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Side Reaction in Peptide Synthesis: Modification of Tryptophan During Treatment with Mercury(II) Acetate/2-Mercaptoethanol in Aqueous Acetic Acid

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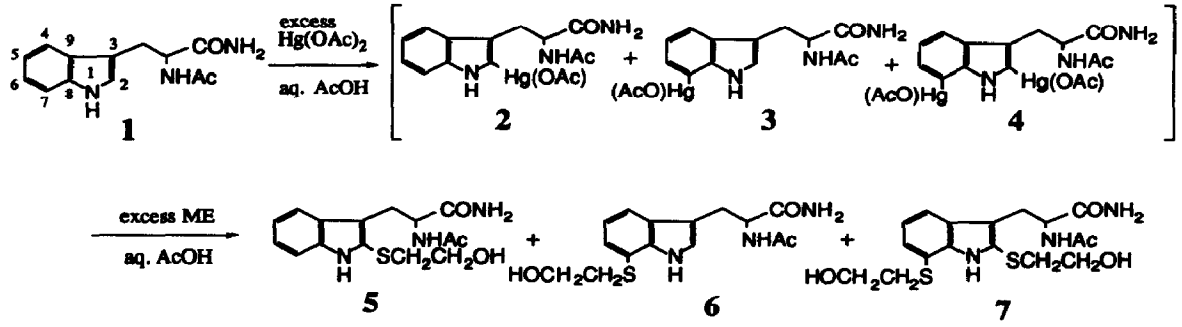
Abstract: *When mercury (II) acetate/2-mercaptoethanol procedure in aq. acetic acid was used to remove the acetamidomethyl group in the peptide synthesis, the Trp residue was modified.*

The acetamidomethyl (Acm) group[1] has been widely used both in solution and solid phase peptide synthesis. The Acm group is stable during the deprotection process with acid such as TFA or HF, but is readily removable by treatment with mercury (II) acetate ($Hg(OAc)_2$), iodine or thiocyanogen. Since 1986, we have been involved in the solution synthesis of biologically active peptides containing several disulfide bonds using a combination of maximum protection strategy and HF deprotection procedure, in which the Acm group was used as the protecting group of the Cys residues. The protecting groups were removed with HF except for the Acm groups which were removed by treatment with $Hg(OAc)_2$ (1.1-2.2 eq./Cys(Acm)) in 5% AcOH. Excess Hg was removed from the product by gel filtration on Sephadex G-25 after treatment with a large excess of 2-mercaptoethanol (ME) for over 20 h. However, when Trp-containing peptides were treated with excess $Hg(OAc)_2$ (5eq./Cys(Acm)) in 5% AcOH, several side products were formed showing different UV spectra from that of the desired product. Mass spectrometric analysis and UV analysis as well as peptide mapping of these products suggested that the Trp residues had been modified by ME. In the present communication, we report the structure of the side products and the optimal conditions to suppress this side reaction with N-acetyl-L-tryptophanamide[2] (**1**) as the model compound.

To the solution of (**1**) in aqueous AcOH at the concentration of 1 mM, excess $Hg(OAc)_2$ was added with stirring at room temperature. After 2-20 h, ME was added and stirring was continued for 20 h at room temperature. Detailed reaction conditions are listed in Table 1. The reaction mixture was analyzed by reversed-phase HPLC, and the products were isolated by reversed-phase HPLC. The product yields were calculated from peak areas at 220 nm on HPLC .

The treatment of (**1**) with excess $Hg(OAc)_2$/ME in aqueous AcOH produced three products (**5**), (**6**) and (**7**) as shown in Scheme 1. The UV spectrum (in CH_3OH) of these products showed the bathochromic shift, 9 nm for (**5**), 13 nm for (**6**) and 20 nm for (**7**) by comparison with that (λmax = 281 nm) of (**1**). The PD-MS spectrum of products (**5**) and (**6**) displayed the same M^+ value (321), 76 atomic mass units higher than that (245) of (**1**). This mass difference corresponds to an ME moiety ($-SCH_2CH_2OH$). On the other hand, the M^+ value (397) of product (**7**) was 152 atomic mass units higher than that of (**1**), which corresponds to two ME moieties. In the ^1H-NMR spectrum (in DMSO-d_6 270 MHz) of product (**5**), the signal corresponding to the indole proton at position 2 disappeared and additional signals corresponding to an ME moiety appeared; δ 2.90 (dt, J=2.7, 6.8

Hz, 2H, -S$\underline{CH_2}CH_2OH$) and δ 3.49 (t, J=6.8 Hz, 2H, -$SCH_2\underline{CH_2}OH$). As for product (**6**), the signal corresponding to the indole proton at position 7 disappeared and signals corresponding to an ME moiety appeared; δ 2.97 (t, J=6.9 Hz, 2H, -S$\underline{CH_2}CH_2OH$) and δ 3.49 (t, J=6.9 Hz, 2H, -$SCH_2\underline{CH_2}OH$). For product (**7**), two indole protons at position 2 and 7 were not observed but signals corresponding to two ME moieties were observed; δ 2.99 (t, J=6.9 Hz, 4H, -S$\underline{CH_2}CH_2OH$ x 2) and δ 3.53 (t, J=6.9 Hz, 4H, -$SCH_2\underline{CH_2}OH$ x 2). From these results, we confirmed the structures of these products to be N-acetyl-2-[(2-hydroxyethyl)thio]-tryptophanamide[3,4], N-acetyl-7-[(2-hydroxyethyl)thio]tryptophanamide and N-acetyl-2,7-bis[(2-hydroxyethyl)-thio]tryptophanamide for products (**5**), (**6**) and (**7**), respectively. These structures were also confirmed by ^{13}C-NMR spectra[5].



Scheme 1

Next, we investigated the effects of AcOH concentration, the amount of $Hg(OAc)_2$ and the reaction time after addition of $Hg(OAc)_2$ on the formation of ME adduct of Trp. As shown in Table 1, the modification of (**1**) by ME greatly depends on the concentration of AcOH in aqueous solution (Experiments 1 and 10-14), the amount of $Hg(OAc)_2$ (Experiments 2-5 and 8-10) and the reaction time after addition of $Hg(OAc)_2$ (Experiments 5-7). The mechanism of these modifications may be that shown in scheme 1; compound (**1**) reacts with $Hg(OAc)_2$, resulting in the formation of intermediates (**2**), (**3**) and (**4**)[6]. These intermediates react with ME to form products (**5**), (**6**) and (**7**).

The most effective condition for suppressing the modification of Trp is to use 50% AcOH even if excess $Hg(OAc)_2$ is used with compound(**1**) (Experiment 14).

We next examined these modification reactions with [Cys(Acm)^{3,14}]-somatostatin, H-Ala-Gly-Cys(Acm)-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys(Acm)-OH. As shown in Fig. 1, similar modification of the Trp residue was observed when the peptide was treated with excess $Hg(OAc)_2$ (20 eq./Cys(Acm)) followed by treatment with excess ME (30eq./$Hg(OAc)_2$) in 1% AcOH (Fig. 1 (i)). On the other hand, this modification was almost completely suppressed when the reaction was carried out in 50% AcOH as in the case of compound(**1**) (Fig. 1 (ii)). Therefore, for the general deprotection procedure of the Acm group during the synthesis of Trp-containing peptides, we recommend the use of 1-2 eq. of $Hg(OAc)_2$ against 1 Acm group in 50% AcOH under which complete cleavage of the Acm group and complete suppression of the modification of the Trp residue are expected. By applying these conditions, we were able to obtain highly homogeneous free Cys peptides such as ω-agatoxin IVA, a 48 amino acid peptide having eight Cys residues[7]. Detailed synthesis of ω-agatoxin IVA will be reported elsewhere.

Table 1. Modification of N-acetyl-L-tryptophanamide under various conditions

Experiment	Conc.of AcOH (%)	Amount of $Hg(OAc)_2$ (eq.)*a	Reaction time (h)*b	Reaction time (h)*c	Yield(%)			Recovery of 1(%)
					5	6	7	
1	0.1	40	2	20	38.8	9.7	6.2	43.5
2	1	0.5	2	20	5.2	0.2	0.1	93.7
3	1	1	2	20	5.2	0.7	0.1	92.9
4	1	2	2	20	9.6	1	0.1	88.4
5	1	5	2	20	16.9	2.6	1.3	77.7
6	1	5	5	20	37.5	4.2	3.6	53.0
7	1	5	20	20	38.4	6.9	12.3	40.2
8	1	10	2	20	22.6	4.6	2.2	69.1
9	1	20	2	20	18.9	9	3.2	66.6
10	1	40	2	20	17.4	17	4.3	57.9
11	5	40	2	20	14.5	8.5	1.3	73.1
12	10	40	2	20	7.6	5.5	0.5	84.4
13	20	40	2	20	4.5	1.8	0.1	92.3
14	50	40	2	20	3.3	0.1	N.D.	95.4

In all experiments, compound (1) was dissolved in aqueous AcOH at the concentration of 1 mM and the amounts of ME used were fixed at 1200 eq. against (1). *a : Equivalent to (1), *b: Reaction time after addition of only $Hg(OAc)_2$, *c : Reaction time after treatment with $Hg(OAc)_2$ followed by adding ME., N.D. : not detected

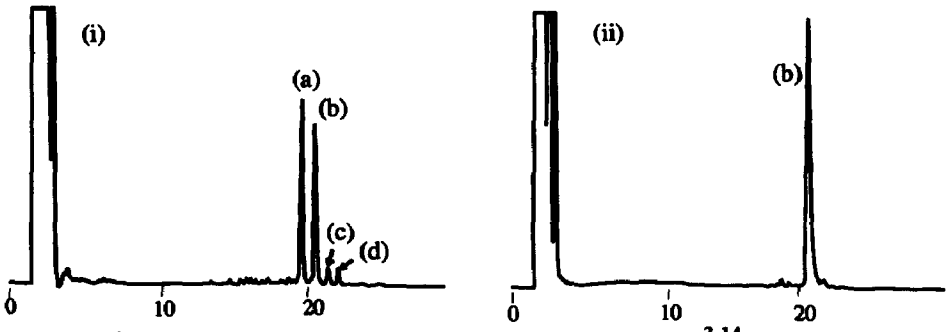


Fig.1 HPLC profile of the reaction mixture after treatment of [Cys(Acm)^{3,14}]-somatostatin with excess $Hg(OAc)_2$/ME
(i) Treatment in 1% aqueous AcOH, (ii) treatment in 50% aqueous AcOH, (a) and (d) mono-ME adduct on Trp residue of reduced somatostatin, (b) reduced somatostatin, (c) bis-ME adduct on Trp residue of reduced somatostatin[8]

In summary, the Trp residue in a Cys(Acm)-containing peptide was found to be modified with ME at position 2,7 or both 2 and 7 when the Acm residue was removed with $Hg(OAc)_2$ in aqueous AcOH followed by treatment with a large excess of ME. This side reaction could be suppressed almost completely when the

concentration of AcOH was increased to 50%. If we succeed in introducing an ME moiety onto Trp residues selectively at position 2 or 7, this reaction can be used to study the structure activity relationship of biologically active peptides containing Trp residues.

REFERENCES AND NOTES

1. Veber D.F.; Milkowski J.D.; Varga S.L.; Denkewalter R.G. and Hirschmann R. *J.Amer.Chem.Soc.*,**1972**, *94*, 5456-5461
2. Compound **1** : ^{13}C-NMR (in DMSO-d_6 67.5 MHz) δ 22.6 ($CO\underline{C}H_3$), 27.8 (β-CH_2), 53.2 (α-CH), 110.4 (C-3) 111.2 (C-7), 118.2 (C-5), 118.5 (C-4), 120.8 (C-6), 123.4 (C-2), 127.3 (C-9), 136.0 (C-8), 168.8, 173.7; UV (CH_3OH) λ_{max} 290, 281, 273 (sh) nm.
3. Product **5** : ^{13}C-NMR (in DMSO-d_6 67.5 MHz) δ 22.6, 28.0, 37.9 ($S\underline{C}H_2CH_2OH$), 53.5, 60.2 ($SCH_2\underline{C}H_2OH$), 110.7, 115.6, 118.6, 119.0,121.7, 126.4, 127.5, 136.7, 168.8, 173.5 ;UV (CH_3OH) λ_{max} 284(sh), 290, and 299 nm. UV, ^{1}H-NMR and^{13}C-NMR spectra of Product (*5*) were also identical to those of the authentic product which was synthesized by Ohno's method (see reference. 4).
4. Syntheses of Trp (2 thioether) derivatives were previously reported by two methods, one method is the synthesis by the reaction of cyclic intermediates of Trp with proper thiol (cf. Savige W.E. and Fontana A.*Int. J. Peptide Protein Res.*, **1980**, *15*, 102-112 and Ohno M.; Tanaka S.; Shieh T.C. and Spend T.F. *J. Org. Chem.*, **1984**, *49*, 5069-5072), and the other is the synthesis by the reaction of Trp with S-halide. (cf. Wieland Th. and Sarges R. *Liebigs Ann. Chem.* **1962**, *658*, 181-193 , Fontana A.; Marchiori F.; Rocchi R. and Pajetta P. *Gazz. Chim. Ital.*, **1966**, *96*, 1301-1312 , Wieland Th.; Urries J.D.; Indest H. and Faulstich H. *Liebigs Ann. Chem.* **1974**, 1570-1579 and Sieber P.; Kamber B.; Riniker B. and Rittel W. *Helv. Chim. Acta*, **1980**, *63*, 2358-2363)
5. Product **6** :^{13}C-NMR (in DMSO-d_6 270 MHz) δ 22.6, 27.8, 36.0 ($S\underline{C}H_2CH_2OH$), 53.1, 60.2 ($SCH_2\underline{C}H_2OH$), 111.4, 116.5, 118.1, 118.8, 124.0, 124.4, 127.7, 136.3, 169.0, 173.6 ; UV (CH_3OH) λ_{max} 301(sh), 294 and 284(sh) nm.
   Product **7** :^{13}C-NMR (in DMSO-d_6 270 MHz) δ 22.6, 28.1, 36.1 ($S\underline{C}H_2CH_2OH$), 38.0 ($S\underline{C}H_2CH_2OH$), 53.5, 60.1 ($SCH_2\underline{C}H_2OH$), 60.2 ($SCH_2\underline{C}H_2OH$), 116.2, 116.3, 118.3, 119.2, 124.9, 127.6, 127.8, 136.9, 168.9, 173.3 ; UV (CH_3OH) λ_{max} 310(sh), 301, 291(sh) nm.
6. Interaction of Trp and Trp-containing protein with $Hg(OAc)_2$ was previously reported by Ramachandran et. al., but further reactivity of these compounds was not described. (cf. Ramachandran L.K. and Witkop B. *Biochemistry*, **1964**, *3*, 1603-1611)
7. Mintz, I.M.; Venema, V.J.; Swiderek, K.M.; Lee, T.D.; Bean, B.P.and Adams, M.E. *Nature*, **1992**, *355*, 827-829.
8. Characterizations of product (a), (b), (c) and (d) were carried out by peptide mapping, PD-MS spectrometric analysis and sequence analysis. HPLC conditions: Instrument, Shimadzu LC6A; column, YMC ODS A-302 (4.6 x 150 mm); flow rate, 1.0 ml/min; detection, 220 nm; gradient, from 20% to 35% CH_3CN in 0.1% aq. TFA over 25 min.

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