Available online at www.sciencedirect.com
ScienceDirect
Tetrahedron Letters
Tetrahedron Letters 48 (2007) 5107–5110

Synthesis of protected norcysteines for SPPS compatible with Fmoc strategy

Manoj P. Samant and Jean E. Rivier*

The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA

Received 12 January 2007; revised 11 May 2007; accepted 14 May 2007
Available online 18 May 2007

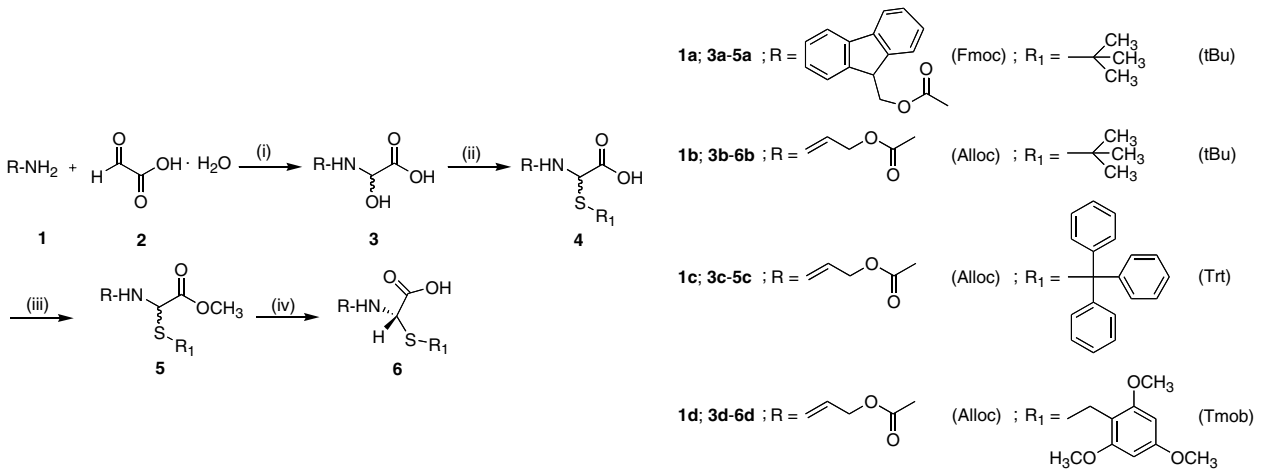
Abstract—We report the synthesis of racemic Alloc-Ncy(Tmob)–OH, the resolution of its methyl ester and demonstrate its application to form a norcystine bridge in octreotide-amide using the Fmoc strategy on solid phase. *N*-Alloc and *S*-Tmob protections of norcysteine (Ncy) were found to be a preferred choice for Fmoc strategy over three other protected norcysteines synthesized, that is, Fmoc-Ncy(*t*Bu)–OH, Alloc-Ncy(*t*Bu)–OH, and Alloc-Ncy(Trt)–OH.

Norcysteine (Ncy) or α-thiolglycine (H_2N–CH(SH)–COOH) is an unnatural amino acid possessing an electronegative sulfur atom attached directly to the α-carbon atom. Recently, we described the synthesis of Boc-D,L-Ncy(Mob)–OH, the resolution of its methyl ester, and the introduction of both D- and L-Ncy in cyclic gonadotropin-releasing hormone (GnRH) analogs.[1] Boc-Ncy(Mob)–OH is compatible with SPPS using Boc strategy and can be introduced in peptides as a bridge head to constrain peptide conformation via norcysteine-containing disulfide bridges that are shorter in ring size than the cystine bridges by one or two methylene groups. Herein we describe the synthesis of N- and S-protected norcysteines for Fmoc strategy.

In studies directed toward the protected norcysteines for Fmoc strategy, the α-amino group of norcysteine was protected with Fmoc[2] and Alloc,[3] as both can be easily removed during SPPS under basic and neutral conditions, respectively. The sulfhydryl function was protected either with *t*Bu[4]/Trt[5]/Tmob[6] for their stability during deprotection of Fmoc/Alloc groups and for their lability[7] after the cleavage of the desired peptide from the resin or during the cleavage step. The synthesis of racemic N- and S-protected norcysteines (**4a**–**d**) is illustrated in Scheme 1. In short, refluxing Fmoc- or Alloc-carbamate (**1**) and glyoxylic acid monohydrate (**2**) in acetone for 5 h or stirring in diethylether at room temperature (rt) overnight yielded the α-hydroxy intermediate (**3**). The reaction of *tert*-butylthiol/tritylthiol/2,4,6-trimethoxybenzylthiol[8] in toluene with **3** in the presence of PTSA afforded the racemic N- and S-protected norcysteines (**4a**–**d**). We attempted to separate the enantiomers of **4a**–**d** by stereoselective enzymatic resolution of their methyl esters **5a**–**d** using papain.[1] Methyl esters (**5a**–**d**), which are the preferred substrate for the papain-catalyzed hydrolysis,[9] were obtained by reacting the racemic amino acids (**4a**–**d**) with trimethylsilyldiazomethane.[10] Our attempts to enzymatically resolve the racemic methyl esters **5a** and **5c** using papain were unsuccessful. The enzymatic resolution of **5a** and **5c** with α-chymotrypsin and subtilisin also failed, and may be attributed to the bulkier Fmoc/Trt groups, which may affect the substrate recognition. However, we were successful in resolving racemic methyl esters **5b** and **5d** using papain[1] to optically enriched protected L-norcysteines **6b** and **6d**[11] with enantiomeric excess of 80% and >98%, respectively. The papain catalyzed hydrolysis was carried out in phosphate buffer (pH 6.2) containing 60% CH_3CN at 25 °C and monitored by chiral RP-HPLC using chiralcel® OD-RH™ column (Fig. 1). After 24 h, the reaction mixture contained 50% acid according to chiral RP-HPLC and was quenched by adding acetic acid. The crude products containing unreacted D-methyl esters and the resolved L-acids (**6b** and **6d**) were separated by column chromatography.[12]

Keywords: Norcysteine; Norcystine; SPPS; Somatostatin; Octreotide.
* Corresponding author. Tel.: +1 858 453 4100; fax: +1 858 552 1546; e-mail: jrivier@salk.edu

0040-4039/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2007.05.082



Scheme 1. Synthesis of N- and S-protected norcysteines. Reagents and conditions: (i) acetone, reflux, 5 h or diethylether, rt, overnight; (ii) *tert*-butylthiol/tritylthiol/2,4,6-trimethoxybenzylthiol, benzene, PTSA, Dean–Stark, reflux, 6 h; (iii) Me_3SiCHN_2, benzene/methanol (4:1); (iv) papain, CH_3CN/buffer (3:2), pH 6.2.

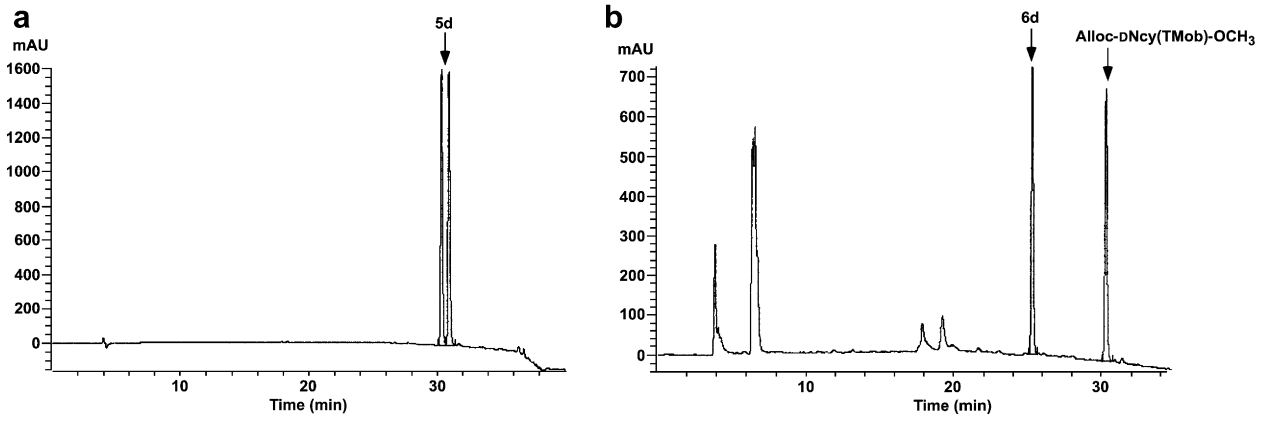


Figure 1. Chiral HPLC profile of the enzymatic hydrolysis of racemic Boc-Alloc-Ncy(Tmob)-OCH_3 (**5d**). Reaction progress (a) initial, retention time (t_R) for racemic ester is 30.34 min and 30.96 min (b) after 24 h, t_R for resolved Alloc-Ncy(Tmob)–OH is 25.32 min and t_R for unreacted Alloc-D-Ncy(Tmob)-OCH_3 is 30.31 min. The HPLC assay conditions for the chiralcel® OD-RH™ reversed-phase column (0.46 cm × 15 cm): buffer A, 0.1% TFA in H_2O, buffer B, CH_3CN in A; gradient elution from 10% B to 70% B in 30 min and then 95% B in 2 min at a flow rate of 0.5 mL/min. UV detection, 0.1 AUFS at 210 nm.

The compatibility of **6b** and **6d** in SPPS to form a norcystine bridge was investigated in somatostatin analog octreotide-amide.[13] All of the peptides **7**–**9** (Table 1) were synthesized manually on a 2,4-dimethoxybenzhydrylamine resin (DMBHA-resin,[14] substitution 0.25 mmol/g resin) or Rink Amide AM resin (Novabiochem, substitution, 0.70 mmol/g resin) using the Fmoc strategy.[15]

Each coupling reaction was achieved using a threefold excess of amino acid, and utilizing *N*,*N*′-diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole(HOBt)-mediated activation of the carboxyl group in DMF. Piperidine treatment (25% piperidine in DMF) was used for the Fmoc removal and the Alloc deprotection was performed under neutral conditions, that is, $Pd(PPh_3)_4$/$PhSiH_3$/DCM[16] in the presence of argon. The fully protected peptido resins [Boc-DPhe^1-Ncy(*t*Bu)^2-Phe^3-DTrp(Boc)^4-Lys(Boc)^5-Thr(*t*Bu)^6-Ncy(*t*Bu)^7-Thr(*t*Bu)^8-resin and Boc-DPhe^1-Ncy(Tmob)^2-Phe^3-DTrp(Boc)^4-Lys(Boc)^5-Thr(*t*Bu)^6-Ncy(Tmob)^7-Thr(*t*Bu)^8-resin] were cleaved with the cocktail mixture of TFA/H_2O/EDT/TIS (94:2.5:2.5:1) for 3 h to give the crude peptide **8** and disulfide-reduced form of the peptide **9**, respectively. The excess of TFA was then removed in vacuo and the crude peptides were precipitated by the addition of excess of *tert*-butyl methyl ether. Analysis of the crude peptides by RP-HPLC and mass spectroscopy revealed deprotection of Tmob, but not *t*Bu protection from the sulfhydryl function of Ncy. The linear precursor peptide DPhe^1-Ncy^2-Phe^3-DTrp^4-Lys^5-Thr^6-Ncy^7-Thr^8-NH_2 obtained from the Boc-DPhe^1-Ncy(Tmob)^2-Phe^3-DTrp(Boc)^4-Lys(Boc)^5-Thr(*t*Bu)^6-Ncy(Tmob)^7-Thr(*t*Bu)^8-resin was oxidatively cyclized in the presence of I_2[17] to give the crude cyclic peptide **9** (Fig. 2).

Our attempts to remove *t*Bu protection from peptide **8** using DMSO/TFA mixtures[18] or using Hg(II) acetate[19] failed with noticeable side products on RP-HPLC. Further experiments were directed toward on-resin

Table 1. Characterization of peptides

No.	Peptides	Ring size	Purity (%)		t_R[c] (min)	MS[d] $(M+H)^+$	
			HPLC[a]	CZE[b]		Calcd.	Obsd.
7	Cyclo$_{(2\text{-}7)}$[DPhe^1-Cys^2-Phe^3-DTrp^4-Lys^5-Thr^6-Cys^7-Thr^8-NH_2] (Octreotide-amide)	20	99	99	17.4	1032.436	1032.404
8	DPhe^1-Ncy(*t*Bu)^2-Phe^3-DTrp^4-Lys^5-Thr^6-Ncy(*t*Bu)^7-Thr^8-NH_2	—	98	99	35.8	1118.546	1118.502
9	Cyclo$_{(2\text{-}7)}$[DPhe^1-Ncy^2-Phe^3-DTrp^4-Lys^5-Thr^6-Ncy^7-Thr^8-NH_2]	18	99	99	14.9	1004.405	1004.341

[a] Percentage purity determined by HPLC using buffer A: TEAP, pH 2.30, buffer B: 60% CH_3CN/40% A under gradient conditions (20% to 50% B over 30 min), at a flow rate of 0.2 mL/min on a Vydac C_{18} column (0.21 cm × 15 cm, 5 μm particle size, 300 Å pore size). Detection at 214 nm.

[b] Percentage purity determined by capillary zone electrophoresis (CZE) using a Beckman P/ACE System 2050 controlled by an IBM Personal system/2 model 50Z; field strength of 15 kV at 30 °C. Buffer, 100 mM sodium phosphate (85:15, H_2O:CH_3CN), pH 2.50, on an Agilent μSil bare fused-silica capillary (75 μm i.d. × 40 cm length). Detection at 214 nm.

[c] Retention times under HPLC conditions described above.

[d] Mass spectra (MALDI-MS) were measured on an ABI-Voyager DE-STR instrument using saturated solution of α-cyano-4-hydroxycinnamic acid in 0.3% trifluoroacetic acid and 50% acetonitrile as matrix. The calculated [M+H] of the monoisotope was compared with the observed $[M+H]^+$ monoisotopic mass.

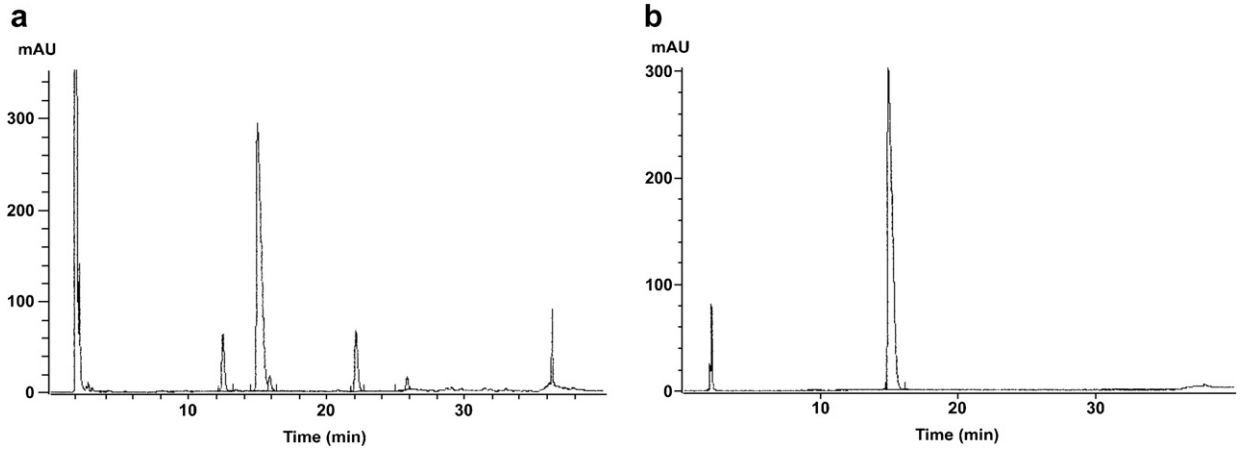


Figure 2. RP-HPLC profile of (a) crude and (b) purified peptide (**9**). RP-HPLC conditions: buffer A, TEAP pH 2.30; buffer B, 60% CH_3CN/40% A; gradient elution from 20% to 50% buffer B in 30 min at a flow rate of 0.2 mL/min on a Vydac C_{18} column (0.21 cm × 15 cm, 5 μm particle size, 300 Å pore size). UV detection, 0.1 AUFS at 210 nm.

oxidative deblocking of *t*Bu protection using $Tl(tfa)_3$.[20] The fully protected Boc-DPhe^1-Ncy(*t*Bu)^2-Phe^3-DTrp(Boc)^4-Lys(Boc)^5-Thr(*t*Bu)^6-Ncy(*t*Bu)^7-Thr(*t*Bu)^8-DMBHA resin was treated with $Tl(tfa)_3$ (1.2 equiv), 3 h in DMF-anisole (19:1) at 0 °C. The cleavage of the peptido resin with a cocktail mixture of TFA/H_2O/TIS (95:2.5:2.5) followed by post cleavage workup did not show the desired [Ncy^2, Ncy^7]octreotide-amide (**9**) on RP-HPLC.

All of the crude peptides (**7**–**9**) were purified[21] by RP-HPLC in at least two different solvent systems (TEAP pH 2.25 and 0.1% TFA on C_{18} silica). The analytical techniques used for the characterization of the analogs in Table 1 included RP-HPLC with two different solvent systems (0.1% TFA and TEAP pH 2.30) and capillary zone electrophoresis (CZE). Mass spectrometric analysis supported the identity of the intended structures (Table 1). Additionally, the structure of **9** was confirmed by coinjection experiment on RP-HPLC with [Ncy^2, Ncy^7]octreotide-amide synthesized in parallel using resolved Boc-Ncy(Mob)–OH[1] and Boc strategy.

In conclusion, we have shown that Alloc-Ncy(Tmob)–OH is compatible with the Fmoc strategy as an alternative to Boc-Ncy(Mob)–OH (used in the Boc strategy) for introducing a norcystine bridge in peptides by SPPS. The optical purity, easy removal of the N^{α}-Alloc protection (during chain elongation step), and the sulfhydryl Tmob protection (during the cleavage of peptide from resin) clearly demonstrate that Alloc-Ncy(TMob)–OH is a preferred choice for Fmoc-based SPPS.

Acknowledgments

We thank Ron Kaiser and Charleen Miller for technical assistance, Thomas Baiga and Dr. Joseph Noel for ^1H, ^{13}C NMR, and chiral HPLC analyses. We thank Dr. David Chatenet for critical reading of the manuscript. We thank Debbie Doan for manuscript preparation. This work was supported by NIH Grants DK059953 and DK026741. J.R. is the Dr. Frederik Paulsen Chair in Neurosciences Professor.

Supplementary data

Experimental procedures for the synthesis of amino acids are given in Scheme 1 and for the synthesis of peptide analog **9** in Table 1. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2007.05.082.

References and notes

1. Samant, M. P.; Rivier, J. E. *Org. Lett.* **2006**, *8*, 2361–2364.
2. Carpino, L. A.; Han, G. Y. *J. Am. Chem. Soc.* **1970**, *92*, 5748–5749.
3. Stevens, C. M.; Watanabe, R. *J. Am. Chem. Soc.* **1950**, *72*, 725–727.
4. Callahan, F. M.; Anderson, G. W.; Paul, R.; Zimmerman, J. E. *J. Am. Chem. Soc.* **1963**, *85*, 201–207.
5. Zervas, L.; Theodoropoulos, D. M. *J. Am. Chem. Soc.* **1956**, *78*, 1359–1363.
6. Munson, M. C.; Garcia-Echeverria, C.; Albericio, F.; Barany, G. *J. Org. Chem.* **1992**, *57*, 3013–3018.
7. Moroder, L.; Musiol, H.-J.; Schaschke, N.; Chen, L.; Hargittai, B.; Barany, G.2.6.6 Thiol Group. In *Methods of Organic Chemistry: Synthesis of Peptides and Peptidomimetics*; Goodman, M., Felix, A., Moroder, L., Toniolo, C., Eds.; Houben-Weyl: New York, 2002; Vol. E 22a, pp 384–423.
8. Vetter, S. *Synth. Commun.* **1998**, *28*, 3219–3223.
9. Miyazawa, T.; Iwanaga, H.; Yamada, T.; Kuwata, S. *Biotechnol. Lett.* **1994**, *16*, 373–378.
10. Compounds **3** and **5** were obtained in quantitative yields from **1** and **4**, respectively. Compounds **4a–d** and **5a–d** were purified by column chromatography. The racemic amino acids **4a–d** were obtained in 35–65% yield after purification (**4a** = 65%, **4b** = 54%, **4c** = 47% and **4d** = 35%). The crude **4d** contained approximately 15% of insoluble polymeric material.
11. Compound **6d**: oil; $[\alpha]_D^{25}$ +42.40 (*c* 1.0, MeOH); ^{1}H NMR (300 MHz, DMSO-d_6): 7.96 (d, 1H, $J = 8.7$ Hz), 6.21 (s, 2H), 5.98–5.85 (m, 1H), 5.32 (dd, 1H, $J = 17.1$, 1.2 Hz), 5.19 (dd, 1H, $J = 10.5$, 1.2 Hz), 5.12 (d, 1H, $J = 8.7$ Hz), 4.51 (d, 2H, $J = 5.1$ Hz), 3.76 (s, 2H), 3.75 (s, 9H); ^{13}C NMR (75 MHz, DMSO-d_6): 170.03, 160.23, 158.34, 158.30, 155.03, 133.34, 133.29, 117.07, 105.77, 90.67, 64.64, 56.17, 55.68, 55.16, 22.46; HRMS Calcd for $C_{16}H_{21}NO_7S$: 394.0931 ($M+Na^+$). Found: 394.0920 ($M+Na^+$), 2.8 ppm error.
12. The unreacted D-methyl esters were eluted with a mixture of EtOAc/hexane (25:75) and the resolved Alloc-Ncy(*t*Bu/Tmob)–OH were eluted with EtOAc/MeOH (85:15).
13. Bauer, W.; Briner, U.; Doepfner, W.; Haller, R.; Huguenin, R.; Marbach, P.; Petcher, T. J.; Pless, J. *Life Sci.* **1982**, *31*, 1133–1140.
14. Penke, B.; Rivier, J. *J. Org. Chem.* **1987**, *52*, 1197–1200.
15. Chan, W. C.; White, P. D. *Fmoc Solid Phase Peptide Synthesis*; Oxford University Press: New York, 2000.
16. Thieriet, N.; Alsina, J.; Giralt, E.; Guibe, F.; Albericio, F. *Tetrahedron Lett.* **1997**, *38*, 7275–7278.
17. Rivier, J.; Erchegyi, J.; Hoeger, C.; Miller, C.; Low, W.; Wenger, S.; Waser, B.; Schaer, J.-C.; Reubi, J. C. *J. Med. Chem.* **2003**, *46*, 5579–5586.
18. Cuthbertson, A.; Indrevoll, B. *Tetrahedron Lett.* **2000**, *41*, 3661–3663.
19. Nishimura, O.; Kitada, C.; Fujino, N. *Chem. Pharm. Bull.* **1978**, *26*, 1576.
20. Fujii, N.; Otaka, A.; Funakoshi, S.; Bessho, K.; Watanabe, T.; Akaji, K.; Yajima, H. *Chem. Pharm. Bull. (Tokyo)* **1987**, *35*, 2339–2347.
21. The RP-HPLC purified peptide analogues **8** and **9** were obtained in 43% and 32% yields, respectively.