Mechanistic Insights into the Photophysics of Ortho-hydroxyl GFP Core Chromophores

Wei-wei Guo, Ye-guang Fang, Qiu Fang, Gang-long Cui

Key Laboratory of Theoretical and Computational Photochemistry, Ministry of Education, College of Chemistry, Beijing Normal University, Beijing 100875, China

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Herein we have employed the MS-CASPT2//CASSCF method to study the S1 excited-state intramolecular proton transfers (ESIPTs) of recently synthesized ortho-hydroxyl GFP core chromophores, i.e. OHIM, CHBDI, and MHBID, and their excited-state relaxation pathways. We have found that in OHIM and CHBDI, the ESIPT process is associated with small barriers of 3.4 and 4.2 kcal/mol; while, in MHBID, it becomes essentially barrierless. Moreover, we have found two main S1 excited-state radiationless channels. In the first one, the enol S1 species decays to the S0 state via the enol S1/S0 conical intersection after overcoming considerable barriers of 7.0 and 7.7 kcal/mol in OHIM and CHBDI (however, in MHBID, it is nearly barrierless). In the second one, the keto S1 species is first generated through the ESIPT event; then, it is de-excited into the S0 state in the vicinity of the keto S1/S0 conical intersection. These energetically allowed excited-state decay channels rationalize experimentally observed ultralow fluorescence quantum yields. The insights gained from the present work may help to guide the design of new ortho-hydroxyl GFP core chromophores with improved fluorescence emission and brightness.

Key words: Excited states, Mechanism, Conical intersections, GFP chromophores

I. INTRODUCTION

Green fluorescent proteins (GFPs) have a lot of ubiquitous applications in molecular biology, for example, as powerful bio-imaging tools [1–7]. In the past years, a broad range of GFP variants have been developed; however, their brightness and stability remain not optimal [8, 9]. To enhance their overall performances and design better GFP chromophores, many experimental and theoretical studies have been carried out to investigate the working mechanism of both natural and chemically synthesized GFP core chromophores [10–29].

Natural GFP core chromophores with a para-hydroxyl group e.g. p-HBDI in different surroundings have been extensively studied both experimentally and computationally (see FIG. 1) [30–36]. Recently, GFP core chromophores with an ortho-hydroxyl group have come into the focus of experimental and computational research, for example o-HBDI [37–40]. This ortho-substitution allows the formation of an intramolecular hydrogen bond, which causes a qualitatively different excited-state behavior. Hsieh et al. studied the photochemistry of o-HBDI and its analogues in FIG. 1 using ultrafast spectroscopic techniques and observed an ultrafast excited-state intramolecular proton transfer from the hydroxyl to imidazole group within 25 fs, and a very low cis-trans photoisomerization quantum yield of ca.5%. [39]. This is totally different from the excited-state behavior of natural GFP core chromophores, wherein there is no excited-state intramolecular proton transfer and the cis-trans quantum yield is close to ca. 50%. This intriguing excited-state dynamics was recently explained by Cui et al. with the use of high-level static electronic structure calculations and nonadiabatic dynamics simulations [41]. It was found

FIG. 1 Several para- and ortho-hydroxyl GFP chromophores (p-HBDI and o-HBDI) and their corresponding chemically locking variants (p-LHBDI and o-LHBDI) studied experimentally and computationally.
that the $S_1$ state is of charge-transfer character (which facilitates the excited-state proton transfer) and the low cis-trans photoisomerization quantum yield is caused due to the fact that the $S_1/S_0$ conical intersection in charge of the $S_1$ excited-state deactivation is within the cis region.

Moreover, ortho-hydroxyl GFP chromophores are experimentally found to have ultralow fluorescence quantum yields in solution, e.g. ca. $3.3 \times 10^{-3}$ for o-HBDI in toluene (see FIG. 1) [37–40]. Motivated by the concept that structural rigidity may help to increase the quantum yield of fluorescence emission, Hsu et al. recently synthesized a set of structurally locked para- and ortho-hydroxyl GFP core chromophores, p-LHBDI and o-LHBDI, in which the rotation around the C4–C5 bond becomes forbidden. The locking causes a distinctly different excited-state behavior for p-LHBDI and o-LHBDI in FIG. 1. It significantly enhances the quantum yield of fluorescence emission of the ortho-hydroxyl GFP chromophores, e.g. $3.3 \times 10^{-3}$ for o-HBDI vs. $0.18$ for o-LHBDI; whereas, it makes no difference to the quantum yield of fluorescence emission of the para-hydroxyl GFP chromophores, ca. $10^{-4}$ of p-HBDI vs. $1.2 \times 10^{-4}$ of p-LHBDI [40]. These unusual excited-state behaviors of chemically locked para- and ortho-hydroxyl GFP chromophores have been rationalized using combined electronic structure calculations and nonadiabatic dynamics simulations on a locked o-LHBI [42]. It was found that the $S_1$ keto species is responsible for the fluorescence emission of the unlocked and locked ortho-hydroxyl GFP chromophores, and chemical locking does not change their $S_1$ and $S_0$ potential energy surfaces of the enol-keto tautomerization; however, the chemical tailoring heavily prevents the $S_1$ keto species from approaching its nearby keto $S_1/S_0$ conical intersections so that the fluorescence quantum yield of the locked ortho-hydroxyl GFP chromophores is enhanced compared with that of the unlocked ones. Very recently, Mandal and coworkers have further explored the effects of different functional groups at the para position of ortho-hydroxyl GFP chromophores on their excited-state dynamics (OHIM, CHBDI, and MHBDI in FIG. 2) [43].

Spectroscopically, OHIM only shows a charge-transfer (CT) band; CHBDI shows comparable charge- and proton-transfer (CT and PT) bands; MHBDI shows a stronger PT band and a much weaker CT band. These distinct spectroscopic properties are ascribed to different excited-state energy levels and acidity constants in OHIM, CHBDI, and MHBDI, which is regulated by these functional groups. In addition, they have observed very low fluorescence quantum yields for these three ortho-hydroxyl GFP chromophores in solution. However, the detailed photophysical mechanisms of these three GFP chromophores in particular their excited-state decay pathways are elusive.

In this work, we employ high-level electronic structure methods (CASSCF and MS-CASPT2) to map the $S_1$ and $S_0$ potential energy profiles of these three ortho-hydroxyl GFP core chromophores i.e. OHIM, CHBDI, and MHBDI that are relevant to the excited-state intramolecular proton transfers, and the excited-state deactivations from both enol and keto regions. On the basis of the present results, a lot of new mechanistic insights are gained, which are helpful for the design of new ortho-hydroxyl GFP core chromophores with improved fluorescence performance [43].

II. COMPUTATIONAL METHODS

Minima, conical intersections, minimum-energy potential (MEP) energy profiles, and linearly interpolated internal coordinate (LIIC) paths of OHIM, CHBDI, and MHBDI are computed using the state-averaged complete active space self-consistent field (CASSCF) method in which equal state weights are used for the lowest five roots. In the CASSCF computations, the active space consists of 10 electrons in 8 orbitals, which include 10 π electrons in 8 π and π* orbitals (see FIGs. S1–S3 in supplementary materials). Since the CASSCF method does not adequately capture electron correlation energy, the multi-state complete active space second-order perturbation approach (MS-CASPT2) [44, 45] is utilized to re-evaluate the energies of all optimized structures, MEP and LIIC paths. In the MS-CASPT2 computations, a larger active space, i.e. 14 electrons in 12 orbitals, is used (see FIGs. S1–S3 in supplementary materials); the Cholesky decomposition technique with unbiased auxiliary basis sets is used for accurate two-electron integral evaluation [46]; the ionization potential-electron affinity (IPEA) shift is set to zero [47]; the imaginary shift technique (0.2 a.u.) is employed to avoid intruder-state issues [48]; the polarizable continuum model (PCM) is employed to implicitly account for solvent effects [49]. This combined MS-CASPT2/CASSCF computational protocol has been recently demonstrated to be able to give accurate description for excited-state structures and energetics of medium-size polyatomic molecules [50–55]. The 6-31G* basis set is employed throughout
FIG. 3 Optimized enol and keto minimum-energy and conical intersection structures of OHIM in and between the \( S_0 \) and \( S_1 \) states. Also shown are selected bond lengths (in \( \text{Å} \)) and dihedral angles (in degree) optimized at the CASSCF(10, 8) level. The chosen atom numbering scheme is indicated in S0-ENOL.

all computations in this work [56, 57]. All CASSCF and MS-CASPT2 computations are performed using MOLCAS8.0 [58, 59].

III. RESULTS AND DISCUSSION

A. Minima in the \( S_0 \) and \( S_1 \) states

In the \( S_0 \) state, OHIM, CHBDI, and MHBBDI have the enol and keto tautomers, which are referred to as S0-ENOL and S0-KETO in FIG. 3–5. In these structures, the five- and six-membered rings are almost coplanar. The intramolecular \( N_7\cdots H_1\cdots O_2 \) or \( N_7\cdots H_1\cdots O_2 \) hydrogen bonds benefit molecular planarity. Structurally, the central C4–C5 and C5–C6 bond lengths of S0-ENOL are computed to be 1.436 and 1.347 \( \text{Å} \) for OHIM, 1.448 and 1.360 \( \text{Å} \) for CHBDI, 1.452 and 1.358 \( \text{Å} \) for MHBBDI, respectively, indicating that the C4–C5 and C5–C6 bonds are of typical single- and double-bond characters. When the H1 atom is transferred from the O2 to N7 atom, the C4–C5 (C5–C6) bond length of S0-KETO decreases (increases) to 1.387 (1.402) \( \text{Å} \) for OHIM, 1.386 (1.404) \( \text{Å} \) for CHBDI, and 1.396 (1.391) \( \text{Å} \) for MHBBDI, respectively. Moreover, the C3–C4 and C6–N7 bond lengths are found to increase and decrease from S0-ENOL to S0-KETO in OHIM, CHBDI, and MHBBDI (see FIGs. 3–5). Of course, the most significant structural change in the ground-state enol-keto tautomerization process i.e. from S0-ENOL to S0-KETO is the marked shortening of the C3–O2 bond length, namely from 1.334 \( \text{Å} \) to 1.237 \( \text{Å} \) for OHIM, from 1.339 \( \text{Å} \) to 1.237 \( \text{Å} \) for CHBDI, and from 1.335 \( \text{Å} \) to 1.236 \( \text{Å} \) for MHBBDI. Energetically, S0-KETO of OHIM, CHBDI, MHBBDI is 5.7, 2.4, 4.9 kcal/mol, respectively, higher than S0-ENOL at MS-CASPT2/CASSCF level (see Table I).

In the \( S_1 \) state, the enol and keto minima of OHIM, CHBDI, and MHBBDI, which are referred to as S1-ENOL and S1-KETO in FIGs. 3–5, are structurally very different from their \( S_0 \) minima. In comparison with S0-ENOL of OHIM, CHBDI, MHBBDI, the C5–C6 bond is elongated by 0.078, 0.046, 0.116 \( \text{Å} \) in S1-ENOL. However, the C4–C5 bond is complicated. From S0-
FIG. 5 Optimized enol and keto minimum-energy and conical intersection structures of MHBDI in and between the S\(_1\) and S\(_0\) states. Also shown are selected bond lengths (in Å) and dihedral angles (in degree) optimized at the CASSCF(10, 8) level. The chosen atom numbering scheme is indicated in S0-ENOL.

ENOL to S1-ENOL, it increases by 0.005 and 0.011 Å in OHIM and CHBDI, while, it decreases by 0.078 Å in MHBDI. The C3–C4 and C6–N7 bond lengths are separately elongated and shortened to different extent from S\(_0\) to S\(_1\) minima (see FIG. 3–5). The C3–O2 bond length also becomes shorter, from 1.334 (1.339) [1.335] Å in S0-ENOL to 1.291 (1.286) [1.300] Å in S1-ENOL in OHIM (CHBDI) [MHBDI]. Moreover, the O2–H1⋯N7 hydrogen-bonding in OHIM, CHBDI, MHBDI is clearly enhanced in the S\(_1\) state, as indicated by the H1⋯N7 distances of 1.809, 1.827, 1.820 Å in S0-ENOL and 1.579, 1.528, 1.637 Å in S1-ENOL. Energetically, S1-ENOL of OHIM, CHBDI, MHBDI is 59.7, 56.6, 68.8 kcal/mol higher than S0-ENOL at MS-CASPT2 level (see Table I).

By contrast, when going from the S0-KETO to S1-KETO minima, the C4–C5 (C5–C6) bond length increases (decreases) by 0.086 (0.036) Å in OHIM, 0.085 (0.036) Å in CHBDI, and 0.088 (0.036) Å in MHBDI, respectively. Similarly, the variations of the C3–C4 and C6–N7 bond lengths are also complicated. In both OHIM and CHBDI, these two bond lengths are all shortened a little from S0-KETO to S1-KETO; whereas, in MHBDI, the C3–C4 bond length is elongated (the C6-N7 bond length still decreases). The C3–O2 bond length is nearly constant from S0-KETO to S1-KETO, 1.237 versus 1.228 Å in OHIM, 1.237 versus 1.232 Å in CHBDI, and 1.236 versus 1.235 Å in MHBDI. At MS-CASPT2 level, S1-KETO is computed to be 60.5, 57.8, and 62.1 kcal/mol higher than S0-ENOL for OHIM, CHBDI, and MHBDI, respectively.

B. Enol-keto tautomerization

Relaxed minimum-energy reaction paths show that the S\(_1\) enol-keto tautomerization processes in OHIM and CHBDI are associated with small barriers, which are computed to be 3.4 and 4.2 kcal/mol at MS-CASPT2/CASSCF level in the left and middle panels of FIG. 6. For these ESIPT processes in OHIM and CHBDI, the S\(_1\) potential energy first increases to its maximum at the transition states and then decreases with the decreasing of the N7–H1 distance i.e. from S1-ENOL to S1-KETO. By contrast, the S\(_1\) enol-keto tautomerization process in MHBDI is essentially barrierless as shown in the right panel of FIG. 6. Therefore, in the S\(_1\) state, there forms an equilibrium between the enol and keto species in OHIM and CHBDI if no other decay channels exist; while, such feature is not available in MHBDI. Moreover, single-point calculations along the relaxed S\(_1\) paths yield unrelaxed S\(_0\) energy profiles, which show that when going from the enol to keto species, the S\(_0\) potential energy rises monotonously, thereby confirming that the enol-keto tautomerization is unfavorable in the S\(_0\) state. Finally, there is no S\(_1\)/S\(_0\) crossing discovered during the tautomerization processes either in the S\(_1\) or S\(_0\) states (FIG. 6). Relaxed S\(_0\) minimum-energy reaction paths for the S\(_0\) enol-keto tautomerization processes are presented in FIG. S4 (in supplementary materials), which are qualitatively similar to those shown in FIG. 6.

C. Conical intersections and excited-state decay paths

As in similar ortho-hydroxyl GFP chromophores, there are two kinds of S\(_1\)/S\(_0\) conical intersections for OHIM, CHBDI, and MHBDI, which are independently optimized at the CASSCF level and labeled as S1S0-ENOL-1, S1S0-ENOL-2, S1S0-KETO-1, and S1S0-KETO-2 (FIGs. 3–5).

The two enol S\(_1\)/S\(_0\) conical intersection structures of OHIM, CHBDI, and MHBDI are different qualitatively in the sense of the rotation around the C5–C6 bond between the five- and six-membered rings. In both cases, the imidazole ring is almost perpendicular to the five-membered ring (C4C5C6N7 dihedral angles of OHIM, CHBDI, MHBDI: 103.4°, 101.5°, 105.0° for S1S0-ENOL-1 and −104.7°, −100.2°, −108.1° for S1S0-ENOL-2), whereas the C3C4C5C6 moiety is essentially planar (dihedral angle: −0.7°, 1.3°, 0.8° for S1S0-ENOL-1 and 0.0°, −0.1°, −1.1° for S1S0-ENOL-2). In addition, one can see that the central C5–C6 and C4–C5 bonds of S1S0-ENOL-1 and S1S0-ENOL-2 are significantly longer and shorter than those of the
FIG. 6 MS-CASPT2//CASSCF computed minimum-energy reaction paths (kcal/mol) of the $S_1$ excited-state intramolecular proton transfers in OHIM (left), CHBDI (middle), and MHBBDI (right) along the N7-H1 reaction coordinates. Also shown are the unrelaxed ground-state energy profiles obtained from the corresponding $S_0$ single-point calculations.

eol $S_1$ minimum in OHIM (CHBDI) (e.g. 1.455 and 1.379 (1.461 and 1.373) Å of S0S1-ENOL-1 versus 1.425 and 1.441 (1.406 and 1.459) Å of S1-ENOL; see FIG. 3 and FIG. 4). In comparison, in MHBBDI, both central C5–C6 and C4–C5 bonds merely change a little from S1-ENOL to S10-ENOL-1 or S10-ENOL-2 (see FIG. 5). At MS-CASPT2 level, the $S_1$ and $S_0$ energies at S10-ENOL-1 (S10-ENOL-2) are computed to be 55.3 and 51.5 (53.3 and 48.3) kcal/mol for OHIM, 54.7 and 51.0 (53.4 and 48.4) kcal/mol for CHBDI, and 62.5 and 56.6 (66.4 and 59.0) kcal/mol for MHBBDI, respectively.

The MS-CASPT2//CASSCF computed $S_1$ minimum-energy reaction paths with respect to the rotation of the C4C5C6N7 dihedral angle show that the excited-state deactivation process along the path from S1-ENOL to S10-ENOL-1 is complicated in OHIM, CHBDI, and MHBBDI. In both OHIM (CHBDI), the $S_1$ system will encounter a barrier of 7.0 (7.7) kcal/mol when the C4C5C6N7 dihedral angle approaches ca. 50° (see the top panels of FIG. 7 and FIG. 8). After overcoming these $S_1$ barriers, both $S_1$ and $S_0$ states become close to each other in energy. In the end, the system arrives at a quasi-degenerate $S_1$/S0 conical intersection region. By contrast, for MHBBDI, such deactivation path from S1-ENOL to S10-ENOL-1 is essentially barrierless, ca. 0.2 kcal/mol at MS-CASPT2 level. Similarly, in the end, both $S_1$ and $S_0$ states approach to each other in energy. Near these regions, the $S_1$ system can easily hop to the $S_0$ state due to much small energy gaps.

The two keto $S_1$/S0 conical intersections also differ mainly in the sense of the rotation around the central C4–C5 bond close to the six-membered ring (FIGs. 3–5). These structures are qualitatively different from their enol $S_1$/S0 conical intersections. The local surroundings of the C5–C6 bond remain almost planar in S10-KETO-1 and S10-KETO-2 in OHIM, CHBDI, and MHBBDI: the C4C5C6N7 dihedral angle is close to its value in the keto $S_1$ minimum S1-KETO (C4C5C6N7: 2.7 [2.5] (3.4) for S0S1-KETO-1 and −4.3 [−4.2] (−4.1) for S10-KETO-2 in OHIM [CHBDI] (MHBBDI)); whereas, the C3C4C5C6 moiety twists a lot so that the five- and six-membered rings become perpendicular to each other (dihedral angle: −86.2° [−87.9°] (−85.5°) for S10-KETO-1 and 83.5° [85.7°] (85.9°) for S10-KETO-2). Moreover, one can see from FIG. 3 and FIG. 4 that the central C4–C5 bond lengths of S10-KETO-1 and S10-KETO-2 of OHIM and CHBDI change subtly compared with their $S_1$ keto minima (e.g. 1.472 (1.467) Å of S10-KETO-1 versus 1.473 (1.471) Å of S1-KETO in OHIM (CHBDI)); however, the corresponding C5–C6 bond lengths decrease more than 0.01 Å (e.g. 1.351 (1.348) Å of S10-KETO-1 versus 1.366 (1.368) Å of S1-KETO in OHIM (CHBDI)). Differing from the situation in OHIM and CHBDI, in MHBBDI, the C5–C6 bond length is nearly
constant from the $S_1$ keto minimum $S_1$-KETO to $S_1$-KETO-1 and $S_1$-KETO-2 (1.355 Å in $S_1$-KETO vs. 1.353 Å in $S_1$-KETO-1 vs. 1.355 Å in $S_1$-KETO-2); whereas, the C4-C5 bond length is reduced more than 0.01 Å (see FIG. 5). At MS-CASPT2 level, the $S_1$ and $S_0$ energies at $S_1$-KETO-1 ($S_1$-KETO-2) are computed to be 56.2 and 49.5 (56.0 and 50.8) kcal/mol for OHIM, 56.1 and 51.4 (55.6 and 47.2) kcal/mol for CHBDI, and 53.7 and 47.3 (55.8 and 49.7) kcal/mol for MHBDI, respectively.

Analogously, the $S_1$ excited-state deactivation paths from $S_1$-KETO to $S_1$-KETO-2 in OHIM, CHBDI, and MHBDI have been explored at the MS-CASPT2//CASSCF level. As shown in the bottom panels of FIGs. 7–9, there are small barriers of 1.3, 2.2, and 2.4 kcal/mol separating the $S_1$ keto minima $S_1$-KETO of OHIM, CHBDI, MHBDI from their $S_1$/S0 conical intersection regions i.e. $S_1$-KETO-2. Once getting through these the $S_1$ barriers, both $S_1$ and $S_0$ states become close to each other in energy. Eventually, the system arrives at a quasi-degenerate $S_1$/S0 conical intersection region, where the energies of the $S_1$ and $S_0$ states are computed to be close to each other.

D. Discussion

On the basis of the present results, one can find that there are two excited-state radiationless pathways for OHIM, CHBDI, and MHBDI upon photoexcitation to the initially populated $S_1$ state, which are summarized in FIG. 10. When the system relaxes to the $S_1$ minimum from the Franck-Condon region, it can choose to nonradiatively decay to the $S_0$ state either via the enol $S_1$/S0 conical intersection or via the keto $S_1$/S0 conical intersection as a result of the $S_1$ excited-state intramolecular proton transfer. To arrive at the enol $S_1$/S0 conical intersections, in OHIM and CHBDI, the $S_1$ system needs to overcome sizable barriers of 7.0 and 7.7 kcal/mol, respectively; whereas, in MHBDI, the corresponding barrier is reduced to 0.2 kcal/mol at MS-CASPT2 level. Similarly, in OHIM and CHBDI, there are small barriers associated with the $S_1$ excited-state intramolecular proton transfers, which are estimated to be 3.4 and 4.2 kcal/mol, respectively; while, it becomes essentially barrierless in MHBDI. Once reaching the $S_1$ keto species, the system can get to the keto $S_1$/S0 conical intersection after overcoming a small barrier of 1.3, 2.2, and 2.4 kcal/mol for OHIM, CHBDI, and MHBDI, respectively.

Experimentally, the quantum yield of the fluorescence emission is measured to be close to and less than $10^{-3}$ in these three ortho-hydroxyl GFP chromophores [43]. These ultralow values imply that there exist energetically allowed $S_1$/S0 conical intersections and efficient radiationless excited-state decay channels. However, these excited-state decay pathways are ambiguous experimentally. In the present work, two relevant $S_1$/S0 conical intersection structures are determined. They are located in the enol and keto region, respectively. In
FIG. 10. Suggested photophysical and photochemical mechanisms of three studied ortho-hydroxyl substituted GFP chromophores, i.e., OHIM, CHBDI, and MHBDI (energies in kcal/mol).

OHIM and CHBDI, the excited-state decay paths from the enol $S_1$ minima to the nearby enol $S_1/S_0$ conical intersection structures are associated with sizable barriers of 7.0 and 7.7 kcal/mol; in contrast, those paths from the keto $S_1$ minima to the nearby keto $S_1/S_0$ conical intersection structures have much smaller barriers, 1.3 and 2.2 kcal/mol at MS-CASPT2 level (see Fig. 10). Different from the situation of OHIM and CHBDI, in MHBDI, the excited-state decay paths from both enol and keto $S_1$ minima to their enol and keto $S_1/S_0$ conical intersection structures are very efficient due to small barriers (0.2 kcal/mol for the enol case and 2.4 kcal/mol for the keto case). These energetically allowed $S_1$ excited-state deactivation channels are mainly responsible for the ultralow fluorescence quantum yields observed in these three ortho-hydroxyl GFP core chromophores [43].

Finally, one should be noted that in our calculations, solvent effects are only considered implicitly and the steric interaction between solvents and solutes is ignored. In realistic solvent surroundings, the barriers associated with these excited-state decay paths should be to certain extent increased because these pathways involve large conformational changes with regard to the rotation of the central chemical bonds. Nevertheless, these excited-state pathways are still approachable in OHIM, CHBDI, and MHBDI [43].

In addition, spectroscopically, OHIM and CHBDI shows a strong CT band in hexane, EtOAc, ACN, and MeOH solution [43]. Differently, CHBDI also exhibits a comparable PT band in hexane and EtOAc solution, which is however very much weaker in ACN and MeOH solution. In stark contrast, MHBDI shows a strong PT band in hexane, EtOAc, ACN, MeOH solution. In addition, there is a very small CT band in ACN and MeOH solution. On the basis of thermodynamic and kinetic parameters, it is experimentally inferred that the excited-state intramolecular proton transfer process (ESIPT) in OHIM is thermodynamically and kinetically unfavorable due to the large endothermicity and the small reduction of $pK_a$ from ground- to excited state (9.96 to 8.47); thus, only a CT band is observed. For CHBDI, this ESIPT process is assumed to be favorable kinetically due to more reduction of $pK_a$ (9.30 to 6.37); however, it becomes more unfavorable thermodynamically as the polarity increases (larger endothermicity). Therefore, comparable CT and PT bands are seen in hexane and EtOAc solution; but, much weaker PT band is observed in ACN and MeOH solution. By contrast, the ESIPT process is favored both thermodynamically and kinetically due to large exothermicity and significant reduction of $pK_a$ from ground- to excited state (7.70 to 0.40); as a result, one can see a clear PT band in four studied solvents [43]. These thermodynamic and kinetic analyses are qualitatively consistent with our present electronic structure calculations. Our computational results reveal that there is a barrier for the $S_1$ ESIPT process of OHIM (CHBDI), which is computed to be 3.4 (4.2) kcal/mol at MS-CASPT2 level; whereas, the $S_1$ ESIPT process is essentially bar-
rierless in MHBID (see Fig. 6). Among them, the ESIPT in MHBID is the most efficient; those in OHIM and CHBDI become less efficient as a result of the existence of small barriers (ca. 4.0 kcal/mol). However, one should note that the S1 ESIPT processes in OHIM and CHBDI are also allowed energetically considering their small barriers. Furthermore, one can find that our MS-CASPT2 computations show that the S1 ESIPT process is a little endothermic in OHIM and CHBDI (ca. 1.0 kcal/mol); whereas, it is very exothermic in MHBID, ca. 6.7 kcal/mol, which is close to experimental value of 8.4 kcal/mol [43].

As discussed above, for OHIM and CHBDI, there exist small barriers for the S1 ESIPT process, and the S1 keto species is a little higher than the S1 enol species in energy; thus, the latter population should be much less than the former one. Moreover, the excited-state decay paths for the S1 keto species are much more efficient than those for the S1 enol species (barriers: ca. 1.0 kcal/mol vs. 7.0 kcal/mol); thus, the S1 enol species can survive for a much longer time than the S1 keto species. Taken these reasons, the CT band emitted by the S1 enol species is dominant in OHIM and CHBDI, which is consistent with recent experiments [43]. For MHBID, the S1 keto species is dominant due to the barrierless ESIPT process; thus, the fluorescence emission is mainly caused by the S1 keto species. This agrees with experimental observation that a strong PT band is seen in four solvent effects [43]. Finally, one can easily find that the fluorescence emission of the PT band in CHBDI and of the CT band in MHBID is very sensitive to solvents used (for example, the CT band of MHBID disappears in hexane solvents [43]). So, these spectroscopic behaviors should be not ruled only by the intrinsic photophysics and photochemistry of these isolated ortho-hydroxyl GFP chromophores. They could be also heavily affected by extrinsic solute-solvent interactions, which is however outside of the scope of the present work.

IV. CONCLUSION

We have employed the MS-CASPT2//CASSCF method to systematically study the S1 excited-state intramolecular proton transfers (ESIPTs) of three recently synthesized ortho-hydroxyl GFP core chromophores, i.e. OHIM, CHBDI, and MHBID, and their S1 excited-state relaxation pathways from both enol and keto regions [43]. We have found that the experimentally observed CT and PT bands are caused by the S1 enol and keto species, respectively. Mechanistically, we have figured out that the ESIPT processes in OHIM and CHBDI have small barriers; while, that in MHBID is essentially barrierless. Moreover, we have found two main S1 excited-state radiationless channels, which are mainly responsible for experimentally observed ultralow fluorescence quantum yields of these ortho-hydroxyl GFP core chromophores. The present computational work enriches our knowledge of the photophysics and photochemistry of GFP core chromophores and could help us synthesize better ones with excellent photoluminescence performance.

Supplementary materials: Active orbitals in CASSCF and MS-CASPT2 computations, additional figures and tables, and Cartesian coordinates of all optimized structures are shown.

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