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General and Facile Route to Isomerically Pure Tricyclic Peptides using Templated Tandem CLIPS/CuAAC Cyclizations

Gaston J. J. Richelle, Sumeet Ori, Henk Hiemstra, Jan H. van Maarseveen,* and Peter Timmerman*

Abstract: *We report a one-pot ligation-cyclization technology for the rapid and clean conversion of linear peptides into tricyclic peptides using tetravalent scaffolds containing two benzyl bromides and two alkyne functionalities that react via CLIPS/CuAAC reactions with cysteines and azides in the peptide. Flexibility in the scaffolds is key to the formation of isomerically pure products, since flexible* ***T4****$_1$ and* ***T4****$_2$-scaffolds mostly promote formation of single isomeric tricycles, while rigid* ***T4****$_3$ and* ***T4****$_4$-scaffolds resist formation of clean products. There seems no limitation to the number and type of amino acids present, since 18 canonical amino acids were successfully implemented. We also observed that azides at the peptide termini and cysteines in the center gave better results than vice versa.*

Cyclic peptides have emerged as a promising class of therapeutics[1–4] showing a wide therapeutic window that ranges from antifertility[5] to anticancer[6–8] and antiviral[9–11] applications. As the number of cyclic peptides entering clinical trials has drastically increased over the last years,[6] the search for novel synthetic routes to multicyclic peptides has gained serious interest.[12–23]

In 2005, our group introduced "CLIPS-technology" (**C**hemical **LI**nkage of **P**eptides onto **S**caffolds), involving tandem ligation-cyclization of tris(cysteine)-containing linear peptides with α,α',α''-tribromomesitylene (**T3**) to give the corresponding bicyclic peptides.[12] Heinis et al. applied this technology in phage display libraries, reaching diversity levels as high as 10^9 – 10^{13} different bicycles.[13] Biological evaluation against various proteins[13–16] identified potent bicyclic peptides with K_i values in the nM-range. In some cases, the best inhibitors displayed only (sub)μM activities,[24] suggesting that higher-order structures, like tricycles, may be needed to reach improved activity levels. *Vancomycin*, a last-resort antibiotic glycopeptide wrapped around an aromatic core provides an illustrative example of such a complex architecture (Figure 1).[25] Currently, complex syntheses impede the large-scale production of vancomycin-like drugs, and complicates structural diversification processes aimed at optimizing their biological activities.[26]

Examples of synthetic tricyclic peptides have been reported, as both Ruchala[17] and Wu[18] used either a T_h- or a D_{2h}-symmetrical scaffold to assemble linear peptides into tricycles, while Suga,[19] Nuijens[20] and our group[21] used various types of backbone cyclization in combination with **T3**-CLIPS. These and

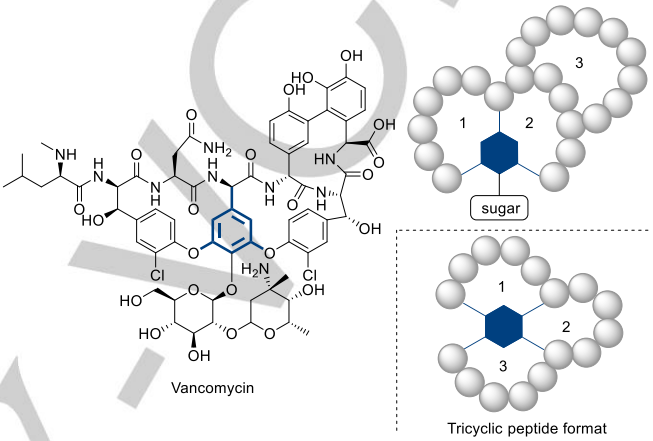

Figure 1. Structure of *vancomycin* and a tricyclic peptide format inspired by its multicyclic structure.

other examples emphasize the interest in multicyclic peptides, despite the fact that formation of multiple isomers, laborious reaction protocols as well as difficulties in generating structural diversity remain a delicate issue. CLIPS-reactions of tetrakis(cysteine)-containing peptides with 1,2,4,5-tetrabromodurene (**T4**) would provide a straightforward route to manufacturing tricycles. However, this reaction yields a mixture of six different regioisomers,[18] which renders this strategy not suitable for a therapeutic development trajectory (Figure 2a).

Here we describe a novel ligation-cyclization strategy that combines CLIPS with copper-catalyzed azide-alkyne cyclization (CuAAC). We observed that CuAAC-chemistry is fully compatible with both peptide[27,28] and CLIPS[22,23] chemistry and does not require protection of any of the amino acid side chains (Figure 2b). Four different **T4**-scaffolds were synthesized containing two CLIPS- and two CuAAC-functionalities (Figure 2c), whereby for the latter we choose for alkynes at the scaffold (circumvents undesired risk of explosion with multiple azides on low-molecular-weight compounds) and azides in the peptides (Fmoc azido amino acids are readily available[29]). All four **T4**$_x$-scaffolds display very different numbers of rotatable bonds connecting the alkynes to the core (4/2/1/0 for **T4**$_{1/2/3/4}$, respectively). This allowed us to evaluate how this ''flexibility'' governs the outcome of the reactions. Moreover, **T4**$_1$ and **T4**$_2$ have a freely rotatable bond (aryl-aryl bond for **T4**$_1$, aryl-amide bond for **T4**$_2$), which should prevent formation of regioisomeric products, while such rotation is not possible for the rigid **T4**$_3$ and **T4**$_4$. Synthesis routes for **T4**$_1$ and **T4**$_2$ are depicted in Scheme 1, for details see SI sections 1 and 2.

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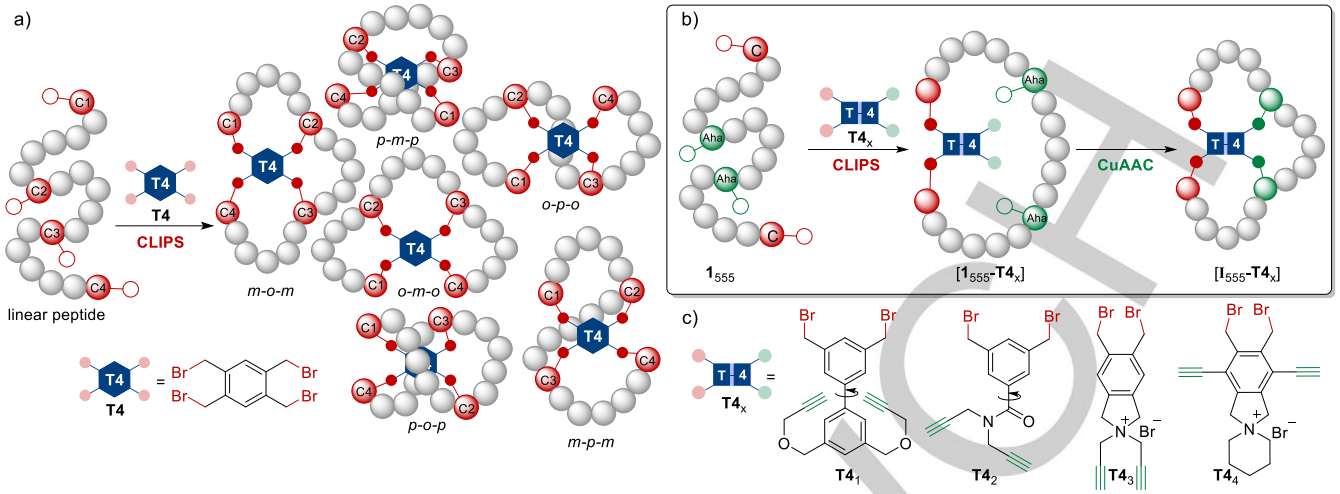
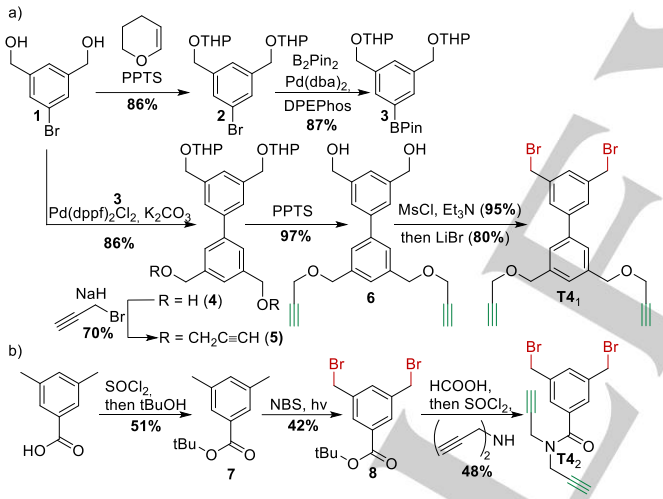


Figure 2. a) Reaction of linear peptides containing four cysteines (red circles) with **T4** give a mixture of six isomers (*o* = ortho, *m* = meta, *p* = para); b) Schematic representation of the CLIPS/CuAAC ligation-cyclization of linear peptides containing two thiols (red open circles) and two azides (green open circles) that react with either two bromomethyl groups (red hazy circles) and two alkynes (green hazy circles) on a **T4**$_x$-scaffold (x = 1-4) to first generate a monocyclic CLIPS-peptide (e.g. [**1**$_{555}$-**T4**$_x$]) and subsequently a tricyclic peptide (e.g. [**I**$_{555}$-**T4**$_x$]) with two thioether linkages (red filled circles) and two triazole linkages (green filled circles); c) Chemical structures of the **T4**$_x$-scaffolds, either with free (**T4**$_1$ and **T4**$_2$) or blocked internal rotation (**T4**$_3$ and **T4**$_4$).

After optimization of the reaction conditions (SI sections 4 and 5), the linear peptide (**1**$_{333}$, Figure 3a; for peptide overview, see Table 1), with 3x3 amino acids separating the two reactive cysteines (**C**)



Scheme 1. a) Synthesis of **T4**$_1$ scaffold; b) Synthesis of **T4**$_2$ scaffold; dba = dibenzylidene acetone, DIPEA = *N*,*N*-diisopropylethylamine, DMAP = 4-dimethylaminopyridine, DPEPhos = bis[(2-diphenylphosphine)phenyl] ether, dppf = (diphenylphosphino)ferrocene, PPTS = pyridinium *p*-toluenesulfonate.

and azides (**Aha** = azidohomoalanine), was reacted with flexible scaffolds **T4**$_1$ and **T4**$_2$, which resulted in complete conversion into the CLIPS-products within 30 min (Figure 3b). Then, a pre-incubated mixture of $CuSO_4$/THPTA/NaAsc in H_2O was added to trigger the CuAAC cyclizations, which led to complete conversion into the expected tricyclic peptides within minutes (Figure 3c). Double triazole formation was confirmed by the fact that TCEP incubation, which reduces azides to amines did not cause any change, while addition of TCEP prior to the CuAAC-reaction showed complete double reduction (MW-52 Da) in ~12 h. UPLC-analysis of the CuAAC reactions showed a slightly better profile for **T4**$_1$ as compared to **T4**$_2$, as judged from the elevated baseline for **T4**$_2$. This is likely due to increased polymerization of the mono- and bicyclic intermediates as a result of restricted flexibility of **T4**$_2$ compared to **T4**$_1$. Purification by RP-HPLC yielded the tricyclic peptides [**I**$_{333}$-**T4**$_1$] (28%) and [**I**$_{333}$-**T4**$_2$] (18%) based on crude linear precursor. CLIPS-reaction of **1**$_{333}$ and **T4**$_3$ resulted in formation of the desired monocycle, however, CuAAC reaction afforded the tricyclic peptide as a mixture of two isomers, along with severe side product formation. This supports our view of flexibility in the linkers being essential for obtaining clean and isomerically pure products. Successful conversion of other 333-peptides (**2**$_{333}$ to **9**$_{333}$, Table 1) using both **T4**$_1$ and **T4**$_2$ proved that the technology does tolerate a wide range of different canonical amino acids in the peptides (18 in total: A/D/E/F/G/H/I/K/L/N/Q/P/R/S/T/V/W/Y, SI 8.7). Changing the order to a CuAAC/CLIPS process was not successful due to

Table 1. Overview of all peptides evaluated in this study; for UPLC-MS data of the CLIPS/CuAAC reactions, see SI section 8.7.

Code	*Sequence*	*Code*	*Sequence*	*Code*	*Sequence*	*Code*	*Sequence*
1$_{333}$	**C**QWG[**Aha**]KAS[**Aha**]FSE**C**	**6**$_{333}$	**C**HPY[**Aha**]RQV[**Aha**]TVD**C**	**11**$_{222}$	**C**ES[**Aha**]FA[**Aha**]KK**C**	**16**$_{444}$	**C**QWGA[**Aha**]KASE[**Aha**]FSEK**C**
2$_{333}$	**C**NSN[**Aha**]SKE[**Aha**]TWN**C**	**7**$_{333}$	**C**DHV[**Aha**]KFY[**Aha**]RHD**C**	**12**$_{222}$	A**C**GS[**Aha**]FE[**Aha**]KN**C**G	**17**$_{555}$	**C**QWGAS[**Aha**]KASEV[**Aha**]FSEKG**C**
3$_{333}$	**C**QYR[**Aha**]KIL[**Aha**]KGR**C**	**8**$_{333}$	**C**NEG[**Aha**]SHN[**Aha**]GIK**C**	**13**$_{222}$	NA**C**EE[**Aha**]FK[**Aha**]KS**C**	**18**$_{333}$	[**Aha**]QWG**C**KAS**C**FSE[**Aha**]
4$_{333}$	CAIP[**Aha**]RYR[**Aha**]NVT**C**	**9**$_{333}$	**C**QLQ[**Aha**]GSY[**Aha**]RFI**C**	**14**$_{111}$	**C**Q[**Aha**]K[**Aha**]F**C**	**19**$_{111}$	[**Aha**]Q**C**K**C**F[**Aha**]
5$_{333}$	**C**THW[**Aha**]QEK[**Aha**]SGN**C**	**10**$_{222}$	**C**QW[**Aha**]KA[**Aha**]FS**C**	**15**$_{111}$	**C**E[**Aha**]F[**Aha**]K**C**		

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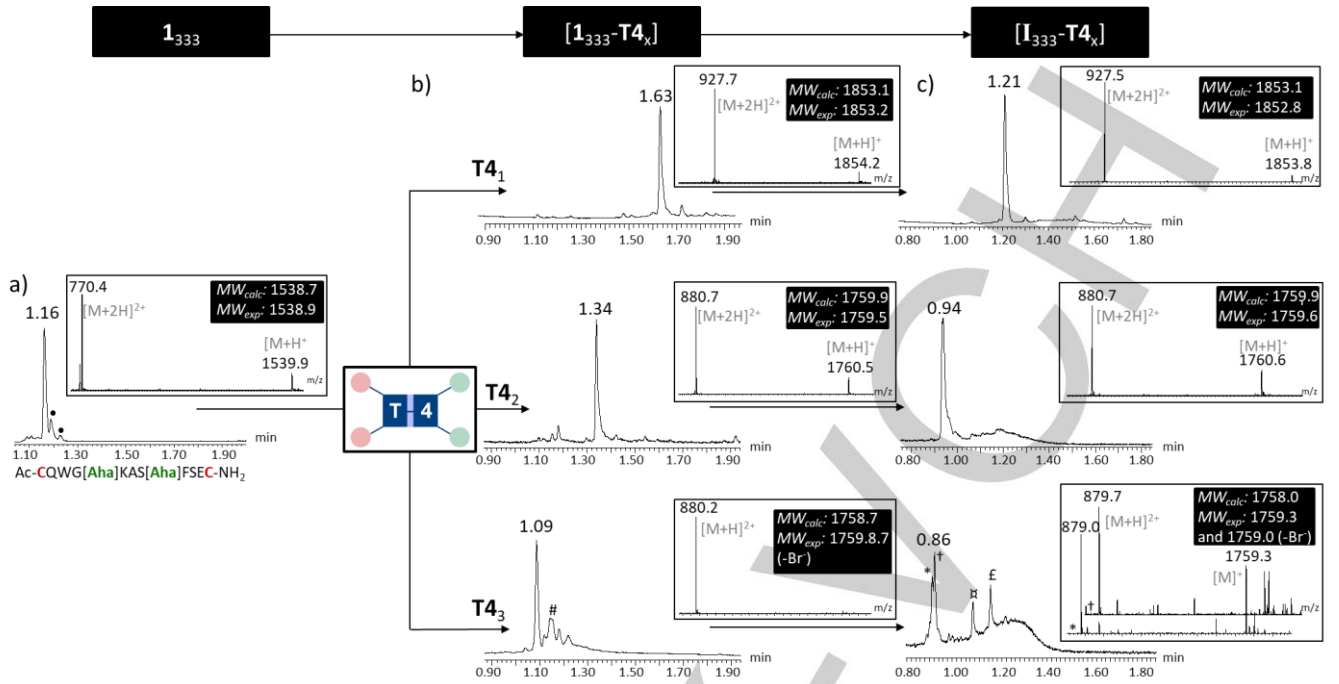

Figure 3. a) UPLC-MS of crude linear peptide $\mathbf{1}_{333}$; CLIPS (b) and CuAAC (c) UPLC reaction analysis of $\mathbf{1}_{333}$ with $\mathbf{T4}_1$, $\mathbf{T4}_2$ and $\mathbf{T4}_3$; MW of $\mathbf{T4}_3$ measured minus Br^-; • = deletion products present in crude linear peptide; # = excess scaffold; ¤ = mono-CuAAC intermediate; £ = S-S oxidized monocycle; */† = two isomers.

unwanted copper-induced cysteine S-S oxidation during CuAAC.

We then studied 222-peptides, where for peptide $\mathbf{10}_{222}$ only [$\mathbf{X}_{222}$-$\mathbf{T4}_1$] was formed as a single tricycle (Figure 4a), while for [$\mathbf{X}_{222}$-$\mathbf{T4}_2$] a second isomer was observed in an otherwise clean chromatogram (Figure 4b). We believe that the presence of the second isomer originates from restricted rotation caused by the tertiary amide bond in [$\mathbf{X}_{222}$-$\mathbf{T4}_2$], for which the energy barrier is significantly higher than for rotation around the aryl-aryl bond in [$\mathbf{X}_{222}$-$\mathbf{T4}_1$].[30,31] With $\mathbf{T4}_3$ reactions, again two isomeric products were observed along with severe side product formation (Figure 4c). For peptides $\mathbf{11}_{222}$ to $\mathbf{13}_{222}$ (SI 8.7), we observed the same pattern, *i.e.* clean formation of the monocyclic CLIPS-products [$\#_{222}$-$\mathbf{T4}_x$] with all four scaffolds. While tricycles on $\mathbf{T4}_{1/2}$ were formed in a clean manner, severe side products/polymer formation for $\mathbf{T4}_3$ and no detectable products for $\mathbf{T4}_4$ were observed. This reveals that it is mainly the loop size and choice of scaffold rather than the nature of the amino acids in the loops that governs the outcome of the reactions.

Subsequently, we studied the cyclization of peptide $\mathbf{14}_{111}$ (SI 8.7). Even though the CLIPS-product [$\mathbf{14}_{111}$-$\mathbf{T4}_1$] was cleanly formed, tricycle [$\mathbf{XIV}_{111}$-$\mathbf{T4}_1$] now also gave a mixture of two isomers (ratio 2:3), likely due to restricted rotation around the aryl-aryl bond, which was also the case for scaffold $\mathbf{T4}_2$. It seems that the azides and alkynes in [$\mathbf{14}_{111}$-$\mathbf{T4}_{1/2}$] cannot easily reach each other causing additional intermolecular CuAAC reactions to overtake. CuAAC of [$\mathbf{15}_{111}$-$\mathbf{T4}_1$] gave slightly better results, but also here two isomers were obtained, illustrating that restricted aryl-aryl bond rotation in [$\#_{111}$-$\mathbf{T4}_1$] tricycles seems a general phenomenon. To further investigate this, we studied the cyclization of single azide peptides **C**E[**Aha**]FAK**C** and **C**EAF[**Aha**]K**C** with scaffold $\mathbf{T4}_1$, which showed complete conversion into single isomeric peptides (see SI section 7a), showing the low rotation barrier around the aryl-aryl bond in the bicyclic intermediate becoming more hindered only in the last cyclization step when bis(azido)peptides are cyclized.

We also investigated ligation-cyclization of peptides $\mathbf{16}_{444}$ and $\mathbf{17}_{555}$. Conversion into isomerically pure tricycles [$\mathbf{XVI}_{444}$-$\mathbf{T4}_x$] and [$\mathbf{XVII}_{555}$-$\mathbf{T4}_x$] was successful for both $\mathbf{T4}_1$ and $\mathbf{T4}_2$ (SI 8.7), albeit that several deletion variants present in the linear peptides were also detected in the tricyclic products.

Finally, we studied two sequence variants of peptides $\mathbf{1}_{333}$ and $\mathbf{14}_{111}$, in which the positions of the Cys- and Aha-residues were interchanged, while the rest of the sequence was kept the same. Reaction of peptide $\mathbf{18}_{333}$ with either scaffold $\mathbf{T4}_{1/2}$ cleanly gave both the tricyclic products [$\mathbf{XVIII}_{333}$-$\mathbf{T4}_{1/2}$] as a single isomer (Figure 5a). The product from reaction with the rigid scaffold $\mathbf{T4}_3$ showed still the formation of two different isomers as expected. Cyclizations of monocycles like [$\mathbf{18}_{333}$-$\mathbf{T4}_{1/2}$] are apparently far

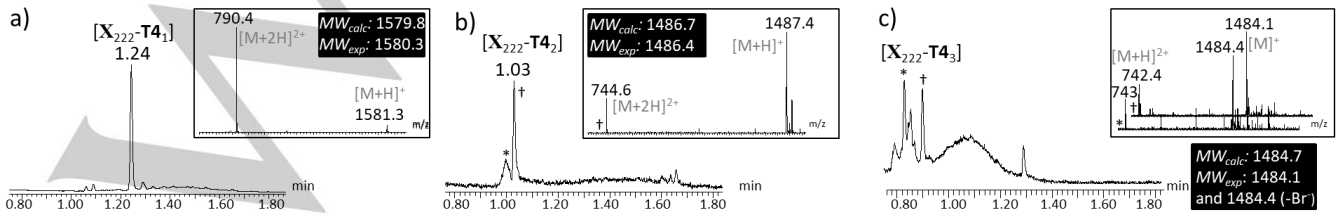

Figure 4. a) UPLC CuAAC reaction analysis of $\mathbf{10}_{222}$ on $\mathbf{T4}_1$ (a), $\mathbf{T4}_2$ (b) and $\mathbf{T4}_3$ (c); */† = two isomers.

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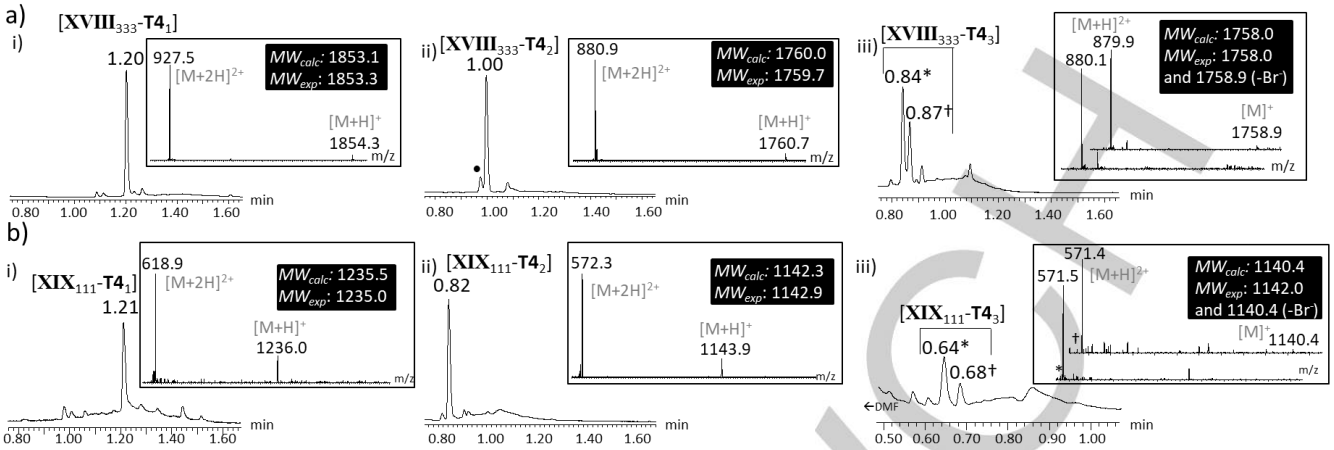

Figure 5. UPLC CuAAC analysis of peptide $\mathbf{18}_{333}$ (a) and $\mathbf{19}_{111}$ (b) with $\mathbf{T4}_1$ (i), $\mathbf{T4}_2$ (ii) or $\mathbf{T4}_3$ (iii); • = tricycle thioether oxidation; */† = two isomers.

more efficient when the azides are located outside the ring at the flexible termini instead of inside the monocycle. This becomes particularly apparent when reacting peptide $\mathbf{19}_{111}$ with either $\mathbf{T4}_1$ or $\mathbf{T4}_2$ (Figure 5b) giving fairly clean formation of the corresponding tricyclic peptides [$\mathbf{XIX}_{111}$-$\mathbf{T4}_{1/2}$], implying that Cys-Aha interchange provides easy access to small tricyclic peptides that are otherwise difficult to manufacture.

In summary, we developed a widely applicable one-pot technology for the locking of linear peptides into isomerically pure tricycles. No restrictions were observed as to the nature and number of amino acids in the loops (except for additional Cys and Met which were not investigated). The technology is currently under investigation for high-diversity generation to enable screening and identification of therapeutically relevant tricyclic peptides.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: peptide • cyclization • tricycle • CLIPS • CuAAC • single • isomer • one-pot

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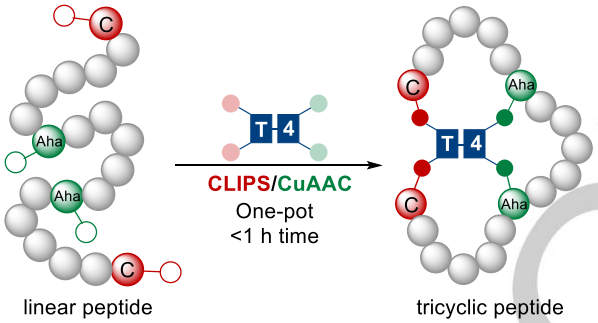
Accessing single isomeric tricyclic peptides: Rotatable tetravalent scaffolds containing both two bromomethyl- and two alkyne-functionalities were reported that enable locking of linear peptides containing two Cys- and two Aha-residues via a one-pot ligation-cyclization procedure into tricyclic topologies, with no limitations observed in number and type of amino acids displayed in the loops.



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