Synthesis of 2'- and 2"-*O*-Acylated Maltotriosides as Potential Fluorescence-quenched Substrates for α-Amylase

Vito Ferro, Morten Meldal* and Klaus Bock
Carlsberg Laboratory, Department of Chemistry, Gamle Carlsberg Vej 10, DK-2500 Valby, Denmark

Several 2'- and 2"-*O*-acylated maltotrioside derivatives have been prepared as substrates for use in fluorescence-quenched assays of α-amylase. These maltotriosides carry a quenching group [2-(4-hydroxy-3-nitrophenyl)ethyl] at the reducing end and a fluorescent donor (2-aminobenzoyl) at either of the non-reducing D-glucose units. The quenching groups were introduced *via* silver triflate-promoted glycosylation, whilst several methods were investigated for the selective introduction of the fluorescent group. Attempted enzymic acylation of the maltotriosides with subtilisin in neat dimethylformamide gave the 2'- and 2"-*O*-acylated derivatives as the major products. These were shown to be the products of chemical rather than enzymic acylation, in contrast to the analogous literature reaction with maltotriose. Intramolecular quenching of the compounds was demonstrated by measuring the increase in fluorescence upon hydrolysis.

α-Amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) catalyses the hydrolysis of 1,4-α-D-glucan linkages of glucose polymers such as starch to produce maltose and larger oligosaccharides.[1] The substrate molecules are attacked at the internal bonds and α-amylases are thus classified as endo-enzymes.[2] α-Amylases are widely distributed in mammals, higher plants, fungi and bacteria[3] and they are of great commercial importance in the brewing and starch industries.[1] Furthermore, the determination of amylase activity in human serum is of diagnostic value in various diseases.[4]

Numerous methods for measuring α-amylase activity have been developed, based on different principles and using various substrates.[5] The more common of these utilize modified maltooligosaccharides with a fluorogenic group as the aglycone. However, these assays require the use of coupled enzymes[5] (*e.g.*, α- or β-glucosidase) to liberate the fluorogenic group. The measurement of activity is thus complicated by the complex mixture of products and the variety of reaction mechanisms operating.[5] In addition, problems arise from the hydrolysis of the substrate by the coupled enzymes. Recently, several substrates have been synthesized which are fluorogenic maltooligosaccharides having modifications at the non-reducing terminus, in order to confer some resistance to hydrolysis by the coupled enzymes.[6-8]

It was decided to investigate the synthesis of internally quenched fluorogenic substrates for assaying α-amylase activity. In such substrates a fluorescent donor is placed near one end of the substrate and an acceptor is placed near the other end. The fluorescence of this type of substrate is initially quenched by intramolecular resonance-energy transfer[9] between the donor and acceptor which is dependent upon the distance between the two, but as the enzyme cleaves the substrate the fluorescence increases. Assays based on this concept have recently been successfully developed for some proteolytic enzymes.[10-12] A fluorescence-quenched assay for α-amylase would offer the advantage of high sensitivity[9,10] and would eliminate the need for coupled enzymes.

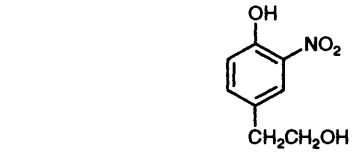
Results and Discussion

A substrate was designed based on the anthraniloyl/3-nitrotyrosine donor/acceptor pair[10] which provides excellent spectral overlap of the fluorescent emission of the donor with the absorption of the acceptor, and thus efficient quenching.[9] The synthetic scheme was developed with maltotriose as a model compound because it is relatively inexpensive and it is the minimum substrate for α-amylase.[1] The scheme required the glycosylation of the acceptor chromophore 2-(4-hydroxy-3-nitrophenyl)ethanol **1**, which was prepared in 51% yield by the lanthanum(III) nitrate-catalysed nitration[13] of 2-(4-hydroxyphenyl)ethanol, with maltotriose to form the trisaccharide **2**, followed by selective introduction of an anthraniloyl group on the non-reducing terminal D-glucose unit. The introduction of an anthraniloyl group on the central D-glucose unit should also provide a suitable substrate.

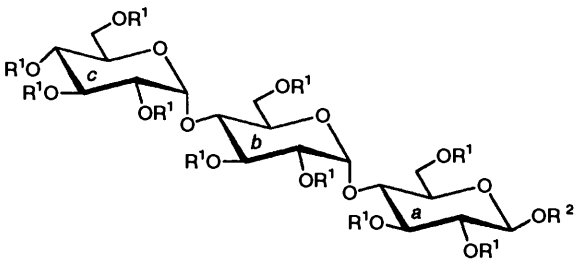
Initially, benzyl β-maltotrioside **3** was synthesized as a model compound to allow us to examine the selectivity of acylations with anthranilic acid derivatives. Maltotriose undecaacetate **4**[14] was converted into the bromide **7** and a glycosylation was attempted with benzyl alcohol at low temperature (−40 °C) by using silver trifluoromethanesulfonate(triflate) as the promoter. However, under these conditions a complex mixture of products resulted (TLC). As the peracetylated bromide **7** was considered to be unstable under the glycosylation conditions, the perbenzoylated derivative **10** was used instead. Treatment of maltotriose with an excess of benzoyl chloride in pyridine at room temperature for 2 days resulted in the formation of an approximately 1:1 mixture of **10** and the decabenzoate **11**. The position of the free hydroxy group at O-3 was confirmed by ^{1}H NMR spectroscopy of the bromide **8** derived from compound **11**. The difficulty in benzoylating at the O-3 position is presumably similar to that reported for maltose[15] and is attributed to the presence of an intramolecular hydrogen bond between the O-3 and O-2' hydroxy groups.[16]

Maltotriose could, however, be completely benzoylated in good yield by heating it with an excess of benzoyl chloride in pyridine at reflux for 16 h in the presence of 4-(dimethylamino)pyridine (DMAP). The resultant mixture of anomers **10** quantitatively gave the desired bromide **9** on subsequent treatment with HBr in acetic acid. Silver triflate-promoted glycosylation of bromide **9** with the alcohol **1** or benzyl alcohol in dichloromethane at −10 °C for 2 to 5 h then gave the glycosides **5** and **6** in 84 and 83% yield, respectively. Subsequent debenzoylation with methanolic sodium methoxide then furnished the required glycosides **2** in 75 (after HPLC) and **3** in 98% yield.

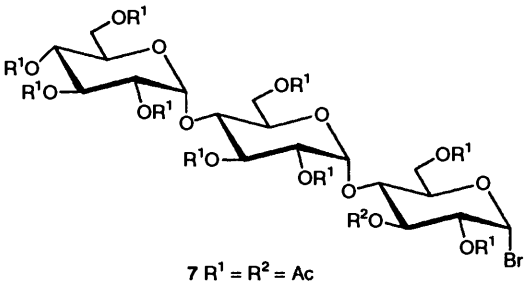
We then attempted to introduce selectively an anthraniloyl group onto the model trisaccharide **3**. Reaction of compound **3**



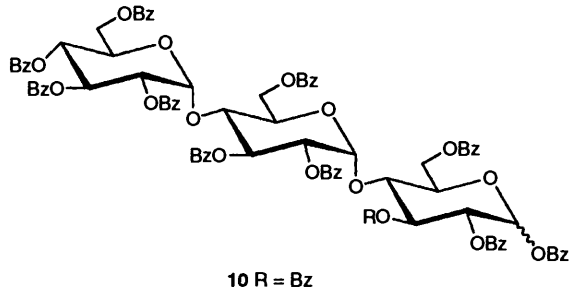
1



2 R^1 = H, R^2 = Ar
3 R^1 = H, R^2 = Bn
4 R^1 = R^2 = Ac
5 R^1 = Bz, R^2 = Ar
6 R^1 = Bz, R^2 = Bn



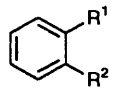
7 R^1 = R^2 = Ac
8 R^1 = Bz, R^2 = H
9 R^1 = R^2 = Bz



10 R = Bz
11 R = H

with the activated ester **12***[10] in pyridine at room temperature gave only a trace of acylated material which, according to ^{1}H NMR spectroscopy, was a mixture of several products. Elevation of the reaction temperature to 45 °C resulted in more acylation but reduced selectivity (TLC, NMR). An attempt was also made to acylate compound **3** with 2-(sulfinylideneamino)-benzoyl chloride **13**[17] in pyridine; however, a complex mixture of products was obtained (TLC), due to lack of selectivity.

To obtain the desired selectivity attention was therefore focussed on enzyme-catalysed acyl-transfer reactions.[18] Recently, proteolytic enzymes have been used in combination with activated esters (usually trichloroethyl esters) to acylate regioselectively di- and oligosaccharides in anhydrous organic solvents.[19-21] The report by Klibanov and co-workers[19] of the monoacylation of maltotriose at the O-6″ position with the protease subtilisin and trichloroethyl butyrate in anhydrous dimethylformamide (DMF) was particularly encouraging.



12 R^1 = NHBoc, R^2 = CO_2Dhbt
13 R^1 = N=S=O, R^2 = COCl
14 R^1 = NH_2, R^2 = $CO_2CH_2CCl_3$
15 R^1 = NHBoc, R^2 = C(O)-Leu-OCH_2CCl_3
16 R^1 = NHBoc, R^2 = $CO_2CH_2CCl_3$

Boc = *tert*-butoxycarbonyl
Dhbt = 4-oxo-3,4-dihydro-1,2,3-benzotriazin-3-yl

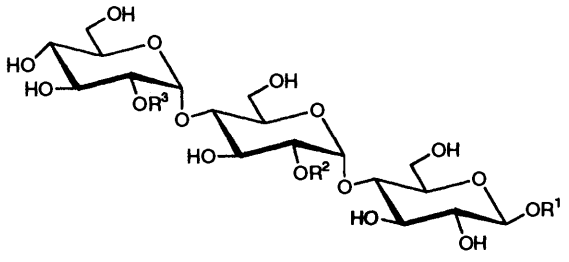
2,2,2-Trichloroethyl 2-aminobenzoate **14** was prepared from the chloride **13** and 2,2,2-trichloroethanol, and the acylation of compound **3** with compound **14** catalysed by subtilisin in dry DMF at 37 °C was attempted. However, there was no detectable reaction after 5 days (the presence of active enzyme was confirmed by removal of an aliquot and assaying for hydrolysis of a chromogenic peptide substrate).[22] It was considered at this stage that the ester **14** may be poorly accommodated in the active site of the enzyme.[23] The substrate specificity of subtilisin was recently determined, and a strong preference for leucine in the S_1 binding pocket of the enzyme was demonstrated.[24] A more appropriate substrate for the enzyme should therefore be a leucine derivative such as **15** or **17**. In the case of compound **17**, a successful acylation could be followed by removal of the *tert*-butoxycarbonyl (Boc) protecting group and the coupling of an anthraniloyl residue to it *via* the activated ester **12**.

Boc-Leu-OCH_2CCl_3
17

The ester **17** was prepared in quantitative yield by dicyclohexylcarbodiimide (DCC)–DMAP-mediated coupling of Boc-Leu-OH with trichloroethanol. Deprotection of the product **17** with 95% aq. trifluoroacetic acid (TFA) and coupling with the anthranilate **12** then furnished compound **15** in excellent yield (97%). Incubation of compound **3** with an excess of the ester **17** and subtilisin in DMF at 37 °C for 7 days resulted in the formation of a mixture of acylation products (TLC). These were separated from the starting material by column chromatography, and were then treated with 95% aq. TFA to remove the Boc groups. Analytical HPLC showed the mixture to contain two major products and several minor ones. The products were then purified by semi-preparative reversed-phase HPLC and their structures determined by a combination of 1D ^{1}H and ^{13}C NMR and 2D shift-correlation spectroscopy (homo- and heteronuclear COSY). The two major products, isolated as their trifluoroacetate salts in yields of 13 and 7%, respectively, were found to be compounds **18** and **19**. Acylation had taken place at the O-2′ and O-2″ hydroxy groups, respectively, in contrast to the earlier report for the acylation of maltotriose with trichloroethyl butyrate.[19]

The trisaccharide **3** was similarly incubated with the ester **15**. Acylation also occurred and analytical HPLC again showed the presence of two major products, presumably compounds **20** and **21**. Similar results were obtained when compound **2** was treated with compound **15** and the two major products, isolated in 13 and 6% yield, were identified as the salts **22** and **23**, respectively. Compounds **22** and **23**, with a fluorescence donor and an acceptor on either side of a scissile bond, fulfil the requirements for potential fluorescence-quenched substrates for α-amylase.

* The m.p. of compound **12** and its precursor, 2-(*tert*-butoxycarbonylamino)benzoic acid, were inadvertedly interchanged (typographical error).[10] Compound **12** has a m.p. of 149–150 °C, whilst the precursor has a m.p. of 155–156 °C.



18 R^1 = Bn, R^2 = Leu·TFA, R^3 = H
19 R^1 = Bn, R^2 = H, R^3 = Leu·TFA
20 R^1 = Bn, R^2 = Leu-ABz·TFA, R^3 = H
21 R^1 = Bn, R^2 = H, R^3 = Leu-ABz·TFA
22 R^1 = Ar, R^2 = Leu-ABz·TFA, R^3 = H
23 R^1 = Ar, R^2 = H, R^3 = Leu-ABz·TFA
24 R^1 = Bn, R^2 = ABz·TFA, R^3 = H
25 R^1 = Bn, R^2 = H, R^3 = ABz·TFA
26 R^1 = Ar, R^2 = ABz·TFA, R^3 = H
27 R^1 = Ar, R^2 = H, R^3 = ABz·TFA

ABz = 2-aminobenzoyl

The selectivities observed in the acylation were inconsistent with those in previously reported enzyme-mediated acylations[18] and pointed towards chemical, rather than enzyme-mediated, acylation. Indeed, when reactants **3** and **17** were shaken in DMF at 37 °C for several days in the absence of enzyme, compounds **18** and **19** were once again formed in the same ratio (analytical HPLC). It was thus clear that the acylations were not subject to enzymic catalysis but were due to the reactivity of the 2′- and 2″-OH groups of the trisaccharides and to that of the activated esters. Interestingly, Klibanov and co-workers[19] have also reported the acylation of various monosaccharides with monochloroethyl esters of some *N*-acylamino acids. In these cases an excess of monosaccharide rather than activated ester was used, and some acylation was reported at the secondary hydroxy groups.

The greater reactivity of the secondary 2-OH group over both the secondary 3-OH and the primary 6-OH has been observed previously in glucopyranose oligomers such as cyclodextrins.[25,26] Recently, it has been suggested, by studies of semiempirical molecular orbital calculations (AM1) on α-D-glucopyranose and β-maltose,[27] that the secondary alcohol functions (2-OH and 3-OH) are more acidic than the primary alcohol (6-OH), which is consistent with experimental findings.[25,26,28] The acidities of the 2-OH and 3-OH groups were found to be similar, and it was proposed that the experimentally observed greater reactivity of the 2-OH over the 3-OH may be due to hydrogen bonding from the 2-OH to the 3-OH of the adjacent sugar residue.[27]

It was subsequently decided to repeat the reported[19] subtilisin-catalysed acylation of maltotriose with trichloroethyl butyrate in DMF. In our hands some acylation occurred in the presence of the enzyme upon incubation at 37 °C for 5 days, as judged by TLC. The acylation products, isolated by column chromatography in 18% yield as an inseparable mixture, were analysed by ^{1}H and ^{13}C NMR spectroscopy and this revealed a complex mixture of products as opposed to the reported[19] greater than 95% selectivity in favour of the 6″-*O*-butyryl derivative. The reaction was then repeated without the enzyme but there was no detectable reaction at 37 °C. However, upon heating of the mixture at 60 °C for 24 h, substantial acylation occurred. The monoacylation products obtained (26%) were similar to those from the enzymic reaction, but they were present in a different ratio (^{1}H NMR). A complex mixture of diacylated products was also obtained (28%).

Finally, the ester **16**, which should be more reactive[10] than the corresponding free amine **14**, was prepared by the DCC–DMAP-mediated coupling of 2-(*tert*-butoxycarbonylamino)-benzoic acid with trichloroethanol. Compound **16** was stirred with compound **3** in DMF at 37 °C for 5 days, without added enzyme. Substantial acylation occurred (TLC) and analytical HPLC once again indicated two major products, presumably compounds **24** and **25**. Two other products were also present to a significant extent. The reaction was repeated with glycoside **2**; however, in this case the acylation was surprisingly slow at 37 °C so the reaction mixture was heated at 60 °C for 2 days. Analytical HPLC indicated a similar product profile and the 2′- and 2″-*O*-acylated products **26** and **27** were isolated in 10 and 4% yield, respectively, after HPLC. The increased temperature also resulted in the formation of a significant amount of higher-R_f material (presumably diacylated products). Compounds **26** and **27** are also potential fluorescence-quenched substrates for α-amylase.

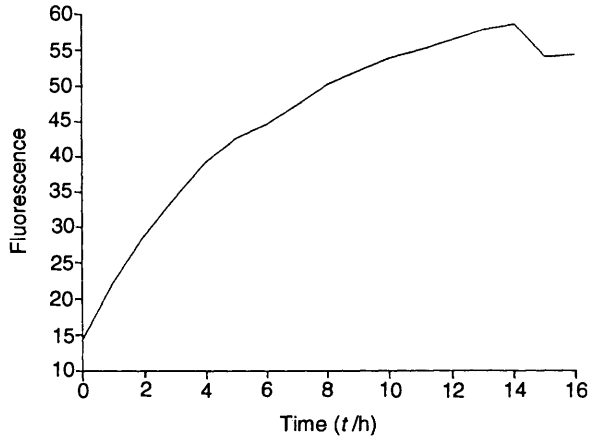


Fig. 1 Progress curve for the enzymic hydrolysis of compound **23** with Novamyl 1500 MG at 60 °C in 40 mmol dm^{-3} sodium acetate, 1 mmol dm^{-3} calcium chloride, pH 5.5

The fluorescence of 1 μmol dm^{-3} aqueous solutions of compounds **22**, **23**, **26** and **27** were measured at 25 °C (emission at 420 nm upon excitation at 320 nm).[10,24] To demonstrate the internal quenching of the compounds the increase in fluorescence upon hydrolysis was determined. Initially this was done chemically. For example, the hydrolysis of a 1 μmol dm^{-3} solution of compound **22** was effected by heating it with 1 mol dm^{-3} HCl at 60 °C overnight. The fluorescence of the hydrolysate, after neutralization, showed a 9.5-fold increase. Hydrolysis of the compounds was also carried out enzymically with Novamyl 1500 MG, a commercially available amylase from Novo Nordisk A/S, as shown in Fig. 1 for compound **23**. Hydrolyses were carried out at 60 °C with 1 μmol dm^{-3} solutions of the substrates in 40 mmol dm^{-3} sodium acetate buffer containing 1 mmol dm^{-3} $CaCl_2$, pH 5.5. Appropriate control experiments were also performed (*e.g.*, without enzyme) to ensure that no chemical hydrolysis was occurring at pH 5.5. Indeed, at neutral or higher pH chemical hydrolysis of the ester group was observed, as shown in Fig. 2 for compound **23**. All the substrates showed an increase in fluorescence of from 2.5- to 8-fold. The enzyme-mediated hydrolyses were somewhat slow, indicating the maltotrioses are poorer substrates for α-amylase than are longer maltooligosaccharides.[29] Further work is in progress to prepare longer substrates, *e.g.*, maltoheptaosides, which are better substrates for α-amylase,[29] by a modified method and to evaluate these substrates in α-amylase assays.

In conclusion, it has been shown that the 2′- and 2″-OH groups of maltotriose derivatives are the most reactive. It was therefore possible to prepare, with good selectivity but modest yields, 2′- and 2″-*O*-acylated maltotriosides, including compounds **22**, **23**, **26** and **27** which are potential fluorescence-quenched substrates for α-amylase. It was also shown that, in reactions carried out in the presence of subtilisin with some

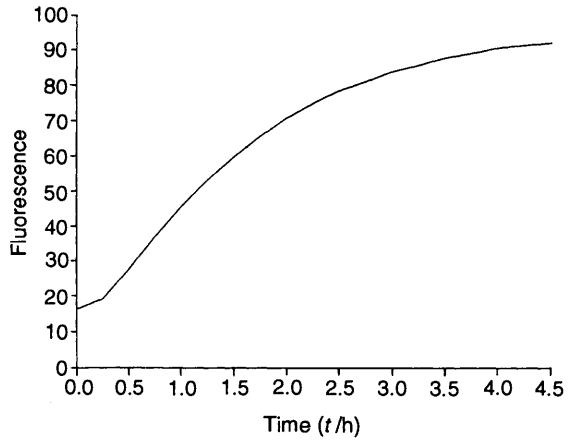


Fig. 2 Progress curve for the chemical hydrolysis of compound **23** at 60 °C in 40 mmol dm^{-3} sodium acetate, 1 mmol dm^{-3} calcium chloride, pH adjusted to 7.5

trichloroethyl esters, the acylation observed was non-enzymic. An examination of the previously reported enzymic acylation of maltotriose[19] also revealed that the selectivity obtained is not as good as that suggested in the literature (at least in our hands and certainly not comparable to that obtained with monosaccharides), and that acylation can also be effected without the enzyme by raising the temperature. Due caution must therefore be exercised in such reactions. The reactivity of the activated ester used must be sufficient to favour the desired transformation,[18] but not too great as to allow chemical acylation to take place, as has been shown here with maltotriosides.

Experimental

General.—M.p.s were determined on a Büchi apparatus and are uncorrected. Optical rotations were determined on a Perkin-Elmer 241 polarimeter at ambient temperature and are given in units of 10^{-1} deg cm^2 g^{-1}. NMR spectra were recorded using a Bruker AM-500 spectrometer or a Bruker AMX-600 spectrometer at 300 K for solutions in $CDCl_3$ ($CHCl_3$ as the standard: δ_H 7.27, δ_C 77.0) or D_2O (internal acetone as the standard: δ_H 2.225, δ_C 31.0) ($CDCl_3$ unless stated otherwise). *J* Values are given in Hz. The assignment of signals was carried out using 2D shift-correlation spectroscopy (homo- and hetero-nuclear COSY). ^1H NMR chemical shifts of overlapping signals were obtained from the centre of the cross-peaks in the COSY spectra. Carbohydrate units are indicated by *a*, *b* and *c* with *a* at the reducing end. Electrospray mass spectra were obtained on a Fisons VG Quattro instrument. Fluorescence measurements were performed on a Perkin-Elmer LS 50 luminescence spectrometer. Microanalyses were performed by LEO Pharmaceutical Products, Denmark. HPLC-grade solvents were purchased from Labscan Ltd (Dublin, Ireland) and, where necessary, were dried by standard procedures and stored over 3 Å molecular sieves. DMF was fractionally distilled under reduced pressure and stored over 3 Å molecular sieves. Light petroleum was the 60–80 °C fraction. Flash chromatography was performed on short columns (10–15 cm) of Merck silica gel 60 (230–400 mesh) under a positive pressure with specified eluents. Vacuum liquid chromatography (VLC) was performed with Merck silica gel 60H. TLC was on Merck silica gel 60 F_{254} aluminium-backed sheets. Compounds were visualized by spraying with 10% aq. sulfuric acid and heating (carbohydrates) or spraying with 10% ammonium molybdate in 2 mol dm^{-3} sulfuric acid and then heating (general). Semi-preparative HPLC separations were performed on a Waters 600E HPLC system using a Delta-Pak C_{18} 15 μ 300 Å (25 mm × 20 cm) column with a flow rate of 10 cm^3 min^{-1} and detection at 215 and 280 nm using a Waters 991 photodiode array detector. Analytical HPLC chromatograms were obtained using a Waters RCM 8 mm × 10 cm module with a Waters 8 NV C_{18} 4 μ 60 Å column. Solvent system A: 0.1% TFA in water; B: 0.1% TFA in 90% acetonitrile–10% water. Subtilisin A was from Novo Nordisk. Prior to use, the enzyme was dissolved in 0.1 mol dm^{-3} Bicine buffer containing 2 mmol dm^{-3} $CaCl_2$, the pH adjusted[30,31] to 7.8, and the solution was then lyophilized. The presence of active enzyme in the reaction mixtures was checked by removal of an aliquot and assaying for hydrolysis of Suc-Ala-Ala-Pro-Phe-NH-Ph-NO_2.[22] Novamyl 1500 MG was a gift from Novo Nordisk.

Maltotriose Undecabenzoate **10**.—Maltotriose (3.0 g, 6.0 mmol) and DMAP (250 mg) were dissolved in pyridine (30 cm^3) and the solution as cooled to 0 °C. Benzoyl chloride (9.6 g, 7.9 cm^3, 69 mmol) was added and the mixture was stirred at 0 °C for 1 h and then was allowed to warm to room temperature. More pyridine (10 cm^3) was added and the mixture was then heated at reflux for 16 h. The mixture was cooled and water was added dropwise until all the solids had dissolved. The mixture was stirred for 0.5 h and then was poured into ice–water. The organic layer was separated and the aqueous phase was extracted with dichloromethane. The combined organic phases were washed successively with dil. HCl, water and brine, dried ($MgSO_4$), and concentrated to give a yellow oil (14 g). VLC [EtOAc–light petroleum (2:3)] then gave, as a mixture of anomers, the *perbenzoate* **10** (8.3 g, 85%) as a foam (Found: C, 68.9; H, 4.8. $C_{95}H_{76}O_{27}$ requires C, 69.2; H, 4.6%).

Deca-O-benzoyl-α-maltotriosyl Bromide **9**.—3 Å Molecular sieves were added to a solution of the perbenzoate **10** (3.3 g, 2.0 mmol) in dichloromethane (10 cm^3) and the mixture was stirred (0.5 h). A solution of 33% HBr in acetic acid (3.6 cm^3, 20 mmol) was added and the mixture was stirred for 1 h at 25 °C before being diluted with dichloromethane and poured into ice–water. The organic phase was separated and the aqueous phase was extracted with dichloromethane. The combined organic phases were washed successively with saturated aq. $NaHCO_3$, water and brine, dried ($MgSO_4$), and concentrated to give a foam (3.2 g, 100%), used without further purification in the next step. A small portion was purified by flash chromatography [EtOAc–light petroleum (2:3)] to give the pure *bromide* **9** as a foam, $[\alpha]_D$ +112 (*c* 0.6, $CHCl_3$) (Found: C, 65.6; H, 4.5. $C_{88}H_{71}BrO_{25}$ requires C, 65.7; H, 4.4%). ^1H and ^{13}C NMR data are presented in Tables 1, 3 and 5.

Benzyl Deca-O-benzoyl-β-maltotrioside **6**.—A mixture of silver triflate (594 mg, 2.0 mmol), benzyl alcohol (430 mg, 0.41 cm^3, 4.0 mmol) and 3 Å molecular sieves in dichloromethane (8 cm^3) under argon was stirred for 0.5 h and was then cooled to −10 °C. A solution of the bromide **9** (3.2 g, 2.0 mmol) in dichloromethane (5 cm^3) was added and the mixture stirred at −10 °C for 2 h. 2,4,6-Trimethylpyridine (2,4,6-collidine) (290 mg, 0.32 cm^3, 2.4 mmol) was added and the mixture was allowed to warm to room temperature. Filtration (Celite) and concentration of the filtrate gave a residue, which was purified by VLC [EtOAc–light petroleum (2:3)] to give the *glycoside* **6** (2.7 g, 83%) as a glass, $[\alpha]_D$ +49 (*c* 0.4, $CHCl_3$) (Found: C, 69.5; H, 4.9. $C_{95}H_{78}O_{26}$ requires C, 69.8; H, 4.8%). ^1H and ^{13}C NMR data are presented in Tables 1, 3 and 5.

Benzyl β-Maltotrioside **3**.—The decabenzoate **6** (2.50 g, 1.53 mmol) was suspended in dry methanol (10 cm^3). Methanolic sodium methoxide (2 cm^3; 1 mol dm^{-3}) was then added and the mixture was stirred at room temperature until dissolution was

Table 1 ^{1}H NMR chemical-shift data for compounds **2**, **3**, **5**, **6**, **8** and **9**

Compound (unit)	1-H	2-H	3-H	4-H	5-H	6-H^A	6-H^B	Selected other
2[a] *a*	4.46	3.27	3.72	3.59	3.53	3.74	3.90	2.95 (ArCH_2), 3.91 and 4.12 (OCH$_2$), 7.11,
b	5.38	3.60	3.93	3.63	3.82	3.75	3.84	7.59 and 8.04 (ArH)
c	5.39	3.57	3.68	3.42	3.70	3.84	3.84	
3[a] *a*	4.54	3.34	3.74	3.64	3.56	3.92	3.80	4.76 and 4.94 (PhCH_2)
b	5.38	3.62	3.95	3.65	3.85	3.85	3.77	
c	5.39	3.60	3.69	3.42	3.72	3.85	3.85	
5[c] *a*	4.77	5.29	5.70	4.45	4.10	4.73	5.05	2.81 (ArCH_2), 3.69 and 4.16 (OCH$_2$), 10.31
b	5.65	5.14	5.97	4.43	4.50	4.67	4.79	(ArOH)
c	5.81	5.33	6.15	5.73	4.43	4.29	4.50	
6[c] *a*	4.91	5.50	5.77	4.60	4.18	4.86	5.16	4.79 and 5.00 (PhCH_2)
b	5.74	5.23	6.06	4.53	4.61	4.76	4.86	
c	5.90	5.43	6.25	5.83	4.54	4.41	4.54	
8[b] *a*	6.71	4.87	4.37	4.06	4.46	4.72	4.87	
b	5.79	5.32	6.09	4.49	4.65	4.78	4.94	
c	5.90	5.39	6.22	5.78	4.60	4.39	4.53	
9[c] *a*	6.78	5.08	6.17	4.55	4.69	4.80	5.05	
b	5.70	5.17	6.01	4.48	4.50	4.66	4.80	
c	5.81	5.31	6.15	5.73	4.42	4.27	4.44	

[a] Measured at 500 MHz in D_2O. [b] Measured at 600 MHz in $CDCl_3$. [c] Measured at 500 MHz in $CDCl_3$.

Table 2 ^{1}H NMR chemical-shift data for compounds **18**, **19**, **22**, **23**, **26** and **27**[a]

Compound (unit)	1-H	2-H	3-H	4-H	5-H	6-H^A	6-H^B	Other
18 *a*	4.50	3.32	3.58	3.66	3.54	3.79	3.93	0.97 (δ-H), 0.98 (ε-H), 1.81 (γ-H), 1.76 and
b	5.58	4.86	4.22	3.80	3.87	3.87	3.87	1.92 (β-H), 4.22 (α-H), 4.76 and 4.94
c	5.44	3.59	3.68	3.43	3.73	3.77	3.86	(PhCH_2)
19 *a*	4.53	3.35	3.72	3.64	3.55	3.78	3.92	0.98 (δ-H), 1.01 (ε-H), 1.84 (γ-H), 1.76 and
b	5.37	3.59	3.79	3.69	3.82	3.82	3.88	1.94 (β-H), 4.22 (α-H), 4.76 and 4.94
c	5.64	4.85	3.91	3.54	3.75	3.75	3.83	(PhCH_2)
22 *a*	4.33	3.20	3.68	3.67	3.47	3.77	3.90	0.97 (δ-H), 1.00 (ε-H), 1.86 (γ-H), 1.88
b	5.68	4.82	4.19	3.80	3.88	3.90	3.90	(β-H), 2.96 (ArCH_2), 3.93 and 4.13 (OCH$_2$),
c	5.44	3.59	3.70	3.43	3.73	3.79	3.87	4.72 (α-H), 7.14–8.06 (ArH)
23 *a*	4.44	3.27	3.70	3.60	3.51	3.77	3.91	0.98 (δ-H), 1.01 (ε-H), 1.86 (γ-H), 1.88
b	5.35	3.58	3.92	3.75	3.84	3.78	3.93	(β-H), 2.96 (ArCH_2), 3.94 and 4.15 (OCH$_2$),
c	5.72	4.80	3.87	3.55	3.76	3.81	3.91	4.75 (α-H), 7.14–8.06 (ArH)
26 *a*	4.37	3.15	3.50	3.64	3.53	3.77	3.92	2.93 (ArCH_2), 3.90 and 4.11 (OCH$_2$),
b	5.70	5.02	4.37	3.86	3.93	3.78	3.88	7.11–8.28 (ArH)
c	5.46	3.60	3.71	3.44	3.75	3.89	3.89	
27 *a*	4.43	3.24	3.65	3.56	3.47	3.72	3.88	2.96 (ArCH_2), 3.94 and 4.14 (OCH$_2$),
b	5.31	3.49	3.70	3.71	3.81	3.85	3.85	7.14–8.16 (ArH)
c	5.78	4.95	4.09	3.62	3.82	3.87	3.92	

[a] Measured at 500 MHz in D_2O.

Table 3 ^{1}H NMR coupling constants for compounds **2**, **3**, **5**, **6**, **8** and **9**[a]

Compound (unit)	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6A}$	$J_{5,6B}$	$J_{6A,6B}$
2[b] *a*	7.9	9.4	9.2	9.7	2.0	5.1	
b	4.0	9.9	9.2	9.2			
c	3.9		9.7	9.7			
3[b] *a*	8.0	9.3			2.1	5.2	
b	3.8	9.2	9.2				
c	3.7	9.9	9.5	9.5			
5[d] *a*	7.6	9.5	9.2	9.3	2.1	4.4	12.2
b	3.9	10.0	9.8	9.8	1.9	3.0	12.4
c	3.9	10.5	10.0	9.6		4.1	13.4
6[d] *a*	7.5	9.1	9.1	9.2	2.1	6.6	12.1
b	3.8	9.9	9.6		3.0		11.5
c	3.9	10.5	10.0	9.6	3.9		13.2
8[c] *a*	3.9	9.6	9.6	9.1	2.0	4.4	12.5
b	4.8	9.6	9.3	8.3	2.0	3.8	12.3
c	3.8	10.5	10.0	10.2	3.0	3.4	12.4
9[d] *a*	3.8	9.9	9.5	9.5			13.4
b	3.6	10.1	8.3	8.2			
c	3.9	10.5	10.1	9.9	3.2		12.5

[a] Observed first-order values ± 0.2 Hz. [b] Measured at 500 MHz in D_2O. [c] Measured at 600 MHz in $CDCl_3$. [d] Measured at 500 MHz in $CDCl_3$.

Table 4 ^{1}H NMR coupling constants for compounds **18**, **19**, **22**, **23**, **26** and **27**[a]

Compound (unit)	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6A}$	$J_{5,6B}$	$J_{6A,6B}$
18 *a*	8.0	9.4	8.9	9.4	2.1	5.2	12.3
b	4.0	10.4	9.2	9.2			
c	3.9	9.9	9.3	9.3	1.9	5.1	
19 *a*	8.0	8.7	9.2	9.5	2.0		12.3
b	3.8	9.9	9.2	9.2		4.2	
c	3.9	10.3	9.7	9.7			
22 *a*	8.1	8.5					
b	4.0	8.9	10.2				
c	3.9	10.0		9.4			
23 *a*	7.9	9.1	9.3	9.4			
b	3.7						
c	4.0						
26 *a*	8.0	9.2	9.0	9.4	2.0	5.6	10.0
b	4.0	10.2	9.4				
c	4.0	9.8	9.5	9.5			
27 *a*	8.0	9.5	9.2	9.2			
b	4.0	10.0	10.0				
c	4.0	10.0	10.0	9.5			

[a] Observed first order values ± 0.2 Hz. Measured at 500 MHz in D_2O.

Table 5 ^{13}C NMR data for compounds **2**, **3**, **5**, **6** and **9**[a]

Compound (unit)	C-1	C-2	C-3	C-4	C-5	C-6	Selected other
2[b] *a*	102.9	73.72	77.0	77.8	75.3	61.5	34.7 (Ar*C*H$_2$), 71.1 (O*C*H$_2$)
b	100.3	72.3	74.1	77.6	72.0	61.3	
c	100.6	72.5	73.68	70.1	73.5	61.3	
3[b] *a*	101.8	73.8	77.0	77.8	75.3	61.5	72.24 (Ph*C*H$_2$)
b	100.2	72.21	74.1	77.5	72.0	61.2	
c	100.5	72.5	73.6	70.1	73.5	61.2	
5[c] *a*	100.5	72.0	74.8	73.7[d]	73.0	63.0	34.6 (Ar*C*H$_2$), 69.5 (O*C*H$_2$)
b	96.6	70.7	71.7	73.6[d]	70.1	63.0	
c	96.7	70.9	69.9	69.0	69.1	62.2	
6[c] *a*	98.6	72.2	75.0	73.6	72.8	63.1	70.2 (Ph*C*H$_2$)
b	96.4	70.7	71.6	73.6	70.0	63.0	
c	96.7	70.8	70.0	69.0	69.1	62.2	
9[c] *a*	86.4	71.9	72.5	72.7	73.2	62.1	
b	97.0	70.7	71.6	73.5	70.3	62.8	
c	96.8	70.8	69.9	69.0	69.1	62.2	

[a] Measured at 125.8 MHz. [b] In D_2O as solvent. [c] In $CDCl_3$ as solvent. [d] Assignments may be reversed.

Table 6 ^{13}C NMR data for compounds **18**, **19**, **22**, **23**, **26** and **27**[a]

Compound (unit)	C-1	C-2	C-3	C-4	C-5	C-6	Selected other
18 *a*	101.8	74.1	77.4	77.2	75.3	61.5	21.9 (C-δ), 22.2 (C-ε), 24.8 (C-γ),
b	97.0	74.7	71.4	76.8	71.9	61.0	39.6 (C-β), 52.3 (C-α), 72.3 (Ph*C*H$_2$),
c	100.3	72.4	73.59	70.1	73.56	61.3	171.3 (C=O)
19 *a*	101.9	73.7	77.0	78.0	75.4	61.5	22.1 (C-δ), 22.2 (C-ε), 24.9 (C-γ),
b	100.3	72.5	74.6	76.3	71.9	61.0	39.6 (C-β), 52.3 (C-α), 72.3 (Ph*C*H$_2$),
c	97.0	74.9	71.0	70.3	73.5	61.3	171.2 (C=O)
22 *a*	102.8	74.2	77.4	75.2	75.1	61.2	21.2 (C-δ), 23.0 (C-ε), 25.4 (C-γ),
b	96.3	74.1	71.5	76.9	71.9	61.5	34.7 (Ar*C*H$_2$), 39.7 (C-β), 52.6 (C-α),
c	100.4	72.4	73.6	70.1	73.5	61.1	71.0 (O*C*H$_2$), 170.0 and 174.4 (C=O)
23 *a*	102.9	73.6	77.0	78.0	75.3	61.5	21.4 (C-δ), 22.9 (C-ε), 25.4 (C-γ),
b	100.4	72.7	74.6	75.1	71.7	61.1	34.7 (Ar*C*H$_2$), 39.9 (C-β), 52.7 (C-α),
c	96.7	74.4	71.0	70.2	73.4	61.3	71.0 (O*C*H$_2$), 170.0 and 174.4 (C=O)
26 *a*	102.8	74.1	77.1	76.9	75.2	61.5	34.7 (Ar*C*H$_2$), 71.0 (O*C*H$_2$), 166.8 (C=O)
b	97.1	74.3	71.5	77.0	71.9	61.3	
c	100.5	72.5	73.61	70.1	73.58	61.1	
27 *a*	102.9	73.7	76.9	77.6	75.3	61.4	34.7 (Ar*C*H$_2$), 71.1 (O*C*H$_2$), 167.7 (C=O)
b	100.1	72.6	74.2	75.9	71.8	61.3	
c	96.9	74.3	71.1	70.2	73.4	61.1	

[a] Measured at 125.8 MHz in D_2O.

complete (4 h). The solution was stirred for a further 2 h, neutralized (Amberlite IRC-50, H^+), filtered and concentrated. The residue was partitioned between water and diethyl ether, and the aqueous phase was then concentrated to give the deprotected *glycoside* **3** (0.89 g, 98%) as a foam, $[\alpha]_D$ +98 (*c* 0.2, water) (Found: C, 47.7; H, 6.6. $C_{25}H_{38}O_{16}$·2H_2O requires C,

47.6; H, 6.7%). ^{1}H and ^{13}C NMR data are presented in Tables 1, 3 and 5.

2-(4′-*Hydroxy*-3′-*nitrophenyl*)*ethanol* **1**.—$NaNO_3$ (1.28 g, 15 mmol) and $La(NO_3)_3$·$6H_2O$ (65 mg, 0.15 mmol) were dissolved in a mixture of water (12 cm^3) and conc. HCl (12 cm^3). A solution of 2-(4-hydroxyphenyl)ethanol[32] (2.07 g, 15 mmol) in diethyl ether (50 cm^3) was added and the mixture was stirred vigorously for 3 h. The ethereal layer was separated and the aqueous layer was extracted with $CHCl_3$. The combined organic phases were washed with water, dried ($MgSO_4$) and concentrated. VLC [EtOAc–light petroleum (1:1)] of the residue then gave 2-(4-hydroxy-3-nitrophenyl)ethanol **1** as yellow needles (1.40 g, 51%), m.p. 56 °C (from Et_2O–light petroleum) (lit.,[33] 56 °C); δ_H[500 MHz; $(CD_3)_2SO$] 2.69 (t, *J* 6.5, CH_2Ar), 3.59 (t, OCH_2), 4.62 (br s, OH), 7.04 (d, $J_{5',6'}$ 8.5, 5′-H), 7.41 (dd, $J_{2',6'}$ 2, 6′-H), 7.73 (d, 2′-H) and 10.61 (br s, ArOH); δ_C[125 MHz; $(CD_3)_2SO$] 37.2 (*C*H_2Ar), 61.6 (OCH_2), 118.8 (C-5′), 144.7 (C-2′), 131.1 (C-3′), 136.2 (2 C, C-1′ and -6′) and 150.4 (C-4′).

2-(4-*Hydroxy*-3-*nitrophenyl*)*ethyl Deca*-O-*benzoyl*-β-*maltotrioside* **5**.—A mixture of silver triflate (256 mg, 0.86 mmol), the alcohol **1** (132 mg, 0.72 mmol) and 3 Å molecular sieves in dichloromethane (3 cm^3) under argon was stirred for 0.5 h and was then cooled to −10 °C. A solution of the bromide **9** (1.40 g, 0.87 mmol) in dichloromethane (3 cm^3) was then added and the mixture was stirred at −10 °C for 5 h. 2,4,6-Collidine (105 mg, 0.115 cm^3, 0.86 mmol) was then added and the mixture was allowed to warm to room temperature. Filtration (Celite) and concentration of the filtrate gave a yellow foam (2.04 g), which was purified by VLC [toluene–light petroleum–EtOAc, (5:4:1)] to give the *glycoside* **5** (1.03 g, 84%) as a yellow, amorphous solid, $[\alpha]_D$ +70 (*c* 0.4, $CHCl_3$) (Found: C, 67.4; H, 4.7. $C_{96}H_{79}NO_{29}$ requires C, 67.4; H, 4.7%). ^{1}H and ^{13}C NMR data are presented in Tables 1, 3 and 5.

2-(4-*Hydroxy*-3-*nitrophenyl*)*ethyl* β-*Maltotrioside* **2**.—The decabenzoate **5** (540 mg, 0.31 mmol) was debenzoylated with methanolic sodium methoxide as described for compound **6** to give a yellow residue (313 mg). Purification by semi-preparative reversed-phase HPLC then gave the deprotected *glycoside* **2** (158 mg, 75%) as a yellow, amorphous solid, $[\alpha]_D$ +36 (*c* 0.1, water); *m/z* 668.1 (M − H) ($C_{26}H_{39}NO_{19}$ requires M, 669.2). ^{1}H and ^{13}C NMR data are presented in Tables 1, 3 and 5.

2′,2′,2′-*Trichloroethyl* 2-*Aminobenzoate* **14**.—A mixture of anthranilic acid (2.5 g, 18 mmol) and thionyl dichloride (5.3 cm^3, 8.7 g, 73 mmol) in benzene (40 cm^3) under nitrogen was heated at reflux (1.5 h). The resultant clear, yellow solution was concentrated to give, presumably, 2-(sulfinylideneamino)-benzoyl chloride **13**[17] as a yellow oil. This oil was dissolved in dichloromethane (20 cm^3), and 2,2,2-trichloroethanol (3.3 g, 2.1 cm^3, 22 mmol), triethylamine (2.6 g, 3.6 cm^3, 26 mmol) and DMAP (200 mg) were added. The mixture was stirred under nitrogen for 16 h and was then poured into water. The organic phase was separated and the aqueous phase was extracted with dichloromethane. The combined organic phases were washed successively with dil. HCl, water, saturated aq. $NaHCO_3$ and brine, dried ($MgSO_4$), and concentrated to give a yellow, oily residue. VLC [light petroleum ⟶ EtOAc–light petroleum (1:9)] then gave the ester **14** (3.9 g, 80%) as needles, m.p. 64.5–65.5 °C (from light petroleum) (lit.,[34] 63 °C); δ_H(500 MHz) 4.96 (s, CH_2), 5.66 (br s, NH_2), 6.72–6.77 (m, 3- and 5-H), 7.37 (ddd, $J_{3,4}$ 8, $J_{4,5}$ 8, $J_{4,6}$ 2, 4-H), 8.04 (dd, $J_{5,6}$ 8.5, 6-H); δ_C(125 MHz) 73.9 (CH_2), 95.3 (CCl_3), 109.2 (C-1), 116.7 and 116.8 (C-3 and -5), 131.4 (C-6), 135.0 (C-4), 150.8 (C-2) and 166.1 (C=O).

N$^\alpha$-(tert-*Butoxycarbonyl*)-L-*leucine* 2′,2′,2′-*Trichloroethyl Ester* **17**.—Boc-Leu-OH (2.0 g, 8.7 mmol) was dissolved in CH_2Cl_2–CCl_4 (1:1; 50 cm^3). 2,2,2-Trichloroethanol (1.94 g, 1.25 cm^3, 13.0 mmol), DCC (1.80 g, 8.7 mmol) and DMAP (100 mg) were added and the mixture was stirred for 3 h. The mixture was cooled (ice-bath), then was filtered, and the filtrate was concentrated to give a yellow oil (4.6 g). Flash chromatography [EtOAc–light petroleum (1:9)] then gave the *ester* **17** (3.14 g, 100%) as fine needles, m.p. 52–53 °C (from aq. EtOH); $[\alpha]_D$ −23 (*c* 0.5, $CHCl_3$) (Found: C, 43.2; H, 6.1. $C_{13}H_{22}Cl_3NO_4$ requires C, 43.0; H, 6.1%); δ_H(500 MHz) 1.00 and 1.02 (2 d, *J* 1.6, δ- and ε-H), 1.48 (s, Me_3), 1.56–1.62 and 1.79–1.87 (2 H, 2 m, β-H_2), 1.70–1.77 (m, γ-H), 4.44–4.50 (m, α-H), 4.69 and 4.94 (AB quartet, *J* 12.5, CH_2CCl_3) and 4.91 (d, *J* 8, NH); δ_C($CDCl_3$) 21.7 and 22.7 (C-δ and -ε), 24.8 (C-γ), 28.2 (Me_3), 41.3 (C-β), 52.1 (C-α), 74.2 (*C*H_2CCl$_3$), 80.1 (Me$_3$*C*), 94.6 (CCl_3), 155.3 (NCO_2) and 171.9 (C=O).

N$^\alpha$-[2-(tert-*Butoxycarbonylamino*)*benzoyl*]-L-*leucine* 2′,2′,2′-*Trichloroethyl Ester* **15**.—The ester **17** (100 mg, 0.27 mmol) was dissolved in dichloromethane (2 cm^3) and the solution was cooled to 0 °C. TFA (0.3 cm^3) was added and the mixture was stirred for 1 h at 25 °C. Toluene was added and the mixture was concentrated to give, presumably, the trifluoroacetate salt as a crystalline solid. This solid was dissolved in DMF (2 cm^3) and Boc-ABz-ODhbt **12**[10] (105 mg, 0.27 mmol) and powdered potassium carbonate (20 mg, 0.14 mmol) were added, resulting in the appearance of a yellow colour. The mixture was stirred for 1 h and was then concentrated. The residue was treated with diethyl ether, then was filtered, and the filtrate was concentrated to give a pale yellow oil (200 mg). Flash chromatography [EtOAc–light petroleum (1:4)] then gave the *amide* **15** (129 mg, 97%) as an oil which crystallized on prolonged storage in the refrigerator as needles, m.p. 67–69 °C (from aq. EtOH); $[\alpha]_D$ −13 (*c* 0.8, $CHCl_3$) (Found: C, 49.8; H, 5.7. $C_{20}H_{27}Cl_3N_2O_5$ requires C, 49.8; H, 5.6%); δ_H(500 MHz) 1.05 (d, *J* 2.7, δ-H), 1.07 (d, *J* 2.9, ε-H), 1.55 (s, Me_3), 1.75–1.81 and 1.87–1.93 (2 H, 2 m, β-H_2), 1.82–1.87 (m, γ-H), 4.74 and 4.96 (AB quartet, *J* 11.9, CH_2CCl_3), 4.92–4.97 (m, α-H), 6.52 (d, *J* 7.9, NH amide), 7.04 (ddd, $J_{4,5}$ 7.8, $J_{5,6}$ 7.8, $J_{3,5}$ 1.1, 5-H), 7.49 (ddd, $J_{3,4}$ 8.5, $J_{4,6}$ 1.3, 4-H), 7.53 (dd, 6-H), 8.41 (d, 3-H) and 9.98 (s, N*H*Boc); δ_C(125 MHz) 21.9 and 22.8 (C-δ and -ε), 25.1 (C-γ), 28.3 (Me_3), 41.2 (C-β), 51.1 (C-α), 74.4 (*C*H_2CCl$_3$), 80.4 (Me$_3$*C*), 94.4 (CCl_3), 118.9 (C-1), 119.9 (C-3), 121.4 (C-5), 126.6 (C-6), 132.9 (C-4), 140.4 (C-2), 153.0 (NCO_2) and 168.8 and 171.2 (C=O).

2′,2′,2′-*Trichloroethyl* 2-(tert-*Butoxycarbonylamino*)*benzoate* **16**.—A solution of 2-(*tert*-butoxycarbonylamino)benzoic acid[10] (237 mg, 1.0 mmol), 2,2,2-trichloroethanol (150 mg, 0.1 cm^3, 1.0 mmol), DCC (206 mg, 1.0 mmol) and DMAP (25 mg) in CH_2Cl_2–CCl_4–THF (5:5:1; 11 cm^3) was stirred (overnight, room temp.). The mixture was cooled (ice-bath), then was filtered, and the filtrate was concentrated. Flash chromatography [light petroleum ⟶ EtOAc–light petroleum (1:9)] of the residue then gave the *ester* **16** (330 mg, 90%) as needles, m.p. 102 °C (from MeOH) (Found: C, 45.8; H, 4.3. $C_{14}H_{16}Cl_3NO_4$ requires C, 45.6; H, 4.4%); δ_H(500 MHz) 1.57 (s, Me_3), 5.00 (s, CH_2), 7.09 (dd, $J_{4,5}$ 7.4, $J_{5,6}$ 8.0, 5-H), 7.61 (ddd, $J_{3,4}$ 8.6, $J_{4,6}$ 1.6, 4-H), 8.17 (dd, 6-H), 8.51 (d, 3-H) and 10.01 (s, NH); δ_C(125 MHz) 28.3 (Me_3), 74.3 (CH_2), 80.8 (Me$_3$*C*), 94.8 (CCl_3), 113.0 (C-1), 119.0 (C-3), 121.4 (C-5), 131.2 (C-6), 135.4 (C-4), 142.8 (C-2), 152.7 (NCO_2) and 166.2 (C=O).

General Procedure for Acylation.—The trisaccharide (~0.1 mmol) and the trichloroethyl ester (0.3–0.5 mmol) were

dissolved in dry DMF (3 cm^3). Lyophilized subtilisin A preparation (25 mg) was added, and the mixture was sonicated for 1 min and then shaken at 250 rpm at 37 °C for 5–7 days. The enzyme was removed by filtration and the filtrate was concentrated under reduced pressure. The acylation products were separated from the starting materials by flash chromatography [EtOAc–MeOH–water (16:4:1)] and were then treated with 95% aq. TFA for 10 min at 25 °C. Toluene was added and the mixture was concentrated. The residue was analysed by analytical HPLC and, as required, purified by semi-preparative reversed-phase HPLC by using a linear gradient of 0–100% solvent B in 100 min to give the major products as their trifluoroacetate salts, as amorphous solids.

The reaction of trisaccharide **3** *with ester* **17**. The major products, prepared and isolated as described above, were the 2′-*O*-ester **18** (13%), $[\alpha]_D$ +63 (*c* 0.4, water); *m/z* 820.7 (M − H) ($C_{33}H_{50}F_3NO_{19}$ requires M, 821.3); and the 2″-*O*-ester **19** (7%), $[\alpha]_D$ +50 (*c* 0.2, water); *m/z* 820.6 (M − H). ^1H and ^{13}C NMR data are presented in Tables 2, 4 and 6. The reaction was repeated as above without the addition of enzyme. Compounds **18** and **19** were again obtained as the major products in a similar ratio (analytical HPLC).

The reaction of trisaccharide of **2** *with ester* **15**. The major products, prepared and isolated as described above, were the 2′-*O*-ester **22** (13%), $[\alpha]_D$ +95 (*c* 0.4, water); *m/z* 1014.7 (M − H) ($C_{41}H_{56}F_3N_3O_{23}$ requires M, 1015.3); and the 2″-*O*-ester **23** (6%), $[\alpha]_D$ +57 (*c* 0.1, water); *m/z* 1014.7 (M − H). ^1H and ^{13}C NMR data are presented in Tables 2, 4 and 6.

The reaction of trisaccharide **2** *with ester* **16**. The reaction was carried out as described above without the addition of enzyme and then heating of the mixture at 60 °C for 2 days. The major products, isolated as described above, were the 2′-*O*-ester **26** (10%), $[\alpha]_D$ +115 (*c* 0.3, water); *m/z* 901.6 (M − H) ($C_{35}H_{45}F_3N_2O_{22}$ requires M, 902.2); and the 2″-*O*-ester **27** (4%), $[\alpha]_D$ +104 (*c* 0.6, water); *m/z* 901.5 (M − H). ^1H and ^{13}C NMR data are presented in Tables 2, 4 and 6.

The Reaction of Maltotriose with Trichloroethyl Butyrate.—Maltotriose (100 mg, 0.2 mmol) and trichloroethyl butyrate[20] (90 mg, 0.41 mmol) were dissolved in dry DMF (1 cm^3). Subtilisin preparation (25 mg) was added, and the mixture was sonicated for 1 min and then shaken at 250 rpm at 37 °C for 5 days. The enzyme was removed by filtration, the filtrate was concentrated, and the residue was subjected to flash chromatography [EtOAc–MeOH–water (16:4:1)] to give the acylation products as an amorphous solid (20 mg, 18%). ^1H NMR analysis indicated a mixture of products were present.

The reaction was repeated as described above but without the presence of enzyme, on a 30 mg scale; however, no products were detected (TLC). The temperature was then raised to 60 °C and the mixture was heated for 24 h. Acylation was detected by TLC. The solvent was evaporated off and the residue was subjected to flash chromatography [EtOAc–MeOH–water (16:4:1)] to give a complex mixture (^1H NMR) of diacylated products (11 mg, 28%) and monoacylated products (9 mg, 26%). ^1H NMR analysis of the monoacylated products indicated they were similar to those obtained in the enzymic reaction, but the ratio of products differed.

Acknowledgements

We wish to thank Mr. Bent O. Petersen, Ms. Hanne Christiansen, Dr. Hanne Grøn and Dr. Kees Rodenburg for valuable technical assistance and discussions, and Ms. Susanne Refn for the preparation of 2-(4-hydroxy-3-nitrophenyl)ethanol.

References

1 *Handbook of Amylases and Related Enzymes. Their Sources, Isolation, Methods. Properties and Applications*, The Amylase Research Society of Japan, Pergamon Press, Tokyo, 1988.
2 *Enzyme Nomenclature*, IUB, Academic Press, Orlando, 1984.
3 W. M. Fogarty, in *Microbial Enzymes and Biotechnology*, ed. W. M. Fogarty, Applied Science, London, 1983, p. 1.
4 M. Ogawa, *Shokaki Geka*, 1980, **3**, 1115.
5 *Methods of Enzymatic Analysis*, ed. J. Bergmeyer, Verlag Chemie, Weinheim, 3rd edn., 1984, vol. 4, p. 146.
6 T. Usui, K. Ogawa, H. Nagai and H. Matsui, *Anal. Biochem.*, 1992, **202**, 61; H. Matsui, H. Kawagishi and T. Usui, *Biochim. Biophys. Acta*, 1990, **1035**, 90.
7 K. Ishimaru, Y. Kamezono, S. Teshima and Y. Hayashi, *Biosci. Biotechnol. Biochem.*, 1992, **56**, 1552; K. Ishimaru, Y. Kamezono and N. Hanayama, *Biosci. Biotechnol. Biochem.*, 1992, **56**, 1136.
8 S. Tokutake, T. Oguma, K. Tobe, K. Kotani, K. Saito and N. Yamaji, *Carbohydr. Res.*, 1993, **238**, 193; S. Tokutake, R. Uchida, K. Kotani, K. Saito and N. Yamaji, *Carbohydr. Res.*, 1993, **238**, 109; S. Tokutake, S. Saito and N. Yamaji, *Anal. Sci.*, 1992, **8**, 669; S. Tokutake, K. Kotani, K. Saito and N. Yamaji, *Chem. Pharm. Bull.*, 1992, **40**, 2531.
9 A. Yaron, A. Carmel and E. Katchalski-Katzir, *Anal. Biochem.*, 1979, **95**, 228.
10 M. Meldal and K. Breddam, *Anal. Biochem.*, 1991, **195**, 141.
11 G. T. Wang, E. Matayoshi, H. J. Huffaker and G. A. Krafft, *Tetrahedron Lett.*, 1990, **31**, 6493; E. D. Matayoshi, G. T. Wang, G. A. Krafft and J. Erickson, *Science*, 1990, **247**, 954.
12 L. L. Maggiora, C. W. Smith and Z.-Y. Zhang, *J. Med. Chem.*, 1992, **35**, 3727.
13 M. Oertani, P. Girard and H. B. Kagan, *Tetrahedron Lett.*, 1982, **23**, 4315.
14 J. H. Pazur, *Methods Carbohydr. Chem.*, 1962, **1**, 337.
15 I. M. E. Thiel, J. O. Deferrari and R. A. Cadenas, *Justus Liebigs Ann. Chem.*, 1969, **723**, 192.
16 R. Khan, *Adv. Carbohydr. Chem. Biochem.*, 1981, **39**, 213.
17 J. Garín, P. Merino, J. Orduna, T. Tejero and S. Uriel, *Tetrahedron Lett.*, 1991, **32**, 3263.
18 For a recent review see K. Faber and S. Riva, *Synthesis*, 1992, 895.
19 S. Riva, J. Chopineau, A. P. G. Kieboom and A. M. Klibanov, *J. Am. Chem. Soc.*, 1988, **110**, 584.
20 G. Carrea, S. Riva, F. Secundo and B. Danieli, *J. Chem. Soc., Perkin Trans. 1*, 1989, 1057.
21 S. Cai, S. Hakomori and T. Toyokuni, *J. Org. Chem.*, 1992, **57**, 3431.
22 H. Grøn, L. M. Bech, S. Branner and K. Breddam, *Eur. J. Biochem.*, 1990, **194**, 897.
23 Y. Takeuchi, S. Noguchi, Y. Satow, S. Kojima, I. Kumagai, K. Miura, K. T. Nakamura and Y. Mitsui, *Protein Eng.*, 1991, **4**, 501.
24 H. Grøn, M. Meldal and K. Breddam, *Biochemistry*, 1991, **32**, 6011.
25 J. Pitha, C. T. Rao, B. Lindberg and P. Seffers, *Carbohydr. Res.*, 1990, **200**, 429.
26 C. T. Rao, B. Lindberg, J. Lindberg and J. Pitha, *J. Org. Chem.*, 1991, **56**, 1327.
27 M. E. Brewster, M.-j. Huang, E. Pop, J. Pitha, M. J. S. Dewar, J. J. Kaminski and N. Bodor, *Carbohydr. Res.*, 1993, **242**, 53.
28 R. Gelb, L. Schwartz and D. Laufer, *Bioorg. Chem.*, 1982, **11**, 274.
29 E. H. Ajandouz, J.-i. Abe, B. Svensson and G. Marchis-Mouren, *Biochim. Biophys. Acta*, 1992, **1159**, 193.
30 A. M. Klibanov, *CHEMTECH*, 1986, **16**, 354.
31 A. Zaks and A. M. Klibanov, *J. Biol. Chem.*, 1988, **263**, 3194.
32 K. Tomita and H. A. Lardy, *J. Biol. Chem.*, 1960, **235**, 3292.
33 H. M. Woodburn and C. F. Stuntz, *J. Am. Chem. Soc.*, 1950, **72**, 1361.
34 R. P. Staiger and E. B. Miller, *J. Org. Chem.*, 1959, **24**, 1214.

Paper 4/00581C
Received 31*st January* 1994
Accepted 20*th April* 1994