

A Unique Family of Rigid Analogues of the GFP Chromophore with Tunable Two-Photon Action Cross-Sections for Biological Imaging**

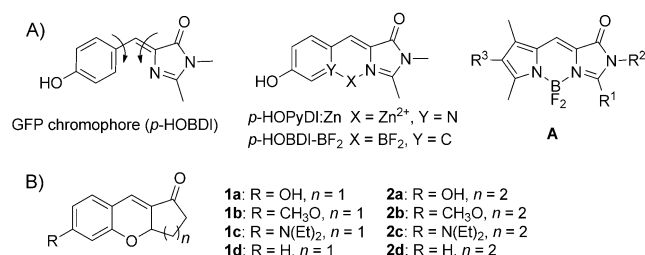
Lin Yuan, Weiyang Lin,* Hua Chen, Sasa Zhu, and Longwei He

The green fluorescent protein (GFP) and related genetically encoded fluorescent proteins have made a great impact on the fundamental research of life science. As a fluorescent tag, GFP is invaluable for studies of protein localization, dynamics, and function. Furthermore, GFP has been engineered as fluorescent sensors for a wide variety of targets including pH, metal ions, and small biomolecules.^[1–7] The chromophore in GFP is *p*-hydroxybenzylideneimidazolone (*p*-HOBDI; Scheme 1 A). However, *p*-HOBDI alone does not exhibit signifi-

cant fluorescence owing to the free rotation of the aryl–alkene bond and the *E/Z* isomerization of the double bond in solution.^[8–11] We performed time-dependent density functional theory (TD-DFT) calculations on the *E/Z* isomers of *p*-HOBDI, and the data indicate that it could show strong fluorescence in the *Z*-isomer (a large oscillator strength: $f = 0.9321$) and relatively weak fluorescence in the *E*-isomer ($f = 0.002$; Supporting Information, Figure S1). These results are in good agreement with the previous reports.^[9,12] To improve the fluorescence of *p*-HOBDI, it is necessary to put it in a rigid environment, such as in the barrel structure of GFP. Toward this end, several elegant approaches have been

employed by encapsulation,^[10,13] reversible locking with metal ions (for example *p*-HOPyDI:Zn)^[8,14] and hydrogen bonds,^[15] or irreversible locking with a BF₂ group (for example *p*-HOBDI-BF₂ and compound A).^[9,14] Two-photon dyes have attracted intense attention in the recent years in light of their useful applications in diverse fields such as two-photon fluorescence microscopy, localized release of bioactive species, photodynamic therapy, optical power limiting, three-dimensional (3D) optical data storage, and microfabrication.^[16–25] In particular, two-photon excitation fluorescence (TPEF) microscopy, where the fluorescence is triggered by spatially confined two-photon excitation, bears several advantages over the conventional one-photon fluorescence microscopy, including three-dimensional imaging of living tissues, reduced photodamage to biosamples, increased tissue penetration, and negligible background fluorescence. To obtain two-photon fluorescence images with sufficient brightness, two-photon dyes with efficient two-photon excitation action cross-section are required (σ' is the two-photon absorption cross-section times the fluorescence quantum yield: $\sigma' = \sigma \times \Phi$). Some *p*-HOBDI rigid analogues with good one-photon properties have been reported;^[8–10,13–15] however, their two-photon properties are unknown. To employ GFP chromophore analogues as two-photon fluorescent platforms for the development of two-photon fluorescent sensors, it is necessary to develop GFP chromophore analogues with tunable two-photon action cross-sections.

Herein, we describe the development of a unique family of rigid analogues of the GFP chromophore, which we call GCTPOC (Scheme 1 B). Compared to the original GFP chromophore and the reported rigid analogues of GFP chromophore (Scheme 1 A), GCTPOC compounds exhibit several distinct features. First, from the structural point of view, in GCTPOC, an oxygen-bridge is employed to lock the chromophore system in a rigid conformation, which is similar to the *Z*-isomer of GFP chromophore, to prevent the free rotation of the aryl–alkene bond and the *E/Z* isomerization of the double bond. Second, the two nitrogen atoms in the imidazolinone ring of the GFP chromophore are replaced as carbon atoms. Consequently, the amide function group in the GFP chromophore is turned into a carbonyl group in GCTPOC, which has better electron-withdrawing ability. Thus, GCTPOC should be a more efficient push–pull system than the GFP chromophore. This is desirable for dyes with asymmetric structures to have good two-photon properties.^[16,19] Third, from the optical point of view, the two-photon properties of the above mentioned rigid analogues of the GFP chromophore have not yet been reported. In contrast, our GCTPOC display favorable two-photon properties. By combining these characteristics, we named the new



Scheme 1. A) Structures of the GFP chromophore and some representative rigid GFP chromophore analogues. B) Structures of GCTPOC compounds. The novel two-photon rigid analogues of the GFP chromophore feature an oxygen bridge and carbon substituents compared to the classic GFP chromophore (*p*-HOBDI).

[*] L. Yuan, Prof. W. Lin, H. Chen, S. Zhu, L. He
 State Key Laboratory of Chemo/Biosensing and Chemometrics
 College of Chemistry and Chemical Engineering, Hunan University
 Changsha, Hunan 410082 (China)
 E-mail: weiyanglin@hnu.edu.cn

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compounds as GCTPOC, which refers to GFP chromophore two-photon analogues with an oxygen bridge and carbon substituents. Although the synthesis of GCTPOC analogues has been reported,^[26] to the best of our knowledge, their optical properties were completely unknown. Herein, we reveal the optical properties of GCTPOC for the first time. Thus, from the optical point of view, GCTPOC is a unique family of conformationally restricted analogues of the GFP chromophore with tunable two-photon action cross-sections.

TD-DFT calculations were conducted to examine the potential effect of the position of the hydroxy group on the fluorescence properties. When the hydroxy group is at the 2-position (*para*-position), the oscillator strength is maximal (Supporting Information, Table S1), indicating that the hydroxy group at the 2-position may be optimal for the fluorescence, consistent with the position of the hydroxy group at the GFP chromophore (Scheme 1A). To investigate the structure two-photon properties, we further designed a series of GCTPOC derivatives (**1a–2d**, Scheme 1B). The novel type of compounds have different electron-donating groups at the *para*-position, which may have an impact on the nature of the push–pull system. Thus, we envisioned that these new compounds should have distinct optical properties. GCTPOC derivatives, **1a–d** and **2a–d**, possess a five- or six-membered carbon ring, respectively.

The dyes **1a–d** and **2a–d** were prepared by reaction of the substituted salicylaldehyde with 2-cyclopenten-1-one/2-cyclohexen-1-one by a Baylis–Hillman reaction^[26] (Supporting Information, Scheme S1). The structures of all the compounds were characterized by ¹H NMR and ¹³C NMR spectroscopy and HRMS.

The new dyes were readily soluble in PBS at micromolar concentrations suitable for optical measurement and imaging applications. The absorption, one-photon, and two-photon fluorescence spectra of **1a–d** and **2a–d** in PBS are shown in Figure 1 (for photophysical data, see the Supporting Information, Table S2). Compound **1a** displays maximal absorp-

tion and emission peaks at 395/445 (extinction coefficient $\epsilon > 20000 \text{ L mol}^{-1} \text{ cm}^{-1}$) and 513 nm, respectively, in PBS (Figure 1). Interestingly, these properties resemble those of the classic green fluorescence protein ($\lambda_{\text{abs}} = 395/475 \text{ nm}$, $\lambda_{\text{ems}} = 509 \text{ nm}$, $\epsilon = 21000 \text{ L mol}^{-1} \text{ cm}^{-1}$).^[27] The large Stokes shift of compound **1a**, just like that of fluorescent proteins, may be attributed to the excited-state proton transfer (ESPT).^[28] Furthermore, like GFP chromophore, the absorption profiles of the dye **1a** is pH-dependent. With the enhancement of pH from 3 to 11, the absorption band at around 392 nm, ascribed to the phenolic form of **1a**, undergoes a red-shift to a peak at around 445 nm, attributed to the phenolate form of **1a** (Supporting Information, Figure S2A). There is a well-defined isosbestic point at 403 nm in the absorption spectra, indicating the equilibrium of the phenolic and phenolate forms of **1a** (Supporting Information, Figure S2B). Excitation of dye **1a** at 445 nm affords one-photon fluorescence spectrum with a maximal emission at 513 nm ($\Phi = 0.29$). The TPEF of compound **1a** displays a maximal emission peak at 512 nm (Figure 1C), and the shape of TPEF spectrum well resembles that of the one-photon fluorescence spectrum (Supporting Information, Figure S3). Significantly, compound **1a** in the phenolate form exhibits excellent two-photon properties with a two-photon cross-section (σ) above 800 GM and a two-photon excitation action cross-section (brightness, σ') above 230 GM (Supporting Information, Table S2). The two-photon cross-section (σ) per molecular weight (σ/MW) of dye **1a** is 3.96, indicating that the dye is potentially useful for bioimaging applications.^[19] Compound **2a**, the six-membered ring analogue of dye **1a**, shows similar excellent two-photon properties (Supporting Information, Figures S3–S5) with a two-photon cross-section above 810 GM and a two-photon excitation action cross-section above 270 GM (Supporting Information, Table S2). Notably, these data are significantly larger than those of the fluorescent proteins.^[29] A brief comparison of some representative conformationally locked GFP-chromophore analogues reported in the literature^[8–10,13–15] and in this work is given in the Supporting Information, Table S3. However, the comparison of the two-photon properties of dyes **1a**, **2a** with those of the rigid GFP chromophore analogues such as *p*-HOPyDI:Zn, *p*-HOBDI-BF₂, and compound **A** is not possible, as surprisingly the two-photon properties of them were not revealed in previous reports. Thus, our dyes **1a** and **2a** are a unique type of GFP chromophore rigid analogue with excellent two-photon properties.

Dyes **1d** and **2d**, which bear no electron-donating group at the *para*-position, display weak fluorescence and negligible two-photon excitation action cross-section (Figure 1; Supporting Information, Table S2). In contrast, dyes **1c** and **2c**, which contain a strong electron-donating group, diethylamino at the *para*-position, exhibit strong fluorescence and large two-photon excitation action cross-section, highlighting the significance of the push–pull character for two-photon properties.

We further examined the photophysical properties of compound **1b**, the methoxy analogue of dye **1a**. The maximal absorption peak of **1b** is at 386 nm, which is blue-shifted when compared to that of **1a**. However, the fluorescence quantum

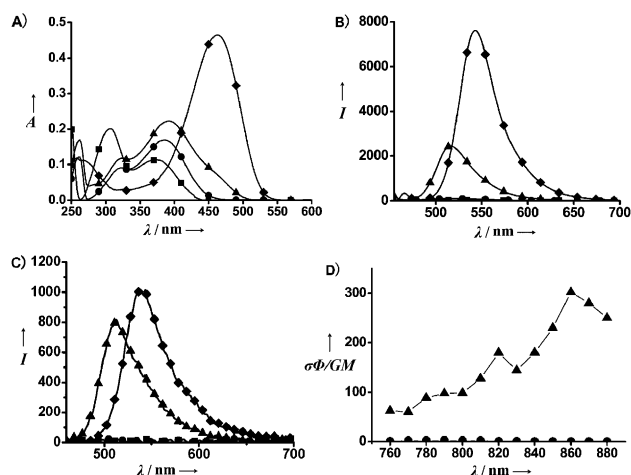
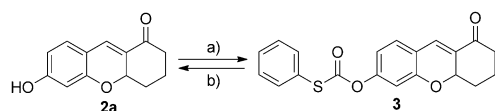


Figure 1. The absorption (A), one-photon fluorescence (B), and two-photon fluorescence (C) spectra of compounds **1a** (▲), **1b** (●), **1c** (◆), and **1d** (■) (5 μM) in PBS containing 1% EtOH). D) Two-photon excitation action cross-section of **1a** (▲) and **1b** (●) in PBS containing 1% EtOH.

yield of **1b** is 0.008, significantly less than that (0.29) of **1a**. Notably, the two-photon excitation action cross-section (σ') of **1b** is only less than 1 GM, which is drastically reduced when compared to that (232 GM) of compound **1a** (Figure 1D; Supporting Information, Table S2). It is known that the phenolate form of fluorescent proteins is fluorescent, whereas the phenolic form of fluorescent proteins is essentially non-fluorescent.^[28,30] Thus, analogously, the extremely low two-photon excitation action cross-section of **1b** may be attributed to the alkyloxy substituent of **1b**, which precludes the formation of the fluorescent phenolate form upon excitation by the ESPT mechanism.^[28,30]

There is a striking distinction in TPEF (more than 300-fold) between the compounds **1a** and **1b** (Supporting Information, Figure S5A). Thus, importantly, these data suggest that the two-photon properties of the new dye **1a** are tunable by modifications on the hydroxy group. The similar relationship between compounds **2a/2b** is also noted (Supporting Information, Table S2, Figure S5B).

Significantly, the above findings that there is a drastic distinction between the two-photon properties of compounds **1a/1b** and **2a/2b**, which implies that the new family of conformationally locked GFP chromophore two-photon analogues represented by **1a** and **2a** may be exploited as novel two-photon based platforms for the design of two-photon fluorescent sensors for biological imaging applications in living tissues by easy modifications on the hydroxy group.^[31] This is further encouraged by the observation that cells stained with **2a** displayed strong fluorescence and those stained with **2b** showed almost no fluorescence (Supporting Information, Figure S6). To demonstrate the use of our two-photon dyes as effective platforms, for proof-of-concept, we further engineered compound **3** (Scheme 2) as a novel



Scheme 2. The design and synthesis of two-photon fluorescence turn-on thiol sensor **3**: a) 4-Nitrophenyl chloroformate, then PhSH; b) RSH.

candidate of two-photon fluorescence turn-on sensor by simple modifications on the hydroxy group of platform **2a** for fluorescence imaging of thiols in living tissues. Small-molecular-weight biological thiols play an important role in many biological processes. However, abnormal levels of thiols are associated with various diseases including liver damage, skin lesions, and slowed growth.^[32] Thus, it is of great interest to monitor biological thiols in living systems by fluorescent sensors.^[33]

The compound **3** is stable in the solid form, and it can be stored for more than one year in a freezer without any hydrolysis. As designed, the free sensor **3** is essentially nonfluorescent in PBS (25 mM, pH 7.4, 1% EtOH), and its fluorescence intensity did not display observable changes for 5 days (Supporting Information, Figure S7). In contrast, the addition of a representative thiol, glutathione (GSH), elicits a significant fluorescence enhancement at 516 nm (Fig-

ure 2A; Supporting Information, Figure S7). In good agreement, a large (up to 30-fold) fluorescence increase in the two-photon fluorescence profile is observed (Supporting Information, Figure S8). The pseudo first-order rate constant of

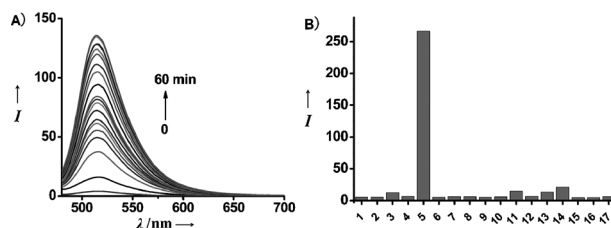


Figure 2. A) The one-photon fluorescence spectra of the sensor **3** (5 μ M) incubated with GSH (5 mM) for 0–60 min in PBS containing 1% EtOH. B) The fluorescent intensity of the sensor **3** (5 μ M) at 516 nm in the presence of various biological relevant analytes (1 mM) for 1 h in PBS containing 10% EtOH. 1) free, 2) Ala, 3) Arg, 4) Gly, 5) GSH, 6) Leu, 7) Phe, 8) Ser, 9) Tyr, 10) Val, 11) Glu, 12) Asn, 13) Lys, 14) H_2O_2 , 15) glucose, 16) Ca^{2+} , 17) Zn^{2+} .

sensor **3** for GSH, cysteine, and homocysteine (100 equiv) were determined to be $k' = 0.05828$, 0.06082 , and 0.04421 min^{-1} , respectively (Supporting Information, Figures S9–S11). It seems that the sensor reacted almost equally well with GSH, cysteine, and homocysteine. The second-order rate constant of sensor **3** for GSH was determined to be $k = 0.04843 \text{ L mol}^{-1} \text{ min}^{-1}$ (Supporting Information, Figure S12). Titration experiments indicated that the emission intensity of sensor **3** at 516 nm is linearly proportional to the amount of GSH (2–200 μ M) and cysteine (2–250 μ M; Supporting Information, Figure S13) with a detection limit ($\text{S/N} = 3$) of 0.80 and 0.75 μ M, respectively, in pH 7.4 PBS containing 1% EtOH as a cosolvent, suggesting that the probe is potentially useful for quantitative determination of thiol concentrations over a large dynamic range.

The reaction product between compound **3** and thiols was isolated and confirmed to be compound **2a** by mass spectrometry (Supporting Information, Figure S14). Furthermore, as shown in Figure 2B and the Supporting Information, Figure S15, the sensor **3** is highly selective to typical thiols (for example GSH, cysteine, and homocysteine) over other biorelevant species, such as Ala, Arg, Leu, Phe, Ser, Tyr, Val, Glu, Asn, Lys, H_2O_2 , glucose, Ca^{2+} , Zn^{2+} , Mg^{2+} , Cd^{2+} , Fe^{2+} , Cu^{2+} , Co^{2+} , NO, F^- , Cl^- , Br^- , I^- , AcO^- , N_3^- , NO_2^- , NO_3^- , SCN^- , SO_3^{2-} , SO_4^{2-} , CN^- , and CO_3^{2-} in PBS containing 10% EtOH, suggesting that the sensor is promising for applications in biological systems.

Encouraged by the above prominent features of the sensor **3** and the advantages of two-photon fluorescence microscopy, we decided to examine the feasibility of the sensor to detect endogenous thiols in living tissues by two-photon fluorescence microscopy. After incubation with 5.0 μ M sensor **3** for 1 hour, a fresh mouse liver slice was washed with PBS. The dye-stained liver slice displays bright two-photon fluorescence (Figure 3a; Supporting Information, Figure S16c), consistent with the two-photon fluorescence turn-on response as observed in solution (Supporting Information, Figure S8). Moreover, the two-photon fluorescence images at

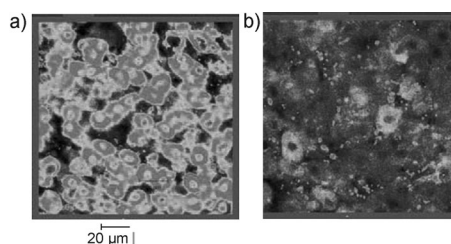


Figure 3. Two-photon fluorescence images (with a magnification at 20 $\times$) of a mouse liver slice that was a) not treated or b) pretreated with NEM (5 mM) for 1 hour before being stained with 5 μ M sensor **3** for 1 h. The two-photon fluorescence emission was collected at between 470 and 570 nm upon excitation at 780 nm with a femto-second pulse. Scale bar = 20 μ m. (For a color version, see the Supporting Information, Figure S22.)

the different depths (0–180 μ m) show that the sensor can image thiols distribution in each xy plane along the z direction (Supporting Information, Figure S17). The observation that the sensor can endogenously image thiols in living tissues at a depth of 180 μ m is in good agreement with the large two-photon excitation action cross-section of the dye **2a** (the reaction product of the sensor with thiols). Control experiments indicated that the compound **2a**-stained liver slice (positive control) displayed strong fluorescence (Supporting Information, Figure S16a) and compound **2b**-stained liver slice (negative control) exhibited almost no fluorescence (Supporting Information, Figure S16b). Furthermore, in another control experiment, a fresh mouse liver slice was pre-treated with N-ethylmaleimide (NEM, a thiol-reactive agent), and further incubated with the sensor **3**, and the two-photon fluorescence decreased significantly (Figure 3b; Supporting Information, Figure S16d), suggesting the selective reaction of the sensor with thiols.

After we had demonstrated that the sensor could monitor endogenous thiols in the isolated tissues (in vitro), we then proceeded to investigate whether our sensor could stain endogenous thiols in intact tissues in living mice (in vivo). 200 nmol sensor **3** was injected into the living mice via a tail vein, and the mice were anesthetized and sacrificed by cervical dislocation at 1.5 h post-injection. The organs such as liver, lung, heart, and kidney were then quickly removed and washed with cold PBS buffer and imaged with a one-photon in vivo imaging system. The results indicate that the liver and kidneys have stronger fluorescence than other organs such as lung and heart (Supporting Information, Figure S18). The tissue slices (thickness 400 μ m) of the liver and kidney were then prepared and subjected to two-photon fluorescence microscopic analysis. The sensor could stain endogenous thiols in the kidney and liver sections with two-photon fluorescence at a depth up to 190 μ m (Supporting Information, Figures S19–S22). Furthermore, in general, the two-photon fluorescence in the kidney slices is brighter than that in the liver slices (Supporting Information, Figure S19–20), which is consistent with the one-photon in vivo fluorescence images (Supporting Information, Figure S18).

In summary, we have developed a unique family of GFP chromophore rigid analogues, GCTPOC, based on a novel design strategy by modifications on the GFP chromophore

with an oxygen-bridge and carbon atoms. Significantly, the two-photon action cross-sections of GCTPOC is tunable by easy modifications on the hydroxy group of GCTPOC compounds, such as **1a** and **2a**, indicating that **1a** and **2a** could be exploited as novel platforms to design two-photon fluorescent sensors. Based on the platform **2a**, the novel two-photon fluorescent thiol sensor **3** was constructed. We have further demonstrated that the two-photon fluorescence turn-on sensor **3** could stain endogenous thiols both in vitro and in vivo. The design strategy will lead to the development of many GFP chromophore rigid analogues, and the robust two-photon fluorescent platforms GCTPOC will open new avenues for the development of two-photon fluorescent sensors as molecular tools for studies in living systems.

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