Characterization of the Cytotoxic Activity of Nitric Oxide Generating *N*-Nitroso Compounds

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The NO-generating abilities of aromatic *N*-nitroso compounds (nitrosoureas, nitrosamides and nitrosamines), and *N*-acetyl-*S*-nitroso-DL-penicillamine at ambient temperature were compared by employing the Griess reaction. 3,3-Dibenzyl-1-(4-tolyl)-1-nitrosourea showed the greatest NO-generating ability among the tested compounds. The NO-generating ability of the aromatic *N*-nitrosoureas and the *N*-nitrosamides was greater than that of the *N*-nitrosamines, presumably reflecting differences in electrostatic repulsion between the carbonyl oxygen and nitroso oxygen in these compounds. In addition, a conjugative effect between the aromatic ring carbon and neighboring nitrogen influences the NO-generating ability; the conjugative effect in the case of *N*-nitrosoureas and *N*-nitrosamides having an *ortho*-alkyl substituted aromatic ring, or *N*-nitrosamines having a bulky *N*-group, such as *tert*-butyl, is decreased by an increase in steric hindrance around the nitroso group. The N–NO bond then becomes more stable. The NO-generating ability was related to the reciprocal of the ID_{50} value for growth inhibition of cultured L-5178 Y cells by the aromatic *N*-nitroso compounds. On the other hand, NO production from the aliphatic *N*-nitroso compounds was not observed under our conditions, and these *N*-nitroso compounds did not show effective cytotoxic activity.

Key words nitric oxide; nitrosourea; nitrosamide; nitrosamine; cytotoxicity; *N*-acetyl-*S*-nitroso-DL-penicillamine

It has been established that nitric oxide (NO) is produced by NO synthase and plays an important role as a messenger for blood vessels[1] and neurons.[2] The radical species, NO, has recently been found to be liberated from activated macrophages and to inhibit tumor cells.[3] The cytotoxic effect of many aliphatic *N*-nitroso compounds on tumor cells is known to result from the DNA binding of alkylating species generated during metabolic decomposition.[4] We recently confirmed[5] the generation of NO from aromatic *N*-nitrosoureas (**1a**,**b**,**d**,**e**, **2a**,**c**,**f**) (Fig. 1) at ambient temperature by trapping of NO as a nitrosyl complex of tetraphenylporphyrinatocobalt(II). Thus, the cytotoxicity originating from NO is expected to be potent in these aromatic *N*-nitrosoureas having NO-generating ability, compared with that in aliphatic *N*-nitrosoureas.

In this work, we report on the relationship between the structural characteristics of aromatic *N*-nitroso compounds and NO generation, as well as the relationship between the NO-generating ability and the cytotoxic action of these compounds.

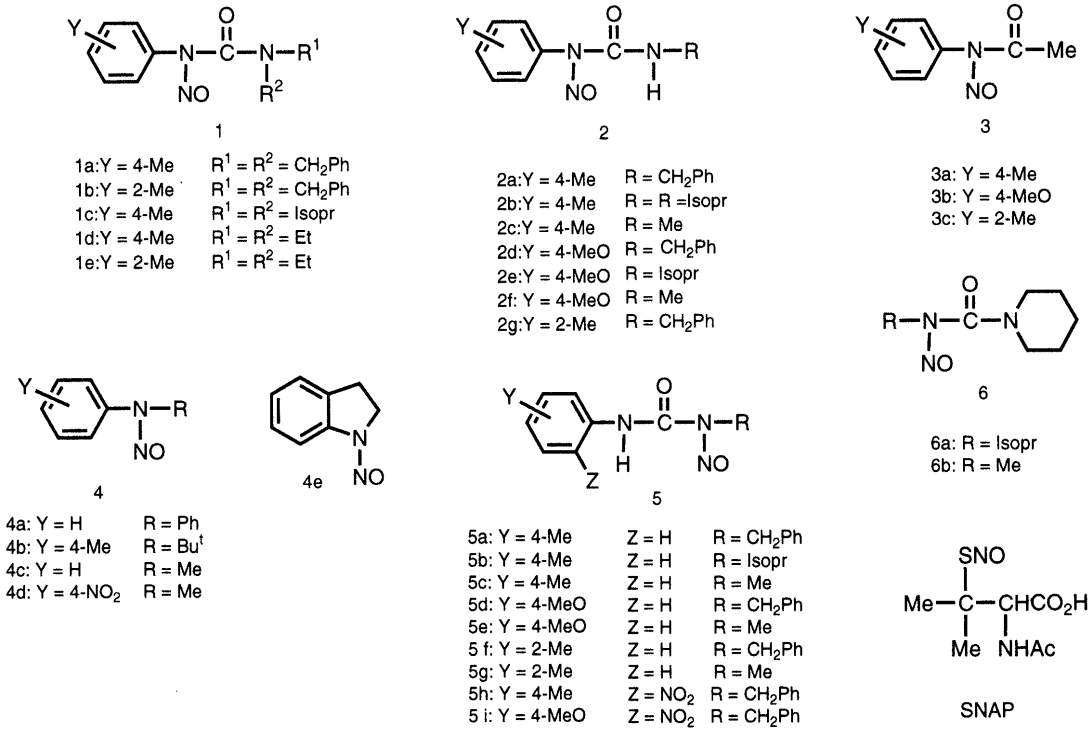

Fig. 1. Structures of Nitroso Compounds

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Results and Discussion

Structural Requirement for NO Generation by *N*-Nitroso Compounds The NO-generating ability was determined as the quantity of NO_2^- produced from $1—10\times10^{-3}$ M of a nitroso compound (Fig. 1) in $CHCl_3$. The ability was classified on the basis of that of *N*-acetyl-*S*-nitroso-DL-penicillamine (SNAP), which is known to generate NO and has been used to relax smooth muscle.[6,7] The aromatic *N*-nitrosoureas (**1**—**3**) and SNAP were decomposed in $CHCl_3$ at 37 °C for 2 h. However, a 4 h decomposition time was required for **1e** to produce a detectable amount of NO_2^-. Further, 7 h was required for NO_2^- to be determined with stable *N*-nitrosamines (**4a, c, d**). The formation of NO_2^- from *N*-nitrosamine (**4b**) or aliphatic *N*-nitrosoureas (**5, 6**) was not observed within 7 h. The NO-generating ability shown in Table 1 is represented as the amount of NO_2^- generated per 100 mM in 2 h.

The NO-generating abilities of aromatic *N*-nitrosoureas (**1, 2**) and aromatic *N*-nitrosamides (**3**) were evidently greater than those of *N*-nitrosamines (**4**) and aliphatic *N*-nitrosoureas (**5, 6**) among these *N*-nitroso compounds. In addition, the trisubstituted *N*-nitrosoureas (**1**) were better than the disubstituted ones (**2**), as exemplified by **1a** and **2a**.

Among the trisubstituted *N*-nitrosoureas (**1**), the NO-generating ability changed appreciably with the size of the substituents at the ureido-N^3 nitrogen; that is, compounds **1a, b**, having dibenzyl groups, showed a greater ability than **1d, e**, having diethyl groups. However, the difference in the NO-generating ability of the disubstituted *N*-nitrosoureas, for example, between 3-benzyl-1-(4-tolyl)-1-nitrosourea (**2a**) and 3-methyl-1-(4-tolyl)-1-nitrosourea (**2c**), was slight, regardless of the substituent size.[5] On the other hand, compound **2f**, having an electron-releasing *p*-MeO group, showed great NO-generating ability. The ability of the *N*-nitrosamide (**3b**) having an MeO group, on the contrary, was somewhat lower than that of **3a**. Among the *N*-nitrosamines **4a**—**c**, compound **4a**, having two phenyl groups, displayed a much greater NO-generating ability than **4b, c**, having alkyl groups such as Bu^t or Me. The N–NO bond of aromatic *N*-nitrosoureas (**1, 2**) and aromatic *N*-nitrosamides (**3**) is more susceptible to cleavage compared with that of the *N*-nitrosamines (**4**). In the case of these amide derivatives, liberation of NO under thermal conditions seemed to be related to electrostatic repulsion between the carbonyl oxygen and nitroso oxygen.[8] The production of NO_2^- from the aliphatic *N*-nitrosoureas (**5, 6**) could not be observed at 37 °C in 7 h, because the aliphatic *N*-nitrosoureas were only slightly decomposed at 37 °C in $CHCl_3$, or even if they do decompose, they may do so through formation of a diazo alkane by a diazo ester rearrangement[9] without NO generation. In the case of *N-tert*-butyl-*N*-nitrosoaniline (**4b**), too, no NO_2^- production was observed under the same conditions. The geometry of some *N*-nitrosoanilines has been examined by electronic spectroscopic analysis[10] of their aromatic ring carbon–anilino nitrogen (C–N) bond. The NO-generating ability of **4a**—**e** could be well explained by the geometry about the C–N bond.[10] That is to say, the NO-generating ability decreased in going from derivatives having a planar or a near-planar geometry to those having a twisted geometry; **4e** > **4a** > **4c** ≫ **4b**. The contribution of the twisted geometry of **4b** is enhanced by steric hindrance, and the N–NO bond becomes more stable as a result of localization of the unshared pair of electrons on the anilino nitrogen and the weakening of the conjugation with the π electrons of the aromatic ring.

The *p*-Me group of the aromatic ring of the *N*-nitroso compounds may also weaken the conjugation of the N–NO and the ring for the same reason. That is, the NO-generating ability of the *p*-substituted derivatives is high compared with that of the corresponding *o*-substituted compounds; **1a** and **1b**, **1d** and **1e**, **3a** and **3c**. Compound **4d** with a strongly electron-attracting *p*-NO_2 group showed the highest NO-generating ability compared to that of other compounds (**4**). Consequently, the degree of conjugative effect, which influences the N–NO bond, is determined by the geometry, which reflects the localization of the unshared pair of electrons. The N–NO bond of aliphatic *N*-nitroso compounds, which can not delocalize the unshared pair of electrons on the nitrogen bonded with the nitroso group, is stable at 37 °C in $CHCl_3$.[11]

Biological Activity of *N*-Nitroso Compounds Next, we investigated the cytotoxic effect (ID_{50}) of *N*-nitroso compounds (**1**—**4**), including their by-products (**5, 7**) and related compounds (**6**),[8] to see whether it has any relationship to the NO-generating ability. Compounds **5**[11,12] and **7**[13,14] are by-products formed by thermal decomposition of compounds **2** and **1**, respectively. The bioactivities of these compounds were determined by measuring the growth inhibition of cultured L-5178 Y cells and the results are listed in Table 2.

The ID_{50} values of aromatic *N*-nitrosoureas (**1, 2**) were

Table 1. NO-Generating Ability[a] of *N*-Nitroso Compounds

	Compd.[b]	NO-generating ability
1a	4-Tol-N(NO)CON(Bn)$_2$	4.79 (8.63)
1b	2-Tol-N(NO)CON(Bn)$_2$	2.12 (3.85)
1c	4-Tol-N(NO)CON(Isopr)$_2$	0.60 (1.09)
1d	4-Tol-N(NO)CON(Et)$_2$	0.35 (0.63)
1e	2-Tol-N(NO)CON(Et)$_2$	0.22 (0.39)[c]
2a	4-Tol-N(NO)CONHBn	1.06 (1.92)
2c	4-Tol-N(NO)CONHMe	1.07 (1.93)
2f	4-MeO-Ph-N(NO)CONHMe	2.52 (4.55)
3a	4-Tol-N(NO)COMe	0.87 (1.56)
3b	4-MeO-Ph-N(NO)COMe	0.75 (1.36)
3c	2-Tol-N(NO)COMe	0.07 (0.12)
4a	Ph-N(NO)Ph	0.02 (0.03)[d]
4b	4-Tol-N(NO)-Bu^t	0.00 (0.00)[d]
4c	Ph-N(NO)-Me	0.01 (0.02)[d]
4d	4-NO_2-Ph-N(NO)-Me	0.11 (0.20)[d]
4e	*N*-Nitrosoindoline	0.03 (0.05)[d]
5a—i	Alkyl-N(NO)CONH-Aryl	0.00 (0.00)[d]
6a	Isopr-N(NO)CON(CH$_2$)$_5$	0.00 (0.00)[d]
6b	Me-N(NO)CON(CH$_2$)$_5$	0.00 (0.00)[d]
SNAP		0.55 (1.00)

a) Concentration of each *N*-nitroso compound and SNAP for thermal decomposition in $CHCl_3$ was $1-10\times10^{-3}$ M. Reactions were carried out at 37 °C for 2 h, followed by detection as NO_2^- using the Griess method. The values are amounts of NO_2^- generated *via* NO from the 100 mM $CHCl_3$ solution of the compound, and those in parenthesis are the ratio to SNAP. Data for **1a**,**b**,**d**,**e**, **2a**,**c**, and SNAP are from reference 5. *b*) Tol = methylphenyl, Bn = benzyl. *c*) Reacted for 4 h. *d*) Reacted for 7 h.

Table 2. Cytotoxic Activity against L-5178 Y Cell Line[a] of *N*-Nitroso and Related Compounds

	Compd.[b]	ID_{50} mg/ml	ID_{50} $\times 10^{-5}$ M		Compd.[b]	ID_{50} mg/ml	ID_{50} $\times 10^{-5}$ M
1a	4-Tol–N(NO)CON(Bn)$_2$	6.3	1.8	**5a**	Bn–N(NO)CONH(4-Tol)	100.0	37.1
1b	2-Tol–N(NO)CON(Bn)$_2$	3.6	1.0	**5b**	Isopr–N(NO)CONH(4-Tol)	75.0	33.9
1c	4-Tol–N(NO)CON(isopr)$_2$	1.0	4.1	**5c**	Me–N(NO)CONH(4-Tol)	57.0	29.3
1d	4-Tol–N(NO)CON(Et)$_2$	11.0	4.8	**5d**	Bn–N(NO)CONH(4-MeO—Ph)	29.0	10.0
1e	2-Tol–N(NO)CON(Et)$_2$	12.0	5.2	**5e**	Me–N(NO)CONH(4-MeO–Ph)	51.0	24.5
2a	4-Tol–N(NO)CONHBn	20.0	7.6	**5f**	Bn–N(NO)CONH(2-Tol)	>100.0	37.1
2b	4-Tol–N(NO)CONH(isopr)	15.0	6.6	**5g**	Me–N(NO)CONH(2-Tol)	25.0	13.0
2c	4-Tol–N(NO)CONHMe	17.0	8.6	**5h**	Bn–N(NO)CONH(2-NO$_2$-4-Tol)	>100.0	10.0
2d	4-MeO–Ph–N(NO)CONHBn	17.0	6.0	**5i**	Bn–N(NO)CONH(2-NO$_2$-4-MeO–Ph)	>100.0	>30.3
2e	4-MeO–Ph–N(NO)CONH(isopr)	4.0	1.6	**6a**	Isopr–N(NO)CON(CH$_2$)$_5$	>100.0	50.2
2f	4-MeO–Ph–N(NO)CONHMe	3.0	1.4	**6b**	Me–N(NO)CON(CH$_2$)$_5$	71.0	42.3
2g	2-Tol–N(NO)CONHBn	16.0	6.1	**7a**	4-Tol–N=N–N(Bn)$_2$	>100.0	37.1
3a	4-Tol–N(NO)COMe	2.4	1.4	**7b**	4-Tol–N=N–N(isopr)$_2$	21.0	9.5
3b	4-MeO–Ph–N(NO)COMe	4.0	2.1	**7c**	4-Tol–N=N–N(Et)$_2$	5.0	2.6
4a	Ph–N(NO)Ph	2.5	6.3	**7d**	2-Tol–N=N–N(Et)$_2$	>100.0	18.2
4b	4-Tol–N(NO)Bu^t	70.5	36.7	**7e**	4-Tol–N=N–NH(isopr)	31.0	17.2
				7f	4-Tol–N=N–NHMe	27.0	18.2

a) L-5178 Y Leukemia cells were cultured in a stoppered tube in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C. The growth-inhibitory effect was determined as the ratio of cell numbers, which were counted visually with a microscope, in treated and control groups (%, treated/control) after incubation of 10^5 cells/ml for 48 h with various concentrations of each sample. To express the results, the ID_{50} (50%-inhibitory) value was calculated by probit diagramming analysis. Mitomycin C, employed as a cytotoxic activity standard showed an ID_{50} of 1.0×10^{-7} M (34.1 ng/ml) on the cultured L-5178 Y leukemia cells under our experimental conditions. *b*) Tol = methylphenyl, Bn = benzyl.

in the range of 1.0×10^{-5} to 8.6×10^{-5} M, and those of *N*-nitrosamides (**3**) were also in this range. On the other hand, the aliphatic *N*-nitrosoureas (**5**, **6**) showed values larger than 10.0×10^{-5} M. Among the *N*-nitrosoanilines (**4**), the *N*,*N*-diphenyl derivative (**4a**) was more active than the *N*-*tert*-butyl derivative (**4b**). The cytotoxic action of aromatic *N*-nitroso compounds (**1**—**3**, **4a**) is apparently potent.

3,3-Dialkyl-1-(2- or 4-tolyl)triazenes (**7a**—**d**)[13,14] were formed as by-products in the thermal decomposition of the aromatic trisubstituted *N*-nitrosoureas (**1**). The cytotoxic activity of these triazenes, except for the 3,3-diethyl derivative (**7c**), was inferior to that of the parent compounds (**1**). The reason for the effectiveness ($ID_{50}/2.6 \times 10^{-5}$ M) of the triazene (**7c**), however, is not clear. The ID_{50} of the related monoalkyltriazenes (**7e**, **f**), by-products of the aromatic disubstituted *N*-nitrosoureas (**2**), were 17.7×10^{-5} and 18.2×10^{-5} M, respectively. The activity of the aliphatic nitroso compounds, 3-alkyl-1-aryl-3-nitrosoureas (**5a**—**g**),[10,12] formed by a 1,3-nitroso shift of 3-alkyl-1-aryl-1-nitrosoureas (**2**), was also inferior to that of compounds **2**. Other aliphatic *N*-nitroso compounds (**5h**, **i**, **6**) showed no bioactivity.

The relationship between the NO generation and the cytotoxic activity was investigated and is shown in Fig. 2. The values of NO_2^-, shown as the amount of NO generation per 2 h with each *N*-nitroso compound (**1**—**6**) divided by the amount of NO generation with SNAP (0.55) per 2 h, were related to the reciprocal of the ID_{50} values for the *N*-nitroso compounds (correlation constant = 0.713, $(1/ID_{50}) \times 10^5 = 6.7024 \times 10^{-2} + 0.1768([NO_2^-]$ from compounds **1**—**6**$]/[NO_2^-]$ from SNAP$]$)), except for that of **1a**. Compound **1a** is assumed to be decomposed rapidly before acting effectively on the cells, because it is extremely labile under the experimental conditions. The *N*-nitrosoacetanilides (**3**) showed great activity. Though

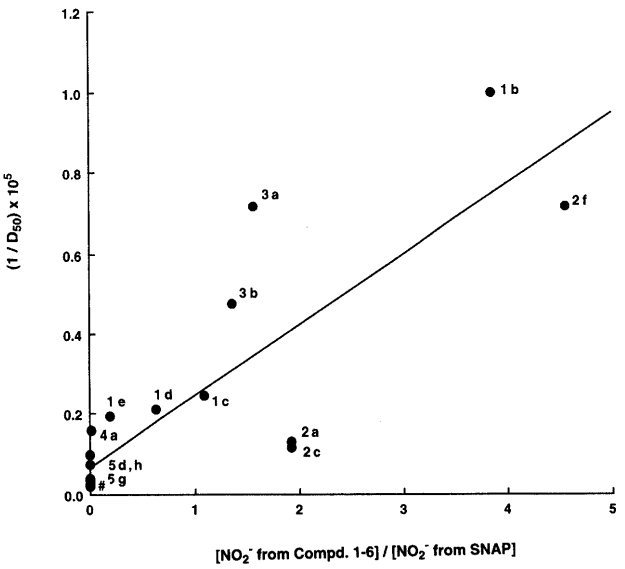

Fig. 2. Relationship between NO Generation and Cytotoxic Activity

The figure shows the NO-generating ability per 2 h and $1/ID_{50}$ from Tables 1 and 2, except for the data for **1a**. The NO-generating abilities of **1e** and **4a** were converted into values per 2 h. The symbol # in the figure shows the data for **4b**, **5a**—**c**, **e**, **f**, **i** and **6a**, **b**.

the ID_{50} value of benzenediazonium acetate, probably produced by the thermal rearrangement of **3**,[15] has not been investigated, the greater part of the action of these compounds is ascribed to the NO radical generated from **3**. Although the possibility of bioactivity *via* the incorporation of the *N*-nitroso compound itself must be considered, the cytotoxicity towards L-5178 Y leukemia cells appears to depend mainly on NO or NO-originated species, because the reciprocal of ID_{50} tends to increase with the NO-generating ability.

The participation of NO was reported to involve inhibition of the action of a ribonucleotide reductase

obtained from L 1210 lymphoma in mice.[16] However, another report found no consistent relationship between the quantity of NO produced by activated macrophages and the tumoricidal activity.[17] In the present work, the quantity of NO generated from the *N*-nitroso compounds was found to be proportional to the ID_{50} value for the cultured cells (Fig. 2). It has recently been reported that, when superoxide (O_2^-) is present in an NO-generating system, peroxynitrite anion ($ONOO^-$) is produced, and this causes DNA damage by deamination or oxidation.[18] In the case of the aromatic *N*-nitroso compounds, we consider that the cytotoxic activity is a result of deamination of DNA bases by the *S*-nitroso derivatives or the iron–nitrosyl complex formed by the reaction of generated NO with sulfur proteins containing iron.

Experimental

Electronic spectra (UV) were recorded on a Hewlett Packard 8452A spectrophotometer.

Materials 3,3-Dialkyl-1-aryl-1-nitrosoureas (**1**), 3-alkyl-1-aryl-1-nitrosourea (**2**),[8,11–14,19] *N*-phenyl-*N*-nitrosoacetamide (**3**),[10,13,20] *N*-nitrosoanilines (**4a, b, e**),[8,10] 3-alkyl-1-aryl-3-nitrosoureas (**5, 6**),[10,12] and 3,3-dialkyl-1-tolyltriazenes (**7a—d**)[13,14] were prepared according to the methods described in our previous papers. *N*-Nitrosamines (**4c, d**) and monoalkyltriazenes (**7e, f**) were purchased from Wako Pure Chemical Co., Ltd., (Osaka). SNAP was purchased from Alexis Co., Ltd., (California).

Determination of NO Generation from *N*-Nitroso Compounds Determination of NO generation was performed by means of the Griess method using the NO measurement apparatus described in the previous paper.[5] For example, 3,3-dibenzyl-1-(4-tolyl)-1-nitrosourea (**1a**) (3 mg, 8.35×10^{-3} mmol) was dissolved in $CHCl_3$ (2 ml), and the solution was kept at 37 °C for 2 h. The NO gas evolved from the reaction mixture was fed into a solution of *N*-(1-naphthyl)ethylenediamine (Griess reagent)[5] (2 ml). In this step, the NO was oxidized to NO_2^- *via* NO_2 under the aerobic condition, and the thus-formed NO_2^- was converted into an azo compound *via* the Griess reaction. This azo compound showed the maximal absorption at 546 nm in the visible spectrum.[21] The quantity of NO_2^- was determined by comparison with a standard curve of known amounts of $NaNO_2$. The results are listed in Table 1.

Cytotoxic Activity Cytotoxic activity was examined by the method described in the literature.[22] L-5178 Y Leukemia cells were cultured in a stoppered tube in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C. The growth-inhibitory effect was determined as the ratio of the cell numbers which were counted visually with a microscope in the treated and control groups (%, treated/control) after incubation of 10^5 cell/ml for 48 h with various concentrations of compounds. The results were expressed as the ID_{50} (50% inhibiting) value calculated by probit diagramming analysis and are shown in Table 2.

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