

Abstract

Jeong, Euigyung. Aminoalkoxyazobenzene Dyes – Studies Leading to the Mechanism of Mutagenesis (Under the direction of Dr. Harold Stanley Freeman).

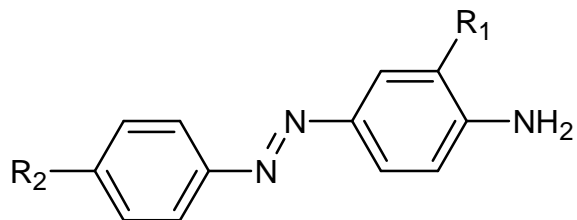
This study is an extension of previous work in our laboratories pertaining to the effects of substituents on the mutagenicity of aminoazobenzene-based dyes (cf. **52** - **66**). A key outcome of the previous work was the recognition that bulky alkoxy substituents placed *ortho* to the primary amino (-NH₂) group significantly reduced or removed mutagenicity. In this regard, the *ortho*-OCH₂CH₂OH group gave a significant reduction in mutagenicity, with the notable exception of the dye in which R₁ = OCH₂CH₂OH and R₂ = N(CH₂CH₂OH)₂ (dye **66**). Interestingly, this dye was not mutagenic following reductive cleavage of the azo group, suggesting that metabolism involving the N(CH₂CH₂OH)₂ group was associated with mutagenic activity. It is not clear, however, how the combination R₁ = OCH₂CH₂OH and R₂ = N(CH₂CH₂OH)₂ contributes to the anomalous behavior of dye **66**.

With the above-mentioned results in mind, the present thesis pertains to the synthesis of three analogs of dye **66** (cf. **67** - **69**) and the determination of their mutagenicity. These novel structures permit an assessment of the importance of the R₂ = N(CH₂CH₂OH)₂ group in producing a mutagenic response when R₁ = OCH₂CH₂OH, as this group is replaced by: 1) NHCH₂CH₂OH (dye **67**), 2) N(CH₂CH₂OAc)₂ (dye **68**), and 3) N(CH₂CH₂OH)₂ (dye **69**).

The target dyes were synthesized by diazotizing and coupling the appropriate alkoxyated anilines to the corresponding *N*-substituted anilines, and their structures were established by ¹H NMR and high resolution FAB and ESI mass spectrometry. Surprisingly, all three dyes were obtained as viscous oils that could not be induced to crystallize.

Mutagenicity was assessed in Salmonella strain TA98 with and without S9 enzyme activation, since mutagenicity was observed in dye **66** only in that strain. In this way, the effects of the structural changes would be most evident.

The results of the mutagenicity assessment indicated that removing one of the *N*-hydroxyethylamino groups (cf. dye **67**) causes an increase in mutagenicity, while either acetylating the *N,N*-bis(hydroxyethyl)amino group to give dye **68** or increasing the length of the hydroxyethyl groups to give dye **69** removed mutagenicity. These results suggest that the presence of a free *N*-hydroxyethyl group in the coupler moiety is responsible for the mutagenicity of parent dye **66**.



	<u>R1</u>	<u>R2</u>
52	OCH ₃	H
53	OCH ₂ CH ₃	H
54	OCH ₂ CH ₂ CH ₃	H
55	OCH ₂ CH ₂ CH ₂ CH ₃	H
56	OCH ₂ CH ₂ OH	H
57	OCH ₃	N(CH ₂ CH ₃) ₂
58	OCH ₂ CH ₃	N(CH ₂ CH ₃) ₂
59	OCH ₂ CH ₂ CH ₃	N(CH ₂ CH ₃) ₂
60	OCH ₂ CH ₂ CH ₂ CH ₃	N(CH ₂ CH ₃) ₂
61	OCH ₂ CH ₂ OH	N(CH ₂ CH ₃) ₂
62	OCH ₃	N(CH ₂ CH ₂ OH) ₂
63	OCH ₂ CH ₃	N(CH ₂ CH ₂ OH) ₂
64	OCH ₂ CH ₂ CH ₃	N(CH ₂ CH ₂ OH) ₂
65	OCH ₂ CH ₂ CH ₂ CH ₃	N(CH ₂ CH ₂ OH) ₂
66	OCH ₂ CH ₂ OH	N(CH ₂ CH ₂ OH) ₂
67	OCH ₂ CH ₂ OH	NH(CH ₂ CH ₂ OH)
68	OCH ₂ CH ₂ OH	N(CH ₂ CH ₂ OAc) ₂
69	OCH ₂ CH ₂ OH	N(CH ₂ CH ₂ CH ₂ OH) ₂

**AMINOALKOXYAZOBENZENE DYES – STUDIES LEADING TO
THE MECHANISM OF MUTAGENESIS**

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BIOGRAPHY

Euigyung Jeong was born on April 30, 1976 in Puyo, Korea. He was raised in Puyo and graduated from Puyo High School in February 1995. He undertook undergraduate studies in Fiber and Polymer Engineering, Hanyang University, Seoul, Korea and received a Bachelor of Science degree in February 2001. In August 2002, he entered North Carolina State University and began studies towards a Master of Science degree in Textile Chemistry under the direction of Dr. Harold S. Freeman.

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S9 activation 70

I. Introduction

1. Azo Dyes

1.1. Introduction

Azo dyes are by far the most important class of industrial dyes both in number and amount produced [1]. Reasons for the prominence of azo dyes arise from the fact that: 1) they are tinctorially strong, having about twice the strength of anthraquinone dyes which are the next most important class of dyes; 2) they are usually easy to manufacture in a chemical plant from cheap, readily available starting materials; 3) they cover the entire color spectrum; and 4) they have good fastness properties. The first two features give azo dyes greater cost effectiveness than most other dye groups, particularly anthraquinone dyes. Moreover, the ease of preparation from a host of readily available intermediates allows azo dyes to meet almost any commercial need [2].

1.2. Structures

Azo dyes contain one or more azo groups ($\text{—N}=\text{N—}$) which are linked to sp^2 hybridized carbon atoms. Based on the number of such groups, the dyes are known as mono-, dis-, tris-, tetrakis-, (etc.) azo dyes. The azo group is normally attached to two aromatic groups. The resultant dyes exist in the *trans* form which is more stable than the *cis* form [2].

Azo dyes are usually classified into subgroups to indicate an appropriate method of synthesis. A “shorthand” classification, known as Winther symbols [3], uses letters A, D, E, Z, and M, to represent the dye intermediates and the synthetic method used:

A = Diazotizable amine

D = Tetrazotizable diamine

E = Coupler capable of reacting with one diazonium ion

Z = Coupler capable of reacting with more than one diazonium ion

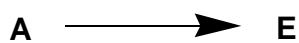
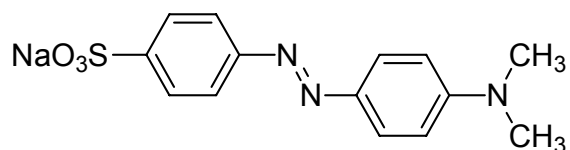
M = Coupler capable of diazotization after coupling

Arrows are used to indicate the direction of the coupling reaction. Thus, A → E dyes are monoazo dyes formed by diazotization of component A and reaction with coupler E. If both A and E are aromatic, the dye is a carbocyclic azo dye. On the other hand, if A and/or E is heterocyclic, the dye is a heterocyclic azo dye.

1.2.1. Monoazo dyes

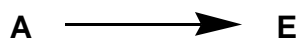
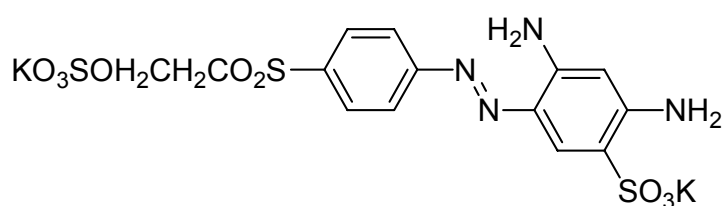
Some examples of monoazo dyes are shown in Figure 1. Examples of carbocyclic azo dyes of type A → E are C.I. Acid Orange 52, which is synthesized from the sodium salt of sulfanilic acid and *N,N*-dimethylaniline and C.I. Reactive Yellow 201, which is synthesized from 2-[(*p*-aminophenyl)-sulfonyl]ethyl potassium sulfate and the potassium salt of *m*-phenylenediamine-4-sulfonic acid.

Heterocyclic azo dyes of type A → E are prepared by disazotation of aromatic components A and reaction with heterocyclic coupler E. An example is C.I. Disperse Blue 339, which is synthesized from 2-amino-5-nitrothiazole and *N,N*-diethyl-*m*-aminotoluene.



(Sulfanilic acid, sodium salt) (*N,N*-Dimethylaniline)

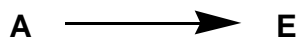
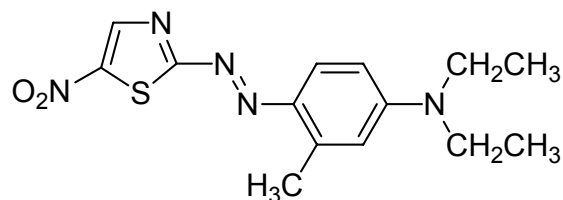
C.I. Acid Orange 52



(2-[(*p*-Aminophenyl)sulfonyl]ethyl potassium sulfate)

(*m*-Phenylenediamine-4-sulfonic acid, potassium salt)

C.I. Reactive Yellow 201



(2-Amino-5-nitrothiazole) (*N,N*-Diethyl-*m*-aminotoluene)

C.I. Disperse Blue 339

Figure 1. Structures of some A \rightarrow E monoazo dyes.

1.2.2. Disazo dyes

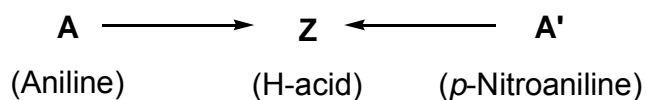
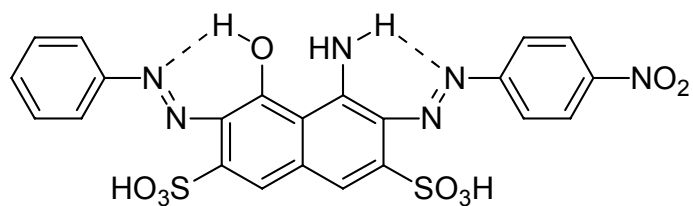
Examples of this group of dyes are shown in Figure 2. Disazo dyes of type $A \rightarrow Z \leftarrow A'$ dyes are prepared by diazotization of components A and A' and reaction with coupler Z. An example is C.I. Acid Black 1 which is synthesized from H-acid, aniline, and *p*-nitroaniline.

Disazo dyes are also represented by the $A \rightarrow M \rightarrow E$ system which has an almost endless number of combinations and can cover colors from red to green with appropriate selections. An example is C.I. Reactive Brown 1, which uses sulfanilic acid, aniline, Cleve's acid and cyanuric chloride.

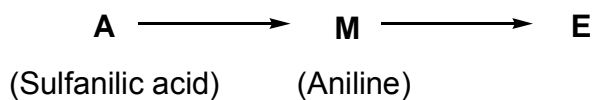
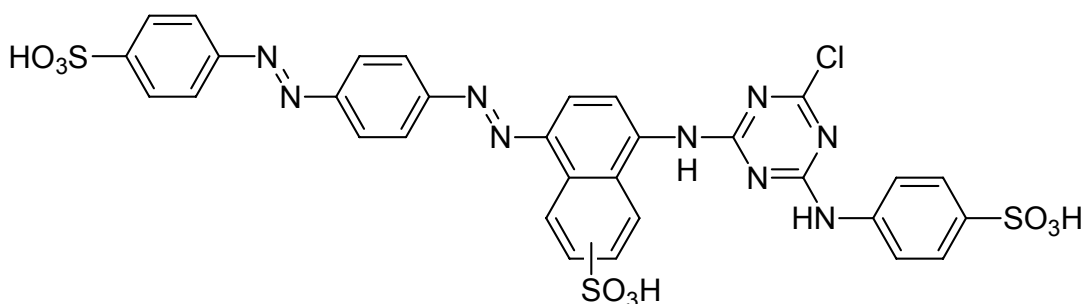
A third type of disazo dyes are $E \leftarrow D \rightarrow E'$ dyes. These dyes are usually synthesized via tetrazotization of a suitable diamine followed by coupling twice. In this regard, E and E' may be the same, as illustrated in C.I. Basic Brown 2.

1.2.3. Trisazo and polyazo dyes

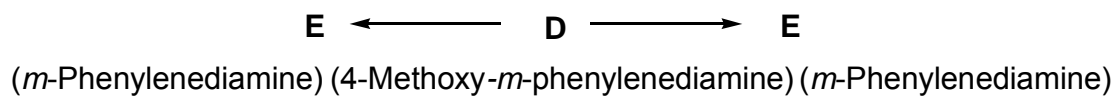
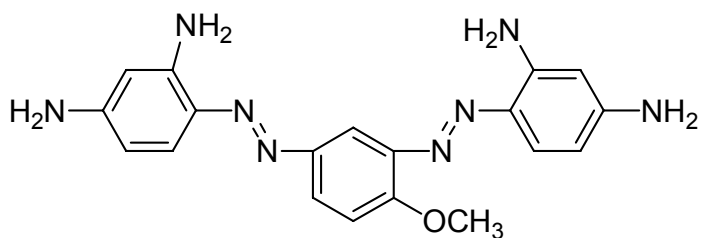
The synthesis of these dyes requires at least three coupling reactions. These dyes have dull colors and include olive green, navy blue, and black colors. Some examples are shown in Figure 3.



C.I. Acid Black 1



C.I. Reactive Brown 1



C.I. Basic Brown 2

Figure 2. Structures of some disazo dyes.

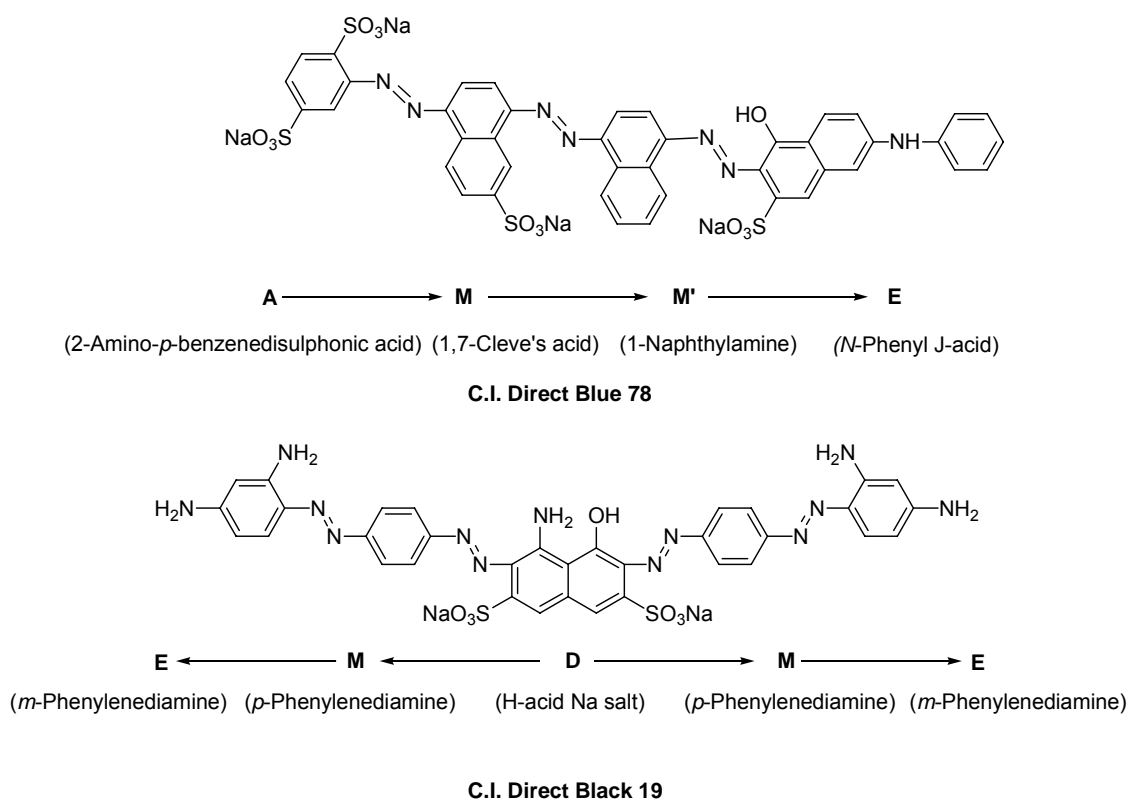
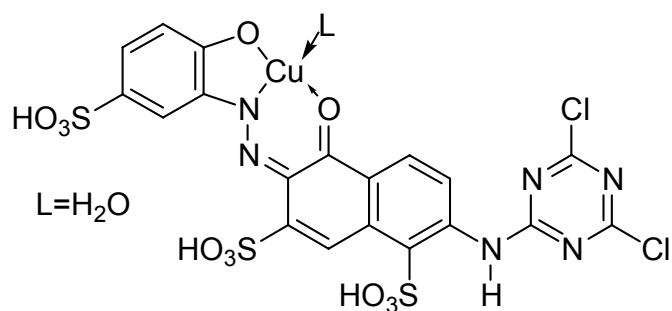


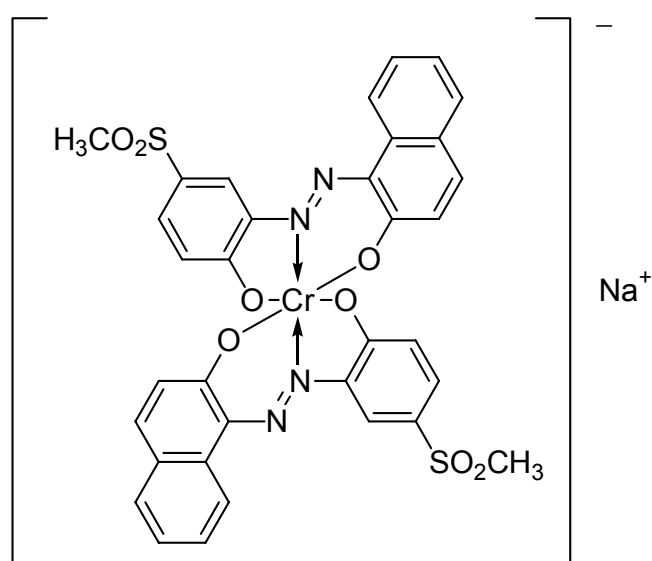
Figure 3. Structures of some trisazo and tetrakisazo dyes.

1.2.4. Metal-complexed dyes

In certain cases, the lightfastness of azo dyes can be improved by making a metal-complexed structure. Commonly used metals for preparing metal-complexed azo dyes are chromium, cobalt, and copper. Copper forms planar 1:1 complexes and polyazo copper complexes are used as direct dyes [4]. Cobalt and chromium, which are trivalent metals, combine with tridentate ligands to form 1:1 and 1:2 complexes. These dyes are usually used for protein and polyamide fiber dyeing. Some examples are shown in Figure 4.



C.I. Reactive Red 6



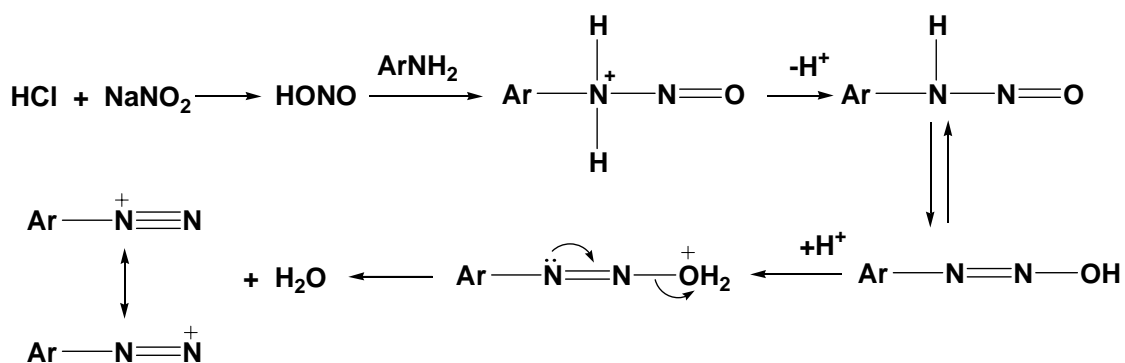
C.I. Acid Violet 78

Figure 4. Structures of some metal complexed azo dyes.

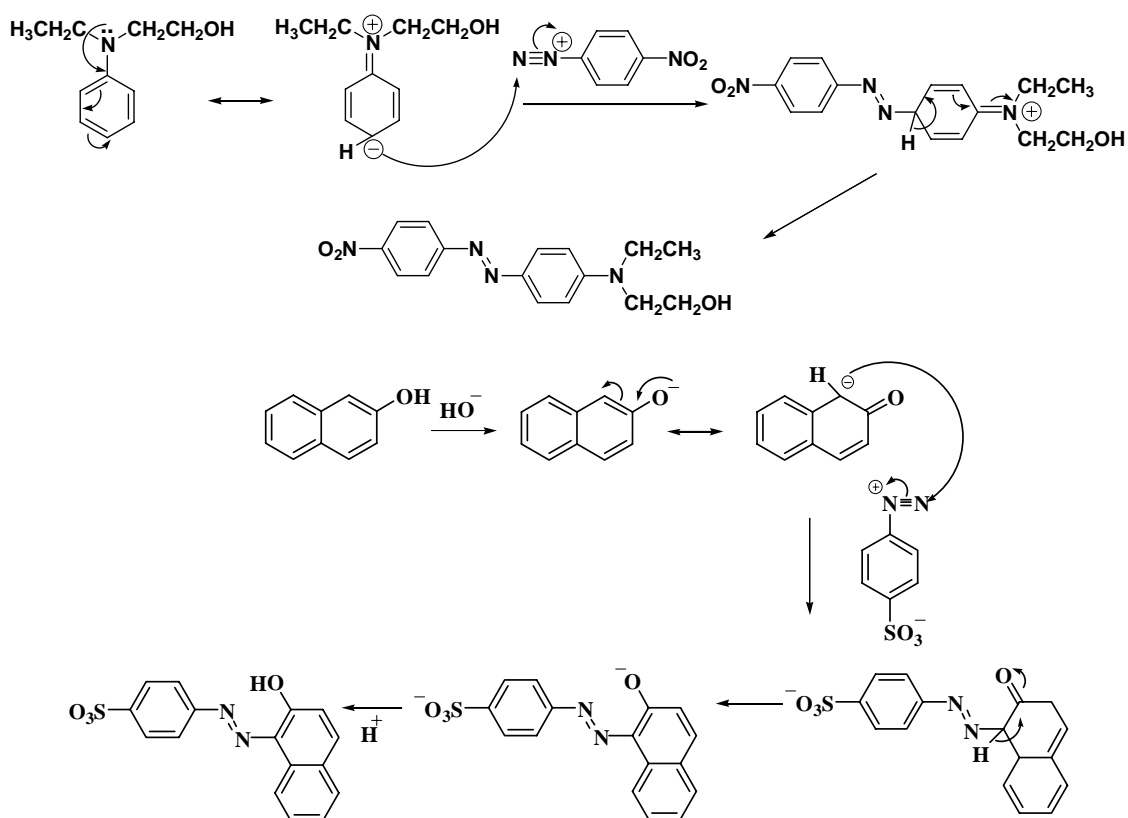
1.3. Synthesis

Generally, azo dyes are synthesized by diazotization of a primary aromatic amine followed by coupling of the resultant diazonium compound with an electron-rich ring system. The primary aromatic amine is usually diazotized with nitrous acid, which is normally generated *in situ* from hydrochloric acid and

sodium nitrite (see Scheme 1). A diazonium compound, a weak electrophile, will react only with highly electron-rich aromatic species such as phenols/naphthols and arylamines. Even phenolic compounds should be ionized for the coupling reaction to be effected. Therefore, phenols and naphthols are coupled in an alkaline medium, while aromatic amines are coupled in a slightly acidic medium. Slightly acidic pH during amine coupling can provide optimum stability to the diazonium salt without amine deactivation via protonation. Reaction schemes for diazo coupling with aromatic amines and phenols are shown in Scheme 2.



Scheme 1. Mechanism for the diazotization of an aromatic amine.



Scheme 2. Mechanism for the coupling process involving aromatic amines and phenolic compounds.

Coupling components with both amino and hydroxyl groups, such as H-acid, can be coupled twice. The first coupling is carried out in an acidic medium to form an azo bond in the ring containing the amino group. Then, the pH is raised to ionize the hydroxyl group and form an azo bond in the ring containing the hydroxyl group (see Figure 6). The availability of a number of diazo and coupling components means that an extensive number of azo dyes is possible. This enormous synthetic versatility enables azo dyes to be developed to suit

virtually any application. Syntheses of selected azo dyes are shown in Figures 5-9.

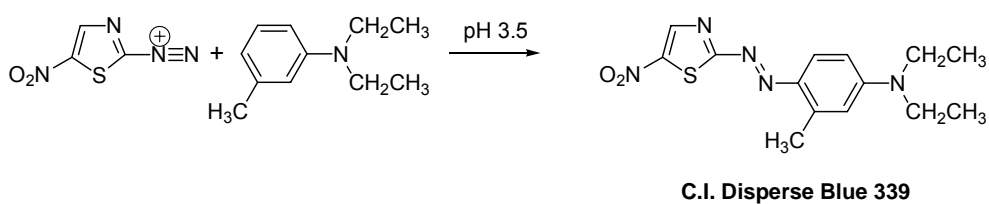
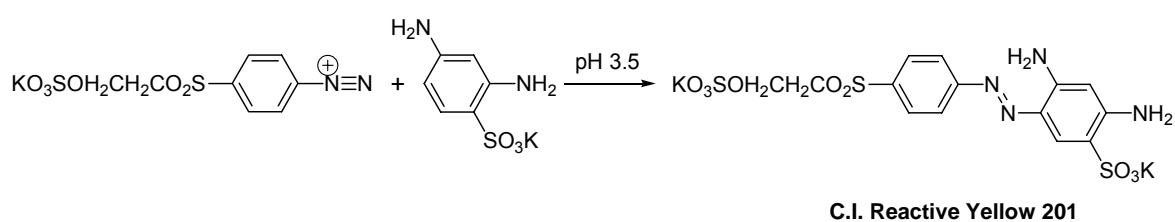
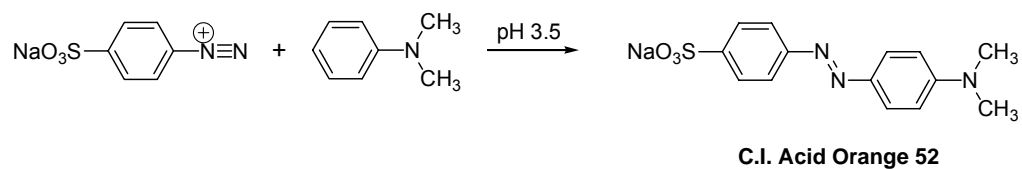


Figure 5. Examples of monoazo dye syntheses.

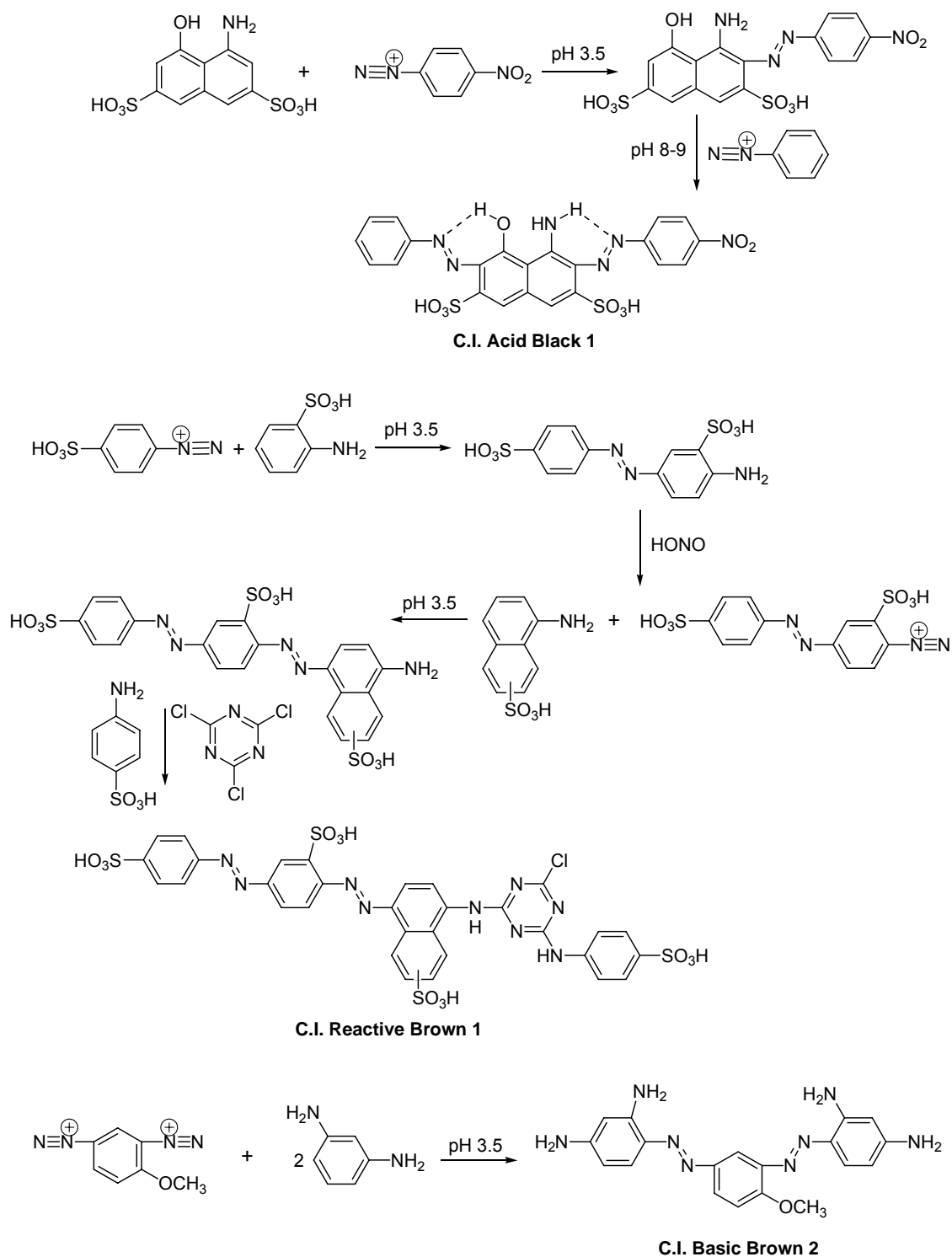


Figure 6. Examples of disazo dye syntheses.

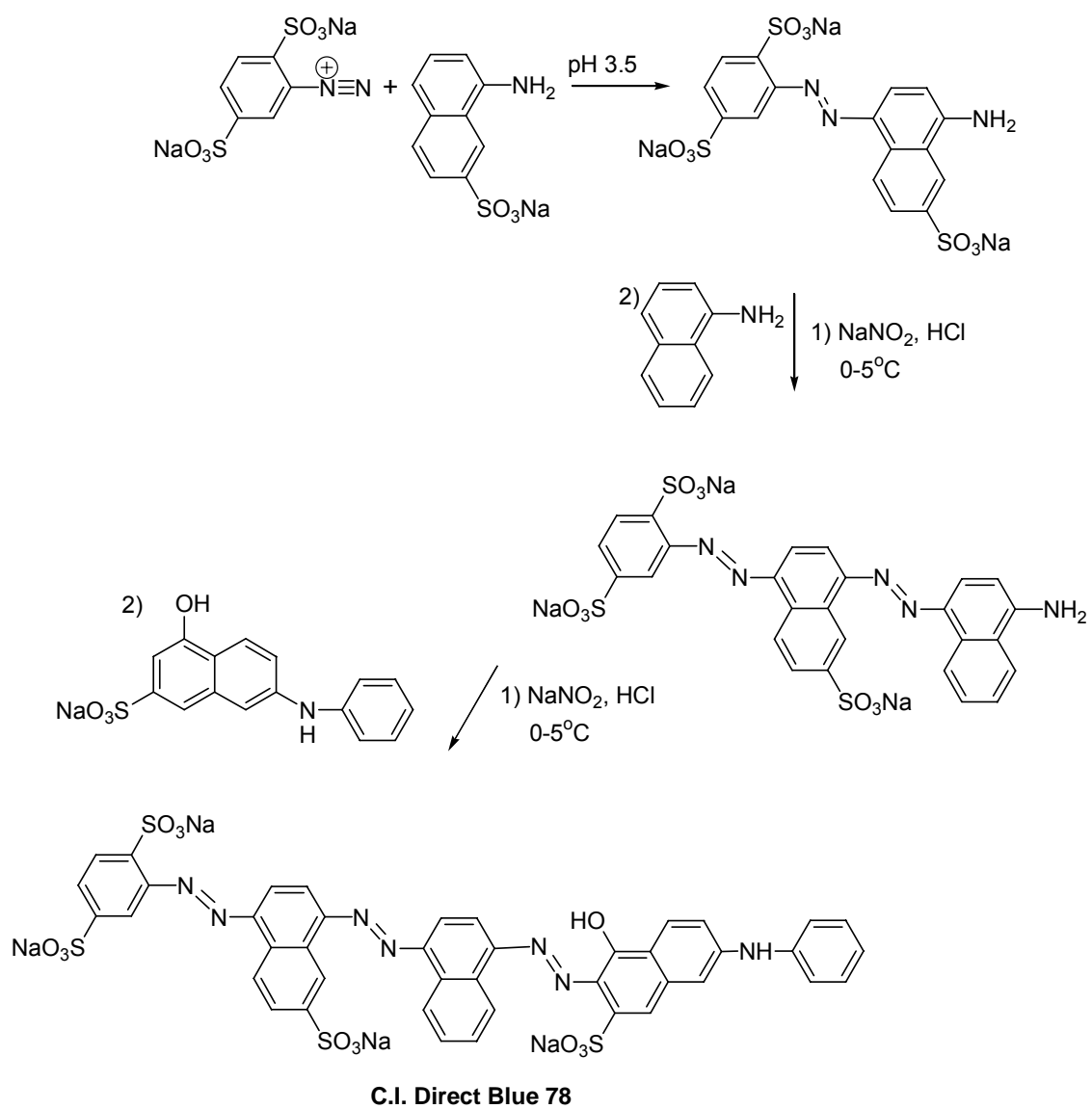


Figure 7. The synthesis of C.I. Direct Blue 78.

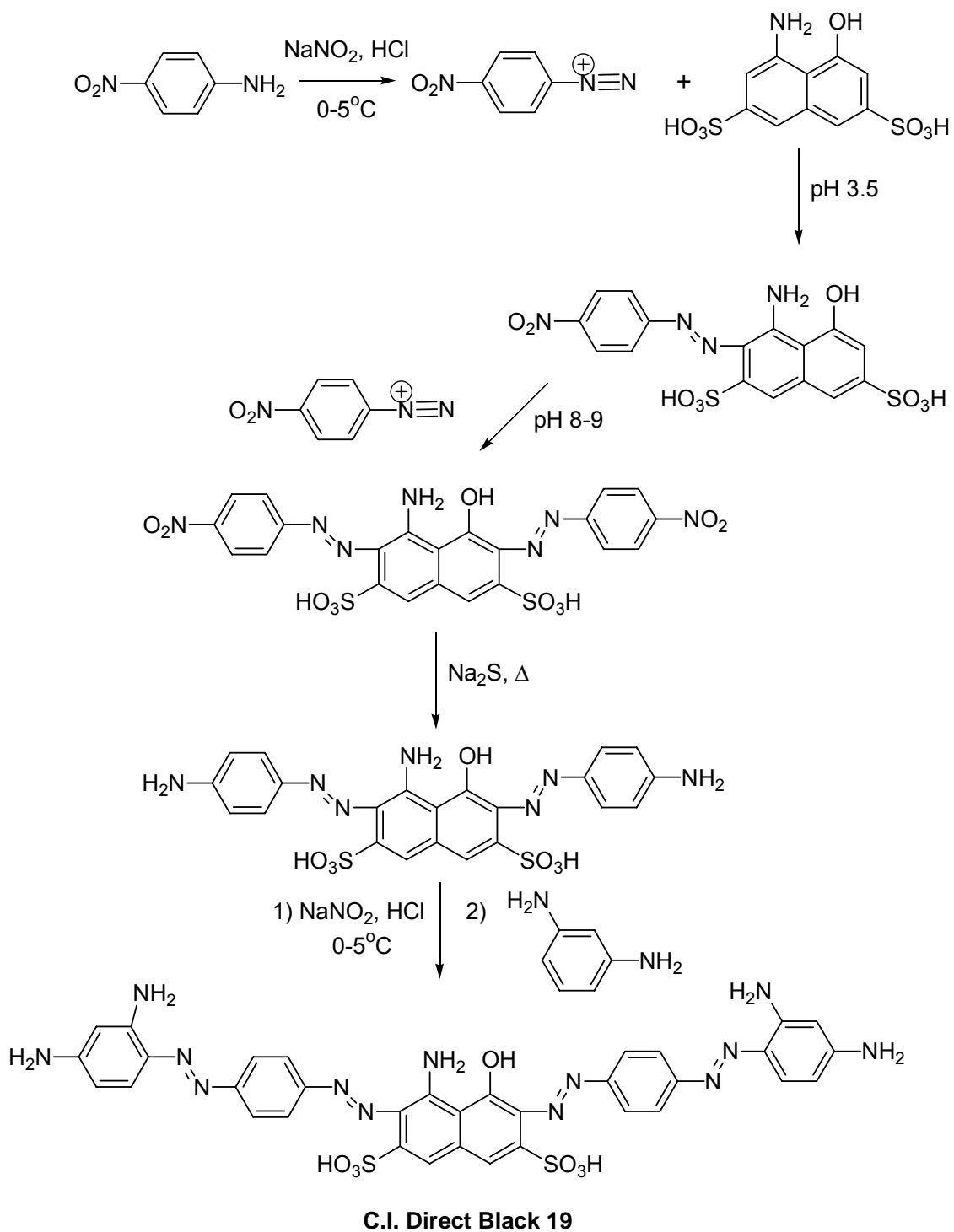


Figure 8. The synthesis of C.I. Direct Black 19.

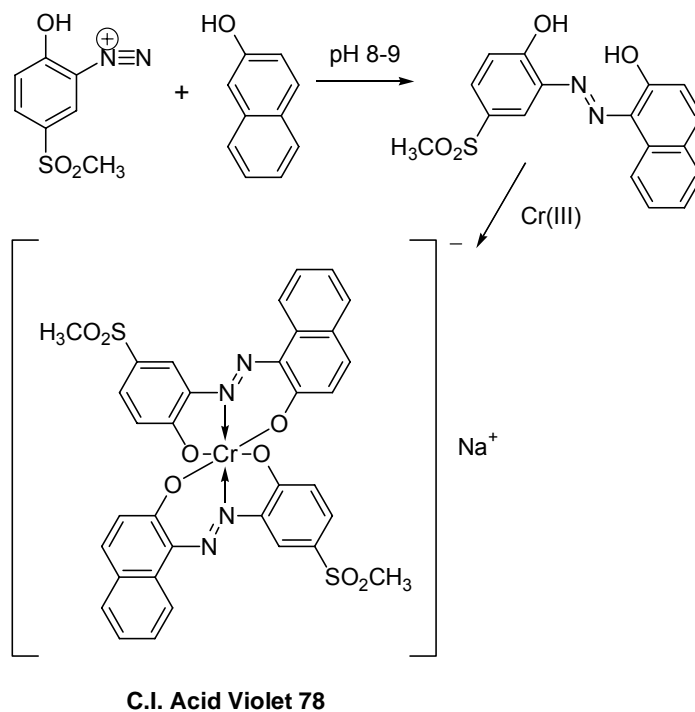
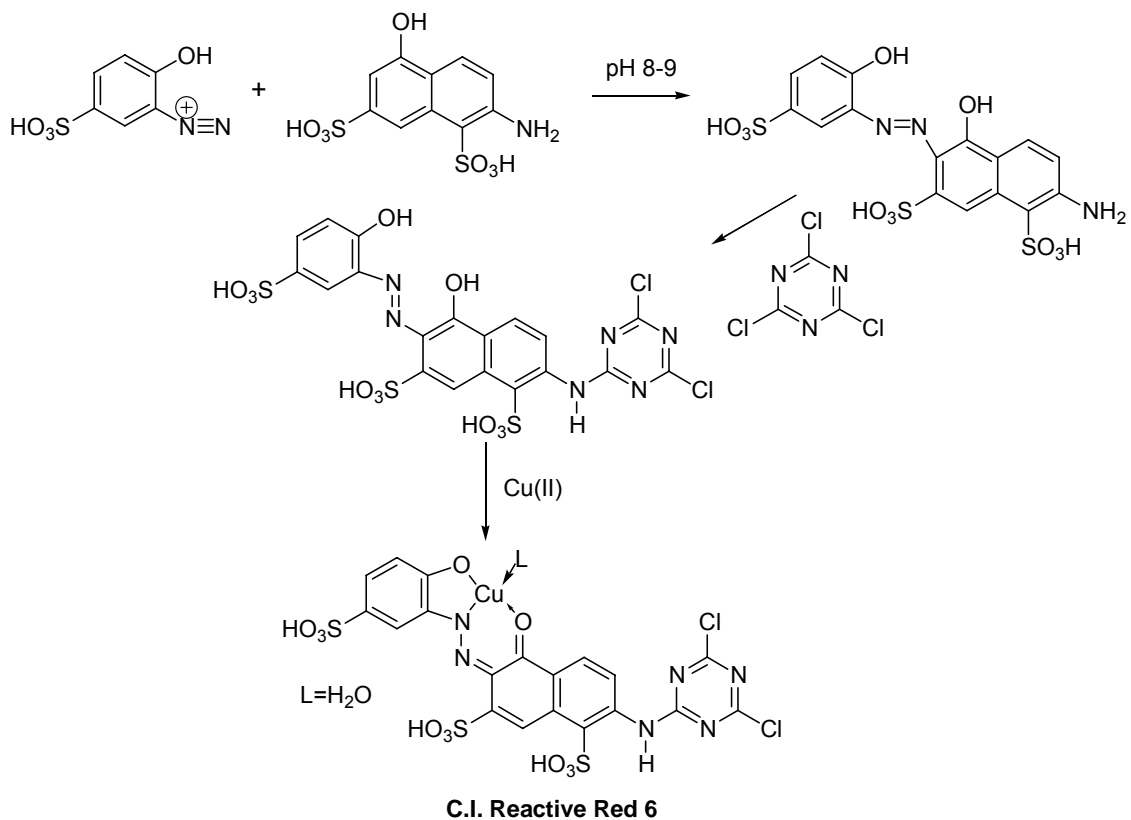


Figure 9. Examples of syntheses of metal complexed azo dyes.

1.4. Color vs. Structure in Azo Dyes

Azo dyes can cover the entire color spectrum from yellow to blue, even black, and the color of these dyes can be predicted from their chemical structures. Mostly, dye color versus constitution is affected by the presence of electron-donating and withdrawing groups. Monoazo dyes with different colors are shown in Figure 10. Normally, dyes containing two benzene rings and an azo group have yellow or orange colors. Yellow dye **1** contains weak to moderate ring activating groups (NHAc, OH, CH₃) on both benzene rings, while orange dye **2** contains a stronger electron-donating amino group on one benzene ring and a stronger electron-withdrawing nitro group in the other ring. This electron push and pull effect gives dye **2** a longer conjugated system than dye **1** and as a result, the dye absorbs at a longer wavelength (bathochromic shift) and has an orange color. A further bathochromic shift can also be seen in red dye **3** and blue dye **4**. A much stronger electron-donor (*N*-ethyl, *N*-hydroxyethyl amino group) was introduced to dye **3** to give an orange to red shift. The combination of a strong electron-donor and a very strong electron-acceptor gives blue dye **4**. A blue color can also be obtained without using a complex acceptor group, by introducing a simple thiazole group (dye **5**).

Disazo dyes with different colors are shown in Figure 11. By introducing more rings, dye colors are changed from yellow to blue because of more extended conjugation. Dyes **7** and **8** contain a naphthalene ring instead of a benzene ring and a bathochromic shift occurs. The presence of the hydrazone form of dye **8** causes a bathochromic shift versus dyes **6-7**. A blue color was

obtained for dye **9** by introducing two naphthalene rings. A violet color can be made via metal complexation, as shown earlier in Figure 9. A black dye can be obtained by increasing the number of azo groups (see Figure 12).

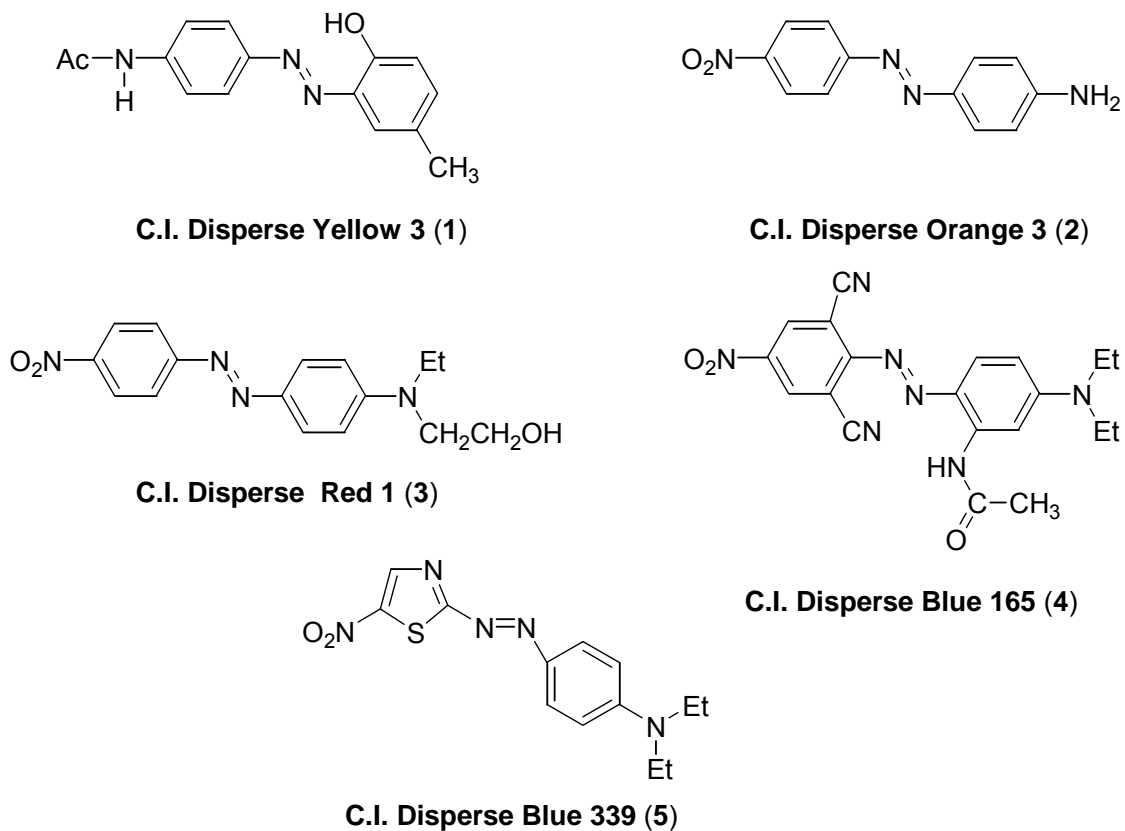


Figure 10. Examples of monoazo dyes with different colors.

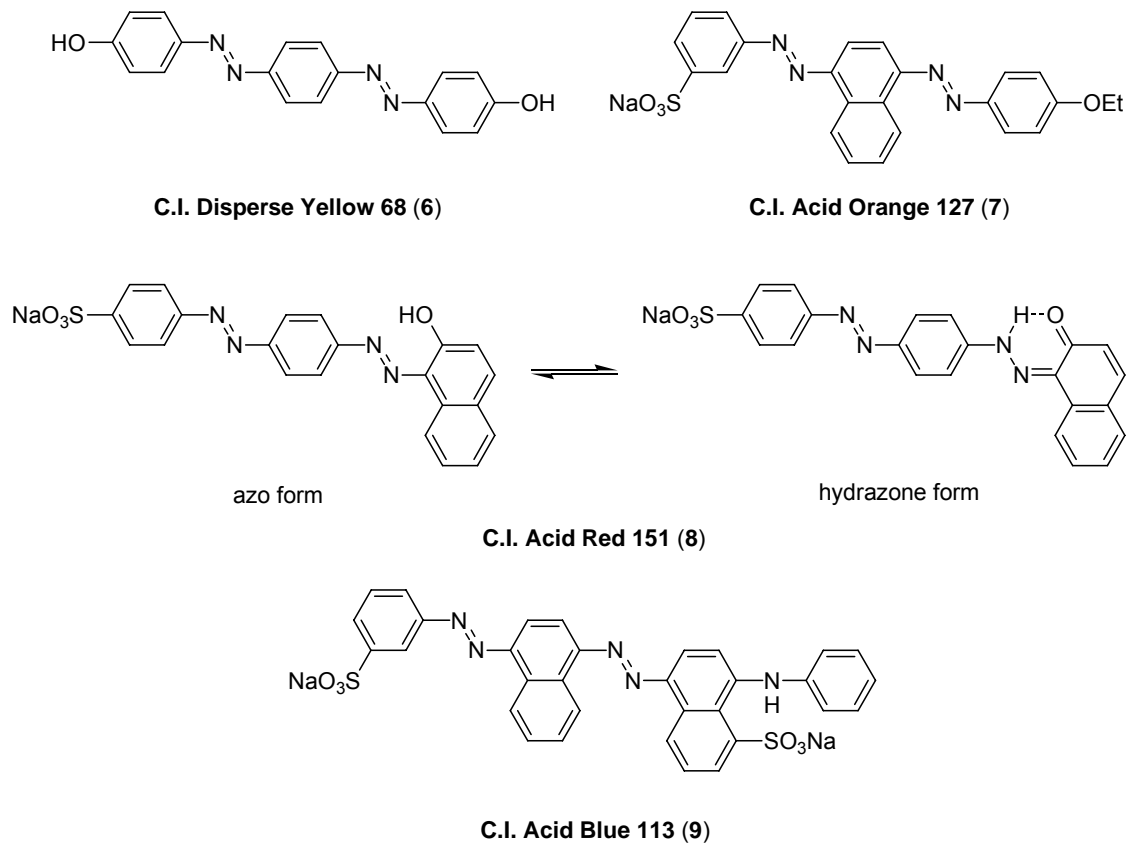


Figure 11. Examples of disazo dyes with different colors.

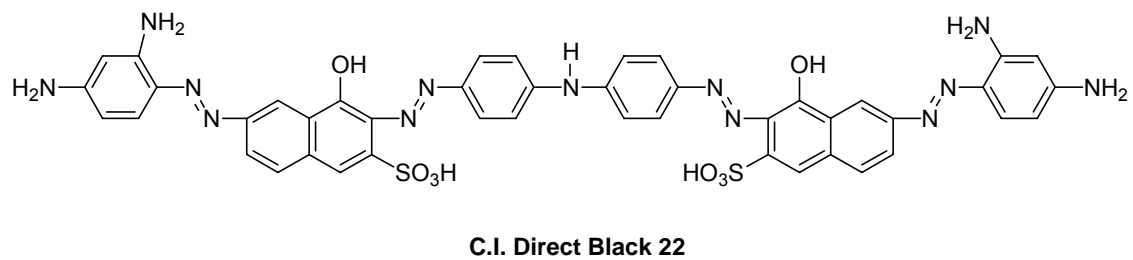


Figure 12. The example of a black polyazo dye.

2. Toxicity

2.1. Acute Toxicity vs. Chronic Toxicity and Genotoxicity

Hodgson and Levi defined acute toxicity as “adverse effects on, or mortality of, organisms following soon after a brief exposure to a chemical agent” [5]. Adverse effects that occur within two weeks of exposure are considered as acute toxicity. The exposure itself may involve one episode or more [6]. Acute toxicity can be measured by tests for dermal irritation and sensitivity, eye irritation, photoallergy, and phototoxicity [5]. However, it is normally measured by LD₅₀ or LC₅₀ tests. The LD₅₀ is the estimated dose of a toxicant that causes death to 50% of the test population. The LC₅₀ is the estimated concentration of a toxicant that causes death to 50% of the test population.

Chronic toxicity is defined as “adverse effects manifested after a long time period of uptake of small quantities of the toxicant in question” [6]. Chronic toxicity requires smaller doses than those causing acute toxicity and is significantly affected by an organism’s life span. Chronic toxicity may cause carcinogenesis, teratogenesis, and/or behavioral changes [5].

Genotoxicity, which is a type of chronic toxicity, is a general term employed by genetic toxicologists when referring to adverse interactions between DNA and various substances to produce a heritable change in the cell or organism. In humans, such changes are associated with birth defects, carcinogenesis, teratogenesis, and other type of diseases.

Carcinogenesis is “the process encompassing the conversion of normal cells to neoplastic cells and the further development of these neoplastic cells into a tumor” [5]. Carcinogenesis is characterized by three steps: initiation, promotion, and progression (see Figure 13). In initiation, the DNA of the target cell is activated by an electrophile. In promotion, the cell’s ability to become cancerous is increased by another chemical agent. In progression, a malignant (cancerous) tumor is formed. However the cell’s ability to recognize damaged DNA and repair it delays and interrupts this three-stage process. This makes chemically induced carcinogenesis still relatively rare [5].

Teratogenesis refers to the production of a damaged or deformed offspring and a reduction in fertility, i.e. fewer pregnancies or fewer successful pregnancies. Due to the species dependent nature of teratogenesis, it is hard to test for or predict. However, it is generally considered that teratogenesis involves exposure to a toxicant during the very earliest stages of embryo development [5].

Behavioral changes due to chronic toxicity may be associated with motor and glandular responses to changes in environment [6]. In addition, subchronic toxicity is due to “chronic exposure to quantities of a toxicant that do not cause any evident toxicity for a time period that is extended but is not so long as to constitute a significant part of the lifespan of the species in question” [6].

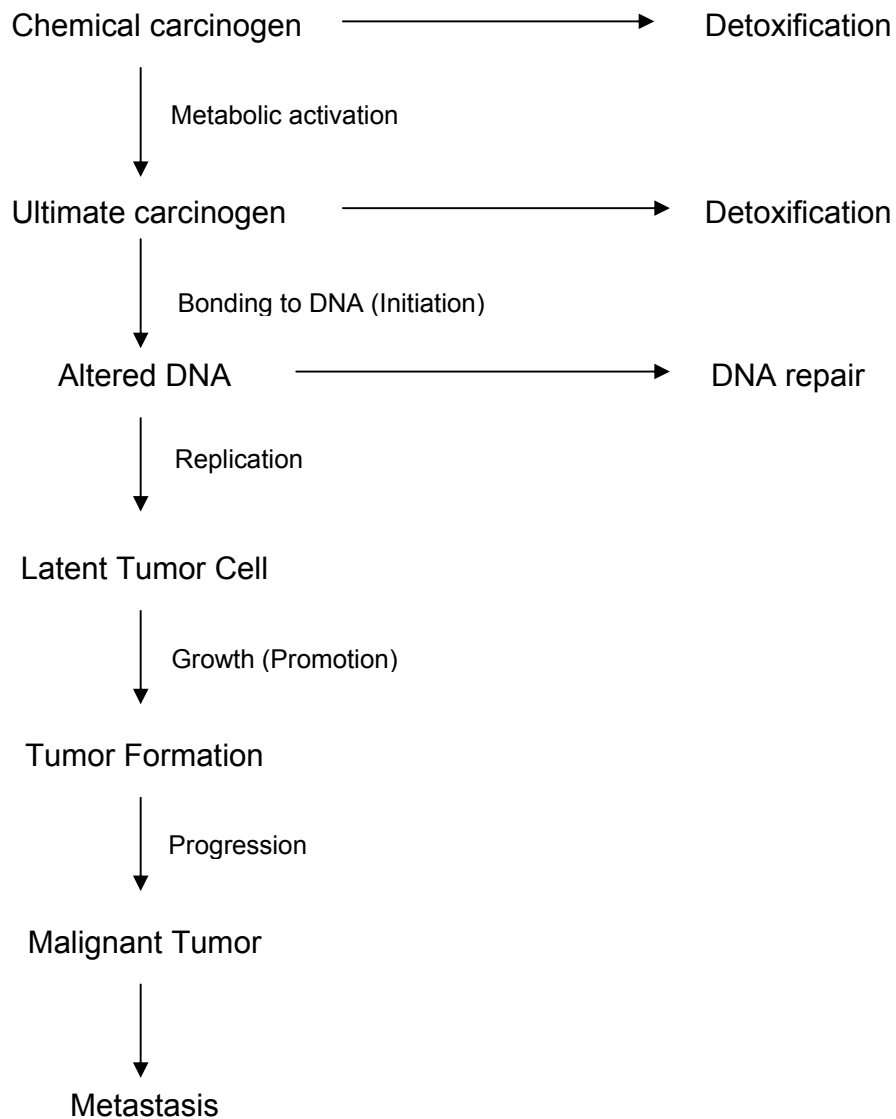


Figure 13. Sequence of events in chemical carcinogenesis [5].

2.2. Mutagenicity

Mutations are heritable changes in a cell's DNA (deoxyribonucleic acid). A chemical that can cause such changes is called a mutagen and the process involving mutations is called mutagenesis [6]. DNA is a linear macromolecule

that stores the genetic code for almost all organisms. The genetic code includes four nitrogenous bases: adenine, guanine, cytosine, and thymine. Three bases are arranged in groups which are called codons, each specifying an amino acid. During cell division, DNA replicates itself, so mutations to DNA are inheritable by offspring [7].

Mutations are usually of three general types: point mutations, chromosomal mutations, and genomic mutations. A point mutation is the change in the nucleotide sequence of one or more codons by exchange, deletion, or addition of a base. The two types of exchange point mutations are called base pair substitutions. A transversion point mutation involves replacement by a different type of base, e.g. a purine (adenine or guanine) for a pyrimidine (cytosine or thymine), while a transition point mutation involves replacement of one base by another base of the same kind, e.g., a pyrimidine for pyrimidine. Deletions or additions of bases can cause gaps or breaks in DNA, or a phenomenon known as a frameshift mutation, in which a codon, the DNA triplet code, is shifted out of sequence (see Figure 14). Chromosomal mutations are morphological alterations in the structure of the chromosome by deletions, translocations, duplications, and inversions of parts of the DNA chain and can often be seen microscopically during cell division. They result from breakage of the chromosome and an incorrect reunion. Genomic mutations cause changes in the number of chromosomes in the genome. A cell may have more or less of a certain chromosome. These mutations may cause serious conditions in humans such as Down's syndrome [7].

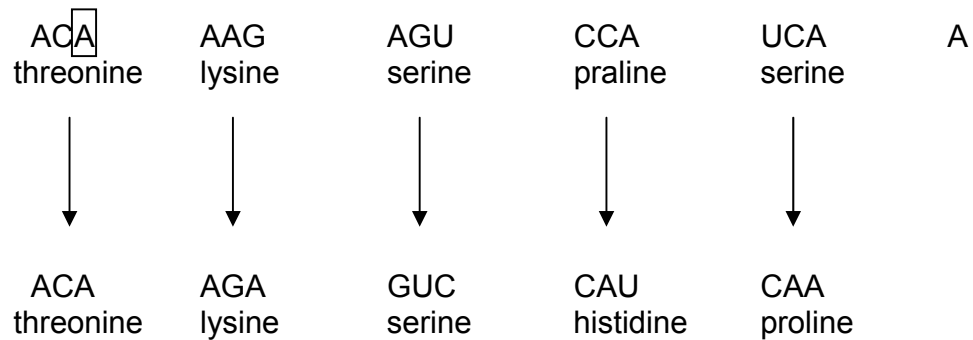


Figure 14. Frameshift mutation in which the base adenine is deleted.

Mutations to DNA can occur in many ways. DNA changes may be caused by chemical agents or when the cell attempts to repair such change. There are four major sources of DNA changes:

- (1) hydrolytic damage, i.e., reactions involving water
- (2) adduct formation, in which chemicals form covalent bonds to DNA
- (3) strand breakage
- (4) exposure to electromagnetic radiation

Hydrolytic damage and adduct formation lead to point mutations, while strand breakage often causes chromosomal aberrations. Electromagnetic radiation involves ultraviolet light, cosmic radiation, and natural background exposure to ionizing radiation [7].

Cell responses to DNA damage are varied, including death or repair processes. There are three general types of DNA repair mechanisms: (1) error-free repair, where the DNA is restored to its previous condition; (2) excision repair, where the damaged section is removed and then a new section is synthesized in its place; and (3) error-prone repair, where DNA replication

proceeds despite the damage. Error-prone repair is a major source of mutations, and is the basis for many short-term mutagenicity assays, because bacteria are very vulnerable to this type of repair. DNA mutation is regarded as a crucial first step in the onset of cancer [8].

DNA mutations can be induced directly by some chemicals. Direct-acting agents include epoxides, aromatic N-oxides, alkyl sulphonic esters, lactones, and many nitro-substituted compounds. Most of these compounds are electrophiles, a key point since DNA has many nucleophilic centers that can react with them. Some chemicals can be converted to electrophiles by metabolic activation. These indirect-acting chemicals include polycyclic aromatic hydrocarbons, aromatic amines, alkyl and acylnitrosamines, aromatic azo compounds, and aliphatic vinyl compounds [7].

2.3. Mutagenicity Test Methods

The most directly useful data are obtained via measurement of genotoxicity in whole living organisms. However, such *in vivo* experiments are time consuming, requiring up to 3 years for comprehensive testing, and are exorbitantly expensive. For example, it has been suggested that the total cost of *in vivo* testing of 33 benzidine-based azo dyes would be at least US \$100 million [61]. In addition, the *in vivo* tests also have been criticized because they only measure lethality and require large numbers of animals to perform. Moreover, they are species dependent and it is often difficult to extrapolate results to humans. Hence, testing procedures that are effective, rapid, inexpensive, and do

not require the sacrifice of large numbers of animals are needed. *In vitro* mutagenicity screening methods have been developed for these reasons. They use microorganisms (bacteria) or isolated tissues as substitutes for whole animal studies. The following are examples of tests that are usually used to provide mutagenicity test data on azo dyes.

2.3.1. The Salmonella mutagenicity assay and the Prival modification

The most commonly used test is the reverse mutation assay employing bacteria. Specially engineered bacteria are treated with the test substance to determine if a second or reverse mutation can be induced or suppressed. Testing for reversion from amino acid auxotrophy to prototrophy is the most commonly used genetic marker for this test. Prototrophs, or “wild types” are bacteria that can synthesize certain amino acids from inorganic sources, while auxotrophs are bacteria that cannot perform these syntheses. Both the standard Salmonella mutagenicity assay and Prival modification use the latter type of reverse mutation on several strains of *Salmonella typhimurium* that have mutations in the operon coding for histidine synthesis [9].

The Salmonella/mammalian microsome assay, often called the Ames test, is now the most widely used protocol as an initial screening test procedure for new experimental compounds. It was introduced in 1975 by Dr. Bruce Ames and his colleagues [10]. They observed that most mutagens were also carcinogens and the extent of a chemical’s adverse interaction with DNA is related to its carcinogenic potential due to the susceptibility of DNA to chemical mutagenesis.

The Ames test is an *in vitro* method that commonly uses one or more of five strains of *Salmonella typhimurium*. There are seven strains of *Salmonella typhimurium* that are widely used in a mutagenicity test and are designed to detect different mutations. TA98 and TA1538 are designed for frameshift mutations, TA100 and TA1535 are used to detect base pair substitution, and TA97 and TA1537 are used for base pair substitution and some frameshift mutations. TA102 detects mutagens that other strains cannot detect, such as formaldehyde [10]. These strains cannot produce the amino acid histidine, an essential component for growth. Thus, the bacteria are unable to multiply unless a suitable mutagen causes the proper type of reverse mutation in the histidine gene. Mutagenic activity can be measured quantitatively by simply counting the number of colonies present after incubating bacteria with the test compound and other necessary test additives for a standard length of time. The change in the bacteria is called a reverse mutation and the colonies that form are called revertant colonies. Generally, the compound is considered mutagenic when the number of revertant colonies is more than twice that of the base count. By the number of the revertant colonies produced, the compound is characterized as either a non mutagen, a weak mutagen or a strong mutagen. The test is repeated using various doses of the test components for each bacterial strain employed and other components are added to the test mixture to optimize the mutagenic activity of the test compound. A solvent is added to facilitate adequate mixing, and an enzyme may also be added to metabolize the test compound, since the bacteria are incapable of metabolizing compounds to

electrophiles. The enzyme is important because many compounds are mutagenic once they are metabolized in the liver and other tissues, rather than functioning as a direct mutagen. The rat liver microsomal enzyme system, S9, is used in the Salmonella microsome assay and is induced by administering a mixture of poly chlorinated biphenyls prior to sacrificing the animals to isolate the required tissues.

The Ames test is sensitive to mutagens for the following reasons:

- (1) Large populations (over 1 million) of *Salmonella typhimurium* bacteria are exposed to the test compound,
- (2) The bacteria are genetically designed to grow in the presence of a mutagen,
- (3) The bacteria have a mutation that increases cell-wall permeability to large hydrophobic molecules,
- (4) Most strains of *Salmonella typhimurium* have error-prone DNA repair mechanisms,
- (5) The mutations in the DNA are highly susceptible to attack by electrophilic compounds.

Benzidine-based dyes are known to be carcinogenic in several mammalian species, including humans. However, benzidine and certain benzidine-based dyes are not mutagenic in the Salmonella microsome test.

Prival and Mitchell modified the standard Ames test in 1982 to insure the liberation of the parent diamines and the maximum possible mutagenic activity in each of the four dyes initially studied [11]. This is a better protocol for benzidine-

based dyes, especially benzidine-based disazo dyes and is called the Prival, or preincubation, modification. There are five differences between the standard mutagenicity test and the Prival modification:

- (1) Uninduced hamster liver S9 is employed instead of induced rat liver S9,
- (2) Three times the amount of S9 used in the standard method is added,
- (3) Flavin mononucleotide (FMN) is used to facilitate reductive-cleavage,
- (4) Four times the amount of glucose 6-phosphate and exogenous glucose 6-phosphate dehydrogenase (NADH) are also added to facilitate reductive-cleavage, and,
- (5) A 30 minute pre-incubation step is employed before the addition of top agar.

All of the changes are considered necessary for optimal mutagenic activity to be observed. It is especially important that the reducing agent FMN is included in the assay, since the reduction of azo compounds can occur in mammals [12-16]. While one might argue for the use of synthesized reduction products directly in the Ames test, this is not practical since the reduction products are not stable in many cases. Hence the Prival modification, which enables reduction and metabolic activation to be carried out *in situ*, is a valuable method when testing azo compounds. Generally, the preincubation test is employed in conjunction with the Ames test. If a compound displays a weak or negative mutagenic response in the standard test with rat liver S9, the Prival method should also be conducted to lower the risk of a false negative outcome.

3. Genotoxicity of Aromatic Amines and Monoazo Dyes

3.1. Aromatic Amines

Aromatic amines are important intermediates for the synthesis of azo dyes. It was recognized that some of them are carcinogenic, when certain workers in the dye manufacturing plants developed bladder cancer as a consequence of their occupational exposure to aromatic amines of the type shown in Figure 15 [17].

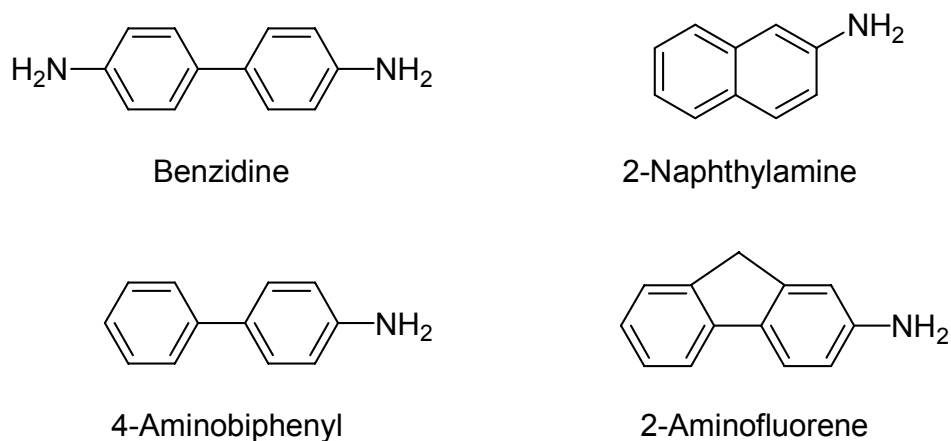


Figure 15. Carcinogenic aromatic amines.

Aromatic amines become carcinogenic by metabolic conversion to the corresponding electrophiles, which may interact with DNA to form a covalent bond. A two-stage metabolic activation is applied to aromatic amines for carcinogenicity. *N*-hydroxylation occurs first to form a hydroxylamine or an arylhydroxylamic acid, and then *O*-acylation occurs to yield acyloxy amines that

are highly reactive towards cellular nucleophiles such as DNA (Figure 16) [18, 19, 20].

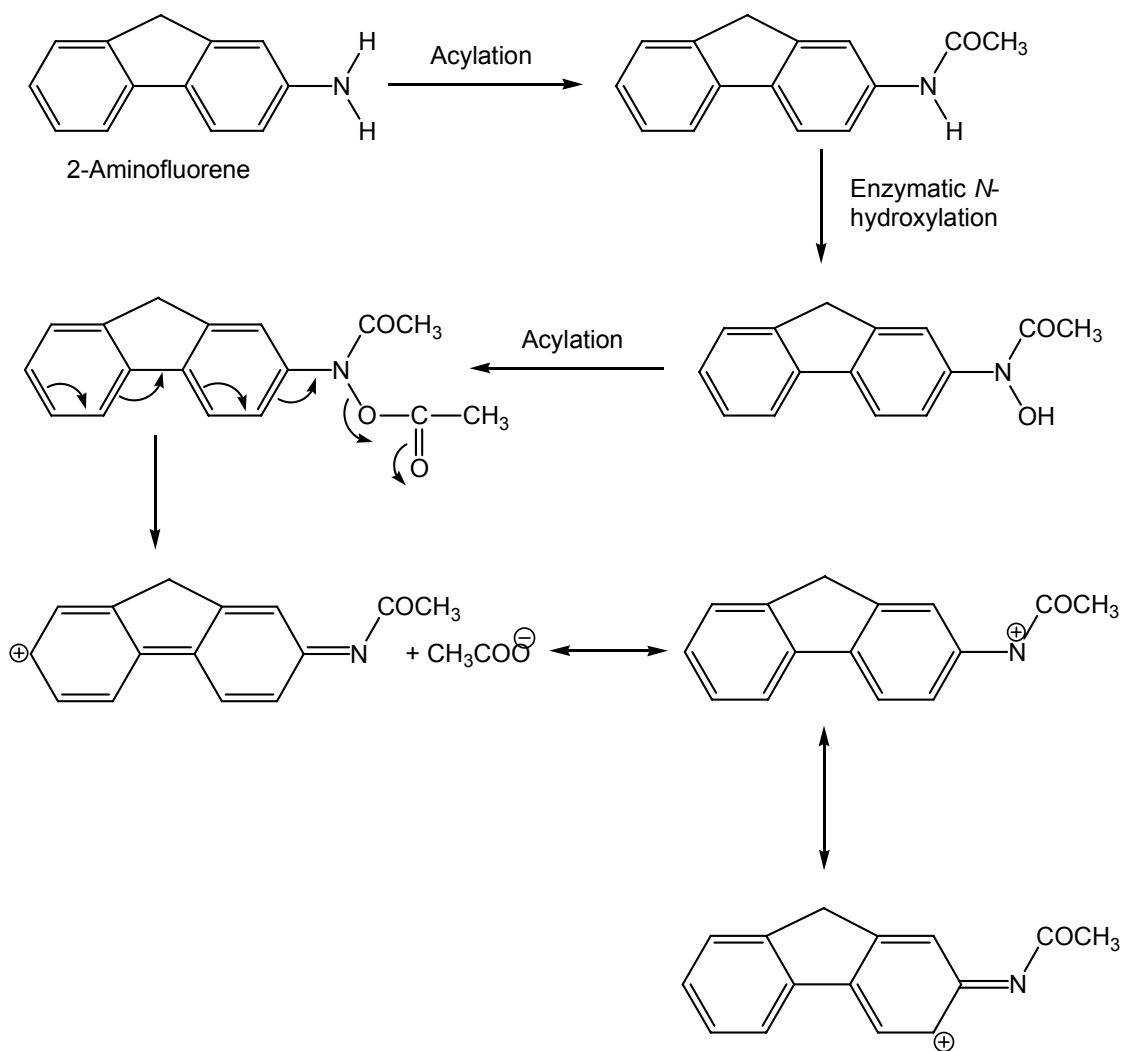


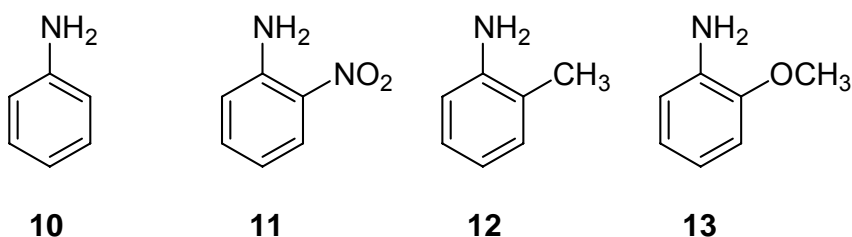
Figure 16. The metabolism of aromatic amines.

The acyloxy group will be eliminated to form the arylnitrenium ion and resonance structures that are carbocations. The arylnitrenium ion is regarded as the ultimate carcinogenic form of aromatic amines. Hence, the observed mutagenicity can be a function of the ease of elimination of the acyloxy group

and on the strength of the N–O bond. A compound will have high carcinogenic potential when the reactive electrophile is stabilized by resonance. This stabilization gives the reactive species a better chance of remaining active during transfer from the activation site to the target site on DNA [18].

3.1.1. Anilines and substituted anilines

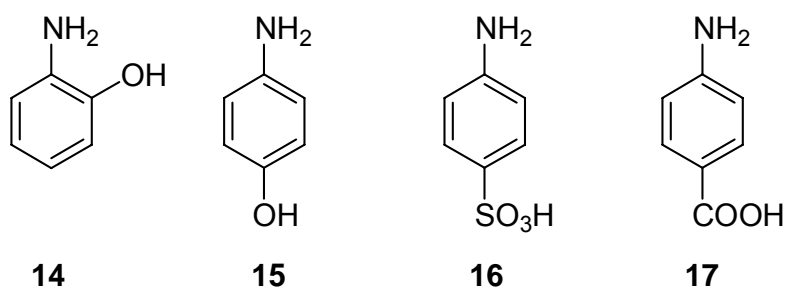
Anilines **10-13** are commonly used as intermediates in dyestuff synthesis, primarily as diazo compounds but also as coupling compounds. Aniline itself is carcinogenic in laboratory animals, even though it is nonmutagenic in the Ames test [21].



The mutagenic behavior of aniline compounds can be changed by the electronic character of ring substituent groups. Ring activating groups *ortho* or *para* to the amino group of a mono-substituted aniline enhance the stability of the corresponding arylnitrenium ions, while ring deactivating groups destabilize arylnitrenium ions and as a result lower mutagenicity. These observations can be illustrated by comparing *ortho*-nitroaniline (**11**) to *ortho*-toluidine (**12**) and *ortho*-anisidine (**13**). *ortho*-Anisidine (**13**) and *ortho*-toluidine (**12**) have ring-activating groups, while *ortho*-nitroaniline (**11**) has a deactivating group. *ortho*-Anisidine (**13**) is mutagenic in the Ames test with metabolic activation [22]. *ortho*

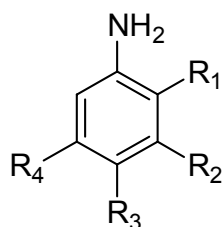
-Toluidine (**12**) is Ames negative with/without metabolic activation, but it is a potent carcinogen in rats and mice, producing several types of tumors [21, 23, 24]. *ortho*-Nitroaniline (**11**) is nonmutagenic in the Ames test [25]. The mutagenicity of *meta*- or *para*-nitroaniline may be explained by the transformation of the nitro group to an active species rather than by electronic effects.

Mutagenicity of mono-substituted anilines can also be influenced by increasing their hydrophilicity. *ortho*-Aminophenol (**14**) is weakly mutagenic in the Ames test, while *para*-aminophenol (**15**) is not. Sulfonated aniline (**16**) and *para*-aminobenzoic acid (**17**) are also nonmutagenic. The water solubility caused by the sulfonate group or carboxylate group makes these mono-substituted anilines more readily excreted from the body. It is also possible that the sulfonated aniline (**16**) is too hydrophilic to penetrate lipophilic tissue. Both factors probably contribute to the Ames negative outcome of this substituted aniline [18].



The effect of *ortho*-substitution on the enzymatic oxidation of the related anilines has been studied [20, 26]. Most notably, Zimmer et. al. [27] tested several di-substituted and tri-substituted anilines (**18-22**), and found that 2,3-,

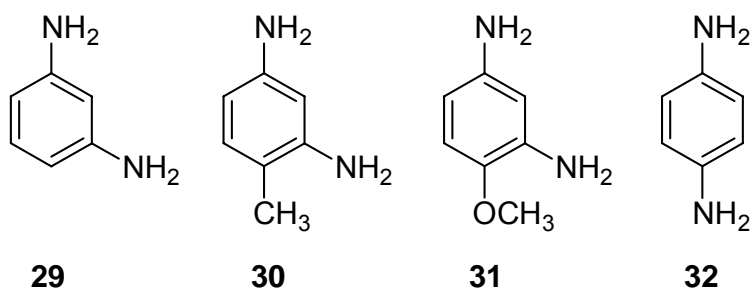
2,4-, 2,5-, and 3,4-dimethylaniline, and 2-methyl-4-fluoroaniline were weak mutagens, while 2,4,5-trimethylaniline, 2-methyl-4-chloroaniline, 2-methyl-4-bromoaniline, 4-methyl-2-chloroaniline, 4-methyl-2-bromoaniline, and 2-ethyl-4-chloroaniline (**23-29**) were strong mutagens in the standard Ames test. These results are consistent with previous reports which indicate that hydrophobic anilines are generally mutagenic when they contain methyl and/or methoxy groups in *ortho* positions.



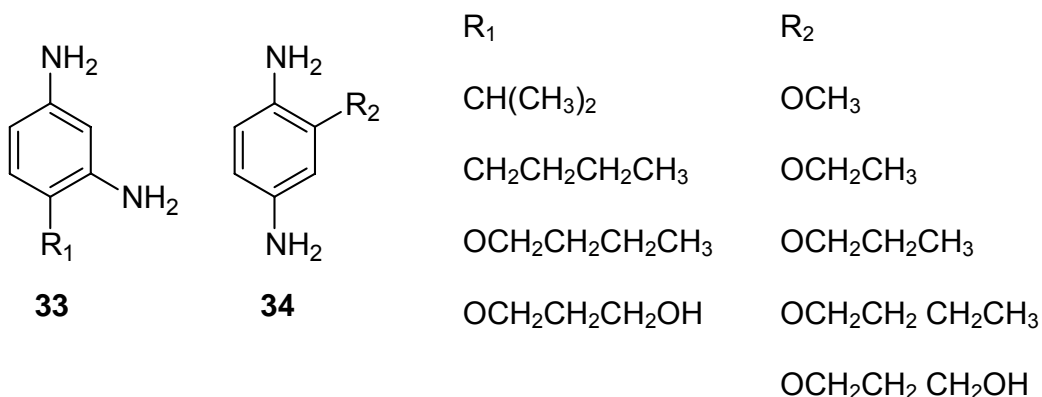
	R ₁	R ₂	R ₃	R ₄	Mutagenicity
18	CH ₃	CH ₃	H	H	Weak
19	CH ₃	H	CH ₃	H	Weak
20	CH ₃	H	H	CH ₃	Weak
21	H	CH ₃	CH ₃	H	Weak
22	CH ₃	H	F	H	Weak
23	CH ₃	H	CH ₃	CH ₃	Strong
24	CH ₃	H	Cl	H	Strong
25	CH ₃	H	Br	H	Strong
26	Cl	H	CH ₃	H	Strong
27	Br	H	CH ₃	H	Strong
28	CH ₂ CH ₃	H	Cl	H	Strong

3.1.2. Phenylenediamine derivatives

Phenylenediamines are commonly used in the formation of hair dyes [28] or as coupling components in the synthesis of azo dyes. Some of the most widely used examples are *meta*-phenylenediamine (**29**), 2,4-diaminotoluene (**30**), 2,4-diaminoanisole (**31**), and *para*-phenylenediamine (**32**), all of which are indirect-acting mutagens in the Ames test [29-32] and animal carcinogens [33, 34].



Shahin et al. [35, 36] and Freeman et al. [37, 38] reported that the mutagenicity of *meta*- and *para*-phenylenediamines can be lowered or removed by the incorporation of bulky alkyl or alkoxy groups *ortho* to one of the amino groups (cf. **33-34**). In compound **33**, mutagenicity was removed when $\text{R} = \text{CH}(\text{CH}_3)_2$, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$, $\text{OCH}_2\text{CH}_2\text{OH}$. In compound **34**, mutagenicity was lowered as the size of the substituent R_2 increased.

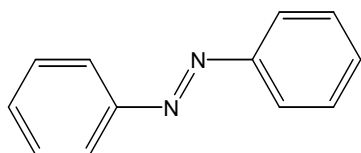


3.2. Monoazo dyes

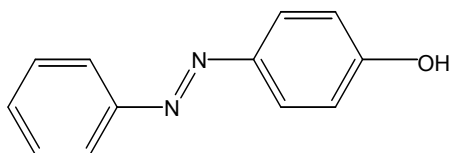
Spitz et. al. [39] suggested that azobenzene (**35**) is noncarcinogenic, but the National Cancer Institute [40] designated that it is a carcinogen. Since azobenzene is not an aromatic amine, the formation of an arylnitrenium ion would require that the azo group undergo reductive-cleavage to produce aniline.

The mutagenic behavior of azobenzene can be changed by varying substituent groups on the aromatic rings. For instance, the introduction of a hydroxyl group removes mutagenicity. C. I. Solvent Yellow 7 (**36**), C. I. Solvent Orange 1 (**37**), and C. I. Acid Orange 6 (**38**) are nonmutagenic in the Ames test [41, 42]. Removal of mutagenicity may arise from the ability of the hydroxyl groups to enhance excretion from the body through conjugation. Nitro derivatives of azobenzene are often mutagenic. C. I. Mordant Orange 1 (**39**), 4-nitroazobenzene (**40**) and 3-methoxy-4-nitrobenzene (**41**) were reported to be

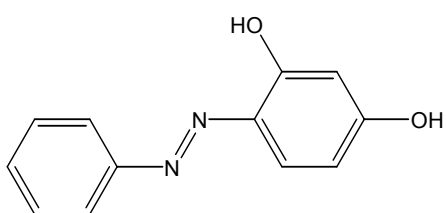
mutagenic [43, 44]. The nitro group can be reduced by enzymes to groups that cause adverse interactions with DNA.



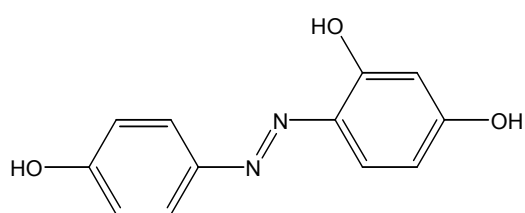
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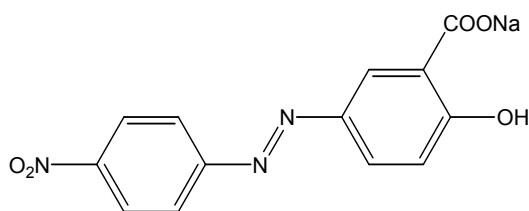
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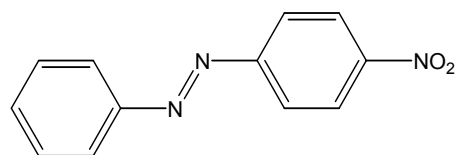
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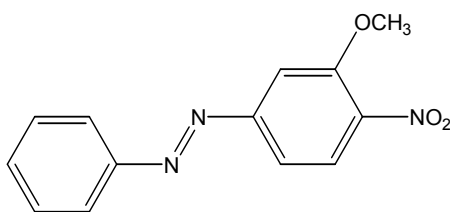
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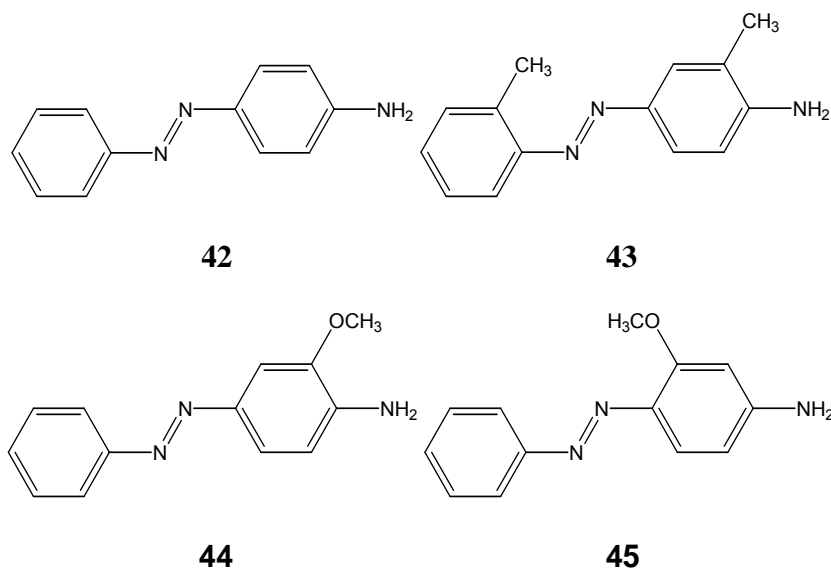
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41

Aminoazobenzene dyes have been evaluated for mutagenicity and carcinogenicity and many of them are genotoxic. 4-Aminoazobenzene (**42**) itself is mutagenic [45]. Like aniline derivatives, methyl and methoxy-substituted

aminoazobenzenes are carcinogenic and mutagenic. *ortho*-Aminoazotoluene (**43**) is a good example [46, 47]. The position of methoxy groups also affects the carcinogenicity of aminoazobenzene derivatives. Kojima et. al. [48] reported that 2-methoxy-4-aminoazobenzene (**44**) is noncarcinogenic, while 3-methoxy-4-aminoazobenzene (**45**) is a potent carcinogen in rats. It has been suggested that *ortho* substitution at the azo bond by a methoxy group stabilizes the *N*-hydroxy derivative of the dye after metabolic activation.



In a separate study, 4-aminoazobenzene (**42**) and related compounds were mutagenic or carcinogenic, while 2-aminoazobenzene was found to be nonmutagenic [49]. This can be explained by the formation of a benzotriazole group from the intermediate nitrenium ions instead of covalent bonding with DNA (Figure 17) [50].

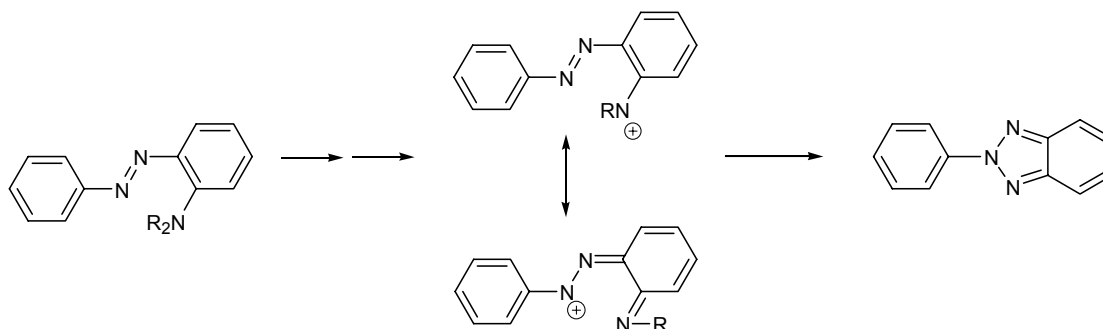
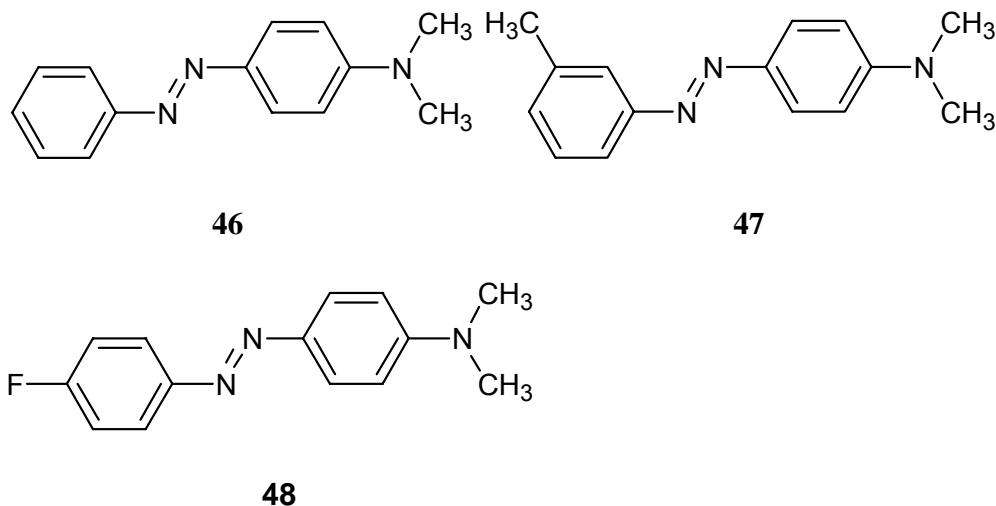


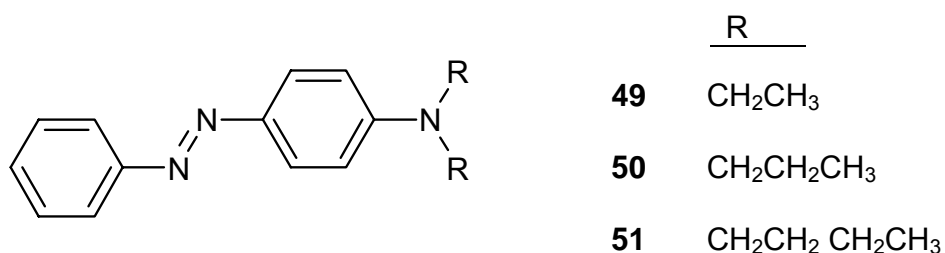
Figure 17. The formation of a benzotriazole system following metabolism (R = H, CH₃).

C.I. Solvent Yellow 2 (**46**) is a well-known carcinogenic monoazo compound [45]. 3'-Methyl-4-dimethylaminoazobenzene (**47**) and 4'-fluoro-4-dimethylaminoazobenzene (**48**) are also carcinogenic in laboratory animals.



Several reports pertain to the effects of alkyl groups in *N,N*-dialkyl-4-aminoazobenzenes. As the size of the alkyl group increases, the genotoxicity of the dye decreases [52, 53, 54]. For instance, *N,N*-diethyl-4-phenylazoaniline (C.

I. Solvent Yellow 56, **49**), *N,N*-(di-*n*-propyl)-4-phenylazoaniline (**50**) and *N,N*-(di-*n*-butyl)-4-phenylazoaniline (**51**) are noncarcinogenic. Miller [55] reported that *N,N*-dimethyl-4-aminoazobenzene derivatives in which one of the *N*-methyl groups is substituted by a hydroxyethyl or benzyl group were not carcinogenic. These results suggest that the metabolism of the amino group may be hindered by the bulky substituents.



Esancy et. al. [56] reported the effects of alkoxy substituents on the mutagenicity of some aminoazobenzenes and their reductive-cleavage products (c.f. Table 1). In this work, the mutagenicity of a series of dyes (**52-66**) was normally reduced or removed with increasing the size of the alkoxy substituent and by the substitution of hydrogen in the 4'-position with N(CH₂CH₃)₂ or N(CH₂CH₂OH)₂ group. When R₂ = H, mutagenicity of the parent dye **52** decreased, as the size of the substituent *ortho* to the primary amino group increased (**53-56**). When R₂ = N(CH₂CH₃)₂ (**57-61**), mutagenicity was ultimately removed and the lowest mutagenicity was obtained with R₁ = CH₂CH₂OH. When R₂ = N(CH₂CH₂OH)₂, mutagenicity was also reduced with increasing R₁ group. However, dye **66** was more mutagenic than other dyes in the group. This was an

unexpected outcome, as the $-\text{CH}_2\text{CH}_2\text{OH}$ group gave the lowest mutagenicity level in the other two series.

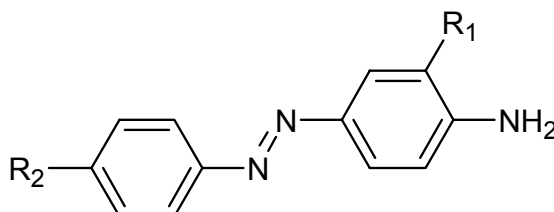


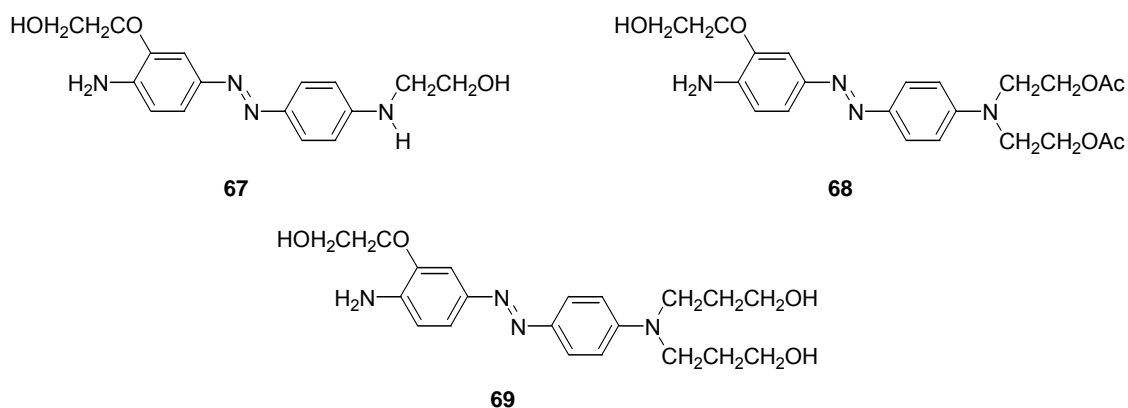
Table 1. Mutagenicity test results for some aminoazobenzenes

Dye	R ₁	R ₂	TA98 +S9	TA98 -S9	TA100 +S9	TA100 -S9
52	OCH ₃	H	77,065	NEG	38,317	NEG
53	OCH ₂ CH ₃	H	13,802	NEG	4,937	NEG
54	OCH ₂ CH ₂ CH ₃	H	18,919	NEG	6,664	NEG
55	OCH ₂ CH ₂ CH ₂ CH ₃	H	4,983	NEG	2,502	NEG
56	OCH ₂ CH ₂ OH	H	1,348	NEG	67	NEG
57	OCH ₃	N(CH ₂ CH ₃) ₂	7	NEG	?	NEG
58	OCH ₂ CH ₃	N(CH ₂ CH ₃) ₂	?	NEG	NEG	NEG
59	OCH ₂ CH ₂ CH ₃	N(CH ₂ CH ₃) ₂	NEG	NEG	NEG	NEG
60	OCH ₂ CH ₂ CH ₂ CH ₃	N(CH ₂ CH ₃) ₂	?	NEG	NEG	NEG
61	OCH ₂ CH ₂ OH	N(CH ₂ CH ₃) ₂	?	NEG	NEG	NEG
62	OCH ₃	N(CH ₂ CH ₂ OH) ₂	390	NEG	NEG	NEG
63	OCH ₂ CH ₃	N(CH ₂ CH ₂ OH) ₂	NEG	NEG	NEG	NEG
64	OCH ₂ CH ₂ CH ₃	N(CH ₂ CH ₂ OH) ₂	NEG	NEG	NEG	NEG
65	OCH ₂ CH ₂ CH ₂ CH ₃	N(CH ₂ CH ₂ OH) ₂	NEG	NEG	NEG	NEG
66	OCH ₂ CH ₂ OH	N(CH ₂ CH ₂ OH) ₂	1,052	NEG	?	NEG

4. Proposed Research

Dye **66** had the highest mutagenicity level of series **62-66**, although the incorporation of a $\text{CH}_2\text{CH}_2\text{OH}$ group gave the lowest level of mutagenicity in the other two series of dyes (**52-56** and **57-61**). These results suggested that changing R_2 from H or $\text{N}(\text{CH}_2\text{CH}_3)_2$ to $\text{N}(\text{CH}_2\text{CH}_2\text{OH})_2$ was responsible for the mutagenicity of dye **66**.

With the above-mentioned results in mind, the present thesis pertains to the synthesis of three analogs of dye **66** (cf. **67-69**) and the determination of their mutagenicity. These novel structures permit an assessment of the importance of the $\text{R}_2 = \text{N}(\text{CH}_2\text{CH}_2\text{OH})_2$ in producing a mutagenic response when $\text{R}_1 = \text{OCH}_2\text{CH}_2\text{OH}$, as this group is replaced by: 1) $\text{NHCH}_2\text{CH}_2\text{OH}$ (dye **67**), 2) $\text{N}(\text{CH}_2\text{CH}_2\text{OAc})_2$ (dye **68**), and 3) $\text{N}(\text{CH}_2\text{CH}_2\text{CH}_2\text{OH})_2$ (dye **69**).



II. Experimental

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 thick layer chromatography were Whatman 60Å silica gel plates with a 0.1 mm layer and fluorescent indicator. The plates for thin layer chromatography were Whatman PE silica gel plates with UV₂₅₄ indicator. The structures of intermediates were confirmed by ¹H NMR and the structures of the target dyes were confirmed by ¹H NMR and high resolution FAB (dye **67**) or ESI mass spectrometry (dyes **68** and **69**). ¹H NMR spectra were recorded on a Varian Mercury 400 MHz spectrometer. The standard Ames test employing TA98 with and without S9 was employed in this study. Mutagenicity testing was conducted by the EPA genetic toxicology laboratory, RTP, NC.

2. Synthesis of Dyes and Intermediates

2.1. N-(2-Hydroxy-4-nitrophenyl)acetamide (**70**)

2-Amino-5-nitrophenol (20 g, 0.129 mol) was dissolved in 100 mL of pyridine and then acetic anhydride (28.58g, 0.28 mol) was added. The mixture was stirred at 60°C for 2 h. The resulting solution was cooled to RT and poured

into 300 mL of water and the mixture was filtered to collect the product. The solid was washed with water and vigorously stirred with a solution of 5.33 g NaOH in 280 mL H₂O at 0 °C for 5 min to remove the O-acetyl moiety [57]. The red solution was filtered and the filtrate was neutralized quickly with 4N HCl (18 mL). The yellow solid was collected by filtration and dried to give 13 g (51.2%) of product having m.p. 260-264 °C. ¹H-NMR (DMSO-d₆): δ 10.98 (s, 1H), δ 9.52 (s, 1H), δ 8.26-8.29 (d, 1H), δ 7.68-7.72 (dd, 1H), δ 7.65-7.66 (d, 1H), δ 2.17 (s, 1H). TLC: R_f = 0.44 (ethyl acetate:hexane = 1:1).

2.2. N-(4-Nitro-2-hydroxyethoxyphenyl)acetamide (71)

Cain and Atwell's method [58] was used to prepare this compound. Compound **70** (11.0 g, 0.056 mol) was dissolved in 50 mL of DMF and 2-bromoethanol (8.0 g, 0.064 mol), anhydrous K₂CO₃ (8.9g, 0.064 mol), and KI (150 mg) were added. The mixture was stirred at 100 °C for 2 h and 8.0 g of 2-bromoethanol and 8.9 g of K₂CO₃ 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 and the resultant product was mixed well with dilute NH₄OH solution (5 mL conc. NH₄OH/95 mL H₂O). The crude product was collected by filtration and recrystallized from EtOH/ H₂O (3:7) to give 13g (96.4%) of yellow needles having m.p. 164-167 °C. ¹H-NMR (DMSO-d₆): 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 (ethyl acetate:hexane = 8:2).

2.3. *N*-(2-(2-Hydroxyethoxy)-4-aminophenyl)acetamide (72)

Compound **71** (5 g, 0.021 mol) was dissolved in 100 mL of EtOH and 150 mg of Pd(OH)₂/C was added. Hydrazine monohydrate (5 g, 0.105 mol) was added dropwise and the mixture was stirred under reflux for 1 h. Pd(OH)₂/C was removed by filtration and the filtrate was evaporated to dryness. The transparent oil was crystallized from EtOAc to give 3.5 g (79.3%) of nearly colorless prisms having m.p. 135-137 °C. ¹H-NMR (DMSO-d₆): 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 (acetone:hexane = 1:3).

2.4. *N,N*-bis(3-hydroxypropyl) aniline (73)

The procedure of Jones and coworkers [59] was used for the synthesis of this compound. Aniline (1.0 g, 0.011 mol), 3-chloropropanol (10.4 g, 0.110 mol), CaCO₃ (2.2 g, 0.022 mol) and 140 mL of H₂O were mixed and stirred under reflux for 50 h. The pH was adjusted to 10 with 2N NaOH on cooling, and the mixture was extracted 3 times with 75 mL of CH₂Cl₂. After drying with Na₂SO₄, the solvent was removed and the resultant brown oil was purified by silica gel column chromatography using EtOAc/Hexane (8:2). The product was crystallized from EtOAc/Hexane (8:2) to give 1.7 g (73.8%) of colorless prisms having m.p. 116-119 °C. ¹H-NMR (DMSO-d₆): δ 7.10-7.13 (m, 2H), δ 6.65-6.65 (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 (ethyl acetate:hexane = 8:2).

2.5. 4-((3-(2-Hydroxyethoxy)-4-amino)phenylazo) *N*-(2-hydroxyethyl)-aniline (67)

Compound **72** (3 g, 0.0165 mol) was stirred with 15 mL of water, as a small amount of crushed ice was added directly and an ice bath was used to cool the mixture at 0 °C. HCl (2N, 20.6 mL, 0 °C) was added and the amine was diazotized by adding NaNO₂ (1N, 16.5 mL, 0.0165 mol) 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 nitrous acid. If there was no color change, additional 1N NaNO₂ was added and the reaction was continued for 30 min and the solution was checked again with KI/starch paper. If there was a color change from white to brown, sulfamic acid was added until there was no color change. The solution was purified by adding some activated carbon with stirring, followed by filtration with cooling in an ice bath.

N-(2-Hydroxyethyl)aniline (2.5 g, 0.0165 mol) was suspended with stirring in 30 mL of water as 20 % HCl (2 mL) was added slowly at 0 °C. NaOAc (5.4 g, 0.066 mol) was added to the solution with stirring. The cold 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. The acetyl group was removed in a solution of 4.9 g NaOH, 49.2 mL 95% EtOH, 14.8 mL water by stirring the solution for 5 h at 100 °C. The reaction mixture was cooled to RT and the pH of this mixture was lowered to 7 with 20% HCl. The solvent was removed at reduced pressure and the residue was dissolved in acetone to remove salts. The brown oil produced after the

evaporation of acetone was purified by using thick layer chromatography plates and EtOAc/toluene (8:2) eluent and 50 mg of the dye in 3 mL of acetone. The product (**67**) was extracted from the silica gel mixture with acetone to give 28 mg (56%) of dye. ¹H-NMR (DMSO-d₆): 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 = 0.49 (acetone:hexane = 1:3), λ_{max} = 455nm.

2.6. 4-((3-(2-Hydroxyethoxy)-4-amino)phenylazo)*N,N*-phenyldiethanolamine diacetate (68**)**

Compound **72** (3 g, 0.0165 mol) was stirred with 15 mL of water, as a small amount of crushed ice was added directly and an ice bath was used to cool the mixture at 0 °C. HCl (2N, 20.6 mL, 0 °C) was added and the amine was diazotized by adding NaNO₂ (1N, 16.5 mL, 0.0165 mol) 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 nitrous acid. If there was no color change, additional 1N NaNO₂ was added and the reaction was continued for 30 min and the solution was checked again with KI/starch paper. If there was a color change from white to brown, sulfamic acid was added until there was no color change. The solution was purified by adding some activated carbon with stirring, followed by filtration with cooling in an ice bath.

N-phenyldiethanolamine diacetate (4.4 g, 0.0165 mol) was suspended with stirring in 30 mL of water as 20 % HCl (2 mL) was added slowly at 0 °C. NaOAc (5.4 g, 0.066 mol) was added to the solution with stirring. 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. The acetyl group was removed in a solution of 4.9 g NaOH, 49.2 mL 95% EtOH, 14.8 mL water by stirring the solution for 5 h at 100 °C. The reaction mixture was cooled to RT and the pH of this mixture was lowered to 7 with 20% HCl. The solvent was removed at reduced pressure and the residue was dissolved in acetone to remove salts. The brown oil produced after the evaporation of acetone was purified by using thick layer chromatography plates and acetone/hexane (1:3) eluent and 50 mg of the dye in 3 mL of acetone. The product (**68**) was extracted from the silica gel mixture with acetone to give 2.5 mg (5%) dye. ¹H-NMR (DMSO-d₆): 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 = 0.63 (acetone:hexane = 1:3), λ_{max} = 454nm.

2.7. 4-((3-(2-Hydroxyethoxy)-4-amino)phenylazo) *N,N*-bis(3-hydroxypropyl)-aniline (69)

Compound **72** (3 g, 0.0165 mol) was stirred with 15 mL of water, as a small amount of crushed ice was added directly and an ice bath was used to cool the mixture at 0 °C. HCl (2N, 20.6 mL, 0 °C) was added and the amine was diazotized by adding NaNO₂ (1N, 16.5 mL, 0.0165 mol) 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 nitrous acid. If there was no color change, additional

1N NaNO₂ was added and the reaction was continued for 30 min and the solution was checked again with KI/starch paper. If there was a color change from white to brown, sulfamic acid was added until there was no color change. The solution was purified by adding some activated carbon with stirring, followed by filtration with cooling in an ice bath.

Compound **73** (3.45 g, 0.0165 mol) was suspended with stirring in 30 mL of water as 20 % HCl (2 mL) was added slowly at 0 °C. NaOAc (5.4 g, 0.066 mol) was added to the solution with stirring. The cold 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. The acetyl group was removed in a solution of 4.9 g NaOH, 49.2 mL 95% EtOH, 14.8 mL water by stirring the solution for 5 h at 100 °C. The reaction mixture was cooled to RT and the pH of this mixture was lowered to 7 with 20% HCl. The solvent was removed at reduced pressure and the residue was dissolved in acetone to remove salts. The brown oil produced after the evaporation of acetone was purified by using thick layer chromatography plates and acetone/hexane (1:3) eluent and 50 mg of the dye in 3 mL of acetone. The product (**69**) was extracted from the silica gel mixture with acetone to give 30 mg (60%) dye. ¹H-NMR (DMSO-d₆): 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 (acetone:hexane = 1:3), λ_{max} = 463nm.

III. Results and Discussion

1. Synthesis

The reaction scheme used for the synthesis of dyes **67-69** is shown in Figure 18. The chemistry is based on that of a previous study [60]. Compound **70** was obtained in two steps from **74**, and was acetylated by treating with excess acetic anhydride in pyridine to give the diacetylated derivative. Then, the O-acetyl group was removed by treatment with cold 4N NaOH for 5 min. Compound **71** was prepared by using 2 molar equivalents of 2-bromoethanol in *N,N*-dimethyl formamide and KI as a catalyst. Compound **72** was reduced with hydrazine monohydrate and Pd(OH)₂/C. Diazotization of compound **72** and coupling of the resultant diazonium compound with *N*-(2-hydroxyethyl)aniline, *N,N*-bis(3-hydroxypropyl)aniline, and *N*-phenyldiethanolamine diacetate were accomplished under slightly acidic conditions, pH 4-5. To remove the acetyl groups to give final dyes, alkaline hydrolysis was employed for the dyes **67** (56% yield) and **68** (60% yield), while acidic hydrolysis was used for dye **69** (5% yield). The low isolated yield of dye **69** was due to the formation of a mixture produced by the acid-induced hydrolysis of one or both the ester groups.

All three dyes were purified by thick layer chromatography and dye structures were confirmed by ¹H NMR and high resolution FAB (dye **67**) or ESI mass spectrometry (dyes **68** and **69**).

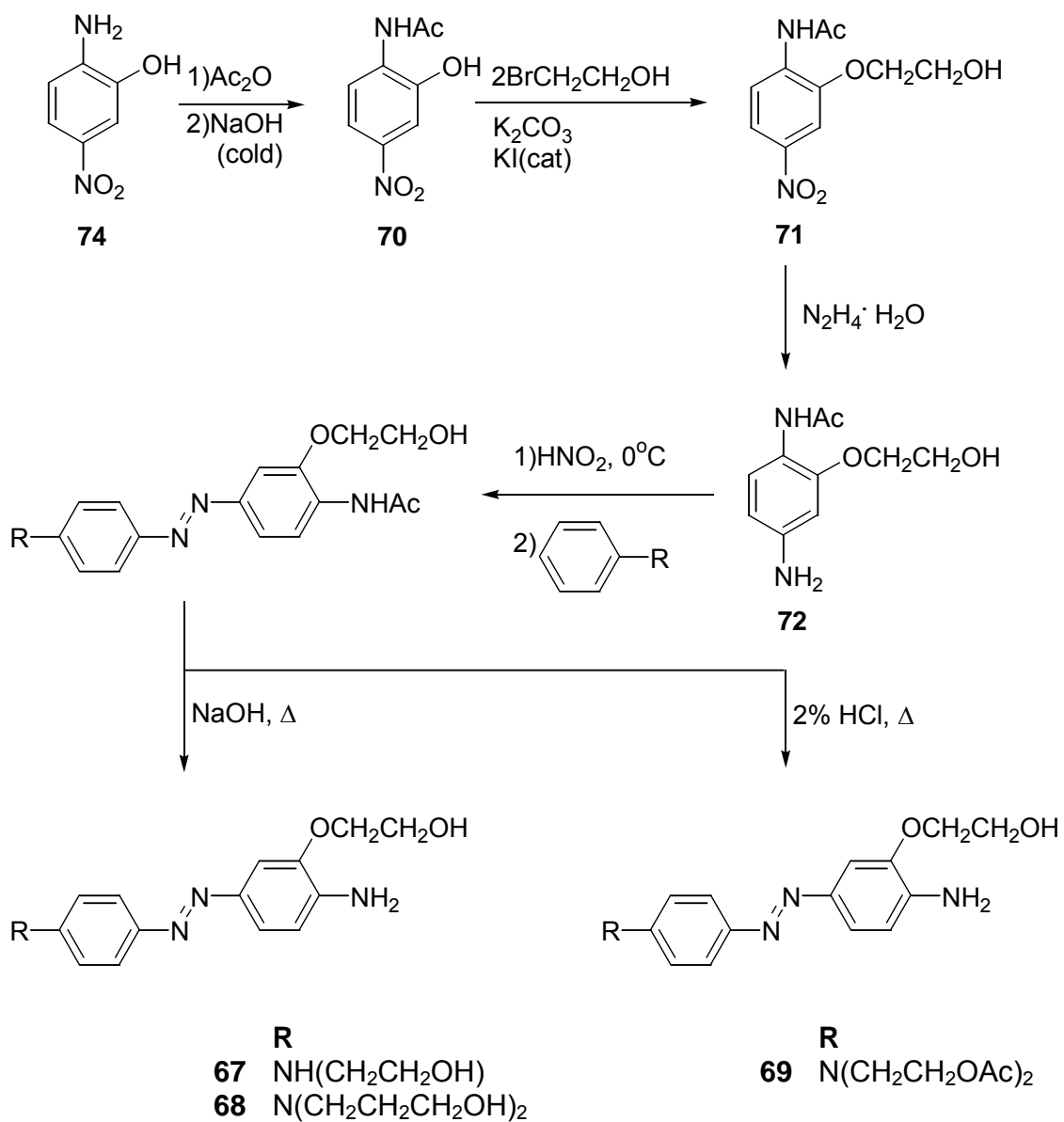


Figure 18. Reaction scheme for the synthesis of **67-72**.

2. NMR Spectra of Dyes and Dye Intermediates

NMR spectra of dyes and dye intermediates **67-73** are shown in Figures 19-25. The spectrum of **70** contains a singlet at δ 2.17 ppm (3H) arising from the methyl group of the acetyl group, demonstrating that the acetylation of amine group of **74** has occurred. The spectrum of **71** contains triplets at δ 5.14-5.18 ppm (1H) and δ 4.16-4.19 ppm (2H) and a multiplet at δ 3.77-3.82 ppm (2H) arising from the hydroxyl and methylene groups of hydroxyethoxy moiety, confirming the hydroxyethoxylation of hydroxyl group in **70**. The spectrum of **72** contains a multiplet at δ 4.91-4.95 ppm (3H) from the primary amino group and an upfield shift in the position of protons **b** and **c** (2H), which is consistent with reduction of the nitro group in **71**. The spectrum of **73** contains triplets at δ 4.51-4.53 ppm (2H) and δ 3.30-3.34 ppm (4H), a quartet at δ 3.44-3.47 ppm (4H) and a multiplet at δ 1.61-1.68 ppm (4H) arising from hydroxyl and methylene groups of hydroxypropoxy group following hydroxypropoxylation of amine group in aniline. The spectrum of dye **67** contains a doublet at δ 7.57-7.60 ppm (2H), a multiplet at δ 6.65-6.70 ppm (3H), and quartets at δ 3.55-3.60 ppm (2H) and δ 3.16-3.20 ppm (2H) arising from protons **h** and **i** and two methylene groups in the part of the dye. The spectrum of dye **68** contains doublets at δ 7.57-7.60 ppm (2H) and δ 6.87-6.90 ppm (2H) and a singlet at δ 2.01 ppm (6H) arising from protons **h** and **i** and two methyl groups in the coupler of the dye. The spectrum of dye **69** contains doublets at δ 7.62-7.64 ppm (2H) and δ 6.76-6.78 ppm (2H), a triplet at δ 4.58-4.60 ppm (2H), and a multiplet at δ 1.70-1.73 ppm (4H) and

arising from protons h and i and methylene group (k) and two hydroxyl groups (m) in *N,N*-bis(3-hydroxypropyl)aniline part (coupler) of the dye.

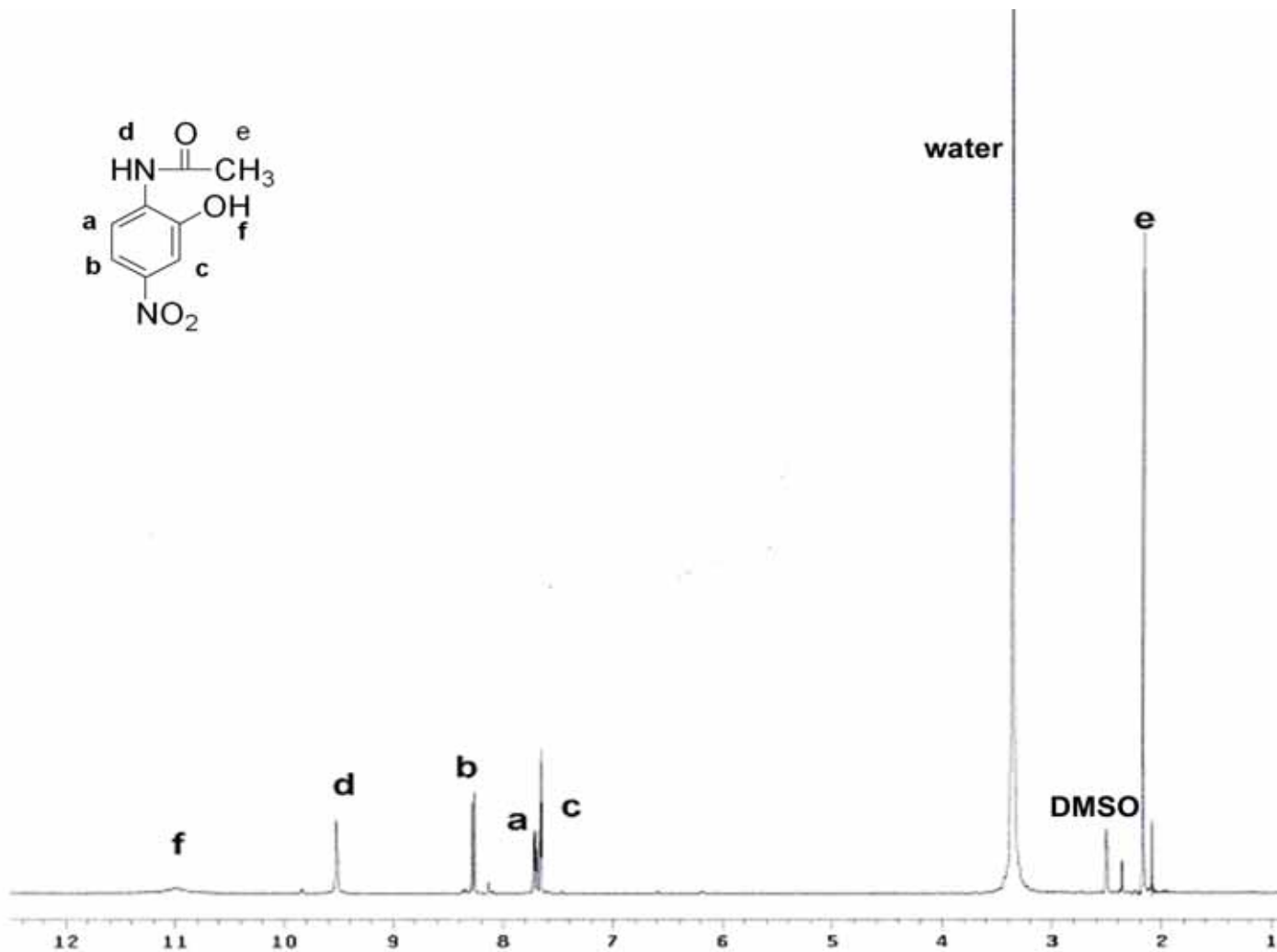


Figure 19. NMR spectrum of *N*-(2-hydroxy-4-nitrophenyl)acetamide (70).

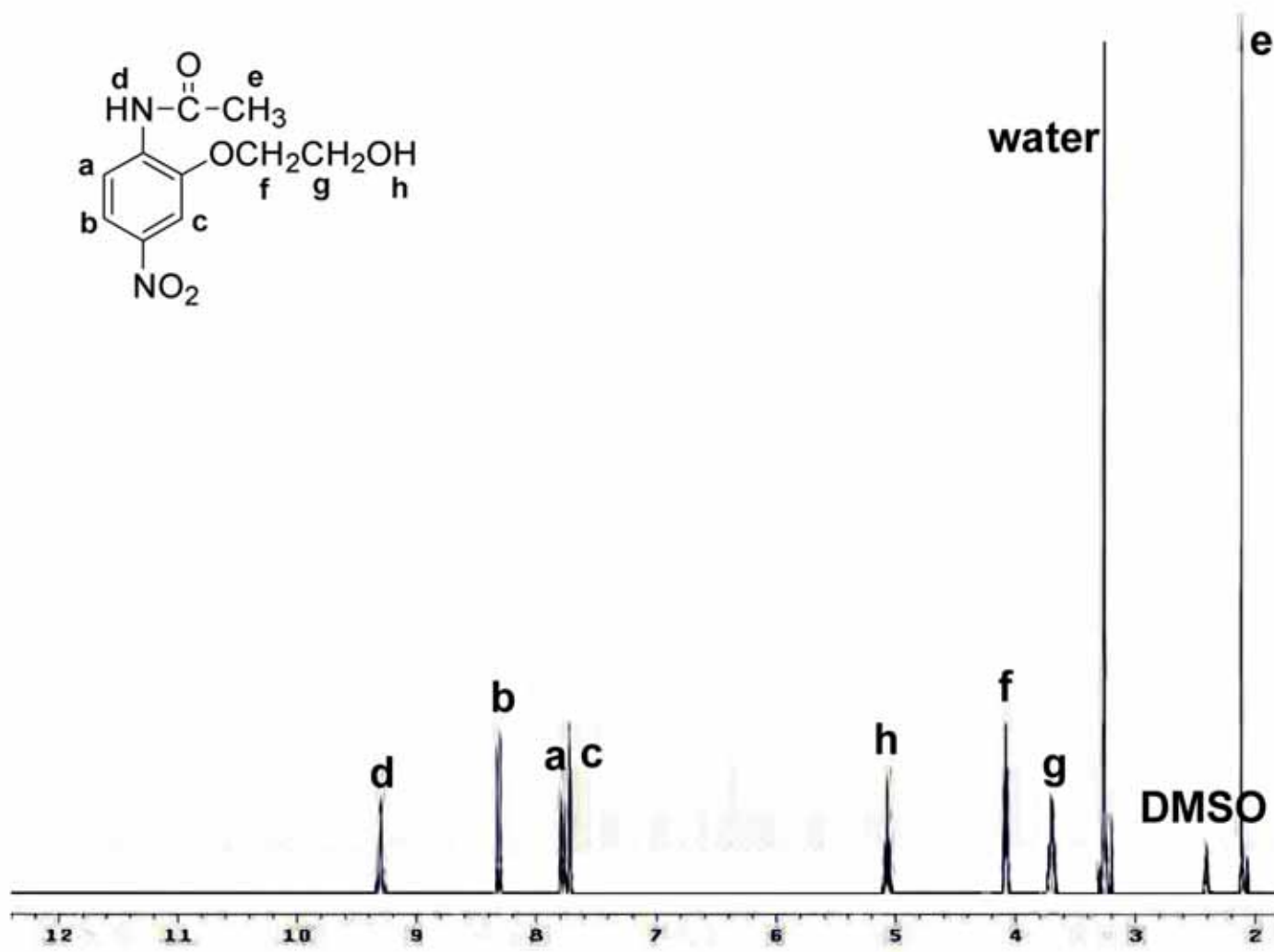


Figure 20. NMR spectrum of *N*-(4-nitro-2-hydroxyethoxyphenyl)acetamide (71).

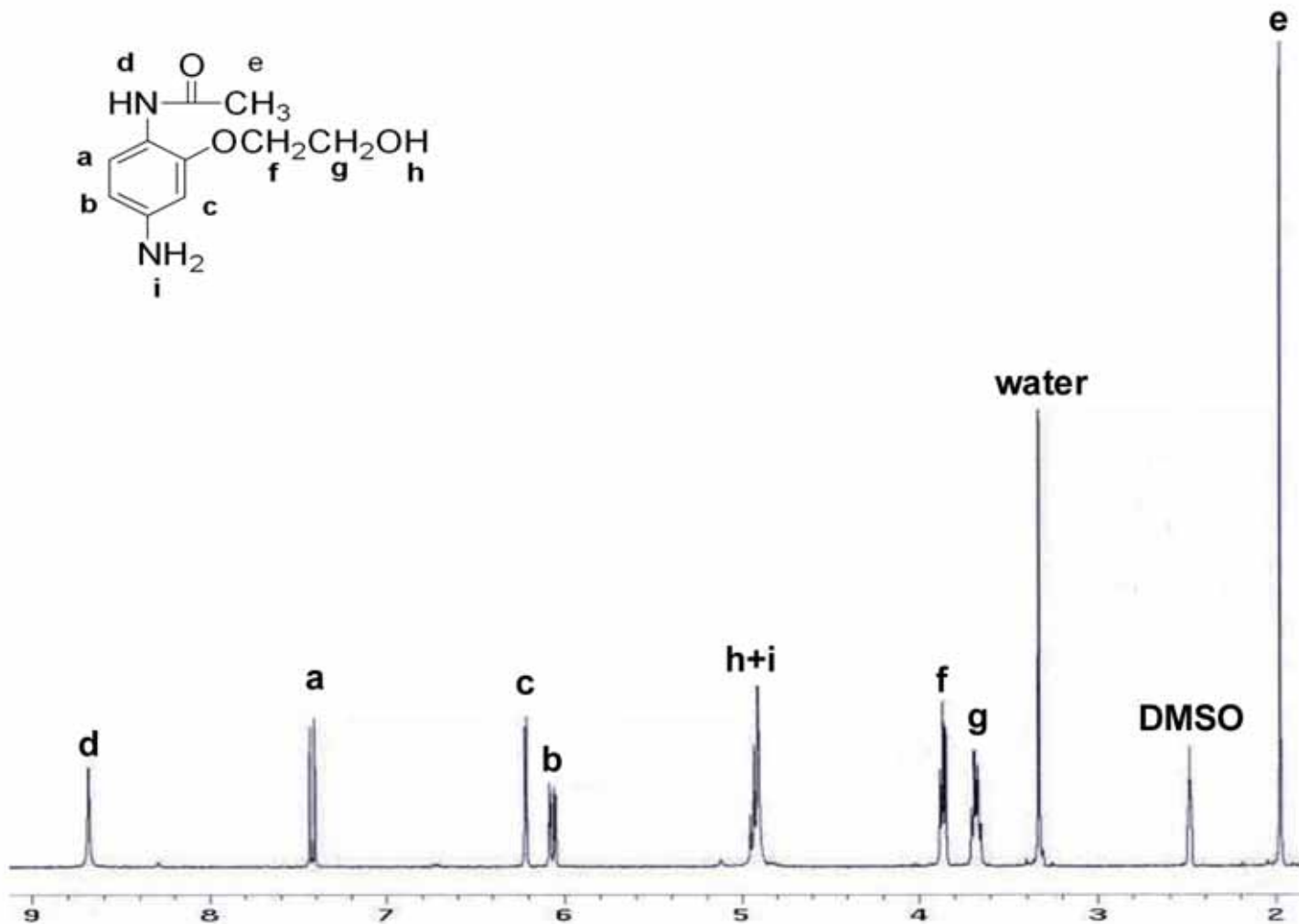


Figure 21. NMR spectrum of *N*-(2-(2-hydroxyethoxy)-4-aminophenyl)acetamide (72).

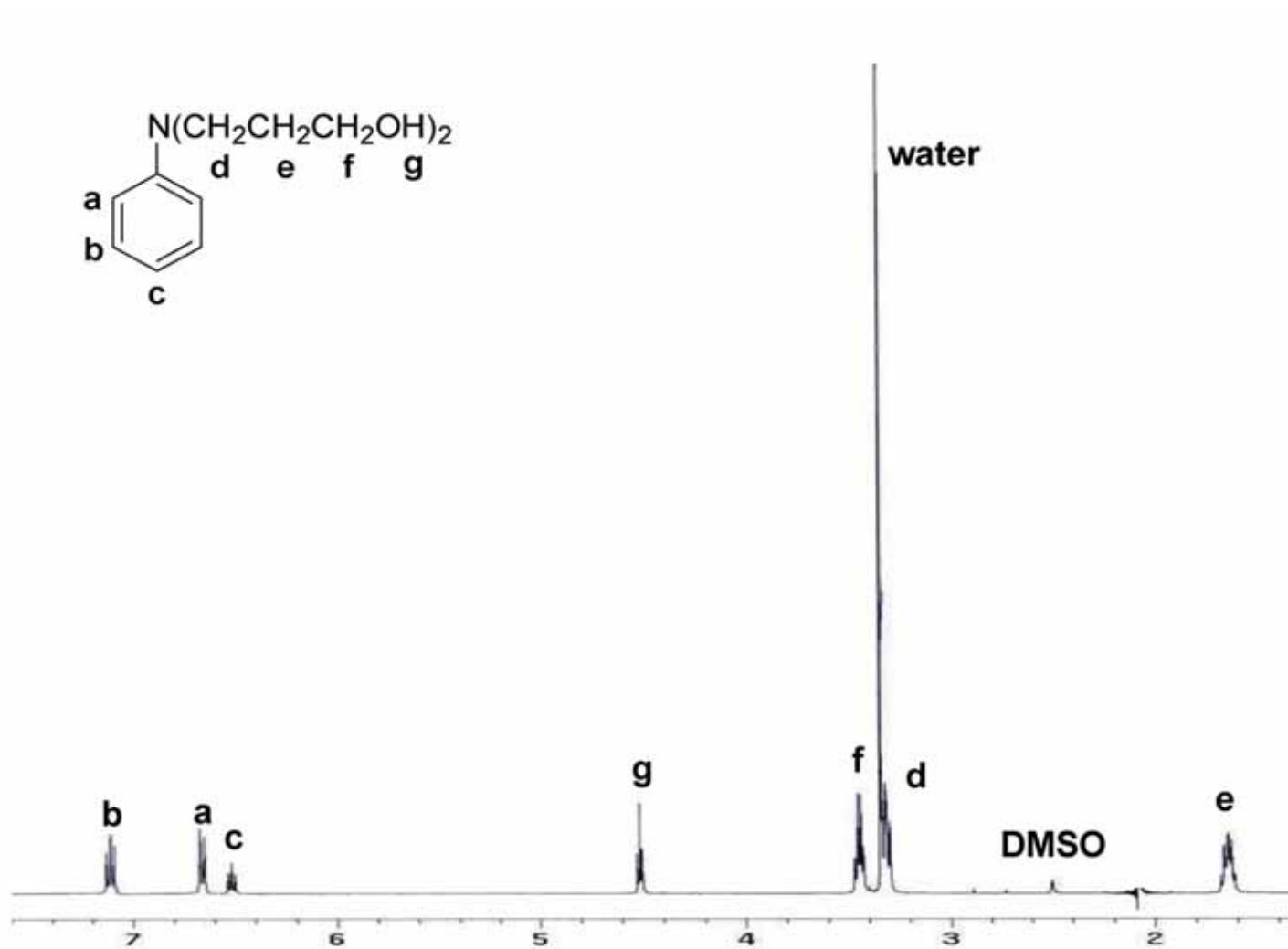


Figure 22. NMR spectrum of *N,N*-bis(3-hydroxypropyl) aniline (73).

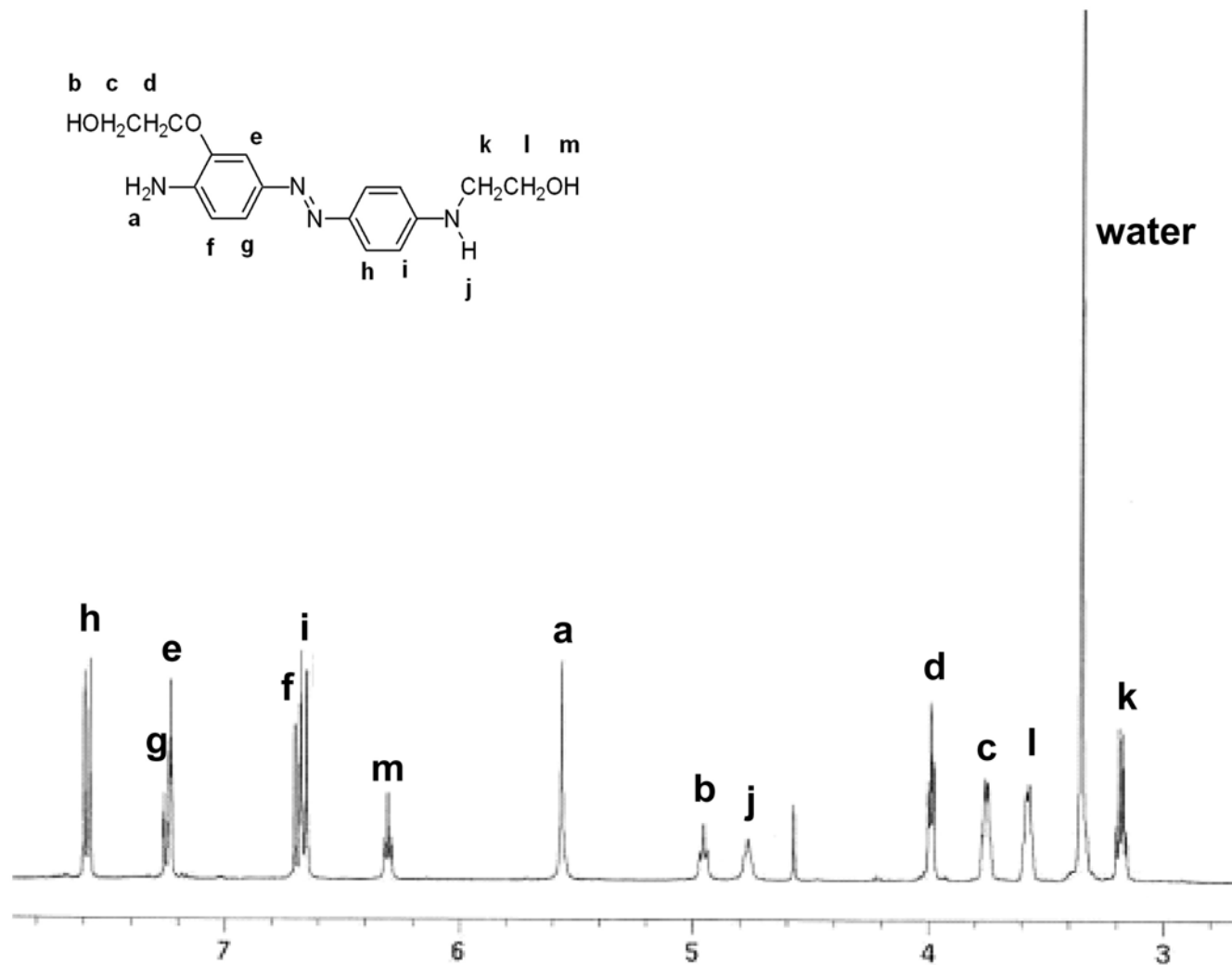


Figure 23. NMR spectrum of 4-((3-(2-hydroxyethoxy)-4-amino)phenylazo)-*N*-(2-hydroxyethyl)-aniline (**67**).

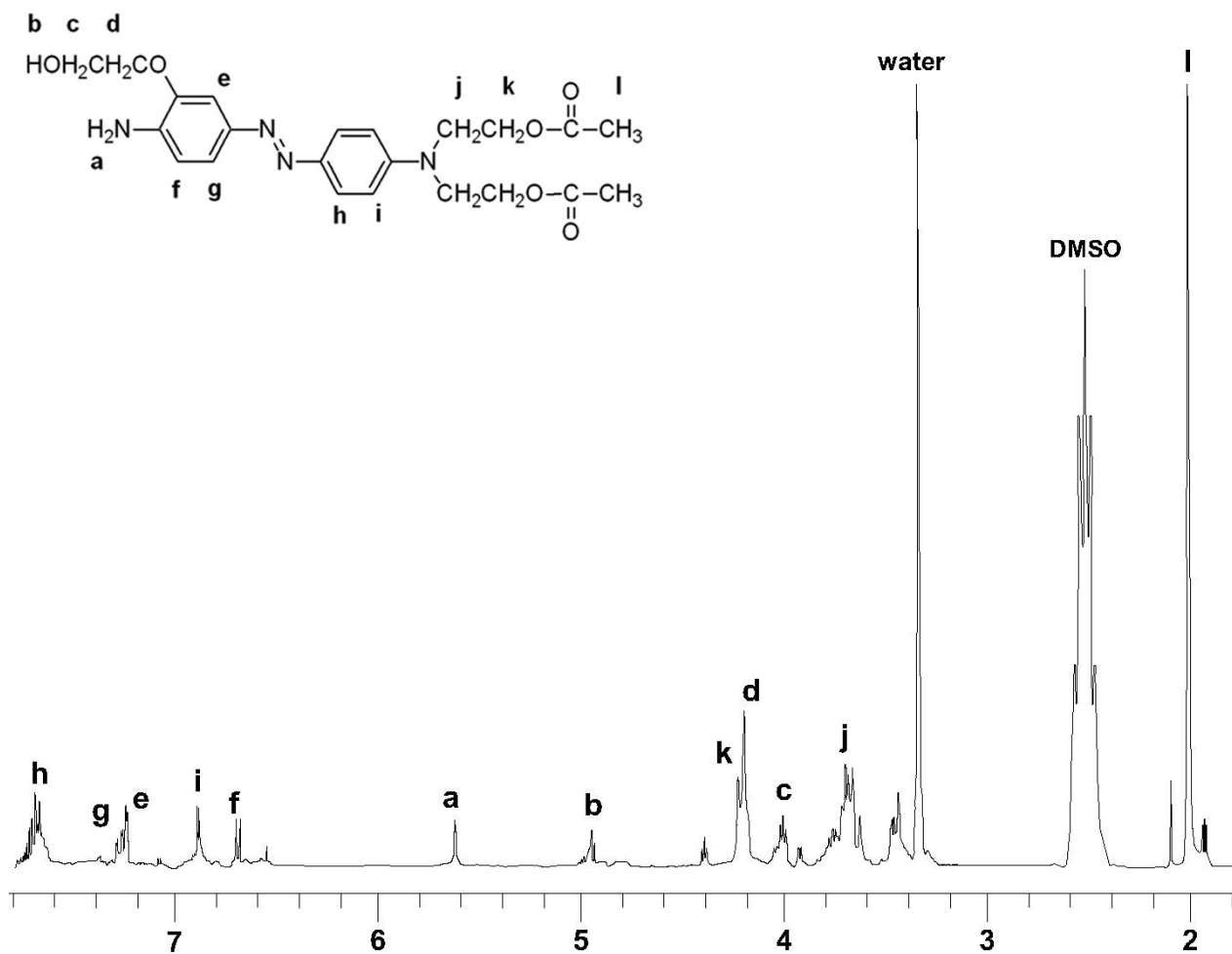


Figure 24. NMR spectrum of 4-((3-(2-hydroxyethoxy)-4-amino)phenylazo)-*N,N*-phenyldiethanolamine diacetate (**68**).

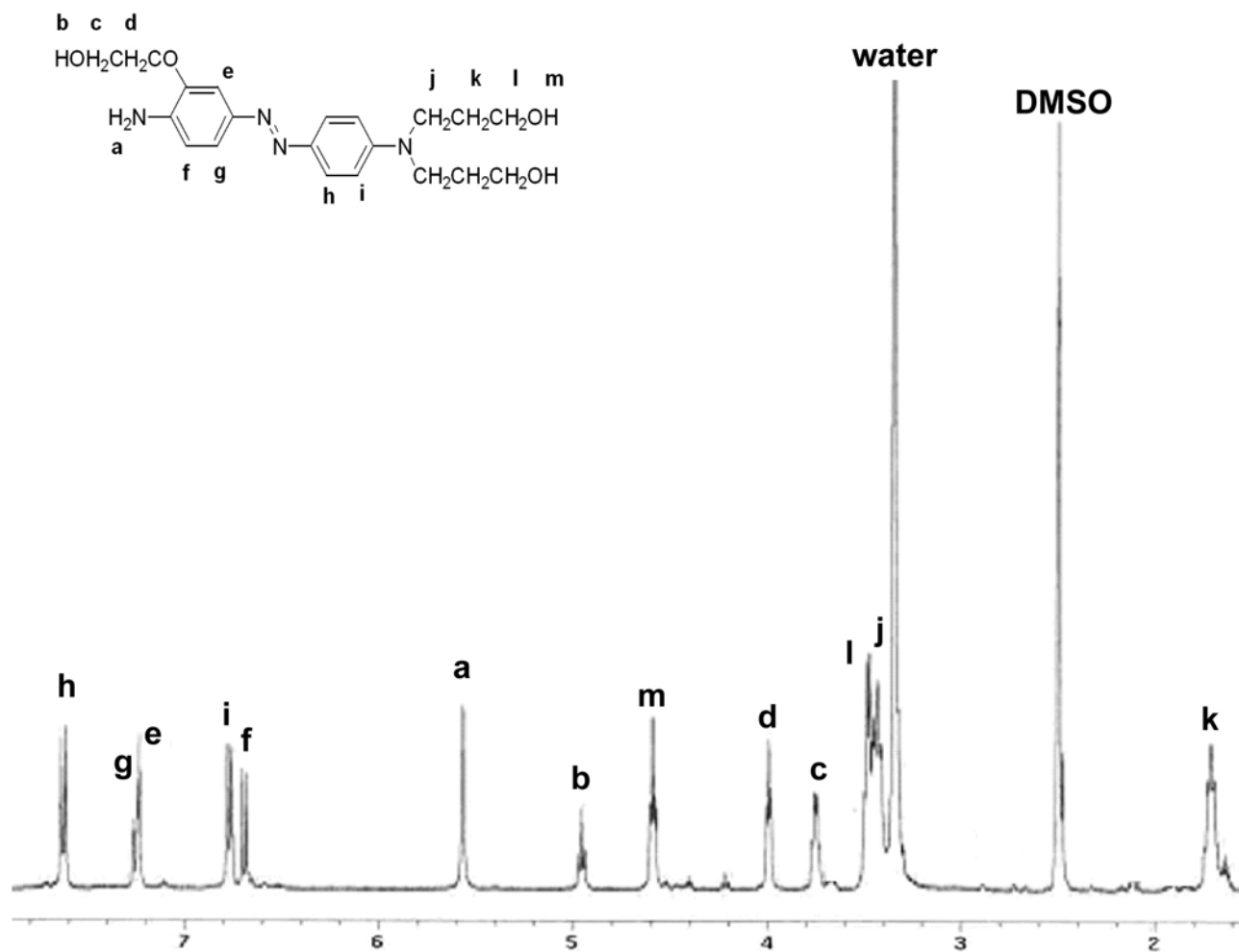


Figure 25. NMR spectrum of 4-((3-(2-hydroxyethoxy)-4-amino)phenylazo)-*N,N*-bis(3-hydroxypropyl)aniline (**69**).

3. Mass Spectrometry of Dyes

High resolution FAB mass spectrometry was used to analyze the structure of dye **67**. Dye **67**, which has MW 316.15, formed an $[M\cdot]^+$ ion and its high resolution mass spectrum had the base peak at $m/z = 316.15$. The peaks at $m/z = 283.18$ and 327.20 are from PEG, which was used as a part of the matrix. The peaks of $m/z = 289.08$ and 307.10 are NBA peaks that also arise from the matrix system. The mass spectrum is shown in Figure 26.

High resolution ESI mass spectrometry was used to analyze structures of dyes **68** and **69**. Dye **68**, which has MW 444.48, underwent protonation to give a species with charge of +1. The peak of the mass spectrum of this dye was $m/z = 445.2$ ($[M+1]^+$). The peaks at $m/z = 290.0886$ and 609.2771 are lock mass standard peaks, which were used for an accurate measurement. The mass spectrum is shown in Figure 27.

Dye **69**, which has MW 388.46, underwent protonation to give a species with charge of +1. The peak of the mass spectrum of this dye was $m/z = 389.2$ ($[M+1]^+$). The peak at $m/z = 290.0886$ is a lock mass standard peak. The mass spectrum is shown in Figure 28.

[Mass Spectrum]
Data : 040926-HR Date : 07-Oct-2004 22:23
Sample: FAB(+), Hi Res, DYE 1', C16H20N4O3, M+=316.1535, NBA/PEG400/MeC12
Note : 10,000R, MF, cycle=12s, 280-330amu, 66 s
Inlet : Direct Ion Mode : FAB+
Spectrum Type : Normal Ion [EF-Linear]
RT : 0.50 min Scan# : (2,5)
BP : m/z 283.1757 Int. : 17.62
Output m/z range : 280.0000 to 330.0000 Cut Level : 0.00 %

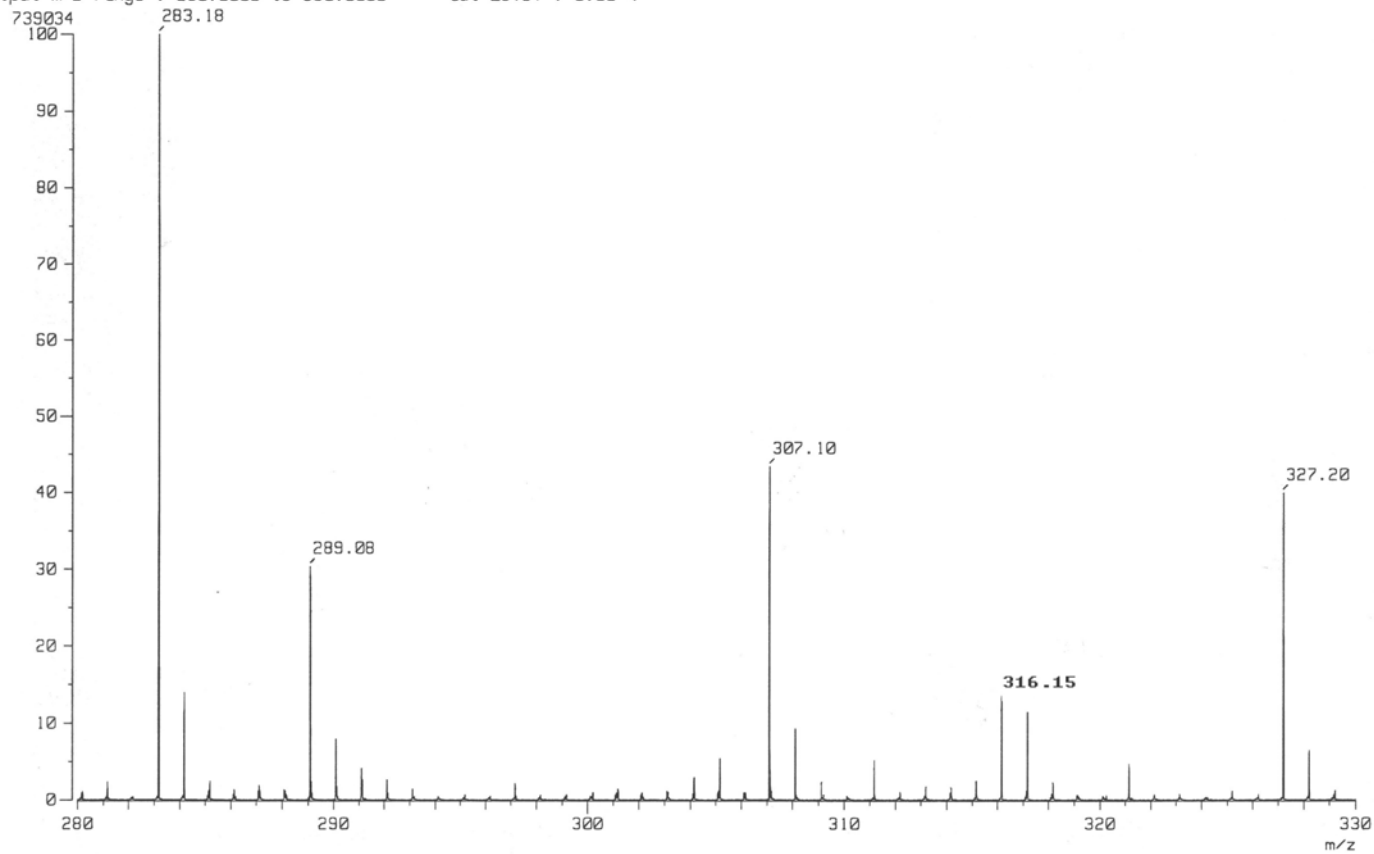


Figure 26. FAB mass spectrum of dye 67.

DYE 2 EJ-2

gw-716-dye2 43 (2.227) AM (Cen,4, 80.00, Ht,7880.0,290.09); Cm (28:44)

16-JUL-2004

TOF MS ES+
2.26e4

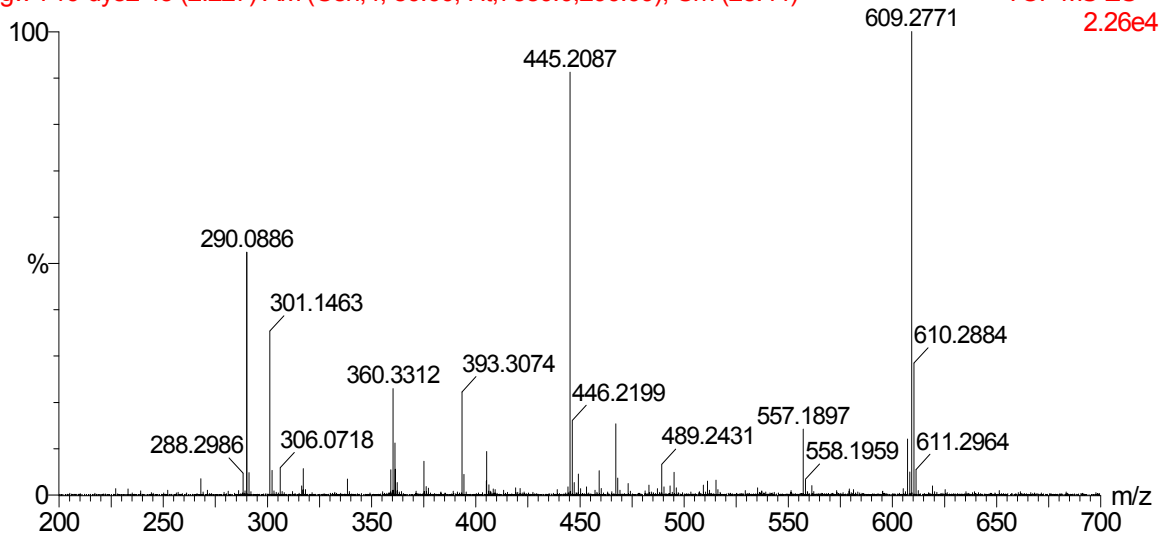


Figure 27. ESI mass spectrum of dye **68**.

DYE 3 EJ-3

gw-716-dye3 63 (3.287) AM (Cen,4, 80.00, Ht,7880.0,290.09); Cm (63:72)

16-JUL-2004

TOF MS ES+
1.97e4

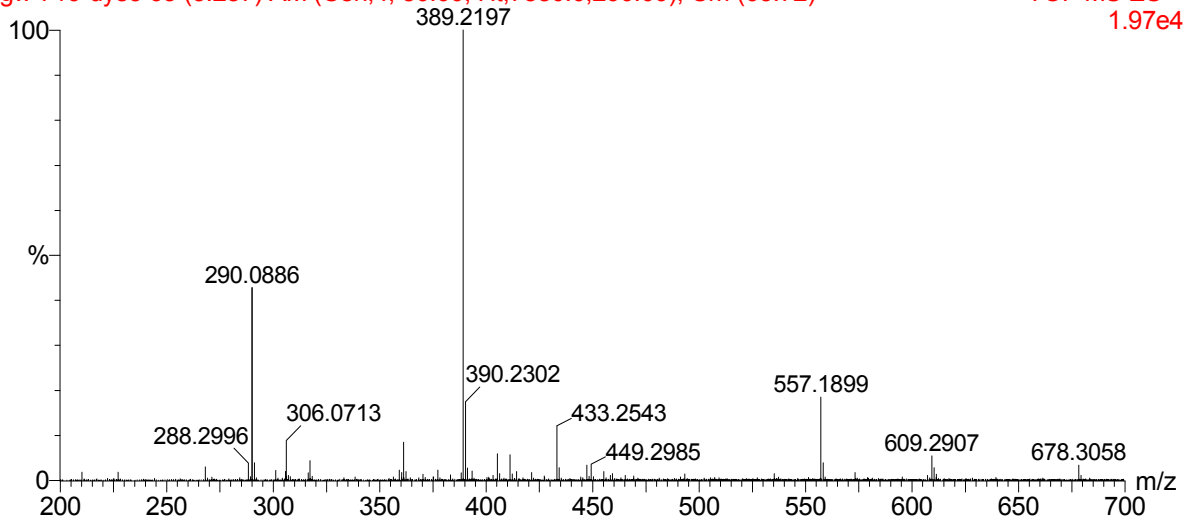


Figure 28. ESI mass spectrum of dye **69**.

4. Absorption Spectra and Colors

The visible absorption spectra of dyes **67** - **69** were recorded in DMSO, the results of which are summarized in Table 2 and Figures 29-31. The λ_{\max} value for dye **69** was slightly higher than that of dyes **67** and **68**. The bathochromic shift associated with dye **69** suggests that adding a C-atom to the N-hydroxy-ethyl groups increases the basicity of the amino group, thus making it a slightly better electron donor. As would be expected, the 3 dyes gave yellow colors in DMSO, as they lack a conjugated electron-donor/acceptor system.

Table 2. Absorption spectral data for dyes **67** – **69**

Dye	λ_{\max}^1	E_{\max}	Color ¹
67	455	17,300	Yellow
68	454	20,900	Yellow
69	463	19,300	Yellow

¹In DMSO

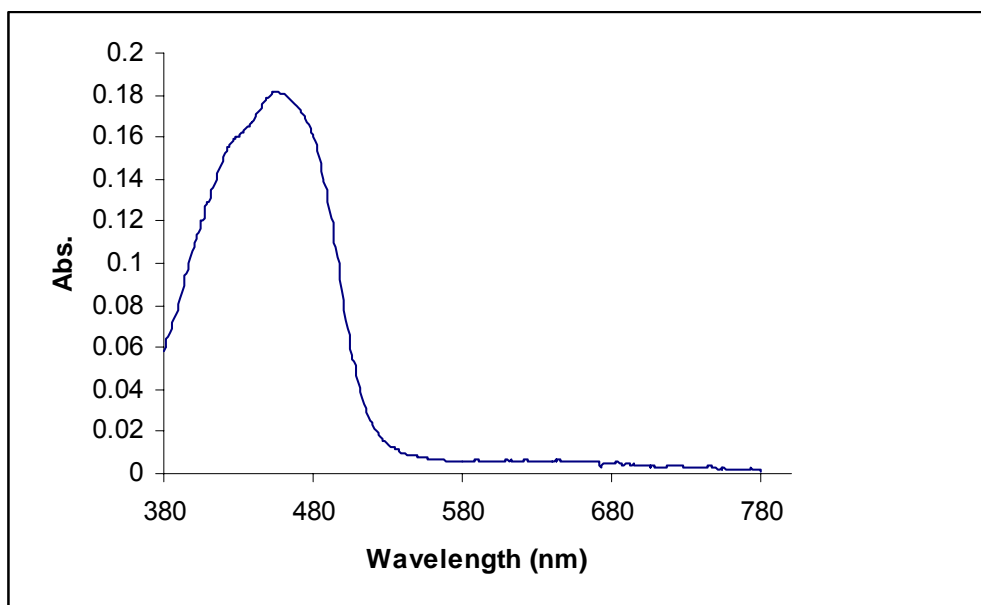


Figure 29. Absorption spectrum of dye 67.

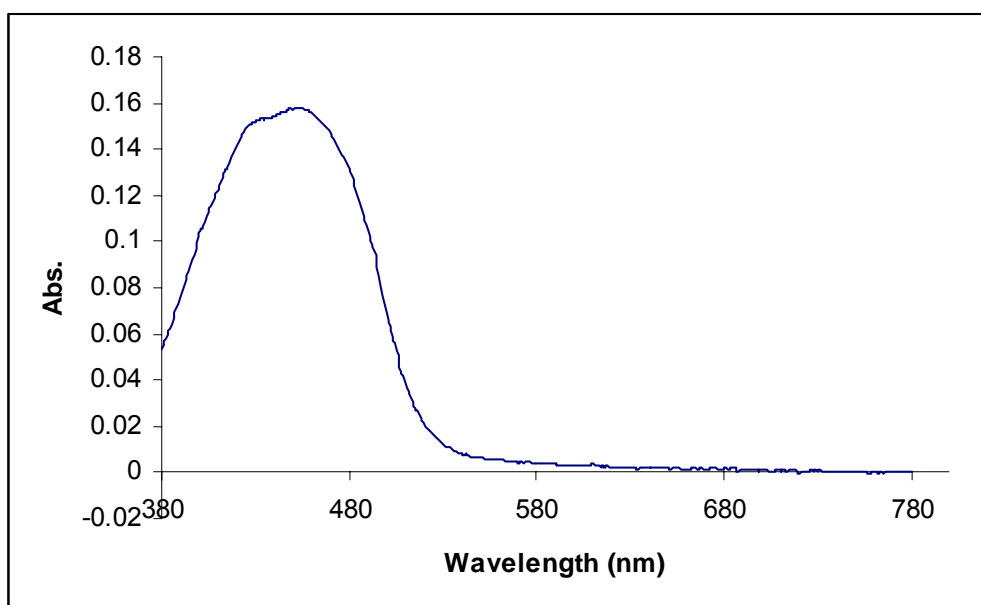


Figure 30. Absorption spectrum of dye 68.

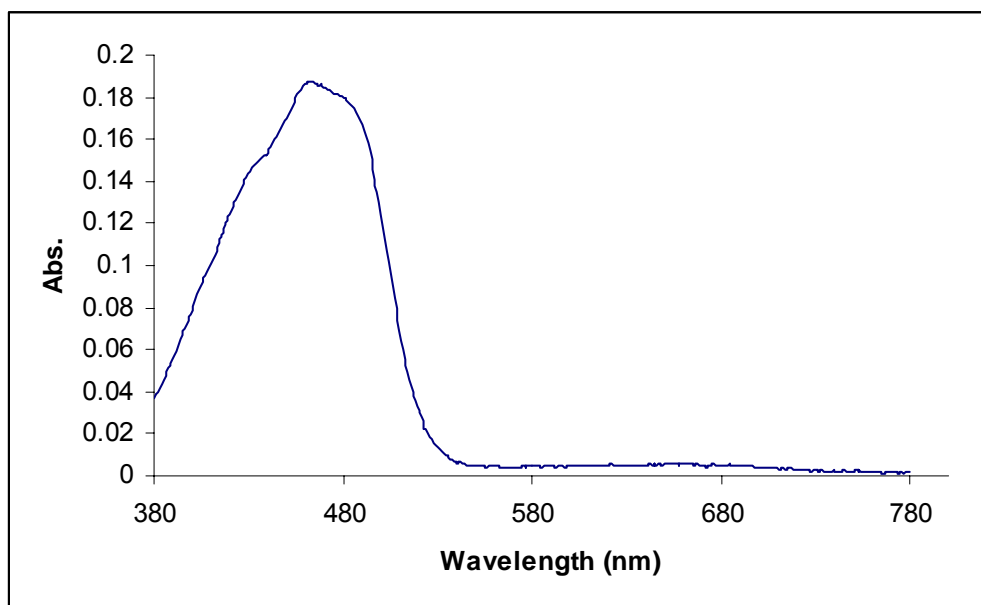


Figure 31. Absorption spectrum of dye **69**.

5. Mutagenicity Data

Dose response curves for dyes **66** - **69** are shown in Figures 32-37. The background count was obtained from the number of revertant colonies counted in a control test where no dye was present.

The test results at 0 - 0.4 μ M dose levels are shown in Figures 33-35. Dye **67** was positive (mutagenic) in TA98 with/without S9 metabolic activation, while dyes **68** and **69** were negative (nonmutagenic) in TA 98 with/without S9 metabolic activation. The result showed that dye **67** gave more than twice the number of revertant colonies versus the background count in TA98 with/without S9 activation, indicating that its mutagenicity did not require enzyme activation. The revertant count arising dye **67** increased as the dose level increased.

When compared to our previous data for dye **66** (Figure 32), the present results indicate that dye **67** is more mutagenic than dye **66**. These results suggest that dehydroxyethylation of dye **66** plays a role in the metabolic activation of dye **66**, just as demethylation of *N,N*-dimethyl-4-aminoazobenzene to give *N*-methyl-4-aminoazobenzene is a key process [62]. The increase in the number of revertant colonies associated with dyes **68** and **69**, versus the background levels, was small. Neither dye gave more than twice the number of revertant colonies versus the background count in TA98 with/without S9 activation, at the 0 - 0.4 μ M dose level. These results indicate that the length of the *N*-hydroxyalkyl group affects mutagenicity, as changing the C-2 group to the C-3 group removes mutagenicity.

Dose response curves at 0 - 4.0 μM dose levels of dye **68** and **69** are shown in Figures 36-37. The revertant counts were higher than those obtained at lower dose levels but neither dye produced revertant levels significantly exceeding the background count with/without S9 activation. Although the results in Figure 36 indicate that the number of revertants produced by dye **68** were slightly more than twice the background count at the top dose level, the slope value was not significant. It is possible that the observed doubling of the background count at 4.0 μM may be due to the killing of enough bacteria cells that histidine was released, which allowed a few colonies to grow when they otherwise would not. With these points in mind, both dyes were judged to be nonmutagenic at both dose levels employed in this study.

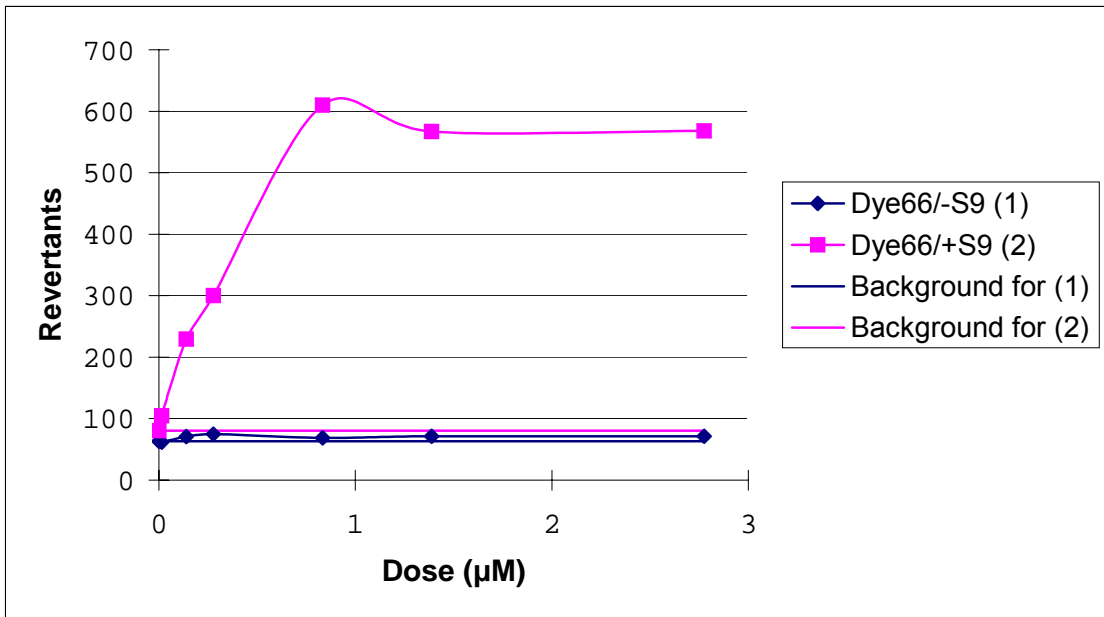


Figure 32. Dose response curves at 0 - 2.8 µM dose levels for dye 66 from the standard mutagenicity assay using TA98 with/without S9 activation.

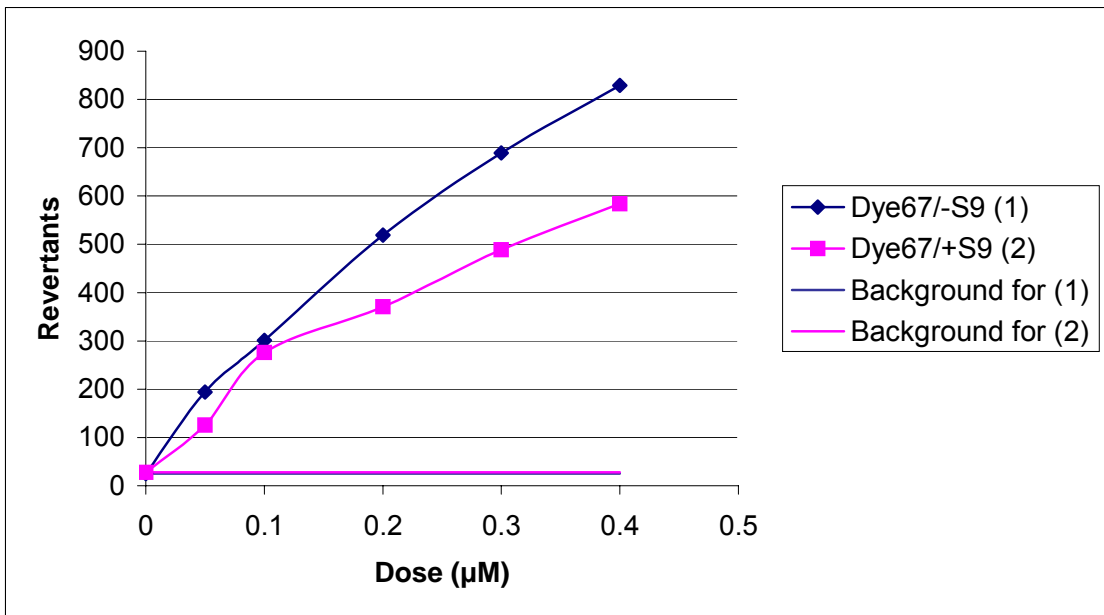


Figure 33. Dose response curves at 0 - 0.4 µM dose levels for dye 67 from the standard mutagenicity assay using TA98 with/without S9 activation.

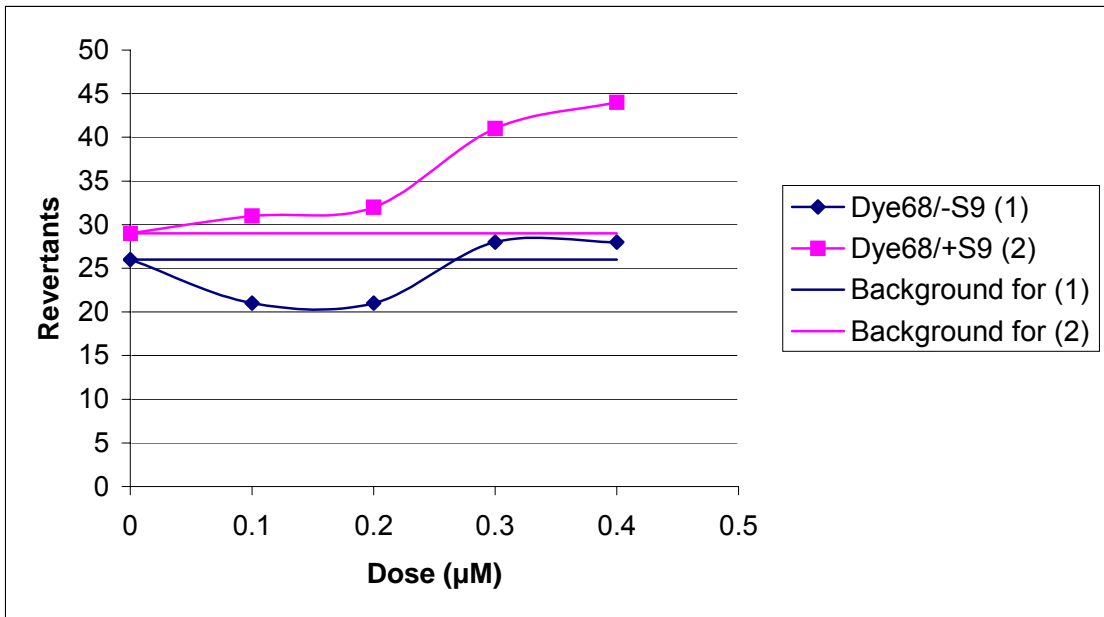


Figure 34. Dose response curves at 0 - 0.4 µM dose levels for dye **68** from the standard mutagenicity assay using TA98 with/without S9 activation.

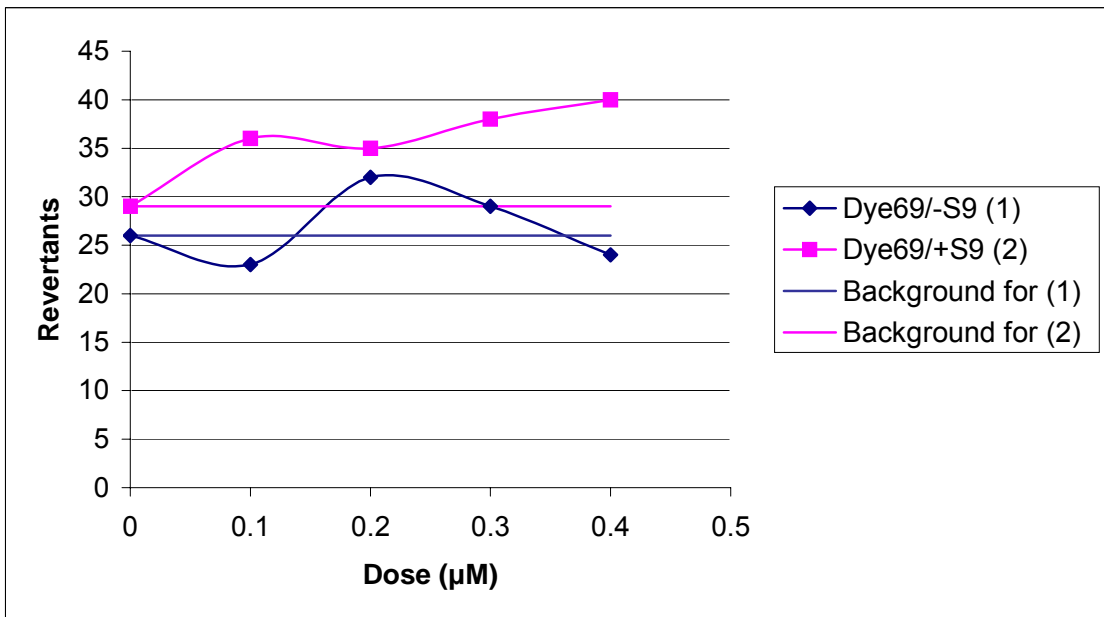


Figure 35. Dose response curves at 0 - 0.4 µM dose levels for dye **69** from the standard mutagenicity assay using TA98 with/without S9 activation.

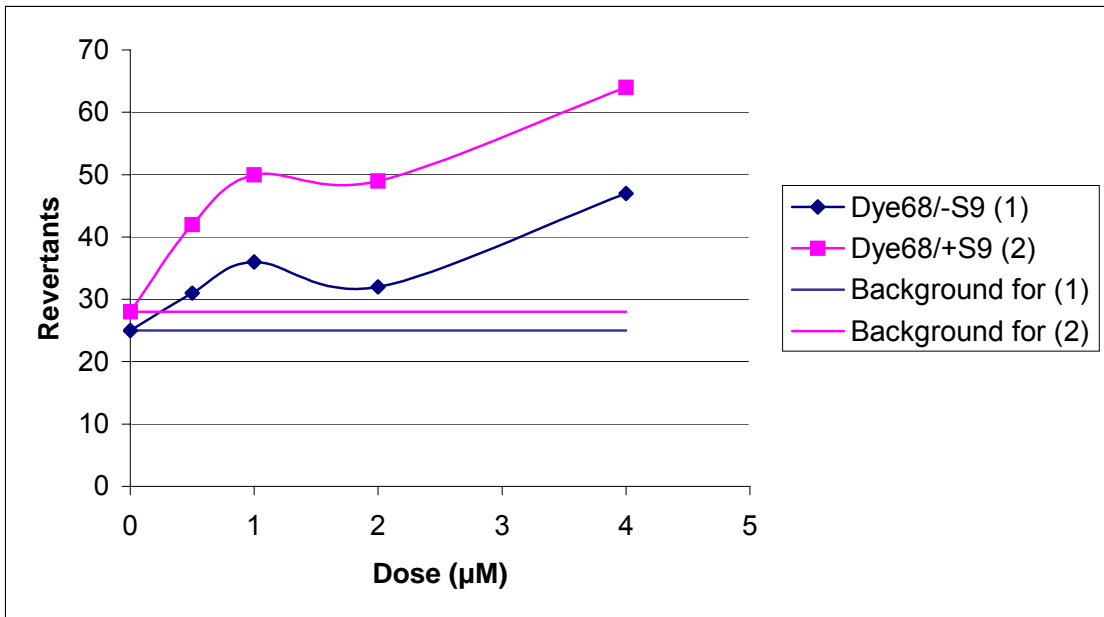


Figure 36. Dose response curves at 0 - 4.0 µM dose levels for dye **68** from the standard mutagenicity assay using TA98 with/without S9 activation.

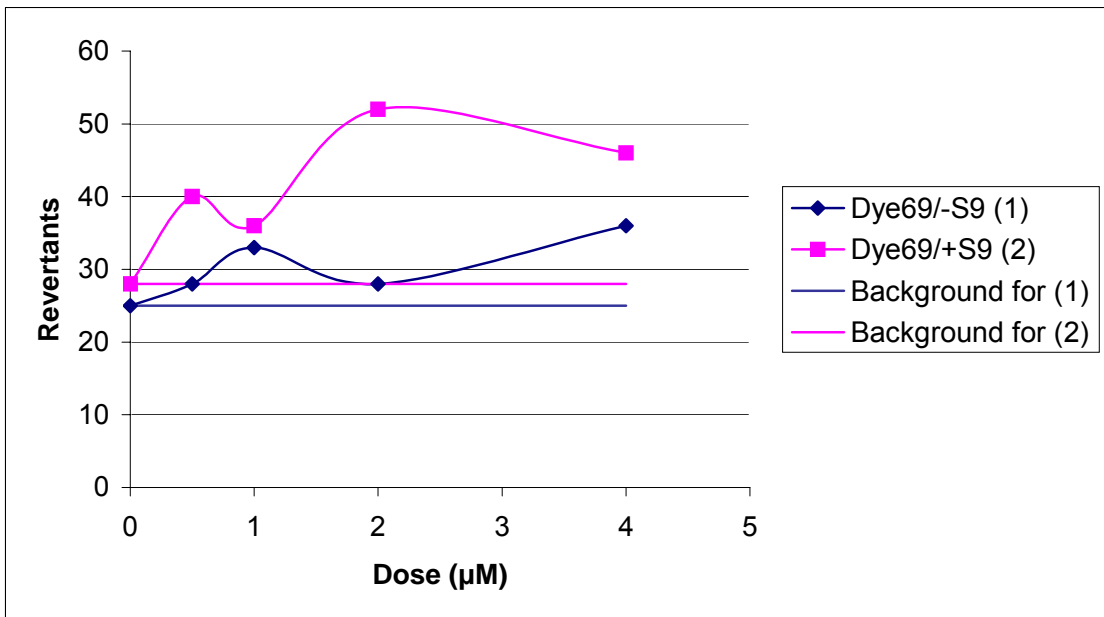


Figure 37. Dose response curves at 0 - 4.0 µM dose levels for dye **69** from the standard mutagenicity assay using TA98 with/without S9 activation.

IV. Conclusions

The results of this study indicate that the synthesis of the target novel dyes was successful, albeit in relatively low yields. In the case of dye **68**, a key contributor to the low yield was the challenge of producing the dye from a reaction sequence that required the hydrolysis of an -NHAc group in the presence of an $N(\text{CH}_2\text{CH}_2\text{OAc})_2$ moiety. In this regard, a tight constraint on the acid hydrolysis step was a limiting but essential factor in getting the target dye. Once obtained in pure form, the dyes were amenable to high-resolution mass spectrometry using either FAB or ESI mass spectrometry.

The results of this study also expand the knowledge base pertaining to the use of bulky alkoxy substituents *ortho* to the primary amino (-NH₂) group of aminoazobenzene-based dyes as an approach to preventing mutagenicity. In this regard, it is clear that the combination of an *ortho*-OCH₂CH₂OH group in the diazo component and an *N*-hydroxyethyl group in the coupler moiety should be avoided. The results of this study also suggest that removing one of the *N*-hydroxyethylamino in the parent dye causes an increase rather than a decrease in mutagenicity. In the present starting dye, either acetylating the free -OH groups in the *N,N*-bis(hydroxyethyl)amino moiety or increasing the chain length is needed to remove mutagenicity. Further, these results suggest that the presence of a free *N*-hydroxyethyl group in the coupler moiety is responsible for the mutagenicity of the parent dye, which was the key question at the beginning of this study.

As future work in this area, the following studies are recommended:

1. Confirm that the structural changes have not introduced mutagenicity in TA100. This is important since the parent dye (**66**) was nonmutagenic in that strain.
2. Characterize the dyeing and fastness properties of the new nonmutagenic dyes.
3. Identify the actual mutagenic species in dyes containing the *N*-hydroxyethyl group in the coupler when the *ortho*-hydroxyethoxyl group is also present in the diazo component.
4. Conduct a computational study involving dyes **66**, **67**, **68**, and **69** to determine whether differences in structural and electronic properties of these dyes correlate with the ability of a free *N*-hydroxyethyl group in the coupler moiety to cause mutagenicity.

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