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Synthesis and properties of bifunctional chloroalkyl nitrosamines with an intercalating moiety

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Abstract—Three *N*-nitroso-*N*-(arylcarbonyloxymethyl)-3-chloropropylamines were synthesized, and their chemical and biological properties were studied. All arylcarboxylates intercalated with double-stranded DNA, and their mutagenicity and DNA cross-linking activity were affected by their ring structure. The DNA interstrand cross-link formation increased dose dependently after treatment with the acridine analog. The anthraquinone analog showed the highest bacterial mutagenicity among the three nitrosamines in *Salmonella typhimurium* TA100, while in *Salmonella typhimurium* TA92, which can detect cross-linking agents, the acridine analog showed the highest mutagenicity. This agreed with the result of a cross-linking assay. These results suggest that the three-ring aromatic moiety gives DNA-intercalating ability to cross-linkable chloropropyl nitrosamine, and the acridine analog is considered as a possible new antitumor lead compound.

1. Introduction

Chloroethyl nitrosoureas are clinically effective antitumor alkylating agents.[1] Antitumor *N*-nitrosoureas such as 1,3-bis(2-chloroethyl)-1-nitrosourea have a chloroethyl moiety as a common bifunctional group, and decompose under physiological conditions to generate chloroethyldiazohydroxides similarly to the decomposition of carcinogenic *N*-nitroso compounds.[1] Of the two geometric isomers, (*E*)-chloroethyldiazohydroxide reacts with DNA to form an alkylated base,[2] and a second alkylation can lead to DNA cross-links due to the presence of the chloro leaving group.[1,2] DNA interstrand cross-links prevent separation of the DNA double strands in the replication process, which eventually inhibits DNA synthesis, resulting in a cytotoxic effect.[3]

Among *N*-nitroso compounds, chloroethyl nitrosocarbamates were developed as potential antitumor alkylators in addition to many *N*-nitrosoureas,[4] but many *N*-nitrosamines are carcinogens, and thus, hardly considered as antitumor agents. We have studied the chemical and biological properties of carcinogenic *N*-nitrosamines.[5,6] Subsequently, we have been interested in bifunctional nitrosamines, which could act as DNA cross-linking agents in vivo, and have antitumor activity. *N*-Nitroso cross-linkers possess high reactivity toward cellular components, but simultaneous protein carbamoylation may occur in the case of *N*-nitrosoureas, which is thought to be the cause of their side effects.[7] In a previous study, we synthesized chloroalkyl nitrosamines as mimics of antitumor chloroethylnitrosoureas.[8] These compounds are expected not to carbamoylate cellular components because of their nitrosamine structure. The chlorinated nitrosamines **1**–**3** (Fig. 1) showed direct mutagenicity, with activity higher

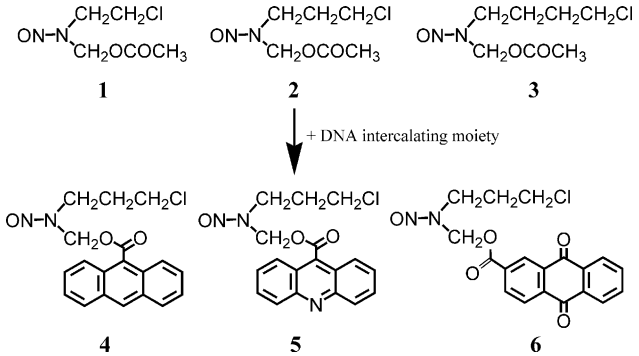

Figure 1. Structures of *N*-nitroso-*N*-(acetoxymethyl)-ω-chloroalkylamines (**1**–**3**) and nitrosamines synthesized in this study (**4**–**6**).

Keywords: Nitrosamine; DNA cross-link; Antitumor agent.
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than the parent α-acetoxy nitrosamines.[8] In a further study with various bacterial strains having DNA repair ability, the mechanism of mutation of chlorinated nitrosamines involved DNA alkylation as well as DNA cross-linking.[9]

Anticancer alkylating agents are important for cancer chemotherapy, and new drugs with more effective and more selective activity are desired.[10] Recently, hybrid alkylating molecules with an intercalating or minor groove binding moiety were produced to alter sequence specificity or DNA binding affinity.[11–14] DNA intercalation is thought to be DNA-selective, and compounds with intercalating activity showed stronger activity than simple cross-linking agents.[12] In this study, chloropropyl nitrosamines with an aromatic ring system as the intercalating moiety were synthesized (Fig. 1, **4**–**6**) to enhance the DNA affinity of chloropropyl nitrosamine, and their chemical and biological behaviors was compared with their structures.

2. Results and discussion

2.1. Chemistry

A three-ring aromatic system was selected as the minimum structural moiety for the intercalator, and anthracene,[15] acridine,[11] or anthraquinone[16] structures were introduced into the structures of cross-linkable chloropropyl nitrosamine as carriers for DNA in vivo (Fig. 1).

The nitrosamines were expected to intercalate DNA at first, then to release an active form of nitrosamine by hydrolysis of the ester, and finally, the alkyldiazonium ion was expected to alkylate DNA (Fig. 2). The molecule presented here was characterized by the detection of activated nitrosamine, which is released only after ester bond cleavage at the intercalation site.

Three novel arylcarboxylates of chloropropyl nitrosamines were synthesized according to the reported

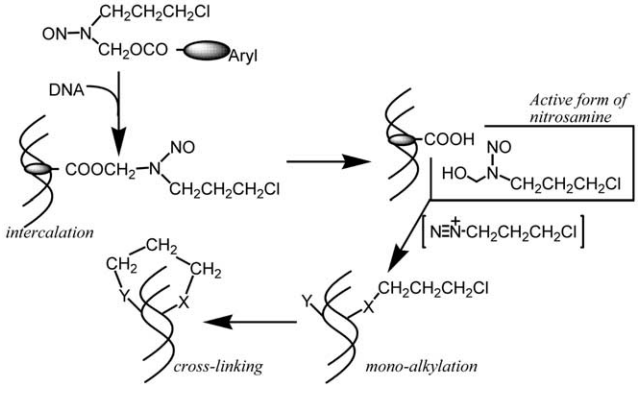

Figure 2. Possible mechanism of intercalation, alkylation, and cross-linking of DNA by chloropropyl nitrosamines having an aromatic ring system.

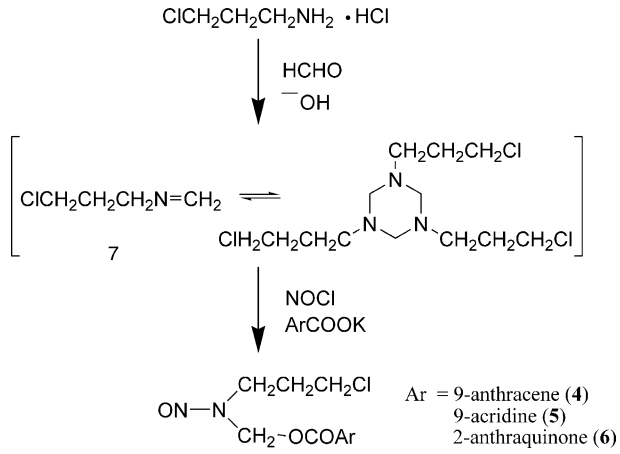

Figure 3. Synthesis of *N*-nitroso-*N*-(arylcarbonyloxymethyl)-3-chloropropylamines.

method (Fig. 3).[17,18] *N*-3-Chloro-*N*-methylenepropylamine (**7**) was synthesized by the reaction of 3-chloropropylamine with formaldehyde, and treatment with nitrosyl chloride, followed by addition of the corresponding potassium arylcarboxylate. The products were purified by silica gel column chromatography, and in the case of **5**, the crude solids obtained by column chromatography were further recrystallized.

The rate of decomposition of the nitrosamines in an aqueous solution was determined by incubating an acetonitrile solution of each nitrosamine mixed with a phosphate buffer. When the amount of nitrosamine was analyzed by reverse-phase HPLC, the nitrosamines were shown to decompose exponentially with time. Arylcarboxylate esters were more unstable than the parent acetate ester (half life = 25 h pH = 7.4),[8] and **5** decomposed the most rapidly. The half lives of the nitrosamines at pH 7.4 were as follows: **4**, 1.6 h; **5**, 0.11 h; **6**, 3.7 h. The mechanism of decomposition seems to be base-catalyzed hydrolysis of carboxylate. The stability of the arylcarboxylate can be explained by the leaving ability of the arylcarboxylate anion as compared to acetate anion. That is, arylcarboxylate anion is a weaker base than acetate anion, as determined by the acidity of their conjugate acids.

2.2. DNA intercalating activity

A competitive ethidium displacement assay[19] was performed to determine the DNA binding ability of the nitrosamines. All nitrosamines intercalated with DNA, but the activities were weaker than that of amsacrine, a common intercalator. Among the nitrosamines tested, **6** showed a higher binding affinity than either **4** or **5**. The nitrosamine **2**, which does not have intercalating moiety, showed no activity in this assay (Fig. 4).These results suggested that nitrosamines **4**–**6** have DNA intercalating activity due to their aromatic ring systems, but the differences in their activities were very small.

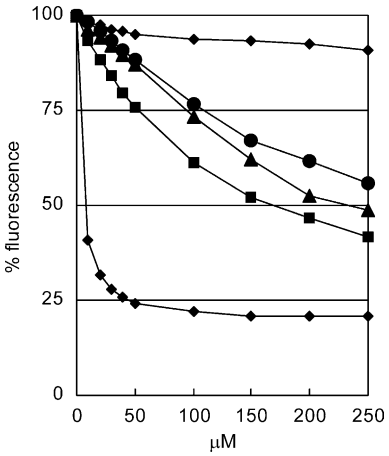

Figure 4. Competitive ethidium-DNA fluorescence displacement assay of **2** (◆), **4** (●), **5** (▲), **6** (■), and amsacrine (◆).

2.3. DNA interstrand cross-linking activity

The ability of nitrosamines to form DNA interstrand cross-links was determined using plasmid DNA.[20] In the samples treated with **5**, double-stranded DNA due to cross-link formation increased dose dependently; that is **5** clearly formed DNA interstrand cross-links in plasmid DNA (Fig. 5, lanes 3–5). On the other hand, **6** showed no activity in this test system (Fig. 5, lanes 6–8). The activity of **5** was weaker than that of cisplatin, a common cross-linker (Fig. 5, lanes 9–10) but was similar to that of **2**.[9] These results suggests that introduction of aromatic ring systems does not affect the interstrand cross-linking activity of nitrosamines.

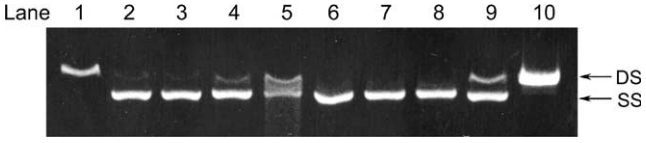

Figure 5. Cross-linking activity of **5** and **6** toward plasmid DNA. Lane 1; nondenatured DNA, lane 2; denatured DNA, lane 3; treated with 0.1 mM **5** for 48 h, lane 4; treated with 1 mM **5** for 48 h, lane 5; treated with 10 mM **5** for 48 h, lane 6; treated with 0.1 mM **6** for 48 h, lane 7; treated with 1 mM **6** for 48 h, lane 8; treated with 10 mM **6** for 48 h, lane 9; treated with 0.1 mM cisplatin for 6 h, lane 10; treated with 0.01 mM cisplatin for 6 h. DS and SS are bands of double-stranded and single-stranded DNA, respectively.

2.4. Mutagenicity

Bacterial mutagenicity was assayed in three *Salmonella typhimurium* strains TA100, TA98, and TA92. In TA100, which can detect base pair change mutation, **4**–**6** had positive activity; and the activity of **6** was higher than that of **2**[8,9] (Fig. 6A), followed by **4** and **5**. In TA98, which can detect frameshift mutation, **5** and **6** showed stronger mutagenicity than **4**, but only **6** showed killing effect (Fig. 6B). This results suggested that **6** intercalated more effectively, which agreed well with the results in an intercalating assay. In TA92 (+*uvrB*, pKM101), which can detect cross-linking agents, **5** showed higher mutagenicity than **2**. (Fig. 6C) The order of mutagenicity in strain TA92 agreed with the result of a cross-linking assay using plasmid DNA. From these results, acridine analog **5** was expected to act as an more effective DNA cross-linker than parent compound, **2**, because of the introduction of intercalating acridine structure.

There are many factors that affect the biological activity of nitrosamines, such as stability, intercalating activity, alkylating activity, and cross-linking activity. In this study, cross-linkable chloropropyl nitrosamines demonstrated DNA affinity, but intercalating ability and stability in an aqueous solution did not contribute to enhanced cross-linking activity. Nevertheless, cross-linking activity and bacterial mutagenicity differed with the structure of the ring system in chloropropyl nitrosamines. Stable **6**, with the highest intercalating activity and the highest mutagenicity in TA100 and TA98, was not a cross-linker, while unstable **5**, with weaker mutagenicity than **6** in TA100, had stronger mutagenicity in TA92 and also showed cross-linking activity. If the

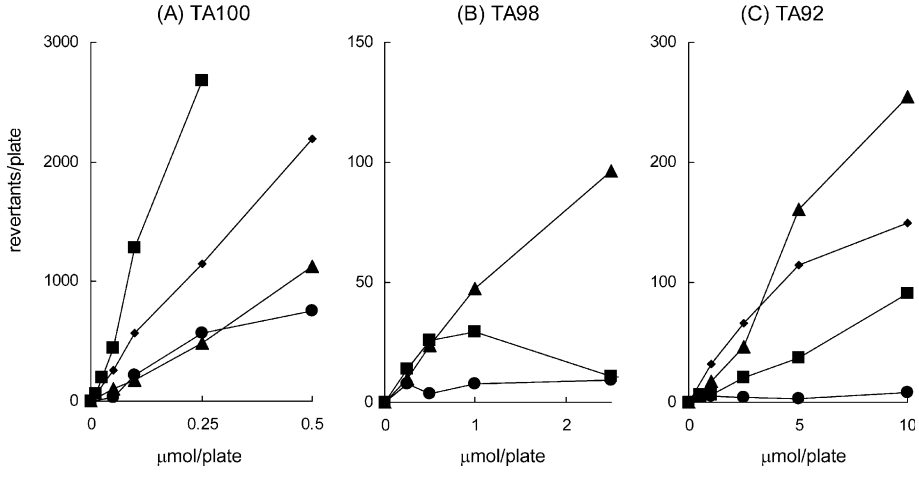

Figure 6. Mutagenicity of *N*-nitroso-*N*-(arylcarbonyloxymethyl)-3-chloroalkylamines in *Salmonella typhimurium* TA100, TA98, and TA92: (◆) **2**; (●) **4**; (▲) **5**; (■) **6**.

nitrosamines react following the mechanism in Figure 2, and if intercalating activity does not affect the activity of nitrosamines, no difference in alkylating and cross-linking activity among nitrosamines **4**–**6** should be expected. But the difference in activities of nitrosamines **5** and **6** is clear, and from these results, base sequence selectivity of DNA intercalation can possibly explain the difference between **5** and **6**. Further study is needed to confirm the contribution of structure of the bifunctional nitrosamines with aromatic ring systems on base sequence specificity of DNA alkylation or DNA cross-link formation.[21]

3. Conclusion

N-Nitroso-*N*-(acetoxymethyl)-ω-chloroalkylamines are DNA cross-linkers, which are expected to be antitumor lead compounds. To enhance their DNA affinity further, three *N*-nitroso-*N*-(arylcarbonyloxymethyl)-3-chloropropylamines were synthesized, and their chemical and biological behavior was tested. Arylcarboxylates were more unstable in aqueous solution than the acetate ester, and the acridine analog decomposed most rapidly. The DNA binding ability of the nitrosamines was determined by a competitive DNA-ethidium fluorescence quenching assay, and all arylcarboxylates intercalated to double-stranded DNA, and their activities were affected by ring structure or substituents. Furthermore, the ability of the nitrosamines to form DNA interstrand cross-links was determined using plasmid DNA, and cross-link formation increased dose dependently after treatment with the acetate and the acridine analog. When bacterial mutagenicity was assayed in *Salmonella typhimurium* TA92, which can detect cross-linking agents, the acridine analog showed the highest mutagenicity; this agreed with the result of a cross-linking assay using plasmid DNA. These results suggested that the three-ring aromatic moiety gave DNA-intercalating ability to cross-linkable chloropropyl nitrosamine, and the acridine analog that formed DNA cross-links efficiently is considered as a possible new antitumor lead compound. Although the cross-linkable chloropropyl nitrosamines achieved DNA affinity, DNA intercalation was not a driving force behind the cross-link formation. Difference in base sequence specificity of DNA alkylation or DNA cross-link formation derived from aromatic ring structure may contribute to the activity of the nitrosamines in vivo.

4. Experimental

4.1. Chemistry

^{1}H NMR spectra were measured on Jeol JNM-A500 and JNM-ECP600 spectrometers using tetramethylsilane as an internal reference. The abbreviations for signal patterns are as follows: s, singlet; t, triplet; q, quartet; qu, quintet; se, sextet; br, broad; m, multiplet. MS spectra were determined on a Jeol JMS-700 spectrometer. HPLC analysis was performed on a system consisting of a Shimadzu LC-10AD pump, a Shimadzu SPD-10A UV detector, and a Shimadzu C-R6A chromatopac. Fluorescence intensity was recorded on a Hitachi F-4000 fluorescence spectrophotometer.

Most reagents used were purchased from Wako Pure Chemical Ind. (Osaka, Japan) as the purest grade available. 3-Chloropropylamine hydrochloride was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Silica gel 60 was purchased from Merck & Co. (Rahway, NJ, USA). Nitrosyl chloride (NOCl) was synthesized by the reported method,[22] and was used without further purification.

4.1.1. Chloro-*N*-methylenepropylamine 7. 3-Chloropropylamine hydrochloride (2.6 g, 20 mmol) was stirred with formalin (1.7 mL, 21 mmol) in an ice bath; then aqueous sodium hydroxide (1.7 g/10 mL) was dropped slowly into the solution. After reaction for 5 h, the reaction solution was extracted with diethyl ether. The ether layer was washed with water and dried with anhydrous sodium sulfate, then evaporated, which gave 0.6 g of pale yellow oil. Yield: 95.7%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.90 (2H, q, $J = 6.6$ Hz, C–CH_2–C), 2.59 (2H, t, $J = 7.0$ Hz, CH_2–N), 3.34 (2H, br, N=CH_2), 3.62 (2H, t, $J = 6.6$ Hz, Cl–CH_2). From NMR data, the compound **7** exists as a trimer[17,23] at ambient temperature as shown in Figure 3. The product was used without further purification for the following synthesis.

4.1.2. *N*-Nitroso-*N*-(9-anthracenecarbonyloxymethyl)-3-chloropropylamine 4. A dichloromethane (CH_2Cl_2) solution of NOCl (12.1 mL, 5.7 mmol) was stirred in an ice bath, then the solution of **7** (0.6 g/CH_2Cl_2 10 mL, 5.7 mmol) was added dropwise. To the solution, 1.5 g of potassium 9-anthracenecarboxylate (5.8 mmol) was added, and stirred overnight. The suspension was filtered, and the filtrate was evaporated under reduced pressure. The yellow oil obtained was purified by silica gel column chromatography to give 0.17 g of yellow oil. Yield: 8.3%. ^{1}H NMR (600 MHz, $CDCl_3$): δ 2.02 (1.7H, qu, $J = 6.3$ Hz, *E*-C–CH_2–C), 2.36 (0.3H, qu, $J = 6.5$ Hz, *Z*-C–CH_2–C), 3.49 (1.7H, t, $J = 6.3$ Hz, *E*-CH_2Cl), 3.61 (0.3H, t, $J = 6.0$ Hz, *Z*-CH_2Cl), 3.88 (1.7H, t, $J = 7.1$ Hz, *E*-CH_2N), 4.62 (0.3H, t, $J = 6.9$ Hz, *Z*-CH_2N), 5.86 (0.3H, s, *Z*-CH_2O), 6.72 (1.7H, s, *E*-CH_2O), 7.49–7.53 (2H, m, arom-H), 7.55–7.58 (2H, m, arom-H), 7.98 (0.6H, d, $J = 8.8$ Hz, *Z*-arom-H), 8.05 (3.4H, dt, $J = 9.6$, 1.1 Hz, *Z*-arom-H), 8.56 (0.15H, s, *Z*-arom-H), 8.59 (0.85H, s, *E*-arom-H). EI-MS m/z: 356 (M^+), 358 ([M+2]$^+$). FAB-HR-MS m/z: 356.0905 (calcd for $C_{19}H_{17}ClN_2O_3$: 356.0928 (M^+)).

4.1.3. *N*-Nitroso-*N*-(9-acridinecarbonyloxymethyl)-3-chloropropylamine 5. An NOCl solution (13.3 mL, 9.0 mmol) was stirred in an ice bath, then the solution of **7** (0.95 g/CH_2Cl_2 10 mL, 9.0 mmol) was added dropwise. To the solution, potassium 9-acridinecarboxylate (2.4 g,

9.2 mmol) was added, and the mixture was stirred overnight. The suspension was filtered and the filtrate evaporated under reduced pressure. The yellow oil obtained was purified by silica gel column chromatography to give a yellow solid. The solid was recrystallized from hexane and chloroform, to give 0.27 g of pale yellow needles. Yield: 8.4%. Mp: 93.0–94.0 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.99–2.04 (1.7H, m, *E*-C–CH_2–C), 2.36 (0.3H, qu, $J = 6.6$ Hz, *Z*-C–CH_2–C), 3.49 (1.7H, t, $J = 6.0$ Hz, *E*-CH_2Cl), 3.61 (0.3H, t, $J = 6.0$ Hz, *Z*-CH_2Cl), 3.88 (1.7H, t, $J = 7.1$ Hz, *E*-CH_2N), 4.62 (0.3H, t, $J = 6.8$ Hz, *Z*-CH_2N), 5.87 (0.3H, s, *Z*-CH_2O), 6.70 (1.7H, s, *E*-CH_2O), 7.62–7.65 (2H, m, arom-H), 7.81–7.85 (2H, m, arom-H), 7.97 (0.3H, d, $J = 8.8$ Hz, *Z*-arom-H), 8.01 (1.7H, d, $J = 8.8$ Hz, *E*-arom-H), 8.26–8.30 (2H, m, arom-H). EI-MS *m*/*z*: 357 (M^+), 359 ($[M+2]^+$). FAB-HR-MS *m*/*z*: 358.0932 (calcd for $C_{19}H_{18}ClN_2O_3$: 358.0958 ($[M+H]^+$)).

4.1.4. *N*-Nitroso-*N*-(2-anthraquinonecarbonyloxymethyl)-3-chloropropylamine 6. An NOCl solution (13.5 mL, 9.6 mmol) was stirred in an ice bath, then the solution of **7** (1.0 g/CH_2Cl_2 10 mL, 9.6 mmol) was added dropwise. To the solution, potassium 2-anthraquinonecarboxylic acid (2.3 g, 7.9 mmol) was added and stirred overnight. The suspension was filtered and the filtrate was evaporated under reduced pressure. The yellow oil obtained was purified by silica gel column chromatography to give 0.11 g of yellow solid. Yield: 4.1%. Mp: 92.0–94.5 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.04 (1.8H, qu, $J = 6.0$ Hz, *E*-C–CH_2–C), 2.38–2.41 (0.2H, m, *Z*-C–CH_2–C), 3.52 (1.8H, t, $J = 6.1$ Hz, *E*-CH_2Cl), 3.60–3.75 (0.2H, m, *Z*-CH_2Cl), 3.86 (1.8H, t, $J = 7.0$ Hz, *E*-CH_2N), 4.57 (0.2H, t, $J = 6.7$ Hz, *Z*-CH_2N), 5.69 (0.2H, s, *Z*-CH_2O), 6.54 (1.8H, s, *E*-CH_2O), 7.84–7.86 (2H, m, arom-H), 8.33–8.37 (2H, m, arom-H), 8.32–8.46 (2H, m, arom-H), 8.88 (0.1H, s, *Z*-arom-H), 8.96 (0.9H, s, *E*-arom-H). EI-MS *m*/*z*: 386 (M^+), 388 ($[M+2]^+$). FAB-HR-MS *m*/*z*: 387.0714 (calcd for $C_{19}H_{16}ClN_2O_5$: 387.0748 ($[M+H]^+$)).

4.1.5. Decomposition of nitrosamines. Fifty microliter of an acetonitrile (CH_3CN) solution of *N*-nitroso-*N*-(arylcarbonyloxymethyl)-3-chloroalkylamine was added to 450 μL of 0.05 M phosphate buffer (pH 7.4), and the solution was incubated at 37 °C (final concentration: 10 mM). At constant intervals, 5 μL of the solution was injected into the HPLC system, and the amount of nitrosamine remaining was analyzed on a LiChrosorb RP-18 column (10 μm), eluted with a 70:30 mixture of CH_3CN and water.

4.2. DNA intercalating assay

DNA intercalating activity was assayed by a competitive ethidium displacement assay.[19] To a mixture of calf thymus DNA and ethidium bromide at pH 7.0, an aliquot of a dimethyl sulfoxide (DMSO) solution of nitrosamine was added, and the intensity of fluorescence (λ_{EX} 520 nm, λ_{EM} 600 nm) was recorded. Remaining fluorescence was expressed as a percentage of the initial fluorescence. The final fluorescence was obtained by subtraction of the value measured in the absence of ethidium bromide from the value measured in the presence of ethidium.

4.3. Interstrand cross-linking assay

The assay procedure was performed according to the method of Hartley et al.[20] and the method reported previously.[9] DMSO was freshly distilled from calcium hydride before use (bp_{14} 76 °C). Linearized pBluescript® DNA (280 ng) was treated with the test compound dissolved in DMSO at pH 7.4, 37 °C for 48 h. After the reaction, the DNA was precipitated and was lyophilized to dryness. The redissolved DNA was heated at 95 °C for 5 min, and then chilled in an ice bath immediately. The DNA samples were separated by 1% agarose gel electrophoresis, and the DNA bands were visualized with ethidium staining.

4.4. Mutagenicity of nitrosamines

4.4.1. Bacterial strains. A culture of *Salmonella typhimurium* TA100 and TA98 was kindly provided by Dr. B. N. Ames, University of California, Berkeley, USA, and TA92 was kindly provided by Dr. T. Nohmi, National Institute of Health Sciences, Tokyo, Japan.

4.4.2. Mutation assay. The method for mutation assays was the same as reported previously.[8] All data reported are representative of at least three experiments using duplicate plates for each dose level.

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