SYNTHESIS AND BIOLOGICAL EVALUATION OF A TRISACCHARIDE REPEATING UNIT DERIVATIVE OF *STREPTOCOCCUS PNEUMONIAE* 19A CAPSULAR POLYSACCHARIDE

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Abstract: *Streptococcus pneumoniae* (SP) is a common human pathogen associated with a broad spectrum of diseases and it is still a leading cause of mortality and morbidity worldwide, especially in children. Moreover, SP is increasingly associated with drug resistance. Vaccination against the pathogen may thus represent an important strategy to overcome its threats to human health. In this context, revealing the molecular determinants of SP immunoreactivity may be relevant for the development of novel molecules with therapeutic perspectives as vaccine components. Serogroup 19 comprises the immune-cross reactive types 19F, 19A, 19B and 19C and it accounts for a high percentage of invasive pneumococcal diseases, mainly caused by serotypes 19F and 19A. Herein, we report the synthesis and biological evaluation of an aminopropyl derivative of the trisaccharide repeating unit of SP 19A. We compare two different synthetic strategies, based on different disconnections between the three monosaccharides which make up the final trisaccharide, to define the best approach for the preparation of the trisaccharide. Synthetic accessibility to the trisaccharide repeating unit lays the basis for the development of more complex biopolymer as well as saccharide conjugates. We also evaluate the binding affinity of the trisaccharide for anti-19A and anti-19F sera and discuss the relationship between the chemical properties of the trisaccharide unit and biological activity.

1. Introduction

Streptococcus pneumoniae (SP) represents a relevant cause of infections associated with high mortality and morbidity: invasive pneumococcal disease (IPD) indeed still shows a high incidence especially in children and in the elderly. Capsular polysaccharides (CPSs) are the primary determinants of the pathogenicity of the bacterium, and account for the classification of SP in more than 90 serotypes.[1] A limited subset of serotypes is responsible for the majority of pneumococcal infections, and representatives of such subsets are contained in commercial licensed vaccines (for example PCV7, Prevnar 7 - Wyeth Pharmaceuticals, contains serotypes 4, 6B, 9V, 14, 18C, 19F and 23F). Indeed, capsular polysaccharides (CPSs) are immunogenic, and the generation of type-specific antibodies to CPS is protective.[2] The pattern of predominant IPD associated serotypes, subjected to a natural fluctuation over time, contains also serotypes of low immunogenicity, such as 6, 14, 19 and 23, where low immunogenicity unfortunately does not equate to low virulence, especially in immune-naive hosts.[3] Consequently, a lower vaccination efficacy has been observed for these serotypes.[4] This is probably not associated to the absolute antibody concentration generated by the vaccine towards each single different serotype, but, more likely, to the increased amount of antibodies required for killing less immunogenic serotypes. Serogroup 19, which comprises the immune-cross reactive types 19F, 19A, 19B and 19C, belongs to this group, and deserves particular attention since it globally accounts for a high percentage of IPD. Serogroup 19 IPD are mainly caused by serotypes 19F and 19A, and, in particular, type 19F is one of the most common causes of IPD in children.[5] The low immunogenicity of this serotype can be explained by the thickness of the 19F capsule and increased resistance to complement deposition, which is the

event required to opsonize pneumococci, facilitate phagocytosis and pathogen clearance. Serogroup 19 has also attracted the interest of the research community because it represents one of the most significant cases to investigate cross-protective immunity. Capsules of serotypes 19F and 19A are isopolymers, differing only in one glycosidic linkage (glucose to rhamnose, Figure 1). The high similarity of the two capsular structures suggested the inclusion of only SP 19F in the formulation of the first glycoconjugate vaccine PCV7, since antibodies to some CPS may cross-react with related types providing protection against additional types. Indeed, this is what happened for the vaccine-type 6B, included in PCV7, since 6B-induced antibodies resulted able to cross protect against the structurally similar 6A CPS, with high effectiveness against 6A disease.[6] Unfortunately, antibodies elicited by 19F antigen present in PCV7 provided limited cross-reactive protection against 19A disease, with the consequence of increasing non-vaccine 19A serotype carriage and virulence among population in a process defined "serotype replacement".[7] Indeed, most of the PCV7 recipients achieved a significant concentration of antibodies for the vaccine-associated serotype, but the absence of 19A opsonophagocytic activity indicates that such antibodies are not-functional against 19A.[8] The immunogenicity of the19F vaccine serotype, and the level of cross-opsonophagocytic antibodies can be influenced by the conjugation method used to connect the antigenic saccharide fragment to the T-helper peptide, like reductive amination *vs* cyanylation.[9] The lack of antibody-related cross-protection between serotypes 19F and 19A may be alternatively related to conformational differences between the two CPS structures.[10] Of note, the problem to induce protection against 19A disease was overcome after the replacement of PCV7 with PCV13, that contains antigenic CPSs of both serotypes 19A and 19F. Remarkably, a higher level of serotype 19F IgG was found in the sera of patients immunized with PCV13 with respect to PCV7 recipients, suggesting a contribution of cross-reactive 19A antibodies to the higher 19F opsonophagocytic activity titers induced by PCV13.[8]

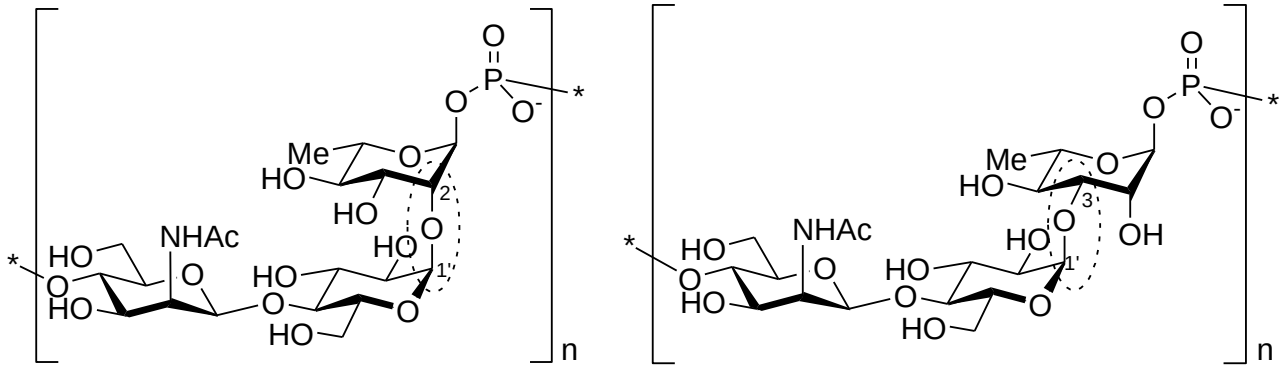
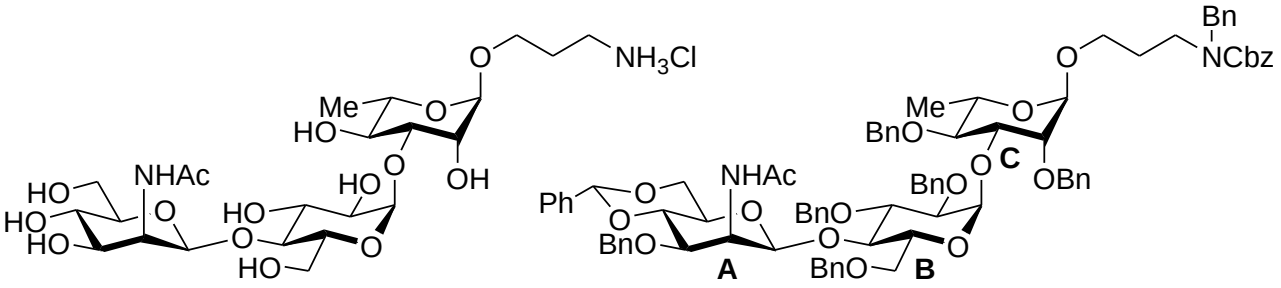


Figure 1: Structures of serotypes **19F** and **19A** capsular polysaccharides

Molecular approaches investigating the structural and chemical determinants of the cross reactivity between 19F and 19A serotypes have never been reported. Nonetheless, this knowledge may be useful to elucidate the mechanism responsible for immunoreactivity. 19F and 19A CPSs are linear biopolymers made up of trisaccharide repeating units linked through phosphodiester bridges. Each trisaccharide is composed by a β-D-Man*p*NAc-(1→4)-α-D-Glc*p* disaccharide linked to C2 or C3 of an α-L-Rha unit respectively (Figure 1). In this framework, we report the synthesis of compound **1**, the trisaccharide repeating unit of SP 19A, functionalized at the reducing end with an aminopropyl linker, in turn obtained from protected trisaccharide **2** (Figure 2). Our strategy is based on the development of a new route for the synthesis of an aminopropyl functionalized rhamnosyl acceptor, compound **3** (Scheme 1). Furthermore, in search of the most straightforward approach towards 19A trisaccharide, we explored two alternative synthetic strategies, based on different disconnections between the three monosaccharides which make up the final trisaccharide. In particular,

trisaccharide **1** was assembled with higher yields when the α-Glc-(1→3)-Rha disaccharide was glycosylated with a glucose moiety, followed by epimerization at C2.
Finally, we evaluated the binding affinity of trisaccharide **1** towards anti-19A and anti-19F sera, to investigate the role of the carbohydrate portion of the repeating unit in the antibody binding affinity. Trisaccharide **1** showed a similar and moderate activity towards both sera, indicating that a limited cross recognition exists at the level of the single repeating unit.



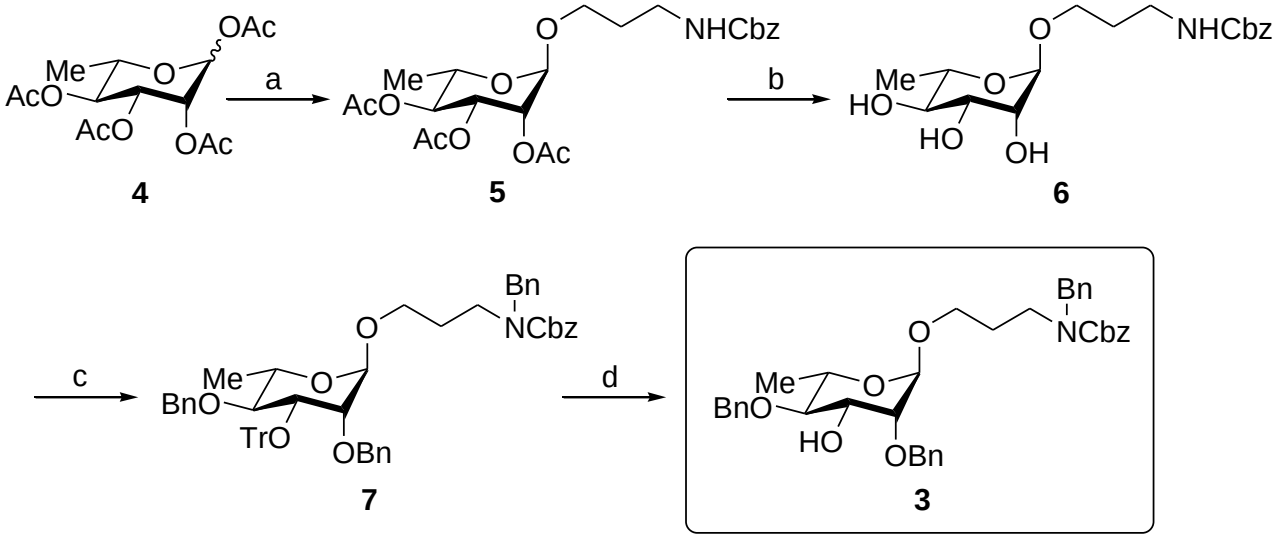
1 **2**

Figure 2: Structures of the target compound **1** and its precursor **2**

2. Results and discussion

2.1 Chemistry

A key point in our synthetic strategy towards compound **1** has been the preparation of protected trisaccharide **2** as the direct precursor of the target derivative. Compound **2** is a very versatile molecule, which allows access to both the trisaccharide repeating unit of SP 19A (the goal of this work), and, in principle, to oligomeric and/or shifted fragments of SP 19A CPS. Elongation at the upstream residue of the trisaccharide can be performed after selective reductive opening of the benzylidene group. The functionalization at the reducing end with a 3-aminopropyl linker has been designed to allow conjugation to carrier proteins[11] or the preparation of multivalent systems[12,13] appropriate for the *in-vivo* evaluation of the immunogenic activity of 19A CPS-related saccharide antigens. In this frame of thoughts, we have planned the synthesis of rhamnosyl acceptor **3**, with the aminopropyl linker already installed,[14] in order to avoid the glycosylation of the aglycon acceptor at a later stage of the synthetic route.

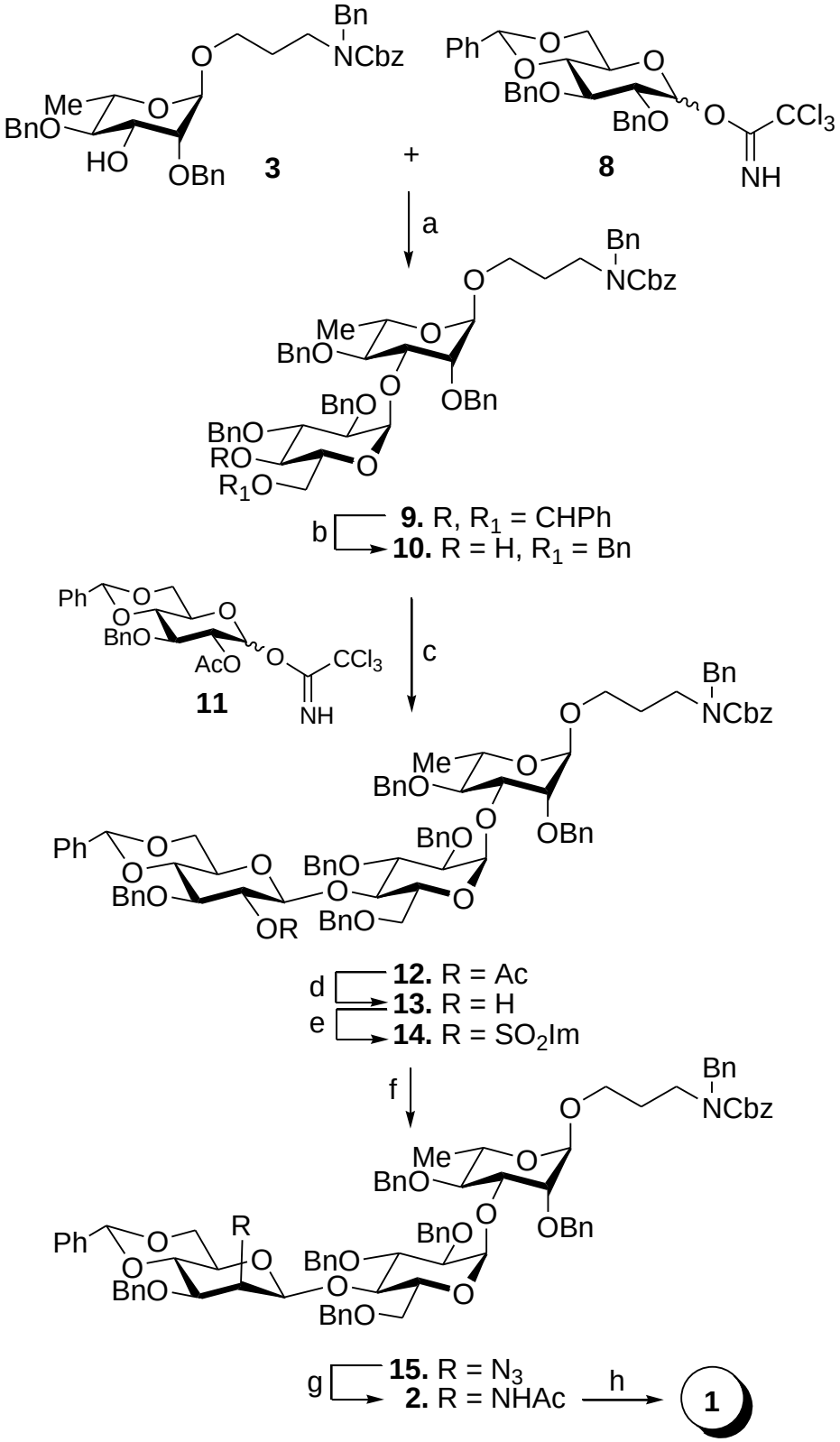


4 **5** **6** **7** **3**

Scheme 1: *Reagents and conditions:* a. *N*-Z-3-aminopropanol, $BF_3{\cdot}Et_2O$, DCM, 0 °C to rt, 75%; b. MeONa, MeOH, 93%; c. TrCl, Py, 60 °C; BnBr, NaH, 64%; CF_3COOH, DCM/MeOH, 90%.

To this aim, tetraacetyl rhamnopyranoside **4**[15]was glycosylated with *N*-Z-3-aminopropanol in the presence of boron trifluoride etherate to give the rhamnose aminopropyl glycoside **5** in 75% yield (Scheme 1). Zemplen deacetylation afforded deprotected rhamnoside **6** (93%), which was

regioselectively tritylated at position 3 by treatment with trityl chloride at high temperature, and then benzylated in 64% yield over two steps. Finally, the trityl group was removed by treatment with trifluoroacetic acid to give rhamnoside acceptor **3** in 90% yield.



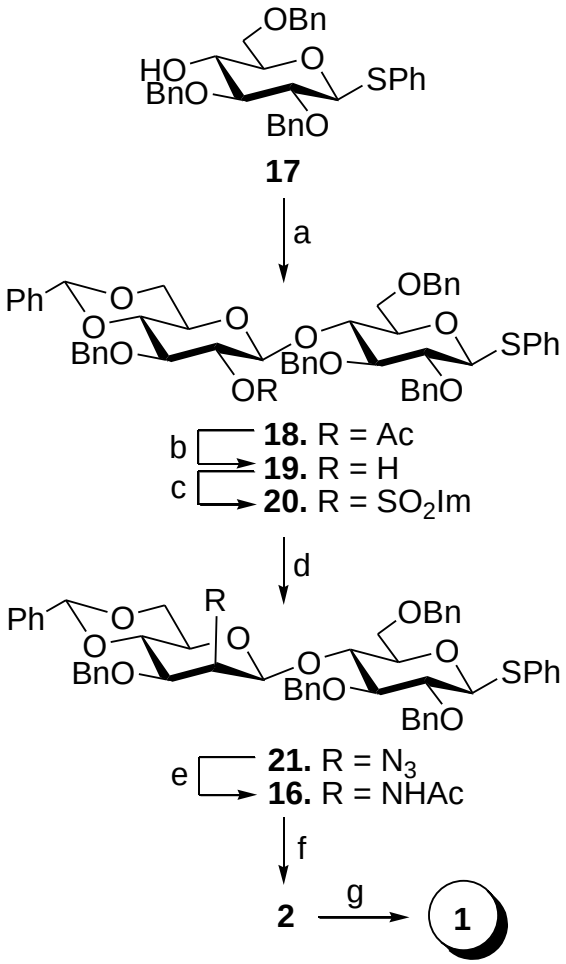
Scheme 2: *Reagents and conditions:* a. TESOTf, DCM, - 20 °C, 93%; b. Et_3SiH, $BF_3{\cdot}Et_2O$, DCM, 0 °C, *ms*, 60%; c. TMSOTf, DCM, - 20 °C, *ms*, 88%; d. MeONa, MeOH, DCM, 89%; e. Im_2SO_2, NaH, DMF, - 40 °C, 85%; f. NaN_3, DMF, 80 °C, 80%; g. Zn, $AcOH/Ac_2O$, THF, 62%; h. H_2, $Pd(OH)_2$, HCl, AcOEt, MeOH, quant.

Two different disconnection strategies are possible for the construction of the 19A trisaccharide repeating unit (Figure 2), and all the syntheses previously reported are based on a *A*-*B* + *C* approach, where a preformed β-ManNAc-(1→4)-Glc (*A*-*B*) disaccharide is coupled with a rhamnosyl acceptor (*C*).[16,17,18,19] Based on our previous experience on the synthesis of the trisaccharide related to SP 19F CPS,[20,21] we first followed the alternative *B*-*C* + *A* pathway in which an α-Glc-(1→3)-Rha (*B*-*C*) disaccharide is initially formed in high selectivity, then β-glycosylated with a glucose moiety (*A*) which is finally epimerized to *N*-acetyl-mannosamine.

Rhamnosyl acceptor **3** was thus glycosylated at position 3 with 2,3-*O*-benzyl-4,6-*O*-benzylidene glucosyl trichloroacetimidate donor **8**[22] under the catalysis of triethylsilyl triflate (Scheme 2). The aminopropyl disaccharide **9** was recovered in excellent yield (93%) and complete α-selectivity. Reductive opening of the benzylidene acetal to the corresponding 6-*O*-benzyl ether was next accomplished by treatment of **9** with triethylsilane in the presence of boron trifluoride-diethyl ether complex to give disaccharide acceptor **10** in good yield.

The desired trisaccharide scaffold was obtained through a high yield glycosylation between the 2-*O*-acetyl-3-*O*-benzyl-4,6-*O*-benzylidene glucosyl trichloroacetimidate donor **11**[23] and disaccharide acceptor **10** to give compound **12**. The β-selectivity was guaranteed by the anchimeric assistance offered by the acetyl group at position 2 of glucose **11**. Trisaccharide **12** was finally subjected to the synthetic sequence that allows *gluco* to *manno* epimerization. The acetyl group was initially removed to give unprotected **13** through Zemplen de-acetylation. Compound **13** was then reacted with sulfonyldiimidazole in the presence of sodium hydride to yield saccharide **14**, which was subjected to nucleophylic displacement with sodium azide to give mannoside **15**. The newly established *manno* configuration was confirmed by the broad ^{1}H-NMR singlet for the anomeric proton of mannose. Finally, the azido group was reduced with Zinc in the presence of acetic acid/acetic anhydride to give the fully protected trisaccharide **2**, which upon hydrogenolysis gave the target trisaccharide **1** in quantitative yield. Overall, the desired trisaccharide **1** was obtained starting from the properly protected monosaccharide donors **8** and **11** and the rhamnosyl acceptor **3** in 18% overall yield over 8 steps.

With the goal of developing a solid protocol to trisaccharide **1**, we next planned to test the feasibility of the alternative *A*-*B* + *C* disconnection strategy, which offers the advantage to reduce the number of steps on the already formed trisaccharide scaffold. To this aim, we decided to exploit a new synthetic strategy to obtain thio-disaccharide **16** for the glycosylation of the aminopropyl rhamnosyl acceptor **3**. This approach is based on our consolidated protocols for the construction of the β-mannoside linkage (Scheme 3). In this framework, disaccharide **18** was initially formed in a stereoselective fashion through a high yield glycosylation (86%) between phenylthio glucoside **17**[24] and trichloroacetimidate donor **11**. Next, epimerization at the C2' of disaccharide **18**, and the introduction of the acetamido group gave compound **16** in 35% overall yield over 4 steps. In detail, compound **18** was initially de-acetylated to **19**, then the hydroxy group was activated in high yield as imidazylate and subjected to azide displacement with sodium azide to give mannoside **21**, followed by azide reduction and *N*-acetylation. Glycosylation with rhamnosyl acceptor **3** was promoted using silver triflate–*N*-iodosuccinimide system as previously described,[19] and gave protected trisaccharide **2** in satisfactory yields but low stereoselectivity (α/β = 1:2). Compound **2** was finally quantitatively deprotected to the target compound **1**. The overall yield of the second synthetic strategy to compound **1**, starting from the suitable building blocks **11**, **17** and **3**, is 6% over 7 steps. In general, this A-B + C strategy shows an efficient and easy linear synthesis of the β-mannosylated thioglycosyl-donor **16**, but suffers from moderate yields and low stereoselectivity in the final glycosylation of rhamnose **3**.

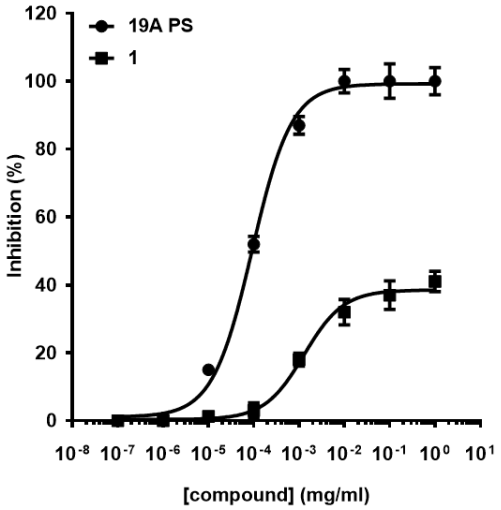


Scheme 3: *Reagents and conditions:* a. **11**, TESOTf, DCM, - 20 °C, *ms*, 86%; b. MeONa, MeOH, DCM, 75%; c. Im_2SO_2, NaH, DMF, - 40 °C, 86%; d. NaN_3, DMF, 80 °C 83%; e. Zn, AcOH/Ac_2O, THF, 66%; f. **3**, AgOTf, NIS, DCM, - 35 °C to - 10 °C, α/β: 60%, α: 20%) g. H_2, $Pd(OH)_2$, HCl, AcOEt, MeOH, quant.

2.2 Biology

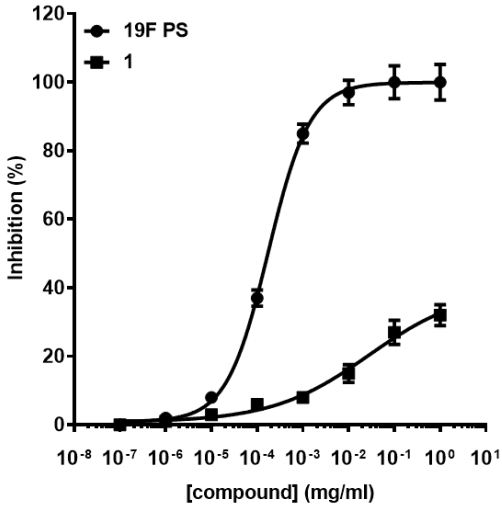
The ability of increasing concentrations (from 10^{-7}-10^{0} mg/mL) of the newly synthetized trisaccharide to inhibit the binding between 19A polysaccharide coated onto plates (positive control) and the anti 19A rabbit polyclonal antibody was evaluated by competitive ELISA. To evaluate the cross-reactivity against 19F serotype, competitive ELISA was done using native 19F polysaccharide and 19F reference serum. Figure 3 shows the inhibition curves obtained with compound **1**, under evaluation in both systems. The relative efficacy of compound **1** was calculated by measuring the maximum effect elicited in each system, while the concentration that produces 50% of the maximum effect (EC_{50}) was taken as indirect index of its relative potency (Figure 3). As expected the natural polysaccharide exhibited higher efficacy (100% inhibition at 10^{-1} mg/mL) and affinity (EC_{50} = $9.1x10^{-5}$ mg/mL) than synthetized compound (41% inhibition at 10^{0} mg/mL and EC_{50} =1.3 x 10^{-3}) confirming that saccharide chain length seems to be important for their biological activity. The low effectiveness of the newly synthetized compound could be related to its relative weak avidity, since short chain lengths saccharide antigens, like a trisaccharide, have decreased strength of antibody-antigen binding. The single repeating unit of 19A polysaccharide displayed inhibitory properties also in 19F system. The trisaccharide was slightly both more effective and potent in 19A than in 19F system (41% and 32% of inhibition for 19A and 19F respectively; EC_{50} 1.3 x 10^{-3} and 2.7 x 10^{-2} for 19A and 19F respectively). These data suggest that differences in structures of the 19A and 19F trisaccharides are almost negligible at the repeating

unit level, and a level of cross reactivity exists. It is reasonable to speculate that saccharide fragments with chain length longer than compound **1**, resulting in more complex structures, would contain multiple epitopes leading to an increase in specificity for 19A serum and a reduction in cross-reactivity versus 19F.



Compound	EC_{50} ± SEM (mg/mL)	Max inhibition[a] (%)
19A PS	$(9.1 \pm 1.2) \times 10^{-5}$	100
1	$(1.3 \pm 0.02) \times 10^{-3}$	41

[a] The maximum inhibition elicited by each compound at 1 mg/ml.



Compound	EC_{50} ± SEM (mg/mL)	Max inhibition[a] (%)
19F PS	$(1.7 \pm 0.006) \times 10^{-4}$	100
1	$(2.7 \pm 0.3) \times 10^{-2}$	32

[a] The maximum inhibition elicited by each compound at 1 mg/ml.

Figure 3. Results of the Elisa experiments with compound **1**. Concentration/response curves of compound **1** on the inhibition of the binding between the 19A (on the left) or 19F (on the right) native polysaccharides, coated onto the plates, and the anti-19A or anti-19F antibodies, respectively, were evaluated by a competitive ELISA method.

3. Conclusions

In conclusion, the synthesis of compound **1**, an aminopropyl derivative of the trisaccharide repeating unit of SP 19A, has been developed exploiting rhamnosyl acceptor **3**, already functionalized with an aminopropyl linker. We developed a new and more efficient synthetic route to the rhamnosyl acceptor, which allows to obtain compound **3** in 40% overall yield over four steps. Two different synthetic strategies were used to build trisaccharide **1**, allowing a direct comparison among the two protocols. Based on our results, we suggest that the protocol based on the *B*-*C* + *A* strategy is more effective than the *A*-*B* + *C* one. The overall yield of assembly was around 20% for the first protocol, in contrast to the more modest 6% of the second approach, which is limited by the low selectivity in the glycosylation between disaccharide *A*-*B* and rhamnoside **3** (*C*). The results confirmed that the stereoselectivity of the reaction of α-glucosylation is a function of the protecting groups on glucose, and the use of 4,6-*O*-benzylidene glucosyl donors, protected with no participating groups at the 2-position, are usually α-selective.[25] Indeed, the use of 4,6-*O*-benzylidene glucosyl donor **8** allowed the formation of the α-product in excellent yield. Overall, the first approach to trisaccharide **1** is solid and highly reproducible. Furthermore, the protected trisaccharide **2** is a valuable intermediate for the synthesis of shifted fragments of the CPS of SP 19A: the elongation of the trisaccharide at the upstream residue is functional for the synthesis of oligomers functionalized at the downstream residue with the aminopropyl linker, useful for conjugation to proteins or multivalent scaffolds. We have also showed that compound **1**, which possesses moderate inhibitory activity towards anti-19A antibodies, displays a comparable activity also towards anti-19F antibodies. This data suggests that the two sera are not capable of discriminating small differences in the structure of 19F and 19A trisaccharides. Since differences in

conformational preferences have been described for the repeating units of SP 19A and 19F,[10] it is reasonable to assume that longer and structured fragments are needed to significantly affect the binding specificity of the antibodies to the saccharide antigens.

4. Experimental Section:

4.1 Synthetic procedures

Standard laboratory procedures were followed to carry out the reactions and to prepare dry solvents.[26] Optical rotations were measured with a Perkin-Elmer 241 polarimeter at 20 °C. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AVANCE-500 spectrometer at a sample temperature of 298 K.[27] Mass spectrometric analyses were performed on a Thermo Quest Finnigan LCQ™DECA ion trap mass spectrometer; equipped with a Finnigan ESI interface. High-resolution mass spectra were collected by electrospray ionization (ESI) spectroscopy on a QTof SYNAPT G2Si Mass Spectrometer. NaH was washed with hexane three times prior to use.

*4.1.1.Synthesis of N-(benzyloxycarbonyl)aminopropyl 2,3,4-tri-O-acetyl-α-L-rhamnopyranoside (**5**)*

$BF_3 \cdot Et_2O$ (5.0 mL, 39.45 mmol) was slowly added through a dropping funnel to a solution at 0 °C under argon of compound **4** (2.28 g, 6.86 mmol) and N-CBz-aminopropanol (3.59 g, 17.15 mmol) in dry CH_2Cl_2 (70 mL). The reaction was stirred at room temperature, monitored by TLC (hexane/ethyl acetate, 1:1) and appeared to be complete after 12 h. The reaction was washed with saturated $NaHCO_3$ solution (2 x 100mL), and the combined aqueous phases extracted with AcOEt (2 x 100 mL). The combined organic layers were dried over Na_2SO_4 and concentrated. Purification by flash chromatography (hexane/AcOEt, 6:4) gave pure **5** (2.48 g, 75 %) as a colorless oil. $[\alpha]_D^{20}$ = -43.6 (*c* = 0.5 in chloroform); ^{1}H NMR ($CDCl_3$): δ = 7.40-7.30 (m ,5H, arom.), 5.29 (dd, 1 H, $J_{2,3}$ = 3.5 Hz, $J_{3,4}$=10.0 Hz, H-3), 5.25 (dd, 1 H, $J_{1,2}$ = 1.7 Hz, $J_{2,3}$ = 3.5 Hz, H-2), 5.13 (s, 2H, CH_2Ph), 5.08 (t, 1 H, $J_{3,4}$ = $J_{4,5}$= 10.0 Hz, H-4), 4.95-4.88 (m, 1 H, NH), 4.73 (*br* s, 1 H, H-1), 3.91 – 3.83 (m, 1 H, H-5), 3.81-3.74 (m, 1 H, H-a), 3.54-3.47 (m, 1H, H-a'), 3.37-3.29 (m, 2 H, 2 H-c), 2.17 (s ,3 H, CH_3CO), 2.06 (s, 3 H, CH_3CO), 2.01(s, 3 H, CH_3CO), 1.92-1.80 (m, 2 H, 2 H-b), 1.24 (d, 3 H, $J_{5,6}$ = 6.3Hz, 3 H-6); ^{13}C NMR ($CDCl_3$): δ = 170.2 (C=O), 170.0 (C=O), 169.9 (C=O), 156.4 (C=O, Cbz), 136.6 (arom), 128.5-128.1 (5 C arom), 97.5 (C-1), 71.1 (C-4), 69.8 (C-2), 69.1 (C-3), 66.7 (CH_2Ph), 66.5 (C-5), 65.8 (C-a), 38.4 (C-c), 29.6 (C-b), 20.9 (CH_3), 20.8 (CH_3), 20.7 (CH_3), 17.4(C-6). MS (ESI) *m/z* (%): 504.1 (100) $[M+Na]^+$. HRMS (ESI): *m/z* calcd for $C_{23}H_{31}NO_{10}Na$ 504.1846 $[M+Na]^+$, found 504.1836.

*4.1.2.Synthesis of N-(benzyloxycarbonyl)aminopropyl α-L-rhamnopyranoside (**6**)*

Compound **5** (2.40 g, 4.98 mmol) was dissolved in dry dichloromethane (50 mL) and sodium methoxide in dry methanol (0.2 M solution, 12 mL) was added. The reaction was stirred for 3h at room temperature, then it was neutralized with an ion exchange resin (Dowex 50 × 8, H^+ form), filtered and concentrated. The crude was subjected to flash chromatography (CH_2Cl_2/MeOH, 9:1) to give compound **6** (1.64 g, 93 %) as a colorless oil. $[\alpha]_D^{20}$ = -38.5 (*c* = 0.5 in chloroform) ^{1}H NMR (MeOD): δ =7.40-7.28 (m, 5H, arom.), 5.09 (*br* s,2H, CH_2Ph), 4.67 (*br* s, 1H, H-1), 3.83-3.80 (m,1H, H-2), 3.77-3.70 (m, 1H, H-a), 3.66 (dd, 1 H, $J_{2,3}$ = 3.3 Hz, $J_{3,4}$ = 9.5 Hz, H-3), 3.61-3.55 (m, 1H, H-5), 3.47-3.41 (m, 1H, H-a), 3.38 (t, 1 H, $J_{3,4}$ = $J_{4,5}$ =9.5 Hz, H-4), 3.28-3.18 (m, 2H, 2 H-c), 1.83-1.75 (m, 2H, 2 H-b), 1.27 (d, 3 H, $J_{5,6}$ = 6.4Hz, 3 H-6); ^{13}C NMR (MeOD): δ = 157.5 (C=O), 137.0 (arom), 128.1-127.4 (5 C arom), 100.3 (C-1), 72.6 (C-4), 71.0 (C-3), 70.9 (C-2), 68.4 (C-5), 66.0 (CH_2Ph), 64.5 (C-a), 37.6 (C-c), 29.4 (C-b), 16.6 (C-6). MS (ESI) *m/z* (%): 378.1 (100) $[M+Na]^+$, 732.8 (12) $[2M+Na]^+$. HRMS (ESI): *m/z* calcd for $C_{17}H_{25}NO_7Na$ 378.1529 $[M+Na]^+$, found 378.1526.

*4.1.3. Synthesis of N-benzyl-N-benzyloxycarbonyl-3-aminopropyl 2,4-di-O-benzyl-3-O-trityl-α-L-rhamnopyranoside (**7**)*

A mixture of **6** (1.60 g, 4.50 mmol), trityl chloride (2.51 g, 9.00 mmol) and dry pyridine (15 mL) was stirred at 60 °C for 20 h. After the addition of Et_3N (2 mL), the reaction was diluted with EtOAc (50 mL) and washed with HCl 1N (2 x 50 mL). The combined aqueous phases were extracted with AcOEt (3 x 40 mL), and then the combined organics were washed with satd. $NaHCO_3$ soln. (1 x 60 mL), dried over Na_2SO_4 and evaporated under reduced pressure. To a solution of the crude and benzyl bromide (3.2 mL, 27 mmol) in dry DMF (50 mL), NaH (60 % in oil, 1.21 g, 31.5 mmol) was added portionwise at 0 °C. The reaction was warmed to room temperature. After 5 h, an additional amount of NaH (60 % in oil, 0.34 g, 9.00 mmol) was added and the reaction stirred for 12 h. The mixture was quenched by carefully addition of MeOH (5 mL), then diluted with HCl 1N (100 mL), and extracted with AcOEt (3 x 100 mL). The combined organics were washed with brine (2 x 150 mL), dried over Na_2SO_4 and evaporated. The crude was purified through flash chromatography (hexane/ AcOEt, 82:25) to give product **7** (2.5 g, 64 %) as a light yellow viscous oil. $[\alpha]_D^{20}$ = -8.7 (*c* = 0.5 in chloroform)

^{1}H NMR ($CDCl_3$): δ = 7.65-7.10 (m, 35H, arom.), 5.25-5.00 (m, 3H, CH_2Ph), 4.80-4.65 (m, 1H, CH_2Ph), 4.55-4.35 (m, 3H, H-1 and CH_2Ph), 4.30-4.15 (m, 2H, CH_2Ph), 4.10 (dd, 1H, $J_{2,3}$ = 2.6 Hz, $J_{3,4}$ = 9.2 Hz, H-3), 3.90-3.70 (m, 1H, H-4), 3.65-3.35 (m, 2H, H-5 and H-a), 3.35-3.05 (m, 3H, H-a and 2 H-c), 2.45-2.25 (m, 1H, H-2), 1.75-1.55 (m, 2H, 2 H-b), 1.33(d, 3H, $J_{5,6}$ = 6.2 Hz, 3 H-6); ^{13}C NMR ($CDCl_3$): δ = 156.8 (C=O), 145.1-127.0 (42 C, arom), 97.2 (C-1), 87.4 (C trityl), 80.5 (C-4), 77.9 (C-2), 75.3 (CH_2Ph), 73.8 (C-3), 71.9 (CH_2Ph), 69.1 (C-5), 67.2 (CH_2Ph), 65.0 (C-a), 51.0 (CH_2Ph), 45.1-44.1 (m, C-c), 28.6-28.0 (m, C-b), 18.4 (C-6); MS (ESI) *m/z* (%): 890.5 (100) $[M+Na]^+$. HRMS (ESI): *m/z* calcd for $C_{57}H_{57}NO_7Na$ 890.4033 $[M+Na]^+$, found 890.4029.

*4.1.4. Synthesis of N-benzyl-N-benzyloxycarbonyl-3-aminopropyl 2,4-di-O-benzyl-α-L-rhamnopyranoside (**3**)*

To a solution of compound **7** (0.90 g, 1.04 mmol) in 21 mL of DCM/MeOH (6:1, v/v), trifluoroacetic acid (0.60 mL, 7.88 mmol) was added dropwise. The reaction was stirred at room temperature for 5 h, then quenched to neutrality through addition of TEA. The solvent was evaporated under reduced pressure, and the crude purified by flash chromatography (hexane/ AcOEt, 82:25) to give rhamnoside **3** (0.58 g, 90 %) as a colorless oil. $[\alpha]_D^{20}$ = -13.8 (*c* = 0.1 in chloroform)

^{1}H NMR ($CDCl_3$): δ = 7.43-7.13 (m, 20 H, arom), 5.23-5.14 (m, 2H, CH_2Ph), 4.92 (d, 1H, J = 11.1 Hz, CH_2Ph), 4.81-4.70 (m, 2H, H-1 and CH_2Ph), 4.67 (d, 1H, J = 11.1 Hz, CH_2Ph), 4.63-4.43 (m, 3H, CH_2Ph), 3.97-3.87 (m, 1H, H-3), 3.74-3.17 (m ,3H, H-2,5 and H-a), 3.48-3.25 (m, 4H, H-4, H-a and 2 H-c), 1.87-1.70 (m, 2H, 2 H-b), 1.33 (d, 3H, $J_{5,6}$ = 6.2 Hz, 3 H-6); ^{13}C NMR ($CDCl_3$): δ = 156.2 (C=O), 138.6-127.3 (24 C, arom), 97.0 (C-1), 82.3 (C-4), 78.6 (C-2), 75.1 (CH_2Ph), 73.0 (CH_2Ph), 71.7 (C-3), 67.2 (2C, C-5 and CH_2Ph), 65.0 (C-a), 50.5 and 50.7 (d, NCH_2Ph), 44.5 and 43.7 (d, C-c), 28.3 and 27.8 (C-b), 18.0 (C-6); MS (ESI) *m/z* (%): 684.4 (100) $[M+Na]^+$. HRMS (ESI): *m/z* calcd for $C_{38}H_{43}NO_7Na$ $[M+Na]^+$ 648.2937, found 648.2936.

*4.1.5. Synthesis of N-benzyl-N-benzyloxycarbonyl-3-aminopropyl 2,3-di-O-benzyl-4,6-O-benzylidene-α-D-glucopyranosyl-(1→3)-2,4-di-O-benzyl-α-L-rhamnopyranoside (**9**)*

A solution of glucosyl trichloroacetimidate **8** (0.70 g, 1.20 mmol) and rhamnoside **3** (0.30 g, 0.48 mmol) in DCM (16 mL) was cooled at –20 °C, then triethylsilyl trifluoromethanesulfonate (0.1 M solution in DCM, 0.95 mL) was added dropwise. After 1,5 h the reaction was quenched by the addition of TEA, and allowed to warm to room temperature. The reaction was concentrated, then purified by flash chromatography (hexane/AcOEt, 8:2) to give disaccharide **9** (0.47 g, 93 %) as an oil. $[\alpha]_D^{20}$ = +3.8 (*c* = 1 in chloroform). ^{1}H NMR ($CDCl_3$): δ = 7.50-7.14 (m, 35H, arom.), 5.56 (s, 1H, CHPh), 5.18 (*br* s, 2H, CH_2Ph), 5.14 (d, 1H, $J_{1',2'}$ = 3,4 Hz, H-1'), 4.98-4.93 (m, 2H, CH_2Ph),

4.85-4.41 (m, 9H, H-1 and CH_2Ph), 4.21-4.04 (m, 4H, H-3, 6' and $2H_s$), 3.90-3.81 (m, 1H, H-2), 3.69-3.55 (m, 6H, H-a, 4, 5, 2', 6' and 1H), 3.44-3.22 (m, 3H, 1 H-a and 2 H-c), 1.84-1.69 (m, 2H, 2 H-b), 1.31 (*br* d, 3H, 3 H-6). ^{13}C NMR ($CDCl_3$): δ = 163.3 (C=O), 138.6-126.2 (42C, arom.), 101.3 (PhCH), 98.1 (C-1), 96.3 (C-1'), 82.6, 80.2, 79.2, 78.5, 76.7(C-3), 75.5 (2C, C-2 and CH_2Ph), 75.1 (CH_2Ph), 73.7 (CH_2Ph), 73.2 (CH_2Ph), 69.0 (C-6'), 68.4, 67.2 (CH_2Ph), 65.1 (C-a), 63.0, 50.52 and 50.75 (NCH_2Ph),43.73 and 44.50 (C-c), 27.81 and 28.30 (C-b), 18.0 (C-6). MS (ESI) *m/z* (%): 1079.1 (100) $[M + 1 + Na]^+$. HRMS (ESI): *m/z* calcd for $C_{65}H_{69}NO_{12}Na$ 1078.4717 $[M+Na]^+$, found 1078.4712.

*4.1.6. Synthesis of N-benzyl-N-benzyloxycarbonyl-3-aminopropyl 2,3,6-tri-O-benzyl-α-D-glucopyranosyl-(1→3)-2,4-di-O-benzyl-α-L-rhamnopyranoside (**10**)*

Compound **9** (0.45 g, 0.43 mmol) and 4 Å m.s. (0.45 g) were dissolved in DCM (10 mL), stirred at room temperature for 15 minutes, then the suspension was cooled at 0 °C. Triethylsilane (0.63 mL, 4.30 mmol) was added, followed by the slow dropwise addition of $BF_3.Et_2O$ (0.27 mL, 2.15 mmol). The reaction was stirred at 0 °C for 2 h, then quenched with triethylamine, diluted with DCM, filtered over celite, and concentrated *in vacuo*. The residue was purified by flash chromatography (Hexane/AcOEt, 8:2) to afford compound **10** (0.27 g, 60%) as an oil. $[\alpha]_D^{20}$ = +10.8 (*c* = 1 in chloroform) ^1H NMR ($CDCl_3$): δ = 7.40-7.20 (m, 35H, arom), 5.21-5.13 (m, 3H, H-1' and CH_2Ph), 5.00-4.80 (m, 2H, CH_2Ph), 4.84-4.83 (m, 11H, H-1 and CH_2Ph), 4.14-4.06 (m, 1H, H-3), 4.04-3.97 (m, 1H, H-5'), 3.96-3.80 (m, 2H, H-2, 3'), 3.74-3.45 (m, 7H, H-a, 4, 5, 2', 4', 6'a, 6'b), 3.42-3.19 (m, 3H, 2 H-c and 1 H-a), 2.25-2.05 (*br* s, 1H, OH), 1.81-1.65 (m, 2H, 2 H-b), 1.33 (*br* s, 3H, 3 H-6). ^{13}C NMR ($CDCl_3$): δ = 156.4 (C=O), 138.8-127.3 (42C, arom.), 98.2 (C-1), 95.0 (C-1'), 81.3 (C-3'), 80.1, 79.4, 76.0 (C-3), 75.5 (C-2), 75.2 (2C, CH_2Ph), 73.4 (CH_2Ph), 73.2 (CH_2Ph), 73.0 (CH_2Ph), 71.2, 70.2 (C-5'), 69.5 (C-6'), 68.4, 67.2 (CH_2Ph of Cbz), 65.1 (C-a), 50.5 and 50.8 (NCH_2Ph), 43.7 and 44.5 (C-c), 27.8 and 28.3 (C-b), 18.1 (C-6). MS (ESI) *m/z* (%): 1080.1 (100) $[M+Na]^+$. HRMS (ESI): *m/z* calcd for $C_{65}H_{71}NO_{12}Na$ 1080.4874 $[M+Na]^+$, found 1080.4883.

*4.1.7. Synthesis of N-benzyl-N-benzyloxycarbonyl-3-aminopropyl 2-O-acetyl-3-O-benzyl-4,6-O-benzylidene-β-D-glucopyranosyl-(1→4)-2,3,6-tri-O-benzyl-α-D-glucopyranosyl-(1→3)-2,4-di-O-benzyl-α-L-rhamnopyranoside (**12**)*

A suspension of 2-*O*-acetyl-glucosyl trichloroacetimidate **11** (0.42 g, 0.78 mmol), disaccharide **10** (0.23 g, 0.22 mmol) and 4 Å m.s. (0.23 g) in DCM (7 mL) was stirred for 0.15 min at room temperature, then cooled at –20 °C. Triethylsilyl trifluoromethanesulfonate (0.1 M solution in DCM, 0.44 mL) was added dropwise and the disappearance of the starting material was followed by TLC (Toluene/Acetone, 7:3; hexane/AcOEt, 7:3). After 1.5 h, the reaction was quenched with triethylamine, diluted with DCM, filtered over Celite, and the solvent evaporated. The crude product was purified by flash chromatography (hexane/AcOEt, 8:2) to give **12** (0.28 g, 88%) as an amorphous solid. $[\alpha]_D^{20}$ = +5.9 (*c* = 1 in chloroform). ^1H NMR ($CDCl_3$): δ = 7.53-7.10 (m, 45H, arom.), 5.48 (s, 1H, PhCH), 5.23-5.13 (m, 3H, H-1' and CH_2Ph), 4.97-4.82 (m, 4H, H-2'' and CH_2Ph), 4.76 (d, 2H, CH_2Ph), 4.72-4.53 (m, 7H, H-1 and CH_2Ph), 4.52-4.44 (m, 3H, H-1'' and NCH_2Ph), 4.29-4.22 (m, 1H, CH_2Ph), 4.17-4.11 (m, 1H, H-6a''), 4.08-3.99 (m, 1H, H-3), 3.99-3.91 (m, 3H, H-3', 4', 5'), 3.91-3.82 (m, 1H, H-2), 3.74-3.53 (m, 6H, H-a, 4, 5, 2', 6a', 4''), 3.51-3.36 (m, 3H, H-6b', 3'', 6b''), 3.35-3.21 (m, 3H, 1 H-a and 2 H-c), 3.18-3.10 (m, 1H, H-5''), 1,82 (s, 3H, $COCH_3$), 1.81-1.67 (m, 2H, 2 H-b), 1.22 (*br* d, 3H, 3 H-6). ^{13}C NMR ($CDCl_3$): δ = 168.9 (C=O), 139.3-126.0 (54C, arom.), 101.1 (CHPh), 100.8 (C-1''), 98.2 (C-1), 96.5 and 96.3 (C-1'), 81.6 (C-4''), 80.1, 79.9, 79.2, 78.7, 77.4 (C-3), 76.7, 76.1 (C-2), 75.0 (CH_2Ph), 74.9 (CH_2Ph), 74.0 (CH_2Ph), 73.6 (CH_2Ph), 73.3 (2C, C-2'' and CH_2Ph), 73.2 (CH_2Ph), 70.7, 68.6 (C-6''), 68.2, 67.6 (C-6'), 67.2 (CH_2Ph), 65.9 (C-5''), 65.1 (C-a), 50.8 and 50.5 (NCH_2Ph), 44.6 and 43.7 (C-c), 28.3 and 27.9 (C-b), 20.8 (CH_3CO), 18.0 (C-6). MS (ESI) *m/z* (%): 1463.5 (100) $[M + 1 + Na]^+$. HRMS (ESI): *m/z* calcd for $C_{87}H_{93}NO_{18}Na$ 1462.6290 $[M+Na]^+$, found 1462.6276.

*4.1.8. Synthesis of N-benzyl-N-benzyloxycarbonyl-3-aminopropyl 3-O-benzyl-4,6-O-benzylidene-β-D-glucopyranosyl-(1→4)-2,3,6-tri-O-benzyl-α-D-glucopyranosyl-(1→3)-2,4-di-O-benzyl-α-L-rhamnopyranoside (**13**)*

To a stirred solution of **12** (0.27 g, 0.19 mmol) in DCM/MeOH 1:1 (6 mL) sodium methoxide in methanol (1 M solution, 0.19 mL) was added. The reaction was stirred for 48 h at room temperature, then it was neutralized with an ion exchange resin (Dowex 50 × 8, H^+ form), filtered and concentrated. The crude product was subjected to flash chromatography (hexane/AcOEt, 7:3) to give pure **13** (0.24 g, 89%) as an amorphous solid. $[\alpha]_D^{20}$ = +15.3 (*c* = 1 in chloroform). ^{1}H NMR ($CDCl_3$): δ = 7.52-7.16 (m, 45H, arom.), 5.48 (s, 1H, PhCH), 5.24-5.11 (m, 2H, H-1' and CH_2Ph), 5.00-4.61 (m, 10H, H-1 and CH_2Ph), 4.61-4.41 (m, 4H, CH_2Ph), 4.37 (d, 1H, $J_{1'',2''}$ = 7,5 Hz, H-1''), 4.33-4.28 (m, 1H, CH_2Ph), 4.12-3.95 (m, 5H, H-3, 3', 4', 5', 6a''), 3.89-3.80 (m, 1H, H-2), 3.80-3.74 (m, 1H, H-6a'), 3.74-3.58 (m, 5H, H-a, 4, 5, 2'), 3.58-3.51 (t, 1H, $J_{3'',4''}$ = $J_{4'',5''}$ = 9.3 Hz, H-4''),3.51-3.37 (m, 3H, H-6b', 3'', 6b''),3.37-3.21 (m, 4H, H-a, 2'' and 2 H-c), 3.11-3.04 (m, 1H, H-5''), 1.85-1.60 (m, 3H, 2 H-b and OH), 1.35 (d, 3H, J = 5.7 Hz, 3 H-6). ^{13}C NMR ($CDCl_3$): δ = 157.7 (C=O), 128.4-126.0 (54C, arom), 103.4 (C-1''), 101.2 (CHPh), 98.3 (C-1), 94.5 (*br* s, C-1'), 81.2 (C-4''), 80.6, 80.3 (C-3''), 80.0, 79.1 (C-2'), 77.4, 76.1 (C-3), 75.2 (2C, C-2, 2''), 75.1-73.3 (6C, CH_2Ph), 70.0 (C-5'), 68.7 (C-6''), 68.4, 68.2 (C-6'), 67.2 (CH_2Ph), 66.1 (C-5''), 65.2 (C-a), 50.6 (*br* s, NCH_2Ph), 44.5 and 44.4 (C-c), 27.9 and 27.6 (C-b), 18.0 (C-6). MS (ESI) *m/z* (%): 1420.7 (100) $[M+NaM+Na]^+$. HRMS (ESI): *m/z* calcd for $C_{85}H_{91}NO_{17}Na$ 1420.6185 $[M+Na]^+$, found 1420.6194.

*4.1.9. Synthesis of N-benzyl-N-benzyloxycarbonyl-3-aminopropyl 3-O-benzyl-4,6-O-benzylidene-2-O-(N-imidazole-1-sulfonyl)-β-D-glucopyranosyl-(1→4)-2,3,6-tri-O-benzyl-α-D-glucopyranosyl-(1→3)-2,4-di-O-benzyl-α-L-rhamnopyranoside (**14**)*

NaH (60 % in oil, 0.070 g, 1.76 mmol) was added to a stirred solution of compound **13** (0.23 g, 0.16 mmol) in dry DMF (3.5 mL) at room temperature. After 1 h, the suspension was cooled at –40 °C and 1,1'-sulfonyl-diimidazole (0.22 g, 1.12 mmol) in dry DMF (1.5 mL) was added. After 24 h the reaction mixture was quenched with MeOH and allowed to warm to room temperature, then diluted with water (40 mL). The mixture was extracted with AcOEt (3 x 50 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and evaporated. Flash chromatography (hexane/AcOEt, 7:3) of the crude product gave trisaccharide **14** (0.21 g, 85%) as an amorphous solid. $[\alpha]_D^{20}$ = +1.8 (*c* = 1 in chloroform). ^{1}H NMR ($CDCl_3$): δ = 7.94 (*br* s, 1H, Im), 7.53-7.10 (m, 45H, arom), 7.00 (m, 2H, Im), 5.46 (s, 1H, PhCH), 5.25-5.12 (m, 3H, H-1' and CH_2Ph), 4.97 (d, 1H, J = 11.1 Hz, CH_2Ph), 4.85-4.58 (m, 10H, H-1and CH_2Ph), 4.58-4.44 (m, 4H, H-2'' and CH_2Ph), 4.32 (d, 1H, $J_{1'',2''}$ = 7,9 Hz, H-1''), 4.23 (dd, 1H, $J_{5'',6''}$ = 4,9 Hz, $J_{6a'',6b''}$ = 10.6 Hz, H-6a''), 4.16-4.09 (m, 1H, CH_2Ph), 4.09-3.80 (m, 5H, H-2, 3, 3', 4', 5'), 3.75-3.52 (m, 5H, H-a, 4, 5, 2',4''), 3.52-3.20 (m, 7H, H-a', c, c', 6a', 6b', 3'', 6b''), 3.10-2.98 (m, 1H, H-5''), 1.91-1.69 (m, 2H, 2 H-b), 1.29-1.21 (*br* d, 3H,3 H-6). ^{13}C NMR ($CDCl_3$): δ = 155.6 (C=O), 139.1-136.5 (9C, arom), 136.8 (C Im),129.2-126.0 (46C, arom.), 118.6 (C Im), 101.4 (CHPh), 98.6 (C-1''), 98.2 (C-1), 97.1 (*br* s, C-1'), 85.7 (C-2''), 81.9 (C-4''), 80.3 (*br* s, C-4), 79.5 (C-3'), 79.2 (C-2'), 78.4 (*br* s, C-3), 76.7 (C-3''), 76.5 (*br* s, C-2), 76.2 (C-4'), 75.2 (2C, CH_2Ph), 74.6 (*br* s, CH_2Ph), 74.3 (CH_2Ph), 73.6 (CH_2Ph), 73.1 (*br* s, CH_2Ph), 70.2 (C-5'), 68.4 (C-6''), 68.3 (C-5), 67.3 (C-6'), 67.2 (CH_2Ph), 65.7 (C-5''), 65.2 (C-a), 50.8 and 50.5 (NCH_2Ph), 44.6 and 43.7 (C-c), 28.4 and 27.9 (C-b), 18.0 (C-6). MS (ESI) *m/z* (%): 1550.3 (100) $[M+NaM+Na]^+$. HRMS (ESI): *m/z* calcd for $C_{88}H_{93}N_3O_{19}NaS$ 1550.6022 $[M+Na]^+$, found 1550.6055.

*4.1.10. Synthesis of N-benzyl-N-benzyloxycarbonyl-3-aminopropyl 2-azido-3-O-benzyl-4,6-O-benzylidene-2-deoxy-β-D-mannopyranosyl-(1→4)-2,3,6-tri-O-benzyl-α-D-glucopyranosyl-(1→3)-2,4-di-O-benzyl-α-L-rhamnopyranoside (**15**)*

To a stirred solution of **14** (0.20 g, 0.13 mmol) in dry DMF (4 mL), sodium azide (0.085g, 1.30 mmol) was added and the resulting solution was heated at 80 °C. After 5 h, the reaction was cooled to room temperature, diluted with H_2O and extracted with AcOEt (3 x 40 mL). The combined organic layers were dried over Na_2SO_4, filtered and evaporated. The crude product was purified by flash chromatography (hexane/AcOEt, 75:25) to give compound **15** (0.15g, 80%) as colourless oil $[\alpha]_D^{20}$ = -4.1 (*c* = 1 in chloroform). ^{1}H NMR ($CDCl_3$): δ = 7.63-7.08 (m, 45H, arom.), 5.52 (s, 1H, PhCH), 5.25-5.16 (m, 2H, CH_2Ph), 5.13 (d, 1H, $J_{1',2'}$ = 3.4 Hz, H-1'), 5.06 (d, 1H, J = 10.5 Hz, CH_2Ph), 4.92 (d, 1H, J = 11.5 Hz, CH_2Ph), 4.88-4.82 (m, 2H, CH_2Ph), 4.82-4.75 (m, 2H, CH_2Ph), 4.74-4.45 (m, 8H, H-1 and CH_2Ph), 4.34 (*br* s, 1H, H-1''), 4.19-3.95 (m, 6H, H-3, 3', 4', 5', 6a'' and 1H x CH_2Ph), 3.88 (t, 1H, $J_{3'',4''} = J_{4'',5''}$ = 9.4 Hz, H-4''), 3.85-3.76 (m, 1H, H-2), 3.75-3.58 (m, 4H, H-a, 4, 5, 2'), 3.56-3.48 (m, 2H, H-2'', 6b''), 3.48-3.20 (m, 6H, H-a', 6a', 6b', 3'' and 2 H-c), 3.00-2.92 (m, 1H, H-5''), 1.88-1.72 (m, 2H, 2 H-b), 1.33 (d, 3H, $J_{5,6}$ = 5.8 Hz, 3 H-6). ^{13}C NMR ($CDCl_3$): δ = 156.6 and 156.1 (C=O), 137.9-126.0 (54C, arom.), 101.5 (CHPh), 99.7 (C-1''), 98.4 (C-1), 95.5 (*br* s, C-1'), 80.5 (C-3'), 79.8 (C-4), 79.0 (C-2'), 78.5 (C-4''), 76.9 (C-3), 76.7 (C-4'), 76.4 (C-3''), 75.9 (C-2), 75.1 (CH_2Ph), 74.7 (CH_2Ph), 73.7 (CH_2Ph), 73.6 (CH_2Ph), 73.5 (CH_2Ph), 72.5 (CH_2Ph), 69.7 (C-5'), 68.6 (C-5), 68.4 (C-6''), 68.2 (C-6'), 67.2 (CH_2Ph), 67.1 (C-5''), 65.2 (C-a), 63.2 (C-2''), 50.8 and 50.6 (NCH_2Ph), 44.6 and 43.7 (C-c), 28.3 and 27.9 (C-b), 18.0 (C-6). MS (ESI) *m/z* (%): 1446.4 (100) $[M + 1 + Na]^+$. HRMS (ESI): *m/z* calcd for $C_{85}H_{90}N_4O_{16}Na$ 1445.6250 $[M+Na]^+$, found 1445.6246.

*4.1.11. Synthesis of phenyl 2-O-acetyl-3-O-benzyl-4,6-O-benzylidene-β-D-glucopyranosyl-(1→4)-2,3,6-tri-O-benzyl-1-thio-β-D-glucopyranoside (**18**)*

Glucosyl trichloroacetimidate **11** (0.31 g, 0.57 mmol), phenylthio glucoside **17** (0.20 g, 0.37 mmol) and 4Å molecular sieves (0.20 g) were diluted in DCM (4 mL). The suspension was cooled at –20 °C, then triethylsilyl trifluoromethanesulfonate (0.1 M solution in DCM, 0.74 mL) was added dropwise. The reaction was monitored by TLC (toluene/acetone, 9:1). After 1 h the reaction was quenched by the addition of TEA, diluted with AcOEt, and filtered over a Celite pad. After evaporation of the solvent, then crude was purified by flash chromatography (hexane/AcOEt, from 8:2 to 7:3) to give disaccharide **18** (0.29 g, 86 %) as an amorphous white solid. $[\alpha]_D^{20}$ = +20.6 (*c* = 1 in chloroform). ^{1}H NMR ($CDCl_3$): δ = 7.65-7.20 (m, 30H, arom), 5.50 (s, 1H, PhCH), 5.02-4.95 (m, 2H, H- 2' and CH_2Ph), 4.92-4.70 (m, 5H, CH_2Ph), 4.68-4.62 (m, 3H, H-1,1' and CH_2Ph), 4.52 (d, 1H, J = 12.0 Hz, CH_2Ph), 4.15 (dd, 1H, $J_{5',6'a}$ = 5.0 Hz , $J_{6'a,6'b}$ = 10.5 Hz, H-6'a), 3.98 (t, 1H, $J_{3,4}$ = $J_{4,5}$ = 9.4 Hz, H-4), 3.79-3.77 (m, 2H, 2 H-6), 3.68 (t, 1H, $J_{3',4'} = J_{4',5'}$ = 9.3Hz, H-4'), 3.65-3.56 (m, 2H, H-3, 3'), 3.52-3.43 (m, 2H, H-2, 6'b), 3.38(dt, 1H, $J_{4,5}$ = 9.4 Hz, $J_{5,6}$ = 2.6 Hz, H-5), 3.21 (dt, 1H, $J_{4',5'}$ = 9.3 Hz, $J_{5',6'a}$= 5.0 Hz, $J_{5',6'b}$ = 9.8 Hz, H-5'), 1,97 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$): δ = 169.1 (C=O), 138.2-126.0 (36C, arom.), 101.2 (CHPh), 100.8 (C-1'), 87.4 (C-1), 84.7 (C-3), 81.7 (C-4'), 80.2 (C-2), 79.0 (C-5), 78.6 (C-3'), 76.7 (C-4), 75.5 (CH_2Ph), 75.4 (CH_2Ph), 74.1 (CH_2Ph), 73.6 (CH_2Ph), 73.3 (C-2'), 68.6 (C-6'), 67.9 (C-6), 66.1 (C-5'), 20.9(CH_3). MS (ESI) *m/z* (%): 947.6 (100) $[M+Na]^+$. HRMS (ESI): *m/z* calcd for $C_{55}H_{56}O_{11}NaS$ 947.3441 $[M+Na]^+$, found 947.3439.

*4.1.12. Synthesis of phenyl 3-O-benzyl-4,6-O-benzylidene-β-D-glucopyranosyl-(1→4)-2,3,6-tri-O-benzyl-1-thio-β-D-glucopyranoside (**19**)*

To a stirred solution of **18** (0.28 g, 0.30 mmol) in DCM (6 mL) sodium methoxide in methanol (0.1 M solution, 0.60 mL) was added. The reaction was stirred for 17 h at room temperature, then it was neutralized with an ion exchange resin (Dowex 50 × 8, H^+ form), filtered and concentrated. The crude product was subjected to flash chromatography (hexane/AcOEt, 8:2) to give pure **19** (0.20 g, 75%) as an amorphous white solid. $[\alpha]_D^{20}$ = +0.20 (*c* = 1 in chloroform). ^{1}H NMR ($CDCl_3$): δ = 7.60-7.27 (m, 30H, arom.), 5.49 (s, 1H, CHPh), 5.33 (s, 1H, OH), 4.98-4.94 (m, 2H, CH_2Ph), 4.87-4.78 (m, 3H, CH_2Ph), 4.74-4.60 (m, 5H, H-1,1' and CH_2Ph), 4.10-4.01 (m, 2H, H-3, 6a), 3.97 (dd, 1H, $J_{5',6'a}$ = 5 Hz, $J_{6'a,6'b}$ = 10.4 Hz, H-6'a), 3.87-3.84 (m, 1H, H-6b), 3.68 (dd, 1H, $J_{3,4} = J_{4,5}$ = 8.8 Hz,

H-4), 3.63-3.48 (m, 6H, H-2, 4, 5, 2', 3', 6'), 3.18-3.12 (m, 1H, H-5'). ^{13}C NMR ($CDCl_3$): δ = 138.8-126.0 (36 C, arom.), 103.6 (C-1'), 101.2 (CHPh), 87.5 (C-1), 85.5 (C-4), 81.3, 80.5, 80.4, 78.8, 77.0 (C-3), 75.5, 75.3 (2 C, CH_2Ph), 74.6 (CH_2Ph), 73.6 (CH_2Ph), 68.6 (C-6'), 68.5 (C-6), 66.4 (C-5'). MS (ESI) 905.3 (100) $[M+Na]^+$, 1787.9 (40) $[2M+Na]^+$. HRMS (ESI): *m/z* calcd for $C_{53}H_{54}O_{10}NaS$ 905.3335 $[M+Na]^+$, found 905.3331.

*4.1.13. Synthesis of phenyl 3-O-benzyl-4,6-O-benzylidene-2-O-(N-imidazole-1-sulfonyl)-β-D-glucopyranosyl-(1→4)-2,3,6-tri-O-benzyl-1-thio-β-D-glucopyranoside (**20**)*

NaH (60 % in oil, 0.13 g, 3.30 mmol) was added to a stirred solution of compound **19** (0.19 g, 0.22 mmol) in dry DMF (6 mL) at room temperature. After 1 h, the suspension was cooled at –40 °C and 1,1'-sulfonyl-diimidazole (0.44 g, 2.20 mmol) in dry DMF (3 mL) was added. After 2 h the reaction mixture was quenched with MeOH and allowed to warm to room temperature, then diluted with AcOEt (40 mL) and washed with brine (2 x 30 mL). The organic layers were dried over Na_2SO_4, filtered and evaporated. Flash chromatography (hexane/AcOEt, 8:2) of the crude product gave compound **20** (0.19 g, 86%) as a foamy solid. $[\alpha]_D^{20}$ = -10.6 (*c* = 1 in chloroform). ^{1}H NMR ($CDCl_3$): δ = 7.89 (s, 1H, H imidazole), 7.59-7.22 (m, 31H, arom.), 7.03 (s, 1H, H imidazole), 5.49 (s,1H, CHPh), 4.89-4.75 (m, 6H, CH_2Ph), 4.64-4.58 (m, 3H, H-1, 1' and 1H of CH_2Ph), 4.52 (t, 1H, $J_{1',2'} = J_{2',3'} = 8.7$ Hz, H-2'), 4.42 (d, 1H, J =11.8 Hz,1H of CH_2Ph), 4.26 (dd, 1H, $J_{5,6'a} = 5.0$ Hz, $J_{6'a,6'b} = 10.5$ Hz, H-6'a), 4.06 (t, 1H, $J_{3,4} = J_{4,5} = 9.5$ Hz, H-4), 3.70-3.44 (m, 7H, H-2, 3, 6a, 6b, 3', 4', 6'b), 3.18-3.07 (m, 2H, H-5, 5'). ^{13}C NMR ($CDCl_3$): δ = 138.7-126.0 (38 C, 2 C imidazole and 36 C arom), 118.5 (C imidazole), 101.5 (CHPh), 98.4 (C-1'), 87.5 (C-1), 85.5 (C-2'), 84.1, 81.9, 80.3, 78.1 (C-5), 77.0, 75.6, 75.5 (CH_2Ph), 75.4 (CH_2Ph), 74.5 (CH_2Ph), 73.6 (CH_2Ph), 68.4 (C-6'), 67.6 (C-6), 65.9 (C-5'). MS (ESI) *m/z* (%): 1035.2 (100) $[M+Na]^+$. HRMS (ESI): *m/z* calcd for $C_{56}H_{56}N_2O_{12}NaS_2$ 1035.3172 $[M+Na]^+$, found 1035.3165.

*4.1.14. Synthesis of phenyl 2-azido-3-O-benzyl-4,6-O-benzylidene-2-deoxy-β-D-glucopyranosyl-(1→4)-2,3,6-tri-O-benzyl-1-thio-β-D-glucopyranoside (**21**)*

To a stirred solution of **20** (0.18 g, 0.18 mmol) in dry DMF (3.5 mL), sodium azide (0.12 g, 1.80 mmol) was added and the resulting solution was heated at 85 °C. After 4 h, the reaction was cooled to room temperature, diluted with brine (30 mL) and extracted with AcOEt (3 x 20 mL). The combined organic layers were dried over Na_2SO_4, filtered and evaporated. The crude product was purified by flash chromatography (hexane/AcOEt, 75:25) to give compound **21** (0.13 g, 83%) as a foamy white solid. $[\alpha]_D^{20}$ = -23.9 (*c* = 1 in chloroform). ^{1}H NMR ($CDCl_3$): δ = 7.60-7.28 (m, 30H, arom.), 5.53 (s, 1H, CHPh), 5.01 (d, 1H, J = 10.5 Hz, 1H of CH_2Ph), 4.88-4.76 (m, 4H, CH_2Ph), 4.73-4.70 (m, 2H, H-1' and 1H of CH_2Ph), 4.68-4.64 (m, 2H, H-1 and 1H of CH_2Ph), 4.50 (d, 1H, J = 10.5 Hz, 1H of CH_2Ph), 4.05-3.99 (m, 2H, H-3, 6'a), 3.94 (t, 1H, $J_{3',4'} = J_{4',5'} = 9.5$ Hz, H-4'), 3.86 (dd, 1H, $J_{1',2'} = 1.1$ Hz, $J_{2',3'} = 3.6$ Hz, H-2'), 3.81-3.78 (m, 2H, 2 H-6), 3.72 (t, 1H, $J_{3,4} = J_{4,5} = 8.9$ Hz, H-3), 3.58-3.49 (m, 4H, H-2, 5, 3', 6'b), 3.09-3.04 (m, 1H, H-5'). ^{13}C NMR ($CDCl_3$): δ = 138.8-126.0 (36C, arom.), 101.5 (CHPh), 100.3 (C-1'), 87.5 (C-1), 85.0 (C-4), 80.3, 78.5, 76.5 (C-4'), 77.4 (C-3), 76.7, 75.5 (2C, CH_2Ph), 73.7 (CH_2Ph), 72.8 (CH_2Ph), 68.8 (C-6), 68.3 (C-6'), 67.3 (C-5'), 63.6 (C-2'). MS (ESI) *m/z* (%): 930.3 (100) $[M+Na]^+$, 1837.5 (20) $[2M+Na]^+$. HRMS (ESI): *m/z* calcd for $C_{53}H_{53}N_3O_9NaS$ 930.3400 $[M+Na]^+$, found 930.3403.

*4.1.15. Synthesis of phenyl 2-acetamido-3-O-benzyl-4,6-O-benzylidene-2-deoxy-β-D-glucopyranosyl-(1→4)-2,3,6-tri-O-benzyl-1-thio-β-D-glucopyranoside (**16**)*

A mixture of **21** (0.12 g, 0.13 mmol) and Zinc (0.43 g, activated with aq. 2% $CuSO_4$) in THF/Ac_2O/AcOH 3:2:1 (5 mL) was stirred for 1h at room temperature. The reaction was diluted with AcOEt and filtered over a Celite pad. Satd. aq. $NaHCO_3$ was added (30 mL) and, after separation, the aqueous phases were extracted with AcOEt (2 x 20mL). The combined organics

were dried over $NaSO_4$, filtered and concentrated. Flash chromatography (Hexane/AcOEt, 6:4) of the crude product gave pure **16** (0.080 g, 66%) as a foam. $[\alpha]_D^{20}$ = -34.0 (*c* = 1 in chloroform) ^{1}H NMR ($CDCl_3$): δ = 7.60-7.24 (m, 30H, arom.), 5.58 (*br* d, 1H, J = 9.1 Hz, NH), 5.50 (s, 1H, CHPh), 4.89-4.86 (m, 3H, CH_2Ph), 4.77-4.67 (m, 5H, H-1', 2' and CH_2Ph), 4.65 (d, 1H, $J_{1,2}$ = 9.7 Hz, H-1), 4.57-4.53 (m, 2H, CH_2Ph), 4.18-4.06 (m, 2H, H-4,6'a), 3.84-3.77 (m, 2H, 2 H-6), 3.66-3.58 (m, 3H, H-3, 4', 6'b), 3.54-3.51 (m, 2H, H-2, 3'), 3.46-3.44 (m, 1H, H-5), 3.22-3.15 (m, 1H, H-5'), 1.87 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$): δ = 170.4 (C=O), 139.0-126.1 (36C, arom.), 101.6 (CHPh), 100.1 (C-1'), 87.5 (C-1), 85.3, 80.6, 78.7 (C-5), 78.6, 76.5 (C-4), 75.8, 75.4 (CH_2Ph), 75.2 (CH_2Ph), 73.5 (CH_2Ph), 71.5 (CH_2Ph), 68.7 (C-6), 68.6 (C-6'), 67.1 (C-5'), 50.4 (C-2'), 23.2 (CH_3). MS (ESI) *m/z* (%): 946.4 (100) $[M+Na]^+$, 1869.7 (75) $[2M+Na]^+$. HRMS (ESI): *m/z* calcd for $C_{55}H_{57}NO_{10}NaS$ 946.3601 $[M+Na]^+$, found 946.3605.

*4.1.16. Synthesis of N-benzyl-N-benzyloxycarbonyl-3-aminopropyl 2-acetamido-3-O-benzyl-4,6-O-benzylidene-2-deoxy-β-D-mannopyranosyl-(1→4)-2,3,6-tri-O-benzyl-α-D-glucopyranosyl-(1→3)-2,4-di-O-benzyl-α-L-rhamnopyranoside (**2**)*

*From compound **15**:* A mixture of **15** (0.14 g, 0.10 mmol) and Zinc (0.44 g, activated with aq. 2% $CuSO_4$) in THF/Ac_2O/AcOH 3:2:1 (5 mL) was stirred for 3h at room temperature. The reaction was diluted with AcOEt and filtered over a Celite pad. Satd. aq. $NaHCO_3$ was added (30 mL) and, after separation, the aqueous phases were extracted with AcOEt (2 x 20mL). The combined organics were washed with brine, dried over $NaSO_4$, filtered and concentrated. Flash chromatography (Hexane/AcOEt, 7:3) of the crude product gave pure **16** (0.091 g, 62%) as an amorphous glassy solid.

*From compound **16**:* A solution of **16** (0.06 g, 0.065 mmol) and **3** (0.08 g, 0.13 mmol) in dry DCM (2 mL) containing 4Å molecular sieves (0.15 g) was stirred at room temperature for 0.5 h.The suspension was cooled to -35 °C, and then NIS (0.022 g, 0.097 mmol) followed by AgOTf (8 mg, 0.033 mmol) were added. After the addition, the reaction was allowed to warm to -10 °C and was stirred at that temperature. After 0.45 h, TLC (Hexane/AcOEt, 6:4) showed the disappearances of the donor. The reaction was diluted with DCM (30 mL) and filtered over a Celite pad. The organic solution was then washed with 10 % aq. $Na_2S_2O_3$ (30 mL) and satd aq. $NaHCO_3$ (30 mL). The organics were then dried over $NaSO_4$, filtered and concentrated. The residue was purified by flash chromatography (Hexane/AcOEt, 7:3) to give first α-**2** (0.018 g), followed by β-**2** (0.037 g) with an overall glycosylation yield of 60%. $[\alpha]_D^{20}$ = +0.77 (*c* = 0.5 in chloroform). ^{1}H NMR ($CDCl_3$): δ = 7.59-7.01 (m, 45H, arom.), 5.53-5.43 (m, 2H, 1H x CH_2Ph and NH), 5.23-5.17 (m, 2H, CH_2Ph), 5.15 (d, 1H, $J_{1',2'}$ = 2.9 Hz, H-1), 4.95 (d, 1H, J = 11.7 Hz, 1 x CH_2Ph), 4.89-4.74 (m, 4H, CH_2Ph), 4.74-4.46 (m, 10H, H-1, 2" and CH_2Ph), 4.43 (*br* s, 1H, H-1"), 4.25-4.11 (m, 2H, H-6a'' and 1 x CH_2Ph), 4.07-4.01 (m, 2H, H-3, 3'), 3.99-3.92 (m, 2H, H-4', 5'), 3.84 (br d, 1H, H-2), 3.75-3.58 (m, 5H, H-a, 4, 5, 2', 6b''), 3.55 (t, 1H, $J_{3",4"} = J_{4",5"}$ = 9.6Hz, H-4''), 3.47-3.20 (m, 6H, H-a', 6a', 6b', 3'' and 2 H-c), 3.13-3.03 (m, 1H, H-5''), 1.87-1.73 (m ,5H, 2 H-b and CH_3CO), 1.32 (d, 3H, $J_{5,6}$ = 6.0 Hz ,3 H-6). ^{13}C NMR ($CDCl_3$): δ = 170.25 (C=O), 156.6 and 156.1 (C=O), 139.4-126.1 (54C, arom.), 101.6 (CHPh), 99.4 (C-1''), 98.3 (C-1), 96.3 (C-1'), 80.8 (C-4'), 79.9 (C-4), 79.4 (C-2'), 78.7 (C-4''). 77.6 (*br* s, C-3'), 76.3 (C-2), 75.8 (C-3''), 75.7 (C-3), 74.9 (CH_2Ph),74.8 (CH_2Ph), 73.6 (CH_2Ph), 73.4 (CH_2Ph), 73.3 (CH_2Ph), 71.3 (CH_2Ph), 70.2 (C-5'), 68.7 (C-6''), 68.5 (C-5), 68.0 (C-6'), 67.2 (CH_2Ph), 67.0 (C-5''), 65.1 (C-a), 50.8 and 50.6 (2C, C-2'' and NCH_2Ph), 44.6 and 43.7 (C-c), 28.3 and 27.9 (C-b), 23.1 (CH_3CO), 18.1(C-6). MS (ESI) *m/z* (%): 1461.9 (100) $[M+Na]^+$. HRMS (ESI): *m/z* calcd for $C_{87}H_{94}N_2O_{17}Na$ 1461.6450 $[M+Na]^+$, found 1461.6449.

*4.1.17. Synthesis of 3-aminopropyl 2-acetamido-2-deoxy-β-D-mannopyranosyl-(1→4)-α-D-glucopyranosyl-(1→3)-α-L-rhamnopyranoside hydrochloride salt (**1**)*

Compound **2** (0.080 g, 0.056 mmol) in AcOEt/MeOH/0.02M HCl, 1:1:1 (9 mL) was hydrogenolyzed over $Pd(OH)_2$ (0.070 g) for 4 days. The mixture was filtered over pleated filter

paper, the filtrate was concentrated to 1 mL, and then lyophilized to give trisaccharide **1** (0.034 g, 97%) as an amorphous white solid. $[\alpha]_D^{20}$ = -19.6 (*c* = 0.5 in water). ^{1}H-NMR (D_2O): δ = 4.98 (d, 1H, $J_{1',2'}$= 3.7 Hz, H-1'), 4.81 (*br* d, 1H, $J_{1'',2''}$ = 1.3 Hz, H-1''), 4.77 (*br* d, 1H, $J_{1,2}$ = 1.7 Hz, H-1), 4.47 (dd, 1H, $J_{2'',2''}$ = 1.4 Hz, $J_{2'',3''}$ = 4.4Hz, H-2''), 4.09-4.05 (m, 1H, H-2), 3.98-3.92 (m, 1H, H-5'), 3.89-3.34 (m, 15H), 3.10-2.95 (m, 2H, 2 H-c), 1.99 (s, 3H, CH_3CO), 1.95-1.87 (m, 2H, H-b), 1.23 (d, 3H, 3 H-6). ^{13}C NMR (D_2O): δ = 175.4 (C=O), 99.4 (2C, C-1, 1''), 95.5 (C-1'), 78.6, 76.5 (C-5''), 75.9 (C-3), 71.9 (C-3''), 71.5 (C-3'), 71.2 (C-2'), 70.2 (2C), 68.8, 66.8 (C-2), 66.7, 64.9 (C-a), 60.4 (C-6''), 59.7 (C-6'), 53.3 (C-2''), 37.4 (C-c), 26.7 (C-b), 22.0 (CH_3CO), 16.7 (C-6). MS (ESI) *m/z* (%): 609.3 (100) $[M+Na]^+$. HRMS (ESI): *m/z* calcd for $C_{23}H_{43}N_2O_{15}$ 587.2663 $[M+H]^+$, found 587.2664.

4.2. Biological test

Competitive ELISA assay: 96-well flat-bottomed plates were incubated overnight at 4-8°C with a mixture of *S. pneumoniae* CPS 19A (1 mg/mL, Statents serum Institute, Artillerivej, Denmark) or 19F (1 mg/mL,Sanofi-Aventis, France) and methylated human serum albumin (1 mg/mL). A solution of foetal calf serum (5%) in phosphate-buffered saline supplemented with Brij-35 (0.1%) and sodium azide (0.05%) was applied to the plates for blocking of nonspecific binding sites. The plates were incubated overnight at 4-8°C with a solution (1:200) of rabbit anti-19A or 19F, used as reference serum (Statents serum Institute, Artillerivej, Denmark). When trisaccharide was tested, it was added to each well immediately before the addition of the reference serum. The plates were then incubated with alkaline phosphatase conjugate goat anti-rabbit IgG (Sigma-Aldrich, Milan, Italy), stained with *p*-nitrophenylphosphate, and the absorbance was measured at 405 nm with an Ultramark microplate reader (Bio-Rad Laboratories S.r.l., Milan, Italy).

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