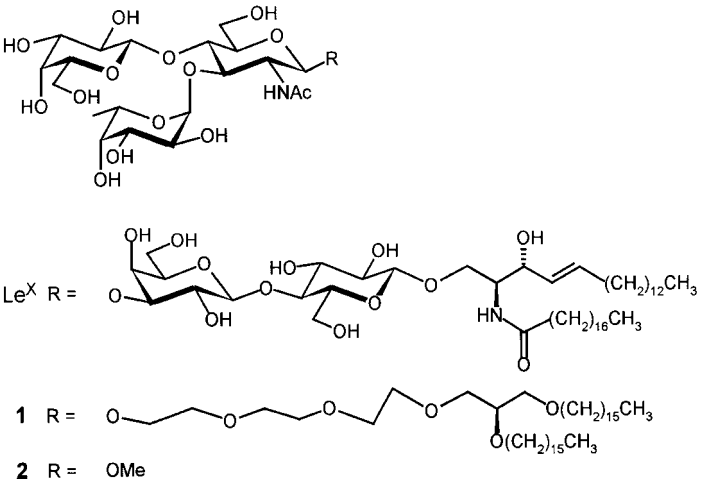
Carbohydrate – Carbohydrate Recognition between Lewis^X Glycoconjugates**

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

Dedicated to Professor Hans-Jürgen Quadbeck-Seeger on the occasion of his 60th birthday

Weak polyvalent interactions play a large part in biological systems.[1] Whereas many examples are now known of protein – protein and protein – carbohydrate interactions, hardly any studies exist of carbohydrate – carbohydrate recognition. Polyvalent, intercellular carbohydrate – carbohydrate contacts add up to forces that are relevant for cell adhesion. So far it has not been established conclusively how far the glycosphingolipid (GSL) microdomains (lipid clusters) formed on the membrane from GSLs through lateral segregation play a part.[2] Side-by-side contacts of the GSLs stabilize the microdomains, head-to-head contacts between GSLs from different membranes bring about cell adhesion.[2b] The GSL microdomains can be identified indirectly from a count of aggregated cells[3] or from the distribution of GSL antibodies on cell membranes.[4] Recently, GSL domains with diameters of 20 nm were observed directly.[5]

The aggregation of cells by means of homotypic head-to-head interactions was mainly investigated for the Lewis^X (Le^X) GSL, which bears the trisaccharide D-Galβ(1 $\rightarrow$4)[L-Fucα(1 $\rightarrow$3)]D-GlcNAcβ(1 $\rightarrow$OR) as its head group.[2–4] It was also shown that Le^X oligosaccharides which are not anchored in the membrane are able to undo these cell – cell contacts.[3] A direct NMR spectroscopic observation of Le^X – Le^X dimers is not possible as the dimers are mainly dissociated in solution owing to the weak adhesive force.[6] The transient bond of a low molecular weight ligand to a high molecular weight receptor can, however, be detected with NMR spectroscopy by means of transfer NOEs.[7] Carbohydrates exhibit high off-rates upon receptor binding, therefore they are very suitable for such measurements.[8] However, the method can be also employed for the quantification of the membrane affinity of low molecular weight compounds.[9] As the NOEs of an oligosaccharide bonded transiently to a receptor in the membrane are strongly negative, the very weak affinities of the carbohydrate – carbohydrate recognition should also be detectable. The native Le^X GSL was reduced to two model compounds which bear only the groups relevant for homotypic recognition (**1** and **2**). To avoid the physical separation of the membrane components favored by ceramide,[10] 1,2-di-*O*-hexadecyl-*sn*-glycerin serves as a flexible membrane anchor



in **1**; in addition, lactose was replaced by a triethylene glycol spacer.

Compound **1** can insert into phosphatidylcholine membranes and serves as a high molecular weight receptor with which low molecular weight **2** interacts (Figure 1). Several model membranes in the fluid Lα phase were tested. Experiments with DMPC liposomes[11] or with disk-shaped DMPC/DHPC bicells[12] (DHPC = dihexanoylphosphatidylcholine), however, remained unsuccessful.[13] At 300 K and therefore below the temperature of phase transition for the formation of bicells, the DMPC/DHPC mixture (3/1, molar) forms liposomes.[14] Similar to cholesterol in native membranes, the short-chain membrane component DHPC increases the fluidity of the membrane. The surface curvature of the liposomes (diameter approximately 20 nm) and the membrane fluidity suppress side-to-side contacts of the GSLs and thus the formation of insoluble glycolipid aggregates. Therefore the addition of 6 mg of **1** to a solution of DMPC/DHPC liposomes (3/1, 300 K, 50 mg in 0.5 mL D_2O, 10 mM $CaCl_2$) does not lead to the formation of precipitate. Compound **1** inserts in the model membrane, and spin diffusion is observed for the positive intramolecular cross-signals in the NOESY spectrum[15] (negative NOE). In the ^{1}H NMR spectrum **2** is equivalent in chemical shift to **1**; however, owing to its small relative molecular mass, **2** shows negative cross-signals in the NOESY spectrum (positive NOE). During the stepwise

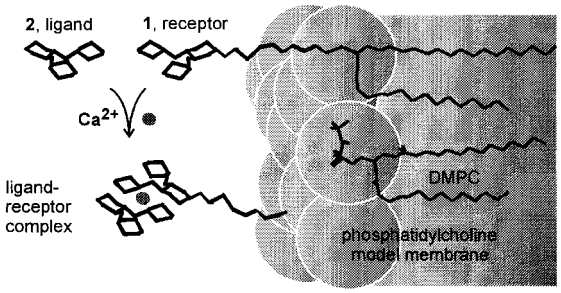


Figure 1. The low molecular weight ligand **2** coordinates transiently to the membrane-bound **1**, which functions as a receptor. The change in average rotational diffusion of trisaccharides with identical chemical shifts is quantified by NMR spectroscopy from NOESY spectra. DMPC = dimyristoylphosphatidylcholine.

[*] Dr. A. Geyer, Dipl.-Chem. C. Gege, Prof. Dr. R. R. Schmidt
Fakultät für Chemie M716 der Universität
D-78457 Konstanz (Germany)
Fax: (+49) 7531-88-3898
E-mail: armin.geyer@uni-konstanz.de

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addition of **2** (3.6 – 36 mM) to a solution of **1** in DMPC/DHPC liposomes, the NOESY cross-signals of the two shift-equivalent trisaccharides add up. As a consequence of the transient binding of **2** to **1**, the measured intensities of the cross-signals, which directly reflect the average molecular mobility of **1** and **2**, are distinctly more positive than the sum of the cross-signals of separate solutions of **1** and **2** in DMPC/DHPC liposomes. From the difference, the slowing down of the mean rotational diffusion of **2** can be quantified.[16] The very weak adhesive forces between **1** and **2** can only be detected because of the strong dependency of the NOE on the molecular mobility. Several intensive intramolecular NOEs served as experimental values. The calcium dependence found in cell assays was also observed, as the addition of EDTA reduces the NOE intensity to the value expected in the absence of Le^X-Le^X contacts. Clouding or even formation of precipitate did not occur. The affinity constant between the Le^X trisaccharides was found to be $2-3\ M^{-1}$ if one assumes the concentration of **1** to be equal to the receptor concentration.

The Le^X-Le^X dimers can only be thermodynamically stabilized in water by means of hydrophobic contacts between pyranose rings and concomitant reorganization of the solvating envelope.[17] Previous models[18] assume either complete desolvatization of the calcium ion or a coordination number below seven—both are very improbable for the weak bond of a neutral trisaccharide ligand to calcium ions.[6, 19] The contact of the two Le^X trisaccharides can be stabilized by complementary hydrophobic surfaces. Thus, the rigid solution conformation of the Le^X trisaccharide[20] allows an antiparallel dimerization with a hydrophobic contact between the α face of galactose and β face of *N*-acetyl glucosamine. The calcium ion can coordinate transiently twice to three oxygen atoms (Fuc-O2, Fuc-O3, Gal-O6) in the two Le^X trisaccharides and bridge them; the remaining coordination sites of the calcium ion can be saturated by water. A similar bridging, C_2-symmetric coordination is known for calcium from a crystal structure analysis.[21]

The experimental strategy introduced here reduces a complex biological recognition to a minimalized ligand – receptor system that allows the observation of the calcium-dependent head-to-head dimerization between Le^X glycoconjugates. This is only one example for the homotypic recognition between glycoconjugates. By variation of the dissolved component or of that anchored in the membrane, the affinities between other glycoconjugates are also quantifiable under standardizable experimental conditions.

Experimental Section

The glycoconjugates **1** and **2** were obtained by the reaction of the Le^X trisaccharide imidate[22] with the triethylene glycol spacer[23] or with methanol and subsequent deprotection (see ref. [24]).

The mixture of DMPC and DHPC (3/1, molar) was prepared once and dissolved in portions of 50 mg in 0.5 mL D_2O for the experiments. The triethylene glycol membrane anchor in **1** showed no affinity towards calcium ions. This was examined for sialyl-Lewis^X which was joined to model membranes (sodium lauryl sulfate micelles, dodecylphosphocholine micelles and DMPC/DHPC bicells) with spacers of different lengths.[25] Furthermore, in the concentration range between 5 and 15 mM no lipid clusters are formed from side-to-side contacts of **1**. This was established by independent titration experiments in which the systematic variation of the concentration of **1** or $CaCl_2$ did not lead to a change of the line shape in the ^1H NMR spectrum of **1**.

All NMR experiments were carried with a Bruker DRX-600 spectrometer. The 2D spectra were recorded in the phase-sensitive mode with TPPI (time-proportional phase incrementation). The NOESY data matrices contained 2 K items in $f2$ and 256 items in $f1$. For processing, the matrix elements of the indirect dimension were filled with zeros and an apodization was carried out with a square sinus function shifted by $\pi/2$ in both dimensions.

The calculation of the affinity constant for the carbohydrate-carbohydrate interaction ($K_a = [\mathbf{1}\cdot\mathbf{2}]/[\mathbf{1}][\mathbf{2}]$, where $[\mathbf{1}\cdot\mathbf{2}]$ is the concentration of the calcium-bridged Le^X-Le^X complex) was made under the assumption, which was justified at this degree of dilution, that the complex is mainly present in its dissociated form: $[\mathbf{2}] = [\mathbf{2}]_{total}$. Calcium ions were present in surplus, and the ratio $[\mathbf{1}\cdot\mathbf{2}]/[\mathbf{2}]$ was calculated from the ratio of cross- to diagonal signal in the NOESY spectrum (cf. ref. [16]).

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[11] Stable, unilamellar vesicles can be generated directly from DMPC in water. The two sets of signals (2:1) in the ^1H NMR spectrum allow an estimation of the average liposome size of approximately 20 nm (L. Calzolai, E. Gaggelli, A. Maccotta, G. Valensin, *J. Magn. Reson. B* **1996**, *112*, 228 – 235).
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[13] Compound **1** inserts into the bilayers of the DMPC liposomes with its alkyl chains, and within a few hours considerable formation of precipitate is observed, which can be put down to the formation of stable GSL domains. Upon addition of calcium ions an insoluble precipitate forms immediately. The amount of the deposit is much larger than for DMPC liposomes without **1**. In the case of the DMPC/DHPC bicells (310 K), an anisotropic molecular rotation is observed for **2** that is independent of the concentrations of **1** and the calcium ions in the examined concentration range. The dipolar couplings are visible in the DQF-COSY spectrum as additional cross-signals (see also P. J. Bolon, J. H. Prestegard, *J. Am. Chem. Soc.* **1998**, *120*, 9366 – 9367). The ^1H NMR resonance signals of **1** on the surface of the bicells are broadened considerably.
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Chelated Enolates of Amino Acid Esters—Efficient Nucleophiles in Palladium-Catalyzed Allylic Substitutions**

Uli Kazmaier* and Franz L. Zumpe

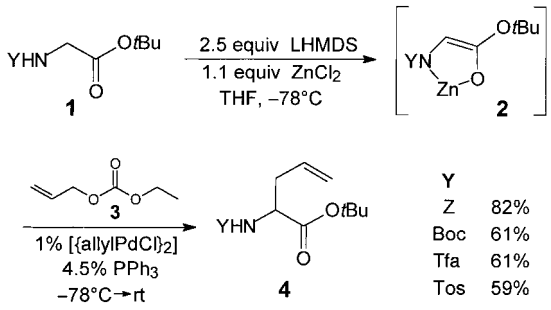
Dedicated to Professor Ulrich Schmidt on the occasion of his 75th birthday

Because of the very mild reaction conditions required, transition metal catalyzed reactions are becoming more and more popular in organic synthesis.[1] As many of these reactions tolerate a variety of functional groups, they are especially suited for the synthesis of complex molecules (natural products, etc.). Palladium, which can be used for cyclizations, cross-coupling reactions, or allylic substitutions,[2] holds a predominant position among the transition metals. Especially allylic substitutions are of particular interest, because asymmetric variations of these reactions are possible.[3] In addition to heteronucleophiles mainly symmetrical stabilized carbanions like malonates are used as C-nucleophiles. It is very advantageous that during the C–C bond forming step only one stereogenic center is generated in the allyl moiety. Thus, its configuration can be controlled quite easily.

With the use of unsymmetrical C-nucleophiles like β-keto esters[4] or imines of amino acid esters,[5] mixtures of the diastereomeres are usually obtained. This is due to the configurational lability of the allylated nucleophiles. Thus, considerably better results are obtained with alkylated derivatives.[6] Trost et al. recently reported the use of substituted azlactones as nucleophiles, giving rise to α-alkylated γ,δ-unsaturated amino acids in a highly stereoselective fashion.[7]

In contrast to the extremely well investigated reactions of stabilized "soft" carbanions, there are only a few reports concerning unstabilized enolates, for example those of ketones or esters, although the resulting products are often more interesting. The reactions of these enolates seem to be limited to only a few substrates. In the case of enolates of ketones, the best results are obtained with tin[8] and boron enolates.[9] These enolates attack the terminal positions of the allyl moiety *trans* to the palladium atom. In contrast, lithium enolates of esters preferentially attack the central position of the allyl moiety,[10] giving rise to cyclopropane derivatives.[11] The main problems with the conversion of these unstabilized enolates are probably due to coordination to the palladium atom, what might lead to inactive complexes.

This prompted us to investigate chelated enolates of amino acid esters **2** (Scheme 1) as nucleophiles in palladium-catalyzed allylic substitutions. Chelation causes a marked



Scheme 1. Synthesis of the unsaturated amino acid ester **4**. LHMDS = lithium bis(trimethylsilyl)amide, Z = benzyloxycarbonyl, Boc = *tert*-butoxycarbonyl, Tfa = trifluoroacetyl, Tos = toluene-4-sulfonyl.

enhancement of thermal stability without having any negative influence on the reactivity of these enolates. Due to the fixed geometry of the enolates, their conversions often proceed with a high degree of stereoselectivity. Therefore, we are investigating conversions of these enolates providing non-natural amino acids. We are especially interested in reactions which cannot be carried out with "normal" nonchelated enolates. Thus, chelated enolates of amino acid esters undergo

[*] Priv.-Doz. Dr. U. Kazmaier, F. L. Zumpe
Organisch-Chemisches Institut der Universität
Im Neuenheimer Feld 270, D-69120 Heidelberg (Germany)
Fax: (+49) 6221 56-4205
E-mail: ck1@popix.urz.uni-heidelberg.de

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