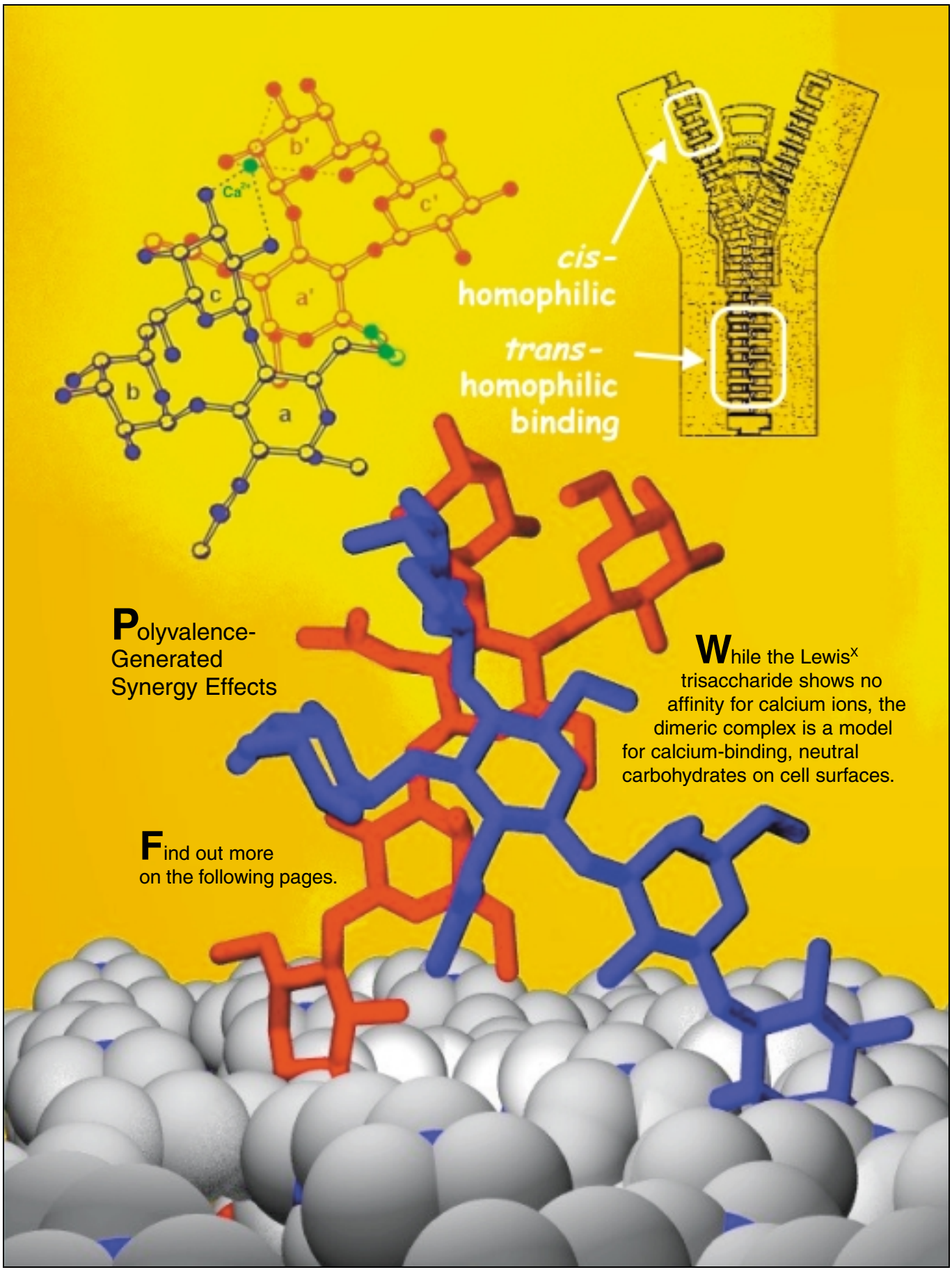

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Calcium-Dependent Carbohydrate–Carbohydrate Recognition between Lewis^X Blood Group Antigens**

Armin Geyer,* Christian Gege, and Richard R. Schmidt*

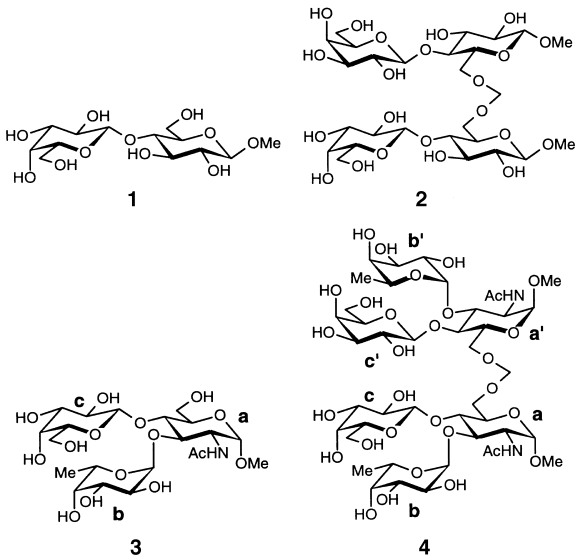
Dedicated to Professor Horst Kessler on the occasion of his 60th birthday

Lewis^X (Le^X) is the terminal trisaccharide moiety of numerous cell-surface glycolipids and glycoproteins which are involved in selectin-mediated cell–cell adhesion and recognition processes.[1] In addition to protein–carbohydrate binding, there are adhesive forces between cells which are based on direct carbohydrate–carbohydrate interactions, for instance on homophilic Le^X–Le^X binding.[2] Molecular details of the homophilic recognition between neutral carbohydrate moieties are incompletely understood because of chemical complexity of the cell membrane. Ligand–receptor complexes with weak affinity constants between singular entities (K_a values in the mM^{-1} range) multiply to polyvalent intercellular adhesive forces of high affinity.[3] The experimental characterization of the synergistic multiplication of weak carbohydrate–carbohydrate affinities to considerable intercellular forces is an analytical challenge. Protein-mediated binding and charge effects must be excluded; separation of membrane components can even be induced by their differing phase transition properties.[4]

The cation-dependent clustering of membrane-anchored sialylated,[5] and also neutral,[6] Lewis glycolipids has been described. However, in isotropic solution, no self-assembly of Le^X glycoconjugates or significant metal complexation was detectable.[7] The very weak calcium affinity of the neutral Le^X oligosaccharides obviously requires their tethering to a lipid bilayer in order to make clustering detectable.[8] Yet, conformational details of the Le^X clusters could not be determined until now.

We describe here how we have mimicked the preorientation of Le^X moieties with a flexible spacer between two Le^X trisaccharides. Six rotatable bonds permit several relative orientations of the Le^X moieties. In the presence of calcium, a single mode of self-assembly was observed by NMR spectroscopy and for the first time conformational details of a Le^X cluster were quantified. The cooperative calcium complexation of a simple Le^X cluster contrasts the ion-binding behaviour of other carbohydrate clusters.

Mono- and oligosaccharides, such as the methyl lactoside **1** (Scheme 1), have a weak calcium affinity. The lactose moieties of **2** are kept in close vicinity by the methylene spacer but no sterical restrictions force the disaccharide moieties of **2** into a prearranged conformation. The two



Scheme 1. Two methyl lactosides **1** were covalently tethered in the bis-disaccharide **2**. Similarly, two Le^X trisaccharides **3** lead to the bis-trisaccharide **4**. The methylene spacers in **2** and **4** keep the homotopic moieties in close vicinity without restraining their relative orientations. **a** = *N*-acetylglucosamine, **b** = fucose, **c** = galactose.

covalently tethered lactoses **2** have an affinity for calcium ions with a complex formation constant K_a of 8 M^{-1}.[9] The K_a value is 7 M^{-1} for the monomeric lactoside **1**. The affinity of the bis-disaccharide **2** for calcium equals to the affinity of disaccharide **1** for calcium ions; therefore, the average affinity per lactose is divided by approximately two (Figure 1). Lactose lacks any cooperativity for calcium binding and the affinity of the bivalent receptor **2** is much lower than the multiplied monomeric affinities ($K_{bi} \ll K_{mono}K_{mono}$; $\Delta G_{bi} > \Delta G_{mono} + \Delta G_{mono}$), thereby avoiding the accumulation of cationic charges. Although crystal structures with selective coordination of lactose to calcium salts are known,[10] lactose binds calcium only weakly in isotropic solution.

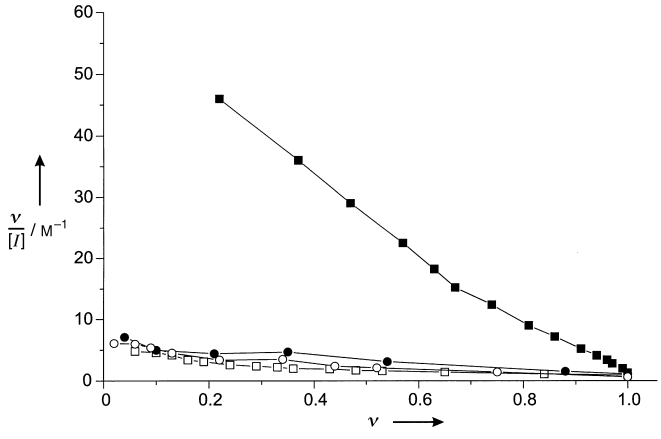


Figure 1. Scatchard plots[20] of **1** (○), **2** (●), **3** (□), and **4** (■). The fraction of complexed carbohydrate (ν) is plotted against the ratio of ν/I (I = concentration of uncomplexed calcium ions). The affinity constant K_a [M^{-1}] is obtained from the y axis by linear extrapolation to $\nu = 0$. Only compound **4** yields a straight line, with an affinity constant of $K_a = 55$ M^{-1}. Secondary binding sites are populated at high concentrations of calcium ions and a reduced slope is observed at $\nu > 0.7$. Weak affinities below $K_a = 10$ M^{-1} are slightly overestimated because full complexation ($\nu = 1$) is not reached without secondary effects playing a dominant role.

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The Le^X trisaccharide **3** behaves differently. The calcium affinity of **3**, which is weak in methanol ($K_a = 6\,M^{-1}$) and not detectable in water, multiplies to one of the highest affinities measured for a neutral oligosaccharide[11] when two Le^X trisaccharides are covalently linked through O6 of *N*-acetylglucosamine (**4**, $K_a = 55\,M^{-1}$, Figure 2). The affinity of **4** as a receptor for calcium ions is even detectable in water ($K_a = 5-10\,M^{-1}$). The Le^X trisaccharide moieties cooperatively assemble and form a new receptor site with a significantly enhanced

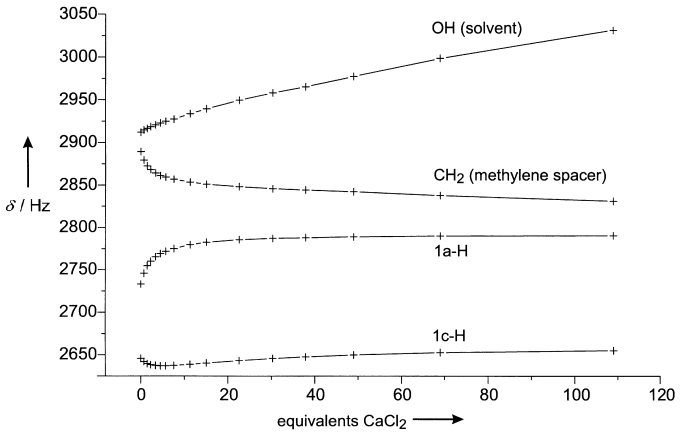

Figure 2. The chemical shifts (^{1}H NMR spectroscopy) of **4** in [D$_4$]methanol are plotted against the amount of calcium chloride added. A strong chemical shift dependence of the proton resonances is observed below ten equivalents of calcium chloride. The complexation of calcium freezes the conformational equilibrium of the trisaccharide moieties of **4**, and all chemical shifts show calcium dependence. The conformational effect of the calcium complexation causes a highfield shift for 1c-H which is inverted at higher calcium levels due to the nonselective binding of calcium to **4**. All curves, except for the solvent resonance, show a double exponential dependence on the calcium concentration.

affinity for calcium ions ($K_{bi} \geq K_{mono} K_{mono}$). The two Le^X trisaccharides show this cooperativity of calcium binding in isotropic solution and this allows proof to be obtained spectroscopically for the existence of homophilic carbohydrate–carbohydrate recognition.[12]

The solution structure of **4** was investigated by NMR spectroscopy.[13] As expected for a flexible spacer, no NOE contacts are visible between the homotopic trisaccharide moieties of **4** in the absence of calcium ions. The trisaccharide moieties are separated by six single bonds and the NOE cross-signal intensities resemble the intensities of **3**. These six single bonds become constrained in the calcium complex of **4**. The ROESY[14] spectrum of the calcium complex of **4** exhibits new NOE contacts (Figure 3). The additional NOE contacts prove the self-assembly of the homotopic trisaccharide moieties into a defined receptor conformation. The high on- and off-rates of calcium-ion complexation[15] average the expected hexasaccharide signal set to a trisaccharide signal set which contains the additional NOE results. The NOE contacts are caused by average proton–proton distances of less than four ångstroms and indicate hydrophobic contacts between the two trisaccharide units of **4**. They were quantified according to the two-spin approximation:[16] 2a-H–2c-H (approximately 3.0 Å), 1a-H–6b-CH_3 (approximately 3.2 Å), and 6b-CH_3–2a-COCH_3 (approximately 3.5 Å). The new cross signals identify

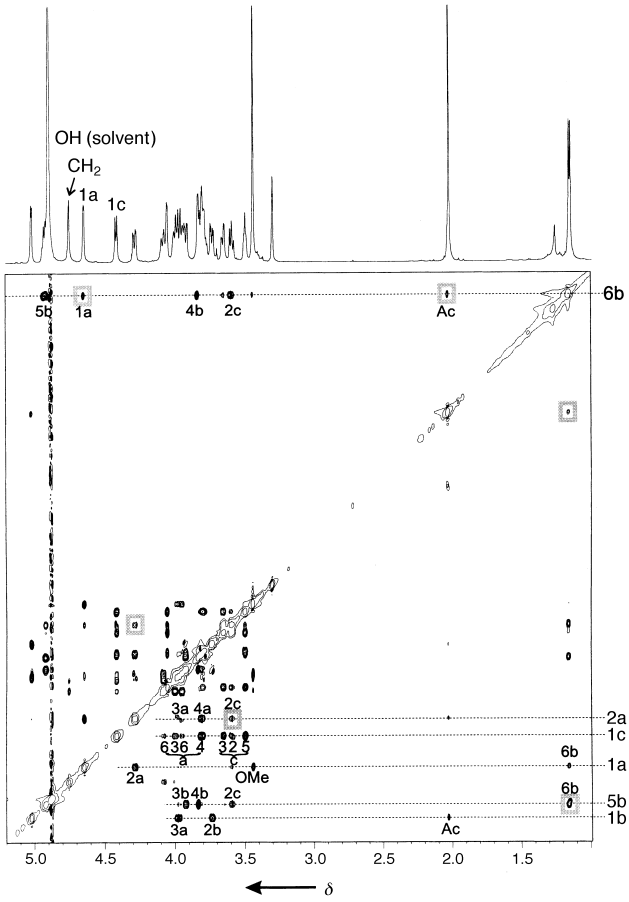

Figure 3. Compensated ROESY spectrum (600 MHz, 4 kHz spin lock, 200 ms mixing time, CD_3OD, 300 K) of **4** in the presence of 15 equivalents of calcium chloride. Rotating frame NOE interactions, which are not visible in the absence of calcium chloride, are indicated by gray boxes. Other cross signals than those indicated do not change their intensities relative to the ROESY spectrum of **4** without calcium chloride. The cross-signal intensities correspond to average interproton distances between 1.8 and 4 Å.

a cross-shaped, approximately 90°, relative orientation of the trisaccharide units of **4** (Figure 4).[17] The hydrophobic contact stabilizes a neighbouring polar cation-binding site. This only polar contact site between the trisaccharide moieties is a hydrophilic cavity which is flanked by the five hydroxyl groups of rings c, b′, and c′ (Figure 4). The calcium ion fits into the NOE-derived structure with Ca–O distances of approximately 2.5 Å.[18]

Le^X glycosphingolipids are oriented at the outer layer of cellular membranes. *cis*-Homophilic contacts between Le^X glycosphingolipids stabilize glycolipid microdomains within one membrane. *trans*-Homophilic contacts mediate the cell–cell adhesion between the outer membrane layers of different cells. Parallel[19] or antiparallel[8] molecular recognition between individual Lewis glycolipids was proposed to explain the two processes. The cross-shaped orientation of the covalently tethered Le^X trisaccharide moieties in **4** allows for *cis*- and *trans*-homophilic contacts between Le^X glycoconjugates without resorting to different recognition phenomena. Thus, pairs of dimers from adjacent cell membranes can stack above each other and stabilize cell–cell contacts (Figure 5).

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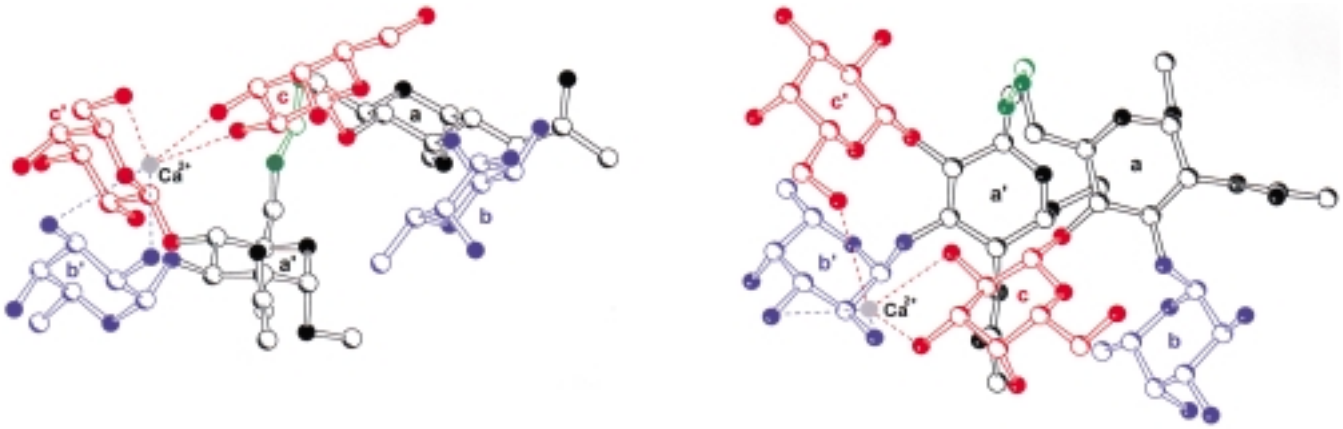


Figure 4. Solution conformation of the calcium complex of **4**. The Le^X trisaccharide populates one well-defined conformational minimum, as known from previous experimental studies.[21] Torsional restraints kept the glycosidic torsions of fucose (b) and galactose (c) at this minimum in a molecular dynamics simulation of **4**. The rotational mobility about the covalent tether was not restricted and the experimental NOE values were included as weak additional restraints. The exocyclic torsion of *N*-acetylglucosamine (a) populates the *gauche*–*trans* rotamer in pyranose a and the *gauche*–*gauche* rotamer in ring a′. The methylene spacer linking the *N*-acetylglucosamines is shown in green. Rings a and a′ are coplanar (left) and assume a cross-shaped relative orientation, which is more easily visible from a different direction (right). The position of the calcium ion is tentatively assigned at the hydrophilic contact site of the trisaccharide moieties of **4**.

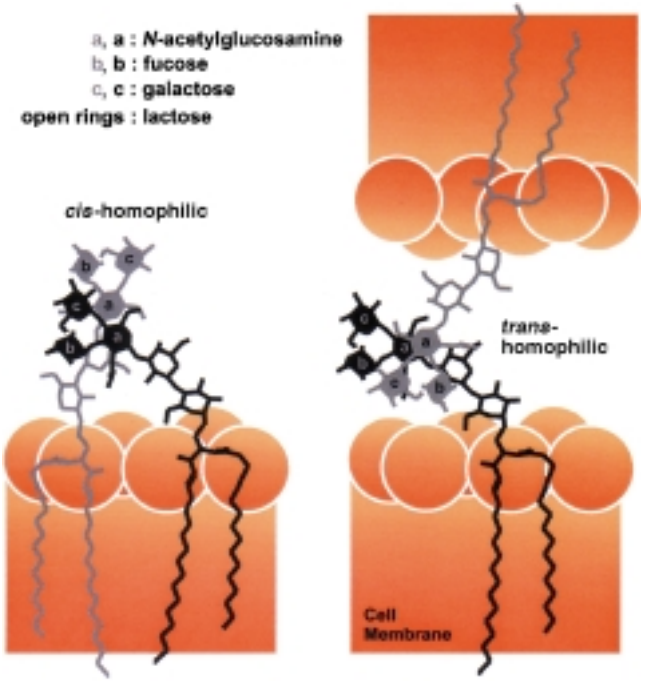
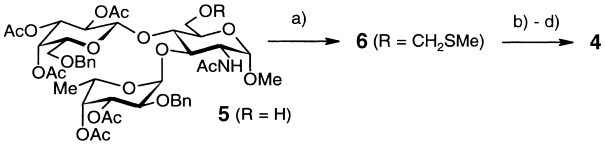


Figure 5. Two Le^X pentasaccharides (lactoses: open pyranose rings, Le^X trisaccharides: filled pyranose rings) are sketched with the relative orientation of the Le^X trisaccharide headgroups as determined from the calcium complex of **4**. The structures show the proposed Le^X–Le^X interaction between glycolipids which are anchored to the same membrane (*cis*-homophilic contact) or which are anchored to different membranes (*trans*-homophilic contact).



Scheme 2. a) Dimethyl sulfide, dibenzoylperoxide, 2,6-lutidine (89%); b) **5**, *N*-iodosuccinamide, trifluoromethanesulfonic acid, 4 Å molecular sieves, CH_2Cl_2 (58%); c) Pd/C, H_2; d) NaOMe, MeOH (90% over two steps).

Experimental Section

Synthesis of **4**: The Le^X trisaccharide **5** (Scheme 2) was assembled from the monosaccharide building blocks using the trichloroacetimidate method.[5b] This selectively deprotected trisaccharide was transformed into the thioacetal **6**. An iodonium-mediated reaction with **5** gave the dimer which was deprotected to **4**.

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[1] a) R. A. Dwek, *Chem. Rev.* **1996**, *96*, 683–720; b) S. Hakomori, *Cancer Res.* **1996**, *56*, 5309–5318.
[2] a) I. Eggens, B. Fenderson, T. Toyokuni, B. Dean, M. Stroud, S. Hakomori, J. *Biol. Chem.* **1989**, *264*, 9476–9484; b) N. V. Bovin in *Glycosciences* (Eds.: H.-J. Gabius, S. Gabius), Chapman & Hall, Weinheim, **1997**, 277–289; c) S. Hakomori, K. Handa, K. Iwabuchi, S. Yamamura, A. Prinetti, *Glycobiology* **1998**, *8*, xi-xix; d) B. G. Davis, J. *Chem. Soc. Perkin 1* **1999**, 3215–3237.
[3] a) U. Dammer, O. Popescu, P. Wagner, D. Anselmetti, H.-J. Günther-odt, G. N. Misevic, *Science* **1995**, *267*, 1173–1175; b) M. Mammen, S.-K. Choi, G. M. Whitesides, *Angew. Chem.* **1998**, *110*, 2908–2953; *Angew. Chem. Int. Ed.* **1998**, *37*, 2754–2794; c) K. Matsura, A. Tsuchida, Y. Okahata, T. Akaike, K. Kobayashi, *Bull. Chem. Soc. Jpn.* **1998**, *71*, 2973–2977.
[4] P. M. Macdonald, *Acc. Chem. Res.* **1997**, *30*, 196–207.
[5] a) J. Vogel, G. Bendas, U. Bakowsky, G. Hummel, R. R. Schmidt, U. Kettmann, U. Rothe, *Biochim. Biophys. Acta* **1998**, *1372*, 205–215; b) C. Gege, J. Vogel, G. Bendas, U. Rothe, R. R. Schmidt, *Chem. Eur. J.* **2000**, *6*, 111–122.
[6] M. Boubelik, D. Floryk, J. Bohata, L. Draberova, J. Macak, F. Smid, P. Draber, *Glycobiology* **1998**, *8*, 139–146.
[7] a) M. R. Wormald, C. J. Edge, R. A. Dwek, *Biochem. Biophys. Res. Commun.* **1991**, *180*, 1214–1221; b) B. Henry, H. Desvaux, M. Pristchepa, P. Berthault, Y.-M. Zhang, J.-M. Mallet, J. Esnault, P. Sinaÿ, *Carbohydr. Res.* **1999**, *315*, 48–62.
[8] A. Geyer, C. Gege, R. R. Schmidt, *Angew. Chem.* **1999**, *111*, 1569–1571; *Angew. Chem. Int. Ed.* **1999**, *38*, 1466–1468.

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[9] The binding affinity is defined as $K_a = [\text{complex}]/([\text{sugar}][\text{cation}])$. NMR titrations were performed for **1**, **2**, and **3** at concentrations of 14, 17, and 10 mM in CD_3OD. About 100 equivalents of calcium chloride were added in portions and several well-separated chemical shifts were analysed. Experimental errors are below $\pm 3\,M^{-1}$. Compound **4** was investigated at 5 mM and at 20 mM, no significant differences were observed. Below temperatures of 250 K, severe signal broadening indicates higher aggregates of **4** in the presence of calcium chloride. The NMR titration of **4** in D_2O was performed at a concentration of 10 mM. Weak calcium affinities were observed for the hybrid oligosaccharide of lactose and Le^X ($K_a = 20\,M^{-1}$) and for a bistrisaccharide with the Le^X moieties anomerically linked by 1,3-propanediol through six rotatable bonds ($K_a = 17\,M^{-1}$).

[10] C. E. Bugg, J. *Am. Chem. Soc.* **1973**, *95*, 908 – 913; W. J. Cook, C. E. Bugg, *Acta Crystallogr. Sect. B* **1973**, *29*, 907 – 909.

[11] Higher affinities were observed for rigid pyranose rings with an *ax-eq-ax* arrangement of hydroxy groups. See: S. J. Angyal, *Adv. Carbohydr. Chem. Biochem*. **1989**, *47*, 1 – 43.

[12] A. P. Davis, R. S. Wareham, *Angew. Chem.* **1999**, *111*, 3160 – 3179; *Angew. Chem. Int. Ed.* **1999**, *39*, 2978 – 2996.

[13] Compounds **1** – **4** were fully assigned by homo- and heteronuclear NMR methods. All NMR spectra were acquired at 600 MHz and 300 K.

[14] Compensated ROESY spectra were acquired with a pulsed 4 kHz spin lock and a 200 ms mixing time. See: a) A. A. Bothner-By, R. L. Stephens, J. Lee, C. D. Warren, R. W. Jeanloz, *J. Am. Chem. Soc.* **1984**, *106*, 811 – 813; b) A. Bax, D. G. Davis, *J. Magn. Reson.* **1985**, *63*, 207 – 213; c) C. Griesinger, R. R. Ernst, *J. Magn. Reson.* **1987**, *75*, 261 – 271.

[15] J. K. Beattie, M. T. Kelso, *Aust. J. Chem*. **1981**, *34*, 2563 – 2568.

[16] Spin diffusion is neglectable under these experimental conditions and the volume integral of each cross peak correlates to a single interproton distance (two-spin approximation). The offset-corrected cross-peak intensities were calibrated according to the r^{-6}-dependence of the NOE with isotropic tumbling of **4**. See: A. Kumar, G. Wagner, R. R. Ernst, K. Wüthrich, *J. Am. Chem. Soc.* **1981**, *103*, 3654 – 3658.

[17] The experimental interproton distances were included as weak ($7\,kcal\,mol^{-1}\,Å^{-2}$) restraints in molecular dynamics simulations of **4**. Structure sampling and energy minimization were carried out according to: Z.-H. Jiang, A. Geyer, R. R. Schmidt, *Angew. Chem*. **1995**, *107*, 2730 – 2734; *Angew. Chem. Int. Ed. Engl*. **1995**, *34*, 2520 – 2524. Figure 4 was drawn with SCHAKAL (E. Keller, University of Freiburg, Germany).

[18] The residual octahedral coordination sphere of calcium can be saturated by water. See: A. K. Katz, J. P. Glusker, S. A. Beebe, C. W. Bock, *J. Am. Chem. Soc.* **1996**, *118*, 5752 – 5763.

[19] a) G. Siuzdak, Y. Ichikawa, T. J. Caulfield, B. Munoz, C.-H. Wong, K. C. Nicolaou, *J. Am. Chem. Soc.* **1993**, *115*, 2877 – 2881; b) N. Kojima, B. A. Fenderson, M. R. Stroud, R. I. Goldberg, R. Habermann, T. Toyokuni, S. Hakomori, *Glycoconjugate J.* **1994**, *11*, 238 – 248.

[20] R. P. Junghans, *Immunology Today* **1999**, *20*, 401 – 406.

[21] Average glycosidic torsions are $\phi = 40°$ ($\phi = H^{1\prime}\text{-}C^{1\prime}\text{-}O^{3}\text{-}C^{3}$) and $\psi = 30°$ ($\psi = C^{1\prime}\text{-}O^{3}\text{-}C^{3}\text{-}H^{3}$) for fucose and $\phi = 47°$ ($\phi = H^{1\prime}\text{-}C^{1\prime}\text{-}O^{4}\text{-}C^{4}$) and $\psi = 17°$ ($\psi = C^{1\prime}\text{-}O^{4}\text{-}C^{4}\text{-}H^{4}$) for galactose. The Le^X trisaccharide moiety is found in a well-defined solution conformation without populating secondary minima. A. Geyer, G. Hummel, T. Eisele, S. Reinhardt, R. R. Schmidt, *Chem. Eur. J.* **1996**, *2*, 981 – 988.

Solid-Phase Synthesis of Doubly Labeled Peptide Nucleic Acids as Probes for the Real-Time Detection of Hybridization**

Oliver Seitz*

The mutual recognition of two complementary nucleic acid strands is the molecular basis for most of the current approaches in oligonucleotide-based diagnostics and therapy. Much work has therefore been devoted to the development of methods that allow for detection of the hybridization event. The hybridization assays that are most commonly used employ a solid phase in order to facilitate the separation of bound from unbound analytes.[1] In contrast, assays that proceed in homogeneous solution avoid problems such as nonspecific adsorption and the tedious washing protocols associated with heterogeneous assays.[2] In addition, homogeneous assays can provide a means to perform real-time measurements in vitro and even in vivo.[3] Conventional DNA-based probes, however, are susceptible to nucleolytic degradation which complicates their use in living cells. Herein, a method for real-time DNA measurements is presented that takes advantage of the enzyme-stable DNA-analogue peptide nucleic acid.[4] It is demonstrated that the application of a highly orthogonal protecting-group strategy, in combination with chemoselective conjugation reactions, allows for the rapid and automatable solid-phase synthesis of doubly labeled PNA probes suitable for homogeneous DNA detection.

Peptide nucleic acids (PNAs) bind with a remarkably high affinity and selectivity to complementary nucleic acids.[5] The structure of the resulting duplexes is similar to that of the corresponding DNA · DNA or DNA · RNA duplex molecules. However, there are striking differences between the structure of DNA and PNA single strands. For single-stranded PNA oligomers the analysis of the temperature-dependent UV absorbance reveals a sigmoidal melting curve.[6] The observed hypochromicity can reach values up to 30 %, which indicates that, in PNA single strands, base stacking is a favourable process. We reckoned that, due to this inter- or intramolecular association, a fluorescence-donor and a fluorescence-quencher group appended to the unhybridized PNA molecule **1** would be positioned in close proximity (Figure 1).[7] In analogy to the DNA-based molecular beacons **2**, which were introduced by the pioneering work of Kramer and Tyagi, collisional quenching and the distance-dependent fluorescence resonance energy transfer (FRET) would diminish the fluorescence of the donor label.[8a] Hybridization with complementary nucleic acids should induce a structural reorgan-

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