary amylase has been obtained and is also presented.

Earlier studies[6] have shown that *macerans* amylase effects a transfer of the glucosyl units of cyclohexaamylose to methyl α-D-glucoside to form methyl α-D-maltoheptaoside which, in turn, *via* redistribution reactions,[7] is converted into a homologous series of methyl glycosides. Since in the *macerans* digest no reducing co-substrate is present, the reducing series of malto-oligosaccharides is not produced and the difficulty of separating the reducing and non-reducing compounds as experienced by Peat, *et al.*,[3] is not encountered. The individual methyl glycosides are resolved easily by chromatography on paper or on cellulose powder columns with a *n*-butyl alcohol–ethyl alcohol–water system (4:1:1 by volume).[8]

The methyl β-D-glycosides of the malto-oligosaccharides also have been prepared by use of the *macerans* enzyme. In this case the cosubstrate employed was methyl β-D-glucoside. From a digest of methyl β-D-glucoside and cyclohexaamylose with *macerans* amylase, methyl β-D-glucoside and methyl β-D-maltoside were isolated in crystalline form and methyl β-D-maltotrioside and methyl β-D-maltotetraoside in amorphous form. The apparent R_f values for the β-series of compounds, for the α-series and for the unsubstituted malto-oligosaccharides are recorded in Table I.

TABLE I

APPARENT R_f VALUES[a] OF MALTO-OLIGOSACCHARIDES AND THEIR METHYL GLYCOSIDES

	Reducing compound	Methyl α-D-series	Methyl β-D-series
Glucose	0.58	0.86	0.87
Maltose	.24	.54	.55
Maltotriose	.07	.22	.24
Maltotetraose		.06	.07

[a] Three ascents of the solvent system *n*-butyl alcohol–ethyl alcohol–water (4:1:1 by volume) were employed. The apparent R_f value is the distance traveled by the compound divided by the total height of the paper.

Of the methyl α-D-glycosides, methyl α-D-glucoside, methyl α-D-maltotrioside and methyl α-D-maltotetraoside were obtained in crystalline form. The methyl α-D-maltoside was chromatographically pure but has not yet been crystallized. The specific rotation of the methyl α-D-maltoside is +184° and agrees with a value reported by Peat, *et al.*[3] Paper chromatographic mobility and the nature of the products of acid and enzyme hydrolysis of the compound substantiate the methyl α-D-maltoside structure for this non-reducing compound. Yeast maltase and fungal amyloglucosidase cleaved the α-D(1→4)-glucosidic bond of the maltoside to yield D-glucose and methyl α-D-

(1) Published with the approval of the Director as Paper No. 910 Journal Series, Nebraska Agricultural Experiment Station. Supported in part by a grant from the National Science Foundation. Presented before the Division of Biological Chemistry at the 133rd National Meeting of the American Chemical Society, San Francisco, Calif., April 13, 1958.

(2) Fulbright exchange scholar sponsored, in part, by Takamine Laboratory, Division of Miles Laboratories, Inc., Clifton, N. J.

(3) S. Peat, W. J. Whelan and G. Jones, *J. Chem. Soc.*, 2490 (1957).

(4) Y. Inouye, K. Onodera, I. Karasawa and Y. Nishisawa, *J. Agr. Chem. Soc. (Japan)*, **26**, 631 (1952).

(5) S. Peat, W. J. Whelan and W. R. Rees, *J. Chem. Soc.*, 44 (1956).

(6) D. French, M. L. Levine, E. Norberg, P. Nordin, J. H. Pazur and G. M. Wild, THIS JOURNAL, **76**, 2387 (1954).

(7) E. Norberg and D. French, *ibid.*, **72**, 1202 (1950).

(8) We are indebted to Dr. R. E. Reeves, School of Medicine, Louisiana State University, New Orleans, for suggesting this solvent system and for a sample of crystalline methyl β-D-maltoside.

glucoside but did not hydrolyze the methyl glucosidic bond of the compound.

The specific rotation and the melting point of the crystalline methyl α-D-maltotrioside were +202° and 145–147°. Crystalline salivary amylase hydrolyzed the maltotrioside in a pattern very similar to that for the hydrolysis of maltotriose,[9] *i.e.*, bond 1[10] of the trioside and triose were hydrolyzed at a faster rate than bond 2. Use has been made of the difference in rate of hydrolysis of the two bonds in the trioside to obtain two hydrolytic fragments which aided in the characterization of the non-reducing compound as methyl α-D-maltotrioside. These were methyl α-D-glucoside and maltose, the former being identified by its melting point and X-ray diffraction pattern and the latter by its specific rotation and X-ray diffraction pattern.

The methyl α-D-maltotetraoside crystallized as a dihydrate with a specific rotation of +201° and a melting point of 170–172°. Paper chromatographic evidence indicates that the methyl α-D-maltotetraoside was hydrolyzed by salivary amylase in a manner quite similar to maltotetraose.[11] The major products of hydrolysis of the tetraoside were maltose and methyl α-D-maltoside, tentatively identified by paper chromatography. Small amounts of reducing compounds which appear to be glucose and maltotriose also were detected on the chromatograms. The concentration of maltotriose in the hydrolysate was greater than that of glucose indicating that the terminal bond 1[10] was hydrolyzed at a somewhat faster rate than the terminal bond 3. On the basis of the chromatographic evidence the rate constants for the hydrolysis of the terminal bonds of the tetraoside are much smaller than the constant for the hydrolysis of the central bond. These patterns of hydrolysis of the maltotrioside and maltotetraoside by salivary amylase differ from those reported by Peat, *et al.*[3] Apparently, in the experiments of the investigators mentioned above, the slow rate of hydrolysis of the terminal bonds of the tetraoside and bond 1 of the trioside escaped detection.

Enzymatic hydrolysis of methyl β-D-maltoside by yeast maltase and fungal amyloglucosidase occurred by routes similar to those for the methyl α-D-maltoside. Likewise, salivary amylase hydrolyzed methyl β-D-maltotrioside and methyl β-D-maltotetraoside by patterns very similar to those for the α-series of compounds.

Experimental

Enzymes.—*Macerans* amylase was prepared from *Bacillus macerans* by methods previously described.[12] The enzyme sample used in this study possessed 1.4 units[13] of activity per ml. It was free of hydrolytic activity as indicated by the absence of reducing products in digests of methyl α-D-glucoside and cyclohexaamylose. Crystalline salivary amylase[14] was the gift of Dr. Jytte Muus. In our experiments, a solution of the enzyme containing 28 SKB units[15] per ml. was employed for hydrolyzing the triosides and a solution containing 9.0 SKB units per ml. for hydrolyzing the tetraoside. Yeast maltase of *Saccharomyces cerevisiae* was prepared by a published procedure[16] with slight modifications. Amyloglucosidase of *Aspergillus niger* was obtained in a highly purified form by a chromatographic procedure employing cellulose ion exchange material.[17] The details of the preparation of this enzyme will be published elsewhere.

Preparation of the Methyl Glycosides.—Four grams of methyl α-D-glucoside and 4 g. of cyclohexaamylose[18] were dissolved in 80 ml. of water and mixed with 20 ml. of *macerans* amylase solution (1.4 units per ml.). Aliquots of the digest were examined for reaction products by paper chromatography (solvent system, *n*-butyl alcohol–ethyl alcohol–water, 4:1:1 by volume). The non-reducing compounds were detected with permanganate-periodate reagent[18] and reducing compounds with copper sulfate and phosphomolybdic acid reagents.[11] In the *macerans* digest there was produced a series of non-reducing compounds (R_f values shown in Table I) but no reducing compounds. A plot of the R_f values in Table I *versus* degree of polymerization as outlined by French and Wild[19] resulted in a straight line relationship. As pointed out by French and Wild such a linear relationship indicates strongly that structural irregularities do not exist in the series of compounds.

The digest of non-reducing compounds was concentrated to a volume of approximately 10 ml., mixed with 5 g. of washed cellulose powder,[20] and taken to dryness in a vacuum desiccator. The material was stirred with a small volume of a solution of *n*-butyl alcohol–ethyl alcohol–water (4:1:1 by volume) and introduced on a cellulose column (70 g.; 300 mm. × 40 mm.) which had been thoroughly washed with the above solvent mixture. The development of the column was continued with the *n*-butyl alcohol–ethyl alcohol–water solvent until 250 fractions (10 ml.) were collected. Aliquots of these fractions were heated with diphenylamine reagent[11] to locate the tubes containing carbohydrates and the identities of the compounds in the various fractions were then determined by qualitative paper chromatography. Pure methyl α-D-glucoside was present in fractions 21 to 38, pure methyl α-D-maltoside in fractions 42 to 78, pure methyl α-D-maltotrioside in fractions 90–135 and pure methyl α-D-maltotetraoside in fractions 162–195. The fractions containing the individual pure components were combined, concentrated under vacuum to a small volume and allowed to stand at room temperature. Methyl α-D-glucoside, methyl α-D-maltotrioside, methyl α-D-maltotetraoside were obtained in crystalline form from the solutions. Methyl α-D-maltoside came out of solution as a sirup which after evaporation of the solvent was dried in a vacuum desiccator to constant weight. The yields of compounds were as follows: methyl α-D-glucoside, 0.6 g.; methyl α-D-maltoside, 0.5 g.; methyl α-D-maltotrioside, 0.4 g.; methyl α-D-maltotetraoside 0.3 g.

A similar procedure was used for preparing the methyl β-D-glycoside series of malto-oligosaccharides. In this experiment 4 g. of methyl β-D-glucoside was used as the co-substrate. The chromatographic behavior of the methyl β-series of compounds was quite similar to that of the α-series (see Table I). The methyl β-D-glycosides of D-glucose and maltose were obtained in crystalline form while those of maltotriose and maltotetraose were obtained in amorphous form.

Methyl α-D-Glucoside.—The compound with R_f value 0.86 isolated from the enzymatic digest of methyl α-D-glucoside and cyclohexaamylose proved to be methyl α-D-glucoside. The melting point of the compound was 167° and the mixed melting point with an authentic sample of methyl α-D-glucoside was 166°. The specific rotation of +157° agrees with the literature value for methyl-α-D-glucoside. An X-ray diffraction pattern[21] of the material isolated from

(9) J. H. Pazur and T. Budovich, *Science*, **121**, 702 (1955).

(10) The glucosidic bonds in the oligosaccharides are numbered consecutively beginning with the α-D-(1→4)-bond joining the reducing glucose unit (or methyl glucoside residue) to the remainder of the molecule.

(11) J. H. Pazur, *J. Biol. Chem.*, **205**, 75 (1953).

(12) D. French, M. L. Levine, J. H. Pazur and E. Norberg, THIS JOURNAL, **71**, 353 (1949).

(13) E. B. Tilden and C. S. Hudson, *J. Bact.*, **43**, 527 (1942).

(14) J. Muus, *Compt. rend. trav. Lab. Carlsberg*, **28**, 317 (1953).

(15) R. M. Sandstedt, E. Kneen and M. J. Blish, *Cereal Chem.*, **16**, 712 (1939).

(16) R. Willstätter and E. Bamann, *Z. physiol. Chem.*, **151**, 242 (1926).

(17) M. B. Rhodes, P. R. Azari and R. E. Feeney, *J. Biol. Chem.*, **230**, 399 (1958).

(18) R. U. Lemieux and H. F. Bauer, *Anal. Chem.*, **26**, 920 (1954).

(19) D. French and G. M. Wild, THIS JOURNAL, **75**, 2612 (1953).

(20) Solka-Floc BW-200, Brown Co., Berlin, N. H.

(21) All X-ray patterns were obtained by Professor W. C. Robison, University of Nebraska Instrumentation Laboratory, Lincoln, Neb.

the enzymatic digest was identical to the pattern of methyl α-D-glucoside.

Methyl α-D-Maltoside.—The compound with R_f value 0.54 was non-reducing and possessed a specific rotation of +184°. In an acid hydrolysate of the compound (0.05 *N* HCl at 100° for 1 hour) two reducing fragments, one with R_f value identical to that of D-glucose and the other with R_f value identical to that of maltose were detected by paper chromatography. The concentration of maltose in the hydrolysate was considerably lower than that of D-glucose even in the early stages of hydrolysis, indicating that the α-D-(1→4)-glucosidic bond in the compound is hydrolyzed more rapidly than the methyl glucosidic bond.

The compound (0.05 g.) dissolved in 0.5 ml. of water was treated with 0.5 ml. of a solution of fungal amyloglucosidase. After a 6-hour incubation period the sample was heated at 100° for 5 minutes to inactivate the enzyme and the compounds in the hydrolysate were separated on cellulose powder column (7 g.; 230 mm. × 12 mm.) with the *n*-butyl alcohol–ethyl alcohol–water solvent system. From the combined eluates in tubes 23–32, there was obtained a crystalline compound with m.p. 166° and with X-ray diffraction pattern identical to that of methyl α-D-glucoside. From the eluates in tubes 38–50 there was obtained a crystalline reducing compound with specific rotation of +53°. The compound yielded an X-ray diffraction pattern identical to that of D-glucose.

Methyl α-D-Maltotrioside.—The crystalline non-reducing compound with R_f value 0.22 and specific rotation of +202° is evidently methyl α-D-maltotrioside. The melting point of the compound was 145–147°.

Anal. Calcd. for $C_{19}H_{34}O_{16}$: C, 44.05; H, 6.56. Found: C, 43.77; H, 6.90.

The X-ray diffraction data on the compound were: 12.01[22]–70,[23] 9.64–60, 7.93–50, 7.12–80, 6.47–80, 5.45–50, 5.19–60, 4.80–50, 4.56–40, 4.37–40, 4.08–100, 3.77 (double)–80, 3.58–70, 3.42–50, 3.19–30, 3.08–30, 2.94–30, 2.81–20.

In preliminary experiments, it was found that the methyl α-D-maltotrioside was hydrolyzed by crystalline salivary amylase to D-glucose, maltose, methyl α-D-glucoside and methyl α-D-maltoside. This action pattern on methyl maltotrioside is very similar to that observed earlier for the action of salivary amylase on maltotriose.[9] A sample of 0.12 g. of the trioside in 2.0 ml. of water was treated with 2.0 ml. of salivary amylase solution (28 SKB units per ml.). After a 12-hour incubation period at room temperature, the products in the hydrolysate were separated on a cellulose powder column (20 g.; 220 mm. × 22 mm.) with *n*-butyl alcohol–ethyl alcohol–water (4:1:1 by volume) solvent. Paper chromatograms of the eluate showed that tubes 25 to 36 contained a compound with R_f value of methyl α-D-glucoside. The samples from these tubes were combined, concentrated to a small volume and allowed to stand at room temperature. On further evaporation of the solvent a crystalline compound with m.p. 166° and with an X-ray diffraction pattern identical to that of methyl α-D-glucoside was obtained. The solution from tubes 91 to 135 contained a reducing compound with R_f value typical for maltose. There was obtained from the combined solution from these tubes a crystalline product with a specific rotation of +129° and with an X-ray diffraction pattern identical to that of maltose.

Methyl α-D-Maltotetraoside.—As indicated in Table I, the R_f value of the fourth member of the series of compounds was 0.06. The specific rotation of +201° for the crystalline product is lower than that reported by Peat, *et al.*,[3] indicating that the compound contains some solvent of crystallization. Quantitative values for the elemental composition of the compound agree with the values for a dihydrate of the compound.

Anal. Calcd. for $C_{25}H_{44}O_{21}.2H_2O$: C, 41.92; H, 6.72. Found: C, 41.85; H, 6.68.

The specific rotation calculated on the anhydrous basis is +212° and is in good agreement with the literature value.[3] The X-ray diffraction data on the crystalline compound were: 8.54[22]–70[23], 7.86–50, 7.34–10, 6.47–20, 5.87 (double)–80, 5.10–100, 4.87–80, 4.48–60, 4.05–50, 3.85–100, 3.63–80, 3.35–40, 3.14–50, 3.02–70, 2.85–60, 2.76–20, 2.68–30, 2.62–40, 2.56–10, 2.49–20.

A sample of 0.005 g. of the compound dissolved in 0.1 ml. of water was treated with 0.1 ml. of salivary amylase (9.0 SKB units per ml.) for 3 hours. Analysis of the 0- and 3-hour aliquots by paper chromatography showed the presence of three reducing compounds in the 3-hr. digest. The one present in highest concentration had an R_f value of 0.42 (R_f value of maltose under the same conditions 0.42) and the other two present only in trace amounts with R_f values of 0.68 and 0.35 (R_f values of D-glucose and maltotriose, 0.67 and 0.35, respectively).

Methyl β-D-Glucoside.—The fastest moving compound in the enzymatic digest of methyl β-D-glucoside and cyclohexaamylose proved to be methyl β-D-glucoside. The m.p. of this compound was 105°; mixed m.p. with authentic methyl β-D-glucoside was 105°. The specific rotation of −33° and the data for X-ray diffraction diagram of the compound agree with the values for methyl β-D-glucoside.

Methyl-β-D-Maltoside.—The second member of the β-series of compounds also was obtained in crystalline form; m.p. 110° and specific rotation +84°. These values agree with the literature values for methyl β-D-maltoside.[24] The X-ray diffraction pattern of this compound was identical to that obtained for pure methyl β-D-maltoside.[8]

The compound (0.01 g.) dissolved in 0.1 ml. of water was incubated with 0.1 ml. of yeast maltase solution at 30° for 0, 1, 2, 4 and 6 hours. In the 1-hr. hydrolysate there was detected by paper chromatography a reducing compound and a non-reducing compound which are, in all likelihood, D-glucose and methyl β-D-glucoside. The concentration of these compounds increased progressively up to the 6-hr. sample.

Methyl β-D-Maltotrioside and Methyl β-D-Maltotetraoside.—The third and fourth members of the β-series were also obtained in chromatographically pure form; R_f values for these compounds were 0.24 and 0.07, respectively. On the basis of chromatographic behavior and the experience in the preparation and characterization of the α-series of compounds these compounds are probably methyl β-D-maltotrioside and methyl β-D-maltotetraoside. Reducing fragments detected by paper chromatography in hydrolysates of these compounds with salivary amylase were D-glucose and maltose from the trioside and D-glucose, maltose and maltotriose from the tetraoside. Detailed structural information on these two compounds has not yet been obtained.

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(22) Interplanar spacings, Å., CuKα radiations.

(23) Relative intensities on the basis of 100 for the strongest line.

(24) T. Schoch, E. J. Wilson, Jr., and C. S. Hudson, THIS JOURNAL, **64**, 2871 (1942).