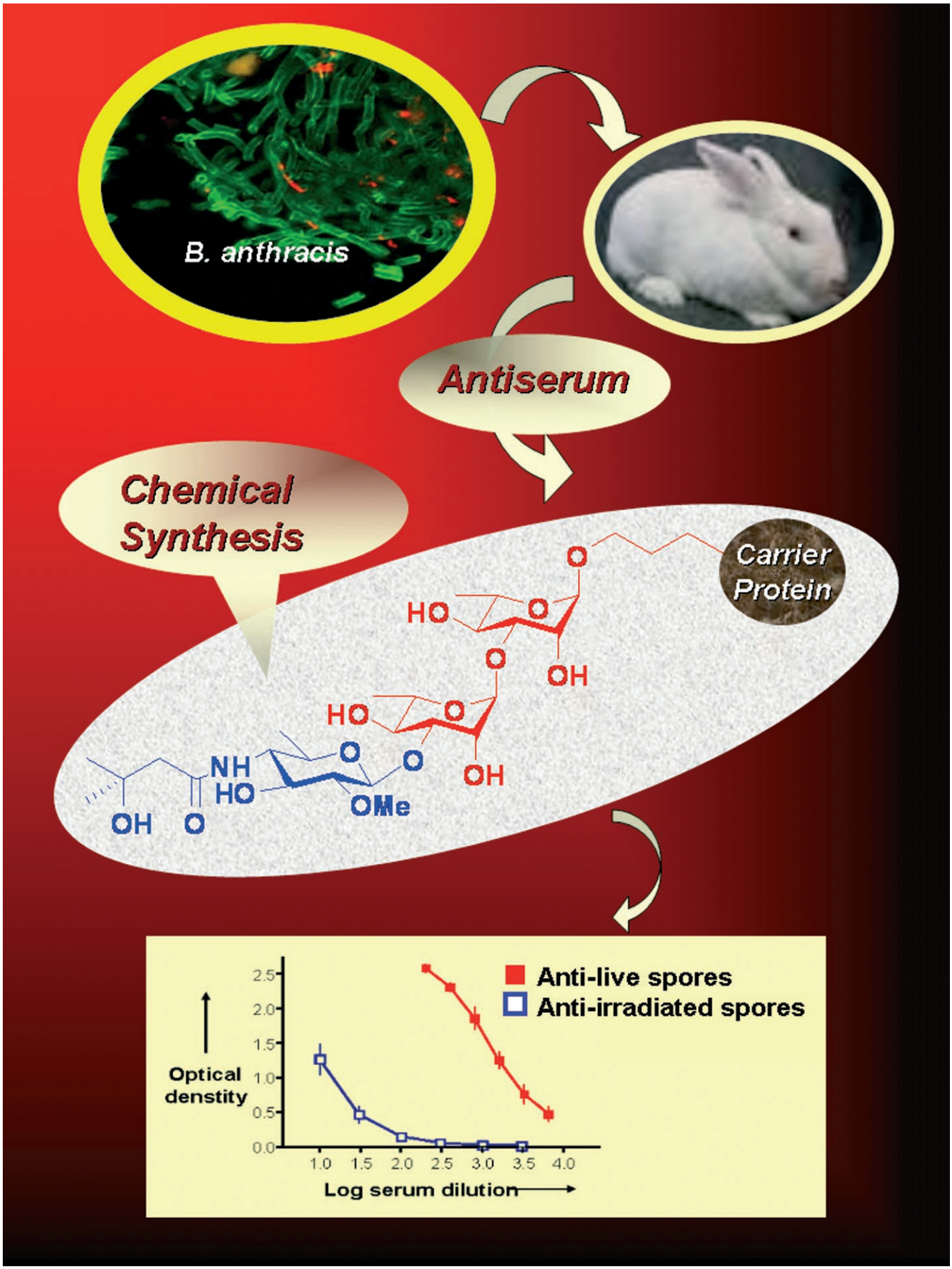
DOI: 10.1002/chem.200601245



Synthesis and Antigenic Analysis of the BclA Glycoprotein Oligosaccharide from the *Bacillus anthracis* Exosporium

Alok S. Mehta,[a, d] Elke Saile,[b, d] Wei Zhong,[a, d] Therese Buskas,[a] Russell Carlson,[a] Elmar Kannenberg,[a] Yvonne Reed,[c] Conrad P. Quinn,*[b] and Geert-Jan Boons*[a]

Abstract: The glycoprotein BclA is an important constituent of the exosporium of *Bacillus anthracis* spores. This glycoprotein is substituted with an oligosaccharide composed of a β-L-rhamnoside substituted with the previously unknown terminal saccharide, 2-*O*-methyl-4-(3-hydroxy-3-methylbutanamido)-4,6-dideoxy-D-glucopyranose, also referred to as anthrose. Anthrose has not been found in spores of *B. cereus* and *B. thuringiensis*, making it a potential species-specific marker for *B. anthracis*. In order to study the antigenicity of anthrose, efficient syntheses of an anthrose-containing trisaccharide and a series of structurally related analogues were developed. The analogues lacked either the methyl ether at C-2 or contained modified C-4 amino functionalities of anthrose. The synthetic compounds were equipped with an aminopropyl spacer to facilitate conjugation to the carrier proteins mariculture Keyhole Limpet Hemocyanin (mcKLH) and bovine serum albumin (BSA). Serum antibodies of rabbits immunized with live or irradiated spores of *B. anthracis* Sterne $34F_2$ were able to recognize the synthetic trisaccharide–mcKLH conjugate. The specificity of the interaction was confirmed by competitive inhibition with the free- and BSA-conjugated trisaccharides. Inhibition using the trisaccharide analogues demonstrated that the isovaleric acid moiety of anthrose is an important structural motif for antibody recognition. These data demonstrate that 1) anthrose is a specific antigenic determinant of the *B. anthracis* Sterne spore; 2) this antigen is presented to the immune system of rabbits receiving the anthrax live-spore vaccine; 3) synthetic analogues of the oligosaccharide retain the antigenic structure; and 4) the antigenic region is localized to specific terminal groups of the oligosaccharide. Collectively these data provide an important proof-of-concept step in the synthesis and development of spore-specific reagents for detection and targeting of non-protein structures in *B. anthracis*.

Keywords: anthrax · glycoconjugates · oligosaccharides · vaccines

Introduction

Bacillus anthracis is a gram-positive, spore-forming bacterium that causes anthrax in humans and other mammals.[1,2] Because of the high resilience of *Bacillus anthracis* spores to extremes of their environment they can persist for many years until encountering a signal to germinate.[3,4] When spores of *B. anthracis* are inhaled or ingested they may germinate and establish populations of vegetative cells which release anthrax toxins, often resulting in the death of the host.[5] The relative ease by which *B. anthracis* may be weaponized and the difficulty in early recognition of inhalation anthrax due to the non-specific nature of its symptoms were demonstrated by the deaths of five people who inhaled spores from contaminated mail.[6–8] Consequently, considerable efforts are being directed towards the development of early disease diagnostics and there is a renewed interest in anthrax vaccines. Sterile, cell-free vaccines containing the

[a] A. S. Mehta, W. Zhong, Dr. T. Buskas, Dr. R. Carlson, Dr. E. Kannenberg, Dr. G.-J. Boons
Complex Carbohydrate Research Center, University of Georgia
315 Riverbend Road, Athens, GA 30602 (USA)
Fax (+1) 706-542-4412
E-mail: gjboons@ccrc.uga.edu

[b] Dr. E. Saile, Dr. C. P. Quinn
Centers for Disease Control and Prevention
Microbial Pathogenesis and Immune Response Laboratory
Meningitis and Special Pathogens Branch, NCID/DBMD
1600 Clifton Road, MS D-11, Atlanta, GA 30333 (USA)
Fax: (+1) 404-639-2835
E-mail: cquinn@cdc.gov

[c] Y. Reed
Centers for Disease Control and Prevention
Scientific Resources Program, 1600 Clifton Road, MS D-11
Atlanta, GA 30333 (USA)

[d] A. S. Mehta, Dr. E. Saile, W. Zhong
These authors contributed equally to this work.

Supporting information for this article is available on the WWW under http://www.chemeurj.org/ or from the author.

protective antigen (PA) component of anthrax toxin have proven safe and effective.[9,10] The anthrax vaccine that provides the most comprehensive protection is, however, the *B. anthracis* Sterne $34F_2$ live-spore vaccine.[11,12] Although not licensed for human use in the US or EU, the live-spore vaccine has proven highly efficacious as a veterinary vaccine and similar live-spore preparations have been used extensively in humans and animals in eastern Europe and Asia.[13] Although these live-spore vaccines may elicit lower anti-toxin antibodies than the licensed cell-free anthrax vaccines, their documented efficacy is attributed to additional adjuvant properties and as yet undefined protective epitopes contributed by the spores or outgrowing vegetative cells.[14] It is feasible, but as yet unexplored, that specific carbohydrate antigens may contribute to the enhanced efficacy of the live spore vaccines.

Spores of *B. anthracis* are enclosed by a prominent loose fitting layer called the exosporium, which consists of a paracrystalline basal layer composed of a number of different proteins and an external hair-like nap.[15–19] The filaments of the nap are formed by the highly immunogenic glycoprotein BclA, which has a long, central collagen-like region containing multiple X-X-Gly repeats where X can be any amino acid.[20] Almost all of the repeating units contain a threonine (Thr) residue, which provides sites for potential glycosylation.[21,22] Recently, it was shown that the BclA glycoprotein contains an *O*-linked saccharide, the structure of which was determined by a combination of NMR spectroscopy and mass spectrometry.[23] The oligosaccharide is probably attached to the protein through a GalNAc moiety, which was lost during the hydrazine-mediated release from the BclA glycoprotein.[23] The structure of the tetrasaccharide is depicted in Figure 1. The previously unknown non-reducing terminal saccharide, 2-*O*-methyl-4-(3-hydroxy-3-methylbutanmido)-4,6-dideoxy-D-glucopyranose, was named anthrose and has not been found in spores of *B. cereus* and *B. thuringiensis*, making it a potential species-specific marker for *B. anthracis*. It may also be a new target for therapeutic intervention or vaccine development.[23]

In this paper, we report the synthesis of an anthrose-containing trisaccharide and a series of structurally related analogs. We demonstrate that 1) serum of rabbits immunized by live or irradiated spores of B. *anthracis* Sterne $34F_2$ recognize the trisaccharide **1**, which is derived from the glycoprotein BclA; 2) the antigenic nature of the trisaccharide can be altered by modification of specific side groups in the terminal glycosyl structure; and 3) a 3-methyl butyryl substituent is essential for recognition by anti-spore antiserum.

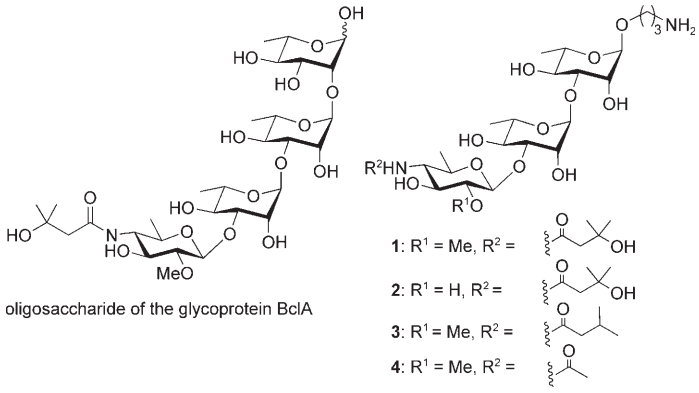


Figure 1. Oligosaccharide of glycoprotein BclA and synthetic targets.

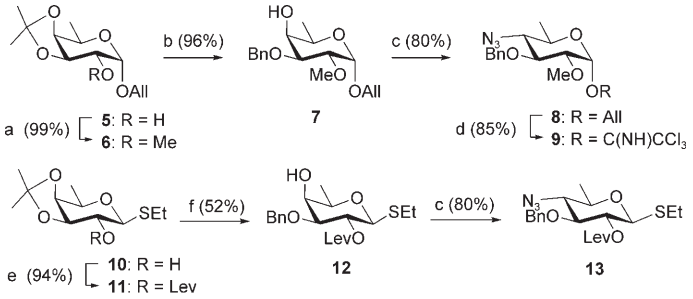
Results and Discussion

Synthesis: To study the immunological properties of the oligosaccharide of BclA, we examined whether antisera from rabbits immunized with live or irradiated spores of *B. anthracis* Sterne $34F_2$ were able to recognize the synthetic anthrose-containing BclA oligosaccharide[24–27] and selected analogues. Although challenging, chemical synthesis offers an opportunity to obtain almost every oligosaccharide target in sufficient quantity and purity for these biological studies. Furthermore, chemical synthesis has the advantage that a target compound can be equipped with an artificial spacer for convenient conjugation to a carrier protein, and offers opportunities for obtaining analogues for structure–activity relationship studies.

Compounds **1**–**4** (Figure 1) were selected as targets for chemical synthesis. Compound **1** is derived from the oligosaccharide of BclA and contains an intact anthrose moiety. Compound **2** lacks the methyl ether at C-2 and derivatives **3** and **4** contain modified C-4 amino functionalities of anthrose. We anticipated that compound **1** conjugated to bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) would be attractive material for determining whether live or irradiated spores of *B. anthracis* Sterne $34F_2$ can induce an anti-carbohydrate antibody response, and derivatives **2**–**4** valuable to examine which chemical moieties of anthrose are critical for binding with antibodies.

Compounds **1**–**4** were synthesized from monosaccharide precursors **14**, **15** and **9** or **13** (Schemes 1 and 2). Thus, glycosyl donor **14**[28] can be coupled with a benzyloxycarbonyl protected amino propyl spacer to give compound **16**, which immediately can be used in a subsequent glycosylation with rhamnoside **15** to give disaccharide **17**. After removal of the levulinoyl (Lev) ester of **17**, the resulting glycosyl acceptor can be coupled with an appropriately protected anthrose donor. The benzoyl ester at C-2 of **15** will ensure that only α-glycosides will be obtained during glycosylation due to neighboring group participation.

The anthrose moieties of target compounds **1**–**4** are linked through a β-glycoside to the C-3 hydroxyl of the rhamnoside. Thus, an obvious strategy to introduce this moiety would be the use of a glycosyl donor which carries a selectively removable ester at C-2. At a late stage of the synthesis, this protecting group can be removed to reveal an alcohol, which can then be methylated. However, this strategy is complicated by the fact that the methylation has to be performed under neutral or mildly acidic conditions due to the presence of a number of base sensitive ester protecting



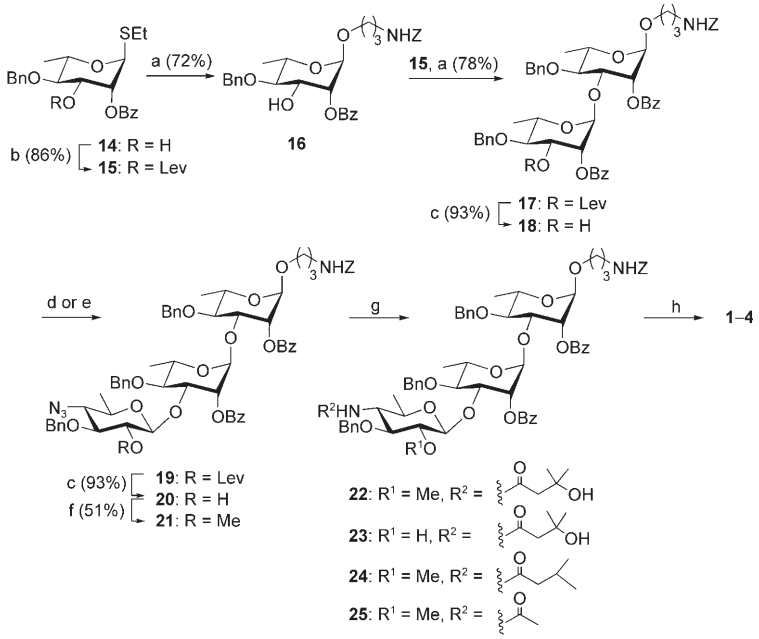
Scheme 1. a) MeI, NaH, DMF, RT; b) 1) 60% HOAc in H_2O, 90°C, 2) Bu_2SnO, MeOH, reflux, 3) CsF, BnBr, DMF, RT; c) 1) Tf_2O, pyridine, CH_2Cl_2, 0°C, 2) NaN_3, DMF, 40°C; d) 1) $PdCl_2$, NaOAc, 90% HOAc/H_2O, RT, 2) trichloroacetonitrile, DBU, CH_2Cl_2, RT; e) levulinic acid, DCC, DMAP, CH_2Cl_2, RT; f) 1) 60% aq. HOAc, 90°C, 2) Bu_2SnO, toluene, reflux, 3) Bu_4NBr, BnBr, toluene, reflux.

groups. In general, such procedures provide relatively low yields of product, especially when applied to a complex compound. Alternatively, the methyl ether can be introduced at the monosaccharide stage by using strongly basic conditions; however, this approach may suffer from the formation of anomeric mixtures during the introduction of the anthrose glycoside. In order to examine both strategies, glycosyl donors **9** and **13** were prepared and coupled with glycosyl acceptor **18**. Compounds **9** and **13** contain an azido moiety at C-4, which at a late stage of the synthesis can be reduced to an amine and then acylated with different reagents to provide compounds **1**–**4**.

Glycosyl donor **9** was synthesized from selectively protected allyl 6-deoxygalactoside **5** (Scheme 1).[29] Thus, methylation of the C-2 hydroxyl of **5** could easily be accomplished by treatment of **5** with methyl iodide in the presence of sodium hydride to give compound **6** in a yield of 99%. The 3,4-*O*-isopropylidene acetal of **6** could easily be removed by using aqueous acetic acid to give a diol, which was selectively benzylated at C-3 to give compound **7**, by first stannylene acetal formation by reaction with dibutyltin oxide in refluxing methanol followed by treatment with benzyl bromide and CsF in DMF.[30,31] Next, an azido group was introduced at C-4 with inversion of configuration to give compound **8** by conversion of the hydroxyl of **7** into a triflate by reaction with triflic anhydride and pyridine followed by displacement with sodium azide in DMF.[32] Fully protected **8** was converted into trichloroacetimidate **9** by removal of the anomeric allyl ether by treatment with $PdCl_2$ and NaOAc followed by reaction of the resulting lactol with trichloroacetonitrile in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU).[33,34]

Glycosyl donor **13** was synthesized from known thioglycoside **10**.[35] Thus, a levulinoyl (Lev) ester at C-2 of compound **10** was installed by treatment with levulinic acid, 1,3-dicyclohexylcarbodiimide (DCC), and 4-(dimethylamino)pyridine (DMAP) in CH_2Cl_2 to give compound **11** in excellent yield.[36] Next, the isopropylidene acetal of **11** was removed by treatment with aqueous acetic acid to give the corresponding diol. Attempts to selectively benzylate the C-3 hydroxyl of this compound by intermediate stannylene acetal formation, using conditions described for the preparation of **7**, gave **12** in a low yield due to cleavage of the Lev ester. However, a moderate yield of **12** was obtained when the stannene acetal formation was performed by refluxing the diol and dibutyltin oxide in toluene followed by treatment with benzyl bromide and tetrabutylammonium bromide (Bu_4NBr). Finally, triflation of **12** followed by nucleophilic displacement with sodium azide gave the required thioglycosyl donor **13**.

Next, attention was focused on the preparation of rhamnosyl acceptor **18** and installment of the anthrose moiety. Thus, an *N*-iodosuccinimide/trifluoromethanesulfonic acid (NIS/TfOH) mediated glycosylation[37] of thioglycosyl donor **14** with benzyloxycarbonyl protected aminopropanol gave spacer modified **16** as only the α-anomer. No self-condensation of **14** was observed due to a much higher glycosyl acceptor reactivity of *N*-benzyloxycarbonylaminopropanol. Compound **16** was immediately used in a second glycosyla-



Scheme 2. a) $HO(CH_2)_3NHZ$, NIS, TfOH, CH_2Cl_2, 0°C; b) levulinic acid, DCC, DMAP, CH_2Cl_2, RT; c) NH_2NH_2·HOAc, MeOH, CH_2Cl_2, RT; d) **13**, NIS, TfOH, CH_2Cl_2, 0°C, 76%; e) **9**, BF_3·Et_2O, MeCN, −40°C, 86% α/β 1:4; f) MeI, Ag_2O, Me_2S, THF, RT; g) 1) 1,3-propanedithiol, TEA, pyridine, H_2O, 2) for **22**, **23**, **24**, HOAt, HATU, DIPEA, RT, 61–76%, for **25**, Ac_2O, pyridine, RT, **22**: 63%, **23**: 78%, **24**: 61%, **25**: 66%; h) 1) NaOMe, MeOH, RT, 2) Pd/C, H_2 (g), *t*BuOH/H_2O/AcOH 40:1:1, RT, **1**: 98%, **2**: 96%, **3**: 94%, **4**: 92%.

tion with glycosyl donor **15**, using NIS/TfOH as the activator to give disaccharide **17** in a good yield. Next, the levulinoyl ester of **17** was selectively removed by treatment with hydrazine acetate,[36] to afford glycosyl acceptor **18** in a yield of 93%. Coupling of trichloroacetimidate **9** with **18** in the presence of $BF_3{\cdot}Et_2O$ in acetonitrile at −40°C gave trisaccharide **21** in a good yield (86%) as a 1:4 mixture of α/β-anomers. In this case, the modest β-selectivity was achieved by the formation of an intermediate α-nitrilium ion.[38,39] Anomerically pure **22** was obtained after reduction of the azido group of **21** to give an amine, which was acylated with 3-hydroxy-3-methyl-butyric acid using *O*-(7-azabenzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate/1-hydroxy-7-azabenzotriazole/diisopropylethylamine (HATU/HOAt/DIPEA) as the activating reagent.

As expected, an NIS/TfOH mediated coupling of thioglycosyl donor **13** with acceptor **18** gave trisaccharide **19** as only the β-anomer due to the neighboring group participating Lev ester at C-2. The Lev group of **19** was selectively removed by treatment with hydrazine acetate[36] and the hydroxyl of the resulting trisaccharide **20** was methylated by treatment with methyl iodide and freshly prepared Ag_2O in the presence of dimethyl sulfide. Despite a prolonged reaction time, the product was obtained in a modest yield of 51%. Thus, the advantage of using glycosyl donor **13** in trisaccharide formation was off-set by a low yielding methylation reaction.

Reduction of the C-4″ azido moiety of **21** followed by the coupling with 3-hydroxy-3-methyl-butyric acid gave compound **22**. Deprotection of **22** could easily be accomplished by a two-step procedure entailing removal of the benzoyl esters using sodium methoxide in methanol, followed by cleavage of the benzyl ethers and benzyloxycarbamate by hydrogenation over Pd/C in a mixture of *tert*-butanol/water/acetic acid.

Analogue **2**, lacking a methyl ether at C-2 of anthrose, was prepared by reduction of the azido group of **20** followed by introduction of the 3-hydroxy-3-methyl-butyric acid moiety and deprotection using standard procedures. Compounds **3** and **4** were obtained by reduction of the azido moiety of **21** followed by acylation of the resulting amine using appropriate reagents to give compounds **24** and **25**, which were deprotected using standard procedures.

Preparation of carbohydrate–protein conjugates: Trisaccharide **1** was linked to the carrier protein mariculture Keyhole Limpet Hemocyanin (mcKLH; Pierce Biotechnology, Rockford, IL) for immunological evaluation. To this end, the amino functionality of trisaccharide **1** was derivatized with an acetyl thioacetic acid moiety by reaction with *S*-acetylthioglycolic acid pentafluorophenyl ester to afford the corresponding thioacetate derivative, which after purification by size-exclusion chromatography, was directly de-*S*-acetylated using 7% ammonia (g) in DMF just prior to conjugation. The de-*S*-acylation was performed under a strict argon atmosphere to prevent formation of the corresponding disulfide. KLH was activated with succinimidyl 3-(bromoacetamido) propionate (SBAP) in a sodium phosphate buffer (pH 7.2) containing 0.15 M sodium chloride and then purified by a centrifugal filter device with a nominal molecular-weight limit of 30 kDa. The bromoacetyl activated KLH (KLH-BrAc) was subsequently incubated with the thiolated trisaccharide in a 0.1 mM sodium phosphate buffer (pH 8.0) containing 5 mM ethylene diamine tetraacetate (EDTA). The afforded glycoconjugate (KLH-BrAc-**1**) carried 1042 copies of trisaccharide **1** per KLH molecule as determined by Lowry's protein concentration test and quantitative carbohydrate analysis by HPAEC-PAD. For the purpose of evaluating the binding specificity of antibodies raised against the *B. anthracis* spores, the thiol derivative of trisaccharide **1** was conjugated to maleimide activated BSA (BSA-MI, Pierce Endogen, Inc.) in a phosphate buffer (pH 7.2) containing sodium azide and EDTA. After a reaction time of 2 h, the glycoprotein was purified using a centrifugal filter device with a nominal molecular weight cut-off of 10 kDa. The average number of trisaccharide copies per BSA molecule was determined to be 18:1. The same conjugation method and thiolated derivatives of trisaccharides **2**, **3**, and **4** were used to give the corresponding BSA-MI-**2**, BSA-MI-**3**, and BSA-MI-**4** glycoconjugates with a sacchride/protein ratio of 10:1, 9:1, and 4:1, respectively.

Antibody binding analyses: To explore the immunogenicity of the saccharide moieties of BclA, rabbits were immunized four times at biweekly intervals with live or irradiated spores of *B. anthracis* Sterne $34F_2$. First, it was investigated whether the post-immune sera have the ability to recognize the synthetic anthrose-containing trisaccharide **1**. For this purpose, an ELISA was performed whereby microtiter plates were coated with the KLH-BrAc-**1** conjugate and serial dilutions of sera added. An anti-rabbit IgG antibody labeled with horseradish peroxidase was employed as a secondary antibody for colorimetric detection (OD, optical density). Binding was observed between the antisera and KLH–trisaccharide conjugate whereas no interaction was detected for native KLH, indicating that the saccharide epitopes of BclA are antigenic (Figure 2a). Rabbits immunized with irradiated Sterne$34F_2$ spores elicited lower but detectable titers of anti-saccharide antibodies. The fact that the irradiated spores elicited IgG antibodies indicates that the saccharide epitopes were not damaged during the irradiation process.

Next, the specificity of the interaction of the antisera with the KLH-BrAc-**1** conjugate was further investigated using a competitive inhibition ELISA. Thus, microtiter plates were coated with the KLH-BrAc-**1** conjugate, and serial dilutions of antisera mixed with free trisaccharide **1** were added. As depicted in Figure 2b, a six-fold excess of trisaccharide **1** (as compared to a concentration of trisaccharide used for coating microtiter wells), resulted in a significant drop in OD at all serum dilutions tested. Also, increasing the excess of the competing trisaccharide **1** resulted in a further reduction in OD. It is evident that the inhibition is dose dependent, thus demonstrating that the interaction of the elicited antibodies

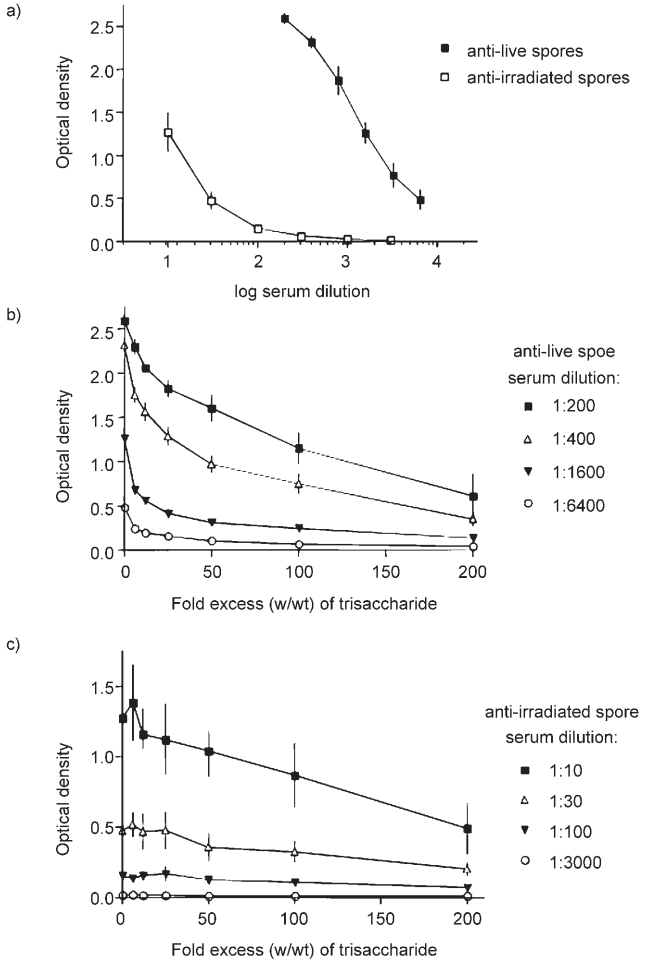


Figure 2. ELISA and competitive inhibition of anti-live and anti-irradiated spore antiserum. Microtiter plates were coated with KLH-BrAc-**1** conjugate (0.5 μg per mL conjugate, corresponding to 0.03 μg per mL trisaccharide). Rabbit anti-live (1:200→1:6400 diluted) or anti-irradiated (1:10→1:3000 diluted) spore *B. antracis* Sterne 34F_2 antisera were applied to coated microtiterplates (a). For the inhibition assay the serum was first mixed with free trisaccharide **1** (0–200-fold excess, wt/wt) (b and c). Unspecific binding was tested with uncoated wells with 200-fold "excess" trisaccharide or 200-fold "excess" KLH (data not shown). The data are reported as the means ±SD of triplicate measurements.

with **1** is specific. The interaction of antisera from rabbits immunized with irradiated spores with **1** could also be inhibited in a dose response manner (Figure 2c).

Having established that Sterne 34F_2 spores are able to induce an anti-carbohydrate antibody response, we sought to further evaluate which structural motifs of the anthrose moiety are critical for antibody recognition. To this end, the ability of BSA-MI-**1** and BSA-MI-conjugates of the three structural analogues **2**, **3**, and **4** to inhibit the interaction of the antisera with KLH-BrAc-**1** was determined (Figure 3). For these experiments, BSA conjugates were employed in an effort to conserve synthetic material. Microtiter plates were again coated with the KLH-BrAc-**1** conjugate and treated with an antisera dilution of 1:1600. The importance of the 2′′-*O*-methyl ether of anthrose was established using the BSA-MI-**2** conjugate. This conjugate carries trisaccharide analogue **2**, which lacks the 2′′-*O*-methyl ether but has an intact *N*-(3-hydroxy-3-methyl-butyryl) moiety at C-4′′. As shown in Figure 3, this conjugate is a potent inhibitor of antibody binding with as low as a 2-fold weight excess eliciting >95% reduction in reporter signal, compared to the BSA-MI-conjugate carrying the native trisaccharide **1**, for which no significant difference in inhibition was observed in the concentration range investigated. These data indicate that the methyl ether is not critical for anti-spore antibody binding. To elucidate the importance of the 3-hydroxy-3-methyl-butyryl moiety of anthrose, conjugates BSA-MI-**3** and BSA-MI-**4** were prepared. Trisaccharide **3** carries a 3-methyl-butyryl moiety at the C-4′′, thus only lacking the hydroxyl group of the native C-4-moiety of the anthrose monosaccharide, whereas trisaccharide **4** is *N*-acetylated at the C-4′′, thus lacking most of the 3-hydroxy-3-methyl-butyryl moiety. Interestingly, a two-fold excess of trisaccharide **3** reduced OD by 85% compared to the control. In contrast, a similar concentration of analogue **4** resulted in reduction in OD of only 17%. Very high concentrations of BSA-MI-**4** were required to achieve considerable inhibition (a 500-fold excess of BSA-MI-**4** resulted in a 50% drop in OD, data not shown). These results indicate that the 4′′-(3-methylbutyryl)-moiety is an important structural motif of the authentic saccharide epitope on the surface of *B. anthracis* Sterne spores.

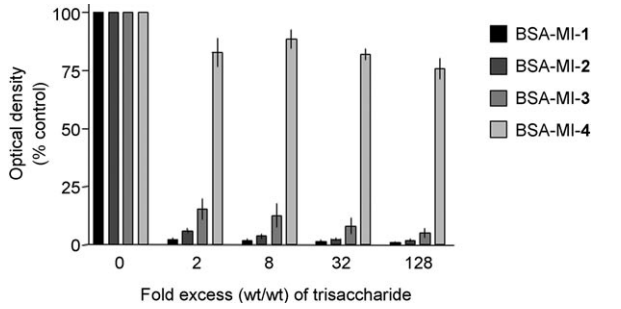


Figure 3. Competitive inhibition of anti-live spore antiserum binding to synthetic anthrose-containing trisaccharide by synthetic analogue conjugates. Microtiter plates were coated with KLH-BrAc-**1** conjugate (0.5 μg per mL conjugate corresponding to 0.03 μg per mL trisaccharide). Rabbit anti-live spore *B. anthracis* Sterne 34F_2 antiserum (1:1600 dilute) was first mixed with BSA–trisaccharide conjugates (0–128-fold excess, wt/wt based on carbohydrate concentration) and then applied to the coated microtiter plate. Unconjugated BSA mixed with antiserum did not have any effect (data not shown). OD values were normalized for the OD values obtained without BSA-trisaccharide conjugate (0-fold "excess", 100%). Non-specific binding was tested with uncoated wells containing antiserum and buffer (data not shown). The data are reported as the means ±SD of triplicate measurements.

Conclusion

The significance of the observations described in this paper is two-fold. First, we have demonstrated that, by using anti-live spore antisera and anti-irradiated spore antisera, the anthrose-containing trisaccharide of BclA is antigenic and exposed on the surface of *B. anthracis* Sterne 34F_2 spores

when presented in rabbits. Second, we have located an important antigenic component of this reactivity in the terminal 3-methyl-butyryl structures of the saccharide and confirmed its specificity using synthetic saccharide analogues. These data provide an important proof-of-concept step in the development of spore-specific reagents for detection and targeting of non-protein structures in *B. anthracis*. These structures may in turn provide a foundation for directing immune responses to spore structures during the early stages of the *B. anthracis* infection process. During the preparation of this manuscript Seeberger and co-workers reported that the anthrax oligosaccharide conjugated to KLH could elicit antibodies that recognize *B. anthracis* spores.[40] Our data are complementary to these findings in that *B. anthracis* spores elicit anti-carbohydrate antibodies, which may be harnessed for diagnosis. Ongoing studies will demonstrate whether these and additional saccharide structures are present and accessible on the spores from other *B. anthracis* isolates, including the highly virulent *B. anthracis* Ames and other B. *anthracis* cured of virulence plasmids pXO1 and pXO2.

Experimental Section

General: ^{1}H NMR spectra were recorded in $CDCl_3$ or D_2O on Varian Merc-300 or Varian Inova-500 spectrometers equipped with Sun workstations at 300 K. TMS (δ_H 0.00) or D_2O (δ_H 4.67) was used as the internal reference. ^{13}C NMR spectra were recorded in $CDCl_3$ or D_2O at 75 MHz on a Varian Merc-300 spectrometer, respectively, using the central resonance of $CDCl_3$ (δ_C 77.0) as the internal reference. COSY, HSQC, HMBC and TOCSY experiments were used to assist signal assignment of the spectra. The different monosaccharide units are referred to as a, b, and c, respectively, with a denoting the reducing end monosaccharide. Mass spectra were obtained on Applied Biosystems Voyager DE-Pro MALDI-TOF (no calibration) and Bruker Daltonics 9.4T (FTICR, external calibration with BSA). Optical rotatory power was obtained on Jasco P-1020 polarimeter at 300 K.

Chemicals were purchased from Aldrich or Fluka and used without further purification. CH_2Cl_2, acetonitrile and toluene were distilled from calcium hydride; THF from sodium; and MeOH from magnesium and iodine. Mariculture keyhole limpet hemocyanin (mcKLH), maleimide activated bovine serum albumin (BSA-MI), and succinimidyl 3-(bromoacetamido)propionate (SBAP) were purchased from Pierce Endogen, Rockford, IL. Aqueous solutions are saturated unless otherwise specified. Molecular sieves were activated at 350 °C for 3 h in vacuo. All reactions were performed under anhydrous conditions under argon and monitored by TLC on Kieselgel 60 F254 (Merck). Detection was by examination under UV light (254 nm) and by charring with 10% sulfuric acid in methanol. Silica gel (Merck, 70–230 mesh) was used for chromatographies. Iatrobeads 6RS-8060 was purchased from Bioscan.

General procedure for levulination: A solution of DCC (6 equiv) and DMAP (0.015 equiv) in CH_2Cl_2 was added under argon to a solution of **10** or **14** (1 equiv) and levulinic acid (10 equiv) in CH_2Cl_2 (at a concentration of 0.06 mol saccharide per L). The reaction mixture was stirred at room temperature for 2 h, and then filtered through Celite. The filtrate was washed twice with water. The organic layer was dried ($MgSO_4$), filtered, and concentrated to dryness. Purification of the crude product by column chromatography on silica gel afforded the desired product **11** or **15**.

General procedure for isopropylidene removal: A solution of **6** or **11** (1 equiv) in acetic acid/water (3:2, at a concentration of 0.5 mol saccharide per L) was heated under reflux at 90 °C for 15 min, and then concentrated to dryness. The residue was co-distilled with toluene twice. Purification of the crude product by column chromatography on silica gel afforded the desired diol product.

General procedure for introduction of the C-4 azide group: Trifluoromethanesulfonic anhydride (1.5 equiv) was added slowly at 0 °C to a solution of **7** or **12** (1 equiv) in pyridine (10 equiv) and dry CH_2Cl_2 (at a concentration of 0.2 mol saccharide per L). The reaction mixture was stirred at 0 °C for 1 h, and then diluted with CH_2Cl_2. The solution was washed with H_2O and saturated $NaHCO_3$. The organic layer was dried ($MgSO_4$), filtered, and concentrated to dryness. To a solution of this residue in DMF (at a concentration of 0.08 mol saccharide per L) was added sodium azide (5 equiv). The reaction mixture was stirred at 40 °C overnight, and then concentrated to dryness. The residue was dissolved in ethyl acetate, and the solution was washed with saturated $NaHCO_3$ and brine. The organic layer was dried ($MgSO_4$), filtered, and concentrated to dryness. Purification of the crude product by column chromatography on silica gel afforded the desired product **8** or **13**.

General procedure for cleavage of the levulinoyl ester: A solution of hydrazine acetate (1 equiv) in dry MeOH (0.4 mol L^{-1}) was added under argon to a solution of **17** or **19** (1 equiv) in dry CH_2Cl_2 (at a concentration of 0.04 mol saccharide per L). The reaction mixture was stirred at room temperature for 4 h, and then concentrated to dryness. The residue was dissolved in CH_2Cl_2, and then washed with water. The organic layer was dried ($MgSO_4$), filtered, and concentrated to dryness. Purification of the crude product by column chromatography on silica gel afforded the desired product **18** or **20**.

General procedure for azide reduction and introduction of C-4″ moiety: TEA (15 equiv) was added to a solution of **20** or **21** (1 equiv) and 1,3-propanedithiol (20 equiv) in pyridine (at a concentration of 0.014 mol saccharide per L) and H_2O (at a concentration of 0.1 mol saccharide per L). The reaction mixture was stirred at room temperature overnight, and then concentrated to dryness. The residue was co-evaporated with toluene twice and ethanol twice. Purification of the crude product by column chromatography on silica gel (CH_2Cl_2/MeOH/TEA 100:5:1) afforded the free amine compounds. β-Hydroxyisovaleric acid or isovaleric acid (2 equiv) was activated by HOAt (4 equiv) and HATU (4 equiv) in DMF (at a concentration of 0.01 mol saccharide per L) for 1 h, and then DIPEA (8 equiv)was added. The resulting yellow solution was added dropwise to the free amine compound (1 equiv) in DMF (at a concentration of 0.02 mol saccharide per L). The reaction mixture was stirred at room temperature for 4 h, and then concentrated to dryness. Purification of the crude product by column chromatography on silica gel afforded the desired product **22**, **23** or **24**. Alternatively, a solution of free amine (1 equiv) in Ac_2O (2 equiv), pyridine (2 equiv) and DMAP (0.1 equiv) was stirred at room temperature overnight, and then concentrated to dryness. The residue was co-evaporated with toluene twice. Purification of the crude product by column chromatography on silica gel afforded the desired product **25**.

General precedure for global deprotection: NaOMe (pH 8–10) was added to a solution of **22**, **23**, **24** or **25** in dry MeOH (at a concentration of 0.06 mol saccharide per L). The reaction mixture was stirred at room temperature overnight, and then neutralized by the addition of Dowex 650 H^+. The suspension was filtered through Celite, and washed with MeOH/CH_2Cl_2 1:1. The combined filtrates were concentrated to dryness. Purification of the crude product by column chromatography on silica gel afforded the desired deacetylated product. To a solution of the partially deprotected compound in *tert*-butanol/H_2O/AcOH (40:1:1, 0.01 mol L^{-1}) was added Pd/C (cat.) under an atmosphere of hydrogen. The reaction mixture was stirred at room temperature overnight, and then filtered through Celite. The filtrate was concentrated to dryness. Purification of the crude product by Iatro beads afforded the desired product **1**–**4**.

Allyl 3,4-*O*-isopropylidene-2-*O*-methyl-α-D-fucopyranoside (6): NaH (3.25 g, 67.63 mmol, 50% in mineral oil) was added to a solution of **5** (8.26 g, 33.81 mmol) in DMF (90 mL). The reaction mixture was stirred at 0 °C for 1 h, and then methyl iodide (4.21 mL, 67.62 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 6 h, and then poured into ice water. The solution was extracted with CH_2Cl_2 (100 mL) and washed with water (100 mL). The organic layer was dried

($MgSO_4$), filtered, and concentrated to dryness. Purification of the crude product by column chromatography on silica gel (hexane/EtOAc 4:1) afforded the desired product **6** as colorless oil (8.66 g, 99%). R_f=0.74 (hexane/EtOAc 2:1); $[\alpha]_D^{27}$=+67.7 (c=3.6 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.26 (d, 3H, $J_{5,6}$=6.3 Hz, H-6), [1.29, 1.47, 2×s (CH_3CCH_3)], 3.30 (dd, 1H, $J_{1,2}$=3.6, $J_{2,3}$=8.1 Hz, H-2), 3.44 (s, 3H, OCH_3), 3.94–4.10 (m, 3H, H-4, H-5, OCH_2CHCH_2), 4.14 (dd, 1H, J= 5.4, 12.9 Hz, OCH'_2CHCH_2), 4.18 (dd, 1H, $J_{2,3}$=8.1, $J_{3,4}$=5.7 Hz, H-3), 4.88 (d, 1H, $J_{1,2}$=3.6 Hz, H-1), 5.16 (dd, 1H, J=1.2, 10.2 Hz, OCH_2CHCH_2), 5.28 (dd, 1H, J=1.5, 17.1 Hz, $OCH_2CHCH'_2$), 5.87 (m, 1H, OCH_2CHCH_2); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.2 (C-6), [26.3, 28.3 (CH_3CCH_3)], 58.5 (OCH_3), 63.1 (C-5), 68.2 (OCH_2CHCH_2), 75.7 (C-4), 76.0 (C-3), 79.1 (C-2), 95.3 (C-1), 108.7 (CH_3CCH_3), 117.9 (OCH_2CHCH_2), 133.6 (OCH_2CHCH_2); MALDI-TOF/MS: m/z: calcd for $C_{13}H_{22}O_5Na$: 281.1365; found: 281.7 $[M+Na]^+$.

Allyl 3-*O*-benzyl-2-*O*-methyl-α-D-fucopyranoside (7): Treatment of **6** (8.66 g, 33.53 mmol) in acetic acid/water (40.2 mL/26.8 mL) as described in the general procedures gave the diol as a white solid (7.39 g, 33.86 mmol, quantitative). R_f=0.30 (CH_2Cl_2/MeOH 19:1); $[\alpha]_D^{27}$=+4.9 (c=2.5 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.21 (d, 3H, $J_{5,6}$= 6.6 Hz, H-6), 2.56 (s, 1H, OH), 3.40 (s, 3H, OCH_3), 3.47 (dd, 1H, $J_{1,2}$= 3.0, $J_{2,3}$=9.6 Hz, H-2), 3.75 (s, 1H, H-4), 3.89–3.97 (m, 2H, H-3, H-5), 4.00 (dd, 1H, J=6.3, 12.6 Hz, OCH_2CHCH_2), 4.14 (dd, 1H, J=3.6, 12.9 Hz, OCH'_2CHCH_2), 4.99 (d, 1H, $J_{1,2}$=3.0 Hz, H-1), 5.16 (d, 1H, J= 10.5 Hz, OCH_2CHCH_2), 5.28 (d, 1H, J=17.1 Hz, $OCH_2CHCH'_2$), 5.87 (m, 1H, OCH_2CHCH_2); ^{13}C NMR (75 MHz, $CDCl_3$): δ 16.1 (C-6), 57.7 (OCH_3), 65.6 (C-5), 68.2 (OCH_2CHCH_2), 69.4 (C-3), 71.5 (C-4), 77.9 (C-2), 94.5 (C-1), 117.9 (OCH_2CHCH_2), 133.8 (OCH_2CHCH_2); MALDI-TOF/MS: m/z: calcd for $C_{10}H_{18}O_5Na$: 241.1052; found: 241.7 $[M+Na]^+$.

Dibutyltin oxide (8.43 g, 33.86 mmol) was added to a solution of the diol (7.39 g, 33.8 mmol) in dry MeOH (300 mL). The reaction mixture was heated under reflux until the solution became clear. After cooling to room temperature, the reaction mixture was concentrated to dryness.

Benzyl bromide (4.0 mL, 33.86 mmol) and CsF (5.15 g, 33.86 mmol) were added to a solution of the residue in DMF (130 mL). The reaction mixture was stirred at room temperature overnight, and then concentrated to dryness. The residue was dissolved in CH_2Cl_2 (100 mL), and the solution was washed with H_2O (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated to dryness. Purification of the crude product by column chromatography on silica gel (hexane/EtOAc 3:1) afforded the desired product **7** as colorless oil (10.03 g, 32.53 mmol, 96%). R_f= 0.34 (hexane/EtOAc 2:1); $[\alpha]_D^{27}$=+86.6 (c=2.0 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.21 (d, 3H, $J_{5,6}$=6.5 Hz, H-6), 3.45 (s, 3H, OCH_3), 3.55 (dd, 1H, $J_{1,2}$=3.5, $J_{2,3}$=9.5 Hz, H-2), 3.59–3.78 (m, 2H, H-3, H-5), 3.86 (dd, 1H, $J_{2,3}$=7.0, $J_{3,4}$=7.0 Hz, H-4), 3.99 (dd, 1H, J=7.0, 13.0 Hz, OCH_2CHCH_2), 4.12 (dd, 1H, J=5.5, 13.0 Hz, OCH'_2CHCH_2), 4.61 (d, 1H, J=12.0 Hz, $PhCH_2$), 4.72 (d, 1H, J=12.0 Hz, $PhCH'_2$), 4.94 (d, 1H, $J_{1,2}$=3.5 Hz, H-1), 5.15 (d, 1H, J=10.5 Hz, OCH_2CHCH_2), 5.26 (d, 1H, J=17.0 Hz, $OCH_2CHCH'_2$), 5.89 (m, 1H, OCH_2CHCH_2), 7.19–7.29 (m, 5H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.1 (C-6), 58.9 (OCH_3), 65.3 (C-4), 68.2 (OCH_2CHCH_2), 70.2 (C-3), 72.7 ($PhCH_2$), 77.5 (C-2), 77.9 (C-5), 95.5 (C-1), 117.9 (OCH_2CHCH_2), [127.7, 127.8, 128.4, 133.9 (C_{arom})], 138.3 (OCH_2CHCH_2); MALDI-TOF/MS: m/z: calcd for $C_{17}H_{24}O_5Na$: 331.1521; found: 331.2 $[M+Na]^+$.

Allyl 4-azido-3-*O*-benzyl-4,6-dideoxy-2-*O*-methyl-α-D-glucopyranoside (8): Treatment of **7** (10.03 g, 32.53 mmol) in pyridine (28.6 mL, 0.33 mol) and CH_2Cl_2 (160 mL) with trifluoromethanesulfonic anhydride (8.2 mL, 48.66 mmol) followed by treatment of triflate residue in DMF (400 mL) with sodium azide (10.40 g, 0.16 mol) was performed according to the general procedure to give compound **8** as colorless oil (8.67 g, 80%). R_f= 0.41 (hexane/EtOAc 5:1); $[\alpha]_D^{27}$=+130.5 (c=2.4 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.30 (d, 3H, $J_{5,6}$=6.5 Hz, H-6), 3.02 (t, 1H, $J_{3,4}$= 9.0, $J_{4,5}$=10.0 Hz, H-4), 3.27 (dd, 1H, $J_{1,2}$=3.5, $J_{2,3}$=9.5 Hz, H-2), 3.44 (s, 3H, OCH_3), 3.52 (m, 1H, H-5), 3.72 (t, 1H, $J_{2,3}$=9.5, $J_{3,4}$=9.0 Hz, H-3), 3.98 (dd, 1H, J=7.0, 13.0 Hz, OCH_2CHCH_2), 4.12 (dd, 1H, J=5.0, 13.0 Hz, OCH'_2CHCH_2), 4.72 (d, 1H, J=10.5 Hz, $PhCH_2$), 4.84 (d, 1H, J=10.5 Hz, $PhCH'_2$), 4.89 (d, 1H, $J_{1,2}$=3.5 Hz, H-1), 5.18 (d, 1H, J= 10.5 Hz, OCH_2CHCH_2), 5.28 (d, 1H, J=17.5 Hz, $OCH_2CHCH'_2$), 5.87 (m, 1H, OCH_2CHCH_2), 7.19–7.35 (m, 5H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 18.4 (C-6), 58.7 (OCH_3), 66.1 (C-5), 67.9 (C-4), 68.2 (OCH_2CHCH_2), 75.5 ($PhCH_2$), 79.8 (C-3), 82.3 (C-2), 94.8 (C-1), 118.3 (OCH_2CHCH_2), [127.8, 128.2, 128.4, 133.6 (C_{arom})], 138.2 (OCH_2CHCH_2); MALDI-TOF MS: m/z: calcd for $C_{17}H_{23}N_3O_4Na$: 356.16; found: 356.7 $[M+Na]^+$.

Ethyl 3,4-*O*-isopropylidene-2-*O*-levulinoyl-1-thio-β-D-fucopyranoside (11): Treatment of **10** (1.34 g, 5.40 mmol) and levulinic acid (5.53 mL, 54.00 mmol) in CH_2Cl_2 (90 mL) with DCC (6.69 g, 32.42 mmol) and DMAP (9.90 mg, 0.081 mmol) in CH_2Cl_2 (9 mL) according to the general procedure gave compound **11** as colorless oil (1.76 g, 94%). R_f= 0.71(hexane/EtOAc 1:1); $[\alpha]_D^{27}$=+1.3 (c=0.7 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.19 (t, 3H, J=7.5 Hz, SCH_2CH_3), 1.28 (s, 3H, CH_3), 1.35 (d, 3H, $J_{5,6}$=7.0 Hz, H-6), 1.49 (s, 3H, CH'_3), 2.12 (s, 3H, $CH_3C(O)CH_2CH_2C(O)O$), 2.53–2.78 (m, 6H, $CH_3C(O)CH_2CH_2C(O)O$, SCH_2CH_3, $CH_3C(O)CH_2CH_2C(O)O$), 3.78–3.82 (m, 1H, H-5), 3.98 (dd, $J_{3,4}$=5.5, $J_{4,5}$=2.5 Hz, H-4), 4.06 (dd, 1H, $J_{2,3}$=7.5, $J_{3,4}$=5.5 Hz, H-3), 4.25 (d, 1H, $J_{1,2}$=10.0 Hz, H-1), 4.92 (dd, 1H, $J_{1,2}$=10.0, $J_{2,3}$=7.5 Hz, H-2); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.7 (SCH_2CH_3), 16.8 (C-6), [23.8, 26.4 (CH_3)], [27.8, 28.1 ($CH_3C(O)CH_2CH_2C(O)O$, SCH_2CH_3)], 29.8 ($CH_3C(O)CH_2CH_2C(O)O$), 38.0 ($CH_3C(O)CH_2CH_2C(O)O$), 71.8 (C-2), 72.7 (C-5), 76.4 (C-4), 77.2 (C-3), 82.2 (C-1), 171.7 ($CH_3C(O)CH_2CH_2C(O)O$), 206.3 ($CH_3C(O)CH_2CH_2C(O)O$); MALDI-TOF/MS: m/z: calcd for $C_{16}H_{26}O_6SNa$: 369.13; found: 369.5 $[M+Na]^+$.

Ethyl 3-*O*-benzyl-2-*O*-levulinoyl-1-thio-β-D-fucopyranoside (12): Treatment of **11** (1.75 g, 5.05 mmol) in acetic acid/water (6.0 mL/4.0 mL) according to the general procedure for isopropylidene removal gave the diol as a white solid (1.55 g, quantitative). R_f=0.38(CH_2Cl_2/MeOH 19:1); $[\alpha]_D^{27}$=−3.5 (c=1.2 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.19 (t, 3H, J=7.5 Hz, SCH_2CH_3), 1.28 (d, 3H, $J_{5,6}$=6.0 Hz, H-6), 2.13 (s, 3H, $CH_3C(O)CH_2CH_2C(O)O$), 2.51–2.86 (m, 6H, $CH_3C(O)CH_2CH_2C(O)O$, SCH_2CH_3, $CH_3C(O)CH_2CH_2C(O)O$), 3.57–3.68 (m, 2H, H-3, H-5), 3.75 (d, J=2.7 Hz, H-4), 4.32 (d, 1H, $J_{1,2}$=9.9 Hz, H-1), 4.98 (t, 1H, $J_{1,2}$=9.9, $J_{2,3}$=9.3 Hz, H-2); ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.8 (SCH_2CH_3), 16.6 (C-6), 23.7 (SCH_2CH_3), 28.2 ($CH_3C(O)CH_2CH_2C(O)O$), 29.8 ($CH_3C(O)CH_2CH_2C(O)O$), 38.4 ($CH_3C(O)CH_2CH_2C(O)O$), [71.5, 71.8 (C-2, C-4)], 73.8 (C-3), 74.7 (C-5), 82.7 (C-1), 172.7 ($CH_3C(O)CH_2CH_2C(O)O$), 206.3 ($CH_3C(O)CH_2CH_2C(O)O$); MALDI-TOF/MS: m/z: calcd for $C_{13}H_{22}O_6SNa$: 329.10; found: 330.2 $[M+Na]^+$.

Dibutyl tin oxide (1.26 g, 5.06 mmol) was added to a solution of the diol (1.55 g, 5.06 mmol) in dry toluene (50 mL). The reaction mixture was heated under reflux with a Dean-Stark apparatus for 3 h, and then cooled to 60°C. Benzyl bromide (0.60 mL, 5.06 mmol) and tetrabutylammonium iodide (1.68 g, 5.06 mmol) were added and the resulting reaction mixture was heated under reflux for 3 h. After cooling to room temperature, the reaction mixture was concentrated to dryness. The residue was dissolved in EtOAc (50 mL), and the resulting solution was washed with H_2O (50 mL). The organic layer was dried ($MgSO_4$) filtered, and concentrated to dryness. Purification of the crude product by column chromatography on silica gel (hexane/EtOAc 2:1) afforded the desired product **12** as colorless oil (1.04 g, 52%). R_f=0.43 (hexane/EtOAc 1:1); $[\alpha]_D^{27}$= −4.0 (c=0.8 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.16 (t, 3H, J=7.5 Hz, SCH_2CH_3), 1.28 (d, 3H, $J_{5,6}$=6.3 Hz, H-6), 2.12 (s, 3H, $CH_3C(O)CH_2CH_2C(O)O$), 2.48–2.76 (m, 6H, $CH_3C(O)CH_2CH_2C(O)O$, SCH_2CH_3, $CH_3C(O)CH_2CH_2C(O)O$), 3.43–3.54 (m, 2H, H-3, H-5), 3.75 (d, J=3.0 Hz, H-4), 4.23 (d, 1H, $J_{1,2}$=9.9 Hz, H-1), 4.57 (d, 1H, J= 12.0 Hz, $PhCH_2$), 4.61 (d, 1H, J=11.1 Hz, $PhCH'_2$), 5.14 (t, 1H, $J_{1,2}$=9.6, $J_{2,3}$=9.6 Hz, H-2), 7.19–7.31 (m, 5H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.7 (SCH_2CH_3), 16.6 (C-6), 23.4 (SCH_2CH_3), 28.1 ($CH_3C(O)CH_2CH_2C(O)O$), 29.9 ($CH_3C(O)CH_2CH_2C(O)O$), 37.9 ($CH_3C(O)CH_2CH_2C(O)O$), [69.1, 69.2 (C-2, C-4)], 71.7 ($PhCH_2$), 74.5 (C-3), 79.7 (C-5), 82.9 (C-1), [127.9, 128.1, 128.5, 137.5 (C_{arom})], 171.7 ($CH_3C(O)CH_2CH_2C(O)O$), 206.3 ($CH_3C(O)CH_2CH_2C(O)O$); MALDI-TOF/MS: m/z: calcd for $C_{20}H_{28}O_6SNa$: 419.15; found: 419.5 $[M+Na]^+$.

Ethyl 4-azido-3-*O*-benzyl-4,6-dideoxy-2-*O*-levulinoyl-1-thio-β-D-glucopyranoside (13): Treatment of **12** (0.50 g, 1.26 mmol) in pyridine (1.0 mL, 12.61 mmol) and CH_2Cl_2 (6.5 mL) with trifluoromethanesulfonic anhydride (0.32 mL, 1.90 mmol) followed by treatment of triflate residue in

DMF (16 mL) with sodium azide (0.41 g, 6.31 mmol) according to the general procedure for introduction of the C-4 azide group gave compound **13** as colorless oil (0.42 g, 79%). R_f=0.32 (hexane/EtOAc 4:1); $[\alpha]_D^{27}$=+13.1 (c=0.5 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.67 (t, 3H, J=7.5 Hz, SCH_2CH_3), 1.30 (d, 3H, $J_{5,6}$=5.7 Hz, H-6), 2.10 (s, 3H, $CH_3C(O)CH_2CH_2C(O)O$), 2.44–2.49 (m, 2H, $CH_3C(O)CH_2CH_2C(O)O$), 2.57–2.67 (m, 4H, SCH_2CH_3, $CH_3C(O)CH_2CH_2C(O)O$), 3.11–3.24 (m, 2H, H-4, H-5), 3.48 (t, $J_{3,4}$=9.0, $J_{2,3}$=9.0 Hz, H-3), 4.27 (d, 1H, $J_{1,2}$=9.9 Hz, H-1), 4.68 (d, 1H, J=11.1 Hz, PhCH_2), 4.71 (d, 1H, J=11.1 Hz, PhCH'_2), 4.94 (dd, 1H, $J_{1,2}$=9.9, $J_{2,3}$=9.0 Hz, H-2), 7.22–7.29 (m, 5H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.8 (SCH_2CH_3), 18.7 (C-6), 23.9 (SCH_2CH_3), 28.0 ($CH_3C(O)CH_2CH_2C(O)O$), 29.8 ($CH_3C(O)CH_2CH_2C(O)O$), 37.8($CH_3C(O)CH_2CH_2C(O)O$), 67.7 (C-4), 72.2 (C-2), 74.9 (PhCH_2), 75.1 (C-5), 82.3 (C-3), 83.2 (C-1), [127.9, 128.0, 128.2, 128.4, 137.5 (C_{arom})], 171.5 ($CH_3C(O)CH_2CH_2C(O)O$), 206.1 ($CH_3C(O)CH_2CH_2C(O)O$); MALDI-TOF/MS: m/z: calcd for $C_{20}H_{27}N_3O_5SNa$: 444.15; found: 444.1 [M+Na]$^+$.

Ethyl 2-*O*-benzoyl-4-*O*-benzyl-3-*O*-levulinoyl-1-thio-α-L-rhamnopyranoside (15): Treatment of **14** (4.93 g, 12.25 mmol) and levulinic acid (12.5 mL, 122.50 mmol) in CH_2Cl_2 (180 mL) with DCC (15.18 g, 73.57 mmol) and DMAP (22.45 mg, 0.18 mmol) in CH_2Cl_2 (18 mL) according to the general procedure for levulination gave compound **15** as colorless oil (5.29 g, 86%). R_f=0.34 (hexane/EtOAc 3:1); $[\alpha]_D^{27}$=−18.9 (c=2.6 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.23 (t, 3H, J=7.2 Hz, SCH_2CH_3), 1.33 (d, 3H, $J_{5,6}$=6.0 Hz, H-6), 2.02 (s, 3H, $CH_3C(O)CH_2CH_2C(O)O$), 2.35–2.39 (td, 2H, J=6.9, 9.6 Hz, $CH_3C(O)CH_2CH_2C(O)O$), 2.46–2.72 (m, 4H, SCH_2CH_3, $CH_3C(O)CH_2CH_2C(O)O$), 3.58 (t, $J_{4,5}$=9.3, $J_{3,4}$=9.6 Hz, H-4), 4.18 (m, 1H, H-5), 4.59 (d, 1H, J=11.1 Hz, PhCH_2), 4.66 (d, 1H, J=11.1 Hz, PhCH'_2), 5.22 (s, 1H, H-1), 5.28 (dd, 1H, $J_{2,3}$=3.3, $J_{3,4}$=9.6 Hz, H-3), 5.51 (dd, 1H, $J_{1,2}$=1.5, $J_{2,3}$=3.3 Hz, H-2), 7.19–8.00 (m, 10H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.9 (SCH_2CH_3), 18.0 (C-6), 25.4 (SCH_2CH_3), 27.9 ($CH_3C(O)CH_2CH_2C(O)O$), 29.7 ($CH_3C(O)CH_2CH_2C(O)O$), 37.8-($CH_3C(O)CH_2CH_2C(O)O$), 68.3 (C-5), 72.5 (C-2), 72.6 (C-3), 74.9 (PhCH_2), 78.9 (C-4), 81.9 (C-1), [127.8, 127.9, 128.4, 128.5, 129.7, 129.8, 133.4, 137.9 (C_{arom})], 165.5 (PhC(O)O), 171.7 ($CH_3C(O)CH_2CH_2C(O)O$), 206.2 ($CH_3C(O)CH_2CH_2C(O)O$); MALDI-TOF/MS: m/z: found: 524.1 [M+Na]$^+$; MALDI-FTICR/MS: m/z: calcd for $C_{27}H_{32}O_7SNa$: 523.1766; found: 523.1761 [M+Na]$^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl 2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (16): Glycosyl donor **14** (3.79 g, 9.42 mmol), 3-(*N*-benzyloxycarbonyl)aminopropanol (3.94 g, 18.83 mmol) and 4 Å powdered molecular sieves (7.73 g) in CH_2Cl_2 (150 mL) in the presence of NIS (2.33 g, 10.36 mmol) and TfOH (0.166 mL, 1.88 mmol) were reacted according to the general procedure for NIS glycosylation to give compound **16** as white solid (3.73 g, 72%). R_f=0.26 (hexane/EtOAc 2:1); $[\alpha]_D^{27}$=+11.3 (c=1.8 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.32 (d, 3H, $J_{5,6}$=6.0 Hz, H-6), 1.73 (m, 2H, $OCH_2CH_2CH_2NHZ$), 2.11 (d, 1H, J=4.5 Hz, OH), 3.24 (dd, 2H, J=6.3, 12.6 Hz, $OCH_2CH_2CH_2NHZ$), 3.36–3.45 (m, 2H, $OCH_2CH_2CH_2NHZ$, H-4), 3.65–3.75 (m, 2H, $OCH'_2CH_2CH_2NHZ$, H-5), 4.12 (dd, 1H, $J_{2,3}$=3.3, $J_{3,4}$=8.4 Hz, H-3), 4.69 (d, 1H, J=11.1 Hz, PhCH_2), 4.76 (s, 1H, H-1), 4.79 (d, 1H, J=11.1 Hz, PhCH'_2), 4.85 (broad, 1H, NH), 5.02 (s, 2H, PhCH_2OC(O)), 5.25 (dd, 1H, $J_{1,2}$=1.5, $J_{2,3}$=3.3 Hz, H-2), 7.18–7.99 (m, 15H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ 18.2 (C-6), 29.6 ($OCH_2CH_2CH_2NHZ$), 38.6 ($OCH_2CH_2CH_2NHZ$), 65.6 ($OCH_2CH_2CH_2NHZ$), 66.6 (C-5), 67.6 (PhCH_2OC(O)), 70.5 (C-3), 73.2 (C-2), 75.2 (PhCH_2), 81.6 (C-4), 97.5 (C-1), [127.9, 128.1, 128.4, 129.7, 129.9, 130.4, 133.3, 136.6, 138.1 (C_{arom})], 156.3 (PhCH$_2$OC(O)), 166.3-(PhC(O)O); MALDI-TOF/MS: m/z: found: 572.9 [M+Na]$^+$; MALDI-FTICR/MS: m/z: calcd for $C_{31}H_{35}NO_8Na$: 572.2260; found: 572.2259 [M+Na]$^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl *O*-(2-*O*-benzoyl-4-*O*-benzyl-3-*O*-levulinoyl-α-L-rhamnopyranosyl)-(1→3)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (17): Glycosyl donor **15** (3.04 g, 6.07 mmol), glycosyl acceptor **16** (3.03 g, 5.51 mmol) and 4 Å powdered molecular sieves (6.07 g) in CH_2Cl_2 (100 mL) in the presence of NIS (1.51 g, 6.71 mmol) and TfOH (0.11 mL, 1.22 mmol) was treated according to the general procedure for the linker glycosylation to give compound **17** as colorless oil (4.26 g, 78%). R_f=0.34 (hexane/EtOAc 2:1); $[\alpha]_D^{27}$=+23.6 (c=1.8 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.11 (d, 3H, $J_{5,6}$=6.0 Hz, H-6b), 1.28 (d, 3H, $J_{5,6}$=6.0 Hz, H-6a), 1.72 (m, 2H, $OCH_2CH_2CH_2NHZ$), 1.99 (s, 3H, $CH_3C(O)CH_2CH_2C(O)O$), 2.32–2.39 (m, 2H, $CH_3C(O)CH_2CH_2C(O)O$), 2.50–2.67 (m, 2H, $CH_3C(O)CH_2CH_2C(O)O$), 3.22 (dd, 2H, J=6.0, 12.3 Hz, $OCH_2CH_2CH_2NHZ$), 3.39–3.49 (m, 2H, $J_{3,4}$=9.6, $J_{4,5}$=9.6 Hz, $OCH_2CH_2CH_2NHZ$, H-4b), 3.56 (t, $J_{3,4}$=9.3, $J_{4,5}$=9.3 Hz, H-4a), 3.62–3.72 (m, 2H, $OCH'_2CH_2CH_2NHZ$, H-5a), 3.84 (m, 1H, H-5b), 4.17 (dd, 1H, $J_{2,3}$=3.0, $J_{3,4}$=9.0 Hz, H-3a), 4.45 (d, 1H, J=11.4 Hz, PhCH_2), 4.50 (d, 1H, J=11.4 Hz, PhCH'_2), 4.66 (d, 1H, J=10.8 Hz, PhCH''_2), 4.80 (broad, 2H, H-1a, NH), 4.95 (d, 1H, J=10.8 Hz, PhCH'''_2), 4.99 (s, 2H, PhCH_2OC(O)), 5.06 (s, 1H, H-1b), 5.29 (d, 1H, $J_{2,3}$=3.3 Hz, H-2a), 5.32 (dd, 1H, $J_{2,3}$=3.0, $J_{3,4}$=9.6 Hz, H-3b), 5.52 (dd, 1H, $J_{1,2}$=1.8, $J_{2,3}$=3.0 Hz, H-2b), 7.05–8.00 (m, 25H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 17.8 (C-6b), 18.2 (C-6a), 28.0 ($CH_3C(O)CH_2CH_2C(O)O$), 29.7 ($OCH_2CH_2CH_2NHZ$, $CH_3C(O)CH_2CH_2C(O)O$)), 37.8($CH_3C(O)CH_2CH_2C(O)O$), 38.5 ($OCH_2CH_2CH_2NHZ$), 65.6 ($OCH_2CH_2CH_2NHZ$), 66.5 (PhCH_2OC(O)), 67.9 (C-5a), 68.6 (C-5b), 70.7 (C-2b), 71.8 (C-3b), 72.7 (C-2a), [73.9, 75.8 (PhCH_2)], 78.2 (C-4b), 79.3 (C-3a), 79.8 (C-4a), 97.0 (C-1a), 99.7 (C-1b), [127.5, 127.7, 127.9, 128.2, 128.3, 128.4, 128.5, 129.5, 129.6, 129.7, 129.8, 133.4, 136.6, 137.9, 138.0 (C_{arom})], 156.3 (PhCH$_2$OC(O)), [165.3, 166.1 (PhC(O)O)], 171.7 ($CH_3C(O)CH_2CH_2C(O)O$), 206.2 ($CH_3C(O)CH_2CH_2C(O)O$); MALDI-TOF/MS: m/z: found: 1011.6 [M+Na]$^+$; MALDI-FTICR/MS: m/z: calcd for $C_{56}H_{61}NO_{15}Na$: 1010.3939; found: 1010.3932 [M+Na]$^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl *O*-(2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranosyl)-(1→3)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (18): Treatment of **17** (4.26 g, 4.31 mmol) in CH_2Cl_2 (100 mL) with hydrazine acetate (397 mg, 4.31 mmol) in MeOH (10 mL) according to the general procedure for cleavage of the levulinoyl ester gave compound **18** as white solid (3.56 g, 93%). R_f=0.42 (hexane/EtOAc 2:1); $[\alpha]_D^{27}$=+21.9 (c=2.2 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.17 (d, 3H, $J_{5,6}$=6.0 Hz, H-6b), 1.26 (d, 3H, $J_{5,6}$=6.0 Hz, H-6a), 1.72 (m, 2H, $OCH_2CH_2CH_2NHZ$), 3.22 (d, 2H, J=6.0 Hz, $OCH_2CH_2CH_2NHZ$), 3.30–3.41 (m, 2H, $J_{3,4}$=9.6, $J_{4,5}$=9.3 Hz, $OCH_2CH_2CH_2NHZ$, H-4b), 3.54 (t, 1H, $J_{3,4}$=9.3, $J_{4,5}$=9.3 Hz, H-4a), 3.62–3.71 (m, 2H, $OCH'_2CH_2CH_2NHZ$, H-5a), 3.78 (dd, 1H, $J_{4,5}$=9.3 Hz, $J_{5,6}$=6.0 Hz, H-5b), 4.04 (dd, 1H, $J_{2,3}$=2.1, $J_{3,4}$=9.6 Hz, H-3b), 4.18 (dd, 1H, $J_{2,3}$=3.0, $J_{3,4}$=9.0 Hz, H-3a), 4.57–4.63 (m, 3H, PhCH_2), 4.77 (s, 1H, H-1a), 4.86 (d, 1H, J=10.8 Hz, PhCH'_2), 4.99 (s, 2H, PhCH_2OC(O)), 5.11 (s, 1H, H-1b), 5.28 (d, 1H, $J_{2,3}$=3.0 Hz, H-2a), 5.33 (dd, 1H, $J_{2,3}$=2.1 Hz, H-2b), 7.12–8.01 (m, 25H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.9 (C-6b), 18.1 (C-6a), 29.5 ($OCH_2CH_2CH_2NHZ$), 38.5 ($OCH_2CH_2CH_2NHZ$), 65.6 ($OCH_2CH_2CH_2NHZ$), 66.5 (PhCH_2OC(O)), 67.9 (C-5a), 68.3 (C-5b), 69.8 (C-3b), 72.8 (C-2b), 73.1 (C-2a), [74.0, 75.6 (PhCH_2)], 77.6 (C-3a), 80.3 (C-4a), 81.1 (C-4b), 97.1 (C-1a), 99.5 (C-1b), [127.7, 127.8, 127.9, 128.2, 128.3, 128.4, 128.5, 129.6, 129.7, 129.8, 133.2, 133.3, 137.8, 138.1 (C_{arom})], 156.3 (PhCH$_2$OC(O)), [165.8, 165.9 (PhC(O)O)]; MALDI-TOF/MS: m/z: found: 913.5 [M+Na]$^+$; MALDI-FTICR/MS: m/z: calcd for $C_{51}H_{55}NO_{13}Na$: 912.3571; found: 912.3559 [M+Na]$^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl *O*-(4-azido-3-*O*-benzyl-4,6-dideoxy-2-*O*-levulinoyl-β-D-glucopyranosyl)-(1→3)-*O*-(2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranosyl)-(1→3)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (19): Glycosyl donor **13** (80 mg, 0.19 mmol), glycosyl acceptor **18** (151 mg, 0.17 mmol) and 4 Å powdered molecular sieves (0.23 g) in CH_2Cl_2 (3 mL) in the presence of NIS (47 mg, 0.21 mmol) and TfOH (3 μL, 0.034 mmol) was treated according to the general procedure for the linker glycosylation to give compound **19** as colorless oil (161 mg, 76%). R_f=0.30 (hexane/EtOAc 2:1); $[\alpha]_D^{27}$=+15.3 (c=0.8 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.89 (d, 3H, $J_{5,6}$=6.5 Hz, H-6c), 1.06 (d, 3H, $J_{5,6}$=6.5 Hz, H-6b), 1.27 (d, 3H, $J_{5,6}$=6.5 Hz, H-6a), 1.73 (m, 2H, $OCH_2CH_2CH_2NHZ$), 1.88 (s, 3H, $CH_3C(O)CH_2CH_2C(O)O$), 1.99–2.10 (m, 2H, $CH_3C(O)CH_2CH_2C(O)O$), 2.12–2.22 (m, 2H, $CH_3C(O)CH_2CH_2C(O)O$), 2.76 (m, 1H, H-5c), 2.91 (t, 1H, $J_{3,4}$=9.5, $J_{4,5}$=10.0 Hz, H-4c), 3.17–3.23 (m, 3H, $OCH_2CH_2CH_2NHZ$, H-3c), 3.41–3.44 (m, 2H, $OCH_2CH_2CH_2NHZ$, H-4a), 3.56 (t, 1H, $J_{3,4}$=9.5, $J_{4,5}$=9.5 Hz, H-4b), 3.66–3.74 (m, 3H, $OCH'_2CH_2CH_2NHZ$, H-5a, H-5b), 3.97 (dd, 1H, $J_{2,3}$=3.0, $J_{3,4}$=9.5 Hz, H-3a), 4.20 (m, 2H, H-1c, H-3b), 4.45 (d,

1H, J=12.0 Hz, PhCH_2), 4.53 (d, 1H, J=11.5 Hz, PhCH'_2), 4.61 (d, 1H, J=12.0 Hz, PhCH''_2), 4.63 (d, 1H, J=11.5 Hz, PhCH'''_2), 4.71 (d, 1H, J=11.5 Hz, PhCH''''_2), 4.77 (s, 1H, H-1a), 4.84 (m, 2H, NH, PhCH'''''_2), 4.95 (t, 1H, $J_{1,2}$=8.0, $J_{2,3}$=10.5 Hz, H-2c), 5.00 (s, 2H, PhCH_2OC(O)), 5.14 (s, 1H, H-1b), 5.30 (d, 1H, $J_{2,3}$=3.0 Hz, H-2a), 5.32 (d, 1H, $J_{2,3}$=3.0 Hz, H-2b), 7.13–8.05 (m, 30H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.7 (C-6c), 17.9 (C-6b), 18.1 (C-6a), 27.6 ($CH_3C(O)CH_2CH_2C(O)O$), 29.6 ($CH_3C(O)CH_2CH_2C(O)O$), 31.6 ($OCH_2CH_2CH_2NHZ$), 37.3 ($CH_3C(O)CH_2CH_2C(O)O$), 38.5 ($OCH_2CH_2CH_2NHZ$), 65.6 ($OCH_2CH_2CH_2NHZ$), 66.6 (PhCH$_2$OC(O)), 67.2 (C-4c), 67.8 (C-5a), 68.6 (C-5b), 70.6 (C-5c), 71.9 (C-2a), 72.7 (C-2b), [73.4, 74.3, 74.4 (PhCH$_2$)], 75.4 (C-2c), 77.2 (C-3b), 78.0 (C-3a), 79.6 (C-4a), 80.2 (C-4b), 80.9 (C-3c), 97.2 (C-1a), 98.8 (C-1b), 100.3 (C-1c), [127.0, 127.3, 127.8, 128.0, 128.1, 128.2, 128.3, 128.4, 128.5, 129.8, 129.9, 130.1, 133.0, 133.3, 133.4, 136.6, 137.5, 137.9, 138.6 (C_{arom})], 156.3 (PhCH$_2$OC(O)), [165.7, 165.8 (PhC(O)O)], 171.1 ($CH_3C(O)CH_2CH_2C(O)O$), 206.1 ($CH_3C(O)CH_2CH_2C(O)O$); MALDI-TOF/MS: m/z: found: 1271.7 [M+Na]$^+$; MALDI-FTICR/MS: m/z: calcd for $C_{69}H_{76}N_4O_{18}$Na: 1271.5052; found: 1271.4893 [M+Na]$^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl *O*-(4-azido-3-*O*-benzyl-4,6-dideoxy-β-D-glucopyranosyl)-(1→3)-*O*-(2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranosyl)-(1→3)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (20): Treatment of **19** (116 mg, 0.093 mmol) in CH_2Cl_2 (2.3 mL) with hydrazine acetate (8.6 mg, 0.093 mmol) in MeOH (0.23 mL) according to the general procedure for cleavage of the levulinoyl ester gave compound **20** as white solid (100 mg, 93%). R_f=0.36 (hexane/EtOAc 2:1); $[\alpha]_D^{27}$=+11.0 (c=0.06 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.88 (d, 3H, $J_{5,6}$=6.0 Hz, H-6c), 1.13 (d, 3H, $J_{5,6}$=6.0 Hz, H-6b), 1.26 (d, 3H, $J_{5,6}$=5.5 Hz, H-6a), 1.73 (m, 2H, $OCH_2CH_2CH_2NHZ$), 2.74 (m, 1H, H-5c), 2.83 (t, 1H, $J_{3,4}$=9.5, $J_{4,5}$=10.0 Hz, H-4c), 3.12 (t, 1H, $J_{2,3}$=9.0, $J_{3,4}$=9.5 Hz, H-3c), 3.22 (m, 2H, $OCH_2CH_2CH_2NHZ$), 3.30 (t, 1H, $J_{1,2}$=8.0, $J_{2,3}$=9.0 Hz, H-2c), 3.40 (m, 1H, $OCH_2CH_2CH_2NHZ$), 3.49 (t, 1H, $J_{3,4}$=9.0, $J_{4,5}$=10.0 Hz, H-4b), 3.54 (t, 1H, $J_{3,4}$=9.0, $J_{4,5}$=10.0 Hz, H-4a), 3.64–3.71 (m, 2H, $OCH'_2CH_2CH_2NHZ$, H-5a), 3.78 (m, 1H, H-5b), 4.05 (dd, 1H, $J_{2,3}$=3.0, $J_{3,4}$=9.5 Hz, H-3b), 4.08 (d, 1H, $J_{1,2}$=8.0 Hz, H-1c), 4.21 (dd, 1H, $J_{2,3}$=3.0, $J_{3,4}$=9.0 Hz, H-3a), 4.56 (d, 1H, J=10.5 Hz, PhCH_2), 4.60 (d, 1H, J=10.5 Hz, PhCH'_2), 4.66 (d, 1H, J=11.0 Hz, PhCH''_2), 4.71 (d, 1H, J=12.0 Hz, PhCH'''_2), 4.75 (d, 1H, J=12.0 Hz, PhCH''''_2), 4.76 (s, 1H, H-1a), 4.83 (broad, 1H, NH), 4.92 (d, 1H, J=11.0 Hz, PhCH'''''_2), 5.00 (s, 2H, PhCH_2OC(O)), 5.12 (s, 1H, H-1b), 5.32 (d, 1H, $J_{2,3}$=3.0 Hz, H-2a), 5.36 (d, 1H, $J_{2,3}$=3.0 Hz, H-2b), 7.19–8.01 (m, 30H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.9 (C-6c), 18.1 (C-6a, C-6b), 29.6 ($OCH_2CH_2CH_2NHZ$), 38.5 ($OCH_2CH_2CH_2NHZ$), 65.6 ($OCH_2CH_2CH_2NHZ$), 66.6 (PhCH$_2$OC(O)), 66.9 (C-5c), 67.9 (C-5a), 68.7 (C-5b), 70.6 (C-4c), 72.5 (C-2a, C-2b), 74.6 (C-2c), [74.7, 75.0, 75.3 (PhCH$_2$)], 75.4 (C-3b), 77.6 (C-3a), 80.1 (C-4a, C-4b), 82.2 (C-3c), 97.1 (C-1a), 99.0 (C-1b), 103.0 (C-1c), [127.8, 127.9, 128.0, 128.1, 128.2, 128.3, 128.4, 128.5, 128.6, 129.8, 130.0, 133.3, 133.4, 136.6, 137.8, 138.0 (C_{arom})], 156.4 (PhCH$_2$OC(O)), [165.6, 165.8 (PhC(O)O)]; MALDI-TOF/MS: m/z: found: 1172.7 [M+Na]$^+$; MALDI-FTICR/MS: m/z: calcd for $C_{64}H_{70}N_4O_{16}$Na: 1173.4685; found: 1173.4588 [M+Na]$^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl *O*-(4-azido-3-*O*-benzyl-4,6-dideoxy-2-*O*-methyl-β-D-glucopyranosyl)-(1→3)-*O*-(2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranosyl)-(1→3)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (21)

Method A: Sodium acetate (0.63 g, 7.68 mmol) and $PdCl_2$ (0.38 g, 2.14 mmol) was added to a solution of **8** (0.594 g, 1.78 mmol) in AcOH/H_2O 9:1 (60 mL). The reaction mixture mixture was stirred at room temperature overnight, and then filtered through Celite. The filtrate was concentrated to dryness and the residue was co-evaporated with toluene (2× 60 mL). Purification of the crude product by column chromatography on silica gel (hexane/EtOAc 2:1) afforded the hemiacetal compound. To a solution of this compound in CH_2Cl_2 (30 mL) was added trichloroacetonitrile (1.79 mL, 17.85 mmol) and DBU (0.11 mL, 0.74 mmol). The reaction mixture was stirred at room temperature for 5 h, and then concentrated to dryness. Purification of the crude product by column chromatography on silica gel (hexane/EtOAc 2:1 +0.5% TEA) afforded imidate donor **9** as an α/β mixture 9:1 (0.622 g, 85%). A mixture of acceptor **18** (1.22 g, 1.37 mmol), donor **9** (0.622 g, 1.51 mmol) and 4 Å powdered molecular sieves (1.85 g) in dry acetonitrile (23 mL) was stirred at 0°C for 1 h, and then cooled to −40°C. A solution of $BF_3{\cdot}Et_2O$ (0.28 mL, 2.27 mmol) was added slowly. The mixture was stirred at −40°C for 1 h, and then neutralized with triethylamine. The solution was filtered through Celite, washed with MeOH/CH_2Cl_2 5:95 (20 mL), and the combined filtrates were concentrated to dryness. Purification of the crude product by column chromatography (hexane/EtOAc 3:1) on silica gel afforded the desired product **21** as α/β 1:4 mixture (1.38 g, 86%).

Method B: Methyl iodide (0.20 mL, 3.24 mmol) and silver(I) oxide (0.37 g, 1.60 mmol) were added to a solution of **20** (93 mg, 0.08 mmol) in THF (2 mL). Dimethyl sulfide (1 μL, 0.014 mmol) was added as catalyst. The flask was wrapped by aluminium foil to exclude light. The reaction mixture was stirred at room temperature overnight, and then filtered through Celite. The filtrate was concentrated to dryness. Purification of the crude product by column chromatography (hexane/EtOAc 3:1) on silica gel afforded the desired product **21** as colorless oil (48 mg, 51%). R_f=0.56 (hexane/EtOAc 2:1); $[\alpha]_D^{27}$=+82.0 (c=0.2 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.83 (d, 3H, $J_{5,6}$=6.0 Hz, H-6c), 1.12 (d, 3H, $J_{5,6}$=6.0 Hz, H-6b), 1.24 (d, 3H, $J_{5,6}$=5.5 Hz, H-6a), 1.73 (m, 2H, $OCH_2CH_2CH_2NHZ$), 2.74 (m, 1H, H-5c), 2.84 (t, 1H, $J_{3,4}$=10.5, $J_{4,5}$=10.0 Hz, H-4c), 2.91 (t, 1H, $J_{1,2}$=8.0, $J_{2,3}$=9.0 Hz, H-2c), 3.11 (t, 1H, $J_{2,3}$=9.0, $J_{3,4}$=9.5 Hz, H-3c), 3.22 (m, 2H, $OCH_2CH_2CH_2NHZ$), 3.36 (s, 3H, OCH_3), 3.39 (m, 1H, $OCH_2CH_2CH_2NHZ$), 3.50 (t, 1H, $J_{3,4}$=9.5, $J_{4,5}$=10.0 Hz, H-4b), 3.54 (t, 1H, $J_{3,4}$=9.5, $J_{4,5}$=10.0 Hz, H-4a), 3.67 (m, 2H, $OCH'_2CH_2CH_2NHZ$, H-5a), 3.76 (m, 1H, H-5b), 4.10 (dd, 1H, $J_{2,3}$=3.0, $J_{3,4}$=9.5 Hz, H-3b), 4.20 (dd, 1H, $J_{2,3}$=3.0, $J_{3,4}$=9.5 Hz, H-3a), 4.31 (d, 1H, $J_{1,2}$=8.0 Hz, H-1c), 4.52 (d, 1H, J=11.0 Hz, PhCH_2), 4.59 (d, 1H, J=10.5 Hz, PhCH'_2), 4.65 (d, 1H, J=11.0 Hz, PhCH''_2), 4.72 (d, 1H, J=11.5 Hz, PhCH'''_2), 4.75 (d, 1H, J=12.0 Hz, PhCH''''_2), 4.76 (s, 1H, H-1a), 4.80 (d, 1H, J=10.5 Hz, PhCH'''''_2), 4.82 (broad, 1H, NH), 5.00 (s, 2H, PhCH_2OC(O)), 5.14 (s, 1H, H-1b), 5.30 (d, 1H, $J_{2,3}$=3.0 Hz, H-2a), 5.38 (d, 1H, $J_{2,3}$=3.0 Hz, H-2b), 7.19–8.02 (m, 30H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.8 (C-6c), 18.0 (C-6b), 18.1 (C-6a), 29.6 ($OCH_2CH_2CH_2NHZ$), 38.5 ($OCH_2CH_2CH_2NHZ$), 60.4 (OCH_3), 65.6 ($OCH_2CH_2CH_2NHZ$), 66.6 (PhCH$_2$OC(O)), 67.3 (C-5c), 67.9 (C-5a), 68.6 (C-5b), 70.2 (C-4c), 72.7 (C-2a), 73.2 (C-2b), [74.2, 75.2, 75.5 (PhCH$_2$)], 75.9 (C-3b), 78.0 (C-3a), 80.0 (C-4a), 80.5 (C-4b), 82.5 (C-3c), 84.3 (C-2c), 97.1 (C-1a), 99.2 (C-1b), 102.9 (C-1c), [127.6, 127.8, 127.9, 128.0, 128.2, 128.3, 128.4, 128.5, 129.7, 129.8, 129.9, 130.1, 133.0, 133.3, 137.8, 137.9, 138.2 (C_{arom})], 156.3 (PhCH$_2$OC(O)), [165.5, 165.8 (PhC(O)O)]; MALDI-TOF/MS: m/z: found: 1187.8 [M+Na]$^+$; MALDI-FTICR/MS: m/z: calcd for $C_{65}H_{72}N_4O_{16}$Na: 1187.4841; found: 1187.4715 [M+Na]$^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl *O*-(4-(3-hydroxy-3-methylbutanamido)-3-*O*-benzyl-4,6-dideoxy-2-*O*-methyl-β-D-glucopyranosyl)-(1→3)-*O*-(2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranosyl)-(1→3)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (22): Treatment of **21** (0.71 g, 0.61 mmol), 1,3-propanedithiol (1.26 mL, 12.55 mmol) in pyridine (43 mL) and H_2O (6.1 mL) with TEA (1.28 mL, 9.15 mmol) according to the general procedure for azide reduction and introduction of C-4″ moiety gave free amine(0.69 g, 99%). Treatment of the free amine (0.47 g, 0.41 mmol) in DMF (20 mL) with β-hydroxyisovaleric acid (88 μL, 0.82 mmol) which was activated with HOAt (0.23 g, 1.64 mmol) and HATU (0.62 g, 1.64 mmol) in DMF (10 mL) for 1 h, and then added DIPEA (5.71 mL, 3.28 mmol) gave compound **22** as colorless oil (0.32 g, 63%) and its α-isomer (76 mg, 15%). R_f=0.26 (hexane/EtOAc 1:1); $[\alpha]_D^{27}$=+9.4 (c=0.3 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.73 (d, 3H, $J_{5,6}$=5.5 Hz, H-6c), 1.09 (s, 3H, $(CH_3)_2C(OH)CH_2C(O)NH$), 1.12 (d, 3H, $J_{5,6}$=6.0 Hz, H-6b), 1.18 (s, 3H, $(CH'_3)_2C(OH)CH_2C(O)NH$), 1.24 (d, 3H, $J_{5,6}$=5.5 Hz, H-6a), 1.74 (m, 2H, $OCH_2CH_2CH_2NHZ$), 1.99 (d, 1H, J=15.0 Hz, $(CH_3)_2C(OH)CH_2C(O)NH$), 2.09 (d, 1H, J=15.0 Hz, $(CH_3)_2C(OH)CH'_2C(O)NH$), 2.91 (m, 1H, H-5c), 2.98 (t, 1H, $J_{1,2}$=8.0, $J_{2,3}$=8.5 Hz, H-2c), 3.15 (t, 1H, $J_{2,3}$=8.5, $J_{3,4}$=9.0 Hz, H-3c), 3.22 (m, 2H, $OCH_2CH_2CH_2NHZ$), 3.38 (s, 3H, OCH_3), 3.39 (m, 2H, $OCH_2CH_2CH_2NHZ$, H-4c), 3.52 (t, 1H, $J_{3,4}$=9.0, $J_{4,5}$=9.5 Hz, H-4b), 3.54 (t, 1H, $J_{3,4}$=9.0, $J_{4,5}$=9.5 Hz, H-4a), 3.67 (m, 2H, $OCH'_2CH_2CH_2NHZ$, H-5a), 3.76 (m, 1H, H-5b), 4.12 (dd, 1H, $J_{2,3}$=3.5, $J_{3,4}$=9.0 Hz, H-3b), 4.21 (dd, 1H, $J_{2,3}$=3.0, $J_{3,4}$=9.0 Hz, H-3a), 4.34 (d, 1H, $J_{1,2}$=8.0 Hz, H-1c), 4.48 (d, 1H, J=11.0 Hz, PhCH_2), 4.54 (d, 1H, J=11.0 Hz, PhCH'_2),

4.60 (d, 1H, J=10.5 Hz, PhCH''_2), 4.71 (d, 1H, J=12.5 Hz, PhCH'''_2), 4.77 (s, 1H, H-1a), 4.83 (d, 1H, J=11.5 Hz, PhCH''''_2), 4.85 (broad, 1H, NH), 4.95 (d, 1H, J=11.0 Hz, PhCH'''''_2), 5.00 (s, 2H, PhCH_2OC(O)), 5.15 (s, 1H, H-1b), 5.30 (d, 1H, $J_{2,3}$=3.0 Hz, H-2a), 5.39 (d, 1H, $J_{2,3}$=3.5 Hz, H-2b), 7.14–8.00 (m, 30H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 17.7 (C-6c), 17.8 (C-6b), 18.1 (C-6a), 29.2 (OCH$_2$CH_2CH$_2$NHZ), [29.3, 29.7 ((CH_3)$_2$C(OH)CH$_2$C(O)NH)], 38.5 (OCH$_2$CH$_2$CH_2NHZ), 47.7 ((CH$_3$)$_2$C(OH)CH_2C(O)NH), 55.7 (C-4c), 60.3 (OCH$_3$), 65.6 (OCH$_2$CH$_2$CH$_2$NHZ), 66.6 (PhCH$_2$OC(O)), 67.9 (C-5a), 68.6 (C-5b), 69.4 ((CH$_3$)$_2$C(OH)CH$_2$C(O)NH), 70.6 (C-5c), 72.8 (C-2a), 73.1 (C-2b), [73.5, 74.1, 75.5 (PhCH_2)], 76.1 (C-3b), 78.1 (C-3a), 79.8 (C-3c), 80.0 (C-4a), 80.5 (C-4b), 84.4 (C-2c), 97.1 (C-1a), 99.2 (C-1b), 103.0 (C-1c), [127.5, 127.7, 127.8, 127.9, 128.0, 128.1, 128.2, 128.3, 128.4, 128.5, 129.7, 129.8, 129.9, 130.2, 133.0, 133.3, 137.9, 138.3, 138.5 (C_{arom})], 156.4 (PhCH$_2$OC(O)), [165.6, 165.9 (PhC(O)O)], 172.2 ((CH$_3$)$_2$C(OH)CH$_2$$C$(O)NH); MALDI-TOF/MS: m/z: found: 1261.4; MALDI-FTICR/MS: m/z: calcd for $C_{70}H_{82}N_2O_{18}Na$: 1261.5460; found: 1261.5427 [M+Na]$^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl *O*-(4-(3-hydroxy-3-methylbutanamido)-3-*O*-benzyl-4,6-dideoxy-β-D-glucopyranosyl)-(1→3)-*O*-(2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranosyl)-(1→3)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (23): Treatment of **20** (21 mg, 0.018 mmol), 1,3-propanedithiol (0.04 mL, 0.40 mmol) in pyridine (1.28 mL) and H_2O (0.92 mL) with TEA (0.03 mL, 0.27 mmol) according to the general procedure for azide reduction and introduction of C-4″ moitety gave free amine (20 mg, 98%). Treatment the free amine (20 mg, 0.018 mmol) in DMF (2 mL) with β-hydroxyisovaleric acid (4 μL, 0.037 mmol) which was activated with HOAt (10 mg, 0.074 mmol) and HATU (28 mg, 0.074 mmol) in DMF (1 mL) for 1 h, and then added DIPEA (26 μL, 0.15 mmol) gave compound **23** as colorless oil (17 mg, 78%). R_f=0.61 (hexane/EtOAc 1:2); ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.79 (d, 3H, $J_{5,6}$= 6.5 Hz, H-6c), 1.11 (s, 3H, (CH_3)$_2$C(OH)CH$_2$C(O)NH), 1.12 (d, 3H, $J_{5,6}$= 6.5 Hz, H-6b), 1.14 (s, 3H, (CH'_3)$_2$C(OH)CH$_2$C(O)NH), 1.25 (d, 3H, $J_{5,6}$=5.5 Hz, H-6a), 1.74 (m, 2H, OCH$_2$CH_2CH$_2$NHZ), 2.06 (d, 1H, J= 15.0 Hz, (CH$_3$)$_2$C(OH)CH_2C(O)NH), 2.14 (d, 1H, J=15.0 Hz, (CH$_3$)$_2$C(OH)CH'_2C(O)NH), 2.98 (m, 1H, H-5c), 3.21(m, 3H, OCH$_2$CH$_2$CH_2NHZ, H-3c), 3.36–3.42 (m, 3H, OCH_2CH$_2$CH$_2$NHZ, H-2c, H-4c), 3.52 (t, 1H, $J_{3,4}$=9.0, $J_{4,5}$=9.5 Hz, H-4b), 3.54 (t, 1H, $J_{3,4}$=9.0, $J_{4,5}$=10.0 Hz, H-4a), 3.68 (m, 2H, OCH'_2CH$_2$CH$_2$NHZ, H-5a), 3.77 (m, 1H, H-5b), 4.08 (dd, 1H, $J_{2,3}$=3.0, $J_{3,4}$=9.0 Hz, H-3b), 4.14 (d, 1H, $J_{1,2}$= 7.5 Hz, H-1c), 4.21 (dd, 1H, $J_{2,3}$=3.0, $J_{3,4}$=9.0 Hz, H-3a), 4.49 (d, 1H, J= 11.0 Hz, PhCH_2), 4.57 (d, 1H, J=11.0 Hz, PhCH'_2), 4.60 (d, 1H, J= 10.5 Hz, PhCH''_2), 4.67 (d, 1H, J=11.0 Hz, PhCH'''_2), 4.73 (d, 1H, J= 11.0 Hz, PhCH''''_2), 4.77 (s, 1H, H-1a), 4.86 (broad, 1H, NH), 4.94 (d, 1H, J=10.5 Hz, PhCH'''''_2), 5.00 (s, 2H, PhCH_2OC(O)), 5.13 (s, 1H, H-1b), 5.32 (d, 1H, $J_{2,3}$=3.0 Hz, H-2a), 5.38 (d, 1H, $J_{2,3}$=3.5 Hz, H-2b), 5.43 (d, 1H, J=9.0 Hz, (CH$_3$)$_2$C(OH)CH$_2$C(O)NH), 7.19–8.02 (m, 30H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.7 (C-6c), 17.9 (C-6b), 18.1 (C-6a), [29.3, 29.4 ((CH_3)$_2$C(OH)CH$_2$C(O)NH)], 29.7 (OCH$_2$CH_2CH$_2$NHZ), 38.5 (OCH$_2$CH$_2$CH_2NHZ), 47.8 ((CH$_3$)$_2$C(OH)CH_2C(O)NH), 55.3 (C-4c), 65.6 (OCH$_2$CH$_2$CH$_2$NHZ), 66.6 (PhCH$_2$OC(O)), 67.9 (C-5a), 68.7 (C-5b), 69.5 ((CH$_3$)$_2$$C$(OH)CH$_2$C(O)NH), 70.9 (C-5c), 72.7 (C-2a), 72.8 (C-2b), [72.5, 74.6, 75.4 (PhCH_2)], 74.9 (C-2c), 77.2 (C-3b), 78.0 (C-3a), 79.6 (C-3c), 80.0 (C-4a), 80.1 (C-4b), 97.1 (C-1a), 99.1 (C-1b), 103.1 (C-1c), [127.8, 127.9, 128.0, 128.1, 128.2, 128.3, 128.4, 128.6, 129.7, 129.8, 130.0, 133.1, 133.3, 137.9, 138.0, 138.4 (C_{arom})], 151.7 (PhCH$_2$OC(O)), [165.7, 165.9 (PhC(O)O)], 172.3 ((CH$_3$)$_2$C(OH)CH$_2$$C$(O)NH); MALDI-TOF/MS: m/z: calcd for $C_{69}H_{80}N_2O_{18}Na$: 1247.5304; found: 1249.7 [M+Na]$^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl *O*-(4-(3-methylbutanamido)-3-*O*-benzyl-4,6-dideoxy-2-*O*-methyl-α-D-glucopyranosyl)-(1→3)-*O*-(2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranosyl)-(1→3)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (24): The azide of compound **20** was reduced as described in the general procedures. Treatment of the free amine (0.12 g, 0.11 mmol) in DMF (5 mL) with DIPEA (0.15 mL, 0.86 mmol) and isovaleric acid (24 μL, 0.22 mmol) that was pre-activated with HOAt (57 mg, 0.42 mmol) and HATU (0.16 g, 0.42 mmol) in DMF (2.6 mL) for 1 h, gave compound **24** as colorless oil (78 mg, 0.064 mmol, 61%) and its α-isomer (19 mg, 0.016 mmol, 15%). R_f=0.39 (hexane/EtOAc 1:1); $[\alpha]_D^{27}$= +18.3 (c=0.6 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.73 (d, 3H, $J_{5,6}$=6.0 Hz, H-6c), 0.78 (s, 3H, (CH_3)$_2$CHCH$_2$C(O)NH), 0.82 (s, 3H, (CH'_3)$_2$CHCH$_2$C(O)NH), 1.12 (d, 3H, $J_{5,6}$=6.0 Hz, H-6b), 1.24 (d, 3H, $J_{5,6}$=5.5 Hz, H-6a), 1.71 (m, 2H, OCH$_2$CH_2CH$_2$NHZ), 1.80–1.98 (m, 3H, (CH$_3$)$_2$CHCH_2C(O)NH, (CH$_3$)$_2$CHCH$_2$C(O)NH), 2.92 (m, 1H, H-5c), 2.97 (t, 1H, $J_{1,2}$=7.8, $J_{2,3}$=9.0 Hz, H-2c), 3.15–3.23 (m, 3H, OCH$_2$CH$_2$CH_2NHZ, H-3c), 3.37 (s, 3H, OCH$_3$), 3.39 (m, 2H, OCH_2CH$_2$CH$_2$NHZ, H-4c), 3.51 (t, 1H, $J_{3,4}$=9.0, $J_{4,5}$=9.0 Hz, H-4b), 3.56 (t, 1H, $J_{3,4}$=9.0, $J_{4,5}$=9.0 Hz, H-4a), 3.66 (m, 2H, OCH'_2CH$_2$CH$_2$NHZ, H-5a), 3.76 (m, 1H, H-5b), 4.13 (dd, 1H, $J_{2,3}$=3.0, $J_{3,4}$=9.0 Hz, H-3b), 4.21 (dd, 1H, $J_{2,3}$=3.0, $J_{3,4}$=9.0 Hz, H-3a), 4.34 (d, 1H, $J_{1,2}$=7.8 Hz, H-1c), 4.48 (d, 1H, J=12.0 Hz, PhCH_2), 4.53 (d, 1H, J=10.8 Hz, PhCH'_2), 4.60 (d, 1H, J=10.8 Hz, PhCH''_2), 4.69 (d, 1H, J=12.0 Hz, PhCH'''_2), 4.77 (s, 1H, H-1a), 4.83 (d, 1H, J=10.8 Hz, PhCH''''_2), 4.85 (broad, 1H, NH), 4.96 (d, 1H, J=10.8 Hz, PhCH'''''_2), 5.00 (s, 2H, PhCH_2OC(O)), 5.15 (d, 1H, $J_{1,2}$=1.2 Hz, H-1b), 5.30 (dd, 1H, $J_{1,2}$=1.2, $J_{2,3}$=3.0 Hz, H-2a), 5.39 (dd, 1H, $J_{1,2}$=1.8, $J_{2,3}$=3.0 Hz, H-2b), 7.18–8.06 (m, 30H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 17.6 (C-6c), 17.8 (C-6b), 18.1 (C-6a), [22.4, 22.5 ((CH_3)$_2$CHCH$_2$C(O)NH)], 25.9 ((CH$_3$)$_2$CHCH$_2$C(O)NH), 29.5 (OCH$_2$CH_2CH$_2$NHZ), 38.4 (OCH$_2$CH$_2$CH_2NHZ), 46.2 ((CH$_3$)$_2$CHCHCH$_2$C(O)NH), 55.7 (C-4c), 60.3 (OCH$_3$), 65.6 (OCH_2CH$_2$CH$_2$NHZ), 66.5 (PhCH_2OC(O)), 67.8 (C-5a), 68.5 (C-5b), 70.7 (C-5c), 72.7 (C-2a), 73.1 (C-2b), [73.3, 74.1, 75.5 (PhCH_2)], 76.0 (C-3b), 78.2 (C-3a), 79.7 (C-3c), 79.8 (C-4a), 80.4 (C-4b), 84.3 (C-2c), 97.0 (C-1a), 99.2 (C-1b), 103.0 (C-1c), [127.5, 127.6, 127.7, 127.9, 128.0, 128.1, 128.2, 128.3, 128.4, 128.5, 129.7, 129.8, 129.9, 130.1, 133.0, 133.3, 136.6, 137.9, 138.3, 138.4 (C_{arom})], 156.3 (PhCH$_2$OC(O)), [165.6, 165.9 (PhC(O)O)], 172.2 ((CH$_3$)$_2$CHCH$_2$$C$(O)NH); MALDI-TOF/MS: m/z: found: 1245.4; MALDI-FTICR/MS: m/z: calcd for $C_{70}H_{82}N_2O_{17}Na$: 1245.5511; found: 1245.5510 [M+Na]$^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl *O*-(4-acetamido-3-*O*-benzyl-4,6-dideoxy-2-*O*-methyl-β-D-glucopyranosyl)-(1→3)-*O*-(2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranosyl)-(1→3)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (25): The azide of compound **20** was reduced as described in the general procedures. Treatment the free amine (94 mg, 0.083 mmol) with acetic anhydride (0.016 mL, 0.17 mmol) in pyridine (0.014 mL, 0.17 mmol) and DMAP (1 mg, 0.008 mmol) gave compound **25** as colorless oil (64 mg, 66%) and its α-isomer (17 mg, 17%). R_f=0.25 (hexane/EtOAc 2:3); $[\alpha]_D^{27}$=+7.2 (c=0.4 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.72 (d, 3H, $J_{5,6}$=6.0 Hz, H-6c), 1.12 (d, 3H, $J_{5,6}$=6.0 Hz, H-6b), 1.23 (d, 3H, $J_{5,6}$=6.5 Hz, H-6a), 1.70 (s, 3H, CH_3C(O)NH), 1.73 (m, 2H, OCH$_2$CH_2CH$_2$NHZ), 2.90 (m, 1H, H-5c), 2.97 (t, 1H, $J_{1,2}$=8.0, $J_{2,3}$= 8.5 Hz, H-2c), 3.12 (t, 1H, $J_{2,3}$=8.5, $J_{3,4}$=9.5 Hz, H-3c), 3.22 (m, 2H, OCH$_2$CH$_2$CH_2NHZ), 3.33 (t, 1H, $J_{3,4}$=9.5, $J_{4,5}$=10.0 Hz, H-4c), 3.38 (s, 3H, OCH$_3$), 3.39 (m, 1H, OCH_2CH$_2$CH$_2$NHZ), 3.51 (t, 1H, $J_{3,4}$=9.0, $J_{4,5}$=10.0 Hz, H-4b), 3.54 (t, 1H, $J_{3,4}$=9.5, $J_{4,5}$=8.5 Hz, H-4a), 3.67 (m, 2H, OCH'_2CH$_2$CH$_2$NHZ, H-5a), 3.75 (m, 1H, H-5b), 4.13 (dd, 1H, $J_{2,3}$= 3.0, $J_{3,4}$=9.0 Hz, H-3b), 4.21 (dd, 1H, $J_{2,3}$=2.5, $J_{3,4}$=9.5 Hz, H-3a), 4.34 (d, 1H, $J_{1,2}$=8.0 Hz, H-1c), 4.48 (d, 1H, J=11.5 Hz, PhCH_2), 4.53 (d, 1H, J=11.0 Hz, PhCH'_2), 4.59 (d, 1H, J=10.5 Hz, PhCH''_2), 4.70 (d, 1H, J= 12.0 Hz, PhCH'''_2), 4.77 (s, 1H, H-1a), 4.81 (d, 1H, J=11.0 Hz, PhCH''''_2), 4.83 (broad, 1H, NH), 4.95 (d, 1H, J=10.5 Hz, PhCH'''''_2), 5.00 (s, 2H, PhCH_2OC(O)), 5.15 (s, 1H, H-1b), 5.30 (d, 1H, $J_{2,3}$=2.5 Hz, H-2a), 5.40 (d, 1H, $J_{2,3}$=3.0 Hz, H-2b), 7.19–8.00 (m, 30H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 17.5 (C-6c), 17.8 (C-6b), 18.1 (C-6a), 23.5 (CH_3C(O)NH), 29.7 (OCH$_2$CH_2CH$_2$NHZ), 38.5 (OCH$_2$CH$_2$CH_2NHZ), 55.9 (C-4c), 60.3 (OCH$_3$), 65.6 (OCH$_2$CH$_2$CH$_2$NHZ), 66.6 (PhCH$_2$OC(O)), 67.8 (C-5a), 68.5 (C-5b), 70.7 (C-5c), 72.8 (C-2a), 73.1 (C-2b), [73.5, 74.2, 75.5 (PhCH_2)], 76.0 (C-3b), 78.3 (C-3a), 79.6 (C-3c), 79.8 (C-4a), 80.5 (C-4b), 84.5 (C-2c), 97.0 (C-1a), 99.3 (C-1b), 103.0 (C-1c), [127.6, 127.8, 127.9, 128.0, 128.2, 128.3, 128.4, 128.5, 129.7, 129.8, 129.9, 130.2, 133.0, 133.3, 136.6, 137.9, 138.3, 138.5 (C_{arom})], 156.3 (PhCH$_2$OC(O)), [165.6, 165.9 (PhC(O)O)], 169.8 (CH$_3$$C$(O)NH); MALDI-TOF/MS: m/z: found: 1204.3; MALDI-FTICR/MS: m/z: calcd for $C_{67}H_{76}N_2O_{17}Na$: 1203.5042; found: 1203.5040 [M+Na]$^+$.

3-Aminopropyl *O*-(4-(3-hydroxy-3-methylbutanamido)-4,6-dideoxy-2-*O*-methyl-β-D-glucopyranosyl)-(1→3)-*O*-(α-L-rhamnopyranosyl)-(1→3)-α-L-rhamnopyranoside (1): Treatment of **22** (138.0 mg, 111.3 μmol) in

MeOH/CH_2Cl_2 (2 mL/2 mL) with NaOMe (pH 8–10) according to the general procedure for global deprotection gave deacetylated product (110.1 mg, 96%). Treatment of the partially deprotected compound (110.1 mg, 106.7 μmol) in *tert*-butanol/H_2O/AcOH (10 mL/0.25 mL/0.25 mL) with a catalytic amount of Pd/C under an atmosphere of hydrogen gave compound **1** as white solid (65.5 mg, 98%). R_f=0.50 (CH_3CN/H_2O/AcOH 40:20:1); ^{1}H NMR (500 MHz, D_2O): δ 1.13 (d, 3H, $J_{5,6}$= 6.0 Hz, H-6c), 1.21 (broad, 12H, $(CH_3)_2C(OH)CH_2C(O)NH$, H-6a, H-6b), 1.92 (m, 2H, $OCH_2CH_2CH_2NH_2$), 2.36 (s, 2H, $(CH_3)_2C(OH)CH_2C(O)NH$), 3.00–3.15 (m, 4H, $OCH_2CH_2CH_2NH_2$, H-2c, H-4c), 3.42–3.52 (m, 5H, $OCH_2CH_2CH_2NH_2$, H-4a, H-4b, H-3c, H-5c), 3.53 (s, 3H, OCH_3), 3.61 (m, 1H, H-5a), 3.69–3.76 (m, 3H, H-3a, H-5b, $OCH'_2CH_2CH_2NH_2$), 3.90 (d, 1H, $J_{3,4}$=10.0 Hz, H-3b), 3.93 (s, 1H, H-2a), 4.17 (s, 1H, H-2b), 4.63 (d, 1H, $J_{1,2}$=8.0 Hz, H-1c), 4.65 (s, 1H, H-1a), 4.93 (s, 1H, H-1b); ^{13}C NMR (75 MHz, D_2O): δ [16.7, 16.8 (C-6a, C-6b)], 17.2 (C-6c), 26.8 ($OCH_2CH_2CH_2NH_2$), [28.2, 28.4 ($(CH_3)_2C(OH)CH_2C(O)NH$)], 37.6 ($OCH_2CH_2CH_2NH_2$), 49.0 ($(CH_3)_2C(OH)CH_2C(O)NH$), 56.7 (C-4c), 60.2 (OCH_3), 65.0 ($OCH_2CH_2CH_2NH_2$), 68.9 (C-5a), 69.4 (C-5b), 69.9 ($(CH_3)_2C(OH)CH_2C(O)NH$), 70.0 (C-2a), 70.3 (C-2b), [70.9, 71.2, 71.4, 72.9 (C-4a, C-4b, C-3c, C-5c)], 78.4 (C-3a), 79.7 (C-3b), 83.4 (C-2c), 99.8 (C-1a), 102.3 (C-1b), 103.8 (C-1c), 174.2 ($(CH_3)_2C(OH)CH_2C(O)NH$); MALDI-TOF/MS: *m*/*z*: found: 649.6; MALDI-FTICR/MS: *m*/*z*: calcd for $C_{27}H_{50}N_2O_{14}Na$: 649.3160; found: 649.3156 $[M+Na]^+$.

3-Aminopropyl *O*-(4,6-dideoxy-4-(3-hydroxy-3-methylbutanamido)-β-D-glucopyranosyl)-(1→3)-*O*-(α-L-rhamnopyranosyl)-(1→3)-α-L-rhamnopyranoside (2): Treatment of **23** (17.0 mg, 13.9 μmol) in MeOH/CH_2Cl_2 (0.5 mL/0.5 mL) with NaOMe (pH 8–10) according to the general procedure for global deprotection gave the deacetylated product (14.0 mg, 99%). Treatment of the partially deprotected compound (14.0 mg, 13.8 μmol) in *tert*-butanol/H_2O/AcOH (2 mL/0.05 mL/0.05 mL) with a catalytic amount of Pd/C under an atmosphere of hydrogen gave compound **2** as white solid (8.1 mg, 96%). R_f=0.30 (CH_3CN/H_2O/AcOH 40:20:1); ^{1}H NMR (300 MHz, D_2O): δ = 1.11 (d, 3H, $J_{5,6}$=6.0 Hz, H-6c), 1.17 (broad, 12H, $(CH_3)_2C(OH)CH_2C(O)NH$, H-6a, H-6b), 1.84 (m, 2H, $OCH_2CH_2CH_2NH_2$), 2.33 (s, 2H, $(CH_3)_2C(OH)CH_2C(O)NH$), 2.96 (m, 2H, $OCH_2CH_2CH_2NH_2$), 3.11 (t, 1H, $J_{3,4}$=7.2, $J_{4,5}$=7.2 Hz, H-4c), 3.27 (t, 1H, $J_{1,2}$=7.8, $J_{2,3}$=8.4 Hz, H-2c), 3.36–3.60 (m, 6H, $OCH_2CH_2CH_2NH_2$, H-4a, H-5a, H-4b, H-3c, H-5c), 3.64–3.75 (m, 3H, H-3a, H-5b, $OCH'_2CH_2CH_2NH_2$), 3.86 (m, 2H, H-2a, H-3b), 4.14 (s, 1H, H-2b), 4.58 (d, 1H, $J_{1,2}$=7.8 Hz, H-1c), 4.63 (s, 1H, H-1a), 4.88 (s, 1H, H-1b); ^{13}C NMR (75 MHz, D_2O): δ = [16.7, 17.2 (C-6a, C-6b, C-6c)], 27.1 ($OCH_2CH_2CH_2NH_2$), [28.2, 28.4 ($(CH_3)_2C(OH)CH_2C(O)NH$)], 37.6 ($OCH_2CH_2CH_2NH_2$), 49.0 ($(CH_3)_2C(OH)CH_2C(O)NH$), 56.7 (C-4c), 65.1 ($OCH_2CH_2CH_2NH_2$), 68.9 (C-5a), 69.1 (C-5b), 69.9 ($(CH_3)_2C(OH)CH_2C(O)NH$), 70.0 (C-2a), 70.3 (C-2b), [71.1, 71.3, 71.4, 73.5 (C-4a, C-4b, C-3c, C-5c)], 74.2 (C-2c), 78.4 (C-3a), 79.7 (C-3b), 99.8 (C-1a), 102.3 (C-1b), 103.6 (C-1c), 174.2 ($(CH_3)_2C(OH)CH_2C(O)NH$); MALDI-TOF/MS: *m*/*z*: found: 635.3; MALDI-FTICR/MS: *m*/*z*: calcd for $C_{26}H_{48}N_2O_{14}Na$: 635.3003; found: 635.3000 $[M+Na]^+$.

3-Aminopropyl *O*-(4,6-dideoxy-4-(3-methylbutanamido)-2-*O*-methyl-β-D-glucopyranosyl)-(1→3)-*O*-(α-L-rhamnopyranosyl)-(1→3)-α-L-rhamnopyranoside (3): Treatment of **24** (47.0 mg, 38.4 μmol) in MeOH/CH_2Cl_2 (0.5 mL/0.5 mL) with NaOMe (pH 8–10) according to the general procedure for global deprotection gave the deacetylated product (39.0 mg, 100%). Treatment of the partially deprotected compound (39.0 mg, 38.4 μmol) in *tert*-butanol/H_2O/AcOH (4 mL/0.1 mL/0.1 mL) with a catalytic amount of Pd/C under an atmosphere of hydrogen gave compound **3** as white solid (22.1 mg, 94%). R_f=0.40 (CH_3CN/H_2O/AcOH 60:20:1); ^{1}H NMR (500 MHz, D_2O): δ = 0.77 (m, 6H, $(CH_3)_2CHCH_2C(O)NH$), 1.06 (d, 3H, $J_{5,6}$=6.0 Hz, H-6c), 1.14 (m, 6H, H-6a, H-6b), 1.84 (broad, 3H, $OCH_2CH_2CH_2NH_2$, $(CH_3)_2CHCH_2C(O)NH$), 1.99 (m, 2H, $(CH_3)_2CHCH_2C(O)NH$), 2.94–2.99 (m, 4H, $OCH_2CH_2CH_2NH_2$, H-2c, H-4c), 3.33–3.46 (m, 5H, $OCH_2CH_2CH_2NH_2$, H-4a, H-4b, H-3c, H-5c), 3.47 (s, 3H, OCH_3), 3.55 (m, 1H, H-5a), 3.63 (dd, 1H, $J_{2,3}$=3.5, $J_{3,4}$=9.5 Hz, H-3a), 3.69 (m, 2H, H-5b, $OCH'_2CH_2CH_2NHZ$), 3.83 (dd, 1H, $J_{2,3}$=3.0, $J_{3,4}$=10.0 Hz, H-3b), 3.87 (s, 1H, H-2a), 4.12 (s, 1H, H-2b), 4.57 (d, 1H, $J_{1,2}$=8.5 Hz, H-1c), 4.61 (s, 1H, H-1a), 4.86 (s, 1H, H-1b); ^{13}C NMR (75 MHz, D_2O): δ = [16.7, 16.8 (C-6a, C-6b)], 17.2 (C-6c), [21.7, 21.8 ($(CH_3)_2CHCH_2C(O)NH$)], 22.3 (CH_3COOH), 26.2 ($(CH_3)_2CHCH_2C(O)NH$), 26.8 ($OCH_2CH_2CH_2NH_2$), 37.6 ($OCH_2CH_2CH_2NH_2$), 45.5 ($(CH_3)_2CHCH_2C(O)NH$), 56.7 (C-4c), 60.2 (OCH_3), 65.1 ($OCH_2CH_2CH_2NH_2$), 69.0 (C-5a), 69.4 (C-5b), 70.0 (C-2a), 70.1 (C-2b), [71.0, 71.3, 71.5, 73.0 (C-4a, C-4b, C-3c, C-5c)], 78.4 (C-3a), 79.8 (C-3b), 83.5 (C-2c), 99.9 (C-1a), 102.3 (C-1b), 103.8 (C-1c), 177.2 (CH_3COOH), 179.7 ($(CH_3)_2CHCH_2C(O)NH$); MALDI-TOF/MS: *m*/*z*: found: 633.2; MALDI-FTICR/MS: *m*/*z*: calcd for $C_{27}H_{50}N_2O_{13}Na$: 633.3211; found: 633.3207 $[M+Na]^+$.

3-Aminopropyl *O*-(4-acetamido-4,6-dideoxy-2-*O*-methyl-β-D-glucopyranosyl)-(1→3)-*O*-(α-L-rhamnopyranosyl)-(1→3)-α-L-rhamnopyranoside (4): Treatment of **25** (27.2 mg, 23.0 μmol) in MeOH/CH_2Cl_2 (0.5 mL/0.5 mL) with NaOMe (pH 8–10) according to the general procedure for global deprotection gave the deacetylated product (22.9 mg, quantitative). Treatment of the partially deprotected compound (22.9 mg, 23.5 μmol) in *tert*-butanol/H_2O/AcOH (4 mL/0.1 mL/0.1 mL) with a catalytic amount of Pd/C under an atmosphere of hydrogen gave compound **4** as white solid (12.1 mg, 92%). R_f=0.45 (CH_3CN/H_2O/AcOH 40:20:1); ^{1}H NMR (500 MHz, D_2O): δ = 1.04 (d, 3H, $J_{5,6}$=5.5 Hz, H-6c), 1.13 (d, 3H, $J_{5,6}$=6.5 Hz, H-6b), 1.16 (d, 3H, $J_{5,6}$=6.5 Hz, H-6a), 1.87 (s, 3H, $CH_3C(O)NH$), 1.89 (m, 2H, $OCH_2CH_2CH_2NH_2$), 2.93–3.08 (m, 4H, $OCH_2CH_2CH_2NH_2$, H-2c, H-4c), 3.34–3.46 (m, 5H, $OCH_2CH_2CH_2NH_2$, H-4a, H-4b, H-3c, H-5c), 3.47 (s, 3H, OCH_3), 3.55 (m, 1H, H-5a), 3.64–3.71 (m, 3H, H-3a, H-5b, $OCH'_2CH_2CH_2NHZ$), 3.83 (d, 1H, $J_{3,4}$= 10.0 Hz, H-3b), 3.87 (s, 1H, H-2a), 4.12 (s, 1H, H-2b), 4.59 (d, 1H, $J_{1,2}$= 8.0 Hz, H-1c), 4.61 (s, 1H, H-1a), 4.87 (s, 1H, H-1b); ^{13}C NMR (75 MHz, D_2O): δ = [16.8, 17.0 (C-6a, C-6b, C-6c)], 22.3 (CH_3COOH), 26.3 ($CH_3C(O)NH$), 26.8 ($OCH_2CH_2CH_2NH_2$), 37.6 ($OCH_2CH_2CH_2NH_2$), 56.9 (C-4c), 60.2 (OCH_3), 65.1 ($OCH_2CH_2CH_2NH_2$), 68.2 (C-5a), 69.0 (C-5b), 69.4 (C-2a), 70.0 (C-2b), [71.0, 71.3, 71.5, 73.0 (C-4a, C-4b, C-3c, C-5c)], 78.4 (C-3a), 79.7 (C-3b), 83.3 (C-2c), 99.9 (C-1a), 102.3 (C-1b), 103.8 (C-1c), 174.8 ($CH_3C(O)NH$), 178.4 (CH_3COOH); MALDI-TOF/MS: *m*/*z*: found: 591.2; MALDI-FTICR/MS: *m*/*z*: calcd for $C_{24}H_{44}N_2O_{13}Na$: 591.2741; found: 591.2737 $[M+Na]^+$.

General procedure for *S*-acetylthioglycolylamido derivatization of the aminopropyl spacer: The oligosaccharide **1** (10 mg, 0.016 mmol) was slurried in dry DMF (500 μL) and SAMA-OPfp (7.2 mg, 0.024 mmol) was added followed by dropwise addition of DIPEA (5.6 μL, 0.032 mmol). After stirring at room temperature for 2 h, the mixture was concentrated, co-evaporated twice with toluene and the residue purified by size-exclusion chromatography (Biogel P2 column, eluated with H_2O containing 1% *n*-butanol) to give, after lyophilization, the corresponding thioacetate (10.6 mg, 0.0144 mmol, 90%) as a white powder. In this manner, the thioacetamido derivatives of compounds **1**–**4** were prepared in yields of 85–95%.

General procedure for *S*-deacetylation: 7% NH_3 (g) in DMF solution (200 μL) was added to a solution of the thioacetate derivative corresponding to trisaccharide **1** (2.6 mg, 3.5 μmol) in ddH_2O (40 μL) and the mixture was stirred under argon atmosphere. The reaction was monitored by MALDI-TOF showing the product peak of $[M+Na]^+$. After 1 h the solvent was evaporated under high-vacuum. The thiol derivatized trisaccharide was further dried under high vacuum for 30 min and then used immediately in conjugation without further purification.

General procedure for the conjugation of thiol derivatized trisaccharides to BSA-MI: The conjugations were performed as instructed by Pierce Endogen Inc. In short, the thiol derivative (2.5 equiv excess to available MI-groups on the protein), deprotected just prior to conjugation as described above, was dissolved in ddH_2O (100 μL) and added to a solution of the maleimide activated protein (2 mg) in conjugation buffer sodium phosphate pH 7.2 containing EDTA and sodium azide (200 μL). The mixture was incubated for 2 h at room temperature and then purified by Millipore Centriplus centrifugal filter devices with a 10 kDa molecular cut-off. All centrifugations were performed at 8°C for 25 min, spinning at 13×g. The reaction mixture was centrifuged and the filter washed with 10 mM Hepes buffer pH 6.5 (3×200 μL). The conjugate was retrieved and taken up in sodium phosphate buffer pH 7.4, 0.15 M sodium chloride (1 mL). This gave glycoconjugates with a carbohydrate/BSA ratio of 18:1 for trisaccharide **1**, 10:1 for 2″-OH-trisaccharide **2**, 9:1 for 4″-isovaleric

acid trisaccharide **3** and 4:1 for trisaccharide 4″-HNAc-trisaccharide **4** as determined by Dubois′ phenol-sulfuric acid total carbohydrate assay, quantitative monosaccharide analysis by HPAEC/PAD and Lowry protein concentration test.

Conjugation of thiol derivatized trisaccharide to KLH-BrAc: A solution of KLH (15 mg) in 0.1 M sodium phosphate buffer pH 7.2 containing 0.15 M NaCl (1.5 mL) was added to a solution of SBAP (6 mg) in DMSO (180 μL). The mixture was incubated for 2 h at room temperature and then purified using Millipore Centriplus centrifugal filter devices with a molecular cut-off of 30 kDa. All centrifugations were performed at 8 °C for 25 min. spinning at 3000 rpm. The reaction mixture was centrifuged off and the filter washed with conjugation buffer (2 × 750 μL). The activated protein was retrieved by spinning at 3000 rpm for 15 min at 8 °C and taken up in 0.1 mM sodium phosphate buffer pH 8.0 containing 5 mM EDTA (2 mL). The activated protein was added to a vial containing de-S-acetylated trisaccharide (2.6 mg) and the mixture was incubated at room temperature for 18 h. Purification was achieved using centrifugal filters as described above for the BSA–MI–trisaccharide conjugates. This gave a glycoconjugate with 1042 trisaccharide residues/KLH molecule as determined by phenol/sulfuric acid total carbohydrate assay, quantitative monosaccharide analysis by HPAEC/PAD and Lowry protein concentration test.

Preparation of *Bacillus anthracis* Sterne 34F$_2$ spores: *Bacillus anthracis* Sterne 34F$_2$ was obtained from the CDC culture collection. Spores of *B. anthracis* Sterne 34F$_2$ were prepared from liquid cultures of PA medium[41] grown at 37 °C, 200 rpm for six days. Spores were washed two times by centrifugation at 10000 × g in cold (4 °C) sterile deionized water, purified in a 50 % Reno-60 (Bracco Diagnostics Inc., Princeton, NJ) gradient (10000 × g, 30 min, 4 °C) and washed a further four times in cold sterile deionized water. After suspension in sterile deionized water, spores were quantified with surface spread viable cell counts on brain heart infusion (BHI) agar plates (BD BBL, Sparks, MD). Spore suspensions were stored in water at −80 °C.

For the preparation of killed spores, 500-μL aliquots of spore suspensions in water, prepared as described above and containing approximately 3 × 10^8 CFU, were irradiated in 2.0 mL Sarstedt freezer tubes (Sarstedt, Newton, NC) in a gammacell irradiator with an absorbed dose of 2 million rads. Sterility after irradiation was monitored by spread-plating 10-μL aliquots of irradiated spore suspension on BHI agar plates. The plates were incubated for 72 h at 37 °C and monitored for colony growth. Absence of growth was taken as an indicator of sterility.

Preparation of antispore antiserum: All antisera were prepared in female New Zealand White rabbits (2.0–3.5 kg) purchased from Myrtle's Rabbitry (Thompson Station, TN). For antiserum production each of two rabbits were inoculated intramuscularly at two sites in the dorsal hind quarters with 0.5 mL of washed live-spore or irradiated spore inoculum (3 × 10^6 total spores). Rabbits were vaccinated at 0, 14, 28, and 42 days. Serum was collected prior to the first immunization (pre-immune serum) and at 7 and 14 d after each injection of antigen. Terminal bleeds were collected 14 d after the last immunization. All animal protocols were approved by the CDC Animal Care and Use Committee (ACUC) and implemented under the direction of the CDC attending veterinarian.

Antibody-binding analyses: Binding of rabbit anti-live spore antiserum to synthetic oligosaccharide conjugates was done by enzyme-linked immunosorbent assay (ELISA). Briefly, Immulon II-HB flat bottom 96-well microtiter plates (Thermo Labsystems, Franklin, MA) were coated with 100 μL per well of the KLH-BrAc-**1** conjugate at a concentration of 0.03 μg per mL of carbohydrate content, corresponding to 0.5 μg per mL by protein content, or by the protein mcKLH by itself (0.5 μg per mL protein) in coating buffer (0.01 M PBS, pH 7.4). Plates were washed three times in wash buffer (0.01 M PBS, pH 7.4, 0.1 % Tween-20) using an ELX405 microplate washer (BioTek Instruments Inc., Winooski, VT). Serial dilutions (100 μL per well) in blocking solution (0.01 M PBS, pH 7.4, 5 % skim milk, 0.5 % Tween-20) of either rabbit anti-spore antiserum from the day 49 bleed or pre-immune serum were then added and plates were incubated for 1 h 37 °C. After incubation the plates were washed three times in wash buffer at which time a goat anti-rabbit IgG horseradish peroxidase conjugate (ICN Pharmaceuticals, Aurora, OH) was added (100 μL per well) and the incubation continued for 1 hour at 37 °C. Plates were then washed three times in wash buffer and 100 μL per well of ABTS peroxidase substrate was added (KPL, Gaithersburg, MD). Color development was stopped after 15 minutes at 37 °C by addition of 100 μL per well of ABTS peroxidase stop solution (KPL, Gaithersburg, MD). Optical density (OD) values were read at a wavelength of 410 nm (490 nm reference filter) with a MRX Revelation microtiter plate reader (Thermo Labsystems, Franklin, MA).

To test for competitive inhibition, the rabbit anti-live spore antiserum or the rabbit anti-irradiated spore antiserum was added together with unconjugated trisaccharide in blocking solution at a 6-, 12-, 25-, 50-, 100-, or 200-fold weight excess compared to weight of carbohydrate used for coating. The negative control consisted of uncoated wells incubated with the respective antiserum plus trisaccharide **1** at a concentration corresponding to "200-fold excess" of trisaccharide.

To explore competitive inhibition by synthetic saccharide analogues conjugated to bovine serum albumin (BSA; Pierce Biotechnology, Rockford, IL), rabbit anti-live spore antiserum was diluted 1:1600 in blocking solution. For each well 100 μL of the serum were mixed with either 100 μL blocking solution or 100 μL of BSA-MI-conjugate in blocking solution with a concentration corresponding to a 2-, 4-, 8-, 16-, 32-, 64-, or 128-fold weight excess of carbohydrate compared to carbohydrate used for coating. The four conjugates tested were: BSA-MI-**1**, BSA-MI-**2**, BSA-MI-**3**, and BSA-MI-**4**. First the serum and then the BSA–saccharide conjugate solutions were added to an uncoated microtiter plate and mixed by pipetting up and down before the well contents were transferred to a coated plate. The microtiter plates were incubated and developed as described above.

The data are reported as the means ± SD of triplicate measurements.

Acknowledgements

This work was supported by NIAID Grant R21 AI059577 from the National Institutes of Health.

[1] M. Mock, A. Fouet, *Annu. Rev. Microbiol.* **2001**, *55*, 647–671.
[2] F. G. Priest in *Bacillus subtilis and other Gram positive bacteria: biochemistry, physiology, and molecular biology* (Eds.: A. L. Sonenshein, J. A. Hoch, R. Losick), American Society for Microbiology, Washington, D.C., **1993**, p. 3–16.
[3] W. L. Nicholson, N. Munakata, G. Horneck, H. J. Melosh, P. Setlow, *Microbiol. Mol. Biol. Rev.* **2000**, *64*, 548–572.
[4] M. Paidhungat, P. Stetlow in *Bacillus subtilis and its closest relatives: from genes to cells* (Eds.: A. L. Sonenshein, J. A. Hoch, R. Losick), American Society for Microbiology, Washington, D.C., **2002**, p. 537–548.
[5] N. S. Duesbery, G. F. Vande Woude, *Cell. Mol. Life Sci.* **1999**, *55*, 1599–1609.
[6] D. B. Jernigan, P. L. Raghunathan, B. P. Bell, R. Brechner, E. A. Bresnitz, J. C. Butler, M. Cetron, M. Cohen, T. Doyle, M. Fischer, C. Greene, K. S. Griffith, J. Guarner, J. L. Hadler, J. A. Hayslett, R. Meyer, L. R. Petersen, M. Phillips, R. Pinner, T. Popovic, C. P. Quinn, J. Reefhuis, D. Reissman, N. Rosenstein, A. Schuchat, W. J. Shieh, L. Siegal, D. L. Swerdlow, F. C. Tenover, M. Traeger, J. W. Ward, I. Weisfuse, S. Wiersma, K. Yeskey, S. Zaki, D. A. Ashford, B. A. Perkins, S. Ostroff, J. Hughes, D. Fleming, J. P. Koplan, J. L. Gerberding, *Emerging Infect. Dis.* **2002**, *8*, 1019–1028.
[7] J. A. Jernigan, D. S. Stephens, D. A. Ashford, C. Omenaca, M. S. Topiel, M. Galbraith, M. Tapper, T. L. Fisk, S. Zaki, T. Popovic, R. F. Meyer, C. P. Quinn, S. A. Harper, S. K. Fridkin, J. J. Sejvar, C. W. Shepard, M. McConnell, J. Guarner, W. J. Shieh, J. M. Malecki, J. L. Gerberding, J. M. Hughes, B. A. Perkins, *Emerging Infect. Dis.* **2001**, *7*, 933–944.
[8] G. F. Webb, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 4355–4356.

[9] A. M. Friedlander, P. R. Pittman, G. W. Parker, *J. Am. Med. Assoc.* **1999**, *282*, 2104–2106.
[10] L. M. Joellenbeck, L. L. Zwanziger, J. S. Durch, B. L. Strom, *The Anthrax vaccine: is it safe? Does it work?*, National Academy Press, Washington, D.C., **2002**.
[11] M. Sterne, *Onderstepoort J. Vet. Sci. Anim. Ind.* **1937**, *9*, 49–67.
[12] M. Sterne, *Onderstepoort J. Vet. Sci. Anim. Ind.* **1937**, *8*, 271–349.
[13] P. C. Turnbull, *Vaccine* **1991**, *9*, 533–539.
[14] F. Brossier, M. Levy, M. Mock, *Infect. Immun.* **2002**, *70*, 661–664.
[15] P. Gerhardt, *Fed. Proc.* **1967**, *26*, 1504–1517.
[16] P. Gerhardt, E. Ribi, *J. Bacteriol.* **1964**, *88*, 1774–1789.
[17] Y. Hachisuka, K. Kojima, T. Sato, *J. Bacteriol.* **1966**, *91*, 2382–2384.
[18] T. C. Beaman, H. S. Pankratz, P. Gerhardt, *J. Bacteriol.* **1971**, *107*, 320–324.
[19] M. J. Kramer, I. L. Roth, *Can. J. Microbiol.* **1968**, *14*, 1297–1299.
[20] P. Sylvestre, E. Couture-Tosi, M. Mock, *Mol. Microbiol.* **2002**, *45*, 169–178.
[21] M. A. Schmidt, L. W. Riley, I. Benz, *Trends Microbiol.* **2003**, *11*, 554–561.
[22] N. Jentoft, *Trends Biochem. Sci.* **1990**, *15*, 291–294.
[23] J. M. Daubenspeck, H. Zeng, P. Chen, S. Dong, C. T. Steichen, N. R. Krishna, D. G. Pritchard, C. L. Turnbough, Jr., *J. Biol. Chem.* **2004**, *279*, 30945–30953.
[24] R. Adamo, R. Saksena, P. Kovac, *Carbohydr. Res.* **2005**, *340*, 2579–2582.
[25] R. Saksena, R. Adamo, P. Kovac, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 615–617.
[26] R. Saksena, R. Adamo, P. Kovac, *Carbohydr. Res.* **2005**, *340*, 1591–1600.
[27] D. B. Werz, P. H. Seeberger, *Angew. Chemie* **2005**, *117*, 6474–6476; *Angew. Chem. Int. Ed.* **2005**, *44*, 6315–6318.
[28] T. Ziegler, *Carbohydr. Res.* **1994**, *253*, 151–166.
[29] M. Liu, B. Yu, X. Wu, Y. Hui, K. P. Fung, *Carbohydr. Res.* **2000**, *329*, 745–754.
[30] S. David, A. Thieffry, A. Veyrieres, *J. Chem. Soc. Perkin Trans. 1* **1981**, 1796–1801.
[31] H. P. Qin, T. B. Grindley, *J. Carbohydr. Chem.* **1996**, *15*, 95–108.
[32] B. Elchert, J. Li, J. H. Wang, Y. Hui, R. Rai, R. Ptak, P. Ward, J. Y. Takemoto, M. Bensaci, C. W. T. Chang, *J. Org. Chem.* **2004**, *69*, 1513–1523.
[33] R. R. Schmidt, *Angew. Chem.* **1986**, *98*, 213–236; *Angew. Chem. Int. Ed. Engl.* **1986**, *25*, 212–235.
[34] R. R. Schmidt, W. Kinzy, *Adv. Carbohydr. Chem. Biochem.* **1994**, *50*, 21–123.
[35] J. Q. Jiang, J. B. Biggins, J. S. Thorson, *Angew. Chem.* **2001**, *113*, 1550–1553; *Angew. Chem. Int. Ed.* **2001**, *40*, 1502–1505.
[36] T. Zhu, G. J. Boons, *Chem. Eur. J.* **2001**, *7*, 2382–2389.
[37] G. H. Veeneman, S. H. van Leeuwen, J. H. van Boom, *Tetrahedron Lett.* **1990**, *31*, 1331–1334.
[38] I. Braccini, C. Derouet, J. Esnault, C. H. Dupenhoat, J. M. Mallet, V. Michon, P. Sinay, *Carbohydr. Res.* **1993**, *246*, 23–41.
[39] Y. D. Vankar, P. S. Vankar, M. Behrendt, R. R. Schmidt, *Tetrahedron* **1991**, *47*, 9985–9992.
[40] M. Tamborrini, D. B. Werz, J. Frey, G. Pluschke, P. H. Seeberger, *Angew. Chem.* **2006**, *118*, 6731–6732; *Angew. Chem. Int. Ed.* **2006**, *45*, 6581–6582.
[41] B. D. Green, L. Battisti, T. M. Koehler, C. B. Thorne, B. E. Ivins, *Infect. Immun.* **1985**, *49*, 291–297.

Received: August 31, 2006

