Pergamon

*Tetrahedron Letters*, Vol. 35, No. 15, pp. 2287-2290, 1994
Elsevier Science Ltd
Printed in Great Britain
0040-4039/94 $6.00+0.00

0040-4039(94)E0289-A

# Synthesis of Enantiomerically pure N-Fmoc-S-Mob-(S)-α-Amino-ε-mercaptohexanoic Acid and its use in Solid Phase Peptide Synthesis

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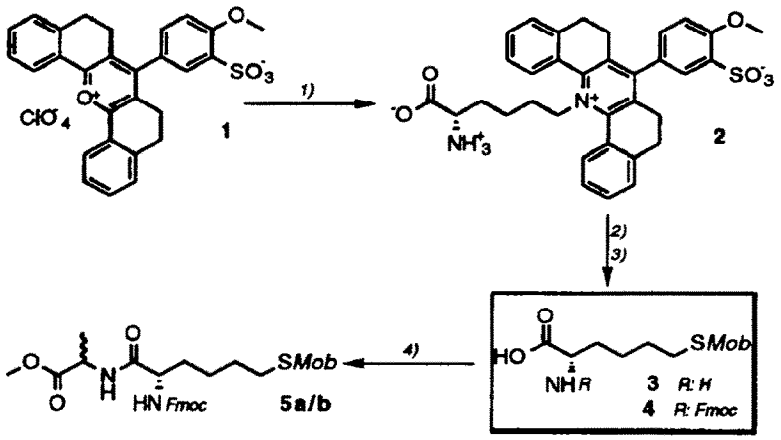
***Abstract***: Enantiomerically pure N-Fmoc-S-Mob (S)-α-amino-ε-mercaptohexanoic acid (Amh) was synthesized in an efficient three step process from (L)-lysine via nucleophilic displacement of the pyridinium salt. An efficient method for Fmoc protection without any racemization is described. Incorporation of this unnatural amino acid into peptides and its complete deprotection was accomplished in high yield using standard procedures.

Sulfur containing amino acids are important constituents in numerous biologically active peptides and proteins. Due to their extraordinary reactivity, mercapto groups are often found in the active sites of enzymes and are crucial for many metabolic processes. Furthermore, sulfur containing amino acids are excellent building blocks in designing conformationally rigid peptide structures. Cysteine, homocysteine and methionine are widely used in biological systems, whereas other homologues have not yet been isolated. Recently, α-amino-ε-mercaptohexanoic acid had been introduced into peptides to stabilize α-helices via intramolecular disulfide bonds.[1] The S-acetamidomethyl (Acm) protected α-amino-ε-mercaptohexanoic acid was obtained in a five step synthesis starting from Boc protected lysine. Although Acm protection of sulfhydryl groups is useful in special cases, acid labile benzylic protecting groups (e.g. trityl (Trt), 4-methoxybenzyl (Mob) or 2,4,6-trimethoxybenzyl (Tmob)) are preferable where simultaneous cleavage of all protecting groups is desired. Moreover, benzylic protection not only eliminates an additional iodine mediated Acm cleavage, but more importantly, sidesteps iodination of tyrosine and histidine residues.[2] In this communication we report an efficient three step synthesis of enantiomerically pure N-(9-fluorenylmethyloxycarbonyl)-S-(4-methoxybenzyl) protected (S)-α-amino-ε-mercaptohexanoic acid (Amh) starting from inexpensive (L)-lysine. The reaction sequence is shown in scheme 1.

Following a protocol from Katritzky *et. al.* the water soluble pyrylium salt (**1**) was used to convert the ε-amino group of (L)-lysine into the corresponding pyridinium salt (**2**).[3] Nucleophilic substitution at the ε-carbon yielded the methoxybenzyl protected aminomercaptohexanoic acid (**3**). Several attempts were made to introduce the Fmoc group using standard procedures.[4] The carbamate (**4**) could only be isolated in 20-30 % yield. However, our finding, that amino acid (**3**), in the presence of chlorotrimethylsilane is pyridine soluble, made it possible to avoid the widely used two phase system (aqueous $Na_2CO_3$ / dioxane or tetrahydrofuran). The transient silylation allowed Fmoc protection in a purely organic medium. Our protection protocol not only gave significantly higher yield (80 %), but also lead to purer product and shorter reaction time. Albericio *et. al.* reported the formation of small amounts of Fmoc-dipeptides when Fmoc chloride was used under phase transfer conditions.[5] The *in situ* silylation circumvents these side reactions. *In situ* silylation of hydroxyl and carboxyl groups to achieve selective N-tritylation of amino acids has been reported earlier.[6]

Because of potential racemization along the reaction sequence, the project's success rested on preserving (L)-lysine's chiral center. To verify the optical purity, we synthesized the dipeptide of Amh with (L)- and (DL)- alanyl-O-methyl ester. Determination of the diastereomeric excess of dipeptides **5a/b** by




***Scheme 1:*** Synthesis of Fmoc-Mob protected (S)-α-amino-ε-mercaptohexanoic acid (**4**). Mob: 4-Methoxybenzyl, Fmoc: 9-Fluorenylmethyloxycarbonyl, 1) (L)-Lysine, pH 10.0-10.5; 2) 4-Methoxybenzylmercaptan; 3) Fmoc-Cl, $Me_3SiCl$-Pyridine; 4) (DL)-Alanine methyl ester·HCl, BOP, HOBT, pyridine.

NMR analysis was not successful due to partially overlapping signals. Nevertheless, baseline separation of the diastereomers could be accomplished using chiral phase HPLC (Pirkle phase).[7] Diastereomeric excess > 99 % was obtained using Fmoc-Mob protected Amh (**4**) which had been synthesized using our transient silyl protection method. In contrast, the Fmoc derivative prepared using the standard two phase system showed nearly 7% racemization (Figure 1). These findings proved that our synthesis is not only efficient, 23 % in three steps, but also yielded enantiomerically pure product. All the compounds were characterized by $^1H$, $^{13}C$-NMR spectroscopy and high resolution FAB-MS.[8]

To verify the use of the Mob protected aminomercaptohexanoic acid (**4**) we investigated the synthesis of two peptides (**6**) and (**7**) using standard solid phase method and deprotection protocols.[9] The incorporation of Amh (**4**) in peptides was accomplished smoothly and with high efficiency on an automated peptide synthesizer using HOBT-BOP activation. Deprotection under acidic and reducing conditions in the presence of a scavenger not only cleaved the t-Butyl, Tmob and Boc protection, but also released the Mob group completely within 6 h which was proven by $^1H$-NMR spectroscopy and FAB-MS analysis of the purified peptides.[10] The amino acid sequences and some characteristic properties of peptides (**6**) and (**7**) are given in table 1.

| Compound | FAB-MS | Amino Acid Analysis (found) |
|---|---|---|
| $H_3N^+$-VKKITA**mh**SIP-$CONH_2$ (**6**) | 1028 | $Amh_{1.0}I_{2.0}K_{2.0}P_{1.0}S_{0.9}T_{1.0}V_{1.0}$ |
| $H_3N^+$-GKKSQQVKKITA**mh**SIP-$CONH_2$ (**7**) | 1687 | $Amh_{1.1}G_{1.0}I_{1.9}K_{4.0}P_{1.0}Q_{2.0}S_{1.8}T_{1.0}V_{1.0}$ |

***Table 1:*** Amino acid sequences and some typical properties of peptides (**6**) and (**7**).

## EXPERIMENTAL DETAILS

Following a literature protocol (L)-lysine was transformed in 50 % yield to the corresponding pyridinium salt (**2**) using the water soluble pyrylium perchlorate (**1**).[3] We found a simple precipitation from methanol-diethyl ether to be sufficient to obtain pure product. Under $N_2$ atmosphere, the pyridinium salt (**2**) was heated for 1h at 70 °C in an aqueous solution of 4 equiv. 4-methoxybenzylmercaptan and 3.5 equiv. NaOH. The by-product was filtered off and amino acid (**3**) was precipitated by acidification (pH 6). The optically active product, purified by continuous ether extraction (Soxlet extractor, 48 h), was obtained in 57 %

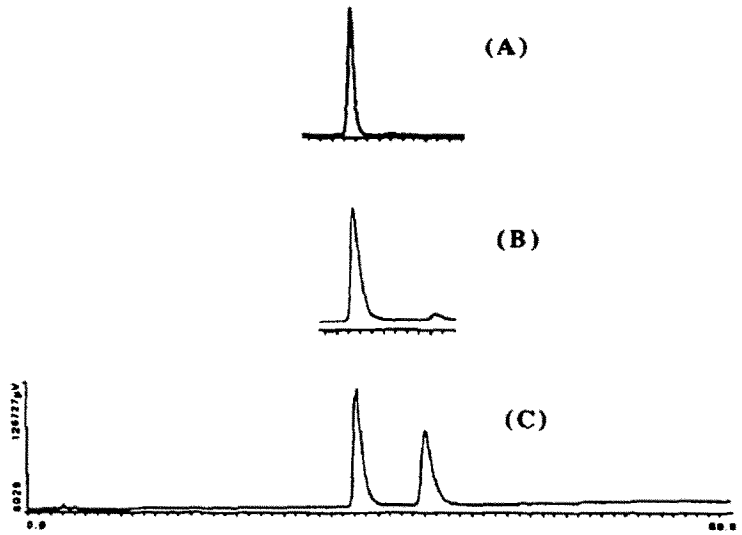



***Figure 1:*** Chiral phase HPLC (Pirkle phase) chromatogram of dipeptide prepared from (L) alanyl O-methyl ester and Fmoc-Mob-Amh (**4**) prepared using our transient silyl protection method (A); of dipeptide prepared from (L) alanyl O-methyl ester and Fmoc-Mob-Amh prepared using conventional two phase reaction conditions (B) and of dipeptides **5a/b** prepared from (DL) alanyl O-methyl ester and Fmoc-Mob-Amh (C).

yield ($[\alpha]^D$: +6.1 (c=0.62, $CH_2Cl_2$), FAB-MS calcd. for $C_{14}H_{22}NO_3S$ ($M+H^+$): 284.1364, found 284.1342).[11] 1.1 equiv. 9-fluorenylmethyl chloroformate was added to a clear solution of amino acid (**3**) in pyridine-chlorotrimethylsilane (19:1) at 0 °C. After stirring for 3 h at r.t. the solvent was distilled off under reduced pressure and the residue, dissolved in dichloromethane was washed with sat. $NH_4Cl$ solution. After column chromatography ($SiO_2$, dichloromethane-methanol 19:1 to 9:1), the optically active product was obtained in 80 % yield ($[\alpha]^D$: +0.3 (c=3.81, $CH_2Cl_2$), FAB-MS calcd. for $C_{29}H_{32}NO_5S$ ($M+H^+$): 506.1991, found 506.1996).[12] The enantiomeric excess of amino acid (**4**) was determined to be >99% based on chiral phase HPLC analysis of the corresponding (L)- and (DL)-alanyl-O-methyl ester derivatives. One equiv. of either the racemate or the optically active (L)-alanine methyl ester hydrochloride suspended in pyridine in presence of 1 equiv. triethylamine was added to a pyridine solution of the amino acid (**4**), 1.5 equiv. HOBT and 1 equiv. BOP and stirred for 3 h at r.t. The solvent was removed under reduced pressure and the residue, dissolved in dichloromethane was washed with sat. $NH_4Cl$ solution. An aliquot of the crude was purified by thin layer chromatography ($SiO_2$, dichloromethane-methanol 19:1). Determination of the diastereomeric excess by NMR analysis of dipeptides **5a/b** was not successful due to partially overlapping methyl ester signals ($^1$H-NMR ($CDCl_3$) d=3.716 and 3.720).[13] Baseline separation of the two pairs of diastereomers (**5a/b**) could be accomplished using chiral phase HPLC with (R)-3,5-dinitrobenzoyl-phenylglycine as the stationary phase (Baker, covalent) and 2-propanol-hexane mixtures as the mobile phase (flow: 1 ml/min, 5-15 % 2-propanol over 60 min). The two diastereomers could be detected by their characteristic UV absorbance at $\lambda$= 265 nm and eluted at 29.6 min (**5a**) and 36.4 min (**5b**).

**Acknowledgement.** This research was supported by National Institutes of Health grant GM39822.

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8 $^{1}H$- and $^{13}C$- NMR spectra were obtained on a General Electric GE-QE 300 at 300 MHz and 75 MHz. Chemical shifts ($\delta$) are reported in ppm, coupling constants (J) in Hz. $^{1}H$- and $^{13}C$-chemical shifts were determined with respect to $CHCl_3$ (7.24 ppm and 77.0 ppm).

9 Peptides were synthesized as carboxamides by using 5-[4-(9-fluorenylmethyloxycarbonyl)-aminomethyl-3,5-dimethoxyphenoxy)] valeric acid (PAL) resin$^{R}$ on a Milligen/Biosearch 9050 peptide synthesizer$^{R}$ using 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry and benzotriazolyl N-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate (BOP) / 1-hydroxybenzotriazole (HOBT) activation. The peptides were cleaved from the resin with trifluoroacetic acid-thioanisole-ethane dithiol-anisole (90:5:3:2) at r.t. for 6 h and precipitated in diethyl ether at - 20 °C.

10 Peptides were purified to homogeneity on a reversed-phase $C_{18}$ HPLC column using water-trifluoroacetic acid (0.1 %) and acetonitrile-trifluoroacetic acid (0.1 %) mixtures. Quantitative amino acid analysis was done on a Beckman 6300 high-performance amino acid analyzer at the Protein Structure Laboratory at University of California, Davis.

11 **$^{1}H$-NMR** ($D_2O$, NaOD): 1.3 (m, 2H, H-C(4)); 1.6 (m, 4H, H-C(3,5)); 2.5 (t, J=7.3 Hz, 2H, H-C(6)); 3.2 (t, J=6.2 Hz, 1H, H-C(2)); 3.7 (s, 2H, H-C(Mob)); 3.8 (s, 3H, H-C(Mob)); 7.0 (d, J=8.2 Hz, 2H, H-C(Mob)); 7.4 (d, J=8.2 Hz, 2H, H-C(Mob)).**$^{13}C$-NMR** ($D_2O$, NaOD): 24.5 (t, C(4)); 28.5 (t, C(5)); 30.6 (t, C(3)); 34.5 (t, C(6)); 34.6 (t, C(Mob)); 55.3 (d, C(2)); 55.9 (q, C(Mob)); 113.0 (d, 2C, C(Mob)); 130.0 (d, 2C, C(Mob)); 130.9 (s, C(Mob)); 157.8 (s, C(Mob)); 183.2 (s, C(1)).

12 **$^{1}H$-NMR** ($CDCl_3$): 1.2-1.8 (m, 6H, H-C(3,4,5)); 2.4 (m, 2H, H-C(6)); 3.6 (s, 2H, H-C(Mob)); 3.8 (s, 3H, H-C(Mob)); 4.2 (m, 1H, H-C(2)); 4.4 (m, 3H, H-C(Fmoc)); 5.5 (s, 1H, H-N(2)); 6.8 (d, J=8.2 Hz, 2H, H-C(Mob)); 7.2 (d, J=8.2 Hz, 2H, H-C(Mob)); 7.3 (t, J=6.6 Hz, 2H, H-C(Fmoc)); 7.4 (t, J=6.6 Hz, 2H, H-C(Fmoc)); 7.6 (d, J=8.2 Hz, 2H, H-C(Fmoc)); 7.7 (d, J=8.2 Hz, 2H, H-C(Fmoc)); 8.5 (s, broad, 1H, H-O(1)). **$^{13}C$-NMR** ($CDCl_3$): 24.3 (t, C(4)); 28.6 (t, C(5)); 30.8 (t, C(3)); 31.8 (t, C(6)); 35.6 (t, C(Mob)); 47.1 (d, C(Fmoc)); 53.6 (d, C(2)); 55.2 (q, C(Mob)); 67.1 (t, C(Fmoc)); 113.9 (d, 2C, C(Mob)); 120.0 (d, 2C, C(Fmoc)); 125.0 (d, 2C, C(Fmoc)); 127.0 (d, 2C, C(Fmoc)); 129.9 (d, 2C, C(Mob)); 130.0 (s, C(Mob)); 141.3 (s, 2C, C(Fmoc)); 143.7 (s, 2C, C(Fmoc)); 156.1 (s, C(Fmoc)); 158.5 (s, C(Mob)); 176.8 (s, C(1)).

13 **$^{1}H$-NMR** ($CDCl_3$): 1.3-1.7 (m, 6H, H-C(3,4,5$_{Amh}$)); 1.4 (d, J=7.2 Hz, 3H, H-C(3$_{Ala}$)); 2.5 (t, J=7.3 Hz, 2H, H-C(6$_{Amh}$)); 3.67 (s, 2H, H-C(Mob)); 3.720 (s, 3H, H-C(OMe)); 3.80 (s, 3H, H-C(Mob)); 4.16 (m, 1H, H-C(Fmoc)); 4.24 (t, J=7.2 Hz, 1H, H-C(2$_{Amh}$)); 4.46 (m, 2H, H-C(Fmoc)); 4.58 (quintet, J=7.2 Hz, 1H, H-C(2$_{Ala}$)); 5.3 (m, 1H, H-N(2$_{Amh}$)); 6.4 (m, 1H, H-N(2$_{Ala}$)); 6.8 (d, J=8.2 Hz, 2H, H-C(Mob)); 7.2 (d, J=8.2 Hz, 2H, H-C(Mob)); 7.3 (t, J=6.6 Hz, 2H, H-C(Fmoc)); 7.4 (t, J=6.6 Hz, 2H, H-C(Fmoc)); 7.6 (d, J=8.2 Hz, 2H, H-C(Fmoc)); 7.7 (d, J=8.2 Hz, 2H, H-C(Fmoc)).

*(Received in USA* 14 *September* 1993; *revised* 5 *January* 1994; *accepted* 3 *February* 1994)