



Cite this: *Chem. Commun.*, 2023, 59, 11397

Received 14th July 2023,
Accepted 30th August 2023

DOI: 10.1039/d3cc03397j

rsc.li/chemcomm

Specific N-terminal attachment of TMTHSI linkers to native peptides and proteins for strain-promoted azide alkyne cycloaddition†

Matt Timmers,^{ib} *^{ab} Wouter Peeters,^c Niels J. Hauwert,^{ib} ^d
Cristianne J. F. Rijcken,^b Tina Vermonden,^{ib} ^a Ingrid Dijkgraaf^{ib} *^c and
Rob M. J. Liskamp^{ib} *^{bce}

The site specific attachment of the reactive TMTHSI-click handle to the N-terminus of peptides and proteins is described. The resulting molecular constructs can be used in strain-promoted azide alkyne cycloaddition (SPAAC) for reaction with azide containing proteins e.g., antibodies, peptides, nanoparticles, fluorescent dyes, chelators for radioactive isotopes and SPR-chips etc.

Selective modification of the N-terminus of peptides or proteins remains a challenge in view of the usually abundant presence of functional and hence reactive side chains of amino acids. However, in many cases site specific N-terminal modification is required to circumvent interference with active or binding domains. These domains play crucial roles in the biological activity of for example antibodies, cell penetrating peptides, signalling or immunologically active peptides and as a consequence should not be modified. Recently, MacDonald *et al.*¹ have developed a highly suitable reagent for specific N-terminal modification of native peptides and proteins. They showed a specific reaction of the 2-pyridinecarboxaldehyde (2PCA) moiety with the N-terminus of peptides and proteins. The aim of this research was to combine this versatile 2PCA reagent with a highly reactive click moiety capable of a fast strain promoted azide alkyne cycloaddition (SPAAC). After N-terminal specific introduction of this novel molecular combination the click moiety can then undergo a mild and efficient reaction with relatively easy accessible azide containing compounds, not requiring the presence of a copper catalyst†. We realised that it might be

challenging to develop a molecular combination of a reactive click moiety and 2PCA followed by its specific N-terminal introduction. However, the relatively easy accessibility of a diversity of azides present in antibodies and other proteins, peptides, nucleic acids, nano particles, fluorescent dyes, chelators for radioactive isotopes, SPR surfaces *etc.* made this the preferred approach instead of synthesis of an azide containing 2PCA reagent, which then has to react with much more difficult accessible click moiety containing antibodies *etc.*

We wished to combine the 2PCA reagent with our recently published tetramethylthiocycloheptyne sulfoximine (TMTHSI)-click handle.² This click reagent is the fastest reacting SPAAC reagent to date. TMTHSI is small, and not sterically crowded to reduce any observer effect, that is a biological response induced by the steric bulk of the reagent on the formed construct.^{2,3} This molecular combination of TMTHSI and 2PCA should then specifically react with the N-terminus of a peptide or protein after which a SPAAC reaction is possible. Thus, we describe the synthesis of TMTHSI-2PCA linker constructs, show the specific N-terminal introduction onto peptides and a protein as well as purification of the obtained molecular constructs. Finally, SPAAC of selected TMTHSI molecular constructs is illustrated especially by the successful preparation of a protein construct from an azide containing protein component and the N-terminal TMTHSI moiety containing Evasin-3 protein.

The first click construct (3) was conveniently obtained in 50% yield by combining piperazine-2PCA (2) and TMTHSI in a reaction with our earlier described TMTHSI-hydroxy succinimide derivative 1 (Scheme 1 and ESI†).

Octreotide 4, a clinically used peptide for the treatment of acromegaly, was used for selective coupling to this new TMTHSI-2PCA reagent 3. Since octreotide is a cyclized peptide, containing several functionalities including a lysine side chain NH₂ and a disulfide bridge, it is a relevant model to evaluate the N-terminal specificity of reagent 3. After reaction with 15 eq. of TMTHSI-2PCA 3, a >90% conversion was achieved based on the decrease of octreotide peak shown by HPLC (Scheme 2, details in ESI,† Section S2).

^a Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht 3584 CG, The Netherlands

^b Cristal Therapeutics, Maastricht 6229 EV, The Netherlands

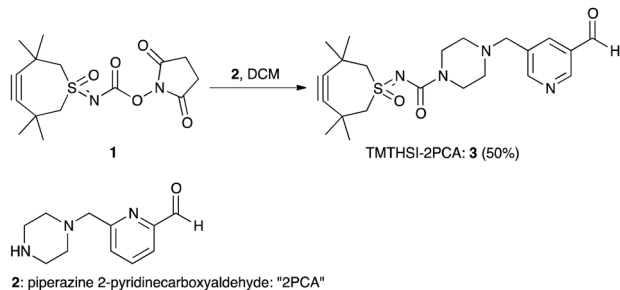
^c Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht 6229 ER, The Netherlands

^d Symeres, Kerkenbos 1013, Nijmegen 6546 BB, The Netherlands

^e School of Chemistry, University of Glasgow, Glasgow G12 8QQ, UK.
E-mail: robert.liskamp@glasgow.ac.uk

† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d3cc03397j>





Scheme 1 Synthesis of TMTHSI-2PCA 3.

Octreotide was selectively functionalized on the N-terminus as was proven by LC-MS (Fig. 1). Not only was the expected mass of the modified octreotide observed, but also proof of the occurrence of cyclization, resulting from the N-terminal specific Edman-degradation-like cyclization reaction⁴ was apparent from the presence of two diastereomers, having the same mass and which could be separated by LC-MS.

Next, we exposed the oncolytic LTX-315 peptide^{5,6} 6 containing several (five) lysine residues to TMTHSI-2PCA 3 (Scheme 3). Here too, a LTX peptide adduct 7 was formed having only one TMTHSI-linker moiety (Fig. 2). As expected, no (stable) Schiff base adducts were formed with the side chains of the lysine residues. As with octreotide, the N-terminal modification of LTX-315 was also apparent from the formation of a diastereomeric mixture (Fig. 2).

Thus, the TMTHSI-2PCA 3 reagent is a suitable reagent for N-terminus site specific introduction of a TMTHSI-containing linker to peptides.

For larger peptides and proteins, a TMTHSI derivative (16) with a larger distance between the aldehyde reacting with the N-terminus and the TMTHSI-moiety was envisioned (Scheme 4), to avoid any steric encumbrance in both the N-terminal specific reaction and the subsequent SPAAC. A suitable distance between the SPAAC moiety and the site of attachment of the reagent in a sizable peptide or protein will be beneficial for the final bioconjugate. The larger distance formed by an ethylene glycol spacer may also be favourable for water solubility of the construct. The

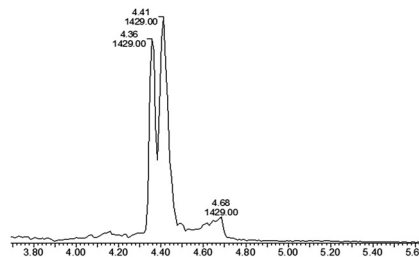
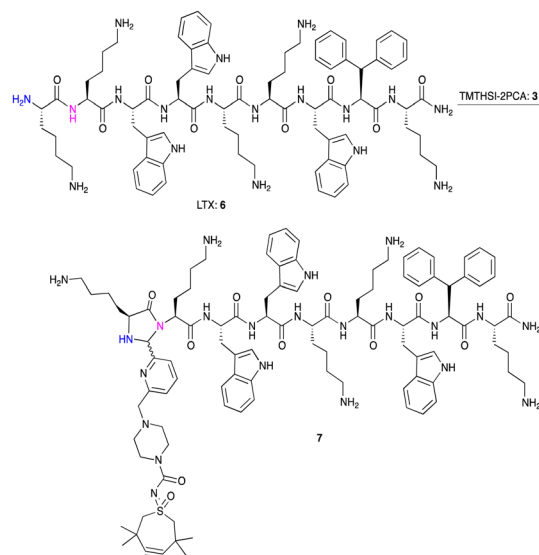


Fig. 1 HPLC-trace of TMTHSI-2PCA reaction with octreotide.



Scheme 3 Reaction of TMTHSI-2PCA 3 with LTX 6 containing several lysine residues.

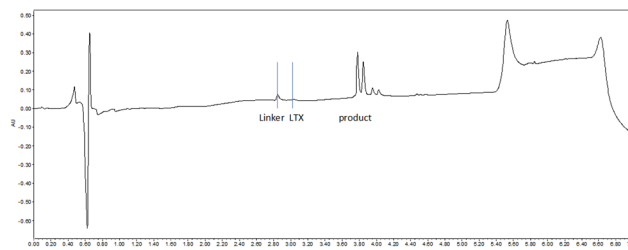
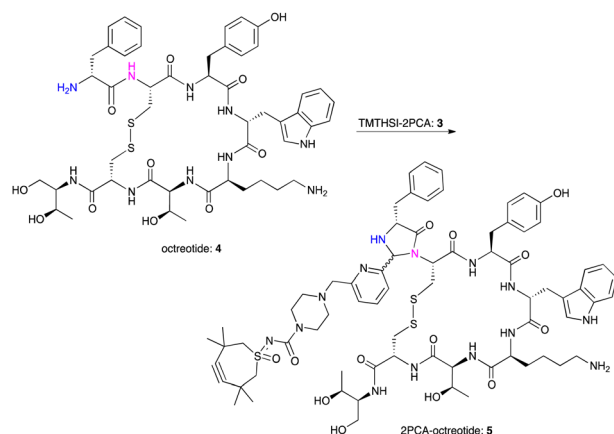


Fig. 2 HPLC trace of the TMTHSI-2PCA reaction with LTX-315.

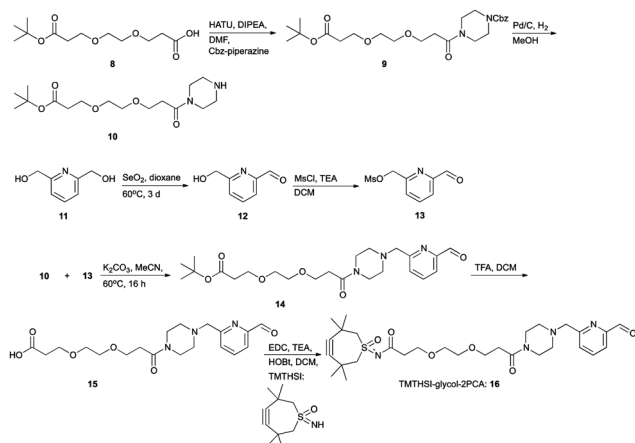


Scheme 2 Reaction of TMTHSI-2PCA 3 with octreotide 4.

TMTHSI-glycol-2PCA derivative 16 was obtained in a straightforward 7-step synthesis in an overall yield of 25% starting from ethylene glycol derivative 8 and pyridine bismethylene alcohol 11 (Scheme 4, details in ESI,[†] Section S3).

This TMTHSI-glycol-2PCA derivative 16 was used in the N-terminal modification of the peptide angiopep2 17 (Scheme 5). Angiopep2 is a conjugate peptide to facilitate intracellular delivery and blood-brain barrier transport of oligonucleotides.^{7–10} The crude reaction mixture 18 required extensive purification to remove the excess of unreacted TMTHSI-glycol-2PCA 16. Fortunately, excess

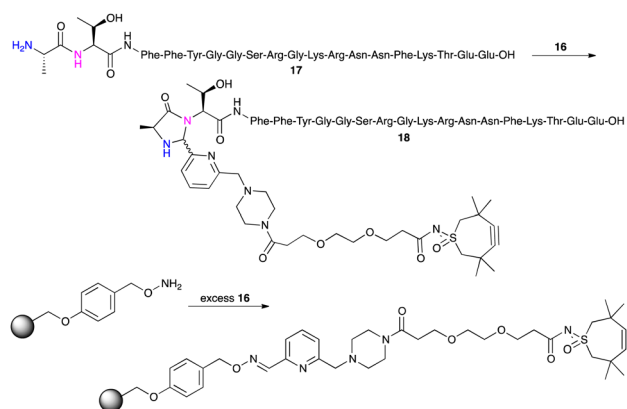
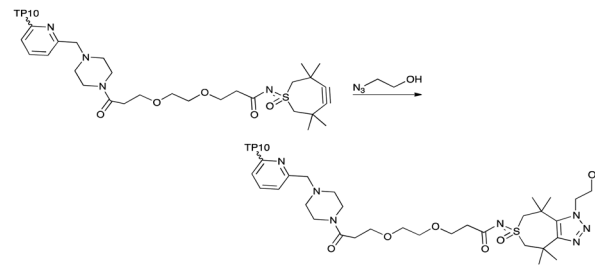


Scheme 4 Synthesis of TMTHSI-glycol-2PCA **16**.

of TMTHSI-glycol-2PCA can be removed by incubation with hydroxylamine-containing beads (Scheme 5). These beads capture the aldehyde of TMTHSI-glycol-2PCA by formation of an oxime, which is illustrated in the work-up of the reaction of peptide TP10 (AGYLLGKINLKALAALAKKIL, membrane-transporting peptide^{11–13}) **17** with hydroxylamine beads (Scheme 5, bottom and ESI,† Section S4).

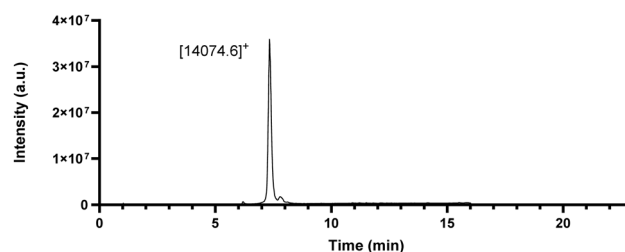
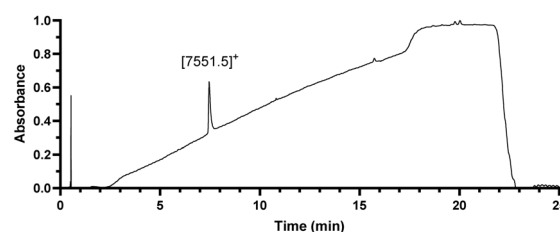
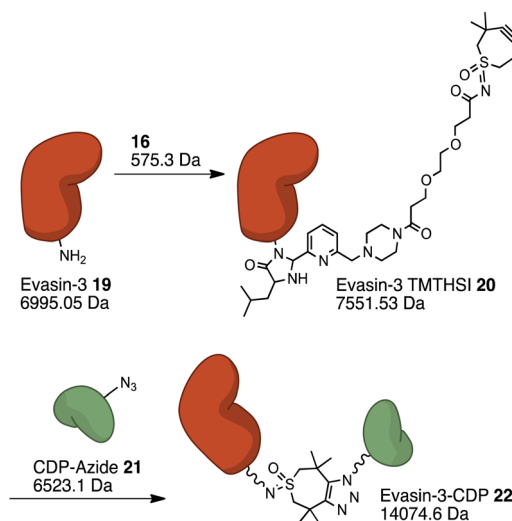
The TMTHSI-moiety integrity and reactivity was monitored by addition of azido-ethanol to the worked-up product of the N-terminal modified TP10 peptide (Scheme 6). This resulted in a complete shift of the diastereomeric peptides to the left, a more polar region of the HPLC-trace indicating a full reactivity of the TMTHSI-modified peptide (ESI,† Section S5).

Finally, TMTHSI-glycol-2PCA was evaluated for its ability to specifically react with the N-terminus of a protein. Here, Evasin-3 **19** was selected, This is a protein which shows a high affinity to CXC-type chemokines, in particular CXCL1 and CXCL8, inflammatory chemokines associated with the development of numerous diseases¹⁴ such as cancer and atherosclerosis. N-terminal modification of **19** with **16** will enable attachment of the bioconjugate to a bacterial chimeric designer protein (CDP)¹⁵ to boost the immune recognition (Fig. 3).

Scheme 5 Reaction of **17** with TMTHSI-glycol-2PCA (top) and capture of excess of TMTHSI-glycol-2PCA **16** using hydroxylamine beads (bottom).

Scheme 6 Click reaction of the TMTHSI-glycol-2PCA peptide construct with azido ethanol.

Thus, 12 equivalents of **16** were reacted with Evasin-3 **19** for 26 hours. While no full conversion of Evasin-3 **19** was observed (ESI,† Section S6), separation on HPLC was possible and therefore we could purify Evasin-3-TMTHSI **20** by preparative HPLC

Fig. 3 Representation of the reaction of Evasin-3 **19** with TMTHSI-glycol-2PCA **16** leading to molecular construct **20** (top HPLC trace of purified **20**), followed by reaction with CDP-azide **21** leading to Evasin-3-CDP **22** (purified **22**, bottom HPLC trace).

in a yield of 40%. Considering that only 12 equivalents of **16** were used and preparative HPLC purification was performed, a yield of 40% of pure product was quite satisfactory.

Next, conjugation with azide-functionalised CDP **21** was carried out (Fig. 3). Two equivalents of Evasin-3-TMTHSI **20** were added dropwise to 1 equivalent of CDP-azide **21**. After reacting for 3.5 hours, the reaction mixture was purified using preparative HPLC. This resulted in a pure Evasin-CDP conjugate **22**, as was confirmed by MS. The yield of this conjugate after purification was 60%, which is certainly acceptable considering loss of product during preparative HPLC.

We have synthesized clickable, N-terminal site-specific constructs TMTHSI-2PCA and TMTHSI-glycol-2PCA. The synthesis of both derivatives proceeded without problems, which certainly is not obvious using very reactive click compounds (e.g., BARAC).¹⁶ These reagents have proven to be N-terminal specific, and the reaction *via* cyclization was demonstrated by observation of the formation of diastereomers on HPLC and confirmed by mass spectrometry. Pleasantly, also in the N-terminal reaction no side products have been observed despite the possible formation of an intermediate diene, in principle capable of reacting with the TMTHSI-moiety.⁴ Furthermore, we found that it was possible to use a much smaller excess of 2PCA-reagent than was earlier reported¹ and that if necessary, excess can be efficiently removed by capture using functionalized (hydroxyl amine containing) beads. This purification strategy together with HPLC purification has been used to determine yields which is an improvement compared to determination of conversion yields solely based on MS.¹

These clickable N-terminal specific constructs can also be used in larger proteins, as was demonstrated by the clicking of Evasin-3 with CDP-azide. These combined findings demonstrate the high potential of these TMTHSI-2PCA reagents for in-solution N-terminal specific introduction of a click handle in peptides and proteins. As such this approach affords pure N-terminally click-reactive peptides and proteins leaving their internal functional domains unaffected. It is expected that this specific bioconjugation strategy will soon enable a wealth of therapeutic and diagnostic applications facilitated by the convenient SPAAC-based possible introduction of a variety of azide containing (bio)molecular constructs originating from antibodies, nucleic acids, nanoparticles, surfaces *etc.*

Conflicts of interest

There are no conflicts to declare.

Notes and references

‡ Copper catalysts are usually considered toxic and therefore have to be removed carefully, before any use of the resulting molecular constructs

in vitro or *in vivo*. In some cases this is even hardly possible, e.g., when a metal chelator has to be introduced for a radio-active isotope which can also bind copper-ions.

- 1 J. I. MacDonald, H. K. Munch, T. Moore and M. B. Francis, One-step site-specific modification of native proteins with 2-pyridinecarboxyaldehydes, *Nat. Chem. Biol.*, 2015, **11**(5), 326–331.
- 2 J. Weterings, C. J. F. Rijcken, H. Veldhuis, T. Meulemans, D. Hadavi, M. Timmers, M. Honing, H. Ippel and R. M. J. Liskamp, TMTHSI, a superior 7-membered ring alkyne containing reagent for strain-promoted azide-alkyne cycloaddition reactions, *Chem. Sci.*, 2020, **11**(33), 9011–9016.
- 3 S. B. Engelsma, L. I. Willems, C. E. Van Paaschen, S. I. Van Kasteren, G. A. Van Der Marel, H. S. Overkleeft and D. V. Filippov, Acylazetines as a dienophile in bioorthogonal inverse electron-demand Diels–Alder ligation, *Org. Lett.*, 2014, **16**(10), 2744–2747.
- 4 F. P. J. T. Rutjes, How to pick a single amine?, *Nat. Chem. Biol.*, 2015, **11**(5), 306–307.
- 5 B. Sveinbjörnsson, K. A. Camilio, B. E. Haug and Ø. Rekdal, LTX-315: a first-in-class oncolytic peptide that reprograms the tumor microenvironment, *Future Med. Chem.*, 2017, **9**(12), 1339–1344.
- 6 H. Zhou, S. Forveille, A. Sauvat, T. Yamazaki, L. Senovilla, Y. Ma, P. Liu, H. Yang, L. Bezu and K. Müller, *et al.*, The oncolytic peptide LTX-315 triggers immunogenic cell death, *Cell Death Dis.*, 2016, **7**(3), e2134.
- 7 H. Gao, S. Zhang, S. Cao, Z. Yang, Z. Pang and X. Jiang, Angiopep-2 and activatable cell-penetrating peptide dual-functionalized nanoparticles for systemic glioma-targeting delivery, *Mol. Pharmaceutics*, 2014, **11**(8), 2755–2763.
- 8 S. Habib and M. Singh, Angiopep-2-Modified Nanoparticles for Brain-Directed Delivery of Therapeutics: A Review, *Polymers*, 2022, **14**(4), 712.
- 9 Y. Lei, S. Chen, X. Zeng, Y. Meng, C. Chang and G. Zheng, Angiopep-2 and cyclic RGD dual-targeting ligand modified micelles across the blood–brain barrier for improved anti-tumor activity, *J. Appl. Polym. Sci.*, 2022, **139**(24), 52358.
- 10 X. X. Shi, W. M. Miao, D. W. Pang, J. S. Wu, Q. S. Tong, J. X. Li, J. Q. Luo, W. Y. Li, J. Z. Du and J. Wang, Angiopep-2 conjugated nanoparticles loaded with doxorubicin for the treatment of primary central nervous system lymphoma, *Biomater. Sci.*, 2020, **8**(5), 1290–1297.
- 11 M. Z. Islam, H. Ariyama, J. M. Alam and M. Yamazaki, Entry of cell-penetrating peptide transport 10 into a single vesicle by translocating across lipid membrane and its induced pores, *Biochemistry*, 2014, **53**(2), 386–396.
- 12 U. Soomets, M. Lindgren, X. Gallet, M. Hällbrink, A. Elmquist, L. Balaspiri, M. Zorko, M. Pooga, R. Brasseur and Ü. Langel, Deletion analogues of transportan, *Biochem. Biophys. Acta*, 2000, **1467**(1), 165–176.
- 13 C. Zhang, W. Ren, Q. Liu, Z. Tan, J. Li and C. Tong, Transportan-derived cell-penetrating peptide delivers siRNA to inhibit replication of influenza virus *in vivo*, *Drug Des., Dev. Ther.*, 2019, **13**, 1059–1068.
- 14 S. S. Denisov, J. H. Ippel, A. C. A. Heinzmann, R. R. Koenen, A. Ortega-Gomez, O. Soehnlein, T. M. Hackeng and I. Dijkgraaf, Tick saliva protein Evasin-3 modulates chemotaxis by disrupting CXCL8 interactions with glycosaminoglycans and CXCR2, *J. Biol. Chem.*, 2019, **294**(33), 12370–12379.
- 15 A. Blanas, H. Karsjens, A. de Ligt, E. J. M. Huijbers, K. van Loon, S. S. Denisov, C. Durukan, D. J. M. Engbersen, J. Groen and S. Hennig, *et al.*, Vaccination with a bacterial peptide conjugated to SARS-CoV-2 receptor-binding domain accelerates immunity and protects against COVID-19, *Science*, 2022, **25**(8), 104719.
- 16 M. F. Debets, J. C. M. van Hest and F. P. J. T. Rutjes, *Org. Biomol. Chem.*, 2013, **11**, 6439–6455.

