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Isolation and structural identification of a direct-acting mutagen derived from *N*-nitroso-*N*-methylpentylamine and Fenton's reagent with copper ion

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ABSTRACT

N-Nitrosodialkylamines show their mutagenicity by forming α-hydroxynitrosamines in the presence of rat S9 mix in the Ames assay. The hydroxyl radical derived from Fe^{2+}–H_2O_2 (Fenton's reagent) with Cu^{2+} activates *N*-nitrosamines, with an alkyl chain longer than a propyl constituent, to a direct-acting mutagen. The reactivity of Fe^{2+}–Cu^{2+}–H_2O_2 on nitrosamines in relation to their metabolic activation is not fully characterized. Here, we report the identification of the direct-acting mutagen derived from *N*-nitroso-*N*-methylpentylamine (NMPe) in the presence of Fe^{2+}, Cu^{2+}, H_2O_2 and nitric oxide (NO), which is a product of nitrosamine metabolism. A dichloromethane extract of the NMPe reaction mixtures was fractionated by silica gel column chromatography several times and by a preparative high performance liquid chromatography (HPLC); we obtained white crystals as a product. The direct-acting mutagen that was isolated was provisionally identified as 5-ethyl-5-nitro-1-pyrazoline 1-oxide by ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, infrared (IR) spectroscopy and X-ray crystallography. To confirm the structure of the mutagen, the authentic compound was synthesized from 2-nitrobutene and diazomethane, followed by *N*-oxidation with *m*-chloroperoxybenzoic acid. The ^1H NMR spectral data from the direct-acting mutagen that was synthesized was identical to the data from the isolated mutagen. Furthermore, the authentic 5-ethyl-5-nitro-1-pyrazoline 1-oxide was mutagenic in *Salmonella typhimurium* TA1535. The results showed that 5-ethyl-5-nitro-1-pyrazoline 1-oxide was a direct-acting mutagen derived from the reaction of NMPe and Fe^{2+}–Cu^{2+}–H_2O_2–NO.

1. Introduction

Mutagenic and carcinogenic *N*-nitrosamines exist as contaminants in foods, tobacco and other products.[1–7] In addition to this environmental exposure, human exposure to *N*-nitrosamines also occurs in the body by nitrosation of amines with nitrite in the presence of acid, or by nitrosation through nitric oxide (NO) generated from macrophages during inflammation or infection.[8–11] *N*-Nitrosodialkylamines induce cancer in almost all experimental animals tested, and the organ specificity in their carcinogenicity is noted.[1,12] Exposure to *N*-nitrosamines is, therefore, suspected to be a cause of human cancer.[13,14]

The *N*-nitrosodialkylamines are mutagenic through metabolic activation by cytochrome P450.[1] The activation mechanism of *N*-nitrosodialkylamines is through hydroxylation at an α-carbon adjacent to the *N*-nitroso group by cytochrome P450. The α-hydroxynitrosamine decomposes spontaneously to yield aldehyde and alkanediazohydroxide, followed by the generation of an alkyldiazonium ion, which alkylates DNA bases. Non-enzymatic degradation of nitrosamines by Fenton's reagent[15–19] or the Udenfriend system[20] has also been reported. However, little is known about the biological role of these non-enzymatic activation processes. Fenton's reagent supplemented with a copper ion (Fe^{2+}–Cu^{2+}–H_2O_2) has been used as an oxidant for the activation of the *N*-nitrosodialkylamines.[19,21,22] The *N*-nitrosodialkylamine in the presence of the Fe^{2+}–Cu^{2+}–H_2O_2 is thought to be activated through a mechanism other than α-hydroxylation.[22] Fe^{2+}–Cu^{2+}–H_2O_2 activates *N*-nitrosodialkylamines with an alkyl chain longer than a propyl constituent to a direct-acting mutagen.[21] The reaction of *N*-nitroso-*N*-methylbutylamine (NMB) produced a higher level of mutagenicity than *N*-nitrosodipropylamine, *N*-nitrosodibutylamine, and *N*-nitroso-*N*-methylpropylamine. The direct-acting mutagen derived from NMB in the presence of Fe^{2+}–Cu^{2+}–H_2O_2 was isolated and analyzed by various spectroscopic methods. However, the crystal of the isolated mutagen was not suitable for

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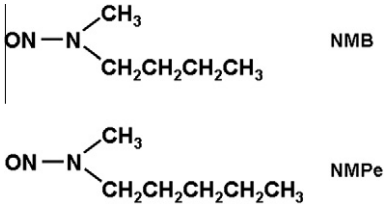


Figure 1. Structure of NMB and NMPe.

X-ray crystallography, and the structure of the direct-acting mutagen is still unknown. In the present study, *N*-nitroso-*N*-methylpentylamine (NMPe) was used to identify the structure of the direct-acting mutagen instead of NMB (Fig. 1). The direct-acting mutagen derived from NMPe was isolated and examined by ^{1}H nuclear magnetic resonance (NMR) spectroscopy, ^{13}C NMR spectroscopy, infrared (IR) spectroscopy and X-ray crystallography. Furthermore, the authentic compound was synthesized and compared with the instrumental data of the direct-acting mutagen isolated, and then was evaluated for the mutagenicity in *Salmonella typhimurium* TA1535.

2. Results

2.1. Effect of metal ions and NO on the mutagenicity of the reaction extract with NMPe in the presence of H_2O_2

The effect of Fe^{2+}, Cu^{2+}, and H_2O_2 on NMPe mutagenicity was investigated (Fig. 2A). NMPe was treated with $FeSO_4{\cdot}7H_2O$, $Cu(OAc)_2{\cdot}H_2O$ and H_2O_2 in 1 M acetate buffer (pH 4.5) under inert atmosphere for 2 h at 37 °C. The reaction mixture was extracted with ethyl acetate. The organic phase was dried over anhydrous sodium sulfate, and the solvent was subsequently evaporated. The reaction extract was assayed for mutagenicity in *S. typhimurium* TA1535. Cu^{2+}–H_2O_2 weakly activated NMPe. However, Fe^{2+}–H_2O_2, Cu^{2+}–Fe^{2+}, or H_2O_2 alone did not activate NMPe (Fig. 2A). The coexistence of both metal ions and H_2O_2 in the reaction enhanced the mutagenicity of the extracts from the reaction with NMPe. The mutagenic potency of the reaction mixture was similar to previous reports of NMB mutagenicity,[21] indicating that the direct-acting mutagen was similar in structure to NMB.

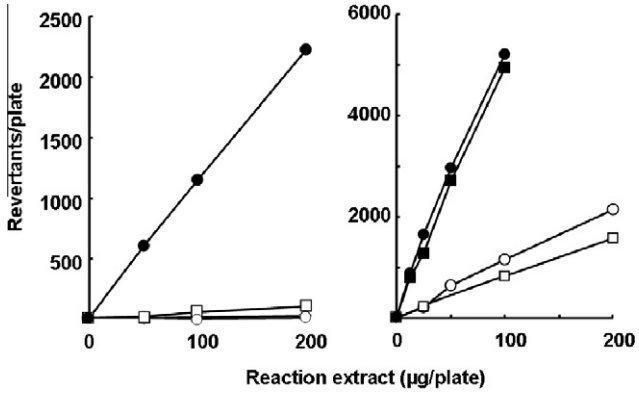


Figure 2. Mutagenicity of the extract from the reaction of *N*-nitrosodialkylamines with Fe^{2+}–Cu^{2+}–H_2O_2 in *S. typhimurium* TA1535. (A) Effect of Fe^{2+} and Cu^{2+} in the presence of H_2O_2 on the mutagenicity of the extract from NMPe treated with Fe^{2+}–Cu^{2+} in *Salmonella typhimurium* TA1535. The complete system contained NMPe, $FeSO_4$, $Cu(OAc)_2$ and H_2O_2 (●). The control system without $FeSO_4$ (□), without $Cu(OAc)_2$ (△), and H_2O_2 alone (○), which all overlapped. (B) Effect of NO in the presence of Fe^{2+}–Cu^{2+}–H_2O_2 on the mutagenicity of the extract from NMB and NMPe treated with Fe^{2+}–Cu^{2+} in *S. typhimurium* TA1535. NMB and Fe^{2+}–Cu^{2+}–H_2O_2 with NO (■) or without NO (□). NMPe and Fe^{2+}–Cu^{2+}–H_2O_2 with NO (●) or without NO (○).

Since NO is involved in the formation of the direct-acting mutagen, NO was introduced to the reaction mixture of *N*-nitrosodialkylamine in the presence of Fe^{2+}–Cu^{2+}–H_2O_2, and tested for mutagenicity in *S. typhimurium* TA1535 (Fig. 2B).[22] NMPe and Fe^{2+}–Cu^{2+}–H_2O_2 in the presence or absence of NO was reacted in 1 M acetate buffer (pH 4.5) as described above. Figure 2B shows that NO addition produced a higher level of mutagenicity of the direct-acting mutagen in the presence of Fe^{2+}–Cu^{2+}–H_2O_2.

2.2. Isolation of a direct-acting mutagen from the reaction of NMPe and Fe^{2+}–Cu^{2+}–H_2O_2–NO

The reaction was conducted as described above. After 2 h, the reaction mixture was extracted consecutively with hexane, CH_2Cl_2, ethyl acetate, and, finally, ethyl acetate with salting out. Each of the organic phases was dried over anhydrous sodium sulfate, and the solvent was subsequently evaporated. The mutagenicity of the CH_2Cl_2 extract showed the highest activity among the organic phases (Fig. 3). The percentage of the mutagenic activity of the CH_2Cl_2 extract as a portion of total mutagenicity was 60%. The CH_2Cl_2 extract was fractionated by silica gel column chromatography twice (R_f value = 0.33, Hexane/CH_2Cl_2/Et_2O = 2:2:1, Detection UV 254 nm), and by a preparative high performance liquid chromatography (HPLC) (Lichrosorb Si 60 10 μm, 7.5 × 250 mm; Hexane/CH_2Cl_2/EtOH = 8:1:0.2, flow rate 2.0 mL/min, Detection UV 254 nm). The fraction showing strong mutagenicity contained a single compound, which was crystallized by cooling, followed by washing with CCl_4, colorless plates, mp. 37.5–38.5 °C, in yield 0.02%. Spectral data of the direct-acting mutagen is shown in Table 1.

The ^{1}H NMR and ^{13}C NMR spectra showed five carbon signals. The ^{1}H NMR spectrum suggested that the mutagen was a cyclic

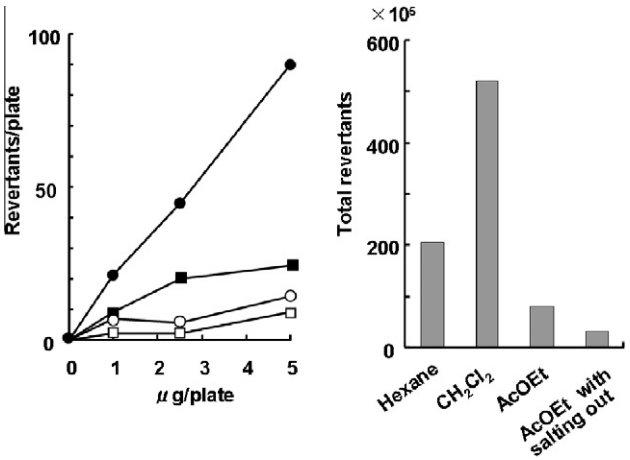


Figure 3. Mutagenicity of the each organic phase in *S. typhimurium* TA1535. Hexane (□), dichloromethane (●), ethyl acetate (■), ethyl acetate with salting out (○).

Table 1
Spectral data of the direct-acting mutagen derived from NMPe.

^{1}H NMR (at r.t., in $CDCl_3$)
1.06 (3H, t, J = 7.4 Hz, —$CH_2\underline{CH_3}$), 2.50 (1H, dq, J = 14.9, 7.4 Hz, —$C\underline{H_2}CH_3$), 2.57 (1H, m, —$C\underline{H_2}CH_3$), 2.60-2.68 (1H, m, H-4), 2.92-2.98 (1H, m, H-4), 4.21-4.35 (2H, m, H-3)
^{13}C NMR (at r.t., in $CDCl_3$)
7.7 (—$CH_2\underline{C}H_3$), 26.4 (—$\underline{C}H_2CH_3$), 29.6 (C-4), 54.7 (C-3), 117.9 (C-5)
IR (cm^{-1}, KBr) 1570, 1521, 1373, 1332
UV (nm, CH_3CN) λ = 221 (ε = 6380)
MS (FAB) [M+1] 160

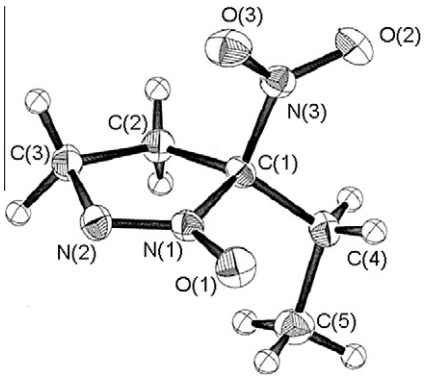


Figure 4. X-ray crystallography of the direct-acting mutagen.

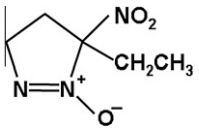


Figure 5. Structure of the direct-acting mutagen.

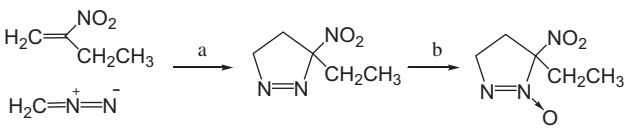
structure because of the complicated couplings. A proton signal at 1.06 ppm integrated three protons with a single peak in triplet and two protons in 2.50–2.57 ppm indicated the presence of an ethyl group adjacent to a carbon atom, not a nitrogen or oxygen atom. The existence of an ethyl group was consistent with 7.7 and 26.4 ppm from ^{13}C NMR. Four proton signals at 4.3 (2H), 3.0 (1H) and 2.6 (1H) ppm, indicated that the direct-acting mutagen was composed of a $-CH_2CH_2-$ structure with a highly-restricted ring component. A carbon peak at 117.9 ppm was a tertiary carbon in the structure. The ^1H and ^{13}C NMR spectra indicated that the direct-acting mutagen was contained the following structure: $-CH_2CH_2-C-CH_2CH_3$. The IR spectra at 1570 and 1373 cm^{-1} were characterized by a nitro or nitrite group. The FAB-MS spectrum of the mutagen gave a molecular ion at m/z 160 [M+1]. The structure of the direct-acting mutagen was also determined by X-ray crystallography. A perspective view of the crystal structure, obtained using ORTEP, showing the atomic numbering is presented in Figure 4. The structure of the direct-acting mutagen was expected to be 5-ethyl-5-nitro-1-pyrazoline 1-oxide (Fig. 5).

2.3. Synthesis of 5-ethyl-5-nitro-1-pyrazoline 1-oxide

To identify the structure of the direct-acting mutagen, 5-ethyl-5-nitro-1-pyrazoline 1-oxide was synthesized. 3-Ethyl-3-nitro-1-pyrazoline was synthesized by the reaction of diazomethane and 2-nitrobutene followed by the *N*-oxidation with *m*-chloroperoxybenzoic acid (MCPBA) (Scheme 1). The ^1H NMR data of the authentic compound was identical to that of the isolated mutagen.

2.4. Mutagenicity of authentic 5-ethyl-5-nitro-1-pyrazoline 1-oxide

The authentic 5-ethyl-5-nitro-1-pyrazoline 1-oxide was assayed for the mutagenicity in *S. typhimurium* TA1535, and we



Scheme 1. Reagents and conditions: (a) −78 °C, 30 min→rt, 3 h; (b) MCPBA/CH_2Cl_2, rt, 12 h.

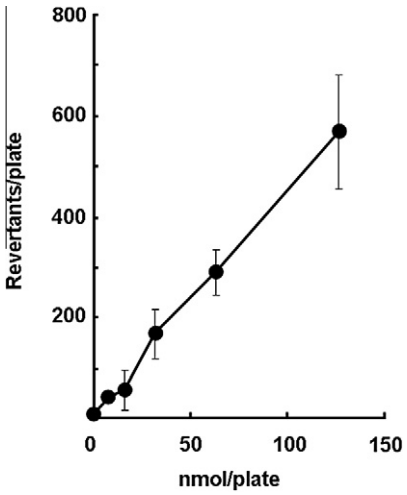


Figure 6. Mutagenicity of the authentic 5-ethyl-5-nitro-1-pyrazoline 1-oxide in *S. typhimurium* TA1535.

confirmed the direct-acting activity (Fig. 6). The data indicated that the 5-ethyl-5-nitro-1-pyrazoline 1-oxide was a direct-acting mutagen derived from NMPe by Fe^{2+}–Cu^{2+}–H_2O_2–NO.

3. Discussion

N-Nitrosodialkylamines are pro-carcinogens that require metabolic activation by cytochrome P450 enzymes to exert their mutagenicity and carcinogenicity.[1] The α-hydroxylation of a carbon atom adjacent to the *N*-nitroso group is believed to result from the metabolic activation of *N*-nitrosodialkylamines, followed by the formation of an alkanediazonium ion, which alkylates DNA. The alkylated DNA causes base substitution mutations during DNA replication.[23]

There are a few reports regarding the oxidation of several *N*-nitrosodialkylamines by Fenton's reagent.[15–19] Fe^{2+}–Cu^{2+}–H_2O_2 reportedly activates *N*-nitrosodialkylamines with alkyl chains longer than propyl groups to a direct-acting mutagen, and the formation of the direct-acting mutagen is induced by a hydroxyl radical and NO.[19,21,22] The structural identification of the direct-acting mutagen was necessary to define the activation mechanism of *N*-nitrosodialkylamines in the presence of Fe^{2+}–Cu^{2+}–H_2O_2.

By eliminating one of the constituents, either Fe^{2+}, Cu^{2+} or H_2O_2, in the presence of NMPe, the mutagenicity of each reaction mixture decreased (Fig. 2A). Fe^{2+}–Cu^{2+}–H_2O_2 activated NMPe efficiently. Since the mutagenic potency was similar to that of NMB,[21] the direct-acting mutagen from NMPe was assumed to have a similar structure to NMB. Furthermore, the mutagenicity of the reaction products from NMPe in the presence of Fe^{2+}–Cu^{2+}–H_2O_2 was similar to NMB. Since NO is involved in the formation of the direct-acting mutagen,[22] NO was added to a reaction mixture of *N*-nitrosodialkylamines in the presence of Fe^{2+}–Cu^{2+}–H_2O_2 (Fig. 2B). The reaction conditions were arranged to isolate the direct-acting mutagen in *S. typhimurium* TA1535. The mutagenicity of the reaction product from NMPe or NMB with Fe^{2+}–Cu^{2+}–H_2O_2 increased when NO was added to the reaction mixture. The direct-acting mutagen derived from the reaction of NMB with Fe^{2+}–Cu^{2+}–H_2O_2 was identical to that derived from the reaction product of NMB with Fe^{2+}–Cu^{2+}–H_2O_2–NO, according to HPLC (data not shown).

The direct-acting mutagen derived from NMPe was isolated by chromatography and a preparative HPLC. The isolated mutagen was analyzed by instrumental data, and finally by X-ray crystallography (Table 1, Fig. 4). The structure of the mutagen was provision-

ally specified as 5-ethyl-5-nitro-1-pyrazoline 1-oxide. Furthermore, the presumed compound, 5-ethyl-5-nitro-1-pyrazoline 1-oxide, was synthesized from diazomethane and 2-nitrobutene, followed by *N*-oxidation. The 3-methyl-3-nitro-1-pyrazoline with a sugar moiety is synthesized from the corresponding nitro olefin and diazomethane by 1,3-dipolar cycloaddition.[24] Selective *N*-oxidation of the 1-pyrazoline at the N1 position by MCPBA was difficult and the yield was low under the present conditions due to the formation of the 2-oxide isomer as the major product. The ^{1}H NMR data of the authentic compound was identical to that of the isolated direct-acting mutagen. The authentic 5-ethyl-5-nitro-1-pyrazoline 1-oxide was mutagenic in *S. typhimurium* TA1535 (Fig. 6). Thus, the direct-acting mutagen derived from NMPe in the presence of Fe^{2+}–Cu^{2+}–H_2O_2–NO was identified as 5-ethyl-5-nitro-1-pyrazoline 1-oxide (Fig. 5). In NMB, the structure of the mutagen was assumed to be 5-methyl-5-nitro-1-pyrazoline 1-oxide. The direct-acting mutagen derived from NMB will be reported soon.

There are a few reports of the formation of cyclic compounds as reactive intermediates derived from *N*-nitrosamines.[25,26] An α-oxidation product of *N*-nitroso-*N*-butyl-3-carboxypropylamine cyclizes to a γ-lactone, which is a direct-acting mutagen.[25] *N*-Nitroso-(2-oxopropyl)propylamine is also metabolized to a methylating agent via oxadiazoline formation.[26] The genotoxicity, particularly the mutagenicity, of some compounds with pyrazolines or pyrazoline *N*-oxides has been infrequently reported.[27,28] The formation of 5-ethyl-5-nitro-1-pyrazoline 1-oxide may occur through γ-oxidation of the pentyl group, followed by demethylation. The γ-oxidation and demethylation is reported to be occurred by rat liver microsomes.[29–31] The hydroxyl radical is generated in our body constantly.[32] Copper and iron are also reported to exist in our body and concerned with some degenerative disease, such as Alzheimer's disease.[33] Thus the 5-ethyl-5-nitro-1-pyrazoline 1-oxide can be formed from NMPe in vivo. The mechanism of mutagenesis is under investigation, and we are trying to detect some adducts with nucleophiles.

N-Nitrosodialkylamine, which has an alkyl chain longer than a propyl group, gives rise to a stable mutagenic product after treatment with Fe^{2+}–Cu^{2+}–H_2O_2–NO. The mechanism of this novel reaction involves the formation of the pyrazoline ring structure in the direct-acting mutagen.

4. Materials and methods

4.1. Chemicals

NMB and NMPe were synthesized as described,[34] and then purified by distillation (NMB: b.p. 87 °C/18 mm Hg, NMPe: b.p. 96–98 °C/11 mmHg). In order to obtain the unknown direct-acting mutagen formed in a trace amount, the *N*-nitrosodialkylamine was dissolved in methanol saturated with sodium hydroxide and the entire solution was stirred overnight at room temperature. The reaction mixture was extracted three times with CH_2Cl_2, and the combined organic phases were dried over Na_2SO_4, filtered, and then evaporated in vacuo to produce a pale yellow oil.[21] Bacto agar and bacto nutrient broth were obtained from Becton Dickinson Microbiology System (Sparks, USA). Sodium ammonium hydrogen phosphate tetrahydrate was purchased from Merck (Darmstadt, Germany). Copper (II) acetate monohydrate ($Cu(OAc)_2{\cdot}H_2O$) was obtained from Kanto Chemical Co. Ltd (Tokyo, Japan). Other reagents used were purchased from Wako Pure Chemical Industries (Osaka, Japan). 2-Nitrobutene was synthesized from 2-nitro-1-butanol as reported.[35,36] Professor B. N. Ames (University of California, Berkeley, USA) kindly provided the *S. typhimurium* TA1535.

4.2. Experimental

Melting points were measured on a Yanagimoto microapparatus and were uncorrected. The FAB-MS was obtained with JEOL JMS-700 mass spectrometer. The NMR experiments were performed with JEOL JNM-LA400, with tetramethylsilane as an internal standard. HPLC was performed using a Shimadzu LC-6A system [SPD-6AV UV/vis spectrometric detector, [Lichrosorb Si 60 (10 μm, 7.5 × 250 mm)]. TLC was performed on precoated Kieselgel 60F_{254} (Merck), and spots were visualized under UV light. Column chromatography was performed on Silica Gel 60 (0.063–0.200 mm, Merck).

4.3. Bacterial mutation assay

The bacterial mutation assay was conducted according to the plate-incorporating method.[37,38] All of the yellow oil obtained above was dissolved into distilled DMSO. Five DMSO solutions with concentrations of 12.5, 25, 50, 100 and 200 μg/50 μL were put into separate test tubes; then, 0.5 mL of 0.1 M sodium phosphate buffer (pH 7.4) and 0.1 mL of a culture of tester strain were added to each test tube, followed by 2 mL of top agar. The mixture from each tube was then poured onto a minimal-glucose agar plate. After incubation for 44 h at 37 °C, the colonies were counted. All plates were prepared in duplicate and the experiments were repeated at least twice. Data represent the means of duplicate determinations. The results were considered positive if the assay produced reproducible and dose-related increases in the number of revertants.[38]

The authentic 5-ethyl-5-nitro-1-pyrazoline 1-oxide was also dissolved in 50 μL of acetonitrile and the assay was performed by the method as described above.

4.4. Effect of metal ion and H_2O_2 on the mutagenicity of NMPe

N-Nitrosodialkylamine in the presence of the oxidation system was treated without each one of the following constituents $FeSO_4{\cdot}7H_2O$, $Cu(OAc)_2{\cdot}H_2O$, H_2O_2· $FeSO_4{\cdot}7H_2O$ (28 mg, 100 μmol) and $Cu(OAc)_2{\cdot}H_2O$ (20 mg, 100 μmol) were added to a solution of NMPe (13 mg, 100 μmol) in acetate buffer (pH 4.5, 2 mL), followed by 2% H_2O_2 (170 μL, 100 μmol). The mixture was incubated for 2 h at 37 °C. The reaction mixture was extracted three times with ethyl acetate 2 mL, and the combined organic phase was washed with 2 mL water, dried over Na_2SO_4, filtered, and evaporated in vacuo to produce a yellow oil. The extraction and the Ames assay were conducted as described above.

4.5. Reaction of *N*-nitrosodialkylamines with Fe^{2+}–Cu^{2+}–H_2O_2–NO for mutation assay

$FeSO_4{\cdot}7H_2O$ (28 mg, 100 μmol) and $Cu(OAc)_2{\cdot}H_2O$ (20 mg, 100 μmol) were added to a solution of *N*-nitrosodialkylamine (100 μmol) in acetate buffer (pH 4.5, 2 mL), followed by 2% H_2O_2 (170 μL, 100 μmol). Then NO gas (2.5 mL 100 μmol) was introduced to the reaction under nitrogen gas, and incubated for 2 h at 37 °C. The reaction mixture was extracted three times with ethyl acetate 2 mL, and the combined organic phase was washed with water 2 mL, dried over Na_2SO_4, filtered, and evaporated in vacuo to produce a yellow oil.

4.6. Reaction of NMPe with Fe^{2+}–Cu^{2+}–H_2O_2–NO for isolation of the direct-acting mutagen

$FeSO_4{\cdot}7H_2O$ (28 g, 100 mmol) and $Cu(OAc)_2{\cdot}H_2O$ (20 g, 100 mmol) were added to a solution of NMPe (13 g, 100 mmol) in acetate buffer (pH 4.5, 1 L), followed by the addition of 10%

H_2O_2 (34 mL, 100 mmol). NO gas (250 mL, 100 mmol) was introduced to the reaction for 1 h under nitrogen atmosphere, and incubated for 2 h at 37 °C. The reaction mixture was extracted twice with hexane 100 mL, twice with dichloromethane 100 mL, twice with ethyl acetate 100 mL, and twice with ethyl acetate 100 mL with salting out. Each organic phase was dried over Na_2SO_4, filtered, and evaporated in vacuo to produce a yellow oil. The CH_2Cl_2 extract showed the highest mutagenicity among the organic extracts. The crude product was purified by column chromatography twice (silica gel 60, hexane:CH_2Cl_2/Et_2O = 2:2:1, UV 254 nm), and by preparative HPLC (Lichrosorb Si60 7.5 × 250 mm 10 μm, hexane: CH_2Cl_2/EtOH = 8:1:0.2, 2.0 mL/min, UV 254 nm) to produce a colorless oil. The oil was crystallized by cooling, followed by washing with CCl_4 (mp 37.5–38.5 °C).

4.7. X-ray data for the direct-acting mutagen

A colorless prismatic crystal with dimensions of 0.48 × 0.20 × 0.07 mm was obtained by cooling, followed by washing with CCl_4. Diffraction data were collected on a crystal of the direct-acting mutagen at 173 K on a Bruker/AXS Smart 1000 diffractometer with graphite monochromated Mo-Kα radiation (λ = 0.71069 Å) in the monoclinic space group $P2_1/_n$ with a = 6.5597(4) Å, b = 9.9753(6) Å, c = 10.9713(8) Å. The structure was identified by direct methods using the program SIR-97, and expanded using Fourier techniques. The non-hydrogen atoms were refined anistropically by full matrix least-squares calculations (R = 0.046 and Rw = 0.046 for 1282 reflections ($I > 3.00\sigma(I)$)).

CCDC-821046 (the direct-acting mutagen) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/data_request/cif (or from the Cambridge Crystallographic Data Center, 12 Union Road Cambridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk).

4.8. Synthesis of 3-ethyl-3-nitro-1-pyrazoline

Diazomethane was generated from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (8.45 g, 0.039 mol) with KOH.[39] 2-Nitrobutene (2.03 g) in diethyl ether 10 mL was slowly added to a solution of diazomethane in diethyl ether at −78 °C, and the mixture was stirred. After 30 min, the reaction was allowed to warm to room temperature. After 3 h, the yellow color disappeared. The reaction mixture was washed twice with water 50 mL and brine 20 mL. The ether phase was dried over Na_2SO_4, filtered, and evaporated in vacuo to produce a yellow oil. The oil was purified chromatographically [Silica Gel 60, hexane/CH_2Cl_2/Et_2O = 4:2:1, UV 254 nm or diphenylamine] to obtain 3-ethyl-3-nitro-1-pyrazoline (yield 42%). UV (nm, CH_3CN) λ = 200 (ε = 4930), 249 (ε = 440), 325 (ε = 75). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.00 (3H, t, J = 7.4 Hz, –CH_2CH_3), 1.86 (1H, ddd, J = 14.5, 8.6, 6.0 Hz, H-4), 2.36 (1H, dq, J = 14.6, 7.4 Hz, –CH_2CH_3), 2.39 (1H, ddd, J = 14.3, 8.9, 5.4 Hz,H-4), 2.58 (1H, dq, J = 14.6, 7.4 Hz, –CH_2CH_3), 4.72–4.88 (2H, m, H-5). ^{13}C NMR ($CDCl_3$, 100 MHz): 7.82 (–CH_2CH_3), 26.0 (–CH_2CH_3), 28.7 (C-4), 78.4 (C-5), 127.8 (C-3). IR (neat) cm^{-1}: 1544, 1352 (NO_2). Anal. Calcd for $C_5H_9N_3O_2$: C, 41.95; H, 6.34; N, 29.36; O, 22.35. Found: C, 42.17; H, 6.30; N, 29.04; O, 22.49.

4.9. Synthesis of 5-ethyl-5-nitro-1-pyrazoline 1-oxide

Small portions of MCPBA 860 mg were added to a solution of 3-ethyl-3-nitro-1-pyrazoline 333 mg in CH_2Cl_2 6 mL at room temperature. After 12 h, the reaction mixture was refluxed and dimethyl sulfoxide 1 mL was added to remove the excess MCPBA. The reaction mixture was washed twice with water 10 mL and brine 5 mL, dried over Na_2SO_4, filtered, and evaporated in vacuo to obtain a residue. The crude product was purified by column chromatography (Silica Gel 60, hexane/CH_2Cl_2/Et_2O = 2:2:1, UV 254 nm, diphenylamine, anisaldehyde/sulphuric acid), by a preparative HPLC (Lichrosorb Si 60, hexane: CH_2Cl_2/EtOH = 8:1:0.4, UV 254 nm), and by a preparative TLC (silica gel, hexane/CH_2Cl_2/Et_2O = 4:4:1, UV 254 nm) to produce a colorless oil (yield 1%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.06 (3H, t, J = 7.4 Hz, –CH_2CH_3), 2.50 (1H, dq, J = 14.9, 7.4 Hz, –CH_2CH_3), 2.57 (1H, m, –CH_2CH_3), 2.60–2.68 (1H, m, H-4), 2.92–2.98 (1H, m, H-4), 4.21–4.35 (2H, m, H-3).

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