ORGANIC LETTERS

2011
Vol. 13, No. 20
5440–5443

Staudinger-Phosphonite Reactions for the Chemoselective Transformation of Azido-Containing Peptides and Proteins

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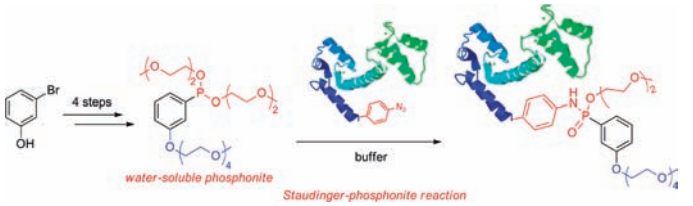
Received July 26, 2011

ABSTRACT



Site-specific functionalization of proteins by bioorthogonal modification offers a convenient pathway to create, modify, and study biologically active biopolymers. In this paper the Staudinger reaction of aryl-phosphonites for the chemoselective functionalization of azido-peptides and proteins was probed. Different water-soluble phosphonites with oligoethylene substituents were synthesized and reacted with unprotected azido-containing peptides in aqueous systems at room temperature in high conversions. Finally, the Staudinger-phosphonite reaction was successfully applied to the site-specific modification of the protein calmodulin.

The selective transformation of a particular functional group in the presence of additional chemical functionalities by chemoselective reactions is an important tool in organic synthesis[1] as well as chemical biology.[2] In addition to simplifying synthetic routes for the synthesis of natural products, these reactions allow the site-specific modification of proteins by selectively conjugating functional modules to proteins, which carry bioorthogonal reporters.[3] In combination with the many advances for the introduction of bioorthogonal functionalities into biopolymers,[4] this concept has proven to be especially useful in the area of proteomic research, in particular for elucidating the role of posttranslational modifications in key biological processes such as cellular recognition and signal transduction.[3,5]

Over the past years many bioorthogonal reactions have been identified and applied to the transformation of azides which can be easily introduced into biopolymers.[2,6] Prominent examples include 1,3-dipolar cycloadditions, such as the Cu(I)-catalyzed reaction of azides with alkynes ("click reaction").[6,7] To address the toxicity of the Cu-catalyst, reactions with highly reactive strained alkynes[8] and Staudinger reactions with phosphines[9] have been

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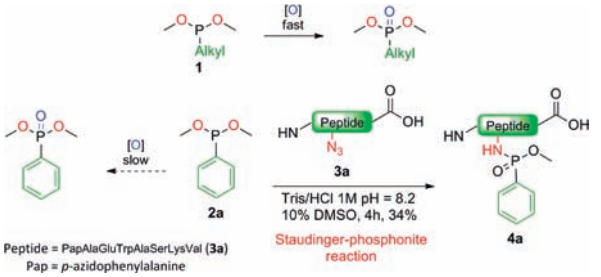
10.1021/ol2020175
Published on Web 09/29/2011

developed, which can be applied even for modifications *in vivo*.[10] Nevertheless, there is still a great demand for the development of new metal-free reactions in aqueous buffers, for which the azide reaction partner is easily accessible, allowing a straightforward conjugation of various functional modules to biomolecules to gain deeper insight into their function and operational mode.

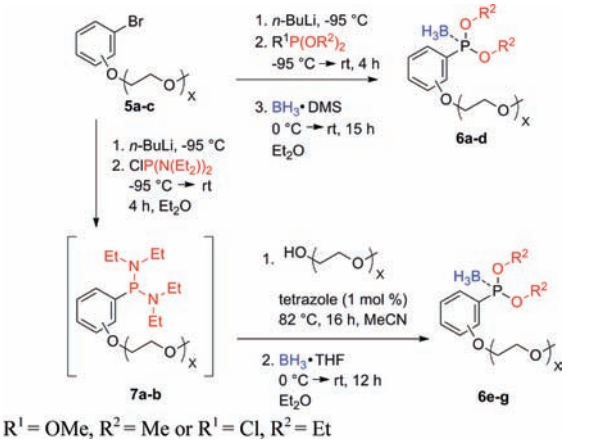
Recently, we have identified the Staudinger-phosphite reaction as a chemoselective transformation of azides under physiological conditions.[11,12] In the current study, we introduce phosphonites as another type of P(III)-reagents for the chemoselective functionalization of azido-biopolymers. Our main motivation was to employ the high intrinsic reactivity of phosphonites in Staudinger reactions, which have been mainly used for transformations in organic solvents in the past.[13] Additionally, phosphonites have the potential to transfer a single functional module or label that is attached to the carbon chain at phosphorus to an azido-containing biopolymer.[12b] However, since alkyl-phosphonites **1** appeared to oxidize rapidly, we focused on aryl-substituted analogues **2**, in which the sp^2-hybridized carbon at phosphorus accounts for a higher stability upon air exposure (Scheme 1).

Scheme 1. Oxidation of Phosphonites and Staudinger-Phosphonite Reaction of Peptides



In our investigations, the reactivity of phosphonites **2** with azides was probed. First, we used the commercially available dimethyl phenylphosphonite (**2a**), which is however only partially soluble in water. Although reactions of benzyl azide in organic solvents proceed in high yields to the corresponding phosphonamidate (see Supporting Information (SI)), which is in accordance with similar transformations from the literature,[13,14] an analogous reaction with a water-soluble unprotected arylazido-peptide **3a** delivered only a very moderate conversion of the peptide (see Scheme 1 and Table 2, entry 1) as monitored by HPLC-MS (see SI). Nevertheless, a phosphonamidate-peptide obtained from **2a** did not show signs of decomposition under physiological (pH 7.6−8.2, 26 h) or HPLC conditions (1% AcOH in AcCN/H_2O, 3 h), indicating the stability of the Staudinger-phosphonite conjugates (see SI).

Table 1. Synthesis of Water-Soluble Phosphonites



entry	BH_3-phosphonite	R^2	X	yield (%)
1	**6a**	Me	3[a]	52
2	**6b**	Me	3[b]	51
3	**6c**	Me	4[a]	44
4	**6d**	Et	3[a]	79
5	**6e**		3[a]	33
6	**6f**		3[a]	55
7	**6g**		4[a]	87[c]

[a] Substituent in *meta* position. [b] Substituent in *para* position. [c] HPLC purified.

To enhance the water solubility of the phosphonite, we decided to synthesize aryl-phosphonites **2b**−**h**, in which different oligo(ethylene glycol)(OEG)-substituents were attached to the aromatic ring and the phosphorus atom. For **2b**−**e**, which contain the OEG-moiety only at the aromatic ring, *meta*- or *para*-bromophenol was reacted with tosylated tri- or tetraethylene glycol to yield **5a**−**c** (see SI). The phosphorus atom was then introduced by halogen−lithium exchange and reaction with either trimethyl phosphite to yield **6a**−**c** or diethyl chlorophosphite for **6d** in yields between 44 and 79% (Table 1). It is important to note that the phosphonites were obtained as borane adducts in a one-pot process to allow easy purification and prolonged storage. Thereby, borane-protected phosphonites could be stored at 4 °C for more than a year without signs of decomposition. Removal of the borane group was accomplished by heating

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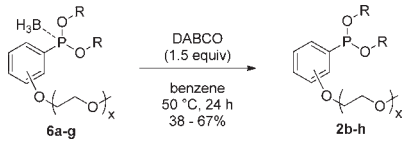
6a–**d** to 50 °C with DABCO delivering phosphonites **2b**–**e** in 46–67% yields (Scheme 2).[15] Subsequent conversion of azido-peptide **3a** with the obtained free phosphonites **2b**–**e** in aqueous buffer at room temperature was again determined by HPLC-MS (Table 2). Peptide conversion with phosphonites carrying methoxy or ethoxy groups at phosphorus was between 28 and 33% with 100 equiv. Only higher amounts of phosphonite (500 equiv) led to a moderate product formation. The best conversion rate of 70% was measured for **2d** (Table 2, entry 6) carrying a tetraethylene glycol chain, which supports the importance of the phosphonite solubility in water for a successful transformation.[16] Along those lines, phosphonite hydrolysis to a phosphinic acid ester was observed by ^{31}P NMR as another limiting factor in the Staudinger-phosphonite reaction. Consequently, we addressed these two issues by introducing OEG-groups at phosphorus to further enhance the water solubility and reduce the probability of nucleophilic attack at the phosphorus.

Scheme 2. Deprotection of Borane-Protected Phosphonites[a]



[a] R: Me, Et, or $(CH_2CH_2O)_xMe$; x = 3 or 4.

For the synthesis of the second set of phosphonites **2f**–**h** bis(diethylamino) phosphines **7a**–**b** were accessed first by reaction of the lithiated aryl species with bis(diethylamino) chlorophosphine (Table 1). The common reaction conditions for the synthesis of phosphites using 1 equiv of tetrazole were found to be inefficient. Reducing the amount of tetrazole to 1 mol % and simultaneous heating of the reaction mixture finally led to a protocol that proved to be very suitable. Borane-protected phosphonites with one (**6e**) or two ethylene glycol units (**6f** and **6g**) at phosphorus were achieved in good yields, further deprotected as stated before and used in reactions with the azido-peptide **3a** (Table 2).

^{31}P NMR measurements revealed that replacing the methoxy group at the phosphorus (in **2b**) with a diethylene glycol chain (in **2g**) increased the solubilized amount of phosphonite from 4% to 14%. The solubility was even further increased by a tetraethylene glycol chain at the aromatic system in **2h**, which led to 45% dissolved phosphonite in buffer after 4 min. Dissolved **2b** was almost completely decomposed after less than 10 min, whereas **2g** already showed higher stability. Remarkably, **2h** displayed the highest stability, in which solubilized phosphonite was present after more than 24 min (Figure 1).[16]

In contrast to the previous conversion studies, **2f**–**h** showed significantly higher conversion rates. Phosphonite **2f** containing ethylene glycol chains at the phosphorus doubled the conversion rate (Table 2, entry 8) as compared to the methoxy derivative **2b** (Table 2, entry 1). The best conversions between 86 and 95% could be achieved with 500 equiv of **2g** and **2h**. It is also important to note that the reaction appeared to be very fast and a conversion of 89% could already be observed after 20 min. Additionally, we could show that the deprotected phosphonite could be used without additional purification, since similar conversions were observed when crude phosphonite **2h** was used in the reaction (Table 2, entry 14).

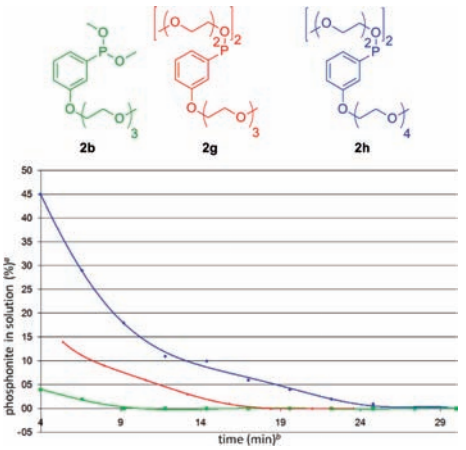


Figure 1. Hydrolysis and solubility of phosphonites. [a]Values were obtained by ^{31}P NMR in Tris/HCl buffer at pH = 8.2 with disodium hydrogen phosphate as external standard. [b]NMR measurements were started at the indicated time.

Since our investigations focused so far on the azido-peptide *conversion*, we next probed if side reactions with the other peptide functionalities occur or if other reaction products than the expected phosphonamidates **4** are formed. Therefore, we prepared a fluorophore-containing peptide **3b** by SPPS incorporation of an ε-NBD-lysine building block into the model peptide. This fluorescent marker allowed the identification and quantification of peptidic byproducts. Subsequent treatment of the peptide **3b** with **2h** revealed that the only by-product formed during this reaction was the corresponding amino-peptide (see SI).

Finally, we attempted to transfer the Staudinger-phosphonite reaction to the protein level. For this purpose we chose calmodulin with a *p*-azidophenylalanine (Pap) at position 2 as the model protein **8**. Calmodulin is a 17 kD calcium-binding protein found in all eukaryotes, which

(15) Deprotected phosphonites can be extracted with *n*-hexane (see SI). This protocol can however lead to lower yields for derivatives with many OEG-groups.

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Table 2. Reactivity of Phosphonites in Buffer

Peptide: AlaGluTrpAlaSerLysVal (**3a**)
or AlaGluAlaSerLysSerLys(NBD)Val (**3b**)

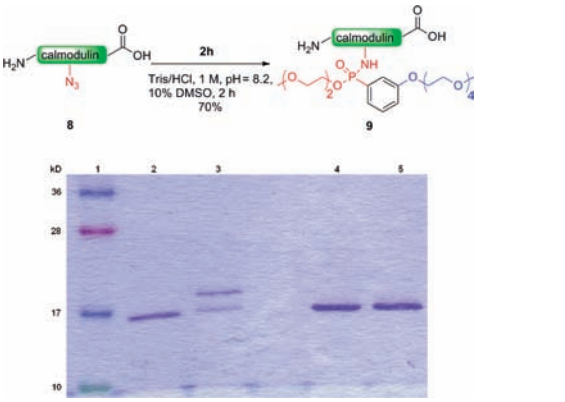
entry	phosphonite	R	X	phosphonite concentration	conversion of **3a** (%)
1	**2b**	Me	3	10 mM	33
2	**2b**	Me	3	50 mM	54
3	**2c**	Me	3[a]	10 mM	28
4	**2c**	Me	3[a]	50 mM	49
5	**2d**	Me	4	50 mM	70
6	**2e**	Et	3	10 mM	29
7	**2e**	Et	3	50 mM	53
8	**2f**		3	10 mM	65
9	**2f**		3	50 mM	85
10	**2g**		3	50 mM	86
11	**2h**		4	10 mM	44
12	**2h**		4	50 mM	95
13	**2h**		4	50 mM	93[b]
14	**2h**		4	50 mM	89[c]
15	**2h**		4	50 mM	83[d]

[a] Substituent in para position. [b] Reaction was performed with crude deprotection product. [c] Reaction time: 20 min. [d] Peptide **3b** was used: 62% product **4i** and 21% amino-peptide was formed (see SI).

plays an important role in many regulatory pathways by controlling various kinases and phosphatases, most notably the CaM-Kinase family.[17] The unnatural amino acid was incorporated into the protein by *in vitro* translation in RF1-deficient *E. coli* lysates using the Amber suppression methodology.[18]

For the reaction with azido-calmodulin **8** we chose phosphonite **2h**, which gave the highest conversion rates for peptides (Table 2). The crude protein reaction mixture was first analyzed by gel electrophoresis (Scheme 3), in which the modified calmodulin product **9** was observed by a clear mobility shift. MALDI-TOF analysis further proved the Staudinger-phosphonite reaction to be successful and indicated an estimated conversion of 70% (see SI). To additionally verify that no unspecific binding between the phosphonite and the protein occurred, we prepared a Pap2Ser mutant of calmodulin **10**, which was incubated with phosphonite **2h** under the same conditions as before. SDS-PAGE analysis of the crude reaction mixture shows only one band for the unmodified calmodulin **10**, which demonstrates that phosphonite **2h** reacted exclusively with the azide and no unspecific interactions between the phosphonite and the protein functionalities occurred.

Scheme 3. Chemoselective Staudinger-Phosphonite Reaction of Azido-Calmodulin **8**[a]



[a] SDS-Page stained with Coomassie; lane 1: Molecular Weight Marker; lane 2: azido-calmodulin **8**; lane 3: azido-calmodulin **8** reacted with **2h** to **9**; lane 4: Pap2Ser mutant **10** of calmodulin; lane 5: Pap2Ser mutant **10** of calmodulin reacted with **2h** (see SI).

In summary, we have developed the first example of a Staudinger-phosphonite reaction for the chemoselective transformation of azido-containing peptides and proteins in aqueous systems. Several water-soluble phosphonites were synthesized and probed with respect to their stability and performance in this bioorthogonal transformation. Currently, we are developing new functional phosphonites with higher stability against hydrolysis and higher solubility for metal-free functionalizations of different biopolymers.

Acknowledgment. The authors acknowledge financial support from the German Science Foundation within the Emmy-Noether program (HA 4468/2-1) and the SFB 765, the Boehringer-Ingelheim Foundation ("Plus 3 program"), and the Fonds der Chemischen Industrie (FCI).

Supporting Information Available. Experimental procedures, compound characterization, and spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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