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Mutagenic and Prophage-inducing Activities of 1-Alkyl-3-nitro-1-nitrosoguanidines[1]

Shigeo Iwahara, Kimie Yanagimachi (née Koshinuma), Shozo Kamiya, Masahiro Nakadate and Ikuo Suzuki[2]

National Institute of Hygienic Sciences[2]

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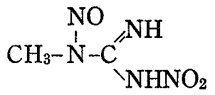
A concurrent test for the mutagenicity and the prophage-inducing activity of 1-alkyl-3-nitro-1-nitrosoguanidines (II) was carried out. In mutagenicity test, mutation from streptomycin-dependent to streptomycin-nondependent in *E. coli* Sd 4 was tested, and in prophage-induction test, induction of lambda-phage in *E. coli* K12 was used.

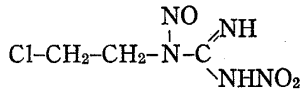
1-(2-Chloroethyl)-3-nitro-1-nitrosoguanidine (CNNG) showed the most potent mutagenicity, though the mutagenicity of 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) was remarkably high in comparison with those of other 1-alkyl-3-nitro-1-nitrosoguanidines (II: R=C_{2-9}). While, in prophage-inducing activity, *n*-propyl (II: R=*n*-C_3H_7) and *n*-butyl (II: R=*n*-C_4H_9) derivatives were most effective, showing larger induction index than MNNG and CNNG.

When the alkyl chain became longer than C_5, both activities were extremely weakened.

It is well known that some of N-nitroso compounds have carcinogenic activity for animals, but on the other hand, they have mutagenic and prophage-inducing activities for bacteria. Among these N-nitroso compounds, 1-methyl-3-nitro-1-nitrosoguanidine (Abbreviation: MNNG) has been claimed to be the most potent mutagen yet discovered for microorganisms.[3,4]



MNNG



CNNG

Recently, MNNG has received much attention from the fact that, when continuously administered by dissolving in drinking water, it induced adenocarcinoma in the glandular stomach of rats with high frequency.[5]

While, Skinner, *et al.*[6] reported that 1-(2-chloroethyl)-3-nitro-1-nitrosoguanidine (Abbreviation: CNNG) showed marked antileukaemic activity against intraperitoneal L 1210 leukaemia. The filament formation of bacteria with these compounds was also reported.[7]

As a matter of fact, many of the compounds showing mutagenic and prophage-inducing actions for bacteria, are carcinogenic and also carcinostatic for animals. For example, a parallel relation exists between the prophage-inducing and carcinostatic activities of mitomycin C and nitrogen mustard derivatives.[8]

1) A part of this work was presented at the 69th Annual Meeting of the Pharmaceutical Society of Japan, Nagoya, April 1969.
2) Location: *Kamiyoga 1-18-1, Setagaya, Tokyo.*
3) J.D. Mandell and J. Greenberg, *Biochem. Biophys. Res. Commun.*, **13**, 575 (1960).
4) E.A. Adelberg, M. Mandel and G.C.C. Chen, *Biochem. Biophys. Res. Commun.*, **18**, 788 (1965).
5) T. Sugimura and S. Fujimura, *Nature*, **216**, 943 (1967); T. Kawachi, K. Kogure, Y. Kamijo and T. Sugimura, *Biochem. Biophys. Acta*, **222**, 409 (1970).
6) W.A. Skinner, H.F. Gram, M.O. Green, J. Greenberg and B.R. Baker, *J. Med. and Pharm. Chem.*, **2**, 299 (1960).
7) W.W. Kilgore and J. Greenberg, *J. Bact.*, **81**, 258 (1961).
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In connection with our investigation to define the structure-activity relationship between carcinogenicity and carcinostatic action in 1-alkyl-3-nitro-1-nitrosoguanidines on mammals now under way in this laboratory, this paper describes the structure-activity relationship in the mutagenicity and the prophage-inducing activity of 1-alkyl-3-nitro-1-nitrosoguanidines on bacteria.

Any concurrent test for both mutagenicity and prophage-inducing activity of a series of compounds on bacteria has not, to our knowledge, been reported.

Material and Method

Synthesis of 1-Alkyl-3-nitro-1-nitrosoguanidines (IIa—k)——All of 1-alkyl-3-nitro-1-nitrosoguanidines were prepared by the method reported by McKay, *et al.*[9a,b] (Chart 1). Some of them are new compounds.

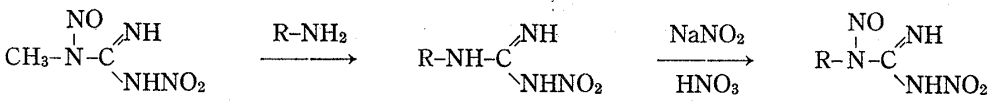

Chart 1

As shown in Chart 1, 1-alkyl-3-nitroguanidines (I) were prepared by the reaction of MNNG with the corresponding primary amines, and the resulting 1-alkyl-3-nitroguanidines were nitrosated with sodium nitrite and nitric acid to yield their N-nitroso derivatives (II). A general process is described with 1-(*n*-heptyl)-3-nitroguanidine (Ig) and with its N-nitroso derivative (IIg).

1-Methyl-3-nitroguanidine (MNG): Colorless needles (from ethanol), mp 159—160° (Reported,[10] 160—161°). 1-Methyl-3-nitro-1-nitrosoguanidine (MNNG): Purchased from the Aldrich Chemical Co. Inc., Milwaukee, Wisconsin, U.S.A.

1-Ethyl-3-nitroguanidine (Ia): Colorless granules (from ethanol), mp 145—146° (Reported,[10] 147°). 1-Ethyl-3-nitro-1-nitrosoguanidine (IIa): Yellowish leaflets (from methanol), mp 116° (decomp.) (Reported,[11] 114°).

1-(*n*-Propyl)-3-nitroguanidine (Ib): Colorless needles (from methanol), mp 97—98° (Reported,[10] 98—99°). 1-(*n*-Propyl)-3-nitro-1-nitrosoguanidine (IIb): Yellowish leaflets (from methanol), mp 118° (decomp.) (Reported,[11] 118°).

1-(*n*-Butyl)-3-nitroguanidine (Ic): Colorless needles (from methanol), mp 84—85° (Reported,[12] 84—85°). 1-(*n*-Butyl)-3-nitro-1-nitrosoguanidine (IIc): Yellowish leaflets (from methanol), mp 121° (decomp.) (Reported,[12] 121°).

1-(iso-Butyl)-3-nitroguanidine (Id): Colorless needles (from methanol), mp 121—122° (Reported,[9] 121—122°). 1-(iso-Butyl)-3-nitro-1-nitrosoguanidine (IId): Yellowish leaflets (from methanol), mp 82—83° (decomp.) (Reported,[5] 82—83°).

1-(*n*-Pentyl)-3-nitroguanidine (Ie): Colorless leaflets (from methanol), mp 97—98° (Reported,[10] 98—99°). 1-(*n*-Pentyl)-3-nitro-1-nitrosoguanidine (IIe): Yellow leaflets (from methanol), mp 124° (decomp.) (Reported,[6] 124°).

1-(*n*-Hexyl)-3-nitroguanidine (If): Colorless needles (from methanol), mp 109—110°. Yield, 35%. *Anal.* Calcd. for $C_7H_{16}O_2N_4$: C, 44.66; H, 8.57; N, 29.77. Found: C, 45.18; H, 8.73; N, 29.80. 1-(*n*-Hexyl)-3-nitro-1-nitrosoguanidine (IIf): Yellowish leaflets (from methanol), mp 113° (decomp.). Yield, 35%. *Anal.* Calcd. for $C_7H_{15}O_3N_5$: C, 38.70; H, 6.96; N, 32.24. Found: C, 39.02; H, 6.99; N, 32.35.

1-(*n*-Heptyl)-3-nitroguanidine (Ig): A solution of 3.0 g (0.0026 mole) of *n*-heptylamine in 3 ml of water was added dropwise to a suspended solution of 3.0 g (0.0026 mole) of MNNG[13] in 3 ml of water with stirring under ice-cooling. The mixture was stirred for 20 min at 0°, the crystals separated were collected and washed with ice-water. The product was recrystallized from methanol to give Ig as colorless needles, mp 111°. Yield, 2.70 g (51%). *Anal.* Calcd. for $C_8H_{18}O_2N_4$: C, 47.50; H, 8.97; N, 27.70. Found: C, 47.15; H, 9.38; N, 27.92.

1-(*n*-Heptyl)-3-nitro-1-nitrosoguanidine (IIg): A solution of 1.0 g (0.014 mole) of sodium nitrite in 2 ml of water was added dropwise to a solution of 1.0 g (0.005 mole) of Ig in 2 ml of conc. nitric acid with

9) *a*) A.F. McKay and G.F. Wright, *J. Am. Chem. Soc.*, **69**, 3028 (1947); *b*) A.F. McKay and J.E. Milks, *ibid.*, **72**, 1610 (1950).
10) T.L. Davis and S.B. Luce, *J. Am. Chem. Soc.*, **49**, 2303 (1929).
11) A.F. McKay, W.L. Otto, G.W. Taylor, M.N. Buchanan and J.F. Crooker, *Canad. J. Res.*, **B28**, 683 (1950).
12) A.F. McKay, *J. Am. Chem. Soc.*, **71**, 1968 (1949).
13) Purchased from Aldrich Chemical Co. Inc., Milwaukee, Winscosin, U.S.A.

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stirring under ice-cooling. The mixture was then stirred for 20 min at 0°. The separated crystals were collected, washed with ice-water, and recrystallized from methanol to give IIg as yellow leaflets, mp 119° (decomp.). Yield, 0.85 g (75%). *Anal.* Calcd. for $C_8H_{17}O_3N_5$: C, 41.55; H, 7.41; N, 30.29. Found: C, 42.01; H, 7.66; N, 29.96.

1-(*n*-Octyl)-3-nitroguanidine (Ih): Colorless leaflets (from aqueous ethanol), mp 108°. Yield, 74%. *Anal.* Calcd. for $C_9H_{20}O_2N_4$: C, 49.98; H, 9.32; N, 25.91. Found: C, 50.74; H, 9.76; N, 26.30.

1-(*n*-Octyl)-3-nitro-1-nitroso-guanidine (IIh): Yellowish leaflets (from ethanol), mp 108—109° (decomp.). Yield, 47%. *Anal.* Calcd. for $C_9H_{19}O_3N_5$: C, 44.07; H, 7.81; N, 28.56. Found: C, 44.73; H, 7.62; N, 28.23.

1-(*n*-Nonyl)-3-nitroguanidine (Ii): Colorless needles (from methanol), mp 105—106°. Yield, 67%. *Anal.* Calcd. for $C_{10}H_{22}O_2N_4$: C, 52.15; H, 9.63; N, 24.33. Found: C, 52.44; H, 9.83; N, 24.25.

1-(*n*-Nonyl)3-nitro-1-nitrosoguanidine (IIi): Yellowish leaflets (from methanol), mp 111—112° (decomp.). Yield, 80%. *Anal.* Calcd. for $C_{10}H_{21}O_3N_5$: C, 46.32; H, 8.16; N, 27.01. Found: C, 46.41; H, 8.25; N, 27.30.

1-Benzyl-3-nitroguanidine (Ij): Colorless leaflets (from aqueous methanol), mp 180°. (Reported,[10] 183°). 1-Benzyl-3-nitro-1-nitrosoguanidine (IIj): Yellow plates (from aqueous methanol), mp 120° (decomp.). Yield, 83%. *Anal.* Calcd. for $C_8H_9O_3N_5$: C, 43.05; H, 4.06; N, 31.38. Found: C, 43.19; H, 3.98; N, 31.10.

Table I. Mutagenic and Prophage-inducing Activities of 1-Alkyl-3-nitro-1-nitrosoguanidines and 1-Alkyl-3-nitroguanidines

Compd. No.	R^1	R^2	Mutation (from SM-dependent to SM-nondependent in *E. coli* Sd 4) Concentraion (μg/ml)	Mutants (per 10^8)	Prophage-induction (lambda-phage in *E. coli* K 12) Concentration (μg/ml)	Index
MNNG	CH_3	NO	20	975.0	10	20
			0	1.0		
CNNG	$ClCH_2CH_2$	NO	20	2117.6	50	220
			0	1.2		
IIa	C_2H_5	NO	200	659.0	20	160
			0	3.4		
IIb	*n*-C_3H_7	NO	500	787.4	50	640
			0	1.3		
IIc	*n*-C_4H_9	NO	500	756.8	100	270
			0	1.5		
IId	iso-C_4H_9	NO	500	82.7	500	3.1
			0	1.3		
IIe	*n*-C_5H_{11}	NO	500	53.5	10	2.1
			0	1.5		
IIf	*n*-C_6H_{13}	NO	200	39.0	—	—
			0	2.1		
IIg	*n*-C_7H_{15}	NO	500	9.7	—	—
			0	1.3		
IIh	*n*-C_8H_{17}	NO	100	3.1	—	—
			0	1.5		
IIi	*n*-C_9H_{19}	NO	20	3.1	—	—
			0	1.4		
IIj	$C_6H_5CH_2$	NO	200	38.7	10	4.7
			0	1.5		
IIk	$C_6H_5CH_2CH_2$	NO	20	79.2	20	4.1
			0	1.8		
MNG	CH_3	H	500	1.0	100	1.4
			0	1.0		
CNG	$ClCH_2CH_2$	H	500	1.4	—	—
			0	1.2		
Ib	*n*-C_3H_7	H	500	1.9	1000	1.0
			0	1.8		

Mutation: The concentration for maximum number of the mutants generated was only noted.
Prophage-induction: The concentration for maximum induction index was only noted.

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1-Phenethyl-3-nitroguanidine (Ik): Colorless needles (from methanol), mp 162° (Reported,[6] 162—164°). 1-Phenethyl-3-nitro-1-nitrosoguanidine (IIk): Yellow leaflets (from methanol), mp 139—140° (decomp.) (Reported,[6] 130—134°).

1-(2-Chloroethyl)-3-nitroguanidine (CNG): Colorless leaflets, mp 115—116°. (Reported,[9b] 116—117°). 1-(2-Chloroethyl)-3-nitro-1-nitrosoguanidine (CNNG): Yellow leaflets (from ethanol), mp 109—110° (decomp.) (Reported[9b] 114.5°). *Anal.* Calcd. for $C_3H_6O_3N_5Cl$: C, 18.35; H, 3.08; N, 35.81. Found: C, 18.41; H, 3.04; N, 36.22.

Mutagenicity Test——In this test, streptomycin-dependent *E. coli* Sd 4 strain[14] was used as the test strain. The method for the detection of induced mutation using the *E. coli* Sd 4 strain have been reported by several authors.[15-17]

The test strain was grown on an agar plate containing 100 μg of streptomycin per ml, at 37° for 48 hr. Cells were washed and suspended in distilled water to a concentration of approximately 10^9 cells per ml.

These test compounds were dissolved in distilled water just before use, and water-insoluble compounds were dissolved in a small amount of ethanol followed by dilution with distilled water. The final concentration of the ethanol did not exceed five per cent.

One mililiter of the cell suspension was mixed with 1 ml of the solution of a test compound, and the mixture was incubated at 37° for 15 min. After the incubation, 0.1 ml of the mixture was spread over the surface of a nutrient agar plate and the plate was incubated at 37°. After the incubation, the number of the streptomycin-nondependent colonies appeared on the plate was counted after 48 hr and again after 72 hr. Viable count of the mixture at the end of the incubation period was also made.

Mutagenic activity of a test compound was indicated as the number of mutant cells per 10^8 survivors. The results were shown in Table I.

Prophage-induction Test——Induction of lambda-phage in *E. coli* K12[18] (streptomycin-resistant) was tested. *E. coli* W3102[18] was used as the indicator strain. Dilution of a test compound similarly made as described in the mutation test.

The broth culture of *E. coli* in logarithmic phase was diluted with fresh broth to a concentration of approximately 10^7 per ml.

A 0.5 ml amount of a cell suspension was mixed with the same volume of the solution of a test compound, and the mixture was incubated at 37° in a water bath. After incubation period for 30 min, the mixture was diluted to 100 times with fresh broth, and 1 ml of this diluted sample solution was incubated for additional 90 min at 37°. At the end of the incubation period, the mature phage were measured by the soft agar layer technique.

The prophage-inducing activity of a test compound was indicated as induction index (test sample-control plaque ratio). The results were tabulated in Table I.

Result and Discussion

From the data tabulated in Table I, structure-activity relationship in the mutagenic and prophage-inducing activities of this type of compounds was obtained as follows.

(1) None of 1-alkyl-3-nitroguanidines (I) lacking an N-nitroso group, showed both mutagenic and prophage-inducing activities.

(2) The chloroethyl derivative (CNNG) showed the most potent mutagenicity, though the mutagenicity of MNNG was remarkably high in comparison with those of other 1-alkyl derivatives (II: R=C_{2-9}).

While, in prophage-inducing activity, *n*-propyl (II: R=*n*-C_3H_7) and *n*-butyl (II: R=*n*-C_4H_9) derivatives were most effective, showing larger induction index than MNNG and CNNG.

When the alkyl chain became longer than C_5, both activities were extremely weakened.

(3) In the mutagenicity of IIc (R=*n*-C_4H_9) and IId (R=iso-C_4H_9), the normal chain compound was far more active than its branched one.

Skinner, *et al.*[6] have reported that, antileukaemic activity of MNNG against Leukaemia L 1210, ascites form, is occasionally active, while that of CNNG is active on all tests. Since carcinogenicity of CNNG is now under investigation, we could not discuss structure-activity

14) Gifted by Dr. V.N. Iyer of Carleton University, Canada.
15) G. Bertani, *Genetics*, **36**, 598 (1950).
16) M. Demerec, G. Bertani and J. Flint, *American Naturalist*, **85**, 119 (1951).
17) V.N. Iyer and W. Szybalski, *J. Applied Microbiol.*, **6**, 23 (1958).
18) Gifted by Dr. M. Yoshikawa of the Institute of Medical Science, The University of Tokyo.

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relationship in mutagenicity, prophage-inducing activity, carcinogenicity and carcinostatic action, of 1-alkyl-3-nitro-1-nitrosoguanidines in this stage. However, as far as this type of compounds is concerned, a remarkably potent mutagen such as CNNG is a candidate for a carcinostatic agent.

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