DOI: 10.1002/cbic.200700501

Synthesis and Immunological Properties of a Tetrasaccharide Portion of the B Side Chain of Rhamnogalacturonan II (RG-II)

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A highly convergent strategy was used for the synthesis of a tetrasaccharide [3-aminopropyl β-L-arabinofuranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→3)]-α-L-arabinopyranoside] portion of the B side chain of the plant cell-wall pectic polysaccharide rhamnogalacturonan II (RG-II). The terminal nonreducing β-L-arabinofuranosyl residue of the target compound was installed by using an arabinofuranosyl donor that was protected with a 3,5-O-(di-tert-butylsilane) group to facilitate nucleophilic attack from the β-face. The synthetic strategy also employed a chemoselective glycosylation of a trichloroacetimidate donor with a thioglycosyl acceptor; this gave a product that could be used immediately in a subsequent glycosylation. The reducing end of the tetrasaccharide contained an aminopropyl group to facilitate conjugation to keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Mice that were immunized with a KLH–tetrasaccharide conjugate produced antibodies that recognized RG-II isolated from Arabidopsis thaliana *cell walls, but did not recognize RG-II obtained from red wine. Our data suggest that the arabinopyranosyl residue exists in the $^4C_1$ conformation in the tetrasaccharide and in* A. thaliana *RG-II, whereas it has the $^1C_4$ conformation in wine RG-II. It is proposed that differences in the conformation of side chain B might account for the ability of antibodies to discriminate between RG-II that was isolated from* Arabidopsis *and wine.*

Introduction

Rhamnogalacturonan II (RG-II) is a complex pectic polysaccharide that is present in the primary cell walls of all vascular plants.[1,2] RG-II has a backbone that is composed of 1,4-linked α-D-galactosyluronic acid (D-Gal*p*A) residues. Four structurally different oligosaccharides (side chains A–D) are attached to the backbone.[1–3] In the primary cell wall, two RG-II molecules are covalently cross-linked to one another by a 1:2 borate diester between each apiofuranosyl residue of side chain A.[4–6] This cross-linking is required for normal plant growth and development, as it is believed to regulate the organization of the pectic network in the cell wall, and to contribute to the mechanical properties of the primary wall.[7] Indeed, changes in the primary structure of RG-II have been shown to result in decreased borate cross-linking of pectin, and lead to abnormal plant growth.[8–10]

Side chain B of RG-II contains the seldom-observed monosaccharides apiose, aceric acid, and 2-*O*-methyl fucose (Figure 1).[1,11,12] The primary sequence of side chain B from RG-IIs of various plants has been determined. Taken together, the results of such studies suggest that variation in the sequence of this side chain result in large part from differences in the substitution pattern of the arabinopyranosyl (Ara*p*) residue.[2] For example, in RG-II that is isolated from sycamore, ginseng and red wine, the Ara*p* is substituted at C2 with β-L-Ara*f*-(1-2)-α-L-Rha*p* and at C3 with α-L-Rha*p*. By contrast, the Ara*p* of *Arabidopsis thaliana*, *Equisetum hyemale*, and *Selaginella kraussiana* RG-IIs lack a saccharide substituent at C3. In RG-IIs from some nonflowering plants, including *Platycerium bifurcatum* and *Psilotum nudum*, 3-*O*-Me-α-L-Rha*p* is linked to C2 and C3 of the Ara*p* moiety.[13] Different substitution patterns of the Ara*p* residue have been shown to affect the ring conformation of this sugar, although the effects on the biological properties of RG-II are not known.[14,15]

Monoclonal antibodies that recognize RG-II will be valuable tools to monitor changes in plant primary cell walls at the cellular and subcellular level.[16,17] Towards this end, we report here the chemical synthesis of tetrasaccharide **1** (Figure 1), which corresponds to a portion of side chain B of RG-II from sycamore, ginseng, and grape. NMR spectroscopic studies established that the Ara*p* residue in **1** has a $^4C_1$ conformation. We show that mice that are immunized with a KLH–tetrasaccharide conjugate produce antibodies that recognize *A. thaliana* RG-II, but not wine RG-II. This unexpected recognition pattern is likely because the 2-linked Ara*p* residue of *A. thaliana* adopts a $^4C_1$ conformation, whereas the 2,3-linked Ara*p* residue of wine RG-II has a $^1C_4$ conformation.

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Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author: copies of ^1H and ^{13}C NMR spectra of synthetic compounds.

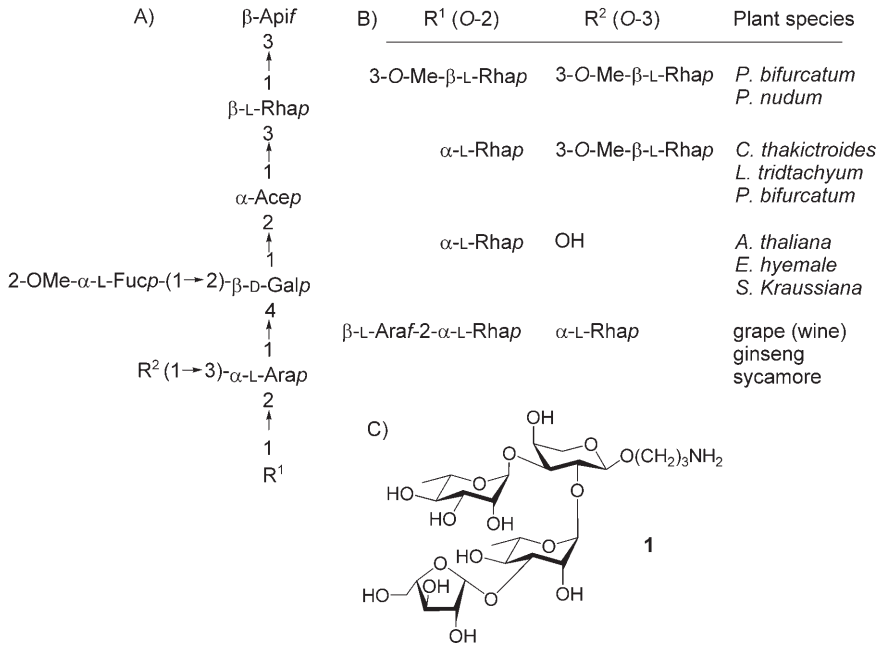
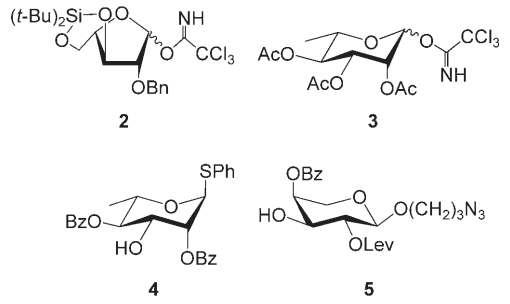


Figure 1. Structural features of the B side chain of RG-II. A) General structures of the B side chain of RG-II; B) substitution pattern of Ara*f* in various plant species; C) structure of target tetrasaccharide **1**.

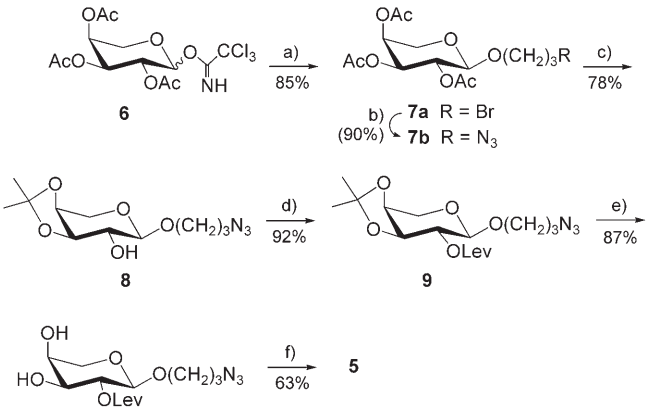
Result and Discussion

We envisaged that target tetrasaccharide **1** could be synthesized by a convergent approach that employs glycosyl donors **2** and **3** and glycosyl acceptors **4** and **5** (Schemes 1–3). A trimethylsilyl trifluoromethanesulfonate (TMSOTf)-promoted glycosylation[18] of **2** with **4** would give disaccharide **11**, which itself can be coupled with disaccharide **13**, to give fully protected tetrasaccharide **14**; deprotection of **14** would give **1**. Disaccharide **13** is obtained by glycosylation of **3** with **5**, followed by selective removal of the levulinoate (Lev; 4-oxopentanoate) ester. A major challenge in the synthesis of **1** is the stereoselective installment of the β-L-arabinofuranoside moiety.[19] We anticipated that high β-selectivity could be achieved by employing arabinofuranosyl donor **2**, which is protected with a 3,5-*O*-(di-*tert*-butylsilane) group.[20] The cyclic protecting group of **2** locks the arabino-L-furanosyl oxacarbenium ion in an E_3 conformation. This favors nucleophilic attack from the β-face because no staggered substituents can be experienced; in other words, an approach from the α-face is not favorable because an eclipsed H2 is encountered. Our synthetic strategy also employs a chemoselective glycosylation of a trichloroacetimidate donor with a thioglycosyl acceptor to give a product that can be used immediately in a subsequent glycosylation by activation with a thiophilic promotor.[21,22] Finally, the Lev group is cleaved with hydrazine that is buffered with acetic acid. These reaction conditions should not affect the acetyl and benzoyl esters.

Monosaccharides **2**, **3**, and **4** were prepared by previously reported procedures (Scheme 1).[14,20,23,24] However, relatively little effort has been directed towards the development of methods for the selective protection of arabinopyranosides. Therefore, an efficient protocol was developed for the preparation of **5** by starting from trichloroacetimidate **6** (Scheme 2).[25] Thus, the trimethylsilyl triflate (TMSOTf)-mediated coupling of **6** with 1-bromopropan-3-ol in dichloromethane gave **7a** as the α-anomer only in a yield of 85%.[18] By contrast, glycosylation reactions with the corresponding thioethyl D-arabinopyranoside or tetra-*O*-acetyl-α/β-D-arabinopyranose by using *N*-iodosuccinimide (NIS)/TMSOTf[26] or (BF_3·Et_2O) as the promoter gave **7a** in low yield. Displacement of the bromide of **7a** with sodium azide in a mixture of acetone and water provided derivative **7b**. The acetyl esters of **7b** were cleaved by treatment with sodium methoxide in methanol and the *cis*-3,4-diol of the result-



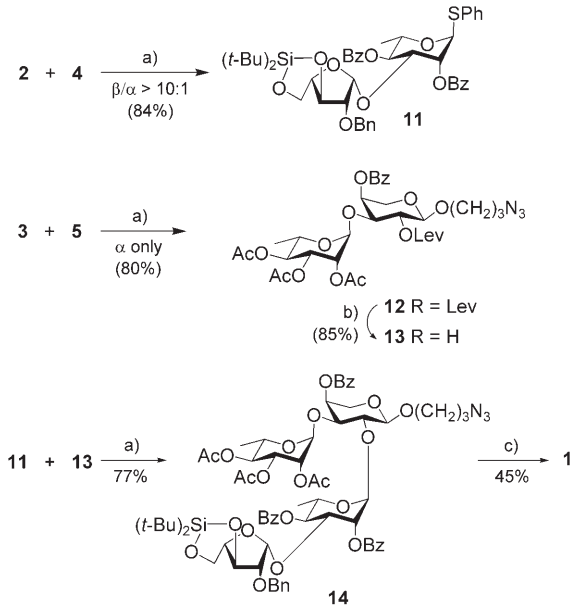
Scheme 1. Monosaccharide building blocks that were used in the synthesis of tetrasaccharide **1**.



Scheme 2. a) TMSOTf, $HO(CH_2)_3BR$, CH_2Cl_2, −70 °C; b) NaN_3, acetone/H_2O, Δ; c) i. NaOMe/MeOH, ii. 2,2-dimethoxypropane, PTSA, CH_2Cl_2; d) levulinic acid, DCC, DMAP, CH_2Cl_2; e) 80% aq. AcOH, 80 °C; f) trimethoxy orthobenzoate, PTSA, CH_2Cl_2 then 80% aq. AcOH, room temperature.

ing compound was selectively protected as its isopropylidene acetal by reaction with 2,2-dimethoxypropane in the presence of *p*-toluene sulfonic acid (PTSA) to give **8** in an overall yield of 78%. The C2 hydroxyl of **8** was protected as a Lev ester to give **9** by reaction with levulinic acid in the presence of 1,3-dicyclohexylcarbodiimide (DCC) and *N*,*N*-dimethylaminopyridine (DMAP) in dichloromethane. Finally, target glycosyl acceptor **5** was obtained by removal of the isopropylidene acetal of **9** by using 80% aqueous acetic acid, followed by treatment with trimethoxy orthobenzoate in the presence of a catalytic amount of PTSA in dichloromethane. The intermediate orthoester that was formed was then regioselectively opened by treatment with 80% aqueous acetic acid to give a C4 benzoate ester. The ^{1}H NMR spectrum of **5** showed a downfield shift in the signals for H2 ($\delta = 5.11$ ppm) and H4 ($\delta = 5.34$ ppm); this confirms the presence of esters at these positions.

We then focused our attention on the synthesis of disaccharides **11** and **13**, which were used for the assembly of tetrasaccharide **14** (Scheme 3). A TMSOTf-promoted glycosylation of



Scheme 3. a) TMSOTf, 4 Å MS, CH_2Cl_2, $-70\,^\circ$C; b) NH_2NH_2–AcOH, CH_2Cl_2/MeOH; c) NIS, TfOH, 4 Å MS, CH_2Cl_2, $-20\,^\circ$C; d) i. TBAF, THF, ii. NaOMe/MeOH, iii. Pd/C, *t*BuOH/AcOH/H_2O.

trichloroacetimidate **3** with α-L-arabinopyranoside acceptor **5** gave **12** only as the α-anomer in a yield of 80% due to neighboring group partifipation by the acetyl ester of **3**. The Lev ester of **12** was selectively removed by treatment with hydrazine acetate in a mixture of dichloromethane and methanol to give glycosyl acceptor **13**. Coupling of trichloroacetimidate **2** with acceptor **4** in the presence of a catalytic amount of TMSOTf gave disaccharide **11** mainly as the β-anomer in a yield of 84% .[14,20] The anomeric configuration of **11** was confirmed by the chemical shift of H1 ($\delta = 5.15$ ppm, $J_{1,2} = 5.4$ Hz) and C1 ($\delta = 97.8$ ppm).[27,28] As expected, the thiophenyl group of **11** was stable under the conditions that were used for the activation of trichloroactimidates. However, in the presence of a thiophilic promoter this functionality is activated and can be used as a glycosyl donor. Thus, **11** was used in the glycosylation of **13** by using NIS/TMSOTf as the promoter[26] in dichloromethane at $-20\,^\circ$C to give tetrasaccharide **14** in a yield of 77%, as only the α-isomer. Finally, deprotection of **14** to give target compound **1** was accomplished by a three-step procedure that involved the removal of the di-*tert*-butylsilane protecting group by treatment with tetra-*n*-butylammonium fluoride (TBAF) in THF, saponification of the acetyl and benzoyl esters by using sodium methoxide in methanol, and finally catalytic hydrogenolysis over Pd/C to remove the benzyl ethers, and to convert the azido moiety into an amine.

An examination of the chemical shift and coupling constant data from the ^{1}H and ^{13}C NMR spectra of **1** established that the central Ara*p* moiety exists in the $^4C_1$ conformation (Table 1).

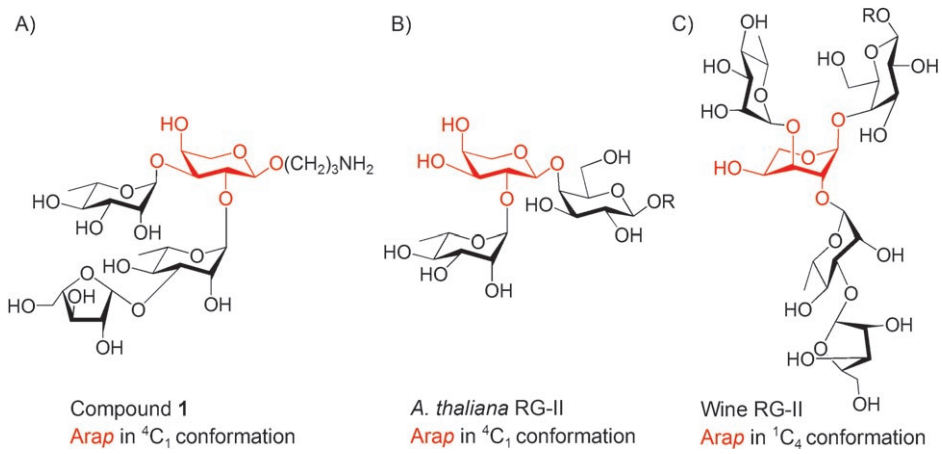
Table 1. Comparison of ^{1}H NMR spectroscopic data for the Ara*p* of tetrasaccharide **1**, Wine RG-II[15] and free α-L-Ara*p*.[15]

	Tetrasaccharide **1**	Wine RG-II	α-L-Arabinopyranose
H1 [ppm]	4.35	5.04	4.49
$J_{H1,H2}$ [Hz]	7.8	3	7.8
$J_{H2,H3}$ [Hz]	8.5	4	9.9
$J_{H3,H4}$ [Hz]	3.5	5	3.6
$J_{H4,H5}$ [Hz]	3.0, 4.0	8, 4	2.1, <2

Thus, $J_{H1,H2}$ and $J_{H2,H3}$ of **1** are larger than 7 Hz, which is indicative of a *trans*-diaxial arrangement of H1, H2 and H3 in a $^4C_1$ chair conformation. Similar coupling constant data of Ara*p* of wine RG-II shows that this moiety adopts a different conformation, which was assigned as a distorted $^1C_4$ conformation. Our previous studies had also shown that the Ara*p* adopts such a conformation when the 2,3-linked Ara*p* of tetrasaccharide **1** is attached to O4 of galactoside, as in the structure of the B side chain of wine RG-II (Scheme 4).[14] On the other hand, the Ara*p* moiety of *A. thaliana* RG-II, which lacks a saccharide substituent at its C3, adopts a regular $^4C_1$ conformation. Taken together these results suggest that the Ara*p* moiety adopts a distorted $^1C_4$ conformation only when bulky substituents are present at its anomeric center and at the C2 and C3 hydroxyls. Thus, tetrasaccharide **1** contains the terminal portion of side chain B from wine RG-II, yet has a conformation that is comparable with side chain B of *A. thaliana* RG-II.

Preparation of carbohydrate–protein conjugates, immunization, and antigenic analysis

For most immunogens, including oligo- and polysaccharides, antibody production requires the cooperative interactions of B cells, helper T cells and antigen-presenting cells.[29] Saccharides alone typically do not activate helper T cells and therefore are weak immunogens. However, the immunogenicity of saccharides is greatly increased by conjugation to carrier proteins such as KLH or detoxified tetanus toxoid.[30–33] Immune responses are further increased by using an adjuvant such as QS-21, Freund's or detoxified lipid A.



Scheme 4. The proposed conformation of Ara*p* (in red) in structures that correspond to A) tetrasaccharide **1**; B) *A. thaliana* RG-II; C) Wine RG-II. Sugar substituents at C1, C2, and C3 are required for the conformational change of Ara*p* from a $^4C_1$ to a $^1C_4$ conformation.

A conjugate of tetrasaccharide **1** with KLH was used to immunize mice, and BSA-linked tetrasaccharide **1** was employed in an enzyme-linked immunosorbent assay (ELISA) to determine antibody titers of antisera. KLH, which is a very large protein (8.0×10^6 Da) that contains many lysines (>6% by mass) for hapten conjugation, has proven to be an attractive carrier protein for mice immunizations. Thus, the carbohydrate–KLH conjugate was prepared by activating the amino propyl spacer of **1** with *S*-acetylthioglycolic acid pentafluorophenyl ester to give the corresponding thioacetate. The thioacetyl derivative was purified by size-exclusion chromatography, de-*S*-acetylated by using ammonia (7%) in DMF (→free thiol), and then conjugated to KLH that had been modified with 3-(bromoacetamido)propionate.[34] The resulting carbohydrate–protein conjugate was found to contain an average of 1700 mol of tetrasaccharide per mol of KLH. The protein content was determined according to the method of Bradford[35] and quantitative carbohydrate analysis was performed by treatment with aqueous trifluoroacetic acid (TFA) to generate the monosaccharides, which were quantified by high-pH anion-exchange chromatography with a pulsed amperometric detector (HPAEC-PAD) by using serial dilutions of rhamnose as a standard.[36] A BSA–**1** conjugate was prepared by reaction of the tetrasaccharide thiol with BSA that had been derivatized with a maleimide-containing linker to give a conjugate with an average of 9 mol of tetrasaccharide per mol of BSA.

Different carrier proteins and linker technologies were used to immunize mice and for ELISA to limit detection of anti-protein and anti-linker antibodies.[34] We also used the small and flexible 3-(bromoacetamido)propionate cross linker to generate KLH–**1**, as it is significantly less antigenic than a maleimide linker, which itself has been reported to suppress antigenic responses against carbohydrate epitopes.[34] Gratifyingly, the KLH–**1** conjugate elicited antibodies against tetrasaccharide **1** (Figure 2A).

An ELISA procedure was developed to determine if the antisera recognized native RG-II. RG-II is a low-molecular-weight anionic polysaccharide that does not bind to regular ELISA plates. Thus, RG-II that was isolated from wine and *A. thaliana* cell walls was modified with biotin by reductive amination with biotin-X-hydrazide in the presence of $NaBH_3(CN)$. The biotinylated RG-II was then bound to NeutrAvidin-coated microtiter plates. Unexpectedly, the anti-sera recognized *A. thaliana* RG-II, albeit at low titers (Figure 2B), but did not bind to red wine RG-II (Figure 2C). Furthermore, there was little correlation between the responses to **1** and *A. thaliana* RG-II and it appears that the antibody populations that were elicited by each mouse have different levels of cross reactivity with *A. thaliana* RG-II.

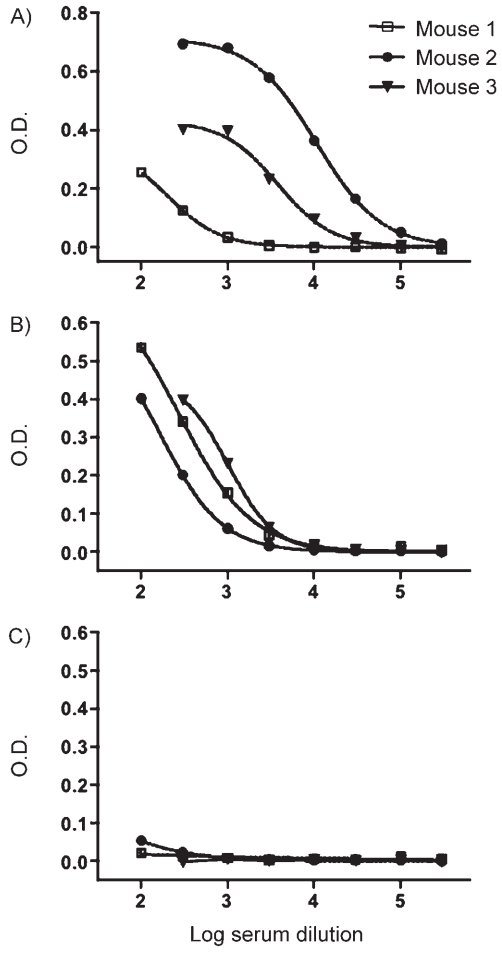


Figure 2. IgG antibody responses of mice that were immunized with a KLH–**1** conjugate one week after the fourth immunization. Plates (96-well) were coated with A) BSA-**1** conjugate; B) biotinylated *Arabidopsis thaliana* RG-II or C) biotinylated wine RG-II and responses were determined by ELISA. The OD values were corrected for blank readings that were obtained from microtiter plates that were coated with BSA (A) or biotin (B, C).

The Ara*p* residue of the B side chain of wine RG-II adopts a $^1C_4$ conformation due to the saccharide substituents at its anomeric center and the C2 and C3 hydroxyl groups (Scheme 4). The glycosyl sequence of tetrasaccharide **1** is comparable to the B side chain of wine RG-II. Nevertheless, the Ara*p* residue of **1** adopts a $^4C_1$ conformation due to the lack of a bulky substituent at its anomeric center.[14,15] Thus, it is likely that wine RG-II is not recognized by antisera that are elicited by **1** because the Ara*p* of the natural and synthetic materials have different conformations. RG-II that was obtained from *A. thaliana*, which lacks the Rha*p* at C3 of the central Ara*p* moiety is recognized by the antisera that are elicited by KLH–**1** because its Ara*p* adopts a $^4C_1$ conformation.[14,15]

The results reported here suggest that the Ara*p* moiety of the B side chain of RG-II has a central role in establishing the conformation of the side chain, and hence it influences its immunological properties. Our data are consistent with previous studies[30-33] that show that determining the epitope selectivities of antibodies often requires a detailed knowledge of the primary sequences and conformational properties of an oligosaccharide. For example, the capsular polysaccharide of group B *streptococcus* serotype III is composed of a (→4)-β-D-Glc*p*-(1→6)-β-D-GlcNAc*p*-(1→3)-β-D-Gal*p*-(1→) backbone that is substituted at C4 of the GlcNAc residue with α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→). Although antibodies bind to the backbone of the polysaccharide, the side chain is essential for antibody recognition. Molecular dynamics simulations suggest that the Neu5Ac stabilizes the helical conformation of the polysaccharide, and that the predominant Neu5Ac-dependant antibody population recognizes the immunodominant sequence in the core of the helix.[37,38] In group B meningococcal polysaccharides, the degree of polymerization (DP) of the α(2→8)-linked oligosialic acids affects the relative distributions of conformers in solution, which in turn influences antibody recognition.[39,40] Thus, in these bacterial polysaccharides, the dihedral angles of glycosidic linkages are affected by the nature and DP of the oligosaccharide. A unique aspect of the conformational epitope that is described here is that differences in conformation are obtained by a change in sugar ring conformation.

Experimental Section

General methods and materials: All solvents employed were of reagent grade and were dried by refluxing over appropriate drying agents. All reactions were performed under argon unless stated otherwise. Reactions were monitored by thin-layer chromatography (TLC) by using silica gel 60 F_{254} and the compounds were visualized with UV light (254 nm) or by treatment with a solution of 10% H_2SO_4 in EtOH. Flash chromatography was performed on 70–230 mesh silica gel. Solvents were evaporated under reduced pressure while maintaining the water bath temperature between 35 and 40 °C. NMR spectra were recorded on Varian spectrometers (models Inova300, Inova500 and Inova600) that were equipped with Sun workstations. ^{1}H NMR spectra were recorded in $CDCl_3$ and referenced to residual $CHCl_3$ at 7.26 ppm. ^{13}C NMR spectra were referenced to the central signal of $CDCl_3$ at 77.0 ppm. MALDI-TOF MS spectra were recorded on a VOYAGER-DE (Applied Biosystems) in the positive ion mode by using 2,5-dihydroxy-benzoic acid in acetonitrile (10 mg mL^{-1}) as a matrix.

3-Bromopropyl 2,3,4-tri-*O*-acetyl-α-L-arabinopyranoside (7): TMSOTf (39.0 μL, 0.215 mmol) was added to a solution of **6** (0.90 g, 2.15 mmol) and 3-bromo-propanol (232 μL, 2.58 mmol) in CH_2Cl_2 (10 mL) at −70 °C in the presence of 4 Å molecular sieves. The temperature of the reaction was raised to −10 °C over a period of 1 h. Et_3N was then added, and stirring was continued for 15 min at room temperature prior to filtration and concentration of the filtrate in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc 2:1, *v*/*v*) to give **7** (0.73 g, 85%). $[\alpha]_D^{25} = +29.4^\circ$ ($c = 0.76$, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 5.25$ (m, 1H; H4), 5.15 (dd, $J = 6.9$, 9.3 Hz, 1H; H2), 5.05 (dd, $J = 3.6$, 9.3 Hz, 1H; H3), 4.42 (d, $J = 6.9$ Hz, 1H; H1), 4.03–3.94 (m, 2H; H5a, $OCH_{2a}CH_2CH_2N_3$), 3.65–3.60 (m, 2H; H5b, $OCH_{2b}CH_2CH_2N_3$), 3.47 (dd, $J = 5.7$, 6.9 Hz, 2H; $CH_2CH_2CH_2N_3$), 2.15–2.00 ppm (m, 11H; 3OAc, $CH_2CH_2CH_2N_3$); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 170.5$, 170.3, 169.8, 101.4, 70.3, 69.4, 67.8, 67.0, 63.4, 32.5, 30.4, 21.1, 21.0, 20.9 ppm; MALDI-TOF HRMS: *m*/*z*: calcd for $C_{14}H_{21}BrO_8Na$: 419.0420; found: 419.0427 $[M+Na]^+$.

3-Azidopropyl 3,4-*O*-isopropylidene-α-L-arabinopyranoside (8): NaN_3 (0.95 g, 14.4 mmol) was added to a solution of compound **7** (1.14 g, 2.88 mmol) in a mixture of acetone/H_2O (15 mL, 2:1 *v*/*v*). The mixture was stirred for 5 h at 70 °C, then the solvent was removed in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc 2:1, *v*/*v*) to give an azido intermediate. MALDI TOF HRMS: *m*/*z*: calcd for $C_{14}H_{21}N_3O_8Na$: 382.1329; found: 382.1338 $[M+Na^+]$. The azido intermediate was dissolved in dry MeOH (15 mL) and treated with NaOMe (pH value adjusted to 12.0). The reaction was stirred at room temperature for 2 h and then neutralized by the addition of the weakly acidic resin Amberlite IRC-50. After filtration and concentration of the filtrate in vacuo, the residue was dried in vacuo for 3 h. PTSA (28.0 mg, 0.15 mmol) and 2,2-dimethoxypropane (1.40 mL, 6.03 mmol) were added to a solution of the intermediate in CH_2Cl_2 (15.0 mL). The reaction mixture was kept stirring for 1 h at room temperature. Et_3N was then added and stirring was continued for 3 min prior to filtration and concentration of the filtrate in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc 1:1, *v*/*v*) to give **8** (0.52 g, 70% over three steps). $[\alpha]_D^{25} + 63.4^\circ$ ($c = 0.4$, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 4.19$ (m, 1H; H4), 4.16 (d, $J = 7.5$ Hz, 1H; H1), 4.12 (dd, $J = 3.0$, 13.2 Hz, 1H; H5a), 4.02 (t, $J = 6.0$ Hz, 1H; H3), 3.86 (m, 1H; $OCH_{2a}CH_2CH_2N_3$), 3.76 (dd, $J = 3.6$, 13.2 Hz, 1H; H5b), 3.60–3.51 (m, 2H; H2, $OCH_{2b}CH_2CH_2N_3$), 3.38 (t, $J = 6.6$ Hz, 2H; $CH_2CH_2CH_2N_3$), 2.93 (br s, 1H; OH), 1.88–1.82 (m, 2H; $CH_2CH_2CH_2N_3$), 1.49 (s, 3H; CH_3), 1.32 ppm (s, 3H; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 110.3$, 102.4, 78.4, 73.7, 73.2, 66.4, 63.2, 48.6, 29.3, 28.1, 26.2 ppm; MALDI-TOF HRMS *m*/*z*: calcd for $C_{11}H_{19}N_3O_5Na$: 296.1325; found: 296.1331 $[M+Na]^+$.

3-Azidopropyl 3,4-*O*-isopropylidene-2-*O*-levulinoyl-α-L-arabinopyranoside (9): To a solution of **8** (0.61 g, 2.23 mmol) in CH_2Cl_2 (15 mL) was added levulinic acid (0.3 mL, 2.68 mmol), DCC (0.80 mg, 3.35 mmol) and DMAP (27.2 mg, 0.22 mmol). The reaction mixture was stirred for 2 h at room temperature, then filtered, and the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc 2:1, *v*/*v*) to give **9** (0.76 g, 92%). $[\alpha]_D^{25} + 26.8^\circ$ ($c = 1.76$, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 5.62$ (d, $J = 5.0$ Hz, 1H; H1), 4.54 (dd, $J = 2.0$, 7.5 Hz, 1H; H3), 4.39 (dd, $J = 2.0$, 5.5 Hz, 1H; H2), 4.21 (d, $J = 8.0$ Hz, 1H; H4), 3.72–3.65 (m, 2H; H5a, H5b), 3.51 (t, $J = 6.0$ Hz, 2H; $OCH_2CH_2CH_2N_3$), 3.35 (t, $J = 6.0$ Hz, 2H; $CH_2CH_2CH_2N_3$), 2.64–2.61 (m, 2H; $CH_3COCH_2CH_2CO$), 2.22–2.16 (m, 2H; $CH_3COCH_2CH_2CO$),

2.15 (s, 3H; $CH_3COCH_2CH_2CO$), 1.79 (t, $J=6.0$ Hz, 2H; $CH_2CH_2CH_2N_3$), 1.46 (s, 3H; CH_3), 1.33 ppm (s, 3H; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=207.4$, 121.2, 109.2, 96.7, 70.8, 70.4, 69.7, 60.7, 59.9, 48.4, 38.2, 30.2, 29.1, 28.1, 26.2, 24.4 ppm; MALDI-TOF HRMS *m/z*: calcd for $C_{16}H_{25}N_3O_7Na$: 394.1693; found: 394.1685 $[M+Na]^+$.

3-Azidopropyl 2-*O*-levulinoyl-α-L-arabinopyranoside (10): Compound **9** (0.72 g, 1.94 mmol) was dissolved in 80% aqueous AcOH (20 mL) and the resulting mixture was stirred for 1 h at 80 °C, after which it was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc 1:4, *v/v*) to give **10** (0.56 g, 87%). $[\alpha]_D^{25}+24.9^\circ$ ($c=0.6$, CH_2Cl_2); ^1H NMR (500 MHz, $CDCl_3$): $\delta=4.89$ (t, $J=6.9$ Hz, 1H; H2), 4.32 (d, $J=5.7$ Hz, 1H; H1), 3.86–3.77 (m, 3H; H4, H5a, $OCH_{2a}CH_2CH_2N_3$), 3.69 (dd, $J=2.4$, 7.8 Hz, 1H; H3), 3.49–3.44 (m, 2H; H5b, $OCH_{2b}CH_2CH_2N_3$), 3.31 (t, $J=6.3$ Hz, 2H; $CH_2CH_2CH_2N_3$), 2.71 (t, $J=6.0$ Hz, 2H; $CH_3COCH_2CH_2CO$), 2.55–2.51 (m, 2H; $CH_3COCH_2CH_2CO$), 2.12 (s, 3H; $CH_3COCH_2CH_2CO$), 1.81–1.75 ppm (m, 2H; $CH_2CH_2CH_2N_3$); ^{13}C NMR (75 MHz, $CDCl_3$): δ 207.5, 172.5, 100.3, 72.5, 71.0, 67.5, 66.0, 64.3, 48.4, 38.1, 29.9, 29.0, 28.2 ppm; MALDI-TOF HRMS: *m/z*: calcd for $C_{13}H_{21}N_3O_7Na$: 354.1380; found: 354.1374 $[M+Na]^+$.

3-Azidopropyl 4-*O*-benzoyl-2-*O*-levulinoyl-α-L-arabinopyranoside (5): To a solution of **10** (1.10 g, 3.32 mmol) in CH_2Cl_2 (15 mL) was added trimethyl orthobenzoate (0.32 mL, 5.0 mmol) and PTSA (28.0 mg, 0.15 mmol). The mixture was stirred for 2 h at room temperature. Then Et_3N (0.3 mL, 2.15 mmol) was added to quench the reaction, and the resulting solution was concentrated in vacuo. The residue was dissolved in 80% aqueous AcOH (20 mL) and stirred at room temperature for 30 min, then concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc 2:1, *v/v*) to give **5** (0.91 g, 63% over two steps). $[\alpha]_D^{25}+87.5^\circ$ ($c=0.31$, CH_2Cl_2); ^1H NMR (500 MHz, $CDCl_3$): $\delta=8.08$–8.05 (m, 2H; Ar), 7.59–7.40 (m, 3H; Ar), 5.34 (m, 1H; H4), 5.11 (dd, $J=6.0$, 7.8 Hz, 1H; H2), 4.52 (d, $J=5.7$ Hz, 1H; H1), 4.14 (dd, $J=4.8$, 12.9 Hz, 1H; H5a), 4.02 (m, 1H; H3), 3.92 (m, 1H; $OCH_{2a}CH_2CH_2N_3$), 3.72 (dd, $J=2.7$, 12.9 Hz, 1H; H5b), 3.58 (m, 1H; $OCH_{2b}CH_2CH_2N_3$), 3.41 (t, $J=6.6$ Hz, 2H; $CH_2CH_2CH_2N_3$), 3.04 (br s, 1H; OH), 2.81–2.76 (m, 2H; $CH_3COCH_2CH_2CO$), 2.65–2.61 (m, 2H; $CH_3COCH_2CH_2CO$), 2.18 (s, 3H; $CH_3COCH_2CH_2CO$), 1.92–1.82 ppm (m, 2H; $CH_2CH_2CH_2N_3$); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=206.9$, 172.5, 166.3, 133.6, 130.1, 129.7, 128.7, 100.2, 72.5, 70.3, 69.9, 66.3, 61.8, 48.4, 38.2, 34.1, 30.0, 29.1, 28.2, 25.2 ppm; MALDI-TOF HRMS: *m/z*: calcd for $C_{20}H_{25}N_3O_8$: 458.1642; found: 458.1649 $[M+Na]^+$.

3-Azidopropyl 2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl-(1→3)-4-*O*-benzoyl-2-*O*-levulinoyl-α-L-arabinopyranoside (12): TMSOTf (15.0 μL) was added to a solution of **3** (262 mg, 0.688 mmol) and **5** (272 mg, 0.625 mmol) in CH_2Cl_2 (5.0 mL) at −70 °C in the presence of 4 Å molecular sieves. The temperature of the reaction was raised to −10 °C over a period of 1 h. Et_3N was then added and stirring was continued for 15 min at room temperature prior to filtration and concentration of the filtrate in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc 2:1, *v/v*) to give **12** (354 mg, 80%). $[\alpha]_D^{25}+75.6^\circ$ ($c=0.48$, CH_2Cl_2); ^1H NMR (500 MHz, $CDCl_3$): $\delta=8.11$–8.07 (m, 2H; Ar), 7.58–7.44 (m, 3H; Ar), 5.39 (m, 1H; H4), 5.22–5.14 (m, 3H; H2, H2′, H3′), 5.02–4.93 (m, 2H; H1′, H4′), 4.52 (d, $J=5.4$ Hz, 1H; H1), 4.25 (dd, $J=5.7$, 12.3 Hz, 1H; H5a), 4.06 (dd, $J=3.3$, 7.5 Hz, 1H; H3), 3.95–3.81 (m, 2H; H5′, $OCH_{2a}CH_2CH_2N_3$), 3.68 (dd, $J=9.6$, 12.3 Hz, 1H; H5b), 3.54 (m, 1H; $OCH_{2b}CH_2CH_2N_3$), 3.45 (t, $J=6.9$ Hz, 2H; $CH_2CH_2CH_2N_3$), 2.86–2.77 (m, 2H; $CH_3COCH_2CH_2CO$), 2.69–2.65 (m, 2H; $CH_3COCH_2CH_2CO$), 2.19 (s, 3H; $CH_3COCH_2CH_2CO$), 2.12 (s, 3H; OAc), 1.95–1.85 (m, 8H; 2×OAc, $CH_2CH_2CH_2N_3$), 0.92 ppm (d, $J=6.0$ Hz, 3H; H6′); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=206.6$, 171.8, 170.3, 170.1, 169.9, 166.0, 133.6, 130.1, 129.6, 128.6, 100.1, 97.6, 73.2, 70.9, 69.9, 69.8, 69.6, 69.0, 68.9, 67.3, 66.6, 66.1, 66.0, 48.4, 38.0, 29.9, 29.2, 28.1, 21.1, 20.93, 20.90, 17.2 ppm; MALDI-TOF HRMS: *m/z*: calcd for $C_{32}H_{41}N_3O_{15}Na$: 730.2538; found: 730.2532 $[M+Na]^+$.

3-Azidopropyl 2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl-(1→3)-4-*O*-benzoyl-α-L-arabino pyranoside (13): Hydrazine acetate (178.1 mg, 1.91 mmol) was added to a solution of **12** (270 mg, 0.382 mmol) in a mixture of CH_2Cl_2 (9 mL) and MeOH (0.9 mL). After being stirred at room temperature for 2 h, the reaction was quenched with acetonylacetone (270 μL, 2.29 mmol) and concentrated in vacuo. The residue was purified by flash silica gel column chromatography (hexane/EtOAc 2:1, *v/v*) to afford **13** (197.7 mg, 85%). $[\alpha]_D^{25}+52.0^\circ$ ($c=0.64$, CH_2Cl_2); ^1H NMR (500 MHz, $CDCl_3$): $\delta=$ 8.11–8.08 (m, 2H; Ar), 7.61–7.44 (m, 3H; Ar), 5.35–5.29 (m, 2H; H2′, H4), 5.15 (dd, $J=2.4$, 10.2 Hz, 1H; H3′), 5.10 (d, $J=1.5$ Hz, 1H; H1′), 5.01 (t, $J=10.2$ Hz, 1H; H4′), 4.28 (d, $J=6.9$ Hz, 1H; H1), 4.23 (dd, $J=2.4$, 13.2 Hz, 1H; H5a), 4.02–3.83 (m, 4H; H2, H3, H5′, $OCH_{2a}CH_2CH_2N_3$), 3.69–3.62 (m, 2H; H5b, $OCH_{2b}CH_2CH_2N_3$), 3.46–3.41 (td, $J=1.8$, 6.6 Hz, 2H; $CH_2CH_2CH_2N_3$), 2.12 (s, 3H; OAc), 1.93–1.91 (m, 8H; 2×OAc, $CH_2CH_2CH_2N_3$), 1.09 ppm (d, $J=6.0$ Hz, 3H; H6′); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=207.2$, 170.1, 169.8, 166.3, 133.4, 129.9, 128.6, 103.4, 98.9, 77.7, 71.1, 70.8, 70.6, 69.7, 69.1, 67.1, 66.7, 63.7, 48.5, 29.2, 20.9, 20.8, 20.7, 17.3 ppm; MALDI-TOF HRMS: *m/z*: calcd for $C_{27}H_{35}N_3O_{13}Na$: 632.2170; found: 632.2176 $[M+Na]^+$.

3-Azidopropyl 2-*O*-benzyl-3,5-*O*-(di-*tert*-butylsilane-diyl)-β-L-arabinofuranosyl-(1→3)-2,4-di-*O*-benzoyl-α-L-rhamnopyranosyl-(1→2)-[2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl-(1→3)]-4-*O*-benzoyl-α-L-arabinopyranoside (14): NIS (34.0 mg) and TfOH (5.0 μL) were added to a solution of **11** (92.0 mg, 0.112 mmol) and **13** (56.5 mg, 0.093 mmol) in CH_2Cl_2 (4.0 mL) at −20 °C in the presence of 4 Å molecular sieves. The temperature of the reaction was raised to 0 °C over a period of 1 h. Then Et_3N was added, and stirring was continued for 15 min at room temperature. The suspension was diluted with CH_2Cl_2 (10 mL), filtered through a pad of Celite, and the filtrate was washed successively with 10% $Na_2S_2O_3$ (15 mL) and sat. aq NaCl (15 mL). The organic layer was dried ($MgSO_4$) and concentrated in vacuo. Chromatography of the residue over silica gel by using hexane/EtOAc (4:1, *v/v*) as the eluent gave **14** (94.7 mg, 77%). $[\alpha]_D^{25}+165.3^\circ$ ($c=0.17$, CH_2Cl_2); ^1H NMR (500 MHz, $CDCl_3$): $\delta=8.11$–8.05 (m, 4H; Ar), 7.61–7.35 (m, 12H; Ar), 7.15–7.09 (m, 4H; Ar), 5.66 (s, 1H; $H2_{rha}$), 5.52 (t, $J=10.0$ Hz, 1H; $H4_{rha}$), 5.42–5.38 (m, 2H; $H2'_{rha}$, $H4_{arap}$), 5.32 (s, 1H; $H1_{rha}$), 5.28 (dd, $J=3.5$, 9.5 Hz, 1H; $H3'_{rha}$), 5.23 (d, $J=5.0$ Hz, 1H; $H1_{araf}$), 5.05 (s, 1H; $H1'_{rha}$), 4.97 (t, $J=$ 10.5 Hz, 1H; $H4'_{rha}$), 4.53 (d, $J=5.0$ Hz, 1H; $H1_{arap}$), 4.43 (AB, $J=$ 13.0 Hz, 1H; $PhCH_2$), 4.39 (dd, $J=3.5$, 10.5 Hz, 1H; $H3_{rha}$), 4.29–4.24 (m, 3H; $PhCH_2$, $H5_{rha}$, $H5a_{arap}$), 4.06 (t, $J=6.0$ Hz, 1H; $H2_{arap}$), 4.00–3.91 (m, 5H; $H3_{araf}$, $H3_{arap}$, $H4_{araf}$, H −$5a_{araf}$, H5′, $OCH_{2a}CH_2CH_2N_3$), 3.82 (qd, $J=4.0$, 9.5 Hz, 1H; $H5'_{rha}$), 3.76 (dd, $J=4.8$, 9.6 Hz, 1H; $H2_{araf}$), 3.68–3.64 (m, 2H; H5 b_{arap}, $OCH_{2b}CH_2CH_2N_3$), 3.47 (t, $J=$ 6.0 Hz, 2H; $CH_2CH_2CH_2N_3$), 3.26 (t, $J=9.5$ Hz, 1H; H5 b_{araf}), 2.12 (s, 3H; OAc), 1.95–1.91 (m, 2H; $CH_2CH_2CH_2N_3$), 1.85 (s, 3H; OAc), 1.77 (s, 3H; OAc), 1.33 (d, $J=6.5$ Hz, 3H; $H6_{rha}$), 0.93 (d, $J=6.0$ Hz, 3H; $H6'_{rha}$), 0.87 (s, 9H; *t*-Bu), 0.77 ppm (s, 9H; *t*-Bu); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=170.2$, 170.1, 169.7, 166.3, 166.1, 165.5, 138.4, 133.6, 133.5, 133.1, 130.23, 130.20, 130.1, 130.0, 129.7, 128.7, 128.6, 128.4, 128.1, 127.4, 127.2, 101.6, 99.1, 98.8, 97.4, 80.1, 77.9, 74.9, 73.8, 72.7, 72.3, 71.1, 70.8, 70.5, 68.9, 68.7, 68.2, 67.7, 67.3, 66.4, 48.6, 29.9, 29.4, 27.4, 27.2, 22.5, 21.2, 20.8, 20.6, 20.1, 17.9, 17.2 ppm; MALDI-TOF HRMS: *m/z*: calcd for $C_{67}H_{83}N_3O_{23}SiNa$: 1348.5187; found: 1348.5180 $[M+Na]^+$.

3-Aminopropyl β-L-arabinofuranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→3)]-α-L-arabinopyranoside

(1): A 1.0 M solution of TBAF in THF (192.0 μL) and AcOH (40.0 μL) were added to a solution of tetrasaccharide **14** (85.0 mg, 0.064 mmol) in THF (5 mL). The mixture was stirred for 18 h, then the solvent was removed in vacuo. The residue was dissolved in CH_2Cl_2 (5 mL) and washed with water (2×3 mL). The organic layer was dried ($MgSO_4$), filtered and the residue was concentrated under reduced pressure. The residue was dried in vacuo for several hours, then it was dissolved in dry MeOH (5 mL) and treated with NaOMe (pH adjusted to 12.0). The reaction was stirred at room temperature for 2 h, then it was neutralized with a weakly acidic resin (Amberlite IRC-50). After filtration and concentration of the filtrate in vacuo, the residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH, 10:1, *v/v*) to afford a product, which was immediately used in the next reaction step. MALDI HRMS: *m/z*: calcd for $C_{32}H_{49}N_3O_{17}$: 770.3062; found: 770.3056 $[M+Na]^+$. Pd/C (10%) was added to a solution of the partially deprotected tetrasaccharide in a mixture of *t*BuOH, AcOH, and H_2O (10:2:1, *v/v/v*, 5.2 mL) under an atmosphere of argon. The mixture was then placed under an atmosphere of H_2 and stirred for 18 h. TLC analyses (*i*PrOH/28% NH_4OH, 2:1, *v/v*) indicated the presence of a single new product. The reaction mixture was filtered through a syringe filter (25 mm, 0.2 μm, PTFE, nonsterile, Fisherbrand, Fisher Scientific) and the residue then washed with AcOH (2 mL). After removal of the solvents by codistillation with toluene, the residue was dried in vacuo for several hours and then purified by Iatrobead column chromatography by using *i*PrOH and 28% NH_4OH (3:1, *v/v*) as eluent. Fractions that contained the products were collected and concentrated in vacuo. The products were dissolved in water (0.5 mL) and brought to pH 4.5 with AcOH and freeze dried to give compound **1** (18.1 mg, 45% over three steps). $[\alpha]_D^{25}$ +117.1° (*c* = 0.5, H_2O); ^1H NMR (500 MHz, $CDCl_3$): δ = 5.01 (d, *J* = 4.2 Hz, 1H; $H1_{araf}$), 4.87 (s, 1H; $H1_{rha}$), 4.82 (s, 1H; $H1'_{rha}$), 4.35 (d, *J* = 7.8 Hz, 1H; $H1_{arap}$), 4.06–4.02 (m, 2H; $H2_{araf}$), 3.91–3.83 (m, $J_{H4,H5a}$ = 3.0 Hz, $J_{H4,H5b}$ = 4.0 Hz, 3H; $H4_{arap}$, $H2_{rha}$), 3.82–3.63 (m, $J_{H3,H4}$ = 3.5 Hz, 10H; $H2'_{rha}$, $H3_{araf}$, $H3_{arap}$, $H4_{rha}$, $H5_{rha}$, $H5'_{rha}$, $OCH_2CH_2CH_2NH_2$), 3.57–3.49 (m, $J_{H2,H3}$ = 8.5 Hz, 3H; $H2_{arap}$), 3.41 (t, *J* = 9.6 Hz, 1H; $H4_{rha}$), 3.31 (t, *J* = 10.2 Hz, 1H; $H4'_{rha}$), 2.86 (t, *J* = 6.6 Hz, 2H; $CH_2CH_2CH_2NH_2$), 1.81–1.75 (m, 2H; $CH_2CH_2CH_2NH_2$), 1.15 (d, *J* = 6.0 Hz, 3H; $H6_{rha}$), 1.12 ppm (d, *J* = 6.6 Hz, 3H; $H6'_{rha}$); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 103.0, 102.1, 101.3, 99.2, 82.2, 81.9, 77.3, 76.8, 76.6, 73.7, 72.4, 71.1, 70.7, 69.8, 69.4, 68.7, 68.1, 66.8, 64.9, 62.2, 49.6, 37.8, 28.9, 17.8, 17.3 ppm; MALDI-TOF HRMS: *m/z*: calcd for $C_{25}H_{45}NO_{17}$: 654.2688; found: 654.2679 $[M+Na]^+$.

Activation of tetrasaccharide 1: *S*-Acetylthioglycolic acid pentafluorophenyl ester (SAMA-OPfp; 3.5 mg, 11.8 μmol) and *N*,*N*-diisopropylethylamine (DIPEA; 4.2 μL) were added to a solution of tetrasaccharide **1** (5.0 mg, 7.9 μmol) in DMF (400 μL). The mixture was stirred for 2 h at room temperature and then concentrated in vacuo. The mixture was chromatographed on a Bio-gel P2 column that was eluted with H_2O that contained 1% *n*-BuOH to give the thioacetyl-activated tetrasaccharide (4 mg). MALDI-ToF: *m/z*: calcd for $C_{29}H_{49}NO_{19}SNa$: 770.8; found: 770.2 $[M+Na]^+$.

Conjugation to maleimide activated BSA: The activated tetrasaccharide (2 mg) was *S*-deacetylated by stirring for 1 h with a solution of ammonia gas in DMF (250 μL, 7%, *w/w*) under an atmosphere of argon. The mixture was then concentrated in vacuo, and the residue was used directly without further purification. Maleimide-activated BSA (2.4 mg, lyophilized powder with conjugation buffer sodium phosphate pH 7.2 that contained EDTA and sodium azide; Pierce Endogen, Rockford, IL, USA) was reconstituted in H_2O (200 μL), and then added to the tetrasaccharide. The conjugation reaction was stirred for 18 h at room temperature, after which the conjugate was purified by using a Millipore Centriplus spin-filter with a molecular weight cut-off of 30 kDa at 4 °C and 3000 rpm for 30 min. After the initial spin-down, the filter was washed with sodium phosphate buffer pH 7.4 (3×1 mL). The conjugate was retrieved by centrifugation at 4 °C and 3000 rpm for 15 min and taken up in sodium phosphate buffer (pH 7.4), and 0.15 M NaCl (1 mL). This gave a glycoconjugate with a tetrasaccharide/BSA ratio of 7:1. The carbohydrate content was determined by heating a mixture of carbohydrate–protein conjugate (50 μL) and aq TFA (2 M, 200 μL) in a sealed tube for 4 h at 100 °C. The solution was then concentrated in vacuo and analyzed by HPAEC-PAD and a CarboPac PA-10 column. Serial dilutions of rhamnose were used as standards. The protein concentration was determined by using the method of Bradford (BioRad).[35]

Conjugation of thiol-derivatized tetrasaccharide to KLH–BrAc: A solution of KLH (2.3 mg) in 0.1 M sodium phosphate buffer pH 7.2 that contained 0.15 M NaCl (200 μL) was added to a solution of SBAP (1 mg) in DMSO (50 μL). The mixture was incubated for 2 h at room temperature and then purified by using a Millipore Centriplus centrifugal filter with a molecular cut-off of 30 kDa. All centrifugations were performed at 8 °C for 25 min. and 3000 rpm. The reaction mixture was centrifuged, and the filter was washed with conjugation buffer (3×1 mL). The activated protein was retrieved by spinning at 3000 rpm for 15 min at 8 °C, and was taken up in 0.1 M sodium phosphate buffer pH 8.0 that contained 5 mM EDTA (200 μL). The activated protein was added to a vial that contained tetrasaccharide (2 mg) that had been de-*S*-acetylated as described above. The conjugation mixture was incubated at room temperature for 18 h. Purification was performed by using centrifugal filters as described to give a glycoconjugate with ~1700 tetrasaccharide residues/KLH molecule. The carbohydrate content was determined by heating a mixture of carbohydrate–protein conjugate (50 μL) and aqueous TFA (2 M, 200 μL) in a sealed tube for 4 h at 100 °C. The solution was then concentrated in vacuo and analyzed by HPAEC-PAD. Serial dilutions of rhamnose were used as standards. Protein content was determined by the method of Bradford (BioRad).[35]

Biotinylation of RG-II: A solution of RG-II (~10 mg) in 20 mM NaOAc, pH 5, that contained $NaBH_3(CN)$ (1 M, 600 μL) was treated for 3 h at 65 °C with biotin-X-hydrazide (25 mg, Sigma). The reaction mixture was then cooled to room temperature, dialyzed against deionized H_2O (3.5 kDa molecular weight cut-off), and freeze-dried.

Generation of polyclonal antibodies to KLH–tetrasaccharide 1 conjugate: Groups of three mice (female BALB/c, age 8–10 weeks, Jackson Laboratories, Bar Harbor, ME, USA) were immunized subcutaneously four times at 2-week intervals. Each boost included carbohydrate (19 μg) in Freund's immunoadjuvant. Serum samples were obtained one week after the final immunization.

Serological assays: Antibody titers were determined by ELISA by using 96-well plates (Costar 3598). Wells were coated with BSA–**1** conjugate (50 μL per well of a 10 μg conjugate per mL solution in H_2O) and then allowed to dry, overnight, at 37 °C. Wells were blocked for 1 h at room temperature with 1% (*w/v*) instant nonfat dry milk (Carnation) in 0.1 M TBS (50 mM Tris–HCl, pH 7.5 that contained 100 mM NaCl). Biotinylated RG-II was bound to NeutrAvidin 96-well plates that were preblocked with Pierce SuperBlock (Pierce Inc.). Wells were coated by treatment for 2 h at room temperature with the biotinylated polysaccharide (50 μL per well of a solution of 10 μg polysaccharide/mL in 0.1 M TBS buffer). The plates were then washed with 0.1 M TBS and incubated for 1 h at room tem-

perature with serial dilutions of mouse serum. The antisera solutions were then removed, and the plates were washed with TBS (3×0.1 M) that contained 0.1% (*w/v*) instant nonfat dry milk. The plates were then incubated for 1 h at room temperature with goat anti-mouse IgG whole-molecule peroxidase-conjugated antibodies (Sigma). The plates were washed as before, enzyme substrate, 3,3′,5,5′-tetramethylbenzidine (Vector Laboratories, Burlingame, CA, USA) was added, and plates were incubated for 20 min at room temperature. The enzymatic reaction was quenched by the addition of sulfuric acid (0.5 N), and A_{450nm} and A_{655nm} were measured.

Acknowledgement

This research was supported by a grant from the NSF Plant Genome Program (DBI 0421683).

Keywords: carbohydrates · conformational epitope · immunology · pectins · rhamnogalacturonan II

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Received: August 28, 2007
Published online on January 14, 2008

