Spectrochimica Acta Part A 71 (2009) 2030–2039

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

Synthesis and spectral properties of polymethine-cyanine dye–nitroxide radical hybrid compounds for use as fluorescence probes to monitor reducing species and radicals

Shingo Sato[a,*], Minoru Tsunoda[a], Minoru Suzuki[a], Masahiro Kutsuna[a], Kiyomi Takido-uchi[a], Mitsuru Shindo[a], Hitoshi Mizuguchi[a], Heitaro Obara[b], Hiroaki Ohya[b]

[a] *Graduate School of Science and Engineering, Yamagata University, 4-3-16 Jonan, Yonezawa-shi, Yamagata 992-8510, Japan*
[b] *Institute for Life Support Technology, Yamagata Promotional Organization for Industrial Technology, 2-2-1 Matsuei, Yamagata 990-2473, Japan*

ARTICLE INFO

Article history:
Received 14 March 2008
Received in revised form 24 July 2008
Accepted 27 July 2008

Keywords:
Cy5
Cy3
Nitroxide radical
ESR intensity
Fluorescence intensity

ABSTRACT

Various hybrid compounds comprised of two types of nitroxide radicals and either a pentamethine (Cy5) or trimethine cyanine (Cy3) were synthesized. The nitroxide radicals were linked either via an ester-bond to one or two *N*-alkyl carboxyl-terminated groups of Cy5, or via two amido-bonds (aminocarbonyl or carbonylamino group) to the 5-position of the indolenine moieties of Cy5 and Cy3. Changes in fluorescence and ESR intensities of the hybrid compounds were measured before and after addition of Na ascorbate in PBS (pH 7.0) to reduce the radicals. Among the hybrid compounds synthesized, those that linked the nitroxide radicals via an aminocarbonyl residue at the 5-position of the indolenine moieties on Cy5 and Cy3 exhibited a 1.8- and 5.1-fold increase in fluorescence intensity with the reduction of the nitroxide segment by the addition of Na ascorbate, respectively. In contrast, fluorescence intensity was not enhanced in the other hybrid compounds. Thus, the hybrid compounds which exhibited an increase in fluorescent intensity with radical reduction can be used in the quantitative measurement of reducing species such as Fe^{2+} and ascorbic acid, and hydroxyl radicals. Because these hybrid compounds have the advantage of fluorescing at longer wavelengths—661 (Cy5) or 568 (Cy3) nm, respectively, they can be used to measure radical-reducing species or radicals either in solution or *in vivo*.

1. Introduction

Previously, hybrid compounds comprised of a nitroxide radical and a fluorophore linked via an amido- or ester-bond as a spacer had been prepared and their use in quantifying radical and radical-reducing species has been evaluated (Fig. 1) [1]. Their fluorescence was mostly faded owing to electron transfer to nitroxide radicals, but was recovered by reducing or radical trapping of the nitroxide radicals. A method for the quantitative measurement of reductants such as Fe^{2+} [1-a], ascorbic acid [1-b], or of radical-trapping species such as hydroxyl radicals [1-c,d,e] was developed by applying this phenomena. The fluorescence technique allows detection of antioxidants in the submicromolar concentration scale while the electron spin resonance (ESR) technique can detect only micromolar concentrations [1-f]. The ESR measurements are limited by the high cost and complexity of the required equipment and by difficulties in acquiring and processing experimental data [1-b]. The fluorescence technique makes it possible to visualize the presence of the radicals or radical-reducing species, in addition to the quantitative measurement of them [1-g].

For the preparation of these hybrid compounds, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) or 2,2,5,5,-tetramethylpyrrolidine-1-oxyl (PROXYL) has been used as the stable nitroxide radical, and aromatic compounds, such as naphthalene [1-b,h,i,j,d] or pyrene [1-a,b,k,l], had been used as the fluorescence molecule. These aromatic compounds have short excitation wavelength (λ_{ex} 300 and 350 nm), and the emission wavelengths (λ_{em}) also are short—390 and 430 nm. Both the excitation and emission wavelengths often overlap the absorption spectra of proteins and the other background compounds [2-a,b,c,d]. It is unclear how hybrid compounds composed of non-aromatic fluorochromes and nitroxide radicals affect the recovery of fluorescence with the reduction of radicals.

We synthesized hybrid compounds composed of well-known fluorescent molecules and nitroxide radicals and evaluated the fluorescence and ESR intensities of the hybrid compounds. In a previous paper [3], three hybrid compounds composed of a well-known fluorophore, umbelliferone, and 2,2,5,5,-tetramethylpyrroline-1-

* Corresponding author. Tel.: +88 238 26 3120; fax: +88 238 26 3120.
E-mail address: shingo-s@yz.yamagata-u.ac.jp (S. Sato).

1386-1425/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.saa.2008.07.045

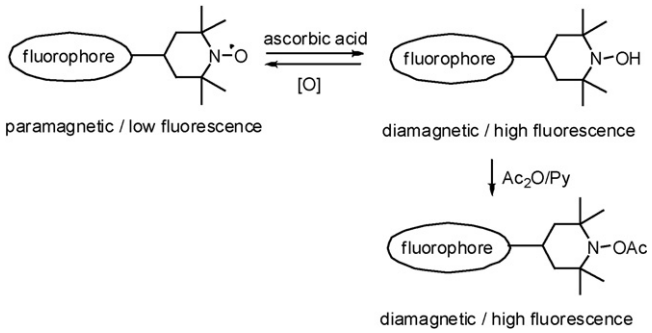


Fig. 1. Mechanism of the fluorescent increase and decrease in a fluorophore nitroxide hybrid compound.

oxyl (TEMN) or TEMPO radicals (**1**, **2**, and **3**) were synthesized and the relationships between their fluorescence and ESR intensities before and after addition of L-ascorbic acid sodium salt (Na ascorbate) were explored (Fig. 2). It appeared that the decay in ESR intensity upon addition of Na ascorbate caused a proportional and parallel increase in fluorescence intensity. Because fluorescence intensity increased in a concentration-dependent manner to a maximal eight- or nine-fold elevation, these two umbelliferylnitroxide radical-hybrid compounds **1** and **2** may be used as fluorescence spin-probes for the quantitative measurement of radicals in biosystems, Fe^{2+} in solution, and ascorbic acid in food products [1-d]. The excitation and emission wavelengths of these compounds were 330 and 443 nm, respectively, which were a little longer than those of pyrene-TEMPO (λ_{em} 430 nm) [1-c]. However, the wavelengths are not long enough to perform measurements in living cells and organisms due to autofluorescence from normal cellular constituents [2-a,b,c,d].

This paper introduces two new hybrid compounds composed of nitroxide radicals and well-known polymethine-cyanine dyes (Cy3 and Cy5), which are typically used as fluorescent labels for proteins, as the fluorescence molecules. Cy3 and Cy5 have the following properties: emission wavelengths of 500 and 650 nm, respectively: sharp and intense absorption bands ($\varepsilon > 100{,}000$): and, in ethanol, quantum fluorescence yield is typically between 0.2 and 0.4 [2-a]. Cy3 and Cy5 are conjugated polymethine fluorophores and differ significantly from aromatic compound such as naphthalene and pyrene which have been used previously in the synthesis of hybrid compounds. The advantage of using cyanine dyes is that these dyes can be tuned to the desired wavelengths by altering the heterocyclic nucleus and the number of double bonds in the polymethine chain [2-e].

2. Results and discussion

First, an ester-linkage between a hydroxyl group of a nitroxide radical (Fig. 3) and the *N*-alkyl carboxyl-terminated group of Cy5 was formed. The *N*-alkyl carboxyl-terminated group generally functions as a spacer for protein binding [2-f]. One of two radicals, 4-hydroxy-TEMPO (**4**) or 3-hydroxymethyl-TEMN (**5**) was bonded either to both or to one of two terminal carboxyl residues. The *N*-alkyl carboxyl residue is composed of either a two- or five-carbon chain. A total of eight hybrid compounds were synthesized (Fig. 4). ESR and fluorescence spectra of these hybrid compounds were measured in phosphate-buffered saline (PBS, 0.1 M, pH 7.0). However, both ESR and fluorescence intensities were much lower than an original nitroxide radical or Cy5, and each intensity value was various. When excess of Na ascorbate was added to a solution of these hybrid compounds, ESR intensity was decayed, however all fluorescence intensities were not enhanced. Hydroxylamine acetate of these hybrid compounds which is a diamagnetic derivative and is not subjected to oxidation (Fig. 1) was also observed no enhancement of fluorescence intensity.

Since the methyl ester of *N*-alkyl-terminated carboxylic acid also did not exhibit fluorescence, the development of this series of hybrid compounds for use as fluorescence probes in the detection of radical reducing species was stopped. Blough and co-workers reported that relatively high rates of quenching by nitroxide radical ($>10^8\ s^{-1}$) are observed over distances as great as 12 Å [1-i]. Unfortunately, our results differed from theirs.

We next planed a design of the hybrid compounds linked at the closer position of the fluorophore with a nitroxide radical (Fig. 5).

Two nitroxide-equivalents were introduced at the 5-position of the indolenine skeleton in both Cy5 and Cy3 via an amido-bond. A methyl group was selected as the *N*-alkyl residue because of unnecessary of the spacer in this study. Based on trial and error, a successful synthesis was finally achieved by the following reaction sequence, as shown in Scheme 1: (1) synthesis of methyl 5-nitroindolenium; (2) reaction with 1,1,3,3-tetraethoxypropane and triethyl orthoformate, leading to 5-nitro-Cy5 (**9**) and 5-nitro-Cy3 (**15**); (3) reduction of the two nitro groups to amino groups (5-amino-Cy5 **10** and -Cy3 **16**); and (4) condensation of **10** and **16** with 3-carboxy-2,2,5,5-tetramethylpyrroline-1-oxyl (3-carboxy-TEMN **7**, Fig. 3).

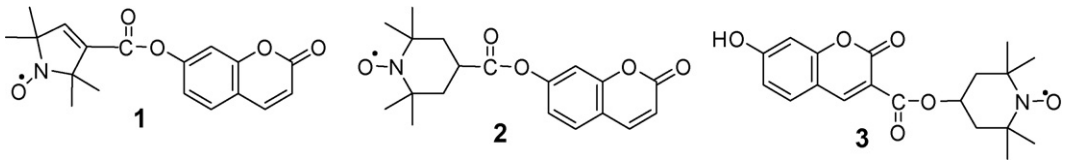


Fig. 2. Umbelliferylnitroxide radical hybrid compounds.

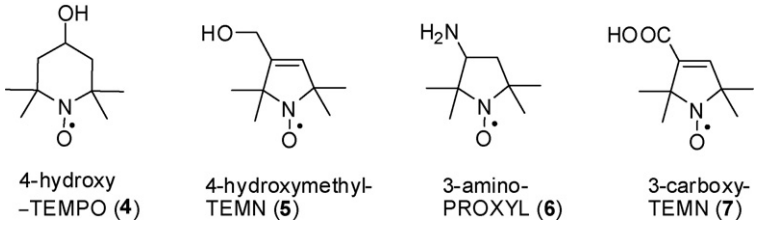


Fig. 3. Nitroxide radicals adapted for the synthesis of hybrid compounds.

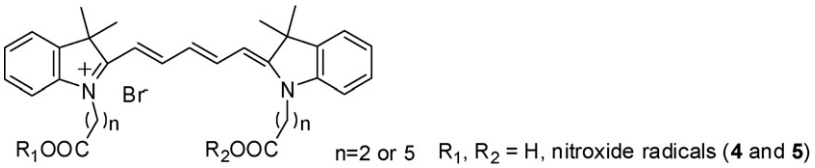


Fig. 4. Cy5-nitroxide hybrid compounds linked between one or both of *N*-alkyl-terminated carboxylic acids and nitroxide radicals (**4** and **5**) via ester bond.

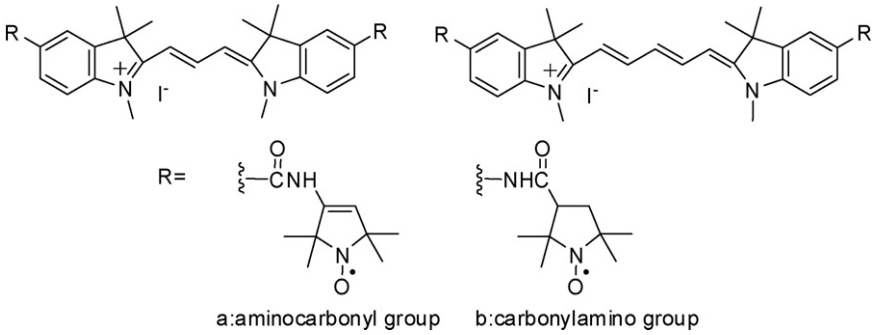
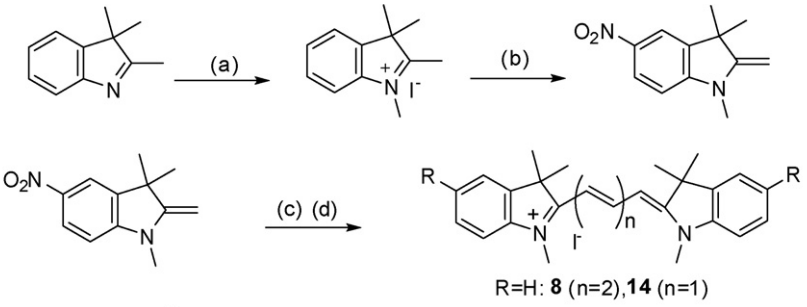
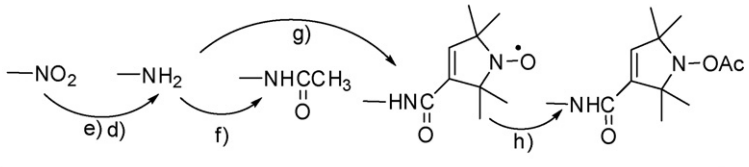


Fig. 5. Cy3, Cy5-nitroxide compounds linked between at the 5-positions of cyanine dyes and nitroxides via two kinds of amido bonds (a: aminocarbonyl group, b: carbonylamino group).

Fluorescence intensities of Cy5 (**8**) in some solvents, as measured, were found to vary (Fig. 6). When acetonitrile was used as a solvent, fluorescence intensity reached maximum; thereafter acetonitrile was employed as the solvent in the fluorescence measurement. Na ascorbate used for the reduction of the hybrid compounds was all dissolved in PBS (0.1 M, pH 7.0). The influence of the fluorescence intensity was explored when a PBS solution of Na ascorbate was added to an acetonitrile solution of Cy5 (**8**) in a ratio of 1:9 (v/v) (Table 1). When various concentrated PBS solutions of Na ascorbate were added to an acetonitrile solution of **8**, the fluorescence intensity did not change much. Therefore, the ratio of the addition of a PBS solution of Na ascorbate to an acetonitrile solution of hybrid compounds was decided to be a 1:9 (v/v), when the hybrid compound was reduced. The ESR and fluorescence spectra of the synthesized bis-5-(TEMN-3-yl-carbonylamino)-Cy5 (**12**) and -Cy3 (**18**) were measured before and after addition of a PBS solution of Na ascorbate (Tables 2 and 3). The relative fluorescent intensity (RFI) of hybrid compound **12** was 0.156 in comparison with Cy5 (**8**, 1.00) and RFI after reduction by the addition of Na ascorbate was 0.150. The RFI of the hybrid compound comprised of Cy3 (**18**) was 0.430 in comparison with Cy3 (**14**, 1.00); RFI after reduction was 0.256. No recovery of the fluorescence intensity of these hybrid compounds was observed. The RFIs of 5-acetamido-Cy5 (**11**) and -Cy3 (**17**) in which an acetyl group was introduced in the place of TEMN, were also 0.09 and 0.22, respectively. Therefore, it was assumed that the low fluorescence intensity was not due to the introduction of nitroxide radicals, but, rather, to the amido-bond. The RFIs of bis-5-nitro-Cy5 (**9**) and -Cy3 (**15**) were 0.564 and 4.51,



Compound (yield)						
	(n=2, Cy5)	**9** (69%)	**10** (38%)	**11** (57%)	**12** (80%)	**13** (43%)
	(n=1, Cy3)	**15** (78%)	**16** (61%)	**17** (64%)	**18** (73%)	–
R						

Scheme 1. Synthesis of hybrid compounds (**12**, **13**, **18**) by condensation of 5-bis-amino-Cy5 (**10**) or -Cy3 (**16**) and 3-carboxy-TEMN (**7**). Reagents and conditions—(a) CH_3I (2.0 equiv.) in toluene at r.t. 58 h; (b) $NaNO_3$ (1.1 equiv.) in conc. H_2SO_4 at 0 °C, 0.5 h; (c) for Cy5: malonaldehydedianilide hydrochloride (0.5 equiv.) in AcOH/Ac_2O, reflux 2 h; for Cy3: triethyl orthoformate (1.0 equiv.) in AcOH/Ac_2O, reflux 1 h; (d) 14% aq. KI, reflux 1 h; (e) $SnCl_2{\cdot}2H_2O$ (12 equiv.) in conc. HCl, reflux 1 h; (f) Ac_2O at r.t. 1 h; (g) 3-carboxy-TEMN (2.5 equiv.)/DCC (2.8 equiv.)/DMAP (0.30 equiv.) in CH_3CN, at 0 °C, r.t. 15 h; (h) 1. hydrazobenzene (9.5 equiv.) in CH_2Cl_2 at r.t. 3 h; 2. Ac_2O in CH_2Cl_2 at r.t. 6.5 h.

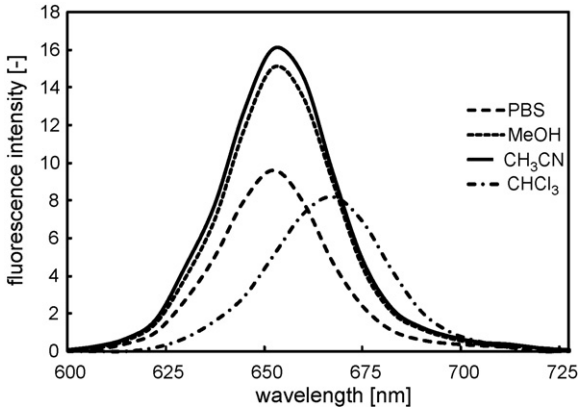


Fig. 6. Fluorescence intensity of Cy5 (**8**) in some solvents (*c* 0.2 μM, λ_{ex} = 630 nm).

Table 1
Influence for the fluorescence intensity of Cy5 (**8** in acetonitrile) after addition of various concentrated solution of Na ascorbate in PBS (0.1 M, pH 7.0) (acetonitrile:PBS = 9:1, v/v)

Solvent	Concentration of Na ascorbate (μM)	Fluorescence intensity (*c* = 0.2 μM)
CH_3CN	–	8.70
	0	7.60
CH_3CN:Na ascorbate in PBS = 9:1	100	7.50
	500	7.55
	2,500	7.45
	12,500	7.35
	62,500	7.25

respectively, and neither bis-5-amino-Cy5 (**10**) nor -Cy3 (**16**) exhibited any fluorescence. These results suggest that the fluorescence intensities of Cy5 and Cy3 substituted with an electron-donating group at the 5-position decrease significantly, while substitution with an electron-withdrawing group results in a small decrease in nitro-Cy5 and a large increase in nitro-Cy3.

The third strategy examined was introduction of the nitroxide radical at the 5-position of both indolenines via substitution of a carbonylamino group with an aminocarbonyl group, which

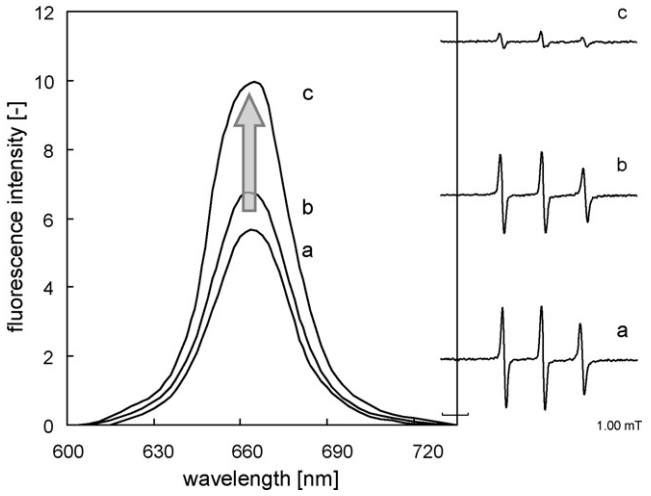


Fig. 7. Changes in fluorescence (λ_{ex} 649 nm) and ESR spectra at 20 min after three concentrations of Na ascorbate in PBS (a: 0 mM, b: 1.0 mM, c: 30 mM) were added to a 20-μM solution of **21** in PBS (pH 7.0) (1:9, v/v).

is more electron attracting (Fig. 5). The synthesis used the following reaction sequence as shown in Scheme 2: (1) synthesis of *N*-methyl 5-carboxy-indolenium iodide; (2) synthesis of bis-5-carboxy-Cy5 and -Cy3; and (3) introduction of 3-amino-PROXYL (**6**) at the carboxyl group occupying the 5-position of the indolenine moiety.

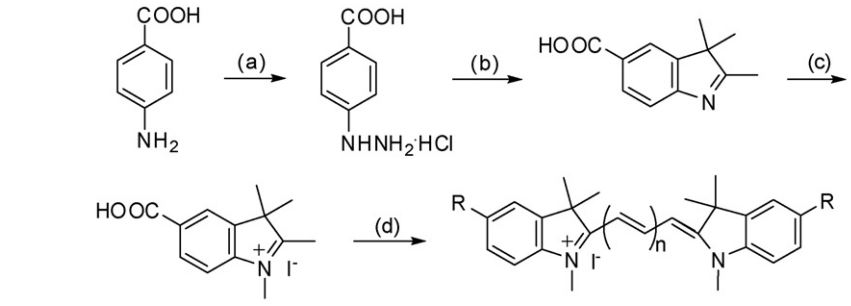
ESR and fluorescence intensities of the synthesized bis-5-(PROXYL-3-yl-aminocarbonyl)-Cy5 (**21**) were measured before and after the addition of 30 mM PBS solution of Na ascorbate (Fig. 7). As expected, after addition of Na ascorbate, the RFI of **21** increased to 1.80-fold with the decay in ESR intensity. The RFI (1.48) of diamagnetic hydroxylamine acetate of **21** (**22**) increased 2.22-fold in comparison with that of paramagnetic **21** (0.668, Table 4). For Cy3, after addition of Na ascorbate, the RFI of **24** increased 5.1-fold, and the RFI of the diamagnetic hydroxylamine acetate (**25**) was increased 6.54-fold compared with the hybrid compound composed of Cy3 (**24**) (Table 4). Next, fluorescence intensity for the hybrid compound **21** composed of Cy5, which is more unstable

Table 2
Absorption and fluorescence spectra of Cy5 (**8**, **9**, **10**, **11**), Cy5-nitroxide radicals hybrid compounds (**12**), and **12** + Na ascorbate

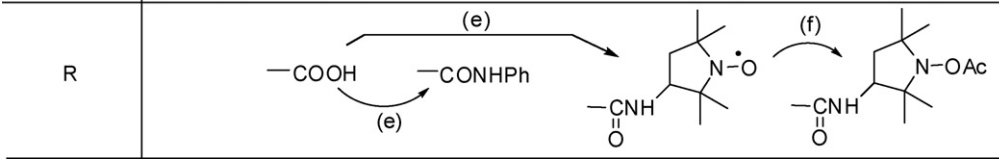
Compound	Absorption (in CH_3CN)		Fluorescence (*c* 0.2 μM in CH_3CN)		
	λ_{max} (nm)	log ε	λ_{ex} (nm)	λ_{em} (nm)	RFI
8	637	5.33	634	650	1.00
9	661	5.32	654	670	0.564
10	694	5.15	–	–	–
11	666	5.23	662	682	0.094
12	669	5.34	665	682	0.156
12 + Na ascorbate	–	–	668	680	0.150

Table 3
Absorption and fluorescence spectra of Cy3 (**14**, **15**, **16**, **17**), Cy3-nitroxide radicals hybrid compounds (**18**), and **18** + Na ascorbate

Compound	Absorption (in CH_3CN)		Fluorescence (*c* 0.2 μM in CH_3CN)		
	λ_{max} (nm)	log ε	λ_{ex} (nm)	λ_{em} (nm)	RFI
14	542	5.10	540	556	1.00
15	565	5.21	562	577	4.51
16	596	4.91	–	–	–
17	573	4.90	572	596	0.22
18	575	5.08	572	596	0.43
18 + Na ascorbate	–	–	572	598	0.26



Compound (yield)				
(n=2,Cy5)	**19** (74% or 66%)	**20** (40%)	**21** (33%)	**22** (43%)
(n=1,Cy3)	**23** (82%)	–	**24** (35%)	**25** (49%)



Scheme 2. Synthesis of hybrid compounds (**21**, **22**, **24**, **25**) by condensation of 5-bis-carboxy-Cy5 (**19**) or -Cy3 (**23**) and 3-amino-PROXYL (**6**). Reagent and conditions—(a) 1. 16% aq. $NaNO_2$ in HCl at 0 °C, 0.5 h; 2. $SnCl_2{\cdot}2H_2O$ (3.0 equiv.) at 0 °C, 0.5 h; (b) 3-methyl-2-butanone/conc. H_2SO_4 in CH_3CN, reflux 7 h; (c) CH_3I (2.0 equiv.) in $CHCl_3$, reflux 7.5 h; (d) for Cy5: malonaldehydedianilide hydrochloride (0.5 equiv.) in AcOH/Ac_2O, reflux 2 h, or 1,1,3,3-tetraethoxypropane (0.55 equiv.) in pyridine, reflux 10.5 h; for Cy3: triethyl orthoformate (1.0 equiv.) in AcOH/Ac_2O, reflux 1 h; (e) aniline (6.5 equiv.)/DCC (3.0 equiv.)/DMAP (0.6 equiv.) in CH_3CN, at 0 °C–r.t., 19.5 h; (f) 1. hydrazobenzene (10 equiv.) in CH_2Cl_2 at r.t. 1.5 h; 2. Ac_2O in CH_2Cl_2, at r.t. 11 h.

than one composed of Cy3 was measured under various pH conditions. Hybrid compound **21** was stable between pH 1 and 6, and fluorescence intensity decreased gradually above pH 6, as shown in Fig. 8. When a 15 mM PBS solution of Na ascorbate was added (1:1, v/v) to a 20 μM PBS solution of **21**, the ESR intensity decreased immediately and the reaction was complete within 15 min (Fig. 9).

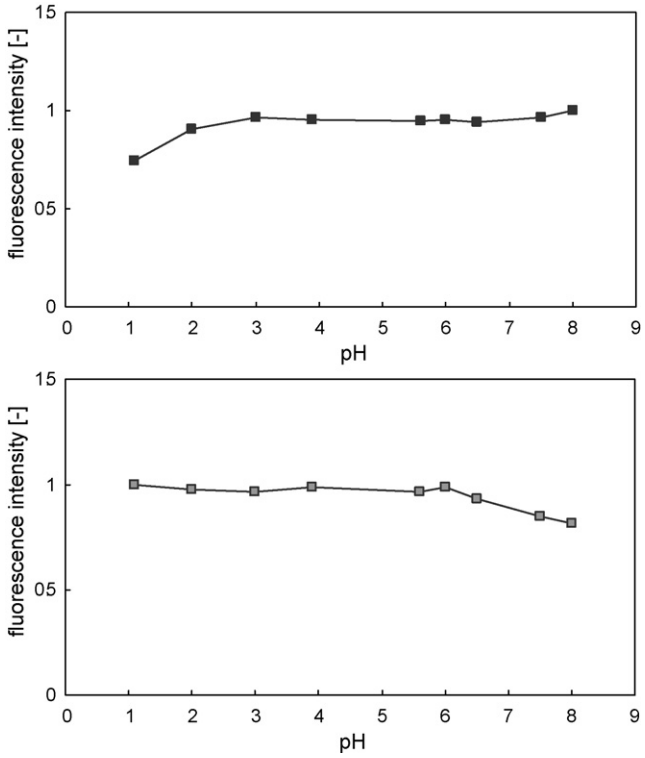


Fig. 8. Effect of pH on the fluorescence intensity of **8** (1.0 μM, upper panel) and **21** (1.0 μM, lower panel).

Table 4
Relative fluorescence intensity of hybrid compounds (**21**, **24**) and their hydroxylamine acetates (**22**, **25**)

Compound	Absorption (in CH_3CN)		Fluorescence (c 0.2 μM in CH_3CN)		
	λ_{max} (nm)	log ε	λ_{ex} (nm)	λ_{em} (nm)	RFI
8	637	5.33	634	650	1.00
19	649	5.31	650	658	0.084
20	653	5.06	652	664	0.80
21	650	5.36	649	661	0.67
22	650	5.37	647	661	1.48
24	555	5.05	555	568	1.00
25	556	5.23	552	568	6.54

The fluorescence intensity of **21** increased in parallel to the decay in ESR (Fig. 10). However, ESR signals were not perfectly removed, that may be due to the reoxidation of the corresponding hydroxylamine by the resulting ascorbate radical [4]. When various concentrations of the Na ascorbate in PBS were added to a 20 μM solution of **21** in PBS, the ESR intensity of **21** decayed in proportion to the Na ascorbate concentration (Fig. 11), while the fluorescence intensity of **21** increased in parallel (Fig. 12).

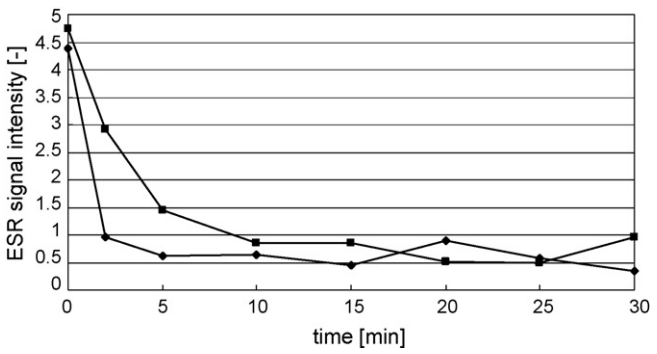


Fig. 9. Time-course of the decay in ESR intensity after addition of 15-mM PBS solution of Na ascorbate to 20-μM PBS solutions of 3-amino-PROXYL (**6**, ♦) and **21** (■) (1:1, v/v), respectively.

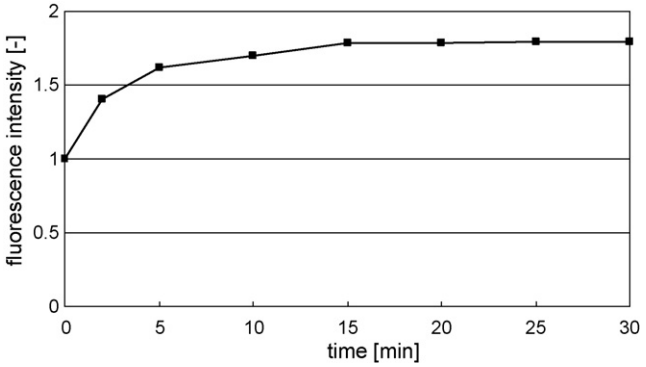


Fig. 10. Time-course of the increase in fluorescence intensity (0.2 μM, λ_{ex} 620 nm) after addition of a 15-mM PBS solution of Na ascorbate to a 20-mM PBS solution of **21** (1:1, v/v).

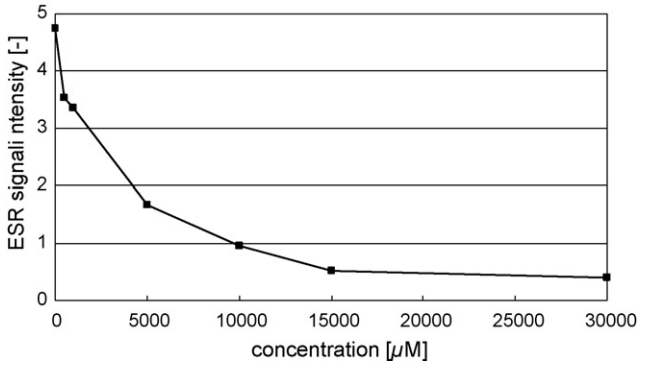


Fig. 11. ESR intensity at 20 min after the addition of various concentrations of a PBS solution of Na ascorbate to a 20-μM PBS solution of **21** (1:1, v/v).

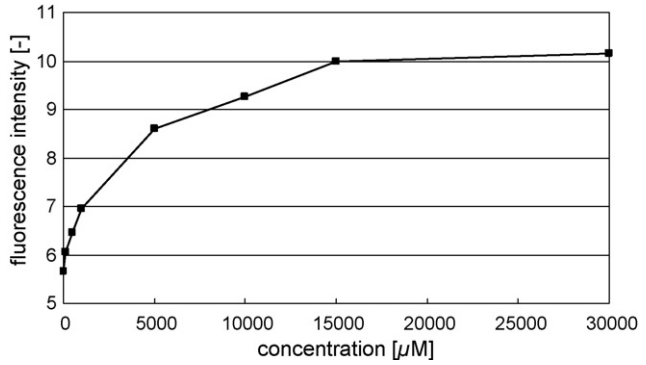


Fig. 12. Fluorescence intensity (0.2 μM, λ_{ex} 620 nm) at 20 min after the addition of various concentrations of a PBS solution of Na ascorbate to a 20-μM PBS solution of **21** (1:1, v/v).

3. Conclusion

Hybrid probes composed of polymethine-cyanine dyes and nitroxide radicals were synthesized and the relationships between their fluorescence and ESR intensities for the hybrid probes were evaluated. Among the hybrid compounds synthesized in the present study, polymethine-cyanine dye–nitroxide radical hybrid probes synthesized by the introduction of a nitroxide radical at the 5-position of the indolenine skeletons of Cy5 and Cy3 via an aminocarbonyl bond (**21** and **24**) showed that fluorescence intensity increased in parallel to a decay in ESR intensity. After reduction of radicals by addition of Na ascorbate, hybrid probes composed of Cy5 and PROXYL radicals (**21**) fluoresced at 661 nm, while Cy3 hybrids (**24**) fluoresced at 568 nm. Thus, these hybrid probes can be used in the quantitative measurement of reducing species such as Fe^{2+}, ascorbic acid, and hydroxyl radicals. In addition, these hybrid compounds can be used to visualize reducing species or radicals *in vivo*. Similar to the phenomenon observed in hybrid compounds composed of condensed-benzene ring fluorophores (naphthalene and pyrene) and nitroxide radicals, fluorescence intensity of the hybrid compounds composed of the conjugated polymethine fluorophores and nitroxide radicals synthesized in this study increased in parallel to the decrease in radicals. Maximum enhancement of the fluorescence intensity was a 6.54-fold of the diamagnetic hydroxylamine acetate (**25**).

4. Experimental

4.1. Apparatus

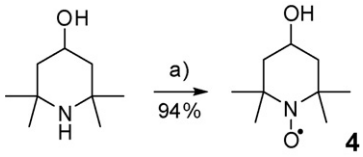
Fluorescence spectra and relative fluorescence intensity were measured using a Hitachi 650-10M fluorescence spectrophotometer. Both the excitation and emission wavelength band passes were both set at 2 nm and range was 1 and all concentrations of the measured samples were 0.2 μM. Absorption spectra were obtained using a Hitachi U-2010 UV-VIS spectrometer. Electron spin resonance 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, 9.2 GHz; modulation amplitude, 1.25 G. All pH measurements were performed using a Horiba M-13 PH meter.

4.2. Synthesis

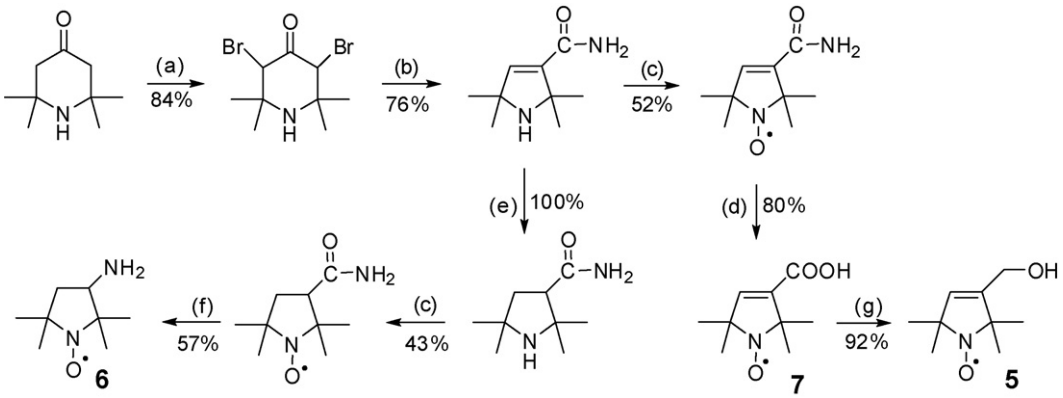
The solvents used in this study were purified by distillation. Reactions were monitored using TLC on 0.25-mm Silica Gel F254 plates (E. Merck), and UV light and a 7% ethanolic solution of phosphomolybdic acid with heat was used as the coloration agents. Flash column chromatography was performed on silica gel (230–400 mesh, Fuji-Silysia Co. Ltd., BW-300) to separate and purify 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 KBr disks. NMR spectra were recorded on a Varian Inova 500 spectrometer using Me_4Si as the internal standard. NMR spectra of the hybrid compounds were measured as the corresponding hydroxylamine induced by the reduction of nitroxyl radicals in the presence of excess of hydrazobenzene. Mass spectral data were obtained by fast-atom bombardment (FAB) using 3-nitrobenzyl alcohol (NBA) as the matrix on a JEOL JMS-AX505HA instrument.

4.2.1. Synthesis of nitroxide radicals

The four nitroxide radicals used in the present study (Fig. 3) were synthesized by conventional methods [5], as shown in Schemes 3 and 4.



Scheme 3. Synthesis of 4-hydroxy-TEMPO (**6**). Reagents and conditions: (a) 30% H_2O_2, EDTA, Na_2WO_4, in H_2O, r.t. 1 day.



Scheme 4. Synthesis of 3-amino-PROXYL (**4**) and 3-carboxy-TEMN (**5**). Reagents and conditions—(a) Br_2 in AcOH, at r.t. 1 day; (b) 25% aq. NH_3, KOH, at 0 °C; (c) 30% H_2O_2, EDTA, $NaWO_4$ in MeOH–H_2O, at r.t., 21 h; (d) 10% aq. NaOH, reflux 1 day; (e) H_2/10% Pd-C, in MeOH, at r.t., 17 h; (f) NaOH, Br_2, H_2O, KOH, at 0–70 °C, 3 h; (g) $LiAlH_4$ (1.15 equiv.) in THF, at 0 °C–r.t., for 24 h.

*4.2.2. Synthesis of hybrid compounds (**8**–**25**)*

The synthesis of Cy5 and Cy3 (**8**, **14**) and their derivatives (**9**–**13** and **15**–**25**) were carried out as shown in Schemes 1 and 2 [2-g,h].

To a solution of *p*-aminobenzoic acid in H_2O and concentrated HCl, which was cooled in an ice-bath, a 16% aqueous $NaNO_2$ solution was added and resulting mixture was stirred for 0.5 h. A mixture of $SnCl_2{\cdot}2H_2O$ (34.0 g, 0.151 mmol) and concentrated HCl (35 mL) was then added, followed by stirring in the ice-bath for 0.5 h. After work-up, the resulting crude white powder, hydradine hydrochloride (11.6 g), was used in the subsequent reaction without any purification. To a solution of HCl salt of hydradine and 3-methyl-2-butanone in CH_3CN concentrated H_2SO_4 (2.5 mL) was added and refluxed for 7 h. After work-up and purification by silica-gel column chromatography (*n*-hexane–AcOEt), 5-carboxyindolenine was afforded as orange crystals in 31% yield from *p*-aminobenzoic acid. 5-Carboxyindolenine and methyl iodide were refluxed in $CHCl_3$ for 7.5 h to give ammonium iodide as a white powder in 32% yield. Methyl 5-carboxyindolenium iodide was refluxed with malonaldehydedianilide hydrochloride (0.5 equiv.) in AcOH and Ac_2O for 2 h or with tetraethoxypropane (0.55 equiv.) in pyridine for 10.5 h, followed by successive purification using silica-gel column chromatography ($CHCl_3$–MeOH–AcOH) to give the corresponding Cy5 as dark-green crystals in the yields of 74% and 66%, respectively. Methyl 5-carboxyindolenium iodide was refluxed with triethyl orthoformate (1.5 equiv.) in pyridine for 2 h, followed by successive purification using silica-gel column chromatography ($CHCl_3$–MeOH–AcOH) to give the corresponding Cy3 as a dark-blue powder in 82% yield.

*4.2.2.1. 2-[5-(1,3,3-Trimethyl-2,3-dihydro-1H-indol-2-ylidene)pent-1,3-dienyl]-1,3,3-trimethyl-3H-indolium iodide (**8**).* Mp = 268–269 °C. IR ν 1496, 1458, 1373, 1333, 1172, 1103 cm^{-1}. ^1H NMR ($CDCl_3$) δ1.68 (s, 12H, $CH_3 \times 4$), 3.61 (s, 6H, $CH_3 \times 2$), 6.28 (d, 2H, *J* 13.9 Hz, –CH= × 2), 6.55 (t, 1H, *J* 12.2, 12.4 Hz, –CH=), 7.25 (m, 2H, ArH × 2), 7.40 (m, 4H, ArH × 4), 7.62 (d, 2H, *J* 7.6 Hz, ArH × 2), 8.33 (t, 2H, *J* 11.7, 14.6 Hz, –CH= × 2). ^{13}C NMR ($CDCl_3$) δ 27.9 ($CH_3 \times 4$), 32.3 (*N*-$CH_3 \times 2$), 49.3 (C3 × 2), 103.8, 126.3, 153.7 (each polymethine-C × 2, ×1, ×2), 173.4 (C2 × 2), 110.3, 122.1, 125.1, 128.5, 140.9, 142.6 (each 2C, indolenylbenzene × 2). FAB-MS (*m*/*z*) 383 $(M)^+$.

*4.2.2.2. 5-Nitro-2-[5-(5-nitro-1,3,3-trimethyl-2,3-dihydro-1H-indol-2-ylidene)pent-1,3-dienyl]-1,3,3-trimethyl-3H-indolium iodide (**9**).* Mp = >300 °C. IR ν 1498, 1457, 1373, 1321, 1300, 1163, 1079 cm^{-1}. ^1H NMR (DMSO-d_6) δ 1.77 (s, 12H, $CH_3 \times 4$), 3.68 (s, 6H, $CH_3 \times 2$), 6.49 (d, 2H, *J* 13.9 Hz, –CH= × 2), 6.73 (t, 1H, *J* 12.2, 12.4 Hz, –CH=), 7.60 (d, 2H, *J* 9.0 Hz, ArH × 2), 8.35 (dd, 2H, *J* 2.2, 8.8 Hz, ArH × 2), 8.50 (t, 2H, *J* 12.7, 13.4 Hz, –CH= × 2), 8.62 (d, 2H, *J* 2.2 Hz, ArH × 2). ^{13}C NMR (DMSO-d_6) δ 26.8 ($CH_3 \times 2$), 32.0 (*N*-$CH_3 \times 2$), 49.1 (C3 × 2), 106.2, 128.2, 156.3 (each polymethine-C × 2, ×1, ×2), 175.1 (C2 × 2), 111.7, 118.6, 125.6, 142.4, 144.3, 148.3 (indolenylbenzene × 2). FAB-MS (*m*/*z*) 473 $(M)^+$.

*4.2.2.3. 5-Amino-2-[5-(5-amino-1,3,3-trimethyl-2,3-dihydro-1H-indol-2-ylidene)pent-1,3-dienyl]-1,3,3-trimethyl-3H-indolium iodide (**10**).* Mp = >300 °C. IR ν 3311, 3216, 1622, 1466, 1334, 1157, 1093 cm^{-1}. ^1H NMR (DMSO-d_6) δ1.60 (s, 12H, $CH_3 \times 4$), 3.49 (s, 6H, $CH_3 \times 2$), 5.29 (br. s, 4H, $NH_2 \times 2$), 6.05 (d, 2H, *J* 14.2 Hz, –CH= × 2), 6.35 (t, 1H, *J* 12.4 Hz, –CH=), 6.57 (dd, 2H, *J* 2.2, 8.5 Hz, ArH × 2), 6.73 (d, 2H, *J* 2.2 Hz, ArH × 2), 7.05 (d, 2H, *J* 8.5 Hz, ArH × 2), 8.06 (t, 2H, *J* 12.9, 13.2 Hz, –CH= × 2). ^{13}C NMR (DMSO-d_6) δ 27.5 ($CH_3 \times 4$), 31.2 (*N*-CH_3), 48.7 (C3 × 2), 101.9, 123.4, 150.5 (each polymethine-C × 2, ×1, ×2), 170.0 (C2 × 2), 108.2, 111.7, 113.0, 133.0, 142.6, 147.2 (each 2C, indolenylbenzene × 2). FAB-MS (*m*/*z*) 413 $(M)^+$.

*4.2.2.4. 5-Acetoamido-2-[5-(5-acetoamido-1,3,3-trimethyl-2,3-dihydro-1H-indol-2-ylidene)pent-1,3-dienyl]-1,3,3,-trimethyl-3H-indolium iodide (**11**).* Mp = >300 °C. IR ν 1675, 1621, 1508, 1473, 1370, 1161, 1099 cm^{-1}. ^1H NMR (DMSO-d_6) δ1.63 (s, 12H, $CH_3 \times 4$), 2.04 (s, 6H, $COCH_3 \times 2$), 3.56 (s, 6H, $CH_3 \times 2$), 6.19 (d, 2H, *J* 13.9 Hz, –CH= × 2), 6.47 (t, 1H, *J* 12.2, 12.4 Hz, –CH=), 7.30 (d, 2H, *J* 8.5 Hz, ArH × 2), 7.43 (dd, 2H, *J* 2.0, 8.5 Hz, ArH × 2), 7.86 (d, 2H, *J* 2.0 Hz, ArH × 2), 8.23 (t, 2H, *J* 12.9, 13.2 Hz, –CH= × 2), 10.05 (s, 2H, NH × 2). ^{13}C NMR (DMSO-d_6) δ 24.2 ($COCH_3$), 27.3 ($CH_3 \times 4$), 31.3 (*N*-$CH_3 \times 2$), 49.0 (C3 × 2), 103.2, 124.9, 153.0 (each polymethine-C × 2, ×1, ×2), 168.4 (C=O × 2), 172.3 (C2 × 2), 111.3, 113.6, 119.2, 136.8, 138.4, 141.5 (each 2C, indolenylbenzene × 2). FAB-MS (*m*/*z*) 497 $(M)^+$.

*4.2.2.5. 5-(2,2,5,5-Tetramethyl-pyrroline-1-oxyl-3-yl)carbonylamino-2-{5-[5-(2,2,5,5-tetramethyl-pyrroline-1-oxyl-3-yl)carbonylamino-1,3,3-trimethyl-2,3-dihydro-1H-indol-2-ylidene]pent-1,3-dienyl}-1,3,3,-trimethyl-3H-indolium iodide (**12**).* Mp = >300 °C. IR ν 1662, 1618, 1508, 1466, 1331, 1159 cm^{-1}. ^1H NMR (DMSO-d_6) δ(PROXYL moiety): 1.20, 1.29 (each s, 12H, $CH_3 \times 8$), 1.64 (s, 12H, $CH_3 \times 4$), 6.54 (s, 2H, –CH= × 2). (Cy5 moiety): 3.57 (s, 6H, $CH_3 \times 2$), 6.19 (d, 2H, *J* 13.9 Hz, –CH= × 2), 6.47 (t, 1H, *J* 12.2, 12.4 Hz, –CH=), 7.32 (d, 2H, *J* 8.5 Hz, ArH × 2), 7.97 (s, 2H, ArH × 2), 8.23 (t, 2H, *J* 13.2 Hz, –CH= × 2), 9.88 (s, 2H, NH × 2). ^{13}C NMR (DMSO-d_6) δ(PROXYL moiety): 25.1, 25.3 (each $CH_3 \times 2$), 66.9, 69.3 (C2, 5), 139.9 (C4), 140.0 (C3). (Indolenine moiety): 27.3 ($CH_3 \times 4$), 31.3 (*N*-$CH_3 \times 2$), 49.0 (C3 × 2), 103.2, 125.0, 153.1 (each

polymethine-C × 2, ×1, ×2), 163.0 (C=O × 2), 172.3 (C2 × 2), 111.1, 114.4, 120.0, 136.5, 138.6, 141.4 (each 2C, indolenylbenzene × 2). FAB-MS (*m*/*z*) 745 $(M)^+$.

*4.2.2.6. 5-(1-Acetoxy-2,2,5,5-tetramethylpyrrolidine -3-yl)carbonylamino-2-{5-[5-(1-acetoxy-2,2,5, 5-tetramethylpyrrolidine-3-yl)carbonylamino-1,3,3-trimethyl-2, 3-dihydro-1H-indol-2-ylidene]pent-1,3-dienyl}-1,3,3,-trimethyl-3H-indolium iodide (**13**).* Mp = 243–246 °C. IR *ν* 2972, 2931, 1751, 1670, 1618, 1508, 1470, 1363, 1331 cm^{-1}. ^1H NMR (DMSO-d_6) *δ* (pyrroline moiety): 1.28 (s, 12H, CH_3 × 4), 1.31, 1.42 (each s, 6H, CH_3 × 4), 6.58 (s, 2H, –CH= × 2). (Cy5 moiety): 1.66 (s, 12H, CH_3 × 4), 2.13 (s, 6H, $COCH_3$ × 2), 3.60 (s, 6H, CH_3 × 2), 6.22 (d, 2H, *J* 13.9 Hz, –CH= × 2), 6.49 (t, 1H, *J* 12.0, 12.4 Hz, –CH=), 7.36 (d, 2H, *J* 8.5 Hz, ArH × 2), 7.60 (dd, 2H, *J* 2.0, 8.7 Hz, ArH × 2), 7.96 (d, 2H, *J* 2.0 Hz, ArH × 2), 8.25 (t, 2H, *J* 12.9, 13.2 Hz, –CH= × 2), 10.03 (s, 2H, NH × 2). ^{13}C NMR (DMSO-d_6) *δ* (pyrroline moiety): 19.1 ($COCH_3$), 22.6, 23.0, 27.6, 28.4 (CH_3 × 8), 68.5, 71.0 (C2,2′,5,5′), 138.9 (C3,3′), 170.9 ($COCH_3$). (Cy5 moiety): 27.6 (CH_3 × 4), 31.3 (*N*-CH_3), 49.0 (C3), 103.3 (polymethine-C × 2), 125.0 (polymethine-C), 153.1 (polymethine-C × 2), 162.4 (C=O × 2), 172.4 (C2 × 2), 111.2, 114.6, 120.2, 136.3, 138.7, 141.4 (each 2C, indolenylbenzene × 2). FAB-MS (*m*/*z*) 831 $(M)^+$.

*4.2.2.7. 5-Carboxy-2-[5-(5-carboxy-1,3,3-trimethyl-2, 3-dihydro-1H-indol-2-ylidene)pent-1,3-dienyl]-1,3,3,-trimethyl -3H-indolium iodide (**19**).* Mp = >300 °C. IR *ν* 3409, 2978, 1676, 1508, 1473, 1362, 1333, 1163 cm^{-1}. ^1H NMR (DMSO-d_6) *δ* 1.68 (s, 12H, CH_3 × 4), 3.62 (s, 6H, CH_3 × 2), 6.34 (d, 2H, *J* 13.9 Hz, –CH= × 2), 6.60 (t, 1H, *J* 12.2, 12.4 Hz, –CH=), 7.45 (d, 2H, *J* 8.5 Hz, ArH × 2), 7.97 (dd, 2H, *J* 1.5, 8.4 Hz, ArH × 2), 8.13 (d, 2H, *J* 1.5 Hz, ArH × 2), 8.38 (t, 2H, *J* 12.9, 13.2 Hz, –CH= × 2). ^{13}C NMR (DMSO-d_6) *δ* 27.1 (CH_3 × 4), 31.6 (*N*-CH_3 × 2), 48.9 (C3 × 2), 104.8 (polymethine-C × 2), 126.9 (polymethine-C × 2), 155.4 (polymethine-C), 167.3 (C=O × 2), 174.4 (C2 × 2), 111.1, 123.6, 127.2, 130.7, 141.4, 146.6 (each 2C, indolenylbenzene × 2). FAB-MS (*m*/*z*) 471 $(M)^+$.

*4.2.2.8. 5-Anilidyl-2-[5-(5-anilidyl-1,3,3-trimethyl-2,3-dihydro-1H-indol-2-ylidene)pent-1,3-dienyl]-1,3,3,-trimethyl-3H-indol ium iodide (**20**).* Mp = 154–158 °C. IR *ν* 3047, 2929, 1728, 1647, 1564, 1468, 1356, 1211, 1163 cm^{-1}. ^1H NMR (DMSO-d_6) *δ* 1.75 (s, 12H, CH_3 × 4), 3.65 (s, 6H, CH_3 × 2), 6.45 (d, 2H, *J* 13.9 Hz, –CH= × 2), 6.63 (t, 1H, *J* 12.4, 12.7 Hz, –CH=), 7.11 (m, 2H, aniline–ArH × 2), 7.36 (m, 4H, aniline–ArH × 4), 7.51 (d, 2H, *J* 8.3 Hz, indolenine–ArH × 2), 7.77 (d, 4H, *J* 7.6 Hz, aniline–ArH × 4), 8.06 (dd, 2H, *J* 1.5, 8.4 Hz, indolenine–ArH × 2), 8.20 (d, 2H, *J* 1.5 Hz, indolenine–ArH × 2), 8.38 (t, 2H, *J* 12.9 Hz, –CH– × 2), 10.19 (s, 2H, NH × 2). ^{13}C NMR (DMSO-d_6) *δ* 27.2 (CH_3 × 4), 31.6 (*N*-CH_3 × 2), 48.9 (C3 × 2), 104.6 (polymethine-C × 2), 126.6 (polymethine-C × 1), 156.8 (polymethine-C × 2), 164.9 (C=O × 2), 174.2 (C2 × 2), 110.8, 122.1, 123.9, 131.0, 141.2, 145.6 (each 2C, indolenylbenzene × 2), 107.1, 120.7, 128.8, 139.3 (aniline × 2). FAB-MS (*m*/*z*) 621 $(M)^+$.

*4.2.2.9. 5-(2,2,5,5-Tetramethylpyrrolidine-1-oxyl -3-yl)aminocarbonyl-2-{5-[5-(2,2,5,5-tetramethylpyrrolidine -1-oxyl-3-yl)aminocarbonyl-1,3,3-trimethyl-2,3-dihydro-1H-indol-2-ylidene]pent-1,3-dienyl}-1,3,3,-trimethyl-3H-indolium iodide (**21**).* Mp = 273–276 °C. IR *ν* 3400, 2972, 2931, 1647, 1604, 1506, 1462, 1356, 1333, 1165 cm^{-1}. ^1H NMR (DMSO-d_6) *δ*(PROXYL moiety): 0.93, 1.07 (each s, 6H, CH_3 × 4), 1.13 (s, 12H, CH_3 × 4), 1.80, 1.89 (each m, 2H, CH_2 × 2), 4.34 (m, 2H, >CH– × 2). (Cy5 moiety): 1.70 (s, 12H, CH_3 × 4), 3.62 (s, 6H, CH_3 × 2), 6.31 (d, 2H, *J* 13.7 Hz, –CH= × 2), 6.57 (t, 1H, *J* 12.2 Hz, –CH=), 7.44 (d, 2H, *J* 8.3 Hz, ArH × 2), 7.96 (d, 2H, *J* 8.3 Hz, ArH × 2), 8.14 (s, 2H, ArH × 2), 8.18 (s, 2H, NH × 2), 8.37 (t, 2H, *J* 12.9 Hz, –CH= × 2). ^{13}C NMR (DMSO-d_6) *δ*(PROXYL moiety): 21.0, 26.0, 26.6, 27.5 (each 2C, CH_3 × 8), 40.6 (C4,4′), 53.5 (C3,3′), 60.4, 65.4 (each 2C, C2,2′,5,5′). (Cy5 moiety): 27.2 (CH_3 × 4), 31.5 (*N*-CH_3 × 2), 48.9 (C3 × 2), 104.4 (C × 2, polymethine-C), 126.4 (C × 2, polymethine-C), 154.8 (polymethine-C), 165.8 (C=O × 2), 174.0 (C2 × 2), 110.7, 121.7, 128.5, 130.7, 141.0, 145.2 (each 2C, indolenylbenzene). FAB-MS (*m*/*z*) 749 $(M)^+$.

*4.2.2.10. 5-(1-Acetoxy-2,2,5,5-tetramethylpyrrolidine -3-yl)aminocarbonyl-2-{5-[5-(1-acetoxy-2,2,5, 5-tetramethylpyrrolidine-3-yl)aminocarbonyl-1,3,3-trimethyl-2, 3-dihydro-1H-indol-2-ylidene]pent-1,3-dienyl}-1,3,3,-trimethyl-3H-indolium iodide (**22**).* Mp = 227–229 °C. IR *ν* 3371, 2970, 2931, 1757, 1647, 1506, 1462, 1356, 1333, 1165 cm^{-1}. ^1H NMR (DMSO-d_6) *δ*(PROXYL moiety): 1.00, 1.16, 1.18, 1.23 (s, each 6H, CH_3 × 8), 1.99 (m, 4H, CH_2 × 2), 4.49 (br. s, 2H, >CH– × 2). (Cy5 moiety): 1.72 (s, 12H, CH_3 × 4), 2.08 (s, 6H, $COCH_3$ × 2), 3.64 (s, 6H, CH_3 × 2), 6.35 (d, 2H, *J* 13.4 Hz, –CH= × 2), 6.61 (t, 1H, *J* 12.0 Hz, –CH=), 7.47 (d, 2H, *J* 8.5 Hz, ArH × 2), 7.84 (dd, 2H, *J* 1.5, 8.4 Hz, ArH × 2), 8.13 (s, 2H, ArH × 2), 8.24 (br. s, 2H, NH × 2), 8.40 (t, 2H, *J* 12.7, 13.4 Hz, –CH= × 2). ^{13}C NMR (DMSO-d_6) *δ*(PROXYL moiety): 19.1 ($COCH_3$), 24.9, 25.0, 26.1 (CH_3 × 8), 40.3 (C4,4′), 54.1 (C3,3′), 62.0, 66.2 (C2,2′,5,5′), 170.8 ($COCH_3$ × 2). (Cy5 moiety): 27.2 (CH_3 × 4), 31.5 (*N*-CH_3 × 2), 48.9 (C3 × 2), 104.4 (polymethine-C × 2), 126.4 (polymethine-C), 154.9 (polymethine-C × 2), 166.0 (C=O × 2), 174.0 (C2 × 2), 110.7, 121.7, 128.5, 130.5, 141.1, 145.3 (each 2C, indolenylbenzene). FAB-MS (*m*/*z*) 835 $(M)^+$.

*4.2.2.11. 2-[3-(1,3,3-Trimethyl-2,3-dihydro-1H-indol-2-ylidene)propenyl]-1,3,3-trimethyl-3H-indolium iodide (**14**).* Mp = 241–243 °C. IR *ν* 1556, 1496, 1452, 1396, 1207, 1105 cm^{-1}. ^1H NMR (DMSO-d_6) *δ*1.69 (s, 12H, CH_3 × 4), 3.65 (s, 6H, CH_3 × 2), 6.47 (d, 2H, *J* 13.7 Hz, –CH= × 2), 7.30 (m, 2H, ArH × 2), 7.45 (m, 4H, ArH × 4), 7.64 (d, 2H, *J* 7.57 Hz, ArH × 2), 8.34 (t, 1H, *J* 13.4, 13.7 Hz, –CH=). ^{13}C NMR ($CDCl_3$) *δ* 28.0 (CH_3 × 4), 32.6 (*N*-CH_3 × 2), 48.8 (C3 × 2), 104.8, 150.6 (each polymethine-C × 1, ×2), 174.2 (C2 × 2), 110.7, 122.0, 125.3, 128.8, 140.3, 142.5 (each 2C, indolenylbenzene). FAB-MS (*m*/*z*) 357 $(M)^+$.

*4.2.2.12. 5-Nitro-2-[3-(5-nitro-1,3,3-trimethyl-2,3-dihydro -1H-indol-2-ylidene)propenyl]-1,3,3-trimethyl-3H-indolium iodide (**15**).* Mp = >300 °C. IR *ν* 1556, 1448, 1388, 1329, 1200, 1097 cm^{-1}. ^1H NMR (DMSO-d_6) *δ* 1.77 (s, 12H, CH_3 × 4), 3.74 (s, 6H, CH_3 × 2), 6.73 (d, 2H, *J* 13.4 Hz, –CH= × 2), 7.71 (d, 2H, *J* 8.8 Hz, ArH × 2), 8.40 (dd, 2H, *J* 2.2, 8.8 Hz, ArH × 2), 8.44 (t, 1H, *J* 13.4 Hz, –CH=), 8.62 (d, 2H, *J* 2.2 Hz, ArH × 2). ^{13}C NMR (DMSO-d_6) *δ* 27.2 (CH_3 × 4), 32.4 (*N*-CH_3 × 2), 49.3 (C3 × 2), 106.0, 151.9 (each polymethine-C × 2, ×1), 176.8 (C2 × 2), 112.4, 118.6, 125.7, 142.0, 144.9, 148.1 (each 2C, indolenylbenzene). FAB-MS (*m*/*z*) 447 $(M)^+$.

*4.2.2.13. 5-Amino-2-[3-(5-amino-1,3,3-trimethyl-2,3-dihydro -1H-indol-2-ylidene)propenyl]-1,3,3-trimethyl-3H-indolium iodide (**16**).* Mp = 243–246 °C. IR *ν* 3321, 3226, 1616, 1560, 1471, 1390, 1356, 1211, 1167, 1107 cm^{-1}. ^1H NMR (DMSO-d_6) *δ* 1.60 (s, 12H, CH_3 × 4), 3.51 (s, 6H, CH_3 × 2), 5.32 (br. s, 4H, NH_2 × 2), 6.16 (d, 2H, *J* 13.7 Hz, –CH= × 2), 6.58 (dd, 2H, *J* 2.2, 8.5 Hz, ArH × 2), 6.73 (d, 2H, *J* 2.0 Hz, ArH × 2), 7.08 (d, 2H, *J* 8.3 Hz, ArH × 2), 8.09 (t, 1H, *J* 13.5 Hz, –CH=). ^{13}C NMR (DMSO-d_6) *δ* 27.8 (CH_3 × 4), 31.2 (*N*-CH_3 × 2), 48.7 (C3 × 2), 101.1, 145.6 (each polymethine-C × 2, ×1), 170.8 (C2 × 2), 108.1, 112.0, 113.1, 132.8, 142.1, 147.5 (each 2C, indolenylbenzene × 2). FAB-MS (*m*/*z*) 387 $(M)^+$.

*4.2.2.14. 5-Acetoamino-2-[3-(5-acetoamino-1,3,3-trimethyl-2, 3-dihydro-1H-indol-2-ylidene)propenyl]-1,3,3,-trimethyl-3H-indolium iodide (**17**).* Mp = >300 °C. IR *ν* (KBr) 3251, 3209, 3080, 1676, 1624, 1560, 1466, 1394, 1201, 1115 cm^{-1}. ^1H NMR (DMSO-d_6)

δ 1.64 (s, 12H, $CH_3 \times 4$), 2.05 (s, 6H, $COCH_3 \times 2$), 3.60 (s, 6H, $CH_3 \times 2$), 6.37 (d, 2H, *J* 13.7 Hz, –CH= × 2), 7.37 (d, 2H, *J* 8.5 Hz, ArH × 2), 7.54 (dd, 2H, *J* 1.7, 8.5 Hz, ArH × 2), 7.83 (d, 2H, *J* 2.0 Hz, ArH × 2), 8.24 (t, 1H, *J* 13.4, 13.7 Hz, –CH=), 10.16 (s, 2H, NH × 2). ^{13}C NMR (DMSO-d_6) δ 24.2 ($COCH_3$), 27.6 ($CH_3 \times 4$), 31.5 (*N*-$CH_3 \times 2$), 49.0 (C3 × 2), 102.6 (polymethine-C × 2), 148.4 (polymethine-C), 168.5 (C=O × 2), 173.4 (C2 × 2), 111.7, 113.6, 119.4, 137.2, 138.2, 141.1 (each 2C, indolenylbenzene × 2). FAB-MS (*m*/*z*) 471 $(M)^+$.

*4.2.2.15. 5-(2,2,5,5-Tetramethylpyrroline-3-yl)carbonylamino -2-{3-[5-(2,2,5,5-tetramethylpyrroline-3-yl)carbonylamino-1,3, 3-trimethyl-2,3-dihydro-1H-indol-2-ylidene]propenyl}-1,3,3, -trimethyl-3H-indolium iodide (**18**).* Mp = >300 °C. IR ν 3298, 2973, 1672, 1624, 1560, 1454, 1390, 1205, 1107 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ(PROXYL moiety): 1.20, 1.29 (each s, 12H, $CH_3 \times 8$), 6.61 (s, 2H, –CH= × 2). (Cy3 moiety): 1.65 (s, 12H, $CH_3 \times 4$), 3.60 (s, 6H, $CH_3 \times 2$), 6.36 (d, 2H, *J* 13.7 Hz, –CH= × 2), 7.38 (d, 2H, *J* 8.5 Hz, ArH × 2), 7.68 (dd, 2H, *J* 1.7, 8.7 Hz, ArH × 2), 7.93 (d, 2H, *J* 1.7 Hz, ArH × 2), 8.24 (t, 1H, *J* 13.2, 13.7 Hz, –CH=), 9.99 (s, 2H, NH × 2). ^{13}C NMR (DMSO-d_6) δ(PROXYL moiety): 25.2, 25.4 (each $CH_3 \times 2$), 67.0, 69.4 (C2, 5), 139.8 (C4), 140.3 (C3). (Cy3 moiety): 27.6 ($CH_3 \times 4$), 31.6 (*N*-$CH_3 \times 2$), 49.1 (C3 × 2), 102.8 (polymethine-C × 2), 148.4 (polymethine-C), 163.2 (C=O), 173.5 (C2 × 2), 111.6, 114.6, 120.4, 137.0, 138.5, 141.0 (each 2C, indolenylbenzene × 2). FAB-MS (*m*/*z*) 719 $(M)^+$.

*4.2.2.16. 5-Carboxy-2-[3-(5-carboxy-1,3,3-trimethyl-2,3-dihydro -1H-indol -2-ylidene)propenyl]-1,3,3,-trimethyl-3H-indolium iodide (**23**).* Mp = >300 °C. IR ν (KBr) 3429, 2970, 2929, 1720, 1556, 1469, 1392, 1193, 1105 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ 1.71 (s, 12H, $CH_3 \times 4$), 3.67 (s, 6H, $CH_3 \times 2$), 6.57 (d, 2H, *J* 13.4 Hz, –CH= × 2), 7.55 (d, 2H, *J* 8.5 Hz, ArH × 2), 8.03 (dd, 2H, *J* 1.5, 8.3 Hz, ArH × 2), 8.15 (d, 2H, *J* 1.5 Hz, ArH × 2), 8.38 (t, 1H, *J* 13.4 Hz, –CH=). ^{13}C NMR (DMSO-d_6) δ27.4 ($CH_3 \times 4$), 32.0 (*N*-$CH_3 \times 2$), 49.0 (C3 × 2), 104.5, 150.9 (each polymethine-C × 2, ×1), 111.7, 123.5, 127.8, 130.8, 141.0, 146.4 (each 2C, indolenylbenzene × 2) FAB-MS (*m*/*z*) 445 $(M)^+$.

*4.2.2.17. 5-(2,2,5,5-Tetramethylpyrrolidine-1-oxyl-3 -yl)aminocarbonyl-2-{3-[5-(2,2,5,5-tetramethylpyrrolidine-1 -oxyl-3-yl)aminocarbonyl-1,3,3-trimethyl-2,3-dihydro-1H-indol- 2-ylidene]propenyl}-1,3,3,-trimethyl-3H-indolium iodide (**24**).* Mp = >300 °C. IR ν 3400, 2974, 2933, 1651, 1562, 1458, 1388, 1211, 1112 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ(PROXYL moiety): 0.96, 1.08, 1.14, 1.15 (each s, 6H, $CH_3 \times 8$), 1.83, 1.92 (each m, 2H, $CH_2 \times 2$), 4.37 (m, 2H, >CH– × 2). (Cy3 moiety): 1.707, 1.710 (each s, 6H, $CH_3 \times 4$), 3.67 (s, 6H, $CH_3 \times 2$), 6.52 (d, 2H, *J* 13.4 Hz, –CH= × 2), 7.25 (br. s, 2H, OH × 2), 7.53 (d, 2H, *J* 8.3 Hz, ArH × 2), 8.00 (dd, 2H, *J* 1.5, 8.3 Hz, ArH × 2), 8.16 (d, 2H, *J* 1.5 Hz, ArH × 2), 8.33 (s, 2H, NH × 2), 8.35 (t, 1H, *J* 13.4 Hz, –CH=). ^{13}C NMR (DMSO-d_6) δ(PROXYL moiety): 20.7, 25.7, 26.3, 27.1 ($CH_3 \times 4$), 40.3 (C4), 53.2 (C3), 60.0 and 65.0 (C2, 5). (Cy3 moiety): 27.3 ($CH_3 \times 4$), 31.6 (*N*-$CH_3 \times 2$), 48.7 (C3 × 2), 103.7, 155.8 (each polymethine-C × 2, ×1), 165.6 (C=O × 2), 175.1 (C2 × 2), 110.9, 121.6, 128.3, 131.1, 140.3, 144.7 (each 2C, indolenylbenzene × 2). FAB-MS (*m*/*z*) 723 $(M)^+$.

*4.2.2.18. 5-(1-Acetoxy-2,2,5,5-tetramethylpyrrolidine -3-yl)aminocarbonyl-2-{3-[5-(1-acetoxy-2,2,5,5 -tetramethylpyrrolidine-3-yl)aminocarbonyl-1,3,3-trimethyl-2, 3-dihydro-1H-indol-2-ylidene]propenyl}-1,3,3,-trimethyl-3H- indolium iodide (**25**).* Mp = >300 °C. IR ν 3435, 2973, 2933, 1749, 1651, 1560, 1456, 1392, 1207 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ(PROXYL moiety): 1.00, 1.15 (each s, 6H, $CH_3 \times 4$), 1.17 (s, 12H, $CH_3 \times 4$), 1.95 (m, 4H, $CH_2 \times 2$), 4.48 (m, 2H, >CH– × 2). (Cy3 moiety): 1.72 (s, 12H, $CH_3 \times 4$), 2.07 (s, 6H, $COCH_3 \times 2$), 3.67 (s, 6H, $CH_3 \times 2$), 6.52 (d, 2H, *J* 13.4 Hz, –CH= × 2), 7.54 (d, 2H, *J* 8.5 Hz, ArH × 2), 7.99 (dd, 2H, *J* 1.0, 8.3 Hz, ArH × 2), 8.11 (s, 2H, ArH × 2), 8.29 (br. s, 2H, NH × 2), 8.36 (t, 1H, *J* 13.4, 13.7 Hz, –CH=). ^{13}C NMR (DMSO-d_6) δ(PROXYL moiety): 19.1, 170.8 (OAc × 2), 26.1 (br, $CH_3 \times 4$), 40.7 (br, C4 × 2), 53.9 (br, C3 × 2), 61.9, 66.3 (C2, 5 × 2). (Cy3 moiety): 27.4 ($CH_3 \times 4$), 31.9 (*N*-$CH_3 \times 2$), 49.0 (C3 × 2), 104.1, 150.4 (each polymethine-C × 2, ×1), 111.2, 121.9, 128.6, 131.2, 140.7, 145.2 (each 2C, indolenylbenzene × 2), 166.0 (C=O × 2), 175.5 (C2 × 2). FAB-MS (*m*/*z*) 809 $(M)^+$.

4.3. Preparation of stock solutions

Stock solutions (0.001 M) of Cy5 and Cy3 derivatives **8**–**25** were prepared in 10 mL of CH_3CN and were kept at 4 °C in a refrigerator. Buffer solutions in the pH range of 5.6–8.0 were prepared using 0.1 M PBS, while buffer solutions with pH values ranging from 1.09 to 5.5 were prepared using 1 M AcONa and 1 M HCl. Each pH solution was prepared to the concentration of 20 μM by each buffer solution.

4.4. Fluorescence measurement

The above stock solutions were diluted with acetonitrile to 0.2 μM and subjected to the measurement.

Fluorescence measurement of the hybrid compounds after reduction with Na ascorbate was as follows. A 20-μM PBS solution of the hybrid compound and a 15-mM PBS solution of Na ascorbate were mixed in a ratio of 1:1 (v/v). And after the recognition of the reduction of the radicals by ESR measurement, this sample solution was diluted with acetonitrile to 0.2 μM and then was subjected to the measurement of the fluorescence intensity.

4.5. ESR measurement

Sample solutions were prepared with 0.1 M PBS (pH 7.0) to 20 μM and were subjected to the measurement of the ESR. This 20-μM PBS sample solution and a 15-mM PBS of Na ascorbate were mixed in a ratio of 1:1 (v/v) for reduction of the nitroxide radicals and the resulting mixed solution was subjected to the measurement of the ESR.

Acknowledgements

The authors wish to thank Professors Tateaki Ogata for the use of a JEOL JES-FR30 ESR spectrometer, and Mr. Hiroya Masukawa and Mr. Yuki Takaoka for their technical assistance.

References

[1] (a) J.-L. Chen, S.-J. Zhuo, Y.-Q. Wu, F. Li, L. Freug, C.-Q. Zhu, Spectrochim. Acta Part A 63 (2006) 438–443;
(b) E. Lozinsky, V.V. Martin, T.A. Berezina, A.I. Shames, A.L. Weis, G.I. Liktenshtein, J. Biochem. Biophys. Methods 38 (1999) 29–42;
(c) X.-F. Yang, X.-Q. Guo, Analyst 126 (2001) 1800–1804;
(d) X.-F. Yang, X.-Q. Guo, Anal. Chim. Acta 434 (2001) 169–177;
(e) S. Pou, Y.-I. Huang, A. Bhan, V.S. Bhadti, R.S. Hosmane, S.Y. Wu, G.-L. Cao, G.M. Rosen, Anal. Biochem. 212 (1993) 85–90;
(f) N. Medvedeva, V.V. Martin, A.L. Weis, G.I. Likhttenshten, J. Photochem. Photobiol. A 163 (2004) 45–51;
(g) T. Kobayashi, Y. Urano, M. Kamiya, T. Ueno, H. Kojima, T. Nagano, J. Am. Chem. Soc. 129 (2007) 6696–6697;
(h) N.V. Blough, D.J. Simpson, J. Am. Chem. Soc. 110 (1988) 1915–1917;
(i) S.A. Green, D.J. Simpson, G. Zhou, P.S. Ho, N.V. Blough, J. Am. Chem. Soc. 112 (1990) 7337–7346;
(j) J.L. Gerlock, P.J. Zacmanidis, D.R. Bauer, D.J. Simpson, N.V. Blough, I.T. Almeen, Free Radic. Res. Commun. 10 (1990) 119–121;
(k) H. Wang, D. Zhang, X. Guo, L. Zhu, Z. Shuai, D. Zhu, Chem. Commun. (2004) 670–671;
(l) T. Kalai, J. Jeko, K. Hideg, Tetrahedron Lett. 45 (2004) 8395–8398;
(m) N. Soh, Y. Katayama, M. Maeda, Analyst 126 (2001) 564–566;
(n) J.A. Green II, L.A. Singer, J. Chem. Phys. 58 (1973) 2690–2695;

(o) Y. Katayama, N. Soh, M. Maeda, Chem. Physchem. 2 (2001) 655–661;
(p) J.A. Green II, L.A. Singer, J. Am. Chem. Soc. 96 (1974) 2730–2732;
(q) T. Hirano, K. Kikuchi, T. Nagano, Rev. Fluoresc. (2004) 55–73;
(r) K. Kikuchi, H. Takakusa, T. Nagano, Trends Anal. Chem. 23 (2004) 407–415;
(s) G.I. Likhtenstein, K. Ishii, S. Nakatsuji, Photochem. Photobiol. 83 (2007) 871–881 (review).
[2] (a) L.A. Ernst, R.K. Gupta, R.B. Mujumbar, A.S. Waggoner, Cytometry 10 (1989) 3–10;
(b) J.J. Aubin, Histchem. Cytochem. 27 (1979) 367–443;
(c) R. DeBiaso, G. Bright, L.A. Ernst, D.L. Taylor, A.S. Waggoner, J. Cell Biol. 105 (1987) 1613–1622;
(d) R.F. Murphy, M. Roederer, Flow cytometric analysis of endocyclic pathways, in: D.L. Taylor, A.S. Waggoner, R.F. Murphy, F. Lanni, R.R. Birge (Eds.), Applications of Fluorescence in the Biomedical Sciences, Alan R. Liss, Inc., New York, 1986, pp. 545–566;
(e) S.R. Mujumber, R.B. Mujumbar, C.M. Grant, A.S. Waggoner, Bioconjug. Chem. 7 (1996) 356–362;
(f) R.B. Mujumbar, L.A. Ernst, S.R. Mujumber, C.J. Lewis, A.S. Waggoner, Bioconjug. Chem. 4 (1993) 105–111;
(g) O.V. Kharitonova, Z.M. Alieva, S.M. Makin, J. Org. Chem. USSR (Engl. Transl.) 10 (1992) 1732–1736;
(h) A. Toutchkine, P. Nalbant, K. Hahn, Bioconjug. Chem. 13 (2002) 387–391.
[3] S. Sato, M. Suzuki, T. Soma, M. Tsunoda, Spectrochim. Acta Part A 70 (2008) 799–804.
[4] A.A. Bobko, I.A. Kirilyuk, I.A. Grigor'ev, J.L. Zweier, V.V. Khramtsov, Free Radic. Biol. Med. 42 (2007) 404–412.
[5] (a) E.G. Rozantsev, in: H. Ulrich (Ed.), Free Nitroxyl Radicals, Plenum Press, New York, 1970;
(b) E.J. Rauckman, G.M. Rosen, M.B. Abou-Donia, J. Org. Chem. 41 (1976) 564–565;
(c) S. Sato, T. Kumazawa, S. Matsuba, J.-I. Onodera, M. Aoyama, H. Obara, H. Kamada, Carbohydr. Res. 334 (2001) 215–222.