Novel 6-formylpterin derivatives: chemical synthesis and O_2 to ROS conversion activities

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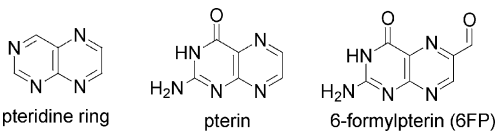
6-Formylpterin (6FP) has been demonstrated to have strong neuroprotective effects against transient ischemia-reperfusion injury in gerbils. Also it has been shown that in rats, 6FP protected retinal neurons even when it was administered after the ischemic insult. Since there is a significant need for such a compound that effectively suppresses the events caused by the lack of oxygen supply, 6FP has attracted further investigation. Unfortunately, however, 6FP is hardly soluble in water at neutral pH and in organic solvents because of its self-assembling ability. Although a several mM solution of 6FP is available in alkaline water, it is unstable. In the present study, a novel chemical derivatization of 6FP has been developed which maintains the formyl group on the 6-position of 6FP, which is essential for the physiological activities of 6FP, and increases solubility in water and organic solvents. In the method, the 2- and 3-positions of 6FP were modified by a three component coupling reaction: 6FP was subjected to the reaction with acid chloride and *N*,*N*-dimethylformamide. The derivatives synthesized here, 2-(*N*,*N*-dimethylaminomethyleneamino)-6-formyl-3-pivaloylpteridine-4-one **1**, 2-(*N*,*N*-dimethylamino-methyleneamino)-6-formyl-3-isobutyrylpteridine-4-one **2**, and 2-(*N*,*N*-dimethylamino-methyleneamino)-6-formyl-3-*o*-toluoylpteridine-4-one **3**, showed high solubility in water (1.0–5.6 mM) and organic solvents. The O_2 conversion property has also been determined for the derivative **1**. Using an oxygen electrode, it has been found that O_2 is consumed in the presence of **1** and NADH at around pH 7.4 and that the rate of O_2 consumption is enhanced by UV-A irradiation. Electron paramagnetic resonance (EPR) analysis coupled with DMPO spin trapping has also revealed that in the presence of NADH, **1** converts O_2 to $^{\bullet}O_2^-$, which is further reduced to $^{\bullet}OH$. By UV-A illumination in the analogous systems, $^1O_2$ formation was observed. These results are similar to those reported previously for 6FP.

Introduction

Ischemia-reperfusion injury (IRI) occurs in cardiac infarction, brain infarction, organ transplantation, and so on, resulting in apoptotic cell death.[1] Protective agents against IRI have been important research targets in pharmaceutical studies over the past two decades.[2] We have demonstrated that 6-formylpterin (6FP), which is known as a xanthine oxidase inhibitor, has potent neuroprotective effects against transient IRI in gerbils.[3a] Also we have shown the similar effects of 6FP in rat retinal IRI.[3b]

Many pteridines are endogenous: they are important electron transfer compounds in biological systems. They act as coenzymes and participate in important biological functions.[4,5] 6FP is one of the pteridine derivatives (Scheme 1).



Scheme 1 Structures of pteridine derivatives.

6FP generates reactive oxygen species (ROS) from molecular oxygen (O_2) in the presence of reducing agents such as NAD(P)H.[6] Recently 6FP was shown to produce singlet oxygen ($^1O_2$) under irradiation of long-wavelength ultraviolet light (UV-A) in aqueous solutions.[7]

Pteridines are generally insoluble compounds. This nature is due to strong intermolecular hydrogen bonds which lead to the self-assembling structure of pteridines.[8] To overcome this drawback, a number of trials have been reported.[9,10] The intermolecular hydrogen bonds are formed by amino and amide functions on the pteridine ring and it is known that protection of the N- or O-atoms of the amino or the amide function increases solubility in water and organic solvents.[9] Similarly, acylation of the amino group affects the solubility.[10] 6FP is also poorly soluble in water at neutral pH and in organic solvents for the same reason. Although a several mM solution of 6FP is available in alkaline water, it decomposes into pterin-6-carboxylic acid and 6-hydroxymethylpterin

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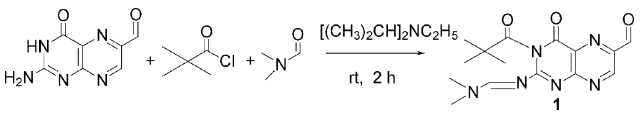
by the Cannizzaro reaction or photooxidation. For the clinical application of 6FP, 6FP must be modified, possibly by the introduction of bulky groups onto the ring, so that it becomes soluble in water. Also, it is worthwhile to explore the detailed chemical properties of 6FP to reveal the relationship between its physiological activities and its chemical natures.

We assume that maintaining the formyl group at the 6-position is essential for the notable physiological activities of 6FP, and in the present study, therefore, we developed novel chemical derivatization methods which not only improve 6FP solubility but maintain its physiological activity.

Results and discussion

First, a coupling reaction of three components was performed. When 6FP (1.0 mmol) was subjected to the reaction with pivaloyl chloride (3.5 mmol) and *N*,*N*-dimethylformamide (DMF) (12 ml) in the presence of *N*,*N*-diisopropylethylamine (4.5 mmol) at room temperature, 2-(*N*,*N*-dimethylaminomethyleneamino)-6-formyl-3-pivaloylpteridine-4-one **1** was obtained in a yield of 95% as a 2- and 3-position modified 6FP derivative as shown in Scheme 2. **1** was identified by MS, IR, ^{1}H-NMR, ^{13}C-NMR, and elemental analysis.

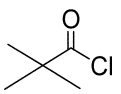
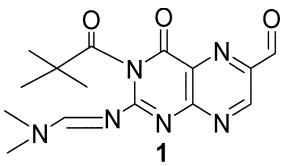
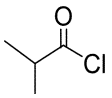
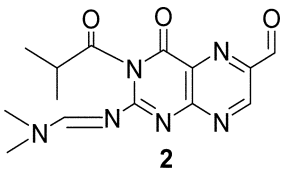
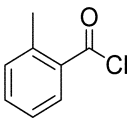
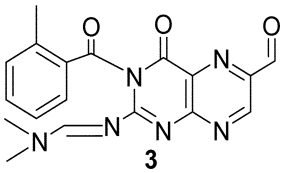
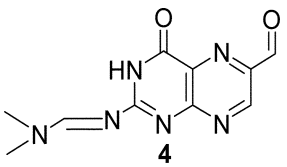
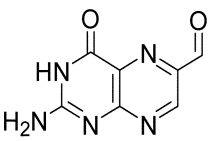


Scheme 2 The coupling reaction of 6FP with pivaloyl chloride and DMF in the presence of *N*,*N*-diisopropylethylamine.

Next, we used isobutyryl chloride, propionyl chloride and *o*-toluoyl chloride instead of pivaloyl chloride in the analogous reaction. The results are summarized in Table 1.

The reaction of isobutyryl chloride with 6FP and DMF resulted in 2-(*N*,*N*-dimethylaminomethyleneamino)-6-formyl-3-isobutyrylpteridine-4-one **2** in a yield of 88% (Entry 2). The reaction of *o*-toluoyl chloride with 6FP and DMF gave rise to 2-(*N*,*N*-dimethylaminomethyleneamino)-6-formyl-3-*o*-toluoylpteridine-4-one **3** in a yield of 52% (Entry 3). When *N*,*N*-diisopropylethylamine or acid chloride was absent, or when DMF was absent and 2-butanone was used as a solvent, 6FP also did not react. When the reaction was stopped in 20 minutes, 2-(*N*,*N*-dimethylaminomethyleneamino)-6-formylpteridine-4-one[11] **4** was isolated by column chromatography. Additionally, although the reaction of **4** with pivaloyl chloride produced **1** in the presence of *N*,*N*-diisopropylethylamine in DMF, the reaction did not proceed

Table 1 Coupling reactions of 6FP with acid chlorides and DMF and the water solubility of 6FP and the 6FP derivatives obtained

Entry	Acid chloride	Product	Yield (%)	Water solubility/mM
1		 **1**	95	3.8
2		 **2**	88	5.6
3		 **3**	52	1.0
4		 **4**		2.3
5		 **6FP**		0.02

in $CHCl_3$. According to these results, the first step of this reaction would be the activation of DMF and probably the Vilsmeyer type activated complex is involved. The reaction of the activated complex with the amino group on the 2-position of 6FP would give **4**. Then the reaction of the complex with the 3-position of **4** proceeds and gives **1** (Scheme 3).

These 6FP derivatives showed high solubility in methanol, ethanol, and other organic solvents. The water solubilities of these derivatives are summarized in Table 1, indicating that the 2- and 3-position modified 6FP derivatives have high water solubility and are more soluble than **4** in which only the 2-position is modified. These results suggest that the strong intermolecular hydrogen bonds between 6FP molecules are eliminated by these modifications. **3** showed lower solubility than **1**, **2** and **4** probably because of the hydrophobic nature of the toluoyl substituent (Entry 3).

We further examined the reaction of 6FP derivative **1** with O_2. In a previous paper, we have shown that the oxygen concentration of the solution containing NADH decreased linearly and dose dependently when 6FP was added to the solution.[6b] Using a similar method, we measured the O_2 consumption activity of **1** using oxygen electrodes. In Fig. 1, a comparison of the O_2 consumption by **1** with that by 6FP is depicted. The solution **a** contained 2 mM of **1** (pH 7.4) and the solution **b** contained 2 mM of 6FP (pH 8.3). Arrow 1 indicates the time of NADH addition to the solution. Arrows 2 and 3 indicate the time of irradiation with fluorescence light and UV-A light, respectively. The rate of O_2 consumption by **1** was lower than that by 6FP. However, both O_2 consumption rates were accelerated by irradiation with fluorescence light and UV-A light. In these experiments, the rate of O_2 consumption by **1** at physiological pH (pH 7.4) showed the same tendency as that of 6FP.

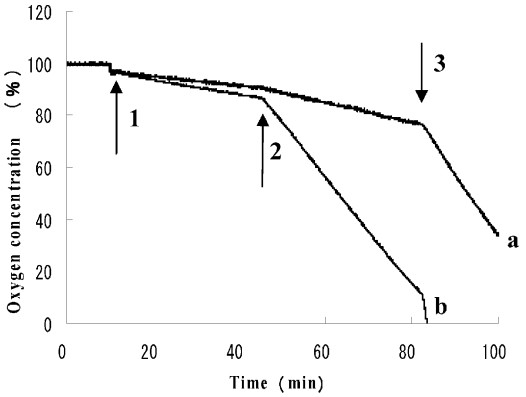
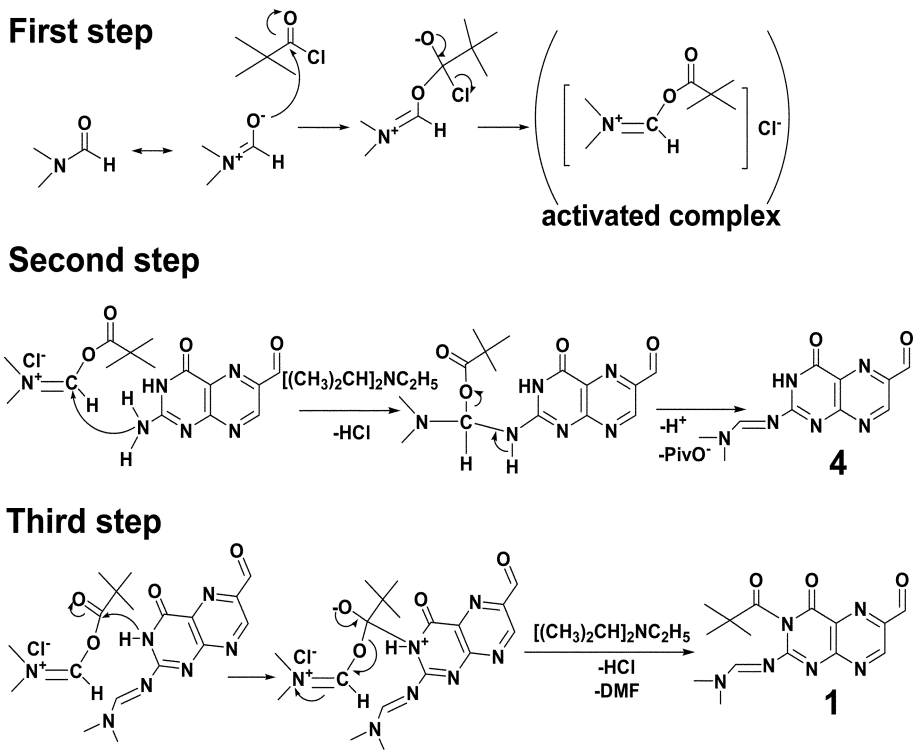


Fig. 1 O_2 consumption in the solution containing 6FP (2 mM) or **1** (2 mM) and NADH (6 mM). The time course of the changes in oxygen concentration measured by oxygen electrodes is shown. **a**: Mixture of **1** and NADH in PBS (pH 7.4); **b**: mixture of 6FP and NADH in PBS (pH 8.3). Arrow 1 indicates the addition of NADH to the solutions **a** and **b**. Arrow 2 indicates irradiation with fluorescence light. Arrow 3 indicates irradiation with UV-A light.

We investigated the mechanism for the above reaction by the EPR spin trapping technique using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin trap. EPR spectra obtained from the reaction of **1** in the presence of NADH are shown in Fig. 2. After adding **1** to the solution containing NADH, prominent EPR spectra were obtained (Fig. 2(a)), consisting of a quartet with a 1 : 2 : 2 : 1 intensity ratio with hyperfine splitting constants (hfsc's) of a(N) = 1.49 mT and a(βH) = 1.49 mT which is assignable to DMPO-OH,[12] indicative that the signals were the results of trapping hydroxyl radicals (•OH). These signals were totally quenched by superoxide dismutase (SOD) which converts



Scheme 3 A plausible reaction mechanism for the conversion of 6FP to **1** in the presence of pivaloyl chloride and *N*,*N*-diisopropylethylamine in DMF.

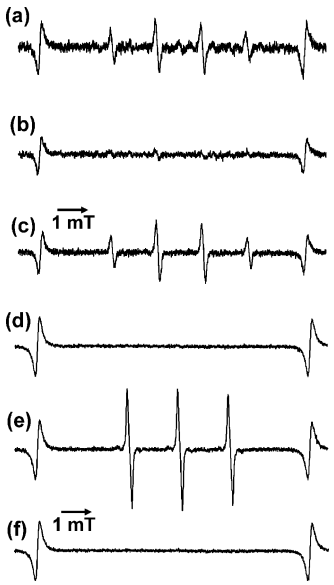


Fig. 2 (a), (b) and (c): EPR spectra obtained from a PBS solution containing **1** (2 mM), NADH (6 mM) and DMPO (1.47 M) under fluorescence light. (a) Mixture of **1**, NADH and DMPO; (b) mixture of **1**, NADH and DMPO in the presence of SOD (2000 units ml^{-1}); (c) mixture of **1**, NADH, DMPO, SOD (2000 units ml^{-1}), $FeSO_4$ (0.1 mM) and DTPA (1 mM) as a chelating agent. (d), (e) and (f): EPR spectra obtained under UV-A irradiation from a PBS solution containing **1** (50 μM) and 4-oxo-TEMP (1 mM). (d) Without **1**; (e) mixture of **1** and 4-oxo-TEMP; (f) mixture of **1**, 4-oxo-TEMP, and NaN_3 (100 μM) as a scavenger of $^1O_2$. Signals appearing at both high and low field correspond to Mn^{2+} installed in the EPR cavity as a reference.

the superoxide anion radical ($^{\bullet}O_2^-$) to H_2O_2 in the presence of a proton source (Fig. 2(b)), indicating that the signals were generated by the DMPO-trapping of $^{\bullet}OH$ converted from $^{\bullet}O_2^-$. When $FeSO_4$ was added to the reaction mixture of **1**, NADH and SOD, a 1 : 2 : 2 : 1 quartet of hydroxyl radical signals was observed (Fig. 2(c)). It should be noted that in the presence of ferrous ion (Fe^{2+}), $^{\bullet}OH$ is generated from H_2O_2 *via* the iron-catalyzed Fenton reaction.[13] Consequently these results show that $^{\bullet}O_2^-$ generated by the reaction of **1** and NADH under light illumination was converted to H_2O_2 by SOD. In summary, in the presence of **1** and NADH, O_2 was converted to $^{\bullet}O_2^-$ and then to $^{\bullet}OH$. The reaction of **1** with O_2 under UV-A irradiation was also investigated by analogous EPR analysis. When the solution containing **1** and 4-oxo-2,2,6,6-tetramethylpiperidine (4-oxo-TEMP) was irradiated with UV-A light, a 1 : 1 : 1 triplet (a(N) = 1.61 mT) of 4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy (4-oxo-TEMPO) was observed (Fig. 2(e)). It should be noted that 4-oxo-TEMP has been used as a $^1O_2$ detector.[14] These signals were completely quenched by sodium azide, a scavenger of $^1O_2$ (Fig. 2(f)). This result demonstrates that the signals were derived from the reaction of 4-oxo-TEMP with produced $^1O_2$, and that **1** has the ability to generate $^1O_2$ under UV-A light irradiation.

Conclusions

We synthesized novel 6FP derivatives and succeeded in increasing the water and organic solvent solubility of 6FP. The NH and NH_2 of 6FP were modified in one single action in one pot. The water solubility of the derivatives was hundreds of times larger than that of 6FP. Water solubility is an important factor for the practical use of 6FP as a pharmacologically active substance in living systems. We ascertained that the derivatives reduce O_2 to generate reactive oxygen species in the same way as 6FP. We believe that these functions are deeply related to the physiological activity of 6FP. Other modifications of 6FP and their effects on the physiological and pharmacological activities of the derivatives are now under investigation.

Experimental

Materials

6FP was obtained from Sankyo Kasei Kogyo (Hiratsuka, Japan). *N*,*N*-Diisopropylethylamine, *o*-toluoyl chloride and 4-oxo-2,2,6,6-tetramethyl-4-piperidine (4-oxo-TEMP) were purchased from SIGMA Chemical Co. (St. Louis, MO, USA), *N*,*N*-dimethylformamide (DMF) and isobutyryl chloride from Wako (Osaka, Japan), pivaloyl chloride from Tokyo Kasei (Tokyo, Japan), NADH from Oriental Yeast (Tokyo, Japan), and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) from Labotec Co. (Tokyo, Japan).

Synthesis

2-(*N*,*N*-Dimethylaminomethyleneamino)-6-formyl-3-pivaloylpteridine-4-one (1). 6FP (191.1 mg, 1.0 mmol), *N*,*N*-diisopropylethylamine (780 μl, 4.5 mmol) and pivaloyl chloride (430 μl, 3.5 mmol) were mixed in DMF (12 ml) with stirring under nitrogen for 2 h at room temperature. During this time, the suspended solid 6FP dissolved. The mixture was evaporated *in vacuo* and the resultant brown oil was purified by column chromatography eluting with chloroform–methanol (99 : 1) to give the title compound **1** as a yellow solid (312.4 mg, 95%), mp 178–179 °C. δ_H(600 MHz; $CDCl_3$) 10.21 (1H, s, CHO), 9.32 (1H, s, 7-H), 9.02 (1H, s, NCHN), 3.29 (3H, s, CH_3), 3.18 (3H, s, CH_3) and 1.43 (9H, s, ^{t}Bu). δ_C(150 MHz; $CDCl_3$) 191.1, 183.8, 160.5, 159.7, 157.9, 157.8, 148.9, 143.1, 130.3, 44.1, 42.2, 36.2 and 27.9. (Found: C, 54.77; H, 5.46; N, 25.15; O, 14.33%; M^+, 330.1448. $C_{15}H_{18}N_6O_3$ requires C, 54.54; H, 5.49; N, 25.44; O, 14.53%; M, 330.3421). ν_{max}/cm^{-1} 1766, 1693 and 1639 (CO). The water solubility (3.8 mM) was determined by UV-spectroscopy.

When the reaction was stopped after 20 min, 2-(*N*,*N*-dimethylaminomethyleneamino)-6-formylpteridine-4-one **4** was isolated by column chromatography as a yellow solid. δ_H(600 MHz; $(CD_3)_2SO$) 12.26 (1H, bs, NH), 10.02 (1H, s, CHO), 9.13 (1H, s, 7-H), 8.91 (1H, s, NCHN), 3.28 (3H, s, CH_3) and 3.15 (3H, s, CH_3). (Found: M^+, 246.0874. $C_{10}H_{10}N_6O_2$ requires M, 246.0865). The water solubility (2.3 mM) was determined by UV-spectroscopy.

2-(*N*,*N*-Dimethylaminomethyleneamino)-6-formyl-3-isobutyrylpteridine-4-one (2). 6FP (192.0 mg, 1.0 mmol), *N*,*N*-diisopropylethylamine (780 μl, 4.5 mmol) and isobutyryl chloride (370 μl, 3.5 mmol) were mixed in DMF (6 ml) with stirring under nitrogen for 2 h at room temperature. During this time, the suspended solid 6FP dissolved. The mixture was evaporated *in vacuo* and the resultant brown oil was purified by column chromatography eluting with chloroform–methanol (99 : 1) to

give the title compound **2** as a yellow solid (277.7 mg, 88%), mp 192–193 °C. δ_H (600 MHz; $CDCl_3$) 10.22 (1H, s, CHO), 9.32 (1H, s, 7-H), 9.00 (1H, s, NCHN), 3.29 (3H, s, CH_3), 3.21 (1H, m, CH), 3.16 (3H, s, CH_3) and 1.37 (6H, d, *J* 7.2, iPr). δ_C (150 MHz; $CDCl_3$) 191.1, 180.1, 160.4, 159.4, 157.9, 157.7, 148.8, 143.1, 130.5, 42.0, 40.3, 35.9 and 18.3. (Found: C, 52.99; H, 5.14; N, 26.58; O, 15.00%; M^+, 316.1290. $C_{14}H_{16}N_6O_3$ requires C, 53.16; H, 5.10; N, 26.57%; M, 316.3155). ν_{max}/cm^{-1} 1778, 1697 and 1643 (CO). The water solubility (5.6 mM) was determined by UV-spectroscopy.

2-(*N*,*N*-Dimethylaminomethyleneamino)-6-formyl-3-*o*-toluoylpteridine-4-one (3). 6FP (190.0 mg, 1.0 mmol), *N*,*N*-diisopropylethylamine (780 µl, 4.5 mmol) and *o*-toluoyl chloride (460 µl, 3.5 mmol) were mixed in DMF (6 ml) with stirring under nitrogen for 2 h at room temperature. During this time, the suspended solid 6FP dissolved. The mixture was evaporated *in vacuo* and the resultant brown oil was purified by column chromatography eluting with chloroform–methanol (99 : 1) to give the title compound **3** as a yellow solid (187.8 mg, 52%), mp 140–144 °C. δ_H (600 MHz; $CDCl_3$) 10.23 (1H, s, CHO), 9.34 (1H, s, 7-H), 8.94 (1H, s, NCHN), 7.59 (1H, d, *J* 7.8, Ph), 7.48 (1H, t, *J* 7.2, Ph), 7.37 (1H, d, *J* 7.2, Ph), 7.23 (1H, t, *J* 7.5, Ph), 3.20 (3H, s, CH_3), 2.88 (3H, s, CH_3) and 2.77 (3H, s, $PhCH_3$). δ_C (150 MHz; $CDCl_3$) 191.2, 168.7, 160.7, 159.2, 158.1, 158.0, 148.9, 143.1, 142.5, 133.7, 132.2, 131.2, 131.1, 130.6, 126.4, 41.9, 35.6 and 21.9. (Found: M^+, 364.1286. $C_{18}H_{16}N_6O_3$ requires M, 364.3582). ν_{max}/cm^{-1} 1743, 1695 and 1633 (CO). The water solubility (1.0 mM) was determined by UV-spectroscopy.

Oxygen consumption measurements with an oxygen electrode

The O_2 consumption during the reaction of **1** in the presence of NADH was measured polarographically using a Clarke oxygen electrode (model 5300; Yellow Springs Instruments; Yellow Springs, OH, USA) at 37 °C in a 4 ml reaction mixture. The oxygen concentration was calibrated with air-saturated phosphate-buffered saline (PBS), assuming that the dissolved oxygen concentration in 1 ml of PBS was initially 200 nmol ml^{-1}.[15] The 6FP solution was prepared by diluting 60 mM 6FP in 0.1 N NaOH solution with PBS buffer.

EPR analysis

EPR spectra obtained from 1 in the presence of NADH

To determine whether **1** produces ROS in the presence of NADH and what species are generated, EPR spectroscopy combined with spin trapping with DMPO was employed. For the sample preparation, a solution containing DMPO (1.47 M) was employed. Diethylenetriaminepentaacetic acid (DTPA) (1 mM) was added to the solution to avoid baseline contamination. The EPR spectra were recorded on a Model JES-TE300 spectrometer (JEOL Ltd.; Tokyo, Japan). The EPR settings were as follows: microwave power: 5 mW; field: 334.5 ± 5 mT (9.4160 GHz); modulation: 0.079 mT; time constant: 0.03 s; amplitude: 500; and sweep time: 2 min. Sample solutions were placed in a flat quartz EPR aqueous cell fixed in the cavity of the EPR spectrometer. The hyperfine splitting constants (hfsc's) were calculated based on the Mn^{2+} marker, which was inserted into the cavity of the EPR spectrometer.

Measurements of $^1O_2$ production under UV-A irradiation in an aqueous solution of 1

To examine whether **1** produces $^1O_2$ under UV-A irradiation, EPR spectroscopy was used and 4-oxo-2,2,6,6-tetramethylpiperidine (4-oxo-TEMP), which reacts with $^1O_2$ and produces a stable nitroxide radical, 4-oxo-2,2,6,6-tetramethyl-4-piperidinyloxy (4-oxo-TEMPO), was employed as a $^1O_2$ detector. The solution of **1** and 4-oxo-TEMP was placed in a flat quartz EPR aqueous cell, which was fixed in the cavity of the EPR spectrometer. The EPR spectrum recording started after 10 s of UV-A irradiation in a microwave cavity with a light focused from a RUVF-203F xenon arc-lamp (Radical Research Co., Tokyo, Japan) with a 360 nm band path filter operating at 200 mW. The EPR spectra were recorded with the following EPR parameters: microwave power: 5 mW; field: 329 ± 5 mT (9.2331 GHz); modulation: 0.079 mT; time constant: 0.03 s; amplitude: 320; and sweep time: 2 min. The intensity of 4-oxo-TEMPO and the hfsc's were calculated in a similar way to that shown above. In some experiments, sodium azide was used as a scavenger of $^1O_2$.

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