ORGANIC LETTERS

2006
Vol. 8, No. 11
2361−2364

Norcystine, a New Tool for the Study of the Structure–Activity Relationship of Peptides

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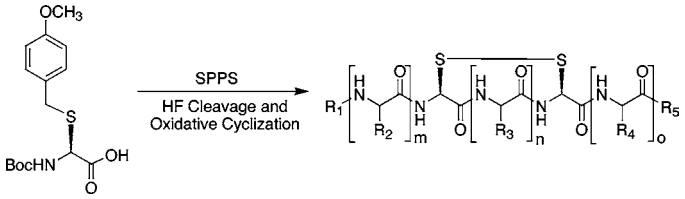
Received March 17, 2006

ABSTRACT



Norcysteine (Ncy) is an unnatural amino acid possessing an electronegative sulfur atom directly attached to the α-carbon atom. We describe 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 GnRH analogues.

Cystine is commonly found in biologically active peptides and proteins where it plays the critical role of stabilizing their secondary and tertiary conformations. During the past several decades, chemists have mimicked the intramolecular disulfide bridges in natural or de novo designed peptides and small proteins with the goal of improving biological activities. The most common mimics include lactam (−NH-CO−),[1,2] thioether (−CH_2-S-CH_2−),[3] methylenedithioether (−S-CH_2-S−),[4,5] dicarba (−CH_2-CH_2−),[6] monoselenide (−S-Se−),[7] diselenide (−Se-Se−),[8] and trisulfides (−S-S-S−).[9] Cystine bridges (C_α−CH_2−S−S−CH_2−C_α) have been extended by one or two methylene groups (C_α−CH_2−CH_2−S−S−CH_2−CH_2−C_α) by introducing one or two homocysteines in peptides and proteins.[7,10,11] There is no report describing the introduction of one or two norcysteines to form disulfide bridges such as −C_α−S−S−CH_2−C_α−, −C_α−CH_2−S−S−C_α−, or −C_α-S−S−C_α−. We hypothesized that shorter disulfide bridges than those resulting from the oxidation of the side chains of two cysteines would affect the overall conformation of a peptide or protein and modulate their biological activities.

N-protected α-mercaptoamino acids (or α-thiolamino acids) are reported in the literature[12−14] and are of interest in relationship to certain antibiotics such as gliotoxin, sporidesmin, aranotin, chaetocin, and quinomycins.[15,16] Our successful use of α-aminoglycine (Agl)[17−19] as a bridge head

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10.1021/ol0606740 CCC: $33.50
Published on Web 04/28/2006

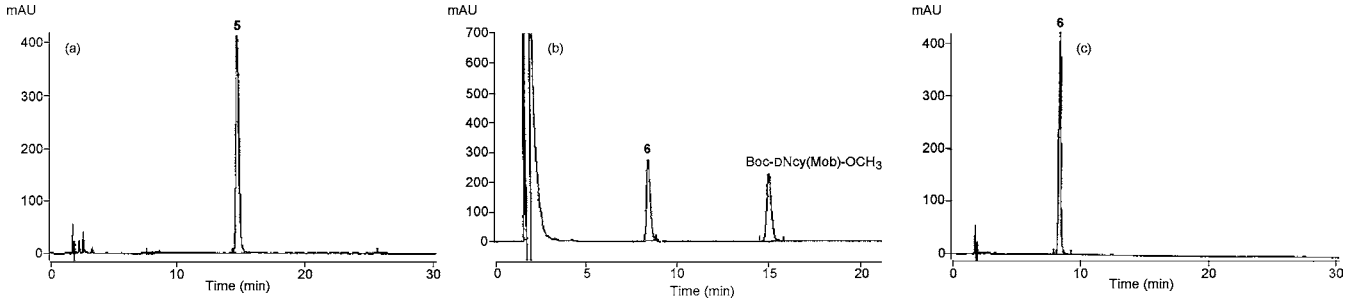
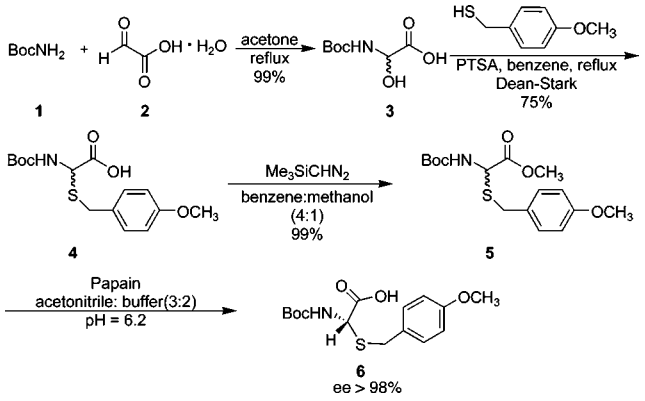


Figure 1. HPLC profile of the enzymatic hydrolysis of racemic Boc-Ncy(Mob)-OCH_3 (**5**). Reaction progress: (a) initial, (b) after 24 h, and (c) after workup and purification. RP-HPLC conditions: buffer A, 0.1% TFA in H_2O; buffer B, 0.1% TFA in 80% CH_3CN/20% H_2O; gradient elution from 40 to 70% buffer B in 30 min at a flow rate of 0.2 mL/min; UV detection, 0.1 AUFS at 214 nM.

in some cyclic analogues of the gonadotropin releasing hormone (GnRH)[20] suggested a possible use of norcysteine (Ncy) to constrain peptide conformations in general and that of GnRH in particular. In this communication, we report a synthetic pathway to a chiral derivative of norcysteine suitable for solid-phase peptide synthesis and demonstrate its compatibility in analogues of GnRH antagonists.

The racemic α-(4-methoxybenzylthio)-*N*-(*tert*-butoxycarbonyl)glycine or Boc-D,L-Ncy(Mob)-OH (**4**) was synthesized by a modified procedure reported for the synthesis of α-isopropylthiohyppuric acid by Zoller et al.[14] In short, refluxing *tert*-butyl carbamate (**1**) and glyoxylic acid monohydrate (**2**) in acetone yielded α-hydroxy intermediate (**3**) which was immediately reacted with 4-methoxybenzylmercaptan (Mob) under Dean−Stark conditions to give the racemic Boc-Ncy(Mob)-OH (**4**) in 75% yield (Scheme 1).

Scheme 1. Synthesis of Boc-Ncy(Mob)-OH



To limit the number of possible diastereomeric peptides containing Ncy, we attempted the stereoselective enzymatic resolution of its derivative Boc-D,L-Ncy(Mob)-OCH_3. Papain, a sulfhydryl protease, was our first choice because it was reported to enantioselectively hydrolyze the L-isomer of *N*-protected α-alkyloxyglycine esters[21] and *N*-protected α-aminoglycine esters.[22] Boc-D,L-Ncy(Mob)-OCH_3 (**5**), a preferred substrate for the papain-catalyzed hydrolysis,[23] was obtained in quantitative yield by reacting the racemic amino acid (**4**) with trimethylsilyldiazomethane. The papain-catalyzed hydrolysis was carried out in phosphate buffer (pH 6.2) containing 60% acetonitrile at 25 °C and monitored by RP-HPLC. After 24 h, the reaction mixture contained 50% methyl ester and 50% acid according to HPLC (Figure 1) and was quenched by adding acetic acid. A simple workup and one purification gave Boc-Ncy(Mob)-OH (**6**) in 90% yield with respect to the L-isomer. The enantiomeric excess was found to be greater than 98% according to chiral HPLC.[24] Our attempts to obtain an optically pure D-isomer from the unhydrolyzed D-methyl ester failed because of substantial racemization during chemical saponification. This observation was analogous to that of Strijtveen et al. for the synthesis of thiolactic acid by chemical hydrolysis of the carboxy ester of the enantiomerically pure (*R*)- or (*S*)-2-(acetylsulfanyl)propanoate.[25] The coupling efficiency of Boc-Ncy(Mob)-OH (**6**) in peptide synthesis was investigated in GnRH analogues.

GnRH (*p*-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) is a highly flexible molecule that exists in solution as an equilibrium mixture of multiple conformers; the most favored is a type II β-turn involving residues 5−8 (Tyr^5-Gly^6-Leu^7-Arg^8).[26,27] In GnRH antagonists, the overall conformational freedom was restricted by cyclization via head-

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Table 1. Characterization of Cyclic GnRH Antagonists

entry	peptide[a]	ring size	% purity HPLC[b]	% purity CZE[c]	t_R[d] (min)	MS[e] (M + H)$^+$ calcd	MS[e] (M + H)$^+$ obsd
7	cyclo$_{(4-10)}$[Ac-D-Nal^1,D-Cpa^2,D-Pal^3,Ncy^4,Arg^5,D-Pal^6,Cys^{10}]GnRH	22	98	98	15.74	1446.6	1446.6
8	cyclo$_{(4-10)}$[Ac-D-Nal^1,D-Cpa^2,D-Pal^3,D-Ncy^4,Arg^5,D-Pal^6,Cys^{10}]GnRH	22	99	99	18.33	1446.6	1446.6
9	cyclo$_{(4-10)}$[Ac-D-Nal^1,D-Cpa^2,D-Pal^3,Cys^4,Arg^5,D-Pal^6,Ncy^{10}]GnRH	22	99	99	16.96	1446.6	1446.5
10	cyclo$_{(4-10)}$[Ac-D-Nal^1,D-Cpa^2,D-Pal^3,Cys^4,Arg^5,D-Pal^6,D-Ncy^{10}]GnRH	22	99	99	17.67	1446.6	1446.5
11	cyclo$_{(4-10)}$[Ac-D-Nal^1,D-Cpa^2,D-Pal^3,Ncy^4,Arg^5,D-Pal^6,Ncy^{10}]GnRH	21	97	98	16.37	1432.6	1432.5

[a] IUPAC rules are used for nomenclature except for the following: Ac = acetyl; Nal = 3-(2-naphthylalanine); Cpa = 4-chlorophenylalanine; Pal = 3-(3-pyridyl)alanine; Ncy = norcysteine. [b] Percentage purity determined by HPLC using buffer A (TEAP, pH 2.30) and buffer B (60% CH_3CN/40% A) under gradient conditions (25−55% B over 30 min), at a flow rate of 0.2 mL/min on a Vydac C_{18} column (0.21 × 15 cm, 5 μm particle size, 300 Å pore size). Detection at 214 nm. [c] 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 a Agilent μSil bare fused-silica capillary (75 μm i.d. x 40 cm length). Detection at 214 nm. [d] Retention times under HPLC conditions described above. [e] Mass spectra (MALDI-MS) were measured on an ABI-Voyager DE-STR instrument using a saturated solution of α-cyano-4-hydroxycinnamic acid in 0.3% trifluoroacetic acid and 50% acetonitrile as the matrix. The calculated [M + H]$^+$of the monoisotope was compared with the observed [M + H]$^+$ monoisotopic mass.

to-tail[28,29] or through the side chain encompassing the residues 5−8, with a goal to identify a consensus bioactive conformation or to improve the antagonist potency. The compatibility of a number of side chain to side chain bridges has been systematically explored,[20,30,31] and so far, only two different lactam-bridged constraints, cyclo$_{(4-10)}$ and cyclo$_{(5-8)}$, were found to be well tolerated both in vivo and in vitro.[32,33] Among the cyclic GnRH antagonists with a disulfide bridge,[30,33−35] the cyclo$_{(4-10)}$[Ac-D-Nal^1,D-Cpa^2,D-Pal^3,-Cys^4,Arg^5,D-Pal,^{6}Cys^{10}]GnRH[32] with a 23-membered ring was potent in both in vitro and in vivo assays and was chosen for the present study.

All of the Ncy-containing GnRH analogues shown in Table 1 were synthesized either manually or automatically on a *p*-methylbenzhydrylamine resin (MBHA resin) using the Boc strategy.[36] All of the individual Boc-protected amino acids were incorporated in a sequential manner utilizing *N*, *N*′-diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt)-mediated activation of the carboxyl group in DMF. Trifluoroacetic acid (TFA) treatment (60% TFA in DCM) was used for Boc removal, and the *N*-terminal acetylation was performed by using an excess of acetic anhydride in DCM. The protected peptido resins were cleaved and deprotected in anhydrous HF (1.5 h at 0−5 °C) in the presence of a scavenger (anisole, 10% v/v) and methyl sulfide (5% v/v).[24] The cyclization (oxidation) of the fully deprotected peptide was carried out with iodine.[19] The crude peptides were purified by RP-HPLC in at least two different solvent systems (triethylammoniumphosphate (TEAP), pH 2.25, and 0.1% TFA on C_{18} silica).[24] The analytical techniques used for the characterization of the analogues 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.

Analogues **7**, **9**, and **11** were synthesized with the resolved Boc-Ncy(Mob)-OH (**6**). The RP-HPLC and MALDI analyses of the crude peptides (obtained after HF cleavage and I_2 cyclization) indicated that a single stereoisomer was formed, and the coupling reaction proceeded without racemization at the α-carbon. The HPLC profile of the crude peptide **11** is shown in Figure 2. Because the starting amino acid Boc-D-Ncy(Mob)-OH for the synthesis of analogues **8** and **10**

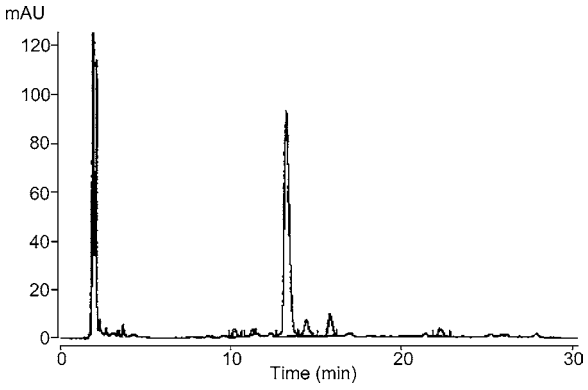

Figure 2. HPLC profile of crude peptide **11**. RP-HPLC conditions: buffer A, TEAP pH 2.30; buffer B, 60% CH_3CN/40% A; gradient elution from 30 to 60% buffer B in 30 min at a flow rate of 0.2 mL/min; UV detection, 0.1 AUFS at 214 nM.

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could not be obtained, we synthesized the diastereomeric mixture of these peptides with the racemic Boc-D,L-Ncy-(Mob)-OH (**4**) at positions 4 and 10, respectively. The two isomers were then separated by RP-HPLC. The stereochemistry at the Ncy residue in analogues **8** and **10** was confirmed by comparison of the HPLC retention times (Table 1) of each diastereomer with those of the analogues synthesized with the resolved Boc-Ncy(Mob)-OH (**6**), i.e., analogues **7** and **9**, respectively. Analogues **7** and **9** coeluted on HPLC with the first eluting diastereomers of the pairs (**7** + **8** and **9** + **10**) obtained by the incorporation of unresolved Boc-D,L-Ncy(Mob)-OH (**4**). This confirmed that analogues **8** and **10** contained the D-enantiomer of Ncy at position 10.

The yields of these peptides (Table 1) containing the unnatural disulfide bridge were comparable to that of the homologous peptides with the natural disulfide bridge.[24] Peptides were stored as TFA salts at −20 °C and were stable; no decomposition products were identified on HPLC after six months.

All of the GnRH analogues (Table 1) were tested in vitro in a reporter gene assay in HEK-293 cells expressing the human GnRH receptor and a stably integrated luciferase reporter gene as previously described.[37] The antagonism of the GnRH-induced response by each analogue was determined and reported as an IC_{50} value, the concentration required to suppress the response in the reporter gene assay by 50%. In this assay, analogues **7** (IC_{50} = 37 nM), **8** (IC_{50} = 33 nM), and **11** (IC_{50} = 33 nM) are half as potent as the reference cyclic GnRH antagonist cyclo$_{(4-10)}$-[Ac-DNal^1,DCpa^2,DPal^3,Cys^4,Arg^5,DPal^6,Cys^{10}]GnRH^{32} (IC_{50} = 13 nM). Analogues **9** (IC_{50} = 290 nM) and **10** (IC_{50} = 340 nM) were less potent.

In conclusion, we have successfully demonstrated the applicability of norcysteine (α-thiolglycine) to shorten disulfide bridges in peptides by one or two methylene groups. This amino acid and its derivatives complement our present armamentarium of unnatural amino acids to study the structure−activity relationships of bioactive peptides containing disulfide bridges and to understand the role of disulfide bridges in protein structure and function.

Acknowledgment. We thank Ron Kaiser (Salk Institute) and Charleen Miller (Salk Institute) for technical assistance, William Low (Salk Institute) and Dr. Wolfgang Fisher (Salk Institute) for MS analysis, and Thomas Baiga (Salk Institute) and Dr. Joseph Noel (Salk Institute) for ^1H, ^{13}C NMR, and chiral HPLC analyses. We thank Dr. Judit Ercheygi (Salk Institute) and Dr. David Chatenet (Salk Institute) for critical reading of the manuscript. We thank Dr. Glenn Croston (Ferring Research Institute Inc.) and Doley Hong (Ferring Research Institute Inc.) for in vitro assay data of GnRH analogues. We thank Debbie Doan (Salk Institute) for manuscript preparation. This work was supported by NIH Grants HD-039899 and DK-059953. J.R. is the Dr. Frederik Paulsen Chair in Neurosciences Professor.

Supporting Information Available: Detailed experimental procedures and spectral and analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

OL0606740

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