Abstract

The DNA recognition properties of minor groove-binding polyamides make them logical candidates for a number of biologically relevant applications and their scope of use is further expanded by the same structural features and synthetic accessibility that allowed their development from distamycin. The modular nature of polyamide structure and function, combined with amenability to solid phase synthesis, has facilitated the construction of polyamide conjugates bearing a diverse set covalently-linked chemical species.

Polyamide-linked moieties have been employed to supplement the DNA recognition profile of polyamides (*intercalator conjugates*), to modify their biological activities (*activator peptide conjugates*), and to facilitate detection of targeted DNA sequences (*fluorophore conjugates*). The growing utility of fluorophore-polyamide conjugates is evidenced by their use in chromosome staining and cellular localization studies. The physical properties of these conjugates often show marked variations with respect to the covalent modification strategy employed as well as the chemical nature of the fluorophore. As a result, multiple iterations of conjugate design may be required, depending on the application envisioned.

Chapter 2 summarizes the synthesis of structurally distinct classes of fluorophore-hairpin polyamide conjugates. The physical properties and immediate applications of these compounds are discussed in Chapters 3 and 4. Chemically distinct fluorescent probes and modification strategies are described, with an emphasis on the C-terminal *tail* and pyrrole N-methyl *ring* positions as sites for fluorophore attachment.

Background and Significance

The ability of minor groove-binding polyamides to specifically target a wide range of predetermined DNA sequences with relatively high affinities logically suggests their use in the localization of other chemical species to particular sites on the DNA double helix. Another advantage offered by polyamides used in this role is their compatibility with condensed DNA structures and protein-DNA complexes, including chromosomes and nucleosome particles, without the need for sample pretreatment. Additionally, the development of solid phase methods for polyamide synthesis, with Boc[1] or Fmoc[2] protecting group strategies, on several chemical supports[1,3,4], has allowed a diverse set of molecules to be covalently linked to polyamides. To date, peptides, carbohydrates, DNA, RNA, DNA-alkylators, intercalators, biotin, and an array of fluorophores have been attached to several polyamide motifs. These conjugates have been used in numerous biological applications, including the control of gene expression through both activation and inhibition of transcription, fluorescent detection of DNA in homogenous solution, and chromosome staining (Figure 2.1A-C).

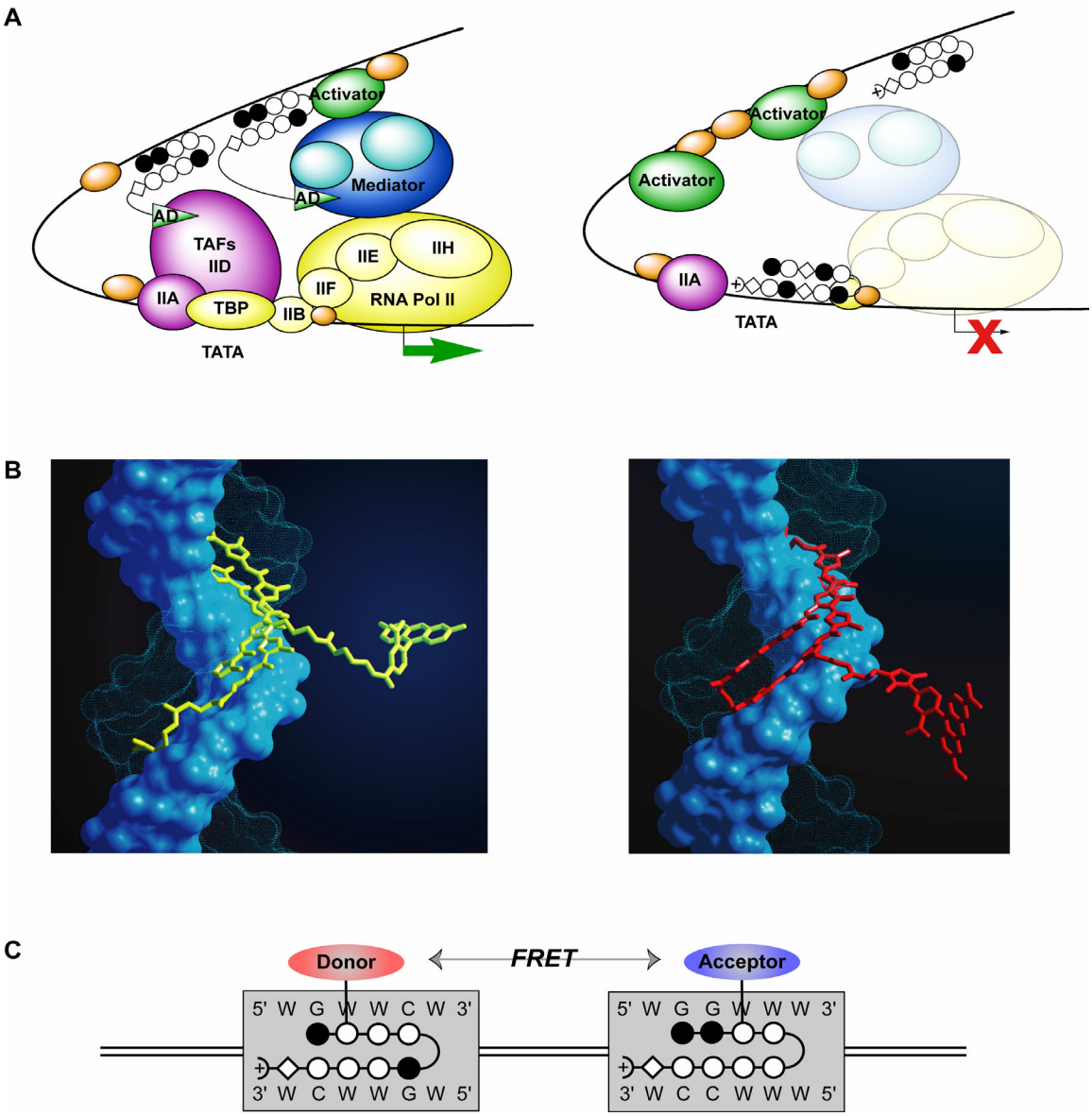

Figure 2.1 **(A)** Cartoon illustrations of transcriptional activation (*left*) and inhibition (*right*) by peptide-polyamide conjugates. **(B)** Molecular representation of fluorescein- (*left*) and TMR- (*right*) hairpin polyamide conjugates bound to DNA. **(C)** Ball and stick schematic of FRET between minor groove-associated polyamides.

Strategies for Covalent Modification of Hairpin Polyamides

The hairpin motif presents three structural features suitable for covalent modification by reactive fluorescent probes—the C-terminal *tail*, the γ-*turn*, and the

N-methyl position of pyrrole *ring* subunits (Figure 2.2). The site of fluorophore attachment and the chemical nature of the linker often have pronounced effects on the spectroscopic characteristics, DNA-binding properties, and cellular localization of fluorescent conjugates.

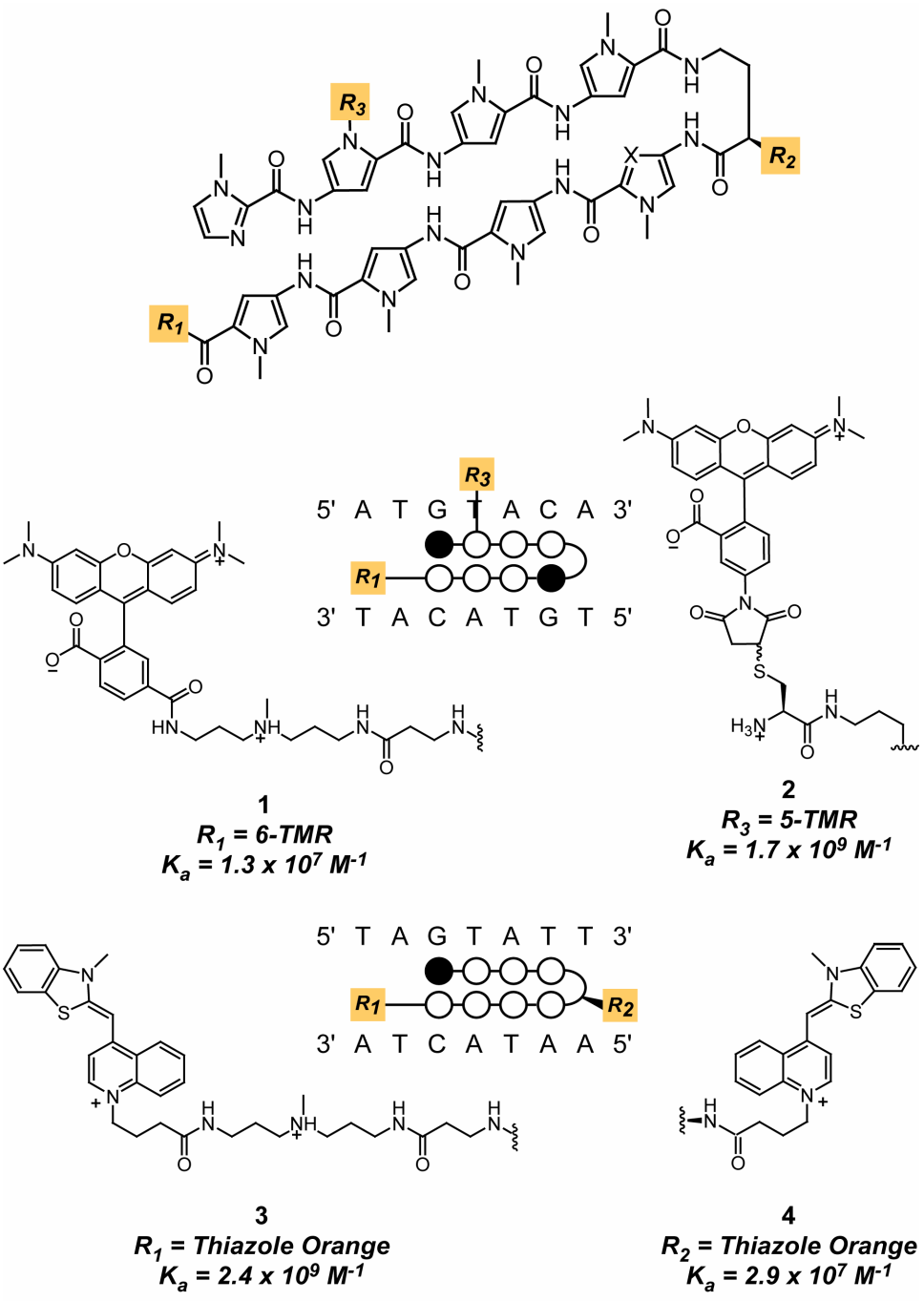

Figure 2.2 Modification strategies for synthesis of hairpin polyamide conjugates.

Polyamide Ring Conjugates

Derivatization of the N-methyl position of Py residues was first envisioned as a means of improving the often reduced affinity of polyamide conjugates. It was hoped that orienting the fluorophore away from the minor groove would minimize unfavorable contacts with the sugar phosphate backbone of the double helix (Figure 2.1B). The construction of ring conjugates requires orthogonally protected, N-substituted pyrrole building blocks, compatible with existing solid phase synthetic protocols. The first incarnation of these pyrrole building blocks employed an Fmoc-protected aminopropyl moiety as the N-substituent.[12] The synthetic route to this monomer (Figure 2.3) is straightforward; however, its amenability to scale up and overall yield are limited by handling difficulties associated with the key zwitterion

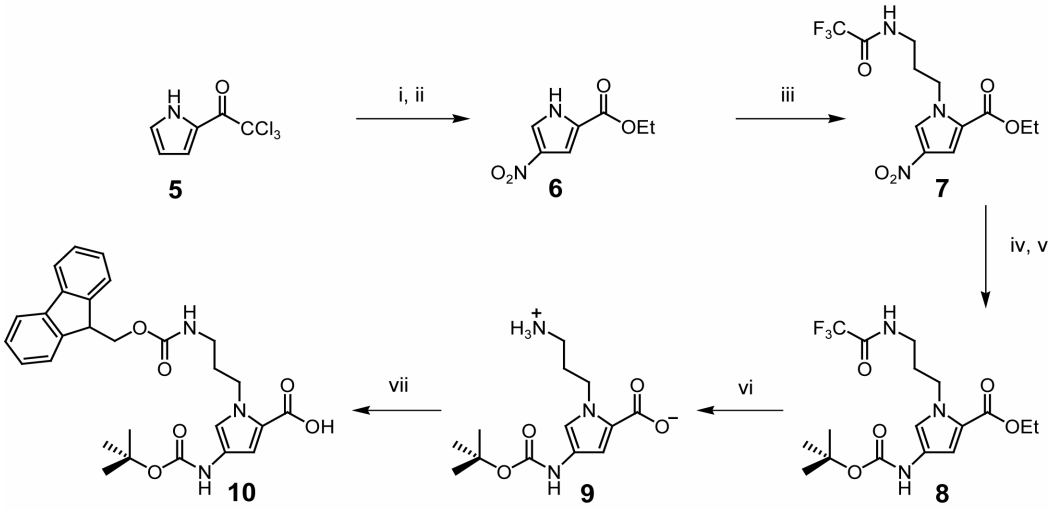

Figure 2.3 Synthesis of N-(Fmocaminopropyl)pyrrole building block. (i) HNO_3, Ac_2O; (ii) EtOH, NaOEt; (iii) 3-bromotrifluoroacetamide, KI, K_2CO_3, acetone, reflux; (iv) H_2, Pd-C, EtOAc; (v) Boc_2O, 10% K_2CO_3, *p*-dioxane; (vi) KOH, EtOH; (vii) Fmoc-OSu, 10% K_2CO_3, *p*-dioxane.

intermediate **9** and the necessity of column chromatography for purification of the finished monomer **10**. The above scheme typically gives yields of 15-20% over

seven steps. The protecting group exchange used to install the Fmoc group as the last step limits opportunities for optimization and subsequent column purification restricts recoveries to gram-scale. Additionally, this building block is not compatible with polyamides containing an Fmoc-protected chiral turn and can prove problematic if novel residues, requiring elevated coupling temperatures, are to be included in the target polyamide.

The above drawbacks were addressed in the next generation of N-functionalized pyrrole monomers by using the more robust phthalimide protecting group. While this group does not allow on-resin modification like its Fmoc progenitor, it is compatible with the chiral γ-turn and can withstand prolonged exposure to elevated temperatures. Initial synthetic routes to this monomer relied on benzyl ester protecting group for the 2-carboxylate of the pyrrole ring. Removal of this group under reductive conditions proved problematic on multigram scales, requiring non-catalytic amounts of palladium and prolonged reaction times. Scale-up resulted in decomposition. A 2-(trimethylsilyl)ethyl ester protecting group, on the other hand, proved more practical, allowing efficient synthesis on multigram scales. Stepwise optimization of the reaction scheme provided two equally efficient routes to the phthalimidopropyl pyrrole monomer in 23-25% yield over six steps, on multigram scales (Figure 2.4). Intermediate **13** has proven particularly useful in the construction of other N-substituted pyrrole derivatives by late-stage alkylation with bromides or iodides.

The phthalimide monomer **15** is readily incorporated into standard Boc-based solid phase synthesis on PAM resin (Figure 2.5). Coupling efficiencies are high and

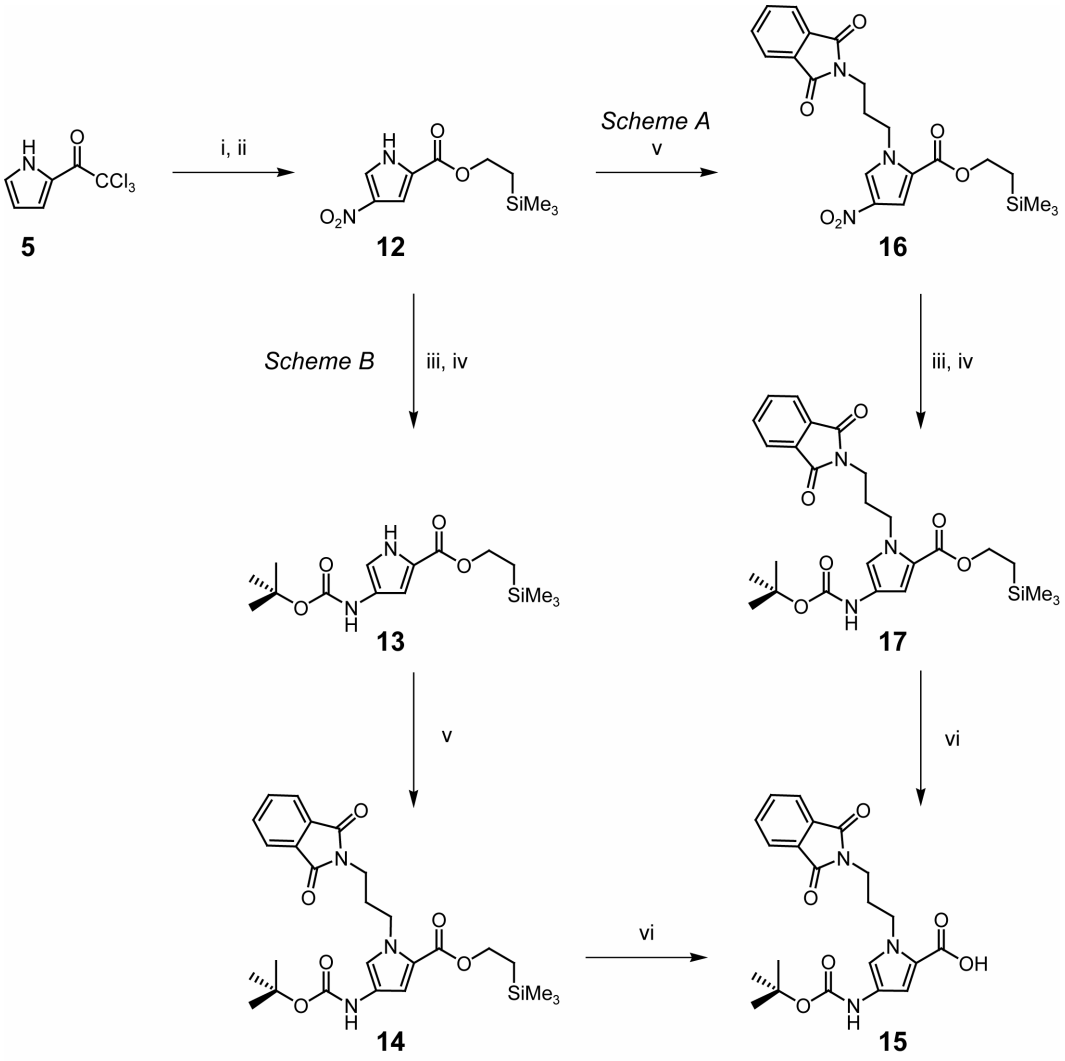

Figure 2.4 Syntheses of N-(phthalimidopropyl)pyrrole building block. (i) HNO_3, Ac_2O; (ii) 2-(trimethylsilyl)ethanol, Na^0; (iii) H_2, Pd-C, EtOAc; (iv) Boc_2O, $NaHCO_3$, EtOAc; (v) N-(3-bromopropylphthalimide), Bu_4NI, K_2CO_3, acetone, relux; (vi) TBAF, THF, 0 ^oC.

the yields of support-bound polyamides, such as **P1** and **P2,** are usually high. Phthalimide deprotection and resin cleavage are effected simultaneously by treatment of the support-bound polyamide with dimethylaminopropylamine (Dp). The crude cleaved products, **18** and **19**, were purified by solid phase extraction and reacted with commercially available amine-reactive cyanine probes. Conjugates

were purified by reversed phase HPLC with modest recoveries. This strategy, combining the phthalimide monomer with PAM resin was used to attach cyanine fluorophores to eight ring hairpin polyamides possessing C-terminal β-Dp tails, **20**-**24** (Figure 2.5).

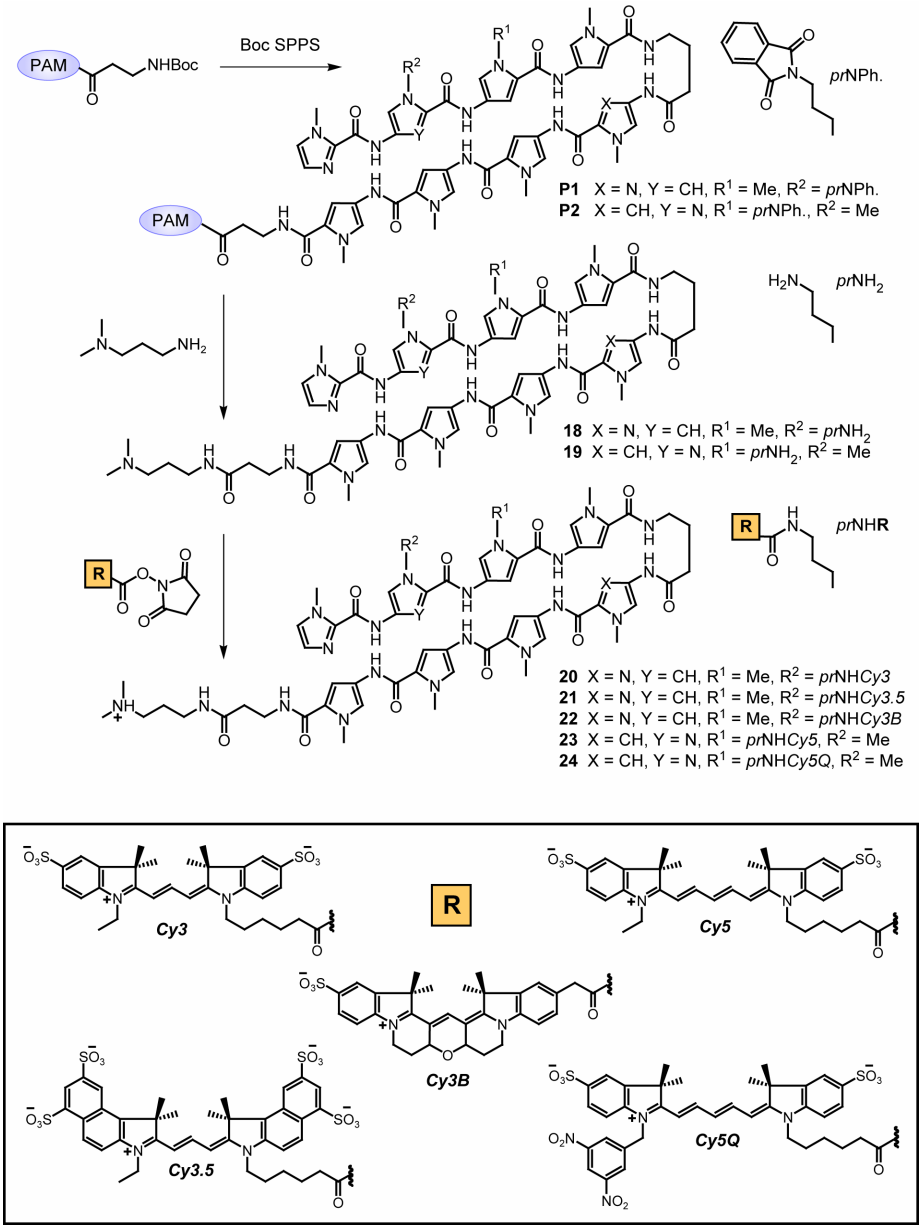

Figure 2.5 Synthesis of cyanine-hairpin polyamide conjugates. Standard Boc-based solid phase protocols[1] were employed in conjunction with N-(phthalimidopropyl)pyrrole building block, on PAM resin.

Polyamide Tail Conjugates

By far the most versatile structural component of hairpin polyamides is the C-terminal tail. The aliphatic Dp moiety, generated by ammonolysis of PAM resin-bound polyamide, can be replaced with tails bearing reactive groups by using any number of commercially available di- or triamines for resin cleavage. This strategy was used in the synthesis of hairpin polyamides, **1** and **27**-**29**, bearing 6-tetramethylrhodamine (6-TMR) and cyanine probes (Figure 2.6). A standard linker and a longer ethylene glycol linker were examined for each probe with the aim of minimizing fluorophore-DNA contacts in the polyamide-DNA complex. Linker modification using the above strategy is easily achieved by attaching amino acid residues to the PAM resin before initiating polyamide synthesis. Following cleavage of the polyamide intermediates, **25** and **26** from the solid support, crude products were purified by solid phase extraction and coupled with commercially available probes.

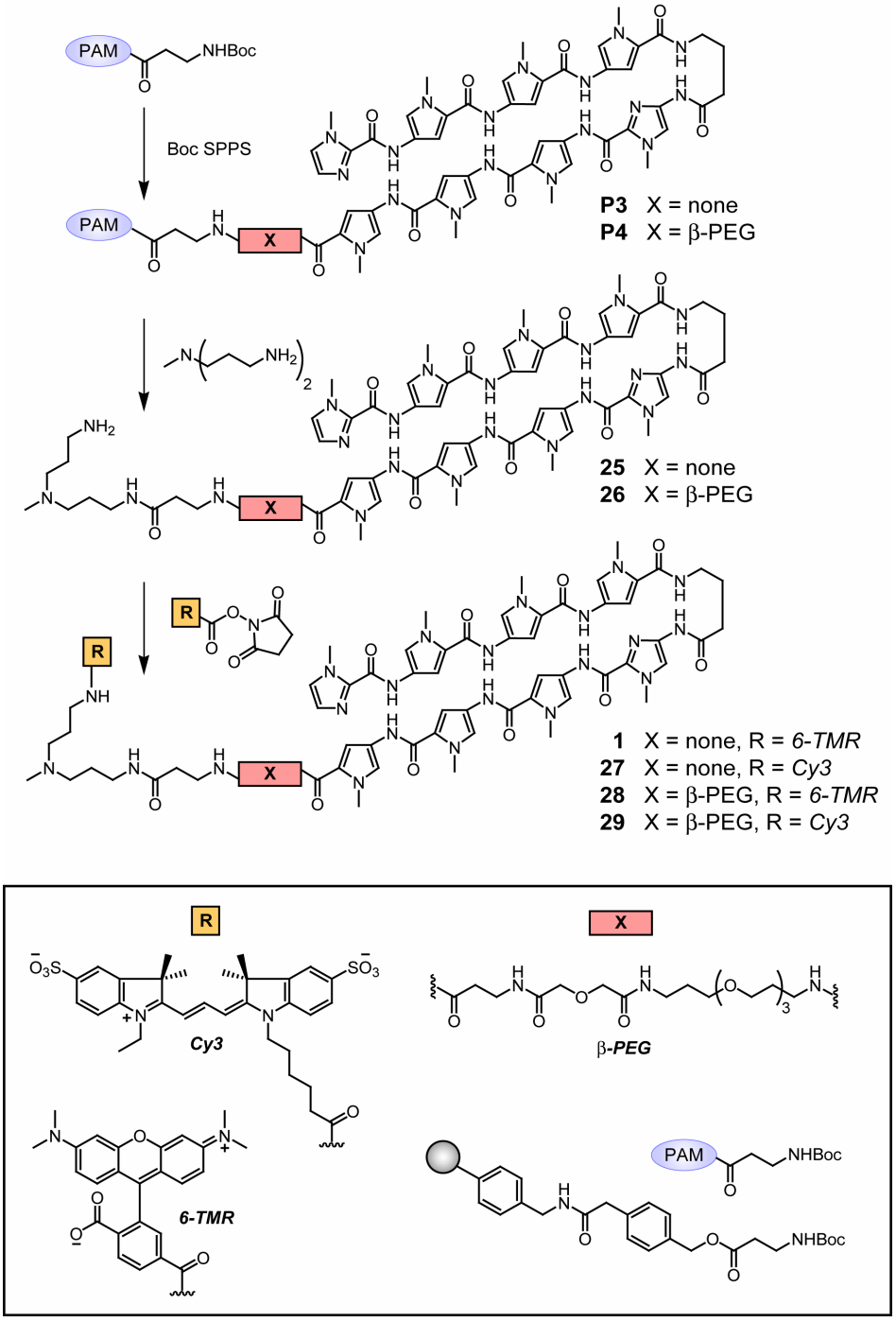

Figure 2.6 Synthesis of fluorophore conjugates containing C-terminal β-tails using PAM resin.

The C-terminal β-alanine residue present in conjugates described thus far originated from synthetic practicality, as it allowed commercial Boc-β-PAM resin to be used for polyamide synthesis; however, this residue imposes T, A selectivity at the tail and can have deleterious effects on cellular uptake. Polyamide synthesis using oxime resin allows the C-terminal b to be removed while still providing access to the reactive tails described above.[4] This resin was used to prepare hairpin conjugates, **31**-**33**, targeting the centromere repeat sequence for chromosome staining in live cells (Figure 2.7).

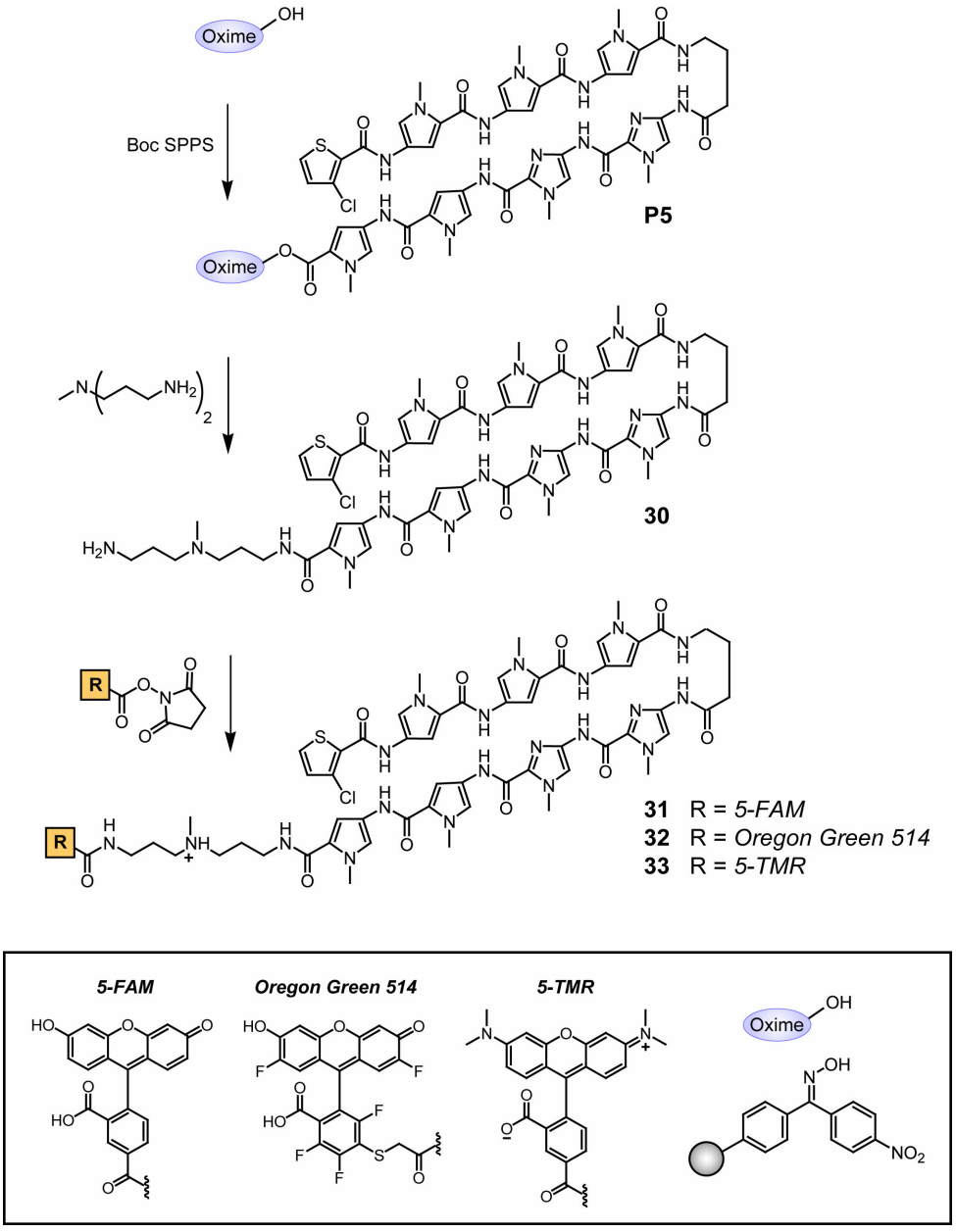

Figure 2.7 Synthesis of C-terminal fluorophore conjugates using Oxime resin.

Polyamide recovery from oxime resin is outstanding; however, this resin is not stable to prolonged treatment with concentrated solutions of trifluoroacetic acid (TFA) commonly used with PAM resin for deprotection of Boc protecting groups. More dilute TFA solutions (25%) can be used for Py residues but deprotection of

support bound Im requires more stringent conditions (50%). Repeated cycles of Im deprotection, therefore, reduce the overall yields of Im-rich polyamides substantially. This problem can be circumvented by incorporating Im as dimers with Py or β, or by using a more acid stable solid support. The hydrazine safety-catch resin is well suited to this role and can be used with Boc or Fmoc chemistries. Following elaboration of the target polyamide on the solid phase by standard protocols, resin cleavage is facilitated by oxidation of the linker in the presence of the desired di- or triamine. This synthetic strategy was used to prepare a fluorescein conjugate, **35**, designed to inhibit synthesis of the human MDR gene (Figure 2.8). While yields for this particular support are comparable to PAM and oxime resins when Dp is used for cleavage, recovery of reactive polyamide intermediates like **34** is frequently diminished, possible due to chelation of the amine by the copper reagent. Despite lower overall yields, this resin does provide a last resort for constructing imidazole-rich polyamide conjugates without C-terminal β-tails.

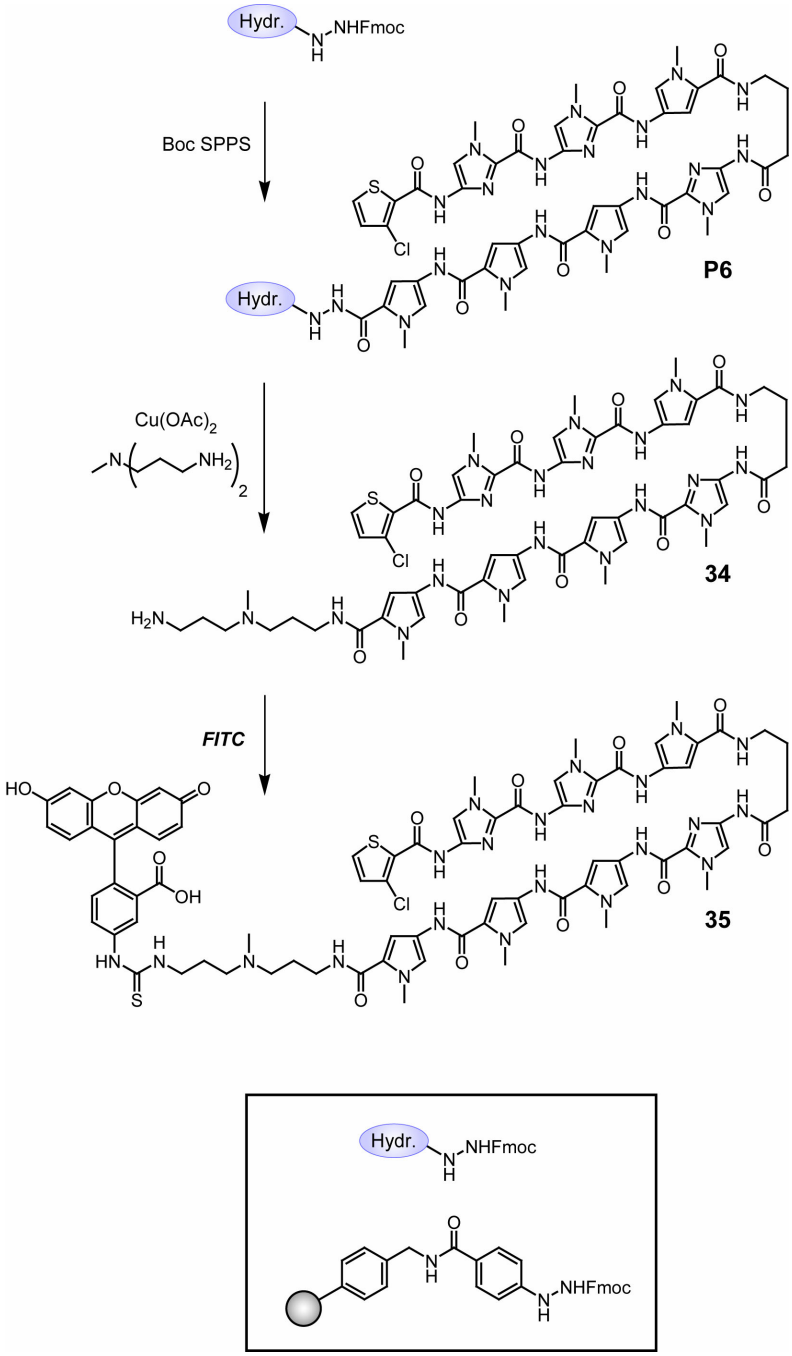

Figure 2.8 Synthesis of tail fluorophore conjugates using hydrazine resin.

Self-Quenched TMR-Polyamide Ring Conjugates

The absence of polyamide-compatible on-resin deprotection conditions for the N-(phthalimidopropyl)pyrrole building block has immediate implications for the design of ring conjugates. Longer chemical linkers are often desirable with regard to improving the binding affinities of polyamide conjugates and incorporation of charged groups is often used to enhance the solubility of fluorescent conjugates. These issues are readily addressed by the C-terminal modification techniques already described; however, tail conjugates often show reduced affinities relative to their ring-modified counterparts and ring conjugates have also exhibited another desirable property–fluorescence quenching.

Linker modification in ring conjugates was therefore achieved by combining solution and solid phase techniques, as in the synthesis of TMR- and fluorescein-hairpin conjugates **38** and **39** (Figure 2.9). Following synthesis of the target polyamide, **P7**, on PAM resin, cleavage with Dp yields reactive polyamide intermediate **36** which was acylated with a protected cysteine amino acid. Isolation of **37** from the crude reaction mixture was accomplished by solid phase extraction (SPE), in good yield. Excesses of cysteine and coupling reagent were used to ensure high yield and purification by SPE provided enhanced recovery relative to preparative HPLC. (This scheme relies on the purity of the support-bound polyamide **P7**, and in cases where solid phase synthesis does not give a single product, preparative HPLC should be performed before subsequent reactions are performed.) The protected intermediate **36** was then treated with TFA and modified with thiol-reactive maleimide derivatives of 5-TMR and fluorescein. These probes

selectively react with thiols in the presence of amines, at neutral pH, allowing the introduction of an additional charged group on the linker. The scheme illustrated in

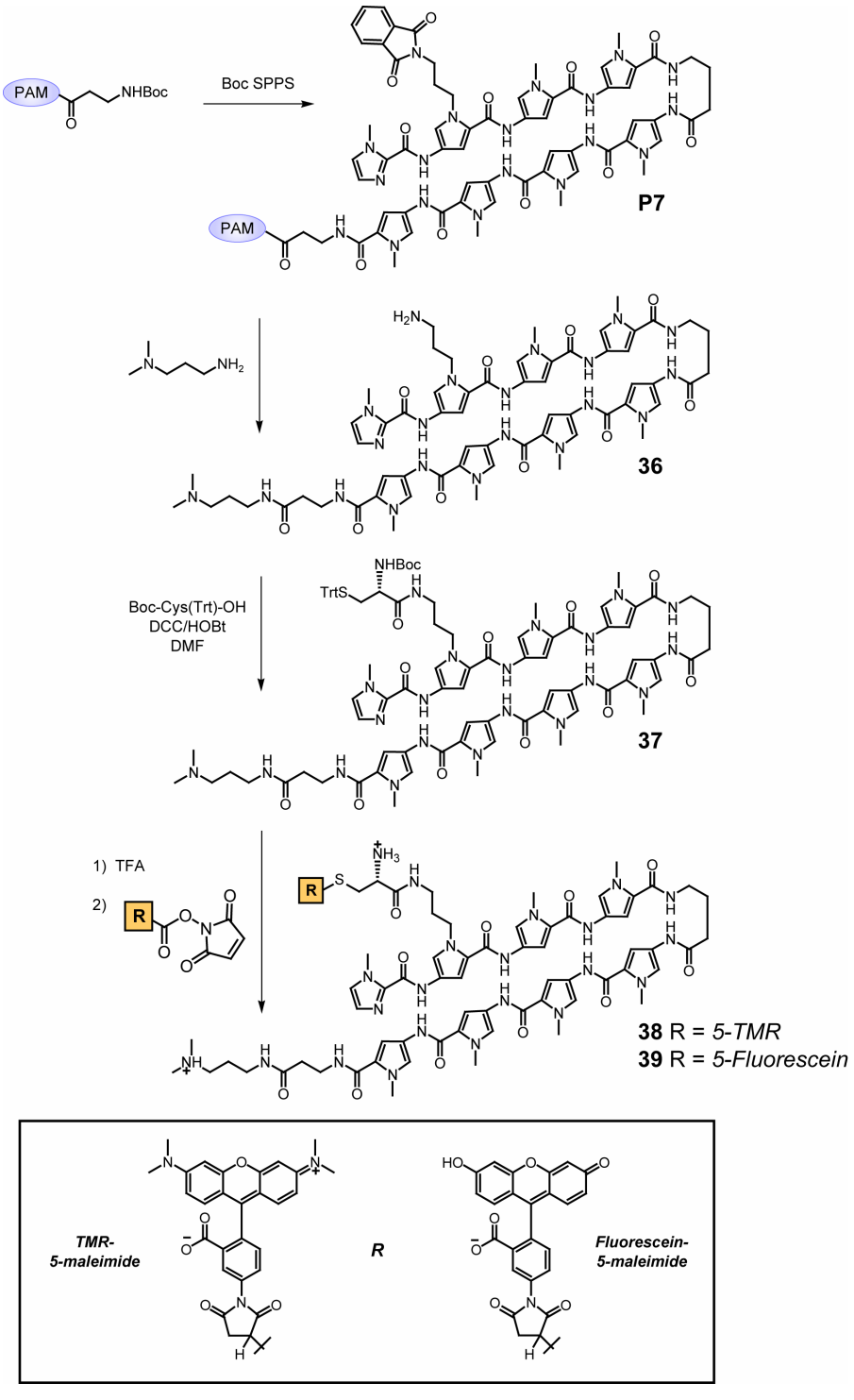

Figure 2.9 Synthesis of self-quenched TMR-hairpin polyamide conjugates.

Figure 2.9 was applied to several distinct polyamide scaffolds, affording quenched fluorophore conjugates targeting different DNA sequences (Figure 2.10).

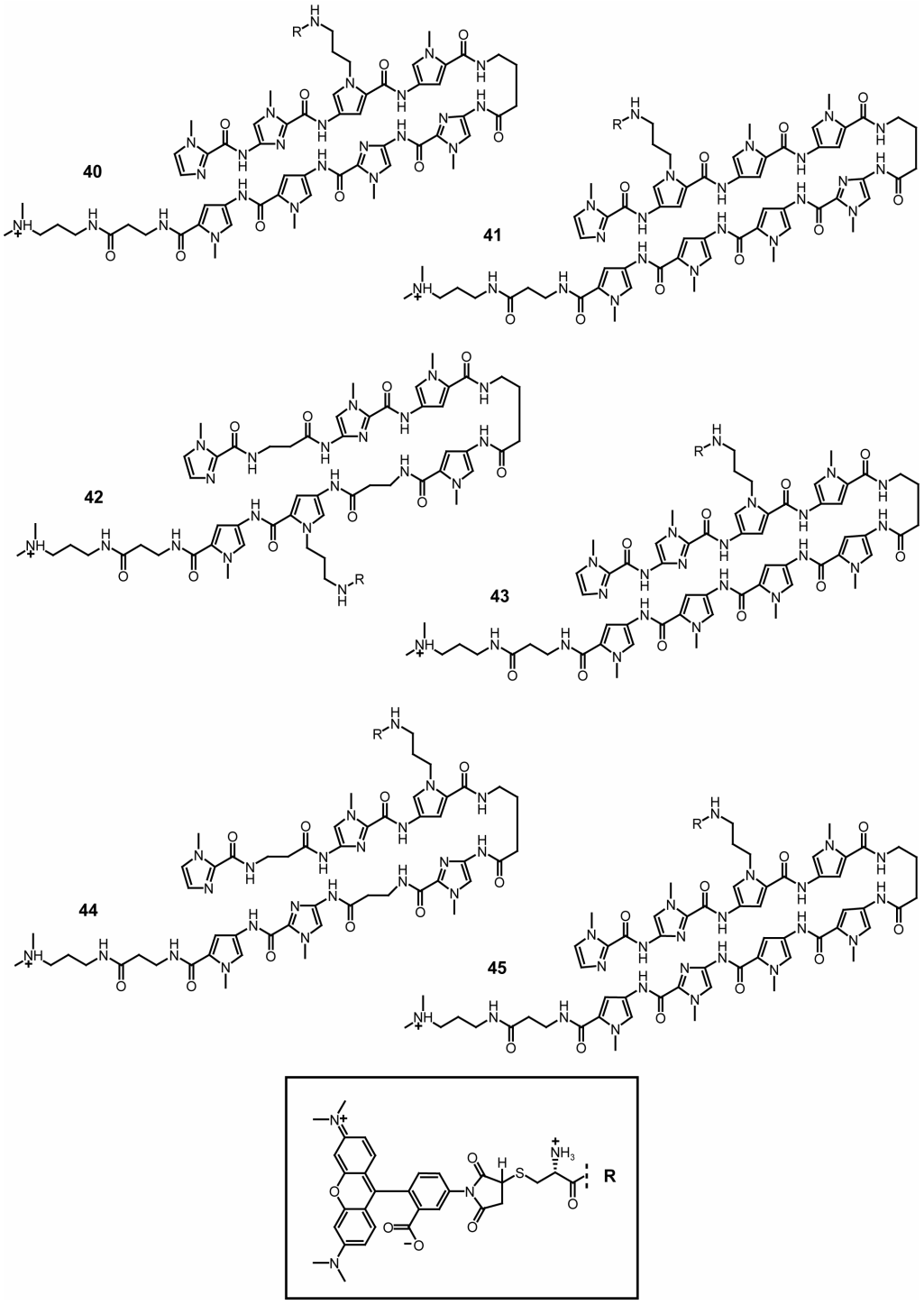

Figure 2.10 Chemical structures of self-quenched TMR-polyamide conjugates.

Experimental

Materials. 2-pyrrolyl trichloromethyl ketone (2-(trichloroacetyl)pyrrole), 2-(trimethylsilyl)ethanol, sodium, 10% Palladium/C, di-*t*-butyl dicarbonate (Boc-anhydride), N-(3-bromopropyl)phthalimide, trifluoroacetic anhydride, 3-bromopropylamino hydrobromide, Fmoc-succinimidyl ester, tetrabutylammonium iodide, and tetrabutylammonium fluoride were purchased from Aldrich. Ethyl acetate (EtOAc), acetone, tetrahydrofuran (THF), and acetic anhydride (Ac_2O) were reagent grade from EM. Amine reactive cyanine probes were purchased from Amersham and reactive fluorescein and rhodamine probes were obtained from Molecular Probes. All reagents were used without further purification.

^{1}H NMR and ^{13}C NMR spectra were recorded using a Varian Mercury instrument operating at 300 MHz. Chemical shifts are reported in parts per million relative to residual solvent signal. IR spectra were recorded on a Perkin-Elmer FTIR spectrometer. High-resolution EI mass spectra were recorded at the Mass Spectrometry Laboratory at the UCLA Mass Spectrometry Facility, as well as at the Mass Spectrometry Center at Caltech. Thin-layer chromatography (TLC) was performed on precoated silica gel 60 F_{254} plates. Reagent grade chemicals were used unless otherwise indicated.

UV spectra were measured in water on a Beckman model DU 7400 spectrophotometer. MALDI-TOF and ESI mass spectrometry data was collected by the Protein and Peptide Microanalytical Facility at The California Institute of Technology, or obtained manually using a Voyager MALDI system or a Finnegan LCQ ESI apparatus. Unless otherwise indicated HPLC analysis was performed

using a Beckman Gold Nouveau system using a Rainin C_{18}, Micosorb MV, 5 μm, 300 × 4.6 mm reversed phase column using 0.1% (wt/v) TFA with acetonitrile as eluent and a flow rate of 1.0 mL/min, gradient elution 1.25% acetonitrile/min. Preparatory reversed phase HPLC was performed on a Beckman HPLC with a Waters DeltaPak 25 × 100 mm, 100 m C_{18} column equipped with a guard, 0.1% (wt/v) TFA/0.25% acetonitrile/min. Solid phase extraction cartridges were purchased from Waters Chromatography. Water was from either a Millipore MilliQ water purification system or RNase free water from a USB.

Monomer Synthesis—N-(Fmocaminopropyl)pyrrole Building Block

Ethyl 4-nitro-pyrrole-2-carboxylate (6). 2-(trichloroacetyl)pyrrole (200 g = 0.941 mol) was dissolved in acetic anhydride (0.9L). The resulting solution was stirred vigorously at -15 °C and fuming nitric acid (90 mL) was cautiously added. The mixture was then slowly allowed to warm to ambient temperature over a period of 6 hours and was subsequently poured onto ice water (3.5 L) to yield a white suspension. The precipitate was collected by vacuum filtration and recrystalized from 9:1 $CHCl_3$/EtOH to yield a finely divided white solid (100 g, 41.2%). This material was used without further purification.

The white solid (75 g = 0.291 mol) was suspended in EtOH (300 mL) and a solution of NaOEt (4.1 g = 0.6 mol) in EtOH (100 mL) was added. The resulting mixture was stirred for 8 hours and quenched with 10% H_2SO_4. The mixture was then cooled at - 20°C for 2 hours and product was collected by vacuum filtration as a white crystalline solid (50.8 g, 95%). TLC (5:2 hexanes/EtOAc) R_f = 0.2. ^{1}H NMR

(DMSO-d_6) δ 13.2 (br s, 1H), 8.1 (d, 1H, J = 1.4), 7.3 (d, 1H, J = 1.3), 4.3 (q, 2H, J = 7.0), 1.3 (t, 3H, J = 7.0).

Ethyl 4-nitro-1-(trifluoroacetamidopropyl)pyrrole-2-carboxylate (7). 3-bromoaminopropyl hydrobromide (15 g = 0.069 mol) was suspended in CH_2Cl_2 (150 mL). This suspension was cooled to 0 °C with stirring and DIEA (30 mL) was added. Trifluoroacetic anhydride (12.7 mL = 0.09 mol) was slowly added to the cold solution and the mixture was stirred for 6 hours. The solution was then washed with 1M citric acid (2 x 150 mL), 5% $NaHCO_3$ (2 x 150 mL), and brine (2 x 150 mL). The organic layer was isolated, dried over $MgSO_4$, filtered, and concentrated *in vacuo* to yield a yellow oil that solidified upon standing (10.4 g, 64%). This material was used without further purification or characterization.

The yellow solid above (9 g = 0.038 mol) was dissolved in acetone (50 mL) and **7** (5.9 g = 0.032 mol), K_2CO_3 (8.9 g = 0.064mol), and Bu_4NI (3.5 g = 0.009 mol) were added. The resulting suspension was stirred vigorously under refluxing conditions for 14 hours. The crude mixture was concentrated *in vacuo* and taken up in $CHCl_3$ (150 mL) and washed with water (3 x 250 mL). The organic phase was isolated, dried over $MgSO_4$, filtered, and concentrated to yield a dark oil. The oil was flashed over silica gel using 1:1 hexanes/EtOAc and the eluents were concentrated to yield a light yellow oil, which solidified upon co-evaporation with 1:1 petroleum ether/ CH_2Cl_2 (7 g, 65%). TLC (5:2 hexanes/EtOAc) R_f = 0.2. ^{1}H NMR ($CDCl_3$) δ 7.7 (d, 1H, J = 1.8), 7.5 (d, 1H, J = 1.8), 7.4 (br s, 1H), 4.5 (t, 2H, J = 7.0), 4.4 (q, 2H, J = 7.1), 3.4 (q, 2H, J = 6.0), 2.2 (quintet, 2H, J = 6.2), 1.4 (t, 3H, J = 7.1).

Ethyl 4-[(*t*-butoxycarbonyl)amino]-1-(trifluoroacetamidopropyl)pyrrole-2-carboxylate (8). **7** (5 g = 0.015 mol) was dissolved in EtOAc (30 mL) and Boc_2O (4.9 g = 0.0225 mol) was added. A suspension of 10% Pd-C (0.7 g) in EtOAc (20 mL) was then added and the resulting mixture was stirred vigorously under H_2 (300 psi) for 14 hours. The mixture was filtered over celite and the supernatant was concentrated to yield a yellow oil. This oil was taken up in CH_2Cl_2 and flashed over silica gel using 7:3 toluene/EtOAc. The eluent was co-evaporated with 1:1 CH_2Cl_2/petroleum ether to afford a white solid (6 g, 98%). TLC (7:3 toluene/EtOAc) R_f = 0.4. ^{1}H NMR ($CDCl_3$) δ 7.8 (br s, 1H), 7.3 (d, 1H, J = 1.6), 6.7 (d, 1H, J = 1.6), 6.4 (s, 1H), 4.4 (t, 2H, J = 6.3), 4.3 (q, 2H, J = 7.2), 3.3 (q, 2H, J = 5.6), 2.1 (quintet, 2H, J = 6.2), 1.5 (s, 9H), 1.4 (t, 3H, J = 7.2).

4-[(*t*-butoxycarbonyl)amino]-1-(aminopropyl)pyrrole-2-carboxylic acid (9). **8** (4.6 g = 0.011 mol) was dissolved in MeOH (20 mL) and 5M NaOH (20 mL) was added. This mixture was stirred for 6 hours at 37 °C. The mixture was then cooled to 0 °C and neutralized with 1M HCl. The solution was then concentrated *in vacuo* to remove MeOH and the resulting aqueous suspension was washed with EtOAc (2 x 100 mL). The organic phase was isolated, dried over $MgSO_4$, filtered and concentrated to yield a yellow oil, which solidified upon co-evaporation with 1:1 CH_2Cl_2/petroleum ether, to yield a tan solid (1.7 g, 55%). ESI-MS (MeOH) [M+H] = 283.4; calculated [M+H] = 283.1.

4-[(*t*-butoxycarbonyl)amino]-1-[(fluorenylmethoxycarbonyl)propylamine] pyrrole-2-carboxylic acid (10). Zwitterion **9** (1.5 g = 0.005 mol) was dissolved in 10% K_2CO_3 (5 mL) and Boc_2O (1.7 g = 0.0075 mol) in *p*-dioxane (5 mL) was added. The resulting mixture was stirred for 4 hours and cooled in an ice bath. The cold solution was acidified to pH 3 with 1N HCl and washed with CH_2Cl_2 (2 x 30 mL). The organic layers were pooled, dried over $MgSO_4$, filtered, and concentrated to yield a gummy solid. Co-evaporation with 1:1 CH_2Cl_2/petroleum ether afforded a slightly tan crystalline solid (2.2 g, 90%). ESI-MS (MeOH) [M+H] = 506.6, [M+Na] = 528.5; calculated [M+H] = 506.3, [M+Na] = 529.2.

Monomer Synthesis—N-(Phthalimidopropyl)pyrrole Building Block

(2-Trimethylsilyl)-ethyl 4-nitropyrrole-2-carboxylate (12). To a cooled (-15 ºC) solution of 2-(trichloroacetyl)pyrrole (250 g, 1.18 mol) in acetic anhydride (1 L) in a 4 L flask equipped with a mechanical stirrer was added fuming nitric acid (135 mL) over a period of 1 hour. The reaction mixture was then slowly allowed to warm to ambient temperature and was stirred for an additional 6 hours. The mixture was then poured onto ice water (5 L) and the resulting suspension was stirred vigorously and filtered to yield a white solid. The solid was azeotroped from toluene and recrystalized from 95:5 (v:v) $CHCl_3$/EtOH to yield the nitrated product as a tan solid (168.5 g, 0.654 mol, 55% yield): TLC (5:2 hexanes/ethyl acetate; silica) R_f 0.5; ^{1}H NMR (DMSO-d_6) δ 8.34 (d, 1 H, J = 1.5 Hz), 7.67 (d, 1 H, J = 1.5 Hz); ^{13}C NMR (DMSO-d_6) δ 173.5, 137.7, 128.5, 122.0, 115.0, 94.5; EI-MS m/e 255.9215 (M^+

255.9209 calculated for $C_6H_3Cl_3N_2O_3$); IR (ν_{max} cm^{-1}; $CHCl_3$) 3317, 1676, 1551, 1519, 1405, 1379, 1316, 1149, 814, 742, 730.

To a well-stirred solution of the above solid (100 g, 0.388 mol) in THF (300 mL) was added a solution of Na^0 (0.897 g, 0.039 mol) and 2-(trimethylsilyl)ethanol (50 g, 0.423 mol) in THF (200 mL). The resulting mixture was stirred for 6 hours and quenched with concentrated H_2SO_4 (2.2 mL, 0.039 mol). The reaction mixture was then concentrated *in vacuo* to yield a slightly yellow solid. The solid was suspended in cold (0 ºC) dichloromethane (100 mL) and petroleum ether (300 mL) was added. The resulting suspension was then filtered to yield **12** (91.5 g, 0.356 mol, 92% yield) as a finely divided white solid: TLC (3:2 hexanes : ethyl acetate; silica) R_f 0.5; ^1H NMR (DMSO-d_6) δ 8.06 (d, 1 H, J = 1.5 Hz), 7.19 (d, 1 H, J = 1.5 Hz), 4.32 (t, 2 H, J = 8.3 Hz), 1.04 (t, 2 H, J = 8.3 Hz), 0.04 (s, 9 H); ^{13}C NMR (DMSO-d_6) δ 160.1, 137.2, 124.9, 123.8, 109.8, 63.5, 17.7, -0.7; EI-MS m/e 256.0879 (M^+ 256.0879 calculated for $C_{10}H_{16}N_2O_4Si$); IR (ν_{max} cm^{-1}; $CHCl_3$) 3260, 1690, 1512, 1372, 1320, 1202, 836, 752.

(2-Trimethylsilyl)ethyl-4-[(*t*-butoxycarbonyl)amino]pyrrole-2-carboxylate (13). 10% Palladium on activated carbon (6 g) was added to a well-stirred solution of **12** (30.8 g, 0.120 mol) in EtOAc (500 mL) and EtOH (2.5 mL). The resulting suspension was stirred under H_2 (500 psi) for 8 hours. The reaction mixture was then filtered over celite and the supernatant was concentrated *in vacuo* to yield a yellow oil. The oil was resuspended in EtOAc (240 mL) and Boc-anhydride (39.3 g, 0.180 mol) was added, followed by 1M $NaHCO_3$ (240 mL). The biphasic mixture

was stirred for 4 hours and the layers separated. The aqueous layer was washed with EtOAc (3 x 250 mL) and the organic layers were combined, suspended over sodium sulfate, filtered and concentrated to yield a yellow oil. The oil was dissolved in dichloromethane (100 mL) and petroleum ether (400 mL) and cooled at -20 ºC for 1 hour. The resulting suspension was filtered to yield **13** as a finely divided white solid (29.8 g, 0.0913 mol, 76% yield): TLC (3:2 hexanes/ethyl acetate; silica) R_f 0.8; ^{1}H NMR (DMSO-d_6) δ 11.5 (s, 1 H), 9.1 (s, 1 H), 6.94 (s, 1 H), 6.58 (s, 1 H), 4.24 (t, 2 H, J = 8.4 Hz), 1.42 (s, 9 H), 1.01 (t, 2 H, J = 8.4 Hz), 0.03 (s, 9 H); ^{13}C NMR (DMSO-d_6) δ 161.0, 153.3, 125.6, 120.1, 113.2, 105.9, 79.0, 62.1, 29.0, 17.9, -0.6; EI-MS m/e 326.1656 (M^+ 326.1662 calculated for $C_{15}H_{26}N_2O_4Si$); IR (ν_{max} cm^{-1}; $CHCl_3$) 3313, 2955, 2899, 1693, 1682, 1590, 1558, 1403, 1368, 1250, 1218, 1167, 1110, 1058, 969, 860, 838, 763.

(**2-Trimethylsilyl)ethyl-4-[(*t*-butoxycarbonyl)amino]-1-(phthalimidopropyl)-pyrrole-2-carboxylate (14).** To a solution of **13** (21.4 g, 0.042 mol) in acetone (85 mL) was added N-(3-bromopropyl)phthalimide (22.5 g, 0.084 mol), tetrabutylammonium iodide (3.1 g, 0.008 mol), and potassium carbonate (8.71 g, 0.063 mol). The resulting suspension was stirred vigorously at reflux for 24 hours. The reaction mixture was then concentrated *in vacuo* to yield a yellow solid. The solid was suspended in chloroform (500 mL) and washed with water (4 x 500 mL). The chloroform layer was isolated, dried over sodium sulfate, filtered and reconcentrated to yield a yellow oil. The oil was purified by flash chromatography on silica gel using 98:2 (v:v) dichloromethane/diethyl ether as the eluent to yield **14** as a

gummy yellow solid (13.9 g, 0.027 mol, 65% yield): TLC (95:5 dichloromethane/ethyl ether; silica) R_f 0.4; ^{1}H NMR (DMSO-d_6) δ 9.12 (s, 1 H), 7.82 (m, 4 H), 7.21 (d, 1 H, *J* = 0.9 Hz), 6.60 (d, 1 H, *J* = 1.8 Hz), 4.25 (t, 2 H, *J* = 7.2 Hz), 4.13 (t, 2 H, *J* = 8.4 Hz), 3.55 (t, 2 H, *J* = 6.6 Hz), 1.95 (p, 2 H, *J* = 7.2 Hz), 1.42 (s, 9 H), 0.95 (t, 2 H, *J* = 8.1 Hz), 0.02 (s, 9 H); ^{13}C NMR (DMSO-d_6) δ 168.5, 160.7, 153.2, 134.8, 132.4, 123.9, 123.5, 118.8, 108.4, 79.1, 62.0, 46.5, 35.8, 31.1, 29.0, 17.7, -0.6; EI-MS m/e 513.2286 (M^+ 513.2295 calculated for $C_{26}H_{35}N_3O_6Si$); IR (ν_{max} cm^{-1}; $CHCl_3$) 3351, 2954, 1772, 1719, 1587, 1551, 1398, 1367, 1248, 1168, 1088, 838, 720.

4-[(*t*-butoxycarbonyl)amino]-1-(phthalimidopropyl)pyrrole-2-carboxylic acid (15). To a cooled (0 ºC) solution of **14** or **17** (10.0 g, 0.020 mol) in anhydrous THF (100 mL) was added 1M tetrabutylammonium fluoride in THF (23.4 mL, 0.023 mol) via syringe under a positive pressure of Ar. The mixture was stirred at 0 ºC, under Ar, for 1 hour and slowly allowed to warm to ambient temperature. The reaction was stirred an additional 8 hours and was quenched with 0.1 M citric acid (100 mL). The resulting mixture was washed with EtOAc (3 x 300 mL) and the organic washes were combined, suspended over Na_2SO_4, filtered over a short silica plug, and concentrated *in vacuo* to yield **15** as a white solid (7.6 g, 0.018 mol, 92% yield): TLC (3:2 hexanes/ethyl acetate; silica) R_f 0.1; ^{1}H NMR (DMSO-d_6) δ 12.06 (s, 1H), 9.08 (s, 1 H), 7.81 (m, 4 H), 7.18 (s, 1 H), 6.56 (s, 1 H), 4.25 (t, 2 H, *J* = 7.2 Hz), 3.55 (t, 2 H, *J* = 6.6 Hz), 1.94 (p, 2 H, *J* = 6.9 Hz), 1.42 (s, 9 H); ^{13}C NMR (DMSO-d_6) δ 168.5, 162.2, 153.2, 134.8, 132.4, 123.6, 123.5, 119.3, 118.6, 108.6, 79.1, 46.5,

35.8, 31.2, 29.0; EI-MS m/e 413.1587 (M^+ 413.1587 calculated for $C_{21}H_{23}N_3O_6$); IR (ν_{max} cm^{-1}; $CHCl_3$) 3339, 2978, 1771, 1710, 1588, 1550, 1467, 1397, 1366, 1244, 1160, 759, 720.

(2-Trimethylsilyl)-ethyl-4-nitro-1-(phthalimidopropyl)pyrrole-2-carboxylate (16). To a solution of **12** (15.2 g, 0.059 mol) in anhydrous acetone (0.115 L) was added finely ground K_2CO_3 (12.3 g, 0.077 mol), tetrabutylammonium iodide (6.6 g, 0.018 mol), and N-(3-bromopropyl)phthalimide (20.7 g, 0.077 mol). The resulting suspension was stirred vigorously at reflux for 1.5 days. The crude reaction mixture was then concentrated *in vacuo* and resuspended in $CHCl_3$ (0.3 L) and washed with water (2 x 0.3L). The $CHCl_3$ solution was suspended over $MgSO_4$, filtered, and concentrated to yield a yellow oil. The oil was purified by flash chromatography on silica using gradient elution from 5:1 (hexanes : ethyl acetate) to 3:1 (hexanes : ethyl acetate) (v:v). **16** (17.2 g, 0.039 mol, 65% yield) was obtained as an amorphous white solid: TLC (5:2 hexanes : ethyl acetate; silica) R_f 0.4; ^{1}H NMR (DMSO-d_6) δ 8.32 (d, 1 H, *J* = 2.1 Hz), 7.82 (m, 4 H), 7.21 (dd, 1 H, *J* = 0.6, 2.1 Hz), 4.37 (t, 2 H, *J* = 7.2 Hz), 4.22 (t, 2 H, *J* = 8.4 Hz), 3.58 (t, 2 H, *J* = 6.6 Hz), 2.06 (quint, 2 H, *J* = 6.9 Hz), 1.00 (t, 2 H, *J* = 8.4 Hz), 0.01 (s, 9 H); ^{13}C NMR (DMSO-d_6) δ 168.6, 159.8, 135.0, 134.9, 132.3, 129.5, 123.5, 122.8, 112.5, 63.5, 48.1, 35.5, 30.3, 17.6, -0.6; IR (ν_{max} cm^{-1}; $CHCl_3$) 3134, 2954, 1772, 1714, 1540, 1509, 1397, 1369.1319, 1252, 1103, 1081, 861, 840, 752, 721.

(2-Trimethylsilyl)ethyl-4-[(*t*-butoxycarbonyl)amino]-1-(phthalimidopropyl)-pyrrole-2-carboxylate (17). 10% Palladium on activated carbon (3 g) was added to a well-stirred solution of **16** (15.1 g, 0.034 mol) in 10:1 ethanol : ethyl acetate (v:v; 165 mL). The resulting suspension was stirred under H_2 (300 psi) for 6 hours. The crude mixture was filtered over Celite and the supernatant was concentrated *in vacuo* to yield a yellow oil. The oil was dissolved in ethyl acetate (80 mL) and Boc-anhydride (14.9 g, 0.068 mol) was added. Saturated sodium bicarbonate (80 mL) was then added and the biphasic mixture was stirred for 2.5 hours. The ethyl acetate layer was isolated and washed with saturated sodium bicarbonate (3 x 250 mL), 5% citric acid (3 x 250 mL), and brine (1 x 250 mL). The ethyl acetate solution was then suspended over sodium sulfate, filtered, and concentrated to a yellow oil. The oil was filtered over a short silica plug using 5:2 hexanes : ethyl acetate as the eluent. **17** (14.6 g, 0.028 mol, 84% over two steps) was obtained as a white solid: TLC (5:2 hexanes /ethyl acetate; silica) R_f 0.5; ^{1}H NMR (DMSO-d_6) δ 9.12 (s, 1 H), 7.82 (m, 4 H), 7.21 (d, 1 H, J = 0.9 Hz), 6.60 (d, 1 H, J = 1.8 Hz), 4.25 (t, 2 H, J = 7.2 Hz), 4.13 (t, 2 H, J = 8.4 Hz), 3.55 (t, 2 H, J = 6.6 Hz), 1.95 (p, 2 H, J = 7.2 Hz), 1.42 (s, 9 H), 0.95 (t, 2 H, J = 8.1 Hz), 0.02 (s, 9 H); ^{13}C NMR (DMSO-d_6) δ 168.5, 160.7, 153.2, 134.8, 132.4, 123.9, 123.5, 118.8, 108.4, 79.1, 62.0, 46.5, 35.8, 31.1, 29.0, 17.7, -0.6; EI-MS m/e 513.2286 (M^+ 513.2295 calculated for $C_{26}H_{35}N_3O_6Si$); IR (ν_{max} cm^{-1}; $CHCl_3$) 3351, 2954, 1772, 1719, 1587, 1551, 1398, 1367, 1248, 1168, 1088, 838, 720.

Cyanine-Polyamide Ring Conjugate Synthesis (20-24)

Previously described solid phase protocols were employed for polyamide synthesis using Boc-protected pyrrole and imidazole amino acids on PAM resin (0.6 mmol/g).[1] N-(Phthalimidopropyl)pyrrole amino acid **15** (2 equiv) and HBTU (1.1 equiv) were dissolved in anhydrous DMF to final concentrations of ~0.4 M. N,N-diisopropylethylamine (DIEA, 5 equiv) was then added and the mixture was agitated for 20 minutes at 40 °C. The solution was then poured onto neutralized, deprotected resin and the resulting slurry was shaken at room temperature for 4 hours. Activation of monomer **15** was also accomplished with DCC and HOBt.

Polyamide resins **P1** and **P2** (100 mg), prepared by methods indicated above, were cleaved by agitation with neat Dp (1 mL) at 40 °C for 4 hours. (Overnight mixing at ambient temperatures was also effective for cleavage.) The crude cleavage mixtures were filtered and diluted with 0.1% TFA (aq) and acetonitrile to a final volume of 5 mL. These solutions were loaded onto preconditioned SPE cartridges (C_{18} bonded phase) and washed with a 4:1 (v:v) mixture of 0.1% TFA and acetonitrile. Products were eluted with MeOH and concentrated from toluene to yield **18** and **19** as slightly yellow solids. The purity and identity of these intermediates were assessed by analytical HPLC and MALDI-TOF MS and the compounds were used without further purification.

Intermediates **18** and **19** (0.5 μmol) were dissolved in anhydrous DMF (450 μL) and DIEA (50 μL). To these solutions were added pre-packaged amine-reactive cyanine probes (~1 mg). The resulting solutions were agitated in the absence of light, at ambient temperature, for 4 hours. (Commercial probes Cy3, Cy3.5, Cy3B,

Cy5, and Cy5Q were obtained as succinimidyl esters from Amersham and used as received.) Crude products, with the exception of **21**, were purified by preparative HPLC using C_{18} bonded phase with 0.1% TFA and acetonitrile as mobile phases. The Cy3.5 fluorophore contains four sulfate groups that render it negatively charged, making separation under acidic conditions ineffective. This compound was therefore purified on the same stationary phase with 100 mM ammonium acetate and MeOH as the mobile phases.

ImPy(*pr*NHCy3)PyPy-γ-ImPyPyPy-β-Dp (20). UV-Vis (H_20) λ_{max} nm (ε M^{-1} cm^{-1}): 313 (69,500), 555 (75,000). MALDI/TOF MS (monoisotopic) [M+H] 1877.60 (1877.81 calcd for $C_{91}H_{112}N_{24}O_{17}S_2$).

ImPy(*pr*NHCy3.5)PyPy-γ-ImPyPyPy-β-Dp (21). UV-Vis (H_20) λ_{max} nm (ε M^{-1} cm^{-1}): 319 (69,500), 585 (96,000). MALDI/TOF MS (monoisotopic) [M+4H] 2138.73 (2138.76 calcd for $C_{99}H_{117}N_{24}O_{23}S_4$).

ImPy(*pr*NHCy3B)PyPy-γ-ImPyPyPy-β-Dp (22). UV-Vis (H_20) λ_{max} nm (ε M^{-1} cm^{-1}): 310 (69,500), 566 (113,000). MALDI/TOF MS (monoisotopic) [M] 1807.30 (1807.80 calcd for $C_{91}H_{106}N_{24}O_{15}S$).

ImImPy(*pr*NHCy5)Py-γ-PyPyPyPy-β-Dp (23). UV-Vis (H_20) λ_{max} nm (ε M^{-1} cm^{-1}): 318 (69,500), 653 (127,000). MALDI/TOF MS (monoisotopic) [M+H] 1904.21 (1903.83 calcd for $C_{93}H_{114}N_{24}O_{17}S_2$).

ImImPy(*pr*NHCy5Q)Py-γ-PyPyPyPy-β-Dp (24). UV-Vis (H_20) λ_{max} nm (ε M^{-1} cm^{-1}): 314 (69,500), 653 (102,000). MALDI/TOF MS (monoisotopic) [M+H] 2055.90 (2055.81 calcd for $C_{98}H_{114}N_{26}O_{21}S_2$).

Tail Conjugate Synthesis Using β-Ala PAM Resin (1, 27-29)

Polyamide resin **P3** was prepared as described above and resin **P4** was obtained by acylating commercial b-PAM resin with an N-protected ethylene glycol amino acid. Briefly, a diamino-ethylene glycol derivative was monoprotected with Boc anhydride and then reacted with diglycolic anhydride. The polyamide resins (100 mg) were cleaved with 3,3'-diamino-N-methyldipropylamine (Dp_2, 1 mL) at 40 °C for 4 hours. The crude products were purified by SPE, as described above, to yield **25** and **26**. These intermediates (0.5-1 μmol) were then dissolved in a 9:1 (v:v) mixture of anhydrous DMF and DIEA to final concentrations of ~ 1mM, and amine-reactive probes, 6-TMR and Cy3 (~1.5 euiv) were added. (6-TMR obtained as succinimidyl ester from Molecular Probes. The resulting solutions were agitated in the absence of light, at ambient temperature, for 4 hours. Products were purified using standard HPLC conditions; however, better separation was observed when C_8 was employed as the stationary phase, especially in the case of the PEG-linked conjugates.

ImPyPyPy-γ-ImPyPyPy-β-Dp_2-6TMR (1). UV-Vis (MeOH) λ_{max} nm (ε M^{-1} cm^{-1}): 310 (69,500), 556 (91,000). ESI-MS [M+H] 1677.8 (1677.75 calcd for $C_{85}H_{96}N_{24}O_{14}$).

ImPyPyPy-γ-ImPyPyPy-β-Dp_2-Cy3 (27). UV-Vis (H_20) λ_{max} nm (ε M^{-1} cm^{-1}): 310 (69,500), 554 (140,000). ESI-MS [M+H] 1877.4 (1876.80 calcd for $C_{91}H_{111}N_{24}O_{17}S_2$).

ImPyPyPy-γ-ImPyPyPy-β-PEG-β-Dp_2-6TMR (28). UV-Vis (MeOH) λ_{max} nm (ε M^{-1} cm^{-1}): 300 (69,500), 548 (95,000). ESI-MS [M+H] 2067.2 (2066.97 calcd for $C_{101}H_{127}N_{27}O_{21}$).

ImPyPyPy-γ-ImPyPyPy-β-PEG-β-Dp_2-Cy3 (29). UV-Vis (H_20) λ_{max} nm (ε M^{-1} cm^{-1}): 310 (69,500), 554 (106,000). ESI-MS [M+H] 2266.4 (2266.02 calcd for $C_{108}H_{142}N_{27}O_{24}S_2$).

Tail Conjugate Synthesis Using Oxime Resin (31-33)

Polyamide synthesis on Kaiser oxime resin was carried out according to published protocols.[4] The synthesis and properties of 3-chlorothiophene-2-carboxylic acid are described in detail in Chapter 5. Boc-γ-Im-OH (2.5 equiv) dimer was used in solid phase synthesis with HBTU (1 equiv) in anhydrous DMF. Boc-Im deprotection was effected by shaking with 50% TFA (in CH_2Cl_2) for 25 minutes and Boc-Py deprotection was accomplished with 25% TFA (in CH_2Cl_2) for 25 minutes. Polyamide resin **P5** (200 mg) was cleaved by treatment with Dp_2 (2 mL) at 40 °C for 4 hours. The crude product was purified by preparative HPLC and intermediate **30** was identified by MALDI-TOF MS. The appropriate HPLC fractions were lyophilized to dryness and used to prepare conjugates **31**-**33**.

Intermediate **30** (1 μmol) was dissolved in a 9:1 (v:v) solution of DMF and DIEA and mixed with amine-reactive probes 5-FAM, Oregon Green 514, and 5-TMR (2 equiv) in the absence of light, at ambient temperature, for four hours. (These probes were all obtained as succinimidyl esters from Molecular Probes.) The crude products were purified by preparative HPLC under standard conditions.

CtPyPyPy-γ-ImImPyPy-Dp$_2$-5FAM (31). UV-Vis (H_20) λ_{max} nm (ε M^{-1} cm^{-1}): 311 (69,500), 447 (20,000). MALDI-TOF MS (monoisotopic) [M] 1588.6 (1588.53 calcd for $C_{77}H_{77}Cl\,N_{20}O_{15}S$).

CtPyPyPy-γ-ImImPyPy-Dp$_2$-OrGr514 (32). UV-Vis (H_20) λ_{max} nm (ε M^{-1} cm^{-1}): 322 (69,500), 489 (20,100). MALDI-TOF MS (monoisotopic) [M+H] 1226.17 (1725.47 calcd for $C_{78}H_{74}ClF_5N_{20}O_{15}S_2$).

CtPyPyPy-γ-ImImPyPy-Dp$_2$-5TMR (33). UV-Vis (H_20) λ_{max} nm (ε M^{-1} cm^{-1}): 309 (69,500), 565 (66,500). MALDI-TOF MS (monoisotopic) [M+H] 1644.10 (1643.63 calcd for $C_{81}H_{88}ClN_{22}O_{13}S$).

Tail Conjugate Synthesis Using Hydrazine Resin (35)

Synthesis on hydrazine resin was performed according to developed protocols (M. A. Marques, P. B. Dervan, unpublished data). Standard Boc-based protocols were used to generate polyamide resin **P6** (500 mg) which was cleaved with $Cu(OAc)_2$ (50 mg), pyridine (0.1 mL), and Dp_2 (3 mL).

CtImImPy-γ-ImPyPyPy-Dp$_2$-FITC (35). CtPyPyPy-γ-ImImPyPy-Dp$_2$-5TMR (33). UV-Vis (H_20) λ_{max} nm (ε M^{-1} cm^{-1}): 303 (69,500), 453 (20,300). MALDI-TOF MS (monoisotopic) [M+H] 1621.92 (1621.52 calcd for $C_{76}H_{77}ClN_{22}O_{14}S_2$).

TMR-Polyamide Ring Conjugate Synthesis (38-45)

The synthetic route used to prepare these conjugates is described for polyamide resin **P7** leading to compounds **38** and **39**; however, this protocol is general and similar conditions were used to prepare **40**-**43**. Polyamide resin **P7** was

synthesized as described for cyanine conjugates above. The resin was cleaved by treatment with Dp at 40 °C overnight. Crude product was purified by HPLC or SPE and the resulting intermediate **36** was lyophilized to a white solid. Boc-Cys(Trt)-OH (30 μmol) and HBTU (15 μmol) were dissolved in anhydrous DMF (0.9 mL) and DIEA (0.1 mL) and the solution was heated at 40 °C for 20 minutes before being added to a dry aliquot of **36** (2 μmol). The resulting solution was stirred at 40 °C for an additional 6 hours and the crude product was isolated using SPE.

The protected intermediate **37** was then dissolved in a 1:1 (v:v) mixture of TFA and CH_2Cl_2 and triethylsilane (3 equiv) was added dropwise. The solution was stirred at ambient temperature for 25 minutes and concentrated *in vacuo* to yield a yellow residue. The residue was dried under high vacuum overnight and dissolved in DMF to final concentration of ~20 mM. Maleimide-fluorescent probes (Molecular Probes, 1.1 equiv) were then added and the solutions were stirred in the absence of light, at ambient temperature, for 4 hours. Products **38**-**40** were purified by preparative HPLC using standard conditions, though, again better separation was observed using C_8 as the stationary phase.

ImPy(*pr*NHCys-5TMR)PyPy-γ-PyPyPyPy-β-Dp (38). MALDI-TOF MS (monoisotopic) [M + H] 1850.54 (1850.81 calcd for $C_{92}H_{108}N_{25}O_{16}S$).

ImPy(*pr*NHCys-5FI)PyPy-γ-PyPyPyPy-β-Dp (39). MALDI-TOF MS (monoisotopic) [M + H] 1850.54 (1795.71 calcd for $C_{88}H_{96}N_{23}O_{18}S$).

ImImPy(*pr*NHCys5-TMR)Py-γ-ImImPyPy-β-Dp (40). MALDI-TOF MS (monoisotopic) [M + H] 1853.6 (1853.8 calcd for $C_{89}H_{105}N_{28}O_{16}S$).

ImPy(*pr*NHCys-5TMR)PyPy-γ-ImPyPyPy-β-Dp (41). MALDI-TOF MS (monoisotopic) [M + H] 1851.74 (1851.81 calcd for $C_{91}H_{107}N_{26}O_{16}S$).

ImβImPy-γ-PyβPy(*pr*NHCys-5TMR)Py-β-Dp (42). MALDI-TOF MS (monoisotopic) [M + H] 1749.9 (1749.79 calcd for $C_{85}H_{105}N_{24}O_{16}S$).

ImImPy(*pr*NHCys5-TMR)Py-γ-PyPyPyPy-β-Dp (43). MALDI-TOF MS (monoisotopic) [M + H] 1851.8 (1851.81 calcd for $C_{91}H_{107}N_{26}O_{16}S$)

ImβImPy(*pr*NHCys-5TMR)-γ-ImβImPy-β-Dp (44). MALDI-TOF MS (monoisotopic) [M + H] 1751.6 (1751.78 calcd for $C_{83}H_{103}N_{26}O_{16}S$).

ImImPy(*pr*NHCys5-TMR)Py-γ-PyPyImPy-β-Dp (45). (monoisotopic) [M + H] 1852.7 (1852.63 calcd for $C_{90}H_{106}N_{27}O_{16}S$).

For **38**, **40**, **41**, **43**, and **45** UV (H_2O) λ_{max} nm (ε M^{-1} cm^{-1}) 312 (68 800), **42** and **44**, UV (H_2O) λ_{max} nm (ε M^{-1} cm^{-1}) 300 (51 600), **38, 40-45**, Vis (H_2O) λ_{max} nm (ε M^{-1} cm^{-1}) 563 (65 700).

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