Stereoselective Thioglycoside Syntheses. Part 6.† Aryl 4-Thiomalto-oligosaccharides as Chromogenic Substrates for Kinetic Studies with α-Amylase

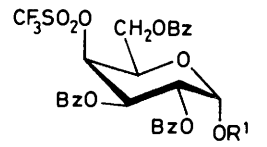
Michèle Blanc-Muesser, Jacques Defaye,* and Hugues Driguez
Centre de Recherches sur les Macromolécules Végétales, Centre National de la Recherche Scientifique Grenoble, 68 X, 38402 St-Martin-d'Hères, France
Guy Marchis-Mouren and Christiane Seigner
Institut de Chimie Biologique, Université d'Aix-Marseille, Place Victor Hugo, 13331 Marseille, France

Nucleophilic bimolecular substitution, of either *o*- or *p*-nitrophenyl 2,3,6-tri-*O*-benzoyl-4-*O*-trifluoromethylsulphonyl-α-D-galactopyranoside (**1**) or (**2**) with the sodium salt of 1-thio-α-D-glucopyranose in hexamethylphosphoramide afforded, after the usual deprotection sequences, *o*- and *p*-nitrophenyl 4-thio-α-maltosides (**7**) and (**8**). A similar synthetic scheme with (**1**) and the 1-α-thiolate of 4-thiomaltose (**12**) led to *o*-nitrophenyl 4,4′-dithio-α-maltotrioside (**15**). These 4-thio-oligosaccharides and their corresponding oxygen analogues were used, in comparative assays, as chromogenic substrates with porcine and human pancreatic α-amylases. In both series, enzymic velocity was higher for the maltotrioside derivatives than for the maltodisaccharides. *o*-Nitrophenyl glycosides behave as better substrates than the corresponding *para* isomers. Replacement of intersaccharide oxygen atoms by sulphur increased slightly the Michaelis constant, but had a negative effect on the hydrolysis rate. As a consequence, 4-thiomaltosyloligosaccharides were less sensitive substrates for pig pancreatic α-amylase as compared with their *O*-glycosyl counterparts. However, as the former class of compounds is split exclusively at the chromogenic site, they appear to be substrates of interest for direct kinetic studies with α-amylases.

α-Amylase [(1 ⟶ 4)-α-D-glucan 4-glucanohydrolase, E.C. 3.2.1.1], an enzyme widely distributed in animals, plants and micro-organisms,[2] acts mostly on starch to give maltose and α-limit dextrins as final products.[3] Partly owing to the molecular complexity of its substrates and to variations in the action pattern of enzymes from various sources,[4] the mechanism of action of α-amylase is still incompletely understood and, as a result, reliable characterization and evaluation processes have still to be found. Nitrophenyl α-malto-oligosaccharides, which can release a chromophoric aglycone under enzymic catalysis, have been recently developed.[5-7] However, a limitation of their use in kinetic studies is their possible cleavage[8] at sites not concerned with the release of the chromophore. Coupled α-glucosidase assays have been proposed to overcome this difficulty.[7] Another approach lies in substrate analogues which would be susceptible to the enzyme action exclusively at the aglyconic bond. In view of the established resistance of 1-thioglycosides to enzymic hydrolysis, aryl 4-thiomalto-oligosaccharides were seen as a solution to this problem and this paper deals with the synthesis of *o*- and *p*-nitrophenyl 4-thio-α-maltoside (**7**) and (**8**) and *o*-nitrophenyl 4,4′-dithio-α-maltotrioside (**15**) and their behaviour with human and porcine pancreatic α-amylase.

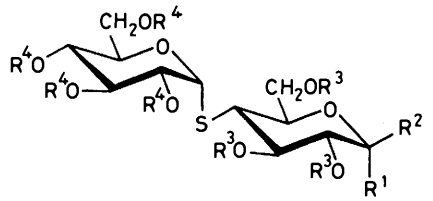
Results and Discussion

Previous papers in this series[9,10] described the stereoselective synthesis of *O*-aryl, *S*-aryl glycosides and glycosyl 1-thioglycosides using the concept of nucleophilic enhancement of a phenoxide or a sulphide anion in a dipolar aprotic solvent, hexamethylphosphoramide (HMPA). This approach, which led further to the preparation of 4-thio(1 ⟶ 4)-linked disaccharides,[11,12] is now extended to aryl 4-thiomaltosides and an aryl 4,4′-dithiomaltotrisaccharide.



(**1**) $R^1 = C_6H_4NO_2-o$

(**2**) $R^1 = C_6H_4NO_2-p$



(**3**) $R^1 = OC_6H_4NO_2-o$, $R^2 = H$, $R^3 = Bz$, $R^4 = Ac$
(**4**) $R^1 = OC_6H_4NO_2-p$, $R^2 = H$, $R^3 = Bz$, $R^4 = Ac$
(**5**) $R^1 = OC_6H_4NO_2-o$, $R^2 = H$, $R^3 = R^4 = Ac$
(**6**) $R^1 = OC_6H_4NO_2-p$, $R^2 = H$, $R^3 = R^4 = Ac$
(**7**) $R^1 = OC_6H_4NO_2-o$, $R^2 = R^3 = R^4 = H$
(**8**) $R^1 = OC_6H_4NO_2-p$, $R^2 = R^3 = R^4 = H$
(**9**) $R^1 = H$, $R^2 = Cl$, $R^3 = R^4 = Ac$
(**10**) $R^1 = SBz$, $R^2 = H$, $R^3 = R^4 = Ac$
(**11**) $R^1 = SAc$, $R^2 = H$, $R^3 = R^4 = Ac$
(**12**) $R^1 = SNa$, $R^2 = R^3 = R^4 = H$

Bz = benzoyl

Either *o*- or *p*-nitrophenyl 2,3,6-tri-*O*-benzoyl-α-D-galactopyranoside[10] was quantitatively esterified with trifluoromethanesulphonic anhydride in pyridine. The resulting C-4 trifluoromethanesulphonates (**1**) or (**2**) were then allowed

† Part 5, J. Defaye, H. Driguez, E. Ohleyer, C. Orgeret, and C. Viet, *Carbohydr. Res.* 1984, in the press; Part of a presentation at the 1st International Symposium on Use of Enzymes in Food Technology, Versailles, France, 5—7 May 1982 (see ref. 1).

Table 1. ^{1}H N.m.r. spectral date (δ) for ring protons for the acylated α-(1 ⟶ 4)-thiodisaccharides (**3**), (**4**), (**6**), (**9**), (**10**), and (**11**), and the *O*-acylated α-(1 ⟶ 4)-thiotrisaccharides (**13**) and (**14**) in deuteriochloroform. *J* values are in Hz[a,b,c]

Proton	Compounds							
	(3)	(4)	(6)	(9)	(10)	(11)	(13)	(14)
1-H	6.06 d ($J_{1,2}$ 3.5)	6.09 d ($J_{1,2}$ 3.5)	5.78 d ($J_{1,2}$ 3.5)	5.29 d ($J_{1,2}$ 8.5)	6.41 d ($J_{1,2}$ 5.0)	6.15 d ($J_{1,2}$ 5.0)	6.05 d ($J_{1,2}$ 3.5)	5.79 d ($J_{1,2}$ 4.0)
2-H	5.34 dd ($J_{2,3}$ 10.0)	5.33 dd ($J_{2,3}$ 10.0)	4.95 dd ($J_{2,3}$ 10.0)	4.98 t ($J_{2,3}$ 8.5)	5.19 dd ($J_{2,3}$ 10.0)	5.10 dd ($J_{2,3}$ 10.0)	5.35 dd ($J_{2,3}$ 10.0)	4.90 dd ($J_{2,3}$ 10.0)
3-H	6.28 t ($J_{3,4}$ 10.0)	6.25 t ($J_{3,4}$ 10.0)	5.75 br t ($J_{3,4}$ 11.0)	5.25 dd ($J_{3,4}$ 11.0)	5.38 t ($J_{3,4}$ 10.0)	5.24 t ($J_{3,4}$ 10.0)	6.28 t ($J_{3,4}$ 10.0)	5.71 t ($J_{3,4}$ 10.0)
4-H	3.41 br t ($J_{4,5}$ 11.0)	3.37 t ($J_{4,5}$ 11.0)	3.12 t ($J_{4,5}$ 11.0)	3.12 t ($J_{4,5}$ 11.0)	3.10 br t ($J_{4,5}$ 11.0)	3.04 br t ($J_{4,5}$ 10.5)	3.38 br t ($J_{4,5}$ 11.0)	3.08 br t ($J_{4,5}$ 11.0)
5-H	4.46 m ($J_{5,6a}$ 2.0)	4.24—4.40 m[c] ($J_{5,6a}$ 2.0)	3.94 o ($J_{5,6a}$ 2.0)	5.77 o ($J_{5,6a}$ 2.0)	4.04 o ($J_{5,6a}$ 2.0)	3.92 o[c] ($J_{5,6a}$ 2.5)	4.44 m ($J_{5,6a}$ 2.5)	
6-H_a	4.85 dd[c] ($J_{a,b}$ 12,0)	4.86 dd[c] ($J_{a,b}$ 12.0)	4.59 dd ($J_{a,b}$ 12.0)	4.68 dd ($J_{a,b}$ 12.0)	4.57 dd ($J_{a,b}$ 12.0)	4.55 dd[c] ($J_{a,b}$ 12.0)	4.85 dd ($J_{a,b}$ 12.0)	
6-H_b	4.65 dd[c] ($J_{5,6b}$ 7.0)	4.58 dd[c] ($J_{5,6b}$ 8.0)	4.14—4.29 m	4.21 dd ($J_{5,6b}$ 6.5)	4.22—4.30 m	4.23 dd[c] ($J_{5,6b}$ 5.0)	4.69 dd ($J_{5,6b}$ 7.0)	
1′-H	5.85 d ($J_{1,2}$ 6.0)	5.84 d ($J_{1,2}$ 6.0)	5.93 d ($J_{1,2}$ 6.0)	5.93 d ($J_{1,2}$ 6.0)	5.94 d ($J_{1,2}$ 6.0)	5.90 d ($J_{1,2}$ 6.0)	5.74 d ($J_{1,2}$ 6.0)	5.83 d ($J_{1,2}$ 6.0)
2′-H	4.97 dd ($J_{2,3}$ 10.5)	4.97 dd ($J_{2,3}$ 10.0)	4.98 dd ($J_{2,3}$ 10.0)	4.96 dd ($J_{2,3}$ 10.0)	4.99 dd ($J_{2,3}$ 10.0)	4.97 dd ($J_{2,3}$ 10.5)	4.82 dd ($J_{2,3}$ 10.0)	4.84 dd ($J_{2,3}$ 10.0)
3′-H	5.19 dd ($J_{3,4}$ 10.0)	5.20 dd ($J_{3,4}$ 9.5)	5.27 dd ($J_{3,4}$ 9.5)	5.26 t ($J_{3,4}$ 10.0)	5.29 t ($J_{3,4}$ 10.0)	5.27 dd ($J_{3,4}$ 10.0)	5.24 t ($J_{3,4}$ 10.0)	5.34 br t ($J_{3,4}$ 10.0)
4′-H	5.03 t ($J_{4,5}$ 10.0)	5.03 t ($J_{4,5}$ 9.5)	5.08 t ($J_{4,5}$ 9.5)	5.07 t ($J_{4,5}$ 10.0)	5.09 t ($J_{4,5}$ 10.0)	5.07 t ($J_{4,5}$ 10.0)	2.97 t ($J_{4,5}$ 10.0)	3.04 br t ($J_{4,5}$ 11.0)
5′-H	4.29—4.41 m	4.24—4.40 m[c]	4.14—4.29 m ($J_{5,6a}$ 4.0)	4.2—4.26 m ($J_{5,6a}$ 4.0)	4.22—4.30 m ($J_{5,6a}$ 4.0)	4.19—4.26 m[c] ($J_{5,6a}$ 4.0)	4.26—4.38 m	
6′-H_a	4.29—4.41 m[c]	4.24—4.40 m[c]	4.33 dd ($J_{a,b}$ 12.0)	4.35 dd ($J_{a,b}$ 12.0)	4.34 dd ($J_{a,b}$ 12.0)	4.33 dd[c] ($J_{a,b}$ 12.0)	4.56—4.64 m	
6′-H_b	4.10 m[c]	4.14 m[c]	4.14 dd ($J_{5,6b}$ 2.0)	4.12 dd ($J_{5,6b}$ 12.0)	4.13 dd ($J_{5,6b}$ 2.0)	4.11 dd[c] ($J_{5,6b}$ 2.0)	4.26—4.38 m	
1″-H							5.84 d ($J_{1,2}$ 6.0)	5.88 d ($J_{1,2}$ 6.0)
2″-H							4.93 dd ($J_{2,3}$ 10.0)	4.96 dd ($J_{2,3}$ 10.0)
3″-H							5.23 t ($J_{3,4}$ 10.0)	5.26 t ($J_{3,4}$ 10.0)
4″-H							5.05 t ($J_{4,5}$ 10.0)	5.08 t ($J_{4,5}$ 10.0)
5″-H							4.16—4.26 m	
6″-H_a							4.26—4.38 m ($J_{a,b}$ 12.0)	
6″-H_b							4.10 dd ($J_{5,6b}$ 2.0)	

[a] The primed numbers refer to the protons of the second α-D-glucopyranosyl unit, the double-primed numbers to the third α-D-glucopyranosyl unit. [b] Abbreviations: d, doublet, dd, doublet of doublets; m, multiplet; o, octet; t, triplet. [c] The assignments may be reversed in the same column.

to react smoothly at 0 °C for about 4 h with the sodium salt of 1-thio-α-D-glucopyranose leading, after the usual *O*-acetylation procedure, to the expected peracylated nitrophenyl α-4-thiodisaccharides (**3**) and (**4**) with respective yields of 51 and 49%. Characterization of both disaccharides is straightforward from an examination of their ^{1}H n.m.r. spectra (Table 1), which show distinctly, in each case, both anomeric protons with the expected deshielding for the *O*-aryl substitution. The respective anomeric coupling constants of 3.5 and 6 Hz for the *O*-glycosyl and *S*-glycosyl[11] moieties in both disaccharides are in agreement with the expected α-(D) configuration at both anomeric sites. De-*O*-acylation with sodium methoxide in methanol of acyldisaccharides (**3**) and (**4**) afforded *o*- and *p*-nitrophenyl 4-thio-α-maltoside (**7**) and (**8**) as solid foams, which needed, however, a further re-acetylation, to produce (**5**) and (**6**), and de-*O*-acetylation sequence in order to get rid of the simultaneously formed methyl benzoate.

A similar stepwise procedure was used for the synthesis of *o*-nitrophenyl α-4,4′-dithiomaltotrioside (**15**), the key compound for this synthesis being the hepta-*O*-acetyl-1-*S*-acetyl-1,4-dithio-α-maltose (**11**). This 1,4-dithiodisaccharide is now readily available from octa-*O*-acetyl-4-thio-β-maltose.[11] The action of dichloromethyl methyl ether in the presence of boron trifluoride–diethyl ether on this compound, according to the procedure developed by Bognár and co-workers,[13] led to an 83% yield of the corresponding crystalline β-chloride (**9**) which was further converted, by the action of sodium thiobenzoate in HMPA at room temperature, into 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-1-*S*-benzoyl-1,4-α-D-dithiomaltose (**10**) in 57% yield. The anomeric configuration for this disaccharide is easily inferred from its ^{1}H n.m.r. spectrum (Table 1), which shows two doublets at δ 6.41 and 5.94 for 1-H and 1′-H respectively with vicinal couplings of 5 and 6 Hz, in agreement, in the 1-thioglycoside series,[9] with an α-(D) anomeric configuration at both sites.

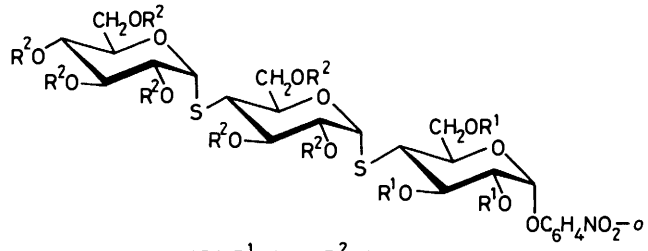
Activation of the anomeric sulphur atom in the 1-thio-

Table 2. Kinetic parameters for the hydrolysis of nitrophenyl malto-oligosaccharides (**7**), (**8**), and (**15**)—(**18**)

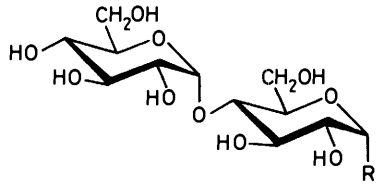
Substrate	K_M (mM)	kat mol^{-1}	kat mol^{-1}/K_M
α-D-Glc*p*-(1 ⟶ 4)-*S*-α-D-Glc*p*-$C_6H_4NO_2$-*o* (**7**)	1.15	0.043	0.0374
α-D-Glc*p*-(1 ⟶ 4)-*S*-α-D-Glc*p*-$C_6H_4NO_2$-*p* (**8**)	9.73	0.057	0.005 86
α-D-Glc*p*-(1 ⟶ 4)-*S*-α-D-Glc*p*-(1 ⟶ 4)-*S*-α-D-Glc*p*-$C_6H_4NO_2$-*o* (**15**)	1.69	3.30	1.952
	4.4*	4.05*	0.920*
α-D-Glc*p*-(1 ⟶ 4)-α-D-Glc*p*-$C_6H_4NO_2$-*o* (**16**)	0.47	0.187	0.398
α-D-Glc*p*-(1 ⟶ 4)-α-D-Glc*p*-$C_6H_4NO_2$-*p* (**17**)	14	0.134	0.0096
α-D-Glc*p*-(1 ⟶ 4)-α-D-Glc*p*-(1 ⟶ 4)-α-D-Glc*p*-$C_6H_4NO_2$-*o* (**18**)	0.54	30.68	56.81

* Assays using human pancreatic α-amylase. All other assays were carried out using porcine pancreatic α-amylase.[15]

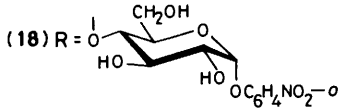
benzoate (**10**) by the action of sodium methylate in methanol, followed by a direct attempt at displacement of the C-4 triflate in the *o*-nitrophenyl α-D-galactopyranoside (**1**) in HMPA, unexpectedly failed. However, when the thiobenzoate (**10**) was previously converted into the corresponding 1-thioacetyl derivative (**11**), through successive sodium methylate de-*O*-acylation followed by the action of an acetic anhydride–pyridine mixture, the corresponding thiolate (**12**) exhibited the expected nucleophilic reactivity with the 4-*O*-triflylgalactoside (**1**). This led us to suspect that methyl benzoate may inhibit the condensation reaction of (**12**) with the triflate (**1**), probably through competition with the sulphur nucleophile.



(**13**) R^1 = Bz, R^2 = Ac
(**14**) R^1 = R^2 = Ac
(**15**) R^1 = R^2 = H



(**16**) R = $OC_6H_4NO_2$-*o*
(**17**) R = $OC_6H_4NO_2$-*p*



The resulting *o*-nitrophenyl 4,4′-dithiotrisaccharide derivative was isolated from the condensation mixture, after pyridine–acetic anhydride *O*-acetylation, as its hepta-*O*-acetyl tri-*O*-benzoyl derivative (**13**) in 67% yield. This dithiotrisaccharide derivative showed an almost first-order ^{1}H n.m.r. spectrum at 250 MHz (Table 1), which enabled assignment of most of the proton resonances and, noteworthy, of the three anomeric protons 1-H, 1′-H and 1″ H with respective vicinal couplings of 3.5, 6, and 6 Hz, thus confirming the α-(D) configuration at all three anomeric sites. A further characterization of this dithiotrisaccharide was made through its conversion into its corresponding deca-*O*-acetyl derivative (**14**) which showed a similarly well resolved ^{1}H n.m.r. spectrum with almost identical anomeric coupling constants.

As already noticed,[14] *o*-nitrophenyl α-D-glycosides appear to be extremely sensitive to base, since migration of the aglycone may occur. De-*O*-acetylation of the per-*O*-acetylated dithiomaltotrioside (**14**) into (**15**) was thus managed using minimum amounts of sodium methoxide in a large volume of methanol.

Enzymic hydrolysis of *o*- and *p*-nitrophenyl 4-thio-α-maltoside (**7**) and (**8**) and *o*-nitrophenyl 4,4′-dithio-α-maltotrioside (**15**) were compared, in kinetic experiments with mammalian α-amylases, with their *O*-glycosyl analogues, *o*- and *p*-nitrophenyl α-maltoside[10] (**16**) and (**17**) and *o*-nitrophenyl α-maltotrioside[6] (**18**) (Table 2). Although Michaelis constants are not supposed to reflect entirely the affinity of an enzyme for its substrate, cross comparison of K_M values by pairs between compounds (**7**), (**16**); (**8**), (**17**); and (**15**), (**18**) shows, in a rough estimation, that the enzymic affinity is not appreciably modified with 4-thiomaltosides as compared with their *O*-glycosyl analogues. However, as determined from reciprocal Lineweaver–Burk plots, α-amylase had about 10—30 times more affinity for *o*-nitrophenyl disaccharides (**7**) and (**16**) when compared with their *p*-nitrophenyl isomers (**8**) and (**17**), while the specific activity was at the same time modified to a lesser extent. Similar behaviour was observed by Wallenfels *et al.*[7] with human pancreatic and salivary α-amylases.

Comparison by pairs between Michaelis constants of *o*-nitrophenyl 4-thio-α-maltoside (**7**), the corresponding thiomaltotrioside derivative (**15**), and their oxygen analogues (**16**) and (**18**) (Table 2) suggests furthermore that these substrates are bound with the same efficiency to the active site of the enzyme. However, the release of the nitrophenyl aglycone, through enzymic catalysis, appears to be seriously affected, depending both on the length of the oligosaccharide chain, and the presence of intersaccharide sulphur linkages. Thus, enzymic activity on *o*-nitrophenyl α-maltotrioside (**18**) is enhanced by a factor of 170, as compared with the *o*-nitrophenyl maltoside (**16**). A similar comparison with the 4,4′-dithiotrisaccharide (**15**), and the thiodisaccharide (**7**), results only in an 80-fold enhancement of this activity. Nitrophenyl 4-thiomaltosyl oligosaccharides thus exhibit a lower sensitivity with pancreatic human and porcine α-amylases[15] than their nitrophenyl maltosyl analogues, but these substrates can be split exclusively at the aglyconic bond. Thus *o*-nitrophenyl α-maltoside (**16**), when subjected to the action of pancreatic porcine α-amylase, at a substrate:enzyme ratio of 400:1, shows production of the expected maltose, but together with *o*-nitrophenyl α-D-glucopyranoside and maltotriose. In a comparative experiment, *o*-nitrophenyl 4-thio-α-maltoside (**7**) leads exclusively to formation of *o*-nitrophenol and 4-thiomaltose. This comparative behaviour is an argument in favour of the use of aryl thio-oligosaccharides as substrate analogues for kinetic activity evaluations of α-amylase, and possibly of other glycanases.[16]

Experimental

Solutions were dried (Na_2SO_4) and evaporated under reduced pressure at temperatures below 45 °C. T.l.c. was performed on silica gel (Merck F 254, Merck, Darmstadt, Germany). Preparative chromatography used silica gel (Merck 60, 70—230 Mesh). Optical rotations were determined with a Quick polarimeter (Roussel et Jouan) at room temperature. The ^1H n.m.r. spectra (Table 1) were recorded at 250 MHz with a Cameca (Thomson-CSF Paris) spectrometer. Assignments were confirmed by double irradiation or the INDOR technique. The chemical shifts are reported in δ relative to internal $SiMe_4$ and the coupling constants (in parentheses) are in Hz.

o- *and* p-*Nitrophenyl* 2,3,6-*Tri*-O-*benzoyl*-4-O-*trifluoromethylsulphonyl*-α-D-*galactopyranoside* (**1**) *and* (**2**).—To a solution of either *o*- or *p*-nitrophenyl 2,3,6-tri-*O*-benzoyl-α-D-galactopyranoside[10] (735 mg, 1.2 mmol) in pyridine at 0 °C was added trifluoromethanesulphonic anhydride (1 ml, 5.9 mmol). After being stirred for 30 min at 0 °C and then for 2 h at ambient temperature, the reaction mixture was diluted with dichloromethane (100 ml) and washed successively with ice-cold aqueous solutions of potassium hydrogen sulphate (10%; 2 × 100 ml) and sodium hydrogen carbonate (saturated; 100 ml), and water (100 ml). Aqueous washings were then back-extracted with dichloromethane (2 × 100 ml). The dried organic solutions gave, after concentration, the syrupy 4-*O*-triflyl derivatives (**1**) and (**2**) which were used without further purification or characterization.

o- *and* p-*Nitrophenyl* 2,3,6-*Tri*-O-*benzoyl*-4-S-(2,3,4,6-*tetra*-O-*acetyl*-α-D-*glucopyranosyl*)-4-*thio*-α-D-*glucopyranoside* (**3**) *and* (**4**).—2,3,4,6-Tetra-*O*-acetyl-1-*S*-acetyl-1-thio-α-D-glucopyranose[11] (406 mg, 1 mmol) was dissolved in methanol (10 ml) containing sodium methoxide (1M; 1.1 ml). After being kept overnight at room temperature, the solution was evaporated. The residue, dried *in vacuo* over phosphorus pentaoxide, was suspended in HMPA (3 ml) and either of the above trifluoromethane sulfonates (**1**) [for (**3**)] or (**2**) [for (**4**)] was then added. After 4 h at room temperature, one major component was observed in each case on t.l.c. [chloroform–methanol–water (65:25:4 v/v/v)]. Acetylation overnight by addition of acetic anhydride–pyridine (3:2 v/v; 5 ml) to the reaction mixture, and addition of dichloromethane (100 ml), followed by the usual extraction process,[11] led to an oil which was purified on a silica gel column (35 g) with chloroform–diethyl ether (6:1 v/v) as eluant which gave *compound* (**3**) [from (**1**)] (498 mg, 51%) $[\alpha]_D^{20}$ +181° (*c* 1.3 in chloroform) (Found: C, 58.7; H, 4.9; N, 1.4; S, 3.2. $C_{47}H_{45}NO_{19}S$ requires C, 58.81; H, 4.72; N, 1.46; S, 3.34%), or *compound* (**4**) [from (**2**)] (470 mg, 49%), m.p. 108—115 °C (from diethyl ether); $[\alpha]_D^{20}$ +210° (*c* 0.38 in chloroform) (Found: C, 58.9; H, 4.7; N, 1.4; S, 3.5%).

o- *and* p-*Nitrophenyl* 2,3,6-*Tri*-O-*acetyl*-4-S-(2,3,4,6-*tetra*-O-*acetyl*-α-D-*glucopyranosyl*)-4-*thio*-α-D-*glucopyranoside* (**5**) *and* (**6**).—To a solution of the acylated 4-thiodisaccharide (**3**) [for (**5**)] or (**4**) [for (**6**)] (100 mg, 0.1 mmol) in methanol (40 ml) was added sodium methoxide in methanol (0.7M; 0.13 ml). After 78 h, the solution was neutralized with Amberlite IR 120 (H^+), filtered, and concentrated to dryness. Acetylation and work-up as described[11] led to a syrup, which was purified on a silica gel column [35 g; chloroform–diethyl ether (6:1 v/v) as eluant] to give *compound* (**5**) [from (**3**)] (43 mg, 50%), $[\alpha]_D^{20}$ +240° (*c* 0.64 in chloroform) (Found: C, 49.4; H, 5.1; N, 1.8; S, 4.1. $C_{32}H_{39}NO_{19}S$ requires C, 49.67; H, 5.08; N, 1.81; S, 4.14%), or *compound* (**6**) [from (**4**)] (62 mg, 77%), $[\alpha]_D^{20}$ +256° (*c* 0.67 in chloroform) (Found: C, 50.4; H, 5.3; N, 1.7; S, 3.95%).

o- *and* p-*Nitrophenyl* 4-S-α-D-*Glucopyranosyl*-4-*thio*-α-D-*glucopyranoside* (**7**) *and* (**8**).—To solutions of each peracetylated aryl 4-thiomaltoside (**5**) [for (**7**)] and (**6**) [for (**8**)] (50 mg, 0.06 mmol) in methanol (30 ml) was added sodium methoxide in methanol (0.7M; 0.01 ml). After 78 h, neutralization with Amberlite IR 120 (H^+), evaporation, and freeze-drying led to the *aryl* 4-*thiodisaccharides* (**7**) and (**8**) as solid foams: (**7**) [from (**5**)] (26 mg, 100%), $[\alpha]_D^{20}$ +213° (*c* 0.15 in water) (Found: C, 44.7; H, 5.6; N, 2.6; S, 5.9. $C_{18}H_{25}NO_{12}S{\cdot}0.5H_2O$ requires C, 44.26; H, 5.36; N, 2.87; S, 6.56%), and (**8**) [from (**6**)] (25 mg, 96%), $[\alpha]_D^{20}$ +181° (*c* 0.11 in water) (Found: C, 43.5; H, 5.7; N, 2.6; S, 6.3. $C_{18}H_{25}NO_{12}S{\cdot}H_2O$ requires C, 43.46; H, 5.47; N, 2.81; S, 6.44%).

2,3,6-*Tri*-O-*acetyl*-4-S-(2,3,4,6-*tetra*-O-*acetyl*-α-D-*glucopyranosyl*)-4-*thio*-β-D-*glucopyranosyl Chloride* (**9**).—A solution of 1,2,3,6-tetra-*O*-acetyl-4-*S*-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)-4-thio-β-D-glucopyranose[11] (500 mg, 0.72 mmol) in dichloromethane (1 ml), containing dichloromethyl methyl ether (1 ml) and boron trifluoride–diethyl ether (0.05 ml), was kept for 75 min at room temperature, then concentrated to dryness under reduced pressure. The solid residue was dissolved in dichloromethane (50 ml) and washed with ice-cold saturated aqueous sodium hydrogen carbonate. After being dried and concentrated the solution gave the *chloride* (**9**) (403 mg, 83%), m.p. 163—167 °C (from diethyl ether); $[\alpha]_D^{20}$ +138° (*c* 0.55 in chloroform) (Found: C, 46.5; H, 5.2; Cl, 5.5; S, 4.5. $C_{26}H_{35}ClO_{16}S$ requires C, 46.53; H, 5.26; Cl, 5.28; S, 4.78%).

2,3,6-*Tri*-O-*acetyl*-1-S-*benzoyl*-4-S-(2,3,4,6-*tetra*-O-*acetyl*-α-D-*glucopyranosyl*)-1,4-*dithio*-α-D-*glucopyranose* (**10**).—To a solution of thiobenzoic acid (0.14 ml, 1.2 mmol) in HMPA (4 ml) was added sodium hydride (50% in oil dispersion; 63 mg, 1.31 mmol) followed by the chloride (**9**) (350 mg, 0.52 mmol). After being kept overnight at room temperature, purification of the whole reaction mixture on two successive silica gel columns [65 g and 35 g; diethyl ether–hexane (4:1 v/v)] led to the *dithiodisaccharide* (**10**) (230 mg, 57%), $[\alpha]_D^{20}$ +238° (*c* 0.47 in chloroform) (Found: C, 51.3; H, 5.3; S, 8.1. $C_{33}H_{40}O_{17}S_2$ requires C, 51.29; H, 5.22; S, 8.30%).

2,3,6-*Tri*-O-*acetyl*-1-S-*acetyl*-4-S-(2,3,4,6-*tetra*-O-*acetyl*-α-D-*glucopyranosyl*)-1,4-*dithio*-α-D-*glucopyranose* (**11**).—A solution of the 1,4-dithiodisaccharide (**10**) (230 mg, 0.3 mmol) in methanol (3 ml) containing sodium methoxide in methanol (0.7M; 0.44 ml) was kept for 6 h under nitrogen. Concentration, followed by acetylation of the residue with acetic anhydride–pyridine (1:1 v/v; 2 ml), extraction as described,[11] and purification on a silica gel column (35 g) with diethyl ether as eluant, afforded the *peracetylated* 1,4-*dithiodisaccharide* (**11**) (142 mg, 70%), $[\alpha]_D^{20}$ +223° (*c* 0.87 in chloroform) (Found: C, 47.0; H, 5.4; S, 8.4. $C_{28}H_{38}O_{17}S_2$ requires C; 47.32; H, 5.39; S, 9.02%).

o-*Nitrophenyl* S-(2,3,4,6-*Tetra*-O-*acetyl*-α-D-*glucopyranosyl*)-(1 ⟶ 4)-S-(2,3,6-*tri*-O-*acetyl*-4-*thio*-α-D-*glucopyranosyl*)-(1 ⟶ 4)-2,3,6-*tri*-O-*benzoyl*-4-*thio*-α-D-*glucopyranoside* (**13**).—To a solution of the octa-acetyldithiodisaccharide (**11**) (150 mg, 0.21 mmol) in methanol (2 ml) was added sodium methoxide in methanol (0.7M, 0.3 ml), and the solution was kept at room temperature overnight, before concentration under reduced pressure. To a solution of the dried resulting sodium thiolate (**12**) in HMPA was added the triflate (**1**) (314 mg, 0.42 mmol) and the reaction mixture was kept for 2 h at room temperature under nitrogen, after which time t.l.c. showed a major component [R_F 0.65; chloroform–methanol–water (65:25:4 v/v/v)]. Acetylation and work-up as described for (**7**) and (**8**) led to a residue which was purified by chromatography on a silica gel column (35 g) with elution with diethyl ether, and gave *compound* (**13**) (179 mg, 67%), $[\alpha]_D^{20}$ +216° (*c* 0.31 in

chloroform) (Found: C, 55.9; H, 4.8; N, 1.1; S, 5.0. $C_{59}H_{61}NO_{26}S_2$ requires C, 56.05; H, 4.86; N, 1.11; S, 5.07%).

o-*Nitrophenyl* S-(2,3,4,6-*Tetra*-O-*acetyl*-α-D-*glucopyranosyl*)-(1 ⟶ 4)-S-(2,3,6-*tri*-O-*acetyl*-4-*thio*-α-D-*glucopyranosyl*)-(1 ⟶ 4)-2,3,6-*tri*-O-*acetyl*-4-*thio*-α-D-*glucopyranoside* (**14**).—To a solution of the acylated 4,4′-dithiotrisaccharide (**13**) (158 mg, 0.12 mmol) in methanol (65 ml) was added sodium methoxide in methanol (0.7M, 0.2 ml). Work-up and acetylation as described for (**5**) and (**6**), followed by chromatographic purification on a silica gel column with elution with diethyl ether, gave the *peracetylated aryl dithiotrisaccharide* (**14**) (109 mg, 81%), $[\alpha]_D^{20}$ +206° (*c* 0.32 in chloroform) (Found: C, 49.0; H, 5.2; N, 1.2; S, 6.0. $C_{44}H_{55}NO_{26}S_2$ requires C, 49.02; H, 5.14; N, 1.30; S, 5.95%).

o-*Nitrophenyl* S-α-D-*Glucopyranosyl*-(1 ⟶ 4)-S-4-*thio*-α-D-*glucopyranosyl*-(1 ⟶ 4)-4-*thio*-α-D-*glucopyranoside* (**15**).—Sodium methoxide in methanol (0.7M, 0.06 ml) was added to a solution of the peracetylated aryl dithiotrisaccharide (**14**) (109 mg, 0.1 mmol) in methanol (60 ml). After being stirred overnight, the solution was neutralized with Amberlite IR 120 (H^+). Filtration through a silica gel column [10 g; chloroform–methanol–water (65:25:4 v/v/v)] and freeze-drying of the sample led to the *aryl* 4,4′-*dithiomaltotrioside derivative* (**15**) which was obtained as a white solid foam (46 mg, 80%), $[\alpha]_D^{20}$ +228° (*c* 0.14 in water) (Found: C, 42.6; H, 5.5; N, 2.0; S, 9.2. $C_{24}H_{35}NO_{16}S_2 \cdot H_2O$ requires C, 42.66; H, 5.52; N, 2.07; S, 9.44%).

Enzymes and Enzymic Assays.—Porcine pancreatic α-amylase was purified from an aqueous extract according to the method of Granger *et al.*,[15] concentrated by ultrafiltration through a PM10 membrane (Amicon) up to a concentration of 1—10 mg ml^{-1}, and kept at 5 °C; concentration was determined by measuring the absorbance at 280 nm according to the relation $\varepsilon_{280}^{1\%} = 25$ cm^{-1}. Before use, the enzyme was dialysed against a Tris-HCl buffer solution of pH 7.4 (10 mM) containing calcium chloride (1mM). Human pancreatic α-amylase was a gift from Dr. C. Figarella (Marseille).

The liberation of nitrophenols from the substrate analogues was measured with a Carry 14 recording spectrophotometer at 410 nm with $\varepsilon_{410} = 2.4$ cm^2 μmol^{-1} for *o*-nitrophenol and $\varepsilon_{410} = 11$ cm^2 μmol^{-1} for *p*-nitrophenol. The optimal fit of data was taken from a linear least-squares procedure. K_M and *V* values were obtained from double reciprocal plots of Lineweaver–Burk curves. Analyses of the reaction products were carried out by t.l.c. on silica gel using chloroform–methanol–water (65:30:4 v/v/v) as eluant or by high-pressure liquid chromatography with a reverse-phase C_{18} Merck (10 μ) analytical column. In this case the reaction mixture (1 ml) was filtered through a 'Septlak' precolumn which was washed with methanol (2 ml). The solvent was evaporated and the residue dissolved in methanol–water (20:80 v/v). The flow rate of the eluant was 2 ml min^{-1}. Detection of nitrophenyl derivatives was performed with a u.v. detector (Waters model 450) at 310 nm. Components not having the nitrophenyl moieties were detected with a differential refractometer detector (Waters model R 401).

Acknowledgements

The authors thank Professor K. Wallenfels for a generous gift of compound (**18**), Mr. M. Paillet for his skilful technical assistance during the chemical syntheses, and Mrs. M. L. Dheu-Andries and Mr. H. Reutenauer for the n.m.r. spectral measurements.

References

1 M. Blanc-Muesser, J. Defaye, H. Driguez, G. Marchis-Mouren, and C. Seigner, in 'Use of Enzymes in Food Technology,' ed. P. Dupuy, Technique et Documentation Lavoisier, Paris, 1982, p. 127.
2 J. A. Thoma, J. E. Spradlin, and S. Dygert, in 'The Enzymes,' ed. P. Boyer, Academic Press, New York, 1971, vol 5, pp. 115—189.
3 P. Nordin and D. French, *J. Am. Chem. Soc.*, 1958, **80**, 1145.
4 R. Bird and R. H. Hopkins, *Biochem. J.*, 1954, **56**, 86.
5 B. K. Gillard, H. C. Markman, and S. A. Feig, *Clin. Chem. (Winston-Salem, N. C.)*, 1977, **23**, 2279.
6 K. Wallenfels, P. Földi, H. Niermann, H. Bender, and D. Linder, *Carbohydr. Res.*, 1978, **61**, 359.
7 K. Wallenfels, G. Laule, and B. Meltzer, *J. Clin. Chem. Clin. Biochem.*, 1982, **20**, 581.
8 J. F. Robyt and D. French, *J. Biol. Chem.*, 1970, **245**, 3917.
9 M. Blanc-Muesser, J. Defaye, and H. Driguez, *Carbohydr. Res.*, 1978, **67**, 305.
10 M. Apparu, M. Blanc-Muesser, J. Defaye, and H. Driguez, *Can. J. Chem.*, 1981, **59**, 314.
11 M. Blanc-Muesser, J. Defaye, and H. Driguez, *J. Chem. Soc., Perkin Trans. 1*, 1982, 15.
12 D. Rho, M. Desrochers, L. Jurasek, H. Driguez, and J. Defaye, *J. Bacteriol.*, 1982, **149**, 47.
13 I. Farkas, I. F. Szabó, R. Bognár, and D. Anderle, *Carbohydr. Res.*, 1976, **48**, 136.
14 D. Horton and A. E. Luetzow, *Chem. Commun.*, 1971, 79.
15 M. Granger, B. Abadie, Y. Mazzei, and G. Marchis-Mouren, *FEBS Lett.*, 1975, **50**, 276.
16 J. Defaye, H. Driguez, E. Ohleyer, C. Orgeret, E. Seillier, and C. Vidaud, in 'Sciences et Industries du Bois,' Ministère de l'Industrie et de la Recherche, Paris, 1983, pp. 23–40.

Received 7th November 1983; *Paper* 3/1975