Site-specific functionalisation of proteins by a Staudinger-type reaction using unsymmetrical phosphites†‡

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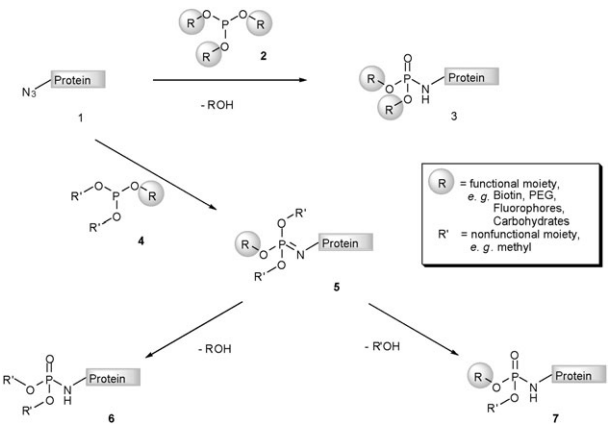
Unsymmetrical phosphites react efficiently in a Staudinger reaction with *p*-azido-phenylalanine, which can be applied for the biotinylation of proteins, thereby expanding the scope of the chemoselective Staudinger-phosphite reaction of aryl azides with symmetrical phosphites to the corresponding phosphoramidates.

The development of methods that allows the decoration of biopolymers with functional probes serving either to visualise biological processes or to alter specifically their behaviour holds mutual interest for biologists and chemists.[1–5] Various biochemical methods for the installation of an unnatural chemical reporter on biological molecules have been developed,[6–9] which could be addressed in a chemo-selective transformation.[4,10–12] Most commonly, azides are employed as chemical reporters in such bioorthogonal reactions. The formation of triazoles as a result of a [3 + 2]-cycloaddition between azides and alkynes was shown to be very efficient,[10,12] but it usually requires the presence of cytotoxic Cu(I) catalysts, thus limiting its *in vivo* applicability.[4,13] A strain-promoted triazole formation was introduced as a metal-free alternative, which however inevitably introduces a large unnatural linkage between the macromolecules and the functional module, that may influence biological properties.[14–16] The Staudinger ligation, which is based on the reaction between azides and phosphines, has also shown great potential in the labelling of biomolecules *in vivo*,[11,17] although sometimes the oxidation of phosphines limits its utility.[18] Recently, we have introduced the Staudinger-phosphite reaction as an alternative, chemoselective transformation of azides, which utilises the reactivity of air-stable phosphites, and can be performed in water at room temperature.[19] Another advantage of the reaction is the phosphoramidate linkage, which mimics natural occurring phosphoric esters. In addition to the previously published modifications of polypeptide azides **1** with symmetrical phosphites **2**, we report herein the first examples of Staudinger-phosphite ligation between an azido amino acid and easily accessible unsymmetrical phosphites **4** carrying only one functionalised substituent (Scheme 1). The obvious advantage of using unsymmetrical ligation partners results from the molecular economy, especially for complicated functionalities, and the steric properties. The presence of three bulky substituents on the phosphorous may hinder quantitative conversion of shielded azido groups. The proximity of two identical functionalities also may cause interactions, *e.g.* an autoquench in the case of flourescence dyes. However, a potential pitfall is associated with it, lying in the possible and undesired hydrolysis of a functional substituent in phosphorimidate intermediate **5**, leading to an unfunctionalised side product **6**. Depending on the choice of substituents (R and R′), different fractions of phosphoramidate products **6** and **7** are expected to be obtained.



Scheme 1 Functionalisation of azido-proteins **1** with symmetrical **2** and unsymmetrical **4** phosphites.

Although the coupling between azides and unsymmetrical phosphites has been applied in the preparation of small molecules in organic solvents,[20–25] including unnatural mono- and dinucleotides,[22–24] the utilisation of the transformation as means for modification of macromolecules in aqueous media has so far not been investigated. Furthermore, the exact distribution of phosphoramidate products of these transformations was often not addressed nor quantified. Therefore, we decided to investigate the relative propensities of simple substituents to be expelled during the hydrolysis of phosphorimidate **5**. We synthesised unsymmetrical model phosphites that contained two methyl groups in addition to a short (**4a**), long (**4b**), branched (**4c**) or functionalised (**4d**) hydrocarbon chain, as well as phenyl (**4e**) and benzyl (**4f**) substituents. All of these compounds were prepared in moderate to good yields from the easily accessible phosphoramidate **8**

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‡ Electronic supplementary information (ESI) available: Experimental procedures and analytical data. See DOI: 10.1039/b926818a

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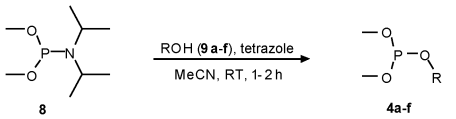
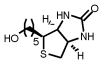
Table 1 The preparation of unsymmetrical phosphites



Phosphite	ROH	Isolated yield (%)
4a	EtOH	89
4b	DecOH	94
4c	*i*PrOH	90
4d		49[a]
4e	PhOH	93
4f	BnOH	87

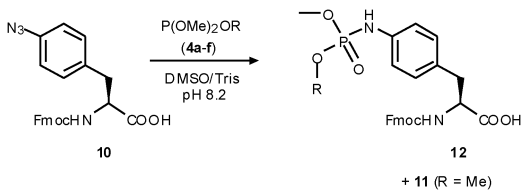
[a] For details on isolation and storage of the biotin phosphite **4d**, see ESI‡.

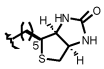
and alcohols **9a–f** *via* tetrazole-mediated substitution at a phosphorous atom (Table 1).[26–28] Unsymmetrical phosphites **4a–f** were reacted with *N*-Fmoc-*p*-azido-phenylalanine **10** in aqueous DMSO to ensure the solubility of all phosphites **4**, and the ratio between dimethylphosphoramidate **11** and a mixed phosphoramidate **12** was monitored using LC-UV.[29] From the results compiled in Table 2, it can be seen that alkyl substituents of phosphites **4a–d** (entries 1–4) are less prone to hydrolysis than methyl groups, whereas phenyl (**4e**) and benzyl groups (**4f**) (entries 5 and 6) are relatively good leaving groups.

Phosphorimidate hydrolysis has been discussed in the literature. Based on ^{18}O-labelling experiments, it was suggested that the hydrolysis proceeds *via* nucleophilic attack partially at the sp^3 hybridised α-carbon (if present) and partially at the phosphorous atom.[30,31]

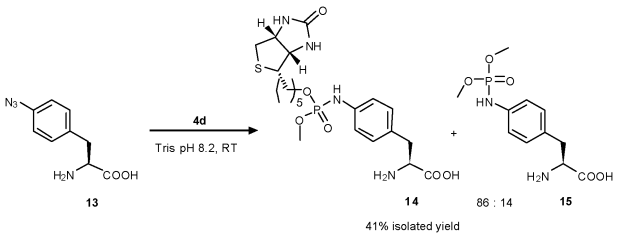
If this were to be the case, substituents containing more electrophilic α-carbons should be prone to an enhanced attack by nucleophiles, and more stabilised alkoxides should be expelled from the pentavalent phosphorous transition state

Table 2 Reaction of the mixed phosphites **4a–f** with model compound **10**



Entry	Phosphite	R	**11**[a] (%)	**12**[a] (%)	Error[b] (%)
1	**4b**	Dec	15	85	1
2	**4c**	*i*Pr	21	79	2
3	**4d**		23	77	1
4	**4a**	Et	29	71	1
5	**4e**	Ph	50	50	2
6	**4f**	Bn	72	28	2

10 (10 mM) was reacted with **4a–f** (30 mM) in DMSO containing 10% of Tris buffer (1 M, pH 8.2) for 6 h at RT. Conversion: [a] fraction of products calculated based on LC-UV analysis, see ESI‡ for details; [b] reactions were repeated at least three times; error = standard deviation of the performed measurements.



Scheme 2 The biotinylation of amino acid **13** in Tris buffer (pH 8.2).

more readily. The results obtained for the transformation of alkyl- and phenyl-substituted phosphites are in agreement with the predicted trend. However, the observation of the benzyl group (**4f**) leaving with unexpected ease suggests the involvement of an alternative mechanism, in which heterolysis of a C–O bond to form a stabilised carbocation occurs as the first step. This result is in agreement with a previous report that questioned the common belief for general Arbuzov reactions to proceed *via* S_N2 mechanism.[32]

Additionally, to confirm that the distribution of products **11** and **12** does not depend on the stage of conversion, azide **10** was incubated with phosphite **4e**, and the reaction was monitored at intervals of one hour. The product distribution was consistent throughout the course of transformation, suggesting that secondary reactions of phosphoramidates do not occur in the system (ESI‡). Since the ultimate advantage of a chemoselective Staudinger-phosphite reaction is the possibility for its conduction in aqueous media, water soluble *p*-azido-phenylalanine **13** was reacted with the biotin phosphite **4d** in Tris buffer without the addition of co-solvents (Scheme 2). Biotinylated phosphoramidate **14** was the major product in this transformation, and the fraction of **14** with respect to phosphoramidate byproduct **15** (86 : 14) was even greater than that observed in the buffer containing DMSO for the Fmoc protected amino acid (Scheme 2). When the transformation was repeated on a slightly larger scale, biotinylated amino acid **14** was isolated in good yield (41%) and its identity was confirmed by HRMS and NMR.

Encouraged by these results, we applied **4d** to the biotinylation of a model protein (**16**) containing a single *p*-azido-phenylalanine residue, which was incorporated *via* unnatural protein translation in *E. coli* lysate.[19] Since **16** presented limited water solubility,[33] it was incubated with **4d** in phosphate buffer containing DMSO. In order to probe the occurrence of non-specific binding between protein **16** and the biotin module, the protein was incubated with the chemically inert alcohol precursor to **4d**, biotinol **9d**. Modification of protein **16** was performed overnight at ambient temperature and was followed by SDS-PAGE separation and subsequent transfer of the protein content onto a PVDF membrane. The membrane was then subjected to incubation with a streptavidin horseradish peroxidase conjugate, and the interactions of streptavidin with biotin, locally present on the surface of the membrane, were detected in a luminol-based luminescence assay (Fig. 1A). As can be deduced from the figure, biotin was incorporated into protein **16** (lanes 1 and 2) in the presence of phosphite **4d**. On the other hand, as expected, incubation of the protein with biotinol (lane 3) or the reaction buffer (lane 4) did not result in protein biotinylation. The

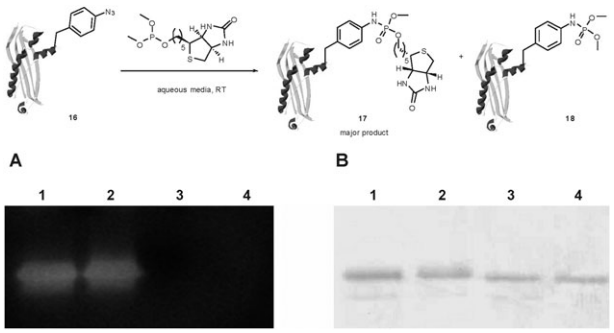


Fig. 1 The biotinylation of azido-protein **16** in aqueous media. (A) Luminescence image of streptavidin-recognised protein on a Western blot; (B) Western blot stained with Ponceau S; lanes 1, 2: **16** reacted with **4d**; lane 3: **16** incubated with **9d**; lane 4: **16** incubated without **4d** or **9d**.

membrane was stained with Ponceau S (Fig. 1B) in order to visualise the total protein content and ensure proper protein transfer from the gel. Additionally, MS analysis performed on the whole protein (upon incubation with **4d**) revealed the covalent nature of the linkage between the protein and the biotin moiety (ESI‡).

In conclusion, unsymmetrical phosphites appear to be useful reagents for the modification of proteins *via* aqueous Staudinger-phosphite based ligations. Thereby, we showed that not only symmetrical but also unsymmetrical phosphites can be used as efficient modifiers of aryl azides. With an application of model reagents, it was presented that, with the exception of benzyl groups, a general trend can be observed for the ability of substituents on a phosphite to depart from phosphorimidates generated as primary products of the Staudinger-phosphite reaction. An unsymmetrical phosphite substituted with two methyl groups and a biotin moiety was prepared in good yield, and was successfully applied as a reagent for biotinylation of a model azido-protein in aqueous solution. The applicability of the method for the biotinylation of proteins was confirmed by the strong affinity of streptavidin to a site-specific biotin phosphoramidate protein conjugate, as demonstrated in a Western blot assay. Thereby, it appears that the Staudinger-phosphite ligation is a promising metal-free alternative to commonly utilised bioconjugation methods. Currently, we are further working on the preparation of phosphites for modification of azido-proteins with different labels and functionalities.

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