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Synthesis of an anthrose derivative and production of polyclonal antibodies for the detection of anthrax spores

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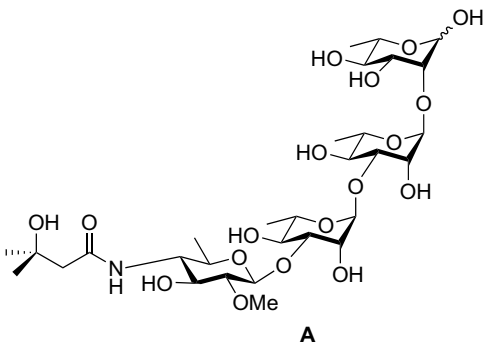
Abstract—A straightforward synthesis of a derivative of anthrose, the non-reducing terminal fragment of the antigenic tetrasaccharide from *Bacillus anthracis*, was achieved starting from D-galactose. This hapten is able to induce a highly specific and sensitive immune response in rabbit when attached to a carrier protein.

Keywords: Anthrose; *Bacillus anthracis*; Antigenic carbohydrate; Antibody; Immunoassays

1. Introduction

Bacillus anthracis is a spore-forming Gram positive bacteria causing anthrax, an often deadly disease, affecting mainly domesticated and wild animals. Until recently, only humans in contact with infected animals or contaminated soils, contracted the illness. In most cases, infection occurs in the form of skin disease, which is much less serious than when inhalation is involved. The recent use of *B. anthracis* spores as a biological weapon has stressed the need for efficient vaccines and detection systems.[1]

The spore of *B. anthracis* is surrounded by a layer called exosporium which is composed of a paracrystalline basal layer and an external hairlike nap.[2] The key component of the latter is a glycoprotein called BclA (*Bacillus* collagen-like protein of *anthracis*) which has been shown to react with most antibodies raised against anthrax spores. Daubenspeck et al.[2] have recently shown that multiple copies of tetrasaccharide **A** (Chart 1) are linked to the collagen-like region of BclA. This linear tetrasaccharide contains a unique nonreducing terminal sugar which has not yet been found in other species, the so-called anthrose. Recently, using a photogenerated glycan array, Wang et al.[3] have discovered that rabbit IgG antibodies elicited by *B. anthracis* spores specifically recognize this tetrasaccharide chain. It has



A

Chart 1. Structure of the *Bacillus anthracis* antigenic tetrasaccharide.[2]

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been suggested that this characteristic antigen may be an interesting target for the development of a vaccine or antibody-based detection system.

Several syntheses[4,5] of tetrasaccharide analogues of **A** and one of its related trisaccharide[6] have been reported in the literature. Two of these required the use of rare and expensive D-fucose as starting material in the preparation of the anthrose building block.[4,6] In a more lengthy strategy,[5] D-mannose has been used. Very recently, Guo and O'Doherty[7] have proposed a *de novo* asymmetric approach starting from 2-acetylfuran to prepare the anthrose building block as well as the L-rhamnose unit.

With the aim of developing an antibody-based detector, we have undertaken the synthesis of tetrasaccharide **A** and analogues thereof. This work reports a straightforward preparation of the anthrose building block starting from relatively cheap D-galactose [during the preparation of this manuscript, D. Crich reported a synthesis of the antigenic tetrasaccharide side chain from BclA starting also from D-galactose][8] which is suitable for use as glycosyl donor for further elongation. Moreover, using a derivative of anthrose, we raised polyclonal antibodies in rabbits and developed a sensitive and specific competitive enzyme immunoassay (EIA) which has potential as powerful and useful tool permitting both qualitative and quantitative detection of anthrose and its derivatives.

2. Results and discussion

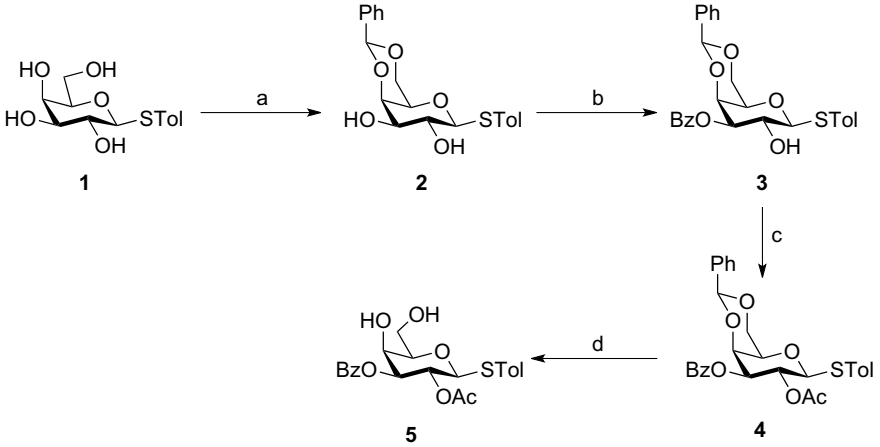
2.1. Preparation of anthrose derivative 16

The known *p*-methylphenyl 1-thio-β-D-galactopyranoside **1** was obtained in three steps from D-galactose according to the procedure reported by Wong and co-workers.[9] Reaction of **1** with benzaldehyde dimethyl acetal furnished the 4,6-*O*-benzylidene derivative **2**.[10] Selective benzoylation was performed with benzoyl cyanide in the presence of triethylamine as a base at −70 °C. Esterification occurred mainly at O-3, as expected for a β-D-galactopyranoside,[11] affording **3** in high yield (89%). Further O-acetylation at O-2, followed by acid-catalyzed O-debenzylidenation gave **5** in 77% overall yield (Scheme 1).

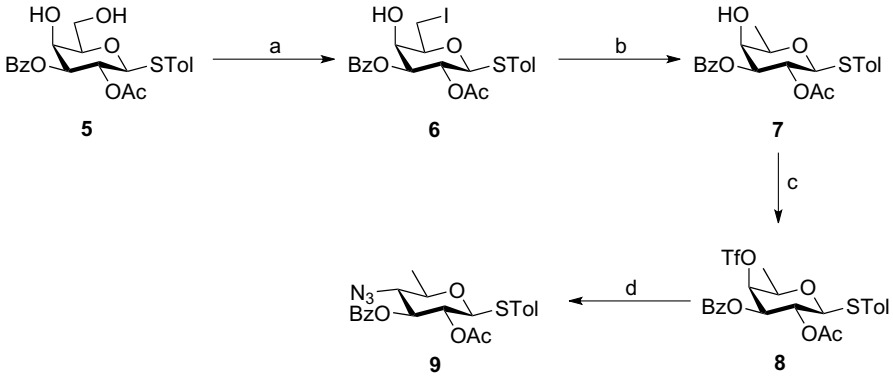
Transformation of the primary hydroxy group of diol **5** into a deoxy functionality was carried out by a two-step reaction sequence. The C-6 hydroxy group was first regioselectively iodinated. Preliminary attempts using Garegg–Samuelsson's conditions[12] (triphenylphosphine/imidazole/iodine in toluene at 40 °C) afforded **6** in poor yield (28%) together with unreacted **5**. Use of elevated temperatures (80 °C) did not provide the monoiodinated derivative **6** in higher yield, and led mainly to the iodination of both hydroxy groups. Recently, Firouzabadi et al.[13] reported the iodination of several alcohols using the reagent *N*-halosaccharin-triphenylphosphine to generate phosphonium halides as reactive phosphonium species in Mitsunobu reactions. In our hands, *N*-iodosaccharine appeared to be a good alternative to Garegg–Samuelsson procedure in the *galacto* series when regioselectivity is needed. Diol **5** was allowed to react with *N*-iodosaccharine-triphenylphosphine (12 equiv) in dichloromethane to afford the desired 6-deoxy-6-iodo-galactopyranoside derivative **6** in 67% yield.

Catalytic hydrogenation with Pd/C of **6** afforded 6-deoxygalactopyranoside **7**. Subsequent reaction of **7** with triflic anhydride followed by the nucleophilic displacement of the resulting 4-*O*-triflate in **8** with azide[14] gave the expected thioglycoside donor **9** in 85% yield over two steps (Scheme 2).

At this stage, a spacing arm bearing a masked amino group was introduced at the anomeric position to allow subsequent ligation to a carrier protein.



Scheme 1. Reagents and conditions: (a) benzaldehyde dimethyl acetal, *p*TsOH, MeCN, 5 h; (b) BzCN, Et_3N, CH_2Cl_2, MeCN, −70 °C, 2 h; (c) Ac_2O, pyridine, 12 h; (d) AcOH/water, 50 °C, 18 h.



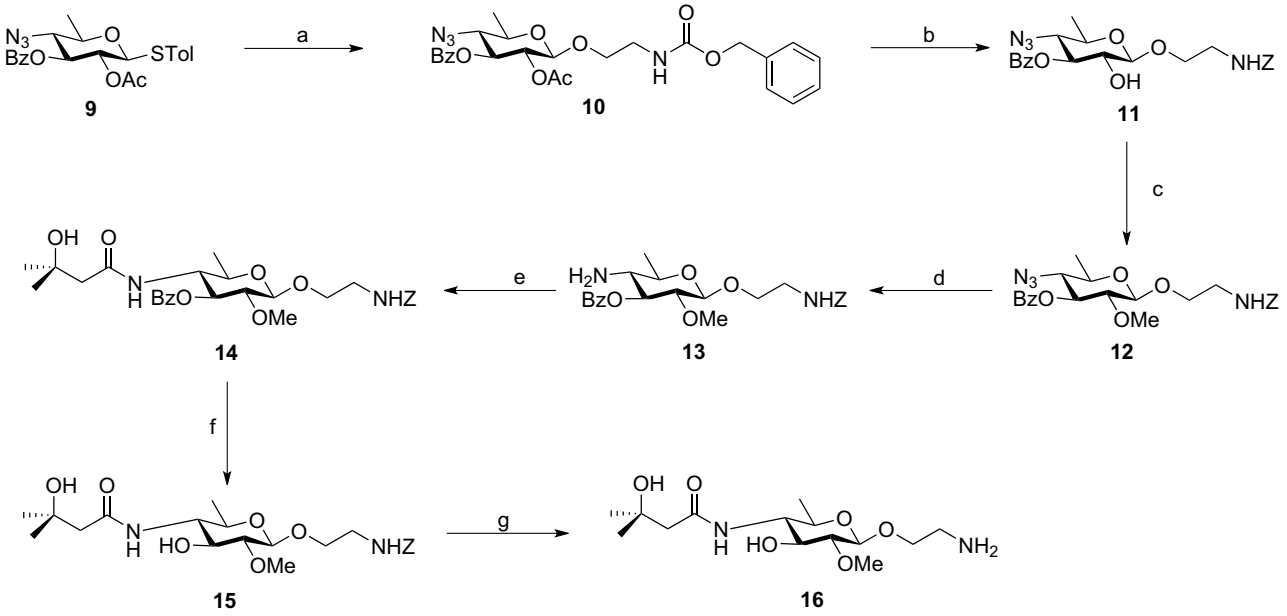
Scheme 2. Reagents and conditions: (a) Ph_3P, *N*-iodosaccharine, CH_2Cl_2, reflux, 20 h; (b) H_2, Pd/C, $NaHCO_3$, DMF, 12 h; (c) Tf_2O, pyridine, CH_2Cl_2, −20 °C, 2 h; (d) NaN_3, DMF, 80 °C, 1 h.

Benzyl *N*-(2-hydroxyethyl)carbamate was reacted with thioglycoside **9** in the presence of NIS and triflic acid as catalysts, resulting in the desired β-linked glycoside **10**. Selective deacetylation at O-2 of **10** was performed by acid-catalyzed methanolysis[15] to afford **11** in 70% yield. The characteristic methoxy group of anthrose was then introduced following the procedure reported by Porter and co-workers[16] by the reaction of **11** with iodomethane in the presence of silver(I) oxide affording the expected derivative **12** in 91% yield.

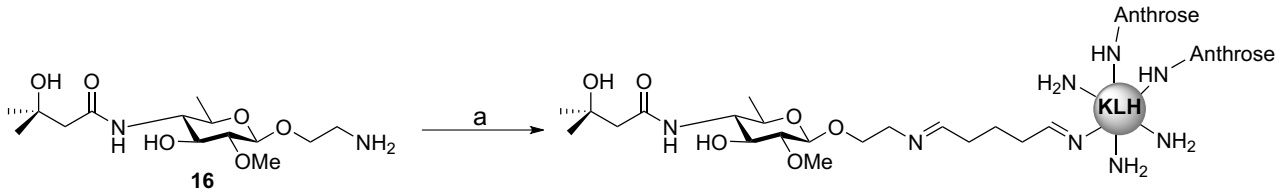
Reduction of the azide in **12** was achieved with sodium borohydride in the presence of nickel chloride to ensure the integrity of the carbamate group as previously described by Alpe and Oscarson,[17] affording amine **13**. Treatment of **13** with 3-hydroxy-3-methylbutyric acid in the presence of *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-(4,5-*b*)-pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) gave **14** in 66% overall yield (2 steps). Finally, 3-*O*-debenzoylation using base-catalyzed methanolysis conditions followed by catalytic hydrogenation of the carbamate group gave the target spacer arm equipped anthrose derivative **16** (Scheme 3).

2.2. Synthesis of an anthrose–KLH conjugate and investigation of the immune response

Due to the haptenic nature of anthrose, preparation of an immunogen with a carrier protein was necessary to obtain antibody production. Glutaraldehyde was used to ensure covalent coupling between the amino groups



Scheme 3. Reagents and conditions: (a) $HOCH_2CH_2NHCOOCH_2Ph$, TfOH, NIS, CH_2Cl_2, 0 °C, 45 min; (b) AcCl/MeOH, CH_2Cl_2, 0 °C to rt, 24 h; (c) Ag_2O, MeI, CH_2Cl_2, 8 d; (d) $NaBH_4$, $NiCl_2{\cdot}6H_2O$, EtOH, CH_2Cl_2, 1 h; (e) $HO(CH_3)_2CCH_2COOH$, HATU, DIPEA, CH_2Cl_2, 18 h; (f) MeONa, MeOH, 1 h; (g) H_2, AcOH, $Pd(OH)_2$, EtOH, 5 h.



Scheme 4. Reagents and conditions: (a) KLH (Keyhole Limpet Haemocyanin) carrier protein, glutaraldehyde 25%, phosphate buffer (pH 7.4), overnight, 4 °C.

of the carrier protein and the anthrose derivative **16** (Scheme 4).

With the anthrose derivative-KLH conjugate in hand, rabbits were immunized and boostered every two months. Rabbits were bled on a weekly basis after each booster. Antibody titre was measured by enzymatic immunoassay (EIA) using acetylcholinesterase (AChE) as tracer. The latter was prepared by covently coupling anthrose derivative **16** to AChE through a succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) linker to ensure a full immunoreactivity of the enzymatic tracer, and to avoid specific recognition of the spacer arm by antibodies (Scheme 5).

A good antibody titre (above 1/100,000) was measured for both immunized rabbits from the first booster injection onwards. This indicates a strong immunogenic potency of anthrose–KLH immunogen. Using the anthrose–AChE tracer, we optimized the dilution of antisera for the assay and performed standard curves using anthrose derivative **16** as a standard. As shown in Figure 1, the sensitivity at B/B_0 50% was satisfactory at 2.6 ng mL^{-1}.

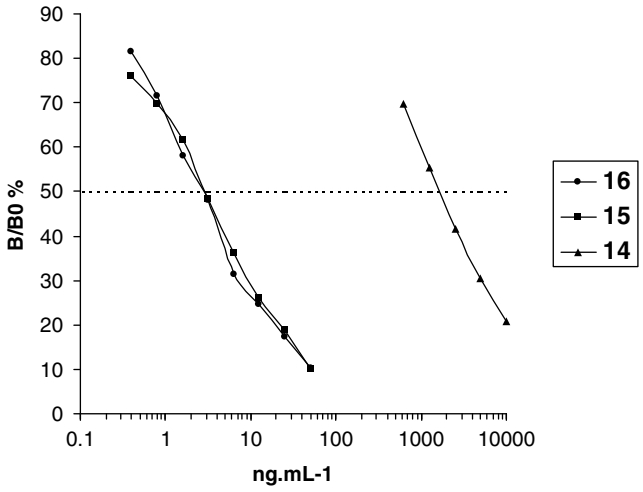
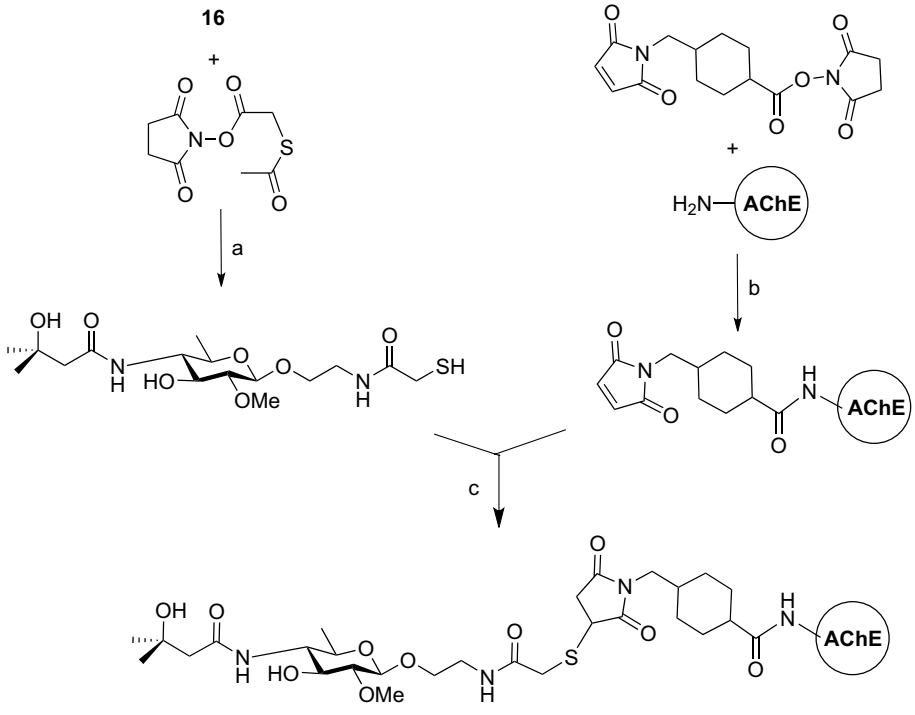


Figure 1. Standard enzyme immunoassay titration curves for **14**, **15** and **16**.



Scheme 5. Reagents and conditions: (a) borate buffer (pH 9), 20 °C, 1 h, then NH_2OH; (b) Ref. 19; (c) 18 h, 4 °C.

Table 1. Relative cross-reactivity obtained with **12**, **14** and **15** taking **16** as reference

Compound	B/B_0 50% (ng mL^{-1})	CR (%)
15	2.9	127
14	1711	0.26
12	>10,000	0.04

Moreover, a number of natural sugars (D-fucose, 6-deoxy-D-glucose, D-galactose, D-galactosamine, D-glucose, D-glucosamine) and three intermediates of the synthesis (**12**, **14** and **15**) were tested as competitors to better check the specificity of the assay. The results expressed in terms of percentage of cross-reactivity (CR) [(dose of **16** B/B_0 50% (M)/dose of analogue B/B_0 50% (M)) × 100] are summarized in Table 1.

Analogue **15** exhibited a full cross-reactivity, the sensitivity at B/B_0 50% being only slightly superior to that of anthrose derivative **16** (6 nM for compound **15** vs 8.1 nM for **16**), showing that the linker used for the preparation of the anthrose–KLH glycoconjugate is only negligibly involved in the recognition by the antibodies. None of the natural sugars, nor compound **12**, exhibited significant cross-reactivity demonstrating that the C-4 butanamido substituent of anthrose is strictly necessary for antibody recognition. On the other hand, the poor recognition of **14** indicates that modification at C-3 of anthrose has a decisive effect on the antibody recognition. These results support the hypothesis that the epitope recognized by the antibodies corresponds to the western moiety of the monosaccharide.

The results reported here clearly demonstrate that polyclonal antibodies directed against an analogue of a monosaccharide unit, specific of *B. anthracis* spores, can be obtained using the newly described hapten conjugate. We anticipate that such conjugate will be helpful in the development of immunological tools allowing the detection of anthrax spores.

3. Experimental

3.1. General methods

All reactions were monitored by TLC on Kieselgel 60 F254 (E. Merck). Detection was obtained by charring with vanillin. Silica Gel (E. Merck, 240–400 mesh) was used for chromatography. Solutions were concentrated under diminished pressure. Optical rotations were measured with a JASCO DIP-370 digital polarimeter, using a sodium lamp ($\lambda = 589$ nm) at 20 °C. All NMR experiments were performed at 300.13 and 500.13 MHz using Bruker DMX300 and DRX500 spectrometers equipped with a *Z*-gradient unit for pulsed-field gradient spectroscopy. Assignments were performed by stepwise identification using COSY, successive RELAY, HSQC and HMBC experiments using standard pulse programmes from the Brucker library. Chemical shifts are given relative to external TMS with calibration involving the residual solvent signals. When D_2O was used, TMS was used as internal standard reference in a previous ^{13}C NMR experiment performed in the same experimental conditions. The length of the 90° pulse was approximatively 7 μs (^1H NMR) and 10 μs (^{13}C NMR), respectively. 1D NMR spectra data were collected using 16 K data points. 2D experiments were run using 1 K data points and 512 time increments. The phase sensitive (TTPI) sequence was used and processing resulted in a 1K*1K (real–real) matrix. A 45° flip angle (3.5 μs) and a total recovery time of 5 s were used to ensure complete relaxation of the protons and quantitative measurements. The digital integration of the transformed spectra was performed after polynomial baseline correction.

Low resolution ESI mass spectra were obtained on a hybrid quadrupole/time-of-flight (Q-TOF) instrument, equipped with a pneumatically assisted electrospray (Z-spray) ion source (Micromass). High resolution mass spectra were recorded in positive mode on a ZabSpec TOF (Micromass, UK) tandem hybrid mass spectrometer with EBETOF geometry. The compounds were individually dissolved in 1:1 water–MeCN at a concentration of 10 μg cm^{-3}, and then infused into the electrospray ion source at a flow rate of 10 mm^3 mn^{-1} at 60 °C. The mass spectrometer was operated at 4 kV whilst scanning the magnet at a typical range of 4000–100 Da. The mass spectra were collected as continuum profile data. Accurate mass measurement was achieved using polyethylene glycol as internal reference with a resolving power set to a minimum of 10,000 (10% valley).

Elemental analyses were performed at the Service de Microanalyse of Université de Champagne-Ardennes in Reims, France. The samples were previously dried under diminished pressure for one week.

Acetylcholinesterase (AChE, EC 3.1.1.7) from the electric organs of the electric eel *Electrophorus electricus*, was purified by affinity chromatography as reported.[18] The modified Ellman's reagent was a soln of acetylthiocholine iodide (enzyme substrate) and 5,5-dithiobis-2-nitrobenzoic acid (chromogen) in phosphate buffer (pH 7.4). All reagents used for the enzyme immunoassay (EIA) were diluted in the following buffer (EIA buffer) 0.1 M potassium phosphate (pH 7.4) containing 0.15 M NaCl, 0.1% bovine serum albumin (BSA) and 0.01% sodium azide. The washing buffer was 10 mM phosphate (pH 7.4) containing 0.05% Tween 20. Solid phase EIA was performed on 96-well microtiter plates (immunoplate Maxisorb with certificate, Nunc, Roskilde Denmark) with as specialized microtitration equipment using automatic plate washer (ELX 405, Bio-Tek Instruments) and automatic plate-reader (Multiskan Ex labsystem thermo life sciences).

3.2. Synthesis of anthrose A

3.2.1. *p*-Methylphenyl 4,6-*O*-benzylidene-1-thio-β-D-galactopyranoside (2). *p*-Toluenesulfonic acid was added to a soln of **1** (3.75 g, 13.11 mmol) and benzaldehyde dimethyl acetal (2.4 mL, 15.73 mmol) in MeCN (50 mL) until the reaction mixture reached pH 3. The latter was stirred at room temperature for 5 h. Et_3N was added dropwise and the solvents were removed under diminished pressure. The residue was dissolved in CH_2Cl_2 and washed with saturated aq $NaHCO_3$ (3×60 mL) and brine (2×60 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated to give compound **2** (4.90 g, 100%) as a white powder which was used in the next step without further purification: R_f 0.1 (7:3 cyclohexane–EtOAc); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.59 (d, 2H, J 8 Hz, $SC_6H_4CH_3$), 7.41–7.33 (m, 5H, C_6H_5), 7.12 (d, 2H, $SC_6H_4CH_3$), 5.50 (s, 1H, H-7), 4.46 (d, 1H, $J_{1,2}$ 9 Hz, H-1), 4.38 (dd, 1H, $J_{5,6a}$ 1.5 Hz, $J_{6a,6b}$ 12.5 Hz, H-6a), 4.20 (dd, 1H, $J_{3,4}$ 1.1 Hz, $J_{4,5}$ 3.3 Hz, H-4), 4.02 (dd, 1H, $J_{5,6a}$ 1.5 Hz, H-6b), 3.70–3.60 (m, 2H, H-2, H-3), 3.54 (m, 1H, H-5), 2.55–2.52 (m, 2H, OH-2, OH-3), 2.36 (s, 3H, $SC_6H_4CH_3$); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 138.5–126.6 ($SC_6H_4CH_3$, C_6H_5), 101.4 (C-7), 86.9 (C-1), 75.3 (C-4), 73.7 (C-2, C-3), 69.9 (C-5), 69.3 (C-6), 68.7 (C-2, C-3), 21.2 ($SC_6H_4CH_3$); HRESIMS: calcd for $C_{20}H_{22}NaO_5{}^{32}S$ 397.1086; found, m/z 397.1085 $[M+Na]^+$.

3.2.2. *p*-Methylphenyl 3-*O*-benzoyl-4,6-*O*-benzylidene-1-thio-β-D-galactopyranoside (3). Compound **2** (10.52 g, 28.13 mmol) was dissolved in a mixture of dry MeCN (92 mL), CH_2Cl_2 (92 mL) and Et_3N (46 mL). The mixture was cooled to −70 °C and a soln of benzoyl cyanide (4.06 g, 30.94 mmol) in dry CH_2Cl_2 (70 mL) was added dropwise under argon. After 2 h, the mixture was washed with saturated $NaHCO_3$ (120 mL). The aq layer was extracted with CH_2Cl_2 (3×50 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash chromatography (7:3 cyclohexane–EtOAc) to give **3** (11.98 g, 89%) as a white powder: R_f 0.45 (7:3 cyclohexane–EtOAc); $[\alpha]_D$ +15 (c 0.22, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.07 (d, 2H, J 8 Hz, $SC_6H_4CH_3$), 7.65–7.38 (m, C_6H_5, $OCOC_6H_5$), 7.11 (d, 2H, $SC_6H_4CH_3$), 5.51 (s, 1H, H-7), 5.23 (dd, 1H, $J_{2,3}$ 9.7 Hz, $J_{3,4}$ 3.3 Hz, H-3), 4.65 (d, 1H, $J_{1,2}$ 9.5 Hz, H-1), 4.51 (dd, 1H, $J_{4,5}$ <1 Hz, H-4), 4.41 (dd, 1H, $J_{6a,6b}$ 12.4 Hz, $J_{5,6a}$ 1.3 Hz, H-6a), 4.14 (t, 1H, H-2), 4.05 (dd, 1H, $J_{5,6b}$ 1.3 Hz, H-6b), 3.66 (m, 1H, H-5), 2.38 (s, 3H, $SC_6H_4CH_3$); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 166.3 ($OCOC_6H_5$), 139.0–126.0 ($SC_6H_4CH_3$, C_6H_5, $OCOC_6H_5$), 100.6 (C-7), 87.7 (C-1), 75.2 (C-3), 73.8 (C-4), 69.7 (C-5), 69.1 (C-6), 65.7 (C-2), 21.2 ($SC_6H_4CH_3$); HRESIMS: calcd for $C_{27}H_{26}NaO_6{}^{32}S$ 501.1348; found, m/z 501.1361 $[M+Na]^+$. Anal. Calcd for $C_{27}H_{26}O_6S$: C, 67.76; H, 5.48. Found: C, 67.46; H, 5.18.

3.2.3. *p*-Methylphenyl 2-*O*-acetyl-3-*O*-benzoyl-4,6-*O*-benzylidene-1-thio-β-D-galactopyranoside (4). To a soln of **3** (3.14 g, 6.57 mmol) in pyridine (10 mL) was added Ac_2O (5 mL). The reaction mixture was stirred at room temperature overnight and was quenched by the addition of MeOH (30 mL) at 0 °C, then concentrated. The residue was dissolved in CH_2Cl_2 (30 mL). The organic layer was washed with saturated $KHSO_4$ (1×15 mL), saturated $NaHCO_3$ (1×15 mL) and water (1×15 mL), dried over Na_2SO_4, filtered and concentrated to give **4** (3.18 g, 93%) as a white powder which was used in the next step without further purification: R_f 0.5 (7:3 cyclohexane–EtOAc); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.03 (d, 2H, J 8 Hz, $SC_6H_4CH_3$), 7.61–7.37 (m, C_6H_5, $OCOC_6H_5$), 7.09 (d, 2H, $SC_6H_4CH_3$), 5.58 (t, 1H, $J_{1,2} = J_{2,3}$ 9.8 Hz, H-2), 5.51 (s, 1H, H-7), 5.26 (dd, 1H, $J_{3,4}$ 3 Hz, H-3), 4.78 (d, 1H, H-1), 4.56 (dd, 1H, $J_{4,5}$ <1 Hz, H-4), 4.43 (dd, 1H, $J_{6a,6b}$ 12.3 Hz, $J_{5,6a}$ <1 Hz, H-6a), 4.07 (dd, 1H, $J_{5,6b}$ <1 Hz, H-6b), 3.68 (m, 1H, H-5), 2.39 (s, 3H, $SC_6H_4CH_3$), 2.06 (s, 3H, CH_3CO); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 168.9 (CH_3CO), 165.9 ($OCOC_6H_5$), 139.0–126.0 ($SC_6H_4CH_3$, C_6H_5, $OCOC_6H_5$), 100.8 (C-7), 84.9 (C-1), 73.9 (C-3), 73.4 (C-4), 69.5 (C-5), 68.9 (C-6), 66.5 (C-2), 21.1 ($SC_6H_4CH_3$), 20.7 (CH_3CO); HRESIMS: calcd for $C_{29}H_{28}O_7Na$ ^{32}S 543.1453; found, m/z 543.1437 $[M+Na]^+$.

3.2.4. *p*-Methylphenyl 2-*O*-acetyl-3-*O*-benzoyl-1-thio-β-D-galactopyranoside (5). Compound **4** (1 g, 1.92 mmol) was dissolved in 4:1 AcOH–water (12 mL) and heated at 50 °C. After 18 h of stirring, the mixture was extracted with CH_2Cl_2 (5×40 mL) and the organic layers were combined and washed with saturated aq $NaHCO_3$ (6×100 mL), brine (2×100 mL), dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash chromatography (4:1 cyclohexane–EtOAc) to give **5** (0.69 g, 83%) as a white powder: R_f 0.4 (4:1 cyclohexane–EtOAc); $[\alpha]_D$ +84 (c 0.16, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.02 (d, 2H, J 8 Hz, $SC_6H_4CH_3$), 7.60–7.35 (m, $OCOC_6H_5$), 7.14 (d, 2H, $SC_6H_4CH_3$), 5.52 (t, 1H, $J_{1,2} = J_{2,3}$ 10 Hz, H-2), 5.15 (dd, 1H, $J_{3,4}$ 3 Hz, H-3), 4.75 (d, 1H, H-1), 4.35 (m, 1H, H-4), 3.92 (m, 2H, H-6a, H-6b), 3.71 (t, 1H, $J_{5,6a} = J_{5,6b}$ 5.5 Hz, H-5), 3.09 (br s, 1H, OH), 2.71 (br s, 1H, OH), 2.35 (s, 3H, $SC_6H_4CH_3$), 2.01 (s, 3H, CH_3CO); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 169.6 (CH_3CO), 165.8 ($OCOC_6H_5$), 139.0–128.0 ($SC_6H_4CH_3$, $OCOC_6H_5$), 86.6 (C-1), 77.9 (C-5), 75.5 (C-3), 68.1 (C-4), 67.4 (C-2), 62.6 (C-6), 21.1 ($SC_6H_4CH_3$), 20.8 (CH_3CO); HRESIMS: calcd for $C_{22}H_{24}O_7Na{}^{32}S$ 455.1158; found, m/z 455.1140 $[M+Na]^+$; Anal. Calcd for $C_{22}H_{24}O_7S$: C, 61.10; H, 5.59. Found: C, 61.29; H, 5.35.

3.2.5. *p*-Methylphenyl 2-*O*-acetyl-3-*O*-benzoyl-6-deoxy-6-iodo-1-thio-β-D-galactopyranoside (6). To **5** (300 mg, 0.69 mmol) in dry CH_2Cl_2 (50 mL) was added successively at room temperature triphenylphosphine (2.17 g, 8.28 mmol) and freshly prepared[13] *N*-iodosaccharine (2.56 g, 8.28 mmol). The reaction mixture was stirred at reflux for 20 h. The reaction mixture was cooled, saturated aq $NaHCO_3$ (20 mL) was added and the mixture was stirred for 5 min. Iodine was added in portions (about 300 mg each). When the CH_2Cl_2 supernatant remained iodine-coloured, the reaction mixture was stirred for an additional 10 min. Excess of iodine was removed by the addition of saturated aq $Na_2S_2O_3$ (20 mL). The mixture, diluted with CH_2Cl_2 (30 mL), was extracted with water (2 × 40 mL). The organic layer was then dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash chromatography (9:1 cyclohexane–EtOAc) to give **6** (252 mg, 67%) as a white powder: R_f 0.7 (7:3 cyclohexane–EtOAc); $[\alpha]_D$ +77 (*c* 0.24, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.03 (d, 2H, *J* 8 Hz, $SC_6H_4CH_3$), 7.60–7.28 (m, $OCOC_6H_5$), 7.17 (d, 2H, $SC_6H_4CH_3$), 5.46 (t, 1H, $J_{1,2} = J_{2,3}$ 10 Hz, H-2), 5.18 (dd, 1H, $J_{3,4}$ 3 Hz, H-3), 4.73 (d, 1H, H-1), 4.48 (br s, 1H, H-4), 3.87 (t, 1H, $J_{5,6a} = J_{5,6b}$ 6.9 Hz, H-5), 3.42 (d, 2H, H-6a, H-6b), 2.37 (s, 3H, $SC_6H_4CH_3$), 2.05 (s, 3H, CH_3CO); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 169.6 (CH_3CO), 165.6 ($OCOC_6H_5$), 139.0–128.0 ($SC_6H_4CH_3$, $OCOC_6H_5$), 86.8 (C-1), 78.8 (C-5), 75.3 (C-3), 67.9 (C-4), 66.9 (C-2), 21.2 ($SC_6H_4CH_3$), 20.8 (CH_3CO), 1.27 (C-6); HRESIMS: calcd for $C_{22}H_{23}O_6Na^{32}SI$ 565.0158; found, *m*/*z* 565.0148 $[M+Na]^+$. Anal. Calcd for $C_{22}H_{23}IO_6S$: C, 48.72; H, 4.27. Found: C, 48.78; H, 4.22.

3.2.6. *p*-Methylphenyl 2-*O*-acetyl-3-*O*-benzoyl-1-thio-β-D-fucopyranoside (7). To compound **6** (120 mg, 0.22 mmol) in dry DMF (40 mL) was added 20% palladium-on-charcoal catalyst (68 mg, 1.31 mmol) and $NaHCO_3$ (37 mg, 0.44 mmol). The reaction mixture was stirred overnight in a hydrogen atmosphere under 25 bars using Parr's apparatus. The catalyst was filtered on Celite 521 and the filtrate was concentrated. The residue was dissolved in CH_2Cl_2 (50 mL). The organic layer was extracted with water (3 × 25 mL), dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash chromatography (4:1 cyclohexane–EtOAc) to give **7** (92 mg, 76%) as a white powder: R_f 0.3 (4:1 cyclohexane–EtOAc); $[\alpha]_D$ +25 (*c* 0.15, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.03 (d, 2H, *J* 8 Hz, $SC_6H_4CH_3$), 7.60–7.41 (m, $OCOC_6H_5$), 7.16 (d, 2H, $SC_6H_4CH_3$), 5.44 (t, 1H, $J_{1,2} = J_{2,3}$ 10 Hz, H-2), 5.15 (dd, 1H, $J_{3,4}$ 3 Hz, H-3), 4.70 (d, 1H, H-1), 4.06 (br s, 1H, H-4), 3.84 (q, 1H, $J_{5,6}$ 6.5 Hz, H-5), 2.36 (s, 3H, $SC_6H_4CH_3$), 2.02 (s, 3H, CH_3CO), 1.39 (d, 3H, H-6); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 169.5 (CH_3CO), 165.8 ($OCOC_6H_5$), 139.0–128.0 ($SC_6H_4CH_3$, $OCOC_6H_5$), 86.3 (C-1), 75.9 (C-3), 74.6 (C-5), 70.0 (C-4), 67.3 (C-2), 21.1 ($SC_6H_4CH_3$), 20.8 (CH_3CO), 16.4 (C-6); HRESIMS: calcd for $C_{22}H_{24}O_6Na^{32}S$ 439.1191; found, *m*/*z* 439.1211 $[M+Na]^+$. Anal. Calcd for $C_{22}H_{24}O_6S$: C, 63.44; H, 5.81. Found: C, 63.08; H, 5.92.

3.2.7. *p*-Methylphenyl 2-*O*-acetyl-4-azido-3-*O*-benzoyl-4,6-dideoxy-1-thio-β-D-glucopyranoside (9). To a soln of **7** (1.5 g, 3.61 mmol) in dry CH_2Cl_2 (40 mL) containing pyridine (780 μL) under argon was added dropwise at −20 °C a soln of trifluoromethanesulfonic anhydride (898 μL, 5.42 mmol) in dry CH_2Cl_2 (10 mL). The reaction mixture was stirred at −20 °C for 2 h. The mixture was then diluted in CH_2Cl_2 (40 mL) and extracted with water (40 mL). The organic layer was washed with saturated aq $KHSO_4$ (40 mL), saturated aq $NaHCO_3$ (40 mL) and brine, dried over Na_2SO_4, filtered and concentrated. Triflate derivative **8** (1.93 g) was obtained as a white powder and was used in the next step without purification. To a soln of **8** (1.93 g, 3.52 mmol) in dry DMF (50 mL) was added sodium azide (916 mg, 14.09 mmol). The reaction mixture was stirred at 80 °C for 1 h, then concentrated. The residue was dissolved in EtOAc (70 mL). The organic layer was washed with water (30 mL) and brine (30 mL), dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash chromatography (4:1 cyclohexane–EtOAc) to give **9** (1.35 g, 85% yield over two steps) as a white powder: R_f 0.5 (4:1 cyclohexane–EtOAc); $[\alpha]_D$ +2 (*c* 0.27, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.03 (d, 2H, *J* 8 Hz, $SC_6H_4CH_3$), 7.62–7.28 (m, $OCOC_6H_5$), 7.16 (d, 2H, $SC_6H_4CH_3$), 5.41 (t, 1H, $J_{2,3} = J_{3,4}$ 9.6 Hz, H-3), 5.07 (t, 1H, $J_{1,2}$ 9.6 Hz, H-2), 4.71 (d, 1H, H-1), 3.50 (dq, 1H, $J_{4,5}$ 9.6 Hz, $J_{5,6}$ 6.9 Hz, H-5), 3.38 (t, 1H, H-4), 2.37 (s, 3H, $SC_6H_4CH_3$), 2.06 (s, 3H, CH_3CO), 1.46 (d, 3H, H-6); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 169.3 (CH_3CO), 165.6 ($OCOC_6H_5$), 139.0–128.0 ($SC_6H_4CH_3$, $OCOC_6H_5$), 85.8 (C-1), 74.9 (C-3, C-5), 70.2 (C-2), 65.8 (C-4), 21.1 ($SC_6H_4CH_3$), 20.6 (CH_3CO), 18.5 (C-6); HRESIMS: calcd for $C_{22}H_{23}N_3O_5Na^{32}S$ 464.1256; found, *m*/*z* 464.1268 $[M+Na]^+$. Anal. Calcd for $C_{22}H_{23}N_3O_5S$: C, 59.85; H, 5.25; N, 9.52. Found: C, 59.19; H, 4.81; N, 8.20.

3.2.8. *N*-Benzyloxycarbonylaminoethyl 2-*O*-acetyl-4-azido-3-*O*-benzoyl-4,6-dideoxy-β-D-glucopyranoside (10). To a soln of **9** (1 g, 2.27 mmol) and benzyl *N*-(2-hydroxyethyl)carbamate (890 mg, 4.54 mmol) in dry CH_2Cl_2 (100 mL) containing molecular sieves 4 Å was added *N*-iodosuccinimide (1.02 g, 4.54 mmol). The reaction mixture was stirred under argon for 1 h at room temperature, then cooled at 0 °C. Trifluoromethanesulfonic acid (100 μL, 1.14 mmol) was added. After 45 min, the reaction was quenched by the addition of Et_3N. The molecular sieves were filtered and the reaction mixture was diluted with CH_2Cl_2 (100 mL). The

organic layer was washed with saturated aq $Na_2S_2O_3$ (60 mL), water (60 mL), dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash chromatography (4:1 cyclohexane–EtOAc) to give **10** (813 mg, 70%) as an oil: R_f 0.3 (7:3 cyclohexane–EtOAc); $[\alpha]_D$ −6 (*c* 0.13, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz): δ 8.02–7.31 (m, $OCOC_6H_5$, $OCH_2C_6H_5$), 5.39 (t, 1H, $J_{2,3} = J_{3,4}$ 9.7 Hz, H-3), 5.23 (br s, 1H, NH), 5.12 (s, 2H, $OCH_2C_6H_5$), 5.08 (dd, 1H, $J_{1,2}$ 8.0 Hz, $J_{2,3}$ 9.7 Hz, H-2), 4.52 (d, 1H, H-1), 3.86 (m, 1H, OCH_2CH_2NHZ), 3.70 (m, 1H, OCH_2CH_2NHZ), 3.43 (m, 4H, H-4, H-5, OCH_2CH_2NHZ), 1.89 (s, 3H, CH_3CO), 1.41 (d, 3H, $J_{5,6}$ 6.0 Hz, H-6); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 169.6 (CH_3CO), 165.6 ($OCOC_6H_5$), 156.3 ($NHCOOCH_2C_6H_5$), 140.6–128.0 ($NHCOOCH_2C_6H_5$, $OCOC_6H_5$), 100.7 (C-1), 73.6 (C-3), 71.6 (C-2), 70.9 (C-5), 69.3 (OCH_2CH_2NHZ), 66.7 ($NHCOOCH_2C_6H_5$), 65.9 (C-4), 40.8 (OCH_2CH_2NHZ), 20.4 (CH_3CO), 18.2 (C-6); HRESIMS: calcd for $C_{25}H_{28}N_4NaO_8$ 535.1805; found, *m*/*z* 535.1783 $[M+Na]^+$. Anal. Calcd for $C_{25}H_{28}N_4O_8$: C, 58.59; H, 5.51; N, 10.93. Found: C, 59.05; H, 5.70; N, 10.45.

3.2.9. *N*-Benzyloxycarbonylaminoethyl 4-azido-3-*O*-benzoyl–4,6-dideoxy-β-D-glucopyranoside (11). To a soln of **10** (1.11 g, 2.17 mmol) in dry CH_2Cl_2 (70 mL) was added dropwise a soln of acetyl chloride (4.6 mL, 65.04 mmol) in MeOH (120 mL). The reaction mixture was stirred at 0 °C for 15 min, then at room temperature for 24 h. The organic layer was washed with saturated aq $NaHCO_3$ (40 mL), water (40 mL), then dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash chromatography (7:3 cyclohexane–EtOAc) to give compound **11** (713 mg, 70%) as an oil: R_f 0.3 (3:2 cyclohexane–EtOAc); $[\alpha]_D$ −34 (*c* 0.11, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz): δ 8.07–7.31 (m, $OCOC_6H_5$, $OCH_2C_6H_5$), 5.46 (br s, 1H, NH), 5.24 (t, 1H, $J_{2,3} = J_{3,4}$ 9.4 Hz, H-3), 5.06 (s, 2H, $OCH_2C_6H_5$), 4.34 (d, 1H, $J_{1,2}$ 7.7 Hz, H-1), 3.88 (m, 1H, OCH_2CH_2NHZ), 3.71 (m, 1H, OCH_2CH_2NHZ), 3.57 (m, 1H, H-2), 3.39 (m, 4H, H-5, OCH_2CH_2NHZ, OH), 3.30 (t, 1H, $J_{3,4} = J_{4,5}$ 9.4 Hz, H-4), 1.38 (d, 3H, $J_{5,6}$ 6.0 Hz, H-6); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 166.5 ($OCOC_6H_5$), 156.7 ($NHCOOCH_2C_6H_5$), 136.4–128.1 ($NHCOOCH_2C_6H_5$, $OCOC_6H_5$), 102.9 (C-1), 76.2 (C-3), 72.8 (C-2), 70.7 (C-5), 69.9 (OCH_2CH_2NHZ), 66.8 ($NHCOOCH_2C_6H_5$), 65.9 (C-4), 41.0 (OCH_2CH_2NHZ), 18.3 (C-6); HRESIMS: calcd for $C_{23}H_{26}N_4NaO_7$ 493.1699; found, *m*/*z* 493.1694 $[M+Na]^+$. Anal. Calcd for $C_{23}H_{26}N_4O_7$: C, 58.72; H, 5.57; N, 11.91. Found: C, 58.37; H, 5.57; N, 11.89.

3.2.10. *N*-Benzyloxycarbonylaminoethyl 4-azido-3-*O*-benzoyl-6-dideoxy-2-*O*-methyl-β-D-glucopyranoside (12). To a soln of **11** (620 mg, 1.32 mmol) in dry CH_2Cl_2 (50 mL) were added silver(I) oxide (1.53 g, 6.60 mmol). The reaction mixture was stirred at room temperature for 1 h. Iodomethane (822 μL, 13.2 mmol) was then added dropwise and the reaction mixture was stirred at room temperature for 8 days. Silver(I) oxide was filtered over Celite 521 and the filtrate was concentrated. The residue was purified by flash chromatography (4:1 cyclohexane–EtOAc) to give **12** (581 mg, 91%) as an oil: R_f 0.5 (3:2 cyclohexane–EtOAc); $[\alpha]_D$ −6 (*c* 0.11, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz): δ 8.11–7.29 (m, $OCOC_6H_5$, $OCH_2C_6H_5$), 5.31 (t, 2H, NH, $J_{2,3} = J_{3,4}$ 9.7 Hz, H-3), 5.13 (s, 2H, $OCH_2C_6H_5$), 4.40 (d, 1H, $J_{1,2}$ 7.7 Hz, H-1), 3.92 (m, 1H, OCH_2CH_2NHZ), 3.79 (m, 1H, OCH_2CH_2NHZ), 3.42 (m, 6H, H-5, OCH_2CH_2NHZ, OCH_3), 3.30 (t, 1H, $J_{3,4} = J_{4,5}$ 9.7 Hz, H-4), 3.24 (dd, 1H, H-2), 1.41 (d, 3H, $J_{5,6}$ 6.1 Hz, H-6); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 165.5 ($OCOC_6H_5$), 156.3 ($NHCOOCH_2C_6H_5$), 136.5–128.0 ($NHCOOCH_2C_6H_5$, $OCOC_6H_5$), 103.2 (C-1), 81.6 (C-2), 74.8 (C-3), 70.6 (C-5), 69.6 (OCH_2CH_2NHZ), 66.7 ($NHCOOCH_2C_6H_5$), 66.0 (C-4), 60.7 (OCH_3), 41.1 (OCH_2CH_2NHZ), 18.2 (C-6); HRESIMS: calcd for $C_{24}H_{28}N_4NaO_7$ 507.1856; found, *m*/*z* 507.1839 $[M+Na]^+$.

3.2.11. *N*-Benzyloxycarbonylaminoethyl 3-*O*-benzoyl-4,6-dideoxy-4-(3-hydroxy-3-methylbutanamido)-2-*O*-methyl-β-D-glucopyranoside (14). To a soln of **12** (400 mg, 0.83 mmol) in a mixture of dry CH_2Cl_2 (12 mL) and EtOH (58 mL) was added $NaBH_4$ (63 mg, 1.65 mmol) and a catalytic amount of $NiCl_2{\cdot}6H_2O$. The reaction mixture was stirred at room temperature for 1 h and concentrated. The residue was dissolved in CH_2Cl_2 (50 mL). The organic layer was washed with water (20 mL) and brine (20 mL), dried over Na_2SO_4, filtered and concentrated. Compound **13** (291 mg) was used in the next step without purification. To a soln of compound **13** (291 mg, 0.64 mmol) in dry CH_2Cl_2 (15 mL) were added dropwise in the following order: 3-hydroxy-3-methylbutanoic acid (121 μL, 0.96 mmol), HATU (365 mg, 0.96 mmol) then DIPEA (159 μL, 0.96 mmol). The reaction mixture was stirred at room temperature under argon for 18 h. The residue was diluted with CH_2Cl_2 (20 mL). The organic layer was washed with saturated aq $NaHCO_3$ (2 × 10 mL), water (10 mL), dried over Na_2SO_4, filtered and concentrated. The residue was purified by flash chromatography (1:1 cyclohexane–EtOAc) to give **14** (305 mg, 66% yield over two steps) as a white powder: R_f 0.5 (3:7 cyclohexane–EtOAc); $[\alpha]_D$ +26 (*c* 0.08, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz): δ 8.05–7.31 (m, $OCOC_6H_5$, $OCH_2C_6H_5$), 6.17 (d, 1H, $J_{NH,\ H4}$ 10.1 Hz, $NHCOCH_2C(CH_3)_2OH$), 5.39 (m, 1H, $NHCOOCH_2C_6H_5$), 5.16 (dd, 1H, $J_{2,3}$ 9.4 Hz, $J_{3,4}$ 10.1 Hz, H-3), 5.11 (s, 2H, $OCH_2C_6H_5$), 4.40 (d, 1H, $J_{1,2}$ 7.7 Hz, H-1), 4.11 (q, 1H, $J_{3,4} = J_{4,5} = J_{NH,\ H4}$ 10.1 Hz, H-4), 3.92 (m, 1H, OCH_2CH_2NHZ), 3.79 (m, 1H, OCH_2CH_2NHZ), 3.48 (s, 3H, OCH_3), 3.40 (m, 3H, H-5, OCH_2CH_2NHZ), 3.31 (dd,

1H, H-2), 2.25 (d, 1H, J 14.7 Hz, NHCOCH_2C(CH$_3$)$_2$-OH), 2.17 (d, 1H, NHCOCH_2C(CH$_3$)$_2$OH), 1.29 (d, 3H, $J_{5,6}$ 6.2 Hz, H-6), 1.10 (s, 3H, NHCOCH$_2$C(CH_3)$_2$-OH), 0.98 (s, 3H, NHCOCH$_2$C(CH_3)$_2$OH); ^{13}C NMR (CDCl$_3$, 125 MHz): δ 172.5 (NHCOCH$_2$C(CH$_3$)$_2$OH), 167.3 (OCOC$_6$H$_5$), 156.4 (NHCOOCH$_2$C$_6$H$_5$), 136.5–128.0 (NHCOOCH$_2$$C_6H_5$, OCO$C_6H_5$), 103.2 (C-1), 81.5 (C-2), 75.0 (C-3), 71.6 (C-5), 69.6 (OCH$_2$CH$_2$-NHZ), 69.3 (NHCOCH$_2$$C$(CH$_3$)$_2$OH), 66.7 (NHCOO-$CH_2C_6H_5$), 60.9 (OCH$_3$), 54.6 (C-4), 47.9 (NHCO-CH$_2$C(CH$_3$)$_2$OH), 41.1 (OCH$_2$$CH_2$NHZ), 29.1 (NH-COCH$_2$C($CH_3$)$_2$OH), 29.0 (NHCOCH$_2$C($CH_3$)$_2$OH), 17.8 (C-6); HRESIMS: calcd for $C_{29}H_{38}N_2NaO_9$ 581.2475; found, m/z 581.2476 [M+Na]$^+$.

3.2.12. *N*-Benzyloxycarbonylaminoethyl 4,6-dideoxy-4-(3-hydroxy-3-methylbutanamido)-2-*O*-methyl-β-D-glucopyranoside (15). A soln of sodium methoxide (1.1 mL, 1 M in MeOH) was added dropwise at 0 °C to a soln of **14** (155 mg, 0.28 mmol) in MeOH (15 mL). The reaction mixture was stirred at room temperature for 1 h. An acidic resin (Amberlite IR 120 H$^+$) was added to neutralize MeONa. The resine was filtered and the solvent was removed. The residue was then purified by flash chromatography (1:9 cyclohexane–EtOAc) to give **15** (82 mg, 65 %) as a white powder: R_f 0.1 (1:9 cyclohexane–EtOAc); $[\alpha]_D$ −16 (c 0.13, CHCl$_3$); ^{1}H NMR (CDCl$_3$, 500 MHz): δ 7.39–7.30 (m, 5H, OCH$_2$C$_6$$H_5$), 6.26 (d, 1H, $J_{NH,H4}$ 8.3 Hz, NHCOCH$_2$C(CH$_3$)$_2$OH), 5.44 (m, 1H, NHCOOCH$_2$C$_6$H$_5$), 5.14 (s, 2H, OCH_2C$_6$H$_5$), 4.29 (d, 1H, $J_{1,2}$ 7.8 Hz, H-1), 3.93–3.63 (m, 3H, H-4, OCH_2CH$_2$NHZ), 3.61 (s, 3H, OCH$_3$), 3.57–3.46 (m, 4H, H-3, H-5, OCH$_2$CH_2NHZ), 3.03 (dd, 1H, $J_{2,3}$ 8.7 Hz, H-2), 2.41 (s, 2H, NHCOCH_2-C(CH$_3$)$_2$OH), 1.34 (s, 3H, NHCOCH$_2$C(CH_3)$_2$OH), 1.33 (s, 3H, NHCOCH$_2$C(CH_3)$_2$OH), 1.30 (d, 3H, $J_{5,6}$ 6.0 Hz, H-6); ^{13}C NMR (CDCl$_3$, 125 MHz): δ 173.0 (NHCOCH$_2$C(CH$_3$)$_2$OH), 156.4 (NHCOOCH$_2$C$_6$H$_5$), 136.5–128.0 (NHCOOCH$_2$$C_6H_5$), 103.3 (C-1), 83.8 (C-2), 74.3 (C-3), 70.8 (C-5), 69.9 (NHCOCH$_2$-C(CH$_3$)$_2$OH), 69.4 (OCH$_2$CH$_2$NHZ), 66.7 (NHCOO-CH$_2$C$_6$H$_5$), 60.8 (OCH$_3$), 57.0 (C-4), 48.7 (NHCO-CH$_2$C(CH$_3$)$_2$OH), 41.2 (OCH$_2$$CH_2$NHZ), 29.8 (NH-COCH$_2$C($CH_3$)$_2$OH), 29.2 (NHCOCH$_2$C($CH_3$)$_2$OH), 17.9 (C-6); HRESIMS: calcd for $C_{22}H_{34}N_2NaO_8$ 477.2213; found, m/z 477.2201 [M+Na]$^+$. Anal. Calcd for $C_{22}H_{34}N_2O_8$: C, 58.14; H, 7.54; N, 6.16. Found: C, 58.21; H, 7.70; N, 6.12.

3.2.13. Ethylamino 4,6-dideoxy-4-(3-hydroxy-3-methylbutanamido)-2-*O*-methyl-β-D-glucopyranoside (16). To a soln of **15** (82 mg, 0.18 mmol) in EtOH (18 mL) was added AcOH (12 mL) and a catalytic amount of Pd(OH)$_2$. The reaction mixture was stirred at room temperature under hydrogen atmosphere. The reaction was controlled by mass spectrometry. After 5 h, the reaction was complete. The mixture was then centrifuged and the supernatant concentrated. The residue was dissolved in water (20 mL) and washed with CHCl$_3$ (15 mL). The organic layer was extracted with water (5 × 10 mL). The aq layers were combined and concentrated. The residue was purified on a resin cation exchanger (Bio-RAD, AG MP-50 Resin, analytical grade 100–200 mesh). A glass column (400 mm × 15 mm) was packed with the resin (25 mL) which was washed with water and then acidified to pH 1 with a soln of HCl (1 N). An acidified aq soln of the residue (5 mL, pH 1) was added on the top of the column. The resin was then washed with water (2 × 50 mL) and **16** was eluted with a soln of 10% ammonia (3 × 50 mL). After lyophilization, compound **16** (43 mg, 75%) was obtained as a yellow solid: ^{1}H NMR (D$_2$O, 500 MHz): δ 4.52 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1), 4.00 (m, 1H, OCH_2CH$_2$NH$_2$), 3.84 (m, 1H, OCH_2CH$_2$NH$_2$), 3.65 (m, 1H, H-4), 3.62 (s, 3H, OCH$_3$), 3.60–3.54 (m, 2H, H-3, H-5), 3.13 (dd, 1H, $J_{1,2} = J_{2,3}$ 8.5 Hz, H-2), 3.06 (m, 2H, OCH$_2$-CH_2NH$_2$), 2.47 (s, 2H, NHCOCH_2C(CH$_3$)$_2$OH), 1.32 (s, 3H, NHCOCH$_2$C(CH_3)$_2$OH), 1.31 (s, 3H, NHC-OCH$_2$C(CH_3)$_2$OH), 1.24 (d, 3H, $J_{5,6}$ 5.9 Hz, H-6); ^{13}C NMR (CDCl$_3$, 125 MHz): δ 176.1 (NHCOCH$_2$C(CH$_3$)$_2$OH), 104.0 (C-1), 85.2 (C-2), 75.0 (C-3), 72.9 (C-5), 72.2 (OCH$_2$CH$_2$NH$_2$), 71.0 (NHCOCH$_2$$C$(CH$_3$)$_2$OH), 62.2 (OCH$_3$), 58.6 (C-4), 50.9 (NHCOCH$_2$C(CH$_3$)$_2$OH), 41.8 (OCH$_2$$CH_2NH_2$), 30.3 (NHCOCH$_2$C($CH_3$)$_2$OH), 30.1 (NHCOCH$_2$C($CH_3$)$_2$-OH), 18.9 (C-6); HRESIMS: calcd for $C_{14}H_{28}N_2NaO_6$ 321.2026; found, m/z 321.2023 [M+Na]$^+$.

3.3. Immunoassay

3.3.1. Antiserum production. For the production of antibodies, the synthetic anthrose derivative **16** was covalently linked to KLH (Keyhole Limpet Haemocyanin) using glutaraldehyde which cross-links primary amino groups of the carrier protein to the spacer arm of the monosaccharide analogue.

Anthrose derivative **16** (1.85 mg, 5.8 μmol) dissolved in 50% MeOH was reacted with KLH (20 mg, 0.29 μmol) and glutaraldhehyde (25 μL, 25% in water) in phosphate buffer (0.1 M, 5 mL, pH 7.4). The reaction mixture was stirred overnight at 4 °C in the dark before aliquoting and keeping frozen at 4 °C.

Rabbits (Blanc de Bouscat, Evic, France) were immunized and boostered every two months with immunogen (1 mg in complete Freund's adjuvant) in multiple subcutaneous injections according to the procedure described by Vaitukaitis.[19] Rabbits were bled from the central ear artery before the first immunization (serum S_0), then on a weekly basis after each booster (serums S_1, S_2, …). The sera were kept at 4 °C in the presence of sodium azide (0.01% final concentration).

3.3.2. Preparation of the enzymatic tracer. The tracer was obtained by covalently coupling anthrose derivative **16** to AChE using the procedure previously described for haptens[20] and proteins.[21] In the first step, a thiol group was introduced into hapten **16** by the reaction of its primary amino group with *N*-succinimidyl-*S*-acetyl thioacetate (SATA). Briefly, a soln of SATA (2.3 mg, 10 μmol) and anthrose derivative **16** (0.32 mg, 1 μmol) in a borate buffer (250 μL, 0.1 M, pH 8.5) was stirred for 1 h at 20 °C. The thiolated derivative was purified using a Sep-Pak-C18 cartridge (Waters, Milford, USA) before deprotecting the thiol function in the presence of hydroxylamine. The enzyme conjugate was obtained by mixing the thiolated–anthrose derivative **16** (0.8 nmol) with AChE-SMCC (0.1 nmol) prepared as previously described[21] for 18 h reaction at 4 °C. The enzyme conjugate was purified by molecular sieve chromatography on a Bio-Gel A 1.5 M column (90 × 1.5 cm Bio-Rad) eluted with EIA buffer and was stored at −20 °C until use.

3.3.3. Competitive EIA procedure. Competitive EIA was performed in 96-well microtitre plates coated with mouse monoclonal anti-rabbit immunoglobulin antibodies to ensure separation between the free and bound moieties of the enzymatic tracer during the immunological reaction. The assay was performed in a total vol of 150 μL, each reagent—enzymatic tracer, diluted rabbit antisera and standard (anthrose derivative **16**, natural sugars or intermediates **12**, **14** and **15**)—being added in a 50 μL volume. The optimal working dilution of antiserum was previously determined by serial dilution experiment.

After 18 h of immunological reaction at 4 °C, the plates were washed with the washing buffer and AChE's substrate (200 μL, Ellman's reagent) was added in each well. After 1 or 2 h of gentle shaking in the dark at room temperature, the absorbance of each well was measured at 414 nm. The results were expressed in terms of $(B/B_0) \times 100$ as function of concentration (logarithmic scale) where B and B_0 represent bound enzymatic activity in the presence and absence of the competitor (antigen or analogues), respectively. A linear log–logit transformation was used to fit the calibration curve. The sensitivity of the assay was characterized by the dose of standard inducing a 50% lowering of the binding observed in the absence of competitor (B/B_0 50%). Non-specific binding represents less than 0.1% of the total enzyme activity. All experiments were made in duplicate and quadruplicate for B_0.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2007.11.030.

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