A synthesized GFP analogue emits via a hydrogen-bonding system

Juntao Kang, Guiyan Zhao, Jingwei Xu* and Wei Yang* 

The State Key laboratory of Electroanalytic Chemistry, Changchun Institute of Applied Chemistry, No. 5625, Renming Street, Changchun, Jilin, China, 130022

jwxu@ciac.jl.cn; yangwei@ciac.jl.cn

Synthesis of the molecules.

The synthesis pathway for o-HBMO and o-HBDI followed Scheme 1 in main text that starts from 2-hydroxybenzaldehyde. The detailed conditions are listed below. The product of each step was identified and confirmed using mass spectrometer and NMR. The chemical or solvent amounts given in following is from one of the multiple experiments as examples.

1. Synthesis of 2-formylphenyl acetate

2-Hydroxybenzaldehyde (10 g) was dissolved in dry ether (50 mL) and acetic anhydride (15 mL). K2CO3 (15 g) was then added. The resulting mixture was stirred for 3h and then filtered under reduced pressure. The solvents of the filtrate were removed in vacuum. Small amount of water was added and the resulted solution was placed under -20 °C overnight. 2-Formylphenyl acetate (12.1 g) precipitated as white solid.

2. Synthesis of (4Z)-4-(2-hydroxybenzylidene)-2-methyloxazol-5(4H)-one (o-HBMO)

A mixture of 2-formylphenyl acetate (13.12 g), 2-acetamidoacetic acid (10.24 g), and sodium acetate (6.56 g) in acetic anhydride (16 mL) is stirred at 80 °C in a 100 mL single-mouth flask overnight. Then alcohol (20 mL) is added slowly and the mixture was on standing at room temperature for 2 h to allow the yellow solid product precipitate out. The product was filtered and then washed with
10 mL of ice-cold alcohol twice and 10 mL of boiling water twice. The remaining solvents were removed in vacuum to get pure product (9.75 g).

3. Synthesis of (4Z)-4-(2-hydroxybenzylidene)-1,2-dimethyl-1H-imidazol-5(4H)-one (o-HBDI)

The product o-HBMO (1.6 g) from the last step was suspended in 20 mL alcohol and 12 mL aqueous methylamine (33%, g/g). K₂CO₃ (2 g) was added and the mixture was refluxed at 80 °C for 5 h. The product was concentrated under reduced pressure and further purified by chromatography on silica column with ether as the fluid. A light yellow solid product (1.19 g) was obtained at the end.
Figure S1 The infrared spectra of o-HBMO in solid state (solid line) and in water-free chloroform (dashed line). A signal of lactone was expected around 1750 cm\(^{-1}\) for oxazolone-like isomer of o-HBMO while a signal of amide was expected around 1660 cm\(^{-1}\) for its coumarin-like isomer. The experimental result showed two signals at 1683 and 1711 cm\(^{-1}\) for the molecule at solid state, which suggested that the solid should be a mixture of a coumarin-like and an oxazolone-like isomers. When the sample was dissolved in water-free chloroform, these two IR signals shifted to higher wavenumbers at 1696 and 1716 cm\(^{-1}\), respectively, closer to the expects from a lactone-like structure. More important, a signal for OH vibration clearly appeared at 3685 cm\(^{-1}\). This signal was not from remaining water since 1) a newly-opened water-free chloroform was used and 2) typically the peak for water has a broader shape. The IR results demonstrated the existence of oxazolone-like isomer in solution and indicated that the molecule might apply varied configurations under different conditions.
Figure S2. The $^{13}$C chemical shifts of oxazolone-like isomer of $o$-HBMO (A) and its coumarin-like isomer (B) predicted by the $^{13}$C NMR prediction function in ChemDraw (version 10?) are shown (the software labels the high-confident predictions as blue). The most significant difference between two predictions is on the carbon linked to the hydroxyl group, a chemical shift of 157 ppm in oxazolone-like isomer and 120 ppm in coumarin-like isomer. There are three resonances for oxazolone-like isomer while only two for coumarin-like isomer with predicted chemical shifts over 150 ppm. The observed $^{13}$C spectra (C) clearly show three resonances over 150 ppm. Despite of the uncertainties of the predictions and the measurements, the results strongly supported the oxazolone-like
structure in solution. The samples were in 1:1 water-tetrahydrofuran (in order to enhance the sample concentrations since the solubility in pure water is limited) with a concentration of 10 mM. The sample was first detected at pH 7 (bottom). Then NaOH was added leading to a pH >12 (middle). And finally HCl was added into the same sample to drop the pH <5 (top). The sample was stored at room temperature one day after every pH adjustment. The results confirmed that o-HBMO was stable and kept intact during our whole experiment processes since none of our other measurements lasted more than several hours.

A N-H correlated HSQC spectrum has also been collected using a saturated sample in 1:1 water-tetrahydrofuran (the concentration was estimated to be ~30 mM, $^{15}$N isotope concentration ~0.3 mM, much higher than the requirement for HSQC-type experiments). 128 scans (typically 16 scans are sufficient at this concentration level) were collected for each FID. No N-H crosspeak was observed (data not shown). We believe that this is due to that the major component in solution is not the coumarin-like isomer. A fast exchange of the active proton with the solvent proton would also weaken the signal but we do not think this effect would completely abolish the signal.

Overall, based on the IR, $^{13}$C NMR, and $^{15}$N-$^1$H HSQC results, the molecule applies, at least partially applies, the oxazolone-like structure in water solution as shown in the main text. Consistent with our conclusion, dozens of reactions of this structure have been reported.
Figure S3. The absorption maximum of \( o \)-HBMO is 316 nm at pH 4 and 336 nm at pH 13. The absorption of \( o \)-HBDI red shifts to 375 nm at pH 4 and 444 nm at pH 12, corresponding to an observed solvent color transition from light yellow at low pHs to brown at high pHs. The absorption of \( o \)-HBDI is very similar to that of the enzyme digested GFP, which has absorptions at 380 nm at acidic pHs and 445 nm at basic pHs. This suggests that the \( \pi \)-conjugated system of \( o \)-HBDI has a higher similarity to that of the GFP chromophore. (A) The UV-Vis absorption of \( o \)-HBMO undergoes a pH-dependent change with a dichroism point at about 334 nm. The pHs (from top to bottom at 316 nm) are 8.5, 10.2, 10.4, 10.6, 10.8, 11, and 13, respectively. (B) The absorption decrease at 316 nm following the pH increase gives a transition pH of 10.6 ± 0.1 for \( o \)-HBMO. The resulted Hill coefficient is 2.4 ± 0.4. These correspond well with the fluorescence study. (C) The UV-Vis absorption of \( o \)-HBDI undergoes a pH-dependent change with a dichroism point at 402 nm. The pHs (from bottom to top at 450 nm) are 6.5, 7.5, 8.4, 9.0, 9.2, 9.4, 9.6, 9.8, 10.2, and 11.5, respectively. (D) The absorption changes at 375 nm (◆) and 444 nm (●) give a transition pH of 9.1 ± 0.1 for \( o \)-HBDI. The resulted Hill coefficient is 0.7 ± 0.1. The concentrations for all measurements are ~40 \( \mu \)M. The solid lines in the B and D panels, as well as the solid line in the Figure 1 inset in the main text, are generated using the following Hill equation:

\[
I = I_0 \times \frac{10^{-pH \times n}}{10^{-pH \times n} + 10^{-pH_0 \times n}} + c
\]

Where \( I \) is the signal at the observed pHs; \( I_0 \) is the total signal change; \( c \) is the signal at high pH; \( pH_0 \) is the transition pH (pK\(_a\)); and \( n \) is the Hill coefficient. The Hill coefficient \( n \) deviated from 1 indicates cooperative binding or other processes accompanying the proton association and dissociation.
Figure S4. The mass spectra of \( o \)-HBMO in H\(_2\)O (bottom) and D\(_2\)O (top) are shown. There is only one Dalton mass differences observed, suggesting that there is only one active hydrogen atom in \( o \)-HBMO. Therefore, the cooperativity observed by fluorescence and UV-vis studies cannot be from the binding of multiple protons, but should be the proton binding accompanied with H-bond formation. The observed mass (204.070 in H\(_2\)O and 205.077 in D\(_2\)O) corresponds well with the theoretical value (204 for proton additive in H\(_2\)O), confirming the success of synthesis and the purity of the product. The spectra were collected using a Bruker autoflexTOF/TOF mass spectrometer at Changchun Institute of Applied Chemistry. The molecule was dissolved in 20 mM phosphate buffer at pH 6 prepared using either H\(_2\)O or D\(_2\)O.
Figure S5. The pH-dependent fluorescence change of o-HBMO is reversible. The fluorescence of o-HBMO decreased with the pH enhancement (black). When HCl was added into the sample, the fluorescence was restored (red). The dilution effect (volume increase ~15% by the HCl addition) was not normalized.
Figure S6. The 1D $^1$H NMR spectrum of o-HBDI in chloroform shows a resonance from OH group at about 14 ppm with a relative area of 0.8 (the singlet peak at right as 1). The signal cannot be observed in water solutions.
Figure S7. An NOE interaction opposite of the diagonal peaks was observed for the hydroxyl proton (8.08 ppm) and the methyl protons (2.2 ppm) of o-HBMO, suggesting the hydroxyl group is located close to the nitrogen atom of the oxazol ring instead of the carboxyl oxygen atom. The spectrum was collected using a Bruker 600 MHz NMR spectrometer at Changchun Institute of Applied Chemistry with a mixing time of 600 ms. There were 2048 and 64 data points at D1 and D2 dimensions, respectively, with spectra widths of ~8 ppm at both dimensions. The sample (~15 mM) was dissolved in chloroform.