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Synthesis and fluorescence properties of six fluorescein-nitroxide radical hybrid-compounds

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ABSTRACT

Six fluorescein-nitroxide radical hybrid-compounds (**2ab**, **3ab**, **4**, and **5**) were synthesized by the condensation of 5- or 6-carboxy-fluorescein and 4-amino-TEMPO (**2ab**), 5- or 6-aminofluorescein and 4-carboxy-TEMPO (**3ab**), and fluorescein and 4-carboxy-TEMPO (**4**), or by reaction of the 3-hydroxyl group of fluorescein with DPROXYL-3-ylmethyl methanesulfonate (**5**). Fluorescence intensities (around 520 nm) after reduction of the radical increased to 1.43-, 1.38-, and 1.61-folds for **2a**, **2b** and **3b** respectively; **3a** alone exhibited a decrease in intensity on reduction. Since **4** was readily solvolyzed in PBS or even methanol to afford fluorescein and 4-carboxy-TEMPO, its fluorescence change could not be measured. Hybrid compound **5** containing an ether-linkage between the fluorescein phenol and 3-hydroxymethyl-DPROXYL hydroxyl centers, was stable and on reduction, showed a maximum increase (3.21-fold) in relative fluorescence intensity in PBS (pH 5.0), despite its remarkably low absolute fluorescence intensity.

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1. Introduction

Fluorescein has been used as a fluorescent dye for over 30 years. With no reports on the organ toxicity of fluorescein to the best of our knowledge, this dye considered safe. Recently, various excellent fluorescein derivatives have been developed by Nagano and co-workers for use as fluorescent probes based on the photo-induced electron transfer (PeT) theory [1]. It has been shown that the fluorescence is controlled by intramolecular PeT between the xanthene and benzene rings of fluorescein, and that the fluorescence can be switched on and off by changing the electron density of the benzene-ring.

Active radical oxygen species such as hydroxyl radicals formed *in vivo* show various pathological behaviors linked to the development of cancer and Alzheimer's disease [2–4]. They are, however, unstable and react very rapidly once formed. Using fluorescence techniques it is possible to both detect and quantify radicals or radical-reducing species such as ascorbic acid (AsA) [5,6]. We have also designed and synthesized several new hybrid compounds consisting of a nitroxide radical and well-known fluorophores, such as umbelliferone [7] and polymethine-cyanine dyes (Cy3 and Cy5) [8], and measured the increase in fluorescence of these hybrid compounds upon radical reduction, with the goal of developing a probe to monitor reducing species or radicals in biological systems. The fluorescence intensity of these hybrid compounds was restored after the addition of excess AsA, For the hybrid compound 7-*O*-[2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO)-4-carbonyl]umbelliferone, adding excess AsA increased the fluorescence intensity 9.1-fold [7]. Similarly, for 5.5′-bis-[2,2,5,5-tetramethylpyrrolidine-*N*-oxyl (PROXYL)-3-aminocarbonyl]Cy3 and 5.5′-bis-(PROXYL-3-aminocarbonyl)Cy5, the increase in fluorescence intensity was 5.1-fold and 1.8-fold, respectively [8]. Fluorophores bearing a nitroxide radical show weak fluorescence; however, after reduction of the radical, the fluorescence is restored significantly. In addition to umbelliferone and polymethine-cyanine dyes, fluorescein has been a commonly used fluorescent-dye *in vivo* because of its long-excitation and emission wavelengths, high-quantum yield (λ_{ex} = 490 nm, λ_{em} = 515 nm, Φ 0.85) [9,1c], low-toxicity, and water-solubility. Thus, we were interested in using fluorescein- nitroxide radical hybrid compounds after radical reduction as probes to explore the behavior of radical-reducing species *in vivo*, by exploiting the fluorescence restoration property [10]. Hybrid compound **1** (Fig. 1), comprising fluorescein and a nitroxide radical, has recently been reported by Bottle's group [11]. Here an isoindoline-nitroxide radical was bound to the lower benzene ring of fluorescein without a spacer, and a 2.2-fold increase in fluorescence was observed after the addition of AsA. This compound was then successfully used in the fluorescent staining of living cells. However, the synthesis of **1** requires at least eight steps, and even the best overall yield is only 4%; the complexity of the synthesis of the isoindoline radical precludes its ready availability [12].

Here we describe the design and synthesis of six new hybrid compounds in which TEMPO is linked at the 5- or 6-position of the benzene ring of fluorescein *via* amide bonds (CONH or NHCO) (**2ab** and **3ab**), and TEMPO or 2,2,5,5-tetramethyl-3-pyrroline-1-oxyl (3.4-dehydro-PROXYL, DPROXYL) is linked to a hydroxyl group of the xanthene moiety *via* ester or ether bonds (**4** and **5**). Our previous hybrid probes consisted of a nitroxide radical directly bonded to the fluorophore *via*

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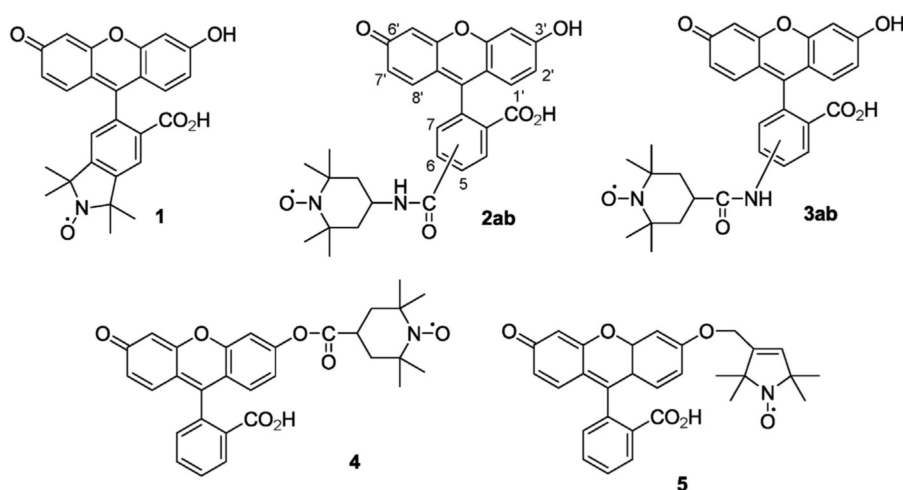


Fig. 1. Flourescein-nitroxide radical hybrid-compounds.

a spacer. Here, **2ab** and **3ab** are designed such that a nitroxide radical is bonded not to the xanthene moiety of the fluorophore, but to the lower benzene ring. The PeT theory predicts that the xanthene fluorescence will be suppressed due to the high highest occupied molecular orbital (HOMO) of the nitroxide radical. However, since the HOMO of the hydroxylamine formed by reduction of the nitroxide radical is lower in energy, reduction increases the fluorescence intensity. Hybrid compounds **4** and **5** are such that a nitroxide radical is directly bonded to the fluorophore. Additionally, we document the fluorescence characteristics of the TEMPO and DPROXYL radical hybrids at varying pH levels, and the increases in intensity of their fluorescence after the addition of excess AsA, from the viewpoint of using them as probes for the redox status *in vivo*.

2. Results and discussion

2.1. Synthesis

We undertook the condensation of 5- or 6-carboxyfluorescein [**9a**,**b** (Scheme S1)] and 4-amino-TEMPO (Scheme S2), as shown in Scheme 1. Accordingly, **9a** (**9b**) was condensed with 4-amino-TEMPO in the presence of dicyclohexylcarbodiimide (DCC, 1.2 equiv) and 1-hydroxybenzotriazole hydrate (HOBt·H_2O, 1.2 equiv) in dry DMF to afford **2a** (**2b**) in 47.2% (10.6%) yield.

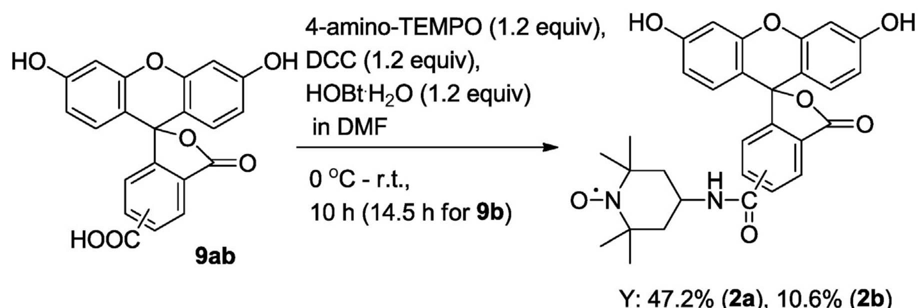
Next, synthesis of hybrid compounds **3ab** bearing the reversed amide linkage as **2ab** was carried out, as shown in Scheme 2. Since the yield of **2b** from the condensation reaction was low (10.6%), 5- and 6-amino-3.10-di-*O*-acetylfluoresceins (**13ab**, Scheme S4) in which two phenol groups were acetylated, were prepared to improve solubility. Condensation of **13a** (**13b**) and 4-carboxy-TEMPO (Scheme S3) was conducted by reacting DCC and *N*, *N*-dimethyl-4-aminopyridine (DMAP) in dry CH_2Cl_2, followed by saponification (NaOH in MeOH), to afford **3a** (**3b**) in 64.4% (56.6%) yield.

We then focused on the introduction of a nitroxide radical into the 3-hydroxyl position of the xanthene moiety of fluorescein. Since we previously found that the increase in fluorescence intensity was greater for hybrids bearing a TEMPO group than for those with a PROXYL group [7], we used TEMPO as a nitroxide radical in the present study. We designed a hybrid compound wherein the TEMPO group was bonded to the phenol residue of the xanthene moiety as in our previous study [7,8]. The desired compound **4** was synthesized in 39% yield (Scheme 3) by direct condensation of fluorescein and 4-carboxy-TEMPO. However, because **4** was rapidly solvolyzed in PBS (pH 6.0) and even in methanol, its relative fluorescence intensity (RFI) could not be measured.

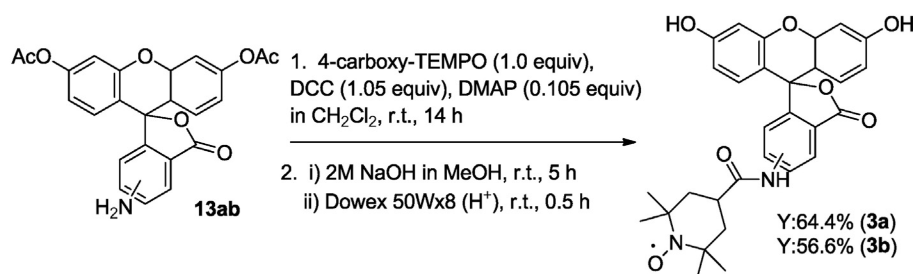
Based on the experience with compound **4**, we next designed hybrid compound **5** in which the 3-hydroxyl group on the fluorescein and a nitroxide radical are linked by an ether group. The substitution reaction of fluorescein with DPROXYL-3-ylmethyl methanesulfonate (Scheme S6) was conducted using sodium ethoxide as a base catalyst to give the desired compound **5** in 62% yield (Scheme 4).

2.2. Analysis

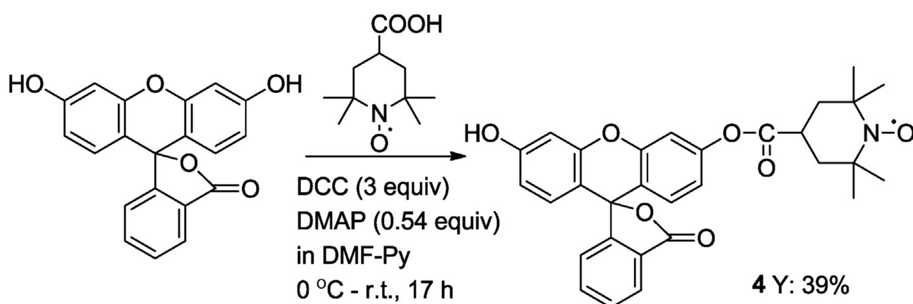
Reduction reactions were carried out by the addition of 20 mM AsA in 0.1 M PBS (pH 7.0) to 20 μM of the sample in 0.1 M PBS (pH 7.0), in 1:1 (v/v) ratio. It was confirmed by ESR spectroscopy that each radical **2ab** and **3ab** disappeared completely 1 h after the addition of AsA



Scheme 1. Synthesis of 2a and 2b.



Scheme 2. Synthesis of 3a and 3b.



Scheme 3. Synthesis of 3-*O*-(TEMPO-4-carbonyl) fluorescein [4].

(Figs. S6–13), while that of sample **5** almost disappeared at 2 h (Figs. S14–16).

On the basis these results, fluorescence intensities were measured before, and at 2 h (for **2ab** and **3ab**) or 3 h (for **5**) after the addition of AsA. Fluorescence measurements on the reaction mixtures were performed after dilution to 0.1 μM with 0.1 M NaOH aqueous solution, because this was known to maximize the fluorescence intensity of fluorescein [1c,9b,11]. The results are summarized in Table 1.

The fluorescence intensities of **2ab** and **3ab** were high (20–34 a.u.), but, that of **5** was significantly lower (1.12 a.u.) because of the lower degree of dissociation [9b,11]. In hybrid compounds **2ab** and **3ab**, where amide group binding to the 5- or 6- position of the benzene ring had little effect on the fluorescence properties. The directionality of the amide linkage (CONH or NHCO) did have an effect as the NHCO spacer group led to a decrease in fluorescence intensity, particularly for **3a**, after reduction.

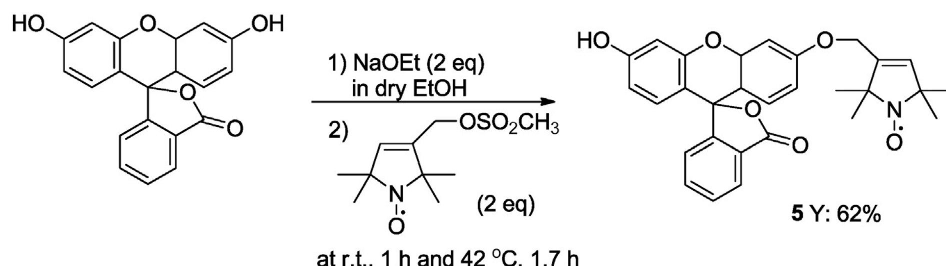
As the fluorescence intensity of fluorescein is affected by solution pH due to changes in the degree of dissociation [9b,11], the fluorescence intensity of **5** was measured at the various pH levels (3.0–10.0) before and after the addition of AsA (Table 2). At higher pH (6.0–10.0), the fluorescence intensity decreased (1.45–0.93 a.u.), but, the rate of change was larger after reduction of the radical (2.1–3.2 a.u., RFI: 1.82 to 2.81-fold increase). The largest increase in RFI (3.21-fold) was found at pH 5.0, but, its fluorescence intensity showed very low 1.12 a.u. The smallest increase (1.82-fold) was seen at pH 10.0.

Next, the fluorescence intensity of each sample was measured in 0.1 M PBS (pH 6.0) before and after the addition of AsA. The results are summarized in Table 3. The results were analogous to those measured in 0.1 M NaOH aqueous solution. Fluorescence intensities at pH 6 were decreased a little in comparison to those in the alkaline solution, and the RFI values, before and after AsA addition were increased as follows: **2a**, from 1.14 to 1.43; **2b**, 1.15 to 1.38; **3b**, 1.05 to 1.61; and **5**, 1.15 to 2.37. The maximum increase was observed for **5** (2.37-fold). The fluorescence intensity before and after reduction of the radicals of **2ab** and **3b** was 7- to 10-fold higher than that of **5**.

The photographs of the fluorescence of **2a** (1), **2b** (2), **3a** (3), **3b** (4), and **5** (5) under $UV_{365\ nm}$ light irradiation (a) before and (b) after the addition of 20 mM of AsA in PBS (pH 6.0, 1:1, v/v) are shown in Fig. S18. The fluorescence of fluorescein with a nitroxide radical bonded to the benzene ring was high. But, the increase in fluorescence after reduction of the radical were difficult to detect with the naked eye (Figs. S18–1, 2, 4). The fluorescence of **5** was markedly decreased, but, the restoration of the fluorescence after reduction was largest at the neutral pH, and could be confirmed with the naked eye (Fig. S18–5).

3. Conclusion

Six new hybrid compounds consisting of a fluorescein label having the potential advantages of high-fluorescent quantum yield, water-solubility, low-toxicity and a nitroxide radical were synthesized. A TEMPO



Scheme 4. Synthesis of 3-*O*-(3.4-dehydroPROXYL-3-methyl)fluorescein [5].

Table 1
Relative fluorescence intensity (RFI) of **2ab**, **3a**, and **5**, before and at 2 h (**2ab**, **3a**) and 3 h (**5**) after the addition of 20 mM AsA in PBS (1:1, v/v).

	Fluorescence (c = 0.1 μM in 0.1 M NaOH aq.)		
Compound	λ_{ex} [nm]	λ_{em} [nm]	Fluorescence intensity [a.u.] (RFI)
2a	492	521	33.5
Reduced **2a**	492	521	38.3 (1.14)
2b	492	516	31.0
Reduced **2b**	492	516	35.5 (1.15)
3a	490	515	24.1
Reduced **3a**	490	515	20.1 (0.83)
3b	491	516	19.6
Reduced **3b**	491	516	20.6 (1.05)
5	453	518	1.12
Reduced **5**	453	518	1.29 (1.15)

radical was bonded at the 5- or 6-position of the benzene-ring of fluorescein *via* an amide spacer (CONH) (**2ab**), or the reversed amide spacer (NHCO) (**3ab**) [7]. Additionally, TEMPO or DPROXYL radicals were bonded to the 3-hydroxyl group of the fluorescein xanthene moiety *via* an ester or an ether linkage (**4**, **5**). Compound **4** was rapidly destroyed by hydrolysis in solution, and hence, its ESR spectrum could not be measured; **3a** was unusable as a fluorescent radical probe due to the decrease in its fluorescence intensity upon reduction of the radical. The RFIs of **2a**, **2b**, and **3b** were small (1.43-, 1.38-, and 1.61-fold increases on reduction at pH 6), although their fluorescence intensity was sufficiently high for measurement. The largest RFI increase upon reduction was observed in the case of **5** (2.37-fold increase at pH 6), but, its fluorescence intensity was the smallest [varying from 0.945 a.u. (unreduced) to 2.243 a.u. (reduced)], though, its fluorescence change was visible. Recently, compound **1**, a highly fluorescent fluorescein nitroxide species containing an isoindoline nitroxide in the lower benzene ring, was found to show a 2.2-fold increase in fluorescence intensity at pH 7.4 after AsA reduction [10]. The RFI increase due to the reduction of **5** was essentially the same as that of **1**; however, its fluorescent intensity was low, but, measurable. Compound **5** is the best candidate as a probe for exploring the behavior of radical-reducing species *in vivo* for our six compounds, but further improvements are necessary for practical use. Yamada and co-workers had examined in detail the detection of AsA using hybrid probes consisting of a naphthalene and three nitroxides with different reactivities [5d]. They had evaluated that DiPy {7-aza-3.11-dioxadispiro[5.1.5^8.3^6]hexadecane-7-oxyl} showed higher reactivity for AsA than TEMPO and was best nitroxide as a bio-probe for the diagnosis of disease states. We here reported the relation between the conjugation of fluorescein with a nitroxide

Table 2
Fluorescence intensity of **5** before and at 3 h after the addition of 20 mM AsA at different pH levels in 0.1 M PBS (pH 3.0–5.0:citrate-Na_2HPO_4, pH 6.0–10.0: Na_2HPO_4-NaH_2PO_4).

	Fluorescence (c = 0.1 μM in 0.1 M PBS)		
pH	λ_{ex} [nm]	λ_{em} [nm]	Fluorescence intensity [a.u.] (RFI)
3.0	453	534	0.062
3.0	453	534	0.179 (2.89)
4.0	453	534	0.166
4.0	453	534	0.508(3.06)
5.0	453	535	0.350
5.0	453	535	1.124 (3.21)
6.0	453	518	0.945
6.0	453	518	2.243 (2.37)
7.0	453	519	0.930
7.0	453	519	2.115 (2.27)
8.0	453	518	1.023
8.0	453	518	2.874 (2.81)
9.0	453	519	1.484
9.0	453	519	3.248 (2.19)
10.0	453	519	1.451
10.0	453	519	2.645 (1.82)

Table 3
Relative ratio of the fluorescence intensities of **2ab** and **3ab** before and at 2 h after the addition of 20 mM AsA in PBS (1:1, v/v).

	Fluorescence [c = 0.1 μM in 0.1 M PBS (pH 6.0)]		
Compound	λ_{ex} [nm]	λ_{em} [nm]	Fluorescence intensity [a.u.] (RFI)
2a	492	522	11.16
Reduced **2a**	492	521	15.93 (1.43)
2b	492	518	14.06
Reduced **2b**	492	518	19.47 (1.38)
3a	490	513	12.56
Reduced **3a**	489	514	0.950 (0.08)
3b	489	516	10.04
Reduced **3b**	489	516	16.19 (1.61)

radical and the fluorescent increase after reduction of the radical. As an improvement for the detection of AsA in living cells, in the compound **5**, conversion of the nitroxide radical moiety from DPROXYL to the more reactive DiPy is necessary.

4. Experimental

4.1. Synthesis

The solvents used in the reaction were purified by distillation. Reactions were monitored by TLC on 0.25-mm silica gel F254 plates (E. Merck). UV radiation, and a 7% ethanolic solution of phosphomolybdic acid with heating, were used for coloration. Flash column chromatography was performed on silica gel (silica-gel 60, 40–50 μm, Kanto Chemical Co. Inc.) to separate and purify the reaction products. Melting points were determined using an ASONE micro-melting point apparatus and uncorrected values are reported. IR spectra were recorded on a Horiba FT-720 IR spectrometer using a KBr disk. NMR spectra were recorded on a JEOL ECX-500 spectrometer using Me_4Si as the internal standard. NMR spectra of the hybrid compounds were measured for the corresponding hydroxylamines, which were prepared by the reduction of nitroxide radicals in the presence of excess of hydrazobenzene (Wako pure Chemical Industries, Ltd.). Mass spectra were obtained by fast-atom bombardment (FAB) using 3-nitrobenzyl alcohol (NBA) as the matrix on a JEOL JMS-AX505HA instrument. After drying at 80 °C under reduced pressure for more than 2 h, each product was subjected to elemental analysis, on a Perkin-Elmer PE 2400 II instrument.

The nitroxide radicals and fluorescein derivatives used in the present study were synthesized by conventional methods, as shown in Supporting Information (Schemes S1–5).

4.1.1. 5- and 6-(TEMPO-4-aminocarbonyl)fluorescein (2a and 2b)

To a stirred solution of **9a** (158 mg) and 4-amino-TEMPO (86.2 mg) in dry DMF (2 mL), DCC (104 mg) and HOBt·H_2O (68 mg) were added in an ice-bath under argon atmosphere. The mixture was stirred at room temperature for 10 h. The reaction mixture was filtered and washed with $CHCl_3$. After the removal of organic solvents, the residue was purified by silica-gel column chromatography ($CHCl_3$-MeOH = 10:1) to afford **2a** (65.2 mg, 47.2%). By the similar way as **2a**, **2b** (14.6 mg, Y: 10.6%) was afforded from **9b** (383 mg).

Data for **2a**: yellow powder. Mp = 273–277 °C (dec.). IRν (KBr) 3282, 2975, 2938, 1741, 1644, 1612, 1540, 1508, 1457, 1321, 1241, 1180, 1114 cm^{-1}. ^{1}H NMR (DMSO-d_6 + hydrazobenzene, 500 MHz) δ 1.09 (s, 6H, $CH_3 \times 2$), 1.11 (s, 6H, $CH_3 \times 2$), 1.53 (t, J = 12.5, 2H, CH_2), 1.76 (m, 2H, CH_2), 4.23 (m, 1H, >CH), 6.54 (dd, J = 2.0, 8.5, 2H, ArH), 6.57 (d, J = 8.5, 2H, ArH), 6.69 (d, J = 2.0, 2H, ArH), 7.35 (d, J = 8.0, 1H, ArH), 8.22 (dd, J = 1.0, 8.0, 1H, ArH), 8.47 (d, J = 1.0, 1H, ArH), 8.54 (d, J = 8.0, 1H, NHCO), 10.20 (brs, 2H, OH × 2). ^{13}C NMR (DMSO-d_6 + hydrazobenzene, 125 MHz) δ 19.9, 32.9, 41.4, 44.8, 58.1, 83.6, 102.5, 109.3, 112.9, 123.6, 124.4, 126.7, 129.3, 135.0, 136.6, 152.1, 154.8, 159.9, 164.2, 168.5. *Anal.* Calcd for $C_{30}H_{29}N_2O_7 \cdot H_2O$: C, 65.80;

H, 5.71; N, 5.12. Found: C, 65.74; H, 5.68; N, 5.08. FABMS 530 $(M + H)^+$. g = 2.0059. aN = 1.70 mT.

Data for **2b**: yellow powder. Mp = 274–277 °C (dec.). IRν (KBr) 3264, 2977, 2938, 1747, 1637, 1610, 1542, 1508, 1457, 1380, 1367, 1322, 1268, 1241, 1214, 1180, 1112 cm^{-1}. ^{1}H NMR (DMSO-d_6 + hydrazobenzene, 500 MHz) δ 1.01 (s, 6H, CH_3), 1.04 (s, 6H, CH_3), 1.40 (t, J = 12.2, 2H, CH_2), 1.65 (m, 2H, CH_2), 4.13 (m, 1H, >CH), 6.55 (dd, J = 2.1, 8.8, 2H, ArH), 6.58 (d, J = 8.8, 2H, ArH), 6.69 (d, J = 2.1, 2H, ArH), 7.96 (s, 1H, ArH), 8.06 (d, J = 8.1, 1H, ArH), 8.16 (dd, J = 1.5, 8.1, 1H, ArH), 8.36 (brd, J = 7.9, 1H, NHCO), 10.20 (brs, 2H, OH × 2). ^{13}C NMR (DMSO-d_6 + hydrazobenzene, 125 MHz) δ 19.8, 32.7, 41.4, 44.6, 58.1, 83.5, 102.4, 109.4, 112.9, 122.3, 124.9, 128.4, 129.4, 129.8, 140.9, 152.0, 152.8, 159.8, 163.9, 168.2. *Anal.* Calcd for $C_{30}H_{29}N_2O_7 \cdot H_2O$: C, 65.80; H, 5.71; N, 5.12. Found: C, 65.66; H, 5.79; N, 5.09. FABMS 530 $(M + H)^+$. g = 2.0058. aN = 1.70 mT.

4.1.2. 5- and 6-(TEMPO-4-carbonylamino)fluorescein (*3a* and *3b*)

To a stirred solution of 4-carboxy-TEMPO (139 mg) and **13a** (300 mg) in dry CH_2Cl_2 (5 mL), DCC (151 mg) and DMAP (9 mg) were added in an ice bath under argon atmosphere. After stirring at room temperature for 14 h, the reaction mixture was filtered and washed with $CHCl_3$. After the removal of organic solvents, the residue was purified by silica-gel column chromatography ($CHCl_3$) to afford 3.10-di-*O*-acetyl **3a** (329 mg, 77.0%). To a stirred solution of acetate in MeOH (3 mL), 1 mL of 2 N NaOH aqueous solution was added and the mixture was stirred at room temperature for 5 h. After being acidified by the addition of Dowex® 50Wx8 (H^+) resin, the reaction mixture was filtered and washed with MeOH. After the removal of MeOH, the residue was purified by silica-gel column chromatography ($CHCl_3$-MeOH = 10:1) to afford **3a** (237 mg, 83.6%, overall yield: 64.4%). By the similar way as **3a**, **3b** (208 mg, overall yield: 56.6%) was afforded from **13b** (300 mg).

Data for **3a**: yellow prism (from EtOH). Mp = 262–264 °C (dec.). IRν (KBr) 3448, 2929, 1734, 1541, 1508, 1458, 1338, 1240, 1176, 1111 cm^{-1}. ^{1}H NMR (DMSO-d_6 + hydrazobenzene, 500 MHz) δ 1.08 (s, 12H, CH_3 × 4), 1.59 (t, 2H, J = 12.4, CH_2), 1.66 (d, 2H, J = 12.4, CH_2), 2.77 (m, 1H, >CH), 6.54 (dd, 2H, J = 2.3, 8.3, H2′,7′), 6.58 (d, 2H, J = 8.3, H1′,8′), 6.67 (d, 2H, J = 2.3, H4′,5′), 7.18 (d, 1H, J = 8.3, H7), 7.82 (dd, 1H, J = 8.3, 2.5, H6), 8.33 (d, 1H, J = 2.3, H4), 10.70 (br. s, 2H, OH × 2). ^{13}C NMR (DMSO-d_6 + hydrazobenzene, 125 MHz) δ 19.9 (×2), 32.8 (×2), 36.7, 42.2 (×2), 57.6 (×2), 83.7, 102.5 (×2), 110.1(×2), 112.0 (×2), 118.1(×2), 120.1, 124.7, 126.6, 127.3, 146.9, 150.1(×2), 150.2, 159.7 (×2), 169.0 (C = O), 174.6 (C = O). *Anal.* Calcd for $C_{30}H_{29}N_2O_7 \cdot 0.1H_2O$: C, 67.81; H, 5.54; N, 5.27. Found: C, 67.56; H, 5.47; N, 5.21. FABMS (m/z) 530 $(M + H)^+$. g = 2.0058. aN = 1.70 mT.

Data for **3b**: Yellow powder. Mp = 235–239 °C. IRν (KBr) 3431, 2978, 2937, 1734, 1610, 1541, 1508, 1466, 1338, 1178, 1115 cm^{-1}. ^{1}H NMR (DMSO-d_6 + hydrazobenzene, 500 MHz) δ 1.01 and 1.02 (each s, 6H, CH_3 × 4), 1.44 (t, 2H, J = 12.1, CH_2), 1.56 (d, 2H, J = 11.4, CH_2), 2.67 (m, 1H, >CH), 6.55 (dd, 2H, J = 2.3, 8.3, H2′,7′), 6.59 (d, 2H, J = 8.3, H1′,8′), 6.66 (d, 2H, J = 2.3, H4′,5′), 7.53 (d, 1H, J = 1.5, H7), 7.78 (dd, 1H, J = 8.4, 1.5, H5), 7.91 (d, 1H, J = 8.3, H4), 10.24 (br. s, 2H, OH × 2). ^{13}C NMR (DMSO-d_6 + hydrazobenzene, 125 MHz) δ 19.7 (×2), 32.7 (×2), 36.7, 41.9 (×2), 57.5 (×2), 82.4, 102.5 (×2), 109.9 (×2), 113.0 (×2), 120.1, 120.2, 120.7, 125.9, 129.1, 129.3, 145.9, 151.9 (×2), 154.9, 159.7 (×2), 168.7 (C = O), 174.8 (C = O). *Anal.* Calcd for $C_{30}H_{29}N_2O_7 \cdot 1.5H_2O$: C, 64.74; H, 5.80; N, 5.03. Found: C, 65.05; H, 5.80; N, 5.03. FABMS (m/z) 530 $(M + H)^+$. g = 2.0059. aN = 1.70 mT.

4.1.3. 3′–O–(TEMPO-4-carbonyl)fluorescein (*4*)

To a stirred solution of 4-carboxy-TEMPO (100 mg) and fluorescein (166 mg) in dry DMF (0.7 mL) and dry pyridine (0.7 mL), DCC (309 mg) and DMAP (33 mg) were added in an ice bath under argon atmosphere. After stirring at room temperature for 17 h, the reaction mixture was filtered and washed with $CHCl_3$. After the removal of halogen solvents, the residue was purified by silica-gel column chromatography ($CHCl_3$) to afford **4** (100 mg, Y: 39.0%).

Pale-yellow powder. Mp = 174–176 °C. IRν (KBr) 3527, 3176, 2975, 2937, 1766, 1731, 1612, 1498, 1465, 1427, 1315, 1288, 1245, 1166 cm^{-1}. ^{1}H NMR (DMSO-d_6 + hydrazobenzene, 500 MHz) δ 1.10 (s, 6H, CH_3 × 2), 1.08 (s, 6H, CH_3 × 2), 1.56 (t, J = 12.8, 2H, CH_2), 1.89 (m, 2H, CH_2), 2.92 (m, 1H, >CH), 6.58 (dd, J = 1.9, 8.7, 1H, ArH), 6.60 (dd, J = 0.9, 8.7, 1H, ArH), 6.71 (dd, J = 0.9, 1.9, 1H, ArH), 6.81 (d, J = 8.7, 1H, ArH), 6.89 (dd, J = 2.3, 8.7, 1H, ArH), 7.23 (d, J = 2.3, 1H, ArH), 7.35 (dt, J = 1.0, 7.6, 1H, ArH), 7.73 (dt, J = 7.6, 7.5, 1H, ArH), 7.80 (dt, J = 7.6, 7.5, 1H, ArH), 8.02 (dt, J = 1.0, 7.5, 1H, ArH), 10.24 (brs, 1H, OH). ^{13}C NMR (DMSO-d_6 + hydrazobenzene, 500 MHz) δ 19.5, 32.7, 34.6, 41.3, 57.6, 82.3, 102.5, 109.3, 110.6, 113.3, 116.8, 118.3, 124.3, 125.1, 126.1, 129.3, 129.4, 130.6, 136.0, 151.4, 151.8, 152.1, 152.5, 160.0, 168.8, 173.6. *Anal.* Calcd for $C_{30}H_{28}NO_7 \cdot 1.5H_2O$: C, 66.53; H, 5.77; N, 2.57. Found: C, 66.20; H, 5.87; N, 2.57. FABMS (m/z) 515 $(M + H)^+$.

4.1.4. 3′-O-(3.4-dehydroPROXYL-3-methyl)fluorescein (*5*)

To a suspension of fluorescein (332 mg) in dry EtOH (5 mL), NaOEt (136 mg) was added under argon atmosphere and the suspension solution was cleared. To a stirred solution, methanesulfonate (496 mg) was added by portions at room temperature. After stirring at room temperature for 1 h, the reaction mixture was stirred at 42 °C for 1.7 h. After standing at room temperature, the reaction mixture was poured into an ice-1 M HCl solution and extracted with AcOEt for three times. The extract was separated and purified by silica-gel column chromatography (toluene-AcOEt-AcOH = 6:1:0.2) to afford **5** (300 mg, Y: 62%).

Yellow powder. Mp = 130–132 °C (dec.). IRν (KBr) 3448, 2975, 2929, 1734, 1541, 1508, 1458, 1249, 1184, 1111 cm^{-1}. ^{1}H NMR (DMSO-d_6 + hydrazobenzene, 500 MHz) δ (PROXYL moiety): 1.09 and 1.15 (each s, 6H, CH_3 × 2), 4.61 (s, 2H, CH_2), 5.66 (s, 1H, olefinic H), (fluorescein moiety): 6.63 (d, J = 9.1, 1H, H8′), 6.69 (s, 1H, H4′ or H5′), 6.81 (d, J = 7.5, 1H, H1′), 6.71 (d, J = 9 0.1, 1H, H7′), 7.26 (s, 1H, H4′ or H5′), 7.26 (d, J = 7.4, 1H, H7), 7.28 (d, J = 7.5, 1H, H2′), 7.78 (t, J = 7.4, 1H, H6), 8.00 (d, J = 7.4, 1H, H4′), 10.19 (s, 1H, OH). ^{13}C NMR (DMSO-d_6 + hydrazobenzene, 125 MHz) δ (PROXYL moiety): 24.493, 24.512, 25.546, 25.575 (CH_3 × 4), 66.44 and 68.50 (C1″, 4″), 139.79 (C3″), 149.86 (C2″), 64.76 (C5″), (fluorescein moiety): 82.72 (C1), 101.36 (C5′), 102.16 (C4′), 111.04 (C9′), 111.60 (C12′), 112.36 (C7′), 112.74 (C2′), 123.92 (C7), 124.12 (C4), 126.03 (C3), 128.75 (C8′), 128.83 (C1′), 130.08 (C5), 135.56 (C6), 151.74 (C10′), 151.79 (C11′), 152.35 (C8), 159.54 (C6′), 160.01 (C3′), 168.60 (C2). *Anal.* Calcd for $C_{29}H_{26}NO_6$: C, 71.89; H, 5.41; N, 2.89. Found: C, 71.55; H, 5.69; N, 2.71. FABMS 485 $(M + H)^+$. g = 2.0056. aN = 1.62 mT.

4.2. Analysis

4.2.1. Apparatus

Fluorescence spectra and relative fluorescence intensity were measured using a Hitachi F7000 fluorescence spectrophotometer. Both the excitation and emission wavelength band passes were set at 5 nm and the range was 1. Each sample was measured at a concentration of 0.1 μM. Absorption spectra were obtained using a Hitachi U-3000 UV–VIS spectrometer. Electron spin resonance (ESR) spectra were obtained on a JEOL JES-FR30 ESR spectrometer. Samples were drawn into quartz capillaries, the bottoms of the capillaries were sealed and the capillaries were placed in standard 2-mm-i.d. quartz ESR tubes. The ESR spectrometer settings were as follows: microwave power, 4.0 mW; frequency, 9.5 GHz; and, modulation amplitude, 1.25 G. All pH measurements were performed using a Fisher AB15 pH meter.

4.2.2. ESR measurement

A 20 μM sample in 0.1 M PBS (pH 7.0 or 6.0) and a 20 mM AsA in 0.1 M PBS (pH 7.0 or 6.0) were mixed in a ratio of 1:1 (v/v). The

resulting solution was drawn into quartz capillaries and sealed and measured ESR spectroscopy for a constant period of time.

4.2.3. Fluorescence measurement

The above resulting solution was diluted with 0.1 M PBS or NaOH aqueous solution until a 0.1 μM and subjected to the fluorescence measurement.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.saa.2016.06.021.

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