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# Fluorophores related to the green fluorescent protein

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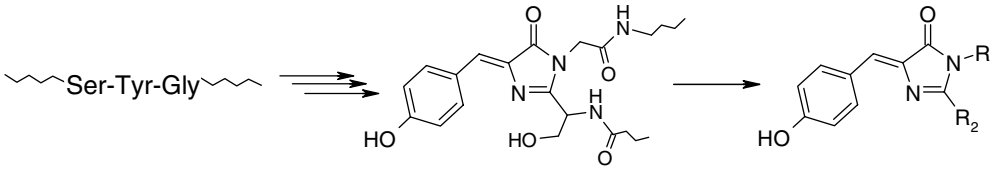
**Abstract**—Imidazolin-5-one derivatives and isosteres (oxazolinones, butenolides, and pyrrolinones) of the 4-hydroxybenzylidene-imidazolinone chromophore of the GFP have been synthesized and their photophysical properties have been investigated.


The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has found many applications in molecular biology, because the chromophore is an integral part of the protein sequence. Consequently, in situ expression of GFP results in an intrinsically fluorescent protein that can be fused to another protein and used to report on protein expression and transport within cells.[1–3] Wild-type GFP autocatalytically generates the 4-hydroxybenzylidene-imidazolinone chromophore, shown in Figure 1, via a posttranslational internal cyclization of the Ser[65]-Tyr[66]-Gly[67] tripeptide followed by 1,2-dehydrogenation of the tyrosine.[4,5] The absorption spectrum of wild-type GFP contains two main bands at about 395 and 475 nm, and these are usually assigned to the neutral and the anionic forms of the chromophore, respectively. The emission spectrum shows an intense fluorescence emission band, centered at 508 nm with a high quantum yield ($\Phi = 0.79$).[6] It is now well known that when it is isolated from the protein, the chromophore totally loses its fluorescence.[4,6] Optimization of the fluorescence properties of the chromophore might lead to efficient fluorescent dyes. In a previous paper[7] we described the photophysical properties of imidazolinone derivatives with significant fluorescence quantum yields. The aim of this work is the synthesis and the study of the spectroscopic properties of some hydrophobic imidazolin-5-ones (compounds **1**, $R_2 = Ar$) and their isosteres (oxazolinones **2**, pyrrolinones **3**, and butenolides **4**), when compared with the parent chromophore illustrated in Figure 1.

The oxazolinones **2** were synthesized from aroylglycine[8] and aromatic aldehydes in sodium acetate–acetic anhydride mixture, whereas butenolides **4** were synthesized




GFP chromophore

**Figure 1.** Structural formula of the wild-type GFP chromophore and its derivatives.

*Keywords*: Green fluorescent protein; Imidazolinones; Oxazolinones; Butenolides; Pyrrolinones; Fluorescence.
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**Table 1.** Synthesis of compounds **6** with R = *i*-Bu

| Compds | $R_1$ | $R_2$ | Method A yield (%) | Method B yield (%) |
|---|---|---|---|---|
| **6b** | CN | H | 10 | a |
| **6g** | $CO_2Me$ | 4-$NO_2$ | 28 | a |
| **6h** | $CO_2Me$ | 4-CN | 23 | 62 |
| **6i** | CN | 4-NHAc | 20 | a |
| **6j** | $NO_2$ | H | 30 | 67 |
| **6k** | $NO_2$ | 4-OMe | a | 76 |

a: not performed.

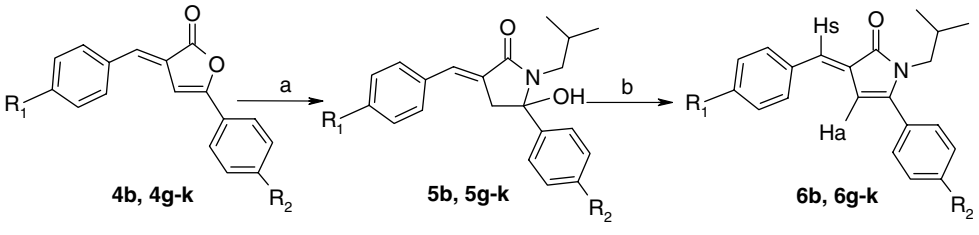
from β-aroylpropionic acids[9] with aromatic aldehydes in sodium acetate–acetic anhydride mixture. Imidazolin-5-ones derivatives were prepared as described in our previous papers.[7,10] The butenolides **4** were allowed to react with ammonium acetate in concentrated ammonia to yield the corresponding pyrrolinones **3** (30–90%).[11] N-substitution, besides specific electronic effects, significantly increased their solubility in aprotic solvents ($CHCl_3$, dioxane). When heating the butenolides with primary amines (e.g., isobutylamine), the N-substituted pyrrolinones **6** were obtained in low yields (⩽30%, Method A[12], Table 1). However a second step of heating the intermediate **5** in acetic acid was needed to afford dehydro compounds **6**. Using a microwave apparatus (Scheme 1, Method B[13]), the compounds could be obtained with better yields after recrystallization in ethanol (62–76%).

Within each class of analogues **1**–**4**, the aryl unsubstituted derivatives **1a**–**4a** were considered as reference compounds. Monosubstituted ($R_1 = H$, referred to **d** or $R_2 = H$, referred to **b**, **c**, **j**), or disubstituted derivatives (**e**, **f**, **g**, **h**, **i**, **k**) were studied (Table 2). They bear either electron-withdrawing groups (CN, $CO_2Me$, $NO_2$) or donor groups (OMe, NHAc) in $R_2$, mainly in *para* position. Taking into account our previous results with the imidazolone series,[7] only electron-withdrawing groups were introduced on the $Ar_1$ aromatic ring to increase the fluorescence emission properties.

The $^1H$ NMR data of the H styryl proton (Hs) pointed out the following comments: (i) as the corresponding imidazolones[14] and oxazolones[15] are proved to be *E*-isomers ($Ar_1$ and CO in *trans* position), we focused attention on both pyrrolinones and butenolides for which a similar configuration was ascertained by NMR (NOESY experiments[16]). It is noteworthy that in all cases (compounds **1**–**4** and **6**), only one signal could be observed for this proton, supporting the existence of only one single structure for these compounds, (ii) specific deshielding effects could be clearly identified on the styryl proton, as the result of the presence of oxygen (X = O) in both the oxazolone and butenolide series (compare **2** and **1**, **4** and **3** in Fig. 2, $\Delta = +0.31$ and $+0.50$ ppm, respectively). Similar deshielding effects were found by N-substitution at the pyrrolinone ring (compare **3** and **6**, $\Delta = +0.30$ ppm). These data highlight modulation of the oxygen carbonyl electronegativity, which may account for direct effects on Hs deshielding (electronic delocalization), or indirect effects (anisotropic field), (iii) within the series of butenolides **4**, introducing a second electron-withdrawing group in $Ar_2$ (compounds **4g** and **4h**) dramatically enhanced the deshielding of the styryl proton ($\delta = 7.98$ ppm). It supports a strong electronic delocalization in these systems, as illustrated by the highest value of the extinction coefficient at the absorption maximum found for the butenolide **4g** ($\varepsilon = 38{,}300\ M^{-1}\ cm^{-1}$).

As all the compounds had a low solubility and were nearly not fluorescent in water, the photophysical properties of all the compounds (concentrations of approximately 3 μM) were determined in 1,4-dioxane at 20 °C. This solvent is an aprotic solvent with a very small dielectric constant ($\xi = 2.2$ at 25 °C), commonly referred to as a good simulator of the hydrophobic environment of a chromophore buried inside a protein matrix. Figure 3 shows the absorbance and emission spectra of compounds **1**–**4e**, which are representative of the four series of derivatives. Imidazolin-5-ones **1** and oxazolinones **2** have structured absorption spectra, characterized by a main absorption peak with a shoulder. The spectra of pyrrolinones **3** and butenolides **4** are not structured at all, and are largely red shifted. They also show greatly increased widths at half maximum ($\Delta\lambda$).

The detailed photophysical properties of all the compounds are listed in Table 2. The extinction coefficient $\varepsilon$ of the compounds was determined at the absorption maximum and the fluorescence quantum yield $\phi$ was determined as described in our previous paper.[7] Steady-state absorption spectra were recorded on a Cary IV spectrophotometer. Steady-state fluorescence spectra were obtained on an SLM 48000 spectrofluorometer,




**Scheme 1.** Synthesis of N-substituted pyrrolinones under microwaves. Reagents and conditions: (a) *i*-$BuNH_2$ 1.2 equiv, μω (NORMATRON$^R$ 112 reactor), acetonitrile, 10 min; (b) AcOH, μω, 5 min.

**Table 2.** Photophysical data of compounds **1**, **2**, **3**, **4**, and **6**

| Compd | Class | R | $R_1$ | $R_2$ | Mp (lit.) (°C)[a] | $\varepsilon \cdot 10^{-3}$ ($M^{-1}\,cm^{-1}$) | $\lambda_{max}^{abs}/\lambda_{max}^{em}$ (nm) | $\phi$ | $\varepsilon \cdot \phi \cdot 10^{-3}$ ($M^{-1}\,cm^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| **1a** | Imidazolones | H | H | H | 265 (274)[b] | 13.1 | 384/444 | 0.001 | 0. 02 |
| **1b** | | H | CN | H | 285 | 24.8 | 393/464 | 0.158 | 3.92 |
| **1c** | | H | $CO_2Me$ | H | 266 | 28.8 | 394/461 | 0.159 | 4.58 |
| **1d** | | H | H | 3,4-DiOMe | 234 | 23.0 | 390/455 | 0.034 | 0.78 |
| **1e** | | H | CN | 3,4-DiOMe | 285 | 26.5 | 405/482 | 0.189 | 5.01 |
| **1f** | | H | $CO_2Me$ | 3,4-DiOMe | 274 | 32.2 | 403/478 | 0.295 | 9.49 |
| **1g** | | H | $CO_2Me$ | 4-$NO_2$ | 304 | 25.3 | 405/510 | 0.258 | 6.53 |
| **2a** | Oxazolones | — | H | H | 171 (168)[b] | 32.0 | 363/447 | 0.002 | 0.06 |
| **2b** | | — | CN | H | 287[b] | 34.2 | 369/426 | 0.028 | 0.96 |
| **2c** | | — | $CO_2Me$ | H | 195[b] | 41.6 | 370/nf[c] | 0 | 0 |
| **2d** | | — | H | 3,4-DiOMe | 165 (168)[b] | 37.1 | 378/447 | 0.020 | 0.74 |
| **2e** | | — | CN | 3,4-DiOMe | 249 | 38.1 | 393/489 | 0.421 | 16.0 |
| **2f** | | — | $CO_2Me$ | 3,4-DiOMe | 208 | 40.0 | 391/474 | 0.348 | 13.9 |
| **2i** | | — | CN | 4-NHAc | 244 | 33.0 | 389/461 | 0.540 | 17.8 |
| **3a** | Pyrrolinones | H | H | H | 222 (226)[b] | 14.6 | 422/539 | 0.012 | 0.17 |
| **3b** | | H | CN | H | 225 | 18.9 | 438/569 | 0.294 | 5.55 |
| **6b** | | *i*-Bu | CN | H | 140 | 10.6 | 440/598 | 0.153 | 1.62 |
| **3c** | | H | $CO_2Me$ | H | 229 | 15.2 | 437/544 | 0.100 | 1.52 |
| **3d** | | H | H | 3,4-DiOMe | 232 | 18.6 | 431/542 | 0.108 | 2.01 |
| **3e** | | H | CN | 3,4-DiOMe | 301 | 19.1 | 457/585 | 0.188 | 3.59 |
| **3f** | | H | $CO_2Me$ | 3,4-DiOMe | 271 | 19.0 | 454/574 | 0.363 | 6.89 |
| **6g** | | *i*-Bu | $CO_2Me$ | 4-$NO_2$ | 155 | 14.4 | 442/594 | 0.245 | 3.53 |
| **6h** | | *i*-Bu | $CO_2Me$ | 4-CN | 147 | 13.0 | 438/589 | 0.192 | 2.49 |
| **3i** | | H | CN | 4-NHAc | 281 | 19.9 | 456/584 | 0.260 | 5.17 |
| **6i** | | *i*-Bu | CN | 4-NHAc | 150 | 13.0 | 450/606 | 0.218 | 2.83 |
| **6j** | | *i*-Bu | $NO_2$ | H | 145 | 12.7 | 456/601 | 0.033 | 0.42 |
| **6k** | | *i*-Bu | $NO_2$ | 4-OMe | 159 | 12.5 | 465/616 | 0.018 | 0.22 |
| **4a** | Butenolides | — | H | H | 153 (155)[b] | 23.4 | 442/493 | 0.007 | 0.16 |
| **4b** | | — | CN | H | 274 | 30.1 | 400/494 | 0.027 | 0.81 |
| **4c** | | — | $CO_2Me$ | H | 182 | 24.8 | 401/495 | 0.007 | 0.17 |
| **4d** | | — | H | 3,4-DiOMe | 149 (147)[b] | 31.0 | 406/495 | 0.004 | 0.12 |
| **4e** | | — | CN | 3,4-DiOMe | 233 | 31.1 | 428/531 | 0.450 | 14.0 |
| **4f** | | — | $CO_2Me$ | 3,4-DiOMe | 191 | 31.1 | 423/522 | 0.355 | 11.0 |
| **4g** | | — | $CO_2Me$ | 4-$NO_2$ | 295 | 38.3 | 411/493 | 0.002 | 0.08 |
| **4h** | | — | $CO_2Me$ | 4-CN | 277 | 34.7 | 404/501 | 0.002 | 0.07 |
| **4i** | | — | CN | 4-NHAc | 285 | 31.2 | 424/521 | 0.437 | 13.6 |
| **4j** | | — | $NO_2$ | H | 290 (292)[b] | 22.3 | 416/518 | 0.083 | 1.86 |
| **4k** | | — | $NO_2$ | 4-OMe | 276 | 30.3 | 438/550 | 0.043 | 1.30 |

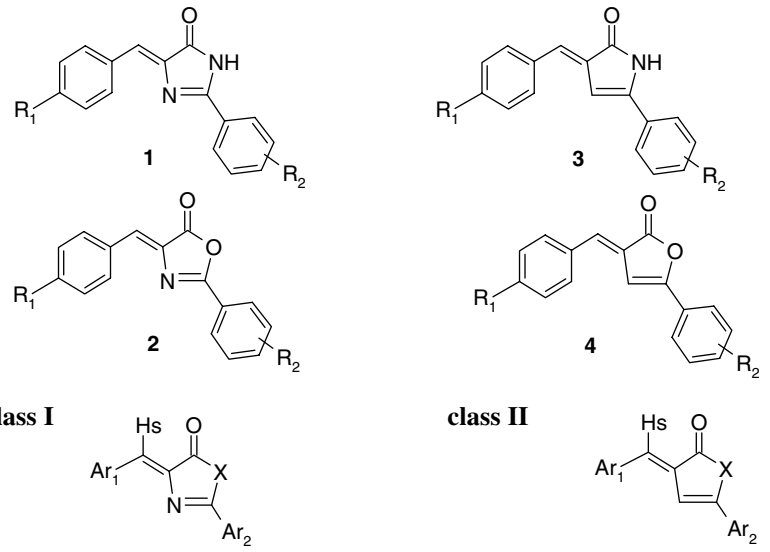
[a] The microanalyses were in satisfactory agreement with calculated values.
[b] Compounds described in the literature.
[c] Nonfluorescent.

with 4 nm excitation and emission bandwidths. The fluorescence quantum yields were determined at 20 °C from the area under the fluorescence spectra, taking as reference a solution of quinine sulfate in 0.1 M $H_2SO_4$ ($\phi_{QS} = 0.51$ at this pH)[17] or fluorescein in 0.1 M NaOH ($\phi_F = 0.95$)[18] of the same absorbance at the excitation wavelength.

A first analysis focused on the imidazolones **1**. When compared with the nonsubstituted imidazolone **1a**, introducing a substituent in *para* position of one (**1b**, **1c**, **1d**), or both aromatic rings (**1e**, **1f**, **1g**) significantly increased simultaneously the extinction coefficient $\varepsilon$, the $\lambda_{max}^{abs}$, $\lambda_{max}^{em}$ (bathochromic effects), and more dramatically the quantum yield (300 times). The most important substitution effect was found with the dimethoxy phenyl derivative **1f** bearing an electron-withdrawing group on the second aromatic ring ($R_1 = CO_2Me$).

When considering data for the third class of isosteric compounds (pyrrolinones **3** and **6**), they support the following comments: (i) a similar but less pronounced increase in $\varepsilon$ by aromatic substitution in $R_2$, (ii) additive bathochromic effects on $\lambda_{max}^{abs}$ induced by each aromatic substituent (compare **3a** with **3e**, **3f**, **3i**, $\Delta\lambda \sim +30$ nm, or with **3b**, **3c**, **3d**, $\Delta\lambda \sim +15$ nm), (iii) N-substitution by an *i*-Bu group caused the strongest bathochromic effect on $\lambda_{max}^{em}$ (~600 nm), but significantly decreased the quantum yield (compare **6b** and **3b**, **6i** and **3i**, respectively), (iv) all the substitutions at the phenyl rings dramatically increased the quantum yield, with the dimethoxy derivative **3f** as the most potent compound within this series.

The oxazolone series (NH → O isosteric replacement) showed high values of $\varepsilon$ (more than 30,000 $M^{-1}\,cm^{-1}$). However the compound **2c**, with the highest $\varepsilon$ value, was




| | Imidazolones | Oxazolones | Pyrrolinones | | Butenolides |
|---|---|---|---|---|---|
| | **1** | **2** | **3** | **6** | **4** |
| X | NH | O | NH | N-*i*Bu | O |
| Hs (ppm) | 7.02 ± 0.12 | 7.33± 0.08 | 7.15± 0.05 | 7.45± 0.07 | 7.65± 0.07 |

**Figure 2.** Derivatives (imidazolin-5-ones **1**) and isosteres (oxazolinones **2**, pyrrolinones **3**, and butenolides **4**) of the 4-hydroxybenzylidene-imidazolinone chromophore.

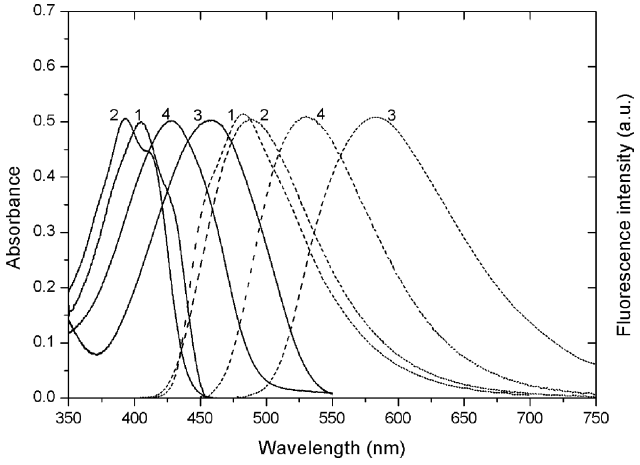



**Figure 3.** Room-temperature steady-state absorption spectra (solid) and fluorescence emission spectra (dashed) of the compounds **1e**, **2e**, **3e**, and **4e** (≈3 μM) in dioxane. The absorption and fluorescence spectra were normalized to approximately the same maximum amplitude.

not fluorescent. When considering fluorescence quantum yield values within this series, **2e**, **2f**, and **2i** were selected as the most interesting compounds.

Similar features were found in the butenolide series highlighting the most promising compounds **4e**, **4f**, and **4i** (increased $\varepsilon$ and $\phi$ values). In addition, a profound increase in the $\varepsilon$ value was observed with butenolides bearing two instead of one electron-withdrawing groups in both $Ar_1$ and $Ar_2$ (compounds **4g** and **4h**, $\varepsilon > 34,000\,M^{-1}\,cm^{-1}$), in relation with existing highly delocalized π-conjugated systems, and illustrated by highly deshielded styryl protons. However, due to their very low fluorescence quantum yields ($\phi = 0.002$), this type of compounds was no more investigated.

The chromophore isolated by enzymatic digestion of GFP lost totally its emissive properties.[6] In addition simpler imidazolinones in which tyrosine vicinal α-aminoacids have been replaced by alkyl chains ($R = R_2 =$ alkyl, see Fig. 1)[6,19] were not fluorescent at room temperature ($\phi < 0.0001$). Only few papers deal with optimization of luminescence properties of some oxazolones and imidazolones.[20,21] These works showed some beneficial effects of electron-withdrawing group in part $R_1$, but nothing has been reported on part $R_2$ of these compounds.

When considering the most promising compounds within each series, a common type of substitution of aromatics was found for **1e–4e**, **1f–4f**, and **2i–4i** highlighting the combined beneficial effects of an electron donor group in $Ar_2$ ($R_2 =$ OMe, NHAc) and an electron-withdrawing group in $Ar_1$ ($R_1 =$ CN, $CO_2Me$, $NO_2$). Among them was selected **4e** as a good compromise between high values of $\varepsilon$ ($31,000\,M^{-1}\,cm^{-1}$) and emission of fluorescence at 531 nm with a high fluorescence quantum yield of 0.45. N-substituted pyrrolinones **6i–j** seemed to be promising, because of the important red shift observed with emission fluorescence at more

than 600 nm, but disappointing concerning the value of $\varepsilon$ (about 13,000 $M^{-1}\,cm^{-1}$).

In conclusion, we synthesized and investigated new interesting structural analogues of the GFP chromophore with good fluorescence quantum yields in a hydrophobic environment ($\phi > 0.24$), and large range of emission wavelengths ($461 < \lambda_{max}^{em} < 594$ nm). Most promising compounds were found in both oxazolone (**2e**, **2f**, **2i**) and butenolide (**4e**, **4f**, **4i**) series. Some of them may constitute a good starting point for building hydrophobic fluorescent markers of biological materials. Several derivatives may allow very efficient fluorescence resonance energy transfer (FRET) and will be useful for a wide panel of biosensor applications.

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9. Schueler, F. W.; Hanna, C. *J. Am. Chem. Soc.* **1951**, *73*, 3528–3530, Analytical data for representative compounds of butenolide class: **4e**: mp 233 °C. $^1$H RMN (DMSO, 300 MHz) $\delta$ 3.84 (s, 3H, OC$H_3$); 3.87 (s, 3H, OC$H_3$); 7.11 (d, $J = 8.2$, 1H, ArH); 7.33 (s, 1H, Ha); 7.4–7.5 (m, 2H, ArH); 7.56 (s, 1H, Hs); 7.94 (d, $J = 8.3$, 2H, ArH); 7.94 (d, $J = 8.3$, 2H, ArH). Anal. Calcd C% 72.06, H% 4.54, N% 4.20; found C% 72.46, H% 4.40, N% 4.25. **4f**: mp 191 °C. $^1$H RMN (DMSO, 300 MHz) $\delta$ 3.87 (s, 3H, OC$H_3$); 3.90 (s, 3H, OC$H_3$); 3.91 (s, 3H, $CO_2$C$H_3$); 7.15 (d, $J = 8.8$, 1H, ArH); 7.37 (s, 1H, Ha); 7.4–7.55 (m, 2H, ArH); 7.58 (s, 1H, Hs); 8.00 (d, $J = 8.6$, 2H, ArH); 8.07 (d, $J = 8.6$, 2H, ArH). Anal. Calcd C% 68.85, H% 4.95, O% 26.20; found C% 68.51, H% 4.96, O% 26.41. **4i**: mp 285 °C. $^1$H RMN (DMSO, 200 MHz) $\delta$ 2.11 (s, 3H, C$H_3$); 7.39 (s, 1H, Ha); 7.58 (s, 1H, Hs); 7.76 (d, $J = 8.7$, 2H, ArH); 7.88 (d, $J = 8.6$, 2H, ArH); 7.95 (d, $J = 8.7$, 2H, ArH); 8.05 (d, $J = 8.6$, 2H, ArH); 10.28 (s, 1H, NHAc). Anal. Calcd with 0.9$H_2O$ C% 69.31, H% 4.60, N% 8.09; found C% 69.27, H% 4.58, N% 8.19.
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11. General procedure for compounds **3**: Compound **4e** (150 mg, 0.6 mmol), dry ammonium acetate (56 mg, 0.72 mmol), and 30% ammonia solution (94 μL, 0.72 mmol) were suspended in MeOH (3 mL). The mixture was placed in a sealed tube, stirred, and heated to 90 °C for 5 h. The solution was evaporated to dryness and $CH_2Cl_2$ (5 mL) was added. The product **3e** was filtered and washed with water. Yield 73%. Mp 301 °C. $^1$H NMR (DMSO, 200 MHz) $\delta$ 3.85 (s, 3H, OC$H_3$); 3.88 (s, 3H, OC$H_3$); 6.85 (s, 1H, Ha); 7.0–7.2 (m, 2H, Hs and ArH); 7.4–7.7 (m, 2H, ArH); 7.90 (d, $J = 8.4$, 2H, ArH); 7.99 (d, $J = 8.5$, 2H, ArH); 10.65 (s, 1H, NH ech./$D_2O$). Anal. Calcd with 0.2$H_2O$ C% 71.50, H% 4.92, N% 8.34; found C% 71.48, H% 4.74, N% 8.43. **3f**: mp 271 °C. $^1$H NMR (DMSO, 200 MHz) $\delta$ 3.81 (s, 3H, OC$H_3$); 3.84 (s, 3H, OC$H_3$); 3.87 (s, 3H, $CO_2$C$H_3$); 6.82 (s, 1H, Ha); 7.0–7.2 (m, 2H, Hs and ArH); 7.4–7.6 (m, 2H, ArH); 7.91 (d, $J = 8.3$, 2H, ArH); 8.00 (d, $J = 8.3$, 2H, ArH); 10.59 (s, 1H, NH ech./$D_2O$). Anal. Calcd with 0.4$H_2O$ C% 67.70, H% 5.36, N% 3.76; found C% 67.56, H% 5.28, N% 3.90. **3i**: mp 281 °C. $^1$H NMR (DMSO, 200 MHz) $\delta$ 2.07 (s, 3H, C$H_3$); 6.83 (s, 1H, Ha); 7.10 (s, 1H, Hs); 7.67 (d, $J = 8.8$, 2H, ArH); 7.83 (d, $J = 8.8$, 2H, ArH); 7.86 (d, $J = 8.4$, 2H, ArH); 7.96 (d, $J = 8.4$, 2H, ArH); 10.17 (s, 1H, NH ech./$D_2O$); 10.59 (s, 1H, NH ech./$D_2O$). Anal. Calcd with 0.6$H_2O$ C% 70.62, H% 4.80, N% 12.36; found C% 70.66, H% 4.64, N% 12.44.
12. Method A: Compound **4g** (500 mg, 1.83 mmol) was suspended in $CH_3CN$ (5 mL) and 0.184 mL of isobutylamine was added. The mixture was placed in a sealed tube and heated to 90–100 °C during 4 h. The solution was evaporated to dryness under reduced pressure and the residue was washed with $Et_2O$. The intermediate **5g** was obtained as a white powder. $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$ 0.79 (d, $J = 6.5$, 3H, $CH_2CH(CH_3)_2$); 0.82 (d, $J = 6.5$, 3H, $CH_2CH_C(CH_3)_2$); 1.71 (m, 1H, $CH_2CH_C(CH_3)_2$); 2.74 (dd, $J_{HAHc} = 8.1$ and $J_{HAHB} = 13.7$, 1H, $CH_AH_B$-$CH_C(CH_3)_2$); 3.29 (dd, $J_{HBHC} = 7.5$ and $J_{HAHB} = 13.7$, 1H, $CH_AH_BCH_C(CH_3)_2$); 3.48 (d, $J = 2.2$, 2H, $CH_2$ pyrrole); 3.55 (s, 1H, OH); 3.92 (s, 3H, $CO_2CH_3$); 7.43 (d, $J = 8.4$, 2H, ArH); 7.52 (m, 1H, Hs); 7.43 (d, $J = 9$, 2H, ArH); 7.99 (d, $J = 8.4$, 2H, Ar2); 8.25 (d, $J = 9$, 2H, ArH). MS (ES): 447 (M+Na).
The intermediate **5g** was placed in a sealed tube and AcOH (3 mL) was added. The mixture was heated at 90–100 °C for 4 h. After evaporation, the red powder **6g** was washed with EtOH. Yield 28%. Mp 155 °C. $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ 0.75 (d, $J = 6.6$, 6H, $CH_2$-$CH(CH_3)_2$); 1.62 (m, 1H, $CH_2CH(CH_3)_2$); 3.57 (d, $J = 7.6$, 1H, $CH_2CH(CH_3)_2$); 3.94 (s, 3H, $CO_2CH_3$); 6.31 (s, 1H, Ha); 7.56 (s, 1H, Hs); 7.6–7.8 (m, 4H, ArH); 8.09 (d, $J = 8.4$, 2H, ArH); 8.25 (m, 2H, ArH). Anal. Calcd with 0.3$H_2O$ C% 67.08, H% 5.53, N% 6.80; found C% 67.03, H% 5.55, N% 6.74.

13. Method B: Compound **4j** (500 mg, 1.83 mmol) was suspended in $CH_3CN$ (5 mL) and 0.184 mL of isobutylamine was added. The mixture was placed in a sealed tube, stirred, and submitted to microwave irradiation (~300 W) until dissolution (~10 min). The solution was evaporated to dryness under reduced pressure and AcOH (3 mL) was added. Then the solution was submitted to microwave irradiation (~300 W) for 5 min. After concentration, EtOH was added and the red powder **6j** was filtered and recrystallized in EtOH. Yield 67%. Mp 145 °C. $^1$H NMR ($CDCl_3$, 200 MHz) $\delta$ 0.75 (d, $J = 6.6$, 6H, $CH_2CH(CH_3)_2$); 1.64 (m, 1H, $CH_2CH(CH_3)_2$); 3.56 (d, $J = 7.6$, 1H, $CH_2CH(CH_3)_2$); 6.15 (s, 1H, Ha); 7.43 (s, 1H, Hs); 7.4–7.6 (m, 4H, ArH); 7.77 (d, $J = 9$, 2H, ArH); 8.27 (d, $J = 9$, 2H, ArH). Anal. Calcd C% 72.40, H% 5.79, N% 8.04; found C% 72.52, H% 5.95, N% 8.13.
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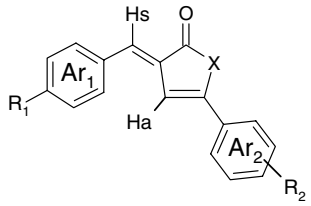
16. Structures of compounds **3**, **4**, and **6** have been unambiguously assigned by NOESY experiments: strong correlations have been observed between Ha and Ar1 and between Hs and $Ar_1$ whereas no correlation has been observed between Ha and Hs.

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