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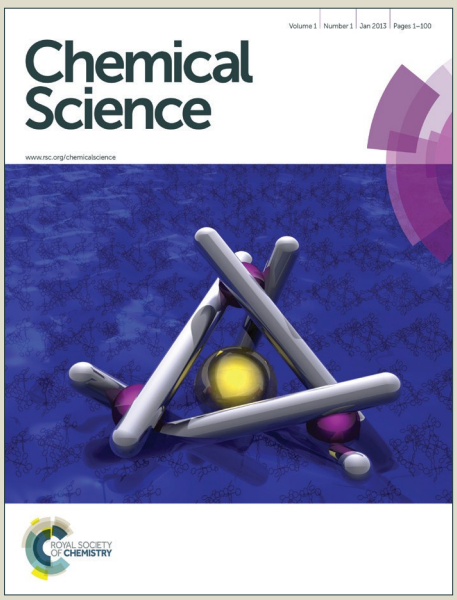

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EDGE ARTICLE

A straightforward method for automated Fmoc-based synthesis of bio-inspired peptide crypto-thioesters†

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Despite recent advances, the direct Fmoc-based solid phase synthesis of peptide α-thioesters for convergent synthesis of proteins by native chemical ligation (NCL) remains a challenge in the field. We herein report on a simple and general methodology enabling access to peptide thioester surrogates. A novel C-terminal *N*-(2-hydroxybenzyl)cysteine thioesterification device based on an amide-to-thioester rearrangement was developed, and the resulting peptide crypto-thioesters can be directly used in NCL reactions with fast *N*→*S* shift kinetics at neutral pH. These fast kinetics arise from our bio-inspired design, *via* intein-like intramolecular catalysis. Due to a well positioned phenol moiety, an impressive > 50 fold increase in kinetic rate is observed compared to an *O*-methylated derivative. Importantly, the synthesis of this new device can be fully automated using inexpensive commercially available materials and does not require any post synthetic steps prior to NCL. We successfully applied this new method to the synthesis of two long naturally-occurring cysteine-rich peptide sequences.

Introduction

Peptide α-thioesters are key intermediates for the convergent synthesis of proteins through native chemical ligation (NCL), a reaction that has revolutionized the field.[1] While peptide thioesters can be directly synthesized through Boc-based solid-phase peptide synthesis (SPPS), their access *via* the more widely used Fmoc-based strategy is not straightforward, due to the instability of the thioester moiety to repeated piperidine treatments used for Fmoc deprotection. Most Fmoc-based methodologies developed to date rely on a post-SPPS conversion of a piperidine-stable precursor into a thioester, but despite considerable efforts, no universal strategy has emerged.[2] Thanks to their simple implementation, methods such as Dawsons's *N*-acylurea (Nbz)[3] and Liu's hydrazides[4] thioester precursors have recently become popular, but are still associated with several limitations.[5] Thus, a reliable and straightforward route to peptide α-thioesters via Fmoc-SPPS is highly desirable.

A number of strategies based on β- or γ-mercapto amide thioesterification devices have recently appeared that exploit an amide-to-thioester rearrangement[6,7] (Fig. 1A), which is reminiscent of the first step of intein-promoted *in vivo* protein splicing.[8] In most cases, the *N*→*S* acyl shift is reversible and requires acidic conditions to proceed efficiently, meaning that peptides have to be first converted into thioesters prior to NCL, which is optimal at pH ~7. A few systems, referred as crypto-thioesters, are able to operate under NCL conditions thus enabling one-pot reactions (thioester formation and NCL). Cysteine-prolyl esters (CPE) developed by Aimoto[7d] (Fig. 1B-b) rely on an elegant intramolecular *O*→*N* shift to displace the amide-thioester equilibrium under NCL conditions, but with slow ligation kinetics. Although simple to implement, the α-methyl cysteine crypto-thioesters recently introduced by Offer[7j] (Fig. 1B-e) are impaired by similarly slow kinetics. In both cases, the limiting factor is likely the disfavored *trans*-to-*cis* isomerization of the amide bond to generate geometrically feasible intramolecular attack of the thiolate. Use of *N*,*N*-disubstituted β-mercapto amides can overpass this limitation by disfavoring the unproductive *trans* conformer. The symmetrical *bis*-sulfanylethylamides (SEA, Fig. 1B-c) introduced by Melnyk[7g] and Liu[7h] can be used in NCL at pH 6-7 with relatively fast kinetics, but work better at lower pH. Otaka's *N*-sulfanylethylanilides[7e] (Sealides, Fig. 1B-d) exploit the electronic effect of anilide to enhance the electrophilicity of the amide bond. They are efficient NCL substrate at neutral pH only when using a phosphate-based buffer, probably arising from pseudo-intramolecular acid-base catalysis, but the exact mechanism remains elusive.[9] Interestingly, while we were preparing this paper a method appeared based on an *N*-ethyl cysteine device featuring a free carboxylic acid, which can act as a crypto-thioester. The α-acid group was shown to accelerate the reaction compared to an α-amide-containing analogue, with an optimum of ligation kinetics at pH 5.5, but with very slow kinetics at pH 7.[10]

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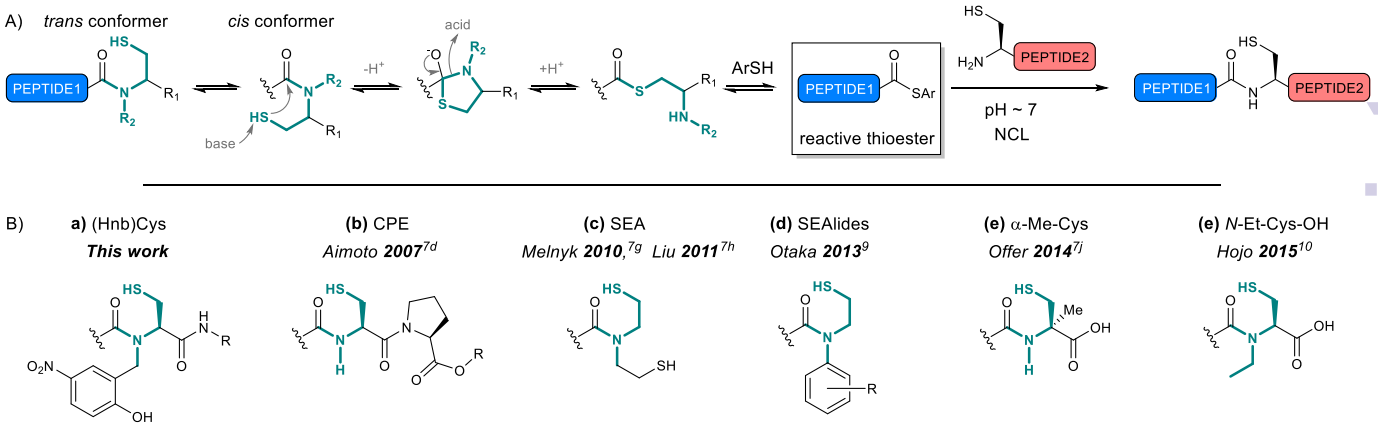


Fig. 1 *N→S* shift-based in situ synthesis of peptide thioesters for NCL. A) Mechanism. B) Crypto-thioesters bearing thioesterification devices operating under NCL conditions.

We herein report on the design and optimization of a simple and general methodology based on an *N*-(2-hydroxybenzyl) cysteine device, which can be automatically assembled on solid phase using inexpensive commercially available materials. Peptides bearing this device show fast *N→S* shift kinetics under NCL conditions around neutral pH, likely arising from a biomimetic intein-like intramolecular catalysis mechanism. The synthesis of these novel crypto-thioesters is straightforward and does not require an additional step prior to NCL. We believe that this accessible and robust Fmoc-based thioesterification device provides a significant advance to chemical protein synthesis.

Results and discussion

We based our design on *N*-alkyl cysteine[7c] as a scaffold for the design of our thioesterification device, as it can be readily assembled on solid phase from inexpensive and commercially available materials.[11] However, its *N→S* shift is usually very slow at neutral pH,[12] preventing its direct use in NCL. We focused our efforts on addressing this challenge by taking inspiration from the mechanism of *in vivo* protein splicing promoted by class 1 inteins. The first step of this process consists of a self-catalyzed *N→S* acyl shift of an amine into a thioester,[8] where a highly conserved histidine residue plays a pivotal role. This *N*-protonated His is believed to catalyze *C*-*N* bond scission through polarization and then protonation of the nitrogen leaving group (Fig. 2A).[13] We reasoned that the *N*-alkyl group of cysteine could be functionalized by an appropriate intramolecular proton donor able to mimic the catalytic histidine side chain, resulting in a much faster *N→S* shift at neutral pH. Accordingly, we designed an *N*-2-hydroxybenzyl cysteine device (Fig. 2B) leading to a hypothetical thermodynamically favored six-membered transition state for intramolecular *N*-protonation. Key to this design is a phenol group with a pKa close to neutral, as this would result in a low energy cost for the intramolecular proton transfer at pH 7.

We expected an additional advantage from the 2-hydroxybenzyl group: acceleration of *N*-acylation *via* *O*-acylation followed by intramolecular *O→N* shift (Fig. 2C), as in the *N*-acylation of 2-hydroxy-5-methoxybenzyl (Hmb) protected amines.[14] This could overcome a common drawback encountered with most *N→S* acyl shift-based thioesterification devices developed so far, the difficult *N*-acylation of a secondary amine,[7g-h,15] to introduce the first residue of the peptide sequence.

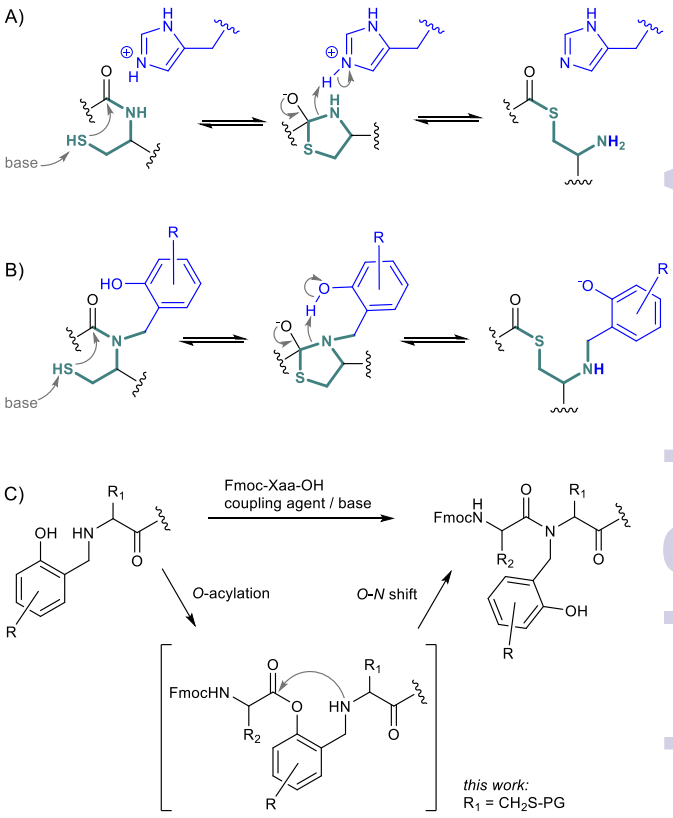
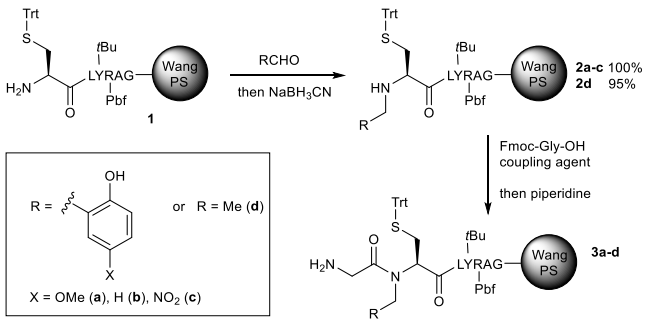


Fig. 2 Rational design of *N*-(2-hydroxybenzyl) cysteine thioesterification devices. A) Mechanism of intein self-catalyzed *N→S* shift in water. B) Bio-inspired putative mechanism for self-catalysis of *N→S* shift in *N*-acyl-*N*-(2-hydroxybenzyl) cysteine peptides in water. C) Mechanism of intramolecular *O→N* shift in organic solvents shared by *N*-Hmb peptides and the new *N*-(2-hydroxybenzyl) cysteine thioesterification devices. PG: protecting group.

Optimization of the hydroxybenzyl group.

Three different 2-hydroxybenzyl groups were introduced through an automated reductive amination protocol (compounds **2a-c**) and were compared with a reference *N*-ethyl cysteine device (**2d**). For this preliminary study, a reporter peptide (LYRAG-NH_2) was introduced C-terminal to cysteine to facilitate analysis by LC-MS after TFA-mediated deprotection and cleavage from the resin. Excellent results were obtained with a 2-hydroxy-5-nitrobenzyl (Hnb) group[16] giving quantitative *N*-acylation yields for a Gly residue under mild amide coupling conditions (HBTU/HOBt) (table 1, entry 5), while the ethyl-substituted compound (table 1, entry 7) was unreactive. Hmb or unsubstituted 2-hydroxybenzyl groups (table 1, entries 1 and 3) showed only moderate assistance. This results are in line with literature:[16] Alewood and collaborators already observed much superior acyl transfer efficiencies of 2,6- and 2,5-Hnb compared to Hmb. Moreover, the pKa of Hnb hydroxyl group was close to 7 (~6.5, see supplementary information, Fig. S11†), in line with our requirements discussed above for efficient biomimetic catalysis. We accordingly selected (Hnb)Cys as our preferred scaffold.

Table 1 Screening of different *N*-(2-hydroxybenzyl) groups for their ability to enhance the *N*-acylation yield of a solid-supported *S*-trityl-protected *N*-substituted cysteine. PS: polystyrene.



Entry	Resin	Coupling agent[a]	Product	Yield [%][b]
1	**2a**	HBTU/HOBt	**3a**	< 5[c]
2	**2a**	HATU	**3a**	45[c]
3	**2b**	HBTU/HOBt	**3b**	11
4	**2b**	HATU	**3b**	12
5	**2c**	HBTU/HOBt	**3c**	> 99
6	**2c**	HATU	**3c**	> 99
7	**2d**	HBTU/HOBt	**3d**	< 1
8	**2d**	HATU	**3d**	12
9	**2d**	PyBrop	**3d**	1
10	**2d**	HATU/DMAP[d]	**3d**	13

[a] All reactions were conducted for 2 h, in DMF, using 10 equiv. Fmoc-Xaa-OH, 9.5 equiv. coupling agent and 20 equiv. iPr$_2$NEt. [b] Yields determined after TFA-mediated cleavage of the peptide resin by HPLC analysis of the amine-to-amide conversion. [c] Estimated yields due to closely eluting HPLC peaks. [d] 0.1 equiv. DMAP.

Optimization of the thiol protecting group of cysteine and linkage to the resin.

When exploring the scope of peptides bearing our (Hnb)Cys thioesterification device, we observed an instability upon purification and storage due to premature spontaneous *N*→*S* shift. Fortunately, protection of the cysteine thiol as an *S*-S*t*Bu disulfide, stable to Fmoc-SPPS and readily removed under NCL conditions,[17] resolved this problem (see supplementary information, pS24-S29†).[18] As the use of a reporter peptide was no longer needed at this point, we looked at introducing the Cys(S*t*Bu) as either the α-acid or amide, using a Wang type or a Rink's amide linker, respectively. Disappointingly, in the former case we were confronted to Cys epimerization[19] and β-elimination followed by addition of piperidine.[20] In the latter case, we observed a TFA-catalyzed hydrolysis[21] leading to a mixture of amide- (**7**) and acid-terminated (**8**) compounds (see supplementary information pS27-S29 for details†). Simple incorporation of a Gly spacer between Rink's linker and the device solved these problems. Preliminary evaluation of NCL reactions with model cysteinyl peptide **6** showed that this additional residue (compound **9**) did not affect NCL kinetics compared to cysteine α-amide **7**, the reaction being even slightly faster than for α-acid **8** (supplementary information, Fig. S57†). As expected cysteine deprotection was very fast, showing complete removal of the S*t*Bu group within minutes.

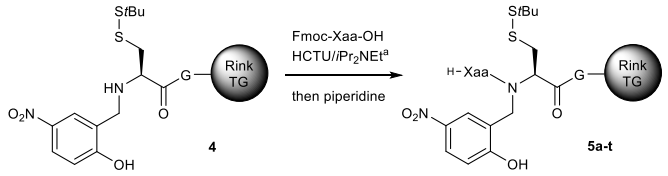
Study of the *N*-acylation of the device.

N-Acylation of the optimized device was then examined for the twenty proteogenic amino acids, using a standard SPPS protocol (table 2). In most cases coupling yields were good to excellent. No significant epimerization was observed, except for Fmoc-Cys(Trt) (7%), which is well known for its propensity for racemization.[22] Six residues showed modest *N*-acylation yields (< 50%). In these difficult cases, we looked at further optimizing the coupling avoiding the use of sensitive or costly reagents, and keeping in mind an easy-to-automate procedure. Three successive couplings of Fmoc-Ser(*t*Bu) resulted in an excellent yield (85%, entry 16). Five-fold coupling of the more demanding Fmoc-Val led to a good 65 % yield (entry 21). Alternatively, single coupling under microwave heating at 70°C led to an excellent 92% yield for Fmoc-Val (entry 22) and 78% for Fmoc-Ile (entry 24). Importantly, the unreacted secondary amine could be quantitatively capped by acetylation, giving a byproduct that can be readily removed during the peptide precipitation step following TFA-based cleavage from the resin.[23]

Mechanistic insights.

We next wanted to assess the validity of our intein-inspired design hypothesis that should result in self-catalysis of the *N*→*S* shift at neutral pH through intramolecular protonation. Here, we synthesized a model peptide equipped with a 2-methoxy-5-nitrobenzylcysteine device (**12**), designed to inhibit the acid-base properties of the phenol group while not being expected to lead to major changes in terms of steric hindrance compared to Hnb (peptide **9**). We were delighted to see that

Table 2 Acylation of resin **4** with the 20 different protected proteogenic amino acids under automated coupling conditions. TG: Tentagel.



Entry	Xaa	Product	Nb of couplings[b]	Yield [%][c]
1	Gly	**5a**	1	97
2	Asp(O*t*Bu)	**5b**	1	94
3	Ala	**5c**	1	90
4	Glu(O*t*Bu)	**5d**	1	89[d]
5	Cys(Trt)	**5e**	1	86[e]
6	Met	**5f**	1	85
7	Asn(Trt)	**5g**	1	80[f]
8	His(Trt)	**5h**	1	77
9	Phe	**5i**	1	77
10	Gln(Trt)	**5j**	1	72
11	Leu	**5k**	1	71
12	Trp(Boc)	**5l**	1	69
13	Arg(Pbf)	**5m**	1	67
14	Tyr(*t*Bu)	**5n**	1	58
15	Ser(*t*Bu)	**5o**	1	49
16	Ser(*t*Bu)	**5o**	3	85
17	Lys(Boc)	**5p**	1	45
18	Thr(*t*Bu)	**5q**	1	19
19	Pro	**5r**	1	19
20	Val	**5s**	1	16
21	Val	**5s**	5	65
22	Val	**5s**	1	92[g]
23	Ile	**5t**	1	12
24	Ile	**5t**	1	78[g]

[a] Reactions were conducted at a 0.025 mmol scale on a Prelude synthesizer, at RT for 30 min, in NMP/DMF 5:1, using 10 equiv. Fmoc-Xaa-OH, 9.5 equiv. HCTU and 20 equiv. iPr_2NEt. [b] When indicated, the coupling step was repeated under the same conditions. [c] Yields determined after TFA-mediated cleavage of the peptidyl resin and subsequent HPLC analysis of the amine-to-amide conversion. [d] Concomitant formation of pyroglutamate during TFA cleavage. [e] 93:7 mixture of L-Cys/D-Cys. [f] Incomplete deprotection of the Trt group. [g] Coupling at 70°C.

the hydroxyl group does very efficiently catalyze the *N*→*S* acyl shift: > 50-fold rate enhancement of NCL was measured for **9** compared to **12** under NCL conditions (Fig. 3), supporting our hypothesis.[24]

Moreover, ligation of **9** at different pHs showed the *N*→*S* acyl shift / NCL process to be fastest at pH ~6.5, and slightly slower at lower pH (supplementary information, Fig. S67†). This fits with the pKa of the Hnb group (6.5), suggesting the need for a subtle balance between phenol group protonation and thiol group deprotonation (cysteine pKa ~8.5) in influencing the *N*→*S* shift. To eliminate a possible *N*→*O* shift mechanism, the thiol was protected with a stable acetamidomethyl (Acm) group (**13**). This NCL-unreactive compound confirms the crucial role of the SH group and provides further data to suggest an intramolecular *N*→*S* shift mechanism. Critically, this latter finding also highlights the potential of (Hnb)Cys thioester precursors for successive ligations in the N-terminus-to-C-terminus (N-to-C) direction. We are currently evaluating latent crypto-thioesters that could be activated by deprotection of a NCL-stable cysteine protecting group, as has been exploited for related *N*-Methyl Cys,[11,25] CPE,[26] SEA[17] and Sealides[27] thioester precursors.

Study of the NCL with model peptides.

We next turned our attention to the effectiveness of our new thioesterification device in NCL reactions for a range of thioesters bearing different amino acids C-terminal to the (Hnb)Cys device (Fig. 3). Note that we used relatively dilute conditions for the peptide reactants (~1 mM) and low concentrations of 4-mercaptophenylacetic acid catalyst (MPAA, 25 mM).[28] Relatively fast NCL kinetics were observed using Ala (peptide **9**) and Ser (**10**) crypto-thioesters, as the reactions were complete after 24 h at 37°C. For comparison to NCL kinetics data available in the literature, we determined apparent second order kinetic constants, with results in the 0.03-0.06 $M^{-1}.s^{-1}$ range, in line with reported values for standard NCL with preformed thioesters.[29] These data validate the rapid *N*→*S* shift promoted by our device. Ligation at valine (crypto-thioester **11**) was 10-times slower, as we had anticipated from the steric hindrance of its side chain.[30] However, increasing MPAA concentration to 300 mM,

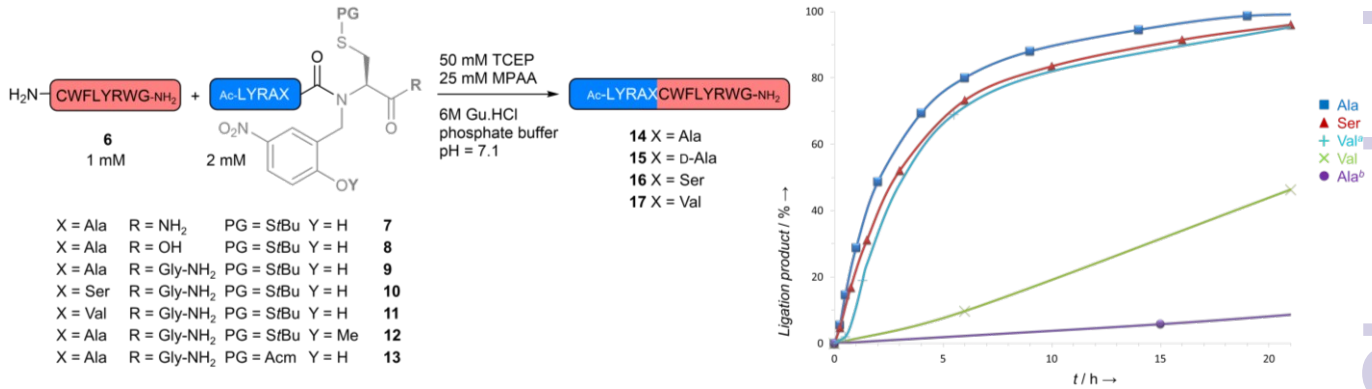


Fig. 3 NCL of model crypto-thioester peptides **7**-**11** bearing a *N*-(Hnb) cysteine device, and an analog (**12**) with the hydroxyl group masked as a methyl ether. [a] 300 mM MPAA, pH 6.6, 50 °C. [b] Hnb methyl ether (**12**).

lowering the pH close to the optimum (6.6) and heating to 50 °C led to kinetics comparable to Ala and Ser crypto-thioesters. The latter result suggests that the method could be applied in routine NCL even for peptide sequences known to be kinetically-demanding.

Potential epimerization during the *N*→*S*-shift / NCL process was rigorously examined by synthesizing a D-Ala-containing product (**15**) as an HPLC standard. Only trace epimerization (< 0.4 %) was detected in the crude NCL mixture starting from the L-Ala crypto-thioester **9**. We also examined hydrolysis of the crypto- thioesters into the corresponding acid. All reactions were clean and did not show significant amounts of hydrolysis (< 4% for **9** after 24h and using excess crypto-thioester).

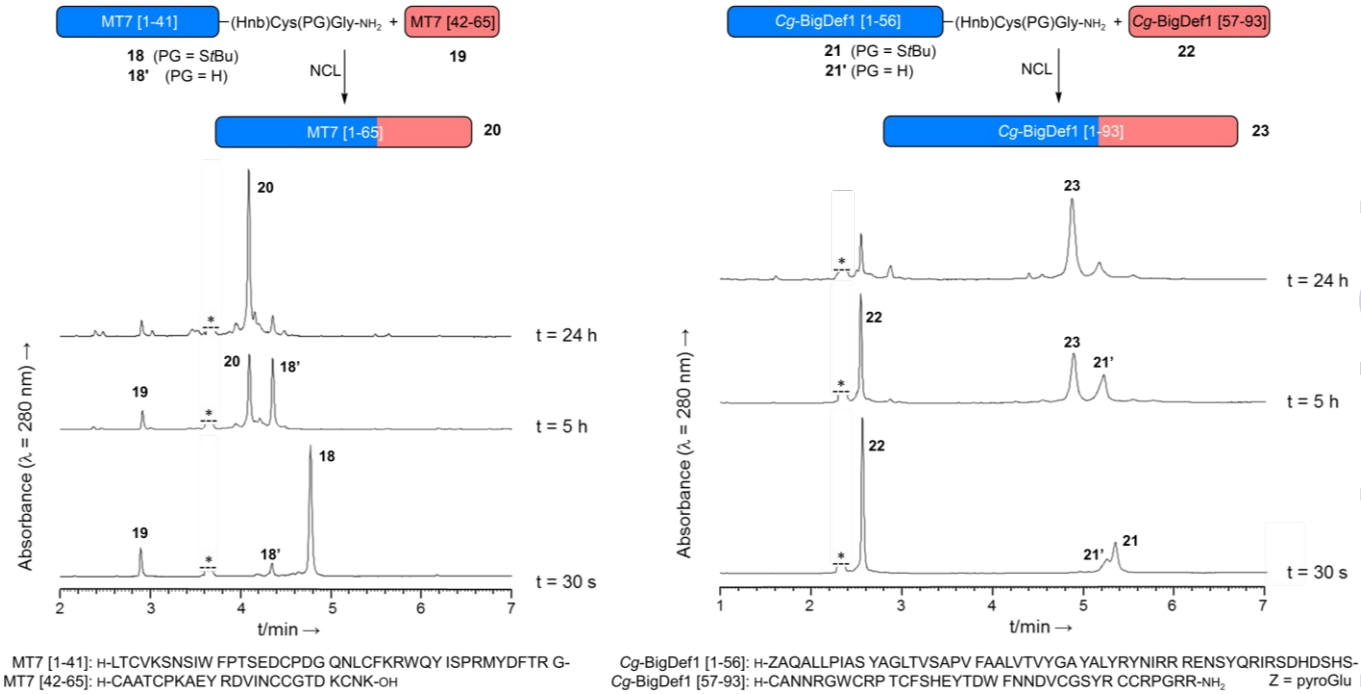
Application of the methodology to two cysteine-rich peptides.

Importantly, our new method was applied to the synthesis of two long naturally-occurring cysteine-rich peptide sequences, MT7 and Cg-BigDef1. Muscarinic toxin 7 (MT7), 65 residues, was isolated from green mamba venom.[31] MT7 naturally forms a three-finger-fold stabilized by four disulfide bonds. Big defensin 1 (Cg-BigDef1) is a 93-residue host-defense peptide isolated from Japanese oyster composed of a hydrophobic N-terminal domain and a β-defensin-like cationic C-terminal domain containing six cysteine residues involved in three disulfide bridges.[32]

These two peptides sequences were chosen as challenging examples to demonstrate the applicability of our methodology, as their syntheses *via* two segments NCL require long thioesters, 41 and 56 amino acids, respectively. In addition, the N-terminal Cg-BigDef1 domain is a "difficult sequence",[33] hard to synthesize by Fmoc SPPS.[34] Both crypto-thioesters (**18** and **21**) were synthesized and purified in good yields (13% and 7%, see supplementary information, p S86 and S92†). The two ligations were complete overnight at 1 mM concentrations, and showed clean HPLC profiles (Fig. 4). Finally, pure reduced forms of MT7 (**20**) and Cg-BigDef1 (**23**) were isolated after HPLC purification in 14% and 18% yields, respectively (supplementary information, figures S85 and S92†), demonstrating the utility of the (Hnb)Cys method.

Conclusions

In conclusion, we introduced a novel C-terminal thioesterification device, (Hnb)Cys(S*t*Bu) that can rapidly rearrange into a thioester at neutral pH, allowing its use in NCL even under demanding conditions. Fast kinetics likely results from an internal catalysis mechanism provided by a well-positioned phenol moiety arising from a bio-inspired intein-like design. Synthesis from inexpensive commercially available materials and further peptide elongation is straightforward, and can be fully automated on a peptide synthesizer without requiring a post-synthetic step, thus constituting a major advance in the field. Applicability of the strategy was demonstrated by the synthesis of two long naturally occurring cysteine-rich peptide sequences. Further work is ongoing in our laboratory for its application to other synthetically demanding targets.



MT7 [1-41]: H-LTCVKSNSIW FPTSEDCPDG QNLCFKRWQY ISPRMYDFTR G-
MT7 [42-65]: H-CAATCPKAEY RDVINCCGTD KCNK-OH

Cg-BigDef1 [1-56]: H-ZAQALLPIAS YAGLTVSAPV FAALVTVYGA YALYRYNIRR RENSYQRIRS DHDSHS-
Cg-BigDef1 [57-93]: H-CANNRGWCRP TCFSHEYTDW FNNDVCGSYR CCRPGRR-NH2 Z = pyroGlu

Fig 4 Application of the *N*-(2-hydroxy-5-nitrobenzyl)cysteine thioesterification device to the NCL-based syntheses of two long demanding peptide sequences.

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Acknowledgements

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