

Solid-Phase Synthesis of C-Terminus Cysteine Peptide Acids

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ABSTRACT: Cysteine (Cys) is a key amino acid in many therapeutic peptides. For research and industrial purposes, solid-phase peptide synthesis is the method of choice for the preparation of most peptides. The solid-phase synthesis of C-terminal Cys peptide acids is problematic because it is accompanied by a side reaction, namely, the abstraction of α -H from the Cys residue, which leads to the formation of three side products: the epimer and two *N*-piperidinyl-Ala epimer peptides. Here, we used a chlorotrityl chloride resin to conduct a rational and in-depth study of this side reaction. The following variables were examined: removal of the fluorenylmethoxycarbonyl (Fmoc) group by different bases, the presence or absence of an acid rectifier for buffering the base, and thiol side-chain protection. In conclusion, the use of Fmoc-Cys protected with tetrahydropyran (Thp) and 4-methoxytrityl (Mmt) along with 30% 4-methylpiperidine in 0.5 M OxymaPure-DMF for Fmoc removal assures minimization of the side reaction, as demonstrated in a model peptide and confirmed for the elongation of somatostatin.

KEYWORDS: epimerization, 4-methylpiperidine, racemization, side reaction, solid-phase peptide synthesis

INTRODUCTION

Cysteine (Cys) is one of the least abundant amino acids in all organisms,^{1–4} accounting for 0.98, 0.94, and 2.3% of all amino acids in Archaea, Bacteria, and Humans, respectively.⁴ However, it is a highly conserved residue that is often observed in catalytic, regulatory, cofactor binding sites, and other functional sites in proteins.^{2,5} Given that Cys residues are used to improve the pharmacological properties of peptides, they are ideal templates for drug construction.^{3,6} For instance, the C-terminal Cys of the peptides buforin II and lysin is modified to increase the antimicrobial activity of these compounds.⁷ In recent years, 13 Cys-containing peptide-based drugs have been approved by the FDA and EMA, which include Voxzogo, which was the last drug of this nature approved (in 2021) to date. C-terminal Cys peptide acids and amides are a key class of biologically active peptides, which include prenylated and farnesylated peptides.^{7–9}

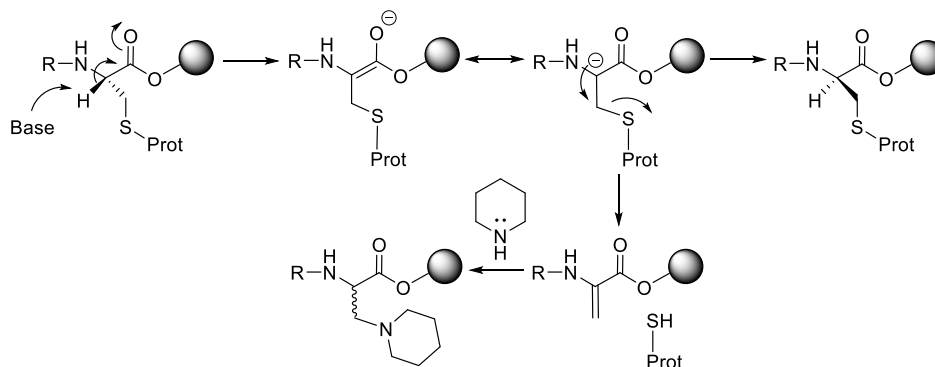
The solid-phase approach [solid-phase peptide synthesis (SPPS)],¹⁰ using a fluorenylmethoxycarbonyl (Fmoc)/*tert*-butyl (tBu) protection scheme,^{11,12} is the method of choice for the preparation of peptides in both research and production modes. Although cystine/Cys-containing peptides are not an exception, their synthesis brings about more challenges than that of homodetic peptides where only the peptide bond is present.^{3,13,14} The disulfide bond is formed mostly when the elongation of the peptide chain has taken place. Although this formation can occur while the peptide is still anchored to the resin, the most efficient way to achieve this bond is when the unprotected peptide has been cleaved from the resin.¹⁵ The difficulty around the Cys-containing peptides increases in molecules that carry multi-disulfide bonds. Regarding side-chain protection, two or three protecting groups are commercially available for the remaining trifunctional amino acids. In contrast, there are more than 10 Fmoc-Cys protected

derivatives, thereby reflecting not only the richness but also the complexity associated with the synthesis of these peptides.^{13,16}

Furthermore, the presence of the thiol and the disulfide bridge in the structure of Cys-containing peptides favors a large number of side reactions.¹⁷ Thus, the thiol group in Cys can be oxidized to sulfonic derivatives, and the alpha carbon is also very prone to racemization. Given that the S-protecting group (S-Prot) moiety is a good leaving group, the presence of a base (e.g., piperidine to remove the Fmoc group) can cause an elimination reaction with the concomitant formation of dehydroalanine (Dha). The latter can, in turn, undergo a Michael addition of the piperidine, rendering *N*-piperidinyl-Ala or even the same thiol derivative, thus restoring the initial structure of the molecule.¹⁸ Both reactions take place with total loss of the chiral integrity of the amino acid. The formation of the disulfide bridge from the two thiol molecules is a reversible reaction, which in the case of peptides with multi-disulfides can lead to the so-called scrambling and formation of other regioisomers, depending on the thermodynamics.¹⁵

Cys in the C-terminal position is particularly prone to undergo side reactions. The first evidence of this was described by one of our groups in 1987, in a seminal work related to the use of the *S*-*t*-butyl-sulfonyl (StBu) group for the SPPS of Cys-containing peptides using the Fmoc strategy.¹⁹ That study concluded that “when StBu is located in the C-terminal position, a side reaction prevents the formation of the desired

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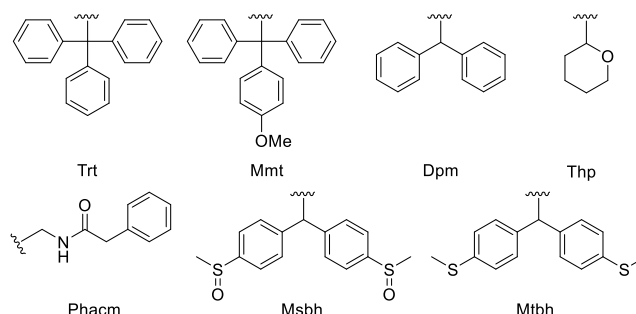
Scheme 1. Mechanism of Epimerization/Racemization and Formation of *N*-Piperidinyl Ala Derivative with the Cys Residue at the C-Terminal Position in the SPPS Scheme

product". At that time, we were not able to attribute the peak with 36 (in fact, it was 37) mass units fewer than expected to the *N*-piperidinyl-Ala derivative. In 1996, we identified the side reaction and reported on its dependence on C-terminus anchoring to the resin, much more with an ester as in the Wang or chlorotrityl chloride (CTC) resins than with an amide as in the case of PAL linker.¹⁸ Regarding thiol side-chain protecting groups, the side reaction was much more prominent with acetamidomethyl (Acm) than with trityl (Trt).^{18,20} The loss of chirality in the C-terminal Cys residue was also studied by us and other groups in work related to the development of new thiol side-chain Cys protecting groups.^{18,20–24} The minimization of epimerization is crucial in the large-scale synthesis of C-terminal Cys acid peptides because this side product could be difficult to remove from the target, and even its small presence can ruin a synthetic production campaign.

In addition to the quality of the leaving groups, exemplified in the case of *S*tBu, the two side reactions are associated with the abstraction of α -H of the C-terminal Cys by a base. The reprotonation of the species formed leads to epimerization/racemization. On the other hand, the carbanion can cause an elimination reaction with the thio derivative as the leaving group to render a dehydroalanine residue, which can react with nucleophiles, such as piperidine, to afford the *N*-piperidinyl-Ala peptide epimers (Scheme 1). Although there is some controversy, the dehydroalanine peptide resin can also react with the thio derivative leaving group, with concomitant restoration of the previous Cys(protected) derivative but accompanied by epimerization/racemization.^{25,26}

Here, we conducted a rational study of the two reactions, epimerization/racemization and *N*-piperidinyl-Ala formation, comparing different elongation strategies (C-terminus anchoring vs side-chain anchoring), the use of different bases to remove the Fmoc group, the presence or absence of an acid rectifier for buffering the base during the deprotection step, and the myriad of Cys protecting groups commercially available. Figure 1 shows the Cys protecting groups used in this study.

Trt, probably the most common Cys side-chain protecting group, is removed with 5–10% of trifluoroacetic acid (TFA) in dichloromethane (DCM).²⁷ 4-Methoxytrityl (Mmt) is more acid labile than Trt and is removed with 1–2% TFA-DCM.²⁸ Mmt and Trt are not compatible because it is not possible to remove Mmt in the presence of Trt. Diphenylmethyl (Dpm) requires a high content of TFA (60–90% TFA-DCM) and is compatible with Mmt.²⁹ Thus, it is possible to remove Mmt in the presence of Dpm. Cleavage of the Thp group requires a

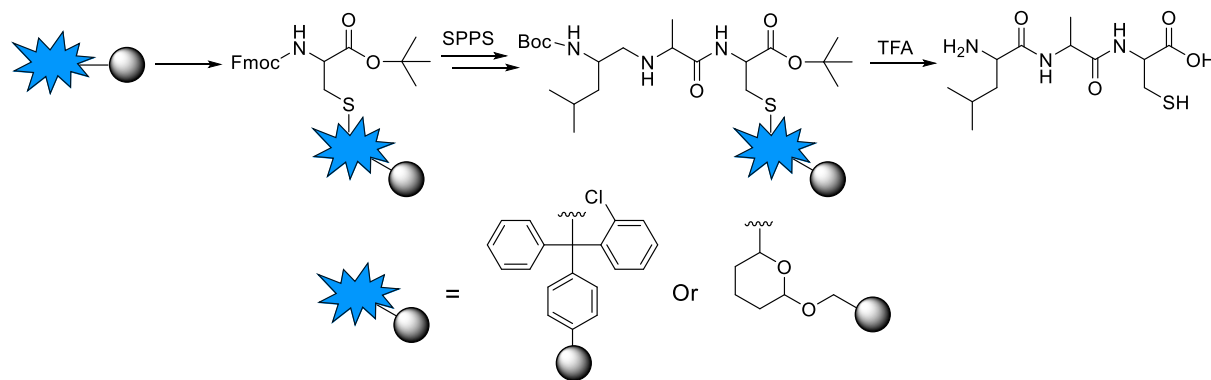
**Figure 1.** Cys protecting groups used in this work.

similar amount of TFA as cleavage of the Trt group, and Thp-protected Cys residues proved considerably less prone to racemization than Trt.²⁴ Phenylacetamidomethyl (Phacm) belongs to the same family as Acm (structure not shown), and it can be removed in the presence of oxidizing reagents [I_2 or *N*-chlorosuccinimide (NCS)] to directly render the disulfide bridge.^{30,31} In our hands (data not shown), Phacm gives cleaner crude peptides than Acm. In addition, Phacm can be enzymatically removed by penicillin G acylase (PGA). 4,4'-Bis(methylsulfinyl)benzhydryl (Msbh) belongs to the family of safety-catch protecting groups.³² In this regard, it is stable during peptide elongation and cleavage of the peptide with TFA from the resin, and it is acid cleavable only after reduction to 4,4'-bis(methylthio)benzhydryl (Mtbh). Fmoc-Cys(Mtbh)-OH has not been reported in the literature, but its synthesis is described in the Experimental Section.

RESULTS AND DISCUSSION

A Cys residue at the C-terminus of a peptide contains two protecting groups to be considered. One is the carboxylic group, which very often is the linker that binds Cys to the solid support, and the other corresponds to the thiol side chain. The question raised concerns the effect of these two kinds of protecting groups on masking the acidity of the α -proton of the Cys residue. This masking can be done either by electronic effects (electron-donating) or by the introduction of steric congestion around the α -proton. This is evidenced by the observation that the CTC resin shows less tendency toward these side reactions (epimerization and *N*-piperidinyl adduct formation) compared to Wang resin, and this is also corroborated with respect to diketopiperazine (DKP) formation, which is much more severe in the case of Wang resin.

Scheme 2. Side-Chain Anchoring of Fmoc-Cys-OtBu on CTC and DHP Resins



Side-Chain Anchoring. Although most peptides are synthesized in solid-phase starting by anchoring the carboxylic group of the C-terminus to the solid support, it is also possible to start by attaching trifunctional amino acids through the side chain.^{33,34} In the case of the Cys residue, the literature provides an example of Cys side-chain anchoring to the xanthenyl linker (XAL),^{33,35} which is structurally similar to Sieber resin.³⁶

In the current work, Fmoc-Cys-OtBu was anchored to the CTC resin and the dihydro-pyranyl (DHP) resin (Scheme 2). The latter mimics Thp protection for the thiol of the Cys residue, and the Thp-protected Cys shows less tendency to racemize in comparison with the Trt protecting group.^{37,38}

After elongation to H-Leu-Ala-Cys(resin)-OtBu, using 20% piperidine-DMF (1 min +7 min) for Fmoc removal, and the cleavage of the tripeptide from the resin, HPLC analysis showed 16% of racemization for CTC resin and 23% for DHP resin. These results suggested that side-chain anchoring using *t*Bu as C-carboxylic protecting groups is not favored versus the C-carboxylic anchoring to minimize racemization, thus reaffirming the importance of the hindrance to mask the acidity of the α -proton. The formation of the *N*-piperidinyll adducts in these two cases was not further investigated.

Screening of Bases for Fmoc Removal on the CTC Resin. Next, using the CTC resin and Trt as the protecting group for Cys and the peptide Boc-Leu-Ala-Cys(Trt)-O-CT-resin as a model, several secondary cyclic amines, namely, piperidine (PP), 4-methylpiperidine (4 MP),^{39,40} piperazine (PZ), pyrrolidine (PY), and morpholine (MOP),^{41,42} were studied (Figure 2).⁴³ Furthermore, in some cases, an acid

prepared by treatment of the peptide Boc-Leu-Ala-Cys(*St*Bu)-O-2-CT resin with 20% piperidine-DMF for 24 h. Given its tendency to give these side reactions, *St*Bu was used as the Cys protecting group.

The reaction of the *St*Bu peptide resin with 20% piperidine-DMF led to the disappearance of the Cys-containing peptide, with the formation of two adducts in epimer forms. As expected, one adduct was resulted from the attack of the piperidine on the dehydroalanine (A) and the other from the attack of the thiol-containing peptide on the same dehydroalanine (B), which is affording a thioether similar to the moiety present in nisin peptides (Figure 3).^{52–54}

Next, the formation of the side products was studied using Boc-Leu-Ala-Cys(Trt)-O-2-CT resin as a model peptide, and the most common cyclic secondary amines were used to remove the Fmoc group (Table 1).

As expected, adduct formation occurred to a lesser extent than the epimerization and was more pronounced when a more concentrated base solution was used. Piperidine (pKa, 11.22) (#1,2) gave more side reactions than 4-methylpiperidine (pKa, 10.69) (#3,4) and piperazine (pKa, 9.82) (#5). These results are in agreement with those previously reported in the literature.^{20,55} Due to solubility issues, the latter was tested only at 10% concentration in DMF-ethanol (EtOH) (9:1). Interestingly, pyrrolidine (pKa, 11.31) (#6,7), which has a similar pKa to that of piperidine and is considered greener,⁵⁶ yielded a much worse result. The less hindrance of pyrrolidine may account for this difference. Morpholine (pKa, 8.36) (#8,9), which is much less basic than the rest of the bases, was practically devoid of the side reaction. The addition of OxymaPure (0.5–1.0 M) in all cases resulted in a reduction in the extent of the side reactions.

Somatostatin Elongation Using Selected Bases for Fmoc Removal. First, linear somatostatin (H-AGCKNFFWKFTFTSC-OH) and linear D-¹⁴Cys-somatostatin (H-AGCKNFFWKFTFTSc-OH) were synthesized. The separation of the peptides in HPLC was then studied using artificial mixtures of the D and L epimers in proportion (1:9, 1:19, and 1:99). As shown in Figure 4, around 1% of the D epimer was detected.

Four syntheses of somatostatin were carried out using two treatments (10 min +20 min) for Fmoc cleavage (standard control conditions) with the following bases: 20% piperidine-DMF, 20% 4 MP-DMF, 20% 4 MP in 1 M OxymaPure-DMF, and 20% morpholine-DMF. The HPLC analysis of the final somatostatin from the four syntheses (Figure 5), in terms of purity, indicated that 20% piperidine-DMF gave the best

Piperidine	4-Methylpiperidine	Piperazine	Morpholine	Pyrrolidine
pKa = 11.22	pKa = 10.69	pKa ₁ = 9.82 pKa ₂ = 5.68	pKa = 8.36	pKa = 11.31

Figure 2. Cyclic amines used in this study to remove the Fmoc group and their corresponding pKa.^{49–51}

rectifier, OxymaPure, was added to achieve a buffer impact, which has been demonstrated to reduce the formation of other side products such as aspartimides and δ -lactams.^{44–48}

First, references of H-Leu-Ala-L/D-Cys-OH and their corresponding *N*-piperidinyll-Ala adducts were prepared and characterized by HPLC and LC-MS. The two epimers were synthesized using L- and D-Cys (Trt), and the adducts were

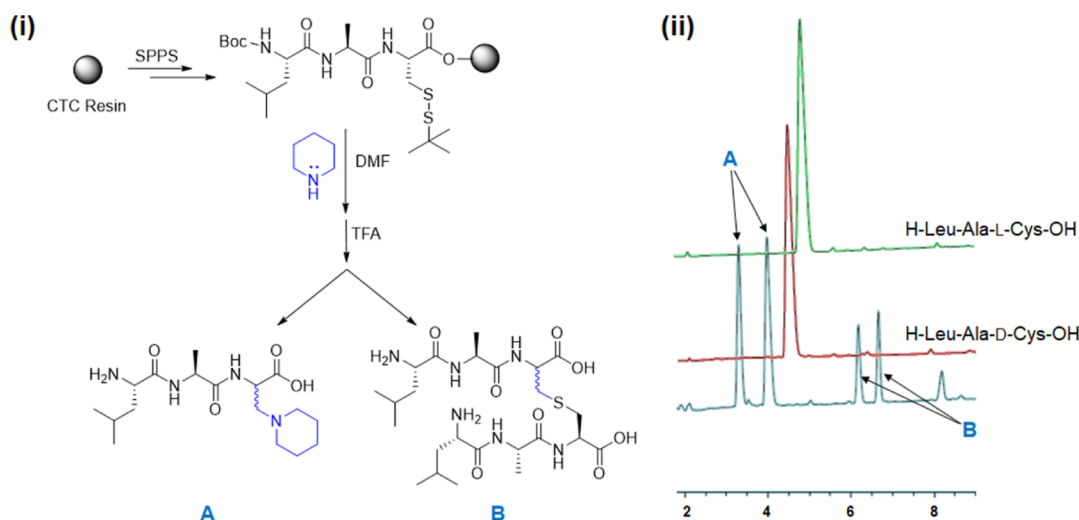


Figure 3. (i) Synthetic scheme for the preparation of the *N*-piperidinyl adduct (A) and an additional thioether adduct (B) and (ii) HPLC separation of the adducts from L and D tripeptides.

Table 1. Epimerization and Adduct Formation after Treatment of Boc-Leu-Ala-Cys(Trt)-O-2-CT Resin with Amines for 24 h

#	condition		D/L- β -substituted Ala ^a		
	(% in DMF) base	oxymaPure (M)	epimer (%)	adduct 1 (%)	adduct 2 (%)
1	20% piperidine		5.7	2.2	2.3
2	10% piperidine		3.6	1.2	1.7
3	20% 4-methylpiperidine		2.5	0.3	0.2
4	10% 4-methylpiperidine		1.6	0.3	0.2
5	10% w/v piperazine [DMF-EtOH (9:1)]		2.2	ND ^b	ND ^b
6	20% pyrrolidine		28.3	4.8	9.2
7	10% pyrrolidine		16.9	1.9	3.0
8	20% morpholine		<0.1	ND ^b	ND ^b
9	10% morpholine		<0.1	ND ^b	ND ^b
10	20% piperidine	0.5	3.5	0.4	0.5
11	20% piperidine	1	3.3	0.7	0.3
12	10% piperidine	0.5	2.3	<0.1	<0.1
13	10% piperidine	1	1.2	<0.1	<0.1
14	20% 4-methylpiperidine	1	1.5	0.2	0.2
15	10% 4-methylpiperidine	1	0.8	0.2	0.1

^aAdducts corresponding to piperazine and morpholine could not be determined (^bNot determined). However, the formation of the adducts was less important than the epimerization whenever this side reaction was identified, and this value was taken mainly to evaluate the extent of the side reaction.

results. Although 4 MP is described to perform similarly to piperidine, the quality of the final peptide, although acceptable, was lower compared to that achieved with piperidine. This observation was reinforced when 4 MP was used in a 1 M OxymaPure-DMF solution, where the presence of deletion peptides indicated less effectivity in removing the Fmoc group. Finally, 20% morpholine-DMF gave very poor results. All the extra peaks are presumably due to incomplete Fmoc removal.

Study of the Side Reactions After the Incorporation of Each Fmoc-Amino Acid. Next, to determine the amino acid cycles in which the formation of the side reaction could be most prevalent, somatostatin was synthesized. After removal of each Fmoc group by standard control conditions (10 min +20

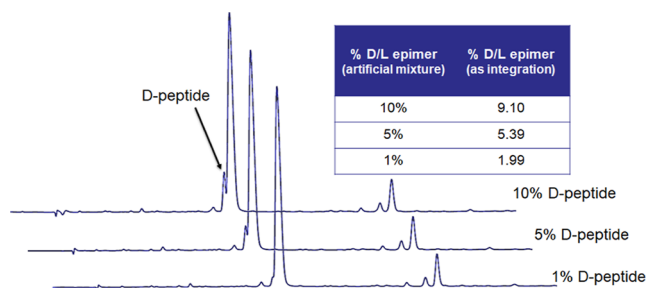


Figure 4. HPLC of mixtures of linear D-¹⁴Cys-somatostatin and linear somatostatin in proportion (1:9, 1:19, and 1:99). In the inset table, the result of the integration is presented. HPLC conditions: 25–35% B (MeCN with 0.1% TFA) into A (H₂O with 0.1% TFA) over 15 min. Peaks at higher retention times correspond to the *t*-butylated side products of linear somatostatin.

min), the peptidyl resin was subjected to 8 h stress studies using 20% piperidine-DMF (Scheme 3).

As the two first amino acid cycles [incorporation of Cys(Trt) and Ser(*t*Bu)] cannot be studied directly, in both cases, the peptide was elongated up to the tripeptide, which was then analyzed. Thus, Fmoc was removed from the Cys(Trt) residue with 20% piperidine-DMF (10 min +20 min) in a sample of resin for 8 h stress studies, and then Fmoc-Ser(*t*Bu)-OH was incorporated, followed by removal of the Fmoc group as before and subsequent incorporation of Fmoc-Thr(*t*Bu)-OH. The H-Thr-Ser-Cys-OH tripeptide obtained after Fmoc removal and cleavage was studied by HPLC. The second cycle (after incorporation of Ser(*t*Bu)) was also analyzed after treatment of the dipeptide H-Ser(*t*Bu)-Cys(Trt)-O-2-CT resin with 20% piperidine-DMF for 8 h stress studies, and then a further addition of Fmoc-Thr(*t*Bu)-OH was done with subsequent Fmoc removal and cleavage (Table 2).

The results in Table 2 show that side reactions were less likely to occur during the removal of Fmoc after the incorporation of the first amino acid [Cys(Trt)] (#1) when compared to Fmoc removal after the second [Ser(*t*Bu), #2] and the third [Thr(*t*Bu), #3] incorporations, respectively. The analysis continued with the rest of the amino acids. However, after the incorporation of the third amino acid [Thr(*t*Bu)], the presence of the side reactions was also analyzed without

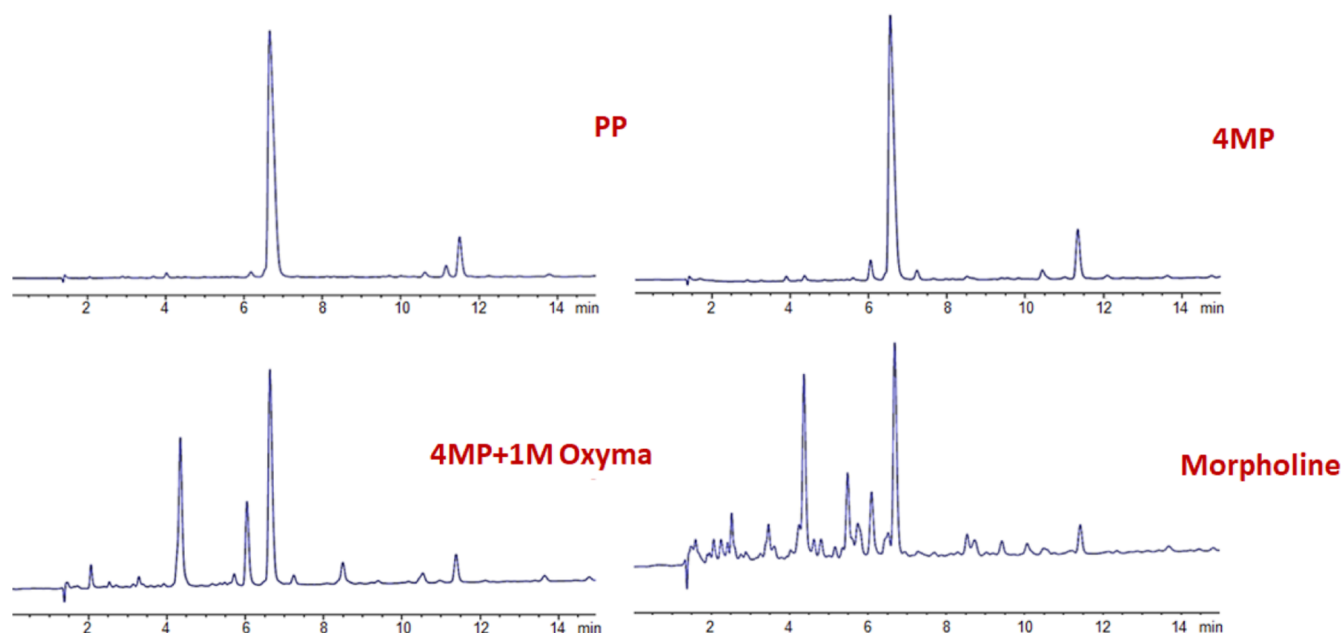


Figure 5. HPLC of somatostatin synthesized using different bases, as indicated in the text. HPLC conditions: 25–35% B (MeCN with 0.1% TFA) into A (H₂O with 0.1% TFA) over 15 min. Peaks at higher retention times correspond to the *t*-butylated side products of linear somatostatin.

Scheme 3. Study of Side Reactions after the Incorporation of Each Fmoc-Amino Acid in the Somatostatin Elongation Using C-Terminus Cys(Trt)

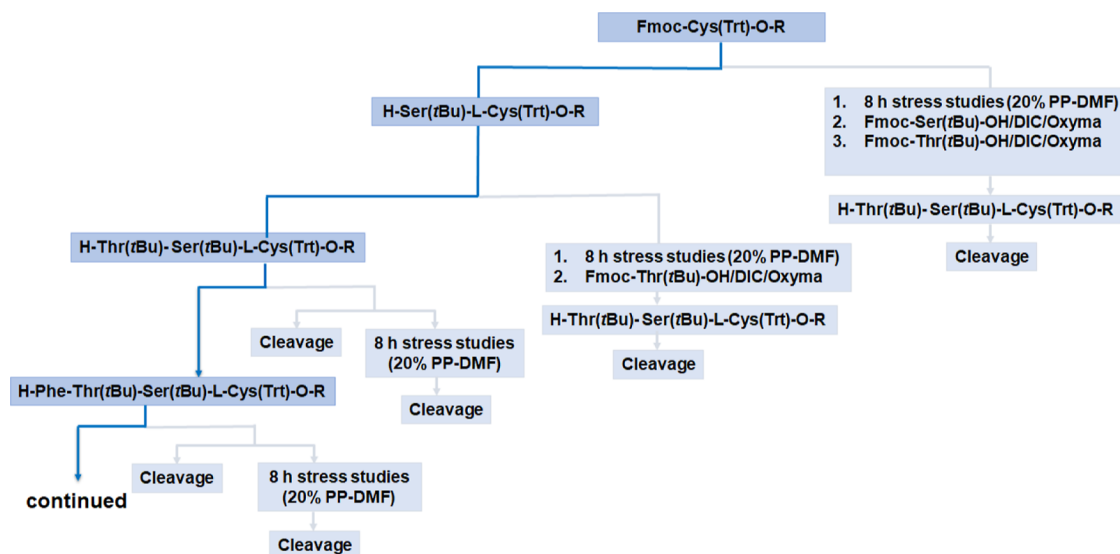


Table 2. Analysis of the Side Reactions (Epimerization and Adduct) after Incorporation and Fmoc Removal on the Tripeptide H-Leu-Ala-Cys-OH as Calculated by HPLC

#	peptidyl-resin	20% PP-DMF (stress studies)	extra AA coupled	epimer (%)	adducts (%)
1	H-Cys(Trt)-O-2CT-resin	8 h	Ser + Thr	0.5	0.7
2	H-Ser(tBu)-Cys(Trt)-O-2CT-resin	8 h	Thr	2.5	1.4 and 1.3
3	H-Thr(tBu)-Ser(tBu)-Cys(Trt)-O-2CT-resin	8 h		3.1	1.1 and 0.8

extended piperidine treatment (8 h stress studies), only after Fmoc removal by standard control conditions (10 min +20 min) (see the column labeled “synthesis” in Table 3).

Data from Table 3 confirm that the first amino acid cycles had a greater tendency to render side reactions. This observation can be interpreted on the basis that the growing peptide chain exerts a kind of protection on the α -H of the Cys residue, and the shorter the peptide, the less the protection.

However, after completion of the sequence, around 1% of the epimer was found in the standard syntheses.

Screening of Different Thiol Side-Chain Protecting Groups. Next, several commercially available protecting groups (Figure 1) were screened using Boc-Leu-Ala-L-Cys(PG)-O-2-CT resin as the model and following the strategy shown in Scheme 4, which was similar to that in Scheme 3. First, Cys(Trt) was evaluated, and the presence of

Table 3. Side Reactions Formed after Each Fmoc-Amino Acid Coupling in the Synthesis of Linear Somatostatin^c

#	peptide	epimer (%)		adducts (%)	
		synthesis ^a	20% PP-DMF (8 h stress studies)	synthesis ^a	20% PP-DMF (8 h stress studies)
1 ^b	H-TSC-OH	-	3.1	0.8	1.1 and 1.8
2	H-FTSC-OH	0.4	2.0	0.8	1.1 and 1.8
3	H-TFTSC-OH	0.6	1.9	0.2	0.5
4 ^d	H-KTFTSC-OH	0.3	1.8	0.3 and 0.2	0.7 and 0.8
5	H-WKTFTSC-OH	-	1.5	0.2	0.1 and 0.1
6	H-FWKTFTSC-OH	-	-	0.4 and 0.2	0.5 and 0.6
7	H-FFWKTFTSC-OH	-	-	0.4 and 0.2	0.4 and 0.1
8	H-NFFWKTFTSC-OH	-	-	-	-
9	H-KNFFWKTFTSC-OH	-	-	-	-
10	H-CKNFFWKTFTSC-OH	1.4	1.9	-	-
11 ^d	H-GCKNFFWKTFTSC-OH	0.5	1.9	-	-
12	H-AGCKNFFWKTFTSC-OH	1.2	2.2	-	1.0

^aFmoc removal was carried out by standard control conditions. ^bData from #3, Table 2. ^cThe “-” in this case means not determined. ^dThe fluctuation of the data attributes to the inherent difficulty in chromatographic integration.

Scheme 4. Study of Side Reactions after Each Fmoc-Amino Acid Coupling in the Tripeptide Elongation Using Selected Protecting Groups for the Cys Residue

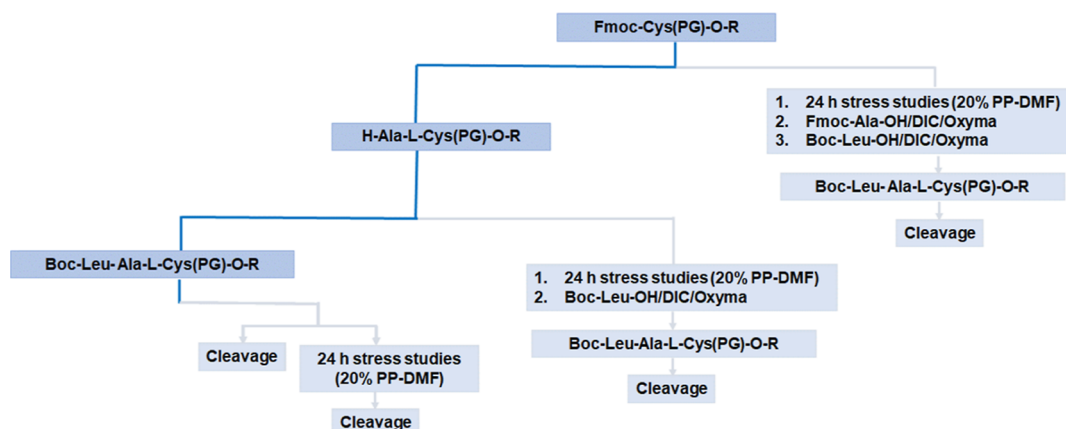


Table 4. Analysis of the Side Reactions (Epimerization and Adduct Formation) After Incorporation of Each Amino Acid and Fmoc Removal on the Tripeptide H-Leu-Ala-Cys-OH, as Calculated by HPLC

#	peptide	20% PP-DMF (stress studies)	extra AA coupled	epimer (%)	adducts (%)
1	H-Cys(Trt)-O-2CT	24 h	Ala + Leu	0.5	
2	H-Ala-Cys(Trt)-O-2CT	24 h	Leu	4.7	3.0 and 2.3
3	Boc-Leu-Ala-Cys(Trt)-O-2CT	24 h		7.1	2.9 and 3.6

the side products was studied after the incorporation of each amino acid. However, after the first three cycles, the occurrence of side reactions was examined at the level of the tripeptide H-Leu-Ala-L-Cys-OH.

The results shown in Table 4 again confirm that the side reactions did not take place during the removal of the Fmoc group after the incorporation of the first amino acid. Again, during the removal of the Fmoc group of the second and third residues, side reactions became more prevalent. The lower tendency of side reactions observed during the piperidine treatment of the Cys residue alone can be attributed to electronic effects. Thus, it is possible to conclude that *N*-acylation leads to greater tendency for Cys isomerization compared to H-Cys(PG)-O-2-CT resin.

Next, the different protecting groups (Figure 1) were studied only to the tripeptide level. Thus, the Boc-Leu-Ala-Cys(PG)-O-2-CT resin was constructed (the two Fmoc groups were removed following treatments of 1 min +7 min)

and then treated with 20% piperidine-DMF for 24 h stress studies. After cleavage from the resin, the peptide was analyzed by HPLC (Table 5).

Taking into consideration the two side reactions in Table 5, Mmt and Thp outperformed the other protecting groups. In the case of Thp, this observation confirms previous findings by our group²⁴ that Thp-protected Cys residues are less prone to epimerization.

Elongation of Somatostatin Using Trt, Mmt, and Thp as Protecting Groups for the C-Terminal Cys Residue. Somatostatin is one of the most important peptide-based APIs. The relevance of having an optimized synthesis of this peptide has been recently illustrated by the group of Shi and Fang,⁵⁷ where an elegant strategy was developed by the solid-phase preparation of the hydrazide peptide and its reaction with NaNO₂ to give the corresponding acyl azide. The azide derivative reacts with a thiol to afford the thioester, which further reacts with β -mercaptoethanol to render the β -hydroxy

Table 5. Base Treatment of the Tripeptide Boc-Leu-Ala-Cys(PG)-O-2-CT Resin (PG Shown in Figure 1) and Release. Analysis of the Side Reactions (Epimerization and Adduct Formation) as Calculated by HPLC^c

#	protecting group (PG)	epimer (%)		adducts (%)	
		synthesis ^a	20% PP-DMF (24 h stress studies)	synthesis ^a	20% PP-DMF (24 h stress studies)
1	Trt	0.1	7.1	-	2.9 and 3.6
2	Mmt	0.1	5.2	-	1.4 and 2.7
3	Dpm	-	7.5	-	1.8 and 2.2
4	Thp	-	4.7	-	1.4 and 1.4
5	Mtbh	-	5.1	-	5.0 and 6.0
6	Msbh	0.1	7.0	-	8.4 and 8.4
7	Phacm	ND ^b	ND ^b	-	3.4 and 4.6

^aThe tripeptide was cleaved directly without extended piperidine treatment. ^bNot determined. ^cThe “-” in this case means not found.

thioester. The thioester later suffers a rearrangement to render the ester which is finally hydrolyzed. Thus, somatostatin was synthesized using Trt, Mmt, and Thp as the side-chain protecting groups of the C-terminal Cys and Trt for ³Cys. In addition to Mmt and Thp, which were the most promising protecting groups from the previous experiment (# 2 and 4, Table 5), synthesis was also performed with Trt as it is the most commonly used Cys protecting group. For the three growing peptides with different S-protecting groups during chain elongation, Fmoc was removed by two treatments with 20% piperidine-DMF (10 min +20 min) during peptide chain elongation. Given that the experiments described in Table 1 showed that the presence of both 4 MP and OxymaPure reduced the magnitude of side reactions in all cases, for Mmt and Thp, syntheses were also carried out using 30% 4 MP in 0.5 M OxymaPure-DMF (# 3 and 5, Table 6). The amount of 4 MP was increased up to 30% because the previous synthesis of somatostatin using 20% 4 MP in 1 M OxymaPure-DMF (Figure 5) showed the presence of deletion peptides.

Samples from these five protected linear somatostatin-2-CT resins were additionally treated with the corresponding base for 8 h stress studies (to simulate additional Fmoc removal steps), cleaved and analyzed.

In addition to the epimerization, the total of the two adducts, as determined by LC–MS, was <1% (#2 and #4), and they were not detected for #3 and #5. In all cases, the purity of the final product was excellent. Thus, the results of this set of experiments confirmed the previous results of the present work. Thp (#4) and Mmt (#2) outperformed Trt (#1), and the use of 30% 4 MP in 0.5 M OxymaPure-DMF (#3 and #5) gave better results than 20% piperidine-DMF (#2 and #4) (Figure 6).

CONCLUSIONS

The synthesis of C-terminal Cys peptide acids using the Fmoc/*t*Bu strategy is accompanied by two side reactions, namely, the epimerization at the C-terminus Cys and the formation of *N*-piperidinyl-Ala-containing peptides. Both side reactions are triggered by the abstraction of α -H by the base (normally piperidine) used to remove the Fmoc group. The corresponding carbanion can initiate the epimerization or can form a dehydroalanine residue through a β -elimination reaction, where the S-protecting group moiety is the leaving group. The dehydroalanine residue, which is an α,β -unsaturated system, reacts with nucleophiles such as the piperidine to afford a *N*-piperidinyl-Ala-containing peptide. Due to the formation of a chiral center, the two side reactions are translated into the formation of three side products, namely, the epimer at the Cys residue, and the two epimers with the *N*-piperidinyl-Ala residue. The presence of these side products hinders the purification step, thus potentially jeopardizing the production campaign in the case of an Active Pharmaceutical Ingredient (API). This drawback can be greater in the case of the epimer, which, due to its structural similarity with the parent peptide, can be more difficult to identify and therefore to remove from the API.

It is well known that these side reactions depend on the electronic effect. Thus, these reactions do not take place during the synthesis of C-terminal Cys peptide amides due to the electron-donating effect of the amide-N-atom. In the present work, we confirm that the steric effect also plays an important role in masking the acidity of the α -H. Accordingly, it is widely known that Wang-type resins are more prone to give these side reactions than 2-CT resins.^{18,20} Here, we have demonstrated that side-chain anchoring of the Cys residue to a 2-CT resin gives many more side reactions compared to when the Cys residue is anchored to the same resin through the carboxylic group.

The extension of the reaction is also dependent on the basicity/nucleophilicity of the amine. Thus, piperidine gave more side reactions than 4 MP and piperazine, while morpholine, as the less basic amine, led to less side reactions. Interestingly, pyrrolidine, which has a pKa that is practically the same as that of piperidine (11.31 vs 11.22), gave higher extent of side products than piperidine. This could be explained by greater nucleophilicity of pyrrolidine. This observation is consistent with the reported enhancement of DKP formation, and other important side reactions during peptide synthesis when pyrrolidine is used instead of piperidine.⁵⁶

As observed, these side reactions occurred in all cycles. However, during the removal of Fmoc from the first amino acid (Cys), the side reaction was less important than for the subsequent amino acids (up to position 4), and then, it prevailed again to a lesser extent. This could be interpreted by

Table 6. Analysis of Epimerization by HPLC for the Somatostatin Syntheses Using Different Cys Protecting Groups

#	protecting group (PG)	synthesis		additional base treatment	
		10 min + 20 min	epimer (%)	8 h stress studies	epimer (%)
1	Trt	20% PP	1.6	20% PP	2.5
2	Mmt	20% PP	0.9	20% PP	1.5
3	Mmt	30% 4 MP + 0.5 M OxymaPure	0.5	30% 4 MP + 0.5 M OxymaPure	1.2
4	Thp	20% PP	0.8	20% PP	1.4
5	Thp	30% 4 MP + 0.5 M OxymaPure	0.4	30% 4 MP + 0.5 M OxymaPure	1.0

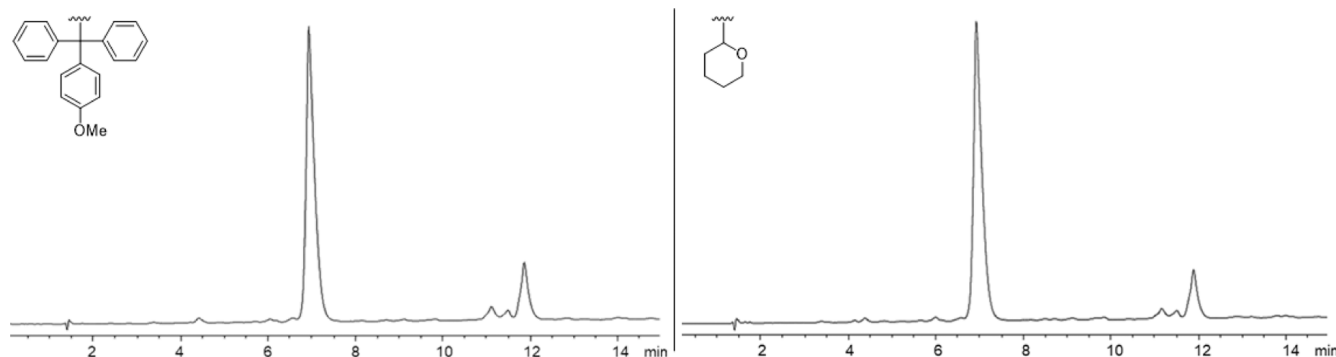


Figure 6. HPLC of somatostatin synthesized using Mmt and Thp as side-chain protecting groups and 30% 4 MP in 0.5 M OxymaPure-DMF for Fmoc removal. HPLC conditions: 25–35% B (MeCN with 0.1% TFA) into A (H₂O with 0.1% TFA) over 15 min. Peaks at higher retention times correspond to the *t*-butylated side products of linear somatostatin.

electronic effects in the C-terminal Cys and by the steric effect of the growing peptide chain in later cycles.

Although one of our groups and others have described that 4 MP gives a very similar performance to piperidine, the results presented herein showed that its performance is slightly inferior. This could be remedied by extending the deprotection time and/or increasing the percentage of 4 MP in the removal mixture.

In the same context, the use of an acid rectifier such as OxymaPure (in this work), or HOBt, for buffering the basic solutions and minimizing side reactions such as those reported here, or aspartimide formation, and the double hit when incorporating a Fmoc protected amino acid on a Pro residue deserve further attention because they could minimize side reaction formation. However, at the same time, the use of such rectifiers may also prevent the complete removal of the Fmoc group, as demonstrated herein.

Finally, the nature of the thiol protecting group is also key for the extension of the side reaction. This work has demonstrated that Thp and Mmt are better choices for minimizing the side reaction compared to Trt. Phacm, being from the Acn family, showed the poorest performance as a protecting group in that regard. Thp had already been described to be more appropriate than Trt for minimizing the side reactions, while Acn appeared to be worse.

To conclude, the use of Thp or Mmt in conjunction with 30% 4 MP in 0.5 M OxymaPure-DMF assures the minimization of side reactions. For large-scale synthesis, this combination is also an excellent starting point, but careful optimization for each peptide should be carried out. As demonstrated again in this study, the removal of Fmoc is a more demanding reaction than originally believed and is often an overlooked area in peptide synthesis. Thus, and for the case of somatostatin, while 20% 4 MP in 1 M OxymaPure-DMF did not render acceptable results, the use of 30% 4 MP in 0.5 M OxymaPure-DMF did.

SPPS is a multifactorial process where all variables must be considered in order to optimize the purity of the final peptide.

EXPERIMENTAL SECTION

General Information. All solvents and reagents used in the experiments were bought from commercial suppliers and used without any purification unless otherwise mentioned. Fmoc amino acids and CTC and DHP resins were purchased from Iris Biotech GmbH (Marktredwitz, Germany). Carbo-dimides were gifts from Luxembourg Bio-Technologies, Ness

Zion, and piperidine was supplied by Sigma-Aldrich (St. Louis, Missouri, USA). DMF and HPLC-quality CH₃CN were purchased from SRL (CRD-SRL, India). Milli-Q water was used for RP-HPLC analyses. Analytical HPLC was performed on an Agilent 1100 system using a Phenomenex AerisTMC18 (3.6 μ m, 4.6 mm \times 150 mm) column with a flow rate of 1.0 mL/min and UV detection at 220 nm. Chemstation software was used for data processing. Buffer A: 0.1% TFA in H₂O; buffer B: 0.1% TFA in CH₃CN. LC–MS was performed on an Ultimate 3000, AerisTM 3.6 μ m-wide pore column, Phenomenex C₁₈ (4.6 mm \times 150 mm) column (system 2). Buffer A: 0.1% formic acid in H₂O; buffer B: 0.1% formic acid in CH₃CN, flow 1.0 mL/min, and UV detection 220 nm. NMR spectra were recorded on Bruker AVANCE III 400 MHz spectrometers using CDCl₃ as a solvent and tetramethylsilane (TMS) as an internal standard. Chemical shifts (δ) are reported in ppm, and spin–spin coupling constants (*J*) are given in Hz. Abbreviations to denote the multiplicity of a particular signal are s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Purification of the reaction products was carried out by column chromatography using silica gel (60–120 mesh) and the eluent EtOAc/hexane. Solvents were removed under reduced pressure using a Buchi rotary evaporator.

General Procedure for the Synthesis of Peptides. All peptides were synthesized following the standard Fmoc/*t*Bu-based solid-phase synthesis protocol (SPPS). 2-Chlorotriethyl chloride (2-CT) resin (0.08 mmol), with a loading of 1.6 mmol/g, was used as a solid support for the synthesis. Initially, the resin was washed with DCM (3 \times 1 min). The incorporation of the first Fmoc-protected amino acid was achieved by stirring the mixture of Fmoc-Cys(PG)-OH (0.8 equiv.) and DIEA (10 equiv.) in 700 μ L of DCM with 2-CT resin for 1 h at rt. After 1 h, capping of unreacted chlorotriethyl groups on the resin was done by adding MeOH (50 μ L) to the loaded resin for 30 min. After a wash with DCM (3 \times 1 min) and DMF (3 \times 1 min), the Fmoc group was deprotected by treatment of the resin with 20% piperidine-DMF (1 mL, 1 min + 7 min), followed by washing with DMF. The next protected Fmoc-amino acid (3 equiv.) was incorporated using DIC (3 equiv) and OxymaPure (3 equiv) as coupling reagents in DMF for 1 h at rt. This was repeated until the final peptide was achieved. Cleavage was performed by treating the dried peptidyl resin either with TFA/TIS/H₂O (95:2.5:2.5) for 45 min at rt or with TFA/TIS/H₂O/anisole (90:2.5:2.5:5) for 1 h at rt in the case of linear somatostatin. The cleavage mixture

was then precipitated with Et₂O and centrifuged, and the pellet was redissolved in H₂O/MeCN (1:1) for analysis by HPLC and LC–MS.

Side-Chain Anchoring of Fmoc-Cys-OtBu on 2-CT Resin and DHP Resin. *Synthesis of Fmoc-Cys-OtBu.* Fmoc-Cys-OtBu was synthesized following the procedures as reported earlier,³⁴ with minor modifications regarding the use of reagents. First, Fmoc-Cystine-OH (505.0 mg, 0.738 mmol) was dissolved in a 6 mL mixture of *tert*-butanol and pyridine (7:5), followed by the addition of DCM (10 mL). The reaction mixture was then cooled to 0°C and treated with POCl₃ (6.4 equiv.) under stirring for 30 min. After this, the mixture was brought to rt and stirred for 5 h to afford Fmoc-Cystine-OtBu. The reaction mixture was then quenched with ice water, followed by extraction with ethyl acetate. The combined organic layer thus obtained was washed with water, 5% NaHCO₃ solution, and brine in a successive manner. Finally, the crude product was collected over Na₂SO₄, filtered, evaporated under reduced pressure, and used for the next step directly. Fmoc-Cystine-OtBu was dissolved in MeCN (9 mL), followed by the addition of 1N HCl (30 mL) and Zn powder (40 equiv.) for reduction at rt. The reaction was instantaneous. The reaction mixture was filtered immediately using Celite, followed by extraction with ethyl acetate and water. The organic layer was collected over NaSO₄, filtered, and evaporated under reduced pressure to afford Fmoc-Cys-OtBu. White solid, yield = 473.0 mg.

Side-Chain Anchoring of Fmoc-Cys-OtBu on CTC Resin. Fmoc-Cys-OtBu (1.0 equiv., 0.1 mmol) was dissolved in (700 μ L) DCM with DIEA (2 equiv., 0.2 mmol) and poured onto the CTC resin (0.1 mmol), which was swollen in DCM. The suspension was stirred at rt, and the progress of the reaction was monitored over time by HPLC. Every 30 min, 1 μ L of suspension was withdrawn and filtered, and the filtrate obtained was then dried, dissolved in MeCN, and injected to be analyzed by HPLC. The reaction was complete in 1 h, as reflected by the disappearance of Fmoc-Cys-OtBu. Next, MeOH (50 μ L) was added to the stirring suspension to cap any unreacted 2-CT groups on the resin for. After 15 min, the Fmoc-Cys-OtBu-loaded resin was filtered, washed (DCM), and dried. A small-scale cleavage of the loaded resin was done using TFA/TIS/DCM (2:2:96) for 20 min. The crude obtained after removing TFA was dissolved in MeCN and analyzed by HPLC and LC–MS.

Side-Chain Anchoring of Fmoc-Cys-OtBu on DHP Resin. The solution of Fmoc-Cys-OtBu (3 equiv., 0.06 mmol) and *p*-toluenesulfonic acid (0.1 equiv., 0.06 mmol) in (500 μ L) DCM was added to DHP-HM resin (0.06 mmol) at rt for 1 h. The loading was completed in 1 h, and the resin was then filtered, washed (DCM), and dried. A mini cleavage of the loaded resin was done by treatment with TFA/TIS/DCM (2:2:96) for 20 min. The crude obtained after removing TFA was dissolved in MeCN and analyzed by HPLC and LC–MS.

Synthesis of Boc-Leu-Ala-Cys(2-CT-Resin/DHP-Resin)-OtBu. Fmoc was deprotected from Fmoc-Cys(2-CT resin)-OtBu (60 mg, 0.1 mmol) and Fmoc-Cys(DHP resin)-OtBu (30 mg, 0.1 mmol) by 20% piperidine in DMF (1 min +7 min). Fmoc-Ala-OH (0.1 mmol) was dissolved in DMF and incorporated into both loaded resins using DIC (0.1 mmol) and Oxyma (0.1 mmol) as coupling reagents for 1 h at rt. This was repeated for the coupling of Boc-Leu-OH to achieve Boc-Leu-Ala-Cys(2-CT resin)-OtBu and Boc-Leu-Ala-Cys(DHP resin)-OtBu. The mini cleavages were performed using TFA/

TIS/H₂O and processed as per the general procedures above. The crude peptides obtained were analyzed by HPLC and LC–MS.

Study of Side Reactions on C-Terminal Cys in Boc-Leu-Ala-Cys(PG)-CT Resin. *Preparation of D/L- β -Substituted Alanine Adducts from Boc-Leu-Ala-Cys(StBu)-O-2-CT Resin Using Different Bases.* The peptide Boc-Leu-Ala-Cys(StBu)-O-2-CT (0.32 mmol) resin was synthesized as per the standard Fmoc SPPS protocols described in the general procedure above. After synthesis, the peptidyl resin was divided into five equal portions, taking 10 mg from each. These 5 \times 10 mg portions were then treated with five distinct solutions of X % of base in DMF (1 mL) for 24 h stress studies at rt. Five bases, namely, 20% piperidine, 20% 4-methylpiperidine, 20% pyrrolidine, 20% morpholine, and 10% piperazine in DMF-ethanol (9:1), were tested. After treatments, the peptidyl resins were washed with DMF (5 times), dried, cleaved using TFA/TIS/H₂O (95:2.5:2.5), and analyzed by HPLC and LC–MS.

Study of Side Reactions on C-Terminal Cys in the Model Peptide Boc-Leu-Ala-Cys(Trt)-O-2-CT Resin Using Different Bases. Boc-Leu-Ala-Cys(Trt)-O-2-CT resin (0.08 mmol) was synthesized as per the standard Fmoc SPPS protocols described in the general procedure above. After synthesis, each 10 mg of peptidyl resin (of 15 portions) was then treated with 15 different solutions of base in DMF (1 mL) or solutions of base and OxymaPure in DMF (1 mL) for 24 h stress studies at rt. The types of base solution used included piperidine (10 and 20%), 4 MP (10 and 20%), pyrrolidine (10 and 20%), morpholine (10 and 20%), and piperazine [10% in DMF-ethanol (9:1)]. The combinations of base and OxymaPure used included 10% piperidine with 0.5 M and 1 M OxymaPure, 20% piperidine with 0.5 M and 1 M OxymaPure, 10% 4 MP with 1 M OxymaPure, and 20% 4 MP with 1 M OxymaPure. After the treatments, all the 15 portions of resins were washed with DMF (5 times), dried, cleaved using TFA/TIS/H₂O (95:2.5:2.5), and analyzed by HPLC and LC–MS.

Study of Side Reactions after Each Fmoc-Amino Acid Coupling in the Model Peptide Boc-Leu-Ala-Cys(Trt)-O-2-CT Resin. Boc-Leu-Ala-Cys(Trt)-O-2-CT resin (0.16 mmol) was synthesized using the Fmoc-based SPPS protocols described in the general section above. The Fmoc deprotection condition used during chain elongation was 20% piperidine-DMF (1 min +7 min). After synthesis, the tripeptidyl resin was washed (DMF and DCM), dried, and cleaved (TFA/TIS/H₂O), and the crude product thus obtained was analyzed by HPLC and LC–MS. The Boc-Leu-Ala-Cys(Trt)-O-2-CT resin (10 mg) was subjected to treatment with 1 mL of 20% piperidine-DMF for 24 h stress studies, followed by washing (DMF and DCM), drying, cleavage (TFA/TIS/H₂O), and analysis.

During the synthesis, after the incorporation of the first Fmoc-amino acid, 10 mg of Fmoc-Cys(Trt)-O-2-CT resin was withdrawn and treated with 1 mL of 20% piperidine-DMF for 24 h stress studies. Next, the resin was washed (DMF), and Fmoc-Ala-OH and Boc-Leu-OH were incorporated successively following the DIC/OxymaPure protocol until the Boc-Leu-Ala-Cys(Trt)-O-2-CT resin was achieved. The tripeptidyl resin was then washed (DMF and DCM), dried, cleaved (TFA/TIS/H₂O), and analyzed.

Similarly, after the incorporation of the second Fmoc-amino acid and subsequent Fmoc deprotection, 10 mg of H-Ala-Cys(Trt)-O-2-CT resin was withdrawn and treated with 1 mL of 20% piperidine-DMF for 24 h stress studies. After washing

with DMF, Boc-Leu-OH was incorporated to achieve the Boc-Leu-Ala-Cys(Trt)-O-2-CT resin using the DIC/OxymaPure protocol, as mentioned in the general procedure. The tripeptidyl resin was then washed (DMF and DCM), dried, cleaved (TFA/TIS/H₂O), and analyzed.

STUDY OF SIDE REACTIONS ON C-TERMINAL CYS IN LINEAR SOMATOSTATIN

Synthesis of Linear Somatostatin Using Different Fmoc Removal Conditions. Four batches of protected linear somatostatin, [H-Ala-Gly-Cys(Trt)-Lys(Boc)-Asn(Trt)-Phe-Phe-Trp(Boc)-Lys(Boc)-Thr(*t*Bu)-Phe-Thr(*t*Bu)-Ser(*t*Bu)-L-cys(Trt)-O-2-CT resin], were synthesized as per the standard Fmoc SPPS protocols described in the general procedure above. All were prepared at the same scale (0.16 mmol), and in all cases, the incorporation of the first amino acid was achieved by stirring the mixture of Fmoc-Cys(Trt)-OH (2 equiv.) and DIEA (6 equiv.) in DCM (1 mL) with 2-CT resin for 1 h at rt. Four different base combinations for Fmoc deprotection, namely, 20% piperidine, 20% 4 MP, 20% 4 MP in 1 M OxymaPure, and 20% morpholine, were used for the four batches of protected linear somatostatin-2-CT resin. In all cases, Fmoc deprotection was achieved using 10 min +20 min treatments (standard control conditions). All the syntheses were checked by cleaving the dried peptidyl resins with TFA/TIS/H₂O/anisole (90:2.5:2.5:5) for 1 h at rt.

Study of Side Reactions after Each Fmoc-Amino Acid Coupling in Linear Somatostatin Synthesis. Protected linear somatostatin H-Ala-Gly-Cys(Trt)-Lys(Boc)-Asn(Trt)-Phe-Phe-Trp(Boc)-Lys(Boc)-Thr(*t*Bu)-Phe-Thr(*t*Bu)-Ser(*t*Bu)-L/D-Cys(Trt)-2-CT resin (0.8 mmol) was synthesized using the Fmoc-based SPPS protocols described in the general section above. The D-peptide was synthesized mainly for the development of the method for separating L/D from or within each peptide. Fmoc deprotection was achieved using 20% piperidine-DMF (10 min +20 min). After each amino acid incorporation and/or coupling, 15 mg of resin was kept aside in a separate syringe for further studies. That is, at the end, 14 peptidyl resins were studied: Fmoc-Cys(Trt)-O-2-CT, H-Ser(*t*Bu)-Cys(Trt)-O-2-CT, and H-Thr(*t*Bu)-Ser(*t*Bu)-Cys(Trt)-O-2-CT until the final H-Ala-Gly-Cys(Trt)-Lys(Boc)-Asn(Trt)-Phe-Phe-Trp(Boc)-Lys(Boc)-Thr(*t*Bu)-Phe-Thr(*t*Bu)-Ser(*t*Bu)-L/D-Cys(Trt)-O-2-CT resin. These resins were washed with DMF (three times) and DCM (three times) and then dried. Next, 5 mg of each of the 14 peptidyl resins was cleaved (using TFA/TIS/H₂O or TFA/TIS/H₂O/anisole) individually, and the crude products obtained were analyzed by HPLC and LC–MS. The residual 10 mg of resin was taken from each of the 14 peptidyl resins and treated individually with 1 mL of 20% piperidine-DMF for 8 h stress studies at rt. For Fmoc-Cys(Trt)-O-2-CT resin, after 8 h stress studies with base, Fmoc-Ser(*t*Bu)-OH and Fmoc-Thr(*t*Bu)-OH were incorporated successively following the DIC/OxymaPure protocol until Fmoc-Thr(*t*Bu)-Ser(*t*Bu)-Cys(Trt)-2-CT resin was achieved. For the H-Ser(*t*Bu)-Cys(Trt)-O-2-CT resin, after 8 h stress studies with base, Fmoc-Thr(*t*Bu)-OH was incorporated following the DIC/OxymaPure protocol until the complete linear somatostatin peptidyl resin was achieved. The tripeptidyl resins obtained in this manner were cleaved after Fmoc deprotection and then washed and dried. The crude products obtained were analyzed by HPLC and LC–MS.

INFLUENCE OF THIOL PROTECTING GROUP ON SIDE REACTIONS OF C-TERMINAL CARBOXYLIC ACID CYS

Synthesis of Fmoc-Cys(Mtbh)-OH. Fmoc-Cys-OH (100 mg, 1 equiv.) and Mtbh (80 mg, 1 equiv.) were placed in a round-bottomed flask equipped with a stirrer bar. Neat TFA (1 mL) was then added and stirred for 1 h at rt. After that, TFA was evaporated, washed with *n*-hexane, and cooled to 0 °C (3 times) and kept under vacuum to dry. The crude product was dissolved in MeCN/H₂O (20:80) and freeze-dried to afford a light blue fluffy solid, yield = 167.0 mg (96%), HPLC *t*_R = 13.54 min; ¹H NMR (600 MHz, CDCl₃): δ = 7.75 (d, *J* = 7.2 Hz, 2H; ArH), 7.59 (dd, *J* = 7.5 Hz, 2H; ArH), 7.38 (dd, *J* = 6.6 Hz 2H; ArH), 7.25 (m, *J* = 7.6 Hz 6H; ArH), 7.13 (t, *J* = 7.9 Hz, 4H; ArH), 5.61 (d, *J* = 7.9 Hz 1H), 5.28 (s, 1H), 5.13 (s, 1H), 4.58 (dd, *J* = 7.03 Hz, 1H), 4.40 (dd, *J* = 7.07 Hz, 2H), 4.22 (t, *J* = 6.89 Hz, 1H), 2.89 (dd, *J* = 7.07 Hz, 1H), 2.81 (dd, *J* = 6.01 Hz, 1H), 2.41 (s, 3H), 2.39 (s, 3H) ¹³C NMR (150 MHz, CDCl₃): 174.8, 155.9, 143.8, 143.6, 141.3, 137.8, 137.2, 128.7, 127.8, 127.1, 126.7, 125.1, 125.0, 120.0, 67.4, 53.4, 47.1, 34.1, 29.7, 15.7.

LC–MS: *m/z*: calcd For C₃₃H₃₁NO₄S₃: 624.13 [M + Na]⁺; found, 624.10 [M + Na]⁺

Study of Side Reactions on C-Terminal Cys in Boc-Leu-Ala-Cys(PG)-O-2-CT Using Different Protecting Groups. Boc-Leu-Ala-Cys(PG)-O-2-CT resins were synthesized using six distinct Fmoc-protected Cys residues, where PG stands for protecting groups Mmt, Thp, Dpm, Mtbh, Phacm, and Msbh. The syntheses were performed as per the standard Fmoc SPPS protocols described in the general procedure above, and 20% piperidine-DMF was used for Fmoc deprotection (1 min +7 min) during chain elongation. After synthesis, the peptidyl resins were washed (DMF, DCM), dried, cleaved, and analyzed.

Each of the Boc-protected tripeptidyl resins (10 mg) with different Cys residues was treated separately with 1 mL of 20% piperidine-DMF. The peptidyl resins were then treated with base for 24 h stress studies at rt. The treated resins were then washed (DMF and DCM), dried, cleaved (TFA/TIS/H₂O), and analyzed.

Reduction of Msbh in Boc-Leu-Ala-Cys(Msbh)-O-2-CT Resin. 1% Me₂S solution (1 mL) in TFA was added to the Boc-Leu-Ala-Cys(Msbh)-O-2-CT resin (20 mg) at 0 °C under stirring. Next, NH₄I (300 mg) was added, and the reaction mixture was stirred for 45 min. Thereafter, the reaction mixture was filtered, precipitated with cold Et₂O, and centrifuged, and the pellet was redissolved in H₂O for reduction with tris(2-carboxyethyl)phosphine (TCEP) for 25 min at rt. The progress of the reduction was monitored by HPLC and LC–MS.

Study of Side Reactions on C-Terminal Cys in Linear Somatostatin Resin Using Different Protecting Groups and Different Fmoc Removal Conditions. Protected linear somatostatin H-Ala-Gly-Cys(Trt)-Lys(Boc)-Asn(Trt)-Phe-Phe-Trp(Boc)-Lys(Boc)-Thr(*t*Bu)-Phe-Thr(*t*Bu)-Ser(*t*Bu)-Cys(PG)-2-CT resins were synthesized using four distinct Fmoc-protected Cys residues on the C-terminus, where PG stands for protecting groups Trt, Mmt, and Thp. The syntheses were performed as per the standard Fmoc SPPS protocols described in the general procedure above. Three protected linear somatostatin-2-CT resins were synthesized using three different Cys(PG) residues [PG = Trt, Mmt, and Thp] at the

C-terminal Cys and using 20% piperidine-DMF (10 min +20 min) for Fmoc deprotection. Along with these three, two additional protected linear somatostatin-2-CT resins were synthesized using two distinct Cys(PG) residues [PG = Mmt and Thp] at the C-terminus. In these two cases, 30% 4 MP in 0.5 M OxymaPure-DMF (10 min +20 min) was used for Fmoc deprotection. After the syntheses, all five peptidyl resins were washed (DMF and DCM), dried, cleaved (TFA/TIS/H₂O/anisole), and analyzed.

Three of the protected linear somatostatin-2-CT resins (10 mg from each) with three different Cys residues [PG = Trt, Mmt, and Thp] at the C-terminus were treated separately with 1 ml of a solution of 20% piperidine-DMF. The pre-swelled resins were treated with the base for 8 h stress studies at rt. Similarly, 10 mg of two of the protected linear somatostatin-2-CT resins with two different Cys residues [PG = Mmt and Thp] at the C-terminus were treated separately with 1 ml of a solution of 30% 4 MP in 0.5 M OxymaPure-DMF for 8 h at rt. After treatments, all the five peptidyl resins were washed with DMF (5 times), dried, cleaved (TFA/TIS/H₂O/Anisole), and analyzed.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.oprd.2c00321>.

HPLC, LC–MS, and NMR characterization data of compounds (PDF)

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Author Contributions

The strategy was designed by all of the authors. Experimental work was performed by S.N.M and A.C. All of the authors discussed the results and prepared the manuscript. All authors approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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