Bioinspired peptide stapling with lanthionine ketenamine esters

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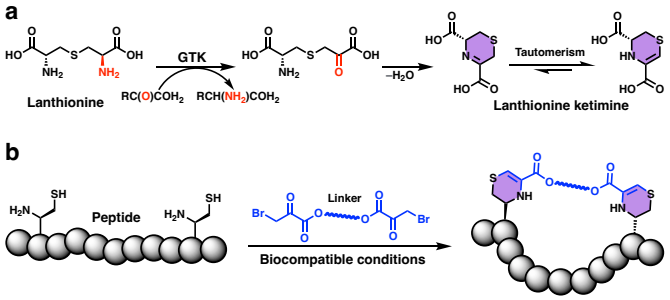
Stapling of peptides renders them ideal drug candidates. The individual characteristics of synthetic staple moities impact directly on the peptide's final properties. We report a bioinspired ketenamine-based peptide staple resembling the natural metabolite lanthionine ketenamine. The strategy is orthogonal to canonical amino acids, proceeds in water and allows for tailored linkers.

Constraining the conformational flexibility of a peptide has significant advantages for the development of peptide-based drug candidates.[1, 2] Locking the peptide in its active conformation increases the affinity to the target protein by reducing the entropic barrier of binding. Preventing the peptide from adopting a large variety of alternative conformations decreases the undesired ability to bind to off-target proteins and thus increases selectivity and metabolic stability.

A powerful strategy for the generation of constrained peptides is two-component stapling.[3, 4] Although this strategy is frequently used in the context of α-helical peptides, the term encloses all two-component peptide macrocyclizations where one component is a peptide and the other a double-reactive linker molecule. We recently reported a two-component stapling strategy using 1,2-aminothiol residues in peptides and the reagent 2,6-dicyanopyridine.[5] Although the approach is fully biocompatible, the resulting staple is unnatural and has potentially undesirable metal-binding properties.[6] Other biocompatible stapling strategies such as double strain-promoted azide–alkyne cycloaddition (SPAAC) generate even larger unnatural and hydrophobic staples that may interfere with target interactions.[7] In the present study, we introduce a bioinspired stapling reaction that results in a natural lanthionine ketenamine-based peptide staple.

Lanthionine ketenamine is a metabolite found in the mammalian brain and central nervous system.[8] Due to imine-enamine tautomerism, it is often described as either a ketimine or a ketenamine. Its biosynthetic pathway involves transamination of the nonproteinogenic amino acid lanthionine, followed by cyclic imine formation (Scheme 1a).[9] Lanthionine ketenamine esters have been studied as neurotrophic, neuroprotective, and anti-neuroinflammatory agents.[10]

The chemical synthesis of lanthionine ketenamine and its ester derivatives results from the reaction between *N*-terminal cysteines and α-bromopyruvates.[11] In order to employ this simple transformation for peptide stapling, we developed doubly reactive staples containing two α-bromopyruvate functional groups and studied their reactivity with peptides containing two 1,2-aminothiols (Scheme 1b).



Scheme 1 (a) Conversion of lanthionine into its ketimine analogue by glutamine transaminase K (GTK) and an α-keto acid acceptor. The enamine is the predominant tautomer (lanthionine ketenamine). (b) Bioinspired peptide stapling strategy explored in this study. The reaction between 1,2-aminothiols and α-bromopyruvates results in lanthionine ketenamine ester linkages between amino acids.

We first explored this reaction with staple **b** and an oxytocin derivative, **1,** in which the central cysteine was replaced by our unnatural amino acid Dys (Figure 1a). Our previously described amino acid, Dys, is a L-2,3-diaminopropionic acid (Dab) with its side-chain amine amidated to L-cysteine.[5] This slight peptide modification allows the presence of two 1,2-aminothiol groups in oxytocin at the same positions as the naturally occurring disulfide bridge (Figure 1a). Staple **b** links two α-bromopyruvates via a butyl chain. Simple addition of peptide **1** and staple **b** in a 2:3 ratio in aqueous buffer at physiological pH yielded cyclic peptide **1b** in 71% yield after 1 hour (Figure 1b). The major by-product was a double stapled species which is considered inevitable using two-component peptide stapling strategies at reasonably high concentrations.[3] We isolated **1b** using RP-HPLC purification under acidic conditions (Figure S28).

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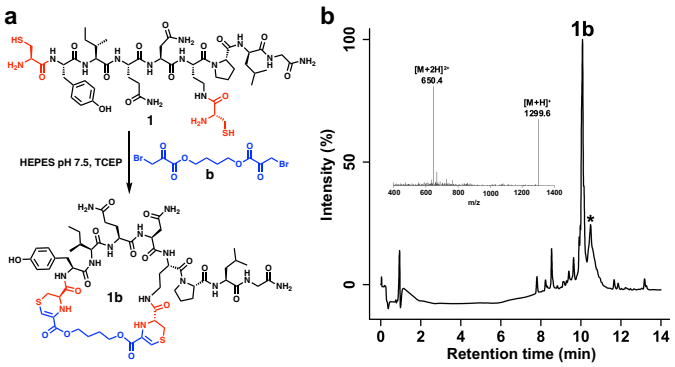

Figure 1 (a) Synthesis of **1b** from peptide **1** and linker **b**. (b) Crude LC-MS of **1b** after 1 h of reaction. The asterisk (*) indicates the double-reacted by-product.

In order to explore the scope of this bioinspired and biocompatible stapling reaction, we synthesised three more peptides, representing positively charged (**2**), negatively charged (**3**) and neutral (**4**) peptide sequences (Figure 2). In addition, we explored a shorter staple **a** and the longer polyethylene glycol (PEG)-based staple **c** (Figure 2). We incubated all peptides **1**-**4** with staples **a**-**c** under identical conditions and analysed the reaction mixtures by LC-MS after 1 and 24 hours (Table 1). All reactions displayed the desired stapled peptide after 1 h in yields (LC-MS) ranging from 71% to 96%. Yields slightly increased over 24 hours, indicating superior stability in aqueous solution and reaction completion in only a few hours.

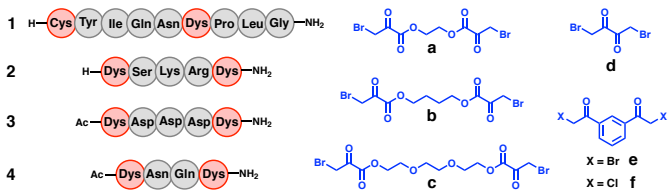

Figure 2 Peptides (**1**-**4**) and staples (**a**-**f**) investigated in this study.

We additionally explored alternative staples **d**-**f** for all peptides (Figure 2). Staple **d** is commercially available and represents the shortest possible way to link two α-bromoketones in a single molecule. Staples **e** and **f** employ an aromatic ring to link two α-bromoketones. Staples **e** and **f** were also designed to directly compare the difference in reactivity of bromo and chloro substituents. All three linkers yielded the expected cyclic peptides after 1 hour reaction in yields ranging from 35% to 97% (Table 1). However, in stark contrast to staples **a**-**c**, the resulting peptides showed significant degradation after 24 hours (Table 1). Attempts to isolate and characterise peptide **1e** as a representative example of this compound class proved unsuccessful.

The instability of peptides stapled with **d**-**f** was surprising, particularly in direct comparison to the very stable peptides containing linkers **a**-**c**. To further investigate the cause of the degradation, we aimed to synthesise the simple product of 2-bromoacetophenone and cysteamine as a model system (Scheme S7).[12] A compound isolated from this reaction was derivative **6**, which represents an oxidised form of the desired compound (Supporting Information). Sulfur oxidation was previously described for lanthionine ketenamine esters.[13] NMR studies of **6** further revealed an enamine tautomer in which the alkene proton can be replaced by deuterium at neutral pD (Figure S18-S23). In addition, we found that peptide **1e** can be detected as the major product in LC-MS after 4 days, if the reaction is carried out under nitrogen atmosphere (data not shown), further indicating that degradation of peptides equipped with linkers **d**-**f** is likely related to oxidation.

Table 1 Stapling yield (%) and stability of investigated peptides[a]

Peptide	Staple					
	a	b	c	d	e	f
1	77 (98)	71 (86)	72 (78)	58 (51)	80 (35)	84 (38)
2	89 (94)	96 (96)	87 (90)	62 (0)	35 (2)	97 (5)
3	73 (86)	89 (94)	75 (92)	85 (3)	72 (0)	59 (0)
4	80 (92)	88 (89)	75 (94)	85 (56)	84 (1)	91 (7)

[a] Yield (LC-MS) of macrocyclic peptides after 1 h and 24 h (in parentheses). Peptide (600 μM) and staple (900 μM) were incubated in 10 mM HEPES pH 7.5, 4 mM TCEP. Colour code indicates full (green), partial (orange) or no (red) stability over 24 h.

In order to prove the structure of the linker domain and investigate the potential tautomerism of the lanthionine ketenamine esters we synthesised compound **5** (Figure 3), which represents a simple model of a peptide stapled by **b**. NMR spectra of **1b** proved too complex to investigate this equilibrium (data not shown), hence, NMR spectra of **5** served as a simpler model system. Spectra recorded in CD_3CN clearly indicated the enamine species without any evidence for an imine (Figure S16-S17). Spectra recorded in 50% D_2O:CD_3CN at pD 7 (Figure S11-S15) indicated the same species (assignments reported in Figure 3 and Table S3). Most importantly, we did not observe a replacement of the alkene proton by deuterium, indicating strong stability of the enamine tautomer. This lack of exchange in **5** is in stark contrast to derivative **6**, where we observed such an exchange (Figure S18-S23), which might be a reason for the observed difference in stability.

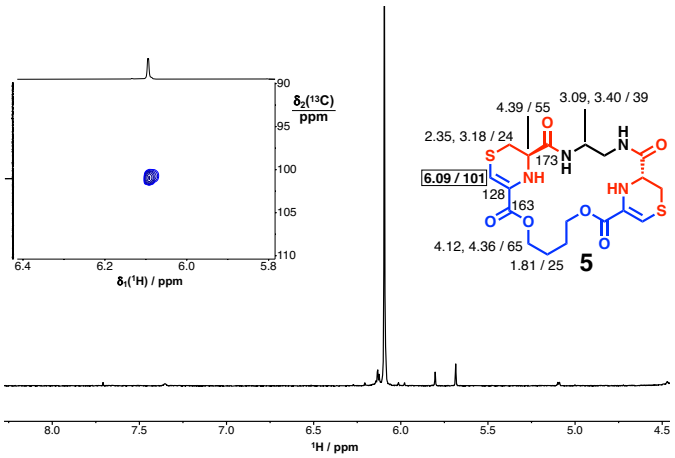

Figure 3 Structure of compound **5** and NMR assignments proving the enamine tautomer. Selected areas of ^{1}H and ^{1}H,^{13}C-HSQC (insert) NMR spectra are shown. Assignments of ^{1}H and ^{13}C peaks are indicated.

In summary, we present a peptide stapling strategy resembling lanthionine ketenamine esters as novel peptide staples. The strategy is extremely versatile, allowing for tailored aliphatic and PEG-based linkers. Stapled peptides can be accessed in water under biocompatible conditions in a few hours and isolated using standard chromatography. Our studies show that the resulting lanthionine ketenamine linkers strongly resemble an enamine instead of an imine tautomer, indicating that lanthionine ketimines should more precisely be described as lanthionine ketenamines.

There are no conflicts to declare.

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