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Action Mechanism of Anti-AH13 Activity of 1,3-Diaryl-1-nitrosoureas and Related Compounds

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Administration of 1,3-diaryl-1-nitrosoureas (I) prolonged the life span of rats inoculated intraperitoneally with ascites hepatoma AH13 cells. Active species produced from compound I were aryl diazonium ion, aryl isocyanate and nitrosonium ion. These species reacted with adenine, guanine, L-lysine, and the SH group of *N*-acetyl-DL-penicillamine. A possible anti-AH13 action mechanism of 1,3-diaryl-1-nitrosoureas is proposed to be as follows. The nitrosoureas (I) permeate quickly into the cytoplasm or nucleus, and decompose to give an active intermediate, an aryl diazonium ion, which reacts with nucleic acids of tumor cells to form a deoxyribonucleic acid (DNA) adduct, followed by tumor cell death. DNA repair of other damaged cells is inhibited by aryl isocyanate and nitrosonium ion liberated from compound I, which reacts with repair enzyme to form carbamoylated and *S*-nitrosated enzyme.

Keywords——1,3-diaryl-1-nitrosourea; AH13; DNA adduct; DNA damage; aryl diazonium ion; DNA repair; aryl isocyanate; nitrosonium ion

1-Alkyl-1-nitrosoureas [$RN(NO)CONH_2$] are well known as potent carcinogens and some of them have been used in studies of cancer in animal models. On the other hand, some 3-substituted 1-(2-chloroethyl)-1-nitrosoureas [$ClCH_2CH_2N(NO)CONHR$] are highly effective against mouse lymphoid leukemia L1210.[1] Thus, the reactions of these nitrosoureas *in vitro* and *in vivo* have been well studied. However, the biological activity of 1,3-diaryl-1-nitrosoureas [$ArN(NO)CONHAr'$] has not been clarified.

This paper describes the antitumor activities of 1,3-diaryl-1-nitrosoureas (Ia—g) and related compounds against rat ascites hepatoma AH13 and mouse lymphoid leukemia L1210. A possible anti-AH13 action mechanism of these 1,3-diaryl-1-nitrosoureas is proposed on the bases of their chemical reactions with nucleic acid bases such as adenine and guanine, and with α-amino acids such as L-lysine *N*-acetyl-DL-penicillamine.

Table I shows the antitumor activities of 1,3-diaryl-1-nitrosoureas (Ia—g) against AH13 and L1210. Since aryl isocyanates [ArNCO] and aryl diazonium ions [ArN_2^+] seemed to be the active species from our chemical investigation on the decomposition of 1,3-diaryl-1-nitrosoureas,[2] the corresponding aryl isocyanates (IIa—d) and aryl diazonium salts (IIIa—c) were also tested for antitumor and cytological activities. Compounds Ia—f prolonged the life span of AH13-bearing rats, but did not prolong the life span of L1210-bearing mice. Compound Ig was inactive against AH13. Only compound Id was slightly active against L2110 with a maximum $T/C\%$ of 129. After administration of compounds Ia—g, their cytological effect against AH13 cells was observed under a microscope as follows.

When AH13 cells were mixed with these compounds in the rat peritoneal cavity, the nucleus first contracted, then expanded and burst, leading to cell death; thus, the total cell number of tumor cells in the peritoneal fluid decreased. Aryl isocyanates (IIa, c, d) did not prolong the life span of AH13-bearing rats, though compound IIb prolonged the life span of AH13-bearing rats slightly. Moreover, administration of aryl diazonium salts (IIIa—c), did not prolong the life span of AH13-bearing rats as much as that of 1,3-diaryl-1-nitrosoureas,

TABLE I. Antitumor Activities of 1,3-Diaryl-1-nitrosoureas and Related Compounds

	Compd. No.	R (*p*-)	R′ (*p*-)	X	MTD[a] (mg/kg)	AH13 Evaluation[b] Dose (mg/kg)	60 d surv.	*T/C* (%)	L1210 Evaluation[c] Dose (mg/kg)	*T/C* (%)
$RC_6H_4N(NO)CONHC_6H_4R'$ (I)	Ia	H	H		100	5	2/6	>364	3.75	119
						10	2/6	>390	7.5	106
						20	1/6	>322	15	112
									30	toxic
	Ib	Cl	Cl		>250	12.5	2/6	>383	100	115
						25	3/6	>537	200	112
						50	2/6	>370	400	38
	Ic	OCH_3	OCH_3		100	5	3/6	>392	50	113
						10	0/6	132	100	96
						20	0/6	202	200	toxic
	Id	CH_3	CH_3		>250	5	2/6	>294	50	129
						10	0/6	126	100	113
						20	1/6	>247	200	toxic
	Ie	OCH_3	H		250	12.5	0/6	116	50	120
						25	0/6	202	100	123
						50	2/6	>308	200	120
	If	CH_3	Cl		100	5	0/6	85	6.25	111
						10	1/6	>185	12.5	115
						20	1/6	>198	25	toxic
	Ig	OCH_3	Cl		50	2.5	0/6	87	6.25	104
						5	0/6	87	12.5	113
						10	0/6	87	25	116
RC_6H_4NCO (II)	IIa	H			100	5	0/6	99		
						10	0/6	102		
						20	0/6	117		
	IIb	Cl			>250	12.5	1/6	>184		
						25	0/6	72		
						50	1/6	>187		
	IIc	OCH_3			>250	12.5	0/6	107		
						25	0/6	120		
						50	0/5	110		
	IId	NO_2			>250	25	0/4	91		
						50	0/6	97		
$RC_6H_4N_2^+X^-$ (III)	IIIa	H		PF_6	50	2.5	0/6	83	25	95
						5	0/6	105	50	105
						10	0/6	125	100	toxic
	IIIb	Cl		PF_6	100	2.5	0/6	120	100	104
						5	1/4	>303		
						10	1/6	>283		
	IIIc	NO_2		BF_4	>250	12.5	0/6	149		
						25	0/6	152		
						50	0/6	161		

a) Maximum tolerated dose (MTD) on single intraperitoneal administration, 3 d after intraperitoneal inoculation of 10^6 AH13 cells/rat.
b) All compounds were administered intraperitoneally once a day on days 3—7 after intraperitoneal inoculation of 10^6 AH13 cells/rat.
c) All compounds were administered intraperitoneally on days 2 and 6 after intraperitoneal inoculation of 10^5 L1210 cells/mouse.

and had no effect on L1210-bearing mice. After administration of aryl isocyanates (IIa—d), no cytological effect against AH13 cells was observed under a microscope. However, after administration of aryl diazonium salts (IIIa—c), a cytological effect against AH13 cells

TABLE II. Combination Therapy Experiment

Compd. No.	Dose (mg/kg)[b]	60-d survivors	T/C %
IIIa[a]	10	0/6	125
IIa[a]	20	0/6	117
IIIa+IIa	10+20	1/6	>194
IIIc	50	0/6	132
CyIC[c]	10	0/6	135
IIIc+CyIC	50+10	1/6	>186

a) Data for IIIa and IIa are taken from Table I.
b) All compounds were administered intraperitoneally once a day on days 3—7, after intraperitoneal inoculation of 10^6 AH13 cells/rat. When combination therapy was employed, isocyanates (IIa, CyIC) were administered intraperitoneally 30 min after intraperitoneal administration of aryl diazonium salts (IIIa, c).
c) Cyclohexyl isocyanate.

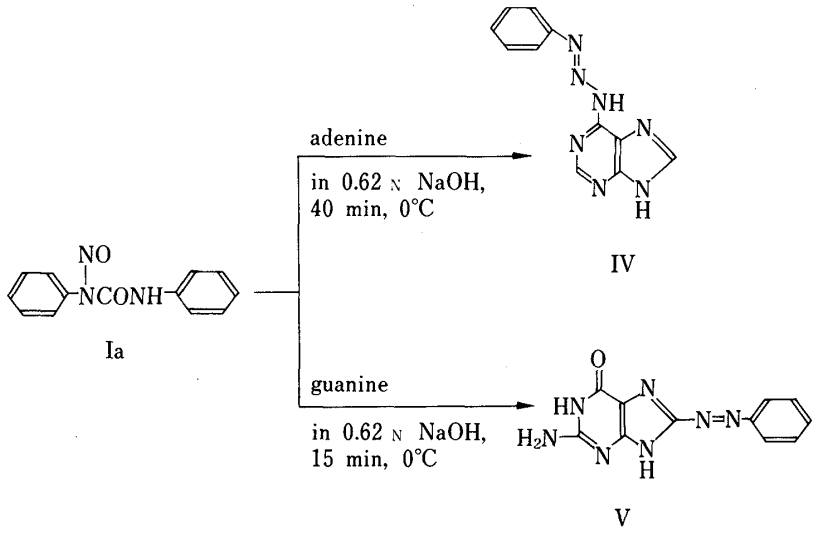

Chart 1

appeared, as observed in the case of 1,3-diaryl-1-nitrosoureas (Ia—g), though more slowly. The difference might be due to a difference in their ability to permeate through the cell membrane. These data of compounds Ia—g and aryldiazonium salts (IIIa—c) indicated that the active intermediates of compounds Ia—g may be the corresponding aryl diazonium ions.

Thus, in order to investigate the role of another possible active species, aryl isocyanates [ArNCO], combination therapy was carried out. Table II shows the antitumor activity in combination therapy with aryl diazonium salts (IIIa, c) and isocyanates (IIa, cyclohexyl isocyanate (CyIC)) in AH13-bearing rats. Phenyl diazonium salts (IIIa, c) did not increase the life span of AH13-bearing rats (Table II), whereas in the combination with isocyanates (IIa, CyIC), they did increase it synergistically. 2-Chloroethyl isocyanate [$ClCH_2CH_2NCO$] acts as an inhibitor of the repair of damaged deoxyribonucleic acid (DNA)[3] and cyclohexyl isocyanate probably inhibits repair.[4] 2-Chloroethyl isocyanate and cyclohexyl isocyanate are active inhibitors of DNA polymerase II (replication enzyme), and the inhibitory action is probably the result of reaction with critical amino acid moieties at or near the active site of the enzyme.[5]

Consequently, we examined the chemical reactivity of compounds Ia—g. When an ethanol solution of compounds Ia—g was left for 3 d at room temperature in the presence of

2-naphthol, the corresponding 1-phenylazo-2-naphthols were produced (Table III). This is evidence for the formation of aryl diazonium ions from the nitrosoureas (Ia—g).

The reaction of compound Ia with adenine and guanine was then examined. Adenine and guanine are slightly soluble in ethanol, so that the reactions were tried under alkaline conditions. In 0.62 N sodium hydroxide solution, compound Ia reacted with adenine to give (*E*)-6-(3-phenyl-2-triazen-1-yl)purine (IV) in 11% yield and with guanine to give 8-phenylazoguanine (V) in 75% yield (Chart 1).

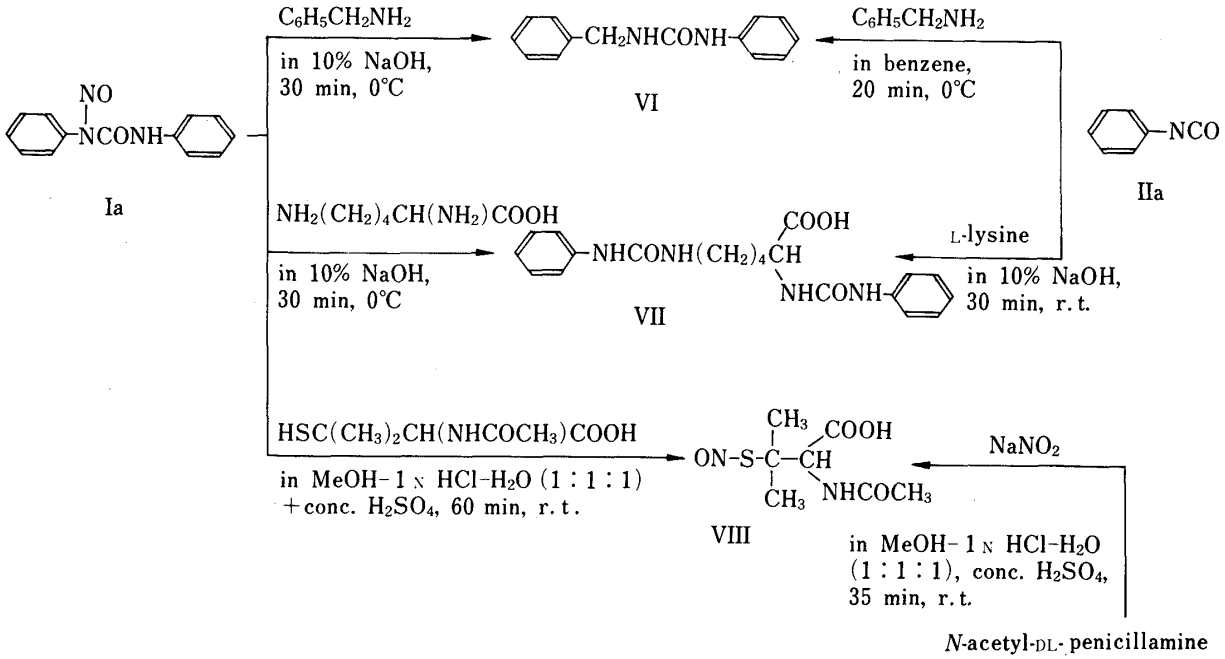

Chart 2

The reaction of compound Ia with benzylamine and L-lysine was then examined to check the carbamoylation activity. As shown in Chart 2, compound Ia reacted with benzylamine and L-lysine in 10% sodium hydroxide solution to give 1-benzyl-3-phenylurea (VI) and with L-lysine to give N^{α},N^{ε}-bis(phenylcarbamoyl)lysine (VII) as phenyl isocyanate (IIa) itself did. Moreover, compound Ia reacted with *N*-acetyl-DL-penicillamine as a model compound of cysteine to give *N*-acetyl-*S*-nitroso-DL-penicillamine (VIII).

A possible action mechanism of 1,3-diaryl-1-nitrosoureas against AH13 is proposed in Chart 3. The nitrosoureas (I) permeate quickly into the cytoplasm or nucleus and decompose to give an active intermediate, aryl diazonium ion (III′), which reacts with nucleic acids of tumor cells to form DNA adducts, followed by tumor cell death; some damaged cells are repaired enzymatically. Another intermediate, aryl isocyanate (II), reacts with significant amino acid moieties in the active site of the repair enzyme to form carbamoylated enzyme. Moreover, nitrosonium ion liberated from 1,3-diaryl-1-nitrosoureas (I) reacts with SH groups of the enzyme to inhibit DNA repair. As a result, damaged cells cannot be repaired and treated rats survive longer than control rats.

Experimental

Materials——1,3-Diaryl-1-nitrosoureas (Ia—g) were prepared by previously reported methods:[6] 1,3-diphenyl-1-nitrosourea (Ia); 1,3-bis(4-chlorophenyl)-1-nitrosourea (Ib); 1,3-bis(4-methoxyphenyl)-1-nitrosourea (Ic); 1,3-di(4-tolyl)-1-nitrosourea (Id); 3-phenyl-1-(4-methoxyphenyl)-1-nitrosourea (Ie); 3-(4-chlorophenyl)-1-(4-tolyl)-1-nitrosourea (If); 3-(4-chlorophenyl)-1-(4-methoxyphenyl)-1-nitrosoureas (Ig). The following materials were obtained

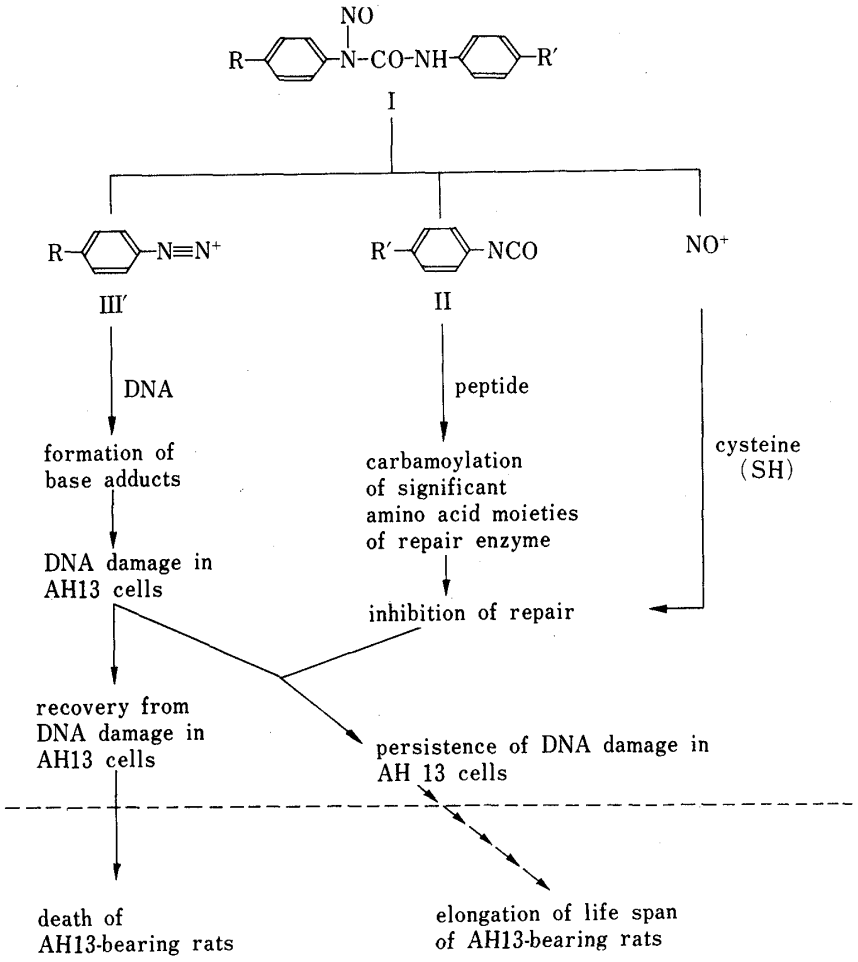

Chart 3. A Possible Action Mechanism of 1,3-Diaryl-1-nitrosoureas

commercially: phenyl isocyanate (IIa) and *p*-chlorophenyl isocyanate (IIb) from Tokyo Chemical Industry Co., Ltd., Tokyo; *p*-methoxyphenyl isocyanate (IIc) and *p*-nitrophenyl isocyanate (IId) from Eastman Kodak Co., New York, U.S.A.; benzenediazonium hexafluorophosphate (IIIa) from Aldrich Chemical Co., Inc., Milwaukee, U.S.A.; *p*-chlorobenzenediazonium hexafluorophosphate (IIIb) from Tokyo Chemical Industry Co., Ltd., Tokyo; *p*-nitrophenyldiazonium fluoroborate (IIIc) from Koch-Light Laboratories Ltd., Colnbrook, Bucks, England; cyclohexyl isocyanate (CyIC) from Tokyo Chemical Industry Co., Ltd., Tokyo; benzylamine and L-lysine from Wako Pure Chemical Industries, Ltd., Tokyo; *N*-acetyl-DL-penicillamine from Aldrich Chemical Co., Inc., Milwaukee, U.S.A.

Test System for Antitumor Activity against AH13 or L1210 Cells *in Vivo*——Details were described in our previous paper.[7] Antitumor activity was evaluated in terms of survival rate at the 60th d after inoculation and $T/C\%$ (T=mean survival time of treated animals, C=mean survival time of control animals). Toxic effect on Donryu rats and cytological effect on AH13 cells were examined in one experiment to estimate roughly the maximum tolerated dose (MTD) and the cytological effect on AH13 cells in peritoneal fluid of rats administered each compound at the MTD. MTD was estimated by observation for 1 week after a single injection, and cytological effect was assessed in terms of abnormal mitotic figures of cells in mitosis by microscopic observation of the treated fluid.

Chemical Experiments——All melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Visible spectra were measured on a Shimadzu UV-240 spectrophotometer. Infrared (IR) spectra were recorded on a JASCO IR A-102 spectrophotometer. ^{1}H-Nuclear magnetic resonance (NMR) spectra were measured with a Varian EM 360A spectrometer, and ^{13}C-NMR spectra were measured with a JEOL FX-200 spectrometer, using tetramethylsilane as an internal standard in dimethylsulfoxide-d_6 (DMSO-d_6).

Reaction of 1,3-Diaryl-1-nitrosoureas (Ia—g) with 2-Naphthol——Ethanol solution (3 ml) of a test compound (Ia—g) (7.5 μmol) was mixed with a similar solution of 2-naphthol (15 μmol in 3 ml). The mixture was left for 3 d at room temperature; the visible absorption spectra of the mixture showed λ_{max} at 460—490 nm due to 1-arylazo-2-naphthol.[8] From the calibration curve, the yield of each azo-dye was calculated.

Reaction of 1,3-Diphenyl-1-nitrosourea (Ia) with Adenine——Adenine (150 mg, 1.11 mmol) was dissolved in

TABLE III. Formation of 1-Arylazo-2-naphthols in the Reaction of 1,3-Diaryl-1-nitrosoureas (Ia—g) with 2-Naphthol in Ethanol

Compd. No.	λ_{max} (nm) in 460—490 nm	Yield of azo dye[a] (%)
Ia	478	25.3
Ib	476	95.3
Ic	458	20.7
Id	477	2.8
Ie	461	19.1
If	475	83.1
Ig	462	15.8

a) The yields of 1-arylazo-2-naphthols were spectrophotometrically determined using calibration curves measured in ethanol.

0.62 N sodium hydroxide solution (10 ml). Powdered Ia (250 mg, 1.04 mmol) was added to the solution at 0 °C. The mixture was kept at 0 °C for 40 min, then filtered to remove the unreacted nitrosourea, and the filtrate was neutralized with 1 N hydrochloric acid. The precipitate which formed was filtered off, and washed repeatedly with water and chloroform. The precipitate was then dissolved in methanol, and the solution was filtered. The filtrate was evaporated to dryness to yield (*E*)-6-(3-phenyl-2-triazen-1-yl)purine, (IV), which was identical with an authentic sample prepared according to the reported method.[9] Yellow powder, mp 251—253 °C. Yield, 21 mg (11%).

Reaction of 1,3-Diphenyl-1-nitrosourea (Ia) with Guanine——Guanine (150 mg, 1 mmol) was dissolved in 0.62 N sodium hydroxide solution (10 ml). Powdered Ia (250 mg, 1.04 mmol) was added to the solution at 0 °C. The mixture was kept at 0 °C for 15 min, then filtered to remove the unreacted nitrosourea. The filtrate was neutralized with 1 N hydrochloric acid. The precipitate which formed was filtered off, and dissolved in 1 N sodium hydroxide solution. The solution was neutralized with 1 N hydrochloric acid. After filtration, the product was washed with water, and air-dried to give 8-(phenylazo)guanine, (V), which was identical with an authentic sample prepared according to the reported method.[10] Red solid, mp >280 °C. Yield, 179 mg (75%).

Reaction of 1,3-Diphenyl-1-nitrosourea (Ia) with Benzylamine——Benzylamine (1 ml) was dissolved in 10% sodium hydroxide solution (20 ml). Powdered Ia (500 mg, 2.07 mmol) was added to the solution at 0 °C. The mixture was kept at 0 °C for 30 min, then filtered. The precipitate was washed with ether repeatedly. This product was identical with authentic 1-benzyl-3-phenylurea (VI) prepared by the reaction of phenyl isocyanate and benzylamine in benzene. Colorless needles, mp 168—170 °C. Yield, 115 mg (11%).

Reaction of 1,3-Diphenyl-1-nitrosourea (Ia) with L-Lysine——L-Lysine (500 mg, 3.42 mmol) was dissolved in 10% sodium hydroxide solution (5 ml). Powdered Ia (1 g, 4.15 mmol) was added, and the solution was kept for 30 min at room temperature. The mixture was filtered to separate the unreacted nitrosourea and produced diphenylurea. The filtrate was extracted with ether, and the aqueous layer was acidified with hydrochloric acid. The solution was evaporated to dryness, and the residue was extracted with ethanol. The ethanol solution was chromatographed on Kieselgel 60 (chloroform : methanol = 9 : 1) to give N^{α},N^{β}-bis(phenylcarbamoyl)lysine[11] (VII), which was identical with an authentic sample prepared by the reaction of phenyl isocyanate and L-lysine in an alkaline solution. IR ν_{max}^{Nujol} cm^{-1}: 3300 (NH), 1650—1640 (CO), 745 (phenyl). ^{13}C-NMR δ: 174.38 (COOH), 155.24 (–NHCONH–), 154.83 (–NHCONH–), 140.60 (phenyl-Cl), 140.28 (phenyl-Cl), 128.58 (phenyl-C2), 128.48 (phenyl-C2), 121.06 (phenyl-C4), 120.77 (phenyl-C4), 117.58 (phenyl-C3), 117.54 (phenyl-C3), 52.30 (>CH–), 31.74 (–CH_2–), 29.50 (–CH_2–), 22.55 (–CH_2–). *Anal.* Calcd for $C_{20}H_{24}N_4O_4 \cdot 5/6H_2O$: C, 60.29; H, 6.42; H, 13.76. Found: C, 60.14, H, 6.49; N, 14.03. Yellow needles, mp 99—101 °C (dec.). Yield, 13 mg (1%).

Reaction of 1,3-Diphenyl-1-nitrosourea (Ia) with *N*-Acetyl-DL-penicillamine——*N*-Acetyl-DL-penicillamine (500 mg, 2.6 mmol) was dissolved in a mixture of methanol, 0.5 N HCl and conc. H_2SO_4 (5 : 10 : 0.5 ml). Powdered Ia (600 mg, 2.49 mmol) was added, and the solution was kept at 25 °C for 1 h with vigorous stirring. The precipitate was filtered off, and recrystallized from methanol–water. The product was identical with authentic *N*-acetyl-*S*-nitroso-DL-penicillamine (VIII), prepared by the reported method.[12] Deep green crystals with red reflections, mp 152—154 °C (dec.). Yield, 32 mg (6%).

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