[Chem. Pharm. Bull. 24(7)1485—1489(1976)]

UDC 547.556.3'555.04 : 542.951.1

Studies on N-Hydroxy-4-aminoazo Dyes. I. Synthesis of N-Hydroxy-N-methyl-4-aminoazo Dyes and the Acyl Derivatives, and Their Degradation in Alkaline Solution[1]

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(Received September 8, 1975)

Alkaline hydrolysis of N-benzoyloxy-N-methyl-4-aminoazobenzene (II) in the presence of ascorbic acid gave N-hydroxy-N-methyl-4-aminoazobenzene (I) which has been estimated as proximate compound of hepatocarcinogenic N-methyl- or N,N-dimethyl-4-aminoazobenzene. Several acyl derivatives of I including acetyl derivative were synthesized. Alkaline treatment of I or II in the presence of dissolved oxygen, yield 4-nitroazobenzene (IV) and 4,4'-bisphenylazo-azoxybenzene (V), probably through intermediate forms, nitron and hydroxylamine compound.

N-Hydroxy-N-methyl-4-aminoazobenzene (I) or its O-esters have been estimated as a proximate or ultimate compounds of N-methyl- or N,N-dimethyl-4-aminoazobenzene in the course of hepatocarcinogenesis in rats by the compounds.[3,4] As a model for such ultimate compound, N-benzoyloxy-N-methyl-4-aminoazobenzene (II) has been already synthesized by Poirier, *et al.*[4] but synthesis of free N-hydroxy derivative (I) was unsuccessful because of the unstable charactor of the compound. However, we found that II was easily hydrolized in basic solution and gave I, but I was unstable to oxidation. Therefore, addition of an antioxidant to the solution stabilizes I and make it possible to isolate free N-hydroxy compound I.

In this paper we are reporting (a) synthesis of I and its acyl derivatives as well as their 4'-methoxycarbonyl derivatives, and (b) degradative process of these compounds in alkaline solution.

As shown by the spectra in Fig. 1, color of the ethanol solution of II or 4'-methoxycarbonyl-N-benzoyloxy-N-methyl-4-aminoazobenzene (III) changed at the room temperature immediately from yellow to red purple or blue by addition of aqueous alkali. This bluish color fade out in a few minutes and the solution became yellowish again. Final products from II were identified as 4-nitroazobenzene (IV) and 4,4'-bisphenylazo-azoxybenzene (V). However, if the oxygen in the solution is previously removed by evacuation, or by replacement with nitrogen gas, or by addition of ascorbic acid, bluish color of the solution was retained infinitely (Fig. 2). The product from the neutralized solution of II or III was identified as N-hydroxy-N-methyl-4-aminoazobenzene (I) or its 4'-methoxycarbonyl derivative (VII). Several acylates of I and VII were prepared by treating I or VII with acyl chloride and pyridine. Physical charactors of these products were summarized in Table I. Solution of I colored bluish in pH higher than 13 and readily oxidized to afford nitro compound IV and azoxy compound V.

Possible route of degradation of N-acyloxy compounds to nitro and azoxy compounds in alkaline solution was shown in Chart 1. In the first step of the reaction, N-acyloxy com-

1) A part of this work is supported by a Grant-in-Aid for Sientific Research from the Ministry of Education, Science and Culture.

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3) *a)* J.A. Miller, *Cancer Res.*, **30**, 559 (1970); *b)* K. Sato, L.A. Poirier, J.A. Miller, and E.C. Miller, *ibid.*, **26**, 1678 (1966); *c)* P.G. Wislocki, J.A. Miller, and E.C. Miller, *ibid.*, **35**, 880 (1975).

4) L.A. Poirier, J.A. Miller, E.C. Miller, and K. Sato, *Cancer Res.*, **27**, 1600 (1967).

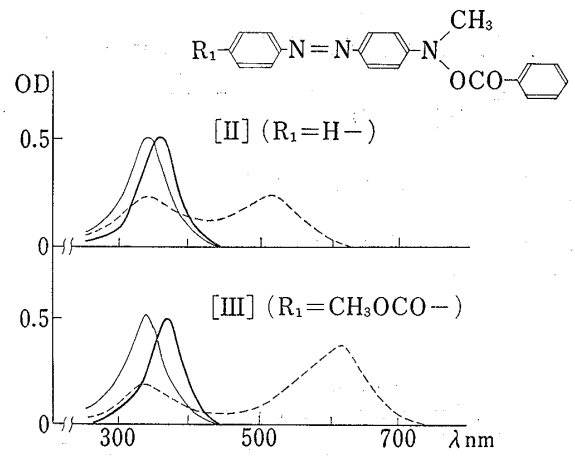


Fig. 1. Absorption Spectra of II and III

——: 1.7×10^{-3}mM in EtOH,
- - - - -: in 80% EtOH–1N KOH (30 sec after addition of alkali)
——: in 80% EtOH–1N KOH (30 min after addition of alkali)

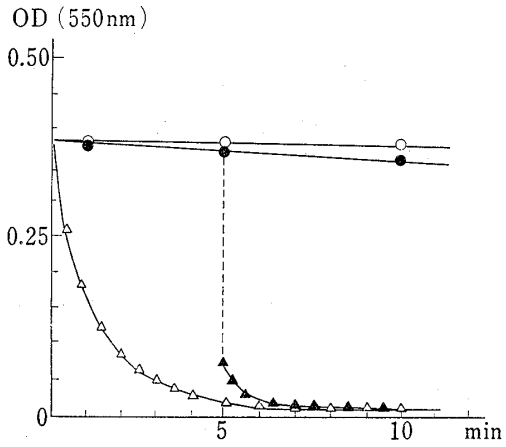
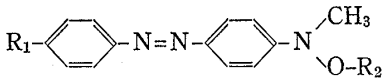


Fig. 2. Stability of I in Alkaline Solution in the Presence and Absence of Oxygen

To 4 volume of solution of II (1.5×10^{-2} mM) in EtOH was added 1 volume of 5N KOH. The solution was kept at room temperature. —△—: in air atomosphere; —○—: same as above but in the addition of 1 mg ascorbic acid/ml; —●—: in N_2 atomosphere; —▲—: to the solution in N_2 atomosphere, 30% H_2O_2 solution (0.01 ml/ml) was added.

TABLE I. Physical Charactors of N-Hydroxy-N-methyl-4-aminoazobenzene Derivatives



Compound No.	R_1	R_2	mp(decomp.) °C	UV λ_{max}^{EtOH} nm (ε)	IR ν_{max}^{KBr} cm^{-1}	NMR($CDCl_3$) δ(ppm) ($N-CH_3$)	Mass $M^+(m/e)$
I	H-	H-	171—174	382	3500—3200(OH)	3.81(s)	227
II	H-	C_6H_5CO-	89— 91	357 (2.1×10^4)	1750(CO)	3.41(s)	331
III	H_3COCO-	C_6H_5CO-	125—127	370 (2.4×10^4)	1750, 1710(CO)	3.45(s)	389
VII	H_3COCO-	H-	164—167	407 (2.0×10^4)	3600—3200(OH) 1720(CO)	3.17[a](s)	285
IX	H-	CH_3CO-	74— 76	353 (1.7×10^4)	1775, 1760(CO)	3.28(s)	269
X	H_3COCO-	CH_3CO-	127—129	369 (2.9×10^4)	1770, 1720(CO)	3.35(s)	327
XI	H_3COCO-	p-CH_3-C_6H_4CO-	113—115	380 (2.2×10^4)	1750, 1720(CO)	3.45(s)	403
XII	H_3COCO-	p-NO_2-C_6H_4CO-	106—108	370 (2.0×10^4)	1755, 1720(CO) 1525(NO_2)	3.48(s)	434

a) in d_6-dimethyl sulfoxide

pound is hydrolized to give N-methylhydroxylamine derivative which represents bluish color in the alkaline solution. Substituent effect on the velocity of the alkaline hydrolysis of N-acyloxyazo dyes is in accordance to Hammet's law as indicated in Fig. 3. 4′-Methoxycarbonyl group decreases the velocity.

N-Hydroxy derivative is readily oxidized by dissolved oxygen in the alkaline solution affording nitro and azoxy derivatives. It has been known that nitron derivative is formed by oxidation of N-alkylphenylhydroxylamine[5,6] or by condensation of phenylhydroxylamine and aldehyde.[5] A nitron derivative thus formed is usually unstable, *e.g.* benzylnitron is easily hydrolized in neutral solution to give benzylhydroxylamine and formaldehyde.[7] There-

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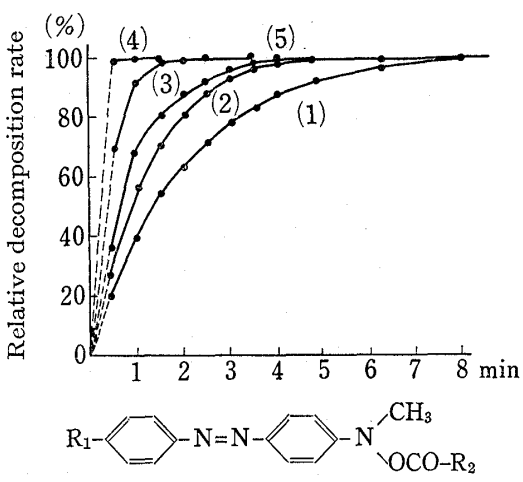
7) L.L. Poulsen, F.F. Kadlubar, and D.M. Ziegler, *Arch. Biochem. Biophys.*, **164**, 774 (1974).

fore, it was suggested that intermediate compound(s) from I to nitro and azoxy derivatives is assumed to be nitron and/or hydroxylamine derivative of the azo dye. Formation of nitron derivative in the present reaction was indicated from the following results. As shown in Table II, alkaline treatment of I gave predominantly 4-nitroazobenzene (IV), whereas N-hydroxy-4-aminoazobenzene (VII) gave a larger amount of azoxy derivative (V) than nitro compound (IV). However, when equimolar HCHO was added to a solution of VIII to form nitron derivative, the ratio of IV to V became equivalent to that of those products from I. Addition of the higher amount of HCHO yielded the higher amount of nitro derivative (IV). Therefore, it can be suggested that nitro and azoxy derivatives from I are mainly derived respectively from nitron and hydroxylamine intermediates. A spot comprising a compound suspected as nitron was detected on the thin-layer chromatogram of the products either from solution of I (1 to 2 min after addition of KOH) or from the reaction mixture of hydroxylamine derivative VIII and HCHO. By two dimensional chromatography of a product from I, a spot comprising nitron derivative in the first development decomposed by the sec-



Compound No.	R_1	R_2
(1) [XI]	CH_3OCO-	p-CH_3-C_6H_4-
(2) [III]	CH_3OCO-	C_6H_5-
(3) [X]	CH_3OCO-	CH_3-
(4) [XII]	CH_3OCO-	p-NO_2-C_6H_4-
(5) [II]	H-	C_6H_5-

Fig. 3. Relationship between Rate of Degradation of N-Acyloxyazo Dye in Alkaline Solution and the Structure of Its O-Substituent

To 0.1 ml ethanol containing azo dye (5×10^{-3} mmole) was added 5ml of 2 mM KOH–50% ethanol containing sodium ascorbate. Absorbance (X-XII and III; 610 nm, II; 550 nm) of the reaction mixture was measured periodically and relative absorbance to a plateau value was recorded.

TABLE II. Formation of Nitro and Azoxy Derivative from N-Hyroxy-4-aminoazo Dyes, and Effect of Formaldehyde

Compound (molar ratio)	% yield of 4-Nitroazobenzene (IV)	% yield of 4,4′-Bisphenyl-azo-azoxybenzene (V)	Ratio of IV/V
N-OH-MAB (I)	64	30	2.1
N-OH-AB (VIII)	30	61	0.5
N-OH-AB+HCHO(1:1)	66	25	2.6
N-OH-AB+HCHO(1:200)	70	13	5.4

The experiment was carried out in the same procedure as described for alkaline treatment of II in the text. HCHO was added to a dye solution 5 min before the addition of alkali.

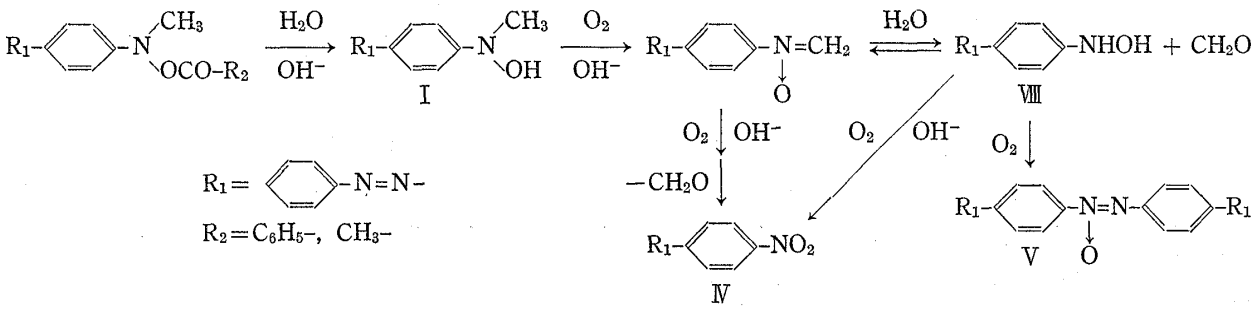


Chart 1. Possible Mechanism for Degradation of N-Acyloxy-N-methyl-4-aminoazobenzene in Alkali

ond development to give a spot whose *Rf* value was identical to that of hydroxylamine derivative VIII. Mass spectrum of I or VII showed a peak (M^+-2) which corresponded to the molecular weight of nitron derivative from these N-hydroxy-N-methyl-4-aminoazo dyes. As indicated in Chart 1, nitron derivative may be oxidized in two ways, one of which is direct oxidation to nitro derivative and another is hydrolysis to hydroxylamine derivative followed by oxidation to azoxy and nitro derivatives. Presence of N-hydroxy-4-aminoazobenzene in the intermediate step of alkaline degradation of I was shown by TLC. Although N-hydroxy-4-aminoazobenzene gave azoxy and nitro derivatives in alkaline solution as like phenylhydroxylamine,[8,9] alkaline degradation of I may take place mainly through nitron intermediate considering from the results shown in Table II. As indicated in Chart 1, HCHO can be generated in the degradation of I. Presence of HCHO in the final reaction mixture of II was confirmed colorimetrically by chromotropic acid reaction and by Nash's reaction.[10]

In the present experiment we obtained the compounds which were supposed to be a proximate or ultimate form of N,N-dimethyl-4-aminoazobenzene or N-methyl-4-aminoazobenzene. Reactivity of these synthetic compounds to biological materials[11] and their mutagenic activity to bacterias[12] have been or will be reported in elsewhere.

Experimental

All melting points were determined on Yanagimoto melting point apparatus and uncorrected. Infrared (IR) (KBr), ultraviolet (UV) and visible, and mass spectra were measured on Hitachi spectrometers, ER1-S2, EPS-3T, and RMU-7M models, respectively. Nuclear magnetic resonance (NMR) spectra were recorded with Hitachi-Perkin-Elmer, R-20A spectrometer.

Thin-layer chromatography (TLC) on Silica gel H (E. Merck, Germany) was performed with solvent systems consist of benzene and acetone of the following mixing ratio, 2: 1, 5: 1, and 10: 1. Column chromatography was carried out on a column of Wako-gel C-200 as the absorbent and benzene–acetone (10: 1) as the eluent. Physical charactors of a compound with asteric were shown in Table I.

N-Hydroxy-N-methyl-4-aminoazobenzene (I)*——N-Benzoyloxy-N-methyl-4-aminoazobenzene (II) was prepared, according to the method by Poirier, *et al.*[4] Crude product of II was purified by reprecipitations using acetone and 50% aqueous EtOH to give pure II (yield, 9.2%), pale yellow tiny needles, mp 89—91° [lit.[4] 89—91°]; IR ν_{max}^{KBr} cm^{-1}: 1750 (C=O); NMR ($CDCl_3$) δ: 7.1—8.25 (14H, m, aromatic protons), 3.41 (3H, s, CH_3); UV λ_{max}^{EtOH} nm: 357; Mass Spectrum *m/e*: 331 (M^+).

To a solution of II (0.5 mmole) in 80 ml of 99% EtOH was added 20 ml of 5N KOH containing 50 mg of ascorbic acid. The solution colored bluish, was kept at room temperature for 5 min, neutrallized with 1N HCl, and extracted with AcOEt (100 ml × 2). The AcOEt extract was washed with saturated $NaHCO_3$ solution (100 ml), and with water, and then dried over anhydrous Na_2SO_4. Residual product of the extract was dissolved in acetone and precipitated with 50% EtOH to give I (40% yield from II), yellow-orange amorph. *Anal.* Calcd. for $C_{13}H_{13}ON_3$ (I): C, 68.70; H, 5.77; N, 18.49. Found: C, 69.12, 68.91; H, 5.27, 5.11; N, 18.66, 18.68.

4′-Methoxycarbonyl-N-benzoyloxy-N-methyl-4-aminoazobenzene (III)*——4′-Methoxycarbonyl-N-methyl-4-aminoazobenzene (XIII) was prepared from N-methylaniline and 4-aminomethylbenzoate in the usual coupling procedure. Crude dye was recrystallized from benzene to give XIII, orange needles, mp 171—173° (62% yield). IR ν_{max}^{KBr} cm^{-1}: 3400 (NH), 1720 (C=O); NMR ($CDCl_3$) δ: 6.5—8.2 (8H, m, aromatic protons), 3.93 (3H, s, $OCOCH_3$), 3.25 (1H, s, NH), 2.9 (3H, s, N–CH_3); UV λ_{max}^{EtOH} nm: 430; Mass Spectrum *m/e*: 285 (M^+). *Anal.* Calcd. for $C_{15}H_{15}O_2N_3$: C, 66.90; H, 5.61; N, 15.61. Found: C, 66.57; H, 5.50; N, 15.52.

To a suspension of XIII (2.9 g, 0.01 mole) in dioxane (50 ml) was added a solution of benzoyl peroxide (2.4 g, 0.01 mole) in 30 ml of dioxane in a rate to keep the temperature of the solution between 15—20°. The reaction mixture was stirred for 12 hr in a refregerator and was poured into 200 ml of cold water. Precipitated dye was collected by filtration and dried. The crude dye was chromatographed on silica gel column. Eluent comprising the first orange band was evaporated to dryness below 20° to give orange needles which was purified by reprecipitation with benzene and petroleum benzine to give orange tiny needles of III (0.6 g, 16% yield). *Anal.* Calcd. for $C_{22}H_{19}O_4N_3$: C, 67.85; H, 4.92; N, 10.79. Found: C, 67.91; H, 4.86; N, 10.86.

8) Y. Ogata, Y. Sawaki, J. Mibae, and T. Morimoto, *J. Am. Chem. Soc.*, **86**, 3854 (1964).
9) N.V. Sidgwich, "The Organic Chemistry of Nitrogen," Oxford Press, 1937, p. 162.
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11) Y. Hashimoto and M. Degawa, *Gann*, **66**, 215 (1975).
12) T. Yahagi, M. Degawa, Y. Seino, T. Matsushima, M. Nagao, T. Sugimura, and Y. Hashimoto, *Cancer Letters*, **1**, 91 (1975).

4′-Methoxycarbonyl-N-hydroxy-N-methyl-4-aminoazobenzene (VII)*——Benzoyloxy compound III was hydrolized in the similar way to the hydrolysis of II. Crude N-hydroxy compound was purified by reprecipitation with acetone and 50% EtOH to afford VII, tiny orange needles (40% yield). *Anal.* Calcd. for $C_{15}H_{15}O_3N_3$: C, 63.15; H, 5.30; N, 14.73. Found: C, 63.03; H, 5.20; N, 14.53.

N-Acyloxy Derivatives of N-Methyl-4-aminoazobenzene (IX)* and 4′-Methoxycarbonyl-N-methyl-4-aminoazobenzenes (X—XII)*——Acyl chloride (0.5 mmole) was added to a solution of N-hydroxy compound I or VII (0.5 mmole) in pyridine (2 ml) and benzene (5 ml) with stirring under cooling in an ice bath. The reaction mixture was kept for 3 to 5 hr and extracted with benzene–hexane (1: 5, 30 ml). The extract was washed several times with cold water and was dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo* and the residual material was washed with a small amount of cold EtOH and purified by precipitation with acetone and 50% aqueous EtOH.

N-Acetoxy-N-methyl-4-aminoazobenzene (IX)*; *Anal.* Calcd. for $C_{15}H_{15}O_2N_3$: C, 66.90; H, 5.61; N, 15.61. Found: C, 66.76; H, 5.76; N, 15.52.

4′-Methoxycarbonyl-N-acetoxy-N-methyl-4-aminoazobenzene (X)*; *Anal.* Calcd. for $C_{17}H_{17}O_4N_3$: C, 62.37; H, 5.24; N, 12.84. Found: C, 62.41; H, 5.21; N, 13.13.

4′-Methoxycarbonyl-N-(4-methylbenzoyloxy)-N-methyl-4-aminoazobenzene (XI)*; *Anal.* Calcd. for $C_{23}H_{21}O_4N_3$: C, 68.47; H, 5.25; N, 10.42. Found: C, 67.60; H, 5.21; N, 11.39.

4′-Methoxycarbonyl-N-(4-nitrobenzoyloxy)-N-methyl-4-aminoazobenzene (XII)*; *Anal.* Calcd. for $C_{22}H_{18}O_6N_4$: C, 60.82; H, 4.18; N, 12.90. Found: C, 61.22; H, 4.32; N, 12.53.

N-Hydroxy-4-aminoazobenzene (VIII) was prepared from 4-nitroazobenzene by the method descrived by Sato, *et al.*[3b] The crude product was recrystallized from EtOH to give VIII, mp 193—195° [lit.[3b] 195—197°]; IR ν_{max}^{KBr} cm^{-1}: 3250 (NH), 3125 (OH).

Oxidative Products of N-Benzoyloxy-N-methyl-4-aminoazobenzene (III) in Alkaline Solution——To a solution of II (0.5 mmole) in 80 ml EtOH was added 5 N KOH (20 ml) and kept it at room temperature. The color of the solution turned to bluish immediately after the addition of alkali and then changed to yellow after a few minutes. After 10 hr, yellow precipitate formed in the solution was collected by filtration and recrystallized from acetone to give red needles of 4,4′-bisphenylazo-azoxybenzene (V), mp 215—217°, 30% yield; IR ν_{max}^{KBr} cm^{-1}: 1450 (NO); Mass Spectrum *m/e*: 406 (M^+). *Anal.* Calcd. for $C_{24}H_{18}ON_6$: C, 70.92; H, 4.46; N, 20.68. Found: C, 70.27; H, 4.24; N, 20.40.

The filtrate was extracted with EtOAc (100 ml). The EtOAc extract was washed with 2 N HCl (50 ml) and with water, and then dried over anhydrous Na_2SO_4. Evaporation of the solvent gave yellow solid residue which was recrystallized from acetone to afford red needles of 4-nitroazobenzene (IV), 64% yield, mp 132—133° [lit.[3b] 133—134°]; IR ν_{max}^{KBr} cm^{-1}: 1520, 1340 (NO_2); Mass Spectrum *m/e*: 227 (M^+). *Anal.* Calcd. for $C_{12}H_9O_2N_3$: C, 63.43; H, 3.99; N, 18.49. Found: C, 63.43; H, 3.93; N, 18.63.

Detection of HCHO——To a solution of N-benzoyloxy-N-methyl-4-aminoazobenzene (II) or its 4′-methoxycarbonyl derivative (III) (1 mg) in 2.4 ml EtOH was added 0.6 ml 5N KOH. The reaction mixture was kept at room temperature for 1 hr, washed well with benzene, and the aqueous layer was used for assay. Aliquot of 0.1 ml of the solution was determined by chromotropic acid reaction and by Nash's method.[10] Bluish color in the former and yellowish color in the later reaction showed the presence of HCHO in the solution.