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 ID₅₀ 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 vessels¹⁾ 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 confirmed⁵⁾ 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 $1\text{--}10 \times 10^{-3}$ 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*-nitrosoareas (**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*-nitrosoareas (**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*-nitrosoareas (**1, 2**) and aromatic *N*-nitrosamides (**3**) were evidently greater than those of *N*-nitrosamines (**4**) and aliphatic *N*-nitrosoareas (**5, 6**) among these *N*-nitroso compounds. In addition, the trisubstituted *N*-nitrosoareas (**1**) were better than the disubstituted ones (**2**), as exemplified by **1a** and **2a**.

Among the trisubstituted *N*-nitrosoareas (**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*-nitrosoareas, for example, between 3-benzyl-1-(4-tolyl)-1-nitrosoarea (**2a**) and 3-methyl-1-(4-tolyl)-1-nitrosoarea (**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*-nitrosoareas (**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*-nitrosoareas (**5, 6**) could not be observed at 37 °C in 7 h, because the aliphatic *N*-nitrosoareas 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 rearrangement⁹⁾ 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 analysis¹⁰⁾ 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*-nitrosoareas (**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	<i>N</i> -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 $1\text{--}10 \times 10^{-3}$ 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		Compd. ^{b)}		ID ₅₀	
		mg/ml	× 10 ⁻⁵ M			mg/ml	× 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 10⁵ cells/ml for 48 h with various concentrations of each sample. To express the results, the ID₅₀ (50%-inhibitory) value was calculated by probit diagramming analysis. Mitomycin C, employed as a cytotoxic activity standard showed an ID₅₀ 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 (ID₅₀/2.6 × 10⁻⁵ M) of the triazene (**7c**), however, is not clear. The ID₅₀ 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 NO₂⁻, 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 ID₅₀ values for the *N*-nitroso compounds (correlation constant = 0.713, (1/ID₅₀) × 10⁵ = 6.7024 × 10⁻² + 0.1768([NO₂⁻ from compounds **1**—**6**]/[NO₂⁻ 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/ID₅₀ 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 ID₅₀ 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 ID₅₀ 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 ID₅₀ value for the cultured cells (Fig. 2). It has recently been reported that, when superoxide (O₂⁻) 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 CHCl₃ (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 NO₂⁻ via NO₂ under the aerobic condition, and the thus-formed NO₂⁻ 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 NO₂⁻ was determined by comparison with a standard curve of known amounts of NaNO₂. The results are listed in Table I.

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 10⁵ cell/ml for 48 h with various concentrations of compounds. The results were expressed as the ID₅₀ (50% inhibiting) value calculated by probit diagramming analysis and are shown in Table 2.

References

- 1) Palmer R. M. J., Ferrige A. G., Moncada S., *Nature* (London), **327**, 524–526 (1987).
- 2) Radomski M. W., Palmer R. M. J., Moncada S., *Br. J. Pharmacol.*, **92**, 639–646 (1987).
- 3) Higuchi M., Higashi N., Taki H., Osawa T., *J. Immunol.*, **144**, 1425–1431 (1990).
- 4) Lawley P. D., Orr D. J., Shah S. A., Farmer P. B., Jarman M., *Biochem. J.*, **135**, 193–201 (1973).
- 5) Tanno M., Sueyoshi S., Miyata N., Nakagawa S., *Chem. Pharm. Bull.*, **44**, 1849–1852 (1996).
- 6) Field L., Dilts R. V., Ravichandran R., Lenhert P. G., Carnahan G. E., *J. Chem. Soc., Chem. Commun.*, **1978**, 249–250.
- 7) a) Gibsor A., Babbidge R., Brave S. R., Hart S. L., Tucker A. J., Wallace P., Moore P. K., *Br. J. Pharmacol.*, **107**, 715–721 (1992); b) Radomski M. W., Rees D. D., Dutra A., Moncada S., *ibid.*, **107**, 745–749 (1992).
- 8) Tanno M., Sueyoshi S., *Chem. Pharm. Bull.*, **35**, 1353–1359 (1987).
- 9) Lepoivre M., Flaman J.-M., Henry Y., *J. Biol. Chem.*, **267**, 22994–23000 (1992).
- 10) D'Agostino J. T., Jaffe H. H., *J. Am. Chem. Soc.*, **92**, 5160–5166 (1970).
- 11) Tanno M., Sueyoshi S., *Chem. Pharm. Bull.*, **35**, 1360–1371 (1987).
- 12) Tanno M., Sueyoshi S., Kamiya S., *Chem. Pharm. Bull.*, **38**, 49–54 (1990).
- 13) Sueyoshi S., Tanno M., *Chem. Pharm. Bull.*, **33**, 488–496 (1985).
- 14) Tanno M., Sueyoshi S., Kamiya S., *Chem. Pharm. Bull.*, **38**, 2644–2649 (1990).
- 15) Ruchardt C., Tan C. C., *Chem. Ber.*, **103**, 1774–1783 (1970).
- 16) Keller R., Bassetti S., Keist R., Mulsch A., Klausner S., *Biochem. Biophys. Res. Commun.*, **184**, 1364–1371 (1992).
- 17) Stuehr D. J., Nathan C. F., *J. Exp. Med.*, **169**, 1543–1555 (1989).
- 18) deRoja-Walker T., Tamir S., Ji H., Wishnok J. S., Tannenbaum S. R., *Chem. Res. Toxicol.*, **8**, 473–477 (1995).
- 19) Tanno M., Sueyoshi S., Miyata N., *Chem. Pharm. Bull.*, **42**, 1760–1767 (1994).
- 20) Hey D. H., Stuart-Webb J., Williams G. H., *J. Chem. Soc.*, **1952**, 4657–4665.
- 21) Saltzmann B. E., Mendenhall A. L., Jr., *Anal. Chem.*, **36**, 1300–1304 (1964).
- 22) Ikehara T., Umeji (Ohtsuka) M., Yanoma S., Yoshimoto K., Nishikawa Y., *Chem. Pharm. Bull.*, **27**, 2016–2020 (1979).