Bioorganic & Medicinal Chemistry Letters, Vol.3, No.4, pp. 739-742, 1993
Printed in Great Britain

0960-894X/93 $6.00 + .00

SYNTHESIS OF HOMOLOGS OF CYSTEINE SUITABLE FOR PEPTIDE AND PROTEIN CROSSLINKING

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(*Received in USA* 11 *December* 1992)

Abstract: Practical syntheses of two homologs of cysteine are reported: (R)-2-amino-4-thio-butanoic acid and (R)-2-amino-5-thio-pentanoic acid, starting from L-methionine and L-ornithine respectively. These derivatives contain protecting groups which are amenable to solid phase peptide synthesis and expand the repertoire currently available for peptide and protein crosslinking.

Disulfide bonds are a frequently used motif for stabilizing specific, biologically active peptide conformations for pharmacological studies.[1] Cysteine has been the most widely used amino acid for forming these disulfide linkages due to the fact that it is naturally occurring and available with a wide range of protecting groups for peptide synthesis. In not all cases, however, is the cystine linkage the appropriate length needed to bridge certain distances in a potentially conformationally restricted peptide chain. In these cases it is essential to have a series of homologs of cysteine available to choose from for introducing conformational rigidity into peptides. In this paper we report a practical approach to the synthesis of two homologous derivatives of cysteine: (R)-2-amino-4-thio-butanoic acid and (R)-2-amino-5-thio-pentanoic acid. The introduction of fluorenylmethyloxycarbonyl (Fmoc) protection at the α-amino-group, and the acetamidomethyl (Acm) sulfhydryl protecting group makes these derivatives important in both solid phase peptide synthesis as well as for disulfide bond formation under mild conditions.

The (R)-2-amino-4-thio-butanoic acid derivative (1) was synthesized in three steps without purification of the intermediates in a 54% overall yield based on L-methionine (Figure 1).[2] We found that it was critical in this series to remove all traces of ammonia before step 3, or difficulties in protecting the α-amino group were encountered.

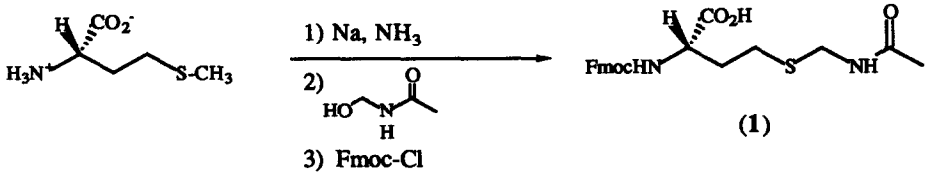

Figure 1. The synthesis of (R)-2-amino-4-thiol-butanoic acid derivative **1** from L-methionine.

In a typical reaction L-methionine (6.7 mmol) was dissolved in liquid ammonia (approx. 40 ml) and sodium (20.1 mmol) was added over 15 min until the reaction mixture remained blue. The reaction was stirred for 30 min, quenched by slow addition of ammonium acetate (10.75 mmol), and the ammonia evaporated. The

residue was dissolved in TFA (30 ml) and treated with hydroxymethylacetamide (6.7 mmol) at RT for 45 min. This reaction was monitored for free sulfhydryl groups using dithiobis(2-nitrobenzoic acid) (Ellman's test).[3] The TFA was removed *in vacuo*, the pH was adjusted to 8-9, and the residue was dissolved in H_2O (200 ml) and lyophilized to dryness (2X). The residue was dissolved in 10% Na_2CO_3 (100 ml), dioxane (56 ml) was added and the solution was cooled to 0°C. The mixture was treated with fluorenylmethyloxycarbonyl chloride (6.7 mmol), and the reaction was stirred for 4 hr at 0°C and RT for 18 hr. The reaction was extracted with Et_2O (2X), the aqueous layer acidified to pH 2-3, and extracted with EtOAc (3X). The EtOAc extracts were dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was purified on silica (5% MeOH, 2% HOAc, 93% CH_2Cl_2) to yield (**1**) in 54% overall yield from L-methionine. Compound (**1**) was recrystallized from MeOH/H_2O to yield a white solid, mp 134°C. All spectral data are consistent with the structure of **1**.[4]

The synthesis of next higher homolog of cysteine, (R)-2-amino-4-thio-pentanoic acid, involved the initial quarternization of the ε-amino group of N-α-boc-L-ornithine (**2**) with pyrillium salt **3**.[5] We found that one equivalent of the amino acid could be used in this procedure for every equivalent of **3**. This allowed us to save valuable amino acids as compared to a previously reported procedure for lysine quarternization.[5] The pyridinium derivative **4** was then treated with a thiolate nucleophile to yield derivative **5** (Figure 2). We found that unless the α-amino group of ornithine was *t*-Boc protected, 40-50% of an unwanted cyclization to proline occurred during this step. This derivative is suitable for solid phase peptide synthesis using a *t*-Boc strategy, or was converted into derivative **6** for use in a Fmoc based synthesis strategy.[6]

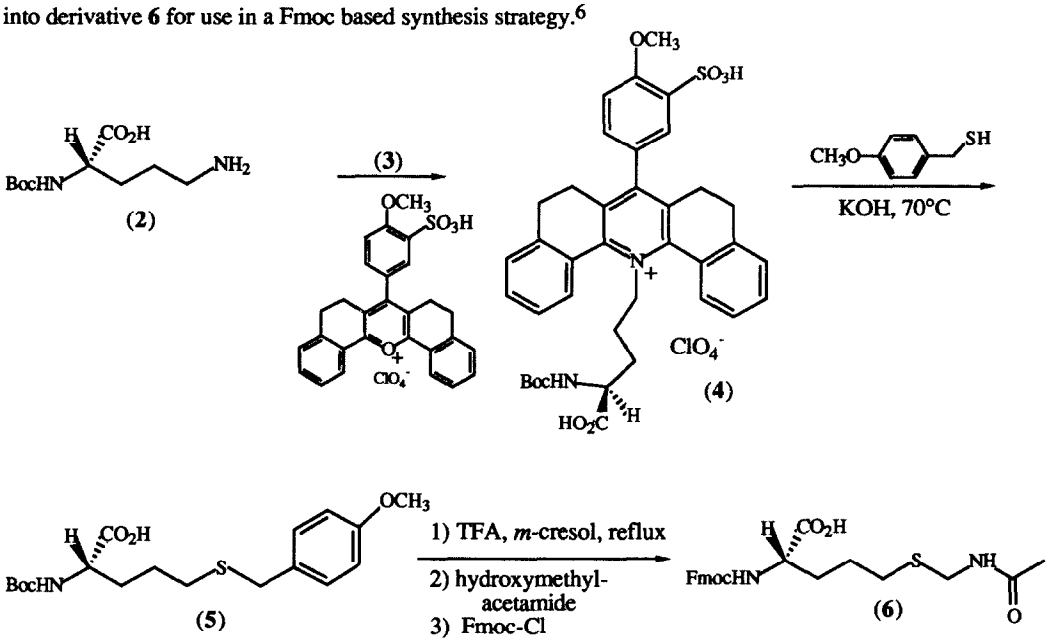

Figure 2. The synthesis of (R)-2-amino-5-thiolpentanoic acid derivatives **5** and **6** from N-α-boc-L-ornithine.

In a typical reaction N-α-boc-L-ornithine (1.1 mmol) was dissolved in a H_2O (10 ml), the pH was brought to 10 with 10M NaOH, and **3** (1.1 mmol) was added in portions keeping the pH at 11. After 2 days at

RT, the pH was brought to 3 with $HClO_4$ (10%), the solution was decanted from the gummy precipitate, and the gum was precipitated from hot EtOH with ether to yield **4** (0.76 mmol) in 69%.[7] Pyridinium salt **4** (0.58 mmol) was added to a solution of 4-methoxybenzyl mercaptan (1.7 mmol) and KOH (2.9 mmol) in H_2O (10 ml) under nitrogen and the mixture was heated to 70°C for 2 hr.[8] The precipitate was filtered, the filtrate extracted with Et_2O (2X), and the pH of the aqueous layer was brought to 3 with 1N HCl. The mixture was extracted with EtOAc (3X), and the EtOAc layers were concentrated and purified on silica (3% MeOH, 2% HOAC, 95% CH_2Cl_2) to give **5** in 75% yield.[9]

A degassed solution of **5** (0.20 mmol) and *m*-cresol (2.0 mmol) in TFA (3 ml) was refluxed for 24 hr. The mixture was concentrated, H_2O (2 ml) was added,the pH was brought to 4, and the solution was extracted with Et_2O (3X).[10] The aqueous layer was concentrated, the residue was dissolved in TFA (3 ml) and hydroxymethylacetamide (0.40 mmol) was added. After 30 min at RT the mixture was concentrated, dissolved in H_2O (5 ml), and the pH adjusted to 7. Na_2CO_3 (0.8 g) was added, followed by dioxane (6 ml), and fluorenylmethyl chloroformate (0.6 mmol) was added at 0°C. The mixture was stirred at 0°C for 2 h, RT for 12 h and extracted with Et_2O (2X). The pH of the aqueous layer was brought to 3 and extracted with EtOAc (3X). The EtOAc layers were concentrated and purified on silica (3% MeOH, 1.5% HOAc, 95.5% CH_2Cl_2) to give **6** in a 52% yield.[11]

In conclusion, two homologs of cysteine have been prepared in good yield starting from readily available amino acid starting materials. The reactions began with chiral amino acids and no racemization was detected during any of the amino acid manipulations.[12] The procedure for the preparation of homocysteine derivative **1** can easily be modified to incorporate a wide variety of amine and sulfhydryl protecting groups. Derivatives **1** and **6** can be used in solid phase peptide synthesis using an Fmoc-based strategy, whereas **5** can be used in the conventional *t*-Boc approach to peptide synthesis. Such straightforward syntheses of higher homologs of cysteine increases the repertoire available for disulfide crosslinking in peptide and protein structures.

Acknowledgements: We thank the generous financial support of the Monsanto Agricultural Company and the National Institutes of Health (1 R29 GM46936-01).

Abbreviations: Acm = acetamidomethyl, HOAc = acetic acid, *t*-boc = *t*-butyloxycarbonyl, Fmoc = fluorenylmethyloxycarbonyl, Et_2O = diethyl ether, EtOAc = ethyl acetate, TFA = trifluoroacetic acid

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4. Spectral data for compound (**1**): ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.9(s, 3H), 2.1(m, 2H), 2.7(m, 2H), 4.3(m, 6H), 6.0(d, J=4 Hz, 1H), 6.47(t, J=3 Hz, 1H), 7.3(td, J=1, 7 Hz, 2H), 7.4(td, J=1, 7 Hz, 2H), 7.7(d, J=7 Hz, 2H), 7.8(d, J=7 Hz, 2H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 22.6, 28.3, 32.6, 41.4, 48.4, 54.2, 68.0, 121.1, 126.5, 128.4, 129.0, 142.9, 145.4, 145.6, 159.1, 173.6, 176.0; MS (FAB) 429.0 (MH^+); $[\alpha]^{29}_D$ -17.3° (*c* 2.6 MeOH).

5. a) Katritzky, A. R.; Yang, Y. K., *J. Chem. Soc. Perkin Trans. II* **1984**, 885-889. b) Jackson, D.; King, D.; Chmielewski, J.; Singh, S.; Schultz, P., *J. Am. Chem. Soc*, **1991**, *113*, 9391.

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7. Spectral data are consistent for all intermediates in this synthesis.

8. The solution of KOH and 4-methoxybenzyl mercaptan in H_2O was preformed by stirring for 30 min under N_2.

9. Spectral data for compound (**5**): ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.4(s, 9H), 1.6-2.0(m, 4H), 2.42(t, J=7 Hz, 2H), 3.65(s, 2H), 3.79(s, 3H), 4.32(bt, 1H), 5.03(bd, 1H), 6.84(td, J=2, 9 Hz, 2H), 7.21(td, J=2, 9 Hz, 2H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 25.0, 28.4, 30.7, 31.7, 35.6, 53.2, 55.4, 80.5, 114.3, 130.3, 130.7, 156.1, 159.1, 177.9; MS (FAB) 392.3 ($M+Na^+$).

10. The extractions were monitored using the Ellman's reagent to insure that all of the thiol-derivative remained in the water layer.[3]

11. Spectral data for compound (**6**): ^{1}H NMR (D_6-acetone, 200 MHz) δ 1.7-1.9 (m, 4H), 1.9(s, 3H), 2.6(t, J=7 Hz, 2H), 4.2-4.7(m, 6H), 6.8(d, J=9 Hz, 1H), 7.3(t, J=7 Hz, 2H), 7.4(t, J=7 Hz, 2H), 7.5-7.7 (m, 1H), 7.7(d, J=7 Hz, 2H), 7.9(d, J=7 Hz, 2H); ^{13}C NMR (D_6-acetone, 50 MHz) δ 23.0, 26.8, 30.2, 31.8, 41.0, 48.2, 54.6, 67.4, 121.2, 126.6, 128.4, 128.9, 142.5, 145.4, 145.6, 157.6, 170.8, 174.6; MS (FAB) 465.0 ($M+Na^+$); $[\alpha]^{29}_D$ +2.1° (*c* 0.5 $CHCl_3$).

12. As determined by the ^{19}F NMR spectra of the Mosher's amides of compounds **1** and **6**.[13]

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