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An Approach to the Design of Non-Mutagenic Azo Dyes: 1. The Identification of Non-Mutagenic Precursors and Potential Metabolites*

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SUMMARY

This paper describes a strategy for the development of non-mutagenic dyes by identifying and elaborating some non-genotoxic precursors. The approach presented makes use of the numerous published articles which describe the results of the mutagenicity and carcinogenicity testing of azo dyes and their intermediates.

A number of chemical intermediates have been evaluated in this study as potential replacements for known carcinogenic dye intermediates which have been used in the past to produce dyes possessing good physical and chemical properties. Several of the intermediates examined were found to be non-mutagenic, and were converted to azo dyes which were themselves non-mutagenic.

The results of this study suggest that the development of non-carcinogenic azo dyes could be accomplished by employing an approach which would utilize

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non-mutagenic intermediates and also take into consideration the potential genotoxicity of the metabolites resulting from reductive cleavage of the azo linkages.

1 INTRODUCTION

In the years since the discovery of the carcinogenicity of benzidine (**1**), 2-naphthylamine (**2**), and certain azo dyes derived from them, several articles have appeared which describe the mutagenicity, carcinogenicity, and the potential risk to man of numerous synthetic dyes. Most of these outline the results of *in vitro* evaluations (primarily the Ames assay[1]) of azo dyes; much less attention has been afforded to the anthraquinones[2-4] and dyes of other classes. A detailed review of the literature in this important area is beyond the scope of this paper; however, a brief review of some articles which are most relevant to the present investigation is presented.

About 20 years ago, the Toxic Substances Control Act was signed into law in the USA. Since then a number of papers have been published which outline the governmental regulations[5-7] controlling the distribution and use of dyes in food, drug, and cosmetic products. In fact, in all cases (including textile use) where dyes and their intermediates have been positively identified as carcinogens, the manufacture of such materials has been banned in the USA and the UK.

Much of the research on the ecological aspects of synthetic dyes has involved the use of short-term *in vitro* tests to infer the potential carcinogenic risk to humans. Two papers of that type by Longstaff[8,9] discuss the utility of *in vitro* and *in vivo* evaluations of dyes in determining potential carcinogenic risk to man. In those papers, it is suggested:

(1) that the Ames test (Salmonella/Microsome Mutation Assay) be given preference over BHK21 Cell Transformation assays as a short-term test for predicting carcinogenicity;

(2) that only those dyes and dye intermediates which have proved to produce malignant tumors in at least two species of laboratory animals be labeled as a carcinogenic risk to man;

(3) that all valid carcinogenicity testing involve male and female laboratory animals for a duration of at least two-thirds the animals' expected lifetime (i.e. 16 months for mice, and 2 years for rats);

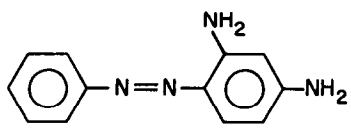
(4) that oral administration of the test compounds involve testing at three or more dose levels; and

(5) that subcutaneous injections, bladder implantations of test compounds, and the use of promoting agents such as croton oil are inappropriate protocols for studies involving a determination of the potential risk of dyes to man.

Three recent papers from Stanford Research Institute summarize[10–12] an attempt to gather information for a computer database which could be used to predict the potential toxicity of nitro, azo, and anthraquinone dyes. The latter two papers review about 200 articles which describe the results of the toxicity testing of a large number of synthetic dyes but do not include a further useful review[13] of the genotoxicity of food, drug, and cosmetic colors and other dyes.

The International Agency for Research on Cancer (IARC) has reported[14–16] an evaluation of the carcinogenic risk of some aromatic amines and related nitro compounds and azo dyes to man. It seems clear from these reports that, as a general rule, azo dyes which are based on mutagenic aromatic amines and which also lack at least one sodium sulfonate (—SO_3Na) group (cf. **3–6**) pose a risk to man and this point is also supported by more recent work.[16–18] On the other hand, those dyes which contain two or more hydrophilic —SO_3Na groups (cf. **7–9**) are non-mutagenic and non-carcinogenic. There are some exceptions to this latter point but in these cases the observed toxicity has been attributed to the presence of toxic impurities rather than to the dye molecules themselves.

Gregory[17] has recently proposed some structure–carcinogenicity relationships to account for the animal carcinogenicity data of an earlier



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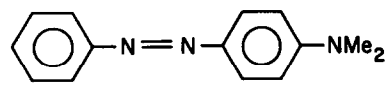

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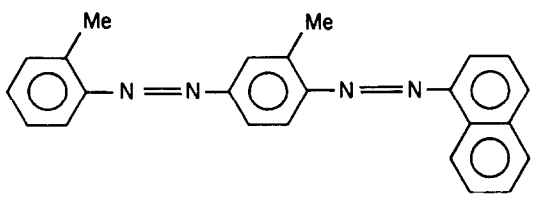

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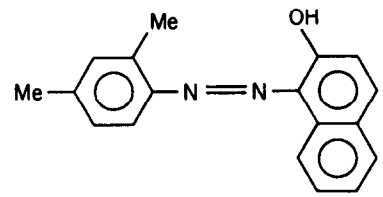

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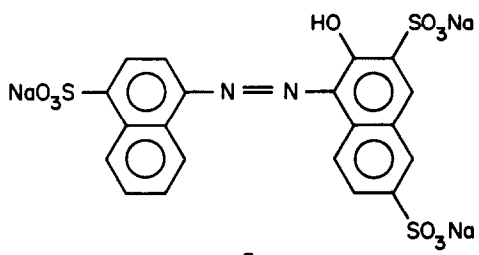

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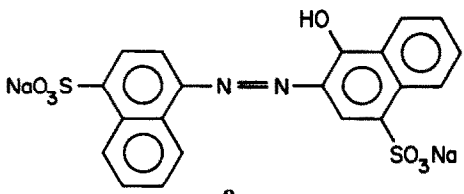

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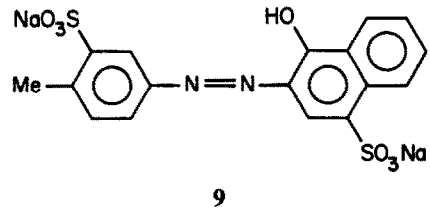

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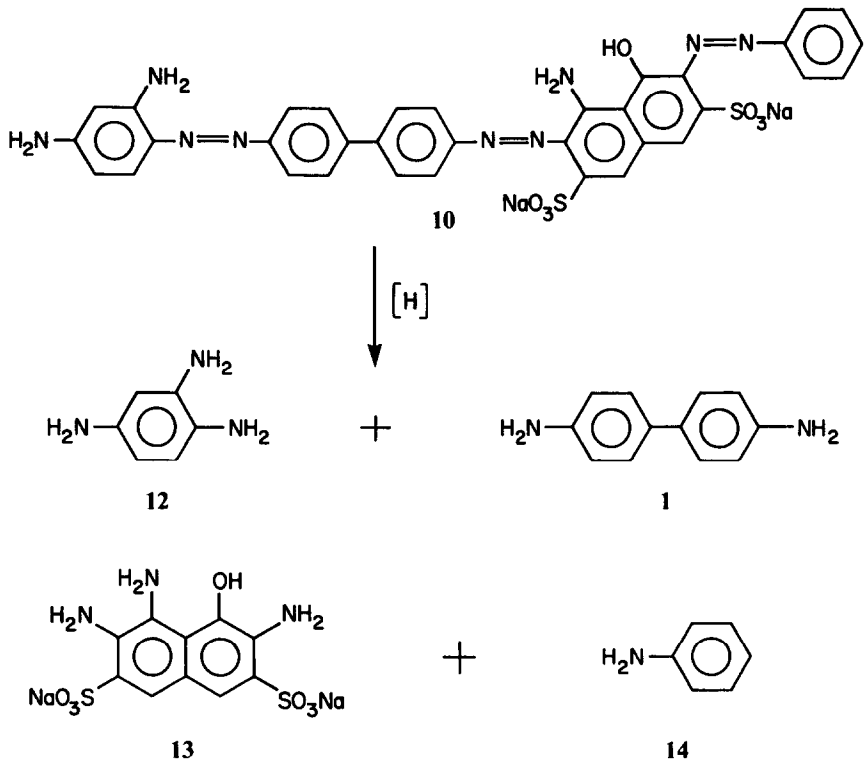
paper by Longstaff.[8] He suggests that those azo dyes which resist reductive cleavage of the azo linkage are likely to be pro-carcinogens themselves, and that those susceptible to reductive cleavage produce an aromatic amine which is likely to be a pro-carcinogen. It was also suggested that the insolubility of azo pigments prevents such a breakdown, even where the dye exists in the hydrazone form.

Whilst the literature contains a large number of papers which describe the toxicity (or potential toxicity) of synthetic dyes, there is little published work on attempts to use that information to design new synthetic dyestuffs.

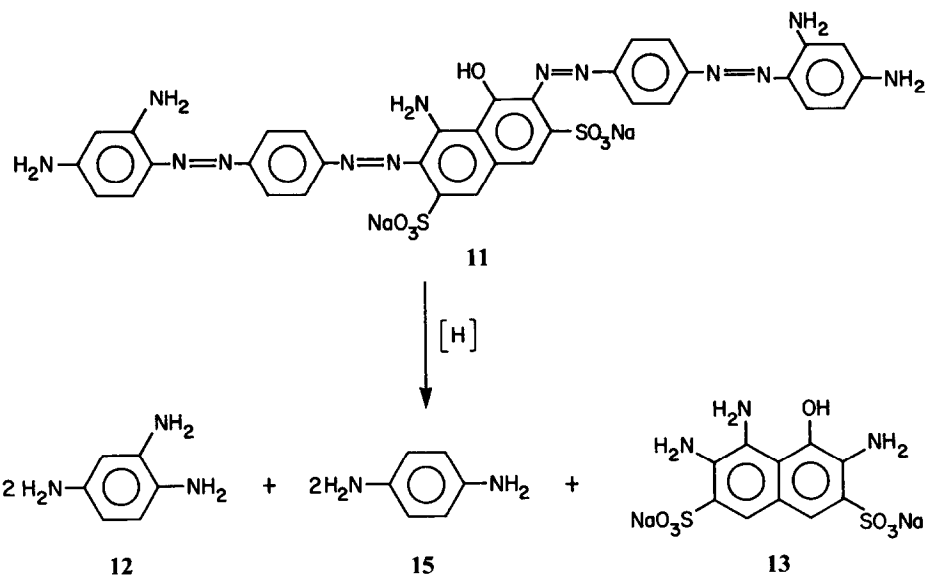
This present paper is concerned with the use of the available literature, and the development of new mutagenicity data from our own research studies, to formulate strategies for the generation of new dyestuffs whose design includes toxicological considerations. The approach is based on two basic assumptions: (1) the use of non-mutagenic and non-carcinogenic chemical intermediates will produce non-genotoxic dyes in their intact form; and (2) the dyes prepared will be non-genotoxic upon metabolic breakdown if the potential reductive-cleavage products are non-mutagenic and non-carcinogenic.

Our interest in this area resulted in part from a need for suitable replacements of Direct Black 38 (**10**) (Scheme 1) and Direct Black 19 (**11**) (Scheme 2) as dyes in certain ink formulations. Both compounds are based on intermediates which are known mutagens and/or carcinogens, and give positive results in mutagenicity/carcinogenicity tests. It is generally accepted that azo dyes, particularly water-soluble dyes, undergo metabolism to produce a mixture of the amines used as diazo components (cf. **1**, **14** and **15**) in the dye synthesis and of the aminated derivatives (**12** and **13**) of the coupling components (Schemes 1 and 2). We have evaluated a number of commonly used aromatic amines, coupling components, and some metabolites which would result from the reductive cleavage of a typical azo dye. The results of that evaluation were used as the basis for an approach to the development of non-mutagenic dyes and to design a few monoazo dyes which would serve as a simple test of such an approach.

Shortly after we started this work, a series of papers by Shahin and co-workers which describe the toxicity testing of dyes and dye intermediates[18–23] included a report[23] of successful attempts to remove the

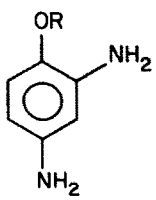


Scheme 1. Reductive-cleavage products of Direct Black 38.



Scheme 2. Reductive-cleavage products of Direct Black 19.

mutagenicity of aromatic amines such as **16–18** by incorporating certain alkyl groups. Interestingly, whereas the R = H member of each of the three series of amines was quite mutagenic, the *N*-propyl, *N*-butyl, and *N*-hydroxyethyl analogs were reportedly devoid of mutagenicity in each of the five tester strains of Salmonella bacteria. It is unclear (and the authors provided no explanations) why increasing the hydrophobic character of those compounds lowers their toxicity and it is possible that steric rather than polar effects account for these results. We will describe in a future communication our attempts to incorporate the results of Shahin's research into our dye synthesis studies.



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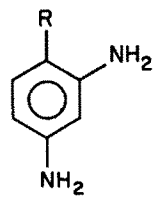

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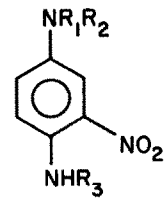

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R = H, Me, Et, Pr, i-Pr, Bu, CH_2CH_2OH

2 RESULTS AND DISCUSSION

Figures 1 and 2 show the diazo components and the coupling components, respectively, which were used in this investigation. Compounds **19**, **20**, **22**, and **25** were examined as possible replacements for the well-known carcinogen 2-naphthylamine. Although this intermediate is not required for the synthesis of dyes **10** and **11**, it was once a quite prevalent residue among the cellulose-substantive Direct Black dyes. In general the quinolines appear to be non-mutagenic by the Standard Ames test.[1,24] The nitroamine **25**, however, is clearly a mutagen. The remainder of the compounds of Table 1 were examined as possible replacements of *p*-phenylenediamine and aniline. Although aniline (**14**) proved non-mutagenic in our results, it has been shown to be a pro-carcinogen in certain animal models.[25] Compounds **23**, **24**, **26**, **31**, and **32** appear to be non-mutagenic (Table 1) by both the Standard Ames test and the Prival modification.[26]

The results from the evaluation of the coupling components of Fig. 2 suggest that a non-mutagenic potential replacement of the *m*-phenylenediamine moiety of dyes **10** and **11** exists only in compound **33**. The other analogs (**36–37**) and derivatives (**34–35** and **38–39**) of *m*-phenylenediamine were mutagens, with the possible exception of compound **38**, which showed weak activity.

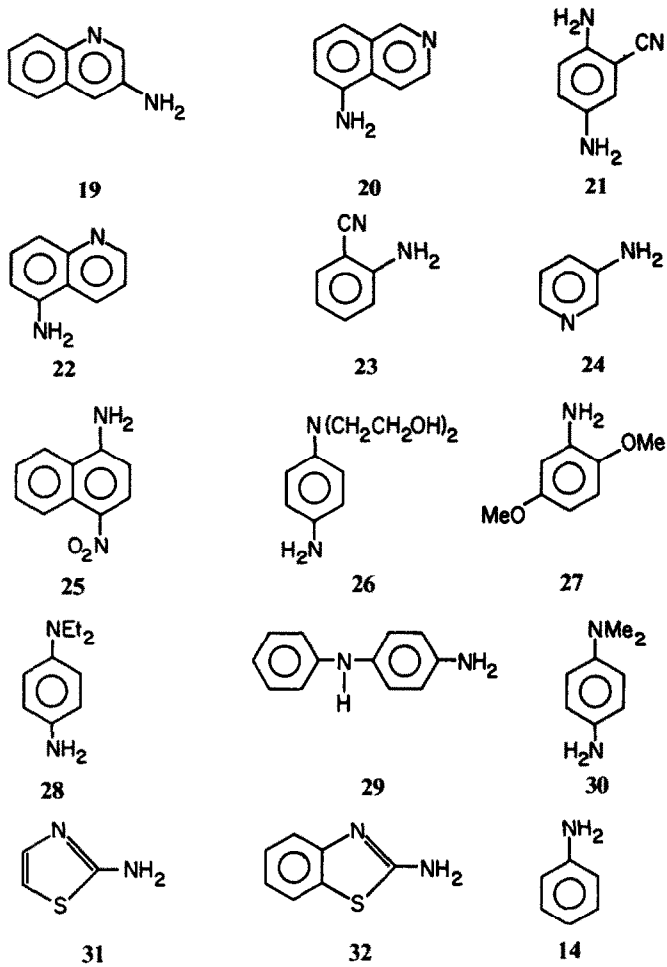


Fig. 1. Diazo components evaluated in short-term assays in this investigation.

The results of our evaluation of the commonly used naphthalenesulfonic acids **40–46** suggest that all are either non-mutagenic or only very weak mutagens. These results are consistent with an earlier report[27] which describes the removal of the mutagenicity of some aromatic amines by sulfonation. It may be that the slight activity exhibited by compounds **41–45** is due to impurities, as the samples used in our assays were not 100% pure.

A few compounds (**47–50**; Fig. 3) were tested which would result from a reductive-cleavage reaction of an azo dye; based on the test results, each would be an undesirable metabolite.

Eight monoazo dyes employing a non-mutagenic coupling component were synthesized. The diazo component was produced from aniline or from *p*-carboxyaniline (to assist with interpreting the ^{1}H-NMR spectra). Figure 4

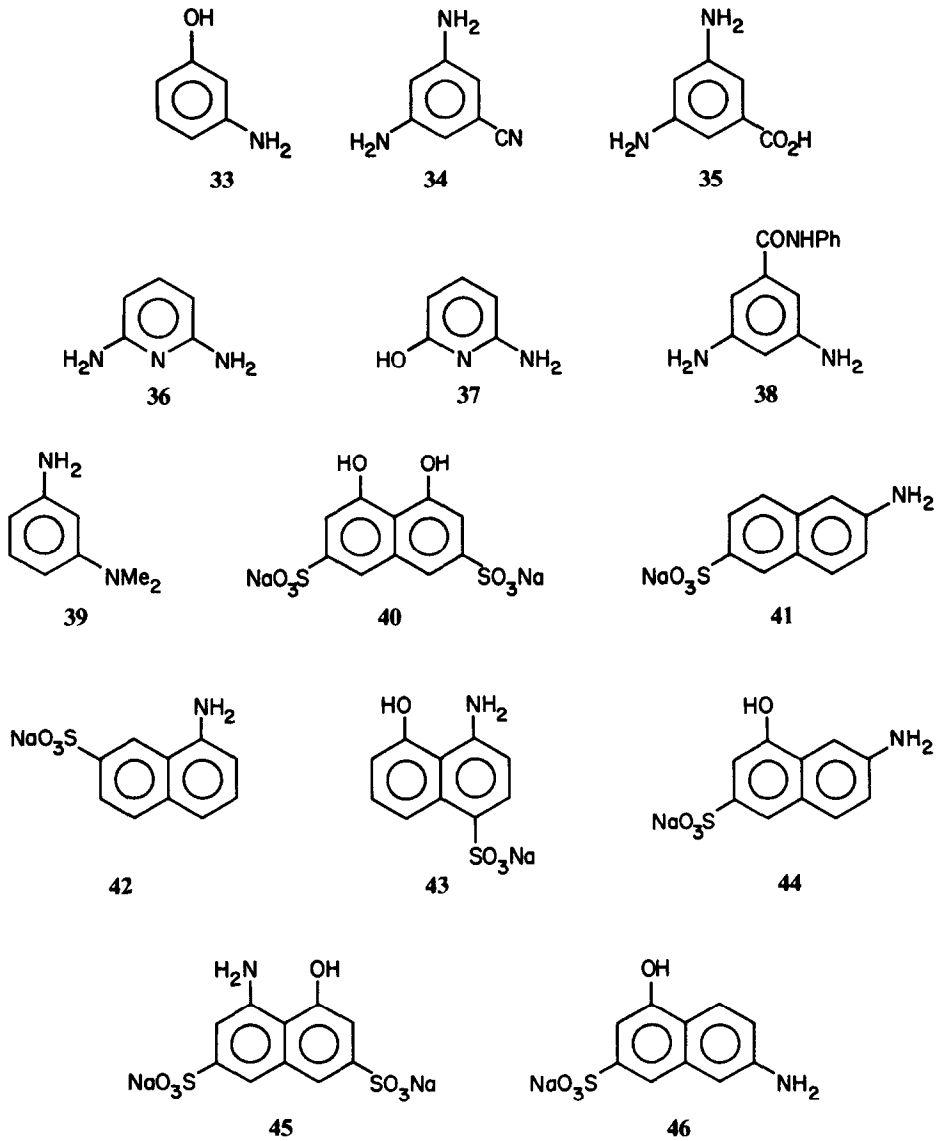


Fig. 2. Coupling components evaluated in short-term tests in this investigation.

shows the structures of the dyes examined in our short-term tests, and Table 1 outlines the results of the mutagenicity testing of those dyes (**51–58**). Of this group, only two of the dyes (**53**, **58**) exhibited a level of mutagenicity which merits mentioning. This activity resulted in the Prival modification of the Ames test, which would be expected to produce the reductive-cleavage products **59** and **14** from dye **53**, and compounds **60** and **14** from dye **58**. The

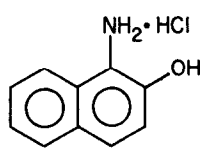
TABLE 1
Mutagenicity Data of the Test Compounds

	Test compound	*Mutagenicity (Revertants μmol^{-1})*	
		Standard assay + S9	*Prival modification*
14	Aniline	Negative	Negative
19	3-Aminoquinoline	Negative	—
20	5-Aminoisoquinoline	21	—
21	2-Cyano-*p*-phenylenediamine	422	—
22	5-Aminoquinoline	42	—
23	2-Cyanoaniline	Negative	—
24	3-Aminopyridine	0·7	—
25	4-Nitro-1-naphthylamine	507	1 665
26	4-Amino-*N*,*N*-bis(2-hydroxyethyl)aniline	Negative	Negative
27	2,5-Dimethoxyaniline	42·1	17·7
28	4-Amino-*N*,*N*-diethylaniline	1 007	689
29	4-Aminodiphenylamine	Negative	154
30	4-Amino-*N*,*N*-dimethylaniline	Negative (toxic)	—
31	2-Aminothiazole	4·1	—
32	2-Aminobenzothiazole	Negative	—
33	3-Aminophenol	1·7	—
34	3,5-Diaminobenzonitrile	467	—
35	3,5-Diaminobenzoic acid	520	—
36	2,6-Diaminopyridine	Negative (toxic)	—
37	2-Amino-6-hydroxypyridine	Negative	—
38	*N*-Phenyl-3,5-diaminobenzamide	149	31
39	*N*,*N*-Dimethyl-*m*-phenylenediamine	1 233–4 678 (Not linear)	2 274 (Linear)
40	Chromotropic acid	Negative	Negative
41	Broenner's acid	4·9	7·7
42	Cleve's acid	4·9	12·5
43	S-acid	13·8	24·4
44	Gamma acid	15·0	32·6
45	H-acid	Negative	Negative
46	J-acid	5·2	41
47	1-Amino-2-naphthol	Negative (toxic)	Negative
48	4-Amino-1-naphthol	1 413	Negative
49	1,2,4,5-Tetraaminobenzene	54 296	12 338
50	3,5-Diaminosalicylic acid	4 988	—
51	4-Amino-5-hydroxy-8-phenylazo-2,7-naphthalenedisulfonic acid disodium salt	Negative	Negative
52	4-Amino-8-(4-carboxyphenylazo)-5-hydroxy-2,7-naphthalenedisulfonic acid trisodium salt	Negative	Negative

(*continued*)

TABLE 1—*contd.*

	Test compound	*Mutagenicity (Revertants μmol^{-1})*	
		Standard assay + S9	*Prival modification*
53	6-Amino-4-hydroxy-3-phenylazo-2-naphthalenesulfonic acid monosodium salt	83	431
54	4-Amino-8-(carboxyphenylazo)-5-hydroxy-1-naphthalenesulfonic acid monosodium salt	Negative	Negative
55	7-Amino-4-hydroxy-1-phenylazo-2-naphthalenesulfonic acid monosodium salt	16·1	67·8
56	8-Amino-5-(4-carboxyphenylazo)-2-naphthalenesulfonic acid disodium salt	6·7	Negative
57	4-Amino-3-(4-carboxyphenylazo)-5-hydroxy-1-naphthalenesulfonic acid disodium salt	Negative	Negative
58	6-Amino-4-hydroxy-5-phenylazo-2-naphthalenesulfonic acid monosodium salt	27·4	547



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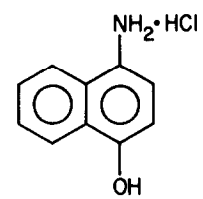

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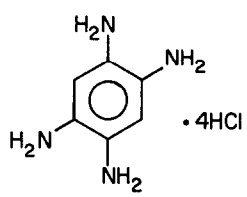

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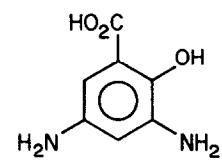

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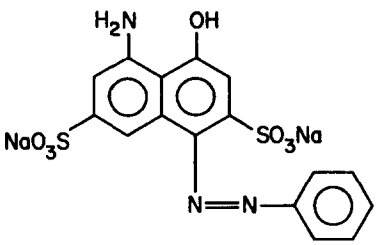
Fig. 3. Aminated coupling components isolated and evaluated in short-term tests in this investigation.



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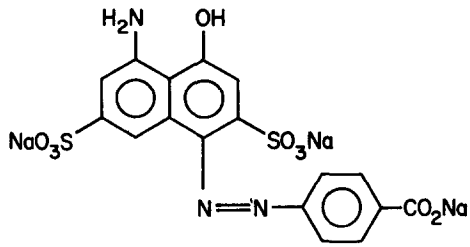

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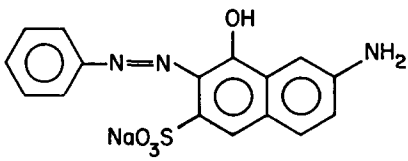

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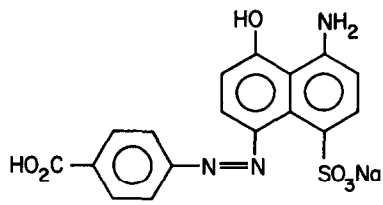

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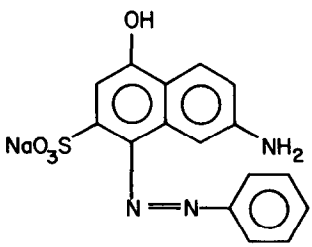

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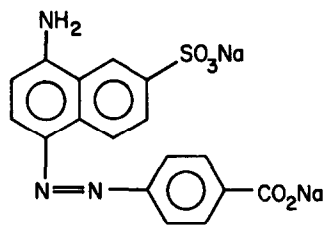

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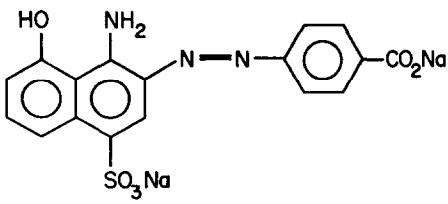

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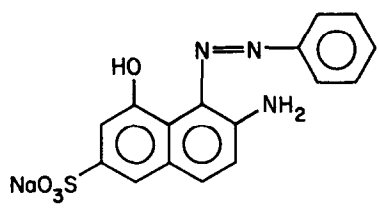

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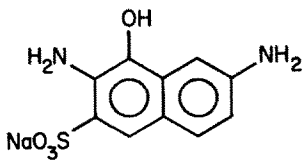
Fig. 4. Monoazo dyes derived from non-mutagenic intermediates, which were evaluated in short-term tests in this investigation.



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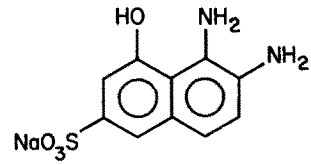

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actual mutagen has not been isolated. It is interesting that the isomeric dye **55** was much less active in these tests. Obviously, animal studies would be required before one could say conclusively that dyes based on Gamma acid should be avoided.

Work is in progress in our laboratory to expand the series of dyes to include disazo and trisazo derivatives.

3 EXPERIMENTAL

3.1 Chemicals

The test compounds were obtained from Aldrich Chemical Company (940 West Saint Paul Avenue, Milwaukee, Wisconsin 53233, USA), Mobay Chemical Corporation (Dyes and Pigments Division, PO Drawer 2855 CRS, Rock Hill, SC 29731, USA), or prepared in these laboratories. The purity of each compound was determined with the aid of a Waters Analytic HPLC instrument, and by TLC on silica gel with an appropriate eluent. The structures of the compounds which we synthesized were confirmed by ^{1}H-NMR spectroscopy using a Varian EM390 90 MHz spectrometer.

Five of the diamines (**21**, **34**, **35**, **38**, and **50**) employed in this investigation were prepared from the corresponding commercially available nitro precursors. Compounds **21**, **34**, **35**, and **50** were made via the catalytic reduction of 2-amino-5-nitrobenzonitrile, 3,5-dinitrobenzonitrile, 3,5-dinitrobenzoic acid, and 3,5-dinitrosalicylic acid, respectively. Compound **38** was made in two steps (acylation of aniline followed by catalytic reduction) from 3,5-dinitrobenzoyl chloride. Palladium (5%) on charcoal was used as a catalyst in the reductions and MeOH was used as the solvent.

The aromatic amines were converted to the corresponding hydrochlorides prior to Ames testing. This was accomplished by dissolving the amine in EtOAc and adding 1–4 moles of HCl per mole of amine, depending upon the number of —NH_2 groups in the molecule.

The dyes were synthesized by a previously described procedure,[28] and the structures were determined with the aid of a Brucker 250 MHz NMR spectrometer.

3.2 Mutagenicity assays

The standard Salmonella/microscope mutagenicity assays were conducted according to the method of Ames and coworkers.[1,24] The S9 liver homogenate was obtained from rats which were treated with Aroclor 1254 five days prior to the S9 preparation. The level of S9 in the S9 mix was 40 μg ml^{-1}, and each plate was treated with either 0·5 ml of S9 mix or 0·5 ml of 0·1M-phosphate buffer. The test compounds were dissolved in DMSO or in distilled water and assayed for mutagenicity in the TA1535, TA1537, TA1538, TA98, and TA100 strains.

The Prival modification[26] of the Ames test was employed as a confirmatory assay. The procedure described in that paper was employed without modification, using the five basic bacteria strains.

4 CONCLUSIONS

The results of this investigation suggest that a number of analogs of diazo components and naphthalenesulfonic acid derivatives which are commonly used in the synthesis of azo dyes satisfactorily passed these initial tests to assess their potential risk to man. However, confirmatory tests in animal models, to measure carcinogenicity, are necessary before more definitive conclusions can be made.

The mutagenicity data obtained from the evaluation of the dyes prepared in this investigation suggest that an amino derivative of most naphthalenesulfonic acids would be a safe metabolite to generate as a result of the reductive cleavage of an azo linkage. Those data further suggest that the development of dyes from non-genotoxic aromatic amines (diazo components) and coupling components is a logical approach to developing non-carcinogenic dyestuffs for food, drug, cosmetic, and textile use.

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