VIP Azide Conjugation

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Alkyne Phosphonites for Sequential Azide–Azide Couplings**

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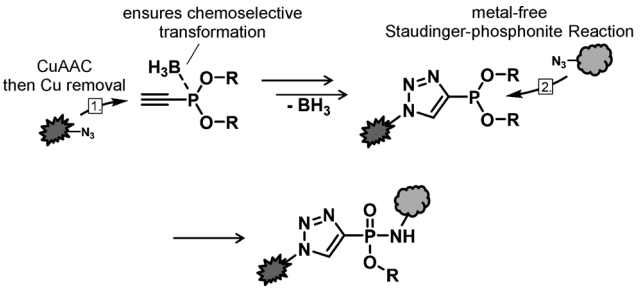
Dedicated to the Bayer company on the occasion of its 150th anniversary

Modern research often demands the modular conjugation of molecular systems with functional modules including fluorophores, purification tags, solubility enhancers, isotopic labels, or biologically active ligands, which enable their functional analysis, tracking in complex environments, and aids in several medicinal and pharmaceutical applications. Such molecular systems span several orders of size and complexity and range from small molecules and natural products to polymers, particles, or surfaces in material research and to even whole living organisms in the life sciences.[1] A common way to achieve the functionalization of these systems relies on the incorporation of highly reactive functional groups, which can be easily introduced either by synthetic or biochemical methods. Ideally, these groups are chemically inert to other given functionalities,[2] while still displaying a high level of intrinsic reactivity. Furthermore, these functional groups should be able to form reliably new chemical bonds in high yields as defined for click reactions[3] and with excellent chemoselectivity.[4]

Among many reactive functional groups that meet these criteria, azides have certainly been the most popular, as they can be reacted either with alkynes to form triazoles by 1,3-dipolar cycloaddition[5] or with P^{III} reagents by Staudinger reactions. The development of copper-catalyzed (CuAAC)[6] and strain-promoted azide–alkyne cycloaddition[7] as well as the use of phosphines[8] and phosphites[9] in Staudinger ligations led to numerous labeling and conjugation applications.[10] Azide groups can be introduced easily into small molecules, polymers, and materials by organic synthesis.[11] Along those lines, the high tolerance of azides towards a number of other organic transformations whilst still offering a high reactivity to ensure efficient conversions leads to the ubiquitous application of CuAAC[12] and the commercial availability of many azide-containing functional building blocks.

Herein, we introduce a reagent which allows the sequential coupling of two different azido compounds in polar, unpolar, and aqueous solvents by a short reaction sequence. This approach allows the modular coupling of readily available azido-containing functional building blocks by a final metal-free conjugation step. To achieve this formal azide–azide, coupling we wanted to combine the CuAAC[6a] with the metal-free Staudinger phosphonite reaction recently advanced in our laboratory.[13] Our proposal led to the design of an alkyne directly attached to borane-protected phosphonite (Scheme 1). The borane protecting group fulfills several purposes, as it ensures that no homo-coupling occurs during



Scheme 1. Borane-protected alkyne phosphonites for the sequential coupling of two different azides.

the first CuAAC step and also protects the phosphorus during the subsequent copper removal. Furthermore, the alkyne–phosphonite moiety not only saves unnecessary spacer atoms between the conjugates, but it also leads to the formation of a sp^2 P bond after CuAAC, which is assumed to stabilize the free phosphonite against oxidation.[13]

We started our investigations with the known synthesis of the borane-protected alkyne-phosphonite **1** by the addition of alkynyl Grignard reagent to diethyl chlorophosphite (Scheme 2), followed by addition of borane.[14] Careful optimization of the reaction conditions by elongating the reaction times, slightly increasing the reaction temperature, and optimizing the final purification by flash chromatography yielded building block **1** in an excellent yield of 92%. The obtained alkyne–phosphonite **1** was then applied in CuAAC

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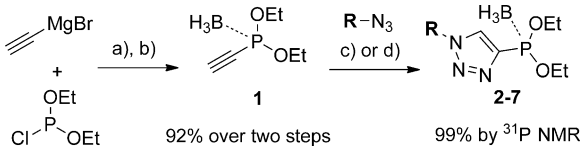
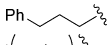
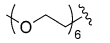
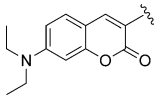
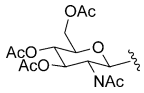
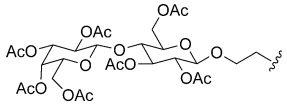
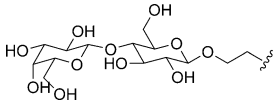


Scheme 2. Synthesis of borane-protected triazole phosphonites **2–7**. a) THF, −96 °C, 30 min, then RT, 1 h. b) BH_3, 0 °C to RT, 1.5 h. c) $Cu(CH_3CN)_4PF_6$, THF, 16 h, 40 °C. d) $CuSO_4$, THPTA, sodium ascorbate, H_2O/*t*BuOH 1:1, RT, 14 h. THPTA = tris(3-hydroxypropyltriazolylmethyl)amine.

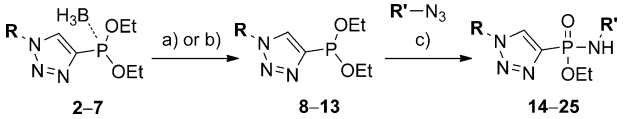
reactions with different azides, allowing the synthesis of several borane-protected triazole–phosphonites **2–7** with alkyl-, PEG-, coumarin-, and protected as well as unprotected carbohydrate substituents (Table 1). The CuAAC proceeded in all cases in quantitative conversions as determined by ^{31}P NMR under standard CuAAC conditions and in very good to excellent isolated yields (Scheme 2).[6a] The 1,3-dipolar cycloaddition was either performed in aqueous solution with copper(II) sulfate as copper source and sodium ascorbate as reducing agent or in THF with tetrakis(acetonitrile)copper(I) hexafluorophosphate, depending on the solubility of the azide. At this stage more than 99.98 % of copper was removed during chromatographic purification, which was verified for **2**, **5**, and **7** by ICP-MS. Thereby, a metal-free environment for the Staudinger phosphonite reaction is ensured, which prevents often impossible purifications of the final conjugates, especially when azido (bio)polymers are used in the second azide coupling.

Table 1: Reaction scope for functionalized borane-protected phosphonites.

Entry	Borane phosphonite	R	Yield [%]
1	**2**		93[a]
2	**3**		89[b]
3	**4**		96[a]
4	**5**		74[a]
5	**6**		77[a]
6	**7**		75[b]

[a] $[Cu(CH_3CN)_4]PF_6$, THF, 16 h, 40 °C. [b] $CuSO_4$, THPTA, sodium ascorbate, H_2O/*t*BuOH 1:1, RT, 14 h.

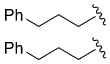
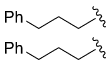
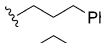
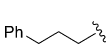
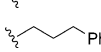
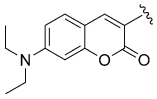
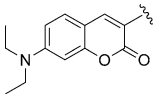
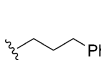
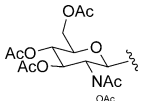
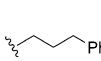
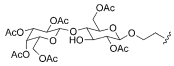
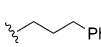
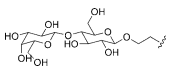
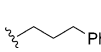
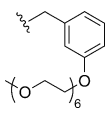
phosphonites **8–13** in over 96 % conversion as determined by ^{31}P NMR spectroscopy (see the Supporting Information). Alternatively, the deprotection can be performed in nontoxic toluene in similar conversions of over 94 % as tested for borane-protected phosphonites **2** and **3**. After changing to a more polar solvent, triazole–phosphonites **8–12** were reacted with a second azide in a Staudinger-phosphonite reaction to form the desired phosphonamidates **14–23** (Scheme 3) in very good yields from 69–94 %, in which the nature of the triazole substituent did not influence the Staudinger reaction (Table 2, entries 1–10).



Scheme 3. Synthesis of phosphonamidates **14–25**. a) DABCO, benzene, 50 °C, 20 h, > 96 %. b) DABCO, DMSO, 70 °C, 20 h, 81 %. c) MeCN, CH_2Cl_2, DMSO, RT, 10 to 24 h, then H_2O, 24 h, or water (15 % DMSO), RT, 20 h.

Owing to insufficient solubility of the borane-protected lactose phosphonite **7**, unpolar solvents could not be used for the borane deprotection. Therefore, we had to change the solvent to dimethyl sulfoxide, in which the borane depro-

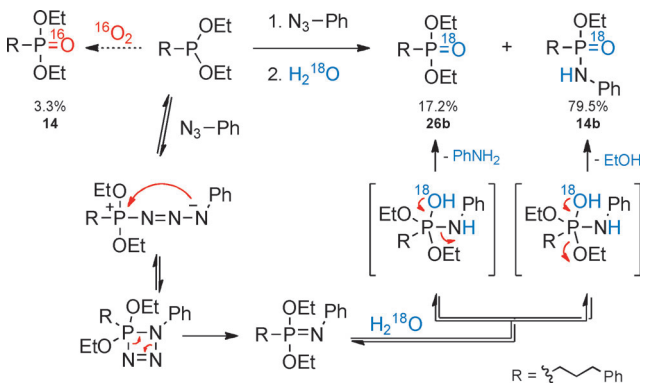
Table 2: Reaction scope for phosphonamidates (**14–25**) obtained by the Staudinger phosphonite reaction.

Entry	Phosphonite	Phosphonamidate	R	R′	Yield [%]
1	**8**	**14**			74[a]
2	**8**	**15**			88[a]
3	**8**	**16**			91[a]
4	**8**	**17**			84[a]
5	**9**	**18**			78[b]
6	**9**	**19**			83[b]
7	**10**	**20**			69[a]
8	**10**	**21**			94[a]
9	**11**	**22**			82[a]
10	**12**	**23**			80[a]
11	**13**	**24**			51[c]
12	**9**	**25**			74[d]

[a] The reaction was performed in MeCN. [b] In CH_2Cl_2. [c] In DMSO. [d] In water (15 % DMSO); 98 % conversion by HPLC-MS.

tection needed slightly higher temperature to convert the borane–phosphonite adduct completely. Nevertheless, the free phosphonite **13** could be generated in 81% conversion as determined by ^{31}P NMR and subsequently be used in Staudinger phosphonite reactions to deliver the unprotected triazole–lactose–phosphonite **24** in 51% yield after HPLC purification (Table 2, entry 11). Finally, we applied the Staudinger phosphonite reaction of azides to phosphonamidates not only in organic solvents but also under aqueous conditions by reaction of a water soluble PEG-substituted benzyl azide[15] with an excess of phosphonite **3** (Table 2, entry 12). HPLC analysis of the crude reaction revealed that the reaction proceeds in very high azide conversion of 98% without any byproduct formation. The corresponding phosphonamidate **25** was isolated in 74% yield.

Although the Staudinger phosphonite reactions proceed in very good overall yields, we were intrigued by lower yield (circa 70%) for reactions of triazole–phosphonites **8** and **10** with phenyl azide compared to about 90% yield obtained for other alkyl azides (Table 2). Analysis of these reactions leading to phosphonamidate products **14** and **20** revealed the formation of about 18% phosphonate as byproduct. This P^V compound is formally the oxidation product of the phosphonite starting material, but as it only occurs during the Staudinger reaction with phenyl azides, we assumed that an oxidation with molecular oxygen is not the reason for its formation. Alternatively, the phosphonate byproduct could be generated during the hydrolysis of the phosphonimidate if the arylamine is eliminated instead of an alkoxy group. To look further into the mechanism, we performed the hydrolysis of the phosphonimidate with ^{18}O-labeled water. NMR and mass analysis of the crude reaction mixture revealed the formation of 17.2% ^{18}O-labeled phosphonate **26b**, 3.3% unlabeled phosphonate **26a**, as well as 79.5% ^{18}O-labeled phosphonamidate **14b** (Scheme 4). This implies that 84% of the formed phosphonate **26** is formed during the phosphonimidate hydrolysis, in which aniline acts as leaving group (Scheme 4). Changing the solvent of the Staudinger phosphonite reaction from acetonitrile to more polar solvents, such as dimethyl sulfoxide or water, or less polar solvents, such as dichloromethane, did not influence the ratio of phosphonate to phosphonamidate. If alkyl or benzyl azides were used instead of phenyl azide, no amine byproduct was formed during the Staudinger phosphonite reaction. Consequently, these experiments point towards the conclusion that the phosphonate formation depends on the better leaving group propensity of the aryl amine as opposed to the alkyl amine in the phosphinimidate during hydrolysis.



Scheme 4. ^{18}O-Labeling experiments to confirm the origin of phosphonates formed during the Staudinger phosphonite reaction.

Next we wanted to probe the influence of the electronic properties of the azides on the Staudinger phosphonite reaction. Therefore we lowered the concentration of both reactants to 0.15 mM and looked at the conversion rates of phosphonite **2** after 8 h with phenyl and different alkyl azides. At this concentration and time point, phenyl azide reacted completely. In contrast, alkyl azides showed that after 42 h at room temperature still 17% benzyl, 28% primary and 58% secondary azide were present, which pointed towards both an electronic and a steric influence on the reaction rate. The benzyl and primary azides could be brought to complete conversion within further 48 h by increasing the temperature to 40 °C, whereas the secondary azides needed nine full days and stepwise heating up to finally 60 °C for complete conversion. For this study, it should be noted that under the normal concentration of 1 M all reactions were complete in less than 24 h.

Finally, we intended to apply the CuAAC–Staudinger azide–azide coupling to the metal-free glycosylation of polyglycerols, which have previously been successful as multivalent scaffolds for the presentation of carbohydrate ligands in lectin binding studies.[16] As polyglycerols contain many nucleophilic side chains that act as a chelating agent for copper ions, a copper-free functionalization method is of great interest. For this purpose we chose azido polyglycerol (azido PG; **27**) with an average molar mass of 10 kDa and 68% azide functionalization as substrate for the Staudinger phosphonite reaction. This azido polymer was reacted with the unprotected lactose triazole–phosphonite **13** to the corresponding phosphonamidate **28** (Figure 1 a). After dialysis of the crude reaction mixture the glycosylated polyglycerol **28** was obtained in 78% yield and good purity (> 94% determined by ^{1}H NMR spectroscopy). Alternatively, the acetyl protected carbohydrate **12** could be coupled analogously to azido PG **27** followed by acetyl deprotection.[17] In our case, however, this two-step method led to significantly lower yields and was not further investigated.

Surface plasmon resonance (SPR) is, among others, a widely used method to study receptor–ligand binding and screening for potential inhibitory molecules. The glycosylated polyglycerol **28** was subsequently tested by this method for its binding propensity to immobilized peanut agglutinin (PNA; Figure 1). As a positive control we included a linear commercial available Thomsen–Friedenreich (TF) antigen (Galβ1-3GalNAc, Figure 1 b) conjugated to polyacrylamide (PAA). PNA is known to bind specifically to terminal galactose residues of glycans and therefore to lactose as well.[18] To address possible nonspecific interactions with the polyglycerol core, unmodified polyglycerol azide (PG-N_3) was applied as a control for which no binding could be observed. This was in contrast to the lactose-modified polyglycerol **28**, for which strong binding to PNA in a concentration range from 0.1 to 10 μM and even higher RU values than the TF

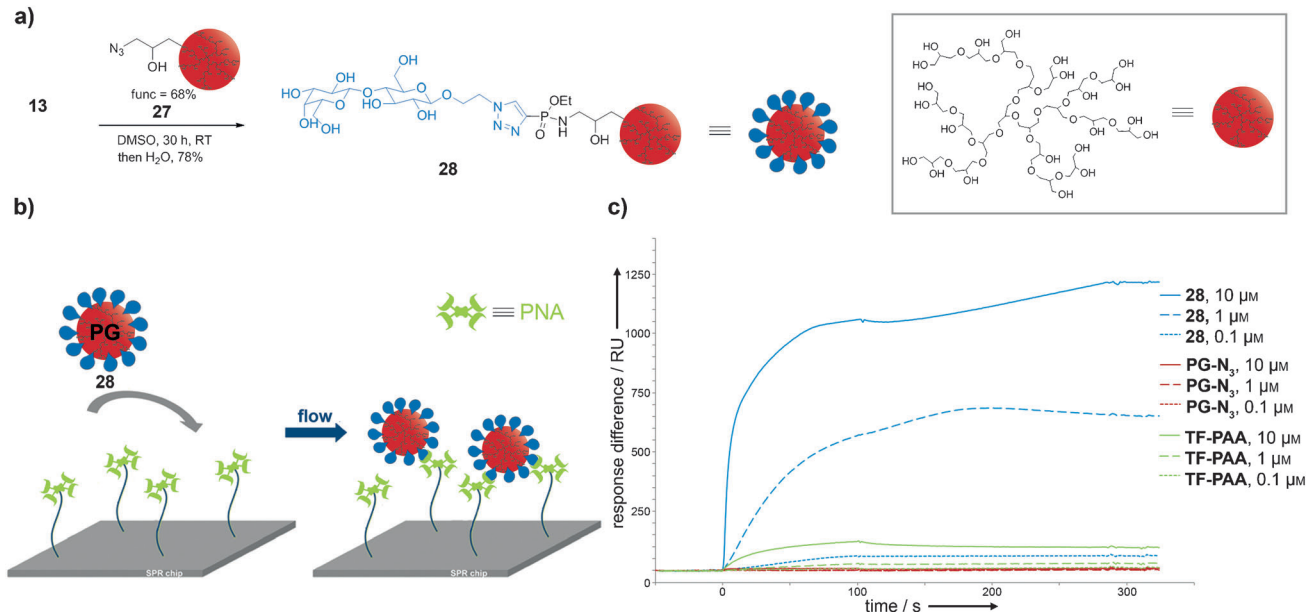


Figure 1. a) Lactose-conjugation to azido polyglycerol **27** with unprotected lactose triazole phosphonite **13**. b) Glycopolymer binding to peanut agglutinin (PNA) by surface plasmon resonance. c) Concentration-dependent binding of lactose-functionalized PG **28** (blue lines), PG-N_3 (negative control, red lines), and TF-PAA (positive control, green lines) to PNA. For more information, see the Supporting Information.

antigen were observed (Figure 1c). This result was in accordance with experiments using CuAAC-conjugated carbohydrate-PGs, in which it has been demonstrated that modified polyglycerols usually excel in multivalent binding of lectins.[19]

In conclusion, we introduce alkyne–phosphonites as versatile reagents that allow the selective step-by-step coupling of two different straightforwardly accessible azides by combining CuAAC with the metal-free Staudinger reaction. We could demonstrate that a variety of azide building blocks containing PEG, protected and unprotected carbohydrates and a coumarin fluorophore could be converted, in very good to excellent yields in organic solvents as well as in an aqueous system, to give rise to a characteristic 1,3-triazole phosphonamidate moiety. Our method offers a very broad and versatile applicability, as the functional entities are not limited to either hydrophobic or hydrophilic azido building blocks. Finally, we demonstrated the use of our azide–azide coupling in a metal-free conjugation of unprotected carbohydrates to an azido polyglycerol. This method does not require additional protecting group manipulations after the coupling and yielded carbohydrate–polymer conjugates, which showed excellent binding in a SPR-based peanut agglutinin–carbohydrate binding assay.

We strongly believe that the azide–azide coupling product has great potential for modular conjugation in organic synthesis, especially as no cytotoxic metals are used in the final conjugation step. Experiments are currently underway in our laboratory, which address the application of these building blocks in bioorganic labeling reactions.

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