Evaluation of newly synthesised benzo[*a*]phenoxazinium chlorides as fluorescent probes using *Saccharomyces cerevisiae* as model organism

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Abstract: The potential as fluorescent probes of two recently synthesised water-soluble benzo[*a*]phenoxazinium salts, mono- or dissubstituted with carboxylic ester groups as terminal of propyl substituents at the amine of position 9, were studied by fluorescence microscopy employing intact, live cells. With the aim of assessing the compounds cellular staining pattern and potential specificity, co-localization experiments were performed using yeast mutants expressing green fluorescent protein (GFP)-tagged proteins that localize to specific intracellular location/organelles. It was found that both compounds display a very high affinity for the vacuolar membrane, being the dissubstituted compound especially important due to its higher fluorescence at lower, less toxic concentrations.

Keywords: NIR fluorescent probes; Nile Blue; benzo[*a*]phenoxazine derivatives; fluorescence microscopy.

1. Introduction

The use of fluorescent probes is a well-established practice in many scientific and technologic areas with several decades, being these molecules important in the field of science and medicine. Nevertheless, developing new fluorophores is fundamental to overcome the limitations associated with the commercially available fluorescent probes.[1,2]

In this context, benzo[*a*]phenoxazine derivatives are preferred as labels due to their photostable nature,

high molar absorption coefficient and fluorescence in the near-infrared spectral region (NIR) compared to other dyes. Considering the synthetic and analytic importance of these heteroaromatic structures several groups have reported different types of compounds with a variety of combinations.[3]

Nile Red, the 9-diethylamino-5*H*-benzo[*a*]phenoxazine-5-one, is a dye with a neutral and oxidized system that allows an electronic delocalization between the amine and the carbonyl group. The fluorescence intensity of this fluorophore and its emission wavelength is highly indicative of the solvent polarity.[4] Due to this solvatochromic behaviour, Nile Red is a dye especially important in the study of membranes and lipid droplets in apolar environments. Several uses of this compound in bio-imaging have been reported, including the measurement of lipids in microalgae with applications in biodiesel production,[5] and the evaluation of cytoplasmic lipid droplets in tumor cells.[6] Recently, Prifti *et al.* reported a novel Nile Red derivative that specifically reacts with SNAP (*S*-nitroso-*N*-acetylpenicillamine), which is attached to a plasma transmembrane target receptor. This compound when free undergoes self-quenching and therefore it is non-fluorescent. Upon reaction a covalent bond is formed between the SNAP-tag biomolecule and the dye, which in turn inserts into the membrane, a lipophilic apolar environment that result in a fluorescent probe. As a result, this fluorogenic compound allows identifying numerous SNAP-tagged proteins.[7]

Nile Blue, *N*-(5-amino-9*H*-benzo[*a*]phenoxazine-9-ylidene)-*N*-ethyletanaminium, first synthesized by Mohlau and Uhlmann in 1896,[3] possesses a positively charged benzophenoxazine system, being more hydrophilic than the Nile Red dye. Benzo[*a*]phenoxazinium salts have been reported as important biological probes, being used in covalent staining of carboxylic acids,[8] amino acids,[9] proteins,[10] peptides, DNA,[11] microbial polyesters,[12] and polymeric nanocapsules as potential drug delivery systems.[13] Nevertheless, its most common application is the non-covalent staining, mainly through hydrophobic interactions, with uses in nucleic acid staining in blotting experiments and assays employing intact live cells.[14]

Taking this into account, we report two newly synthesised benzo[*a*]phenoxazinium chlorides possessing ethyl ester groups as terminal substituents at 9-position of the polycyclic system.[15] Based on the photophysical behaviour, it was decided to evaluate its capability as a fluorescent probe using *Saccharomyces cerevisiae* as a model organism. Fluorescence microscopy was used to determine the intracellular distribution of benzo[*a*]phenoxazine derivatives within the organelles of *S. cerevisiae*. *Saccharomyces cerevisiae* W303-1A was used as wild-type and one mutant strain from this yeast was used to localize the vacuolar membrane (*S. cerevisiae* W303-1A-pDF01-VBA1-YEGFP). A mutant

yeast expressing chromosomally-tagged GFP fusion protein (*S. cerevisiae* BY4741-SEC66-GFP) derived from the *S. cerevisiae* BY4741 (ATCC 201388) was used to localize the endoplasmic reticulum membrane.[16]

2. Experimental

2.1. Fluorescence microscopy studies

In advance of each experiment the required strain was grown in the appropriate agar plates thus allowing the use of a young culture every time. From this culture, biomass was taken to prepare a cell suspension that was then incubated overnight at 30 °C and 120 rpm (incubator Certomat H), in liquid medium until the culture reaches an optical density at 640 nm ($O.D._{640nm}$) of approximated 0.5-0.6. An aliquot of this culture was incubated with the respective dye at room temperature for the period indicated below in each case, then centrifuged at 3000 rpm for 5 minute. The pellet was resuspended in 200 µL of phosphate-buffered saline solution (PBS) and centrifuged again, at 3000 rpm for 3 minutes. The pellet obtained in this second centrifugation was resuspended in 20 µL of PBS and the sample was observed by fluorescence microscopy. A 6 µL aliquot of the sample to be observed was put in a microscope slide and a cover glass was placed applying a slight pressure.

Fluorescence imaging of the preparation was performed with Leica DM5000 microscope, in all objective lenses. Live-cell experiments were processed using the LAS AF software (Leica microsystems, Germany), and the images presented here were obtained with the Leica DFC350 FX Digital Camera. Recordings were performed using a 100×1.3 NA oil-immersion objective and under red, green, blue and DIC (*Differential Interference Contrast*) filters. An eyepiece magnification of 10×, the magnification of the objective (100×) and the application of a numerical aperture of 1.25 resulted in a 1250× overall magnification for all presented images.

For each assay a control was performed, in which the same conditions used in the experiments with the fluorescent probe were applied to each strain, but instead of the dye only DMSO was employed. These allowed us to observe the cellular morphology before treating with our fluorescent probes. The DMSO concentration used varied between 0.06% and 0.25%. All treatment conditions were performed in two independent experiments and the images presented are representative of the results obtained.

3. Results and discussion

4-Ethoxy-*N*-(5-((3-hydroxypropyl)amino)-9*H*-benzo[*a*]phenoxazin-9-ylidene)-4-oxobutan-1-aminium chloride **1** and 4-ethoxy-*N*-(4-ethoxy-4-oxobutyl)-4-oxo-*N*-(5-(propylamino)-9*H*-benzo[*a*]phenoxazin-9-ylidene)butan-1-aminium chloride **2** were synthesised by the reaction of ethyl 4-((3-hydroxy-4-nitrosophenyl)amino)butanoate hydrochloride or diethyl 4,4'-((3-hydroxy-4-nitrosophenyl)azanediyl)dibutanoate hydrochloride with 3-(naphthalen-1-ylamino)propan-1-ol or *N*-propylnaphthalen-1-amine in acid media, in ethanol under reflux conditions (Figure 1). The required nitrosophenol hydrochloride was obtained by the nitrosation of ethyl 4-((3-hydroxyphenyl)amino)butanoate or diethyl 4,4'-((3-hydroxyphenyl)azanediyl)dibutanoate with sodium nitrite in the presence of hydrochloric acid, in a mixture of ethanol-water as the solvent. The required *N*-alkylnaphthalen-1-amines were prepared by the *N*-alkylation of naphthalen-1-amine with 3-hydroxy-1-bromopropane or 1-bromopropane.[15]

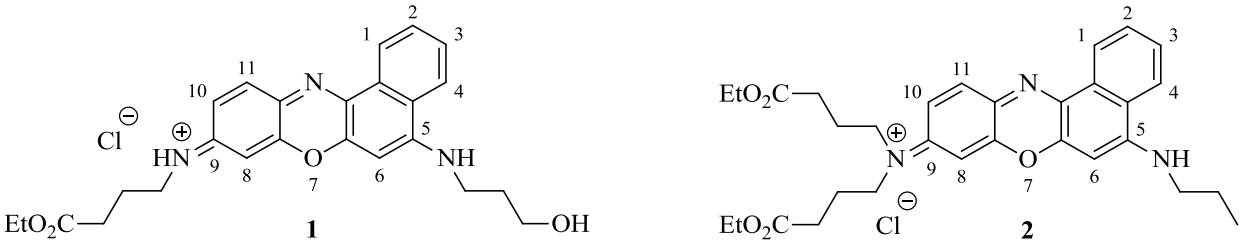

Figure 1. Structures of the functionalised benzo[*a*]phenoxazinium chlorides **1** and **2**.

The two recently synthesised water-soluble benzo[*a*]phenoxazinium chlorides **1** and **2** obtained by our research group, were evaluated as fluorescent probes by fluorescence microscopy using the yeast *Saccharomyces cerevisiae* as model organism. Compounds **1** and **2** display relative fluorescence quantum yields (Φ_F) of 0.38 and 0.31 in dry ethanol acidified with trifluoroacetic acid (TFA), and 0.15 and 0.55 in buffered aqueous solution at pH = 2, respectively. Oxazine 1 was used as a standard (Φ_F = 0.11 in ethanol)[17] at 575 nm excitation.[15]

As presented in Figure 2 for the wild-type strain, W303-1A, dye **1** displays a high affinity to the vacuolar membrane (or an increased intensity when bounded to these membranes). Images obtained for strain W303-1A-pDF01-VBA1-YEGFP show a co-localization between the GFP-tagged protein and the red-fluorescence images, confirming that compound **1** is targeting the vacuolar membrane. The

treatment of this strain with dye **1** results in the formation of aggregates/vesicles derived from the vacuole membrane identified by the white arrow, still tagged with GFP. This effect is observed in 73% of the considered cells (750 cells counted for both phenotypes, i.e. with intact GFP-tagged vacuolar membrane and with all the GFP associated with these aggregates/vesicles). These structures may be vesicles originated from the vacuole membrane, but further studies are required in order to verify our perspective and to understand the reason why these structures are originated.

Furthermore, when *S. cerevisiae* BY4741-SEC66-GFP cells were treated with this dye a significant co-localization of both green- and red-fluorescence was not observed, being only detected in 13% of the cells (470 cells considered in two independent experiments).

ATPases are multi-subunit complexes responsible for ATP synthesis, being found in membranes inside eukaryotic cells coupled to an H^+-pump that generates an electrochemical gradient across the membrane where it is localized (called proton motif force, *pmf*). These H^+/ATPases systems present in vacuolar membranes function by pumping protons into the organelle acidifying its interior.[18] *N*,*N*-dicyclohexylcarbodiimide, DCCD,[19] was used as decoupling agent of the H^+/ATPase system in order to determine the influence of the membrane potential and energy availability in the compound staining pattern and fluorescence intensity (Figures 2 and 3). *S. cerevisiae* W303-1A cells were incubated with 0.4 mM DCCD for 10 minutes at room temperature, then compound **1** was added and the cell suspension was left for incubation for additional 20 minutes. As it can be observed in Figure 2, the vacuolar membranes in cells previously treated with DCCD are stained displaying an intense red fluorescence, showing that the specificity and fluorescence of dye **1** is not affected by pH variations of the target organelle. Also, some red-fluorescent granules adjacent to the vacuolar membrane are distinguished (white arrow), revealing that the vacuolar membrane dynamic or / and its structure is being affected by the treatment with both compound **1** and DCCD or by the DCCD itself.

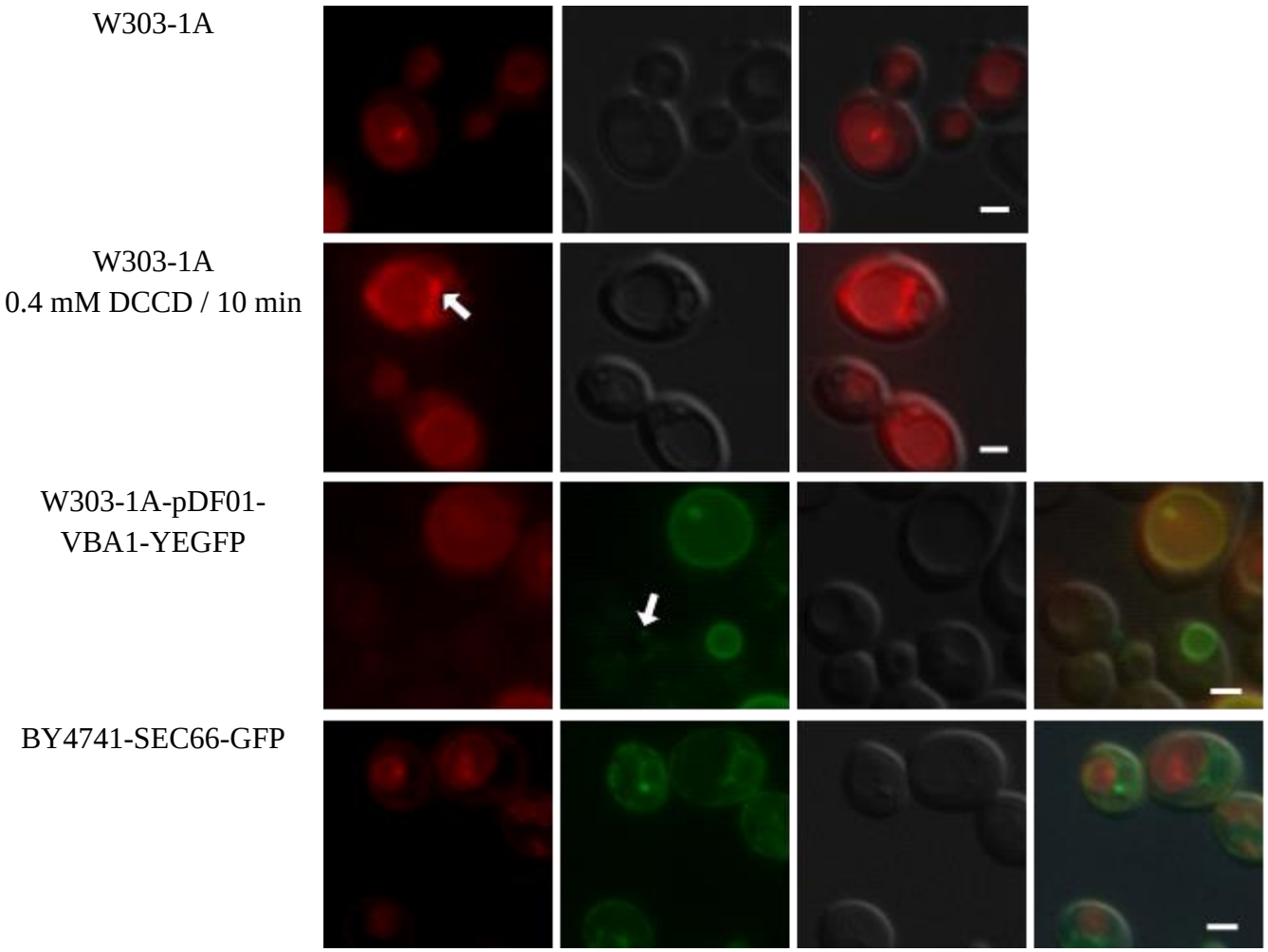

Figure 2. Images of *Saccharomyces cerevisiae* cells treated with dye **1** (25 μM, 20 minutes) at room temperature. The effect of the H^+/ATPase pump inhibitor, DCCD, in the staining pattern of **1** is presented. Red fluorescence is due to the compound, green fluorescent images refer to the GFP-tagged proteins and grey images were obtained with the DIC filter. The last image in each row is the merge of either red-fluorescent and DIC images (W303-1A, wild type) or red, green and DIC filters (mutant strains). Scale bar represents 2 μm.

In Figure 3, the images obtained for the cellular staining of the benzo[*a*]phenoxazinium salt **2** are presented. This dye also exhibits a high affinity for the vacuole membrane, but in lesser extent than **1** as seen in images on top for *S. cerevisiae* W303. In fact, only 71% of the cells have their vacuoles stained. On the other hand, some unidentified red-fluorescent structures are also observed in 15% of the cells

treated with **2** (white arrows), and 14% of the cells presented red-fluorescent aggregation dots, similar to those seen for *S. cerevisiae* W303-1A-pDF01-VBA1-YEGFP treated with **1** (red arrow).

Although in many cases it seems that the compound is being accumulated (in the form of dots) around the endoplasmic reticulum, co-staining experiments with *S. cerevisiae* BY4741-SEC66-GFP need to be performed to sustain this conclusion. Co-localization assays with *S. cerevisiae* W303-1A-pDF01-VBA1-YEGFP were performed to confirm our findings that benzo[*a*]phenoxazinium chloride **2** is predominantly targeting the vacuole. The formation of aggregates / vesicles derived from the vacuolar membrane still tagged with GFP, observed for staining with dye **1**, were also identified for treatment with **2** (not shown), but in a greater extent, being detected in 63% of the cells (500 cells counted for both phenotypes, i.e. with intact GFP-tagged vacuolar membrane and with all the GFP associated with these aggregates/vesicles).

As it was done for compound **1**, the effect of DCCD was also studied for compound **2** in *S. cerevisiae* W303-1A cells. It was noticed that when cells are previously treated with DCCD, the amount of cells with stained vacuoles decreases to 32% (green arrow) while the number of cells containing the referred red aggregates (red arrow) displayed a very significant increase to 68% (**A**). In general, these cells do not display a specific staining pattern, being the compound distributed within the cell without a representative pattern (in **B** an image is shown as an example). The other yet unidentified structures are not clearly distinguished in these cells due to the presence of the red “dots”.

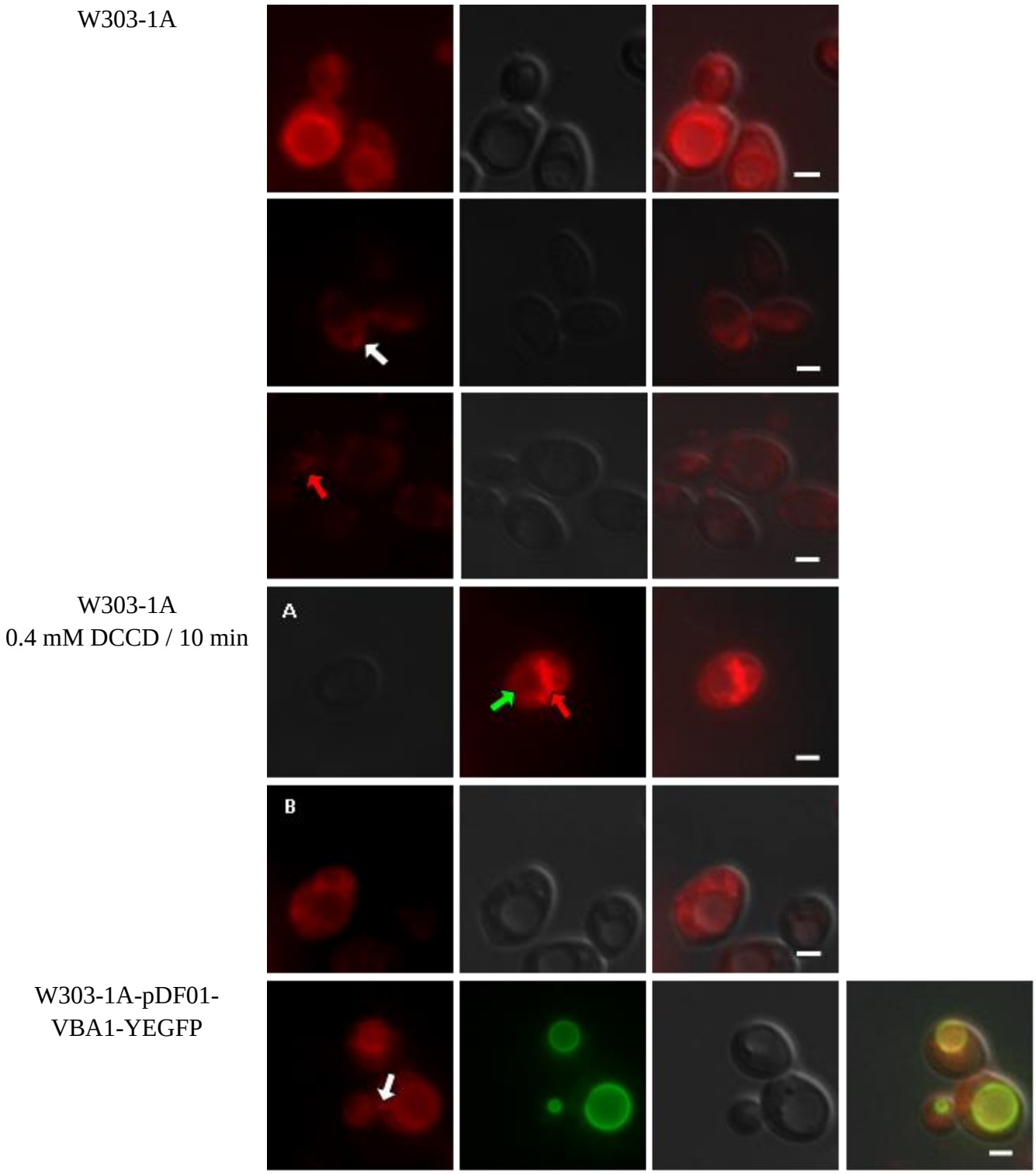

Figure 3. Images of *Saccharomyces cerevisiae* cells treated with dye **2** (12.5 μM, 10 minutes) at room temperature. The effect of the H^+/ATPase pump inhibitor, DCCD, in the staining pattern of **2** is presented. Red fluorescence is due to the compound, green fluorescent images refer to the GFP-tagged proteins and grey images were obtained with the DIC filter (*differential interference contrast*). The last

image in each row is the merge of either red-fluorescent and DIC images (W303-1A, wild type) or red, green and DIC filters (mutant strains). Scale bar represents 2 μm.

Conclusions

Newly synthesised benzo[*a*]phenoxazinium chlorides possessing ester groups as terminals in substituents at 9-amine position were evaluated as fluorescent probes. Our findings reveal the excellent potential of the benzo[*a*]phenoxazinium chloride **1** as a vacuolar membrane fluorescent probe, although a very small percentage of the cells may also have their endoplasmic reticule stained. Compound **2** also displays an important affinity to the vacuole, but in a less significant manner, being its staining pattern much more affected by the depolarization of the cells inner membranes than the one observed for dye **1**.

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