Investigation of cysteine as an activator of *sidechain* N→S acyl transfer and tail-to-sidechain cyclization

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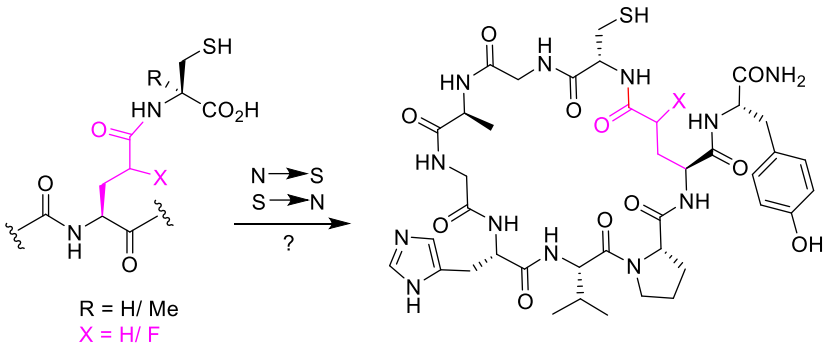

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Abstract N→S acyl transfer is a popular method for the post-synthesis production of peptide C_α-thioesters for use in Native Chemical Ligation and for the synthesis of head-to-tail cyclic peptides. Meanwhile thioester formation at the sidechain of aspartic or glutamic acids, leading to tail-to sidechain cyclized species, is less common. Herein we explore the potential for cysteine to function as a latent thioester when appended to the sidechain of glutamic acid. Initial insights gained through study of C-terminal β-alanine as a model for the increased chain length were ultimately applied to peptide macrocyclization. Our results emphasize the increased barrier to acyl transfer at the Glutamic acid sidechain and indicate how a slow reaction, facilitated by cysteine itself, may be accelerated by fine tuning of the stereoelectronic environment.

Key words cyclic peptides, native chemical ligation, acyl transfer

Due to the increasing importance of cyclic peptides as robust pharmaceutical scaffolds for otherwise metabolically fragile bioactive peptides,[1] a vast number of chemical and biological methods have been reported for their synthesis. Despite the multitude of methods accessible to the synthetic chemist,[2] chemoselective ligation technologies continue to lead the way in the quest for scalable and cost-effective protocols.[3] The use of Native Chemical Ligation (NCL, Fig. 1a) to effect cyclisation of a linear precursor, adorned with a C-terminal thioester and N-terminal cysteine, is arguably the most common approach since it can be conducted in water, using unprotected peptides, and employ precursors of synthetic or biological origin. Post-synthesis N→S acyl transfer is becoming a common method for production of the required thioester component and several devices have been developed around the β-amino thiol scaffold of cysteine (Cys) to help facilitate it.[4] We have mainly studied the N→S acyl transfer activity of native peptide sequences in particular, where cysteine itself promotes the reaction (Fig.1b).

Having demonstrated the cysteine promoted head-to-tail cyclization using synthetic[5] and biologically[6] produced precursors we turned our attention to more challenging topologies such as the tail-to-sidechain cyclisation depicted in Figure 1c. This mode of cyclisation is often found in lariat (lasso) peptides which have not yet proved amenable to chemical synthesis,[7] and is particularly interesting because a sidechain thioester cannot be introduced by using an intein. Furthermore, conducting thioester formation in aqueous solution using fully unprotected, or tethered, lariat precursors may allow the peptide to adopt a more native conformation prior to cyclization. Melnyk and co-workers previously showed that acyl transfer, when conducted at the sidechain of aspartic acid (Asp) or glutatamic acid (Glu), was slow relative to C-terminal thioester formation, when using a *bis*-2-sulfanylethylamino (*bis*-SEA) functionalised peptide.[8] The difference in reactivity also formed the basis for kinetically controlled thioester formation.[9] We were keen to establish whether slow thioester formation was also observed at the side chain of Asp/Glu when cysteine

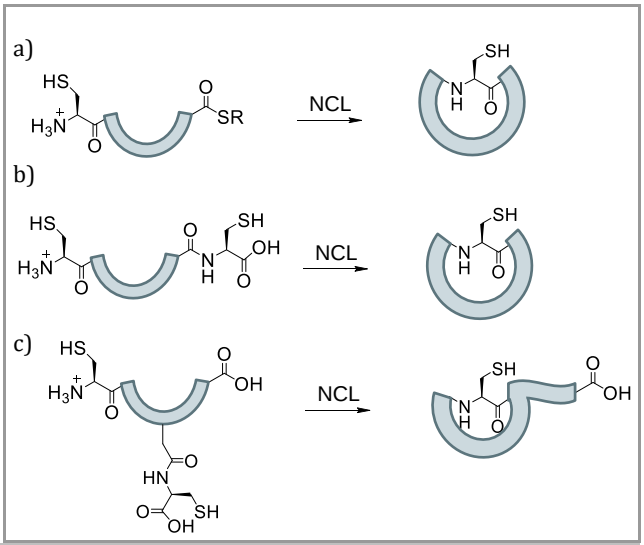
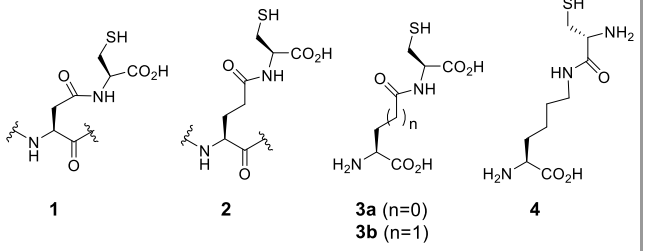


Figure 1 a) Head-to tail peptide cyclisation enabled by NCL. b) A C-terminal cysteine residue promotes *N→S* acyl transfer, providing transient access to the required thioester for cyclisation via NCL. c) Tail-to-sidechain cyclisation using a thioester precursor installed on an amino acid sidechain



Scheme 1. Desired peptide motifs derived from Asp (**1**) or Glu (**2**) could be introduced using e.g **3a/b** and an expanded genetic code. Dipeptide **4** has already been genetically encoded, enabling side-chain ubiquitination using NCL.

was used to promote the initial *N→S* acyl shift and, if so, what could be done to remedy it. Specifically, we set out to examine the *N→S* acyl transfer behaviour of branched dipeptide motifs **1** and **2** (Scheme 1) within the context of a synthetic peptide sequence. If successful the hope would be that these should be genetically encodeable via building blocks such as **3a/b**. Interestingly several branched dipeptides including **4**,[10] which enables site-specific ubiquitination via NCL with ubiquitin thioesters, have already been genetically encoded in bacteria employing orthogonal pyrrolysyl tRNA/tRNA synthetase pairs suggesting that this route could well be plausible.

To establish how dramatically the additional methylene groups between the sidechain carboxyl group of, for example, glutamic acid and the peptide backbone influence the reactivity of cysteine towards N→S acyl shift, we first compared the reactivity glycine terminated model peptide **5a** with β-alanine terminated peptides **6a** and **7a** (Figure 2), which served as models for the increased chain length.[11] We were pleasantly surprised to find that thioester **6b** could be cleanly formed from β-alanine terminated **6a**. However formation of **6b** was significantly slower when compared with the corresponding Gly-Cys-OH motif (**5a**), taking up to 72 h to reach >70% conversion at 60°C.

Although not surprising, since the Melnyk group reported reaction times of 144 h for thioesterification/cyclisation (at 37°C) with a sidechain SEA motif,[8] this raised concerns about the use of cysteine itself as a facilitator N→S acyl shift when applied to the amino acid side chain and re-emphasised the important contribution of the α-amino group of the adjacent *non-scissile* amide in mediating the reactivity of native peptide sequences. Keen to reconstitute glycine-like reactivity at β-alanine terminated peptide we additionally explored the use of model peptide **7a** containing αMethyl cysteine because it is more susceptible to thiolysis than cysteine itself and, as a commercially available amino acid, it is simple to introduce relative to common alternatives.[12] Furthermore, since the desired dipeptide motifs that would need to be introduced in order to effect side-chain activation (e.g. **3**) are not naturally occurring there was no need to retain a strict cysteine scaffold. The model reaction employing **7a** was extremely encouraging showing almost complete consumption of the starting material within 6 h.

We postulated that the reduction in reaction rate, when using β-Ala, was likely due to the increased distance between the reaction site and the proximal electron withdrawing amide nitrogen atom. To explore this further we examined whether reactivity could be restored through introduction of an electronegative atom, such as fluorine, placed alpha to the carbonyl group of the scissile amide bond (Figure 3). Consequently we prepared fluorinated peptides **8a** and **9a**. Monofluorinated **8a** was prepared as an epimeric mixture from racemic 2-fluoro-β-alanine. The epimers were almost completely separated by HPLC, appeared configurationally stable under the reaction conditions and both behaved similarly in reactions. Analysis of reaction mixtures after 24 h indicated that the fluoro-β-alanine residues installed at the C-terminus indeed accelerated the rate of thioester formation relative to β-alanine itself. However what also became evident was that thioesters **8b** and **9b** were unstable and more prone to hydrolysis. After 24 h the initially formed thioester product **8b** was almost completely hydrolysed at 60 °C. Hydrolysis could be reduced, but not abolished, by conducting the reaction at 50 or 40 °C (Figure 3a) but, when using **9a**, a thioester product could not be observed at all. However the reactive nature of the 2-fluoro-β-Ala-Cys-OH motif prompted us to investigate the reaction further since we were confident that, by intercepting the intermediate with a nucleophilic species such as hydrazine or creating a more stable product via *in-situ* cyclisation,[5d] we could ultimately create a potentially useful process. Then,

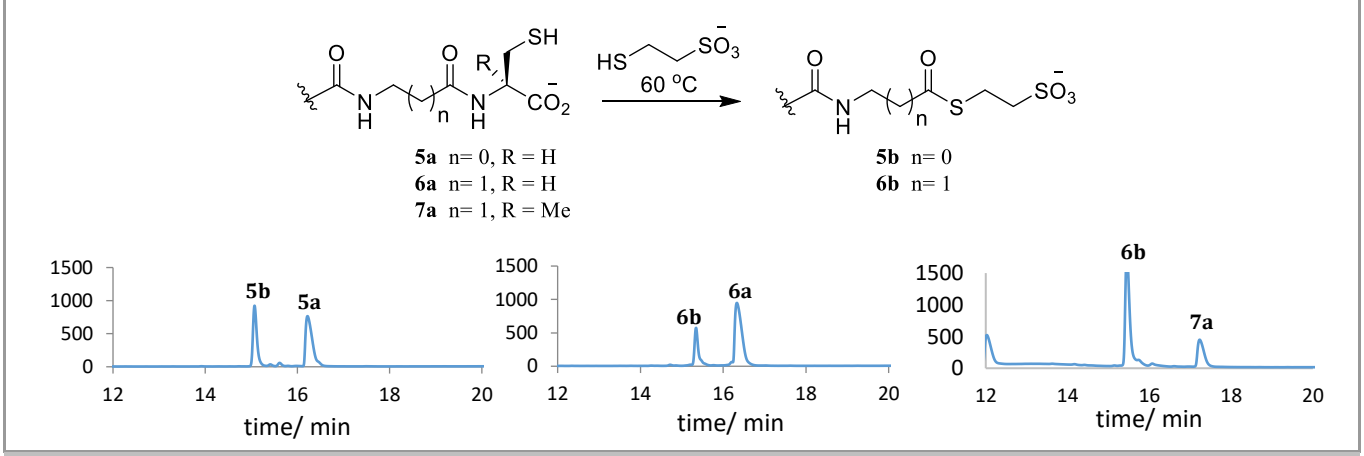


Figure 2 a) comparison of MESNa thioester formation across Gly-Cys-OH (H-MEELKYSGC-OH, **5a**), βAla-Cys-OH (H-MEELYKSβAC-OH, **6a**), and βAla-αMeCys-OH (H-MEELYKSβAαMeC-OH, **7a**) terminated peptides after 6 h at 60 °C

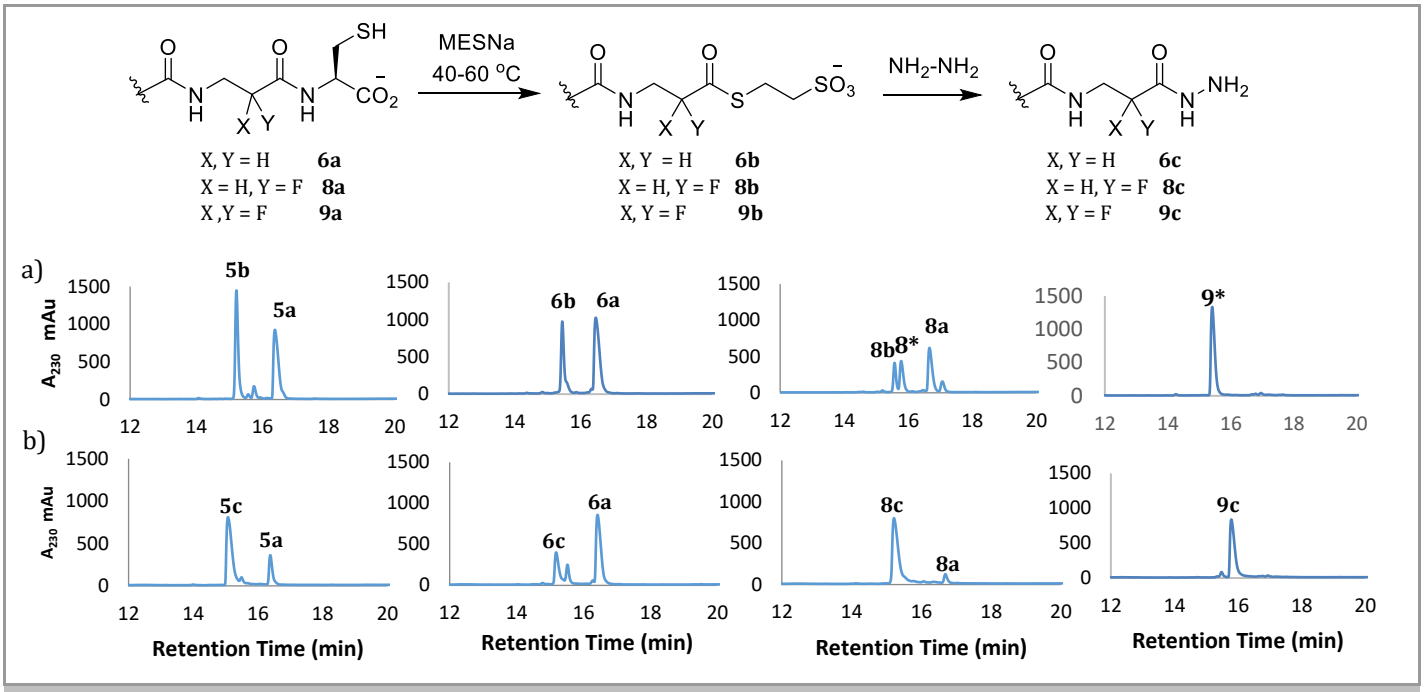


Figure 3 Cysteine promoted thioester and hydrazide formation at glycine (**5a**), β-alanine (**6a**), and fluorinated analogues (**8a**, **9a**) a) After 24 h at 50 °C in the absence of hydrazine. b) After 24 at 50 °C in the presence of hydrazine. * corresponds to the hydrolysis product as identified by LC-MS.

as expected, **8a** and **9a** formed acyl hydrazides **8c**/**9c** cleanly and near quantitatively at 50 °C after 24 when the reaction mixture was supplemented with 5% w/v hydrazinium acetate,[13] appearing superior both β-Ala-Cys-OH and Gly-Cys-OH terminated peptides (Figure 3b). Curious as to whether the successful outcome indicated that cyclisation may prove more productive than thioester formation a model peptide corresponding to an analogue of the plasmin inhibitory peptide, Agardhipeptin A,[14] was readily assembled. As with **8a**, the racemic monofluorinated β-Ala residue was employed but, in contrast to **8a**, the Agardhipeptin A analogue **10a** did not yield an easily separable mixture of epimers. Since this was considered to be of little consequence for the purpose of the experiment the progress of the reaction was monitored using the summed peak areas of each epimer for the starting material and product. In the cyclisation reaction only the linear precursor containing a monofluorinated β-Ala residue gave rise to a cyclic peptide (Figure 4) whereas the precursor containing the difluorinated β-Ala showed only hydrolysis under identical reaction conditions.

Overall, the results of model experiments using β-alanine and fluorinated derivatives were extremely enlightening showing, not only how an additional methylene group placed between the scissile amide bond and the adjacent α-amino group reduced reactivity, but additionally how reactivity could be restored by introduction of a single fluorine atom. Whilst thioesters **8b**, and **9b** appeared unstable, we demonstrated how they could be efficiently trapped by added nucleophiles such as hydrazine, which may well find new applications in the future. However, regardless of how encouraging the cyclisation of **10a** appeared, we were not keen to progress the monofluorinated β-alanine derivatives as acyl transfer precursors. Since the fluorine atom would be retained by the product, a scalable and enantioselective synthesis of a corresponding monofluorinated glutamic/aspartic acid would need to be conducted. Although enantioselective routes to monofluorinated glutamic acids exist from hydroxyproline,[15] pyroglutamic acid derivatives,[16] and more recently from the γ-aldehyde,[17] their synthesis is difficult to scale, employing expensive and/or toxic metals and catalysts. Furthermore, having already achieved acceptable results with **6a** and improved results with **7a** we felt sufficiently confident to explore the reactivity of the γ-glutamyl cysteine containing peptides themselves. Due to the potential for aspartimide formation upon activation of aspartic acid derivative **1** we initially focussed on the synthesis and reactivity of the γ-glutamyl cysteine motif **2**.

Previous investigations employing cysteine as the sole facilitator of acyl transfer indicated that, for optimal reactivity,

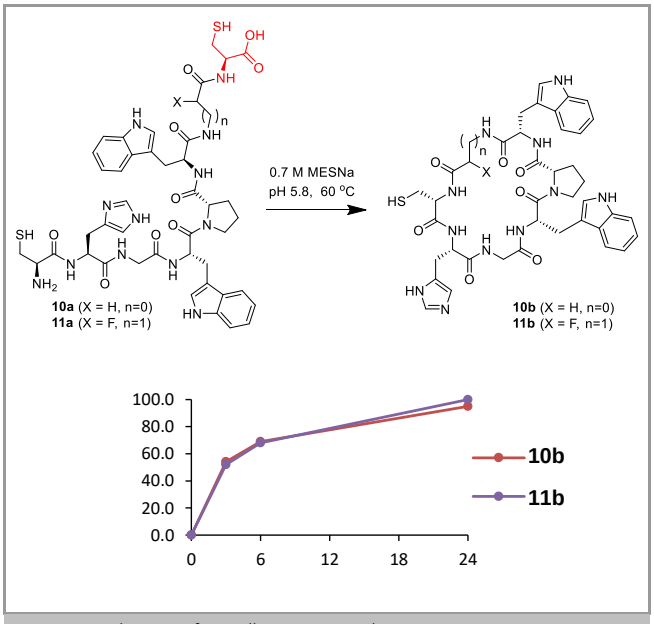
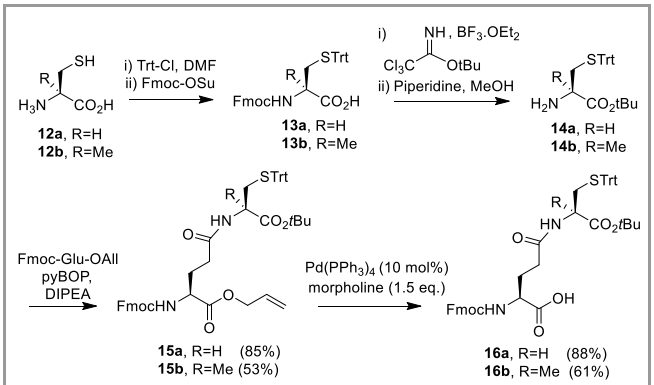


Figure 4 Cyclisation of Agardhipeptin A analogues in 0.7 M MESNa at 60 °C from linear precursors H-CHGWPWGX-OH, where X = Gly (**10a**) or 2-fluoro β-alanine (**11a**).



Scheme 2. Synthesis of suitably protected branched dipeptides **16a** and **16b**.

the branching cysteine residue should possess a free carboxyl group.[18] Consequently it was important to differentiate the α-carboxyl groups of cysteine and glutamic acid in the dipeptide building block such that the α-carboxyl group of glutamic acid could be unmasked for solid phase peptide synthesis while the α-carboxyl group of cysteine remained protected (Scheme 2). Suitably protected dipeptide building blocks **16a/b** were readily prepared from available H-Cys-OH, or H-αMeCys-OH, and Fmoc-Glu-OAll. Briefly, the free amino acids were converted to the STrt/Nα-Fmoc protected derivatives **13a/b** by overnight reaction with trityl chloride in DMF followed by reaction of the crude product with Fmoc succinimide in 1:1 water/dioxane in the presence of excess Na_2CO_3 (Fmoc-Cys(Trt)-OH is also commercially available).[12] Introduction of the cysteinyl Cα *tert*-butyl ester was next introduced according to the method reported by Schmidt,[19] using *tert*-butyl 2,2,2-trichloroacetimidate (TBTA) in the presence of $BF_3.OEt_2$ to afford fully the protected amino acid. Subsequent Fmoc deprotection with piperidine in DCM liberated the free α-amino group for coupling to the γ-carboxyl of commercially available Fmoc-Glu-OAll, which was efficiently completed using pyBOP as coupling reagent in the presence of DIPEA.[20] The desired building blocks, Fmoc-Glu[Cys(Trt)OtBu]-OH (**16a**) and Fmoc-Glu[αMeCys(Trt)OtBu]-OH (**16b**) were ultimately prepared upon Pd^0 catalysed cleavage of the allyl ester from **15a/b**.[21] Whether using Cys or αMeCys identical procedures could be used throughout and, with the exception of coupling to the glutamic acid derivative and Allyl deprotection, yields for the αMeCys derivatives were comparable.

Dipeptides **16a** and **16b** were next incorporated in place of Glu8 into the nine N-terminal residues of antimicrobial lariat peptide microcin J25 to afford **17** and **18** respectively (Figure 5). Although this partial sequence is incapable of forming the characteristic lariat structure of microcin J25,[22] it served as a suitable model system to test the tail-to-sidechain cyclization. In agreement with preceding results cyclization of **17** similarly showed that the desired peptide macrocycle could be formed in greater that 70 % yield (as judged by HPLC) over 72 h at 50 °C, validating the use of β-alanine as a model system. While encouraging, we ultimately believed that the long reaction time at elevated temperature would be generally detrimental to the formation of sidechain thioesters and tail-to-sidechain cyclisation reactions in more complex samples. Encouragingly peptide **18** also behaved similarly to the preceding model experiments. While the reaction to form **19**[23] took nearer 24 h to reach 92% conversion at 50 °C (compared with ca. 6 h at 60 °C in β-Ala experiments) it appeared that thioester formation had advanced significantly within 6 h, as indicated by the appearance and subsequent consumption of the MESNa thioester intermediate (marked with an asterisk in Fig. 5b). It is likely that NCL cyclisation under non-ideal conditions (pH <7) is the reason for the accumulation of the MESNa thioester.

In conclusion cysteine, when installed at the side chain carboxyl group of glutamic acid, was found to be a willing facilitator of N→S acyl transfer, albeit with reduced efficiency when compared with presentation at the C-terminus. Based on previous work by Melnyk and co-workers, as well as our own examination of β-Ala terminated model peptides this was not particularly unexpected, however reactivity could be reconstituted by employing more reactive acyl transfer precursors **7a** and **18** containing recently described α-methylcysteine. Interestingly, researchers have mainly sought to elevate the reactivity of thioester precursors towards N→S acyl transfer by modification of the cysteine-like C-terminus. In contrast here we have shown that N→S acyl transfer can also be enhanced by electronic activation of the adjacent residue offering new opportunities, more generally, to promote controlled fragmentation of peptides under mild conditions. Installation of a single α-fluorine atom conferred Gly-Cys like properties on otherwise sluggish β-Ala-Cys terminated peptides, whereas introduction of a geminal difluoro motif appeared too reactive to support thioester formation. Nevertheless fluorinated amino acid building blocks (FlXaa)

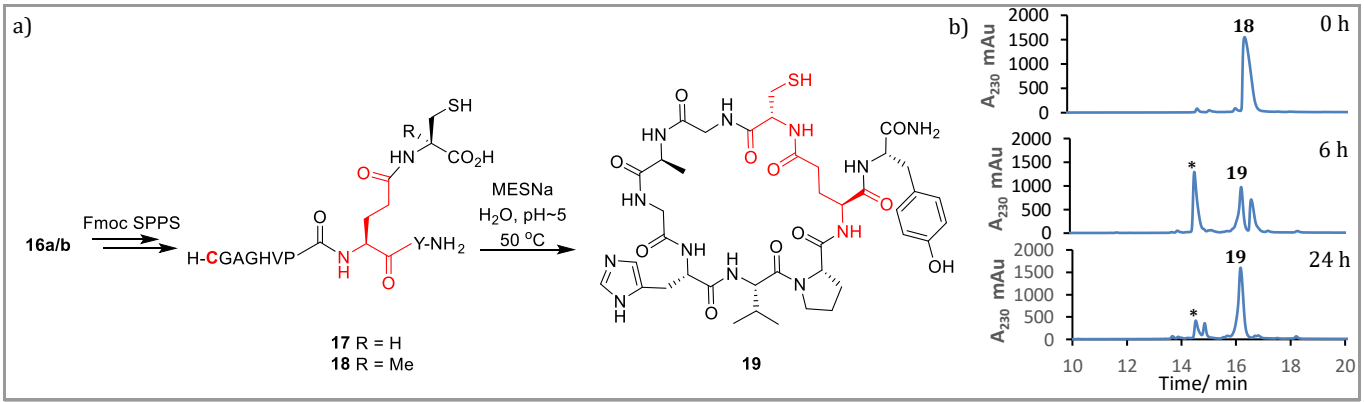


Figure 5 a) cyclisation of branched precursor peptides **17** and **18** at 50 °C in the presence of 0.7 M MESNa. b) HPLC monitoring of the cyclisation of **18** to afford **19** over 24 h at 50 °C. The peak marked with an asterisk is the MESNa thioester intermediate.

may yet serve as useful promoters of N→S acyl transfer across FlXaa-Cys motifs in future.

Overall the selective activation of the glutamic acid sidechain using unprotected peptides in aqueous solution, and in the context of McJ25, paves the way to a potentially viable route to lariat peptides. Selective acyl transfer reactions, likely working in combination with a tethering strategy to template the lariat fold,[24] may ultimately bring this fascinating class of peptides under the routine command of the synthetic chemist.

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Supporting Information

YES (this text will be updated with links prior to publication)

Primary Data

NO (this text will be deleted prior to publication)

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(20) ***Synthesis of dipeptides 15a/b***. Cysteine derivative **14a** (0.437 g, 1.04 mmol) was dissolved in anhydrous DCM (6.0 mL). Fmoc-Glu-OAllyl (0.512 g, 1.25 mmol) and pyBOP (0.595 g, 1.5 mmol) were added, followed by DIPEA (0.362 mL, 2.1 mmol) and the reaction mixture was stirred at room temperature for 1 h. The reaction mixture was then diluted with EtOAc (50 mL) and washed with sat. aq. $KHSO_4$ (1 x 15 mL) and sat aq. $NaHCO_4$ (2 x 15 mL). The organic phase was separated, dried ($MgSO_4$), and evaporated to afford the crude product as a white foam. Purification by flash column chromatography over silica, Eluent toluene/ EtOAc (5:1) afforded **15a** (0.719 g; 85%) as a white foam, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (2H, d, *J* = 8.0, 2xAr-H), 7.62 (2H, d, *J* = 7.4, 2xAr-H), 7.44-7.15 (ca.15H, m(overlapped by $CDCl_3$ signal), ArH), 6.15 (1H, d, *J* = 7.6, CH allyl), 5.93-5.85 (1H, m, CH allyl), 5.74 (1H, d, *J* = 8.0, CH allyl), 5.35-5.24 (2H, 2 x d, 2 x NH), 4.64 (2H, s(br), CH_2-allyl), 4.52-4.50 (1H, m, CH_α), 4.43-4.38 (3H, m, CH_α, CH_2-Fmoc), 4.23 (1H, t, J=7.0, CH-Fmoc), 2.79-2.54 (2H, m $CH_{2\beta}$-cys), 2.33-1.97 (4H, m, $CH_{2\beta}$-Glu and $CH_{2\gamma}$-Glu), 1.45 (9H, s, *t*Bu). ESI+ MS (*m/z*) calculated for $C_{49}H_{50}N_2O_7S$ 810.33 found $[MH]^+$ 811.5 Da and $[MNa]^+$ 833.5 Da. For dipeptide **15b**: (procedure as above, 53% isolated as a white foam) ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.78 (2H, d, *J* = 7.5, 2xAr-H), 7.61 (2H, d, *J* = 7.4, 2xAr-H), 7.42-7.17 (ca.15H, m (overlapped by $CDCl_3$ signal), ArH), 6.39 (1H, s (br), CH allyl), 5.86-5.83 (2H, m, 2 x CH allyl), 5.35-5.21 (2H, 2 x d, 2 x NH), 4.63 (2H, d(br), CH_2-allyl) 4.42-4.37 (3H, m, CH_α-Glu, CH_2-Fmoc) 4.23 (1H, t(b), CH-Fmoc), 3.04 (1H, d, J = 11.3 Hz, half $CH_{2\beta}$-αMecys), 2.60 (1H, d, J = 11.3 Hz, half $CH_{2\beta}$-αMecys), 2.36-2.05 (4H, m, $CH_{2\beta}$-Glu and $CH_{2\gamma}$-Glu), 1.45 (12H, s (br), CH_3, *t*Bu). ESI+ MS (*m/z*) calculated for $C_{50}H_{52}N_2O_7S$ 824.35 found $[MH]^+$ 825.5 Da and $[MNa]^+$ 847.5 Da

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(23) Lyophilized peptide **18** was dissolved in ddH_2O to a final concentration of 2 mg/mL. 0.5 ml was transferred to a sterile Eppendorf tube and water (0.3 mL) was added followed by 1 M sodium phosphate buffer (pH 5.8, 0.1 mL), TCEP.HCl (5 mg) and sodium 2-mercaptoethanesulfonate (MESNa, 0.1 g). The reaction mixture was shaken (700 rpm) in an Eppendorf thermomixer at 50

°C for 24 h. The cyclic peptide was then purified from the reaction mixture by preparative HPLC. t_R = 26.5 min, and lyophilized to afford the pure product as fluffy white solid: ESI^+ MS (*m/z*) calc. 912.4 found $[MH]^+$ 913.2 Da.

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