Chemoselective Reactions

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Chemoselective Staudinger-Phosphite Reaction of Azides for the Phosphorylation of Proteins**

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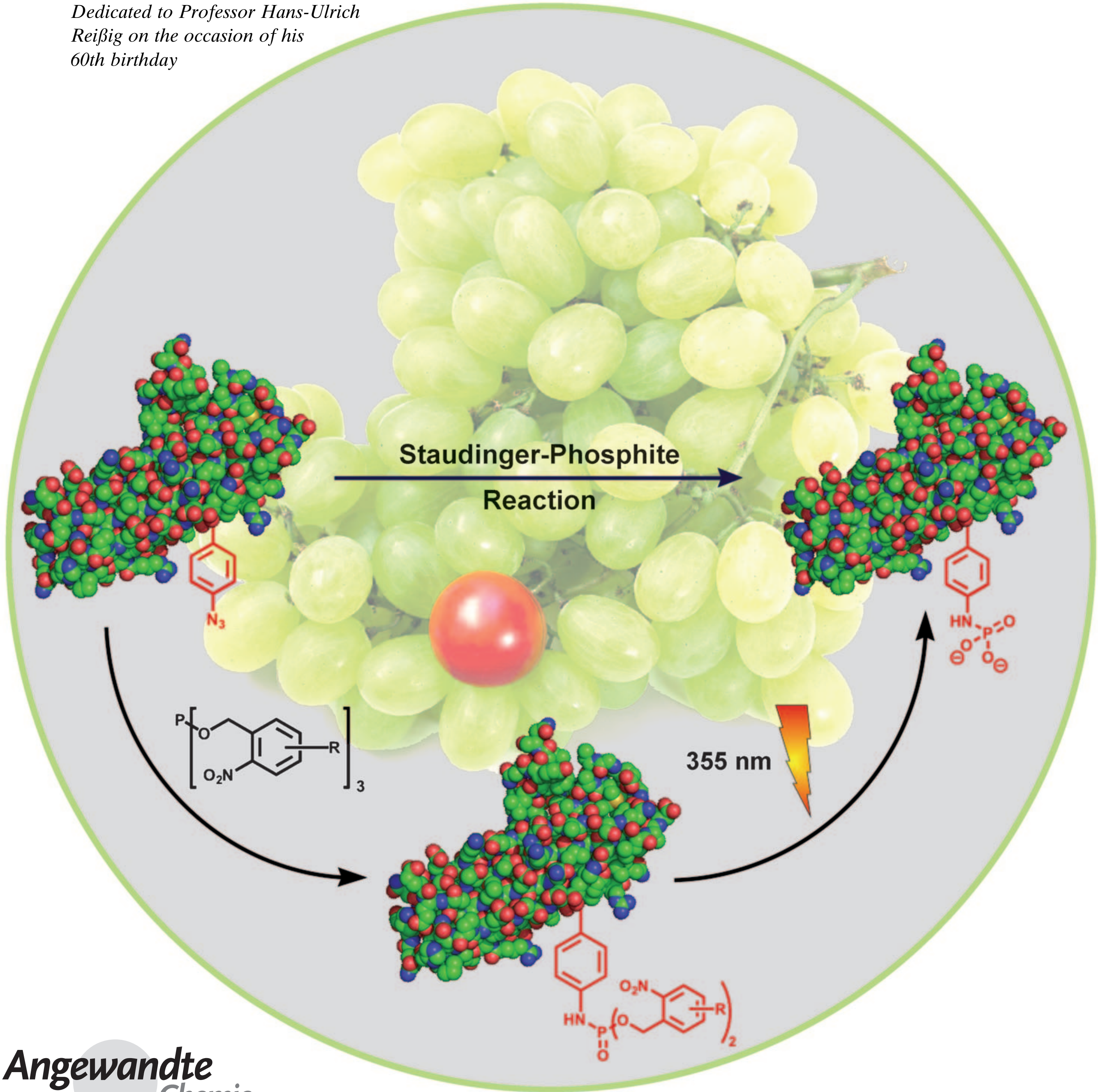
Dedicated to Professor Hans-Ulrich Reißig on the occasion of his 60th birthday



Chemoselective reactions have become important tools in chemical research as well as in modern life sciences.[1,2,3a] They are used in the synthesis, for instance, of modified proteins for biological studies and thereby help in the evaluation of posttranslational modifications, such as phosphorylation or glycosylation, in signal transduction and regulation.[3] In addition, biophysical probes or other functional modules can be introduced into complex biomolecules, even within a cellular environment, to visualize biological processes or specifically alter their functional behavior.[1–3]

For biological applications, a chemoselective reaction must transform a single chemical functionality within a complex biomolecule under mild aqueous conditions at ambient temperature. Furthermore, for full spatial control of the location of the desired modification unit within the target biopolymer, reactions are particularly useful, in which both reaction partners are nonnatural, since they can address a unique chemical functionality within a complex biopolymer. Several of such bioorthogonal[4] reactions have been identified and employed within the last years, which rely on the introduction of nonnatural functionalities, commonly referred to as chemical reporters,[2a,4] into biological molecules.[5,6]

Among these chemoselective reactions, azide transformations are very popular, since various biochemical techniques exist that deliver azide-containing biopolymers. These methods include auxotrophic expression and nonnatural protein translation as well as metabolic and enzymatic processes.[5,6] Examples for chemoselective azide reactions are the Cu^{I}-catalyzed ("click chemistry")[7,8] and strain-promoted [3+2] cycloaddition,[9] both of which employ alkyne substrates for the reaction with azides by the formation of triazoles. Although employed frequently, these reactions still have some disadvantages, in particular the use of toxic Cu^{I} catalysts, which limits in vivo applicability, and the introduction of large modification units in the linkage between biopolymers and the functional modules.[10] Another chemoselective strategy, the Staudinger ligation,[11] utilizes the reactivity of the Staudinger reaction. In this reaction azides **1** react with P^{III} compounds, namely phosphines **2**, to give iminophosphoranes **3** (Scheme 1 A). To suppress hydrolysis of the P=N bond to give amine **4**,[12] Bertozzi et al. have positioned an intramolecular electrophilic trap on phosphine **5**, which reacts with the nucleophilic iminophosphorane nitrogen (Scheme 1 B). This chemoselective modification strategy has found widespread application in labeling[4,13] and immobilization[14] of DNA and proteins even within living animals,[15] although sometimes phosphine oxidation limits the application of this reaction.[10]

We have now identified another Staudinger-type reaction for the chemoselective functionalization of azides, that can occur in high yields under mild conditions in complex biological molecules (Scheme 1 C).[16,17] This reaction consists of a two-step process, in which the formation of phosphorimidate **7** from phosphite **6** and azide **1** is followed by hydrolysis to give phosphoramidate **8**. Although this reaction is known[18] and has been used previously, for instance, in the synthesis of DNA oligomers with phosphoramidate linkages in THF or pyridine,[18b,19] it has to our knowledge not been considered as a chemoselective reaction for the modification of peptides or proteins. In addition, Staudinger-phosphite reactions have not been carried out in pure water or buffers, which is a requisite for advanced peptide and protein modifications.

Our first goal was to determine the scope and applicability of this transformation under mild reaction conditions for peptide modifications. We observed that the Staudinger reaction of phenyl azide (**1a**) with symmetrical phosphites **6** occurs at room temperature in various solvents including CH_2Cl_2, dimethylformamide (DMF), dimethylsulfoxide (DMSO), and even pure water, although some of the starting materials are not completely soluble (Table 1).[20] Most importantly, during the hydrolysis no P−N cleavage is observed, as in the analogous reaction with phosphines, but instead a primary phosphoramidate **8** is formed under ambient temperatures in yields of 80–90 % (Table 1, entries 1–5). It is important to note that the hydrolysis also proceeds under biphasic conditions in nonpolar solvents; however, longer reaction times may be required.

Next, we applied the Staudinger-phosphite reaction to the chemoselective modification of azide-containing peptides with readily available phosphites. These model peptides contained several functional groups present in proteins in addition to a commercially available azido-Phe unit; they were synthesized by solid-phase peptide synthesis (SPPS). The resin-bound peptides were cleaved from the support by treatment with trifluoroacetic acid (TFA), and the unprotected phenylazidopeptides **1b** and **1c** were purified by HPLC. Peptides **1b** and **1c** were treated with tributyl- and triethylphosphite, respectively. The Staudinger-phosphite reaction proceeded in DMSO with only minimal amounts of aniline peptides originating from P−N bond cleavage and along with rearranged products.[16] After full azide conversion, peptides **8c** and **8d** were purified by HPLC and isolated in good overall yields (Table 1, entries 6 and 7).[21] Remarkably, the peptide containing a Cys residue was modified only at the azide function.

We then turned our attention to a potentially biologically relevant functional group that can be introduced into proteins by the chemoselective reaction itself. Charged phosphoramidates **11** closely resemble the biologically very relevant phosphorylated tyrosine residues in **12**, and may hence

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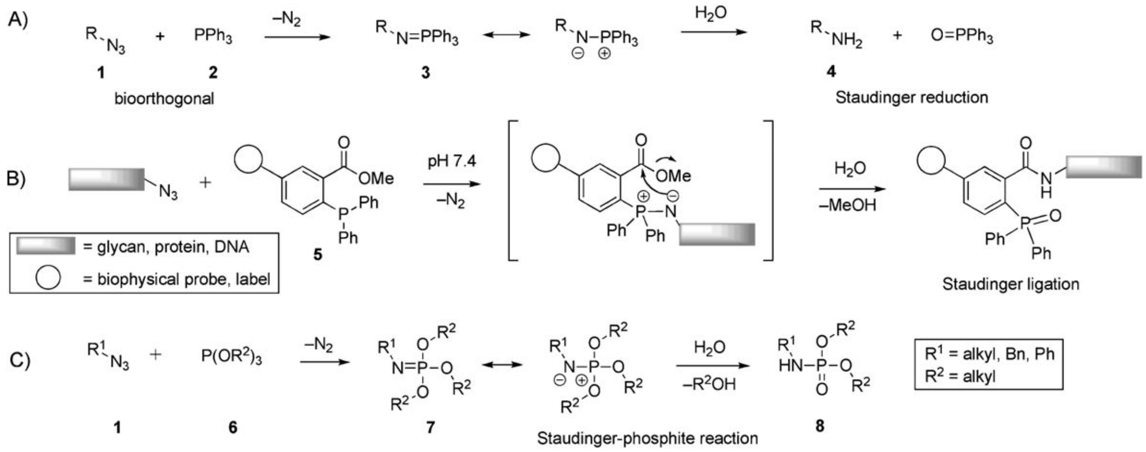
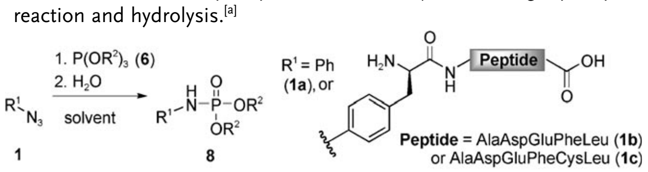
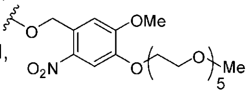


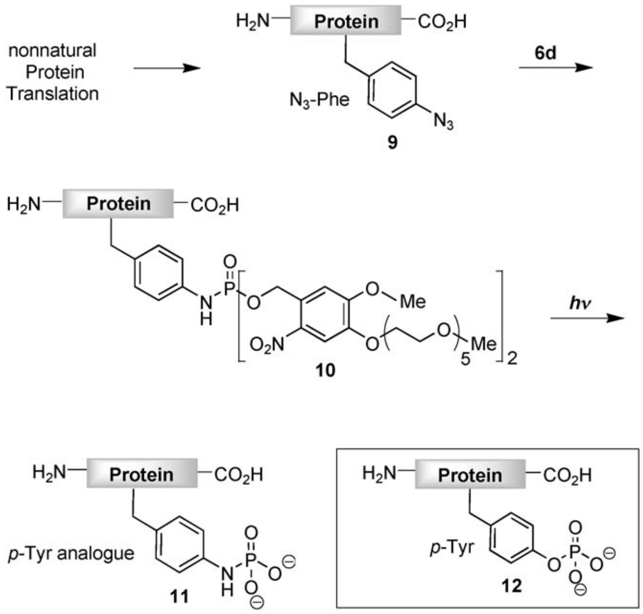
Scheme 1. A) Staudinger reaction of azides with phosphines followed by the hydrolysis of the resulting iminophosphoranes to give amines (Staudinger reduction). B) Staudinger ligation. C) Staudinger-phosphite reaction followed by hydrolysis of the resulting phosphorimidates to give phosphoramidates.

Table 1: Formation of phosphoramidates **8** by a Staudinger-phosphite reaction and hydrolysis.[a]



Entry	Azide	Phosphite, R^2	Solvent	Product	Yield [%]
1	**1a**	**6a**, Me	CH_2Cl_2	**8a**	84
2	**1a**	**6a**, Me	DMF	**8a**	88
3	**1a**	**6a**, Me	H_2O	**8a**	78
4	**1a**	**6b**, Et	CH_2Cl_2	**8b**	90
5	**1a**	**6b**, Et	H_2O	**8b**	80
6	**1b**	**6c**, *n*Bu	DMSO	**8c**	63
7	**1c**	**6b**, Et	DMSO	**8d**	39
8	**1b**	**6d**, 	Tris buffer pH 8.2	**8e**	50

[a] Reagents and conditions: 1. Phosphite **6** (1–10 equiv), RT, 6–24 h; 2. H_2O, RT, 0–48 h. For further details see the Experimental Section and the Supporting Information.

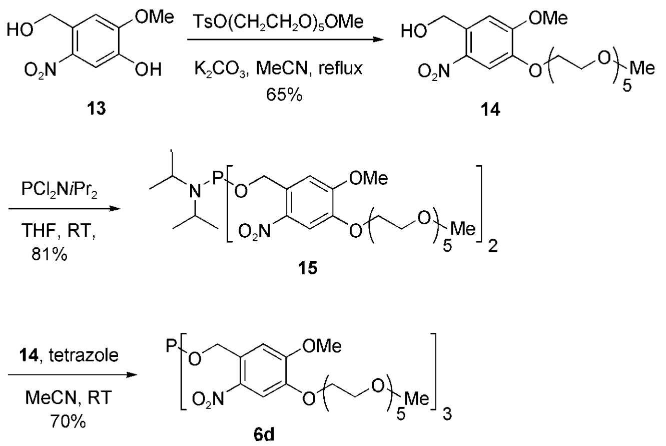


Scheme 2. Two-step conversion of azidophenylalanine residues in proteins into the corresponding phosphotyrosine analogues. For further details see the Supporting Information.

serve as phosphate ester mimics, in which the naturally occurring oxygen substituent is replaced by an NH group (Scheme 2). Phosphoramidates **11** could be in theory accessed by mild light-induced saponification of 2-nitrobenzyl esters **10**, and the latter can be obtained from the reaction of phenylazido-containing proteins **9** with symmetrical 2-nitrobenzylphosphites.

Since tris(2-nitrobenzyl)phosphite, prepared by a known protocol,[22a] is only poorly soluble in water, we focused on the synthesis of phosphites with attached ethylene glycol units to overcome this problem. In the synthesis outlined in Scheme 3 phosphite **6d** was prepared in three steps from readily available alcohol **13**[23] via intermediate **14**. Although **6d** could be synthesized from **14** and PCl_3 or $P(NAlk_2)_3$ in one step, we found that the yields were much higher when phosphoramidite **15** was isolated from the reaction between $PCl_2N(i\text{Pr})_2$ and two equivalents of **14**, before addition of a third equivalent of **14** resulted in the final product. Phosphite **6d** with fifteen ethylene glycol units displayed excellent solubility in water (> 60 mM).

Phosphite **6d** was then treated with peptide **1b** in aqueous buffers at pH 7.4–8.2 at room temperature. The reaction proceeded with almost quantitative conversion to **8e** in less than 8 hours, and aniline hydrolysis products accounted for less than 7% of the material (Figure 1). Peptide **8e** was isolated by preparative HPLC (Table 1, entry 8) to test its stability and to probe the rates of light-induced saponification. Peptide **8e** was stable for at least 72 hours in aqueous buffers (pH 7.4–8.2) in the absence of light, whereas solutions



Scheme 3. Preparation of water-soluble phosphite **6d**. For details see the Experimental Section and the Supporting Information.

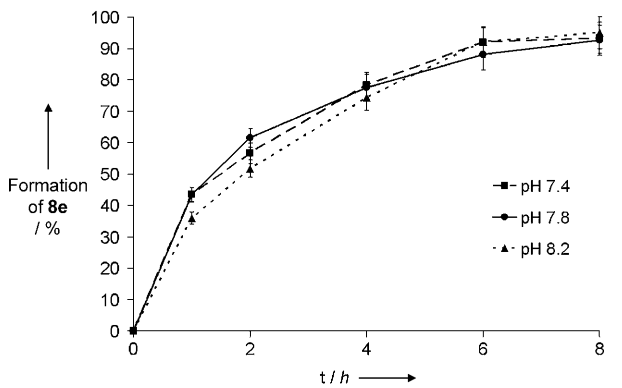


Figure 1. Formation of phosphoramidate **8e** from peptide **1b** (50 μM) and phosphite **6d** (5 mM) in buffered solutions at room temperature. Conversion of **1b** into **8e** was based on LC-UV analysis. For details see the Experimental Section and the Supporting Information.

of **8e** irradiated with a 355 nm laser for 90 seconds underwent complete saponification (data not shown).

Next, we determined whether the Staudinger-phosphite reaction can be employed for site-specific phosphorylation in proteins, even at nonnatural sites.[22,24] For this purpose we employed the azido-Phe protein **9**, which can be obtained by nonnatural protein translation using the *amber*-suppression-based orthogonal system,[5] as the reaction partner for phosphite **6d**.[25]

As a model protein, the naturally non-phosphorylated 17 kDa protein SecB **9′**, which contains a single *p*-azido-Phe residue at position 156 in the protein sequence followed by a C-terminal His tag, was prepared by expression in a cell-free orthogonal protein translation system (see the Supporting Information). After purification of His tag, azido-SecB was reacted with phosphite **6d** at pH 8.0. Full conversion of the azide to phosphoramidate **10′** was observed and verified by protein electrophoresis; the gel shift of the modified protein corresponded to the molecular weight of phosphite **6d** (Figure 2A). In addition, the resulting phosphoramidate

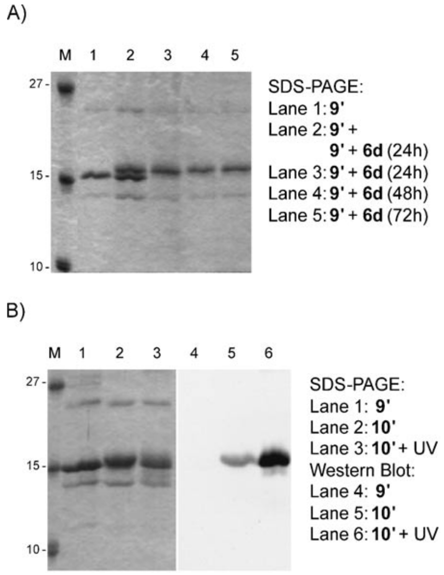


Figure 2. A) Analysis of reaction mixtures obtained upon incubation of **9** (12.5 μM) in the presence of **6d** (5 mM) by +SDS-PAGE (Coomassie staining). B) SDS-PAGE (Coomassie staining) analysis (lanes 1–3) and anti-phosphotyrosine antibody recognition (Western blot) assay (lanes 4–6) performed on the isolated proteins **9′** and **10′**, as well as on the mixture obtained upon irradiation (355 nm) of a solution of **10′** for 90 s.

moiety in **10′** was completely stable in solution for up to 72 hours, since no decay was observed in the protein gel.

To test the behavior of the phosphoramidate as a mimic of a phosphorylated protein, we saponified the phosphoramidate ester in **10′** to give **11′** under irradiation with a 355 nm laser and applied a phosphotyrosine-specific antibody to the SecB proteins **9′**, **10′**, and **11′** in a Western blot analysis (Figure 2B). A strong response to the phosphorylation mimic in **11′** and no recognition of the azide functionality in **9′** was evident by luminol-based visualization of the antibody. A slight interaction was noticed for the phosphoramidate ester in **10′**, which we attribute to an undesired partial photolysis of the reactive protecting groups. Further studies to investigate this interaction are currently underway.

In summary we have shown that the Staudinger-phosphite reaction is suitable for the metal-free, chemoselective transformation of azides in peptides and proteins. This Staudinger reaction is very easy to perform as it utilizes phosphites, which can be prepared by standard organic synthesis protocols and are stable against oxidation upon exposure to air. Chemoselective transformations by the Staudinger-phosphite reaction proceed in various solvents and buffers at room temperature, conditions suitable for quantitative modification reactions in proteins. Upon combination with light-sensitive phosphites, phosphoramidate esters can be hydrolyzed to yield analogues of phosphorylated Tyr residues in proteins, which can be recognized by phosphotyrosine-specific antibodies. Current investigations in our laboratory aim to apply this chemoselective phosphorylation strategy to study biologically relevant signaling processes.

Experimental Section

Synthesis of azido-peptides **1b** and **1c:** The peptides were synthesized on an ABI 433A peptide synthesizer using standard amide coupling conditions (HBTU/HOBt; Fmoc protocol) on a Wang resin with Fmoc-*p*-azido-Phe-OH as the last residue. Peptides were cleaved from the solid support with 95% TFA and purified by semipreparative HPLC.

General procedure for the Staudinger-phosphite reaction of azidopeptides **1b** and **1c:** A solution of the azidopeptide in DMSO or in a buffer (0.2 $mL\,mg^{-1}$ peptide) was treated with phosphite **6** (5–10 equiv), and the reaction mixture was stirred at room temperature for 6–24 h. Without further workup phosphoramidate-peptides **8c–e** were isolated from the respective reaction mixtures by preparative HPLC followed by lyophilization. For LC-HRMS analysis see the Supporting Information.

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