Photoinduced Upregulation of Calcitonin Gene-Related Peptide in A549 Cells through HNO Release from a Hydrophilic Photocontrollable HNO Donor

Kazuya Matsuo, Hidehiko Nakagawa,* Yusuke Adachi, Eri Kameda, Kazuyuki Aizawa, Hiroki Tsumoto,† Takayoshi Suzuki,‡ and Naoki Miyata*

Department of Organic and Medicinal Chemistry, Graduate School of Pharmaceutical Sciences, Nagoya City University; 3–1 Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467–8603, Japan.
Received April 16, 2012; accepted May 12, 2012

Nitroxyl (HNO), a one-electron-reduced form of nitric oxide, has various biological activities, including a cardioprotective effect. Here, we first synthesized another, more hydrophilic photocontrollable HNO donor (3), which can release HNO in a spatially and temporally controlled manner, and then examined the properties of our series of compounds as practical HNO donors in a cellular system under photocontrol. We selected compound 2 as the preferred donor, and used it to show that calcitonin gene-related peptide (CGRP) can be upregulated in A549 cells *via* photocontrolled HNO release. This result demonstrates the suitability of this photocontrollable HNO donor for biological investigations.

Key words nitroxyl; photo-uncaging; nitric oxide; retro hetero Diels–Alder reaction; chemical biology

Since nitric oxide (NO) was found to be biologically synthesized in mammalian systems,[1,2] many researchers have studied its signaling pathways and bioactivities. The term 'nitric monoxide' actually covers three different entities, which are nitrosyl cation (NO^+), nitric oxide, and nitroxyl (anion).[3–7] Nitroxyl (HNO), which is a one-electron-reduced form of NO, has been less well studied than the former two species, but has attracted more interest in recent years. It can activate sarcoplasmic reticulum Ca^{2+}-ATPase (SARCA)[8] and ryanodine receptor (RyR),[9,10] and induce positive inotropy.[11] Its mechanism of action is thought to be different from those of common cardiovascular drugs. Furthermore, HNO was reported to have cardioprotective activity owing to a preconditioning-like effect.[12] Therefore, HNO is expected to be a candidate for treatment of various heart disorders.[13] Vasodilating action of HNO has been reported; the mechanism is not clear, but there are many possibilities, including soluble guanylate cyclase (sGC) upregulation,[14,15] and K^+ channel activation,[16,17] which are different from those in the case of NO. It has also been suggested[18,19] that the mechanism of vasodilation by HNO partly involves a pathway mediated by calcitonin gene-related peptide (CGRP). CGRP is a neuropeptide consisting of 37 amino acids, produced by alternative mRNA splicing of the calcitonin gene, and shows vasodilatory action and ionotropic and chronotropic activities. CGRP signaling is transduced through G-protein-coupled receptor (GPCR), leading to production of cAMP.[11]

Inspired by the cycloadduct formation between 9,10-dimethylanthracene and acyl nitroso compounds reported by King and colleagues,[20,21] we have developed photocontrollable HNO donors, compounds **1** and **2**[22,23] (Fig. 1). These donors release HNO in response to photoirradiation, unlike previously reported HNO donors (Chart 1). Although Angeli's salt[24,25] ($Na_2N_2O_3$) is a widely used HNO donor and releases HNO, together with a little NO, at physiological pH, its release of HNO is spontaneous and not controllable, except by temperature. Furthermore, Angeli's salt releases NO, an oxidized form of HNO, under acidic conditions, so that pH control is important. Piloty's acid[26] ($PhSO_2NHOH$) and cyanamide[27] (NH_2CN) also offer only uncontrolled HNO release. King and colleagues reported a useful type of HNO donor, acyloxy nitroso compounds,[28] which have the property of slow release of HNO; they have been useful to elucidate HNO actions,[29] but the HNO release is still uncontrollable.

Photocontrol of HNO release permits precise temporal and spatial control of HNO treatment, and therefore photocontrollable HNO donors are expected to be superior tools to examine the biological effects of HNO, *e.g.*, in cell cultures. Here, we developed another hydrophilic photocontrollable HNO donor, compound **3**, and examined the suitability of our series of compounds (Fig. 1) for use as practical HNO donors in cellular systems under photocontrol.

Experimental

Synthesis of Compound 3. General Methods Melting points were determined using a Yanaco micro melting point apparatus or a Büchi B-545 melting point apparatus and were left uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra and carbon nuclear magnetic resonance (^{13}C-NMR) spectra were recorded on a JEOL JNM-LA500 or JEOL JNM-A500 spectrometer in the indicated solvent. Chemical shifts (δ) are reported in parts per million (ppm) relative to the internal standard tetramethylsilane (TMS). Elemental analysis was performed with a Yanaco CHN CORDER NT-5 analyzer, and all values were within $\pm$0.4% of the calculated values. UV/vis spectra were measured using an Agilent 8453 spectrometer. IR spectra were recorded on an Avatar360. GC-MS analyses were performed on a Shimadzu GCMS-QP2010. FAB-MS were recorded on a JEOL JMS-SX102A mass spectrometer. Reagents and solvents were purchased from Aldrich, Tokyo Kasei Kogyo, Wako Pure Chemical

The authors declare no conflict of interest.

† Present address: *Kyoto University Graduate School of Pharmaceutical Sciences; 46–29 Yoshida-Shimo-Adachi-cho, Sakyo-ku, Kyoto 606–8501, Japan.*

‡ Present address: *Kyoto Prefectural University of Medicine; 13 Taisho-gun Nishitakatsukasa-cho, Kita-ku, Kyoto 403–8334, Japan.*

* To whom correspondence should be addressed. e-mail: deco@phar.nagoya-cu.ac.jp; miyata-n@phar.nagoya-cu.ac.jp

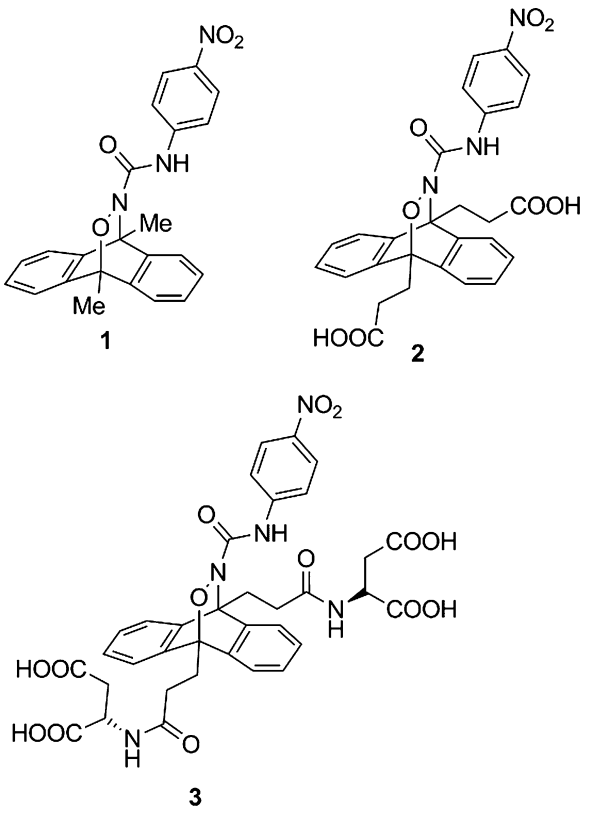


Fig. 1. Photocontrollable HNO Donors in This Study

Industries, and Kanto Kagaku and used without further purification. Flash chromatography was performed using Silica Gel 60 (particle size 0.046–0.063 mm) supplied by Merck.

Di-*tert*-butyl 3,3′-(9,10-Anthracenediyl)bis(2-propenoate) (5) A solution of **4** (3360 mg, 10 mmol), *t*-butyl acrylate (29 mL, 200 mmol), Et_3N (28 mL, 200 mmol), $Pd(OAc)_2$ (246.96 mg, 1.10 mmol), and tri-*o*-tolylphosphine (669.6 mg, 2.20 mmol) in *N*,*N*-dimethylformamide (DMF) (100 mL) was heated at 120°C for 4 h. After cooling, water and AcOEt were added. The organic solution was evaporated under reduced pressure to give a residue, which was purified by silica gel flash chromatography (AcOEt–*n*-hexane=1 : 10) to give **5** (4163 mg, 97%) as a yellow solid: ^{1}H-NMR ($CDCl_3$, 500 MHz, δ; ppm) 8.51 (2H, d, J=16.1 Hz), 8.26 (4H, dd, J=3.2, 6.8 Hz), 7.53 (4H, dd, J=3.2, 6.8 Hz), 6.32 (2H, d, J=16.1 Hz), 1.63 (18H, s).

Di-*tert*-butyl 9,10-Anthracenedipropanoate (6) $Pd(OAc)_2$ (89.8 mg, 0.40 mmol) was added to a stirred solution of **5** (1722.2 mg, 4.0 mmol) in DMF (300 mL) at 80°C, and HCOOK (1776.6 mg, 21.1 mmol) was added. The reaction mixture was stirred for 29 h. Then, AcOEt and water were added to the cooled mixture. The AcOEt layer was separated, washed with brine, and dried over Na_2SO_4. The solvent was evaporated and the residue was purified by silica gel flash column chromatography (toluene–*n*-hexane=1 : 4) to give 1046.4 mg (60%) of **6** as a yellow solid: ^{1}H-NMR ($CDCl_3$, 500 MHz, δ; ppm) 8.33 (4H, dd J= 3.2, 6.8 Hz), 7.54 (4H, dd, J=3.2, 6.8 Hz), 3.92 (4H, t, J=8.51 Hz), 2.69 (4H, t, J=8.51 Hz), 1.49 (18H, s).

9,10-Anthracenedipropanoic Acid (7) Eleven milliliters of trifluoroacetic acid (TFA) was added to a solution of **6** (1046 mg, 2.41 mmol) in 20 mL of CH_2Cl_2. The reaction mixture was stirred at room temperature for 1 h. The reaction mixture was concentrated by evaporation *in vacuo* and the residue was suspended in *n*-hexane. Filtration and recrystallization from tetrahydrofuran (THF)–*n*-hexane gave 591 mg (76%) of **7** as a yellow solid: ^{1}H-NMR (DMSO, 500 MHz, δ; ppm) 12.33 (2H, br), 8.36 (4H, dd J=3.0, 7.0 Hz), 7.59 (4H, dd, J=3.2, 6.9 Hz), 3.86 (4H, t, J=8.2 Hz), 2.62 (4H, t, J=8.1 Hz).

(*S*,*S*)-Di-*tert*-butyl 2-(3-{10-[2-(1-*tert*-Butoxycarbonylmethyl-2-isopropoxy-ethylcarbamoyl)ethyl]anthracen-9-yl}propionylamino)butanedioate (8) A solution of **7** (200 mg, 0.621 mmol), H-Asp(OtBu)–OtBu·HCl (350 mg, 1.24 mmol), EDCI (357.1 mg, 1.86 mmol), HOBt·H_2O (285.3 mg, 1.86 mmol), and Et_3N (0.17 mL) in 35 mL of DMF was stirred at room temperature for 3 h. The reaction mixture was poured into water and extracted with AcOEt. The organic layer was separated, washed with brine, and dried over Na_2SO_4. Filtration, evaporation of the solvent *in vacuo*, and purification of the residue by silica gel flash column chromatography (AcOEt–*n*-hexane= 0 : 1 to 1 : 1) gave 281.6 mg (58%) of **8** as a yellow solid: ^{1}H-NMR (DMSO, 500 MHz, δ; ppm) 8.42 (2H, d J=7.9 Hz), 8.37 (4H, dd, J=3.3, 5.3 Hz), 7.59 (4H, dd, J=3.0, 5.4 Hz), 4.55 (2H, q, J=7.3 Hz), 3.79 (4H, t J=8.2 Hz), 2.65 (2H, m), 2.55 (4H, overlap), 1.42 (36H, s).

(*S*,*S*)-2-(3-{10-[2-(1-*tert*-Butoxycarbonylmethyl-2-isopropoxy-ethylcarbamoyl)ethyl]anthracen-9-yl}propionylamino)butanedioic Acid (9) Two milliliters of TFA was added to a solution of **8** (265.2 mg, 0.34 mmol) in 15 mL of CH_2Cl_2. The reaction mixture was stirred at room temperature for 24 h. The mixture was concentrated by evaporation *in vacuo* and the residue was dissolved in TFA (15 mL). The solution was stirred at room temperature for 12 h, then evaporated *in vacuo* and the residue was recrystallized from THF–*n*-hexane to give 151.9 mg (74%) of **9** as a yellow solid: ^{1}H-NMR (CD_3OD, 500 MHz, δ; ppm) 8.40 (4H, dd, J=3.3, 5.1 Hz), 7.54 (4H, dd, J=3.0, 5.0 Hz), 4.75 (2H, t, J=6.9 Hz), 3.95 (4H, t, J=7.9 Hz), 2.71 (4H, m), 2.70 (4H, m).

***N*-(Tetrahydropyran-2-yl)oxy-*N*′-(4-nitrophenyl)urea (11)** To a solution of NH_2OTHP (936 mg, 8 mmol) in dehydrated THF (16 mL) was added dropwise **10** (1313 mg, 8 mmol) at 0°C. The reaction mixture was stirred for 40 min at 0°C. The solvent was removed, and purification of the residue by silica gel flash chromatography (AcOEt–*n*-hexane=2 : 3) gave 2256 mg (100%) of **11** as a yellow solid: ^{1}H-NMR ($CDCl_3$, 500 MHz, δ; ppm) 8.63 (1H, s), 8.21 (2H, d, J=9.0 Hz), 7.63 (2H, d, J=9.0 Hz), 7.33 (1H, s), 4.86 (1H, m), 4.09 (1H, m), 3.66 (1H, m), 1.90 (2H, m), 1.64 (4H, m).

***N*-Hydroxy-*N*′-(4-nitrophenyl)urea (12)** To a solution of **11** (1950 mg, 6.9 mmol) in MeOH (150 mL) was added TsOH·H_2O (119 mg, 0.69 mmol). The reaction mixture was stirred for 23 h at room temperature. The solution was concentrated under reduced pressure, and purification of the residue by silica gel flash chromatography (AcOEt–*n*-hexane=3 : 1) gave 1068 mg (79%) of **12** as a yellow solid: ^{1}H-NMR (DMSO-d_6, 500 MHz, δ; ppm) 9.51 (1H, s), 9.29 (1H, s), 9.16 (1H, s), 8.16 (2H, d, J=9.5 Hz), 7.92 (2H, d, J=9.5 Hz).

(*S*,*S*)-9,10-[Dihydro-*N*-4-nitrophenyl-9,10-(epoxyimino)-11-carbamylanthracene-9,10-(propionylamino)-butanedioic Acid (3) To a suspension of **9** (70 mg, 0.127 mmol) and $NaIO_4$ (54.2 mg, 0.253 mmol) in 10 mL of acetone was added a solution of **12** (49.9 mg, 0.253 mmol) in 7 mL of acetone and 10 mL of H_2O over a period of 20 min

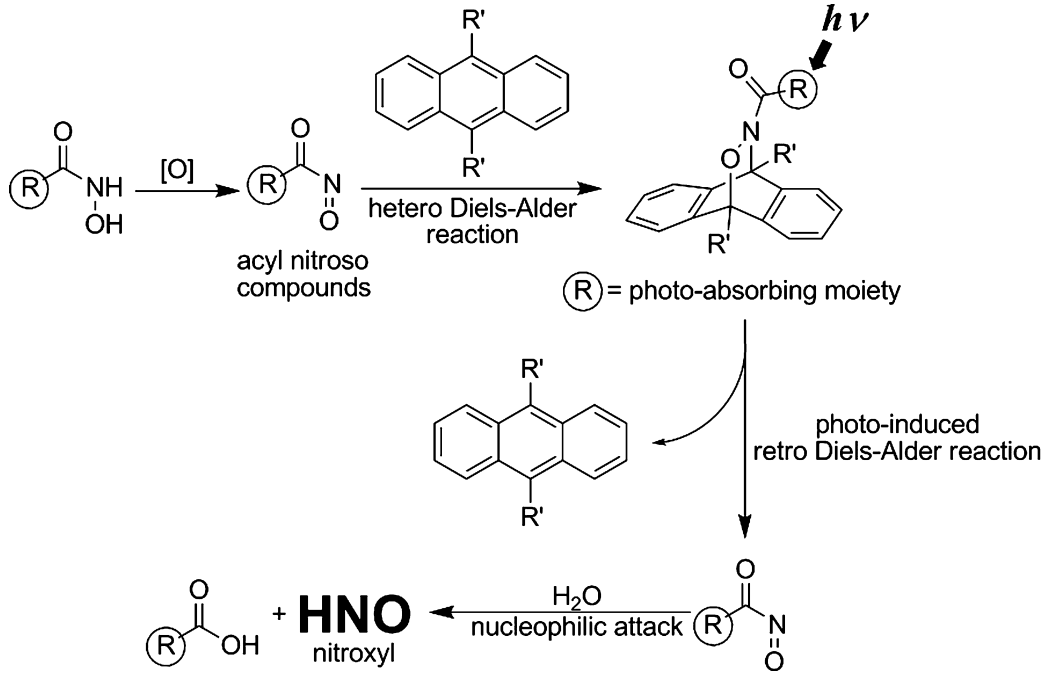


Chart 1. Hetero Diels–Alder Reaction and Photo-Induced Retro Diels–Alder Reaction

at 0°C. The reaction mixture was stirred for 5 h at 0°C, then poured into water and extracted with AcOEt. The organic layer was washed with 4 N HCl and brine, and dried over Na_2SO_4. Filtration, concentration *in vacuo*, and precipitation with *n*-hexane gave 48.5 mg (51%) of **3** as a light yellow solid: decomp. point 100.8–102.1°C; UV/Vis (DMSO–Milli Q=1 : 9): $\varepsilon_{323\,nm}=1.22\times10^4\,M^{-1}\,cm^{-1}$; ^{1}H-NMR (DMSO-$d_6$, 500 MHz, δ; ppm) 9.39 (1H, br), 8.55 (1H, d, J=7.6 Hz), 8.38 (1H, d, J=8.2 Hz), 8.09 (2H, d, J=9.1 Hz), 7.76 (2H, d, J=9.1 Hz), 7.55 (4H, m), 7.30 (4H, m), 4.64 (2H, m), 2.50 (2H, overlap), 3.16 (2H, m), 2.55–2.78 (8H, m); ^{13}C-NMR (DMSO-d_6, 125 MHz, δ; ppm); 173.15, 172.58, 172.28, 171.90, 171.73, 171.55, 158.88, 145.02, 141.89, 128.85, 127.28, 126.98, 126.39, 125.53, 124.66, 118.54, 112.36, 81.13, 66.07, 48.96, 48.79, 36.11, 35.91, 30.99, 28.96, 24.90, 22.22; MS (FAB) *m/z*: 748 ($[M+H]^+$); *Anal.* Calcd for $C_{35}H_{33}N_5O_{14}\cdot3H_2O$: C, 52.43; H, 4.90; N, 8.74. Found: C, 52.46; H, 4.83; N, 8.63.

Photoirradiation Photoirradiation with UV A (UVA) was performed by using the light source (100 W mercury lamp) of a fluorescence microscope (Olympus BX60/BX-FLA) with a WU filter (330–380 nm band-pass filter). Photoirradiation *in vitro* was performed under Ar- or He-purged anaerobic conditions, and that in cellular experiments was done under aerobic conditions. The light intensity was attenuated to 1.5% with a combination of a 6% ND filter and a 25% ND filter.

Detection of N_2O by Gas Chromatography A solution of compound **1** (1.5 μmol) in a mixed solution of dimethyl sulfoxide (DMSO) (2700 μL) and 50 mM Tris buffer pH 7.5 (300 μL), a solution of compound **2** (1.5 μmol) in DMSO (300 μL) and 50 mM Tris buffer pH 7.5 (2700 μL), or a solution of compound **3** (1.5 μmol) in 50 mM Tris buffer pH 7.5 (3000 μL) was placed in a 4-mL cuvette sealed with a rubber septum. For HNO scavenging experiments, 2-mercaptoethanol (1.05 μL) was added to the solution. The sample solution was photoirradiated with UVA, or allowed to stand without photoirradiation for the designated time at room temperature. The cuvette was then shaken and placed in an ice bath for 30 min for equilibration of N_2O in the headspace of the cuvette without further decomposition of the donor compound. An aliquot of the reaction headspace gas (50 μL) was injected onto a Shimadzu GC-2010 gas chromatograph equipped with a mass spectrometer (QP2010, Shimadzu Corp., Kyoto, Japan) and an Rt-QPLOT column (0.32 mm × 15 m) (Restek, Bellefonte, PA, U.S.A.) attached with a 15-m inactivated fused silica capillary (total 30 m). The GC injector was operated with a split ratio of 0.1 at 200°C. The carrier gas (He) flow rate was set at 2.2 mL/min. The GC oven was held at 35°C. The MS interface was set to 280°C. N_2O formation was calculated based on the decomposition of Angeli's salt. On the chromatogram in GC-MS analysis, the peak of N_2O was partly overlapped with the large CO_2 peak in many cases. To obtain the peak area of N_2O, the area of N_2O on the tail of the CO_2 peak was determined by calculation.

Reaction between HNO and 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, Sodium Salt (Carboxy-PTIO). N_2O Detection in Fig. 3 A solution of Angeli's salt (1.5 μmol) or compound **2** (1.5 μmol) with carboxy-PTIO (1.5 μmol) in a mixed solution of DMSO (300 μL) and 50 mM Tris buffer pH 7.5 (2700 μL) was placed in a 4-mL cuvette sealed with a rubber septum. The sample solution was incubated for 30 min at 37°C. The cuvette was then shaken and placed in an ice bath for 30 min for equilibration of N_2O in the headspace of the cuvette without further decomposition of the donor compound. An aliquot of the reaction headspace gas was analyzed by the same method as described above.

Reaction of 1-Hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC7), Angeli's Salt, or Compound 2 with Carboxy-PTIO *in Vitro* A solution of NOC7 (1.5 μmol), Angeli's salt (1.5 μmol), or compound **2** (1.5 μmol) with carboxy-PTIO (1.5 μmol) in a mixed solution

of DMSO (300 μL) and 50 mM Tris buffer pH 7.5 (2700 μL) was placed in a 5 mL vial. The sample solutions were incubated without photoirradiation for 30 min at 37°C, and photographed.

Cell Culture A549 cells were obtained from RIKEN Cell Bank and grown in Dulbecco's modified Eagle's medium (DMEM) containing penicillin and streptomycin (100 U/mL) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified CO_2 incubator. When confluent, cells were detached in trypsin solution, washed with DMEM, centrifuged at 125×***g*** for 5 min, and then resuspended and subcultured according to the standard protocol. For the experiments, the cells were washed with DMEM twice and maintained in DMEM without FBS at 37°C for 4 h, before treatment with chemicals.

Detection of CGRP Release in A549 Cells in Response to HNO Released from the Donors A549 cells were plated at 2×10^5 per dish and cultured in 6-cm tissue culture dishes. After reaching confluence, cells were treated with chemicals (final DMSO concentration=0.09% in the culture medium) and/or exposed to photoirradiation (UVA) for 10 min, then washed with DMEM without FBS, and incubated in the culture media in a humidified CO_2 incubator at 37°C for 24 h. The treated cells were washed with D-PBS, homogenized in 2 N acetic acid, heated at 90°C for 10 min, and centrifuged. The supernatant was dried *in vacuo*, and then dissolved in the assay buffer. CGRP concentrations were determined using a CGRP (human) EIA Kit from Cayman Chemical (Ann Arbor, MI, U.S.A.).

Results

First, we modified our previous hydrophilic HNO donor (compound **2**),[23] which has two carboxylate groups, to examine whether a compound bearing four carboxylate groups (compound **3**) would be superior in a cellular environment (Fig. 1). Briefly, the hydrophilic anthracene (**7**) was synthesized by the reported method,[30] and conjugated with two molecules of protected L-aspartic acid by condensation reaction to obtain the highly hydrophilic anthracene (**9**) (Chart 2). The *N*-hydroxyurea derivative with a *p*-nitrophenyl group was converted into the corresponding acyl nitroso compound *in situ* by oxidation, and then trapped with the synthesized anthracene derivative *via* hetero Diels–Alder reaction to obtain compound **3** (Chart 3). Compounds **1**–**3** have a *p*-nitrophenyl group as the photo-absorbing moiety, and their absorption spectra indicated that they would be activated by UVA (330–380 nm) irradiation (see Supplemental information).

We confirmed photoinduced release of HNO from compounds **1**–**3** and compared their ability to release HNO *in vitro*. The most distinctive feature of HNO chemistry is dimerization reaction to form N_2O (nitrous oxide).[31] N_2O can be easily measured by gas chromatography-mass spectrometry (GC-MS), and most studies on HNO donors have used N_2O measurements to evaluate HNO production. So, we measured N_2O to compare HNO production from the compounds, as previously reported.[22] Compound **1** was evaluated in a solution of DMSO–Milli Q water (=9 : 1), compound **2** in a solution of DMSO–Milli Q water (=1 : 9), and compound **3** in 50 mM Tris buffer (pH 7.5), due to their different solubility characteristics. The formation of N_2O from compounds **1**–**3** was found to be accelerated by photoirradiation, but the extent of acceleration was less for compound **3** in comparison with compounds **1** and **2** (Fig. 2). The conversion rates in the dark and under photoirradiation were also calculated from the absorption change at 400 nm, which is characteristic for anthracene derivatives, and we confirmed that photoirradiation-induced conversion of compound **3** was less efficient, although the conversion was accelerated by photoirradiation (see Fig. S1 in the Supplemental information). Considering these results and the solubility of the compounds in aqueous solution, we selected compound **2** for further studies on the cellular effect of photoinduced HNO release. Compound **2** was also found to be the most stable among the synthesized donors in the dark.

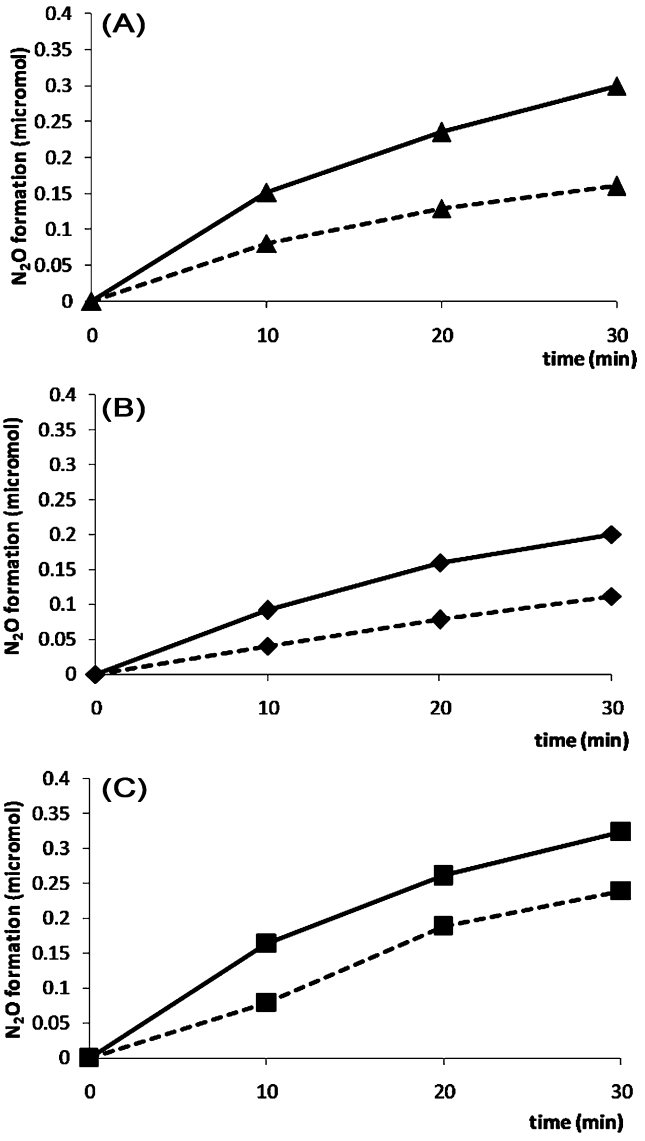
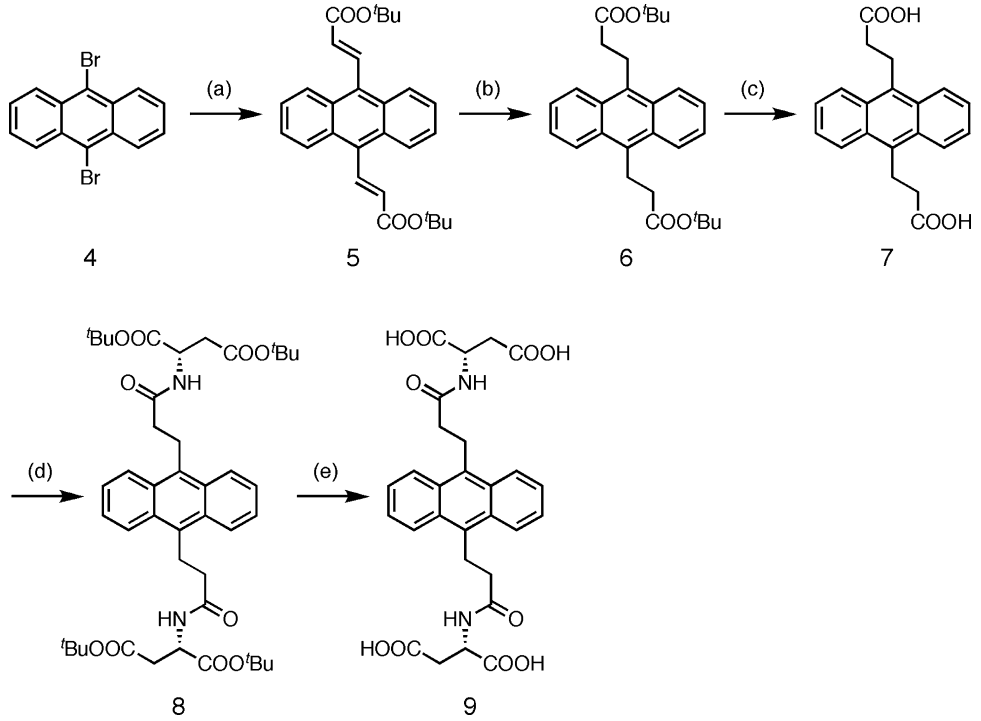


Fig. 2. Amounts of N_2O Released from Compounds **1** (A), **2** (B), and **3** (C) Measured by GC-MS

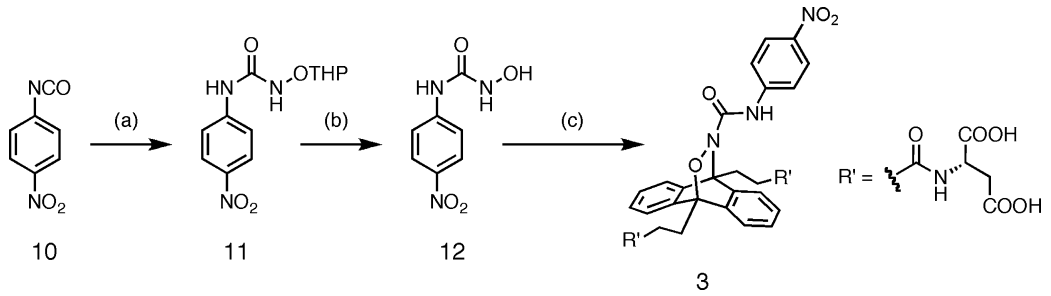
Solid lines indicates the results obtained under photoirradiation, and broken lines indicate those obtained in the dark.

To evaluate compound **2**, as a controllable HNO donor in biological systems, we focused on intracellular induction of CGRP. CGRP is produced in many primary-cultured cells, as well as some cultured cell lines, including A549 cells.[32] So, we treated A549 cells with compound **2** and evaluated the intracellular level of CGRP in the cells. To measure CGRP concentration, we employed a CGRP measurement kit from



(a) *t*-Butyl acrylate, *o*-tolylphosphine, $Pd(OAc)_2$, Et_3N, DMF, 120°C, 97% yield; (b) HCOOK, $Pd(OAc)_2$, DMF, 60°C, 60% yield; (c) TFA, CH_2Cl_2, 76% yield; (d) H-Asp(O*t*Bu)-O*t*Bu·HCl, HOBt·H_2O, EDCI, DMF, 58% yield; (e) TFA, 74% yield. DMF=*N*,*N*-dimethylformamide, TFA=trifluoroacetic acid, HOBt=1-hydroxybenzotriazole, EDCI=1-ethyl-3-(3-dimethiaminopropyl)carbodiimide hydrochloride.

Chart 2



(a) NH_2OTHP, dry THF, 100% yield; (b) TsOH·H_2O, MeOH, 79% yield; (c) **9**, $NaIO_4$, acetone/H_2O, 50% yield. THP=2-tetrahydropyranyl, TsOH=*p*-toluenesulfonic acid.

Chart 3

SPI Bio (Cayman Chemical, Ann Arbor, MI, U.S.A.). Angeli's salt was used as a positive control, and NOC7,[33] a widely used NO donor, was used to confirm the effect of NO on CGRP production. *N*-Acetyl-L-cysteine (NAC) was used as an HNO trap.[34]

We initially considered the use of carboxy-PTIO[35] as a scavenger for NO to discriminate the effect of NO from that of HNO itself. However, previous reports[15,36] have suggested that HNO from Angeli's salt might react with carboxy-PTIO to form NO. Therefore, we investigated whether carboxy-PTIO is a suitable reagent for selectively trapping NO, before conducting cellular experiments. If HNO does not react with carboxy-PTIO, this agent would be a powerful tool for selectively studying the effects of HNO. Angeli's salt (0.50 mM) or compound **2** (0.50 mM) was incubated with or without carboxy-PTIO (0.50 mM) for 30 min at 37°C in the dark, and N_2O formation was determined by means of the GC-MS method (Fig. 3). The amount of N_2O derived from Angeli's salt was found to be significantly reduced, but N_2O derived from compound **2** was less affected by carboxy-PTIO. Simultaneously, the experimental solution of Angeli's salt with carboxy-PTIO, which is originally blue, was bleached, probably due to conversion of carboxy-PTIO to carboxy-PTI by one-electron reduction, whereas the solution of compound **2** with carboxy-PTIO remained blue (Fig. 4). This result means that carboxy-PTIO does not work as a pure NO scavenger, but is also a scavenger for HNO. So, we did not use it in our CGRP assay experiments.

The results of CGRP assay in A549 cells are shown in Figs. 5–8. First, we confirmed that HNO treatment of A549 cells upregulates cellular CGRP. Angeli's salt, a typical spontaneous HNO donor, dramatically increased the level of CGRP (Fig. 5) at 50 μM, but NOC7 was ineffective (see Supplementary information). Moreover, the increase of CGRP by Angeli's salt was not observed in the presence of NAC (2 mM), a scavenging reagent for HNO (Fig. 6). These results indicate that HNO induces CGRP production in A549 cells, but NO does not.

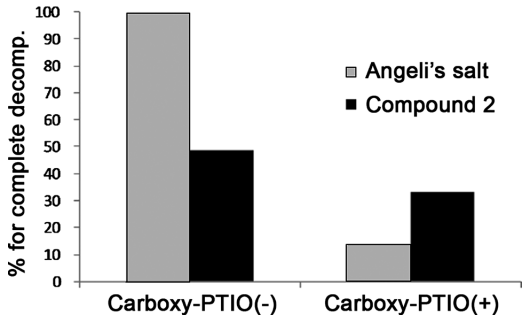


Fig. 3. Amounts of N_2O Release from Angeli's Salt and Compound **2** in the Absence or Presence of Carboxy-PTIO Measured by GC-MS, as a Percentage of the Amounts Corresponding to Complete Decomposition

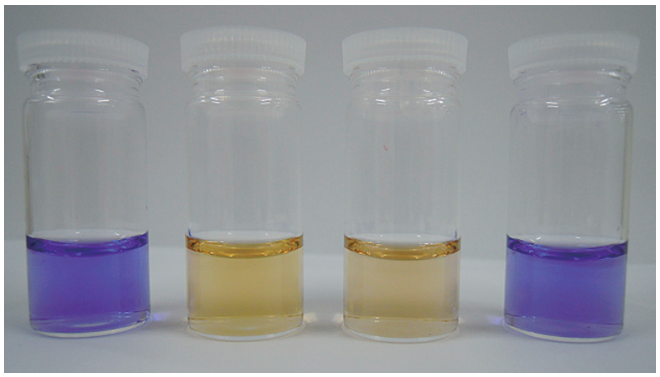


Fig. 4. Photograph of Reaction Mixtures Containing Carboxy-PTIO only (Far Left), Carboxy-PTIO+NOC7 (Second Left), Carboxy-PTIO+Angeli's Salt (Second Right), and Carboxy-PTIO+Compound **2** (Far Right)

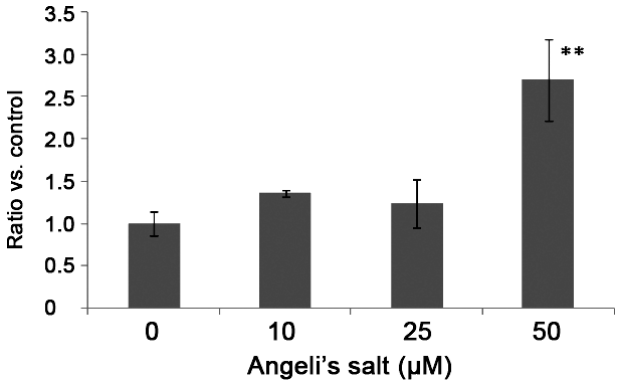


Fig. 5. Effect of HNO on CGRP Level in A549 Cells Using Angeli's Salt as a HNO Generator

$^{**}p<0.01$ when compared with 0 μM by ANOVA and Bonferroni-type multiple *t*-test (parametric, $n=3$–6).

Next, our photocontrollable HNO donor, compound **2**, was examined. In the dark, the CGRP level was not significantly changed (Fig. 7), but upon photoirradiation, it was increased (Fig. 8A). These results indicate that compound **2** photoirradiation-dependently induced CGRP upregulation. Furthermore, NAC abolished the effect of compound **2** (Fig. 8B).

Overall, these results indicate that HNO from compound **2**, as well as Angeli's salt, accelerated CGRP production in A549 cells. They also confirm that compound **2** can function as photocontrollable HNO donor under biological conditions.

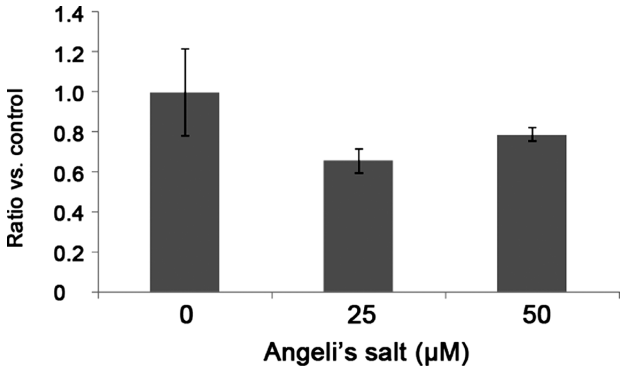


Fig. 6. Change of CGRP Level in A549 Cells in the Presence of Angeli's Salt+NAC (2 mM) ($n=3$)

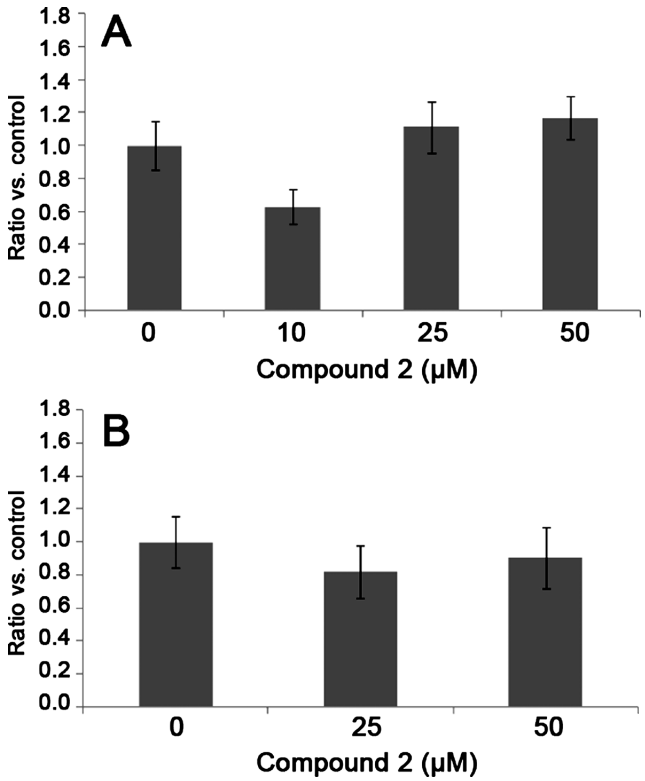


Fig. 7. Change of CGRP Level in A549 Cells in the Presence of Compound **2** (A) and Compound **2**+NAC (B) in the Dark ($n=3$)

Discussion

We synthesized a novel, highly hydrophilic photocontrollable HNO donor compound **3**, based on our previously reported compounds **1** and **2**. Although the highly hydrophilic nature of **3** was expected to be advantageous for biological applications, compound **3** was found to be unstable under ambient conditions even in the dark. This might be partly due to the linker length of the carboxyl groups. In the previous report, we suggested that intramolecular interaction of the carboxylic acid group with the heterocyclic part of the molecule would contribute to the unexpected stability of compound **2**.[23] But, in compound **3**, the carboxylic acid might be located too far from the core part of the molecule to permit significant intramolecular interaction. Therefore, we chose compound **2** for further evaluation because of its stability in aqueous solution.

HNO formation from the donor compounds in response to

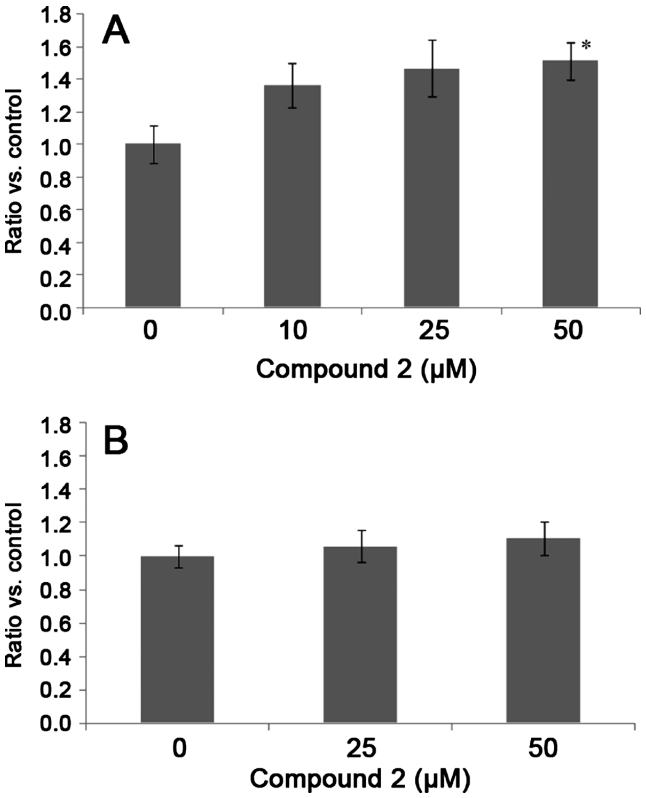


Fig. 8. Change of CGRP Level in A549 Cells in the Presence of Compound **2** as a HNO Generator (A) and in the Presence of Compound **2**+NAC (B), under Photoirradiation

*$p<0.05$ when compared with 0 μM by ANOVA and Bonferroni-type multiple *t*-test (parametric, $n=3–6$).

photoirradiation is believed to occur *via* retro Diels–Alder type reaction, followed by hydrolysis of the resulting acylnitroso intermediate (Chart 1). Although retro Diels–Alder reaction as a photochemical process is forbidden according to the Woodward–Hoffmann rule, we considered that the absorbed energy from photoirradiation could be partly converted into thermal (internal) energy of the molecule, for example, through a non-radiative transition-like process. We previously showed that the conjugated system is important for this type of retro Diels–Alder reaction.[22] This seems to be a unique example of a photo-triggered retro Diels–Alder reaction.

To assess the effect of HNO released from compound **2** on cellular CGRP regulation, we chose A549 human alveolar basal epithelial carcinoma cells, which have been reported to produce CGRP.[32] Considering that compound **2** exhibited HNO release upon photoirradiation *in vitro* (Fig. 2B) and Angeli's salt induced CGRP upregulation in A549 cells (Figs. 5, 6), the increase of CGRP in the cells treated with compound **2** in combination with photoirradiation was considered to have been induced *via* HNO formation. Suppression of this effect by NAC, an HNO trap, was considered to support this conclusion although NAC may have several effects on the cells. The results also imply that HNO might act as an important regulatory factor of pathways in which CGRP is involved.

The generation of CGRP in response to HNO has so far been observed only in *in vivo* and *ex vivo* experiments. In this report, we show that HNO released from compound **2** could induce CGRP upregulation in the A549 cell line. Therefore, photocontrolled release of HNO from compound **2** should be an effective approach for *in vitro* mechanistic studies of the biological effects of HNO and CGRP.

In our hands, carboxy-PTIO was unsuitable for HNO scavenging experiments. It has been suggested that the rate of HNO release might influence the reactivity between carboxy-PTIO and HNO, and a high HNO release rate might result in a complex reaction.[37] Angeli's salt is a spontaneous HNO releaser and its release rate of HNO is considered to be concentration-dependent, being high in the early stage and then exponentially decreasing. The combination of Angeli's salt and carboxy-PTIO was often used in previous studies, but this reactivity had not been taken into consideration, so at least some of the reported findings might need to be reassessed. Furthermore, Angeli's salt might not work as an efficient HNO donor in the presence of carboxy-PTIO. On the other hand, unirradiated compound **2** did not show a significant reaction with carboxy-PTIO, as indicated by the lack of color change. Considering that compound **2** slowly releases HNO even in the dark, as shown in Fig. 2, this might mean that controlled HNO release from HNO donors such as compound **2** is compatible with carboxy-PTIO.

In conclusion, we confirmed that hydrophilic photocontrollable HNO donors release HNO upon photoirradiation in an aqueous environment. Further, we found that photoinduced HNO release from compound **2** induces an increase of CGRP production in cultured A549 cells. Therefore, compound **2** is expected to be a useful chemical tool for *in vitro* studies of the biological functions of HNO and CGRP.

Acknowledgments This work was supported in part by Grants-in-Aid for Scientific Research on Innovative Areas (Research in a Proposed Research Area) (No. 21117514 to H.N.) and Grants-in-Aid for Scientific Research (No. 22590103 to H.N.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1) Palmer R. M. J., Ferrige A. G., Moncada S., *Nature* (London), **327**, 524–526 (1987).
2) Ignarro L. J., Buga G. M., Wood K. S., Byrns R. E., Chaudhuri G., *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 9265–9269 (1987).
3) Fukuto J. M., Bartberger M. D., Dutton A. S., Paolocci N., Wink D. A., Houk K. N., *Chem. Res. Toxicol.*, **18**, 790–801 (2005).
4) Paolocci N., Jackson M. I., Lopez B. E., Miranda K., Tocchetti C. G., Wink D. A., Hobbs A. J., Fukuto J. M., *Pharmacol. Ther.*, **113**, 442–458 (2007).
5) Irvine J. C., Ritchie R. H., Favaloro J. L., Andrews K. L., Widdop R. E., Kemp-Harper B. K., *Trends Pharmacol. Sci.*, **29**, 601–608 (2008).
6) Switzer C. H., Flores-Santana W., Mancardi D., Donzelli S., Basudhar D., Ridnour L. A., Miranda K. M., Fukuto J. M., Paolocci N., Wink D. A., *Biochim. Biophys. Acta*, **1787**, 835–840 (2009).
7) Fukuto J. M., Switzer C. H., Miranda K. M., Wink D. A., *Annu. Rev. Pharmacol. Toxicol.*, **45**, 335–355 (2005).
8) Lancel S., Zhang J., Evangelista A., Trucillo M. P., Tong X. Y., Siwik D. A., Cohen R. A., Colucci W. S., *Circ. Res.*, **104**, 720–723 (2009).
9) Cheong E., Tumbev V., Abramson J., Salama G., Stoyanovsky D. A., *Cell Calcium*, **37**, 87–96 (2005).
10) Tocchetti C. G., Wang W., Froehlich J. P., Huke S., Aon M. A., Wilson G. M., Di Benedetto G., O'Rourke B., Gao W. D., Wink D. A., Toscano J. P., Zaccolo M., Bers D. M., Valdivia H. H., Cheng H., Kass D. A., Paolocci N., *Circ. Res.*, **100**, 96–104 (2007).

11) Paolocci N., Katori T., Champion H. C., St John M. E., Miranda K. M., Fukuto J. M., Wink D. A., Kass D. A., *Proc. Natl. Acad. Sci. U.S.A.*, **100**, 5537–5542 (2003).
12) Pagliaro P., Mancardi D., Rastaldo R., Penna C., Gattullo D., Miranda K. M., Feelisch M., Wink D. A., Kass D. A., Paolocci N., *Free Radic. Biol. Med.*, **34**, 33–43 (2003).
13) Kass D. A., *Nat. Med.*, **15**, 24–25 (2009).
14) Fukuto J. M., Hobbs A. J., Ignarro L. J., *Biochem. Biophys. Res. Commun.*, **196**, 707–713 (1993).
15) Zeller A., Wenzl M. V., Beretta M., Stessel H., Russwurm M., Koesling D., Schmidt K., Mayer B., *Mol. Pharmacol.*, **76**, 1115–1122 (2009).
16) Irvine J. C., Favaloro J. L., Kemp-Harper B. K., *Hypertension*, **41**, 1301–1307 (2003).
17) Irvine J. C., Favaloro J. L., Widdop R. E., Kemp-Harper B. K., *Hypertension*, **49**, 885–892 (2007).
18) Miranda K. M., Katori T., Torres de Holding C. L., Thomas L., Ridnour L. A., McLendon W. J., Cologna S. M., Dutton A. S., Champion H. C., Mancardi D., Tocchetti C. G., Saavedra J. E., Keefer L. K., Houk K. N., Fukuto J. M., Kass D. A., Paolocci N., Wink D. A., *J. Med. Chem.*, **48**, 8220–8228 (2005).
19) Paolocci N., Saavedra W. F., Miranda K. M., Martignani C., Isoda T., Hare J. M., Espey M. G., Fukuto J. M., Feelisch M., Wink D. A., Kass D. A., *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 10463–10468 (2001).
20) Xu Y., Alavanja M.-M., Johnson V. L., Yasaki G., King S. B., *Tetrahedron Lett.*, **41**, 4265–4269 (2000).
21) Zeng B. B., Huang J., Wright M. W., King S. B., *Bioorg. Med. Chem. Lett.*, **14**, 5565–5568 (2004).
22) Adachi Y., Nakagawa H., Matsuo K., Suzuki T., Miyata N., *Chem. Commun.* (Camb.), 5149–5151 (2008).
23) Matsuo K., Nakagawa H., Adachi Y., Kameda E., Tsumoto H., Suzuki T., Miyata N., *Chem. Commun.* (Camb.), **46**, 3788–3790 (2010).
24) Angeli A., *Gazz. Chim. Ital.*, **27**, 357–367 (1897).
25) Dutton A. S., Fukuto J. M., Houk K. N., *J. Am. Chem. Soc.*, **126**, 3795–3800 (2004).
26) Piloty O., *Ber. Dtsch. Chem. Ges.*, **29**, 1559–1567 (1896).
27) Nagasawa H. T., DeMaster E. G., Redfern B., Shirota F. N., Goon D. J. W., *J. Med. Chem.*, **33**, 3120–3122 (1990).
28) Sha X., Isbell T. S., Patel R. P., Day C. S., King S. B., *J. Am. Chem. Soc.*, **128**, 9687–9692 (2006).
29) Miller T. W., Cherney M. M., Lee A. J., Francoleon N. E., Farmer P. J., King S. B., Hobbs A. J., Miranda K. M., Burstyn J. N., Fukuto J. M., *J. Biol. Chem.*, **284**, 21788–21796 (2009).
30) Zeng B., King S. B., *Synthesis*, **2002**, 2335–2337 (2002).
31) Bazylinski D. A., Hollocher T. C., *Inorg. Chem.*, **24**, 4285–4288 (1985).
32) Juaneda C., Dumont Y., Quirion R., *Trends Pharmacol. Sci.*, **21**, 432–438 (2000).
33) Adachi Y., Hashimoto K., Ono N., Yoshida M., Suzuki-Kusaba M., Hisa H., Satoh S., *Eur. J. Pharmacol.*, **324**, 223–226 (1997).
34) Shoeman D. W., Shirota F. N., DeMaster E. G., Nagasawa H. T., *Alchol*, **20**, 55–59 (2000).
35) Akaike T., Yoshida M., Miyamoto Y., Sato K., Kohno M., Sasamoto K., Miyazaki K., Ueda S., Maeda H., *Biochemistry*, **32**, 827–832 (1993).
36) Ellis A., Lu H., Li C. G., Rand M. J., *Br. J. Pharmacol.*, **134**, 521–528 (2001).
37) Samuni U., Samuni Y., Goldstein S., *J. Am. Chem. Soc.*, **132**, 8428–8432 (2010).