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Peroxynitrite generation from a NO-releasing nitrobenzene derivative in response to photoirradiation†

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Photocontrollable $ONOO^-$ generation from a nitrobenzene derivative was demonstrated. The designed compound released NO in response to photoirradiation, and the resulting semiquinone reduced molecular oxygen to generate $O_2^{\bullet -}$; reaction of the two generated $ONOO^-$, as confirmed with an $ONOO^-$ fluorescent probe, HKGreen-3.

Reactive oxygen species (ROS) play key functions in many pathophysiological processes. Among them, peroxynitrite ($ONOO^-$), formed by reaction between nitric oxide (NO) and superoxide ($O_2^{\bullet -}$) under diffusion control ($k = 6.7 \times 10^9$ M^{-1} s^{-1}),[1–3] has the greatest oxidizing power and cytotoxicity. $ONOO^-$ is involved in the progression of a wide variety of diseases, including diabetes,[4,5] degenerative neurological disorders,[6,7] and cardiovascular disturbance.[8–11] On the other hand, $ONOO^-$ is also implicated in cell signalling[12–15] and biodefence. $ONOO^-$ generators would be useful not only as tools for investigation of the pathophysiological functions of this species, but also as candidate anticancer agents, due to their cytotoxicity. However, the only $ONOO^-$ donor that has been reported so far is 3-(4-morpholinyl)sydnonimine hydrochloride (SIN-1), which generates equal amounts of $O_2^{\bullet -}$ and NO, leading to $ONOO^-$ formation.[16] The generation of $ONOO^-$ occurs by spontaneous autolysis, but for biological applications, it would be desirable for the release of $ONOO^-$ to be controllable in a time- and site-dependent manner.

Previously, we have reported photoinduced NO release from 4-substituted 2,6-dimethylnitrobenzene derivatives exposed to photoirradiation.[17] It was suggested that the nitro group in those compounds is not in a planar conformation, owing to the steric hindrance of the methyl groups at the two *ortho*-positions.

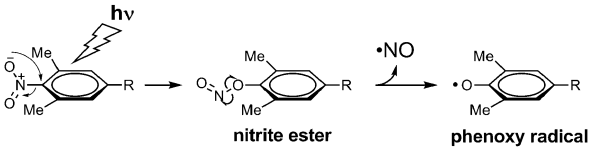

Fig. 1 Mechanism of photoinduced NO release from nitrobenzene.

The consequent twisted conformation is considered to facilitate isomerization reaction of the nitro group to nitrite ester, which is the key reaction for NO release. In this reaction, after homolysis of the nitrite ester to release NO, an oxyl radical is considered to be formed (Fig. 1). In the light of this finding, we designed and synthesized a novel 2,6-dimethylnitrobenzene derivative bearing a phenol group (**1**). Based on our previous findings, compound **1** is expected to be converted to a semiquinone-like form upon NO release in response to photoirradiation. As it is known that semiquinone derived from benzoquinone can convert O_2 to $O_2^{\bullet -}$ by one-electron reduction,[18] it is expected that the semiquinone-like form derived from **1** after release of NO will generate an equivalent amount of $O_2^{\bullet -}$ with formation of quinone-type compound **2** (Fig. 2). So, we expected that this compound might act as a novel photocontrollable $ONOO^-$ generator.

Compound **1** was prepared as shown in Scheme 1. The structure and purity of **1** were confirmed by ^{1}H-NMR, ^{13}C-NMR, mass spectrometry and elemental analysis.

To confirm NO release as the first step of $ONOO^-$ generation from **1** upon photoirradiation, we used an ESR spin trapping

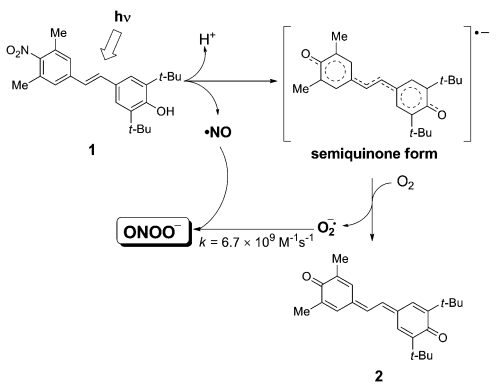

Fig. 2 Plausible mechanism of $ONOO^-$ generation from **1**.

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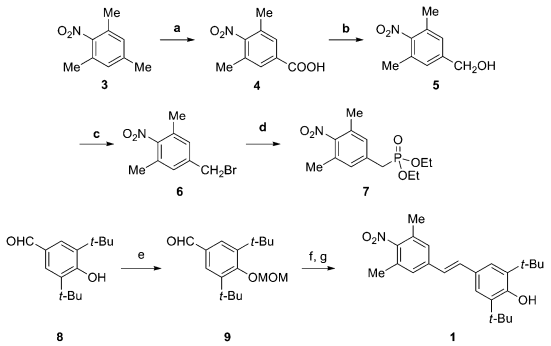


Scheme 1 *Reagents and conditions*: (a) CrO_3, AcOH; (b) $NaBH_4$, BF_3 etherate; (c) PBr_3; (d) $P(OEt)_3$, $(n\text{-}Bu)_4NI$, 120 °C; (e) CH_3OCH_2Cl, DIPEA, DMAP, CH_2Cl_2; (f) **7**, NaH, THF; (g) HCl, AcOEt. DIPEA = diisopropylethyl-amine, DMAP = *N*,*N*-dimethyl-4-aminopyridine.

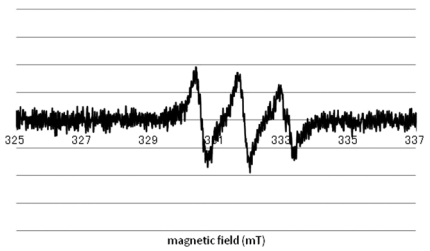
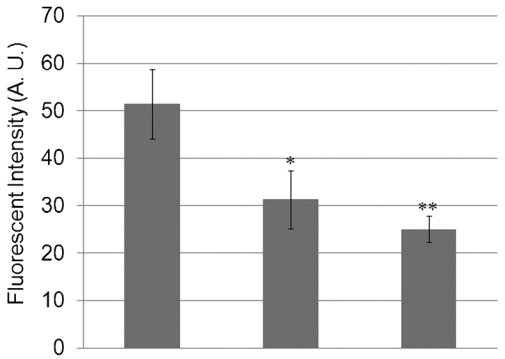


Fig. 3 ESR spectra of the [$(MGD)_2$–Fe–NO] complex after photo-irradiation (330–380 nm) in the presence of **1**. Samples contained 100 μM **1**, 1.5 mM $FeSO_4$ and 6 mM MGD (in PBS, pH 7.5, containing 1% DMF); ESR spectra were recorded after photo-irradiation for 15 min under an argon atmosphere with a modulation width of 1.25 G and a microwave power of 10 mW.

method with an iron *N*-methylglucamine dithiocarbamate (Fe–MGD) complex, which traps NO to yield the NO–Fe–MGD complex and exhibits a broad three-line signal at around 330 mT in 1 GHz ESR spectroscopy. An aqueous solution (1% DMF, PBS, pH 7.0) of **1** was irradiated with 330–380 nm (UV-A) light (100 W Hg lamp) under an argon atmosphere in the presence of the Fe–MGD complex. After irradiation, ESR spectra of the solution showed typical triplet signals assigned to the NO–Fe–MGD complex, suggesting that **1** released NO upon UV-A irradiation under an argon atmosphere (Fig. 3).

To examine the effect of the substituent at the *ortho*-position of the phenol structure on NO formation, we synthesized a variety of compounds bearing different functional groups at the *ortho*-position of the phenol moiety (Me, Et, *i*-Pr, F, and OMe) (synthetic methods are described in ESI†). From the results of NO spin trapping experiments, the intensity of the NO–Fe–MGD signal was found to be dependent on the substituent (Fig. S1a, ESI†), and the extent of NO formation under photoirradiation was highest for **1** among these compounds (Table S1, ESI†). It was also confirmed that **1** was the most efficiently decomposed upon photoirradiation, by means of HPLC analysis.

To investigate the reaction after NO release, we conducted NO spin trapping experiments under aerobic conditions. When **1** was irradiated in the presence of the Fe–MGD complex under aerobic conditions, the NO–Fe–MGD signal



	(a)	(b)	(c)
1 10 μM	+	+	–
HKGreen-3 10 μM	+	+	+
Photoirradiation	+	+	+
Under argon	–	+	–

Fig. 4 Detection of $ONOO^-$ generation from **1** using HKGreen-3. Samples in potassium phosphate buffer (10 mM, pH 7.5, containing 1% DMF) were irradiated for 15 min. The fluorescence intensity was determined at 535 nm with excitation at 520 nm. $^{**}p < 0.01$ when compared with (a), $^{*}p < 0.05$ when compared with (a) by ANOVA and Bonferroni-type multiple *t*-test ($n = 3$).

was attenuated (Fig. S1b, ESI†). However, despite the aerobic conditions, the signal was restored by addition of superoxide dismutase (SOD) (Fig. S1c, ESI†). These results indicate that the reaction of NO and $O_2^{\bullet-}$ competes with the reaction of NO and Fe–MGD. Unfortunately, $O_2^{\bullet-}$ itself could not be detected by means of ESR spin-trapping because DEPMPO, which is a typical spin-trapping reagent for detection of $O_2^{\bullet-}$, reacted with $O_2^{\bullet-}$ too slowly ($k = 24\ M^{-1}\ s^{-1}$) as compared with the reaction of $O_2^{\bullet-}$ with NO ($k = 6.7 \times 10^9\ M^{-1}\ s^{-1}$) (data not shown). Nevertheless, these results obtained by means of Fe–MGD spin trapping indicated that NO detection was affected by $O_2^{\bullet-}$, and thus implied $O_2^{\bullet-}$ generation from the semiquinone-like radical anion.

Next, we tried to detect $ONOO^-$ generation from **1** with a fluorescent probe, HKGreen-3,[19] which fluoresces upon reaction with $ONOO^-$. When a solution of **1** (10 μM) and HKGreen-3 (10 μM) was irradiated, the fluorescence was found to be increased. Furthermore, the fluorescence increase was suppressed by irradiation under an argon atmosphere (Fig. 4). These results indicated that **1** generates $ONOO^-$ in response to photoirradiation, and $ONOO^-$ generation by **1** requires atmospheric O_2.

We next investigated the photo-decomposition products of **1**. For this investigation, we synthesized a quinone-type compound **2**, which was expected to be formed in the putative reaction process. The preparation of **2** is shown in ESI.† When an irradiated solution of **1** was analyzed by HPLC, **2** was detected as the main product (Fig. 5). However, benzaldehyde derivatives, which are probably generated by oxidative cleavage at the olefin moiety of **1**, were detected in addition to **2** (Fig. S2, ESI†). The redox potential of **2** was determined as *ca.* −0.8 V (*versus* an Ag/AgCl electrode) by cyclic voltammetry, indicating that the resultant quinone-type compound **2** is unlikely to be reduced to its semiquinone-like form again

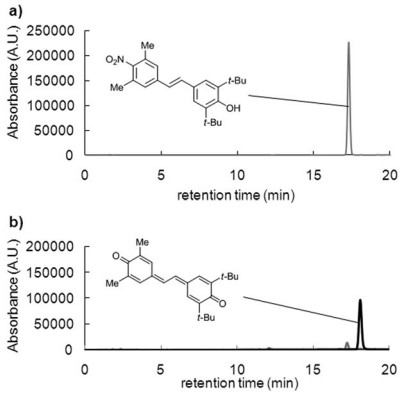


Fig. 5 Detection of **2**, the photo-decomposition product of **1**, using reverse-phase HPLC: (a) a solution of **1** (100 μM) was examined by means of HPLC; (b) an irradiated (15 min) solution of **1** (100 μM) was examined by means of HPLC.

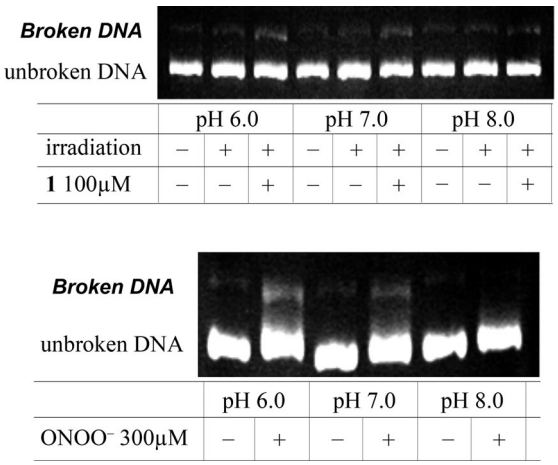


Fig. 6 DNA strand break assay by using **1** (upper panel), or $ONOO^-$ solution (lower panel).

under physiological conditions, so would not generate an excess amount of $O_2^{\bullet -}$.

As $ONOO^-$ eventually decomposes to NO_2^- or NO_3^-, the concentration of NO_2^-/NO_3^- in the irradiated solution of **1** was determined by means of the 2,3-diaminonaphthalene fluorescence method.[20] The amount of NO_2^-/NO_3^- formed was found to correspond to *ca.* 20% of the initial concentration of **1** after 15 min irradiation, and this is consistent with the amount of **2** formed as determined by HPLC after photoirradiation (Table S2, ESI†).

We also examined DNA cleavage by photoirradiation in the presence of **1** *in vitro*. It is known that ONOOH (pK_a = 6.8, protonated form of $ONOO^-$) decays by homolysis of its peroxo bond, generating $^{\bullet}OH$ and nitrogen dioxide ($^{\bullet}NO_2$), and shows OH-like DNA cleavage reactivity. So, we conducted a DNA cleavage assay under various pH conditions (pH = 6.0, 7.0 and 8.0). DNA strand breaks were observed on photoirradiation in the presence of **1**, and the activity was found to be diminished at high pH (pH = 8.0), like that of $ONOO^-$ (Fig. 6). From these results, it was suggested that $ONOO^-$ generated from **1** in response to photoirradiation oxidatively damages DNA.

In the present study, we have demonstrated photocontrollable $ONOO^-$ generation from a novel 2,6-dimethylnitrobenzene bearing a conjugated phenol moiety (**1**). NO release from **1** was detected by means of an ESR spin trapping method. $ONOO^-$ generation in response to photoirradiation of **1** was detected with an $ONOO^-$-specific fluorescent probe, HKGreen-3. The results of the DNA strand break assay with **1** indicate that **1** shows $ONOO^-$-like DNA cleavage reactivity. To our knowledge, this is the first example of a photocontrollable $ONOO^-$ generator. It is expected be a useful tool for time- and site-specific generation of $ONOO^-$.

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