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C-Terminal Acetylene Derivatized Peptides *via* Silyl-Based Alkyne Immobilization

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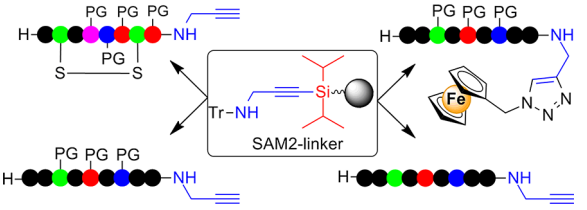
ABSTRACT



A new Silyl-based Alkyne Modifying (SAM)-linker for the synthesis of *C*-terminal acetylene-derivatized peptides is reported. The broad scope of this SAM2-linker is illustrated by manual synthesis of peptides that are side-chain protected, fully deprotected, and disulfide-bridged. Synthesis of a 14-meric $(KLAKLAK)_2$ derivative by microwave-assisted automated SPPS and a one-pot cleavage click procedure yielding protected 1,2,3-triazole peptide conjugates are also described.

From the dawn of resin-based peptide synthesis in 1963,[1] the field has advanced to the current level that allows the preparation of polypeptides, and their use in various ligation strategies to afford even entire proteins.[2,3] As a result of these advancements, peptides are being used in biomedical research,[4] in catalysis,[5] and in advanced materials,[6] to name a few. In light of the many applications of peptides, new synthetic methods for a more convenient preparation of diverse peptides are desired. One of the most prominent diversification methods recently utilized is the solid-phase compatible CuI/D*i*PEA 1,3-dipolar alkyne–azide cycloaddition reaction, as initially described by Meldal and co-workers.[7] This reaction is used not only in peptide conjugation[8] but also across the entire chemical landscape.[9]

In view of the widespread applications of this so-called click reaction and the availability of silyl-based protecting groups for acetylene moieties,[10] we pursued the preparation of a linker that is based on a silyl-protected alkyne and that can be used in standard solid-phase peptide synthesis (SPPS). The first generation of this class of silyl-based alkyne modifying (SAM)-linkers was recently described by our group; after fully automated microwave-assisted SPPS, fluoride-mediated cleavage yielded a *C*-terminal arylacetylene derivatized peptide.[11] Notably, SAM-linkers allow orthogonal click modifications: side-chain positioned acetylene moieties can be clicked during the peptide-chain assembly and orthogonally to the silyl-protected acetylene moiety that is used for immobilization; this can

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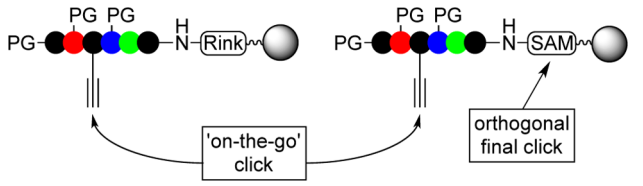

Figure 1. Click modifications possible by Rink Amide (*left*) and SAM (*right*) derivatized solid supports.

be conjugated in a final cleavage-click procedure[12] (Figure 1). Generally speaking, SAM-linkers are particularly useful in those cases where *N*-terminal or side-chain click modifications of peptides are detrimental for their activity, or when an additional final click-conjugation reaction is required that has to be orthogonal to side-chain or *N*-terminal based labeling reactions.

In principle, our first SAM-linker already contained some important properties: it was conveniently prepared on gram scale and showed general SPPS compatibility. However, the presence of an arylacetylene makes the linker incompatible with acids that are required to remove protecting groups in Fmoc/*t*-Bu peptide chemistry. Acid-catalyzed hydration of the carbon−carbon triple bond generates an acetophenone derivative, which can be used in other ligation strategies, but not in the desired click reaction with an azide anymore. Thus, we prepared an improved version of the SAM-linker that is stable toward acid treatments, especially applicable for peptides that need a free *N*-terminus for their activity.[13]

Herein, this versatile and economic second member of the family of SAM-linkers is presented. In order to assess the compatibility of silyl-based alkyne immobilization of the SAM2-linker with SPPS, five nonapeptides containing 18 proteinogenic amino acid derivatives were synthesized using manual Fmoc-based SPPS. In this focused library, acid-stable (Acm for Cys), highly acid-labile (Mtt or Tr for Lys or His and Cys, respectively), and standard (Pbf, Boc, *t*-Bu) protecting groups were used to investigate a wide spectrum of side-chain protection (Table 1). This library was used to assess various cleavage conditions. Lastly, compatibility of the SAM2-linker with on-resin disulfide-bond formation and with microwave-assisted automated SPPS was investigated.

Synthesis of SAM2-linker **1** was performed in three steps with an overall yield of 62%. Propargylamine **2** was protected using triphenylmethylchloride **3**, affording Tr-protected propargylamine **4** in high yield.[14] Subsequently, **4** was treated with *n*-butyllithium in the presence of (3-cyanopropyl)diisopropylchlorosilane (Cl-CPDIS) **5** to yield bis-protected propargylamine **6** with a yield of 90%. Finally, the cyano-group of trityl-protected silyl-alkyne **6**

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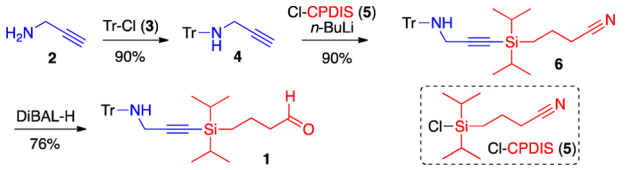
Scheme 1. Synthesis of SAM2-Linker **1**



was reduced with DiBAL-H followed by acidic hydrolysis to yield corresponding aldehyde **1** in good yields (Scheme 1). After this, SAM2-linker **1** was immobilized on a TentaGel HL NH_2 solid support (loading: 0.54 mmol/g) *via* reductive amination using 1 equiv of aldehyde **1** and 1.4 equiv of $NaB(OAc)_3H$. An IR spectrum of linker-loaded resin **7** showed a faint peak at 2172 cm^{-1}, which can be assigned to the carbon−carbon triple bond (see Supporting Information). Unprotected amino groups were acylated with acetic anhydride, followed by Tr-removal using a mixture of DCM/TFA/TIS (94:4:2)[15] and coupling of Fmoc-Leu-OH and Fmoc-Phe-OH using standard SPPS procedures at ambient conditions, resulting in a loading of 0.33 mmol/g as determined by Fmoc-determination. Thus, an overall yield of 61% was achieved with respect to the amount of used linker **1** in seven steps. As peptide elongation steps usually proceed quantitatively, the determined yield most likely correlates to the immobilization of linker **1**.

Table 1. Amino Acid Composition of the Peptide Library

peptide	sequence
a	H-Phe-Val-**Tyr**(*t*-Bu)-Gly-**Gln**(Tr)-Ala-**Cys**(Acm)-Leu-Phe-N(H)CH_2CCH
b	H-Phe-Val-**Thr**(*t*-Bu)-Gly-**Cys**(Tr)-Ala-**Arg**(Pbf)-Leu-Phe-N(H)CH_2CCH
c	H-Phe-Val-**Asp**(O*t*-Bu)-Gly-**Lys**(Mtt)-Ala-**Trp**(Boc)-Leu-Phe-N(H)CH_2CCH
d	H-Phe-Val-**Lys**(Boc)-Gly-**His**(Tr)-Ala-**Glu**(O*t*-Bu)-Leu-Phe-N(H)CH_2CCH
e	H-Phe-Val-**Ser**(*t*-Bu)-Gly-**Asn**(Tr)-Ala-**Met**-Leu-Phe-N(H)CH_2CCH

Parallel synthesis of the focused peptide library was performed on five equal batches of Fmoc-Leu-Phe-SAM2-TG using 20% piperidine/NMP for Fmoc-removal and a mixture of Fmoc-AA(PG)-OH/HOBt/TBTU/D*i*-PEA for coupling reactions (see Supporting Information). Each coupling was analyzed with a Kaiser test[16] to determine completion of the acylation reaction. Fmoc-determination of the completed peptide chains confirmed the stability of SAM2-linker **1** during SPPS (see Supporting Information). After final Fmoc-deprotection, fluoride-mediated cleavage with 0.1 M TMAF·$4H_2O$ in DMF/MeOH (9:1) yielded 77−89% of crude materials (70−94% purity). Subsequent

(15) For the list of abbreviations, see Supporting Information.
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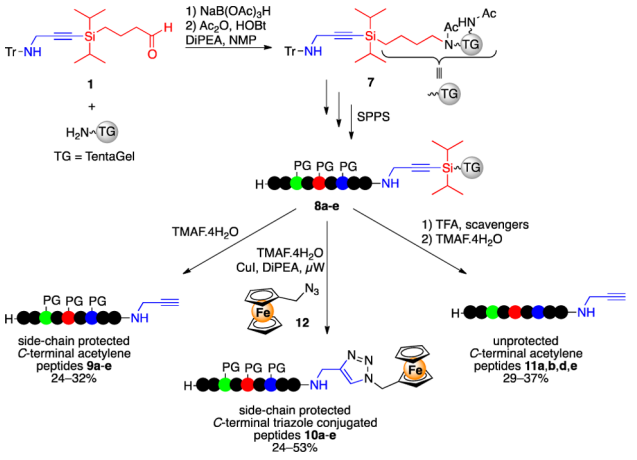
purification by preparative HPLC provided side-chain protected, acetylene derivatized peptides **9a**–**e** in 24–32% yield (Scheme 2). Importantly, except for Asp(O*t*-Bu), all side-chain protecting groups—e.g. Trp(Boc), Lys(Mtt), or Cys(Tr)—remained intact during cleavage and purification. Since Glu(O*t*-Bu) did not release its protecting group upon fluoride treatment, we assume aspartimide formation followed by hydrolysis to be the route to *tert*-butyl ester cleavage in Asp(O*t*-Bu).

After this successful synthesis of protected acetylene-derivatized peptides, compatibility of the SAM2-linker with CuAAC was investigated by applying a one-pot cleavage click procedure for the direct synthesis of peptide conjugates. For this, azidomethylferrocene **12**, an air- and water-stable, redox-active metallocene, was used as a substrate.[17] In short, peptide-loaded resin was combined with a methanolic solution of TMAF·4H_2O, **12**, D*i*PEA, and CuI and was heated under microwave irradiation to 60 °C for 30 min. The click-cleaved products were concentrated to dryness and directly subjected to HPLC purification affording side-chain protected, ferrocene-labeled peptide conjugates **10a**–**e** in 24–53% yield (Table 2). Again, all side-chain protecting groups, excluding Asp(O*t*-Bu), were stable toward these reaction conditions. Thus, the one-pot cleavage click reaction is a highly promising route for the synthesis of *C*-terminally modified peptide conjugates.

After this, the stability of the SAM2-linker toward treatment with TFA/scavengers was investigated. For this, all resin-bound library members were treated with a mixture of TFA/H_2O/TIS/TIA/2-ME (82.5:10:2.5:2.5:2.5, % v/v) at ambient conditions for 1.5 h. Since no peptide material was isolated from the TFA solutions, linker **4** is shown to be stable against treatment with a concentrated TFA/scavenger cocktail, making this SAM2-linker a highly acid-stable alkyne-modifying linker. For comparison, commonly used di-/trialkoxy BAL linkers release peptides upon treatment with 1–30% TFA.[18] After washing the side-chain deprotected resin-bound peptides with D*i*PEA, fluoride cleavage yielded the corresponding deprotected, acetylene-derivatized peptides in 29–37% yield after purification. Methionine oxidation in peptide **11e** was avoided using 1,2-ethylenedithiol instead of 2-mercaptoethanol in the deprotection cocktail. Whereas Cys containing peptide **11b** did not show acceptable purity after fluoride cleavage, Cys(Acm) containing peptide **11a** was cleanly obtained. Therefore, the use of this amino acid is recommended for the synthesis of free thiol functions.[19]

Scheme 2. Solid-Phase Synthesis of the Peptides and Chemical Diversification[a]



[a] Yields of the purified peptides are given.

Lastly, SAM2 solid-support **7** was applied in the synthesis of two biologically relevant peptides: the disulfide-bridged somatostatin analogue octreotate and the antimicrobial 14-meric $(KLAKLAK)_2$ sequence. The peptide chain of octreotate analogue **13** was assembled manually

Table 2. Experimental Data of *C*-Terminally Modified Peptides

		side-chain protected				unprotected	
		9a-e X = -NH		**10a-e** X = -NH-triazole-Fc		**11a-e** X = -NH	
peptide	sequence	yield (%)[a]	*m/z* obsd. (calcd.) species	yield (%)[a]	*m/z* obsd. (calcd.) species	yield (%)[a]	*m/z* obsd. (calcd.) species
a	H-FVYGQACLF-X	27	1475.8 (1475.7) $[M+H]^+$	36	1694.9 (1694.8) $[M+H]^+$	37	1155.2 (1155.5) $[M+H]^+$
b	H-FVTGCARLF-X	26	1600.7 (1600.8) $[M+H]^+$	53	1841.7 (1841.8) $[M+H]^+$	--	--
c	H-FVDGKAWLF-X	26	1476.0 (1475.8) $[M-t\text{-Bu}+2H]^+$	24	1716.7 (1716.8) $[M-t\text{-Bu}+2H]^+$	35	1119.4 (1119.6) $[M+H]^+$
d	H-FVKGHAELF-X	24	1482.9 (1482.8) $[M+H]^+$	51	1723.8 (1723.8) $[M+H]^+$	37	1084.4 (1084.6) $[M+H]^+$
e	H-FVSGNAMLF-X	32	1321.0 (1320.7) $[M+H]^+$	26	1561.9 (1561.7) $[M+H]^+$	29	1022.4 (1022.4) $[M+H]^+$
13	S—S H-fCFwKTCT-X	36	1382.9 (1382.7) $[M+H]^+$	--	--	--	--
14, 15	H-$(KLAKLAK)_2$-X	(37)	2184.8 (2184.4) $[M+Na]^+$	--	--	37	781.2 (781.1) $[M+2H]^{2+}$

[a] Reported yields are after HPLC-purification, crude yields were typically in the range of 77-89% (only for **9a-e**, see Supporting Information).

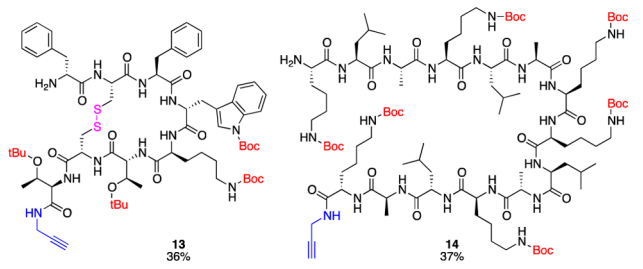


Figure 2. Structure of octreotate analogue **13** and 14-meric $(KLAKLAK)_2$ sequence **14**.

as described above. Fmoc-Cys(Tr)-OH was chosen in order to follow disulfide-bond formation qualitatively with Ellman's reagent.[20] Having removed the trityl groups, the disulfide-bridge was formed on the resin using $Tl(CF_3COO)_3$ at ambient conditions.[21] Subsequent cleavage with TMAF·$4H_2O$ and purification by preparative HPLC yielded side-chain protected, disulfide-bridged octreotate **13** in 36% yield (Figure 2). In addition, we assessed the compatibility of SAM2 resin **7** with microwave-assisted automated synthesis in the preparation of the 14-meric $(KLAKLAK)_2$ sequence, an antimicrobial apoptosis inducing peptide.[22] Using Fmoc-Lys(Boc)-SAM2-TentaGel (loading: 0.30 mmol/g), automated SPPS was performed. After completion of the reaction sequence a loading of 0.18 mmol/g was determined by Fmoc quantification after the 27 reaction steps that were needed to complete the synthesis of the peptide. Cleavage of the side-chain protected peptide from the resin was done by treatment with excess TMAF·$4H_2O$ in DMF/MeOH (9:1, v/v) using microwave irradiation (T_{max} = 50 °C) for 30 min. After HPLC purification, side-chain protected acetylene-modified $(KLAKLAK)_2$ peptide **14** was obtained in 37% yield. A small amount of peptide **14** (1 mg) was treated with TFA/H_2O/TIS (95:2.5:2.5), and HPLC analysis of this deprotected product **15** showed high purity; MS analyses of peptides **14** and **15** confirmed formation of both the desired protected and deprotected products.

In summary, a second generation silyl-based alkyne-modifying linker is described which is generally applicable in Fmoc/*t*-Bu-based solid-phase peptide synthesis. This SAM2-linker yields *C*-terminal prop-2-yn derivatized peptide carboxamides upon cleavage with tetramethylammonium fluoride (TMAF). Except for Asp(O*t*-Bu), compatibility of protected peptides with fluoride cleavage is shown in the synthesis of a focused peptide-library containing a broad variety of proteinogenic, side-chain protected amino acid derivatives. A cleavage click procedure allows a one-step synthesis of protected 1,2,3-triazole peptide conjugates in moderate to good yields after purification. In addition, this SAM2-linker is the first generally applicable, highly acid-stable silyl-based alkyne modifying linker yielding deprotected, acetylene-modified peptides after fluoride cleavage. Furthermore, the linker is compatible with $Tl(CF_3COO)_3$ mediated disulfide-bond formation and applicable in the synthesis of peptides of intermediate length as shown for the 14-meric $(KLAKLAK)_2$ sequence. We expect that the SAM2-linker will find widespread applications in the synthesis of *C*-terminally functionalized peptide bioconjugates.

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Supporting Information Available. Detailed experimental procedures for on-resin preparations, NMR spectra of compounds **1** and **6**, IR spectrum of SAM2-loaded resin **7**, and HPLC chromatograms as well as ESI mass spectra of all peptides presented in this study are provided. 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.