Arch Pharm Res Vol 25, No 1, 39-44, 2002

Archives of Pharmacal Research
http://apr.psk.or.kr

Synthesis, Characterization and *In Vitro* Identification of N^7-Guanine Adduct of 2-Bromopropane

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(Received October 30, 2001)

Recently, we have reported that 2-bromopropane might have an immunotoxic potential in rats when exposed for 28 days. In the present studies, the possibility of 2ĺ-deoxyguanosine adduct formation by 2-bromopropane was investigated *in vitro* to elucidate molecular mechanism of 2-bromopropane-induced immunosuppression. N^7-Guanine adduct of 2'-bromopropane (i.e., N^7-isopropyl guanine) was chemically synthesized and structurally characterized by analysis of UV, ^{1}H-NMR, ^{13}C-NMR, COSY and fast atom bombardment mass spectrometry to use as a reference material. Incubation of 2'-deoxyguanosine with an excess amount of 2-bromopropane in PBS buffer solution, pH 7.4, at 37°C for 16 h, followed by a thermal hydrolysis, produced a detectable amount of N^7-isopropyl guanine by an HPLC and UV analysis. The present results suggest that 2-bromopropane might form a DNA adduct in N^7 position of 2'-deoxyguanosine at a physiological condition.

Key words: 2-Bromopropane, DNA adduct, Immunotoxic, N^7-Isopropyl guanine

INTRODUCTION

2-Bromopropane, $CH_3CHBrCH_3$, is a major component of the mixture of SPG-6AR and Solvent 5200 which is widely utilized as a cleansing solvent in electronic factories. It has been reported that a number of female workers, exposed occupationally to 2-bromopropane, were diagnosed with amenorrhea and male workers with oligospermia in Korea (Kim *et al.* 1996). Following this mass intoxication of workers, many extensive studies have been performed to assess toxic effects of 2-bromopropane on mutagenicity and reproductive organs (Maeng and Yu 1997, Ichihara *et al.* 1997, Kamijima *et al.* 1997, Lim *et al.* 1997, Yu *et al.* 1997, Yu *et al.* 1999a, Yu *et al.* 1999b, Ishikawa *et al.* 2001, Yu *et al* 2001a). Most recently, we have found that 2-bromopropane might also have immunotoxic potential in rats when treated for 28 days (Jeong *et al.* 2002). Meanwhile, the molecular mechanisms of toxicity of 2-bromopropane have never been studied. In the Salmonella Ames test, 2-bromopropane induced mutagenicity in TA 100 and TA 1535 strains (Maeng and Yu 1997), indicating a possibility of adduct formation by 2-bromopropane with DNA. On the other hand, the chromosomal aberration analysis did not reveal positive results in Chinese hamster lung cells, nor the micronucleus test in the bone marrow of rats. Therefore an identification of specific 2-bromopropane-induced DNA adducts is critical to understand the mechanism of its toxic effects. It is strongly expected that the bromide of 2-bromopropane can undergo an S_N2-type substitution at the carbon on bromine. Highly nucleophilic sites in DNA, such as N^7 of guanine and N^3 of adenine, are most likely to participate in these reactions.

This paper reports chemical synthesis and purification of N^7-guanine adduct of 2-bromopropane (i.e., N^7-isopropyl guanine), and full structural characterization of N^7-isopropyl guanine by analysis of UV, ^{1}H-NMR, ^{13}C-NMR, COSY and fast atom bombardment (FAB) mass spectrometry. In addition, *in vitro* adduct formation of 2-bromopropane with 2'-deoxyguanosine at physiological conditions was reported. Incubation of 2'-deoxyguanosine with an excess amount of 2-bromopropane in PBS buffer

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solution, pH 7.4, at 37°C for 16 h, followed by thermal hydrolysis, produced a detectable amount of N^7-isopropyl guanine.

MATERIALS AND METHODS

Materials

2-Bromopropane (purity, 99+%) and dimethyl formamide (DMF) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Guanosine and 2'-deoxyguanosine were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC grade acetonitrile (ACN) was obtained from Fisher Scientific (Fair Lawn, NJ). Thin-layer chromatography (TLC) and column chromatography were performed with Kieselgel 60 F_{254} (Merck) and silica gel (Kieselgel 60, 70-230 mesh, Merck), respectively. All other reagents used were of reagent grade commercially available.

Synthesis of N^7-isopropyl guanine

Synthesis of N^7-isopropyl guanine was carried out as described in the literature (Brooks and Lawley 1961), with some modification schemed in Fig. 1. Guanosine (1.00 g, 3.53 mmol) was dissolved in anhydrous DMF (15 ml) in a 100 ml round bottomed flask under an anhydrous atmosphere. An excess amount of 2-bromopropane (6.2 ml, 66.2 mmol) was added, and the mixture was stirred for 3 hr at 85°C under an argon atmosphere. Following that the mixture was cooled to room temperature and the insoluble material was filtered off. Then the filtrate was collected and evaporated to dryness under a reduced pressure. The residue was dissolved in 1 M aqueous HCl solution (20 ml), and the solution was heated to 100°C for 1 h. The mixture was cooled to room temperature, and 10% aqueous $NaHCO_3$ solution was added until the pH of the solution became 5. The resulting precipitate was filtered, collected, and purified by a silica gel column chromatography with an elution of CH_2Cl_2/MeOH (9:1, v:v) to give a colorless solid, which was recrystallized twice in water to afford a colorless crystal (86 mg, 0.45 mmol).

TLC (CH_2Cl_2:MeOH = 3:1, v:v), R_f = 0.46

Mp >300°C

^{1}H-NMR (250 MHz, DMSO-d_6): ppm 1.45 (d, 6H, J = 6.7 Hz, H-11), 4.77 (m, 1H, J = 6.7 Hz , H-10), 6.15 (s, 2H, H-2), 8.00 (s, 1H, H-8), 10.82 (s, 1H, H-1)

^{13}C-NMR (62.5 MHz, DMSO-d_6): ppm 22.82, 49.23, 107.87, 140.92, 152.80, 154.51, 160.55

FAB MS: $[M+H]^+$ = 194

Reaction of 2'-deoxyguanosine with 2-bromopropane at physiological condition

The reaction of 2-bromopropane and 2'-deoxyguanosine at physiological condition was carried out by the previously reported methods (Selzer and Elfarra 1996, Tretyakova *et al.* 1997), with some modification. 2'-Deoxyguanosine (2.0 mg) was dissolved in PBS buffer solution (pH 7.4, 1.0 ml) in a 10 ml vial. 2-Bromopropane (0.3 ml) was added to the vial via microsyringe injection, and incubated for 16 hr at 37°C. After a white precipitate formed was removed by filtration, diethyl ether (1.0 ml) was added to the vial. Then the mixture was extracted three times to remove unreacted 2-bromopropane. The remained aqueous solution was heated to 100°C for 30 min and analyzed by an HPLC as described below.

HPLC analyses

HPLC analyses were performed with a 10 μl injection volume on a Waters XTerra® 5 μm ODS reverse-phase C_{18} analytical column (250 × 4.6 mm i. d.), using a two Waters 510 pumps gradient-controlled HPLC system equipped with Waters photo diode array detector (Model 996) and Waters autosampler (Model 717) and dual-channel UV detection at 254 nm and 280 nm. Use of an isocratic condition in 7.5% ACN in aqueous 50 mM ammonium formate buffer solution, pH 6.9, at a flow rate of 1 ml/min afforded the best resolution of the reaction mixture.

NMR spectrometry

The ^{1}H and COSY NMR spectra were obtained on a Bruker ARX-250 spectrometer at 250 MHz, and chemical shifts were reported in ppm relative to the proton resonance of tetramethyl silane (TMS). The ^{13}C-NMR spectra were obtained at 62.5 MHz, and chemical shifts were recorded in ppm relative to the carbon resonance of TMS.

FAB mass spectrometry

The FAB mass spectrometry was conducted using a high resolution magnet sector mass spectrometer (Autospec. Micromass Co., UK) operated at 70 eV by a glycerol matrix.

RESULTS AND DISCUSSION

Synthesis and characterization of N^7-isopropyl guanine

Synthetic methods of N^7-isopropyl guanine are summarized in Fig. 1.

Guanosine was reacted with an excess amount of 2-bromopropane in DMF to afford an unstable intermediate. Then the reactant was heated to 100°C for 1 h in 1 M aqueous HCl solution (acidic hydrolysis) in order to hydrolyze carbohydrate moiety on guanosine skeleton to afford N^7-isopropyl guanine as a colorless crystal in 12.6% total

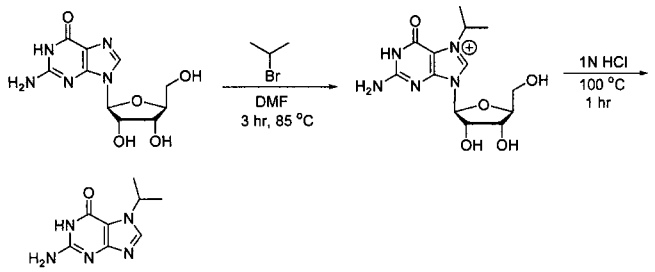

Fig. 1. Synthetic scheme of N^7-isopropyl guanine

yield after purification by a silica gel column chromatography and recrystallization in water. Since the reactivity of this type of reaction is not high, the yield obtained was relatively low. To enhance the yield, several other methods (Selzer and Elfarra 1996, Tretyakova *et al.* 1997) were tried, but failed to increase the yield. During preparation, unreacted guanosine could be easily removed by filtration since guanosine has poor solubility in DMF at room temperature. And some remained guanosine could be mostly removed in water, since water solubility of guanosine is much better than that of N^7-isopropyl guanine. Finally, the crude product was purified by a silica gel chromatography and recrystallization in water.

Fig. 2 shows the ^{1}H-NMR spectrum of prepared N^7-isopropyl guanine.

From the spectrum the chemical shift of H-11 could be found at 1.45 ppm as a doublet with coupling constant of 6.7 Hz, and integration value was corresponding to 6 H. At 4.77 ppm, chemical shift of H-10 was present as a multiplet having coupling constant of 6.7 Hz with integration value of 1 H. Chemical shift of H-2 was present at 6.15 ppm as a singlet (2 H) and H-8 was present at 8.00 ppm as a singlet (1 H). It has been reported that the chemical shift of H-8 on N^7-substituted guanine usually appears at between 8.0 - 8.1 ppm whereas that of H-8 on

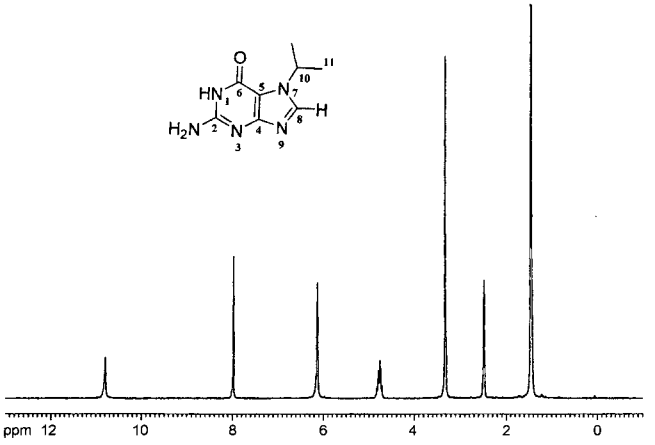

Fig. 2. 250 MHz ^{1}H-NMR spectrum of N^7-isopropyl guanine

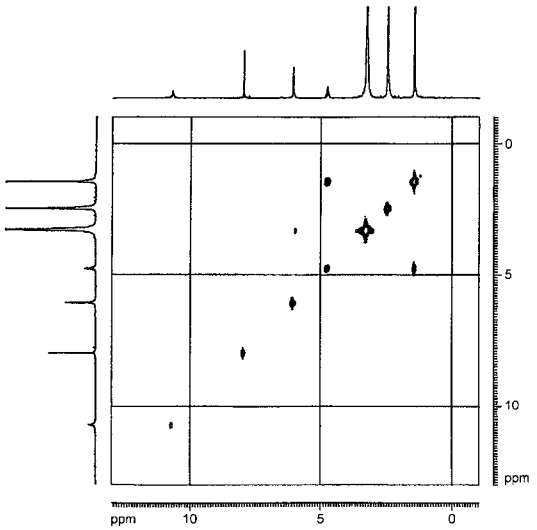

Fig. 3. Two dimensional COSY ^{1}H-NMR spectrum of N^7-isopropyl guanine

N^9-substituted guanine usually appears at between 7.8 - 7.9 ppm (Kjellberg and Liljenberg 1986). Therefore, the result indicates that the prepared compound has a correct structure as we expected. Chemical shift of H-1 was present at 10.82 ppm as a singlet (1 H). The signal assignments were confirmed by the COSY NMR experiments as shown in Fig. 3. As expected, only one cross-peak was observed at a position of 1.45 and 4.77 ppm, which indicated that H-11 and H-10 were coupled to each other, but other protons were not coupled.

In the ^{13}C-NMR spectrum of prepared N^7-isopropyl guanine, chemical shifts corresponding seven carbons on N^7-isopropyl guanine were observed. Chemical shifts of alkyl carbons on N^7-isopropyl guanine were found at 22.82 and 49.23 ppm, and those of aromatic carbons were observed at 107.87, 140.92, 152.82, 154.51 and 160.55 ppm. In FAB mass spectrum, molecular ion including hydrogen $[M+H]^+$ peak was observed at 194, which was consistent with molecular weight of 193 of N^7-isopropyl guanine. All spectral evidence was consistent with N^7-isopropyl guanine structure.

Fig. 4 shows HPLC chromatograms of the prepared N^7-isopropyl guanine and 2'-deoxyguanosine. HPLC system was operated in an isocratic elution of 7.5% ACN in aqueous 50 mM ammonium formate buffer solution, pH 6.9, at a flow rate of 1 ml/min at 280 nm detection. In the HPLC chromatogram of N^7-isopropyl guanine, only one peak was observed at a retention time of 10.1 min, which indicated that the prepared compound was a single compound. An HPLC chromatogram with a retention time at 4.5 min of 2'-deoxyguanosine was also obtained as a reference compound for the comparison of chromatogram with those of products in a reaction with 2'-deoxyguano-

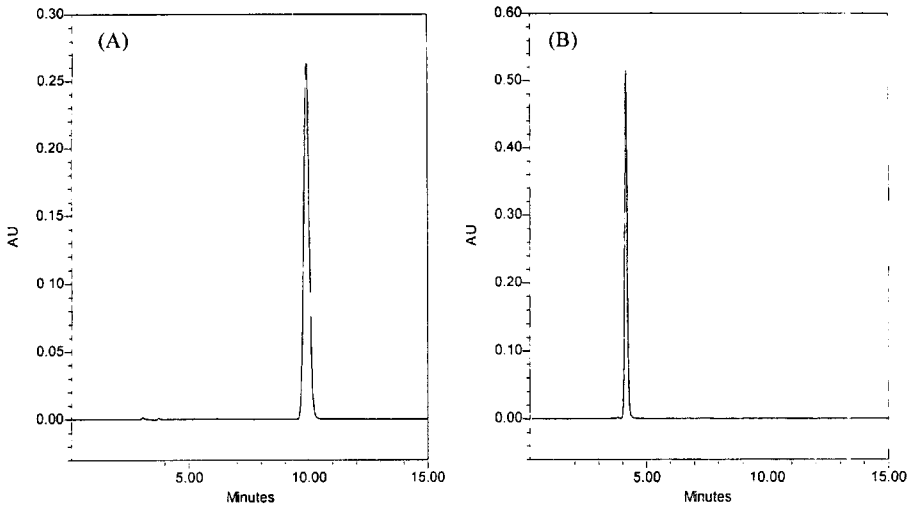

Fig. 4. HPLC chromatogram of N^7-isopropyl guanine (A) and 2'-deoxyguanosine (B)

sine and 2-bromopropane at the physiological condition.

Reaction of 2'-deoxyguanosine with 2-bromopropane at physiological condition and identification of adduct products

For the preparation and identification of adduct products of 2-bromopropane and 2'-deoxyguanosine reacted at a physiological condition, 2'-deoxyguanosine and excess amount of 2-bromopropane was incubated in PBS buffer solution, pH 7.4, at 37°C for 16 hr. After 1 h of incubation, the colorless solution became cloudy and formed a white precipitate, which gradually increased until the time of termination of reaction. At present the chemical entity of the precipitate has not been identified. After removal of the white precipitate and unreacted 2-bromopropane, the aqueous solution was heated to 100°C for 30 min to hydrolyze carbohydrate moiety (i.e., thermal hydrolysis), and the aqueous solution was analyzed by HPLC. This reaction was repeated three times to confirm the reproducibility.

Fig. 5 shows an HPLC chromatogram of products reacted with 2'-deoxyguanosine and 2-bromopropane at a physiological condition. From the chromatogram, two major peaks with retention times of 3.7 (peak **1**) and 5.8 min (peak **2**) and several minor peaks including retention time of 10.1 min (peak **3**) were observed. The peak **3** is almost the same as that of N^7-isopropyl guanine shown in Fig. 4(A). In addition, the UV spectrum of the compound corresponding to the peak **3** is in accordance with that of N^7-isopropyl guanine, which indicated that the compound

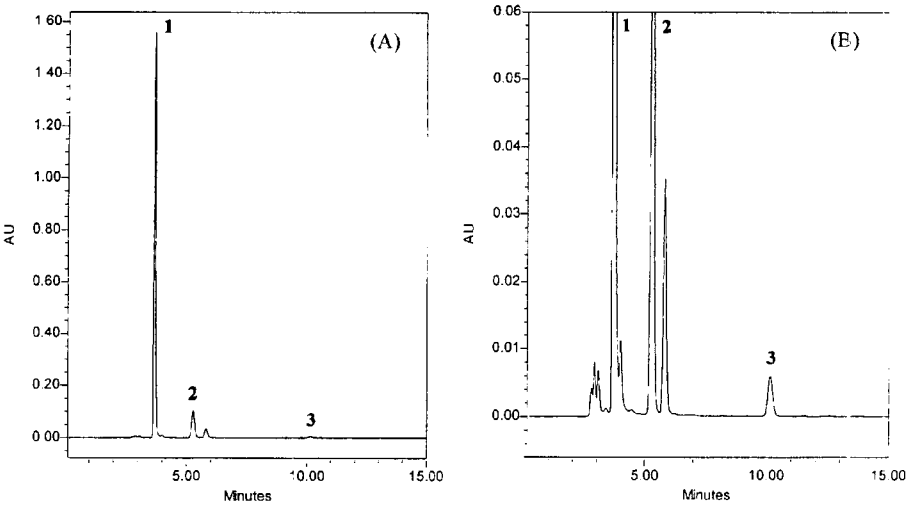

Fig. 5. HPLC chromatogram of products reacted with 2'-deoxyguanosine and 2-bromopropane at the physiological condition. (A) AUFS = 1.60, (B) AUFS = 0.06. Peak **1**; unidentified compound, peak **2**; unidentified compound, peak **3**; N^7-isopropyl guanine, respectively.

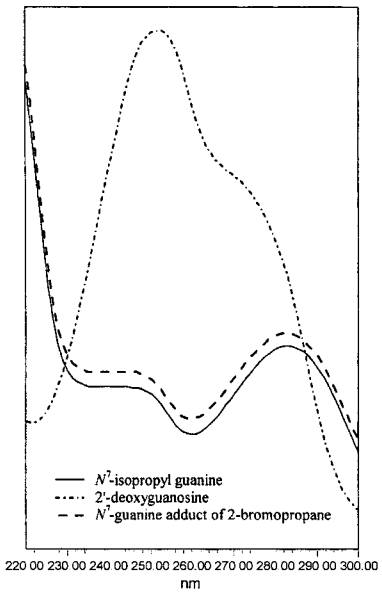

Fig. 6. UV spectra of N^7-isopropyl guanine, 2'-deoxyguanosine and N^7-guanine adduct of 2-bromopropane

corresponding to the peak **3** is N^7-isopropyl guanine (Fig. 6).

Surprisingly, the peak corresponding 2'-deoxyguanosine having a retention time of 4.5 min in Fig. 4(B) was completely disappeared and a new major peak at retention time of 3.7 min (peak **1**) was appeared. It indicated that almost of 2'-deoxyguanosine might be changed to a compound corresponding the peak at retention time of 3.7 min (peak **1**). At present, we do not have a structural clue for a compound corresponding this peak. It was only identified that a white precipitate formed during incubation of 2'-deoxyguanosine and 2-bromopropane in PBS buffer solution was a compound corresponding the peak at retention time of 3.7 min (peak **1**). In addition, it is possible that the compounds corresponding the peaks of retention time 5.2 (peak **2**) and 5.8 min might be N^1-isopropyl 2'-deoxyguanosine, N^2-isopropyl 2'-deoxyguanosine and/or O^6-isopropyl 2'-deoxyguanosine (Selzer and Elfarra 1996). Further studies are now under investigation for the identification and characterization of the unidentified products.

For further characterization of the compound corresponding to the peak **3** and differentiation of the peak **1** between a peak corresponding 2'-deoxyguanosine, authentic samples of 2'-deoxyguanosine and N^7-isopropyl guanine were co-injected with products which reacted with 2'-deoxyguanosine and 2-bromopropane at a physiological condition, which is shown in the HPLC chromatogram in Fig. 7.

From the comparison of HPLC chromatogram in Fig. 5 and Fig. 7, a new peak in Fig. 7 was generated at a retention time of 4.5 min (peak **4**) which was corresponding to 2'-deoxyguanosine, and amount and height of the peak at a retention time of 10.1 min (peak **3**) was increased, which indicated that the compound corresponding to the peak **3** is N^7-isopropyl guanine. For further confirmation, HPLC analyses of those co-injected compounds were performed in various solvent systems, which always afforded a single peak around retention time of 10 min. These results also indicated that the compound corresponding to the peak **3** is N^7-isopropyl guanine.

Although neither the chromosomal aberration analysis in Chinese hamster lung cells nor the micronucleus test in the bone marrow of rats showed positive results, a recent

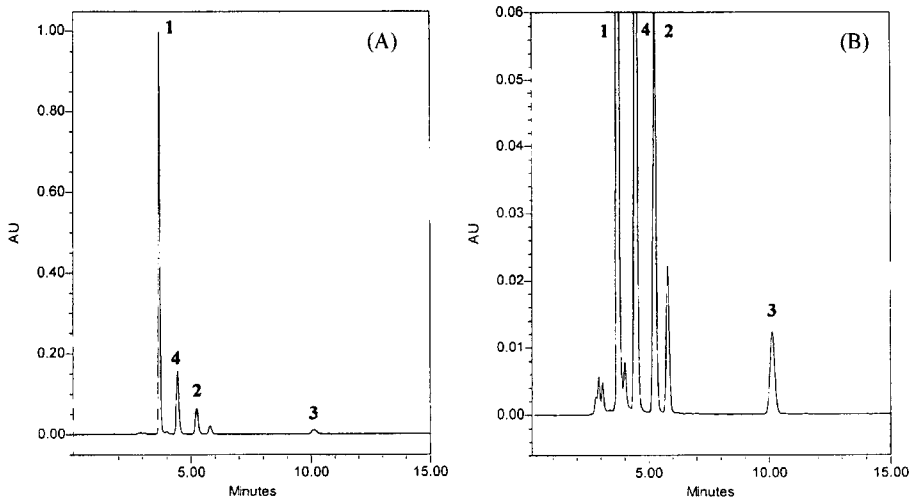

Fig. 7. HPLC chromatogram of co-injection of N^7-isopropyl guanine, 2'-deoxyguanosine and products reacted with 2'-deoxyguanosine and 2-bromopropane at the physiological condition. (A) AUFS = 1.00, (B) AUFS = 0.06. Peak **1**; unidentified compound, peak **2**; unidentified compound, peak **3**; N^7-isopropyl guanine, peak **4**; 2'-deoxyguanosine, respectively.

report showed an induction of micronuclei formation in preimplantation mouse embryos after maternal treatment with 2-bromopropane accompanied by a decrease in embryo cell number (Ishikawa *et al.* 2001). In addition, 2-bromopropane induced apoptotic death of testicular germ cells through the Bcl-2 family genes and the Fas signaling system (Yu *et al.* 2001b). Therefore, our present results suggest that the DNA damage by 2-bromopropane might be involved in toxicity induced by 2-bromopropane.

In conclusion, we synthesized and characterized N^7-isopropyl guanine, and demonstrated the formation of N^7-isopropyl guanine as an adduct product with a reaction of 2'-deoxyguanosine and 2-bromopropane at the physiological condition. These results would be useful for understanding and studying the molecular mechanism of toxic effect of 2-bromopropane. At present, however, we are not confident that the adduct formation to N^7-isopropyl guanine is responsible for the immunotoxic effect of 2-bromopropane. The adduct formation in immune cells by 2-bromopropane is under investigation at immunotoxic doses to determine a possible contribution of N^7-isopropyl guanine in 2-bromopropane-induced immunosuppression.

ACKNOWLEDGEMENTS

This work was supported by a grant (2000-2-21800-001-3) from the Korea Science and Engineering Foundation.

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