JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
J. Am. Chem. Soc., 1996, 118(12), 3053-3054, DOI:10.1021/ja9538501

JA9538501-334 revised JAN - 9 1996 J3054-1
RECEIVED

SUPPLEMENTARY MATERIAL
for the manuscript entitled

JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Design and Evaluation of a Peptidyl Fluorescent Chemosensor for Divalent Zinc

Grant K. Walkup and Barbara Imperiali*

General Information

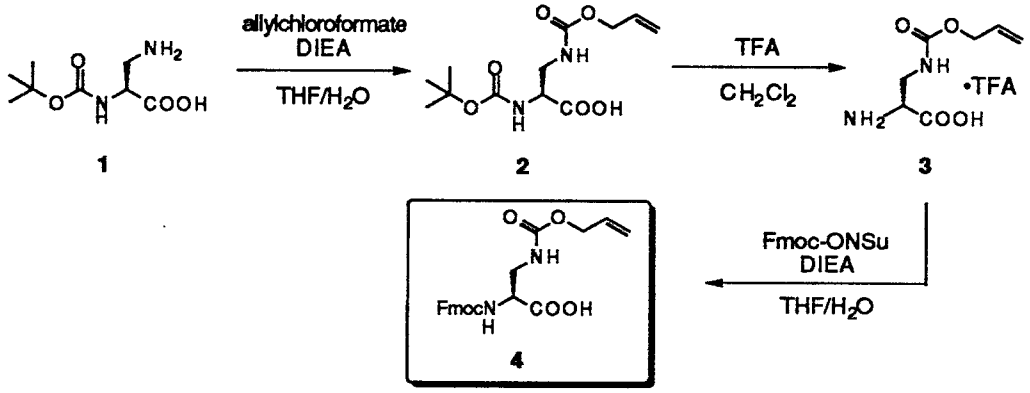
Reagents used were purchased from Aldrich Chemical Co. or Fluka. Protected amino acid derivatives and reagents were obtained from Milligen (Perseptive) Biosearch, except Fmoc-L-Baa(alloc)-OH which was synthesized as outlined below. Fluorescence experiments were performed on a SLM-Aminco SPF 500c spectrofluorometer at ambient temperature. UV-Vis spectra were obtained on a Shimadzu UV160U, or a Beckman DU 7500 recording spectrophotometer.

All solutions and buffers employed in assays were composed of high-purity (18 Ω cm) water obtained from a Milli-Q (Millipore) deionization purification system, and were handled in acid-washed HDPE containers. To prevent metal catalyzed- and auto-oxidation of ZNS-1, buffer solutions were sparged with argon for one half hour then hydrogen for fifteen minutes immediately prior to use.

Metal titrations were performed by the addition of aliquots of concentrated stock solutions. The concentration of these stocks were determined by complexometric titrations performed in triplicate with standard EDTA solutions (Aldrich) and an appropriate colorometric indicator.[1]

Synthesis of Fmoc-L-Baa(alloc)-OH

The amino acid derivative (**4**) was synthesized from the precursor N^{α}-Boc-L-β-amino alanine[2,3] *via* the route outlined in Scheme I.



Scheme I. Strategy for the synthesis of Fmoc-Baa(alloc)-OH.

J3054-2

Boc-L-Baa(alloc)-OH (**2**) A solution of the Boc-amine (**1**) was prepared by dissolving 4.33 g (21.2 mmol) in 100 mL of 50% v/v THF in deionized water, assisted by sonication at 50 °C. Sodium carbonate (2.46 g, 23.2 mmol) in 7 mL of water was added, and the resultant slurry was cooled to 0 °C with constant stirring. Allyl chloroformate (2.25 mL, 21.2 mmol) and diisopropylethylamine (3.66 mL, 21.2 mmol) were added in alternating portions over a period of 20 minutes, after which the ice bath was removed and the reaction vessel let stir an additional 1.5 hr. The organic solvent was removed under reduced pressure, and additional deionized water added to increase the volume to 250 mL. Citric acid was added until pH 3.2, and the solution extracted with CH_2Cl_2 (5 x 200 mL). The organic layers were combined, dried over $MgSO_4$, and the solvent removed under reduced pressure to give a clear oil.. Traces of contaminating lactam were separated by chromatography on silica gel (95:4:1, $CHCl_3$/MeOH/AcOH) to afford 4.60 g (75% yield) of a clear, colorless oil. R_F (85:15:3, $CHCl_3$/MeOH/AcOH) = 0.47. R_F (95:5:1, CH_2Cl_2/MeOH/AcOH) = 0.20. IR (NaCl, neat) cm^{-1} 1164.0 (m), 1253.6 (m), 1517.4 (m), 1532.4 (m), 1694.5 (s), 1711.4 (s), 1728.4 (s), 2977.5 (m). ^1H NMR (300 MHz, DMSO-d_6) δ 1.40 (s, 9H), 3.98 (m, 1H), 4.42 (d, 2H), 5.27 (d, 1H), 5.92 (m, 1H), 6.92 (d, 1H), 7.2 (t, 1H). ^{13}C NMR (DMSO-d_6) δ 28.7, 43.2, 64.9, 72.9, 78.8, 117.4, 134.1, 162.8, 171.8, 172.7.

L-Baa(alloc)-OH • TFA (**3**) A 100-mL round bottom flask was charged with a stir bar, Boc-L-Baa(alloc)-OH (**2**, 4.55 g, 15.8 mmol), and CH_2Cl_2 (15 mL), then chilled to 0 °C with constant stirring. Trifluoroacetic acid (TFA, 10 mL, 130 mmol) was added drop wise over 15 minutes. After an additional 15 minutes the reaction was allowed to come up to room temperature and let stir for 2 hours. Excess TFA was removed under a stream of nitrogen, then the solvent was removed in vacuo to give a clear gum. Lyophilization of this residue from benzene gave **3** as a clear oil (4.63g, 97% yield). R_F (1:1:1:1, n-butanol/AcOH/H_2O/EtOAc) = 0.54. R_F (1:1:1, n-butanol/AcOH/H_2O) = 0.4. ^1H NMR (300 MHz, CD_3OD) δ 3.58 (d, 1H), 3.73 (d, 1H), 4.1 (m, 1H) 4.53 (d, 2H), 5.18 (d, 1H), 5.28 (d, 1H), 5.88 (m, 1H). ^{13}C NMR (D_2O) δ 43.6, 66.6, 73.6, 118.0, 133.0, 165.3, 170.5.

Fmoc-L-Baa(alloc)-OH (**4**) The carbamate amine **3** (370 mg, 1.21 mmol) was dissolved in 2:1 THF/H_2O (12 mL), requiring the assistance of vigorous stirring and sonication. Diisopropylethylamine (DIEA, 465 μL, 2.66 mmol) was added until the solution was slightly basic to litmus. Under vigorous stirring, *N*-(9-Fluorenylmethoxycarbonyl)succinimide (Fmoc-ONSu, 816 mg, 2.42 mmol) was added in alternating portions with DIEA (420 μL, 2.42 mmol) over 30 minutes. The reaction was allowed to stir an additional four hours. The organic phase was removed in vacuo, and the remaining aqueous solution was increased in volume to ~100 mL, and acidified with citric acid to pH 3. This was extracted with diethyl ether (2 x 50 mL), and the organic phase discarded. The aqueous phase was further extracted with CH_2Cl_2 (4 x 100 mL), the organic phases combined, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to afford a clear oil. Purification by silica gel chromatography (95:5:3 $CHCl_3$ / MeOH /

J3054-3

AcOH), followed by lyophilization from benzene gave **4** (465 mg, 96% yield) as a white powder. R_F (CH_2Cl_2 / MeOH / AcOH, 95:5:3) = 0.24. R_F ($CHCl_3$ / MeOH / AcOH, 85:15:3) = 0.51. IR (CH_2Cl_2, NaCl) cm^{-1} 1407.1 (m), 1450.1 (m), 1523.8 (s), 1712.9 (s), 3044.3 (w), 3066.4 (w). ^1H NMR (300 MHz, DMSO-d_6) δ 3.25-3.45 (m, 3H), 4.0-4.4 (m, 3H), 4.44 (d, 2H), 5.14 (d, 2H), 5.25 (d, 2H), 5.85 (m, 1H), 7.2-7.6 (m, 4H), 7.70 (d, 2H), 7.88 (d, 2H), 12.7 (s, 1H). ^{13}C NMR (D_2O) δ 42.8, 47.2, 54.9, 67.4, 100.2, 118.0, 120.2, 125.5, 127.4, 128.0, 132.9, 144.0, 141.5, 157.3, 163.9, 172.8.

Peptide synthesis

General. Peptides were synthesized on a Milligen 9050 peptide synthesizer at a 0.125 mmol scale. The support used was in all cases PAL-PEG-PS (Millipore) thus providing carboxy-terminal primary amides. Couplings were performed at a concentration of 0.3 M acylating reagent and HOBT, in a volume sufficient to achieve a three-fold excess of amino acid to resin-bound amine. Pentafluorophenyl ester / 1-hydroxybenzotriazole (Pfp/HOBT) chemistry was employed for all residues except Fmoc-L-Baa(alloc)-OH which was coupled by *in situ* active ester generation using HOBT / *N,N'*-diisopropylcarbodiimide (HOBT / DIPCDI) activation. All coupling reactions were allowed to proceed for greater than 45 minutes, and were monitored for completion with the Enhanced Monitoring Option (EMO, Milligen). A coupling judged to be incomplete (< 97%) was double coupled for an additional 45 minutes. After successful coupling cycles, any residual amines were acylated by a 10 min wash with 0.3 M acetic anhydride/HOBT solution in 9:1 DMF / dichloromethane. Removal of the Fmoc group was performed with piperidine (20% v/v in DMF) with a standard wash duration of 7 minutes. This time was extended if an inefficient deblock was detected by EMO. After addition of the final residue, the amino-terminus was acetyl-capped (DMF / acetic anhydride / triethylamine, 4 mL:63 μL:94 μL) for 0.5 h then washed with DMF (5 x 10 mL) and MeOH (5 x 10 m). Residual solvent was removed under reduced pressure.

Alloc removal. The method of Kates et al.[4] was employed with some minor modifications. A typical procedure for removal of the alloc group was as follows. Under a blanket of nitrogen, a 20-mL plastic, stoppered vial was charged with resin from the completed peptide synthesis (300 mg, 0.21 meq/g, 63 μmol), and 5 mL of a solvent cocktail ($CHCl_3$ / morpholine / AcOH, 90:5:5). The resin was allowed to swell 10 min, to which tetrakis(triphenylphosphine)palladium (200 mg, 173 μmol) was added under a blanket of nitrogen. The vial was capped, shielded from light, and placed on a wrist-action shaker at low speed for 2 h. The resin was filtered, washed with $CHCl_3$ (5 x 10 mL), and a palladium-chelating cocktail (DMF / diethyldithiocarbamic acid • $3H_2O$ / triethylamine, 25 mL:225 mg:250 μL). Traces of this solution were removed with a basic wash (0.5% v/v triethylamine in DMF),

J3054-4

and a final wash with methanol. The resin was transferred to a clean plastic vial, and the residual solvent removed under reduced pressure.

Dansyl coupling. Lyophilized resin taken directly from the alloc-removal procedure (280 mg, 0.21 meq/g, 59 μmol), was allowed to swell in DMF (5 mL). Dansyl chloride (159 mg, 590 μmol) was added, followed by triethylamine (82 mL, 590 μmol). The vial was capped under a blanket of nitrogen, and placed on a wrist-action shaker at low speed for 2 h. The resin was filtered, washed with a basic wash (5 x 10 mL, see above), washed with DMF (5 x 10 mL), then finally washed with MeOH (5 x 10 mL). The resin was transferred to a clean 20 mL polyethylene vial, and residual solvent was removed under reduced pressure prior to peptide cleavage.

Peptide cleavage and purification. Peptides were cleaved after fluorophore coupling using 10 mL of Reagent K[5] (trifluoroacetic acid / H_2O / ethanedithiol / thioanisole / phenol, 82.5:5:5:5:2.5) with a 2 h incubation period. The resin was filtered, concentrated to ca. 2 mL volume, and precipitated with ether / hexane 2:1 at -20 °C for 30 min. The supernatant was decanted, and the peptides triturated with ether / hexane 2:1 (5 x 50 mL). The resultant solid was resuspended in water (20 mL), lyophilized, and then purified by reverse phase (C_{18}) high-performance liquid chromatography (HPLC), gradient elution with 0.1% v/v TFA in acetonitrile (solvent B) added to 0.1% v/v TFA in water (solvent A). The reduced (dithiol) and oxidized (intramolecular disulfide) forms of the peptide are separable by HPLC. Both forms of the peptide were collected and characterized, then combined and submitted together to a reduction protocol.

Peptide reduction.. The reduction was performed by dissolving the combined HPLC purified peptides in 0.1 M phosphate buffer, pH 8.0, 50 mM dithiothreitol (DTT), 10mM EDTA, 6 M guanidine, and incubating at 25 °C, 1 h. The reduction mixture was loaded onto a C_{18} Sep-Pak (Millipore), and buffer salts and DTT were eluted with 10 mL water. The reduced peptide was eluted with 30% acetonitrile in water. Acetonitrile was removed from this solution under a stream of nitrogen until the sample could be frozen at 0 °C, whence it was lyophilized. This was resuspended in high-purity water that had been sparged with argon 0.5 h then hydrogen 0.5 h. to delay oxidation. The resulting stock solution was periodically analyzed by HPLC and no significant (> 2%) disulfide formation was detected.

Peptide characterization. ZNS-1: Ac-Tyr-Gln-Cys-Gln-Tyr-Cys-Glu-Lys-Arg-Baa(dns)-Ala-Asp-Ser-Ser-Asn-Leu-Lys-Thr-His-Ile-Lys-Thr-Lys-His-Ser-NH_2

t_R (oxidized, linear gradient 0-70% B over 25 min) = 18.1 min.

t_R (reduced, linear gradient 0-70% B over 25 min) = 18.8 min.

t_R (oxidized, linear gradient 20-40% B over 25 min) = 10.6 min.

t_R (reduced, linear gradient 20-40% B over 25 min) = 13.5 min.

Electrospray-MS (oxidized) MW Calcd. for $C_{138}H_{212}O_{42}N_{42}S_3$ 3227.6; Obs. 3227.2

J30545

Electrospray-MS (reduced) MW Calcd. for $C_{138}H_{214}O_{42}N_{42}S_3$ 3229.7; Obs. 3228.9

The concentration of stock solutions ZNS-1 was determined by reaction with Ellman's reagent, 2,5'-dithiobis(2-nitrobenzoic acid) (DTNB)[6]. These assays were performed in triplicate, with excellent agreement (< 5% error) between replicate runs. With this knowledge, the extinction coefficient of the dansyl chromophore was determined at 333 nm for ZNS-1 to be 5.3 x 10^3 M^{-1} cm^{-1} in 50 mM Hepes, pH 7.0. This value was used for all subsequent quantitations of ZNS-1.

Emission fluorescence assay. Assays were performed at pH 7.0 in 50 mM Hepes buffer, 0.5 M NaCl in a stoppered 750 μL (1cm x 3mm) quartz fluorometer cell. The concentration of ZNS-1 in a given assay was determined by measurement of the unique absorbance of the dansyl chromophore (ε 333 nm = 5.3 x 10^3). Blank scans were taken against the same buffer immediately prior to ZNS-1 addition. Emission spectra were accumulated at 1 nm intervals with the following parameters: excitation wavelength = 333 nm, emission wavelength = 400-750 nm, excitation band pass = 4 mm, emission band pass = 2 mm, lamp potential = 975 V, gain = 10, filter (time constant) = 3. To mask the effects of adventitious metal ions present prior to the initiation of metal titration, small aliquots of EDTA were added (in increments of 0.5 μM) until overlaying emission spectra were observed, and the wavelength of maximum emission was 560 nm. The EDTA concentration at the initiation of the metal titration was never in excess of 4 μM (< 0.001% of the Mg^{2+}, and < 4% of the Co^{2+} subsequently added). With the concentrations of Zn^{2+} and Co^{2+} present over the course of a titration, and the relative stability constants of EDTA for these species, at most 10% of the Zn^{2+} would be complexed by EDTA.[7] Divalent metal ions were added to the assay from stock solutions prepared in unbuffered water. These included a 2.83 M solution of $MgCl_2$, a 0.0969 M solution of $CoCl_2$, and a 100 μM solution of $ZnCl_2$.

References

(1) *Vogel's Textbook of Quantitative Inorganic Analysis*; Bassett, J.; Denney, R. C.; Jeffery, G. H. Mendham, J.; William Clowes: London, 1978; pp 223-402 .

(2) Arnold, L. D.; Kalantar, T. H.; Vederas, J. C. *J. Am. Chem. Soc.* **1985**, *107*, 7105-9.

(3) Kucharczyk, N.; Badet, B.; Goffic, F. L. *Synth. Comm.* **1989**, *19*, 1603-1609.

(4) Kates, S. A.; Daniels, S. B.; Albericio, F. *Anal. Biochem.* **1993**, *212*, 303-310.

(5) King, D. S.; Fields, C. G.; Fields, G. B. *Int. J. Pept. Protein Res.* **1990**, *36*, 255-266.

(6) Riddles, P. W.; Blakeley, R. L.; Zerner, B. *Meth. Enzymol.* **1983**, *91*, 49-60.

(7) O'Sullivan, W. J.; Smithers, G. W. *Meth. Enzymol.* **1979**, *63*, 294.