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Boc-S-methylbenzyl-(S)-2-amino-6-mercaptohexanoic acid: Preparation and application to the synthesis of a large cyclic disulfide peptide

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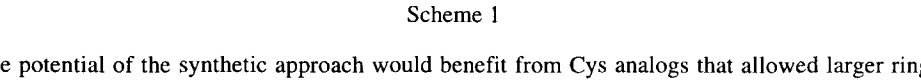
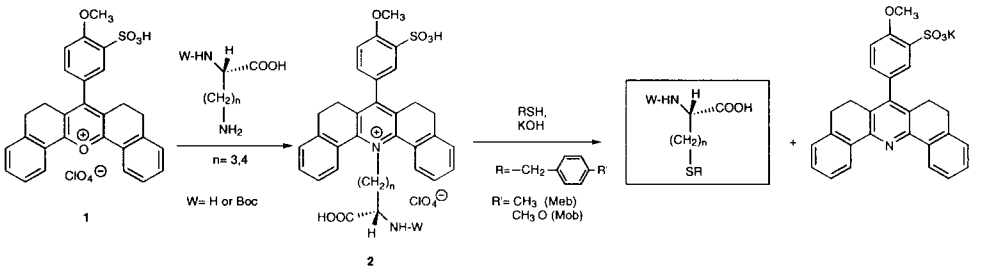
Abstract: A derivative of 2-amino-6-mercaptohexanoic acid (Amh) suitable for Boc solid phase synthesis has been prepared by conversion of the ε-NH_2 group of lysine into a 4-methylbenzyl thioether. The derivative has been used in the synthesis of a 22-residue peptide with a 62-atom cyclic disulfide. Amh(Meb) is stable to acidolysis but can be conveniently deprotected, with simultaneous disulfide formation, by treatment with diphenyl sulfoxide and trichloromethylsilane.

A variety of reasons have made cyclic disulfide peptides attractive targets for peptide synthesis.[1] In addition to reproducing natural peptides containing internal disulfide bridges (e.g., oxytocin, vasopressin, calcitonin), it is interesting to introduce disulfide units in linear peptide molecules and thus induce bioactive-like conformations with improved binding and biological action.[2] An example is the G-H loop of foot-and-mouth virus (FMDV), serotype C-S8c1,[3] a highly mobile region on the virus surface (residues 134-155 of viral protein VP1) that is the target for antibodies involved in the neutralization of infectivity. We have modeled this antigenic site by means of restricted mobility analogs such as the $Cys^{136,153}$ cyclic disulfide[4,5] (Scheme 1, Xaa=Cys^6), a prospective vaccine candidate with interesting structural features.



Scheme 1

The potential of the synthetic approach would benefit from Cys analogs that allowed larger ring sizes and/or a broader repertory of conformational arrangements than those provided by Cys pairs. One example is the restricted mobility disulfides derived from L-penicillamine (*(S)* β,β-dimethyl-cysteine).[7] An alternative approach, leading to larger size disulfides, uses Cys analogs with the side chain extended by several methylene units. Homocysteine (Hcy, *(S)* 2-amino-4-mercaptobutyric acid), the simplest analog of this type, can be prepared as Boc-Hcy(tBu)-OH from methionine[8] and is also commercially available as Boc-Hcy(Meb)-OH. Longer side chain Cys analogs are in principle available by a procedure first described by Katritzky et al.[9,10] In this method, reaction of a pyrilium salt (**1**) with lysine (either free or Boc-protected at the α-NH_2) produces a pyridinium salt (**2**) which can undergo nucleophilic attack by a thiol to give an *S*-protected 2-amino-6-mercaptohexanoic acid (Amh) derivative (Scheme 2). The same procedure leads to *S*-protected Boc-amino-5-mercaptopentanoic acid (Amp) from Boc-ornithine. In a recent application of this method,[11] an intramolecular disulfide was generated from two Amh(Mob) residues, incorporated as Fmoc-Amh(Mob) derivatives. The choice of Mob as *S*-protecting group would seem to be largely determined by the commercial availability of 4-methoxybenzylthiol. The Mob group, however, is not quite the ideal choice for either of the two usual protocols of solid phase peptide synthesis.[12] In Fmoc strategy,[13] on the one hand,

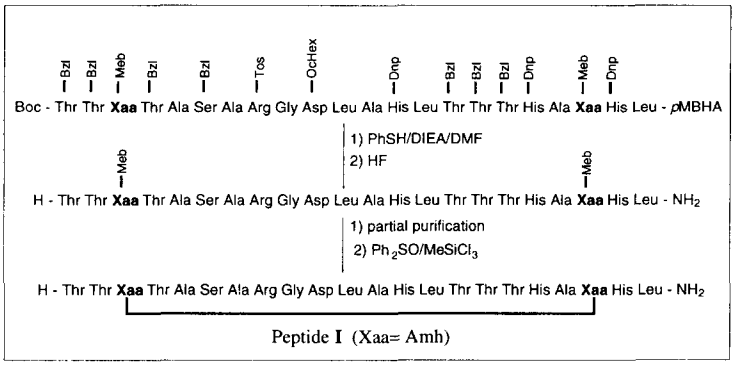


Scheme 2

full deprotection of Amh(Mob) requires acidolysis times (e.g., 90% TFA, 6 h, r.t.)[11] that significantly defeat the advantages of this synthetic approach (i.e., minimal exposure of the growing peptide chain to strong acid). On the other hand, in the more classical Boc[13] strategy, which uses acidolysis to deprotect the N^{α} group, *S*-Mob becomes only marginally stable to repetitive TFA treatment. We have observed significant loss of the side chain protection of Amh(Mob), detectable by tlc or the Ellman[14] thiol test, after just 1 h exposure to TFA. This problem may be tolerable if the *S*-Mob-protected residues are near the N-terminus (i.e., introduced at latter stages of the synthesis and thus subjected to only a few cycles of acid deprotection). However, for a large disulfide peptide, early deprotection of an Amh(Mob) residue near the C-terminus on repetitive exposure to acid is likely to cause side reactions.

To overcome this problem, we decided to use the more acid stable *S*-4-methylbenzyl (Meb) protecting group. 4-Methylbenzylthiol, the corresponding *S*-nucleophile (Scheme 2), was readily prepared from 4-methylbenzyl bromide and thiourea.[15] This thiol (19 mmol) was converted to its potassium salt by treatment with 2 M KOH (16 mL) in water for 30 min at r.t. under N_2, then reacted with 5.5 mmol of the pyridinium salt **2** derived from lysine (Scheme 2; n= 4, W=H), for 3 h at 85 °C under N_2. The resulting amino acid was isolated as the zwitterion H-Amh(Meb)-OH[16] by precipitation at pH 6. Prolonged (>24 h) exposure to TFA of an aliquot of this product did not reveal any loss of the Meb protection. The zwitterion was next treated with Boc_2O to give quantitatively Boc-Amh(Meb)-OH.[17] This derivative was then used in the synthesis of a cyclic disulfide analog (**I**) of the G-H loop of FMDV (Scheme 1, Xaa=Amh) in which the Amh[136,153] disulfide bridge defines a large 62-atom ring.

Peptide **I** was synthesized (Scheme 3) as a C-terminal carboxamide on *p*MBHAR[18] using standard Boc[4,12] chemistry.[19] The HF crude product had the expected amino acid composition and ran as a major peak in analytical HPLC (Fig. 1A). However, its mass was 210 units higher than expected for **I** in the dithiol form. This suggested that, contrary to what would be expected for Cys(Meb), the two Meb protecting groups (105 mass units each) were still on the peptide. Attempts to deprotect Amh(Meb) at higher temperature (25°C) and/or stronger acid conditions (95% HF-5% p-cresol, 2 h) were unsuccessful. The hypothesis that the Meb group was released but immediately recaptured by the thiol group of Amh -more basic than that of Cys due to its distance from the electron-withdrawing NH and CO groups- was tested by running the HF reaction in the presence of stronger scavengers such as 1,4-dimethoxybenzene (10% v/v; 2 h, r.t.), again with negative results. It was therefore concluded that Amh(Meb) was stable to acidolysis and that an alternative deprotection method was needed.



Scheme 3

Akaji et al.[20] have described an efficient method of intramolecular disulfide formation by simultaneous deprotection-oxidation of *S*-protected Cys derivatives with Ph_2SO-$MeSiCl_3$ in TFA. We applied this procedure to partially (Meb) protected **I** (after RPLC purification (Fig. 1B)), with good results. Optimal deprotection/oxidation conditions were 3 mM peptide, 30 mM Ph_2SO, 300 mM PhOMe and 300 mM $MeSiCl_3$ in TFA for 10 min, r.t. HPLC analysis of the reaction (Fig. 1C) showed a faster-eluting peak (*ca.* 13 min) identified by ESMS as the target cyclic peptide **I** (2544 mass units). The twin peaks eluting next were assigned to the parallel and antiparallel dimers of **I** based on their ESMS. The last peak of the chromatogram was non-peptidic. Peptide **I** was isolated from the reaction by addition of Et_2O (10 vol) and extraction into 1 M HOAc; it was purified to homogeneity by RPLC (Fig. 1D), in 30% yield from purified Meb-protected **I**.

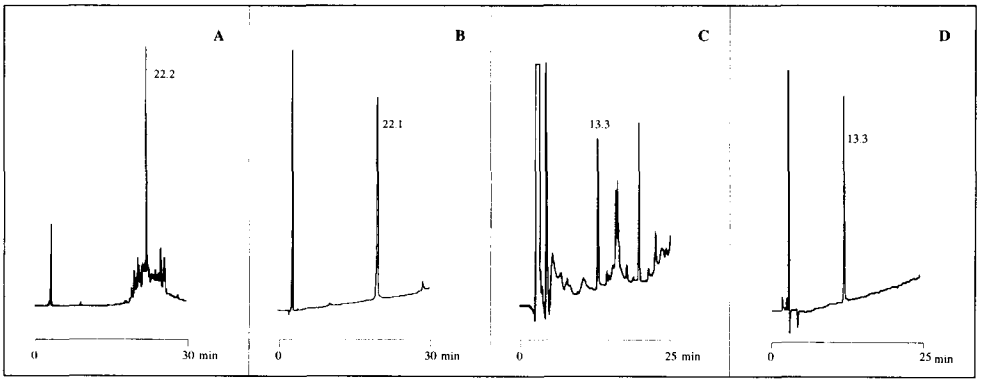


Figure 1. HPLC analysis of peptide **I** cyclization. A: partially protected [Amh^{136,153} (Meb)] **I**, HF crude; B: same after RPLC purification; C: crude from $Ph_2SO/MeSiCl_3$ deprotection/oxidation; D: purified cyclic **I**. HPLC conditions: Panels A and B: 5-65% MeCN into H_2O (+0.05% TFA) in 30 min; Panels C and D: 5-80% MeCN into H_2O (+0.05% TFA) in 25 min. UV detection at 220 nm.

In conclusion, Boc-Amh(Meb)-OH is a suitable new derivative for the Boc solid phase of Amh-based intramolecular disulfides larger than the typical Cys-Cys pairs. *S*-Meb compares favorably to *S*-Mob in terms of stability to acid deprotection conditions. Its somewhat unexpected resistance to HF acidolysis is readily

overcome by the $Ph_2SO/MeSiCl_3$ deprotection/oxidation method, which has recently been shown[5] to be also applicable to solid phase conditions.

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6. Abbreviations used in this paper for amino acids and peptides follow the rules of the IUPAC-IUB Comission of Biochemical Nomenclature in *Eur. J. Biochem.* **1984**, 138, 9-37 and *J. Biol. Chem.* **1989**, 264, 633-673. The following additional abbreviations are used: Amh: 2-amino-6-mercaptohexanoic acid; Amp: 2-amino-5-mercaptopentanoic acid; Boc, *tert*-butoxycarbonyl; BOP, benzotriazole-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate; Bzl, benzyl; cHex, cyclohexyl; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; Dnp, 2,4-dinitrophenyl; ESMS, electrospray mass spectrometry; Hcy, homocysteine; HOAc, acetic acid; HPLC, high performance liquid chromatography; *p*-MBHAR, *p*-methylbenzhydrylamine resin; Meb: *p*-methylbenzyl; MeCN, acetonitrile; Mob: *p*-methoxybenzyl; RPLC, reverse phase liquid chromatography; tBu, *tert*-butyl; TFA, trifluoroacetic acid; Tos, *p*-toluenesulfonyl. The amino acid symbols used denote the L configuration.
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16. ^{1}H-NMR (0.1 M NaOD, δ, DSS): 1.00-1.50 (m, 6H), 2.10 (s, 3H), 2.30 (t, 2H), 2.97 (t, 1H), 3.52 (s, 2H), 7.0-7.1 (m, 4H)
17. Yellowish oil; FAB-MS ($M+H^+$): 366; ^{1}H-NMR ($CDCl_3$, δ, TMS): 1.44 (s, 9H), 1.5-2.0 (m, 6H), 2.32 (s, 3H), 2.40 (t, 2H), 3.65 (s, 2H), 4.3 (broad, 1H), 5.02 (bd, 1H), 7.1-7-2 (m, 4H).
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19. Side chain protections were Bzl (Ser, Thr), cHex (Asp), Tos (Arg), Dnp (His) and Meb (Amh). The main part of the sequence was assembled in the automatic mode in an Applied Biosystems 430A synthesizer using single couplings with 2-fold molar excess of Boc-amino acid symmetric anhydrides. Residues Ala^{138} to Thr^{134}, located near the N-terminus and prone to incomplete couplings, were incorporated in the manual mode using BOP-mediated couplings. The fully protected [$Amh(Meb)^{136,153}$]VP1(134-155) resin was first treated with PhSH-DIEA-DMF (3:3:4; 6 x 1 h) to remove the Dnp group from His, followed by TFA (2 x 5 min) to deprotect the N-terminus, then submitted to acidolysis in HF-p-cresol (9:1, 1 h, 0°C).
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