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Coupling of sterically demanding peptides by β -thiolactone-mediated native chemical ligation†

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The ligation of sterically demanding peptidyl sites such as those involving Val–Val and Val–Pro linkages has proven to be extremely challenging with conventional NCL methods that rely on exogenous thiol additives. Herein, we report an efficient β -thiolactone-mediated additive-free NCL protocol that enables the establishment of these connections in good yield. The rapid NCL was followed by *in situ* desulfurization. Reaction rates between β -thiolactones and conventional thioesters towards NCL were also investigated, and direct aminolysis was ruled out as a possible pathway. Finally, the potent cytotoxic cyclic-peptide axinastatin 1 has been prepared using the developed methodology.

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Introduction

Despite the major advances in the synthetic coupling of peptides and proteins such as the use of Native Chemical Ligation (NCL) procedures¹ and metal free desulfurization,² the accomplishment of ligations³ involving amino acids with sterically demanding side chains continues to be problematic (Fig. 1a). In the previous reports by Bertozzi,⁴ Danishefsky⁴ and others,⁴ the ligation sites involving valine or proline were furnished in low yield and required an extended reaction time. This challenge is particularly relevant for the synthesis of membrane proteins in which amino acids such as Val, Ile, Leu, and Ala constitute more than one-third of the transmembrane domains.⁵ Thus, the need to develop methodologies for the ligation of these residues is tremendous. Although considerable efforts have been directed towards improving the ligation efficiency at sterically hindered sites,^{6,7} such as the elegant report by Dong⁸ on the internal activation of thioesters promoting the occurrence of ligation at proline sites, the problem remains and continues to place severe limitations on the ability to access membrane proteins by NCL.^{9,10} Herein we describe a protocol that utilizes readily available β -thiolactones to facilitate the challenging peptide ligation at sterically demanding Val–Ala, Val–Leu, Val–Val, and Val–Pro sites (Fig. 1b). This allows the connection of two bulky peptidyl residues, through a one-pot

additive-free NCL followed by desulfurization, and provides a significant advance in protein synthesis.

NCL is typically initiated by adding an exogenous thiol additive such as 4-mercaptophenylacetic acid¹¹ (MPAA 1) (Fig. 2a); a reversible transthioesterification between an activated thioester and cysteine provides a tethered peptidyl adduct, which then undergoes a facile N–S shift to form a new amide bond. There are two critical drawbacks associated with the use of 1. When the coupling partners involve bulky side-chains, the MPAA thiol exchange outcompetes the forward N–S shift, resulting in the disintegration of the tethered peptide and low yields. In addition, it necessitates the inclusion of a step to remove the thiol additive prior to the desulfurization. Although there are multiple procedures for simplified MPAA removal,^{7,11}

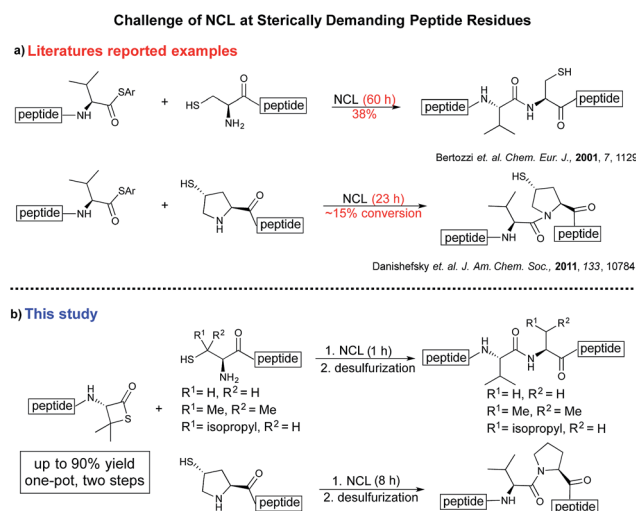


Fig. 1 The coupling of sterically hindered peptides with β -thiolactone by NCL.

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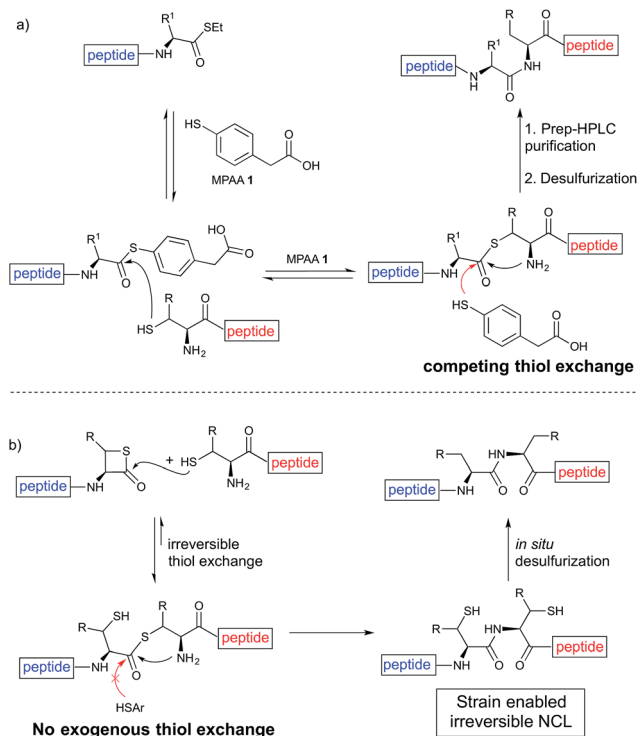


Fig. 2 Complication of the NCL when an external thiol activator is added.

reverse phase preparative HPLC purification remains the most effective technique. The additional purification and subsequent requisite lyophilization further lower the reaction yield and efficiency.

Results and discussion

We envisioned that the constraints mentioned above could be overcome if β -thiolactones¹² were used as thioester surrogates¹³ (Fig. 2b). Considering that the concept of using macrothiolactones as thioesters has been elegantly illustrated by the Nishiuchi group,¹⁴ the relief of the β -thiolactone ring strain might promote ligation without pre-activation. Furthermore, difficulty of regenerating the thietane could circumvent the need for the thiol additive, which might not only enable *in situ* desulfurization, but it might also prevent the reversible trans-thioesterification and improve the reactivity at the sterically hindered sites. This accessible methodology could provide a significant advance in protein synthesis and eliminate the additional steps typically required for peptide ligation, while achieving the synthesis of sterically challenging proteins and peptides with high efficiency. Pioneering work,¹⁵ elegantly illustrated by Crich and co-workers, has demonstrated the connections of single amino acids in the presence of exogenous thiol activators, however the investigation of β -thiolactones in the context of polypeptides as well as at sterically hindered sites has been elusive.¹⁶ Our approach commenced with the cyclization of commercially available Boc-L-cysteine and Boc-L-penicillamine (Boc-Cys and Boc-Pen respectively)^{15,17} to furnish

thiolactones **2** and **3** (ref. 18) in high yield (Table 1). Under the standard NCL conditions with the presence of MPAA, the mixture of thietan **2** and cysteine ester **4** produced the dipeptide **5** in 82% yield. The ligation between **2** and **4** in the absence of **1** was completed within 30 min, and the dipeptide **5** was obtained. Subsequent *in situ* desulfurization cleanly provided Ala-Ala dipeptide **6**. Although β -thiolactones are known to undergo reactions at their carbon sites,¹⁹ we did not observe any by-products. After screening, the use of Na_2HPO_4 buffer provided the best result, furnishing the dipeptide **6** in 84% yield over two steps. The addition of $\text{Gn} \cdot \text{HCl}$ lowered the reaction yield, and the presence of TCEP did not change the ligation outcome. Under the optimized reaction conditions, the scope of the reaction was investigated. It was found that L-cysteine boosted the reaction yield to 90% when coupled to thiolactone **2** (Table 1, entry 1). The reaction between **2** and L-Pen²⁰ **9c** smoothly yielded the Ala-Val dipeptide **10c**. The coupling of thiolactone **2** and selenocysteine **9d** generated the desired product **10b** in two steps.²¹ Interestingly, the mass spectrum of the initial ligated dipeptide was messy, and no meaningful mass peaks could be identified; however, after the desulfurization, the mass spectrum shows that dipeptide **10b** can be detected as the major product. We believed that our observation was caused by the poor solubility of the intermediate dipeptide. The penicillamine-derived thiolactone **3** was next investigated. It was reacted with cysteine to provide the corresponding dipeptide **10d** in 81% yield. The thiolactone-bearing dipeptide **7**, prepared using a literature reported procedure,¹⁵ was mixed with dipeptide **9g** in the NCL buffer to produce tetrapeptide **10l** in excellent yield (80%). Finally, the Pen dipeptide **8** was examined. The tripeptide **10m** and tetrapeptides **10n** and **10o** could be prepared in good yield. To further explore the reaction scope and limitations, we embarked on studies of more extended peptides (Table 2). The thiolactone bearing peptides were prepared using an EDC/HOObt coupling method²³ followed by global sidechain deprotection. The peptides **11–15** were generated without epimerization.²⁴ Ligation between peptidyl fragments **11** and **16a** provided polypeptide **17a** in good yield. The reaction between **12** and **16a** afforded **17b** in 86% yield after two steps. The reaction between thiolactone **11** and a 3-mercapto-leucine-bearing peptide²⁵ **16b** provided the desired product **17c** in high yield. Interestingly, considering the notorious difficulty of amide bond generation between a valine and valine or proline residues,⁴ we attempted to construct Val-Val and Val-Pro bonds using our protocol. The coupling between **12** and **16c** occurred smoothly and the overall Val-Val linked polypeptide **17e** was produced in good yield over two steps (76%). Finally, the possibility of ligating valine and proline constructs was examined. The linkage of **12** and **16d** was successfully achieved in 57% yield in a one-pot fashion. A few more examples of coupling between thiolactones and 4-mercapto-prolines were probed, and the yields varied based on the sequences (42% for **17g** and 32% for **17h**). The β -thiolactone mediated ligation has a wide functional group tolerance; a variety of amino acid residues have been investigated, and the side-chains of Asp, Lys, Arg, His, Thr, Glu, Gln, and Tyr do not interfere with the NCL (Table 2 entries 9 and 10). It is worth



Table 1 Scope of the β -thiolactone mediated one-pot NCL and desulfurization method^a

$\text{BocHN} \begin{matrix} \text{R} \\ \text{S} \\ \text{O} \end{matrix} + \text{HS} \begin{matrix} \text{O} \\ \text{C} \\ \text{NH}_2 \end{matrix} \text{OMe} \xrightarrow{\text{a)}} \text{HS} \begin{matrix} \text{O} \\ \text{C} \\ \text{NH} \end{matrix} \begin{matrix} \text{SH} \\ \text{O} \\ \text{OMe} \end{matrix} \xrightarrow{\text{b)}} \text{Product 6}$

$\text{R} = \text{H}$ **2**
 $\text{R} = \text{Me}$ **3**

Entry	Thiolactones	Ligating amino acids/peptides	Products	Yield (%)
1				90%
2	2			53%
3	2			80%
4				81%
5	2		Boc-Ala-Ala-OMe 10f	77%
6	3	9f	Boc-Val-Ala-Ala-OMe 10h	67%
7	3		Boc-Val-Ala-Phe-OMe 10i	69%
8		4	Boc-Phe-Ala-Ala-OMe 10j	71%
9	7	9f	Boc-Phe-Ala-Ala-Ala-OMe 10k	79%
10	7	9g	Boc-Phe-Ala-Ala-Phe-OMe 10l	80%
11		4	Boc-Phe-Val-Ala-OMe 10m	62%
12	8	9f	Boc-Phe-Val-Ala-Ala-OMe 10n	72%
13	8	9g	Boc-Phe-Val-Ala-Phe-OMe 10o	77%

^a Reagents and conditions: (a) buffer (0.1 M Na₂HPO₄, 0.01 M TCEP, pH = 7.8), rt, 1–8 h; (b) desulfurization buffer (0.5 M TCEP pH = 7.2, 0.1 M VA-044, *t*-BuSH), 37 °C, 2 h.

noting that among all the ligations described in Table 2, the initial NCL conversions were quantitative or near-quantitative;²⁶ it was the subsequent desulfurization that significantly reduced the overall reaction yield. More specifically, regarding the sequence **17h** in entry 8 which gave the lowest yield, the precursor product (bis-thiol) was isolated in 54% yield prior to desulfurization. Considering the yield reduction from prep-

HPLC purification and the known obstacle of thiol removal required for hydrophobic residues,²⁷ this method still provides a fundamental improvement over current protocols.

Intrigued by the facile β -thiolactone reactivity, we next investigated the reaction mechanism and rate. Reaction rates of the thiolactone and other known activated thioesters^{10,28} were compared (Fig. 3a). By monitoring with LC-MS, the ligation of



Table 2 Studies of the β -thiolactone mediated one-pot NCL method with polypeptides^{22a}

1. EDC, HOObt
2. TFA, TIPS, H₂O

11-15

Entry	Thiolactones	Ligating peptides	Products	Yield (%)
1		t BuSS-CH ₂ -H-Cys-Gln-Leu-Glu-Tyr-OH 16a	H-ALQPF AA QLEY-OH 17a	84%
2		t BuSS-CH ₂ -H-Cys-Gln-Leu-Glu-Tyr-OH 16a	H-ALQPF VA QLEY-OH 17b	86%
3		MeSS-CH(CH ₃)-CO-Gln-Ala-Glu-Gly-OH 16b	H-ALQPF AL QAEG-OH 17c	73%
4		MeSS-CH(CH ₃)-CO-Gln-Ala-Glu-Gly-OH 16b	H-ALQPF VL QAEG-OH 17d	81%
5		HS-CH(CH ₃)-H-Pen-Gln-Leu-Glu-Tyr-OH 16c	H-ALQPF VV QLEY-OH 17e	76%
6		HS-CH(CH ₃)-Gln-Ala-Glu-Gly-OH 16d	H-ALQPF VP QAEG-OH 17f	57%
7		HS-CH(CH ₃)-Gln-Leu-Glu-Tyr-OH 16e	H-ALQPF VP QLEY-OH 17g	42%
8		HS-CH(CH ₃)-Gln-Leu-Glu-Tyr-OH 16e	H-ALQPF VP QLEY-OH 17h	32% (54%)
9		t BuSS-CH ₂ -H-Cys-Gln-Leu-Glu-Tyr-OH 16a	H-ADFHT VA QLEY-OH 17i	82%
10		t BuSS-CH ₂ -H-Cys-Gln-Leu-Glu-Tyr-OH 16a	H-ARFKT VA QLEY-OH 17j	83%

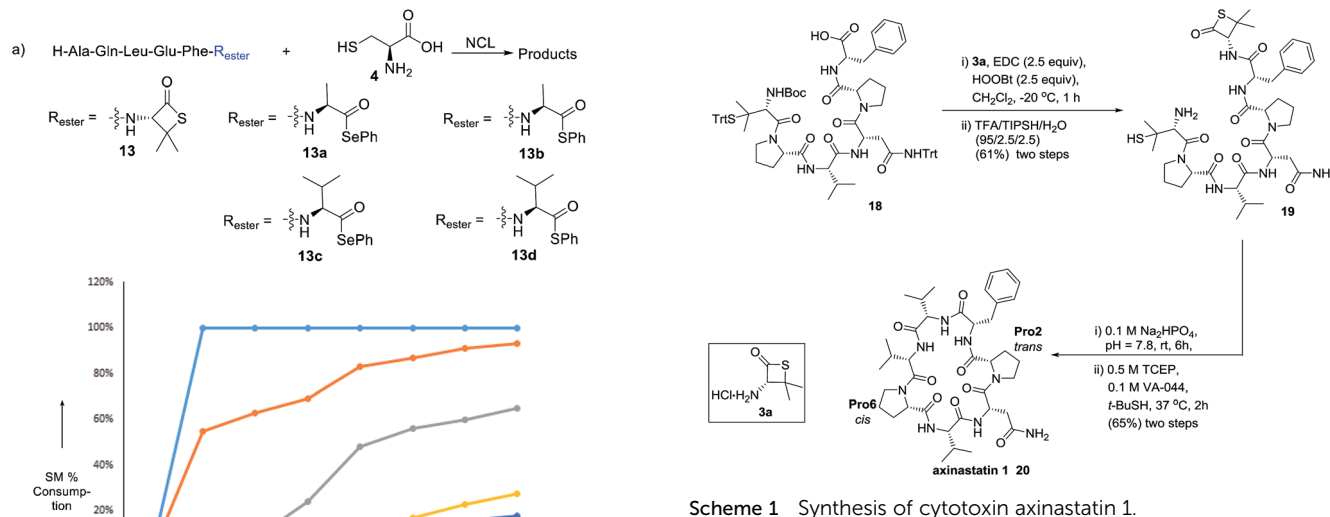
^a Reagents and conditions: (a) EDC (2.5 equiv.), HOObt (2.5 equiv.), CH₂Cl₂, -20 °C, (b) TFA/H₂O/TIPS = 95/2.5/2.5; NCL conditions: 0.1 M Na₂HPO₄, 0.01 M TCEP, pH = 7.8, rt, 1–8 h; desulfurization: buffer (0.5 M TCEP pH = 7.2, 0.1 M VA-044, *t*-BuSH), 37 °C, 2–3 h. * = isolated yield after the initial NCL shown in parentheses.

the *l*-cysteine and thiolactone-bearing peptide **13** was determined to be completed within 5 min. Analogously, peptides that possessed phenyl selenoesters **13a/13c** and phenyl thioesters **13b/13d** were subjected to identical reaction conditions. After 5 min, alanine phenyl selenoester **13a** was consumed by 55% and alanine phenyl thioester **13b** was converted by 4%. More intriguingly, the analogous valine phenyl selenoester **13c** and thioester **13d** were merely consumed by 4% and 1% respectively during the same period. This experiment illustrated that β -thiolactone was a superior thioester compared to existing activated thioesters regarding the reaction rate. Using a cross-over experiment, we probed whether the reaction proceeded through an NCL process or an undesired aminolysis pathway (Fig. 3b). A combination of peptides **13**, **16a** and **16f** were mixed in the NCL buffer. After 60 min, two major peaks appeared in the LC trace. One peak corresponded to peptide **17i**, which is the desired NCL product, and another was identified as the unreacted peptide **16f**. At 120 min, the trace amounts of **13** and

16a were consumed, furnishing **17i** exclusively. The direct aminolysis product **17j** was not observed, which suggested that thietan-mediated peptide ligation did indeed proceed by the NCL pathway.²⁹

With our enhanced understanding of the strain facilitated peptide ligation, we explored an expansion of the reaction scope and application by pursuing the synthesis of a cyclic peptide axinastatin **1** (**20**) with our methodology (Scheme 1). Axinastatin **1** is a naturally occurring cyclic heptapeptide which was isolated in minute quantities from a marine sponge (4.5×10^{-5} % yield).³⁰ It shows potent cytotoxicity against a leukemia cancer cell line (ED₅₀ = 0.21 μ g mL⁻¹). Structurally, **20** contains three valines and two prolines in the seven amino acid backbone. Importantly, Pro2 and Pro6 of the naturally occurring axinastatin **1** are in *trans* and *cis* configurations, respectively. The synthesis herein commenced with the preparation of **18** using an Fmoc solid phase peptide synthesis with a TGT resin, and the thiolactone **19** was produced through the coupling of **3a** and the





Scheme 1 Synthesis of cytotoxin axinastatin 1.

Conclusions

In summary, we have successfully developed a novel one-pot protocol for peptide ligations at sterically demanding sites utilizing the strained β -thiolactone. The strain not only greatly accelerates the reaction and enables *in situ* thiol removal, but also provides a practical method for the otherwise challenging ligation linking sterically hindered peptidyl residues. The reaction scopes and limitations have been evaluated and discussed, and the β -thiolactones proved to be a much more rapid thioester surrogate compared to conventional thioesters. Furthermore, the mode of the reaction has been verified to be an NCL pathway. The versatility of the method was demonstrated in part by the synthesis of axinastatin 1. Overall, this strain-driven thietan-mediated ligation provides a powerful tool for the synthesis of peptides and proteins. In particular, our method could make the hydrophobic regions of membrane proteins³³ more synthetically accessible. Studies towards the synthesis of complex membrane proteins using our approach will be reported in due course.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

- 1 P. Dawson, T. Muir, I. Clark-Lewis and S. Kent, *Science*, 1994, **266**, 776–779.

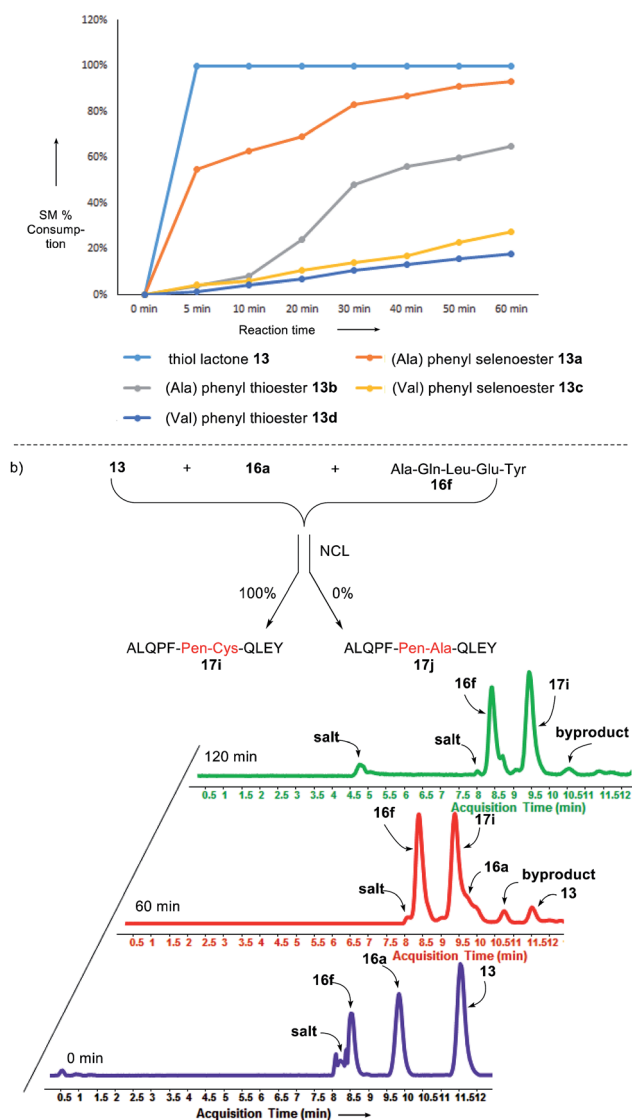


Fig. 3 Reaction rate and mechanistic investigation. Reagents and conditions: NCL condition for (a) and (b): 0.1 M Na_2HPO_4 , 0.01 M TCEP, pH = 7.8, rt.

peptide **18** without noticeable epimerization.²³ The thiolactone **19** was subjected to a one-pot NCL followed by desulfurization to successfully produce the cyclic peptide **20** intramolecularly in good yield. The high resolution MS, ^1H , and ^{13}C NMR data of synthesized axinastatin 1 are identical to the reported ones.³¹ The proper proline conformations were confirmed by 2D NMR experiments.³²



- 2 L. Z. Yan and P. E. Dawson, *J. Am. Chem. Soc.*, 2001, **123**, 526–533; Q. Wan and S. Danishefsky, *Angew. Chem., Int. Ed.*, 2007, **46**, 9248–9252.
- 3 C. Unverzagt and Y. Kajihara, *Chem. Soc. Rev.*, 2013, **42**, 4408–4420; A. Tejada, J. Brailsford, Q. Zhang, J. Shieh, M. Moore and S. Danishefsky, *Top. Curr. Chem.*, 2014, **362**, 1–26; C. Hackenberger and D. Schwarzer, *Angew. Chem., Int. Ed.*, 2008, **47**, 10030–10074; L. Raibaut, N. Ollivier and O. Melnyk, *Chem. Soc. Rev.*, 2012, **41**, 7001–7015.
- 4 L. Marcaurelle, L. Mizoue, J. Wilken, L. Oldham, S. Kent, T. Handel and C. Bertozzi, *Chem.–Eur. J.*, 2001, **7**, 1129–1132; S. Shang, Z. Tan, S. Dong and S. Danishefsky, *J. Am. Chem. Soc.*, 2011, **133**, 10784–10786; H. Ding, A. Shigenaga, K. Sato, K. Morishita and A. Otaka, *Org. Lett.*, 2011, **13**, 5588–5591.
- 5 T. Sato, *Biopolymers*, 2016, **106**, 613–621.
- 6 M. Ulmschneider and M. Sansom, *Biochim. Biophys. Acta, Biomembr.*, 2001, **1512**, 1–14; B. Nilsson, L. Kiessling and R. Raines, *Org. Lett.*, 2000, **2**, 1939–1941; R. Hondal, B. Nilsson and R. Raines, *J. Am. Chem. Soc.*, 2001, **123**, 5140–5141; M. Gieselman, L. Xie and W. Donk, *Org. Lett.*, 2001, **3**, 1331–1334; J. Bode, R. Fox and K. Baucom, *Angew. Chem., Int. Ed.*, 2006, **45**, 1248–1252; J. Canosa and P. Dawson, *Angew. Chem., Int. Ed.*, 2008, **47**, 6851–6855; X. Li, H. Lam, Y. Zhang and C. Chan, *Org. Lett.*, 2010, **12**, 1724–1727; T. Durek and P. Alewood, *Angew. Chem., Int. Ed.*, 2011, **50**, 12042–12045; N. Ollivier, J. Vicogne, A. Vallin, H. Drobecq, R. Desmet, O. Mahdi, B. Leclercq, G. Goormachtigh, V. Fafeur and O. Melnyk, *Angew. Chem., Int. Ed.*, 2012, **51**, 209–213; R. Thompson, X. Liu, N. García, P. Pereira, K. Jolliffe and R. Payne, *J. Am. Chem. Soc.*, 2014, **136**, 8161–8164; M. Jbara, M. Seenaiaha and A. Brik, *Chem. Commun.*, 2014, **50**, 12534–12537; J. Tailhades, N. Patil, M. Hossain and J. Wade, *J. Pept. Sci.*, 2015, **21**, 139–147; M. Raj, H. Wu, S. Blosser, M. Vittoria and P. Arora, *J. Am. Chem. Soc.*, 2015, **137**, 6932–6940; F. Burlina, G. Papageorgiou, C. Morris, P. D. Whitee and J. Offer, *Chem. Sci.*, 2014, **5**, 766; J. Blanco-Canosa, B. Nardone, F. Albericio and P. Dawson, *J. Am. Chem. Soc.*, 2015, **137**, 7197–7209; P. M. Shelton, C. Weller and C. Chatterjee, *J. Am. Chem. Soc.*, 2017, **139**, 3946–3949; C. Rao and C. Liu, *Org. Biomol. Chem.*, 2017, **15**, 2491–2496; Y. Asahina, T. Kawakami and H. Hojo, *Chem. Commun.*, 2017, **53**, 2114–2117.
- 7 N. Mitchell, L. Malins, X. Liu, R. Thompson, B. Chan, L. Radom and R. Payne, *J. Am. Chem. Soc.*, 2015, **137**, 14011–14014; O. Reimann, C. Smet-Nocca and C. Hackenberger, *Angew. Chem., Int. Ed.*, 2015, **54**, 306–310.
- 8 Y. Gui, L. Qiu, Y. Li, H. Li and S. Dong, *J. Am. Chem. Soc.*, 2016, **138**, 4890–4899.
- 9 J. Li, S. Tang, J. Zheng, C. Tian and L. Liu, *Acc. Chem. Res.*, 2017, **50**, 1143–1153; S. Kent, *Bioorg. Med. Chem.*, 2017, **25**, 4926–4937; H. Burke, L. McSweeney and E. Scanlan, *Nat. Commun.*, 2017, **8**, 15655.
- 10 D. Bang, B. L. Pentelute and S. Kent, *Angew. Chem., Int. Ed.*, 2006, **45**, 3985–3988; J. Zheng, H. Cui, G. Fang, W. Xi and L. Liu, *ChemBioChem*, 2010, **11**, 511–515.
- 11 T. Moyal, H. Hemantha, P. Siman, M. Refua and A. Brik, *Chem. Sci.*, 2013, **4**, 2496–2501; J. Shimko, C. Howard, M. Poirier and J. Ottesen, *Methods Mol. Biol.*, 2013, **981**, 177–192.
- 12 S. Aubry, G. Aubert, T. Cresteil and D. Crich, *Org. Biomol. Chem.*, 2012, **10**, 2629–2632; A. Noel, B. Delpéch and D. Crich, *J. Sulfur Chem.*, 2013, **34**, 104–141; A. Noel, B. Delpéch and D. Crich, *J. Org. Chem.*, 2014, **79**, 4068–4077; M. Michael, T. Brandon, Y. Wang, C. Geoffrey and G. Doherty, *J. Am. Chem. Soc.*, 2014, **136**, 10814–10820; X. Liu, Y. Wang and G. Doherty, *Asian J. Org. Chem.*, 2015, **4**, 994–1009.
- 13 G. Kenner, W. McDermott and R. Sheppard, *J. Chem. Soc. D*, 1971, 636–637; B. Backes and J. Ellman, *J. Am. Chem. Soc.*, 1994, **116**, 11171–11172; E. George, R. Novick and T. Muir, *J. Am. Chem. Soc.*, 2008, **130**, 4914–4924; G. Fang, J. Wang and L. Liu, *Angew. Chem., Int. Ed.*, 2012, **51**, 10347–10350; S. Panda, C. Hall, A. Oliferenko and A. Katritzky, *Acc. Chem. Res.*, 2014, **47**, 1076–1087.
- 14 S. Tsuda, T. Yoshiya, M. Mochizuki and Y. Nishiuchi, *Org. Lett.*, 2015, **17**, 1806–1809.
- 15 D. Crich and S. Kasinath, *J. Org. Chem.*, 2009, **74**, 3389–3393; Aubry, K. Sasaki, L. Eloy, G. Aubert, P. Retailleau, T. Cresteil and D. Crich, *Org. Biomol. Chem.*, 2011, **9**, 7134–7143.
- 16 The homocysteine derived γ -thiolactone was also investigated in the preparation of polymers, see: Z. Fan, Y. Zhang, J. Jia and X. Li, *RSC Adv.*, 2015, **5**, 16740–16747.
- 17 M. Suzuki, K. Makimura and S. Matsuoka, *Biomacromolecules*, 2016, **17**, 1135–1141.
- 18 Thiolactone 2 can be stored at 0 °C for at least one month, and pen-thiolactone 3 can be stored indefinitely in the refrigerator.
- 19 A. Noel, B. Delpéch and D. Crich, *Org. Biomol. Chem.*, 2012, **10**, 6480–6483.
- 20 C. Haase, H. Rohde and O. Seitz, *Angew. Chem., Int. Ed.*, 2008, **47**, 6807–6810.
- 21 N. Metanis, E. Keinan and P. E. Dawson, *Angew. Chem., Int. Ed.*, 2010, **49**, 7049–7053; K. Cergol, R. Thompson, L. Malins, P. Turner and R. Payne, *Org. Lett.*, 2014, **16**, 290–293.
- 22 Reactions were generally carried out at 3.0 mM. Reactions carried out in diluted buffer required a longer reaction time (0.2 mM, 20 h), yet the yields are nearly identical compared to the ones in more concentrated buffers. See the ESI† for experiment details.
- 23 S. Sakakibara, *Biopolymers*, 1995, **37**, 17–28.
- 24 The D-Phe incorporating polypeptide 12a was prepared, and its LC-MS trace does not match with the one from corresponding L-Phe bearing peptide 12 produced via the EDC coupling protocol. This observation further validated that EDC/HOObt coupling conditions did not lead to epimerization. See the ESI† section VI for details.
- 25 Z. Harpaz, P. Siman, K. Kumar and A. Brik, *ChemBioChem*, 2010, **11**, 1232–1235; Z. Tan, S. Shang and S. Danishefsky, *Angew. Chem., Int. Ed.*, 2010, **49**, 9500–9503.
- 26 See the ESI† for selected NCL crude LC-MS traces.



- 27 B. Fierz, S. Kilic, A. Hieb, K. Luger and T. Muir, *J. Am. Chem. Soc.*, 2012, **134**, 19548–19551.
- 28 N. Mitchell, S. Kulkarni, L. Malins, S. Wang and R. Payne, *Chem.–Eur. J.*, 2017, **23**, 946–952; L. Malins, N. Mitchell and R. Payne, *J. Pept. Sci.*, 2014, 64–77; L. Raibaut, M. Cargoet, N. Ollivier, Y. Chang, H. Drobecq, E. Boll, R. Desmet, J. Monbaliu and O. Melnyk, *Chem. Sci.*, 2016, **7**, 2657–2665.
- 29 No reaction was observed when mixing **13** and alanine bearing **16f** in NCL buffer for 4 h.
- 30 G. Pettit, C. Herald, M. Boyd, J. Leet, C. Dufresne, D. Doubek, J. Schmidt, R. Cerny, J. Hooper and K. Rützler, *J. Med. Chem.*, 1991, **34**, 3339–3340.
- 31 R. Konat, B. Mathä, J. Winkler and H. Kessler, *Liebigs Ann./Recl.*, 1995, 765–774.
- 32 See the ESI† for structure determination details.
- 33 R. Igotz, B. Kelly, R. Davis and J. Massagué, *Proc. Natl. Acad. Sci. U. S. A.*, 1986, 6307–6363.

