Chemoenzymatic Approach to the Preparation of Regioselectively Modified Cyclodextrins. The Substrate Specificity of the Enzyme Cyclodextrin Glucosyltransferase (CGTase) *

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The synthesis of α-maltosyl fluorides substituted at the 6- or 6′-position with H, F, Br, OMe, OAc is described, together with the preparation of the 4-thio-α-maltosyl fluoride and 5-thio-α-D-glucosyl fluoride. These compounds were obtained by chemical or enzymatic procedures and their structures have been established by ^{13}C NMR spectroscopy. The glycosyl fluorides have been tested as substrates for the enzyme CGTase under coupling and condensation conditions. It has been found that all the compounds tested are substrates in coupling reactions. Only three of them led to higher oligosaccharides with a modified maltosyl residue as repeating unit, but only the 6′-*O*-Me and 6′-*O*-acetyl fluorides were transformed into cyclic compounds. Under the conditions used, 6^A, 6^C, 6^E-tri-*O*-methylcyclomaltohexaose, 6^A, 6^C, 6^E-tri-*O*-methylcyclomaltoheptaose, and 6^A, 6^C, 6^E, 6^G-tetra-*O*-methylcyclomaltooctaose were isolated in 42, 13 and 16% yield, respectively. The 6′-*O*-acetylmaltosyl fluoride afforded 6^A, 6^C, 6^E-tri-*O*-acetylcyclomaltohexaose and a mixture of partially acetylated cyclomaltoheptaoses in only low yields.

By this approach, new insights have been obtained on the specificity of the catalytic site of CGTase of *Bacillus macerans* and new routes for the preparation of regioselectively modified cyclodextrins have been developed.

The increasing world demand for cyclodextrins ensures that the enzyme CGTase will become manufactured on an industrial scale. More than 10 CGTase genes have been cloned in the past 4 years, and the first three-dimensional structure of this protein (from *Bacillus circulans*) has recently been published.[3]

The use of substrate-analogues would be helpful in determining the nature of the amino acids involved in the active site of the enzyme. Some information about the specificity of the acceptor subsites has already been obtained, using the coupling reaction between an α-cyclodextrin and various acceptors.[4–6] Recently, to define the specificity of the donor part of the catalytic site, modified α-cyclodextrins have been used.[7,8] These approaches have shown that acceptor subsites T and U (Fig. 1) may accommodate various modified glycosyl acceptors, but also that modified glucosyl residues do not fit in subsites S and R of the donor part of the active site.

To investigate simultaneously the specificity of both parts, it was thought that modified glycosyl fluorides would be useful since Hehre *et al.* demonstrated, 7 years ago, that α-maltosyl fluoride and, to a lesser extent, α-D-glycosyl fluoride were substrates for CGTase.[9] This enzyme, by autocondensation and cyclization, led to linear and cyclic maltodextrins.

For these reasons, we have chosen to prepare α-maltosyl fluoride **1** and its analogues modified at the 6- or 6′-position. This paper deals with the syntheses of 6-deoxy **2**, 6-deoxy-6-fluoro **3**, 6-bromo-6-deoxy **4**, 6-*O*-methyl **5**, 6′-deoxy **6**, 6′-deoxy-6′-fluoro **7**, 6′-bromo-6′-deoxy **8**, 6′-*O*-methyl **9** and 6′-*O*-acetyl **10** maltosyl fluorides. We also reported the synthesis of 4-thiomaltosyl fluoride **11** and 5-thio-α-D-glucosyl fluoride **13**, together with the enzymatic behaviour of compounds **1**–**11**.

Results and Discussion

A reliable procedure has been developed for the preparation of α-maltosyl fluoride modified at C-6 or C-6′ with a high degree of purity for use in enzymatic experiments. In most cases, the initial steps involve the synthesis of acetylated maltose derivatives with a free hydroxy group at position 6 or 6′. After modification at these positions, the synthesis of the corresponding acetylated α-maltosyl fluoride was achieved by treatment with a hydrogen fluoride–pyridine mixture. De-*O*-acetylated fluorides were generated with sodium methoxide in methanol.

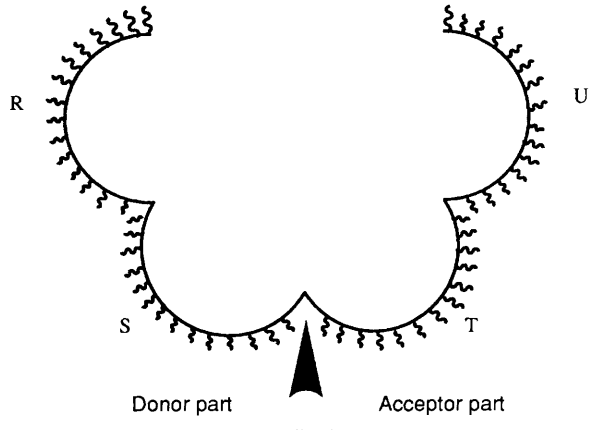
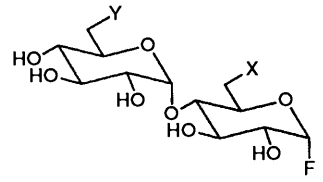


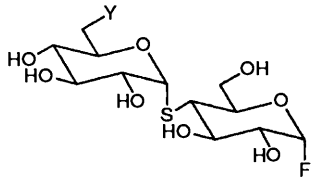
Fig. 1

The common precursor to maltosyl fluorides modified at C-6 was the hepta-*O*-acetylmaltose **17** which has a free hydroxy group ready for selective substitution. This compound was synthesized from 1,6-anhydromaltose hexaacetate **14**. The selective opening of the 1,6-anhydro ring was performed with dichloromethyl methyl ether in the presence of zirconium tetrachloride. Hexa-*O*-acetyl-6-*O*-formylmaltosyl chloride **15** thus formed was treated with acetic acid and silver acetate and then, without purification, by hydrochloric acid in methanol for the selective hydrolysis of the 6-*O*-formyl group. This sequence of reactions,

* Preliminary reports on this work have been presented (see refs. 1 and 2).



1 X = Y = OH
2 X = H, Y = OH
3 X = F, Y = OH
4 X = Br, Y = OH
5 X = OMe, Y = OH
6 X = OH, Y = H
7 X = OH, Y = F
8 X = OH, Y = Br
9 X = OH, Y = OMe
10 X = OH, Y = OAc



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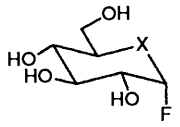
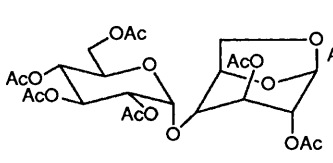


12 X = O
13 X = S

which led to compound **17** in good overall yield from the 1,6-anhydromaltose **14** (59%), competes with the known methods of Asp and Lindberg,[10] Bognar *et al.*,[11] Fujimaki and Kuzuhara,[12] and is similar to that recently developed by Bock and Pedersen.[13]



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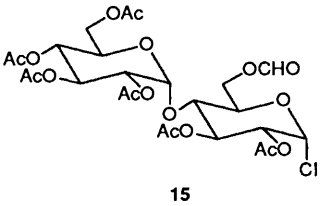

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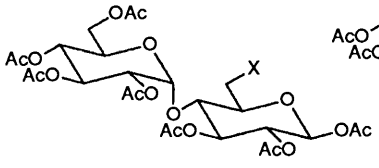
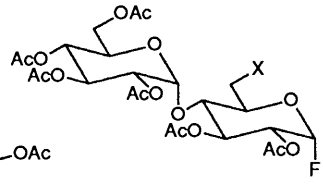
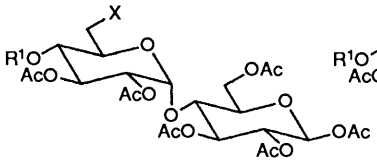


16 X = OCHO
17 X = OH
18 X = H
19 X = I
20 X = F
21 X = Br
22 X = OMe

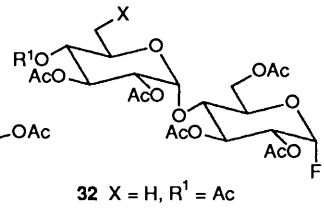


23 X = H
24 X = F
25 X = Br
26 X = OMe

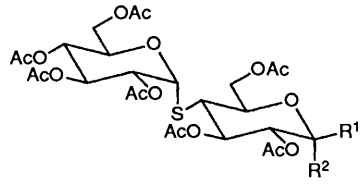
Hepta-*O*-acetyl-6-deoxymaltose **18** was obtained from compound **17** in two steps, through the 6-iodomaltose **19**, according to the method of Guerrera and Weill.[14] Preparation of the 6-fluoro compound **20** was achieved by the action of *N*,*N*-diethylaminosulphur trifluoride (DAST)[15] on the 6-position of heptaacetate **17**. The expected product **20** was obtained in 77% yield. Compound **17** was also converted into the 6-bromo derivative **21** by treatment with *N*-bromosuccinimide (NBS) (96%). Then, hexa-*O*-acetyl-6-*O*-methylmaltose **22** was synthesized in high yield (90%) by treatment, under pressure, of compound **17** with methyl trifluoromethanesulphonate in the presence of hindered base.[16] This method is more convenient than that using diazomethane as methylating agent.[17] Under the latter conditions compound **22** was isolated in only modest yield (40%). The maltose derivatives **18** and **20**–**22** were converted into the corresponding fluorides **23**–**26** by use of a mixture of anhydrous hydrogen fluoride and pyridine (7:3 v/v).[18] All the compounds were obtained in high yield except the acetylated 6-*O*-methylmaltosyl fluoride **26** which was synthesized in only 30% yield. Preparation of the fluoro derivatives was first attempted by using neat anhydrous hydrogen fluoride[19] as described for the synthesis of α-maltosyl fluoride **1**, but this resulted in the formation of less polar compounds attributed to monosaccharides obtained by splitting of interglycosidic bonds. All these fluorides were crystallized and analysed by NMR spectroscopy.



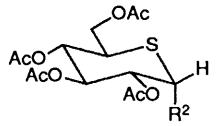
27 X = OH, R^1 = Ac
28 X = H, R^1 = Ac
29 X = F, R^1 = Ac
30 X = OMe, R^1 = Ac
31 X = Br, R^1 = Bz



32 X = H, R^1 = Ac
33 X = F, R^1 = Ac
34 X = OMe, R^1 = Ac
35 X = Br, R^1 = Bz
36 X = Br, R^1 = Ac



37 R^1 = OAc, R^2 = H
38 R^1 = H, R^2 = F



39 R^2 = OAc
40 R^2 = OH
41 R^2 = F

Following a similar pathway, the synthesis of maltosyl fluorides modified at the 6′-position started from the hepta-*O*-acetylmaltose **27** which has a free hydroxy group in the 6′-position. This compound was easily obtained through a selective cleavage of the 4′,6′-*O*-benzylidene ring of the maltose derivative and conventional treatment as described by Takeo and Shinmitsu.[20] 6′-Deoxy-, 6′-deoxy-6′-fluoro-, and 6′-*O*-methylmaltose derivatives **28**–**30** were obtained with similar yields by the methods already described for compounds **18**, **20**, and **21**, respectively. Acylated 6′-bromo-6′-deoxymaltose, compound **31**, was prepared in 70% yield by treatment of the 4′,6′-*O*-benzylidene derivative with NBS.[20] Crystalline acetylated maltosyl fluorides **32**–**34** were prepared in 60, 85 and 85% yield, respectively. Acylated fluoride **35**, obtained in amorphous form in 51% yield, was fully characterized by treatment with sodium methoxide in dry methanol followed by acetylation with an acetic anhydride–pyridine mixture. The expected crystalline fluoride **36** was isolated in 77% yield.

The retrosynthetic scheme for the synthesis of 6′-*O*-acetylmaltosyl fluoride **10** by sequential protection–deprotection steps classically used in organic synthesis is not obvious. In an attempt to overcome these problems we explored the regioselective acetylation of maltosyl fluoride **1** *via* enzymatic transesterification. During the last three years several reports on enzyme-catalysed regioselective acylation of mono- and disaccharides have been published, these reactions requiring organic solvents and activated esters.[21,22] We selected to use vinyl acetate as the active ester, which was first introduced in such enzymatic reactions in 1987,[23] and subtilisin, which was known to catalyse, in pyridine or *N*,*N*-dimethylformamide (DMF) solution, the transesterification on the 6′-position of maltose.[22] In pyridine, with the conditions described in the Experimental section, the expected fluoride **10** was isolated in 56% yield; the only by-product was the starting material **1** which was recovered in 40% yield.

Treatment of per-*O*-acetylated-4-thio-β-maltose **37**[24] as

already described for acetylated maltose modified at the 6- or 6′-position led to the fluoro compound **38** in 70% yield.

An original strategy for the synthesis of 5-thioglucosyl fluoride **41** was devised since only penta-*O*-acetyl-5-thio-α-D-glucose **39** was known.[25] Selective *O*-deacetylation at the anomeric position was obtained by treatment of compound **39** in DMF with hydrazine hydrate.[26] Compound **40** was isolated in crystalline form in 88% yield. Reaction of DAST with the free anomeric group of compound **40** led to the fluoride **41** in 81% yield.

All these α-maltosyl fluorides **23–26**, **32–34**, **36**, **38** and **41** were de-*O*-acetylated with sodium methoxide in methanol to give the corresponding unprotected pure compounds in quantitative yield as shown by HPLC. The reactions were performed just before the enzymatic incubations.

In the first set of experiments, α-maltosyl fluorides **1–13** were tested as acceptors in a coupling reaction catalysed with CGTase using α-CD as donor. The enzymatic mixture was analysed by HPLC using a μ-Bondapak NH_2 column. All the chromatographic profiles were identical and revealed the presence of linear oligosaccharides. These experiments confirm already reported data: the amino acids of subsites T and U do not establish essential bindings with primary hydroxy groups of maltosyl residues.*

However, under autocondensation conditions, only the incubation of modified maltosyl fluorides **9–11** led to alternated oligosaccharides with the starting modified disaccharide as the repeating unit. In all the other experiments only modified maltoses resulting from the hydrolysis of the C–F bound of the corresponding fluorides could be detected.

These data show that all the modifications made at the 6-position of the maltose residue prevent binding in subsite S. The modifications allowed are only the acetylation and the methylation of the primary hydroxy group of the non-reducing unit which may accommodate the specificity of subsite R.

After incubation of 6′-*O*-methylmaltosyl fluoride **9** with CGTase for 20 h, the reaction mixture was freed of enzyme by heating at 100 °C and spinning at 14 000 rpm. The residue was then purified by preparative HPLC using a reversed-phase C_{18} silica gel column with a water–methanol mixture (65:35 v/v) as eluent. Tri-*O*-methyl-α-cyclodextrin **43** was isolated in 42% yield together with tetra-*O*-methyl-γ-cyclodextrin **44** and the unexpected tri-*O*-methyl-β-cyclodextrin **45** in 16 and 13% yield, respectively. To attempt to determine how this compound was obtained, since from the coupling conditions it seems that a 6-*O*-methylglucosyl residue cannot fit into subsite S, cyclodextrin **43** was incubated with CGTase in the presence or in the absence of 6′-*O*-methylmaltosyl **46**, or its corresponding fluoride **9** and the time-course of the enzymatic reaction was followed and analysed by HPLC on a μ-Bondapak NH_2 column. At first it was shown that, under the conditions used, α-cyclodextrin **43** was not split in the absence of maltosyl acceptors **9** and **46**. When equimolecular amounts of substrates **43** and 6′-*O*-methylmaltosyl fluoride **9** were incubated in the presence of CGTase, a mixture of 6′-*O*-methylmaltose **46**, α-cyclodextrin **43**, β-cyclodextrin **45** and γ-cyclodextrin **44** was obtained in the apparent proportions 39:21:1:6, respectively (Scheme 1). Linear malto-oligosaccharides **42** were also produced but were not characterized. The enzymatic disproportionation in the presence of 6′-*O*-methylmaltose **46** led to the same complex mixture but in the proportions 45:8:8:1, respectively. If the production of γ-CD can be straightforwardly explained, the formation of β-CD remains unclear. One can speculate that the 6-*O*-methylglucosyl unit may occupy subsite S only when positive interactions exist for subsites R, T and U. This hypothesis fits with the fact that 6-*O*-methylmaltosyl fluoride **5** was not a substrate. From a linear alternating maltodecaose, 6,6″-di-*O*-methylmaltotriose could be released and the formation of the β-CD **45** could occur.

* It is worthy of note that, under the conditions used and in contrast to the behaviour of **12**, 5-thio-α-D-glucosyl fluoride **13** was spontaneously hydrolysed in the presence or in the absence of enzyme.

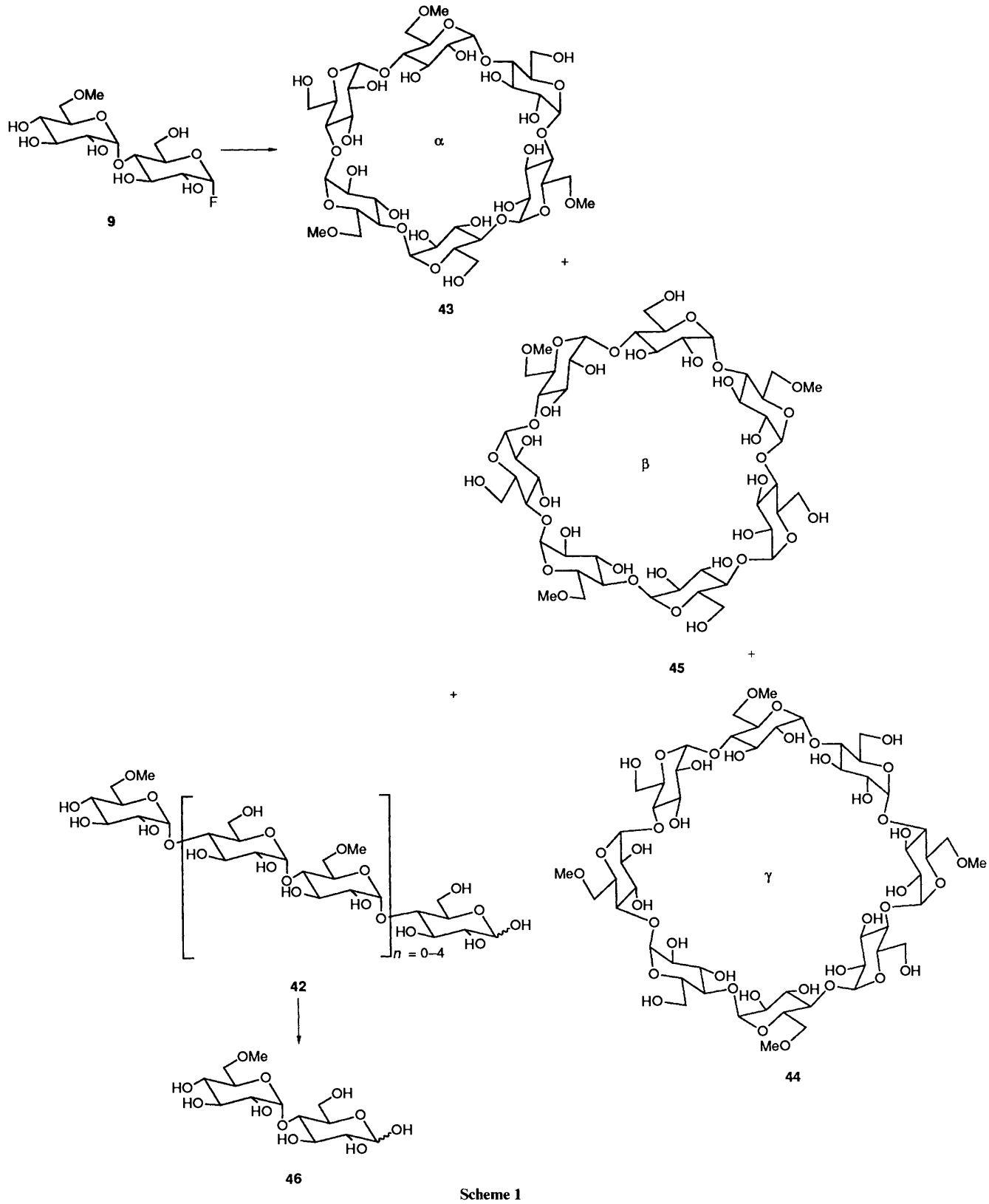
Experimental

General Methods.—CGTase (EC 3.2.1.19, *B. macerans*) was a gift from Amano Co. Ltd., α-amylase (EC 3.2.1.1, Taka-amylase) and subtilisin (EC 3.4.21.14, *B. subtilis*) were from Sigma Chemical Co. Subtilisin was used under the precautions described in ref. 22. M.p.s were measured on a Zeiss apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 261 polarimeter. NMR spectra were recorded with a Bruker AC 300 spectrometer. Chemical shifts are given in ppm downfield from $SiMe_4$, and *J* values are in Hz. Low-resolution mass spectra were recorded on a Nermag R.10.10.C spectrometer using chemical ionization (Cl) or fast-atom bombardment (FAB) modes. High-resolution mass spectrometry was performed for compounds **44** and **45** on a ZAB 2 SEQ (VG) spectrometer using the FAB(+) technique. TLC was performed on silica gel F254 (Merck) with detection by UV light and/or charring with a sulphuric acid–methanol mixture. Usual work-up means successive washing of the organic phase with ice-cold aq. solutions of potassium hydrogen sulphate (10%, w/v), sodium hydrogen carbonate (saturated), and water. Water washings were usually back-extracted, and solutions were dried (sodium sulphate), then evaporated under reduced pressure at temperature below 45 °C. Solvents such as DMF, pyridine, 1,2-dimethoxyethane, dichloromethane, 1,2-dichlorethane and tetrachloromethane were distilled and kept over calcium hydride. The homogeneity of the deacetylated glycosyl fluorides was checked by HPLC on a μ-Bondapak NH_2 column (Waters Ass. Milford MA 01757) with acetonitrile–water (7:3 v/v) as eluent. Light petroleum refers to the fraction boiling in the range 40–65 °C.

1,2,3-*Tri*-O-*acetyl*-4-O-(2,3,4,6-*tetra*-O-*acetyl*-α-D-*glucopyranosyl*)-β-D-*glucopyranose* **17**.—To a stirred solution of hexa-*O*-acetyl-1,6-annhydro-β-maltose **14**, prepared as described[27] (5 g, 8.7 mmol), and dichloromethyl methyl ether (20 cm^3) in dichloromethane (100 cm^3) was added zirconium tetrachloride (2.5 g). After 3 h at 60 °C, the cold mixture was filtered through Celite. Work-up led, after evaporation, to compound **15** (6.7 g) which could be used without purification in the next step.

To a solution of the above chloride **15** (6.7 g) in acetic acid–acetic anhydride (51 cm^3; 50:1 v/v) was added silver acetate (5 g). After being kept for 12 h at 50 °C, the mixture was filtered through Celite, the filtrate was evaporated, and the residue was co-evaporated with toluene (3 × 50 cm^3). Work-up led, after evaporation, to the peracetylated product **16**, which was treated with hydrochloric acid (100 mm^3) in methanol (400 cm^3); after 3 h at room temperature, the solution was evaporated, the residue was mixed with dichloromethane, washed with ice-cold saturated aq. sodium hydrogen carbonate, dried and evaporated. Compound **17** was isolated by flash chromatography [eluent ethyl acetate–light petroleum (1:1 v/v)]. Crystallization from diethyl ether gave pure *compound* **17** (3.25 g, 59% from **1**) with m.p. 145–147 °C (from diethyl ether–light petroleum) [lit.,[10] 140–141 °C (EtOH–water, 1:9)] Found: C, 48.75; H, 5.5. $C_{26}H_{36}O_{18}$ requires C, 49.06; H, 5.70%).

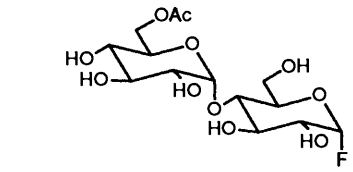
1,2,3-*Tri*-O-*acetyl*-6-*deoxy*-6-*iodo*-4-O-(2,3,4,6-*tetra*-O-*acetyl*-α-D-*glucopyranosyl*)-β-D-*glucopyranose* **19**.—A solution of compound **17** (2.3 g, 3.6 mmol) in acetonitrile (75 cm^3) was



Scheme 1

treated with iodine (1.97 g, 7.75 mmol), triphenylphosphine (2.15 g), and imidazole (1.15 g) at 90 °C for 5 min. The mixture was evaporated, the residue was dissolved in dichloromethane, and the solution was washed with water. Purification by chromatography with ethyl acetate–light petroleum (1:1 v/v) gave the expected *compound* **19**, which was crystallized from diethyl ether (2.1 g, 80%), m.p. 130–131 °C (lit.,[14] 131–132 °C).

1,2,3-*Tri*-O-*acetyl*-6-*deoxy*-6-*fluoro*-4-O-(2,3,4,6-*tetra*-O-*acetyl*-α-D-*glucopyranosyl*)-β-D-*glucopyranose* **20**.—Hepta-*O*-acetylmaltose **17** (350 mg, 0.55 mmol) was dissolved in a cold solution of anhydrous glyme (3 cm^3; −10 °C). After dropwise addition of DAST (300 mm^3, 2.4 mmol) in glyme (900 mm^3) the reaction mixture was heated at 60 °C for 3–4 h. To the cold solution (0 °C) was added methanol (500 mm^3) and, after



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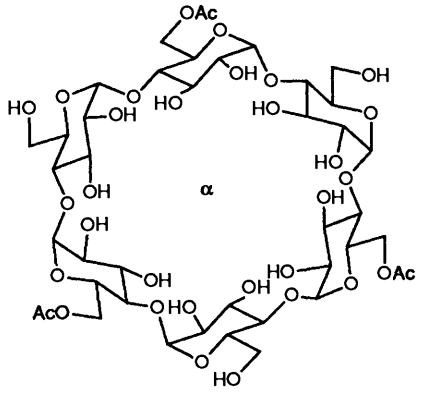

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evaporation, the residue was dissolved in dichloromethane and the organic phase was washed with cold water (3 × 5 cm^3). Purification on a silica gel column with ethyl acetate–light petroleum (2:3 v/v) gave the expected *compound* **20** (270 mg, 77%), which was crystallized from diethyl ether, m.p. 180–181 °C (Found: C, 49.0; H, 5.4; F, 3.0. $C_{26}H_{35}FO_{17}$ requires C, 48.90; H, 5.52; F, 2.97%); $[\alpha]_D$ +55° (*c* 1.0 in $CHCl_3$); δ_C(75 MHz; $CDCl_3$) 95.3 (C-1′), 91.5 (C-1), 80.8 (C-6, $J_{6,F}$ 180), 75.2, 71.0, 70.1, 69.7, 69.3, 68.7 and 68.2 (C-2, -2′; C-3, -3′; C-4, -4′; C-5, -5′) and 61.6 (C-6′).

1,2,3-*Tri*-O-*acetyl*-6-*bromo*-6-*deoxy*-4-O-(2,3,4,6-*tetra*-O-*acetyl*-α-D-*glucopyranosyl*)-β-D-*glucopyranose* **21**.—A solution of hepta-*O*-acetylmaltose **17** (312 mg, 0.49 mmol) and NBS (175 mg, 1 mmol) in dichloromethane (5 cm^3) was treated with triphenylphosphine (257 mg, 2 mmol) at room temperature for 10 min. The reaction was quenched by addition of methanol (1 cm^3), diluted with dichloromethane, and washed with ice-cold water (3 × 100 cm^3). Purification on a silica gel column with ethyl acetate–light petroleum (1:1 v/v) and crystallization from diethyl ether yielded *compound* **21** (330 mg, 96%), m.p. 131–132 °C (Found: C, 44.5; H, 4.9; Br, 11.3. $C_{26}H_{35}BrO_{17}$ requires C, 44.64; H, 5.04; Br, 11.42%); $[\alpha]_D$ +52° (*c* 1.0 in $CHCl_3$); δ_C(75 MHz; $CDCl_3$) 95.2 (C-1′), 90.9 (C-1), 74.9, 72.8, 72.6, 70.8, 69.8, 69.1, 68.5 and 67.8 (C-2, -2′, C-3, -3′, C-4, -4′, C-5, -5′), 61.6 (C-6′) and 32.2 (C-6).

1,2,3-*Tri*-O-*acetyl*-4-O-(2,3,4,6-*tetra*-O-*acetyl*-α-D-*glucopyranosyl*)-6-O-*methyl*-β-D-*glucopyranose* **22**.—In a steel container containing a solution of compound **17** (1 g, 1.57 cm^3) in dichloromethane (23 cm^3) were added 2,6-di-*tert*-butylpyridine (6.9 cm^3) and methyl triflate (1.8 cm^3). After 2 h at 80 °C under magnetic stirring, the cold solution was washed with water, purified by column chromatography [ethyl acetate–light petroleum (2:3 v/v)], and the expected product **22** was crystallized from diethyl ether (1 g, 97%), m.p. 136–137 °C; (lit.,[17] 135–136 °C); δ_C(75 MHz; $CDCl_3$) 95.0 (C-1′), 91.7 (C-1), 75.4, 74.9, 71.2, 70.5, 70.2, 69.5, 68.1 (C-2, -2′, C-3, -3′, C-4, -4′, C-5, -5′, C-6), 61.5 (C-6′) and 59.5 (OMe).

2,3-*Di*-O-*acetyl*-6-*deoxy*-4-O-(2,3,4,6-*tetra*-O-*acetyl*-α-D-*glucopyranosyl*)-α-D-*glucopyranosyl Fluoride* **23**.—In a plastic bottle, a solution of 1,2,3-tri-*O*-acetyl-6-deoxy-4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)-β-D-glucopyranose **18** (235 mg, 0.38 mmol) prepared as described,[14] in pyridine (500 mm^3), was treated with a mixture of hydrogen fluoride–pyridine (7:3 v/v, 4 cm^3) at 0 °C. After 1 h at room temperature, the solution was poured into a plastic beaker (1 dm^3) containing ice (250 cm^3) and dichloromethane (50 cm^3) and was neutralized (pH paper) by addition of sodium hydrogen carbonate. The organic phase was then washed twice with cold water, dried, and evaporated. Column chromatography [ethyl acetate–light petroleum (1:2 v/v)] of the residue gave *compound* **23**, which was crystallized from diethyl ether (160 mg, 72%), m.p. 163–164 °C (Found: C, 49.9; H, 5.7; F, 3.3. $C_{24}H_{33}FO_{15}$ requires C, 49.65; H, 5.72; F, 3.27%); $[\alpha]_D$ +93° (*c* 1.0 in $CHCl_3$); δ_C(75 MHz; $CDCl_3$) 103.6 (C-1, $J_{1,F}$ 232 Hz), 95.7 (C-1′), 61.9–78.4 (C-2 to C-5 and C-2′ to C-5′) and 18.8 (C-6).

2,3-*Di*-O-*acetyl*-6-*deoxy*-6-*fluoro*-4-O-(2,3,4,6-*tetra*-O-*acetyl*-α-D-*glucopyranosyl*)-α-D-*glucopyranosyl Fluoride* **24**.—A solution of compound **20** (150 mg, 0.23 mmol) in pyridine (350 mm^3) was treated with hydrogen fluoride–pyridine mixture (2.5 cm^3) at 0 °C for 40 min. After work-up as described above for compound **23**, column chromatography [ethyl acetate–light petroleum (2:3 v/v)] of the residue gave *compound* **24**, which was crystallized from diethyl ether (110 mg, 80%), m.p. 170–171 °C (Found: C, 48.0; H, 5.6; F, 5.9. $C_{24}H_{32}F_2O_{15}$ requires C, 48.16; H, 5.39; F, 6.34%); $[\alpha]_D$ +93° (*c* 1.0 in $CHCl_3$); δ_C(75 MHz; $CDCl_3$) 103.9 (C-1, $J_{1,F}$ 236), 95.1 (C-1′), 80.8 (C-6), 71.8–68.2 (C-2 to C-5 and C-2′ to C-5′) and 61.7 (C-6′).

2,3-*Di*-O-*acetyl*-6-*bromo*-6-*deoxy*-4-O-(2,3,4,6-*tetra*-O-*acetyl*-α-D-*glucopyranosyl*)-α-D-*glucopyranosyl Fluoride* **25**.—A solution of compound **21** (200 mg, 0.28 mmol) in pyridine (400 mm^3) was treated with hydrogen fluoride–pyridine mixture (3 cm^3) at 0 °C for 1 h. After work-up as described above for compound **23**, column chromatography [ethyl acetate–light petroleum (1:1 v/v)] of the residue gave *compound* **25** which was crystallized from diethyl ether (135 mg, 71%), m.p. 184–185 °C (Found: C, 43.5; H, 5.1; Br, 11.9; F, 2.7. $C_{24}H_{32}BrFO_{15}$ requires C, 43.71; H, 4.89; Br, 12.11; F, 2.88%); $[\alpha]_D$ +98° (*c* 1.0 in $CHCl_3$); δ_C(75 MHz; $CDCl_3$) 103.6 (C-1, $J_{1,F}$ 236), 95.3 (C-1′), 72.5–67.9 (C-2 to C-5 and C-2′ to C-5′), 61.8 (C-6′) and 32.7 (C-6).

2,3-*Di*-O-*acetyl*-6-O-*methyl*-4-O-(2,3,4,6-*tetra*-O-*acetyl*-α-D-*glucopyranosyl*)-α-D-*glucopyranosyl Fluoride* **26**.—A solution of compound **22** (200 mg, 0.3 mmol) in pyridine (500 mm^3) was treated with hydrogen fluoride–pyridine mixture (3 cm^3) at 0 °C. After 20 min at room temperature, work-up as already described, followed by column chromatography [ethyl acetate–light petroleum (2:3 v/v)], led to *compound* **26**, which was crystallized from diethyl ether (60 mg, 30%), m.p. 134–135 °C (Found: C, 49.1; H, 5.7; F, 3.1. $C_{25}H_{35}FO_{16}$ requires C, 49.18; H, 5.78; F, 3.11%); $[\alpha]_D$ +86° (*c* 1.0 in $CHCl_3$); δ_C(75 MHz; $CDCl_3$) 103.8 (C-1, $J_{1,F}$ 233), 95.1 (C-1′), 72.0–68.1 (C-2 to C-6 and C-2′ to C-5′), 61.4 (C-6′) and 59.5 (OMe).

1,2,3,6-*Tetra*-O-*acetyl*-4-O-(2,3,4-*tri*-O-*acetyl*-6-*deoxy*-6-*fluoro*-α-D-*glucopyranosyl*)-β-D-*glucopyranose* **29**.—A solution of compound **27** (300 mg, 0.47 mmol) in glyme (3 cm^3) was cooled to −10 °C, when a solution of DAST (250 mm^3) in glyme (850 mm^3) was added. Treatment and work-up as described for the synthesis of compound **20**, followed by chromatography on silica column with ethyl acetate–light petroleum (2:3 v/v), led to *compound* **29**, which was crystallized from diethyl ether (280 mg, 93%), m.p. 183–184 °C (Found: C, 48.9; H, 5.4; F, 3.0. $C_{26}H_{35}FO_{17}$ requires C, 48.90; H, 5.52; F,

2.97%); $[\alpha]_D$ +54° (*c* 1.0 in $CHCl_3$); δ_C(75 MHz; $CDCl_3$) 95.6 (C-1'), 91.4 (C-1), 82.7 (C-6', $J_{6,F}$ 180), 75.4–67.7 (C-2 to C-5 and C-2' to C-5') and 62.4 (C-6).

1,2,3,6-*Tetra*-O-*acetyl*-4-O-(2,3,4-*tri*-O-*acetyl*-6-O-*methyl*-α-D-*glucopyranosyl*)-β-D-*glucopyranose* **30**.—A solution of compound **27** (1 g, 1.57 mmol) was treated as described for the synthesis of compound **22**. Chromatography on silica gel with ethyl acetate–light petroleum (1:1 v/v) as eluent afforded *compound* **30**, which was crystallized from diethyl ether (1 g, 97%), m.p. 170–171 °C (Found: C, 49.7; H, 6.0. $C_{27}H_{38}O_{18}$ requires C, 49.84; H, 5.88%); $[\alpha]_D$ +64° (*c* 1.0 in $CHCl_3$); δ_C(75 MHz; $CDCl_3$) 95.6 (C-1'), 91.4 (C-1), 75.4–68.7 (C-2 to C-5 and C-2' to C-5'), 62.6 (C-6) and 59.6 (OMe).

2,3,6-*Tri*-O-*acetyl*-4-O-(2,3,4-*tri*-O-*acetyl*-6-*deoxy*-α-D-*glucopyranosyl*)-α-D-*glucopyranosyl Fluoride* **32**.—A solution of 1,2,2',3,3',4',6-hepta-*O*-acetyl-6'-deoxy-β-maltose **28** (200 mg, 0.32 mmol), prepared as described,[20] in pyridine (450 mm^3) was treated with hydrogen fluoride–pyridine mixture (3.5 cm^3) at 0 °C for 2 h. Work-up and chromatography on silica gel with ethyl acetate–light petroleum (2:3 v/v) as eluent yielded *compound* **32**, which was crystallized from diethyl ether (110 mg, 60%), 168–170 °C (Found: C, 49.7; H, 5.9; F, 2.4. $C_{24}H_{33}FO_{15}$ requires C, 49.65; H, 5.72; F, 3.27%); $[\alpha]_D$ +106° (*c* 1.0 in $CHCl_3$); δ_C(75 MHz; $CDCl_3$) 103.7 (C-1, $J_{1,F}$ 232), 95.6 (C-1'), 73.4–66.7 (C-2 to C-5 and C-2' to C-5'), 68.1 (C-6) and 17.4 (C-6').

2,3,6-*Tri*-O-*acetyl*-4-O-(2,3,4-*tri*-O-*acetyl*-6-*deoxy*-6-*fluoro*-α-D-*glucopyranosyl*)-α-D-*glucopyranosyl Fluoride* **33**.—A solution of compound **29** (200 mg, 0.31 mmol) in pyridine (450 mm^3) was treated with hydrogen fluoride–pyridine mixture (3.5 cm^3) at 0 °C for 40 min. Work-up and chromatography on silica gel with ethyl acetate–light petroleum (1:1 v/v) as eluent yielded *compound* **33**, which was crystallized from diethyl ether (160 mg, 85%), m.p. 165–166 °C (Found: C, 48.2; H, 5.5; F, 6.4. $C_{24}H_{32}F_2O_{15}$ requires C, 48.16; H, 5.39; F, 6.34%); $[\alpha]_D$ +102° (*c* 1.0 in $CHCl_3$); δ_C(75 MHz; $CDCl_3$) 103.8 (C-1, $J_{1,F}$ 236), 95.6 (C-1'), 82.7 (C-6'), 72.0–67.7 (C-2 to C-5 and C-2' to C-5') and 62.0 (C-6).

2,3,6-*Tri*-O-*acetyl*-4-O-(2,3,4-*tri*-O-*acetyl*-6-O-*methyl*-α-D-*glucopyranosyl*)-α-D-*glucopyranosyl Fluoride* **34**.—A solution of compound **30** (300 mg, 0.46 mmol) in pyridine (650 mm^3) was treated with hydrogen fluoride–pyridine mixture (5 cm^3) at 0 °C for 25 min. Work-up and chromatography on silica gel with ethyl acetate–light petroleum (1:2 v/v) as eluent yielded *compound* **34**, which was crystallized from diethyl ether (240 mg, 85%), m.p. 137–138 °C (Found: C, 48.8; H, 5.8; F, 3.0. $C_{25}H_{35}FO_{16}$ requires C, 49.18; H, 5.78; F, 3.11%); $[\alpha]_D$ +107° (*c* 1.0 in $CHCl_3$); δ_C(75 MHz; $CDCl_3$) 103.8 (C-1, $J_{1,F}$ 232), 95.6 (C-1'), 71.9–69.8 (C-2 to C-5 and C-2' to C-6') and 59.7 (OMe).

2,3,6-*Tri*-O-*acetyl*-4-O-(2,3,4-*tri*-O-*acetyl*-6-*bromo*-6-*deoxy*-α-D-*glucopyranosyl*)-α-D-*glucopyranosyl Fluoride* **36**.—A solution of 1,2,2',3,3',6-hexa-*O*-acetyl-4'-*O*-benzoyl-6'-bromo-6'-deoxy-β-maltose **31** (200 mg, 0.26 mmol), prepared as described,[20] in pyridine (400 mm^3) was treated with hydrogen fluoride–pyridine (3 cm^3) at room temperature for 45 min. Work-up and chromatography on silica gel with ethyl acetate–petroleum (1:2 v/v) as eluent yielded *compound* **35** (100 mg, 51%); (Cl) *m/z* 721 $(M + H)^+$.

Without further characterization, the acylated compound **35** (100 mg, 0.14 mmol) was treated in methanol (3 cm^3) with 1 mol dm^{-3} sodium methoxide (100 mm^3). After neutralization (pH paper) with Amberlite IR 120 (H^+), filtration and concentration to dryness, the residue was acetylated with pyridine–acetic anhydride (1:1 v/v; 7 cm^3). After the usual work-up, *compound* **36** was isolated after chromatography on silica gel with ethyl acetate–light petroleum (2:3 v/v) as eluent and crystallization from diethyl ether (73 mg, 77%); m.p. 174–175 °C (Found: C,44.1; H, 4.9; Br, 12.0; F, 2.9. $C_{24}H_{32}BrFO_{15}$ requires C, 43.71; H, 4.89; Br, 12.11; F, 2.88%); $[\alpha]_D$ +100° (*c* 1.0 in $CHCl_3$); δ_C(75 MHz; $CDCl_3$) 103.7 (C-1, $J_{1,F}$ 236), 95.6 (C-1'), 72.1–69.0 (C-2 to C-5 and C-2' to C-5'), 62.4 (C-6) and 31.3 (C-6').

2,3,6-*Tri*-O-*acetyl*-4-S-(2,3,4,6-*tetra*-O-*acetyl*-α-D-*glucopyranosyl*)-4-*thio*-α-D-*glucopyranosyl Fluoride* **38**.—A solution of acetylated 4-thiomaltose **37** (1 g, 1.4 mmol), prepared as described,[24] in pyridine (500 mm^3) was treated in hydrogen fluoride–pyridine (5 cm^3) at room temperature for 2 h. Work-up and chromatography on silica gel with ethyl acetate–light petroleum (2:3 v/v) as eluent, followed by crystallization from diethyl ether–hexane, yielded *compound* **38** in quantitative yield; m.p. 97–100 °C (Found: C, 47.5; H, 5.1; F, 3.0; S, 4.8. $C_{26}H_{35}FO_{16}S$ requires C, 47.70; H, 5.39; F, 2.90; S, 4.89%); $[\alpha]_D$ +203° (*c* 1.0 in $CHCl_3$); δ_C(75 MHz; $CDCl_3$) 104.1 (C-1, $J_{1,F}$ 232), 82.7 (C-1'), 71.8–68.1 (C-2, -3, -5 and C-2' to C-5'), 63.1 (C-6), 61.6 (C-6') and 43.1 (C-4).

2,3,4,6-*Tetra*-O-*acetyl*-5-*thio*-α-D-*glucopyranose* **40**.—To a solution of 1,2,3,4,6-penta-*O*-acetyl-5-thio-α-D-glucopyranose **39** (750 mg, 1.85 mmol), prepared as described,[25] in DMF (5 cm^3) was added hydrazine acetate (250 mg, 1.2 mol equiv.).[26] After 10 min at 50 °C, the reaction mixture was diluted with ethyl acetate and washed with brine. The extract was dried and evaporated. Chromatography on silica gel with ethyl acetate–light petroleum (1:2 v/v) as eluent yielded *compound* **40**, which was crystallized from diethyl ether (590 mg, 88%); m.p. 113–114 °C (Found: C, 46.5; H, 5.9; S, 8.6. $C_{14}H_{20}O_9S$ requires C, 46.15; H, 5.53; S, 8.79%); $[\alpha]_D$ +135° (*c* 1.0 in $CHCl_3$); δ_C(75 MHz; $CDCl_3$) 75.0 (C-1), 72.2–70.4 (C-2 to C-4), 61.2 (C-6) and 38.0 (C-5); δ_H(300 MHz; $CDCl_3$) 5.45 (t, 3-H), 5.20 (t, 4-H), 5.10 (d, $J < 3$, 1-H), 5.05 (dd, *J* 10, 2-H), 4.30 (dd, 6-H^a), 4.00 (dd, 6-H^b), 3.75 (s, OH) and 3.60 (m, 5-H).

2,3,4,6-*Tetra*-O-*acetyl*-5-*thio*-α-D-*glucopyranosyl Fluoride* **41**.—A solution of compound **40** (100 mg, 0.27 mmol) in 1,2-dichloroethane (2 cm^3) was cooled to −10 °C, and then DAST (150 mm^3) was added slowly. After 25 min at room temperature, the reaction mixture was treated as described for the synthesis of compound **20**. Chromatography on silica gel with ethyl acetate–light petroleum (1:3 v/v) as eluent afforded *compound* **41**, which was crystallized from diethyl ether (80 mg, 81%), m.p. 102–103 °C (Found: C, 45.9; H, 5.3; F, 5.4. $C_{14}H_{19}FO_8S$ requires C, 45.89; H, 5.23; F, 5.19%); $[\alpha]_D$ +106 (*c* 1.0 in $CHCl_3$); δ_C(75 MHz; $CDCl_3$) 91.1 (C-1, $J_{1,F}$ 226), 73.9 (C-2, $J_{2,F}$ 23.4), 71.2 and 70.1 (C-3 and -4), 60.6 (C-6) and 39.6 (C-5).

General Procedure for the Synthesis of Glycosyl Fluorides **1–9** *and* **11–13**.—Peracetylated maltosyl fluorides (100 mg) were treated in methanol (30 cm^3) with 1 mol dm^{-3} sodium methoxide (100 mm^3). After 2 h at room temperature, neutralization with Amberlite IRN 77, evaporation to dryness, and freeze-drying led in quantitative yield to the expected fluorides **1–9**, **11–13**. All the compounds were pure by TLC and HPLC and were immediately used in enzymatic experiments.

6'-O-*Acetyl*-α-*maltosyl Fluoride* **10**.—Vinyl acetate (20 mg, 0.22 mmol) and freeze-dried substilisin (5.5 mg) in buffer phosphate (0.1 mol dm^{-3}; pH 7.8) were added to a solution of α-maltosyl fluoride **1** (55 mg, 0.16 mmol) in pyridine (500 mm^3). The suspension was stirred at 45 °C for 2 days, then the enzyme was removed by filtration and the mixture was evaporated to dryness. Chromatography on silica gel with chloroform–methanol (8:2 v/v) as eluent led to *compound* **10** (35 mg,

60%), δ_C(75 MHz; CD_3OD) 172.9 (CO), 108.7 (C-1, $J_{1,F}$ 230), 130.1 (C-1′), 81.1 (C-4), 74.9 (C-3′), 74.7 (C-3), 74.2 and 74.1 (C-2′ and -5′), 72.7 (C-2), 72.3 (C-5), 71.6 (C-4′), 65.1 (C-6′), 61.7 (C-6) and 20.7 (Ac).

Enzymatic Incubation with CGTase.—Coupling reactions for the specificity of the acceptor part of the active site. Each potential substrate (5 mg) was added to α-cyclodextrin (5 mg) in phosphate buffer (0.1 mol dm^{-3}; pH 7, 1 cm^3). Then to this solution was added CGTase (635 U cm^{-3}; 20 mm^3) and the mixture was treated to 45 °C for 1 h. The reaction mixture was analysed by HPLC under the conditions described in General Methods. Relevant controls were used. All chromatogram patterns except that for the 5-thioglycosyl fluoride **13**, were compatible with the formation of linear malto-oligosaccharides.

Specificity of the donor part of the active site. The fluoride (~50 mmol dm^{-3}) in phosphate buffer (0.1 mol dm^{-3}; pH 7.1) was incubated with CGTase (20 mm^3) at 45 °C for 12 h. The reaction mixture was then analysed by HPLC. Negative results were confirmed by addition of more enzyme and longer incubation times.

$6^A,6^C,6^E$-*Tri*-O-*methylcyclohexaamylose* **43**, $6^A,6^C,6^E,6^G$-*tetra*-O-*methylcyclooctaamylose* **44** *and* $6^A,6^C,6^E$-*tri*-O-*methylcycloheptaamylose* **45**. CGTase (635 U cm^{-3}; 1.1 cm^3) was added to a solution of compound **9** (750 mg, 2.12 mmol) in phosphate buffer (0.2 mol dm^{-3}; pH 7; 40 cm^3). The mixture was heated to 40 °C for 20 h. Under those conditions, the presence of the fluoride **9** cannot be detected by HPLC. The reaction was stopped by boiling for 5 min, proteins were eliminated by spinning (14 000 rpm; 20 min) and the supernatant was freeze-dried. HPLC on a C_{18} reversed-phase column (10 mm^3; μ-Bondapak, 19 × 300 mm, Waters Associates) with water–methanol (65:35 v/v) as eluent allowed separation of the crude mixture into its components: linear malto-oligosaccharides **42** (234 mg) were the more mobile compounds; the *cyclohexaamylose* **43** (303 mg, 42%) was then eluted, m.p. 303–306 °C (from water) (Found: C, 44.6; H, 6.7. $C_{39}H_{66}O_{30}\cdot 2H_2O$ requires C, 44.67; H, 6.71%); $[\alpha]_D$ +40° (*c* 0.5 in water); (FAB$^+$) *m/z* 1039 $(M + Na)^+$ and 1015 $(M + H)^+$; δ_C(75 MHz; D_2O) 102.5 and 102.3 (C-1 and -1′), 82.3 (C-4 and -4′), 74.4 (C-3 and -3′), 73.0 (C-5), 72.7 (C-2 and -2′), 71.8 (C-6′), 71.7 (C-5′), 61.4 (C-6) and 59.5 (OMe). Primed carbons are those of the methylated units; the *cycloheptaamylose* **45** (95 mg, 13%) was the next compound, m.p. 270–273 °C (Found: C, 38.4, H, 6.2. $C_{45}H_{76}O_{35}\cdot 2NaHPO_4$* requires C, 38.1; H, 5.7%); $[\alpha]_D$ +124° (*c* 0.5 in water); (FAB$^+$) for $C_{45}H_{76}O_{35}Na$ *m/z* 1199.41 ± 2.8 ppm $(M + Na)^+$; δ_C(75 MHz; D_2O) 103.6 and 103.0 (C-1 and -1′), 82.7 and 82.0 (C-4 and -4′), 74.5 (C-2), 74.3 (C-3), 73.7 (C-5′), 72.0 (C-6′), 71.7 (C-5), 61.4 (C-6) and 58.8 (OMe). Primed carbons are these of the methylated units; the *cyclooctaamylose* **44** (118 mg, 16%) was the last compound eluted, m.p. 250–253 °C (Found: C, 33.3, H, 5.2. $C_{52}H_{88}O_{40}\cdot 2(Na_2HPO_4 + NaH_2PO_4)$* requires C, 33.2; H, 5.0%); $[\alpha]_D$ +127° (*c* 0.5 in water); (FAB$^+$) for $C_{52}H_{88}O_{40}Na$ *m/z* 1375.47 ± 3.4 ppm $(M + Na)^+$; δ_C(75 MHz; D_2O) 103.9 and 103.8 (C-1 and -1′), 82.7 and 83.3 (C-4 and -4′), 74.5 (C-2 and -2′), 74.0 (C-3 and -3′), 73.7 (C-5′), 72.0 (C-6′), 71.7 (C-5), 61-5 (C-6) and 58.7 (OMe).

4-O-(6-O-*Methyl*-α-D-*glucopyranosyl*)-D-*glucopyranose* **46**. Linear malto-oligosaccharides **42** (100 mg) were incubated in the presence of α-amylase (54 U mg^{-1}, 5 mg) in phosphate buffer (0.1 mol dm^{-3}; pH 7; 5 cm^3) for 1 h at 40 °C. After inactivation of the enzyme, the mixture was centrifuged and freeze-dried. The only detected compound was identical with the one obtained by de-*O*-acetylation of disaccharide **30** as described for the corresponding fluoride **34** (Found: C, 41.8; H, 72.4. $C_{13}H_{24}O_{11}\cdot H_2O$ requires C, 41.71; H, 7.00%); $[\alpha]_D$ +110° (*c* 1.35 in water); δ_C(75 MHz; D_2O) 99.8 (C-1′), 96.1 (C-1β), 92.2 (C-1α), 77.5, 77.3, 76.5, 74.8, 74.4, 73.5, 73.1, 71.9, 71.6, 71.2, 70.2 and 69.8 (C-2, -2′, -3, -3′, -4, -4′, -5, -5′ and -6′), 61.1 (C-6) and 59.0 (OMe).

6^A, 6^C, 6^E-*Tri*-O-*acetylcyclohexaamylose* **47**. CGTase (635 U cm^{-3}; 400 mm^3) was added to a solution of compound **10** (200 mg, 0.52 mmol) in phosphate buffer (0.2 mol dm^{-3}; pH 7; 1 cm^3). The mixture was heated to 40 °C for 48 h. After work-up as described for compound **43** and HPLC with water–methanol (1:4 v/v) as eluent *compound* **47** was obtained (6 mg, 3%); (FAB$^+$) *m/z* 1121 $(M + Na)^+$ and 1099 $(M + H)^+$; δ_C(75 MHz; D_2O) 174.9 (CO), 102.5 and 102.0 (C-1 and -1′), 82.4 and 81.8 (C-4 and -4′), 74.0 (C-2 and -2′), 72.7 (C-5), 72.4 (C-3 and -3′), 70.4 (C-5′), 64.8 (C-6′), 60.6 (C-6) and 20.9 (Ac).

* Except for compound **43** which was crystallized from water, elemental analytical data for compounds **44**, **45** and **47** may fit with the inclusion of sodium hydrogen phosphate in their cavities. This fact was confirmed by high resolution mass spectroscopy for compounds **44** and **45**.

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