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COMMUNICATION

Red-shifted and pH-Responsive Imidazole-based Azo Dyes with Potent Antimicrobial ActivityDaniela Dantas^a, Ana I. Ribeiro^{a,b}, Filipe Carvalho^a, Eva Gil-Martins^c, Renata Silva^c, Fernando Remião^c, Andrea Zille^b, Fátima Cerqueira^{d,e,f,g}, Eugénia Pinto^{g,h} and Alice M. Dias^{*a}Received 00th January 20xx,
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A novel route is described to obtain 2-aminoimidazole azo dyes with unique substituents pattern in the heteroaryl unit that provides halochromic properties, exhibiting vibrant colours that change from magenta to deep blue. Potent antimicrobial properties against infectious yeasts were demonstrated. No cytotoxicity was detected for concentrations lower than 16 $\mu\text{g}\cdot\text{mL}^{-1}$.

Halochromic molecules have received significant attention for smart materials development due to their capacity to exhibit a visual colour change when exposed to pH variations stimuli. The most explored classes of halochromic dyes are the phthalides, triarylmethanes and fluorans, whereas azobenzenes have been more extensively studied as photochromic dyes¹. Despite the impressive advances in the development of photoswitches applications^{2,3}, azo dyes are still one of the most popular group of pH-indicators¹. Hydroxyazo or aminoazo dyes have been usually used as pH indicators as significant spectral differences can be found between their acid and base forms. For the amino-substituted azobenzenes, when protonation takes place at nitrogen of the azo-group, the resulting azonium ion exhibits a bathochromic shift of the absorption peaks¹. On the contrary, hypsochromic shifts result from protonation of peripheral

nitrogens (ammonium ions). In general, the presence of amino substituents can red-shift the $\pi\pi^*$ band of the parent azobenzene, found in the UV-region (~ 320 nm), to > 400 nm. Recently, azonium ions derived from amino-substituted azo dyes with *ortho*-groups exhibited unusual far-red/near-infrared absorptions^{2,3}. However, the use of this new generation of aminoazo dyes has been hampered by laborious and demanding syntheses, as diazotation is often unsuitable due to the steric congestion around the azo bond.

Incorporating heterocyclic moieties in azo dyes affords functional advantages and unusual spectral properties over conventional azobenzenes, such as enhanced colouring properties, tinctorial strength, thermal stability, and more positive solvatochromic behavior⁴. They also offer broader structural diversity, basic sites and H-bonding interactions⁵. An additional interest in incorporating heterocyclic moieties into the azo dye scaffold is due to the improved bioactivity of the conjugated derivatives^{4,6,7}. Among the different heterocycles, imidazole nucleus has become one of the most important synthons in medicinal chemistry due to its broad range of chemical and biological properties⁸. Its particular relevance in the design of new imidazole-based antifungal agents^{9,10} is another important area of research since the development of antimicrobial resistance, the emergence of new pathogens and the spread of the existing ones represent a global crisis^{11,12}. The conventional synthesis procedure of imidazole azo dyes mainly involves coupling of a diazonium salt, obtained from diazotization of the aromatic or heterocyclic amine, with the more electron-rich nucleophile segment^{13,14}. Like classical azobenzenes, azoimidazoles show absorptions at 336 – 369 nm ($\pi\pi^*$), and 450 – 460 nm ($n\pi^*$)⁵. Thus, as they mostly exhibit colours in the yellow-red range, more red-shifted imidazole-based azo dyes are still needed. Additionally, to the best of the authors knowledge, imidazole-containing azo dyes exhibiting halochromic properties are scarce in the literature.

2-Aminoimidazole skeleton is a unique building block for the design of small-molecule drugs as modulators of different

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targets¹⁵. This scaffold represents an important bioisostere of guanidine, acylguanidine, benzamidine and triazole groups. Classical construction of the 2-aminoimidazole moiety by condensation of α -aminoketones with cyanamides, or α -haloketones with guanidine derivatives, suffer from unstable precursors and narrow application scope¹⁶. More recent approaches involve the reaction between propargylic amines and carbodiimides^{17,18}, but major drawbacks include the use of expensive catalysts, harsh conditions and availability of reagents. Functionalization of imidazole derivatives is another approach¹⁸, but it usually requires multiple steps, protection/deprotection strategies, and activation of C2 for the introduction of the amine. Herein, a totally novel, versatile and affordable method for the synthesis of a new class of azo dyes containing a 2-aminoimidazole moiety is reported. The novel red-shifted azoimidazole dyes presented both halochromic, and potent antifungal properties. Their quite unusual colours change from magenta to blue, which rely on the uncommon substituents pattern in the imidazole scaffold.

Amidrazones have been widely described given their vast applications in chemistry, particularly in the synthesis of heterocycles and azo precursors¹⁸⁻²¹. It was previously reported that these compounds were readily oxidized when exposed to the air²², but similar studies are quite rare in the literature. In a previous work of our research group, imidazole-based amidrazones **1** were synthesized and exhibited potent antimicrobial properties²³⁻²⁵. It was also observed that amidrazones **1** quickly developed intense colours in contact with the air, which prompt the interest in studying its reactivity upon other oxidants. It was found that the oxidation of **1** with silver nitrate (AgNO_3) was immediate and afforded dark red solids, identified as nitrate salts of azoimidazoles **2** (**2.HNO₃**) (Fig. 1). Analysis of these solids by atomic absorption spectroscopy enabled to quantify the Ag^0 present (41.0%), which led to estimate the yields of compounds **2a.HNO₃** and **2b.HNO₃** as 63 and 80 %, respectively. All the solid Ag^0 was removed by centrifugation, or filtration, and reused in other applications. The structures of azoimidazoles **2.HNO₃** were assigned by the proton (^1H), carbon-13 (^{13}C) and two dimensional (2D) nuclear magnetic resonance (NMR) data. The proton signal of the imidazole 2-H appears at δ 7.9 – 8.0 ppm, showing a +1 ppm shift relative to the corresponding H-2 signal of amidrazone **1**. The 5-amino signal at δ 7.8 – 7.9 ppm, also shows a +2 ppm shift when compared with the 5-amino group of **1**. The phenyl H_o -signals also appeared highly deshielded (+0.8 ppm) due to the conjugation with the azo group. Finally, the presence of two sharp signals at δ 9.2 and 8.8 ppm identified the $\text{C}=\text{NH}_2^+$ group. The structures of **2.HNO₃** salts are also supported by ^{13}C and 2D NMR spectra (distortionless enhancement by polarization transfer (DEPT), heteronuclear multiple quantum correlation (HMQC) and heteronuclear multiple bond correlation (HMBC)) shown in SI. The neutralization of **2b.HNO₃** was then performed by adding dimethylamine (DMA), Et_3N (triethylamine), or NaOH. As expected, Et_3N or NaOH afforded the neutral forms of the azoimidazoles **2** with excellent yields (96%). Surprisingly, the colour of the **2b.HNO₃** solution immediately changed from red to a deep magenta in the presence of DMA to give a product identified as the 2-aminoimidazoles **3b-DMA**. Afterwards, the

reaction was successfully reproduced with piperidine (Pip), affording **3b-Pip**. After optimizing the reaction conditions, differently substituted compounds **3** were obtained in 55-74% yields (Fig. 1). The use of neutral azoimidazoles **2** as alternative reagents was also successfully tested, but they afforded lower yields (33–57%) of compounds **3**. The 2-aminoimidazoles **3** were characterized by ^1H , ^{13}C and 2D NMR, infra-red (IR) and mass (MS) spectroscopy. The ^1H NMR spectrum of **3b-DMA** showed absence of the 2-H imidazole proton. Additionally, the methyl signal that typically appears at δ 3.4-3.5 ppm in imidazoles **1** and **2** was significantly shifted to δ 3.3 ppm. Moreover, in the HMBC spectra (SI), this methyl group showed two three-bonds correlations with carbon signals found at δ 162.06 and 163.36 ppm, which were assigned to the imidazole C-2 and C-5 carbon atoms. The correlations of the 2-dimethylamino protons confirmed the C-2 attribution. In addition, the azobenzene unit was well demonstrated by phenyl signals at δ 7.6 ppm, δ 7.5 and δ 7.3 ppm. The broad singlets at δ 8.8-8.9 and δ 5.3-5.4 ppm, assigned to the iminic NH and the 5-amino groups, also supported the structures **3**.

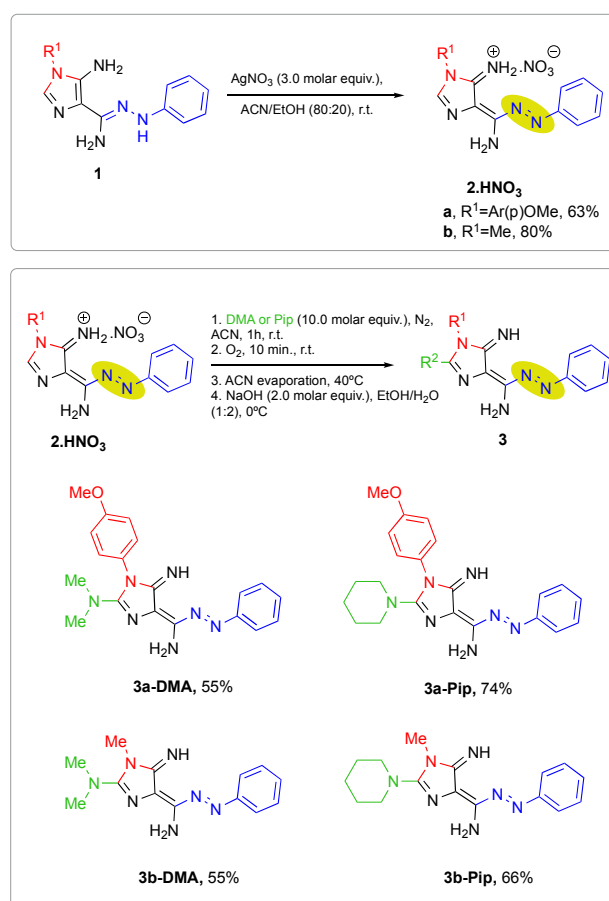


Fig. 1. Synthesis of azoimidazoles **2** and **3**.

A crystal obtained from compound **3b-Pip** was characterized by X-ray analysis, and crystallography data are reported in SI (SI-Table 6 – 11). The molecular structure (Fig. 2) reveals a planar conjugated system extending from the 2-aminoimidazole nucleus to the *trans* azobenzene unit, showing an intramolecular H-bond between 5-NH proton and N7 nitrogen

that compares with reference azo dyes²⁶. The internal angles of the imidazole ring show minor differences, compared with reference azoimidazoles²⁷. However, C4-C5 bond length is 1.490 Å (0.13 Å longer), and the lengths found for C5-N13 and C4-C6 bonds are 1.270 and 1.377 Å, supporting the two exocyclic double bonds at C-4 and C-5 positions²⁸. In addition, the C6-N2 bond length (1.380 Å) agrees with the sp³ character of the 6-NH₂ group, while the C5-N12 bond length (1.270 Å) confirmed the sp² geometry of the 5-imino nitrogen.

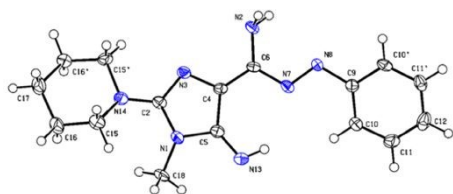


Fig. 2. Crystal structure of compound **3b-Pip** (deposited in Cambridge Structural Database with number CCDC2237834).

The proposed mechanism for the reaction of compounds **2** with DMA and Pip (Fig. 3) involves a first step of neutralization, followed by nucleophilic attack of secondary amine to the imidazole C-2 atom, giving the amidrazone **4**. This intermediate should oxidize easily in the presence of atmospheric O₂ to generate the corresponding azo derivative **3**.

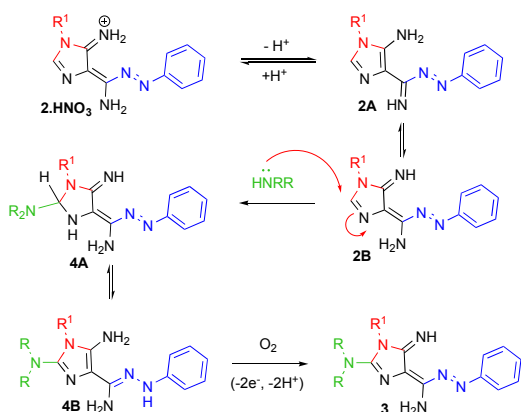


Fig. 3. Proposed mechanism for synthesis of azoimidazoles **3**.

The dark-purple solids **3** were assessed by UV-vis spectroscopy. Two medium bands were detected in the UV (286 and 333 nm), and a strong band was observed in the visible region around 570 nm (SI-Table 3). This band is red-shifted from classical azobenzenes or azoimidazoles by ~100 or ~300 nm (comparing with nπ* or ππ* transitions, respectively). It also exhibited large molar extinction coefficients (ε) (27x10³ M⁻¹cm⁻¹). The solvatochromism was evaluated in different solvents, but the absorption spectra showed very similar λ_{max}. However, hypochromic effects were detected in THF and EtOH, while hyperchromic shifts were observed in DCM and ACN. Then, the absorption spectra were obtained upon titration with HCl and NaOH. A significant colour change from magenta (alkaline media) to blue (acidic media) occurred (Fig. 4). Titration with HCl caused substantial bathochromic and hyperchromic shifts

of the visible bands (~30 nm for compounds **3a**, and ~20 nm for **3b**), while titration with NaOH presented hypsochromic shifts. The pK_a constants were determined and the 6.25 - 7.22 values obtained, which fit in the range of physiological pH variations.

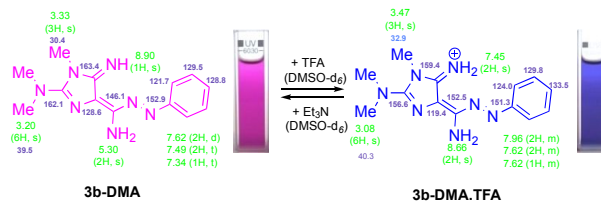


Fig. 4. Colour changing from the addition of TFA (blue) or Et₃N (purple) to **3b-DMA** and corresponding NMR data.

The effect of adding trifluoroacetic acid (TFA) to **3b-DMA** was evaluated by ¹H NMR spectroscopy in deuterated dimethyl sulfoxide (DMSO-d₆) (Fig. 4). Although relevant downfield-shifts occurred for the phenyl and methyl protons in the protonated form, the most significant changes occurred at the NH protons. Amino and imino peaks of the neutral form, observed at δ 5.30 ppm (2H) and 8.90 ppm (1H), were replaced by two broad bands at δ 8.66 ppm (2H) and 7.45 ppm (2H). In the ¹³C spectra, the C-6 signal suffered a great downfield shift (+6 ppm), whereas imidazole and dialkylamino carbon signals shifted to higher fields. Thus, protonation should occur on the 5-imino nitrogen, as the aromaticity of the imidazole ring increases. However, the splitting of the phenyl signals in the ¹H NMR spectra suggested that ammonium-azonium tautomerism in solution might contribute to the colorimetric properties. Moreover, the ammonium ion might increase the push-pull effects and turn the absorption towards longer wavelengths. Interestingly, the unique 6-amino and 5-imino substituents compare with the ortho-amino and methoxy groups of the known red-shifted azobenzenes^{2,3} in relative position and H-bonding pattern.

The antifungal and antibacterial activity of the new compounds was evaluated (Table 1 and SI-Table 3). For *Candida* spp., all compounds showed a higher antifungal activity against *Candida krusei*, with equal minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) values, indicating a yeasticidal activity. These are interesting results since *C. krusei* is intrinsically resistant to fluconazole, but is also able to develop acquired resistance to other azoles²⁹. Additionally, the activity in *C. albicans* is the same for clinical isolates that are susceptible or resistant to azoles. Besides that, derivatives **3** were even more potent against *Cryptococcus neoformans* (MIC and MLC of 2 μg·mL⁻¹), with the exception of the compound **3a-DMA**. *C. neoformans* is responsible for a high mortality rate among infected individuals and drug resistance is an actual problem³⁰. Although filamentous fungi as *Aspergillus* spp. were not very sensitive to the compounds, their activity against the dermatophytes tested (particularly *Trichophyton rubrum* and *T. mentagrophytes*, frequently involved in superficial mycoses³¹) was moderate, with the MIC from 64 to 16 μg·mL⁻¹. The new compounds were also active against emerging infectious agents, such as *Scedosporium* spp.³² or *Fusarium solani* exhibiting a broad spectrum of antifungal resistance³³. Concerning the antibacterial activity, compounds inhibited the

growth of *Staphylococcus aureus* but had a neglectable effect on *Escherichia coli*.

Table 1. Antimicrobial activity of compounds **2** and **3**.^a

	MIC (MLC) $\mu\text{g}\cdot\text{mL}^{-1}$					
	2a	2b	3a-DMA	3a-Pip	3b-DMA	3b-Pip
<i>C. albicans</i> ATCC 10231	64 (256)	64 (128)	32 (64)	16 (32)	32 (64)	16 (32)
<i>C. albicans</i> DSY294 (S)	32 (256)	32 (128)	32 (64)	16 (32)	16 (32)	16 (32)
<i>C. albicans</i> DSY296 (R)	32 (256)	16 (128)	16 (64)	16 (32)	16 (32)	16 (32)
<i>C. glabrata</i> DSY562 (S)	64 (256)	64 (128)	64 (128)	32 (64)	32 (64)	16 (32)
<i>C. glabrata</i> DSY565 (R)	64 (256)	64 (128)	64 (128)	32 (64)	32 (64)	16 (32)
<i>C. krusei</i> ATCC 6258	4 (4)	4 (4)	4 (4)	4 (4)	4 (4)	4 (4)
<i>C. neoformans</i> CECT1078	4 (4)	2 (4)	2 (4)	2 (2)	2 (2)	2 (2)
<i>T. rubrum</i> FF5	64 (128)	64 (128)	64 (64)	32 (32)	16 (32)	16 (32)
<i>T. mentagrophytes</i> FF7	32 (64)	64 (64)	32 (32)	32 (32)	32 (32)	16 (16)

^a Only part of the results are shown. Complete version is in SI-Table 3.

Compounds cytotoxicity was evaluated in HaCat cells by the (neutral red) NR uptake, resazurin reduction and sulforhodamine B binding assays, 24 h after exposure (SI-Figure 3). A concentration-dependent cytotoxic effect was detected for all the tested compounds, and for all the performed assays. SI-Table 5 illustrates the compounds concentrations above which a significant cytotoxic effect was detected by the NR uptake assay, which demonstrated to be the most sensitive method in evaluating the cytotoxic effects of these compounds. In conclusion, a simple and mild method for the synthesis of novel azoimidazoles was developed. The products were obtained with very good yields after simple purification. The unusual red-shifted absorption bands and significant halochromism near the physiological pH, as well as the promising antifungal activity and the lack of relevant cytotoxicity against human keratinocytes, allow these accessible dyes to pave the way for future development of smart materials in the areas of chemical biology tools, and (bio)medical and textile applications.

Conflicts of interest

There are no conflicts to declare.

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