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Acid-Labile Cys-Protecting Groups for the Fmoc/*t*Bu Strategy: Filling the Gap

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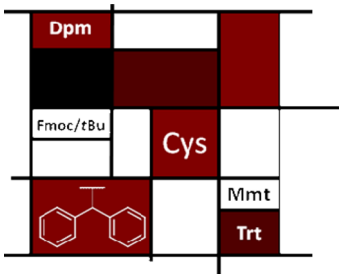
ABSTRACT



To address the existing gap in the current set of acid-labile Cys-protecting groups for the Fmoc/*t*Bu strategy, diverse Fmoc-Cys(PG)-OH derivatives were prepared and incorporated into a model tripeptide to study their stability against TFA. *S*-Dpm proved to be compatible with the commonly used *S*-Trt group and was applied for the regioselecive construction of disulfide bonds.

Since the early days of peptide chemistry, the effective synthesis of natural or non-natural isomers, analogues, or *de novo* designed peptides with complex disulfide bridge patterns has been a demanding task. The oxidative folding[1] of fully deprotected linear peptides is a desirable and commonly applied approach for the synthesis of complex Cys-rich peptides. However, achievement of the desired disulfide bond connectivity through this approach is not always affordable. To overcome these challenging syntheses, a myriad of protecting groups for the β-thiol group of Cys, along with efficient regioselective protection schemes, have been developed.[2]

In recent years, several acid-labile Cys-protecting groups have been developed for the Fmoc/*t*Bu strategy (Figure 1).[3] Most of these are highly sensitive to acid, the *S*-Trt group being one of the most commonly used in the Fmoc/*t*Bu approach. In contrast, the *S*-Mob group requires a high TFA concentration and harsh conditions (high temperature and long reaction times) to be fully removed. In this regard, the current gap between *S*-Trt and *S*-Mob groups captured our attention and prompted us to browse through acid-labile protecting groups to find Cys-protecting groups that, ideally, could be quantitatively removable under mild acidic conditions and, simultaneously, show compatibility with *S*-Trt for their further application in synthetic strategies for the preparation of Cys-rich peptides. Thus, three distinct scaffolds, namely diphenylmethyl, biphenylmethyl, and benzyl groups, were selected and finely tuned for this purpose. Twelve Fmoc-Cys(PG)-OH (**1a**–**l**) were prepared and incorporated into the model tripeptide Fmoc-Ala-Cys(PG)-Leu-NH_2 (**2a**–**l**),

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(1) (a) Moroder, L.; Besse, D.; Musiol, H. J.; Rudolph-Böhner, S.; Siedler, F. *Biopolymers (Pept. Sci.)* **1996**, *40*, 207. (b) Narayan, M.; Welker, E.; Wedemeyer, W. J.; Scheraga, H. A. *Acc. Chem. Res.* **2000**, *33*, 805. (c) Anfinsen, C. B. *Biochem. J.* **1972**, *128*, 737.

(2) (a) Barany, G.; Merrifield, R. B. Solid-phase peptide synthesis. In *The Peptides*; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1979; Vol. 2, pp 1–84. (b) Andreu, D.; Albericio, F.; Solé, N. A.; Munson, M. C.; Ferrer, M.; Barany, G. In *Methods in Molecular Biology: Peptide Synthesis Protocols*; Pennington, M. W., Dunn, B. M., Eds.; Humana Press Inc.: Totowa, NJ, 1994; Vol. 35, pp 91–169.

(3) (a) Isidro-Llobet, A.; Álvarez, M.; Albericio, F. *Chem. Rev.* **2009**, *109*, 2455–2504. (b) Boulègue, C.; Musiol, H. J.; Prasad, V.; Moroder, L. *Chem. Today* **2006**, *24*, 24.

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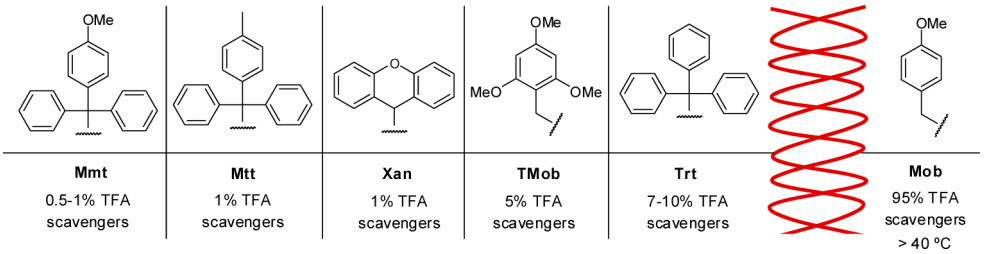
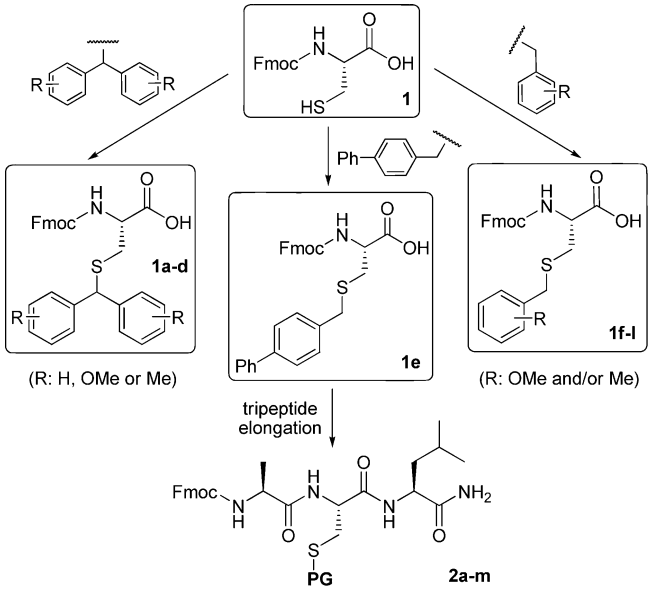


Figure 1. Acid-labile Cys-protecting groups for Fmoc/*t*Bu chemistry.

and their lability against TFA was studied and compared with the *S*-Mob group (**2m**) (Scheme 1).

Scheme 1. Preparation of Fmoc-Cys(PG)-OH Derivatives and Their Successive Incorporation into a Standard Tripeptide

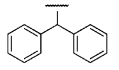
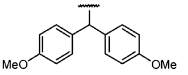
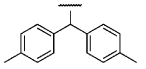
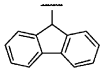
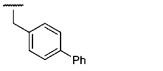
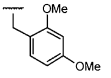
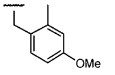
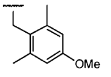
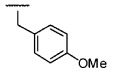


After elongation on a Sieber amide resin, the standard tripeptides were cleaved from the resin and TFA-lability studies were carried out in solution with a range of reaction times and temperatures, in the presence of 2.5% TIS and 2.5% H_2O as scavengers. The tripeptides were then analyzed by RP-HPLC to determine the percentage of deprotected Cys (Table 1).

As expected, the *S*-Mob protecting group was stable against diluted TFA treatments and required a high concentration of TFA, a longer reaction time, and an increase of temperature up to 40 °C to be totally removed (**2m**). Pleasantly, the diphenylmethyl (Dpm) group **1a**, along with two decorated benzyl moieties—**1h** with a *p*-methoxy and an *o*-methyl group and **1i** with two *o*-methoxy groups—exhibited the desired lability against TFA (**2a**, **2h**–**i**), while the others were not labile to TFA treatments (**2d**–**f**, **2j**) or were highly sensitive to TFA (**2b**–**c**, **2g**, **2k**–**l**). Furthermore, the three protecting groups **1a**, **1h**, and **1i** were stable under *S*-Trt cleavage conditions (10% TFA, **2a**, **2h**–**i**). This outcome demonstrates the compatibility of these groups with *S*-Trt for regioselective disulfide construction.

Among the three promising protecting groups for Cys, the easily synthetically accessible *S*-Dpm was chosen as an alternative to the *S*-Mob group for advanced studies.[4]

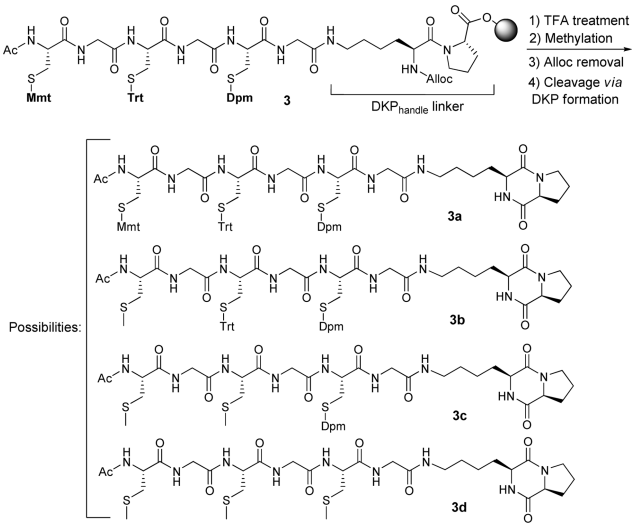
Table 1. TFA-Lability Study of the Tripeptides **2**

	PG	TFA (%)	temp (°C)	reaction time	deprotected Cys (%)
a		10	25	5 min	0
		60	25	1 h	100
b		10	25	5 min	100
c		10	25	5 min	29
		20	25	30 min	100
d		10	25	5 min	0
		95	40	2 h	0
e		10	25	5 min	0
		95	40	2 h	0
f		10	25	5 min	0
		95	40	2 h	0
g		10	25	5 min	17
		20	25	30 min	100
h		10	25	5 min	0
		50	25	1 h	100
i		10	25	5 min	0
		50	25	1 h	100
j		10	25	5 min	0
		95	25	1 h	21
k		10	25	5 min	7
		20	25	30 min	100
l		10	25	5 min	9
		20	25	30 min	100
m		10	25	5 min	0
		95	40	2 h	100

(4) *S*-Mob and *S*-Dpm groups are not compatible. Under the cleavage conditions of *S*-Dpm, 22% *S*-Mob removal was observed.

Although *S*-Dpm was described as a Cys-protecting group by Photaki et al. back in 1970,[5] use of this group in Fmoc/*t*Bu chemistry has not been tackled until now.

Scheme 2. Compatibility Study of Protecting Groups



Before further studies, the absence of racemization during Cys(Dpm) incorporation was proven (see Supporting Information (SI)). Next, the compatibility of *S*-Dpm with *S*-Trt and the highly sensitive acid-labile *S*-Mmt groups were thoroughly examined through a single experiment. Thus, a hexapeptide, which contained three Cys residues, was elongated onto a DKP$_{handle}$ linker, which allowed total free acid cleavage[6] (Scheme 2, hexapeptide **3**). After TFA treatment, the free thiol groups were methylated, and the Alloc group was then removed from the α-*N* of the Lys residue, and the peptidyl-resin was treated with piperidine/THF to render the *C*-terminal DKP$_{handle}$-protected hexapeptides (**3a**–**d**). At 10% TFA in the presence of 2.5% TIS as a scavenger, the *S*-Trt and *S*-Mmt groups were fully removed on solid phase. In contrast, *S*-Dpm was stable under these acidic conditions, requiring up to 90% TFA and 2.5% TIS for its entire removal (Table 2). It is worth mentioning that in any case the selective removal of *S*-Mmt vs *S*-Trt was not achieved, thereby showing the incompatibility of these protecting groups.[7]

After confirming the compatibility of *S*-Dpm with the *S*-Trt and *S*-Mmt groups, we were encouraged to apply the *S*-Dpm/*S*-Trt and *S*-Dpm/*S*-Mmt combinations in the protection scheme for the regioselective construction of intra- and intermolecular disulfide bridges. Thus, the regioselective syntheses of a double-chain bis-cystinyl

Table 2. Lability Study of the Acid-Labile *S*-Mmt, *S*-Trt, and *S*-Dpm Groups on Solid Phase[a]

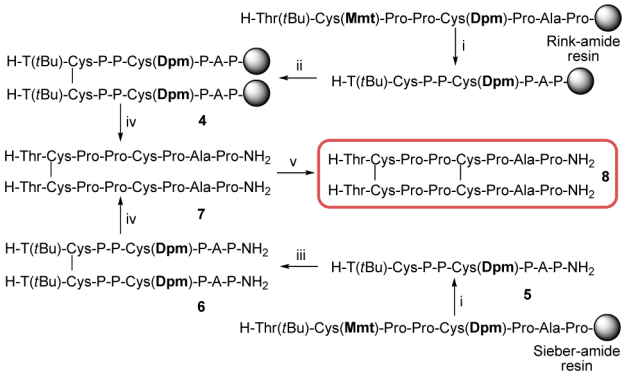
TFA (%)	scavenger	reaction time	**3a** (%)	**3b** (%)	**3c** (%)	**3d** (%)
1	–	3 × 5 min	45	45	10	–
2	–	3 × 5 min	25	50	25	–
10	2.5% TIS	3 × 5 min	–	–	100	–
60	2.5% TIS	1 h	–	–	30	70
90	2.5% TIS	1 h	–	–	–	100

[a] All experiments were carried out with 15 mg of peptidyl-resin.

fragment 225–232/225′–232′ of the human immunoglobulin G1 (IgG1)[1a,8] combining *S*-Mmt and *S*-Dpm, along with the preparation of the α-conotoxin ImI[9] combining the *S*-Trt and *S*-Dpm groups, were carried out.

The hinge fragment of IgG1 was accomplished following two strategies in parallel (Scheme 3). In the first approach (Rink-amide resin), the *S*-Mmt group was selectively removed by diluted TFA treatments and the first disulfide bond was achieved by piperidine/DMF (1:4) on solid phase. The anchored *S*-Dpm-protected dimer **4** was then treated with TFA/TIS/H_2O (95:2.5:2.5) for 1 h at 25 °C, and the resultant fully deprotected intermediate **7** was redissolved in DMSO/phosphate buffer (1:4) at pH 9 to render the final cyclic parallel dimer **8** (see SI).

Scheme 3. Regioselective Syntheses of Hinge Fragment of IgG1 Combining *S*-Mmt and *S*-Dpm Protecting Groups[a]



[a] (i) TFA/TIS/CH_2Cl_2 (5:2.5:92.5) (5 × 1 min); (ii) piperidine/DMF (1:4); (iii) H_2O/ACN (1:9), 20% DMSO at pH 9; (iv) TFA/TIS/H_2O (95:2.5:2.5) for 1 h at 25 °C; and (v) DMSO/phosphate buffer (1:4) at pH 9 and 25 °C.

In the second approach (Sieber-amide resin), the linear partial *S*-Dpm-protected peptide **5** was obtained by diluted TFA treatments. Subsequently, the first disulfide bond was accomplished in solution to render the protected dimer intermediate **6**. The second disulfide bridge was achieved as described before to render the bis-cystinyl parallel dimer **8** (Figure 2).

(5) Photaki, I.; Taylor-Papadimitrious, J.; Sakarellos, C.; Mazarakis, P.; Zervas, L. *J. Chem. Soc. (C)* **1970**, 2683.

(6) Góngora-Benítez, M.; Cristau, M.; Giraud, M.; Tulla-Puche, J.; Albericio, F. *Chem. Commun.* **2012**, *48*, 2313.

(7) Although it has been reported in the literature that *S*-Mmt can be selectively removed in the presence of *S*-Trt, actually it has no practical use because the safety window is so narrow that conditions should be carefully optimized and these are, therefore, not of general application.

(8) Wünsch, E.; Moroder, L.; Göhring-Romani, S.; Musiol, H.-J.; Göhring, W.; Bovermann, G. *Int. J. Peptide Protein Res.* **1988**, *32*, 268.

(9) (a) McIntosh, J. M; Yoshikami, D.; Mahe, E.; Nielsen, D. B.; Rivier, J. E.; Gray, W. R.; Olivera, B. M. *J. Biol. Chem.* **1994**, *269*, 16733. (b) Nielsen, J. S.; Buczed, P.; Bulaj, G. *J. Pept. Sci.* **2004**, *10*, 249.

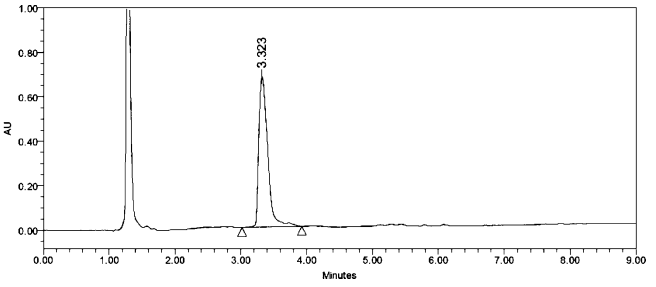
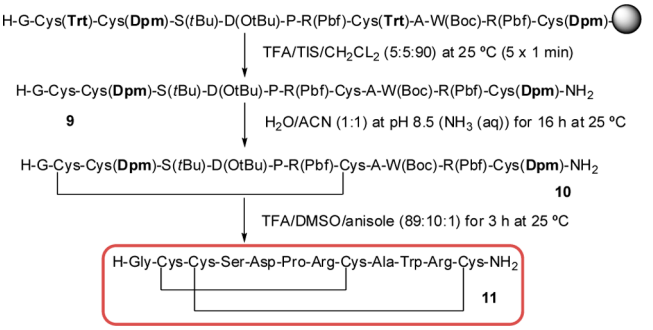


Figure 2. RP-HPLC analysis of the synthesized bis-cystinyl parallel dimer **8** from the Sieber-amide approach.

Although oxidative folding conditions can be carefully refined to provide a major isomer, the regioselective synthesis of the two-disulfide-containing α-conotoxin family[10] members allows the correct construction of disulfide bond pattern present in their biologically active isomers to be ensured.[9,11] Thus, the α-conotoxin ImI, a 12-mer peptide, which contains two disulfide bridges (2Cys-8Cys and 3Cys-12Cys), was prepared by combining two *S*-Dpm and two *S*-Trt for the protection of the Cys residues (Scheme 4). After completion of the peptide elongation

Scheme 4. Regioselective Synthesis of α-Conotoxin ImI Combining *S*-Trt and *S*-Dpm Protecting Groups



on a Sieber-amide resin, the partial *S*-Dpm-protected intermediate **9** was cleaved from the resin by diluted TFA treatments and the construction of the first disulfide bond was achieved in H_2O/ACN (3:7) at pH 8 for 16 h at 25 °C, as determined by RP-HPLC analysis (see SI). At this

(10) (a) Myers, R. A.; Zafaralla, G. C.; Gray, W. R.; Abbott, J.; Cruz, L. J.; Olivera, B. M. *Biochemistry* **1991**, *30*, 9370. (b) Arias, H. R.; Blanton, M. P. *Int. J. Biochem. Cell Biol.* **2000**, *32*, 1017. (c) Janes, R. W. *Curr. Opin. Pharmacol.* **2005**, *5*, 280.

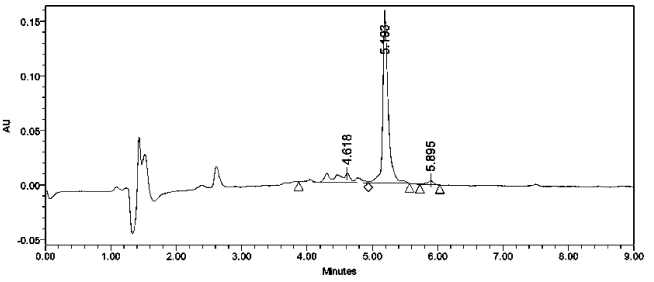


Figure 3. RP-HPLC analysis of the synthesized α-conotoxin ImI **11** combining *S*-Dpm and *S*-Trt protecting groups.

point, various conditions were attempted to obtain the final bicyclic peptide. When the total deprotection and oxidation steps were performed consecutively, a mixture of two isomers was identified by RP-HPLC and RP-HPLC-ESMS analysis, while a single peak corresponding to the expecting isomer **11** was observed when the two steps were performed following a one-pot strategy (Figure 3).

In summary, the *S*-Dpm protecting group is an alternative to the *S*-Mob group. *S*-Dpm can be fully deblocked under the standard conditions used for cleavage and total deprotection steps in Fmoc chemistry, and it is fully compatible with two commonly used acid-labile protecting groups such as *S*-Trt and *S*-Mmt. Here we successfully applied *S*-Dpm for the regioselective synthesis of peptides containing intra- and intermolecular disulfide bonds. These results could be extrapolated to the other two protecting groups **1h** and **1i**.

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Supporting Information Available. Experimental procedures, compound characterization, RP-HPLC analyses, and spectral data of the Fmoc-Cys(PG)-OH derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

(11) (a) Walewska, A.; Bulaj, G. Mechanisms of the Oxidative Folding of Small, Disulfide-Rich Peptides. In *Oxidative Folding of Proteins and Peptides*; Moroder, L., Ed.; Royal Chemical Society: Cambridge, U.K., 2008. (b) Bulaj, G.; Olivera, B. M. *Antioxid. Redox Signal.* **2008**, *10*, 141.

The authors declare no competing financial interest.