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Synthesis and characterization of selected 4,4′-diaminoalkoxyazobenzenes

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ABSTRACT

The role of the $-N(CH_2CH_2OH)_2$ group in producing a mutagenic response from 4-((3-(2-hydroxyethoxy) 4-amino)phenylazo)-*N*,*N*-bis(2-hydroxyethyl)aniline has been investigated. To accomplish this goal, a group of substituted 4,4′-diaminoazobenzene dyes was synthesized, and their structures were confirmed using 1H NMR, TOF-LC-ESI mass spectrometry, and combustion analysis. Mutagenicity was determined using the standard Ames test in *Salmonella* strains TA98, TA100, and TA1538 with and without S9 enzyme activation. The results of this study provide evidence that the mutagenicity of the parent dye arises from the metabolic cleavage of *N*-hydroxyethyl groups to give the corresponding $-NHCH_2CH_2OH$ and $-NH_2$ substituted monoazo dyes as direct-acting mutagens. All 5 of the dyes studied were mutagenic at various levels with and without S9 enzyme activation in TA1538. In addition, the results show that removing one *N*-hydroxyethyl group and capping both −OH groups in the parent dye did not affect mutagenicity, whereas removing both *N*-hydroxyethyl groups produced a strong direct-acting mutagen in all three bacterial strains. Increasing the length of the *N*-alkyl chain from two to three carbon atoms eliminated mutagenicity in TA98 without S9 activation.

1. Introduction

The potential of organic dyes and their intermediates to adversely impact human health and the environment has moved toxicological considerations to the forefront of their molecular design. In this regard, the process of designing new azo dyes takes into consideration the potential to form mutagenic or carcinogenic metabolites. Azo dyes often exhibit mutagenicity via an activation step such as reductive-cleavage of the azo linkage [1]. This process generates the corresponding aromatic amines and can lead to oxidation of free NH_2 groups (e.g. *N*-hydroxylation) in route to DNA-reactive electrophilic species. Not surprisingly, the precise mechanism associated with the genotoxicity of azo dyes is strongly affected by changes in their molecular structure [1].

It is well known that the mutagenicity of aromatic amines can be influenced by structural modification. For instance, it has been reported that the mutagenicity of *meta*- and *para*-phenylenediamines can be lowered or removed by placing bulky alkyl or alkoxy groups *ortho* to one of the amino groups [2–5] (cf. **1**–**2**, Fig. 1). In both cases, mutagenicity was removed when R-groups such as $CHMe_2$, $CH_2CH_2CH_2CH_3$ (n-Bu), $OCH_2CH_2CH_2CH_3$ (OBu), $O(CH_2)_2OH$ were present.

The effects of alkoxy substituents on the mutagenicity of 15 aminoazobenzenes (Fig. 2) have also been reported [4,5], as part of an investigation aimed at designing nonmutagenic analogs of C.I. Direct 17 by modifying the structure of the hydrophobic monoazo component of this dye. In that study, when $R_2 = H$, mutagenicity decreased as the size of the substituent *ortho* to the primary amino group increased and when $R_2 = NEt_2$, mutagenicity was ultimately removed when $R_1 = (CH_2)_2OH$. However, when $R_2 = N(CH_2CH_2OH)_2$, mutagenicity dropped initially with increasing size of the R_1 group, but the dye having $R_1 = (CH_2)_2OH$ was more mutagenic than the other dyes in this 5-member group. This was an unexpected outcome because it had been shown that the incorporation of an $-O(CH_2)_2OH$ group gave the lowest level of mutagenicity in the other two series of dyes. Further, it was shown that the reductive cleavage of dye having $R_1 = (CH_2)_2OH$ and $R_2 = N(CH_2CH_2OH)_2$ (dye **3**) afforded the pair of nonmutagenic amines shown in Fig. 3. These results suggested that reductive-cleavage was not responsible for the mutagenicity of dye **3** and that this dye exhibited mutagenicity with its azo bond intact.

Building on the previously described study, and with the goal of furthering understanding of the structural requirements for mutagenic response and lack thereof in the design of new aminoazobenzene dyes, the objective of the present study was to determine

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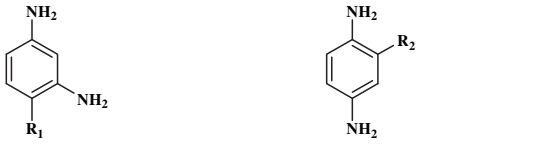


Fig. 1. Phenylenediamines containing alkyl or alkoxy groups *ortho* to an amino group.

the role of the 4′-$N(CH_2CH_2OH)_2$ group in producing a mutagenic response when an $-OCH_2CH_2OH$ is incorporated into the 3-position. The synthesis of dyes **4**–**7** (Fig. 4), arising from modifications of the $-N(CH_2CH_2OH)_2$ group, was conducted, and the resultant novel dyes were characterized using ^1H NMR, high-resolution mass spectrometry, and combustion analyses. These new compounds permitted an assessment of the importance of the number of *N*-hydroxyethyl groups (dyes **4** and **5**), the length of *N*-hydroxyalkyl chains (dye **6**), and the presence of free –OH groups (dye **7**) in the mutagenicity of parent dye **3**. Mutagenicity of the target dyes was assessed using three strains of *Salmonella typhimurium*.

2. Experimental

2.1. General

All of the chemicals used as starting materials in this study were purchased from Aldrich Chemical Co., Milwaukee, WI. All of the solvents used were obtained from Fisher Scientific, Pittsburgh, PA, except for DMF, which was purchased from Aldrich Chemical Co. The plates used for thin layer chromatography were Whatman PE silica gel plates with UV_{254} indicator. ^1H-NMR spectra were recorded on a Brucker 500 MHz spectrometer and mass spectra were recorded on an Agilent Technologies 6210 LC–TOF mass spectrometer. Visible absorption spectra were recorded in MeOH, using a Varian Cary 3 UV–Visible spectrophotometer. The λ_{max} values are those selected by the software connected to spectrophotometer. The standard *Salmonella* tests employing TA98, TA100, and TA1538 with and without S9 were conducted in this study [6]. Mutagenicity testing was conducted at the US EPA, Research Triangle Park, North Carolina. Elemental analyses were performed by the Analytical Service Laboratory in the Soil Science Department of North Carolina State University.

2.2. Synthesis of dyes and intermediates

2.2.1. 4-((3-(2-Hydroxyethoxy)-4-amino)phenylazo)-N,N-bis (2-hydroxyethyl)-aniline (*3*)

Compound **10** (1 g, 4.8 mmol) was stirred with H_2O (5 mL), as a small amount of crushed ice was added and an ice bath was used to cool the mixture to 0 °C. HCl (2 N, 7 mL, 0 °C) was added and the phenylazoamine was diazotized by adding $NaNO_2$ (1 N, 5.5 mL, 4.8 mmol) slowly at 0 °C. After stirring at 0 °C for 30 min, the diazonium salt solution was checked with KI/starch paper for the presence of HNO_2 and solid sulfamic acid was added to destroy excess HNO_2. The solution was clarified by adding activated carbon with stirring and the adsorbent was removed by filtration, with simultaneous cooling of the filtrate in an ice bath.

Compound **11** (0.9 g, 5 mmol) was stirred with 10 mL H_2O as HCl (20%, 3 mL) was added slowly at 0 °C. To this solution, NaOAc (1.8 g, 0.022 mol) was added with stirring. The cold solution of diazo compound prepared above was added to this solution, keeping the temperature at 0 °C. After stirring for 24 h at 0 °C, the yellow solid was collected by filtration. The acetyl group was removed in a solution of NaOH (1.4 g), 95% EtOH (14. mL), H_2O (7 mL) by stirring for 2 h at 100 °C. The reaction mixture was cooled to RT and adjusted to pH 7 using 20% HCl. The solvent was removed at reduced pressure and the residue was extracted with DMF to remove salts. The brown oil obtained after DMF removal was crystallized from acetone/H_2O to give 1 g (58%) dye **3** having m.p. 128 °C (lit. [8] m.p. 132–134 °C). ^1H NMR (DMSO-d_6): δ 7.64–7.67 (d, 2H), δ 7.27–7.37 (m, 2H), δ 6.70–6.81 (m, 3H), δ 4.94–4.99 (t, 1H), δ 4.81–4.85 ppm (t, 2H), δ 3.99–4.03 (t, 2H), δ 3.74–3.80 (q, 2H), δ 3.51–3.71 (m, 8H), and TLC: $R_f = 0.53$ (2-Propanol:Toluene = 1:3).

2.2.2. 4-((3-(2-Hydroxyethoxy)-4-amino)phenylazo)-N-(2-hydroxyethyl)aniline (*4*)

Compound **10** (1 g, 4.8 mmol) was diazotized and the resultant solution clarified using activated carbon in the manner described above for the synthesis of dye **3**. Compound **12** (0.7 g, 5 mmol) was suspended in H_2O (10 mL) with stirring as HCl (20%, 3 mL) was added slowly at 0 °C. NaOAc (1.8 g, 0.022 mol) was added to this solution with stirring and the cold solution of diazo compound prepared in the previous step was added to this solution, keeping the temperature at 0 °C. After stirring for 24 h at 0 °C, the yellow solid was collected by filtration. Deacetylation, workup and dye isolation were the same as outlined above for dye **3**, to give 0.9 g (57%) dye **4** having m.p. 106–107 °C. ^1H NMR (DMSO-d_6): δ 7.57–7.60 (d, 2H), δ 7.26 (d, 1H), δ 7.23–7.24 (dd, 1H), δ 6.65–6.70 (m, 3H), δ 6.29–6.32 (t, 1H), δ 5.56 (s, 2H), δ 4.94–4.97 (t, 1H), δ 4.76 (s, 1H), δ 3.98–4.00 (t, 2H), δ 3.75–3.77 (q, 2H), δ 3.55–3.60 (q, 2H), δ 3.16–3.20 (q, 2H). TLC: $R_f = 0.33$ (EtOAc). Calculated for $C_{16}H_{20}N_4O_3$: C, 60.75; H, 6.37; N, 17.71. Found: C, 60.57; H, 6.83; N, 17.19.

2.2.3. 4-((3-(2-Hydroxyethoxy)-4-amino)phenylazo) aniline (*5*)

Compound **16** (0.7 g, 2.3 mmol) was dissolved in a mixture of H_2O (5 mL) and DMF (1 mL). NaOH (1 N) was added to adjust the mixture to pH 10 and the mixture was heated to 45 °C. Na_2S (0.36 g) was added and the mixture was stirred for 30 min, adjusted to pH 4 with conc. HCl and filtered. The filtrate was neutralized using NaOH (1 N) and EtOAc (30 mL) was added. The organic layer was separated and stirred as HCl gas was added. The light purple solid was collected to give 0.7 g dye **5** (87%) as the hydrochloride. The hydrochloride (0.1 g) was stirred with EtOH (50 mL) and NH_4OH (3 mL) was added to the mixture until dissolution occurred. The resultant solution was clarified using activated carbon and the

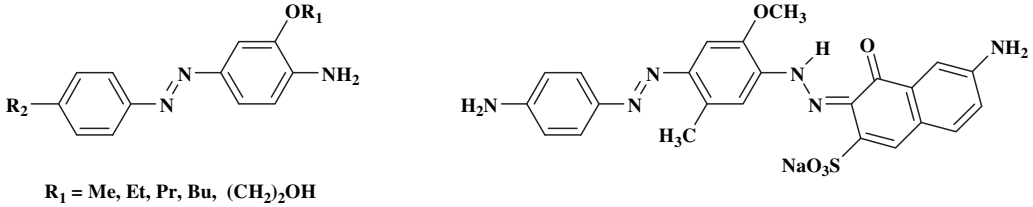


Fig. 2. Substituted aminoazobenzenes evaluated to account for C.I. Direct Black 17 mutagenicity [4].

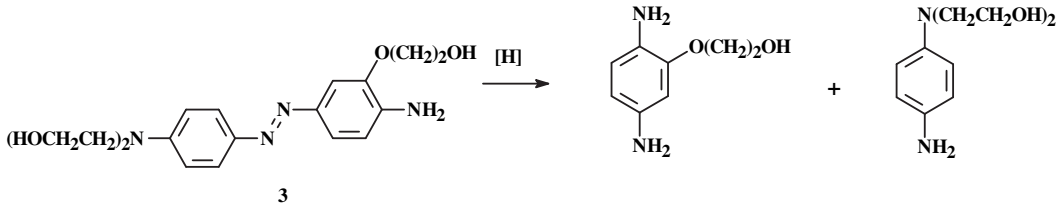


Fig. 3. Reductive-cleavage of dye **3** leading to nonmutagenic aromatic amines.

solvent was removed at reduced pressure. Dye 5 was crystallized from acetone/H_2O to give 0.07 g (87%) having m.p. 82–83 °C. ^{1}H NMR (DMSO-d_6): δ 7.50–7.54 (d, 2H), δ 7.20–7.27 (m, 3H), δ 6.68–6.72 (d, 1H), δ 6.60–6.64 (d, 2H), δ 5.72 (s, 2H), δ 5.52 (s, 2H), δ 4.78–4.81 (t, 1H), δ 3.96–3.99 (t, 2H), δ 3.73–3.76 (t, 2H). TLC: $R_f = 0.52$ (EtOAc). Calculated for $C_{14}H_{16}N_4O_2$: C, 61.75; H, 5.92; N, 20.58. Found: C, 61.89; H, 6.45; N, 18.45.

2.2.4. 4-((3-(2-Hydroxyethoxy)-4-amino)phenylazo)-N,N-bis(3-hydroxypropyl) aniline (**6**)

Compound **10** (1 g, 4.8 mmol) was diazotized and the resultant solution clarified using activated carbon in the manner described above for the synthesis of dye **3**. Compound **13** (1.4 g, 5 mmol) was suspended with stirring in H_2O (10 mL) as HCl (20%, 3 mL) was added slowly at 0 °C. NaOAc (1.8 g, 0.022 mol) was added to the solution with stirring. The cold solution of diazo compound prepared in step 1 was added to this solution, keeping the temperature at 0 °C. After stirring for 24 h at 0 °C, the yellow solid was collected by filtration. Deacetylation, workup and dye isolation were the same as outlined above for dye **3**, to give 1.1 g (60%) dye **6** having m.p. 99–100 °C. ^{1}H NMR (DMSO-d_6): δ 7.62–7.64 (d, 2H), δ 7.24–7.27 (m, 2H), δ 6.76–6.78 (d, 2H), δ 6.68–6.70 (d, 1H), δ 5.57 (s, 2H), δ 4.94–4.97 (t, 1H), δ 4.58–4.60 (t, 2H), δ 3.98–4.01 (t, 2H), δ 3.73–3.77 (q, 2H), δ 3.41–3.50 (m, 8H), δ 1.70–1.73 (m, 4H). TLC: $R_f = 0.36$ (2-Propanol:Toluene = 1:3). Calculated for $C_{20}H_{28}N_4O_4$: C, 61.84; H, 7.27; N, 14.42. Found: C, 60.94; H, 7.86; N, 14.02.

2.2.5 4-(3-(2-Hydroxyethoxy)-4-amino)phenylazo-N,N-bis (2-acetoxyethyl)aniline (**7**)

Iron oxide hydroxide (Fe_xO_y) was prepared first [11]. $FeCl_3$ (2 g) was dissolved in H_2O (250 mL) and NaOH (2 M, 18.8 mL) was added drop wise to adjust the pH to 7-8. The mixture was heated to 60 °C over 2 h and stirred for 12 h at pH <8. The reddish brown precipitate was collected by filtration and dried to give 1.3 g product. Fe_xO_y was activated by adding water 30 min before it was used.

Compound **23** (0.74 g, 1.6 mmol), Fe_xO_y (300 mg), and EtOH (100 mL) were mixed with stirring and heated to 70 °C. Hydrazine monohydrate (0.45 g, 9.0 mmol) was added slowly and the mixture was stirred for 5 h at 70 °C. The catalyst was removed and the filtrate was evaporated to dryness under reduced pressure. The crude product was purified by dry column chromatography on silica gel using 2-PrOH:Toluene (1:3) to give 0.21 g (30%) dye **7** having m.p. 84 °C. ^{1}H NMR (DMSO-d_6): δ 7.66–7.68 (d, 2H), δ 7.28–7.30 (dd, 1H), δ 7.25–7.26 (d, 1H), δ 6.87–6.90 (d, 2H), δ 6.70–6.71 (d, 1H), δ 5.63 (s, 1H), δ 4.93–4.97 (t, 1H), δ 4.20–4.23 (m, 4H), δ 3.70–3.76 (t, 2H), δ 3.66–3.67 (m, 6H), δ 2.01 (s, 6H). TLC: $R_f = 0.56$ (2-Propanol:Toluene = 1:3). Calculated for $C_{16}H_{20}N_4O_3$: C, 59.45; H, 6.35; N, 12.61. Found: C, 59.24; H, 6.95; N, 11.88.

2.2.6. N-(2-Hydroxy-4-nitrophenyl)acetamide (**8**)

2-Amino-5-nitrophenol (20 g, 0.13 mol) was dissolved in pyridine (100 mL) and stirred at room temperature (RT) as acetic anhydride (28.6 g, 0.28 mol) was added all at once. The mixture was heated to and stirred at 60 °C for 2 h. The solution was cooled to RT and poured into H_2O (300 mL), and the mixture was filtered to collect the product. The solid was washed with H_2O and vigorously stirred with H_2O (280 mL) containing NaOH (5.3 g) at 0 °C for 5 min to remove the O-acetyl group [7]. The red solution was filtered and the filtrate was neutralized quickly with 4-N HCl (18 mL). The yellow solid was collected by filtration and dried to give 13 g (51%) of product **8** having m.p. 264 °C (lit. [8] m.p. 269 °C). ^{1}H NMR (DMSO-d_6): δ 10.98 (s, 1H), δ 9.52 (s, 1H), δ 8.26–8.29 (d, 1H),

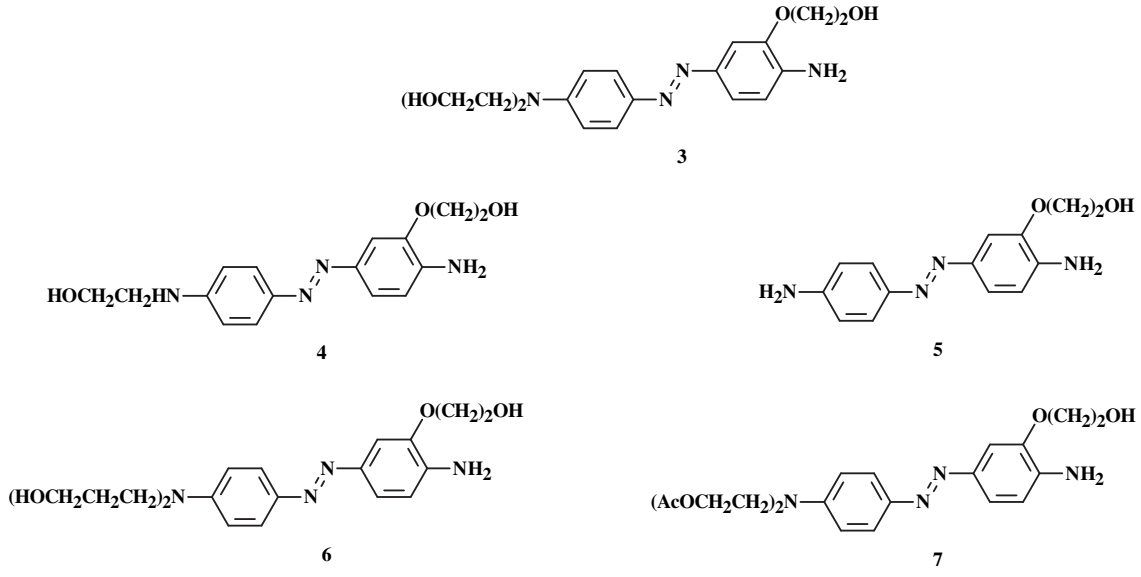


Fig. 4. Molecular structures of dye **3** and four analogs synthesized in this study.

δ 7.68–7.72 (dd, 1H), δ 7.65–7.66 (d, 1H), δ 2.17 (s, 1H). TLC: $R_f = 0.44$ (EtOAc:Hexane = 1:1).

*2.2.7. N-(4-Nitro-2-hydroxyethoxyphenyl)acetamide (**9**)*

Compound **8** (11.0 g, 0.056 mol) was dissolved in DMF (50 mL) and 2-bromoethanol (8.0 g, 0.064 mol), anhydrous K_2CO_3 (8.9 g, 0.064 mol), and KI (0.15 g) were added. The mixture was stirred and heated to 100 °C over 10 min and stirred for 2 h, whereupon additional 2-bromoethanol (8.0 g) and K_2CO_3 (8.9 g) were added. Heating was continued at 100 °C for 2 h and the cooled mixture was filtered to remove salt. The filtrate was evaporated to dryness under reduced pressure and the product was mixed well with dilute NH_4OH solution (5 mL conc. NH_4OH/95 mL H_2O). The crude product was collected by filtration and recrystallized twice from EtOH/H_2O (3:7) to give 13 g (96%) of yellow needles having m.p. 168 °C (lit. [9] m.p. 167–171 °C). ^{1}H NMR (DMSO-d_6): δ 9.39 (s, 1H), δ 8.39–8.42 (d, 1H), δ 7.85–7.89 (dd, 1H), δ 7.80–7.81 (d, 1H), δ 5.14–5.18 (t, 1H), δ 4.16–4.19 (t, 2H), δ 3.77–3.82 (q, 2H), δ 2.20 (s, 3H). TLC: $R_f = 0.50$ (EtOAc).

*2.2.8. N-(2-(2-Hydroxyethoxy)-4-aminophenyl)acetamide (**10**)*

Catalytic hydrogenation was performed on a solution of compound **9** (5 g, 0.02 mol) in EtOH (100 mL) using 20% Pd(OH)$_2$/C (0.15 g). After 1 h, the catalyst was removed by filtration and the filtrate was evaporated to dryness under reduced pressure. The transparent oil crystallized from EtOAc to give 4.1 g (94%) of nearly colorless prisms having m.p. 136–137 °C. ^{1}H NMR (DMSO-d_6): δ 8.68 (s, 1H), δ 7.41–7.44 (d, 1H), δ 6.21–6.22 (d, 1H), δ 6.05–6.08 (dd, 1H), δ 4.91–4.95 (m, 3H), δ 3.85–3.88 (t, 1H), δ 3.67–3.71 (q, 2H), δ 1.98 (s, 3H). TLC: $R_f = 0.17$ (2-Propanol:Toluene = 1:3).

*2.2.9. N,N-bis(3-hydroxypropyl)aniline (**13**)*

Aniline (1.0 g, 0.011 mol), 3-chloropropanol (10.4 g, 0.110 mol), $CaCO_3$ (2.2 g, 0.022 mol) and H_2O (150 mL) were mixed and stirred under reflux for 50 h. The solution was adjusted to pH 10 using 2 N NaOH, on cooling to RT, and the mixture was extracted 3 times with CH_2Cl_2 (75 mL). After drying the organic layer with Na_2SO_4, activated carbon was added and the mixture stirred for 5 min. Activated carbon was removed by filtration and the solvent was evaporated at reduced pressure. The resultant brown oil was crystallized from EtOAc/Hexane (4:1) to give 1.7 g (74%) colorless prisms having m.p. 116–117 °C (lit. m.p 116–118 °C [10]). ^{1}H NMR (DMSO-d_6): δ 7.10–7.13 (m, 2H), δ 6.65–6.68 (m, 2H), δ 6.50–6.54 (m, 1H), δ 4.51–4.53 (t, 2H), δ 3.44–3.47 (q, 4H), δ 3.30–3.34 (t, 4H), δ 1.61–1.68 (m, 4H). TLC: $R_f = 0.40$ (EtOAc:Hexane = 4:1).

*2.2.10. 2-(2-Nitrophenoxy)ethanol (**14**)*

2-Nitrophenol (20 g, 0.143 mol) was dissolved in a solution of NaOH (5.72 g) in H_2O (40 mL). 2-Chloroethanol (11.5 g, 0.143 mol) was added slowly with stirring and the mixture was stirred under reflux for 5 h. The reaction mixture was cooled to RT, stirred with NaOH (5%, 50 mL) and extracted with CH_2Cl_2 (300 mL). The organic layer was stirred for 5 min after adding activated carbon. After filtration and CH_2Cl_2 removal, compound **14** was produced as a light yellow oil (14 g, 53%). ^{1}H NMR (DMSO-d_6): δ 7.84–7.88 (dd, 1H), δ 7.60–7.67 (m, 1H), δ 7.36–7.39 (d, 1H), δ 7.11–7.15 (t, 1H), δ 4.96 (s, 1H), δ 4.19–4.23 (t, 2H), δ 3.76–3.79 (t, 2H). TLC: $R_f = 0.5$ (EtOAc:Hexane = 1:1).

*2.2.11. 2-(2-Aminophenoxy)ethanol (**15**)*

Catalytic hydrogenation was performed on a solution of compound **14** (10 g, 0.055 mol) in EtOH (100 mL) using 20% Pd(OH)$_2$/C (0.15 g). After 1 h, the catalyst was removed by filtration, and the filtrate was evaporated to dryness under reduced pressure to give a light brown solid. The solid was recrystallized from 1-PrOH:Petroleum ether to give 7.7 g (93%) white needles having m.p. 86–87 °C. ^{1}H NMR (DMSO-d_6): δ 6.73–6.36 (d, 1H), δ 6.60–6.70 (m, 2H), δ 6.47–6.51 (m, 1H), δ 4.88–4.93 (t, 1H), δ 4.79 (s, 2H), δ 3.88–3.92 (t, 2H), δ 3.68–3.74 (q, 2H), δ 1.98 (s, 3H). TLC: $R_f = 0.4$ (EtOAc).

*2.2.12. 4-((3-(2-Hydroxyethoxy)-4-amino)phenylazo) nitrobenzene (**16**)*

4-Nitroaniline (0.5 g, 3.6 mmol) and HCl (2 N, 9 mL) were mixed with stirring, heated to 40 °C, and cooled to 0 °C when a clear solution formed. The amine was diazotized by adding $NaNO_2$ (1 N, 3.6 mL) at 0 °C. After stirring at 0 °C for 1 h, the diazonium salt solution was checked with KI/starch paper for the presence of HNO_2. Additional $NaNO_2$ (1 N, 0.5 ml) was added and diazotization was continued for 30 min. At that point, solid sulfamic acid was added to destroy excess HNO_2. The solution was clarified by adding activated carbon with stirring, followed by filtration with cooling of the filtrate in an ice bath.

Compound **15** (0.57 g, 3.7 mmol) and HOAc (5 mL) were mixed with stirring. The cold solution of diazo compound prepared above was added to this solution, keeping the temperature at 0 °C and

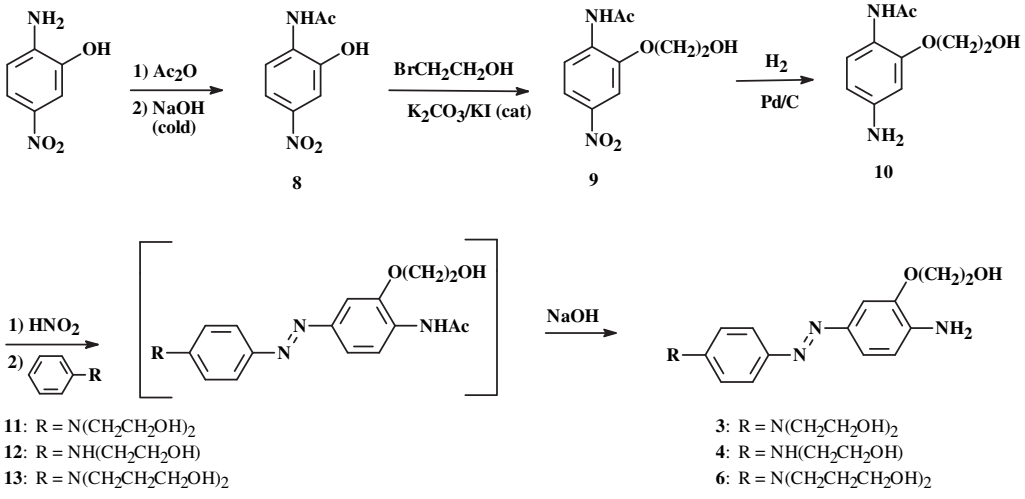


Fig. 5. Reaction sequence used to synthesize dyes **3**, **4**, and **6**.

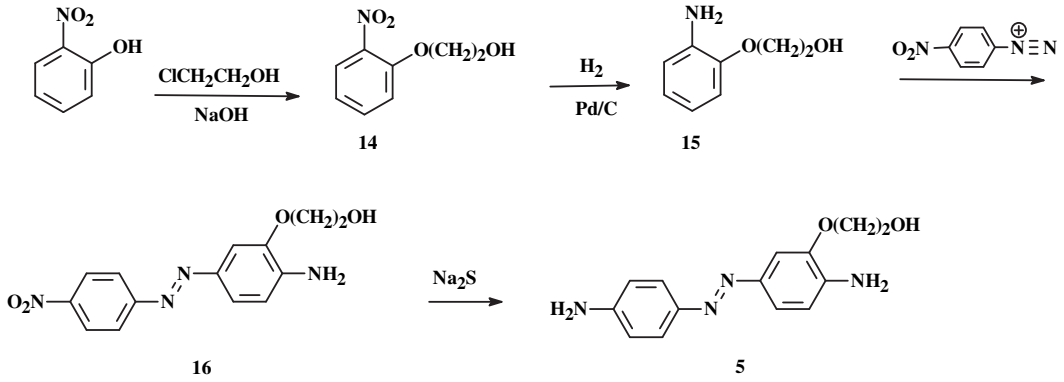


Fig. 6. Reaction sequence used in the synthesis of dye **5**.

maintaining pH 3 by adding NaOAc. After stirring for 24 h at 0 °C, the orange solid was collected by filtration to give 0.99 g (91%) compound **16** having m.p. 198 °C. ^{1}H NMR (DMSO-d_6): δ 8.32–8.36 (d, 2H), δ 7.92–7.96 (d, 2H), δ 7.53–7.57 (dd, 1H), δ 7.51–7.53 (d, 1H), δ 6.82–6.86 (d, 1H), δ 5.55 (s, 1H), δ 4.05–4.08 (t, 2H), δ 3.75–3.78 (t, 2H). TLC: $R_f = 0.67$ (EtOAc).

2.2.13. *N,N′-1,3-Phenylenebisacetamide (**17**)*

m-Phenylenediamine (15.6 g, 0.144 mol) was dissolved in HOAc (50 mL) and then acetic anhydride (32.4 g, 0.327 mol) was added gradually. The mixture was stirred for 30 min and H_2O (100 mL) added. The white solid was collected by filtration and dried to give 26.2 g (94%) compound **17** having m.p. 196–198 °C. ^{1}H NMR (DMSO-d_6): δ 9.88 (s, 2H), δ 7.86 (s, 1H), δ 7.24–7.26 (d, 2H), δ 7.18–7.12 (m, 1H), δ 2.02 (s, 6H). TLC: $R_f = 0.36$ (EtOAc).

2.2.14. *N,N′-(4-Nitro-1,3-phenylene)bisacetamide (**18**)*

Compound **17** (26.2 g, 0.14 mol) was dissolved in conc. H_2SO_4 (98.3 mL) at 20 °C. A mixture of HNO_3 (9 mL) and H_2SO_4 (9.8 mL) was added gradually at 5–10 °C, and the solution was stirred for 15 min. The solution was poured into ice water (500 mL) and the product was collected and dried to give 28.8 g (89%) **18** having m.p. 223–224 °C. ^{1}H NMR (DMSO-d_6): δ 10.48 (s, 1H), δ 10.28 (s, 1H), δ 8.13–8.15 (d, 1H), δ 7.96–8.00 (2, 1H), δ 7.50–7.54 (dd, 1H), δ 2.10 (s, 6H). TLC: $R_f = 0.67$ (EtOAc).

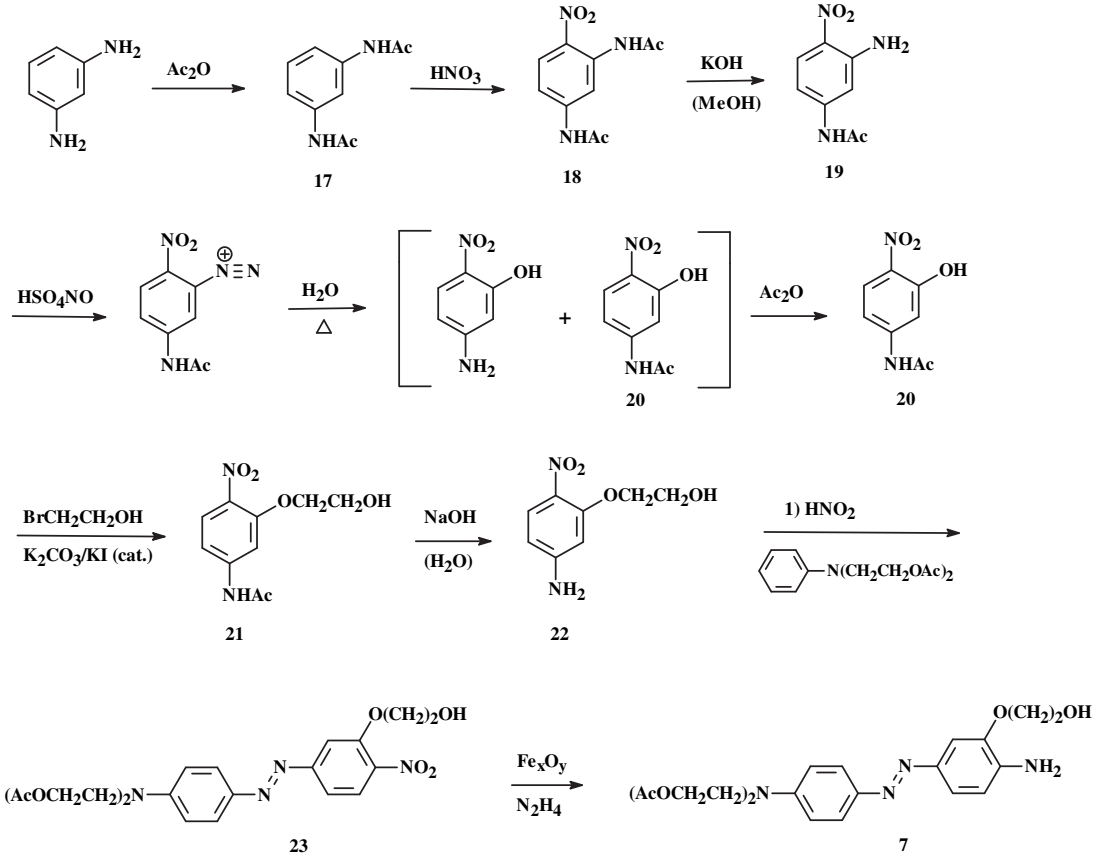


Fig. 7. Reaction sequence used in the synthesis of dye **7**.

Table 1
Absorption spectral data for dyes **3**–**7** in MeOH.

Dye	λ_{max}(nm)	E_{max} {L/(mole-cm)}
3	450	35,200
4	448	33,200
5	407	29,700
6	459	34,600
7	450	30,800

*2.2.15. N-(3-Hydroxy-4-nitrophenyl)acetamide (**20**)*

Compound **18** (20 g, 0.084 mol), MeOH (100 mL), and KOH (4.6 g) were stirred at RT for 2 h whereupon conc. HCl (8.8 g) and H_2O (100 mL) were added and the mixture was cooled to 10 °C. The product was collected by filtration and dried to give 14.4 g (87%) **19**. Compound **19** (10 g, 0.052 mol), HOAc (60 mL), and MeCN (15 mL) were stirred at 40 °C, and the mixture was cooled to 5 °C. Conc. H_2SO_4 (18 mL) was cooled and $NaNO_2$ (3.9 g) was added gradually at < 20 °C, then the mixture was heated to 70 °C with stirring and cooled after dissolution. The solution of nitrosylsulfuric acid was added to the mixture of **19** in HOAc/MeCN over 30 min, under N_2, at 5–10 °C. After stirring for 10 min, the solution was added to H_2O (40 mL) and conc. H_2SO_4 (30 mL) with stirring, at 120 °C, and then stirred for 10 min. After cooling this solution, NaOAc was added to achieve pH 4, and the solvents were evaporated. Salt was removed by dissolving the organic components in acetone followed by filtration. Evaporating the solvent afforded 6 g of a mixture of *N*-(3-hydroxy-4-nitrophenyl)acetamide (**20**) and 3-hydroxy-4-nitroaniline. The latter component was acylated by adding acetic anhydride (8.6 g) and pyridine (50 mL) to the mixture and stirring for 2 h at 60 °C. Pyridine was removed under reduced pressure and the concentrate was mixed with cold NaOH (5.33 g) and H_2O (280 mL), then stirred for 5 min and filtered. The filtrate was neutralized with 4 N HCl and the mixture was filtered and dried to give 3.9 g (39%) **22**, having m.p. 205 °C. ^{1}H NMR (DMSO-d_6): δ 10.92 (s, 1H), δ 10.37 (s, 1H), δ 7.91–7.95 (d, 1H), δ 7.61–7.63 (d, 1H), δ 7.02–7.06 (dd, 1H), δ 2.10 (s, 3H). TLC: R_f = 0.28 (EtOAc:Hexane = 1:1).

*2.2.16. N-(4-Nitro-3-hydroxyethoxyphenyl)acetamide (**21**)*

Compound **20** (2.0 g, 0.010 mol) was dissolved in DMF (20 mL) and 2-bromoethanol (1.5 g, 0.012 mol), anhydrous K_2CO_3 (1.6 g, 0.012 mol), and KI (150 mg) were added. The mixture was stirred at 100 °C for 2 h and 2-bromoethanol (1.45 g) and K_2CO_3 (1.6 g) were added. Heating was continued at 100 °C for 2 h, and the mixture was cooled to RT and filtered to remove salt. The filtrate was evaporated to dryness and the product was mixed well with dilute NH_4OH solution (5 mL conc. NH_4OH/95 mL H_2O). The solid was collected by filtration and recrystallized from EtOH/H_2O (3:7) to give 2.3 g (96%) **21** having m.p. 121 °C. ^{1}H NMR (DMSO-d_6): δ 10.42 (s, 1H), δ 7.90–7.94 (d, 1H), δ 7.64–7.66 (d, 1H), δ 7.22–7.26 (dd, 1H), δ 4.87–4.90 (t, 1H), δ 4.00–4.03 (t, 2H), δ 3.71–3.74 (t, 2H), δ 2.10 (s, 3H). TLC: R_f = 0.47 (EtOAc).

*2.2.17. 3-Hydroxyethoxy-4-nitroaniline (**22**)*

Compound **21** (2 g, 8.3 mmol), EtOH (31 mL), H_2O (16 mL), and NaOH (3.2 g) were mixed and boiled for 1 h. The mixture was cooled overnight and the precipitant was collected by filtration to give 1.6 g (93.1%) **22**, having m.p. 76–77 °C. ^{1}H NMR (DMSO-d_6): δ 7.73–7.80 (d, 1H), δ 6.50 (s, 2H), δ 6.38–6.40 (d, 2H), δ 6.16–6.20 (dd, 1H), δ 4.81–4.84 (t, 1H), δ 4.00–4.03 (t, 2H), δ 3.70–3.73 (t, 2H). TLC: R_f = 0.62 (EtOAc).

*2.2.18. 4-((3-(2-Hydroxyethoxy)-4-nitro)phenylazo) N,N-bis(2-acetoxyethyl) aniline (**23**)*

Compound **22** (1.55 g, 7.8 mmol) was mixed with 2 N HCl (24.4 mL), heated to 40 °C, and cooled to 0 °C. To the clear solution, $NaNO_2$ (1 N, 7.9 mL) was added slowly at 0 °C. After stirring at 0 °C for 1 h, the diazonium salt solution was checked with KI/starch paper for the presence of HNO_2. Sulfamic acid was added to destroy excess HNO_2 and the solution was clarified by adding activated carbon with stirring. The adsorbent was removed by filtration with simultaneous cooling of the filtrate in an ice bath.

N,N-bis(2-Acetoxyethyl)aniline (2.1 g, 8.0 mmol) was dissolved in HOAc (10 mL). The cold solution of diazo compound prepared in the previous step was added to this solution, keeping the temperature at 0 °C and maintaining pH 4 by adding NaOAc. After stirring for 24 h at 0 °C, the red solid was collected by filtration and the crude dye was purified by flash column chromatography (silica gel, EtOAc:Toluene = 5:2) to give 2.1 g (59%) **23** having m.p. 84 °C. ^{1}H NMR (DMSO-d_6): δ 8.00–8.04 (d, 1H), δ 7.81–7.85 (d, 2H), δ 7.63–7.65 (d, 1H), δ 7.41–7.45 (dd, 1H), δ 6.96–7.00 (d, 2H), δ 4.90–4.93 (t, 1H), δ 4.27–4.30 (t, 2H), δ 4.22–4.25 (t, 2H), δ 3.72–3.76 (m, 6H), δ 1.98 (s, 6H). TLC: R_f = 0.4 (EtOAc).

2.3. Mutagenicity testing

The methods of Maron and Ames [6] using strains of *S. typhimurium* were used to examine the mutagenic potential of the compounds. To top agar (2–3 mL) at 47–50 °C was added ~10^8 bacteria (TA98, TA100, or TA1538) from a fresh culture, test compound in DMSO (0.1 mL) at the appropriate concentration (0–2 mg), and rat liver S9 mixture (0.5 mL, when activation was employed). This combination was mixed gently and poured on plates containing minimal-glucose agar (25 mL). After solidification of the top agar for ~20 min, the plates were inverted and incubated at 37 °C for 72 h. The revertant colonies were then counted.

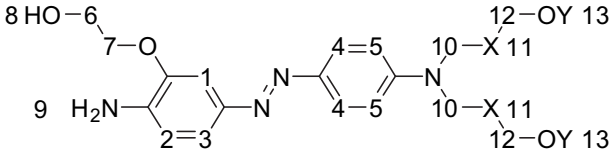
Mutagenicity results were designated positive or negative based on the criteria of Claxton and co-workers [12]. A dye was judged positive (mutagenic) when revertant counts increased with dose

Table 2
Absorption maxima of dyes **3**–**7** in solvents with different relative polarity.

Solvent	EtOAc	Acetone	DMF	MeCN	EtOH	MeOH
Rel. Pol.[a]	0.228	0.355	0.404	0.460	0.654	0.762
Dye	λ_{max}(nm)	λ_{max}(nm)	λ_{max}(nm)	λ_{max}(nm)	λ_{max}(nm)	λ_{max}(nm)
3	436	443	455	443	457	450
4	412	416	448	416	449	448
5	407	410	440	408	409	407
6	439	446	456	449	460	459
7	416	437	449	419	451	450

[a] [18].

Table 3
Comparisons of NMR spectra of dyes **3**–**7**.



Proton	Dye **3** X = 0, Y = H	Dye **4** X = 0,Y = H,11 = NH	Dye **5** X = 0,Y = H,10 = H	Dye **6** X = CH_2, Y = H	Dye **7** X = 0, Y = Ac
1	7.25–7.26 (d)	7.25–7.26 (d)	7.23–7.24 (d)	7.25–7.26 (d)	7.25–7.26 (d)
2	6.69–6.71 (d)	6.69–6.71 (d)	6.69–6.71 (d)	6.69–6.71 (d)	6.70–6.72 (d)
3	7.27–7.28 (dd)	7.27–7.28 (dd)	7.25–7.26 (dd)	7.27–7.28 (d)	7.28–7.29 (dd)
4	7.62–7.64 (d)	7.58–7.60 (d)	7.52–7.54 (d)	7.62–7.64 (d)	7.65–7.67 (d)
5	6.78–6.80 (d)	6.65–6.67 (d)	6.61–6.63 (d)	6.77–6.78 (d)	6.88–6.90 (d)
6	3.73–3.78 (m)	3.75–3.79 (m)	3.74–3.80 (m)	3.73–3.78 (m)	3.74–3.78 (m)
7	3.99–4.01 (t)	3.99–4.01 (t)	3.99–4.01 (t)	3.99–4.01 (t)	3.99–4.01 (t)
8	4.91–4.93 (t)	4.91–4.93 (t)	4.91–4.93 (t)	4.91–4.93 (t)	4.91–4.93 (t)
9	5.55 (s)	5.53 (s)	5.53 (s)	5.54 (d)	5.60 (s)
10	3.57–3.60 (t)	3.16–3.19 (t)	5.72 (s)	3.43–3.45 (t)	3.68–3.70 (t)
11	N/A	6.26–6.28 (1H, t)	N/A	1.69–1.74 (m)	N/A
12	3.48–3.52 (m)	3.56–3.59 (m)	N/A	3.48–3.50 (m)	4.20–4.22 (t)
13	4.79–4.81 (t)	4.72–4.74 (t)	N/A	4.55–4.57 (t)	2.00 (s)

levels as determined by the statistical models of Stead and co-workers [13] and Bernstein and co-workers [14].

3. Results and discussion

3.1. Dye synthesis

The reaction scheme used for the synthesis of dyes **3**, **4**, and **6** is shown in Fig. 5. The starting point is 2-hydroxy-4-nitroaniline, which was converted to compound **8** in two steps, viz. acetylation with acetic anhydride and removal of the O-acetyl group by treatment with cold NaOH. Compound **9** was prepared by alkylating **8** with 2-bromoethanol at 100 °C using KI as a catalyst. The heating rate was important in this procedure. When the reaction mixture was heated slowly (over 30–45 min) to 100 °C, the ratio of the target product (**9**) to the corresponding deacetylated by-product was almost 1:1. However, when the reaction temperature was reached in 10 min, a very small amount of the by-product was produced and it was readily removed by recrystallization. Compound **10** was obtained by catalytic hydrogenation of **9** in the presence of 20% $Pd(OH)_2/C$. The reaction yield (94%) was significantly higher than that (79%) reported previously [15]. Diazotization of compound **10** was followed by coupling with *N*,*N*-bis(2-hydroxyethyl)aniline (**11**), *N*-(2-hydroxyethyl)aniline (**12**), and *N*,*N*-bis(3-hydroxypropyl)aniline (**13**) at pH 4-5 to give intermediate azo compounds that were deacetylated groups to give the target dyes. Dye purification by crystallization from acetone/H_2O gave semi-solids that completely solidified over 1–3 weeks.

The reaction sequence used to synthesize dye **5** is shown in Fig. 6. 2-Nitrophenol was alkylated with 2-chloroethanol to give **14**, which, in turn, was subjected to catalytic hydrogenation. 4-Nitroaniline was diazotized and coupled to compound **15** at pH 3 to give azo compound **16**. Reduction of the nitro group using Na_2S yielded dye **5**.

The reaction sequence used to synthesize dye **7** is outlined in Fig. 7. *m*-Phenylenediamine was diacetylated with acetic anhydride to give compound **17**, nitrated to give compound **18**, and converted to compound **19** by selective deacetylation using KOH/MeOH [16]. Compound **19** was diazotized and then hydroxylated by heating the diazo compound in H_2O. The *N*-acetyl group was restored by reaction with acetic anhydride in pyridine to give compound **20** as the sole product. Compound **20** was alkylated with 2-bromoethanol and hydrolyzed using aqueous NaOH to give compound **22**.

After diazotization of compound **22**, coupling of the resultant diazonium compound to *N*,*N*-bis(2-acetoxyethyl)aniline was accomplished at pH 4–5. Compound **23** was reduced to give the target dye **7**, by using iron oxide/hydroxide as a catalyst and hydrazine monohydrate as a hydrogen source; however, the yield was low (30%). These results are consistent with a previous report [17] pertaining to the selective reduction of nitrophenylazobenzenes, where it was found that azo reductive-cleavage competed with nitro-group reduction. Weak electron-donating groups and electron-withdrawing groups increased nitro group reduction, whereas strong electron-donating groups increased reductive-cleavage of the azo bond. Hence, due to the strong electron-donating character of the $-N(CH_2CH_2OAc)_2$ group, the reduction of compound **23** favors azo reductive-cleavage rather than nitro group reduction. Because it was also reported that higher reaction temperatures favored nitro-group reduction over azo reductive cleavage, 70 °C was the reaction temperature used for the reduction of compound **23**.

3.2. Analyses

Visible absorption spectral data for dyes **3**–**7** are summarized in Table 1. After observing a large λ_{max} difference between dye **5** and the other dyes in EtOH, visible spectra were recorded in solvents of different relative polarity (H_2O relative polarity = 1) and the results are shown in Table 2. The much lower λ_{max} value for dye **5** versus the other 4 dyes was not observed in DMF but there was no clear correlation between λ_{max} and solvent polarity. It is evident, however, that solvents other than DMF produced λ_{max} values $\leq$ 410 nm for dye **5**.

Table 4
Summary of high-resolution ESI mass spectra for dyes **3**–**7**, and **23**.

Dye	Exact mass	$[M+H]^{+}$, [a]	$[M+H]^{+}$, [b]
3	360.1798	361.1876	361.1875
4	316.1535	317.1613	317.1605
5	272.1273	273.1351	273.1346
6	388.2111	389.2189	389.2183
7	444.2009	445.2087	445.2088
23	474.1751	475.1829	475.1837

[a] Calculated.
[b] Observed.

Table 5
Mutagenicity test results for dyes **3**–**7** from the standard *Salmonella* mutagenicity assay.

Dye	TA98/-S9	TA98/+S9	TA100/-S9	TA100/+S9	TA1538/-S9	TA1538/+S9
	Rev/mg (Mut)[a]	Rev/mg (Mut)[a]	Rev/mg (Mut)[a]	Rev/mg (Mut)[a]	Rev/mg (Mut)[a]	Rev/mg (Mut)[a]
3	(−)	(−)	(−)	(−)	14.5(+)	33.3(+)
4	(−)	(−)	(−)	(−)	53.8(+)	17.7(+)
5	612.5(+)	4541.0(+)	2800.0(+)	813.0(+)	2383.0(+)	8456.0(+)
6	(−)	42.3(+)	(−)	(−)	11.0(+)	104.3(+)
7	(−)	(−)	(−)	(−)	52.9(+)	71.9(+)
3[b]	(−)	1052(+)	(?)	(−)	635(+)	NT[c]

[a] (Mut) = mutagenicity designation; (−) negative, (+) positive, (?) equivocal.
[b] Data recorded from ref. [4].
[c] NT = Not tested.

Comparisons of ^{1}H NMR spectral data for dyes **3**–**7** are shown in Table 3. The position of peaks for protons 1–3 and 6–9 were the same or differed little in each case, but peaks for protons 4–5 and 10–13 differed with changing nature of the substituted amino group. In the spectra of dyes **3**–**7**, peaks 4 and 5 moved up field as the number of hydroxyethyl groups decreased. In the spectrum of dye **6**, which has hydroxypropyl groups instead of hydroxyethyl groups (dye **3**), peaks for protons 4 and 5 were very close to or the same as those arising from dye **3**. In the spectrum of dye **7**, peaks for protons 4 and 5 shifted downfield slightly, as the two −OH groups were capped by acetyl groups. Peaks for protons 10–13 occupied different positions, because of changes in the nature of side chains.

High resolution ESI mass spectrometry was used to help confirm the structures of dyes **3**–**7** and **23**. A summary of the mass spectral data for dyes **3**–**7** and **23** is given in Table 4. All spectra contained $[M+H]^+$ species as the base peak. In some cases, a $[M+2H]^{2+}$ or $[M+Na]^+$ peak also appeared. The possible sources of sodium are the glass containers used and trace amounts of NaCl from neutralization of reaction media during dye synthesis. The spectrum of dye **3** contained a peak at $m/z = 121.0483$ for the internal standard and the base peak ($[M+H]^+$) at $m/z = 361.1875$. The spectrum of dye **4** included an $[M+2H]^{2+}$ peak at $m/z = 159.0836$ and the base peak $[M+H]^+$ at $m/z = 317.1605$. The spectrum of dye **5** had a small internal standard peak at $m/z = 663.45102$, the base peak ($[M+H]^+$) at $m/z = 273.1346$, and a small $[M+Na]^+$ peak at $m/z = 295.1170$. The spectrum of dye **6** contained an $[M+2H]^{2+}$ peak at $m/z = 195.1122$ and the base peak ($[M+H]^+$) at $m/z = 389.2183$. The spectrum of dye **7** had a small $[M+2H]^{2+}$ peak at $m/z = 223.1079$, a small sodiated peak of a dimerized dye at $m/z = 911.3914$, a $[M + Na]^+$ peak at $m/z = 467.1903$, and the base peak ($[M+H]^+$) at $m/z = 445.2088$. The spectrum of compound **23** contained an $[M+H]^+$ peak at $m/z = 475.1837$, an $[M+Na]^+$ peak at $m/z = 497.1657$, and a sodiated peak for dimerized dye at $m/z = 971.3402$ with very similar intensities.

3.3. Mutagenicity

Dyes **3**–**7** were examined in the standard *Salmonella* mutagenicity test [6] using bacterial strains TA98, TA100, and TA1538 with and without S9 activation, the results of which are summarized in Table 5. Results were designated as (+) for mutagenic and as (−) for nonmutagenic, and the numbers shown are slope values based on the Bernstein statistical model [14], which indicate the level of mutagenicity. A mutagenic dye with a large slope value is more mutagenic than a mutagenic dye with a smaller slope value.

Dye **3** was not mutagenic in TA98 or TA100 with or without S9 activation. However, dye **3** was mutagenic in TA1538 with and without S9 activation. Surprisingly, test results in TA98 with S9 activation differ from previous work [4], in which it was reported that dye **3** was quite mutagenic in TA98 with S9 activation. In that study dye **3** was also mutagenic in TA1538 with S9 activation, but it was determined to be more mutagenic than in the present study. It is possible that a sampling error led to the previous outcomes because the present results were checked three times.

Dye **4** was not mutagenic in TA98 and TA100 with or without S9 activation but was mutagenic in TA1538 with and without S9 activation. The higher slope value in the absence of S9 suggest that losing one hydroxyethyl group from the $-N(CH_2CH_2OH)_2$ group of dye **3** increases mutagenicity.

Dye **5** was a direct-acting mutagen in TA98 with and without S9 activation and unlike the other dyes in this study, was strongly mutagenic in TA100 and TA1538 with and without S9 activation. The slope values of dye **5** are much larger than those of the other dyes in this study. This indicates that losing two hydroxyethyl groups from the $-N(CH_2CH_2OH)_2$ moiety of dye **3** transform the parent dye into a strong direct-acting mutagen in all 3 strains, whereas losing one hydroxyethyl group from $-N(CH_2CH_2OH)_2$ does not.

Dye **6** was mutagenic in TA98 with S9 activation and in TA1538 with and without S9 activation. Mutagenicity in TA1538 was higher with S9 activation. Dye **6** contains hydroxypropyl groups instead of hydroxyethyl groups, and it was expected that increasing the length of the alkyl chains would make de-hydroxyalkylation more difficult, resulting in dye **6** being less mutagenic than dye **3**. However, the results indicate that increasing the length of the hydroxyalkyl group leads to higher mutagenicity.

Dye **7** was mutagenic only in TA1538 with and without S9 activation. It is shown that the slope values of dye **7** is larger than those of dye **3**, suggesting that the capped (acetylated) hydroxyethyl groups lead to slightly increased mutagenicity.

4. Conclusions

A group of aminoalkoxyazobenzene dyes were readily synthesized, albeit in a relatively low yield of the dye made using a step involving a competition between nitro group reduction and azo bond reductive-cleavage. In this regard, using iron oxide/hydroxide as a catalyst and heating the reaction mixture were essential to obtaining the target dye.

Mutagenicity test results of the present compounds have shed light on the structural basis for the mutagenic activity of 4-((3-(2-hydroxyethoxy)4-amino)phenylazo)-*N*,*N*-bis(2-hydroxyethyl) aniline. The results of this study have shown that the mutagenicity of this dye arises from the metabolic cleavage of *N*-hydroxyethyl groups to give 4-((3-(2-hydroxyethoxy)4-amino)phenylazo)-*N*-(2-hydroxyethyl)aniline and 4-((3-(2-hydroxyethoxy)4-amino) phenylazo)aniline as direct-acting mutagens and that the

mutagenicity of 4-((3-(2-hydroxyethoxy)4-amino)phenylazo)-*N*,*N*-bis(2-hydroxyethyl)aniline was less than previously reported.

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