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Synthesis of Alaninyl and *N*-(2-Aminoethyl)glycinyl Amino Acid Derivatives Containing the Green Fluorescent Protein Chromophore in Their Side Chains for Incorporation into Peptides and Peptide Nucleic Acids

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Artificial amino acids carrying either the chromophore of the Green Fluorescent Protein (GFP) or a modification as their side chains have been synthesized: Boc-protected alaninyl derivatives and Fmoc-protected *N*-(2-aminoethyl)glycine-functionalized amino acids were obtained and could be applied in solid-phase peptide synthesis. The incorporation of the GFP chromophore into *N*-(2-aminoethyl)glycine-PNA was achieved and fluorescence was studied as a function of hybridization with complementary DNA.

Introduction

Since 1992, when the Green Fluorescent Protein (GFP) was cloned for the first time, it has become widely used as a highly efficient tool in cell biology,[1] thanks to its interesting photophysics, which allows monitoring of protein movements and interactions inside the living cell by various techniques such as Fluorescence Recovery After Photobleaching (FRAP), Fluorescence Loss In Photobleaching (FLIP), Fluorescence Localization After Photobleaching (FLAP), and Fluorescence Resonance Energy Transfer (FRET).[2] The photophysical properties of GFP and its homologues are the products of interactions between the remarkable small chromophore **1** and its immediate protein environment. Fluorescence of the isolated chromophore in solution is quenched by radiationless internal conversion, but appears upon freezing.[3] Fast internal conversion is also responsible for photobleaching of the GFP fluorescence. A rotation mechanism has been suggested (Figure 1): either *cis*-*trans* isomerization, a rotation of the phenyl group, or a combination of both phenomena results in a decay of the chromophore's excited state, so fluorescence can only be observed if the fast internal conversion is suppressed by restriction of the chromophore flexibility, as is provided by the rigid protein environment.[4] The role of the protonation state of the chromophore **1** in internal conversion is still a matter of debate, as is the role of stacking interactions vs. the role of the hydrogen bond network for the restriction of the chromophore flexibility.[5]

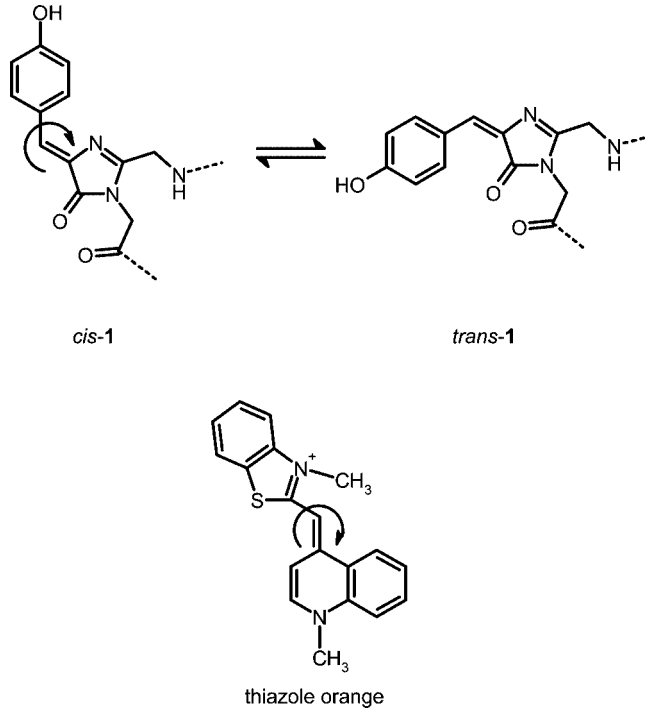

Figure 1. The proposed fluorescence quenching mechanism of the GFP chromophore **1** and comparison with thiazole orange.

Thiazole orange is a chromophore with a similar mechanism for fluorescence quenching through *cis*-*trans* isomerization; it shows a strong increase in fluorescence emission upon intercalation in DNA, a property that has been used to test for single-point mutations in DNA.[6] This raises the question of the minimal environment required for the small GFP chromophore (GFPC) to show fluorescence and to display the interesting photophysical properties we know

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from the GFP. To answer this question, here we present the synthesis of two GFP chromophores and their functionalization as alaninyl and *N*-(2-aminoethyl)glycinyl amino acids for incorporation into regular peptides and into *N*-(2-aminoethyl)glycine-PNA[7] by solid-phase peptide synthesis (Figure 2). From the incorporation of the GFP chromophore into peptides and nucleic acid analogues we might expect to learn about the interactions between the GFP chromophore and the protein environment, and one future prospect might be the application of the fluorescence properties of the GFP chromophore **1** to test for DNA binding.

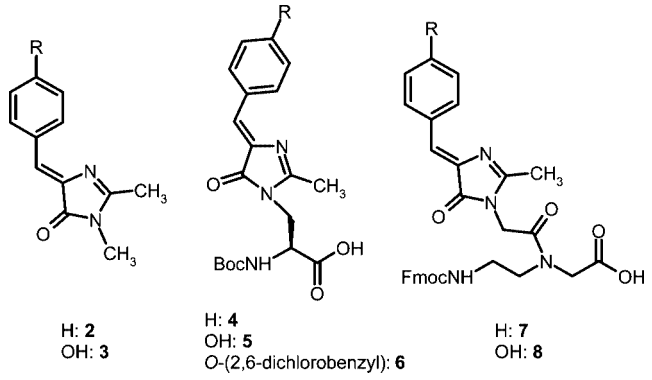

Figure 2. GFP chromophores **2** and **3** and their incorporation into alanyl derivatives **4**–**6** and *N*-(2-aminoethyl)glycinyl amino acids **7** and **8**.

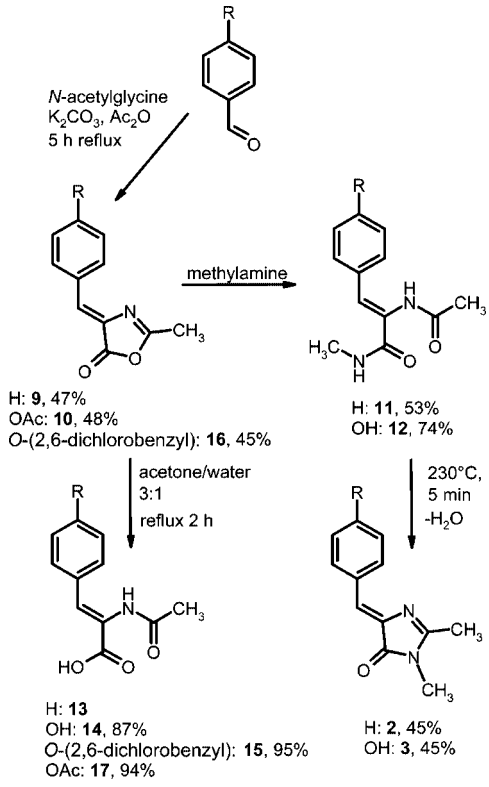

Figure 3. Synthesis of the 3-methyl-GFP chromophores **2** and **3**.

Results and Discussion

Synthesis

The synthesis of the 3-methyl-GFP chromophores **2** and **3** was accomplished through preparation of the corresponding azlactones **9** and **10** by literature procedures (Figure 3).[8] The azlactones were then transformed into the corresponding amides **11** and **12** by treatment with methylamine, followed by thermal cyclization at 230 °C under vacuum.[9] On application of the literature procedures for the synthesis of the GFP chromophores **2** and **3** in one step starting from the azlactones **9** and **10**, the major products turned out to be the acids **13** and **14**.[10] The moderate yields of the thermal conversion of the amides into the GFP chromophores were the result of the low thermal stabilities of the GFP chromophores, which did not allow complete conversion.

The synthesis of the alaninyl GFP chromophores **4**–**6** (Figure 4) started from the corresponding acids **13**–**15** (Figure 3), which were in turn synthesized from azlactones **9**, **10**, and **16** by mild hydrolysis in acetone/water. The hydrolysis of the acetyl group of **10** required further treatment with potassium hydroxide after hydrolysis of the azlactone moiety in acetone/water. The acids **13**–**15** were then coupled with enantiomerically pure *N*-α-Boc-L-diaminopropionic acid[11] **18** by use of the DCC/HOBt activation technique[12] in 73–90% yields (Figure 4), and the obtained amides **19**–**21** were converted into the GFPC-functionalized alanine monomers **4**–**6** by thermal cyclization in DMF in the presence of catalytic amounts of DBU. The low temperature and the presence of catalytic amounts of base reduced the thermal degradation, giving 74–86% yields and complete conversion. As a minor product, **22** (10–20% yield, characterized by NMR, EI-MS) was probably formed from the products **4**–**6** by intramolecular nucleophilic attack of an adjacent carbonyl group with the 4-imidazolone anion as the leaving group. The enantiomeric excess was determined to be > 95% by HPLC after derivatization with enantiomerically pure alanine. In addition, the phenol functionality of the (OH)GFPC building block **5** was protected with dichlorobenzyl bromide to yield **6** (63%) once more.[13]

The synthesis of the GFPC-functionalized *N*-(2-aminoethyl)glycine (aeg) monomers **7** and **8** (Figure 5) required the three-step syntheses of GFPC acetic acids **23** and **24**, starting from acids **13** and **17** by coupling with glycine methyl ester by the DCC/HOBt activation procedure, thermal cyclization to afford the GFPC acetic acid methyl esters **27** and **28**, and basic saponification in the presence of LiOH to give the corresponding GFPC acetic acids **23** and **24** in 25% and 10% overall yields.

The GFPC-functionalized Fmoc-aeg-monomers **7** and **8** were synthesized by a recently developed strategy for the synthesis of highly acid-labile building blocks for study of charge transfer in nucleic acids.[14] In analogy with this procedure, the GFPC acetic acids **23** and **24** were coupled with Fmoc-aeg-OMe (**29**) by the HBTU/HOBt activation method and were saponified with retention of the Fmoc group in the presence of lithium iodide[15] to provide **7** and

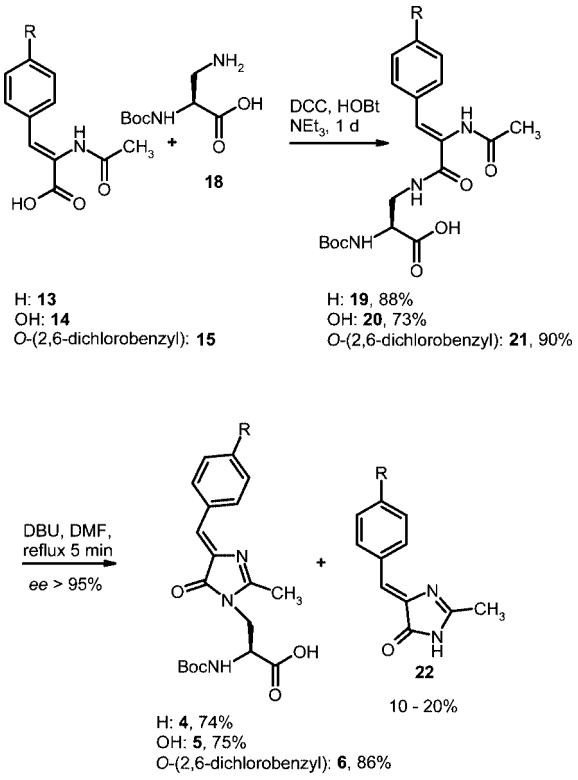

Figure 4. Synthesis of Boc-L-alaninyl GFP chromophores **4**–**6**.

8 in 51% and 12% overall yields. The remarkably low yield for the coupling of (OH)GFPC acetic acid **24** with Fmoc-aeg-OMe (**29**) might be because of the requirement for prior phenol protection.

The (H)GFPC-functionalized Fmoc-aeg monomer **7** was introduced into a PNA oligomer through solid-phase synthesis by use of the Fmoc/Bhoc strategy, with a recently developed method that allows for the synthesis of *N*-(2-aminoethyl)glycine-PNA on acid-labile Sieberamide resin for this purpose.[14] The PNA oligomer Ac-t-t-t-t-(H)GFP-g-g-c-g-g-c-Lys-Lys-Gly-NH_2 (**32**) was synthesized on a 5 μM scale and was obtained in 9% yield after HPLC purification.

For solid-phase peptide synthesis by the Boc strategy one has to allow for the fact that the GFP chromophore is not stable to treatment with TFMSA, so a suitable linker and side chain protecting group strategy was required. Alternatively, it should also be possible to transform the Boc-protected alaninyl monomers **4**–**6** into the corresponding Fmoc-protected monomers in order to use them with conventional side chain protection methods. (The direct synthesis of the Fmoc-protected monomers starting from N_α-Fmoc-diaminopropionic acid is not possible because of the low thermal stability of the Fmoc group.)

Fluorescence Properties

The synthesized GFP chromophores showed typical UV spectra and fluorescence behavior, with almost no fluorescence being observed in methanol, water, or ethyl acetate. Interestingly, though, fluorescence was observed in highly viscose solvents such as glycerin (Figure 6), in which the typical blue fluorescence of (H)GFPC (GFP mutant Y66F, λ_{exc} = 360 nm, λ_{em} = 442 nm) was observed for **30**, with λ_{exc} = 350 nm, λ_{em} = 436 nm. On freezing of a solution of **30**

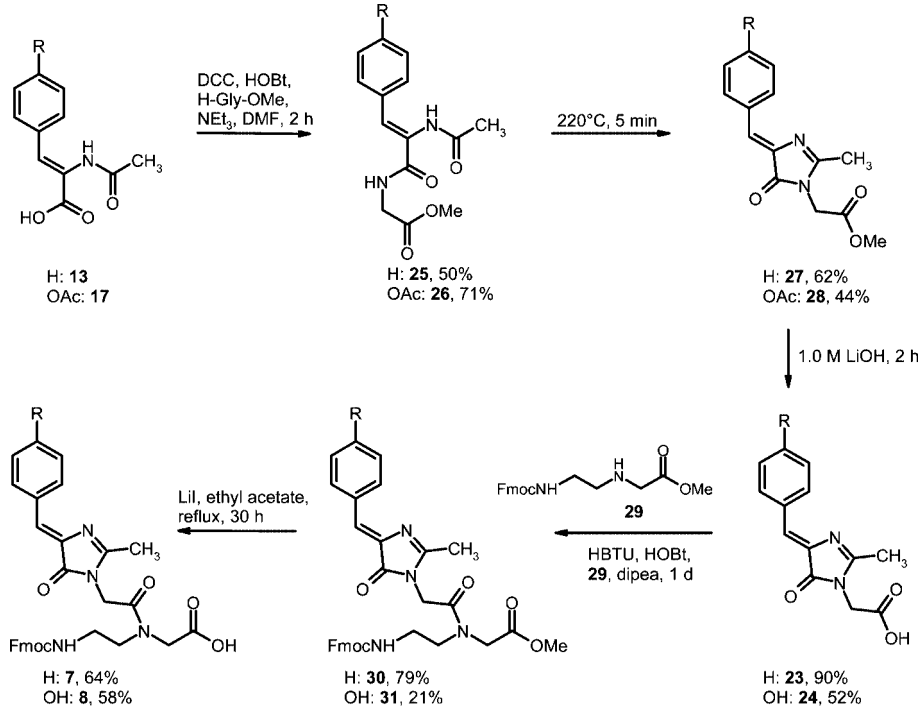

Figure 5. Synthesis of *N*-(2-aminoethyl)glycine-functionalized GFP chromophores **7** and **8**.

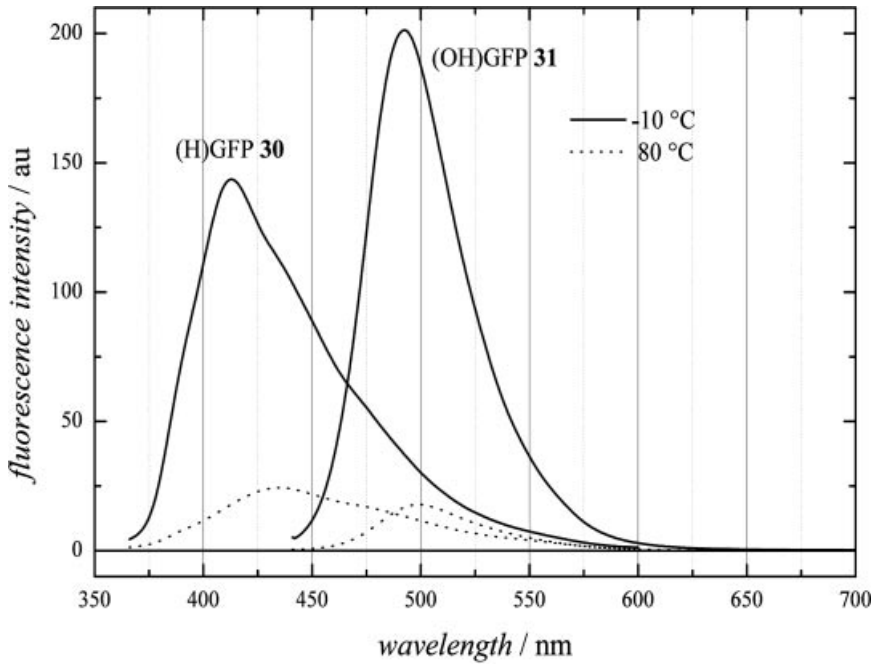

Figure 6. Temperature dependences of the fluorescence emissions of Fmoc-aeg-H(GFPC)-OMe (**30**) [λ_{exc} = 350 nm, A_{350nm} = 0.9, λ_{em} = 436 nm, Φ_{rel} = 1 (80 °C) and λ_{em} = 413 nm, Φ_{rel} = 5 (–10 °C)] and of Fmoc-aeg-(OH)GFPC-OMe (**31**) [λ_{exc} = 430 nm, A_{430nm} = 1.6, λ_{em} = 499 nm, Φ_{rel} = 1 (80 °C) and λ_{em} = 492 nm, Φ_{rel} = 11 (–10 °C)] in glycerin (+ 0.1% NEt_3 for **31**) at 80 °C and in glycerin glass at –10 °C. A = absorption; Φ = fluorescence quantum yield.

to –10 °C the fluorescence intensity increased by a factor of five with a strong blue shift of the fluorescence maximum of 23 nm (Figure 6).

Analogous behavior was found for the (OH)GFP chromophore **31**. In glycerin with 0.1% NEt_3, typical green fluorescence with λ_{exc} = 430 nm, λ_{em} = 499 nm was observed (wild-type GFP, λ_{exc} = 395/475 nm, λ_{em} = 509 nm), while on freezing to –10 °C the fluorescence intensity strongly increased by a factor of 11, with a slight blue shift of the fluorescence maximum of 7 nm.

The PNA oligomer **32**, containing the (H)GFP chromophore, formed an antiparallel double strand with the complementary DNA **33** (5′-G-C_2-G-C_2-A_5-3′). Adenine was chosen as the counterbase for the (H)GFP chromophore. Temperature-dependent UV spectroscopy yielded a melting temperature of 71 °C for the duplex **32**+**33**, with a small

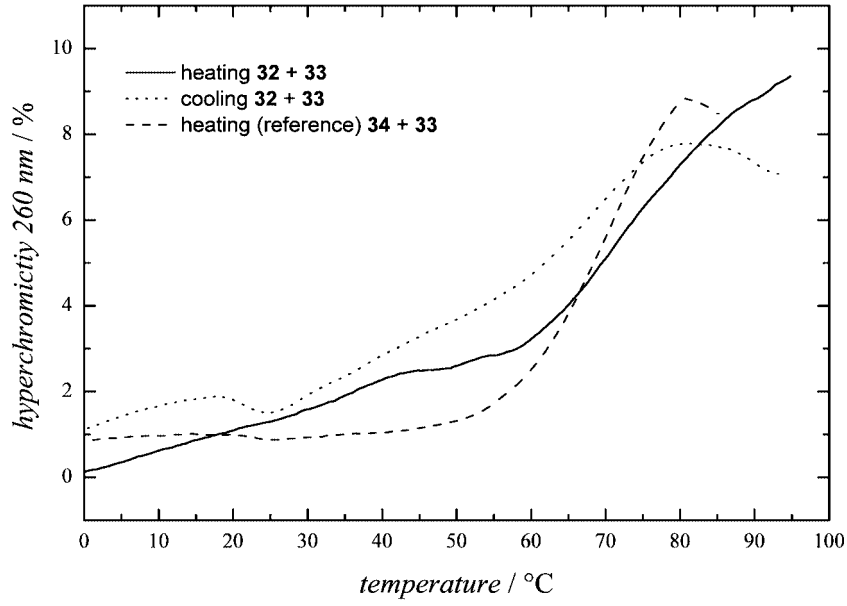

Figure 7. UV melting curve of oligomer **32** after hybridization with complementary DNA **33** (5′-G-C_2-G-C_2-A_5-3′), each 3 μm in 10 mm NaH_2PO_4 buffer, 0.1 M NaCl, pH 6.85. The binding of Uronium-t-t-t-t-t-g-g-c-g-g-c-Lys-Lys-Gly-NH_2 (**34**) with DNA **33** is also shown, as a reference.[17]

heating–cooling hysteresis of 3 °C (Figure 7). In comparison, the UV experiment with the reference oligomer **34** + **33**, with replacement of the (H)GFP chromophore by thymine – therefore giving the canonical A-T base pair – resulted in a melting temperature of 70 °C. Overall, this indicates a well stacked (H)GFP chromophore.[16]

CD spectroscopy with the DNA/PNA pairing complex **32** + **33** showed typical temperature-dependent bands at the optical transitions of the nucleobases (Figure 8, top) indicating the formation of a B-DNA-like, right-handed pairing complex. At the optical transitions of the (H)GFP chromophore (Figure 8, bottom), centered around 293 nm (+3 mdeg) and 347 nm (–3 mdeg), significant CD bands were found, their temperature dependency consistent with the UV melting behavior (Figure 9). The appearance of CD bands at the optical transitions of the (H)GFP chromophore once more supported the assumption of a well stacked chromophore buried inside the DNA/PNA double strand.

The fluorescence spectrum of oligomer **32** upon excitation at 350 nm showed broad emission maxima at 413 nm, 440 nm, and 510 nm (Figure 10). This fluorescence was sensitive to the addition of complementary DNA **33**, which produced an increase in fluorescence intensity by a factor of two and the upraising of a sharp emission band at 417 nm with shoulders at 440 and 506 nm. In comparison, the fluorescence of the free (H)GFP chromophore (**27** as reference) showed a weak emission at 398 nm with 1/12 of the intensity of the double strand.

The emission bands at 413 nm and 440 nm appeared close to the emission of the Y66F mutant of the GFP containing the (H)GFP chromophore (442 nm) and are consistent with the fluorescence behavior of the chromophore monomer **30** observed in glycerin at 80 °C (440 nm) and –10 °C (413 nm). In comparison to the free chromophore **27** in methanol, with an emission maximum at 398 nm, the typical blue fluorescence requires the incorporation of the chromophore into the base stack. Interestingly, a more red-

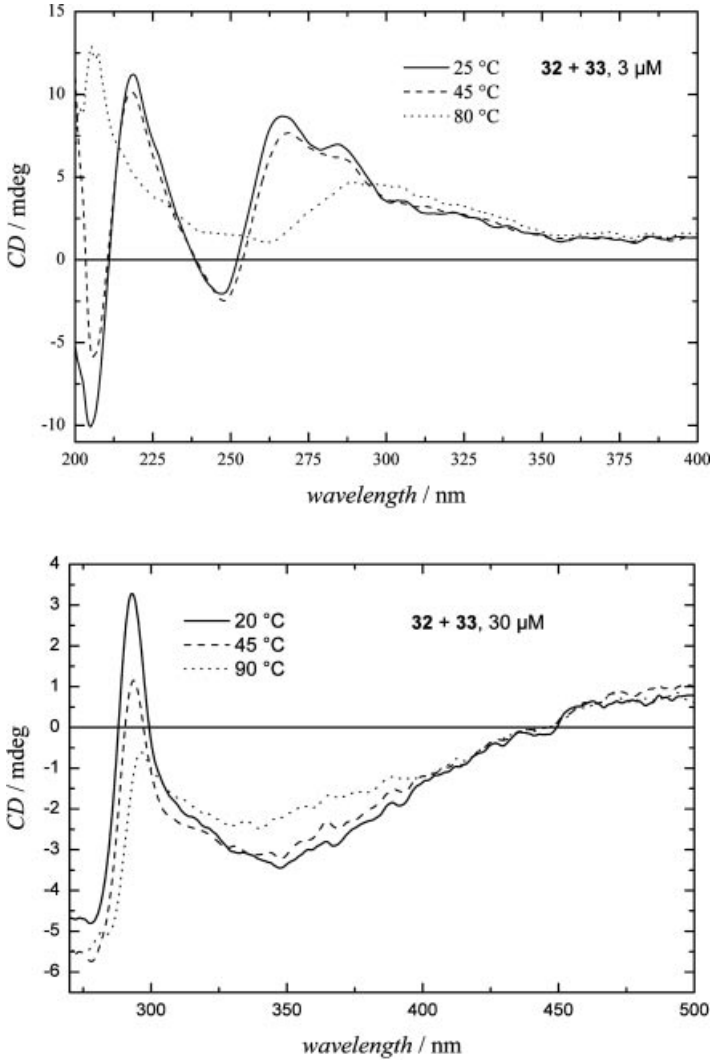

Figure 8. *Top:* CD spectra of oligomer **32** after hybridization with complementary DNA **33**, each 3 µm in 10 mm NaH_2PO_4 buffer, 0.1 M NaCl, pH 6.85. *Bottom:* Same pairing complex, but oligomer and DNA each 30 µm in the same buffer. The concentration needed to be increased for monitoring of the CD effect at the optical transitions of the (H)GFP chromophore since its absorption coefficient ($\varepsilon_{350nm} \approx 9 \cdot 10^3$ cm^2·mol^{-1}) is much smaller than that of the double strand ($\varepsilon_{260nm} \approx 206 \cdot 10^3$ cm^2·mol^{-1}).

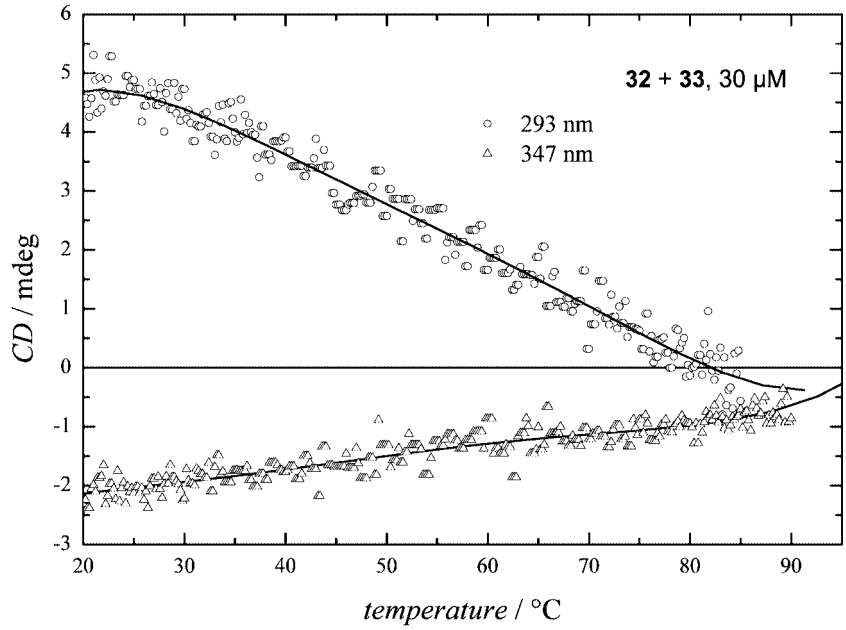

Figure 9. Temperature dependency of the CD effect at the optical transitions of the (H)GFP chromophore at 293 nm and 347 nm. Oligomer **32** + DNA **33** each 30 μm in 10 mm NaH_2PO_4 buffer, 0.1 M NaCl, pH 6.85.

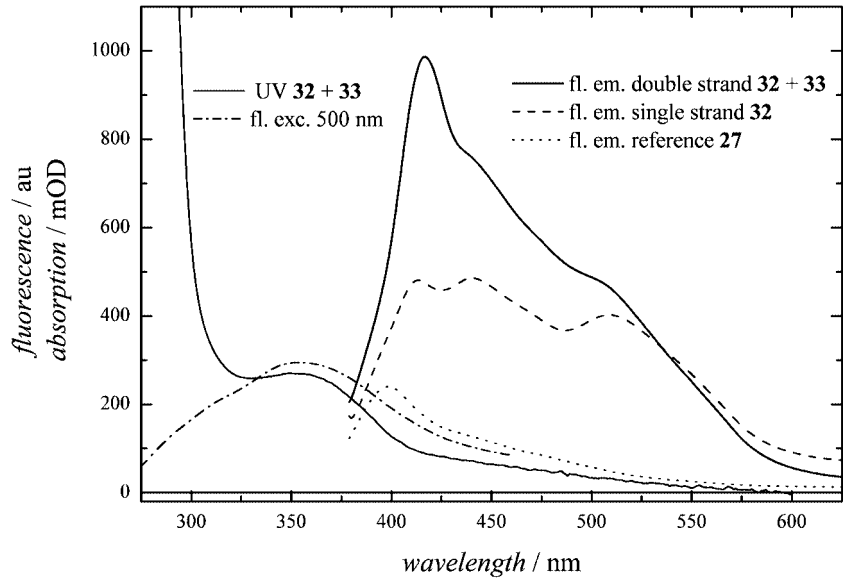

Figure 10. Fluorescence and UV spectra of oligomer **32** depending on hybridization; concentrations of oligomer **32** and DNA **33** each 30 μm in 10 mm NaH_2PO_4 buffer, 0.1 M NaCl, pH 6.85. *UV absorption*: λ_{max} = 356 nm; *fluorescence emission*: λ_{exc} = 350 nm, A_{350nm} = 0.27; single strand λ_{em} = 413, 440, 510 nm, Φ_{rel} = 6; double strand Φ_{rel} = 12; reference (H)GFPC-methyl acetate **27**, λ_{exc} = 350 nm, A_{350nm} = 0.27 in methanol, λ_{em} = 398 nm, Φ_{rel} = 1; *fluorescence excitation*: **32**+**33**, λ_{em} = 500 nm (fix), λ_{max} = 356 nm (scanned). A = absorption, Φ = fluorescence quantum yield.

shifted band, at 506–510 nm, was found both for duplex **32**+**33** and for single-strand **32**, and might result from π-stacking inside the base stack as indicated by UV and CD spectroscopy. On switching from single-strand **32** to duplex **32**+**33** the fluorescence emission spectra changed not only in fluorescence intensity but also in the emission band shape, which might be the result of the transition from a less ordered single strand with several conformations to the well defined double helix structure. The (H)GFP chromophore was clearly identified as the source of the red-shifted fluorescence by collection of a fluorescence excitation spectrum at 500 nm, revealing a total overlap with the UV absorption spectra with λ_{max} = 356 nm. Like the fluorescence emission, the UV ground state absorption was slightly red-shifted (8 nm) in relation to that of the free chromophore **27** with λ_{exc} = 348 nm. It is known from mutagenesis experiments that stacking of the chromophore with an aromatic amino acid as in T203F/H/Y mutations yields GFP variants

with red-shifted (15–20 nm) fluorescence emission maxima. X-ray structure analysis revealed coplanar stacking of the aromatic amino acid in position 203 with the GFP chromophore with a distance of 3.3–3.8 Å, comparable with the raise of 3.4 Å in the DNA base stack.[18] Nevertheless, the overall low quantum yield ($\Phi < 0.5\%$)[19] indicates that blocking of the radiationless deactivation channels additionally requires the incorporation of the chromophore into the hydrogen bond network of the protein. It remains an open question whether the hydrogen bond network inside the GFP protein barrel blocks the radiationless deactivation through a protonation mechanism or simply by more efficient restriction of the chromophore flexibility.[5,20]

Conclusions

The synthesis and characterization of two GFP chromophores has been performed. In order to incorporate them into alaninyl or *N*-(2-aminoethyl)glycinyl peptide nucleic acids the chromophores were prepared as the side chains of the corresponding amino acids. Incorporation into an *N*-(2-aminoethyl)glycine-PNA oligomer was achieved by carefully optimized solid-phase peptide synthesis. The double strand stability of an *N*-(2-aminoethyl)glycine-PNA functionalized with the GFP chromophore with a complementary DNA was investigated by UV and CD spectroscopy, while – since fluorescence of the GFP strongly depends on nearest-neighbor interactions and shielding from solvent – fluorescence within this model system was also investigated, with the appearance of blue fluorescence with sensitivity to the presence of the DNA counter-strand being observed. Interestingly, an additional green fluorescence was found and assumed to be caused by π-stacking interactions. Unfortunately, the overall quantum yield was very low, indicating insufficient blocking of the radiationless internal conversion by simple π-stacking, which might limit utility in molecular biology diagnostics.

Experimental Section

General Methods: Infrared spectra were recorded on a Perkin–Elmer FT-IR 1600 instrument. NMR spectra were recorded on a Varian Unity 300 or a Varian Inova 600 spectrometer. UV absorption spectra were recorded with a Jasco V-550 spectrometer; all extinction coefficients are given in $cm^2 \cdot mol^{-1}$. Fluorescence emission and excitation spectra were collected with a Jasco FP 6200 instrument. Fluorescence emission spectra were not corrected for the wavelength dependency of the detector sensitivity, and fluorescence excitation spectra were not corrected for the wavelength dependency of the lamp emission intensity. ESI mass spectra were recorded with a Finnigan LCQ iontrap spectrometer and high-resolution mass spectra (HR-ESI) were recorded on a Bruker FTMS-7 APEX® IV 70e FT-ICR spectrometer. Reversed-phase HPLC was performed with a Pharmacia Biotech Äkta Basic 900 system [eluents: A: deionized water (18 $M\Omega \cdot cm^{-1}$) + 0.1% TFA, B: acetonitrile/water 8:2 + 0.1% TFA], analytical HPLC was carried out with a Grom-Sil 80 ODS, 250 × 4.6 mm, 4 μm, 80 Å, C-18 column (0.5 mL min^{-1}), and preparative HPLC was done on a J'sphere ODS-H80, 250 × 20 mm ID, 8–4 μm, 8 nm, C18 (YMC) column (10 mL min^{-1}). DNA was purchased from Carl Roth GmbH.

Analytical Data: The NMR analysis of compounds **7**, **8**, **30**, and **31** suffered from high rotation barriers caused by the tertiary amide bond of the *N*-acyl-(2-aminoethyl)glycine, which produced broadening and partly doubling of ^1H and ^{13}C NMR signals.[21] Mostly, a complete set of NMR assignments was obtained separately for each rotamer with the aid of H,H-COSY, HSQC, and HMBC experiments. The 2-methyl group in the 4-imidazolone moiety was found to be quite acidic, as is known from the literature.[22] H/D exchange takes place within some hours and needs to be taken into account for 2D NMR spectroscopy. The enantiomeric excesses were determined by deprotection of monomer **4**–**6** with TFA followed by coupling with enantiomerically pure D- and L-Ala-OSu and analytical HPLC. In all cases the enantiomeric excess was higher than 95%.

Synthesis of 3-Methyl-GFP Chromophores

3-Methyl-(H)GFPC (2): The GFPC-precursor **11** (218 mg, 1.00 mmol, 1.0 equiv.) was heated quickly to 230 °C under vacuum in an oil bath and stirred for 5 min at 230 °C. After cooling, the black residue was extracted with hexane (10 × 5 mL) to yield an orange solution, which was purified on silica gel (70 g, ethyl acetate/hexane 3:1) to provide the chromophore **2** (89 mg, 45%) as light red crystals. R_F (ethyl acetate/hexane 3:1) = 0.49. ^1H NMR (200 MHz, $CDCl_3$): δ = 2.34 (s, 3 H, CH_3), 3.11 (s, 3 H, NCH_3), 7.04 (s, 1 H, acryl-H), 7.34 (m, 3 H, phenyl-H), 8.06 (d, J = 8.1 Hz, 2 H, phenyl-H) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 15.7 (CH_3-2), 26.6 (CH_3-3), 127.2 (CH), 128.7 (CH), 130.0 (CH), 132.1 (CH), 134.1 (C-phenyl), 138.7 (C-5), 162.6 (C-2), 170.7 (CO-4) ppm. UV/Vis (methanol): λ_{max} (ε) = 236, 293, 350 nm. EI-MS: *m/z* (%): 200 (100) $[M]^+$. HR-ESI: *m/z* $[2M + H]^+$ = calcd. for $C_{12}H_{12}N_2O$ 401.1972; found: 401.1971.

3-Methyl-(OH)GFPC (3): Acrylamide **12** (298 mg, 1.27 mmol, 1.0 equiv.) was heated quickly to 230 °C under vacuum in an oil bath and stirred at 230 °C for 5 min. After cooling, the residue was extracted with hot (70 °C) ethyl acetate (8 × 5 mL) to provide an orange raw product (238 mg) that was purified on silica gel (80 g ethyl acetate/hexane 3:1) to give the chromophore **3** (124 mg, 45%) as bright yellow crystals. R_F (ethyl acetate/hexane 3:1) = 0.36. ^1H NMR (200 MHz, [D$_6$]DMSO, 35 °C): δ = 2.32 (s, 3 H, CH_3), 3.09 (s, 3 H, NCH_3), 6.84 [d, $^3J_{H,H}$ = 8.4 Hz, 2 H, phenyl-H], 6.88 (s, 1 H, acryl-H), 8.07 (d, $^3J_{H,H}$ = 8.4 Hz, 2 H, phenyl-H), 9.84 (br. s, 1 H, phenyl-OH) ppm. ^{13}C NMR (50 MHz, [D$_6$]DMSO, 35 °C): δ = 15.6 (CH_3), 26.9 (CH_3), 116.4 (2 × CH-phenyl), 125.9 (C), 126.8 (CH-methylene), 134.7 (2 × CH-phenyl), 136.6 (C), 159.9 (C), 163.3 (CO), 170.9 (CO) ppm. UV/Vis (methanol): λ_{max} (ε) = 248, 371 nm. EI-MS: *m/z* (%): 216 (100) $[M]^+$. HR-ESI: *m/z* $[M + H]^+$ = calcd. for $C_{12}H_{12}N_2O_2$ 217.0972; found: 217.0971.

2-Acetylamino-*N*-methylcinnamide (11): (H)Azlactone **9** (1.64 g, 8.76 mmol, 1.0 equiv.) was dissolved in ethanolic methylamine (8 M, 15 mL) and the mixture was stirred for 1 d. The product precipitated in small crystals and was separated by filtration, and the title compound **11** (1.02 g, 53%) was obtained as a colorless solid after washing of the crystals with hexane and drying in vacuo. R_F (ethyl acetate/methanol 7:1) = 0.38. ^1H NMR (200 MHz, [D$_6$]DMSO, 35 °C): δ = 2.00 (s, 3 H, $COCH_3$), 2.67 (d, $^3J_{H,H}$ = 5.2 Hz, 3 H, CH_3), 7.05 (s, 1 H, acryl-H), 7.28–7.45 (m, 3 H, phenyl-H), 7.52 (d, $^3J_{H,H}$ = 8.0 Hz, 2 H, phenyl-H), 7.87 (br. s, 1 H, NHMe), 9.30 (s, 1 H, NHCO) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 22.8 (Ac-CH_3), 26.1 (CH_3), 127.4 (CH), 128.4 (2 × CH-phenyl), 129.2 (2 × CH-phenyl), 130.1 (CH-methylene), 134.2 (C-phenyl), 165.2 (CO), 169.2 (CO) ppm. UV/Vis (methanol): λ_{max} (ε) = 216, 275 nm.

EI-MS: *m/z* (%): 218 (40) $[M]^+$. HR-ESI: *m/z* $[M + H]^+$ = calcd. for $C_{12}H_{14}N_2O_2$ 219.1128; found: 219.1126.

2-Acetamido-4-hydroxy-*N*-methylcinnamide (12): (OAc)Azlactone **10** (1.00 g, 4.01 mmol, 1.0 equiv.) was dissolved in aqueous methylamine solution (40%, 10 mL) and the mixture was stirred for 5 h. The solution was reduced in vacuo, water (10 mL) was added, and the residue was dissolved with heating (100 °C). After the solution had been cooled at 4 °C overnight, light yellow crystals (745 mg) had precipitated and were separated by filtration. These crystals were dissolved in water (25 mL, 100 °C) and precipitated by addition of acetic acid (10 drops) to provide the desired product **12** (710 mg, 74%) as colorless crystals. R_F (ethyl acetate/methanol 7:1) = 0.30. ^{1}H NMR (200 MHz, [D$_6$]DMSO, 35 °C): δ = 2.00 (s, 3 H, $COCH_3$), 2.66 (d, $^3J_{H,H}$ = 6.0 Hz, 3 H, NCH_3), 2.90–3.80 (br. s, 1 H, OH), 6.87 (d, $^3J_{H,H}$ = 8.6 Hz, 2 H, phenyl-H), 7.03 (s, 1 H, acryl-H), 7.39 (d, $^3J_{H,H}$ = 8.6 Hz, 2 H, phenyl-H), 7.74 (d_{br}, $^3J_{H,H}$ = 6.0 Hz, 1 H, NH), 9.16 (s, 1 H, NHCO) ppm. ^{13}C NMR (50 MHz, [D$_6$]DMSO, 35 °C): δ = 23.2 (CH_3), 26.6 (CH_3), 115.9 (2 × CH-phenyl), 125.3 (C), 126.6 (C), 129.8 (CH-methylene), 131.7 (2 × CH-phenyl), 158.3 (C), 166.4 (CO), 170.6 (CO) ppm. UV/Vis (methanol): λ_{max} (ε) = 226, 299 nm. EI-MS: *m/z* (%): 234 (40) $[M]^+$. HR-ESI: *m/z* $[M + H]^+$ = calcd. for $C_{12}H_{14}N_2O_3$ 235.1077; found: 235.1076.

Synthesis of Alaninyl GFP Chromophores

Boc-L-Ala-(H)GFPC-OH (4): Precursor **19** (800 mg, 2.04 mmol) was dissolved in DMF (5 mL) and the solution was flushed with nitrogen for 10 min. DBU (5 drops) was added and the solution was heated under reflux for 5 min. The solvent was removed under vacuum and the crude orange product was purified on silica gel (100 g, ethyl acetate/methanol 7:1 + 0.5–2% acetic acid) to provide amino acid **4** (562 mg, 74%) as light red crystals. R_F (ethyl acetate/methanol 7:1 + 2.0% acetic acid) = 0.35. ^{1}H NMR (300 MHz, CD_3OD): δ = 1.33 (s, 9 H, *t*Bu), 2.44 (s, 3 H, CH_3; prone to H/D exchange), 3.85 (m, 1 H, Hβ), 4.08 (m, 1 H, Hβ′), 4.42 (m, 1 H, Hα), 7.02 (s, 1 H, acryl-H), 7.39 (m, 3 H, phenyl-H), 8.05 (d, $^3J_{H,H}$ = 6.6 Hz, 2 H, phenyl-H) ppm. ^{13}C NMR (75 MHz, CD_3OD): δ = 18.0 (CH_3-2, prone to H/D exchange), 28.6 (Boc-CH_3), 44.1 (β-CH_2), 54.5 (α-CH), 80.7 (Boc-C), 128.2 (CH), 129.7 (CH), 131.3 (CH), 133.2 (CH), 135.4 (C), 139.5 (C), 157.5 (C), 165.4 (C), 172.5 (CO), 174.9 (CO) ppm. ESI-MS: *m/z* (%): 372 (60) $[M - H]^-$, 396 (100) $[M + Na]^+$, 769 (40) $[2M + Na]^+$. HR-ESI: *m/z* $[M + H]^+$ = calcd. for $C_{19}H_{23}N_3O_5$ 374.1711; found: 374.1713.

Boc-L-Ala-(OH)GFPC-OH (5): Precursor **20** (160 mg, 0.40 mmol, 1.0 equiv.) was dissolved in DMF (6 mL) and the system was flushed with nitrogen for 15 min. The solution was heated at reflux for 15 min, the solvent was removed in vacuo, and the residue was cleaned on silica gel (50 g, ethyl acetate/methanol 7:1 + 0.5–2% acetic acid) to provide amino acid **5** (142 mg, 75%) as a bright yellow solid. R_F (ethyl acetate/methanol 7:1 + 2% acetic acid) = 0.30. ^{1}H NMR (300 MHz, CD_3OD): δ = 1.31 (s, 9 H, *t*Bu), 2.42 (s, 3 H, CH_3), 3.83 (m, 1 H, Hβ), 4.06 (m, 1 H, Hβ′), 4.36 (m, 1 H, Hα), 6.81 (d, $^3J_{H,H}$ = 8.1 Hz, 2 H, phenyl-H), 6.99 (s, 1 H, acryl-H), 7.94 (d, $^3J_{H,H}$ = 8.1 Hz, 2 H, phenyl-H) ppm. ^{13}C NMR (50 MHz, CD_3OD): δ = 17.8 (CH_3), 28.7 (CH_3), 44.7 (β-CH_2), 55.7 (α-CH), 80.5 (Boc-C), 116.7 (2 × CH-phenyl), 126.9 (C), 128.6 (CH), 129.5 (CH), 135.5 (2 × CH-phenyl), 136.9 (C), 141.5 (CH), 157.5 (C), 161.5 (C), 163.4 (C), 172.8 (CO), 175.4 (CO) ppm. UV/Vis (methanol): λ_{max} (ε) = 249, 371 nm. ESI-MS: *m/z* (%): 390 (85) $[M + H]^+$, 412 (35) $[M + Na]^+$. HR-ESI: *m/z* $[M + H]^+$ = calcd. for $C_{19}H_{23}N_3O_6$ 390.1660; found: 390.1660.

Boc-L-Ala-(OBzl)GFPC-OH (6): **A**) Boc-L-Ala-(OH)GFP-OH (**5**, 34 mg, 87 μmol, 1.0 equiv.) was dissolved in methanol (250 μL), aqueous NaOH (1 M, ≈ 250 μL) was added to provide pH 11, and 2,5-dichlorobenzyl bromide (25 mg, 105 μmol, 1.2 equiv.) dissolved in methanol (1000 μL) was then added. Each day, further 2,5-dichlorobenzyl bromide (10 mg) and NaOH (1 M, to keep the pH at ca. 11) were added. After 3 d the reaction was terminated by addition of HCl (1 M, ≈ 100 μL, final pH ≈ 5.5), the solvent was removed in vacuo, and the crude product was purified on silica gel (14 g, ethyl acetate/methanol 8:1 + 1.0% acetic acid) to provide the title compound **6** (30 mg, 63%) as a bright yellow solid.

B) Boc-L-Ala-(OBzl)GFPC-precursor **21** (1.80 g, 3.18 mmol) was dissolved in DMF (5 mL) and the system was flushed with nitrogen for 10 min. DBU (5 drops) was added, the solution was heated under reflux for 5 min, the solvent was removed under vacuum, and the orange crude product was purified on silica gel (100 g, ethyl acetate/methanol 7:1 + 0.5–2% acetic acid) to provide the title compound **6** (1.51 g, 86%) as an orange solid. R_F (ethyl acetate/methanol 7:1 + 2% acetic acid) = 0.45. ^{1}H NMR (300 MHz, CD_3OD): δ = 1.36 (s, 9 H, *t*Bu), 2.42 (s, 3 H, CH_3), 3.89 (dd, $^2J_{H,H}$ = 15 Hz, $^3J_{H,H}$ = 10 Hz, 1 H, Hβ), 4.09 (dd, $^2J_{H,H}$ = 15 Hz, $^3J_{H,H}$ = 5.2 Hz, 1 H, Hβ′), 4.47 (m, 1 H, Hα), 5.35 (s, 2 H, benzyl-H), 7.03 (s, 1 H, acryl-H), 7.09 (d, $^3J_{H,H}$ = 7.6 Hz, 2 H, phenyl-H), 7.35–7.48 (m, 3 H, phenyl-H), 8.10 (d, $^3J_{H,H}$ = 7.6 Hz, 2 H, phenyl-H) ppm. ^{13}C NMR (75 MHz, CD_3OD): δ = 18.0 (CH_3, prone to H/D exchange), 28.6 (3 × Boc-CH_3), 43.6 (β-CH_2), 53.8 (α-CH), 66.3 (CH_2), 80.8 (Boc-C), 116.0 (CH), 128.5 (C), 128.6 (CH), 129.7 (CH), 132.2 (CH), 133.1 (C), 135.3 (CH), 137.4 (C), 138.0 (C), 157.8 (C), 162.2 (C), 163.7 (C), 172.5 (C) ppm. UV/Vis (methanol): λ_{max} (ε) = 225, 248, 370 nm. ESI-MS: *m/z* (%): 548 (100) $[M + H]^+$. HR-ESI: *m/z* $[M + H]^+$ = calcd. for $C_{26}H_{27}Cl_2N_3O_6$ 548.1350; found: 548.1354.

2-Acetamido-4-hydroxycinnamic Acid (14): 4-Acetoxy-2-acetamidocinnamic acid (**17**, 2.10 g, 8.0 mmol, 1.0 equiv.) was suspended in water (7 mL) and a solution of KOH (1.56 g, 2.5 equiv.) in water (3 mL) was added slowly at 0 °C. The resulting orange solution was stirred (5 h) at 0 °C and acidified with concentrated HCl (37%) at 0 °C, and the precipitate was filtered off, washed with cold HCl (0.1 M), and dried in vacuo. The title compound **14** (1.65 g, 7.44 mmol, 93%) was obtained as light pink crystals. R_F (ethyl acetate/methanol 7:1) = 0.15. ^{1}H NMR (200 MHz, [D$_6$]DMSO, 35 °C): δ = 1.96 (s, 3 H, $COCH_3$), 6.79 (d, $^3J_{H,H}$ = 8.1 Hz, 2 H, phenyl-H), 7.18 (s, 1 H, acryl-H), 7.48 (d, $^3J_{H,H}$ = 8.1 Hz, 2 H, phenyl-H), 9.22 (s, 1 H, NH), 9.90 (br. s, 1 H, phenyl-OH) ppm. ^{13}C NMR (50 MHz, [D$_6$]DMSO, 35 °C): δ = 22.5 (CH_3), 115.4 (2 × CH-phenyl), 124.0 (C), 124.6 (C), 131.7 (2 × CH-phenyl), 132.4 (C-methylene), 158.6 (C), 166.6 (CO), 169.1 (CO) ppm. UV/Vis (methanol): λ_{max} (ε) = 225, 301 nm. EI-MS: *m/z* (%): 221 (40) $[M]^+$. HR-ESI: *m/z* $[M + H]^+$ = calcd. for $C_{11}H_{11}NO_4$ 222.0761; found: 222.0760.

2-Acetamido-3-[4-(2,6-dichlorobenzyloxy)phenyl]cinnamic Acid (15): Potassium acetate (2.5 g, 25 mmol, 0.8 equiv.) was dried in vacuo with melting for 15 min. Aldehyde **35** (8.0 g, 28 mmol, 1.0 equiv.) and *N*-acetylglycine (3.6 g, 31 mmol, 1.1 equiv.) were suspended in acetic acid anhydride (15 mL) and the system was flushed with nitrogen (15 min). The resulting mixture was heated under reflux until no aldehyde **35** was detectable by TLC (5 h), the solution was kept in the fridge (4 °C) overnight, ice water (100 mL) was then added, and the precipitated product was filtered off and washed with aqueous K_2CO_3 (1 M, 2 × 100 mL). The brown residue was dissolved in dichloromethane, silica gel (50 mL) was added, the solvent was removed in vacuo, and the product was eluted with ethyl acetate/hexane 1:3 and dried in vacuo. This crude product **16** was dissolved in acetone/water 3:1 with heating and heated at reflux for

2 h. After the system had been stirred overnight at room temperature, the product precipitated, and was filtered off, washed with ethyl acetate/hexane, and dried in vacuo to provide the title compound **15** (4.6 g, 43%) as a light yellow solid. ^{1}H NMR (300 MHz, [D$_6$]DMSO, 35 °C): δ = 1.99 (s, 3 H, CH$_3$), 5.28 (s, 2 H, CH$_2$), 7.10 (d, $^3J_{H,H}$ = 8.0 Hz, 2 H, phenyl-H), 7.22 (s, 1 H, acryl-H), 7.40–7.62 (m, 5 H, phenyl-H), 9.31 (s, 1 H, NH), 12.50 (br. s, 1 H, OH) ppm. ^{13}C NMR (75 MHz, [D$_6$]DMSO, 35 °C): δ = 22.5 (CH$_3$), 62.8 (CH), 64.9 (CH$_2$), 102.1 (CH), 114.6 (CH), 125.4 (C), 126.8 (C), 128.7 (CH), 131.3 (C), 131.4 (CH), 136.0 (C), 159.0 (C), 166.5 (C), 169.0 (C) ppm. EI-MS: *m*/*z* (%): 379.2 (50) [M]$^+$. HR-ESI: *m*/*z* [M + Na]$^+$ = calcd. for $C_{14}H_{10}Cl_2O_2$ 402.0270; found: 402.0269.

2-Acetamido-4-acetoxycinnamic Acid (17): (OAc)Azlactone **10** (11.4 g, 46.5 mmol, 1.0 equiv.) was heated under reflux in acetone/water 3:1 (120 mL) for 4 h, the solution was kept at 4 °C overnight, and the precipitate was filtered off with a Buchner funnel, washed with cold water (30 mL), cold acetone (10 mL), and cold ethyl acetate (20 mL), and dried in vacuo to provide the title compound **17** (11.5 g, 43.7 mmol, 94%) as light yellow crystals. ^{1}H NMR (300 MHz, [D$_6$]DMSO, 35 °C): δ = 1.99 (s, 3 H, CH$_3$), 2.27 (s, 3 H, CH$_3$), 7.17 (d, $^3J_{H,H}$ = 8.1 Hz, 2 H, phenyl-H), 7.23 (s, 1 H, acryl-H), 7.65 (d, $^3J_{H,H}$ = 8.1 Hz, 2 H, phenyl-H), 9.42 (s, 1 H, NH), 12.5 (br. s, 1 H, COOH) ppm. ^{13}C NMR (125.7 MHz, [D$_6$]DMSO, 35 °C): δ = 20.8 (CH$_3$), 22.5 (CH$_3$), 121.9 (2 × CH-phenyl), 127.3 (C), 124.6 (C), 130.2 (C-methylene), 130.9 (2 × CH-phenyl), 132.4 (C), 150.8 (C), 166.3 (CO), 169.0 (CO), 169.2 (CO) ppm. UV/Vis (methanol): λ_{max} (ε) = 225, 301 nm. ESI-MS: *m*/*z* (%): 264.1 (20) [M + H]$^+$, 286.1 (100) [M + Na]$^+$, 549.2 (35) [2M + Na]$^+$. HR-ESI: *m*/*z* [M + H]$^+$ = calcd. for $C_{13}H_{13}NO_5$ 264.0867; found: 264.0866.

Boc-L-Ala-GFPC-Precursor 19: 2-Acetamidocinnamic acid (**13**, 900 mg, 4.40 mmol, 1.05 equiv.) and HOBt (770 mg, 5.7 mmol, 1.3 equiv.) were dissolved in DMF (3 mL). DCC (850 mg, 4.11 mmol, 1.0 equiv.) dissolved in DMF (2 mL) was added at 0 °C and the solution was stirred for 60 min at 0 °C and for 120 min at room temperature. The precipitated urea was filtered off and washed with DMF (18 mL) until the urea was colorless, (*S*)-3-amino-2-(*tert*-butoxycarbonylamino)propionic acid (**18**, 1.00 g, 4.90 mmol, 1.1 equiv.), and triethylamine (1.2 mL, 890 mg, 8.8 mmol, 2.0 equiv.) were added to the obtained yellow solution, and the resulted suspension was stirred until a clear solution was obtained (1 d). The solvent was removed in vacuo and the crude product was purified on silica gel (170 g, ethyl acetate/methanol 7:1, + 0.5–1.5% acetic acid) to provide the title compound **19** (1.42 g, 88%) as a colorless solid. R_F (ethyl acetate/methanol 7:1 + 1% acetic acid) = 0.18. ^{1}H NMR (200 MHz, [D$_6$]DMSO, 35 °C): δ = 1.39 (s, 9 H, *t*Bu), 2.00 (s, 3 H, COCH$_3$), 3.30–3.48 (m, 2 H, Hβ, Hβ′), 3.75 (m, 1 H, Hα), 6.44 (d, $^3J_{H,H}$ = 5.6 Hz, 1 H, NHBoc), 7.08 (s, 1 H, acryl-H), 7.28–7.44 (m, 3 H, phenyl-H), 7.54 (d, $^3J_{H,H}$ = 8.3 Hz, 2 H, phenyl-H), 8.30 (br. s, 1 H, N*H*CH$_2$), 9.56 (s, 1 H, NHCO) ppm. ^{13}C NMR (50 MHz, [D$_6$]DMSO, 35 °C): δ = 22.6 (CH$_3$), 28.1 (Boc-CH$_3$), 42.8 (β-CH$_2$), 54.2 (α-CH), 77.8 (Boc-C), 128.0 (CH), 128.4 (CH), 129.2 (CH), 129.8 (CH), 134.0 (CH), 155.2 (C), 164.4 (C), 169.5 (CO), 173.3 (CO) ppm. UV/Vis (methanol): λ_{max} (ε) = 217, 277 nm. ESI-MS: *m*/*z* (%): 391 (100) [M – H]$^-$, 414 (100) [M + Na]$^+$. HR-ESI: *m*/*z* [M + Na]$^+$ = calcd. for $C_{19}H_{25}N_3O_6$ 414.1636; found: 414.1638.

Boc-L-Ala-(OH)GFPC-Precursor 20: 2-Acetamido-4-hydroxycinnamic acid (**14**, 370 mg, 1.67 mmol, 1.05 equiv.) and HOBt (300 mg, 2.22 mmol, 1.3 equiv.) were dissolved in DMF (1 mL), DCC (310 mg, 1.50 mmol, 1.0 equiv.) dissolved in DMF (1 mL) was added at 0 °C, the resulting solution was stirred for 1 h at 0 °C and for 45 min at room temperature, and the precipitated urea was filtered off and washed with DMF (6 mL). (*S*)-3-Amino-2-(*tert*-butoxycarbonylamino)propionic acid (**18**, 332 mg, 1.62 mmol, 1.05 equiv.) and triethylamine (500 μL, 360 mg, 3.60 mmol, 2.5 equiv.) were added to the DMF solution, the resulting suspension was stirred until a clear solution was obtained (1 d), the solvent was removed in vacuo, and the crude product was purified on silica gel (50 g, ethyl acetate/methanol 7:1, + 0.5–2% acetic acid) to provide the title compound **20** (446 mg, 73%) as a colorless solid. R_F (ethyl acetate/methanol 7:1 + 2% acetic acid) = 0.15. ^{1}H NMR (200 MHz, CD$_3$OD): δ = 1.42 (s, 9 H, *t*Bu), 2.15 (s, 3 H, COCH$_3$), 3.64 (m, 2 H, Hβ,β′), 4.20 (m, 1 H, Hα), 6.79 (d, $^3J_{H,H}$ = 8.0 Hz, 2 H, phenyl-H), 7.19 (s, 1 H, acryl-H), 7.40 (d, $^3J_{H,H}$ = 8.0 Hz, 2 H, phenyl-H) ppm. ^{13}C NMR (50 MHz, CD$_3$OD): δ = 21.8 (CH$_3$), 27.3 (CH$_3$), 43.0 (β-CH$_2$), 53.3 (α-CH), 77.2 (Boc-C), 114.5 (2 × CH-phenyl), 123.9 (C), 125.8 (C), 128.1 (CH-methylene), 130.3 (2 × CH-phenyl), 154.4 (C), 157.3 (C), 164.3 (C), 168.5 (C), 177.0 (CO) ppm. UV/Vis (methanol): λ_{max} (ε) = 229, 300 nm. ESI-MS: *m*/*z* (%): 406 (95) [M – H]$^-$, 430 (95) [M + Na]$^+$. HR-ESI: *m*/*z* [M + H]$^+$ = calcd. for $C_{19}H_{25}N_3O_7$ 408.1765; found: 408.1766.

Boc-L-Ala-(OBzl)GFPC-Precursor 21: Acrylic acid **15** (2.50 g, 6.6 mmol, 1.05 equiv.) and HOBt (1.10 g, 8.1 mmol, 1.3 equiv.) were dissolved in DMF (8 mL), DCC (1.29 g, 6.3 mmol, 1.0 equiv.) dissolved in DMF (2 mL) was added at 0 °C, and the solution was stirred for 30 min at 0 °C and for 60 min at room temperature. The precipitated urea was filtered off and washed with DMF (25 mL) until the urea was colorless, (*S*)-3-amino-2-(*tert*-butoxycarbonylamino)propionic acid **18** (1.41 g, 6.9 mmol, 1.1 equiv.) and triethylamine (1.5 mL, 1.33 mg, 13 mmol, 2.0 equiv.) were added to the obtained yellow solution, and the resulting suspension was stirred until a clear solution was obtained (1 d). The solvent was removed in vacuo and the crude product was purified on silica gel (200 g, ethyl acetate/methanol 7:1, + 0.5–1.5% acetic acid) to provide the title compound **21** (3.21 g, 90%) as a colorless solid. ^{1}H NMR (300 MHz, [D$_6$]DMSO, 35 °C): δ = 1.38 (s, 9 H, Boc), 2.05 (s, 3 H, CH$_3$), 3.31–3.42 (m, 2 H, β-H), 3.90 (m, 1 H, α-H), 5.21 (s, 2 H, CH$_2$), 6.63 (br. s, 1 H, NH), 7.05 (s, 1 H, acryl-H), 7.06 (d, $^3J_{H,H}$ = 8.0 Hz, 2 H, phenyl-H), 7.40–7.60 (m, 5 H, phenyl-H), 8.12 (br. s, 1 H, NH), 9.40 (s, 1 H, NH), 12.00 (br. s, 1 H, OH) ppm. ^{13}C NMR (50 MHz, [D$_6$]DMSO, 35 °C): δ = 22.7 (CH$_3$), 28.1 (3 × Boc-CH$_3$), 41.8 (β-CH$_2$), 54.1 (α-CH), 64.9 (CH$_2$), 78.0 (Boc-C), 114.6 (CH), 127.2 (C), 128.0 (C), 128.3 (CH), 128.7 (CH), 131.0 (CH), 131.4 (C), 131.6 (CH), 136.0 (C), 155.3 (C), 158.6 (C), 165.0 (C), 169.3 (C), 172.4 (C) ppm. ESI-MS: *m*/*z* (%): 588.5 (100) [M + Na]$^+$. HR-ESI: *m*/*z* [M + Na]$^+$ = calcd. for $C_{26}H_{29}Cl_2N_3O_7$ 588.1275; found: 588.1277.

4-(2,6-Dichlorobenzyloxy)benzaldehyde (35): 4-Hydroxybenzaldehyde (10 g, 90 mmol, 1.1 equiv.) and 2,6-dichlorobenzyl bromide (19.5 g, 80 mmol, 1.0 equiv.) were dissolved in dichloromethane (60 mL), DBU (21 mL, 1.5 equiv.) was added, and the resulting mixture was stirred for 17 h at room temperature. The reaction was stopped by addition of acetic acid (3 mL, 0.6 equiv.), the solvent was removed in vacuo, ethyl acetate (100 mL) was added, and the mixture was stirred for 10 min. The resulting suspension was filtered off and the organic layer was extracted with aqueous K_2CO_3 solution (0.1 M, 2 × 100 mL), washed with brine (100 mL), and dried with Na_2SO_4 to provide the title compound **35** (14.4 g, 64%) as a light brown solid. This product could be used without further purification for the Erlenmeyer azlactone synthesis, but for NMR analysis a small sample was recrystallized from ethyl acetate/hexane 1:6, 5 mL·g^{-1} to provide the title compound as long, colorless needles. R_F (ethyl acetate/hexane 1:1) = 0.80. ^{1}H NMR (200 MHz, CDCl$_3$): δ = 5.39 (s, 2 H, CH$_2$), 7.12 (d, $^3J_{H,H}$ = 8.0 Hz, 2 H,

phenyl-H), 7.20–7.42 (m, 3 H, phenyl-H), 7.88 (d, $^3J_{H,H}$ = 8.0 Hz, 2 H, phenyl-H), 9.92 (s, 1 H, HCO) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 65.3 (CH_2), 115.0 (CH), 128.5 (CH), 130.3 (C), 130.8 (CH), 131.2 (C), 132.0 (CH), 137.0 (C), 163.7 (C), 190.8 (CHO) ppm. EI-MS: *m*/*z* (%): 280.2 (10) $[M]^+$, 159.1 (100) [benzyl cation]. HR-ESI: *m*/*z* $[M + Na]^+$ = calcd. for $C_{14}H_{10}Cl_2O_2$ 302.9950; found: 302.9948.

Synthesis of (2-Aminoethyl)glycinyl GFPC Chromophores

Fmoc-aeg-(H)GFPC-OH (7): Fmoc-aeg-(H)GFPC-OMe (**30**, 463 mg, 0.83 mmol) was dissolved in ethyl acetate (20 mL), LiI (480 mg, 3.6 mmol) was added, and the obtained solution was heated under reflux for 25 h. After the mixture had cooled to room temperature, ethyl acetate (200 mL) was added and the organic layer was washed with $NaHSO_3$/HCl (0.5 M, pH 2, 3 × 100 mL) and saturated NaCl/HCl (pH 2, 2 × 100 mL) and dried with Na_2SO_4. After purification on RP-18 silica gel (methanol/water 7:3) title compound **7** (0.30 g, 0.53 mmol, 64%) was obtained as a red solid. M.p. 118 °C; NMR: two rotamers 1:1. ^{1}H NMR (300 MHz, $[D_6]$DMSO, 35 °C): δ = 8.20 (d, $^3J_{H,H}$ = 8.1 Hz, 2 H, CH-phenyl), 7.88 (d, $^3J_{H,H}$ = 7.2 Hz, 2 H, CH-Fmoc), 7.68 (t, $^3J_{H,H}$ = 6.6 Hz, 2 H, CH-Fmoc), 7.48–7.30 (m, 7 H, CH-Fmoc, CH-Fmoc, CH-phenyl, CH-phenyl), 6.99 (s, 1 H, CH-methylene), 4.62 (s, 1 H, CH_2-GFP), 4.46 (s, 1 H, CH_2-GFP), 4.38–4.28 (m, 2 H, CH_2-Fmoc), 4.28–4.21 (m, 1 H, CH-Fmoc), 3.97 (s, 2 H, CH_2COOH), 3.49 (t, $^3J_{H,H}$ = 6.9 Hz, 2 H, CH_2–N), 3.16–3.12 (m, 2 H, CH_2–NH), 2.20 (s, 1.5 H, CH_3), 2.18 (s, 1.5 H, CH_3) ppm. ^{13}C NMR (150 MHz, $[D_6]$-DMSO, 35 °C): δ = 170.4 (COOH), 169.6 (CO-GFP), 167.0 (CH_2*C*ON), 164.3 (N=*C*–N), 156.4 (OCON), 143.9 (2 × C-Fmoc), 140.7 (2 × C-Fmoc), 138.8 (CH=*C*–N), 134.0 (C-phenyl), 131.9 (2 × CH-phenyl), 130.0 (CH-phenyl), 128.7 (2 × CH-phenyl), 127.6 (2 × CH-Fmoc), 127.1 (2 × CH-Fmoc), 125.1 (CH-methylene), 125.0 (2 × CH-Fmoc), 120.1 (2 × CH-Fmoc), 65.5 (CH_2-Fmoc), 47.0 (CH-Fmoc), 46.7 (CH_2–N, *C*H_2COOH), 40.3 (CH_2-GFP), 39.4 (CH_2–NH), 15.2 (CH_3) ppm. IR (KBr): $\tilde{\nu}$ = 3441, 1718, 1653, 1458, 1253, 742 cm^{-1}. UV/Vis (methanol): λ_{max} = 347, 299, 289, 264, 210 nm. ESI-MS: *m*/*z* (%) = 565.2 (100) $[M - H]^-$, 679.1 (90) [M + TFA]$^-$, 1131.2 (25) $[2M - H]^-$, 1697.3 (10) $[3M - H]^-$. HR-ESI-MS: *m*/*z* $[M + H]^+$ = calcd. for $C_{32}H_{30}N_4O_6$ 567.2238; found: 567.2239.

Fmoc-aeg-(OH)GFPC-OH (8): Fmoc-aeg-(OH)GFPC-OMe (**31**, 394 mg, 0.66 mmol) was dissolved in ethyl acetate (17 mL), LiI (408 mg, 3.1 mmol) was added, and the resulting solution was heated under reflux for 25 h. After removal of the solvent under reduced pressure, $NaHSO_3$ (106 mg, 1.5 equiv.) in HCl (1.0 M, 4 equiv.) was added. Methanol (≈ 2 mL) was then added to the suspension until a clear solution had been obtained, and this was purified on RP-18 silica gel (methanol/water 7:3). The title compound **8** (223 mg, 0.38 mmol, 58%) was obtained as a yellow solid. M.p. 196 °C; NMR: two rotamers 1.4:1. *First rotamer* (major component): ^{1}H NMR (300 MHz, $[D_6]$DMSO, 35 °C): δ = 8.08 (d, $^3J_{H,H}$ = 9.0 Hz, 2 H, phenyl-CH), 7.88 (d, $^3J_{H,H}$ = 7 Hz, 2 H, Fmoc-CH), 7.69 (d, $^3J_{H,H}$ = 7 Hz, 2 H, Fmoc-CH), 7.41 (t, $^3J_{H,H}$ = 7 Hz, 3 H, Fmoc-CH, NH), 7.33 (t, $^3J_{H,H}$ = 7 Hz, 2 H, Fmoc-CH), 6.91 (s, 1 H, CH=C), 6.85 (d, $^3J_{H,H}$ = 9.0 Hz, 2 H, phenyl-CH), 4.44 (s, 2 H, GFP-CH_2), 4.36 (d, $^3J_{H,H}$ = 6 Hz, 2 H, Fmoc-CH_2), 4.24 (t, $^3J_{H,H}$ = 6 Hz, 1 H, Fmoc-CH), 3.97 (s, 2 H, CH_2COOH), 3.49 (m, 2 H, CH_2–N), 3.26 (m, 2 H, CH_2–NH), 2.15 (s, 3 H, CCH_3) ppm. ^{13}C NMR (150 MHz, $[D_6]$DMSO, 35 °C): δ = 170.3 (COOH), 169.5 (GFP-*CO*), 167.4 (GFP-CH_2–*CO*), 162.0 (C=N), 159.6 (phenyl-*C*–OH), 156.3 (Fmoc-CO), 143.8 (2 × Fmoc-C), 140.6 (2 × Fmoc-C), 135.9 (*C*=CH), 134.0 (2 × phenyl-CH), 127.5 (2 × Fmoc-CH), 127.0 (2 × Fmoc-CH), 125.8 (*CH*=C), 125.2 (phenyl-*C*–CH), 125.9 (2 × Fmoc-CH), 120.0 (2 × Fmoc-CH), 115.7 (2 × phenyl-CH), 65.4 (Fmoc-CH_2), 47.8 (*C*H_2COOH), 46.7 (Fmoc-CH), 46.6 (CH_2–N), 41.1 (GFP-CH_2), 37.9 (CH_2–NH), 14.9 (*C*H_3C) ppm. *Second rotamer*: ^{1}H NMR (300 MHz, $[D_6]$DMSO, 35 °C): δ = 8.08 (d, $^3J_{H,H}$ = 9.0 Hz, 2 H, phenyl-CH), 7.88 (d, $^3J_{H,H}$ = 7 Hz, 2 H, Fmoc-CH), 7.67 (d, $^3J_{H,H}$ = 7 Hz, 2 H, Fmoc-CH), 7.41 (t, $^3J_{H,H}$ = 7 Hz, 2 H, Fmoc-CH), 7.33 (t, $^3J_{H,H}$ = 7 Hz, 3 H, Fmoc-CH, NH), 6.91 (s, 1 H, CH=C), 6.85 (d, $^3J_{H,H}$ = 9.0 Hz, 2 H, phenyl-CH), 4.60 (s, 2 H, GFP-CH_2), 4.28 (d, $^3J_{H,H}$ = 6 Hz, 2 H, Fmoc-CH_2), 4.24 (t, $^3J_{H,H}$ = 6 Hz, 1 H, Fmoc-CH), 4.17 (s, 2 H, CH_2COOH), 3.36 (m, 2 H, CH_2–N), 3.13 (m, 2 H, CH_2–NH), 2.18 (s, 3 H, CCH_3) ppm. ^{13}C NMR (150 MHz, $[D_6]$DMSO, 35 °C): δ = 170.9 (COOH), 169.5 (GFP-*CO*), 166.9 (GFP-CH_2–*CO*), 161.8 (C=N), 159.6 (phenyl-C–OH), 156.1 (Fmoc-CO), 143.8 (2 × Fmoc-C), 140.7 (2 × Fmoc-C), 136.0 (*C*=CH), 134.0 (2 × phenyl-CH), 127.5 (2 × Fmoc-CH), 127.0 (2 × Fmoc-CH), 125.7 (*CH*=C), 125.2 (phenyl-*C*–CH), 125.9 (2 × Fmoc-CH), 120.0 (2 × Fmoc-CH), 115.7 (2 × phenyl-CH), 65.4 (Fmoc-CH_2), 47.1 (*C*H_2COOH), 46.7 (Fmoc-CH), 46.6 (CH_2–N), 40.6 (GFP-CH_2), 37.9 (CH_2–NH), 15.0 (*C*H_3C) ppm. IR (KBr): $\tilde{\nu}$ = 3412, 1707, 1646, 1599, 1516, 1447, 1252, 850, 746 cm^{-1}. UV/Vis (methanol): λ_{max} (OD) = 370 (0.3), 300 (0.14), 223 (0.1) → same sample: UV/Vis (+ 0.01% NEt_3): λ_{max} (OD) = 431 (0.22), 386 (0.22), 300 (0.15), 228 (3.0) nm. ESI-MS: *m*/*z* (%) = 583.2 (100) $[M + H]^+$, 581.1 (90) $[M - H]^-$, 695.0 (100) $[M + CF_3COO^-]^-$. HR-ESI-MS: *m*/*z* $[M + H]^+$ = calcd. for $C_{21}H_{30}N_4O_7$ 583.2187; found: 583.2187.

(H)GFPC-Acetic Acid (23): Aqueous LiOH (1.0 M, 12 mL, 2 equiv.) was slowly added (15 min) to a stirred solution of (H)GFPC-methyl acetate (**27**, 1.50 g, 5.8 mmol, 1 equiv.) in methanol (60 mL). After conversion was complete (TLC, 2 h), aqueous HCl (1.0 M, 6 mL, 1 equiv.) was added and methanol was removed under reduced pressure. The obtained aqueous suspension was acidified with several drops HCl (12 M) and the precipitated product was filtered off and washed with a small amount of cold aqueous HCl solution (0.1 M) and dried in vacuo to provide product **27** (1.27 g, 5.2 mmol, 90%) as a yellow solid. R_F (ethyl acetate/methanol 7:1) = 0.2 (product), 0.86 (starting material); m.p. 79 °C (combustion). ^{1}H NMR (300 MHz, CD_3OD): δ = 8.11 (dd, $^3J_{H,H}$ = 7.8 Hz, 2 H, CH-phenyl), 7.46–7.39 (m, 3 H, CH-phenyl), 7.08 (s, 1 H, CH-methylene), 4.45 (s, 2 H, CH_2), 2.35 (s, 3 H, CH_3; prone to H/D exchange) ppm. ^{13}C NMR (75.5 MHz, CD_3OD): δ = 171.7 (CO), 171.0 (COOH), 164.8 (*C*CH_3), 139.3 (*C*CH), 135.3 (C-phenyl), 133.3 (2 × CH-phenyl), 131.5 (CH-phenyl), 129.7 (2 × CH-phenyl), 128.6 (methylene-CH), 42.2 (CH_2), 15.5 (CH_3 prone to H/D exchange) ppm. IR (KBr): $\tilde{\nu}$ = 3500, 1715, 1645, 1413, 1260, 770, 688, 608 cm^{-1}. UV/Vis (methanol): λ_{max} = 346, 291, 233, 205 nm. ESI-MS: *m*/*z* (%) = 243.0 (100) $[M - H]^-$, 487.1 (50) $[2M - H]^-$, 489.2 (100) $[2M + H]^+$. HR-ESI-MS: *m*/*z* $[M + H]^+$ = calcd. for $C_{13}H_{12}N_2O_3$ 245.0921; found: 245.00920.

(OH)GFPC-Acetic Acid (24): (OAc)GFPC-Methyl acetate (**28**, 2.00 g, 6.33 mmol) was dissolved in methanol (100 mL) and aqueous LiOH (1.0 M, 22.2 mL, 3.5 equiv.) was added slowly (1 h). After the system had been stirred for 2 h at room temperature, aqueous HCl (1.0 M, 19 mL, 3.0 equiv.) was added and methanol was removed under reduced pressure. To complete precipitation, further aqueous HCl (1.0 M, 9.5 mL, 1.5 equiv.) was added. The obtained suspension was purified on RP-18 silica gel (methanol/water 2:3) and the title compound **24** (860 mg, 3.3 mmol, 52%) was obtained as an orange solid. M.p. 160 °C. ^{1}H NMR (300 MHz, $[D_6]$DMSO, 35 °C): δ = 8.07 (d, $^3J_{H,H}$ = 8.7 Hz, 2 H, CH-phenyl), 7.91(s, 1 H, CH), 6.82 (d, $^3J_{H,H}$ = 8.7 Hz, 2 H, CH-phenyl), 4.35 (s, 2 H, CH_2), 2.26 (s, 3 H, CH_3) ppm. ^{13}C NMR (75.5 MHz, $[D_6]$DMSO, 35 °C): δ = 169.5 (CO), 169.4 (CO), 161.2 (NC=N), 159.7 (phenyl-*C*–OH),

135.6 (CH=*C*), 134.1 (2× phenyl-CH), 126.2 (*C*H=C), 125.1 (phenyl-C), 115.7 (2× phenyl-CH), 41.2 (CH_2), 15.0 (CH_3) ppm. IR (KBr): $\tilde{\nu}$ = 3426, 1595, 1383, 1251, 1165, 837 cm^{-1}. UV/Vis (methanol): λ_{max} = 370, 246 nm. ESI-MS: *m*/*z* (%) = 259.1 (100) $[M - H]^-$, 518.8 (12) $[2M - H]^-$. HR-ESI-MS: *m*/*z* $[M + H]^+$ = calcd. for $C_{13}H_{12}N_2O_4$ 261.0870; found: 261.0870.

(H)GFPC-Methyl Acetate Precursor 25: α-Acetamidocinnamic acid (**13**, 10 g, 48.7 mmol) was dissolved in DMF (100 mL), DCC (10.6 g, 1.05 equiv.) was added, and the system was stirred for 30 min at 0 °C and for 1 h at room temperature. Glycine methyl ester hydrochloride (7.96 g, 63.4 mmol 1.3 equiv.) and DIPEA (33.4 mL, 4 equiv.) were added and the system was again stirred overnight. The resulting suspension was filtered, the solid residue was washed with DMF (50 mL), the combined organic layers were reduced to dryness and taken up with ethyl acetate (300 mL), and the organic layer was extracted with saturated $KHCO_3$ solution (3× 100 mL) and HCl solution (0.1 M, 3× 100 mL). The title compound **25** was obtained from the acidic water layer as a yellow solid (2.80 g, 10.1 mmol, 21%) without further purification. The crude product from the basic layer was cleaned by filtration through silica gel (30 g silica gel, ethyl acetate/methanol 9:1 + 0.5% triethylamine, 1 L) to provide further title compound **25** as a yellow solid (3.92 g, 14.2 mmol, 29%). R_F (ethyl acetate/methanol 9:1 + 0.5 triethylamine) = 0.53; m.p. 117 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.91 (s, 1 H, NH), 7.58–7.48 (m, 1 H, N*H*CH_2), 7.37–7.29 (m, 5 H, CH-phenyl), 6.96 (s, 1 H, CH), 4.01 (d, $^3J_{H,H}$ = 5.7 Hz, 2 H, CH_2), 3.68 (s, 3 H, OCH_3), 2.01 (s, 3 H, C*H*$_3$CONH) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.7 (CO), 170.5 (CO), 166.2 (CO), 133.4 (phenyl-C), 130.1–128.5 (CH=*C*–N, 5× phenyl-CH), 52.3 (OCH_3), 41.5 (CH_2), 22.9 (NHCO*C*H$_3$) ppm. IR (KBr): $\tilde{\nu}$ = 3445, 1635, 1376, 1203, 573 cm^{-1}. UV/Vis (methanol): λ_{max} = 208, 281, 327 nm. ESI-MS: *m*/*z* (%) = 299.1 (20) $[M + Na]^+$, 574.8 (100) $[2M + Na]^+$, 275.1 (100) $[M - H]^-$, 550.8 (30) $[2M - H]^-$. HR-ESI-**MS**: *m*/*z* $[M + Na]^+$ = calcd. for $C_{14}H_{16}N_2O_4$ 299.1002; found: 299.1003.

(OAc)GFPC-Methyl Acetate Precursor 26: 2-Acetamido-4-acetoxycinnamic acid (**17**, 10 g, 38 mmol) was dissolved in DMF (100 mL), and DCC (7.84 g, 38 mmol) dissolved in DMF (10 mL) was added slowly (5 min) at 0 °C with stirring in an ice bath. The resulting solution was stirred at 0 °C for 30 min and for 1 h at room temperature, and the formed urea was filtered off and washed with DMF (40 mL). Triethylamine (21.2 mL, 152 mmol, 4.0 equiv.) and glycine methyl ester hydrochloride (6.18 g, 49 mmol, 1.3 equiv.) were added, the DMF solution was stirred overnight, the solvent was removed in vacuo, and the obtained solid was dissolved in HCl (0.1 M, 60 mL) and extracted several times with chloroform (150 mL overall). Silica gel (30 g) was added to the organic layer, which was reduced to dryness, and the product was eluted from the silica gel (ethyl acetate/methanol 95:5 + 2% triethylamine), concentrated, and dried in vacuo. The title compound **26** (8.92 g, 27 mmol, 71%) was obtained as a yellow solid. R_F (ethyl acetate/methanol 95:5 + 2% triethylamine) = 0.29 (product); m.p. 94 °C. ^{1}H NMR (300 MHz, CD_3OD): δ = 7.55 (d, $^3J_{H,H}$ = 8.7 Hz, 2 H, CH-phenyl), 7.22 (s, 1 H, CH), 7.14 (d, $^3J_{H,H}$ = 8.7 Hz, 2 H, CH-phenyl), 4.02 (s, 2 H, CH_2), 3.73 (s, 3 H, OCH_3), 2.27 (s, 3 H, C*H*$_3$CO), 2.11 (s, 3 H, C*H*$_3$CONH) ppm. ^{13}C NMR (75.5 MHz, CD_3OD): δ = 173.3 (CO), 171.8 (CO), 170.9 (CO), 168.3 (CO), 152.7 (phenyl-*C*-OAc), 132.8 (CH=*C*), 131.7 (2× phenyl-CH), 130.6 (CH), 129.9 (phenyl-C), 123.1 (2× phenyl-CH), 52.6 (OCH_3), 42.3 (CH_2), 22.7 (NHCO*C*H$_3$), 20.9 (OCO*C*H$_3$) ppm. IR (KBr): $\tilde{\nu}$ = 3272, 1756, 1658, 1534, 1371, 1205, 1014, 910, 655 cm^{-1}. UV/Vis (methanol): λ_{max} = 358, 281, 216 nm. ESI-MS: *m*/*z* (%) = 357.1 (15) $[M + Na]^+$, 590.8 (100) $[2M + Na]^+$, 1024.3 (20) $[3M + Na]^+$, 333.1 (100) $[M - H]^-$, 378.8 (70) $[M + HCOO]^-$, 666.6 (30) $[2M - H]^-$. HR-ESI-MS: *m*/*z* $[M + H]^+$ = calcd. for $C_{16}H_{18}N_2O_6$ 335.1238; found: 335.1238.

(H)GFPC-Methyl Acetate (27): Under vacuum, (H)GFPC-methyl acetate precursor **25** (2.80 g, 10.1 mmol) was quickly heated up to 220 °C in an oil bath and stirred at 220 °C for 10 min. The crude product was dissolved in DCM and cleaned on silica gel (110 g, ethyl acetate/pentane 1:2), and the title compound **27** was obtained as a yellow solid (1.62 g, 6.3 mmol, 62%). R_F (ethyl acetate/hexane 1:2) = 0.2 (product), 0.0 (starting material); m.p. 112 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.11 (dd, $^3J_{H,H}$ = 8.1 Hz, 2 H, CH-phenyl), 7.43–7.32 (m, 3 H, CH-phenyl), 7.11 (s, 1 H, CH-methylene), 4.37 (s, 2 H, CH_2), 2.93 (s, 3 H, OCH_3), 2.31 (s, 3 H, CH_3) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 170.0 (CO), 168.0 (*C*OOMe), 161.3 (*C*CH_3), 137.9 (*C*CH), 133.9 (C-phenyl), 132.2 (2× CH-phenyl), 130.3 (CH-phenyl), 128.7 (2× CH-phenyl), 128.2 (methylene-CH), 52.8 (OCH_3), 41.2 (CH_2); 15.5 (CH_3) ppm. IR (KBr): $\tilde{\nu}$ = 3500, 1743, 1645, 1561, 1406, 1363, 1232, 1144, 981, 904, 775, 700, 610 cm^{-1}. UV/Vis (methanol): λ_{max} = 348, 290, 234, 209 nm. ESI-MS: *m*/*z* (%) = 257.1 (40) $[M - H]^-$, 259.1 (60) $[M + H]^+$, 281.0 (100) $[M + Na]^+$, 538.8 (95) $[2M + Na]^+$. HR-ESI-MS: *m*/*z* $[M + H]^+$ = calcd. for $C_{14}H_{14}N_2O_3$ 259.1077; found: 259.1077.

(OAc)GFPC-Methyl Acetate (28): Under vacuum, (OAc)GFPC-methyl acetate precursor **26** (5.3 g, 15.9 mmol) was quickly heated up to 200 °C in an oil bath and stirred at 200 °C for 12 min. The crude product was dissolved in DCM (5 mL) and cleaned on silica gel (ethyl acetate/pentane 1:1), and the title compound **28** (2.2 g, 7.0 mmol, 44%) was obtained as a yellow solid. M.p. 75 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.14 (d, $^3J_{H,H}$ = 8.7 Hz, 2 H, CH-phenyl), 7.12 (d, $^3J_{H,H}$ = 8.7 Hz, 2 H, CH-phenyl), 7.07 (s, 1 H, CH), 4.36 (s, 2 H, CH_2), 3.75 (s, 3 H, OCH_3), 2.30 (s, 3 H, C*H*$_3$CO), 2.28 (s, 3 H, CH_3) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 169.9 (CO), 169.0 (CO), 167.9 (CO), 161.5 (NC=N), 151.9 (phenyl-*C*-OAc), 137.9 (CH=*C*), 133.4 (2× phenyl-CH), 131.7 (phenyl-C), 126.9 (*C*H=C), 121.9 (2× phenyl-CH), 52.8 (OCH_3), 41.2 (CH_2), 21.1 (OCO*C*H$_3$), 15.5 (CH_3) ppm. IR (KBr): $\tilde{\nu}$ = 1747, 1646, 1367, 1216, 980, 908, 609 cm^{-1}. UV/Vis (methanol): λ_{max} = 351, 292, 236, 203 nm. ESI-MS: *m*/*z* (%) = 339.1 (90) $[M + Na]^+$, 654.9 (100) $[2M + Na]^+$. HR-ESI-MS: *m*/*z* $[M + H]^+$ = calcd. for $C_{16}H_{16}N_2O_5$ 317.1132; found: 317.1132.

Fmoc-aeg-(H)GFPC-OMe (30): (H)GFPC-Acetic acid (**23**, 0.61 g, 2.5 mmol, 1.3 equiv.), Fmoc-aeg-OMe·HCl[14] (**29**, 0.75 g, 1.9 mmol, 1.0 equiv.), HBTU (0.95 g, 2.5 mmol, 1.3 equiv.), and HOBt (0.52 g, 3.8 mmol, 2.0 equiv.) were dissolved in DMF (25 mL). DIPEA (1.65 mL, 5 equiv.) was added and the resulting solution was stirred for 14 h at room temperature. The solvent was removed in vacuo, and the obtained solid was dissolved in ethyl acetate (100 mL), washed with $NaHCO_3$ (0.5 M, 100 mL), brine (100 mL), saturated NH_4Cl solution (100 mL), and brine (100 mL), and dried with Na_2SO_4. After purification on silica gel (ethyl acetate, 600 mL) title compound **30** was obtained as a yellow solid (0.87 g, 1.5 mmol, 79%). M.p. 165 °C; NMR: two rotamers 2:1. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.10 (d, $^3J_{H,H}$ = 10.8 Hz, 2 H, CH-phenyl), 7.72 (d, $^3J_{H,H}$ = 9.6 Hz, 2 H, CH-Fmoc), 7.57 (t, $^3J_{H,H}$ = 6.9 Hz, 2 H, CH-Fmoc), 7.43–7.24 (m, 7 H, CH-Fmoc, CH-Fmoc, CH-phenyl, CH-phenyl), 7.09 (s, 0.66 H, CH-methylene), 7.07 (s, 0.33 H, CH-methylene), 5.85 (t, $^3J_{H,H}$ = 6.0 Hz, 0.66 H, NH), 5.30 (t, $^3J_{H,H}$ = 5.7 Hz, 0.33 H, NH), 4.50 (d, $^3J_{H,H}$ = 6.3 Hz, 1.3 H, CH_2-Fmoc), 4.36 (d, $^3J_{H,H}$ = 8.7 Hz, 2.7 H, CH_2-Fmoc, CH_2-GFP), 4.19 (t, $^3J_{H,H}$ = 5.4 Hz, 1 H, CH-Fmoc), 3.99 (s, 2 H, C*H*$_2$COOMe), 3.75 (s, 2.0 H, OCH_3), 3.72 (s, 1.0 H, OCH_3), 3.53 (m, 2 H, CH_2–N), 3.40–3.32 (m, 2 H, CH_2–NH), 2.32 (s, 2.0 H, CH_3), 2.15 (s, 1.0 H, CH_3) ppm. ^{13}C NMR (150 MHz, CD_3OD):

first rotamer (major component): δ = 170.4 (COOMe), 169.8 (CO-GFP), 167.1 (CH_2CON), 162.4 (N=*C*–N), 156.6 (O*C*ON), 143.6 (2 × C-Fmoc), 141.3 (2 × C-Fmoc), 138.2 (CH=*C*–N), 134.1 (C-phenyl), 132.2 (2 × CH-phenyl), 130.1 (1 CH-phenyl), 128.6 (2 × CH-phenyl), 127.7 (2 × CH-Fmoc), 127.6 (CH-methylene), 127.0 (2 × CH-Fmoc), 124.9 (2 × CH-Fmoc), 120.0 (2 × CH-Fmoc), 66.6 (CH_2-Fmoc), 52.6 (OCH_3), 48.9 (CH_2–N, CH_2COOMe), 47.3 (CH-Fmoc), 40.9 (CH_2-GFP), 39.2 (CH_2–NH), 15.4 (CH_3) ppm; *second rotamer*: δ = 170.3 (COOMe), 169.8 (CO-GFP), 167.8 (N*C*O–CH_2), 162.4 (N=*C*–N), 156.61 (OCON), 143.8 (2 × C-Fmoc), 141.3 (2 × C-Fmoc), 138.2 (CH=*C*–N), 134.0 (C-phenyl), 132.2 (2 × CH-phenyl), 130.2 (1 × CH-phenyl), 128.6 (2 × CH-phenyl), 127.7 (2 × CH-Fmoc), 127.6 (CH-methylene), 127.0 (2 × CH-Fmoc), 125.1 (2 × CH-Fmoc), 119.9 (2 × CH-Fmoc), 66.8 (CH_2-Fmoc), 53.0 (OCH_3), 48.9 (CH_2–N, CH_2COOMe), 47.1 (CH-Fmoc), 41.5 (CH_2-GFP), 39.2 (CH_2–NH), 15.6 (CH_3) ppm. IR (KBr): $\tilde{\nu}$ = 3412, 2928, 1750, 1722, 1663, 1470, 1210, 1045, 745 cm^{-1}. UV/Vis (methanol): λ_{max} = 347, 299, 289, 264, 210 nm. Fluorescence (glycerin): λ_{exc} = 350 nm, $\lambda_{max,\ em}$ = 413, 436, 477 nm. ESI-MS: *m*/*z* (%) = 581.2 (25) $[M + H]^+$, 1182.8 (100) $[2M + Na]^+$, 624.9 (100) $[M + TFA]^-$, 1204.7 (70) $[2M + TFA]^-$. HR-ESI-MS: *m*/*z* $[M + H]^+$ = calcd. for $C_{33}H_{32}N_4O_6$ 581.2395; found: 581.2396.

Fmoc-aeg-(OH)GFPC-OMe (31): (OH)GFPC-Acetic acid (**24**, 508 mg, 1.95 mmol, 1.1 equiv.), Fmoc-aeg-OMe·HCl[14] (**29**, 694 mg, 1.77 mmol, 1.0 equiv.), and HBTU (740 mg, 1.95 mmol, 1.1 equiv.) were dissolved in DMF (15 mL). DIPEA (1.22 mL, 920 mg, 7.1 mmol, 4 equiv.) was added, the resulting solution was stirred for 2 d, the solvent was removed under reduced pressure, and the residue was dissolved in ethyl acetate (100 mL), washed (3 × 100 mL 0.5 M NH_4Cl, 3 × 100 mL 0.5 M $NaHCO_3$, 1 × 100 mL brine), and dried with Na_2SO_4. The crude product was purified on silica gel (200 mL, ethyl acetate) and the title compound **31** (0.223 g, 0.37 mmol, 21%) was obtained as a yellow solid. M.p. 174 °C; NMR: two rotamers 2:1. *First rotamer* (major component): ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.89 (d, $^3J_{H,H}$ = 8.7 Hz, 2 H, phenyl-CH), 7.70 (d, $^3J_{H,H}$ = 6.9 Hz, 2 H, Fmoc-CH), 7.57 (d, $^3J_{H,H}$ = 6.6 Hz, 2 H, Fmoc-CH), 7.35 (t, $^3J_{H,H}$ = 6.9 Hz, 2 H, Fmoc-CH), 7.27 (t, $^3J_{H,H}$ = 6.6 Hz, 2 H, Fmoc-CH), 6.95 (s, 1 H, CH=C), 6.76 (d, $^3J_{H,H}$ = 8.7 Hz, 2 H, phenyl-CH), 5.91 (t, $^3J_{H,H}$ = 6.0 Hz, 1 H, NH), 4.48 (d, $^3J_{H,H}$ = 6.3 Hz, 2 H, Fmoc-CH_2), 4.35 (s, 2 H, GFP-CH_2), 4.19 (t, $^3J_{H,H}$ = 6.3 Hz, 1 H, Fmoc-CH), 3.98 (s, 2 H, CH_2COOMe), 3.71 (s, 3 H, OCH_3), 3.52–3.49 (m, 2 H, CH_2–N), 3.41–3.39 (m, 2 H, CH_2–NH), 2.11 (s, 3 H, CCH_3) ppm. ^{13}C NMR (150 MHz, CD_3OD): δ = 170.4 (GFP-CO), 169.7 (*C*OOMe), 167.7 (GFP-CH_2–*CO*), 160.4 (C=N), 158.7 (phenyl-*C*–OH), 156.8 (Fmoc-CO), 143.6 (2 × Fmoc-C), 141.3 (2 × Fmoc-C), 135.7 (*C*=CH), 134.5 (2 × phenyl-CH), 128.7 (*CH*C), 127.8 (2 × Fmoc-CH), 127.1 (2 × Fmoc-CH), 126.4 (phenyl-*C*–CH), 124.9 (2 × Fmoc-CH), 120.0 (2 × Fmoc-CH), 115.9 (2 × phenyl-CH), 66.7 (Fmoc-CH_2), 52.6 (OCH_3), 50.0 (CH_2COOMe), 49.0 (CH_2–N), 47.2 (Fmoc-CH), 41.0 (GFP-CH_2), 39.2 (CH_2–NH), 15.4 (CH_3C) ppm. *Second rotamer*: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.89 (d, $^3J_{H,H}$ = 8.7 Hz, 2 H, phenyl-CH), 7.72 (d, $^3J_{H,H}$ = 6.9 Hz, 2 H, Fmoc-CH), 7.55 (d, $^3J_{H,H}$ = 6.6 Hz, 2 H, Fmoc-CH), 7.35 (t, $^3J_{H,H}$ = 6.9 Hz, 2 H, Fmoc-CH), 7.27 (t, $^3J_{H,H}$ = 6.6 Hz, 2 H, Fmoc-CH), 6.95 (s, 1 H, CH=C), 6.76 (d, $^3J_{H,H}$ = 8.7 Hz, 2 H, phenyl-CH), 5.39 (t, $^3J_{H,H}$ = 5.7 Hz, 1 H, NH), 4.35 (s, 2 H, GFP-CH_2), 4.33 (d, $^3J_{H,H}$ = 6.3 Hz, 2 H, Fmoc-CH_2), 4.19 (t, $^3J_{H,H}$ = 6.3 Hz, 1 H, Fmoc-CH), 4.16 (s, 2 H, CH_2COOMe), 3.71 (s, 3 H, OCH_3), 3.52–3.49 (m, 2 H, CH_2–N), 3.37–3.31 (m, 2 H, CH_2–NH), 2.27 (s, 3 H, CCH_3) ppm. ^{13}C NMR (150.8 MHz, CD_3OD): δ = 170.3 (GFP-CO), 169.7 (*C*OOMe), 167.7 (GFP-CH_2–*CO*), 160.4 (C=N), 158.7 (phenyl-*C*–OH), 156.8 (Fmoc-CO), 143.8 (2 × Fmoc-C), 141.3 (2 × Fmoc-C), 135.5 (*C*=CH), 134.5 (2 × phenyl-CH), 129.0 (*CH*C), 127.7 (2 × Fmoc-CH), 127.1 (2 × Fmoc-CH), 126.4 (phenyl-*C*–CH), 125.0 (2 × Fmoc-CH), 120.0 (2 × Fmoc-CH), 115.9 (2 × phenyl-CH), 66.9 (Fmoc-CH_2), 53.0 (OCH_3), 50.0 (CH_2COOMe), 49.0 (CH_2–N), 47.2 (Fmoc-CH), 41.5 (GFP-CH_2), 38.9 (CH_2–NH), 15.2 (CH_3C) ppm. IR (KBr): $\tilde{\nu}$ = 3405, 1703, 1601, 1513, 1448, 1252, 750 cm^{-1}. UV/Vis (methanol): λ_{max} (OD) = 370 (0.83), 300 (0.46), 265 (0.8), 221 (1.0) → same sample: UV/Vis (+ 0.01% NEt_3): λ_{max} (OD) = 431 (0.86), 300 (0.43), 264 (0.88), 227 (3.3). Fluorescence (glycerin + 0.01% NEt_3): $\lambda_{exc.}$ = 430 nm, $\lambda_{em.}$ = 499 nm. ESI-MS: *m*/*z* (%) = 597.3 (40) $[M + H]^+$, 1193.4 (100) $[2M + H]^+$. HR-ESI-**MS**: *m*/*z* $[M + H]^+$ = calcd. for $C_{33}H_{32}N_4O_7$ 597.2344; found: 597.2346.

PNA Oligomer Ac-t-t-t-t-(H)GFPC-g-g-c-g-g-c-Lys-Lys-Gly-NH_2 (32): The solid-phase peptide synthesis was performed on a 5 μM scale in a plastic syringe by the Fmoc/Bhoc strategy on Sieberamide resin as described elsewhere.[14] Commercially available amino acids Fmoc-aeg-thymine-OH, Fmoc-aeg-guanine(Bhoc)-OH, Fmoc-aeg-cytosine(Bhoc)-OH, Fmoc-Lys(Mtt)-OH, and Fmoc-Gly-OH on Sieberamide resin (0.12 mmol·g^{-1}) were used for the oligomer synthesis with use of the HBTU/HATU activation technique. Double coupling was performed in cases involving the coupling of guaninyl amino acids following a thyminyl or guaninyl amino acid. Double coupling was not required for introduction of Fmoc-aeg-(H)GFPC-OH (**7**). Oligomer **32** was cleaved from the resin by continuous flow with 2% trifluoroacetic acid, 4% triethylsilane in 1,1,1,3,3,3-hexafluoropropan-2-ol/dichloromethane 1:2 and was obtained (1.5 mg, 9% yield) after preparative HPLC purification. HR-ESI-MS: $[M]^+$: calcd. for $C_{141}H_{184}N_{64}O_{41}$ 3429.4280; found: 3429.4306, $[M + H]^+$: calcd. 3430.4329; found: 3430.4329, $[M + 3H]^{3+}$: calcd. 1144.1500; found: 1144.1510, $[M + 4H]^{4+}$: calcd. 858.3643; found: 858.3647; HPLC: analytical: 10% → 40% in 30 min (Grom25), t_R = 26.00 min. UV: ε_{260nm} = 96.9·10^3 [approximated by nearest neighbor approach with ε_{260nm} = 9.0·10^3 for the (H)GFP chromophore]. UV/Vis (pH 6.85, phosphate buffer): λ_{max} (ε/10^3) = 350 (9.0), 260 (97.0), 204 (204) nm. UV-melting: pairing with complementary DNA **33** (5′-G-C-C-G-C-C-A-A-A-A-A-3′, ε_{260nm} = 110.5·10^3) each 3 μM in 10 mm phosphate buffer pH = 6.85, NaCl (0.10 M): H_{260nm} = 10% at T_m = 71.0 °C, heating–cooling hysteresis 3 °C; **CD**: pairing with complementary DNA **33** each 3 μm in phosphate buffer (10 mm) pH = 6.85, NaCl (0.10 M): temperature-dependent band at 265 nm (10 mdeg at 20 °C), melting temperature ca. 75 °C; pairing with complementary DNA **33** each 30 μm in 10 mm phosphate buffer pH = 6.85, NaCl (0.10 M): temperature-dependent band at 293 nm (3 mdeg at 20 °C) and 347 nm (–3 mdeg, 20 °C); fluorescence: oligomer **32** with and without complementary DNA **33** (5′-G-C_2–G–C_2–A_5-3′) each 30 μm in phosphate buffer (10 mm) pH 6.85, NaCl (0.10 M); excitation: 350 nm (OD = 0.27), 20 nm bandwidth, emission: single strand 413, 441, 510 nm. pairing complex: 416, 440, 505 nm, quantum yield < 0.5%.

Abbreviations: aeg = *N*-(2-aminoethyl)glycine, Bhoc = benzhydryloxycarbonyl, Boc = *tert*-butyloxycarbonyl, DAP = 2-aminopropionic acid, DBU = 1,3-diazabicyclo[5.4.0]undecane, Fmoc = 9-fluorenylmethoxycarbonyl, GFPC = Green Fluorescent Protein chromophore, HFIP = 1,1,1,3,3,3-hexafluoropropan-2-ol, HATU = *O*-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, HOAt = 1-hydroxy-7-azabenzotriazole, Mtt = monomethyltrityl, PNA = peptide nucleic acid, OD = optical density, TES = triethylsilane, TFA = trifluoroacetic acid, TFMSA = trifluoromethanesulfonic acid, Z = benzyloxycarbonyl.

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