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Azide reduction during peptide cleavage from solid support – the choice of thioscavenger?

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Peptide azides acquired growing impact because of application in bioconjugation via 'click chemistry' or Staudinger ligation. Furthermore, there are many methods established in organic synthesis addressing the reduction of azides to amines, but no observation of a reductive transformation of peptide azides during SPPS cleavage was yet reported. In the present study, the reduction of peptide azides during SPPS cleavage was investigated depending on the choice of thioscavenger, reacting as reductive species. First observed for short PNA/peptide conjugates the occurring extensive side reaction was also validated for one of the applied azide amino acid building blocks and was further investigated by applying different cleavage cocktails to a series of peptides varying in hydrophobicity and position of the azide moiety in the oligomer sequence. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: azide reduction; ligation methods; peptide cleavage; side reaction; scavenger; SPPS

The azide moiety obtained outstanding importance in chemical biology because it serves as the reactive functional group in two of the most important 'bioconjugation' techniques [1–4]. In the 'click chemistry' approach introduced by *Sharpless et al.* in 2001 [5], later including the *Huisgen* 1,3-dipolar cycloaddition between an azide and a terminal alkyne using copper catalysts, combines the advantages of a fast, simple to use, easy to purify and regiospecific reaction that is insensitive to oxygen and water [6–8]. The *Staudinger* ligation developed by *Saxon, Bertozzi et al.*, often considered as complementary to 'click chemistry' with respect to the applied solvents, exploits a smooth reaction between an azide functionality and a phosphane to form a phospha-aza-ylide [9]. Nevertheless, when dealing with the ligation of rather hydrophilic peptide segments or polar PNA moieties the application of the 'click'-approach has frequently been reported [10–13]. Thereby, the azide groups are mostly already incorporated in peptide segments during the SPPS, and meanwhile, several azide amino acid building blocks have been designed for that purpose [14,15]. In classical organic synthesis, there exists a wide variety of straightforward and chemoselective methods for the reduction of azides to amines [16–24]. These reactions naturally acquire special interest as amino group precursors for the synthesis of amino acid building blocks and are even applied for the functionalisation of resin-bound peptides [25,26].

Herein, a significant reductive side reaction of azide functionalised peptides and PNAs during cleavage from solid support is reported (Figure 1). The azides were reduced to amines during acidic cleavage with a mixture of TFA, water, silane scavenger and 1,2-ethanedithiol (EDT). Various cleavage conditions applied to an azide containing amino acid, peptides and peptide/PNA chimera clearly indicated that otherwise standard cleavage cocktails in peptide chemistry cause serious conversion of azide containing target oligomers.

Starting point for our investigation was the preparation of azide functionalised chimera of *N*-(2-aminoethyl)glycine PNAs (aeg-PNAs) and peptides. Applying the cocktail for oligomer cleavage from solid support, azide to amine reduction was observed as a significant side reaction. The short peptide/aeg-PNA azides $N_3(CH_2)_2CO$-caccXKK-NH_2 (**1**) and $N_3(CH_2)_2CO$-gtggXKK-NH_2 (**2**) (X = 5,5-diiodo-allylglycine; a = aeg(adenine), t = aeg(thymine); g = aeg(guanine); c = aeg(cytosine)) were designed for 1,3-dipolar 'click'-application.

In SPPS only the reduced amine-species were isolated applying a TFA (92.5%)/water (2.5%)/triisopropylsilane (TIS) (2.5%)/EDT (2.5%) (*v*/*v*/*v*/*v*) cleavage cocktail (Table 1). This mixture was selected instead of the standard TFA/*m*-cresol conditions used to protect PNA from side reactions with benzhydryl cations (Bhoc-cleavage from the exocyclic amines) because of the volatility of all components [27].

The short PNA-segments were not easily purified by precipitation during postcleavage work-up in order to remove scavenger and other hydrophobic impurities. Direct HPLC-purification of the cleavage cocktail was actually unfavourable because of the remaining amount of *m*-cresol (up to 25%) that diminishes the peptide/PNA interaction with the stationary phase during chromatography. Therefore, the TFA/water/TIS/EDT cocktail was extensively used for successful cleavage of peptide/PNA conjugates lacking an azide residue [(Schneggenburger and Diederichsen,unpublished)]. The application of the TFA/water/TIS/EDT cleavage cocktail to additional peptide/PNA or peptide azides

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Journal of PeptideScience

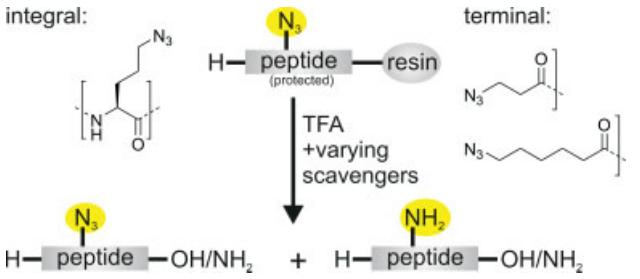

Figure 1. Azide reduction within peptides varying the cleavage cocktail composition, peptide polarity and position of the azide moiety. The azide was incorporated integral as amino acid or *N*-terminal by acylation.

3–**6** mainly differing from oligomers **1** and **2** in length and sequence also led to mixtures of the reduced amine and the azide species as derived from analytical HPLC and high resolution mass spectrometry (Figure 2). In contrast, the usage of a TFA (95%)/*m*-cresol (5%) mixture or a TFA (95%)/water (2.5%)/TIS (2.5%) cocktail for the SPPS-cleavage reaction only preserved the respective azide compound (Table 1).

The postulated side-reaction observed for several azide containing peptide/PNA conjugates was verified by conversion of azide amino acid **7** to ornithine **8** with 39% yield applying the TFA (95%)/water (2.5%)/TIS (2.5%) and TFA (92.5%)/water (2.5%)/TIS (2.5%)/EDT (2.5%) cleavage conditions for 2 h (Figure 3). The reduction product generated under cleavage conditions was compared by HPLC and NMR analysis with independently obtained Fmoc-Orn-OH. A substantial reduction of azide **7** was obtained for the EDT-containing cleavage cocktail yielding ornithine **8** with 39% conversion (Figure 4 and Supporting Information). The cleavage mixture lacking EDT had no influence on the artificial building block.

Considering a possible reducing agent within the cleavage cocktails, azide reduction by thiol compounds or by silanes is known from literature but only rarely reported to play a decisive role in synthesis [28–36]. Dithiothreitol (DTT), glutathione and mercaptoethanol, for example, have been used for quantitative reduction of 3′-azidothymines under physiological conditions and for efficient reduction of azido groups in carbohydrates under basic conditions [31,34]. In addition, DTT was even applied for the quantitative reduction of free peptide azides and for the reversible azide to amine conversion of resin-bound peptides both under basic conditions [32,33]. On the other hand, a quantitative azide reduction of aryl azides by triethylsilane under radical conditions and thiol catalysis has recently been reported. A radical mechanism for silane based reduction is described for alkynyl azides. Nevertheless, the active radical silane species also reacts with aliphatic azides [24,35,36].

Water can be used as a moderately efficient scavenger for peptides without sensitive amino acids like Cys, Met, Trp and Arg. Nevertheless, even for uncomplicated sequences water is often recommended to be combined with silane derivatives like TES or TIS. For cleavage of peptides containing sensitive amino acids, EDT and DTT are still the most common and efficient scavengers [37–39]. With the assumption that the typical amount of EDT present in various cleavage cocktails is responsible for the occurring azide reduction, peptide octamers **9**–**13** varying in polarity, positioning and connectivity of the azide moiety have been synthesised (Table 2).

Cleavage of azide containing peptide octamers from the solid support was investigated using three different TFA/water/TIS scavenger cocktails with differing thiol component. Using DTT and thioanisol next to EDT notably in all reactions a certain amount of the peptide species with reduced azide moiety was produced (Table 2). Azide reduction was highly pronounced for all peptides in the case of the EDT-containing cocktail. In general, reduction was suppressed most efficiently using DTT as thioscavenger. It also seems to be a general trend that oligomers **9**, **10** and **13** with terminal azide functionality were reduced more easily. Furthermore, increasing hydrophobicity (**9** > **11** > **13**) facilitates azide to amine conversion. Positioning of a tryptophan residue that is known to easily undergo oxidation processes next to the azide side chain (oligomer **13**) does not have a noteworthy influence on the azide reduction [40].

In conclusion, we have reported an azide to amine conversion observed as an extensive side reaction during peptide cleavage from solid support. With EDT as thioscavenger cleavage of peptide azides led up to 50% loss of desired product. DTT should be the thioscavenger of choice for peptide sequences containing azide groups and amino acid side chains that are sensitive to the usual cleavage conditions. PNA cleavage might be beneficial applying standard TFA/*m*-cresol (limited to 5%) cleavage instead of thioscavengers at all. The significance studying the thioscavengers with respect to azide reduction is given by the increasing application of azide functionalised peptides and PNAs in various ligation methods.

Experimental Part

Amino acids Fmoc-Orn(N_3)-OH, Fmoc-5,5-diiodoallylglycine-OH and building blocks 3-azido-propionic acid and 6-azido-hexanoic

Table 1. Peptides and peptide/PNA oligomers submitted to different cleavage conditions

Azide containing oligomers	TFA/H_2O/TIS	TFA/H_2O/TIS/EDT	TFA/*m*-cresol
$N_3(CH_2)_2CO$-caccXKK-NH_2 (**1**)	n.d[a]	amine[b]	n.d.
$N_3(CH_2)_2CO$-gtggXKK-NH_2 (**2**)	n.d.	amine	n.d.
$N_3(CH_2)_5CO$-gcaccKK-NH_2 (**3**)	azide	29/71[c]	azide
$N_3(CH_2)_5CO$-cgtggKK-NH_2 (**4**)	azide	50/50	azide
Fmoc-Orn(N_3)GKGLKK-NH_2 (**5**)	azide	37/63	azide
Fmoc-Orn(N_3)GK(G-Fmoc)GLKK-NH_2 (**6**)	azide	18/82	azide

Significant reduction to the respective amine was observed as a side reaction with TFA/water/TIS/EDT.
[a] Not determined.
[b] Only the mentioned species were observed.
[c] Amine/azide ratio as estimated by analytical HPLC.

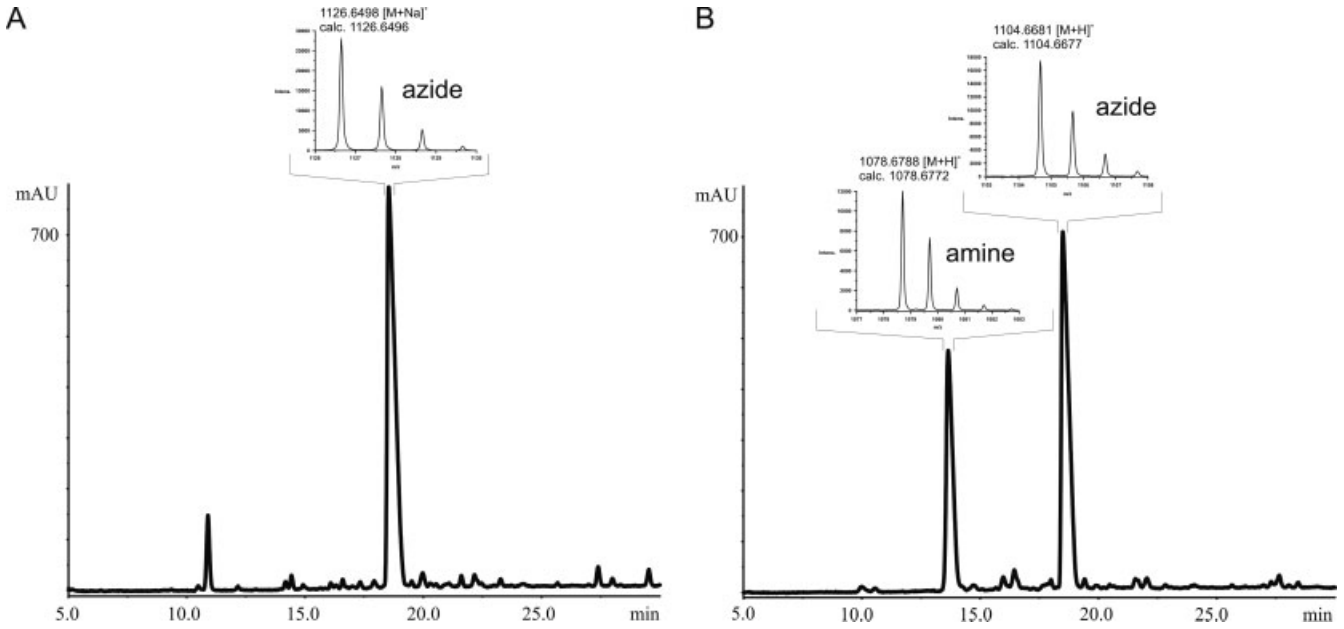

Figure 2. HPLC analysis [gradient A (0.1% TFA in water) → B (0.1% TFA in MeCN/water 8 : 2): 25–80% B in 30 min] of the crude peptide **5** with corresponding HR-MS data after cleavage from the solid support under (A) TFA/*m*-cresol cleavage conditions preserving the azide compound and (B) TFA/water/TIS/EDT cleavage cocktail producing a mixture of the azide and the reduced species.

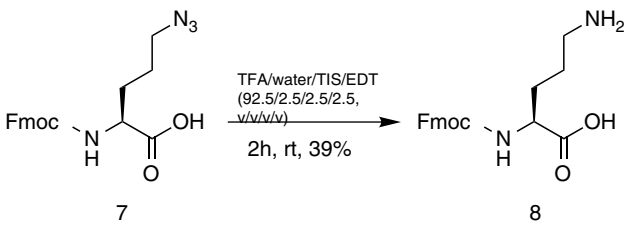

Figure 3. Reduction of azide amino acid **7** under the conditions of a SPPS-cleavage cocktail.

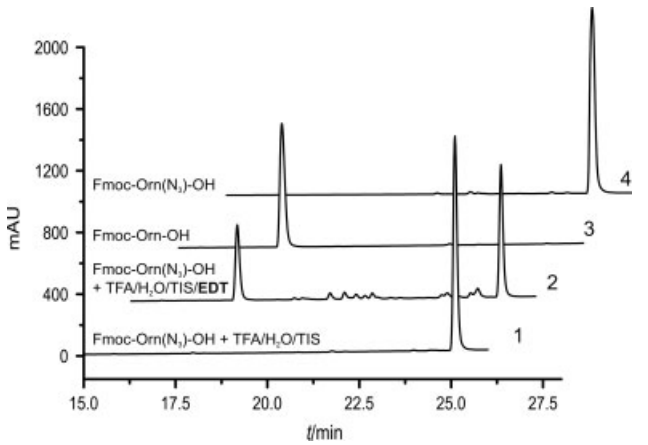

Figure 4. HPLC analysis [gradient A (0.1% TFA in water) → B′ (0.1% TFA in MeCN): 0–80% B in 30 min] investigating the influence of the cleavage cocktails TFA/H_2O/TIS (trace 1) and TFA/H_2O/TIS/EDT (trace 2) on Fmoc-Orn(N_3)-OH (**7**). Fmoc-Orn-OH (**8**) (trace 3) and azide **7** (trace 4) are depicted for comparison.

acid, used in SPPS, were synthesised following procedures described elsewhere [14,15,41]. The analytical data can be found in the Supporting Information. For materials and methods, manual and automated microwave assisted SPPS, analytical data of the monomer reaction depicted in Figure 3 (NMR and HPLC analysis) and the analytical data of the peptide compounds **1**–**6** and **9**–**13** please also note the Supporting Information.

Peptide Synthesis

Peptides **1**–**6** were manually synthesised via Fmoc-SPPS on a preloaded Fmoc-Lys(Boc)-NovaSyn TGR® resin (0.203 mmol/g) at scales of 5 µmol for peptide/PNA conjugates **1**–**4** and 10 µmol for peptides **5, 6**. Peptides **9**–**13** were also synthesised via Fmoc-SPPS but on a preloaded H-Ala-Wang resin (0.1 mmol, 0.42 mmol/g) using an automated peptide synthesiser (Liberty, CEM) with a microwave reaction cavity (Discover, CEM). The precleavage treatment was accomplished by alternate washing (10×) with DCM and MTBE (each 5×) followed by drying over KOH. The TGR (PEG)-resin samples were swollen in DCM (20 min) prior to cleavage cocktail treatment. To the dry Wang resin fractions the respective cleavage mixture was added directly. The following cleavage cocktail compositions have been applied to the resin-bound peptides for 2 h at room temperature. Peptides **1**–**6**: (i) TFA/water/TIS (95 : 2.5 : 2.5 *v/v/v*); (ii) TFA/water/TIS/EDT (90 : 2.5 : 2.5 : 5 *v/v/v/v*); (iii) TFA/*m*-cresol (95 : 5 *v/v*). Peptides **9**–**13**: (i) TFA/water/TIS/EDT (92.5 : 2.5 : 2.5 : 2.5 *w/w/w/w*); (ii) TFA/TIS/water/DTT (92.5 : 2.5 : 2.5 : 2.5 *w/w/w/w*); (iii) TFA/TIS/water/thioanisol (92.5 : 2.5 : 2.5 : 2.5 *w/w/w/w*). The cleavage mixtures were concentrated or dried in a nitrogen stream. Whenever possible, the crude peptides were precipitated using cold MTBE, diethyl ether or pentane also to avoid effects of increasing amount of thiol upon concentration and evaporation. Precipitated fractions were centrifuged at −20 °C, the supernatant was removed and the resulting peptide pellets were dried over KOH and dissolved in water with a little amount of acetonitrile when needed. Peptide fractions that were not possible to precipitate were directly dissolved for RP-HPLC.

Journal of PeptideScience

Table 2. Sequence product correlation giving the amine to azide ratio of the systematically designed peptides **9**–**13** dependent on the applied thiol scavenger, peptide polarity and position of the azide moiety

Azide containing oligomers	TFA/H_2O/TIS/EDT	TFA/H_2O/TIS/DTT	TFA/H_2O/TIS/thioanisol
$N_3(CH_2)_5CO$-LPFGVYA-OH (**9**)	50/50	15/86	17/83
$N_3(CH_2)_5CO$-SYNKGTA-OH (**10**)	35/65	13/87	17/83
H-GLPFOrn(N_3)VYA-OH (**11**)	20/73	8/92	17/83
H-GSYNKOrn(N_3)TA-OH (**12**)	13/87	4/96	14/86
$N_3(CH_2)_5CO$-WIPGVYA-OH (**13**)	44/56	13/87	7/93

Peptide purification and characterisation

HPLC analysis was either performed by using a Pharmacia Äkta basic instrument (GE Healthcare, London, UK) with a pump type P-900, variable wavelength detector UV-900 applying a linear gradient of A (0.1% TFA in water) to B (0.1% TFA in MeCN/water 8 : 2) or by using a Jasco semi-micro HPLC system (Jasco, Groß-Umstadt, Germany) equipped with two PU-2085Plus pumps, a diode array multi-wavelength detector MD-2010Plus, a CO-2060Plus column thermostat and an AS-2055Plus auto sampler device applying a linear gradient of A (0.1% TFA in water) to B′ (0.1% TFA in MeCN). Ultra-pure water was derived by water purification device 'Simplicity' (Millipore, Bedford, UK). Peptides were analysed using a Jasco Reprosil 100 ODS-A column, RP-C18, 250 × 4.6 mm, 5 µm, 100 Å, with a flow rate of 1 ml min^{-1}; a Jasco Reprosil 100 ODS-A column, RP-C18, 150 × 3.0 mm, 5 µm, 100 Å, with a flow rate of 0.6 ml min^{-1} or a YMC J'spere column ODS-H80, RP-C18, 250 × 4.6 mm, 4 µm, 80 Å, with a flow rate of 1 ml min^{-1}.

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Supporting information

Supporting information may be found in the online version of this article.

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