Peptide macrocyclisation via intramolecular interception of visible-light-mediated desulphurisation†

Frances R. Smith,a Declan Meehan,a Rhys C. Griffiths,a Harriet J. Knowles,a Peiyu Zhang,b Huw E. L. Williams,c Andrew J. Wilson bd and Nicholas J. Mitchell,*a

Synthetic methods that enable the macrocyclisation of peptides facilitate the development of effective therapeutic and diagnostic tools. Herein we report a peptide cyclisation strategy based on intramolecular interception of visible-light-mediated cysteine desulphurisation. This method allows cyclisation of unprotected peptides in an aqueous solution via the installation of a hydrocarbon linkage. We explore the limits of this chemistry using a range of model peptides of increasing length and complexity, including peptides of biological/therapeutic relevance. The method is applied to replace the native disulphide of the peptide hormone, oxytocin, with a proteolytically/redox-stable hydrocarbon, and internal macrocyclisation of an MCL-1-binding peptide.

Introduction

Despite increasing interest in peptide therapeutics within the pharmaceutical industry over the past three decades, relatively few peptides have made it through pre-clinical development to challenge the dominance of small-molecule pharmaceuticals and protein-based biologics.1 This is primarily due to the low membrane permeability and poor stability of linear peptides.2 Conversely, constrained macrocyclic peptides display enhanced stability relative to their linear counterparts and higher binding affinities due to a rigid conformation. Furthermore, macrocyclic peptides offer an effective tool to enable selective interference of the myriad of traditionally intractable protein–protein interactions (PPIs) that mediate cellular biochemistry.3,4 Thus, this modality of therapeutic bridges the gap between the advantageous physicochemical properties of small molecules and the exceptional activity, specificity, and bioavailability inherent to protein-based biologics. While numerous synthetic methods have been developed to enable effective peptide macrocyclisation, biocompatible chemistries that work with readily accessible building blocks in environmentally acceptable solvents are still required to enable the significant impact of macrocyclic peptides as a next-generation drug discovery tool to be realised.

Many of the synthetic methods developed to cyclise peptides5–8 take inspiration from nature and utilise the canonical residues, cysteine (Cys) and lysine (Lys), or the native N- and C-terminal functionality to form disulphide,5 thioether,9,10 and amide bonds (Fig. 1). Cyclisation via amide bond formation has been applied using both synthetic methods (peptide ligation11–13) and biological approaches exploiting intein chemistry (e.g., SICLOPPS9,14), mRNA display,10 and ligase enzymes.16 Due to the nucleophilicity of the thiol sidechain, numerous Cys-selective reactions (beyond disulphide and thioether formation) have been developed/repurposed for peptide cyclisation strategies.

Peptide macrocyclisation strategies:

Fig. 1 Commonly applied peptide macrocyclisation strategies; interception of desulphurisation as an approach to macrocyclisation of unprotected peptides.
cyclosisation. These include the formation of bicycles\textsuperscript{37} via alkylation scaffolds,\textsuperscript{18-21} bridging Cys residues using perfluoroaryl bromines/fluorine displacement\textsuperscript{22-23} (and alternative bridging groups\textsuperscript{24,25}), thiol coordination to bismuth,\textsuperscript{26} thiol-addition chemistry,\textsuperscript{27-28} and desulfurative replacement of a disulfide bridge.\textsuperscript{29} Incorporation of non-standard amino acids enables the exploitation of bioorthogonal chemistry such as azide–alkyne cycloaddition\textsuperscript{30,31} (i.e., ‘Click Chemistry’), Staudinger ligation\textsuperscript{32} and azide-phosphinite chemistry.\textsuperscript{33} Simple imine\textsuperscript{34} and oxime\textsuperscript{35} bond formation has been utilized, as well as more complex transition metal (TM)-catalysed\textsuperscript{36-41} and multi-component chemistry.\textsuperscript{42} Radical reactions such as thiolyne\textsuperscript{43-45} and, more recently, decarboxylative photoredox catalysis\textsuperscript{46} and C–H alkylation\textsuperscript{47} have also been successfully applied. However, among the broad range of available synthetic techniques, Grubbs’ ruthenium (Ru)-catalysed ring-closing olefin metathesis (RCM)\textsuperscript{48} reaction has found global application as a method-of-choice for peptide cyclisation due to effective formation of a proteolytic, hydrolytic, and redox-stable hydrocarbon linkage.\textsuperscript{49-53}Whilst an undeniably powerful method, RCM is usually conducted on protected peptides in organic solvent, and access to the saturated hydrocarbon necessitates reduction under harsh conditions. New, operationally simple techniques that retain the benefits of RCM, but that work effectively in more sustainable solvents, would offer an impactful alternative to this universally popular method.

We recently reported a novel strategy for the site-selective modification of peptides and proteins via visible-light-mediated desulfurative C(sp\textsuperscript{3})–C(sp\textsuperscript{3}) bond formation.\textsuperscript{54-55} Desulfurisation of Cys (and alternative non-proteinogenic thiol-containing amino acids\textsuperscript{56-59}) can be applied post-peptide ligation as an elegant method to access a broad range of ligation junctions and facilitate chemical protein synthesis.\textsuperscript{56,59-67} A widely used free-radical-mediated Cys desulfurative protocol,\textsuperscript{60} developed by Danishefsky and co-workers, proceeds via a thiophosphoranyl radical species generated using a radical initiator (VA-044) to form a thyl radical from the thiol sidechain of Cys in the presence of the water-soluble phosphine, tris(2-carboxyethyl)phosphine hydrochloride (TCEP). \textsuperscript{61–63} \textsuperscript{\beta}-Scission of the thiophosphoranyl radical\textsuperscript{62} produces a peptide ‘alanyl’ radical which, in the presence of a suitable thiol additive (e.g. glutathione), will abstract an H-atom to yield the residue, alanine (Ala), at the ligation junction.\textsuperscript{64} In previous work, we demonstrated desulfurisation using an iridium(m) photocatalyst (PC) and employed alkynes to intercept the alanyl radical species, enabling installation of Lys sidechains carrying natural modifications as well as effective mimics of this modified sidechain.\textsuperscript{65-67} This reaction is initiated via excitation of the Ir(m) PC by a photon of visible light. The activated catalyst is then reduced by the thiol group producing a thyl radical cation which forms a thyl radical on deprotonation. In the presence of TCEP, the thiophosphoranyl radical is formed; \textsuperscript{\beta}-scission of this species produces the ‘alanyl’ radical\textsuperscript{48} which is trapped by the alkene. Due to the requirement to out-compete H-atom abstraction during this process, a large excess of the alkene is required (a minimum of 200 equivalents). By installing an appropriate alkene into a peptide containing a Cys residue, we postulated that intramolecular trapping of the radical produced upon desulfurisation may proceed preferentially to H-atom abstraction, essentially allowing us to use an equimolar equivalent of the alkene (Fig. S1f). The cyclic peptide radical produced during this reaction will be quenched to form the macrocyclic product; H-atom transfer (HAT) from the thiol group of remaining starting peptide is a likely pathway. The resulting thyl radical can then continue the cycle or be reduced by the catalyst and protonated.\textsuperscript{68} Oxygen in the buffer has also been identified as an oxidant for the catalyst during Ru-mediated desulfurisation.\textsuperscript{69}

The chemoselectivity of this chemistry should ensure that the reaction enables efficient cyclisation of unprotected peptides in aqueous solution. If realised, this technique would be a valuable addition to the toolkit available to researchers for the production of cyclic peptides.

**Results and discussion**

**Optimisation of reaction conditions and radical trap exploration**

Initial trials of this strategy focused on the utilisation of both 2-methylallyl and allyl moieties to facilitate peptide cyclisation in the presence of a Cys residue (Table 1). Addition of the alanyl radical to a 2-methylallyl group would generate a tertiary radical intermediate previously shown to improve the efficiency of this chemistry;\textsuperscript{55} however, diastereomers of the desired macrocycle will be produced due to the methyl branch of the linkage. Employing an allyl group as the radical trap would result in a single product, however, the reaction will be less effective and may not fully out-compete H-atom abstraction. Both of these strategies were initially explored. Amino acid building blocks 1 and 2 were synthesised via alkylation of Boc-Ser-OH with 1-bromo-3-methylbut-3-ene and allyl bromide, respectively (ESI†). These residues were incorporated into a simple peptide sequence using solid phase peptide synthesis (SPPS) to afford peptides 3 (H–S(O-2-methylallyl)AFAC-NH\textsubscript{2}) and 4 (H–S(O Allyl) AFAC-NH\textsubscript{2}). Both peptides (3/4, 0.5 mM) were subjected to desulfurisation conditions in 9:1 conjugation buffer (6 M Gdn·HCl, 0.1 M Na\textsubscript{2}HPO\textsubscript{4}, pH 7.5–8.0) and acetonitrile (MeCN) in the presence of a phosphine (TCEP; 5 mM) and an Ir(m) PC (5 mol%, [Ir(dF(CF\textsubscript{3}))ppy\textsubscript{3}(dtbpy)]PF\textsubscript{6}), under irradiation of blue light (450 nm) using inexpensive blue LED light strips (see ESI† for details). The reaction progress was monitored using analytical HPLC. Excess phosphine was employed to ensure that the thyl radical did not interfere with the reaction via thiol–ene radical addition.\textsuperscript{69} Promisingly, we observed complete and clean conversion of the starting peptide 3 within 10 min. As anticipated, two products were observed, each with the same mass. Purification of the material via preparative HPLC was followed by NMR analysis which determined that the two products were diastereomers of the desired cyclised peptide, isolated in an excellent combined yield of 77% (5a/5b, 53% and 24%, respectively) (Table 1, entry 1). No resonances were observed corresponding to the allyl group in the \textsuperscript{1}H NMR spectrum, which precludes the misidentification of the desulfurised starting peptide as the product; a concern considering these structures share the same molecular weight.
Quantitative conversion of the starting sequence to cyclised product, with negligible formation of the linear desulphurised by-product (formed via the alanyl radical abstracting an H-atom), is a gratifying initial result that allows high-yielding and rapid access to macrocyclic peptides. However, the formation of diastereomers is not ideal. Peptide 4 carries an allyl group as the radical trap; while this moiety would generate a less stable secondary radical upon addition of the alanyl radical, our previous results gave us confidence that intramolecular trapping should still out-compete H-atom abstraction. Under the conditions described, the starting peptide (4) was consumed within 60 min leading to the production of a major product (72% conversion to product by analytical HPLC, entry 2). It was noted that the reaction was equally effective without using degassed buffer; therefore, this step was omitted from the protocol. Prior to scaling the reaction up for isolation, a brief optimisation study was undertaken. It was observed that a reduction in the equivalents of TCEP led to a dramatic decrease in conversion to the product (Entry 3; 38% conversion to product 6), as did decreasing the mol% loading of the PC from 5 to 1 mol% (entry 4). When scaling up to an isolable yield it was observed that the conditions detailed in entry 2 were not optimal; an increase in the equivalents of TCEP to 50 (25 mM) was necessary to maintain high conversion to product. Using these adjusted conditions (entry 5) the desired product (6) was isolated in 78% yield and characterised via MS and NMR spectroscopy. The remaining mass balance for these reactions was the linear desulphurised by-product. No peptide degradation or epimerisation was observed over the course of the reaction.

Due to the need to synthesise the allyl-protected serine (Ser) building block, a more readily accessible option was sought. The commercially available amino acid, Fmoc-allyl-Gly-OH (alGly, alG), would afford a cyclic peptide with a butyl hydrocarbon linker. Therefore, Fmoc-protected alGly was incorporated into a model peptide (H-(alG)AFAC-NH2; 7a) and cyclised using the optimised conditions based on entry 5, Table 1 with a decrease in the PC loading to 1 mol% (entry 6), a change which did not hinder conversion to the product. The reaction proceeded to completion as expected over 45 min and the desired product (8a) was isolated in an excellent yield of 79% by preparative HPLC (Fig. 2). The following conditions were therefore identified as optimal: 0.5 mM peptide, 1 mol% PC, 25 mM TCEP, in 10% MeCN/6 M Gdn·HCl, 0.1 M Na2HPO4, pH 7.5–8.0 (degassing step omitted). The reaction was then repeated using a PhotoRedOx Box equipped with a 34 mW LED strips, photochem set up 1 (ESI†). Peptide products isolated by preparative HPLC. * Reaction conducted in 10% acetonitrile (MeCN)/6 M Gdn·HCl, 0.1 M Na2HPO4, pH 7.5–8.0. † LED strips, photochem set up 1 (ESI†). ‡ Diastereomeric ratio calculated via analytical HPLC. ‡‡ Combined yield from both diastereomers. ‡§ Reaction completed in 15 min using a PhotoRedOx box (photochem set up 2 – ESI†).
To further explore the optimised reaction, we considered buffer composition, the phosphine additive, and alternative desulfurisation conditions. Cyclisation of model 7a in PBS did not proceed cleanly (Fig. S55†), while employing HEPES buffer did lead to clean conversion of the starting peptide, however, the undesired desulfurised linear by-product was the dominant product (Fig. S66†). Thus, we conclude that cyclisation is most effective in a buffer containing a high concentration of chaotropic salt. Replacing TCEP with the water soluble phosphine, 3,3′,3″-phosphane-triyl-tris(benzenesulfonic acid) trisodium salt (TPPTS) did not lead to effective conversion of the starting peptide 7a (Fig. S57†). Employing 1,3,5-triaz-7-phosphaadamantane (PTA) initially appeared to eliminate the production of the undesired linear desulfurised by-product (Fig. S58†). However, repetition of the reaction on an isolable scale and purification by preparative HPLC revealed the by-product and other impurities eluting under the broader product peak (Fig. S59–S62†) which ultimately limited the isolated yield. Finally, the peptide H-(alG)AFAC-OH (7b) was synthesised on 2-CTC resin to probe the cyclisation on peptides bearing a C-terminal carboxylic acid. While conversion to the desired product was observed (Fig. S68†), the ratio of by-product to macrocycle was less favourable when compared to C-terminal amide peptides. In addition, for this simple model, the retention times for the desired product and by-product were very similar making separation a significant challenge.

Several methods for the desulphurisation of Cys residues have been reported in the literature, these include; photo-induced desulphurisation60 using a ruthenium PC61 photo-desulphurisation in flow,62 accelerated desulphurisation using tetraethylborate (NaBEt4),63 desulfurative borylation,64 and the exploitation of phosphite64 and phosphine-dependent pathways.65 We attempted to adapt and explore two appropriate examples.66,67 However, both the use of NaBEt464 and a ruthenium photocatalyst65 failed to improve on our reported conditions (Fig. S63 and S64–S67†).

To enable the synthesis of peptide macrocycles with longer hydrocarbon linkages, the amino acid pentenyl glycine (pGly, pG) was incorporated into the N-terminus of a simple model hydrocarbon linkage, the amino acid pentenyl glycine (pGly, pG) was incorporated into the N-terminus of a simple model hydrocarbon linkage, the amino acid pentenyl glycine (pGly) was incorporated into the N-terminus of a simple model hydrocarbon linkage, the amino acid pentenyl glycine (pGly) was incorporated into the N-terminus of a simple model hydrocarbon linkage, the amino acid pentenyl glycine (pGly) was incorporated into the N-terminus of a simple model hydrocarbon linkage, the amino acid pentenyl glycine (pGly) was incorporated into the N-terminus of a simple model hydrocarbon linkage, the amino acid pentenyl glycine (pGly) was incorporated into the N-terminus of a simple model hydrocarbon linkage. In addition, for this simple model, the reten-

Fig. 2 (A) Cyclised peptide 8a from H-(alG)AFAC-NH2 (7a); (B) analytical HPLC of the crude cyclisation of 7a (blue trace) overlaid with peptide H-(alG)AFAC-NH2 (9), inset – ESI MS showing the mass of the desired cyclised product (8a); (C) 1H NMR spectrum for linear peptide 7a (red trace) and peptide macrocycle 8a (blue trace) showing the absence of the allylic protons.

Fig. 3 Hα region for the TOCSY NMR spectrum of 8a confirming successful formation of the macrocycle.
the C-terminus, effective N- to C-terminal macrocyclisation was realised for all sequences explored to yield 17-membered macrocycles (wrt the number of bonds within the macrocycle) in moderate to excellent yields (21–27, 48–74% isolated yield). The model peptides explored carried the majority of the 20 canonical amino acids, demonstrating the tolerance of this reaction to the diverse chemical functionality displayed across the proteome. Interestingly, switching the terminal residues eliminated or hindered conversion to the desired product for this size of macrocycle (Table S1†).

To explore macrocyclisation of a peptide carrying the radical trap residue at an internal position, peptide 20 was synthesised with alGly as the penultimate N-terminal residue. For this model, cyclisation was successful in high yield (27, 82%). Moving to longer sequences we explored several 6, 7, & 8-residue peptides carrying a range of proteinogenic residues (28–38) to yield 20, 23, and 26-membered peptide macrocycles in moderate to excellent yield (39–48, 32–76%, Fig. 5). All macrocyclic products were characterised by analytical HPLC, MS and 1H NMR; CD spectroscopy was run for examples of each size of macrocycle (ESI†). Switching the N- and C-terminal residues for model 29 (H-CKISY(alG)-NH2) had no effect on yield for this size of macrocycle, unlike the smaller model peptides explored (Table S1†). In several cases of low or negligible yield (e.g., 40a) acetylation of the N-terminus reinstated a moderate yield (40b, 48%). To confirm the effect that buffer composition has on the isolated yield, cyclisation of model 28 was carried out in PBS. Conversion to the desired product was again not as effective in this buffer compared to cyclisation in conjugation buffer. However, for this model, the ratio of product to desulphurised linear by-product was comparable to that of the conjugation buffer example. Additionally, PTA was revisited for the low-yielding model 31. While this additive did not give an improvement in yield for model 7a, we found that it improved the conversion to the product for the more complex model 31, increasing the yield from 37% to 52% (41). This result indicates that, while TCEP gives more favourable results for the majority of sequences, alternative phosphines can be employed.

A final 14-residue model peptide (49) was prepared to explore the preparation of larger (44-membered) macrocycles. Under the optimised cyclisation conditions the starting peptide was fully consumed to an inseparable mixture of the desired cyclised product and the desulphurised linear by-product. Increasing the loading of the PC to 5 mol% resulted in formation of the postulated phosphonium by-product observed for acrylamide model 13 (Fig. S144–S146†). This by-product (50) was isolated in 58% yield; 31P NMR analysis gave a single phosphorus resonance at 36.6 ppm. Our strategy is, therefore, highly effective for the formation of peptide macrocycles up to a 26-membered ring, and tolerates the full range of proteinogenic chemical functionality found across the proteome. Access to larger macrocycles may be possible, but will be dependent on successful separation of the desired product from the linear desulphurised by-product.

**Macrocyclisation of therapeutic peptides**

To apply this technology to biomedically relevant peptides, the hormone oxytocin was synthesised with alGly replacing one of the Cys residues in the peptide to install a hydrocarbon ‘brace’ in place of the native disulphide (carba-oxytocin). This
Modification has been previously explored to enhance the proteolytic and hydrolytic stability of this peptide.\textsuperscript{70} Positioning alGly at the N-terminus and Cys at an internal position for this model (51, ESI\textsuperscript{†}) failed to afford the desired product when applying the cyclisation conditions, instead producing desulphurised linear material only. Interestingly, Scanlan, Petracca and co-workers observed the same challenge when attempting to cyclise oxytocin via a thiol–ene reaction using an N-terminal alGly residue.\textsuperscript{71} We found that switching the positions of the Cys and alGly residues (sequence 52) re-instated the cyclisation, affording the desired macrocycle in moderate yield (53, 52\% isolated yield, Fig. 5).

Fig. 5 Starting peptides (28–38) and the macrocyclic peptide products formed via desulphurative C–C bond formation (39–48); carba-oxytocin (53); internally ‘stapled’ MCL-1 binding peptides (57–59), initial positions of alGly and Cys indicated within the sequence of these products; macrocycle size indicated.
Finally, to explore internal peptide ‘stapling’ using this strategy, we selected a region of a BH3 protein – BID50–102, that binds MCL-1 to regulate apoptosis.5,7 This PPI is known to play a significant role in cancer development and progression.5,7 Three sequences (54–56) were synthesised with alGly and Cys positioned to afford either an internal i, i + 4 staple (57, 59; Fig. 5 and ESIFT) or i, i + 7 staple (58; Fig. 5 and ESIT), fixing the length of either one or two full helical turns of the peptide, respectively. In addition, peptide macrocycle 26 is a head-to-tail macrocycle representing the i, i + 4 binding region of these longer sequences. The staple for all three peptides was successfully formed under the standard conditions, albeit in lower yield than the N- to C-terminal cyclisations previously explored (57–59). The alpha-helical structure of peptide 59 was assessed via CD spectroscopy. While macrocyclisation did increase the helicity by a few percent compared to the native BH3 sequence (22% compared to 19%), the starting peptide carrying the alGly glycine residue had a relatively high helical content (38%, Fig. S159T). This can be rationalised by considering the strain on the macrocycle imposed by the linker, and the fact that the native residues glutamine (Gln) and Ser were switched for alGly and Cys, respectively. These original residues have higher helical propensities relative to their replacements. Competitive inhibition studies (measured via fluorescence anisotropy) using MCL-1 and the fluorophore-labelled WT BH3 sequence demonstrated slightly lower inhibitory potency (26 ± 4 mM) for 59 compared to BID-wt (7.4 ± 0.9 mM) (Fig. S160T).

Conclusions

Herein, we report a powerful method for the cyclisation of peptides via desulphurative C(sp3)–C(sp3) bond formation. Our approach is operationally simple, effective ‘on the bench’ under ambient conditions with irradiation of blue light, utilising readily available starting building blocks. The reaction is rapid, high yielding (for most cases studied) on unprotected peptides in aqueous solution and tolerant to all proteinogenic chemical functionality. The technique enables the preparation of a range of macrocycle sizes and extends to internal macrocyclisation (peptide stapling). This technology presents a more sustainable alternative to the widely employed RCM, offering an effective new method for peptide cyclisation.

Data availability

The experimental procedures and compound/peptide characterisation data can be found in the ESI†

Author contributions

R. C. G. and N. J. M. conceived the project, F. R. S., R. C. G., D. M., H. J. K., P. Z., and H. E. L. W. carried out the experimental work. A. J. W. and N. J. M. supervised the work, N. J. M. wrote the manuscript. All authors contributed to writing the manuscript.

Conflicts of interest

There are no conflicts to declare.