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RESEARCH ARTICLE

A general strategy to enhance donor-acceptor molecules using solvent-excluding substituents

Conner A. Hoelzel,[a] Hang Hu,[c,d] Charles H. Wolstenholme,[a] Basel A. Karim,[a,b] Kyle T. Munson,[a] Kwan Ho Jung,[a] Prof. Yu Liu,[a,e] Prof. Hemant P. Yennawar,[b] Prof. John B. Asbury,[a] Prof. Xiaosong Li,*[c] Prof. Xin Zhang*[a,b]

Abstract: While organic donor-acceptor (D–A) molecules are widely employed in multiple areas, application of a large number of D-A molecules could be limited due to an inherent polarity sensitivity that inhibits photochemical processes. Here we present a facile chemical modification to attenuate solvent-dependent mechanisms of excited state quenching through addition of a β-carbonyl-based polar substituent. Our results reveal a mechanism wherein the β-carbonyl substituent creates a structural buffer between the donor and the surrounding solvent. Through computational and experimental analyses, we demonstrate that β-carbonyl simultaneously attenuates two distinct solvent-dependent quenching mechanisms. Using the β-carbonyl substituent, we demonstrate improvements in the photophysical properties of commonly used D–A fluorophores and their enhanced performance in biological imaging. In summary, this work discovers the β-carbonyl substituent as a general method to enhance the photophysical properties of D-A molecules, thus potentiating its application in a broad spectrum of D–A molecules and their excited state chemistry in polar environments.

Introduction

Organic donor-acceptor (D–A) molecules possess a common motif of both an electron donating and withdrawing group separated by a conjugated π system (Figure 1a). Such D–A type molecules are widely employed in an array of fields within chemical and biological sciences provided the unique metal-free, spatiotemporal control over their reactivity and behavior with low-energy, visible light. In organic synthesis, D–A molecules are employed as photolabile protecting groups for acidic functional groups[1] and as photoredox catalysts to unlock unique chemical transformations.[2] In biological research, D–A type fluorophores have been utilized as labeling reagents to image the behavior, expression, and localization of biological macromolecules, in particular via fluorescence microscopy.[3] Yet the application of D–A type molecules is limited in many regards often due to low fluorescence quantum yields, short excited state lifetimes, and an inherent polarity sensitivity that leads to rapid non-radiative decay. The mechanisms governing the quenching of D–A excited states include internal conversion to a lower energy, non-emissive excited state, aggregation,[4] intermolecular hydrogen bonding,[5] twisted intramolecular charge transfer (TICT),[6] and heat loss to the solvent upon intramolecular charge transfer, known as external conversion (EC)[7] (Figure 1b). However, the task of engineering practical and generalized methods to control these dynamics remains persistent.

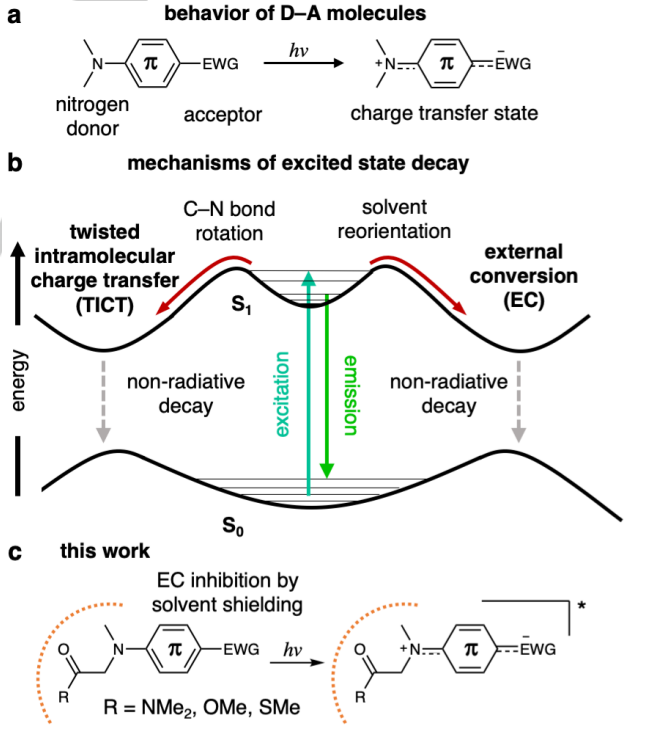

Figure 1. Proposed β-carbonyl donor attenuates solvent-mediated excited state decay. a) Common scaffold of D-A molecules. b) Established mechanisms of thermal, non-radiative excited state decay. c) Proposed role of β-carbonyl based donors to attenuates solvent-mediated excited state decay. (EWG: electron withdrawing group).

[a] C. Hoelzel, C. Wolstenholme, B. Karim, K. Munson, K. Jung, Prof. Y. Liu, Prof. J. Asbury, Prof. X. Zhang
Department of Chemistry
Pennsylvania State University
University Park, PA 16802, United (United States of America)
E-mail: xuz31@psu.edu
[b] B. Karim, Prof. H. Yennawar, Prof. X. Zhang
Department of Biochemistry and Molecular Biology
Pennsylvania State University
University Park, PA 16802 (United States of America)
[c] H. Hu, Prof. X. Li
Department of Chemistry
University of Washington
Seattle, WA 98105 (United States of America)
E-mail: xsli@uw.edu
[d] H. Hu
Molecular Engineering and Sciences Institute
University of Washington
Seattle, WA 98105 (United States of America)
[e] Prof. Y. Liu
Dalian Institute of Chemical Physics
Dalian 116023 (China)

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In recent years, a focus has been placed on achieving the above goal in order to develop more effective fluorophores with improved fluorescence quantum yields and photostability for application in biological and photoorganic chemistry.[8] In particular, small azacyclic donors have been used to inhibit TICT in D–A and rhodamine based fluorophores, improving their application in super-resolution microscopy.[8c, 8d] Additionally, N-cyclohexanol substituted amino donors have been shown to inhibit solvent-solute interactions with the excited state, attenuating the effects of EC in aqueous solutions.[8b] Each of these works address a single mechanism of excited state quenching. However, as TICT and other solvent-dependent quenching mechanisms operate independently of one another, it is desirable to develop a unique and generally applicable chemical modification to control solvent interactions with the excited state that would act cooperatively with these other established methods to prevent excited state non-radiative decay.

In line with the established theme of perturbing amino donors to improve the photophysical and photochemical properties of D–A molecules, our chemical modifications were aimed at shielding an amino auxochrome from solvent interaction through appendage of a polar functional group at the β position relative to the nitrogen donor (Figure 1c). We hypothesized that such a modification should effectively improve the photophysical properties of D–A molecules by minimizing the contribution of EC to the overall rate of excited state quenching. Using D–A fluorophores as model systems and their fluorescence quantum yield (Φ_f) as an evaluation, we demonstrate that inclusion of a β-carbonyl effectively attenuates the influence of solvent-dependent quenching mechanisms on the rate of non-radiative decay in D–A fluorophores. Crystallographic and empirical characterizations in tandem with computational modeling support a structure-based argument for attenuation of solvent interaction with the excited state, thus inhibiting EC. Further, we show that incorporation of the β-carbonyl effectively inhibits TICT by increasing the excited state rotational energy barrier of the donating group, allowing us to propose a mechanism in which the polarity of the β-carbonyl acts to simultaneously control two independent means of excited state quenching. In this work, we demonstrate the application of this β-carbonyl modification to enhance brightness of fluorophores in live cell imaging. These results promote us to envision that polar auxiliaries, represented by the β-carbonyl, offer a general strategy to mitigate multiple mechanisms of non-radiative quenching and expand the use of D–A type molecules in a wide range of applications that utilize excited state chemistry.

Results and Discussion

β-carbonyl enables improved photophysical behavior in a model D–A fluorophore, NBD.

Our investigations into the designed β-carbonyl donor began with the synthesis of a library of fluorophores using the canonical D–A scaffold, 4-dialkylamino-7-nitro-benzoxadiazole (NBD) (Figure 2a). NBD served as a valuable model system for initial explorations provided its ease of functionalization and polarity sensitivity, allowing for rapid and quantifiable observation of changes in fluorescence properties with derivatization.[9] On the basis of dimethylamino-NBD **1**, the β-carbonyl donor was installed in the form of amide derivatives of sarcosine to serve as the donating group (compounds **2** and **3**). We observed 6 and 9-fold increases in fluorescence brightness (B) for compounds **2** and **3** in ethanol (B = 2,750 and 3,909, respectively) when compared to 1 (B = 485) (Figure 2b, Table S1 and Figure S1). Furthermore, the increases in brightness for **2** and **3** persist in a nonpolar solvent such as a 1,4-dioxane, where solvent-solute interactions are weak and EC is generally suppressed, albeit the fold change is reduced (2.5- and 3.7-fold for **2** and **3**, respectively). While brightness is influenced by both fluorescence quantum yield and molar absorptivity, our photophysical characterization found the improved brightness was solely due to the increase of fluorescence quantum yields instead of molar absorptivity (Table S1). Further, we removed the carbonyl component by synthesizing **S20**, an NBD derivative comprised of a 2-methylamino-ethyl methyl ether donor with a similar molecular weight of the β-carbonyl donor. A fluorescence quantum yield of 0.09 in ethanol was determined for **S20** (Table S1), compared to 0.18 for **3**, indicating the necessity of the β-carbonyl to confer the observed effect.

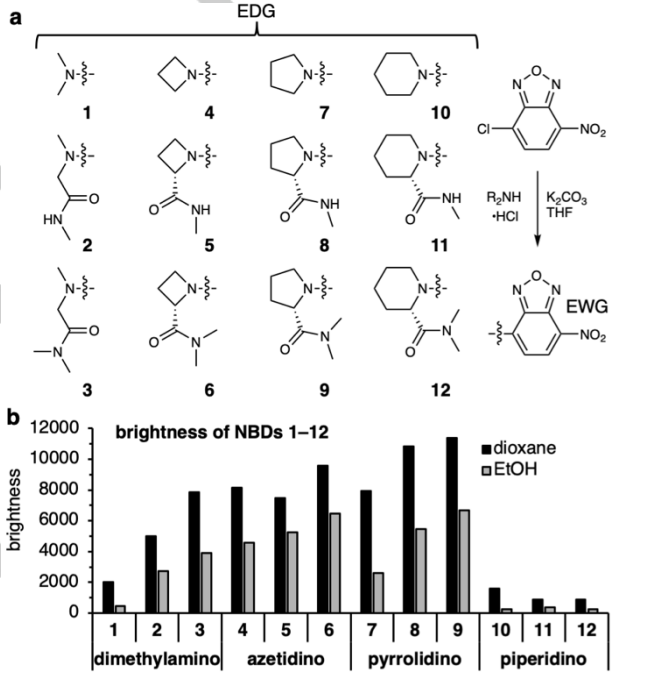
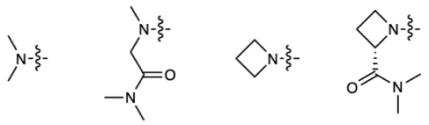


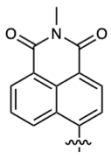
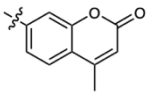
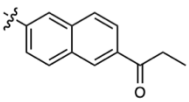
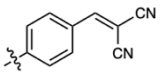
Figure 2. β-carbonyl donors enable enhanced photophysical properties in a model D–A fluorophore, NBD. a) The amide based β-carbonyl NBD library. b) Brightness of NBDs **1**–**12** as determined in ethanol and 1,4-dioxane. (brightness = $\Phi_f \cdot \varepsilon$). Fluorescence quantum yields were measured relative to a fluorescein standard (Φ_{std} = 0.79 in 0.1 M aqueous NaOH).

We next sought to explore whether the effect of the β-carbonyl can be expanded to small azacyclic donating groups (Figures 2a-b, Table S1 and Figure S1).[8c, 8d] Of these donors, azetidine is known to enhance fluorescence quantum yield and brightness via the inhibition of TICT. In ethanol where the contribution of EC to the overall rate of non-radiative decay is pronounced, both β-carbonyl derivatives of azetidino-NBD **5** and **6** reveal notable increases in brightness (B = 5,248 and 6,492, respectively) and Φ_f (Φ_{EtOH} = 0.29 and 0.31, respectively) over the azetidino control **4** (B = 4568, Φ_{EtOH} = 0.24). However, in nonpolar 1,4-dioxane, where TICT is the most prominent mechanism of fluorescence quenching, both **5** and **6** display no increase in fluorescence quantum yield ($\Phi_{dioxane}$ = 0.49 and 0.52,

Table 1. β-carbonyl-enabled enhancement of photophysical properties in D–A fluorophores.



A *B* *C* *D*

Scaffold	Donor, **compound**	Solvent Conditions	λ_{abs} (nm)	ε ($M^{-1} \cdot cm^{-1}$)	λ_{em} (nm)	Φ_f ± % error
	*A*, **13**	EtOH	419	9730	540	0.01 ± 8.1%
		H_2O	436	6780	n.d.	n.d.
	B, **14**	EtOH	420	9490	532	0.11 ± 8.5%
		H_2O	428	7470	548	0.05 ± 3.1%
	*A*, **15**	EtOH	367	20880	449	0.84 ± 8.3%
		H_2O	372	16380	467	0.21 ± 1.5%
	B, **16**	EtOH	363	12680	440	0.95 ± 7.6%
		H_2O	367	11970	455	0.98 ± 1.0%
	C, **17**	EtOH	363	19340	451	0.94 ± 2.1%
		H_2O	353	14760	469	0.91 ± 0.4%
	D, **18**	EtOH	356	14810	449	0.98 ± 0.2%
		H_2O	355	13930	456	0.99 ± 0.6%
	*A*, **19**	EtOH	360	17920	491	0.71 ± 3.2%
		H_2O	356	5190	526	0.25 ± 4.7%
	B, **20**	EtOH	360	15400	485	0.82 ± 2.0%
		H_2O	368	14390	514	0.76 ± 4.6%
	*A*, **21**	Glycerol/MeOH (4:1)	442	55130	489	0.016 ± 3.9%
	B, **22**	Glycerol/MeOH (4:1)	433	52250	483	0.017 ± 2.0%

respectively) relative to **4** ($\Phi_{dioxane}$ = 0.55). These results suggest the β-carbonyl acts to prevent solvent-mediated quenching mechanisms, in particular EC. For pyrrolidino-based donors **7**–**9** that are known to undergo TICT,[8c, 8d] increasing fluorescence brightness and quantum yield are observed in ethanol (Φ_{EtOH} = 0.10 for **7** and 0.28 for **9**). In nonpolar 1,4-dioxane, this trend persists ($\Phi_{dioxane}$ = 0.31 for **7** and 0.47 for **9**), suggesting a potential role of β-carbonyl to inhibit TICT. β-carbonyl functionalization fails to improve upon the behavior of the piperidino derivatives **10**–**12**, likely due to the low-energy, twisted conformation stably present in the ground state.[8d] In all cases, Beer's law plot was used to ensure that all compounds were soluble at the concentrations and solvents of measurement, suggesting that the effect of β-carbonyl was not due to the change of solubility (Figure S16). Importantly, the observable increases in fluorescence quantum yield and brightness of the β-carbonyl derivatives of NBD are consistent with the measured fluorescence lifetimes of these fluorophores, corroborating the presented data (Table S2). Collectively, these data suggest that the β-carbonyl donor effectively increases fluorescence quantum yield of NBD with respect to the corresponding dialkylamino controls. Thus, the β-carbonyl donor could represent a novel class of simple and effective modifications to increase fluorescence quantum yield of D–A fluorophores.

β-carbonyl is generally applicable to enhance fluorescence quantum yield of D-A fluorophores.

In addition to NBD, we elected to study several representative D–A scaffolds, including 4-dialkylamino-1,8-naphthalimide (naphthalimide), 7-dialkylamino-4-methylcoumarin (coumarin), 2-dialkylamino-6-propionylnaphthalene (PRODAN), and 4-dialkylamino-(2,2-dicyanovinyl)benzylidene (DCV). The results of the photophysical characterizations are outlined in Tables 1 and S3.

For naphthalimide, an approximate 10-fold increase in Φ_f was observed for the β-carbonyl derivative **14** (Φ_{EtOH} = 0.11) over the dimethylamino control **13** in ethanol (Φ_{EtOH} = 0.01). In water, an observable Φ_f of **14** was determined to be 0.05 while such a

value could not be ascertained for 13 due to an immeasurable fluorescence intensity (Figure S2). For coumarin, the β-carbonyl derivative **16** reveals Φ_f increases in both ethanol (from 0.84 to 0.95) and water (from 0.21 to 0.98), despite the considerably high efficiency of the dimethylamino control **15** in organic solvents (Figure S3). Given that coumarin-based probes are commonly used in microscopy and other analytical techniques with fluorescence readouts, we sought to explore whether an azetidino β-carbonyl donor would improve their performance with respect to the benchmark azetidino donors.[8c] We found that while the fold change is modest, the β-carbonyl auxiliary increases Φ_f of coumarin **18** compared to the alkyl counterpart **17** in both ethanol (from 0.94 to 0.98) and water (from 0.91 to 0.99). This increase in Φ_f is also observed with the incorporation of the β-carbonyl donor on the PRODAN scaffold (**19** and **20**), with approximately 3-fold enhancement in water (from 0.25 to 0.76; Figure S4). However, an increase in Φ_f was not observed in both dilute (ethanol) and viscous solvent (80:20 v/v glycerol:methanol, viscosity= 132 cp[10]) when we applied the β-carbonyl donor to the representative molecular-rotor DCV scaffold (**21** and **22**; Figure S5), suggesting the limited effect of β-carbonyl on molecular-rotor fluorophores. Taken together, these data along with NBD suggest the β-carbonyl donor can be generally applicable to increase fluorescence quantum yield of D–A type fluorophores.

β-carbonyl increases the excited state energy barrier for rotation, inhibiting TICT.

Our realization of the broad scope of β-carbonyl donors with respect to D–A fluorophores warranted an investigation into the underlying mechanism that governs the improved fluorescence quantum yields. Given the observed increases in brightness for the β-carbonyl NBD derivatives in 1,4-dioxane, we first set out to determine the relative contribution of TICT to the overall rate of non-radiative decay. *In silico* calculation of potential energy surface upon rotation about the C–N bond of the donating amine in vacuum indicated that incorporation of the β-carbonyl in **3** increased the required activation energy to achieve the TICT state by 2.5-fold over the dimethylamino control 1 (E_a = 3.77 and 1.50 kcal/mol for **3** and **1**, respectively) (Figures 3 and S6). Previous work of azetidino type donors showed that TICT inhibition was the

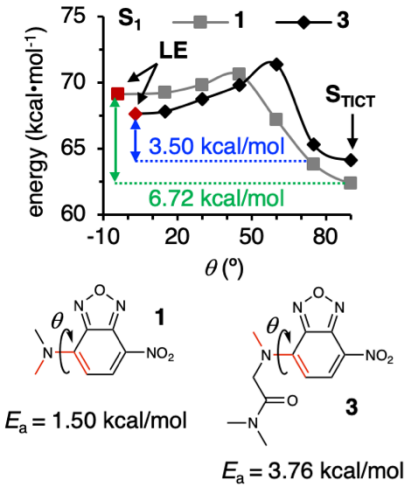


Figure 3. TICT inhibition reduces excited-state decay with β-carbonyl functionalization. Potential energy surface scan for rotation (indicated by the red arrow) about highlighted bond in **1** and **3**. Red markers correspond to the calculated LE geometry.

result of reduced 1,3-allylic strain, allowing the donors to attain a more planar structure with respect to the aromatic π motif.[8c, 8d] Such a mechanism, however, is not apparent in the case of the β-carbonyl donors as the calculated geometry of the local excited state (LE) reveals that the donors of **1** and **3** adopt a roughly equivalent dihedral angle (Figure 3). Instead, calculation of the dipole moment of the excited and ground states (μ_E and μ_G, respectively) indicates a lesser overall difference between these values with β-carbonyl functionalization, supporting a model wherein intramolecular charge transfer in the excited state is inhibited (Figure S6c). This result suggests that the increase in requisite energy for rotation is caused by the inductive electron withdrawing nature of the carbonyl group, which would give rise to a destabilization of the amino radical cation of the TICT state.[8e] This is further supported by the lesser overall difference in the driving energy between LE and TICT states of **3** (3.50 kcal/mol) compared to that of **1** (6.72 kcal/mol) (Figure 3).

β-carbonyl enables a solvent exclusion mechanism, inhibiting EC.

While TICT inhibition sufficiently describes the observed increases in fluorescence emission of β-carbonyl donors in non-polar solvents, the increases in fluorescence quantum yield and brightness persist upon functionalization of azetidino based donors (**6** and **18**) where TICT is already mitigated. Particularly, this effect is most prevalent in polar, hydrogen-bonding solvents such as ethanol and water. Previous work shows that the employment of a hydrophobic, 4-(hydroxycyclohexyl)amino donor acted to exclude solvent interaction with the donating amine in aqueous environments, yielding improved fluorescence intensity.[8b] In contrast to this hydrophobic exclusion, we envisioned a mechanism wherein the carbonyl would shield the amino donor from solvent interaction, functioning as a sacrificial hydrogen-bond acceptor.

As transient infrared spectroscopic analysis reveals no perturbance of the β-carbonyl in the excited state structure of 3 (Figure S7), key to such a hypothesis is the structural orientation of the β-carbonyl relative to the nitrogen donor in the ground state. To this end, we solved crystal structures of both **3** for NBD and **14** for naphthalimide (details can be found in Tables S7-S14 for **3** and Tables S15-S22 for **14**). Analyses of these structures show that the carbonyl is oriented toward the nitrogen donor at a distance of 2.7 Å, placing it in proper spatial position to prevent solvent from accessing the nitrogen donor (Figures 4a and S8). The structural consistency between **3** and **14** suggests that the orientation of the β-carbonyl is relatively independent of the chromophore employed.

With this structural information in mind, we began assessing the solvent-solute interactions that take place upon excitation of the naphthalimide derivative **14** in comparison to **13**. *In silico* analyses of solvent interaction with **13** and **14** were conducted by calculating the change in electron density ($\Delta\rho$) during excitation with the inclusion of an explicit ethanol solvent molecule, which was geometrically optimized near the donating group with both solvent and dispersion effects taken into account. The results of these calculations show that electron density shifts about the ethanol's hydroxyl upon excitation of **13** (Figure 4b, left), suggesting that a ground state hydrogen bond between the solvent and the dimethylamino donor is broken (Figure 4c, left). However, no electron density shift was observed when the β-carbonyl derivative **14** was subjected to the same analysis (Figure

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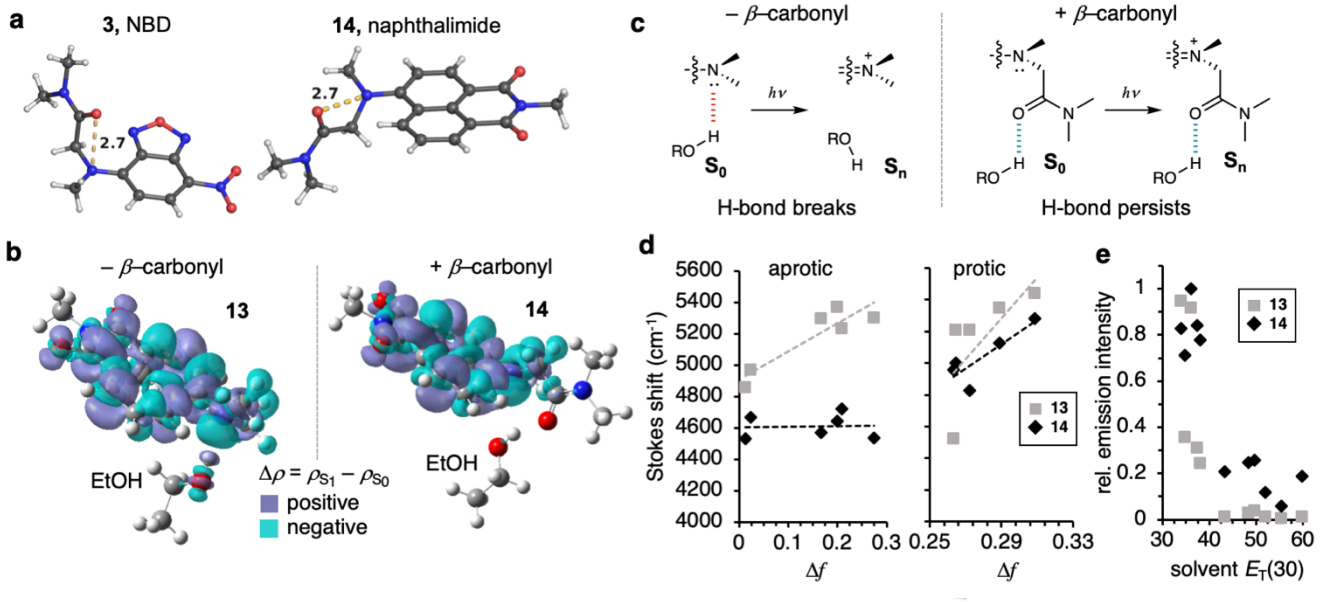

Figure 4. Solvent shielding collectively reduce environmental sensitivity with β-carbonyl functionalization. a) Crystal structures of **3** and **14**. Labeled distances (Å) correspond to yellow dashes. b) Electron density difference during excitation calculated for **13** and **14** with an explicit ethanol solvent molecule geometrically optimized about the donating group. c) Broken H-bond upon excitation of **13**, but not **14**. d) Lippert-Mataga plot of **13** and **14** in both aprotic (dimethylformamide, toluene, 1,4-dioxane, tetrahydrofuran, ethyl acetate, methyl *t*-butyl ether) and protic (methanol, ethanol, *i*-propanol, *n*-butanol, 2,2,2-trifluoroethanol) solvents. e) Relative emission intensity plotted against solvent $E_T(30)$ for the solvents characterized.

4b, right), indicating that the ground state hydrogen-bond between the ethanol and the carbonyl oxygen persists (Figure 4c, right). Finally, this model shows that the ethanol molecule migrates away from the nitrogen donor of **13** during emission (left panel of Figure S9a), indicating a transfer of energy from the excited state of **13** in the form of heat. By contrast, the ethanol molecule remained fixed in place during emission of **14** (right panel of Figure S9a), suggesting the inhibition of thermal non-radiative decay. In addition to naphthalimide, similar observations were made when NBD derivatives **1** and **3** are subject to the same computational analyses (Figure S9b). Taken together, these models suggest that the β-carbonyl effectively shields the nitrogen donor from interacting with the surrounding solvent, preventing quenching of the charge transfer state via EC.

In support of the computational model, spectroscopic analyses of napthalimides **13** and **14** were conducted in several solvents of varying polarity and H-bonding capacity. According to the Lippert-Mataga equation (Equation 1), solvatochromism can be quantified by linear fit of Stokes' shift ($\nu_{abs}-\nu_{em}$, cm^{-1}) as a function of the empirical solvent polarity parameter, Δf.[11]

(Equation 1) $$(\nu_{abs}-\nu_{em}) = \Delta f \cdot (\mu_G-\mu_E)^2 / a^3 + \text{const.}$$

In this equation, the slope of the line (m) is directly related to the square of the difference in dipole moment between μ_G and μ_G and indirectly related to the cube of the radius of the Onsager cavity (a), or the solvent shell. Our data show that this slope is decreased for the β-carbonyl derivative **14** with respect to the dimethylamino control **13** in both protic (m = 7675.2 and 12,593.0, respectively) and aprotic (m = 37.3 and 1,784.3, respectively) solvents (Figures 4d and S10). Based on the obtained structural data (Figure 4a) along with the computational model of solvent shielding (Figures 4b and S9), we suggest that this decrease in slope is the result of an increase in the radius of the solvent shell a, in addition to the calculated decrease in the difference between μ_G and μ_G (Figure S6c). Furthermore, brightness of **14** is less sensitive to solvent polarity than **13**, based on the relationship between the relative fluorescence intensity and the solvent $E_T(30)$ (Figure 4e).[7] Collectively, these data suggest that the spatial orientation of the β-carbonyl effectively mitigates solvent-induced fluorescence quenching by shielding the nitrogen donor from interaction with its local environment.

Polarity of β-carbonyl controls fluorescence quantum yield.

Because the polarity of β-carbonyl is the key towards inhibiting both the EC and TICT pathways, we aimed to finely tune this property to control the fluorescence quantum yields and brightness of fluorophores. As the β-carbonyl is engaged in polar interactions with the solvent, we hypothesized that modulation of the electron density of the carbonyl oxygen would allow for control over the strength of these interactions. As such, NBD derivatives **23**–**26** were synthesized and the photophysical properties were characterized in ethanol (Figure 5a and S11, Tables S4 and S6). We predicted that carbonyls with lesser polarity such as the aldehyde (**23**) and ketone (**24**) would offer weaker H-bond acceptors, conferring lower fluorescence quantum yields compared to the more polar carbonyls represented by the amide (**3**), ester (**25**), and thioester (26) derivatives. As expected, the quantum yield values of **23** (Φ_{EtOH} = 0.05) and **24** (Φ_{EtOH} = 0.14) are lower

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relative to that of **3** (Φ_{EtOH} = 0.18), **25** (Φ_{EtOH} = 0.26), and **26** (Φ_{EtOH} = 0.28). Consistent to these measurements, brightness of these fluorophores shows a similar trend (Figure 5a). In compound **23** and **24**, the aldehyde and ketone functional groups have been used as potent d-PET quenchers when attached to fluorophores.[3b, 12] We found that the fluorescence quantum yield of **23** and **24** were higher than that of the control compound **1** (Φ_{EtOH} = 0.02), suggesting the lower fluorescence quantum yield of **23** and **24** were largely due to the lower polarity of β-carbonyl.

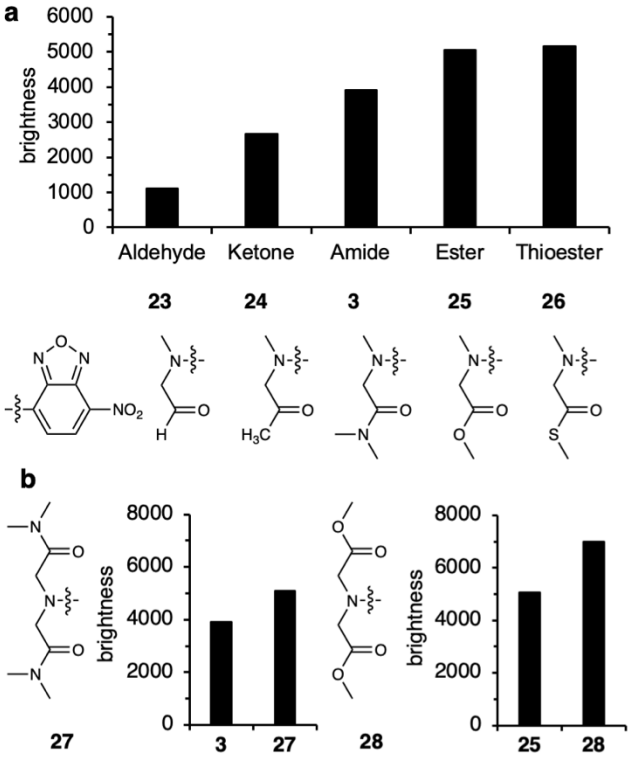

Figure 5. Control of quantum efficiency though β-carbonyl derivation. a) Efficiency of β-carbonyl NBD derivative **25**-**28** quantified in terms of brightness. b) Brightness of bis-β-carbonyl derivatives **27** and **28** compared to the corresponding mono counterparts. Fluorescence quantum yields were measured relative to a fluorescein standard (Φ_{std} = 0.79 in 0.1 M aqueous NaOH).

Further, we rationalized that an alternative approach to amplify the solvent-shielding effect of β-carbonyl is to install an additional β-carbonyl. To this end, we synthesized and characterized the bis-β-carbonyl derivatives of NBD, **27** and **28**. Indeed, both the bis-β-amide **27** and the bis-β-ester **28** display a significant improvement relative to the mono analogs **3** and **25** in both fluorescence quantum yield and brightness (Figures 5b and S12, Tables S5-S6). It is also noted that the bis-β-carbonyl mimics metal binding domain, we added EDTA to chelate possible metal ion that could be bound to bis-β-carbonyl and found no change in brightness, suggesting the bis-β-carbonyl group barely binds with metal ions (Figure S13). Taken together, these data indicate that photophysical behavior of β-carbonyl functionalized D–A fluorophores can be controlled by tuning the solvent-shielding effect of the β-carbonyl.

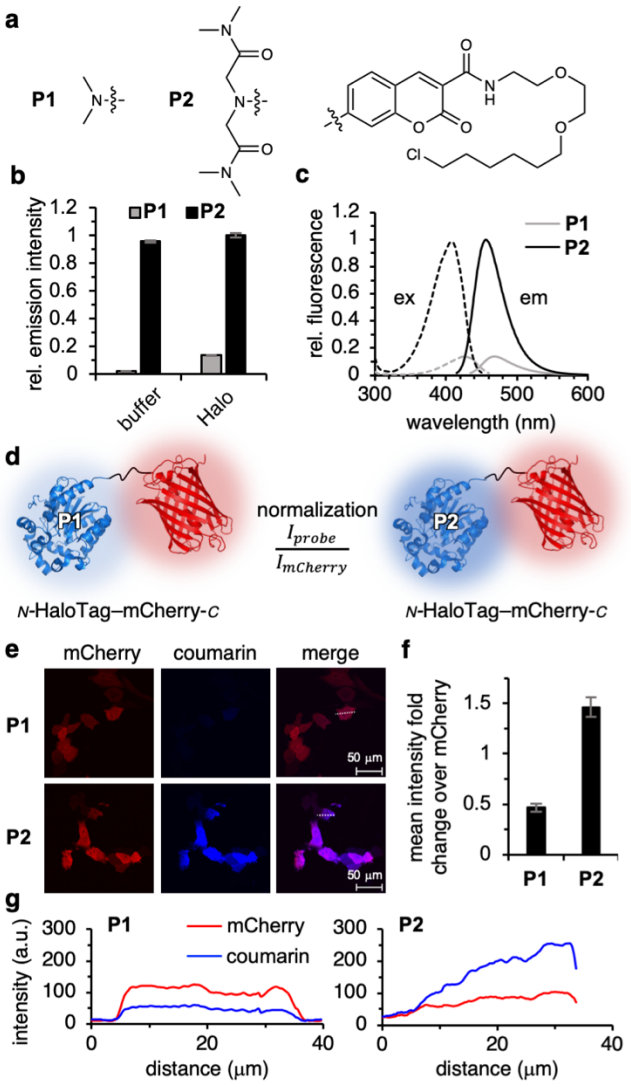

Figure 6. Improved brightness of a bis-β-amide coumarinyl probe in cellular imaging. a) Structures of **P1** and **P2**. b) In vitro characterization of **P1** and **P2** (5 μM) fluorescence intensity in 50 mM tris•HCl buffer (100 mM NaCl, pH 7.5) and in conjugation with purified HaloTag protein (25 μM) in tris buffer (standard error, n = 3). c) Excitation (dashed lines) and emission spectra (solid lines) of **P1** (grey) and **P2** (black) upon conjugation with purified HaloTag protein in tris•HCl buffer. d) Normalization strategy to account for expression level in cell imaging analysis of **P1** and **P2** by ratio of mean pixel intensity of probe (I_{probe}) to that of the mCherry ($I_{mCherry}$) domain in a transiently expressed N-HaloTag–mCherry-C fusion protein. e) Live-cell confocal microscopy of HeLa cells transiently expressing an N-HaloTag–mCherry-C fusion protein for 24 h in the presence of P1 or P2 (0.5 μM) after a single wash. f) Quantified mean pixel intensity fold-change of probe signal relative to that of mCherry (standard error, n = 3). g) Intensity profiles of mCherry (red) and probe (blue) signal corresponding to the marked cross sections (white dotted lines) in panel f.

Enhance brightness for live cell imaging using the bis-β-amide modification.

We envision a wide range of applications can be enabled by the β-carbonyl modification on D–A molecules. As an example, we aimed to develop β-carbonyl functionalized fluorophores for general labeling of proteins in live cell imaging using fluorescence microscopy. Due to higher fluorescence quantum yield and brightness, we expect such fluorophores would require less laser power to obtain desired signal-to-

noise ratios. To this end, coumarinyl probes for HaloTag[13] **P1** and **P2** were synthesized with a dimethylamino control and bis-β-amide donor, respectively (Figure 6a). The bis-β-amide derivative was chosen over the ester and thioester counterparts given the known instability of the latter in vivo. In vitro characterization of **P1** and **P2** indicates a greater than 5-fold increase in fluorescence intensity upon conjugation to purified HaloTag protein (Figure 6b-c). HeLa cells transiently expressing a HaloTag-mCherry fusion protein were incubated with **P1** or **P2** prior to imaging. Brightness of each image was quantified by the fold-change of mean pixel intensity over that of mCherry to account for expression level differences (Figure 6d). The results reveal an approximate 3-fold change in emission intensity of **P2** over **P1** (Figures 6e-g and S14). Similarly, 4-dialkylamino-7-sulfonylbenzoxadiazole derivatives **SP1** and **SP2** reveal an approximate 1.5-fold change in emission intensity with inclusion of the β-carbonyl donor when subjected to the same experimental conditions and analysis as described above (Figure S15). These results show that β-carbonyl functionalized fluorophores exhibit enhanced brightness in biological imaging applications.

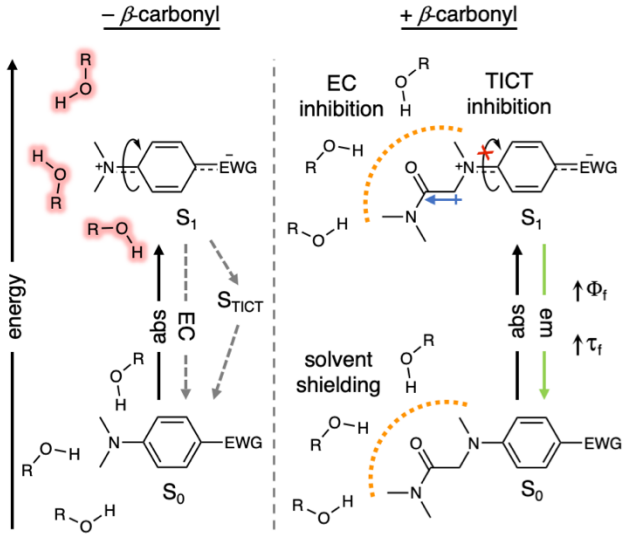

Figure 7. Proposed mechanism governing the enhancement of photophysical behavior in D–A fluorophores with β-carbonyl functionalization. Non-radiative decay mechanisms indicated by grey, dashed arrows.

Conclusion

In summary, we demonstrate that the β-carbonyl modified donor is a general method to improve upon the photophysical properties of D–A type molecules through the inhibition of excited state non-radiative decay. Our efforts have elucidated a mechanistic wherein the addition of a polar auxiliary to an amino donating in the form of a carbonyl effectively attenuates the contributions of TICT and EC to overall rate of excited state decay (Figure 7). This unique mechanism not only distinguishes the β-carbonyl from other existing modifications to D–A type molecules, but also makes the β-carbonyl additive to other modifications such as small azacyclic donating groups. We show that these functional groups improve upon the brightness of several classes of D–A scaffolds, and further that these properties can be finely tuned by perturbation of the carbonyl itself. Our demonstration of the improved brightness of these dyes in a cellular setting offers a single application of the β-carbonyl in practice, but still many opportunities remain. We suggest this modification can be used broadly to improve signal-to-noise ratios in analytical techniques that employ a D–A chromophore for fluorescence read-out. Further, we propose that these donors can be used to improve the kinetics of D–A based photolabile protecting groups or the turnover and longevity of photoredox catalysts in polar environment.

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Keywords: excited state • fluorescence • twisted intramolecular charge transfer • external conversion • donor-acceptor molecule

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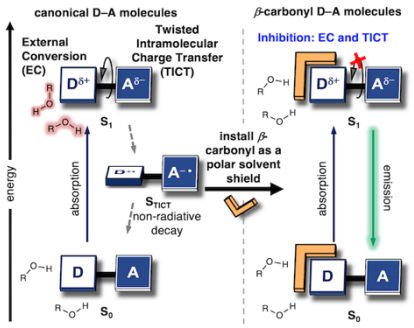

The presented work outlines the characterization and application of a novel means to improve the photophysical behavior of donor-acceptor molecules through installation of a polar, solvent-shielding auxiliary to an amino donating group. This β-carbonyl substitute generally improves fluorescence quantum yield of donor-acceptor fluorophores, enabling brighter labeling probes for live-cell imaging.

Institute and/or researcher Twitter usernames: @psu_chemistry, @kfzhangxin