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Photochemical Activities of *N*-Nitroso Carboxamides and Sulfoximides and Their Application to DNA Cleavage

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Abstract: *N*-Nitroso compounds containing benzene, fluorene or fluorenone rings were synthesized. Photolysis of these compounds with 312-nm UV light provided the NO• species, the presence of which was corroborated by use of an EPR method and of 2-phenyl-4,4,5,5-tetramethylimidazolin-1-oxyl 3-oxide (PTIO) as a trapping agent. During irradiation of *N*-methyl-*N*-nitroso-9-fluorenone carboxamide (**14c**) in the absence of PTIO, it underwent decomposition followed by recombination to give the heterocyclic nitric oxide radical **15**. Incorporation of intercalating moieties endowed the *N*-nitroso compounds with DNA-cleaving ability through single-strand scission upon UV irradiation in a phosphate buffer (pH 5.0–8.0) under aerobic conditions.

Keywords: DNA cleavage · EPR spectroscopy · nitric oxide · radical reactions

Introduction

Nitric oxide radical (NO•) is a colourless gas with a solubility of 2–3 mM in water. It may react with a variety of atoms and radical species.[1] Its importance increased dramatically after Furchgott,[2] Ignarro,[3] Murad[4] and their co-workers reported its physiological roles in humans. Nevertheless, direct applications of this radical species encounter problems because of its instability and the inconvenience of handling of its aqueous solutions. Use of "NO•-masked" compounds (i.e., NO• donors) is a feasible alternative. Some NO• donors may be heat- or light-sensitive; others may decompose in the presence of oxidants or metal-ion catalysts.[5] *N*-Nitrosamines, for example, can function as NO• donors through homolytic fission of the N–NO bond.[6,7]

We set out to develop *N*-nitroso carboxamides and sulfoximides as efficient NO• donors under control conditions, and then to apply their derivatives as a new class of DNA-cleaving agents with potential for gene therapy. Our first step was to understand their photochemistry and to use UV light as the "trigger." The second step was to incorporate intercalating moieties into *N*-nitroso carboxamides and to study their abilities to nick DNA.

NO• within cells plays a major role as a neurotransmitter and a regulator of blood pressure.[8,9] Recent reports indicate that NO• species cause damage to DNA and mutation in a human lymphoblastoid cell line in cell culture. Macrophages also use nitric oxide as a cytotoxic agent that can cause DNA damage.[10–12] Nitric oxide radical can deaminate deoxynucleosides and deoxynucleotides; it can also intact with DNA under physiological conditions.[13,14] Here we report our success in developing *N*-nitroso carboxamides and sulfoximides[15] as NO• donors triggered by UV light. Furthermore, our results indicate that irradiation of these compounds caused homolytic fission at the N–NO bond and that the resultant NO• species cleaved DNA through single-strand scission.

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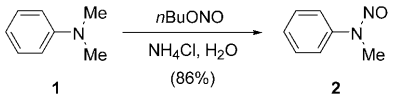
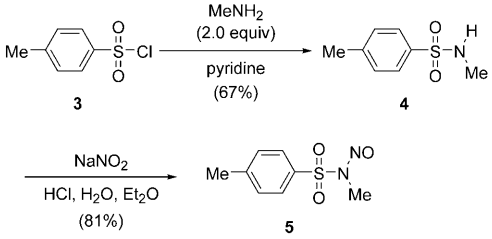
Results

Syntheses of *N*-nitroso compounds: We synthesized a series of *N*-nitroso compounds from readily available starting materials by established methods.[16–19] Accordingly, *N*-methyl-*N*-nitrosoaniline (**2**) was obtained in 86% yield by *N*-demethylation/*N*-nitrosation of *N*,*N*-dimethylaniline (**1**) with *n*BuONO (Scheme 1). Compound **5**, bearing an *N*-nitroso



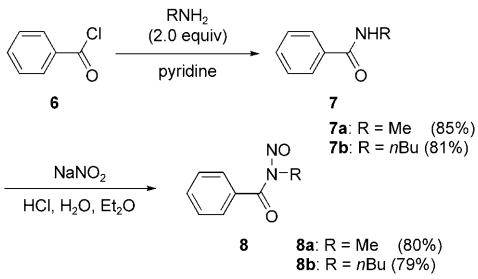
Scheme 1.

moiety attached to a sulfonyl group, was generated in 54% yield by amidation of *p*-toluenesulfonyl chloride (**3**) followed by *N*-nitrosation with $NaNO_2$ (Scheme 2).



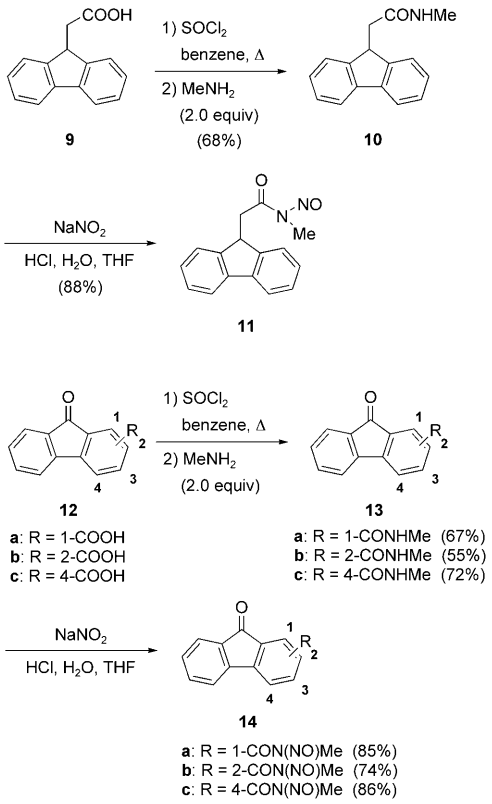
Scheme 2.

Compounds **8**, **11** and **14**, each containing an N–NO moiety attached to a carbonyl group, were prepared from the corresponding acid or acyl chloride in two steps in 41–61% overall yields (see Schemes 3 and 4). Compounds **11**



Scheme 3.

and **14** incorporated fluorene or fluorenone moieties, respectively, which can function as intercalating species for DNA.[20] In the 9-fluorenone carboxamides **14a–c**, the *N*-methyl-*N*-nitroso carboxamide moiety was placed variously



Scheme 4.

at a C-1, a C-2, or a C-4 position. Accordingly, the N–NO moiety might approach DNA from different angles.

Detection of radicals resulting from NO˙ species: To detect the nitric oxide radical (NO˙) intermediate, we used UV light ($\lambda > 300$ nm) to irradiate a solution of **14c** containing 2-phenyl-4,4,5,5-tetramethylimidazolin-1-oxyl 3-oxide (PTIO). The reagent PTIO can oxidize the NO˙ radical to form 2-phenyl-4,4,5,5-tetramethylimidazolin-1-oxyl (PTI) and NO_2˙ radicals.[21,22] Over 15 min of irradiation, the colour of the solution gradually changed from dark blue to brown. We detected a set of EPR signals as shown in Figure 1a. The *g* value and hyperfine splitting constants ($g = 2.0061$, $a_N1 = 9.65$ G, $a_N3 = 4.32$ G) were consistent with those of PTI as shown in Figure 1b.[22] These results clearly indicate that the NO˙ species was generated in the reaction medium.

Production of the NO˙ radical can be inferred from our successful detection of the PTI signal, the appearance of which was found to be time-dependent (see Figure 2a–e). At the start, the reaction mixture was placed in the dark and the EPR spectrometer exclusively showed the signal of PTIO (Figure 2a). After the mixture had been irradiated with UV light for various time periods, the EPR signals corresponded to combinations of PTIO and PTI as shown in Figure 2b–d. After 15 min, the detected EPR signal shown in Figure 2e could be fully superimposed on the simulated signal for pure PTI shown in Figure 2j. Moreover, use of the

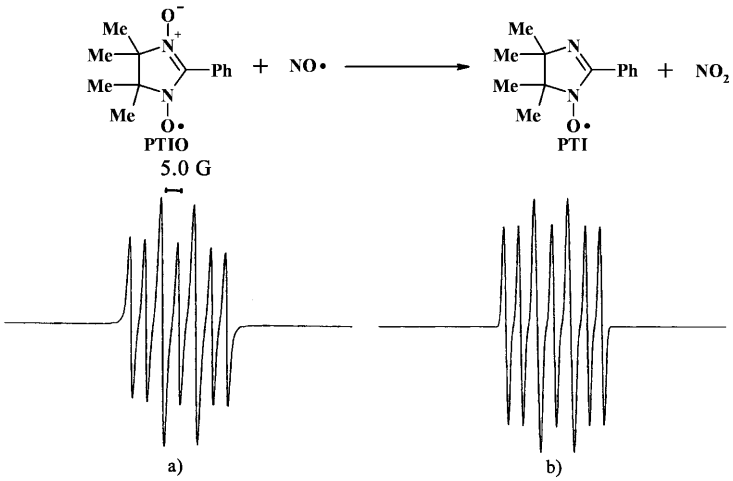


Figure 1. EPR spectra of PTI obtained a) by photolysis of **14c** in the presence of PTIO, and b) by computer simulation.

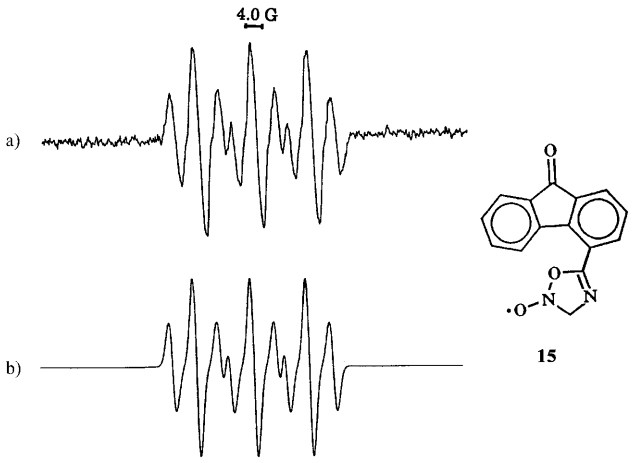


Figure 3. EPR spectra of radical **15** obtained a) by photolysis of **14c**, and b) by computer simulation. These two spectra are superimposable.

Simfonia EPR program allowed us to determine the ratios of PTIO/PTI by comparing the detected signals shown in Figure 2b–d with the simulated signals shown in Figure 2g–i, respectively.

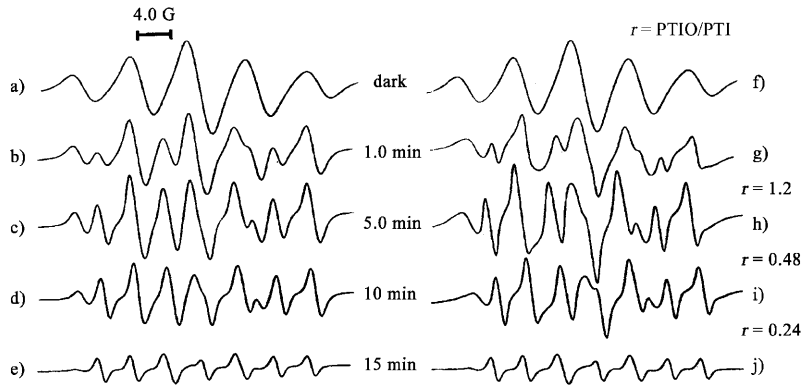


Figure 2. EPR spectra a)–e) of PTI were obtained by photolysis of a mixture containing **14c** and PTIO 1:1 in THF under different conditions: a) in the dark at $t=0$, b) $t=1.0$ min, c) $t=5.0$ min, d) $t=10$ min, and e) $t=$ 15 min. These spectra are superimposable with those (i.e., spectra f–j, respectively) obtained by computer simulation. Spectrum f), obtained by use of the Sinfonia program, represents the signal from pure PTIO and spectrum j) that from PTI; spectra g)–i) were identified as combinations of PTIO and PTI signals in the ratios of 1.2, 0.48, and 0.24, respectively.

Similarly, we repeated the EPR experiments with **14c** under the same photolytic conditions but without addition of PTIO. After 3.0 min, we detected a complicated signal as shown in Figure 3a. Its g value ($=2.0062$) and hyperfine splitting constants ($a_N=13.34$ G, $a_H=5.571$ G) agreed well with a simulated spectrum of nitric oxide radical **15**, shown in Figure 3b.

DNA-cleaving ability of *N*-nitroso compounds: The *N*-nitroso compounds **2**, **5**, **8a**, **8b**, **11** and **14a–c**, in sodium phosphate buffer (pH 5.0, 6.0, 7.0, and 8.0) containing THF (10%) and the supercoiled circular ϕX174 RFI DNA (form I; 50 μM/base pair), were irradiated with 312 nm UV light (16 W) at room temperature for 2.0 h. Results from gel electrophoresis on 1.0% agarose with ethidium bromide staining showed that these *N*-nitroso agents exhibited apparent DNA-cleaving activities and gave the relaxed circular DNA (form II). The ratios of form II to form I ranged from 0.61–99 (Table 1).

Furthermore, we performed two control experiments with the *N*-nitroso carboxamide **14c** to investigate its photolytic DNA-cleaving mechanism. For the first control experiment, the same reaction was repeated, except in the dark. For the second control experiment, sodium azide was added to the photolytic reaction mixture as a scavenger of singlet oxygen. Our data, listed in the last column of Table 1, indicate that single-strand cleavage did not occur in the dark [(form II)/-(form I) = 0.12], so the UV light was functioning as a "trigger" to initiate the cleaving process. On the other hand, the DNA-cleaving ability was not reduced dramatically after the addition of sodium azide (see the second-last row of Table 1). Accordingly, singlet oxygen did not participate in this reaction.

Our results indicate that the DNA-cleaving abilities of the *N*-nitroso compounds follow the order: **14c** > **14a** > **14b** > **11** > **5** > **8a** > **2** > **8b** (see Figure 4). The better potencies of **14a–c** and **11** relative to the other compounds could be the result of their structures containing intercalating moieties. Out of all the *N*-nitroso compounds, *N*-methyl-*N*-nitroso-9-fluorenone-4-carboxamide (**14c**) exhibited the highest

Table 1. Single-strand cleavage of supercoiled circular ϕX174 RFI DNA (form I) to relaxed circular DNA (form II) by photolysis of *N*-nitroso compounds with 312-nm UV light at room temperature over 2.0 h.

Compound[a]	pH	% Form I[b]	% Form II[b]	Form II/Form I
2	6.0	48	52	1.1
5	6.0	34	66	1.9
8a	6.0	36	64	1.8
8b	6.0	50	50	1.0
11	6.0	30	70	2.3
14a	6.0	5.0	95	19
14b	6.0	6.0	94	16
14c	5.0	none	>99	>99
14c	6.0	3.0	97	32
14c	7.0	3.0	97	32
14c	8.0	22	78	3.6
none	6.0	88	12	0.14
14c[c]	6.0	5.0	95	19
14c[d]	6.0	89	11	0.12

[a] A sodium phosphate buffer (Na_2HPO_4 and NaH_2PO_4, 0.10 M) containing an *N*-nitroso compound (500 μM), 50 μM/base pair of form I DNA (molecular weight 3.50×10^6, 5386 base pairs in length). [b] Analysed by gel electrophoresis with 1.0% agarose and ethidium bromide staining. [c] Sodium azide (50 mM) was added. [d] In the dark.

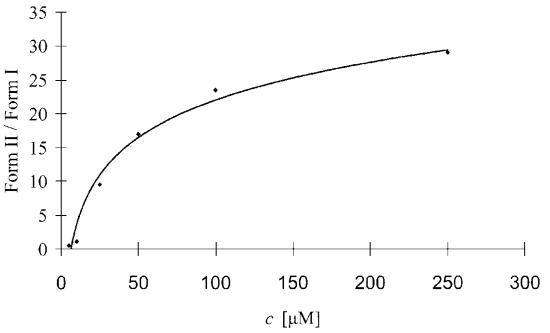


Figure 5. Dose measurement of the DNA-cleaving ability of **14c** in a sodium phosphate solution (pH 6.0) with irradiation with 312-nm UV light room temperature over 2.0 h.

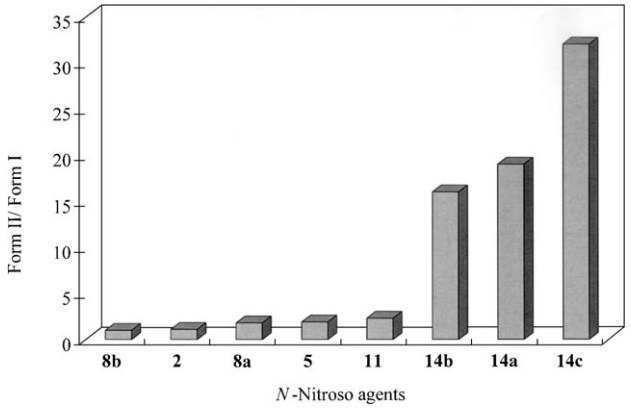


Figure 4. Cleavage of supercoiled circular ϕX174 RFI DNA (form I) by the *N*-nitroso agents to produce the relaxed circular DNA (form II) in a sodium phosphate buffer (pH 6.0) under aerobic conditions and with photolysis with 312-nm UV light at room temperature over 2.0 h.

DNA-cleaving ability. At pH 6.0, **14c** was able to cleave DNA with a (form II)/(form I) ratio of 1.1 at concentrations as low as 5.0 μM (see Figure 5).

Site specificity of DNA cleavage by compound 14c: To explore our findings relating to DNA cleavage in terms of site specificity, we used polyacrylamide gel electrophoresis to analyse the DNA cleavage pattern achieved with **14c** under photolytic conditions. Mixtures containing ^{32}P-end-labelled DNA fragments and **14c** (2000, 1000, and 500 μM) in sodium phosphate buffer (pH 6.0) containing THF (10%) were preincubated for 30 min. The double-stranded 16-mer d(5′-^{32}P-GGACGAAGGATTACGT) · d(ACGTAATCCTTCGTCC) was prepared with a DNA synthesizer. Irradiation of the mixture with UV light (312 nm), followed by piperidine treatment at 95 °C for 30 min, gave the autodiagram as shown in Figure 6. The cleavage sites occurred predominantly at the guanine position.

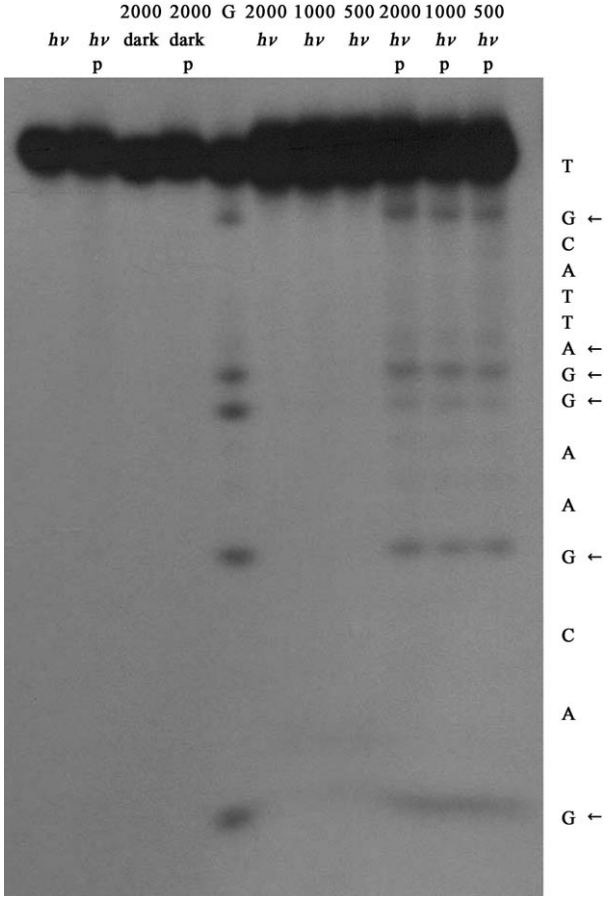
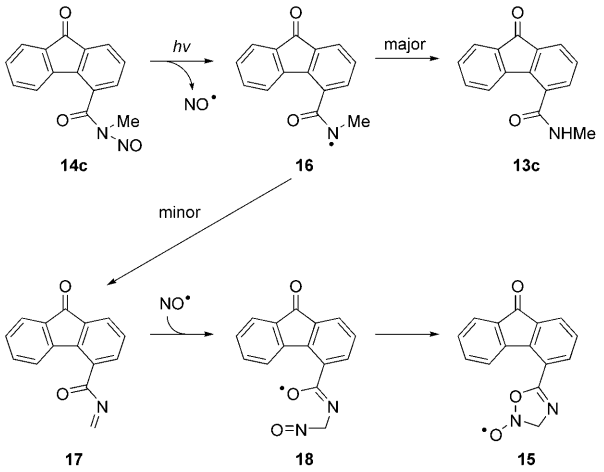


Figure 6. Autoradiogram of a denaturing polyacrylamide gel representing the cleavage of the 16-mer d(5′-^{32}P-TGCATTAGGAAGCAG) · d(ACGTAATCCTTCGTCC) after photolysis of **14c** at 2000, 1000, and 500 μM. p: DNA fragments with piperidine treatment; A + G and G: nucleotide residue determined by the Maxam–Gilbert chemical sequence; *hν*: 312-nm UV light.

Discussion

Formation of nitric oxide radical 18: A plausible mechanism for the photochemical transformation of the *N*-nitroso carboxamide **14c** into the nitric oxide radical **15** is illustrated in Scheme 5. Upon irradiation with UV light, **14c** decomposes



Scheme 5.

to give the amidyl radical **16** and an NO• radical. The presence of the NO• radical was confirmed in the control EPR experiment involving the use of the trapping agent PTIO. Disproportionation then takes place in the amidyl radical **16** to give the carboxamide **13c** in 91% yield, together with the unstable conjugated ketone **17**. The NO• radical produced in situ then adds to **17** in a 1,4-fashion to afford the oxy-radical **18**. Finally intramolecular cyclization occurs in **18** to afford the nitric oxide radical **15** through a 5-*exo-trig* mode.

Potencies, intercalation, computer simulation, and site specificities of *N*-nitroso compounds: DNA plays a crucial role in cells because of its ability to carry the genetic information. It is hence generally chosen as the biological target of anti-cancer agents, some of which exhibit intercalating or cleaving ability or both.[23,24] There is widespread interest in exploration of new compounds that can cleave DNA in unique and controllable ways.[25,26] These controllable conditions include the application of heat,[27] light,[28,29] metal ions,[30,31] enzymes[32,33] etc. to initiate the process of free radical formation. The resultant radicals then damage DNA. The DNA-cleaving agents under our development included **2**, **5**, **8**, **11** and especially compounds **14**. We adopted the *N*-nitrosofluorenones **14a–c** because they possessed both intercalating moieties and photocleavable N–NO bonds. UV light was applied as the trigger to initiate the process of generation of NO• radicals, which were responsible DNA for scission.

To understand the geometric effects on the intercalation of the cleaving species into DNA, we carried out the graphic molecular modelling[34] of oligodeoxyribonucleotides accompanied by the *N*-nitroso agent. Our computational results are shown in Figure 7a–c, with the oligodeoxyribonucleotide assigned a d(TGCATTAGGAAGCAG) sequence on the basis of the sequence in Figure 6. After the energy minimization of the host–guest complex shown in Figure 7a, we obtained the oligodeoxyribonucleotide intercalated by **14c** (see Figure 7b). This stable complex allowed hydrogen bonds to exist between the carbonyl group of **14c** and the C-2 amino group of guanine (Figure 7c and d]. Our computational simulation results thus explain the outcome that compounds **14a–c** exhibited greater cleaving potencies than compounds **2**, **5**, **8a**, **8b** and **11**.

According to the gel electrophoresis result shown in Figure 3, **14c** predominantly cleaved the double-stranded 16-mer DNA at the guanine residue. Our findings coincide with the properties associated with nitric oxide.[35] Recent studies—based on determination of deaminated bases such as hypoxanthine and xanthine—have revealed that, in intact DNA, guanine is relatively more reactive than adenine and cytosine.[36] The highly preferential cleavage at guanine residues may be due to fast deamination of guanine and its rapid depurination.[35]

Conclusions

A series of *N*-nitroso compounds containing intercalating moieties were designed and synthesized. Upon UV irradiation, these agents released active NO• species, the presence of which was corroborated by an EPR method. These *N*-nitroso compounds functioned as UV-triggered DNA cleavage agents. The fluorenone-containing nitroso compounds exhibited high DNA-nicking abilities at concentrations as low as 5.0 μM with site specificity at guanine residues.

Experimental Section

General procedures: Ethyl acetate, hexanes and tetrahydrofuran (THF) were purchased from Mallinckrodt Chemical Co. Ethyl acetate and hexanes were dried and distilled from CaH_2; THF was dried by distillation from sodium and benzophenone under nitrogen. The following compounds and reagents were purchased from Aldrich Chemical Co.: *n*-butylamine, *N*,*N*-dimethylaniline, ethanol, 9-fluoreneacetic acid, 9-fluorenone-1-carboxylic acid, 9-fluorenone-2-carboxylic acid, 9-fluorenone-4-carboxylic acid, methylamine (2.0 M in THF), *N*-methyl-*p*-toluenesulfonamide (**4**), *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (**5**), *N*-methylbenzamide (**7a**) and *N*-*n*-butylbenzamide (**7b**). *N*-Methyl-*N*-nitrosoaniline (**2**) was provided by Sinova Chemical Co. The following compounds and reagents were purchased from Merck, Inc.: benzoyl chloride, *n*-butyl nitrite, HCl (37% wt. in water), pyridine, sodium nitrite, thionyl chloride and toluene-4-sulfonyl chloride. Supercoiled circular ϕX174 RFI DNA and pBR322 DNA were purchased from New England Biolabs Co. Agarose gel was purchased from Sigma Chemical Co.

Analytical thin layer chromatography (TLC) was performed on precoated plates (silica gel 60 F-254), purchased from Merck, Inc. Purification by gravity column chromatography was carried out with Merck Reagents silica gel 60 (particle size 0.063–0.200 mm, 70–230 mesh ASTM).

Infrared (IR) spectra were measured on a Bomem Michelson Series FT-IR spectrometer. The wavenumbers reported are referenced to the poly-

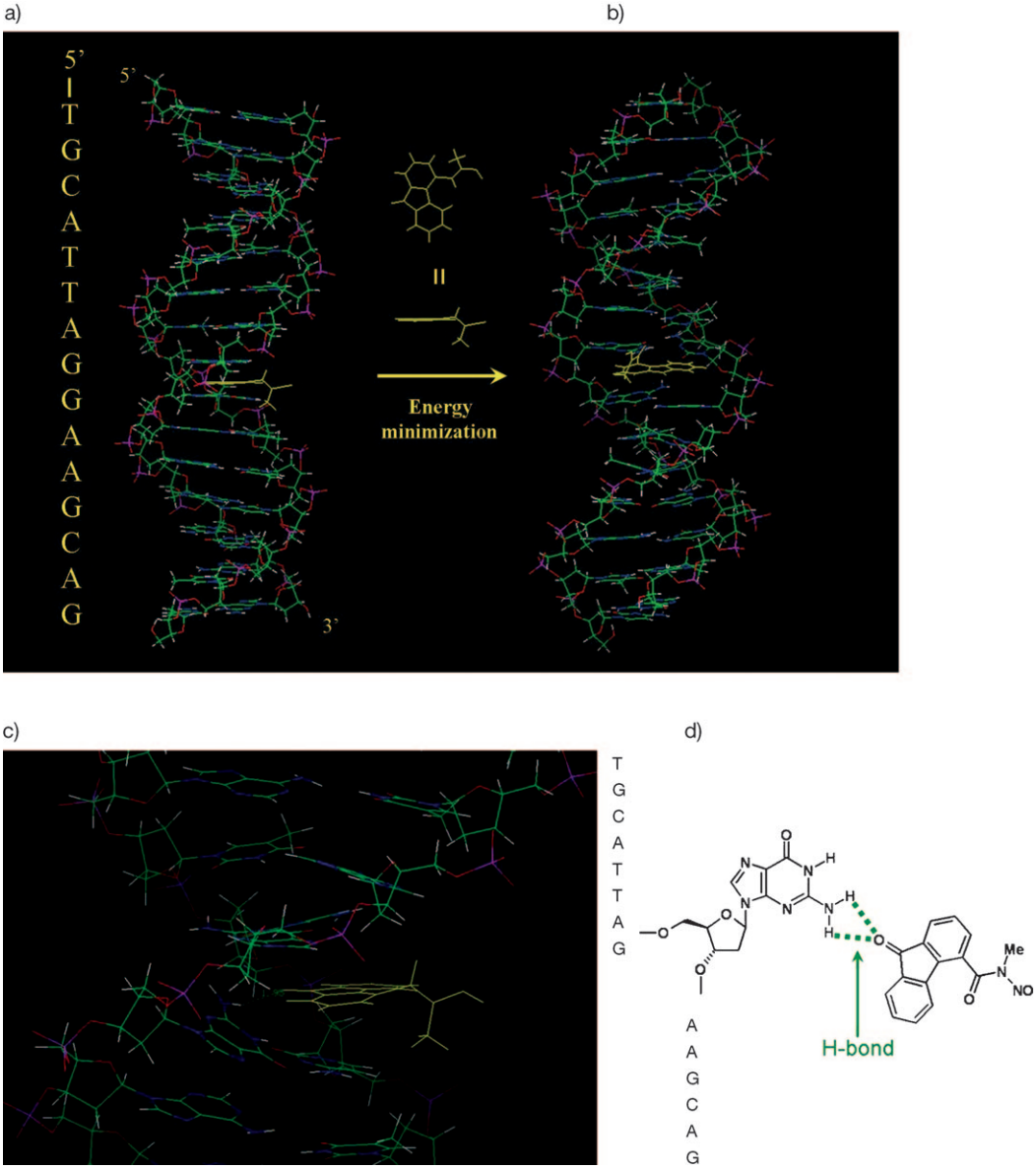


Figure 7. A host–guest complex was obtained by computer simulation and energy minimization with MM2 until the root mean square derivative reached 0.01 kcal mol^{-1} Å. It contains a double-stranded DNA with one d(TGCATTAGGAAGCAG) strand sequence. The DNA was intercalated by **14c**: a) before energy minimization, and b) after energy minimization. c) A close view of a hydrogen bond between the carbonyl group of **14c** and the C-2 amino group residue. d) Chemical structure depicting the hydrogen bonding.

styrene 1601 cm^{-1} absorption. Absorption intensities are recorded by the following abbreviations: s, strong; m, medium; w, weak; br, broad. Proton NMR spectra were obtained on a Varian Unity 400 (400 MHz) spectrometer with use of $CDCl_3$ as solvent and tetramethylsilane as an internal standard. Carbon-13 NMR spectra were performed on a Varian Unity 400 (100 MHz) spectrometer with use of $CDCl_3$ as solvent. Carbon-13 chemical shifts are referenced to the centre of the $CDCl_3$ triplet (δ 77.0 ppm). Multiplicities are recorded by the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; *J*, coupling constant (hertz). The EPR spectra were recorded on a Brucker EMX-10 X-Band spectrometer and the hyperfine splitting constants were measured in gauss. Elemental analyses were carried out on a Heraeus CHN-O RAPID element analyser. The DNA cleavage experiments were carried out in autoclaved glassware or Eppendorf tubes. Photolytic experiments were carried out at 37 °C by use of UV light (312 nm, 16 W) with an instrument from Fisher Biotech. Purities of all products were >99.5 %, as checked by GC.

General procedure 1 for the preparation of *N*-aryl-*N*-nitrosobenzamides: HCl (37 %, 4.0 equiv) was added to a solution of the *N*-arylbenzamide (1.0 equiv) in diethyl ether (1.0 mL). The reaction mixture was stirred vigorously in a water/ice bath at 10 °C. Sodium nitrite (4.0 equiv) in water (1.0 mL) was then added over a period of 5.0 min and the reaction mixture was then stirred at room temperature for 4.0 h. It was then worked up with water and extracted with diethyl ether (3×5.0 mL). The combined organic layer was washed with saturated aqueous NaCl, dried over solid $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (10 % EtOAc/hexanes as eluent) to provide the desired *N*-aryl-*N*-nitrosobenzamide.

***N*-Methyl-*N*-nitrosobenzamide (8a)**: General procedure 1 was followed with use of *N*-methylbenzamide (**7a**, 165 mg, 1.22 mmol), diethyl ether (1.0 mL), HCl (37 %, 481 mg, 4.88 mmol) and sodium nitrite (338 mg, 4.88 mmol) in water (1.0 mL) to give **8a** (160 mg, 0.975 mmol, 80 %) as a yellow oil: TLC R_f=0.68 (20 % EtOAc/hexanes); ^{1}H NMR ($CDCl_3$,

400 MHz): $\delta = 3.31$ (s, 3H; CH_3), 7.47–7.59 (m, 3H; ArH), 7.78 ppm (d, $J = 8.0$ Hz, 2H; ArH); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 26.85$, 128.16, 130.73, 132.54, 132.71, 172.90 ppm; IR (neat): $\tilde{\nu} = 3064$ (w, CH_3), 2948 (w, ArH), 1705 (m), 1600 (w), 1499 (m), 1343 (m), 1166 (s), 968 (s), 898 (w), 8.03 cm^{-1} (s). The compound's spectroscopic characteristics are consistent with those reported for the same compound.[37]

***N*-*n*-Butyl-*N*-nitrosobenzamide (8b)**: General procedure 1 was followed with use of *N*-*n*-butylbenzamide (**7b**, 217 mg, 1.23 mmol), diethyl ether (1.0 mL), HCl (37%, 481 mg, 4.88 mmol) and sodium nitrite (338 mg, 4.88 mmol) in water (1.0 mL) to give **8b** (200 mg, 0.970 mmol, 79%) as a yellow oil: TLC $R_f = 0.58$ (20% EtOAc/hexanes); ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 0.91$ (t, $J = 7.2$ Hz, 3H; CH_3), 1.26–1.31 (m, 2H; CH_2), 1.42–1.49 (m, 2H; CH_2), 3.92 (t, $J = 7.2$ Hz, 2H; CH_2), 7.41–7.53 (m, 3H; ArH), 7.71–7.73 ppm (m, 2H; ArH); IR (neat): $\tilde{\nu} = 2963$ (m, ArH), 2936 (w), 1594 (m), 1568 (s), 1372 (s), 1282 (s), 1174 (s), 1087 (m), 905 (m), 669 (s) cm^{-1}. The compound's spectroscopic characteristics are consistent with those reported for the same compound.[37]

General procedure 2 for the preparation of *N*-methylarylamides: Thionyl chloride (2.0 equiv) was added to a solution of the aryl acid (1.0 equiv) in benzene (1.0 mL). The reaction mixture was heated at reflux for 4.0 h. The excess of thionyl chloride was distilled off along with benzene. After the residue had cooled to room temperature, methylamine in THF (2.0 M, 2.0 equiv) was added slowly into the reaction flask. The reaction mixture was stirred for about 30 min. The reaction mixture was worked up with water and extracted with CH_2Cl_2 (3×10 mL). The combined organic layer was washed with saturated aqueous NaCl, dried over $MgSO_{4\,(s)}$, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (3.0% MeOH/CH_2Cl_2 as eluent) to provide the desire *N*-methyl-*N*-nitrosoarylamide.

***N*-Methyl-9-fluoreneacetamide (10)**: General procedure 2 was followed with use of 9-fluoreneacetic acid (**9**, 75.2 mg, 0.331 mmol) in benzene (1.0 mL), thionyl chloride (78.5 mg, 0.662 mmol) and methylamine in THF (2.0 M, 0.33 mL, 0.66 mmol). The residue was purified by column chromatography (3.0% MeOH/CH_2Cl_2 as eluent) to give **10** (53.1 mg, 0.224 mmol, 68%) as a white solid: TLC $R_f = 0.35$ (5.0% MeOH/CH_2Cl_2); m.p. 180–182 °C (recrystallized from CH_2Cl_2); ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 2.54$ (d, $J = 7.6$ Hz, 2H; CH_2), 2.85 (d, $J = 4.8$ Hz, 3H; CH_3), 4.50 (t, $J = 7.6$ Hz, 1H; CH), 5.43 (s, 1H; NH), 7.26–7.75 ppm (m, 8H; ArH); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 26.42$, 40.94, 44.00, 119.92, 124.44, 127.11, 127.39, 140.65, 146.41, 171.96 ppm; IR (neat): $\tilde{\nu} = 3446$ (w), 3286 (m, NH), 3040 (w, CH_3), 1634 (s), 1585 (m), 1276 (m), 1164 (m), 968 (w), 732 (s), 629 (s) cm^{-1}; MS: *m*/*z* (%): 237 $[M]^+$ (56), 180 (7), 178 (100), 176 (11), 166 (8), 165 (43), 163 (7), 152 (7), 151 (4); elemental analysis (%) calcd for $C_{16}H_{15}NO$: C 80.98, H 6.37, N 5.90; found: C 80.79, H 6.24, N 5.86.

General procedure 3 for the preparation of *N*-methyl-*N*-nitrosoarylamides: HCl (4.0 equiv) was added to a solution of an *N*-methylarylamide (152 mg, 1.0 equiv) in THF (1.0 mL). The reaction mixture was stirred vigorously in a water/ice bath at 10 °C, and sodium nitrite (4.0 equiv) in water (1.0 mL) was then added over a period of 5.0 min. After the reaction mixture had been stirred at room temperature for 4.0 h, the reaction mixture was worked up with water and extracted with diethyl ether (3×5.0 mL). The combined organic layer was washed with saturated aqueous NaCl, dried over $MgSO_{4\,(s)}$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (3.0% MeOH/CH_2Cl_2 as eluent) to provide the desire *N*-methyl-*N*-nitrosoarylamide. Because of thermal lability, the resultant *N*-nitroso compounds were unable to procceed a HRMS detection.

***N*-Methyl-*N*-nitroso-9-fluoreneacetamide (11)**: General procedure 3 was followed, with use of **10** (30.3 mg, 0.128 mmol) in THF (1.0 mL), HCl (37%, 50.5 mg, 0.512 mmol) and sodium nitrite (35.3 mg, 0.512 mmol) in water (1.0 mL). The residue was purified by column chromatography (3.0% MeOH/CH_2Cl_2 as eluent) to give **11** (29.9 mg, 0.112 mmol, 88%) as a yellow solid: TLC $R_f = 0.77$ (3.0% MeOH/CH_2Cl_2); m.p. 121–125 °C (recrystallized from CH_2Cl_2); ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 3.17$ (s, 3H; CH_3), 3.68 (d, $J = 6.4$ Hz, 2H; CH_2), 4.68 (t, $J = 6.4$ Hz, 1H; CH), 7.25–7.78 ppm (m, 8H; ArH); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 25.79$, 39.23, 42.89, 120.03, 124.46, 127.27, 127.34, 127.59, 140.87, 146.08, 175.76 ppm; IR (neat): $\tilde{\nu} = 3453$ (br), 1726 (s), 1623 (w), 1495 (m), 1307 (w), 1206 (m), 1060 (m), 989 (s), 821 (m), 750 cm^{-1} (s); MS: *m*/*z* (%): 267 $[M+1]^+$ (6), 266 $[M]^+$ (6), 238 (10), 237 (25), 236 (16), 191 (13), 189 (11), 165 (100), 152 (12), 57 (19); elemental analysis (%) calcd for $C_{16}H_{14}N_2O_2$: C 72.17, H 5.30, N 10.52; found: C 72.40, H 5.42, N 10.41.

***N*-Methyl-9-fluorenone-1-carboxamide (13a)**: General procedure 2 was followed with use of 9-fluorenone-1-carboxylic acid (**12a**, 151 mg, 0.672 mmol) in benzene (1.0 mL), thionyl chloride (159 mg, 1.34 mmol) and methylamine in THF (2.0 M, 0.67 mL, 1.3 mmol) to give **13a** (106 mg, 0.447 mmol, 67%) as a yellow solid: TLC $R_f = 0.52$ (3.0% MeOH/CH_2Cl_2); m.p. 135–138 °C (recrystallized from CH_2Cl_2); ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 3.05$ (d, $J = 4.4$ Hz, 3H; CH_3), 7.25–7.58 (m, 6H; ArH), 8.18–8.21 (m, 1H; ArH), 10.01 ppm (s, 1H; NH); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 26.61$, 119.96, 122.69, 124.86, 129.41, 129.89, 132.42, 132.71, 133.97, 135.02, 135.68, 143.34, 145.38, 164.49, 195.54 ppm; IR (neat): $\tilde{\nu} =$ 3268 (m, NH), 3093 (w, CH_3), 1691 (s), 1665 (s), 1606 (s), 1575 (s), 1407 (m), 1102 (m), 924 (s), 739 (s) cm^{-1}; MS: *m*/*z* (%): 237 $[M]^+$ (35), 209 (12), 208 (79), 207 (57), 181 (16), 180 (100), 152 (19), 151 (64), 150 (43), 75 (8); elemental analysis (%) calcd for $C_{15}H_{11}NO_2$: C 75.94, H 4.67, N 5.90; found: C 76.05, H 4.87, N 6.05.

***N*-Methyl-9-fluorenone-2-carboxamide (13b)**: General procedure 2 was followed with use of 9-fluorenone-2-carboxylic acid (**12b**, 229 mg, 1.03 mmol) in benzene (1.0 mL), thionyl chloride (244 mg, 2.05 mmol) and methylamine in THF (2.0 M, 1.03 mL, 2.06 mmol) to give **13b** (134 mg, 0.565 mmol, 55%) as a yellow solid: m.p. 232–234 °C (recrystallized from CH_2Cl_2); TLC $R_f = 0.41$ (5.0% MeOH/CH_2Cl_2); ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 3.05$ (d, $J = 4.8$ Hz, 3H; CH_3), 6.22 (s, 1H; NH), 7.35–8.08 ppm (m, 7H; ArH); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 24.46$, 119.07, 119.81, 120.43, 122.16, 128.08, 131.47, 132.02, 132.51, 133.44, 133.55, 141.32, 144.29, 163.61, 190.66 ppm; IR (neat): $\tilde{\nu} = 3260$ (br, NH), 3089 (w, CH_3), 1691 (s), 1664 (s), 1584 (s), 1471 (m), 1448 (m), 1189 (m), 923 (s), 738 (s) cm^{-1}; MS: *m*/*z* (%): 237 $[M]^+$ (37), 209 (12), 208 (80), 207 (52), 181 (14), 180 (100), 151 (66), 150 (34), 125 (3), 75 (7); elemental analysis (%) calcd for $C_{15}H_{11}NO_2$: C 75.94, H 4.67, N 5.90; found: C 75.71, H 4.51, N 6.10.

***N*-Methyl-9-fluorenone-4-carboxamide (13c)**: General procedure 2 was followed with use of 9-fluorenone-4-carboxylic acid (**12c**, 199 mg, 0.888 mmol) in benzene (1.0 mL), thionyl chloride (213 mg, 1.79 mmol) and methylamine in THF (2.0 M, 0.893 mL, 1.78 mmol) to give **13c** (151 mg, 0.636 mmol, 72%) as a yellow solid: TLC $R_f = 0.37$ (3.0% MeOH/CH_2Cl_2); m.p. 219–221 °C (recrystallized from CH_2Cl_2); ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 3.11$ (d, $J = 5.2$ Hz, 3H; CH_3), 6.11 (s, 1H; NH), 7.26–7.49 (m, 4H; ArH), 7.66–7.76 ppm (m, 3H; ArH); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 26.81$, 123.84, 124.26, 125.33, 128.96, 129.50, 131.85, 132.87, 134.06, 134.89, 135.13, 141.09, 142.89 ppm; IR (neat): $\tilde{\nu} =$ 3276 (br, NH), 1719 (s), 1636 (s), 1469 (m), 1413 (m), 1326 (m), 1164 (m), 944 (m), 734 (s), 675 (m) cm^{-1}; MS: *m*/*z* (%): 237 $[M]^+$ (100), 179 (17), 155 (12), 154 (47), 136 (41), 107 (15), 91 (12), 90 (10), 89 (15), 77 (17); elemental analysis (%) calcd for $C_{15}H_{11}NO_2$: C 75.94, H 4.67, N 5.90; found: C 75.89, H 4.61, N 5.95.

***N*-Methyl-*N*-nitroso-9-fluorenone-1-carboxamide (14a)**: General procedure 3 was followed, with use of **13a** (80.1 mg, 0.338 mmol) in THF (1.0 mL), HCl (37%, 133.2 mg, 1.35 mmol) and sodium nitrite (93.2 mg, 1.35 mmol) in water (1.0 mL) to give **14a** (76.4 mg, 0.287 mmol, 85%) as a yellow solid: m.p. (recrystallized from CH_2Cl_2) 105–107 °C; TLC $R_f =$ 0.85 (3.0% MeOH/CH_2Cl_2); ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 3.36$ (s, 3H; CH_3), 7.27–7.62 ppm (m, 7H; ArH); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 26.12$, 120.60, 122.15, 124.53, 127.72, 129.63, 131.40, 131.93, 133.33, 135.08, 135.19, 143.62, 144.36, 172.72, 191.95 ppm; IR (neat): $\tilde{\nu} = 3046$ (w, CH_3), 1713 (s), 1607 (m), 1586 (m), 1505 (s), 1402 (m), 1350 (m), 1186 (m), 980 (s), 728 (s) cm^{-1}; MS: *m*/*z* (%): 267 $[M+1]^+$ (11), 259 (38), 237 (100), 207 (62), 179 (32), 151 (32), 77 (28), 57 (38), 41 (27), 23 (77); elemental analysis (%) calcd for $C_{15}H_{10}N_2O_3$: C 67.67, H 3.79, N 10.52; found: C 67.79, H 3.91, N 10.48.

***N*-Methyl-*N*-nitroso-9-fluorenone-2-carboxamide (14b)**: General procedure 3 was followed, with use of **13b** (51.2 mg, 0.216 mmol) in THF (1.0 mL), HCl (37%, 83.5 mg, 0.846 mmol) and sodium nitrite (59.6 mg, 0.846 mmol) in water (1.0 mL) to give **14b** (42.6 mg, 0.160 mmol, 74%)

as a yellow solid: TLC $R_f = 0.83$ (5.0% MeOH/CH_2Cl_2); m.p. 130–133 °C (recrystallized from CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 3.32$ (s, 3H; CH_3), 7.37–7.94 (m, 2H; ArH), 7.96–8.00 ppm (m, 5H; ArH); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 26.88$, 120.02, 121.24, 124.60, 126.55, 130.27, 133.27, 133.69, 134.57, 135.00, 137.39, 143.02, 147.88, 171.63, 192.20 ppm; IR (neat): $\tilde{\nu} = 1705$ (s), 1611 (s), 1494 (s), 1398 (s), 1338 (m), 1180 (m), 1031 (w), 943 (s), 893 (m), 662 (s) cm^{-1}; MS: *m*/*z* (%): 267 $[M+1]^+$ (0.14), 266 $[M]^+$ (0.56), 225 (8), 224 (55), 208 (19), 207 (83), 179 (26), 151 (100), 45 (18), 30 (13); elemental analysis (%) calcd for $C_{15}H_{10}N_2O_3$: C 67.67, H 3.79, N 10.52; found: C 67.51, H 3.85, N 10.42.

***N*-Methyl-*N*-nitroso-9-fluorenone-4-carboxamide (14c)**: General procedure 3 was followed, with use of **13c** (0.100 g, 0.422 mmol) in THF (1.0 mL), HCl (37%, 167 mg, 1.69 mmol) and sodium nitrite (117 mg, 1.69 mmol) in water (1.0 mL) to give **14c** (96.5 mg, 0.362 mmol, 86%) as a yellow solid: TLC $R_f = 0.83$ (3.0% MeOH/CH_2Cl_2); m.p. 140–144 °C; ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 3.40$ (s, 3H; CH_3), 7.20–7.54 (m, 5H; ArH), 7.69–7.82 ppm (m, 2H; ArH); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta =$ 26.14, 122.49, 124.64, 126.23, 128.47, 128.70, 128.87, 134.17, 134.78, 134.84, 134.97, 142.16, 142.32, 173.10, 192.33 ppm; IR (neat): $\tilde{\nu} = 1712$ (s), 1606 (m), 1500 (m), 1348 (m), 1172 (m), 1090 (m), 1011 (s), 830 (m), 783 (m) cm^{-1}; MS: *m*/*z* (%): 267 $[M+1]^+$ (25), 239 (16), 220 (22), 208 (24), 207 (100), 180 (26), 179 (25), 152 (20), 151 (22), 23 (22); elemental analysis (%) calcd for $C_{15}H_{10}N_2O_3$: C 67.67, H 3.79, N 10.52; found: C 67.65, H 3.89, N 10.43.

Photolysis of *N*-methyl-*N*-nitroso-9-fluorenone-4-carboxamide (14c) in the presence of 2-phenyl-4,4,5,5-tetramethylimidazolin-1-oxyl 3-oxide (PTIO): A solution containing PTIO (2.35 mg, 0.0101 mmol) and **14c** (2.71 mg, 0.0101 mmol) in THF (1.0 mL) was placed in an EPR cell at room temperature. The cell was introduced into the cavity of an EPR spectrometer and the solution was photolysed by use of UV light ($\lambda >$ 300 nm). After different periods of time, the resultant EPR signals were detected as shown in Figure 1a–e. Furthermore, computer-simulated EPR spectra of PTIO/PTI with different ratios are shown in Figure 1f–i.

Photolysis of *N*-methyl-*N*-nitroso-9-fluorenone-4-carboxamide (14c): A solution of **14c** (2.71 mg, 10.1 μmol) in THF (1.0 mL) was placed in an EPR cell at room temperature. The cell was introduced into the cavity of an EPR spectrometer and the solution was photolysed by use of UV light ($\lambda > 300$ nm). After 8.0 min, a strong signal of PTI was detected as shown in Figure 1a. Furthermore, a computer-simulated EPR spectrum was obtained as shown in Figure 1b.

General procedure for DNA cleavage by use of *N*-nitroso compounds: The reaction mixture containing supercoiled circular ϕX174 RFI DNA stock solution (50 μM/base pair, 1.0 μL), an *N*-nitroso compound (0.50 mM, 1.0 μL) and phosphate buffer (8.0 μL) in a Pyrex vial was irradiated with UV light (312 nm) under aerobic conditions at 37 °C. Gel-loading buffer (6×, 2.5 μL) was added to the reaction mixture, which was then slowly added to the slots of the submerged agarose gel (1.0%). The gel was subjected to electrophoresis with a constant voltage (~100 mV) in a buffer until the bromophenol blue and xylene cyanol FF had migrated by the appropriate distances. After the gel had been allowed to stand in double distilled water overnight, it was examined by UV light and then photographed.

Control DNA cleavage experiments: Sodium azide (500 mM, 1.0 μL) in a phosphate buffer (pH 6.0, 7.0 μL) was added to a reaction mixture containing supercoiled circular ϕX174 RFI DNA stock solution (50 μM/base pair, 1.0 μL) and **14c** (1.00 mM, 1.0 μL) in a Pyrex vial. The mixture was then irradiated as described. Electrophoresis was then performed as described in the General procedure for DNA cleavage.

Dose measurement experiments with *N*-methyl-*N*-nitroso-9-fluorenone-4-carboxamide (14c): A reaction mixture containing supercoiled circular ϕX174 RFI DNA stock solution (50 μM/base pair, 1.0 μL), **14c** (500, 250, 100, 50.0, 10.0, 5.00, 1.00 or 0.50 μM, 1.0 μL) and a phosphate buffer (pH 6.0, 8.0 μL) in a Pyrex vial was irradiated as described in the General procedure. Electrophoresis was then performed as described in the General procedure for DNA cleavage by use of *N*-nitroso compounds. The NIH 1.60 image program, provided by Dr. R. Wayne of the National Institutes of Health, U.S.A., was used for the quantitative analysis of DNA cleavage.

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