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Synthesis of protected norcysteines for SPPS compatible with Fmoc-strategy

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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 norcystine (Ncy) were found to be a preferred choice for Fmoc-strategy over three other protected norcysteines synthesized *i.e.* Fmoc-Ncy(tBu)-OH, Alloc-Ncy(tBu)-OH and Alloc-Ncy(Trt)-OH.

Keywords

norcystine; norcystine; SPPS; somatostatin; octreotide

Norcystine (Ncy) or α -thiolglycine ($\text{H}_2\text{N}-\text{CH}(\text{SH})-\text{COOH}$) 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) analogues.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* norcystine-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 towards the protected norcysteines for Fmoc-strategy, the α -amino group of norcystine was protected with Fmoc² and Alloc,³ as both can be easily removed during SPPS under basic and neutral conditions, respectively. The sulfhydryl function was protected either with tBu⁴/Trt⁵/Tmob⁶ for their stability during deprotection of Fmoc/Alloc groups and for their lability⁷ 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-trimethoxybenzylthiol⁸ 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

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Supplementary Data: Experimental procedures for the synthesis of amino acids in Scheme 1 and for the synthesis of peptide analog **9** in Table 1.

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stereoselective enzymatic resolution of their methyl esters **5a-d** using papain.¹ The 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 papain¹ 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% CH₃CN at 25 °C and monitored by chiral RP-HPLC using chiralcel® OD-RH™ column, Figure 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

The compatibility of **6b** and **6d** in SPPS to form a norcystine bridge was investigated in somatostatin analogue 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 3-fold 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 *i.e.* Pd(PPh₃)₄/PhSiH₃/DCM¹⁶ in the presence of argon. The fully protected peptido-resins [Boc-D-Phe¹-Ncy(tBu)²-Phe³-D-Trp(Boc)⁴-Lys(Boc)⁵-Thr(tBu)⁶-Ncy(tBu)⁷-Thr(tBu)⁸-resin and Boc-D-Phe¹-Ncy(Tmob)²-Phe³-D-Trp(Boc)⁴-Lys(Boc)⁵-Thr(tBu)⁶-Ncy(Tmob)⁷-Thr(tBu)⁸-resin] were cleaved with the cocktail mixture of TFA:H₂O: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 tBu protection from the sulfhydryl function of Ncy. The linear precursor peptide D-Phe¹-Ncy²-Phe³-D-Trp⁴-Lys⁵-Thr⁶-Ncy⁷-Thr⁸-NH₂ obtained from the Boc-D-Phe¹-Ncy(Tmob)²-Phe³-D-Trp(Boc)⁴-Lys(Boc)⁵-Thr(tBu)⁶-Ncy(Tmob)⁷-Thr(tBu)⁸-resin was oxidatively cyclized in the presence of I₂¹⁷ to give the crude cyclic peptide **9**, Figure 2.

Our attempts to remove tBu protection from peptide **8** using DMSO/TFA mixtures¹⁸ or using Hg(II) acetate¹⁹ failed with noticeable side-products on RP-HPLC. Further experiments were directed towards on-resin oxidative deblocking of tBu protection using Tl(tfa)₃.²⁰ The fully protected Boc-D-Phe¹-Ncy(tBu)²-Phe³-D-Trp(Boc)⁴-Lys(Boc)⁵-Thr(tBu)⁶-Ncy(tBu)⁷-Thr(tBu)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:H₂O:TIS (95:2.5:2.5) followed by post cleavage workup did not show the desired [Ncy², Ncy⁷]octreotide-amide (**9**) on RP-HPLC.

All of the crude peptides (**7-9**) were purified²¹ by RP-HPLC in at least two different solvent systems (TEAP pH 2.25 and 0.1% TFA on C₁₈ 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 [Ncy², Ncy⁷]

octreotide-amide synthesized in parallel using resolved Boc-Ncy(Mob)-OH¹ 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- 3 and 5 were obtained in quantitative yields from 1 and 4, respectively. 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.
- 6d. oil; [α]_D²⁵ = +42.40 (c = 1.0, MeOH); ¹H NMR (300 MHz, DMSO-d₆): 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); ¹³C NMR (75 MHz, DMSO-d₆): 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 C₁₆H₂₁NO₇S: 394.0931 (M + Na⁺), Found: 394.0920 (M + Na⁺), 2.8 ppm error.
- The unreacted D-methyl esters were eluted with a mixture of EtOAc:hexane (25:75) and the resolved Alloc-Ncy(tBu/Tmob)-OH were eluted with EtOAc:MeOH (85:15).
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21. The RP-HPLC purified peptide analogues 8 and 9 were obtained in 43% and 32% yields, respectively.

Figure 1.

Chiral HPLC profile of the enzymatic hydrolysis of racemic Boc-Alloc-Ncy(TMob)-OCH₃ (**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-DNcy(TMob)-OCH₃ 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 H₂O, buffer B, CH₃CN in A; gradient elution from 10% B – 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.

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% CH₃CN/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 C₁₈ column (0.21 cm × 15 cm, 5 μm particle size, 300 Å pore size). UV detection, 0.1 AUFS at 210 nm.

Scheme 1.

Synthesis of N- and S- protected norcysteines.

(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) Me₃SiCHN₂, benzene:methanol (4:1); (iv) Papain, CH₃CN:buffer(3:2), pH 6.2.

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 ₍₂₋₇₎ [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(tBu) ² -Phe ³ -DTrp ⁴ -Lys ⁵ -Thr ⁶ -Ncy(tBu) ⁷ -Thr ⁸ -NH ₂	-	98	99	35.8	1118.546	1118.502
9	cyclo ₍₂₋₇₎ [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

^aPercentage purity determined by HPLC using buffer A: TEAP, pH 2.30, buffer B: 60% CH₃CN/40% A under gradient conditions (20% to 50% B over 30 min), at flow rate of 0.2 mL/min on a Vydac C18 column (0.21 cm × 15 cm, 5 μm particle size, 300 Å pore size). Detection at 214 nm.

^bPercentage 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, H₂O:CH₃CN), pH 2.50, on a Agilent μSil bare fused-silica capillary (75 μm i.d. × 40 cm length). Detection at 214 nm.

^cRetention times under HPLC conditions described above.

^dMass 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.