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Cite this: *Org. Biomol. Chem.*, 2012, **10**, 6211

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PAPER

Chemoselective Staudinger-phosphite reaction of symmetrical glycosyl-phosphites with azido-peptides and polygycerols†‡

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Received 27th January 2012, Accepted 18th May 2012
DOI: 10.1039/c2ob25207d

In this paper we present the synthesis of glyco-phosphoramidate conjugates as easily accessible analogs of glyco-phosphorous esters *via* the Staudinger-phosphite reaction. This protocol takes advantage of synthetically accessible symmetrical carbohydrate phosphites in good overall yields, in which ethylene or propylene linkers can be introduced between phosphorous and galactose or lactose moieties. The phosphites were finally applied for the chemoselective reaction with azido-peptides and polyazido(poly)glycerols.

The covalent attachment of single or oligomeric carbohydrates represents a functional natural modification for proteins and (phospho-)lipids, responsible for the regulation of molecular recognition, immune response, pathogen interaction and intracellular transport.[1] In addition to the commonly occurring linkages of Asn (N-linked) or Ser/Thr amino acids (O-linked glycosylation) to the anomeric center of saccharides, glycans can also be linked to phosphoresters, which often play a distinct role as virulence factors in pathogens (*e.g.* leishmania), several bacteria and yeasts.[2] Additionally, glyco-phosphordiesters are components of capsular polysaccharides (CPS) of Gram negative and Gram positive bacteria, in which mono- or oligosaccharides can be connected *via* interglycosidic phosphodiesters (*e.g.* in *Neisseria meningitidis* type A or *Haemophilus influenzae* type C), *via* glycerol phosphates (in *N. meningitidis* type Z) or *via* alditol phosphates (in *H. influenzae* type A).[3]

Over the last years, several protocols have been developed for the synthesis of homogeneously glycosylated peptides, since their isolation from natural sources proves to be very difficult. These strategies aim to obtain either glycopeptides containing natural or unnatural linkages, while the latter are often easier to synthesise and could furthermore lead to higher biological activity and greater stability against enzymatic cleavage.[2,4] It is important to note that in particular glycosyl-phosphite precursors have found considerable attention as key intermediates in the synthesis of glyco-phosphates and glycoconjugates.[5]

Recently, our group has contributed to these methodologies by using the Staudinger-phosphite reaction[6] between azides and phosphites to deliver phosphoramidate-linked glycoconjugates.[4c,7] Specifically, α- or β-linked azido-monosaccharides were reacted with protected Ser-phosphitylated peptides on solid support, which yielded glyco-phosphoramidates under very good retention of the anomeric linkage and in good overall isolated yields as easily accessible N-analogs of glyco-phosphorester-linked glycopeptides, which proved to be stable under physiological conditions (Scheme 1A).[8]

In the current paper, we intended to broaden the applicability of the Staudinger-phosphite reaction to the synthesis of phosphorester-linked glycoconjugates by employing easily accessible azido-peptides and polymers as core substrates for the reaction with symmetrical glycosyl-phosphite derivatives (Scheme 1B). This reversion in the functionalities of the starting materials features several potential advantages: Due to the ease of azide-incorporation in many functional (bio-)polymeric core structures by synthetic[9] as well as biochemical[10] protocols, a chemoselective glycan-conjugation by the Staudinger-phosphite reaction would become possible. Thereby, additional acidic protecting group manipulations, which are necessary for the protection of other nucleophiles present in the synthesis of phosphite-containing core structures, could be avoided. Second, the use of symmetrical phosphites results in the attachment of two glycans to the core, which points towards the generation of multivalent scaffolds for recognition studies in a straightforward manner.

Consequently, we report our recent studies for the synthesis of symmetrical mono- and disaccharide-phosphites (galactose and

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†This article is part of the *Organic & Biomolecular Chemistry* 10th Anniversary issue.

‡Electronic supplementary information (ESI) available: Synthetic protocols for the synthesis of glycosyl-phosphites, Staudinger-phosphite reactions with peptides and PG, SPR measurements, characterization of the synthesised compounds. See DOI: 10.1039/c2ob25207d

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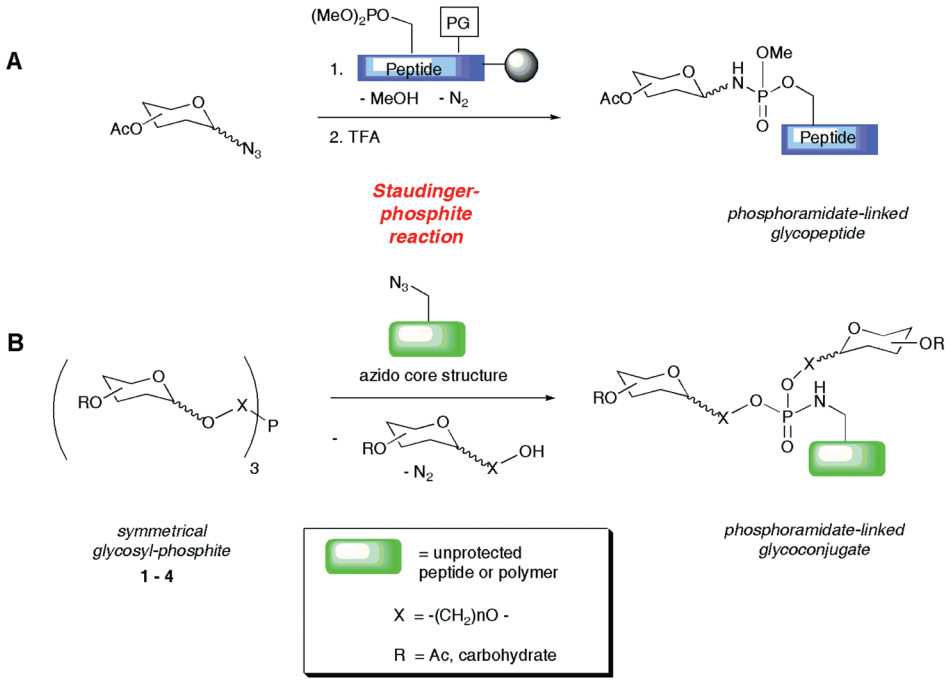
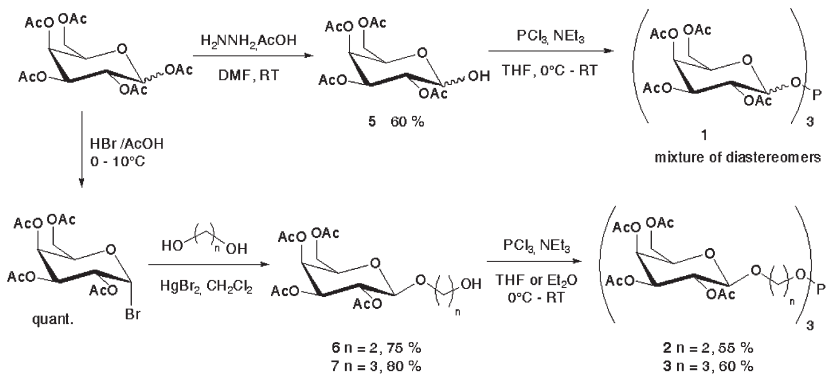


Scheme 1 Staudinger-phosphite reaction for the synthesis of glycoconjugates. (A) Reaction of glycosylazides with protected phosphitylated peptides;[8] (B) Reaction of symmetrical glycosyl-phosphites with azido core structures.



Scheme 2 Synthesis of symmetrical galactose phosphites **1**–**3**.

lactose) with different linker lengths, their Staudinger-phosphite reactions with scaffolds like polyazido(poly)glycerols or unprotected azido-peptides as well as the elucidation of the carbohydrate-functionalized polymers in first lectin-binding interactions.

Results and discussion

Synthesis of the symmetrical glycosyl phosphites

At the outset of our studies, we focused on the development of a synthetic protocol for symmetrical glycosyl-phosphites, allowing the linkage between the carbohydrate and the phosphorous to be varied. Therefore, in addition to the direct connection of the anomeric position to the phosphorous in galactose **1**, ethylene and propylene spacers were probed in galactoses **2** and **3** (Scheme 2).

For the synthesis of glycosyl-phosphite **1** peracetylated galactose was selectively deprotected at the anomeric glycosidic position to **5** with hydrazinium acetate at room temperature.[11] Phosphite **1** was obtained *via* reaction with stoichiometric amounts of PCl_3 and triethyl amine at 0 °C in THF as a inseparable mixture of α,β-anomers. Separation of the diastereomers failed due to low stability of the P(III)-compound. The identity of the crude phosphite **1** was confirmed by ^{31}P-NMR, IR and HRMS and it was directly used without further purification in the reaction with polymeric azides later (see ESI‡ for details).

In order to achieve glycosyl-phosphites **2** and **3** with a defined stereochemistry at the anomeric position, peracetylated galactose was converted into the anomeric bromide by treatment with HBr–AcOH and subsequently glycosylated with ethylenediol or propylenediol under mercury bromide catalysis delivering β-linked glycans **6** and **7** in 60% and in 75% yield.[12]

After isolation alcohols **6** and **7** were dried under vacuum and treated with freshly distilled PCl_3 and Et_3N in anhydrous THF or Et_2O at 0 °C. The reaction was monitored both by ^{31}P-NMR and by TLC. Finally, the galactosyl-phosphites **2** and **3** were obtained in good yields of 55% and 60% (overall yield 41% and 48%). In contrast to phosphite **1**, compounds **2** and **3** were stable during purification by column chromatography (see ESI‡ for details). For the synthesis of **2** and **3**, other electrophilic P(III) sources like $P(N^iPr_2)_3$ were also tested; however, the instability of the phosphites at elevated temperature, which is required for the phosphite synthesis with $P(N^iPr_2)_3$ presented itself as a limiting factor in this reaction. The pure phosphite reagents **2** and **3** could be handled on air and stored in the freezer for at least eight weeks without any signs of decomposition, while showing sensitivity in solution especially under elevated temperature or in solvents containing traces of acid (*e.g.* chloroform).

Reaction of glycosyl phosphites with peptides

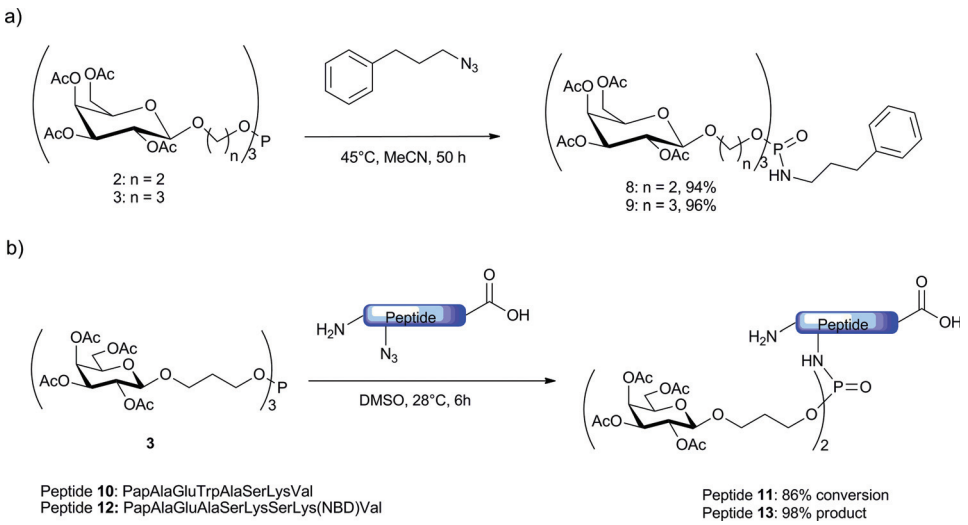
The reactivity of the phosphites was first probed with small organic azides. Thus, phosphites **2** and **3** were reacted with (3-azidopropyl)benzene at 45 °C for 50 h. After deprotection, the corresponding phosphoramidates **8** and **9** were obtained in 94% and 96% isolated yield (Scheme 3a). For later applications on the modification of azido-containing peptides, the reactivity of amino acids bearing alkyl or aryl azides in the side chain was then tested. Fmoc-protected *para*-azido-Phe was reacted with the phosphite **3** in DMSO. To our delight the conversion to the dibranched phosphoramidate was complete after 6 h at 28 °C as monitored by HPLC-MS (see Scheme S1 in ESI‡). In contrast, the reaction of **3** with the aliphatic Fmoc-protected ε-azido-Lys demanded elevated temperature (40 °C) and longer reaction times (24 h) for full conversion.

In addition to the reaction with single azido-containing amino acids, we probed the Staudinger-phosphite reaction of glycosyl-phosphites with unprotected azido-peptides. First, we investigated the conversion of the aryl-azido-containing peptide **10** to the glyco-phosphoramidate (Scheme 3b). Peptide **10** was obtained by standard SPPS, coupling *para*-azido-Phe as the last amino acid.[13] This experiment already revealed a very good conversion of the azido-peptide **10** to the peptidic phosphoramidate **11** of 86% in DMSO at room temperature, as determined by HPLC-MS with a deuterium-labeled peptide as internal standard (see ESI‡).[14] In order to further demonstrate the chemoselectivity of the Staudinger-phosphite reaction[6,13] another fluorescence-labeled peptide **12** with an NBD-group attached to the ε-amino group of Lys prepared, in which the NBD-Lys was introduced as Fmoc-protected building block. As illustrated in Fig. 1, the reaction resulted in a clean product formation and a quantitative transformation of the azide peptide **10** to the phosphoramidate **13** without any side product formation.

Staudinger-phosphite reaction of multifunctional dendritic azides

After the successful transformation of azido-amino acids and peptides, we next turned our attention to the reaction of azido-polymers with glycosyl-phosphites. The bisfunctionality of the resulting phosphoramidate products renders the reaction very attractive for the generation of polymeric scaffolds for multivalency binding studies. We therefore probed the applicability of the Staudinger-phosphite reaction on multifunctional dendritic core structures, in which the latter have been demonstrated previously as an excellent core structure for the presentation of multivalent ligands.[15]

Initially, we tested the reaction of a small phosphite with polyglycerol (PG) azide (10 kDa, degree of functionalisation (DF) = 20%, 2.7 mmol N_3-groups per g) that was obtained from multifunctional dendritic glycerols,[16] in which the terminal hydroxyl groups were transformed into azides by mesylation followed by nucleophilic substitution with sodium azide. Reaction of this polymer with $P(OMe)_3$ at 40 °C in wet DMF resulted in a quantitative conversion to the phosphoramidate as detected by NMR and IR (data not shown).



Scheme 3 (a) Synthesis of glycosyl phosphoramidates **8** and **9** *via* Staudinger-phosphite reaction of **2** and **3**; (b) Reaction of glycosyl-phosphite **3** with azido-peptides. Pap = *p*-azidophenylalanine, NBD = 4-amino-7-nitrobenzo-2-oxa-1,3-diazole.

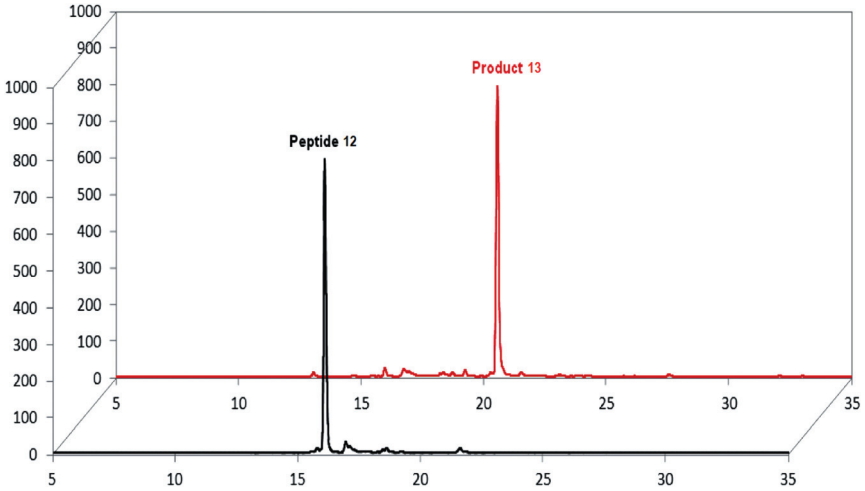
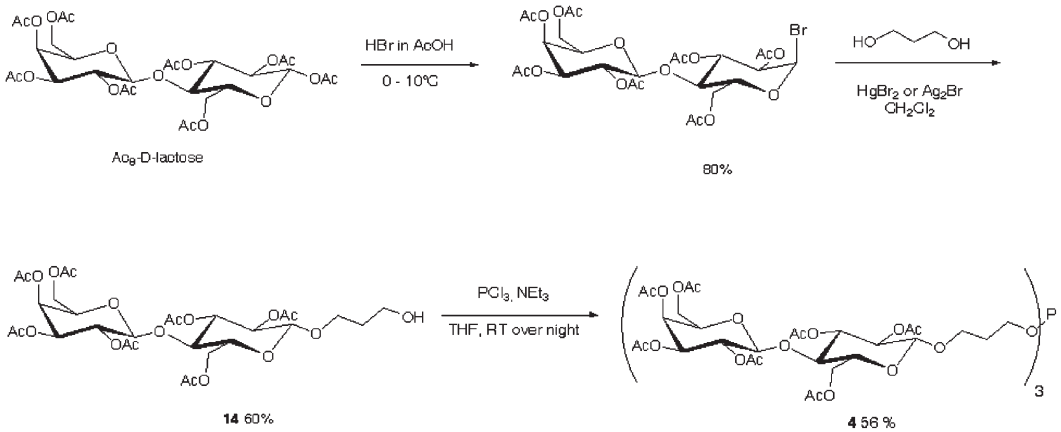


Fig. 1 HPLC-fluorescence traces of the reaction of **3** with peptide **12** (see also Scheme 3b).



Scheme 4 Synthesis of lactose phosphite **4**.

After this successful transformation, we synthesised lactose-phosphite **4** as another symmetrical carbohydrate for lectin–peanut agglutinin (PNA) binding studies, since lactose-conjugates were previously shown to exhibit significantly better binding than the corresponding galactose derivatives.[17] The lactose-phosphite **4** could be obtained in 27% yield over two steps, whereas longer reaction times for the introduction of the propane linker were needed in comparison to the synthesis of galactose-phosphite **3** (Scheme 4). About 10% of the alcohol **14** was still present in the purified phosphite **4**, which however did not disturb the later Staudinger reaction. Replacing $HgBr_2$ with Ag_2CO_3 in the glycosylation reaction led to a similar outcome of the reaction.[12]

Staudinger-phosphite reactions of phosphites **1**–**4** with two different PG azides **A** and **B** (**A**: core 7.7 kDa, DF 100%, 13.5 mmol N_3-groups per g, and **B**: core 12.6 kDa, DF 98%, 13.2 mmol N_3-groups per g) were performed in wet DMSO at 40 °C for 48 h, while adding the phosphites in two portions (Fig. 2). The polymers were purified *via* dialysis and the loading was determined by ^{1}H-NMR. The conversions as determined by NMR ranked from 23–91% with respect to the azido groups. The conversion rates were mainly dependant on the linker and the carbohydrate used (Fig. 2 and 3). Since the addition of fresh phosphites did not result in higher loadings, we assumed sterical reasons for the different observed conversions, resulting in a dense carbohydrate shell surrounding the polydendritic core. The carbohydrate moieties were deprotected with NaOMe–MeOH to yield approximately 2 mg of each desired compound. The deprotection was quantitative according to ^{1}H-NMR and IR. Since the glyco-polymers presented insufficient water solubility for reliable lectin binding studies, two polyglycerols C and D with a lower azide-substitution (**C**: core 7.7 kDa, DF = 30%, 4.05 mmol N_3-groups per g and **D**: core 10.6 kDa, DF = 32%, 4.32 mmol N_3-groups per g) containing free hydroxy groups as solubility mediators were prepared and submitted to the Staudinger phosphite reaction with lactose phosphite **4**. Although the azide conversion was still incomplete, the deprotected products possessed sufficient water solubility to be suitable for binding studies, as we could demonstrate in a first test lectin binding assay. In this study, the inhibitory effect of the glycan-conjugated PGs on PNA binding to the immobilized Thomsen–Friedenreich (TF) antigen was probed *via* a competitive surface plasmon resonance (SPR) binding assay and we obtained inhibition values of about 60% with both polymers **4C** and **4D** (Fig. 3).

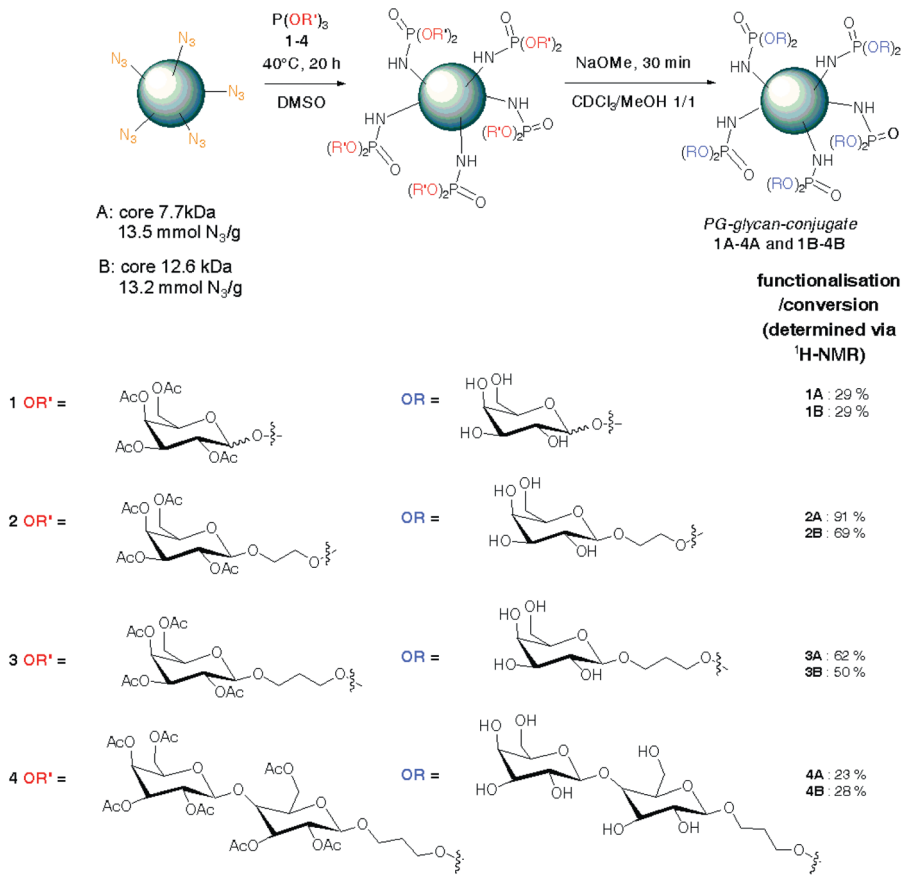


Fig. 2 Functionalisation of polyazido(poly)glycerols with phosphites **1**–**4**. Reaction rates are given for the conversion of azides (for additional information see the ESI‡).

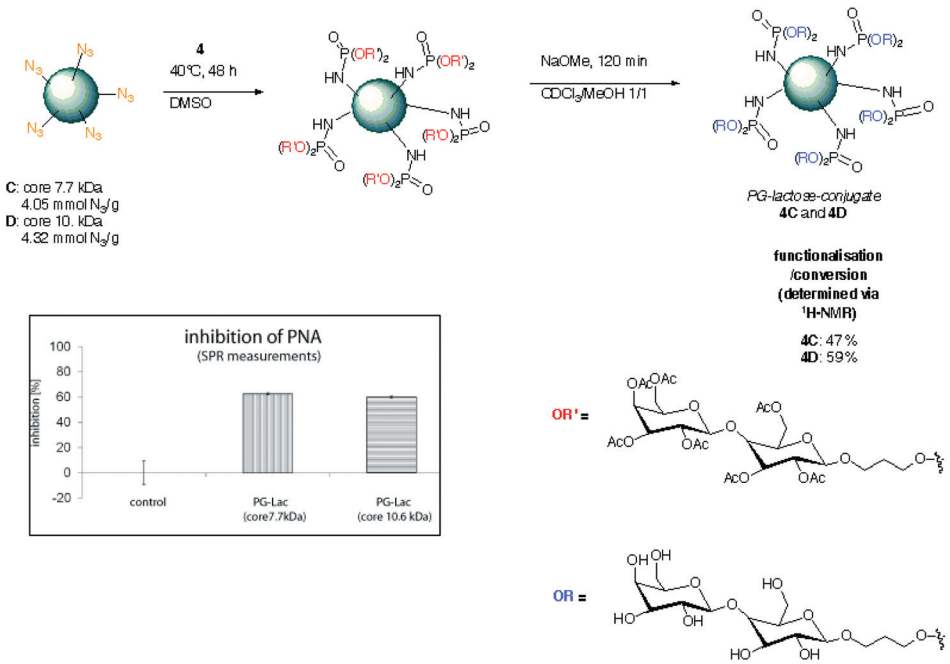


Fig. 3 Binding studies of polylactose(poly)glycerols **4C** and **4D**. Reaction rates are given for the conversion of azides (for additional information see the ESI‡).

Summary and conclusion

Taken together we present here the synthesis of different symmetrical glycosyl-phosphites with three carbohydrate side chains. These phosphite building blocks were applied in chemoselective Staudinger-phosphite reactions with a variety of substrates, including peptides and polyglycerol-azides. Generally, the reactions are easy to conduct and proceeded in high conversions. The carbohydrate phosphites **2–4** present themselves as easy to use reagents for the introduction of carbohydrate residues on biomolecules or on polymers as tools for delivery and recognition studies. Purification after the short ligation/deprotection protocol can simply be done *via* dialysis in water. Consequently, we believe that the reagents will be particularly useful for researchers in the fields of modern organic chemistry, material science and chemical biology due to the simplicity of application to complex structures.

An advantage of our methodology towards other reactions used in biochemistry labs such as copper catalysed click chemistry is, besides the avoiding of transition metals, the introduction of two carbohydrate residues as analogs of glyco-phosphodiesters. Given the CPS-Structure of many pathogens and the multivalent recognition patterns of most glycan binding molecules, this presents a distinct advantage in such studies, which will be in the focus of future investigations.

Acknowledgements

This work was funded by the Deutsche Forschungsgemeinschaft (SFB 765 – Multivalency as chemical organization and action principle), the Emmy Noether Programm (HA 4468/2-2), the Boehringer-Ingelheim Foundation (Plus 3 Programm), the Fonds der Chemischen Industrie and the COST Action CM0802, for the financial support and the Swiss National Science Foundation for the postdoctoral fellowship to TM (PBEZP2-133274).

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