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Synthesis and photophysical properties of novel succinimidyl benzazole derivatives, evaluated by *Candida albicans* ATCC 10231 fluorescent staining

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ABSTRACT

New fluorescent succinimidyl benzazole derivatives were synthesised and successfully used to stain *Candida albicans* ATCC 10231 cells. The dyes were characterised by means of infrared, ^{13}C and ^{1}H NMR spectroscopies and elemental analysis. UV–Vis and steady-state fluorescence in solution were also applied to characterise their photophysical behaviour. The novel dyes were fluorescent in the yellow–green region by a phototautomerism in the excited state (ESIPT) with a large Stokes shift (9065–10962 cm^{-1}). Dual fluorescence could also be observed depending on the solvent polarity. The present dyes were used as new probes by means of culture methodology or direct staining to study the micro-morphology of *Candida albicans*.

Fluorescent probes with improved properties such as higher light, air and temperature stability during storage, and which can be used as a fast, economic and efficient method to stain fungal cells always present great interest.[1–8] Particular attention has been given to benzazoles as fluorescent probes,[9–12] since these compounds show a large Stokes shift through an excited state intramolecular proton transfer (ESIPT) mechanism.[13,14] In the ESIPT mechanism (Fig. 1), UV light absorption by the enol (E) produces the excited enol (E*), which is quickly converted to an excited keto tautomer (K*) by intramolecular proton transfer since the hydrogen becomes more acidic and the nitrogen becomes more basic in the excited state. The excited keto tautomer (K*) decays, thereby emitting fluorescence, to a keto tautomer in the ground state (K). Since the enol conformer (E) is more stable than the keto tautomer in the ground state, the initial enol form is regenerated without any photochemical change.[14]

Fungemia is a frequent disease in humans, especially among neonates, the elderly and HIV patients.[15] *Candida albicans* is an opportunistic fungal pathogen, and is the most common fungal infection in hospitalised patients.[16,17] *C. albicans* belongs to the normal human microflora and can drive systemic life-threatening disease in cancer, immunosuppressed, post-surgical and trauma patients.[18] Several fluorescent dyes have been used to indicate

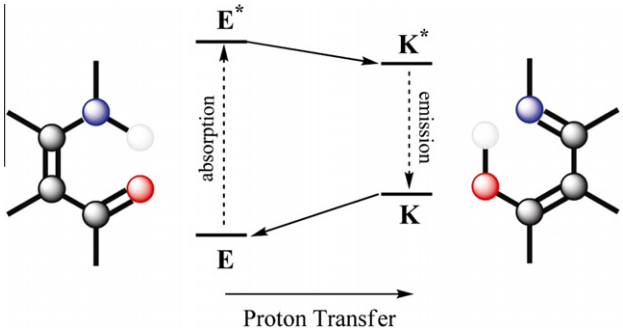

Figure 1. ESIPT mechanism.

yeast physiological states, which are used in novel methods for in vitro antifungal susceptibility tests.[19–24] Morphological changes in *C. albicans* can be followed by direct fluorescence staining with suitable dyes to visualise the different features of cellular activities.[25] However, pathogenic fungi, like *C. albicans*, require accurate morphophysiological analysis for diagnostic and therapeutic purposes. The study of *C. albicans* morphogenesis is very significant since its ability to switch between its budding and filamentous forms is crucial for its pathogenicity.[26,27] In this way, the development of new probes which are able to show morphological changes, viability and metabolic activities is of extreme relevance.

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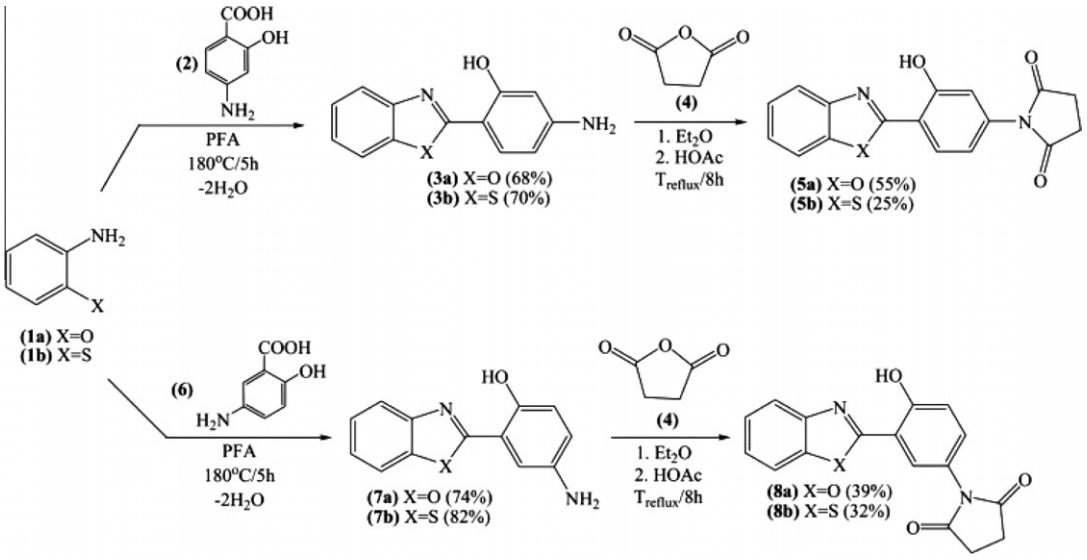

Figure 2. Synthesis of the succinimidyl benzazole derivatives **5a**–**b** and **8a**–**b**.

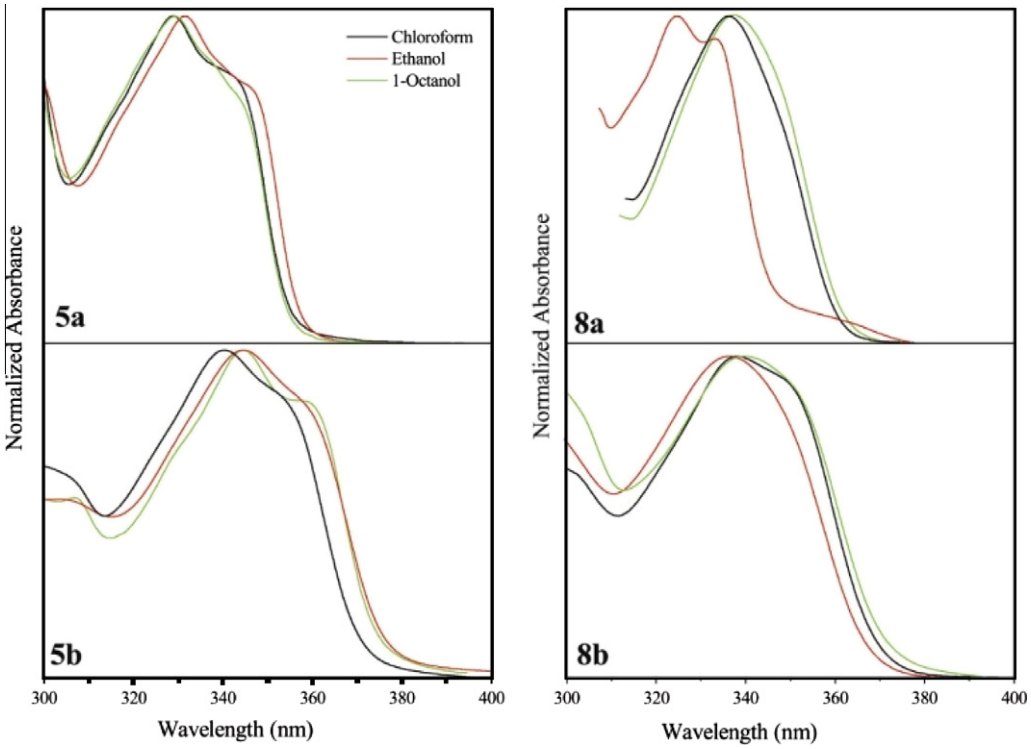

Figure 3. Normalised UV–Vis absorption spectra of the succinimidyl benzazole derivatives **5a**–**b** and **8a**–**b**.

Succinimidyl derivatives[28,29] have been used to label proteins in biological processes,[30] fluorescence microscopy[31] and apoptosis studies in disease.[32–34] Although the benzazole dyes have been used as fluorescent probes and in staining protocols, the major difficulty usually involves dye solubility with lower antifungal properties, which provide more safety as probes in fungal cell cultures.[10–12] In this way, we present in this Letter the synthesis and photophysical characterisation of novel succinimidyl benzazole dyes, which could increase dye solubility in the aqueous phase regarding non-substituted precursors, and the staining of *C. albicans* cell structures using simple protocols.

The benzazole precursors were prepared using a previously described methodology.[14] The succinimidyl derivatives were prepared according to the scheme in Figure 2. To a solution of the corresponding benzazole dye in diethyl ether (20 ml) was added an equimolar amount of the succinic anhydride (2) which was at room temperature for 2 h. The obtained yellow precipitate was filtered, dried and used without further purification. In a second reaction step, this precipitate was heated in glacial acetic acid (25 mL) at reflux for 8 h. The mixture was poured into ice and filtered. The crude product was washed with water and cold diethyl ether and dried at 60 °C. Purification was performed by column

Table 1
Relevant fluorescence emission data of the succinimidyl benzazole derivatives

Dye	Solvent	UV–Vis absorption		Fluorescence emission					
				Enol			Keto		
		λ_{max} (nm)	ε_{max} ($\times 10^4$ l mol^{-1} cm^{-1})	λ_{max} (nm)	$\Delta\lambda_{ST}$ (nm/cm^{-1})	φ_{fl}	λ_{max} (nm)	$\Delta\lambda_{ST}$ (nm/cm^{-1})	φ_{fl}
5a	Chloroform	329	2.86	374	45/3657	0.015	474	145/9298	0.174
	Ethanol	332	2.37	370	38/3093	0.011	473	141/8979	0.040
	1-Octanol	329	2.46	—	—	—	472	143/9209	0.102
5b	Chloroform	345	2.95	—	—	—	502	157/9065	0.103
	Ethanol	345	2.80	389	44/3279	0.026	—	—	—
	1-Octanol	340	3.16	387	47/3572	0.029	501	161/9452	0.022
8a	Chloroform	341	1.17	—	—	—	508	167/9640	0.156
	Ethanol	343	0.57	424	81/5570	0.076	—	—	—
	1-Octanol	324	1.31	409	85/6414	0.006	490	166/10456	0.025
8b	Chloroform	339	1.92	—	—	—	515	176/10081	0.061
	Ethanol	336	0.86	357	21/1751	0.044	—	—	—
	1-Octanol	340	1.95	396	56/4159	0.006	542	202/10962	0.059

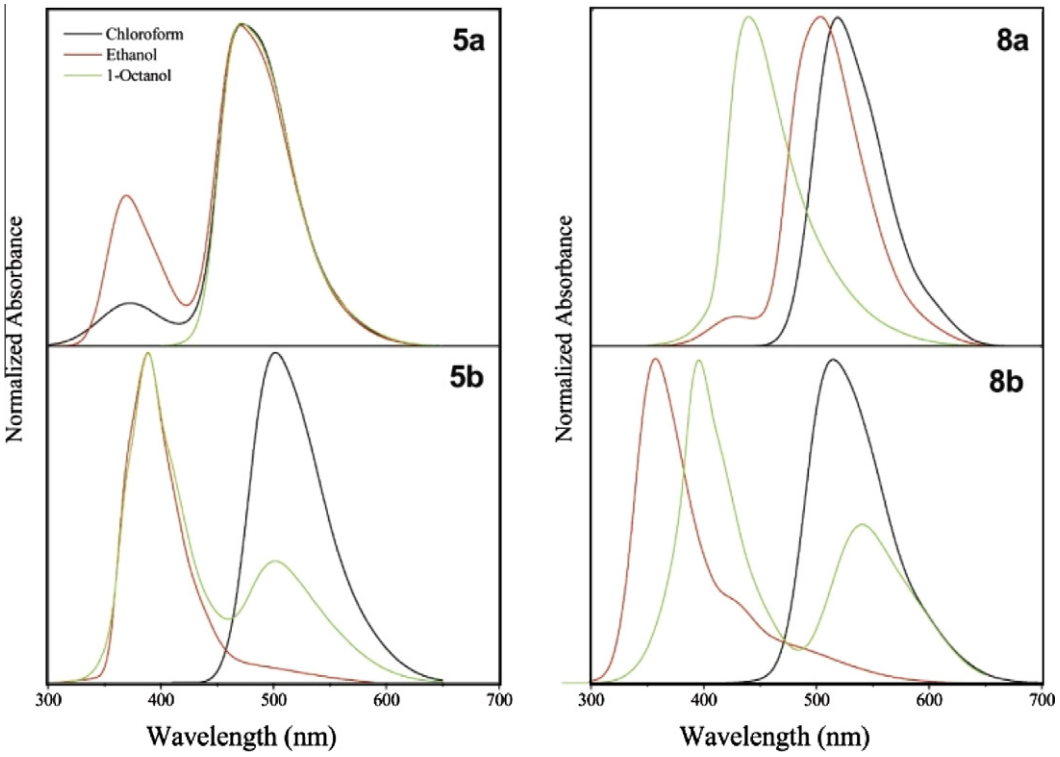

Figure 4. Normalised fluorescence emission spectra of the succinimidyl benzazole derivatives **5a**–**b** and **8a**–**b**.

chromatography using chloroform as the eluent or by recrystallisation using ethanol.

The UV–Vis absorption emission spectra were made in chloroform, ethanol and 1-octanol and were normalised (Fig. 3). 1-Octanol was used in this protocol to simulate an apolar organic environment, which could lead to selective staining of the hydrophobic portion of the cell wall. All the experiments were performed at room temperature (25 °C) at a concentration of 10^{-6} M. The relevant data from the UV–Vis and fluorescence emission are summarised in Table 1 (see also Supplementary Tables 1 and 2).

As it can be seen in Figure 3, the dyes present an absorption maximum located at 329–348 nm with molar absorptivity coefficient ε values (10^4 l mol^{-1} cm^{-1}) in agreement with π–π^* transitions. The observed difference in these values can probably be associated with the difference in planarity of the dyes.[14]

Figure 4 presents the fluorescence emission spectra of the succinimidyl derivatives. As expected, a dual fluorescence emission could be observed for all dyes in different solvents, since the ESIPT mechanism is quite dependent on solvent polarity. Dye **5a** presents in chloroform and ethanol a main emission band located at 470 nm and an additional one, blue shifted, located at 368 nm. One main emission band located at 470 nm was observed in 1-octanol. Usually, the dual fluorescence emission presents a band at higher wavelengths attributed to an excited keto tautomer (*K*), which arises from the enol conformer (E_I) in the excited state and a blue shifted one due to the conformational forms which are stabilised in solution and present normal relaxation (E_{II-IV}) (Fig. 5).[14,35]

Dye **5b** presents one main band in chloroform and ethanol located at 502 and 389 nm, ascribed to the ESIPT and the normal band, respectively. On the other hand, a dual fluorescence emission

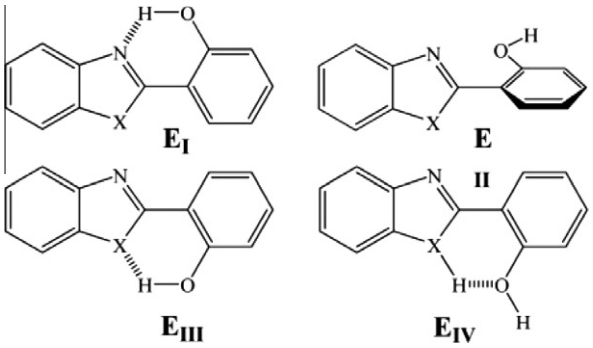

Figure 5. Enol conformer stabilised in solution in the ground state.

could be observed in 1-octanol, with the main band at around 387 nm (normal band) and a red shifted one located at 501 nm (ESIPT band). It is worth mentioning that the intensities of the emission bands are changed in relation to those observed with the dye **5a** in chloroform and ethanol.

Derivatives **8a–b** present in chloroform one main band located at 508 nm (**8a**) and on at 515 nm (**8b**) ascribed to the ESIPT band. Ethanol seems to stabilise the conformers which decay by normal relaxation, since one main band, blue shifted in relation to the ESIPT band, could be observed with the dyes **8a–b** located at 424 and 350 nm, respectively. A dual fluorescence emission could be observed using 1-octanol. The ESIPT bands are located at around 490–542 nm, while the blue shifted ones are located at 369–409 nm. The observed results indicate that changes in the heteroatom, as well as in the solvent polarity, play a fundamental role in the photophysical behaviour of the succinimidyl benzazole derivatives.

Vegetative cells of *C. albicans* ATCC 10231 were obtained by culture on YEPD agar medium (1% yeast extract, 1% peptone, 4% D-glucose, 2% agar) with incubation at 37 °C for 48 h. The inoculum was prepared for the experiment from 24 h cultures grown at the same temperature. The *C. albicans* culture was activated by means of three repetitive culture steps. A preliminary assay was performed to observe cellular viability (24 h-old cultures) (Fig. 6F). Two different protocols were used to analyse the cells by epifluorescence microscopy.[36]

Both staining protocols presented similar behaviour. A small difference could be observed in the fluorescence intensity distribution over the cellular structures. Figure 6 presents the images obtained from Protocol A, with a better image resolution and suitable fluorescence intensity. The results probably indicate a higher specificity of the dyes for intracellular components from the cellular wall or other membrane structures. The *C. albicans* ovoid cells present fluorescence emission in the green or blue region after staining with the dyes presented in this work (Figs. 6 and 7). In a preliminary test to study cellular viability, intense fluorescence could be observed in some cells (Fig. 6F), which possibly indicates a selective characterisation of different cell metabolic states.

Protocol B was more sensitive to dye concentration (Fig. 7A and B). Higher dye content in relation to the one used in this protocol led to irregular impregnation, with dye deposition on the fungal structures and glass slide surfaces. A dye content of 65 μM produced better image resolution, where some topographic details

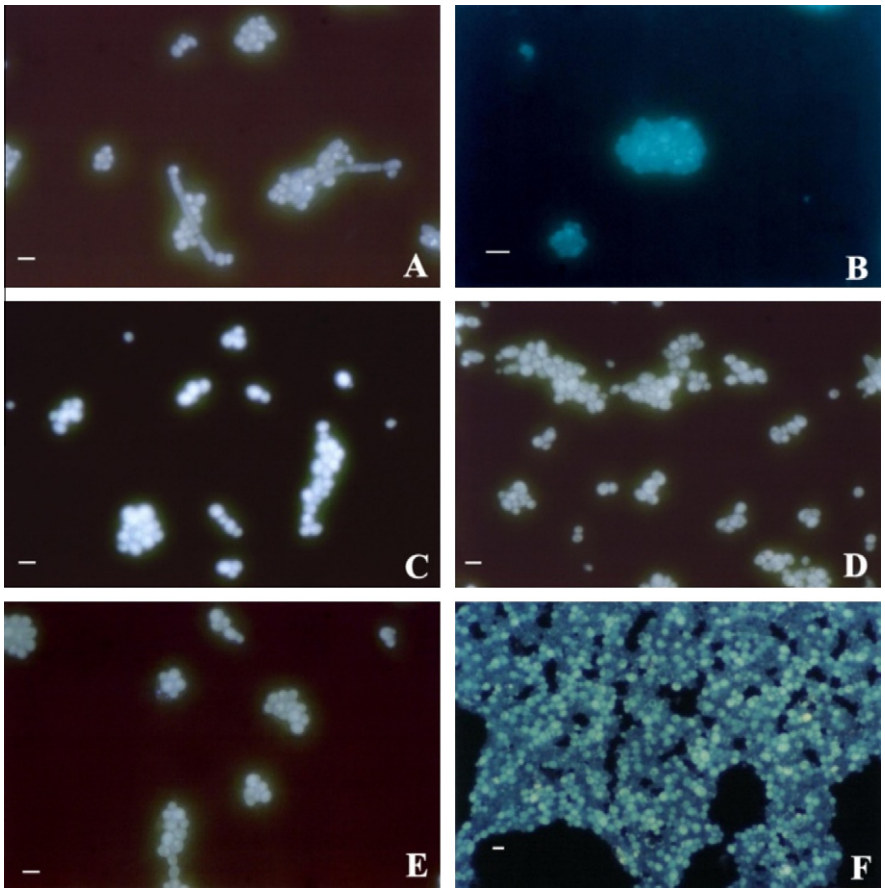

Figure 6. *Candida albicans* morphology examined by epifluorescence microscopy after staining with the dyes **3a** (A), **7a** (B), **5a** (C), **5b** (D), **8b** (E) and **8b** (F) (preliminary assay to observe cellular viability) using Protocol A and a V-2A filter (Bar = 10 μm).

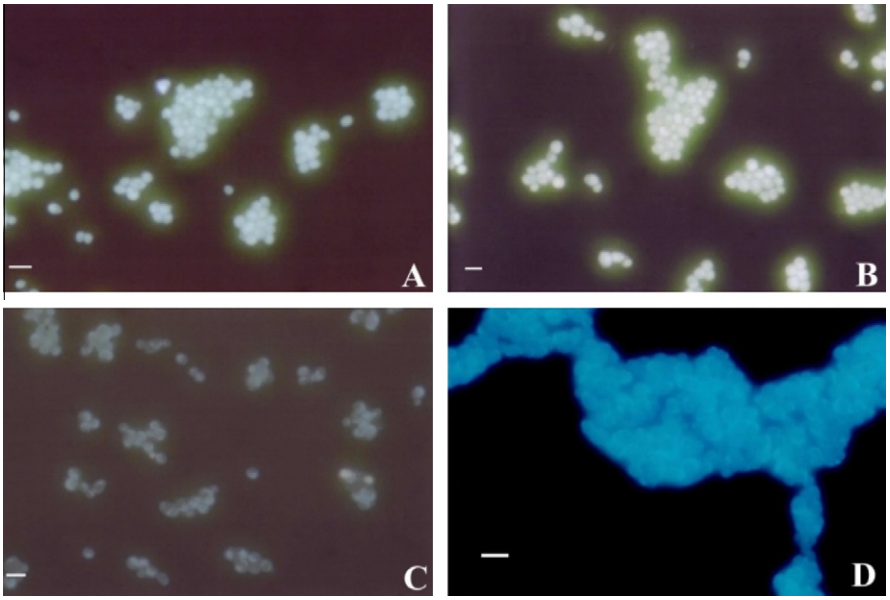

Figure 7. *Candida albicans* morphology examined by epifluorescence microscopy after staining (Protocol B, dye concentration 65 μM) with dyes **5a** (A) and **8b** (B) (V-2A filter). The unstained sample (C) and one stained with Calcofluor white (D) prepared with the same methodology used for the dyes **5a** and **8b** (V-2A filter) are also presented for comparison (Bar = 10 μm)

of the cell surface could be observed. In (Fig. 7C, an image from the unstained slide is also shown which presents weak blue intrinsic fluorescence. Calcofluor white was used for comparison (Protocol B) under similar experimental conditions, however at a different dye concentration, and presented worse resolution and excess brightness. Since the experimental conditions used in the staining protocols were inadequate for succinimidyl ring opening, the benzazole derivatives were used in their 'non-active' forms (closed ring), which forms non-covalent bonds between the dyes and the *C. albicans* cell structures.

All the dye solutions used for staining *C. albicans* cells presented photophysical stability when tested after six months of storage at room temperature. The succinimidyl derivatives **5a–b** and **8a–b** presented similar staining properties when compared to their precursors (Fig. 6A–B).The slides with the stained cells also kept their fluorescence after four months of storage at room temperature. This photophysical behaviour can be very useful for teaching methodology in mycology studies. Additional studies are in progress to use the succinimidyl benzazole derivatives in antifungal activity assays to observe morphological changes associated with antifungal effects, such as loss of cell viability and cell growth.

In conclusion, four new fluorescent succinimidyl benzazole derivatives were synthesised and used to stain *C. albicans* ATCC 10231 cells. The new probes are fluorescent in the yellow-green region by an intramolecular proton transfer mechanism (ESIPT) with a large Stokes shift (9065–10962 cm^{-1}). The dyes were successfully used as new dyes by means of a culture method or by direct staining to study the micromorphology of *C. albicans*.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2011.04.026.

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36. *Protocol A (the culture protocol)*: An initial solution of the dyes in dimethylsulphoxide (40 mM) was diluted in culture media (Sabouraud dextrose agar, 1:100 v/v) at 60 °C. After its gelification, the *C. albicans* (24 h culture) was inoculated and kept at 35 °C for 24 h. In the following stage, one drop of distilled water was placed on the centre of a clean glass slide. The stained treated cells were taken and spread over the drop of water and dried at room temperature (25 °C). Protocol B (the direct staining protocol): An initial solution of the dye was prepared in 1-octanol/ethanol (1:9 v/v) in the following concentrations: 5.0 and 2.5 mM and 65 μM. The *C. albicans* cells were directly squashed with the dye solutions and dried at room temperature (25 °C). It is worth mentioning that the selection of 1-octanol in this protocol was to simulate an apolar organic environment, which could lead to selective staining of the hydrophobic portion of the cell wall. The excess dye was removed by washing with ethanol. In this step, an unstained slide was used for comparison. A slide stained with Calcofluor white (1 mM) was also used as a standard to detect chitin-rich areas of the cell wall.