Synthesis and Properties of Novel Bifunctional Nitrosamines with ω-Chloroalkyl Groups

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Novel *N*-nitroso-*N*-(acetoxymethyl)-ω-chloroalkylamines were synthesized and their chemical and biological properties were evaluated. The nitrosamines were expected to decompose through ω-chloroalkyldiazohydroxides in aqueous solution, and then to alkylate various cellular macromolecules. *N*-Nitroso-*N*-(acetoxymethyl)-2-chloroethylamine rapidly decomposed in aqueous solution, and the reaction rate was apparently independent of the pH of the solution. On the other hand, the rate of decomposition of chloropropyl and chlorobutyl homologs was pH-dependent, and increased in alkaline solution. When mutagenicity was assayed in *Salmonella typhimurium* TA1535 and TA92 for preliminary evaluation, all three compounds were directly mutagenic. The mutagenicity in *Salmonella typhimurium* TA1535, which can detect base-pair change mutation, clearly showed that these compounds induced DNA alkylation *in vivo*. The increase of alkyl chain length in chloroalkyl compounds increased the mutagenic activity, and the activities were stronger than those of the corresponding simple α-acetoxy nitrosamines lacking a chloro group, *N*-nitroso-*N*-(acetoxymethyl)alkylamines. Furthermore, the positive result in TA92 suggested that chlorinated nitrosamines cross-linked DNA like antitumor chloroethylnitrosoureas and that they are expected to be new lead compounds for antitumor agents.

Key words ω-chloroalkyl nitrosamine; antitumor agent; DNA cross-linking; DNA alkylation; mutagenicity

There are various carcinogens in the environment, such as polycyclic aromatic hydrocarbons, aromatic amines and *N*-nitrosamines.[1—3] Among many carcinogenic chemicals, *N*-nitroso compounds are important not only because they are present in the environment, but also because they can be formed under the acidic conditions in the stomach,[3] or under neutral *in vivo* conditions by the action of activated macrophages on amines.[4]

N-Nitrosodialkylamines are a group of environmental carcinogens and are metabolized *in vivo* by cytochrome P450. The metabolites, α-hydroxy nitrosamines, decompose spontaneously to alkanediazohydroxides and then to alkyldiazonium ions.[5] Alkanediazohydroxides and alkyldiazonium ions can react with biological nucleophiles in nucleic acids and proteins. The resulting DNA alkylation is thought to be an important reason why *N*-nitroso compounds show carcinogenic and/or mutagenic activities.[5] Since α-hydroxy nitrosamines are unstable in aqueous solution, α-acetoxy nitrosamines[6] and α-hydroperoxy nitrosamines[7,8] have been used as precursors for α-hydroxy nitrosamines in research on the chemical and biological activities of *N*-nitrosamines. The α-hydroxy nitrosamines were isolated by deoxygenation of α-hydroperoxy nitrosamines,[9,10] and their chemistry and mutagenicity have been elucidated.[10] Furthermore, the mutagenicity and alkylating activity of simple alkanediazohydroxides, which are the ultimate alkylating agents produced from *N*-nitroso compounds, were examined using their potassium salts as the precursors.[11,12]

While many *N*-nitroso compounds are considered as carcinogens, some *N*-nitrosoureas have antitumor activities.[13] Chloroethyl *N*-nitrosoureas (CENUs) such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and chlorozotocin 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. Of the two geometrical isomers, (*E*)-chloroethyldiazohydroxide can react with DNA to form alkylated base,[14] and the second alkylation reaction due to the presence of the chloro leaving group can lead to DNA cross-links.[13] DNA interstrand cross-links prevents separation of DNA double strands in replication process, which eventually inhibits DNA synthesis, resulting in a cytotoxic effect.

We synthesized *N*-nitrosamines having a chloroethyl group to evaluate their chemical and biological properties. In the present study, in order to investigate the properties of activated *N*-nitrosodialkylamines having a chloroalkyl group with a longer alkyl chain, we synthesized *N*-nitroso-*N*-(acetoxymethyl)-ω-chloroalkylamines (**1**—**3**, Fig. 1). The nitrosamines were expected to hydrolyze and decompose to chloroalkyldiazohydroxides in an aqueous solution, then to alkylate various cellular macromolecules (Fig. 2). To confirm the reaction of the nitrosamines with DNA, the stability in an

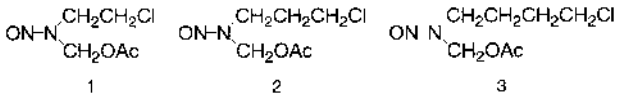

Fig. 1. Structures of *N*-Nitroso-*N*-(acetoxymethyl)-ω-chloroalkylamines Synthesized in This Study

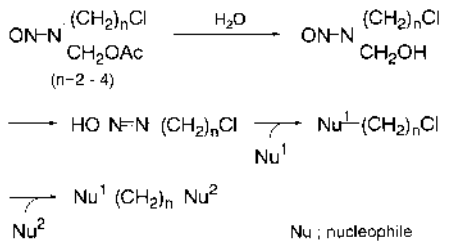

Fig. 2. Possible Reaction of *N*-Nitroso-*N*-(acetoxymethyl)-ω-chloroalkylamines with Nucleophiles

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Dedicated to the memory of Dr. Kyosuke Tsuda.

aqueous solution and mutagenicity towards two bacterial strains were assayed and the results were compared with those of simple nitrosamines without a chloro moiety to elucidate the reaction mechanism of chloroalkyl nitrosamines under physiological conditions.

Experimental

^{1}H-NMR spectra were measured on JEOL JNM-A500 (500 MHz) and JNM-GX270 (270 MHz) spectrometers using tetramethylsilane as an internal reference. The abbreviations for signal patterns are as follows: s, singet; t, triplet; q, quartet; se, sextet; br, broad; m, multiplet. UV spectra were recorded on a Hitachi U-3210 spectrometer. MS spectra were determined on a JEOL JMS-DX303 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. Most reagents used were purchased from Wako Pure Chemical Ind. (Osaka, Japan) as the purest grade available. 2-Chloroethylamine hydrochloride, 3-chloropropylamine hydrochloride and 4-amino-1-butanol were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Kieselgel 60 (230—400 mesh) and sodium ammonium hydrogenphosphate were purchased from Merck & Co. (Rahway, NJ, U.S.A.). Dimethyl sulfoxide (DMSO) was freshly distilled from calcium hydride before use (bp_7 58 °C). Bacto agar and nutrient broth were purchased from Difco Laboratories (Detroit, MI, U.S.A.).

***N*-Nitroso-*N*-(acetoxymethyl)-2-chloroethylamine (1)**[15] 2-Chloroethylamine hydrochloride 5.5 g (54 mmol) was dissolved in 150 ml of acetic acid, and 5 ml of formalin (HCHO 62 mmol) was added. The solution was stirred in an ice bath and aqueous sodium nitrite (7.5 g/20 ml, $NaNO_2$ 109 mmol) was added. Reaction temperature was kept below 10 °C for 6 h, and 50 ml of water was added to the resulting solution and it was extracted with dichloromethane. The organic layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The yellow oil obtained was purified by silica gel column chromatography to give 1.2 g of yellowish oil. Yield: 12.2%. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 2.06 (3H, S, Z-C$\underline{H}_3$), 2.15 (3H, S, *E*-C$\underline{H}_3$), 3.57 (2H, t, J=6.2 Hz, *E*-C$\underline{H}_2$Cl), 3.89 (2H, t, J=6.0 Hz, *Z*-C$\underline{H}_2$Cl), 3.92 (2H, t, J=6.3 Hz, *E*-C$\underline{H}_2$N), 4.63 (2H, t, J=6.3 Hz, *Z*-C$\underline{H}_2$N), 5.40 (2H, s, *Z*-C$\underline{H}_2$O), 6.24 (2H, s, *E*-C$\underline{H}_2$O). *E* : *Z*=89.6 : 10.4.

***N*-Nitroso-*N*-(acetoxymethyl)-3-chloropropylamine (2)** 3-Chloropropylamine hydrochloride 5.8 g (50 mmol) was dissolved in 150 ml of acetic acid, and 5 ml of formalin (HCHO 62 mmol) was then added. The solution was stirred in an ice bath and was treated with aqueous sodium nitrite solution (6.9 g/20 ml, $NaNO_2$ 100 mmol). Reaction temperature was kept below 10 °C for 6 h, and 50 ml of water was added to the resulting solution and it was extracted with dichloromethane. The organic layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The yellow oil obtained was purified by silica gel column chromatography to give 2.02 g of yellowish oil. Yield: 20.7%. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.97 (2H, q, J=6.7 Hz, *E*–C–C$\underline{H}_2$–C), 2.07 (3H, s, *Z*-C$\underline{H}_3$), 2.15 (3H, s, *E*-C$\underline{H}_3$), 2.30 (2H, m, *Z*–C–C$\underline{H}_2$–C), 3.48 (2H, t, J=6.3 Hz, *E*-C$\underline{H}_2$Cl), 3.62 (2H, t, J=6.3 Hz, *Z*-C$\underline{H}_2$Cl), 3.73 (2H, t, J=7.0 Hz, *E*-C$\underline{H}_2$N), 4.43 (2H, t, J=6.8 Hz, *Z*-C$\underline{H}_2$N), 5.37 (1H, s, *Z*-C$\underline{H}_2$O), 6.19 (1H, s, *E*-C$\underline{H}_2$O). *E* : *Z*=90.1 : 9.9. UV λ_{max} (CH_3CN) nm (ε): 231 (6900), 369 (110). MS *m/z*: 194 (M^+), 196 ($[M+2]^+$). *Anal*. Calcd for $C_6H_{11}ClN_2O_3$: C, 37.03; N, 14.40; H, 5.70. Found: C, 36.73; N, 14.22; H, 5.53.

***N*-Nitroso-*N*-(acetoxymethyl)-4-chlorobutylamine (3)** 4-Amino-1-butanol 4.7 ml (50 mmol) was added dropwise to 4 ml of thionyl chloride (55 mmol) in an ice bath. When the dark brownish solution was allowed to warm to 50 °C, gas evolution was observed then the solution was evaporated to dryness. The resulting residue was dissolved in 15 ml of ethanol. After refluxing for 30 min, the solution was filtered through activated carbon. Evaporation of the resulting solution gave 3.5 g of yellow gel. ^{1}H-NMR (270 MHz, DMSO-d_6) δ: 1.74 (4H, m, C-C$\underline{H}_2$C$\underline{H}_2$-C), 2.80 (2H, se, J=6.4 Hz, C$\underline{H}_2$-NH_3^+), 3.67 (2H, t, J=6.4 Hz, C$\underline{H}_2$–Cl), 8.11 (3H, br, $N\underline{H}_3^+$). Yield: 44.7%. Crude 4-chlorobutylamine hydrochloride 3.5 g (*ca*. 25 mmol) was dissolved in 80 ml of acetic acid, and 2.5 ml of formalin (HCHO 31 mmol) was added. The solution was stirred in an ice bath and aqueous sodium nitrite (3.5 g/10 ml, $NaNO_2$ 50 mmol) was added. Reaction temperature was kept below 10 °C for 15 h, and 50 ml of water was added to the resulting solution and it was extracted with dichloromethane.The organic layer was dried over anhydrous sodium sulfate, then evaporated under reduced pressure. The pale yellow oil obtained was purified by silica gel column chromatography to give 0.66 g of yellowish oil. Yield: 12.7%. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.67 (4H, m, *E*-C-C$\underline{H}_2$-C$\underline{H}_2$-C), 1.85 (2H, m, *Z*-C$\underline{H}_2$-C-Cl), 1.98 (2H, m, *Z*-C$\underline{H}_2$-C-N), 2.06 (3H, s, *Z*-C$\underline{H}_3$), 2.14 (3H, s, *E*-C$\underline{H}_3$), 3.52 (2H, t, J=6.1 Hz, *E*-C$\underline{H}_2$Cl), 3.60 (2H, t, J=7.3 Hz, *Z*-C$\underline{H}_2$Cl), 3.61 (2H, t, J=7.4 Hz, *E*-C$\underline{H}_2$N), 4.30 (2H, t, J=7.0 Hz, *Z*-C$\underline{H}_2$N), 5.36 (1H, s, *Z*-C$\underline{H}_2$O), 6.16 (1H, s, *E*-C$\underline{H}_2$O). *E* : *Z*=90.1 : 9.9. MS *m/z*: 208 (M^+), 210 ($[M+2]^+$). *Anal*. Calcd for $C_7H_{13}ClN_2O_3$: C, 40.29; N, 13.43; H, 6.28. Found: C, 39.99; N, 13.19; H, 6.21.

Decomposition of Nitrosamines Ten μl of an acetonitrile (CH_3CN) solution of *N*-nitroso-*N*-(acetoxymethyl)-ω-chloroalkylamines was added to 990 μl of 0.1 M phosphate buffer (pH 5.0, 7.4 and 9.0), and the solution was incubated at 37 °C (final concentration; 1 mM). At 15 min 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 mixtures of CH_3CN and water.

Bacterial Strains A culture of *Salmonella typhimurium* TA1535 was kindly provided by Dr. B. N. Ames, University of California, Berkeley, U.S.A., and TA92 was kindly provided by Dr. T. Nohmi, National Institute of Health Sciences, Tokyo, Japan.

Mutation Assay The medium used for overnight culture of bacterial strains was a nutrient broth medium (NB) containing 6 g of nutrient broth and 5 g of NaCl per liter. The minimal media used for mutation assays were the same as reported by Maron and Ames.[16] The agar plates contained 30 ml of the minimal medium with 1.5% Bacto agar.

The bacteria were grown in 5 ml of NB for 15.5 h at 37 °C. Test compounds were diluted in DMSO. To a tube containing 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.4) and 0.1 ml of a culture of bacterial tester strain, 0.1 ml of sample solution was added, then the mixture was shaken immediately. Two milliliters of top agar was added to the mixture (0.6% agar in 0.6% NaCl) at 45 °C, and it was poured onto a minimal agar plate. After incubation at 37 °C for 2 d, the colonies on the plate were counted manually, and the number of spontaneous revertant colonies was subtracted from that of the induced revertants. The concentration of the test compounds was expressed as μmol/plate. All data reported are representatives of at least three experiments using duplicate plates for each dose level.

Results and Discussion

DNA alkylation and DNA cross-linking are classical mechanisms for activity of cancer chemotherapeutic agents such as mitomycin C and carmustine (BCNU), currently used in clinical treatment. These agents alkylate DNA by reaction with one electrophilic center, allowing a neighboring nucleophile in the alkylated DNA to react with a second electrophilic center to eventually form a DNA cross-link.

The chloroethyl moiety is a common bifunctional group antitumor cross-linking agents. CENUs are one group of effective chemotherapeutic agents with a chloroethyl group, and many analogs have been developed in an effort to improve DNA-directing reactivity and reduce side effects.[13] *N*-Nitrosamides including CENUs show their biological activities by alkylation and carbamoylation, and protein carbamoylation is thought to be the cause of their side effects. In contrast, *N*-nitrosamines degrade *in vivo* by releasing aldehydes and alkylate cellular nucleophiles, with no possibility of carbamoylation. *N*-Nitrosamines are thought to be environmental carcinogens, and little has been reported about their antitumor activity. We are interested in the behavior of nitrosamines having a chloroethyl, or chloroalkyl group with a longer carbon chain because of their potential as lead compounds for novel antitumor agents.

The synthesis of compound **1** and its effect on increasing the life span of tumor-bearing mice has been already reported,[15] but little is known about its precise biological activity. We have additionally synthesized nitrosamines with chloropropyl and chlorobutyl groups, compounds **2** and **3** respectively, and the properties for **2** and **3** were compared with **1**, and the effect of alkyl-chain length and the chloro leaving group was examined.

As one of the key chemical properties, the rate of decom-

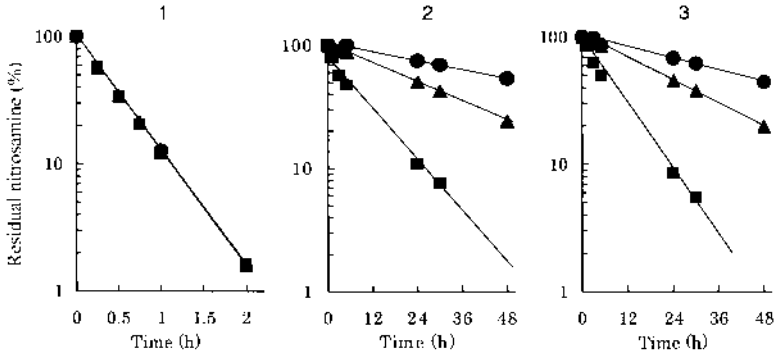

Fig. 3. Decomposition of *N*-Nitroso-*N*-(acetoxymethyl)-ω-chloroalkylamines in Aqueous Solutions
●, pH 5.5; ▲, pH 7.4; ■, pH 9.0.

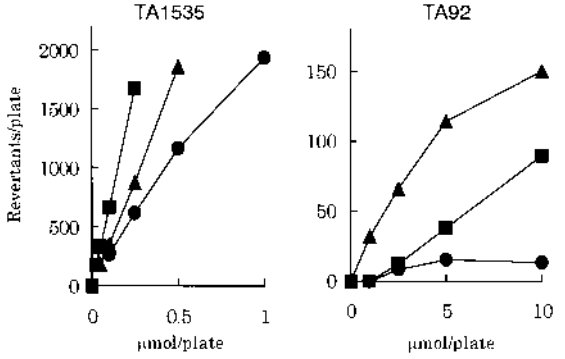

Fig. 4. Mutagenicity of *N*-Nitroso-*N*-(acetoxymethyl)-ω-chloroalkylamines in *Salmonella typhimurium* TA1535 and TA92
●,**1**; ▲,**2** ; ■,**3**.

Table 1. Relative Mutagenicity of *N*-Nitroso-*N*-(acetoxymethyl)-ω-chloroalkylamines in *Salmonella typhimurium* TA1535

Compound		Relative mutagenicity (revertants/μmol)
ON–N(CH_2)$_n$Cl, CH_2OAc	**1** (n=2)[a]	2320
	2 (n=3)[a]	3700
	3 (n=4)[a]	6650
ON–N(CH_2)$_n$$CH_3$, CH_2OAc	n=1[b]	530
	n=2[b]	450
	n=3[b]	620

a) This paper. *b*) Ref. 6.

position of these nitrosamines in aqueous solution was determined. The concentration of the nitrosamines decreased exponentially in phosphate buffer, which indicates that the nitrosamines time-dependently decomposed, as shown in Fig. 3. Compound **1** rapidly decomposed in aqueous solution, and the rate was apparently independent of the pH of the solution. On the other hand, compounds **2** and **3** were comparably stable and decomposed pH-dependently, and the rate of the reaction increased under alkaline conditions. The half-lives of **1**, **2** and **3** under physiological conditions (pH 7.4) were calculated as 0.3, 25 and 21 h, respectively. The stability of **2** and **3** in aqueous solution and the effect of pH on their decomposition were clearly different from those of **1**, which suggested that the mechanism of decomposition of **2** and **3** was different from that of **1**. That is, **2** and **3** decomposed more rapidly at higher pH, because the acetyl group was hydrolyzed through base catalysis and this step determined the overall rate of decomposition. Compound **1** decomposed pH-independently because of the chloroethyl group, since there is a neighboring group effect as observed in nitrogen mustards, which accelerates decomposition under aqueous conditions.

The mutagenicity of compounds **1**—**3** was assayed in *Salmonella typhimurium* TA1535 and TA92 as a preliminary evaluation of their biological activity. All compounds were directly mutagenic, and the mutagenic potency was linearly related to the concentration of the chemicals (Fig. 4). A specific mutagenicity per μmol of chemical was defined by the slope of the linear part of the dose–mutagenicity relationship, and was calculated by the least squares method. Table 1 shows the specific mutagenicity for three series of chemicals in *Salmonella typhimurium* TA1535 strains in comparison to that for *N*-nitroso-*N*-(acetoxymethyl)alkylamine lacking a chloro group.[6] In the TA1535 strain, which can detect base-pair change mutations, an increase of alkyl chain length potentiated the mutagenic activity. The activities were stronger than those of the corresponding simple α-acetoxy nitrosamines without a chloro group. These results suggested that the nitrosamines reacted with bacterial DNA, and induced DNA alkylation *in vivo* more frequently than simple α-acetoxy nitrosamines did. One of the reasons why the mutagenicity of **1** was weaker than those of **2** and **3** was that the replacement by chlorine enhanced the reactivity due to a neighboring group effect. Thus, **1** rapidly decomposed in aqueous conditions, and the chance to react with DNA decreased *in vivo*, which weakened its mutagenicity in bacteria. Compounds **2** and **3** seem to have sufficient stability to react with bacterial DNA, and their alkylating activity may be reflected in their mutagenic potency.

Although chlorinated nitrosamines were reported as a product of nitrosation of spermidine in the presence of chloride ion[17] and as a product from reaction of hydroxylated nitrosamine and chloride ion,[18] there are few reports about chlorinated *N*-nitroso compounds except CENUs. Haloethylnitrosoureas form haloethyldiazotates as ultimate electrophiles, and the *E*-isomers of diazotates are reported to form DNA cross-links in contrast to the *Z*-isomer. Furthermore, *E*-2-chloroethyldiazotate exhibited activity against P388 leukemia *in vivo*.[19] The chloroalkylnitrosamines synthesized in this study may also form chloroalkyldiazotates,

and they are expected to act as cross-linking agents. *Salmonella typhimurium* TA92 retains excision repair capabilities and is commonly used for detection of cross-linking agents, so we chose this assay at first for evaluating whether the compounds have cross-linking activity that is essential for antitumor activity. Compound **1** showed weaker mutagenicity in TA92 than the corresponding α-acetoxy nitrosamine lacking a chloro group (data not shown), which was attributed to its instability. On the other hand, compound **2** showed higher mutagenicity than the corresponding α-acetoxy nitrosamine lacking a chloro group, but compound **3** showed the same mutagenic potency as the corresponding α-acetoxy nitrosamine lacking a chloro group (data not shown). Compound **2** showed a weaker mutagenicity than compound **3** towards the TA1535 strain, but a higher mutagenicity towards the TA92 strain. These results suggested that not only a chloroethyl group but also a chloropropyl group could behave as an antitumor bifunctional group, and suggested the possibility that chlorinated nitrosamine derivatives could be applicable as lead compounds for antitumor agents. Further investigation is in progress to study in detail whether ω-chloroalkyl α-acetoxymethyl nitrosamines form DNA cross-links similar to antitumor chloroethylnitrosoureas.

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