ABSTRACT


Until the 1970s, benzidine and some of its analogs were widely used for the synthesis of direct dyes and organic pigments. It was then that benzidine and its analogs were shown to be carcinogenic and as a consequence, dyes based on them could no longer be manufactured for use on textiles in the United States and Europe. The commercial significance of benzidine-based colorants has led to the search for viable nonmutagenic analogs of this important intermediate.

This dissertation is concerned with the synthesis and evaluation of direct dyes and pigments in which diamines 4,4’-diamino-para-terphenyl (DATP) and 4,4’-diamino-para-quaterphenyl (DAQP) were employed as potential alternatives to benzidine. DATP (113) was synthesized by direct nitration of para-terphenyl and reduction of the resultant dinitro compound using hydrazine and Pd catalyst. DAQP (117) was synthesized in three steps from biphenyl: 1) simultaneous nitration and iodination, 2) Cu-induced coupling of 4-iodo,4’-nitrobiphenyl to give 4,4’-dinitro-para-quaterphenyl (116), 3) reduction DNQP to the target diamine (117) using SnCl₂/HCl.

![Diamines used in this study](image-url)
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<td>4,4(^\prime)-diamino-\textit{para}-quaterphenyl (DAQP) ((117))</td>
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Organic dyes were prepared by coupling DATP (cf. 121-125) or DAQP (cf. 127-131) to the widely used dye intermediates: naphthionic acid, J-acid, H-acid, chromotropic acid and Chicago acid. Diazotizations were conducted at 0-5°C using conc. HCl or H\(_2\)SO\(_4\). Couplings were conducted by adding the tetrazonium salt to the couplers at pH 8-9. Analogs of the commercially important dye C.I. Direct Black 38 were also made from DATP (cf. 126) and DAQP (cf. 132). These two dyes were synthesized through a four-step procedure: 1) diazotization of the diamines, 2) coupling with H-acid at 10°C and pH 2, 3) coupling with the diazonium salt of aniline at pH 8-9, and 4) addition of \textit{meta}\textendash phenylenediamine at 10°C and pH 7-8. The purity of all dyes was assessed by TLC and electrospray mass spectrometry (ESMS) was used to confirm dye structures. Results of visible absorption spectra showed that dyes made from DATP had red, blue and brown colors (\(\lambda_{\text{max}}\) 476.0–585.0 nm), while those from DAQP had red, purple and brown colors (\(\lambda_{\text{max}}\) 473.0-574.0 nm). Consequently, it was clear that increasing the number of phenylene groups caused a hypsochromic shift in the color of the disazo direct dyes.

Organic pigments were prepared by coupling or condensing DATP (cf. 133-137) or DAQP (cf. 138-142) with intermediates such as 2-mercapto-4,6-dihydroxypyrimidine, acetoacetanilide, 1-phenyl-3-methyl-5-pyrazolone, 5-
acetoacetylaminobenzimidazolone and 4-[(2,5-dichlorophenyl)azo]-3-hydroxy-2-naphthoyl chloride. In this case, DATP gave yellow, orange and purple colors ($\lambda_{\text{max}}$ 382.0-546.0 nm), while those from DAQP had yellow, orange and purple colors ($\lambda_{\text{max}}$ 349.0-535.0 nm), making it clear that increasing the number of phenylene groups also caused a hypsochromic shift in the color of organic pigments.

The standard Ames test and Prival modification were used to assess mutagenicity. The results showed that DATP was mutagenic, while DAQP was nonmutagenic. Most of the dyes based on DATP (cf. 121-123, 125) and DAQP (cf. 128-131) were nonmutagenic in the Ames test. However, dyes 124 and 126, which are based on DATP and dyes 127 and 132, which are based on DAQP, were mutagenic. The Prival modification of Ames test showed that dyes 121 and 127 were mutagenic, with the former giving a much higher number of revertants. All pigments based on DATP and DAQP were nonmutagenic, which may be accounted for in part by the extremely low solubility of the pigments in the test media.

The results of this study indicated that both diamines can be used to make disazo and azomethine organic colorants; however due to the mutagenicity of DATP, only DATQ could be accepted as a synthetic intermediate, despite the lack of mutagenicity in organic pigments derived from the former diamine. Further, the high melting and decomposition temperatures associated with organic pigments derived from DAQP suggest that they are suitable for applications requiring high performance pigments, such as the coloration of fibers
and plastics during melt extrusion. On the other hand, the low light stability of
dyes derived from DAQP could limit their use in commerce. In this case,
however, it was of interest to show that the experimental properties of dyes
derived from coupling DATP and DAQP validated results from molecular
modeling studies, which predicted that the resultant dyes would have lower $\lambda_{\text{max}}$
values than the parent dye, Congo Red (cf. 121, n=0). In this regard, $\lambda_{\text{max}}$ values
recorded in DMF for Congo Red and its two homologs were 529nm (n=0), 516
nm (n=1), and 502 nm (n=2).
121 (n=1), 127 (n=2)

122 (n=1), 128 (n=2)

123 (n=1), 129 (n=2)

124 (n=1), 130 (n=2)

125 (n=1), 131 (n=2)

126 (n=1), 132 (n=2)
PHENYLENE HOMOLOGS OF BENZIDINE: MUTAGENICITY AND USE IN THE SYNTHESIS OF ORGANIC DYES AND PIGMENTS

By

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BIOGRAPHY

Jinlong Wang was born on November 10, 1973 in Xinbin, China. He was reared in Xinbin and graduated from Xinbin High School in July 1992. In September 1992, he began his undergraduate studies in the Department of Chemical Engineering, Hefei University of Technology, Hefei, China and received a Bachelor of Science degree in June 1996. In September 1996, he began his graduate studies in the Department of Fine Chemicals, College of Chemical Engineering, Dalian University of Technology, Liaoning, China and received a Master of Science degree in July 1999.

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On August 21, 2000, he married Xiaojie Li who currently works for a company in Chicago, IL.
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List of Abbreviations

BON.................................................................................................β-Hydroxynaphthoic acid
DAP ................................................................................................. 1,4-Diaminophenylene
DABP ................................................................................................ Diaminobiphenyl
DAQP .........................................................................................4,4’-Diamino-para-quaterphenyl
DATP ..............................................................................4,4’-Diamino-para-terphenyl
DNQP ..........................................................................................4,4’-Dinitro-para-quaterphenyl
DNTP .........................................................................................4,4’-Dinitro-para-terphenyl
ESMS ..........................................................................................Electrospray mass spectrometry
FMN ................................................................................................ Flavin mononucleotide
G6P ............................................................................................... Glucose-6-phosphate
G6PDH .......................................................... Glucose-6-phosphatedehydrogenase
MALDI .......................................................... Matrix assisted laser desorption/ionization
MMS .............................................................................. Microsomal salt solution
NADH .......................................................................................... β-Nicotinamide adenine dinucleotid
NADP .......................................................................................... β-Nicotinamide adenine dinucleotide phosphate
PAH ................................................................................................ Polycyclic aromatic hydrocarbons
S9 ........................................................ A 9000 x g supernatant of rat liver
TEOF .......................................................................................... Triethylorthoformate
TLC .............................................................................................. Thin layer chromatography
VBME .......................................................................................... Vogel-Bonner Medium E
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I. Introduction

1. Nitration of aromatic compounds

1.1 Introduction

Nitration is defined as the reaction between a nitrating agent and an organic compound that results in one or more nitro (-NO₂) groups becoming chemically bonded to an atom in the starting compound [1]. This process is very important in azo dye chemistry since the resulting nitro compounds are readily reduced to the corresponding amines, which are key intermediates in azo dye synthesis.

The first reaction of this type involving an aromatic system was the nitration of benzene itself. In 1834, Mitscherlich [2] prepared nitrobenzene by treating benzene with fuming nitric acid. Subsequently, this reaction was found to be applicable to other aromatic compounds and could be facilitated by sulfuric acid.

By employing nitric acid as the nitrating agent, C-, O- and N- nitration can be achieved (Figure 1). In this paper only C-nitration will be discussed.

![Reaction of nitric acid with C-, O- and N-centers.](image)
For the nitration of most aromatics, typically there are two liquid phases: an organic phase and an acid phase. A large interfacial area between the phases is needed to expedite transfer of the reactants to the interface. The site of the main reactions is often at or close to the interface. To provide large interfacial areas, a mechanical agitator is frequently used.

Nitric acid in both aqueous and organic solvent systems is used for nitrating more reactive compounds, while less reactive and more difficultly dissolved compounds require "mixed acid", the combination of nitric and sulfuric acid, as the nitrating agent. The ratio of these mixed acids varies from 35% nitric-65% sulfuric acid to 83% nitric-17% sulfuric acid. Sulfuric acid serves as the solvent for carrying out the reaction. It was initially considered as a dehydrating agent for withdrawing water formed in the reaction and thus shifting the equilibrium towards product [3]. Later researchers showed that sulfuric acid reacts with nitric acid molecules to form the nitronium ion (-NO₂⁺), which is the actual nitrating agent.

While nitration can be a highly exothermic process, the heat of reaction varies with the molecule that is nitrated. In addition, the mechanism of a given nitration reaction depends on the reactant and operating conditions. The reaction species involved usually are either ionic or free radical. An ionic species is commonly associated with aromatics, heterocycles, phenols and amines. Nitration of paraffin, cycloparaffins and olefins frequently involves a free-radical process.
1.2 Mechanism of the nitration reactions

The nitration of aromatic compounds is an electrophilic substitution reaction and is commonly carried out with nitric acid and other nitronium ion “carriers” such as acetyl nitrate, dinitrogen pentoxide, nitryl chloride, \(N\)-nitropyridine, and tetranitromethane [4].

Ingold and Hughes [5, 6] studied the kinetics and mechanism of aromatic nitrations and proposed the \(\text{NO}_2^+\)-based mechanism shown in Figure 2. This mechanism has been accepted since about 1950 for the nitration of most aromatic hydrocarbons and numerous other compounds in which high concentrations of nitric acid or mixed acids are used.

\[
\begin{align*}
\text{HNO}_3 & \quad + \quad \text{HA} \quad \Leftrightarrow \quad \text{H}_2\text{NO}_3^{\ominus} \quad + \quad \text{A}^{\ominus} \quad (1) \\
\text{H}_2\text{NO}_3^{\ominus} & \quad \Leftrightarrow \quad \text{NO}_2^{\ominus} \quad + \quad \text{H}_2\text{O} \quad (2) \\
\text{ArH} & \quad + \quad \text{NO}_2^{\ominus} \quad \Leftrightarrow \quad \text{ArHNO}_2^{\ominus} \quad (3) \\
\text{ArHNO}_2^{\ominus} & \quad + \quad \text{A}^{\ominus} \quad \Leftrightarrow \quad \text{ArNO}_2 \quad + \quad \text{HA} \quad (4)
\end{align*}
\]

Figure 2. Ingold-Hughes mechanism for the nitration of aromatic compounds.

The first two steps of this mechanism involve the acid catalyzed conversion of nitric acid (\(\text{HNO}_3\)) to \(-\text{NO}_2^+\). \(\text{NO}_2^+\) then reacts with an aromatic compound to form a \(\sigma\)-complex intermediate (step (3)). The last step (4) is the loss of a proton to give rearomatization and regeneration of the acid catalyst.
This mechanism accounts for the observed electronic effects of different substituents. It is known that electron-donating groups on a benzene ring increase its reactivity and direct the incoming nitro group to the ortho and/or para positions, while electron-withdrawing groups decrease reactivity and direct the nitro group to the meta position.

An unusual case is the nitration of chlorobenzene. While the existence of a chloro group decreases the reactivity of benzene ring; however, nitration of chlorobenzene yields ortho-chloro and para-chloro nitrobenzenes. The HOMO of chlorobenzene is lower than that of benzene, which makes chlorobenzene less reactive; however, the electron-donating property of the chloro group directs the incoming nitro group to ortho- and para- positions.
1.3 Examples of the nitration reactions

The nitro group is almost always introduced into the aromatic ring by direct nitration using either concentrated nitric acid or a mixture of concentrated nitric and sulfuric acids. Other nitrating agents are also employed for the nitration of aromatics (Table 1) [4].

Table 1. Electrophilic nitrating agents.

<table>
<thead>
<tr>
<th>NO$_2^+$ Carrier</th>
<th>Acid Catalyst</th>
<th>NO$_2^+$ Carrier</th>
<th>Acid Catalyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNO$_3$</td>
<td>$\text{H}_2\text{SO}_4$</td>
<td>(CH$_3$)$_2$C(CN)ONO$_2$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\text{H}_2\text{SO}_4\cdot\text{SO}_3$</td>
<td>(CH$_3$)$_2$SiONO$_2$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\text{H}_3\text{PO}_4$</td>
<td>RC(O)ONO$_2$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polyphosphoric acid (PPA)</td>
<td>NO$_2$F</td>
<td>BF$_3$,PF$_5$,AsF$_5$</td>
</tr>
<tr>
<td></td>
<td>$\text{HClO}_4$</td>
<td>NO$_2$Cl</td>
<td>HF,AlCl$_3$,TiCl$_4$</td>
</tr>
<tr>
<td></td>
<td>HF, HF-BF$_3$, BF$_3$</td>
<td>N$_2$O$_3$</td>
<td>BF$_3$</td>
</tr>
<tr>
<td></td>
<td>CH$_3$SO$_3$H</td>
<td>N$_2$O$_4$</td>
<td>H$_2$SO$_4$,AlCl$_3$,FeCl$_3$, BF$_3$,SbF$_5$,AsF$_5$, IF$_5$</td>
</tr>
<tr>
<td></td>
<td>CF$_3$SO$_3$H</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R$_F$SO$_3$H, FSO$_3$H</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Solid acids (polystyrene-sulfonic acid)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AgNO$_3$,NaNO$_3$</td>
<td>$\text{FeCl}_3$, BF$_3$, AlCl$_3$, CF$_3$CO$_2$H</td>
<td>NO$_2$O$_5$</td>
<td>BF$_3$</td>
</tr>
<tr>
<td>KNO$_3$,NH$_4$NO$_3$, Ti(NO$_3$)$_4$</td>
<td></td>
<td>NO$_2^+$BF$_4^-$,NO$_2^+$PF$_6^-$ and other salts</td>
<td>N-Nitropyridinium salts</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-Nitropyrazole</td>
<td></td>
</tr>
<tr>
<td>RONO$_2$</td>
<td>$\text{H}_2\text{SO}_4$, BF$_3$</td>
<td>N$_2$O$_5$</td>
<td>BF$_3$</td>
</tr>
<tr>
<td>C$_2$H$_5$ONO$_2$</td>
<td>AlCl$_3$, SnCl$_4$, SbCl$_5$, FeCl$_3$</td>
<td>NO$_2^+$BF$_4^-$,NO$_2^+$PF$_6^-$ and other salts</td>
<td>N-Nitropyridinium salts</td>
</tr>
<tr>
<td>CH$_3$ONO$_2$</td>
<td>BF$_3$</td>
<td>9-Nitroanthracene</td>
<td>HF-TaF$_5$</td>
</tr>
<tr>
<td>CH$_3$ONO$_2$</td>
<td>BF$_3$</td>
<td>C$_6$(CH$_3$)$_6$NO$_2^+$</td>
<td></td>
</tr>
</tbody>
</table>
1.3.1 The nitration of benzene and its analogs

Mononitration of benzene is easily accomplished and nitrobenzene can be produced in high yield on an industrial scale at \(~50^\circ\text{C}\) by using mixed acid [7]. The introduction of a second NO\(_2\) group requires more rigorous conditions, since the first introduced NO\(_2\) group deactivates the benzene ring. The main product of dinitration is \textit{meta}-dinitrobenzene (Figure 3).

The formation of \textit{meta}-dinitrobenzene is accompanied by a small amount of \textit{ortho}- and \textit{para}-dinitro isomers. Industrially, these secondary products are removed by treating the reaction mixture with sodium sulfite to give water soluble derivatives (Figure 4) [8].
The groups present in substituted benzenes influence the position in which the NO$_2$ group enters during nitration. Activating groups tend to direct the NO$_2$ group to ortho- and para- positions, while deactivating groups direct it to the meta-position (Table 2) [9].

Table 2. The effects of ring substituents on aromatic nitration.

<table>
<thead>
<tr>
<th>Property</th>
<th>ortho- and para- Directing groups</th>
<th>meta- Directing groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strength</td>
<td>Strong</td>
<td>Medium</td>
</tr>
<tr>
<td>Substituents</td>
<td>-NR$_2$</td>
<td>-NHR</td>
</tr>
<tr>
<td>Activity</td>
<td>Activating groups</td>
<td>Deactivating groups</td>
</tr>
</tbody>
</table>

Several examples can be used to illustrate substituents effects. One is the nitration of aromatic amines. When directly nitrated by nitric or mixed acid, the amino group of aniline is protonated and the resulting ammonium ion is both a meta-directing and deactivating group. In the case of ortho-anisidine, the methoxy group and ammonium group reinforce attack para to the methoxy group and meta to the NH$_2$ group (Figure 5) [10].

Figure 5. Nitration of ortho-anisidine.
To form ortho- and para- nitroanilines and to avoid ring oxidation, the NH$_2$ group of aniline itself must be protected. This is accomplished by converting aniline to the corresponding amide using acetic anhydride or other suitable acylating agents such as para-toluenesulfonyl chloride. The nitration of the acetanilide occurs mainly at the para- position [11], and hydrolysis of the amide group produces the target compound (Figure 6).

![Figure 6. The formation of para-nitroaniline.](image)

1.3.2 The nitration of naphthalene

Naphthalene undergoes nitration under milder conditions than benzene and gives predominantly 1-nitronaphthalene, which is a major source of 1-aminonaphthalene. By increasing the severity of the reaction conditions, di-nitration [12], tri-nitration and tetra-nitration [13] can be achieved (Figure 7). The lack of 1, 6- and 1, 7-dinitro naphthalenes is due to the preference of the NO$_2$ group for the α-position [14].
Nitration of naphthalenesulfonyl acids provides an important method for the synthesis of intermediates such as 8-nitronaphthalene-1, 3, 6-trisulfonic acid (T-nitro-acid) (Figure 8) [15]. The nitro substituted naphthalene is readily converted to amino compounds that are widely used as coupling components for azo dyes.
1.3.3 The nitration of anthraquinone

The nitration of anthraquinone in the presence of sulfuric acid produces mainly 1-nitroanthraquinone. By-products, mainly dinitroanthraquinone and 2-nitroanthraquinone, are also formed and are difficult to remove. It was found that by nitration of anthraquinone in the presence of phosphoric acid, 1-nitroanthraquinone could be produced in about 60% yield with a very high degree of purity (≥90%) (Figure 9) [16].

![Anthraquinone and 1-Nitroanthraquinone](image)

**Figure 9. Nitration of anthraquinone in the presence of phosphoric acid.**

Dinitration of anthraquinone yields a mixture of 1, 5-, 1, 8-, 1, 6-, and 1, 7-dinitroanthraquinones. The 1, 5- and 1, 8- isomers are important dyestuff intermediates. Auge and coworkers [17] reported that by adjusting the molar ratios of nitric acid to anthraquinone from 10:1 to 80:1, 1, 5- and 1, 8-dintroanthraquinone are produced.
1.3.4 The nitration of polycyclic aromatic hydrocarbons

Some polycyclic aromatic hydrocarbons (PAH) can be nitrated to form mono or poly nitro-substituted compounds. For instance, nitration of pyrene, chrysene and fluorene using nitrogen dioxide (NO₂) gives mono-substituted products (Figure 10) [18]. Pitts and coworkers [19] reported similar results by using dinitrogen pentoxide (N₂O₅) as the nitrating agent; however, in this case nitrochrysene is not formed.

![Figure 10. Nitration of some PAH using NO₂.](image)
The nitration of fluoranthene using $\text{N}_2\text{O}_5$ in $\text{CCl}_4$ solution at 25°C gives two major products (Figure 11) [20].

![Figure 11. Nitration of fluoranthene by using $\text{N}_2\text{O}_5$.](image)

1.3.5 The nitration of heterocyclic aromatic compounds

Nitro groups can be introduced into heterocyclic aromatic compounds to produce intermediates used in dyestuff and pharmaceutical synthesis. Since the starting compounds are easily oxidized under strong oxidizing agents, concentrated nitric acid is often avoided.

The nitration of pyrrole by nitric acid in acetic anhydride produced a mixture of 2- and 3-nitropyrrrole (Figure 12) [21], in which the 2-isomer is dominant. The actual nitrating agent is $\text{CH}_3\text{COONO}_2$.

![Figure 12. Nitration of pyrrole.](image)
A directing group may be used when a specific product is preferred. For example, Blatt and coworkers [22] reported a method for the synthesis of pure 3-nitrothiophene (Figure 13). For this case, thiophene was chlorosulfonated, and the product was nitrated and then dechlorosulfonated.

![Figure 13. A 3-step synthesis of 3-nitrothiophene.](image)

2-Amino-5-nitrothiazole is used as an azo dye intermediate and it can be made by nitration of 2-aminothiazole. Since the later compound has poor stability in acid solutions, 2-aminothiazole bisulfite, which is stable in acids, was employed as the starting material [23]. The nitration gave a high yield (≥90%) of the target compound.

Crivello [24] reported the nitration of benzothiazole with inorganic nitrate in trifluoroacetic anhydride (Figure 14).

![Figure 14. The nitration of benzothiazole.](image)
Another compound that is used in dye synthesis is 2-amino-6-nitrobenzothiazole. This dye intermediate can be made by the initial acetylation of 2-aminobenzothiazole, followed by nitration and hydrolysis (Figure 15) [25].

Pyridine is very difficult to nitrate on its carbon atoms and 3-nitropyridine is obtained only in poor yield (~6%) even under vigorous conditions (H$_2$SO$_4$-SO$_3$-KNO$_3$, at 300°C) [26]. Ring activating groups such as amino, alkoxy and alkyl groups facilitate C-atom nitration. For example, 2, 4, 6-trimethylpyridine can be nitrated in good yield (Figure 16) [27].
2. Reduction of aromatic nitro compounds

2.1 Introduction

The amino group is probably the most important group in dyestuff chemistry. It is present in many coupling components and is widely used as an auxochrome in dye molecules. More importantly, it is essential to the formation of azo dyes by diazotization and coupling.

The amino group is most often introduced to aromatic rings by the reduction of a nitro group. There are several commercial processes for reducing a nitro group to an amino group, best known of which is the Béchamp method. This reaction employs iron in water containing hydrochloric, formic or acetic acid (Figure 17) [8]. The importance of this method has declined in recent years due to environmental considerations associated with the disposal of iron sludge.

\[
2\text{ArNO}_2 + 5\text{Fe} + 4\text{H}_2\text{O} \rightarrow 2\text{ArNH}_2 + \text{Fe}_3\text{O}_4 + 2\text{Fe(OH)}_3
\]

Figure 17. Béchamp reduction of aromatic nitro compounds.

Other specialized methods for the synthesis of amines by reduction include the Zinin reduction, in which sulfides in alkaline media are used, bisulfite reductions, electrochemical reductions, reductions using metal amalgams or hydrides, catalytic hydrogenation, and benzidine rearrangement following zinc reduction in alkaline media [28].
2.2 The mechanism of reduction reactions

2.2.1 The Béchamp process

The Béchamp reduction reaction given in Figure 17 is oversimplified and the reaction is depicted more completely by the sequence in Figure 18 [29].

\[
2RNO_2 + FeCl_2 + 6Fe + 10H_2O \rightarrow 2RNH_3Cl + 7Fe(OH)_2 \tag{1}
\]

\[
RNO_2 + 6Fe(OH)_2 + 4H_2O \rightarrow RNH_2 + 6Fe(OH)_3 \tag{2}
\]

\[
Fe(OH)_2 + 2Fe(OH)_3 \rightarrow Fe_3O_4 + 4H_2O \tag{3}
\]

Figure 18. The Béchamp reduction sequence.

The amine hydrochloride produced in step (1) may react further with iron and the nitro compound to form the free amine (step (4)).

\[
6RNH_3Cl + 3Fe + RNO_2 \rightarrow 7RNH_2 + 3FeCl_2 + 2H_2O \tag{4}
\]

2.2.2 Catalytic hydrogenation

In catalytic hydrogenation, the nitro group is reduced by molecular hydrogen in the presence of a catalyst. The difference in the rate of hydrogenation of the nitro group versus the aromatic ring is so large that the hydrogenation is easily regulated to avoid ring reduction.
The mechanism of catalytic hydrogenation has been investigated by many researchers and the mostly accepted mechanism for the reduction of nitro compounds was proposed by Haber in 1898 (Figure 19) [30].

![Figure 19. The catalytic hydrogenation of a nitro group.](image)

2.2.3 Benzidine rearrangement

Benzidine rearrangement refers to the acid-catalyzed conversions of hydrazoarenes into diaminobiphenyls or aminodiarylamines and usually is carried out in aqueous or ethanolic solutions of hydrochloric or sulfuric acid. Finar [31] summarized the benzidine rearrangement mechanism (Figure 20) in 1973. The nitro compound is first reduced to hydrazobenzene; then under acid conditions, the product is protonated and finally undergoes rearrangement through a concerted transition state [32]. The final step involves losing a pair of hydrogen ions to regain aromaticity.
2.3 Example of reduction reactions

2.3.1 The Béchamp reaction

This process is generally adaptable and economically competitive with other types of reductions. It can be conducted at atmospheric pressure, which makes it suitable for use in small batch operations. The reductions are usually carried out in aqueous or aqueous-alcoholic media. Iron and hot water react under essentially neutral conditions, although the addition of a catalytic amount of acids such as hydrochloric, formic, and acetic acids can greatly accelerate the reaction rate. Despite the complexity of the reaction, the amines are usually produced in good yield (Table 3) [14].
### Table 3. Application of the Béchamp reduction.

<table>
<thead>
<tr>
<th>Starting material</th>
<th>Product</th>
<th>Acid used</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>meta-Nitrotoluene</td>
<td>meta-Toluidine</td>
<td>Formic</td>
<td>87</td>
</tr>
<tr>
<td>4-Nitrobiphenyl</td>
<td>4-Aminobiphenyl</td>
<td>Formic</td>
<td>95</td>
</tr>
<tr>
<td>1-Chloro-3-nitrobenzene</td>
<td>Meta-Chloroaniline</td>
<td>Formic</td>
<td>95</td>
</tr>
<tr>
<td>2-Chloro-6-nitrotoluene</td>
<td>3-Chloro-ortho-toluidine</td>
<td>Formic</td>
<td>~100</td>
</tr>
<tr>
<td>para-Nitroaniline</td>
<td>para-Phenylenediamine</td>
<td>FeCl₃</td>
<td>93</td>
</tr>
<tr>
<td>para-Nitrophenol</td>
<td>para-Aminophenol</td>
<td>HOAc</td>
<td>97</td>
</tr>
<tr>
<td>2-Nitro-para-cresol</td>
<td>2-Amino-para-cresol</td>
<td>HCl</td>
<td>81~83</td>
</tr>
<tr>
<td>4-Methyl-2-nitroanisole</td>
<td>5-Methyl-ortho-anisidine</td>
<td>Formic</td>
<td>91</td>
</tr>
<tr>
<td>1,4-Dimethoxy-2-nitrobenzene</td>
<td>2,5-Dimethoxyaniline</td>
<td>Formic</td>
<td>91.8</td>
</tr>
<tr>
<td>para-Nitrobenzoic acid</td>
<td>para-Aminobenzoic acid</td>
<td>HCl</td>
<td>97</td>
</tr>
<tr>
<td>2-Chloro-5-nitrobenzoic acid</td>
<td>5-Amino-2-chlorobenzoic acid</td>
<td>HCl</td>
<td>90~91</td>
</tr>
<tr>
<td>meta-Nitrobenzenesulfonic acid</td>
<td>Metanilic acid</td>
<td>HCl</td>
<td>89</td>
</tr>
<tr>
<td>3-Nitro-5-sulfosalicylic acid</td>
<td>3-Amino-5-sulfosalicylic acid</td>
<td>-</td>
<td>~ 76</td>
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<tr>
<td>5-Nitro-ortho-toluenesulfonic acid</td>
<td>5-Amino-ortho-toluenesulfonic acid</td>
<td>HCl</td>
<td>93~95</td>
</tr>
<tr>
<td>1,3-Dichloro-4-nitrobenzene</td>
<td>2,4-Dichloroaniline</td>
<td>HCl</td>
<td>95.5</td>
</tr>
<tr>
<td>4-Chloro-2-nitroanisole</td>
<td>5-Chloroaniline</td>
<td>Formic</td>
<td>79.5</td>
</tr>
<tr>
<td>1-Chloro-2,5-dimethoxy-4-nitrobenzene</td>
<td>4-Chloro-2,5-dimethoxyaniline</td>
<td>-</td>
<td>89.8</td>
</tr>
<tr>
<td>2-Chloro-4-nitroaniline</td>
<td>2-Chloro-para-phenylenediamine</td>
<td>FeCl₃</td>
<td>92</td>
</tr>
<tr>
<td>Bis(para-nitrophenyl) sulfone</td>
<td>4,4'-Sulfonyldianiline</td>
<td>Formic</td>
<td>60</td>
</tr>
</tbody>
</table>

Besides iron, agents such as zinc, tin, titanium trichloride, stannous chloride, and ferrous sulfate can be used as the reducing agent [33]. In the laboratory reduction of nitro aromatics, stannous chloride is often used. Although this agent is expensive, it can be used to reduce some aromatic nitro compounds that contain groups sensitive to other reducing agents [34].
2.3.2 Catalytic hydrogenation

Catalytic hydrogenation is the most efficient method for the large scale manufacture of many aromatic amines. The catalysts used for the reduction are based on one or more of the group VIII metals such as platinum, rhodium-platinum, palladium, and nickel [28]. Other than hydrogen, hydrazine, formic acid, ammonium formate, or triethylammonium formate can be used for catalytic hydrogen transfer [33]. Hydrogenation of aromatic nitro compounds is very fast, and the limiting step is often hydrogen transfer to the catalyst. Therefore, usually more catalyst than actually necessary is used [35].

Heterogeneous and homogeneous catalysts can be used for hydrogenation of a nitro group, and they generally contain the same type of metal. In the case of homogeneous catalysts, the metals are present in the form of complexes or clusters with various organic or inorganic ligands. Because they are easy to use and recover for reuse, heterogeneous catalysts are preferred. In addition, the reduction can be carried out in the vapor phase, or in the liquid phase.

Aniline has been made commercially by numerous processes and catalysts such as supported copper, cobalt, palladium, and nickel have been employed. Because of their higher activity, the latter two require the use of inhibitors to prevent ring reduction. Vapor phase processes employ either fixed or fluid-bed reactors, and copper on silica is typically used as the catalyst [36]. In this case, aniline was produced in at least 99% yield [28]. For less volatile compounds, only liquid phase processing is appropriate. An example is the
reduction of 1-nitronaphthalene using platinum/activated carbon at temperatures of 150-250°C and pressures of 50-300 bars to produce 1-naphthylamine in 99.5% with 99.7% purity (Figure 21) [37].

![Figure 21. The catalytic hydrogenation of 1-nitronaphthalene.](image)

Interestingly, when hydrogenation of nitro aromatics is carried out under acidic conditions, the intermediate N-phenylhydroxylamines will undergo a Bamberger rearrangement to form para-aminophenols [38]. For the commercial synthesis of para-aminophenol from nitrobenzene, dilute sulfuric acid is usually employed and the Pt/C catalyst also gives a low yield of aniline byproduct (Figure 22) [38, 39].

![Figure 22. Hydrogenation of nitrobenzene under acidic conditions.](image)

Halo-anilines are important starting materials for the manufacture of dyestuffs and pigments by disazotization and coupling. These amines can be prepared by hydrogenating the corresponding nitro compounds. During the
reduction, dehalogenation must be avoided. This is facilitated by inhibiting or poisoning the catalysts [40]. Hildreth and Haslam [41] reported that molten chlorinated aromatic nitro compounds can be reduced in the melt using a catalyst and a controlled amount of thiophene as a dechlorination inhibitor (Figure 23). This method gives good yield (~96%).

![Figure 23. Hydrogenation of 2-chloro-4-nitroaniline.](image)

Although heterogeneous catalysts are widely used in the hydrogenation of nitro aromatics, their homogeneous counterparts can be used for small-scale syntheses. The latter type catalysts are also used in the synthesis of compounds containing multiple functional groups, which demonstrates their mild conditions and high selectivity. Various ruthenium complexes were found to be effective catalysts for the hydrogenation of nitro aromatics and its uniqueness is illustrated in the hydrogenation of only one of the nitro groups of \textit{para}-dinitrobenzene (Figure 24) [42].

![Figure 24. Selectively reduction of \textit{para}-dinitrobenzene by a homogeneous catalyst.](image)
2.3.3 Zinin reduction

The method of reducing aromatic nitro compounds with divalent sulfur is known as the Zinin reduction [43]. This reaction can be carried out in a basic medium by using sulfide, polysulfide, or hydrosulfide as the reducing agent (Figure 25).

\[ 4\text{ArNO}_2 + 6\text{Na}_2\text{S} + 7\text{H}_2\text{O} \rightarrow 4\text{ArNH}_2 + 3\text{Na}_2\text{S}_2\text{O}_3 + 6\text{NaOH} \]
\[ \text{ArNO}_2 + \text{Na}_2\text{S}_2 + \text{H}_2\text{O} \rightarrow \text{ArNH}_2 + \text{Na}_2\text{S}_2\text{O}_3 \]
\[ 4\text{ArNO}_2 + 6\text{NaSH} + \text{H}_2\text{O} \rightarrow 3\text{Na}_2\text{S}_2\text{O}_3 + 4\text{ArNH}_2 \]

Figure 25. Variation of the Zinin reduction reaction.

Compared to reduction by iron or catalytic hydrogenation, the advantage of the Zinin reduction of nitro aromatics is its low reduction potential and thus functional groups other than the nitro group are less likely to be reduced. Although this method is more expensive than the Béchamp process, it is still used to reduce compounds such as nitrophenols [44], nitroanthraquinones [45], and nitro azobenzenes (Figure 26) [46].

Figure 26. Some examples of the Zinin reduction reaction.
An important application of the Zinin reduction is the partial reduction of polynitro aromatic compounds to give a particular isomer in good yield. The reduction of meta-nitrobenzene to meta-nitroaniline offers an example (Figure 27) [47].

![Figure 27. Selective reduction of meta-dinitrobenzene.](image)

2.3.4 Alkaline reduction of nitro compounds

When nitrobenzene is treated with finely divided zinc in alkaline solutions, it gives a hydrobenzene that is converted to benzidine in cold hydrochloric acid solution.

The reaction process may initially involve the evolution of hydrogen, and then the formed zinc hydroxide reacts with sodium hydroxide to give a zinc oxide salt (Figure 28) [29].

\[
\text{Zn} + 2\text{H}^+ + 2\text{OH}^- \rightarrow \text{Zn(OH)}_2 + \text{H}_2 \\
\text{Zn(OH)}_2 + 2\text{NaOH} \rightarrow \text{Na}_2\text{ZnO}_2 + 2\text{H}_2\text{O}
\]

![Figure 28. The reactions of Zn under alkaline conditions.](image)

Thus the reduction of nitrobenzene to the hydrazo compound may be represented by the following equation:
The reduction gives anilines as one of the by-products and is influenced by the reaction conditions such as temperature. Freeman and coworkers [48] reported that the desired reduction to the hydrazo intermediate takes place at 70-80°C, and that increasing the temperature to 115°C gave only the corresponding aniline (Figure 29).

Figure 29. Alkaline reduction of nitro compounds using Zn/NaOH.

The benzidine rearrangement can also be accomplished by catalytic hydrogenation under alkaline conditions, and then followed by acidic rearrangement [49]. In this case, 3,3'-dimethylbenzidine can be obtained from ortho-nitrotoluene in 60-70% yield. The key step in this process is the formation of hydrazo intermediates from the corresponding nitro compounds [50].
3 Direct dyes

3.1 Introduction

Direct dyes are colorants that are able to dye cellulose fibers without the need for a pre-treatment of the fibers with mordants [51]. The first direct dye for cotton was Congo Red which was discovered by Professor Paul Boettiger in 1884 [52]. The direct dye molecule can interact with cellulose via hydrogen bonding and van de Waals forces. Unfortunately, this type bonding of direct dyes to cellulose is not always sufficient to yield good wash fastness.

Direct dyes are economical to manufacture, and they provide a simple and relatively inexpensive way of dyeing cellulose fibers. In addition to their use on cotton and rayon, direct dyes are important in dyeing of leather and the coloration of paper [53].

Direct dyes are available in all colors and in large numbers. However, most of these dyes give dull shades. This is due to the broad absorption in the visible region, which is due to an extended conjugation system and multiple chromophoric and auxochrome groups in the dye structure.

Benzidine-based direct dyes once constituted an important category of direct dyes. However, they pose a significant risk to human health and are no longer produced in most countries. Research in the potential replacements for benzidine has been carried on by scientists in a number of laboratories [54, 55, 56].
3.2 The structure of direct dyes

The majority of direct dyes are azo compounds, mostly disazo and trisazo in nature. Direct dyes are long, narrow, and flat in molecular structure, which allows them to enter the pores of cellulose and interact with cellulose chains readily. The affinity between dye and fiber can be increased by increasing the size of the conjugated system by introducing further azo groups [51]. The long, linear and planar structure also allows the dye molecules to have multiple numbers of interactions with the polymer chains [57].

The presence of sodium sulfonate (-SO$_3$Na) groups gives most direct dyes the water solubility that is important for dyeing. When the dye is dissolved in water, ionization to –SO$_3^-$ takes place.

Since the degree of direct dye exhaustion from the dye bath to the fiber depends on the relative affinity of the dye to these two heterogeneous phases, direct dyes usually contain a minimum number of -SO$_3$Na groups. Also, minimum solubility allows an acceptable level of wash fastness to be achieved [58].

Other substituents that direct dyes contain are hydroxyl and amino groups. These electron-rich groups are capable of forming hydrogen-bonds with the hydroxyl groups on cellulose fiber. An example of a dye containing such groups is C.I. Direct Blue 2.

\[
\begin{align*}
\text{OH} & \quad \text{H}_2\text{N} & \quad \text{SO}_3\text{Na} \\
\text{N} & \quad \text{N} & \quad \text{N} & \quad \text{OH} & \quad \text{NH}_2 \\
\text{C.I. Direct Blue 2} & & & & \text{NaO}_3\text{S} & \quad \text{SO}_3\text{Na}
\end{align*}
\]
3.3 Direct dye synthesis

3.3.1 Diazotization reaction

Azo direct dyes are synthesized by diazotization of one or more primary aromatic amines followed by coupling of the resultant diazonium salt with an activated system. The most common method used to convert primary aromatic amines to diazonium salts is to treat the amine with nitrous acid (HNO₂) at 0-5°C (Figure 30) [59]. Nitrous acid is generally obtained by combining an aqueous mineral acid such as hydrochloric acid with sodium nitrite (NaNO₂). The nitrous acid reacts with aniline to generate an N-nitroso intermediate and the latter then tautomerizes to the diazo hydroxide. Protonation of the hydroxyl group followed by the elimination of water generates the diazonium salt. Weakly basic amines such as para-nitroaniline and 2, 4-dinitroaniline are diazotized in concentrated sulfuric acid, where the nitrosating agent is NO⁺HSO₄⁻ (nitrosyl sulfuric acid) [10, 59].

Figure 30. The mechanism of diazotization process of aniline.
3.3.2 Coupling reaction

The coupling reaction is an electrophilic aromatic substitution process (Figure 31) [51, 59]. Because diazonium ions are relatively weak electrophilic reagents, only aromatic compounds that carry electron-donating groups such as NH$_2$, OH, and NHR can be used as coupling components. Coupling with amines under slightly acidic conditions provides stability for the diazonium salt without deactivating the amino group by protonation. Phenols and naphthols are coupled under alkaline conditions. By increasing the pH, the hydroxyl group is converted to the phenolate ion, which is more electron-donating. Thus, the coupling reaction rate is increased.

![Diagram of the coupling reaction of diazonium salt with phenolate ion and aniline.](image-url)

Figure 31. The coupling reaction of diazonium salt with phenolate ion and aniline.
3.3.3 Approaches

There are several ways for the diazonium salts and the coupling components to produce numerous direct azo dyes. A “convenient shorthand” classification known as Winther symbols [53] uses the arrow symbol combined with the following symbols to indicate the nature of the reacting components:

- **A**: Diazonium component (amine).
- **E**: Coupler with one coupling center.
- **D**: Tetrazotizable amine.
- **M**: Coupler with diazotizable amine.
- **Z**: Coupler that couples twice.

Using this nomenclature, the synthesis of monoazo dyes can be represented by $A \rightarrow E$. More complex azo dyes can be also built up by combining two or more diazotization and coupling sequences.
3.3.3.1 Monoazo direct dyes

Many of the monoazo dyes still used today are based on J-acid (6-amino-1-naphthol-3-sulfonic acid) as the coupler. One example is C.I Direct Red 118 [60].

The coupler was prepared by condensation of J-acid with 3-nitrobenzoyl chloride followed by reduction of the nitro group, and coupling with benzenediazonium chloride in alkaline solution to complete the synthesis of the dye (Figure 32).

![Chemical structure of C.I. Direct Red 118](image)

Figure 32. The synthesis of C.I. Direct Red 118.

3.3.3.2 Disazo direct dyes

Disazo direct dyes can be synthesized in several ways. According to the Winther symbols, they can be classified in the following categories. Thus, \( A \rightarrow Z \leftarrow A' \) molecules, also known as primary disazo dyes, are formed first by diazotization of compound \( A \) and coupling with \( Z \). The synthesis is completed by
disazotization of A’ and coupling with A→Z. An example is C.I. Direct Orange 18, which is obtained from resorcinol coupled with diazotized dehydrothio-para-toluidinesulfonic acid and benzenediazonium chloride (Figure 33) [60].

![Chemical structure of C.I. Direct Orange 18]

**Figure 33. The synthesis of C.I. Direct Orange 18.**

Secondary disazo dyes classified as A→M→E. About 150 direct dye structures are listed in this class. Type A components are selected from substituted anilines or naphthamines. M is a coupler containing a diazotizable amino group and is chosen from aniline or α-naphthylamines, and E is chosen from J-acid, H-acid, Chicago acid (8-amino-1-naphthol-5, 7-disulfonic acid), γ-acid (7-amino-1-naphthol-3-sulfonic acid), and Schaeffer’s acid (2-naphthol-6-sulfonic acid). The most useful dyes are those having E components based on J-acid itself or as an N-substituted derivative. An example is the synthesis of C.I. Direct Red 61 (Figure 34) [60]. Sulfaninic acid was diazotized and coupled with
aniline under weak acidic conditions to form A→E. the later is diazotized and coupled with N-substituted J-acid under alkaline conditions.

The third type of disazo dyes can be classified as E←D→E'. Many dyes of this type had been based on benzidine. Other diamines that have been employed in direct dye synthesis are 2,2'-dimethylbenzidine (ortho-toluidine), 2,2'-dichlorobenzidine, 2,2'-dimethoxybenzidine (dianisidine), 4,4'-diaminodiphenylurea, 4,4'-diaminodiphenylthiourea, 1,5-diaminonaphthalene, and 4,4'-diaminostilbene. Type E components have been chosen from substituted naphthols and phenols such as J-acid, H-acid, and salicylic acid [61]. One dye belonging to this type is C.I. Direct Blue 14 (Figure 35) [62].

Figure 34. The synthesis of C.I. Direct Red 81.
3.3.3.3 Trisazo direct dyes

One important example of trisazo direct dye is C.I. Direct Black 38. Its structure can be illustrated by $E \leftrightarrow D \rightarrow Z \leftrightarrow A$. The synthesis of this dye is given in Figure 36 [63]. Benzidine was tetrazotized and coupled with H-acid at 10-12°C for several hours. Then the diazonium salt of aniline was added to the above product at 5°C. Finally, pure meta-phenylenediamine was added to complete the reaction.
3.3.3.4 Polyazo direct dyes

Polyazo direct dyes are less commonly used today. The dyes produced have dull colors, such as brown, navy blue, and black. One such dye is C.I. Direct Black 19. The synthesis of this dye is shown in Figure 37 [64].
Figure 37. The synthesis of C.I. Direct Black 19.
3.4 Application of direct dyes

The objective of direct dyeing is to obtain a uniform coloration of the mass of fibers constituting the textile material and to match a pre-specified color. Dyeing with direct dyes is carried out under neutral to slightly acidic solution conditions. The dyebath is gradually heated to the boil and held at this temperature to maximize dye diffusion into the fiber. High temperature also helps to promote diffusion and leveling of the dyes. Without the presence of electrolyte, some direct dyes will not dye cellulose at all, while all commercial direct dyes are adsorbed readily if salt is added to the dye bath. This is because of the negative charge (zeta potential) on the fiber surface, which causes repulsion of dye anions at the fiber surface. Although the positive sodium ions of the dyes can interact with the anionic fiber surface, their concentration is insufficient to neutralize the negative surface charge. The addition of electrolyte to the dye bath neutralizes the surface charge, allowing the dye anions to approach the fiber. Suitable electrolytes are common salt (sodium chloride) or Glauber’s salt (sodium sulphate) [65].

The neutralizing effect is not the only reason for the ability of the added salt to drive the direct dye into the fiber. The introduction of a strong electrolyte to a dye bath can significantly reduce the solubility of the dyes in the bath and cause the aggregation of dyes [57]. In practice, dye bath exhaustion is controlled by the addition of electrolyte and the regulation of the dye bath temperature.
3.4.1 Dyeing behavior

Direct dyes having similar chemical structures may have quite different application and fastness properties, and vary greatly in their behavior during the dyeing process. This observation led to the classification of direct dyes according to their dyeing properties. The most common grouping of direct dyes is the Society of Dyers and Colourists (SDC) system. Based on their leveling ability and their response to increases in the dyeing temperature and to addition salt during exhaust dyeing, direct dyes are divided into the following three classes [66]:

Class A. These dyes are relatively low molecular weight mono- and bisazo dyes with good water solubility, which minimizes aggregation in solution. They have good leveling or migration properties. Their substantivity to the fiber and salt sensitivity are low, so they require considerable amounts of salt for good exhaustion and have low washfastness.

Class B. These dyes are often bisazo or trisazo dyes and have higher molecular weights than class A dyes. They are salt sensitive but are not self-leveling, since leveling can be controlled by adding salt, they are called salt controllable dyes.

Class C. These dyes are not self-leveling and are highly sensitive to salt. Temperature control is required for leveling. These dyes usually are polyazo dyes with few sulfonate groups and have high affinity to cellulose. They have a high degree of aggregation in the solution which is accentuated by electrolyte presentation. So leveling is achieved by a careful and gradual increase in dye bath temperature.
3.4.2 Dyeing Methods

Direct dyes are applied by both exhaust and continuous methods. In practice, the dyeing procedure employed depends on the type of equipment, the fiber form and the exhaustion rate of the dyes chosen for dyeing. The exhaust dyeing procedures in laboratory may be described by the following steps [66, 67, 68]:

1. The fabric is well wet in water, and put into the dye bath containing appropriate amount of dye dissolved in water.
2. With good stirring, the dye bath is gradually heated to the boil or to the highest practical temperature, and held there for 30-60 minutes. The dye bath volume is maintained by frequent additions of hot water.
3. Common salt or Glauber’s salt is added to enhance the dye exhaustion, and the dye bath is cooled to a predetermined temperature, since most direct dyes have more affinity for the cellulose below 93°C.
4. The dyed fabric is rinsed with cold water to remove unfixed dye.
5. Usually an after-treatment is applied at the end of dyeing to improve wash fastness. Then the fabric is dried.

Continuous dyeing is most economic for production of large amounts of dyed fabric in a single color. Most continuous dyeing processes can be divided into four stages [69]:
1. The direct dye solution is applied to the fabric by padding. The pad bath conditions should be chosen carefully to avoid tailing when dye mixtures are used. In general, the best results are obtained with Class B dyes [58].

2. Dye fixation, this is usually done in hot air or steam.

3. Wash off unfixed dyes and auxiliary chemicals.

4. The dyed goods are dried using steam heated cylinders.

Besides fully continuous dyeing, there are a number of semi-continuous dyeing processes in which the fabric is continuously impregnated in the dye bath, while the fixation of the dyes is actually a batch process.

3.4.3 After treatment

Direct dyes are frequently after-treated to overcome their poor wash fastness. Some after-treatments can cause a change in hue that impedes shade correction and color matching, and the process can be difficult and costly to carry out. Many of the direct dye after-treatments have limited utility and reactive dyes are usually used to give high wash fastness.

Direct dyes that have a free amino group can be diazotized and then developed with β-naphthol, 2, 4-diaminotoluene or other coupling agents (Figure 38) [67, 69]. Since the diazotized dye is sensitive to light and heat, the coupling step is immediately carried out. The size of the dye molecule is increased in this
procedure, and wash fastness is greatly improved. It should be mentioned that introducing a new azo group causes a bathochromic shift in the color of the dye. Amino resins and crosslinking finishing agents usually improve the wet fastness of the direct dyes [69, 70, 71]. The reaction of cellulose chains with resins/crosslinking agents coats the dyed fiber with a layer of cross-linked resin and thus restricts the dye’s ability to be washed out the fiber.

Another way to improve the wash fastness of direct dyes involves after-treatment with cationic fixing agents [72, 73, 74]. The cationic compounds interact with the sulfonate groups in direct dyes to form a large, complex salt molecule with low solubility (Figure 39). This makes the dye more difficult to wash out.

Figure 38. After-treatment by coupling with diazonium salt.
Certain direct dyes such as C.I. Direct Green 34 containing hydroxyl groups ortho and ortho’ to the azo group can form a sparingly soluble complex with metal ions, e.g., Cu$^{2+}$, Cr$^{3+}$, and Co$^{2+}$ (Figure 40) [75]. The light fastness and wash fastness of direct dyes can be improved by this treatment (Table 4). Copper is not environmentally friendly. In order to minimize the amount of Cu$^{2+}$ in the effluent, most metallizable dyes are pre-metallized by the dye manufacturers.
Figure 40. Formation of the copper complex of C.I. Direct Green 34.

Table 4. Copper complexes of direct dyes and their fastness properties.

<table>
<thead>
<tr>
<th>Dye name</th>
<th>S.D.C washfastness</th>
<th>S.D.C. Lightfastness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Treated</td>
</tr>
<tr>
<td>C.I. Direct Blue 156</td>
<td>4</td>
<td>4-5</td>
</tr>
<tr>
<td>C.I. Direct Orange 94</td>
<td>-</td>
<td>3-4</td>
</tr>
<tr>
<td>C.I. Direct Red 174</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>C.I. Direct Blue 8</td>
<td>2</td>
<td>2-3</td>
</tr>
<tr>
<td>C.I. Direct Blue 159</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td>C.I. Direct Blue 156</td>
<td>4</td>
<td>4-5</td>
</tr>
<tr>
<td>C.I. Direct Blue 51</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>
4. Azo Pigments

4.1 Introduction

While the chromophore in azo pigments is identical to that in azo dyes, these colorants are insoluble in water. Because of the relatively easy synthesis and the good technical performance, azo pigments constitute the largest group of organic pigments, both with respect to the number of different chemical structures and to the total production volume [51]. They are mostly yellow, orange, and red colorants. Azo pigments are so diverse in type, tinctorial characteristics, and physical properties that their application is found in nearly all fields of color use.

The first commercial use of the diazotization and coupling reaction in the field of azo pigments involved producing colorants such as C.I. Pigment Red 1 [76]. Pigments color various substrates by being physically attached to their surfaces or by incorporation at the polymer stage. Their application is more complex than coloration with dyes, since they have to go through a molecular disperse phase.

C.I. Pigment Red 1
4.2 Crystal structures

The early work on the crystal structure analysis of azo pigments was completed on β-naphthol pigments by single crystal X-ray diffraction techniques [77]. The crystal structures of azo pigments derived from acetoacetanilide have also been determined by single crystal X-ray diffraction techniques [78,79]. By determining the hydrogen atom positions and the various bond lengths, which are two key factors in deciding the tautomeric structure, it was pointed out that these pigments also exist in hydrazone form (Figure 41). In this case, the hydrazone tautomer has three intramolecular hydrogen bonds, causing the pigment molecule to be planar and thus more stable. Christie and coworkers investigated the crystal structures of some diarylide yellow pigments [80]. The results showed that these pigments also exist in the bisketo-hydrazone tautomeric form. The intramolecular hydrogen bonding was also observed.

![Diagram of Azo and Hydrazo Forms](image)

**Figure 41. Tautomerism in azo pigments derived from acetoacetanilide.**
Unlike dyes, pigments remained as crystalline particles throughout the application process. Thus, the properties such as the hue and the stability of the pigment to solvent, heat and light is determined by the crystal structures. By using modified methods of synthesis and various finishing techniques, the technical properties of a particular pigment can be dramatically changed as a result of the modification in its particle size which determines its physical form or crystal structure.

Carr [81] concluded that many factors can affect the physical properties of the pigments. These factors can be the method of coupling, pH value, reaction temperature, the concentration of the reactants, the efficiency of the mixing of the reactants, the rate of coupling, the presence of resins and/or surfactants and/or solvents during the coupling, the addition of resins and/or surfactants and/or solvents after coupling, the heat treatment of the aqueous pigment slurry, the drying method and the drying temperature. These factors may modify the physical properties of the particles by preventing the crystals from growing to excessively large particles or modify the particle surfaces so that they are more readily wetted by various media.

One of the most important activities in organic pigment technology is the continual quest for pigments with improved color strength and brightness. It was pointed out that the color strength increases with decreasing particle size in the submicron region [82]. The color strength passes through a maximum within the particle size range from 0.1 to 0.01 micron. Thus it sets a limit to the potential
color strength of pigment dispersions, and it is also important to know how close existing pigment types approach this limit.

Azo pigments with particle size of the order of 0.1 micron or less are highly transparent. Although this is desirable in many circumstances, when it comes to the application of pigments where good hiding power or opacity is required, transparency becomes a disadvantage. To increase the opacity, it requires that pigment particles should have larger size than usual. To give optimum light scattering for the desired opacity, a large proportion of pigment particles should be in a narrow size range around 0.2 to 0.6 micron [83]. Therefore, opacity is achieved at the expense of color strength.

4.3 Classifications

Azo pigments may be classified according to the chromophores they contain. In addition, azo pigments are distinguished by the nature of the coupling components, which have a significant influence on the pigment properties, such as hue and fastness [84].

4.3.1 Acetoacetanilide-based pigments

Acetoacetanilide derivatives are typical coupling components for yellow and orange monoazo and disazo pigments. Pigments belonging to this group are usually called “Hansa Yellows” [69]. The first commercial Hansa Yellow was produced in 1910 [6]. Some Hansa Yellows are listed in Table 7.
This type of yellow pigments shows excellent fastness to light, water, acid, and alkali and has good resistance to weathering. This is due to intramolecular hydrogen bonding formed in the hydrazone form of the pigment. They bleed in most solvents, migrate in many plastic materials, and are sensitive to heat. These results suggest that mainly van der Waals’ forces are involved in intermolecular interactions.

Table 5. Some commercial Hansa Yellow pigments.

<table>
<thead>
<tr>
<th>C.I. Pigment Yellow</th>
<th>X</th>
<th>Y</th>
<th>Z</th>
<th>W</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NO2</td>
<td>CH3</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>2</td>
<td>NO2</td>
<td>Cl</td>
<td>CH3</td>
<td>CH3</td>
</tr>
<tr>
<td>5</td>
<td>NO2</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>6</td>
<td>NO2</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
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<td>9</td>
<td>NO2</td>
<td>CH3</td>
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<td>65</td>
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<td>OCH3</td>
<td>OCH3</td>
<td>H</td>
</tr>
<tr>
<td>74</td>
<td>OCH3</td>
<td>NO2</td>
<td>OCH3</td>
<td>H</td>
</tr>
</tbody>
</table>

The most important disazo yellow pigments are called diarylides, and are made from benzidine and its analogs [85]. The first pigment of this family appeared on the market in 1938 and an example is C.I. Pigment Yellow 63.
The pigment is synthesized by the tetrazotization of 3,3'-dichlorobenzidine, then coupling with two equivalents of acetoacetanilide (Figure 42) [86]:

![Chemical structure of C.I. Pigment Yellow 63](image)

Figure 42. Synthesis of C.I. Pigment Yellow 63.

The diarylide pigments show considerably superior tinctorial strength and transparencies compared to the monoarylide pigments, and are widely used in printing inks. They also exhibit improved fastness to solvents and heat, which is a
consequence of increasing their molecular size [87]. However, their light fastness is inferior to the monoaryldies.

4.3.2 Pyrazolone-based pigments

As coupling components, pyrazolone couplers render pigments a slightly redder shade than acetoacetarylamines, while the two types of pigments have similar fastness properties [88]. These pigments encompass yellow, orange, and red colors, and their preparation is similar to that of arylide azo pigments.

An example of the mono azo pyrazolone pigment is C.I. Pigment Yellow 10.

\[
\begin{align*}
\text{C.I. Pigment Yellow 10} \\
\end{align*}
\]

Disazo pyrazolone pigments have better solvent-fastness than their monoazo counterparts and are used more widely. An important pigment in this series is C.I. Pigment Orange 13. This pigment is suitable for application in rubber due to its high color intensity and soft texture [84].

\[
\begin{align*}
\text{C.I. Pigment Orange 13} \\
\end{align*}
\]
4.3.3 β-Naphthol-based pigments

The first β-naphthol azo pigment can be traced to 1880, and was produced by coupling diazotized para-nitroaniline with β-naphthol on cotton [89]. These monoazo pigments possess colors that are yellowish and medium red to bordeaux, maroon, and violet. Their good durability, excellent chemical resistance, and acceptable non-bleeding properties make them useful for many fields, including inks for the packaging industry. C.I. Pigment Red 22 is a representative of these pigments. It is synthesized by first diazotization of para-nitro-ortho-toluidine and then coupling it with the anilide of β-hydroxynaphthoic acid (BON). To get good pigments, the coupling reaction is usually carried out in a neutral or a buffered acid medium [84].

![C.I. Pigment Red 22](image)

BON acid is used as the coupling component for in combination with various diazonium salts to form red pigments [90, 91]. The sodium salt forms of the resultant colorants dissolve easily in water. They are then precipitated by the addition of calcium, barium, strontium, or manganese anions to render the required insolubility [92]. Some of these pigments are shown in Figure 43. They
have high color strength and brightness, good light fastness, high bleeding resistance, and high dispersing properties. These excellent physical properties enable them to be used as printing inks.

![Diagram of the chemical structure of pigments.]

<table>
<thead>
<tr>
<th>C.I. Pigment Red</th>
<th>R₁</th>
<th>R₂</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>48:1</td>
<td>CH₃</td>
<td>Cl</td>
<td>Ba</td>
</tr>
<tr>
<td>48:2</td>
<td>CH₃</td>
<td>Cl</td>
<td>Ca</td>
</tr>
<tr>
<td>48:3</td>
<td>CH₃</td>
<td>Cl</td>
<td>Sr</td>
</tr>
<tr>
<td>48:4</td>
<td>CH₃</td>
<td>Cl</td>
<td>Mn</td>
</tr>
</tbody>
</table>

Figure 43. Red azo lake pigments.
5. Mutagenicity/Carcinogenicity of aromatic amines and azo colorants

5.1 Introduction

Various organic substances can undergo adverse interactions with DNA to produce a hereditable change in the cell or organism. Such changes will cause birth defects, carcinogenesis, teratogenesis, and other types of diseases. Consequently, studies involving the relationships between chemical structures and mutagenicity/carcinogenicity have long been of interest [93]. The most commonly used method for generating mutagenicity data is the Salmonella assay, which is also known as the Ames test [94]. This method is used as a cost-effective and relatively quick way to predict the potential carcinogenicity of chemical compounds.

Chemicals can damage DNA by undergoing reactions that form covalent bonds to DNA and thus initiate mutations leading to carcinogenicity. In this regard, the chemicals must first reach DNA to have the ability to react with DNA. However, if chemicals are “too reactive”, i.e. they can polymerize or hydrolyze spontaneously, or react with non-critical cellular constituents before they can reach target DNA, they are not carcinogenic [95].

Besides its chemical structure, the carcinogenicity of a compound is also dependent on its physical properties, such as molecular weight, physical state, and solubility.
5.2 Aromatic amines

Aromatic amines are important intermediates for the synthesis of organic colorants and some of them have been recognized as carcinogens since 1895, when Rehn found that certain workers in the dye industry developed bladder cancer as a consequence of their long exposure to aromatic amines of the type shown in Figure 44 [96].

Figure 44. Examples of carcinogenic aromatic amines.

The carcinogenicity of aromatic amines has been attributed to their metabolic conversion to the corresponding electrophiles, which may interact with DNA to form a covalent bond. Aromatic amines (a) undergo a two-stage metabolic activation for carcinogenicity. The first step is $N$-hydroxylation to form an arylhydroxylamine (or an arylhydroxylamic acid) (b) and the second step involves $O$-acylation (the acyl group maybe acetyl) to yield acyloxy amines (c)
that are highly reactive towards cellular nucleophiles such as DNA (Figure 45) [95, 97, 98].

Figure 45. The metabolism of aromatic amines.
The departure of the acyloxy group then leads to formation of the arylnitrenium ion (e) and its resonance structure (f), the carbocation. The arylnitrenium ion (e) is considered as the ultimate carcinogenic form of aromatic amines. So the mutagenicity is a function of the leaving potential of the acyloxy group which is dependent on the strength of the -N-O bond. A compound often has higher carcinogenicity potential if the reactive electrophile can be stabilized by resonance. This is because the reactive species has a better chance of remaining active during transport from the site of activation to the target macromolecule [95].

5.2.1 Aniline derivatives

Aniline derivatives are frequently used as dye intermediates, and aniline itself is one of the most widely used amines. Aniline is nonmutagenic in the Ames test; however, it is a carcinogen to laboratory animals such as rats [99].

Mutagenicity of mono-substituted anilines can be influenced by the electronic effects of the substituent groups. The presence of the electron-donating group ortho- or para- to the amino group will stabilize the arylnitrenium ions, while placement of electron-withdrawing group at these positions will destabilize the arylnitrenium ions and lower genotoxicity responses [98]. This may be illustrated by comparing mutagenicity of ortho-toluidine and ortho-anisidine to ortho-nitroaniline. ortho-Toluidine and ortho-anisidine have ring activating groups, whereas nitro group functions as a deactivating group. ortho-Toluidine and ortho-anisidine are carcinogens, but are negative in the Ames
The nitro-substituted anilines such as ortho-nitroaniline are not mutagenic. The mutagenicity of meta- and para-nitroaniline can not be explained by the electronic effects, but may due to the transformation of the nitro group itself to an active species by other enzyme systems.

![Chemical structures of ortho-nitroaniline, ortho-Toluidine, and ortho-Anisidine](image)

It has been shown that substitution ortho to amino groups could prevent or lower mutagenicity by introducing steric hindrance to the required enzymatic oxidation of the aromatic amines. Several di-substituted and tri-substituted anilines were tested for mutagenicity to develop this concept. 2,6-Diethylaniline and 2,4,6-trimethylaniline were weakly mutagenic in strain TA100 when 20% S9 mixture was used.

Zimmer and coworkers also reported that 2,3-, 2,4-, 2,5-, 3,4-dimethylaniline, and 2-methyl-4-fluoroanilline 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 were strong mutagens in the Salmonella typhimurium mutagenicity assay.
These results indicated that mutagenicity is a general property of hydrophobic aniline derivatives containing methyl and/or methoxy groups in the ortho positions.

<table>
<thead>
<tr>
<th></th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>Mutagenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>CH₃</td>
<td>CH₃</td>
<td>H</td>
<td>H</td>
<td>weak</td>
</tr>
<tr>
<td>4</td>
<td>CH₃</td>
<td>H</td>
<td>CH₃</td>
<td>H</td>
<td>weak</td>
</tr>
<tr>
<td>5</td>
<td>CH₃</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
<td>weak</td>
</tr>
<tr>
<td>6</td>
<td>H</td>
<td>CH₃</td>
<td>CH₃</td>
<td>H</td>
<td>weak</td>
</tr>
<tr>
<td>7</td>
<td>H</td>
<td>CH₃</td>
<td>H</td>
<td>F</td>
<td>weak</td>
</tr>
<tr>
<td>8</td>
<td>CH₃</td>
<td>H</td>
<td>Cl</td>
<td>H</td>
<td>strong</td>
</tr>
<tr>
<td>9</td>
<td>CH₃</td>
<td>H</td>
<td>Br</td>
<td>H</td>
<td>strong</td>
</tr>
<tr>
<td>10</td>
<td>Cl</td>
<td>H</td>
<td>CH₃</td>
<td>H</td>
<td>strong</td>
</tr>
<tr>
<td>11</td>
<td>Br</td>
<td>H</td>
<td>CH₃</td>
<td>H</td>
<td>strong</td>
</tr>
<tr>
<td>12</td>
<td>CH₂CH₃</td>
<td>H</td>
<td>Cl</td>
<td>H</td>
<td>strong</td>
</tr>
</tbody>
</table>

Anilines containing hydrophilic groups such as carboxyl or sulfonic groups show little or no mutagenicity. This is because the hydrophilic character leads to poor absorption of the anilines in lipophilic tissue (13, 14), and the resultant anilines are easily excreted if absorbed [95].

13 (para-Aminobenzoic acid) 14 (Sulfanilic acid)
5.2.2 Phenylendiamines

Phenylenediamines are used as coupling components for azo dyes and as the main aromatic amine in hair dye formulations [104]. Some of the important diamines are shown below (15-18). All of them are mutagenic in the Ames test.

Shahin and coworkers [105,106] reported that the incorporation of bulky substituents ortho to one of the amino groups of meta-phenylenediamine (19-22) lowered or removed its mutagenicity. Freeman and coworkers [107] evaluated para-phenylenediamines (23-27) with alkoxy groups ortho to one of the amino groups. They found that as the size of the substituent increased, mutagenicity decreased.

\[ R \]

\[
\begin{align*}
19 & \quad \text{CH(CH}_3)_2 \\
20 & \quad \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3 \\
21 & \quad \text{OCH}_2\text{CH}_2\text{CH}_2\text{CH}_3 \\
22 & \quad \text{OCH}_2\text{CH}_2\text{OH}
\end{align*}
\]
5.2.3. Benzidine and its analogs

Before the 1970s, benzidine and its analogs (28-30) were among the most important intermediates used in dye and pigment manufacturing [106,108]. All of these benzidine derivatives are carcinogenic in laboratory animals and mutagenic in the Ames test [109]. Messerly and coworkers [110] assumed that an increase in mutagenicity correlated to a decrease in basicity in both TA100 and TA98.
Introducing other groups ortho to the amino groups in benzidine also influences mutagenicity. 3,3’-Biphenyldicarboxylic acid (31) and its sodium salt are much less carcinogenic than benzidine and analogs 28 and 30 [109]. This suggests that intramolecular hydrogen bonding formed between the ortho carboxyl group and amino group reduces the metabolic potential of the amino groups. Ashby and coworkers [111] reported that the mutagenicity of benzidine could be lowered or removed by incorporation of sulfonic groups (32).

3,3’,5,5’-Tetramethylbenzidine (33) is non-mutagenic in the Ames test [112, 113]; Ashby and coworkers [111] reported that 3,5-dimethyl-4-aminobiphenyl (34) was nearly as mutagenic as 4-aminobiphenyl. They argued that although the methyl groups caused a steric effect to the amino group, the electron-donating properties of the methyl groups assist the formation of an electrophilic species in the molecule during pre-incubation. Interestingly, Boche and coworkers [114] recently reported that compound 34 was non-mutagenic. They concluded that this was because the double-alkylation ortho to the amino group causes a steric hindrance which prevented the metabolism of this compound. The conflicting results given by these authors may due to the different pre-incubation methods.
5.3 Azo colorants

Azo dyes and pigments are extensively used in the textile, printing, leather, coating, ink, drug, and food industries. These compounds may be activated by a number of reduction and oxidation processes before interacting with DNA [115]. For instance, the azo group is metabolized by reductive-cleavage to aromatic amines, followed by oxidation of free amino groups, or the azo linkage to produce electrophilic molecules.

The cleavage of azo compounds has been known since the discovery of the first sulfanilamide drug in 1935 (Figure 46) [116]. The azo group was reduced to the corresponding amino groups by enzymes such as cytochrome oxidase.

![Figure 46. The metabolism of Prontosil Red by intestinal enzymes.](image-url)
5.3.1 Monoazo colorants

Azobenzene is a simple example of this family of compounds. It has been designated as a carcinogen by National Cancer Institute (NCI). Different substituent groups on the aromatic rings of these colorants influence their mutagenic properties.

Several nitro-containing azo dyes are reported to be mutagenic. Brown and coworkers [117] reported that C.I. Mordant Orange 1 (35) was a direct-acting mutagen. 4-Nitroazobenzene (36) and 3-methoxy-4-nitrobenzene (37) were also mutagenic [118]. The nitro group can be reduced by enzymes and activated to form groups that covalent bonding with DNA.

![Chemical structures](image)

Many benzene amine-containing azo dyes have been evaluated for mutagenicity and 4-aminoazo disperse dyes (38-42) were found to be mutagenic [119, 120].

![Chemical structures](image)
On the other hand, 2-aminoazo benzene/naphthalene disperse dyes were reported to be nonmutagenic [121]. Gregory [122] suggested that this is due to the formation of benzotriazoles from the intermediate nitrenium ions instead of interaction with DNA (Figure 47).

![Figure 47. The formation of benzotriazoles as metabolic products, where R=H, Me.](image)

Freeman and coworkers [123] studied the effect of alkoxy substituents on the mutagenicity of some aminoazobenzenes and their reductive-cleavage products (43-57). In general, mutagenicity decreased as the size of the substituent ortho to the primary amino group was increased. Substitution of hydrogen atom by an N(CH$_2$CH$_3$)$_2$ or N(CH$_2$CH$_2$OH)$_2$ group in the 4'-position significantly lowers or removes the mutagenicity. Also, the reductive-cleavage products of the corresponding mutagenic dyes were either nonmutagenic or considerably less mutagenic than the dyes themselves in the standard mutagenicity assay. Thus, the mutagenicity of aminoazobenzene dyes can not be solely accounted for from the properties of their reductive-cleavage products.
<table>
<thead>
<tr>
<th>R1</th>
<th>R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCH₃</td>
<td>H</td>
</tr>
<tr>
<td>OCH₂CH₃</td>
<td>H</td>
</tr>
<tr>
<td>OCH₂CH₂CH₃</td>
<td>H</td>
</tr>
<tr>
<td>OCH₂CH₂CH₂CH₃</td>
<td>H</td>
</tr>
<tr>
<td>OCH₂CH₂OH</td>
<td>H</td>
</tr>
<tr>
<td>OCH₃</td>
<td>N(CH₂CH₃)₂</td>
</tr>
<tr>
<td>OCH₂CH₃</td>
<td>N(CH₂CH₃)₂</td>
</tr>
<tr>
<td>OCH₂CH₂CH₃</td>
<td>N(CH₂CH₃)₂</td>
</tr>
<tr>
<td>OCH₂CH₂CH₂CH₃</td>
<td>N(CH₂CH₃)₂</td>
</tr>
<tr>
<td>OCH₂CH₂OH</td>
<td>N(CH₂CH₃)₂</td>
</tr>
<tr>
<td>OCH₃</td>
<td>N(CH₂CH₂OH)₂</td>
</tr>
<tr>
<td>OCH₂CH₃</td>
<td>N(CH₂CH₂OH)₂</td>
</tr>
<tr>
<td>OCH₂CH₂CH₃</td>
<td>N(CH₂CH₂OH)₂</td>
</tr>
<tr>
<td>OCH₂CH₂CH₂CH₃</td>
<td>N(CH₂CH₂OH)₂</td>
</tr>
<tr>
<td>OCH₂CH₂OH</td>
<td>N(CH₂CH₂OH)₂</td>
</tr>
</tbody>
</table>
Certain dyes based on β-naphthol, such as C.I. Solvent Orange 7, are also mutagenic [124]. These dyes predominantly exist in the hydrazone form, which is more easily reduced to amines than their azo counterparts (Figure 48) [122].

Some monoazo pigments based on β-naphthol are also mutagenic (58-61) [125], even though they have very low water solubility and only a fraction of molecules available for enzyme metabolism. One of the metabolic cleavage products is 1-amino-2-naphthol, which is a nonmutagen [125]. So the genotoxic effect may be produced by the aniline derivatives that are formed by reductive-cleavage, or may because the naphthalene ring changes the nature of the metabolism of these colorants.
5.3.2 Polyazo colorants

Disazo and trisazo dyes require reductive-cleavage of the azo groups to show mutagenicity [115]. Gut microflora, liver enzymes, certain bacteria, and tissues containing the enzyme azo reductase have been employed by researchers to demonstrate the metabolism of these azo dyes [126-129]. The results showed that the azo linkages could be reduced to mutagenic aromatic amines (Figure 49) [130], leading to an indirect route of exposure to a carcinogenic precursor. In the case of C.I. Direct Black 38, the established carcinogen benzidine is produced.
5.3.3 Benzidine-based colorants

The mutagenicity of benzidine-based azo colorants has been studied by a number of investigators. In many cases, the mutagenic components were the reduction products such as benzidine, ortho-toluidine, and ortho-dianisidine [131].

Prival and coworkers [132] tested a series of benzidine-based dyes by using a modification of the standard Ames assay, in which Flavin mononucleotide (FMN), hamster liver S9 and a pre-incubation step were used to facilitate the reduction and detection of the resultant aromatic amines. They reported that C.I. Direct Red 2, C.I. Acid Red 85, C.I. Direct Blue 1, C.I. Direct Blue 15, and C.I. Direct Brown 95 (62-66) were clearly mutagenic, while C.I. Direct Blue 218 (67), which is the copper complex of C.I. Direct Blue 15, was nonmutagenic. This suggests that the metal complex prevents reductive cleavage of the azo linkages.
Reid and coworkers [133] examined the mutagenic activity of a group of azo dyes based on benzidine and its congeners. The metabolic activation of the dyes was done through sequential reduction and oxidation and the mutageicity was assayed with *Salmonella typhimurium* strain TA1538. The result showed that 15 of the 17 tested compounds were mutagenic. The mutagenic dyes including Congo Red, C.I. Direct Orange 6, C.I. Direct Red 39, C.I. Direct Red 46, C.I. Direct Blue 10, C.I. Direct Blue 25, C.I. Direct Violet 32, and C.I. Direct Black 38 (68-75).
C.I. Direct Red 46

C.I. Direct Blue 10

C.I. Direct Blue 25

C.I. Direct Violet 32

C.I. Direct Black 38
The mutagenicity of azo pigments based on benzidine has also been reported [132, 133, 134]. In this work, colorant such as C.I. Pigment Yellow 12 (76) was shown to be nonmutagenic, although it is derived from the mutagenic intermediate 3,3'-dichlorobenzidine [109]. Presumably the reason is the lack of solubility in the test medium [132].
5.4 The design of nonmutagenic azo colorants

The commercial importance of benzidine-based colorants has led many scientists to search for nonmutagenic analogs of benzidine [106, 130]. Emphasis has been placed on identifying and utilizing intermediates that also gave non-mutagenic colorants.

5.4.1 Replacing benzidine-based azo dyes
5.4.1.1 Dyes from 7-amino-3-(4-aminophenyl)quinoline

One of the first attempts associated with the design of benzidine-free dyes was the synthesis of 7-amino-3-(4-aminophenyl)quinoline (77) (Figure 50). This diamine was tetrazotised and combined with various coupling components to form disazo and trisazo dyes (78, 79) [135]. The dyes have shades in scarlet and green. They showed substantivity to cellulose, but they did not give good washfastness and lightfastness.

Figure 50. The synthesis of 7-amino-3-(4-aminophenyl)quinoline.
5.4.1.2 Dyes from 4,4'-diaminobenzanilide

Studies in this area involved introducing a bridging group between the two phenyl rings of the benzidine structure (80). In this case, $X=-\text{CH}_2, -\text{O}, -\text{NH}, -\text{CONH}, -\text{CO}, \text{ and } -\text{NHCONH}$. The incorporation of these groups causes the loss of co-planarity of the two phenyl residues and may reduce the substantivity of the dyes derived from these intermediates. In addition, the conjugated dye system is shortened by the isolating groups, sometimes causing a hypsochromic shift in color. C.I. Direct Black 166 (81), which is derived from 4,4'-diaminobenzanilide, is an example of that can be used as a replacement for C.I. Direct Black 38 [136].
5.4.1.3 Dyes from 2-(3'-aminophenyl)-5-aminobenzotriazole

2-(3'-Aminophenyl)-5-aminobenzotriazole (82) can be synthesized by cyclization of aminoazobenzene in the presence of copper (II) acetate [137]. This diamine is nongenotoxic [115]. An example of a dye prepared from this intermediate is the direct blue dye 83 [138], which has been considered as substituent for C.I. Direct Blue 2.
5.4.1.4 Dyes from 2-(4’-aminophenyl)-5-aminobenzimidazole

Replacement of benzidine by this diamine (84) and using it with key coupling components led to the synthesis of C.I. Direct Black 177 (85) [139], which can be used in non-thermal ink-jet printing systems.

![Chemical structure of 84 and 85](image.png)

5.4.1.5 Dyes from 5, 5’-diamino-2, 2’-bipyridine

In the design of non-carcinogenic azo dyes, it is important to ensure that the dye intermediates and the reductive-cleavage products are non-mutagenic. Based on this approach, diamine 86 was synthesized and evaluated by the Ames test [130]. The result showed that this diaminobipyridine was much less genotoxic than benzidine itself and that certain derived dyes (87, 88) were also less genotoxic than those made from benzidine.
5.4.1.6 Dyes from 3,3'-dialkyl/dialkoxybenzidine

Following the discovery that the mutagenicity of aromatic amines could be lowered or eliminated by incorporating bulky alkyl or alkoxy substituents ortho to the amino group [106], Hunger and coworkers [140, 141] developed benzidine analogs (89) based on this concept. These intermediates were employed by Bauer and coworkers [142] for the synthesis of non-mutagenic water soluble disazo dyes (90, 91) that were suitable for giving wet fast shades on natural and synthetic fibers.
5.4.2 Replacing benzidine-based azo pigments

Owing to their high stability and insolubility in the media in which they are applied, azo pigments (92-94) derived from benzidine analogs are considered non-genotoxic and are still prepared on a large scale. However, these pigments pose an occupational risk from exposures to the benzidines during their synthesis and may pose health risks if not all of the diamine is converted to pigment [115]. As a result, some studies were conducted to develop pigments based on new diamines.
5.4.2.1 Pigments from 3,3’-dialkyl/alkoxybenzidine

Freeman and coworkers [143] employed these intermediates in the synthesis of non-mutagenic pigments 95-102. These yellow to golden-yellow bisazomethine pigments possessed satisfactory thermal stability, light fastness and hue.
5.4.2.2 Pigments from 2,2',5,5'-tetra-substituted benzidines

Freeman and coworkers [144] reported that introducing substituents in the 2,2'-positions of benzidine caused a hypsochromic shift in colorants produced therefrom 103. The substituents produce steric strain across the biphenyl linkage, and reduce the \( \pi \)-orbital overlap across the biphenyl linkage relative to when protons were present in these positions. This work led to the synthesis of 2,2',5,5'-tetra-substituted benzidine analogs (104, 105) [48] and their use in the synthesis of disazo and bisazomethine pigments, e.g., 106-110 [145].
II. Proposed research

In view of prior studies, new research is proposed that pertains to the synthesis of aromatic diamines and their conversion to potential replacements for currently used direct dyes and azo pigments that are derived from mutagenic diamines. The following sections provide examples of the proposed studies.

1 Synthesis of benzidine homologs

1.1 4,4'-Dinitro-para-terphenyl (112)

Studies conducted in this area involve evaluating the prior approaches to synthesizing this intermediate. Initially examined was the work of France and coworkers [146] who used fuming HNO$_3$ in glacial HOAc to nitrate para-terphenyl and obtained 4,4'-dinitro-para-terphenyl in 43% yield. Also examined was the work of Culling and coworkers [147] who studied the nitration of para-terphenyl in glacial HOAc with fuming HNO$_3$. Their procedure gave ~ 30% yield of 4,4'-dinitro-para-terphenyl, which was much lower than that of France and coworkers. They suggested that the dinitro-para-terphenyl was derived from 4-nitro-para-terphenyl, and if the latter gave the same ratio of ortho- and para- isomers, the optimum yield of 4,4'-dinitro-para-terphenyl would be about 30%. Similarly, Hammond and coworkers [148] synthesized 4,4'-dinitro-para-terphenyl by adding fuming HNO$_3$ dissolved in HOAc to a refluxing solution of para-terphenyl in glacial HOAc over a period of 15 minutes. Then refluxing was continued for another 75 minutes. After recrystallizing the products from pyridine, its melting
point was 274-275°C and the yield was 32%. Their NMR spectra in DMSO-d$_6$ showed an aromatic singlet superimposed on an A2B2 quartet.

Vanallan [149] adopted a method for the nitration of $para$-terphenyl that used nitrobenzene as the solvent. In this case, $para$-terphenyl in nitrobenzene was treated with fuming HNO$_3$ (d1.50) causing a temperature rise to 90°C and dissolution of the starting material. On cooling, the product separated in pure form. Compared to other nitrating methods, this approach required much less solvent and did not involve heating the reaction mixture. Also it gave a higher yield (59%).

1.2 4,4'-Diamino-$para$-terphenyl (113)

Sawicki and coworkers [150] synthesized 4,4'-diamino-$para$-terphenyl by reducing the corresponding dinitro compound with SnCl$_2$·2H$_2$O in conc. HCl. After refluxing for 3 h, the yellow needles became colorless plates. Following isolation, the colorless plates (91%) were crystallized from xylene, 2-methoxyethanol or aqueous acetone, to give a melting point 248-249°C. Hammond and coworkers [148] used similar methods to synthesize 4,4'-diamino-$para$-terphenyl. They dissolved 4,4'-dinitro-$para$-terphenyl in glacial HOAc and stirred the solution at reflux as SnCl$_2$·2H$_2$O in conc. HCl was slowly added over 15 min, then refluxing was continued for 15 min. Following purification, a light brown product (74%) was obtained. The NMR spectrum in DMSO-d$_6$ included a singlet at 7.69 downfield of an A2B2 quartet at 7.61, 7.48, 6.88 and 6.77 with a ratio of the integrated intensities being 2:1.
Khromov-Borisov and coworkers [151] published a quicker process for the reduction of 4,4'-dinitro-\textit{para}-terphenyl. The mixture of 4,4'-dinitro-\textit{para}-terphenyl and Raney nickel in ethylene glycol was heated to 165-170°C, then hydrazine hydrate was added dropwise to avoid intense foaming. The reaction was continued for several minutes, to give a creamy colored product (100%), melting point 240°C.

1.3 4,4'-Dinitro-\textit{para}-quarterphenyl (116)

Scheinbaum [152] reported that the nitration of \textit{para}-quarterphenyl with CH$_3$CONO$_2$ generated 4-nitro-\textit{para}-quarterphenyl and 4,4'-dinitro-\textit{para}-quarterphenyl, without producing \textit{ortho}- or \textit{meta}- nitration products. Also examined was the method of Hammond and coworkers [148] who the nitration of \textit{para}-quarterphenyl in glacial HOAc and nitrobenzene mixture using fuming HNO$_3$. When the yellow crystals were recrystallized from chlorobenzene, the yield was 33%.

Harley-Mason and Mann [153] investigated the direct nitration of \textit{para}-quarterphenyl, and found that the starting material was either unchanged or converted to a mixture of polynitro derivatives. In another route adopted by these authors, biphenyl was mixed with I$_2$ and heated at reflux for 2 h, with HNO$_3$ added during the first hour. This reaction gave 4-iodo,4'-nitrobiphenyl as long, pale yellow needles (29%), having melting point 212-214°C. 4-Iodo,4'-nitrobiphenyl was heated with copper bronze at 235-245°C for 6 h. The dinitro
product was obtained as bright yellow needles (6.7%) and the melting point was 317-320°C. The authors concluded that this was the optimum set of conditions.

1.4 4,4'-Diamino-para-quaterphenyl (117)

Harley-Mason and Mann [153] used SnCl₂ to reduce 4,4'-dinitro-para-quaterphenyl. In this case, 4,4'-dinitro-para-quaterphenyl and SnCl₂ were stirred at reflux in HOAc, as dry HCl was slowly bubbled through the mixture for 5 h. The precipitated chlorostanate was decomposed with 20% aqueous NaOH, and the liberated diamine was washed and then sublimed at 310-320°C /0.01 mm. The product was obtained as colorless plates, and melting point was 312-315°C. Hammond and coworkers [148] used the same procedure to conduct the reduction. However, they used a different after-treatment step. The precipitate was first boiled with 20% aqueous NaOH, filtered, washed and recrystallized from 10% aqueous pyridine. The diamine was obtained as a light buff colored solid and the yield was 60%. It had a melting point of 304°C with charring.

In the present study, DATP (113) and DAQP (117) will be synthesized from para-terphenyl and biphenyl according to Figure 51 and examined as potential benzidine replacements in direct dye and pigment formation.
Figure 51. Proposed synthesis of DATP (a) and DAQP (b).
2. Direct dyes

Direct dyes based on DATP and DAQP will be synthesized and evaluated. The two intermediates will be tetrazotized and coupled with some widely used dye intermediates to generate a group of new direct dyes (Figure 52). The dye structures will be confirmed using electrospray mass spectrometry (ESMS), and the fastness properties of these dyes will be assessed using standard AATCC test methods [154].

Figure 52. The proposed direct dye structures (where n=1 and 2).
3. Organic pigments

Organic pigments based on DATP and DAQP will be synthesized and evaluated (Figure 53). The structures of the pigments will be confirmed by matrix assisted laser desorption/ionization (MALDI) mass spectrometry and elemental analysis.

Figure 53. The proposed structures of pigments (where n=1 and 2).
4. Mutagenicity testing

This research will include an assessment of the mutagenicity of 4,4’-diamio-para-terphenyl (DATP), 4,4’-diamino-para-quaterphenyl (DAQP). The mutagenicity of the two compounds will be analyzed to evaluate the effects of increasing the number of phenyl rings in the diamines by comparing them with that of para-phenylenediamine and benzidine. Also the mutagenicity of the target direct dyes and pigments will be determined.

Mutagenicity will be assessed using the Salmonella mammalian assay (Ames test). In this study, Salmonella typhimurium strains TA98 and TA100 will be used with and without S9 enzyme activation. The Prival modification of the standard assay will also be used for the mutagenicity test of azo direct dyes and pigments. This variation of the Ames test enhances the metabolic breakdown of azo dyes such as Congo Red [131], which do not show significant mutagenicity in the Ames test [155].
III. Experimental

1. General information

The para-terphenyl and diaminobiphenyl dihydrochloride (DABP·HCl, 98%) used in this study was obtained from TCI America, Portland, OR and all the other compounds were obtained from Aldrich Chemical Company, Milwaukee, WI. DABP·HCl was converted to the free diamine using 5% NaOH solution and then extracted into EtOAc and treated with activated carbon. The free diamine was collected by removing the solvent by evaporation under pressure. TLC plates used were Whatman® PE silica gel plates with UV254 indicator.

The structures of the benzidine homologs and related intermediates were confirmed by $^1$H NMR and EI mass spectrometry. The structures of dyes were confirmed by electrospray mass spectrometry and those of organic pigments were confirmed by MALDI mass spectrometry. The purity of the organic pigments was also determined by combustion analysis. $^1$H NMR spectra were recorded on a General Electrical GN 300 MHz spectrometer and EI mass spectra were recorded on a Hewlett-Packard 5985B GC mass spectrometer. Melting points were recorded on a Mel-Temp apparatus and are uncorrected.

Mutagenicity testing was conducted using the standard Ames test [93], and the Prival modification [130]. Salmonella typhimurium strains TA98 and TA100 were employed in these studies.
2. Synthesis of intermediates, direct dyes and pigments

2.1 4,4′-Dinitro-para-terphenyl (112)

\textit{para}-Terphenyl (111) (10 g, 43.4 mmol) and 25 ml nitrobenzene were mixed at room temperature, and stirred vigorously as fuming HNO\textsubscript{3} (12 ml, 0.286 mol) was added. This caused boiling and the evolution of a reddish brown gas. After 30 min, the reaction mixture was allowed to cool to room temperature. TLC showed only one component, $R_f = 0.27$ (toluene:hexane/4:1). The product was collected by filtration, washed with acetone and water, and dried under vacuum at 40°C. The light yellow solid (5.10 g, 36.7\%) had mp 282-284°C (dec.). $^1$H-NMR (DMSO-d\textsubscript{6}): $\delta$ 7.98 (s, 4H), $\delta$ 8.05-8.08 (d, 4H), $\delta$ 8.33-8.35 (d, 4H). EI MS: m/z (relative intensity), 320.1 ([M+], 100), 275.1 (72.0), 228.1 (25.0).

2.2 4,4′-Diamino-para-terphenyl (113)

4,4′-Dinitro-para-terphenyl (1 g, 3.12 mmol) and Pd(OH)\textsubscript{2}/C catalyst (20\% wt.) were suspended in ethylene glycol (50 ml) and the mixture was heated to 165-170°C. Hydrazine monohydrate (1.6 ml, 33 mmol) was added over 10 min and the temperature was raised to 175-180°C and kept there for 1.75 h. TLC showed only one component, $R_f = 0.47$ (toluene:ethyl acetate/1:1). Activated carbon was added and after stirring for about 15 min, the mixture was filtered while hot. Upon cooling down, a creamy-colored solid precipitated from the filtrate. The solid was collected by filtration and dried under vacuum at 40°C. The synthesis gave 0.78 g (96\%) product having mp 243-245°C (dec.). Elemental analysis. Calculated for C\textsubscript{18}H\textsubscript{16}N\textsubscript{2}: C, 83.04; H, 6.19; N, 10.76. Found: C, 82.63;
H, 6.29; N, 10.84. $^1$H-NMR (DMSO-$d_6$): $\delta$ 5.21 (s, 4H), $\delta$ 6.63-6.65 (d, 4H), $\delta$ 7.53 (s, 4H). EI MS: m/z (relative intensity), 260.2 ([M$^+$], 100), 232.1 (7.5), 130.1 (47.0).

2.3 4-iodo,4'-nitrobiphenyl (115)

Biphenyl (114) (60 g, 0.39 mol) and I$_2$ (30 g, 0.12 mol) were mixed and heated to the boil as HNO$_3$ (144 ml, d1.42) was added over 1 h. After an additional 1 h, the reaction was cooled, added to water and the yellow solid was collected by filtration. The solid was washed with water and extracted 3 times with hot EtOH (180 ml). Recrystallization from toluene (400 ml) and drying under vacuum at 40°C gave long, pale yellow needles of 4-iodo,4'-nitrobiphenyl (40.7 g, 32.2%), mp 218-220°C. TLC showed only one component, $R_f = 0.41$ (toluene:hexane/1:1). $^1$H-NMR (DMSO-$d_6$): $\delta$ 7.58-7.60 (d, 2H), $\delta$ 7.88-7.90 (d, 2H), $\delta$ 7.94-7.96 (d, 2H), $\delta$ 8.29-8.31 (d, 2H). EI MS: m/z (relative intensity), 325.0 ([M$^+$], 100), 295.0 (10.5), 152.1 (32.0).

2.4 4,4'-Dinitro-para-quaterphenyl (116)

4-iodo,4'-nitrobiphenyl (115) (39 g, 0.12 mol) was heated to 240°C, and copper bronze (10.9 g, 0.172 mol) was added. The mixture was stirred 6 h at 235-245°C and cooled. The product was pulverized and extracted 3 times with boiling toluene (200 ml). The residue was boiled with nitrobenzene (200 ml) and filtered. The yellow solid that precipitated after cooling was collected by filtration, and was then extracted once more with boiling nitrobenzene (100 ml). The
collected product was dried under vacuum at 40°C to give 3.93 g (8.26%), mp 317-320°C (dec.). TLC showed only one component, \( R_f = 0.35 \) (toluene:hexane/4:1). \(^1\)H-NMR (DMSO-d\(_6\)): \( \delta \) 7.95 (s, 8H), \( \delta \) 8.05-8.07 (d, 4H), \( \delta \) 8.33-8.35 (d, 4H), \( \delta \) 8.29-8.31. EI MS: m/z (relative intensity), 396.1 ([M\(^+\)], 100), 302.1 (48.0), 151.0 (23.0).

2.5 4,4’-Diamino-para-quaterphenyl (117)

A mixture of 4,4’-dinitro-para-quaterphenyl (116) (2.7 g, 6.8 mmol), \( \text{SnCl}_2\cdot2\text{H}_2\text{O} \) (13.04 g, 57.8 mmol), and \( \text{HOAc} \) (200 ml) was stirred under reflux for 5 h while dry HCl gas was slowly passed through it. The precipitate was collected by filtration, washed with water, and stirred with 20% NaOH solution. The liberated diamine was boiled with 40% aqueous pyridine solution, washed with water, and dried under vacuum at 40°C. The yield was 2.14 g (93.4%), mp >400 °C (dec.). \(^1\)H-NMR (DMSO-d\(_6\)): \( \delta \) 5.22 (s, 4H), \( \delta \) 6.65 (d, 4H), \( \delta \) 7.39-7.42 (d, 4H), \( \delta \) 7.59-7.69 (dd, 4H). EI MS: m/z (relative intensity), 336.2 ([M\(^+\)], 100), 168.1 (19).

2.6 5-Acetoacetylaminobenzimidazolone (118)

5-Aminobenzimidazolone (2.7 g, 18 mmol) was mixed with 50% aq. EtOH (20 ml) and heated to 85°C. Diketene (1.25 ml, 16 mmol) was added over 10 min, during which time the solid dissolved and the temperature rose to 86-87°C. A light brown solid began to precipitate, and after 1 h the mixture was filtered at room temperature and the solid was washed with 50% aq. EtOH (30 ml). The product was dried under vacuum at 40 °C to give 2.58 g (63.2%), mp
>400°C (dec.). EI MS: m/z (relative intensity), 233 ([M⁺], 53), 175 (72), 149 (100).

2.7 4-[(2,5-Dichlorophenyl)azo]-3-hydroxy-2-naphthoic acid (119)

A mixture of 2,5-dichloroaniline (8.10 g, 50 mmol) in water (10 ml) and HCl (30%, 15 ml, 0.46 mol) was stirred at 60°C for 20 min. Ice (50 g) was added to the mixture, and at 0-2°C NaNO₂ (3.45 g, 50 mmol) dissolved in water (15 ml) was slowly added. The diazotization step was continued for 30 min and the solution was filtered to remove a small amount of insoluble material. The diazonium salt solution was added drop wise to 3-hydroxy-2-naphthoic acid (10 g, 65 mmol) dissolved in water (200 ml) containing 30% NaOH (5 ml, 17 mmol) and Na₂CO₃ (17 g, 0.16 mol), at a rate needed to maintain the temperature at 0-2°C. The coupling step was continued for 4 h, and the monoazo dye was isolated by acidification with 30% HCl (20 ml, 0.65 mol). The dye was collected by filtration, washed with water and dried under vacuum at 40°C. The yield was 16.60 g (91.9%), mp 256-259 °C (dec.). EI MS: m/z (relative intensity), 360 ([M⁺], 36), 215(38), 159 (100).

2.8 4-[(2,5-Dichlorophenyl)azo]-3-hydroxy-2-naphthoyl chloride (120)

4-[(2,5-Dichlorophenyl)azo]-3-hydroxy-2-naphthoic acid (119) (7.2 g, 20 mmol) was suspended in chlorobenzene (20 ml), and heated to 60°C. To this mixture were added DMF (0.25 ml, 3.25mmol) and PCl₅ (3.25 g, 15.6 mmol). The temperature rose to 75-80°C and the reaction was held at this temperature for 1
At that point, additional PCl₅ (3 g, 14.4 mmol) was added and the temperature was kept at 75-80°C for 2 h. The resulting acid chloride was collected by filtration, washed with chlorobenzene (20 ml) and dried under vacuum at 40°C. The yield was 5.33 g (70.4%), mp 274-276°C (dec.). EI MS: m/z (relative intensity), 378 ([M⁺, 3), 215(45), 159 (100).

2.9 Direct dyes from 4,4′-diamino-para-terphenyl (121-125)

4,4′-Diamino-para-terphenyl (113) (0.26 g, 1 mmol) was dispersed in water (4 ml) containing 5g ice, and a mixture of conc. HCl (0.67 ml) and water (4 ml) was added. The temperature was cooled to 0-5°C using an ice bath, and NaNO₂ (0.138 g, 2 mmol) dissolved in water (5 ml) was added drop wise. Activated carbon was slowly added with stirring and the mixture was filtered while maintaining the temperature at 0-5°C. The clear yellow tetrazonium salt solution was added to solutions of various couplers with stirring at 0-5°C. The couplers used to make direct dyes and their amounts were naphthionic acid (0.483 g, 2.1 mmol, 97%), J-acid (0.557 g, 2.1 mmol, 97%), H-acid (0.741 g, 2.1 mmol, 96%), chromotropic acid (0.801 g, 2 mmol), and Chicago acid (0.823 g, 2.1 mmol, 87%). These couplers were dissolved in water (20 ml) at pH 8-9 by using 1% NaOH solution. Coupling was continued overnight at room temperature and the dyes were precipitated by adding solid NaCl. The dyes were collected by filtration and dried. All dyes were desalted by extracting with DMF, filtration of salt, and solvent evaporation. All dyes were analyzed by TLC, using n-
BuOH:EtOH:NH₄OH:pyridine (4:1:3:2) as the eluent. The Rᵣ values were 0.47 (121), 0.49 (122), 0.35 (123), 0.30 (124), and 0.27 (125).

2.10 Dye 126 from 4,4'-diamino-para-terphenyl

4,4'-Diamino-para-terphenyl (113) (0.26 g, 1 mmol) was dispersed in water (4 ml) containing 5g ice, and a mixture of conc. HCl (0.67 ml) and water (4 ml) was added. The temperature was cooled to 0-5°C using an ice bath, and NaNO₂ (0.138 g, 2 mmol) dissolved in water (5 ml) was added drop wise with stirring over 10 min. After stirring for 30 min, sulfamic acid was added to destroy excess HNO₂. Activated carbon was added with stirring and the mixture was filtered while maintaining the temperature at 0-5°C. H-acid (0.355 g, 1 mmol 96%) was stirred with water (20 ml) and 10% Na₂CO₃ to give pH 6, and the solution was added drop wise over 3 h to the tetrazonium salt solution at 10°C and pH 2. After the addition, the reaction mixture was stirred for 6 h, maintaining pH 2-2.5 by adding 10% Na₂CO₃ solution.

Aniline (0.093 g, 1 mmol) was suspended in water (10 ml), and then conc. HCl (0.25 ml, 3 mmol) was added. NaNO₂ solution (30%, 0.069 g, 1 mmol) was added drop wise to the aniline solution at 0-5°C. The solution was stirred for 15 min, and excess HNO₂ was decomposed by the addition of small amount of sulfamic acid. Activated carbon was added with stirring and the mixture was filtered. Diazotized aniline was added to the solution from the first coupling and Na₂CO₃ solution (10%) was added to give pH 8-9. The resulting solution was stirred at 3-5°C for 1h to complete the second coupling step. meta-
Phenylenediamine (0.108 g, 1 mmol) dissolved in water (5 ml) and added and the solution was stirred for 3 h at pH 7-8 and 10°C. The dye was precipitated by adding solid NaCl and the mixture was adjusted to pH 6-7 by adding 10% Na₂CO₃ solution. The dye was collected by filtration, dried, and desalted by DMF extraction. The resulting dye was dried under vacuum at 40°C and analyzed by TLC using n-BuOH:EtOH:NH₄OH:pyridine (4:1:3:2) as the eluent. The Rₓ value was 0.47.

2.11 Direct dyes from 4,4’-diamino-para-quarterphenyl (127-131)

Concentrated H₂SO₄ (2 ml) was cooled to 0-5°C and NaNO₂ (0.138 g, 2 mmol) was added slowly to avoid producing a yellow solution or a yellow gas. After the addition of NaNO₂, the temperature was gradually raised to 20°C, giving a clear solution. 4,4’-Diamino-para-quarterphenyl (117) (0.336 g, 1.0 mmol) was added slowly at 0-5°C and the solution became dark green and thick. The tetrazonium salt solution was stirred for 2 h and poured onto 26.4 g ice to give a yellow mixture. A small amount of sulfamic acid was added to destroy excess HNO₂, and the tetrazonium salt solution was diluted with 300 ml cold water, filtered and added drop wise to the couplers. The couplers used to make direct dyes and their amounts were naphthionic acid (0.483 g, 2.1 mmol, 97%), J-acid (0.557 g, 2.1 mmol, 97%), H-acid (0.747 g, 2.1 mmol, 96%), chromotropic acid (1.197 g, 2.1 mmol, 60%), and Chicago acid (0.823 g, 2.1 mmol, 87%). These couplers were dissolved in water (40 ml) and adjusted to pH 8-9 by using dilute NaOH solution. Coupling was continued overnight at room temperature and the
dyes were precipitated by adding solid NaCl. The dyes were collected by filtration and dried. All dyes were desalted by extracting into DMF, filtration and solvent removal. Dyes were analyzed by TLC using n-BuOH:EtOH:NH₄OH:pyridine (4:1:3:2) as the eluent. The R_f values were 0.54 (127), 0.53 (128), 0.39 (129), 0.39 (130), and 0.30 (131).

2.12 Dye 132 from 4,4'-diamino-para-quaterphenyl

Concentrated H₂SO₄ (2 ml) was cooled to 0-5°C and NaNO₂ (0.138 g, 2 mmol) was added slowly to avoid producing yellow solution or a yellow gas. After the addition of NaNO₂, the temperature was gradually raised to 20°C, giving a clear solution. 4,4'-Diamino-para-quarterphenyl (117) (0.336 g, 1.0 mmol) was added slowly at 0-5°C and the solution became dark green and thick. The tetrazonium salt solution was stirred for 2 h and poured onto 26.4 g ice to give a yellow mixture. A small amount of sulfamic acid was added to destroy excess HNO₂, and the tetrazonium salt solution was diluted with 300 ml cold water, filtered to remove the impurities. H-acid (0.355 g, 1 mmol 96%) was stirred with water (20 ml) and 10% Na₂CO₃ solution to give pH 6, and the solution was added drop wise over 3 h to the tetrazonium salt solution at 10°C and pH 2. After the addition, the solution was stirred for about 6 h, maintaining pH 2-2.5 by adding 10% Na₂CO₃ solution.

Aniline (0.093 g, 1 mmol) was suspended in water (10 ml) and conc. HCl (0.25 ml, 3 mmol) was added. NaNO₂ solution (30%, 0.069 g, 1 mmol) was added drop wise to the resultant solution at 0-5°C. The solution was stirred for 15
min, and excess HNO₂ was decomposed by the addition of small amount of sulfamic acid. Activated carbon was added with stirring and the mixture was filtered. The diazotized aniline was added to the solution from the first coupling and Na₂CO₃ solution (10%) was added to give pH 8-9. The resultant solution was stirred at 3-5°C for 1 h to complete this reaction. \textit{meta}-Phenylenediamine (0.108 g, 1 mmol) was dissolved in water (5 ml) and added to the solution from the second coupling. The resultant solution was stirred for 3 h at pH 7-8 and 10°C. The dye was precipitated by adding solid NaCl and the mixture was adjusted to pH 6-7. The dye was desalted by extracting into DMF, filtration and solvent removal. The dye was dried under vacuum at 40°C to give 0.35 g product. TLC using \textit{n}-BuOH:EtOH:NH₄OH:pyridine (4:1:3:2) showed \( R_f \) value = 0.53.

2.13 Pigment 133 from 4,4’-diamino-\textit{para}-terphenyl

2-Mercapto-4,6-dihydroxypyrimidine (0.294 g, 2 mmol, 98%) was added to a stirred solution of 4,4’-diamino-\textit{para}-terphenyl (113) (0.26 g, 1 mmol) in DMF (10 ml) at 25°C. After stirring the solution for a further 30 min, triethylorthoformate (0.302 g, 2 mmol, 98%) was added. The reaction mixture was stirred for 3 h at 25°C and then heated to 80°C over 30 min. After 1 h, the temperature was raised to 120°C over 30 min, and after 2 h, the mixture was allowed to cool to 80°C. The product was collected by filtration and washed with hot methanol (50 ml) and hot water (100 ml) and dried under vacuum at 40°C. The procedure gave 0.38 g (66.8%) yellow pigment, mp >400°C. Elemental analysis. Calculated for C₂₈H₂₀N₆O₄S₂: C, 59.14; H, 3.55; N, 14.78. Found: C, 60.25; H, 3.94; N, 14.50.
2.14 Pigment 134 from 4,4′-diamino-para-terphenyl

4,4′-Diamino-para-terphenyl (0.26 g, 1 mmol) was dispersed in water (4 ml) containing 5 g ice, and a mixture of conc. HCl (0.67 ml) and water (4 ml) was added. The temperature was cooled to 0-5°C and NaNO₂ (0.138 g, 2 mmol) dissolved in water (5 ml) was added drop wise with stirring over 10 min. Sulfamic acid was added to destroy excess HNO₂. Activated carbon was added with stirring and the mixture was filtered while maintaining the temperature at 0-5°C. The clear yellow tetrazonium salt solution was added drop wise, at 0-5°C and pH 6, to a suspension of acetoacetanilide (0.365 g, 2 mmol, 97%) obtained by dissolution of acetoacetanilide in water (40 ml) containing NaOH (0.13 g, 6.4 mmol), followed by precipitation with HOAc (0.32 ml, 5.6 mmol) and NaOAc·3H₂O (1.73 g, 12.8 mmol). The coupling reaction was stirred overnight and then heated to the boil. The pigment mixture was filtered hot, and the product boiled 3 times with hot water, filtered and dried under vacuum at 40°C. TLC showed only one component, R_f = 0.24 (toluene) and the yellow pigment (0.40 g, 62.8%) had mp 319-321°C (dec.). Elemental analysis. Calculated for C₃₈H₃₂N₆O₄: C, 71.68; H, 5.07; N, 13.20. Found: C, 71.50; H, 5.02; N, 13.20.

2.15 Pigment 135 from 4,4′-diamino-para-terphenyl

4,4′-Diamino-para-terphenyl (0.26 g, 1 mmol) was dispersed in water (4 ml) containing 5 g ice and a mixture of conc. HCl (0.67 ml) and water (4 ml) was added. The temperature was cooled to 0-5°C and NaNO₂ (0.138 g, 2 mmol) dissolved in water (5 ml) was added drop wise with stirring over 10 min. Sulfamic
acid was added to destroy excess HNO₂. Activated carbon was added with stirring and the mixture was filtered while maintaining the temperature at 0-5°C. The clear yellow tetrazonium salt solution was added drop wise, at 0-5°C and pH 6, to a suspension of 1-phenyl-3-methyl-5-pyrazolone (0.352 g, 2 mmol, 99%) obtained by dissolution of 1-phenyl-3-methyl-5-pyrazolone in water (40 ml) containing NaOH (0.13 g, 6.4 mmol), followed by precipitation with HOAc (0.32 ml, 5.6 mmol) and NaOAc·3H₂O (1.73 g, 12.8 mmol). The coupling reaction was stirred overnight and the resultant mixture was heated to the boil. The pigment mixture was filtered hot, and the product boiled 3 times with hot water, filtered and dried under vacuum at 40°C. TLC showed only one component, Rₓ=0.27 (toluene), and the orange pigment (0.45 g, 71.7%) had mp 303-305°C (dec.).

2.16 Pigment 136 from 4,4'-diamino-para-terphenyl

4,4'-Diamino-para-terphenyl (0.26 g, 1 mmol) was dispersed in water (4 ml) containing 5 g ice, and a mixture of conc. HCl (0.67 ml) and water (4 ml) was added. The temperature was cooled to 0-5°C, and NaNO₂ (0.142 g, 2 mmol, 97%) dissolved in water (5 ml) was added drop wise with stirring over 10 min. Sulfamic acid was added to destroy excess HNO₂. Activated carbon was added with stirring and the mixture was filtered while maintaining the temperature at 0-5°C. The clear yellow tetrazonium salt solution was added drop wise, at 0-5°C and pH 6, to a suspension of 5-acetoacetylaminobenzimidazolone containing a small amount of Triton X-100 and Surfynol 104 PG-50. The suspension was obtained by dissolving 5-acetoacetylaminobenzimidazolone (0.521 g, 2.1 mmol)
in water (40 ml) containing NaOH (0.19 g, 19.2 mmol), Triton X-100 (0.02 g) and Surfynol 104 PG-50 (0.015 g), followed by precipitation using HOAc (0.48 ml, 8.4 mmol) and NaOAc·3H₂O (2.59 g, 19.2 mmol). The coupling reaction was stirred overnight and then heated to the boil. The pigment mixture was filtered and the solid was dried under vacuum at 40°C. The dried pigment was boiled with DMF (5 ml) for 30 min. After cooling the mixture to 50°C, the pigment was collected by filtration, washed with water and dried in vacuum at 40°C. The yellow pigment (0.59 g, 80.5%) had mp >400°C. Elemental analysis. Calculated for C₄₀H₃₂N₁₀O₆: C, 64.16; H, 4.31; N, 18.71. Found: C, 63.94; H, 4.32; N, 17.90.

2.17 Pigment 137 from 4,4′-diamino-para-terphenyl

4-[(2,5-Dichlorophenyl)azo]-3-hydroxy-2-naphthoyl chloride (0.76 g, 2 mmol) was dispersed in dry chlorobenzene (20 ml) and added to a solution of 4,4′-diamino-para-terphenyl (0.26 g, 1 mmol) in chlorobenzene (10 ml) at 70°C. The mixture was then heated to the boil over a 1-h period and the condensation step was continued for 8 h. The mixture was filtered hot and the pigment was washed with chlorobenzene. After drying under vacuum at 40°C. the red pigment (0.73 g, 76.8%) had mp >400°C. Elemental analysis. Calculated for C₅₂H₃₂Cl₄N₆O₄: C, 65.98; H, 3.41; N, 8.88. Found: C, 64.48; H, 3.25; N, 8.59.
2.18 Pigment 138 from 4,4'-diamino-para-quaterphenyl

2-Mercapto-4,6-dihydroxypyrimidine (0.294 g, 2 mmol, 98%) was added to a stirred solution of 4,4'-diamino-para-quaterphenyl (117) (0.336 g, 1 mmol) in DMF (20 ml) at 25°C. After stirring the solution for a further 30 min, triethylorthoformate (0.302 g, 2 mmol, 98%) was added. The reaction mixture was stirred for 3 h at 25°C and then heated to 80°C over 30 min. After 1 h, the temperature was raised to 120°C over 30 min, and after 2 h, it was allowed to drop to 80°C. The product was collected by filtration and washed with hot methanol (50 ml) and hot water (100 ml) and dried under vacuum at 40°C. This gave 0.45 g (68.2%) yellow pigment, mp > 400°C.

2.19 Pigment 139 from 4,4'-diamino-para-quaterphenyl

Concentrated H₂SO₄ (2 ml) was cooled to 0-5°C and NaNO₂ (0.138 g, 2 mmol) was added slowly to avoid producing a yellow solution or a yellow gas. After the addition of NaNO₂, the temperature was gradually raised to 20°C, giving a clear solution. 4,4'-Diamino-para-quarterphenyl (117) (0.336 g, 1.0 mmol) was added slowly at 0-5°C and the solution became dark green and thick. The tetrazonium salt solution was stirred for 2 h and poured onto 26.4 g ice to give a yellow mixture. A small amount of sulfamic acid was added to destroy excess HNO₂, and the tetrazonium salt solution was diluted with 300 ml cold water, filtered and added drop wise, at 0-5°C and pH 6, under the surface of a suspension of acetoacetanilide (0.380g, 2.1 mmol) obtained by dissolving of acetoacetanilide in 20 ml water containing NaOH (0.13 g, 3.2 mmol), followed by
precipitation using HOAc (0.32 ml, 5.6 mmol) and NaOAc·3H₂O (1.73 g, 12.8 mmol). The coupling reaction was carried out for 12 h and the reaction mixture was heated to the boil. The pigment mixture was filtered hot, and the product was boiled 3 times with hot water, filtered and dried under vacuum at 40°C. TLC showed only one component, Rf = 0.24 (toluene), and the yield of this yellow pigment was 0.64 g (89.8%), mp 274-278°C (dec.). Elemental analysis. Calculated for C₄₄H₃₆N₆O₄: C, 74.14; H, 5.09; N, 11.79. Found: C, 73.77; H, 5.01; N, 11.77.

2.20 Pigment 140 from 4,4′-diamino-para-quaterphenyl

Concentrated H₂SO₄ (2 ml) was cooled to 0-5°C. NaNO₂ (0.138 g, 2 mmol) was added slowly to avoid producing a yellow solution or a yellow gas. After the addition of NaNO₂, the temperature was gradually raised to 20°C, giving a clear solution. 4,4′-Diamino-para-quarterphenyl (117) (0.336 g, 1.0 mmol) was added slowly at 0-5°C and the solution became dark green and thick. The tetrazonium salt was stirred for 2 h and poured onto 26.4 g ice to give a yellow mixture. A small amount of sulfamic acid was added to destroy excess HNO₂, and the tetrazonium salt solution was diluted with 300 ml cold water, filtered and added drop wise, at 0-5°C and pH 6, under the surface of a suspension of 1-phenyl-3-methyl-5-pyrazolone (0.370 g, 2.1 mmol) obtained by dissolving 1-phenyl-3-methyl-5-pyrazolone in 40ml water containing NaOH (0.13 g, 6.4 mmol), followed by precipitation using HOAc (0.32 ml, 5.6 mmol) and NaOAc·3H₂O (1.73 g, 12.8 mmol). The coupling reaction was carried out
overnight and the reaction mixture was heated to the boil. The pigment mixture was filtered hot, boiled 3 times with hot water, filtered and dried under vacuum at 40°C. TLC showed only one component, \( R_f = 0.28 \) (toluene). The yield of this orange pigment was 0.63 g (88.9%), mp 298-300°C (dec). Elemental analysis. Calculated for \( \text{C}_{44}\text{H}_{34}\text{N}_8\text{O}_2 \): C, 74.77; H, 4.85; N, 15.85. Found: C, 74.50; H, 4.92; N, 15.85.

2.21 Pigment 141 from 4,4'-diamino-\( \text{para} \)-quarterphenyl

Concentrated \( \text{H}_2\text{SO}_4 \) (2 ml) was cooled to 0-5°C and NaNO\(_2\) (0.138 g, 2 mmol) was added slowly to avoid producing a yellow solution or a yellow gas. After the addition of NaNO\(_2\), the temperature was gradually raised to 20°C, giving a clear solution. 4,4'-Diamino-\( \text{para} \)-quarterphenyl (117) (0.336 g, 1.0 mmol) was added slowly at 0-5°C and the solution became dark green and thick. The tetrazonium salt solution was stirred for 2 h and poured onto 26.4 g ice to give a yellow mixture. A small amount of sulfamic acid was added to destroy excess HNO\(_2\), and the tetrazonium salt solution was diluted with 300 ml cold water, filtered and added drop wise, at 0-5°C and pH 6 to the surface of a suspension of 5-aceto-acetylaminobenzimidazolone containing a small amount of Triton X-100 and Surfynol 104 PG-50. The suspension was obtained by dissolution of 5-aceto-acetylaminobenzimidazolone (0.521 g, 2.1 mmol) in 40 ml water containing NaOH (0.19 g, 19.2 mmol), Triton X-100 (0.02 g), and Surfynol 104 PG-50 (0.015 g), followed by precipitation with HOAc (0.48 ml, 8.4 mmol) and NaOAc·3H\(_2\)O (2.59 g, 19.2 mmol). The coupling reaction was carried out overnight and the
product was isolated by filtration and dried under vacuum at 40°C. The dried pigment was boiled in 10 ml DMF for 30 min. After cooling the mixture to 50°C, the pigment was collected by filtration, washed with water and dried under vacuum at 40°C. The yield of this yellow pigment was 0.56 g (67.9%), mp >400°C.

2.22 Pigment 142 from 4,4’-diamino-para-quaterphenyl

4-[(2,5-Dichlorophenyl)azo]-3-hydroxy-2-naphthoyl chloride (0.76 g, 2 mmol) was dispersed in dry chlorobenzene (20 ml) and added to a solution of 4,4’-diamino-para-terphenyl (0.336 g, 1 mmol) in chlorobenzene (10 ml) at 70°C. The reaction mixture was then heated to the boil over 1 h and the condensation step was continued for 8 h. The mixture was filtered hot, and the pigment was washed with chlorobenzene and dried under vacuum at 40°C. The yield of this red pigment was 0.73 g (71.4%), mp >400°C.
2. Dyeing procedures

2.1 Dye application

A 1% (owf) dyeing was carried out at pH 7 using a 60:1 liquor ratio. Mercerized cotton fabric (5 g) was wet out with hot water and excess water was removed and the fabric was added to a dyebath consisting of direct dye (0.05 g) and water (285 ml) at 60°C. The temperature was raised to 95°C and maintained for 30 min. Na$_2$SO$_4$ solution (10%, 15 ml) was added to the dyebath and dyeing was continued for another 30 min. The fabric was removed, rinsed with cold water and dried.

3. Fastness testing

3.1 Washfastness determination

The washfastness of dyed fabric was evaluated using AATCC test method 61-1996 No. 2A [154]. Multifiber fabric was employed (5.0 × 15.0 cm) from Test Fabrics, Inc., and was attached to each fabric sample. The testing conditions employed are shown below.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>49°C (120°F)</td>
</tr>
<tr>
<td>Total Volume</td>
<td>150 ml</td>
</tr>
<tr>
<td>Percent AATCC Detergent in Total Volume</td>
<td>0.15%</td>
</tr>
<tr>
<td>Available Chlorine in Total Volume</td>
<td>None</td>
</tr>
<tr>
<td>Number of Steel Balls</td>
<td>50</td>
</tr>
<tr>
<td>Time</td>
<td>45 min</td>
</tr>
</tbody>
</table>
The dried fabric was evaluated for color change using AATCC Evaluate Procedure 2 [154] and staining of adjacent undyed multifiber fabric using AATCC Evaluate Procedure 1 [154]. The change in color was evaluated by using the grey scale for color change, and staining of cotton was evaluated using the grey scale for staining. The rating scale was 1 (poor) to 5 (excellent).

3.2 Lightfastness determination

The lightfastness of dyed fabric was evaluated by using AATCC test method 16-1998 option E (Water-cooled Xenon-Arc lamp, Continuous Light) [154]. The fabric employed was 7.0 × 12.0 cm (2.75 × 4.7 in.), with the exposed area measuring not less than 3.0 × 3.0 cm (1.2 × 1.2 in.). The test conditions are shown below.

<table>
<thead>
<tr>
<th>Light Source:</th>
<th>Xenon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamp Cooling:</td>
<td>Water</td>
</tr>
<tr>
<td>Black Panel Temperature:</td>
<td>63±1°C (145±2°F)</td>
</tr>
<tr>
<td>Dry Bulb Temperature:</td>
<td>43±2°C (110±4°F)</td>
</tr>
<tr>
<td>Relative Humidity (%):</td>
<td>30±5%</td>
</tr>
<tr>
<td>Light Cycle, hours:</td>
<td>Continuous, 24 hours</td>
</tr>
<tr>
<td>Filter Type Outer:</td>
<td>Soda Lime</td>
</tr>
<tr>
<td>Filter Type Inner:</td>
<td>Borosilicate</td>
</tr>
</tbody>
</table>

The fabric was evaluated for color change with the aid of the grey scale for color change, and the rating scale was 1 (poor) to 5 (excellent).
3.3 Color measurements

The dyed fabric was evaluated by reflectance methods to obtain a numerical representation of the color of the samples. The instrument used was Spectraflash SF600 PLUS spectrophotometer and the software was DCI Instruments Program for Chroma-Calc Version 2.2.

The illuminant used was D65, which simulates daylight conditions, and uses the CIE 10° standard observer. Before making measurements, the spectrophotometer was calibrated using a black tile and a white tile. For these measurements, an aperture plate with illuminated area at 3 mm (small area view) was employed. The samples were folded twice to fit the aperture size and were measured twice and the data averaged.

CIE L’, a’, b’ values were recorded for each sample, where L’ represents the light/darkness value, a’ represents the red/green value, and b’ represents the yellow/blue value. The color strength of the dyed fabric was calculated using the Kubelka-Munk equation at the wavelength of maximum absorption [154]:

\[
K/S = \frac{(1.0 - R_\lambda)}{2.0 R_\lambda}
\]

where:

- \( R_\lambda \) is the reflectance of the fabric at \( \lambda_{\text{max}} \), recorded as decimal fraction.
3.4 Mutagenicity testing

The *Salmonella* mammalian mutagenicity assay is based on the use of several selected *Salmonella typhimurium* strains that revert from histidine dependence to histidine independence at an increased frequency in the presence of a mutagen [156]. In this test, *Salmonella* strains TA98 and TA100 were used. Strain TA100 is derived from TA1535 by the introduction of the plasmid pKM101 which increases the sensitivity of mutagen detection by enhancing error-prone DNA repair. The presence of this plasmid makes TA100 respond to both some frame-shift mutagens and base-pair substitution mutagens. TA98 is derived from TA1538 by the introduction of plasmid pKM101 and can detect various frame-shift mutagens. The test procedure was performed in the following way:

To 2-3 ml of top agar (maintained at about 47-50°C) was added approximately $10^8$ bacteria from a fresh culture, approximately 0.5 ml metabolic activation mixture, between 0.01 ml and 0.1 ml of a solution of test compound or solvent. This mixture was mixed gently and poured on plates that contained about 25 ml of minimal-glucose agar. After the top agar had solidified (about 20 minutes), the plates were inverted and incubated at 37°C for 72 h. The plates were scored for the number of revertant colonies and for the presence of a background lawn of auxotrophic bacteria.
IV. Results and Discussion

1. Synthesis of intermediates, direct dyes and pigments

1.1 4,4’-Dinitro-para-terphenyl (112)

Vanallan’s nitration method [149] was adopted in the present study, and the product yield was essentially the same (37%). This structure of the dinitro compound was confirmed by 1H NMR and EI mass spectrometry.

1.2 4,4’-Diamino-para-terphenyl (113)

When the procedure of Khromov-Borisov and coworkers’ [151] was repeated in our laboratory, the yield and purity were lower. Therefore, Pd (OH)$_2$/C catalyst was used instead of Raney nickel in Figure 54. The reduction step gave almost a quantitative yield and TLC showed a single product.

![Figure 54. The synthesis of 4,4’-diamino-para-terphenyl (113).](image-url)
1.3 4,4′-Dinitro-para-quaterphenyl (116)

The method of Harley-Mason and Mann (Figure 55) [153] was assessed and found to give a similar yield. The product was confirmed by $^1$H NMR and EI mass spectrometry.

![Synthesis of 4,4′-dinitro-para-quaterphenyl (116)]

Figure 55. The synthesis of 4,4′-dinitro-para-quaterphenyl (116).

1.4 4,4′-Diamino-para-quaterphenyl (117)

When the method of Hammond and coworkers (Figure 56) [146] was adopted for the reduction of 116, a high yield (93.4%) was obtained. This intermediate was confirmed by 1H NMR and EI mass spectrometry.

![Synthesis of 4,4′-diamino-para-quaterphenyl (117)]

Figure 56. The synthesis of 4,4′-diamino-para-quaterphenyl (117).
1.5 5-Acetoacetylaminobenzimidazolone (118)

5-Aminobenzimidazolone was converted to coupler 118 by the reaction of diketene at 85°C (Figure 57). The yield of 63% was lower than that reported (90%) ([157]). The structure was confirmed by mass spectrometry.

![Figure 57. Synthesis of 5-acetoacetylaminobenzimidazolone (118).](image)

1.6 4-[(2,5-Dichlorophenyl)azo]-3-hydroxy-2-naphthoyl chloride (120).

4-[(2,5-Dichlorophenyl)azo]-3-hydroxy-2-naphthoic acid was synthesized in two steps: 1) coupling 2,5-dichloroaniline diazonium chloride with 3-hydroxy-2-naphthoic acid and 2) reaction of the resultant naphthoic acid with PCl₅ [144] (Figure 58). The reactions gave satisfactory overall yield and the structures of 119 and 120 were confirmed by mass spectrometry.

![Figure 58. Synthesis of 4-[(2,5-dichlorophenyl)azo]-3-hydroxy-2-naphthoyl chloride (120).](image)
1.7 Direct dyes from 4,4'-diamino-para-terphenyl (121-126)

Tetrazotization of 4,4'-diamino-para-terphenyl was carried out using NaNO₂/HCl at 0-5°C. The tetrazotized solutions were added to an alkaline solution of 5 couplers (Naphthionic acid, J-acid, H-acid, Chromotropic acid, and Chicago acid) to give the target direct dyes (Figure 57). A C.I. Direct Black 38 analog was also synthesized by coupling the tetrazonium salt of 4,4'-diamino-para-terphenyl with H-acid under acidic conditions, followed by coupling under alkaline conditions with the diazonium ion from aniline, finally it was coupled with meta-phenylenediamine to form the target dye structure (Figure 59). The disazotization and couplings were straightforward and except for dye 126, the yields were >80%.

Figure 59. Synthesis of dyes from 4,4'-diamino-para-terphenyl. (a) HCl/NaNO₂; (b) Naphthionic acid (121); (c) J-acid (122), H-acid (123), Chromotropic acid (124); (d) Chicago acid (125).
Figure 60. The synthesis of dye 126 from 4,4'-diamino- para-terphenyl.

1.8 Direct dyes from 4,4'-diamino-para-quaterphenyl (127-132)

Since 4,4'-diamino-para-quaterphenyl had too low solubility in HCl, tetrazotization was carried out in conc. H\textsubscript{2}SO\textsubscript{4}. The tetrazotized diamine was added to an alkaline solution of 5 couplers (Naphthionic acid, J-acid, H-acid, Chromotropic acid, and Chicago acid) to give the target direct dyes (Figure 61). C.I. Direct Black 38 analog was also synthesized by coupling the tetrazonium salt of 4,4'-diamino-para-quaterphenyl with H-acid under acidic condition, coupling with the diazonium salt of aniline under alkaline condition, and then coupling with meta-phenylenediamine to form the target dye (Figure 62). Dyes 127 (22%), 130 (29%), 132 (37%) were obtained in low yield, while the other dyes gave yields over 80%.
Figure 61. Synthesis of dyes from 4,4'-diamino-para-quaterphenyl. (a) NaNO₂/Conc. H₂SO₄; (b) Naphthionic acid (127); (c) J-acid (128), H-acid (129), Chromotropic acid (130); (d) Chicago acid (131)

Figure 62. The synthesis of dye 132 from 4,4'-diamino-para-quaterphenyl.
1.9 Pigments from 4,4’-diamino-para-terphenyl (133-137) [144, 145]

4,4’-Diamino-para-terphenyl was condensed with 2-mercapto-4,6-dihydroxypyrimidine and triethylorthoformate (TEOF) in DMF to form the bisazomethine pigment (133). Disazonaphthol pigment (137) was synthesized by the reaction of the diamine with the corresponding azo dye acid chloride (120). Pigments 134-136 were synthesized by tetrazotization of 4,4’-diamino-para-terphenyl followed by coupling with acetoacetylanilide, 1-phenyl-3-methyl-5-pyrazolone or 5-acetoacetylaminobenzimidazolone (Figure 63). In most cases, the coupling was carried out at 0-5°C; however, coupling with 5-acetoacetylaminobenzimidazolone was conducted at room temperature. Also, Triton X-100 and Surfynol 104 PG-50 were added to the suspension of 5-acetoacetylaminobenzimidazolone before adding the tetrazonium solution. For pigment 136, a treatment involving hot DMF was used. The reactions gave 60-90% yield and the structures were confirmed by mass spectrometry or elemental analysis.
Figure 63. Synthesis of pigments from 4,4'-diamino-para-terphenyl. (a) 2-mercapto-4,6-dihydroxypyrimidine (133); (b) 4-[(2,5-dichlorophenyl)azo]-3-hydroxy-2-naphtoyl chloride (137); (c) HCl/NaNO₂; (d) acetoacetylanilide (134); (e) 1-phenyl-3-methyl-5-pyrazolone (135); (f) 5-acetoacetylaminobenzimidazolone (136).

1.10 Pigments from 4,4'-diamino-para-quaterphenyl(138-142)

4,4'-Diamino-para-quaterphenyl was condensed with 2-mercapto-4,6-dihydroxypyrimidine and triethylorthoformate (TEOF) in DMF to form the bisazomethine pigment (138). Disazonaphthol pigment (142) was synthesized by the reaction of diamine with the corresponding azo dye acid chloride (120). The other pigments (139-141) were synthesized by tetrazotization of 4,4'-diamino-para-quaterphenyl in conc. H₂SO₄, followed by coupling with acetoacetylanilide, 1-phenyl-3-methyl-5-pyrazolone or 5-acetoacetylaminobenzimidazolone (Figure 64). In most cases, the coupling was carried out at 0-5°C; however, coupling with
5-acetoacetylaminobenzimidazolone was carried out at room temperature. Also, Triton X-100 and Surfynol 104 PG-50 were added to the suspension of 5-acetoacetylaminobenzimidazolone before adding the tetrazonium solution. For pigment 141, a treatment involving hot DMF was used. The reactions gave 60-90% yield and the structures were confirmed by MALDI mass spectrometry or elemental analysis.

Figure 64. Synthesis of pigments from 4,4'-diamino-para-quaterphenyl. (a) 2-mercapto-4,6-dihydroxypyrimidine (138); (b) 4-[(2,5-dichlorophenyl)azo]-3-hydroxy-2-naphtoyl chloride (142); (c) NaNO2/ Conc. H2SO4; (d) acetoacetylanilide (139); (e) 1-phenyl-3-methyl-5-pyrazolone (140); (f) 5-acetoacetylaminobenzimidazolone (141).

2. Mass spectrometry

2.1 EI mass spectrometry of intermediates

As the oldest and best characterized of all the ionization methods, EI mass spectrometry [158] was used to help confirm the structures of
intermediates made in this study. In this regard, the mass spectra for intermediates 112, 113, and 115-120 are shown in Figure 65-72.

For 4,4’-dinitro-para-terphenyl (112), which has MW 320.1 (C_{18}H_{12}N_{2}O_{4}), the base peak in the spectrum corresponded to the ion-radical [M]^{+} (m/z = 320). Other key peaks resulted from the loss of one NO_{2} group to give m/z = 275.1 and the loss of two NO_{2} groups to give m/z = 228.1.

For 4,4’-diamino-para-terphenyl (113), which has MW 260.2 (C_{18}H_{16}N_{2}), the base peak in the spectrum corresponded to the ion-radical [M]^{+} (m/z = 260).

For 4-iodo,4’-nitrobiphenyl (115), which has MW 325.0 (C_{12}H_{8}I(NO_{2}), the base peak in the spectrum corresponded to the ion-radical [M]^{+} (m/z = 325). The loss of the NO_{2} and iodo groups gave m/z = 152.1.

For 4,4’-dinitro-para-quaterphenyl (116), which has MW 396.1 (C_{24}H_{16}N_{2}O_{4}), the base peak in the spectrum corresponded to the ion-radical [M]^{+} (m/z = 396). The successive loss of NO_{2} groups gave m/z = 350.1 and m/z = 304.1.

For 4,4’-diamino-para-quarterphenyl (117), which has MW 336.4 (C_{24}H_{20}N_{2}), base peak ([M]^{+}) in the spectrum corresponded to the ion-radical [M]^{+} (m/z = 233). A peak arising from the rupture of the bond in the center of this molecule was also observed (cf. m/z = 168.1).

For 5-acetoacetylaminobenzimidazolone (118), which has MW 233.2 (C_{11}H_{11}N_{3}O_{3}), the spectrum showed the molecular ion m/z = 233 as the base peak. Loss of the acetoacetyl group gave the fragment m/z = 148.
For 4-[(2,5-dichlorophenyl)azo]-3-hydroxy-2-naphthoic acid (119), which has MW 361.2 (C_{17}H_{10}Cl_{2}N_{2}O_{3}), the spectrum contained [M]^+ = 360 as the base peak. Loss of the 2,5-dichlorophenyl group gave m/z = 215, and cleavage at the azo bond gave m/z = 159.

For 4-[(2,5-dichlorophenyl)azo]-3-hydroxy-2-naphthoyl chloride (120), which has MW 379.62 (C_{17}H_{9}Cl_{3}N_{2}O_{2}), the spectrum contained [M]^+ = 379 as the base peak. The loss of a chloro group gave m/z = 342, and cleavage at the azo bond gave m/z = 159.

### 2.2 Electrospray mass spectrometry of direct dyes

Since electrospray mass spectrometry (ESMS) has proved to be the method of choice for analyzing sulfonated dyes [159], this technique was used to help confirm the direct dye structures made in the present study. As examples, the mass spectra for dyes 121-131 are shown in Figures 73-82. In each case, the major analyte peaks corresponded to signals for m/2 species.

For dye 121, which has MW 772.76 (C_{38}H_{26}N_{6}Na_{2}O_{6}S_{2}), the loss of 2 Na^+ ions gave a species with charge of 2^- . The signal corresponding to m/2 = 363.5 ([M-2Na]^2-) was the base peak in the spectrum, while a weak (<5% relative intensity) signal was observed for the [M-2Na+H]^+ species (see Figure 73).

Similarly, the loss of 2 Na^+ ions from dye 122 (MW 804.75, C_{38}H_{26}N_{6}Na_{2}O_{6}S_{2}) gave a structure with charge of 2^- . The signal corresponding to m/2 = 379.2 ([M-2Na]^2-) was the base peak in the spectrum. In this case, a
more intense (15% relative intensity) signal was observed for the \([M-2Na+H]^+\) species (see Figure 74).

For dye 123, which has MW 1008.83 (C\(_{38}H_{24}N_6Na_4O_{14}S_4\)), the loss of 4 Na\(^+\) ions and a gain of 2 H\(^+\) ions gave a structure with charge of 2\(^-\). The resulting signal corresponded to m/2 = 459.4. The \([M-4Na+2H]^{2-}\) species had a relative intensity of 65\% (see Figure 75).

For dye 124, which has MW 1010.80 (C\(_{38}H_{24}N_6Na_4O_{14}S_4\)), the loss of 4 Na\(^+\) ions and a gain of 2 H\(^+\) ions gave a structure with charge of 2\(^-\). The resulting signal for the base peak corresponded to m/2 = 459.4. The \([M-4Na+2H]^{2-}\) species had a relative intensity of 50\% (see Figure 76).

For dye 125, which has MW 1008.83 (C\(_{38}H_{24}N_6Na_4O_{14}S_4\)), the loss of 4 Na\(^+\) ions and a gain of 2 H\(^+\) ions gave the signal at m/2 = 459.3. This \([M-4Na+2H]^{2-}\) species had a relative intensity of 45\% (see Figure 77).

For dye 127, which has MW 848.85 (C\(_{44}H_{30}N_6Na_2O_6S_2\)), the loss of 2 Na\(^+\) ions gave a species with charge of 2\(^-\). This peak corresponded to the \([M-2Na]^{2-}\) ion (m/2 = 400.53). The base peak in the spectrum was 362.10 (see Figure 78).

For dye 128, which has MW 880.85 (C\(_{44}H_{30}N_6Na_2O_8S_2\)), the loss of 2 Na\(^+\) ions gave a structure with charge of 2\(^-\). This signal corresponded to the \([M-2Na]^{2-}\) ion (m/2 = 417.4). In this case, a more intense (15\% relative intensity) signal was observed for the \([M-Na]^-\) ion. The base peak in the spectrum was 569.47 (see Figure 79).

For dye 129, which has MW 1084.93 (C\(_{44}H_{28}N_6Na_4O_{14}S_4\)), the loss of 4 Na\(^+\) ions gave a structure with charge of 4\(^-\). The signal corresponding to m/4 =
248.80 ([M-4Na]^{4-}) was the base peak. The loss of 3 Na^+ ions gave m/3 = 336.45 ([M-3Na]^{3-}) and the loss of 2 Na^+ ions gave m/2 = 519.06 ([M-2Na]^{2-}).

For dye 130, which has MW 1086.90 (C_{44}H_{26}N_{6}Na_{4}O_{14}S_{4}), the loss of 3 Na^+ ions gave the [M-3Na]^{3-} ion (m/3 = 338.26). The loss of 2 Na^+ ions gave m/2 = 520.50 ([M-2Na]^{2-}). The base peak in the spectrum was 321.29 (see Figure 81).

For dye 131, which has MW 1084.93 (C_{44}H_{28}N_{6}Na_{4}O_{14}S_{4}), the loss of 2 Na^+ ions gave a structure with charge of 2^- and the signal corresponding to m/2 = 519.25 ([M-2Na]^{2-}). The base peak in the spectrum was 519.25 (see Figure 82).

2.3 MALDI mass spectrometry of organic pigments

Matrix assisted laser desorption/ionization (MALDI) mass spectrometry [160] was used to analyze organic pigments 133-140. While this technique was developed as a method for generating MW information on biomolecules, it was regarded as comparable to the field desorption method used previously in our laboratory for azo and azomethine pigments [161]. The mass spectra of pigments 133-135 and 140 are shown in Figures 83-86.

For pigment 133, which has MW 568.62 (C_{28}H_{20}N_{6}O_{4}S_{2}), protonation gave a structure with charge of 1^+ and a signal corresponding to m/z = 569.9. Similarly, pigments 134 (MW 636.70, C_{38}H_{32}N_{6}O_{4}), 135 (MW 630.70, C_{38}H_{30}N_{8}O_{2}), and 140 (MW 706.8, C_{44}H_{34}N_{8}O_{2}) gave m/z = 636.9, 631.9, and 707.7, respectively.
Figure 65. EI mass spectrum of 4,4'-dinitro-para-terphenyl (112).
Figure 66. EI mass spectrum of 4,4'-diamino-para-terphenyl (113).
Figure 67. EI mass spectrum of 4-iodo,4'-nitro biphenyl (115).
Figure 68. EI mass spectrum of 4,4'-dinitro-para-quaterphenyl (116).
Figure 69. El mass spectrum of 4,4'-diamino-para-quaterphenyl (117).
Figure 70. EI mass spectrum of 5-acetoacetylaminobenzimidazolone (118).
Figure 71. El mass spectrum of 4-[(2,5-dichlorophenyl)azo]-3-hydroxy-2-naphthoic acid (119).
Figure 72. EI mass spectrum of 4-[(2,5-dichlorophenyl)azo]-3-hydroxy-2-naphthoyl chloride (120).
Figure 73. Electrospray ionization mass spectrum of dye 121.

Figure 74. Electrospray ionization mass spectrum of dye 122.
Figure 75. Electrospray ionization mass spectrum of dye 123.

Figure 76. Electrospray ionization mass spectrum of dye 124.
Figure 77. Electrospray ionization mass spectrum of dye 125.

Figure 78. Electrospray ionization mass spectrum of dye 127.
Figure 79. Electrospray ionization mass spectrum of dye 128.

Figure 80. Electrospray ionization mass spectrum of dye 129.
Figure 81. Electrospray ionization mass spectrum of dye 130.

Figure 82. Electrospray ionization mass spectrum of dye 131.
Figure 83. Positive ion MALDI mass spectrum of pigment 133.

Figure 84. Positive ion MALDI mass spectrum of pigment 134.
Figure 85. Positive ion MALDI mass spectrum of pigment 135.

Figure 86. Positive ion MALDI mass spectrum of pigment 140.
3. Absorption Spectra and Colors

The visible absorption spectra of dyes 121-125 were recorded in distilled water, the results of which are summarized in Table 6 and Figure 87-91. When DATP was used as the diamine, Chicago acid gave the highest $\lambda_{\text{max}}$ followed by H-acid, Chromotropic acid, J-acid and Naphthionic acid. The $\lambda_{\text{max}}$ values for these dyes were calculated using PISYSTEM and compared with the experimental data. In addition, PISYSTEM generated absorption intensity in terms of the oscillator strength. The results showed good agreement between the experimental and calculation values. For the calculations, the phenyl rings were rotated to give a 25° dihedral angle and dyes 122, 123, 124 and 125 were drawn in the hydrazone rather than the azo form.

Table 7 shows the effects of different solvents on color and $\lambda_{\text{max}}$. The visible spectra of the dyes 121-126 were recorded in DMF (Figure 92-97) and the comparisons were made with results in distilled water. The comparisons are listed in Table 7. We found that these dyes had a higher $\lambda_{\text{max}}$ in DMF than in water. This may be explained by the increase the percentage of the hydrazone form in DMF.

In Table 8 and Figure 98-103, the absorbance data for dyes based on DAQP (127-132) show the effects of the couplers on color and $\lambda_{\text{max}}$. Results similar to those for dyes based on DATP were obtained, Chicago acid gave the highest $\lambda_{\text{max}}$, followed by H-acid, Chromotropic acid, Naphthionic acid, and J-acid.
In Table 9, the absorbance data in DMF are presented to show the effects of the two diamines. When the same couplers were used, dyes based on DATP gave higher $\lambda_{\text{max}}$ than those based on DAQP. These results showed that by increasing the number of phenylene rings, a hypsochromic shift was observed.

Absorption spectra data for pigments based on DATP (133-137) and DAQP (138-142) in DMF are shown in Table 10 and Figure 104-113. These pigments had a low solubility in DMF, so only $\lambda_{\text{max}}$ data were recorded. Similar to data on direct dyes based on DATP and DAQP, pigments based on DATP gave higher $\lambda_{\text{max}}$ values than those based on DAQP.

Table 6. Absorption spectral data for dyes based on DATP.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Coupler</th>
<th>Color</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$E_{\text{max}}$ L·mol$^{-1}$·cm$^{-1}$</th>
<th>PISYSTEM calculations</th>
<th>Oscillator strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>121</td>
<td>Naphthionic acid</td>
<td>Orange</td>
<td>477.0</td>
<td>1.84×10$^4$</td>
<td>477.0</td>
<td>1.895</td>
</tr>
<tr>
<td>122</td>
<td>J-acid</td>
<td>Red</td>
<td>516.0</td>
<td>3.38×10$^4$</td>
<td>521.8</td>
<td>2.882</td>
</tr>
<tr>
<td>123</td>
<td>H-acid</td>
<td>Blue</td>
<td>566.0</td>
<td>6.31×10$^4$</td>
<td>555.2</td>
<td>2.839</td>
</tr>
<tr>
<td>124</td>
<td>Chromotropic acid</td>
<td>Violet</td>
<td>564.0</td>
<td>4.61×10$^4$</td>
<td>524.4</td>
<td>2.739</td>
</tr>
<tr>
<td>125</td>
<td>Chicago acid</td>
<td>Blue</td>
<td>578.0</td>
<td>5.14×10$^4$</td>
<td>542.4</td>
<td>2.877</td>
</tr>
</tbody>
</table>
Table 7. Absorption spectral data for dyes based on DATP in water and DMF.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Coupler</th>
<th>H₂O</th>
<th>DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</td>
<td>E&lt;sub&gt;max&lt;/sub&gt; L·mol⁻¹·cm⁻¹</td>
</tr>
<tr>
<td>121</td>
<td>Naphthonic acid</td>
<td>477.0</td>
<td>1.84×10⁴</td>
</tr>
<tr>
<td>122</td>
<td>J-acid</td>
<td>516.0</td>
<td>3.38×10⁴</td>
</tr>
<tr>
<td>123</td>
<td>H-acid</td>
<td>566.0</td>
<td>6.31×10⁴</td>
</tr>
<tr>
<td>124</td>
<td>Chromotropic acid</td>
<td>564.0</td>
<td>4.61×10⁴</td>
</tr>
<tr>
<td>125</td>
<td>Chicago acid</td>
<td>578.0</td>
<td>5.14×10⁴</td>
</tr>
<tr>
<td>126</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 8. Absorption spectral data for dyes based on DAQP in DMF.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Coupler</th>
<th>Color</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>E&lt;sub&gt;max&lt;/sub&gt; L·mol⁻¹·cm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>127</td>
<td>Naphthonic acid</td>
<td>Red</td>
<td>502.0</td>
<td>2.95×10⁴</td>
</tr>
<tr>
<td>128</td>
<td>J-acid</td>
<td>Red</td>
<td>498.0</td>
<td>4.03×10⁴</td>
</tr>
<tr>
<td>129</td>
<td>H-acid</td>
<td>Purple</td>
<td>554.0</td>
<td>2.85×10⁴</td>
</tr>
<tr>
<td>130</td>
<td>Chromotropic acid</td>
<td>Purple</td>
<td>541.0</td>
<td>2.45×10⁴</td>
</tr>
<tr>
<td>131</td>
<td>Chicago acid</td>
<td>Blue</td>
<td>574.0</td>
<td>2.41×10⁴</td>
</tr>
<tr>
<td>132</td>
<td>-</td>
<td>Brown</td>
<td>473.0</td>
<td>3.66×10⁴</td>
</tr>
</tbody>
</table>
Table 9. Absorption spectral data for dyes based on DATP and DAQP in DMF.

<table>
<thead>
<tr>
<th>Coupler</th>
<th>Dye</th>
<th>$\lambda_{\text{max}}$ (nm) in DMF</th>
<th>Dye</th>
<th>$\lambda_{\text{max}}$ (nm) in DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthionic acid</td>
<td>121</td>
<td>516.0</td>
<td>127</td>
<td>502.0</td>
</tr>
<tr>
<td>J-acid</td>
<td>122</td>
<td>518.0</td>
<td>128</td>
<td>498.0</td>
</tr>
<tr>
<td>H-acid</td>
<td>123</td>
<td>576.0</td>
<td>129</td>
<td>554.0</td>
</tr>
<tr>
<td>Chromotropic acid</td>
<td>124</td>
<td>571.0</td>
<td>130</td>
<td>541.0</td>
</tr>
<tr>
<td>Chicago acid</td>
<td>125</td>
<td>585.0</td>
<td>131</td>
<td>574.0</td>
</tr>
<tr>
<td>-</td>
<td>126</td>
<td>476.0</td>
<td>132</td>
<td>473.0</td>
</tr>
</tbody>
</table>

Table 10. Absorption spectral data for pigments based on DATP and DAQP in DMF.

<table>
<thead>
<tr>
<th>Pigment</th>
<th>$\lambda_{\text{max}}$ (nm) in DMF</th>
<th>Pigment</th>
<th>$\lambda_{\text{max}}$ (nm) in DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>133</td>
<td>382.0</td>
<td>138</td>
<td>349.0</td>
</tr>
<tr>
<td>134</td>
<td>414.0</td>
<td>139</td>
<td>408.0</td>
</tr>
<tr>
<td>135</td>
<td>468.0</td>
<td>140</td>
<td>467.0</td>
</tr>
<tr>
<td>136</td>
<td>415.0</td>
<td>141</td>
<td>411.0</td>
</tr>
<tr>
<td>137</td>
<td>546.0</td>
<td>142</td>
<td>535.0</td>
</tr>
</tbody>
</table>
Figure 87. Absorption spectrum of dye 121 in water.

Figure 88. Absorption spectrum of dye 122 in water.

Figure 89. Absorption spectrum of dye 123 in water.
Figure 90. Absorption spectrum of dye 124 in water.

Figure 91. Absorption spectrum of dye 125 in water.

Figure 92. Absorption spectrum of dye 121 in DMF.
Figure 93. Absorption spectrum of dye 122 in DMF.

Figure 94. Absorption spectrum of dye 123 in DMF.

Figure 95. Absorption spectrum of dye 124 in DMF.
Figure 96. Absorption spectrum of dye 125 in DMF.

Figure 97. Absorption spectrum of dye 126 in DMF.

Figure 98. Absorption spectrum of dye 127 in DMF.
Figure 99. Absorption spectrum of dye 128 in DMF.

Figure 100. Absorption spectrum of dye 129 in DMF.

Figure 101. Absorption spectrum of dye 130 in DMF.
Figure 102. Absorption spectrum of dye 131 in DMF.

Figure 103. Absorption spectrum of dye 132 in DMF.

Figure 104. Absorption spectrum of pigment 133 in DMF.
Figure 105. Absorption spectrum of pigment 134 in DMF.

Figure 106. Absorption spectrum of pigment 135 in DMF.

Figure 107. Absorption spectrum of pigment 136 in DMF.
Figure 108. Absorption spectrum of pigment 137 in DMF.

Figure 109. Absorption spectrum of pigment 138 in DMF.

Figure 110. Absorption spectrum of pigment 139 in DMF.
Figure 111. Absorption spectrum of pigment 140 in DMF.

Figure 112. Absorption spectrum of pigment 141 in DMF.

Figure 113. Absorption spectrum of pigment 142 in DMF.
4. Fastness properties

The results of fastness tests are summarized in Table 11. The rating scale was 1 (poor) to 5 (excellent). While still low, lightfastness was generally better for dyes based on DATP (dyes 122, 123, 124, 125 and 126), probably because the structures of these dyes are more linear than dye 127, 128, 129, 130, 131 and 132, which are based on DAQP. All these dyes gave similar wash fastness except dye 124 and dye 129. Dye 129 had the highest wash fastness, while dye 124 had the lowest wash fastness. Also staining on cotton was better when the less linear dyes 127, 128, 129, 130, 131 and 132 were employed.

Table 11. Fastness data for the dyes prepared in this study.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Light Fastness</th>
<th>Wash Fastness</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Change in Shade</td>
<td></td>
<td>Staining on Cotton</td>
<td>Staining on Wool</td>
</tr>
<tr>
<td>121</td>
<td>1.0</td>
<td>3.5</td>
<td>1.5</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>122</td>
<td>2.0</td>
<td>3.0</td>
<td>1.0</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>123</td>
<td>2.0</td>
<td>2.5</td>
<td>1.5</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>124</td>
<td>2.5</td>
<td>2.0</td>
<td>2.0</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>125</td>
<td>2.0</td>
<td>2.5</td>
<td>1.5</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>126</td>
<td>2.0</td>
<td>3.5</td>
<td>2.0</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>127</td>
<td>1.0</td>
<td>2.5</td>
<td>4.0</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>128</td>
<td>1.0</td>
<td>2.5</td>
<td>2.0</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>129</td>
<td>1.0</td>
<td>4.0</td>
<td>2.0</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>130</td>
<td>1.5</td>
<td>2.5</td>
<td>2.5</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>131</td>
<td>1.0</td>
<td>3.0</td>
<td>2.0</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>132</td>
<td>1.0</td>
<td>3.0</td>
<td>3.0</td>
<td>4.0</td>
<td></td>
</tr>
</tbody>
</table>
5. Color values

5.1 L*, a*, b* values for direct dyes

The L*, a*, b* values for the dyed fabrics based on DATP and DAQP are reported in Table 12. In general, the light/darkness value, L, increased for dyes based on DAQP (127-132) compared to their counterparts based on DATP (121-126). Except for dye 127, the other dyes based on DAQP had higher red character (high a* values) than those based on DATP.

Table 12. L*, a*, b* Values for dyes 121-132.

<table>
<thead>
<tr>
<th>Dye</th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
</tr>
</thead>
<tbody>
<tr>
<td>121</td>
<td>56.14</td>
<td>46.29</td>
<td>41.68</td>
</tr>
<tr>
<td>127</td>
<td>71.12</td>
<td>26.42</td>
<td>29.04</td>
</tr>
<tr>
<td>122</td>
<td>32.66</td>
<td>38.2</td>
<td>-1.77</td>
</tr>
<tr>
<td>128</td>
<td>45.83</td>
<td>44.3</td>
<td>3.76</td>
</tr>
<tr>
<td>123</td>
<td>30.4</td>
<td>13.38</td>
<td>-24.18</td>
</tr>
<tr>
<td>129</td>
<td>40.05</td>
<td>19.22</td>
<td>-21.24</td>
</tr>
<tr>
<td>124</td>
<td>34.68</td>
<td>10.84</td>
<td>-29.09</td>
</tr>
<tr>
<td>130</td>
<td>48.62</td>
<td>18.65</td>
<td>-23.04</td>
</tr>
<tr>
<td>125</td>
<td>31.12</td>
<td>10.89</td>
<td>-27.34</td>
</tr>
<tr>
<td>131</td>
<td>44.53</td>
<td>15.21</td>
<td>-25.59</td>
</tr>
<tr>
<td>126</td>
<td>33.14</td>
<td>6.08</td>
<td>-3.65</td>
</tr>
<tr>
<td>132</td>
<td>49.23</td>
<td>13.97</td>
<td>-8.33</td>
</tr>
</tbody>
</table>
5.2 K/S spectra for dyed fabrics

Table 13 shows K/S values for dyed cotton. The results indicated the same trend observed for the absorbance values recorded in solution. The fabrics dyed with dyes from DAQP reflected a hypsochromic shift compared to those dyed with dyes from DATP.

Table 13. K/S data for dyeings generated on cotton.

<table>
<thead>
<tr>
<th>Dye</th>
<th>λ_max (nm) of dyed fabrics</th>
<th>K/S</th>
</tr>
</thead>
<tbody>
<tr>
<td>121</td>
<td>500 (360)</td>
<td>3.21 (0.21)</td>
</tr>
<tr>
<td>127</td>
<td>360</td>
<td>0.21</td>
</tr>
<tr>
<td>122</td>
<td>530 (520)</td>
<td>5.75 (2.38)</td>
</tr>
<tr>
<td>128</td>
<td>590 (570)</td>
<td>4.96 (2.19)</td>
</tr>
<tr>
<td>123</td>
<td>590 (570)</td>
<td>3.24 (0.76)</td>
</tr>
<tr>
<td>129</td>
<td>570</td>
<td>0.76</td>
</tr>
<tr>
<td>130</td>
<td>590 (570)</td>
<td>4.79 (1.40)</td>
</tr>
<tr>
<td>124</td>
<td>590 (570)</td>
<td>3.24 (0.76)</td>
</tr>
<tr>
<td>131</td>
<td>590 (580)</td>
<td>4.79 (1.40)</td>
</tr>
<tr>
<td>125</td>
<td>570</td>
<td>2.27</td>
</tr>
<tr>
<td>132</td>
<td>560</td>
<td>0.48</td>
</tr>
</tbody>
</table>
6. Mutagenicity assessments

6.1 Mutagenicity of benzidine and its homologs

Figure 114-117 shows the dose response curves for the diamines tested. The background count was established by the number of revertant colonies counted for a control test in which no diamine was present. A mutagenic response was recorded if the number of revertant colonies counted were at least twice the background count.

![Dose-Response curve](image)

Figure 114. Dose response for four diamines using the standard mutagenicity assay and TA98 without S9 activation.
Figure 115. Dose response for four diamines using the standard mutagenicity assay and TA98 with S9 activation.

Figure 116. Dose response for four diamines using the standard mutagenicity assay and TA100 without S9 activation.
Figure 117. Dose response for four diamines using the standard mutagenicity assay and TA100 with S9 activation.

As can be seen from Figures 114-117, all diamines were negative in TA100, all diamines were negative in TA98 without metabolic activation except DATP, and all diamines except DAQP were mutagenic in TA98 with S9 metabolic activation. The results showed that DATP gave the highest number of reverant colonies in TA98 with/without S9 activation and its mutagenicity did not require enzyme activation. For the benzidine homologs, it was anticipated that increasing the number of the phenylene rings between the amino groups would decrease mutagenicity. This was not the case for DATP, although its analog (DAQP) was nonmutagenic. Our results were similar to that of Wuebbles and coworkers [162], who reported that DATP was mutagenic and DAQP was nonmutaginic.

Although DATP showed good dose-response at the range of 30-100 µg, when the dose level reached 300 µg, toxicity to the bacteria was observed. Thus, a lower dose range was adopted for testing DATP and the results are shown in Figure 118.
These results were similar to those obtained at higher doses (Figure 114, 115). It is interesting that at 1-5 µg levels, this compound was negative, while when it reached 10 µg, a significant increase in colonies versus dose occurred. It is possible that only the amount of the compound around the bacteria reached the concentration required to interact with the bacteria to cause a mutagenic response.

Also diamines DATP and DAQP were tested by using the preincubation protocol developed by Prival and Mitchell [130]. The results were showed in Figures 119 and 120, where it can be seen that DAQP was negative throughout the dose range in strains TA98 and TA100, while DATP gave positive results in both strains.
Figure 119. Dose response for DATP and DAQP using the Prival mutagenicity assay and TA98 with S9 activation.

Figure 120. Dose response for DATP and DAQP using the Prival mutagenicity assay and TA100 with S9 activation.
6.2 Mutagenicity of direct dyes derived from DATP and DAQP

Direct dyes based on DATP and DAQP were evaluated in the standard *Salmonella mammalian* mutagenicity assay [93]. Also Congo Red, dyes 121 and 127 were tested by using the Prival preincubation protocol [130]. Figure 121-132 showed the dose response curves for dyes tested.

It was found that dyes 121-123, 125 and 127-131 were negative in TA98 with and without S9 activation. However, dye 124, which is based on Chromotropic acid, was weakly mutagenic in TA98 (Figure 121, 122). Dye 126, which is the Direct Black 38 analog based on DATP, gave a very high positive result in TA98 with S9 activation (Figure 125). After the dose reached 300 µg, this dye showed toxicity to the bacteria. Dye 126 also gave a good dose-response curve without S9 activation, confirming that it was Ames positive. Dye 132, which is the Direct Black 38 analog based on DAQP, was Ames positive in TA98 with S9 activation, and after the dose reached 100 µg, this dye showed toxicity to the bacteria (Figure 126). Dye 132 showed a dose-response curve without S9 activation until the dose reached 300 µg, where toxicity was observed. All dyes were negative in TA100.
Figure 121. Dose response for dyes 121-125 using the standard mutagenicity assay and TA98 without S9 activation.

Figure 122. Dose response for dyes 127-131 using the standard mutagenicity assay and TA98 without S9 activation.
Figure 123. Dose response for dyes 121-125 using the standard mutagenicity assay and TA98 with S9 activation.

Figure 124. Dose response for dyes 127-131 using the standard mutagenicity assay and TA98 with S9 activation.
Figure 125. Dose response for dye 126 using the standard mutagenicity assay and TA98 with/without S9 activation.

Figure 126. Dose response for dye 132 using the standard mutagenicity assay and TA98 with/without S9 activation.
Figure 127. Dose response for dyes 111-115 using the standard mutagenicity assay and TA100 without S9 activation.

Figure 128. Dose response for dyes 127-131 using the standard mutagenicity assay and TA100 without S9 activation.
Figure 129. Dose response for dyes 121-125 using the standard mutagenicity assay and TA100 with S9 activation.

Figure 130. Dose response for dyes 127-131 using the standard mutagenicity assay and TA100 with S9 activation.
Figure 131. Dose response for dye **126** using the standard mutagenicity assay and TA100 with/without S9 activation.

Figure 132. Dose response for dye **132** using the standard mutagenicity assay and TA100 with/without S9 activation.
Figure 133. Dose response for Congo Red, dye 121 and 127 using the Prival mutagenicity assay and TA98 with S9 activation.

Figure 134. Dose response for Congo Red, dye 121 and 127 using the Prival mutagenicity assay and TA100 with S9 activation.
Figure 135. Dose response of Congo Red, dye 121 and 127 using the Prival mutagenicity assay and TA98 with S9 activation (dose range=30-1000 µg).

Figure 136. Dose response for Congo Red, dye 111 and 117 using the Prival mutagenicity assay and TA100 with S9 activation (dose range=30-1000 µg).
The Prival test results showed that Congo Red and dye 121 were positive in strain TA98, with dye 121 having higher mutagenicity (Figure 133). Dye 121 gave a dose-response curve until the dose reached 30 µg, then it showed toxicity to the bacteria at 50 µg. Congo Red gave a positive dose-response curve throughout the dose range except at 5 µg. Dye 127 was negative throughout these dose ranges. Dye 121 gave a dose response curve with strain TA100 and when the dose reached 30 µg, it was positive. Congo Red gave similar result and it was positive when the dose reached 5 µg. Dye 127 was negative throughout these dose ranges (Figure 134). At higher dose ranges, all three dyes were positive in strain TA98 (Figure 135). In this case, the most mutagenic dye was Congo Red. Congo Red gave a positive dose-response curve until the dose reached 500 µg, where the compound was toxic to the bacteria. In the case of dye 121, toxicity to the bacteria was observed throughout the dose ranges (30-1000 µg) and the dye was still mutagenic. Dye 127 showed mutagenicity when dose reached 300 µg. However, it was weakly mutagenic compared to the other two dyes. Congo Red and dye 121 were positive in strain TA100 but dye 127 was negative in this case.
6.3 Mutagenicity testing of pigments derived from DATP and DAQP

Figures 137 and 139 show the dose response curves for pigments 133-137 based on DATP and the dose response curves for pigments 138-142 based on DAQP are shown in Figure 138 and 140. These results are from the Prival Test in bacteria strains TA 98 and TA100. All pigments were negative in both strains, which may be due in part to the very low solubility of the pigments in the test media.

![Dose-Response Curve](image)

**Figure 137.** Dose response for pigments 133-137 using the Prival mutagenicity assay and TA98 with S9 activation.
Figure 138. Dose response for pigments 138-142 using the Prival mutagenicity assay and TA98 with S9 activation.

Figure 139. Dose response for pigments 133-137 using the Prival mutagenicity assay and TA100 with S9 activation.
Figure 140. Dose response for pigments 138-142 using the Prival mutagenicity assay and TA100 with S9 activation.
V. Conclusions

Diamines 4,4′-diamino-para-terphenyl (DATP) and 4,4′-diamino-para-quaterphenyl (DAQP) were synthesized and evaluated as potential replacements for benzidine in direct dye and pigment synthesis. It was found that both diamines couple with couplers that have been frequently used to prepare direct dyes and pigments. It was clear that increasing the number of phenylene groups in the benzidine moiety gave a hypsochromic shift in the color of the resultant colorants. The dyes included analogs of the commercial colorants Congo Red (121, n=0), which were made from DATP (cf. 126) and DAQP (cf. 132). In this case, it was of interest to note that the visible absorption properties of the three dyes validated results from molecular modeling studies, which predicted that the new dyes would have lower $\lambda_{\text{max}}$ values than the parent dye. In this regard, $\lambda_{\text{max}}$ values recorded in DMF for Congo Red and its two homologs respectively were 529nm (n=0), 516nm (n=1), and 502nm (n=2).

Structures of the direct dyes derived from DATP (cf. 121-125) were confirmed by negative ion electrospray spectrometry (ESMS). The peaks observed corresponded to the formation of m/2 species. Similarly, the structures of novel organic pigments from both diamines were confirmed with the aid of matrix assisted laser desorption/ionization (MALDI) mass spectrometry and elemental analysis.

Results of mutagenicity tests involving the standard (Ames) and preincubation (Prival) assays showed that DATP was mutagenic in TA98 with S9 activation, while both diamines were negative in TA100 with or without S9
activation. Also, in the case of DATP, S9 activation was not required for mutagenicity in TA98. The Prival assay gave similar results for the mutagenicity of DATP and DAQP.

DATP was very mutagenic, while DAQP was nonmutagenic over the entire dose range used. For dyes based on DATP, those derived from naphthionic acid, J-acid, H-acid and Chicago acid were negative in TA98 in the standard assay. In the case of DAQP, only the disazo dye derived from naphthionic acid and the C.I. Direct Black 38 analog were mutagenic in TA98 in the Ames assay. Both dyes were negative in TA100 in the standard assay.

Results from the Prival assay showed that dyes derived from coupling DATP or DAQP to naphthionic acid were mutagenic in TA98. The former dye was also mutagenic in TA100; however, the latter was negative. In the Prival assay involving DATP and DAQP, these two dyes gave a higher mutagenicity than in the standard assay. This indicated that reductive-cleavage of the present disazo dyes is critical to producing the active species, which is known to be the case for Congo Red. All pigments were negative in the Prival test in TA98 and TA100, due at least in part to the very low solubility of the pigments in the test media.

The result of this study indicated that DATP and DAQP could be used in organic dye and pigment synthesis. Although DATP was much easier to tetrazotize than DAQP, mutagenicity test results showed that its mutagenicity is higher than benzidine and suggested that it should not be used as benzidine replacement. Therefore, it is likely that only DAQP would be used industrially.
However, it is possible that the low light stability of dyes derived from DAQP would have limited commercial use.

Interestingly, the high melting points and decomposition temperatures associated with organic pigments based on DAQP suggested that these colorants would be suitable for applications requiring high performance pigments, such as the coloration of fibers and plastics during melt extrusion.
VI. List of References


Appendix A. Protocols used in the *Salmonella* Reverse Mutation Assay

1.1 VBME salt solution

Measure 600 ml of deionized water into a clean 2 L Erlenmeyer flask. Add a magnetic stirring bar and heat the water on a hot plate with stirring. When the temperature reaches about 45°C, add slowly in the following order:

- 10 g Magnesium sulfate
- 100 g Citric acid monohydrate
- 500 g Potassium phosphate
- 175 g Sodium ammonium phosphate

Allow each compound to dissolve before adding the next. After all compounds have dissolved, pour the solution into a 1 L graduated cylinder and adjust the volume to 1 L using deionized water. Filter the solution through Whatman #1 filter paper using a Buchener funnel. After completing filtration, pour the filtrate into clean 500 ml bottles labeled “50X VBME solution”. Autoclave the bottles, loosely capped, for 30 minutes at 121°C on slow exhaust. After the bottles and contents have cooled, tighten caps and store. The solutions may not be stored longer than 2 months.

1.2 Histidine/Biotin solution

Measure 1 L of deionized water into a beaker. Add 0.0976 g of d-biotin, cover with aluminum foil and heat with stirring until biotin dissolves. Remove flask from hot plate and allow solution to cool. Add 0.0525 g 1-histidine HCl and stir to dissolve. Sterilize by filtering through a sterile 0.45 micron filter into a sterile
Erlenmeyer flask or other 500 ml reservoir. Label as “his/bio solution” and store in refrigerator no longer than 3 months.

1.3 VBME plates.

Remove the his/bio solution from the refrigerator, allow coming to room temperature. Measure 2655 ml of deionized water and pour into a 4-liter flask. Weigh 45 g of agar and add to the water; add a magnetic stirbar and cover the flask with aluminum foil; swirl to mix. Place the flask on a hot plate and heat at a medium-high temperature, while stirring with stirbar, until agar dissolves. After the agar is dissolved, autoclave the flask for 20 minutes at 121±2°C on slow exhaust. Allow the solution to cool at room temperature for a few minutes and then add 200 ml dextrose solution to the agar solution. Place the flask in a 48±2°C water bath and allow it to cool for 15 minutes. Using sterile technique, measure 60ml of 50X VBME solution into a sterile graduated cylinder. Pour the VBME solution into the agar solution and swirl to mix. After mixing, check the agar solution for any precipitates that might form. Discard the solution of there are any precipitates.

Cool the flask in the water bath for an additional 15 minutes. Using sterile technique, measure 30 ml of the his/bio solution in a sterile graduated cylinder. Add this solution to the agar solution and swirl to mix. Return flask to water bath. Using an automatic plate pourer (SOP MB-045), dispense agar into sterile 100×15 mm Petri dishes, 28 to 30 ml per dish. After agar solidifies, shake off excess condensate from the lids, pack in bags and store the VBME plates at 5±2°C for no longer than 30 days.
2. **Oxoid nutrient broth No. 2 solution**

   Dissolve 25 g of Oxoid nutrient broth #2 in water in an aluminum foil-covered Erlenmeyer flask over medium heat with stirring. Dispense into 20 ml flasks using a sterile pipette. Cap the flasks. Autoclave for 20 minutes at 121°C on slow exhaust. Store in refrigerator for up to 2 months.

3. **Top agar**

   Add 6 g of agar and 5 g of NaCl to a 2 L flask. Measure 1 L of deionized water in a graduated cylinder and pour into the flask. Carefully add a magnetic stirbar and swirl the mixture. Cover the flask with aluminum foil and heat over medium-high heat on a hot plate with stirring until the mixture dissolves.

   Fill the test tube racks with the 13×100 mm glass tubes. Dispense 2.2 ml agar per tube with the Cornwall syringe. Cap the tubes with the Bacti-Capall caps and autoclave for 20 minutes at 121±2°C on slow exhaust. Tubes can be stored at room temperature for 1 month or in a 4±2°C refrigerator for 2 months. Before use, melt the contents in an autoclave and cool to 45°C in water bath.

4. **Ames strain culture**

4.1 **Preparation of Ames tester strain master plates**

   Thaw a frozen permanent. Apply a drop of culture using a 1 ml pipette or 10 cc syringe and 21 g needle to each plate to be streaked. Sterilize the inoculating loop over the Bunsen burner flame and streak out culture for single colony isolation. Incubate the plates for 48 hours at 37±2°C. Master plates are stored in the refrigerator at 4±2°C for 3 to 4 weeks.
4.2 Preparation of Ames strain overnight culture

Label each flask to be used with appropriate strain name. Sterilize inoculating loop with flame. Select one colony from the master plate and inoculate the appropriately pre-labeled flask. Place the flask in the shaker incubator and incubate for 16 hours at 37±2°C with constant agitation (low speed, 100rpm).

5 Preparation of S9 mixture

5.1 Preparing S9 mixture for standard Ames test

Calculate the amount of S9 mixture for the assay. Weigh out the G-6-P. The amount needed is 1.41 mg per ml of S9 mixture. Weigh out the NADP. The amount needed is 3.06 mg per ml of S9 mixture. Transfer the compounds into a sterile polypropylene dilution tube and set aside. The amount of microsomal salt solution (MSS) needed is 0.02 ml per ml of S9 mixture. Using sterile technique, deliver the amount with a sterile pipette into sterile Erlenmeyer flask. The amount of phosphate buffer needed is one half of the total volume of S9 mixture. Determine the amount of S9 required from the optimization information. Add the volumes of the S9, MSS and buffer to be used in the mixture. Subtract the sum from the total volume of mixture needed. The remainder is the amount of sterile deionized water to be added to the G-6-P and NADP. Add water to the G-6-P and NADP in the polypropylene tubes, cap and shake to dissolve. Add these solutions to the mixture. The S9 is added last and this procedure is performed in the hood using sterile technique. The mixture is placed on ice and is kept on ice for the duration of the assay.
5.2 Preparing S9 mixture for the Prival modification of Ames Assay

Calculate the amount of S9 mixture needed for the assay. The volume can be determined by multiplying the number of plates requiring S9 activation by the volume of S9 mixture used per plate (0.5 ml) for the Prival modification of Ames assay. A recipe for 100 ml of S9 mixture can be showed in Table 1. the components are added in the following order: microsomal salt solution (MSS), phosphate buffer, cofactors, balance of water and S9 fraction. The S9 mixture is placed on ice and is kept chilled for the duration of the assay and is discarded afterwards.

Table 1. The components of 100 ml S9 mixture.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume or mass in 100 ml</th>
<th>Concentration in S9 Mix</th>
</tr>
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<tbody>
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<tr>
<td>MgCl₂/KCl (MSS)</td>
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<tr>
<td>G6P</td>
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<td>S9</td>
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Appendix B. Raw data for mutagenicity in Ames and Prival tests
Table 2. Results for 1,4-diaminophenylene (DAP) from Ames test.

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Table 3. Results for Diaminobiphenyl (DABP) from Ames test.

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Table 4. Results for DATP (113) from Ames test.

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Table 5. Results for DAQP (117) from Ames test.

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Table 6. Results for DATP (1-50 µg) from Ames test.

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Table 7. Results for DATP from the Prival assay.

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Table 9. Results for dye 121 from Ames test.

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Table 10. Results for dye 122 from Ames test

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Table 11. Results for dye 123 from Ames test.

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<td>134</td>
</tr>
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<td>35</td>
<td>29.5</td>
<td>141</td>
<td>127</td>
</tr>
<tr>
<td>1000</td>
<td>34</td>
<td>25</td>
<td>144.5</td>
<td>124</td>
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</table>
Table 12. Results for dye 124 from Ames test.

<table>
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<tr>
<th>Dose (µg)</th>
<th>TA98 (-)</th>
<th>TA98 (+)</th>
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<th>TA100 (+)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>30</td>
<td>43</td>
<td>82</td>
<td>108</td>
</tr>
<tr>
<td>30</td>
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</tr>
<tr>
<td>50</td>
<td>62.5</td>
<td>53.5</td>
<td>119</td>
<td>132</td>
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<tr>
<td>100</td>
<td>80.5</td>
<td>66.5</td>
<td>134</td>
<td>119.5</td>
</tr>
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<td>83.5</td>
<td>55</td>
<td>149</td>
<td>94</td>
</tr>
<tr>
<td>500</td>
<td>78</td>
<td>51</td>
<td>162</td>
<td>108.5</td>
</tr>
<tr>
<td>1000</td>
<td>95</td>
<td>56</td>
<td>139</td>
<td>104</td>
</tr>
</tbody>
</table>

Table 13. Results for dye 125 from Ames test.

<table>
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<tr>
<th>Dose (µg)</th>
<th>TA98 (-)</th>
<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>29</td>
<td>37</td>
<td>144</td>
<td>196</td>
</tr>
<tr>
<td>30</td>
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<td>39</td>
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<td>197</td>
</tr>
<tr>
<td>50</td>
<td>30.5</td>
<td>40</td>
<td>189</td>
<td>181</td>
</tr>
<tr>
<td>100</td>
<td>40</td>
<td>32</td>
<td>178.5</td>
<td>191</td>
</tr>
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<td>27.5</td>
<td>38</td>
<td>176.5</td>
<td>200</td>
</tr>
<tr>
<td>500</td>
<td>26.5</td>
<td>40</td>
<td>177.5</td>
<td>189.5</td>
</tr>
<tr>
<td>1000</td>
<td>38</td>
<td>21.5</td>
<td>192</td>
<td>190</td>
</tr>
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</table>
Table 14. Results for dye 126 from Ames test.

<table>
<thead>
<tr>
<th>Dose (µg)</th>
<th>TA98 (-)</th>
<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>29</td>
<td>37</td>
<td>144</td>
<td>196</td>
</tr>
<tr>
<td>30</td>
<td>37</td>
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<td>202</td>
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<td>1768</td>
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<td>196</td>
<td>292</td>
</tr>
<tr>
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<td>186</td>
<td>398</td>
<td>177</td>
<td>236.5</td>
</tr>
<tr>
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<td>281</td>
<td>81.5</td>
<td>202</td>
<td>230</td>
</tr>
</tbody>
</table>

Table 15. Results for dye 127 from Ames test.

<table>
<thead>
<tr>
<th>Dose (µg)</th>
<th>TA98 (-)</th>
<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>29</td>
<td>95</td>
<td>105</td>
</tr>
<tr>
<td>30</td>
<td>26</td>
<td>35</td>
<td>104.5</td>
<td>113</td>
</tr>
<tr>
<td>50</td>
<td>29</td>
<td>37</td>
<td>117</td>
<td>122</td>
</tr>
<tr>
<td>100</td>
<td>37</td>
<td>44</td>
<td>120</td>
<td>117</td>
</tr>
<tr>
<td>300</td>
<td>42.5</td>
<td>57</td>
<td>129.5</td>
<td>123</td>
</tr>
<tr>
<td>500</td>
<td>50</td>
<td>45.5</td>
<td>126.5</td>
<td>129</td>
</tr>
<tr>
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<td>67.5</td>
<td>60</td>
<td>131</td>
<td>129</td>
</tr>
</tbody>
</table>
Table 16. Results for dye **128** from Ames test.

<table>
<thead>
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<th>Dose (µg)</th>
<th>TA98 (-)</th>
<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>27</td>
<td>39</td>
<td>112</td>
<td>110</td>
</tr>
<tr>
<td>30</td>
<td>26</td>
<td>31.5</td>
<td>121</td>
<td>110</td>
</tr>
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<td>50</td>
<td>28</td>
<td>36</td>
<td>119</td>
<td>118.5</td>
</tr>
<tr>
<td>100</td>
<td>31</td>
<td>51.5</td>
<td>122</td>
<td>125</td>
</tr>
<tr>
<td>300</td>
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<td>144</td>
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<tr>
<td>500</td>
<td>25</td>
<td>45.5</td>
<td>137</td>
<td>143</td>
</tr>
<tr>
<td>1000</td>
<td>26.5</td>
<td>60</td>
<td>120.5</td>
<td>134</td>
</tr>
</tbody>
</table>

Table 17. Results for dye **129** from Ames test.

<table>
<thead>
<tr>
<th>Dose (µg)</th>
<th>TA98 (-)</th>
<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>27</td>
<td>39</td>
<td>112</td>
<td>110</td>
</tr>
<tr>
<td>30</td>
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<tr>
<td>50</td>
<td>25</td>
<td>36.5</td>
<td>111</td>
<td>130</td>
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<td>100</td>
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<td>37</td>
<td>114.5</td>
<td>113</td>
</tr>
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</tr>
<tr>
<td>500</td>
<td>23.5</td>
<td>31.5</td>
<td>142</td>
<td>118</td>
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<tr>
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<td>25</td>
<td>35.5</td>
<td>122</td>
<td>116</td>
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</table>
Table 18. Results for dye 130 from Ames test.

<table>
<thead>
<tr>
<th>Dose (µg)</th>
<th>TA98 (-)</th>
<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>29</td>
<td>43</td>
<td>130</td>
<td>123</td>
</tr>
<tr>
<td>30</td>
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<tr>
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<td>132</td>
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<tr>
<td>500</td>
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<td>48</td>
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<td>123.5</td>
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<tr>
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<td>124</td>
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Table 19. Results for dye 131 from Ames test.

<table>
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<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>130</td>
<td>123</td>
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<tr>
<td>30</td>
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<tr>
<td>50</td>
<td>32</td>
<td>44</td>
<td>147.5</td>
<td>144</td>
</tr>
<tr>
<td>100</td>
<td>32</td>
<td>50.5</td>
<td>128</td>
<td>124</td>
</tr>
<tr>
<td>300</td>
<td>34</td>
<td>42.5</td>
<td>135</td>
<td>123</td>
</tr>
<tr>
<td>500</td>
<td>37</td>
<td>40</td>
<td>120</td>
<td>122.5</td>
</tr>
<tr>
<td>1000</td>
<td>31</td>
<td>44.5</td>
<td>117</td>
<td>116</td>
</tr>
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</table>
Table 20. Results for dye 132 from Ames test.

<table>
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<th>Dose (µg)</th>
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<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>143</td>
<td>138</td>
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<tr>
<td>30</td>
<td>267.5</td>
<td>109</td>
<td>147</td>
<td>138</td>
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<td>153.5</td>
<td>136</td>
<td>163.5</td>
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<td>100</td>
<td>1528.5</td>
<td>211</td>
<td>141.5</td>
<td>143</td>
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<td>145</td>
<td>180</td>
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<td>84.5</td>
<td>438</td>
<td>146</td>
<td>177</td>
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<tr>
<td>1000</td>
<td>175.5</td>
<td>79.5</td>
<td>134</td>
<td>144</td>
</tr>
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</table>

Table 21. Results for Congo Red from the Prival test.

<table>
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<tr>
<th>Dose (µg)</th>
<th>TA98 (-)</th>
<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>96</td>
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<tr>
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<td>130.5</td>
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<td>3</td>
<td>-</td>
<td>137</td>
<td>-</td>
<td>151</td>
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<tr>
<td>5</td>
<td>-</td>
<td>203.5</td>
<td>-</td>
<td>194.5</td>
</tr>
<tr>
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<td>156</td>
<td>-</td>
<td>175.5</td>
</tr>
<tr>
<td>30</td>
<td>-</td>
<td>271</td>
<td>-</td>
<td>196.5</td>
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<tr>
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<td>298.5</td>
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Table 22. Results for dye 121 from the Prival test.

<table>
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<th>Dose (µg)</th>
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<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
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<td>49</td>
<td>-</td>
<td>96</td>
</tr>
<tr>
<td>1</td>
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</tr>
<tr>
<td>3</td>
<td>-</td>
<td>185</td>
<td>-</td>
<td>145.5</td>
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<td>5</td>
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<td>-</td>
<td>155</td>
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<tr>
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<td>160.5</td>
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<td>554</td>
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<td>193</td>
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<td>50</td>
<td>-</td>
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<td>188.5</td>
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</table>

Table 23. Results for dye 127 from the Prival test.

<table>
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<th>Dose (µg)</th>
<th>TA98 (-)</th>
<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>49</td>
<td>-</td>
<td>96</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>50.5</td>
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<td>128</td>
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<td>-</td>
<td>37</td>
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<td>61</td>
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<td>115</td>
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<td>30</td>
<td>-</td>
<td>59</td>
<td>-</td>
<td>124</td>
</tr>
<tr>
<td>50</td>
<td>-</td>
<td>66</td>
<td>-</td>
<td>109</td>
</tr>
</tbody>
</table>
Table 24. Results for Congo Red (30-1000 µg) from the Prival test.

<table>
<thead>
<tr>
<th>Dose (µg)</th>
<th>TA98 (-)</th>
<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>39</td>
<td>-</td>
<td>145</td>
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<tr>
<td>30</td>
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<td>-</td>
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<td>1358</td>
<td>-</td>
<td>1061</td>
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<td>-</td>
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</tr>
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<td>-</td>
<td>632</td>
</tr>
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</table>

Table 25. Results for dye 121 (30-1000 µg) from the Prival test.

<table>
<thead>
<tr>
<th>Dose (µg)</th>
<th>TA98 (-)</th>
<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>39</td>
<td>-</td>
<td>145</td>
</tr>
<tr>
<td>30</td>
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<tr>
<td>50</td>
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<td>1051</td>
<td>-</td>
<td>241.5</td>
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<td>-</td>
<td>1012</td>
<td>-</td>
<td>270</td>
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<td>300</td>
<td>-</td>
<td>1358</td>
<td>-</td>
<td>294</td>
</tr>
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<td>-</td>
<td>1610.5</td>
<td>-</td>
<td>291</td>
</tr>
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<td>1000</td>
<td>-</td>
<td>1245</td>
<td>-</td>
<td>258.5</td>
</tr>
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</table>
Table 26. Results for dye 127 (30-1000 µg) from the Prival test.

<table>
<thead>
<tr>
<th>Dose (µg)</th>
<th>TA98 (-)</th>
<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>39</td>
<td>-</td>
<td>145</td>
</tr>
<tr>
<td>30</td>
<td>-</td>
<td>60</td>
<td>-</td>
<td>151.5</td>
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<tr>
<td>50</td>
<td>-</td>
<td>67</td>
<td>-</td>
<td>173</td>
</tr>
<tr>
<td>100</td>
<td>-</td>
<td>62</td>
<td>-</td>
<td>141</td>
</tr>
<tr>
<td>300</td>
<td>-</td>
<td>135</td>
<td>-</td>
<td>189.5</td>
</tr>
<tr>
<td>500</td>
<td>-</td>
<td>172</td>
<td>-</td>
<td>210</td>
</tr>
<tr>
<td>1000</td>
<td>-</td>
<td>157</td>
<td>-</td>
<td>184</td>
</tr>
</tbody>
</table>

Table 27. Results for pigment 133 from the Prival test.

<table>
<thead>
<tr>
<th>Dose (µg)</th>
<th>TA98 (-)</th>
<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
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<td>160.5</td>
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<td>66.5</td>
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<td>154</td>
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<td>162.5</td>
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<td>92.5</td>
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<td>160</td>
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<td>50</td>
<td>-</td>
<td>96</td>
<td>-</td>
<td>184</td>
</tr>
</tbody>
</table>
Table 28. Results for pigment 134 from the Prival test.

<table>
<thead>
<tr>
<th>Dose (µg)</th>
<th>TA98 (-)</th>
<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>67</td>
<td>-</td>
<td>141</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>65</td>
<td>-</td>
<td>164.5</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>68.5</td>
<td>-</td>
<td>166</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>76.5</td>
<td>-</td>
<td>149</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>79</td>
<td>-</td>
<td>163.5</td>
</tr>
<tr>
<td>30</td>
<td>-</td>
<td>66</td>
<td>-</td>
<td>156.5</td>
</tr>
<tr>
<td>50</td>
<td>-</td>
<td>64.5</td>
<td>-</td>
<td>162</td>
</tr>
</tbody>
</table>

Table 29. Results for pigment 135 from the Prival test.

<table>
<thead>
<tr>
<th>Dose (µg)</th>
<th>TA98 (-)</th>
<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>67</td>
<td>-</td>
<td>141</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>82</td>
<td>-</td>
<td>158</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>65.5</td>
<td>-</td>
<td>161.5</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>78</td>
<td>-</td>
<td>160.5</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>87.5</td>
<td>-</td>
<td>162.5</td>
</tr>
<tr>
<td>30</td>
<td>-</td>
<td>68</td>
<td>-</td>
<td>157</td>
</tr>
<tr>
<td>50</td>
<td>-</td>
<td>77.5</td>
<td>-</td>
<td>155</td>
</tr>
</tbody>
</table>
Table 30. Results for pigment 136 from the Prival test.

<table>
<thead>
<tr>
<th>Dose (µg)</th>
<th>TA98 (-)</th>
<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>47</td>
<td>-</td>
<td>109</td>
</tr>
<tr>
<td>1</td>
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<td>-</td>
<td>123</td>
</tr>
<tr>
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<td>-</td>
<td>52</td>
<td>-</td>
<td>132.5</td>
</tr>
<tr>
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<td>-</td>
<td>44</td>
<td>-</td>
<td>109.5</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>48</td>
<td>-</td>
<td>129</td>
</tr>
<tr>
<td>30</td>
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<td>-</td>
<td>121.5</td>
</tr>
<tr>
<td>50</td>
<td>-</td>
<td>51</td>
<td>-</td>
<td>122</td>
</tr>
</tbody>
</table>

Table 31. Results for pigment 137 from the Prival test.

<table>
<thead>
<tr>
<th>Dose (µg)</th>
<th>TA98 (-)</th>
<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>47</td>
<td>-</td>
<td>109</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
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<td>139.5</td>
</tr>
<tr>
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<td>-</td>
<td>48</td>
<td>-</td>
<td>125.5</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>62</td>
<td>-</td>
<td>123.5</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>55</td>
<td>-</td>
<td>131</td>
</tr>
<tr>
<td>30</td>
<td>-</td>
<td>58.5</td>
<td>-</td>
<td>128.5</td>
</tr>
<tr>
<td>50</td>
<td>-</td>
<td>61.5</td>
<td>-</td>
<td>118</td>
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</table>
Table 32. Results for pigment 138 from the Prival test.

<table>
<thead>
<tr>
<th>Dose (µg)</th>
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<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>47</td>
<td>-</td>
<td>109</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>61.5</td>
<td>-</td>
<td>137.5</td>
</tr>
<tr>
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<td>68</td>
<td>-</td>
<td>130</td>
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<tr>
<td>5</td>
<td>-</td>
<td>62.5</td>
<td>-</td>
<td>145</td>
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<tr>
<td>10</td>
<td>-</td>
<td>54</td>
<td>-</td>
<td>129</td>
</tr>
<tr>
<td>30</td>
<td>-</td>
<td>64.5</td>
<td>-</td>
<td>129</td>
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<tr>
<td>50</td>
<td>-</td>
<td>61</td>
<td>-</td>
<td>117</td>
</tr>
</tbody>
</table>

Table 33. Results for pigment 139 from the Prival test.

<table>
<thead>
<tr>
<th>Dose (µg)</th>
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<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>70</td>
<td>-</td>
<td>117</td>
</tr>
<tr>
<td>1</td>
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<td>146.5</td>
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<td>3</td>
<td>-</td>
<td>74.5</td>
<td>-</td>
<td>126.5</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>69</td>
<td>-</td>
<td>130.5</td>
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<tr>
<td>10</td>
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<td>77</td>
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<tr>
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<td>126.5</td>
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</tbody>
</table>
Table 34. Results for pigment 140 from the Prival test.

<table>
<thead>
<tr>
<th>Dose (µg)</th>
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<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>70</td>
<td>-</td>
<td>117</td>
</tr>
<tr>
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<td>-</td>
<td>79</td>
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<td>126</td>
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<td>77</td>
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<td>123.5</td>
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<tr>
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<td>-</td>
<td>79</td>
<td>-</td>
<td>137</td>
</tr>
</tbody>
</table>

Table 35. Results for pigment 141 from the Prival test.

<table>
<thead>
<tr>
<th>Dose (µg)</th>
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<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>70</td>
<td>-</td>
<td>117</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>67</td>
<td>-</td>
<td>131.5</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>71</td>
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<tr>
<td>5</td>
<td>-</td>
<td>74</td>
<td>-</td>
<td>130.5</td>
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<td>73.5</td>
<td>-</td>
<td>125.5</td>
</tr>
<tr>
<td>30</td>
<td>-</td>
<td>69</td>
<td>-</td>
<td>142.5</td>
</tr>
<tr>
<td>50</td>
<td>-</td>
<td>62.5</td>
<td>-</td>
<td>130.5</td>
</tr>
</tbody>
</table>
Table 36. Results for pigment 142 from the Prival test.

<table>
<thead>
<tr>
<th>Dose (µg)</th>
<th>TA98 (-)</th>
<th>TA98 (+)</th>
<th>TA100 (-)</th>
<th>TA100 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>70</td>
<td>-</td>
<td>117</td>
</tr>
<tr>
<td>1</td>
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<td>74</td>
<td>-</td>
<td>119.5</td>
</tr>
<tr>
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<td>105.5</td>
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<td>-</td>
<td>67.5</td>
<td>-</td>
<td>133.5</td>
</tr>
<tr>
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<td>-</td>
<td>70</td>
<td>-</td>
<td>113.5</td>
</tr>
<tr>
<td>30</td>
<td>-</td>
<td>73</td>
<td>-</td>
<td>152</td>
</tr>
<tr>
<td>50</td>
<td>-</td>
<td>74.5</td>
<td>-</td>
<td>130</td>
</tr>
</tbody>
</table>