ORGANIC LETTERS

2012
Vol. 14, No. 21
5468–5471

Trimethoxyphenylthio as a Highly Labile Replacement for *tert*-Butylthio Cysteine Protection in Fmoc Solid Phase Synthesis

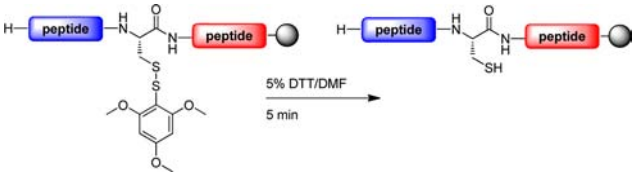
Tobias M. Postma,[†,‡] Matthieu Giraud,[§] and Fernando Albericio*[,†,‡,∥,⊥]

Institute for Research in Biomedicine, Barcelona, 08028, Spain, CIBER-BBN, 08028-Barcelona, Spain, Department of Organic Chemistry, University of Barcelona, 08028, Barcelona, Spain, Lonza Ltd., Visp, VS 3930, Switzerland, and School of Chemistry, University of KwaZulu Natal, 4001-Durban, South Africa

fernando.albericio@irbbarcelona.org

Received September 16, 2012

ABSTRACT



Trimethoxyphenylthio (*S*-Tmp) is described as a novel cysteine protecting group in Fmoc solid phase peptide synthesis replacing the difficult to remove *tert*-butylthio. *S*-Tmp and dimethoxyphenylthio (*S*-Dmp) were successfully used for cysteine protection in a variety of peptides. Moreover, both groups can be removed in 5 min with mild reducing agents. *S*-Tmp is recommended for cysteine protection, as it yields crude peptides of high purity.

Multiple disulfide containing peptides are ubiquitous in nature and therapeutically relevant because of their selective and potent bioactivities (e.g., conotoxins, cyclotides).[1,2] The synthesis of multiple disulfide containing peptides requires the use of orthogonal cysteine (Cys) protection strategies to ensure the correct disulfide connectivity.[3,4] Unfortunately, there is a lack of orthogonal cysteine protecting groups that can be used in routine SPPS under mild conditions.[5]

The concept of Cys protecting groups labile to mild reducing agents is highly promising due to orthogonality to other Cys protecting groups. The commercial Cys protecting group *tert*-butylthio (*S*tBu) is orthogonal to all other Cys protecting groups due to its mild deprotection conditions.[6] This protecting group can be removed with mild reducing agents (e.g., thiols or phosphines) and it is stable to piperidine, hence compatible with Fmoc/tBu peptide synthesis. However, owing to exceedingly long deprotection times (4−24 h), *S*tBu cannot be used in routine SPPS. As a result of the proximity of bulky protecting groups and sensitivity to certain amino acid sequences prone to folding, in some cases this protecting group has proven to be very difficult or even impossible to remove.[7,8] Thus, in previous studies on Linaclotide, a 14-residue peptide containing three disulfide bonds, we observed that *S*tBu groups on Cys2-Cys10 were not possible to remove.[7] Denis and Trifilieff used harsh conditions, heating a peptidyl resin to 135 °C for 24 h; however, they achieved only

[†] Institute for Research in Biomedicine.
[‡] CIBER-BBN.
[§] University of Barcelona.
[∥] Lonza Ltd.
[⊥] University of KwaZulu Natal.

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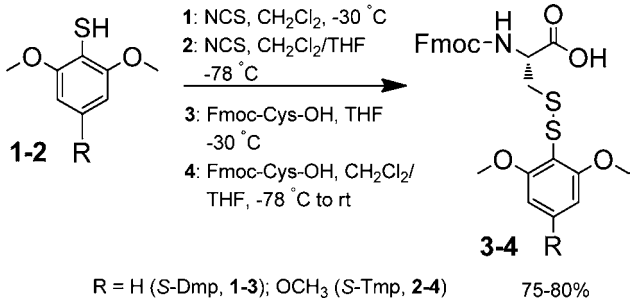
10.1021/ol3025499
Published on Web 10/17/2012

partial *S*tBu deprotection.[8] Additionally, reports of desulfurization of *S*tBu protected Cys to dehydroalanine, by means of prolonged exposure to reducing agents, illustrates the limitations of this protecting group.[9]

Given the importance of having a Cys protecting group removable by reducing agents to fulfill the orthogonal scheme for Cys and the significant limitations of *S*tBu, we have addressed novel reduction labile Cys protecting groups. As scaffolds for the preparation of mixed disulfides we initially studied phenyl and benzyl derivatives. The former were not stable to base while the latter were clearly not stable to acid (data not shown). The benzyl derivatives were discarded, and the phenyl group was modified to contain alkoxy groups on the 2,6 positions in order to study the balance between high lability to reducing agents and base stability (Scheme 1).

Scheme 1. Synthesis of Fmoc-Cys(*S*-Dmp)-OH (**3**) and Fmoc-Cys(*S*-Tmp)-OH (**4**)



The synthesis of Fmoc-Cys(*S*-Dmp)-OH and Fmoc-Cys(*S*-Tmp)-OH is shown in Scheme 1. 2,6-Dimethoxythiophenol was prepared following the literature, and 2,4,6-trimethoxythiophenol was synthesized with minor modifications of the same procedure.[10] The key reaction to the mixed disulfide containing Cys was inspired by a reaction used by Kraus and Jeon where *N*-chlorosuccinimide (NCS) reacts with thiophenol to form a highly reactive sulfenyl chloride.[11]

Fmoc-Cys(*S*-Dmp)-OH was prepared by forming a sulfenyl chloride from 2,6-dimethoxythiophenol. This sulfenyl chloride was subsequently added to a solution of Fmoc-Cys-OH, where nucleophilic attack on the sulfenyl chloride by Cys yields the mixed disulfide. The sulfenyl chloride of 2,4,6-trimethoxythiophenol was unstable at −30 °C and had to be formed in the presence of Fmoc-Cys-OH at −78 °C to obtain Fmoc-Cys(*S*-Tmp)-OH.

In order to compare the efficiency of *S*-Dmp and *S*-Tmp to *S*tBu, and SPPS compatibility, we prepared several model tripeptides (Figure 1). The model tripeptide Fmoc-Ala-Cys-Ala-NH_2 was already used in our laboratory to assess the Cys protecting group on resin. Cys residues

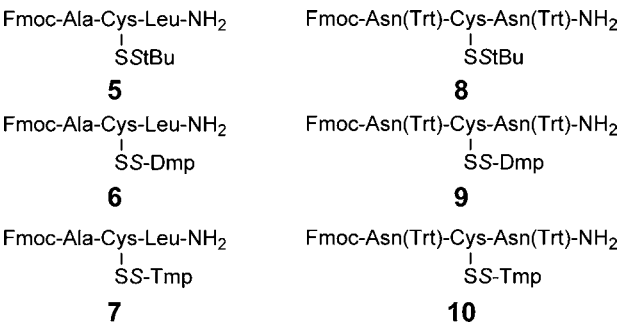


Figure 1. Tripeptides protected with *S*tBu, *S*-Dmp, and *S*-Tmp.

protected with *S*-Dmp, *S*-Tmp, and *S*tBu were incorporated in the model tripeptides by SPPS, using diisopropylcarbodiimide (DIC) and Oxyma Pure with 5 min of preactivation to prevent racemization of the Cys residue.[12]

Table 1. On-Resin Deprotection of Model Tripeptides[a]

peptidyl-resin	deprotection time (min) with BME	deprotection time (min) with DTT
5 (*S*tBu)	180	5% after 24 h
6 (*S*-Dmp)	5	5
7 (*S*-Tmp)	5	5
8 (*S*tBu)	360	2% after 24 h
9 (*S*-Dmp)	5	5
10 (*S*-Tmp)	5	5

[a] β-Mercaptoethanol (BME), Dithiothreitol (DTT). Deprotection conditions: 0.1 M NMM, BME (20%), or DTT (5%) in DMF.

The stability of the protecting groups to piperidine and trifluoroacetic acid (TFA), conditions used in routine SPPS, was evaluated. All protecting groups were found to be stable to 20% piperidine/DMF for 4 h, which is sufficient for routine applications. The peptides were cleaved from the resin with 95% TFA for 1 h at rt. The protecting groups were stable to these conditions.

Subsequently, the lability of these groups to reducing agents was studied. We found that the most efficient deprotection mixtures contained *N*-methylmorpholine (NMM) (0.1 M) and either the malodorous β-mercaptoethanol (BME) (20%) or nonmalodorous dithiothreitol (DTT) (5%) in DMF.[13] Both deprotection mixtures achieved quantitative removal of *S*-Dmp and *S*-Tmp from Fmoc-Ala-Cys(PG)-Leu-NH_2 model tripeptides in 5 min. In contrast, 3 h were required for quantitative *S*tBu removal using BME and practically no deprotection occurred with deprotection mixtures containing DTT (Table 1).

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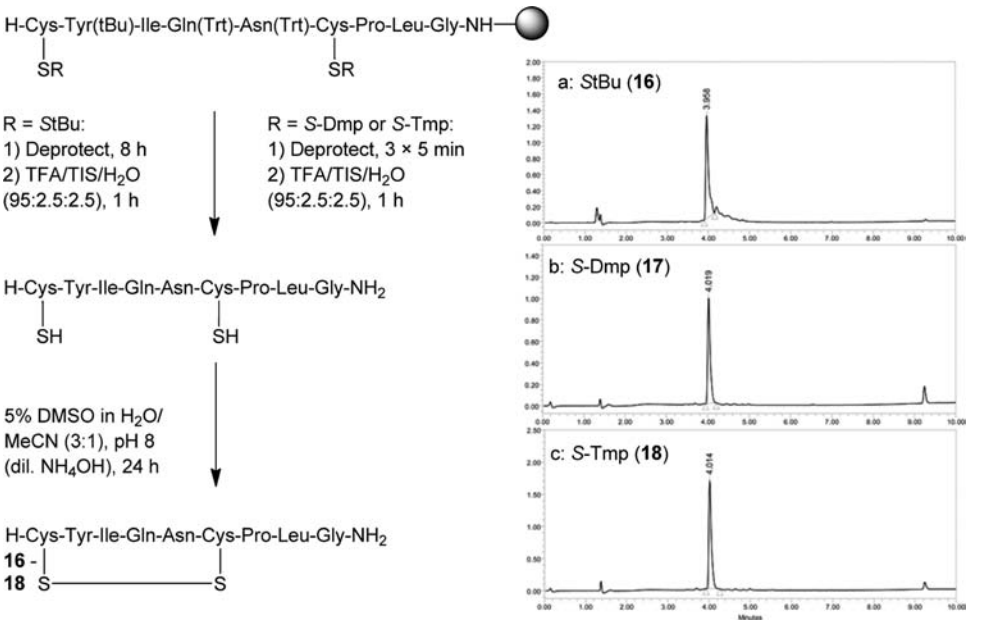
(13) No on-resin reduction was observed with TCEP in DMF and DMF/H_2O mixtures (low DMF solubility). We chose not to work with tributylphosphine to avoid possible desulfurization of Cys (ref 9).

Scheme 2. Oxytocin Synthesis from Linear Oxytocin Protected with *S*tBu (a), *S*-Dmp (b), and *S*-Tmp (c) on a Rink Amide AM Resin[a]



[a] *S*tBu removal: 0.1 M NMM in BME/DMF (1:4). *S*-Dmp/*S*-Tmp removal: 0.1 M NMM in DTT/DMF (5:95).

The deprotection efficiency for the removal of *S*tBu, *S*-Dmp, and *S*-Tmp, when flanked by bulky trityl (Trt) protecting groups, was studied. The model tripeptide Fmoc-Asn(Trt)-Cys(PG)-Asn(Trt)-NH_2 was used to determine whether bulky protecting groups influence deprotection times. The resin containing the peptides was treated with the BME deprotection mixture. The deprotection time for *S*tBu doubled, while those for *S*-Dmp and *S*-Tmp remained unchanged. On the other hand when the DTT mixture was used, both *S*-Dmp and *S*-Tmp were removed very gently in 5 min, while only 5% of *S*tBu was removed after 24 h. For subsequent on-resin deprotection, we applied 3 × 5 min treatments to ensure quantitative removal of *S*-Dmp and *S*-Tmp.

These promising results led us to examine a longer model peptide. For this purpose, we chose oxytocin,[14] an extensively studied nonapeptide containing two Cys residues and currently used as a drug to induce labor. A pair of Cys residues protected with *S*tBu, *S*-Dmp, or *S*-Tmp were incorporated into oxytocin. Treatment of the peptidyl-resin with 20% piperidine/DMF followed by cleavage with TFA/TIS/H_2O (95:2.5:2.5) led to the unexpected monodeprotection of *S*-Dmp (13%) and *S*-Tmp (8%). This process was not observed during the studies with the model tripeptides, and the mechanism of deprotection was unclear. However, we suspected that the monodeprotection was caused by a high concentration of TFA.

To confirm this notion, oxytocin was resynthesized with protecting group *S*-Dmp or *S*-Tmp on a Sieber Amide Resin. Previously, a Rink Amide resin was used. This support requires a cleavage mixture containing 95% TFA, whereas 1−5% TFA is sufficient to achieve peptide cleavage from Sieber Amide resin. The peptidyl-resin containing oxytocin protected with *S*-Dmp or *S*-Tmp was treated with 20% piperidine/DMF followed by cleavage with 1% TFA. Subsequent analysis showed no monodeprotection for either protecting groups, thereby corroborating the instability to high TFA concentrations. The partial instability of these protecting groups to high TFA concentrations is not detrimental for their use in peptide synthesis because in multiple disulfide containing peptides these groups are predominantly removed on-resin. Due to their deprotection mechanism, *S*-Dmp and *S*-Tmp must be removed first in orthogonal Cys protecting group strategies; otherwise the reducing agents required for deprotection would reduce the other disulfide bonds present. Hence, these protecting groups are removed on-resin prior to cleavage. Thus, in SPPS, the end of the synthesis involves removal of the Fmoc group, removal of the Cys protecting groups (*S*-Dmp/*S*-Tmp), and then either oxidation followed by cleavage or cleavage with subsequent oxidation in solution.

Linear oxytocin protected with *S*tBu, *S*-Dmp, or *S*-Tmp was deprotected on-resin (3 × 5 min, 0.1 M NMM in 5% DTT/DMF) for *S*-Dmp and *S*-Tmp, whereas 8 h (0.1 M NMM in 20% BME/DMF) were needed for *S*tBu deprotection (Scheme 2). The linear peptides were cleaved from the resin and oxidized in 5% DMSO in H_2O/CH_3CN (3:1) for 24 h. The peptides protected with *S*tBu, *S*-Dmp, and *S*-Tmp were obtained in 72%, 86%, and 94% purity after lyophilization. Given that the best results were obtained with *S*-Tmp, we focused on this protecting group.

To further demonstrate the applicability of *S*-Tmp, we synthesized the 18-residue T22 peptide containing 4 *S*-Tmp

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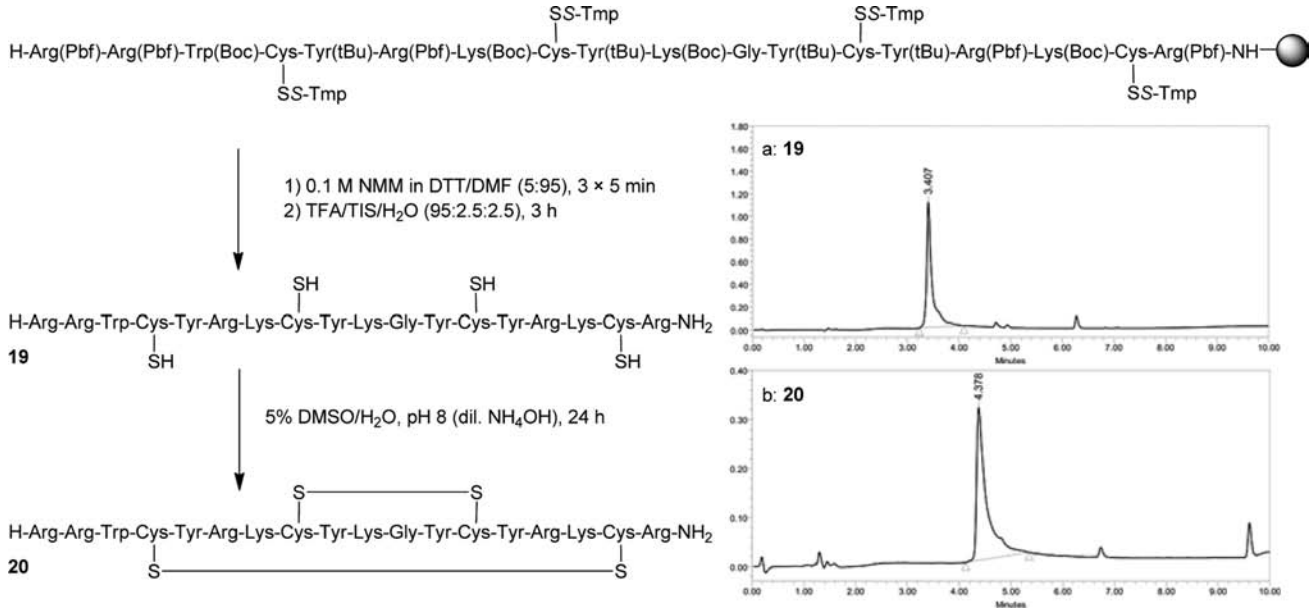
Scheme 3. Synthesis of T22 Using *S*-Tmp Protected Cys on a ChemMatrix Resin



protected Cys residues.[15] In a recent study we demonstrated that the T22 peptide folds into its native conformation by oxidative folding.[16] This peptide contains five arginine and three lysine residues, thus rendering it highly hydrophilic. We were unable to properly synthesize the peptide on a polystyrene based Rink Amide resin and required a hydrophilic polyethylene glycol based ChemMatrix resin for this purpose. Following peptide elongation, *S*-Tmp was removed on-resin (3 × 5 min, 0.1 M NMM, 5% DTT in DMF) and cleaved from the resin with TFA/TIS/H_2O (95:2.5:2.5) for 3 h to ensure complete deprotection of the five Pbf protecting groups (Scheme 3). The linear peptide was oxidized in solution using 5% DMSO in water at pH 8.0 for 24 h at rt and subsequently lyophilized to give crude T22 in 78% purity.

Both *S*-Dmp and *S*-Tmp are stable to base, SPPS coupling conditions, and a low concentration of TFA. In comparison to *S*tBu, *S*-Dmp and *S*-Tmp proved to be superior Cys protecting groups. They are labile to mild reducing agents and have rapid deprotection times of 5 min, which contrasts with the hours required for *S*tBu removal. No changes were observed in the deprotection times when the protecting group was flanked by bulky Trt groups. In concusion, we recommend the use of *S*-Tmp over *S*-Dmp because it showed greater stability, gave the purest product in the synthesis of oxytocin, and was successfully used in T22 synthesis.

Acknowledgment. We are grateful to Miriam Góngora-Benítez from the Institute for Research in Biomedicine for academic input. We thank the European community - FP7-PEOPLE-ITN-2008 for financial support. We also thank Dr. Thomas Bruckdorfer from IRIS Biotech GmbH for fruitful discussions.

Supporting Information Available. Experimental details and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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