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Synthesis and properties of umbelliferone-nitroxide radical hybrid compounds as fluorescence and spin-label probes

Shingo Sato*, Minoru Suzuki, Tomoki Soma, Minoru Tsunoda

Department of Chemistry and Chemical Engineering, Faculty of Engineering, Yamagata University, 4-3-16 Jonan, Yonezawa-shi, Yamagata 992-8510, Japan

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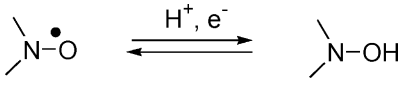
Abstract

Two hybrid compounds **6** and **7**, linked via an ester-bond between the 7-hydroxyl residue of an umbelliferone **1** and a carboxylic acid residue of two nitroxide radicals **3** and **4**, and one hybrid compound **8**, linked via an ester-bond between a 3-carboxylic acid residue of umbelliferone **2** and a hydroxyl residue of nitroxide radical **5**, were synthesized in good yields, and their fluorescence and ESR spectra before and after the addition of L-ascorbic acid sodium salt in PBS (pH 7.0) were measured. The ESR intensities of **6** and **7** were proportionally reduced after the addition of ascorbic acid sodium salt, and their fluorescence intensities were increased maximally by eight- and nine-fold, respectively. However, the fluorescence intensity of **8** was essentially unchanged.

Keywords: Umbelliferone; Nitroxide radical; Hybrid compound; L-Ascorbic acid; ESR; Fluorescence

1. Introduction

Fluorophore-nitroxide radical hybrid compounds have been developed mainly for the detection of radicals or to probe the redox reactions in biological and chemical systems, which were applied to the detection of hydroxyl [1] and nitric oxide [2] radicals, Fe^{2+} [3], and ascorbic acid [4]. In both the radical trapping and fluorescence quenching properties of the nitroxide radical could be turned to advantage in a technique for monitoring radicals and reducing species. The fluorescence from such molecules was almost fully quenched, presumably through an electron exchange mechanism, which both decreased the fluorescence quantum yield and shortened the fluorescence lifetime [5]. All of these fluorophore-nitroxide radical hybrid compounds were linked via an ester-bond between a naphthalene [5–9], pyrene [3] or anthracene carboxylic acid [1,10], and a 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (4-hydroxyTEMPO) or 3-hydroxymethyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (3-hydroxymethylPROXYL). The appearance of fluorescence after reduction of the nitroxide radical to a hydroxylamine (Eq. (1)) in an aqueous solution has been previously observed under acidic conditions [1,3,11] or in organic solvents such as methanol, acetnitrile, or hexane [5–8,12,13], or buffer solution [2,4,14]. However, representative fluorescent dyes such as, fluorescein, umbelliferone, and polymethine cyanine have not been employed as a fluorphore of these hybrid compounds. We have been interested in the decay and enhancement of fluorescence intensity through an electron exchange mechanism between fluorophore and nitroxide radical in a molecule.



(1)

We here wish to report our first observation of an increase in fluorescence intensity and a decrease in ESR intensity along with the chemical reduction of the synthesized umbelliferone-nitroxide hybrid compounds by L-ascorbic acid sodium salt in PBS (pH 7.0) [4]. The usable of PBS as a solvent is important from the view point of applying. In these hybrid compounds, although TEMPO-4-yl coumarin-3-carboxylate has been synthesized and used to study the free radical kinetics of radical polymerization [15], measurements related to the increase in fluorescence with reduction of the nitroxide radical have not been reported.

* Corresponding author. Tel.: +81 238 263121; fax: +81 238 263121.
E-mail address: shingo-s@yz.yamagata-u.ac.jp (S. Sato).

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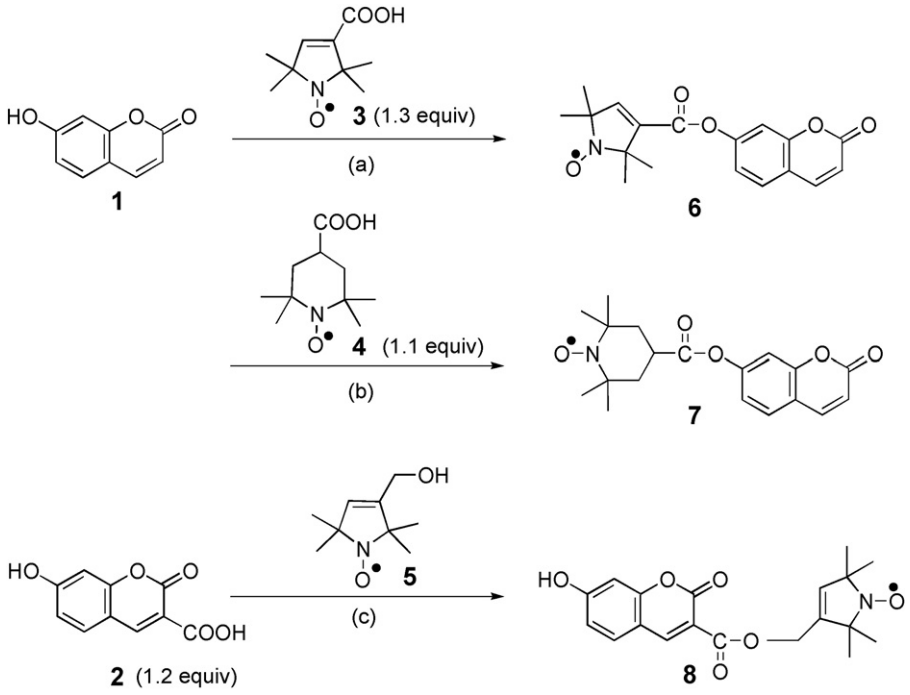

Scheme 1. Synthesis of umbelliferyl nitroxyl radicals (**6**,**7**, and **8**). Reagents and conditions: (a) DCC (1.2 equiv), DMAP (0.1 equiv) in CH_3CN, at 0 °C to r.t. for 7 h, Y: 72%; (b) DCC (1.0 equiv), DMAP (0.1 equiv) in CH_2Cl_2, at 0 °C to r.t. for 18 h, Y: 84%; (c) DCC (1.2 equiv), DMAP (0.3 equiv) in CH_3CN, at r.t. for 18 h, Y: 83%.

2. Results and discussion

We selected two umbelliferones [7-hydroxycoumarin and 7-hydroxycoumarin-3-carboxylic acid (**1** and **2**)] as a fluorophore. Umbelliferone **1** absorbs strongly at 300 ($\log \varepsilon$ 3.9), 305 (3.95), and 325 nm (4.15), respectively, and fluoresces blue ($\lambda_{em} = 455$ nm) in ultraviolet and visible light, and its fluorescence disappears in acidic media ($pK_a = 7.7$) [16]. Umbelliferone **1** is a representative fluorophore-biosensor and has enjoyed extensive use in the past. We also selected 3-carboxy-2,2,5,5-tetramethylpyrroline-1-oxyl (**3**), 4-carboxy-2,2,6,6-tetramethylpiperidine-1-oxyl (4-carboxyTEMPO, **4**), and 3-hydroxymethyl-2,2,5,5-tetramethylpyrroline-1-oxyl (**5**) as nitroxide radicals, which are stable and are used frequently as a spin-probe for monitoring radical and redox reactions in biosystems [17]. The synthesis, as shown in Scheme 1, was carried out by the condensation of a 7-hydroxyl group of **1** and each carboxylic acid of nitroxide **3** or **4**, and a 3-caboxylic acid of **2** and a hydroxyl group of nitroxide **5**, using DCC and DMAP in anhydrous CH_2Cl_2 or CH_3CN to afford hybrid compounds **6**, **7** and **8** in good yield of 72, 84 and 83%, respectively.

The UV–vis and fluorescence spectral data of **1** and **2**, and the synthesized hybrid compounds **6**, **7** and **8** are summarized in Table 1. UV–vis absorption spectra of **6** and **7** were shifted to lower wavelengths at 308 and 310 nm and their maximum absorbance both were observed at 278 and 279 nm. Fluorescence was observed at a very lower intensity at 450 (relative intensity: 3) and 443 (1) nm, compared with **1** [450 nm (53)], as expected. Since the fluorescence intensity of **2** was low (0.28), and that of its hybrid compound **8** was also low (1), it can be concluded that hybrid compound **8** cannot be applied as a fluorescence probe for the measurement of radicals and reducing species. Thus the

Table 1
UV–vis absorption and fluorescence spectra of umbelliferones (**1**, **2**) and the corresponding hybrid compounds (**6**, **7** and **8**)

Compound	Absorption in MeOH		Fluorescence ($c = 1.0$ μM) in PBS (pH 7.0)		
	λ_{max} (nm)	$\log \varepsilon$	λ_{ex} (nm)	λ_{em} (nm)	Relative intensity (−)
1	323	4.19	325	450	53
6	278	4.10	327	450	3
	308	4.01			
7	279	4.13	330	443	1
	310	3.97			
2	292	4.17	307	408	0.28
8	292	4.20	306	422	1

Table 2
ESR signal intensities and *g*-values of the nitroxide compounds (**3**, **4**, **5**, **6**, **7**, **8**; 10 μM) in 0.1 M PBS (pH 7.0)

Compound	Signal intensity	*g*
3	1.8294	2.0058
4	1.6620	2.0052
5	1.2210	2.0050
6	1.5510	2.0050
7	1.6463	2.0052
8	0.9308	2.0050

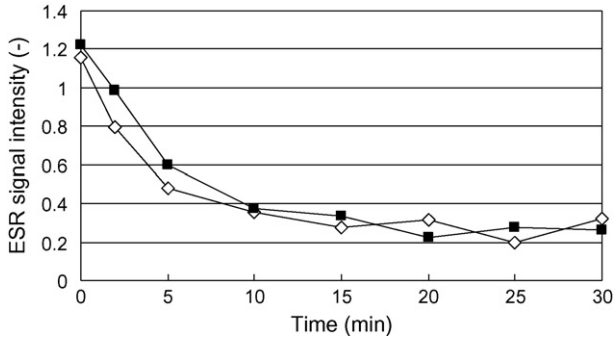

Fig. 1. Time-resolved ESR signal intensity after the addition of 1 mL of 4 mM L-ascorbic acid sodium salt in 0.1 M PBS to 1 mL of 20 μM **3** (■) or **6** (◇) in 0.1 M PBS.

fluorescence of these umbelliferone-nitroxide hybrid compounds (**6**, **7**) was scarcely observed with fluorescence quenching due to nitoxide radicals.

The ESR intensity of **6** (1.55) was observed to be slightly lower than that of **3** (1.83), but the ESR intensity of **7** (1.65) was nearly the same as that of **4** (1.66), as shown in Table 2. Furthermore, the decreasing rate of ESR intensity of **6** and **7** by the addition of L-ascorbic acid sodium salt in 0.1 M PBS (4 mM and 500 μM) was also the same as that of **3** and **4**, respectively, as shown in Figs. 1 and 2. Since the difference in the ESR intensities of **4** and **7** before and after the addition of ascorbic acid was greater than that of **3** and **6**, the rate of reduction of both ESR intensities was equal, and the concentra-

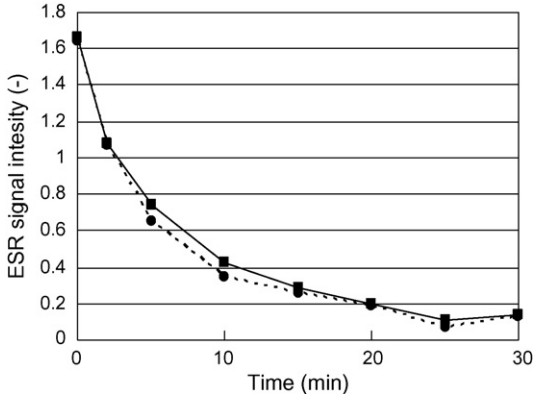

Fig. 2. Time-resolved ESR signal intensity after the addition of 1 mL of 500 μM L-ascorbic acid sodium salt in 0.1 M PBS to 20 μM **4** (■) or **7** (●) in 0.1 M PBS.

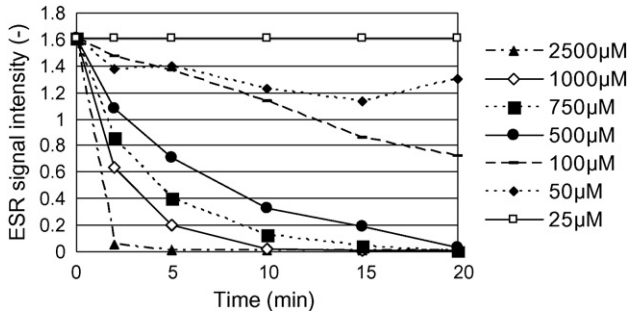

Fig. 3. Time-dependent ESR signal intensity after the addition of various concentration of L-ascorbic acid sodium salt (2500, 1000, 750, 500, 100, 50, 25 μM) in 0.1 M PBS to 20 μM **7** in 0.1 M PBS (volume ratio 1:1).

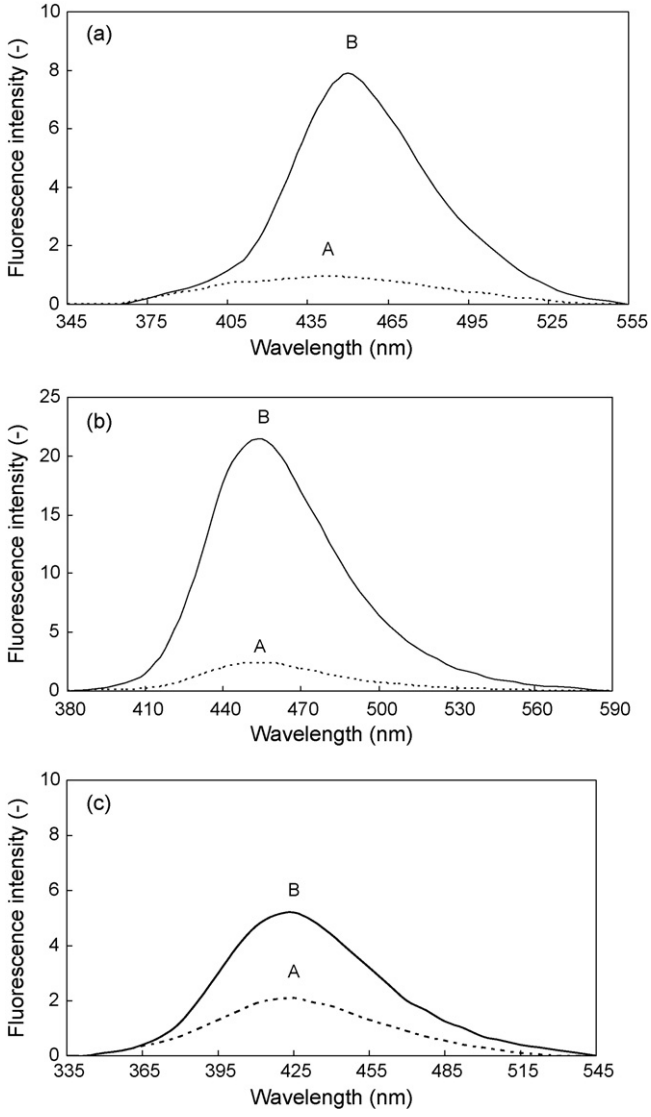

Fig. 4. (a) Fluorescence intensity of **6** after the addition of ascorbic acid sodium salt solution (A: 0 μM, B: 6 mM). (b) Fluorescence intensity of **7** after the addition of L-ascorbic acid sodium salt solution (A: 0 μM, B: 6 mM). (c) Fluorescence intensity of **8** after the addition of L-ascorbic acid sodium salt solution (A: 0 μM, B: 6 mM).

Table 3
Maximum fluorescence intensity of hybrid compounds (**6**, **7** and **8**) after the addition of 6 mM ascorbic acid sodium salt in PBS (pH 7.0) in Fig. 5a–c

Compound	**6**	**7**	**8**
Relative intensity[a]	8.25	9.08	1.66

[a] Fluorescence intensity of each hybrid compound in PBS (pH 7.0) was to be 1.

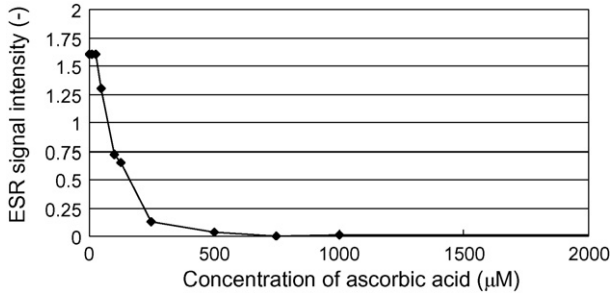

Fig. 5. ESR signal intensity of **7** (20 μM) after the addition of various concentrations of L-ascorbic acid sodium salt.

tion of added ascorbic acid to **4** and **7** (500 μM) was one-eighth over that of **3** and **6** (4 mM), it appears that **7** would be superior to **6** as a radical-spin probe. When an L-ascorbic acid sodium salt solution, at a concentration over 500 μM, was added to a 20 μM PBS of **7** in a volume ratio of 1:1, the radical almost disappeared within 20 min (see Fig. 3). Next, before and after the addition of 6 mM L-ascorbic acid sodium salt in PBS to a 20 μM solution of **6**, **7**, or **8**, the fluorescence intensity of each compound was measured (Fig. 4a–c). The difference in the fluorescence intensity after the addition of L-ascorbic acid sodium salt solution (6 mM) was 9.08-fold for **7**, while that for **6** and **8** was 8.25- and 1.66-fold, respectively (see Table 3). Furthermore, while the fluorescence intensity of **7** was increased in a concentration-dependent manner, in parallel, the ESR intensity of **7** was decreased in a concentration-dependent manner, as shown in Figs. 5 and 6.

Analogous results were also observed for **6**. In the case of Fe(II) in an aqueous solution in place of ascorbic acid in PBS as a reducing agent, the same result was also observed, though the sensitivity was inferior.

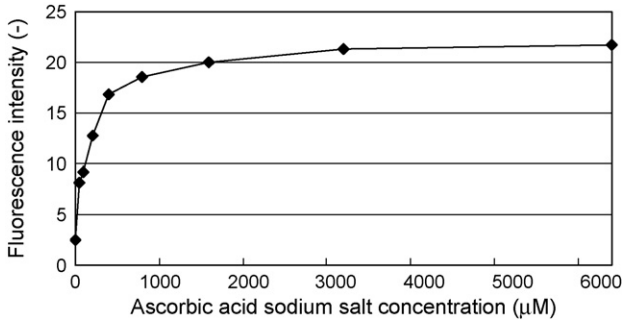

Fig. 6. Fluorescence intensity of **7** (20 μM) after the addition of various concentrations of L-ascorbic acid sodium salt solution.

3. Conclusions

As expected, fluorescence of the synthesized hybrid compounds **6**, **7** and **8** was scarcely observed. By the addition of ascorbic acid sodium salt in 0.1 M PBS (pH 7.0), the ESR intensities of **6** and **7** were smoothly reduced, and their fluorescence intensities were increased concentration dependently in parallel. In the reduction of hybrid compounds **6**, **7** and **8** by L-ascorbic acid sodium salt, the ratio of the enhancement in fluorescence intensity for **7** was 9.08-fold, greater in comparison to that of **6** (8.25) and **8** (1.66) (Table 3). The ESR sensitivity of **7** for L-ascorbic acid sodium salt was also better than that of **6** (Figs. 1 and 2). Since umbelliferone-nitroxide hybrid compounds **6** and **7** have structures that are different from other hybrid compounds developed to date which are linked between a nitroxide radical and an aromatic compound condensed with benzene-rings such as naphthalene and pyrene derivatives and their emission spectra were observed at longer wavelength (443 and 450 nm), they show the potential for application as a fluoresce spin-probe for measuring radicals such as hydroxyl radicals in biosystems, Fe(II) in solution and ascorbic acid in food products.

The fluorescence intensity of the synthetic probes (**6**, **7**) increased in proportion with the decrease in their ESR signal intensity, indicating the conversion of the paramagnetic nitroxide to a diamagnetic hydroxylamine. This linear relationship between the increase in fluorescence intensity and the decrease in ESR signal intensity indicates that the change in fluorescence intensity can serve as a sensitive means for optically detecting radicals or reducing species, such as 4-(1-naphthoyloxy)-TEMPO [8] or 4-(pyrene-1-carboxy)TEMPO [3] or 1-imino nitroxide pyrene [11].

4. Experimental

4.1. Apparatus

Fluorescence spectra and relative fluorescence intensity were measured with a Hitachi 650-10M fluorescence spectrophotometer. The excitation and emission wavelength band passes were both set at 2 nm. Absorption spectra were obtained on 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, which were then sealed at the bottom and placed 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 values were measured with a Horiba M-13 PH meter.

4.2. Synthesis

The solvents used in this study were purified by distillation. Reactions were monitored by TLC, on 0.25 mM Silica Gel F254 plates (E. Merck) using UV light, and a 7% ethanolic solution of phosphomolybdic acid with heat as coloration

agents. For separation and purification, flash column chromatography was performed on silica gel (230–400 mesh, Fuji-Silysia Co., Ltd., BW-300). Melting points were determined on a Shibayama micro-melting point apparatus and are uncorrected. IR spectra were recorded on a Horiba FT-720 IR spectrometer using a KBr disk. NMR spectra were recorded on a Varian Inova 500 spectrometer using Me_4Si as the internal standard. Mass spectral data were obtained by fast-atom bombardment (FAB) using 3-nitrobenzyl alcohol (NBA) as a matrix on a JEOL JMS-AX505HA instrument. Elemental analyses were performed on a Perkin–Elmer PE 2400 II instrument.

Nitroxide radicals (**3**, **4**, and **5**) were synthesized by usual manner [18,19].

*4.2.1. 4-(7-O-Coumarinyl)-oxycarbonyl-2,2,6,6-tetramethylpyrroline-1-oxyl (**6**)*

To a stirred solution of umbelliferone (3-hydroxycoumarin **1**: 50 mg, 0.31 mmol) and 3-carboxy-2,2,5,5-tetramethylpyrroline-1-oxyl (**3**: 68 mg, 0.40 mmol) in dry CH_3CN (8 mL) in an ice bath, DCC (76 mg, 0.37 mmol) and DMAP (3.7 mg, 0.031 mmol) were added and the mixture was stirred at room temperature for 7 h. The resulting precipitates were filtered through a celite pad. The filtrate was evaporated in vacuo and then purified by silica gel column chromatography (2:1 hexane–AcOEt) to give **6** (73 mg, 72%) as a yellow powder, which was recrystallized from MeOH.

Data for **6**: Yellow needles. Mp 161–162 °C. IR ν (KBr) 2979, 2931, 1739, 1620, 1348, 1290, 1269, 1012 cm^{-1}. ^1H NMR ($CDCl_3$ + hydrazobenzene) δ 1.31 and 1.46 (s, each 6H, $CH_3 \times 4$), 6.40 (d, 1H, *J* 9.5 Hz, H-3), 6.88 (s, 1H, H-2′), 7.15 (dd, 1H, *J* 2.0, 8.5 Hz, H-6), 7.17 (d, 1H, *J* 2.0 Hz, H-8), 7.51 (d, 1H, *J* 8.5 Hz, H-5), 7.69 (d, 1H, *J* 9.5 Hz, H-4). ^{13}C NMR ($CDCl_3$ + hydrazobenzene) δ 24.6 and 24.8 ($CH_3 \times 4$), 68.0 (C5′), 69.6 (C2′), 110.4, 116.0, 116.6, 119.8, 128.5, 129.3, 135.5 (C4′), 142.8 (C4), 153.0 (C8), 154.6 (C9), 160.3 (C2), 161.0 (carboxy). FAB-MS (*m/z*) 329 $(M+H)^+$. Anal calcd. for $C_{18}H_{18}NO_5$: C, 65.84; H, 5.53; N, 4.27. Found: C, 65.90; H, 5.28; N, 4.24.

*4.2.2. 3-(7-O-Coumarinyl)-oxycarbonyl-2,2,5,5-tetramethylpiperidine-1-oxyl (**7**)*

To a stirred solution of umbelliferone (3-hydroxycoumarin **1**: 30 mg, 0.19 mmol) and 4-carboxy-2,2,6,6-tetramethylpiperidine-1-oxyl (**3**: 41 mg, 0.21 mmol) in dry CH_2Cl_2 (4.8 mL) in an ice bath, DCC (39 mg, 0.19 mmol) and DMAP (2.3 mg, 0.019 mmol) were added and the mixture was stirred at room temperature for 18 h. The resulting precipitates were filtered through a celite pad. The filtrate was evaporated in vacuo and then purified by silica gel column chromatography (1:1 hexane–AcOEt) to give **7** (55 mg, 84%) as an orange powder, which was recrystallized from MeOH.

Data for **7**: Orange needles. Mp 167–168 °C. IR ν (KBr) 2964, 1747, 1730, 1626, 1142 cm^{-1}. ^1H NMR ($CDCl_3$ + hydrazobenzene) δ 1.20 and 1.25 (s, each 6H, $CH_3 \times 4$), 1.77 (t, 2H, *J* 12.8 Hz, CH_2), 1.97 (m, 2H, CH_2), 2.95 (m, 1H, >CH–), 4.50 (s, 1H, NOH), 6.41 (d, 1H, *J* 9.6 Hz, H-3), 7.04 (dd, 1H, *J* 2.2, 8.4 Hz, H-6), 7.10 (d, 1H, *J* 2.2 Hz, H-8), 7.50 (d, 1H, *J* 8.4 Hz, H-5), 7.70 (d, 1H, *J* 9.6 Hz, H-4). ^{13}C NMR ($CDCl_3$ + hydrazobenzene) δ 19.3 ($CH_3 \times 4$), 32.2 (C2′, 6′), 41.3 (C3′, 5′), 58.3 (C4′), 110.3, 116.0, 116.6, 119.8, 128.5, 142.8 (C4), 153.2 (C8), 154.6 (C9), 160.3 (C-2), 173.2 (carboxy). FAB-MS (*m/z*) 345 $(M+H)^+$. Anal calcd. for $C_{19}H_{22}NO_5$: C, 66.26; H, 6.44; N, 4.07. Found: C, 66.34; H, 6.37; N, 4.06.

*4.2.3. 3-(7-Hydroxycoumarinyl-4-carboxymethyl)-2,2,5,5-tetramethylpyrroline-1-oxyl (**8**)*

Compound **8** was synthesized by condensation of 7-hydroxycoumarinyl-4-acetic acid (**2**) with 3-hydroxylmethyl-2,2,5,5-tetramethylpyrroline-1-oxyl (**5**) following in the same procedure as was used for the synthesis of **6**.

Data for **8**: Yellow powder. Mp 110–112 °C. IR ν (KBr) 1749, 1712, 1610, 1566 cm^{-1}. ^1H NMR ($CDCl_3$ + hydrazobenzene) δ 1.26 and 1.31 (s, each 6H, $CH_3 \times 4$), 4.86 (s, 2H, CH_2), 6.88 (s, 1H, H-2′), 5.73 (s, 1H, olefinic H), 7.35 (m, 2H, ArH), 7.64 (m, 2H, ArH), 8.55 (s, 1H, –CH=). ^{13}C NMR ($CDCl_3$ + hydrazobenzene) δ (PROXYL moiety) 24.4 and 25.4 (each $CH_3 \times 2$), 61.2 (CH_2), 68.3 and 70.3 (C2, 5), 149.1 (C3), 132.3 (C4), (coumarin moiety) 116.8, 117.8, 125.0, 129.6, and 155.2 (benzene ring), 138.6 (C4), 156.5 (C2–C=O), 162.8 (C3–C=O). FAB-MS (*m/z*) 343 $(M+H)^+$. Anal calcd. for $C_{19}H_{20}NO_5$: C, 66.65; H, 5.89; N, 4.09. Found: C, 66.92; H, 6.12; N, 4.14.

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References

[1] X.-F. Yang, X.-Q. Guo, Analyst 126 (2001) 1800–1804.
[2] N. Soh, Y. Katayama, M. Maeda, Analyst 126 (2001) 564–566.
[3] J.-L. Chen, S.-J. Zhuo, Y.-Q. Wu, F.Li.L. Freug, C.-Q. Zhu, Spectrochim. Acta Part A 63 (2006) 438–443.
[4] 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.
[5] N.V. Blough, D.J. Simpson, J. Am. Chem. Soc. 110 (1988) 1915–1917.
[6] J.A. Green II, L.A. Singer, J. Chem. Phys. 58 (1973) 2690–2695.
[7] S.A. Green, D.J. Simpson, G. Zhou, P.S. Ho, N.V. Blough, J. Am. Chem. Soc. 112 (1990) 7337–7346.
[8] 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.
[9] X.-F. Yang, X.-Q. Guo, Anal. Chim. Acta 434 (2001) 169–177.
[10] Y. Katayama, N. Soh, M. Maeda, Chemphyschem 2 (2001) 655–661.
[11] H. Wang, D. Zhang, X. Guo, L. Zhu, Z. Shuai, D. Zhu, Chem. Commun. (2004) 670–671.
[12] J.A. Green II, L.A. Singer, J. Am. Chem. Soc. 96 (1974) 2730–2732.
[13] 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.

[14] T. Kalai, J. Jeko, K. Hideg, Tetrahedron Lett. 45 (2004) 8395–8398.
[15] O.G. Ballesteros, L. Maretti, R. Sastre, J.C. Scaiano, Macromolecules 34 (2001) 6184–6187.
[16] F.M. Dean, Naturally Occurring Oxygen Ring Compounds, Butterworths, London, 1963, pp. 176–219.
[17] M. Aoyama, H. Ohya, S. Sato, T. Ogata, ITE Lett. 5 (5) (2004) 492–495.
[18] E.G. Rozantsev, in: H. Ulrich (Ed.), Free Nitroxyl Radicals, Plenum Press, New York, 1970.
[19] S. Sato, T. Kumazawa, S. Matsuba, J.-i. Onodera, M. Aoyama, H. Obara, H. Kamada, Carbohydr. Res. 334 (2001) 215–222.