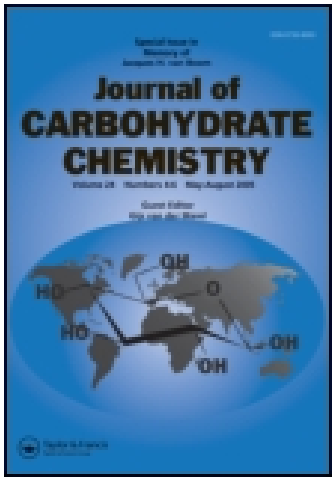

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Synthesis of Laminarin Fragments and Evaluation of a β-(1,3) Glucan Hexasaccaride-CRM_{197} Conjugate as Vaccine Candidate against *Candida albicans*

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Laminarin-CRM_{197} glycoconjugates were previously demonstrated to be immunogenic and confer protection against *Candida albicans* in mice. Laminarin consists of β-(1,3) glucan repeating units, with sporadic β-(1,6) branches. A set of short glucans was used to study the effect of the β-(1,6) branch on the antigenicity of linear β-(1,3) glucans. A linear β-(1,3) glucan hexasaccharide was selected as the best fragment able to inhibit the binding between laminarin and antilaminarin antibodies. The hexamer was then conjugated to CRM_{197} and induced, in mice, significant titers of specific antilaminarin antibodies comparable with those raised by the Lam-CRM_{197} conjugate.

Keywords *Candida albicans*; Glycoconjugate vaccine; Glucans; Delevulinoylation

INTRODUCTION

Deep-seated mycoses represent a serious medical problem, especially in nosocomial settings where mortality due to *Candida* and *Aspergillus* invasive

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infections is approaching 40%.[1] Antifungal therapy is limited by drug toxicity and emergence of resistant strains.[1]

Mucosal infections are also common in nonimmunocompromised subjects; for example, vulvovaginal candidiasis affects a substantial percentage of all women (3% to 5%), and often requires chronic antimycotic treatment.[2] A prophylactic and/or therapeutic vaccine is increasingly being considered as one of the most appropriate options to meet the current pressing medical need.[3]

Fungi are characterized by a cell wall rich in carbohydrates in the form of polysaccharides or glycoproteins[4]; in particular, glucans containing both β-(1,3) and β-(1,6) sequences are highly conserved in most if not all pathogenic fungi, and therefore have been considered as an attractive target for vaccine development. The first evidence of the possible role of fungal glucans as protective antigens was reported by Bromuro et al.,[5] who showed that intact *C. albicans* cells treated to expose glucans on their surface conferred significant anti-*Candida* protection in a mouse model. Subsequent work was then undertaken in order to further explore this hypothesis. Because of their well-known suboptimal immunogenicity, polysaccharides are generally conjugated to carrier proteins in order to transform them into T-dependent antigens able to elicit a response characterized by the prevalence of IgG antibodies and immunological memory.[6]

Considering possible technical issues associated with the large-scale extraction and purification of β-glucans from pathogenic fungi, we addressed the question of whether other biological sources of this type of molecules could be used. In particular, we focused our attention on laminarin (Lam), a β-(1,3) glucan with sporadic β-(1,6) branches, extracted from the brown alga *Laminaria digitata*. We showed that when Lam is conjugated to CRM_{197}, a nontoxic mutant of diphtheria toxin[7] that has been extensively used as a carrier protein for a number of commercially available glycoconjugate vaccines, the resulting Lam-CRM_{197} conjugate is both immunogenic and protective, in a mouse challenge model, against infections induced by *C. albicans*.[8]

Another carbohydrate antigen obtained specifically from *C. albicans* N-glycoproteins is represented by mannans, which have been extensively exploited by other groups for their potential application in the preparation of a fully synthetic conjugate vaccine against candidiases.[9,10]

Indeed, the advantage of targeting β-glucans is represented by their highly conserved presence across almost all pathogenic fungi and the stability of their expression substantially in the entire life cycle of the fungal cells.[4]

In a more recent study we compared CRM_{197} conjugates of two synthetic glucans, a linear one composed of 15 β-(1,3) repeating units (15 mer) and an oligosaccharide with the same backbone but with two β-(1,6) arms spaced by five repeats (17 mer). Only the linear structure conferred protection against *C. albicans.*[11] In the same study we also observed that conjugates of CRM_{197}

with oligosaccharides derived from the linear β-(1,3) glucan curdlan polysaccharide were able to confer a significant level of protection against *C. albicans* in mice. Additionally, it has been demonstrated by microarray analysis that the protective IgG2b mAb named 2G8 specifically recognizes linear β-(1,3) glucan epitopes rather than β-(1,4) or β-(1,6).[12] This prompted us to assume that the protective epitope in the *C. albicans* glucan cell wall is defined by a linear β-(1,3) glucan.

The aim of this study was (a) to address more specifically the effect of β-(1,6) branch on the antigenicity of linear β-(1,3) glucans and (b) to determine what could be the minimal structure of β-(1,3) glucan oligosaccharides capable of efficiently binding to antilaminarin antibodies and whether this structure is immunogenic when conjugated to a carrier protein.

RESULTS AND DISCUSSION

Synthesis of β-glucans

With the scope of studying the role of β-(1,6) linkages in the immunodominant epitope of laminarin, we initially synthesized as methyl glycosides four laminarin fragments, the three glucans **1**–**3** reproducing the branching point of the polysaccharide and the linear trisaccharide **4** (Fig. 1).

Numerous syntheses of β-(1,3) and β-(1,6) glucans have been reported, so the preparation of the four oligosaccharides was achieved by adaptations of literature methods[13–18] in a way that we used only the three monosaccharide building blocks **9**,[19] **8,** and **12**.[20] As a key building block for the introduction of β-(1,3) and β-(1,6) linkages we synthesized from compound **7b** the monosaccharide **8**, possessing two free hydroxyls in the 4 and 6 positions, which allow regioselective glycosylation of the latter[21] and a levulinoyl ester to protect position 3.

The levulinoyl group is commonly used in carbohydrate chemistry both for orthogonal removal in the presence of other esters, such as acetates or benzoates, and to act as a participating group in position 2 during the glycosylation step.[22] Its cleavage is usually carried out with hydrazine acetate or hydrazine buffered with acetic acid,[23–25] which are both carcinogenic reagents. Other reagents, such as hydrogen sulfide at pH 7 or NaH in THF, are also reported,[26] but they have not found large application in orthogonal deprotection. Our efforts to develop a safer method than hydrazine for its selective removal led us to test ethylenediamine-acetic acid as reagent. The deprotection of compound **7b** proceeded very slowly at rt (longer than 24 h) even with four equivalents of base and five equivalents of acid, but could be performed in a reasonable time of 3 to 4 h at 50°C, without migration of benzoate esters at the 2 position, giving in nearly quantitative yield the known 3-hydroxy sugar **7a**.[27]

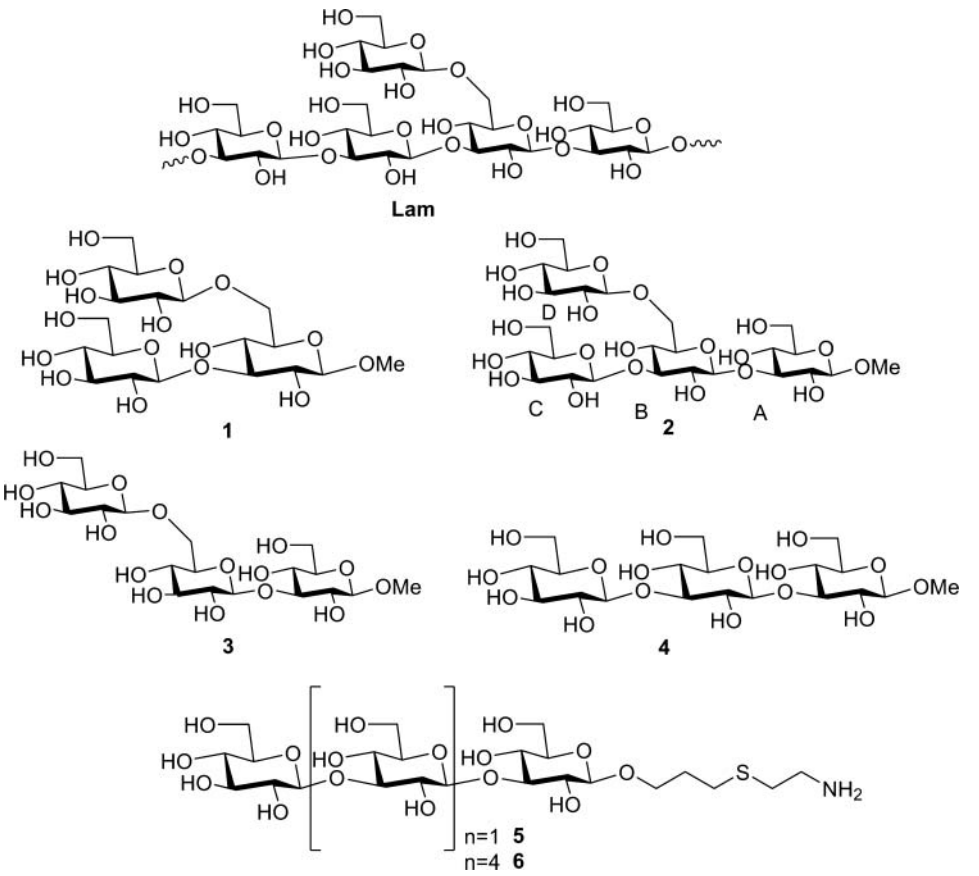


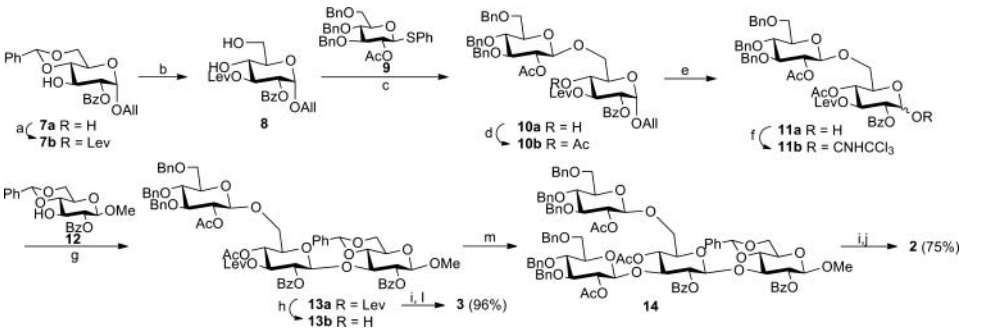
Figure 1: Chemical structure of laminarin and synthetic fragments.

This preliminary result on compound **7b** convinced us to use this method during the synthesis of our glucans.

Compounds **2** and **3** were prepared from the common disaccharide building block **10a**, obtained by glycosylation of the diol **8** with thioglycoside **9** in the presence of NIS-TfOH as promoters (Sch. 1).

The glycosylation reaction occurred with a higher regioselectivity (76% yield) toward position 6 as previously reported with imidate donor.[21] A doublet of doublets at 4.79 ppm in ^{1}H NMR spectrum, assigned to H-2^D with coupling constants $J_{1,2} = 8.1$ Hz and $J_{2,3} = 10.2$ Hz, allowed to ascertain the β-configuration of the newly formed glycosidic bond, as expected in the presence of a participating group at the C-2 position of donor **9**.

After acetylation of the hydroxyl group at C-4, a downfield shift of H-4^B toward 5.0 ppm in the ^{1}H NMR spectrum was observed, confirming that the glycosylation had occurred at the C-6 position. A trisaccharide produced by



Scheme 1: Sequence of reactions leading to tetrasaccharide **2** and trisaccharide **3**. *Reagents*: (a) LevOH, DCC, DMAP, CH_2Cl_2, 95%; (b) 9:1 AcOH-H_2O, 60°C, 93%; (c) NIS, TfOH, CH_2Cl_2, –60°C, 80%; (d) Ac_2O, py, 98%; (e) $PdCl_2$, NaOAc, AcOH, 70%; (f) CCl_3CN, DBU, CH_2Cl_2, 90%; (g) TMSOTf, CH_2Cl_2, 75%; (h) $H_2NCH_2CH_2NH_2$.AcOH, CH_2Cl_2, 50°C, 90%; (i) NaOMe; (j) H_2, 10% Pd-C, MeOH; (m) **9**, NIS, TfOH, CH_2Cl_2, –40°C, 75%.

simultaneous glycosylation of 4 and 6 hydroxyls was also isolated as a minor product (15% yield, as identified by NMR) from the crude mixture. Attempts to glycosylate compound **8** with either peracetylated or perbenzoylated trichloroacetimidate donors and TMSOTf as promoter resulted in a remarkably lower yield due to the concomitant formation of the orthoester as a side product.

The acetylated disaccharide **10b** was converted into trichloroacetimidate **11b** and glycosylated with acceptor **12** using TMSOTf as Lewis acid. The generated β-anomeric center appeared in ^{1}H NMR spectrum as a doublet at 4.4 ppm with $J_{1,2} = 7.8$ Hz.

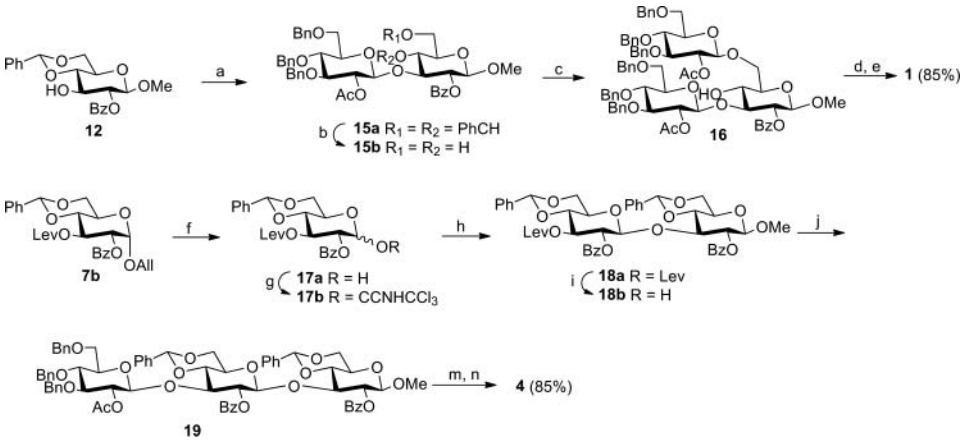
The desired compound **3** was smoothly obtained from trisaccharide **13a** by acyl group removal followed by hydrogenation on 10% Pd-C. Delevulinoylation of trisaccharide **13a** with ethylenediamine-acetic acid (90%) delivered the OH-3^B acceptor **13b**, which was reacted with **9** to furnish tetrasaccharide **14** (Sch. 1).

The four anomeric centers were detected by ^{13}C NMR analysis at 101.14, 100.81, 100.67, and 100.10 ppm, indicating that they all possessed β-configuration.[15,24,28]

Deprotection of **14** by methanolysis and subsequent hydrogenation on 10% Pd-C uneventfully afforded compound **2**.

Trisaccharide **1** was assembled by glycosylation of the monosaccharide acceptor **12** with thioglycoside **9**, followed by hydrolysis of the benzylidene-protecting group and regioselective glycosylation of the primary alcohol of **15b** to yield compound **16** (Sch. 2).

The coupling constants of the three anomeric protons ($J_{1,2} = 8.1$ Hz) unambiguously indicated their β-configuration.



Scheme 2: Sequence of reactions leading to trisaccharide **1** and **4**. *Reagents*: (a) **9**, NIS-TfOH, CH_2Cl_2, –60°C, 82%; (b) TFA-H_2O, CH_2Cl_2, 90%; (c) **9**, NIS, TfOH, CH_2Cl_2, –60°C, 93%; (d) NaOMe, MeOH; (e) H_2, 10% Pd-C, MeOH; (f) 1,5-Cyclooctadiene-bis(methyldiphenylphosphine)-Iridium-hexafluorophosphate, THF; I_2, H_2O, 92%; (g) CCl_3CN, DBU, CH_2Cl_2, 90%; (h) **12**, TMSOTf, CH_2Cl_2, 81%; (i) $H_2NCH_2CH_2NH_2{\cdot}AcOH$, 50°C, 95%; (j) **9**, NIS, TfOH, CH_2Cl_2, –40°C, 80%; (m) NaOMe, MeOH; (n) H_2, 10% Pd-C, MeOH.

For the construction of trisaccharide **4,** compound **7b** was deallylated by isomerization with hydrogen-activated 1,5-cyclooctadiene-bis(methyldiphenylphosphine)-Iridium(I)-hexafluorophosphate catalyst and subsequent oxidative cleavage with I_2, which allowed to obtain **7a** in 92% yield instead of the 60% provided by ally removal with $PdCl_2$ catalyst. Following treatment with trichloroacetonitrile and DBU yielded donor **17b** (Sch. 2).

The preparation of **17b** was accomplished through a more straightforward reaction scheme as compared to the preparation of a similar trichloroacetimidate donor from ethylthio-2-*O*-benzoyl-4,6-di-*O*-benzylidene-6-*O*-*tert*butyldimethylsylil-β-D-glucopyranoside recently described by Mo et al.[28] Conversion to trichloroacetimidate in their case was achieved by hydrolysis of the thioethyl group accompanied by migration of the benzoyl ester to the anomeric position, which needed a further step to rearrange the product to the desired l-hydroxyl derivative before final conversion into the desired trichloroacetimidate.

After glycosylation of **12** with donor **17b,** the synthesized disaccharide **18a** was selectively deprotected at the OH-3^B position by levulinoyl group removal with ethylenediamine-acetic acid to be glycosylated with **9** and deliver trisaccharide **19**.

While conventional proton-proton coupling constants were found for the two β-anomeric protons H-1^A and H-1^D ($J_{1,2} = 7.5$ and 8.1 Hz, respectively), a smaller value ($J_{1,2} = 5.8$ Hz) was noticed for H-1^B. This observation, however,

is consistent with reported coupling constants in similar β-(1,3)-protected oligoglucosides.[29] Acyl group removal and following hydrogenation on 10% Pd-C eventually provided compound **4**.

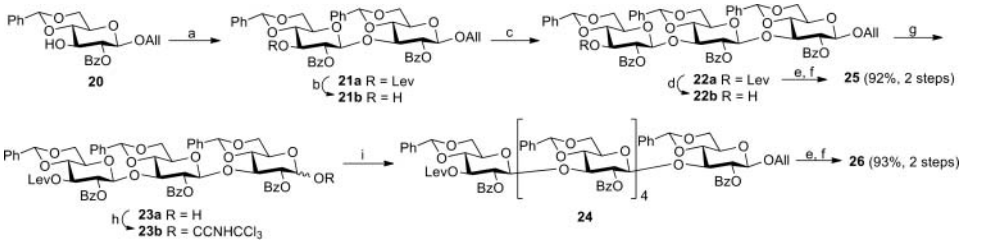
^{1}H NMR analysis of deprotected fragments showed the presence of anomeric protons belonging to β-(1,3) constructs at around 4.70 ppm with $J_{1,2}$ ranging from 7.5 to 8.1 ppm, while the ones coming from β-(1,6) linkages fell at 4.50 ppm with $J_{1,2} = 8.0$ Hz, similarly to what was observed in the laminarin spectrum.[30,31]

In addition, ^{13}C NMR signal for the anomeric position C-1^D involved in β-(1,6) linkage was consistently at 103.6 ppm in all the prepared fragments, while signals attributed to C-1 belonging to β-(1,3) linkages appeared at 103.5 τo 103.6 ppm for C-1^C, at 103.5 ppm for C-1^B, and 103.2 to 103.4 ppm for C-1^A.[15,32]

Since a preliminary competitive ELISA provided the information that the linear trisaccharide was a better inhibitor than the branched oligosaccharides, we synthesized the linear tri- and hexasaccharide **25** and **26**, respectively, as allyl glycosides[33] in view of possible conjugation to carrier protein. For this purpose we used the approach described by Huang et al.,[32] except that levulinoyl ester was introduced for orthogonal removal (Sch. 3).

Glycosylation of monosaccharide **20** with donor **17b** promoted by TMSOTf took place in 75% yield. After delevulinoylation with our method (85%), trisaccharide **22a** was prepared through glycosylation of compound **21b** with donor **17b** (53%). Although the detection of $J_{1,2}$ was not possible in the ^{1}H NMR spectrum, COSY and HMQC experiments allowed to assign the C-1^B signal at 98.4 ppm, confirming the formation of a β-glycosidic bond.

Trisaccharide **22a** was then subjected to levulinoyl ester removal to provide acceptor **22b** (82%), for which H-1^B appeared as a doublet with $J_{1,2}$ = 4.4 Hz in the ^{1}H NMR spectrum and as a signal at 97.9 ppm in



Scheme 3: Sequence of reactions leading to protected trisaccharide **25** and hexasaccharide **26**. *Reagents*: (a) **17b**, TMSOTf, CH_2Cl_2, 75%; (b) $H_2NCH_2CH_2NH_2{\cdot}AcOH$, CH_2Cl_2, 50°C, 85%; (c) **17b**, TMSOTf, CH_2Cl_2, 53%; (d) $H_2NCH_2CH_2NH_2{\cdot}AcOH$, CH_2Cl_2, 50°C, 82%; (e) NaOMe, MeOH; (f) 9:1 AcOH-H_2O, 50°C; (g) 1,5-Cyclooctadiene-bis(methyldiphenylphosphine)-Iridium-hexafluorophosphate catalyst, THF; I_2, H_2O, 70%; (h) CCl_3CN, DBU, CH_2Cl_2, 90%; (i) **22b**, TMSOTf, CH_2Cl_2, 54%.

the ^{13}C NMR spectrum as further validation of the assigned stereochemistry. Allyl cleavage of **22a** with hydrogen activated 1,5-cyclooctadiene-bis (methyldiphenylphosphine)-Iridium(I)-hexafluorophosphate catalyst followed by oxidation with I_2, which worked also in this case with higher efficiency in comparison to single-step deallylation with $PdCl_2$ (70% and 63% of yield, respectively), afforded the donor precursor **23a**. The foregoing 1-hydroxy trisaccharide **23a** was then transformed into donor **23b**, which was used for glycosylation of **22b** to obtain the hexasaccharide **24** in 54% yield. The β-configuration of all the anomeric protons could be derived from ^{13}C NMR analysis where all the anomeric peaks appeared in the range 101.3 to 97.3 ppm.

The utilization of a disaccharide trichloroacetimidate donor closely related to trisaccharide **23**, except for the use of a *tert*-butyldimethylsylil protection at the C-3 position instead of levulinoyl ester, to couple with a 2-*O*-benzoyl-4,6-*O*-dibenzylidene-protected disaccharide acceptor for building up a β-(1,3) glucan tetramer has been reported to give a 4:3 mixture of α-/β-products.[28] The problem was circumvented by the authors replacing the benzoyl group, which failed to provide neighboring group participation with a 4-acethoxy-2,2-dimethylbutanoate ester.

We did not observe any formation of α-glycosylation product in the synthesis of hexasaccharide **24** from our trisaccharide synthons **22b** and **23b**.

Tri- and hexasaccharide **22a** and **24** were deprotected by methanolysis under Zemplen condition and subsequent acid hydrolysis to furnish **25** and **26**, respectively (for $J_{1,2}$ see Experimental section).

For introduction of an amino group to be used for conjugation to protein, the allyl functions of **25** and **26** were subjected to thiol-ene reaction with cysteamine under UV to afford **5** and **6**, respectively (Sch. 4).[34]

Competitive ELISA

Preliminary competitive ELISA experiments on compounds **1**–**4** showed that the linear trisaccharide **4** is the best inhibitor of the binding between Lam and antilaminarin antibodies elicited in the mouse by a Lam-CRM conjugate.

This result supported our hypothesis and previous findings about the linearity of the dominant epitope in *C. albicans* and suggested to us to focus only on nonbranched oligosaccharides. In particular, considering that the 17 mer glucan, which failed to induce protection, contains β-(1,6) linkages separated by five β-(1,3) glucoside units, the 6 mer seemed a promising target as a short glucan. We therefore decided to synthesize both the trimer **5** and the hexamer **6** carrying an amino linker, suitable for conjugation to the carrier protein (Sch. 3).

Competitive ELISA of compounds **1**–**4** and **6** was again performed on plates coated with laminarin as described in the Experimental section. As shown in

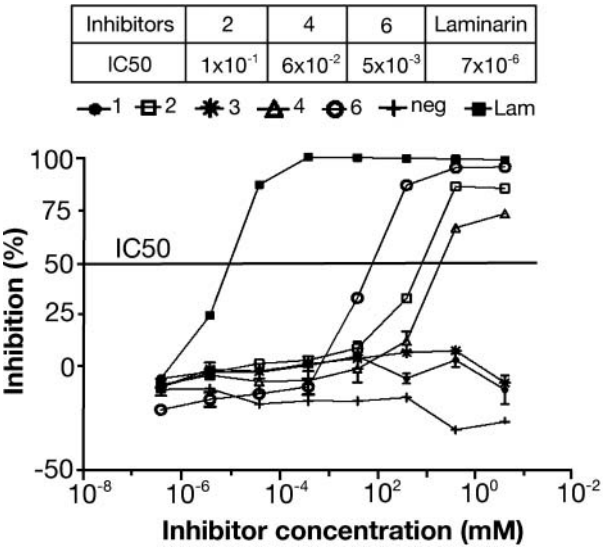


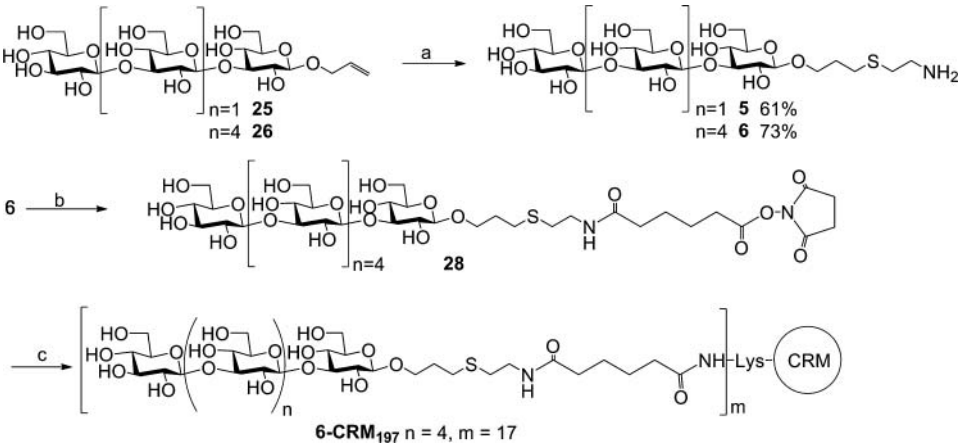
Figure 2: Competitive ELISA of synthetic glucans **1**–**4** and **6**. Meningococcal C polysaccharide was used as negative control (neg). (See Fig. 1 for structures.)

Fig. 2, the hexasaccharide **6** was shown to be the best inhibitor, having an IC_{50} 12-fold lower than trisaccharide **4** and 20-fold lower than tetrasaccharide **2**. Furthermore, compound **6** gave a 95% inhibition at a 4 mMol concentration, while trisaccharide **4** at the same concentration reached an inhibition level of 85% and the β-(1,6)-branched tetrasaccharide **2** only 73%. The remaining two trisaccharides **1** and **3** exhibit no inhibition at the assay concentration.

Conjugation to CRM_{197}

The conjugation of hexasaccharide **6** was carried out in two steps: firstly, the linker-equipped oligosaccharide was reacted with an excess of di-N-hydroxysuccinimidyl adipate in DMSO (Sch. 4) so that formation of only a half ester could be obtained (Sch. 4).[11] The half ester was then purified by precipitation from the reaction mixture with ethyl acetate and reacted with CRM_{197} in phosphate buffer (pH 7). Using a 30:1 molar ratio of carbohydrate/protein, based on spectrophotometric determination of active ester in the oligosaccharide sample, an average loading of 16.8 sugar chains was achieved as determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and confirmed by MALDI-TOF mass spectrometry analysis (Fig. 3).

The conjugation efficiency (57%) was higher than previously reported for the linear 15 mer β-(1,3) glucan (7.5 carbohydrate chains/mol protein, 25% coupling efficiency), although the same reaction stoichiometry was used.[11] This



Scheme 4: Preparation of deprotected trisaccharide **5** and hexasaccharide **6**; synthesis of glycoconjugate **6-CRM$_{197}$**. *Reagents*: (a) cysteamine, hv, H_2O, MeOH; (b) EtN_3, disuccinimidyl adipate, DMSO; (c) CRM_{197}, PBS pH = 7.

observation might indicate a better reactivity of shorter-chain-length oligosaccharides. A similar trend has been observed for the coupling of *Streptococcus pneumonia* type 3 synthetic oligosaccharides to CRM_{197}.[35]

Immunogenicity of β-(1,3) Glucan Hexasaccharide-CRM$_{197}$ Conjugate

Animal experimental guidelines set forth by the Novartis Animal Care Department were followed in the conduct of all animal studies. The β-(1,3) glucan

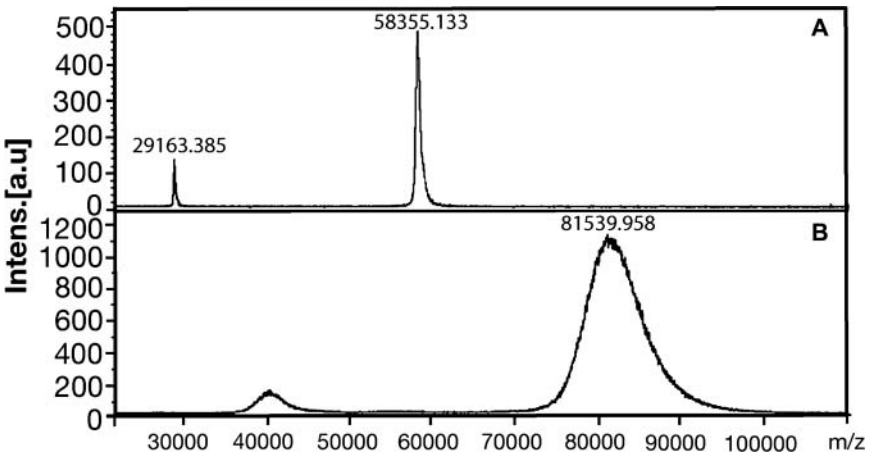


Figure 3: MALDI-TOF spectra of CRM_{197} (**A**) and hexasaccharide(**6**)-CRM_{197} conjugate (**B**).

hexasaccharide-CRM_{197} conjugate was tested in Balb/c mice for its ability to induce antilaminarin antibodies. For this purpose groups of eight mice were immunized with a 5-μg carbohydrate-based dose of Lam- and Hexa-CRM_{197} conjugates, respectively. For Hexa-CRM_{197} a 1-μg dose was used as well, with the aim of studying the dose effect. Vaccines were formulated with the adjuvant MF59, an oil in water emulsion, which was shown to be effective in boosting both cellular and humoral immune response and is commonly used for seasonal flu vaccination.[36] Adjuvant alone in phosphate buffer (PBS) was used as negative controls.

Sera were analyzed by ELISA for their content of antilaminarin IgG. Laminarin was directly coated onto the plates, determining specifically the binding to the carbohydrate antigen, thus excluding any effect of the linker.

After three injections, the hexasaccharide conjugate, at both 1- and 5-μg dosages, induced titers of specific antilaminarin IgG antibody significantly higher as compared to Lam-CRM_{197} (geometric mean titers 18,101 vs. 5191; $p < 0.05$), which induced a level of antibodies in the same range of those previously observed although with a different strain of mice.[11] Interestingly, as shown in Fig. 4, the immunological response of the hexasaccharide conjugate appeared to be more homogeneous in comparison with Lam-CRM_{197}.

The effect of saccharide chain length on glycoconjugate vaccine immunogenicity has been investigated in other independent studies, indicating that chain length and degree of saccharide loading onto the carrier protein are

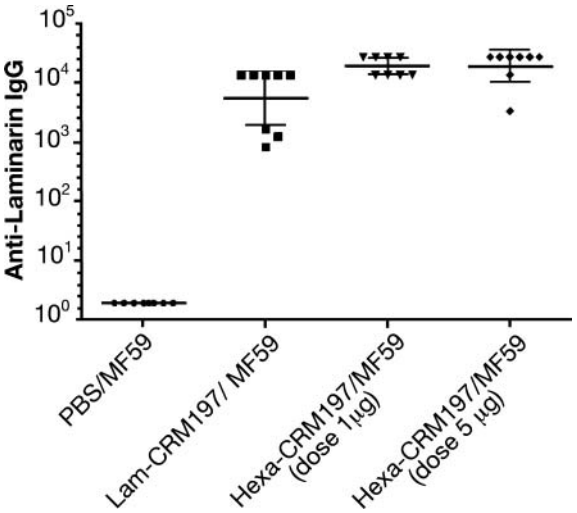


Figure 4: Antibody titers induced in mice by the synthetic β-(1,3) glucan hexasaccharide-CRM_{197} conjugate formulated with adjuvant MF59. Horizontal and vertical bars refer to the geometric means and 95% confidence interval, respectively.

probably two interconnected variables. In particular, glycoconjugates containing short oligosaccharides might work better at higher loading, while increasing the oligosaccharide chain length the optimal glycosylation degree might be lower.[37, 38] Noteworthy, the β-(1,3) glucan hexasaccharide-CRM_{197} conjugate tested in the present study has a loading degree of approximately 17 as compared to a value of 7.5 for the Lam-CRM_{197} conjugate.

CONCLUSIONS

In a previous report we demonstrated that a linear 15 mer synthetic β-(1,3) glucan conjugated to CRM_{197} could be a vaccine candidate against *C. albicans*.[11] Interestingly, the introduction of β-(1,6) branching in each of the five units resulted in loss of protection against the fungus infection. On the other hand, laminarin, whose conjugates with CRM_{197} resulted in conferring protection, contains β-(1,6) branches that are statistically spaced by about 10 residues.[11] Torosantucci et al.[12] showed, through glycoarray analysis, that an IgG murine monoclonal antibody previously reported to control *C. albicans* and *C. neoformans* infections in different animal models[39] specifically recognizes β-(1,3) glucans oligomers. The authors observed the highest binding strength starting from chain lengths of seven sugar residues, which correspond to six repeating units if we consider that the immobilization method used by the authors results in more than 85% of the reducing sugar being in open form.[40]

To further investigate these findings by well-defined synthetic glucans, we reported here an alternative synthesis of laminarin fragments based on regioselective glycosylation at position 6 of 4,6-sugar diols by thioglycoside donors for the construction of the β-(1,6) and novel orthogonal deprotection of 3-*O*-levulinoylated building blocks with ethylenediamine-acetic acid for the introduction of β-(1,3) linkages. Competitive ELISA assay on prepared glucans showed that branching is likely not involved in *C. albicans* epitope recognition as linear fragments possess higher capacity of inhibiting the interaction of laminarin with anti-Lam-CRM_{197} antibodies with respect to the β-(1,6)-branched ones.

In fact, the trisaccharide **4** proved to be a better inhibitor as compared to the β-(1,6)-branched tetrasaccharide **2**. In addition, the linear hexasaccharide **6** resulted in a better inhibitor than trisaccharide **4**. With this information in our hands, which is in line with findings of other authors,[12] the synthetic linear hexamer was conjugated to CRM_{197} for further immunogenicity studies. The β-(1,3) glucan hexasaccharide-CRM_{197} conjugate raised in mice a more homogeneous antibody response with IgG titers significantly higher than the Lam-CRM_{197} vaccine. This outcome suggests that a linear hexasaccharide might be long enough to cover the *C. albicans* epitope.

Following the promising immunogenicity results here described, further studies are planned to evaluate the protection evoked by the β-(1,3) glucan

hexasaccharide-CRM_{197} conjugate, in the context of a larger panel of glycoconjugate vaccine candidates.

EXPERIMENTAL SECTION

General Methods

All chemicals were of reagent grade and were used without further purification. Reactions were monitored by thin-layer chromatography (TLC) on Silica Gel 60 F_{254} (Merck); after exam under UV light, compounds were visualized by heating with 10% (v/v) ethanolic H_2SO_4. In the workup procedures, organic solutions were washed with the amounts of the indicated aqueous solutions, then dried with anhydrous Na_2SO_4 and concentrated under reduced pressure at 30 to 50°C on a water bath. Column chromatography was performed on Silica Gel 60 (Sigma Aldrich, 0.040–0.063 nm). Unless otherwise specified, a gradient 0 → 100% hexane-EtOAc was applied in a Combiflash R_f (Teledyne-Isco) or Flash Master Plus (Biotage) instrument. Solvent mixtures less polar than those used for TLC were used at the onset of separation. ^{1}H NMR spectra were measured at 300 MHz with a Varian Unity Inova 300 or 400 MHz with a Bruker Avance^{III} 400 spectrometer; δ_H values are reported in ppm, relative to internal Me_4Si (δ_H = 0.00, $CDCl_3$ and CD_3OD) or internal acetone (δ_H = 2.22, D_2O); when not suppressed the solvent peak for D_2O was calibrated at 4.79 ppm. ^{13}C NMR spectra were measured at 75 MHz with a Varian Unity Inova 300 or 100 MHz with a Bruker Avance^{III} 400 spectrometer; δ_C values are reported in ppm relative to the signal of $CDCl_3$ (δ_C = 77.0, $CDCl_3$), CD_3OD (δ_C = 49.0, CD_3OD), or internal acetone (δ_C = 30.9, D_2O). Assignments of NMR signals were made by homonuclear and heteronuclear two-dimensional correlation spectroscopy, run with the software supplied with the spectrometers. Sugar moieties in the final compounds **1**–**4** and related building blocks are named using the letters A, B, C, and D based on the tetrasaccharide structure, as depicted in Fig. 1. For hexasaccharide **6** letters A–F are used starting from the aglycon. Nuclei associated with the 3-(2-aminoethylthio)propyl linker are denoted with a prime. Exact masses were measured by electron spray ionization mass spectroscopy, using a Q-Tof *micro* Macromass (Waters) instrument. Palladium chloride and 1,5-Cyclooctadiene-bis(methyldiphenylphosphine)-Iridium-hexafluorophosphate catalyst were purchased from Sigma-Aldrich. Hydrogenation reactions were performed by flow chemistry on an H-Cube (Thalesnano) instrument.

General procedure for glycosylation with trichloroacetimidate donors

To a stirred solution of acceptor (1 mmol) and donor (1.2 mmol) in CH_2Cl_2 (15 mL) containing activated 4 Å MS (0.75 g), TMSOTf (0.1 mmol) was added

at 0°C, under nitrogen atmosphere. The mixture was stirred for 30 min when TLC (2:1 hexane-EtOAc) showed the reaction was complete. Then the mixture was neutralized with triethylamine (0.1 μL) and filtered through a celite pad, and the filtrate concentrated. Chromatography of the residue (hexane-EtOAc) gave the desired product.

General procedure for delevulinoylation

To a solution of the 3-*O*-Lev oligosaccharide (1 mmol) in CH_2Cl_2 (25 mL), ethylenediamine (0.26 mL, 4 mmol) and AcOH (0.29 mL, 5 mmol) were added at 0°C. A white solid was formed and the suspension was stirred for 5 to 6 h at 50°C, when the deprotection was complete (TLC, hexane-EtOAc 2:1). The mixture was concentrated and chromatography of the residue (hexane-EtOAc) yielded the delevulinoylated product.

General procedure for deallylation

A mixture of the 1-*O*-allyl compound (1 mmol) and 1,5-cyclooctadiene-bis(methyldiphenylphosphine)-Iridium(I)-hexafluorophosphate catalyst (2 mg) in dry THF (7 mL) was carefully degassed. The catalyst was activated under hydrogen atmosphere for 2 min, until the catalyst turned from red to pale yellow, and the reaction mixture was stirred at rt for 3 h. When NMR analysis of a small portion showed complete isomerization, water was added in order to get a 4:1 THF:H_2O mixture, followed by iodine (0.5 g, 2 mmol). After 15 min (TLC, 1:1 hexane-EtOAc) the solution was diluted with water and extracted with CH_2Cl_2 (3 × 10 mL). The organic layers were washed with a freshly prepared 5% solution of $Na_2S_2O_3$ until decoloration, dried (Na_2SO_4), filtered, and concentrated. Chromatography of the residue (hexane-EtOAc) afforded the deallylated compound as a mixture of α/β anomers, which was used for the next step.

*Allyl 2-*O*-benzoyl-4,6-*O*-benzylidene-3-*O*-levulinoyl-α-D-glucopyranoside 7b.* To a solution of 3-OH sugar **7a**[26] (2.4 g, 5.8 mmol) in dichloromethane (100 mL), levulinic acid (810 mg, 7 mmol) was added followed by DMAP (850 mg, 7 mmol) and DCC (1.44 g, 7 mmol). The mixture was stirred for 3 h when TLC (2:1 hexane-EtOAc) showed the reaction was complete. The mixture was filtered, and the filtrate was partitioned with aq $NaHCO_3$. Combined organic layers were dried on $MgSO_4$ and concentrated. Chromatography of the residue with 5:1 hexane-EtOAc gave the 3-*O*-Lev product **7b** (2.8 g, 94%). $[\alpha]_D = +77^\circ$ (c = 0.6; $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.07–7.20 (m, 10 H, *Ph*CH, *Ph*CO), 5.88–5.75 (m, 2 H, $CH_2CH{=}CH_2$, incl. t, 5.84, $J = 9.9$ Hz, H-3), 5.56 (s, 1 H, PhC*H*), 5.31–5.09 (m, 4 H, H-2, $CH_2CH{=}CH_2$, incl. s, 5.28, $J_{1,2} = 3.9$ Hz, H-1), 4.34 (dd, $J_{5,6a} = 5.1$, $J_{6a,6b} = 10.1$ Hz, H-6a), 4.25–3.97 (m, 2 H, $CH_2CH{=}CH_2$), 3.85–3.73 (m, 2 H, H-4,6b), 3.65–3.57 (m, 1 H, H-5), 2.65–2.50 (m, 4 H, CH_2CH_2), 2.00 (s, 3 H, CH_3). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 205.75 (CH_2CO), 171.77 (2 C, CH_2COO, CH_3CO), 165.78 (Ph*C*O), 136.86,

134.40, 133.13, 132.53, 131.01, 129.86, 129.78, 129.50, 127.29, 125.24, 124.99 (PhCO, *Ph*CH, =*C*H), 117.65 (=*C*H_2), 101.44 (Ph*C*H), 95.77 (C-1), 79.08 (C-4), 72.17 (C-2), 69.07 (C-3), 68.71 (*C*H_2CH=CH_2), 68.65 (C-6), 62.56 (C-5), 37.87 (*C*H_2CO), 29.53 (CH_3), 27.3 (*C*H_2COO). ESI HR-MS ($C_{28}H_{30}O_9$): m/z = found ($[M+H]^+$ 511.1924; calc 511.1968).

*Allyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→6)-2-*O*-benzoyl-3-*O*-levulinoyl-α-D-glucopyranoside 10a.* A solution of the 3-*O*-Lev monosaccharide **7b** (1.5 g, 2.9 mmol) in 90% AcOH (100 mL) was stirred overnight at 60°C, when TLC (2:1 hexane-EtOAc, 1:4 CH_2Cl_2-EtOAc) showed the reaction was complete. The mixture was coevaporated with toluene and the residue purified on silica gel with CH_2Cl_2-EtOAc 1:4, to afford **8** (1.15 g, 93%).

*Allyl 2-*O*-benzoyl-3-*O*-levulinoyl-α-*D*-glucopyranoside* **8**. $[\alpha]_D$ = +118° (c = 0.1; $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.03–7.41 (m, 5 H, *Ph*CO), 5.87–5.74 (m, 1 H, CH_2CH=CH_2), 5.84 (m, 1 H, H-3), 5.29–5.04 (m, 4 H, H-2, CH_2CH=CH_2, incl. d, 5.21, $J_{1,2}$ = 3.9 Hz, H-1), 4.34–3.96 (m, 2 H, CH_2CH=CH_2), 3.91–3.70 (m, 5 H, H-4,5,6a,b, OH), 2.85–2.40 (m, 4 H, CH_2CH_2), 2.11 (s, 3 H, CH_3), 2.04 (br. s, 1 H, OH). ESI HR-MS ($C_{21}H_{26}O_9$): m/z = found ($[M+H]^+$ 423.1667; calc 423.1655).

To a stirred solution of acceptor **8** (1.12 g, 2.64 mmol) and donor **9**[19] (1.75 g, 3.17 mmol) in CH_2Cl_2 (100 mL) containing activated 4 Å MS, NIS (0.89 g, 3.96 mmol) was added at –60°C, under nitrogen atmosphere, followed by TfOH (60 μL). The mixture was stirred for 6 h at –60°C, when TLC (1:1 hexane-EtOAc) showed the formation of the disaccharide. The mixture was filtered through a celite pad, and partitioned with aq $NaHCO_3$. Chromatography of the concentrated organic layers with 5% CH_2Cl_2-EtOAc gave 1.76 g of product **10a** (76%). $[\alpha]_D$ = +45° (c = 1.2; $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.03–7.14 (m, 20 H, 3 × *Ph*CH_2, *Ph*CO), 5.85–5.72 (m, 1 H, CH_2CH=CH_2), 5.60 (dd, 1 H, $J_{2,3}$ = 9.0, $J_{3,4}$ = 10.2 Hz, H-3^B), 5.29–5.12 (m, 6 H, PhCH_2, CH_2CH=CH_2, incl. d, 5.19, $J_{1,2}$ = 3.6 Hz, H-1^B, dd, $J_{2,3}$ = 10.2 Hz, H-2^B), 4.85–4.45 (m, 6 H, 2 × PhCH_2, incl. dd, 4.79, $J_{1,2}$ = 8.1, $J_{2,3}$ = 10.2 Hz, H-2^D; d, 4.52, H-1^D), 4.20–3.54 (m, 12 H, CH_2CH=CH_2, H-3^D,4^{B,D},5^{B,D},6a^{B,D},6b^{B,D}, OH-4^B), 2.75–2.40 (m, 4 H, CH_2CH_2), 2.07, 1.98 (2 s, 6 H, 2 × CH_3CO). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 207.25 (CH_2*C*O), 172.75 (2 C, CH_2*C*OO, CH_3*C*O), 169.64 (CH_3*C*O), 165.81 (Ph*C*O), 138.12–125.14 (PhCO, *Ph*CH, 3 × *Ph*CH_2, =*C*H), 117.54 (=*C*H_2), 100.60 (C-1^D), 95.89 (C-1^B), 82.79, 77.65, 75.07, 73.54, 72.43, 71.51, 70.63 (9 C, 3 × PhCH_2, C-2^{B,D},3^{B,D},4^{B,D}), 69.20, 68.33, 68.21 (5 C, *C*H_2CH=CH_2, C-6^{B,D},5^{B,D}), 38.26 (*C*H_2CO), 29.56 (*C*H_3CO), 28.32 (*C*H_2COO), 20.88 (*C*H_3CO). ESI HR-MS ($C_{50}H_{56}O_{15}$): m/z = found ($[M+K]^+$ 935.3203; calc 935.3256); found $[M+Na]^+$ 919.3473; calc 919.3513).

*Allyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→6)-4-*O*-acetyl-2-O-benzoyl-3-*O*-levulinoyl-α-D-glucopyranoside 10b.* A solution of the disaccharide **10a** (1.75 g, 1.95 mmol) in 9:1 Ac_2O-pyridine (50 mL) was stirred

overnight. After TLC (1:1 hexane-EtOAc) showed the reaction was complete, the mixture was co-concentrated with toluene and the residue purified with hexane-EtOAc to give compound **10b** in theoretically quantitative yield (1.92 g). $[\alpha]_D = +65^\circ$ (c = 0.3; $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.02–7.16 (m, 20 H, 3 × *Ph*CH_2, *Ph*CO), 5.82–5.65 (m, 2 H, CH_2CH=CH_2, incl. t, 5.71, J = 9.8 Hz, H-3^B), 5.24–5.10 (m, 3 H, CH_2CH=CH_2, incl. d, 5.20, $J_{1,2}$ = 3.6 Hz, H-1^B), 5.02–4.97 (m, 3 H, H-2^B, 2^D, 4^B), 5.79–4.52 (m, 6 H, 3 × PhCH_2), 4.40 (d, 1 H, $J_{1,2}$ = 7.7 Hz, H-1^D), 4.20–4.15 (m, 1 H, CH_{2a}CH=CH_2), 4.05–4.01 (m, 1 H, H-5^B), 3.99–3.97 (m, 2 H, CH_{2b}CH=CH_2, H-6a^B), 3.76–3.60 (m, 4 H, H-3^D,4^D,6b^B,6a^D), 3.53–3.49 (m, 2 H, H-5^D,6b^D), 2.69–2.32 (m, 4 H, CH_2CH_2), 2.07, 1.98 (2 s, 9 H, 3 × CH_3CO). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 205.81 (CH_2CO), 171.69, 170.00 (2 C, CH_2COO, CH_3CO), 169.45, (2 × CH_3CO), 165.64 (Ph*C*O), 138.09–127.60 (PhCO, *Ph*CH, 3 × *Ph*CH_2, =*C*H), 117.65 (=*C*H_2), 101.10 (C-1^D), 94.47 (C-1^B), 82.85, 77.77 (C-3^D,4^D), 75.10 (5^D), 75.00, 74.97, 73.40 (3 × PhCH_2), 72.86, 71.57 (3 C, C-2^B,2^D,4^B), 70.15 (C-3^B), 68.70 (2 C, C-3^D,4^D), 68.53 (*C*H_2CH=CH_2), 68.49 (C-5^B), 68.07 (C-6^D), 67.86 (C-6^B), 37.66 (*C*H_2CO), 29.64 (*C*H_3CO), 27.90 (*C*H_2COO), 20.88, 20.69 (2 × *C*H_3CO). ESI HR-MS ($C_{50}H_{61}O_{21}$): m/z = found ([M+K]$^+$ 977.3340; calc 977.3362), found ([M+Na]$^+$ 961.3647; calc 961.3627).

*2-*O*-Acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→6)-4-*O*-acetyl-2-O-benzoyl-3-*O*-levulinoyl-α-D-glucopyranosyl trichloroacetimidate 11b.* The allyl sugar **10b** (1.8 g, 1.91 mmol) was dissolved in 20:1 AcOH/H_2O (25 mL); then NaOAc (4.2 g, 30.5 mmol) and $PdCl_2$ (288 mg, 1.91 mmol) were added. The reaction was monitored by TLC (1:1 hexane/EtOAc). The mixture was stirred for 24 h, when TLC showed the reaction was complete. The mixture was diluted with CH_2Cl_2 and filtered over celite, washed with satd $NaHCO_3$ until neutralization, dried ($MgSO_4$), filtered, and concentrated. Purification by flash chromatography (hexane-EtOAc) gave 1.35 g of product **11a** (80%), which was used for the next step.

The foregoing 1-OH disaccharide **11a** (1.3 g, 1.52 mmol) was dissolved in CH_2Cl_2 (20 mL) and CCl_3CN (2 mL), to which DBU (60 μL) was added. After 30 min the reaction was complete (1:1 hexane-EtOAc). The mixture was concentrated and after purification with hexane-EtOAc, 1.4 g of product **11b** (92%) was obtained. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.54 (s, 1 H, NH), 7.98–7.16 (m, 20 H, 3 × *Ph*CH_2, *Ph*CO), 6.68 (d, 1 H, $J_{1,2}$ = 3.6 Hz, H-1^B), 5.79 (t, 1 H, J = 9.6 Hz, H-3^B), 5.29 (dd, 1 H, H-2^B), 5.18 (t, 1 H, J = 10.2 Hz, H-2^D), 4.99 (t, 1 H, J = 9.0 Hz, H-4^B), 4.80–4.52 (m, 6 H, 3 × PhCH_2), 4.41 (d, 1 H, $J_{1,2}$ = 8.1 Hz H-1^D), 4.24–3.49 (m, 8 H, H-3^D,4^D,5^{B,D},6a^{B,D},6b^{B,D}), 2.70–2.35 (m, 4 H, CH_2CH_2), 2.07, 2.04, 1.98 (3 s, 9 H, 3 × CH_3CO). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 205.78 (CH_2CO), 171.73 (2 C, CH_2COO, CH_3CO), 169.82, 169.56 (2 × CH_3CO), 165.28 (Ph*C*O), 160.43 (O*C*ONH), 138.14–126.91 (PhCO, 3 × *Ph*CH_2), 100.84 (2 C, C-1^{B,D}), 92.85 (q, CCl_3), 82.85, 77.78, 75.15, 75.00, 73.446,

72.74, 71.55, 70.43 (9 C, 3 × PhCH_2, C-$2^{B,D}$,$3^{B,D}$,$4^{B,D}$), 69.74, 68.54, 67.89, 67.23 (5 C, $CH_2CH{=}CH_2$, C-$5^{B,D}$,$6^{B,D}$), 37.62 (CH_2CO), 29.46 (CH_3CO), 27.64 (CH_2COO), 20.89, 20.66 (2 × CH_3CO). ESI HR-MS ($C_{50}H_{54}Cl_3NO_{15}$): m/z = found ($[M{+}Na{+}H]^{2+}$ 1065.2462; calc 1065.2482).

*Methyl [2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→6)-4-*O*-acetyl-2-O-benzoyl-β-D-glucopyranoyl]-(1→3)-2-O-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranoside 13b.* Reaction of the methyl glycoside **12**[20] and trichloroacetimidate donor **11b** gave the trisaccharide **13a** (73%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.02–7.18 (m, 30 H, 3 × *Ph*CH_2, 2 ×*Ph*CO, *Ph*CH), 5.64 (s, 1H, *Ph*CH), 5.21 (t, 1 H, J = 8.4 Hz, H-3^B), 5.16 (d, 1 H, $J_{1,2}$ = 8.7 Hz, H-1^B), 4.94 (t, 1 H, J = 9.0 Hz, H-2^A), 4.84–4.73 (m, 3 H, H-$2^{B,D}$,4^B), 4.69–4.32 (m, 9 H, 3 × PhCH_2, $6a^A$, incl. d, 4.44, $J_{1,2}$ = 8.1 Hz, H-1^D; d, 4.44, $J_{1,2}$ = 7.8 Hz, H-1^A), 4.24–3.49 (m, 12 H, H-$3^{A,D}$,$4^{A,D}$,$5^{A,B,D}$,$6a^{B,D}$,$6b^{A,B,D}$), 3.67 (s, 3 H, OCH_3), 2.04, 1.99 (2 s, 6 H, 2 × CH_3).

The delevulinoylation procedure was applied to 3-*O*-Lev disaccharide **13a,** yielding product **13b** (91%). $[\alpha]_D = +55^\circ$ (c = 0.3; $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.59–7.14 (m, 30 H, 3 × *Ph*CH_2, 2 × *Ph*CO, *Ph*CH), 5.62 (s, 1H, *Ph*CH), 5.31–4.75 (m, 5 H, H-1^B,$2^{A,B,D}$,4^B), 4.65–4.22 (m, 4 H, 3 × PhCH_2, H-$1^{A,D}$), 4.37 (dd, 1 H, $J_{5,6a}$ = 4.8, $J_{6a,6b}$ = 10.2 Hz, H-$6a^A$), 4.30 (t, 1 H, J = 9.0 Hz, H-3^A), 4.04–3.35 (m, 15 H, H-$3^{B,D}$,$4^{A,D}$,$5^{A,B,D}$,$6a^{B,D}$,$6b^{A,B,D}$, incl. s, 3.41, OCH_3), 2.07, 2.00 (2 s, 6 H, 3 × CH_3). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 170.51 169.57 (2 × CH_3CO), 166.20 164.43 (2 × Ph*C*O), 138.45–125.07 (2 × PhCO, *Ph*CH, 3 × *Ph*CH_2), 102.11 (PhC*H*), 100.89, 100.58, 99.53 (C-$1^{A,B,D}$), 82.53, 78.93, 77.76, 76.36, 74.83, 74.82, 74.40, 73.96, 73.59, 73.29, 72.79, 71.49 (3 × PhCH_2, C-$2^{A,B,D}$,$3^{A,B,D}$,$4^{A,B,D}$), 71.03, 68.47, 68.13, 67.59, 66.43 (6 C, C-$5^{A,B,D}$,$6^{A,B,D}$), 56.80 (OCH_3), 21.31, 20.75 (2 × CH_3CO). ESI HR-MS ($C_{65}H_{68}O_{20}$): m/z = found ($[M{+}Na]^+$ 1191.4202; calc 1191.4243).

*Methyl {[2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl]-(1→3)-[2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl]-(1→6)-2-*O*-benzoyl-4-*O*-acetyl-β-D-glucopyranoyl}-(1→3)-2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranoside 14.* To a stirred solution of acceptor **13b** (150 mg, 0.13 mmol) and donor **9** (96 mg, 0.17 mmol) in CH_2Cl_2 (5 mL) containing activated 4 Å MS, NIS (47 mg, 0.21 mmol) was added at –40°C, under nitrogen atmosphere, followed by TfOH (5 μL). The mixture was stirred for 30 min at –40°C; then TLC (hexane-EtOAc 2:1) showed the reaction was complete. Then the mixture was filtered through a celite pad and partitioned with aq $NaHCO_3$. Chromatography of the concentrated organic layers (hexane-EtOAc) gave 150 mg of product **14** (77%). $[\alpha]_D$ = +45° (c = 0.3; $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.72–7.09 (m, 45 H, 6 × *Ph*CH_2, 2 × *Ph*CO, *Ph*CH), 5.63 (s, 1 H, *Ph*CH), 5.24–4.91 (m, 4 H, H-$2^{A,B,C,D}$), 4.85–4.21 (m, 16 H, 6 × PhCH_2, H-$1^{A,B,C,D}$), 4.37 (dd, 1 H, $J_{5,6a}$ = 4.8, $J_{6a,6b}$ = 10.2 Hz, H-$6a^A$), 4.31–3.35 (m, 22 H, H-$3^{A,B,C,D}$,$4^{A,B,C,D}$,$5^{A,B,C,D}$,$6a^{B,C,D}$,$6b^{A,B,C,D}$,

incl. s, 3.36, OCH_3), 2.07, 2.00, 1.98 (3 s, 9 H, 3 × CH_3). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 169.67, 169.61, 169.44 (3 × CH_3CO), 164.35 164.13 (2 × Ph*C*O), 138.42–126.66 (2 ×PhCO, *Ph*CH, 6 × *Ph*CH_2), 102.06 (PhC*H*), 101.14, 100.88, 100.67, 100.10 (C-$1^{A,B,C,D}$), 82.86, 82.49, 78.75, 78.74, 77.77, 74.98, 74.67, 74.48, 74.46, 74.22, 73.56, 73.35, 73.32, 73.30, 73.29, 73.27, 72.18, 71.93 (20 C, 6 × PhC*H*$_2$, C-$2^{A,B,C,D}3^{A,B,C,D}$,$4^{A,B,C,D}$), 68.75, 68.41, 68.05, 66.45 (8 C, C-$5^{A,B,C,D}$,$6^{A,B,C,D}$), 56.70 (OCH_3), 21.37, 20.38, 20.37 (3 × *C*H_3CO). ESI HR-MS ($C_{94}H_{98}O_{26}$): m/z = found ($[M+Na]^+$ 1665.6268; calc 1665.6244).

*Methyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranoside 15a.* To a stirred solution of acceptor **12** (100 mg, 0.26 mmol) and donor (151 mg, 0.26 mmol) in CH_2Cl_2 (5 mL) containing activated 4 Å MS, NIS (88 mg, 0.4 mmol) was added under nitrogen atmosphere at –60°C followed by TfOH (3 μL). The mixture was stirred for 30 min at –60°C, when it turned red. After 30 min the reaction was finished and one major product was obtained (TLC hexane-EtOAc 2:1). Et_3N (5 μL) was added, the mixture was filtered through a celite pad, and the filtrate concentrated. Chromatography of the residue (98:2 → 95:5 toluene-acetone) gave the disaccharide **15a** (180 mg, 82%). $[\alpha]_D = -6°$ (c = 0.7; $CHCl_3$). ^1H NMR (300 MHz, $CDCl_3$) δ: 8.04–7.10 (m, 25 H, 3 × *Ph*CH_2, *Ph*CO, *Ph*CH), 5.50 (s, 1 H, PhC*H*), 5.27 (t, 1 H, J = 7.8 Hz, H-2^B), 4.93 (dd, 1 H, $J_{1,2}$ = 7.8 Hz, $J_{2,3}$ = 9.6 Hz, H-2^C), 4.68–4.34 (m, 9 H, 3 × PhC*H*$_2$, H-$6a^B$, incl. d, 5.54, H-1^C; d, 4.52, $J_{1,2}$ = 8.4 Hz, H-1^B), 4.15 (t, 1 H, J = 8.7 Hz, H-3^B), 3.84–3.76 (m, 2 H, H-4^B,$6b^B$), 3.66–3.47 (m, 7 H, H-4^C,$5^{B,C}$,$6a^C$, incl. s, 3.47, OCH_3), 3.36–3.30 (m, 2 H, H-$6b^A$, incl. t, 3.33, J = 9.3 Hz, H-3^C), 2.04 (s, 3 H, CH_3CO). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 169.66 (CH_3CO), 164.65 (Ph*C*O), 138.35–125.11 (PhCO, *Ph*CH, 3 × *Ph*CH_2), 102.29 (Ph*C*H), 101.91 (C-1^B), 100.63 (C-1^C), 82.93, 78.94, 77.98, 75.41, 74.98, 73.93, 73.58, 72.53 (C-$2^{B,C}$,$3^{B,C}$,$4^{B,C}$, 3 × Ph*C*H_2), 68.92, 68.64 (C-$6^{B,C}$), 66.70 (2 C, C-$5^{B,C}$), 56.95 (OCH_3), 20.34 (C*H*$_3$CO). ESI HR-MS ($C_{50}H_{52}O_{13}$): m/z = found ($[M+Na]^+$ 883.3266; calc 883.3306).

*Methyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→6)-[2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→3)]-2-*O*-benzoyl-β-D-glucopyranoside 16.* To a solution of disaccharide **15a** (120 mg, 0.14 mmol) in CH_2Cl_2 (5 mL), TFA (100 μL) and water (20 μL) were added. The mixture was stirred overnight, when TLC (9:1 CH_2Cl_2-acetone, 1:1 hexane-EtOAc) showed the reaction was complete. The mixture was concentrated and chromatographed with 95:5 CH_2Cl_2-acetone to yield the debenzylinated disaccharide (95 mg, 88%).

*Methyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-*D*-glucopyranosyl-(1→3)-2-*O*-benzoyl-β-*D*-glucopyranoside* **15b**. $[\alpha]_D = +8°$ (c = 0.3; $CHCl_3$). ^1H NMR (300 MHz, $CDCl_3$) δ: 8.05–7.13 (m, 20 H, 3 × *Ph*CH_2, *Ph*CO), 5.18 (t, 1 H, J = 8.1

Hz, H-2^{B}), 4.95 (t, 1 H, J = 8.1 Hz, H-2^{C}), 4.75–4.42 (m, 8 H, 3 × PhCH_2, incl. 4.46, d, $J_{1,2}$ = 8.4 Hz, H-1^{C}; 4.43, d, $J_{1,2}$ = 8.1 Hz, H-1^{D}), 3.95 (dd, 1 H, $J_{5,6a}$ = 3.3, $J_{6a,6b}$ = 11.7 Hz, H-6a^{B}), 4.82–4.45 (m, 12 H, H-3^{B,C},4^{B,C},5^{B,C},6a^{C},6b^{B,C}, incl. s, 3.47, OCH_3), 1.61 (br. s, 2H, 2 × OH), 1.48 (s, 3 H, CH_3CO). ESI HR-MS ($C_{43}H_{48}O_{13}$): m/z = found ([M+Na]$^+$ 790.3366; calc 790.3412).

To a stirred solution of acceptor **15b** (80 mg, 0.10 mmol) and donor **7** (73 mg, 0.12 mmol) in CH_2Cl_2 (5 mL) containing activated 4 Å MS, NIS (34 mg, 0.15 mmol) was added at –60°C, under nitrogen atmosphere, followed by TfOH (1.5 μL). The mixture was stirred for 30 min at –60°C, when it turned red. After 30 min the reaction was complete (TLC, 9.5:0.5 CH_2Cl_2-acetone, 1:1 hexane-EtOAc). Triethylamine (3 μL) was added, the mixture was filtered through a celite pad, and the filtrate concentrated. Chromatography of the residue (9.5:0.5 toluene-acetone) gave the desired compound **16** (88 mg, 68%). $[\alpha]_D$ = +5° (c = 0.6 $CHCl_3$).^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.04–7.14 (m, 35 H, 6 × *Ph*CH$_2$, *Ph*CO), 5.15 (t, 1 H, J = 8.7 Hz, H-2^{D}), 5.04 (t, 1 H, J = 8.4 Hz, H-2^{B}), 4.94 (t, 1 H, J = 8.1 Hz, H-2^{C}), 4.82–4.28 (m, 16 H, 6 × PhCH_2, H-6a^{B}, incl. d, 4.70, $J_{1,2}$ = 8.1 Hz, H-1^{C}; d, 4.46, $J_{1,2}$ = 8.1 Hz, H-1^{B}; d, 4.34, $J_{1,2}$ = 8.1 Hz, H-1^{D}), 3.76–3.41 (m, 17 H, H-3^{B,C,D},4 ^{B,C,D},5 ^{B,C,D},6a^{C,D},6b^{B,C,D}, incl. s, 3.41, OCH_3), 1.96 (s, 3 H, CH_3CO), 1.55 (s, 3 H, CH_3CO). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 169.44 (2 C, 2 × CH_3CO), 164.67 (Ph*C*O), 138.13–126.88 (PhCO, 6 × *Ph*CH$_2$), 101.65, 101.41, 101.24 (C-1^{B,C,D}), 84.87, 82.77, 77.93, 77.65, 75.69, 75.01, 74.67, 73.57, 73.42, 73.38, 72.20, 71.38 (15 C, C-2^{B,C,D},3^{B,C,D},4^{B,C,D}, 6 × PhCH_2), 69.15, 68.99, 68.70, 68.55 (6 C, C-5^{B,C,D},6^{B,C,D}), 56.59 (OCH_3), 20.87, 20.88 (2 × CH_3CO). ESI HR-MS ($C_{72}H_{77}O_{19}$): m/z = found ([M+Na]$^+$ 1269.5045; calc 1269.5035).

*4,6-*O*-Benzylidene-2-O-benzoyl-3-*O*-levulinoyl-α-D-glucopyranosyl trichloroacetimidate 17b.* Allyl group was removed from sugar **7b** to give **17a** (92%). The foregoing 1-OH sugar **17a** (2.09 g, 4.46 mmol) was dissolved in CH_2Cl_2 (50 mL), to which trichloroacetonitrile (1.45 mL, 13.3 mmol) was added followed by DBU (74 μL, 0.89 mmol). The mixture was stirred for 2 h and monitored by TLC (2:1 hexane-EtOAc). The mixture was concentrated and chromatographed (hexane-EtOAc) to afford 2.1 g of trichloroacetimidate **17b** (84%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.59 (s, 1 H, NH), 8.05–7.26 (m, 10 H, *Ph*CO, *Ph*CH), 6.69 (d, 1 H, $J_{1,2}$ = 3.9 Hz, H-1), 5.89 (t, 1 H, J = 9.6 Hz, H-3), 5.89 (s, 1 H, PhC*H*), 5.37 (dd, 1 H, $J_{2,3}$ =9.9 Hz, H-2), 4.40 (dd, 1 H, $J_{5,6a}$ = 4.8, $J_{6a,6b}$ = 10.2 Hz, H-6a), 4.23–4.08 (m, 1 H, H-5), 3.88 (t, partially overlapped, 1 H, J = 9.9 Hz, H-4), 3.83 (t, partially overlapped, 1 H, J = 10.5 Hz, H-6b), 2.64–2.55 (m, 4 H, CH_2CH_2), 1.98 (s, 3 H, CH_3). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 205.64 (CH_2CO), 171.74 (2 C, CH_2COO, CH_3CO), 165.46 (Ph*C*O), 160.70 (O*C*ONH), 136.56, 134.44, 132.49, 131.06, 129.93, 129.06, 128.86, 127.51, 127.32, 125.01 (PhCO, *Ph*CH), 101.51 (Ph*C*H), 100.94 (C-1), 93.34 (q, CCl_3),

78.40 (C-4), 70.93 (2 C, C-2,3), 68.81 (C-6), 65.17 (C-5), 37.85 (CH$_2$CO), 29.53 (CH$_3$), 27.86 (CH$_2$COO). ESI HR-MS ($C_{27}H_{26}Cl_3NO_9$): m/z = found ([M+Na]$^+$ 636.0529; calc 636.0571).

*Methyl 2-*O*-benzoyl-4,6-*O*-benzylidene-3-*O*-levulinoyl-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranoside 18a*. Acceptor **12** and trichloroacetimidate donor **17b** afforded disaccharide **18a** (81%). $[\alpha]_D$ = +2° (c = 0.8; CHCl$_3$).^{1}H NMR (300 MHz, CDCl$_3$) δ: 7.78–7.24 (m, 20 H, 2 × *Ph*CO, 2 × *Ph*CH), 5.58, (s, 1 H, PhC*H*), 5.34 (s, partially overlapped, 1 H, PhC*H*), 5.32 (t, partially overlapped, 1 H, J = 9.0 Hz, H-3^B), 5.26–5.19 (m, 2 H, H-2^A,2^B), 4.95 (d, 1 H, $J_{1,2}$ = 6.9 Hz, H-1^B), 4.49 (d, 1 H, $J_{1,2}$ = 7.2 Hz, H-1^A), 4.38 (dd, 1 H, $J_{5,6a}$ = 4.8, $J_{6a,6b}$ = 10.8 Hz, H-6a^A), 4.24–4.09 (m, 3 H, H-6a^B,6b^A,3^A), 3.88–3.78 (m, 3 H, H-4^{A,B},6b^B), 3.60–3.45 (m, 2 H, H-5^{A,B}), 3.36 (s, 3 H, OCH$_3$), 3.53–3.29 (m, 4 H, CH$_2$CH$_2$), 1.93 (s, 3 H, CH$_3$CO). ^{13}C NMR (CDCl$_3$, 75 MHz) δ: 205.69 (CH$_2$*C*O), 171.68 (2 C, CH$_2$*C*OO, CH$_3$*C*O), 164.81, 164.50 (2 × Ph*C*O), 137.06–124.99 (2 × *Ph*CO, 2 × *Ph*CH), 102.21 (Ph*C*H), 101.33 (Ph*C*H), 101.16 (C-1^B), 100.53 (C-1^A), 79.16, 78.41, 77.89, 73.11, 72.72, 71.93 (C-2^{A,B},3^{A,B},4^{A,B}), 68.54, 68.50, (C-6^{A,B}), 66.29, 66.14 (C-5^{A,B}), 56.81 (OCH$_3$), 37.77 (CH$_2$CO), 29.54 (CH$_3$), 27.88 (CH$_2$COO). ESI HR-MS ($C_{46}H_{46}O_{15}$): m/z = found ([M+Na]$^+$ 861.2739; calc 861.2734).

*Methyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranoside 19*. From 3-*O*-Lev disaccharide **18a** compound **18b** was obtained (85%). $[\alpha]_D$ = –4° (c = 0.1; CHCl$_3$).^{1}H NMR (300 MHz, CDCl$_3$) δ: 7.85–7.26 (m, 20 H, 2 × *Ph*CO, 2 × *Ph*CH), 5.58, 5.37 (2 s, 1 H each, 2 × PhC*H*), 5.25 (t, 1 H, J = 7.5 Hz, H-2^B), 5.12 (t, 1 H, J = 7.2 Hz, H-2^A), 4.97 (d, 1 H, $J_{1,2}$ = 7.2 Hz, H-1^B), 4.52 (d, 1 H, $J_{1,2}$ = 7.2 Hz, H-1^A), 4.39 (dd, 1 H, $J_{5,6a}$ = 4.8, $J_{6a,6b}$ = 10.8 Hz, H-6a^A), 4.24–4.18 (m, 2 H, H-6a^B,3^A), 3.89–3.36 (m, 10 H, H-3^B,4^{A,B},5^{A,B},6b^{A,B}, incl. s, 3.36, 3 H, OCH$_3$).

To a stirred solution of the foregoing disaccharide acceptor **18b** (140 mg, 0.19 mmol) and donor **7** (123 mg, 0.23 mmol) in CH$_2$Cl$_2$ (5 mL) containing activated 4 Å MS, NIS (64 mg, 0.29 mmol) was added, under nitrogen atmosphere, at –40°C followed by TfOH (5 μL). The mixture was stirred for 30 min at –40°C; then TLC (hexane-EtOAc 2:1) showed the reaction was complete. Then the mixture was filtered through a celite pad and partitioned with aq NaHCO$_3$. Chromatography of the concentrated organic layers (hexane-EtOAc) gave 185 mg of product **19** (80%). $[\alpha]_D$ = +5° (c = 0.3; CHCl$_3$). ^{1}H NMR (300 MHz, CDCl$_3$) δ: 7.78–7.05 (m, 35 H, 2 × *Ph*CO, 2 × *Ph*CH, 3 × *Ph*CH$_2$), 5.54 (s, 1 H, PhC*H*), 5.13 (t, partially overlapped, 1 H, J = 8.1 Hz, H-2^A), 5.08 (t, partially overlapped, 1 H, J = 6.0 Hz, H-2^B), 4.94 (s, 1 H, PhC*H*), 4.84 (t, 1 H, J = 8.1 Hz, H-2^C), 4.82 (d, 1 H, $J_{1,2}$ = 5.8 Hz, H-1^B), 4.31 (m, 9 H, 3 × PhC*H*$_2$, H-6a^A, incl. 4.43, d, $J_{1,2}$ = 7.5 Hz, H-1^C; 4.32, d, $J_{1,2}$ = 8.1 Hz, H-1^A), 4.16–4.08

(m, 2 H, H-6a^{B},3^{A}), 3.92–3.33 (m, 15 H, H-3^{B,C}, H-4^{A,B,C},5^{A,B,C},6a^{C},6b^{A,B,C}, incl. s, 3.36, 3 H, OCH_3), 1.94 (s, 3 H, CH_3CO). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 171.68 (CH_3CO), 169.71, 164.63 (2 × Ph*C*O), 138.35–125.29 (2 × *Ph*CO, 2 × *Ph*CH, 3 × *Ph*CH_2), 102.21 (Ph*C*H), 101.61 (Ph*C*H), 100.86 (C-1^{B}), 99.93, 99.47 (C-1^{A,C}), 83.01, 79.01, 78.00, 77.78, 75.41, 74.96, 74.13, 73.83, 72.48, 70.35, (C-2^{A,B,C},3^{A,B,C},4^{A,B,C}, 3 × Ph*C*H_2), 68.71, 68.69, 68.67 (C-6^{A,B,C}), 66.49, 66.30, 66.01 (C-5^{A,B,C}), 56.85 (OCH_3), 21.94 (CH_3CO). ESI HR-MS ($C_{70}H_{70}O_{19}$): m/z = found ([M+Na]$^+$ 1237.4437; calc 1237.4409).

General procedure for deprotection of 13b, 14, 16, and 19

To a solution of oligosaccharide (0.1 mmol) in MeOH, 1 M NaOMe was added until pH was alkaline. The mixture was stirred overnight, then neutralized with Dowex H$^+$. After filtration, the filtrate was concentrated and purified on silica gel (hexane-EtOAc) to afford the deacetylated product (nearly quantitative yield).

The foregoing oligosaccharide (0.1 mmol) was dissolved in MeOH (10 mL) and flown in H-Cube apparatus with 10% Pd-C cartridge (flow = 0.5 mL/min, t = 20°C, time = 4–8 h), monitored by TLC (1:1 EtOAc-MeOH). The mixture was concentrated and purified in silica gel (1:1 EtOAc-MeOH) to afford the deprotected material, which was dissolved in MilliQ water, filtered through a 0.2-μm Nalgene filter, and freeze-dried (75% to 96%).

Compound 1: ^{1}H NMR (400 MHz, D_2O) δ: 4.72 (d, 1 H, $J_{1,2}$ = 8.0 Hz, H-1^{C}), 4.50 (d, 1 H, $J_{1,2}$ = 8.0 Hz, H-1^{D}), 4.41 (d, 1 H, $J_{1,2}$ = 8.0 Hz, H-1^{B}), 4.23–4.20 (m, 1 H, H-6^{D}), 3.92–3.85 (m, 3 H, H-6b^{D}, 6a^{B,C}), 3.76–3.68 (m, 3 H, H-3^{B},6b^{B,C}), 3.66–3.61 (m, 1 H, H-5^{D}), 3.60–3.55 (m, 6 H, H-4^{B,C,D}, incl. s, 3.56, OCH_3), 3.53–3.40 (m, 6 H, H-2^{B},3^{C,D},5^{B,C,D}), 3.38 (t, 1 H, J = 9.2 Hz, H-2^{C}), 3.34 (t, 1 H, J = 9.1 Hz, H-2^{D}). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 103.61 (C-1^{D}), 103.45 (C-1^{C}), 103.35 (C-1^{B}), 84.93 (C-3^{B}), 76.56, 76.44, 76.17, 76.08 (5 C, C-3^{C,D}, C-4^{A,B,C}), 75.15 (C-5^{D}), 73.98 (C-2^{C}), 73.63 (C-2^{D}), 73.29 (C-2^{B}), 70.11 (C-5$^{B/C}$), 69.12 (C-6^{D}), 68.49 (C-5$^{B/C}$), 61.93 (2 C, C-6^{B,C}), 57.98 (OCH_3). ESI HR-MS ($C_{25}H_{44}O_{21}$): m/z = ([M+K]$^+$ 557.1439; calc 557.1484); found ([M+Na]$^+$ 541.1741; calc 541.1745). This compound was previously synthesized,[41] but NMR data were not reported.

Compound 2: [α]$_D$ = –5° (c = 0.5; H_2O). ^{1}H NMR (400 MHz, D_2O) δ: 4.74 (d, 1 H, $J_{1,2}$ = 7.7 Hz, H-1^{C}), 4.72 (d, 1 H, $J_{1,2}$ = 7.5 Hz, H-1^{B}), 4.50 (d, 1 H, $J_{1,2}$ = 8.0 Hz, H-1^{D}), 4.40 (d, 1 H, $J_{1,2}$ = 7.9 Hz, H-1^{A}), 4.22–4.18 (m, 1 H, H-6^{D}), 3.90–3.85 (m, 4 H, H-6b^{D},6a^{A,B,C}), 3.80–3.63 (m, 6 H, H-3^{A,C},6b^{A,B,C},5^{D}), 3.63–3.54 (m, 5 H, H-2^{B},3^{B}, incl. s, 3.56, OCH_3), 3.52–3.37 (m, 9 H, H-2^{A},3^{D},4^{A,B,C,D},5^{B,C,D}), 3.34 (t, 1 H, J = 9.2 Hz, H-2^{C}), 3.29 (t, 1 H, J = 9.1 Hz, H-2^{D}). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 103.62 (C-1^{D}), 103.47 (C-1^{B,C}), 103.35 (C-1^{A}), 85.99 (C-3^{A}), 85.85 (C-3^{B}), 76.68, 76.55 (C-3^{C,D}), 76.26, 76.22, 75.15, 75.18, (C-4^{A,B,C,D}), 74.12 (C-5^{D}), 73.82 (C-2^{C,D}), 73.55 (C-2^{B}), 73.26 (C-2^{A}), (C-2^{B}), 69.91 (C-5$^{A/B/C}$), 69.39 (C-6^{D}), 68.95, 68.73 (C-5$^{A/B/C}$), 61.35 (3 C,

C-6^{A,B,C}), 57.88 (OCH_3). ESI HR-MS ($C_{25}H_{44}O_{21}$): m/z = found ([M+K]$^+$ 719.2016; calc 719.2012); found ([M+Na]$^+$ 703.2325; calc 703.2273).

Compound 3: $[\alpha]_D = -15^\circ$ (c = 0.5; H_2O). ^{1}H NMR (400 MHz, D_2O) δ: 4.68 (d, 1 H, $J_{1,2}$ = 8.1 Hz, H-1^B), 4.50 (d, 1 H, $J_{1,2}$ = 8.1 Hz, H-1^D), 4.40 (d, 1 H, $J_{1,2}$ = 8.1 Hz, H-1^A), 4.23–4.20 (m, 1 H, H-6a^D), 3.94–3.82 (m, 3 H, H-6b^D,6a^{A,B}), 3.75–3.62 (m, 3 H, H-3^B,6b^{A,B}), 3.69–3.63 (m, 1 H, H-5^D), 3.56 (s, 3 H, OCH_3), 3.54–3.38 (m, 9 H, H-2^B,3^{A,D},4^{A,B,D},5^{A,B,D}), 3.35 (t, 1 H, J = 9.1 Hz, H-2^A), 3.29 (dd, 1 H, $J_{3,4}$ = 9.0 Hz, H-2^D). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 103.64 (C-1^D), 103.59 (C-1^B), 103.37 (C-1^A), 86.03 (C-3^B), 76.53, 76.25, 76.12, 76.06, (5 C, C-3^{A,B}, C-4^{A,B,D}), 75.46 (C-5^D), 73.96 (C-2^B), 73.80 (C-2^D), 73.18 (C-2^A), 70.18, 70.23 (C-5$^{A/B}$), 69.39 (C-6^D), 68.98 (C-5$^{A/B}$), 61.34 (2 C, C-6^{A,B}), 57.88 (OCH_3). ESI HR-MS ($C_{19}H_{34}O_{16}$): found ([M+K]$^+$ 557.1472; calc 557.1484); found ([M+Na]$^+$ 541.1721; calc 541.1745).

Compound 4: ^{1}H NMR (400 MHz, D_2O) δ: 4.76 (d, 1 H, $J_{1,2}$ = 8.4 Hz, H-1^C), 4.73 (d, 1 H, $J_{1,2}$ = 7.7 Hz, H-1^B), 4.40 (d, 1 H, $J_{1,2}$ = 8.3 Hz, H-1^A), 3.94–3.88 (m, 3 H, H-6a^{A,B,C}), 3.78–3.69 (m, 5 H, H-3^{A,C},6b^{A,B,C}), 3.56 (s, 3 H, OCH_3), 3.53–3.34 (m, 9 H, H-2^{A,C},3^B,4^{A,B,C},5^{A,B,C}), 3.38 (dd, 1 H, $J_{3,4}$ = 9.3 Hz, H-2^B). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 103.64 (C-1^C), 103.47 (C-1^B), 103.19 (C-1^A), 85.08, 84.85 (C-3^{A,C}), 76.66 (C-3^B), 76.29, 76.21, 76.05 (C-4^{A,B,C}), 74.11, 73.90, 73.49 (C-2^{A,B,C}), 70.23, 68.82, 68.77 (C-5^{A,B,C}), 61.35 (3 C, C-6^{A,B,C}), 57.90 (OCH_3). ESI HR-MS ($C_{19}H_{34}O_{16}$): m/z = ([M+K]$^+$ 557.1513; calc 557.1484); found ([M+Na]$^+$ 541.1711; calc 541.1745). This compound was previously synthesized,[13] but incomplete NMR data were reported.

*Allyl 2-*O*-benzoyl-4,6-*O*-benzylidene-3-*O*-levulinoyl-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranoside 20.* The general procedure for glycosylation was applied to compounds **20** and **17b**. Yield: 63%. $[\alpha]_D = -6^\circ$ (c = 0.1; $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.76–7.25 (m, 20 H, 2 × *Ph*CO, 2 × *Ph*CH), 5.65–5.54 (m, 2 H, CH_2CH=CH_2, incl. s, 5.58, PhC*H*), 5.34, (s, 1 H, PhC*H*), 5.29 (t, partially overlapped, 1 H, J = 9.6 Hz, H-3^B), 5.27 (t, partially overlapped, 1 H, J = 8.2 Hz, H-2^A), 5.22 (t, partially overlapped, 1 H, J = 8.5 Hz, H-2^B), 5.13–5.00 (m, 2 H, CH_2CH=CH_2), 4.94 (d, 1 H, $J_{1,2}$ = 7.2 Hz, H-1^B), 4.61 (d, 1 H, $J_{1,2}$ = 7.8 Hz, H-1^A), 4.37 (dd, 1 H, $J_{5,6a}$ = 4.9, $J_{6a,6b}$ = 10.5 Hz, H-6a^B), 4.24–4.15 (m, 3 H, H-6a^A,3^B,CH_{2a}CH=CH_2), 3.99–3.95 (m, 1 H, CH_{2b}CH=CH_2), 3.87–3.81 (m, 2 H, H-4^B,6b^B), 3.77 (t, partially overlapped, 1 H, J = 9.6 Hz, H-4^A), 3.71 (t, partially overlapped, 1 H, J = 10.2 Hz, H-6b^A), 3.56–3.43 (m, 2 H, H-5^{A,B}), 2.49–2.33 (m, 4 H, CH_2CH_2), 1.94 (s, 3 H, CH_3CO). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 205.69 (CH_2CO), 171.69 (2 C, CH_2COO, CH_3CO), 164.78, 164.47 (2 × Ph*C*O), 137.06–125.99 (2 × *Ph*CO, 2 × *Ph*CH, *C*H=), 117.54 (=*C*H$_2$), 101.34, 101.20 (2 × Ph*C*H), 101.34 (C-1^B), 100.05 (C-1^A), 79.19 (C-4^B), 78.45 (C-3^A), 77.91 (C-4^A), 73.21 (C-2^A), 72.73 (C-2^B), 71.95 (C-3^B), 69.80 (*C*H$_2$CH=CH_2), 68.68 (C-6^B), 68.52 (C-6^A), 66.35

66.35, (C-5^{B}), 66.13, (C-5^{A}), 37.80 (*C*H_2CO), 29.44 (CH_3), 27.90 (*C*H_2COO). ESI HR-MS ($C_{48}H_{48}O_{15}$): m/z = found ([M+Na]$^+$ 887.2902; calc 887.2891).

*Allyl 2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranoside 21b.* Levulinic group was removed from **21a** as described above. Yield: 85%. White crystals from EtOAc, m.p. 156–157°C. $[\alpha]_D = -15^\circ$ (c = 1.0; $CHCl_3$).^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.83–7.26 (m, 20 H, 2 × *Ph*CO, 2 × *Ph*CH), 5.66–5.54 (m, 2 H, CH_2CH=CH_2, incl. s, 5.55, PhC*H*), 5.35, (s, 1 H, PhC*H*), 5.28 (t, 1 H, J = 7.5 Hz, H-2^{A}), 5.15–5.00 (m, 3 H, CH_2CH=CH_2, incl. t, 5.10, J = 7.5 Hz, H-2^{B}), 4.95 (d, 1 H, $J_{1,2}$ = 7.1 Hz, H-1^{B}), 4.63 (d, 1 H, $J_{1,2}$ = 7.5 Hz, H-1^{A}), 4.37 (dd, 1 H, $J_{5,6a}$ = 4.9, $J_{6a,6b}$ = 10.5 Hz, H-6a^{B}), 4.24–4.18 (m, 3 H, H-3^{A},6b^{B}, CH_{2a}CH=CH_2), 3.99–3.95 (m, 1 H, CH_{2b}CH=CH_2), 3.87–3.79 (m, 3 H, H-3^{B},4^{B},6b^{B}), 3.70 (t, partially overlapped, 1 H, J = 10.1 Hz, H-6b^{A}), 3.66 (t, partially overlapped, 1 H, J = 9.2 Hz, H-4^{A}), 3.58–3.52 (m 1 H H-5^{B}), 3.42–3.35 (m, 1 H, H-5^{A}), 2.35 (br. s, 1 H, OH-3^{B}). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 165.56, 164.59 (2 × Ph*C*O), 137.09–125.92 (2 × *Ph*CO, 2 × *Ph*CH, *C*H=), 117.57 (=*C*H_2), 101.63, 101.40 (2 × Ph*C*H), 100.23 (C-1^{B}), 99.95 (C-1^{A}), 80.46 (C-4^{A}), 79.23 (C-4^{B}), 79.91 (C-3^{A}), 75.22 (C-2^{A}), 73.45 (C-2^{B}), 72.57 (C-3^{B}), 69.77 (*C*H_2CH=CH_2), 68.73 (C-6^{B}), 68.58 (C-6^{A}), 66.32 66.35, (C-5^{B}), 66.00, (C-5^{A}). ESI HR-MS ($C_{43}H_{42}O_{13}$): m/z = found ([M+H]$^+$ 767.2756; calc 767.2702); found ([M+Na]$^+$ 789.2518; calc 789.2523); found ([M+K]$^+$ 805.2308; calc 805.2262).

*Allyl 2-*O*-benzoyl-4,6-*O*-benzylidene-3-*O*-levulinoyl-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-benzylidene β-D-glucopyranoside 22a.* The reaction was carried out with **21b** and **17b** according to the general procedure for glycosylation. Yield: 53%. $[\alpha]_D = -2^\circ$ (c = 0.1; $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.86–7.21 (m, 30 H, 3 × *Ph*CO, 3 × *Ph*CH), 5.66–5.53 (m, 1 H, CH_2CH=CH_2), 5.49 (s, 1 H, PhC*H*), 5.40–5.35 (m, 2 H, PhC*H*, H-3^{C}), 5.27 (t, 1 H, J = 8.6 Hz, H-2^{C}), 5.14–5.01 (m, 4 H, CH_2CH=CH_2, H-2^{B}, incl. d, 5.08, $J_{1,2}$ = 7.4 Hz, H-1^{C}), 4.92–4.88 (m, 2 H, H-2^{A}, H-1^{B}), 4.83 (s, 1 H, PhC*H*), 4.52 (d, 1 H, $J_{1,2}$ = 7.5 Hz, H-1^{A}), 4.32 (dd, 1 H, $J_{5,6a}$ = 4.7, $J_{6a,6b}$ = 10.4 Hz, H-6a^{A}), 4.24–3.99 (m, 6 H, H-6a^{B,C}, 6b^{A},3^{A,B},CH_{2a}CH=CH_2), 3.96–3.91 (m, 1 H, CH_{2b}CH=CH_2), 3.77 (t, 1 H, J = 9.5 Hz, H-4^{C}), 3.73–3.51 (m, 5 H, H-4^{B},5^{B,C},6b^{B,C}), 3.46–3.40 (m, 1 H H-5^{A}), 3.35 (t, 1 H, J = 9.2 Hz, H-4^{A}), 2.49–2.46 (m, 4 H, CH_2CH_2), 1.96 (s, 3 H, CH_3CO). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 205.75 (CH_2*C*O), 171.63 (2 C, CH_2*C*OO, CH_3*C*O), 164.97, 164.52, 164.31 (3 × Ph*C*O), 137.20–125.99 (3 × *Ph*CO, 3 × *Ph*CH, *C*H=), 117.44 (=*C*H_2), 101.66, 101.20, 100.59 (3 × Ph*C*H), 100.59 (C-1^{A}), 99.87 (C-1^{C}), 98.42 (C-1^{B}), 78.71 (C-4^{A}), 78.20 (C-4^{B}), 77.80 (C-4^{C}), 77.20 (C-3^{B}), 75.00 (C-3^{A}), 74.00 (C-2^{A}), 72.72 (C-2^{B}), 72.37 (C-2^{C}), 71.95 (C-3^{C}), 69.69, 68.63, 68.51 (4 C, *C*H_2CH=CH_2, C-6^{A,B,C}), 66.30 (C-5^{A}), 66.13, 65.48 (C-5^{B,C}), 37.86 (*C*H_2CO), 29.46 (CH_3), 27.93 (*C*H_2COO).

ESI HR-MS ($C_{68}H_{66}O_{21}$): m/z = found ($[M+Na]^+$ 1218.4049; calc 1218.4097); found ($[M+K]^+$ 1257.3718; calc 1257.3734).

*2-*O*-Benzoyl-4,6-*O*-benzylidene-3-*O*-levulinoyl-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl trichloroacetimidate 23b.* The removal of allyl group in trisaccharide **22a** (1 g, 0.82 mmol) gave compound **23a** (70%).

The 1-OH trisaccharide **23a** (120 mg, 0.1 mmol) was dissolved in CH_2Cl_2 (5 mL), to which trichloroacetonitrile (30 μL, 0.3 mmol) was added followed by DBU (3 μL, 0.02 mmol). The mixture was stirred for 1 h when TLC (2:1 hexane-EtOAc) showed the reaction was finished. The mixture was concentrated and chromatographed (hexane-EtOAc) to furnish 100 mg of trichloroacedimidate **23b** (76%).

^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.47 (s, 1 H, NH), 7.92–7.22 (m, 30 H, 3 × *Ph*CO, 3 × *Ph*CH), 6.43 (d, 1 H, $J_{1,2}$ = 3.7 Hz, H-1^A), 5.57 (s, 1 H, PhC*H*), 5.46–5.41 (m, 2 H, PhC*H*, incl. t, 5.43, J = 9.1 Hz, H-3^C), 5.31 (t, 1 H, J = 8.7 Hz, H-2^C), 5.16–5.11 (m, 2 H, H-2^B, incl. d, 5.15, $J_{1,2}$ = 7.2 Hz, H-1^C), 5.07 (d, 1 H, $J_{1,2}$ = 4.4 Hz, H-1^B), 4.70 (s, 1 H, PhC*H*), 4.62 (dd, 1 H, $J_{2,3}$ = 9.5 Hz, H-2^A), 4.40 (t, 1 H, J = 9.7 Hz, H-3^A), 4.33–3.94 (m, 5 H, H-3^{B,C},6a^{A,B,C}), 4.08–3.88 (m, 2 H, H-5^{A,B}), 3.79 (t, 1 H, J = 9.6 Hz, H-4^C), 3.81–3.56 (m, 5 H, H-4^B,5^C,6b^{A,B,C}), 3.33 (t, 1 H, J = 9.3 Hz, H-4^A), 2.52–2.51 (m, 4 H, CH_2CH_2), 1.96 (s, 3 H, CH_3CO). ESI HR-MS ($C_{67}H_{62}Cl_3NO_{21}$): m/z = found ($[M+Na]^+$ 1344.2778; calc 1344.2778).

*Allyl bis[2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl-(1→3)]-2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranoside 22b.* The general procedure for delevulinoylation was followed with **22a**. Yield: 82%. $[\alpha]_D$ = +76° (c = 0.2; $CHCl_3$).^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.99–7.15 (m, 30 H, 3 × *Ph*CO, 3 × *Ph*CH), 5.67–5.57 (m, 1 H, CH_2CH=CH_2), 5.46 (s, 1 H, PhC*H*), 5.39 (s, 1 H, PhC*H*), 5.18 (t, 1 H, J = 7.9, H-3^C), 5.15–4.99 (m, 5 H, CH_2CH=CH_2, H-2^{B,C}, incl. d 5.07, $J_{1,2}$ = 7.4 Hz, H-1^C), 4.92 (d, 1 H, $J_{1,2}$ = 4.5 Hz, H-1^B), 4.89 (d, 1 H, J = 8.4 Hz, H-2^A), 4.64 (s, 1 H, PhC*H*), 4.50 (d, 1 H, $J_{1,2}$ = 7.7 Hz, H-1^A), 4.28 (dd, 1 H, $J_{5,6a}$ = 4.7, $J_{6a,6b}$ = 10.4 Hz, H-6a^A), 4.22–3.99 (m, 5 H, H-6a^{B,C},3^{A,B},CH_{2a}CH=CH_2), 3.96–3.89 (m, 2 H, CH_{2b}CH=CH_2, incl. t, 3.91, J = 9.0 Hz, H-4^C), 3.69–3.42 (m, 6 H, H-4^B,5^{B,C},6b^{A,B,C}), 3.41–3.36 (m, 1 H, H-5^A), 3.21 (t, 1 H, J = 9.3 Hz, H-4^A). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 171.12 (CH_3CO), 165.68, 164.65, 164.42 (3 × Ph*C*O), 137.20–126.00 (3 × *Ph*CO, 3 × *Ph*CH, *C*H=), 117.38 (=*C*H$_2$), 101.79, 101.64, 100.44 (3 × Ph*C*H), 99.87 (C-1^A), 98.14 (C-1^C), 97.89 (C-1^B), 80.68 (C-4^B), 78.59 (C-4^A), 77.53 (C-4^C), 76.25 (C-3^B), 74.61 (C-3^C), 74.36 (C-3^A), 74.16 (C-2^A), 72.25, 72.32 (C-2^{B,C}), 69.68 (*C*H$_2$CH=CH$_2$), 68.64, 68.51 (3 C, C-6^{A,B,C}), 66.29 (C-5^A), 65.96 (C-5^B). ESI HR-MS ($C_{63}H_{60}O_{19}$): m/z = found ($[M+Na]^+$ 1143.3581; calc 1143.3567).

*Allyl 2-*O*-benzoyl-4,6-*O*-benzylidene-3-*O*-levulinoyl-β-D-glucopyranosyl-(1→3)-tetrakis[2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl-(1→3)]-2-*O*-benzoyl-4,6-*O*-benzylidene β-D-glucopyranoside 24.* Trisaccharide **22a** (1 g, 0.82 mmol) was subjected to allyl group removal according to the general procedure, yielding compound **23a** (70%).

The 1-OH trisaccharide **23a** (120 mg, 0.1 mmol) was dissolved in CH_2Cl_2 (5 mL), to which trichloroacetonitrile (30 μL, 0.3 mmol) was added followed by DBU (3 μL, 0.02 mmol). The mixture was stirred for 1 h when TLC (2:1 hexane-EtOAc) showed the reaction was finished. The mixture was concentrated and chromatographed (hexane-EtOAc) to furnish 100 mg of trichloroacedimidate **23b** (76%).

*2-*O*-Benzoyl-4,6-*O*-benzylidene-3-*O*-levulinoyl-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-benzylidene-α-D-glucopyranosyl trichloroacetimidate* **23b**. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.47 (s, 1 H, NH), 7.92–7.22 (m, 30 H, 3 × *Ph*CO, 3 × *Ph*CH), 6.43 (d, 1 H, $J_{1,2}$ = 3.7 Hz, H-1^{A}), 5.57 (s, 1 H, PhC*H*), 5.46–5.41 (m, 2 H, PhC*H*, incl. t, 5.43, J = 9.1 Hz, H-3^{C}), 5.31 (t, 1 H, J = 8.7 Hz, H-2^{C}), 5.16–5.11 (m, 2 H, H-2^{B}, incl. d, 5.15, $J_{1,2}$ = 7.2 Hz, H-1^{C}), 5.07 (d, 1 H, $J_{1,2}$ = 4.4 Hz, H-1^{B}), 4.70 (s, 1 H, PhC*H*), 4.62 (dd, 1 H, $J_{2,3}$ = 9.5 Hz, H-2^{A}), 4.40 (t, 1 H, J = 9.7 Hz, H-3^{A}), 4.33–3.94 (m, 5 H, H-3^{B,C},6a^{A,B,C}), 4.08–3.88 (m, 2 H, H-5^{A,B}), 3.79 (t, 1 H, J = 9.6 Hz, H-4^{C}), 3.81–3.56 (m, 5 H, H-4^{B},5^{C},6b^{A,B,C}), 3.33 (t, 1 H, J = 9.3 Hz, H-4^{A}), 2.52–2.51 (m, 4 H, CH_2CH_2), 1.96 (s, 3 H, CH_3CO). ESI HR-MS ($C_{67}H_{62}Cl_3NO_{21}$): m/z = found ([M+Na]$^+$ 1344.2778; calc 1344.2778).

Glycosylation of acceptor **22b** with compound **23b** was performed according to the general procedure, except that 0.5 mmol of TMSOTf was used, to afford **24**. Yield: 54%. $[\alpha]_D = -3^\circ$ (c = 0.1, $CHCl_3$) ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.85–7.14 (m, 60 H, 6 × *Ph*CO, 6 × *Ph*CH), 5.66–5.57 (m, 1 H, CH_2CH=CH_2), 5.53 (s, 1 H, PhC*H*), 5.39 (t, 1 H, J = 9.0, H-3^{F}), 5.38 (s, 1 H, PhC*H*), 5.28 (dd, 1 H, $J_{1,2}$ = 8.3, $J_{1,2}$ = 9.3 Hz, H-2^{C}), 5.19 (t, J = 6.3 Hz, H-2^{B}), 5.15–5.02 (m, 3 H, CH_2CH=CH_2, incl. d 5.06, $J_{1,2}$ = 7.7 Hz, H-1^{F}), 4.99 (d, 1 H, $J_{1,2}$ = 6.0 Hz, H-1^{B}), 4.97–4.71 (m, 13 H, 4 × PhC*H*, H-1$^{C\text{-}F}$,2$^{A,C\text{-}F}$), 4.53 (d, 1 H, $J_{1,2}$ = 7.8 Hz, H-1^{A}), 4.23 (dd, 1 H, $J_{5,6a}$ = 4.7, $J_{6a,6b}$ = 10.4 Hz, H-6a^{A}), 4.23–4.06 (m, 9 H, H-3^{A,B},6a$^{B\text{-}F}$, CH_{2a}CH=CH_2), 3.98–3.89 (m, 5 H, CH_{2b}CH=CH_2, H-3$^{C\text{-}F}$), 3.77 (t, 1 H, J = 9.3 Hz, H-4^{F}), 3.73–3.40 (m, 16 H, H-4$^{C\text{-}F}$,5$^{A\text{-}F}$,6b$^{A\text{-}F}$), 3.33 (t, 1 H, J = 8.5 Hz, H-4^{B}), 3.25 (t, 1 H, J = 9.0 Hz, H-4^{A}), 2.52–2.35 (m, 4 H, CH_2CH_2), 1.96 (s, 3 H, CH_3CO). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 205.83 (CH_2CO), 171.69 (2 C, CH_2COO, CH_3CO), 165.06, 164.67, 164.55, 164.49 (6 × Ph*C*O), 137.22–126.06 (6 × *Ph*CO, 6 × *Ph*CH, *C*H=), 117.50 (=*C*H$_2$), 101.96 (Ph*C*H), 101.25 (C-1), 101.13 (Ph*C*H), 100.96 (2 C, C-1), 100.77 (Ph*C*H), 99.94 (C-1^{A}), 99.16 (C-1^{F}), 98.26 (Ph*C*H), 97.28 (C-1^{B}), 96.94 (Ph*C*H), 96.79 (Ph*C*H), 78.79 (C-4^{A}), 78.36

(C-3), 78.27, 77.83, 77.40 (C-4), 74.94 (C-3^{A}), 74.57, 74.27, 74.15, 74.00 (C-3), 73.01 (C-$2^{A,C-F}$), 72.48 (C-$2^{B,F}$), 72.02 (C-3^{F}), 69.79 ($CH_2CH{=}CH_2$), 68.67, 68.58 (6 C, C-6^{A-F}), 66.33, 66.13, 65.64, 65.47 (C-5^{A-F}), 37.92 (CH_2CO), 29.51 (CH_3), 27.98 (CH_2COO). ESI HR-MS ($C_{128}H_{120}NO_{39}$): m/z = found ($[M+K]^+$ 2319.6982; calc 2319.7044); found ($[M+Na]^+$ 2303.7092; calc 2303.7304)

General procedure for deprotection of 22a and 24

The allyl oligosaccharide (0.1 mmol) was dissolved in 90% AcOH (5 mL). After stirring for 6 h at 50°C the solvent was evaporated, and the material was dissolved in MeOH (5 mL), to which a solution of 1 M NaOMe was added until pH was strongly alkaline (TLC 9:1 CH_2Cl_2-MeOH). The mixture was stirred overnight and then concentrated. The residue was purified on a C18 Isolute SPE column with H_2O. Fractions containing the pure desired compound (NMR) were combined and freeze-dried.

Allyl bis[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranoside 25. Yield: 92%. $[\alpha]_D = -28.2°$ (c = 1.9, $CHCl_3$). ^{1}H NMR (400 MHz, D_2O) δ: 6.00–5.91 (m, 1 H, $CH_2CH{=}CH_2$), 5.39–5.26 (m, 2 H, $CH_2CH{=}CH_2$), 4.76 (d, 1 H, $J_{1,2}$ = 8.3 Hz, H-1^{C}), 4.73 (d, 1 H, $J_{1,2}$ = 8.3 Hz, H-1^{B}), 4.52 (d, 1 H, $J_{1,2}$ = 8.3 Hz, H-1^{A}), 4.39–4.35 (m, 1 H, $CH_{2a}CH{=}CH_2$), 4.23–4.18 (m, 1 H, $CH_{2b}CH{=}CH_2$), 3.90 (d, 3 H, $J_{5,6}$ = 12.0 Hz, H-$6a^{A,B,C}$), 3.78–3.66 (m, 5 H, H-$3^{A,C}$,$6b^{A,B,C}$), 3.53 (t, J = 9.3 Hz, H-2^{C}), 3.52–3.35 (m, 7 H, H-2^{A},$4^{A,B,C}$,$5^{A,B,C}$), 3.41–3.31 (m, 3 H, H-2^{B},$3^{A,C}$). ^{13}C NMR (D_2O, 100 MHz) δ: 133.82 ($CH{=}$), 119.40 ($={C}H_2$), 103.36 (C-1^{C}), 103.12 (C-1^{B}), 101.51 (C-1^{A}), 84.96, 84.77, 76.58, 76.20, 76.13, 74.03, 73.82, 73.47, 71.28 ($CH_2CH{=}CH_2$), 70.15, 68.75, 68.70, 61.28 (3 C, C-6^{A-C}). ESI HR-MS ($C_{21}H_{36}NO_{16}$): m/z = found ($[M+Na]^+$ 567.1930; calc 567.1901).

Allyl pentakis[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranoside 26. Yield: 93%. $[\alpha]_D = -22.2°$ (c = 0.1, $CHCl_3$). ^{1}H NMR (400 MHz, D_2O) δ: 6.00–5.91 (m, 1 H, $CH_2CH{=}CH_2$), 5.39–5.26 (m, 2 H, $CH_2CH{=}CH_2$), 4.76 (d, 4 H, $J_{1,2}$ = 8.0 Hz, H-1^{B-E}), 4.73 (d, 1 H, $J_{1,2}$ = 7.8 Hz, H-1^{F}), 4.52 (d, 1 H, $J_{1,2}$ = 8.3 Hz, H-1^{A}), 4.40–4.39 (m, 1 H, $CH_{2a}CH{=}CH_2$), 4.24–4.19 (m, 1 H, $CH_{2b}CH{=}CH_2$), 3.90 (d, 6 H, $J_{5,6}$ = 12.0 Hz, H-$6a^{A-F}$), 3.79–3.68 (m, 11 H), 3.57–3.30 (m, 18 H). ^{13}C NMR (D_2O, 100 MHz) δ: 133.82 ($CH{=}$), 119.40 ($={C}H_2$), 103.40 (C-1^{F}), 103.12 (4 C, C-1^{B-E}), 101.51 (C-1^{A}), 84.94, 84.77, 76.59, 76.20, 76.13, 74.03, 73.89, 73.84, 73.48, 71.26 ($CH_2CH{=}CH_2$), 70.16, 68.75, 68.66, 61.25 (6 C, C-6^{A-F}). ESI HR-MS ($C_{39}H_{67}NO_{31}$): m/z = found ($[M+H]^+$ 1054.3602; calc 1054.3564).

General procedure for thiol-ene reaction with cysteamine

To a solution of allyl oligosaccharide **25** or **26** (0.1 mmol) in CH_2Cl_2 (0.2 mL), cysteamine (0.3 mmol) dissolved in MeOH (0.2 mL) was added, and the mixture was irradiated in a quartz vial under UV for 12 h. The residue was

purified on a G10 Sephadex column run in an Akta GE Instrument, using water for elution. Fractions containing the pure desired compound (NMR) were combined and freeze-dried.

3-(2-Aminoethylthio)propyl bis[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranoside 5. Yield: 61%. ^{1}H NMR (400 MHz, D_2O) δ: 4.76 (d, 1 H, $J_{1,2}$ = 8.3 Hz, H-1^C), 4.74 (d, 1 H, $J_{1,2}$ = 8.1 Hz, H-1^B), 4.49 (d, 1 H, $J_{1,2}$ = 8.0 Hz, H-1^A), 4.04–3.99 (m, 1 H, H-1′a), 3.92 (d, 3 H, $J_{5,6}$ = 12.0 Hz, H-6a^{A,B,C}), 3.82–3.70 (m, 6 H, H-3^{A,C},6b^{A,B,C},1′b), 3.55 (t, 1 H, J = 9.3 Hz, H-2^C), 3.54–3.40 (m, 8 H, H-2^A,3^B,4^{A,B,C},5^{A,B,C}), 3.36 (t, 1 H, J = 9.1 Hz, H-2^B), 3.21 (t, 2 H, J = 6.8 Hz, H-5′), 2.86 (t, 2 H, J = 6.5 Hz, H-4′), 2.70 (t, 2 H, J = 7.1 Hz, H-3′), 1.96–1.90 (m, 2 H, H-2′). ESI HR-MS ($C_{23}H_{43}NO_{16}S$): m/z = found ([M+Na]$^+$ 622.2115; calc 622.2143).

3-(2-Aminoethylthio)propyl pentakis[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranoside 6. Yield: 73%. ^{1}H NMR (400 MHz, D_2O) δ: 4.80–4.74 (m, 5 H, H-1^{B-F}), 4.48 (d, 1 H, $J_{1,2}$ = 7.8 Hz, H-1^A), 4.02–3.96 (m, 1 H, H-1′a), 3.92 (d, 6 H, $J_{5,6}$ = 12.0 Hz, H-6a^{A-F}), 3.81–3.68 (m, 12 H, H-3$^{A,C-F}$,6b^{A-F},1′b), 3.56–3.38 (m, 18 H, H-2$^{A,C-F}$,3^B,4^{A-F},5^{A-F}), 3.34 (t, 1 H, J = 8.8 Hz, H-2^B), 3.19 (t, 2 H, J = 7.1 Hz, H-5′), 2.84 (t, 2 H, J = 6.4 Hz, H-4′), 2.68 (t, 2 H, J = 7.1 Hz, H-3′), 1.95–1.88 (m, 2 H, H-2′). ^{1}H NMR (400 MHz, D_2O) at 50°C showed the following anomeric signals, δ: 5.05 (d, 3 H, $J_{1,2}$ = 8.0 Hz, 3 × H-1), 5.04 (d, 1 H, $J_{1,2}$ = 5.3 Hz, H-1), 4.74 (d, 1 H, $J_{1,2}$ = 8.1 Hz, H-1^A). ESI HR-MS ($C_{41}H_{73}NO_{31}S$): m/z = found ([M+H]$^+$ 1108.3982; calc 1108.3966).

Conjugation of 6 to CRM_{197}

The amino-oligosaccharide **6** was dissolved in DMSO at a 4 mM amino group concentration, and then triethylamine (10 equiv) and adipic acid N-hydroxysuccinimide-diester (10 equiv) were added. The reaction was kept under gentle stirring at rt for 2 h. The activated oligosaccharides were then separated from the reagents by precipitation with 9 volumes of EtOAc, followed by washing of the precipitate 10 times with EtOAc and drying under vacuum.

Compound 27: ESI HR-MS ($C_{51}H_{85}N_2O_{36}S$): m/z = found ([M+H]$^+$ 1333.4535; calc 1333.4603).

Conjugation was carried out by combining the activated oligosaccharides **27** with CRM_{197} at a molar ratio of 30:1 (moles of active ester per moles of protein) in 70 mM phosphate buffer at pH 7.0. After reaction overnight at rt, the conjugate was purified by ammonium sulfate precipitation (500 mg/mL). Pellet was dissolved in 10 mM phosphate buffer at pH 7.0 and again precipitated with ammonium sulfate (500 mg/mL). After repeating this operation twice, the solid was washed twice with a solution of ammonium sulfate (500 mg/mL) in 10 mM phosphate buffer at pH 7.0, and finally reconstituted with 10 mM phosphate buffer at pH 7.0.

MALDI-TOF mass spectra of CRM_{197} and glycoconjugate **6-CRM_{197}** were recorded by an UltraFlex III MALDI-TOF/TOF instrument (Bruker Daltonics) in linear mode and with positive ion detection. The samples for analysis were prepared by mixing 2.5 μL of product and 2.5 μL of Super DHB matrix; 2.5 μL of each mixture was deposited on a samples plate, dried at rt for 10 min, and subjected to the spectrometer.

Determination of carbohydrate content in glycoconjugates

Total saccharide content in glycoconjugates was determined by ionic chromatography using a CarboPac PA1 column (50 mm × 250 mm) coupled to a CarboPac PA1 guard column and connected to a Dionex ICS3000 system. Dialyzed samples, where free oligosaccharide has been already removed, were diluted at 5 μg/mL of saccharide and treated with trifluoroacetic acid (TFA) at a final concentration of 2 M, heated at 100°C for 2 h, dried, dissolved in 0.5 mL of distilled water, and filtered 0.45 μm before the analysis.

The separation was performed with a flow rate of 1 mL/min using isocratic elution of 40 mM NaOH for 12 min, followed by a washing step with 500 mM NaOH for 5 min. The chromatography was monitored using the pulsed amperometric mode with a gold working electrode and an Ag/AgCl reference electrode; a quadruple-potential waveform for carbohydrates was applied. The chromatographic data were processed using Dionex Chromeleon software. Calibration curve, treated as samples, was set up with glucose (Fluka) in the range 0.5 to 10.0 μg/mL.

Immunochemical Evaluation

ELISA analysis

Ninety-six-well Maxisorp plates (Nunc, Thermo Fisher Scientific) were coated overnight at +4°C with laminarin 5 μg per well in 0.05 M bicarbonate buffer at pH 9.6. After coating, the plates were washed three times with 300 μL per well of TPBS (PBS with 0.05% Tween 20, pH 7.4) and blocked with 100 μL/well of 3% BSA (Sigma-Aldrich) for 1 h at 37°C. Subsequently, each incubation step was followed by a triple TPBS wash. Sera, prediluted 1:25–1:100 in TPBS, were transferred into coated-plates (200 μL) and then serially two-fold diluted followed by 2 h incubation at 37°C. Then 100 μL/well of 1:10,000 in TPBS antimouse IgG alkaline phosphatase conjugated (Sigma–Aldrich) were added and plates were incubated for 1 h at 37°C. Plates were then developed for 30 min at RT with 100 μL per well of 1 mg/mL *p*-nitrophenyl phosphate disodium (Sigma–Aldrich) in 1 M diethanolamine (pH 9.8) and read at 405 nm with a microplate spectrophotometer (Biorad).

Antibody titers were defined as reciprocal of sera dilution using an optical density (OD) of 0.2 as cutoff. Statistical analysis was performed using GraphPad Prism software and applying, on logarithmic transformed data, one-way ANOVA followed by Bonferroni's multiple comparison test.

Competitive ELISA. The protocol described above was followed to prepare laminarin-coated plates. The plate was designed to contain (a) a blank column with TPBS alone, without serum and inhibitors, and (b) a column with serum alone, without inhibitors (b0); the other columns contained both, the serum and the inhibitors, which included also laminarin and the not correlated polysaccharide MenC as positive and negative controls, respectively.

The different competitors (compounds **1**, **2**, **3**, **4,** and **6)** were prediluted to obtain the starting concentration of 4 mM and eight 10-fold dilutions were performed on the plate. Meningococcal C polysaccharide was used as negative control.

The competitors at different concentrations starting from 10 mg/mL concentration were mixed with an equal volume of a fixed dilution of anti-laminarin immune serum, followed by 2 h incubation at 37°C. After primary antibody incubation the general protocol described in the ELISA Analysis section was followed.

All OD lectures were subtracted from the mean value of the blank column (b). The inhibition percentage was expressed as follows:

$$\% \text{ inhibition} = [(B0 - ODx)/B0] * 100,$$

where B0 is the mean values of the b0 column (serum without inhibitor) and ODx is the optical density corresponding to each inhibitor concentration.

IC_{50} was defined as the inhibitor concentration resulting in 50% inhibition of the main reaction.

Fitting of inhibition curves and calculation of IC_{50} values were performed on the Graphpad Prism software using the variable slope model (Graphpad Prism Inc.).

Mice immunization

Animal experimental guidelines set forth by the Novartis Animal Care Department were followed in all animal studies performed. Groups of eight female Balb/c mice were immunized at days 1, 14, and 28 with 5 μg carbohydrate antigens (Lam or **6**) or negative control (PBS), both formulated with MF59 and delivered in a volume of 150 μL by subcutaneous injection. One group of eight female Balb/c mice was immunized with a 1-μg dose of antigen **6**. Bleedings were performed at days 0, 27, and 42.

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