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Bioconjugation

Chemoselective Bioconjugation of Triazole Phosphonites in Aqueous Media

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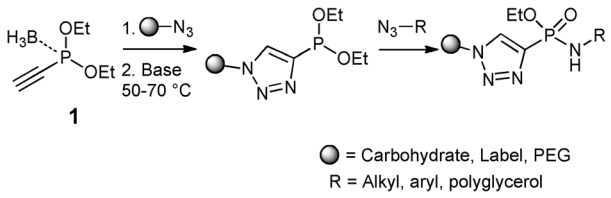
Abstract: Readily accessible and versatile phosphonite building blocks with improved stability against hydrolysis were used for the efficient metal-free functionalization of peptides and proteins in aqueous buffers at low micromolar concentrations. The application of this protocol to the immobilization of a Rasa1-SH2 domain revealed high binding affinity to the human T-cell protein ADAP and supports the applicability of triazole phosphonites for protein modifications without harming their function.

The chemoselective modification of peptides and proteins has become an extremely active field of research,[1] since modified biomolecules are very promising drug candidates as well as important tools to elucidate biological function. With many functional groups present in a natural biological environment, bioorthogonal modification at specific positions is of special interest.[1c] In recent years, many very potent reactions have been developed and applied in this area, in particular the Cu-catalyzed[2] and the strain-promoted[3] alkyne–azide cycloaddition (CuAAC and SPAAC, respectively), the inverse electron demand Diels–Alder reaction with tetrazines,[4] and Staudinger ligations,[5] as well as metal-catalyzed modification protocols such as olefin cross metathesis[6] or the Suzuki–Miyaura cross coupling reaction.[7] Although all of these reactions have become very powerful tools for the chemoselective modification of biomolecules, all of them have their limitations, too. Obstacles, among others, include slow reaction rates, the introduction of large sterically demanding scaffolds at the conjugation site, the requirement of often non-removable toxic metal catalysts, and the instability and therefore difficult accessibility of the reaction partner(s) in buffered solvents due to their high intrinsic reactivity. To address the latter issue, the identification and engineering of new chemoselective modification reactions is still of high interest to ensure high conversions and for the attachment of diverse functional modules to biopolymers.

Our group has recently introduced the Staudinger-phosphite reaction as a metal-free chemoselective modification reaction for azido-containing biomolecules.[8] While phosphites are easy to synthesize using standard phosphoramidite chemistry, it is often difficult to conjugate a single functional module to the biomolecule due to an unselective hydrolysis of the phosphorimidate.[9] To achieve a monofunctionalization of azides, we recently introduced arylphosphonites in Staudinger reactions. These reagents underwent faster reaction rates than phosphites; however, their synthesis involves several steps, which complicates the incorporation of complex functional modules.[10] Subsequently, the synthetic accessibility of phosphonites equipped with fluorophores, labels, poly(ethylene glycol) groups (PEGs), or carbohydrates was greatly enhanced with the use of borane-protected alkyne phosphonite **1** for the metal-free modification of small azido-containing molecules and azido polyglycerol in organic solvents.[11] The alkyne phosphonite **1** contains two chemical reporter units, namely the alkyne and, after its deprotection, the phosphonite moiety. In the first step, the alkyne reacts, in a CuAAC reaction, with an azide carrying the functional module (label, fluorophores). After copper removal and borane deprotection, a reactive triazole phosphonite is generated, which allows a final metal-free Staudinger reaction with a second azido molecule (Scheme 1).



Scheme 1. Sequential azide–azide coupling using alkyne phosphonite **1**.[11]

In our previous publication, we demonstrated the principle applicability of Staudinger reactions with triazole phosphonites in water. A water-soluble benzyl azide was treated at a concentration of 10 mM with 25 equivalents of diethyl triazole phosphonite in water and 15 vol% DMSO. Full conversion of the azide was monitored by HPLC-UV and the desired phosphonamidate product was isolated in 74% yield.[11] This result served as a promising starting point to further develop modular and

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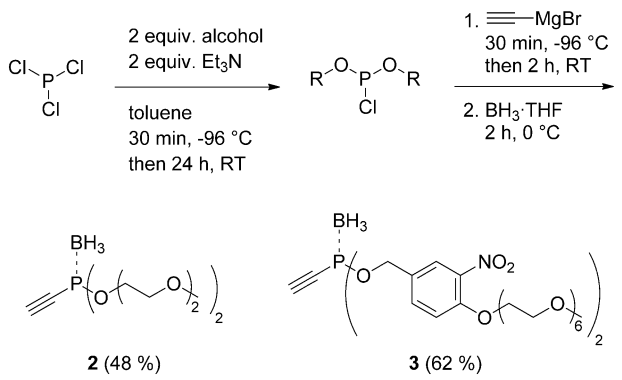
efficient phosphonite reagents for the chemoselective modification of peptides and proteins in aqueous buffer systems.

The crucial parameters to ensure high conversions for the Staudinger-phosphonite reaction in aqueous systems are water solubility[12] and stability against hydrolysis and oxidation. Although arylphosphonites display sufficient oxidation stability due to the sp^2-hybridzed C–P bond, we recently found that exchanging methoxy substituents on phosphorus with diethylene glycol monomethyl ethers led to an increase in solubility and hydrolytic stability.[10] Following this concept, we set out to synthesize the diethylene glycol chain-substituted alkyne phosphonite **2** and, in addition, an electron-poor benzyl-substituted alkyne phosphonite **3** (Scheme 2), to which a hexaethylene glycol chain was attached for increased water solubility.

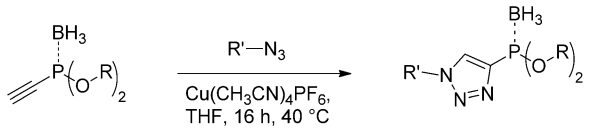


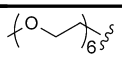
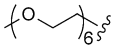
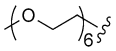
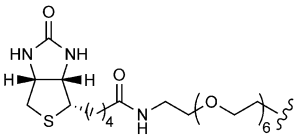
Scheme 2. Synthesis of alkyne phosphonites **2** and **3**.

We started the synthesis from phosphorus trichloride and substituted in the first step two chlorides with the corresponding alcohols in the presence of triethylamine. In the second step, an alkyne–Grignard reagent was added, followed by in situ protection of the phosphonite with borane. This one-pot protocol yielded alkyne phosphonites **2** and **3** in good yields of 48 and 62% (Scheme 2). The borane protection guarantees the stability of the phosphonite during intermediate purification by column or HPLC chromatography and additionally ensures the sequential coupling protocol as mentioned before.

Having three different borane-protected alkyne phosphonites (**1**, **2**, and **3**) in hand, the CuAAC reaction was carried out with different azido compounds. For the hydrolysis and the reactivity study, azido hexaethylene glycol monomethyl ether ($mOEG_6$) was chosen as the first azide reagent to be attached to the alkyne phosphonites **1**, **2**, and **3** for sufficient water solubility for all triazole phosphonite reagents. CuAAC was performed in tetrahydrofuran with tetrakis(acetonitrile)copper(I) hexafluorophosphate as copper(I) source (Table 1). Quantitative formation of borane-protected triazole phosphonites **4**-BH_3, **5**-BH_3, and **6**-BH_3 was confirmed by ^{31}P NMR in all cases. The crude reaction mixtures were purified by column chromatography for **4**-BH_3 or preparative high-performance liquid chromatography for the other phosphonites to yield the desired triazole phosphonite–boranes in good yields (Table 1). Additionally, a biotin-OEG_7-azide was attached to the alkyne phosphonite **3** to deliver biotinylated triazole phosphonite **7**-BH_3, for protein tagging or immobilization. The complete removal of copper was established by inductively coupled plasma mass spectrometry (ICP–MS) for triazole phosphonites **6**-BH_3 and **7**-BH_3. For the removal of the borane protecting group, borane-protected triazole phosphonites **4**-BH_3, **5**-BH_3, and **6**-BH_3 were heated in benzene or toluene to 50 °C with 1,4-diazabicyclo[2.2.2]octane (DABCO) as a base. After 24 h, ^{31}P NMR spectroscopy of the crude reaction mixture indicated full deprotection to the phosphonites **4**–**6**.

Table 1. CuAAC of alkyne phosphonites with azido compounds.



Alkyne phosphonite	R′	Triazole phosphonite–borane	Yield [%]
1		**4**-BH_3	89
2		**5**-BH_3	68
3		**6**-BH_3	71
3		**7**-BH_3	71

Next, we probed the hydrolysis rate of the three differently substituted triazole phosphonites **4**, **5**, and **6** by ^{31}P NMR spectroscopy in Tris buffer (pH 8.2) with 10 vol% DMSO (Figure 1; see the Supporting Information for experimental procedure). As an additional comparison we also measured the hydrolytic stability of the two previously published aryl phosphonites (**8** and **9**).[10] In our measurements we observed that the half-life time of the ethoxy-substituted phosphonite **4** was almost doubled by the incorporation of the *p*-$mOEG_6$-*m*-nitrobenzyl substituents in phosphonite **6**. Furthermore, the most stable phosphonite was the $mOEG_2$ substituted derivative **5**. Most importantly, all three triazole phosphonites **4**, **5** and **6** revealed a significant increase in stability against hydrolysis as compared to the previously published aryl phosphonites **8** and **9**, which hydrolyzed to over 90% within minutes.[10]

The Staudinger reaction of triazole phosphonites **4**, **5**, and **6** was subsequently tested with a model peptide **10**, containing an N-terminal *p*-azidophenylalanine as well as basic (lysine and arginine), polar (serine and glutamine), and acidic (glutamic acid) amino acid residues. Model peptide **13**, with a final concentration of 1 mM, underwent reaction with all three different triazole phosphonites (**4**, **5**, and **6**) under the previously investigated buffer conditions at pH 8.2 (Scheme 3). In accordance with their hydrolysis rate, 25 equivalents of diethyl phosphonite **4** were required to gain full conversion of the azido peptide **10**, whereas with the more stable phosphonites **5** and **6**,

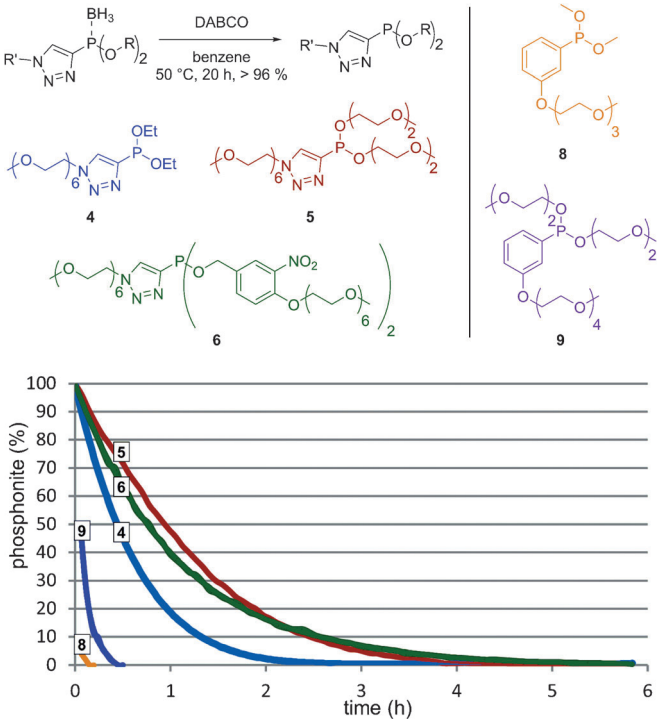
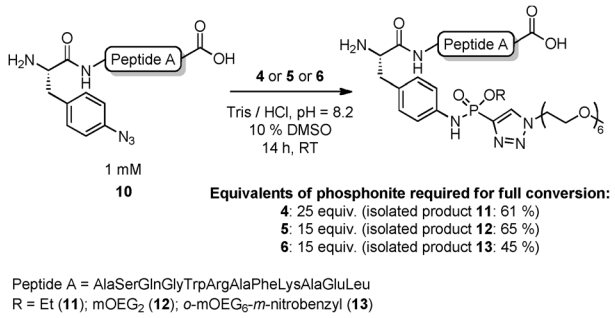


Figure 1. Hydrolysis rate of triazole phosphonites **4**, **5**, and **6** and aryl phosphonites **8** and **9** in Tris/HCl buffer (pH 8.2 w. 10 vol% DMSO), determined by ^{31}P NMR spectroscopy.



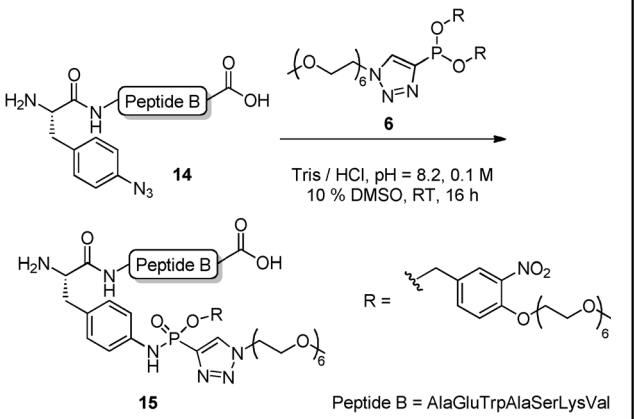
Scheme 3. Modification of azido peptide **10** with different triazole phosphonites.

only 15 equivalents were necessary to fully convert the model peptide **10**. The desired phosphonamidate peptide products **11**, **12**, and **13** could be isolated in good yields of 45–65% after HPLC purification. The only peptidic byproduct detected was the expected reduction product of the azide to the corresponding amine, which is formed during the hydrolysis of the phosphorimidate intermediate of the Staudinger reaction[11] and which was isolated in an average yield of 14±2%.

After the successful aqueous Staudinger-phosphonite reactions with peptides at concentrations of 1 mM, we turned our attention to transformations with phosphonite **6** at lower concentrations to elucidate suitable reaction conditions for protein modifications at lower concentrations. We focused on **6**, because it showed a superior performance in the previous experiment as compared to **4** and its OEG_6 chains ensure water solubility of the triazole phosphonite independently of the polarity of the molecule that is attached to it by CuAAC. To quantify the Staudinger-phosphonite reaction by mass spectrometry, the desired phosphonamidate peptide product was synthesized in acetonitrile with an azido peptide containing two D_3-alanine moieties. The deuterated phosphonamidate peptide **14**-D_6 was added to the crude reaction mixture in a known quantity, which allows quantification by ESI-HRMS. The integrals of the obtained HRMS spectra suggest that full conversion to the phosphonamidate **15** is achieved with an azido peptide concentration of 50 μM and at least 250 equivalents of di-*o*-mOEG$_6$-*m*-nitrobenzyl triazole phosphonite **6** without any side-products detected. Nevertheless, it is unlikely that no reduction of the *p*-azidophenylalanine to the corresponding *p*-aminophenylalanine occurred, as observed before. When the amount of phosphonite was decreased to 100 equivalents, the conversion dropped to a still very good 86%. Lowering the concentration of azido peptide **14** to 25 μM was accompanied by a decrease in the formation of phosphonamidate **15** (34–81%). Thus, higher concentrations of di-*o*-mOEG$_6$-*m*-nitrobenzyl triazole phosphonite **6** (500 equivalents) were necessary to reach a conversion of 85%. With a concentration of 25 μM azido peptide **4** and only 100 equivalents of di-*o*-mOEG$_6$-*m*-nitrobenzyl triazole phosphonite **6**, phosphonamidate formation was observed to be around 50% (Table 2).

To address the applicability of the Staudinger-phosphonite reaction for the modification of functional proteins, we decided to conjugate the biotin phosphonite **7** to a protein domain for a qualitative determination of protein–protein interactions. Specifically, we focused on the interaction of SH2 domains with tyrosine phosphorylated proteins as we demonstrated

Table 2. Staudinger-phosphonite reaction at low concentrations.



Azido peptide [μM]	Phosphonite **6** [equiv]	DMSO [%]	Phosphonamidate yield [%]
50	500	10	100
50	250	10	100
50	250	5	100
50	100	10	86
25	500	5	85
25	250	10	81
25	250	5	77
25	100	5	54

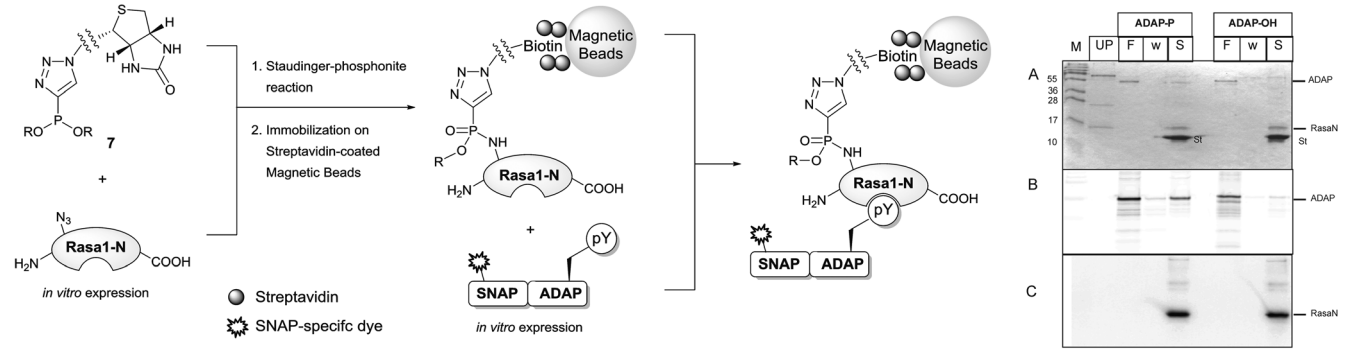


Figure 2. Pull-down of ADAP with immobilized Rasa1-N by biotin conjugation with phosphonite **7**. A) Coomassie stain of the SDS-PAGE with the pull-down samples; B) excitation of the SDS-PAGE at 633 nm to monitor ADAP fluorescence; C) Western blot of the pull-down samples, detection of biotinylation with a Streptavidin-peroxidase conjugate. Abbreviations used: UP = unbound protein (Rasa1-N) prior to pull-down; F = pull-down flow-through; W = wash fraction; S = strip fraction releasing bound ADAP, Rasa1-N and streptavidin (St).

previously that, upon tyrosine phosphorylation, the human T-cell protein ADAP binds to Rasa1 (RasGAP, Ras-GTPase activating protein) with extraordinarily high affinity at an equilibrium rate constant K_d of 100 nM.[13] The main role of Rasa1 is to inactivate the G-protein Ras p21[14] through its C-terminal RasGAP domain, while its N-terminus contains numerous modular binding domains (two SH2 domains, one SH3, one PH, one C2) that allow multiple concerted interactions.[15] The interaction to phosphorylated ADAP is mediated primarily through the Rasa1's N-terminal SH2-domain (Rasa1-N, residues 181–272).

To generate the N-terminal SH2 domain of Rasa1 (Rasa1-N) as an azido protein, we relied on the high-yielding cell-free unnatural protein synthesis[13,16] combined with an amber suppressor-based orthogonal system,[17] to deliver a *p*-azidophenylalanine residue to position 2 of Rasa1-N. The Staudinger-phosphonite reaction with biotin-phosphonite **7** for a later immobilization of Rasa1-N on Streptavidin-coated magnetic beads[18] was performed in 50 mM Tris/HCl, 150 mM NaCl, pH 8.0. Subsequent incubation and pull-down with fluorescence-labeled phosphorylated (ADAP-P) and non-phosphorylated ADAP (ADAP-OH)[13] was conducted to elucidate phosphorylation dependent binding and to probe whether the reaction conditions employed for the Staudinger-phosphonite reaction interfere with the proteins folding state and function. Fluorescence labeling of both ADAP-P and ADAP-OH was carried out by SNAP-tag fusion.[13]

Our results show that Rasa1-N could successfully be modified with phosphonite **7**, as proven by western blot (Figure 2C). Biotinylated protein could only be detected in the strip fractions of the pull-down, implying that the converted protein was completely immobilized on the solid support. Bound biotinylated and unbound non-converted Rasa1-N could be detected in comparable amounts in the respective fractions suggesting a conversion rate of about 50% (Figure 2A). Most importantly, the immobilized protein was able to selectively bind ADAP-P but not ADAP-OH (Figure 2B), showing that the folding state of the SH2 domain was intact, which supports the general utility of the Staudinger-phosphonite reaction for the modification of functional proteins.

In conclusion, we have reported the synthesis of different alkoxy-substituted alkyne and triazole phosphonites, which were successfully applied for the modification of peptides and proteins in aqueous buffers. The stability of triazole phosphonites against hydrolysis was significantly improved by using OEG or electron-deficient benzyl-substituted phosphonites. Furthermore, azido peptides were transformed at lower phosphonite concentrations to reveal the chemoselective modification of azides in aqueous systems. Upon expansion of the Staudinger-phosphonite reaction to applications on the protein level, we could show that phosphonites could successfully be used for the site-specific immobilization of an SH2 domain (Rasa1-N), in which the so-functionalized Rasa1-N binds to its partner ADAP in a phosphorylation-dependent manner. These results demonstrate the applicability of triazole phosphonites as promising chemoselective modification reagents for proteins in aqueous systems.

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Keywords: bioorthogonal · chemoselectivity · phosphonites · protein conjugation · Staudinger reaction

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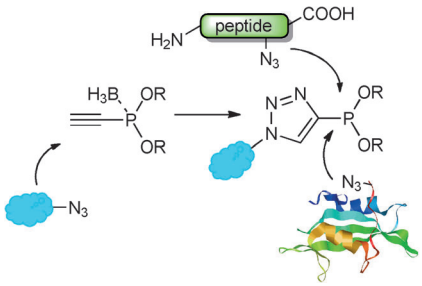
Chemoselective Bioconjugation of Triazole Phosphonites in Aqueous Media



Readily accessible and versatile phosphonite building blocks showed improved stability against hydrolysis and proved to be efficient reactants with azides in metal-free Staudinger reactions for the functionalization of peptides and proteins in aqueous buffers at low micromolar concentrations (see scheme).