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Late-stage macrolactonisation enabled by tandem acyl transfers followed by desulphurisation

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Intramolecular S-acylation of a thiol-installed threonine with a thioester unit, followed by S–O acyl transfer and subsequent desulphurisation, allows the synthesis of lactone peptides. A protocol has been developed enabling the cyclisation of a linear peptide, a reaction which has not been achieved by conventional methods.

In the last decade, the protein-protein interaction (PPI) has been recognized as an event which can modulate biological phenomena, and considerable effort has been devoted to the regulation of PPIs by small molecules. However, the flat and broad surface of proteins can present difficulties to the modulation of PPIs by small molecules. Recently, peptides have received increasing attention as mid-size molecules which can efficiently affect the surfaces involved in PPIs. Among the peptides which can shield interfacial boundaries, cyclic peptides containing macro-lactam or lactone forms have been emerging as potential PPI inhibitors due to the pharmacodynamic properties which are consistent with practical drug use.[1] General access to lactam peptides has taken advantage of the late-stage formation of amide bonds (macrolactamisation) and difficulty associated with macrolactonisation compared to the lactamisation[2] often has led to the synthesis of lactone peptides to employ the macrolactamisation of an ester units-incorporated linear precursor[3]. Both these conventional strategies require the regioselective acylation of an amino (or hydroxyl) group and protection of the functional groups that are not involved in the cyclisation. Consequently, laborious and complicated synthetic operations including a protection-deprotection sequence are necessary, and this has revealed a need for synthetic strategies that don't require such protections. Recent advances in chemoselective ligation technologies, exemplified by native chemical ligation (NCL),[4] has enabled a protection-free macrocyclisation in a highly efficient and selective manner.[5,6] However, the development of a protection-free protocol applicable to the late-stage macrolactonisation is still awaited, although efforts to form ester bonds in lactone peptides have continued.[7]

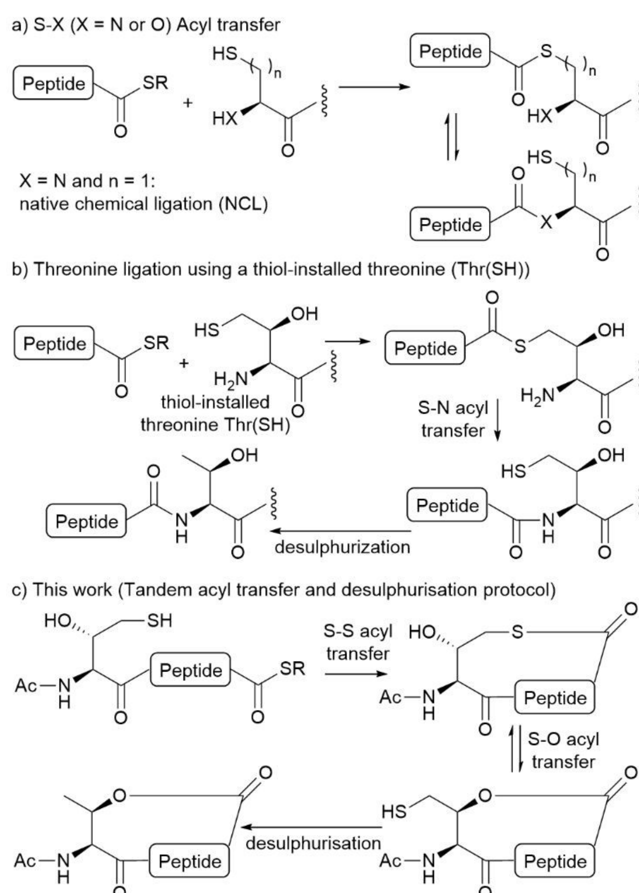

Fig. 1. Acyl transfer-mediated formation of amide and ester bonds.

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† Electronic Supplementary Information (ESI) available: Experimental details and HPLC traces of the reactions. See DOI: 10.1039/x0xx00000x

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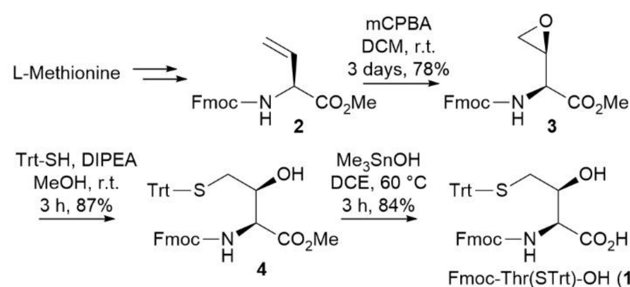
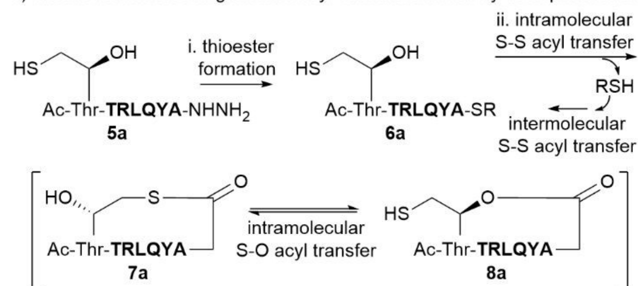
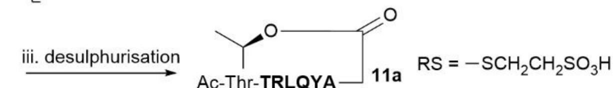
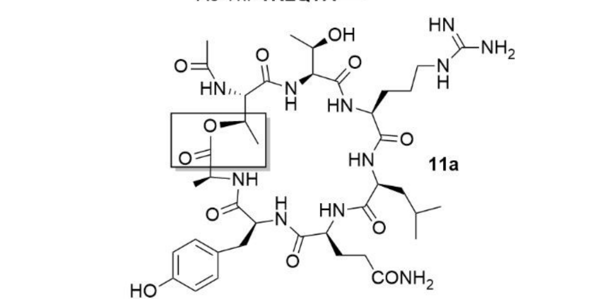


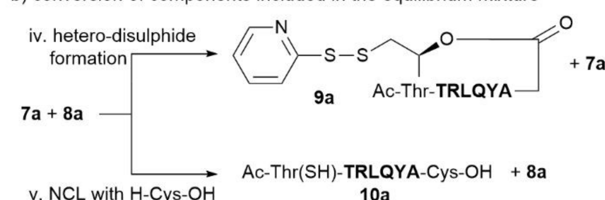
Scheme 1. Access to a protected thiol-containing Thr derivative (Fmoc-Thr(STrt)-OH (**1**))

In this context, we have studied the thioester-mediated chemoselective S–acylation followed by an S–X (X = N or O) acyl transfer (Fig. 1a). In the case of the amino group (X = N), the acyl transfer proceeds apparently irreversibly to generate stable amide bonds, in a process termed NCL (n = 1).[4] However, in the case of a hydroxy group (X = O), where equilibrium between thio and oxyesters is established,[8] the process is not irreversible. Therefore, a loss-of-thiol might interdict the equilibrium, resulting in the accumulation of oxyesters. Among variants of the NCL technology is the threonine (Thr) ligation[9] which employs a sequence of reactions consisting of a thiol-installed Thr (Thr(SH))-mediated NCL-like reaction and subsequent desulphurisation[10] (Fig. 1b). In view of the Thr ligation which features S–N acyl transfer and desulphurisation, we hypothesised that intramolecular S-acylation of a Thr(SH) derivative with thioesters followed by S–O acyl transfer and subsequent desulphurisation, could serve as a protocol for the loss-of-thiol reactivity, and could allow the synthesis of lactone peptides (Fig. 1c). Experimental[8d] and theoretical[8e] investigations disclosed that the acyl transfer of the 1-substituted mercaptoethanol units found in Thr(SH) proceeds without any significant ester hydrolysis, and this prompted us to undertake the research presented here. Our study began with the synthesis of a protected Thr(SH) derivative **1** using Danishefsky's method,[9] in which a combination of N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc) and *S*-triphenylmethyl (Trt) groups was employed for the assembly of amino acids using Fmoc solid-phase peptide synthesis (SPPS). Scheme 1 shows the route to compound **1**. The requisite Fmoc-protected *syn*-epoxide **3** was prepared by oxidation of a vinylglycine derivative **2** with *m*-chloroperoxybenzoic acid (mCPBA) in dichloromethane (DCM). Ring-opening of **3** with triphenyl-methanethiol (TrtSH) in MeOH in the presence of *N,N*-diisopropylethylamine (DIEA) at room temperature gave the sulphenylated compound **4** in 87% isolated yield. Hydrolysis of the methyl ester of **4** using trimethyltin hydroxide[11] in dichloroethane (DCE) at 60 °C proceeded without affecting the Fmoc group to afford **1**, which is requisite for Fmoc SPPS of linear precursor peptides, in 84% isolated yield. Standard Fmoc SPPS protocols using **1** with the hydrazine-treated 2-chlorotriphenyl-methyl (2-ClTrt) resin including a final deprotection step gave the linear peptide hydrazide **5** (Fig. S1 in ESI†).[12]









Scheme 2. i) 10% (v/v) $NaNO_2$ in 6 M Gn·HCl–0.2 M NaPB (pH 3.5) at -10 °C for 30 min, then 200 mM MESNa at 37 °C and pH 6.0 for 30 min, 56% yield. ii) **6a** (1 mM) in 6 M Gn·HCl–0.2 M NaPB at pH 6.5 and 37 °C for 2 h. iii) VA-044 (1.25 mM), TCEP (260 mM) and 5% (v/v) *t*-BuSH at 37 °C and pH 7.4 for 24 h, 75%. iv) 2,2'-dipyridyl disulphide (20 mM) at rt for 14 h. v) cysteine (100 mM), MPAA (50 mM) and TCEP (40 mM) at 37 °C, pH 5.0 for 13 h.

Initial assessment of the lactone formation began with the thioesterification of the model peptide hydrazide, Ac-Thr(SH)-TRLQYA-$NHNH_2$ (**5a**) *via* the corresponding peptide azide.[13] Oxidation of **5a** in 6 M guanidine hydrochloride (Gn·HCl)-0.2 M sodium phosphate buffer (NaPB) containing 10% (v/v) $NaNO_2$ (pH 3.5) at -10 °C for 30 min, followed by treatment with 200 mM sodium mercaptoethanesulfonate (MESNa) at 37 °C and pH 6.0 for 30 min, proceeded efficiently, producing the corresponding peptide thioester **6a** in 56% isolated yield after HPLC purification (Figs. S2 and S3 in ESI†).

Incubation of **6a** (1 mM) in 6 M Gn·HCl-0.2 M NaPB at 37 °C for 2 h under various pH conditions showed that the optimum pH for formation of the (thio)lactone (**7a** and **8a**) is 6.5 (Fig. S4 in ESI†). Rise in reaction temperature to 50 °C induced the formation of a small amount of unidentified side products (Fig. S5 in ESI†). Cyclisation at optimum pH at 37 °C resulted in almost complete disappearance of the linear thioester **6a** giving an 8:2mixture of the thiolactone **7a** and the oxylactone **8b** (Scheme 2a and Figs. 2a and 2b). Characterisation of the lactones shown in Scheme 2b is discussed below. The lactone generating process commences with an intramolecular S-S acyl transfer in **6a**. The reverse of this is the intermolecular reaction

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involving the liberated mercapto-ethanesulfonate (Scheme 2a). The formation of **7a** and **8a** at a substrate concentration of 1 mM was almost complete but the reaction under 10 mM conditions remained incomplete (Fig. S6 in ESI†). The thiolactone species derived from Cys-rich thioester peptides through the intramolecular S-S acyl transfer exhibits electrophilic reactivity superior to that of the parent thioester, and this might be relevant to the conversion of **7a** to **8a** *via* the S–O acyl transfer.[14]

Reaction conditions, suitable for desulphurisation of the equilibrium mixture composed of **7a** and **8a**, were evaluated. Treatment of the above mixture for lactone formation with 2,2'-azobis[2-(2-imidazolin-2-yl)propane] (VA-044) (1.25 mM), TCEP (260 mM) and 5% (v/v) *t*-BuSH at 37 °C and pH 7.4 for 24 h gave the desired desulphurized lactone peptide **11** in 75% conversion yield.[10] The decrease in pH of the reaction resulted in the equilibrium shift to the formation of thiolactone **7a**, and a low conversion yield. Another critical point for the clean conversion is that the radical-mediated desulphurisation process requires operation under anaerobic conditions (Fig. S7 in ESI†). Consequently, the one-pot/sequential conversions of **6a** (1 mM) under optimum conditions (lactone formation: 6 M Gn·HCl–0.2 M NaPB (pH 6.5) at 37 °C for 2 h; desulphurisation: 1.25 mM VA-044, 260 mM TCEP and 5% (v/v) *t*-BuSH in the buffer (pH 7.4) at 37 °C for 24 h) afforded **11a** in 63% isolated yield after HPLC purification (Fig. 2 and Figs. S8 and S9 in ESI†)).

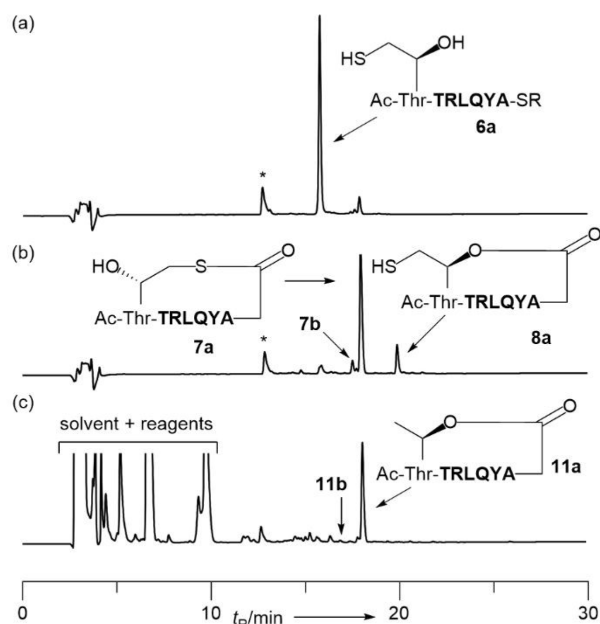

Fig. 2. HPLC trace of the conversion of **6a** to **11a**. (a) Incubation of linear thioester **6a** (1 mM) in 6 M Gn·HCl–0.2 M NaPB at 37 °C and pH 6.5 within 5 min. (b) Incubation for 2 h. A small amount of epimerisation product **7b** was detected. (c) Desulphurisation with 1.25 mM VA-044, 260 mM TCEP and 5% (v/v) *t*-BuSH in 6 M Gn·HCl–0.2 M NaPB at 37 °C pH 7.4 for 24 h. A negligible amount of epimerization product **11b** was detected. Analytical HPLC conditions: linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O, 5% to 45% over 30 min; detection at 220 nm. *Non-peptidic material.

Table 1

Thr(SH)-containing thioester **6** → (S-S acyl transfer) thiolactone **7** ⇅ oxylactone **8** → (desulphurisation) cyclic Thr-lactone peptide **11**

entry	thioesters[a]	isolated yields (%)[b]
1	Ac-Thr(SH)-**TRLQYA**-SR (**6a**)	63 (**11a**)
2	Ac-Thr(SH)-**TRLQY**D**A**-SR (**6b**)	64 (**11b**)
3	Ac-Thr(SH)-**TRLEYA**-SR (**6c**)	62 (**11c**)
4	Ac-Thr(SH)-**TKLQYA**-SR (**6d**)	62 (**11d**)
5	Ac-Thr(SH)-**SRLQYA**-SR (**6e**)	77 (**11e**)
6[c]	Ac-Thr(SH)-**TRLQYV**-SR (**6f**)	34 (**11f**)
7[d]	pGlu-Thr(SH)-**TKLQYA**-SR (**6g**)	74 (**11g**)
8[e]	Ac-Thr(SH)-**TRLYA**-SR (**6h**)	57 (**11h**)
9[e]	Ac-Thr(SH)-**ARYA**-SR (**6i**)	53 (**11i**)

[a]R = $-CH_2CH_2SO_3H$. [b]Unless otherwise noted, each substrate thioester peptide (1 mM) was incubated in 6 M Gn·HCl–0.2 M NaPB (pH 6.5) at 37 °C for 2 h; then desulphurisation was carried out in the presence of 1.25 mM VA-044, 260 mM TCEP and 5% (v/v) *t*-BuSH in 6 M Gn·HCl–0.2 M NaPB (pH 7.4) at 37 °C for 24 h under anaerobic conditions. The reaction was diluted twofold by the addition of 0.1% aqueous TFA and purified by HPLC. [c]Initial cyclization was conducted for 30 h. [d]pGlu = pyroglutamic acid residue. [e]Initial cyclisation was performed at pH 7.0 at 3 h.

Addition of 2,2'-dipyridyl disulphide (20 mM) to the reaction followed by incubation for 2 h led to disappearance of **8a** to afford the pyridyl heterodisulphide peptide **9a** with **7a** apparently unaffected. After a further 22 h, the result was the full conversion of the mixture to **9a** (Scheme 2b). Reduction of the resulting disulphide **9a** with tris(carboxyethyl)phosphine (TCEP) (40 mM) at 37 °C gave a mixture of **7a** and **8a** in a ratio similar to that observed in the cyclisation (Fig. S10 in ESI†).‡ Addition of cysteine (100 mM), 4-mercapto-phenylacetic acid (MPAA) (50 mM) and TCEP (40 mM) gave the NCL product **10a** and disappearance of **7a** (Scheme 2b and Fig. S11 in ESI†). These results allowed us to conclude that **7a** and **8a** are the thiolactone and oxylactone, respectively, existing in equilibrium.

The substrate scope of the developed protocol, including epimerisation was confirmed by the lactone formation of linear thioesters listed in Table 1. Subjecting C-terminal D-Ala peptide Ac-Thr(SH)-**TRLQY**D**A**-$SCH_2CH_2SO_3H$ (**6b**) as substitute for **6a** to the one-pot/sequential process showed that no significant epimerisation during the process is observed (Fig. S12 in ESI† and Figs. 2b and 2c). Side chain functional groups such as carboxylate (glutamate, E in **6c**), amine (lysine, K in **6d**) and alcohol (serine, S in **6e**) were also amenable to the protocol (entries 3-5) and the desired lactone peptides (**11c**-**11e**) were obtained in reasonable yields (Figs. S13 and S14 in ESI†) Esterification of the secondary hydroxyl group of Thr with β-branched amino acids such as Val is difficult, and this encouraged us to attempt the macro-lactonisation of the C-terminal Val peptide **6f**. The conversion of thio- to oxy-ester under desulphurisation step were incomplete but the attempted desulphurisation with the replacement of *t*-BuSH with 20 mM MESNa§ yielded the desired lactone peptide **11f** in 34% isolated yield after HPLC purification (Figs. S15 and S16 in ESI†). Furthermore, no epimerisation was observed during the

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macrolactonisation (Fig. S17 in ESI†) and the cyclisation of linear protected peptide (Ac-Thr-**T**(tBu)**R**(Pbf)**LQ**(Trt)**Y**(tBu)Val-OH (**6f'**), Pbf = 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl; Trt = triphenyl-methyl) under conventional conditions using a 2-methyl-6-nitrobenzoic anhydride (MNBA)–4-dimethylaminopyridine (DMAP)–Et_3N system[15] failed to afford the desired protected cyclic peptide (Fig. S18 in ESI†). A similar trend was observed in the case of cyclisation of **6a** or **6g**[16] where macrolactonisation of the corresponding protected linear peptides failed to give the desired products. The ring size of cyclic peptides appears to affect the ease of cyclisation,[2b] and this is the case for the tandem acyl transfer-desulfurisation protocol developed here. Although all cases have yet to be surveyed, the macrolactonisation of six- and five-residue peptides (**6h** and **6i**) proceeded but those of three- and four-residue peptides failed. (Fig. S19 in ESI†)

In conclusion, tandem acyl transfer of the thiol-installed Thr-containing thioester peptides, and subsequent desulphurization, allows access to macrolactone peptides possessing the esterified Thr structure. This result is difficult to achieve by conventional late-stage macrolactamisation strategies.

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Conflicts of interest

There are no conflicts to declare.

Notes and references

‡ This observation indicates that equilibrium between **7a** and **8a** was already established at 37 °C.

§ Thiolactone **7f** and desulphurisation product **11f** were eluted at the same retention time on HPLC analysis. The use of *t*-BuSH at the desulphurisation step gave **11f** and **7f** that failed to be converted to oxylactone **8f**. In contrast, MESNa allowed for the conversion of the remaining **7f** to the desulphurisation material of **6f** via the opening of thiolactone **8f**; therefore, isolation of the desired **11f** became possible.

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