Color regulation and stabilization of chromophore by Cys69 in photoactive yellow protein active center

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Model compounds of PYP chromophore were synthesized and characterized to investigate the role of the Cys69 residue in the active center, which has the intramolecular NH · · · OC hydrogen bond to the conjugated carbonyl oxygen and thioester linkage of the chromophore. The results of UV-vis and ^{13}C NMR spectroscopy of the model compounds indicated that they delocalized the negative charge of the chromophore and increased the contribution of the quinoid-like resonance structure in the phenolate anion state. Thus, the Cys69 residue plays an important role in the regulation of the color and the stabilization of the chromophore anion in the active center.

Introduction

Photoactive Yellow Protein (PYP) isolated from the purple sulfur bacterium *Halorhodospira halophila* by Meyer in 1985[1] is one of the photoreceptor proteins. PYP has a yellow color ($\lambda_{max} = 446$ nm), and it is considered to function as a blue-light photosensor implicated in the negative phototaxis of the bacterium.[2] Typical photoreceptor proteins such as rhodopsin and bacteriorhodopsin are membrane proteins; however, PYP is water soluble, small (14 kDa), and thermally stable.[3] Therefore, PYP is well studied and its three-dimensional structure has been reported, with its resolution higher than any other photoreceptor proteins. PYP is reported by some groups to be composed of 125 amino acids and one chromophore (4-hydroxycinnamic acid thioester).[4–6] Crystal structure of PYP reported by Borgstahl *et al.* shows a sphere-like structure with six β strands that are in anti-parallel arrangements in the middle, which is sandwiched by five α helices on both sides.[7]

The PYP chromophore is covalently bound to Cys69 of π-loop (residues 63–78) *via* a thiol ester linkage and exists as a phenolate anion state having *E* configuration in the hydrophobic core of the protein.[4–8] The chemical structure and the hydrogen bond network around amino acids (Tyr42, Glu46, and Cys69) and the chromophore are summarized in Fig. 1a.[7,9] Although Glu46 ($pK_a = 4.3$), an acidic residue, is located near the hydroxy group of the chromophore (4-hydroxycinnamic acid thioester, $pK_a = 8.8$),[10] it is proposed that the chromophore exists as a phenolate anion state in the active center of PYP.[5,8] Considering the differences in the pK_a values between phenols and carboxylic acids, this correlation between the deprotonated chromophore and the protonated Glu46 is considered to be distinctive of the active center of PYP. This diagnostic chemical mechanism regulated by the active center of PYP attracted considerable attention.

Point mutation studies of Tyr42 and Glu46 have suggested that hydrogen bonds to a phenolic hydroxy oxygen of the chromophore regulate the electronic properties of the chromophore, the color of PYP, and its stability.[11,12] However, the role of the hydrogen bond between the amide NH in Cys69 and the carbonyl oxygen in the chromophore has been noticed in previous research,[13] but not fully investigated. It was also reported that a point mutation at Cys69 (C69S) had no pigment and could not be reconstituted.[14] In order for PYP to function as a blue-light photosensor, the process of the chromophore binding to Cys69 *via* thioester linkage is considered to be important.

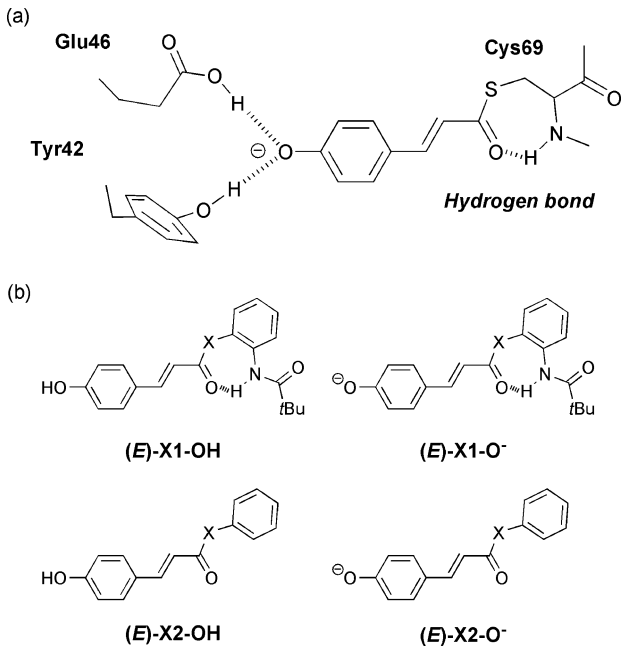

Fig. 1 (a) Schematic diagram around the chromophore at the ground state of Photoactive Yellow Protein and (b) *E* formed model compounds of the chromophore (X = S, O, NH).

To investigate the role of the hydrogen bond between Cys69 and the chromophore, we designed novel model compounds (shown in Fig. 1b) containing an intramolecular hydrogen bond to the carbonyl oxygen in the form of the seven-membered ring like native PYP. In previous work,[15] we synthesized the amide

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linked model compound (X = NH) and confirmed that the intramolecular NH···OC hydrogen bond is formed between the pivaloyl amide NH and the carbonyl C=O of the chromophore. Then, we could investigate the effect of the hydrogen bond to the carbonyl oxygen on the electronic state of the chromophore by using our model compound. In UV-vis spectroscopy, it is observed that the intramolecular NH···OC hydrogen bond contributes to the extension of π-electron delocalization in the part of the chromophore. In this work, we investigate the effects of the sulfur atom of the thioester group, in addition to the hydrogen bond to the carbonyl oxygen, on the chemical properties of the chromophore using the thioester or ester linked model compounds (X = S, O). Through the characterization of these more detailed model compounds, we can elucidate the role of the NH···OC hydrogen bond and sulfur atom (*i.e.* the Cys69 residue) in the active center of PYP.

Results and discussion

Synthesis of model compound

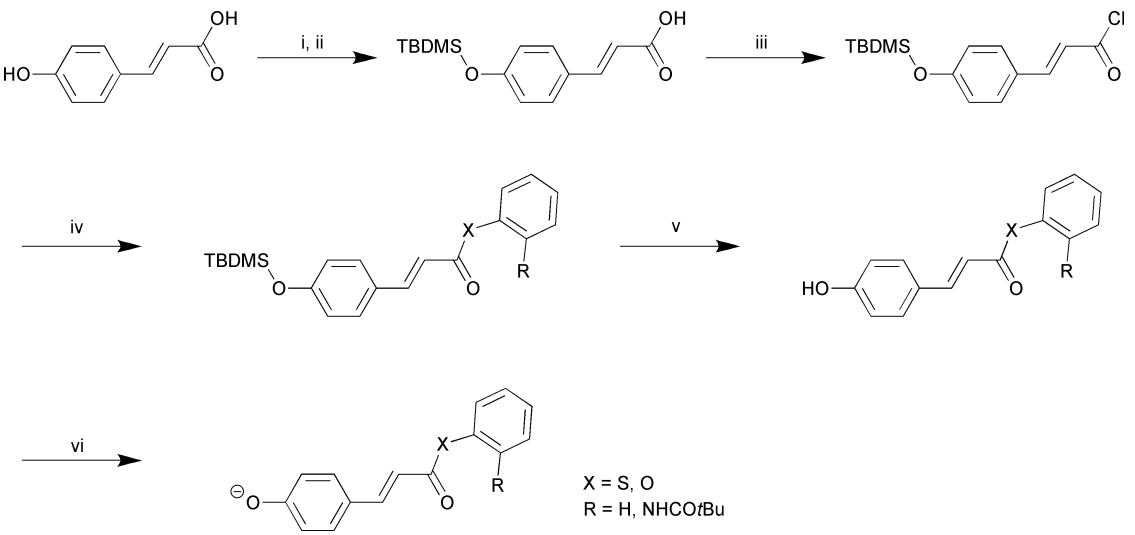
Syntheses of model compounds are shown in Scheme 1. The hydroxy group of 4-hydroxycinnamic acid was protected by treating with *tert*-butyldimethylsilyl triflate. The thioester and ester groups were synthesized by the reaction with a thiol or a phenol *via* an acyl chloride, respectively. Subsequently, the protecting group was removed by hydrogen fluoride.

Deprotonation of the hydroxy group was performed by neutralization with sodium hydroxide in a micellar solution. Unlike the amide linked model compounds (X = NH) reported in previous work,[15] the phenolate anion state of the compounds possessing the thioester or ester group could not be isolated in organic solvents. In the neutralization of the hydroxy group with hydroxide or alkoxide in organic solvents, the thiolate or phenolate anion (*i.e.* the fragment of model compound) was obtained from the reaction solution. This result suggests that the thioester and ester linkages were decomposed by nucleophilic attack of the base in the organic solvent. However, in a micellar solution, the model compound is incorporated into micellar cavity, in which the phenolate anion of the model compound is protected from the nucleophilic attack, and it was observed as stable without decomposition of the linkage for about one day. For various measurements of the model compounds, we employed poly(ethylene glycol) lauryl ether (C12E9) as the detergent since it is nonionic and has relatively little effect on ^{1}H NMR and UV-vis spectroscopy.

Formation of intramolecular NH···OC hydrogen bond in solution

In our previous work,[15] the results of X-ray crystal structure analysis and ^{1}H NMR spectroscopy of the amide linked model compound (X = NH) confirmed the formation of an intramolecular NH···OC hydrogen bond between the pivaloyl amide NH and carbonyl C=O in the 4-hydroxycinnamic acid moiety. In the first step, we confirmed the formation of the intramolecular NH···OC hydrogen bond in (***E***)**-S1-OH** and (***E***)**-O1-OH** by using ^{1}H NMR and IR spectroscopy. The information regarding chemical shifts and temperature coefficients of the amide NH signals in ^{1}H NMR spectra and the amide NH stretching vibrations in IR spectra of (***E***)**-S1-OH** and (***E***)**-O1-OH** are summarized in Table 1. The temperature coefficients of the amide NH signals of (***E***)**-S1-OH** and (***E***)**-O1-OH** were −1.70 and −1.75 ppb/K in chloroform-d_1, respectively. These values were smaller than that for the reference compound **C6H5-NHCO*t*Bu** (−2.38 ppb/K in chloroform-d_1), which has no intramolecular hydrogen bond. These results suggest the formation of an intramolecular NH···OC hydrogen bond to the carbonyl C=O of the chromophore in solution.

However, the coefficients of (***E***)**-O1-OH**, an ester model compound, increased toward changes in the solvent polarity, whereas those of (***E***)**-S1-OH**, a thioester model compound, showed little change in both acetonitrile-d_3 and 10% C12E9 micellar solution (10% D_2O, 80% H_2O). Additionally, the amide NH stretching vibration of (***E***)**-S1-OH** (3405 cm^{-1}) shifts to a lower wavenumber compared with those of (***E***)**-O1-OH** (3459 cm^{-1}) and **C6H5-NHCO*t*Bu** (3455 cm^{-1}) by IR spectroscopy in chloroform solution. These results suggest that a more stable intramolecular NH···OC hydrogen bond is formed in the thioester model (***E***)**-S1-OH** than



Scheme 1 Synthesis of the model compound. (i) TBDMS(OTf), Et_3N; CH_2Cl_2; rt; (ii) K_2CO_3; MeOH/THF/H_2O; rt; (iii) $SOCl_2$; CH_2Cl_2; reflux; (iv) Ar-SH or Ar-OH, Et_3N; CH_2Cl_2; rt; (v) HF·pyridine; THF; rt; (vi) NaOH *aq*; 10% C12E9 micellar solution.

Table 1 Chemical shifts (ppm), temperature coefficients (ppb/K), and stretching vibrations (cm^{-1}) of the model compounds

	Chemical shift (ppm)/Temperature coefficient (ppb/K)			Stretching vibration (cm^{-1})
	in $CDCl_3$	in CD_3CN	in 10% micellar solution (10% D_2O, 80% H_2O)	in $CHCl_3$
(***E***)**-S1-OH**	8.17/−1.70	8.11/−1.02	8.31/−2.43	3405
(***E***)**-O1-OH**	7.59/−1.75	7.72/−2.02	8.21/−6.98	3459

in the ester model (***E***)**-O1-OH**. The downfield shifts of amide NH signal in ^{1}H NMR spectroscopy support this suggestion. In addition, it is considered that the intramolecular NH···OC hydrogen bond in (***E***)**-O1-OH** is not formed or might be weak in polar solutions.

In light of these findings, it is proposed that the difference between bridging atoms (sulfur or oxygen) induces changes in the formation of the intramolecular NH···OC hydrogen bond. Thus, we investigated the conformation of each model compound. Superposition of the optimized structures of model compounds obtained by *ab initio* calculation (B3LYP/6–31++G(d,p))[16–19] is shown in Fig. 2. Superposition of (***E***)**-S1-OH** and (***E***)**-O1-OH** shows that the bond distance between the amide proton and carbonyl oxygen is shorter in the thioester model compound (***E***)**-S1-OH** (1.940 Å) than the ester model compound (***E***)**-O1-OH** (1.972 Å). This conformational difference within a part of the seven-membered ring is due to the change in C(carbonyl)-X-C(Ar) bond angle (106.99 in (***E***)**-S1-OH** and 122.96 in (***E***)**-O1-OH**). These optimized structures support the results obtained from ^{1}H NMR and IR spectroscopy. It is suggested that the thioester model compound (***E***)**-S1-OH** has a conformation that is easy to form an intramolecular hydrogen bond between the amide group and the 4-hydroxycinnamic acid moiety.

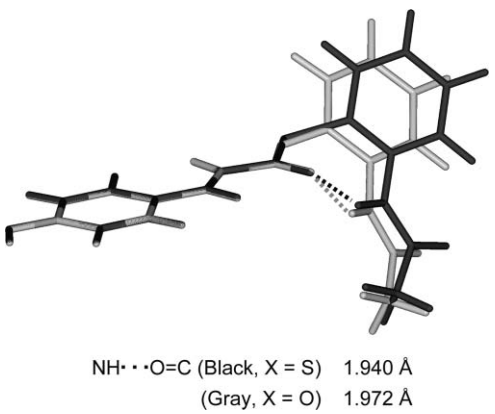


Fig. 2 Superposition of the part of the chromophore of the optimized structure (black: (***E***)**-S1-OH**, gray: (***E***)**-O1-OH**).

The effects of NH···OC hydrogen bond and sulfur atom on p-electron conjugation system of PYP chromophore

^{1}H NMR spectra of model compounds (***E***)**-S1-OH**, (***E***)**-S1-O$^-$**, (***E***)**-O1-OH**, and (***E***)**-O1-O$^-$** in a 10% C12E9 micellar solution (10% D_2O, 80% H_2O) at 303 K are shown in Fig. 3. The chemical shifts of signals (H_a~H_d) derived from the 4-hydroxycinnamic acid moiety show upfield shifts with deprotonation of the hydroxy group. It is noteworthy that the signal of H_d, which is located far away from the hydroxy group, shows an upfield shift. It is suggested that the deprotonation has a significant effect on the π-electron conjugation system of the chromophore.

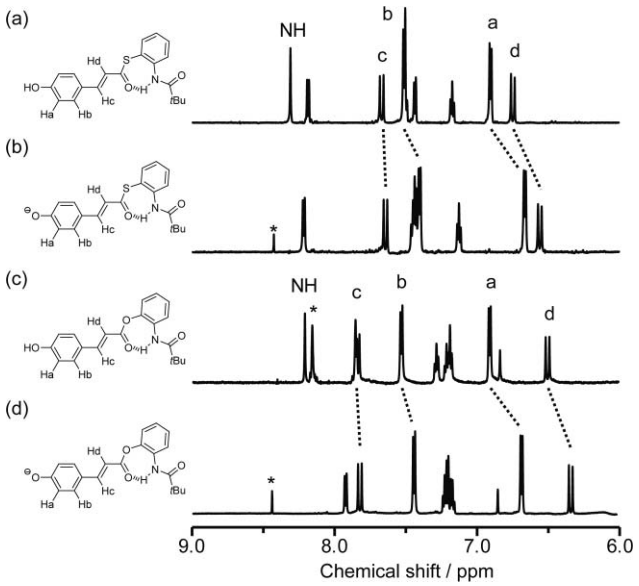


Fig. 3 ^{1}H NMR spectra of (a) (***E***)**-S1-OH**, (b) (***E***)**-S1-O$^-$**, (c) (***E***)**-O1-OH**, and (d) (***E***)**-O1-O$^-$** in a 10% C12E9 micellar solution (10% D_2O, 80% H_2O) at 303 K.

We investigated the detail of this change in the π-electron conjugation system by using UV-vis spectroscopy. The absorption spectra of model compounds obtained in a 10% C12E9 micellar solution at 303 K are shown in Fig. 4, and these spectral data assigned to the π–π* transition of chromophore using the MOPAC program (INDO/S method)[20] are summarized in Table 2. The π–π* transition of the model compounds is located around

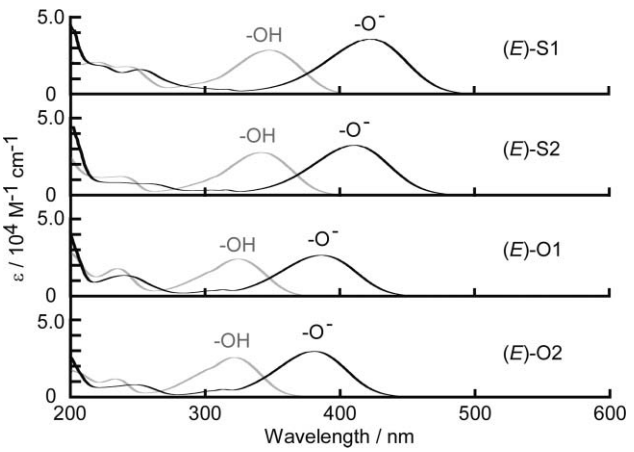


Fig. 4 UV-vis spectra of the model compounds in a 10% C12E9 micellar solution at 303 K. (gray: phenol state, black: phenolate anion state).

Table 2 Absorption maxima of π–π* transition of model compounds in 10% C12E9 micellar solution at 303K

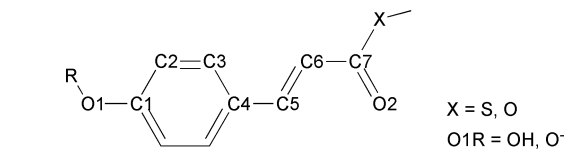
	-OH	-O⁻
(*E*)-**S1**	348	425
(*E*)-**S2**	342	413
(*E*)-**O1**	325	386
(*E*)-**O2**	323	382

330 nm in the phenol state and around 400 nm in the phenolate anion state. It is well known that the extension of π-electron delocalization induces a red shift of the absorption maximum. The absorption maximum is significantly red-shifted according to the deprotonation of chromophore, indicating that the negative charge delocalizes along the skeleton of the chromophore in the phenolate anion state.

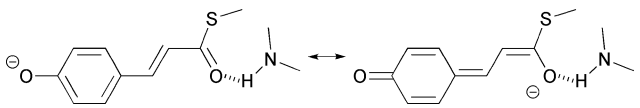
It is observed in both the phenol and phenolate anion states that the absorption maximum of the π–π* transition is red-shifted by the formation of the intramolecular NH···OC hydrogen bond. The absorption maximum of the thioester model compound (*E*)-**S1** is red-shifted by +6 nm in the phenol state and +12 nm in the phenolate anion state compared with those of (*E*)-**S2**. These results indicate that the formation of the intramolecular NH···OC hydrogen bond contributes significantly to the extension of π-electron delocalization along the skeleton of the chromophore, especially in the phenolate anion state. On the other hand, the absorption maximum of the ester model compound (*E*)-**O1** is red-shifted by +2 nm in the phenol state and +4 nm in the phenolate anion state compared with those of (*E*)-**O2**, indicating that the contributions of the intramolecular NH···OC hydrogen bond are smaller in the ester model compounds than in the thioester model compounds. These results reflect the differences in the formation of the intramolecular hydrogen bond between thioester and ester model compounds, as shown in Fig. 2.

The absorption maxima of the thioester model compounds are red-shifted by about +20 nm in the phenol state and about +30~40 nm in the phenolate anion state compared with those of the ester model compounds. It is observed that the extension of π-electron delocalization is induced by the introduction of sulfur into the bridging atom as well.

From the results of UV-vis spectroscopy of the model compound, it is indicated that the intramolecular NH···OC hydrogen bond to the conjugated carbonyl oxygen spatially interacts with the π-electron conjugation system through a different mechanism from substituent effects. Thus, we investigated the changes in the electronic state of the chromophore in more detail using *ab initio* calculation and ^{13}C NMR spectroscopy. The bond lengths of the 4-hydroxycinnamic acid moiety and the Mulliken charges of the hydroxy and the carbonyl oxygen are summarized in Tables 3 and 4. The numbering of the 4-hydroxycinnamic acid moiety are shown in Scheme 2. The bonds of the phenyl ring moiety (C1-C2, C2-C3, C3-C4) have aromatic characteristics in the phenol state. However, the bonds of C1-C2 and C3-C4 are lengthened and those of C2-C3 are shortened in the phenolate anion state, suggesting the contribution of the quinoid-like resonance structure (Scheme 3). It is considered that the shifts between phenol and phenolate anion in ^1H NMR and UV-vis spectroscopy are derived from the contribution of this quinoid-like structure. Additionally, the differences between (*E*)-**X1-O**⁻ and (*E*)-**X2-O**⁻ (X = S, O) show that such contribution is increased by the formation of the



Scheme 2 The numbering of the 4-hydroxycinnamic acid moiety.



Scheme 3 Resonance structure in the phenolate anion state.

Table 3 Bond length (Å) of the 4-hydroxycinnamic acid moiety of the optimized structure

	(*E*)-X1-OH		(*E*)-X2-OH		(*E*)-X1-O⁻		(*E*)-X2-O⁻	
	X = S	X = O	X = S	X = O	X = S	X = O	X = S	X = O
O1-C1	1.364	1.364	1.365	1.366	1.255	1.257	1.258	1.259
C1-C2[a]	1.402	1.402	1.401	1.401	1.460	1.459	1.458	1.458
C2-C3[a]	1.389	1.389	1.390	1.390	1.369	1.371	1.372	1.373
C3-C4[a]	1.411	1.410	1.410	1.410	1.435	1.433	1.432	1.431
C4-C5	1.456	1.457	1.459	1.459	1.410	1.414	1.417	1.419
C5-C6	1.354	1.351	1.352	1.350	1.392	1.387	1.385	1.382
C6-C7	1.468	1.466	1.474	1.471	1.419	1.422	1.430	1.431
C7-O2	1.227	1.223	1.215	1.214	1.243	1.239	1.222	1.225

[a] The average C-C bond lengths in the phenyl ring.

Table 4 Mulliken oxygen atomic charge value of the optimized structure

	(*E*)-X1-OH		(*E*)-X2-OH		(*E*)-X1-O⁻		(*E*)-X2-O⁻	
	X = S	X = O	X = S	X = O	X = S	X = O	X = S	X = O
O1(O-R)	–0.4835	–0.4835	–0.4894	–0.4888	–0.5872	–0.5962	–0.6030	–0.6081
O2(C=O)	–0.3762	–0.4751	–0.3112	–0.4443	–0.4396	–0.5495	–0.3742	–0.5152

Table 5 ^{13}C chemical shift (ppm) of C1 in the model compound (5 mM, 10% C12E9 micellar solution (10% D_2O, 80% H_2O), 303K

	-OH	$-O^-$	$\Delta\delta(O^-\text{-OH})$
(*E*)-S1	163.5	174.0	+10.5
(*E*)-S2	162.7	171.5	+8.8
(*E*)-O1	163.1	171.6	+8.5
(*E*)-O2	162.9	171.3	+8.4

intramolecular NH · · · OC hydrogen bond and the introduction of a sulfur atom into the bridging atom. It is also considered that the intramolecular hydrogen bond increases the negative charge on the conjugated carbonyl oxygen, and influences the π-electron conjugation system of the chromophore through the change in the characteristics of the carbonyl group. For example, the intramolecular hydrogen bond stabilizes the negative charge of the quinoid-like canonical form, as depicted in Scheme 3, and consequently increases the contribution of the quinoid-like structure. The changes in Mulliken atomic charge (shown in Table 4) support this mechanism. The Mulliken oxygen atomic charge values of the carbonyl group are increased by the formation of the intramolecular NH · · · OC hydrogen bond by about 0.06 and 0.03 in the thioester and ester model compounds, respectively.

This change in the electronic state of the phenolate anion was also observed in ^{13}C NMR spectroscopy. The chemical shift of C1, which is directly bound to the phenolic hydroxy oxygen, should undergo a downfield shift by the contribution of the quinoid-like resonance structure since the hybrid orbital between this carbon and the phenolic oxygen changes from sp^3 to sp^2. The ^{13}C chemical shift of the C1 observed in a 10% micellar solution (10% D_2O, 80% H_2O) at 303 K is shown in Table 5. The detection and the assignment of ^{13}C signal were performed by heteronuclear correlation spectroscopy (HMBC). The obtained ^{13}C chemical shifts are shifted downfield by the formation of the intramolecular NH · · · OC hydrogen bond and the introduction of a sulfur atom into the bridging atom, supporting the notion that their effects increase the contribution of the quinoid-like resonance structure shown in Scheme 3.

From these results, the hydrogen bond network from Cys69 NH to the conjugated carbonyl group is considered to have a significant effect on the electronic state of the chromophore. In the native PYP active center, the Cys69 residue plays a crucial role in the color regulation for PYP to function as a blue-light sensor.[2]

Stabilization of phenolate anion state by NH · · · OC hydrogen bond and sulfur atom

In order to investigate the effects of the NH · · · OC hydrogen bond to the conjugated carbonyl oxygen and the sulfur atom of the thioester group on the acidity of the chromophore in the native PYP active center, we performed pH titrations of the model compounds in a micellar solution and determined their pK_a values. The pK_a values of **(*E*)-S1-OH**, **(*E*)-S2-OH**, **(*E*)-O1-OH**, and **(*E*)-O2-OH** at 303 K are 8.2, 8.4, 8.6, and 8.8, respectively. These pK_a values are comparatively small in the phenolic compounds. This is considered to be due to the contribution of the quinoid-like resonance structure proposed from the results of UV-vis and ^{13}C NMR spectroscopy. The pK_a values decreased by the formation of the intramolecular NH · · · OC hydrogen bond (–0.2 units) and the change from oxygen to sulfur in the bridging atom (–0.4 units), suggesting that the phenolate anion of the chromophore is stabilized by their contributions.

In order to investigate in detail the chemical mechanism of the deprotonation, we evaluated the relative difference in acidity (*i.e.* stability of anion state) between the model compounds and carboxylic acid under the influence of a hydrophobic environment. The previous study of the quantum chemical calculation of the chromophore model has reported that the chromophore exhibits the solvatochromism,[21] suggesting that the hydrophobic core of the PYP active center plays an important role in the regulation of the electronic state of the chromophore.

The tetra-*n*-butylammonium acetate (1.0 eq) was added to a THF solution of the phenol model compound (1.0 eq), and the color of the reaction solution changed into yellow. The absorption spectra of the reaction solutions are shown in Fig. 5. These results show a decrease of the absorption band around 310 nm, which is derived from the π–π* transition of the phenol compound, and an increase of a new band around 400 nm. As seen in the results of the absorption spectra in the micellar solution (Fig. 4), this new band is derived from the π–π* transition of the phenolate anion, indicating that the equilibria between phenol and phenolate anion of the model compound are observed in the reaction solution. As mentioned above, the model compounds possessing the thioester or ester group are decomposed in organic solvents by the nucleophilic attack of the base. However, the absorption spectra shown in Fig. 5 had no change for several hours, suggesting that the phenolate anion is stable in the acid-dissociation equilibrium between the model compound and the acetate.

Considering their pK_a values of model compounds, it is suggested that this equilibrium observed in THF solution is different from those in aqueous solution, and it is a specific reaction in the low-polar solvent. It is proposed that the relation of their pK_a values, which represents the stabilities of the anion state,[22–24] between the model compounds and the acetate are largely altered in THF solution compared with when they are in aqueous solution. The chromophore has a tendency to delocalize the negative charge overall along the π-electron conjugation system and stabilize its electronic state. We propose that the delocalization of the negative charge along the extended conjugation system of the chromophore is more stable in low-polar solvents such as THF than the localization at the short conjugation system of the acetate anion.

Populations of the residual phenol, which are calculated by spectral changes between before and after the reaction, are shown in Fig. 5. The obtained values show that this exchange reaction between the chromophore phenol and acetate anion is promoted and this equilibrium is controlled by the contributions of the intramolecular NH · · · OC hydrogen bond and the bridging sulfur atom (Scheme 4). From the results of pH titrations and exchange reactions with the acetate anion of the model compounds, it is concluded that the Cys69 residue, which affects both the hydrogen bond to the carbonyl group and the bridging sulfur atom, is important in the deprotonation of the chromophore in the native PYP active center.

The point mutant PYPs Y42F and Y42A were previously reported by other groups.[11,12] In these studies, the effect of OH · · · O hydrogen bond from Tyr42 to the hydroxy group of the chromophore was removed. The absorption spectra of both Y42F and Y42A are different from the native PYP and have two bands

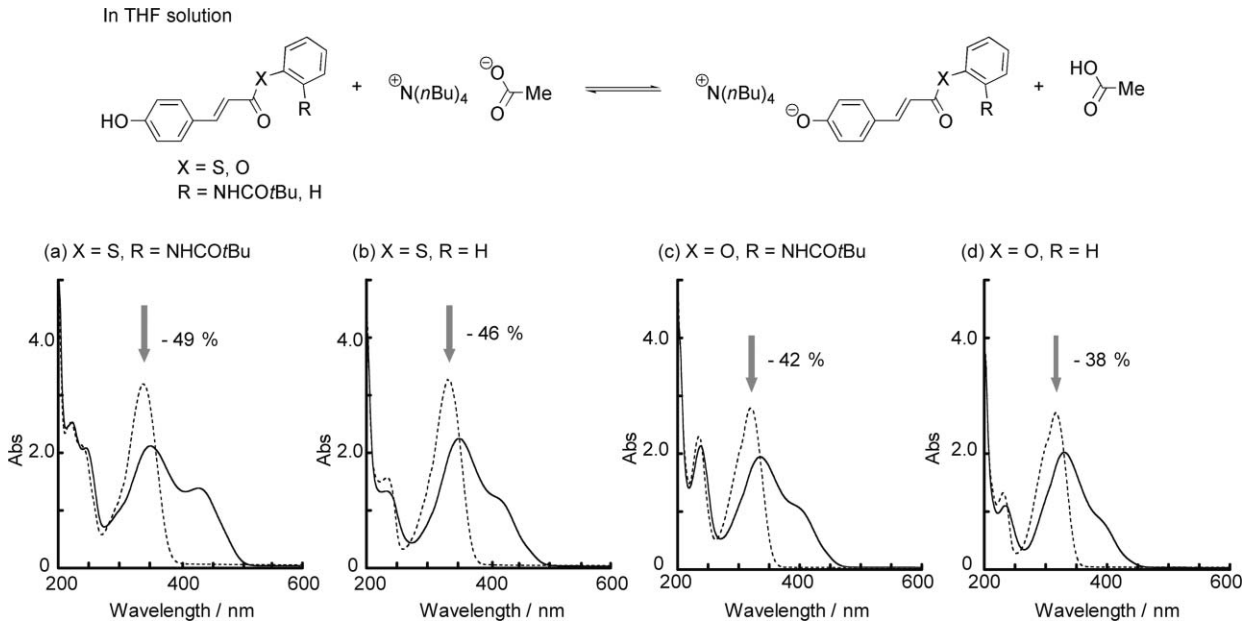
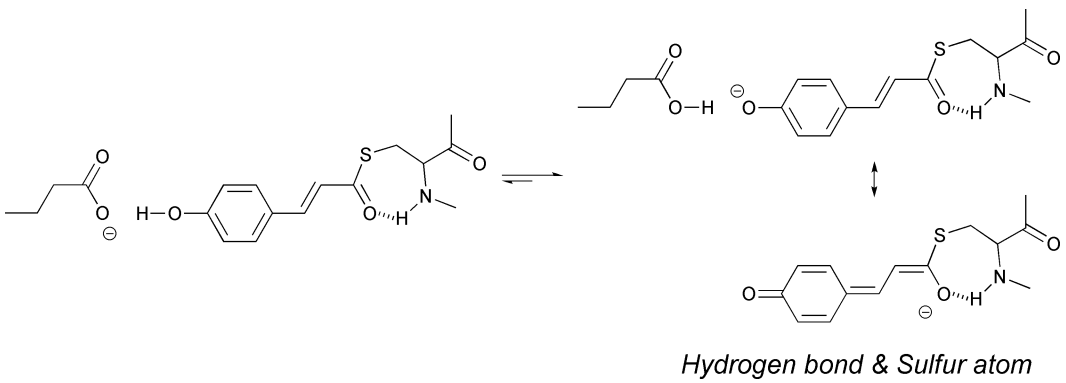


Fig. 5 UV-vis spectra of (a) (*E*)-**S1-OH**, (b) (*E*)-**S1-O**$^-$, (c) (*E*)-**O1-OH**, and (d) (*E*)-**O1-O**$^-$ in both of the phenol of the model compound (dotted line) and the reaction solution with tetra-*n*-butylammonium acetate (solid line) in THF solution at 303 K.



Scheme 4 Proposed mechanism of deprotonation of the chromophore.

at around 450 and 380 nm, which are derived from the phenol and phenolate anion state of the chromophore, respectively. Studies of the model compounds suggest that the chromophore is in equilibrium with the phenol and the phenolate anion in the active center of Y42F and Y42A. In our previous studies, we found that the hydrogen bond to the phenolic hydroxy group lowers the pK_a value of phenol and stabilizes the phenolate anion state.[24,25] Thus, it is considered that the hydrogen bond from Tyr42 to the chromophore is also important in the deprotonation of the chromophore.

Conclusion

We synthesized thioester and ester model compounds containing an intramolecular NH $\cdots$ OC hydrogen bond to the conjugated carbonyl oxygen and investigated the role of Cys69 residue in the native PYP active center. The difference in the bridging atom has an effect on the seven-membered skeleton of the intramolecular hydrogen bond. In the case of the chromophore with the thioester group, a more stable NH $\cdots$ OC hydrogen bond is formed.

The formation of the intramolecular NH $\cdots$ OC hydrogen bond to the conjugated carbonyl oxygen has significant effects on the π-electron conjugation system of the chromophore. In the phenolate anion state, the formation of this hydrogen bond increases the contribution of the quinoid-like resonance structure, which changes the chemical properties of the chromophore (*e.g.* the red-shift of color and anion stabilization). Moreover, it was observed that the relation of the acidity, representing the stabilities of phenolate anion state, between the chromophore model and the acetate anion were largely altered under a hydrophobic environment as compared with in water or in a micellar solution. This result suggests that the chromophore exists stably as a phenolate anion under the influence of incorporation into the hydrophobic active center of PYP and the stabilization of the phenolate anion state by the Cys69 residue (*i.e.* the NH $\cdots$ OC hydrogen bond and sulfur atom).

In conclusion, the Cys69 residue in the active center of PYP is considered to be an essential factor for 4-hydroxycinnamic acid, which is incorporated as its prosthetic group, to function as a chromophore.

Experimental

Materials

All operations were performed under an argon atmosphere. All solvents were dried and distilled under an argon atmosphere before use. 4-Hydroxycinnamic acid was purchased from Tokyo-Kasei Co. *tert*-Butyldimethylsilyl trifluoromethanesulfonate and hydrogen fluoride pyridine were purchased from Aldrich Chemical Company, Inc. Phenol and thiophenol were purchased from Nacalai Tesque, Inc. The syntheses of *N*-(2-hydroxyphenyl)pivalamide and 2,2′-dithiobis(*N*-phenyl-2,2-dimethylpropanamide) were carried out using the same previously reported procedure.[25,26]

Preparation of 4-*tert*-butyldimethylsiloxycinnamic acid

tert-Butyldimethylsilyl trifluoromethanesulfonate (8.60 mL, 37.4 mmol) was added to a suspension of 4-hydroxycinnamic acid (2.03 g, 12.4 mmol) in 40.0 mL of CH_2Cl_2 on an ice bath and then Et_3N (8.00 mL, 57.0 mmol) was added to it. The mixed solution became a homogeneous and the color was changed into light yellow. The solution was stirred for 21 hours. The reaction solution was washed successively with 10% HCl aqueous solution, H_2O, and NaCl aqueous solution, and organic layer was dried with $MgSO_4$. The solution was concentrated under reduced pressure to give bright yellow oil. This oil was dissolved in 1:2 MeOH-THF (12.0 mL) in a round-bottom flask and stirred at room temperature while K_2CO_3 (5.20 g, 37.6 mmol) in 3.0 mL of H_2O was added slowly. The mixture was stirred for 3 hours. The resulting solution was diluted with diethyl ether and successively washed with 10% HCl aqueous solution, H_2O, and saturated NaCl aqueous solution, and this organic layer was dried with Na_2SO_4. The solution was concentrated under reduced pressure to a light yellow powder. The powder was washed with hexane and dried over P_2O_5 under reduced pressure. (Yield: 2.42 g, 68%). Mp 127–129 °C. Anal. Calcd for $C_{15}H_{22}O_3Si$: C, 64.71; H, 7.96. Found: C, 64.82; H, 7.87. ^{1}H NMR (500 MHz, 30 °C, in chloroform-d_1, TMS): δ 7.66 (1H, d), 7.38 (2H, d), 6.78 (2H, d), 6.24 (1H, d), 0.92 (9H, s), 0.16 (6H, s). ^{13}C-NMR (125 MHz, 30 °C, in chloroform-d_1, TMS): δ 172.31, 158.20, 146.68, 129.97, 127.34, 120.53, 114.82, 25.69, 18.33, –4.27. ESI-MS: m/z$^-$; 277.2 (M-H, calcd; 277.12).

Preparation of *N*-(2-mercaptophenyl)pivalamide

2,2′-Dithiobis(*N*-phenyl-2,2-dimethylpropanamide) (1.06 g, 2.544 mmol) was dissolved in 10.0 mL of MeOH and then a large excess of $NaBH_4$ was slowly added until the yellow color changed to pale yellow. Then acetic acid and water were added to pH 4~5. After removal of MeOH under reduced pressure, the desired product was extracted with hexane and diethyl ether. The organic layer was washed with saturated NaCl aqueous solution, and this organic layer was dried with Na_2SO_4. The solution was concentrated under reduced pressure to obtain a white powder. This powder was washed with hexane and dried under reduced pressure. (Yield: 820.2 mg, 77%). Anal. Calcd for $C_{11}H_{15}NOS$: C, 63.12; H, 7.22; N, 6.69. Found: C, 63.43; H, 6.77; N, 6.72. ^{1}H-NMR (500 MHz, 30 °C, in chloroform-d_1, TMS): δ 8.34 (br, 1H), 8.23 (d, 1H), 7.43 (d, 1H), 7.23 (t, 1H), 6.93 (t, 1H), 3.00 (s, 1H), 1.29 (s, 9H).

Preparation of (E)-S-2-(pivalamido)phenyl 3-(4-*tert*-butyldimethylsiloxy-phenyl)prop-2-enethioate

4-*tert*-Butyldimethylsiloxycinnamic acid (792.8 mg, 2.848 mmol) was refluxed with thionyl chloride (2.50 mL, 34.3 mmol) in 10.0 mL of CH_2Cl_2 for 5 hours. The reaction solution was cooled to room temperature, and concentrated to give a yellow oily solution. CH_2Cl_2 was added to it and the solution was concentrated again to remove trace of thionyl chloride. This oil was dissolved in 10.0 mL of CH_2Cl_2 and added to *N*-(2-mercaptophenyl)pivalamide (594.2 mg, 2.839 mmol) in 10.0 mL of CH_2Cl_2. The resulting solution was stirred at room temperature and then Et_3N (3.0 mL, 21.5 mmol) was added to it. The mixed solution was stirred overnight. The resulting solution was diluted with diethyl ether and successively washed with 10% HCl aqueous solution, H_2O, and saturated NaCl aqueous solution, and this organic layer was dried with Na_2SO_4. The solution was concentrated under reduced pressure to obtain a white oil. This oil was dissolved in hexane and cooled in a refrigerator to give a white powder. This powder was washed with hexane and dried under reduced pressure. (Yield: 760.0 mg, 57%). Mp 98–100 °C. Anal. Calcd for $C_{26}H_{35}NO_3SSi$: C, 66.48; H, 7.51; N, 2.98. Found: C, 66.70; H, 7.59; N, 2.97. ^{1}H-NMR (500 MHz, 30 °C, in chloroform-d_1, TMS): δ 8.27 (dd, 1H), 8.10 (br, 1H), 7.61 (d, 1H, 15.57 Hz), 7.41 (d, 2H), 7.39 (td, 1H), 7.37 (dd, 1H), 7.07 (td, 1H), 6.80 (d, 2H), 6.63 (d, 1H, 15.57 Hz), 1.20 (s, 9H), 0.92 (s, 9H), 0.17 (s, 6H). ^{13}C-NMR (125 MHz, 30 °C, in chloroform-d_1, TMS): δ 186.02, 175.56, 157.88, 141.89, 139.27, 134.87, 130.45, 129.50, 125.89, 123.42, 121.44, 119.92, 119.92, 116.50, 38.96, 26.52, 26.50, 24.59, –5.37. ESI-MS: m/z; 492.1 (M + Na, calcd; 492.20).

Preparation of (E)-S-2-(pivalamido)phenyl 3-(4-hydroxyphenyl)-prop-2-enethioate ((E)-S1-OH)

To a solution of (*E*)-*S*-(2-pivalamidophenyl)-3-(4-*tert*-butyldimethylsiloxy-phenyl)prop-2-enethioate (758.0 mg, 1.61 mmol) in 5.0 mL of THF was added 5.0 mL of 2 M HF solution, stirred at room temperature for 5 hours. The resulting solution was diluted with CH_2Cl_2 and successively washed with saturated $NaHCO_3$ aqueous solution, 2% HCl aqueous solution, H_2O, and saturated NaCl aqueous solution, and this organic layer was dried with Na_2SO_4. The solution was concentrated under reduced pressure to give a yellow oil. This oil was dissolved in AcOEt and added hexane to give a white powder, and this powder was dried over P_2O_5 under reduced pressure. (Yield: 359 mg, 63%). Mp. 162–168 °C. Anal. Calcd for $C_{20}H_{21}NO_3S$: C, 67.58; H, 5.95; N, 3.94. Found: C, 67.32; H, 6.00; N, 3.86. ^{1}H-NMR (500 MHz, 30 °C, in chloroform-d_1, TMS): δ 8.33 (dd, 1H), 8.17 (br, 1H), 7.67 (d, 1H, 15.57 Hz), 7.50 (d, 2H), 7.49 (td, 1H), 7.46 (dd, 1H), 7.15 (td, 1H), 6.87 (d, 2H), 6.70 (d, 1H, 15.57 Hz), 5.26 (br, 1H), 1.27 (s, 9H). ^{13}C-NMR (125 MHz, 30 °C, in chloroform-d_1, TMS): δ 187.08, 176.70, 158.48, 142.75, 140.23, 135.89, 131.49, 130.81, 126.60, 124.54, 122.57, 120.84, 117.61, 116.19, 39.99, 27.54. ESI-MS: m/z$^-$; 354.1 (M-H, calcd; 354.12).

Preparation of (E)-S-phenyl 3-(4-*tert*-butyldimethylsiloxy-phenyl)prop-2-enethioate

4-*tert*-Butyldimethylsiloxycinnamic acid (524.5 mg, 1.884 mmol) was refluxed with thionyl chloride (2.00 mL, 27.4 mmol) in 10.0 mL

of CH_2Cl_2 for 5 hours. The reaction solution was cooled to room temperature, and concentrated to give a yellow oily solution. CH_2Cl_2 was added to it and the solution was concentrated again to remove trace of thionyl chloride. This oil was dissolved in 5.0 mL of CH_2Cl_2 and added to thiophenol (0.185 mL, 1.81 mmol) in 5.0 mL of CH_2Cl_2. The resulting solution was stirred at room temperature and then Et_3N (2.80 mL, 20.0 mmol) was added to it. The mixed solution was stirred overnight. The resulting solution was diluted with diethyl ether and successively washed with 2% HCl aqueous solution, H_2O, and saturated NaCl aqueous solution, and this organic layer was dried with Na_2SO_4. The solution was concentrated under reduced pressure to obtain a yellow oil. This oil was dissolved in methanol and cooled in a refrigerator to give a crude yellow powder. This crude product was used for the next reaction without further purification. ^{1}H-NMR (500 MHz, 30 °C, in chloroform-d_1, TMS): δ 7.56 (d, 1H, 15.75 Hz), 7.42 (m, 1H), 7.39 (d, 2H), 7.36 (m, 2H), 7.35 (m, 1H), 6.78 (d, 2H), 6.63 (d, 1H, 15.75 Hz), 0.92 (s, 9H), 0.16 (s, 6H).^{13}C-NMR (125 MHz, 30 °C, in chloroform-d_1, TMS): δ 186.69, 157.19, 140.24, 133.48, 129.05, 128.15, 127.97, 126.75, 126.11, 119.49, 114.88, 24.58, 17.22, −5.37. ESI-MS: m/z; 763.1 (M × 2 + Na, calcd; 763.27).

Preparation of (E)-S-phenyl 3-(4-hydroxyphenyl)prop-2-enethioate ((E)-S2-OH)

To a solution of (*E*)-*S*-phenyl-3-(4-*tert*-butyldimethylsiloxy-phenyl)prop-2-enethioate (490.1 mg, 1.323 mmol) in 10.0 mL of THF was added 7.0 mL of 2 M HF solution, stirred at room temperature for 5 hours. The resulting solution was diluted with diethyl ether and successively washed with saturated $NaHCO_3$ aqueous solution, 2% HCl aqueous solution, H_2O, and saturated NaCl aqueous solution, and this organic layer was dried with Na_2SO_4. The solution was concentrated under reduced pressure to give a yellow oil. This oil was dissolved in diethyl ether and added hexane to give a light yellow powder, and this powder was dried over P_2O_5 under reduced pressure. (Yield: 98.7 mg, 29%). Mp. 117–122 °C. Anal. Calcd for $C_{15}H_{12}O_2S$: C, 70.29; H, 4.72. Found: C, 69.70; H, 4.72. ^{1}H-NMR (500 MHz, 30 °C, in chloroform-d_1, TMS): δ 7.62 (d, 1H, 15.75 Hz), 7.49 (m, 2H), 7.47 (d, 2H), 7.42 (m, 2H), 7.42 (m, 1H), 6.85 (d, 2H), 6.60 (d, 1H, 15.75 Hz), 4.99 (br, 1H). ^{13}C-NMR (125 MHz, 30 °C, in chloroform-d_1, TMS): δ 187.91, 157.91, 141.18, 134.67, 130.51, 129.36, 129.17, 127.91, 127.07, 122.01, 116.14. ESI-MS: m/z$^-$; 255.0 (M-H, calcd; 255.05).

Preparation of (E)-2-(pivalamido)phenyl 3-(4-*tert*-butyldimethylsiloxy-phenyl)acrylate

4-*tert*-Butyldimethylsiloxycinnamic acid (1.013 g, 3.638 mmol) was refluxed with thionyl chloride (2.50 mL, 34.3 mmol) in 15.0 mL of CH_2Cl_2 for 4 hours. The reaction solution was cooled to room temperature, and concentrated to give a yellow oil. CH_2Cl_2 was added to it and the solution was concentrated again to remove trace of thionyl chloride. This oil was dissolved in 15.0 mL of CH_2Cl_2 and added to *N*-(2-hydroxyphenyl)pivalamide (731.6 mg, 3.786 mmol) in 15.0 mL of CH_2Cl_2. The resulting solution was stirred at room temperature and then Et_3N (6.0 mL, 43.0 mmol) was added to it. The mixed solution was stirred overnight. The resulting solution was diluted with diethyl ether and successively washed with 10% HCl aqueous solution, H_2O, and saturated NaCl aqueous solution, and this organic layer was dried with Na_2SO_4. The solution was concentrated under reduced pressure to obtain a yellow oil. This oil was chromatographed on silica gel (3:1:2/CH_2Cl_2:THF:hexane) to obtain a white oil. (Yield: 1.21 g, 73%). ^{1}H-NMR (500 MHz, 30 °C, in chloroform-d_1, TMS): δ 8.13 (dd, 1H), 7.82 (d, 1H, 15.87 Hz), 7.52 (br, 1H), 7.43 (d, 2H), 7.18 (td, 1H), 7.12 (dd, 1H), 7.07 (td, 1H), 6.82 (d, 2H), 6.44 (d, 1H, 15.87 Hz), 1.20 (s, 9H), 0.93 (s, 9H), 0.17 (s, 6H). ^{13}C-NMR (125 MHz, 30 °C, in chloroform-d_1, TMS): δ 175.44, 163.91, 157.75, 146.64, 140.07, 129.28, 129.20, 126.10, 125.43, 123.56, 121.95, 120.94, 119.72, 119.68, 38.81, 26.57, 24.60, 17.26, −5.37. ESI-MS: m/z; 929.1 (M × 2 + Na, calcd; 929.5).

Preparation of (E)-2-(pivalamido)phenyl 3-(4-hydroxyphenyl)-acrylate ((E)-O1-OH)

To a solution of (*E*)-(2-pivalamidophenyl)-3-(4-*tert*-butyldi-methylsiloxy-phenyl)acrylate (1.21 g, 2.67 mmol) in 10 mL of THF was added 10 mL of 2 M HF solution, stirred at room temperature for 5 h. The resulting solution was diluted with CH_2Cl_2 and successively washed with saturated $NaHCO_3$ aqueous solution, 2% HCl aqueous solution, H_2O, and saturated NaCl aqueous solution, and this organic layer was dried with Na_2SO_4. The solution was concentrated under reduced pressure and added hexane to give a white powder, and this powder was dried over P_2O_5 under reduced pressure. (Yield: 734 mg, 81%). Mp. 214–218 °C. Anal. Calcd for $C_{20}H_{21}NO_4$: C, 70.78; H, 6.24; N, 4.13. Found: C, 70.24; H, 6.24; N, 4.13. ^{1}H-NMR (500 MHz, 30 °C, in chloroform-d_1, TMS): δ 8.18 (dd, 1H), 7.88 (d, 1H, 15.57 Hz), 7.59 (br, 1H), 7.52 (d, 2H), 7.26 (td, 1H), 7.19 (dd, 1H), 7.15 (td, 1H), 6.89 (d, 2H), 6.51 (d, 1H, 15.57 Hz), 5.19 (br, 1H), 1.27 (s, 9H). ^{13}C-NMR (125 MHz, 30 °C, in chloroform-d_1, TMS): δ 176.57, 164.93, 158.34, 147.49, 141.20, 130.51, 130.25, 126.85, 126.49, 124.72, 123.13, 121.99, 116.15, 113.43, 39.83, 27.59. ESI-MS: m/z$^-$; 338.0 (M–H, calcd; 338.14).

Preparation of (E)-phenyl 3-(4-*tert*-butyldimethylsiloxy-phenyl)acrylate

4-*tert*-Butyldimethylsiloxycinnamic acid (524.5 mg, 1.884 mmol) was refluxed with thionyl chloride (2.00 mL, 27.4 mmol) in 10.0 mL of CH_2Cl_2 for 5h. The reaction solution was cooled to room temperature, and concentrated to give a yellow oily solution. CH_2Cl_2 was added to it and the solution was concentrated again to remove trace of thionyl chloride. This oil was dissolved in 5.0 mL of CH_2Cl_2 and added to phenol (170.0 mg, 1.81 mmol) in 5.0 mL of CH_2Cl_2. The resulting solution was stirred at room temperature and then Et_3N (2.80 mL, 20.0 mmol) was added to it. The mixed solution was stirred overnight. The resulting solution was diluted with CH_2Cl_2 and successively washed with 2% HCl aqueous solution, H_2O, and saturated NaCl aqueous solution, and this organic layer was dried with Na_2SO_4. The solution was concentrated under reduced pressure to obtain a yellow oil. This oil was dissolved in hexane and cooled in a refrigerator to give a white powder. This powder was washed with hexane and dried under reduced pressure. (Yield: 397.0 mg, 59%). Mp. 67–70 °C. Anal. Calcd for $C_{21}H_{26}O_2Si{\cdot}H_2O$: C, 67.71; H, 7.58. Found: C, 67.33;

H, 7.43. ^{1}H-NMR (500 MHz, 30 °C, in chloroform-d_1, TMS): δ 7.75 (d, 1H, 15.75 Hz), 7.41 (d, 2H), 7.33 (td, 2H), 7.17 (tt, 1H), 7.10 (d, 2H), 6.80 (d, 2H), 6.42 (d, 1H, 15.75 Hz), 0.93 (s, 9H), 0.17 (s, 6H). ^{13}C-NMR (125 MHz, 30 °C, in chloroform-d_1, TMS): δ 164.45, 157.05, 149.74, 145.08, 128.77, 128.20, 126.35, 124.47, 120.51, 119.44, 113.76, 24.58, 17.22, –5.37. ESI-MS: m/z; 731.0 (M × 2 + Na, calcd; 731.32).

Preparation of (E)-phenyl 3-(4-hydroxyphenyl)acrylate ((E)-O2-OH)

To a solution of (*E*)-phenyl-3-(4-*tert*-butyldimethylsiloxyphenyl)acrylate (389.1 mg, 1.098 mmol) in 5.0 mL of THF was added 5.0 mL of 2 M HF solution, stirred at room temperature for 5 hours. The resulting solution was diluted with diethyl ether and successively washed with saturated $NaHCO_3$ aqueous solution, 2% HCl aqueous solution, H_2O, and saturated NaCl aqueous solution, and this organic layer was dried with Na_2SO_4. The solution was concentrated under reduced pressure to give a white powder. This powder was washed with hexane and dried over P_2O_5 under reduced pressure. (Yield: 222 mg, 84%). Mp. 140–144 °C. Anal. Calcd for $C_{15}H_{12}O_3$: C, 74.99; H, 5.03. Found: C, 74.53; H, 4.96. ^{1}H-NMR (500 MHz, 30 °C, in chloroform-d_1, TMS): δ 7.81 (d, 1H, 15.75 Hz), 7.50 (d, 2H), 7.40 (tt, 2H), 7.24 (tt, 1H), 7.17 (dd, 2H), 6.87 (d, 2H), 6.49 (d, 1H, 15.94 Hz), 4.96 (br, 1H). ^{13}C-NMR (125 MHz, 30 °C, in chloroform-d_1, TMS): δ 165.69, 157.80, 150.95, 146.09, 130.26, 129.42, 127.32, 125.70, 121.69, 115.98, 114.96. ESI-MS: m/z^-; 239.0 (M–H, calcd; 239.1).

Deprotonation of phenol model compound in 10% GL micellar solution

The phenol model compound was dissolved in a small amount of THF and to the solution was added poly(ethylene glycol) lauryl ether (C12E9). After removal of THF under reduced pressure, the obtained residue was diluted by degassed water to give a micellar solution. Final concentration is 10% C12E9 aqueous solution. To a micellar solution was added NaOH aqueous solution to give a phenolate anion model compound.

Physical measurements

500 MHz ^{1}H-NMR and 125 MHz ^{13}C-NMR spectra were recorded on a JEOL Lambda spectrometer in 5 mM chloroform-d_1, acetonitrile-d_3, and D_2O (10%) micellar solution. Variable-temperature NMR measurements were made in the range 228 K-303 K, and then the temperature was decreased in each 15 K intervals. HMQC and HMBC spectra were recorded on a Varian UNITY plus 600 MHz spectrometer at 303 K. Tetramethylsilane or sodium 2,2-dimethyl-2-silapentane-5-sulfonate was used as a standard (0 ppm). ESI-mass spectrometric analyses were performed on a Finniganmat LCQ-MS instrument in methanol solution. IR spectra were recorded on a Jasco FT-IR 8300 spectrometer in 5 mM and 1 mM chloroform solution. UV-vis spectra were obtained on a Shimazu UV-3100PC spectrometer in 0.5 mM micellar solution at 303 K. Elemental analysis was performed at the Elemental Analysis Center, Faculty of Scienece, Osaka University. Melting point was measured on a micro melting point apparatus of YANAGIMOTO Co.

pH Titration

The pH of 1 mM solution of each phenol model compound was determined using a Metrohm 716 DMS titrino, which is combined with Metrohm 728 stirrer and a saturated calomel LL micro pH glass electrode. The saturated calomel micro glass electrode was calibrated with the 0.05 M $KHC_6H_4(COO)_2$ buffer (pH = 4.01) and the 0.025 M KH_2PO_4-$NaHPO_4$ buffer (pH = 6.86) at 303 K. The micellar solution was titrated with 0.01 M NaOH aqueous solution at 303 K. The pK_a values was estimated by the following equation: $pK_a = pH - \log[Na^+] + \log\{[phenol]_0 - [Na^+]\}$.

Computational details

The Gaussian03 program[27] was used for geometry optimization and to obtain relative energies of the optimized structures. Final geometry optimization was done at the B3LYP/6–31++G(d,p) level.[16–19] The MOPAC program (WinMOPAC3.9, Fujitsu.)[20] was used for UV-vis absorption spectral analysis (INDO/S) of geometrical optimized model compound.

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