Chemical Synthesis of a Dual Branched Malto-Decaose: A Potential Substrate for α-Amylases

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A convergent block strategy for general use in efficient synthesis of complex α-(1→4)- and α-(1→6)-malto-oligosaccharides is demonstrated with the first chemical synthesis of a malto-oligosaccharide, the decasaccharide 6,6′′′′-bis(α-maltosyl)-maltohexaose, with two branch points. Using this chemically defined branched oligosaccharide as a substrate, the cleavage pattern of seven different α-amylases were investigated. α-Amylases from human saliva, porcine pancreas, barley α-amylase 2 and recombinant barley α-amylase 1 all hydrolysed the decasaccharide selectively. This resulted in a branched hexasaccharide and a branched tetrasaccharide. α-Amylases from Asperagillus oryzae, Bacillus licheniformis *and* Bacillus sp. *cleaved the decasaccharide at two distinct sites, either producing two branched pentasaccharides, or a branched hexasaccharide and a branched tetrasaccharide. In addition, the enzymes were tested on the single-branched octasaccharide 6-α-maltosyl-maltohexaose, which was prepared from 6,6′′′′-bis(α-maltosyl)-maltohexaose by treatment with malt limit dextrinase. A similar cleavage pattern to that found for the corresponding linear malto-oligosaccharide substrate was observed.*

Introduction

α-Amylases (E.C.3.2.1.1) are widespread in all three domains of life—eucarya, bacteria and archaea—and play a key role in carbohydrate metabolism. α-Amylases are endoglycosidases that belong to the glycoside hydrolase family 13 of α-retaining glycosidases.[1] They catalyse the hydrolysis of α-(1→4) linkages in starch and related glucose poly- and oligomers composed of linear α-(1→4) glucan chains with interspersed α-(1→6) branch points.[2] An increased knowledge of their cleavage pattern is of primary importance as these polymers are the most widely used polysaccharides in food and other industries.

α-Amylases hydrolyse a range of differently located glycosidic bonds in polymeric substrates. To orient the scissile bond within the active site of the enzyme, several glucose residues of the substrate that flank the bond to be cleaved are accommodated at an array of subsites which extend into the binding cleft from the catalytic site towards the nonreducing and reducing end. Each substrate glucosyl residue can be thought of as binding to a subsite situated at either side of the catalytic site. The number of subsites and the location of the cleavage site with respect to these subsites dictate the substrate cleavage pattern, as assessed by the structure of the products. While such action patterns of α-amylases have been reported for linear malto-oligosaccharides and short dextrins,[3] the lack of well-defined, pure branched malto-oligosaccharides have hindered the investigation of naturally branched substrates.[4–6] Herein, a strategy is presented for the chemical synthesis of branched malto-oligosaccharides which have subsequently been used as substrates for a series of α-amylases from different origins.

We have recently reported[7] the practical synthesis of new phenyl 6–4′-substituted-1-thio-β-maltosides **2**–**5** (Scheme 1). In combination, building blocks **2**–**5** allow the synthesis of linear and branched malto-oligosaccharides with defined structure and which are identical to any desired part of an amylose or amylopectin molecule. Building blocks **2**–**5** were produced as phenylthio glycosides because we have previously developed[8] an efficient and general method based on the use of *N*-bromosuccinimide (NBS) for the removal of the thiophenyl protecting group at the anomeric position to provide a reducing sugar with all other protecting groups still intact. Further, the use of thioglycosides offers distinct advantages due to their relative stability under chemical conditions typically used for manipulation of other protective groups.[9]

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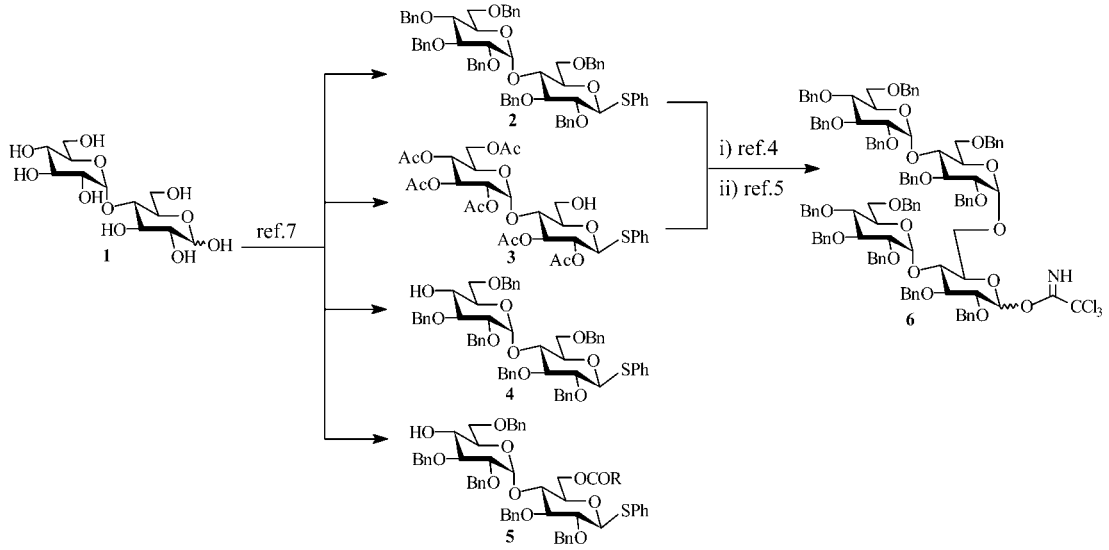


Scheme 1. Maltose derived building blocks **2**–**5** and the branched tetrasaccharide glycosyl donor, **6**.

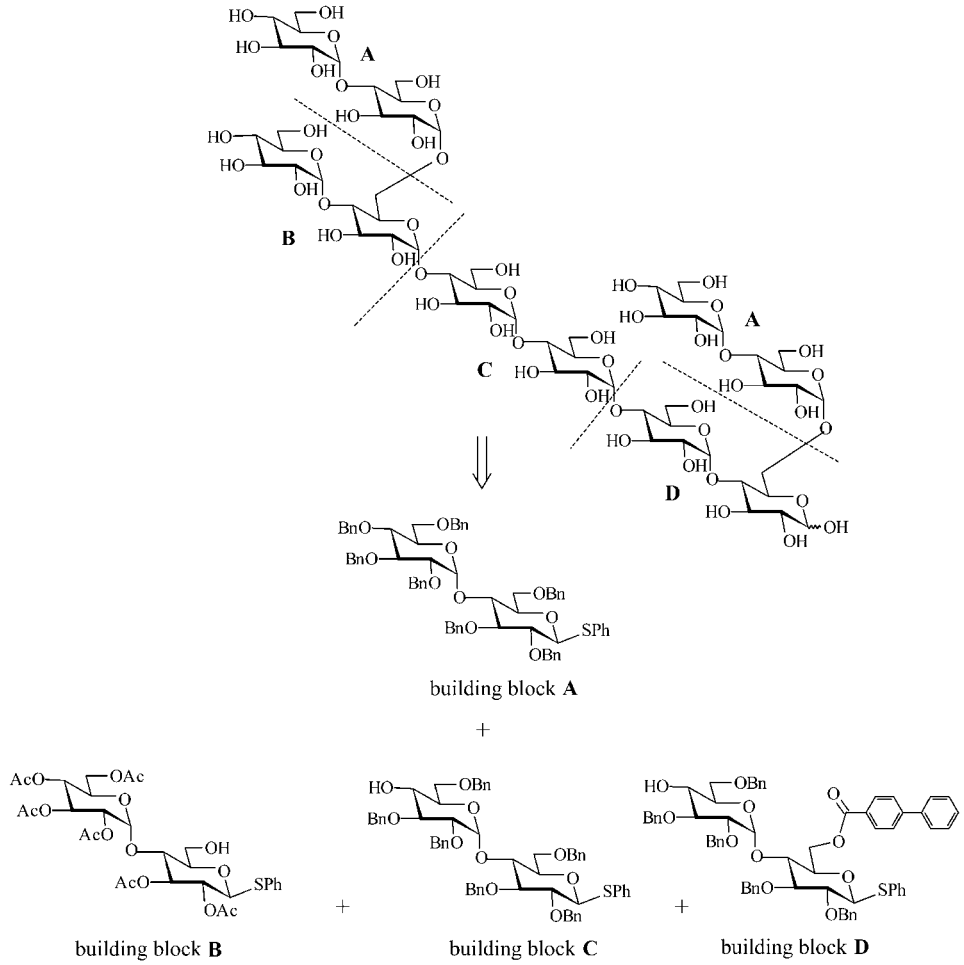
In this paper, we demonstrate the versatility of this set of building blocks in the efficient synthesis of complex α-(1→4)- and α-(1→6)-malto-oligosaccharides. The availability of these complex linear and branched oligosaccharides enables us to investigate the cleavage pattern obtained with seven different α-amylases of mammalian, plant, fungal and bacterial origin.

Results and Discussion

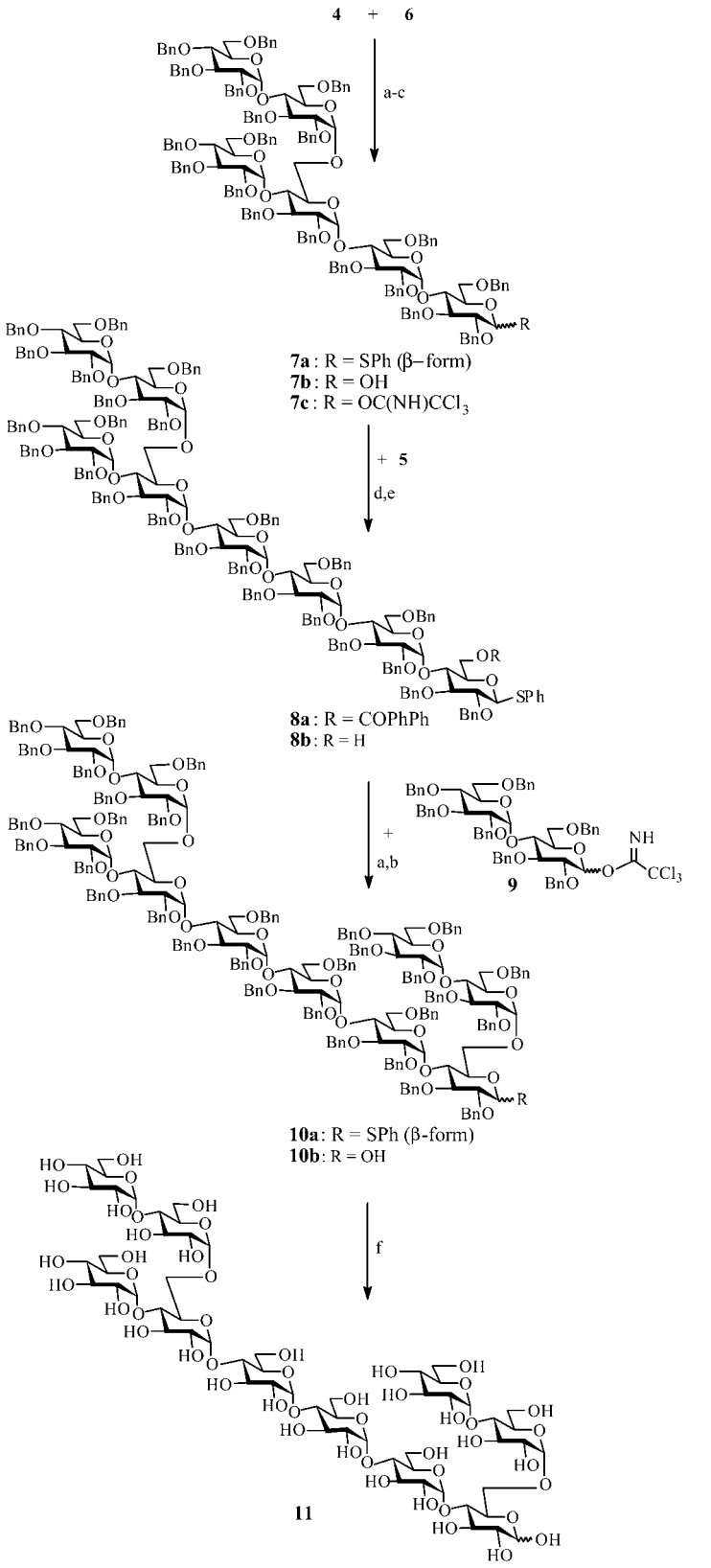
Chemical synthesis of the branched decasaccharide, 6,6′′′′-bis(α-maltosyl)-malto-hexaose

The oligosaccharide 6,6′′′′-bis(α-maltosyl)-maltohexaose (**11**) was chosen as a target molecule for the chemical synthesis of a malto-oligosaccharide with two branch points. The decasaccharide **11** consists of ten glucose units linked by α-(1→4)- or α-(1→6)-linkages and can be retrosynthesized as shown in Scheme 2. These disconnections are logical, because synthesis of the required building blocks **A**–**D** and coupling between some of them (e.g., **A** and **B**) have been previously accomplished.[7,10]

The synthetic pathway for **11** is outlined in Scheme 3. The construction of the glycosyl donor **6** with building blocks **2** and **3** (Scheme 1) has been described earlier.[4,10] Coupling of **6** with the thioglycoside building block acceptor **4** in diethyl ether, by using trimethylsilyl tri-



Scheme 2. Retrosynthesis of the branched decasaccharide, 6,6′′′′-bis(α-maltosyl)-maltohexaose (**11**) via four maltose derived building blocks (**A**–**D**).



Scheme 3. The synthesis pathway for oligosaccharide 6,6′′′′-bis(α-maltosyl)-maltohexaose (**11**). Reagents: a) Trimethylsilyl triflate (TMS-Triflate), Et_2O, 4 Å molecular sieves (MS, activated powder), under Ar, RT, 1.5 h, 52%; b) NBS, acetone/water, RT, 5 min, 94%; c) CCl_3CN, K_2CO_3 anhydrous, THF, 4 Å MS (activated powder), under Ar, RT, 1 h, quantitative; d) TMS-triflate, Et_2O, 4 Å MS (activated powder), under Ar, RT, 1.5 h, 53%; e) 33% MeONa in MeOH, dry MeOH-toluene, RT, 24 h, 93%; f) 10% Pd-C, THF/EtOH/H_2O, under H_2 atmosphere, RT, five days, 78%.

flate (TMS-triflate) as promoter, produced the branched-hexasaccharide thioglycoside **7a**, in 52% yield after chromatographic purification. Small amounts (~5%) of the β-isomer were removed by chromatography. The ^{1}H NMR spectrum of **7a** revealed that the signal due to the anomeric proton of the glucose unit that bears the phenylthio group, H-1$_\beta$, was overlapped by signals from the methylene protons of the benzyl groups. The five doublets at $\delta = 5.38$ ($J_{1,2} = 3.4$ Hz), 5.49 ($J_{1,2} = 3.7$ Hz), 5.54 ($J_{1,2} = 3.6$ Hz), 5.70 ($J_{1,2} = 4.4$ Hz), 5.71 ($J_{1,2} = 3.9$ Hz) correspond to the five remaining internal anomeric protons. The chemical shifts and the characteristic small $J_{1,2}$ couplings for these anomeric protons demonstrate that the newly formed glycosidic linkage has the α-configuration and confirm the stereochemistry of **7a.** Also, the ^{1}H-decoupled ^{13}C NMR spectrum of **7a** contains a diagnostic signal for the anomeric carbon of the glucose unit which bears the phenylthio group; it resonates at $\delta = 87.2$ ppm due to the β-linkage of the phenylthio group. The five additional internal anomeric carbons, which resonate at $\delta = 95.5$, 96.4, 96.6, 96.8 and 97.0 ppm, are in good agreement with the α-configuration.[4,11–13] The phenylthio group of **7a** was efficiently converted to a free HO group by the action of the *N*-bromosuccinimide/acetone/water/ system[8] to provide **7b**, quantitatively. The treatment of **7b** with trichloroacetonitrile in the presence of anhydrous potassium carbonate as catalyst[14,15] afforded an anomeric mixture of the glycosyl trichloroacetimidate derivative **7c**, which was directly used as the glycosyl donor in a coupling reaction with the maltose derivative, **5**. The reaction conditions were similar to those used to obtain **7a**, and produced the octasaccharide derivative **8a**, in 53% yield. ^{1}H and ^{13}C NMR spectra of **8a** confirmed the α-configuration of the newly formed glycosidic linkage. The ^{1}H NMR signals arising from seven interglycosidic anomeric protons were observed as seven doublets resonating at $\delta = 5.40$ ($J_{1,2} = 3.7$ Hz), 5.43 ($J_{1,2} = 3.4$ Hz), 5.47 ($J_{1,2} = 3.7$ Hz), 5.62 ($J_{1,2} = 3.4$ Hz), 5.65 ($J_{1,2} = 3.4$ Hz), 5.69 ($J_{1,2} = 3.6$ Hz) and 5.71 ($J_{1,2} = 3.2$ Hz) ppm. As observed for **7a**, the signal due to the anomeric proton of the glucose unit which bears the phenylthio group, H-1$_\beta$, was overlapped by signals from the methylene protons of the benzyl ether protecting groups. The ^{13}C NMR signals arising from the corresponding seven interglycosidic anomeric carbons were found to resonate at $\delta = 95.1$, 95.9, 96.4, 96.6, 97.0, 97.0 and 97.9 ppm, while the diagnostic signal for the anomeric carbon of the glucose unit that bears the phenylthio group resonated at $\delta = 87.1$ ppm due to the β-linkage of the phenylthio group. Quantitative methanolysis of the biphenyl carboxylate protecting group of the branched octasaccharide derivative **8a**, was achieved by treatment with methanolic methoxide in dry toluene/ methanol (1:1 *v/v*) to give the desired phenyl thioglycoside, **8b**. This compound has a free 6-OH group ready for the introduction of the second α-(1→6)-linkage. Coupling of **8b** with the glycosyl donor **9**[4] by using the same procedures as described for **7a** and **8a**, produced the pro-

tected branched decasaccharide thioglycoside **10a**, in 64% yield. The coupling resulted in the expected α-(1→6)-linkage as verified by ^{1}H and ^{13}C NMR spectroscopy. In the ^{1}H NMR spectrum of **10a**, seven doublets at $\delta = 5.31$ ($J_{1,2} = 3.2$ Hz), 5.40 ($J_{1,2} = 3.2$ Hz), 5.46 ($J_{1,2} = 3.1$ Hz), 5.57 ($J_{1,2} = 3.1$ Hz), 5.63 ($J_{1,2} = 3.6$ Hz), 5.65 ($J_{1,2} = 3.5$ Hz), 5.69 ($J_{1,2} = 3.1$ Hz) ppm and a broad triplet at $\delta = 5.71$ ppm which contained two protons, reflect the nine internal anomeric protons. As observed in compounds **7a** and **8a**, H-1$_\beta$ is overlapped by the signals from the methylene protons of the benzyl ether protecting groups. In the ^{13}C NMR spectrum, a signal at $\delta = 87.9$ ppm reflects the anomeric carbon of the glucose unit that bears the phenylthio group, and the nine signals at $\delta = 95.1$, 95.8, 96.3, 96.5, 96.6, 96.8, 96.8, 97.0 and 97.2 ppm reflect the nine internal anomeric carbons. The phenylthio group of **10a**, was efficiently converted into a free HO group by the action of NBS in aqueous acetone[8] to afford **10b** in 94% yield after purification by flash chromatography on silica gel. Catalytic hydrogenolysis of **10b** to remove the benzyl ether protecting groups was performed under hydrogen in the presence of 10% Pd/C in tetrahydrofuran (THF)/MeOH/H_2O (6:6:1 *v/v*) and gave the branched decasaccharide, **11**, in 78% yield. The MALDI spectrum as well as the ^{1}H and ^{13}C NMR spectra of the anomeric region of **11** is shown in Figure 1. The doublets at $\delta = 4.98$ ($J_{1,2} = 3.8$ Hz) and 4.99 ($J_{1,2} = 3.7$ Hz) ppm (Figure 1b) were assigned to the anomeric protons at the branch points. The corresponding anomeric carbons resonate at $\delta = 99.5$ ppm (Figure 1c). These assignments are consistent with our previous data for such branched oligosaccharides.[4,11–13]

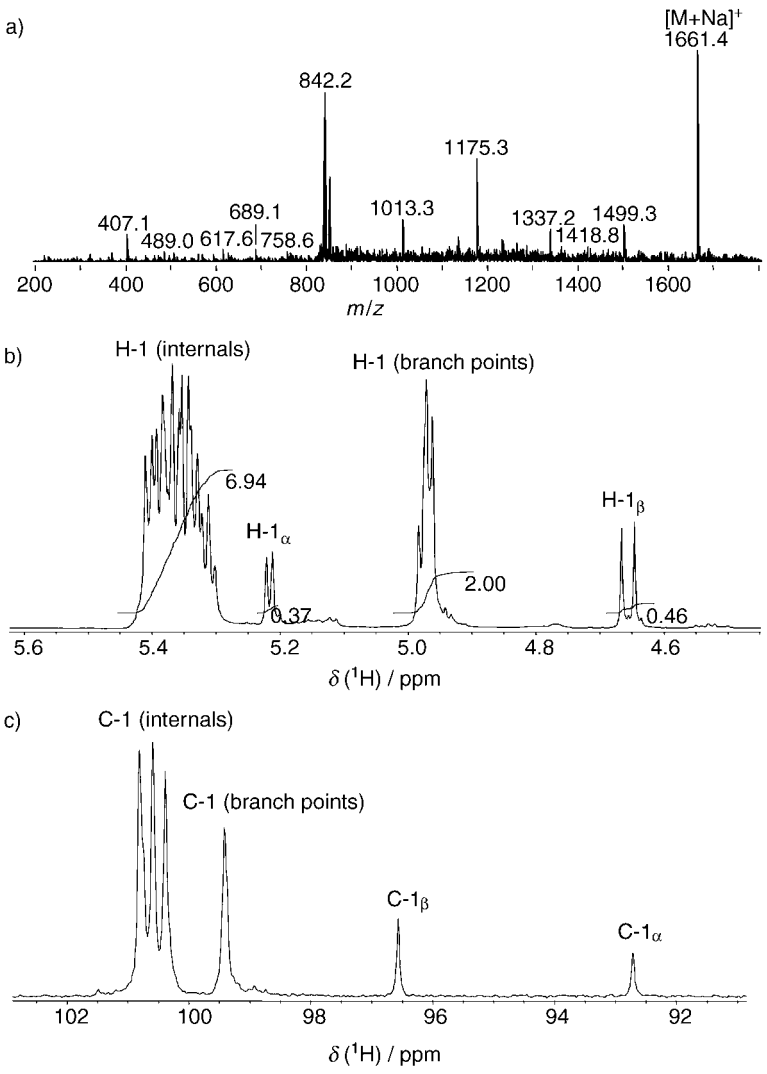


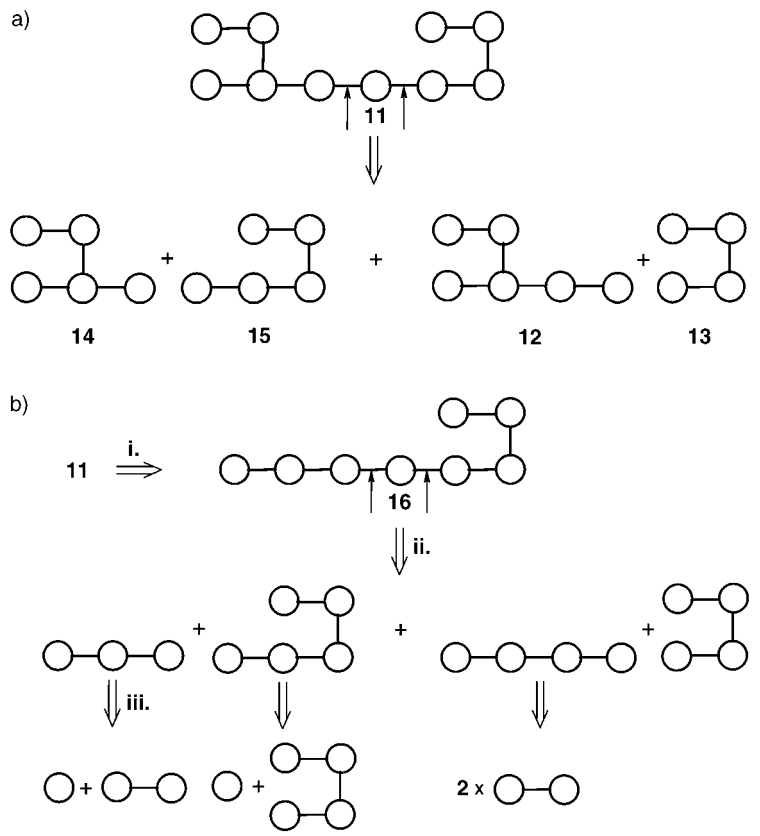
Figure 1. a) ES-MS spectrum of **11**; b) 400 MHz ^{1}H NMR spectrum of the anomeric region of **11** in D_2O; c) 100 MHz ^{13}C NMR spectrum of the anomeric region of **11** in D_2O.

Cleavage pattern of the branched decasaccharide, 6,6′′′′-bis(α-maltosyl)-maltohexaose

The decasaccharide 6,6′′′′-bis(α-maltosyl)-maltohexaose (**11**) (Schemes 3 and 4) holds three glucose moieties between two branch points which represents a likely structure for naturally occurring malto-oligomers. Investigations of this compound as substrate for α-amylases from human saliva (HSA), porcine pancreas (PPA), barley isoenzyme 2 (AMY2), recombinant barley isoenzyme 1 (AMY1), *Asperagillus oryzae* (TAA), *Bacillus licheniformis* (BLA) and *Bacillus sp*. (BSPA) were performed by incubating the individual enzymes with **11** for up to 4 h. The samples were subjected to HPLC and the structures of the products were elucidated by comparison with known standards and by mass spectrometry. The cleavage patterns obtained by incubation of **11** with each of the α-amylases are shown in Scheme 4a, as derived from the results shown in Figure 2. All of the α-amylases were able to cleave **11** between the two branch points. This resulted in the branched hexasaccharide **12** which eluted at 21 min, and the tetrasaccharide **13** which eluted at 12 min (Figure 2). In the mass-spectrum data for PPA (Figure 3) the products can be recognised by signals at 989.2 and 665.1 *m/z* for **12** (M-H) and **13** (M-H), respectively. An additional signal at 701.1 is observed for **13**, which corresponds to the chloride adduct. While PPA, HSA, AMY1 and AMY2 hydrolysed this specific bond selectively, TAA, BLA and BSPA additionally cleaved the adjacent bond thus forming the two pentasaccharides **14** and **15** (Scheme 4a). These eluted at 17 and 16 min, respectively (Figure 2). In the mass spectrum data for BLA (Figure 4) the products are recognised by a single signal with the mass of 827.3 for **14** and **15**. The two pentasaccharides were the minor products of TAA (~10%) and major products formed by both BLA (~87%) and BSPA (~68%; Table 1).

Formation of 6-α-maltosyl-maltohexaose from 6,6′′′′-bis(α-maltosyl)-maltohexaose and cleavage pattern of this single branched substrate

The single branched octasaccharide 6-α-maltosyl-maltohexaose (**16**) was easily obtained by treatment of **11** with limit dextri-



Scheme 4. a) Cleavage pattern of 6,6′′′′-bis(α-maltosyl)-maltohexaose (**11**) obtained with human saliva α-amylase (HSA), porcine pancreas α-amylase (PPA), recombinant barley α-amylase 1 (AMY1), barley α-amylase 2 (AMY2), *Aspergillus oryzae* α-amylase (TAA), *Basillus sp.* α-amylase (BSPA) and *Bacillus licheniformis* α-amylase (BLA). All of the enzymes cleave the substrate to give the branched hexasaccharide **12** and the branched tetrasaccharide **13**. While HSA, PPA, AMY1 and AMY2 exclusively cleave at this specific linkage, TAA, BSPA and BLA cleave also at a second linkage to give two different branched pentasaccharides **14** and **15** (for ratio of cleavage at the different linkages see Table 1). b i) Compound **11** was treated with limit dextrinase to give the octasaccharide **16**. b ii) Compound **16** was treated with the individual enzymes and the initial formed products were maltotriose, maltotetraose, branched tetrasaccharide and branched pentasaccharides. The maltotetraose was further hydrolysed into two maltose molecules and the branched pentasaccharide into glucose and branched tetrasaccharide. b iii) TAA and AMY2 were both able to cleave maltotriose into glucose and maltose.

nase (LD) which specifically hydrolysed the α-1,6 linkage near the nonreducing end of the molecule (Scheme 4 b i). The resulting octasaccharide, **16**, was incubated for up to 24 h with the seven different α-amylases individually. Separation of products by HPLC enabled elucidation of their structures by comparison with known standards and by mass spectrometry. The initial products were maltotriose, maltotetraose, a branched tetrasaccharide and a branched pentasaccharide (Scheme 4 b ii). The branched pentasaccharide was further degraded to glucose and the branched tetrasaccharide. Maltotetraose was hydrolysed to two molecules of maltose. TAA hydrolysed the maltotriose to glucose and maltose (Scheme 4 b iii), whereas this reaction proceeded to a $\leq 1\%$ level for the remaining enzymes within 24 h.

This cleavage pattern of PPA and HSA correlates well with results of earlier experiments obtained with linear maltohexaose as substrate.[16,17] The results suggests that PPA and HSA recognise the six glucose units connected by α-1,4 linkages in **16** regardless of the presence of the short branch at the reducing end. The cleavage pattern of BLA was investigated earlier by using a series of linear molecules, CNP-G4 to CNP-G10.[18] The present degradation of **16** by BLA resembles the pattern resulting from cleavage of CNP-G5 rather than CNP-G6.

In a similar manner, the cleavage pattern obtained with barley AMY1 was studied by using the linear substrates PNP-G5 to PNP-G7 and the degradation pattern compares well to that obtained with PNP-G5.[19,20] Interestingly, these results suggest that a branch at the reducing end of the substrate does not change the reaction pattern. Presumably the branch in this part of the substrate, which is situated at a long distance from the cleavage site, has little impact on recognition. However, the rates of hydrolysis are more than an order of magnitude lower for the branched substrate compared to the linear compound. In a previous study[20] it was shown that the single branched 6′′′-maltotriosyl-maltohexaose was degraded by AMY1 from the nonreducing end to yield 6′′′-maltotriosyl-maltopentaose and glucose only. In this case AMY1 prefers the branch point positioned at subsite $+2$ and not at subsite -3, which is the alternative cleavage site at the reducing end. Interestingly, AMY1 is forced to bind branches in both subsite $+2$ and -3 when cleaving the decasaccharide.

Conclusion

A block synthetic strategy has been used to chemically synthesize the first well-defined branched-maltodecaose that contains two α-(1→6) branch points with a defined spacing (Scheme 3). Furthermore, the cleavage pattern of seven different α-amylases were investigated by using this dual branched substrate, 6,6′′′′-bis(α-maltosyl)-maltohexaose (**11**) (Scheme 4). Interestingly, all seven α-amylases were able to cleave the substrate. This demonstrates that for all tested enzymes a distance of three glucose units between two branch points enables productive binding to the active site cleft. All seven enzymes generated a branched hexasaccharide and a branched tetrasaccharide. The structure of these two oligosaccharides, unambiguously show that all seven enzymes tested cleave the same glycosidic bond in **11**. We conclude that α-amylases are in general able to accommodate and cleave multiply branched oligosaccharides at their active site when the branch points are separated by as few as four glucose units. PPA, HSA, AMY1 and AMY2 were highly specific and exclusively hydrolysed this glycosidic bond. TAA, BLA and BSPA were less specific as demonstrated by their ability to cleave the decasaccharide at a second position. This reaction provided two structurally different branched pentasaccharides. The efficiency for cleavage at this second site in comparison to the first cleavage site was 10%, 87% and 68% for TAA, BLA and BSPA, respec-

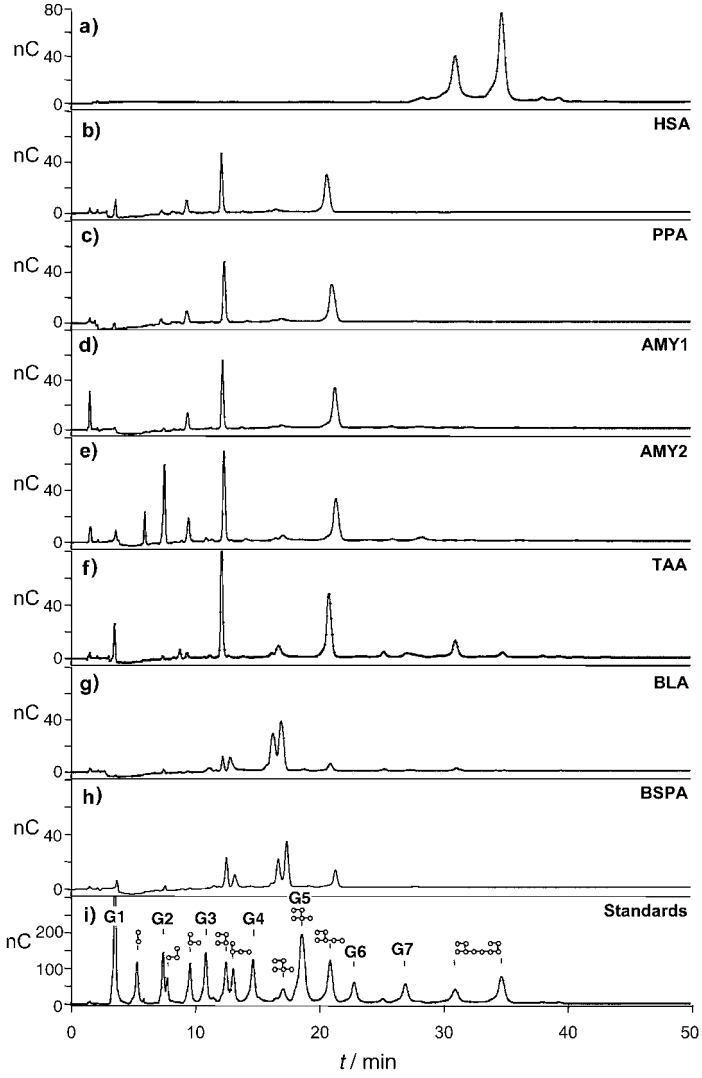


Figure 2. a) Chromatogram of the starting material 6,6′′′′-bis(α-maltosyl)-maltohexaose (**11**). The branched decasaccharide appears in the chromatogram as two distinct peaks that elute at 31 and 35 min; these correspond to either different folds of the substrate or a noncovalent dimer formed from the monomer.[24] b)–e) Chromatograms of cleavage patterns obtained with HSA, PPA, AMY1 and AMY2, respectively, showing specific cleavage of one linkage which results in the formation of the branched hexasaccharide **12** (elutes at 21 min) and the branched tetrasaccharide **13** (elutes at 12 min). e) Formation of isomaltose and maltose which elute at 5 min and 7 min, respectively, is apparent and according to further degradation of the initially formed saccharides. f) TAA exhibits preferential cleavage at one specific site which results in compound **12** and **13**. In addition, the two pentasaccharides **14** and **15** are formed as minor products (~10%) and elute at 17 and 16 min, respectively. g) and h) BSPA and BLA, cleave **11** to mainly give **14** and **15** (~87% and ~68%, respectively) and to a lesser extent **12** and **13** (~13% and ~32%, respectively). i) Standards of linear saccharides (G1–G7) and branched saccharides. The linear saccharides were from Sigma. Isomaltose, isopanose, panose and 6′′-α-glucosyl-maltotriose were from Carlsberg Laboratory. The branched tetrasaccharide was obtained from the hemiacetal of compound **6**: first the phenylthio group was replaced with free OH group followed by hydrogenolysis by using the methods described for **7b** and **11**. The branched pentasaccharide[4] and the branched hexasaccharide were obtained as described previously.[24]

tively. The use of the single branched octasaccharide 6-α-maltosyl-maltohexaose (**16**) as substrate, again demonstrated that the presence of an α-(1→6) branch point at the reducing end of the molecule does not significantly change the bond preference in comparison to the analogous linear substrates.

With the synthesis strategy and the enzyme assay described, it is now possible to gain access to desirable partial structures of glucose polymers which contain α-(1→4)- and α-(1→6) linkages and to test these carbohydrates as substrates for starch degrading enzymes in order to reveal the true nature of the substrate recognition of these enzymes. The demonstrated variation in cleavage site specificity between the different α-amylases provides new opportunities to obtain a desired product profile when a specific α-amylase is used for starch degradation.

Experimental Section

General procedures: Optical rotations were measured with an optical activity Ltd AA-1000 Polarimeter.

All reactions were monitored by TLC on aluminium sheets coated with silica gel $60F_{254}$ (0.2 mm thickness, Merck, Darmstadt, Germany). The presence of reactants and products were monitored by charring with 10% H_2SO_4 in MeOH. Column chromatography was carried out by using silica gel 60, particle size 0.040–0.063 mm, 230–400 mesh ASTM, Merck). Solvent extracts were dried with anhydrous $MgSO_4$ unless otherwise specified.

The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Advance 400 spectrometer at 400 and 100 MHz, respectively. In water, dioxane was used as internal reference (δ_H (dioxane) = 3.75; δ_C (dioxane) = 67.4). In other solvents, δ_H-values were relative to internal Me_4Si, and δ_C-values were referenced to the solvent (δ_C ($CDCl_3$) = 77.0; δ_C (Me_2SO-d_6) = 39.4). MALDI-TOF MS spectra were recorded on a Fisons VG TOFspec E and ESI mass spectra on a Bruker Esquire-LC instrument.

Phenyl 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→6)-[2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl-(1→4)]-2,3-di-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-1-thio-β-D-glucopyranoside (7a): A solution of **4**[7] (4.15 g, 4.26 mmol) and **6**[4] (8.33 g, 4.20 mmol) in dry diethyl ether (200 mL) was stirred for 1 h at RT under Ar in the presence of 4 Å molecular sieves (3 g, activated powder). The stirred mixture was then cooled to −20 °C and a solution of trimethylsilyl trifluoromethanesulfonate (160 μL, 0.87 mmol) in dry diethyl ether (20 mL) was added to it, drop wise. Stirring was continued and the temperature was raised to RT over a period of 1.5 h. After dilution with diethyl ether (150 mL), solid $NaHCO_3$ (5 g) was added and stirring was continued for 10 min. The reaction mixture was filtered through a sea-sand pad and a layer of silica gel. The filtrate was washed successively with saturated aqueous $NaHCO_3$ (3×50 mL), water (3×50 mL) and brine (50 mL), dried and evaporated to dryness. The residue was chromatographed on silica gel (210 g) with diethyl ether/*n*-pentane (2:3 *v/v*) as eluent to give **7a** as white foam (6.11 g, 52%): $[\alpha]_D^{23} = +68.9°$ (c = 1.42, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ = 3.14–4.23 (m, 75H; skeleton-protons and H-1$_\beta$), 5.38 (d, $J_{1,2}$ = 3.4 Hz, 1H; H-1 internal anomeric branch point), 5.49 (d, $J_{1,2}$ = 3.7 Hz, 1H; H-1 internal anomeric), 5.54 (d, $J_{1,2}$ = 3.6 Hz, 1H; H-1 internal anomeric), 5.70 (d, 1H; $J_{1,2}$ = 4.4 Hz H-1 internal anomeric), 5.71 (d, $J_{1,2}$ = 3.9 Hz 1H; H-1 internal anomeric), 6.95–7.61 (m, 100H; Ar-H); ^{13}C NMR ($CDCl_3$): δ = 87.2 (C-1), 95.5, 96.4, 96.6, 96.8, 97.0 (five internal anomeric C), 126.3–138.9 (C-arom, SPh and

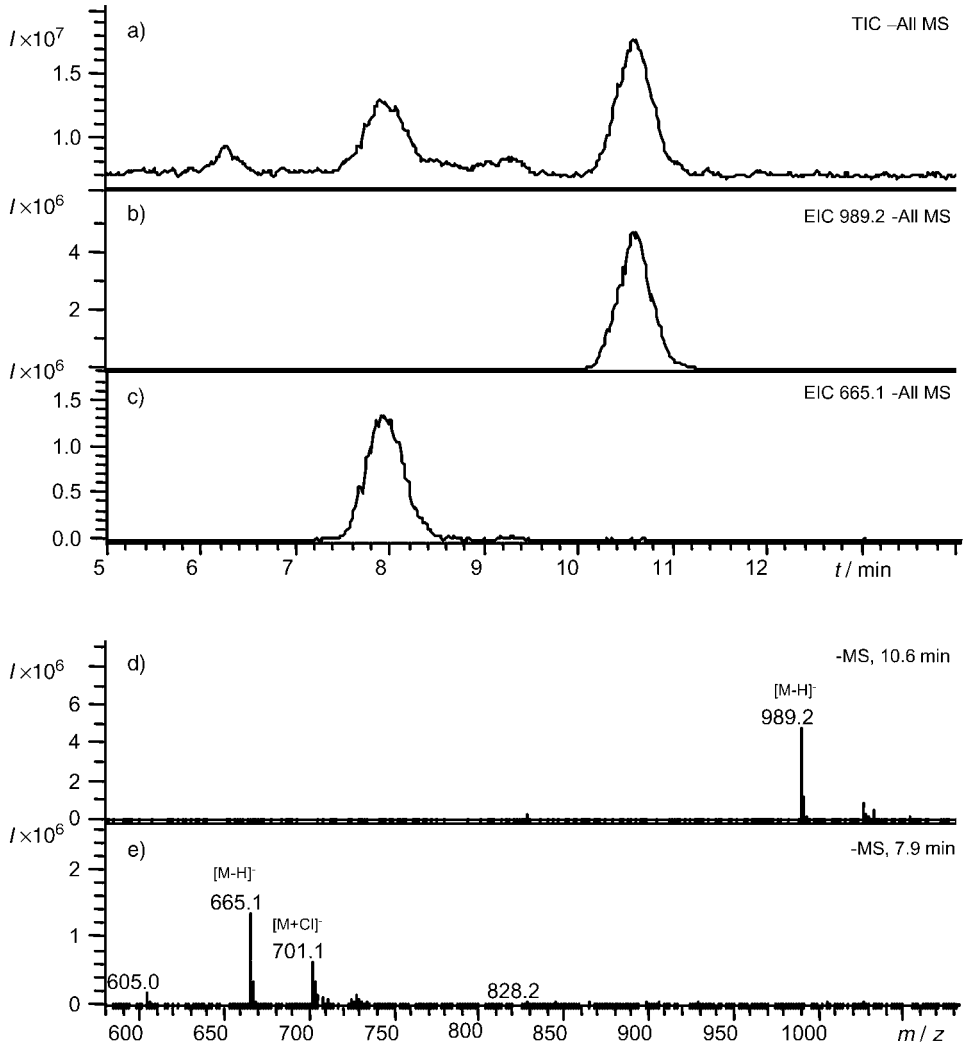


Figure 3. Products obtained after incubation of 6,6‴-bis(α-maltosyl)-maltohexaose (**11**) with PPA for 1 h as detected by LC-MS (ESI, negative mode). a) Total ion current. b) Extracted ion chromatogram. The signal at 10.6 min has a mass of 989.2 which corresponds to a hexasaccharide ([M-H]$^-$; see Figure 3 d). c) Extracted ion chromatogram. The component at 7.9 min shows two signals at 665.1 and 701.1 which corresponds to a tetrasaccharide that is [M-H]$^-$ and [M+Cl]$^-$, respectively (see Figure 3 e).

$PhCH_2$); MALDI-TOF-MS calcd for $C_{175}H_{180}O_{30}S$ [M+Na]$^+$: 2818.4; found: 2818.7.

2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→6)-[2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl-(1→4)]-2,3-di-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α,β-D-glucopyranose (7b): Acetone/water (9:1 *v*/*v*, 150 mL) was added to a solution of **7a** (6.0 g, 2.15 mmol) in acetone (20 mL). NBS (1.15 g, 6.46 mmol) was added to the solution in one portion. After 5 min the reaction was quenched by adding solid $NaHCO_3$ (5 g). The mixture was stirred for 10 min, evaporated in vacuo at RT and then diluted with EtOAc (150 mL). The organic phase was washed with water until neutral pH was reached and then with brine, dried and evaporated to dryness. The residue was chromatographed on silica gel (90 g) with diethyl ether/*n*-pentane (40:60 and 60:40 *v*/*v*) as eluent to give **7b** as a colourless syrup (5.45 g, 94%) as an α/β mixture (3:2): $[\alpha]_D^{23} = +82.1°$ (c = 1.40, $CHCl_3$); ^{1}H NMR ($CDCl_3$) for α-anomer: δ = 5.22 (t, $J_{1,2}$ = 3.2 Hz, $J_{1,OH}$ = 3.0 Hz, 0.6H; H-1α), 5.39 (d, $J_{1,2}$ = 3.3 Hz, 0.6H; H-1 internal anomeric branch point), 5.53 (d, $J_{1,2}$ = 3.1 Hz, 0.6H; H-1 internal anomeric), 5.58 (d, $J_{1,2}$ = 3.6 Hz, 0.6H; H-1 internal anomeric), 5.70 (d, $J_{1,2}$ = 3.5 Hz, 0.6H; H-1 internal anomeric), 5.71 (d, $J_{1,2}$ = 3.4 Hz, 0.6H; H-1 internal anomeric). For β-anomer: δ = 5.38 (d, $J_{1,2}$ = 3.1 Hz, 0.4H; H-1 internal anomeric branch point), 5.52 (d, $J_{1,2}$ = 3.1 Hz, 0.4H; H-1 internal anomeric), 5.57 (d, $J_{1,2}$ = 3.4 Hz, 0.4H; H-1 internal anomeric), 5.70 (d, 0.4H; $J_{1,2}$ = 3.5 Hz, H-1 internal anomeric), 5.71 (d, $J_{1,2}$ = 3.4 Hz,0.4H; H-1 internal anomeric), H-1$_\beta$ was overlapped by signals from the methylene protons of the benzyl groups. ^{13}C NMR ($CDCl_3$) for the α anomer: δ = 90.8 (C-1), 95.3, 96.4, 96.4, 96.4, 97.0 (five internal anomeric C), 126.3–139.0 (C-arom, $PhCH_2$). For the β anomer: δ = 97.4 (C-1), 95.4, 96.6, 96.6, 96.4, 97.0 (five internal anomeric C), 126.3–139.0 (C-arom, $PhCH_2$). MALDI-TOF-MS calcd for $C_{169}H_{176}O_{31}$ [M+Na]$^+$: 2726.2; found: 2727.1.

2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→6)-[2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl-(1→4)]-2,3-di-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α,β-D-glucopyranosyl trichloroacetimidate (7c): A solution of **7b** (5.20 g, 1.92 mmol) in dry CH_2Cl_2 (50 mL) was stirred vigorously with trichloroacetonitrile (6 mL) and K_2CO_3 (3 g) for 18 h at RT under N_2. The mixture was filtered through a sea-sand pad and a layer of silica gel. The filtrate was evaporated to dryness to give **7c** as a colourless syrup (5.48 g, quantitative) as an α/β mixture (1:1), which was used directly for the next step without further purification. ^{1}H NMR ($CDCl_3$): δ = 8.69 (s, 0.5H; NH_β), 8.59 (s, 0.5H; NH_α), 7.30–6.95 (m, 95H; Ar-H), 6.54 (d, $J_{1,2}$ = 3.2 Hz, 0.5H; H-1$_\alpha$), 5.90 (d, $J_{1,2}$ = 7.1 Hz, 0.5H; H-1$_\beta$), 5.71–5.37 (m, 5H; 5 H-1 internal anomeric), 5.02–3.12 (m, 80H; skeleton-protons), ^{13}C NMR ($CDCl_3$): δ = 161.4 (C=NH_α), 161.0 (C=NH_β), 139.0–126.3 (C-arom, $PhCH_2$), 98.1 (C-1$_\beta$), 97.0, 97.0, 96.6, 96.6, 96.5, 96.5, 96.4, 96.4, 95.5, 95.3 (internal anomeric C), 94.1 (C-1$_\alpha$), 91.3 ($CCl_{3,\alpha}$), 91.0 ($CCl_{3,\beta}$).

Phenyl 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→6)-[2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl-(1→4)]-2,3-di-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3-di-*O*-benzyl-6-*O*-(*p*-phenylbenzoyl)-1-thio-β-D-glucopyranoside (8a): The same procedure was used as de-

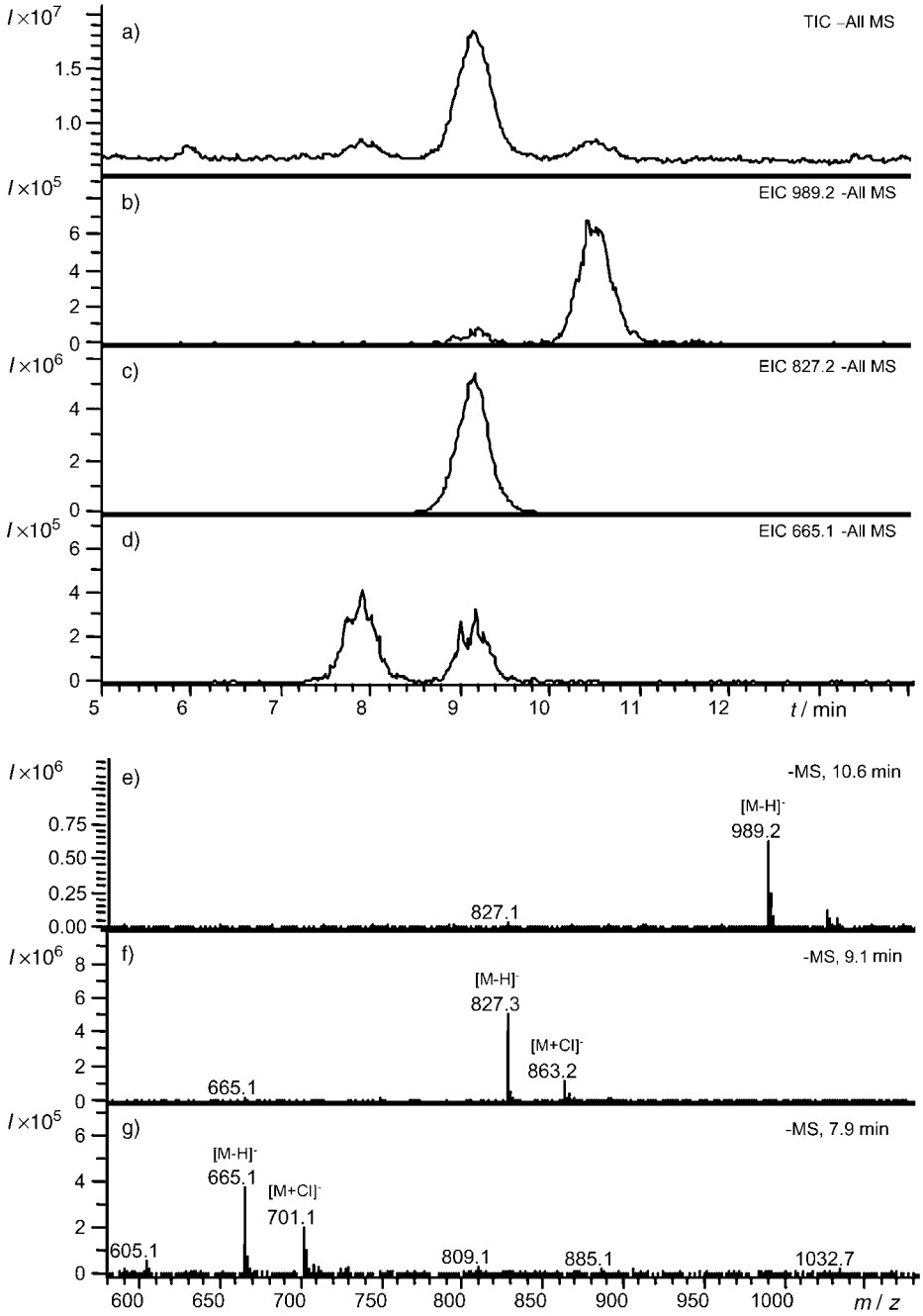
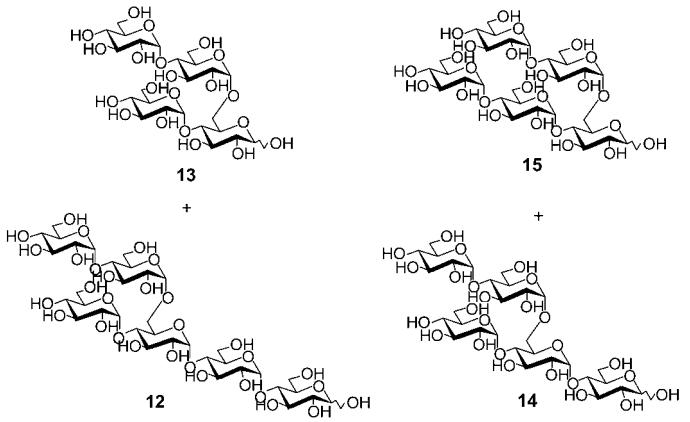


Figure 4. Products obtained after incubation of 6,6‴′-bis(α-maltosyl)-maltohexaose (**11**) with BLA for 1 h as detected by LC-MS (ESI, negative mode). a) Total ion current. b) Extracted ion chromatogram. The signal at 10.5 min has a mass of 929.2 which corresponds to a hexasaccharide ($[M-H]^-$; see Figure 4e). c) Extracted ion chromatogram. The signal at 9.1 min has a mass of 827.2 which corresponds to a pentasaccharide ($[M-H]^-$) and a minor signal with a mass of 863.2 which corresponds to $[M+Cl]^-$ (see Figure 4f). d) Extracted ion chromatogram. The signal at 7.9 min shows two signals at 665.1 and 701.1 (see Figure 4g) which corresponds to a tetrasaccharide that is $[M-H]^-$ and $[M+Cl]^-$, respectively. The signal at 9.1 min is a trace of the pentasaccharides.

scribed for **7a** but using **7c** (5.48 g, 1.92 mmol) and **5**[7] (2.05 g, 1.92 mmol) in dry diethyl ether (150 mL), 4 Å molecular sieves (3 g, activated powder) and trimethylsilyl trifluoromethanesulfonate (53 μL, 0.29 mmol). The residue was chromatographed on a reversed phase silica gel (28 g) by using MeOH/CH_3CN (4:1 *v/v*). This product was further purified on silica gel (180 g) with 30–50% diethyl ether in *n*-pentane as eluent to provide pure **8a** as a white foam (3.82 g, 53%): $[\alpha]_D^{22} = +63.7°$ ($c = 0.73$, $CHCl_3$). ^{1}H NMR ($CDCl_3$): $\delta = 8.11$–6.90 (m, 134H; Ar-H), 5.71 (d, $J_{1,2} = 3.2$ Hz, 1H; H-1 internal anomeric), 5.69 (d, $J_{1,2} = 3.6$ Hz, 1H; H-1 internal anomeric), 5.65 (d, $J_{1,2} = 3.4$ Hz, 1H; H-1 internal anomeric), 5.62 (d, $J_{1,2} = 3.4$ Hz 1H; H-1 internal anomeric), 5.47 (d, $J_{1,2} = 3.7$ Hz, 1H; H-1 internal anomeric), 5.43 (d, $J_{1,2} = 3.4$ Hz, 1H; H-1 internal anomeric branch point), 5.40 (d, $J_{1,2} = 3.7$ Hz, 1H; H-1 internal anomeric branch point). H-1_β was overlapped by the signals from the methylene protons of the benzyl groups. ^{13}C NMR ($CDCl_3$): $\delta = 87.1$ (C-1), 95.1, 95.9, 96.4, 96.6, 97.0, 97.0, 97.9 (seven internal anomeric C), 126.2–145.7 (C-arom, biphenyl, SPh and $PhCH_2$), 165.7 (*CO*-PhPh). MALDI-TOF-MS calcd for $C_{235}H_{238}O_{41}S$ $[M+Na]^+$: 3773.5; found: 3777.8.

Phenyl 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→6)-[2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl-(1→4)]-2,3,-di-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3-di-*O*-benzyl-1-thio-β-D-glucopyranoside (8b): NaOMe (3 mL, 30% in MeOH) was added to a stirring solution of **8a** (2.85 g, 0.76 mmol) in dry MeOH/toluene (100 mL, 1:1 *v/v*) at RT. Stirring was continued for 24 h at RT and the mixture was neutralized by addition of Dowex 50W-X8 (H^+ form, 200–400 mesh, prewashed with EtOH) resin. The resin was filtered off and washed with toluene (4×50 mL). The residue obtained upon evaporation of the combined filtrates was coevaporated with toluene (3×50 mL) and purified by flash chromatography on silica gel (80 g) with 40–60% diethyl ether in *n*-pentane as eluent to give pure **8b** as a colourless syrup (2.50 g, 93%): $[\alpha]_D^{23} = +74.0°$ ($c = 0.96$, $CHCl_3$). ^{1}H NMR ($CDCl_3$): $\delta = 7.60$–6.91 (m, 125H; Ar-H), 5.71 (d, $J_{1,2} = 3.5$ Hz, 1H; H-1 internal anomeric), 5.69 (d, $J_{1,2} = 3.4$ Hz, 1H; H-1 internal anomeric), 5.66 (d, $J_{1,2} = 3.6$ Hz, 1H; H-1 internal anomeric), 5.62 (d, $J_{1,2} = 3.1$ Hz 1H; H-1 internal anomeric), 5.59 (d, $J_{1,2} = 3.6$ Hz, 1H; H-1 internal anomeric), 5.54 (d, $J_{1,2} = 3.5$ Hz, 1H; H-1 internal anomeric), 5.40 (d, $J_{1,2} = 3.4$ Hz, 1H; H-1 internal anomeric

Table 1. Products formed [%] from cleavage of 6,6′′′′-bis(α-maltosyl)-maltohexaose (**11**) by different α-amylases.



HSA	100	–
PPA	100	–
AMY1	100	–
AMY2	100	–
TAA	90	10
BLA	13	87
BSPA	32	68

branch point). H-1$_\beta$ was overlapped by the signals from the methylene protons of the benzyl groups. ^{13}C NMR ($CDCl_3$): $\delta = 87.5$ (C-1), 95.2, 95.9, 96.4, 96.6, 96.9, 97.0, 97.0 (seven internal anomeric C), 126.3–139.0 (C-arom, SPh and $PhCH_2$); MALDI-TOF-MS calcd for $C_{222}H_{230}O_{40}S$ $[M+Na]^+$: 3593.3; found: 3597.0.

Phenyl 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→6)-[2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl)-(1→4)]-2,3-di-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-[2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→6)]-2,3-di-*O*-benzyl-1-thio-β-D-glucopyranoside (10a): The same procedure was used as described for **7a** but using **8b** (2.2 g, 0.62 mmol) and **9**[10] (0.83 g, 0.74 mmol) in dry diethyl ether (80 mL), 4 Å molecular sieves (1 g, activated powder) and trimethylsilyl trifluoromethanesulfonate (20.1 μL, 0.11 mmol). The residue was chromatographed on a reversed phase silica gel (28 g) by using MeOH-CH_3CN (4:1 *v/v*). This product was purified on silica gel (80 g) with 30–50% diethyl ether in *n*-pentane as eluent to give pure **10a** as a white gum (1.78 g, 64%): $[\alpha]_D^{22} = +52.8°$ ($c = 0.97$, $CHCl_3$). ^{1}H NMR ($CDCl_3$): $\delta = 7.56$–6.89 (m, 160H; Ar-H), 5.71 (br t, 2H; H-1 internal anomeric), 5.69 (d, $J_{1,2} = 3.1$ Hz,1H; H-1 internal anomeric), 5.65 (d, $J_{1,2} = 3.5$ Hz, 1H; H-1 internal anomeric), 5.63 (d, $J_{1,2} = 3.6$ Hz 1H; H-1 internal anomeric), 5.57 (d, $J_{1,2} = 3.1$ Hz, 1H; H-1 internal anomeric), 5.46 (d, $J_{1,2} = 3.1$ Hz, 1H; H-1 internal anomeric), 5.40 (d, $J_{1,2} = 3.2$ Hz, 1H; H-1 internal anomeric branch point), 5.31 (d, $J_{1,2} = 3.2$ Hz, 1H; H-1 internal anomeric branch point). H-1$_\beta$ was overlapped by signals from the methylene protons of the benzyl groups. ^{13}C NMR ($CDCl_3$): $\delta = 87.9$ (C-1), 95.1, 95.8, 96.3, 96.5, 96.6, 96.8, 96.8, 97.0, 97.2 (nine internal anomeric C), 125.2–139.0 (C-arom, SPh and $PhCH_2$). MALDI-TOF-MS calcd for $C_{283}H_{292}O_{50}S$ $[M+Na]^+$: 4548.5; found: 4552.6.

2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→6)-[2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl-(1→4)]-2,3,-di-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-[2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→6)]-2,3-di-*O*-benzyl-α,β-D-glucopyranose (10b): The same procedure was used as described for **7b** but using acetone/water (9:1 *v/v*, 15 mL), **10a** (1.6 g, 0.35 mmol) in acetone (5 mL) and NBS (0.19 g, 1.07 mmol). The residue was chromatographed on silica gel (80 g) with 20–30% EtOAc in *n*-pentane as eluent to give **10b** as a colourless syrup (1.48 g, 94%) as an α/β mixture (3:4): $[\alpha]_D^{23} = +80.2°$ ($c = 1.13$, $CHCl_3$). ^{1}H NMR ($CDCl_3$): $\delta = 7.28$–6.89 (m, 155H; Ar-H), 5.73 (d, $J_{1,2} = 3.2$ Hz, 0.5H; H-1 internal anomeric), 5.71 (d, $J_{1,2} = 3.9$ Hz, 1H; H-1 internal anomeric), 5.70 (d, $J_{1,2} = 3.9$ Hz, 1H; H-1 internal anomeric) 5.68 (d, $J_{1,2} = 3.5$ Hz, 1H; H-1 internal anomeric), 5.65 (d, $J_{1,2} = 3.0$ Hz 1H; H-1 internal anomeric), 5.64 (d, $J_{1,2} = 3.0$ Hz, 0.5H; H-1 internal anomeric), 5.62 (d, $J_{1,2} = 3.7$ Hz, 1H; H-1 internal anomeric), 5.61 (d, $J_{1,2} = 4.0$ Hz, 0.5 H H-1 internal anomeric), 5.59 (d, $J_{1,2} = 3.5$ Hz, 0.5H; H-1 internal anomeric), 5.49 (d, $J_{1,2} = 3.3$ Hz, 0.5H; H-1 internal anomeric branch point), 5.44 (d, $J_{1,2} = 3.5$ Hz, 0.5H; H-1 internal anomeric-branch point), 5.39 (d, $J_{1,2} = 3.5$ Hz, 1H; H-1 internal anomeric branch point), 5.13 (t, $J_{1,2} = 3.0$ Hz, $J_{1,OH} = 2.8$ Hz, 0.5H; H-1$_\alpha$). 0.5 H-1$_\beta$ was overlapped by signals from the methylene protons of the benzyl groups. ^{13}C NMR ($CDCl_3$): $\delta = 139.1$–125.1 (C-Ar, *Ph*CH_2), 97.2 (C-1$_\beta$), 97.1, 97.1, 97.1, 97.0, 97.0, 96.6, 96.6, 96.6, 96.4, 96.4, 96.4, 96.4, 96.1, 95.9, 95.9, 95.8, 95.8, 95.1 (18 internal anomeric C, α, β-anomeric mixture), 90.6 (C-1$_\alpha$).

α-D-Glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→6)-[α-D-glucopyranosyl-(1→4)]-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-[α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→6)]-α,β-D-glucopyranose (11): 10% Pd/C (500 mg) was added to a solution of **10b** (940 mg, 0.212 mmol) in THF/EtOH/H_2O (45 mL, 1:1:1 *v/v*) and the reaction mixture was stirred under hydrogen (1 atm) for five days at RT. The catalyst was removed by filtration on a layer of silica gel and the filtrate was concentrated to dryness. The residue was chromatographed on silica gel (85 g) with H_2O/EtOH (10–30:90–70 *v/v*) as eluent and precipitated from EtOH to give **11** (270.3 mg, 78%): $[\alpha]_D^{29}$: +89.8° ($c = 0.334$, H_2O). ^{1}H NMR (D_2O): $\delta =$ 5.41–5.30 (m, 7H; H-1 internal anomeric), 5.21 (d, $J_{1,2} = 3.6$ Hz, 0.35H; H-1$_\alpha$), 4.98–4.96 (m, 2H; H-1 internal anomeric branch point), 4.65 (d, $J_{1,2} = 7.9$ Hz, 0.47H; H-1$_\beta$). ^{13}C NMR (D_2O): $\delta = 100.8$, 100.8, 100.8, 100.8, 100.8, 100.8, 100.6, 100.6, 100.6, 100.6, 100.4, 100.4, 100.4, 100.4 (14×C1$_{\alpha,\beta}$ internal anomeric), 99.4, 99.4, 99.4, 99.4 (4×C1$_{\alpha,\beta}$ internal anomeric branch points), 96.6 (C1$_\beta$), 92.7 (C1$_\beta$), 68.2, 68.2, 68.0, 68.0 (4×C, C-6, α,β-anomer branch points), 61.4, 61.4, 61.4, 61.4, 61.3, 61.3, 61.3, 61.3, 61.3, 61.3, 61.2, 61.2, 61.2, 61.2, 61.2, 61.2 (12×C6, α/β anomer). MALDI-TOF-MS calcd for $C_{60}H_{102}O_{51}$ $[M+Na]^+$: 1662.4; found: 1664.3. ES-MS: 1661.7 $[M+Na]^+$.

Enzymatic hydrolysis of the branched decasaccharide 6,6′′′′-bis(α-maltosyl)-maltohexaose: Reaction mixtures (50 μL) containing **11** (0.1 mM) and enzyme were incubated for up to 4 h (HSA (0.03 mg mL^{-1}), PPA (0.02 mg mL^{-1}), BLA (0.11 mg mL^{-1}) and BSPA (0.04 mg mL^{-1}) in piperazine-1,4-bis(2-ethanesulfonic acid) buffer (PIPES buffer; 20 mM, 20 mm $CaCl_2$, pH 6.9); AMY1 (0.08 mg mL^{-1}),

AMY2 (0.21 mg mL^{-1}) and TAA (0.03 mg mL^{-1}) in sodium acetate buffer (20 mM, 20 mM $CaCl_2$, pH 5.5)). The reactions were stopped by the addition of HCl (2 μL, 1 N) and then neutralised by NaOH (1 μL, 1 N). AMY1 was prepared as described.[21] AMY2 was purified from malt as described.[22] HSA, PPA and TAA were from Sigma. BLA and BSPA were from Novo Nordisk. Enzyme concentration was given from the supplier or determined by amino-acid analysis on protein hydrolysates as described.[20]

Formation of the branched octasaccharide 6-α-maltosyl-maltohexaose and degradation: Reaction mixtures (50 μL) containing **11** (0.1 mM) and limit dextrinase (2 μM) in sodium acetate buffer (20 mM, pH 6.9) were incubated at 37 °C for 10 min. Limit dextrinase was purified from malt essentially as described.[23] α-Amylase was added and the reactions were incubated for up to 4 h (HSA (0.03 mg mL^{-1}), PPA (0.02 mg mL^{-1}), BLA (0.11 mg mL^{-1}) and BSPA (0.04 mg mL^{-1}) in PIPES buffer (20 mM, 20 mm $CaCl_2$, pH 6.9); AMY1 (0.08 mg mL^{-1}), AMY2 (0.21 mg mL^{-1}) and TAA (0.03 mg mL^{-1}) in sodium acetate buffer (20 mM, 20 mM $CaCl_2$, pH 6.9)). The reactions were stopped by HCl addition (2 μL, 1 N) and then neutralised by NaOH (1 μL, 1 N).

High-performance anion-exchange chromatography/pulsed amperometric (HPAE/PAD): Samples were subjected to HPAE by using a Dionex DX 500 system equipped with a GP40 pump and an ED40 pulse amperometric detection (PAD) system equipped with a CarboPack™ PA-100 column (4×250 mm). Aliquots (40 μL) were injected with an S-3500 auto-sampler and the oligosaccharides were separated (flow-rate 1 mL×min^{-1}) by using isocratic NaOH (150 mM) and a linear gradient profile of NaOAc (0–200 mM; 0–60 min).

Liquid chromatography-mass spectrometry (LC-MS): LC-MS was performed on a HP1100 LC coupled to a Bruker Esquire-LC ion trap mass spectrometer. Normal phase conditions were used with a "CC 125/3 Nucleosil 100–3 NH_2" column (Macherey-Nagel, Easton, PA, US). The flow rate was 0.3 mL min^{-1} and a linear gradient of 65–50% acetonitrile in water was used. The mass spectrometer was run in negative mode.

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