EDGE ARTICLE

Converting disulfide bridges in native peptides to stable methylene thioacetals†

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Disulfide bridges play a crucial role in defining and rigidifying the three-dimensional structure of peptides. However, disulfides are inherently unstable in reducing environments. Consequently, the development of strategies aiming to circumvent these deficiencies – ideally with little structural disturbance – are highly sought after. Herein, we report a simple protocol converting the disulfide bond of peptides into highly stable methylene thioacetal. The transformation occurs under mild, biocompatible conditions, enabling the conversion of unprotected native peptides into analogues with enhanced stability. The developed protocol is applicable to a range of peptides and selective in the presence of a multitude of potentially reactive functional groups. The thioacetal modification annihilates the reductive lability and increases the serum, pH and temperature stability of the important peptide hormone oxytocin. Moreover, it is shown that the biological activities for oxytocin are retained.

Introduction

Peptides have recently been enjoying a renewed interest in their application as therapeutic agents.[1] They provide a large chemical space with a diverse array of molecular frameworks for the development of novel therapeutics for a plethora of biomedical applications. However, peptides exhibit inherent drawbacks as drug candidates, due to their conformational flexibility and low stability.[1a,2] Several strategies have evolved to address these unfavourable pharmacokinetics by increasing stability through local or global constraints. This involves the incorporation of non-natural amino acids[3] or amide bond surrogates,[2,4] as well as various cyclisation modes,[5] side chain functionalisations[6] and other modifications.[7,8] These approaches stabilise the secondary and tertiary structure of the peptide by constraining it to a more defined geometry. This increases protease resistance as the vulnerable bonds are less easily accessible.

Disulfide bridges play a crucial role in defining and rigidifying the three-dimensional structure of natural peptides and proteins. However, disulfides are inherently unstable in reducing environments and towards nucleophiles. Reduction or scrambling of the disulfide bonds is caused by trans-hydrogenases, thiol oxidoreductases, disulfide isomerases and other thiol containing agents, such as serum albumin or glutathione.[9] All of them possess the ability to decrease the lifespan of therapeutic disulfide containing agents. Therefore,

disulfide bond engineering emerged as an important strategy to improve the metabolic stability of disulfide-containing peptides, whilst maintaining their biological activity. For instance, replacements of the disulfide group with a lactam,[10] thioether,[11] selenium[12] or dicarba[13] analogues have been reported.[5a] Many of these methods require significant modification of the synthetic building blocks, at the initiation of the synthesis towards the final peptide product, which can be problematic.[11b,14] In addition to the synthetic efforts of a *de novo* approach, these modifications may introduce steric distortions that can negatively impact activity and selectivity.[15]

Concept

Hence, we envisioned a disulfide stapling procedure performed under mild, biocompatible reaction conditions (aqueous media, low to ambient temperature and mild pH) that can be used directly with unprotected native peptides. A key aim of our strategy was to insert a minimal linker into the disulfide bond and maintain the structural integrity necessary for the biological activity of the peptides. We proposed to achieve this by the insertion of a methylene group in between the two sulfur atoms, giving a thioacetal functional group (Scheme 1). Thioacetals are utilised in organic synthesis as a rugged protecting group for carbonyl groups. Chemically, they are inert towards nucleophiles and most reductive conditions.

The thioacetal group has been sporadically previously used to functionally rebridge disulfide bonds within peptide structures.[16] However, these reports suffer from significant shortcomings by using either strongly basic conditions, employing the linker reagent as the solvent or organic and non-biocompatible solvents. Furthermore, these methods were not applied to unprotected native peptides. Moreover, this method would also
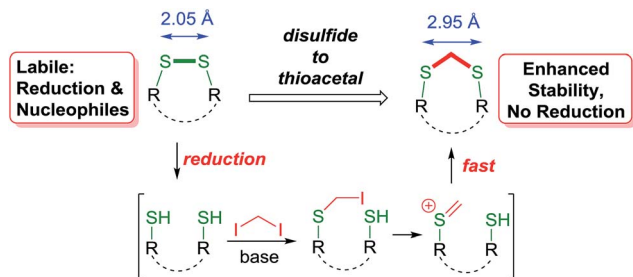
Scheme 1 Thioacetal linkages by methylene insertion into disulfides for enhanced stability.

Scheme 2 Performing the mild one-pot thioacetal transformation on glutathione.

allow the convenient introduction of radiolabeled methylene groups for tracing or imaging purposes. In line with our aims for minimising structural disturbance with the disulfide modification, CH_2X_2 (X = Cl, Br or I) was selected as the smallest possible electrophilic linker. This is complementary to the highly reactive multi-bromo benzylic linkers employed to invoke the cyclisation of prior non-cyclised peptide segments due to the sterically larger size of this linker.[17] For our approach, one end of the reduced disulfide bridge will be alkylated with the electrophilic reagent. A facile ionisation provides a thiocarbenium ion[18] of superior electrophilicity, leading to a rapid cyclisation to the desired methylene thioacetal before any additional reaction with another linker molecule would occur.

Results and discussion

Investigation into a one-pot biocompatible disulfide bond modification

The initial assessment and optimisation of the disulfide to thioacetal conversion, was conducted on N,N'-bis-Boc L-cystine dimethyl ester as a simple model substrate (see ESI†). A completely chemoselective thiol alkylation of the sulfhydryl group forming the desired methylene thioacetal was observed, with no detectable side products. Potentially interfering amino acids were added to the reaction mixture and were innocent bystanders. Diiodomethane was the most efficient linker reagent, although dibromomethane and even dichloromethane reacted. Despite its higher reactivity, it did not result in any undesired side products. A mild base is required for the reaction to proceed as thiols themselves do not react. The reaction performs well at ambient temperature. The addition of an organic co-solvent helps solubilise the linker reagent and THF was favoured due to its miscibility with water and low boiling point. The protocol was completed by a prior disulfide bond reduction using water soluble tris-(2-carboxyethyl)phosphine (TCEP) (Scheme 2).[19] Pleasingly, CH_2I_2 was found to be compatible with TCEP under the reaction conditions, even though it had been reported to impede bioconjugations with maleimides and α-haloacyl groups.[20] For instance, after the reduction of glutathione disulfide (GSSG) to glutathione (GSH), addition of diiodomethane and triethylamine provided the thioacetal modified version GSCSG in 53% yield.
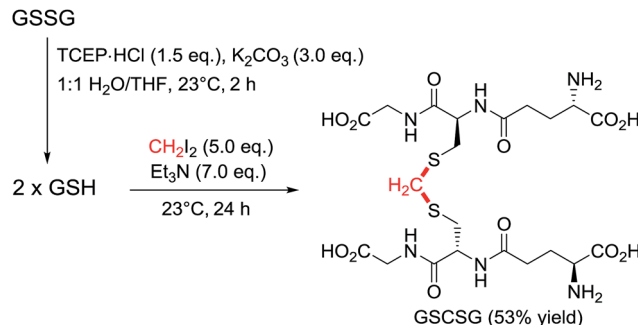
Application of the thioacetal modification to a scope of bioactive peptides

With this simple and effective protocol we aimed to apply this method to bioactive peptides and drugs, containing a plethora of proteinogenic amino acids, to rigorously test the compatibility of our approach (Scheme 3). The selected cyclic peptides have a broad variety of relevant biological activities, which
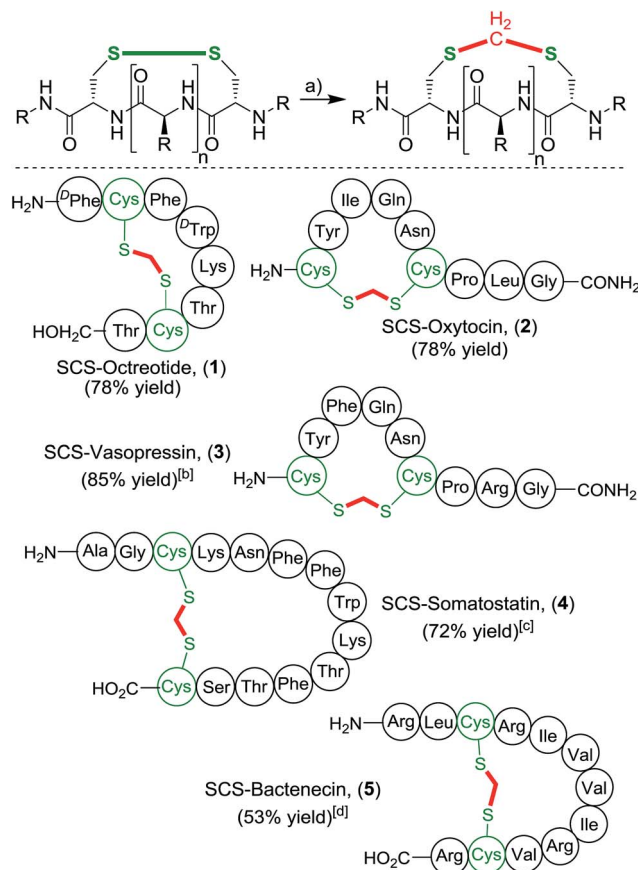
Scheme 3 Employing the thioacetal modification on a variety of cyclic bioactive peptides. Conditions: [a] 1.5 eq. TCEP·HCl, 3.0 eq. K_2CO_3, 5 : 1 H_2O/THF, 23 °C, 2 h, then 4.0 eq. CH_2I_2, 5.0 eq. NEt_3, 23 °C, 9 h; [b] 2.5 eq. CH_2I_2 used; [c] 1.5 eq. TCEP·HCl, 3.0 eq. K_2CO_3, 10 : 1 H_2O/THF, 23 °C, 2 h, then 10 eq. CH_2I_2, 15 eq. NEt_3, 23 °C, 12 h; [d] 8.0 eq. CH_2I_2, 12 eq. NEt_3.

would allow a comparative study of the native parent peptide to the modified analogue. Firstly, we focused on cyclic octapeptide octreotide as more complex prototype substrate. Octreotide is pharmacologically related to somatostatin and is a potent growth hormone inhibitor.[21] It is an important drug marketed for the treatment of a variety of hormone producing tumors.[22] Application of the one-pot methylene insertion protocol provided the desired modified SCS-Octreotide (**1**) in a good yield of 78%. Importantly, the transformation remained efficient under dilute conditions (5 mM) as well as a largely reduced amount of the organic co-solvent (5 : 1 ratio water/THF). Exposure of octreotide to CH_2I_2 and NEt_3 without prior disulfide reduction led to full recovery. No chemical modifications occurred at any other sites, highlighting the high chemoselectivity of the linker protocol. The pharmacologically related natural tetradecapeptide hormone Somatostatin (**4**),[23] performed efficiently under the one-pot transformation to provide the thioacetal bridged analogue in 72% yield. Next, neurohypophysial nonapeptide hormones oxytocin (OXT)[24] and related Arginine Vasopressin (AVP),[25] were targeted. Oxytocin is marketed as a drug to instigate uterine contractions as well to prevent post-partum hemorrhage. Vasopressin constricts the blood vessels to increase arterial blood pressure and regulates the body's retention of water. Both OXT and AVP smoothly underwent the cut-and-sew methylene insertion procedure, affording the desired SCS-analogues **2** and **3** in high yields, 78% and 85% respectively.‡ The antibacterial peptide Bactenecin[26] was chosen as a challenging substrate, due to the presence of multiple nucleophilic guanidine groups of the arginine residues, that potentially could react with the electrophilic linker reagent. Nonetheless, SCS-Bactenecin **4** was isolated in 53% yield without the formation, of detectable side-products.

Besides the illustrated modification of unprotected native peptides, the thioacetal introduction can also be embedded in classical peptide synthesis. For instance, the neurotoxic bicyclic hexadecapeptide α-conotoxin MII containing two critical disulfide bridges is a potent and specific competitive antagonist of neuronal nicotinic acetylcholine receptors.[27] To prevent disulfide scrambling and to ensure correct oxidative folding of α-conotoxin MII following SPPS, an orthogonal 4-monomethoxytrityl/acetamidomethyl (Mmt/Acm) protecting group strategy was pursued (Scheme 4).[28] The thioacetal group proved to be stable towards the acetic acid/iodine deprotection-cyclisation conditions in the formation of **8**. The application of our thioacetal stapling protocol was successfully performed twice, yielding the double modified SCS_2-α-conotoxin MII in a good overall yield.

Stability enhancing thioacetal modification

One key goal of this thioacetal stapling technology was to improve the stability of the modified peptides to various adverse storage and metabolic conditions. The native disulfide bridge is particularly labile under reductive environments and suffers from scrambling reactions in the presence of free thiols. To verify the anticipated reductive stability of the thioacetal modified peptides, OXT and SCS-OXT were incubated at 37 °C in pH 7.0 PBS buffer containing blood levels of L-glutathione
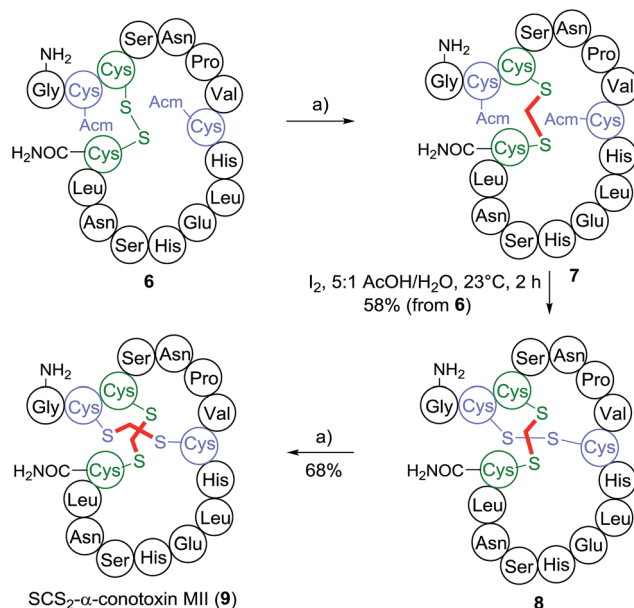
Scheme 4 Synthesis of double thioacetal modified SCS_α-conotoxin MII (**9**) conditions: (a) 1.5 eq. TCEP·HCl, 3.0 eq. K_2CO_3, 10 : 1 H_2O/THF, 23 °C, 2 h, then 6.0 eq. CH_2I_2, 10 eq. NEt_3, 23 °C, 9 h.

(1.0 mM).[29] Under these conditions, the half-life of oxytocin is 8 hours (Fig. 1). In contrast, SCS-OXT is, as expected, completely stable and even after 5 days no degradation was observed.

This confirmed the inherent chemical stability of a thioacetal moiety against reduction. In further tests, we exposed the SCS analogues of OXT and AVP to human serum at 37 °C (Table 1). For both analogues, we found a significant improvement in the serum half-lives. For SCS-OXT the half-life was more than doubled, equating to an additional 40 hours. In addition, SCS-AVP, displayed an approximately 1.5-fold enhancement in the serum half-life in relation to its parent peptide. OXT is included in the WHO's list of essential medicines. A major concern is its thermal liability, particularly in countries where it
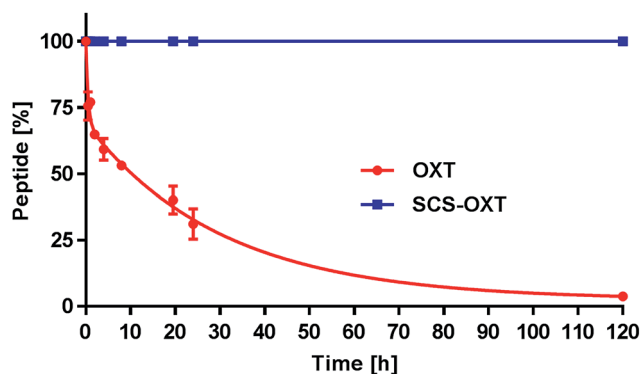

Fig. 1 Reductive stability of SCS-OXT *vs.* OXT (1.0 mM glutathione in pH 7.0 PBS buffer at 37 °C). Data points are displayed as the mean value ± SEM of three independent experiments. Curves for the stability assays were fitted by non-linear, least squares regression analysis using Prism (GraphPad software version 6.07, La Jolla, CA).

Table 1   Serum, temperature and pH-stability of OXT and SCS-OXT[a]

		OXT half-life [d]		SCS-OXT half-life [d]	
Entry	pH	40 °C	55 °C	40 °C	55 °C
1	Serum	1.2 ± 0.2^{b}	—	3.0 ± 0.1^{b}	—
2	2.0	14 ± 0.9	3.2 ± 0.4	27 ± 0.4	3.6 ± 0.4
3	4.5	121 ± 6.2	18 ± 1.3	133 ± 7.0	32 ± 1.7
4	7.0	7.2 ± 0.5	2.0 ± 0.2	34 ± 1.2	8.0 ± 0.4
5	9.0	3.9 ± 0.5	0.9 ± 0.15	8.6 ± 0.8	3.0 ± 0.2

[a] All data are displayed as the mean value $\pm$ SEM (calculated by Prism: GraphPad software version 6.07, La Jolla, CA), of three independent experiments for each pH and temperature variable, and five for the oxytocin serum half-life assay. [b] At 37 °C.

is difficult to ensure proper refrigerated handling and storage, as stability studies indicate that liquid oxytocin formulations rapidly degrade at temperatures greater than 30 °C.[30] Thus, OXT analogues with an enhanced thermal stability profile would be of significant value. Consequently, we undertook a comparative study to compile thermal and pH stability data between SCS-OXT and its unmodified parent peptide (Table 1). The rate of degradation of the peptides were compared by subjecting them to two different temperatures (40 °C and 55 °C) under four pH values (2.0, 4.5, 7.0 and 9.0). Native oxytocin is most stable under slightly acidic or neutral conditions.[30a] Overall, we observed sizeable improvements in stability across the majority of temperature and pH conditions. The most significant gains in stability of the thioacetal modified analogue were obtained at the most medically useful, physiological pH (7.0) increasing the half-life approximately 4 to 5-fold. Even at the best storage pH 4.5, the SCS-OXT displays superior stability at 55 °C. Harsher conditions (high temperature and extremes of pH) were more destructive to both peptides due to the relative ease of peptide bond cleavage under such conditions. Nevertheless, at 55 °C and pH 9.0, our SCS-OXT showed a 3.3-fold increase in half-life.

Evaluation of the biological activities of the thioacetal modified peptides

Although the introduction of the methylene bridge is the smallest possible reinforcement of a disulfide, it involves a minor structural disturbance. The length and the directionality of the disulfide and the methylene thioacetal are slightly different. The impact of such change might level out with the increasing size of the peptide and the length of the loop. We have therefore assessed the binding affinities of the modified OXT and AVP peptides (Table 2). For the oxytocin receptor (OXTR), SCS-OXT demonstrated a slight decrease in binding affinity, ($K_i = 1.41$ nM) compared to OXT ($K_i = 0.24$ nM). For SCS-AVP, the vasopressin receptors $V_{1A}R$ (vasopressor activity), V_2R (water retention) and $V_{1B}R$ (neurotransmitter/neuromodulator)[31] were assessed. Notably, SCS-AVP has a modulated selectivity profile at the different receptors compared to AVP. SCS-AVP displays similar binding affinity ($K_i = 0.77$ nM) as the benchmark standard for the V_{1A} receptor, $[d(CH_2)_5^1, Tyr(Me)^2]$-AVP ($K_i = 0.74$ nM).[32] For the V_2 receptor, SCS-AVP exhibited a slight decrease in affinity ($K_i = 4.52$ nM $vs.$ AVP's $K_i = 1.22$ nM). Concerning the V_{1B} receptor, SCS-AVP binds weaker than the parent AVP ($K_i = 0.87$ nM $vs.$ AVP's $K_i = 0.096$ nM).

Besides binding affinities, the functional activity of the SCS-analogues was also investigated. An *in vitro* uterine contraction assay yielded an $EC_{50} = 4.90$ nM for SCS-OXT, thus remaining a full agonist with a potency of similar magnitude as OXT ($EC_{50} = 1.20$ nM).[33] Prior investigations into the effect of changing the ring size in native OXT demonstrated a significant decrease in the agonist potency of the ring-adjusted analogue by a factor of up to 500.[34] The macrocyclic ring size modification in oxytocin previously involved the carbon framework of the cysteine residues (homocysteine) and not within the disulfide motif. However, our data suggests that increasing the size of oxytocin's 20-membered ring by insertion of the extra methylene group in between the two sulfur atoms does not significantly reduce its agonist potency. Assaying the activation of the V_{1A} receptor by SCS-AVP resulted in an EC_{50} of 290 nM ($EC_{50} = 0.71$ nM for AVP) with both compounds having essentially the same binding affinity (K_i values for SCS-AVP: 0.77 nM, AVP 0.74 nM). Consequently, the thioacetal modification transforms SCS-AVP to a partial agonist at the V_{1A} receptor. Overall, the modified compounds SCS-OXT and SCS-AVP remain biologically active. The thioacetal bridge can alter the selectivity profile of the parent peptides at the different receptors and has the potential to modulate their ligand efficacy.

Table 2   Binding affinity and functional activity of OXT, SCS-OXT, AVP and SCS AVP[a,b]

Entry	Receptor	SCS-OXT	OXT	SCS-AVP	AVP
1	OXTR K_i [nM]	1.41 ± 0.3	0.24 ± 0.04	41 ± 10	16 (ref. 31)
2	OXTR EC_{50} [nM]	4.90 ± 0.34	1.20^{33}	—	—
3	$V_{1A}R$ K_i [nM]	15 ± 3.5	64^{31}	0.77 ± 0.23	0.74 ± 0.14
4	$V_{1A}R$ EC_{50} [nM]	—	—	290 ± 20	0.71 ± 0.10
5	V_2R K_i [nM]	—	—	4.52 ± 0.52	1.22 ± 0.31
6	$V_{1B}R$ K_i [nM]	—	—	0.87 ± 0.24	0.10 ± 0.04

[a] For each assay, five concentrations were performed in duplicate. [b] IC_{50} (or EC_{50}) values were determined by a non-linear, least squares regression analysis using MathIQ™ (ID Business Solutions Ltd., UK) by Eurofins Pharma Discovery Services. Inhibition constants (K_i) were calculated with the Cheng and Prusoff equation using the observed IC_{50} of the tested compound, the concentration of radioligand employed in the assay, and the historical values for the K_D of the ligand.

Conclusions

In conclusion, we have developed an experimentally facile, one-pot transformation reinforcing disulfide linkages in peptides as thioacetals. No synthetic incorporation of engineered amino acids is needed for this stapling process. The protocol operates under mild, bio-compatible conditions (aqueous solution, mild pH and at ambient temperature) and is tolerant towards unprotected amino acids possessing a variety of functional groups. Furthermore, the high site specificity for the insertion into disulfide bonds further enhanced the reactions utility and versatility. The methylene thioacetal bridge completely eliminates the reductive liability of the disulfide group, contributing to an enhanced structural stability. We have exemplarily shown for oxytocin that the thioacetal analogue exhibits superior stability while maintaining binding affinities and functional activities of the same magnitude. This methodology is an additional tool for stapling peptides using natural linking points with potential impact for the medicinal application of bioactive peptides.

Acknowledgements

Notes and references

‡ *General procedure for the synthesis of SCS-OXT*
To a round bottom flask containing a stirrer bar was added 1.00 eq. of oxytocin acetate powder (3.20 mg, 3.20 μmol) and H_2O (3.60 mM). A solution of TCEP·HCl (1.50 eq.) and K_2CO_3 (3.00 eq.) in H_2O (29.0 mM) was added *via* syringe. The reaction was stirred at room temperature for 1.25 hours. The progress of the reaction was monitored by HPLC-MS. Upon completion of this first step of the process, triethylamine (5.00 eq.) in THF (0.30 M) and then diiodomethane (4.00 eq.) in THF (0.25 M) were added sequentially to the reaction mixture *via* microsyringe. The reaction mixture was stirred at room temperature for 6.5 hours. The progress of the reaction was monitored by HPLC-MS. Once adjudged complete, the reaction mixture was diluted with H_2O (5.00 mL) and lyophilised. Then, the crude peptide was purified by RP preparative HPLC, using a linear gradient of 5% B to 50% B over 30 minutes at a flow rate of 25.0 mL min^{-1} with UV detection at 220 nm, 254 nm and 280 nm. (solvent A = 99.9% v/v H_2O and 0.1% v/v formic acid; solvent B = 95% v/v MeCN : 5% v/v H_2O and 0.1% v/v formic acid). The pure product was isolated in 78% yield (2.50 mg) as a fluffy white powder.

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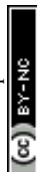
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