David Casto; Thanh Le
Department of Chemistry,
University of Missouri
205 Schlundt Hall
600 S. College Avenue
Columbia, MO 65211

Telephone: (816) 332-0299
E-mail: ptld49@mail.missouri.edu

Professor Rainer Glaser, Associate Editor
The Journal of Organic Chemistry
321 Chemistry Building
601 S. College Avenue
Columbia, MO 65211

April 26, 2011

RE: **Rhodamine-123: A Novel Cationic Mitochondrial Probe**
By David Casto and Thanh Le

Dear Dr. Glaser:
Thank you for your response to our paper, *Rhodamine-123: A Novel Cationic Mitochondrial Probe*. In response to the reviewers' suggestions, we have made the appropriate changes along with a few of our own. The reviewers' comments and our responses are as follows:

Response to Reviewer 6.
[6.1] Replace "non-toxicity" with "nontoxic" on the last line of p.1: suggested word does not agree with the sentence form, instead, restructured sentence and used "low toxicity"

[6.2] Rename "Scheme 1" as "Figure 1": renamed as requested

[6.3] Improve Scheme 3 to illustrate resonance differences: improved by highlighting the specific difference in blue

[6.4] Mention Figure 1 in the section it is given: mentioned as requested

[6.5] Standardize the given units in Table 1: standardized to USD/mg

[6.6] Reword the first sentence of the paragraph below Table 1: rephrased as requested

[6.7] Explain how Rh123 avoids "the process of radical release": explained as requested

[6.8] Complete the final sentence on p. 6: added more detail to the sentence

[6.9] Mentioning the qualities of Rh123 on p.7 is redundant/not necessary: removed redundancy and restructured paragraph

Response to Reviewer 8.

[8.1] Some disagreement in the Introduction and Conclusion as to the manuscript's content and implications – be consistent: restated the purpose and expectations in the last paragraph of the Introduction to correspond with the Conclusion

[8.2] Analysis and comparisons of the molecules presented is somewhat unclear – should use very well-known compounds: we feel that the probes we selected are adequate for comparison and aid our paper in highlighting the advantages of Rh123

[8.3] Data and/or detailed information about toxicity issues are missing: toxicity is not a major issue for mitochondrial probes, thus, we mentioned this in the text as requested (first sentence after Table 1)

[8.4] Figure 2 is unclear from the text and caption: changed to Figure 3; text was changed to better explain the figure

[8.5] Adhere to the formatting guidelines of JOC – use footnotes and endnotes instead of parenthetical citations: reformatted as requested

[8.6] Introduction needs to provide information about the nature and work of fluorescent probes: explained as requested

[8.7] Main criteria for fluorescent probes concept is taken exactly as stated in *Tsien et al. 1989*: rephrased sentence as requested

[8.8] "Mitochondria are found in all eukaryotic cells…" is taken from *invitrogen.com*: rephrased sentence as requested

[8.9] "Rh123 can stain mitochondria directly without the need to pass through…" is taken from *Ludovico et al. 2001*: rephrased sentence and provided citation as requested

[8.10] Explain the concept of "bleaching": changed to "photobleaching"; explained as requested

[8.11] The statement "Our data shows that Rh123…" does not support Figure 2: changed to Figure 3; further explained statement to provide better support for figure

[8.12] Figure 2 is not cited: changed to Figure 3; cited as requested

[8.13] Data to support the statement "...the process gave us a good pure yield" was not provided: statements about the yield were inserted into the Materials and Methods section

[8.14] Explain how the research is important in the Conclusion: some changes were made but overall content remained the same

[8.15] Authors must consider rephrasing and cite other papers that information was taken from: rephrased and cited as requested

Response to Reviewer 9.
[9.1] Put Table 1 in Materials and Methods section: pertinent luminescence data with regards to Rh123 is provided in the text and Figure 2; however, data comparing Rh123 to other probes are provided only in the Results and Discussion section because we did not personally collect that data

[9.2] Materials and Methods section lacks a smooth transition and progressiveness: reformatted paragraph to create a better flow of ideas

[9.3] Add more detail and thoroughness to the conclusion: elaborated on the comments made to provide a greater understanding of the probe

[9.4] Synthesis in Appendix is too general: we were unable to find a more elaborate procedure but we added molar ratio values to make it more specific

Once again, thank you for your consideration of our paper. We appreciate the attention to detail that was put forth by you and the peer reviewers. We hope that the revisions made will put our paper in good standing for publication.

Sincerely,
David Casto and Thanh Le

Rhodamine-123: A Novel Cationic Mitochondrial Probe

David Casto* and Thanh Le*

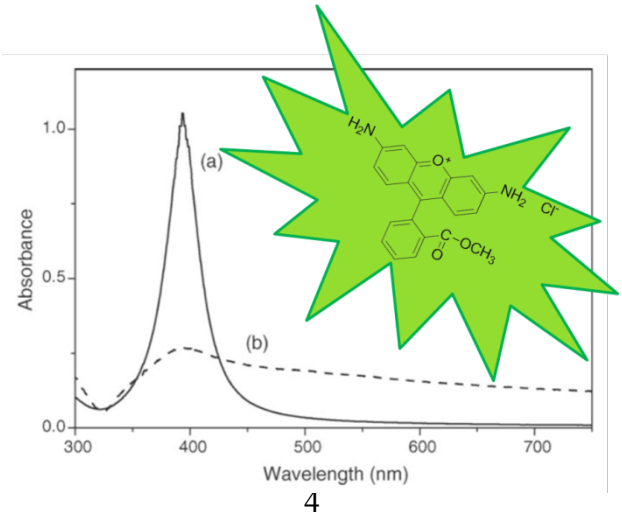
Department of Chemistry, University of Missouri-Columbia, Columbia, Missouri 65201

Email: dmc42d@mail.missouri.edu; ptld49@mail.missouri.edu

Abstract

The molecular fluorescent probe rhodamine-123, also known as Rh123, was newly synthesized to serve as a specific probe for the localization of mitochondria in living cells. The molecular basis for the rhodamine-mitochondria attraction is due to rhodamine-123's aromatic cationic structure, which binds to the membrane of the mitochondria when the net charge is negative. To determine its abilities as a mitochondrial probe, the luminescence properties were measured. These values were then used to compare Rh123 with other competitive mitochondrial probes and as a result, it is believed that Rh123 is ideal not only for its ability to stain for mitochondria, but also for its high extinction coefficient value, low toxicity, ease of use, and low cost.

Graphical Abstract



Introduction

Fluorescence microscopy is a widely used technique that enables the identification of the molecular composition of structures being observed. This technique employs the use of a fluorescent probe, which contains a molecular component known as a fluorophore that absorbs energy at a specific wavelength and then re-emits that energy at a different wavelength.[1] In doing so, the fluorophore enables the fluorescent probe to react to something specific or to localize within a particular region without damaging it.[1] In general, the luminescent properties of fluorescent probes are measured based on values of the excitation and emission wavelengths and the extinction coefficient.[2] With the use of these molecular fluorescent probes, researchers can identify and target specific structures that they want to focus on.

In particular, molecular fluorescent probes for mitochondria are becoming increasingly important. Mitochondria are a part of all eukaryotic cells and have important functions that enable the growth of those cells. As intracellular organelles, they exhibit strong abilities to move within the cell and change shape.[3] Furthermore, they play a cardinal role in the generation of energy essential for the survival and proliferation of eukaryotic cells. Therefore, it is important to have a probe designed specifically for mitochondria, the target molecule. One such probe is rhodamine-123, often abbreviated to Rh123. Rh123 is a yellow-green fluorescent probe that stains for mitochondria in living cells in a membrane potential-dependent fashion. The selective staining of mitochondria by the probe may be due to the attraction of cationic rhodamine molecules by the relatively negative electric potential across the mitochondrial membrane.[4] As a

result, Rh123 can distribute itself into the mitochondrial matrix. The amount of rhodamine accumulation in the mitochondrial matrix is dependent on the membrane potential.[4]

Specifically, here we report the results of a new molecular fluorescent probe that we synthesized: rhodamine-123. To investigate its ability to serve as a mitochondrial probe, we measured its luminescence properties. It is expected that when Rh123 is bound to the mitochondrial membrane, it will fluoresce strongly. Additionally, this strong fluorescence can occur at low concentrations, thus reducing cytotoxic levels.[5] Moreover, Rh123 can stain mitochondria without the aid of other organelles because of its high affinity for specifically the negative mitochondrial membrane.[6] To further illustrate its strengths, this new probe was compared to other competitive mitochondrial probes currently being utilized, such as tetramethylrosamine chloride, MitoTracker orange, and acridine orange 10-nonyl bromide (Figure 1).

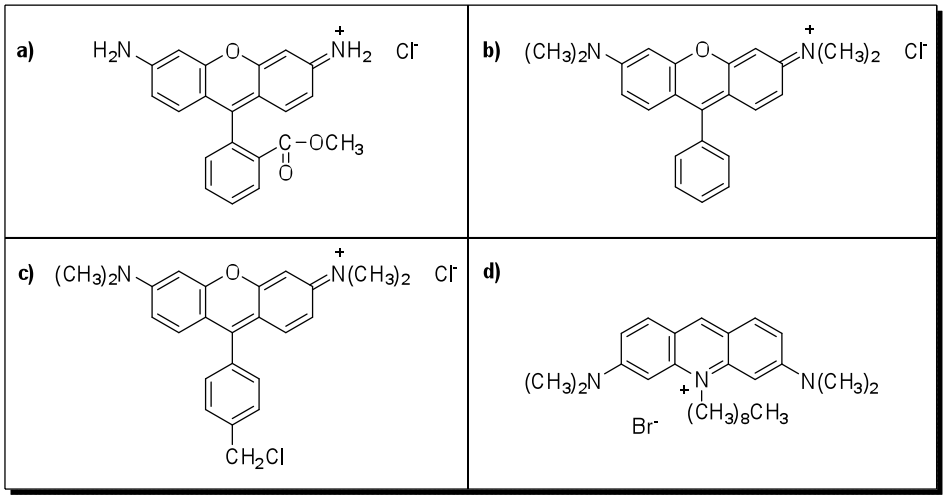
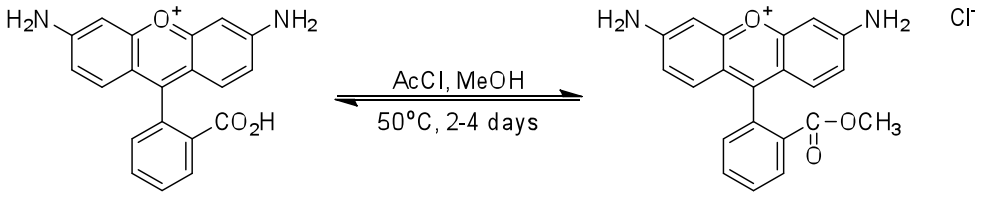


Figure 1. Structures of a) Rhodamine 123, b) Tetramethylrosamine Chloride, c) MitoTracker Orange, and d) Acridine Orange 10-nonyl Bromide

Materials and Methods

The synthesis of the fluorescent probe rhodamine-123 is a single step process with few reactants that only takes 2-4 days, as illustrated in Scheme 1.



Scheme 1. Synthesis of Rhodamine-123

The synthesis occurs when the starting material is reacted with acetyl chloride and methanol and then heated at 50°C. This gives a quantitative yield that does not need purification. A detailed description of the synthesis is provided in the appendix.

Following the synthesis, there was an interest to test the luminescence properties of Rh123. To do this, the excitation and emission data were taken and recorded. The luminescence properties of rhodamine-123 were obtained by Surface enhanced resonance Raman scattering (SERRS). To obtain SERRS, a laser excitation frequency is chosen to coincide with the frequency of the probe to give molecular resonance. The spectrum was recorded by a Spex double monochromator (Model 1403) fitted with a holographic grating of 1800 grooves/mm and a cooled photomultiplier tube (Model R928/115) from Hamamatsu Photonics, Japan.[7] Figure 2 shows the absorption maximum of Rh123 at 507 nm with a fluorescence emission at 529 nm.

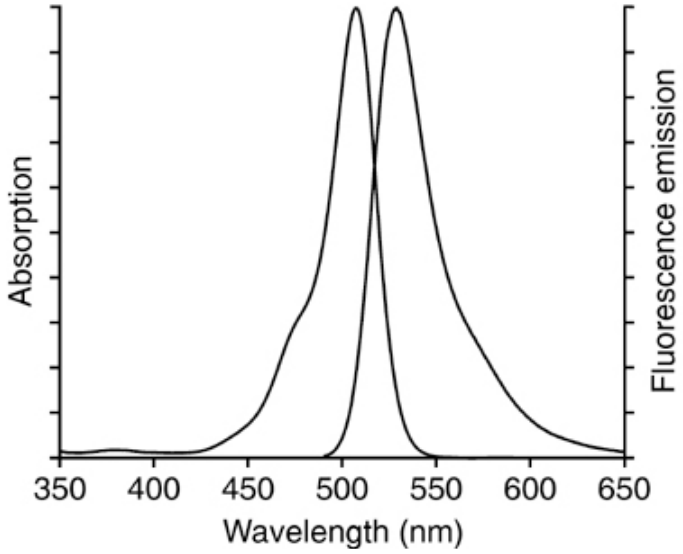
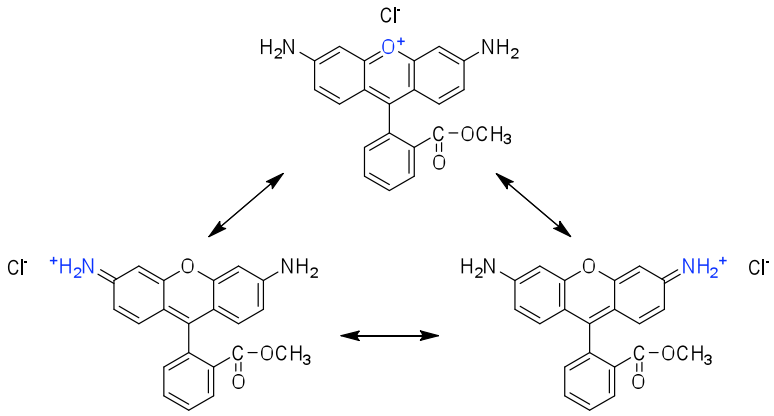


Figure 2. Absorption and Fluorescence Emission Data for Rh123

This data illustrates that Rh123 as a solid will appear red since it absorbs in the green region and will fluoresce greenish-yellow with the emission at 529 nm.

Results and Discussion

First we synthesized the new compound, rhodamine-123, which appears as a cationic probe with three resonant structures (Scheme 2).



Scheme 2. Resonance Structures of Rhodamine-123 (differences indicated in blue)

After synthesizing Rh123, fluorescence spectra were taken and analyzed (Figure 2). This data showed that Rh123 had an emission maximum of 507 nm. This makes the compound fluoresce yellow-green. This data also indicated that Rh123 has an extinction coefficient of 101,000 Lcm^{-1} and a quantum yield of 0.90.

Comparing these values to other mitochondrial probes, as shown in Table 1, it can be seen that rhodamine-123 has a higher extinction coefficient than the comparable probes and is also cheaper.

Compound	***Extinction Coefficient (Lcm^{-1})***	***Excitation (nm)***	***Emission (nm)***	***Price (USD/mg)***
Rhodamine-123	101,000	507	529	3.40
Tetramethylrosamine Chloride	87,000	550	574	5.00
MitoTracker Orange	102,000	551	576	265.00
Acridine Orange 10-nonyl Bromide	84,000	495	519	0.89

Table 1. Comparison of Rh123 to Other Mitochondrial Probes

Similar to most other mitochondrial probes, Rh123 is also very low on toxicity and does not harm cells unless it goes through a process called “bleaching.” Most fluorescent probes may undergo photobleaching, which is where the incident light used is too strong for the fluorophore, thus causing the release of radicals that destroy the fluorophore. However, using a more intense incident beam gives a stronger fluorescence and is easier to detect. Rh123 counters both issues by being more robust than other fluorophores. This means a stronger incident beam can be used on Rh123 to give a stronger fluorescence while still avoiding photobleaching, therefore ideal to use on living cells. As Table 1 depicts, Rh123 has a very high extinction coefficient (EC) comparatively, and even

though it is not the highest, it is still much cheaper than the competitors that have higher EC values.

Our data shows that rhodamine-123 is potential dependent as a cationic probe that binds to the membrane of the mitochondria where the net charge is negative. The more negative the charge, the greater the amount of the probe bound to the membrane, thus giving a stronger fluorescence for that area (Figure 3).

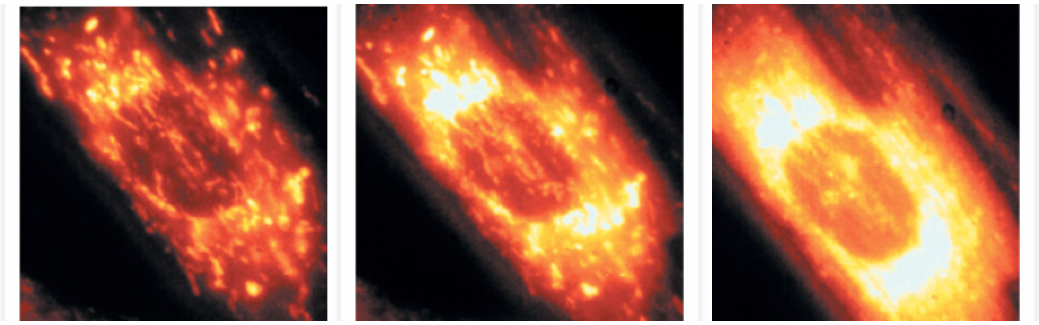


Figure 3. Potential Dependent Accumulation of Rh123 in Rat Cortical Astrocytes[8]

This can measure the membrane potential shift. In its oxidized form, when the probe is excited, fluorescence is given off and can be detected using various instruments. The uptake of Rh123 is also rapid, within a few minutes, compared to other mitochondrial dyes which may take 30 minutes or longer. Although many other probes can also effectively stain mitochondria, the other characteristics of Rh123 mentioned previously make it more effective and easier to use.

Conclusion

From our data, it is evident that we have made and confirmed a new fluorescent probe for mitochondrial membranes. We synthesized rhodamine-123 in a simple one step

process that gave us a good, pure yield. After taking fluorescence data, we confirmed the fluorescence at 529 nm which gives a detectable yellow-green signal that is great for use in living tissues since the yellow-green will stand-out against the reddish tissues.

The data shows that this molecule has a high extinction coefficient that is near or higher than other probes on the market. This gives the advantage of a very high and easy-to-detect signal. It is non-toxic and cheaper than most probes as well. This probe is ideal for use in living tissue and is a great, inexpensive probe for immediate use.

Supplemental Material Available: The appendix contains a detailed description of the synthesis of rhodamine-123 as well as spectroscopic characterization by FT-IR spectroscopy, UV-Vis, and fluorescence spectroscopy. The appendix can be obtained by contacting the authors.

References

[1] Molecular Probes, The Handbook, http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook/Introduction-to-Fluorescence-Techniques.html (Accessed 12 April 2011)

[2] Tsien, R. Fluorescent Probes of Cell Signaling. *Annual Review of Neuroscience*. **1989**, *12*, 227-253

[3] Molecular Probes, The Handbook, http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook/Probes-for-Organelles/Probes-for-Mitochondria.html (Accessed 5 April 2011)

[4] Johnson, L.; Walsh, M.; Chen, L. Localization of mitochondria in living cells with rhodamine 123. *Proc. Natl. Acad. Sci*. USA. **1980**, *77*, 990-994

[5] Petit, P. Flow Cytometric Analysis of Rhodamine 123 Fluorescence during Modulation of the Membrane Potential in Plant Mitochondria. *Plant Physiology*. **1992**, *98*, 279-296

[6] Ludovico, P.; Sansonetty, F.; Corte-Real, M. Assessment of mitochondrial membrane potential in yeast cell populations by flow cytometry. *Microbiology*. **2001**. *147*, 3335-3342

[7] Sarkar, J.; Chowdhurry, J.; Pal, P.; Talapatra, G.B. Ab initia, DFT vibrational calculations and SERRS study of Rhodamine 123 adsorbed on colloidal silver particles. *Vibrational Spectroscopy*. **2006**, *41*, 90-96

[8] Molecular Probes, The Handbook, http://products.invitrogen.com/ivgn/product/R302 (Accessed 12 April 2011)

Supporting Information

Rhodamine-123: A Novel Cationic Mitochondrial Probe

David Casto* and Thanh Le*

Department of Chemistry, University of Missouri-Columbia, Columbia, Missouri 65201

Email: dmc42d@mail.missouri.edu; ptld49@mail.missouri.edu

Table of Contents

Synthesis of Rhodamine-123

The synthesis occurs when one equivalent of acetyl chloride and one equivalent methanol is added to one equivalent of rhodamine free acid. The mixture is then heated and stirred for 2-4 days at 50°C. This solution is then evaporated down to leave the product in a quantitative yield without the need for purification. Rhodamine-123 was purchased from Aldrich Chemical Co. and used after checking the purity by HPLC. All required solutions were prepared with distilled and deionized water from a Milli-Q-plus system of M/S Millipore Corporation, USA.

FT-IR Spectrum of Rhodamine-123

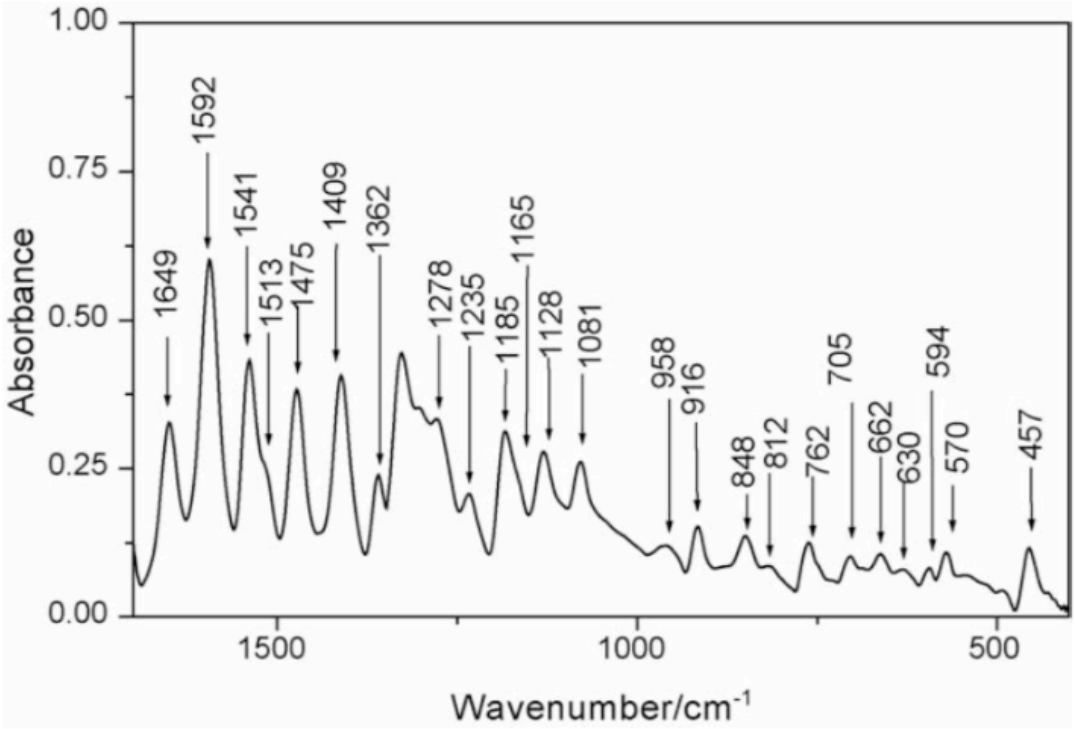


Figure 4. FT-IR spectrum of Rh123 taken in a KBr pellet using a Nicolet Magna-IR 750 spectrometer series II

UV-Vis Spectrum of Rhodamine-123

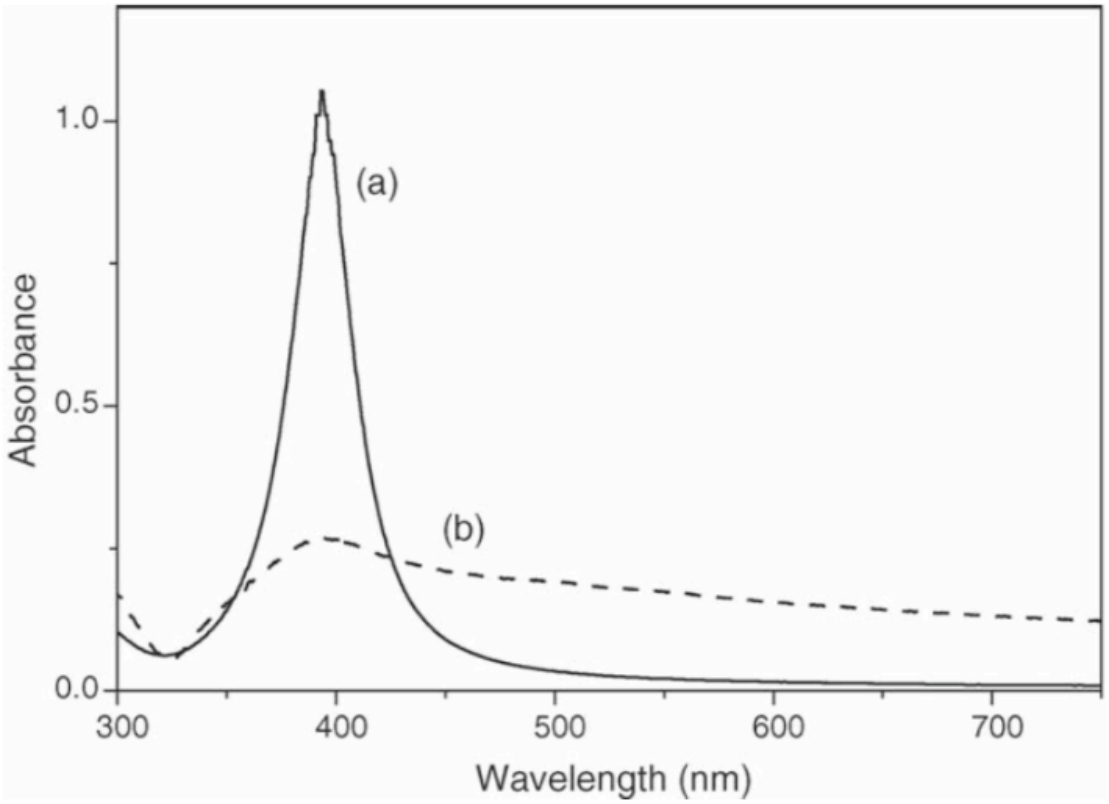


Figure 5. UV-Vis spectrum of Rh123 prepared at 3.0 x 10^{-7} M adsorbate concentration

Fluorescence Spectrum of Rhodamine-123

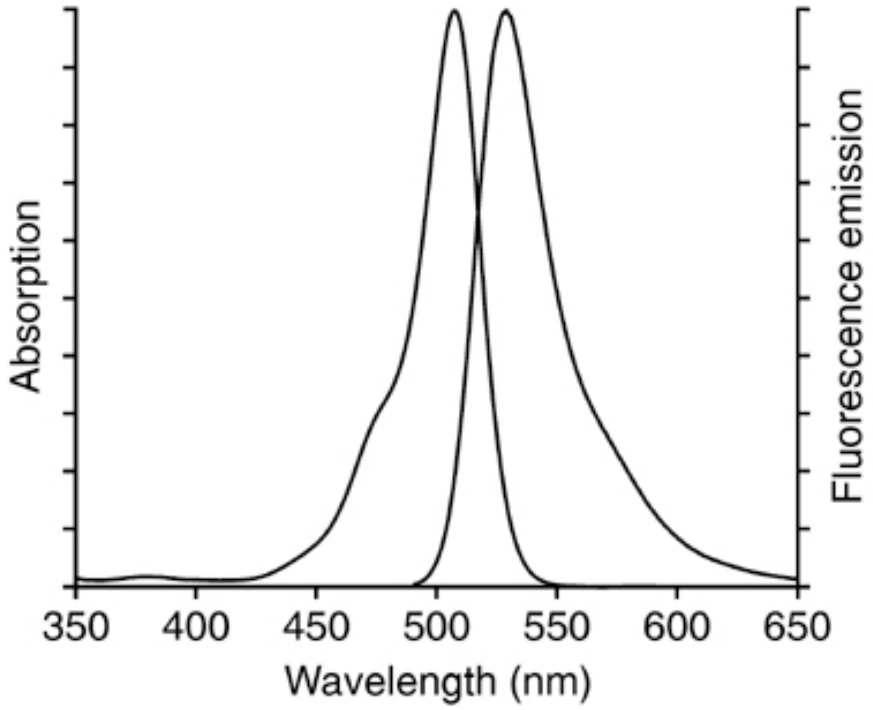


Figure 6. Fluorescence spectrum of Rh123 obtained by dissolving in methanol using an excitation wavelength of 480 nm

Additional References

[1] Ross, J.; Ross, B.; Rubinsztein-Dunlop, H.; McGreary, R. Facile Synthesis of Rhodamine Esters using Acetyl Chloride in Alcohol Solution. *Synthetic Communications*. **2006**, *36:12*, 1745-50

[2] Bolduc, J.; Denizeau, F.; Jumarie, C. Cadmium-Induced Mitochondrial Membrane-Potential Dissipation Does Not Necessarily Require Cytosolic Oxidative Stress: Studies Using Rhodamine-123 Fluorescence Unquenching. *Toxicological Sciences.* **2004**, *77*, 299-306