New Synthetic Amino Acids for the Design and Synthesis of Peptide-Based Metal Ion Sensors

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The syntheses of two new nonstandard amino acids, Flu (**6**) and XBp (**20**), and a new synthesis of Dmd (**12**) are reported. These residues exhibit fluorescence, metal-coordination, and fluorescence-quenching properties, respectively. These building blocks have been incorporated into peptides via solid phase peptide synthesis to afford the prototype for a photoinduced electron transfer-based metal ion chemosensor. The fluorescence of the peptides is modulated upon metal binding. This results from a metal ion-induced conformational change that brings the side chains of the Flu and Dmd amino acids into proximity, thereby favoring photoinduced electron transfer (PET) fluorescence quenching.

The development of chemosensors for the detection of metal ions in aqueous and nonaqueous media continues to be the focus of research in a number of laboratories.[1] In particular, sensors based on fluorescence measurements are desired due to their high sensitivity, with working detection limits typically below 0.1 μM analyte concentrations. Remarkable progress has been achieved in this regard for the determination of intracellular calcium[2−5] and proton[6,7] concentrations. While sensors for other analytes, including Na^+,[8] K^+,[8] Cl^-,[9,10] Mg^{2+},[11] and cAMP,[12] have also been introduced, the need for new strategies for the design of selective fluorosensors continues to exist.[13]

In light of the selectivity and avidity with which proteins bind metal ions,[14] we have focused on the use of polypeptide motifs for the recognition and sensing of divalent metal cations. We have demonstrated that the judicious placement of appropriate ligands in simple polypeptides affords species that fold into more compact structures upon metal complexation.[15,16] Furthermore, we have exploited metal-dependent structure modulation as the basis for trace analyte detection in the development of a peptidyl fluorescent chemosensor for divalent zinc.[17] Recently, a polypeptide-based sensor employing fluorescence resonance energy transfer (FRET) has also been described.[18]

Herein we report the preparation and characterization of peptides that exhibit metal-dependent fluorescence properties resulting from photoinduced electron transfer (PET). The application of PET in a fluorosensor design requires a donor (fluorescence quencher) capable of transferring a single electron to a fluorophore (acceptor) in its excited state to transiently form the radical anion of the fluorophore. Commonly, the electronic properties of the donor are modulated by the analyte to be tested. The lone pair electrons of oxygen- and nitrogen-containing molecules are suitable donors. Indeed, this effect has been exploited in the design of cyclic and noncyclic polyethers, crown ethers,[19−23] cryptands,[24−26] and calixarenes[27−31] for the detection of alkali metals. Similarly, chemosensors incorporating cyclam, thiacyclam rings,[32,33] or dioxotetraaza units[34,35] have been developed for the detection of transition metal ions. The binding of cations

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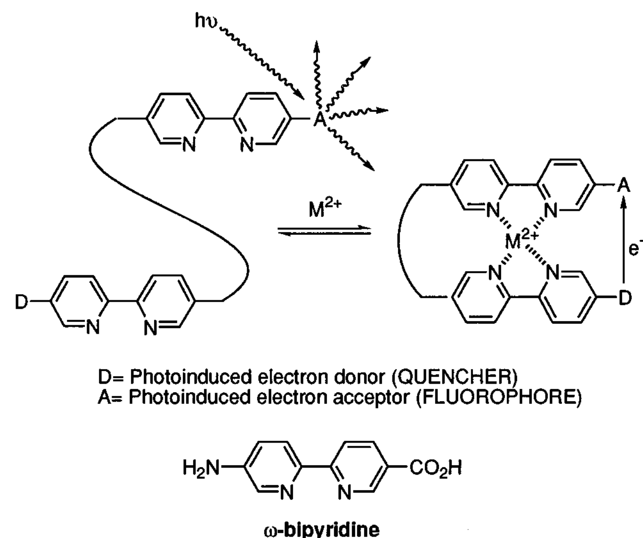


Figure 1.

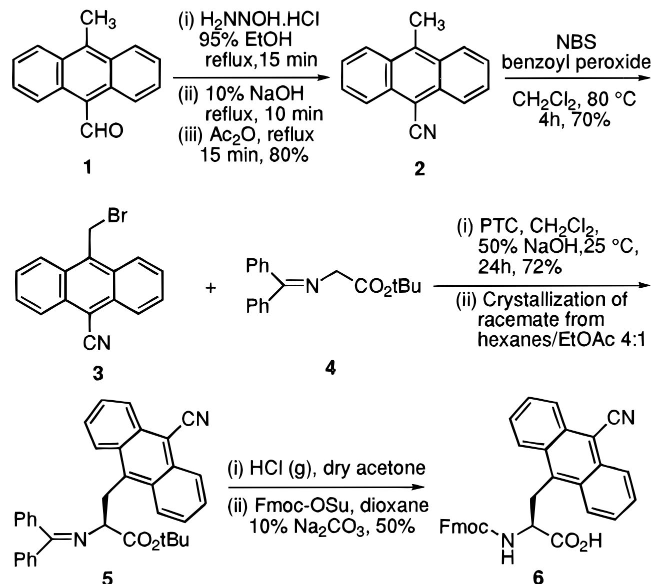
to these macrocycles diminishes or eliminates their ability to quench the donor. The efficiency of electron transfer (fluorescence quenching) is strongly influenced by the distance between the donor and acceptor, as well as the structure of the molecular link. Other factors affecting PET include conformational changes, local polarity modulations, and hydrogen-bonding effects.[36]

Patterned after previous PET-based sensor designs, the system described herein utilizes the cyanoanthracene fluorophore. This moiety exhibits strong and well-characterized emission properties, chemical stability, and sensitivity to electron transfer from chromophores such as the dimethoxybenzyl group.[20] The cyanoanthracene and dimethoxybenzyl groups have been integrated into α-amino acid constructs to allow the facile assembly of these units into functional polypeptides. Additionally, for the sensor design, we have formulated and implemented the ω-bipyridine amino acid illustrated in Figure 1. The bidentate bipyridinyl group of this ω-amino acid residue is well suited for this particular application, since the rigid uncomplexed amino acid maintains the polypeptide in an extended conformation that minimizes chromophore interactions in the absence of metal ions. In contrast, in the presence of a complexing metal analyte, the peptide scaffold undergoes a conformational change to alter the PET transfer properties, thereby generating fluorescence changes that can be monitored. The sensor design is illustrated schematically in Figure 1.

Results and Discussion

Herein we describe the synthesis of two new unnatural amino acids, Fmoc-Flu (**6**) and Fmoc-XBp (**20**). These residues incorporate a cyanoanthracene fluorophore and a metal-coordinating bipyridine derivative. We also describe a new synthesis for L-3,4-dimethoxy-DOPA (**12**).[37-40] This residue is intended to act as the "electron donor" or "quencher" of the cyanoanthracene fluorescence. Judicious incorporation of these three amino acid building blocks into different peptides, via solid phase peptide synthesis (SPPS), affords the prototype for a PET-based chemosensor. This sensor is designed to exhibit a metal ion-induced conformational change which modulates the fluorescence properties of the peptide.

Scheme 1



The synthesis of (S)-2-amino-N^{α}-(9-fluorenylmethyloxycarbonyl)-3-(9-cyanoanthracen-10-yl)propanoic acid (**6**; Fmoc-Flu-OH) begins from the commercially available 10-formyl-9-methylanthracene (**1**) as outlined in Scheme 1. The key step of this synthesis involves a stereoselective alkylation of N-(diphenylmethylene)glycine *tert*-butyl ester (**4**) with the bromide **3** using the phase transfer catalyst (PTC) (8S,9R)-(−)-N-benzylcinchonidinium chloride according to the method of O'Donnell *et al.*[41] The alkylation proceeded with modest asymmetric induction to afford the alkylated product (S)-**5** in 53% ee. Enantiomerically pure material (>99% ee) was obtained after fractional crystallization (hot hexane/EtOAc 4:1) of the racemate from the optically enriched material. Due to the aqueous acid lability of the nitrile group in **5**, deprotection of the imine and ester functionality was effected using anhydrous HCl(g) in dry acetone. Since the amino acid was intended for incorporation into polypeptide sequences using solid phase peptide synthesis methods, the crude amino acid was directly converted to the corresponding 9-fluorenylmethoxycarbonyl (Fmoc) derivative **6** through treatment with (9-fluorenylmethoxycarbonyl)succinimidyl carbonate (Fmoc-OSu) in dioxane and 10% sodium carbonate.

Commercially available 3,4-dimethoxybenzyl alcohol (**7**) was used in the first step of the synthesis of (S)-2-amino-N^{α}-(9-fluorenylmethyloxycarbonyl)-3-(3,4-dimethoxybenz-1-yl)propanoic acid (**12**; Fmoc-L-Dmd-OH) (Scheme 2). The procedure follows the same steps as the synthesis of **6**, except that crystallization to isolate the enantiomerically pure S derivative from the product mixture was not successful. Therefore, the optically pure

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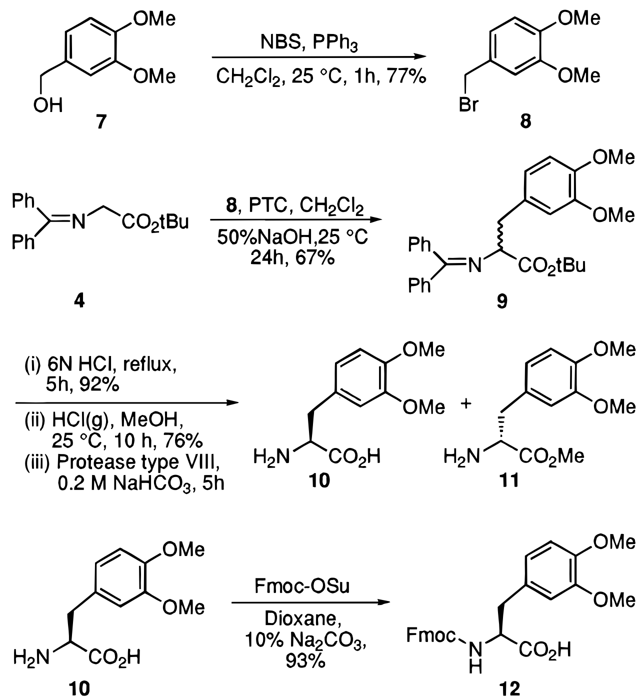
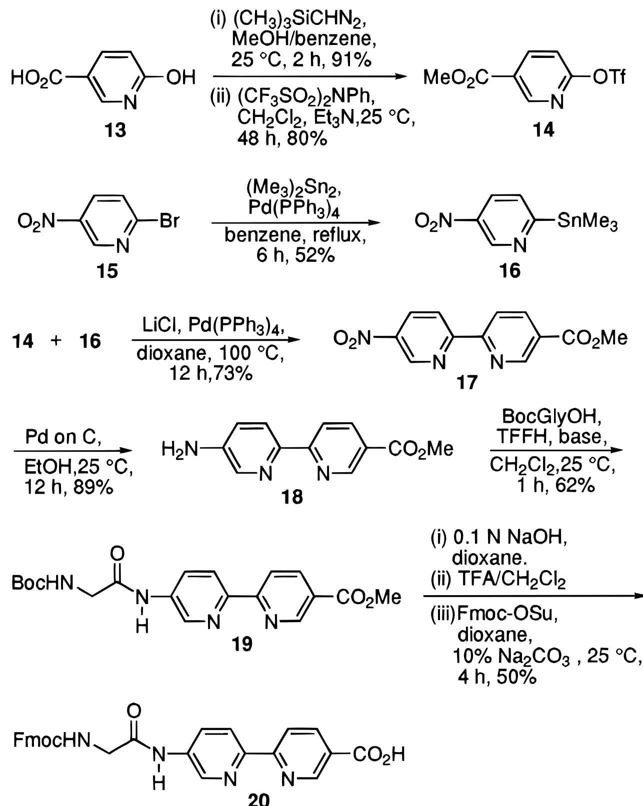
Scheme 2



Scheme 3



material was obtained via the enantioselective hydrolysis of the corresponding methyl ester **11** using commercially available alkaline protease.[42] The enzymatic hydrolysis proceeded smoothly at room temperature and was complete within 5 h. Separation and quantitative recovery of the two enantiomers was accomplished through simple extraction of the D-amino acid ester **11** with chloroform followed by lyophilization of the remaining aqueous layer to afford the L-amino acid **10** (99% ee). The crude L-amino acid **10** was treated with Fmoc-OSu to afford the Fmoc-protected amino acid derivative **12**.

Scheme 3 outlines the synthesis of the metal ligating N^{α}-(9-fluorenylmethyloxycarbonyl)-glycyl-5′-amino-2,2′-bipyridine-5-carboxylic acid (**20**, Fmoc-XBp). The preparation of **20** involved a Stille coupling of the two pyridine derivatives, **14** and **16**. Methyl 2-(trifluoromethylsulfonyl)pyridine-5-carboxylate **14** was synthesized by methylation of the commercially available 2-hydroxypyridine-5-carboxylic acid (**13**) with (trimethylsilyl)diazomethane and subsequent treatment with *N*-phenyltrifluoromethanesulfonamide. Initial attempts to convert the commercially available 2-bromo-5-nitropyridine (**15**) into the tin derivative **16** by treatment with *n*-BuLi followed by trimethylsilyl chloride failed, probably due to the electron-withdrawing effect of the nitro group at the 5-position of the pyridine ring. Ultimately, **16** was obtained by treating **15** with hexamethylditin in benzene at reflux for 6 h.[43,44] The Stille coupling reaction between the triflate **14** and the tin derivative **16** using standard conditions (LiCl, $Pd(PPh_3)_4$, dioxane at reflux)[45] afforded **17**. The nitro compound **17** was subsequently reduced to the amine **18** with H_2/Pd–C in EtOH.[46] Due to the poor reactivity of the aromatic amine functionality in **18**, attempts to protect it as the Fmoc amino acid derivative were singularly unsuccessful with low yields even under forcing conditions. Further, the limited reactivity of this amino acid was deemed unsuitable for performing solid phase coupling reactions. Therefore, the ω-amino acid residue was incorporated into a pseudo-dipeptide "cassette" **19**, wherein Boc-glycine fluoride, generated *in situ*,[47–49] was coupled to the nonreactive aryl amine of the bipyridyl ω-amino acid methyl ester **18**. Removal of the Boc protecting group and hydrolysis of the methyl ester were achieved by treatment with 0.1 N NaOH, followed by TFA. The free amine was subsequently protected with the Fmoc group to afford **20**, ready for incorporation into peptides via SPPS. This reaction sequence was necessitated due to lability of the Fmoc group under the conditions required for ester hydrolysis.

The efficient solid phase synthesis of peptides **FXR01**, **FXR02**, and **FXR03** (Chart 1), incorporating the three nonstandard residues (Flu, Dmd, and XBp) was achieved using macropin technology.[50,51] The successful preparation of these peptides highlights the compatibility of these residues with solid phase methodology. The peptides were prepared with an *N*-terminal acetyl group and a *C*-terminal carboxamide to avoid interactions between the free amine or carboxylic acid and divalent metal ions. These peptides were designed to adopt a random coil

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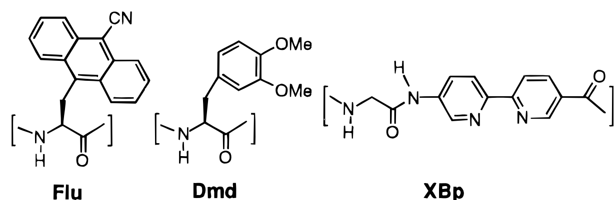
Chart 1

Peptide	Sequence
FXR01	Ac**FluXBp**GlyGlyGly**XBpDmd**GlyNH$_2$
FXR02	Ac**FluXBp**GlyGlyGly**XBpDmd**ThrArgNH$_2$
FXR03	AcArg**FluXBp**GlySerSer**XBpDmd**ThrArgNH$_2$



conformation with the Flu and Dmd amino acids flanking the two XBp residues. In the current designs, the metal-coordinating residues are linked by three α-amino acids. In the absence of metal ions, the peptides are expected to exhibit strong fluorescence due to the side-chain cyanoanthracene fluorophore of the α-amino acid Flu. Furthermore, it is anticipated that, upon coordination of divalent metal cations to the XBp residues, the chromophores on the Flu and Dmd side chains would move into proximity and allow the PET process to occur. This conformational change would result in a decrease in fluorescence from the anthracene moiety.

The first design, **FXR01**, incorporated three glycine residues between the two bipyridine ω-amino acids. The glycine spacer affords flexibility to the peptide chain, allowing it to fold in the presence of divalent metal ions. Unfortunately, a detailed analysis of this peptide in aqueous solution was precluded by its insolubility in water. However, preliminary studies with crude peptide in methanol were promising. Fluorescence measurements for this and all later samples were obtained by excitation at 388 nm, followed by observation of the dominant emission, which is centered between 440 and 470 nm. Notably, fluorescence quenching was observed upon titration with Zn^{2+} and Co^{2+} (Figure 2).

The sequences for peptides **FXR02** and **FXR03** were selected to enhance the aqueous solubility of the peptidyl template while maintaining backbone flexibility. Peptide **FXR02** incorporates three glycines between the two bipyridine ω-amino acids similar to peptide **FXR01**; however, two hydrophilic residues (Thr^8 and Arg^9) were also included to improve solubility. Peptide **FXR03** was designed with even more hydrophilic residues (Arg^1, Ser^5, Ser^6, Thr^8, and Arg^9) to further increase aqueous solubility.

The fluorescence of peptide **FXR02** was measured using a 2.63 μM solution of peptide in 0.01 M PIPES (pH 7.0). The emission properties of **FXR02** are sensitive to the presence of micromolar concentrations of Zn^{2+}. The fluorescence emission of **FXR02** is quenched upon complexation of Zn^{2+} cations (Figure 3a), which may be due to the PET effect. The reversibility of complex formation was assessed with different chelating agents including 1,10-phenanthroline and ethylenediaminetetraacetic acid (EDTA). Upon addition of 1,10-phenanthroline to the **FXR02**–Zn^{2+} complex, the system exhibited total recovery of fluorescence emission (Figure 3b). In contrast,

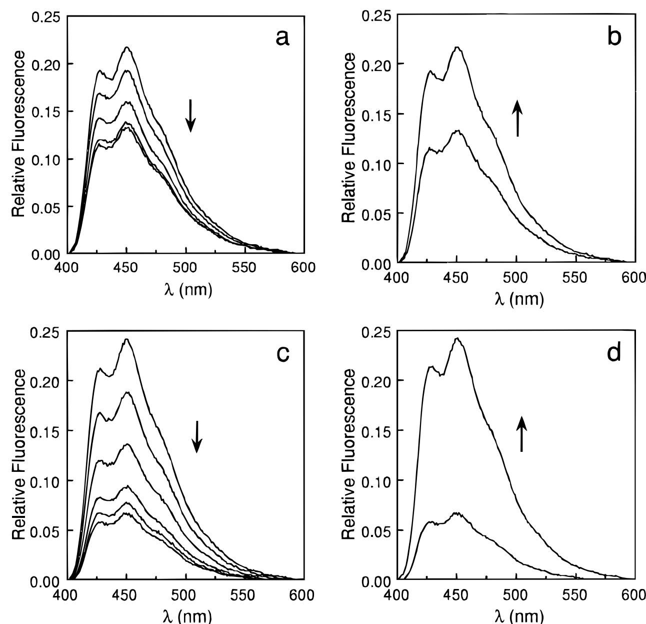


Figure 2. (a) Uncorrected fluorescence emission spectra of a ~1.2 μM soution of **FXR01** in MeOH upon addition of increasing amounts of aqueous $ZnCl_2$: 0, 1.7, 3.4, 5.0, and 6.8 μM $ZnCl_2$, respectively. (b) Recovery of the fluorescence after adding EDTA. (c) Uncorrected fluorescence emission spectra of a ~1.6 μM solution of **FXR01** in MeOH upon addition of increasing amounts of aqueous $CoCl_2$: 0, 1, 2, 3, 7, and 14 μM $CoCl_2$ respectively. (d) Recovery of the fluorescence after adding EDTA. Excitation was performed at 388 nm.

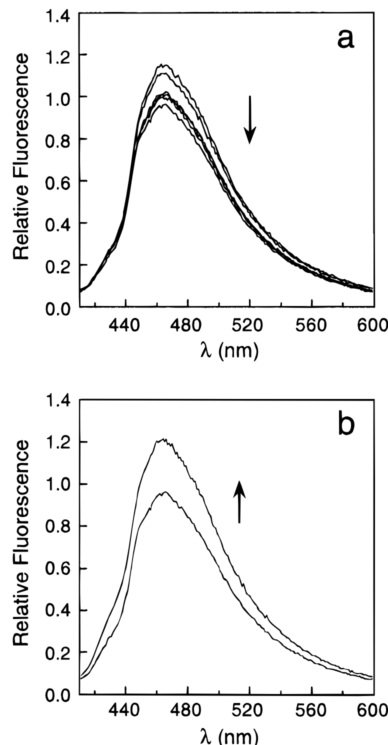


Figure 3. (a) Uncorrected fluorescence emission spectra of a 2.63 μM solution of **FXR02** in 0.01 M PIPES (pH 7.0) upon addition of increasing amounts of aqueous $ZnCl_2$: 0, 1.2, 2.3, 3.9, 5.5, and 24.2 μM $ZnCl_2$, respectively. (b) Recovery of the fluorescence emission after adding 1,10-phenanthroline. Excitation was performed at 388 nm.

addition of EDTA to the peptide–Zn^{2+} system did not result in the recovery of fluorescence emission in aqueous solution. Interestingly, when identical titration studies

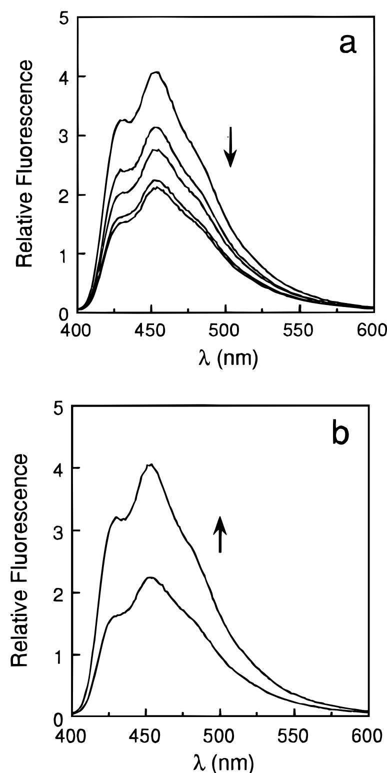


Figure 4. (a) Uncorrected fluorescence emission spectra of a 1.34 μM solution of **FXR02** in MeOH upon addition of increasing amounts of aqueous $ZnCl_2$: 0, 0.8, 1.4, 2.6, and 3.7 μM $ZnCl_2$, respectively. (b) Recovery of the fluorescence emission after adding EDTA. Excitation was performed at 388 nm.

were performed in MeOH (Figure 4) or in 50% MeOH/0.01 M PIPES (pH 7.0) (Figure 5), addition of EDTA completely recovered the fluorescence emission. Furthermore, in 50% MeOH/0.01M PIPES (pH 7.0), although fluorescence was completely recovered (Figure 5b), it was subsequently quenched over a period of 1.5 h (Figure 5c), suggesting that the EDTA quenching effect could be solvent-dependent.

From studies involving different fluorophores, it is known that EDTA acts as an electron donor in aqueous photoinduced electron transfer reactions. For those systems it has been shown that electron transfer from the lone pair of the amine to the excited state of the fluorophore is responsible for fluorescence quenching.[52-54] A similar effect may be responsible for the quenching observed in our system as presented in Scheme 4.

Fluorescence studies with peptide **FXR03** provided results similar to peptide **FXR02** (Figure 5). Fluorescence was measured using a 3.75 μM solution of peptide **FXR03** in 0.01 M PIPES (pH 7.0). The fluorescence emission properties of peptide **FXR03** are more sensitive to the presence of Zn^{2+} compared to peptide **FXR02**. A more significant change in fluorescence is observed with peptide **FXR03** upon addition of metal (Figure 6a). The lower apparent sensitivity of peptide **FXR02** may be due to its lower solubility in aqueous media. Furthermore, the reversibility of the metal complexation was demonstrated by the addition of 1,10-phenanthroline (Figure 6b). The failure to recover fluorescence with EDTA again suggested that this chelating agent participates in an electron transfer quenching reaction. A parallel set of experiments was also carried out in phosphate buffer at pH 7. These studies reveal that there is some intrinsic

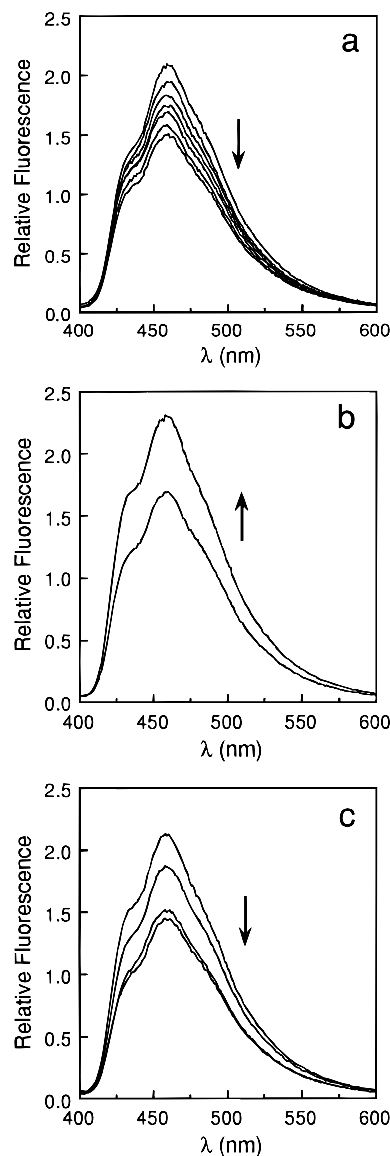
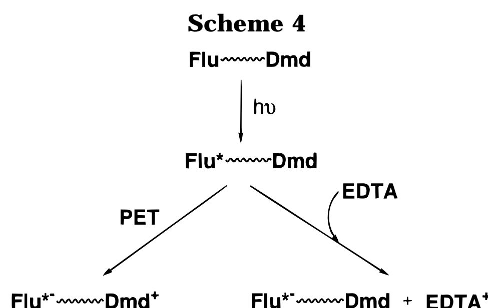


Figure 5. (a) Uncorrected fluorescence emission spectra of a 1.24 μM solution of **FXR02** in 50% MeOH/0.01 M PIPES (pH 7.0) upon addition of increasing amounts of aqueous $ZnCl_2$: 0, 0.6, 1.2, 2.3, 3.5, 4.7, and 5.8 μM $ZnCl_2$, respectively. (b) Recovery of the fluorescence emission after adding EDTA. (c) Quenching of the fluorescence with time after being recovered with EDTA: 0, 30, 60, and 90 min, respectively. Between scans the sample was left in the dark. Excitation was performed at 388 nm.

Scheme 4



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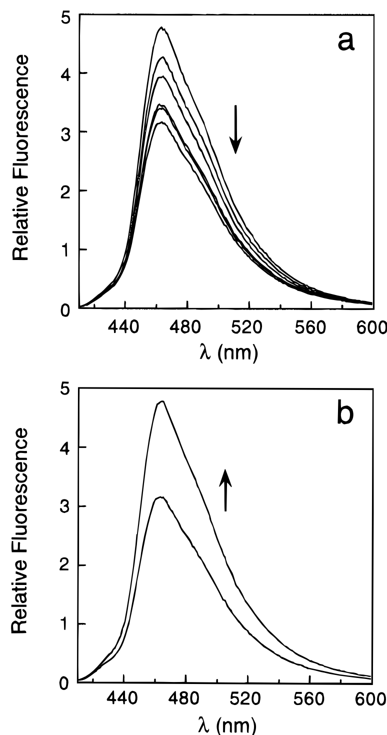

Figure 6. (a) Uncorrected fluorescence emission spectra of a 3.75 μM solution of **FXR03** in 0.01 M PIPES (pH 7.0) upon addition of increasing amounts of aqueous $ZnCl_2$: 0, 2.1, 4.1, 7.2, 10.3, and 14 μM $ZnCl_2$, respectively. (b) Recovery of the fluorescence emission after adding 1,10-phenanthroline. Excitation was performed at 388 nm.

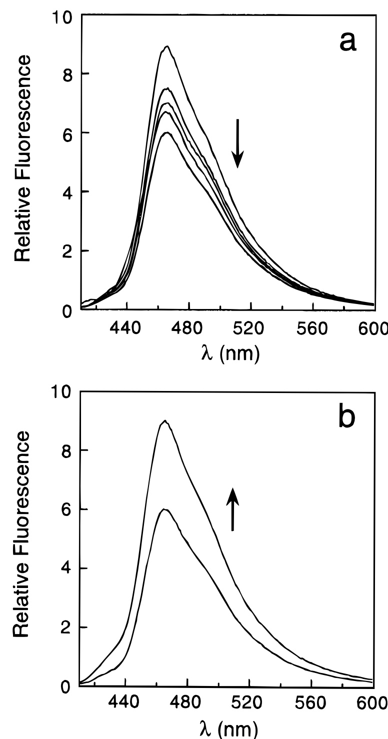

Figure 7. (a) Uncorrected fluorescence emission spectra of a 9.09 μM solution of **FXR03** in 0.01 M phosphate buffer (pH 7.0) upon addition of increasing amounts of aqueous $ZnCl_2$: 0, 9.7, 16.0, 24.0, 35.8, and 47.7 μM $ZnCl_2$, respectively. (b) Recovery of the fluorescence emission after adding 1,10-phenanthroline. Excitation was performed at 388 nm.

fluorescence quenching from the PIPES buffer relative to the phosphate buffer; however, the relative change of metal ion-induced fluoresence and the reversibility with 1,10-phenanthroline is comparable (see Figure 7a and b, respectively). To confirm that the electron transfer was indeed induced by a metal ion-dependent modulation of the peptide structure, rather than direct metal ion or metal ion complex quenching of the cyanoanthracene excited state, the relative fluorescence of a sample of the free cyanoanthracene amino acid (Flu) was examined on treatment with Zn^{2+}, Co^{2+}, and Co^{2+} in the presence of 2,2′-bipyridine. In all cases, even large excesses of the added species failed to effect any significant change in the fluoresence emission properties (data not shown).

In conclusion, we have designed and synthesized a new family of peptide-based metal ion sensors that exhibit a metal ion-modulated PET effect in aqueous solution. These peptides incorporate three nonstandard amino acids. These residues are fully compatible with solid phase peptide synthesis methodology and may be applied in the future toward the design of additional metal ion sensors with complementary cation selectivities. Furthermore, the modular nature of this approach will allow the application of combinatorial methods for signal intensity optimization.

Experimental Section

General Information. Nuclear magnetic resonance (NMR) spectra were recorded at 300 MHz for proton frequency and 75 MHz for carbon frequency. Chemical shifts are reported in δ units (ppm) relative to the solvent used. Optical rotations were recorded at room temperature. Mass spectra were obtained using fast atom bombardment (FAB), electron impact (EI) or plasma desorption (PD) ionization. Thin-layer chromatography was carried out on hard TLC plates with fluorescent indicator from EM Reagents (SiO_2 60, F-254). TLC plates were visualized by UV or 0.2% ninhydrin solution in ethanol followed by heat. Flash column chromatography was performed according to the procedure of Still[55] using J. T. Baker flash silica gel (~40 μm). 10-Formyl-9-cyanoanthracene, 3,4-dimethoxybenzyl alcohol, 2-hydroxypyridine-5-carboxylic acid, 2-bromo-5-nitropyridine, *N*-(diphenylmethylene)glycine *tert*-butyl ester, and (8*S*,9*R*)-(−)-*N*-benzylcinchonidinium chloride were purchased from Aldrich Chemical Co. Tetramethylfluoroformamidinium hexafluorophosphate (TFFH) was prepared by following the method developed by Carpino.[47−49]

10-Methyl-9-cyanoanthracene (2). To **1** (1 g, 4.54 mmol) suspended in 24 mL of 95% EtOH was added hydroxylamine hydrochloride (356 mg, 5.13 mmol, neutralized with Na_2CO_3) in 3.5 mL of warm (75 °C) water. The mixture was heated on a steam bath for 15 min, cooled, and diluted with water. The solid was collected and heated in 10% NaOH for 10 min. The solution was cooled and diluted with water. The solid was collected, and acetic anhydride (20 mL) was added. The resulting solution was refluxed for 15 min and then filtered while still hot. Upon cooling 791 mg (80%) of **2** as yellow crystals (mp 207−208 °C (lit.[56] mp 208−210 °C) appear in the filtrate: R_f = 0.51 (hexane/EtOAc, 8:2); ^{1}H NMR ($CDCl_3$, 300.2 MHz) δ 2.93 (s, 3H), 7.48 (t, 2H, J = 7.5 Hz), 7.57 (t, 2H, J = 7.5 Hz), 8.13 (d, 2H, J = 8.5 Hz), 8.22 (d, 2H, J = 8.5 Hz); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 14.6, 103.8, 117.5, 124.9, 125.3, 125.5, 125.81, 125.83, 125.9, 126.0, 128.0, 128.2, 128.9, 132.4, 137.8; MS *m*/*z* 217 (M^+, 100), 216 (43), 189 (13), 165 (1).

9-(α-Bromomethyl)cyanoanthracene (3). A suspension of **2** (500 mg, 2.3 mmol), *N*-bromosuccinimide (451 mg, 2.5 mmol), and benzoyl peroxide (11 mg, 0.046 mmol) in CCl_4/CH_2Cl_2 (5:1, 8 mL) was degassed four times by the freeze−pump−thaw method and heated to 80 °C in a constant-temperature

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bath for 4 h. The solvent was then removed *in vacuo*, and the crude product was recrystallized from hexanes/$CHCl_3$ to afford 320 mg (67%) of **3** as yellow crystals (mp 201−202 °C):[57] R_f = 0.33 (hexane/EtOAc, 8:2); ^{1}H NMR ($CDCl_3$, 300.2 MHz) δ 5.48 (s, 2H), 7.6−7.8 (m, 4H), 8.3−8.4 (m, 2H), 8.4−8.5 (m, 2H); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 14.6, 103.9, 117.6, 125.2, 125.7, 126.0, 128.1, 129.0, 132.5, 137.9; MS *m/z* 297 (M^+, 3), 295 (5), 216 (100), 189 (13), 163 (4), 108 (4), 94 (8), 87 (4), 74 (6), 63 (5), 50 (6).

***N*-(Diphenylmethylene)-2-amino-3-(9-cyanoanthracen-10-yl)propanoic Acid *tert*-Butyl Ester (5).** *N*-(diphenylmethylene)glycine *tert*-butyl ester (**9**; 248 mg, 0.841 mmol) and (8*S*,9*R*)-(−)-*N*-benzylcinchonidinium chloride (35 mg, 0.084 mmol) were dissolved in CH_2Cl_2 (15 mL). To this was added 1.2 mL of 50% aqueous NaOH and solid 9-(α-bromomethyl)-anthracene (**8**; 311 mg, 1.051 mmol). The reaction mixture was then stirred at room temperature for 24 h. At this time the organic phase was separated and the remaining aqueous phase washed with CH_2Cl_2. The combined organic phases were concentrated under reduced pressure and purified by flash chromatography (eluent hexane/EtOAc 9:1) to afford 387 mg (72% yield) of pure product **5**. The optical purity of the product was determined to be 53%. Recrystallization of the L/D mixture in hexane/EtOAc (4:1) afforded 165 mg of pure L product (mp 162−163 °C): $[\alpha]^{20}_D$ = −341° (0.5, $CHCl_3$, 1 dm path length cell); R_f = 0.36 (hexane/EtOAc, 8:2); ^{1}H NMR ($CDCl_3$, 300.2 MHz) δ 1.49 (s, 9H), 4.17 (dd, 1H, *J* = 14.0, 3.1 Hz), 4.33 (t, 1H, *J* = 12.0 Hz), 4.46 (dd, 1H, *J* = 9.9, 3.2 Hz), 5.8−5.9 (s br, 2H), 6.83 (t, 2H, *J* = 7.5 Hz), 7.02 (t, 1H, *J* = 7.5 Hz), 7.13 (t, 2H, *J* = 7.5 Hz), 7.25 (d, 3H, *J* = 7.9 Hz), 7.46 (t, 2H, *J* = 7.6 Hz), 7.61 (t, 2H, *J* = 7.6 Hz), 8.36 (d, 4H, *J* = 8.6 Hz); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 28.0, 31.5, 66.5, 81.7, 104.9, 117.6, 125.7, 126.1, 126.7, 127.5, 127.8, 128.3, 128.5, 129.7, 130.1, 132.7, 135.0, 138.3, 138.5, 170.4, 170.6; IR ($CHCl_3$) 3060−3012 (m, aromatic C−H), 2978−2912 (m, aliphatic C−H), 2214 (s, C≡N), 1725 (s, C=O), 1619 (m, ring C=C), 1443 cm^{-1} (m, C≡N); MS *m/z* 512 (39), 511 (MH^+, 100), 510 (M^+, 6), 426 (6), 340 (7), 296 (23), 238 (22), 235 (18), 217 (6), 200 (17), 183 (21), 182 (16), 163 (3), 105 (4); HRMS [MH^+] calculated for $C_{35}H_{31}N_2O_2$ 511.239500, observed 511.238554.

Determination of Enantiomeric Excess. The enantiomeric excess of the pure amino acid obtained by hydrolysis of **5** was evaluated by derivatization with 1-fluoro-2,4-(dinitrophenyl)-5-L-alanineamide (FDAA, Marfey's reagent) followed by reversed phase HPLC analysis of the adducts. Ester **5** (2 mg) was heated in 6 N HCl at 100 °C for 1 h. The solution was then neutralized with Na_2CO_3 and lyophilized to dryness. The residue was dissolved in 345 μL of 0.1 M $NaHCO_3$ (10 mM solution of amino acid). The resulting solution was added to 100 μL of freshly prepared solution of 1.4 mg of Marfey's reagent in acetone and kept at 40 °C for 1 h with frequent mixing. After cooling, 2 N HCl was added to make the solution slightly acidic. The mixture was the centrifuged and 10 μL of the supernatant was analyzed by HPLC (C_{18} column; solvent A, H_2O/0.1% TFA; solvent B, MeCN/0.1% TFA). Retention times: *S* derivative 24.06 min; *R* derivative 26.7 min.

(*S*)-2-Amino-N^{α}-(9-fluorenylmethyloxycarbonyl)-3-(9-cyanoanthracen-10-*yl*) propanoic acid (6). To a suspension of **5** (190 mg, 0.372 mmol) in dry acetone (2 mL) was added HCl(g) at 0 °C. The resulting solution was stirred for 15 min at 0 °C and 15 min more at room temperature. The solvent was removed under a stream of argon, and the resulting residue was washed with dry acetone and dried under argon. The mixture was then basified with 10% Na_2CO_3/dioxane (2 mL/5 mL), followed by a solution of Fmoc-ONSu (138 mg, 0.409 mmol) in dioxane (2 mL). The resulting yellow suspension was stirred at room temperature for 6 h. The aqueous phase was extracted with Et_2O (3×), and the aqueous phase was acidified to pH 4 with 2 N HCl and extracted with EtOAc (5×). The combined ethyl acetate phases were dried over Na_2SO_4 and concentrated to give a yellow solid. The residue was dissolved in a small amount of CH_2Cl_2/MeOH and triturated with hexanes. The solid was redissolved in CH_2Cl_2 and concentrated to afford 95 mg of **6** (50%): R_f = 0.41 ($CHCl_3$/MeOH, 8:2); $[\alpha]^{20}_D$ = −148° (0.54, DMF, 0.5 dm path length cell); ^{1}H NMR ($CDCl_3$, 300.2 MHz) δ 3.9−4.1 (m, 3H), 4.33 (dd, 1H, *J* = 14.7, 9.6 Hz), 4.42 (dd, 1H, *J* = 14.4, 5.0 Hz), 4.69 (m, 1H), 7.23 (td, 1H, *J* = 7.5, 0.9 Hz), 7.30 (td, 1H, *J* = 7.6, 1.0 Hz), 7.40 (dd, 1H, *J* = 7.5, 4.0 Hz), 7.42 (dd, 1H, *J* = 76.7, 4.0 Hz), 7.55 (dd, 2H, *J* = 16.8, 7.5 Hz), 7.81 (td, 2H, *J* = 6.9, 0.9 Hz), 7.89 (d, 4H, *J* = 8.1 Hz), 8.40 (d, 2H, *J* = 8.4 Hz), 8.80 (d, 2H, *J* = 9 Hz); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 47.5, 56.7, 67.0, 117.8, 120.7, 125.9, 126.0, 126.1, 126.7, 127.7, 128.3, 129.9, 130.5, 133.4, 140.4, 141.7, 144.6, 144.7, 156.9, 162.9, 173.4. IR (KBr) 3315 (w, N−H), 3072 (w, aromatic C−H), 2205 (m, C≡N), 1694 (s, C=O and amide band), 1445 cm^{-1} (C≡N); MS *m/z* 513 (MH^+, 2), 391 (3), 242 (2), 179 (10); HRMS calculated for $C_{33}H_{24}O_4N_2$ 513.181 433, observed 513.183 600.

3,4-Dimethoxybenzyl Bromide (8). To a solution of 3,4-dimethoxybenzyl alcohol (**7**; 5 g, 29.76 mmol) in CH_2Cl_2 (125 mL) was added PPh_3 (9.4 g, 35.71 mmol) and *N*-bromosuccinimide (5.8 g, 32.74 mmol), and the resulting mixture was stirred for 1 h at room temperature. The reaction volume was then reduced by evaporation and directly passed through a silica gel column (eluent, CH_2Cl_2) to afford 5.3 g (77%) of **8** as white crystals (mp 50−51 °C, (lit.[58] mp 47−50 °C)); R_f = 0.83 (hexane/EtOAc, 4:3); ^{1}H NMR ($CDCl_3$, 300.2 MHz) δ 3.86 (s, 3H), 3.88 (s, 3H), 4.49 (s, 2H), 6.80 (d, 1H, *J* = 8.1 Hz), 6.90 (d, 1H, *J* = 2.1 Hz), 6.94 (dd, 1H, *J* = 8.1, 2.1 Hz); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 34.3, 55.7, 55.8, 110.8, 111.9, 121.4, 130.0, 148.9, 149.0; MS *m/z* 230 (M^+, 2), 151 (100), 137 (10), 107 (10), 94 (29), 80 (39), 65 (12). HRMS [M^+] calculated for $C_9H_{11}BrO_2$ 229.994 241, observed 229.993 400.

***N*-(Diphenylmethylene)-2-amino-3-(3,4-dimethoxybenz-1-yl) propanoic Acid *tert*-Butyl Ester (9).** *N*-(Diphenylmethylene)glycine *tert*-butyl ester (**4**; 1.3 g, 4.33 mmol) and (8*S*,9*R*)-(−)-*N*-benzylcinchonidinium chloride (364 mg, 0.86 mmol) were dissolved in CH_2Cl_2 (80 mL). To this was added 6.5 mL of 50% aqueous NaOH and a solution of **8** (1 g, 4.33 mmol). The reaction mixture was then stirred at room temperature for 36 h. At this time the organic phase was separated and the remaining aqueous phase washed with CH_2Cl_2. The combined organic phases were concentrated under reduced pressure and purified by flash chromatography (eluent hexane/EtOAc/Et_3N, 9:0.75:0.25) to afford 1.290 g (67% yield) of pure product **9**. The optical purity of the product was determined to be 50% ee using a chiral Crownpak CR(+) column (Daicel): flow rate 1 mL/min, 25 °C, 280 nm, eluent 10% MeOH/70% $HClO_4$, *t*-Bu ester D 5.79 min, L 7.70 min; R_f = 0.33 (hexane/EtOAc, 8:2); ^{1}H NMR ($CDCl_3$, 300.2 MHz) δ 1.43 (s, 9H), 3.0−3.1 (m, 2H), 3.61 (s, 3H), 3.81 (s, 3H), 4.06 (dd, 1H, *J* = 8.6, 4.8 Hz), 6.5−6.6 (m, 4H), 6.67 (d, 1H, *J* = 8.1 Hz), 7.2−7.3 (m, 6H), 7.55 (dd, 2H, *J* = 8.2, 1.3 Hz). ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 28.0, 39.0, 55.4, 55.8, 67.9, 110.7, 112.8, 121.7, 127.6, 127.7, 127.8, 127.9, 128.0, 128.1, 128.6, 130.1, 130.8, 136.3, 139.4, 148.3, 170.1, 170.8. IR ($CHCl_3$) 3008−2936 (m, aromatic C−H), 2837 (w, aliphatic C−H), 1725 (s, C=O), 1464 (m, C=N), 1260 (s, C−O−C), 1154 (s), 1140 (s), 1028 cm^{-1} (m, C−O−C); MS *m/z* 446 (MH^+, 49), 390 (97), 344 (29), 294 (11), 238 (100), 193 (44), 182 (18), 165 (40), 151 (86), 137 (11), 107 (11); HRMS [MH^+] calculated for $C_{28}H_{32}NO_4$ 446.233 134, observed 446.233 500.

(D,L)-2-Amino-3-(3,4-dimethoxybenz-1-yl)propanoic Acid (10). The *t*-Bu ester **9** (200 mg, 0.449 mmol) was refluxed in 6 N HCl (3 mL) for 5 h. The hydrolyzed mixture was then cooled, extracted with EtOAc (3 × 5 mL), and concentrated to dryness. The residual material was lyophilized from water overnight to afford 107 mg (92%) of the isomeric mixture of amino acid hydrochlorides: R_f = 0.4 (AcOH/H_2O/*n*BuOH/EtOAc, 1:1:1:1); ^{1}H NMR (D_2O, 300.2 MHz) δ 3.13 (dd, 1H, *J* = 14.7, 7.6 Hz), 3.26 (dd, 1H, *J* = 14.7, 5.5 Hz), 3.81 (s, 6H), 4.25 (dd, 1H, *J* = 7.4, 5.6 Hz), 6.87 (dd, 1H, *J* = 8.5, 1.5 Hz), 6.92 (d, 1H, *J* = 1.7 Hz), 6.99 (d, 1H, *J* = 8.2 Hz); ^{13}C NMR (D_2O, 75.5 MHz) δ 35.3, 54.3, 55.6, 112.1, 112.6, 122.2, 126.9, 147.7, 148.3, 171.7; MS *m/z* 226 (M^+, 100), 209 (13), 180 (22),

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163 (11), 151 (55); HRMS [M$^+$] calculated for $C_{11}H_{16}NO_4$ 226.107 933, observed 226.108 000.

2-Amino-3-(3,4-dimethoxybenz-1-yl)propanoic Acid Methyl Ester (11). Benzene (4 mL) was added to the amino acid hydrochloride (260 mg, 1.15 mmol, 50% ee) and lyophilized. The residue was dissolved in dry MeOH (3 mL) and cooled to 0 °C. Anhydrous hydrogen chloride was passed through the mixture for 10 min. After standing at room temperature overnight, the solvent was removed under reduced pressure. MeOH (3 × 5 mL) was added and removed. The oily residue was the dissolved in 0.2 N $NaHCO_3$ and extracted with $CHCl_3$ (3 × 10 min). The combined organic phases were dried over Na_2SO_4 and concentrated *in vacuo* to yield 210 mg (76%) of a racemic mixture of methyl esters **11**: ^{1}H NMR ($CDCl_3$, 300.2 MHz) δ 2.84 (dd, 1H, J = 13.5, 7.5 Hz), 3.06 (dd, 1H, J = 13.5, 4.8 Hz), 3.74 (s, 3H), 3.87 (s, 6H), 6.73 (s, 1H), 6.76 (s, 1H), 6.82 (d, 1H, J = 7.8 Hz); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 40.3, 51.8, 55.5, 55.6, 111.0, 112.0, 121.1, 129.3, 147.6, 148.6, 175.1; IR ($CHCl_3$) 3360 (w, N—H), 3025−2925 (m, aromatic C—H), 2825 (w, aliphatic C-H), 1731 (s, C=O), 1510 (s, C=C ring), 1259 (s, C—O—C), 1243 (s), 1024 cm^{-1} (m, C—O—C); MS *m/z* 239 (M$^+$, 8), 180 (10), 151 (100), 135 (3), 59 (3). HRMS [M$^+$] calculated for $C_{12}H_{17}NO_4$ 239.115 758, observed 239.115 600.

Enzymatic Resolution of L-3-(3,4-dimethoxybenz-1-yl)-propanoic Acid (10). The optical purity of the underivatized amino acid was determined by resolving the enantiomers directly on a chiral Crownpak CR(+) column (Daicel). A solution of the protease type VIII, Sigma (1.7 mg) in 6.8 mL of 0.2 M $NaHCO_3$ was added to the racemic mixture of the amino acid methyl esters (100 mg, 0.418 mmol). The mixture was agitated on an orbital shaker and monitored by HPLC at 30 min intervals until the ratio of the D-amino acid methyl ester to the L-amino acid methyl ester was >50:1 (∼5 h). At this stage, the mixture was extracted with $CHCl_3$ (6 × 20 mL). The aqueous phase was reduced in volume to remove any chloroform and then lyophilized to yield a mixture of amino acid and carbonate salts. The combined organic layers were dried over Na_2SO_4, and the solvent was evaporated. HPLC analysis of the aqueous phase showed a 100% ee of the L-amino acid **10**, while analysis of the organic residue showed 72% ee of the D-amino acid methyl ester **11**.

***(S)*-2-Amino-*N*$^\alpha$-(9-fluorenylmethyloxycarbonyl)-3-(3,4-dimethoxybenz-1-yl) propanoic Acid (12).** The amino acid/carbonate salt mixtures from above were dissolved in 1.4 mL of 10% Na_2CO_3. (9-Fluorenylmethyl)succinimidyl carbonate (112 mg, 0.331 mmol) was dissolved in 1.4 mL of dioxane and added dropwise to the amino acid solution. The reaction mixture was stirred for 1.5 h. The dioxane was evaporated *in vacuo*, and the aqueous phase was extracted with Et_2O (3 × 5 mL). The aqueous phase was acidified to pH 3 and extracted with EtOAc (5 × 5 mL). The combined organic phases were dried over Na_2SO_4 and concentrated to afford 115 mg (93%) of **12** as a white powder: R_f = 0.56 ($CHCl_3$/MeOH, 8:2); $[\alpha]^{20}_D$ = −28° (0.485, DMF, 0.5 dm path length cell); ^{1}H NMR ($CDCl_3$, 300.2 MHz) δ 3.08 (dd, 1H, J = 8.4, 5.6 Hz), 3.16 (dd, 1H, J = 14.0, 5.1 Hz), 3.79 (s, 3H), 3.83 (s, 3H), 4.19 (t, 1H, J = 6.5 Hz), 4.39 (d, 2H, J = 6.8 Hz), 4.6−4.7 (m, 1H), 5.25 (d, 1H, J = 7.84 Hz), 6.6−6.8 (m, 4H), 7.2−7.4 (m, 4H), 7.54 (t, 2H, J = 6.1 Hz), 7.25 (d, 2H, J = 7.4 Hz); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 25.3, 37.3, 47.0, 55.8, 67.1, 111.2, 112.2, 120.0, 121.4, 125.0, 127.0, 127.7, 127.8, 141.2, 143.5, 143.6, 148.1, 148.8, 155.8; IR ($CHCl_3$) 3332 (w, N—H), 2958−2938 (w), 1687 (s, C=O), 1540 (s, C=C), 1515 (s), 1264 (s, C—O—C), 1235 cm^{-1} (s); MS *m/z* 447 (M$^+$, 13), 391 (4), 282 (61), 179 (66), 165 (17); HRMS [M$^+$] calculated for $C_{26}H_{25}NO_6$ 447.168 188, observed 447.167 600.

Methyl 2-hydroxypyridine-5-carboxylate. (Trimethylsilyl)diazomethane (5 mL, 2.0 M in hexanes, 10 mmol) was added to a suspension of 2-hydroxypyridine-5-carboxylic acid **13** (1.07 g, 7.7 mmol) in MeOH (15 mL) and benzene (54 mL). The resulting solution was stirred at room temperature for 2 h and concentrated *in vacuo* to afford 1.06 g of the methyl ester (91% yield): ^{1}H NMR ($CDCl_3$, 300.2 MHz) δ 3.87 (s, 3H), 6.58 (dd, 1H, J = 9.5, 0.5 Hz), 8.00 (dd, 1H, J = 9.5, 2.4 Hz), 8.19 (dd, 1H, J = 2.4, 0.5 Hz).

Methyl 2-[(trifluoromethyl)sulfonyl]pyridine-5-carboxylate (14). To a suspension of the methyl ester (1g, 6.53 mmol) and *N*-phenyltrifluoromethanesulfonimide (2.3 g, 6.53 mmol) in CH_2Cl_2 (20 mL) was slowly added Et_3N (0.9 mL, 7.18 mmol). The resulting solution was the stirred at room temperature for 48 h. After washing the mixture with 1 N NaOH (2×) and Na_2CO_3, the organic layer was dried and concentrated. Purification by flash chromatography on silica gel (99:1 CH_2Cl_2/MeOH) afforded 1.5 g (80% yield) of triflate **14**:[59] ^{1}H NMR ($CDCl_3$, 300.2 MHz) δ 3.98 (s, 3H), 7.25 (d, 1H, J = 8.5 Hz), 8.49 (dd, 1H, J = 8.5, 2.3 Hz), 9.01 (d, 1H, J = 2.3 Hz); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 53.1, 115.0, 127.0, 142.6, 150.7, 158.4, 164.3.

2-(Trimethylstannyl)-5-nitropyridine (16). A mixture of $Me_3SnSnMe_3$ (1.07 g, 3.27 mmol), 2-bromo-5-nitropyridine (**15**; 500 mg, 2.46 mmol), $Pd(PPh_3)_4$ (25 mg, 0.0213 mmol), and benzene (8 mL) was heated under reflux in a N_2 atmosphere for 12 h. It was then cooled, the benzene concentrated under reduced pressure, and the brownish residue purified by alumina chromatography (eluent, $CHCl_3$) to give 365 mg (52%) of **16** as pale yellow crystals (mp 47−48 °C): R_f = 0.62 (hexane/EtOAc, 8:2); ^{1}H NMR ($CDCl_3$, 300.2 MHz) δ 0.38 (9H, s), 7.69 (1H, d, J = 8.1 Hz), 8.24 (1H, dd, J = 8.1, 2.6 Hz), 9.48 (1H, d, J = 2.4 Hz); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ −9.2, 107.2, 127.2, 131.1, 143.3, 144.1, 184.5; IR ($CHCl_3$) 2987 (w), 1587 (m), 1571 (m), 1447 (w), 1352 (s), 1258 (w), 1216 (s), 1210 (m), 1018 (w), 852 cm^{-1} (s); MS *m/z* 288 (M$^+$cluster, 7), 273 (92), 243 (79), 165 (54), 135 (100), 120 (41). HRMS [M$^+$] calculated for $C_8H_{12}O_2N_2Sn$ 279.994 701, observed 279.993 629.

Methyl 5′-Nitro-2,2′-bipyridine-5-carboxylate (17). Triflate **14** (1.17 g, 4.12 mmol), tin derivative **16** (1.30 g, 4.53 mmol), LiCl (525 mg, 12.36 mmol), and $Pd(PPh_3)_4$ (238 mg, 0.21 mmol) were heated in dioxane at 100 °C for 12 h. The mixture was cooled and filtered to obtain 461 mg of **17** as a pale orange solid (mp 234−235 °C), and the filtrate was concentrated to give a dark solid. The solid was purified by flash column chromatography (silica gel, CH_2Cl_2/MeOH, 98:2) to give 317 mg more of **17** (total yield, 73%). **17** was an insoluble crystal in most of organic and aqueous solvents: R_f = 0.72 ($CHCl_3$/MeOH, 98:2); ^{1}H NMR ($CDCl_3$, 300.2 MHz) δ 4.00 (3H, s), 8.47 (1H, dd, J = 8.4, 2.1 Hz), 8.61 (1H, d, J = 8.4 Hz), 8.62 (1H, d, J = 8.7 Hz), 8.73 (1H, d, J = 8.7 Hz), 9.31 (1H, d, J = 2.1 Hz), 9.50 (1H, d, J = 2.7 Hz); IR (KBr) 3300−3080 (w, aromatic C—H), 1719 (s, C=O), 1520 (m, N=O), 1349 (s, N=O), 856 cm^{-1} (m, C—N); MS *m/z* 259 (M$^+$, 100), 228 (64), 213 (25), 200 (14), 186 (12), 154 (23), 142 (3), 136 (6), 127 (12), 115 (3), 100 (6), 77 (12), 59 (11), 51 (15); HRMS [M$^+$] calculated for $C_{12}H_{10}N_3O_4$ 259.059 306, observed 259.059 900.

Methyl 5′-Amino-2,2′-bipyridine-5-carboxylate (18). The nitro compound **17** (139 mg, 0.54 mmol) was suspended in absolute EtOH (20 mL). Pd/C catalyst was added (14 mg) and the mixture stirred under hydrogen at atmospheric pressure for 12 h. The obtained amine was soluble in EtOH. The reaction mixture was filtered through Celite and the solvent removed to afford 110 mg of **18** as a yellow solid (89%) (mp >250 °C): R_f = 0.52 ($CHCl_3$/MeOH, 8:2); ^{1}H NMR (CD_3OD, 300.2 MHz) δ 3.94 (3H, s), 7.13 (1H, dd, J = 8.6, 2.8 Hz), 8.07 (1H, dd, J = 2.8, 0.5 Hz), 8.13 (1H, dd, J = 8.6, 0.5 Hz), 8.21 (1H, dd, J = 8.4, 0.8 Hz), 8.35 (1H, dd, J = 8.4, 2.2 Hz), 9.09 (1H, dd, J = 2.2, 0.8 Hz); ^{13}C NMR (CD_3OD, 75.5 MHz) δ 52.8, 120.3, 122.0, 124.2, 125.5, 137.2, 139.0, 144.2, 147.7, 151.1, 161.2; IR (KBr) 3430 (w, N—H), 3283−3194 (w, aromatic C—H), 1707 (s, C=O), 1579 (s), 1289 (s), 842 (w, C—H); MS *m/z* 229 (M$^+$, 100), 198 (46), 187 (4), 170 (24), 143 (16), 116 (5), 99 (5), 93 (12), 85 (5), 70 (13), 59 (5); HRMS [M$^+$] calculated for $C_{12}H_{11}O_2N_3$ 229.085 127, observed 229.085 300.

Methyl *N*-Boc-glycyl-5′-amino-2,2′-bipyridine-5-carboxylate (19). *N*-Boc-glycine (344 mg, 1.96 mmol) and TFFH (778 mg, 2.95 mmol) were dissolved in CH_2Cl_2 (18 mL). Pyridine (318 mL, 3.93 mmol) was added, and the solution was stirred at room temperature for 2 h. The resulting mixture was added over a suspension of the amine **18** (150 mg, 0.65

(59) Torrado, A.; López, S.; Alvarez, R.; de Lera, A. R. *Synthesis* **1995**, 285−293.
(60) Biosearch Technical Bulletin No. 9000-02.

mmol) and 2,6-di-*tert*-butyl-4-methylpyridine (403 mg, 1.96 mmol) in CH_2Cl_2 (6 mL) and stirred at room temperature for 30 min (when the reaction is over by TLC). Then, H_2O was added to the suspension and the aqueous phase extracted with CH_2Cl_2 (5×). The combined organic phases were dried over Na_2SO_4 and concentrated. The resulting solid was purified by flash column chromatography (silica gel, $CHCl_3$/MeOH, 98:2) to afford 157 mg of **19** as a yellow powder (62%): $R_f = 0.69$ (CH_2Cl_2/MeOH, 8:2); ^1H NMR ($CDCl_3$, 300.2 MHz) δ 1.46 (9H, s), 3.96 (3H, s), 4.00 (2H, d, $J = 5.7$ Hz), 5.60 (1H, t, $J = 5.7$ Hz), 8.21 (1H, dd, $J = 8.4$, 0.6 Hz), 8.3–8.4 (3H, m), 8.73 (1H, d, $J = 2.1$ Hz), 9.19 (1H, d, $J = 0.6$ Hz), 9.24 (1H, s, br); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 28.3, 45.6, 46.4, 52.3, 120.1, 122.1, 125.2, 127.4, 135.4, 137.9, 140.7, 150.4, 150.6, 156.7, 158.9, 165.8, 168.6; MS *m/z* 387 (MH^+, 100), 258 (34), 230 (14), 173 (9); HRMS [M^+] calculated for $C_{19}H_{23}O_5N_4$ 387.166 845, observed 387.165 300.

***N*$^\alpha$-(9-Fluorenylmethyloxycarbonyl)-glycyl-5′-amino-2,2′-bipyridine-5-carboxylic Acid (20).** The dipeptide **19** (132 mg, 0.34 mmol) was dissolved in a 50% solution of 0.01 N NaOH/dioxane and stirred at room temperature for 4 h. The reaction mixture was acidified to pH 3 and the resulting solid collected and treated with 50% TFA/CH_2Cl_2 for 30 min. The TFA was evaporated under N_2, and the residue washed with CH_2Cl_2 (2×), toluene (1×), and MeOH (1×). The oily residue was dissolved in a mixture of dioxane/10% Na_2CO_3 (1:1, 2 mL), and Fmoc-ONSu (89 mg, 0.26 mmol) in dioxane (0.5 mL) was added over it. The reaction mixture was stirred for 4 h at room temperature. The dioxane was evaporated under reduced pressure and the aqueous phase extracted with Et_2O (3×). The aqueous phase was acidified to pH ~3 and extracted with EtOAc (5×). The combined organic extracts were dried (Na_2SO_4) and evaporated to afford 50 mg (46%) of the *N*-protected dipeptide **20**: $R_f = 0.17$ ($CHCl_3$/MeOH/AcOH, 85:15:5); ^1H NMR (DMSO-d_6, 300.2 MHz) δ 3.87 (2H, d, $J = 6$ Hz), 4.24 (1H, t, $J = 6$ Hz), 4.30–4.33 (2H, m), 7.33 (2H, t, $J = 6.7$ Hz), 7.42 (2H, t, $J = 7.2$ Hz), 7.73 (2H, d, $J = 7.2$ Hz), 7.89 (2H, d, $J = 7.2$ Hz), 8.23 (1H, dd, $J = 9$, 2.4 Hz), 8.35 (1H, dd, $J = 8.4$, 2.1 Hz), 8.42 (2H, d, $J = 8.4$ Hz), 8.87 (1H, d, $J = 2.1$ Hz), 9.10 (1H, d, $J = 2.1$ Hz); ^{13}C NMR (DMSO-d_6, 75.5 MHz) δ 44.1, 46.6, 65.7, 119.6, 120.1, 121.5, 125.2, 126.6, 127.0, 127.6, 128.3, 136.6, 138.0, 140.2, 140.7, 143.8, 148.9, 150.1, 156.6, 157.8, 166.3, 168.8. MS *m/z* 517 (MNa^+, 4), 495 (MH^+, 29), 391 (4), 242 (5), 216 (4), 189 (3), 179 (20), 176 (8), 165 (10); HRMS [M^+] calculated for $C_{28}H_{22}O_5N_4$ 495.166 845, observed 495.169 600.

Macropin Peptide Synthesis. Peptides were synthesized on Macrocrowns purchased from Chiron Mimotopes. Commercially available starting materials and reagents were purchased from Milligen Biosearch, EM Science, Nova Biosearch, or Aldrich Chemical Co. A typical coupling cycle involved a 30 min soak in 20% (v/v) piperidine/DMF followed by a 5 min wash with DMF and three rinses with methanol. After air-drying for 10 min and 5 min soaking in DMF, the pin was submerged in the coupling mixture. Typical coupling were performed in 2 h with Fmoc-protected amino acid pentafluorophenyl esters and *N*-hydroxybenzotriazole (HOBt) at 80 mM concentration each with 450 μL of activated amino acid ester solution per pin. After coupling, the pin was washed once with DMF and three times with methanol. The pin was then air-dried for 10 min to complete a full cycle. Residues Flu, Dmd, and XBp were coupled with 6 equiv of amino acid and HOBt/DIPCDI/DIPEA chemistry in DMF. For these nonstandard amino acids, the coupling time was longer: 5 h for Flu and Dmd and 12 h for XBp. These cycles were repeated until the peptide was complete. Peptides were *N*-acetylated on the solid support by treatment with DMF/Ac_2O/TEA (12 eq). The peptides were deprotected and removed from the solid support by treatment with reagent R: trifluoroacetic acid/thioanisole/ethanedithiol/anisole, 90:5:3:2) for 4 h. The resulting solution was concentrated to ~1 mL and precipitated with ethyl ether/hexane 1:1. The precipitated product was subsequently triturated with ether/hexane 1:1 three times (6 mL). The solvent was decanted and the residue redissolved in H_2O and lyophilized overnight. The peptides were visualized by reversed phase high-pressure liquid chromatography (HPLC) (C_{18}) on a dual-pump system (solvent A, 0.1% TFA/H_2O; solvent B, 0.1% TFA/MeCN), purified on appropiate gradients and identified by mass spectroscopy.

Peptides **FXR02** and **FXR03** were purified by reversed phase HPLC on a semipreparative column (C_{18}) and identified by electrospray mass spectroscopy (MW(**FXR02**) = 1475); MW-(**FXR03**) = 1692).

Fluorescence Studies. A typical fluorescence spectrum was acquired in a 1 cm path length cuvette using a SLM-Aminco SPF-500C spectrofluorometer, with the following parameters: HVA 975, gain 10, $\lambda_{ex} = 388$ nm, $\lambda_{em} = 400$ nm, filter (time constant) 3, and both emission and excitation slit widths 4 nm. The concentrations of the peptide solutions were determined first by quantitative amino acid analysis (QAA) and then spectrophotometrically using the absorption band of the chromophores ($\lambda_{max} = 252$ nm, $\epsilon = 98570$, $\lambda_{max} = 307$ nm, $\epsilon = 54\,569$, $\lambda_{max} = 416$ nm, $\epsilon = 8425$, and $\lambda_{max} = 437$ nm, $\epsilon = 8366$). The methanolic and aqueous solutions of known peptide concentrations were then titrated with standard metal cation solutions at room temperature. Aqueous titrations were performed in 0.01 M PIPES buffer (pH 7.0) and 0.01 M phosphate buffer (pH 7.0). The recorded spectra were analyzed using KaleidaGraph version 3.0 (Abelbeck Software, CA).

Abbreviations

Dmd, (*S*)-2-amino-3-(3,4-dimethoxybenz-1-yl)propanoic acid; DIPEA, diisopropylethylamine; DIPCDI, diisopropylcarbodiimide; EDTA, ethylenediaminetetraacetic acid; FDAA, 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide; Flu, (*S*)-2-amino-3-(9-cyanoanthracen-10-yl)propanoic acid; FRET, fluorescence resonance energy transfer; PET, photoinduced electron transfer; HOBt, *N*-hydroxybenzotriazole; SPPS, solid phase peptide synthesis; PTC, phase transfer catalyst; Fmoc, 9-fluorenylmethoxycarbonyl; PIPES, 1,4-piperazinebis(ethanesulfonic acid); XBp, glycyl-5′-amino-2,2′-bipyridine-5-carboxylic acid.

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Supporting Information Available: ^1H NMR spectra for **5**, **6**, **12**, and **16**–**20** and the ^{13}C NMR spectra for **9** (recorded on a General Electric QE-300 spectrometer) and HPLC traces for peptides **FXR02** and **FXR03** (obtained on a Beckman reversed phase HPLC (C_{18} column)) (11 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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