



Undergraduate Research under Dr. Glaser

University of Missouri-Columbia

Dr. R. Glaser
Professor, University of Missouri-Columbia

RE: Synthesis of the Mitochondria Detecting Molecular Fluorescent Probe Rhodamine B

Dear Dr. Glaser

Thank you for your time and your review of the paper we submitted. We appreciate the constructive criticisms that have been provided by our peers. We have attached a list of revisions done to the paper. We are thankful for your time and recommendation of this paper.

Major Changes

[M.1] We added a detailed description of the interactions between rhodamine B and mitochondria. We also added a brief description of how JC-1 and MitoTracker Green interact with mitochondria.

[M.2] The detailed description of the experimental procedure has been moved to supplemental information

[M.3] Table 1 has been moved to the M&M

[M.4] Scheme 1 has been edited to look less busy, and the S,R, rt, notations have been eliminated

[M.5] One column of table 1 has been deleted since there was no discussion about that particular probe

[M.6] Numerous grammatical errors were found and fixed and many sentences were rewritten for more clarity

[M.7] Scheme 1 was redrawn so the molecules were more spacious and the names of the MFPs were included in the scheme

[M.8] Scheme 2 was redrawn so the molecules are closer to scale and the reagents are less cluttered

Response to Reviewer 12

[12.1] Spelling errors were corrected. The word “stuck” was changed to “struck,” “biomolecules” was changed to “biological molecules” for clarity, and “combing” was changed to “combining”

[12.2] Page numbers were moved to the center of the page

[12.3] Usage of the dye laser to test the fluorescence was added in both the M&M and R&D

[12.4] figure 3 was changed to figure 2 and figure 4 has been changed to figure 3 and cited in the text

[12.5] The URLs contained in the references were information that we used to research rhodamine B, and not supporting evidence like the other references. Accordingly, they have been moved to the bibliography at the end where they are more appropriate.

Response to reviewer 4

[4.1] figures 3 and 4 have been renamed 2 and 3, respectively. They have been cited according to the work from which they came

[4.2] We have greatly revised the paper to fix grammar mistakes and to eliminate colloquial words

Response to reviewer 10

[10.1] scheme 1, scheme 2, table 1, and figure 1 have all been bolded and are now in the appropriate font

[10.2] figures 3 and 4 have been renamed 2 and 3, respectively and have been bolded and are now in the correct font

[10.3] “scheme 2. Rhodamine B synthesis” was done in word and not in ChemDraw

[10.4] The lines mentioned about the R&D have been moved to supplemental information and appropriate spacing has been added

[10.5] The prices of the three probes has been added to the R&D

[10.6] We have added “Rhodamine B is non-flammable and relatively safe to work with, inhaling it would only cause mild irritation” in order to address any hazards

[10.7] appropriate spacing has been placed in between all sections

[10.8] references now have the (1) notation instead of being superscripts

[10.9] The spacing on the first page of both sections is identical, the only difference is that the first page of section has “supporting information” centered at the top

[10.10] We have switched the table of contents, now the page numbers are on the right and the item on that page is listed on the left

We have spent a great deal of time carefully considering every point that was mentioned by our peers and are thankful for their input. We believe their work has led to the strengthening of our paper and we look forward to the possibility of our paper being published.

Best Regards

Jacob Wright and Steven Devine

Synthesis of the Mitochondria Detecting Molecular Fluorescent Probe Rhodamine B

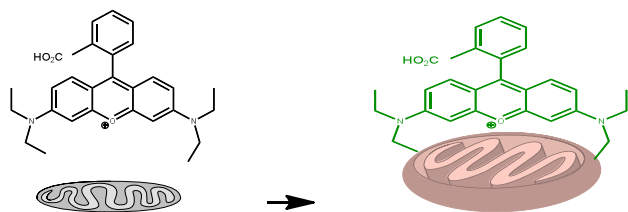
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Abstract

Molecular fluorescent probes are one of the most practical tools in biological research. Enabling viewers to see what is contained in a cell helps to understand what happens in the cell. Contained in this paper is the synthesis of the molecular fluorescent probe rhodamine B. Rhodamine B attaches to active mitochondria which are responsible for the production of ATP. By using characteristic spectroscopy, we were able to gather evidence that supports our conclusion that we successfully synthesized rhodamine B. The unique characteristics of our probe are the wavelengths of the excitation and emission at 554 nm and 574 nm, respectively.

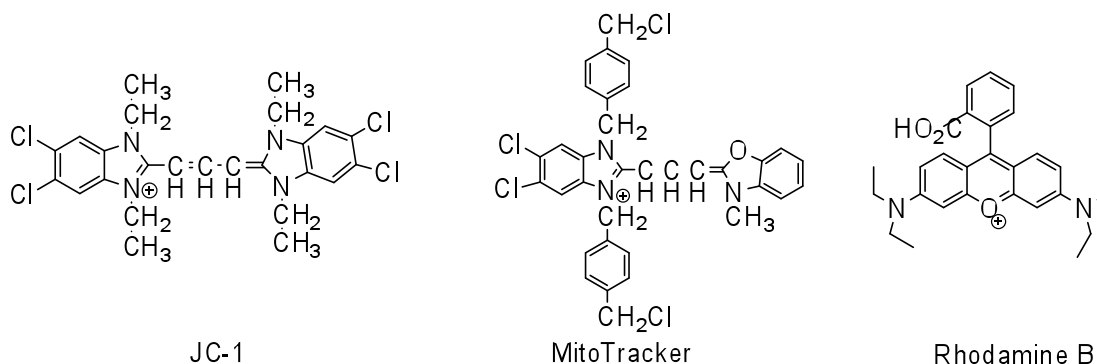


Introduction

Molecular fluorescent probes, MFPs, are organic molecules that are used to identify a biological compound of interest by attaching to it and fluorescing, or emitting a colored light¹. A fluorescent probe is used to either identify or quantify a particular molecule, membrane, or ion. An ideal probe should not emit any light until it is bound with the molecule of interest and should only bind to the molecule of interest. In addition, MFPs should be cheap, safe, and have a high emission coefficient so it can be seen easily. When a MFP is bound to a particular molecule, the probe will undergo a conformational change². When the altered structure of the probe is struck by light of a certain wavelength, the electrons in that molecule go in to a higher energy state, or an excited state³. When the electrons relax, they go down in energy, and the energy lost in the process is emitted as light.

Organisms are very complex beings with a wide range of cells and an enormous range of biological molecules. These molecules can be in various concentrations in different places in numerous species and they are constantly reacting and changing within the body⁴. The body can typically regulate the concentration and placement of these molecules by itself. However, understanding these complex processes is a difficult task because most of the molecules researchers are interested in lack distinct features that could be detected by the human eye. The use of MFPs allows researchers to visually detect the presence of their structures of interest, which in our case are mitochondria⁵. Being able to determine the presence of a structure and its activity, or having the ability to quantify it, could lead to new ways to fight diseases or detect for others.

Here we report the results of our synthesis of rhodamine B which is able to detect active mitochondria in the cell. We will start with the material m-hydroxy-N,N-diethylaniline and react it with anhydphthalic anhydride to produce rhodamine B with a relatively high percent yield, above 50%. We will compare rhodamine B with similar MFPS such as JC-1 and MitoTracker Green(scheme 1).



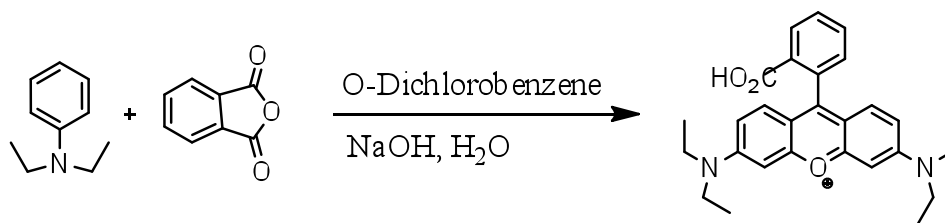
Scheme 1. Probes In Order of Decreasing Fluorescence: JC-1, MitoTracker, and Rhodamine B

Finding a way to synthesize rhodamine B in high yields that is cheap and takes little time and does not create much waste will hopefully reduce the cost of the probe. With a lower cost, the probe will be more readily available to researchers who are interested in studying in this field, and less waste will result in less damage to the environment.

Methods and Materials

Rhodamine B was synthesized by combining m-hydroxy-N,N-diethylaniline, haloaroms, and anhydphthalic anhydride in water⁶. Then with heating in a nitrogen atmosphere, the five

membered heterocyclic ring will decompose, allowing the reaction to proceed (scheme 2). More information can be found in the appendix.



Scheme 2. Rhodamine B Synthesis

Using a dye laser, rhodamine B was found to have a max absorption at a wave length of 554 nm and the emission was at 574 nm (figure 1). The spectrum was obtained by using a 0.1 M solution at a pH of 7.4 while in a phosphate buffer solution. By finding the excitation and absorbance of rhodamine B can help to distinguish it from many similarly structured probes. Table 1 is a list of similar probes for comparison.

Probe	Absorbance (nm)	Emission (nm)
Rhodamine B	554	574
MitoTracker Green	474	524
JC-1	490	590

Table 1. Absorbance and Emission

Results and Discussion

Rhodamine B has proven to be an effective mitochondria binding probe. Rhodamine B will bind to a glycoprotein pump (g-pump) in the transmembrane of active mitochondria. In order for the probe to bind to the g-pump, the ethyl groups on the amines in the first and third benzene rings

will rotate to orient themselves towards the plasma membrane of the cell, away from the mitochondria. This rotation allows the positively charged oxygen in the second ring to move closer to the g-pump since there is less steric hindrance from the ethyl groups. The oxygen will then bind the g-pump at the site of the amino acid cysteine. The particular g-pump that rhodamine will bind to is only found in the transmembrane of mitochondria. JC-1 and MitoTracker Green are shown to bind to an ATP-pump. This pump is mostly found in mitochondria, however there are other similar ATP-pumps distributed throughout the cell. JC-1 and MitoTracker Green will sometimes bind to the pumps not located in the transmembrane of the mitochondria, meaning you will have fluorescence in undesired locations. The ability of rhodamine B to bind to a pump that is only found in mitochondria make it a superior probe to JC-1 and MitoTracker Green.

Excitation and absorbance of rhodamine B and the two other probes were found and are displayed in the table 1. We found the UV-vis spectra and have included it as figure 1. Each material was placed separately under a dye laser, using a thin dye jet as the gain medium. Rhodamine B had an extinction coefficient of $106,000 \text{ M}^{-1} \text{ cm}^{-1}$. Another advantage of rhodamine b, other than its ability to selectively bind mitochondria, is its cost. Our synthesis has made rhodamine B very cheap, about \$45 for a kilogram. JC-1 and mitrotracker green sell for about \$300 and \$400 a kilogram, respectively. Rhodamine B is non-flammable and relatively safe to work with. Inhaling it would only cause mild irritation. The disadvantage is that is has the

smaller stokes shift and thus more difficult to distinguish.

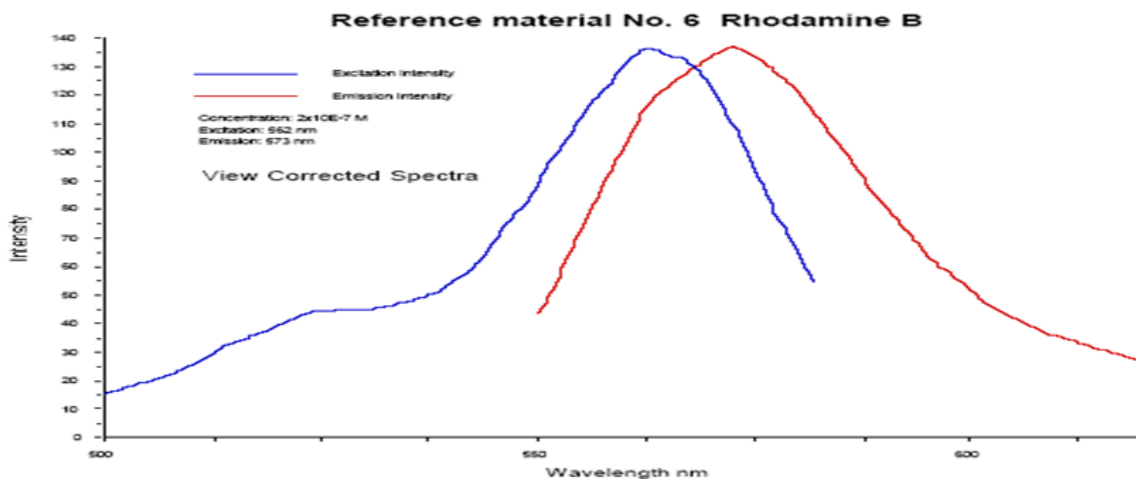


Figure 1. Graph of Absorption and Emission of Rhodamine B

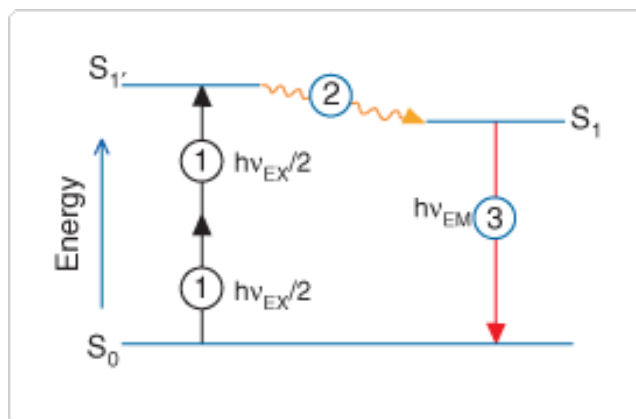


Figure 2. Molecular Orbital Diagram Demonstrating Fluorescence⁵

When electrons are excited from the ground state by UV light, or some other form of energy, they are raised to the HOMO, highest unoccupied molecular orbital, shown as S_0 to S_1 (figure 2).⁵ Next there is a step-down transition to a lower energy level which is shown as path 2 in figure 2. Then electrons in that high energy orbital release their energy (large transition 3), and that energy is emitted in the form of a photon. A larger transition means there is more energy is

in the photon, and the energy in that photon is directly correlated with its color. If the color of the photon emitted is purple then it is a higher energy photon. Conversely if the photon emits red light then it is a lower energy photon (figure 3)⁶.

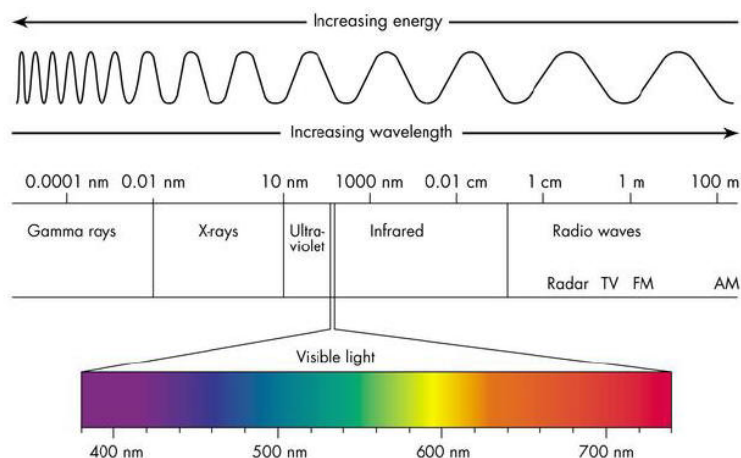


Figure 3. Visible light spectra⁶

The complexity of the delocalization for each molecule produces the largest energy photon when there is the greater difference between S_0 and S_1' , and a small gap between S_1' and S_1 . This can be accounted for in the JC-1 molecule because of the excessive lone pairs from the chlorines. Symmetry of the chlorines in the molecule allows for more prevalent delocalization and creates the specific conditions that create that high energy photon. It is most easily seen when the probes are side by side. JC-1 has the greatest Stokes shift, which correlates to the highest emission photon, also seen numerically in table 1. It has a Stokes shift of 100nm. As previously discussed, conjugation of the JC-1 probe is not the only reason for its fluorescence. It is theorized that the lone pairs on the chlorine and its symmetry are partially responsible for its bright fluorescence. MitoTracker Green is similar to JC-1 in regards to high conjugation and lone pairs on chlorine atoms but lacks the symmetry that JC-1 has and this is thought to be the reason the molecule is

not as strong of an indicator as JC-1. Rhodamine b is very conjugated but has no symmetry or lone pairs. This results in a smaller stroke shift of only 20nm.

Conclusion

We were able to successfully synthesize the mitochondrial probe rhodamine B. It was found that when the reaction was complete there was an 87% yield. This high yield, particularly for an organic reaction, was the result we were looking for. The product formed had an extinction coefficient of $106,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 542.75 nm, which is the defining characteristic of rhodamine B. When placed under UV light it fluoresced a bright green color. Rhodamine B does not fluoresce as brightly as JC-1 or MitoTracker Green, but it is more selective for mitochondria than the other two and is much cheaper to produce, and we feel this makes rhodamine B a superior MFP.

MFPs are an extremely important part of new biological research. By being able to identify a particular target, researchers can gather new information about what is happening inside the cell. Understanding the thousands of processes that occur within the body is the ultimate goal of biology, and probes like rhodamine B will help reach that goal. With rhodamine B's ability to detect for active mitochondria, researchers will be able to determine important characteristics of cancer cells, bacteria, and living cells.

Supplemental Material Available: The appendix contains additional information, including a detailed description of the synthesis and characteristic spectrums including: IR, H-NMR, mass spec, and C-NMR.

References

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- (1) Yasuteru, U. Title Development of Organic Small Molecular Fluorescent Probes That Visualize Intracellular bioactive Molecules: Theoretical Precision Design Based On Photo-induction Electronic Transfer. *Bio jan.* **2007**, *7*, 664
 - (2) Nagano, T. Bioimaging Probes for Reactive Oxygen Species and Reactive Nitrogen Species. *J Clin Biochem Nutr.* **2009**, *45*, 111-124
 - (3) Gasser, M.; Rutishauser, B.; Krug, H.; Gehr, P.; Nelle, M.; Yan, B.; Wick, P. The adsorption of biomolecules to multi-walled carbon nanotubes is influenced by both pulmonary surfactant lipids and surface chemistry. *J Nanobiotech.* **2010**, *8*, 31
 - (4) Terai, T.; Nagano, T. Fluorescent Probes for Bioimaging Applications. *Curr Opin Chem Biol.* **2008**, *12*, 515-521
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 - (6) Zheng, H.; Shang, G.; Yang, S.; Gao, X.; Xu, J. Fluorogenic and Chromogenic Rhodamine Spirolactam Based Probe for Nitric Oxide by Spiro Ring Opening Reaction. *Org let.* **2008**, *10*, 2357-2360

Supporting Information

Synthesis of the Mitochondria Detecting Molecular Fluorescing Probe Rhodamine B

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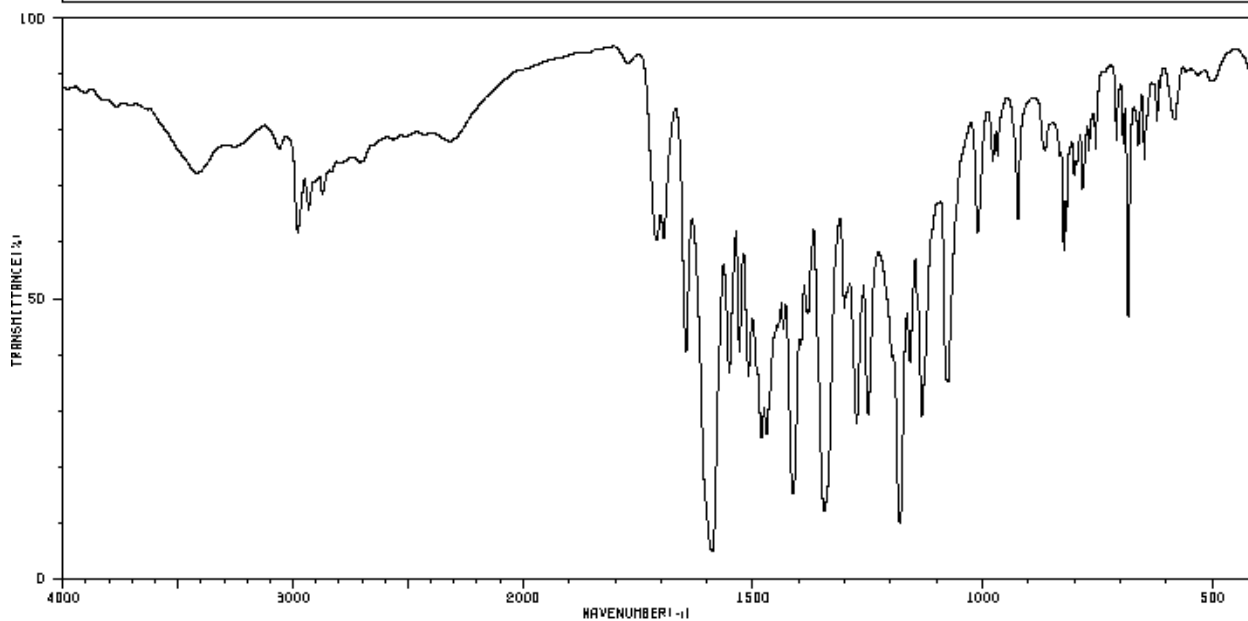
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Synthesis of Rhodamine B

To obtain the new MFP we reacted .38g m-hydroxy-N,N-diethylaniline, 1.50g charged haloaroms, and .48g anhydphthalic anhydride in a 150ml flask with a magnetic stir-bar attached a water separator and condenser, making sure that the reaction was completely protected from standard atmospheric conditions and a nitrogen line was attached, and thermometer inserted. The flask and attachments were then secured on a magnetic hot plate. The nitrogen was then introduced after a thorough inspection and the reaction mixture was then heated to 175°C. We then charged the reaction 5 times in the flask. We then continued the reaction at 170-180°C for 3-4 hours. Heat was turned off and we slowly cooled the reaction mixture to 100°C. We then set up a Buchner vacuum to filter the product, which was in the liquid, and recaptured it by reacting the solute with hydrochloric acid. This was then filtered by the same process except this time the powder substance was the desired product. It was then washed 3 times with 3ml portions of diethyl ether, dried and weighed at .85g and a percent yield of 87 was determined after calculations were completed. 0.479g of rhodamine b was massed and dissolved in 10ml phosphate buffer, in a clean dry Erlenmeyer flask to give a 7.4 pH and UV spectroscopy was then taken.

HIT-NO=7647	SCORE= ()	SDBS-NO=15673	IR-NIDA-29247 : KBR DISC
RHODAMINE B			
C ₂₈ H ₃₁ ClN ₂ O ₃			



3417	70	1688	4	1413	14	1168	37	832	72
2978	58	1551	35	1380	46	1132	27	824	57
2932	64	1529	36	1345	11	1077	34	816	50
2872	66	1610	36	1302	46	1011	58	801	70
1710	58	1481	23	1274	26	978	72	795	72
1693	56	1471	24	1250	26	923	62	763	56
1645	38	1434	42	1181	9	865	72	684	44

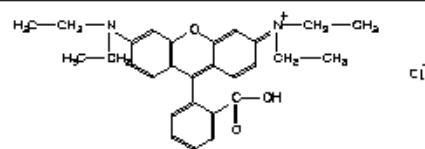


Figure1. Rhodamine B IR: KBr disc

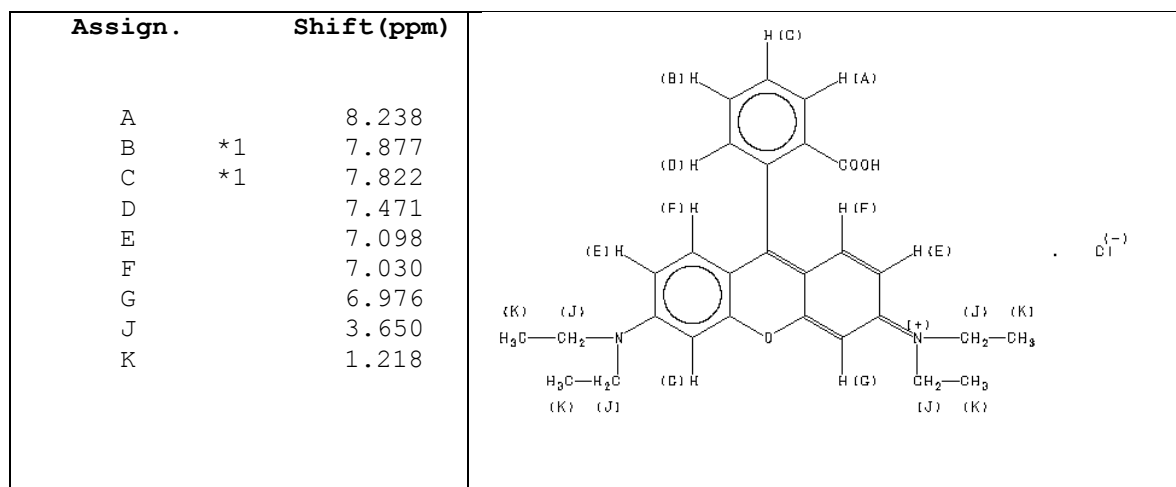
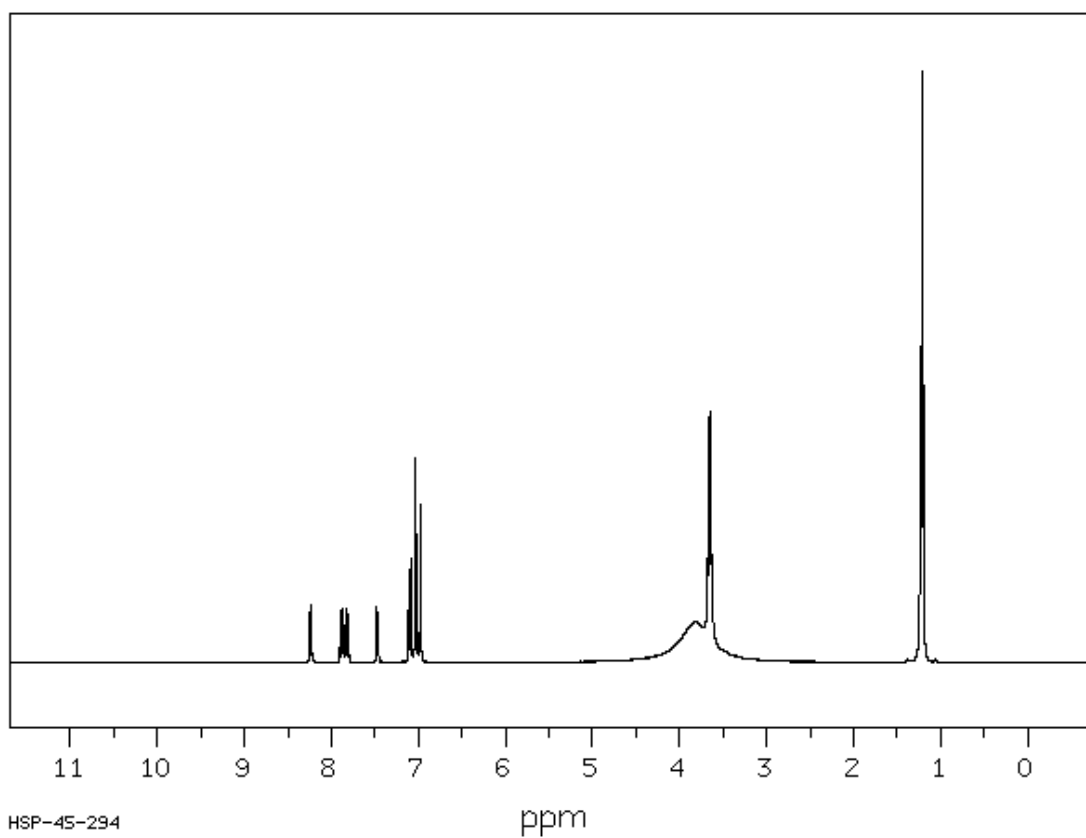


Figure 2. Rhodamine B HNMR with Chemical Shifts

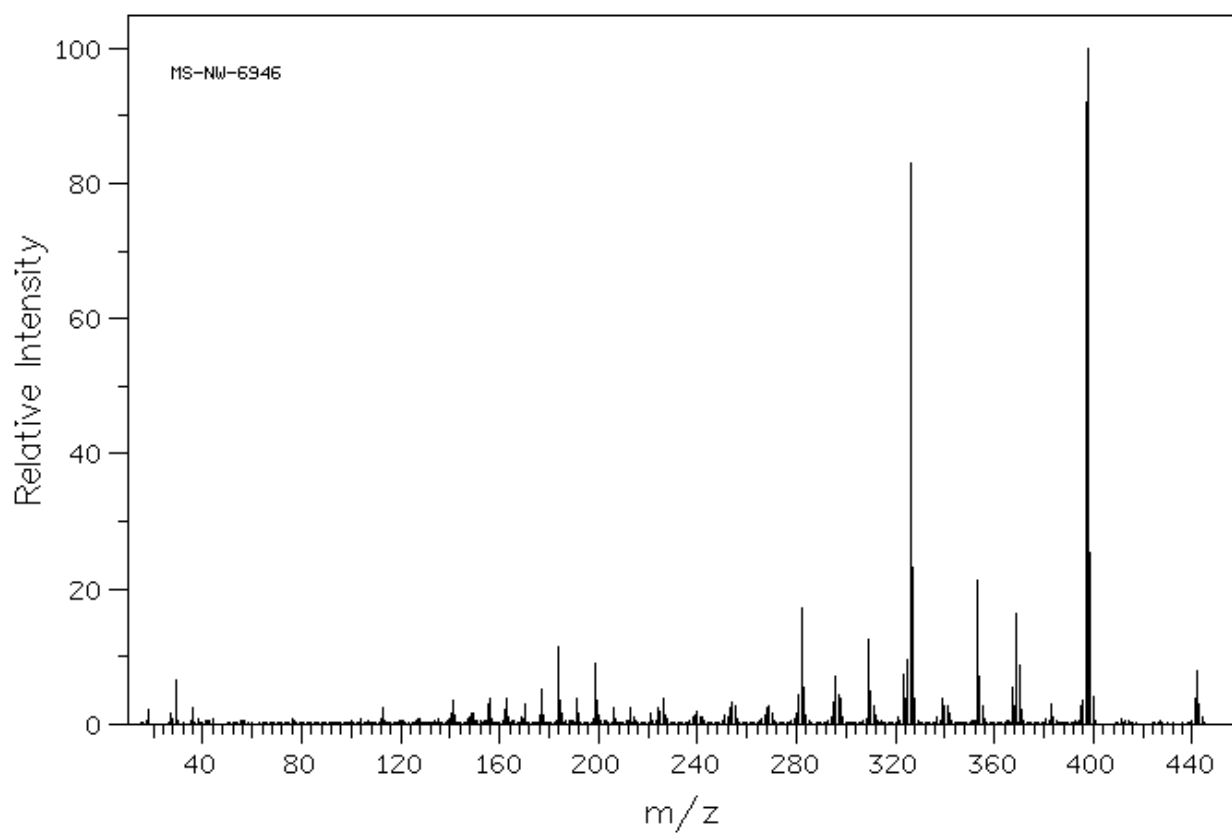
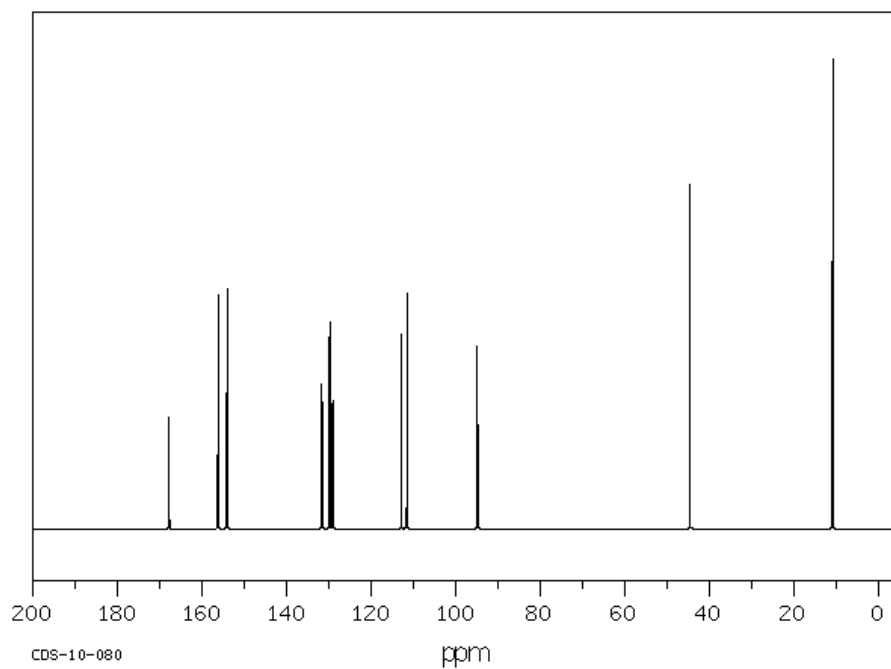


Figure 3. Mass Spectrometer of Rhodamine B



ppm	Int.	Assign.
167.74	235	1
156.25	273	2
156.04	497	3
154.11	511	4
131.76	308	5
131.52	268	6
130.03	406	7
		8
129.72	438	9 *
129.40	266	10 *
128.96	273	11 *
112.88	415	12
111.57	501	13
94.84	389	14
44.58	733	15
10.87	1000	16

Figure 4. CNMR of Rhodamine B with Chemical Shifts

Bibliography

- 1 Zheng, H.; Shang, G.; Yang, S.; Gao, X.; Xu, J. Fluorogenic and Chromogenic Rhodamine Spirolactam Based Probe for Nitric Oxide by Spiro Ring Opening Reaction. *Org Let.* **2008**, *10*, 2357-2360
- 2 http://www.chemistry.nmsu.edu/Instrumentation/fluorescence_Std_4.html (accessed April 5, 2011)
- 3 http://riodb01.ibase.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi (accessed April 5, 2011)
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