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Selective discrimination of closely related monosaccharides at physiological pH by a polymeric receptor

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Abstract—High selectivity for glucose by a polymeric receptor at physiological pH over other 1,2-*cis*-diols, namely mannose and galactose, and over a non-imprinted control polymer is reported. The shape selectivity of the imprinted cavity of the polymer has been examined in competing and non-competing rebinding experiments under saturation conditions applying weak binding interactions at physiological pH. The influence of the orientation of the functional groups during polymerisation resulting in the observed high shape selectivity of the cavity has been discussed.

1. Introduction

Materials combining the high selectivity of enzymes and the advantages of synthetic enzyme mimics can be tailored by using molecular imprinting, a technique which is suitable for developing robust, inexpensive, but highly selective polymers.[1] Molecular imprinting involves pre-organisation of polymerisable functional monomers around a targeted template (e.g. carbohydrates) in solution (formation of a monomer-template assembly), followed by cross-linking polymerisation to stabilise this arrangement in the three-dimensional polymeric material. Template removal yields a polymer matrix containing randomly distributed, immobilised functional monomers with cavities that are complementary to the template, and this matrix can be used to selectively rebind the targeted substrate.

Providing hydrogen or covalent bonds between functional monomer and (derivatised) carbohydrate during polymerisation are demonstrated to lead to carbohydrate imprinted polymers, which are suitable for separation analysis and chromatography.[2,3] Furthermore, Arnold developed a glucose sensing device based on an α-methyl-D-glucoside imprinted polymer applying metal co-ordination as binding force between the functional monomer and the carbohydrate.[4] After polymerisation, the template and the binding metal ion were washed out and the polymer was reloaded with copper(II) prior to use at alkaline pH for glucose sensing. At high pH (>9) the immobilised metal complex releases protons upon binding which can be easily detected by a pH electrode. However, this sensing device is limited by its operation range which is not suitable for sensing carbohydrates at physiological pH values.[5]

Taking advantage of demonstrated recognition capability for *cis*-diols, i.e. glucose, over 1,2 *trans*-diols by metal co-ordinated molecularly imprinted polymers, we are aiming at the preparation of inexpensive, robust enzyme mimics which are operational at $5.5<pH<7.5$. Towards this aim, we demonstrate in this paper for the first time highly selective recognition in-between 1,2-*cis*-diols, i.e. epimeric, underivatised carbohydrates, by molecularly imprinted polymers at physiological pH.

2. Synthesis of the functional monomer

[(4-(*N*-Vinylbenzyl)diethylenetriamine)copper(II)] diformate (**1**), $[Cu^{II}(styDIEN)](HCOO)_2$, was chosen as a suitable and inexpensive functional monomer for binding glucose (**2**) or its epimers galactose (**3**) and mannose (**4**). The synthesis of **1** was conducted according to Scheme 1.†

3. Binding studies in solution

Binding studies of the complex formation by UV–Vis spectroscopy showed that in aqueous solution a 1:1 complex consisting of $[Cu^{II}(styDIEN)]^+$ and carbohydrate is formed at alkaline pH values (pH=12.4),[6] which is not interfered when an excess of carbohydrate is used. Investigation of complex formation of **2**, **3** or **4**, methyl-β-D-glucopyranoside, methyl-α-D-glucopyranoside, 3-*O*-methylglucose and 2-deoxy-D-glucose with **1** showed that both hydroxyl groups

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† The synthesis of **8**–**10** was conducted in close relation to a procedure developed by Anelli,[16] the synthesis of **1** is described here for the first time.

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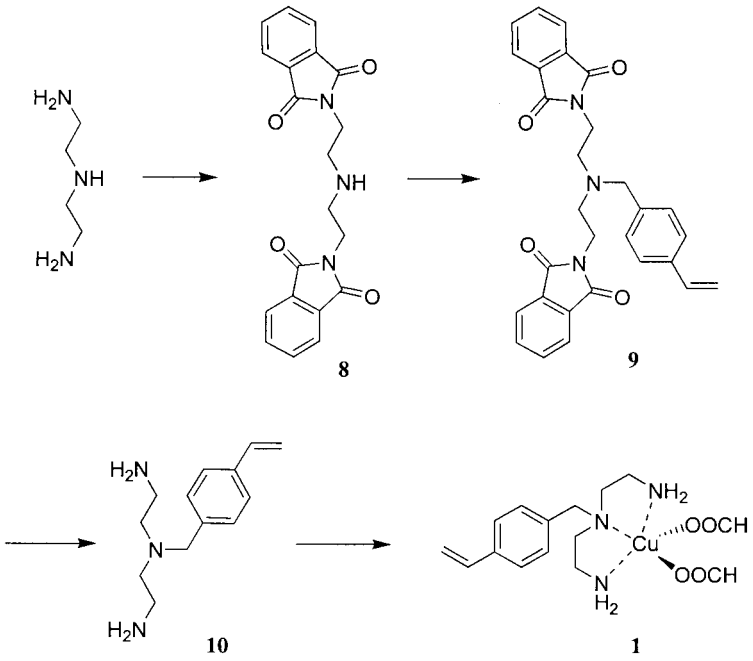
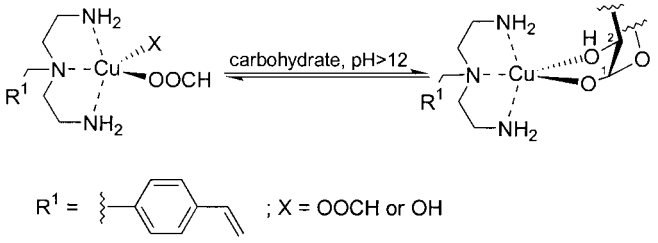


Scheme 1. Synthesis of functional monomer **1**.



Scheme 2. Pre-organisation of **1** and the selected carbohydrate template prior to polymerisation at alkaline pH.

at C^1 and C^2, but not those at C^3, are involved in chelating **1**. The hydroxyl groups of the anomeric carbon of **2**–**4** can be easily deprotonated at high pH values (e.g. $pK_a^{glc}=12.27$),[7] so that preferably a glucosylate, galactosylate or mannosylate anion participates on the complex formation with **1** leading to copper(II)[(4-styryl)DIEN](glucosylate) (**5**), copper(II)[(4-styryl)DIEN](galactosylate) (**6**), or copper-(II)[(4-styryl)DIEN](mannosylate) (**7**). A detailed discussion on the available binding sites of several carbohydrates during complex formation with copper(II) complexes will

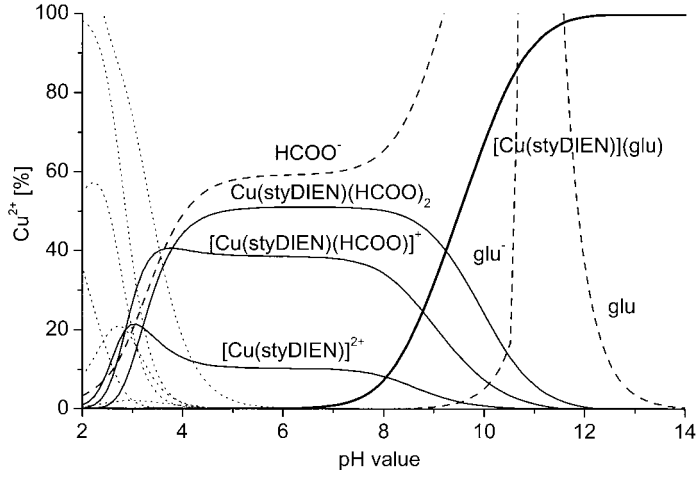


Figure 1. Calculation of species in aqueous solution derived from **1** and **2** (pH=2–14). The species present at pH=2–6 are related to free Cu^{2+}, protonated styDIEN and HCOOH. For clarity their labels are omitted.

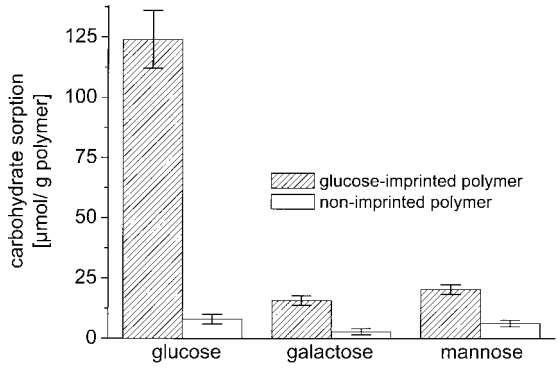


Figure 2. Rebinding capability of glucose- and non-imprinted polymers for **2**–**4** under saturation conditions.

be published elsewhere.[8] The styDIEN ligand (pK_a^{DIEN}= 9.84) is also deprotonated at the pH used for complex formation (Scheme 2).[7]

The apparent binding constants (pK_{app}) of **5**–**7** ($pK_{app,5}$= 3.37, $pK_{app,6}$=3.41, $pK_{app,7}$=3.05) were determined according to Rose and Drago at pH=12.4.[6] As the apparent binding constants are of the same order of magnitude, **1** does not discriminate between **2**–**4**. The composition of the formed ternary complexes, which may exist in several equilibria in aqueous solution, can be calculated from the binding constants of their compounds in dependence on the pH value using the computer program SPE.[9] According to these calculations, only about 90% of **1** form a 1:1 complex with a carbohydrate when a molar ratio of $[Cu^{II}(styDIEN)]^+$/carbohydrate=1:1 is used. This is not favourable for the planned imprinting process since 10% of **1** are not chelated by a carbohydrate and are therefore not able to create specific cavities during polymerisation but will rather generate randomly distributed, non-specific binding sites inside the polymer. 100% Complex formation can be achieved by using a five-fold excess of carbohydrate (Fig. 1).

4. Incorporation of the ternary complex into the polymer

Photo-initiated polymerisation around the pre-organised complexes **5**–**7** (5 mol%) in methanol–water (3:1) using pentaerythritol tetraacrylate (PETA, 95 mol%) as cross-linker and 2,2′-dimethoxy-2-phenylacetophenone as initiator yielded solid macroporous polymers.[10] Photo-initiated polymerisation has already been used for the molecular imprinting technique,[11] but it has not yet been applied to molecularly imprinted polymers using coordinative binding interactions. The functional groups of the template can position their counterparts inside the polymer within 15 s at ambient temperature (25°C). Therefore, this protocol is applicable to carbohydrates, which may decompose or epimerise at elevated temperatures or very alkaline or acidic conditions during a prolonged polymerisation process otherwise.[8] The imprinted polymers were treated prior to rebinding experiments as described.[4] Thermogravimetric analysis confirmed that at least 96.5% of the incorporated copper(II) of **1** can be stripped off and at least 98.4% of that can be reloaded into the polymer after equilibrating with aqueous $CuCl_2$. The high value of reloadable copper(II) indicates a very good accessibility of the created cavity for metal ions. A control polymer without an imprinting carbohydrate was prepared in identical fashion.

5. Rebinding studies at physiological pH

Following the 'bait-and-switch' approach we provided strong interactions during polymerisation while we used weak interactions at physiological pH for the investigation of the rebinding capability of the imprinted polymers.[12] First, we determined the molecular recognition of the free sugars for glucose- and non-imprinted polymers in non-competing batch rebinding studies (Fig. 2). The experiments were conducted with aqueous carbohydrate solutions using seven-fold excess of carbohydrate compared to the number of theoretically available binding sites (100%= 132 µmol carbohydrate/g polymer), which was calculated from the amount of functional monomer immobilised during polymerisation. The remaining carbohydrate concentration in solution was determined after calibration by HPLC.

Glucose has a remarkably good induced fit inside the cavity of a glucose-imprinted polymer, **3** and **4** have not. The control polymer takes up only a small amount of any monosaccharide. The selectivity factors α for glucose over its

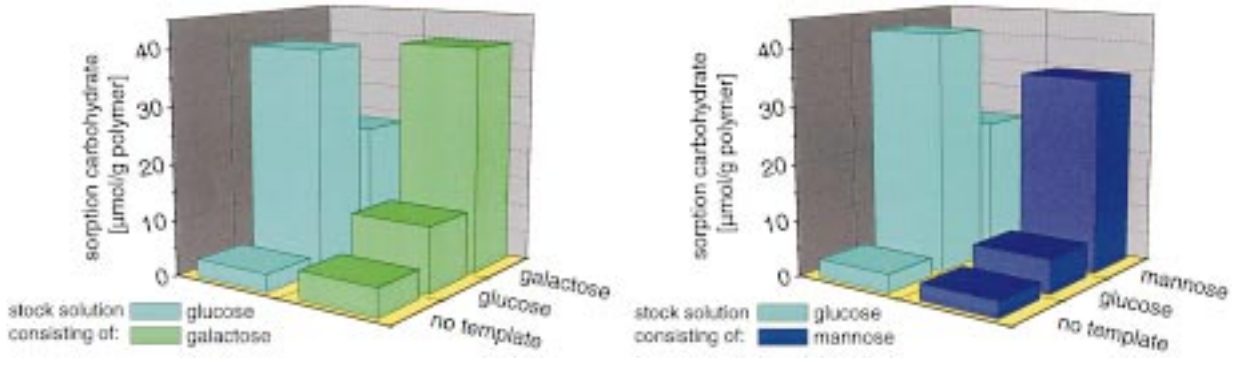


Figure 3. Rebinding capability of carbohydrate- and non-imprinted control polymers for **2**–**4** in competing experiments.

epimers **3** and **4** were determined to be 8.3 and 9.0, respectively.‡

Second, we investigated the recognition capability of the same polymers in competing experiments using equimolar mixtures of **2** and **3** (**4**) in three-fold excess compared to the number of theoretically available binding sites (Fig. 3). The excess sugar was reduced to three-fold due to experimental separation limitations during determination of carbohydrate concentration by GC-MS. Only 43% of the theoretically available sites (uptake of 57 μmol sugar/g polymer) are accessible for carbohydrates under these conditions, as detected by using a pure glucose solution at the same concentration.

The glucose-imprinted polymer exhibits high selectivity for the original template while the non-imprinted polymer is not able to take up a significant amount of sugar at all. A small amount of **3** is taken up by the glucose-imprinted polymer referring to similarity of the shape complementary cavities for **2** and **3**, which differ only in the space around the epimeric carbon atom C^4 close to the opening of this pore-like structured cavity. **2** fits better in a galactose-imprinted polymer than **3** does in a glucose-imprinted cavity. Glucose prefers equatorial arrangements for all hydroxyl groups in its pyranose structure, while this is not possible for its epimers **3** and **4**, where at least the hydroxyl groups of the epimeric carbon atoms C^4 and C^2 occupy axial positions in the main species of the equilibria structures.[13] The interference of the hydroxyl groups at the carbon atoms C^4 and C^2 with the polymer backbone during recognition demonstrates that a glucose-imprinted polymer can take up a small amount of **3**, but even less of **4**, while a galactose- or a mannose-imprinted polymer exhibits a notable induced fit for **2** inside their cavities (the selectivity factor α was determined as α_{23}=5.5, α_{24}=12.4, α_{32}=1.7, α_{42}=1.5).‡ Wulff and his group have demonstrated selective carbohydrate recognition relying on covalent bonding to boronic acid derivatives which were immobilised inside an imprinted cavity.[3,14] In contrast to our results, the selectivity observed in these polymers is mainly due to the orientation of the functional groups while the shape of the cavity is less important during the strong covalent rebinding interactions.

6. Conclusion

The glucose-imprinted polymer presented shows a distinctly higher ability to rebind glucose over other closely related *cis*-1,2-diols, namely mannose and galactose, and a non-imprinted control polymer in equilibrium. Since weak interactions during interaction of monosaccharides and the immobilised functional monomer at physiological pH were provided, the shape of the cavity dominates the demonstrated selectivity. The observed recognition capability also refers to the high influence of the polymerisation procedure, which is mainly responsible for the shape of the created cavity.

‡ The selectivity factor $\alpha_{i,j}$ for two monosaccharides *i* and *j* is defined as $\alpha_{i,j} = (c_{i,j=i} - c_{\text{control},i})/(c_{i,j} - c_{\text{control},j})$, where $c_{i,j}$ is the concentration of a carbohydrate j taken up by an i imprinted polymer. All selectivity factors were calculated from data obtained from at least three independent experiments with two independently prepared polymers. Their 95% confidence limits were less than or equal to ±0.1.

7. Experimental

7.1. General remarks

All reactions were carried out in oven dried glassware. Reactions with moisture-sensitive chemicals were performed under argon. Solvents were dried using standard procedures. Uncorrected melting points: Büchi B-540. NMR: Bruker DRX 400 (^{1}H: 400.1 MHz; ^{13}C: 100.6 MHz) and Bruker AC 200 (^{1}H: 200.1 MHz; ^{13}C: 50.3 MHz); $CDCl_3$ was used as solvent unless otherwise noted. Chemical shifts (δ) are expressed in ppm downfield from tetramethylsilane using the residual non-deuterated solvent as internal standard ($CDCl_3$ δ (^{1}H) 7.24, δ (^{13}C) 77.0; CD_3OD δ (^{1}H) 3.30, δ (^{13}C) 49.0, J in Hz. IR: Bruker IFS 133 V ν in cm^{-1}, samples as KBr pellets. MS: Finnigan MAT SSQ 7000 (CI, EI, FAB). Microanalyses: Elementar Vario EL. UV: J & M TIDAS (software SPECTRALYS, version 1.55), using Suprasil® standard cells (200–2000 nm) with 10 mm thickness and 700 μl volume at 25°C. The measurements were performed between 200 and 900 nm. Analyses by GC were carried out on a Varian Saturn GC/MS 2000 using a DB5 MS column, 30 m×0.25 nm×0.25 μm. Analyses by HPLC were carried out using a Nucleogel® Sugar Pb column (supplied by Macherey-Nagel, Düren, Germany), using 100% water at flow rate=0.3 ml min^{-1} and RI detection.

β-D(+)Glucose (**2**) was obtained from Sigma; potassium carbonate, D-mannose (**4**), methyl-β-D-glucopyranoside and methyl-α-D-glucopyranoside were obtained from Fluka; 0.01N Na_2EDTA, DIEN, hydrazine hydrate, phthalic anhydride, 4-vinylbenzyl chloride, 3-*O*-methylglucose and 2-deoxy-D-glucose were obtained from Aldrich; cupric nitrate trihydrate and D(+)galactose (**3**) were obtained from Merck. All commercially available reagents were used as received from the supplier. Copper formate[15] and 1,5-diphthalimido-3-azapentane (**8**)[16] were prepared as described.

7.1.1. 1,5-Diphthalimido-3-styryl-3-azapentane (9). The compound **9** was prepared in analogy to a published procedure described for 3-benzyl-3-aza-1,5-pentanediamine.[16] A mixture of 34.2 g (0.094 mol) of **8** and 39 g (0.283 mol) of anhydrous K_2CO_3 in 300 ml dry acetonitrile was refluxed with stirring for 15 h. The mixture was refluxed after the addition of 17 ml *p*-vinylbenzyl chloride (0.104 mol) for additional 24 h, filtered and the white precipitate was washed with CH_2Cl_2. The combined organic phases were evaporated, and the residue was dissolved in water and CH_2Cl_2. The organic solution was dried over anhydrous Na_2SO_4, filtrated and the solvents were evaporated. After recrystallisation from acetonitrile, a pale yellow crystalline solid was obtained (35.8 g, 83%). Mp 118–119°C. IR (KBr) 2934, 1711, 1393 cm^{-1}. $C_{29}H_{29}N_3O_4$: calcd C 72.65, H 5.22, N 8.77; found C 72.62, H 5.29, N 8.81. MS (EI): *m/z* (%) 479 (20) [M^+], 319 (100), 160 (8), 117 (66). ^{1}H NMR (200.13 MHz, $CDCl_3$) δ 7.66 (m, 8H), 6.93 (dd, J=18.70, 8.37 Hz, 4H), 6.50 (dd, J=10.83, 17.72 Hz, 1H), 5.51 (dd,

J=17.72, 1.48 Hz, 1H), 5.11 (dd, J=10.83, 1.48 Hz, 1H), 3.73 (m, 4H), 2.77 (m, 4H) 2.59 (s, 2H). ^{13}C NMR (50.32 MHz, $CDCl_3$) δ 168.1, 138.4, 136.6, 135.9, 133.5, 132.3, 129.1, 125.8, 122.9, 112.9, 57.8, 51.7, 35.7.

7.1.2. 4-(*N*-Vinylbenzyl)diethylenetriamine (10). The compound **10** was prepared in analogy to a procedure described for 3-benzyl-3-aza-1,5-pentanediamine.[16] 4.6 g (0.010 mol) of **9** were dissolved in 160 ml ethanol under reflux. 5 ml hydrazine hydrate (98%) was added, the solution refluxed for 1 h and filtered. The solvent was evaporated, 150 ml chloroform was added and after cooling in an ice bath, the precipitated phthalhydrazide was filtered off. Evaporation of $CHCl_3$ gave a pale yellow oil (2.0 g, 91%). IR (thin film) 2935, 1581, 1461 cm^{-1}. $C_{15}H_{21}N_3$: calcd C 79.19, H 9.65, N 19.16; found C 71.13, H 9.77, N 19.09. MS (CI): *m/z* (%) 220 (100) [M^++H], 203 (28), 160 (35), 117 (20), 87 (6). ^{1}H NMR (400.13 MHz, $CDCl_3$) δ 7.21 (dd, J=36.57, 8.15 Hz, 4H), 6.60 (dd, J=17.68, 10.86 Hz, 1H), 5.63 (dd, J=17.62, 1.01 Hz, 1H), 5.12 (dd, J=10.86, 1.01 Hz, 1H), 3.47 (s, 2H), 2.64 (m, 4H), 2.40 (m, 4H), 1.09 (s, 4H). ^{13}C NMR (100.61 MHz, $CDCl_3$) δ 139.0, 136.2, 136.0, 128.6, 125.8, 113.1, 58.6, 57.1, 39.5.

7.1.3. (4-(*N*-Vinylbenzyl)diethylenetriamine)copper(II)-diformate (1). 219 mg (0.997 mmol) of **10** were dissolved in 200 ml dichloroethane. 153 mg (0.996 mmol) copper(II)-formate were added and the resulting mixture was heated up to 60°C for 1 h. After cooling in an ice bath, the blue precipitate was filtered off and dried in vacuum at 40°C (316 mg, 85%). Mp 135.2–136.8°C, hygroscopic. IR (KBr) 2923, 1462, 1376 cm^{-1}. UV λ_{max} 322 and 597 nm. MALDI-TOF (methanol, dithranol) MS: *m/z* (%) 426.7 (37.2) [M^++CH_3OH+Na], 328.6 (16.7) [MH^+−HCOO], 314.6 (13.8), 304.55 (23.2), 281.55 (46.8), 241.7 (14.4), 219.7 (100). $C_{15}H_{23}CuN_3O_4$: calcd C 48.31, H 6.22, N 11.27, Cu 17.06; found C 48.61, H 6.21, N 11.23, Cu 17.07. The copper(II) content was determined by titration with 0.01N EDTA against murexide at pH=2.2 and by photometric titration with 0.01N EDTA.

8. Polymerisation procedure

Typically 0.4 mmol (149.02 mg) of **1** were dissolved with 2.0 mmol (360.34 mg) monosaccharide in water (2.0 ml) at pH=12.4. Then 7.6 mmol (2.678 g) PETA and 0.76 mmol (0.195 g) 2,2′-dimethoxy-phenylacetophenone in methanol (6 ml) were added and the resulting mixture immediately polymerised in UV light by use of a mercury pressure TQ 150 lamp on a petri dish. The resulting blue polymer was dried in a vacuum oven at 30°C for 12 h, grounded and sieved to particles in the range 40–80 μm size. The metal ion and the carbohydrate were removed by addition of 1 M aqueous EDTA and 0.1N HCl. The neutral washed polymer was reloaded for 12 h with 0.1 M $CuCl_2$. The polymer was washed three times with 20 ml water each to remove unbound copper(II) ions and dried for 24 h at 30°C in vacuum.

9. Rebinding procedure under non-competing conditions

100 mg polymer were incubated for 24 h at 25°C with the appropriate aqueous carbohydrate solution and separated by centrifugation. The supernatant solution was filtrated and immediately used for HPLC analysis. The amount carbohydrate in solution was determined by comparison to a calibration curve. The remaining amount of carbohydrate in the polymer was calculated from the difference in concentration between stock and supernatant solution.

10. Rebinding procedure under competing conditions

100 mg polymer were incubated for 24 h with 1 ml aqueous carbohydrate solution (7.2 mg/ml) and centrifuged. 200 μl of the supernatant solution were evaporated, taken up in 200 μl abs. pyridine, silylated with chlorotrimethylsilane (40 μl) and separated by centrifugation. The samples were stored in ice up till analysis. The yields and the compositions of the carbohydrate mixtures in the top solution were obtained by quantitative GC using calibration curves, which were obtained by applying the same protocol to equimolar carbohydrate mixtures of known concentrations for comparison.

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