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Tetrahedron 60 (2004) 11067–11073

Rational design of novel photoinduced electron transfer type fluorescent probes for sodium cation

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Received 31 January 2004; accepted 22 June 2004

Available online 23 August 2004

Abstract—We have developed novel fluorescence probes for sodium cation based on photoinduced electron transfer (PeT). In this study, we rationally designed new probes and succeeded in achieving fluorescence enhancement upon sodium ion binding by reducing the HOMO energy level of the chelator group within the probe molecule. Our new probes show low pH dependency, possibly because of their simple structures. Our results confirm the value of rational probe design based on PeT.

1. Introduction

Fluorescein is highly fluorescent in aqueous media and emits longer wavelength light upon excitation at 490 nm. For these reasons, various fluorescein derivatives have been used as fluorescent tags[1] or fluorescence probes.[2] For example, many fluorescein-based fluorescence probes are available for various cations, such as Fluo-3,[3] ZnAF-2[4] and CoroNa Red[5] (Fig. 1). Most of these probes are based on an aminofluorescein structure containing a selective chelator for the target cation. Why has this structure been retained in so many cation probes? One answer is that many chelators consist of nitrogen atoms.[6] However, another and more important reason is that aminofluorescein is non-fluorescent and becomes fluorescent upon formation of the amidoderivative.[7] In this sense, design of these fluorescence probes is largely empirical, and this limits the design flexibility.

Recently, we showed that the fluorescein molecule is a directly conjugated electron donor and acceptor system.[8] Furthermore, the fluorescence properties of fluorescein derivatives can be controlled by intramolecular photoinduced electron transfer (PeT). PeT is a well-known mechanism through which the fluorescence of a fluorophore is quenched by electron transfer from the donor to the acceptor.[9] In a molecule in which two groups, a fluorophore (electron acceptor) and its fluorescence quencher (electron donor) are located in close proximity and have no ground state interaction with each other, when the fluorophore is photochemically excited, a single electron is transferred

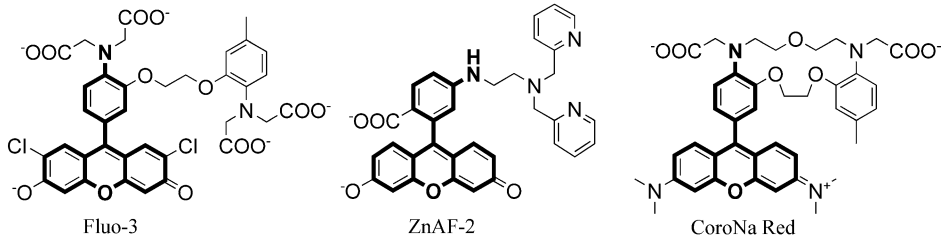

Figure 1. Cation probes based on PeT. Solid lines indicate the aminofluorescein or aminorhodamine platform.

Keywords: Photoinduced electron transfer; Rational design; Fluorescence probe; Sodium cation.
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doi:10.1016/j.tet.2004.06.108

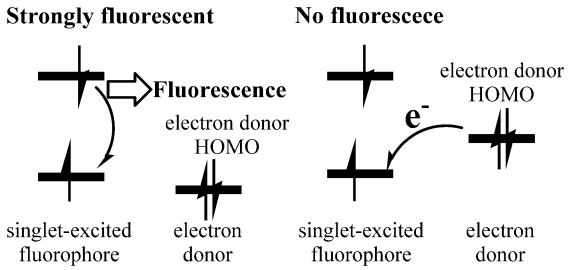


Figure 2. On the left, the HOMO energy level of the electron donor moiety is too low to allow electron transfer. Consequently, emission from the singlet-excited fluorophore is not affected. On the right, the HOMO energy level is enough high to allow electron transfer, so the fluorescence decreases.

from the electron donor to the excited fluorophore. Consequently, the fluorophore loses its energy thermally instead of by emitting fluorescence (Fig. 2).

Indeed, many fluorescence probes for cations as (Fig. 1) are based on PeT. Furthermore, almost all probes that detect cations are structurally based on aminofluorescein. The aniline moiety within aminofluorescein quenches the fluorescence of the singlet excited xanthene dye. However, is aminofluorescein necessary for developing novel probes? Our recent work suggests that the answer to this question is no.

Our report[8] revealed that the rate of intramolecular PeT in the fluorescein molecule follows the Marcus and Rehm–Weller equations and that fluorescein derivatives have characteristically small λ and V values.

Marcus equation:

$$k_{\mathrm{PeT}} = \left(\frac{4\pi^3}{h^2\lambda k_{\mathrm{B}}T}\right)^{1/2} V^2 \exp\left[\frac{-(\Delta G^0_{\mathrm{PeT}} + \lambda)^2}{4\lambda k_{\mathrm{B}}T}\right] \quad (1)$$

λ, reorganization energy; V, electronic coupling matrix element between electron donor and acceptor; $\Delta G^0_{\mathrm{PeT}}$, free energy of electron transfer reaction.

Rehm–Weller equation:

$$\Delta G^0_{\mathrm{PeT}} = E_{\mathrm{ox}} - E_{\mathrm{red}} - \Delta E_{00} - w_{\mathrm{p}} \quad (2)$$

E_{ox}, oxidation potential of electron donor; E_{red}, reduction potential of electron acceptor; ΔE_{00}, singlet excitation energy of fluorophore; w_{p}, work term for charge separation state.

We very recently found that the carboxyl group of fluorescein molecule can be replaced with other substituents such as methyl or methoxyl[10] (Fig. 3). For example, the methyl-substituted derivative called 2-MeTokyoGreen (2-MeTG) was as fluorescent as the traditional fluorescein molecule (Fig. 3). We synthesized many derivatives, and found an excellent relationship[10] between the HOMO energy levels of the benzene moiety (electron donor) and the quantum efficiency (QE) of TGs: a higher-HOMO-energy-level benzene moiety diminishes the fluorescence of xanthene more efficiently (Fig. 3), and the threshold HOMO energy level for fluorescence off/on switching could be precisely determined. Those findings enabled us to control the fluorescence properties of xanthene without an aniline moiety and gave great flexibility in designing novel probes. Using these findings, we tried to develop novel cation probes bearing only O atoms as ligands.

2. Results and discussion

We chose benzo-15-crown-5-ether derivatives as candidates for the electron donor. We calculated the HOMO energy levels of *o*-dimethoxybenzenes as analogues of the benzo-crown-ether moiety. As shown in Figure 3, 3,4-dimethoxybenzoic acid (compound (**1**), whose HOMO energy level is −0.220 hartrees, solid line) seems not to be a good electron donor group; on the other hand, 3,4-dimethoxytoluene ((**2**), −0.206 hartrees, dashed line) seems to be a good donor. So compound (**3**) should emit strong fluorescence (in other words, retention of the carboxyl group has prevented the development of cation probes with a plain benzo-crown-ether), while compound (**4**) should emit little fluorescence. Thus, we synthesized the novel sodium probes, (**10a**) and (**10b**) shown in Scheme 1. In general, the pK_{a} value of the phenolic hydroxyl group of xanthene is about 6[11] and that of 2,7-dichloroxanthene is about 4.5, so it is likely that (**10b**) will be a better probe than (**10a**) under acidic conditions.

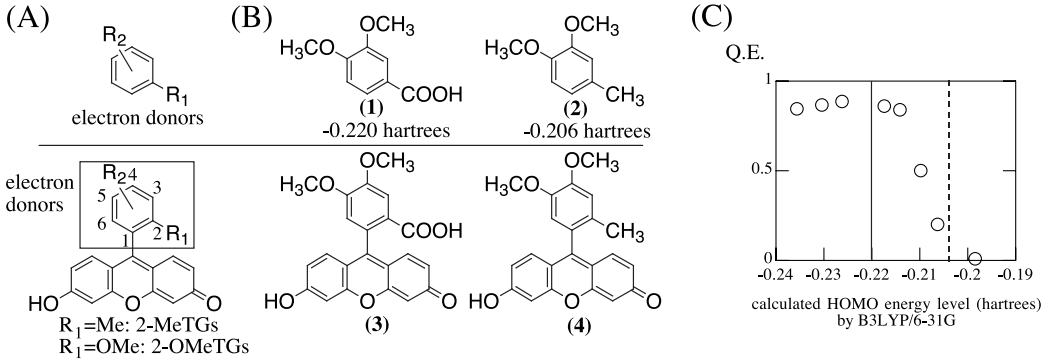
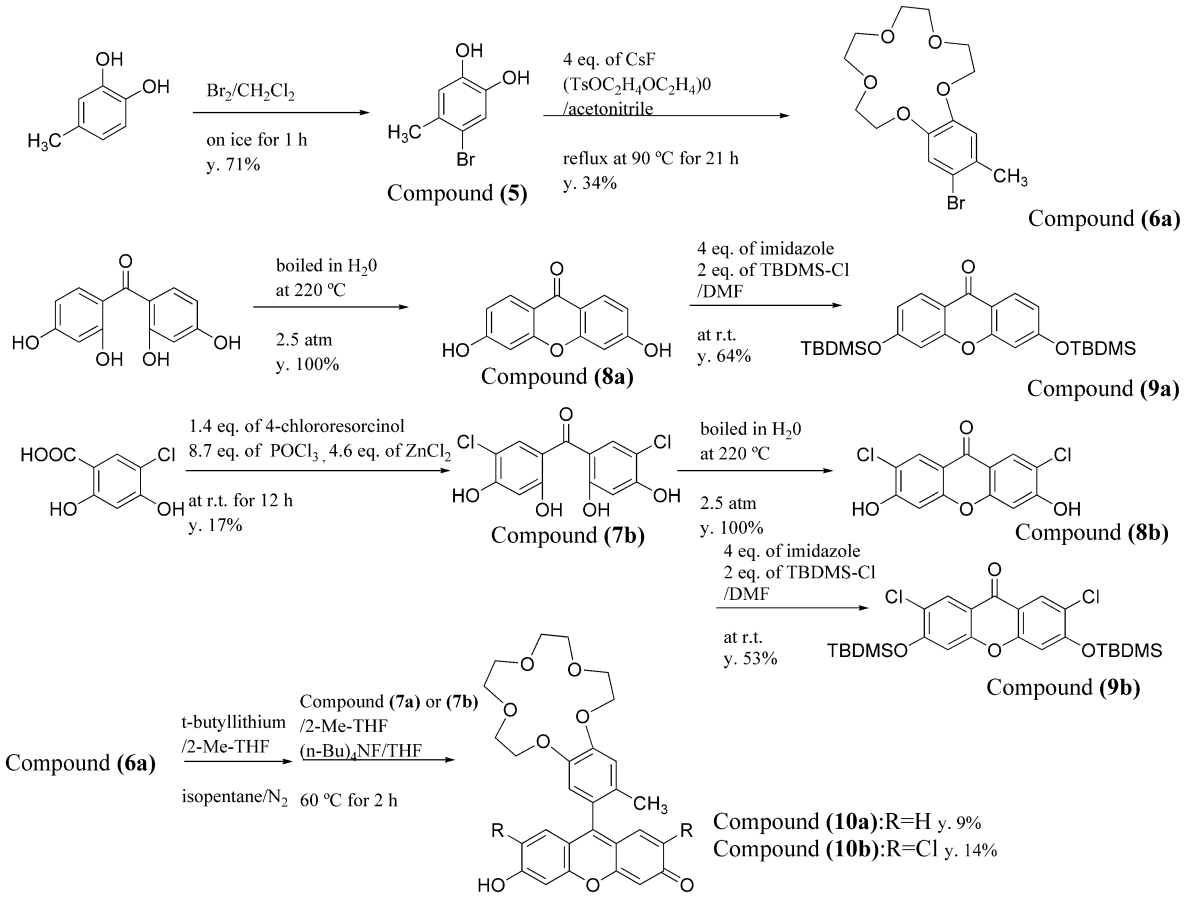


Figure 3. (A) Electron donors and TokyoGreens. (B) Tested analogues for rational design of novel probes. (C) The graph is a plot of the HOMO level of the benzene moiety (electron donor) versus the QE of TGs. HOMO energy levels were calculated by B3LYP/6-31G. The points represent 2-MeTG, 2,4-diMeTG, 2,5-diMeTG, 2-OMeTG, 2-Me, 4-OMeTG, 2-OMe,5-MeTG, 2,4-diOMeTG, and 2,5-diOMeTG, from left to right. The solid line and dashed line indicate the calculated HOMO energy levels of compounds (**1**) and (**2**), respectively. QE of (**3**) and (**4**) can be estimated from the intersection of the respective lines with the best fit to the points on the graph.



Scheme 1. Synthesis of compounds (**10a**) and (**10b**).

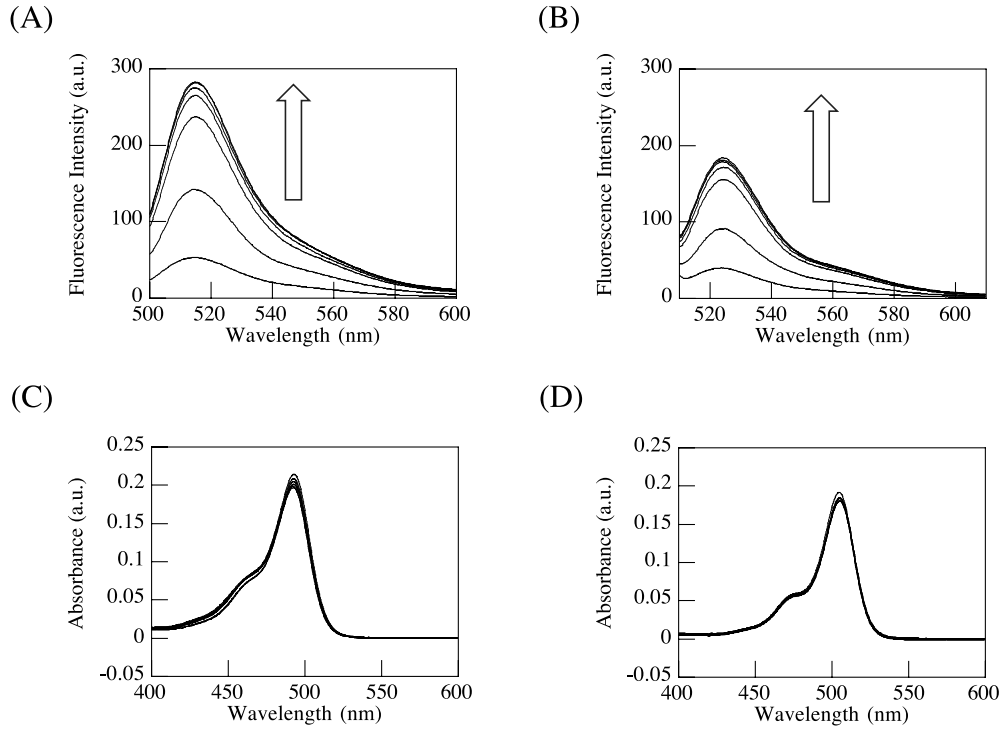


Figure 4. Panels A and B are emission spectra of (**10a**) and (**10b**) excited at 492 and 506 nm, respectively. Panel C and D are absorbance spectra of (**10a**) and (**10b**), respectively. Each sample was measured in aqueous MOPS buffer, pH 7.0, containing 0.5% DMF as a cosolvent. Probes were added as stock solution in DMF, finally 3 μM. [$NaClO_4$] was 0, 200, 700, 1000, 1500, 2000 or 3000 mM. In panel A, Φ_{fl}([$NaClO_4$]=3000 mM)=0.041, Φ_{fl}([$NaClO_4$]=0 mM)=0.008. In panel B, Φ_{fl}([$NaClO_4$]=3000 mM)=0.025, Φ_{fl}([$NaClO_4$]=0 mM)=0.005.

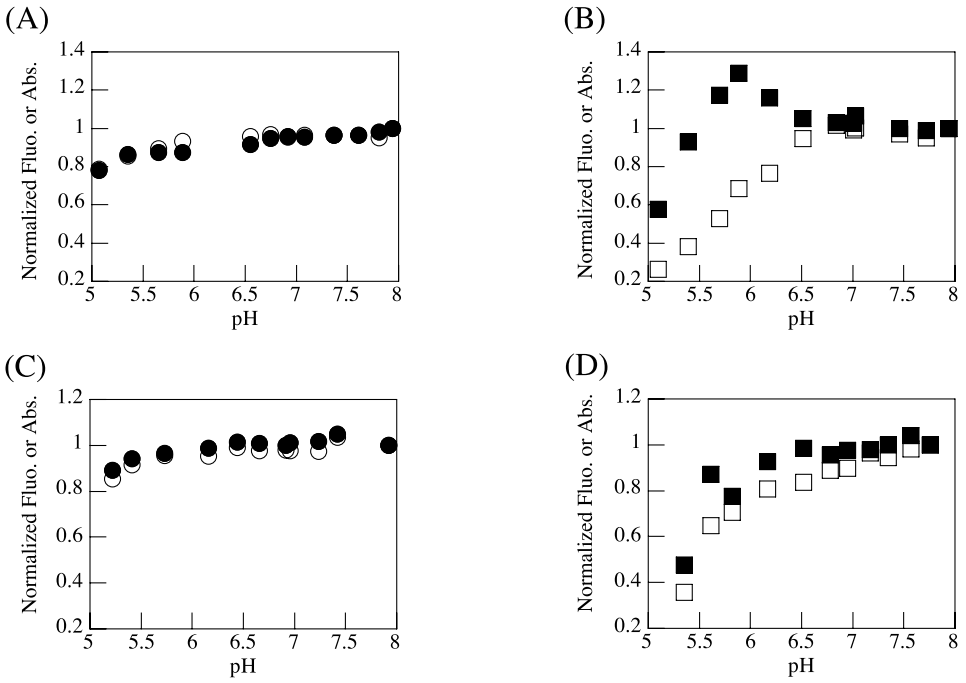


Figure 5. Fluorescence intensity and absorbance were normalized to those at pH 8. Each sample was measured in aqueous MOPS buffer of various pH values, adjusted with trimethylammonium hydroxide, containing 0 or 1000 mM $NaClO_4$, and 0.1% DMF as a cosolvent. The final concentrations of (**10b**) and Sodium Green are 3 and 0.5 μM, respectively. Panels A and C are normalized absorbance (open circle) and normalized fluorescence (closed circle) of (**10b**) in 0 and 1000 mM $NaClO_4$, respectively. Panels B and D are normalized absorbance (open square) and normalized fluorescence (closed square) of Sodium Green in 0 and 1000 mM $NaClO_4$, respectively.

Absorption and emission spectra of (**10a**) and (**10b**) are shown in Figure 4. For both probes, the fluorescence intensity was enhanced by the addition of sodium perchlorate while there was little change in the absorption spectrum, which means that the fluorescence increases of (**10a**) and (**10b**) resulted from increases of QE. The QE of (**10a**) increased from 0.008 to 0.041, that is, about 5-fold, upon binding Na^+. The QE of (**10b**) also increased from 0.005 to 0.025, that is, also 5-fold. Quite similar results were obtained in the case of NaCl addition, so the photochemical properties of (**10a**) and (**10b**) are independent of the counter anion. These fluorescence enhancements are large compared with that of benzos (Böens et al.[12]), which has a plain benzo-crown-ether as the chelator. Benzos may become more or less fluorescent upon binding cations, but PeT-type probes such as (**10a**) and (**10b**) always become more fluorescent. The K_d values for Na^+ are about 0.38 and 0.44 M for (**10a**) and (**10b**), respectively. Fluorescence of (**10a**) and (**10b**), which are distinct from aminofluorescein, could thus be controlled through rational design based on TGs.

We compared the pH dependency of the fluorescence intensity and absorbance of (**10b**) with those of a well-known Na^+ probe, Sodium Green.[13] In panels A and C in Figure 5, in the presence of 0 and 1 M $NaClO_4$, (**10b**) showed plateau profiles in the range of pH 5–8. On the other hand, as shown in panels B and D, the absorbance of Sodium Green decreased drastically below pH 6.5. This pH profile of Sodium Green is distinct from that of dichlorofluorescein, whose pK_a was reported to be below 5; this indicates the existence of the intramolecular stacking of the two fluorophores. The pH dependency of Sodium Green's fluorescence is more complex. In panel B, the fluorescence intensity peaked at pH 5.8 and decreased at lower pH. In panel D, the fluorescence intensity tended to decrease in lower pH. These data clearly show that the fluorescence intensity of Sodium Green is dependent not only on the concentration of Na^+, but also on the pH. Some fluorescent cation probes consist of one chelator and two fluorophores, for example Calcium Green-2[14] and SBFI.[15] The main aim of using two fluorophores in one molecule is to achieve greater fluorescence enhancement by unstacking of the fluorophores upon cation binding. Indeed, Sodium Green has two absorption peaks at 492 and 506 nm, of which the former is thought to correspond to the molecule with intramolecular stacking and the latter to the unstacked molecule. Higher Na^+ concentration makes Abs_{492} lower and Abs_{506} higher. This fluorescence enhancement mechanism provides a low detection limit, but may cause unexpected effects, for example undesirable pH dependency. Our probe design is so simple that there is little possibility of unexpected effects.

We obtained similar results in the case of adding potassium ion (shown in Fig. 6). We employed potassium chloride as the potassium salt. The K_d values for K^+ are 0.29 M for (**10a**) and 0.25 M for (**10b**).

The cation affinity did not differ much between (**10a**) and (**10b**) because the chelator moiety and the xanthene dye are orthogonal and resonantly independent of each other. The K_d values described above imply that the affinities of (**10a**) and (**10b**) for K^+ are higher than those for Na^+, and that full binding to Na^+ enhances the fluorescence of (**10a**) and (**10b**) about 5-fold while full binding to K^+ does so by only

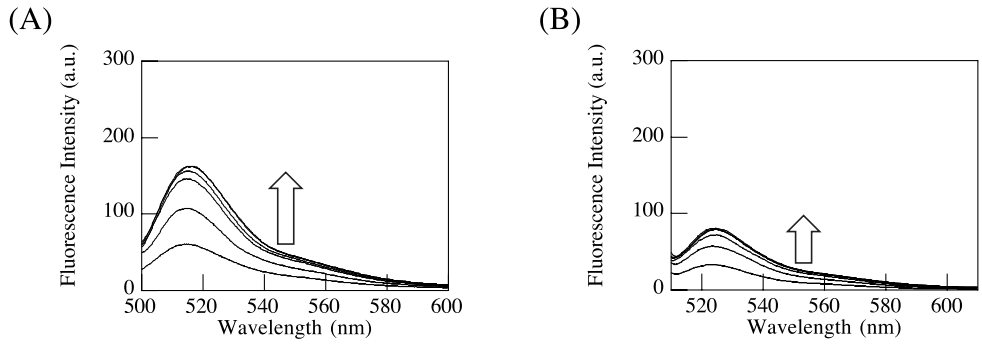


Figure 6. Panels A and B are emission spectra of (**10a**) and (**10b**) excited at 492 and 506 nm, respectively. Each sample was measured in aqueous MOPS buffer, pH 7.0, containing 0.5% DMF as a cosolvent. Probes were added as stock solution in DMF, finally 3 μM. [$NaClO_4$] was 0, 200, 700, 1000, 1800 or 2700 mM. In panel A, Φ_{fl}([$NaClO_4$]=2700 mM)=0.021, Φ_{fl}([$NaClO_4$]=0 mM)=0.008. In panel B, Φ_{fl}([$NaClO_4$]=2700 mM)=0.010, Φ_{fl}([$NaClO_4$]=0 mM)=0.005.

Table 1. Comparison of Na^+ addition and K^+ addition to (**10a**) and (**10b**).

	K_d (M)		F_{max}/F_{min}	
	For Na^+	For K^+	Na^+	K^+
Compound (**10a**)	0.38	0.29	5.3	2.4
Compound (**10b**)	0.44	0.25	4.6	2.4

F_{max}/F_{min}, fluorescence saturated with M^+/fluorescence in M^+-free condition

2–3-fold. Although (**10a**) and (**10b**) have higher affinity for K^+ than Na^+, the electron-withdrawing effect of Na^+ is stronger than that of K^+, so the oxidation potentials of sodium-bound benzo-crown-ether are higher than those of potassium-bound benzo-crown-ether. Consequently, (**10a**) and (**10b**) are more fluorescent in the presence of Na^+ than in the presence of the same concentration of K^+ (Table 1).

To confirm the change of HOMO energy level of the benzo-crown moiety as an electron donor upon Na^+ binding, we measured E_{ox} of 2,3,5,6,8,9,11,12-octahydro-16-methyl-1,4,7,10,13-benzopentaoxacyclopentadecin (**6b**) in acetonitrile containing various concentrations of sodium perchlorate (Fig. 7). The oxidation potential of (**6b**) was shifted to the more positive with the increase of sodium perchlorate content. This indicates that the oxidation potential of (**6b**) was raised upon binding Na^+. The oxidation potential difference between the Na^+-free form and occupied form was about 0.10 V. Thus, we obtained the evidence that the oxidation potential of the electron donors within (**10a**) and (**10b**) was raised upon binding Na^+.

3. Conclusion

By predicting QE from the HOMO energy level of the electron donor, we succeeded in easily designing reasonable PeT-type cation probes. Compounds (**10a**) and (**10b**) become more fluorescent upon binding Na^+, and (**10b**) shows a good pH profile (low pH-dependency). These rationally designed probes are simple, having one chelator as the electron donor and one fluorophore, so that stacking should not be a problem. This strategy can be applied for designing molecules containing other fluorescent dyes, for example, BODIPY[16] and rhodamine.[17] Our strategy should be useful not only for developing new probes, but also for improving existing probes because the key factors for obtaining better probes, for example, the extent of the change of HOMO energy level upon the binding the target and the selectivity of chelator itself, can be controlled by means of minor structural modifications. We intend to search for good pairs of electron donors and fluorophores based on the estimated HOMO levels of electron donor candidates and thereby to develop novel probes for various biomolecules.

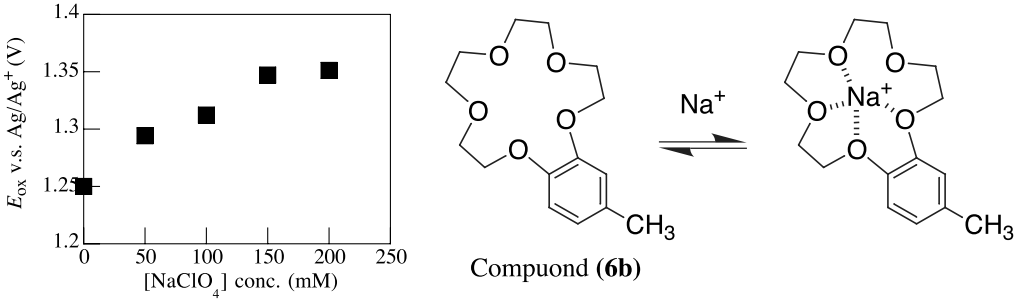


Figure 7. Cyclic voltammetry of compound (**6b**) in acetonitrile containing various concentrations of $NaClO_4$. Ionic strength was kept the same for all solutions with tetrabutylammonium perchlorate.

4. Experimental

4.1. Materials and general instrumentation

General chemicals were of the best grade available, supplied by Tokyo Chemical Industries, Wako Pure Chemical or Aldrich Chemical Company, and were used without further purification. Special chemicals consisted of dimethyl sulfoxide (DMSO, fluorometric grade, Dojindo) and tetrabutylammmonium perchlorate (TBAP, electrochemical grade, dried over P_2O_5 before use, Fluka). Acetonitrile, acetone, *N*,*N*-dimethylformamide (DMF), tetrahydrofuran (THF), methanol, and ethanol were used after appropriate distillation or purification. NMR spectra were recorded on a JNM-LA300 (JEOL) instrument at 300 MHz for ^{1}H NMR. Mass spectra (MS) were measured with a JMS-DX300 (JEOL), for EI, an MS700 (JEOL) for FAB and a JMS-T100LC (JEOL) for ESI-TOF. All experiments were carried out at 298 K, unless otherwise specified.

4.2. Fluorescence properties and quantum efficiency of fluorescence

Steady-state fluorescence spectroscopic studies were performed on an F4500 (Hitachi). UV–visible spectra were obtained on a UV-1600 (Shimadzu), with 0.1 mol L^{-1} NaOHaq (pH 13) as the solvent, unless otherwise specified. Each solution contained up to 0.1% (v/v) DMF as a cosolvent. For determination of the fluorescence (Φ_{fl}), fluorescein in 0.1 mol L^{-1} NaOHaq ($\Phi_{fl}=0.85$) was used as a fluorescence standard.[18] The quantum efficiencies of fluorescence were obtained with the following equation (F denotes fluorescence intensity at each wavelength and $\sum[F]$ was calculated by summation).

$$\Phi_{fl}^{(sample)} = \Phi_{fl}^{(standard)} Abs^{(standard)}/Abs^{(sample)} * \sum[F^{(sample)}]/\sum[\Phi^{(standard)}]$$

4.3. Cyclic voltammetry

Cyclic voltammetry was performed on a 600A electrochemical analyzer (ALS). A three-electrode arrangement in a single cell was used for the measurements: a Pt wire as the auxiliary electrode, a glassy carbon electrode as the working electrode, and an Ag/Ag^+ electrode as the reference electrode. The sample solutions contained 1.0×10^{-3} M sample and 0.1 M tetrabutylammonium perchlorate (TBAP) as a supporting electrolyte in acetonitrile, and argon was bubbled through the solution for 10 min before each measurement.

4.4. Preparation

4.4.1. 4-Bromo-5-methylcatechol (3). To a solution of 4-methylcatechol (28 mmol) in CH_2Cl_2 (100 ml), a mixture of bromine (31 mmol) and CH_2Cl_2 (20 ml) was added dropwise at −80 °C. When a white solid precipitate appeared, addition was stopped to avoid formation of the dibromoderivative (about 15 mmol of bromine was added). The reaction mixture was washed with 100 ml of satd ascorbic acid solution in water, and 100 ml of brine. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give 4.1 g of white solid (yield 71%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.05 (1H, s, aromatic H), 6.76 (1H, s, aromatic H), 5.16 (1H, s, OH), 5.14 (1H, s, OH), 2.27 (3H, s, CH_3); MS (EI) *m*/*z* (%) 204 [M+2], 202 [M+], 18 (100).

4.4.2. 2,3,5,6,8,9,11,12-Octahydro-15-bromo-16-methyl-1,4,7,10,13-benzopentaoxacyclopentadecin (4a). A mixture of 3-bromo-4-methylcatechol (5 mmol), tetra(ethylene glycol) di-*p*-tosylate (5 mmol), CsF (33 mmol), and dry acetonitrile (100 ml) was refluxed at 90 °C for 22 h. Acetonitrile was removed under reduced pressure and the residue was suspended in AcOEt, filtered to remove CsF, and concentrated. The resulting residue was chromatographed (NH-silica gel, AcOEt/MeOH=20/1) to give 0.6 g of white solid (yield 34%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.01 (1H, s, 17-H), 6.74 (1H, s, 14-H), 4.1 (4H, m, 2, 12-H), 3.9 (4H, m, 3, 11-H), 3.7 (8H, m, 5, 6, 8, 9-H), 2.30 (3H, s, CH_3); MS (EI) *m*/*z* (%) 362 [M+2], 360 [M], 228 (100).

4.4.3. 2,3,5,6,8,9,11,12-Octahydro-15-methyl-1,4,7,10,13-benzopentaoxacyclopentadecin (6b). The synthesis of (**6b**) followed that of (**6a**).

4.4.4. 3,3′-Dichloro-2,2′,4,4′-tetrahydroxybenzophenone (7b). *Compound* **7b** was prepared according to the literature.[19]

4.4.5. 3,6-Dihydroxyxanth-9-one (8a). *Compound* **8a** was prepared according to the literature.[20]

4.4.6. 2,7-Dichloro-3,6-dihydroxyxanth-9-one (8b). *Compound* **8b** was prepared according to the literature.[20]

4.4.7. O,O′-Bis(*tert*-butyldimethylsilyl)-3,6-dihydroxyxanth-9-one (9a). *Compound* **9a** was prepared according to the literature.[21]

4.4.8. O,O′-Bis(*tert*-butyldimethylsilyl)-2,7-dichloro-3,6-dihydroxyxanth-9-one (9b). *Compound* **9b** was prepared according to the literature.[21]

4.4.9. 9-(2′,3′,5′,6′,8′,9′,11′,12′-Octahydro-16′-methyl-1′,4′,7′,10′,13′-benzopentaoxacyclopentadecin-15′-yl)-6-hydroxy-3*H*-xanthen-3-one (10a). To a solution of (**6a**) (150 mg) in dry 2-methyltetrahydrofuran (15 ml), a portion of *tert*-butyllithium in *n*-pentane (1.54 N, 1 ml) was added dropwise via a syringe at below −150 °C over 10 min. The mixture was stirred for 30 min at below −150 °C, then a solution of (**9a**) in dry 2-methyltetrahydrofuran (3 ml) was added dropwise via a syringe over 10 min, and the reaction mixture was allowed to warm to room temperature over 1 h. A portion of 2 N HClaq was added, a solution of tetra(*n*-butyl)ammonium fluoride in THF was added, and the reaction mixture was refluxed at 80 °C for 1 h. The mixture was evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2, and the solution was washed with a portion of 2 N HClaq. The organic layer was extracted with five portions of 2 N NaOHaq, and then the aqueous solution was acidified with 2 N HClaq, and

extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, and evaporated under reduced pressure. The resulting residue was chromatographed (silica gel, CH_2Cl_2/methanol=10/2) to give orange-colored solid (yield 8.0%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.09 (2H, d, 1, 8-H, J=1.9 Hz), 7.05 (2H, d, 4, 5-H, J=9.2 Hz), 6.91 (1H, dd, 2, 7-H, J=1.9, 9.2 Hz), 6.86 (2H, s, 1, 14′-H), 6.66 (1H, s, 17′-H), 4.2 (2H, m, 12′-H), 4.1 (2H, m, 2′-H), 4.0 (2H, m, 11′-H), 3.9 (2H, m, 3′-H), 3.8 (4H, m, 5′, 9′-H), 3.77 (4H, s, 6′, 8′-H), 1.95 (3H, s, CH_3) %). ^{13}C NMR (300 MHz, $CDCL_3$) δ 19.4, 68.2, 68.8, 69.6, 70.2, 103.1, 114.2, 115.4, 116.0, 121.5, 132.5, 159.0, 207.1; MS (FAB) *m/z* 515 [MNa+]; HRMS (ESI-Tof) [MNa+] 515.16819, found 515.16644 (−1.75 mmu).

4.4.10. 9-(2′,3′,5′,6′,8′,9′,11′,12′-Octahydro-16′-methyl-1′,4′,7′,10′,13′-benzopentaoxacyclopentadecin-15′-yl)-2,7-dichloro-6-hydroxy-3*H*-xanthen-3-one (10b). The synthesis of (**10b**) followed that of (**10a**) (yield 10%). ^{1}H NMR (300 MHz, DMSO) δ 7.04 (1H, s, 14′-H), 6.84 (1H, s, 17′-H), 6.81 (2H, s, 1, 8-H), 6.26 (2H, s, 4, 5-H), 4.2 (2H, m, 12′-H), 4.0 (2H, m, 2′-H), 3.8 (2H, m, 11′-H), 3.7 (2H, m, 3′-H), 3.6 (4H, m, 5′, 9′-H), 3.6 (4H, s, 6′, 8′-H) 1.94 (3H, s, CH_3). ^{13}C NMR (300 MHz, $CDCL_3$) δ 18.6, 68.0, 68.4, 68.5, 69.3, 70.1, 103.6, 109.3, 114.4, 115.3, 124.3, 126.9, 127.0, 128.1, 132.9, 146.1, 148.6, 149.8, 156.2; MS (FAB) *m/z* 583 [MNa+], 585 ([M+2]Na+); HRMS (ESI-Tof) [MNa+] 583.09024, found 583.08912 (−1.12 mmu).

Acknowledgements

This study was supported in part by the Advanced and Innovational Research Program in Life Sciences from the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government, by Takeda Science Foundation, by Nagase Science and Technology Foundation, by research grants from the Ministry of Education, Science, Sports and Culture of Japan (Grant Nos. 12771349, 13557209, and 14030023 to Y.U.), and by Kowa Life Science Foundation to Y.U.

References and notes

1. (a) Dirks, R. W.; van Gijlswijk, R. P.; Tullis, R. H.; Smit, A. B.; van Minnen, J.; van der Ploeg, M.; Raap, A. K. *J. Histochem. Cytochem.* **1990**, *38*, 467–473. (b) Lau, S. K.; Zaccardo, F.; Little, M.; Banks, P. *J. Chromatogr. A* **1998**, *809*, 203–210.
2. (a) Shoda, T.; Kikuchi, K.; Kojima, H.; Urano, Y.; Komatsu, H.; Suzuki, K.; Nagano, T. *Analyst* **2003**, *128*, 719–723. (b) Takakusa, H.; Kikuchi, K.; Urano, Y.; Kojima, H.; Nagano, T. *Chem. Eur. J.* **2003**, *9*, 1479–1485. (c) Setsukinai, K.; Urano, Y.; Kakinuma, K.; Majima, H. J.; Nagano, T. *J. Biol. Chem.* **2003**, *278*, 3170–3175. (d) Kojima, H.; Urano, Y.; Kikuchi, K.; Higuchi, T.; Nagano, T. *Angew. Chem. Int. Ed.* **1999**, *38*, 3209–3212. (e) Kojima, H.; Sakurai, K.; Kikuchi, K.; Kawahara, S.; Kirino, Y.; Nagoshi, H.; Hirata, Y.; Akaike, T.; Maeda, H.; Nagano, T. *Biol. Pharm. Bull.* **1997**, *20*, 1229–1232. (f) Tanaka, K.; Miura, T.; Umezawa, N.; Urano, Y.; Kikuchi, K.; Higuchi, T.; Nagano, T. *J. Am. Chem. Soc.* **2001**, *123*, 2530–2536.
3. Kao, J. P. Y.; Harootunian, A. T.; Tsien, R. Y. *J. Biol. Chem.* **1989**, *264*, 8179–8184.
4. (a) Hirano, T.; Kikuchi, K.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2002**, *124*, 6555–6562. (b) Hirano, T.; Kikuchi, K.; Urano, Y.; Higuchi, T.; Nagano, T. *Angew. Chem. Int. Ed.* **2000**, *39*, 1052–1054. (c) Hirano, T.; Kikuchi, K.; Urano, Y.; Higuchi, T.; Nagano, T. *J. Am. Chem. Soc.* **2000**, *122*, 12399–12400.
5. Haugland, R. P. *Handbook of Fluorescent Probes and Research Products*, 9th ed. Molecular Probes, Inc.: Eugene, 2002.
6. Martell, A. E.; Hancock, R. D. *Metal Complexes in Aqueous Solutions*; Plenum: New York, 1996.
7. Munkholm, C.; Parkinson, D. R.; Walt, D. R. *J. Am. Chem. Soc.* **1990**, *112*, 2608–2612.
8. Miura, T.; Urano, Y.; Tanaka, K.; Nagano, T.; Ohkubo, K.; Fukuzumi, S. *J. Am. Chem. Soc.* **2003**, *125*, 8666–8671.
9. (a) de Silva, A. P.; Gunaratne, H. Q. N.; Gunnlaugsson, T.; Huxley, A. J. M.; McCoy, C. P.; Rademacher, J. T.; Rice, T. E. *Chem. Rev.* **1997**, *97*, 1515–1566. (b) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265–322.
10. Urano, Y.; Kanda, K.; Kamiya, M.; Ueno, T.; Hirose, K; Nagano, T. Submitted for publication.
11. Chen, S.; Nakamura, H.; Tamura, Z. *Chem. Pharm. Bull.* **1979**, *27*, 475–479.
12. Cielen, E.; Tahri, A.; Ver Heyen, K.; Hoornaert, G. J.; De Schryver, F. C.; Böens, N. *J. Chem. Soc., Perkin Trans. 2*, **1998**, *7*, 1573–1580.
13. (a) Kuhn, M. A.; Haugland, P. R. U.S. Patent 5,405,975, 1995. (b) Kuhn, M. A.; Haugland, P. R. U.S. Patent 5,516,864, 1996.
14. Kuhn, M. A.; Haugland, P. R. U.S. Patent 5,453,517, 1995.
15. Minta, A.; Tsien, R. Y. *J. Biol. Chem.* **1989**, *264*, 19449–19457.
16. Gabe, Y.; Urano, Y.; Kikuchi, K.; Kojima, H.; Nagano, T. *J. Am. Chem. Soc.* **2004**, *126*, 3357–3367.
17. Kojima, H.; Hirotani, M.; Nakatsubo, N.; Kikuchi, K.; Urano, Y.; Higuchi, T.; Hirata, Y.; Nagano, T. *Anal. Chem.* **2001**, *73*, 1967–1973.
18. Nishikawa, Y.; Hiraki, K. *Analytical Methods of Fluorescence and Phosphorescence*; Kyoritsu: Tokyo, 1984, pp 76–80.
19. Kurduker, R.; Subba Rao, N. V. *Proc. Indian Acad. Sci. Sect. A.* **1963**, *57*, 280–287.
20. Grover, P. K.; Shah, G. D.; Shah, R. C. *J. Chem. Sci. (Lond)* **1955**, 3982–3985.
21. Minta, A.; Tsien, R. Y. *J. Biol. Chem.* **1989**, *264*, 8171–8178.