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Original Article

SYNTHESIS AND EVALUATION OF ANTIMICROBIAL PROPERTIES OF *AZO* DYES

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ABSTRACT

Objective: To synthesize*azo* dyesand evaluate their antimicrobial potential.

Methods: A number of *azo* compounds were synthesized *via* diazotization of primary aromatic amine and subsequent coupling with naphthols or other coupling partners. The antimicrobial properties of these *azo* compounds were determined against six microbial species; *Staphylococcus aureus*(ATCC25923), *Escherichia coli* (ATCC10231), *Mycobacterium smegmatis* (clinical strain), *Micrococcus luteus*(ATCC10240), *Pseudomonas aureginosa* (ATCC 9027) and the fungus*Candida* albicans(ATCC10231) using the Kirby-Bauer Standard disc diffusion method. The minimum inhibition concentrations (MIC)were also determined for those compounds that exhibited antimicrobial activity.

Results: Two of the *azo* compounds showed inhibition against microbial agents, with *p*-NAαN in particular exhibiting very good antimicrobial properties. However, *Pseudomonas aureginosa* (ATCC 9027) was resistant against all the *azo* compounds.

Conclusion: *p*-NAαN showed broad spectrum of activity against*Staphylococcus aureus*, *Escherichia coli*, *Mycobacterium smegmatis*, *Micrococcus luteus*and the fungal species *Candida albicans*, with *p*-ABAαN exhibiting activity against *Candida albicans*.

Keywords: *azo*, Diazotization, Antimicrobial, Disc diffusion method, Minimum inhibition concentration

INTRODUCTION

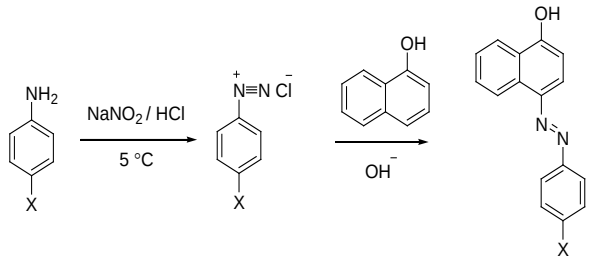
Microbial resistance is one of the world's most pressing public health issues and has therefore become a great challenge as far as delivery of healthcare is concerned [1]. For instance, various strains of organisms are showing resistance towards current antimicrobial agents. Emergence of new strains of organisms is also contributing to the observed resistance to antimicrobial species [2]. This phenomenon has necessitated the quest for new range of antimicrobialsthat willcounter these observed resistances [3].

Although synthetically tractable, [4-6] *azo* dyes have received minimal attention in the search for more effective antimicrobial agents. Despitefew reported cases of antimicrobial properties of *azo* dyes [7-10], their investigation as potential antimicrobial agents has not been fully explored and, very little effort has beeninvested in search for*azo* dyes with microbial inhibition properties.

We present herein our findings, which reveal antimicrobial properties of synthetic *azo* dyes. As part of these preliminary investigations, a number of *azo* compounds were synthesized from diazonium intermediates [11-13] (which wereobtained from the corresponding aromatic amine), andan appropriate coupling partners [14]. These coupling partners included *α*-naphthol, *β*-naphthol, *m*-aminophenol and *p*-aminobenzoic acid. Scheme 1 represents a general route for the synthesis of*azo* dyes. Seven*azo* dyes namely, 4-((4-nitrophenyl)diazenyl)naphthalen-1-ol, 4-(4-hydroxynaphthalen-1-yl)diazenyl)benzoic acid, 1-(4-nitrophenyl)diazenyl)naphthalen-2-ol, 4-((2-hydroxynaphthalen-1-yl)diazenyl)benzoic acid, 4(4-amino-2-hydroxyphenyl)diazenyl)benzoic acid, 4-amino-3-(4-nitrophenyl)diazenyl)benzoic acid and 4-amino-3-((4-carboxylphenyl)diazenyl) benzoic acid were synthesized. These compoundsare coded according to their various reacting components; (*p*-NAαN, *p*-ABAαN, *p*-NAβN, *p*-ABAβN, *p*-ABAγNPhol, *p*-NBA-*p*-NA, *p*-ABA-*p*-ABA)[F] respectively, and are shown in table 1. The synthesized targets were then evaluated for potentialantimicrobial properties using the disc diffusion method [15].

Experimental

All reagents and solvents were obtained from BDH Chemicals as technical or analytical grade. Reaction progress was monitored using thin layer chromatography (*tlc*) technique, which was performed on pre-coated silica gel plate and visualized with UV light or anisaldehyde spray. Compounds were purified by recrystallization from appropriate solvent mixtures. Melting points were determined by open capillary method using Stuart melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded using INTERSPECT spectrometer in the range 400–4000 cm^{-1}. Ultraviolet-visible (UV-vis) spectra were measured on a Perkin Elmer spectrophotometer at 200-800 nm in MeOH, NaOH and acidified methanol.



Scheme 1: General route for the synthesis of *azo* dyes

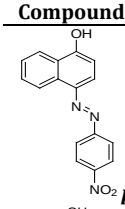
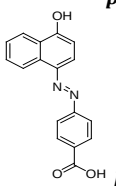
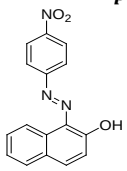
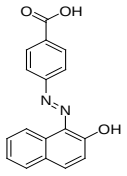
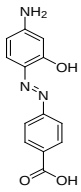
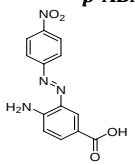
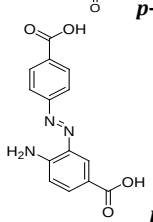
General method for the synthesis of diazonium salt

Amine (15 mmol) was stirred in concentrated hydrochloric acid (20 ml) until a clear solution was obtained. The mixture was cooled to 0–5˚C in an ice bath. A cold solution of sodium nitrite (50 ml) was then added drop-wise to the acidified amine solution, keeping the internal temperature of the mixture below 5˚C. This was stirred for a further 30 min below 5˚C to give the diazonium salt which was used immediately in the coupling reaction.

General method for coupling reactions

A mixture of naphthol (or coupling partner) (15 mmol) in 3 M NaOH (50 ml) was cooled to 5˚C in an ice bath. This was then added to the cold benzenediazonium salt and the resulting mixture stirred slowly below 5˚C for 30 min. Crystals that precipitated were collected by filtration, washed with cold water and dried. Recrystallization from appropriate solvent gave the desired *azo* compound, table 1.

Table 1: Synthesized *azo* compounds and their physical data

Compound	Mol. Formula	Mol. Wt	% Yield	Melting Pt. °C	R_f
 ***p*-NAαN**	$C_{16}H_{11}N_3O_3$	293.28	95.4	123-125 (Water)	0.65
 ***p*-ABAαN**	$C_{17}H_{12}N_2O_3$	292.29	72.2	288-290 (Water)	0.66
 ***p*-NAβN**	$C_{16}H_{11}N_3O_3$	293.28	74.4	245-247 (Water)	0.64
 ***p*-ABAβN**	$C_{17}H_{12}N_2O_3$	292.29	93.3	243-245 (EtOAc)	0.63
 ***p*-ABAγNPhol**	$C_{13}H_{11}N_3O_3$	257.24	87.3	260-262 (EtOH: EtOAc) (90/10, v/v)	0.57
 ***p*-NBA-*p*-NA**	$C_{13}H_{10}N_4O_4$	286.24	88.4	122-125 (EtOAc)	0.63
 ***p*-ABA-*p*-ABA**	$C_{14}H_{10}N_4O_5$	314.25	71.1	190-192 (EtOAc)	0.67

Coding of compounds in the table was derived from the names of two reacting components. [T]

Antimicrobial experiments

The synthesized*azo* dyes were screened against micro bacterialagents, *Staphylococcus aureus*(ATCC 25923), *Escherichia coli*(ATCC 10231), *Mycobacterium smegmatis*(Clinical strain), *Micrococcus luteus*(ATCC 10240), *Pseudomonas aureginosa*(ATCC 9027) and the fungus*Candida albicans*(ATCC10231), using the disc diffusion method to determine their zones of inhibition. The organisms were cultured using tryptone soya broth. Samples were incubated for 24 h, with the bacteria species at 32 °C whiles the fungal species was incubated at 22 °C. Mycobacterium cells however, needed a 48 h incubation period.

Solutions of 20 mg/ml concentrations of the dyes were prepared in methanol, and these were sonicated for approximately 10 min. Sterile filter paper discs (6 mm perforation) were soaked in the solutions for 30 min, and allowed to dry at room temperature. Various concentrations of the test samples in methanol were obtained and used to prepare sterile filter paper discs. 0.1 ml each of the *azo* dye solutions was pipetted onto each tryptone soya plate and evenly spread using a swab stick before leaving to dry for 5 min. The filter paper discs were then placed on the inoculated plates and incubated for 18 h (48 h for *Mycobacterium smegmatis*) and the zones of inhibition determined at the end of the incubation period during which diffusion of the test solution affected the growth of the inoculated microorganism, aiding in determining the zones of inhibition.

Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of compound below which inhibition of micro-organisms would not be observed. These were determined by micro-dilution method which involved preparing the inoculums using 4.6 h broth culture of each microbial strain, adjusted to a turbidity equivalent to 0.5 McFarland Standard, and diluted in nutrient broth media to give cell count of 1.58×10^8 by approximation. Results for the zone of inhibition are shown in table 2.

Table 2: Zone of Inhibition (ZoI) for *azo* dyes at 20 mg/ml

Entry	Compound	S. aureus	E. coli	M. smegmatis	M. luteus	P. aureginosa	C. albicans
1	*p*-NAαN	10.92±1.400	8.37±0.349	26.89±0.465	16.45±1.150	No zone	11.66±0.045
2	*p*-ABAαN	No zone	No zone	No zone	No zone	No zone	6.94
3	*p*-NAβN	No zone	No zone	No zone	No zone	No zone	No zone
4	*p*-ABAβN	No zone	No zone	No zone	No zone	No zone	No zone
5	*p*-ABAγNPhol	No zone	No zone	No zone	No zone	No zone	No zone
6	*p*-NBA-*p*-NA	No zone	No zone	No zone	No zone	No zone	No zone
7	*p*-ABA-*p*-ABA	No zone	No zone	No zone	No zone	No zone	No zone
8	Blank Control	No zone	No zone	No zone	No zone	No zone	No zone

ZoI; n = 3, values are mean±SEM (Standard Error of Mean)

RESULTS

Surprisingly, most of the compounds showed no antimicrobial activity against any of the test organisms, (Entries 3 to 7). It was, however, encouraging to see that *p*-NAαN and *p*-ABAαNshowed levels of inhibition against test organisms, (Entries 1 & 2). This is indicative that α-naphthol moiety may be aiding in the observed inhibitions. The*p*-ABAαN showed inhibition against *Candida albicans* as shown in Entry 2. The*p*-NAαN on the other hand showed inhibition against all the test organisms at 20 mg/ml, except *Pseudomonas aureginosa*, Entry 1. 4-((4-nitrophenyl)diazenyl)naphthalen-1-ol(*p*-NAαN) therefore became our main focus compound for further investigations.

This development led us to further investigate the minimum inhibition concentrations (MIC) that could be achieved with this synthetic dye. Solutions with varied concentrations of *p*-NAαN were prepared for our investigations. It was gratifying to observe that the dye, *p*-NAαN inhibited many test organisms at lower concentrations as shown in table 3.

Table 3: Percentage Zones of Inhibitionof *p*-NAαN against microorganisms at different concentrations

Staphylococcus aureus		*Escherichia coli*		*Mycobacterium smegmatis*		*Micrococcus luteus*		*Candida albicans*	
Conc (mg/ml)	**% ZoI**	**Conc (mg/ml)**	**% ZoI**	**Conc (mg/ml)**	**%ZoI**	**Conc (mg/ml)**	**% ZoI**	**Conc (mg/ml)**	**% ZoI**
10	100.000±0.00	20	100.00±0.000	10	100.00±0.000	10	100.00±0.000	10	100±0.000
8	82.373±4.563	18	95.787±0.166	8	80.300±8.043	8	85.027±5.333	8	83.513±4.5959
6	73.660±4.923	16	90.750±0.392	-	-	6	73.92±7.127	6	78.150±5.575
4	59.900±4.578	14	87.347±0.822	-	-	4	59.783±7.363	4	64.483±1.179
2	48.533±3.678	12	81.3933±1.205	-	-			0.5	38.010±0.362
Cont.	No zone	Cont.	No zone	Cont.	No zone	Cont.	No zone	Cont.	No zone

Cont. = Blank control; ZoI; n =3, values are mean±SEM (Standard Error of Mean)

At 2 mg/ml and 1.5 mg/ml, *p*-NAαN showed inhibition against *Staphylococcus aureus*. Although the dye showed promising inhibition against *E. coli*, the lowest concentration for inhibition of *Escherichia coli* was observed at 12 mg/ml. For *Mycobacterium smegmatis*, inhibition was observed at 10 mg/ml and at 8 mg/ml. *Micrococcus luteus* was inhibited at concentrations as low as 4 mg/ml but, below this concentration, no inhibition was observed. The most interesting revelation involved the fungal species *Candida albicans*, which was observed to be inhibited at concentration of 0.5 mg/ml Based on the above information, the minimum inhibition concentration (MIC) of *p*-NAαN was obtained for the various microorganisms as shown in table 4. Whiles MIC of 0.02 mg/ml was revealed against *C. albicans*, *p*-NAαN showed MIC of 4.08 mg/ml, against the bacterium *M. smegmatis*, the highest MIC out of all the test organisms. This may be due to the acid fast nature and the thick biological membrane that is a feature of*M. smegmatis* [16-17].

Table 4: Minimum Inhibition concentration (MIC) of *p*-NAαN for the various microorganisms

Microbes	Minimum Inhibition Concentration (mg/ml)
S. aureus	1.22±0.096
E. Coli	1.26±0.107
M. Smegmatis	4.06±0.637
M. luteus	0.80±0.817
C. albicans	0.02±0.443

MIC; n = 3, values are mean±SEM

CONCLUSION

Seven *azo* dyes namely 4-((4-nitrophenyl)diazenyl)naphthalen-1-ol, 4-((4-hydroxynaphthalen-1-yl)diazenyl)benzoic acid, 1-((4-nitrophenyl)diazenyl)naphthalen-2-ol, 4-((2-hydroxynaphthalen-1-yl)diazenyl)benzoic acid, 4-((4-amino-2-hydroxyphenyl)diazenyl)benzoic acid, 4-amino-3-((4-nitrophenyl)diazenyl)benzoic acid and 4-amino-3-((4-carboxylphenyl)diazenyl)benzoic acidwere synthesized. Of these, 4-((4-nitrophenyl)diazenyl)naphthalen-1-ol (*p*-NAαN) and 4-((4-hydroxynaphthalen-1-yl)diazenyl)benzoic acid (*p*-ABAαN), have shown antimicrobial activities. 4-((4-nitrophenyl)diazenyl)naphthalen-1-ol (*p*-NAαN)in particular, has been shown to inhibit most strains of microorganisms at lower concentrations. We would therefore like to publish these findings. The results have encouraged us to further investigate synthetic analogues of these *azo* compounds with the hope of improving upon antimicrobial activity and provide firm comparison with standard antimicrobial agents. Results obtained from these structural modifications will form the basis for subsequent publications.

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CONFLICT OF INTERESTS

The authors have no conflict of interest in publication of this article

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