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Epimerization-free access to C-terminal cysteine peptide acids, carboxamides, secondary amides, and esters *via* complimentary strategies†

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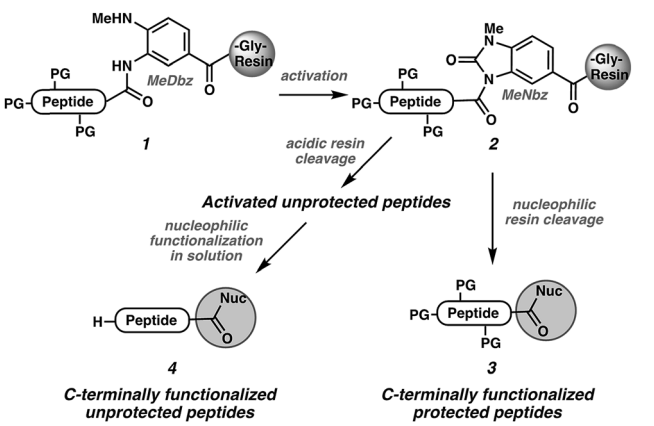
C-Terminal cysteine peptide acids are difficult to access without epimerization of the cysteine α-stereocenter. Diversification of the C-terminus after solid-phase peptide synthesis poses an even greater challenge because of the proclivity of the cysteine α-stereocenter to undergo deprotonation upon activation of the C-terminal carboxylic acid. We present herein two general strategies to access C-terminal cysteine peptide derivatives without detectable epimerization, diketopiperazine formation, or piperidinylalanine side products.

C-Terminal cysteine peptides, including prenylated and farnesylated peptides,[1] disulfide linked peptide toxins,[2] and insulinotropic peptides,[3,4] comprise an important but synthetically challenging class of biologically active peptides. Many of these peptides are modified at the C-terminus. C-terminal modifications such as esters and amides can be critical to maintaining a peptide's active conformation,[5] *in vivo* activity, and pharmacokinetics;[6] therefore, the ability to vary the peptide structure in this location is crucial to drug development efforts.[7] Although several methods have been reported for C-terminal functionalization after solid-phase peptide synthesis (SPPS) is complete,[8] these approaches either result in epimerization when applied to C-terminal Cys peptides[9] or the applicability of the method to C-terminal Cys peptides is not addressed.[10,11] While activation of the C-terminal carboxylic acid can induce epimerization *via* oxazolone formation in most amino acids,[12] cysteine is also prone to epimerization *via* direct deprotonation during its attachment to the resin[13] and upon prolonged or repeated exposure to base (*i.e.*, during peptide elongation *via* Fmoc SPPS).[14] Therefore, even the preparation of simple carboxylic acids or carboxamides of C-terminal cysteine peptides can be fraught with contamination by epimerized products,[1f,g,13a,15] reducing the overall yield and complicating the purification of the target peptides. A method for the epimerization-free synthesis and subsequent C-terminal modification of C-terminal Cys peptides would be highly impactful.

In this work, we report the first mild and convenient method for the epimerization-free diversification of peptides bearing a C-terminal cysteine.[16] Carboxylic acids, primary and secondary amides, and esters are accessed without epimerization or formation of diketopiperazine and piperidinyl-alanine side products.[17] We apply this strategy to the total synthesis of the nicotinic acetylcholine receptor (nAChR) antagonist α-conotoxin ImI.[18] Additionally, we include an alternate strategy employing N-deprotected cysteine derivatives as nucleophiles, and we demonstrate its utility *via* the synthesis of the insect pheromone α-factor.[1]

In the context of our ongoing efforts toward the synthesis of disulfide-linked α- and μ-conotoxins,[19,20] we were concerned about possible epimerization of the C-terminal cysteine during the SPPS. We recently reported a strategy for C-terminal functionalization of non-cysteine peptides involving activation of the methyl-diaminobenzoyl (MeDbz) linker (**1** → **2**)[21] followed by nucleophilic cleavage of the *N*-acyl urea (MeNbz) group[22] to yield various protected (**3**) or unprotected (**4**) peptides (Scheme 1).[23] If this approach were to prove mild enough to enable



Scheme 1 Our strategy for C-terminal functionalization of non-Cys terminated peptides.

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preparation of challenging C-terminal cysteine peptide derivatives, it would establish the MeNbz group as one of the mildest known activated carbonyl intermediates.[24] We were report herein the exploitation of this reactivity to access C-terminal cysteine peptide acids, primary and secondary amides, and esters without epimerization.

For epimerization-free functionalization at Cys, the attachment of the first amino acid,[21b] peptide elongation, linker activation, and nucleophilic attack all must occur without epimerization of the unusually acidic[25] Cys α-stereocenter.

We expected that the parent diaminobenzoyl group would not be sufficiently activating to cause epimerization during prolonged piperidine exposure. Thus, we sought to establish the stereochemical integrity of the Cys residue under these conditions unequivocally. We selected Cys(Trt) for these experiments because of its extreme tendency toward epimerization.[14] Thus, we synthesized tripeptide Boc-Ala-Trp(Boc)-Cys(Trt)-*MeDbzGly-Wang* and exposed it to 20% piperidine/DMF over 2, 4, and 24 h. The peptides were then cleaved under acidic conditions to afford H-AWC-*MeDbz-Gly*-OH. As expected, no epimerization was detected immediately following SPPS or after piperidine exposure at any time point (Fig. SI-2 and SI-3†).[26] Importantly, this is the first report of a linker for which no epimerization is detected at a C-terminal Cys(Trt) residue after treatment with 20% piperidine for 24 h.

With this result in hand, we were poised to evaluate the ability of the activated MeNbz linker to undergo nucleophilic displacement without inducing epimerization of the C-terminal cysteine. We first evaluated epimerization-prone Cys(Trt)-terminated peptides with N and O nucleophiles (**5**). We began with displacement by ammonia because of its small size and the relatively low pK_a of NH_4^+. We were pleased to observe formation of the target peptide (**6**, Nuc = NH_2) with complete conversion and no detectable epimerization in 54% isolated yield (Table 1, entry 1). We next evaluated benzylamine, which has a similar pK_a, but found that treatment of the activated linker with neat $BnNH_2$ led to 16% epimerization (entry 2). However, using only 5 equiv. benzylamine in MeCN, the product was formed with no detectable epimerization (entry 3). We next tested neat butylamine, which is slightly more basic, and 8% epimerization was observed. Reducing the amount of amine and varying the solvent did not improve epimerization in this case (entry 4–6). However, when we reduced the amount of butylamine to 1.1 equiv., we retained reactivity while eliminating epimerization of C-terminal Cys(Trt) (entry 7).

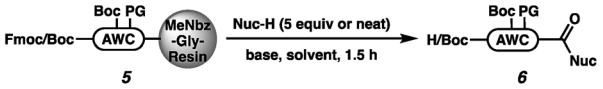
Other commercially available Cys PGs should be less prone to epimerization than Trt. Therefore, we used 5 equiv. $BuNH_2$ (*i.e.*, entry 6 conditions) for the remaining protecting groups. First, we evaluated the Acm group in MeCN (entry 8), finding <1% epimerization in the formation of the C-terminal Cys(Acm) butylamide. Next, we tested Mob, Bn, S*t*Bu, and *t*Bu with $BuNH_2$ in MeCN. In all cases, no epimerization was detected (entries 9–12). Turning our attention to alcohol nucleophiles, we tested MeOH/KO*t*Bu with Cys(Trt) as a benchmark[9] and found 42% epimerization (entry 13). However, in the presence of 5 equiv. Hünig's base (DIEA) in MeOH, no epimerization was observed (entry 14). Because of the lower conversion in this case, we also investigated the use of a 1 : 1 MeOH/phosphate buffer solvent mixture (pH 8). In this case, complete conversion was observed while maintaining no detectable epimerization (entry 15). Even carboxylic acid derivatives with a C-terminal Cys(Trt) can be difficult to access without epimerization.[27] Therefore, water was investigated as a nucleophile in the presence of Hünig's base. In this case, the reaction was slower, but the product acid was observed with <1% epimerization (entry 16).[28] Thus, all nucleophiles tested react with the activated C terminus without any observed epimerization, regardless of the protecting group on cysteine.

Next, we sought to demonstrate the viability of this cleavage strategy in the context of more complex peptides. Because of our interest in disulfide-linked neuroactive peptides,[29] we targeted the C-terminal carboxamide α-conotoxin ImI (**10**), a sub-type selective nicotinic acetylcholine receptor antagonist[30] isolated from the venom of *Conus imperialis* marine snails (Scheme 2).[31,32] Both the C-terminal carboxamide and the correct disulfide bond connectivity are important to the bioactivity of α-ImI (**10**).[31,33] Thus, peptide **7** was activated and cleaved with ammonia to yield the fully protected peptide carboxamide. Acidic removal of protecting groups and HPLC purification gave the reduced peptide **8** in 25% isolated yield.[34] The first disulfide was formed in the presence of air in 1% DMSO in phosphate buffer at pH 8 (peptide **9**). Subsequent iodine treatment removed both Acm groups and induced oxidation to form the native conotoxin (**10**) in 52% isolated yield over both folding steps. Co-injection of with a commercially available standard confirmed the correct folding.[26] Alternatively, on-resin folding with iodine could be followed by MeDbz activation, cleavage with NH_3, and side-chain deprotection to afford conotoxin **10** in 43% isolated yield; however, this approach gives a mixture of folded products, as expected.[35]

We envisioned that situations might exist wherein it would prove advantageous to access the target peptides by using various pre-functionalized cysteine derivatives as nucleophiles for cleavage of MeNbz from the resin.[27,36] For example, this would avoid the need to screen epimerization for each new derivative, it would enable access to authentic standards for peptides generated using direct C-terminal modification approaches, and it would allow incorporation of a very poor or hindered nucleophile. Recently, C-terminal cysteine peptide acids were synthesized using an *N*-(2-hydroxy-5-nitrobenzyl)-cysteine (*N*-Hnb-Cys) crypto-thioester approach.[27] Although this method enabled access to a challenging Pro-Cys linkage at the C terminus, elevated temperatures and long reaction times were required to generate the C-terminal thioester, leading to undesired side products. The stability of esters to this approach was not determined. We envisioned that application of the cysteine elongation tactic to our MeNbz-based C-terminal modification would lead to a convenient alternative method to access challenging targets at ambient temperature and with short reaction times. However, there was no report of an intermolecular[37] NCL-like reaction being conducted with the C-terminally linked peptide still attached to the resin.

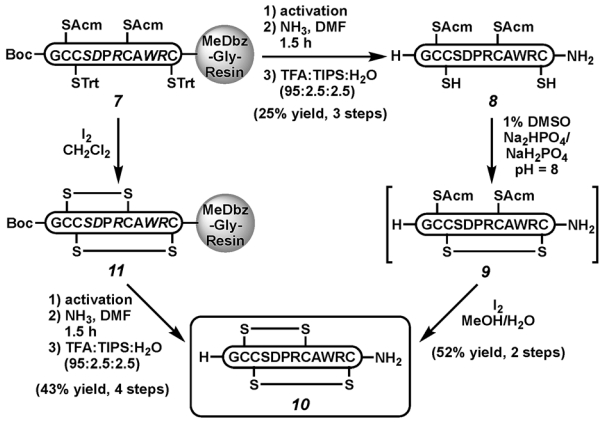
The elongation approach was tested with H-AWA-*MeNbz-Gly-Rink* peptides (**12** and **13**), which were treated with free cysteine,

Table 1 Evaluation of epimerization during nucleophilic cleavage of the MeNbz group in C-terminal cysteine peptides



Entry[a]	PG	Nuc-H	Base (5 equiv.)	Solvent	% conversion	Epimerization[b] (% D-X)
1[c]	Trt	NH_3	—	DMF	>99	<1
2	Trt	$PhCH_2NH_2$	—	$PhCH_2NH_2$	>99	16
3	Trt	$PhCH_2NH_2$	—	MeCN	>99	<1
4	Trt	$BuNH_2$	—	$BuNH_2$	>99	8
5	Trt	$BuNH_2$	—	DMF	>99	10
6	Trt	$BuNH_2$	—	MeCN	>99	9
7[d]	Trt	$BuNH_2$	—	MeCN	>99	<1
8[c]	Acm	$BuNH_2$	—	MeCN	>99	<1
9[c]	Mob	$BuNH_2$	—	MeCN	>99	<1
10[c]	Bn	$BuNH_2$	—	MeCN	>99	<1
11	S*t*Bu	$BuNH_2$	—	MeCN	>99	<1
12[c]	*t*Bu	$BuNH_2$	—	MeCN	>99	<1
13[e]	Trt	MeOH	KO*t*Bu	MeOH	>99	42
14[f]	Trt	MeOH	DIEA	MeOH	>99	<1
15[g]	Trt	MeOH	—	$MeOH/Na_2HPO_{4(aq)}$	>99	<1
16	Trt	H_2O	DIEA	H_2O/MeCN	56	<1

[a] All reactions were performed on 20 mg of resin containing all L amino acids in 200 μL of solvent at ambient temperature (24 ± 1 °C). [b] All PGs were removed prior to epimerization assay unless otherwise noted. [c] Cys(PG) was intact during epimerization assay. [d] 1.1 equiv. of $BuNH_2$ was used. [e] 0.7 equiv. KO*t*Bu. [f] Reaction was conducted for 3 h. [g] Na_2HPO_4/NaH_2PO_4 buffer at pH = 8.



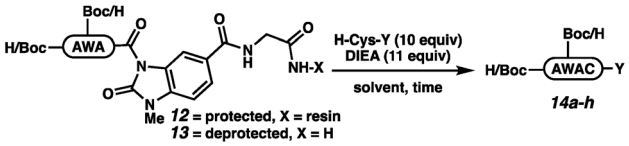
Scheme 2 Synthesis of conotoxin α-Iml (**10**).

H-Cys-OEt, H-Cys-NH_2, or H-Cys-NHBu in the presence of Hünig's base (Table 2). Protected peptides Boc-AW(Boc)AC-OH (**14a**), Boc-AW(Boc)AC-OEt (**14b**), and Boc-AW(Boc)AC-NH_2 (**14c**) were formed with complete conversion (entries 1–3), while Boc-AW(Boc)AC-NHBu (**14d**) was formed with 38% conversion. The elongation was more efficient in solution,[21] and unprotected peptides **14e–h** were accessed with quantitative conversion (entries 5–8). We assumed that the mildly basic reaction conditions would result in rapid S to N acyl transfer upon cysteine thiol addition either on resin or in solution. *In situ* generation of the backbone amide was confirmed by independent synthesis of H-AWAC-OH followed by co-injection with **14e**.[26] The extent of product peptide epimerization was evaluated for the ethyl ester (**14f**), which is the most epimerization-prone derivative. Comparison to a co-injection of H-AWA(D-Cys)-OEt confirmed that the product peptides are not epimerized to any observable extent under the reaction conditions (Fig. SI-057†).

We next executed the cysteine elongation of a series of peptides varying in length and hydrophobicity both on the resin and in solution (Table 3). The unprotected peptide H-AKTWA-*MeNbz-Gly* (**15b**) was functionalized in solution to afford H-AKTWAC-OH (**15c**) with complete conversion in 30 min with no observed side-chain macrocyclization.[23] To enable comparison with the crypto-thioester approach,[27] C-terminal proline-containing peptide **16a** was cleaved from resin using H-Cys-OH to afford protected H-AKTWPC-OH (**16c**) with 10% conversion over 4 h.[38] Repeating this reaction in solution on unprotected peptide (**16b**) led to complete conversion after 1 h at ambient temperature. Elongation of Boc-LYRAGLRAY (**17a**) proceeded with resin cleavage and complete conversion in the presence of DMF and NCL buffer. Hydrophobic peptide **18**, a fragment of amyloid β (Aβ(36–42)),[39] was elongated both on resin (entry 5) and in solution (entry 6). On-resin elongation proved challenging for this substrate (10% conversion), while complete conversion was observed in solution. Overall, for shorter or non-hydrophobic peptides, this chemistry could be executed on resin and in the absence of added thiol. In challenging cases, resin cleavage and then in solution native chemical ligation[40] afforded the target peptides.

To confirm the viability of this approach in the context of a complex natural product, we executed the total synthesis of the insect pheromone α-factor (**21**, Scheme 3), which requires

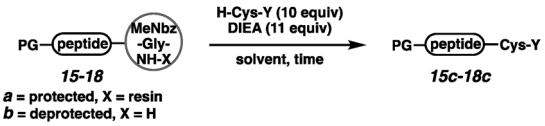
Table 2 C-terminal elongation by nucleophilic attack of cysteine on MeNbz



Entry[a]	Substrate	Y	Solvent	Time (h)	Conversion[b] (%)
1	**12**	OH (**14a**)	(5 : 1) DMF : H_2O	4	>99
2	**12**	OEt (**14b**)	DMF	4	>99
3	**12**	NH_2 (**14c**)	DMF	4	>99
4[c]	**12**	NHBu (**14d**)	DMF	4	>38
5	**13**	OH (**14e**)	(2 : 1) MeCN : H_2O	0.5	>99
6	**13**	OEt (**14f**)	MeCN	0.5	>99
7	**13**	NH_2 (**14g**)	MeCN	0.5	>99
8[c,d]	**13**	NHBu (**14h**)	(25 : 1) MeCN : H_2O	0.5	>99

[a] Unless noted, on resin reactions were performed on 20 mg resin in 500 μL solvent, solution-phase reactions were performed on 20 mg crude peptide in 200 μL solvent, 100 μL of H_2O was added as indicated, rt = 24 ± 1 °C. [b] Conversion based on integration of relevant peaks in HPLC/MS data. [c] Cystine formation was observed. [d] Performed on 3.8 mg of **13** using 520 μL MeCN:H_2O.

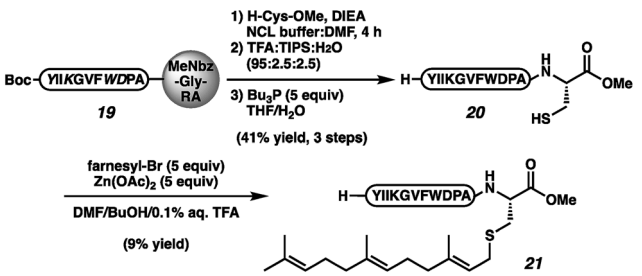
Table 3 Cysteine elongation to generate C-terminal acids, carboxamides, and esters



Entry[a]	PG	Substrate	Peptide	Y	Solvent	Time (h)	Conversion[b] (%)
1[c]	H	**15b**	AKTWA (**15**)	OH	(5 : 1) MeCN : H_2O	0.5	>99
2	Boc	**16a**	AKTWAP (**16**)	OH	(1 : 1) DMF : NCL buffer	4	10
3[d]	H	**16b**	AKTWAP (**16**)	OH	NCL buffer	1	>99
4	Boc	**17a**	LYRAGLRAY (**17**)	Nh_2	(1 : 1) DMF : NCL buffer	4	>99
5	Boc	**18a**	VGGVVI (**18**)	OMe	(1 : 1) DMF : NCL buffer	4	10
6[e]	H	**18b**	VGGVVI (**18**)	OMe	NCL buffer	0.5	>99

[a] Unless noted, on-resin reaction were performed on 20 mg peptide/resin in 500 μL solvent, rt = 24 ± 1 °C, NCL buffer at pH 7.2. [b] Based on integration of relevant peaks in HPLC/MS data. [c] Used 600 μL solvent. [d] Performed on 10 mg of **16b** using 250 μL solvent. [e] Performed on 67 mg of **18b** using 250 μL solvent.

both the C-terminal ester and the prenyl moiety for bioactivity.[1e–h,41] Protected des-farnesyl α-factor was generated by displacement of peptide **19** with cysteine methyl ester. The elongation was conducted in NCL buffer in an effort to reduce cystine-functionalized by-products. However, even under these reducing conditions, cystine-appended α-factor was still observed. Side-chain deprotection, cystine reduction, and HPLC purification afforded peptide **20** in 41% yield over 3 steps. Alkylation per the reported conditions afforded α-factor in 9% yield (**21**).[41]



Scheme 3 Synthesis of α-factor by cysteine elongation.

In summary, we have developed two broadly applicable strategies for the epimerization-free preparation of C-terminal cysteine peptides. The first approach exploits the exceptionally mild activating nature of the *N*-acyl urea group for the direct diversification of the C terminus. Additionally, an alternative strategy wherein cysteine derivatives serve as the nucleophile in a resin-cleaving elongation reaction was also effective. For both strategies, the target peptides are prepared without observation of either diketopiperazine or piperidinylalanine side products. The utility of these methods was demonstrated in the preparation of the disulfide-linked conotoxin α-ImI, bearing a C-terminal cysteine carboxamide and the insect pheromone

α-factor, which possesses a C-terminal cysteine methyl ester. Notably, no previous report has demonstrated successful functionalization of C-terminal cysteine peptides to access carboxylic acids, carboxamides, and other C-terminal derivatives without detectable epimerization of the α-stereocenter.

Conflicts of Interest

The authors declare no conflicts of interest.

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