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Studies on Chemical Carcinogens and Mutagens. XXXIX.[1] Effects of Silicon and Germanium Substitutions on Physicochemical Properties and Cytotoxicity of *N*-Alkyl-*N*-nitrosoureas

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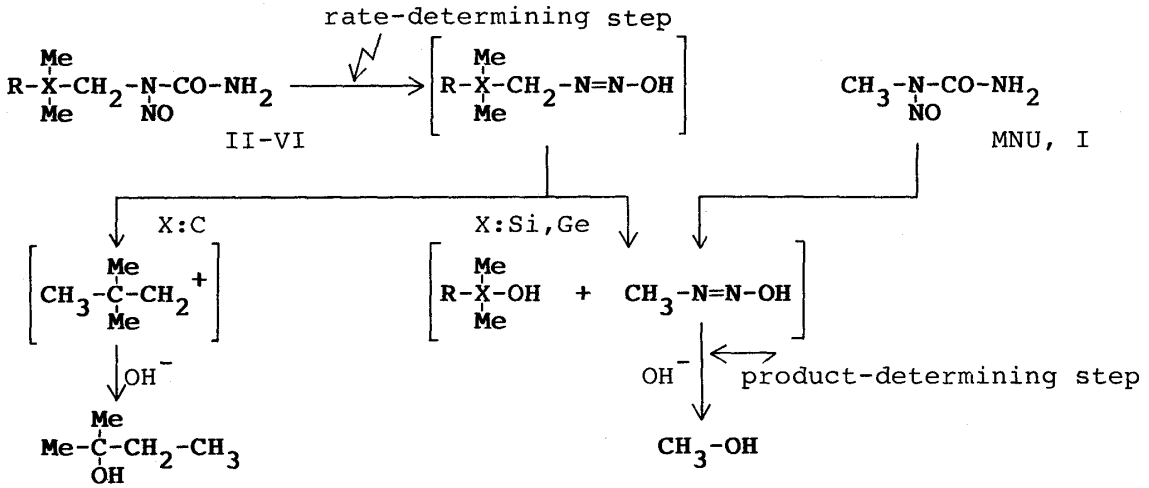
N-Trialkylsilylmethyl- and *N*-trialkylgermylmethyl-*N*-nitrosoureas, which are analogues of *N*-neopentyl-*N*-nitrosourea, were synthesized and tested for cytotoxicity to leukemia L1210 cells. Rates, activation energies, and entropies of activation of the hydrolytic activation of these derivatives were determined. The chemoselectivity and partition properties were also determined in terms of the Swain–Scott's substrate constant and $\log k'$, respectively. In aqueous media, all the Si- and Ge-containing nitrosoureas examined underwent hydrolysis accompanied by the ready cleavage of the Si–C or Ge–C bond to give methanol. From these nitrosoureas, the corresponding diazoalkanes were synthesized and reacted with a carboxylic acid in benzene containing alcohols. The results indicated that the Ge–C bond is more susceptible to hydrolysis than the Si–C bond. More lipophilic dimethylphenylsilyl and -germyl derivatives were more toxic to L-1210 than the corresponding trimethyl derivatives.

Keywords——silicon; germanium; nitrosourea; activation energy; chemoselectivity; substrate constant; diazomethane; cytotoxicity

As previously reported in brief communications,[2,3] the silicon and germanium analogues (III and IV, respectively) of *N*-neopentyl-*N*-nitrosourea (neoPNU, II) undergo hydrolysis at about the same rate as II to form the corresponding alkyldiazohydroxides. The unstable reaction intermediates thus formed are readily hydrolyzed to give methyldiazohydroxide, which is the reaction intermediate of *N*-methyl-*N*-nitrosourea (MNU, I). Thus, the rate-determining steps of III and IV should resemble that of II, whereas their product-determining steps or chemoselectivities are the same as that of I,[2,3] as shown in Chart 1. These conclusions are biologically supported by the fact that the trimethylsilylmethyl derivative (III) showed cytocidal and mutagenic activities almost the same as those of the methyl derivative (I), but different from those of II, in both *E. coli* and Chinese hamster V79 mammalian cells.[4] In this study, two more-lipophilic derivatives containing Si and Ge (V and VI, respectively) were synthesized and tested, together with the compounds so far synthesized, to examine the effects of Si- and Ge-substitutions on the physico-chemical properties and cytotoxicity to cultured leukemia L1210 cells.

Experimental

The nitrosoureas except for V and VI were synthesized by the reported methods.[2,3] All the compounds used were proved to be pure by elementary analysis and nuclear magnetic resonance (NMR) spectroscopy. The ultraviolet (UV) measurements for quantitative analyses were done with a Shimadzu UV 210A spectrophotometer equipped with a temperature controller, SPR-5 (±0.1 °C). Quantitative analysis of methanol produced by hydrolysis of the



X	R	
C	CH_3	*N*-neopentyl-*N*-nitrosourea (neoPNU, II)
Si	CH_3	*N*-trimethylsilylmethyl-*N*-nitrosourea (TMS-MNU, III)
Ge	CH_3	*N*-trimethylgermylmethyl-*N*-nitrosourea (TMG-MNU, IV)
Si	C_6H_5	*N*-dimethylphenylsilylmethyl-*N*-nitrosourea (DMPS-MNU, V)
Ge	C_6H_5	*N*-dimethylphenylgermylmethyl-*N*-nitrosourea (DMPG-MNU, VI)

Chart 1

nitrosoureas was carried out with a Shimadzu GC-8A gas chromatograph equipped with a Porapack Q column.

***N*-Dimethylphenylgermylmethyl-*N*-nitrosourea (DMPG-MNU, VI)**——Chloromethyldimethylchlorogermane (25.0 g),[5] which was prepared by chlorination of trimethylchlorogermane, was dissolved in 30 ml of tetrahydrofuran (THF) and this solution was added dropwise to phenylmagnesium bromide, prepared from 42.0 g of phenyl bromide and 9.7 g of magnesium turnings in 100 ml of THF under a dry N_2 atmosphere. The temperature was maintained at 40—50 °C during the addition. After being refluxed for 1.5 h, the reaction mixture was mixed with aqueous saturated NH_4Cl and acidified with 0.3 N HCl. The whole was extracted with ether and the extract was dried over anhydrous $MgSO_4$. After evaporation of the solvent, the residue was distilled *in vacuo* to give 24.2 g of chloromethyldimethylphenylgermane in 80% yield. bp 93—95 °C/7 Torr. *Anal.* Calcd for $C_9H_{13}ClGe$: C, 47.15; H, 5.72. Found: C, 47.27; H, 5.72. Chloromethyldimethylphenylgermane thus prepared (2.3 g) was stirred with 1.2 g of KOCN and 0.12 g of Et_4NI in 10 ml of dimethylformamide (DMF) and warmed at 100 °C for 40 min. Under ice-cooling, ammonia gas was introduced. The solvent was evaporated off under reduced pressure, then the residue was dissolved in 10 ml of H_2O and extracted with benzene. Crystallization of the benzene extract with benzene–hexane (1 : 1, v/v) gave 0.39 g of *N*-dimethylphenylgermylmethylurea. mp 82—83 °C. *Anal.* Calcd for $C_{10}H_{16}GeN_2O$: C, 47.50; H, 6.38; N, 11.08. Found: C, 47.23; H, 6.11; N, 11.03. The urea thus prepared (0.35 g) was suspended in 8 ml of H_2O and stirred with 0.13 g of $NaNO_2$ at 0 °C. This was acidified with a mixture of 0.09 g of H_2SO_4 and 2 g of ice. The mixture was stirred at 0 °C for 50 min, then the resulting white solids were collected on a filter paper and dissolved in 20 ml of $CHCl_3$. After evaporation of the solvent, the residue was recrystallized from $CHCl_3$–hexane (1 : 2, v/v) to give 0.27 g of VI as white prisms in 89% yield. mp 77 °C. *Anal.* Calcd for $C_{10}H_{15}GeN_3O_2$: C, 42.61; H, 5.34; N, 14.91. Found: C, 42.14; H, 5.03; N, 14.87.

***N*-Dimethylphenylsilylmethyl-*N*-nitrosourea (DMPS-MNU, V)**——Chloromethyldimethylphenylsilane[6] (18.5 g) was treated with 12.2 g of KOCN and 1.2 g of Et_4NI in 10 ml of DMF and then with ammonia in the same manner as described for the preparation of VI. *N*-Dimethylphenylsilylmethylurea was obtained as white prisms in 27% yield. mp 98—99 °C. *Anal.* Calcd for $C_{10}H_{16}N_2OSi$: C, 57.64; H, 7.74; N, 13.45. Found: C, 57.40; H, 7.62; N, 13.66. The urea thus prepared (3.2 g) was treated with 1.6 g of $NaNO_2$ in the same manner as described for the preparation of VI. The *N*-nitrosourea (V) was obtained as white prisms in 70% yield. mp 62—64 °C. *Anal.* Calcd for $C_{10}H_{15}N_3O_2Si$: C, 50.61; H, 6.37; N, 17.70. Found: C, 50.37; H, 6.24; N, 17.92.

Trimethylgermyldiazomethane (VIII) and Trimethylsilyldiazomethane (VII)[7]——*N*-Trimethylgermylmethyl-*N*-nitrosourea (TMG-MNU, IV) (680 mg) was added in portions to 2 ml of 20% KOH at 0 °C over a period of 20 min. The reaction mixture was stirred at 0 °C for 2 h, then extracted with 4 ml of benzene. The benzene extract was washed twice with 5 ml of cold water and dried over $MgSO_4$. The NMR spectrum indicated that the extract contained 20.1% VIII: δ in benzene (ppm); 0.12 (CH_3) and 2.32 (CHN_2). Treatment of 1.08 g of *N*-trimethylsilylmethyl-*N*-nitrosourea (TMS-MNU, III) with 4 ml of 20% KOH gave 8 ml of a benzene solution of 28.2% of VII[7]: δ in benzene (ppm); 0.02 (CH_3) and 2.30 (CHN_2). The benzene solutions of these diazomethanes were used for the esterification experiments without further purification.

Rate of Hydrolyses——The rates were measured in 1/15 M phosphate buffer (pH 6.8) containing 5% dimethyl sulfoxide (DMSO) at appropriate temperatures by following the decrease in UV absorption at appropriate wavelengths: MNU (25.0, 30.0, and 37.0 °C, $\lambda = 244$ nm); neoPNU (25.0, 30.0, and 37.0 °C. $\lambda = 264$ nm); TMS-MNU (25.0, 30.0, and 37.0 °C. $\lambda = 243$ nm); TMG-MNU (30.0, 35.7, and 39.7 °C. $\lambda = 273$ nm); DMPS-MNU (30.1, 35.6, and 39.6 °C. $\lambda = 270$ nm); DMPG-MNU (31.1, 34.9, and 38.3 °C. $\lambda = 270$ nm).

Relative Partition Parameter, log k'——The value of log k' is defined as $\log (R_t - R_0)/R_0$, where R_t and R_0 are the retention times of the nitrosourea in question and that of the solvent used for high-performance liquid chromatographic (HPLC) analysis, respectively. HPLC was carried out with a JASCO TWINCLE type apparatus equipped with a 4.6 mm × 250 mm JASCO SIL C_{18} column at 25 °C by eluting with 60% CH_3OH in H_2O. The values presented in this paper are the means of three experiments.

Esterification of *o*-Nitrobenzoic Acid with Substituted Diazomethanes——Trimethylgermyldiazomethane (0.177 mmol) in 1 ml of benzene was added to 3 ml of 20% alcoholic benzene containing 0.245 mmol of *o*-nitrobenzoic acid. After 1 h at room temperature, the reaction mixture was directly subjected to quantitative analysis by NMR spectroscopy. Quantitation was carried out with reference to a standard amount of toluene added to the reaction mixture. As for the Si-containing diazomethane, 0.217 mmol of trimethylsilyldiazomethane was treated with 0.241 mmol of *o*-nitrobenzoic acid in the same manner as described for the Ge-derivative.

Growth Inhibition of Cultured Leukemia L1210 Cells——The cell line was a gift from Dr. T. Kato of Aichi Cancer Center Research Institute. The cells (1×10^5) were seeded in a 35-mm plastic Petri dish containing 1.95 ml of RPMI 1640 medium. The medium was prepared by mixing the following solutions: 100 ml of distilled water containing 1.02 g of RPMI (Nissui Seiyaku Co., Ltd., Tokyo) and 10 units of kanamycin, 1 ml of dist. water containing 29.2 mg of glutamine, 3.3 ml of dist. water containing 198 mg of $NaHCO_3$, and 10 ml of fetal calf serum (GIBCO, New York). After incubation for 1 d at 37 °C in a CO_2-incubator (5% CO_2 in air), 50 μl of DMSO containing an appropriate amount of a test nitrosourea was added. After 24 h, the cells which were not stained with 0.5% trypan blue solution were counted as surviving cells. The inhibition ratio is given as T/C, where T and C are the numbers of surviving cells in a test dish and a control dish, respectively.

Results

Partition Properties Estimated by HPLC

It has been suggested that, in some series of compounds, cytotoxicity is strongly correlated with the partition properties.[8] Several Si- and Ge-containing nitrosoureas were tested in the present study to establish their partition properties relative to those of the carbon analogues. Since most of the nitrosoureas examined here were not stable enough to allow measurement of the partition between an organic solvent (such as octanol) and aqueous buffer, the relative partition properties were estimated by HPLC in terms of log k' (see Experimental). The values of the relative partition parameter, log k', of *N*-alkyl-*N*-nitrosoureas tested are as follows:

R	log k'	R	log k'
CH_3– (I)	0.116	$CH_3CH_2CH_2CH_2CH_2$–	0.751
CH_3CH_2–	0.224	$CH_3C(CH_3)_2$–CH_2– (II)	0.667
$CH_3CH_2CH_2$–	0.362	$CH_3Si(CH_3)_2$–CH_2– (III)	0.717
$CH_3CH_2CH_2CH_2$–	0.539	$CH_3Ge(CH_3)_2$–CH_2– (IV)	0.733
$CH_3CH(CH_3)$–CH_2–	0.511	$C_6H_5Si(CH_3)_2$–CH_2– (V)	1.073
$C_6H_5CH_2$–	0.520	$C_6H_5Ge(CH_3)_2$–CH_2– (VI)	1.094

As expected, increase in the chain-length of the alkyl moiety makes the compounds more lipophilic and branching of the alkyl moiety makes them slightly less lipophilic. Among the compounds examined, V and VI are about 10 times (in terms of k') more lipophilic than NMU (I). With regard to the effects of Si- and Ge-substitutions, the Ge substitution yields compounds more lipophilic than the corresponding Si analogues (VI *vs.* V and IV *vs.* III), the silicon analogue being more lipophilic than the carbon analogue (III *vs.* II).

Kinetics of Hydrolysis in Aqueous Media

The rates of hydrolysis of compounds I to VI were examined in 1/15 M phosphate buffer (pH 6.8) containing 5% DMSO at 25—40 °C. The activation energy (E_a) and the entropy of

TABLE I. Kinetic Parameters of Hydrolysis of Si- and Ge-Containing *N*-Alkyl-*N*-nitrosoureas in 1/15 M Phosphate Buffer (pH 6.8) Containing 5% Dimethyl Sulfoxide at 37 °C and Swain–Scott's Substrate Constants (s) of These Derivatives

	k_{obs} (/s)	$t_{1/2}$ (min)	k_2 (/M s)[a]	E_a (kcal/mol)	$\Delta S^{\neq}$ (e.u.)[b]	s
MNU (I)	0.743×10^{-3}	15.55	0.74×10^4	31.5	60.5	0.44
neoPNU (II)	1.26×10^{-3}	9.17	1.26×10^4	29.0	53.4	<0
TMS-MNU (III)	1.22×10^{-3}	9.47	1.22×10^4	28.9	53.2	0.44
TMG-MNU (IV)	1.18×10^{-3}	9.78	1.18×10^4	26.0	43.7	0.44
DMPS-MNU (V)	1.27×10^{-3}	9.10	1.27×10^4	31.9	62.9	0.44
DMPG-MNU (VI)	1.42×10^{-3}	8.13	1.42×10^4	29.9	56.8	(NT)

a) Calculated using the p^{OH} value of 7.0, because pH-meter reading for the reaction mixture was 7.0. This estimation is based on the assumption that the addition of DMSO to the phosphate buffer (pH 6.8) affected the pK_a of phosphoric acid, but not the ionic product of water. *b*) Calculated with the k_2 values. Note that the values reported in the communication[2] are those calculated with the k_{obs} values.

activation ($\Delta S^{\neq}$) were calculated from the second order rate constants observed at appropriate temperatures between 25 and 40 °C, the correlation coefficients of the Arrhenius plots being more than 0.999 in all cases. The hydrolysis rates of II to VI are very close to each other, the half-lives ranging from 8.1 to 9.8 min. Since the half-life of MNU is 15.6 min, bulkiness of the alkyl moiety seems to accelerate the hydrolysis of nitrosoureas. The activation energy varies from 26.0 to 31.9 kcal/mol, depending on the structure of the alkyl moiety, but no correlation is apparent with either the electronic or the steric effect of the alkyl group. However, the good linear correlation of activation energy with entropy of activation, as shown in Fig. 1, may indicate that a similar reaction mechanism operates in the hydrolyses of all these derivatives.[9] Hydrolyses of all the Si- and Ge-containing derivatives examined (III to VI) gave methanol quantitatively. The chemoselectivities of these derivatives, evaluated in terms of the substrate constant in the Swain–Scott equation,[2,3] are the same as that of MNU (I), as shown in Table I.

Alkylating Property of Trimethylsilyl- and Trimethylgermyldiazomethanes

In aqueous media, either the Si–C or Ge–C bond is readily hydrolyzed prior to carbocation formation. In order to compare the ease of hydrolytic cleavage of Si–C with that of Ge–C, the esterification of *o*-nitrobenzoic acid was examined using trimethylsilyldiazomethane (VII) and trimethylgermyldiazomethane (VIII) in non-aqueous protic solvents: 20% methanol–benzene, 20% ethanol–benzene, and 20% *tert*-butanol–benzene. Compound VIII was prepared by the same procedure as that used for the preparation of the corresponding Si derivative.[7] The results are shown in Table II. Both metal–carbon bonds were partly cleaved even in *tert*-butanol medium. It is noteworthy that the Ge–C bond is slightly more susceptible to hydrolysis than the Si–C bond.

Growth-Inhibitory Effect on Leukemia L1210

Compounds I to VI were tested for cytotoxicity toward cultured leukemia L1210 cells. As shown in Fig. 2, neoPNU (II) showed no effect under the experimental conditions employed, whereas the corresponding Si and Ge analogues (III and IV) showed cytotoxicity similar in degree to that of MNU (I). The phenyl derivatives (V and VI) were the most toxic among all the derivatives examined. All the Si and Ge derivatives (III to VI) seem to be methylating agents biologically equivalent to MNU, with similar activation rates and the same chemoselectivities. With regard to cytotoxicity, it is of interest that similar extents of toxicity were shown by lipophilic TMS-MNU and TMG-MNU and water-soluble MNU, but an appreciably increased toxicity was shown by the more-lipophilic DMPS-MNU (V) and DMPG-MNU (VI). It is worth noting that no differences in cytotoxicity were apparent

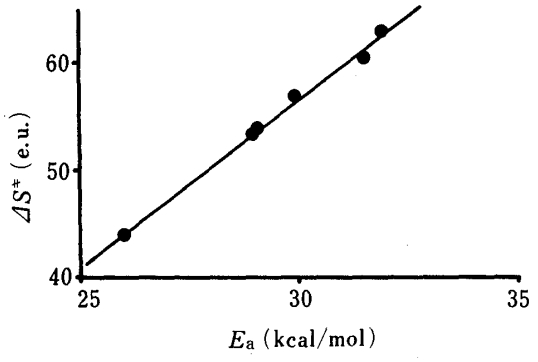


Fig. 1. Correlation of Entropy of Activation ($\Delta S^{\neq}$) at 37 °C with Activation Energy (E_a) for Hydrolysis of Nitrosoureas in Phosphate Buffer (pH 6.8) Containing 5% DMSO

$\Delta S^{\neq} = 3.166\,E_a - 38.410$ ($r = 0.998$; $n = 6$).

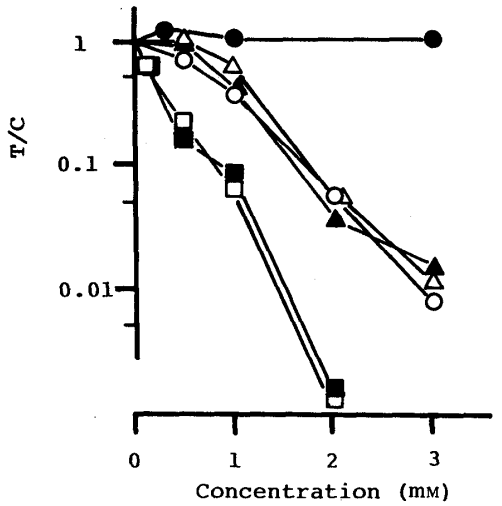


Fig. 2. Inhibitory Effect on Growth of Cultured Leukemia L1210

T/C is the ratio of the number of surviving cells in a test dish to that in a control dish (*ca.* 10^6 cell/ml) after a 24-h incubation. Each value is the average of three experiments. MNU —○—; neoPNU —●—; TMS-MNU —△—; TMG-MNU —▲—; DMPS-MNU —□—; and DMPG-MNU —■—.

TABLE II. Esterification of *o*-Nitrobenzoic Acid with Trimethylsilyldiazomethane (VII) or Trimethylgermyldiazomethane (VIII) at 25 °C for 1 h

$$Me_3\underline{M}\text{–}CHN_2 \longrightarrow [Me_3\underline{M}\text{–}CH_2\text{–}N^+\equiv N] \xrightarrow{o\text{-NBA}} o\text{-}NO_2\text{–}Ph\text{–}COO\text{–}CH_2\text{–}\underline{M}Me_3 \text{ (IX)}$$

$$[CH_3\text{–}N^+\equiv N] \xrightarrow{o\text{-NBA}} o\text{-}NO_2\text{–}Ph\text{–}COO\text{–}CH_3 \text{ (X)}$$

M: Si or Ge. *o*-NBA: *o*-nitrobenzoic acid

Solvent[a)]	MeOH–Benzene	EtOH–Benzene	*tert*-BtOH–Benzene	Benzene
Trimethylsilyldiazomethane (VII)				
IX (Si)	0.0%	8.2%	82.0%	65.0%
X (Si)	83.9%	78.8%	14.5%	31.5%
Trimethylgermyldiazomethane (VIII)				
IX (Ge)	0.0%	0.0%	52.4%	34.6%
X (Ge)	91.5%	95.5%	37.0%	48.1%

a) The content of alcohol was 20% in each solution.

between both the two sets of Si and Ge analogues (III and IV; V and VI).

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