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SYNTHESIS OF SOME NEW TRISAZO DYES AND TOXICITY EVALUATION WITH THE *HYDRACTINIA ECHINATA* TEST SYSTEM

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Abstract:

The synthesis of three new trisazo dyes containing 4,4'-diaminobenzanilide as middle component is presented. The dyes were analyzed by thin layer chromatography (TLC), electronic spectra (VIS) and their structure was confirmed by mass spectroscopy (FABS). The toxicity of the synthesized dyes was evaluated by biological tests, using the process of metamorphosis in the marine Hydrozoon *Hydractinia echinata*. The concentration (termed by MRC_{50}) at which the synthesized dyes (and their precursors) antagonize metamorphosis induction was determined. The obtained results indicate that these dyes exhibit toxicity values which are lying in a low average low scale of toxicity.

Key words: Direct dyes, 4, 4'-diaminobenzanilide, toxicity, Hydractinia echinata

Introduction

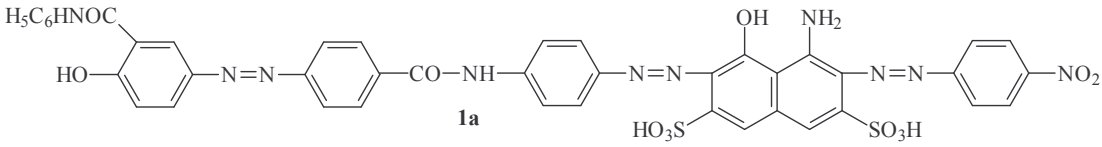
It was proved that in the living organisms, some dyes are able to split one or more azo groups (biodegradation) [1-4], leading to the stoichiometric formation of the corresponding aromatic amines, some of them being known as mutagenic and/or carcinogenic [5-7]. Consequently, the dye's toxicity is often related to the toxicity of the aromatic amines which are used for their synthesis. Any dye which is able to form through cleavage of one or more azo groups one of these toxic amines will fall under the category of banned azo dyes, and the interest in finding alternative compounds is very relevant.

However, the design and the development of a new series of non-toxic dyes presume a complicated strategy, involving a wise choice of the precursors, as well as the development of some procedures of preparation ranging in the present trends of preserving a clean environment. On the other hand, one of the major concerns of the scientific research is the development of new methods for the evaluation of the toxicity. For this reason, the toxicity evaluation by using biological tests on colonies of *Hydractinia echinata* appears as an attractive alternative. This method was applied till now in the case of alkans, alcohols, amino-alcohols, aliphatic primary amines, benzene, benzene derivatives, etc [8-9].

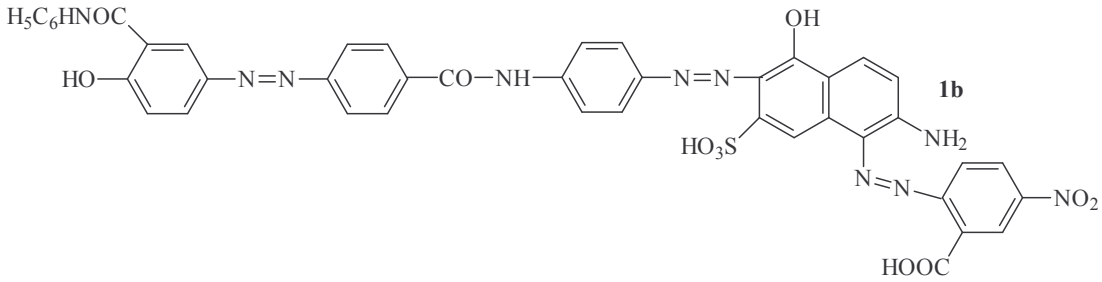
In the present work, the synthesis and the physico-chemical characterization of three new trisazo dyes is presented. The toxicity evaluation of these dyes was performed using biological tests *Hydractinia echinata* colonies.

Results and discussion

Optimum reaction conditions for the synthesis of three new trisazo dyes (**1a, 1b** and **1c**), derived from 4,4'-diaminobenzanilide were established.



1a



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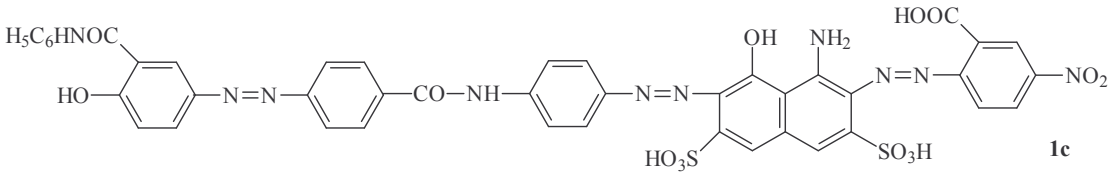

1c

For the synthesis of the dyes **1a, 1b** and **1c**, a three step procedure was developed. The preparation procedure involved the bis-diazotization of 4,4'-diaminobenzanilide by the direct

method, and thereafter two coupling reactions of the resulting bis-diazonium salt with the corresponding alkaline solutions of some aromatic precursors: salicylanilide and a monoazo compound prepared by the coupling reaction of the diazonium salt of *p*-nitroaniline with 1-amino-8-hydroxy-3,6- naphthalenedisulfonic acid (dye **1a**), salicylanilide and a monoazo compound obtained by the coupling reaction of the diazonium salt of 5-nitroantranilic acid with 2-amino-5-naphthol-7-sulphonic acid (dye **1b**). The coupling components used for the synthesis of (dye **1c**) were salicylanilide and a monoazo compound prepared by the coupling reaction of the diazonium salt of 5-nitroantranilic acid with 1-amino-8-naphthol-3,5-disulphonic acid.

During the synthesis, it was noticed that the rate of the coupling reaction was improved and homogeneous final products were obtained when a small quantity of dinaphthyl-methane-disulfonic acid was added to the reaction mixture.

The progress of the coupling reactions was monitored by TLC, which was extremely useful in the identification of the secondary products formed during the coupling reaction and also for the evaluation of the purity of the final dyes. Several eluting systems were tested and the best results were obtained in the case of *i*- propanole: methyl-ethyl-ketone:ammonia 25 % = 4:3:3 (v:v:v) for the control of the progress of the coupling reaction and pyridine: isopropanole: n-butanole: ammonia 25 % = 1:1:1:1 (v:v:v:v), for the analysis of the final products.

The reaction yields and the physico-chemical characteristics of the synthesised dyes are shown in Table 1.

Table 1

REACTION YIELDS AND PHYSICO-CHEMICAL CHARACTERISTICS OF THE TRISAZO DYES **1a, 1b**, and **1c**.

Dye	η [%]	λ_{max}* [nm]	$\varepsilon^{1g/L}_{1cm}$	R_f**	[M-1] *** m/z
1a	91	660	36	0.57	929.87
1b	87	582	38	0.68	892.56
1c	80	694	35	0.43	973.69

* From 0.1 mol/L NaOH aqueous solution;

** Silica gel plates (Merck 60F – 25), pyridine: isopropanole: n-butanole: ammonia 25 % = 1:1:1:1 (v:v:v:v) as eluting system;

*** FAB-MS (-), nitrobenzylic alcohol and glycerol matrix.

The toxicity of the synthesized dyes was evaluated by biological tests, using colonies of Hydrozoon *Hydractinia echinata*. The larvae of *Hydractinia echinata* have an elongated spindle shaped body of about 1 mm in length and a diameter of 100 μm and are easy to get. They consist of about 10 000 cells. They have no mouth, no gut, no extremities, no sense organs but nerve cells which may serve to sense environmental signals. In the laboratory the adult animal produce several thousands eggs per week all year along. Metamorphosis into polyp can be triggered artificially by a three hours treatment with seawater enriched by Cs^+ ions or depleted by Mg^{2+} ions [8,9]. Within one day a larva transforms into a polyp which looks similar to the well known freshwater polyp Hydra. When certain substances (our test substances by instance) are applied during the triggering treatment, induction is antagonised. In this case the larvae remain in the larval stage: they are prevented from completing their life cycle. Thus, the chemical antagonise the species from surviving and this matter of fact is one of the reasons which justify the choice of our testing system. On the other hand, substances which display a strong toxicity in well known test system also display a strong antagonising influence on metamorphosis induction and vice versa.

The results of the biological tests, expressed by the logarithm of the reciprocal value of the metamorphosis reducing concentration, MRC_{50} ($log1/MRC_{50}$), are shown in Table 2.

Table 2

HYDRACTINIA ECHINATA TEST SYSTEM. MEASURED M, AVERAGE CALCULATED VALUE MC, NUMBER OF THE FUNCTIONAL GROUPS AND MOLECULAR WEIGHT G_{MOL} OF THE TESTED DERIVATIVES

No	Compound	M	mC	M-mC	N=N	OH	NH_2	SO_3H	G_{mol}
1	4,4'diaminobenzanilide	3.39							
2	Dye **1a**	3.95*	3.90	0.05	3	2	1	2	930.88
3	Dye **1b**	3.86	3.90	-0.04	3	2	1	1	893.82
4	Dye **1c**	3.89	3.90	-0.01	3	2	1	2	974.89

* average value [3.96; 3.93] which proves the reproducibility of the test system

As it can be seen, the obtained values are very close for all the test substances. When comparing these values with an average middle toxicity scale obtained for other substances studied in the same test system (alkans, alcohols, amino-alcohols, primary aliphatic amines, benzene and benzene derivatives) [8,9] one could notice that the synthesized dyes exhibit toxicity values which are lying in a low average low scale of toxicity.

The tested derivatives contain the azo- and the amidic-(peptidic) group and will lead to concurrent reduction and hydrolysis reactions. The rate of the partial biodegradation products formation will dependent on the rate and intensity of the substrate-enzyme interaction.

In experimental conditions at pH = 8.2, the reaction products are primary aromatic amines of benzene or naphthalene in stoichiometric mixtures. The non-protonated species is more toxic than the protonated one [10], both species have however a contribution to the general toxicity [11-13].

The measured toxicity values, M, are quite close but slightly higher as compared to the value obtained in the case of the central component (e.g. 4,4'-diaminobenzanilide). In this situation one could consider an average calculated toxicity, which could probably undergo some changes when other new experiments on this kind of derivatives will be carried-on.

In the very similar structural conditions depicted in Table 2, the rise of the toxicity values could be assigned to the presence of the nitro group in the *para* position of the studied dyes. This group presents an important withdrawing electron effect onto the closer azo group, the electron's density is lowered and the rate of the reduction reaction will be consequently higher. A similar effect is exercised by the central carbonylic group. However, this effect is probably more intense into the azo group's direction, without the presence of the aminopeptidic group. On the other hand, the azo group's electronic density could also be lowered due to the inner cyclization resulting between the unshared pair of p electrons of the nitrogen atom and an adjacent group (by instance –OH, $-NH_2$, $-SO_3H$ or –COOH) located on the aromatic rings. In this situation, steric hindrances could also appear, between the azo group and an active reaction center.

Nowadays, the methods of toxicity evaluation involve *in vitro* or *in vivo* experiments, on both human and animal subjects, a great volume of work, special equipments, a very long time and often exorbitant costs. For this reason, the development of a method for the toxicity evaluation by using biological tests on colonies of *Hydractinia echinata* appears as an attractive alternative. This method has great advantages: simplicity, accessibility, reproductibilicity, time and

experimental work considerably reduced. The experimental data thus obtained can be generalized by statistical methods. Moreover, the experimental work does not need animal or human subjects.

Materials and Methods

The chemicals employed in this study were obtained from Merck Co and Reactivul Bucureşti. The synthesis and the physico-chemical characterization of 4,4'-diaminobenzanilide was presented in previous work [14].

The Vis spectra were recorded on a Perkin Elmer λ12 spectrophotometer from 0.1 M NaOH aqueous solutions.

The TLC data were taken from silica gel plates (Merck 60F - 25), using CAMAG 10x10 tanks and *i*-propanole:methyl-ethyl-ketone:ammonia 25 % =4:3:3 and 4:3:4 (v:v:v) as eluting system.

Mass spectra were recorded on a Nermag R 10-10 spectrometer using Fast Atomic Bombardment technique and nitrobenzylic alcohol, and triglycerol matrix.

Synthesis of dyes with general structure I.

1. Bis-diazotization of 4,4'-diaminobenzanilide

0.05 moles of 4,4'-diaminobenzanilide were bis-diazotized by the direct method, in concentrate mineral acid, with sodium nitrite as described in previous works [14-16].

2. The first coupling reaction of the bis-diazonium salt of 4,4'-diaminobenzanilide with salicylanilide

An alkaline solution was obtained from 0.054 mol of salicylanilide, 3 g (0.0283 mol) Na_2CO_3, 50 mL water and 20 g (0.15 mol) 30 % NaOH solution. The solution was cooled to 5 ^oC and added dropwise to the bis-diazonium salt of 4,4'-diaminobenzanilide in 45 minutes, at 5 ^oC. The mixture was stirred cold for two hours, and the pH was maintained at 7.5–8.

3. The second coupling reactions (general mode)

A quantity corresponding to 0.051 mol of the corresponding monoazo compounds was suspended in 150 mL water and 3 g dinaphthyl-methane-disulfonic acid. The pH of the mixtures was brought to 7-7.5, the mixtures were cooled to 5 ^oC and were added dropwise into cold suspensions of the disazo compound salicylanilide ←4, 4'-diaminobenzanilide obtained as described in step 2. The reaction mixture was brought to pH 9.5–10 and was stirred for another two hours in the case of dye **1a** and three hours in the case of dye **1b** and **1c**, at 10 ^oC. The pH was maintained in the range of 9.5–10. The coupling reaction was controlled by the drop reaction and by TLC (silica gel plates Merck 60F–25, pyridine: isopropanole: n-butanole: ammonia 25 % = 1:1:1:1 or isopropanole: methyl-ethyl-ketone: ammonia 25 % = 4:2:3 as eluting systems). The final compounds were separated by salting out and

were collected by filtration, washed with water, with 3 % sodium chloride solution, with 2 % dinaphthyl-methane-disulfonic acid solution, dried, and purified by several recrystallizations from water.

Biological tests

The test conditions and the test method were identical to those described in previous work [8-10]. The concentration of the test substance X (mol/L) was varied in such a way that we were able to determine the concentration at which the frequency of the induction of metamorphosis in the hydroid *Hydractinia echinata* was reduced by 50% with respect to a control. That concentration termed MRC_{50} (metamorphosis reduction concentration) was further used as the logarithm of the reciprocal value ($Mlog1/MRC_{50}$). For each concentration, the experiments were performed in triplicate.

$Mlog1/MRC_{50}$ refers to experimentally measured values (M), while mClog1/MRC50 refers to the average calculated toxicity value, mC, considering that the toxicity is constant over a +/- 0,5 logarithmic units range as compared to the calculated toxicity value, C. The calculated average mC value is assessed by the sum of the least squares deviations between the measured toxicity values, M, and the calculated ones.

Conclusions

Three new trisazo dyes derived from 4,4'- diaminobenzanilide have been synthesized and characterized.

The toxicity of the synthesized dyes was evaluated by biological tests, using *Hydractinia echinata* colonies. The synthesized compounds exhibit very close MRC_{50}, values, which are lying in a low average low scale of toxicity.

Due to the very similar structural features of the synthesized dyes, their toxicity values are influenced by the presence of the nitro group located in *para* position.

The aromatic amines resulting from the reduction step and the enzymatic hydrolysis do not determine synergic or additive effects, the individual toxicity values being very close to those of the initial dyes (unpublished results).

The *Hydractinia echinata* test system presents great advantages: simplicity, accessibility, reproductibilicity, time and experimental work considerably reduced.

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