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The Backbone *N*-(4-Azidobutyl) Linker for the Preparation of Peptide Chimera[#]

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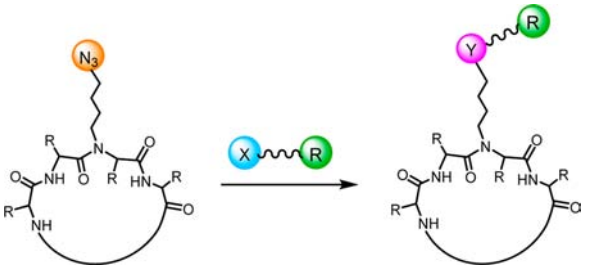
ABSTRACT



A robust synthetic strategy for the introduction of the *N*-(4-azidobutyl) linker into peptides using standard SPPS techniques is described. Based on the example of Cilengitide it is shown that the *N*-(4-azidobutyl) group exerts similar conformational restraints as a backbone *N*-Me group and allows conjugation of a desired molecule either via click chemistry or—after azide reduction—via acylation or reductive alkylation.

The site-specific covalent attachment of "unnatural" moieties, such as fluorophores, radiolabels, affinity labels, or polymers, to peptides has proven useful for a wide variety of applications.[1] The conjugation of peptides with a desired molecule is typically performed at the *N*-terminus or at naturally occurring side-chain functional groups.[2] Cyclic peptides or even some linear peptides without derivatizable groups often require the introduction of additional residues, such as Lys or Cys, to support side-chain-selective conjugation. In the case of cyclic peptides, additional amino acids cannot be introduced, and finding a suitable position for amino acid replacement is not straightforward. Along these lines, bio-orthogonal conjugation methods that target unnatural amino acids are becoming valuable alternatives to the more commonly used Lys- and Cys-based strategies, as they do not involve cumbersome protection and deprotection protocols.[3] However, each of the techniques currently available for peptide modification have specific drawbacks, and generally there is a lack of widely usable and flexible methods.

We envisaged that modification of the backbone amide groups with a functionalized *N*-substituent may be a valuable addition to the chemist's toolbox to perform peptide conjugation. Kessler's group has shown that the introduction of *N*-Me groups in peptide ligands can optimize their activity and receptor selectivity as a result of conformational modulation.[4] Furthermore, backbone *N*-Me groups are common structural motifs in many bioactive peptides isolated from natural sources.[4]

Here we describe the *N*-(4-azidobutyl) group as linker for the attachment of molecules. This *N*-substituent can be

[#] Dedicated to Professor Klaus Burger on the occasion of his 75th birthday.

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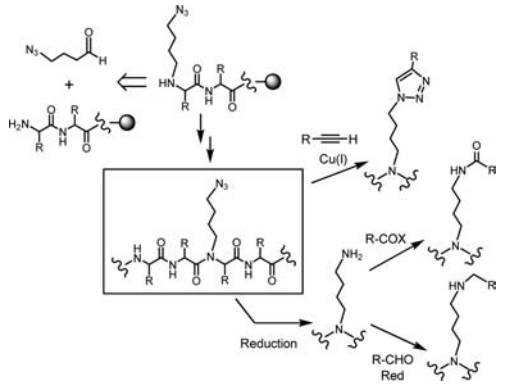
Scheme 1. Peptide Modification through the *N*-(4-Azidobutyl) Linker



introduced into a resin-bound peptide by reductive alkylation with 4-azidobutanal, providing an azide onto which alkyne-functionalized molecules can be grafted by Cu(I)-catalyzed 1,3-dipolar cycloaddition (Scheme 1). Alternatively, the azide group can be reduced to an amine, onto which molecules can be conjugated via amide bond formation or via reductive alkylation. The azide function is stable to common deprotection protocols used in peptide synthesis and chemically inert to side-chain functional groups,[5] thereby minimizing side reactions and simplifying protection schemes.

A few years back, Kirshenbaum et al. showed that *N*-azidopropyl groups are straightforward to incorporate in peptoid sequences using an azido amine as a submonomer reagent, and that azide-functionalized peptoids can be used as substrates for azide−alkyne cycloaddition reactions.[6] However, the submonomer approach is only efficient for the preparation of *N*-substituted Gly oligomers. Also worth mentioning is that there is no reported example in which a peptide with a backbone *N*-azidoalkyl substituent has been obtained.

To demonstrate the applicability of our *N*-(4-azidobutyl) linker strategy, Cilengitide was chosen as a model. This Arg-Gly-Asp (RGD)-peptide is a good example of the difficulty involved in preparing conjugates of small cyclic peptides that do not offer attachment sites and/or that are not amenable to structural modification while preserving biological activity. The RGD-cyclopeptide sequence of Cilengitide, cyclo[RGDf*N*MeV], is the result of systematic research to constrain the RGD motif in its optimum conformation for binding to the $\alpha_v\beta_3$-integrin receptor, which is overexpressed in various malignant cancers and in tumor neovasculature.[6] The functionalization of RGD-cyclopeptide ligands that target this receptor is of great interest, as it allows the conjugation of suitable chemical entities for tumor imaging and therapeutics.[7] However, Cilengitide cannot be conjugated as it is. Among the five amino acids in its cyclic structure, three (RGD) are essential for binding to the receptor, D-Phe is involved in hydrophobic interactions, and *N*MeVal has no derivatizable functional group.[8] Substitution of *N*MeVal by Lys led to cyclo[RGDfK], one of the most conjugated peptide ligands which is used in a number of biomedical applications.[9] However, a decrease in biological activity has to be taken into account when replacing *N*MeVal by Lys, as the *N*-Me group of Val promotes constraints that stabilize the RGD motif in its preferred $\alpha_v\beta_3$-binding conformation.[10]

We report on the synthesis of an analog of cyclo-[RGDf*N*MeV] (**1**) in which the N-Me group of Val is replaced by the *N*-(4-azidobutyl) group (**2**), with minimal perturbation of the original conformation. By preparing various PEG conjugates from **2**, we show that our linker allows conjugation onto cyclic peptides under full conservation of their amino acid sequence.

To obtain the *N*-azidoalkylated cyclopeptide (**2**), its linear pentapeptide precursor (**3**) was prepared by stepwise solid-phase peptide synthesis (SPPS) on 2-chlorotrityl chloride (CTC) resin and then cleaved for subsequent cyclization and side-chain deprotection (Scheme 2). Positioning of the *N*-alkylated residue in the middle of the sequence of **3** minimizes steric hindrance during cyclization and is expected to facilitate this process as a result of backbone preorganization.[11]

The *N*-(4-azidobutyl) group was introduced into the resin-bound peptide by reductive alkylation with 4-azidobutanal in the presence of $NaBH_3CN$. The reaction was tested with various amounts of aldehyde; with 1.5 equiv, most *N*-terminal Val was exclusively *N*-monoalkylated. Taking advantage of the low reactivity of this secondary amine, the small amount of unreacted resin-bound peptide was capped with Ac_2O in order to facilitate the final purification. The foreseeable challenging step was the coupling of Fmoc-D-Phe onto *N*-(4-azidobutylated) Val. The acylation of this sterically demanding residue did not take place under conditions reported to be efficient for coupling D-Phe onto *N*MeVal. Stronger activation methods, such as PyBOP/HOAt and HATU/HOAt, also failed to form the desired product. Finally, this coupling was achieved by activating Fmoc-D-Phe with bis(trichloromethyl)-carbonate (BTC) in the presence of 2,4,6-trimethylpyridine.[12] After three prolonged couplings (15 h), acylation was almost complete and no epimerization was detected (HPLC). Further peptide elongation and cleavage afforded pentapeptide **3**, which was easy to cyclize with EDC and catalytic amounts of 4-DMAP. The Pbf- and ^{t}Bu- groups were then removed, and RP-HPLC purification rendered **2** in 17% overall yield.

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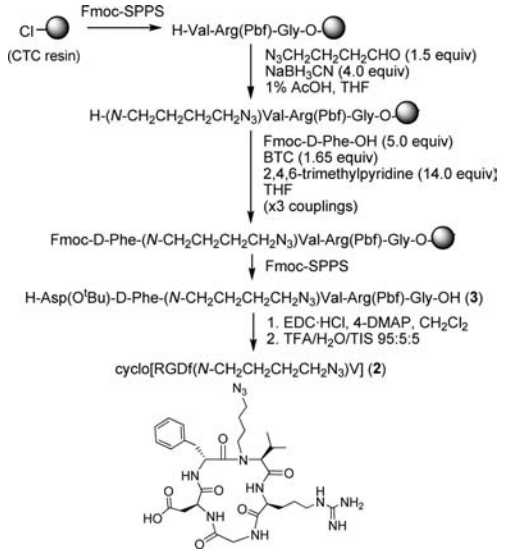
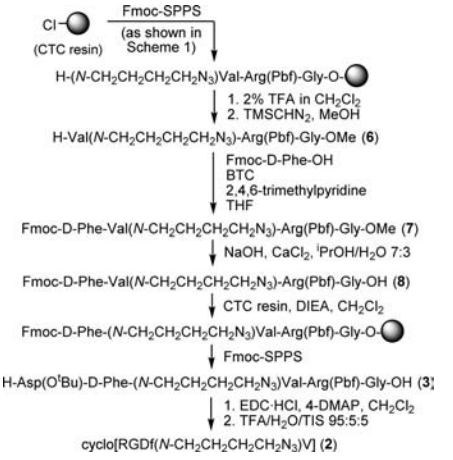
Scheme 2. Small-Scale Synthesis of Cyclo[RGDf(*N*-$CH_2CH_2CH_2CH_2N_3$)V] (**2**)



However, when the SPPS of pentapeptide **3** was performed in a larger amount of resin (> 3 g) and/or with a higher functionalization (> 0.50 mmol g^{-1}), the yields were not as satisfactory. To obtain larger amounts of **3**, an efficient *double SPPS* scheme was developed using the CTC resin for elongation, *de- and reattachment* of fully protected peptide, and final elongation again (Scheme 3). In this approach, the Val-Arg(Pbf)-Gly sequence was assembled followed by reductive alkylation of its *N*-terminus with 4-azidobutanal, as previously described. At this stage, the peptide was cleaved with 2% TFA in CH_2Cl_2, and its *C*-terminus was protected as a methyl ester. The coupling between Fmoc-D-Phe and the *N*-azidoalkylated peptide segment (**6**) was performed in solution using the BTC method. This procedure allowed us to obtain the desired peptide (**7**), which was isolated in 48% yield. Then, the methyl ester of **7** was hydrolyzed under basic conditions in the presence of $CaCl_2$, which is reported to suppress Fmoc- decomposition.[13] Using this additive, the methyl ester was saponified with no detectable Fmoc- decomposition (HPLC-MS). The desired peptide (**8**) was isolated by simple aqueous extraction and loaded again onto the CTC resin. The incorporation of **8** took place with an acceptable yield (i.e., 80% peptide incorporation for an expected functionalization of 0.10 mmol g^{-1}). Further peptide chain elongation and cleavage from the resin yielded pentapeptide **3**, which was cyclized and deprotected as described above. After RP-HPLC purification, the *N*-azidoalkylated cyclopeptide (**2**) was obtained in 18% overall yield.

With the synthesis of **2**, we demonstrate that *N*-(4-azidobutylated) peptides are accessible using standard SPPS protocols that are compatible with common protecting groups used in peptide synthesis. Taking into account

Scheme 3. Large-Scale Synthesis of Cyclo[RGDf(*N*-$CH_2CH_2CH_2CH_2N_3$)V] (**2**)



that the acylation of the *N*-alkylated residue was achieved on a small scale using BTC, we consider that the detour from solid-phase to solution chemistry in the synthesis of **2** was necessary because of the additional steric hindrance exerted by the β-branched side chain of Val and that this change may not be a general requirement for the synthesis of other *N*-(4-azidobutylated) peptides.

It is reasonable to assume that the incorporation of an *N*-(4-azidobutyl) group into a cyclic peptide will exert the same conformational restrictions as a backbone *N*-Me group. The conformation of small cyclic peptides is dictated by their backbone stereochemistry and by the presence of *N*-alkyl groups, rather than by the interactions with or among the amino acid side chains.[14] To test this notion, we performed a detailed NMR study of cyclo[RGDf*N*MeV] (**1**) and its *N*-(4-azidobutylated) analog (**2**). Both peptides had very similar H^N-, H^α-, and C^α-chemical shifts, and their amide protons had almost identical temperature coefficients ($\Delta\delta/\Delta T$) and very similar vicinal scalar coupling constants [$^3J(H^N-H^\alpha)$] (see Supporting Information). The close resemblance of these NMR parameters, which are highly sensitive to conformational changes, indicates that replacement of the *N*-Me group of **1** by our linker provided a minimal perturbation of its conformational state.

To demonstrate the applicability of the *N*-(4-azidobutyl) linker, we prepared several conjugates of **2** with PEG. Conjugate **11** was obtained from **2** by Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition with a polydisperse PEG-alkyne (2 KDa). To obtain conjugates **12** and **13**, the azido group of **2** was first reduced to an amine with the mild Zn/NH_4Cl reducing system. The resulting *N*-(4-aminobutylated) cyclopeptide (**10**) was acylated with a polydisperse PEG-COOSu derivative (2 KDa) to yield conjugate **12**, whereas reductive alkylation of **10** with a polydisperse PEG-propionaldehyde (2 KDa) furnished conjugate **13**. The optimized conditions for each transformation are shown in Scheme 4. Due to the polydispersity of PEG,

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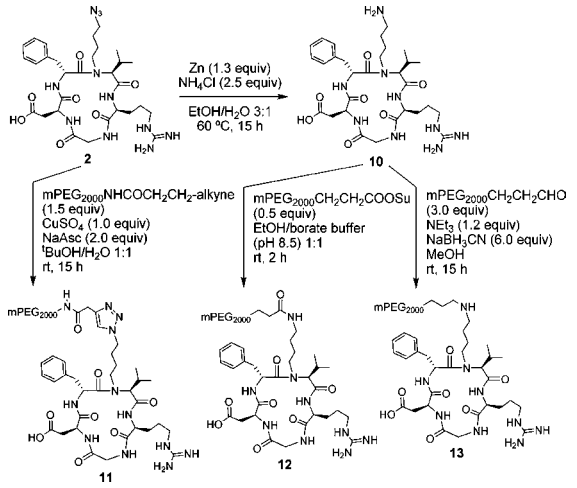
Scheme 4. Synthesis of PEG-Conjugates **11**–**13**



conditions had to be carefully optimized in order to facilitate the RP-HPLC purification of the PEG-conjugates (**11**–**13**).

The PEG-conjugates (**11**–**13**), the *N*-azidoabutylated cyclopeptide (**2**), and cyclo[RGDf*N*MeV] (**1**) were analyzed by circular dichroism (CD), a sensitive technique to monitor changes in a peptide secondary structure. All compounds showed a positive band between 212 and 216 nm (λ_{max}) and a negative band between 230 and 238 nm (λ_{min}) (Figure 1). CD measurements on peptides **2** and **11**–**13** are consistent with data on the peptide **1**, which has a conformation featuring two inverse γ (γ_i) turns and a γ turn.[10]

The biological activity of the PEG-conjugates (**11**–**13**) and cyclo[RGDf*N*MeV] (**1**) was evaluated in cell adhesion inhibition assays. All compounds were tested for their capacity to inhibit the integrin-mediated adhesion of HUVEC endothelial and DAOY glioblastoma cells to their immobilized ligands vitronectin (VN) and fibrinogen (FB) (Table 1). For all the cell/ligand systems, all the compounds inhibited cell adhesion in a concentration-dependent manner, and the same pattern of inhibitory activities was observed. The PEG-conjugates (**11**–**13**) showed IC_{50} values in the low μM range, albeit inferior to those of **1**. The decreased inhibitory activity of **11**–**13** with respect to the parent peptide (**1**) may be attributable to an interference of the PEG chain with the RGD–receptor interaction, resulting in lower binding affinities. Indeed, the reduced biological activity of peptides upon attachment of a bulky PEG chain is an issue of major concern, especially in the case of small peptides.[15]

In conclusion, we have shown that a backbone *N*-(4-azidobutyl) group can be incorporated into a peptide using standard SPPS techniques and allows conjugation at a late stage of the synthesis. Due to the orthogonal properties of the azide, our linker is compatible with side-chain protection strategies, linkers, and resins commonly used in peptide synthesis. Moreover, the chemical versatility of the azide function, which can be reduced to an amine prior to conjugation, allows for the flexible design of peptide conjugates. Along these lines, the possibility of using click chemistry in the conjugation step is an advantageous feature, since it permits conjugation in the presence of side-chain functional groups and thus implies a minimal requirement for protection. On the basis of all these considerations, we strongly believe that our *N*-(4-azidobutyl) linker will have broad utility in peptide chemistry and will widen the application of established conjugation methods.

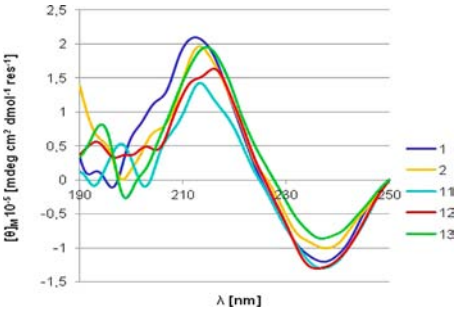


Figure 1. CD spectra of cyclo[RGDf*N*MeV] (**1**), cyclo[RGDf(*N*-$CH_2CH_2CH_2CH_2N_3$)V] (**2**), and compounds **11**–**13** in H_2O at a concentration of 0.5 mM.

Table 1. Adhesion Inhibition Assays of Cyclo[RGDf*N*MeV] (**1**) and Compounds **11**–**13**[a]

	$\alpha_v\beta_3 + \alpha_v\beta_5$		$\alpha_v\beta_3$	
compd	HUVEC on VN	DAOY on VN	HUVEC on FB	DAOY on FB
1	0.37	2.69	0.076	0.44
11	8.90	75.21	0.53	4.36
12	3.06	28.76	0.23	1.77
13	3.40	49.56	0.41	2.71

[a] IC_{50} values are given in μM.

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Supporting Information Available. Experimental details of the syntheses, cellular assays, characterization data, and copies of the HPLC, HRMS, and NMR spectra of selected compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.