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Thioether analogues of the pituitary neuropeptide oxytocin *via* thiol–ene macrocyclisation of unprotected peptides†

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Disulfide bonds are an essential feature of many bioactive peptides, however, they are labile to reducing conditions which can limit therapeutic application. Herein, we report an efficient methodology for peptide macrocyclisation, furnishing thioether mimetics of disulfide linkages *via* thiol–ene click chemistry. Furthermore, this methodology is applied to the efficient synthesis of analogues of the neuropeptide oxytocin and in a highly efficient route to the clinical therapeutic carbetocin.

The chemical synthesis of peptides continues to garner considerable interest due to their burgeoning therapeutic applications, driven by sophisticated advancements in peptide drug development including rational design and phage display.[1] Compared to traditional, small-molecule therapeutics and large biologics, peptides occupy a unique chemical space, often demonstrating improved efficacy and selectivity, as well as reduced toxicity and production costs. However, limitations remain, including poor *in vivo* stability and membrane permeability.[2] Synthetic modifications are critical to addressing these restrictions and in progressing peptide therapeutics to clinical trials. Peptide cyclisation and stapling of α-helical secondary structures has been demonstrated as a viable strategy to surmount limitations of peptide drug stability and improve membrane permeability.[3,4] Common peptide stapling techniques include disulfide or thioester formation, lactamisation and ring-closing metathesis (RCM) and have been applied to the syntheses of a variety of cyclic peptides.[5,6] Click reactions have been widely utilised to facilitate rapid and efficient macrocyclisation of peptides,[7,8] and the development of click chemistry was recently awarded the Nobel Prize. One such reaction, thiol–ene click (TEC) is emerging as highly amenable to such applications.[9] The reaction involves a chemoselective, radical-mediated anti-Markovnikov addition of a thiol onto an alkene, furnishing a robust thioether linkage in high yield and with minimal side products. Mild conditions, fast reaction kinetics and compatibility with both aqueous conditions and a wide range of functional and protecting groups, render it ideal for applications in biorthogonal chemistry. Aimetti *et al.*[10] reported the use of TEC chemistry for one-component on-resin peptide macrocyclisation *via* allyloxycarbonyl (Alloc) or norbornene modifications in the synthesis of an integrin peptide ligand. Use of butenyl and pentenyl terminal alkene side chains in synthesis of short, thioether-tethered cyclic peptides *via* thiol–ene reaction on-resin was reported by Zhao *et al.*[11] Further, TEC has been used in the introduction of photoswitchable staples by Hoppmann *et al.*[12,13] The existing work on TEC for peptide cyclisation has been reviewed by the group.[7,8] However, these methods introduce significant structural changes and hydrophobicity onto the peptide backbone, adversely affecting pharmacokinetic profiles and limiting therapeutic applications.

In contrast to previous methods in structural stabilisation of peptide sequences *via* large modifications using TEC, we aimed to introduce a minimal perturbation to naturally occurring sequences, whilst achieving the known benefits of peptide macrocyclisation. Inspired by recent developments in redox-stable, thioether peptide scaffolds, we focused on the application of thiol–ene chemistry for the synthesis of thioether-bridged peptides as disulfide mimetics, with a particular focus on the pituitary neuropeptide, oxytocin (OT). To this end, the synthesis of various novel thioether analogues of OT *via* thiol–ene chemistry was envisaged (Fig. 1). The thiol–ene reaction provides an optimal disconnection for synthesis of thioether-linked macrocycles. OT is a nonapeptide hormone containing two disulfide bridged Cys residues and with complex neuropsychiatric function, believed to be involved in a range of psychiatric disorders including autism and schizophrenia as well as mood and anxiety disorders.[14,15] Its implication in such disorders and complex neurochemistry has resulted in significant interest in development of the therapeutic potential of OT. As

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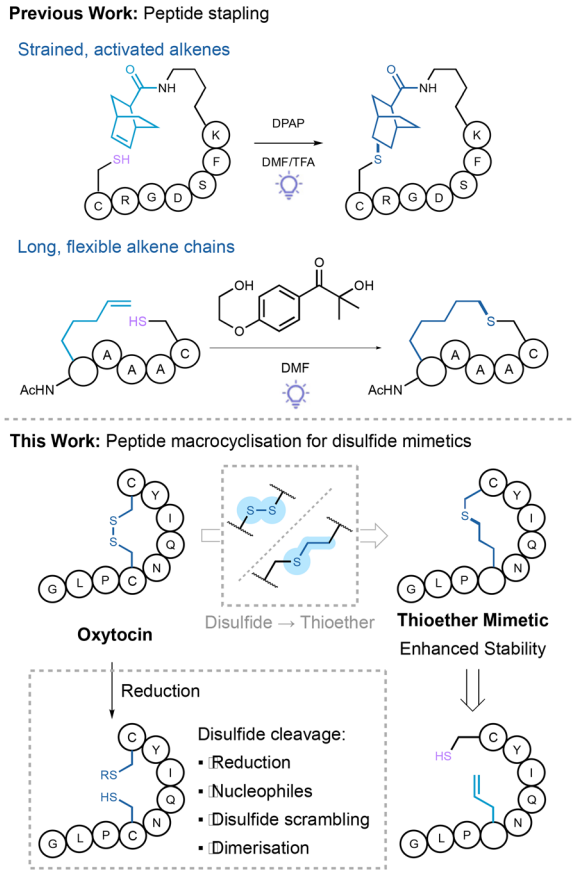

Fig. 1 Previous work using activated alkenes or long alkene side chains, and this work on short disulfide mimetic linkages using TEC.

such, research has been conducted into manipulation of selectivity and physicochemical properties.[16,17] However, the short half-life and poor permeability across the blood–brain barrier of OT still necessitates novel drug design strategies. The substitution of the native disulfide linkage, such as with a thioether, is seen as a viable chemical approach to improvement of the stability of OT.[16–20] Cochrane *et al.* produced a number of OT analogues *via* ring-closing metathesis on unprotected linear precursors, affording all-carbon-, ether- and thioether-containing macrocycles, whilst work by Stymiest *et al.* produced shorter olefin cyclised analogues which showed biological activity when tested on rat uterus stips. Similar selenoether analogues have been synthesised by de Araujo *et al.* showing analgesic properties in a mouse model.[21]

This work presents the use of TEC to produce short disulfide mimic thioether linkages in generation of OT analogues. This presents novel use of thiol–ene chemistry in generation of a biological mimic, rather than structural stabilisation *via* peptide stapling as previously reported. In comparison to previous techniques, TEC offers a rapid (1 h) metal-free methodology for peptide cyclisation. Additionally, due to the radical nature of the reaction, it is sympathetic to the presence of nucleophiles and electrophiles that may interfere in other cyclisation chemistries. The click characteristics of TEC give the additional advantages of aqueous compatibility and remove the need for inert atmosphere.

We first investigated the on-resin cyclisation of fully protected sequences with either Cys residue substituted for a commercially available terminal alkene containing amino acid, Allyl-Gly (Agl). This give two analogues; Agl(6)-OT (**2**) in which the internal Cys(6) has been replaced and Agl(1)-OT (**3**), in which the N-terminal Cys(1) has been substituted (Fig. 2). The peptide sequences were constructed on-resin *via* standard Fmoc-SPPS. Selective removal of the trityl protecting group on the Cys sidechain was achieved upon treatment with 5% trifluoroacetic acid (TFA) solution in DCM, as confirmed qualitatively *via* Ellmann test. The resin was subsequently suspended in DMF, together with catalytic 2,2-dimethoxy-2-phenylacetophenone (DPAP) photoinitiator and 4′-methoxyacetophenone (MAP) photosensitiser, and irradiated at 365 nm for 1 hour with continuous stirring. Following cleavage of the peptide from the resin, analyses showed that the acyclic product was obtained for both analogues, with no macrocyclisation taking place. It is possible that the lack of cyclisation was in part due to disruption of radical chain propagation, since the thiol component cannot freely diffuse when attached to the solid support in this intramolecular thiol–ene variation. The presence of protecting groups may also have been a contributing steric factor. Investigation into on-resin thiol–ene chemistry is ongoing in our laboratory.

Following this initial finding, the alkene-containing peptides were subsequently cleaved from the resin as linear analogues following standard TFA cleavage procedures to facilitate investigation into cyclisation of fully unprotected sequences in solution. The linear peptides were irradiated at 365 nm for 1 hour in the presence of DPAP and MAP (1 equiv. each). The reaction was first performed in deuterated ammonium acetate buffer for ease of reaction monitoring, however this solvent system was found to be suboptimal with only partial conversion to the cyclic product observed for Agl(6)-OT and no conversion observed for Agl(1)-OT (see ESI†). Attempts to perform cyclisation in THF and in methanol gave similar results. This poor repeatability is likely a result of incomplete solvation of the linear peptide starting material. However, upon switching the solvent system to a H_2O/acetonitrile (ACN) (1 : 2) mix with 0.1% TFA, cyclic product **2** was obtained with high conversion (91%). The importance of the buffer/solvent system is supported by the findings of Colak *et al.* in ensuring that the thiol residue remains protonated.[22] With optimised conditions in hand, we investigated the more challenging Agl(1)-OT analogue, displaying an internal cysteine residue. To our delight, in the H_2O (0.1% TFA)/ACN (1 : 2) solvent system, this analogue was successfully cyclised to with conversion of 68%. Indeed, reduced yields have been previously observed for reactions at Cys side chains within a peptide sequence compared to at the termini, and is hypothesised to arise from either the change in Cys pK_a or steric effects.[23] Colak *et al.* have shown that, in the case of model CGGXX and GCGXX sequences, TEC efficiency is decreased for the internal Cys residue, and our findings are in agreement with this observation.[22] Due to the presence of

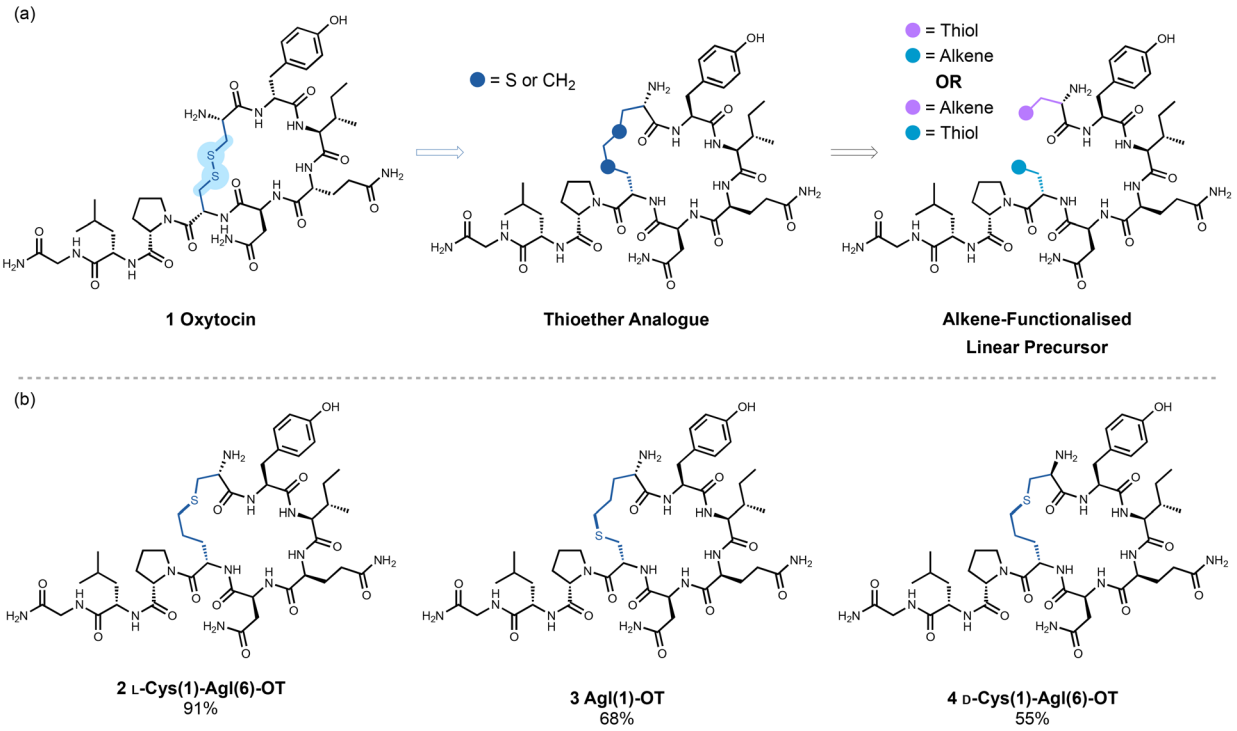

Fig. 2 (a) Structure of oxytocin and thioether analogues obtained *via* TEC. (b) Thioether analogues synthesised.

0.1% TFA in the cyclisation mix, the Cys thiol will be protonated and therefore this effect likely results from steric influence of neighbouring residues.

Following macrocyclisation of both Agl(6)- and Agl(1)-OT peptides bearing all L-stereochemistry, the effects of varying stereochemistry proximal to the thiol residue was investigated. Most peptidases recognise L-amino acids and exchanging and L-AA with the corresponding D-AA can render the peptide more stable to peptidase activity, offering improved stability *in vivo*. The corresponding D-Cys(1)-OT (**4**) analogue was synthesised and investigated under the previously optimised conditions. Gratifyingly, macrocyclization was achieved, albeit with a drop in conversion to 55% relative to the L-Cys analogue.

Importantly for monitoring of this reaction, the mass of the starting material and product are the same, since the reaction amounts to intramolecular hydrothiolation. However, ^{1}H-NMR monitoring of the reaction is facile, as the characteristic alkene peaks are readily observed in the spectrum (Fig. 3) and additionally are readily assigned *via* characteristic coupling constants. The disappearance of these peaks is direct evidence of alkene consumption. In combination with HPLC analysis, this can be used as a preferred method for reaction monitoring.

We next sought to apply the methodology to generation of an OT analogue of the native ring size and we therefore turned to incorporation of vinyl Gly (Vgl). Vgl is particularly prone to both epimerisation and isomerisation during peptide coupling. It was determined that incorporation of the alkene-containing residue at the N-terminal position would facilitate

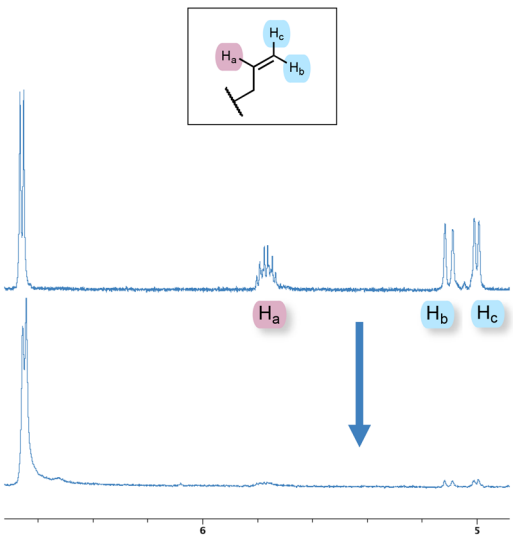

Fig. 3 ^{1}H-NMR analysis of the reaction shows progress *via* consumption of alkene.

attachment of Boc-protected Vgl as the final amino acid in SPPS prior to deprotection and cleavage from the resin, thereby minimising any side-reactions. The Vgl(1)-OT (**5**) peptide was synthesised by Fmoc-SPPS, incorporating Boc-Vgl in the N-terminal position *via* mild, base-free coupling using 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihydroquinoline (IIDQ) over five days (Fig. 4).[19] HPLC analysis of the cleaved peptide revealed high purity of the crude material, and therefore cycli-

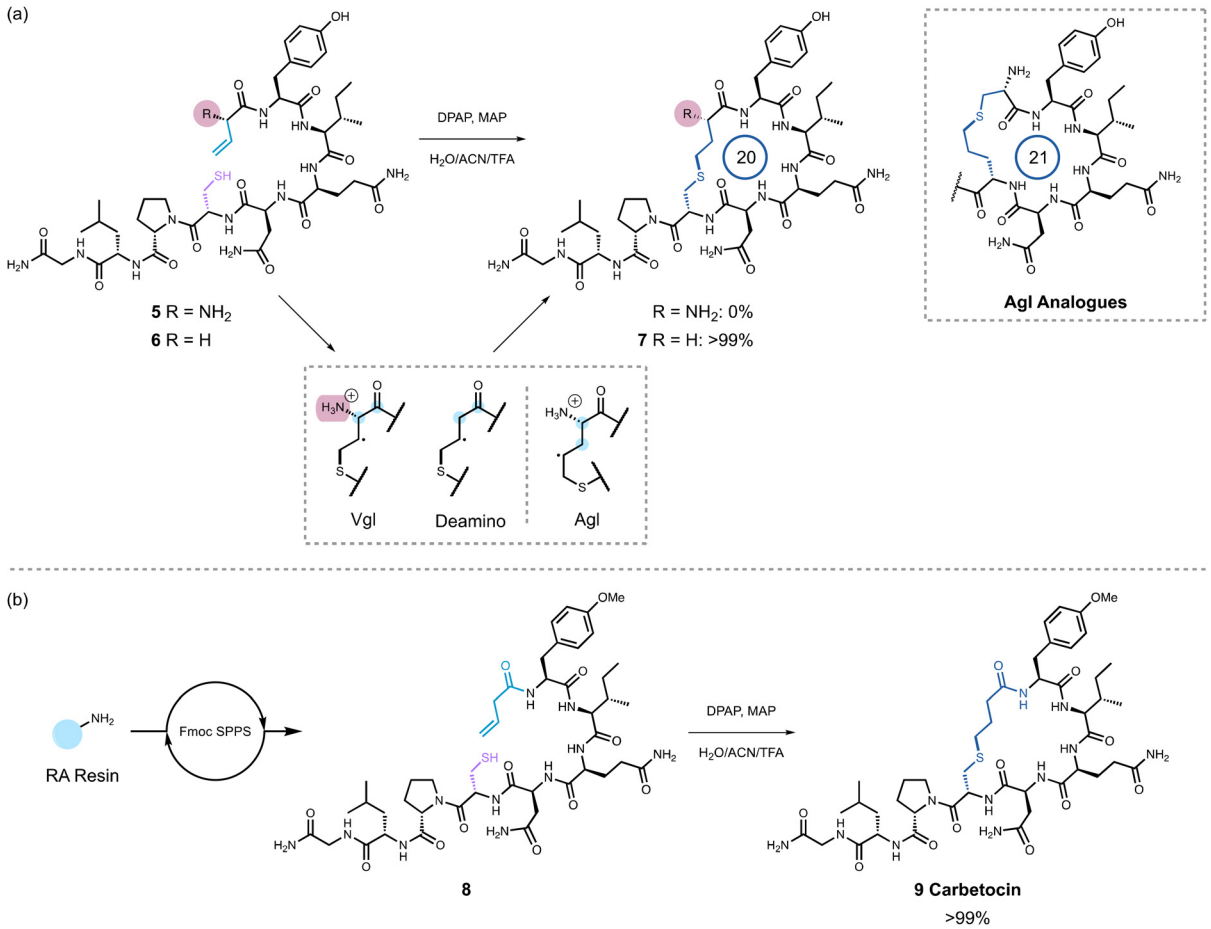

Fig. 4 (a) Cyclisation of Vgl(1) analogue and its deamino analogue and structures of carbon-centered radical intermediates with significant charge transfer contributing centres indicated. (b) Synthesis of carbetocin *via* TEC.

sation was attempted without further purification. Unfortunately, no consumption of the alkene was observed by ^{1}H-NMR analysis. The reduced flexibility of the alkene-containing chain may be partly responsible for the lack of reactivity. Furthermore, since proximal amines have been shown to interfere in TEC,[20] the close proximity of the free N-terminus may also play a role in obstructing macrocyclisation. To further investigate this effect, we synthesised the corresponding deamino analogue (**6**) by coupling but-1-enoic acid in place of the Vgl residue, providing a more flexible alkene that lacks the proximal amine residue. Gratifyingly, this analogue underwent quantitative cyclisation upon irradiation for 1 hour in the presence of DPAP and MAP. The deamino analogue **7** has previously been reported by Adachi *et al.*, showing OT receptor agonist activity in the pM range.[21] Results by Colak *et al.* have shown that allyl amine derivatives (such as Vgl) show significantly reduced efficiency in TEC due to amine proximity, as a result of overlap of the SOMO of the carbon-centered radical intermediate formed with orbital centred at atoms up to 2 bonds away,[22] which is in agreement with our observations.

Following successful cyclisation of the deamino analogue to furnish **7**, we next turned our attention to the synthesis of carbetocin (CT, **9**). The TEC methodology provides an alternative disconnection for the macrocyclisation step in the synthesis of CT, enabling use of widely available and relatively cheap starting materials. On-resin synthesis of the sequence up to the Tyr residue was followed by capping with but-1-enoic acid *via* PyBop coupling. Conveniently, SPPS, followed by cleavage and universal deprotection provided the peptide in sufficient purity for cyclisation without further HPLC purification. The linear peptide precursor **8** was dissolved in the H_2O/ACN (1 : 2) solvent mix with 0.1% TFA, DPAP and MAP and irradiated at 365 nm for 1 hour. Gratifyingly, quantitative conversion >99% was again observed for this analogue. The high reactivity of this analogue in thiol–ene cyclisation is likely due to the highly flexible alkene structure and the absence of an amino group proximal to the alkene. Synthesis of carbetocin *via* this approach offers improved atom economy, in reducing the necessity for protecting groups. Additionally, this route may prove ideal if adapted to in-flow TEC cyclisation. In-flow TEC is also currently under investigation in our laboratory. Whilst UV initiation is a clear caveat for scale-up, flow chemistry could allow for this. Additionally, TEC has been previously reported using visible light.[24]

In conclusion, we have established an efficient methodology for cyclisation of unprotected peptide sequences in solution, employing the thiol–ene click reaction. Cyclisation at both internal and terminal Cys residues was demonstrated, together with a tolerance to variation of stereochemistry at the cyclisation site. Composition of the solvent mix was found to be critical to the success of the process with 0.1% TFA being a requirement for chain propagation. Finally, the methodology was applied to a simplified and highly efficient synthesis of the therapeutic carbetocin. Synthesis of further disulfide peptide analogues is under investigation and is to lead to more stable analogues. Further, we envisage that this approach using simple thioether linkages would be amenable to synthesis of peptide libraries, in particular using peptides of high crude purity produced using thiol resins,[25] work that is currently under investigation.

Author contributions

M. D. N., R. P and E. M. S. devised the study and prepared the manuscript. M. D. N. and R. P. performed the experiments.

Conflicts of interest

There are no conflicts to declare.

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