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Fluorescence on–off switching mechanism of benzofurazans

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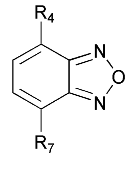
Many fluorescent reagents with a benzofurazan (2,1,3-benzoxadiazole) skeleton have been developed and widely used in bio-analyses. In this study, we try to elucidate the fluorescence on–off switching mechanism of three fluorogenic reagents and their derivatives. Ten 4,7-disubstituted benzofurazans were used for this purpose and the measurements of their fluorescence, phosphorescence, photolysis, and time-resolved thermal lensing signal in acetonitrile were obtained in order to understand the relaxation processes of these compounds. These results indicate that the competition of fluorescence with a fast intersystem crossing or fast photoreaction plays a key role in the fluorescence on–off switching. Semi-empirical molecular orbital calculations show that the existence of the triplet $n\pi^*$ state is responsible for the fast intersystem crossing while the proximity of the reactive second singlet $\pi\pi^*$ state to the first singlet $\pi\pi^*$ state contributes to the fast photoreaction in the excited states.

Introduction

The fluorometric detection method has been widely used in many fields of science because of its high sensitivity and selectivity. To make this method more useful, many fluorescent reagents have been developed.[1] Among such reagents, the 4,7-disubstituted benzofurazans have the advantage of large fluorescence quantum yield (Φ_f) and long absorption/emission wavelengths,[2] which can avoid the interference derived from bio-matrices containing fluorescent compounds.

4,7-Disubstituted benzofurazans (Chart 1) have also a unique character regarding their fluorescence properties; their Φ_f values are dramatically changed by the substituents at the 4- and 7-positions.[3] This characteristic enables us to develop fluorogenic reagents, which are non-fluorescent themselves and react with analytes to form fluorescent adducts. For example, **1** (ref. 4) and **2** (ref. 5) (the original names are described in the Experimental section) are non-fluorescent, but their derivatives with dimethylamine and methylamine (**3** and **4**, respectively) fluoresce.[3] On the other hand, the derivatives of **1** or **2** with other nucleophilic compounds like methanethiol (**5**) and methanol (**6**) are non-fluorescent.[3] Therefore, **1** and **2** can be used as fluorogenic reagents for amines and applied to more than four-hundred bio-analyses.[1a,6] Another compound, **7** (ref. 7), has a different fluorescence characteristic from those of **1** or **2**, that is, **7** is non-fluorescent and its derivative with methanethiol (**8**) is more strongly fluorescent than the derivatives with methylamine (**9**) and methanol (**10**).[3] Taken advantage of this characteristic, **7** has been used as the fluorogenic reagent for more than 50 thiols.[1a,6]



1: R_4 = Cl, R_7 = NO_2
2: R_4 = F, R_7 = NO_2
3: R_4 = NMe_2, R_7 = NO_2
4: R_4 = NHMe, R_7 = NO_2
5: R_4 = SMe, R_7 = NO_2
6: R_4 = OMe, R_7 = NO_2
7: R_4 = F, R_7 = SO_2NH_2
8: R_4 = SMe, R_7 = SO_2NH_2
9: R_4 = NHMe, R_7 = SO_2NH_2
10: R_4 = OMe, R_7 = SO_2NH_2

Chart 1 Chemical structures of the 4,7-disubstituted benzofurazans used in this study.

Some excellent fluorogenic reagents have recently been developed based on the fluorescence on–off switching mechanism,[8] thus the elucidation of the fluorescence on–off switching mechanism in **1**–**10** will allow us to develop rationally new useful fluorogenic reagents with a benzofurazan skeleton. In this article, we report the photophysical and photochemical properties of **1**–**10** and elucidate their fluorescence on–off switching mechanism.

Results and discussion

Relaxation processes

1. Fluorescence. The absorption and fluorescence measurements for **1**–**10** were performed. Their maximum absorption wavelengths (λ_{ab}), molecular extinction coefficients (ε), maximum emission wavelengths (λ_{em}), fluorescence lifetimes (τ_f), average energies dissipated by fluorescence (E_S), and Φ_f values are listed in Table 1. The absorption and fluorescence spectra of **1**–**10** in acetonitrile are shown in Fig. 1. It can be seen that the absorption and fluorescence characteristics of **1**–**10** are significantly affected by the substituents at the 4- and 7-positions of the benzofurazan skeleton.

2. Photoreaction. It has been reported that the photoirradiation of non-substituted benzofurazan in *n*-hexane or water leads to a photochemical reaction to give *cis*–*cis*-1-cyano-4-*N*-oxycyano-buta-1,3-diene with a quantum yield of 0.48 in water. In *n*-hexane, *cis*–*cis*-1-cyano-4-*N*-oxycyano-buta-1,3-diene isomerized to *cis*–*cis*-1-cyano-4-isocyano-buta-1,3-diene.[9] In our previous report,[10] it was also found that non-substituted benzofurazan in acetonitrile undergoes photochemical reactions with a high quantum yield (0.36) during photoirradiation

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Table 1 Photophysical and photochemical properties of **1**–**10** in acetonitrile; maximum absorption wavelength (λ_{ab}), molecular extinction coefficients (ε), maximum emission wavelength (λ_{em}), fluorescence lifetime (τ_f), average energy dissipated by fluorescence (E_S), 0–0 transition energy of T_1 state (E_T), intensity ratio of total signal to that of slow rise component in TRTL signal (U_{slow}/U_{total}), yield of fluorescence (Φ_f), photoreaction (Φ_r), intersystem crossing (Φ_{isc}) and internal conversion (Φ_{ic})

No.	λ_{ab}/nm	ε/M^{-1}cm^{-1}	λ_{em}/nm	τ_f/ns	E_S/cm^{-1}	E_T[a]/cm^{-1}	U_{slow}/U_{total}	Φ_f	Φ_r	Φ_{isc}	Φ_{ic}
1	338	10000	ND[b]	ND	ND	17500	0.130	—	—	0.21	0.79
2	331	8000	ND	ND	ND	ND	0.248	—	0.02	0.41[c]	0.57
3	478	26300	533	0.5	17000	ND	ND	0.01	—	—	0.99
4	458	23000	524	11	18500	ND	ND	0.38	—	—	0.62
5	416	16600	ND	ND	ND	14700	0.0474	—	—	0.09	0.91
6	373	12000	ND	ND	ND	16400	0.121	—	—	0.21	0.79
7	315	4620	ND	ND	ND	ND	ND	—	0.11	—	0.89
8	385	10200	510	4.2	17700	ND	0.172	0.17	—	0.29[d]	0.54
9	425	9700	547	12	17600	ND	ND	0.06	0.09	—	0.85
10	348	6000	460	2.2	20500	ND	ND	0.10	0.05	—	0.85

[a] Measured in the mixture of methanol–ethanol (1 : 1) at 77 K. [b] ND: too weak to be determined. [c] Determined by assuming the E_T values to be 17 500 cm^{-1} (the E_T value of **1**). [d] Determined by assuming the E_T values to be 14 700 cm^{-1} (the E_T value of **5**) since the phosphorescence of **2** and **8** was not detected. But the E_T values affected little the Φ_{isc} values.

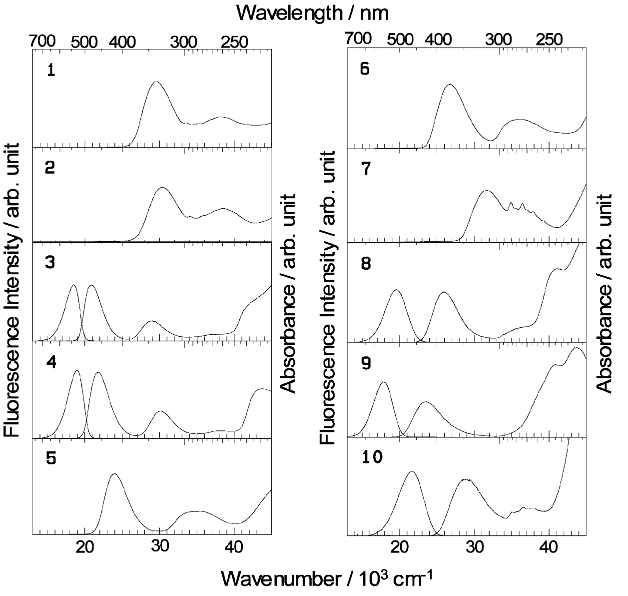

Fig. 1 Absorption and fluorescence spectra of **1**–**10** in acetonitrile at 293 K.

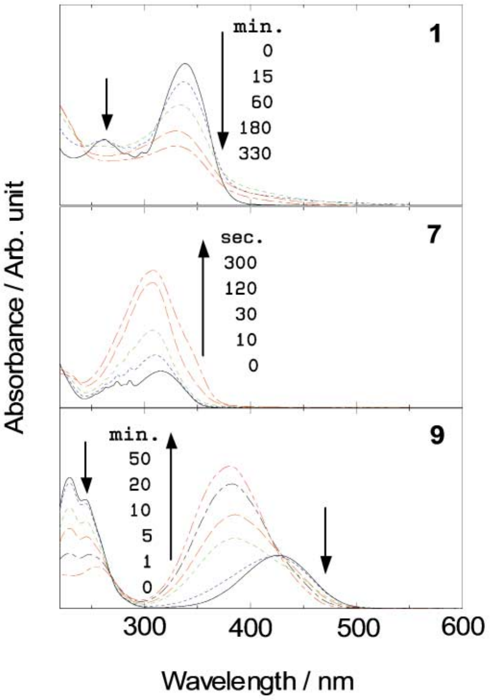

Fig. 2 Changes in absorption spectra of **1**, **7** and **9** as representatives in deaerated acetonitrile upon photoirradiation (**1** and **9**; $\lambda > 350$ nm, **7**; $\lambda > 310$ nm) at 293 K.

and the photochemical reaction from the excited singlet state is one of the main relaxation processes of the 5-substituted benzofurazans. A photoreaction seems to occur even in the excited **1**–**10** and therefore we performed the first photolysis experiments using **1**–**10**. The irradiation was carried out at wavelengths suitable for exciting each benzofurazan from the S_0 state to the S_1 state. As a result, some compounds (especially, **7** and **9**) in acetonitrile were unstable to the photoirradiation and the representative results of the photolysis are shown in Fig. 2. The changes in the absorption spectra of **7** and **9** did not give an isosbestic point, suggesting that the photolysis of these compounds produces a mixture of more than one compound whose composition changes with time. The quantum yields of the photoreactions (Φ_r) of **1**–**10** during the photoirradiation at 275 nm in acetonitrile were accurately determined by using HPLC[10] (for a detailed procedure, see the Experimental section) and are shown in Table 1.

3. Intersystem crossing and internal conversion. Next, the time-resolved thermal lensing (TRTL) measurements[11] in acetonitrile were carried out to clarify the contribution of the intersystem crossing and internal conversion to the fast non-radiative processes in **1**–**10**. For the TRTL measurements, the intensity ratio of the total signal (U_{total}) to that of the slow rise component (U_{slow}) is related to the Φ_{isc} values[11e] in the following equation:

$$\frac{U_{slow}}{U_{total}} = \frac{\Phi_{isc} E_T}{E_{ex} - \Phi_f \langle E_s \rangle - \Phi_r \Delta H} \tag{1}$$

where E_{ex} is the excitation energy, and E_T is the 0–0 transition energy of the lowest triplet state (T_1) (obtained by the phosphorescence measurement using a Hitachi F-4010 spectrofluorometer at 77 K), and ΔH is the total heat of formation of the photoreaction products. $<E_s>$ is the average energy dissipated by the fluorescence from the first singlet excited state,

$$\langle E_s \rangle = \frac{\int \bar{\nu}\ I_f(\bar{\nu})\ d\bar{\nu}}{\int I_f(\bar{\nu})\ d\bar{\nu}} \tag{2}$$

where $I_f(\bar{\nu})$ is the spectral distribution of fluorescence in wavenumber ($\bar{\nu}$). It was confirmed that the effect of the $T_n \longleftarrow T_1$ absorption of **1**–**10** on the TRTL signals at the wavelength (633 nm) of the probe beam could be neglected by measuring the transient absorption spectra of these compounds (data not shown). The results of the TRTL measurements of **1**–**10** are

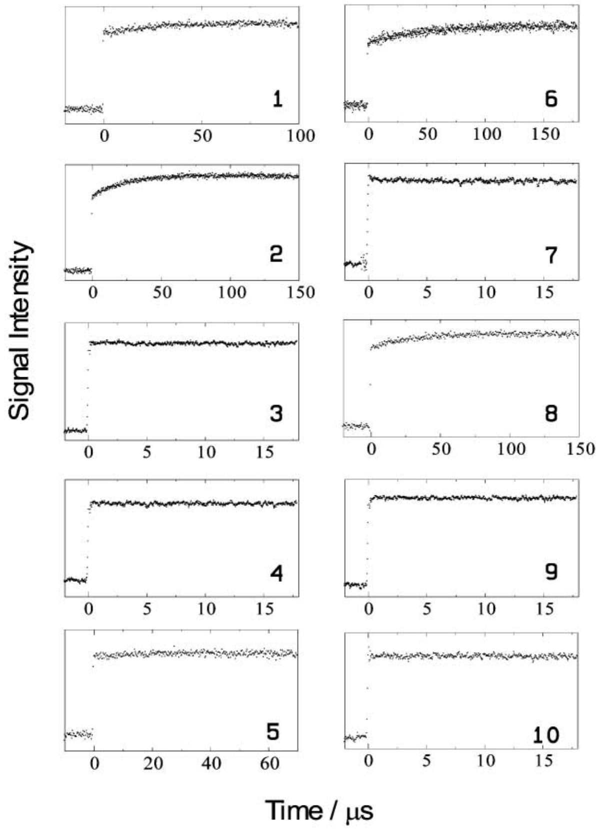

Fig. 3 Time evolution of the time-resolved thermal lensing signals of **1**–**10** in deaerated acetonitrile excited at 355 nm (**1**–**6** and **8**–**10**) and 308 nm (**7**) at 293 K.

shown in Fig. 3. As shown in Fig. 3, the time evolutions of the TRTL signals of **3**, **4**, **7**, **9**, and **10** consist only of a prompt rise component (*i.e.* $U_{\text{slow}}/U_{\text{total}} \approx 0$), indicating that the contribution of the intersystem crossing is negligible in the deactivation processes from the fluorescent state of these compounds. In contrast, the TRTL signals of **1** ($U_{\text{slow}}/U_{\text{total}} = 0.130$), **2** ($U_{\text{slow}}/U_{\text{total}} = 0.248$), **5** ($U_{\text{slow}}/U_{\text{total}} = 0.0474$), **6** ($U_{\text{slow}}/U_{\text{total}} = 0.121$), and **8** ($U_{\text{slow}}/U_{\text{total}} = 0.172$) included the fast and slow rise components, showing the formation of the lowest triplet state. For **1**, **5**, and **6** the formation of the lowest triplet state was also confirmed by the phosphorescence measurement (0–0 transition energy of the lowest triplet state (E_T) are indicated in Table 1 and the phosphorescence spectra are in Fig. 4). The Φ_{isc} values determined by the TRTL measurements are listed in Table 1. For the determination of the Φ_{isc} value of **2** using eqn. (1), the contribution of the photoreaction was neglected, because the Φ_r value (0.02) was sufficiently small. Finally, the quantum yields of the internal conversion (Φ_{ic}) were calculated from the equation $\Phi_{\text{ic}} = 1 - \Phi_f - \Phi_r - \Phi_{\text{isc}}$, and are also listed in Table 1.

Fluorescence on–off switching mechanism

We will first discuss the fluorescence on–off switching mechanism of the reagents (**1** and **2**) for amines and their derivatives (**3**–**6**) of amines, thiols and alcohols. Based on the Φ_f, Φ_{isc} and Φ_{ic} values shown in Table 1, compounds **1**–**6** are classified into two groups: (i) fluorescent **3** ($\Phi_f = 0.01$) and **4** ($\Phi_f = 0.38$), in which only an internal conversion is involved as the non-radiative process; and (ii) non-fluorescent **1**, **2**, **5**, and **6**, in which an efficient intersystem crossing ($\Phi_{\text{isc}} \geq 0.09$) takes place in addition to the internal conversion. This classification leads to the elucidation of the fluorescence on–off switching mechanism in **1**–**6**. Thus, the intersystem crossing occurring in reagents **1** and **2** is inhibited in their amino derivatives, **3** and **4**, consequently, these compounds are fluorescent. In the other derivatives, **5** and **6**, the efficiency of the intersystem crossing is maintained as in the cases of **1** and **2**, leading to the characteristic of **1** and **2** as specific fluorogenic reagents for amines.

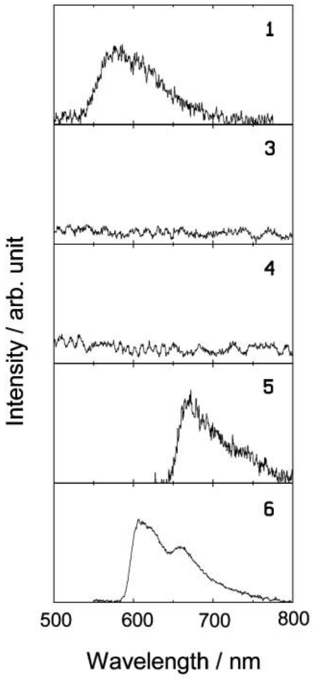

Fig. 4 Phosphorescence spectra of **1** and **3**–**6** as representatives in the mixture of methanol–ethanol (1 : 1) at 77 K.

In contrast, another reagent and its derivatives (**7**–**10**) are divided into three groups based on their relaxation properties: (i) strongly fluorescent **8** ($\Phi_f = 0.17$), in which the photoreaction does not occur; (ii) fluorescent **9** ($\Phi_f = 0.10$) and **10** ($\Phi_f = 0.06$), in which the photoreaction takes place in addition to the internal conversion; and (iii) non-fluorescent **7**, in which a relatively fast photoreaction ($\Phi_r = 0.11$) is involved in addition to the internal conversion. This classification reveals the fluorescence on–off switching mechanism of the fluorogenic reagent, **7** and its derivatives, **8**–**10**, *i.e.*, in **7**–**10**, the photoreaction ought to compete with the fluorescence, and the photoreaction is predominant in **7** but repressed in **8**. However, since the photoreaction is not perfectly repressed in **9** and **10**, these compounds are weakly fluorescent. Therefore, **7** can be used as the selective fluorogenic reagent for thiols.

Molecular orbital calculation

We then calculated the energy levels of **1**–**4**, **7**, and **8** (the reagents and their fluorescent derivatives) to theoretically explain their fluorescence on–off switching mechanisms. The reliability of the calculation methods was examined by comparing the calculated ππ* transition energies with the experimental ππ* excitation energies evaluated by the absorption maximum energy.[10,12] Because the semi-empirical methods require only a short calculation time and provide the precise energy levels of benzofurazans,[10,12b] the PM3 and PM3-CAS/CI(CI = 6) methods were adopted for the geometric optimization and calculation of the energy levels, respectively. The PM3 and PM3-CAS/CI(CI = 6) methods gave good correlation coefficients (r = 0.982) between the calculated ππ* transition energies and the experimental ππ* excitation energies.

The results of the calculations (Table 2 and Fig. 5) also supported the experimentally elucidated fluorescence on–off switching mechanism. In the non-fluorescent **1** and **2**, the triplet *n*π* state (**1**: 4.130 eV, **2**: 3.213 eV) derived from the nitro group is located near the first singlet ππ* state (**1**: 3.489 eV, **2**: 3.673 eV). Especially in **2**, the triplet *n*π* state is more stable than the first singlet ππ* state. Since the intersystem crossing from a singlet ππ* state to a triplet *n*π* state is allowed by the El-Sayed

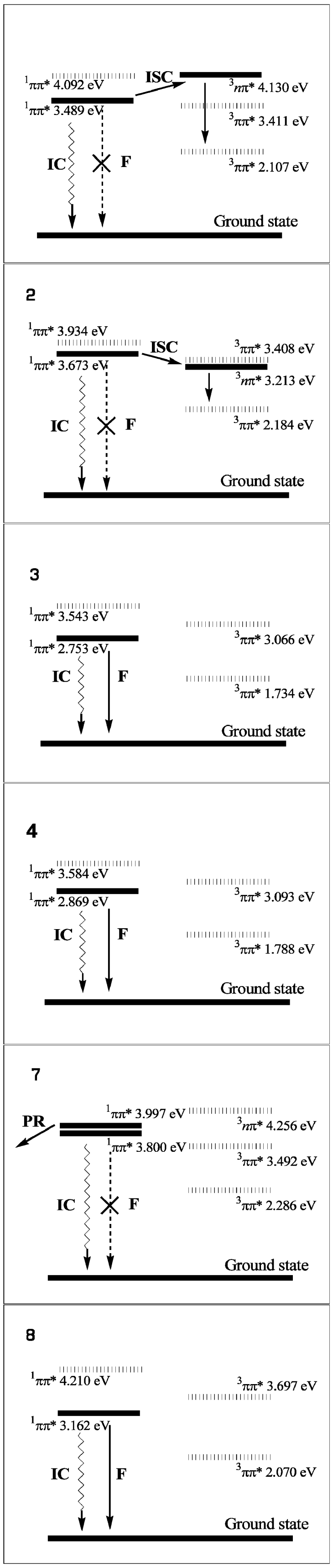

Fig. 5 Energy level diagrams obtained with the molecular orbital calculations and the relaxation processes for **1**–**4**, **7** and **8**; F: fluorescence, IC: internal conversion, ISC: intersystem crossing, and PR: photoreaction.

rule,[13] the fast intersystem crossing occurs in **1** and **2**. In contrast, no triplet $n\pi^*$ state was obtained from the calculations for **3** and **4**, indicating that, in **3** and **4**, the intersystem crossing is prohibited and the fluorescence is emitted. In reagent **7**, the second singlet $\pi\pi^*$ state (3.997 eV) was very close to the first singlet $\pi\pi^*$ state (3.800 eV). Because, in benzofurazans, the second $\pi\pi^*$ state might be the reactive state and the interaction between the first and second singlet $\pi\pi^*$ states promotes a photoreaction,[10] only the fast photoreaction was involved in **7**, although the triplet $n\pi^*$ state (4.256 eV) existed. On the other hand, in fluorescent **8**, the reactive second singlet $\pi\pi^*$ state (4.210 eV) was too high to interact with the first singlet $\pi\pi^*$ state (3.162 eV). Thus, it was suggested by the molecular orbital (MO) calculations that excited **8** was longer-lived than excited **7** and strongly fluoresces.

Conclusion

In this study, the fluorescence on–off switching of **1** and **2** is ascribable to the depression of the intersystem crossing and internal conversion rates accompanying the derivatization with amines, while in **7**, the reduction of the photoreaction and internal conversion rates accompanying the derivatization with thiols plays a key role. This is the first report of the fluorescence on–off switching mechanism occurring in the 4,7-disubstituted benzofurazans. Based on these results, a fluorogenic reagent will be developed.

Experimental

Materials

1 (4-chloro-7-nitro-2,1,3-benzoxadiazole),[4] **2** (4-fluoro-7-nitro-2,1,3-benzoxadiazole),[5] **3** (4-*N*,*N*-dimethylamino-7-nitro-2,1,3-benzoxadiazole),[14] **4** (4-methylamino-7-nitro-2,1,3-benzoxadiazole),[3] **5** (4-methylthio-7-nitro-2,1,3-benzoxadiazole),[3] **6** (4-methoxy-7-nitro-2,1,3-benzoxadiazole),[14] **7** (4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole),[7] **8** (4-methylthio-7-aminosulfonyl-2,1,3-benzoxadiazole),[3] **9** (4-methylamino-7-aminosulfonyl-2,1,3-benzoxadiazole)[3] and **10** (4-methoxy-7-aminosulfonyl-2,1,3-benzoxadiazole)[3] were synthesized and purified as previously reported. Acetonitrile, methanol and ethanol (Kanto) were purified by distillation. Water was purified using a Milli-Q reagent system (Millipore).

Methods

The absorption and fluorescence spectra were measured using a JASCO U-best 50 UV-vis spectrophotometer and a Hitachi F-4010 spectrofluorometer, respectively, at 293 K. Each emission spectrum was obtained by excitation at the maximum absorption wavelength. The fluorescence quantum yield (Φ_f) was determined using quinine sulfate in 0.1 M sulfuric acid (Φ_f = 0.55, excitation wavelength: 355 nm, at room temperature)[15] as the standard. The fluorescence lifetime (τ_f) measurements were carried out using a nitrogen laser (LTB Lasertechnik Berlin, 337 nm, fwhm 350 ps) as the excitation source. The fluorescence decay signals at the wavelength selected by a monochromator (Ritsu MC-20L) were detected by a photomultiplier tube (Hamamatsu H-3284). Signals from the photomultiplier were recorded on a digital storage oscilloscope (Tektronix TDS 520) interfaced to a personal computer (NEC, PC9801). The fluorescence decay curves thus obtained were analyzed by the deconvolution method involving linearization of the single exponential function and least-squares fitting. The detection limit of the method was estimated to be 100 ps.

The photoreaction yields (Φ_r) of **1**–**10** were determined using a spectrofluorometer (Hitachi F-4010) and high performance liquid chromatography (HPLC). This method was established in our previous research[10] and the details are described below. The HPLC consisted of a Hitachi L-6200 pump, a Hitachi L-4000H UV detector, and a Hitachi D-2500 integrator. Each

Table 2 Energy levels of **1**–**4**, **7** and **8** obtained by using PM3 and PM3-CAS/CI(CI = 6) methods for geometric optimization and calculation of energy levels, respectively

No.	$T(\pi,\pi^*)$/eV[a]		$S(\pi,\pi^*)$/eV[a]		$T_n(n,\pi^*)$/eV[a]	$S_n(n,\pi^*)$/eV[a]
1	2.107 (16994[b])	3.411 (27512)	3.489 (28141)	4.092 (33004)	4.130 (33311)	4.141 (33399)
2	2.184 (17615)	3.408 (27487)	3.673 (29625)	3.934 (31730)	3.213 (25915)	3.572 (28810)
3	1.734 (13986)	3.066 (24729)	2.753 (22204)	3.543 (28576)	–[c]	–[c]
4	1.788 (14421)	3.093 (24947)	2.869 (23140)	3.584 (28907)	–[c]	–[c]
7	2.286 (18438)	3.492 (28165)	3.800 (30649)	3.997 (32238)	4.256 (34327)	4.600 (37101)
8	2.070 (16696)	3.697 (29818)	3.162 (25503)	4.210 (33956)	–[c]	–[c]

[a] Relative to the S_0 state. [b] cm^{-1}. [c] Not obtained.

benzofurazan sample was dissolved in acetonitrile and its absorbance was adjusted to 3.00 at 275 nm. The sample solution (3.2 ml) was stirred during the photoirradiation by the monochromatic light at 275 nm in the spectrofluorometer (bandpass: 10 nm). At specific intervals, an aliquot (5 µl) of the solution was subjected to HPLC. Separation of the photoreaction mixture was performed using an analytical column, TOSOH TSKgel ODS-80Ts (250 × 4.6 mm; 5 µm id) and an isocratic eluent (acetonitrile–water) at a flow rate of 1.0 ml min^{-1}. The eluate was monitored by UV detection (275 nm). If S and S' are the peak areas of the benzofurazans, which are included in the aliquot before and after photoirradiation, respectively, and $\varepsilon_{275\ nm}$ is the molecular extinction coefficient at 275 nm, the ratio S'/S varied linearly with the photoirradiation time t (min) according to the following expression:

$$\frac{S'}{S} = 1 - a\varepsilon_{275\ nm}t \qquad (3)$$

The relative photoreaction efficiency a value was calculated from the t value and the observed S'/S value by least-squares analyses. All the least-squares analyses were carried out using Microsoft EXCEL 98. The Φ_r values of **1**–**10** were obtained by comparing their a values with that of the non-substituted benzofurazan ($\Phi_r = 0.36$ in acetonitrile).[10]

The quantum yields of the intersystem crossing (Φ_{isc}) of **1**–**10** were determined using the time-resolved thermal lensing (TRTL) method.[11] For the TRTL measurements, the XeCl excimer laser (308 nm) (LAMBDA PHYSIK, LEXTRA 50, pulse width 17 ns) or the third harmonics (355 nm) of a Nd^{3+} : YAG laser (Spectra Physics, GCR-130, plus width 6 ns) was used as the excitation source. A He–Ne laser (Uniphase, 1103P; 633 nm) was also used as a probe beam for the TRTL measurements. The output signals were converted to a voltage using a digital storage oscilloscope (Tektronix, TDS-744) connected to a personal computer (NEC, PC9821Ap). The absorbance of the sample solution was adjusted to *ca.* 0.10 at the excitation wavelength. A benzophenone–acetonitrile solution ($\Phi_{isc} = 1.0$)[16] was used to confirm the optimized experimental conditions. The TRTL experiments were carried out at 293 K and all the sample solutions were degassed using the freeze–pump–thaw method.

The transient absorption spectra were obtained using a nanosecond laser flash photolysis system. The XeCl excimer laser (308 nm) or the third harmonics (355 nm) of a Nd^{3+} : YAG laser was used as the excitation source. The monitoring light from a xenon lamp (USHIO, 150 W) was focused into a sample cuvette by two convex lenses. The transient signals were recorded on a digitizing oscilloscope (Tektronix, TDS-744) and the timing sequence between the laser pulse and monitoring light was controlled by a digitizing delay pulse generator (Stanford Research System, Model DG-535). In order to improve the signal to noise ratio (S/N), the data averaging was carried out over 5 to 20 shots. The absorbance of each sample solution was adjusted to *ca.* 0.60 at the excitation wavelength.

Computational methods

All the semi-empirical molecular orbital calculations were carried out using the program MOPAC2000 in the WinMOPAC ver. 3.0 package (Fujitsu) with a Gateway 7–800 (CPU: PENTIUM III 800 MHz, RAM: 768 MB). The geometries of **1**–**10** were first completely optimized (keywords PM3 PRECISE) by the eigenvector following routine (keyword EF). The CAS (Complete Active Space) calculations were then performed to obtain the electronic energies of the optimized geometries (keyword C.I. = 6 1SCF).

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