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Photocontrollable Peroxynitrite Generator Based on *N*-Methyl-*N*-nitrosoaminophenol for Cellular Application

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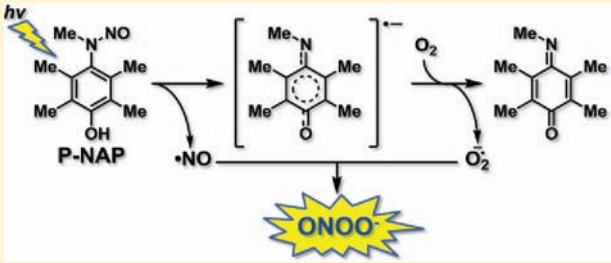
Supporting Information

ABSTRACT: We designed and synthesized a photocontrollable peroxynitrite ($ONOO^-$) generator, P-NAP, which has *N*-methyl-*N*-nitrosoaminophenol structure with four methyl groups introduced onto the benzene ring to block reaction of the photodecomposition product with $ONOO^-$ and to lower the semiquinoneimine's redox potential. The semiquinoneimine intermediate generated by photoinduced release of nitric oxide (NO) reduces dissolved molecular oxygen to generate superoxide radical anion ($O_2^{\bullet-}$), which reacts with NO to afford $ONOO^-$ under diffusion control ($k = 6.7 \times 10^9\ M^{-1}\ s^{-1}$). NO release from P-NAP under UV-A (330−380 nm) irradiation was confirmed by ESR spin trapping. Tyrosine nitration, characteristic of $ONOO^-$, was demonstrated by HPLC analysis of a photoirradiated aqueous solution of P-NAP and *N*-acetyl-L-tyrosine ethyl ester. $ONOO^-$ formation was confirmed with a $ONOO^-$-specific fluorogenic probe, HKGreen-3, and compared with that from 3-(4-morpholinyl)sydnonimine hydrochloride (SIN-1), which is the most widely used $ONOO^-$ generator at present. The photoreaction of P-NAP was influenced by superoxide dismutase, indicating that generation of $O_2^{\bullet-}$ occurs before $ONOO^-$ formation. The quantum yield for formation of duroquinone, the main P-NAP photodecomposition product, was measured as 0.86 ± 0.07 at 334 nm with a potassium ferrioxalate actinometer. Generation of $ONOO^-$ from P-NAP in HCT-116 cells upon photoirradiation was successfully imaged with HKGreen-3A. This is the first example of a photocontrollable $ONOO^-$ donor applicable to cultured cells.



1. INTRODUCTION

Peroxynitrite ($ONOO^-$) is one member of a family of reactive nitrogen species (RNS). It is formed *in vivo* 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] Due to its potent oxidizing power and nitrating activity for biological molecules, $ONOO^-$ is cytotoxic and is associated with the pathogenesis of various diseases.[2] On the other hand, nitrated biomolecules, such as nitrotyrosine- and nitrotryptophan-containing proteins, 8-nitroguanosine, and nitro fatty acids, are associated with essential cell signaling pathways,[3] so $ONOO^-$ may be involved in regulation of these pathways.[4] Consequently, $ONOO^-$ generators are expected to be useful tools for biological research and also candidate anticancer drugs, due to the ability of $ONOO^-$ to activate dendritic cells.[5] The $ONOO^-$ generator most widely used at present is 3-(4-morpholinyl)sydnonimine hydrochloride (SIN-1), which generates equal amounts of $O_2^{\bullet-}$ and NO spontaneously under physiological conditions, leading to $ONOO^-$ formation.[6] However, to study in detail the physiological and pathophysiological roles of $ONOO^-$, it would be preferable to release $ONOO^-$ in a temporally and spatially controlled manner.

We have previously reported DiP-DNB (**1**),[7] having a 2,6-dimethylnitrobenzene structure, as a photocontrollable $ONOO^-$ donor that generates NO by isomerization reaction of the nitro group and subsequent homolysis of nitrite upon photoirradiation.[8] After release of NO from this compound, the resulting semiquinone intermediate reduces O_2 to generate an equivalent amount of $O_2^{\bullet-}$, and reaction of the two generates $ONOO^-$ (Chart 1a), as confirmed with the $ONOO^-$-specific probe HKGreen-3.[9]

However, DiP-DNB showed low water solubility and did not induce tyrosine nitration, a characteristic reaction of $ONOO^-$.[1] Further, HPLC analysis suggested that the stilbene quinone **2** generated from DiP-DNB could react with $ONOO^-$ to form benzaldehyde derivatives (presumably this reaction would be initiated by nucleophilic attack of $ONOO^-$ on the quinone methide moiety of **2**).

To overcome these weaknesses, we focused on the *N*-methyl-*N*-nitrosoaniline structure, which shows high NO-releasing efficiency in response to photoirradiation.[10] By applying our original concept for DiP-DNB to *N*-methyl-*N*-nitrosoaniline,

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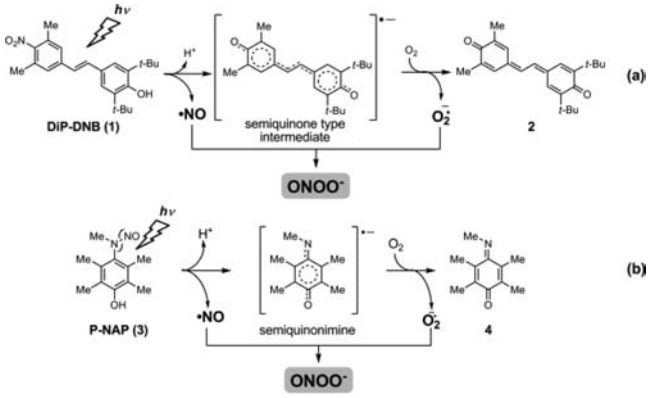
Chart 1. Photocontrollable ONOO⁻ Generator and Proposed Mechanisms of ONOO⁻ Release from DiP-DNB (a) and P-NAP (b)

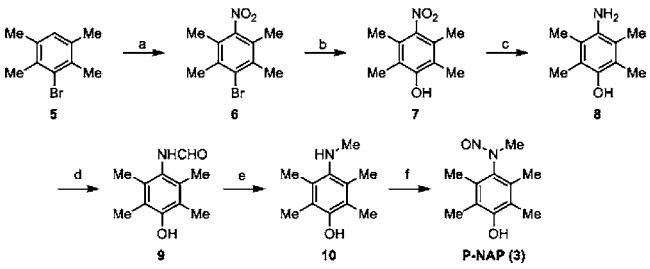


we designed P-NAP (**3**), which would not generate stilbene quinone but would instead be converted to a highly substituted quinoneimine, **4** (Chart 1b). The four methyl groups were introduced at the benzene ring not only to block reaction with $ONOO^-$ due to steric hindrance but also to lower the redox potential of the semiquinoneimine in order to increase the efficiency of $O_2^{\bullet-}$ generation.

2. RESULTS AND DISCUSSION

P-NAP (**3**) was synthesized as shown in Scheme 1. Nitration of bromobenzene derivative **5** afforded the nitrobenzene derivative **6**, which was subjected to Pd-catalyzed hydroxylation[11] to afford the 4-nitrophenol derivative **7**. This product was reduced with Raney Ni to the 4-aminophenol **8**, which in turn was converted to the formamide **9** with Ac_2O and HCOOH. Formamide **9** was reduced with $LiAlH_4$ to give the *N*-methyl-4-aminophenol derivative **10**, which was nitrosated with $NaNO_2$ to afford the desired final product, P-NAP (**3**). Its structure and purity were confirmed by ^1H NMR, ^{13}C NMR, mass spectrometry, and elemental analysis. P-NAP (1 mM) was readily soluble in water in the presence of 1% DMF, whereas over 10% DMF was required to achieve the same concentration of DiP-DNB in water. Therefore, P-NAP should be superior to DiP-DNB in biological applications.

Scheme 1. Synthesis of P-NAP (3)[a]



[a]Reagents and conditions: (a) NO_2BF_4, MeCN; (b) KOH, Pd_2dba_3, 2-di-*tert*-butylphosphino-2′,4′,6′-triisopropylbiphenyl, 1,4-dioxane, H_2O; (c) Raney Ni, MeOH, H_2; (d) HCOOH, Ac_2O, CH_2Cl_2; (e) $LiAlH_4$, THF; (f) $NaNO_2$, AcOH, H_2O. dba = dibenzylideneacetone.

To detect NO release, the first step of $ONOO^-$ generation upon photoirradiation of P-NAP, we used an ESR spin-trapping method with iron *N*-methylglucamine dithiocarbamate complex (Fe-MGD) under an argon atmosphere.[12] Fe-MGD efficiently traps NO to yield NO-Fe-MGD complex, which exhibits a broad three-line signal around 330 mT in 1 GHz ESR spectroscopy. An aqueous solution of P-NAP was irradiated with UV-A light (without attenuation) under an argon atmosphere to avoid any effect of $O_2^{\bullet-}$ in the presence of Fe-MGD complex. The ESR spectrum of the irradiated solution showed typical triplet signals assigned to the NO-Fe-MGD complex (Figure 1a), suggesting that P-NAP released NO upon irradiation. When the solution was irradiated under aerobic conditions, the triplet signals were not observed (Figure 1b). Moreover, the irradiated solution of P-NAP containing superoxide dismutase (SOD), which catalyzes the dismutation of $O_2^{\bullet-}$ into O_2 and H_2O_2 under diffusion control ($k = 2.0 \times 10^9\ M^{-1}\ s^{-1}$),[13] showed the triplet signals even under aerobic conditions (Figure 1c). These results are consistent with the proposed mechanism of $ONOO^-$ generation. That is, NO appeared to be consumed by reaction with $O_2^{\bullet-}$ under diffusion control, under aerobic conditions.

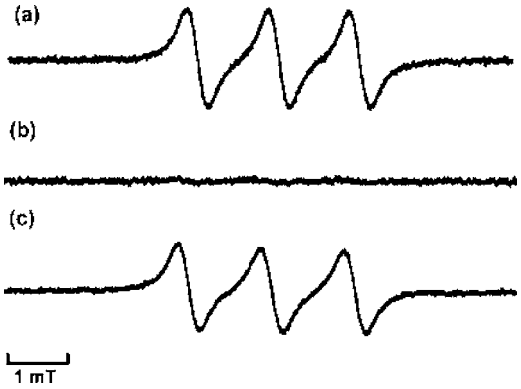


Figure 1. ESR spectra of solutions containing P-NAP (100 μM), $FeSO_4$ (1.5 mM), and *N*-methylglucamine dithiocarbamate (6 mM) (in PBS, pH 7.0, containing 1% DMF) after photoirradiation with UV-A (without attenuation) for 15 min. (a) A solution photoirradiated under an argon atmosphere. (b) A solution photoirradiated under aerobic conditions. (c) A solution containing SOD (1 kunit/mL) photoirradiated under aerobic conditions.

Tyrosine nitration is a characteristic reaction of $ONOO^-$.[1] Therefore, an aqueous solution of P-NAP and *N*-acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt) was irradiated with UV-A (without attenuation) and analyzed by HPLC. As shown in Figure 2b, a peak of *N*-acetyl-L-3-nitrotyrosine ethyl ester (Ac-nitroTyr-OEt) was detected in the irradiated solution. This peak was not detected in the solution irradiated under an argon atmosphere (Figure 2c). Furthermore, neither the solution irradiated without P-NAP nor that incubated in the dark with P-NAP showed a peak of Ac-nitroTyr-OEt (Figure S1). Interestingly, when the solution was irradiated in the presence of SOD, the peak of Ac-nitroTyr-OEt was larger than that in the absence of SOD (Figure 2d). This result is consistent with the report by Beckman et al. that SOD promotes nitration of tyrosine by $ONOO^-$.[14] On the other hand, SOD might suppress $ONOO^-$ generation by our photocontrollable $ONOO^-$ donor, which generates NO and $O_2^{\bullet-}$, by eliminating $O_2^{\bullet-}$. We considered that our results could be explained as follows. To obtain the results in Figure 2, we treated a high concentration of P-NAP (1 mM) with Ac-Tyr-OEt (1 mM) in order to get a clear peak of Ac-nitroTyr-OEt. Excess $O_2^{\bullet-}$ might escape from SOD and combine with NO to form $ONOO^-$. SOD might then facilitate tyrosine nitration by the $ONOO^-$

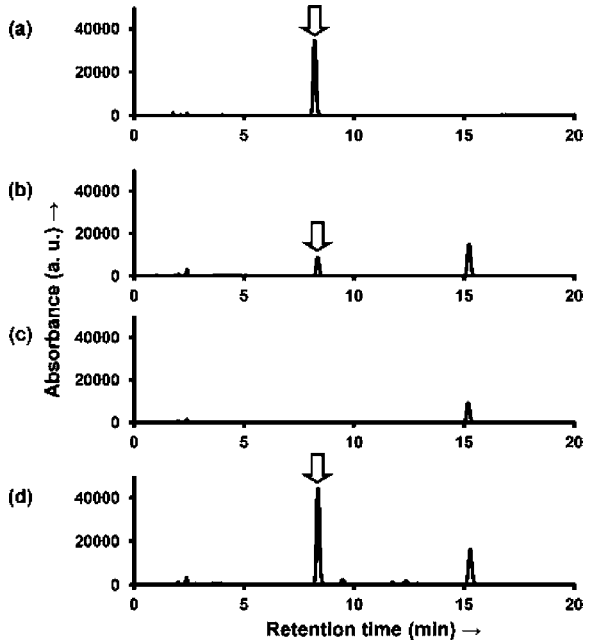
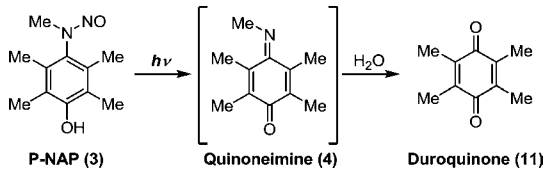


Figure 2. Reversed-phase HPLC chromatograms for detection of Ac-nitroTyr-OEt (arrows). The detector was set at 358 nm. (a) A solution of Ac-nitroTyr-OEt (100 μM). (b) A solution of P-NAP (1 mM) and Ac-Tyr-OEt (1 mM) in PBS (pH 7.0, 2% DMF) after irradiation with UV-A (without attenuation) for 15 min. (c) The same solution as (b), with irradiation under an argon atmosphere. (d) The same solution as (b), but with SOD (1 kunit/mL) after irradiation with UV-A (without attenuation) for 15 min.

formed. When smaller amounts of P-NAP and Ac-Tyr-OEt were used, Ac-nitroTyr-OEt formation was decreased by addition of SOD (Figure S2). Presumably, $O_2^{\bullet-}$ was scavenged by SOD, and it became difficult to generate $ONOO^-$. Beckman et al. also suggested that SOD can catalyze nitration efficiently only with $ONOO^-$, but not with NO, NO_2^-, or NO_2. These results strongly suggest that $ONOO^-$ generated from P-NAP in response to irradiation caused nitration of Ac-Tyr-OEt.

The peak of the main photodecomposition product of P-NAP around 15 min in Figure 2b−d was identified as 2,3,5,6-tetramethyl-1,4-benzoquinone, also called duroquinone (**11**), suggesting that quinoneimine **4**, the expected photodecomposition product of P-NAP, is hydrolyzed to **11** (Chart 2). In our

Chart 2. Expected Formation Pathway of Duroquinone, the Main Photodecomposition Product of P-NAP



study on DiP-DNB, we have discovered that stilbene quinone **2**, a main photodecomposition of DiP-DNB, reacts with $ONOO^-$ to yield 4-hydroxybenzaldehyde derivatives. Further, it was reported that unsubstituted 1,4-naphthoquinone reacts with $ONOO^-$ to yield epoxide.[15] These reactions are undesirable for our purpose, because the generated $ONOO^-$ would be consumed. However, when **11** was treated with an equivalent amount of $ONOO^-$, no decomposition was observed (data not shown). This result implies the importance of the four methyl groups as blocking substituents to prevent the undesired reaction of **11** with $ONOO^-$. Further, although 1,4-benzoquinone derivatives are known to show cytotoxicity due to ROS generation, duroquinone is one of the least cytotoxic 1,4-benzoquinone derivatives, because its four methyl groups prevent repeated redox-driven ROS generation and nucleophilic attack by thiols.[16]

Despite the weak absorbance of P-NAP around 330−380 nm (Figure S3), P-NAP is decomposed by irradiation as effectively as DiP-DNB, which has strong absorbance around the UV-A region. The quantum yield of duroquinone (**11**) formation, Φ_{DQ}, was determined with a potassium ferrioxalate actinometer.[17] The value of observed Φ_{DQ} at 334 nm was 0.86 ± 0.07 for P-NAP. This high value of Φ_{DQ} is considered to explain the efficient decomposition of P-NAP by UV-A light.

For further confirmation of $ONOO^-$ generation, we employed HKGreen-3, a $ONOO^-$-specific fluorogenic probe which fluoresces following nucleophilic attack of $ONOO^-$ on the trifluoromethyl ketone moiety, resulting in NO_2^- elimination. Slight decomposition of HKGreen-3 was observed in control solutions (Figure 3a, i and iii), but the fluorescence was strongly increased upon photoirradiation in the presence of P-NAP (Figure 3a, iii and iv). Furthermore, the fluorescence increase in the presence of P-NAP was suppressed by irradiation under an argon atmosphere or by irradiation under aerobic conditions in the presence of SOD, presumably because $O_2^{\bullet-}$ generation was suppressed (Figure 3a, v and vi). A fluorescence increase was also observed after 2 h incubation in the presence of SIN-1 (Figure 3a, vii and viii). The concentration of $ONOO^-$ generated by P-NAP (10 μM) after irradiation for 15 min was determined to be 4.6 μM, while that generated by SIN-1 (10 μM) after incubation for 2 h, when the fluorescence increase seemed to be saturated (Figure S4), was determined to be 5.1 μM from a standard curve. We also measured the concentration of NO_3^- as the final product of $ONOO^-$ by means of the 2,3-diaminonaphthalene fluorescence method.[18] The amount of NO_3^- in the irradiated solution of P-NAP (10 μM) was determined to be 6.0 μM. When a solution of P-NAP was irradiated for various time periods and at various light intensities, the fluorescence increase was clearly dependent on both irradiation time and light intensity (Figure 3b). Although the concentration of $ONOO^-$ generated from P-NAP was a little less than that from SIN-1, P-NAP allows the amount released to be controlled, while this is not the case with SIN-1.

We also synthesized other *N*-methyl-*N*-nitrosoaminophenol derivatives having reduced numbers of methyl groups at the benzene moiety (Figure S5, compounds **S1**−**S3**) and examined their efficiency of $ONOO^-$ generation upon photoirradiation by using HKGreen-3 (Figure S6). **S1**−**S3** showed lower levels of fluorescence increase than P-NAP. Moreover, these three compounds did not induce nitration of Ac-Tyr-OEt. We considered that the methyl groups may serve not only as blocking groups to prevent undesired reaction of the decomposition product with the produced $ONOO^-$, but also as electron-donating groups to lower the redox potential of the semiquinoneimine intermediate, thereby enhancing generation of $O_2^{\bullet-}$. It was not possible to determine the reduction potentials of the *N*-methylquinoneimine derivatives, due to their instability. However, the reported reduction potentials of the corresponding 1,4-benzoquinone derivatives are as follows: unsubstituted 1,4-benzoquinone, 92 mV; 2,5-dimethyl-1,4-benzoquinone, −76 mV; and duroquinone, −244 mV, while

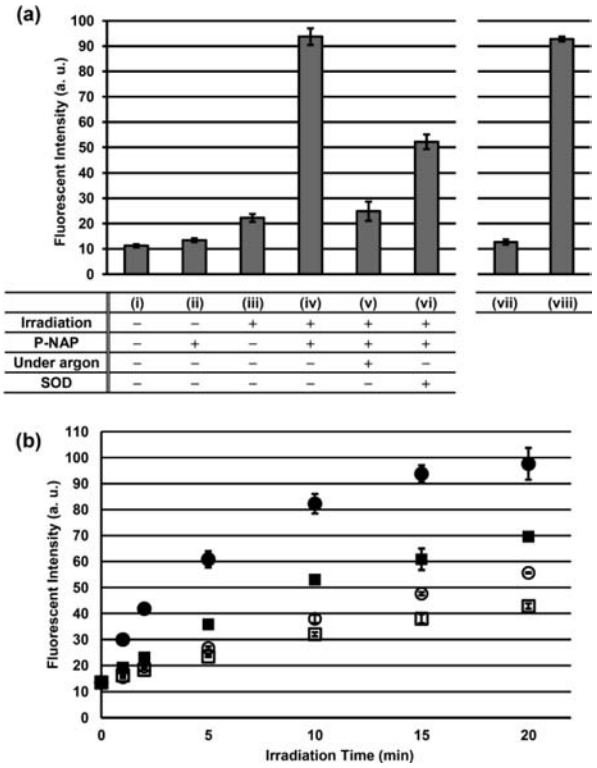


Figure 3. Fluorescence measurement for detection of $ONOO^-$ generation using HKGreen-3. Samples contained P-NAP (10 μM) and HKGreen-3 (10 μM) in PBS, pH 7.4, with 0.2% DMF. The fluorescence intensity of sample solutions was determined at 535 nm with excitation at 520 nm. (a) The fluorescence intensity after irradiation of P-NAP with UV-A light (without attenuation) or incubation for 15 min: (i) solutions without P-NAP incubated in the dark; (ii) solutions including P-NAP incubated in the dark; (iii) solutions without P-NAP after irradiation; (iv) solutions including P-NAP after irradiation; (v) solutions including P-NAP after irradiation under an argon atmosphere; (vi) solutions including P-NAP and SOD (1 kunit/mL) after irradiation; (vii) solutions without SIN-1 incubated at 37 °C for 2 h; and (viii) solutions including SIN-1 (10 μM) incubated at 37 °C for 2 h. $p < 0.01$ for (iv) versus (i)-(iii), (v), and (vi) by multiple t test ($n = 3$). (b) Fluorescence intensity after irradiation of P-NAP for various time periods with various light intensities. Light intensity was not attenuated (●) or attenuated to 25% (■), 6% (○), or 1.5% (□). Error bars were calculated from three independent measurements.

that of molecular dioxygen is −180 mV (vs standard hydrogen electrode).[19] The reduction potentials of the *N*-methylquinoneimine derivatives are expected to be more negative than those of the corresponding 1,4-benzoquinones due to lower electronegativity of the nitrogen atom than that of the oxygen atom, so it seems reasonable that the tetramethyl intermediate can reduce molecular oxygen to produce $O_2^{\bullet-}$.

Next, to examine the DNA cleavage reactivity of P-NAP, an aqueous solution of pBR322 plasmid DNA was photoirradiated with UV-A light (without attenuation) in the presence of P-NAP. The irradiated solution was subjected to 1% agarose gel electrophoresis. After horizontal gel electrophoresis, the gel was stained with ethidium bromide, with UV translumination to visualize DNA. As shown in Figure 4, DNA strand breakage was concentration-dependent and was not observed in the solution irradiated without P-NAP.

Finally, photogeneration of $ONOO^-$ from P-NAP in HCT-116 cells was examined with acetylated HKGreen-3 (HKGreen-3A), which can permeate through the cell membrane. HCT-116 cells were treated with HKGreen-3A and P-NAP and then irradiated with UV-A light (attenuated to 25%) for 15 min. A clear fluorescence increase in the cells was observed by confocal microscopy. In the absence of photoirradiation or P-NAP, little fluorescence was observed in the cells. Further, we conducted MTT assay after incubation in the presence of P-NAP or after photoirradiation of HCT-116 cells. P-NAP showed no cytotoxicity at concentrations less than 100 μM (IC_{50} = 462 μM). Irradiation for 15 min with UV-A attenuated to 25% did show cytotoxicity. In order to obtain clear fluorescence images in Figure 5, we nevertheless conducted irradiation at this level. However, as shown in Figure 3b, $ONOO^-$ could be generated from P-NAP by using light attenuated to the 1.5% level, which was not cytotoxic.

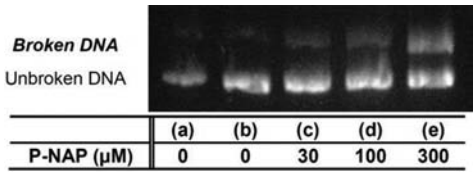


Figure 4. UV translumination photograph of DNA strand-breaking assay after horizontal gel electrophoresis. Samples containing P-NAP and pBR322 plasmid DNA (0.04 mg/mL) in PBS (pH 7.0, containing 0.3% DMF) were photoirradiated with UV-A light (without attenuation) or incubated at room temperature in the dark for 15 min. (a) The solution after incubation in the dark without P-NAP. (b) The solution after irradiation without P-NAP. (c) The solution after irradiation with P-NAP (30 μM). (d) The solution after irradiation with P-NAP (100 μM). (e) The solution after irradiation with P-NAP (300 μM).

P-NAP forms very stable white crystals, and the solution (100 mM) in DMF is stable for at least 3 months when stored at −30 °C in the dark, as determined with a UV−visible photospectrometer. This stability of the compound is favorable for biological applications of P-NAP as a $ONOO^-$ generator.

3. CONCLUSION

We have developed a novel controllable $ONOO^-$ photogenerator, P-NAP, which does not suffer from the disadvantages associated with DiP-DNB (poor water solubility and undesired reaction of the decomposition product with $ONOO^-$). NO generation from P-NAP was confirmed by ESR, and $ONOO^-$ generation was confirmed by the use of a fluorogenic probe, HKGreen-3. Further, P-NAP-mediated tyrosine nitration was detected by means of HPLC analysis. Duroquinone (**11**), which is formed after photoirradiation of P-NAP, is one of the least toxic quinone derivatives due to its four methyl groups. The results of analysis under anaerobic conditions and in the presence of SOD indicated that not only NO but also $O_2^{\bullet-}$ is generated from P-NAP upon irradiation. The results of DNA strand-breaking assay and fluorescence imaging with HKGreen-3A in HCT-116 cells indicated not only that P-NAP could release $ONOO^-$ in cultured cells but also that the generated $ONOO^-$ could act on biomolecules such as DNA. On the basis of these results, we believe that P-NAP will be a useful tool for controllable $ONOO^-$ generation in biological research.

4. EXPERIMENTAL SECTION

General Methods. Melting point was determined with a Büchi 545 melting point apparatus. Proton nuclear magnetic resonance

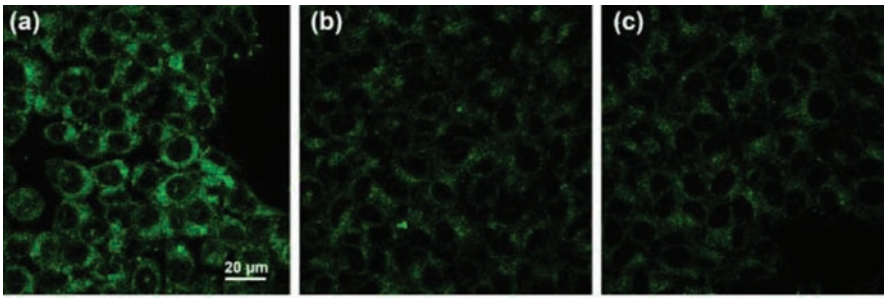


Figure 5. Fluorescence images of $ONOO^-$ generation from P-NAP in HCT-116 cells in the presence of HKGreen-3A. Cultured HCT-116 cells were treated with HKGreen-3A (10 μM) and P-NAP (10 μM). The dishes were then photoirradiated with UV-A light (attenuated to 25%) or incubated in the dark for 15 min. The dishes were observed with a confocal microscope (a) after photoirradiation with P-NAP, (b) after incubation with P-NAP in the dark, and (c) after photoirradiation without P-NAP.

spectra (^{1}H NMR) and carbon nuclear magnetic resonance spectra (^{13}C NMR) were recorded on a JEOL JNM-A500 spectrometer in the indicated solvent. Chemical shifts (δ) are reported in parts per million relative to the internal standard, tetramethylsilane. Elemental analysis was performed with Yanaco CHN CORDER NT-5 analyzer, and all values were within ±0.4% of the calculated values. The MS spectra (EI-GC) were recorded on a JEOL JMS-SX102A mass spectrometer. Ultraviolet−visible light absorbance spectra were recorded on an Agilent 8453 spectrometer. All other reagents and solvents were purchased from Aldrich, Tokyo Kasei Kogyo, Wako Pure Chemical Industries, Nacalai Tesque, Kanto Kagaku, Kishida Kagaku, Junsei Kagaku, and Dojindo and used without purification. Flash column chromatography was performed using silica gel 60 (particle size 0.046−0.063 mm) supplied by Merck.

Synthesis of 6. To a solution of **5** (1.81 g, 8.50 mmol) in MeCN (30 mL) was added NO_2BF_4 (1.49 g, 11.2 mmol, 1.3 equiv). The mixture was stirred at room temperature for 10 min. The reaction mixture was quenched with water and extracted with $CHCl_3$. The organic layer was washed with brine and dried over Na_2SO_4. Filtration and evaporation of the filtrate *in vacuo* gave 2.28 g (quant.) of **6** as a white solid: ^{1}H NMR ($CDCl_3$, 500 MHz; δ, ppm) 2.44 (6H, s), 2.21 (6H, s).

Synthesis of 7. To a solution of **6** (2.28 g, 8.85 mmol) in 1,4-dioxane (20 mL) were added Pd_2dba_3 (84 mg, 0.0917 mmol, 0.01 equiv) and 2-di-*tert*-butylphosphino-2′,4′,6′-triisopropylbiphenyl (75 mg, 0.177 mmol, 0.02 equiv), followed by 1 N KOH (20 mL). The reaction mixture was stirred at 90 °C under a N_2 atmosphere for 24 h. After cooling, the reaction mixture was acidified with 2 N HCl and filtered. The filtrate was extracted with AcOEt. The organic layer was washed with brine and dried over Na_2SO_4. Filtration, evaporation of the filtrate *in vacuo*, and purification of the residue by silica gel flash chromatography (*n*-hexane:AcOEt = 9:1) gave 1.01 g (59%) of **7** as a yellow solid: ^{1}H NMR ($CDCl_3$, 500 MHz; δ, ppm) 4.95 (1H, s), 2.18 (6H, s), 2.15 (6H, s).

Synthesis of 8. To a solution of **7** (1.01 g, 5.19 mmol) in MeOH (15 mL) was added a catalytic amount of Raney Ni. The reaction mixture was stirred at room temperature under a H_2 atmosphere for 14 h and then filtered through Celite. The filtrate was evaporated *in vacuo* to afford 650 mg (76%) of **8** as a brown solid: ^{1}H NMR ($CDCl_3$, 500 MHz; δ, ppm) 4.15 (1H, s), 3.35 (2H, s), 2.20 (6H, s), 2.17 (6H, s).

Synthesis of 9. A mixture of Ac_2O (446 μL, 4.72 mmol, 1.2 equiv) and HCOOH (594 μL, 15.7 mmol, 1.2 equiv) was stirred at room temperature for 1 h. The mixture was added to a slurry of **8** (650 mg, 3.93 mmol) in CH_2Cl_2 (40 mL). The reaction mixture was stirred at room temperature for 10 min, MeOH was added, and the mixture was evaporated *in vacuo* to afford 770 mg (quant.) of **9** as a white solid: ^{1}H NMR ($CDCl_3$, 500 MHz; δ, ppm) 9.21 (1H, s), 8.20 (1H, d, J = 1.2 Hz), 7.97 (1H, s), 2.08 (6H, s), 1.99 (6H, s).

Synthesis of 10. To a slurry of $LiAlH_4$ (940 mg, 25.0 mmol, 6.3 equiv) in dry THF (20 mL) was added a slurry of **9** (770 mg, 3.99 mmol) on an ice−water bath. The reaction mixture was stirred at reflux for 1 h and then allowed to cool, and water (1 mL), 2 N NaOH (2 mL), and water (3 mL) were added successively. The mixture was stirred for 20 min and then filtered through Celite. The filtrate was evaporated *in vacuo*. Purification of the residue by silica gel flash chromatography (*n*-hexane:AcOEt = 4:1) gave 215 mg (30%) of **10** as a pale orange solid: ^{1}H NMR ($CDCl_3$, 500 MHz; δ, ppm) 4.40 (1H, br), 2.63 (3H, s), 2.24 (6H, s), 2.18 (6H, s), 1.60 (1H, br).

Synthesis of P-NAP (3). A solution of **10** (195 mg, 1.09 mmol) in AcOH (5 mL) was added to a solution of $NaNO_2$ (75 mg, 1.09 mmol, 1.0 equiv) in water (6 mL) on an ice−water bath. The reaction mixture was stirred on the ice−water bath for 10 min and then quenched with saturated $NaHCO_3$ and extracted with $CHCl_3$. The organic layer was washed with brine and dried over Na_2SO_4. Filtration, evaporation of the filtrate *in vacuo*, and purification of the residue by silica gel flash chromatography (*n*-hexane:AcOEt = 4:1) gave 147 mg (65%) of **3** as a white solid. This product was recrystallized from AcOEt/MeOH to afford 116 mg of **3** as white needles: mp 162.4−163.9 °C; ^{1}H NMR ($CDCl_3$, 500 MHz; δ, ppm; 3:2 mixture of two rotamers) (*major*) 4.77 (1H, s), 3.96 (3H, s), 2.16 (6H, s), 1.90 (6H, s), (*minor*) 4.84 (1H, s), 3.31 (3H, s), 2.22 (6H, s), 2.01 (6H, s); ^{13}C NMR ($CDCl_3$, 150 MHz; δ, ppm) 152.5, 152.5, 133.9, 132.4, 130.8, 130.7, 120.5, 120.3, 77.5, 40.4, 35.5, 14.9, 14.7, 12.3; MS (FAB) m/z 209 [$(M+1)^+$]. Anal. Calcd for $C_{11}H_{16}N_2O_2$: C, 63.44; H, 7.74; N, 13.45. Found: C, 63.18; H, 7.58; N, 13.29.

Irradiation of Sample Solutions. Photoirradiation in the ultraviolet-A range was performed by using the light source (100 W Hg lamp) of a fluorescence microscope (Olympus BX60/BX-FLA) with a WU filter (330−380 nm band-pass filter) at room temperature. When required, the light intensity was attenuated to 25%, 6%, or 1.5% by inserting attenuation filters.

ESR Analysis by Using Iron Dithiocarbamate Complex. A solution (total volume 200 μL) of $FeSO_4{\cdot}6H_2O$ (1.5 mM), *N*-methylglucamine dithiocarbamate (6 mM), and P-NAP (100 μM) in PBS (pH 7.0, DMF 1%) was photoirradiated (without attenuation) at room temperature for 15 min as a sample for ESR studies. Next, the appropriate SOD (1 kunit/mL) was added or the solution was irradiated under an argon atmosphere. ESR spectra were taken on a JES-RE2X spectrometer (JEOL Co. Ltd., Tokyo, Japan). The measurement conditions were follows: microwave power, 10 mW; frequency, 9.4200 GHz; field, 330 mT; sweep width, 7.5 mT; sweep time, 4 min; modulation width, 0.125 mT; time constant, 0.10 s.

HPLC Analysis for Detection of Ac-NitroTyr-OEt and Duroquinone. Sample solutions (total volume 200 μL) of P-NAP (1 mM) and *N*-acetyl-L-tyrosine ethyl ester (1 mM) in PBS (pH 7.0, containing 2% DMF) were irradiated. An aliquot of each solution (20 μL) was loaded onto a Shenshu Pak C18 column (5 mm; 150 × 4.6 mm) fitted on a Shimadzu HPLC system, and the eluates were monitored with a photodiode array detector. Milli-Q water containing 0.1% TFA (A) and MeCN containing 0.1% TFA (B) were used as developing solvents. Gradient conditions were as follows: 0 min, A 70% and B 30% → 20 min, A 20% and B 80% → 30 min, A 20% and B 80% → 35 min, A 70% and B 30% → 40 min, A 70% and B 30%.

Fluorescence Measurement of HKGreen-3 *in Vitro*. Sample solutions (total volume 1 mL) containing P-NAP (10 μM) or SIN-1 (10 μM) and HKGreen-3 (10 μM) in PBS (pH 7.4, containing 0.2%

DMF) were irradiated or incubated in the dark. The fluorescence intensity was determined with an RF5300-PC (Shimadzu) at 535 nm, with excitation at 520 nm. The slit width was set to 3 nm for both excitation and emission.

Assay for DNA Strand Breaks. Sample solutions (total volume 20 μL) of PBS (pH 7.0) containing 0.04 mg/mL of pBR 322 DNA, 0.3% DMF, and 0, 30, 100, or 300 μM P-NAP were photoirradiated with UV-A (without attenuation) or incubated at room temperature in the dark for 15 min. The reaction mixtures were treated with 5 μL of loading buffer (100 mM TBE buffer, pH 8.3, containing 30% glycerol, 0.1% bromophenol blue). Horizontal gel electrophoresis was carried out in 89 mM TBE buffer (pH 8.3) for 60 min at 100 V. The gel was stained with ethidium bromide (1 mg/mL) for 30 min, destained in TBE buffer for 30 min, and photographed with UV transillumination.

Detection of $ONOO^-$ in HCT-116 Cells. HCT-116 human colon cancer cells were treated in the same manner as in the confocal microscopy experiments. Briefly, the cells were plated on 3 cm glass-bottomed dishes at 1.0×10^5 cells/dish with 2 mL of McCoy's 5A culture medium and then incubated at 37 °C in a humidified 5% (v/v) CO_2 incubator for 2 days. The cells were treated with 10 μM HKGreen-3A and incubated at 37 °C in a humidified 5% (v/v) CO_2 incubator for a further 2 h. The cells were treated with 10 μM P-NAP for 15 min and subjected to photoirradiation for 15 min with the light intensity attenuated to 25%. The dishes were subsequently washed with DMEM three times, and the culture medium was replaced with 2 mL of DMEM. After washing, the cells were subjected to confocal fluorescence microscopy (LSM 510, Carl Zeiss Japan Co. Ltd., Tokyo, Japan).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supporting figures, syntheses of **S1**−**S3**, 1H NMR spectra for **S1**−**S3** and P-NAP, and MTT assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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