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Peptide-directed solid-phase reductive amination

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We report an integrated solid-phase reductive amination/solid-phase fragment condensation (SPFC) strategy for the replacement of amide bonds in peptide sequences with the aminomethylene Ψ[CH_2–NH] amide isostere. *N*-Fmoc-protected C-terminal peptide α-amino aldehydes were prepared and condensed with the N-terminus of peptide sequences assembled on 2-chlorotrityl chloride (CLTR) resin, followed by efficient imine reduction under mild conditions. Epimerization at the reacting α-amino aldehyde was below 10%, typically in the range of 1–7%. Cleavage from the resin under mild acidic conditions afforded peptides in which native amide bonds were selectively replaced by Ψ[CH_2–NH] linkages. In addition, this methodology enables the preparation of suitably protected Ψ[CH_2–NH]-containing peptide fragments that are compatible with further solid-phase, fragment-based, or convergent peptide synthesis and modification strategies. The methodology constitutes a general and versatile tool for selective peptide backbone modification with relevance to the development of peptides and peptidomimetics with improved properties and applications in drug design.

Introduction

Peptides are attractive therapeutic agents but are often limited by intrinsic limitations, including poor metabolic stability, low membrane permeability, and suboptimal pharmacokinetic profiles. To address these challenges, a variety of chemical strategies have been developed. These include side-chain modification, replacement of L-amino acids with D-amino acids or other unnatural amino acids, cyclization, amide *N*-alkylation, *C*α-alkylation, peptoid incorporation, lipidation, and other structural modifications.[1,2] In line with these approaches, we have previously developed solid-phase methodologies for peptoid formation, and [CH_2–S] amide isostere incorporation, while peptide lipidation strategies have also been explored in our group.[3–5]

Solid-phase fragment condensation (SPFC) is a well-established approach for the synthesis of challenging peptide sequences.[6–8] In principle, SPFC relies on the stepwise condensation of peptide fragments onto a resin-bound peptide chain. Conventional SPFC protocols typically employ 2-chlorotrityl chloride (CLTR) resin[9] in combination with the Fmoc/^{t}Bu strategy, using 1-hydroxybenzotriazole (HOBt)/*N*,*N*′-diisopropylcarbodiimide (DIC) or other efficient coupling reagents to promote fragment condensation.[10,11]

In this study, we describe SPFC-based strategies to replace peptide amide bonds with the methylene amino Ψ[CH_2–NH] amide isostere,[12] using C-terminal peptide α-amino aldehydes. This backbone modification can enhance the pharmacokinetic and pharmacodynamic (PK/PD) properties of peptides by improving hydrolytic and proteolytic stability, increasing bioavailability, and, in some cases, enhancing potency or receptor selectivity. Ψ[CH_2–NH] substitutions reduce enzymatic backbone cleavage and may alter peptide conformation. These effects can confer longer *in vivo* half-lives, higher effective concentrations at target sites, and improved functional activity relative to the parent sequences.[13–18]

Although several solid- and solution-phase methods for the replacement of peptide amide bonds with the Ψ[CH_2–NH] isostere have been reported,[14–16,19–24] a general SPFC-based strategy that utilizes C-terminal peptide α-amino aldehydes as alkylating agents to enable amide-to-Ψ[CH_2–NH] replacement directly within peptide sequences has not yet been described. While we previously reported homoserinyl γ-aldehyde-containing peptides in solid-phase reductive amination,[25] the present study investigates peptide-derived C-terminal α-amino aldehydes for direct amide-to-Ψ[CH_2–NH] replacement within peptide backbones. In contrast to the earlier site-specific Hse residue modification strategy, this work employs C-terminal peptide α-amino aldehyde fragments, thereby enabling modular and convergent replacement of native amide bonds. In this case, important methodological adaptations were

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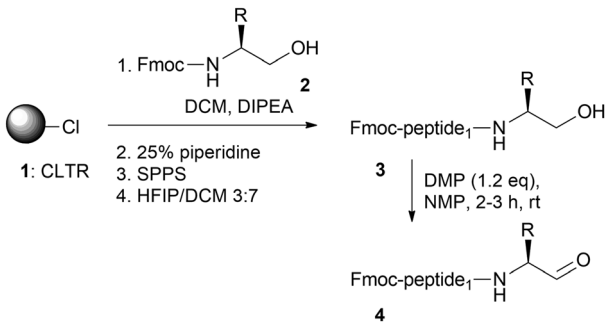
required to achieve faster reaction kinetics and improved control of epimerization at the reacting α-amino aldehyde.

To implement this strategy, Fmoc-protected peptide α-amino alcohols are first synthesized on CLTR resin using the Fmoc/^{t}Bu strategy and then oxidized in solution to the corresponding Fmoc-protected peptide α-amino aldehydes. These aldehydes are subsequently condensed with the N-terminus of resin-bound peptide sequences *via* a simple and efficient solid-phase reductive amination protocol. This approach enables the incorporation of Ψ[CH_2–NH] amide isosteres within peptide backbones with controlled levels of epimerization.

Overall, the reported protocol provides a versatile and reliable platform for the preparation of Ψ[CH_2–NH]-containing peptides and side-chain-protected peptide fragments. The resulting peptides can serve either as the targeted Ψ[CH_2–NH]-modified molecules or be incorporated into larger, synthetically challenging sequences, supporting fragment-based and convergent peptide synthesis strategies. Beyond enabling PK/PD-oriented backbone modification, the methodology represents a generally useful approach for peptide modification with potential relevance to peptidomimetic and peptide-based drug design. It is also readily adaptable to solution-phase peptide chemistry, underscoring its broad utility.

Results and discussion

For the synthesis of peptides where the amide bond is replaced by the Ψ[CH_2–NH] isostere, we rationalized the use of SPFC methodology using of the acid-labile CLTR resin **1** and Fmoc/^{t}Bu strategy.[8] Initially, *N*-Fmoc-protected amino alcohols **2** were synthesized from the corresponding *N*-Fmoc α-amino acid precursors *via* activation with *N*,*N*′-dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS), followed by reduction with sodium borohydride ($NaBH_4$), as previously reported to preserve optical purity.[26–28] The Fmoc-protected amino alcohols **2** were then attached to CLTR resin through their hydroxyl group, allowing the solid-phase synthesis of Fmoc-protected peptide alcohols **3**, by methods already described in literature (Scheme 1).[29] Briefly, **2** was loaded onto the resin in the presence of *N*,*N*-diisopropylethylamine (DIPEA), followed by Fmoc deprotection using standard 25% piperidine treatment, after which conventional solid-phase peptide synthesis was performed. Cleavage from the resin with 30% hexafluoroisopropanol (HFIP) in DCM afforded the Fmoc/side-chain-protected peptide alcohols **3a–3g**.



Scheme 1 General scheme for the synthesis of Fmoc-protected peptide aldehydes though DMP oxidation of solid-phase synthesized Fmoc-protected peptide alcohols.

Previous approaches to peptide aldehyde synthesis rely on resin-bound Weinreb amides, which are reduced with lithium aluminum hydride ($LiAlH_4$) to generate C-terminal peptide aldehydes.[30] Alternatively, Fmoc-protected amino aldehydes have been immobilized on threonyl resins as oxazolidines, followed by Boc protection and solid-phase peptide synthesis.[31] However, these methods typically require harsh conditions and are not compatible with standard Fmoc/^{t}Bu solid-phase synthesis.

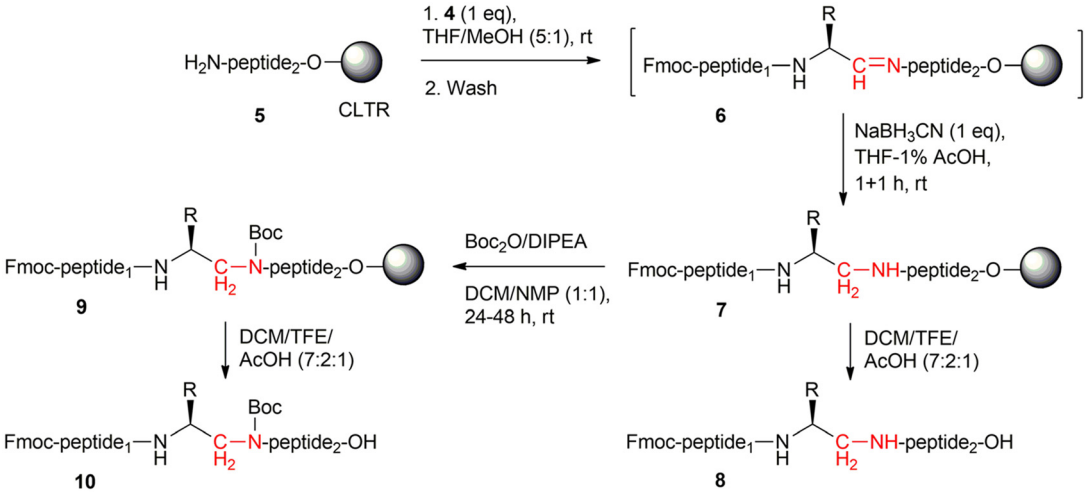
In our case, the Fmoc-protected peptide alcohols **3a–3g** were oxidized to the corresponding Fmoc-protected peptide aldehydes **4a–4g** (Scheme 1) using the Dess–Martin periodinane (DMP), a mild oxidant.[32] Under optimized conditions, 1.2 equiv. of DMP were sufficient to achieve full conversion to the desired **4a–4g** (Table 1) within 2–3 h, at rt. In the case of incomplete reaction, the addition of an extra 20% equivalent of DMP generally afforded full conversion within the next hour.

In the next step, the Fmoc-protected peptide aldehydes **4** reacted with the N-terminus of the pre-formed peptide resin (CLTR) **5** under reductive amination conditions (Scheme 2). For the on-resin reductive amination a two-step approach was developed. In the first step, **4** (1 equiv.) reacted with **5** in THF/MeOH (5 : 1) at rt, a reaction that consistently reached completion within 2 × 15 min, followed by washing the resin with THF to remove unreacted **4**. The resulting resin-bound imine **6** was subsequently reduced using sodium cyanoborohydride ($NaBH_3CN$), a mild reducing agent commonly used in reductive amination processes.[14,15,19,20,33,34] In the optimized protocol, treatment of resin **6** with $NaBH_3CN$ in THF containing 1% AcOH for 1 + 1 h at rt affords complete reduction. In the case of incomplete reaction of peptide aldehyde and/or reduction (identified during the HPLC analysis of the final resin, after acidic treatment and cleavage), a second round of 1 × 15 min imine formation and reduction was sufficient to allow full conversion. The resin-bound peptide fragments **7** were then treated with DCM/2,2,2-trifluoroethanol (TFE)/AcOH (7 : 2 : 1)

Table 1 Fmoc-protected peptide aldehydes **4a–4g** synthesized[a]

A/A	Fmoc-peptide aldehydes
4a	Fmoc-Leu-Val-Glu(O^{t}Bu)-Ser(^{t}Bu)-Gly-Gly-Gly-AL
4b	Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-Aib-AL
4c	Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-Leu-AL
4d	Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-Ile-AL
4e	Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-Phe-AL
4f	Fmoc-Cys(Trt)-Ala-Lys(Boc)-Val-AL
4g	Fmoc-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-Thr(^{t}Bu)-AL

[a] NMR and HPLC of the crude **4a–4g** are presented in Fig. S1–S14.



Scheme 2 On-resin reductive amination between Fmoc-protected peptide aldehydes (as the alkylating agents) and the N-terminus of resin-bound assembled peptides (Method A).

to yield the protected fragments **8**, in which the peptide bond is replaced by the [CH_2–NH] isostere.

By reacting 7 with di-*tert*-butyl dicarbonate (Boc_2O) (10 equiv.)/DIPEA (10 equiv.) in DCM/NMP (1 : 1) for 24–48 h at rt, the Boc group was successfully introduced to afford **9**, which, in the final step, was treated with DCM/TFE/AcOH (7 : 2 : 1) to afford the Ψ[CH_2–N(Boc)] protected peptide fragment **10**.

Using this protocol, we were able to obtain fragments **8a–8g** by reacting **4a–4g** with resin-bound model tetrapeptides possessing a free N-terminus (Table 2) to form the corresponding resin-bound imines **6a–6g**, which were directly subjected to reduction using $NaBH_3CN$ (1 equiv.) in THF–1% AcOH. In most cases, the reduction was completed within 1 + 1 h at rt. An exception was **8b**, where the C-terminal Aib-AL **4b** required two consecutive 24 h reducing periods. The low reactivity was also confirmed when the Fmoc-Aib-AL monomer was used in place of **4b**, supporting that the steric bulk of Aib is responsible for the slower reduction.

An important consideration in this approach is the potential for epimerization at the insertion site (the Cα of the C-terminal amino aldehyde). Thus, in addition to the C-terminal peptide aldehydes **4a** (C-terminal Gly-AL; sterically unhindered, optically inactive) and **4b** (C-terminal Aib-AL; sterically hindered, optically inactive), we evaluated the peptide aldehydes **4c–4g**, which incorporate sterically hindered and optically active C-terminal amino aldehydes, for their susceptibility to epimerization.

It should be noted that preliminary experiments, in which Fmoc-L-Leu-L-Leu-AL and Fmoc-L-Leu-L-Val-AL (as well as the corresponding Fmoc-L-Leu-D-Leu-AL and Fmoc-L-Leu-D-Val-AL analogues) were reacted with the H-Leu-Ala-Phe-Gly-O-CLTR peptidyl resin, afforded each set of diastereomers of H-Leu-D/L-Leu-[CH_2–NH]-Leu-Ala-Phe-Gly-OH and H-Leu-D/L-Val-[CH_2–NH]-Leu-Ala-Phe-Gly-OH, which were separated by HPLC analysis. This allowed the identification of optimized conditions for this two-step approach. Under these conditions: 15 min imine formation at rt in THF/MeOH (5 : 1), followed by washing of the unreacted aldehyde and reduction with $NaBH_3CN$ (1 equiv.) in THF and THF–1% AcOH for 1 + 1 h at rt, the level of epimerization ranged between 2–10%. The presence of the acid catalyst (1% AcOH) ensured complete reduction within 1 h. In cases where the reduction was incomplete, a second reduction step under the same conditions resulted in full conversion.

For the reaction between peptide aldehydes **4c–4g** and the model tetrapeptide resins (Table 2), the resulting fragments **8c–8g** were characterized in terms of purity (including confirmation of complete N-terminal condensation and full reduction) and evaluated for epimerization of the incorporated C-terminal amino aldehyde residue. Epimerization (% Epimer) was assessed by synthesizing the corresponding C-terminal D-analogues of **4c–4g** and the corresponding D-containing **8c–8g**, which allowed chromatographic separation of the diastereomers by HPLC. The results are summarized in Table 2. Overall, most C-terminal residues displayed relatively low levels of epimerization. The lowest was observed for **8g** (Thr(^{t}Bu)-[CH_2–NH]-Leu), <1.0%, whereas slightly higher epimerization was observed for **8c.1** (Leu-[CH_2–NH]-Asp(O^tBu)), **8c.2** (Leu-[CH_2–NH]-Ser(^{t}Bu)) and **8e** (Phe-[CH_2–NH]-Leu), at 2.0–2.2%. Moderate epimerization was observed for **8d** (Ile-[CH_2–NH]-Ser(^{t}Bu)) and **8f** (Val-[CH_2–NH]-Ser(^{t}Bu)), at 3.3–4.3% and 6.0–7.0%, respectively. Repetition of the experiments after six months, using the Fmoc-peptide aldehydes **4a–4g** stored at 4–5 °C, gave epimerization values within ±10% of the original measurements. These results indicate that the peptide aldehydes remain configurationally stable to a degree acceptable for synthetic use over the examined storage period.

To investigate how the rate of reduction influences the extent of epimerization, we performed additional experiments. Resin-bound imine intermediates **6c.2–6g** were first formed and then suspended for 24 h at rt in either: (a) THF and (b) THF–1% AcOH, followed by reduction under the standard conditions ($NaBH_3CN$ in THF–1% AcOH for 1 h at rt). The result-

Table 2 Peptide fragments **8a–8g** synthesized by SPFC method A (Scheme 2),[a] and the corresponding epimerization levels (% Epimer)[b]

Method of preparation	Product obtained	% Epimer[b]
Fmoc-Leu-Val-Glu(O^{t}Bu)-Ser(^{t}Bu)-Gly-Gly-**Gly-AL 4a** + H-Leu-Ala-Phe-Gly-O-CLTR	Fmoc-Leu-Val-Glu(O^{t}Bu)-Ser(^{t}Bu)-Gly-Gly-**Gly-Ψ[CH$_2$–NH]**–Leu-Ala-Phe-Gly-OH **8a**	—
Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Aib-AL 4b** + H-Leu-Ala-Phe-Gly-O-CLTR	Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Aib-Ψ[CH$_2$–NH]**–Leu-Ala-Phe-Gly-OH **8b**	—
Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Leu-AL 4c** + H-Asp(O^{t}Bu)-Tyr(^{t}Bu)-Trp(Boc)-Gly-O-CLTR	Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Leu-Ψ[CH$_2$–NH]**–Asp(O^{t}Bu)-Tyr(^{t}Bu)-Trp(Boc)-Gly-OH **8c.1**	2.1%, 2.2%
Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Leu-AL 4c** + H-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-O-CLTR	Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Leu-Ψ[CH$_2$–NH]**–Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-OH **8c.2**	2.0%, 2.1%
Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Ile-AL 4d** + H-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-O-CLTR	Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Ile-Ψ[CH$_2$–NH]**–Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-OH **8d**	3.3%, 3.6%, 4.3%
Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Phe-AL 4e** + H-Leu-Ala-Phe-Gly-O-CLTR	Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Phe-Ψ[CH$_2$–NH]**–Leu-Ala-Phe-Gly-OH **8e**	2.1%, 2.2%
Fmoc-Cys(Trt)-Ala-Lys(Boc)-**Val-AL 4f** + H-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-O-CLTR	Fmoc-Cys(Trt)-Ala-Lys(Boc)-**Val-Ψ[CH$_2$–NH]**–Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-OH **8f**	6.0%, 6.2%, 7.0%
Fmoc-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-**Thr(^{t}Bu)-AL 4g** + H-Leu-Ala-Phe-Gly-O-CLTR	Fmoc-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-**Thr(^{t}Bu)-Ψ[CH$_2$–NH]**–Leu-Ala-Phe-Gly-OH **8g**	0.7%, 0.8%

[a] 15 min imine formation in THF/MeOH (5 : 1) at rt; 1 + 1 h reduction with $NaBH_3CN$ in THF–1% AcOH at rt. [b] HPLC of the crude **8a–8g** are presented in Fig. S15–S22.

ing epimerization percentages for fragments **8c.2–8g** are summarized in Table 3.

As shown, epimerization in fragments **8c.2–8g** increases significantly when the peptide fragments remain in their imine form for an extended period (24 h), even in the absence of an acid catalyst. This suggests intramolecular deprotonation of the α-hydrogen of the C-terminal amino aldehyde involved in imine bond formation with the N-terminus of the following peptide fragment, leading to enamine formation followed by reprotonation (Scheme 3a). In the presence of 1% AcOH, epimerization is further enhanced, indicating that acid catalysis accelerates the process *via* proton-assisted imine–enamine tautomerization (Scheme 3b).

On the basis of these findings, we further introduce a practical method in which deliberate extension of the time the peptide remains in its imine form prior to reduction promotes epimerization, producing diastereomeric mixtures that can be efficiently separated by HPLC, thus avoiding the need to synthesize the corresponding C-terminal D-amino aldehyde analogues and their final peptides.

Considering the findings so far, the optimized protocol reflects this balance: while acid catalysis increases epimerization, it is also essential for complete and efficient reduction, as its absence led to incomplete reduction in nearly all cases examined. Overall, these results demonstrate that the extent of epimerization strongly depends on the rate at which the initially formed imine is reduced.

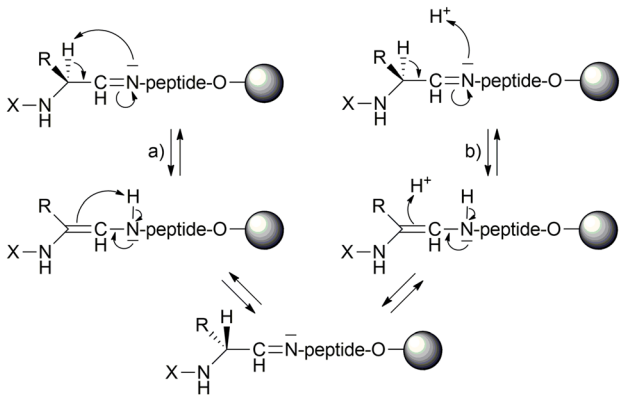
To broaden the available synthetic options, the Fmoc-α-amino aldehyde monomer insertion method outlined in Scheme 4 was also investigated.

In this approach, the Fmoc-amino aldehyde monomer **11** is condensed with the N-terminus of the resin-bound peptide **5**

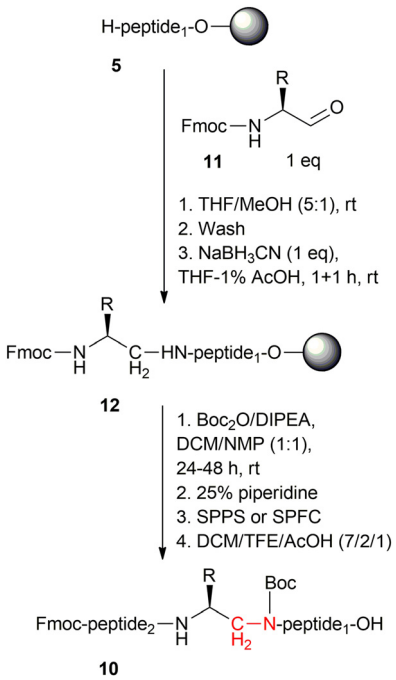
Table 3 Percentage epimerization (% Epimer) of peptide fragments **8c.2–8g** obtained after suspending the corresponding resin-bound imines **6c.2–6g** in THF or THF–1% AcOH for 24 h at rt[a]

Resin-bound peptidyl imine sequence	Ψ[CH$_2$–NH]-containing peptide sequence	% Epimer in THF after 24 h	% Epimer in THF–1% AcOH
Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Leu-[CH=N]**-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-O-CLTR **6c.2**	Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Leu-Ψ[CH$_2$–NH]**-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-OH **8c.2**	15.0%	47.5%
Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Ile-[CH=N]**-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-O-CLTR **6d**	Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Ile-Ψ[CH$_2$–NH]**-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-OH **8d**	7.4%[b]	46.1%
Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Phe-[CH=N]**-Leu-Ala-Phe-Gly-O-CLTR **6e**	Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Phe-Ψ[CH$_2$–NH]**-Leu-Ala-Phe-Gly-OH **8e**	24.8%	
Fmoc-Cys(Trt)-Ala-Lys(Boc)-**Val-[CH=N]**-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-O-CLTR **6f**	Fmoc-Cys(Trt)-Ala-Lys(Boc)-**Val-Ψ[CH$_2$–NH]**-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-OH **8f**	16.2%	49.1%
Fmoc-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-**Thr(^{t}Bu)-[CH=N]**-Leu-Ala-Phe-Gly-O-CLTR **6g**	Fmoc-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-**Thr(^{t}Bu)-Ψ[CH$_2$–NH]**-Leu-Ala-Phe-Gly-OH **8g**	4.1%	

[a] HPLC of the crude epimerized **8c.2–8g** are presented in Fig. S23–S27. All experiments were performed at least three times and this is the maximum measured values. [b] An additional 24 h resin suspension in THF (total 48 h) increased epimerization from 7.4% to 13.8%.



Scheme 3 Plausible epimerization pathway of the C-terminal α-amino aldehyde-derived residue involved in the imine bond formation with the N-terminus of the following peptide fragment *via* imine–enamine tautomerization under non-acidic (a) and acid-catalyzed (b) conditions; X = Fmoc; Fmoc-protected peptide segment.



Scheme 4 Fmoc-amino aldehyde insertion approach Method B evaluated by the synthesis of Fmoc-Cys(Trt)-Ala-Lys(Boc)-Val-Ψ[CH_2–N(Boc)]-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-OH (**10f**).

to form the resin-bound peptidyl imine, which is then reduced to intermediate **12**, bearing the Ψ[CH_2–NH] linkage. This intermediate is subsequently treated with Boc_2O/DIPEA in DCM/DMF (1 : 1) (2 × 24 h, rt), subjected to Fmoc deprotection with 25% piperidine, and further extended by SPPS/SPFC. Cleavage from the resin with DCM/TFE/AcOH (7 : 2 : 1) yields peptide **10** incorporating the Ψ[CH_2–N(Boc)] linkage.

By using this strategy, we initially performed preliminary experiments using Fmoc-L-Ala-AL, Fmoc-L-Leu-AL, and Fmoc-L-Val-AL (as Fmoc-amino aldehydes **11**) in reactions with the H-Leu-Ala-Phe-Gly-O-CLTR resin. The corresponding D-analogues were also employed to enable diastereomer formation and separation by HPLC. Under the optimized conditions, *i.e.* 15 min imine formation in THF/MeOH (5 : 1) at rt followed by resin washing to remove unreacted Fmoc-amino aldehyde and subsequent reduction with $NaBH_3CN$ (1 equiv.) in THF–1% AcOH for 1 h at rt, the epimerization levels were as follows: Ala: 1–3%, Leu: 2–3%, Val: 3–7%. In a comparative study, we tested **4f** (in the synthesis of **10f**), previously identified as the most prone to epimerization *via* Method A peptide sequence. In this alternative approach (Method B, Scheme 4), Fmoc-L-Val-AL was condensed with the resin-bound peptide H-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-O-CLTR, followed by Boc insertion, Fmoc removal, and step-by-step elongation to afford **10f**. The epimerization level measured for **10f** *via* this route (Method B) was 3.0%, lower than the 6.0–7.0% observed for **8f** prepared *via* Method A, or the 5.7% measured for the corresponding **10f** prepared by Method A followed by Boc-insertion. The HPLC chromatograms of the crude **10f** obtained by both Method A and Method B are presented in Fig. 1. In addition, we present the HPLC for **10f** obtained by suspension of the corresponding resin-bound imine in THF–1% AcOH for 24 h at rt, which resulted in a complete epimerization. ESI-MS of the eluted fragment corresponding to **10f** is also presented. The results demonstrate that this second approach provides a complementary synthetic option for the preparation of Ψ[CH_2–NH]-containing peptides and can be particularly advantageous for peptide aldehyde fragments that are especially prone to epimerization.

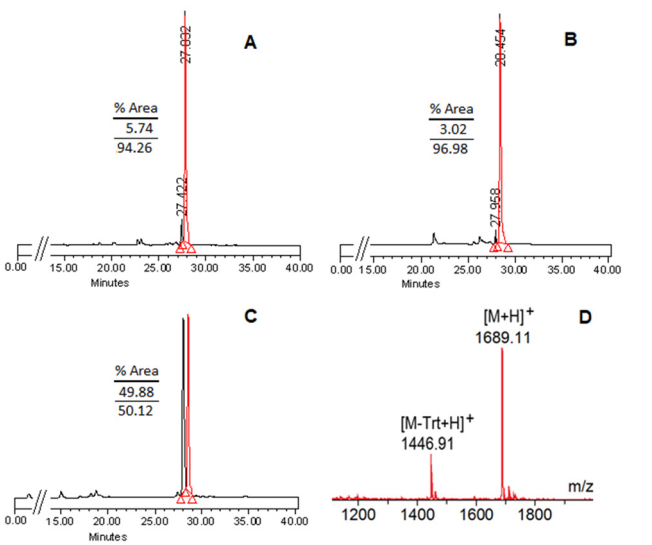


Fig. 1 HPLC chromatograms of crude Fmoc-Cys(Trt)-Ala-Lys(Boc)-Val-Ψ[CH_2–N(Boc)]-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-OH (**10f**) prepared by Method A and Method B: (A) **10f** prepared by Method A (**8f** + **Boc**) (Scheme 2; full trace in Fig. S21); (B) **10f** prepared by Method B (Scheme 4; full trace in Fig. S28); (C) the epimerized **10f** obtained by suspension of the corresponding resin-bound imine in THF–1% AcOH for 24 h at rt (Scheme 3; full trace in Fig. S28); (D) ESI-MS of **10f**.

Table 4 Peptide fragments **13a–13e** synthesized *via* Boc-protection and Direct approaches illustrated in Scheme 5[a]

Reaction performed	Final peptide sequence after cleavage from the resin	Method
Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Leu-AL** + H-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-**Thr(^{t}Bu)-Ψ[CH_2–N(Boc)]**-Leu-Ala-Phe-Gly-O-CLTR	Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Leu-Ψ[CH_2–NH]**-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-**Thr(^{t}Bu)-Ψ[CH_2–N(Boc)]**-Leu-Ala-Phe-Gly-OH **13a**	Boc-protection approach
Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Aib-AL** + H-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Phe-Ψ[CH_2–N(Boc)]**-Leu-Ala-Phe-Gly-O-CLTR	Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Aib-Ψ[CH_2–NH]**-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Phe-Ψ[CH_2–N(Boc)]**-Leu-Ala-Phe-Gly-OH **13b**	Boc-protection approach
Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Leu-Ψ[CH_2–NH]**-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-**Thr(^{t}Bu)-Ψ[CH_2–N(Boc)]**-Leu-Ala-Phe-Gly-O-CLTR + **Boc_2O**/DIPEA	Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Leu-Ψ[CH_2–N(Boc)]**-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-**Thr(^{t}Bu)-Ψ[CH_2–N(Boc)]**-Leu-Ala-Phe-Gly-OH **13c**	Boc-protection approach
Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Aib-AL** + H-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Phe-Ψ[CH_2–NH]**-Leu-Ala-Phe-Gly-O-CLTR	Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Aib-Ψ[CH_2–NH]**-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Phe-Ψ[CH_2–NH]**-Leu-Ala-Phe-Gly-OH **13d**	Direct approach
Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Phe-AL** + H-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Leu-Ψ[CH_2–NH]**-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Sert(^{t}Bu)-O-CLTR	Fmoc-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Phe-Ψ[CH_2–NH]**-Ala-Ser(^{t}Bu)-Ser(^{t}Bu)-**Leu-Ψ[CH_2–NH]**-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Sert(^{t}Bu)-OH **13e**	Direct approach

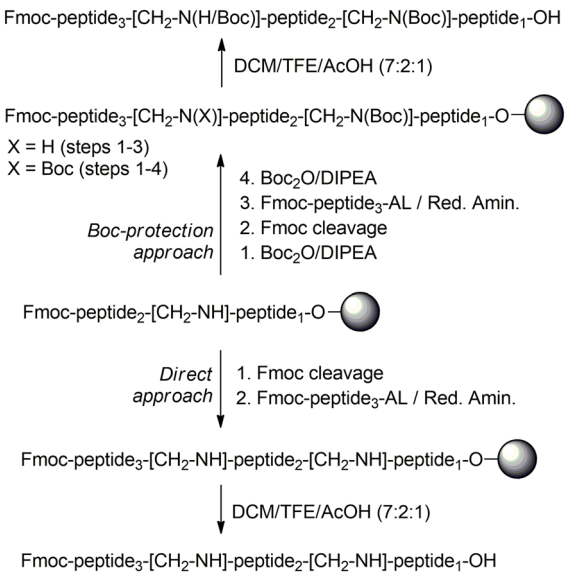
[a] HPLC of the crude products (and intermediates) are presented in Fig. S29–S35.

Besides $NaBH_3CN$, sodium triacetoxyborohydride $NaBH(OAc)_3$ is another widely used mild reducing agent.[35–38] Although we have effectively used this reagent in solid-phase reductive amination with homoserinyl γ-aldehyde-containing peptides,[25] its use in the present study proved problematic as the low reduction rates led to high levels of epimerization (up to 40–50% over 48 h) and, in addition to the expected mono-alkylated Ψ[CH_2–NH] products, generated a high percentage of dialkylation byproducts,[34] for both Method A and Method B. Consequently, $NaBH(OAc)_3$ was not considered further in this work. On the other hand, $NaBH_3CN$ did not exhibit this limitation.

Finally, to highlight the potential of the method for synthesizing longer peptide fragments, we prepared the more complex peptides **13a–13e** (Table 4) using the general approaches outlined in Scheme 5.

In the first approach (Boc-protection approach), the initial Ψ[CH_2–NH] bond was incorporated, followed by on-resin Boc-protection to the generate the corresponding Ψ[CH_2–N(Boc)]. Fmoc-removal of the N-terminal residue enabled further on-resin fragment condensation with the subsequent C-terminal α-amino aldehyde fragment.

Using this approach, the A + B precursor of peptide **13a** (Fig. 2A, S29) and **13a** (Fig. 2B, S30) and the A + B precursor of



Scheme 5 General approaches illustrating the application of SPFC to the synthesis of larger peptide fragments, by the initial protection of the Ψ[CH_2–NH] bond with Boc-group (Boc-protection approach), or the direct insertion of a second Ψ[CH_2–NH] bond (Direct approach).

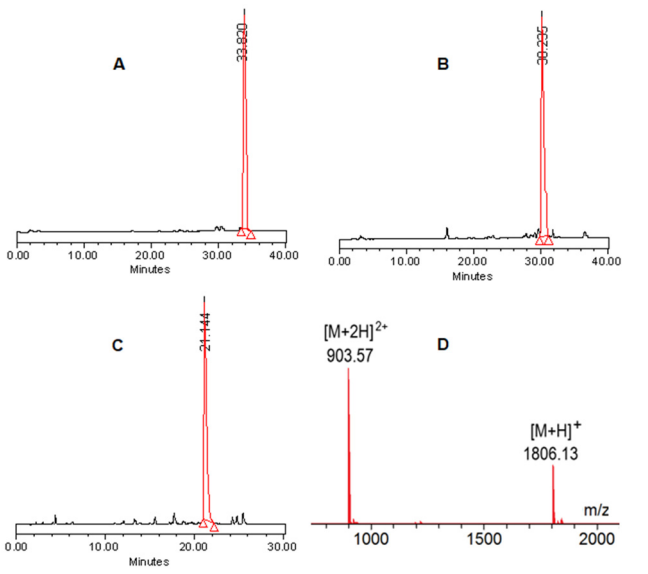


Fig. 2 Representative HPLC chromatograms of crude fragments synthesized *via* Boc-protection approach and Direct approach: (A) Fmoc-Ser(^{t}Bu)-Tyr(^{t}Bu)-Leu-Ser(^{t}Bu)-Thr(^{t}Bu)-Ψ[CH_2–N(Boc)]-Leu-Ala-Phe-Gly-OH (A + B fragment in the synthesis of **13a**) prepared *via* Boc-protection approach (Fig. S29); (B) **13a** (A + B + C fragment) prepared *via* Boc-protection approach (Fig. S30); (C) **13e** prepared by Direct approach (Fig. S35); (D) ESI-MS of **13e**; HPLC chromatograms for all **13a–13e** and ESI-MS spectra are provided in the SI.

peptide **13b** (Fig. S31) and **13b** (Fig. S32) were successfully obtained after treatment of the resin with DCM/TFE/AcOH (7 : 2 : 1). Boc-protection of the resin-bound **13a** and cleavage afforded **13c** (Fig. S33).

In the second approach (Direct approach), fragment condensation was carried out without Boc-protection of the Ψ[CH_2–NH] bond, as demonstrated for **13d** (Fig. S34) and **13e** (Fig. 2C, D and S35). The high purity of the crude products (Fig. S34 and S35) highlights the efficiency and selectivity of rapid imine formation between the C-terminal α-amino aldehyde and the resin-bound N-terminal group, preventing undesired alkylation of the previously installed Ψ[CH_2–NH] linkage.

Conclusions

We report a strategy combining solid-phase reductive amination with solid-phase fragment condensation (SPFC) for the site-specific replacement of native amide bonds in peptide sequences with the aminomethylene Ψ[CH_2–NH] amide isostere. Preformed C-terminal peptide α-amino aldehydes were condensed with resin-bound N-terminal peptides, followed by efficient imine reduction with $NaBH_3CN$, enabling reduction under mild conditions with controlled epimerization, which remained below 10% (typically in the range of 1–7% for optically active substrates). The method is effective for C-terminal peptide α-amino aldehydes encompassing both sterically unhindered and hindered residues, as well as optically inactive and optically active amino acids. For the most epimerization-prone sequences, complementary routes based on solid-phase reductive amination using Fmoc-amino aldehyde monomers followed by classical peptide elongation proved advantageous. Overall, the proposed strategies provide versatile platforms for constructing peptides in which native amide bonds are replaced by Ψ[CH_2–NH]. In addition, they provide suitably protected fragments amenable to further solid-phase or convergent synthesis. Consequently, these methods are well suited for the preparation of larger or synthetically challenging peptide sequences and are broadly applicable to peptide modification, peptide–drug conjugates, and other functionalized peptide constructs.

Experimental

Materials

2-Chlorotrityl chloride resin (CLTR) 100–200 mesh, 1% divinylbenzene (DVB) (loading capacity 1.0–2.0 mmol g^{-1}) and Fmoc-protected amino acids were kindly provided by CBL Patras S.A. (Industrial area of Patras, Building block 1, GR-25018, Patras, Greece). Plastic reactors for Peptide Synthesis (polypropylene syringes equipped with porous polyethylene frits at the bottom) with a pore size of 25 μm were obtained from Carl Roth Gmbh + Co. KG, Karlsruhe, Germany. Tetrahydrofuran (THF) (ACS reagent, Reag. Ph. Eur., ≥99.9%, containing 250 ppm BHT as inhibitor) was purchased from Honeywell Riedel de Haen (Germany). THF was dried over molecular sieves type 4A (beads, diameter 1.7–2.4 mm) 24 h prior to use (Sigma-Aldrich O.M. Ltd, Athens, Greece). Methanol (MeOH) (HPLC grade, ≥99.8%) was purchased from Fischer Chemicals (Thermo Fisher Scientific, Geel, Belgium). All other chemicals were purchased from Sigma-Aldrich O.M. Ltd (Athens, Greece). All chemicals were used without further purification.

Analytical methods

High Performance Liquid Chromatography (HPLC) analysis was performed on a Waters 2695 multisolvent delivery system (Milford, MA, USA), combined with Waters 996 photodiode array detector. The following columns were used: (A) column: purospher RP-8e, 5 μm, 125–4 mm; (B) YMC-Triart C18, 12 nm, S-5 μm, 250–4.6 mm. ESI-MS spectra were recorded at 30 V on a Bruker Amazon SL ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany) in positive ion mode. Data were processed using Bruker Compass DataAnalysis 4.2 software. ^{1}H NMR spectra (600 MHz) and ^{13}C NMR spectra (150.9 MHz) were recorded on a Bruker DPX 600 MHz spectrometer (Bruker BioSpin GmbH, Germany) equipped with a cryoprobe. Spectra were acquired at 25 °C. Chemical shifts (δ) are reported in ppm and were referenced to the residual solvent signals.

Experimental procedures

General protocols for solid-phase peptide synthesis

Solid-phase peptide synthesis was carried out manually, either in plastic peptide synthesis reactors attached to a Visiprep solid-phase extraction vacuum manifold, or in Eppendorf tubes, where the reaction mixture was transferred to either microfilters or polypropylene syringes for washing.

General protocols for solid-phase peptide synthesis (SPPS) on 2-chlorotrityl chloride (CLTR) resin using the Fmoc/^{t}Bu strategy and HOBt/DIC as the coupling system are well documented in the literature.[39–41] Detailed experimental procedures have also been reported in our recent publications, and[3,4,42] are briefly summarized below.

Attachment of the first amino acid on 2-CLTR resin

CLTR (typical loading 1.0–2.0 mmol g^{-1}; taken as 1.0 mmol g^{-1} for calculations) was suspended in dichloromethane (DCM) (10 mL g^{-1} resin) and allowed to swell for 10 min. *N*,*N*′-Diisopropylethylamine (DIPEA, 4 mmol g^{-1} resin) was added, and the resin was shaken for 1 min. The required Fmoc-amino acid (1.5 mmol g^{-1} resin), dissolved in the minimum volume of DCM, was subsequently added, and the resin was gently agitated for 1.5 h at rt. Next, methanol (MeOH, 1 mL g^{-1} resin) and DIPEA (2 mmol g^{-1} resin) were added to cap unreacted trityl chloride groups, and the resin was agitated for an additional 30 min at rt. The resin was filtered and sequentially washed with DCM (2 × 20 mL g^{-1} resin), DCM/MeOH/DIPEA 85 : 10 : 5 (3 × 20 mL g^{-1} resin for 5, 10, 15 min, respectively),

and *N*-methyl-2-pyrrolidone (NMP) (×5). This was directly subjected to Fmoc removal as described below.

Resin loading was estimated on the dried *N*-deprotected amino acid resin (after removal of the *N*-Fmoc group) by measuring the resin weight gain, using the following formula: *S*(wt) = [wt(g) × 1000]/[wt(add) × wt(t)], where *S*(wt): weight gain substitution (mmol g^{-1}); wt(g): weight gained by resin (g); wt(add): molecular weight added to the resin = MW of amino acid minus MW of leaving group (g mol^{-1}); wt(t): total weight of the resin after loading. Using this method, a loading of 0.9–1.0 mmol g^{-1} resin was obtained.

Peptide assembly on CLTR resin

The resin-bound, *N*-deprotected amino acid or peptide (loading ~1.0 mmol g^{-1}) was washed with NMP (2 × 10 min) to afford the pre-swollen resin. For activation, the Fmoc-amino acid (3 equiv. relative to resin loading) and *N*-hydroxybenzotriazole (HOBt, 4.5 equiv.) were dissolved in NMP (5 mL g^{-1} resin) and the mixture was cooled at 4 °C for 15 min. *N*,*N*′-diisopropylcarbodiimide (DIC, 3.6 equiv.) was then added, and the solution was agitated for an additional 15 min at 4 °C. The activated Fmoc-amino acid solution was then added to the pre-swollen resin which was agitated for 3 h at rt. Completion of the coupling reaction was monitored using the Kaiser test method in a small resin sample. In the case of incomplete coupling, recoupling was carried out using a freshly prepared solution of the activated Fmoc-amino acid. Upon completion of the required peptide sequence, the resin was filtered and washed with NMP (×5), isopropanol (iPrOH, ×3), diethyl ether (DEE, ×2) and then dried *in vacuo*.

Removal of *N*-Fmoc group

The resin-bound Fmoc-protected amino acid or peptide was washed with NMP (2 × 10 min) and then treated twice with 25% piperidine in NMP (6 mL g^{-1} resin) for 15 and 30 min at rt. To confirm complete removal of the Fmoc group, two tests were performed. Complete removal of the Fmoc group was first assessed by a Kaiser test. To further confirm deprotection, a resin sample (~2 mg) was treated with 25% piperidine in NMP (20 μL) and heated at 100 °C for 5 min. An aliquot (10 μL) of the resulting solution was spotted onto a TLC plate, developed over a short distance, and examined under UV light for any UV-absorbing material corresponding to Fmoc. If residual Fmoc was detected, the deprotection step was repeated for an additional 20–30 min. Finally, the resin was filtered, washed with NMP (×5), iPrOH (×3), DEE (×2) and dried *in vacuo*.

Acidic cleavage of the C-terminal COOH peptides from CLTR resin

The resin-bound peptides were first washed twice with DCM to remove residual solvents and then treated with a cleavage mixture of DCM/2,2,2-trifluoroethanol (TFE)/acetic acid (AcOH) (7 : 2 : 1) (10–15 mL g^{-1} resin) for 15 min at rt, to afford the fully ^{t}Bu-protected peptides. The cleavage solution was filtered, and the resin was washed with an additional portion of the cleavage mixture, followed by DCM (×2). The combined filtrates were concentrated under reduced pressure to give an oily residue. This was either analysed directly by HPLC, or DEE was added to induce peptide precipitation. The resulting solid was collected by filtration and washed with DEE (×3) and dried *in vacuo* to afford the crude peptide.

Preparation of Fmoc-protected α-amino alcohols (monomers)

Fmoc-protected α-amino alcohols (monomers) were prepared according to previously reported methods, *via* conversion of the Fmoc-amino acid to the corresponding NHS active ester, followed by reduction with $NaBH_4$.[26–28]

Briefly, the Fmoc-amino acid (1 equiv.) and NHS (1.15 equiv.) were dissolved in DCM or DCM/THF (3 : 1) (0.2 M) and to this solution *N*,*N*′-dicyclohexylcarbodiimide (DCC, 1.1 equiv.) was added and the reaction mixture was stirred at rt for 30 min. Then, the reaction mixture was filtered, and the filtering was condensed under reduced pressure at low temperature (<30 °C). The NHS activated Fmoc-amino acid that was obtained was then diluted in THF (0.2 M) and placed in an ice-bath (0–4 °C). $NaBH_4$ (2 eq.) was then added, followed by addition of H_2O to give THF/H_2O (15 : 1) (v/v). The reaction was stirred for 30 min and then the organic solvent was evaporated, and the oily residue was extracted in DCM and water. The organic layer was collected and sequentially washed with 10% aq. citric acid to pH 2–3 and water. The resulting organic layer was dried over magnesium sulphate ($MgSO_4$) and evaporated under reduced pressure to afford the desired Fmoc-protected α-amino alcohols as solids in DEE/hexane (Hex) mixtures.

Preparation of Fmoc-protected α-amino aldehydes (monomers)

Fmoc-protected α-amino aldehydes (monomers) were synthesized according to a previously reported method.[32]

Briefly, Fmoc-amino alcohol (1 equiv.) was dissolved in NMP (0.1–0.2 M) and Dess–Martin (DMP, 1.2 equiv.) was added and the reaction mixture was stirred for 1–2 h at rt. Reaction progress was monitored by TLC. In case of incomplete reaction after 1 h, an additional 20% of the initial amount of DMP was added, leading to complete conversion within the following hour. Upon completion, the reaction mixture was extracted in DCM and water. The organic layer was collected, and the aqueous layer was washed once more with DCM. The combined organic layers were treated with 10% aq. sodium thiosulfate ($Na_2S_2O_3$) under vigorous stirring for 15 min at rt. The organic layer was then separated and sequentially washed with 10% aq. $NaHCO_3$ (×2), water (×2), 10% aq. citric acid to pH 2–3, and water (×3). The resulting organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure to yield the desired Fmoc-protected α-amino aldehydes either as an oily residue or as a solid in DEE/Hex mixtures.

Solid-phase synthesis of Fmoc-protected C-terminal peptide alcohols from CLTR resin

The Fmoc-peptide alcohols **3a–3g** were prepared by loading CLTR resin with the corresponding C-terminal Fmoc-amino alcohols, followed by conventional stepwise SPPS, as already described in the literature.[29,43]

Briefly, the resin (typical loading 1.0–2.0 mmol g^{-1}; taken as 1.0 mmol g^{-1} for calculations) was swollen in DCM (10 mL g^{-1} resin) for 15 min, after which DIPEA (1.3 mmol g^{-1} resin) was added and the resin was agitated for 1–2 min. The Fmoc-amino alcohol (1.0 mmol g^{-1} resin) was then added as a solid, and the mixture was agitated for 24 h at rt. Subsequently, DIPEA (2.0 mmol g^{-1} resin) and MeOH (4 mL g^{-1} resin) were added, and agitation was continued for 2 h at rt. The resin was then sequentially washed and filtered with DCM, DCM/MeOH/DIPEA (80 : 15 : 5, v/v/v; 3 × 15 min), and NMP (×3), followed by peptide elongation using conventional stepwise SPPS protocols (as described above).

Cleavage of Fmoc-peptide alcohols from CLTR resin

The peptidyl resin was initially washed with DCM (×3) and then washed sequentially with increasing concentrations of TFA in DCM (0.2%, 0.3%, up to 0.4%). At each step, the resin–acid mixture was monitored for the release of UV-absorbing material by TLC (UV visualization). Upon detection of slight UV absorbance, the acidic solution was filtered, and the resin was thoroughly washed with DCM (×5) and directly used to the next step (cleavage of Fmoc-peptide alcohol).

Cleavage was performed by treating the resin with either HFIP/DCM (30 : 70, v/v) (10 mL g^{-1} resin) for 45 min at rt or TFE/DCM/triethylsilane (TES) (30 : 65 : 5, v/v/v) (10 mL g^{-1} resin) for 4–5 h at rt. After that time, the resin was filtered and washed with DCM (×3). The combined filtrates were concentrated under reduced pressure, and the resulting oily residue was either triturated with DEE to afford the desired Fmoc-peptide alcohols as solids or directly used in the next step without further purification.

Typical resin loading of the initial amino alcohol on CLTR resin was 0.30–0.35 mmol g^{-1}, and isolated yields of Fmoc-peptide alcohols ranged from 80 to 90%.

Conversion of Fmoc-peptide alcohols to Fmoc-peptide aldehydes

Fmoc-peptide alcohols **3a–3g** were dissolved in NMP to a concentration of 0.05–0.1 M. To this, DMP (1.2 equiv.) was added as a solid, and the reaction mixture was stirred at rt. Reaction progress was monitored by HPLC. Oxidation was typically complete within 2–3 h; if longer reaction times were required, an additional 20% of the initial amount of DMP was added, generally leading to complete conversion within the following hour.

Upon completion, the reaction mixture was diluted with DCM and extracted with water. The aqueous phase was washed with DCM, and the combined organic phases were treated with 10% aq. $Na_2S_2O_3$ under vigorous stirring in an ice-bath for 15 min and another 15 min at rt. After phase separation, the organic layer was sequentially washed with 10% aq. $NaHCO_3$ (×2) and water (×2), acidified to pH 2–3 using 10% aq. citric acid, and washed again with water (×3). The organic phase was dried over $MgSO_4$, filtered, and concentrated under reduced pressure.

The resulting solid or oil was triturated with DEE, affording peptide aldehydes **4a–4g** as white solids. Typical isolated yields ranged from 70 to 80%. NMR spectra, HPLC chromatograms, and the corresponding ESI-MS data for peptide aldehydes **4a–4g** are provided in the SI.

Solid-phase reductive amination between Fmoc-peptide aldehydes (or Fmoc-amino aldehyde monomers) and the N-terminus of peptidyl CLTR resin

The *N*-Fmoc-deprotected peptidyl resin, assembled on CLTR resin *via* classical step-by-step or fragment condensation protocols, was initially swollen and washed in a plastic syringe with anhydrous THF (×3) and then transferred to an Eppendorf tube. A solution of anhydrous THF/MeOH (5 : 1, 5 mL g^{-1} resin) was added, followed by the Fmoc-protected peptide aldehyde **4a–4g** (1.2 equiv., based on a typical resin loading of 1.0 mmol g^{-1}) or the Fmoc-amino aldehyde monomer (1.2 equiv.). The suspension was agitated for 15 min at rt, and the resin was then filtered and washed with anhydrous THF (×3) to remove any unreacted aldehyde.

The resulting resin was then transferred to a new Eppendorf tube, suspended in anhydrous THF containing 1% AcOH (5 mL g^{-1} resin) (or in THF for the comparative non-acidic experiments), and treated with sodium cyanoborohydride ($NaBH_3CN$, 1.0 equiv.). The suspension was agitated for 1 h at rt, after which the resin was returned to a plastic syringe, washed with THF (×3), and the reduction step was repeated under identical conditions. Finally, the resin was sequentially washed with THF (×5), THF/MeOH (5 : 1) (3 × 10 min), THF (×5), NMP (×5), iPrOH (×3), and DEE (×2).

Reaction progress and completion were monitored by HPLC. Briefly, a small resin sample was treated with DCM/TFE/AcOH (7 : 2 : 1) for 15 min and filtered. The filtrate was evaporated under a gentle N_2 stream, and the resulting solid or oily residue was dissolved in MeOH (or, in some cases, THF or NMP) and analyzed by HPLC, without prior purification, to enable detection of unreacted peptide derived from the peptidyl–resin starting material, reaction intermediates, and products. Incomplete imine formation and incomplete reduction were indicated by the presence of imine intermediate, residual unconjugated peptide, and/or aldehyde and peptide fragments resulting from hydrolysis of the peptidyl imine during HPLC analysis. In such cases the imine formation and/or reduction steps were repeated once.

HPLC chromatograms of the crude peptides **8a–8g**, **10f**, and **13a–13e**, as well as chromatograms used for epimerization analysis, are provided in the SI. Corresponding ESI-MS data are also included.

Introduction of Boc group onto methylene amino [CH_2–NH] bonds on solid support

Resin-bound Ψ[CH_2–NH]-containing peptides were initially washed with DCM/NMP (1 : 1) (×3) and then treated with Boc_2O (10 equiv.) and DIPEA (10 equiv.) (based on a typical resin loading of 1.0 mmol g^{-1}) in DCM/NMP (1 : 1) (5 mL g^{-1} resin) at rt. HPLC analysis indicated that after the first 24 h, approximately 15–20% of the Ψ[CH_2–NH] linkages remained unprotected. Therefore, a second treatment with

Boc_2O/DIPEA was necessary, resulting in a total of 2 × 24 h treatment. For peptide fragments containing Aib-derived Ψ[CH_2–NH] linkages, longer reaction times were required (4–5 days at rt). Upon completion, the resin was sequentially washed with NMP (×5), iPrOH (×3), and DEE (×2). Reaction completion was confirmed by HPLC analysis of the peptide following acidic cleavage from the resin, as described previously.

Author contributions

Conceptualization, K. B. and S. M.; methodology, S. M.; validation, S. M.; formal analysis, S. M., M. G., D. T., E. N., A. M. and D. G.; investigation, M. G., D. T., E. N., A. M., and S. M.; resources, S. M. and D. G.; data curation, S. M.; writing—original draft preparation, S. M.; supervision, S. M.; project administration, S. M. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information is available. See DOI: https://doi.org/10.1039/d6ob00034g.

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