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Automated Solid-Phase Synthesis of a β-(1,3)-Glucan Dodecasaccharide

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Abstract: β-Glucans are a group of structurally heterogeneous polysaccharides found in bacteria, fungi, algae and plants. β-(1,3)-D-Glucans have been studied in most detail due to their impact on the immune system of vertebrates. The studies into the immunomodulatory properties of these glucans are typically carried out with isolates that contain a heterogeneous mixture of polysaccharides of different chain lengths and varying degrees of branching. In order to determine the structure–activity relationship of β-(1,3)-glucans, access to homogeneous, structurally-defined samples of these oligosaccharides that are only available through chemical synthesis is required. The syntheses of β-glucans reported to date rely on the classical solution-phase approach. We describe the first automated solid-phase synthesis of a β-glucan oligosaccharide that was made possible by innovating and optimizing the linker and glycosylating agent combination. A β-(1,3)-glucan dodecasaccharide was assembled in 56 h in a stereoselective fashion with an average yield of 88% per step. This automated approach provides means for the fast and efficient assembly of linker-functionalized mono- to dodecasaccharide β-(1,3)-glucans required for biological studies.

Keywords: automation · beta glucans · glycosylation · protecting groups · solid-phase synthesis

Introduction

Among the most abundant polysaccharides in bacteria, fungi, algae and plants are the β-glucans, a heterogeneous class of oligosaccharides composed of D-glucose. β-Glucans play various biological roles, as components of the cell wall or as energy storage polysaccharides. The β-(1,3)-glucans in particular are polymers of D-glucose with a linear β-(1,3)-glycosidic backbone. These polysaccharides can either be linear or have side chains of D-glucose that are either β-(1,4)- or β-(1,6)-linked to the backbone.[1] β-Glucans are recognized by the innate immune system and have been shown to have immunomodulatory,[2] antitumor,[3] antibacterial and antifungal properties.[4] Recent studies identified synthetic β-glucans as the basis for development of carbohydrate vaccines.[5]

Most of the biological studies on β-glucans use material isolated from natural sources. These isolates usually contain a structurally heterogeneous mixture of β-(1,3)-glucan oligosaccharides of variable chain lengths and with varying side chains. Recognition of β-glucans and their interaction with recombinant murine C-type lectin Dectin-1 have been shown to be strongly dependent on the glucan structure.[6] Therefore, studies utilizing isolated β-(1,3)-glucans can lead to inconsistent and sometimes even contradictory results.[7] In order to obtain reliable and reproducible results, the studies concerning the biological roles of β-(1,3)-glucans have to be performed with homogeneous, well-defined oligosaccharide structures that are only accessible via chemical synthesis. Several strategies relying on classical solution-phase syntheses have been developed to produce pure β-glucan oligosaccharides. A linear β-(1,3)-glucan pentasaccharide was accessed using an iterative approach with glycosylation yields ranging from 74 to 89%.[8] Other iterative approaches to β-(1,3)-glucan fragments report problems related to the stereocontrol during glycosylation reactions.[9] Consequently, the solution-phase approaches for longer β-glucan structures typically rely on convergent synthesis strategies employing di-, tetra- and pentasaccharide units. Notable examples are the synthesis of the linear hexasaccharide,[10] and the syntheses of linear hexadeca- and branched heptadecasaccharides by Tanaka and Takahashi.[11] The use of pre-assembled units is efficient but only allows access to glucans of certain chain lengths. Ideally, a strictly modular assembly process would provide maximum flexibility and minimal manual labor. Here, we describe the development of an iterative automated solid-phase synthesis of linear β-(1,3)-glucans that can produce oligosaccharides of any chain length. The power of that approach is highlighted by the synthesis of the linker-functionalized β-(1,3)-glucan dodecasaccharide **1**.

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Results and Discussion

High coupling efficiencies for each glycosylation step are required to synthesize β-(1,3)-glucan oligosaccharides such as dodecasaccharide **1** on solid support (Figure 1). Control of

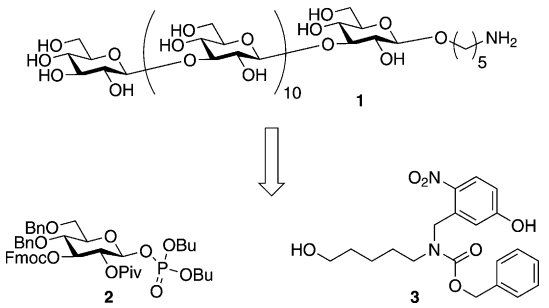
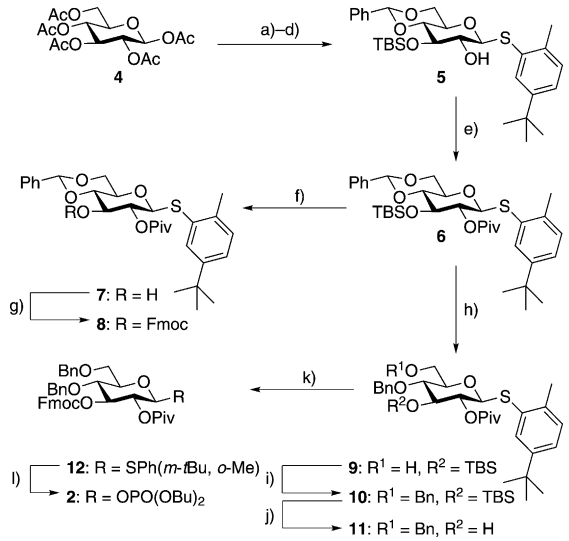


Figure 1. Retrosynthetic analysis of dodecasaccharide **1**.

the anomeric configuration of the newly formed glycosidic bonds is an imperative in order to avoid the formation of complex mixtures of products. The design of the building block including protecting group pattern and anomeric leaving group, the choice of linker and the type of solid support were made mindful of these requirements (Scheme 1).

Thioglucoside **8** was envisioned as a reliable building block to produce β-configured glucans. The anomeric leaving group was chosen since it has already been used for the introduction of β-glucosidic linkages,[12] while the pivaloyl ester in the C2 position was introduced to ensure selective formation of β-glycosidic linkages. The 4,6-*O*-benzylidene



Scheme 1. Synthesis of glycosylating agents **2** and **7**. a) 2-Methyl-5-*tert*-butylthiophenol, $BF_3{\cdot}OEt_2$, CH_2Cl_2, 94%; b) NaOMe, MeOH, quant.; c) benzaldehyde dimethyl acetal, CSA, acetonitrile, 87%; d) TBSCl, imidazole, DMF, 0°C, 69%; e) PivCl, DMAP, pyridine, 79%; f) TBAF·3 H_2O, AcOH, DMF, 97%; g) FmocCl, pyridine, CH_2Cl_2, 97%; h) $BH_3{\cdot}THF$, TMSOTf, CH_2Cl_2, 97%; i) benzyl bromide, NaH, DMF, 78%; j) TBAF·3 H_2O, AcOH, DMF, 85%; k) FmocCl, pyridine, CH_2Cl_2, 91%; l) dibutyl phosphate, NIS, TfOH, 4 Å MS, CH_2Cl_2, 71%.

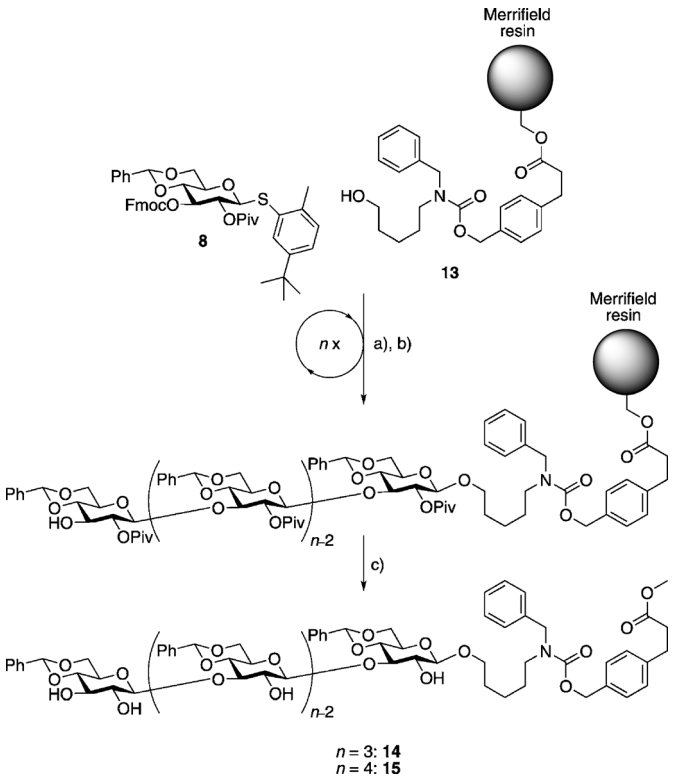
acetal was seen as a suitable protection for the C4 and C6 hydroxyl groups as it has already been successfully employed in several syntheses of β-(1,3)-glucans.[8,10–11] The Fmoc group was chosen for temporary protection of the C3 hydroxyl for of its stability to acids, the ease of assessing the efficiency of each glycosylation step[13] and the mild conditions required for its removal after each coupling step.

Merrifield resin[14] was selected as solid support since it has been employed successfully in the solid-phase synthesis of carbohydrates.[15] The versatility of functionalized Merrifield resin **13** in the synthesis of various oligosaccharides has recently been demonstrated.[15e] The base-labile linker allows for the simultaneous cleavage of the crude products from the solid support and the removal of base-labile protecting groups present on the oligosaccharide. Furthermore, it is stable to the most common activation conditions for thioglycosides.

Building block **8** was synthesized in seven steps starting from β-D-glucose pentaacetate **4** (Scheme 1). Intermediate **5** was prepared according to previously reported procedures.[12,16] Esterification of the C2 hydroxyl group with pivaloyl chloride afforded thioglycoside **6**. Removal of the TBS silyl ether using a buffered solution of TBAF·3 H_2O and glacial acetic acid gave alcohol **7** that was treated with FmocCl to afford thioglycoside building block **8**. Functionalized Merrifield resin **13** was prepared according to previously described procedures.[15e]

The solid phase assembly of β-(1,3)-glucans on functionalized solid support **13** was performed using three equivalents of thioglycoside **8** in three repetitions per glycosylation cycle (Scheme 2). The glycosylations were carried out on the automated synthesizer[15e] by suspending functionalized resin **13** and three equivalents of glycosylating agent **8** in dichloromethane at −40°C before a solution of NIS and TfOH in dichloromethane/acetonitrile (1:1) was added dropwise via a syringe pump. The reaction mixture was agitated using an argon flow while maintaining the temperature for five minutes. Then the temperature was increased to −10°C and the mixture was agitated for another 40 min before draining the reaction vessel using argon pressure. This procedure was executed three times, followed by washing steps using different solvents. Following each glycosylation cycle, the temporary Fmoc protecting group was removed using a 20% (v/v) solution of piperidine in DMF. The reaction vessel was drained and the resin was washed. These steps were repeated until oligosaccharides of the desired chain lengths were assembled (Scheme 2). The crude product was cleaved from the solid support by saponification with sodium methoxide in a mixture of methanol and dichloromethane.

β-(1,3)-Glucan trisaccharide **14** served as the first synthetic target and a context for the optimization of reaction conditions (Scheme 2). The automated elongation cycles were followed by saponification. LC-MS analysis indicated that the main fraction of the crude material contains desired trisaccharide **14** (Figure 2). The contents of minor fractions were identified as various resin/linker degradation side



Scheme 2. General scheme for the automated solid-phase synthesis of β-(1,3)-glucans using thioglycoside **8** and functionalized Merrifield resin **13**. a) 3×3 equiv **7**, NIS, TfOH, CH_2Cl_2/dioxane, −40 °C (5 min) → −10 °C (40 min); b) piperidine (20% v/v), DMF, RT, 15 min (3×); c) NaOMe, MeOH, CH_2Cl_2.

products, while deletion sequences could not be detected. Encouraged by these methodological advances, our attention turned to longer oligosaccharide sequences. Attempts to synthesize linear tetrasaccharide **15** (Scheme 2) or longer glucans, however, were not crowned by success. In all cases the longest structure that was detected by ESI-MS was a trisaccharide. The HPLC chromatograms of the crude products matched that for the synthesis of trisaccharide **14** (Figure 2) and, consequently, this approach was abandoned.

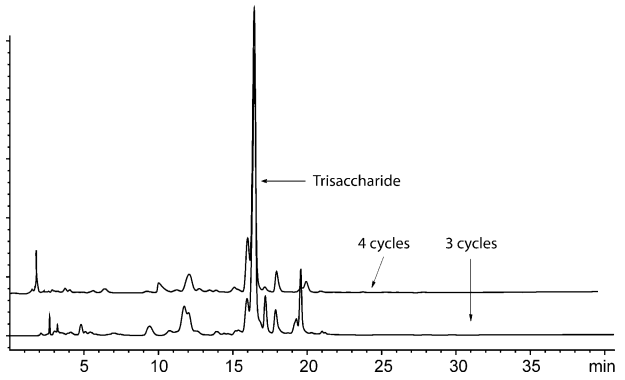
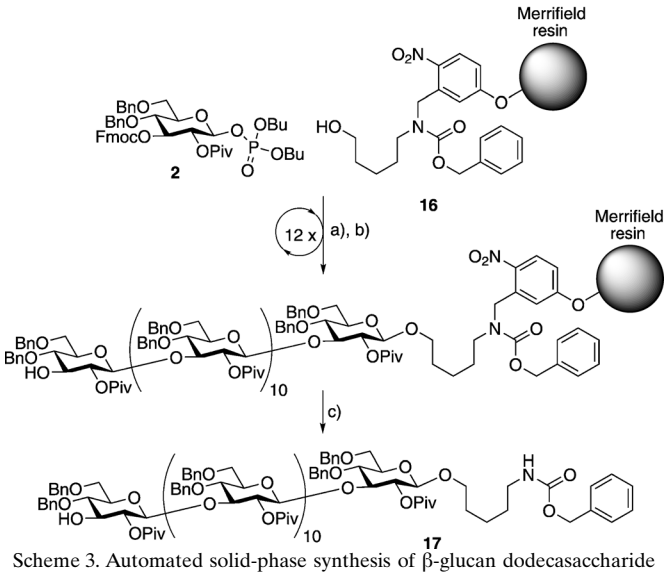


Figure 2. Automated syntheses of linear β-(1,3)-glucan trisaccharide **14** (lower trace) and tetrasaccharide **15** (upper trace): HPLC chromatograms of the crude products. Nucleosil C4, acetonitrile/water 5% (5 min) to 95% (40 min), 254 nm.

It was suspected that the combination of protecting groups on thioglycoside **8** was partly responsible for difficulties in obtaining structures longer than trisaccharides. Previously, Ensley et al. reported conformational distortions in the hexopyranose ring of glucose residues in protected β-(1,3)-glucan structures.[10] Those glucose residues were equipped with a bulky ester protecting group on C2, similar to the pivaloyl group, in combination with a 4,6-*O*-benzylidene acetal. It is conceivable that a conformational distortion in combination with steric hindrance caused by the protecting groups on glycosylating agent **8** prevents chain elongation past the trisaccharide stage.

In order to test this hypothesis, building block **7** was modified by replacing the rigid 4,6-*O*-benzylidene acetal with benzyl ether protecting groups on C4 and C6. The benzyl ether groups allow for more conformational flexibility, increase the reactivity of the glycosylating agent[17] and are stable to both the acidic glycosylation and the basic deprotection conditions. Furthermore, the anomeric leaving group of thioglycoside **8** was replaced by the more reactive dibutylphosphate group, leading to glycosylating agent **2** (Figure 1, Scheme 1).[18] In our hands, functionalized resin **13** was not compatible with the conditions used for glycosyl phosphate activation. Consequently, further optimization relied on functionalized Merrifield resin **16** with a photolabile linker (**3**) as the solid support.[18b] Building block **2** was synthesized from thioglycoside intermediate **6** in five steps, or from starting material **4** in ten steps, respectively. Regioselective opening of the benzylidene acetal yielded monosaccharide **9**, and subsequent benzylation of the resulting free C6 hydroxyl group led to compound **10**. Removal of the silyl ether at C3 gave free alcohol **11** that was transformed into Fmoc carbonate **12**. Conversion of the thioglycoside to a glycosyl phosphate afforded final building block **2** in 42% yield over five steps (Scheme 1). Photolabile linker **3** was synthesized and coupled to the Merrifield resin to obtain linker-functionalized solid support **16** (Scheme 3).[18b]

Glycosylations on functionalized solid support **16** utilized three equivalents of glycosyl phosphate **2** in three repeats for each glycosylation cycle (Scheme 3). For each glycosylation, three equivalents of TMSOTf were added dropwise via a syringe pump to the reaction vessel containing nucleophile and three equivalents of glycosylating agent **2** at −15 °C. The temperature was maintained for 45 min, then raised to 0 °C and kept at 0 °C for 15 min. Following each glycosylation cycle, the temporary Fmoc protecting group was removed using a solution of piperidine in DMF (20% (v/v)). These program modules were performed twelve times, and the reactions were monitored by Fmoc quantification. By collecting the individual solutions resulting from cleavage of each of the Fmoc protecting groups and measuring the absorption of those solutions at 301 nm, the yield of each glycosylation cycle was calculated (see Table S1 in the Supporting Information). While such calculations do not provide exact coupling yields for each cycle, they are a useful estimate of the glycosylation efficiencies and deliver a real time



Scheme 3. Automated solid-phase synthesis of β-glucan dodecasaccharide **17**. a) 3×3 equiv **2**, TMSOTf, CH_2Cl_2, −15 °C (45 min) → 0 °C (15 min); b) piperidine (20 % v/v), DMF, RT, 15 min (3×); c) *hv*, CH_2Cl_2.

feedback on potential problems. The mean calculated glycosylation yield over 12 glycosylation cycles was 89 %.

After completion of the automated synthesis, the crude product was cleaved from the solid support by UV irradiation of the resin in a flow reactor.[18b] The crude mixture was analyzed by HPLC and MALDI-TOF indicating that the main fraction (Figure 3) contains desired dodecasaccharide **17**. Isolation of this HPLC fraction and analysis by NMR established that the structure of this material corresponds to dodecasaccharide **17**. The J_{C1-H1} coupling constants obtained by HSQC measurements confirmed the correct stereochemistry of the desired 1,2-*trans*-glycosidic linkages (J_{C1-H1} = 161 Hz).

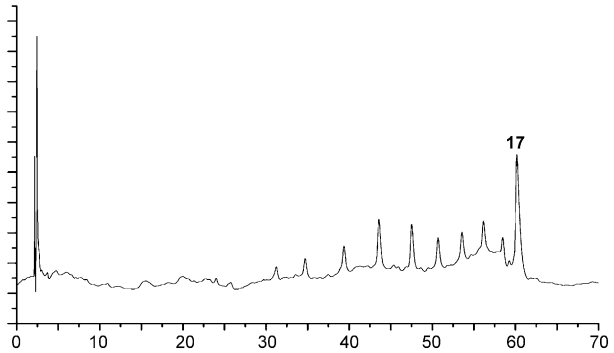
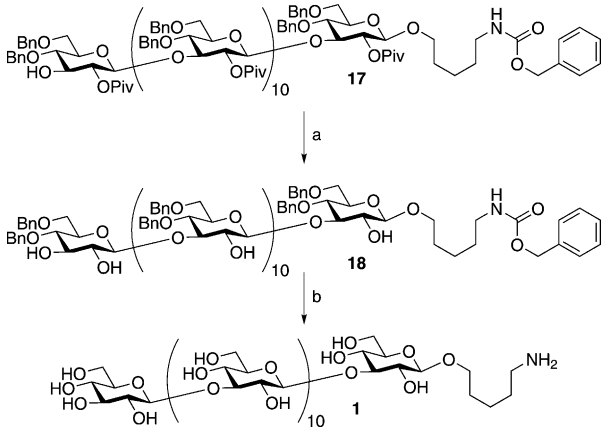


Figure 3. Automated synthesis of dodecasaccharide **17**: HPLC chromatogram of the crude product. YMC-Pack Diol-300, H/EtOAc, 10 % (5 min) to 40 % (70 min), 254 nm.

Dodecasaccharide **17** was obtained in 4.6 % overall yield over 25 steps corresponding to an efficiency of 88.4 % per step. If both Fmoc deprotection and photolytic cleavage from the solid support were to proceed quantitatively, the mean glycosylation yield were 77.4 %. These yields are comparable to previously published solution phase syntheses of β-(1,3)-glucan oligosaccharides.[8,10–11] In contrast to the solution-phase approach, the automated solid phase assembly of dodecasaccharide **17** including release from the solid support was finished within 56 h and required only one purification step. The iterative approach, as opposed to the convergent strategies, facilitates the synthesis of β-(1,3)-glucan structures of any length from monosaccharide to dodecasaccharide with only one monosaccharide building block.

Dodecasaccharide **17** was deprotected in two steps (Scheme 4). First, the pivaloyl esters were removed with sodium methoxide to produce deacylated dodecasaccharide **18**. Finally, all remaining protecting groups were removed by hydrogenation over $Pd(OH)_2$ to afford fully deprotected β-glucan dodecasaccharide **1**. The structure of **1** was confirmed by MALDI-TOF analysis and comparison of the ^{1}H NMR spectrum with isolated curdlan, a naturally occurring linear β-1,3-glucan.[19]



Scheme 4. Global deprotection of **17** to yield dodecasaccharide **1**. a) NaOMe, MeOH, CH_2Cl_2, 56 %; b) $Pd(OH)_2$, H_2, THF, H_2O, AcOH, 52 %.

Conclusion

In summary, an automated approach to the synthesis of β-(1,3)-glucans on Merrifield resin was developed. In the context of this synthesis, the glycosylating agent was optimized, as were the linker and the reaction conditions. Glycosyl phosphate building block **2** and photolabile-linker-functionalized Merrifield resin **16** proved to be a suitable combination for the automated stereocontrolled synthesis of dodecasaccharide **17** with an average yield of 88 % per step. Protected glucan **17** was deprotected in two steps to obtain the target linker-equipped β-(1,3)-glucan dodecasaccharide (**1**). This method allows for the rapid solid phase assembly of well-defined β-(1,3)-glucans of the desired length and supplies the material suitable for further SAR studies on β-(1,3)-glucan oligosaccharides.

Experimental Section

General methods: See Supporting Information.

(2-Methyl-5-*tert*-butylphenyl)-4,6-*O*-benzylidene-3-*O*-tert-butyldimethylsilyl-2-*O*-pivaloyl-1-thio-β-D-glucopyranoside (6): Thioglycoside **5**[14] (7.80 g, 14.32 mmol) was dissolved in anhydrous pyridine (72 mL) under argon, and DMAP (350 mg, 2.86 mmol) was added. The solution was cooled to 0 °C and pivaloyl chloride (4.4 mL, 35.8 mmol) was added dropwise. The reaction was heated to 80 °C and stirred overnight. When TLC analysis indicated complete consumption of the starting material (TLC: cyclohexane/ethyl acetate 9:1), the mixture was diluted with CH_2Cl_2, washed with 1 M HCl (aq) and sat. $NaHCO_3$ (aq) the organic layer was dried over $MgSO_4$ and filtered. The solvent was evaporated in vacuo and the crude material was purified by flash column chromatography (silica gel, cyclohexane/ethyl acetate) to afford **6** (7.09 g, 79%) as a white foam. $R_f = 0.55$ (cyclohexane/ethyl acetate 9:1); $[\alpha]_D^{20} = -33.24$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.56$ (d, $J = 2.0$ Hz, 1H), 7.50–7.46 (m, 2H), 7.39–7.35 (m, 3H), 7.20 (dd, $J = 8.0$, 2.0 Hz, 1H), 7.11 (d, $J = 8.0$ Hz, 1H), 5.52 (s, 1H), 5.14 (dd, $J = 10.2$, 8.3 Hz, 1H), 4.79 (d, $J = 10.2$ Hz, 1H), 4.32 (dd, $J = 10.5$, 4.9 Hz, 1H), 3.99 (t, $J = 8.6$ Hz, 1H), 3.82 (t, $J = 10.2$ Hz, 1H), 3.63 (t, $J = 9.2$ Hz, 1H), 3.52 (td, $J = 9.8$, 5.0 Hz, 1H), 2.33 (s, 3H), 1.31 (s, 9H), 1.28 (s, 9H), 0.80 (s, 9H), 0.01 (s, 3H), −0.05 ppm (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 176.9$, 149.7, 137.1, 135.9, 133.1, 130.0, 129.3, 128.3, 128.0, 126.5, 124.7, 102.3, 87.1, 81.7, 74.4, 73.1, 70.3, 68.8, 39.1, 34.7, 31.5, 27.6, 26.0, 20.3, 18.3, −3.6, −4.6 ppm; IR (thin film): $\tilde{\nu} = 2961$, 2929, 2860, 1741, 1480, 1385, 1249, 1133, 1100, 859, 839, 759 cm^{-1}; HRMS: m/z: calcd for $C_{35}H_{52}O_6SSiNa$ $[M+Na]^+$: 651.3146; found: 651.3168.

(2-Methyl-5-*tert*-butylphenyl)-4,6-*O*-benzylidene-2-*O*-pivaloyl-1-thio-β-D-glucopyranoside (7): Thioglycoside **6** (15.6 g, 24.8 mmol) was dissolved in DMF (125 mL), and the solution was cooled to 0 °C. A mixture of TBAF·3H_2O (31.4 g, 100.0 mmol) and glacial acetic acid (8.5 mL, 149.0 mmol) in DMF (125 mL) was added dropwise, and the solution was warmed to 35 °C and stirred overnight. Upon completion (TLC: cyclohexane/ethyl acetate 7:1), the reaction was cooled to room temperature, diluted with Et_2O, and washed with 1 M HCl (aq) and sat. $NaHCO_3$ (aq). The organic layer was dried over $MgSO_4$ and filtered. The solvent was evaporated in vacuo and the crude material was purified by flash column chromatography (silica gel, cyclohexane/ethyl acetate) to afford **7** (12.4 g, 97%) as a white foam. $R_f = 0.05$ (cyclohexane/ethyl acetate 7:1); $[\alpha]_D^{20} = -30.66$ ($c = 1.0$, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.59$ (d, $J = 1.7$ Hz, 1H), 7.48 (dd, $J = 6.4$, 2.7 Hz, 2H), 7.37 (dd, $J = 4.9$, 1.5 Hz, 3H), 7.24 (dd, $J = 8.1$, 1.8 Hz, 1H), 7.14 (d, $J = 8.0$ Hz, 1H), 5.57 (s, 1H), 5.08–4.97 (m, 1H), 4.78 (d, $J = 10.1$ Hz, 1H), 4.35 (dd, $J = 10.5$, 5.0 Hz, 1H), 3.95 (t, $J = 9.0$ Hz, 1H), 3.84 (t, $J = 10.3$ Hz, 1H), 3.65 (t, $J = 9.3$ Hz, 1H), 3.50 (td, $J = 9.7$, 5.0 Hz, 1H), 2.36 (s, 3H), 1.34–1.27 ppm (m, 18H); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 177.9$, 149.7, 137.0, 136.8, 132.2, 130.1, 129.7, 129.5, 128.5, 126.4, 125.3, 102.0, 87.1, 80.8, 74.1, 72.6, 70.3, 68.7, 39.1, 34.6, 31.5, 27.3, 20.5 ppm; IR (thin film): $\tilde{\nu} = 3473$, 2964, 2868, 1738, 1150, 1098, 996, 760 cm^{-1}; HRMS: m/z: calcd for $C_{29}H_{46}O_6SNa$ $[M+Na]^+$: 537.2281; found: 537.2260.

(2-Methyl-5-*tert*-butylphenyl)-4,6-*O*-benzylidene-3-*O*-fluorenylmethoxycarbonyl-2-*O*-pivaloyl-1-thio-β-D-glucopyranoside (8): Thioglycoside **7** (2.4 g, 4.74 mmol) and FmocCl (1.8 g, 7.10 mmol) were dissolved in CH_2Cl_2 (26 mL) under argon, and pyridine (770 μL, 9.48 mmol) was added dropwise. The reaction was stirred overnight. Upon completion (TLC: cyclohexane/ethyl acetate 3:1), the reaction mixture was diluted with CH_2Cl_2 and washed with 1 M HCl (aq) and sat. $NaHCO_3$ (aq). The organic layer was dried over $MgSO_4$ and filtered. The solvent was evaporated in vacuo and the crude material was purified by flash column chromatography (silica gel, cyclohexane/ethyl acetate) to afford **8** (3.4 g, 97%) as a white foam. $R_f = 0.63$ (cyclohexane/ethyl acetate 3:1); $[\alpha]_D^{20} = -2.53$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.75$ (dd, $J = 7.6$, 0.6 Hz, 2H), 7.61–7.58 (m, 2H), 7.55 (dd, $J = 7.6$, 0.7 Hz, 1H), 7.45 (dd, $J = 6.8$, 3.0 Hz, 2H), 7.42–7.28 (m, 6H), 7.28–7.18 (m, 2H), 7.15 (d, $J = 8.0$ Hz, 1H), 5.57 (s, 1H), 5.32–5.19 (m, 2H), 4.85 (d, $J = 9.8$ Hz, 1H), 4.44–4.33 (m, 2H), 4.31–4.18 (m, 2H), 3.93–3.82 (m, 2H), 3.61 (td, $J = 9.8$, 5.0 Hz, 1H), 2.37 (s, 3H), 1.32 (s, 9H), 1.19 ppm (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 176.8$, 154.6, 149.8, 143.4, 143.2, 141.4, 141.3, 137.0, 136.8, 131.9, 130.2, 130.0, 129.3, 128.4, 128.0, 128.0, 127.4, 127.4, 126.4, 125.6, 125.4, 125.3, 120.1, 120.1, 101.8, 87.5, 78.4, 77.3, 70.6, 70.3, 68.7, 46.7, 39.0, 34.6, 31.5, 27.2, 20.5 ppm; IR (thin film): $\tilde{\nu} = 2964$, 2868, 1752, 1255, 1098, 734 cm^{-1}; HRMS: m/z: calcd for $C_{44}H_{48}O_8SNa$ $[M+Na]^+$: 759.2962; found: 759.2973.

(2-Methyl-5-*tert*-butylphenyl)-4-*O*-benzyl-3-*O*-tert-butyldimethylsilyl-2-*O*-pivaloyl-1-thio-β-D-glucopyranoside (9): Thioglycoside **6** (2.0 g, 3.18 mmol) was coevaporated with toluene three times and subsequently dissolved in CH_2Cl_2 (32 mL) under argon. BH_3·THF (19.1 mL, 85.94 mmol) was added, and the solution was cooled to 0 °C. TMSOTf (290 μL, 1.59 mmol) was added dropwise, and the solution was warmed to room temperature and stirred overnight. After completion (TLC: cyclohexane/ethyl acetate 9:1), the reaction was cooled to 0 °C, quenched by the dropwise addition of methanol and neutralized by the dropwise addition of triethylamine. The volatiles were removed in vacuo and the residue was dissolved in CH_2Cl_2 and washed with water. The organic layer was dried over $MgSO_4$ and filtered. The solvent was evaporated in vacuo and the crude material was purified by flash column chromatography (silica gel, cyclohexane/ethyl acetate) to afford **9** (1.94 g, 97%) as a white foam. $R_f = 0.30$ (cyclohexane/ethyl acetate 9:1); $[\alpha]_D^{20} = -13.40$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.48$ (d, $J = 2.1$ Hz, 1H), 7.37–7.26 (m, 5H), 7.19 (dd, $J = 7.9$, 2.0 Hz, 1H), 7.11 (d, $J = 8.1$ Hz, 1H), 5.09 (dd, $J = 10.0$, 8.4 Hz, 1H), 4.84 (d, $J = 11.7$ Hz, 1H), 4.74 (d, $J = 10.0$ Hz, 1H), 4.65 (d, $J = 11.7$ Hz, 1H), 3.92 (t, $J = 8.4$ Hz, 1H), 3.83 (dd, $J = 12.0$, 1.9 Hz, 1H), 3.67 (dd, $J = 12.0$, 4.1 Hz, 1H), 3.59 (dd, $J = 9.5$, 8.6 Hz, 1H), 3.44 (ddd, $J = 9.5$, 4.5, 2.6 Hz, 1H), 2.33 (s, 3H), 1.30 (s, 9H), 1.29 (s, 9H), 0.89 (s, 9H), 0.08 (s, 3H), 0.06 ppm (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 177.4$, 149.8, 138.1, 135.7, 133.1, 130.1, 128.5, 127.8, 127.5, 127. 5, 124.6, 86.1, 79.4, 78.8, 76.3, 74.8, 73.3, 62.2, 39.3, 34.7, 31.5, 27.8, 26.0, 20.3, 18.1, −3.5, −4.0 ppm; IR (thin film): $\tilde{\nu} = 3493$, 2959, 2929, 2856, 1738, 1480, 1395, 1260, 1157, 1127, 1075, 1037, 838, 779, 733, 697 cm^{-1}; HRMS: m/z: calcd for $C_{35}H_{54}O_6SSiNa$ $[M+Na]^+$: 653.3303; found: 653.3325.

(2-Methyl-5-*tert*-butylphenyl)-4,6-di-*O*-benzyl-3-*O*-tert-butyldimethylsilyl-2-*O*-pivaloyl-1-thio-β-D-glucopyranoside (10): Thioglycoside **9** (1.9 g, 3.01 mmol) was dissolved in DMF (14 mL) under argon. Sodium hydride (130 mg, 5.42 mmol) was added, and the suspension was cooled to 0 °C. Benzyl bromide (1.1 mL, 9.04 mmol) was added dropwise. The solution was warmed to room temperature and stirred for 3 h. Upon completion (TLC: cyclohexane/ethyl acetate 3:1), the reaction was cooled to 0 °C and quenched by the dropwise addition of methanol, diluted with ether and washed with water, NH_4Cl (aq) and water. The organic layer was dried over $MgSO_4$ and filtered. The solvent was evaporated in vacuo, and the crude material was purified by flash column chromatography (silica gel, cyclohexane/ethyl acetate) to afford **10** (1.70 g, 78%) as a white foam. $R_f = 0.67$ (cyclohexane/ethyl acetate 3:1); $[\alpha]_D^{20} = -7.78$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.58$ (d, $J = 2.0$ Hz, 1H), 7.38–7.19 (m, 10H), 7.17 (dd, $J = 7.9$, 2.0 Hz, 1H), 7.09 (d, $J = 8.1$ Hz, 1H), 5.11 (dd, $J = 10.0$, 8.3 Hz, 1H), 4.79 (d, $J = 11.6$ Hz, 1H), 4.70 (d, $J = 10.0$ Hz, 1H), 4.63 (d, $J = 11.6$ Hz, 1H), 4.55 (d, $J = 12.3$ Hz, 1H), 4.50 (d, $J = 12.2$ Hz, 1H), 3.89 (t, $J = 8.3$ Hz, 1H), 3.72–3.68 (m, 2H), 3.63 (dd, $J = 9.4$, 8.4 Hz, 1H), 3.54 (dt, $J = 9.5$, 3.2 Hz, 1H), 2.34 (s, 3H), 1.30–1.27 (m, 18H), 0.89 (s, 9H), 0.07 (s, 3H), 0.03 ppm (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 177.4$, 149.7, 138.3, 138.2, 135.7, 133.8, 129.8, 128.5, 128.4, 128.0, 127.7, 127.7, 127.6, 127.3, 124.2, 86.6, 79.2, 79.0, 76.4, 74.6, 73.6, 73.3, 69.1, 39.2, 34.7, 31.5, 27.8, 26.1, 20.4, 18.1, −3.5, −4.0 ppm; IR (thin film): $\tilde{\nu} = 2958$, 2929, 2858, 1737, 1455, 1362, 1259, 1128, 1096, 882, 838, 778, 734, 697 cm^{-1}; HRMS: m/z: calcd for $C_{42}H_{60}O_6SSiNa$ $[M+Na]^+$: 743.3772; found: 743.3789.

(2-Methyl-5-*tert*-butylphenyl)-4,6-di-*O*-benzyl-2-*O*-pivaloyl-1-thio-β-D-glucopyranoside (11): Thioglycoside **10** (1.7 g, 2.36 mmol) was dissolved in DMF (12 mL), and the solution was cooled to 0 °C. A mixture of TBAF·3H_2O (4.46 g, 14.15 mmol) and glacial acetic acid (1.1 mL, 18.86 mmol) in DMF (12 mL) was added dropwise, and the solution was warmed to 35 °C and stirred overnight. Upon completion (TLC: cyclohexane/ethyl acetate 3:1), the reaction was cooled to room temperature, diluted with Et_2O, and washed with 1 M HCl (aq) and sat. $NaHCO_3$ (aq).

The organic layer was dried over $MgSO_4$ and filtered. The solvent was evaporated in vacuo and the crude material was purified by flash column chromatography (silica gel, cyclohexane/ethyl acetate) to afford **11** (1.22 g, 85%) as a white foam. $R_f = 0.52$ (cyclohexane/ethyl acetate 3:1); $[\alpha]_D^{20} = -30.64$ ($c = 1.0$, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): $\delta = 7.61$ (d, $J = 2.0$ Hz, 1H), 7.35–7.21 (m, 10H), 7.19 (dd, $J = 8.0$, 2.0 Hz, 1H), 7.10 (d, $J = 8.0$ Hz, 1H), 4.92 (dd, $J = 10.0$, 9.1 Hz, 1H), 4.80 (d, $J = 11.1$ Hz, 1H), 4.69–4.51 (m, 4H), 3.79 (t, $J = 8.9$ Hz, 1H), 3.75–3.72 (m, 2H), 3.63 (t, $J = 9.2$ Hz, 1H), 3.49 (ddd, $J = 9.7$, 3.8, 2.5 Hz, 1H), 2.35 (s, 3H), 1.28–1.26 ppm (m, 18H); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 178.4$, 149.7, 138.1, 138.1, 136.4, 133.1, 129.9, 129.1, 128.6, 128.5, 128.2, 128.1, 127.8, 124.8, 86.4, 79.0, 78.2, 77.8, 75.0, 73.7, 72.7, 69.0, 39.1, 34.6, 31.4, 27.3, 20.4 ppm; IR (thin film): $\tilde{\nu} = 3489$, 2962, 2868, 1737, 1480, 1454, 1363, 1280, 1155, 1092, 1045, 822, 736, 698 cm^{-1}; HRMS: m/z: calcd for $C_{36}H_{46}O_6SNa$ $[M+Na]^+$: 629.2907; found: 629.2956.

(2-Methyl-5-*tert*-butylphenyl)-4,6-di-*O*-benzyl-3-*O*-fluorenylmethoxycarbonyl-2-*O*-pivaloyl-1-thio-β-D-glucopyranoside (12): Thioglycoside **11** (1.20 g, 1.98 mmol) and FmocCl (770 mg, 2.97 mmol) were dissolved in CH_2Cl_2 (11 mL) under argon, and pyridine (320 μL, 3.96 mmol) was added dropwise. The reaction was stirred overnight. Upon completion (TLC: cyclohexane/ethyl acetate 3:1), the reaction mixture was diluted with CH_2Cl_2 and washed with 1 M HCl (aq) and sat. $NaHCO_3$ (aq). The organic layer was dried over $MgSO_4$ and filtered. The solvent was evaporated in vacuo and the crude material was purified by flash column chromatography (silica gel, cyclohexane/ethyl acetate) to afford **12** (1.5 g, 91%) as a white foam. $R_f = 0.66$ (cyclohexane/ethyl acetate 3:1); $[\alpha]_D^{20} = +4.68$ ($c = 1.0$, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): $\delta = 7.73$ (d, $J = 7.3$ Hz, 2H), 7.65–7.61 (m, 1H), 7.58 (d, $J = 7.5$ Hz, 2H), 7.41–7.17 (m, 13H), 7.16–7.08 (m, 3H), 5.21–5.14 (m, 2H), 4.76–4.68 (m, 1H), 4.66–4.50 (m, 4H), 4.29 (d, $J = 7.5$ Hz, 2H), 4.20–4.14 (m, 1H), 3.93–3.84 (m, 1H), 3.76–3.70 (m, 2H), 3.59–3.52 (m, 1H), 2.36 (s, 3H), 1.28 (s, 9H), 1.18 ppm (s, 9H); ^{13}C NMR (101 MHz,$CDCl_3$): $\delta = 176.8$, 154.7, 149.7, 143.3, 143.3, 141.3, 141.3, 138.0, 137.7, 136.6, 132.8, 129.9, 129.5, 128.5, 128.4, 128.0, 127.9, 127.8, 127.4, 127.3, 125.3, 125.0, 120.1, 120.1, 86.9, 80.8, 79.1, 75.9, 74.9, 73.7, 70.4, 70.0, 68.6, 46.7, 38.9, 34.6, 31.4, 27.1, 20.4 ppm; IR (thin film): $\tilde{\nu} = 2961$, 2909, 2868, 1752, 1452, 1255, 1133, 1093, 968, 738, 698 cm^{-1}; HRMS: m/z: calcd for $C_{51}H_{56}O_8SNa$ $[M+Na]^+$: 851.3588; found: 851.3616.

Dibutyl 4,6-di-*O*-benzyl-3-*O*-fluorenylmethoxycarbonyl-2-*O*-pivaloyl-β-D-glucopyranoside phosphate (2): Thioglycoside **12** (96 mg, 0.116 mmol), dibutyl phosphate (69 μL, 0.347 mmol) and freshly activated 4 Å molecular sieves were suspended in CH_2Cl_2 (1.3 mL) under argon. The suspension was stirred at room temperature for 20 min, and then cooled to 0°C. NIS (34 mg, 0.151 mmol) was added, and the suspension was stirred for another 20 min at 0°C. TfOH (1 μL, 12 μmol) was added, and the reaction was stirred at 0°C for 1 h. Upon completion (TLC: cyclohexane/ethyl acetate 2:1), the reaction was diluted with CH_2Cl_2, filtered over celite and washed with sat. $NaHCO_3$ (aq) and sat. NaS_2O_3 (aq). The organic layer was dried over $MgSO_4$ and filtered. The solvent was evaporated in vacuo and the crude material was purified by flash column chromatography (silica gel, cyclohexane/ethyl acetate) to afford **2** (71 mg, 71%) as a colorless oil. $R_f = 0.35$ (cyclohexane/ethyl acetate 2:1); $[\alpha]_D^{20} = +22.09$ ($c = 1.0$, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): $\delta = 7.79$–7.73 (m, 1H), 7.60 (dd, $J = 17.2$, 7.2 Hz, 1H), 7.43–7.21 (m, 3H), 7.16 (dd, $J = 6.7$, 2.8 Hz, 1H), 6.25 (d, $J = 3.8$ Hz, 1H), 5.15–5.09 (m, 1H), 4.74–4.45 (m, 1H), 4.39 (dd, $J = 10.4$, 7.5 Hz, 1H), 4.33–4.23 (m, 1H), 4.04 (q, $J = 6.6$ Hz, 1H), 3.97–3.90 (m, 1H), 3.87–3.73 (m, 1H), 3.67 (dd, $J = 10.9$, 1.5 Hz, 1H), 1.72–1.63 (m, 1H), 1.42 (dd, $J = 15.0$, 7.5 Hz, 1H), 1.26 (s, 1H), 1.13 (s, 1H), 0.94 ppm (t, $J = 7.4$ Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 176.8$, 156.1, 143.4, 143.2, 141.4, 137.9, 137.7, 128.6, 128.6, 128.2, 128.1, 128.0, 127.4, 125.3, 125.2, 120.2, 91.8, 80.6, 75.4, 74.8, 73.8, 73.0, 70.8, 70.5, 67.9, 67.6, 67.5, 46.8, 32.4, 32.3, 27.3, 18.8, 13.7 ppm; ^{31}P NMR (243 MHz, $CDCl_3$): $\delta = -2.9$ ppm; IR (thin film): $\tilde{\nu} = 2961$, 2873, 1752, 1452, 1256, 1028, 739 cm^{-1}; HRMS: m/z: calcd for $C_{48}H_{59}O_{12}PNa$ $[M+Na]^+$: 881.3636; found: 881.3629.

Automated solid-phase synthesis

Solution A: *Building block (3×3 equiv per glycosylation cycle)*: Compound **2** (91 mg, 106 μmol) in CH_2Cl_2 (3 mL).

Solution B: *Activator (3×3 equiv per glycosylation cycle)*: TMSOTf (19.2 μL, 106 μmol).

Solution C: *Fmoc deprotection*: piperidine (20% v/v) in DMF.

Module A: *Swelling of resin prior to synthesis*: The reaction vessel was charged with functionalized resin, and CH_2Cl_2 (2 mL) was added. The resin was swelled in CH_2Cl_2 for 2 h. At the beginning of the synthesis, the reaction vessel was drained by argon pressure.

Module B: *Washing before glycosylation*: The resin was washed with THF (6×) and CH_2Cl_2 (6×) at room temperature.

Module C: *Glycosylation*: Solution A (1 mL) was added to the reaction vessel, and the suspension was cooled to −15°C while the resin was being agitated by an argon flow. Upon reaching the set temperature, solution B (1 mL) was added to the reaction vessel. The temperature was maintained for 45 min and then raised to 0°C for 15 min. The reaction vessel was then drained by argon pressure and the resin was washed with CH_2Cl_2 (6×).

Module D: *Washing after glycosylation cycle*: The temperature was raised to 25°C, and the resin was washed with THF (6×) and CH_2Cl_2 (6×).

Module E: *Fmoc deprotection*: The resin was washed with DMF (3×). Solution C (2 mL) was added to the reaction vessel, and the suspension was agitated by an argon flow for 15 min. The reaction vessel was then drained and the solvents were transferred to a fraction collector via argon pressure.

Module F: *Washing after Fmoc deprotection*: The temperature was set to 25°C, and the resin was washed with DMF (6×), THF (6×), AcOH in CH_2Cl_2 (6×), and CH_2Cl_2 (6×).

Dodecasaccharide 17 (Scheme 4): The reaction vessel of the synthesizer was charged with functionalized Merrifield resin **16** (25 mg, loading: 0.47 mmol g^{-1}). Program module A was executed once. Then twelve cycles of the program described in Table 1 were executed by the synthesizer. The resin was removed from the reaction vessel and swelled in CH_2Cl_2. The suspension was irradiated with UV light by delivering the suspension via syringe pump through a FEP tubing (inner diameter: 0.03 inch; volume: 12 mL) wrapped around a UV light source (medium pressure Hg lamp with arc lengths of 27.9 cm and power of 450 W, surrounded by a Pyrex UV filter with 50% transmittance at 305 nm). The resin was slowly injected from a disposable syringe (2 mL) and pushed through the tubing with 15 mL CH_2Cl_2 (flow rate: 300 μL min^{-1}). The tubing was washed with 15 mL CH_2Cl_2/MeOH (1:1 v/v, flow rate: 300 μL min^{-1} for 8 mL and 4 mL min^{-1} for 7 mL), and finally with 15 mL MeOH (flow rate: 4 mL min^{-1}). The suspension leaving the reactor was directed into a filter where the resin was filtered off and washed with CH_2Cl_2/MeOH (1:1 v/v), MeOH and CH_2Cl_2, while the filtrate was collected. The tubing was re-equilibrated with 15 mL CH_2Cl_2using a flow rate of 4 mL min^{-1}. The entire procedure was repeated three times.[18b] (A detailed description of the experimental procedure and setup of the photocleavage can be found on page S20 of the Supporting Information of ref. [18b], as well as in the Supporting Information of this manuscript. This protocol was followed exactly.) The resulting solution was concentrated in vacuo. The crude product was purified by HPLC (YMC-Pack Diol-300, hexane/ethyl acetate, 10% ethyl acetate for 5 min, to 40% ethyl acetate in 70 min) to obtain compound **17** as a colorless oil (2.9 mg, 4.6%). ^1H NMR (600 MHz, $CDCl_3$): $\delta = 7.35$–7.13 (m, 125H), 5.07 (s, 2H), 4.98–4.70 (m, 25H), 4.64–4.55 (m, 10H), 4.55–4.37 (m, 36H), 4.22–4.08 (m, 12H), 3.78–3.26 (m, 53H), 3.17–3.07 (m, 2H), 1.51–1.40 (m, 6H), 1.30–1.00 ppm (m, 108H); ^{13}C NMR (176 MHz, $CDCl_3$): $\delta = 179.3$, 176.7, 176.7, 176.5, 176.2, 156.5, 138.7, 138.6, 138.5, 138.4, 138.3, 138.1, 136.8, 128.7, 128.6, 128.6, 128.5, 128.5, 128.4, 128.3, 128.3, 128.2, 127.9, 127.7, 127.6, 127.6, 101.0, 99.2, 99.0, 78.9, 77.9, 76.5, 76.0, 75.8, 75.5, 75.2, 75.1, 74.9, 74.6, 74.5, 74.4, 74.1, 73.6, 73.5, 73.4, 69.5, 69.3, 69.3, 68.9, 68.8, 66.7, 41.0, 39.1, 38.8, 38.7, 32.1, 29.9, 29.3, 27.4, 27.4, 27.3, 27.2, 23.4, 22.9 ppm; HSQC (700 MHz, $CDCl_3$): $\delta = 101.0$ ($J_{C1,H1} = 161$ Hz, C-1), 99.2 ($J_{C1,H1} = 161$ Hz, C-1), 99.0 ppm ($J_{C1,H1} = 161$ Hz, C-1); MALDI-TOF MS: m/z: calcd for $C_{313}H_{379}NO_{75}Na$ $[M+Na]^+$: 5377.587; found: 5377.612.

Dodecasaccharide 18 (Scheme 4): Compound **17** (2.2 mg, 0.41 μmol) was dissolved in CH_2Cl_2/MeOH (1:1, 0.5 mL). Sodium methoxide (25 mg, 0.46 mmol) was added, and the solution was stirred for two weeks. The

Table 1. Program for one glycosylation/deprotection cycle using modules **B**, **C** and **D**–**F**, executed 12 times to obtain dodecasaccharide **1**.

Program Module	Description	Iterations
B	wash	1
C	glycosylation	3
D	wash	1
E	Fmoc deprotection	3
F	wash	1

reaction mixture was neutralized with Amberlite IR-120 (H^+), and filtered. The volatiles of the filtrate were removed in vacuo to afford compound **18** (1 mg, 56%) as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$): $\delta =$ 7.33–7.13 (m, 125H), 5.21–5.01 (m, 14H), 4.83–4.13 (m, 48H), 3.93–3.26 (m, 74H), 3.16–3.06 (m, 2H), 1.68–1.60 (m, 4H), 1.50–1.41 ppm (m, 2H); MALDI-TOF MS: m/z: calcd for $C_{253}H_{283}NO_{63}Na$ $[M+H]^+$: 4367.893; found: 4369.862.

Dodecasaccharide 1 (Scheme 4): Compound **18** (200 μg, 0.046 μmol) was dissolved in THF/H_2O (300 μL), and $Pd(OH)_2$ (0.5 mg, 20 wt.%) was added. The atmosphere was exchanged with H_2, and the reaction was stirred at RT for five days. The reaction mixture was filtered and concentrated in vacuo. The residue was purified by reversed-phase column chromatography (Waters Sep-Pak SPEVac C18, acetonitrile/water, 0 to 50%) and lyophilized to afford **1** (50 μg, 52%) as a white solid. MALDI-TOF MS: m/z: calcd for $C_{77}H_{131}NO_{61}Na$ $[M+Na]^+$: 2070.723; found: 2070.774. The correct structure of **1** was confirmed by comparison of the ^{1}H NMR spectrum of **1** with a ^{1}H NMR spectrum of isolated Curdlan (linear β-1,3-glucan from *Agrobacterium biobar*).[19]

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