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ARTICLE

Expanding the scope of N→S acyl transfer in native peptide sequences

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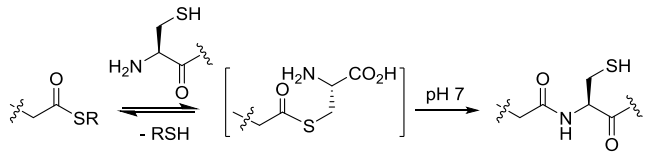
Ben Cowper, Leila Shariff, Wenjie Chen, Samantha M. Gibson, Wei-Li Di and Derek Macmillan*

Understanding the factors that influence N→S acyl transfer in native peptide sequences, and discovery of new reagents that facilitate it, will be key to expanding its scope and applicability. Here, through a study of short model peptides in thioester formation and cyclisation reactions, we demonstrate that a wider variety of Xaa-Cys motifs than originally envisaged are capable of undergoing efficient N→S acyl transfer. We present data for the relative rates of thioester formation and cyclisation for a representative set of amino acids, and show how this expanded scope can be applied to the production of the natural protease inhibitor Sunflower Trypsin Inhibitor-1 (SFTI-1).

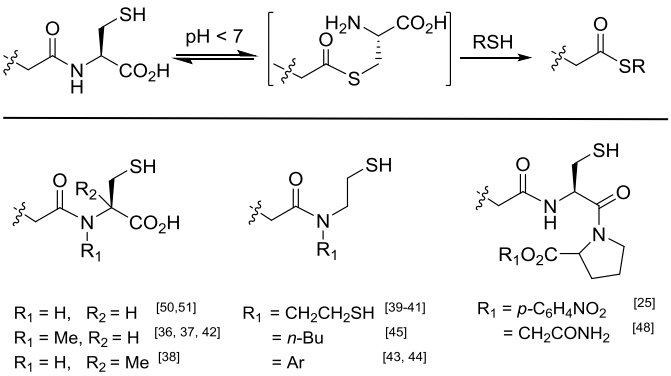
Introduction

Native chemical ligation (NCL, Scheme 1) has emerged as the reaction of choice when it comes to the chemical synthesis of proteins.[1-3] It offers atomic level control of protein sequence and structure and is amenable to the production of both natural[4-7] and unnatural[8, 9] protein products. The thioester component of the NCL reaction is so central to its success that a multitude of methods have emerged for its production by using either Fmoc[10-19] or Boc-based[20-24] solid-phase peptide chemistry approaches. Each method has desirable attributes and, usually, some limitations. This is evidenced by the fact that no single method for thioester synthesis has been universally adopted, although certain methods are more routinely used than others for their reliability in producing long peptide thioesters.



Scheme 1: The process of Native Chemical Ligation (NCL). The peptide thioester (R= alkyl, or aryl), whether pre-assembled or produced in-situ, is central to its success.

One area of thioester synthesis that has received much recent attention is the post-synthesis production of thioesters by N→S acyl transfer,[13, 25-29] owing to its similarity to the intriguing process of protein splicing mediated by inteins, which forms the basis of NCL's biological counterpart, expressed protein ligation (EPL).[30-34] An early observation of the generally poor susceptibility of native peptides to undergo N→S acyl transfer at room temperature or below[26] perhaps prompted examination of several synthetic "devices", which generally invoke the use of tertiary amides of β-amino thiol (cysteine-like) derivatives, to facilitate the process (Scheme 2).[29, 35-48]



Scheme 2: Thioester formation via cysteine mediated reverse NCL and some alternative building blocks that also enable N→S acyl transfer and thioester formation in peptides.

In our laboratory we explored thioester synthesis via N→S acyl transfer in native peptide sequences terminating in a C-terminal cysteine residue.[49] While many of the thioester precursors shown in scheme 2 are reported to facilitate thioester production under milder conditions than when using cysteine alone, the majority of these devices have optimal reactivity under similar reaction conditions (35-40°C, pH<7 rather than

[a] Department of Chemistry, University College London, 20 Gordon Street, London, WC1H 0AJ, UK. E-mail d.macmillan@ucl.ac.uk
[b] Institute of Child Health, UCL, 30 Guilford Street, London, WC1N 1EH, UK

Electronic Supplementary Information (ESI) available: NMR spectra, HPLC traces and LC-MS analysis for selected thioesters and cyclic peptides is available. See DOI: 10.1039/x0xx00000x

45-55°C, pH<7).[38, 39] In our early experiments,[49, 50] reactions were often conducted in aqueous 3-mercaptopropionic acid or 10% acetic acid, with thioester precursors prepared as C-terminal carboxamides (Xaa-Cys-NH_2), which we now know to be significantly less reactive than the corresponding C-terminal carboxylates (Xaa-Cys-OH).[51] The combination of carboxamide-terminated precursor and acidic solvents led us to the conclusion that the reaction was likely only to be practically useful across Gly-Cys, His-Cys, and Cys-Cys motifs.[52]

In fact N→S acyl transfer in native peptides bearing a C-terminal Gly-Cys carboxylate (Gly-Cys-OH) motif occurs to an observable extent at room temperature, this process is currently impractical in light of at least two further influencing factors. The first is that the thioester product has limited stability under the reaction conditions and so thioester formation does not profit from a protracted reaction time. The second is that a mildly reducing environment must be maintained throughout the process and this is more difficult to achieve in a user friendly manner over a longer duration. Furthermore the addition of chemical reducing agents is not without complication.[53] Consequently thioester formation is often conducted at 60°C when this temperature is well tolerated, but we have also reported several successful reactions at temperatures ranging from 45-50°C.[54-57] Additionally, we have demonstrated that peptide hydrazides, which can be formed as "shelf-stable" thioester equivalents can also be formed at lower temperature and are more stable to prolonged reaction times than the corresponding thioester.[57]

An advantage of using a single native amino acid residue to facilitate thioester synthesis is its operational simplicity, requiring only standard procedures, and readily available resins and amino acids. Synthesis monitoring is trivial and standard resin cleavage procedures furnish the desired thioester precursor. A further advantage is that, because the N→S acyl transfer "apparatus" (i.e. a C-terminal Cys residue) is a simple amino acid, the entire peptide precursor can be produced biologically. This potentially addresses a major issue associated with peptide therapeutics, the inefficiency and cost of their synthesis. A potential disadvantage of the process is that it may be limited to particularly reactive peptide sequences, which may necessitate introduction of an amino acid substitution in order to facilitate the chemistry. However, here we show how thioesters can form readily in short peptide sequences terminating in a representative selection of Xaa-Cys-OH motifs. Analysis of the reaction mixtures showed that, in fact, peptides terminating in a greater variety of Xaa-Cys-OH motifs undergo thioester formation and cyclisation at rates similar to motifs that were previously considered to be especially privileged.

Results and Discussion

Factors influencing thioester formation and cyclisation

At first glance one might expect all Xaa-Cys sites within a target peptide or protein to be similarly susceptible to N→S acyl transfer. However, this is not the case and selectivity and efficiency originate from a number of sources. We have shown previously that β-branched amino acids, present as Xaa-Cys-NH_2 or internal Xaa-Cys motifs, are essentially inert to N→S acyl transfer under the reaction conditions. This imparts considerable inherent selectivity at the sequence level.[55] The fact that thiolysis of terminal Xaa-Cys-OH motifs occurs significantly more rapidly than across the corresponding internal Xaa-Cys and Xaa-Cys-NH_2 motifs affords another level of selectivity,[57] which persists whether the C-terminal residue is L-cysteine, D-cysteine or selenocysteine,[56] and appears independent of the peptide sequence (Figures 1a/b).

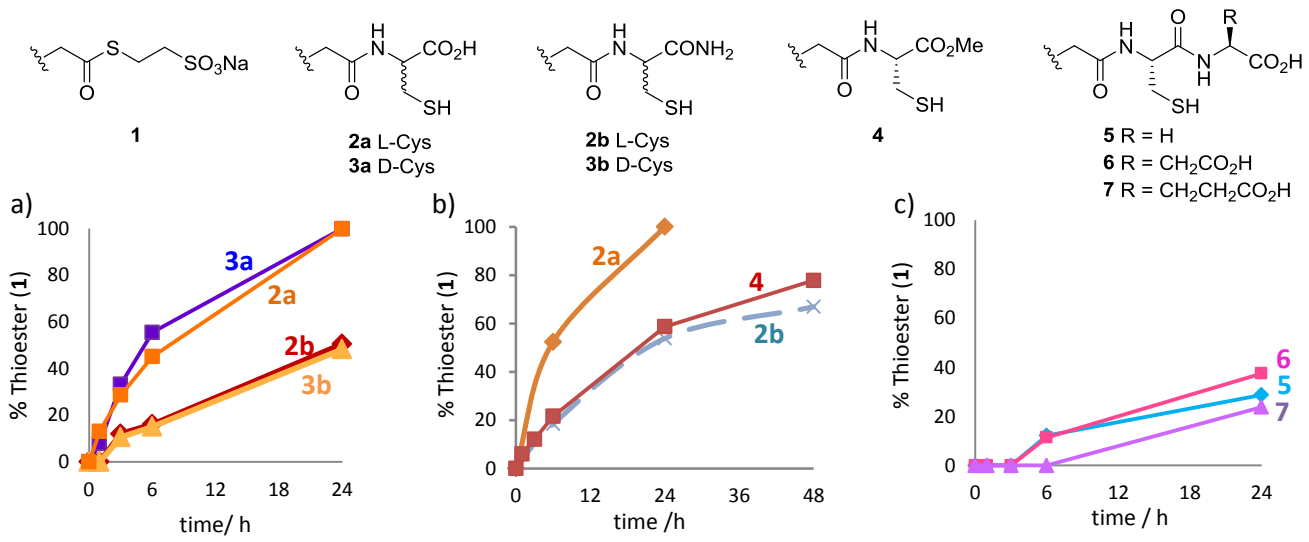


Figure 1. N→S acyl transfer reaction progress, followed by ^{13}C NMR spectroscopy,[26,50] highlights similarities and differences in reactivity between various thioester precursor peptides. a) Thioester formation (sequence H-NPMGYTKEGC-OH) is more rapid when the peptide C-terminus possesses a free carboxyl group **2a/3a** regardless of whether the C-terminal residue is D- or L-configured. b) In precursor H-MEELYKSGC-OH this trend is retained and lower reactivity is also observed with methyl ester **4**. c) Reconstituting the C-terminal carboxyl group through a single amino acid spacer does not restore reactivity. All reactions were conducted at 60°C.

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Interestingly the methyl ester terminated precursor (Xaa-Cys-OMe) also forms a thioester at approximately the same rate as its carboxamide terminated counterpart (Figure 1b).

One explanation for the rate enhancement attributable to the carboxyl group is that the *S*-peptide intermediate formed upon N→S acyl shift is zwitterionic. It is widely appreciated that the zwitterionic form of an amino acid is the most stable in aqueous solution and the electrostatic stability of a zwitterion provides a barrier to peptide bond formation (S→N acyl shift). Consequently the liberated NH_3^+ group of cysteine is likely to be more basic than the corresponding NH_3^+ group from cysteineamide and perhaps unsurprisingly the enhanced reactivity of Xaa-Cys-OH motif is not transmitted through an extended zwitterionic dipeptide leaving group (Figure 1c). In general, if the reaction pH is not adjusted after all of the components (peptide, buffer, MESNa, and TCEP) are mixed, the final pH is between pH 3 and pH 5. However, by lowering the pH of the reaction we might expect that increased protonation of the carboxyl group would reduce any stabilising effect of the zwitterionic form giving rise to less thioester, whereas an Xaa-Cys-NH_2 motif may be positively influenced by the lower pH, favouring *S*-peptide formation. While the rate of thioester production across Xaa-Cys-OH motifs does appear to be reduced as the pH is lowered (Figure 2), thioester formation is not dramatically affected by changes in pH from pH 2-pH 5, possibly because the cysteine carboxyl group (pK_a ~2.0) is still significantly ionised at pH 2.

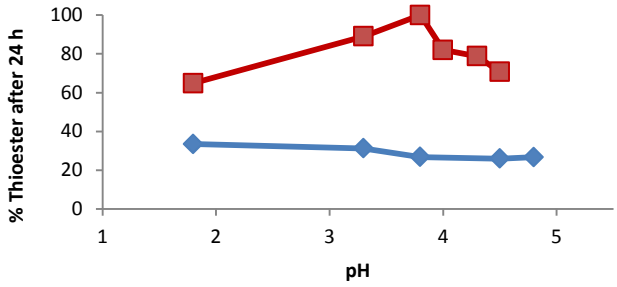

Figure 2. Thioester formation from precursor sequences H-NPMGYTKEGC-OH (red) and H-NPMGYTKEGC-NH_2 (blue) after 24 h as a function of pH was determined by ^{13}C NMR spectroscopy. The red line corresponds to [% thioester + % hydrolysis] since H-NPMGYTKEG-OH presumably arises from hydrolysis of H-NPMGYTKEG-SR. Hydrolysis was not observed in the Xaa-Cys-NH_2 reactions since the quantity of thioester is small (see supporting information for all data).

When N→S acyl shift precedes cyclisation, when the thioester precursor also possesses an N-terminal cysteine residue, the thioester may only be generated fleetingly.[54, 55, 58] Intercepting the thioester with an N-terminal cysteine residue appears to form a cyclic product essentially irreversibly as a result of forming the less reactive internal Xaa-Cys motif. Whether or not the thioester intermediate is observed at all in Cys/Gly/His-Cys-OH terminated peptides depends primarily on the reaction pH and the size of the peptide macrocycle.

For small peptide macrocycles (up to ~15 residues) this allows us to use an expanded set of thiol additives such as 3-mercaptopropanol. 3-mercaptopropanol permits formation of the corresponding thioester although competing S→O acyl transfer compromises the yield of the reaction (Figure 3a). However, this side reaction is not problematic when rapid cyclisation follows thioester formation and offers some practical advantages over using MESNa when scaling up reactions. The high quantities of MESNa employed can only be removed from peptide samples by HPLC, whereas 3-mercaptopropanol can be readily extracted into EtOAc allowing dilute reaction mixtures to be concentrated several hundred-fold prior to purification (Figure 3b).

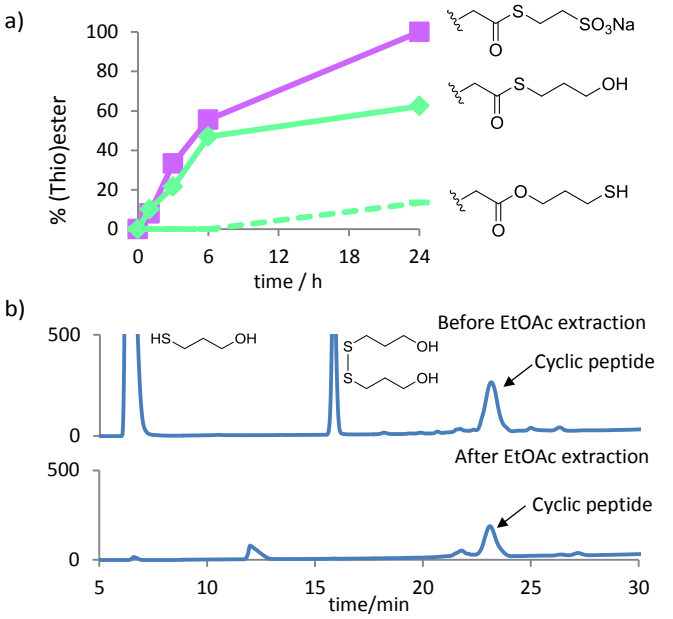

Figure 3. a) 3-mercaptopropanol-mediated thioester formation in model peptide H-NPMGYTKEGC-OH as monitored by ^{13}C NMR spectroscopy. b) Cyclisation of sequence H-CFPDGRCTKSIPPHC-OH[54] after 48 h before (upper trace) and after (lower trace) extraction with EtOAc.

Thioester formation across alternative Xaa-Cys-OH motifs

The enhanced reactivity of Xaa-Cys-OH motifs and potential for "thioester rescue" through cyclisation prompted a reinvestigation of a representative sample of Xaa-Cys-OH motifs towards thioester formation and cyclisation. We were keen to establish whether motifs we had previously considered to react unacceptably slowly showed enhanced reactivity when prepared as C-terminal carboxylates and, if not, whether cyclisation could serve as a means to utilise slowly converting Xaa-Cys-OH motifs without fear of hydrolysis.

To test this hypothesis analogues of a short peptide derived from antimicrobial Agardhipeptin A (*cyclo*-[GWPWGLH]) were prepared with and without an N-terminal cysteine residue. This allowed us to examine thioester formation and cyclisation

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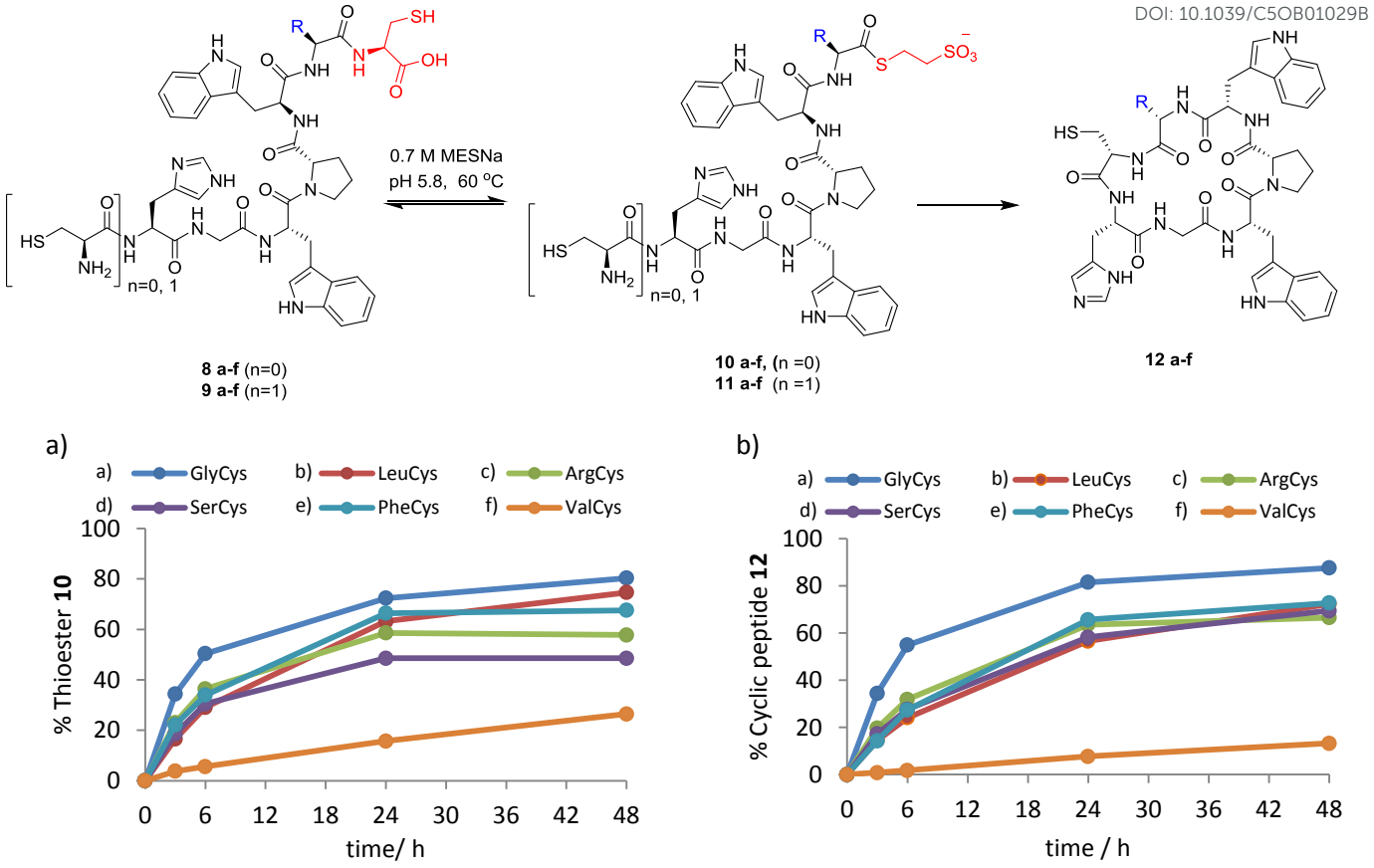

Figure 4: Thioester formation and cyclisation in Agardhipeptin A analogues (*cyclo*-[HGWPWXC]) **8a-f** and **9a-f** respectively. a) Formation of thioesters **10a-f** and (b) Cyclisation products **12a-f** were determined by analytical HPLC.

independently. Consequently six pairs of peptides terminating in Gly-Cys, Leu-Cys, Phe-Cys, Arg-Cys, Ser-Cys, and Val-Cys were prepared as C-terminal carboxylates and subjected to N→S acyl shift at 60 °C (Figure 4). As expected peptides terminating in Gly-Cys (**8a**/**9a**) reacted fastest with linear peptide thioester (**10a**) and cyclic peptide (**12a**) initially forming at near identical rates. This confirmed that, in short peptides, thioester formation was rate-determining with NCL mediated cyclisation occurring almost instantaneously and supports previous ^{13}C labelling studies.[58] Furthermore, the conversion to cyclic peptide appears slightly more efficient than thioester formation from the Gly-Cys-OH terminated precursor, presumably because the cyclic product is stable, whereas Gly-thioesters are more susceptible to hydrolysis as the reaction progresses. Unsurprisingly the Val-Cys-OH terminated peptides (**8f**/**9f**) were least reactive, yet still afforded significant thioester and cyclic peptide products when prepared as the C-terminal carboxylate.

The most surprising outcome was that most Xaa-Cys-OH motifs tested initially formed thioester products and cyclic peptides at a rate to rival the Gly-Cys-OH terminated precursor. Analytical HPLC analysis of the reaction mixture generally showed clean conversion from starting material to product in cyclisation reactions, suggesting that potential side reactions such as hydrolysis were not occurring to a significant extent (see supporting information for HPLC traces). Notably, in most cases, small quantities of unreacted starting material (or starting material and thioester in the case of cyclisation reactions) could be observed after 48 h. Where most Xaa-Cys-OH motifs reacted with similar initial rates, deviations emerged as a consequence of side reactions occurring, mainly thioester hydrolysis. Evidence of "thioester rescue" by cyclisation was particularly apparent in Ser-Cys and Arg-Cys and Gly-Cys terminated precursors where significant reduction in hydrolysis was clearly observed in the cyclisation reactions, supporting the hypothesis that cyclised product is "protected" relative to the free thioester.

Assembly of wild-type Sunflower Trypsin Inhibitor (SFTI-1)

Encouraged by the observation that the Arg-Cys-OH terminated Agardhipeptin A analogue underwent efficient cyclisation, we turned our attention to sunflower trypsin inhibitor-1 (SFTI-1). SFTI-1 is a 14-amino acid cyclic peptide with strong pharmaceutical potential due to its broad-spectrum protease inhibitory activity.[59] We recently demonstrated inhibition by SFTI-1 analogues of human kallikrein-5 (hKLK-5) protease, a viable target for treatment of skin conditions/atopic dermatitis.[54] Notably, SFTI-1 contains Arg2-Cys3 and Ile10-Cys11 junctions, which we had previously considered to be

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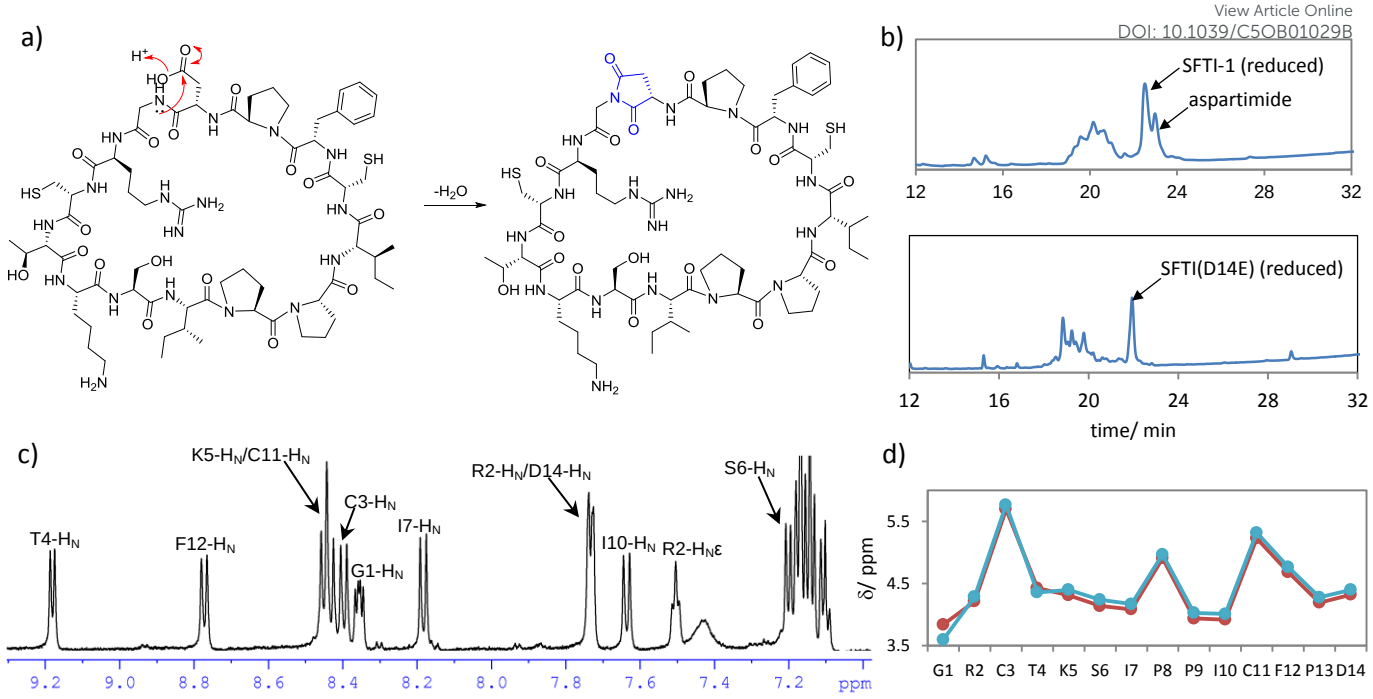

Figure 5. a) Structural reorganisation at the Asp-Gly junction yields an aspartimide-containing side-product. b) Aspartimide formation is significantly reduced at lower (50 °C) reaction temperature and abolished in an SFTI(D14E) mutant (at 60 °C). c) A section of the ^{1}H NMR spectrum of WT SFTI-1, annotated with assignments. d) A comparison of the observed Hα chemical shifts for our wild-type SFTI-1 (blue) with those previously published (red)[63].

poorly compatible with cyclisation via N→S acyl shift relative to Cys/His/Gly-Cys-OH. Consequently when creating putative SFTI-1 inhibitors of hKLK-5 we were compelled to introduce I10G and I10H substitutions in order to aid peptide cyclisation. However the apparent amenability of Arg-Cys to N→S acyl shift should enable production of wild-type SFTI-1. Consequently we sought to produce wild-type SFTI-1 via synthesis, cyclisation and oxidation of a H-CTKSIPPICFPDGRC-OH precursor peptide. Due to the reduced reactivity of an Arg-Cys carboxylate compared with Gly-Cys (Figure 4), we first attempted cyclisation at 60 °C, and monitored the reaction over 48 hours using LC-MS and HPLC. This reaction yielded the expected product, however a side-product less 18 Da in mass accumulated in majority (61% of cyclised material). This modification was consistent with the conversion of aspartic acid to aspartimide (Figure 5a), a common occurrence at Asp-Gly junctions and a likely consequence of the reaction pH and elevated temperature.[60-62] From continued monitoring the reaction it was clear that cyclisation does not protect the peptide from aspartimide formation. As the reaction time increased at 60 °C (>72 h) all of the material could be converted to this undesirable side-product and aspartimide formation could be observed in the product as well as the linear precursor and thioester intermediate.

We previously observed this modification, albeit to a much lesser extent, during cyclisation of SFTI-1 analogue (Gly/His-Cys-OH) precursor peptides at 60°C when the reaction was allowed to proceed over an extended period of time (>48 h). However the enhanced reactivity of these motifs permitted efficient cyclisation at 45°C with minimal aspartimide formation.[54] Consequently we reduced the temperature of wild-type SFTI-1 cyclisation to 50°C, and were pleased to observe accumulation of the desired product in majority (62% of cyclised material) without significantly compromising the reaction rate. Aspartimide formation as the source of the problem was confirmed through synthesis of the D14E mutant which cyclised cleanly (Figure 5b). Although the reaction was successful it is noteworthy that significant quantities of linear precursor and thioester remained after 48 h (Figure 5b). Comparing the overall reaction progress with a His-Cys-OH terminated precursor (Figure 3b) emphasises the important practical consequences of seemingly small differences in reaction rate.

Reaction products were separated using HPLC (see supporting information) and the unmodified peptide was oxidised in air at pH 8.5, yielding 1.9 mg of folded wild-type SFTI-1 from 12 mg of linear precursor (17% isolated yield). The structural integrity of the peptide was confirmed using NMR spectroscopy (Figure 5c), revealing ^{1}H chemical shift assignments in close agreement with previous studies of wild-type SFTI (Figure 5d).[63]

Conclusion

Originally we observed a preference for N→S acyl transfer at Cys/Gly/His-Cys motifs in native peptide sequences.[49] The

enhanced reactivity of these motifs towards thioester formation can be used in many instances to conduct selective acyl transfer reactions at the terminus of peptides containing multiple cysteine residues.[55, 57] However this preference for Cys/Gly/His-Cys is *not* a required consensus motif. Here we have shown that thioesterification is sufficiently fast at additional Xaa-Cys-OH motifs, particularly in shorter peptides, to allow efficient thioester formation and head-to tail cyclisation. Furthermore, in-situ cyclisation can serve to "rescue" a transient thioester from hydrolysis, which can be significant when thioester formation is slow, or especially prone to hydrolysis. Cys/Gly/His-Cys-OH motifs clearly undergo N→S acyl transfer more rapidly with obvious practical advantages so pH, reaction temperature, and peptide concentration may all need to be optimised on a case-by case basis for alternative Xaa-Cys-OH motifs. Cyclisation in itself does not protect these peptides from aspartimide formation in sequences that are especially susceptible (Asp-Gly) in the acidic reaction medium. However we have shown, through the synthesis of native SFTI-1, that this common and troublesome side-reaction can be significantly reduced in an Arg-Cys terminated thioester precursor by lowering the reaction temperature. These new findings should subsequently enable the production of naturally occurring SFTI-1 from a bacterially produced linear precursor, as we have previously shown for the SFTI(I10G) and SFTI(I10H) analogues.[54] Overall our results demonstrate how, even in more challenging cases, N→S acyl transfer in native peptides can be conducted with an expanded repertoire of Xaa-Cys motifs. Gaining an increased understanding of all of the factors that influence N→S acyl transfer in native peptide sequences will only serve to further broaden its application in peptide and protein synthesis.

Experimental Details

All solvents and chemicals were used as received. Column chromatography was carried out using Merck Si 60 (40-63 μm) silica gel and analytical thin layer chromatography was carried out using Merck TLC Silica Gel 60 F254 aluminium-backed plates. Components were visualised using combinations of ultra-violet light, potassium permanganate or ninhydrin. ^{1}H NMR spectra were recorded at 400, 500 or 600 MHz and ^{13}C NMR spectra were recorded at 100, 125 or 150 MHz respectively on Bruker Avance spectrometers. Preparative reversed-phase high performance liquid chromatography (RP-HPLC) was performed using a Dionex Ultimate 3000 system equipped with a Phenomenex Jupiter 10μ Proteo 90A, C_{12}, 250 x 21.2 mm column. Separations involved a mobile phase of 0.1% TFA (v/v) in water (solvent A)/acetonitrile (solvent B) over a 5-60% acetonitrile gradient, and were monitored at wavelengths 230 nm, 254 nm, and 280 nm. Analytical reversed-phase high performance liquid chromatography (RP-HPLC) was performed using a Dionex Ultimate 3000 equipped with a Phenomenex SphereClone 5μ ODS, C_{18}, 250 x 4.6 mm column. Separations involved a mobile phase of 0.1% TFA (v/v) in water (solvent A)/acetonitrile (solvent B) over a 5-95% acetonitrile gradient, and were monitored at wavelengths 230 nm, 254 nm, and 280 nm. Analytical LC-MS was performed using a Waters Acquity UPLC SQD instrument equipped with an Acquity UPLC BEH, C_{18}, 2.1 x 50 mm column. Separations involved a mobile phase of 0.1% formic acid (v/v) in water (solvent A)/acetonitrile (solvent B) over a 5-95% acetonitrile gradient, and were monitored at 254 nm.

Automated Peptide Synthesis of Xaa-Cys-OH model peptides, Agardhipeptin A analogues **8/9a-f** (H-[C]HGWPW**X**C-OH, where X is G, R, L, V, S or F) and SFTI-1 precursors (H-CTKSIPPICFP**D**GRC-OH and H-CTKSIPPICFP**E**GRC-OH) was carried out on an Applied Biosystems ABI 433A automated synthesiser using standard Fmoc amino acids on a 0.05 mmol scale, and employing pre-loaded FmocCys(Trt)-NovaSyn®TGT resin (loading = 0.18 mmolg^{-1}). Model peptides **5-7** were similarly assembled starting from pre-loaded FmocGly-NovaSyn®TGT, FmocAsp(OtBu)-NovaSyn®TGT, or FmocGlu(OtBu)-NovaSyn®TGT resins. C-terminal D-isoform carboxylic acids were produced from NovaSyn®TGA (90 μm) resin (loading = 0.24 mmolg^{-1}) and C-terminal carboxamides were produced from Rink Amide MBHA (100-200 mesh) resin as previously described. The Fast-Moc™ protocol for SPPS was employed, using 10 equivalents of each Fmoc-protected amino acid. Each coupling reaction was carried out using 0.45 M HBTU/HOBt in DMF, and 20% v/v piperidine in NMP for Fmoc deprotection. Cleavage of the assembled peptide chain was carried out in TFA (95% v/v)/1,2-ethanedithiol (2.5%)/H_2O(2.5%) for 4.5 h before filtration and 2 cycles of precipitation into cold ether followed by centrifugation (4000 rpm, 4°C, 15 min). Peptides were purified via preparative RP-HPLC as described above, and fractions containing the desired peptide were identified by LC-MS and lyophilized to yield white powdered solids in 25-70% yields.

*Preparation of methyl ester terminated model peptide **4*** (H-MEELYKSG(^{13}C-1)C-OMe).[64] To Fmoc-Cys-OH (110 mg, 0.304 mmol) in MeOH (3 mL), was added HCl (12 M, 2 drops). The reaction was stirred at room temperature overnight. A white precipitate formed that was dissolved upon addition of acetone (5 mL). The solvents were removed *in vacuo* to afford the product as a white solid (80 mg, 0.224 mmol, 75%) that was used without further purification. R_f 0.82 (1:1 petrol:EtOAc); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.77 (2H, d, J = 7.5, ArH), 7.61 (2H, br d, J = 7.3 ArH), 7.41 (2H, t, J = 7.5, ArH), 7.32 (2H, t, J = 7.5, ArH), 5.79 (1H, d, J = 7.0, NH), 4.69 (1H, m, αH), 4.44 (2H, m, FmocCH_2), 4.24 (1H, t, J = 6.8, FmocCH), 3.79 (3H, s, OCH_3), 3.06 (1H, dd, J = 14.4, 4.7, 1 × CH_2SH), 3.00 (1H, J = 14.4, 4.1, 1 × CH_2SH), 1.38 (1H, t, J = 8.9, SH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.7, 155.8, 143.9, 141.5, 127.9, 127.2, 125.3, 120.2, 67.3, 55.4, 53.0, 47.3, 27.3; LRMS (CI) m/z 358 (14%, M+H$^+$).

Trityl Chloride resin, loading = 1.40 mmolg^{-1} (35.7 mg, 0.05 mmol) was swelled in DMF. A solution of Fmoc-Cys-OMe (80 mg, 0.224 mmol) in CH_2Cl_2 (1.5 mL) was added to the resin. DIPEA (156 μL, 0.896 mmol) was added and the resin shaken for 2 days. MeOH (25 μL) was added and the resin shaken for 5

min. The resin was washed with DMF and CH_2Cl_2 and treated with piperidine in DMF (20% v/v) for 15 min to remove the Fmoc goup. To enable monitoring of the thioesterification process by ^{13}C NMR spectroscopy Fmoc-Gly(^{13}C-1)-OH was introduced,[50] and the peptide was elongated by automated SPPS as described above. The resin was washed with DMF and CH_2Cl_2 and cleaved with Reagent K, comprising TFA (82.5% v/v), phenol (5% w/v), thioanisole (5% v/v), water (5% v/v), EDT (2.5% v/v). Reagent K (4.0 mL) was added and the resin stirred for 30 min. The resin was filtered off and the filtrate added to cold ether to induce precipitation of the peptide. The precipitate was collected by centrifugation (1500 rpm, 4 °C, 15 min), the supernatant removed, and the precipitated peptide resuspended in ether and centrifuged once more. The white precipitate was dissolved in water and then purified by HPLC as described above. Fractions containing the product were identified by LC-MS and lyophilised to afford pure peptide as a white solid (12 mg, 20%) ^{13}C NMR (D_2O, 125 MHz) δ 171.7; Calculated mass 1073.5, observed (ESI-MS) $[MH]^+$ 1074.4 Da.

General Procedure for Thioester Formation

Peptides to be analysed by ^{13}C NMR spectroscopy were dissolved to final concentrations of 1 mg mL^{-1} in 0.1 M sodium phosphate buffer (pH 5.8, prepared in D_2O) containing MESNa (10% w/v), and TCEP.HCl (0.5% w/v). Aliquots of 0.6 mL were dispensed into separate 1.5 mL Eppendorf tubes and shaken in an Eppendorf thermomixer at 60 ºC for 48 h. The contents of each Eppendorf tube, once transferred to an NMR tube were used to obtain an independent estimation of reaction progress. The ^{13}C NMR spectra were acquired at 125 MHz. LC-MS analysis at the end of each reaction was used to confirm the final distribution of peptides present. In order to determine thioester formation at different pH values, pH readings were taken with a Hanna Instruments HI 1330B electrode and pH 210 microprocessor pH meter calibrated to pH 7 and pH 4. For pH variation studies, stock reaction mixtures were neutralised by the addition of aqueous NaOH prior to the addition of buffers.

SFTI-1 cyclisation and oxidation. Backbone cyclisation was carried out in sodium phosphate buffer (pH 5.8, 0.1 M) containing peptide (1 $mgmL^{-1}$), sodium 2-mercaptoethanesulfonate (10% w/v) and tris(2-carboxyethyl)phosphine (0.5% w/v). The samples were heated at 45-60 °C in an Eppendorf thermomixer with agitation (600 rpm) for 48 h, and monitored via analytical RP-HPLC and LC-MS. Backbone-cyclised SFTI-1 was purified via preparative RP-HPLC, as described above, yielding a white powdered solid in 25% isolated yield. Finally, stirring of the cyclic peptide (0.1 $mgmL^{-1}$) in 0.1 M ammonium bicarbonate solution (pH 8.5) at room temperature for 24 h yielded the disulfide-bonded structure. The reaction mixture was lyophilised, dissolved in the minimum volume of water and purified by RP-HPLC. Fractions containing the oxidised peptide were identified by LC-MS, pooled, and lyophilised to afford the target product in 80% yield.

SFTI cyclisation mediated by 3-mercapropropanol was carried out as above employing 10% v/v 3-mercaptopropanol in place of sodium 2-mercaptoethanesulfonate. After 48 h 3-mercaptopropanol was efficiently removed from the reaction mixture by extraction into ethyl acetate (washed with 5 x half of the reaction volume). SFTI(I10H), present in the aqueous phase, was then separated and purified by HPLC as described above.

SFTI-1 NMR spectroscopy. NMR samples were constituted in 20 mM $NaHPO_4$ pH 4.5 supplemented with 10% D_2O. ^1H, ^1H-^1H TOCSY and ^1H-^1H NOESY spectra were acquired at 25°C using a 600 MHz a Bruker Avance III spectrometer.

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