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Tandem ligation at X-Cys and Gly-Gly positions via an orthogonally protected auxiliary group

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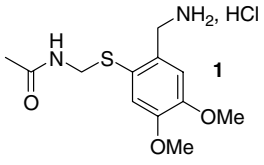
Abstract—4,5-Dimethoxy-2-mercaptobenzylamine (Dmmb) has been protected by acetamidomethyl (Acm) and incorporated into a peptide thioester for use in tandem native chemical ligation. Upon ligation between the thioester and a Cys-peptide, Acm was removed from Dmmb using silver acetate, and a second ligation reaction was done at the Dmmb position. Dmmb removal using TFMSA–TFA effected overall tandem ligation at X-Cys and Gly-Gly.

1. Introduction

Native chemical ligation allows the splicing of unprotected peptides and proteins by amide bonds via reaction of peptide C-terminal thioesters with peptide N-terminal cysteins.[1,2] The original requirement for N-terminal cystein has recently been circumvented by introduction of various auxiliary groups, such as 1-phenyl-2-mercaptoethyl and 4,5-dimethoxy-2-mercaptobenzyl (Dmmb).[3–10] These auxiliary groups have during the solid-phase peptide synthesis been protected by acid-labile groups. Accordingly, the auxiliary mercapto group is deprotected simultaneously with the overall peptide deprotection and peptide cleavage from resin, typically by HF or TFA in Boc- or Fmoc-based peptide synthesis, respectively. Acidic deprotections of the auxiliary mercapto groups however limit the methods to ligation of two peptide fragments, because sequences containing combination of thioesters and unprotected mercapto auxiliary groups will be internally reactive.



The present paper reports on the preparation of *S*-acetamidomethyl 4,5-dimethoxy-2-mercaptobenzylamine hydrochloride, abbreviated Dmmb(Acm) (**1**), along with the use of **1** in peptide synthesis and tandem ligation.[11] It is shown that **1**, similarly to Cys(Acm), can be deprotected by use of Ag(I) or Hg(II).[12] Upon peptide ligations, the Dmmb auxiliary can be removed by treatment with TFMSA/TFA to effect overall tandem ligation at X-Cys and X-Gly.

2. Results and discussion

4,5-Dimethoxy-2-mercaptobenzylamine[13] is commercially available and was S-protected with acetamidomethyl by treatment with *N*-hydroxymethylacetamide in aqueous hydrochloric acid,[14] Scheme 1. Product **1** was isolated in high yield by precipitation from methanol/ether. Use of TFMSA–TFA as catalyst for the transformation was also attempted,[15] but no convenient isolation method of the (mixed) TFMSA–TFA salt could be identified.

While Acm-cleavage from alkyl mercaptan (cysteine) is well established, Acm-cleavages from aromatic mercaptyls are scarcely described.[16] A study of Acm-cleavage from **1** was therefore warranted. In order for easy work-up and analysis, **1** was N-acetylated to give **2**, which was then tested for Acm-cleavage (Scheme 1). Encouragingly, **2** was deprotected

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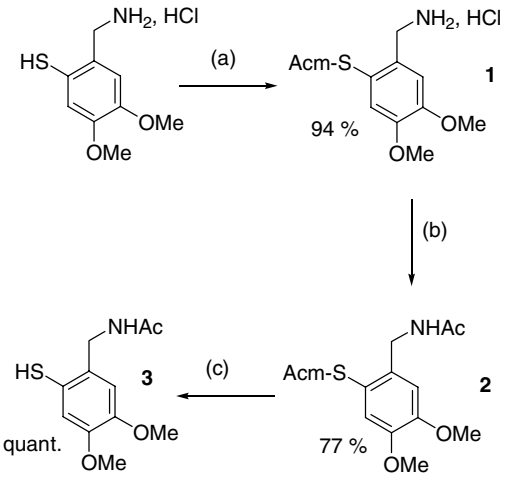
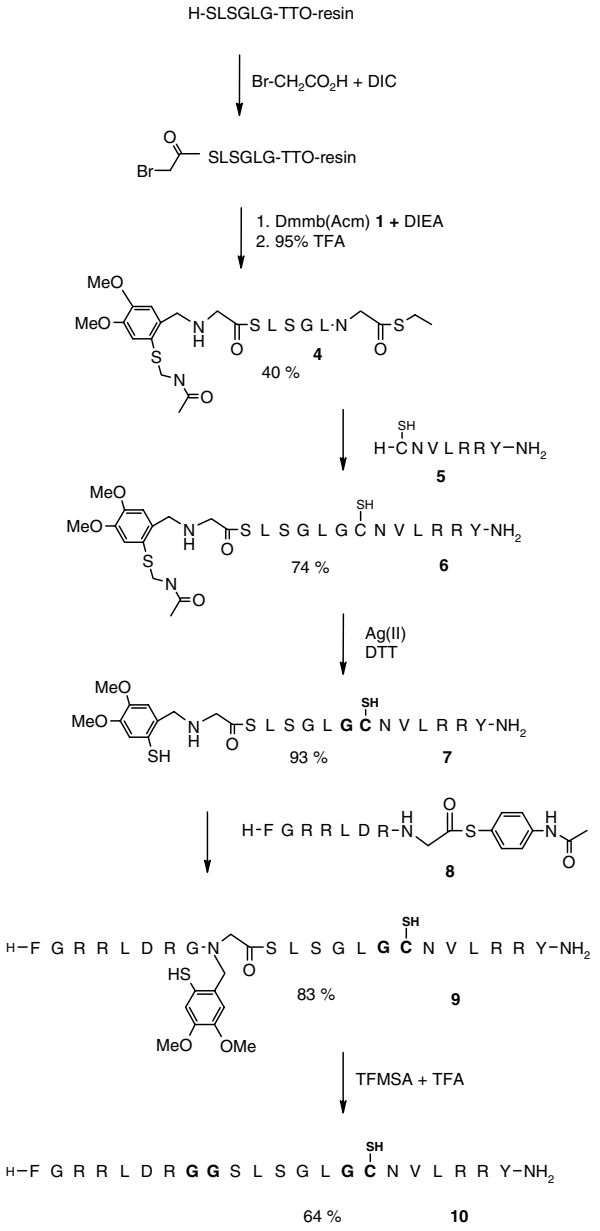


Scheme 1. Reactions and condition: (a) $CH_3CONHCH_2OH$, HCl; (b) Ac_2O, Na_2CO_3; (c) Hg(II), pH 2–3, rt.

smoothly with aqueous Hg(II) at pH 2–3 at room temperature to provide **3**.

In order to study tandem ligation using **1**, peptides **4**, **5**, and **8** were prepared as outlined in Scheme 2. Dmmb(Acm) peptide thioester **4** was prepared by Fmoc-based solid-phase peptide synthesis on a Gly-trithioortho ester resin,[17] via N-terminal bromoacetylation, reaction with Dmmb(Acm) **1**, and cleavage from resin using TFA–TIS. Peptide **4** was isolated in a yield of 40% upon purification by RP-HPLC. Cys-peptide **5** was synthesized by Fmoc-based SPPS on Rink amide linker tentagel. Peptide thioester **8** was prepared by the method of Von Eggelkraut-Gottanka et al.[18]

The ligation between Dmmb(Acm) peptide thioester **4** and Cys-peptide **5** was mediated in 6 M guanidinium chloride at pH 7.5 in the presence of thiophenol and benzyl mercaptan. After 24 h at 25 °C, the ligation product **6** was observed by MALDI-MS and isolated in a yield of 74% after RP-HPLC purification, Figure 1a. The Acm group was removed from peptide **6** by treatment with silver (I) acetate[12,19] followed by DTT to provide peptide **7** in a yield of 93% upon RP-HPLC purification, Figure 1b. The ligation reaction between the Dmmb peptide **7** and thioester **8** was likewise performed in 6 M guanidinium chloride at pH 7.5 in the presence of thiophenol and benzyl mercaptan. After 1 day at 25 °C, the ligation product **9** was observed by MALDI-MS and isolated in a yield of 83% of **9** after RP-HPLC purification, Figure 1c. Finally, the Dmmb group was removed from **9** using 1 M TFMSA + 1 M thioanisol in TFA to give the target peptide **10** (Gly^{12}-Brain Natriuretic $Peptide_{5–26}$, porcine).[20] The yield of the Dmmb removal was 64% after RP-HPLC purification, Figure 1d. Equilibriums between *N*- and *S*-acyl species have previously been described for Dmmb-mediated ligation intermediates,[6] but the major product in the present case with Gly-Gly link at the Dmmb site was the desired compound **10**, as characterized by LC–MS.



Scheme 2. Peptide tandem ligation for preparation of Gly^{12}-brain natriuretic $peptide_{5–26}$, porcine.

3. Conclusions

In conclusion, acetamidomethyl S-protected 4,5-dimethoxy-2-mercaptobenzylamine, Dmmb(Acm) **1**, has been prepared and it has been demonstrated that this removable acyl transfer auxiliary group can be incorporated into peptides by stepwise solid-phase synthesis using Fmoc chemistry. The Acm group could easily be removed from Dmmb by use of mercury (II) or silver (II) acetate. By using Dmmb(Acm) **1** as ligation auxiliary, three peptide segments could be assembled in two subsequent ligations. Upon Dmmb removal by treatment with TFMSA/TFA, tandem native chemical ligation at sites X-Cys and Gly-Gly is thus established. Further work is needed to evaluate the applicability of Dmmb(Acm) for ligation at X-Gly sites.

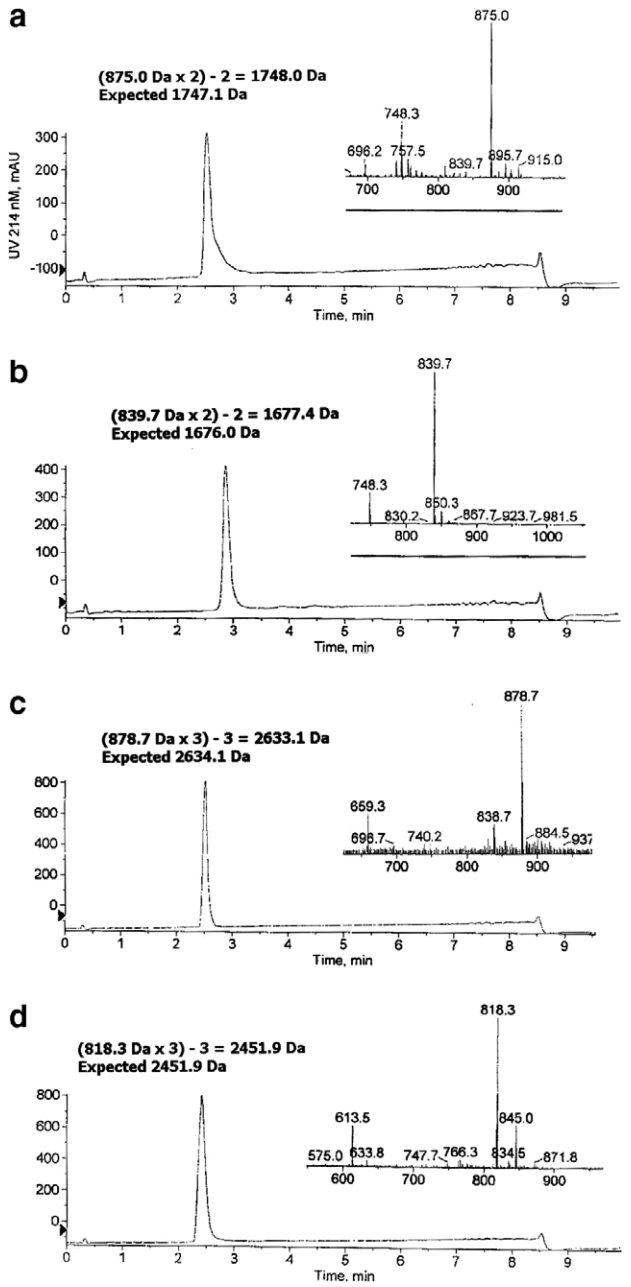


Figure 1. LCMS of peptide products. (a) Gly-Cys ligation product **6**; (b) product **7** by Acm removal; (c) Gly-Dmmb ligation product **9**; (d) final product **10** by Dmmb removal.

4. Experimental

4,5-Dimethoxy-2-mercaptobenzylamine[13] was purchased from Acros Organics. Protected amino acids and other reagents for peptide synthesis were purchased from Novabiochem. Other chemicals were purchased from Sigma–Aldrich. NMR analysis was performed on a Varian 400 MHz instrument. Chemical shifts are reported relative to TMS at 0.0 ppm, and coupling constants J are reported in hertz. Solid-phase peptide synthesis was conducted on an Applied Biosystems 348 peptide synthesizer. MALDI-MS was performed on Voyager-DE from Perseptive Biosystems using a cinnapinic acid matrix, and LC–MS was performed on a Sciex API 100 Single quadroupole mass spectrometer using a XTerra MS C-18 column (5 μm, 3×50 mm). The RP-HPLC separations were performed using the linear gradient 5% buffer B–90% buffer B over 7.5 min at 1.5 ml/min. Analytical RP-HPLC and semi-preparative RP-HPLC were performed using a Waters RCM 8×10 module with C-18 columns, 19×300 and 25×300 mm, respectively. The solvent system for both analytical and semi-preparative RP-HPLC was buffer A (0.1% TFA in water) and buffer B (0.07% TFA in acetonitrile) with detection at 215 nm. The gradient for analytical RP-HPLC was 0–70% buffer B at 1 ml/min over 25 min and for semi-preparative RP-HPLC 10% buffer B for 5 min and then 10–65% buffer B at 4 ml/min over 35 min.

4.1. Dmmb(Acm) HCl 1

4,5-Dimethoxy-2-mercaptobenzylamine hydrochloride (1.93 g, 8.2 mmol) and *N*-hydroxymethylacetamide (1.07 g, 12.1 mmol) were dissolved in water (7.5 ml) under argon, cooled with an ice-bath, and treated with concentrated hydrochloric acid (0.43 ml). The cooling was removed and the mixture was stirred under argon overnight. The reaction was monitored by TLC on silica plates, eluted with *n*-butanol/AcOH/H_2O, 10:2:3, and compound detection by UV-light or sodium nitroprusside (red color with mercapto groups). The solvent was removed in vacuo and the residue was evaporated with dry ethanol four times. The crude product was dissolved in methanol (15 ml), and **1** was isolated by filtration upon precipitation with dry ether and cooling overnight; 2.06 g (94%).

^{1}H NMR, DMSO-d_6: 8.67 (t, 1H, $J = 6.2$), 8.43 (br s, 3H), 7.35 (s, 1H), 7.13 (s, 1H), 4.42 (d, 2H, $J = 6.2$), 4.14 (d, 2H, $J = 5.1$), 3.79 (s, 6H), 1.79 (s, 3H).

^{13}C NMR, DMSO-d_6: 169.3, 149.0, 148.7, 129.2, 124.3, 118.1, 113, 4, 55.8, 55.7, 45.2, 22.4.

4.2. *N*-Acetyl-Dmmb(Acm) 2

Compound **1** (100 mg, 0.32 mmol) was dissolved in 10% sodium carbonate (1 ml) and treated with acetic anhydride (0.10 ml) and the mixture was stirred at rt overnight. The reaction mixture was extracted twice with ethyl acetate, which was washed with water and brine and dried over magnesium sulfate. Evaporation gave 77 mg (77%).

^{1}H NMR, DMSO-d_6: 7.01 (s, 1H), 6.93 (s, 1H), 6.85 (br s, 1H), 6.40 (br s, 1H), 4.58 (d, 2H, $J = 5.5$), 4.55 (d, 2H, $J = 6.6$), 3.88 (s, 3H), 3.87 (s, 3H), 2.03 (s, 3H), 1.92 (s, 3H).

4.3. *N*-Acetyl-Dmmb 3

Compound **2** (15 mg, 0.048 mmol) was dissolved in water (1 ml) and acetic acid was added to adjust pH to approximately 2. Mercuric acetate (15 mg, 0.048 mmol) was added and the reaction was monitored by TLC on silica plates with *n*-butanol/AcOH/H_2O, 10:2:3, using

UV and sodium nitroprusside detection. Precipitation started slowly after 30 min. After 3 h, the mixture was filtered through a short silica pad and evaporated in vacuo to give the mercury adduct of **3** (36 mg, quantitative).

^{1}H NMR, DMSO-d_6: 8.12 (t, 1H, J = 5.5), 7.08 (s, 1H), 6.83 (s, 1H), 4.40 (d, 2H, J = 5.5), 3.71 (s, 3H), 3.64 (s, 3H), 1.87 (s, 3H).

4.4. Peptide 4, Dmmb(Acm)-SLSGLG-SEt

The Dmmb(Acm) peptide thioester **4** was synthesized from Leu-Gly-trithioortho ester resin (0.11–0.14 mmol/g, 1 g) and Fmoc-protected amino acids using DIC/HOAt (3 equiv). Then α-bromoacetic acid (240 mg, 1 mmol) and DIC (160 μl, 0.50 mmol) in DMF were added to the peptide resin (110 μmol). The reaction was left overnight at rt and the resin was washed several times with DMF. To the peptide resin were added Dmmb(Acm) **1** (30.4 mg, 90 μmol) and DIEA (54 μl, 0.29 mmol) in DMF (300 μl). After the protected peptide resin was washed with DMF, NMP, and DCM, the peptide was cleaved from resin and deprotected using 95% TFA. The peptide was purified by RP-HPLC, providing 29.7 mg peptide **4**. Electrospray MS of peptide **4** gave the expected mass (obsd: 887.1 Da; calcd: 887.1 Da).

4.5. Peptide 5, H-CNVLRRY-amide

The Cys-peptide peptide amide **5** was prepared on a Rink amide Tentagel (0.26 mmol/g, 1 g) with couplings mediated by DIC/HOAt. The peptide was cleaved from resin using 95% TFA + 2% TIS and a crude yield of 120 mg was obtained. Part of the crude peptide (80 mg) was purified by semi-preparative RP-HPLC. Peptide **5** was obtained, 10 mg, after purification and lyophilization (LCMS obsd: 923.6 Da; calcd: 922.1 Da).

4.6. Peptide 8, H-FGRRFDRG-SPh-*p*-NHAc

The peptide thioester **8** was synthesized on a Gly-2-chlorotrityl resin (0.6 mmol/g, 1 g) with couplings mediated by DIC/HOAt. The peptide was cleaved from the resin with acetic acid/trifluoroethanol/DCM (1:1:8, v/v/v) and 853 mg of protected peptide was obtained. To the protected peptide (0.045 mmol, 853 mg) were added Py-BOP (3 equiv, 705 mg), DIEA (3 equiv, 0.065 ml), and *p*-acetamidothiophenol (3 equiv, 227 mg) in DCM (280 ml). The solution was stirred overnight at RT. After DCM was evaporated was the peptide was deprotected using 95% TFA and 2% TIS. A yield of 827 mg of crude peptide **8** was obtained after lyophilization. Purification by HPLC of a quarter of the crude material gave 15 mg of peptide **8** (LCMS obsd: 1125.6 Da; calcd: 1125.3 Da).

4.7. Peptide 7 by ligation of peptides 4 and 5 followed by Acm removal

Peptide **4** (5.2 mg, 5.8 μmol) and peptide **5** (5 mg, 5.42 μmol) were dissolved in 6 M guanidinium chloride, 0.1 M sodium phosphate, pH 7.4 (300 μl). Thiophenol (6 μl) and benzyl mercaptan (6 μl) were added and pH was readjusted to 7.5. The solution was stirred at 25 °C for 24 h and MALDI-MS showed that the ligation reaction had occurred and peptide **6** was formed. A yield of 7 mg peptide **6** (74%) was obtained after purification by semi-preparative HPLC, and LC–MS (obsd: 1748.0 kDa; calcd: 1747.1 kDa) confirmed the product. To remove the Acm group the peptide **6** (7 mg, 4 μmol) was dissolved in aqueous 0.1% TFA with 20% acetonitrile (100 μl) and AgOAc (33 mg, 0.2 mmol) was added. The reaction mixture was stirred for 1 h at room temperature. DTT was added to quench the reaction and the supernatant was purified by semi-preparative HPLC. Peptide **7** (6.3 mg, 93%) was obtained after purification and lyophilization, and LC–MS confirmed the expected mass (obsd: 1677.4 Da; calcd: 1676.0 Da).

4.8. Peptide 10 by ligation of peptides 7 and 8 followed by Dmmb removal

Peptide **7** (6.3 mg, 3.7 μmol) and peptide **8** (4.1 mg, 3.6 μmol) were dissolved in 6 M guanidinium chloride, pH 7.4 (200 μl) and pH was adjusted to 7.5. Thiophenol (4 μl) and benzyl mercaptan (4 μl) were added and pH was readjusted to 7.5. The reaction mixture was stirred for 24 h at 25 °C and progress of the formation of peptide **9** was monitored by MALDI-MS. Purification by semi-preparative HPLC gave peptide **9** (8 mg, 83%), characterized by electrospray MS (obsd; 2633.1 Da; calcd; 2634.1 Da). Finally was peptide **9** (5 mg, 1.8 μmol) dissolved in 1 M TFMSA and 1 M thioanisol in TFA (200 μl) for 2 h at 0 °C. The peptide was precipitated with diethyl ether and lyophilized. Purification of peptide **10** by semi-preparative HPLC gave 3 mg (64%) characterized by electrospray MS (obsd: 2451.9 Da; calcd: 2451.9 Da).

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