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Fluorescence characteristics of ionic benzofurazans, 7-substituted-2,1,3-benzoxadiazole-4-sulfonates

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Abstract

Hydrophilic fluorescent derivatization reagents, sensors and probes are of great value because of their use in accurate analyses under water-rich condition. In this study, 13 7-substituted-2,1,3-benzoxadiazole-4-sulfonates (called SBD derivatives) as representatives of ionic benzofurazans were synthesized and their absorption and fluorescence spectra were obtained in solvents of varying polarity. The fluorescence quantum yields (Φ_f) of the investigated compounds were significantly affected by the substituent at the 4-position. Solvent effects on the Φ_f values were also observed. It was found that the combination of the PM3 and PM3-CAS/CI (CI = 6) methods afforded a good relationship between the experimental and calculated $S_1 \leftarrow S_0$ transition energies of the SBD derivatives.

Keywords: Benzofurazan; Benzoxadiazole; Fluorescence; Sensor; Derivatization reagent; Molecular orbital calculation

1. Introduction

In recent years, fluorescence spectroscopy has attracted much attention in the field of biological chemistry because it offers high selectivity and sensitivity. To make the fluorometric method more useful, many fluorescent derivatization reagents, sensors, and probes have been developed [1–3]. Among such compounds, hydrophilic fluorescent molecules are of special value since hydrophobic molecules sometimes deteriorate precision and accuracy of assays. For example, the hydrophobic derivatization reagents decrease the solubility of their fluorescent adducts in water-rich condition which is adopted in reversed-phase high performance liquid chromatography (RP-HPLC) and capillary electrophoresis (CE), resulting in undesirable adsorption of analytes to the stationary phase and the capillary wall. Moreover, even in the handling process for analytes, fluorescent derivatives by a hydrophobic reagent are occasionally adsorbed by the typical sample tube of polypropylene [4]. Furthermore, in cellular studies, the hydrophobic probes would accumulate in the cell membrane rather than locate in the cytosol as may be desired [5]. Thus, the development of hydrophilic fluorescent reagents, sensors and probes are important for the removal of these problems.

The benzofurazan (2,1,3-benzoxadiazole) skeleton is advantageous as a fluorophore [6] because (i) various substituents can be easily introduced into the benzofurazan skeleton [7,8]; (ii) the fluorescence quantum yields

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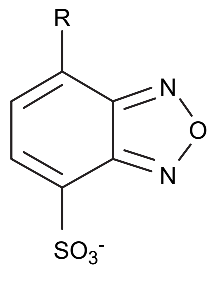
(Φ_f) of compounds containing a benzofurazan skeleton are significantly influenced by substituents [7,8]; (iii) the absorption and fluorescence maximum wavelengths are longer than those with other similar-sized fluorophores such as naphthalene and coumarin. By utilizing these characteristics, several water-soluble derivatization reagents having the benzofurazan skeleton have been developed [4,9–12]. These derivatization reagents are hydrophilic enough to allow us to quantitatively analyze the hydrophobic compounds such as proteins [13], peptides [14] and saturated higher fatty acids [9].

The purpose of the present study is to investigate fluorescence characteristics of 13 water-soluble benzofurazan compounds because of an ionic substituent, sulfo group at the 4-position (Fig. 1), i.e., SBD (2,1,3-benzoxadiazole-4-sulfonate) derivatives. Their systematic names are described in Section 2. In this paper, the SBD derivatives **1**–**13** were obtained and their absorption and fluorescence spectra were measured in media of varying polarity: benzene, chloroform, ethyl acetate, ethanol, methanol, acetonitrile, and water. Semi-empirical molecular orbital calculations were also performed in order to correlate the experimentally observed $S_1 \leftarrow S_0$ transition energies of ionic **1**–**13** with their calculation results.

2. Experimental

2.1. Materials

Acetonitrile, benzene, dichloromethane, ethanol, methanol, ethyl acetate and sodium hydroxide were purchased from Kanto Chemicals (Tokyo, Japan). Fuming sulfuric acid (free SO_3 content: 20%) was obtained from Yotsuhata Kagaku Kogyo (Tokyo, Japan). Dimethylamine solution (50% in water) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Water was purified using a Milli-Q reagent system (Millipore, Bedford, MA, USA). All chemicals were of HPLC or guaranteed reagent grade and were used without further purification.



1 R = SMe
2 R = SEt
3 R = OMe
4 R = OEt
5 R = NH_2
6 R = NHMe
7 R = NMe_2
8 R = NHAc
9 R = H
10 R = Me
11 R = F
12 R = Cl
13 R = Br

Fig. 1. Chemical structures of SBD derivatives used in this study.

2.2. Apparatus

Proton nuclear magnetic resonance (^1H NMR) spectra were obtained on a JEOL JNM-LA500 spectrometer (Tokyo, Japan) with tetramethylsilane as the internal standard (abbreviations used: s = singlet, d = doublet), *J* values are given in Hz. Mass spectra were measured using a Bruker Daltonics (Billerica, MA, USA) esquire 3000 plus (electrospray ionization (ESI) system). UV–visible absorption spectra were measured using a JASCO (Japan Spectroscopic Co., Ltd., Tokyo, Japan) Ubest-50 and SHIMADZU (Kyoto, Japan) UV-1600 spectrometers in seven different solvents (30 μM) at room temperature. Fluorescence spectra were measured using Hitachi (Tokyo, Japan) F-4010 and F-4500 fluorescence spectrophotometers in the solvents (5–30 μM) at room temperature. Fluorescence spectra were obtained by the excitation at the maximum absorption wavelengths. The Φ_f values were determined using quinine sulfate in 0.3 M sulfuric acid ($\Phi_f = 0.55$, excitation: 355 nm, room temperature) as the standard [15].

2.3. Synthesis

7-Methylthio-2,1,3-benzoxadiazole-4-sulfonate (**1**) [7], 7-ethylthio-2,1,3-benzoxadiazole-4-sulfonate (**2**) [7], 7-methoxy-2,1,3-benzoxadiazole-4-sulfonate (**3**) [7], 7-ethoxy-2,1,3-benzoxadiazole-4-sulfonate (**4**) [7], 7-amino-2,1,3-benzoxadiazole-4-sulfonate (**5**) [7], 7-methylamino-2,1,3-benzoxadiazole-4-sulfonate (**6**) [7], 7-acetylamino-2,1,3-benzoxadiazole-4-sulfonate (**8**) [7], 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (**11**) [11], and 7-chloro-2,1,3-benzoxadiazole-4-sulfonate (**12**) [16] were synthesized and purified as previously reported.

2.3.1. 7-N,N-Dimethylamino-2,1,3-benzoxadiazole-4-sulfonate (7)

The compound **11** (100 mg, 0.43 mmol) [11] was dissolved in the mixture of acetonitrile (10 ml) and water (5 ml). After the addition of dimethylamine solution (50% in water, 1 ml), the mixture was refluxed for 2 h. The reaction mixture was evaporated to dryness under reduced pressure and the residue was chromatographed on silica gel with dichloromethane–methanol (5:1) to afford **7** (108 g as *N*,*N*-dimethylammonium salt, 88%) as an orange powder, mp: 172–173 °C (decomp.). ^1H NMR: δ_H (CD_3OD) 7.69 (1H, d, $J = 8.2$), 6.06 (1H, d, $J = 8.2$), 4.51 (6H, s). ESI–MS: m/z 242 (M^-).

2.3.2. 2,1,3-Benzoxadiazole-4-sulfonate (*9*)

2,1,3-Benzoxadiazole (100 mg, 0.83 mmol) [17] was dissolved in fuming sulfuric acid (1 ml). The solution was stirred at 150 °C for 4 h and neutralized with sodium hydroxide solution (1 M) after cooling to room temperature. The reaction mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with ethyl acetate–methanol (5:1) to afford **9** (58 mg as sodium salt, 31%) as a white powder, mp: >280 °C. ^{1}H NMR: δ_H (CD_3OD) 7.91 (1H, d, $J = 8.9$), 7.83 (1H, d, $J = 6.7$), 7.48 (1H, dd, $J = 6.7, 8.9$). ESI–MS: *m/z* 199 (M^-).

2.3.3. 7-Methyl-2,1,3-benzoxadiazole-4-sulfonate (*10*)

4-Methyl-2,1,3-benzoxadiazole (107 mg, 0.80 mmol) [18] was dissolved in fuming sulfuric acid (8 ml). The solution was stirred at 100 °C for 110 min and after cooling to room temperature was neutralized with sodium hydroxide solution (1 M). The reaction mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with ethyl acetate–methanol (5:1) to afford **10** (11 mg as sodium salt, 5.8%) as a white powder, mp: >280 °C. ^{1}H NMR: δ_H (CD_3OD) 7.81 (1H, d, $J = 7.1$), 7.28 (1H, d, $J = 7.1$), 2.66 (3H, s). ESI–MS: *m/z* 213 (M^-).

2.3.4. 7-Bromo-2,1,3-benzoxadiazole-4-sulfonate (*13*)

4-Bromo-2,1,3-benzoxadiazole (22 mg, 0.11 mmol) [19] was dissolved in fuming sulfuric acid (1 ml). The solution was stirred at 150 °C for 4 h and neutralized with sodium hydroxide solution (1 M) after cooling to room temperature. The reaction mixture was evaporated to dryness under reduced pressure, and the residue was chromatographed on silica gel with ethyl acetate–methanol (5:1) to afford **13** (5.2 mg as sodium salt, 16%) as a white powder, mp: >280 °C. ^{1}H NMR: δ_H (CD_3OD) 7.84 (1H, d, $J = 7.3$), 7.80 (1H, d, $J = 7.3$). ESI–MS: *m/z* 277, 279 (M^-).

2.4. Computational methods

We employed PM3 (MNDO-Parametric Method 3) [20] and AM1 (Austin Model 1) [21] because these methods require only a short calculation time and provided the precise energy levels of 4,7-disubstituted benzofurazans [22]. All the PM3 and AM1 semi-empirical molecular orbital calculations were carried out using the programs MOPAC2000 in a WinMOPAC ver. 3.0 package (Fujitsu, Japan) with a Gateway EVF 720.

The geometries of the substituted SBD derivatives in the ground state were first completely optimized [keywords: PRECISE, CHARGE = −1, and (AM1 or PM3)] by the eigenvector following routine [keyword: EF] without considering solvent effects. For several benzofurazan compounds, there were plural stable conformations. We continued the optimization of these compounds until the most stable form was found. The geometries of the SBD compounds with lithium ion as a counter cation (i.e. as $-SO_3^-Li^+$) were also optimized [keywords: PRESCISE, PM3, and EF]. For all the optimization of the geometry of **8**, the keyword MMOK was also used to correct the increase in the barrier to the rotation of the amide linkage. The geometries of SBD derivatives in the first excited singlet state were also optimized by using the additional keyword EXCITED.

For the solvation of the SBD derivatives, the self consistent reaction field (SCRF) approximation and the supermolecule approximation were adopted. For the COSMO (conductor-like screening model) method [23] for the SCRF approximation, the additional keyword EPS = 78.54 (in water) or EPS = 37.5 (in acetonitrile) was used. For the supermolecule approximation [24], six water molecules were placed at a distance ∼3 Å from the oxygen atoms of the $-SO_3^-$ group. The hydrogen atoms of water were oriented toward the oxygen atoms of the $-SO_3^-$ group.

In order to obtain the electronic energies of the optimized geometries, the complete active space (CAS) calculations using the configuration interaction with 400 microstates (number of electrons: 6, number of molecular orbitals: 6) were performed [keywords: C.I. = 6, 1SCF, EF, CHARGE = 0 (or −1), PRECISE, MECI, LARGE, GEO-OK, and (AM1 or PM3)] [25].

3. Results and discussion

3.1. Fluorescence characteristics

We have obtained 13 substituted SBD compounds **1–13** (Fig. 1). For the identification of novel compounds **7**, **9**, **10**, and **13**, NMR and mass spectrometry were used. The absorption and fluorescence spectra of the SBD derivatives **1–13** were measured in benzene, chloroform, ethyl acetate, ethanol, methanol, acetonitrile, and water. They were not soluble in less polar *n*-hexane. Table 1 shows their maximum absorption and emission wavelengths and Φ_f values in the solvents. The absorption and fluorescence spectra of **1–13** in water are shown in Fig. 2 as representatives. These results from Table 1 indicated that the absorption and fluorescence characteristics of **1–13** were significantly affected by the substituent at the 7-position. For example, the maximum absorption wavelengths of three amino derivatives **5–7** are 410–450 nm, while those of three halogen derivatives **11–13** are 310–330 nm. In other words, the amino derivatives **5–7** displayed longer maximum absorption wavelengths than other compounds. These results agree with the previous report that compounds bearing strong electron donating and strong electron accepting substituents have an ICT (intramolecular

Table 1
Fluorescence characteristics of **1–13**; maximum absorption wavelength (λ_{ab}), maximum emission wavelength (λ_{em}), and fluorescence quantum yield (Φ_f) in seven kinds of solvents

	(λ_{ab},λ_{em}/nm) Φ_f						
	Benzene	Chloroform	Ethyl acetate	Ethanol	Methanol	Acetonitrile	Water
1	(387,498) 0.051	(390,489) 0.11	(378,498) 0.30	(376,498) 0.32	(379,509) 0.15	(377,492) 0.37	(383,527) 0.034
2	(387,506) 0.063	(388,490) 0.073	(382,500) 0.30	(377,495) 0.17	(380,506) 0.094	(381,494) 0.17	(386,526) 0.023
3	(351,471) 0.027	(349,509) 0.030	(347,465) 0.045	(345,461) 0.060	(346,469) 0.066	(350,458) 0.053	(349,492) 0.047
4	(355,470) 0.028	(355,452) 0.038	(353,466) 0.056	(348,464) 0.075	(348,470) 0.066	(352,464) 0.063	(353,498) 0.050
5	(414,577) 0.00027	(410,575) 0.00027	(414,571) 0.00073	(413,571) 0.00038	(413,575) 0.0010	(408,566) 0.00074	(411,591) 0.000032
6	(424,572) 0.0010	(420,571) 0.0012	(422,569) 0.0020	(418,562) 0.0025	(420,569) 0.0020	(422,566) 0.0035	(429,596) 0.00010
7	(444,580) 0.0027	(444,578) 0.0034	(437,584) 0.0031	(431,573) 0.0025	(433,574) 0.0010	(463,578) 0.0033	(447,605) 0.000079
8	(364,497) 0.019	(363,499) 0.0025	(363,489) 0.17	(356,482) 0.19	(356,491) 0.14	(360,473) 0.18	(353,512) 0.035
9	–[a]	–[a]	(311,387) 0.00011	(306,382) 0.000076	(306,377) 0.000083	(311,384) 0.00012	(306,389) 0.00017
10	(325,412) 0.00038	(323,406) 0.00027	(322,410) 0.00044	(319,404) 0.00021	(318,409) 0.00023	(321,405) 0.00034	(321,419) 0.00040
11	(321,420) 0.00056	(319,410) 0.00029	(319,409) 0.00042	(318,405) 0.00036	(317,411) 0.00032	(321,407) 0.00056	(317,418) 0.00047
12	(324,407) 0.0047	(324,405) 0.0046	(327,408) 0.0047	(323,400) 0.0014	(322,403) 0.0016	(327,404) 0.0026	(324,414) 0.0032
13	(331,410) 0.0041	(331,408) 0.0057	(329,412) 0.016	(327,402) 0.0054	(325,407) 0.0065	(329,406) 0.0087	(327,412) 0.017

[a] Insoluble.

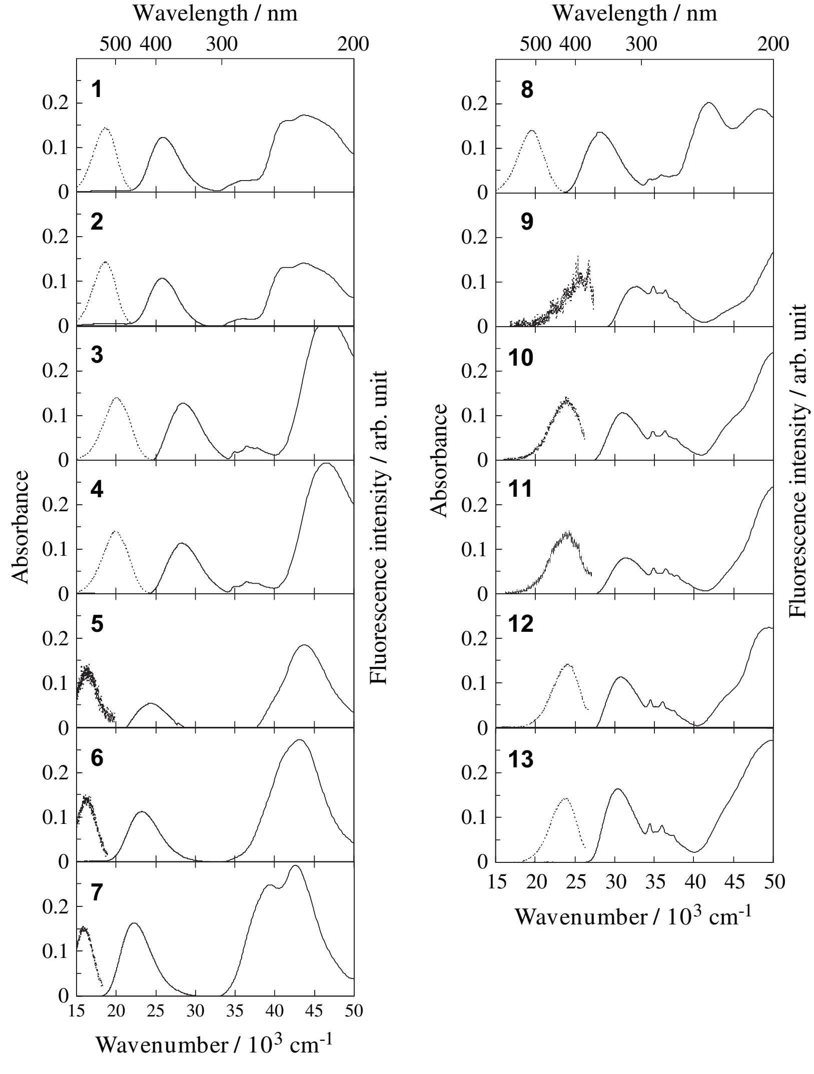


Fig. 2. Absorption (—) and fluorescence (···) spectra of **1–13** in water.

charge transfer) state as the S_1 state and were excited at the longer wavelength [26]. Similarly, the maximum emission wavelengths were influenced by the substituent as indicated in Table 1.

It is also noteworthy that the SBD derivatives **1–13** showed great differences in their Φ_f values. In water, the compounds **1–4**, **8**, and **13** showed large Φ_f values (>0.01), indicating that **1–4**, **8**, and **13** can be used as fluorophores in fluorescent labeling reagents and probes. On the other hand, the compounds **5–7** and **9–11** had small Φ_f values $(0.001 \geq \Phi_f)$. We can find the similar trend in the Φ_f values in other polar solvents such as methanol and acetonitrile. Therefore, the spread of the Φ_f values enables us to develop a fluorogenic reagent, which is non-fluorescent itself and reacts with analytes to form fluorescent adducts. For example, **11** has been used as the fluorogenic reagent for thiols [11] since **11** is nearly non-fluorescent (e.g. in water, $\Phi_f = 0.00040$) whereas its thiol derivatives **1** ($\Phi_f = 0.034$) and **2** ($\Phi_f = 0.023$) are fluorescent. Considering the data in Table 1, we can point out a candidate for another fluorogenic reagent. That is to say, **5** can be used as a fluorogenic reagent for carboxylic acids since the Φ_f values of **5** and its derivative **8** with acetic acid are 0.000032 and 0.035, respectively, in water. In our previous paper [27], it was reported that low Φ_f values were seen for the 5-substituted benzofurazans of which the first absorption band overlapped with the second absorption band having vibrational fine structure. Similarly, the compounds **9–12**, in which the two absorption bands overlapped each other as shown in Fig. 2, were nearly non-fluorescent in all the solvents used in this study.

The fluorescence characteristics of **1–13** were also influenced by solvents. Water gave the longest maximum emission wavelength for **1–8**, indicating that their excited states are of an ICT states. Although acetonitrile has a larger dielectric constant ($\varepsilon = 37.5$) than ethanol ($\varepsilon = 24.6$) and methanol ($\varepsilon = 32.7$), the latter afforded longer emission wavelength in most cases. This result suggests that hydrogen bonding forms between the protic solvents and the SBD derivatives. The Φ_f values of the fluorescent compounds **1**, **2**, and **8** decreased in water as compared with those in ethyl acetate, ethanol, methanol, and acetonitrile. Although the decrease in their Φ_f values in water might be disadvantageous to the applications to sensitive analyses under water-rich condition, the compounds **1**, **2**, and **8** fluoresce strongly enough to be detected fluorometrically even in water.

Recently, novel fluorescent molecular thermometers [28,29] and a glutamine sensing system [30] were developed based on the polarity-sensitive emission characteristics of benzofurazans. The compounds **1**, **2**, and **8** can be utilized in a similar way as the ionic polarity-sensitive fluorophores.

3.2. Semi-empirical molecular orbital calculation

Semi-empirical AM1 and PM3 calculations were performed to correlate the experimental results of wavelengths with the computational results for the ionic compounds **1–13**. For the geometries of ionic **1–13**, three kinds of approaches were adopted as follows: (i) the supermolecular approximation (s.m.a.) by simulating the solvent complexes with six water molecules on the oxygen atoms of a sulfo group of **1–13**, since it has been proposed that the geometry of an ionic compound should be optimized in consideration of the hydrogen bond [24], (ii) COSMO method [23], and (iii) treating **1– 13** as lithium salts by placing the counter cation Li^+ (i.e. $-SO_3^-Li^+$) since **1–13** exist in an undissociated form in organic solvent such as acetonitrile. On the other hand, the CAS/CI (CI = 6) [25] method was used for the energy level calculation.

Table 2 shows the method for calculating the energy levels and the correlation coefficients (r) between the experimental and calculated $S_1 \leftarrow S_0$ energies of **1–13**. Table 3 summarizes the original data of the calculated $S_1 \leftarrow S_0$ transition energies. As shown in Table 2, the original AM1 (method A) and PM3 (method C) methods could not give the reasonable energy levels of **1–13**, shown by r values, 0.852 and 0.883, respectively. Two s.m.a. calculations (methods B and D) gave better relationships ($r = 0.902$ and 0.927, respectively) than the original AM1 and PM3 calculations. Between AM1 and PM3 methods, the PM3 method gave the higher r values than the AM1 method (see methods A–D). Therefore the PM3 method was adopted for further calculations. The combination of the s.m.a. and COSMO methods in the geometric optimization (method E) gave more linear relationship, although the use of COSMO method in the CAS/CI calculations (method F) was disadvantageous.

Table 2
Calculation methods for the ground state of **1–13**; the correlation coefficient (r) between experimental and calculated $S_1 \leftarrow S_0$ energies

Method	Geometrical optimization		Calculation of energy levels		r
	Method	ε[a]	CI	ε[a]	
A	AM1	–	6	–	0.852[b]
B	AM1 (s.m.a.)	–	6	–	0.902[c]
C	PM3	–	6	–	0.883[b]
D	PM3 (s.m.a.)	–	6	–	0.927[c]
E	PM3 (s.m.a.)	78.54	6	–	0.945[c]
F	PM3 (s.m.a.)	78.54	6	78.54	0.849[c]
G	PM3 (salt)	–	6	–	0.976[b]
H	PM3 (salt)	37.5	6	–	0.956[b]
I	PM3 (salt)	37.5	6	37.5	0.934[b]

[a] Dielectric constant used in the COSMO calculations.

[b] Correlation coefficient between experimental $S_1 \leftarrow S_0$ energies in acetonitrile and calculated $S_1 \leftarrow S_0$ energies.

[c] Correlation coefficient between experimental $S_1 \leftarrow S_0$ energies in water and calculated $S_1 \leftarrow S_0$ energies.

Table 3
Calculated $S_1 \leftarrow S_0$ transition energies of **1–13**

Method	Calculated $S_1 \leftarrow S_0$ transition energy/eV												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
A	3.442	3.454	3.750	3.731	3.592	3.595	3.499	3.700	3.975	3.887	3.816	3.874	3.943
B	3.395	3.379	3.679	3.667	3.454	3.408	3.356	3.648	3.900	3.830	3.772	3.815	4.053
C	3.361	3.516	3.477	3.485	3.340	3.342	3.334	3.416	3.736	3.624	3.618	3.577	3.735
D	3.380	3.382	3.579	3.568	3.390	3.378	3.335	3.453	3.803	3.715	3.705	3.656	3.813
E	3.400	3.395	3.575	3.562	3.365	3.372	3.327	3.473	3.809	3.727	3.712	3.663	3.811
F	3.173	3.162	3.101	3.136	2.660	2.708	2.710	2.931	3.506	3.347	3.395	3.182	3.705
G	3.481	3.488	3.702	3.688	3.345	3.308	3.220	3.594	3.999	3.876	3.872	3.720	3.932
H	3.456	3.418	3.700	3.695	3.347	3.361	3.278	3.669	3.886	3.861	3.866	3.717	3.921
I	3.008	3.124	3.106	3.099	2.743	2.605	2.475	2.984	3.601	3.328	3.447	3.241	3.683
In CH_3CN[a]	3.291	3.257	3.545	3.525	3.041	2.940	2.680	3.447	3.990	3.865	3.865	3.795	3.772
In H_2O[a]	3.240	3.215	3.555	3.515	3.019	2.892	2.776	3.515	4.055	3.865	3.914	3.830	3.795

[a] Experimental value.

Among all the methods performed in this study, to treat **1–13** as lithium salts (method G) resulted in the best relationship (Fig. 3, $r = 0.976$) whereas the additional use of the COSMO method (methods H and I) decreased r values to 0.956 and 0.934, respectively. Although there were differences between the experimental and calculated energies as shown Fig. 3, the method G could estimate relative differences in $S_1 \leftarrow S_0$ transition energies of the ionic 4-substituted benzofurazans.

The electronic states of **1–13** in the first excited state were also obtained by the AM1 and PM3 methods with the three approaches. However, no good relationship was given (maximum $r = 0.893$) between the experimental and calculated $S_1 \rightarrow S_0$ transition energies. The main reason for the poor relationship seems to be inaccuracy of optimized chemical structures in the excited state. Other calculation methods are needed for the accurate electronic states of excited benzofurazans.

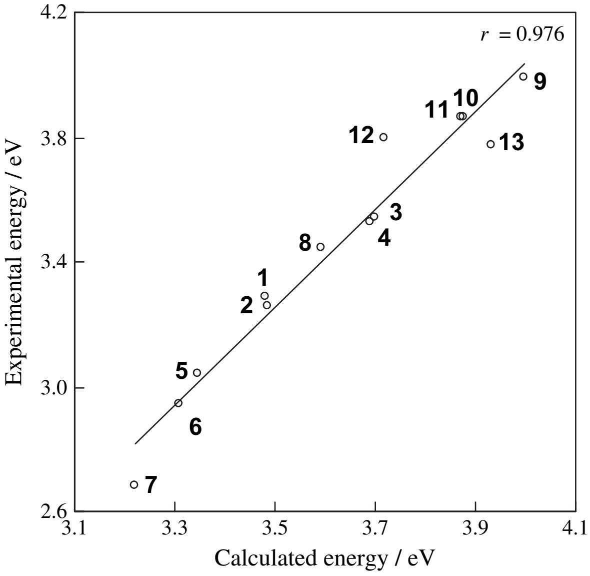


Fig. 3. Correlation of the experimental $S_1 \leftarrow S_0$ energies in acetonitrile and the calculated $S_1 \leftarrow S_0$ energies by the method G.

4. Conclusion

In this paper, 13 SBD derivatives were obtained as representatives of ionic compounds and their fluorescence characteristics were investigated. As a result, their maximum emission wavelengths and Φ_f values were affected by the substituent at the 4-position as well as solvent. The compound **5** is a candidate for an ionic fluorogenic reagent for carboxylic acids. Their $S_1 \leftarrow S_0$ transition energies were calculated by the combination of the PM3 and PM3-CAS/CI (CI = 6) methods on the assumption that the SBD derivatives exist as salts.

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