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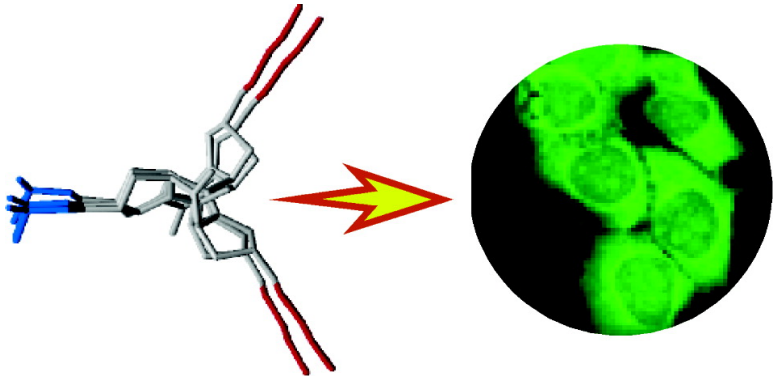

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Cell Penetrating Agents Based on a Polyproline Helix Scaffold

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Abstract: Cell penetrating agents were designed and synthesized that introduce cationic and hydrophobic moieties along the backbone of a polyproline helix (PPII) in an amphiphilic manner. The CD profile has the features that are expected for a PPII helix, demonstrating that the addition of these groups had little effect on the backbone structure. Dramatic increases in uptake were found with MCF-7 cells when up to six guanidinium groups were positioned on the polyproline helix, whereas only modest increases in cellular uptake were observed with the amine-containing polyproline compounds as compared to their flexible counterparts. Amphiphilicity played a key role in the enhanced cell translocation, as scrambled versions of the designed agents, with hydrophobic and cationic groups on all faces of the helix, were only as effective as their flexible peptide counterparts. Interestingly, the most potent agent, P11LRR, demonstrated almost an order of magnitude more efficient cellular uptake as compared to that of the well-studied Tat peptide, with minimal cytotoxicity.

Introduction

Numerous methods have been developed to achieve delivery of therapeutic agents into cells. Cell penetrating peptides (CPPs) that are rich in basic amino acids have become increasingly used in this regard.[1] The most well-studied CPPs are part of transcription factors: the Tat peptide from the cationic domain of HIV-1 Tat[2] and the penetratin peptide from the Antennapedia homeodomain.[3] Polyarginine or polylysine peptides,[4] cationic nuclear localization signal sequences,[5] and cationic moieties linked to scaffolds, such as peptoids,[6] β-amino acid peptides,[7] oligocarbamates,[8] loligomers,[9] and PNA oligomers,[10] have also been studied for cell membrane translocation. CPPs have been shown to deliver varied cargoes into cells from proteins and oligonucleotides to magnetic nanoparticles.[1,11]

Although the cationic nature of CPPs is believed to play a dominant role in cellular uptake, a recent study demonstrated that hydrophobic residues contribute substantially to membrane translocation.[5b] Amphiphilic, cationic peptides have been reported to cross cell membranes; however, this activity is also linked to membrane destabilization.[12] In an effort to systematically investigate the role of hydrophobic residues in cell penetration, we sought a scaffold that would orient both cationic and hydrophobic moieties along a rigid backbone. This is complicated within polyamide structures in that the close positioning of cationic residues does not usually favor a well-folded conformation. Indeed, the α-helical penetratin peptide undergoes a conformational shift upon membrane translocation,[13] and an elegant study with a cationic, β-amino acid peptide indicated a disorganized structure in water.[7] With this in mind, we chose a polyproline helix as the scaffold for our investigations.

In contrast with most of the currently existing CPPs, the polyproline chain adopts a well-defined left-handed type II helix (PPII) with *trans*-proline residues in polar solvents.[14] PPII contains three residues per turn aligning every third ring on the same face of the helix with a pitch of approximately 10 Å per turn. Previously, a proline oligomer was found to be internalized in cells by microscopy, but the extent of cell penetration was not quantitated.[15] Fragments of naturally occurring peptides that

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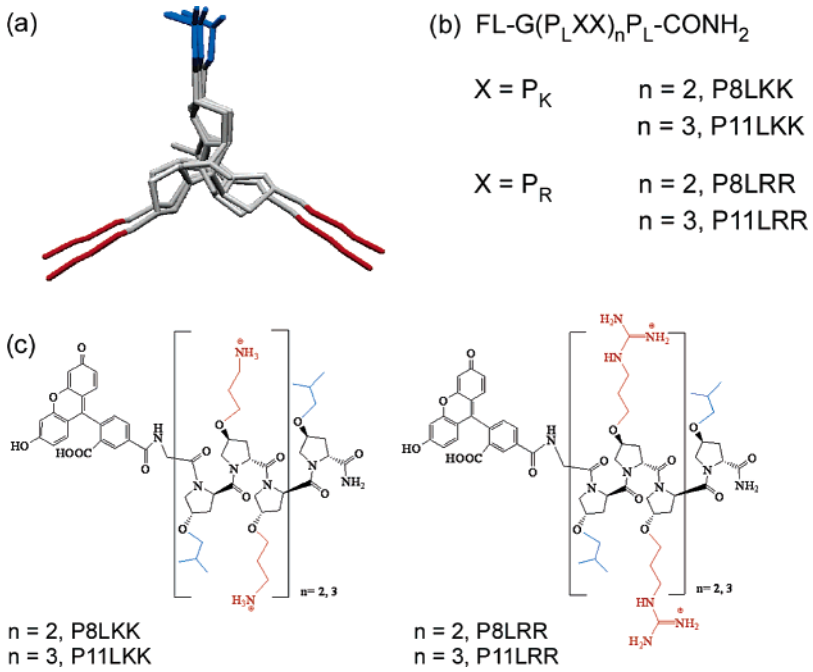

Figure 1. (a) Top view of an amphiphilic polyproline helix containing modified side chains (red, cationic; blue, hydrophobic), (b) sequences, and (c) structures of modified polyproline oligomers containing amino (P_K) or guanidinium (P_R) functionality.

contain a high percentage of proline and arginine residues have also been studied.[16] Tam and co-workers observed cellular uptake with a proline-rich peptide derived from the antimicrobial peptide bactenecin 7.[16a] This peptide was 2−3-fold more effective than the Tat peptide, although a hybrid α-helical and PPII structure was observed. In a complementary study, Giralt and co-workers found that cationic analogues of an amphiphilic, proline-rich peptide, $(VXLPPP)_n$, derived from the γ-Zein protein were able to cross the cellular membrane, although the best was 15-fold less effective than the Tat peptide.[16b] In our design, we sought to maintain a continuous polyproline sequence to ensure a well-folded structure with modifications to the side chain of proline to incorporate the hydrophobic and charged groups.

Results and Discussion

Functionality was incorporated into the PPII helix by O-alkylation of a hydroxyproline monomer to yield a scaffold that displays both hydrophobic (blue) and cationic (red) moieties (Figure 1a). Introduction of an isobutyl group onto hydroxyproline led to a proline-based mimic of leucine (P_L, Figure 1b and c), whereas functionalization with amino or guanidinium groups led to proline-based mimics of lysine (P_K) or arginine (P_R), respectively. Amphiphilic agents were obtained by taking advantage of the 3-fold repeating unit of the PPII helix. In our design, one face of the PPII helix was hydrophobic, while the other two faces contained cationic groups, resulting in agents with a repeating P_LXX (X = P_K or P_R) unit (Figure 1). On the basis of this repeating unit, we developed 7-mer and 10-mer PPII helices—the 7-mer contains three leucine mimics and four basic moieties, whereas the 10-mer contains four leucine mimics and six lysine or arginine mimics. Guanidinium groups within CPPs have been shown to be superior to amino groups as the cationic moiety needed for cellular uptake.[4b] This trend was also evaluated with the PPII helical scaffold with the use of P_K and P_R, in addition to investigating the role of the overall charge on cellular uptake.

Two groups of control molecules were also designed to address the importance of the PPII helical scaffold and amphiphilicity on cell membrane penetration. In this regard, we used flexible tetramers and hexamers of lysine (K4: FL−K_4−NH_2 and K6: FL−K_6−NH_2) and arginine (R4: FL−R_4−NH_2 and R6: FL−R_6−NH_2), and scrambled versions of the designed agents with all compounds containing a fluorescein group (FL). The latter were designed to remove amphiphilicity and contain hydrophobic and cationic groups on all faces of the PPII helix (P8KLr: FL−G$(P_KP_L)_3P_K$−$CONH_2$; P11KLr: FL−G$P_K(P_KP_L)_4$-P_K−$CONH_2$; P11RLr: FL−G$P_R(P_RP_L)_4P_R$−$CONH_2$). In addition, comparisons to the well-studied Tat peptide (FL−G_4-YGRKKRRQRRR−NH_2) were performed.

Studies were initiated with the synthesis of the appropriate hydroxyproline derivatives containing protecting groups appropriate for solid-phase synthesis (Scheme 1, compounds **1**, **2**, and **3**). For the synthesis of the protected leucine mimic (**1**), Cbz−hydroxyproline was deprotonated and treated with 3-bromo-2-methylpropene to provide the desired ether **4** after purification. Hydrogenation of compound **4** simultaneously removed the Cbz protecting group and reduced the double bond, and further treatment with Fmoc−OSu provided the desired compound **1** (Scheme 1a). The synthesis of the protected lysine mimic (**2**) was initiated by deprotonation of Cbz−hydroxyproline, followed by Michael addition with acrylonitrile to provide ether **5**. Protection of **5** as a benzyl ester provided compound **6**, and reduction of the nitrile in the presence of di-*tert*-butyl dicarbonate generated **7**.[17] Compound **7** was hydrogenated, followed

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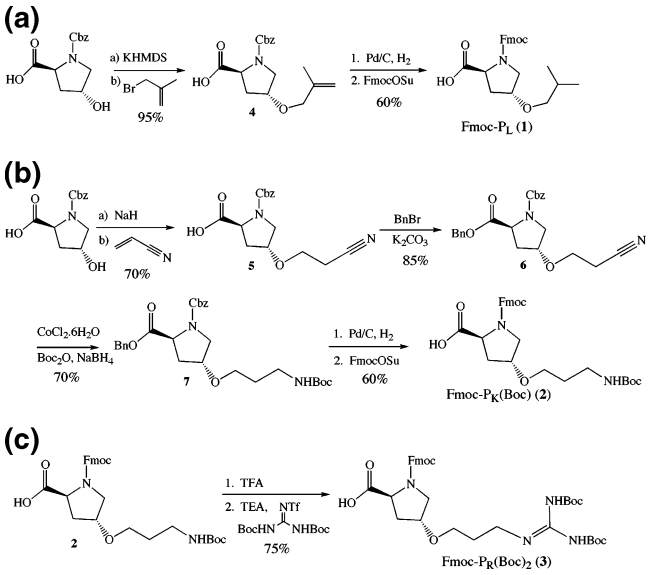
Scheme 1. (a) Synthesis of Fmoc-P_L (**1**), (b) Fmoc-P_K(Boc) (**2**), and (c) Fmoc-$P_R(Boc)_2$ (**3**)



by Fmoc protection, to provide the desired compound **2** (Scheme 1b). Last, the protected arginine mimic (**3**) was prepared from compound **2** by treatment with TFA, followed by reaction with *N*,*N*′-bis(*tert*-butoxycarbony)-*N*′′-triflylguanidine to yield the desired compound **3** (Scheme 1c).[18]

All polyproline derivatives were synthesized on solid support using the Rink resin. An Fmoc-based strategy was employed using HATU as the coupling reagent.[19] Each coupling step was monitored for completion using the chloranil test.[20] The N-terminus of each compound was fluorescently labeled with *N*-hydroxysuccinimide (NHS)−fluorescein while on the solid support. The agents were cleaved from the resin with simultaneous deprotection by treatment with a TFA/triisopropylsilane/water mixture. All compounds were purified to homogeneity by reverse phase HPLC and characterized by mass spectrometry. The series of peptides containing proteinogenic amino acids (K4, K6, R4, R6, and Tat) was synthesized as described above, except that HBTU/HOBT was used as the coupling reagent.

Prior to adding fluorescein to the N-terminus of polyproline derivatives P8LKK and P11LRR, samples of these compounds were cleaved from the resin and purified to homogeneity for analysis by circular dichroism. Polyproline oligomers are known to adopt a type II (PPII) helical conformation in polar solvents with characteristic CD signals that include a strong negative band at 206 nm and a weak positive band at 226 nm.[14b] Analysis of P8LKK and P11LRR lacking fluorescein (P8LKK−FL and P11LRR−FL) by CD provided spectra that corresponded well with the PPII helical structure (Figure 2). These data confirm that side chain extensions to proline do not affect the fold of the backbone, and that the polyproline scaffold maintains its structure even with cationic groups along the same face of the helix.

All cationic agents were evaluated for cellular uptake via flow cytometry using adherent MCF-7 breast cancer cells. For this assay, the cells were incubated with the fluorescently labeled

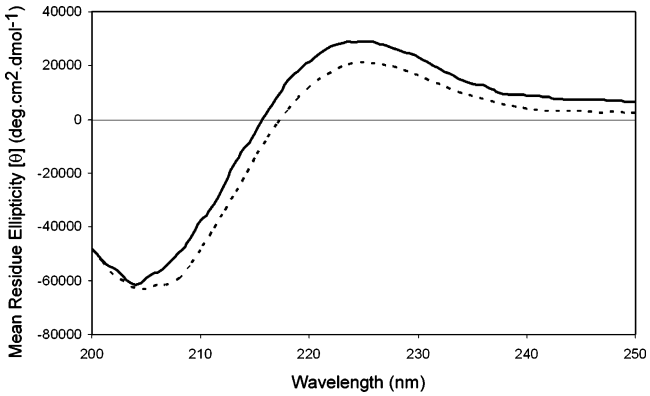


Figure 2. Circular dichroism spectra of P8LKK (solid line) and P11LRR (dashed line) lacking the fluorescein group at 100 μM in PBS buffer (pH 7.4).

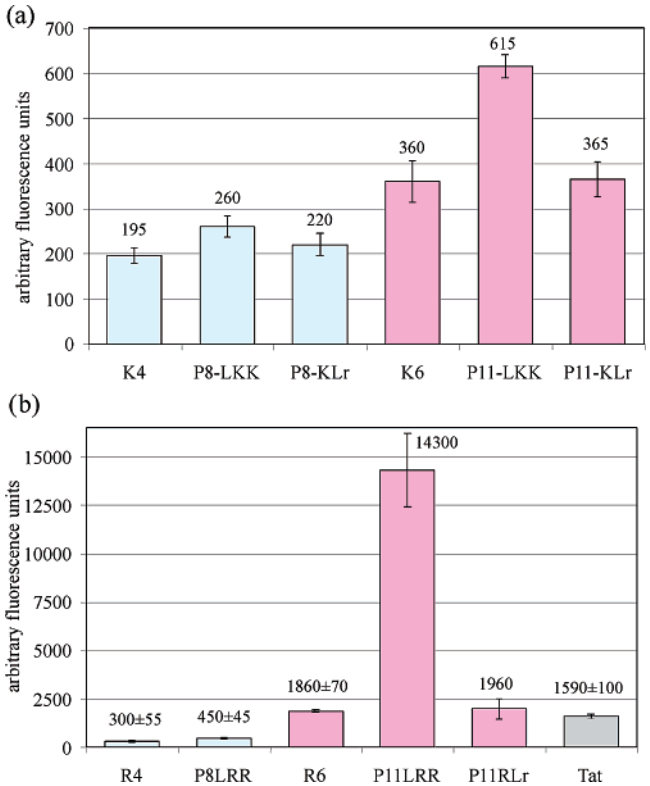


Figure 3. Flow cytometry data for compounds containing (a) amino groups and (b) guanidinium groups and Tat (blue, 4 cationic moieties; pink, 6 cationic moieties).

compounds (50 μM). After a 6 h incubation period, the fluorescent agents were removed from the media, the cells were dislodged, resuspended in media, and analyzed by flow cytometry. Cellular uptake was demonstrated with all compounds analyzed, but a variety of trends could be discerned from the data (Figure 3). More general trends include increased fluorescence of cells treated with the guanidinium-containing agents as compared to their corresponding amine-containing compounds (P8LRR/P8LKK, P11LRR/P11LKK, and P11RLr/P11KLr), and an increased cellular fluorescence with an increasing number of cationic groups (P8LKK/P11LKK and P8LRR/P11LRR), both effects that have been previously observed.[4b]

Trends are also observed that are more specific to the polyproline scaffold. The amine-containing compounds show relatively small variations in uptake (Figure 3a). For instance,

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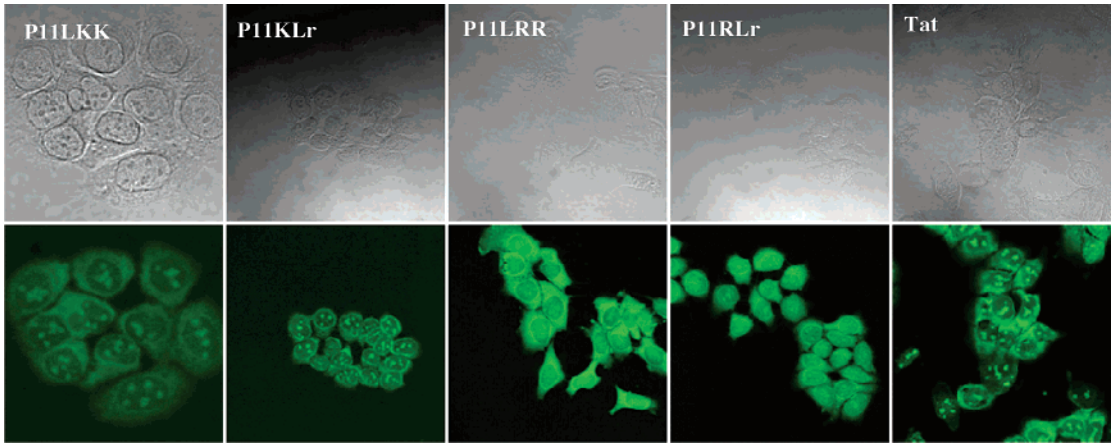

Figure 4. Confocal microscope images of MeOH-fixed MCF-7 cells treated with polyproline derivatives and Tat; (top) transmission images and (bottom) fluorescence images.

the rigid and amphiphilic P8LKK is internalized within MCF-7 cells only slightly (1.3-fold) more effectively than its flexible peptide counterpart K4. However, this small effect is lost when the hydrophobic and cationic moieties are scrambled to remove the amphiphilic character of the polyproline helix (P8KLr). Interestingly, this trend is more significant for the longer amine-containing sequences; cells treated with P11LKK are 1.7-fold more fluorescent than cells treated with the K6 peptide, and removing the amphiphilic character of the polyproline compound (P11KLr) eliminates this enhancement. When the side chain contains a guanidinium moiety, the shorter sequence containing four cationic groups (P8LRR) is only 1.5-fold more effective than the flexible R4 (Figure 3b). A more dramatic difference, however, is noted with the longer sequences; a 7.7-fold increase in cellular uptake is observed with P11LRR as compared to R6, and this effect is lost upon randomizing the amphiphilic character of the agent (P11RLr). Indeed, P11LRR is almost an order of magnitude more effective at cellular penetration than the well-studied Tat peptide, with two less cationic moieties. These data demonstrate the essential roles of rigidity and amphiphilicity for effective cellular uptake, although differences are more easily noted when there are six guanidinium groups within the agents.

To determine the subcellular localization of these compounds, a subset of polyproline compounds and controls was examined by confocal microscopy (Figure 4). MCF-7 cells were treated in a similar fashion as in the flow cytometry experiments, with the exception that the cells were incubated for 4 h with the fluorescein-labeled compounds in LabTek chambered slides. All compounds were found to be localized in both the nucleus and cytoplasm of MeOH-fixed MCF-7 cells. As predicted from flow cytometry, the guanidinium-containing compounds show increased fluorescence as compared to that of the amino series.

To verify that these compounds enter living cells, live confocal images of selected compounds were also obtained (Figure 5). The results of live studies indicate that fluorescence is not due to external binding followed by influx upon fixation, which has been recently reported.[21] Interestingly, nuclear and cytoplasmic staining is evident for P11LRR (Figure 5, left), but a punctate pattern of fluorescence is also observed. The Tat peptide (Figure 5, right) displays a similar uptake profile in live cells as P11LRR, with punctate fluorescence in the cytoplasm, but no penetration of the nucleus was observed.[21]

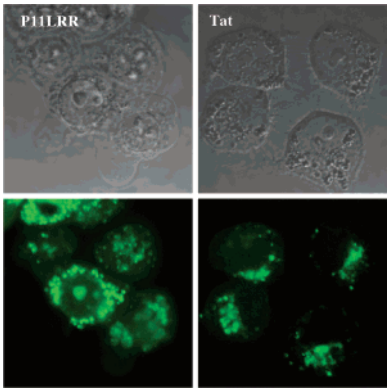

Figure 5. Confocal microscope images of live MCF-7 cells treated with polyproline derivative P11LRR and Tat; (top) transmission images and (bottom) fluorescence images.

To determine if the polyproline compounds were toxic to cells, an MTT assay[22] was performed. Under the conditions used for confocal analysis, all polyproline compounds displayed minimal cytotoxicity to MCF-7 cells (84−99% viability), values that are comparable to those of the polylysine and polyarginine compounds (89−99% viability). These data confirm that the polyproline compounds have the possibility to be safely used to deliver cargo to cells.

Conclusion

We have demonstrated that functionality may be easily incorporated into a polyproline helix by O-alkylation of the side chain of hydroxyproline. A range of modifications can be made, including introduction of hydrophobic and cationic (amino and guanidinium) moieties. These added groups have little effect on the backbone structure since the CD profile has the features that are expected for a PPII helix. Whereas only modest increases in cellular uptake are observed with the amine-containing polyproline compounds as compared to that of their flexible counterparts, more dramatic increases in uptake are found with

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MCF-7 cells when up to six guanidinium groups are positioned on the polyproline helix. Amphiphilicity plays a key role in the enhanced cell translocation since scrambled versions of the designed agents, with hydrophobic and cationic groups on all faces of the helix, are only as effective as their flexible peptide counterparts. Interestingly, the most potent agent, P11LRR, demonstrates almost an order of magnitude more efficient cell uptake as compared to that of the well-studied Tat peptide, with minimal cytotoxicity. These data bode well for the use of the polyproline scaffold in the delivery of therapeutic agents into cells, an avenue under current exploration.

Experimental Section

Amino acids were obtained from Advanced ChemTech, and reagents were purchased from Aldrich. All starting materials were used without purification.

Compound 4. To a solution of Cbz−hydroxyproline (1.0 g, 3.68 mmol) in THF (20 mL) at −40 °C and under N_2 was added KHMDS (15 mL, 0.5 M in toluene, 7.73 mmol), and the mixture was stirred for 30 min. To this mixture was added 3-bromo-2-methylpropene (1.5 mL, 14.7 mmol), and the reaction was allowed to stir for 30 min at −40 °C. The reaction mixture was allowed to warm to room temperature and stirred for an additional 2.5 h. The mixture was cooled to 0 °C, and 10% HCl was added to bring the pH to 1. The resulting solution was extracted with CH_2Cl_2, the organic layer was dried over anhydrous $MgSO_4$, and the solvent was removed in vacuo. The desired product was purified by silica gel column chromatography (95% CH_2Cl_2, 4% MeOH, 1% AcOH) to provide **4** as a colorless oil in 95% yield. MS (EI/CI): 320 *m*/*z* ($M + H^+$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.32 (m, 5H), 5.20 (dd, $J = 8$, 4 Hz, 2H), 4.95 (d, $J = 15$ Hz, 2H), 4.55 (m, 1H), 4.15 (m, 1H), 3.89 (m, 2H), 3.66 (m, 2H), 2.45 (m, 1H), 2.30 (m, H), 1.74 (s, 3H).

Compound 1−Fmoc-P_L. To a solution of **4** (2.0 g, 6.26 mmol) in MeOH (50 mL) was added Pd/C (0.20 g, 10 wt %), and the solution was stirred under 1 atm of hydrogen for 12 h. The solution was filtered through Celite, the solvent was removed in vacuo, and the residue was used in the next step without further purification. A solution of the resulting material (0.88 g, 4.71 mmol) in water/dioxane (18 mL, 1:2) was cooled to 0 °C, and a solution of sodium bicarbonate (1.0 g, 11.8 mmol) in water/dioxane (24 mL, 1:1) was added. To this mixture was added Fmoc−OSu (2.07 g, 6.12 mmol), and the resulting slurry was stirred for 1 h at 0 °C and 3 h at 25 °C. The reaction was treated with 10% HCl to a pH of 1 and extracted with CH_2Cl_2. The organic layer was dried over anhydrous $MgSO_4$, and the solvent was removed in vacuo. The desired product was purified by silica gel column chromatography (97% CH_2Cl_2, 2% MeOH, 1% AcOH) to provide **1** as a white foam in 60% yield. $[\alpha]_D^{22} = -49.2$ (*c* 0.89 $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.90 (t, $J = 6.9$ Hz, 2H), 7.75 (t, $J = 6.9$ Hz, 2H), 7.40 (m, 4H), 4.10 (m, 4H), 3.52 (m, 2H), 3.04 (m, 2H), 2.30 (m, 1H), 2.15 (m, 2H), 1.70 (m, 1H), 0.78 (d, $J = 6.9$ Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 177.7*, 176.4, 155.8, 154.7*, 144.2*, 144.0, 143.9, 141.5, 128.7*, 128.1, 128.0, 127.4, 125.4, 120.4, 120.3, 77.0, 68.8, 68.6*, 59.1, 58.5*, 53.0*, 52.8, 48.1, 37.9, 36.3, 29.8, 20.6 (*indicates minor rotamer). HRMS calcd for $C_{24}H_{27}NO_5$ [$M + H^+$]: 410.1967, found 410.1965.

Compound 5. To a solution of Cbz−hydroxyproline (1.5 g, 5.65 mmol) in THF (20 mL) at 0 °C and under N_2 was added an ice-cooled solution of NaH (0.75 g, 19.8 mmol) in THF (20 mL), and the mixture was stirred at 0 °C for 1.5 h. To this mixture was added acrylonitrile (1.5 mL, 22.6 mmol), and the reaction was allowed to warm to room temperature and stirred for 24 h. The reaction mixture was cooled to 0 °C, and water (100 mL) was added to quench the excess NaH. The THF was removed in vacuo, and 10% HCl was added to bring the solution to a pH of 1. The resulting solution was extracted with EtOAc, the organic layer was dried over anhydrous $MgSO_4$, and the solvent was removed in vacuo. The desired product was purified by silica gel column chromatography (95% CH_2Cl_2, 4% MeOH, 1% AcOH) to provide **5** as a colorless oil in 70% yield. MS (EI/CI): 319 *m*/*z* ($M + H^+$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.34 (m, 5H), 5.15 (m, 2H), 4.54 (m,1H), 3.65 (m, 3H), 2.59 (t, $J = 5.6$ Hz, 2H), 2.40 (m, 1H), 2.15 (m, 2H), 2.39 (m, 2H).

Compound 6. To a solution of **5** (3.82 g, 12.0 mmol) in DMF (50 mL) were added potassium carbonate (4.15 g, 30.0 mmol) and benzyl bromide (1.43 mL, 12.0 mmol). The mixture was stirred for 2 h at 0 °C, allowed to warm to room temperature, and filtered through Celite. To the filtrate was added a saturated aqueous solution of LiCl (100 mL), and the mixture was extracted with diethyl ether. The organic layer was dried over anhydrous $MgSO_4$, and the solvent was removed in vacuo. The desired product was purified by silica gel column chromatography (97% CH_2Cl_2, 3% MeOH) to provide **6** as a colorless oil in 85% yield. MS (EI/CI): 409 *m*/*z* ($M + H^+$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.40 (m, 10H), 5.10 (m, 4H), 4.60 (m, 2H), 3.75 (m, 3H), 3.00 (m, 2H), 2.60 (m, 2H), 2.34 (m, 1H), 2.15 (m, 1H).

Compound 7. Sodium borohydride (3.89 g, 0.103 mol) was added *portion-wise* to a stirred solution of the nitrile **6** (4.20 g, 10.3 mmol), $CoCl_2 \cdot 6H_2O$ (4.90 g, 20.6 mmol), and di-*tert*-butyl dicarbonate (3.55 mL, 0.12 mmol) in methanol (110 mL) at 0 °C. The ice bath was removed from the reaction, and stirring was continued for 4 h. The reaction mixture was filtered through Celite, water (100 mL) was added to the filtrate, and the methanol was removed in vacuo. The resulting solution was extracted with EtOAc, the organic layer was dried over anhydrous $MgSO_4$, and the solvent was removed in vacuo. The desired product was purified by silica gel column chromatography (97% CH_2Cl_2, 3% MeOH) to provide **7** as a colorless oil in 70% yield. MS (EI/CI): 513 *m*/*z* ($M + H^+$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.30 (m, 10H), 5.08 (m, 4H), 4.50 (m, 2H), 4.09 (m, 2H), 3.60 (m, 3H), 3.19 (m, 2H), 2.40 (m, 1H), 2.15 (m, 1H), 1.75 (m,2H), 1.46 (s, 9H).

Compound 2−Fmoc-P_K(Boc). To a solution of **7** (2.77 g, 5.4 mmol) in MeOH (50 mL) was added Pd/C (0.28 g, 10 wt %), and the solution was stirred under 1 atm of hydrogen for 12 h. The solution was filtered through Celite, the solvent was removed in vacuo, and the residue was used in the next step without further purification. A solution of the resulting material (1.33 g, 4.62 mmol) in water/acetone (40 mL, 1:1) was cooled to 0 °C, and sodium bicarbonate (1.36 g, 16.2 mmol) was added. To this mixture was added Fmoc−OSu (2.03 g, 6.0 mmol), and the reaction was stirred for 1 h at 0 °C and 2 h at 25 °C. The reaction was treated with 10% HCl to a pH of 4 and extracted with CH_2Cl_2. The organic layer was dried over anhydrous $MgSO_4$, and the solvent was removed in vacuo. The desired product was purified by silica gel column chromatography (97% CH_2Cl_2, 3% MeOH) to provide **2** as a white foam in 60% yield. $[\alpha]_D^{22} = -40.0$ (*c* 0.58 $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.79 (t, $J = 7.5$ Hz, 2H), 7.62 (t, $J = 7.5$ Hz, 2H), 7.37 (m, 5H), 4.48 (m, 3H), 4.13 (m, 2H), 3.57 (m, 3H), 3.25 (m, 2H), 2.34 (m, 1H), 2.15 (m, 1H), 1.78 (m, 2H), 1.50 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 176.4*, 175.6, 156.2, 155.5, 154.9*, 144.2*, 144.0, 141.5, 128.7*, 128.1, 127.4, 125.4, 120.4, 81.7, 80.1, 68.7*, 68.1, 67.2, 59.0, 58.6*, 52.7, 48.1, 39.4, 37.8, 36.3, 31.1, 29.6 (*indicates minor rotamer). HRMS calcd for $C_{28}H_{34}N_2O_7$ [$M + Na^+$] 533.2264, found 533.2272.

Compound 3−Fmoc-P_R(Boc)$_2$. A solution of **2** (0.56 g, 1.09 mmol) was treated with a solution of TFA in CH_2Cl_2 (10 mL, 1:1), and the mixture was allowed to stir for 2 h at room temperature. The TFA and CH_2Cl_2 were removed in vacuo. The resulting residue was dissolved in dry CH_2Cl_2 (5 mL), and a solution of *N*,*N*′-bis(*tert*-butoxycarbony)-*N*″-triflylguanidine (0.41 g, 1.04 mmol) and triethylamine (0.45 mL, 3.27 mmol) in CH_2Cl_2 (5 mL) was added. The reaction was stirred at room temperature for 5 h, and the mixture was washed with saturated sodium bicarbonate and brine. The organic layer was dried over anhydrous $MgSO_4$, and the solvent was removed in vacuo. The desired product was purified by silica gel column chromatography (95% CH_2Cl_2, 4% MeOH, 1% AcOH) to provide **3** as a white foam in 75% yield.

Table 1. Purification and Mass Spectral Data for Polyproline Compounds

compound	retention time[a] (min)	% purity	mass (exp) ($M + H^+$)
P8LKK	26.9	98	1624
P11LKK	27.2	99	2132
P8KLr	21.3	98	1624
P11KLr	21.5	98	2132
P8LRR	18.7	97	1789
P11LRR	28.5	99	2384
P11RLr	24.8	99	2384

[a] HPLC conditions: 5−70% solvent A using a 40 min gradient on a Vydac C8 analytical column at 1.0 mL/min.

$[\alpha]_D^{22} = -30.6$ (*c* 1.0 $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.79 (t, $J = 7.5$ Hz, 2H), 7.62 (t, $J = 7.5$ Hz, 2H), 7.37 (m, 5H), 4.48 (m, 3H), 4.13 (m, 2H), 3.57 (m, 3H), 3.25 (m, 2H), 2.46−2.20 (m, 2H), 1.78 (m, 2H), 1.50 (s, 18H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 177.2*, 175.9, 163.0, 156.0, 155.8*, 154.75, 153.1, 144.0, 143.8*, 143.7, 141.3, 141.2*, 128.3, 127.8, 127.7*, 127.2, 127.1, 125,1, 120.0, 119.9*, 83.4, 79.8, 68.2, 68.1*, 68.0, 58.2, 57.6*, 51.6*, 51.3, 47.1, 39.8, 36.8, 35.1, 28.8, 28.3, 28.1* (*indicates minor rotamer). HRMS calcd for $C_{34}H_{44}N_2O_7$ [$M + H^+$] 653.3187, found 653.3185.

General Synthesis of Polyproline Compounds. A 10 mL peptide synthesis flask was loaded with 100 mg (0.06 mmol) of Rink amide resin. The resin was washed with CH_2Cl_2 (2 × 4 mL) and DMF (2 × 4 mL). Piperidine (20% in DMF, 4 mL) was added to the reaction flask, the flask was agitated for 30 min, and the piperidine solution was drained. The resin was washed with DMF, CH_2Cl_2, MeOH, CH_2Cl_2, and DMF (2 × 4 mL each). Fmoc-protected amino acids (2.5 equiv, 0.15 mmol) in DMF (4 mL) were added to the reaction flask with HATU (2.5 equiv, 0.15 mmol) and DIEA (2.5 equiv, 0.15 mmol), and the flask was agitated for 4 h. The resin was washed with DMF, CH_2Cl_2, MeOH, CH_2Cl_2, and DMF (2 × 3 mL each). Piperidine (20% in DMF, 4 mL) was added to the reaction flask, the flask was agitated for 30 min, and the piperidine solution was drained. The resin was washed with DMF, CH_2Cl_2, and MeOH (2 × 4 mL each). This procedure was repeated until all amino acids were coupled to the resin.

Coupling of NHS−Fluorescein to Polyproline Compounds. The resin was washed with DMF (2 × 4 mL) and NMP (1 × 4 mL). NHS−fluorescein (1.1 equiv, 0.06 mmol) and DIEA (1.1 equiv, 0.06 mmol) were added, along with 4 mL of NMP. The reaction flask was protected from light with aluminum foil and agitated for 24 h. The resin was washed with NMP, DMF, and MeOH (2 × 4 mL each) and dried in vacuo for 3 h.

Cleavage of Polyproline Compounds from Resin and Purification. A trifluoroacetic acid (TFA) cocktail solution (95% TFA, 2.5% triisopropylsilane, 2.5% water, 4 mL) was added to the resin, and the mixture was agitated for 2 h. The solution was filtered through glass wool into a 50 mL centrifuged tube. The resin was washed with CH_2Cl_2 (3 × 4 mL), and the filtrate was collected into the same tube. The resulting solution was concentrated in vacuo to remove the TFA. The residue was dissolved in cold diethyl ether and placed in the freezer to precipitate the desired compound. The precipitate was collected by centrifugation and washed with cold diethyl ether. The collected precipitate was purified to homogeneity by reverse phase HPLC using a linear 60 min gradient of a solvent system consisting of solvent A (acetonitrile/0.1% TFA) and solvent B (water/0.1% TFA) with a flow rate of 8 mL/min ($\lambda_{214\ nm}$ and $\lambda_{254\ nm}$). All peptides were characterized by MALDI-TOF mass spectrometry, and stock solutions were quantitated by amino acid analysis.

Circular Dichroism Analysis. CD spectra were recorded on a Jasco circular dichroism spectropolarimeter (Model J810) at 25 °C using a 0.1 cm path length quartz cell. The spectra were averaged over three scans taken from 250 to 196 nm with a resolution of 0.1 nm at a scan rate of 50 nm/min. The CD data obtained were processed to convert the data from degrees of rotation to mean residue ellipticity by dividing by the appropriate path length, peptide concentration, and number of residues in the peptide. CD spectra of P8LKK and P11LRR (100 μM) lacking the fluorescent label were taken in PBS buffer, pH 7.4 (140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4).

Cell Culture Experiments. Screening of cellular uptake was carried out on cancerous MCF-7 (breast) cells grown continuously as adherent monolayers in RPMI (Rosewell Park Memorial Institute) culture media supplemented with 10% HIFCS and PS (100 units/mL penicillin and 0.1 mg/mL streptomycin). The cells were grown in a humidified 5% CO_2 atmosphere at 37 °C. The cells were passaged biweekly and discarded after six passages to ensure consistency in uptake.

Flow Cytometry Analysis. Cellular uptake of the fluorescently labeled agents was verified using flow cytometry. The cells were seeded into 24-well plates at a density of 100 000 cells/well in 1 mL of media and grown in a humidified 5% CO_2 atmosphere at 37 °C for 48 h. The cells were then incubated for 6 h in the presence of 84 μL of fluorescently labeled peptides (in sterile water) at a concentration of 50 μM in culture media (170 μL). Following incubation, the cells were washed three times with 100 μL of PD (Ca^{2+}- and Mg^{2+}-deficient phosphate-buffered saline). The cells were gently dislodged from the wells with 100 μL of trypsin (0.25%) and resuspended in 300 μL of the culture media. For each experiment, a control of cells that were not incubated with fluorescent compound was also analyzed. The samples were run in triplicate, and each experiment was repeated twice within the same week. Mean fluorescence values were measured on a BD FACS Caliber Flow Cytometer using an air-cooled laser for excitation of fluorescein at 488 nm.

Confocal Laser Scanning Microscopy. The cells were seeded into LabTek chambered slides at a density of 50 000 cells/well in 1 mL of media and grown for 48 h as described above. The cells were incubated for 4 h with the fluorescent agents using conditions and concentrations described above. The cells were then washed three times with 100 μL of PD (Ca^{2+}- and Mg^{2+}-deficient phosphate-buffered saline). The cells were analyzed live in PBS buffer (250 μL) or by fixation with 100 μL of cold methanol. Fluorescent images were recorded on a Biorad Confocal Laser Scanning Microscope at slow speed using a 60X oil objective and a 488 nm laser line for fluorescein excitation. Images were overlayed using the Thumbs Plus software.

MTT Viability Assay. Cellular toxicity of the fluorescent agents was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. The cells were seeded into 24-well plates at a density of 100 000 cells/well in 1 mL of media and grown in a humidified 5% CO_2 atmosphere at 37 °C for 48 h. The cells were then incubated for 4 h in the presence of 84 μL of fluorescently labeled peptides (in sterile water) at a concentration of 50 μM in culture media (170 μL). Following this incubation, the cells were washed three times with 100 μL of PD (Ca^{2+}- and Mg^{2+}-deficient phosphate-buffered saline) and incubated for 1 h with 0.5 mg/mL of MTT in media. After 1 h, the MTT/media solution was removed, and the precipitated crystals were dissolved in 2-propanol (500 μL). The solution absorbance was read at 580 nm. For each experiment, a control of cells that were not incubated with fluorescent compound was also analyzed. The samples were run in triplicate, and each experiment was repeated twice within the same week. The average absorbance for each sample was calculated and percent viability determined using the following equation: % cell viability = A_{580} treated cells/A_{580} untreated cells × 100.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra for compounds **1**, **2**, and **3**, peptide purity, and cell viability data. This material is available free of charge via the Internet at http://pubs.acs.org.

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