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A convenient synthesis of azido peptides by post-assembly diazo transfer on the solid phase applicable to large peptides

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Abstract—An efficient method for the conversion of solid phase bound peptide amines into azides by a diazo transfer using triflyl azide in the presence of divalent copper ions is described. α-Azido acids and azido peptides—up to 30 amino acids in length—are obtained in good yields and high purities after cleavage from the solid support.

In recent years azides have become of increasing importance in organic chemistry especially for the synthesis of peptides. An azide can be considered as a masked amino group and is used for amine protection adding an extra dimension to protection group orthogonality.[1] Azido acids prepared from α-amino acids have been used in peptide chemistry to overcome difficult coupling reactions.[2] The amine protection offered by azides allows stronger activation of the carboxyl moiety since racemization as a result of oxazolinone formation cannot occur in contrast to the urethane-based amine protection groups. In addition, the formation of diketopiperazines as a notorious side reaction in Fmoc-Wang-based solid phase peptide synthesis is prevented by using azides.[3] Furthermore, azides are versatile precursors in the synthesis of e.g. amides,[4] triazoles, tetrazoles[5] and nitrenes. Until now α-azido acids have been prepared in solution from α-bromo acids by a S_N2 type displacement with sodium azide[2b] or from α-amino acids by diazo transfer[6] using the optimized procedure of copper(II)-catalyzed diazo transfer as developed by Wong.[7]

In this paper the application of the diazo transfer reaction on the solid support for the conversion of solid phase-bound peptide amines into the corresponding azides is described. This methodology provides facile access to unprotected azido peptides, i.e. peptides in which *only* the N-terminal α-amino group is converted into an azide. These azido peptides will be applied in 1,3-dipolar cycloaddition reactions and in amide-forming ligation reactions featuring the Staudinger reaction.[8]

As model compounds for diazo transfer in solution we used phenylalanine and leucine **1** as well as the corresponding methyl esters **3** (Scheme 1).[9] Both amino acids and methyl esters were smoothly converted into their corresponding azido derivatives by the method of Wong.[7] Triethylamine was used as the base instead of K_2CO_3 to prevent ester hydrolysis of **3** and **4**. Water was found not to be essential for an optimal diazo transfer. As a result of the poor water solubility of **4a** and **4b**, triflyl amide could not be removed by simple aqueous extraction, as was described by Pelletier,[3] but extensive chromatographic purification was required hinting at the possible versatility of a solid phase procedure where triflyl amide could be removed simply by washing.

The next step was the conversion of a phenylalanine residue attached to the solid phase into the corresponding azido acid, **2a** (Scheme 1). As was already shown in the case of the methyl esters, the presence of water was not essential and could be replaced by MeOH. The solubility of $CuSO_4$ in MeOH/DCM (9/1) v/v was found to be sufficiently high to ensure an efficient diazo transfer. The diazo transfer could be monitored by the Kaiser test[10] and was found to be complete within 16 h. After cleavage from the resin, azido phenylalanine (**2a**) was obtained in good yield and in high purity. In order to completely remove divalent copper ions from the reaction mixture it was essential to wash the resin with

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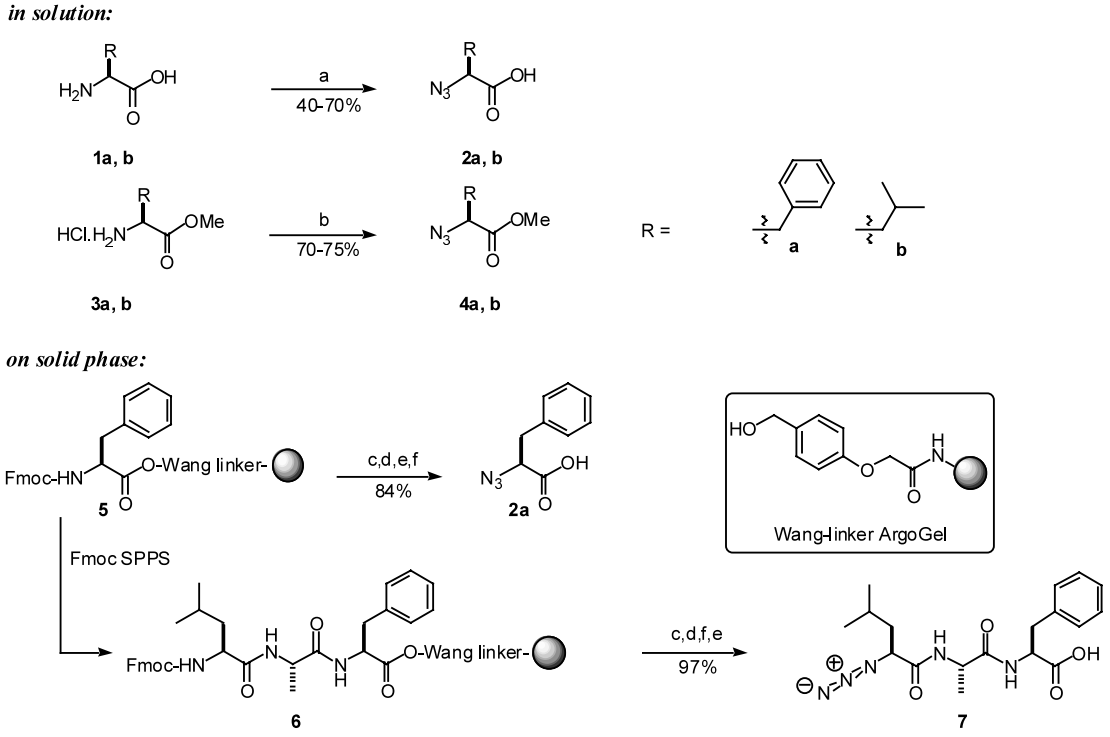


Scheme 1. *Reaction conditions:* (a) CF_3SO_2-N_3, $CuSO_4$, K_2CO_3 in DCM/MeOH/H_2O (9/1/1 v/v/v), rt, 16 h. (b) CF_3SO_2-N_3, $CuSO_4$, TEA in DCM/MeOH (9/1 v/v), rt, 16 h. (c) 20% piperidine in NMP, rt, 3×8 min. (d) CF_3SO_2-N_3, $CuSO_4$, DCM/MeOH (9/1 v/v), rt, at least 16 h. (e) Washing step with 0.02 M diethyldithiocarbamic acid sodium salt, 3×10 min. (f) TFA/DCM (1/1 v/v), rt, 1 h.

a solution of diethyldithiocarbamic acid sodium salt, since residual copper ions led to very broad peaks in the NMR spectra.

The model studies were concluded by treatment of peptide H-Leu-Ala-Phe-O-Wang-ArgoGel with triflyl azide/$CuSO_4$ under the same conditions as H-Phe-O-Wang-ArgoGel. Diazo transfer proceeded smoothly and the azido tripeptide **7** was obtained in good yield and purity (Scheme 1).[11]

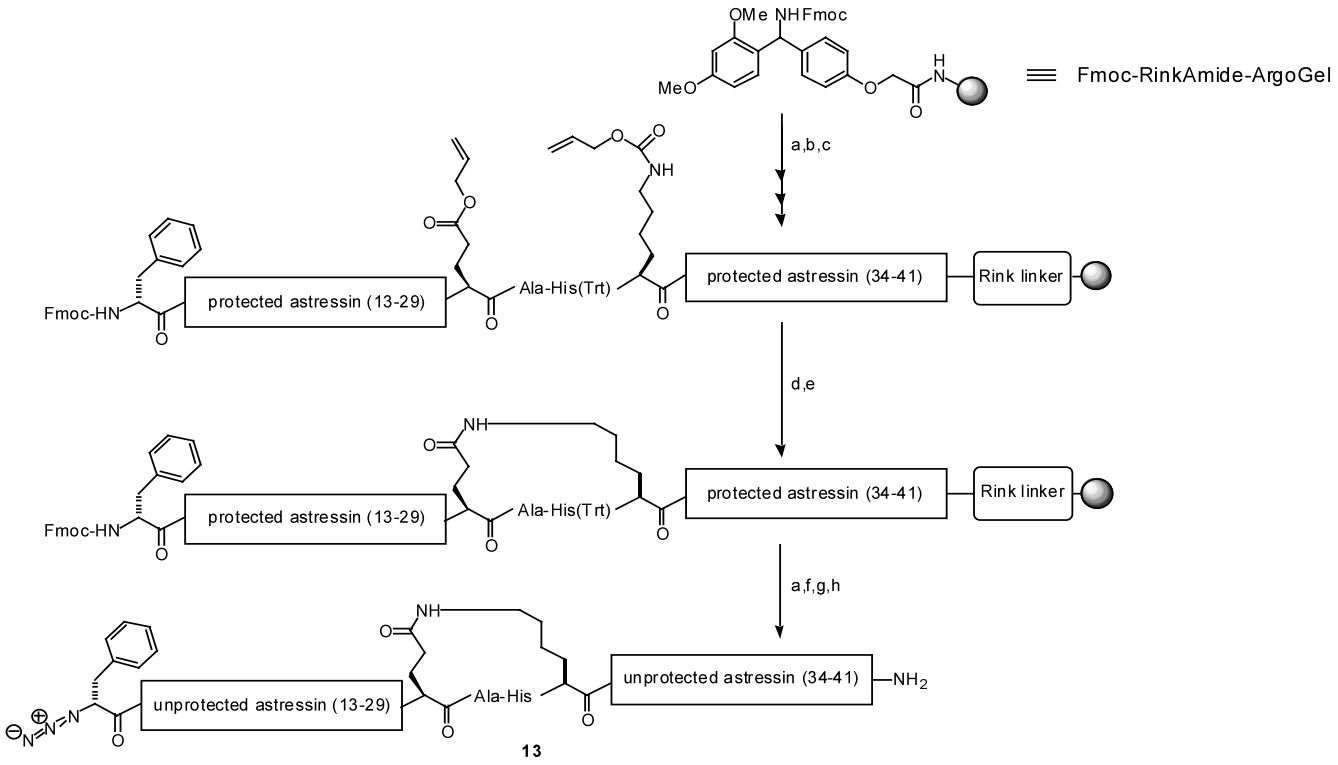
A series of peptides was prepared on solid phase, either on Wang or Rink linkers, and following N-terminal Fmoc deprotection each of them was converted into the corresponding terminal azides using the protocol described above for H-Leu-Ala-Phe-O-Wang-ArgoGel (Table 1). The sequences represent the C-terminal part of human parathyroid hormone[12a] (81–84) **8** and (77–84) **9** respectively, the neuropeptide angiotensin I amide **10**,[12b] the peptide fragment (20–29) derived from amyloid fibril forming human islet amyloid polypeptide **11**,[12c] residues 12–23 of the antimicrobial peptide magainin I amide **12**[12d] and finally, the most active peptide antagonist of corticotropin releasing factor, astressin **13**.[12e] The synthesis of the latter is shown in Scheme 2.

The peptides cover both a wide range of side chain functionalities—including the easily oxidizable methionine—and chain length. Several of these peptides were also selected to investigate the influence of secondary structure (amphiphilic α-helices: **12** and **13** and β-sheets in amyloid fibrils of **11**) on the diazo transfer efficiency. It was found that only the length of the peptide is a major determining factor for a sluggish diazo transfer. Thus, if the length of peptide sequence increased, the concentration of $CuSO_4$ had to be increased up to 5 mg/mL in methanol due to complexation of copper even by the protected peptide, otherwise diazo transfer was incomplete. Treatment with TFA/

Table 1. Azido peptides synthesized in this study

Compound	Sequence	Molecular mass[b] found (calcd)
7	N_3-Leu-Ala-Phe-OH	375.25 (375.19)
8	N_3-Ala-Lys-Ser-Gln-OH	459.30 (459.23)
9	N_3-Val-Leu-Thr-Lys-Ala-Lys-Ser-Gln-OH	900.65 (900.54)
10	N_3-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-NH_2	1321.72 (1321.69)
11	N_3-Ser-Asn-Asn-Phe-Gly-Ala-Ile-Leu-Nle-Ser-NH_2	1060.00 (1060.55)
12	N_3-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Lys-Ser-NH_2	1338.72 (1338.70)
13[a]	N_3-D-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Nle-Ala-Arg-Ala-Glu-Gln-Leu-Ala-Gln-Glu*-Ala-His-Lys*-Asn-Arg-Lys-Leu-Nle-Glu-Ile-Ile-NH_2	3590.20 (3590.23)[c]

[a] Glu*/Lys*: a cyclic lactam formed via the side chains of these two amino acid residues (Scheme 2).
[b] Molecular masses are given as the mono isotopic values.
[c] Average mass value.



Scheme 2. *Reaction conditions:* (a) 20% piperidine in NMP, rt, 3×8 min. (b) Fmoc-Ile-OH/HBTU/HOBt/DIPEA in NMP, rt, 45 min. (c) 29 cycles of Fmoc-deprotection and amino acid coupling. (d) Phenylsilane/$Pd(Ph_3P)_4$ in 1,2-dichloroethane, rt, 16 h. (e) BOP/HOBt/DIPEA in NMP, rt, 16 h. (f) CF_3SO_2-N_3, $CuSO_4$, DCM/MeOH (9/1 v/v), rt, at least 16 h. (g) Washing step with 0.02 M diethyldithiocarbamic acid sodium salt, 3×10 min. (h) TFA/triisopropylsilane/H_2O (95/2.5/2.5 v/v), rt, 3 h.
Protected astressin (13–29): ∼His(Trt)-Leu-Leu-Arg(Pbf)-Glu(O^{t}Bu)-Val-Leu-Glu(O^{t}Bu)-Nle-Ala-Arg(Pbf)-Ala-Glu(O^{t}Bu)-Gln-(Trt)-Leu-Ala-Gln(Trt)∼, protected astressin (34–41): ∼Asn(Trt)-Arg(Pbf)-Lys(Boc)-Leu-Nle-Glu(O^{t}Bu)-Ile-Ile∼, unprotected astressin (13–29): ∼His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Nle-Ala-Arg-Ala-Glu-Gln-Leu-Ala-Gln∼, unprotected astressin (34–41): ∼Asn-Arg-Lys-Leu-Nle-Glu-Ile-Ile-NH_2; Boc: *tert*-butyloxycarbonyl, ^{t}Bu: *tert*-butyl, Nle: norleucine, Pbf: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl, Trt: trityl.

H_2O/triisopropylsilane (TIS) for 3 h did not affect the integrity of the azide functionality and all protective groups were completely removed as was evidenced by ES-MS, HPLC and IR.[13] Sulfur containing scavengers, however, such as 1,2-ethanedithiol could not be used, since the azido functionality was then reduced to the free amine.

In conclusion, we have shown that azido peptides could be synthesized by a post-assembly diazo transfer on the solid phase. Large peptide azides are readily accessible and any secondary structure does not interfere with their formation. Cleavage from the resin did not affect the integrity of the azido functionality and resulted in excellent yields and chemical purities of crude azido peptides. Under present investigation are applications of these peptides in which the azido moiety can be used as an orthogonally reactive group for amide-forming ligation, conjugation and cycloaddition reactions of peptides.

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9. **CAUTION**: We have not experienced any serious problems in handling triflyl azide in spite of its highly explosive nature. However, it should be kept in solution and synthesized prior to use[6,7] in the diazo transfer reaction. **N_3-Phe-OMe** (**4a**): yield 70%, colorless oil; R_f (EtOAc/hexane, 1/1, v/v): 0.80; IR (KBr): υ 2100 cm^{-1}; $[\alpha]_D^{25}$: −39.2 $c=1.4$ $CHCl_3$; ES-MS: $C_{10}H_{11}N_3O_2$, $M=205$, $(M+H)^+$ 206, $(M-CH_3)^-$ 199; ^1H NMR (300 MHz) δ ($CDCl_3$): 3.00 (dd, 1H), 3.18 (dd, 1H), 3.81 (s, 3H), 4.05 (dd, 1H), 7.2–7.4 (m, 5H); ^{13}C NMR (75 MHz) δ ($CDCl_3$): 37.5, 52.6, 63.2, 127.2, 128.6, 129.1, 135.8, 170.5; **N_3-Leu-OMe** (**4b**): yield 75%, colorless oil; R_f (EtOAc/hexane, 1/1, v/v): 0.80; IR (KBr): υ 2100 cm^{-1}; $[\alpha]_D^{25}$: −5.8 $c=2$ $CHCl_3$; ES-MS: $C_7H_{13}N_3O_2$, $M=171$, $(M+H)^+$ 172, $(M-CH_3)^-$ 156; ^1H NMR (300 MHz) δ ($CDCl_3$): 0.98 (2×d, 6H), 1.75–1.95 (m, 1H+2H), 3.80 (s, 3H), 3.85–4.0 (dd, 1H); ^{13}C NMR (75 MHz) δ ($CDCl_3$): 21.7, 22.6, 24.9, 52.5, 59.0, 171.6.
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11. **Typical procedure for diazo transfer on the solid support**: Preparation of N_3-Leu-Ala-Phe-OH (**7**). Fmoc-Leu-Ala-Phe-O-Wang-ArgoGel (**6**) (350 mg resin, 0.345 mmol/g, 0.12 mmol) was synthesized using standard Fmoc/*t*Bu synthesis protocols; coupling reactions were carried out with HBTU/HOBt/DIPEA in NMP. After Fmoc removal by treatment of the resin with 20% piperidine in NMP, an excess of triflyl azide (2 mmol, 16.7 equiv.) in DCM (9 mL) in the presence of a catalytic amount of $CuSO_4$ (2 mg, 12.6 μmol, 0.10 equiv.) in MeOH (1 mL) were added. This reaction mixture was swirled for 18 h at room temperature. The completeness of the diazo transfer could be followed with the Kaiser test; colorless resin beads implied that the conversion of the amino group into the azido functionality had been completed. The resin was subsequently washed with NMP (3×2 min), 0.5% DIPEA in NMP (3×2 min), 0.05 M diethyldithiocarbamic acid sodium salt in NMP (3×10 min), NMP (5×5 min) and DCM (3×3 min). The washing step with diethyldithiocarbamic acid sodium salt is a prerequisite to remove residual divalent copper. The azido peptide was cleaved from the solid support by treatment with TFA in DCM (1/1 v/v, 10 mL) for 1 h at room temperature. After filtration of the resin the TFA solution was evaporated to yield a yellowish oil. Crude yield: 41 mg (97%); HPLC purity: 93% (rt: 21.06 min on Adsorbosphere XL (C8 90 Å 5 μm, 250×4.6 mm) in a linear gradient of 100% buffer A (0.1% TFA in H_2O) to 100% buffer B (0.085% TFA in CH_3CN/H_2O, 95/5 v/v) in 20 min at 1 mL/min); IR (KBr): υ 2100 cm^{-1}; $[\alpha]_D^{25}$: −30 $c=0.1$ $CHCl_3$; ES-MS: $C_{18}H_{25}N_5O_4$, $M=375.19$, $(M+H)^+$ 375.25; ^1H NMR (300 MHz) δ ($CDCl_3$): 1.05 (2×d, 6H), 1.35 (d, 3H), 1.8–2.0 (broad m, 3H), 3.05 (dd, 1H), 3.25 (dd, 1H), 3.83 (dd, 1H), 4.45 (t, 1H), 4.80 (dd, 1H), 6.79 (d, 1H), 7.02 (d, 1H), 7.1–7.3 (m, 5H), 7.81 (broad s, 1H); ^{13}C NMR (75 MHz) δ ($CDCl_3$): 17.7, 21.5, 23.0, 24.9, 37.2, 41.1, 48.4, 62.3, 127.1, 128.5, 129.3, 135.6, 170.0, 171.2, 174.5.
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13. After treatment of the protected and resin-bound peptides with TFA/H_2O/TIS (95/2.5/2.5 v/v/v) for 3 h, the TFA solution was filtered and diluted with methyl *tert*-butyl ether/hexane (1/1 v/v) upon which the azido peptides **8**–**13** precipitated. The precipitates were collected by centrifugation, the pellets were resuspended/centrifuged in methyl *tert*-butyl ether/hexane, 1/1 v/v (three times). Finally, the crude azido peptides were lyophilized from *tert*-BuOH/H_2O (1/1 v/v) or CH_3CH/H_2O (1/1 v/v). The purity and identity were checked by HPLC and ES-MS, respectively. FTIR confirmed the presence of an azide moiety ($\upsilon=2100$ cm^{-1}). The azido peptides were stable if stored at −20°C in the dark.