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Carcinogenic Azo Dyes. XVIII.[1] Syntheses of Azo Dyes related to 3′-Hydroxymethyl-4-(dimethylamino)azobenzene, a New Potent Hepatocarcinogen

Yukio Mori,* Toshiro Niwa, and Kazumi Toyoshi

Gifu College of Pharmacy, 6-1, Mitahora-higashi 5-chome, Gifu 502, Japan

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Twenty azobenzene derivatives structurally related to 3′-hydroxymethyl-4-(dimethylamino)azobenzene (3′-CH_2OH–DAB) were synthesized for the purpose of investigating their mutagenic and carcinogenic effects. They are oxidation products of 3′-Me–DAB, symmetrically substituted azo compounds, their acetyl or chloro derivatives, and their isomers. The 4-(dimethylamino)azobenzenes were prepared by coupling of the corresponding diazonium salts with N,N-dimethylaniline and the azobenzenes by reduction of the corresponding nitrobenzenes with lithium aluminum hydride or zinc in a sodium hydroxide medium. Physical characteristics of these compounds (melting point, elemental analysis, and infrared, ultraviolet, and mass spectral data) are given.

Keywords——carcinogenic aminoazo dye; 3′-methyl-4-(dimethylamino)azobenzene; oxidative metabolite; new potent hepatocarcinogen; symmetrically substituted azo compound; isomer; coupling of diazonium salts with anilines; reduction of nitrobenzenes; mutagenicity; carcinogenicity

The hepatocarcinogenicity of several aminoazo dyes, including 4-(dimethylamino)-azobenzene (DAB) and 4-(methylamino)azobenzene (MAB) is well known, largely because of the extensive studies of Miller and his colleagues.[2] Some carcinogenic aminoazo dyes substituted with a methyl group, such as 3′-Me-DAB and 3′-Me-MAB, are much more potent hepatocarcinogens than either DAB or MAB,[3] while others, 2′-Me-DAB and 4′-Me-DAB are much less potent.[4] The reasons for this variation in carcinogenic activity are not clear. Our earlier experiments indicated that metabolic oxidation occurred at the ring methyl group of 3′-Me-DAB or 3′-Me-MAB to yield the 3′-CH_2OH, 3′-CHO, and 3′-COOH derivatives in rat bile.[5] Recently, we reported that among the metabolites, 3′-CH_2OH-DAB and its N-demethylated derivatives showed potent mutagenicity on *Salmonella typhimurium* TA-98 and TA-100 when tested with rat liver microsomal enzymes[6] and that 3′-CH_2OH-DAB was found to be a new potent hepatocarcinogen in the rat.[7] These results suggest that metabolic oxidation of the 3′-methyl group plays an important role in the carcinogenesis by 3′-Me-DAB. In order to elucidate the contribution of the ring methyl group to the carcinogenic activity of 2′-, 3′-, or 4′-Me-DAB, various azo compounds structurally related to these compounds were prepared, and their mutagenicity, carcinogenicity, and *in vitro* binding with cellular components were investigated. This paper deals with the syntheses of oxidation products of Me-DAB, symmetrically substituted azo compounds, and their acetyl or chloro derivatives.

DAB and azobenzene (AzB) derivatives were synthesized according to the scheme shown in Chart 1. Methyl-, hydroxymethyl, or formyl-DAB was prepared by diazotization of the appropriate toluidine, aminobenzyl alcohol, or aminobenzaldehyde and coupling with N,N-dimethylaniline. On the other hand, the 3,3′- and 4,4′-bis(methyl or hydroxymethyl)azobenzenes were prepared by reduction of the corresponding nitrotoluene or nitrobenzyl alcohol with lithium aluminum hydride or zinc in a sodium hydroxide medium. Reaction with acetic anhydride, acetyl chloride, or thionyl chloride converted these hydroxymethyl derivatives into acetyl esters or chloromethyl derivatives. In the case of chloromethyl-DAB, only the 3′ and 4′ isomers were prepared since 2′-CH_2OH-DAB was converted by treatment with thionyl

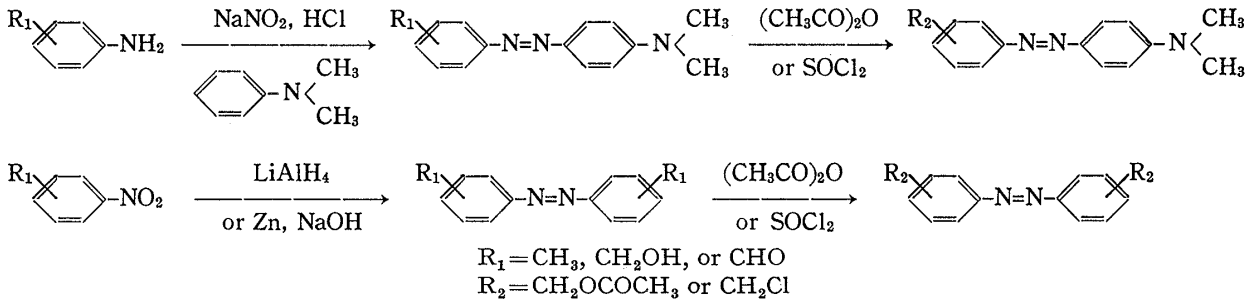

Chart 1. Syntheses of 4-(Dimethylamino)azobenzenes and symmetrically Substituted Azobenzenes

chloride into 2-(*p*-dimethylaminophenyl)indazole.[8] Treatment of 3′-CH_2OH-MAB[5] with 2 molar equivalents of acetic anhydride in benzene gave its N-acetyl compound in 20% yield.

The structural confirmation of these aminoazo or azo compounds was made on the basis of ultraviolet (UV), infrared (IR), and mass (MS) spectral measurements, melting point determination, and elemental analysis. The properties of the synthetic compounds are summarized in Tables I and II. The melting points of Me-DAB,[4] CH_2OH-DAB,[8] CH_2Cl-DAB,[8] and $(Me)_2$-AzB[9] agree with the literature values. Symmetrically substituted azo compounds were prepared for use as model compounds for the corresponding CH_2OH-azo compounds having no 4-amino group. However, 3′-CHO-DAB and 3′-COOH-DAB proved to be less mutagenic[6] and carcinogenic[10] or even inactive when tested by Ames method and when fed at a level equivalent to 0.06% 3′-Me-DAB in the diet for 3 months to Sprague-Dawley rats. Thus, we have not attempted to synthesize 3,3′-bis(CHO or COOH)AzB and its isomers.

Kadlubar *et al.*[11] reported that a two-step enzymic activation mechanism of N-hydroxylation of the amino group followed by esterification of the N-hydroxy function to give a reactive sulfate ester is involved in the carcinogenic action of aminoazo dyes. However, for MAB, 3′-Me-MAB, and 4′-Me-MAB, neither the electrophilic reactivity nor the mutagenicity of their

Table I. Physical Properties of Aminoazobenzene Derivatives

Compound	Yield (%)	mp (°C)	UV λ_{max}^{EtOH} nm (log ε)	IR ν_{max}^{KBr} cm^{-1}	MS M$^+$ *m/z*	Elemental analysis (calculated) C	H	N
2′-Me-DAB	20	66—68	407(4.384)	2840—2900 (CH_3)	239	75.65	7.21	17.43
4′-Me-DAB	27	170—171	406(4.425)	2840—2900 (CH_3)	239	75.13 (75.28	7.16 7.16	17.43 17.56)
2′-CH_2OH-DAB	73	103—104	414(4.441)	3380 (OH)	255	70.34	6.48	16.33
4′-CH_2OH-DAB	70	181—183	410(4.519)	3380 (OH)	255	70.81 (70.57	6.67 6.71	16.29 16.46)
2′-CH_2OAc-DAB	24	124—126	416(4.262)	1730 (–CO–O–) 1240 (CH_3–CO–OR)	297	68.84	6.44	13.90
3′-CH_2OAc-DAB	30	107—108	414(4.461)	1740 (–CO–O–) 1250 (CH_3–CO–OR)	297	68.68	6.34	14.33
4′-CH_2OAc-DAB	64	113—114	412(4.470)	1720 (–CO–O–) 1235 (CH_3–CO–OR)	297	68.80 (68.67	6.55 6.44	14.00 14.13)
3′-CH_2Cl-DAB	15	100—102	412(4.443)	2850—2900 (CH_2)	273:275 =3:1	65.59 (65.81	5.79 5.89	15.52 15.35)
4′-CH_2Cl-DAB	47	142—144	413(4.446)	2850—2900 (CH_2)	273:275 =3:1			
3′-CHO-DAB	51	100—101	410(4.442)	1710 (CO)	253	71.38	6.02	16.52
4′-CHO-DAB	38	178—179	450(4.465)	1700 (CO)	253	71.01 (71.21	5.88 5.98	16.49 16.58)
3′-CH_2OH-MAB-NAc	20	112—113	403(4.374)	1620 (–CO–NHR) 3280 (OH)	283	67.87 (67.82	6.02 6.05	14.69 14.83)

Abbreviations: See Table II.

TABLE II. Physical Properties of symmetrically Substituted Azobenzenes

Compound	Yield (%)	mp (°C)	UV λ_{max}^{EtOH} nm (log ε)	IR ν_{max}^{KBr} cm^{-1}	MS M$^+$ m/z	Elemental analysis (calculated) C	H	N
3,3′-Me_2–AzB	50	50—51	321 (4.188)	—	210	79.94	6.80	13.00
4,4′-Me_2–AzB	54	142—144	330 (4.276)	—	210	79.79 (79.97	6.76 6.71	13.16 13.32)
3,3′-bis(CH_2OH)AzB	23	119—120	321 (4.200)	3160 (OH)	242	69.35	5.87	11.61
4,4′-bis(CH_2OH)AzB	13	233—235	327 (4.286)	3160 (OH)	242	69.23 (69.41	5.85 5.83	11.42 11.56)
3,3′-bis(CH_2OAc)AzB	45	97—98	318 (4.248)	1725 (–CO–O–) 1240 (CH_3–CO–OR)	326	66.10	5.55	8.49
4,4′-bis(CH_2OAc)AzB	10	138—139	324 (4.357)	1750 (–CO–O–) 1257 (CH_3–CO–OR)	326	66.49 (66.25	5.59 5.56	8.69 8.58)
3,3′-bis(CH_2Cl)AzB	65	108—109	319 (4.249)	—	278:280:282 =9:6:1	60.22	4.31	10.07
4,4′-bis(CH_2Cl)AzB	13	181—183	326 (4.386)	—	278:280:282 =9:6:1	60.41 (60.23	4.39 4.33	10.24 10.03)

The following abbreviations are used in Tables I and II: DAB=4-(dimethylamino)azobenzene; MAB=4-(methylamino)azobenzene; AzB=azobenzene.

N-acyloxy derivatives (which are direct mutagens[12]) paralleled the hepatocarcinogenic activity of the parent compounds.[13] Consequently, the activation mechanism at the 4-amino group does not fully explain the different carcinogenic activities of the ring-methylated aminoazo dyes. On the other hand, Dipple[8] reported that 3′-CH_2Cl-DAB, as a model compound for a reactive ester of 3′-CH_2OH-DAB, reacted directly with DNA *in vitro*. 3′-CH_2OH-DAB showed a carcinogenic specificity (in regard to organs and species) similar to that of 3′-Me-DAB, and the isomers (2′- or 4′-substituted DAB) also resembled each other very closely as regards carcinogenic activity.[7,10,14] A similar specificity was also observed in the mutagenic effects on *Salmonella typhimurium* strain TA-98 and TA-100 with liver microsomal enzymes. All the compounds synthesized in the present work were more or less effective, and a parallelism between the carcinogenic and mutagenic activities was observed. 4,4′-Bis(CH_2OAc)AzB was mutagenic without metabolic activation, and was, therefore, a direct mutagen. Details of the carcinogenic and mutagenic experiments will be reported elsewhere.

Experimental

IR spectra were measured with a Jasco model IRA-1 or a Hitachi model 215 spectrometer, UV spectra with a Hitachi model 323, 556, or 181 spectrometer, and MS spectra with a JEOL JMS-D300 mass spectrometer. Melting points were determined with a microscope hot-stage apparatus and are uncorrected.

Chemicals——Toluidines, 3-nitrotoluene, 4-nitrobenzyl alcohol, N,N-dimethylaniline, N-methylaniline, thionyl chloride, and zinc powder were obtained from Wako Pure Chemicals, Ltd., Tokyo. Aminobenzaldehydes (polymer) and 4-nitrotoluene were purchased from Tokyo Kasei Co., Tokyo, and aminobenzyl alcohols and 3-nitrobenzyl alcohol from Aldrich Chemical Co. Inc., Milwaukee, WI. $LiAlH_4$ was obtained from Metallgesellschaft AG. Frankfurt a.M. All other reagents used were of reagent grade.

4-(Dimethylamino)azobenzenes——To the appropriately substituted aniline dissolved in 4 N HCl and cooled to 0—5° with an ice bath was slowly added a solution of $NaNO_2$ (1 mol equiv.) while the temperature was maintained between 0 and 5°. After the addition had been completed, stirring was continued for 15 min, then 1 molar equivalent of N,N-dimethylaniline was added to the diazonium salt solution in one portion. The solution was stirred for 30 min and then neutralized by addition of $CH_3COONa \cdot 3H_2O$ in portions. The resultant precipitates of aminoazo dye were extracted with $CH_3COOC_2H_5$. The extract was washed with water, dried over Na_2SO_4, concentrated under reduced pressure, and chromatographed.

Symmetrically Substituted Azobenzenes——1) Reduction with $LiAlH_4$. Three molar equivalents of $LiAlH_4$ and anhydrous tetrahydrofuran (THF) were placed in a flask fitted with reflux condenser. The mixture was cooled with an ice bath, and the appropriately substituted nitrobenzene dissolved in anhydrous THF was added dropwise with stirring. After the addition had been completed, the mixture was gently

refluxed for 3—22 hr, then extracted with $CH_3COOC_2H_5$. The extract was dried over Na_2SO_4, then concentrated, and the residue was chromatographed.

2) Reduction with Zinc–NaOH. Eight molar equivalents of zinc powder was added in small portions to a mixture of the nitrobenzene dissolved in 99.5% ethanol and 12 N NaOH in a flask fitted with reflux condenser. After the addition had been completed, the mixture was gently refluxed for 15—20 hr, then the insoluble salt was filtered off, and the product was extracted with $CH_3COOC_2H_5$ from the filtrate.

Reaction of the CH_2OH-aminoazo or -azo Compound with Ac_2O, AcCl, or $SOCl_2$——Each isomer was separately treated with an equimolar amount or 2-fold molar excess (in the case of bis(CH_2OH)AzB) of Ac_2O or AcCl and 14—28 molar equivalents of $SOCl_2$ in benzene, pyridine, or $CHCl_3$. After 1—30 hr at room temperature the solutions were evaporated to dryness and the residues were extracted with $CH_3COOC_2H_5$ or benzene. In the case of the chlorination of an aminoazo compound, the residues were slurried in acetone and treated dropwise with 10% (v/v) pyridine in acetone until all the solid had dissolved and the original red color had changed to orange. The extracts were dried over Na_2SO_4 and evaporated to dryness.

Chromatographic Separation and Recrystallization——The compounds were purified by column chromatography on silica gel (Wakogel C-200) or/and by thin-layer chromatography on silica gel (Wakogel B-5F) using benzene (Me–DAB, CH_2OAc–DAB, 3′-CH_2Cl–DAB, Me_2–AzB, bis(CH_2OAc)AzB, and 3,3′-bis(CH_2Cl)AzB), $CHCl_3$–CCl_4=1:4 (CHO–DAB), $CHCl_3$–MeOH=9:1 (bis(CH_2OH)AzB) or benzene–petroleum benzin=1:1 (4,4′-bis(CH_2Cl)AzB) as eluting solvents. Me–DAB, 2′- or 3′-CH_2OAc–DAB, 3′-CH_2OH–MAB–NAc, Me_2–AzB, and bis(CH_2OH)AzB were recrystallized from aqueous ethanol, 4′-CH_2OAc–DAB, 3,3′-bis(CH_2OAc)AzB, and 4,4′-bis(CH_2Cl)AzB from ethanol, 4,4′-bis(CH_2OAc)AzB and 3,3′-bis(CH_2Cl)AzB from methanol, CH_2OH–DAB and CHO–DAB from benzene–petroleum benzin, 3′-CH_2Cl–DAB from petroleum benzin, and 4′-CH_2Cl–DAB from ligroin.

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