

# AZO COMPOUNDS WITH ANTIMICROBIAL ACTIVITY

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Two new disazo compounds containing 4,4'-diaminostilbene-2,2'-disulfonic acid and 4,4'-diaminobenzanilide as middle components were synthesized and characterized by thin layer chromatography (TLC), electronic spectra (VIS), FT/IR and <sup>13</sup>C-NMR spectroscopy. According to the results of the colouristic evaluation, the studied azo compounds can be ranged in the direct dye's class. The individual human skin tolerance for these azo compounds was evaluated by *in vivo* imagistic skin studies and the obtained results indicate that, from dermatologic point of view, the azo compounds may be used as textile dyes. Further, the compounds were evaluated for antibacterial activity against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus vulgaris* by the disk diffusion method. The screening data revealed that the studied azo compounds exhibited antimicrobial activity against most of the tested species, presenting a particular interest against *Staphylococcus aureus* and *Escheria coli*.

**Key words:** Azo dyes, 4,4'-diaminostilbene-2,2'-disulfonic acid, 4, 4'-diaminobenzanilide, antimicrobial activity

## Introduction

Nowadays, synthetic azo compounds are widely used in different application fields, such as medicines, cosmetics, food, paints, plastics, shipbuilding, automobile industry, cable manufacture, etc [1-19].

However, the traditional application field of the synthetic azo dyes still remains the textile industry, and the finishing of fibrous materials in order to impart simultaneously with coloration, antimicrobial properties is of great interest.

This is due to the fact that textile materials undergo biological degradation, and it seems that about 40 % of the damage is due to the effect of microorganisms. The activity of fungi and bacteria results in the reduced mechanical strength of a material, color change, stains and stale odor. In this regard, the use of materials with antimicrobial properties extends the service life of these materials, and avoids damage caused by biological degradation.

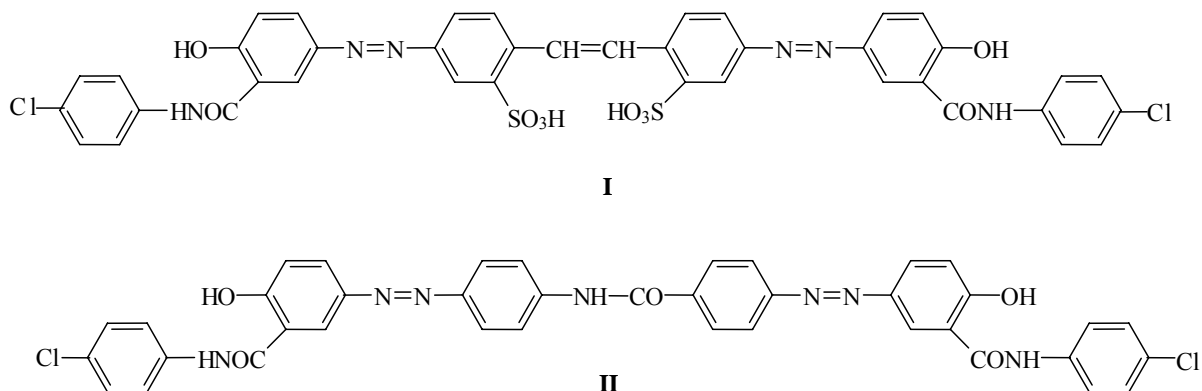
The manufacture of biologically active materials can be performed either by impregnation with antimicrobial compounds, or by chemical reaction (adding antimicrobial compounds by means of chemical bonding to functional groups of the fiber-forming polymers).

Dyes are among the compounds which are suitable for biocidal treatment of textile materials due to the fact that some of them exhibit biological activity, resulting from the presence in their molecule of some antiseptic groups that form a definite type of bonding with the molecules of the fibrous material.

The present study is focused on the possibility of developing new eco friendly azo dyes with good colouristic and application properties, and exhibiting biological activity.

## Results and discussion

Optimum reaction conditions for the synthesis of two new disazo compounds with structure **I** (derived from 4,4'-diaminostilbene-2,2'-disulfonic acid) and structure **II** (derived from 4,4'-diaminobenzanilide) were established.



The synthesis of the azo compounds **I** and **II** was performed by a two step procedure. The preparation procedure involved the bis-diazotization of the corresponding diamine (4,4'-diaminostilbene-2,2'-disulfonic acid and 4,4'-diaminobenzanilide respectively) and thereafter

the coupling reaction (in a 1:2 molar ratio) of the resulting bis-diazonium salt with 4-chlorosalicylanilide.

The bis-diazotization of the two diamines was carried out by the direct method, in a HCl aqueous solution. In the case of 4,4'-diaminostilbene-2,2'-disulfonic acid, the resulting bis-diazonium salt was separated by filtration for the complete removal of the salts, unlike the classical method.

The coupling reactions were performed in an alkaline aqueous medium (pH around 8), in presence of Na<sub>2</sub>CO<sub>3</sub> at a temperature around 10°C, and a 3% excess of coupling component. It was noticed that the direct adding of the alkaline solution of the coupling components to the acid suspension of the bis-diazonium salts of both 4,4'-diaminostilbene-2,2'-disulphonic acid and 4,4'-diaminobenzanilide, was optimum for the synthesis of the disazo compounds **I** and **II**.

The synthesized disazo compounds were obtained in high overall yields and were characterized by means of TLC, VIS, FT/IR and <sup>13</sup>C-NMR spectroscopy. As shown in the experimental part, the FT/IR and <sup>13</sup>C-NMR spectroscopy data agree well with the dye's chemical structure.

The colouristic and the application properties of the disazo compounds **I** and **II** were determined and compared to those exhibited by a classical direct dye, with quite close chemical structure (e.g. C. I. Direct Orange 1).

In the case of the azo compound **I**, the untreated dyeing on cotton was reddish-orange, the dye had good migration and good levelling properties. The percentage uptake on cotton was found to be 68 %. In the case of **II**, the untreated dyeing on cotton was orange and the migration was good, Compound **II** exhibited fair levelling properties and the percentage uptake on cotton was 75 %. The obtained results are presented in Table 1.

As it can be seen in Table 1, the application properties of the disazo dye (**I**) are quite similar to the reference dye Direct Orange 1. Moreover, the after-treatment of the dyed samples with CuSO<sub>4</sub> shows improvements in the case of the wet fastness of dye (**I**). The obtained results indicate that the synthesized dye can be ranged as a direct dye.

**Table 1**

Application properties on cotton of dye (I) and of C. I. Direct Orange 1 (C. I. 22.250).

Fastness:	Disazo dye <b>I</b>		Disazo dye <b>II</b>		C. I Direct Orange 1* (C. I. 22.430)
	Direct dyeing	After treatment with CuSO <sub>4</sub>	Direct dyeing	After treatment with CuSO <sub>4</sub>	Direct dyeing
Light	2-3	3	3	3	2
Water	3-4	4	3-4	4	3-4
Wash 40 °C	2-3	3	2-3	3	1-2
Perspiration (alkaline)	2-3	3	2-3	3	2
Perspiration (acid)	2-3	3	2-3	3	4
Hot pressing	3	3	3	3	3

\* Literature data [20]

Further, the azo compounds **I** and **II** were subjected to *in vivo* imagistic skin evaluation tests, as described in the Experimental part. The possibilities of apparition of adverse effect were monitored with an imagistic skin evaluation, *in vivo*, daily after each application [21-23]. For all the volunteers involved in this study, and for all concentrations applied, any pathological sign were registered. Concerning to skin coloration, no modification was noticed, excepting the transitory color of the studied dyes. As to the skin texture, no peeling effect was registered, and the epidermal desquamative registrations were no more differenced than the blank skin areas. Moreover, during all the period of skin determination, no case of contact dermatitis was registered.

The results of screening antimicrobial activity are given in Table 2. The disazo compounds **I** and **II** were subjected to antibacterial activity screening against two gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus pyogenes*) and three gram-negative bacteria (*Pseudomonas aeruginosa*, *Proteus vulgaris* and *Escherichia coli*) employing the disk diffusion technique. The results show that the studied compounds present antimicrobial activity against most all the tested species, especially against *Staphylococcus aureus* and *Escheria coli*.

**Table 2**

Antimicrobial activities of compounds

Compound	Diameter of zone of inhibition in mm				
	Staphylococcus aureus	Streptococcus pyogenes	Escherichia coli	Pseudomonas aeruginosa	Proteus vulgaris
I	18	10	17	16	14
II	20	18	20	13	15
Septonex	18	17	14	19	16

**Materials and Methods**

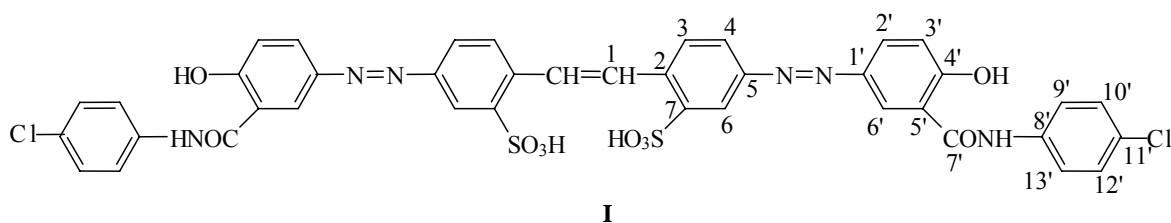
Most of the chemicals used in this study were of analytical grade and were obtained from Merck Co, Fluka Co, Acros Organic Co, Lachema Co, and Aldrich Chemical Co respectively.

The thin layer chromatography (TLC) was performed on silica gel plates (Merck 60F-254) and the eluting system isopropanol: methyl-ethyl-ketone: NH<sub>3</sub> 25%.

Electronic absorption spectra (VIS) of the synthesized dye in 50% aqueous MeOH solution ( $c=1.0 \times 10^{-5}$  mol/dm<sup>3</sup>) were performed on a CECIL CE 7200 spectrophotometer in the range 300–800nm.

The FT/IR spectra were recorded on a JASCO FT/IR-4200 spectrometer in the range 400-4000cm<sup>-1</sup>.

The <sup>13</sup>C-NMR spectra were recorded as solution in DMSO-d<sub>6</sub> using a BRUKER AVANCE DRX-400 spectrometer with tetramethylsilane (TMS) as internal standard.

**Synthesis of the disazo compound I.**

A mixture containing 1.95 g (0.005 mol) of 4,4'-diaminostilbene- 2,2'-disulphonic acid 95%, was bis-diazotised with 0.71 g of 98% solid NaNO<sub>2</sub> (0.0101 mol) at 5°C and a pH value around 1. The obtained bis-diazonium salt was filtered-out, the precipitate was washed with distilled water and then suspended in 20 mL distilled water. The coupling reaction was carried out by direct treating of the bis-diazonium salt suspension with an alkaline solution of 4-

chlorosalicylanilide in a 1:2 molar ratio, at pH=8 and 8-10°C. The coupling reaction was monitored by the conventional method (drop reaction with an alkaline solution of 1-amino-8-naphthol-3,5-disulphonic acid and with the diazonium salt of 4-nitroaniline). The disazo dye was obtained in 82% and was purified by recrystallization from dioxane:pyridine 90:10 (v:v). The homogeneity of the purified dye was confirmed by TLC, using silica gel plates and isopropanol: methyl-ethyl-ketone: ammonia 25 %= 4:2:3 as eluting system.

TLC (silicagel plates 60F-254, *iso*-propanol:methyl-ethyl-ketone:ammonia 25%=4:3:5 (v:v:v)) ( $R_f$ ): 0.62;

VIS (50% aqueous MeOH) ( $\lambda_{max}/nm$ , ( $\epsilon/L mol^{-1} cm^{-1}$ )): 470 (31231);

IR (KBr,  $cm^{-1}$ ): 3432 (stretching of -OH group), 1635(C=O stretching of -CO-NHgroup), 1550( II amide band), 1240( III amide band), 700(-NH deforming of secondary amide), 1180(SO<sub>2</sub> asym. stretching of -SO<sub>3</sub>H group ), 1076(SO<sub>2</sub> sym. stretching of -SO<sub>3</sub>H group), 1336(-OH deforming of -OH group), 824(CH-deforming of aromatic ring), 520,485,476 (skeletal deforming of aromatic ring)

<sup>13</sup>C-NMR (100MHz, (CD<sub>3</sub>)<sub>2</sub>SO,  $\delta/ppm$ ): 125.20(C<sub>1</sub>), 136.96(C<sub>2</sub>), 127.79(C<sub>3</sub>), 123.21(C<sub>4</sub>), 150.73(C<sub>5</sub>), 121.38(C<sub>6</sub>), 138.11(C<sub>7</sub>), 145.99(C<sub>1'</sub>), 126.57(C<sub>2'</sub>), 117.11(C<sub>3'</sub>), 158.02(C<sub>4'</sub>), 119.11(C<sub>5'</sub>), 119.66(C<sub>6'</sub>), 166.13(C<sub>7'</sub>), 133.71(C<sub>8'</sub>), 122.32(C<sub>9'</sub>), 128.57(C<sub>10'</sub>), 129.12(C<sub>11'</sub>)

### **Synthesis of the disazo compound II.**

A suspension of 1.15 g 4,4'-diaminobenzanilide (0,005 mol), 7 ml water, 3 ml (0.27 mol) 35% HCl and 5 g ice was bis-diazotised by the direct method [12].

An alkaline solution containing 0.01 mol of 4-chlorosalicylanilide was added dropwise to the solution of bis-diazonium salt obtained above, in a 45 minutes period. The temperature of the reaction mixture was kept around 5°C and the pH of the reaction was maintained around 8.5 by addition of 0.5 mol/L Na<sub>2</sub>CO<sub>3</sub> aqueous solution. The mixture was stirred for three hours at 5-10°C. The dye was precipitated by salting out with sodium chloride and was separated by vacuum filtration. The reddish-orange disazo dye was obtained in 88% yield.

The homogeneity of the purified dye was confirmed by TLC, using silica gel plates and isopropanol: methyl-ethyl-ketone: ammonia 25 %= 4:3:3 as eluting system.

TLC (silicagel plates 60F-254, *iso*-propanol:methyl-ethyl-ketone:ammonia 25%=4:3:5 (v:v:v)) ( $R_f$ ): 0.68;

VIS (50% aqueous MeOH) ( $\lambda_{max}/nm$ , ( $\epsilon/L mol^{-1} cm^{-1}$ )): 463 (29674);

IR (KBr,  $\text{cm}^{-1}$ ): 3398 (stretching of  $-\text{OH}$  group), 1640 ( $\text{C}=\text{O}$  stretching of  $-\text{CO}-\text{NH}$  group), 1548 (II amide band), 1240 (III amide band), 695 ( $-\text{NH}$  deforming of secondary amide), 1334 ( $-\text{OH}$  deforming of  $-\text{OH}$  group).

The colouristic and application properties, e.g., dyebath uptake, exhaustion degree, migration, levelling and fastness properties of the synthesized dye were determined according to Romanian standards.

The degree of dye exhaustion by cellulose fibres was determined by dyeing samples of bleached cotton fabric at  $95\text{ }^{\circ}\text{C}$ , using 1 % of dye in relation to fibres with addition of 0.5 %  $\text{Na}_2\text{SO}_4$  at the dye liquor at a fibre ratio 30: 1. The dye concentrations in the dyebath before ( $c_1$ ) and after dyeing ( $c_2$ ) were determined spectrophotometrically.

### ***In vivo* skin evaluation**

The evaluation of the possible skin adverse effects of the disazo compounds I and II was performed on a study group of 45 healthy volunteers, all women, aged between 25 – 51 years. The disazo compounds I and II were applied as dilute aqueous solution of 5 %, 2.5 %, 1 % and 0.5 %, on 4 areas of forearms, for 30 days, daily, 1 time a day. In the volunteer selection, the subjects presenting skin pathologic signs (seborrhea dermatitis, psoriasis lesions) were eliminated. However, physiological signs, like photoaging signs, dilatation of sebaceous channel glands, or other physiological signs were admitted. No restrictions regarding to the human skin phototype were considered. For all volunteers, a blank skin area was also chosen, on which we applied distilled water. The possibilities of apparition of adverse effect were monitorized with an imagistic skin evaluation, *in vivo*, daily after the previous application. The tests were performed using a ProDerm II Skin Analyzer. The following parameters were evaluated: the skin coloration (normal or the apparition of a rash); the skin texture (preservation of the normal skin quadrilagde, and eventually irritation signs – a peeling effect), and the possible skin dryness, eventually induced by the disazo compounds (involution of density of sebaceous glands channels).

### ***In vitro* antimicrobial investigations**

The disazo compounds I and II were tested for their antimicrobial activity against the following microorganisms: *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Escherichia coli*, using the cup plate diffusion method [24]. The bacterial organisms were isolated from human being with characteristic infections and diseases. The disazo compounds I and II were dissolved in ethanol, yielding solutions of 0,2 % concentration. Wells (diameter 6 mm) were loaded with 0,1 mL solution of the studied

disazo compounds. The bacterial suspensions were inoculated on sterile Mueller Hinton agar, in each Petri dish with 9 cm diameter. After 24 hours of incubation, the diameters of the inhibition zones were measured (including the 6 mm diameter of the disc). The results were compared with [1- (ethoxycarbonyl) -pentadecyl] -trimethylammonium bromide (Septonex), a commercial antiseptic agent. Inhibitory effect of the compounds was established at zones greater than 12 mm.

## Conclusions

Two new disazo compounds derived from 4,4'-diaminostilbene- 2,2'-disulfonic acid and 4,4'-diaminobenzanilide and 4-chlorosalicylamide, as coupling component were synthesized and their physico-chemical characteristics were determined.

The synthesized azo compounds can be ranged in the direct dye class, due to their colouristic and application properties.

From dermatologic point of view, the new disazo compounds do not present skin adverse effects and so, they can be used as colorants in the textile industry.

The studied azo exhibited good antimicrobial activity against the tested species, especially against *Staphylococcus aureus* and *Escheria coli*.

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