Fluorescence switching by *O*-dearylation of 7-aryloxycoumarins. Development of novel fluorescence probes to detect reactive oxygen species with high selectivity

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Coumarins exhibit fluorescence that is dependent on the nature of their substituents. We have found that the strong fluorescence of 7-hydroxycoumarin in aqueous media is completely lost in 7-aryloxycoumarins. Judging from the relation between the electron-donating character of the substituent at the 7-position and the relative quantum efficiency of fluorescence, we considered that the fluorescence properties of 7-hydroxycoumarin derivatives can be explained in terms of the intramolecular charge transfer (ICT) mechanism. On this basis, we designed and synthesized 7-(4′-hydroxyphenoxy)coumarin, 7-(2′-hydroxyphenoxy)coumarin, 7-(4′-aminophenoxy)coumarin and 7-(2′-aminophenoxy)coumarin, which we expected would be dearylated by highly reactive oxygen species. We found that these nonfluorescent aryloxycoumarins were dearylated to afford highly fluorescent 7-hydroxycoumarin upon reaction specifically with hydroxyl radical ($^{\bullet}OH$), but not superoxide (O_2^{-}), hydrogen peroxide (H_2O_2), or singlet oxygen ($^1O_2$). We suggest that these compounds may be applicable as specific fluorescence probes for hydroxyl radicals in an aqueous environment.

Introduction

Fluorescence detection methods offer high sensitivity and experimental convenience.[1–3] Among many fluorophores, coumarin has been studied for a long time.[4–7] The fluorescence properties of coumarin derivatives depend on their molecular substitution, being influenced by the degree of intramolecular charge transfer (ICT)[8] from the 6- and/or 7-substituents (electron donating) to the coumarin ring (electron accepting).[9,10] Some chemical conversions, including hydrolysis and oxidation, of the derivatives generate strongly fluorescent 7-hydroxycoumarin, and the resultant fluorescence increase has been used for several applications.[11] For example, 4-methyl-7-hydroxycoumarin phosphate monoester has been used for studies of the kinetic behavior of alkaline phosphatase.[12]

We found that 7-phenoxycoumarin (**2e**: Scheme 1) does not fluoresce at all. We thought that this represented an opportunity to develop novel fluorescence probes by using *O*-dearylation as a trigger. We have already reported that *o*- and *p*-phenoxyphenols are *O*-dearylated to yield phenol in the *ipso*-substitution mode by highly reactive oxygen species.[13,14] Therefore, we expected that nonfluorescent 7-(hydroxyphenoxy)coumarins would be *O*-dearylated in the *ipso*-substitution mode to yield 7-hydroxycoumarin upon reaction with highly reactive oxygen species, generating strong fluorescence.

Here we describe the design and synthesis of several derivatives of 7-hydroxycoumarin, **3a**, **3b**, **3c** and **3d** (Scheme 1), as potential fluorescence probes for reactive oxygen species (ROS). Their chemical properties and reactivities towards ROS are also examined.

Experimental

Materials

4-Iodophenol, 4-iodoaniline, 2-iodoaniline, isobutene, 7-hydroxycoumarin, 2′,2′,2′-trifluoroethanol and trifluoromethanesulfonic acid were purchased from Tokyo Kasei Kogyo Co. Ltd. 2-Iodophenol was purchased from Aldrich Chemical Company Inc. All of them were used without further purification. Dichloromethane, benzene, methanol, pyridine and ethyl acetate were used after distillation. Other materials were of the best grade available and were used without further purification.

Instruments

^{1}H-NMR spectra were recorded on a JEOL JNM-LA300 instrument at 300 MHz. Mass spectra (MS) were measured with JEOL JMS-DX 300 and JEOL JMS-SX 102A mass spectrometer. UV–Visible spectra were obtained on a Shimadzu UV-1600. Fluorescence spectroscopic studies were performed on a Hitachi F4500.

Synthesis

4-Iodophenol and 2-iodophenol were *tert*-butylated with isobutene to give 4-*tert*-butoxyiodobenzene and 2-*tert*-butoxyiodobenzene, respectively, according to the standard method.[15] 4-Iodoaniline was acetylated with acetic anhydride to give 4-iodoacetanilide according to the standard method. 7-Methoxycoumarin, 7-benzyloxycoumarin and 7-acetoxycoumarin were synthesized by a general method. The synthetic pathways employed to obtain the new dyes are outlined in Scheme 1.

7-(4′-*tert*-Butoxyphenoxy)coumarin (2a): typical procedure for 2a–2e unless otherwise mentioned.[16] To a solution of potassium *tert*-butoxide (121 mg, 1.10 mmol) in a mixture of benzene (8 ml) and methanol (2 ml) was added 7-hydroxycoumarin (195 mg, 1.20 mmol) at room temperature. When the solid had dissolved completely, the solvent was evaporated *in vacuo* to give 7-hydroxycoumarin potassium salt. Then, to this, cuprous chloride (120 mg, 1.21 mmol) and a solution of 4-*tert*-butoxyiodobenzene (1.31 g, 4.74 mmol) in pyridine (8 ml) were added. The resulting mixture was refluxed under argon for 9 h.

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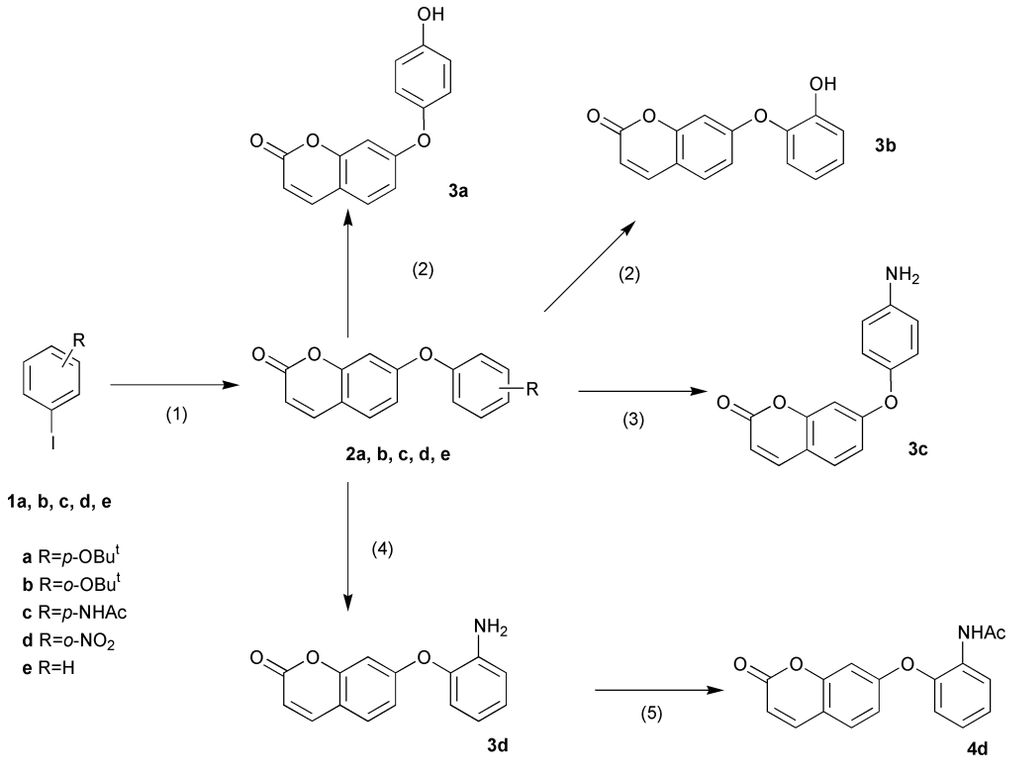


Scheme 1 Synthesis of 7-hydroxycoumarin derivatives. *Reagents and conditions*: (1) cuprous chloride, 7-hydroxycoumarin potassium salt, pyridine, reflux, under argon; (2) trifluoromethanesulfonic acid, 2′,2′,2′-trifluoroethanol, 0 °C; (3) aqueous HCl, reflux; (4) Pd/C, H_2, methanol, room temperature; (5) acetic anhydride, pyridine, room temperature.

After cooling to room temperature, the reaction mixture was acidified with aqueous HCl. The mixture was extracted with dichloromethane (3 × 75 ml). The organic layer was washed with brine, dried over anhydrous sodium sulfate and evaporated *in vacuo*. The residue was purified by silica gel column chromatography, eluting with dichloromethane to give **2a** (135 mg, 39.6%), mp 104.5–105.5 °C. ^{1}H-NMR δ_H ($CDCl_3$; Me_4Si) 1.26 (9H, s, -OBu^t), 6.28 (1H, d, J = 9.5 Hz, 3-H), 6.81 (1H, d, J = 2.4 Hz, 8-H), 6.91 (1H, dd, J = 8.4, 2.4 Hz, 6-H), 6.97–7.05 (4H, m, arom), 7.41 (1H, d, J = 8.4 Hz, 5-H), 7.66 (1H, d, J = 9.5 Hz, 4-H); MS (EI$^+$): *m*/*z* 310 (M^+).

*7-(2′-tert-Butoxyphenoxy)coumarin (**2b**)*. Yield 9.3%, mp 88.5–90.5 °C. The reaction mixture was heated at 200 °C in an autoclave for 1 day. ^{1}H-NMR δ_H ($CDCl_3$; Me_4Si) 1.20 (9H, s, -OBu^t), 6.19 (1H, d, J = 9.5 Hz, 3-H), 6.69 (1H, d, J = 2.4 Hz, 8-H), 6.74 (1H, dd, J = 8.4, 2.4 Hz, 6-H), 7.04–7.13 (4H, m, arom), 7.29 (1H, d, J = 8.4 Hz, 5-H), 7.56 (1H, d, J = 9.5 Hz, 4-H); MS (EI$^+$): *m*/*z* 310 (M^+).

*7-(4′-Acetamidophenoxy)coumarin (**2c**)*. Yield 21.2%, mp 197.0–199.0 °C. The extraction of the reaction mixture was performed with ethyl acetate (4 × 75 ml) and the eluent for the silica gel column chromatography was ethyl acetate. ^{1}H-NMR δ_H ($CDCl_3$; Me_4Si) 2.08 (3H, s, -C*H*$_3$), 6.27 (1H, d, J = 9.5 Hz, 3-H), 6.79 (1H, d, J = 2.4 Hz, 8-H), 6.92 (1H, dd, J = 8.6, 2.4 Hz, 6-H), 7.09 (2H, d, J = 9.1 Hz, 2′-H, 6′-H), 7.65 (1H, d, J = 8.6 Hz, 5-H), 7.73 (2H, d, J = 9.1 Hz, 3′-H, 5′-H), 7.93 (1H, d, J = 9.5 Hz, 4-H); MS (EI$^+$): *m*/*z* 295 (M^+).

*7-(2′-Nitrophenoxy)coumarin (**2d**)*. Yield 14.4%, mp 121.5–123.5 °C. The reaction mixture was heated under reflux for 14.5 h. After purification by silica gel column chromatography, the resulting yellow powder was recrystallized from ethanol. ^{1}H-NMR δ_H ($CDCl_3$; Me_4Si) 6.41 (1H, s, J = 9.5 Hz, 3-H), 7.00–7.05 (2H, m, 6-H and 8-H), 7.39 (1H, d, J = 8.4 Hz, 5′-H), 7.49 (1H, t, J = 8.4 Hz, 4′-H), 7.74–7.78 (2H, m, 3′-H and 5-H), 8.06 (1H, d, J = 9.5 Hz, 4-H), 8.14 (1H, d, J = 6.6 Hz, 2′-H); MS (EI$^+$): *m*/*z* 283 (M^+).

*7-Phenoxycoumarin (**2e**)*. Yield 51.5%, mp 87.5–88.5 °C. The reaction mixture was heated under reflux for 12 h. ^{1}H-NMR δ_H ($CDCl_3$; Me_4Si) 6.29 (1H, d, J = 9.5 Hz, 3-H), 6.84 (1H, d, J = 2.4 Hz, 8-H), 6.92 (1H, dd, J = 8.6, 2.4 Hz, 6-H), 7.08 (2H, d, J = 7.5 Hz, 2′-H and 6′-H), 7.23 (1H, t, J = 7.3 Hz, 4′-H), 7.39–7.44 (3H, m, 3′-H, 5′-H and 5-H), 7.65 (1H, d, J = 9.5 Hz, 4-H); HRMS (EI$^+$): calc. for $C_{15}H_{10}O_3$ (M^+) *m*/*z* 238.0630, obs. 238.0623.

7-(4′-Hydroxyphenoxy)coumarin (3a) and 7-(2′-hydroxyphenoxy)coumarin (3b).[17] To a solution of **2a** (130 mg, 0.419 mmol) or **2b** (10.3 mg, 0.0303 mmol) in 2′,2′,2′-trifluoroethanol (10 ml) was added trifluoromethanesulfonic acid (catalytic amount) and the resulting mixture was stirred at 0 °C under argon for 20 min. Dichloromethane (40 ml) was added and the reaction mixture was washed with water (2 × 30 ml) followed with brine, dried over anhydrous sodium sulfate and evaporated *in vacuo* to give **3a** (91.5 mg, 85.9%), mp 176.5–178.5 °C or **3b** (7.90 mg, quantitative amount), mp 170.5–172.0 °C, respectively. **3a**: ^{1}H-NMR δ_H (d_6-acetone) 6.12 (1H, d, J = 9.7 Hz, 3-H), 6.59 (1H, d, J = 2.4 Hz, 8-H), 6.75 (1H, dd, J = 8.6, 2.4 Hz, 6-H), 6.78–6.95 (4H, m, arom), 7.48 (1H, d, J = 8.6 Hz, 5-H), 7.78 (1H, d, J = 9.7 Hz, 4-H), 8.32 (1H, br s, -O*H*); HRMS (EI$^+$) calc. for $C_{15}H_{10}O_4$ (M^+): *m*/*z* 254.0579, obs. 254.0583, **3b**; ^{1}H-NMR δ_H ($CDCl_3$; Me_4Si) 6.32 (1H, d, J = 9.5 Hz, 3-H), 6.89–7.18 (6H, m, arom, 6-H and 8-H), 7.43 (1H, d, J = 8.4 Hz, 5-H), 7.66 (1H, d, J = 9.5 Hz, 3-H); HRMS (EI$^+$) calc. for $C_{15}H_{10}O_4$ (M^+): *m*/*z* 254.0579, obs. 254.0577.

7-(4′-Aminophenoxy)coumarin (3c).[18] Compound **2c** (67.0 mg, 0.227 mmol) was dissolved in 1.2 M aqueous HCl (20 ml). The resulting mixture was heated under reflux in an autoclave for 3 h. After cooling to room temperature, the reaction mixture was neutralized by addition of aqueous saturated sodium bicarbonate (65 ml). The resultant mixture was extracted with ethyl acetate (4 × 75 ml). The organic layer was washed with brine, dried over anhydrous sodium sulfate and evaporated *in vacuo*. The residue was purified by silica gel column chromatography, eluting with dichloromethane–ethyl acetate (75 : 25) to give **3c** (38.0 mg, 66.2%), mp 132.5–133.5 °C. ^{1}H-NMR

Table 1 Absorbance and fluorescence properties of 7-hydroxycoumarin derivatives[a]

Compound	Absorbance maximum/nm	Emission maximum/nm[b]	10^{-4} Molar absorptivity/ M^{-1} cm^{-1}	Relative quantum efficiency
3a	322	N.D.	1.4	<0.001
3b	320	N.D.	1.1	<0.001
3c	323	N.D.	1.6	<0.001
3d	321	N.D.	1.0	<0.001
2e	322	N.D.	1.5	<0.001
7-Acetoxycoumarin	312	N.D.	0.9	<0.001
7-Benzyloxycoumarin	323	391	0.9	0.41
7-Methoxycoumarin	323	388	1.5	0.42
7-Hydroxycoumarin	326	452	1.3	0.76

[a] All data were obtained in sodium phosphate buffer (0.1 M; pH 7.4). Relative quantum efficiencies were measured as described in the Experimental section. [b] N.D., not detected.

δ_H (d_6-acetone) 4.57 (2H, br s, -NH_2), 6.10 (1H, d, J = 9.5 Hz, 3-H), 6.57 (1H, d, J = 2.4 Hz, 8-H), 6.60–6.78 (5H, m, arom and 6-H), 7.47 (1H, d, J = 8.6 Hz, 5-H), 7.77 (1H, d, J = 9.5 Hz, 4-H); HRMS (EI^+) calc. for $C_{15}H_{11}NO_3$ (M^+): m/z 253.0739, obs. 253.0729.

7-(2′-Aminophenoxy)coumarin (3d). To a solution of **2d** (49.1 mg, 0.174 mmol) in methanol (30 ml) was added 10% palladium/carbon (catalytic amount). The resulting mixture was stirred at room temperature under H_2 for 2.5 h. The palladium/carbon was removed by filtration and the filtrate was evaporated *in vacuo* to give **3d** (18.3 mg, 41.6%), mp 129.5–131.0 °C. ^{1}H-NMR δ_H ($CDCl_3$; Me_4Si) 6.29 (1H, d, J = 9.5 Hz, 3-H), 6.79–7.08 (6H, m, arom, 6-H and 8-H), 7.40 (1H, d, J = 8.6 Hz, 5-H), 7.65 (1H, d, J = 9.5 Hz, 4-H); HRMS (EI^+): calc. for $C_{15}H_{11}NO_3$ (M^+) m/z 253.0739, obs. 253.0735.

7-(2′-Acetamidophenoxy)coumarin (4d). To a solution of **3d** (18.3 mg, 0.0723 mmol) in dichloromethane (10 ml) was added acetic anhydride (150 μl, 1.59 mmol) and pyridine (120 μl, 1.49 mmol). The resulting mixture was stirred at room temperature for 1 h, then evaporated *in vacuo* to give **4d** (18.9 mg, 86.9%), mp 184.5–186.5 °C. ^{1}H-NMR δ_H ($CDCl_3$; Me_4Si) 2.16 (3H, s, -CH_3), 6.34 (1H, d, J = 9.5 Hz, 3-H), 6.85–7.25 (6H, m, arom, 6-H and 8-H), 7.44 (1H, d, J = 8.4 Hz, 5-H), 7.67 (1H, d, J = 9.5 Hz, 4-H); MS (EI^+): m/z 295 (M^+).

Fluorometric analysis

The slit width was 2.5 nm for both excitation and emission. The photomultiplier voltage was 950 V. All 7-hydroxycoumarin derivatives were dissolved in DMF to obtain 10 mM stock solutions. Relative quantum efficiencies of fluorescence of the 7-hydroxycoumarin derivatives were obtained by comparing the area under the corrected spectrum of the test sample excited at 330 nm in sodium phosphate buffer (0.1 M; pH 7.4) with that of a solution of quinine bisulfate excited at 366 nm excitation in 0.05 M H_2SO_4, which has a quantum efficiency of 0.55 according to the literature.[19,20]

Results and discussion

Chemical properties of 7-hydroxycoumarin derivatives

The 7-hydroxycoumarin derivatives (Scheme 1) were obtained according to the reaction scheme shown. The Ullmann coupling between 2-iodoacetanilide and 7-hydroxycoumarin potassium salt proved unsatisfactory, so we obtained **3d** by Ullmann coupling between 2-iodonitrobenzene and 7-hydroxycoumarin potassium salt, followed by reduction of the nitro group.

The absorbance and fluorescence properties of the 7-hydroxycoumarin derivatives are summarized in Table 1. The fluorescence properties varied greatly. The relative quantum efficiencies of fluorescence (ϕ_{fl}) of 7-acetoxycoumarin and the 7-aryloxycoumarins, **3a**, **3b**, **3c**, **3d** and **2e** were less than 0.001, whereas the 7-alkoxycoumarins, 7-benzyloxycoumarin and 7-methoxycoumarin, were 0.41 and 0.42, respectively. It is worth noting that 7-phenoxycoumarin (**2e**), which has a direct connection between the coumarin ring and the phenyl moiety *via* an oxygen atom, did not fluoresce at all, whereas 7-benzyloxycoumarin, which has a methylene linker, showed strong fluorescence.

Although the reason for the low ϕ_{fl} of 7-aryloxycoumarins is not entirely clear, two mechanisms, intramolecular charge transfer (ICT) and photo-induced electron transfer (PET),[8] can be considered as possible explanations for our results. We think that the ICT mechanism is more appropriate than the PET mechanism for the following reasons. The electron-donating character of the benzyl and phenyl groups would be similar in the case of the PET mechanism, since the highest occupied molecular orbital levels on these groups are rather similar, according to PM3 calculations (data not shown). On the other hand, as mentioned above, electron-withdrawing groups at the 3-position and electron-donating groups at the 7-position have been shown to enhance the fluorescence intensity of coumarins.[9,10] If a substituent group at the 7-position has an electron-donating effect on the coumarin ring, the fluorescence intensity would be enhanced, whereas, if the group has an electron-withdrawing effect, the intensity would be weakened. Judging from the Hammett values of substituent groups,[21] acetoxy and phenoxy groups have greater electron-withdrawing inductive effects than methoxy and benzyloxy groups. So, it can be considered that aryloxy and acetoxy groups are less powerful electron donors than alkoxy groups, and this would explain why 7-alkoxycoumarins show some fluorescence, while 7-aryloxycoumarins and 7-acetoxycoumarin do not fluoresce.

Reactivities of 7-hydroxycoumarin derivatives with ROS

ROS (reactive oxygen species) are believed to play key roles in many pathogenic processes, for example carcinogenesis,[22] inflammation[23] and ischemia-reperfusion,[24] as well as in signal transduction pathways.[25–27] Many fluorescence probes for ROS have been developed so far, but none of them show high selectivity among various ROS.[28] There is a clear need to develop novel fluorescence probes with high selectivity.

We examined the reactivities of the 7-aryloxycoumarins **3a**–**3d** towards ROS (O_2^-, H_2O_2, $^{\bullet}OH$ and $^1O_2$) by comparing the fluorescence increase after reaction with each ROS. O_2^- was generated from KO_2, $^{\bullet}OH$ was generated in the Fenton system from ferrous perchlorate and H_2O_2, and $^1O_2$ was generated from 3-(1,4-dihydro-1,4-epidioxy-1-naphthyl)propionic acid (EP-1), which is a $^1O_2$-releasing compound.[29] The results are presented in Table 2. The reactivities of **3a**–**3d** for $^{\bullet}OH$ were much higher

Table 2 Fluorescence increases after the reaction with ROS (arbitrary units)[a]

Compound	O_2^{-} [b]	H_2O_2 [c]	$^{\bullet}OH$ [d]	$^1O_2$ [e]
3a	<1.0	1.8	38	5.7
3b	<1.0	1.6	47	4.0
3c	3.1	<1.0	100	7.3
3d	2.8	<1.0	26	2.0

[a] These reactions were performed at 25 °C unless otherwise noted. The fluorescence intensities were determined at 450 nm with excitation at 370 nm. Dyes (final 10 μM; 0.1% DMF as a cosolvent) were added to sodium phosphate buffer (0.1 M; pH 7.4). [b] KO_2 (final 1 mM) was added and the mixture was stirred for 20 min. [c] H_2O_2 (final 100 mM) was added and the mixture was stirred for 20 min. [d] Ferrous perchlorate (final 500 μM) and H_2O_2 (final mM) were added. [e] EP-1 [3-(1,4-dihydro-1,4-epidioxy-1-naphthyl)propionic acid; final 500 μM] was added and the mixture was stirred at 37 °C for 60 min.

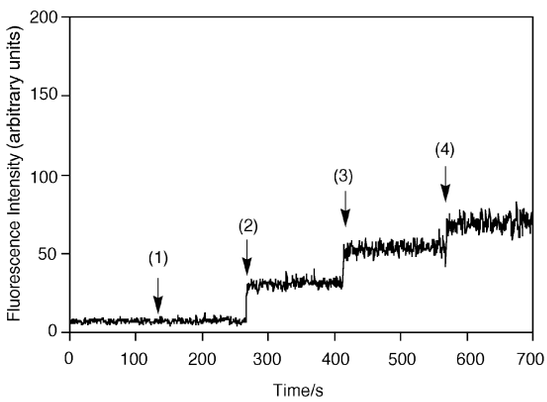
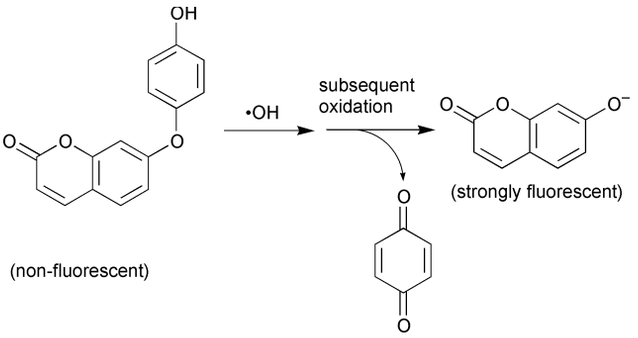


Fig. 1 Time course of fluorescence intensity of **3c** depending on $^{\bullet}OH$ generated from H_2O_2 and ferrous perchlorate. Dyes (final 10 μM; 0.1% DMF as a cosolvent) were added to sodium phosphate buffer (0.1 M; pH 7.4). The fluorescence intensity was determined at 450 nm with excitation at 370 nm. H_2O_2 (final 1 mM) was added at (1) and ferrous perchlorate (final 100 μM) was added three times at (2)–(4).

than those for the other ROS. Further, **3a**–**3d** scarcely reacted even with high concentrations of O_2^{-} and H_2O_2. We confirmed the production of 7-hydroxycoumarin in the reaction with $^{\bullet}OH$ by high-performance liquid chromatography (HPLC) and three-dimensional fluorescence spectroscopy. Thus, **3a**–**3d** all react highly selectively with $^{\bullet}OH$ to yield 7-hydroxycoumarin, which is highly fluorescent. Fig. 1 shows the time course of fluorescence intensity of **3c** after the addition of H_2O_2 at (1) and ferrous perchlorate at (2)–(4). The intensity did not increase upon addition of H_2O_2 alone, but immediately increased upon addition of ferrous perchlorate, which results in generation of $^{\bullet}OH$. In addition, the intensity increase was proportional to the concentration of ferrous perchlorate.

The reason for this high selectivity for $^{\bullet}OH$ is considered to be as follows. A strong oxidizing reagent is required for *O*-dearylation,[13,14] and although O_2^{-}, H_2O_2, $^{\bullet}OH$ and $^1O_2$ are all oxidizing reagents, only $^{\bullet}OH$ has sufficient oxidizing power to abstract a hydrogen atom from a hydroxy group or an electron from an amino group. Therefore, only $^{\bullet}OH$ can mediate *O*-dearylation, and this is why our novel compounds can detect $^{\bullet}OH$ with high selectivity. The proposed reaction scheme is shown in Scheme 2.

To confirm the above hypothesis and to obtain information about the chemical structural requirements for *O*-dearylation upon reaction with $^{\bullet}OH$, we examined the reactivities of **2a**, **2b**, **2c** and **4d**, whose hydroxy or amino groups are protected. None of them reacted with $^{\bullet}OH$. Thus, the structural requirements for *O*-dearylation of **3a**–**3d** are a hydroxy group in the case of **3a** and **3b** and an amino group in the case of **3c** and **3d**.



Scheme 2 *O*-Dearylation reaction of **3a** with $^{\bullet}OH$.

Conclusion

We found that, although 7-hydroxycoumarin is strongly fluorescent, 7-aryloxycoumarins do not fluoresce at all. From the relation between the electron-donating character of the substituent at the 7-position and the relative quantum efficiency of fluorescence, we considered that the fluorescence properties of 7-hydroxycoumarin derivatives can be explained in terms of the intramolecular charge transfer (ICT) mechanism. On this basis, we designed and synthesized novel 7-aryloxycoumarins (**3a**–**3d**) as candidate fluorescence probes for ROS, on the assumption that *O*-dearylation of these compounds by reaction with ROS would act as a trigger for fluorescence increase. Compounds **3a**–**3d** reacted with $^{\bullet}OH$ with high selectivity in an $^{\bullet}OH$-concentration-dependent manner to yield 7-hydroxycoumarin, the *O*-dearylated product, which has strong fluorescence. They showed little reactivity with O_2^{-}, H_2O_2 and $^1O_2$. We are currently using the principles learnt in this study to develop specific fluorescence probes for $^{\bullet}OH$ that display very large fluorescence increases and can be applied *in vivo*.

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