Conjugates of polyhedral boron compounds with carbohydrates
7. Hydrolytic stability of *closo-ortho*-carborane glycoconjugates containing from one to three β-lactosylamine and β-D-galactopyranosylamine residues; estimation of their galectin-binding efficiency with galectin RCA_{120}

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The galectin-binding efficiency (*Ricinus communis* agglutinin, RCA) and the degree of deboronation at 37 °C of glycoconjugates of *ortho*-carboranylacetic acid containing from one to three β-lactosylamine or β-D-galactopyranosylamine residues were estimated; the glycoconjugates are potential agents for boron neutron capture therapy of cancer. Over a period of 24 h, up to ~15% of glycoconjugates underwent deboronation to give glycoconjugates of *nido-ortho*-carborane. With Lac-β-$NH(COCH_2NH)_2COCH_2$-*o*-$CCHB_{10}H_{10}$ as an example, it was demonstrated that the deboronation occurs at both 60 and 5 °C. Glycoconjugates with an *O*-linked β-galactose as a fragment of lactosylamine bind to galectin RCA_{120} much more efficiently (up to ~40 times) than do the corresponding glycoconjugates with a *N*-linked β-galactose residue. The glycoconjugates containing one β-lactosylamine residue bind to galectin RCA approximately two times less efficiently than does lactose; however, introduction of the second and third β-lactosylamine residues into the glycoconjugate increases the binding efficiency by a factor of five to seven due to the cluster effect.

Key words: glycoconjugates, deboronation, galectin RCA_{120}, β-lactosylamine, β-D-galactopyranosylamine, *ortho*-carboranylacetic acid.

In the past few years, intensive efforts have been made to obtain new polyhedral boron compounds. Among them are *closo-ortho*-carborane derivatives containing fragments of amino acids, peptides, porphyrins, and carbohydrates, which are potential agents for boron neutron capture therapy (BNCT) and diagnostics of cancer.[1] *closo-ortho*-Carboranes are known to be inert to acids, oxidants, and reducing agents but unstable in the presence of bases.[2] The degradation mechanism involves a nucleophilic attack on the B(3) or B(6) atom of a *closo-ortho*-carborane derivative followed by its elimination leading to the corresponding *nido-ortho*-carborane.[3]

Several examples of room-temperature degradation of *closo-ortho*-carboranes under neutral conditions in MeOH,[4] MeOH—H_2O,[5,6] DMSO—H_2O, or DMSO—MeOH[7] have been described. The deboronation rate of *closo-ortho*-carborane substantially depends on the substituent structure, the solvent, and the nature of the base (if used).[3—7] Apparently, deboronation is favored by the presence of a carbohydrate fragment in *closo-ortho*-carborane.[8] For instance, in the synthesis of conjugates of lactose amino-functionalized derivatives with *ortho*-carboranylacetic acid in DMSO (10 °C, 20 h), we observed deboronation of the glycoconjugates as a side process,[9,10] while it has been noted earlier[7] that methyl *ortho*-carboranylacetate in DMSO—5% water remained stable at room temperature for 24 h. Moreover, we have shown[11] that the deboronation rate of glycoconjugates in aqueous solutions strongly depends on the glycosidic linkage type (*O*- or *N*-). Glycoconjugate **1a** with the *O*-glycosidic linkage is completely deboronated at 60 °C over 17 h to give a glycoconjugate of *nido-ortho*-carborane **1b** and boric acid, while glycoconjugate **2a** with the *N*-glycosidic linkage is more stable. Complete deboronation of the latter occurs at 60 °C over 165 h, being accompanied by its considerable degradation (Scheme 1).

The present work is devoted to a more detailed study of the stability in water of the conjugates obtained earlier[9,10] from β-lactosylamine or β-D-galactopyranosylamine and *ortho*-carboranylacetic acid, in which the number of carbohydrate residues varies from one to three and spacers differ in length and hydrophilicity (**3a—10a**).

For comparison with data on the stability of glycoconjugate **1a** with the *O*-glycosidic linkage in aqueous solu-

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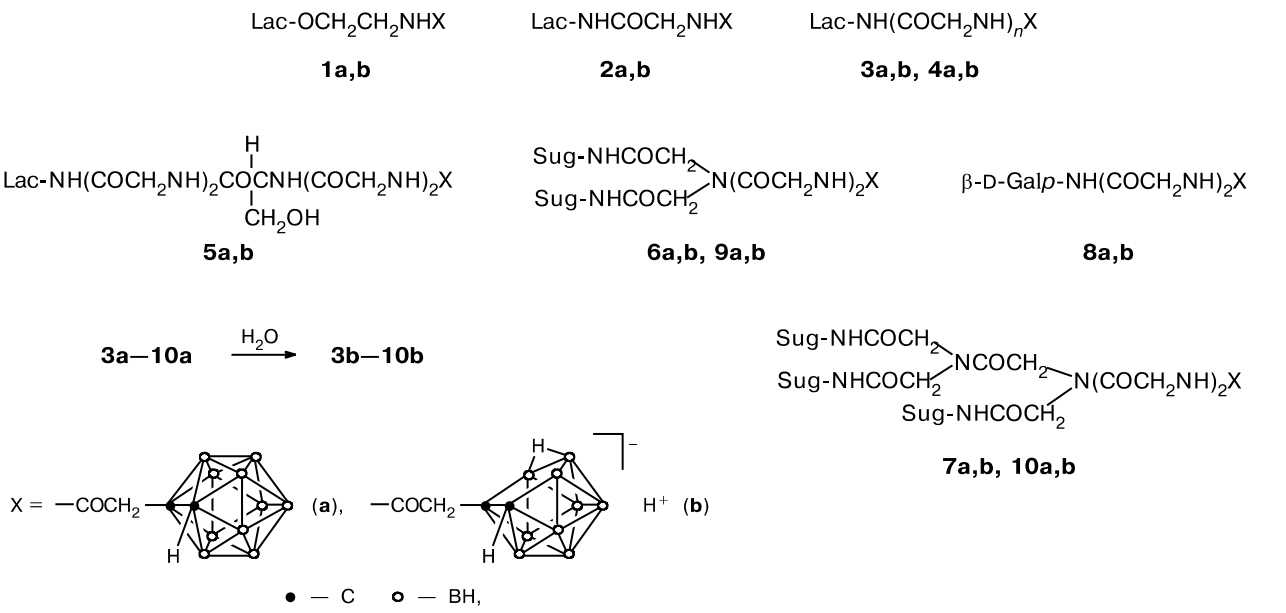
Scheme 1



n = 2 (**3a,b**), 3 (**4a,b**); Lac = β-D-Gal*p*(1-4)β-D-Glc*p*; Sug = Lac (**6a,b**, **7a,b**); β-D-Gal*p* (**9a,b**, **10a,b**)

tion under the conditions of full deboronation (17 h, 60 °C),[11] we carried out deboronation of glycoconjugate **3a** with the *N*-glycosidic linkage under these conditions. Separation of the products using reversed-phase chromatography gave the starting glycoconjugate **3a** (60%), a glycoconjugate of *nido-ortho*-carborane **3b** (~16%), and a complex mixture of compounds eluted from the column with water without retention. According to data from paper electrophoresis, this fraction contains neutral products, positively charged products with the NH_2 group (visualization with ninhydrin), and negatively charged products that are more mobile than *nido-ortho*-carborane glycoconjugate **3b**. The complicated ^{1}H NMR spectrum of this fraction shows characteristic signals for the H(1) proton (δ 4.44, d, J = 7.5 Hz) of the β-galactopyranose residue, for the H(1) proton (δ 5.02, d, J = 9.0 Hz) of the β-glucopyranosylamine residue, and the H(1) protons (δ 5.20, d, J = 3.0 Hz; δ 4.70, d, J = 8.0 Hz) of the α- and β-glucopyranose residues, respectively. The integral intensity ratio (~9 : 7 : 1 : 1) of these signals suggests partial cleavage of the *N*-glycosidic linkage with liberation of free lactose. At the same time, the spectrum contains no signals for the protons of the *closo*- and *nido-ortho*-carboranylacetic acid residues, which was confirmed by ^{11}B NMR spectra. The ^{11}B NMR spectrum of glycoconjugate **3a** kept in D_2O at 5—7 °C for 11 months showed signals characteristic of the *nido-ortho*-carborane and boric acid with virtually no signals for the *closo-ortho*-carborane. The signal for boric acid (δ 19.5) is ~5 times more intense than the signal for one B atom of the *nido-ortho*-carborane residue, which suggests further slow deboronation of the carborane residue with formation of boric acid.

Separation of the complete deboronation products of *closo-ortho*-carborane glycoconjugate **3a** gave two fractions: a fraction containing *nido-ortho*-carborane glycoconjugate **3b** (~30% yield) and the other fraction containing a complex mixture of products similar to a fraction obtained from glycoconjugate **3a** previously kept at 60 °C for 17 h.

Thus, deboronation of *closo-ortho*-carborane glycoconjugate **3a** in water produces *nido-ortho*-carborane glycoconjugate **3b** and a complex mixture of degradation products both on heating and in cold (5 °C), although much more slowly in the latter case. A similar phenomenon has been observed earlier[5] in the deboronation of *closo-ortho*-carboranyl halides to boric and hydrohalic acids in aqueous ethanol.

To characterize the properties of glycoconjugates **3a**—**10a** with various spacers and different number of lactosylamine or galactopyranosylamine residues as potential agents for BNCT of cancer, we determined their degree of deboronation in 0.05 *M* aqueous solutions at 37 °C in 24 h. Reversed-phase chromatography of the deboronation products allowed isolation of the starting glycoconjugates in (80—85)±5% yield and the corresponding *nido-ortho*-carborane glycoconjugates **3b**—**7b**, **9b**, and **10b** in ~9% yield (separation of glycoconjugate **8b** from other degradation products was unsatisfactory). Thus, the glycoconjugate structure seems not to be crucial for the degree of deboronation under these conditions. At the same time,

we noticed a higher degree of deboronation of glycoconjugates in saline (0.9% NaCl) with glycoconjugate **3a** as an example. When kept in aqueous solution at 37 °C for 3 days, glycoconjugate **3a** was recovered in 71% yield, while in the case of 0.9% NaCl, its yield was 62%.

The structures of *nido-ortho*-carborane glycoconjugates **3b**—**7b**, **9b**, and **10b** were confirmed by data from ^{1}H and ^{11}B NMR spectroscopy and mass spectrometry.

The high-resolution mass spectra (HR ESI MS, negative ion mode) of these glycoconjugates contain isotope clusters characteristic of carboranes; the *m/z* ratios of the components of these clusters agree well ($|\Delta| < 5$ ppm) with the calculated values. In the ^{1}H NMR spectra, the protons of the corresponding *closo*- and *nido-ortho*-carborane glycoconjugates have virtually identical chemical shifts, except for the protons of the *ortho*-carboranylacetic acid residue. According to previous data,[7] the *closo-ortho*→*nido-ortho* transformation of the carborane residue results in upfield shifts of the signals for the H atom of the carborane (HC_{carb}) and for the H atoms in the substituent directly bound to the carborane residue; in addition, the ^{11}B NMR spectra of *closo-ortho*- and *nido-ortho*-carboranes differ greatly.[12] In the deboronation of *closo-ortho*-carborane glycoconjugates **3a**—**10a** to the corresponding *nido-ortho*-carborane glycoconjugates **3b**—**10b**, the ^{1}H NMR spectra show upfield shifts of the signals for HC_{carb} (from δ 4.5 to 2.09) and H_2CC_{carb} (from δ 3.3 (singlet) to 2.70 and 2.45 (two doublets)); in the ^{11}B NMR spectra, the signals are shifted from δ −1—−20 to −11—−37. These spectroscopic differences can be used to estimate the degree of deboronation in the initial steps of the hydrolytic process, when the degree of further deboronation of the resulting *nido-ortho*-carborane glycoconjugate is low. For instance, a comparison of the integral intensities of the signals for HC_{carb} and H_2CC_{carb} of the *closo*- and *nido-ortho*-carborane residues in the spectrum of glycoconjugate **3a** showed that its degree of deboronation at 20 °C is ~8% in one day and ~19% in three days.

In addition, we estimated the galectin-binding efficiency of glycoconjugates **3a**—**10a** and lactose from their ability to inhibit agglutination of erythrocytes with the galectin RCA_{120}. We found that the galectin-binding efficiencies of glycoconjugates **3a**—**7a** containing *O*-linked β-galactose residues in lactose is much higher (30—40 times) than those of glycoconjugates **8a**—**10a** containing *N*-linked β-galactose residues (Table 1). A comparison of the inhibitory abilities of lactose and glycoconjugates **3a**—**5a** containing one lactosylamine residue with various spacers showed that lactose is bound to RCA_{120} somewhat better than the above glycoconjugates. Glycoconjugates **6a**, **7a**, **9a**, and **10a** containing two or three sugar residues inhibit agglutination better than the glycoconjugates containing only one lactosylamine (**3a**—**5a**) or galactopyranosylamine residue (**8a**); this is due to their two- or three-point attachment to the lectin (the so-called polyvalent or cluster effect). The minimum concentration of glycoconjugates **3a**—**5a** with one lactosylamine residue decreases by a factor of ~5 to 6 for glycoconjugate **6a** with two lactosylamine residues and by a factor of ~6 to 7 for glycoconjugate **7a** with three lactosylamine residues. It should be noted that the contribution of the third lactosylamine residue to the increase in the galectin-binding efficiency is smaller than that of the second residue. Therefore, introduction of two carbohydrate residues into a glycoconjugate can already be sufficient for the cluster effect to appear.

Table 1. Minimum concentration (C_{min}) of glycoconjugates **3a**—**10a** and lactose required to inhibit the hemagglutination with galectin RCA_{120}

Inhibitor	$C_{min} \cdot 10$/mmol L^{-1}
3a—5a	1.8—2.2
6a	0.37
7a	0.3
8a	60.0
9a	17.6
10a	13.4
Lactose	0.9

Experimental

^{1}H NMR spectra were recorded on a Bruker AM-300 spectrometer (300.13 MHz) in D_2O; ^{11}B NMR spectra were recorded on a Bruker DRX-500 spectrometer. Signals for acetone and $BF_3 \cdot Et_2O$, respectively, were used as the external standards. High-resolution mass spectra (ESI) were measured on a Bruker micrOTOF II instrument[13] in the negative ion mode (capillary voltage 3200 V, scan range *m/z* 50—3000 D, external or internal calibration (Electrospray Calibrant Solution, Fluka)). Experimental and the corresponding calculated *m/z* ratios are given for the most intense peaks of isotope clusters. Samples were dissolved in aqueous methanol and infused through a syringe (flow rate 3 μL min^{-1}, nitrogen as a spraying gas (4 L min^{-1}), interface temperature 180 °C). Optical rotation was determined on a PU-07 polarimeter (Russia). Electrophoresis (30 V cm^{-1}, 1 h) was carried out on an EFA-1 instrument (USSR) using Filtrak FN1 paper in a pyridinium acetate buffer (0.025 *M* Py, pH 4.5). Spots were visualized with ninhydrin and a sequence of reagents KIO_4—$AgNO_3$—KOH (see Ref. 14). Eluates obtained by column chromatography on Silica gel 100 C_{18}-Reversed phase (Fluka) were analyzed using UV absorption at 206 nm.

Hydrolytic stability of *N*-{*N*-[(1,2-dicarba-*closo*-dodecaboran(12)-1-yl)acetyl]diglycyl}-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosylamine (3a). 1. Glycoconjugate **3a** (17.2 mg, 0.025 mmol)[9] was dissolved in water (0.5 mL) and heated at 60 °C for 17 h. The solution was diluted with water (1 mL), applied on the top of a column with silica gel C_{18} (3 g) in water, and eluted with water (120 mL) and then with 25% aqueous MeOH. The UV light-absorbing methanolic fractions were combined, concentrated to 0.5 mL, lyophilized, and dried to give the starting glycoconjugate **3a** (10.3 mg, 60%).

The aqueous fractions eluted with the void volume were combined, concentrated to 0.5 mL, lyophilized, and analyzed using electrophoresis, chromatography, and NMR spectroscopy.

The aqueous fractions containing a homogeneous, negatively charged solute (electrophoresis) eluted with retention on the sorbent were combined, concentrated to 0.2 mL, lyophilized, and dried to give *N*-{*N*-[(7,8-dicarba-*nido*-undecaboran(11)-7-yl)acetyl]diglycyl}-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosylamine (**3b**) (2.8 mg, 16%), amorphous solid, $[\alpha]_D^{23}$ +2.5 (*c* 0.4, H_2O). ^{1}H NMR, δ: 2.10 (br.s, 1 H, HC_{carb}); 2.44 (d, 1 H, H_2CC_{carb}, *J* = 15.0 Hz); 2.68 (d, 1 H, H_2CC_{carb}, *J* = 15.0 Hz); 3.47—3.60 (m, 2 H); 3.61—3.86 (m, 8 H); 3.91 (m, 2 H); 3.96 (br.s, 2 H, CH_2N); 4.03 (s, 2 H, CH_2N); 4.44 (d, 1 H, H(1) Gal, *J* = 7.5 Hz); 5.01 (d, 1 H, H(1) Glc, *J* = 9.0 Hz). ^{11}B NMR, δ: −11.2, −12.2, −14.2, −16.3, −18.7, −20.1, −21.1, −33.1, −37.4 (9 B). MS, *m/z* 629.3533, calculated for $C_{20}H_{42}B_9N_3O_{13}$, $[M - H]^-$: *m/z* 629.3534.

2. The hydrolytic stability of glycoconjugate **3a** in water and 0.9% aqueous NaCl at 37 °C, as well as in D_2O at 20 and 5—7 °C, was estimated in a similar way (deboronation was monitored using ^{1}H and ^{11}B NMR spectroscopy).

Hydrolytic stability of glycoconjugates 4a—10a. Glycoconjugates **4a—10a** (0.01 mmol)[9,10] were dissolved in water (0.2 mL) and kept at 37 °C for 24 h. Separation by column chromatography on silica gel C_{18} (3 g) as described above gave the starting glycoconjugates in (79—85)±5% yields and the corresponding *nido-ortho*-carborane glycoconjugates **4b—7b**, **9b**, and **10b** as amorphous powders in 8—10% yields (glycoconjugate **8b** was not isolated in the individual state).

***N*-{*N*-[(7,8-Dicarba-*nido*-undecaboran(11)-7-yl)acetyl]-triglycyl}-*N*-[4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyl]-amine (4b).** ^{1}H NMR, δ: 2.09 (br.s, 1 H, HC_{carb}); 2.43 (d, 1 H, H_2CC_{carb}, *J* = 15.0 Hz); 2.66 (d, 1 H, H_2CC_{carb}, *J* = 15.0 Hz); 3.45—3.58 (m, 2 H); 3.61—3.84 (m, 8 H); 3.87—3.94 (m, 2 H); 3.97 (br.s, 2 H, CH_2N); 4.03 (br.s, 4 H, 2 CH_2N); 4.44 (d, 1 H, H(1) Gal, *J* = 7.5 Hz); 5.01 (d, 1 H, H(1) Glc, *J* = 9.0 Hz). ^{11}B NMR, δ: −10.9, −11.8, −14.0, −16.4, −18.8, −20.0, −21.1, −33.2, −37.4 (9 B). MS, *m/z* 686.3759, calculated for $C_{22}H_{45}B_9N_4O_{14}$, $[M - H]^-$: *m/z* 686.3750.

***N*-{*N*-[(7,8-Dicarba-*nido*-undecaboran(11)-7-yl)acetyl]-diglycyl-L-seryldiglycyl}-*N*-[4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyl]amine (5b).** ^{1}H NMR, δ: 2.09 (br.s, 1 H, HC_{carb}); 2.43 (d, 1 H, H_2CC_{carb}, *J* = 15.0 Hz); 2.66 (d, 1 H, H_2CC_{carb}, *J* = 15.0 Hz); 3.44—3.57 (m, 2 H); 3.61—3.83 (m, 8 H); 3.84—3.96 (m, 4 H); 3.97 (br.s, 2 H, CH_2N); 3.99 (br.s, 4 H, 2 CH_2N); 4.06 (br.s, 2 H, CH_2N); 4.44 (d, 1 H, H(1) Gal, *J* = 7.5 Hz); 4.48 (t, 1 H, CHN, *J* = 4.5 Hz); 5.01 (d, 1 H, H(1) Glc, *J* = 9.0 Hz). MS, *m/z* 830.4254, calculated for $C_{27}H_{53}B_9N_6O_{17}$, $[M - H]^-$: *m/z* 830.4289.

***N*-{*N*-[(7,8-Dicarba-*nido*-undecaboran(11)-7-yl)acetyl]-diglycyl}-*N*,*N*-bis{*N*-[4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyl]carbamoylmethyl}amine (6b).** ^{1}H NMR, δ: 2.09 (br.s, 1 H, HC_{carb}); 2.43 (d, 1 H, H_2CC_{carb}, *J* = 15.0 Hz); 2.66 (d, 1 H, H_2CC_{carb}, *J* = 15.0 Hz); 3.44—3.57 (m, 4 H); 3.62—3.84 (m, 16 H); 3.88—3.95 (m, 4 H); 3.97 (s, 2 H, CH_2N); 4.20 (m, 4 H, 2 CH_2N); 4.38 (br.s, 2 H, CH_2N); 4.44 (br.d, 2 H, 2 H(1) Gal, *J* = 7.5 Hz); 5.00 (d, 1 H, H(1) Glc, *J* = 9.0 Hz); 5.05 (d, 1 H, H(1) Glc, *J* = 9.0 Hz). MS, *m/z* 1067.5047, calculated for $C_{36}H_{68}B_9N_5O_{25}$, $[M - H]^-$: *m/z* 1067.5031.

***N*-{*N*-[(7,8-Dicarba-*nido*-undecaboran(11)-7-yl)acetyl]-diglycyl}-*N*-{*N*-[4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyl]carbamoylmethyl}-*N*-(*N*,*N*-bis{*N*-[4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyl]carbamoylmethyl}carbamoylmethyl)-amine (7b).** ^{1}H NMR, δ: 2.09 (br.s, 1 H, HC_{carb}); 2.43 (d, 1 H, H_2CC_{carb}, *J* = 15.0 Hz); 2.67 (d, 1 H, H_2CC_{carb}, *J* = 15.0 Hz); 3.42—3.56 (m, 6 H); 3.62—3.84 (m, 24 H); 3.88—3.95 (m, 6 H); 3.97—4.37 (m, 11 H); 4.44 (br.d, 3 H, 3 H(1) Gal, *J* = 7.5 Hz); 4.54 (br.s, 1 H); 4.95—5.07 (m, 3 H, 3 H(1) Glc). MS, *m/z* 1505.6516, calculated for $C_{52}H_{94}B_9N_7O_{37}$, $[M - H]^-$: *m/z* 1505.6526.

***N*-{*N*-[(7,8-Dicarba-*nido*-undecaboran(11)-7-yl)acetyl]-diglycyl}-*N*,*N*-bis[*N*-(β-D-galactopyranosyl)carbamoylmethyl]-amine (9b).** ^{1}H NMR, δ: 2.09 (br.s, 1 H, HC_{carb}); 2.45 (d, 1 H, H_2CC_{carb}, *J* = 15.0 Hz); 2.67 (d, 1 H, H_2CC_{carb}, *J* = 15.0 Hz); 3.61—3.83 (m, 10 H); 3.97 (m, 4 H, 2 H(4), CH_2N); 4.11—4.26 (m, 4 H, 2 CH_2N); 4.39 (br.s, 2 H, CH_2N); 4.93 (d, 1 H, H(1), *J* = 9.0 Hz); 4.98 (d, 1 H, H(1), *J* = 9.0 Hz). ^{11}B NMR, δ: −11.0, −12.0, −14.1, −16.3, −18.7, −20.0, −21.1, −33.1, −37.4 (9 B). MS, *m/z* 743.3969, calculated for $C_{24}H_{48}B_9N_5O_{15}$, $[M - H]^-$: *m/z* 743.3966.

***N*-{*N*-[(7,8-Dicarba-*nido*-undecaboran(11)-7-yl)acetyl]-diglycyl}-*N*-[*N*-(β-D-galactopyranosyl)carbamoylmethyl]-*N*-{*N*,*N*-bis[*N*-(β-D-galactopyranosyl)carbamoylmethyl]carbamoylmethyl}amine (10b).** ^{1}H NMR, δ: 2.08 (br.s, 1 H, HC_{carb}); 2.43 (d, 1 H, H_2CC_{carb}, *J* = 15.0 Hz); 2.66 (d, 1 H, H_2CC_{carb}, *J* = 15.0 Hz); 3.57—3.81 (m, 15 H); 3.97 (m, 5 H, 3 H(4), CH_2N); 4.06—4.52 (m, 10 H); 4.88—4.98 (m, 3 H, H(1)). ^{11}B NMR, δ: −11.0, −12.2, −14.2, −16.3, −18.7, −20.1, −21.0, −33.1, −37.4 (9 B). MS, *m/z* 1019.4944, calculated for $C_{34}H_{64}B_9N_7O_{22}$, $[M - H]^-$: *m/z* 1019.4931.

Inhibition of hemagglutination was carried out according to a standard procedure.[15] A suspension of galectin RCA_{120} (2 mg) in 0.9% aqueous NaCl (1.5 mL) was stirred at room temperature for 1.5 h. The undissolved part of the protein was separated by centrifugation and the solution was kept at 5 °C for at most three days. A part of the solution of galectin was diluted with 0.9% NaCl (degree of dilution was ~10—20) and used to determine the concentration of the lectin that, on fourfold dilution, did not agglutinate a 2% suspension of blood group O human erythrocytes.

For the analysis, 2—5 · 10^{-3} *M* solutions of glycoconjugates **3a—7a** in saline and 15—30 · 10^{-3} *M* solutions of glycoconjugates **8a—10a** were employed; the solutions were stored at −18 °C.

Saline was placed in round-bottomed wells of a plate (25 μL per well). A solution of a glycoconjugate (25 μL) was added to the first well, the resulting solution was stirred, and an aliquot (25 μL) was transferred to the second well. An aliquot (25 μL) of the resulting solution in the second well was similarly transferred to the third well and this procedure was repeated up to the tenth well. A solution of galectin (25 μL) was added to each well and the resulting mixtures were stirred and kept at ~20 °C for 1 h. Then a 2% suspension (25 μL) of erythrocytes in saline was added and stirred; the appearance or absence of agglutination was observed after 3—5 h.

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