Synthesis of Model Chromophores Related to the Gold Fluorescent Protein (GdFP)

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Abstract: The two model chromophores **2** and **3** for the core **1** of the gold fluorescent protein (GdFP) were synthesized from commercially available 2-methyl-3-nitroaniline (**4**) in six synthetic steps and overall yields of 13% and 8%, respectively. The key step of the sequence is the chemoselective, reductive introduction of the amino group after assembly of the *Z*-configured 5-(indol-3-ylmethylene)imidazolin-4-one skeleton of the chromophore. Compound (*Z*)-**2** was shown to undergo a light-initiated *E*/*Z*-isomerization, which allows access also to its *E*-isomer.

Key words: aldol reactions, bioorganic chemistry, heterocycles, indoles, photochemistry

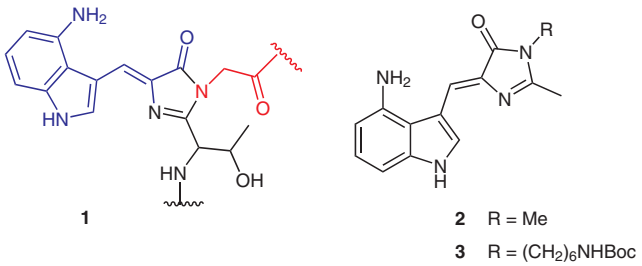

Figure 1 The structure of the chromophore **1** in GdFP as formed from the amino acid triad threonine/4-aminotryptophan/glycine and the structure of the model chromophores **2** and **3** synthesized in this study

The green fluorescent protein (GFP) has revolutionized almost all areas of the biological sciences.[1] The visualization of proteins and protein arrays by its fluorescence has led to literally hundreds of applications in biology, biochemistry and medicine.[2] Biosynthetically, the in vivo formation of the GFP chromophore from the amino acid sequence serine/tyrosine/glycine is well understood[3] and model chromophores of GFP and related proteins have been previously prepared by chemical synthesis.[4]

The search for new fluorescent protein probes is – among others – driven (a) by the desired variation of the fluorescence wavelength at similar excitation wavelengths and (b) by the need for additional information to be collected by the fluorescent probe, e.g., about the polarity of its biological environment. In this respect the recent discovery of a gold fluorescent protein (GdFP) by Budisa et al. is remarkable.[5] The protein was generated by selective pressure incorporation[6] of the non-canonical amino acid 4-aminotryptophan. It contains the chromophore **1** (Figure 1), which is formed from the protein sequence threonine/4-aminotryptophan/glycine. The protein exhibits in aqueous solution an absorption maximum at 466 nm (20 °C, pH 8) and an emission maximum at 574 nm[5] with a remarkably high Stokes shift of ca. 4000 cm^{-1}. This property invites a closer inspection of the chromophore and its photophysical behavior. The 5-(alkylidene)imidazolin-4-one **2** was considered as a reasonable model to elucidate the origin of the Stokes shift and to further study the unrestricted chromophore under varying conditions. In addition, model compound **3** was chosen as a synthetic target because it would allow the incorporation of the chromophore into a given protein via an amide linkage. The syntheses of compounds **2** and **3** are described in this paper.

While there is precedence for the Erlenmeyer azlactone synthesis[7,8] of indoles[9] and the subsequent transformation into the respective imidazolin-4-one,[4,10] the appropriate introduction of the amino group was an open question. We found that it is beneficial to introduce the amino group reductively at the very end of the reaction sequence (Scheme 1). This route allowed us to start from commercially available 2-methyl-3-nitroaniline (**4**) and to follow the procedure by Bergman et al.[11] for the preparation of aldehyde **6** via 4-nitroindole (**5**). Despite extensive optimization the formation of azlactone **7** proceeded only in moderate yields. The best yield was obtained if acetylglycine, NaOAc and Ac_2O were stirred for an hour at 80 °C prior to addition of aldehyde **6**.

The concomitant N-acetylation of indole in the azlactone synthesis could be suppressed if $Ca(OAc)_2$ instead of NaOAc was used as a base.[9e] Yields were lower, however, and we consequently decided to employ the NaOAc method[9b] with the N-deacetylation being likely to occur upon subsequent treatment with amines. Azlactone **7** was obtained as a single diastereoisomer, to which the *Z*-configuration was assigned based on analogy to previous work.[9,12] Further evidence for this assignment was obtained in the course of the conversion of azlactone **7** into products **8** and **9** (vide infra). For our purpose methylamine and *N-tert*-butoxycarbonyl(Boc) protected 1,6-diaminohexane[13] were used as amine nucleophiles. Since the amines not only displace the oxygen atom in the heterocycle but should also deacetylate the indole fragment they were used in excess (3.5 equiv). Yields for imidazo-

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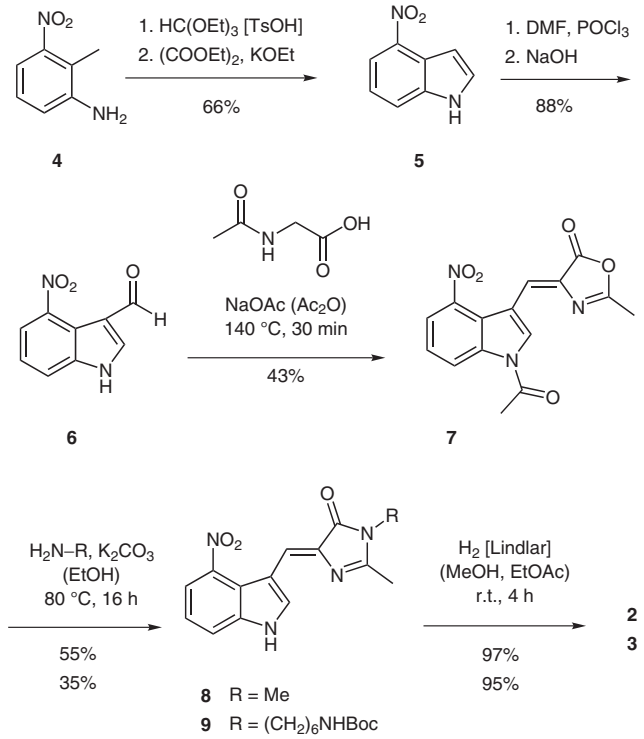


Scheme 1 Synthesis of model compounds **2** and **3** starting from commercially available nitroaniline **4**

lin-4-ones **8** and **9** were expectedly in the moderate range.[4,10] The reaction proceeds stepwise, i.e., via nucleophilic amine-induced azlactone ring opening and subsequent ring closure to the imidazolinone. Using *N*-methylamine as amine, ring-opened intermediate **10** (Figure 2) could be isolated if the reaction was stopped after four hours. The assignment of the two distinct amide NH protons was possible and NOE experiments established the spatial proximity of the NHAc group and the proton at C-2 of the indole.

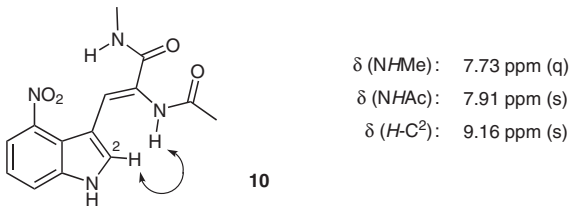


Figure 2 Chemical shift data for the relevant protons in compound **10** and observed NOE contact

The reduction of the nitro group was eventually conducted with hydrogen in the presence of Lindlar catalyst (5% $Pd/CaCO_3/Pb$). Other attempts to produce 4-aminoindoles **2** and **3** from the nitroindoles **8** and **9** met with little success. Reduction experiments with hydrogen and other catalysts or with $SnCl_2$ in EtOH and EtOAc led to partial or complete degradation.

Product **2** was obtained as intensely red colored solid, and **3** as equally intense oil. They are well soluble in organic solvents and UV spectra of compound **2** were recorded (Figure 3). The compound exhibits a hypsochromic extinction maximum relative to GdFP (Tris-buffer: 20 mM tris(hydroxymethyl)aminomethane HCl, pH 8) with the hypsochromic shift depending on the solvent. Absorption maxima were determined to be at 417 nm in MeCN, at 430 nm in H_2O and at 442 nm in EtOH as the solvent. The peak width at half-height of the absorption band is about 4700 cm^{-1}. It is slightly larger than the peak width at half-height of the GdFP band but independent of the solvent. Similarly, the extinction coefficient ε is identical in EtOH and MeCN (ca. 10000 L mol^{-1} cm^{-1}). The fluorescence properties of model compound **2** are currently being studied.

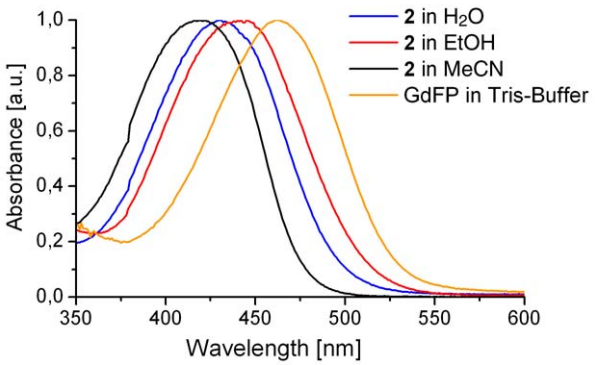
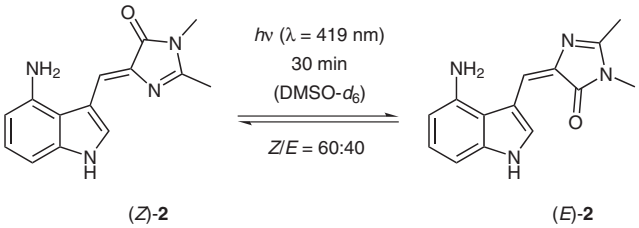


Figure 3 Comparison between the normalized absorption spectra of GdFP and of the model chromophore **2** in various solvents

Upon irradiation at 419 nm (irradiation source: RPR 4190 Å) product (*Z*)-**2** underwent geometrical isomerization into the corresponding *E*-isomer (Scheme 2). Under our conditions (DMSO-d_6 as solvent, 35 °C) a photostationary state was reached after 30 minutes. The *Z*/*E* ratio was determined by NMR as 60:40. There was no indication for other photochemical processes.



Scheme 2 *E*/*Z*-Isomerization of compound (*Z*)-**2**

In conclusion, the model chromophores **2** and **3** were obtained by conventional condensation and functional group transformation chemistry. The azlactone formation and nitro group reduction were closely studied. Optimized conditions are reported for these transformations. Further photophysical and biophysical data of the chromophores will be reported in due course.

All reactions involving water-sensitive chemicals were carried out in flame-dried glassware with magnetic stirring under argon. All solvents, EtOAc, CH_2Cl_2 and MeOH for column chromatography

were distilled prior to use. Acetylglycine was prepared from glycine according to literature precedence.[14] *N*-(*tert*-Butoxycarbonyl)-1,6-diaminohexane was obtained from 1,6-diaminohexane.[13] All other chemicals were commercially available and were used without further purification.

TLC: Merck glass sheets (0.25 mm silica gel 60, F_{254}), eluent given in brackets. Detection by UV or coloration with cerium ammonium molybdate (CAM). NMR: Bruker AV-250, AV-360, AV-500. ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 at ambient temperature, unless stated otherwise. Chemical shifts are reported relative to tetramethylsilane as internal standard. Apparent multiplets which occur as a result of the accidental equality of coupling constants of magnetically nonequivalent protons are marked as virtual (virt.). The multiplicities of the ^{13}C NMR signals were determined by DEPT experiments. IR: PerkinElmer 1600 FT-IR. MS: Finnigan MAT 8200 (EI). UV/Vis: PerkinElmer Lambda 9, Lambda 35. Flash chromatography was performed on silica gel 60 (Merck, 230–400 mesh) (ca. 50 g for 1 g of material to be separated) with the indicated eluent.

4-(*N*-Acetyl-4-nitroindol-3-ylmethylene)-2-methyloxazolin-5-one (7)

A suspension of acetylglycine[14] (358 mg, 3.06 mmol) and NaOAc (233 mg, 2.84 mmol) in Ac_2O (2.50 mL, 2.72 g, 26.6 mmol) was stirred for 1 h at 80 °C. 3-Formyl-4-nitroindole (**6**; 500 mg, 2.63 mmol) was added and the mixture was stirred at 140 °C for 30 min. The dark red mixture was cooled overnight in the refrigerator (4 °C). The brownish precipitate was filtered, washed with H_2O and dried in vacuo at 50 °C. Purification by flash chromatography (CH_2Cl_2–MeOH, 99:1) yielded 306 mg (43%) of azlactone **7** as yellow needles; mp 228–235 °C (dec.); R_f = 0.84 (CH_2Cl_2–MeOH, 95:5).

IR (KBr): 1799 (s), 1722 (s), 1650 (s), 1567 (m), 1519 (s), 1341 (s), 965 (m), 794 (m), 747 (m), 666 cm^{-1} (m).

^{1}H NMR (360 MHz): δ = 2.40 (s, 3 H), 2.79 (s, 3 H), 7.41 (s, 1 H), 7.63 (virt. t, *J* = 8.2 Hz, 1 H), 8.08 (dd, *J* = 8.2, 0.9 Hz, 1 H), 8.80 (dd, *J* = 8.2, 0.9 Hz, 1 H), 8.98 (s, 1 H).

^{13}C NMR (90.6 MHz): δ = 15.4 (q), 24.1 (q), 112.2 (s), 120.1 (s), 121.0 (d), 121.7 (d), 122.3 (d), 125.5 (d), 131.4 (s), 135.8 (d), 136.8 (s), 142.7 (s), 166.3 (s), 166.7 (s), 169.8 (s).

MS (EI, 70 eV): *m*/*z* (%) = 313 (39, [M$^+$]), 271 (40), 225 (6), 201 (13), 184 (13), 154 (14), 145 (14), 44 (19), 43 (100).

HRMS (EI): *m*/*z* calcd for $C_{15}H_{11}N_3O_5$: 313.0699; found: 313.0701.

2,3-Dimethyl-5-(4-nitroindol-3-ylmethylene)imidazolin-4-one (8)

K_2CO_3 (517 mg, 3.74 mmol) was added to a suspension of azlactone **7** (531 mg, 1.70 mmol) and methylamine (40% in H_2O, 0.51 mL, 459 mg, 5.94 mmol) in EtOH (17 mL). The mixture was stirred for 16 h at 80 °C. After addition of H_2O (25 mL), the dark red solution was extracted with CH_2Cl_2 (5 × 25 mL). The combined organic layers were washed with brine (75 mL), dried ($MgSO_4$) and filtered. The product was concentrated in vacuo and purified by flash chromatography (CH_2Cl_2–MeOH, 95:5). Compound **8** was obtained as a dark red solid; yield: 265 mg (55%); mp 240–248 °C (dec.); R_f = 0.18 (CH_2Cl_2–MeOH, 95:5).

IR (KBr): 3155 (m) 1677 (s), 1625 (s), 1560 (m), 1351 (s), 1326 (s), 1138 (s), 775 (m), 735 (m), 558 cm^{-1} (m).

^{1}H NMR (360 MHz): δ = 2.34 (s, 3 H), 3.09 (s, 3 H), 7.37 (virt. t, *J* = 8.0 Hz, 1 H), 7.44 (s, 1 H), 7.91 (dd, *J* = 8.0, 0.9 Hz, 1 H), 8.80 (dd, *J* = 8.0, 0.9 Hz, 1 H), 8.93 (s, 1 H), 12.65 (s, 1 H).

^{13}C NMR (90.6 MHz): δ = 15.2 (q), 26.1 (q), 109.0 (s), 117.8 (s), 118.6 (d), 119.0 (d), 119.4 (d), 121.4 (d), 134.9 (s), 136.4 (d), 138.6 (s), 142.2 (s), 161.2 (s), 169.1 (s).

MS (EI, 70 eV): *m*/*z* (%) = 284 (55, [M$^+$]), 238 (86), 232 (11), 154 (13), 130 (18), 56 (100), 44 (11).

HRMS (EI): *m*/*z* calcd for $C_{14}H_{12}N_4O_3$: 284.0909; found: 284.0910.

UV/Vis (MeCN): λ_{max} (log ε) = 377 nm (4.35).

Isolation of 2-Acetylamino-3-(indol-3-yl)-*N*-methylacrylamide (10)

Upon premature (4 h) addition of H_2O to the above-mentioned reaction mixture (preparation of **8**), a bright yellow solid precipitated, which was not soluble neither in the aqueous nor in the organic layer. The precipitate was filtered, washed with H_2O and CH_2Cl_2, and dried in vacuo; mp >250 °C (dec.).

^{1}H NMR (250 MHz): δ = 2.00 (s, 3 H), 2.67 (d, *J* = 4.5 Hz, 3 H), 7.32 (t, *J* = 8.2 Hz, 1 H), 7.42 (s, 1 H), 7.73 (q, *J* = 4.5 Hz, 1 H), 7.82 (d, *J* = 8.2 Hz, 1 H), 7.84 (d, *J* = 8.2 Hz, 1 H), 7.91 (s, 1 H), 9.16 (s, 1 H), 12.28 (br s, 1 H).

^{13}C NMR (62.9 MHz): δ = 23.0 (q), 26.1 (q), 107.8 (s), 117.6 (d), 117.8 (s), 118.3 (d), 120.8 (d), 122.3 (d), 126.2 (s), 130.7 (d), 138.1 (s), 142.1 (s), 165.3 (s), 169.4 (s).

3-[*N*-(*tert*-Butoxycarbonyl)-6-aminohexyl]-2-methyl-5-(4-nitroindol-3-ylmethylene)imidazolin-4-one (9)

Following the procedure for imidazolinone **8**, azlactone **7** (407 mg, 1.30 mmol) was reacted with *N*-(*tert*-butoxycarbonyl)-1,6-diaminohexane[13] (986 mg, 4.55 mmol) and K_2CO_3 (400 mg, 2.89 mmol) in EtOH (12 mL). After purification by flash chromatography (EtOAc), **9** (211 mg, 35%) was obtained as a yellow solid; mp 125–144 °C; R_f = 0.39 (CH_2Cl_2–MeOH, 95:5).

IR (KBr): 3294 (w), 3159 (m), 2972 (m), 2930 (s), 2858 (m), 1681 (s), 1631 (s), 1558 (m), 1517 (s), 1355 (s), 1324 (s), 1170 (m), 785 (m), 736 (m), 611 cm^{-1} (m).

^{1}H NMR (360 MHz): δ = 1.23–1.31 (m, 4 H), 1.36 (br s, 11 H), 1.48–1.59 (m, 2 H), 2.36 (s, 3 H), 2.89 (q, *J* = 6.4 Hz, 2 H), 3.54 (t, *J* = 7.2 Hz, 2 H), 6.69–6.78 (m, 1 H), 7.38 (virt. t, *J* = 7.9 Hz, 1 H), 7.45 (s, 1 H), 7.91 (dd, *J* = 7.9, 0.9 Hz, 1 H), 7.93 (dd, *J* = 7.9, 0.9 Hz, 1 H), 8.93 (s, 1 H), 12.65 (s, 1 H).

^{13}C NMR (90.6 MHz): δ = 15.3 (q), 25.9 (t), 28.2 (q), 29.3 (t), 39.7 (t), 77.2 (s), 109.1 (s), 117.8 (s), 118.7 (d), 119.1 (d), 119.6 (d), 121.5 (d), 134.8 (s), 136.5 (d), 138.7 (s), 142.3 (s), 160.8 (s), 169.2 (s).

MS (EI, 70 eV): *m*/*z* (%) = 469 (1, [M$^+$]), 439 (1), 413 (1), 368 (1), 323 (1), 266 (1), 241 (2), 224 (3), 197 (4), 162 (5), 132 (3), 116 (5), 112 (4), 98 (4), 86 (3), 56 (33), 44 (34), 41 (100), 39 (31).

HRMS (EI): *m*/*z* calcd for $C_{24}H_{31}N_5O_5$: 469.2325; found: 469.2370.

UV/Vis (MeCN): λ_{max} (log ε) = 377 nm (4.46).

5-(4-Aminoindol-3-ylmethylene)-2,3-dimethylimidazolin-4-one [(*Z*)-2]

Compound **8** (150 mg, 528 μmol) was dissolved in MeOH (2.5 mL) and EtOAc (2.5 mL). Lindlar catalyst (5% Pd/$CaCO_3$/Pb, 26.4 μmol Pd; 56.0 mg) was added and the mixture was stirred vigorously for 4 h under a H_2 atmosphere (1 atm). The mixture was filtered over Celite and washed thoroughly with MeOH (200 mL). The solvent was removed in vacuo and the residue was purified by silica gel chromatography (EtOAc). Compound **2** was obtained as a red solid; yield: 131 mg (97%); mp 240 °C (dec.); R_f = 0.34 (EtOAc).

IR (KBr): 3420 (w), 3344 (m), 3160 (m), 1668 (m), 1627 (s), 1499 (s), 1426 (s), 1329 (s), 780 (m), 612 (m), 553 cm^{-1} (m).

^{1}H NMR (360 MHz): δ = 2.32 (s, 3 H), 3.09 (s, 3 H), 5.09 (s, 2 H), 6.43 (d, *J* = 7.6 Hz, 1 H), 6.77 (d, *J* = 7.6 Hz, 1 H), 6.89 (virt. t, *J* = 7.6 Hz, 1 H), 7.56 (s, 1 H), 8.49 (d, *J* = 1.8 Hz, 1 H), 11.69 (s, 1 H).

^{13}C NMR (90.6 MHz): δ = 15.2 (q), 26.1 (q), 102.2 (d), 107.6 (d), 111.4 (s), 115.2 (s), 121.9 (d), 123.2 (d), 131.7 (d), 133.3 (s), 137.5 (s), 142.6 (s), 158.7 (s), 168.9 (s).

MS (EI, 70 eV): *m/z* (%) = 254 (72, [M$^+$]), 232 (9), 199 (38), 182 (5), 154 (15), 73 (6), 56 (100), 44 (23).

HRMS (EI): *m/z* calcd for $C_{14}H_{14}N_4O$: 254.1168; found: 254.1172.

UV/Vis (MeCN): λ_{max} (log ε) = 419 nm (3.99).

UV/Vis (EtOH): λ_{max} (log ε) = 438 nm (4.00).

_E/Z_-Isomerization of 5-(4-Aminoindol-3-ylmethylene)-2,3-dimethylimidazolin-4-one

(*Z*)-**2** was dissolved in DMSO-d_6 in a NMR tube and irradiated for 30 min at 419 nm (light source: RPR 4190 Å, 35 °C). An equilibrium was established between the two isomers, with an *E*/*Z* ratio of 40:60.

(*Z*)-**2** (60%)

^{1}H NMR (360 MHz): δ = 2.32 (s, 3 H), 3.09 (s, 3 H), 5.09 (s, 2 H), 6.43 (d, *J* = 7.6 Hz, 1 H), 6.77 (d, *J* = 7.6 Hz, 1 H), 6.89 (virt. t, *J* = 7.6 Hz, 1 H), 7.56 (s, 1 H), 8.49 (d, *J* = 1.8 Hz, 1 H), 11.69 (s, 1 H).

(*E*)-**2** (40%)

^{1}H NMR (360 MHz): δ = 2.25 (s, 3 H), 3.14 (s, 3 H), 5.12 (s, 2 H), 6.46 (d, *J* = 7.6 Hz, 1 H), 6.79 (d, *J* = 7.6 Hz, 1 H), 6.91 (virt. t, *J* = 7.6 Hz, 1 H), 7.96 (s, 1 H), 9.38 (d, *J* = 2.8 Hz, 1 H), 11.79 (s, 1 H).

5-(4-Aminoindol-3-ylmethylene)-3-[*N*-(*tert*-butoxycarbonyl)-6-aminohexyl]-2-methylimidazolin-4-one (3)

Following the procedure for chromophore **2**, imidazolinone **9** (130 mg, 277 μmol) and Lindlar catalyst (5% Pd/$CaCO_3$/Pb, 13.9 μmol Pd; 29.5 mg) in MeOH (1.5 mL) and EtOAc (1.5 mL) were stirred vigorously for 4 h under a H_2 atmosphere. After work-up and purification by flash chromatography (EtOAc), chromophore **3** (117 mg, 95%) was obtained as a dark red oil; R_f = 0.54 (EtOAc–MeOH, 90:10).

^{1}H NMR (360 MHz): δ = 1.18–1.29 (m, 4 H), 1.37 (s, 11 H), 1.47–1.60 (m, 2 H), 2.34 (s, 3 H), 2.89 (q, *J* = 6.5 Hz, 2 H), 3.54 (t, *J* = 7.2 Hz, 2 H), 5.11 (br s, 1 H), 6.44 (dd, *J* = 7.7, 0.8 Hz, 1 H), 6.74 (s, 2 H), 6.78 (dd, *J* = 7.7, 0.8 Hz, 1 H), 6.89 (virt. t, *J* = 7.7 Hz, 1 H), 7.57 (s, 1 H), 8.49 (d, *J* = 2.7 Hz, 1 H), 11.72 (d, *J* = 2.7 Hz, 1 H).

^{13}C NMR (90.6 MHz): δ = 15.2 (q), 25.9 (t), 26.1 (t), 28.2 (q), 28.8 (t), 29.1 (t), 29.4 (t), 39.7 (t), 77.2 (s), 102.2 (d), 107.7 (d), 111.5 (s), 115.3 (s), 122.1 (d), 123.2 (d), 131.8 (d), 133.1 (s), 137.6 (s), 142.6 (s), 155.6 (s), 158.2 (s), 168.9 (s).

HRMS (EI): *m/z* calcd for $C_{24}H_{33}N_5O_3$: 439.2583; found: 439.2576.

UV/Vis (MeCN): λ_{max} (log ε) = 421 nm (4.20).

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