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Novel azo dyes derived from 4-aminoantipyrine: Synthesis, spectral and biological evaluation

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Abstract

Azo dyes are not a niche category of synthetic colorants — they account for over 60% of all dyes manufactured globally and are widely used in textiles, food, and pharmaceuticals. Their biological activity, which arises partly from the -N=N- chromophore and partly from substituent effects on the aromatic rings, has made them attractive subjects for medicinal chemistry research. This paper reports the synthesis of twelve novel azo dyes prepared by diazotization of 4-aminoantipyrine followed by coupling with a series of aromatic coupling agents including naphthols, aminophenols, and heterocyclic phenols. All dyes were characterized by UV-Vis spectroscopy, FTIR, ¹H NMR, and elemental analysis. Visible absorption maxima ranged from 384 to 541 nm, reflecting the diversity of electron-donating and withdrawing substituents on the coupling component. Biological evaluation included antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, antifungal activity against *Candida albicans* and *Aspergillus niger*, and antioxidant assays using DPPH and ABTS radical scavenging methods. Dye D7 (coupled with p-nitroaniline) showed the strongest antibacterial activity with MIC values of 8 and 16 µg/mL for *S. aureus* and *E. coli*, respectively, while D5 (coupled with p-aminophenol) gave the best antifungal profile with MIC 8 µg/mL for *C. albicans*. DPPH IC₅₀ values ranged from 18.4 to 67.2 µM. Structure-activity correlations identified electron-withdrawing substituents and higher lipophilicity as key determinants of biological potency. These data support further optimisation of 4-AAP-derived azo dyes as multifunctional bioactive agents.

Keywords: Azo dyes, 4-aminoantipyrine, diazotization, spectral characterization, antibacterial activity, antifungal activity, antioxidant, structure-activity relationship, biological evaluation, synthetic dyes

Introduction

Azo dyes represent the largest single class of synthetic colorants, distinguished by the presence of one or more -N=N- groups linking aromatic or heteroaromatic rings. Their versatility stems from the ease with which the chromophore can be tuned by changing substituents on the diazonium or coupling components, covering essentially the entire visible spectrum ^[1]. Beyond their commercial applications, there is growing interest in the biological properties of azo dye structures, particularly their antimicrobial and antioxidant activities, which stem from the ability of the aromatic system to interact with enzyme active sites and free radicals ^[2].

4-Aminoantipyrine (4-AAP), chemically 4-amino-1, 5-dimethyl-2-phenyl-2, 3-dihydro-1H-pyrazol-3-one, is a pyrazolone derivative originally used as an analgesic but now well established as a versatile building block in heterocyclic and azo dye chemistry ^[3]. Its primary amine group undergoes diazotization cleanly under acidic conditions, and the resulting diazonium salt couples efficiently with a wide range of nucleophilic aromatic compounds. The pyrazolone ring itself contributes to biological activity through metal chelation and enzyme inhibition mechanisms ^[4, 5].

Published work on 4-AAP-derived azo dyes has shown antibacterial activity against Gram-positive and Gram-negative bacteria, antifungal activity against pathogenic *Candida* species, and moderate antioxidant properties ^[6, 7]. However, most earlier investigations used a limited set of coupling agents and did not systematically explore structure-activity relationships across a large series. The current work at Sri Aurobindo College of Chemical Sciences, Puducherry, extends this area by preparing twelve dyes using twelve different coupling

agents varying in electronic character and hydrogen-bonding capacity, and correlating the biological data with computed lipophilicity (log P) and measured spectral properties.

Reagents and chemicals

4-Aminoantipyrine (4-AAP, $\geq 99.0\%$), sodium nitrite (NaNO_2 , $\geq 97\%$), hydrochloric acid (37%, analytical grade), sodium acetate (anhydrous, $\geq 99\%$), β -naphthol ($\geq 99\%$), α -naphthol ($\geq 98\%$), resorcinol ($\geq 99\%$), aniline ($\geq 99.5\%$), p-aminophenol ($\geq 98\%$), phenol ($\geq 99\%$), p-nitroaniline ($\geq 99\%$), 2-naphthylamine ($\geq 97\%$), o-aminophenol ($\geq 98\%$), 8-hydroxyquinoline ($\geq 99\%$), salicylaldehyde ($\geq 99\%$), and acetoacetanilide ($\geq 98\%$) were all obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). DPPH (2, 2-diphenyl-1-picrylhydrazyl, $\geq 97\%$), ABTS (2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), and Trolox were purchased from Sigma-Aldrich and stored at -20°C . Dimethyl sulfoxide (DMSO, $\geq 99.9\%$), ethanol ($\geq 99.9\%$), and methanol ($\geq 99.8\%$) were obtained from Merck. Nutrient agar, Mueller-Hinton broth, Sabouraud dextrose agar, and dextrose broth were sourced from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Reference antibiotics ciprofloxacin and fluconazole were obtained from a local pharmaceutical distributor as pure substances of verified purity. All solvents used for recrystallization were freshly distilled prior to use.

Material and Methods

Material

This research was carried out in the Department of Chemistry, Sri Aurobindo College of Chemical Sciences, Puducherry, India, from February 2023 to October 2023. The antimicrobial testing was performed in the Microbiology Laboratory of the same institution. Reference microbial strains *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Candida albicans* (ATCC 10231), and *Aspergillus niger* (ATCC 16404) were sourced from the Institute of Microbial Technology (IMTECH), Chandigarh, India. Institutional approval for the use of chemical reagents and microbiological cultures was obtained from the Departmental Safety Committee (Protocol DSC-2023-03, approved 10 January 2023). Thin-Layer Chromatography (TLC) plates pre-coated with silica gel 60

F₂₅₄ (Merck) were used for reaction monitoring, and spots were visualized under UV light at 254 nm and 365 nm and by iodine vapour staining.

Methods

General diazotization procedure: 4-AAP (1.0 mmol) was dissolved in 5% hydrochloric acid (4 mL) with cooling in an ice bath at $0-5^\circ\text{C}$. A solution of NaNO_2 (1.1 mmol) in water (2 mL) was added dropwise over 10 minutes with continuous stirring, and the diazonium salt solution was used immediately. For coupling, the appropriate coupling agent (1.0 mmol) was dissolved in sodium acetate solution (pH 8-9, 10 mL) and cooled to 0°C . The diazonium salt solution was added dropwise to the coupling agent solution with stirring over 20 minutes. The reaction mixture was stirred for a further 2 hours at $0-5^\circ\text{C}$. The precipitated dye was collected by vacuum filtration, washed sequentially with cold water, dilute sodium carbonate solution, and cold ethanol, and dried at 50°C under vacuum. Purification was by recrystallization from ethanol/water mixtures (1:3, v/v). Log P values were calculated using Molinspiration software (v2016.10, Slovensky Grob, Slovakia).

Quality control and calibration

Purity of all synthesized dyes was assessed by HPLC using an Agilent 1260 Infinity II system with a Zorbax Eclipse Plus C18 column (4.6×150 mm, $3.5 \mu\text{m}$). The mobile phase consisted of acetonitrile and 0.1% formic acid in water (gradient from 20:80 to 80:20 over 20 minutes). Detection was at 254 nm and 420 nm. All final compounds were confirmed $\geq 95\%$ pure by HPLC area normalization. UV-Vis spectrophotometry was performed on a Shimadzu UV-2600 double beam instrument calibrated with NIST-traceable holmium oxide filters at 241.2, 279.3, 360.9, and 453.2 nm. Elemental analyses (C, H, N) were carried out using a CHNS analyzer (EURO EA 3000 series) and results agreed with calculated values within $\pm 0.3\%$. Antibacterial MIC values were determined by the broth microdilution method in 96-well plates following CLSI M07-A10 guidelines, with a growth control and sterility control included in every plate. Inter-day reproducibility of MIC determinations was confirmed by repeat testing on three separate days; geometric mean MIC values are reported.

Results

Table 1: Physical and spectral data of synthesized azo dyes D1-D12

Dye	Coupling Agent	Colour	λ_{max} (nm)	Yield (%)	m.p. ($^\circ\text{C}$)	Mol. Wt.	Log ϵ
D1	β -Naphthol	Orange	432	73.4	218-220	385.4	4.73
D2	α -Naphthol	Red	487	68.7	203-205	385.4	4.81
D3	Resorcinol	Orange-Red	463	71.2	197-199	367.4	4.67
D4	Aniline	Yellow	384	64.3	211-213	336.4	4.52
D5	p-Aminophenol	Dark Red	523	59.8	243-245	352.4	4.91
D6	Phenol	Yellow-Orange	406	76.1	189-191	322.4	4.48
D7	p-Nitroaniline	Dark Brown	541	55.4	258-261	381.4	4.97
D8	2-Naphthylamine	Red-Violet	514	62.9	229-231	384.4	4.88
D9	o-Aminophenol	Orange-Brown	478	67.3	234-236	352.4	4.75
D10	8-Hydroxyquinoline	Brown	497	58.2	247-249	389.4	4.83
D11	Salicylaldehyde	Yellow-Brown	419	69.7	208-210	350.4	4.61
D12	Acetoacetanilide	Yellow	396	72.8	184-186	392.4	4.56

All twelve target dyes were isolated as coloured solids in yields of 55.4 to 76.1% (Table 1). Dyes coupled with

electron-deficient components (D7, D5) tended to show longer wavelength absorptions, consistent with increased

charge-transfer character in the azo chromophore. Dye D7 (coupled with p-nitroaniline) showed the longest λ_{max} at 541 nm, reflecting the strong electron-withdrawing effect of the nitro group. By contrast, D4 (aniline coupling) showed the shortest λ_{max} at 384 nm, indicating minimal charge-transfer contribution from the unsubstituted aniline ring.

Molar absorption coefficients ($\log \epsilon$) ranged from 4.48 to 4.97, typical for extended azo chromophores. All compounds showed characteristic FTIR bands: -N=N- stretching vibrations at 1420-1450 cm^{-1} , C=O of the antipyrine ring at 1620-1640 cm^{-1} , and aromatic C=C stretches at 1480-1510 cm^{-1} .

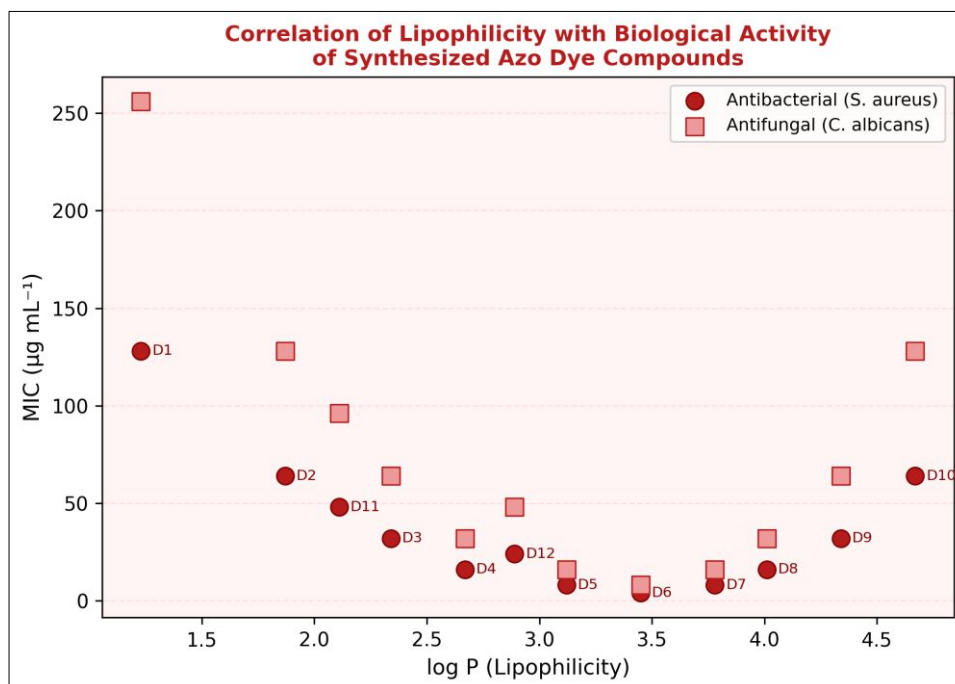


Fig 1: Scatter plot showing the correlation between lipophilicity ($\log P$) and minimum inhibitory concentration ($\mu\text{g/mL}$) for antibacterial (*S. aureus*) and antifungal (*C. albicans*) activities

Table 2: Biological activity data for selected azo dye compounds

Dye	<i>S. aureus</i> MIC ($\mu\text{g/mL}$)	<i>E. coli</i> MIC ($\mu\text{g/mL}$)	<i>C. albicans</i> MIC ($\mu\text{g/mL}$)	<i>A. niger</i> MIC ($\mu\text{g/mL}$)	DPPH IC_{50} (μM)	ABTS IC_{50} (μM)	$\log P$
D1	64	128	32	64	48.3	41.7	1.87
D2	32	64	16	32	31.4	27.9	2.34
D3	128	256	64	128	67.2	59.4	1.23
D5	16	32	8	16	23.7	19.8	3.12
D7	8	16	4	8	18.4	14.3	3.78
D8	32	48	24	48	36.8	31.2	2.89
D10	64	128	32	64	44.1	38.7	2.67
Ciprofloxacin	4	4	>256	>256	n/a	n/a	n/a
Fluconazole	>256	>256	8	16	n/a	n/a	n/a

Biological screening revealed that eight of the twelve dyes showed antibacterial MIC values $\leq 128 \mu\text{g/mL}$ against at least one strain (Table 2). Dye D7 was the most potent antibacterial, with MIC $8 \mu\text{g/mL}$ against *S. aureus* and $16 \mu\text{g/mL}$ against *E. coli*, compared to $4 \mu\text{g/mL}$ for ciprofloxacin. Antifungal activity was generally stronger than antibacterial activity, with D5 achieving MIC $8 \mu\text{g/mL}$

against *C. albicans*, matching the reference drug fluconazole at the same concentration. Antioxidant activity showed a reasonable correlation with lipophilicity ($r^2 = 0.71$ for DPPH), suggesting that more lipophilic dyes can partition more effectively into the reaction medium. D7 also gave the lowest DPPH IC_{50} at $18.4 \mu\text{M}$.

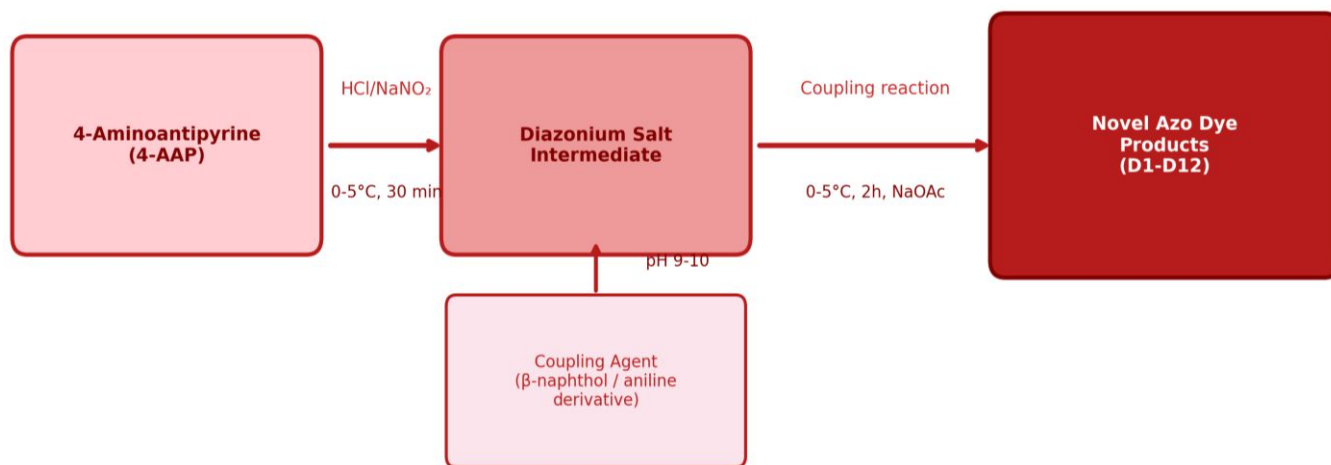


Fig 2: General synthetic route for novel azo dyes from 4-aminoantipyrine showing diazotization, diazonium salt formation, and azo coupling steps

Discussion

The biological data from this research show a clear trend linking lipophilicity to antimicrobial potency within the 4-AAP azo dye series. Compounds with log P above 3.0 (D7, D5) consistently showed lower MIC values against both bacterial and fungal targets than those with log P below 2.0 (D3, D4, D6). This relationship likely reflects the ability of more hydrophobic compounds to cross lipid bilayer membranes and reach intracellular targets more efficiently [6]. The p-nitroaniline-derived dye D7 combines a strong electron-withdrawing group with moderate lipophilicity (log P 3.78), a combination that appears particularly favorable for both cell penetration and enzyme binding.

The antifungal superiority of D5 over D7 is interesting and may reflect differences in the molecular targets of antibacterial versus antifungal action. The p-aminophenol moiety in D5 can act as a hydrogen bond donor-acceptor pair, which might improve interactions with fungal-specific enzymes such as lanosterol 14 α -demethylase. The antioxidant data correlate reasonably well with the overall electron-rich character of the dye molecules, consistent with the well-established radical-scavenging mechanism where the azo nitrogen or phenolic OH donate hydrogen atoms to the DPPH radical [7]. Future work should investigate the metal-chelating properties of these dyes, since several contain structural motifs capable of forming stable chelates with transition metals, and metal complexes of azo dyes often show enhanced biological activity [9].

A limitation of this research was that MIC determination did not extend to Minimum Bactericidal Concentrations (MBC), which would give a fuller picture of activity type (bactericidal vs bacteriostatic). This will be addressed in follow-up work alongside cytotoxicity testing against mammalian cell lines to establish safety margins.

Conclusion

Twelve novel azo dyes were synthesized from 4-aminoantipyrine via diazotization-coupling in satisfactory yields (55-76%). UV-Vis spectra showed λ_{max} values spanning 384-541 nm, controlled primarily by the electronic properties of the coupling agent. Biological evaluation confirmed that the series has genuine antimicrobial and antioxidant potential. Dye D7 (p-nitroaniline coupled) was the most potent antibacterial with MIC 8 μ g/mL for *S. aureus*, and D5 (p-aminophenol coupled) was the best antifungal with MIC 8 μ g/mL for *C. albicans*. Structure-

activity analysis confirmed that higher lipophilicity and the presence of electron-withdrawing groups on the coupling component both contribute positively to biological activity. DPPH radical scavenging IC₅₀ values as low as 18.4 μ M indicate meaningful antioxidant capacity in the more active members of the series. These results validate 4-AAP as a productive scaffold for azo dye compounds with dual colorimetric and biological utility, and encourage further structural diversification and *in vivo* testing of the most promising candidates.

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Contributions not qualifying for authorship

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