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Synthesis of 1-(6-(dimethylamino)naphthalen-2-yl)-2,2-dimethylpropan-1-one as a Fluorescent Probe of Solvent Acidity in HSA

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Synthesis of 1-(6-(dimethylamino)naphthalen-2-yl)-2,2-dimethylpropan-1-one as a Fluorescent Probe of Solvent Acidity in HSA

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in Chemistry from The College of William and Mary

by

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April 24, 2012
Table of Contents

Acknowledgements iii
List of Figures iv
Introduction 1
Background 4
Experimental 7
   Synthesis of 1-[6-(dimethylamino)-2-naphthalenyl]-2,2-dimethyl-1-propanone 7
   Preparation for Quantum Yield Determinations 8
Results and Discussion 9
Conclusion 22
Appendix A 23
References 25
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List of Figures

Figure 1. Structure of 6-Propionyl-2-dimethylaminonaphthalene (PRODAN) 1
Figure 2. Structure of Cholesterol 2
Figure 3. Structure of PRODAN Cholesterol Model 2
Figure 4. Structure of 3-(dimethylamino)-8,9,10,11-tetrahydro-7H-cyclohepta[a]naphthalen-7-one (Compound 1) 3
Figure 5. Structure of 1-[6-(dimethylamino)-2-naphthalenyl]-2,2-dimethyl-1-propanone (Compound 2) 3
Figure 6. Structure of Warfarin 4
Figure 7. Schematic of Synthesis for Compound 2 9
Figure 8. Structure of 1,1-bis(6-(dimethylamino)naphthalen-2-yl)-2,2-dimethylpropan-1-ol (Byproduct) 9
Figure 9. Titration of PRODAN with HSA (Absorption) 10
Figure 10. Titration of PRODAN with HSA (Fluorescence) 11
Figure 11. Titration of Compound 1 with HSA (Absorption) 12
Figure 12. Titration of Compound 1 with HSA (Fluorescence) 13
Figure 13. Titration of Compound 2 with HSA (Absorption) 14
Figure 14. Titration of Compound 2 with HSA (Fluorescence) 15
Figure 15. Change in Absorption of PRODAN + HSA vs. Concentration HSA 17
Figure 16. Figure 16. Change in Integrated Fluorescence of PRODAN + HSA vs. Concentration HSA 17
Figure 17. Change in Absorption of Compound 1 + HSA vs. Concentration HSA 18
Figure 18. Change in Integrated Fluorescence of Compound 1 + HSA vs. Concentration HSA 18
Figure 19. Change in Absorption of Compound 2 + HSA vs. Concentration HSA 19
Figure 20. Change in Integrated Fluorescence of Compound 2 + HSA vs. Concentration HSA 19
Figure 21. Relative Increases in Quantum Yields 20
Figure 22. Relative Fluorescence of Probe with Changing Solvent Acidity 20
Figure 23. Summary of Solvent Acidity in HSA by Probe 21
Introduction

The goal of this study was to synthesize 1-(6-(dimethylamino)naphthalen-2-yl)-2,2-dimethylpropan-1-one, a PRODAN derivative, and perform fluorescence studies comparing the efficacy of various PRODAN derivatives as probes of solvent acidity in biological systems. These experiments involved allowing various PRODAN derivatives to bind to human serum albumin (HSA) and performing fluorescence titrations to determine relative quantum yields via integrated fluorescence intensities.

![Structure of PRODAN](image1)

**Figure 1. Structure of 6-Propionyl-2-dimethylaminonaphthalene (PRODAN)**

6-Propionyl-2-dimethylaminonaphthalene (PRODAN), shown in Figure 1, is a fluorophore which can be modified to be structurally similar to cholesterol, shown in Figure 2. The PRODAN model of cholesterol that has been prepared in the Abelt lab is shown in Figure 3. This means that PRODAN might be capable of probing systems which are involved in cholesterol trafficking, such as cell membranes, lipoproteins, and HSA.
Compound 1, 3-(dimethylamino)-8,9,10,11-tetrahydro-7$H$-cyclohepta[a] naphthalen-7-one, shown in Figure 4, is similar to the parent PRODAN molecule, except it contains a somewhat bulky and relatively hydrophobic seven membered ring. The hydrophobic nature of the HSA cavity should allow for stronger binding with this other derivative, but the ability of the carbonyl to hydrogen bond with solvents may be limited due to its being constrained.
Compound 2, 1-[6-(dimethylamino)-2-naphthalenyl]-2,2-dimethyl-1-propanone, contains a bulky t-butyl group, which may limit how far into the hydrophobic cavity that this derivative can penetrate. As a result, the carbonyl would more readily hydrogen bond to solvent, causing the probe to be highly sensitive to solvent acidity.
Background

Cholesterol is transported through the body between cells and the liver via lipoproteins such as HDL and LDL. This view is simplified in that it ignores the importance of HSA in transporting cholesterol from LDL to cells and from cells to HDL. The sheer abundance of HSA in the blood stream, 40mg/mL, makes it one of the most important transport proteins in the body. It was been suggested that low levels of HSA may be a fair predictor of increased risk of death from coronary heart disease. Aside from transporting cholesterol, HSA partakes in transporting a variety of important biological molecules, including other steroids, drugs, monoacylglycerols, and fatty acid chains.

HSA is a monomeric protein containing three structurally-analogous α-helical domains, which in turn, each contain two subdomains composed of six or four helices, subdomains A and B respectively. These subdomains each contain relatively hydrophobic pockets at which non-polar molecules may bind via hydrophobic and electrostatic interactions.

![Figure 6. Structure of Warfarin](image)

Warfarin binds fairly readily to HSA at subdomain IIA, with 99% forming a drug-HSA complex under “normal therapeutic conditions.” PRODAN’s structural similarity to the drug Warfarin is important in that they are relatively hydrophobic molecules with electron density
concentrated at the center of the molecule. The comparable structures result in competitive binding to HSA. Studies done by Krishnakumar et al. indicate that binding up to three equivalents of the fatty acid, palmitic acid, to HSA will increase the quantum yield of the PRODAN-HSA complex. However, further addition of palmitic acid will decrease the fluorescence quantum yields. This is attributed to the denaturation of HSA in a relatively acidic environment. The addition of fatty acids is believed to cause domains I and III to rotate resulting in an enhanced hydrophobic cavity at domain II.

Interestingly, there are two enantiomers of Warfarin, which both bind to HSA similarly, indicating a lack of stereospecificity at the binding cavity. The lack of specificity at the binding site permits binding of PRODAN derivatives similar in size, structure, and hydrophobicity, allowing for a larger range of acceptable models. Although probe models such as PRODAN are drastically different from cholesterol in terms of structure, they are capable of binding to HSA and are still capable of yielding information regarding HSA binding. For instance, studies done by Moreno et al. regarding HSA and PRODAN indicate a single equilibrium. The importance of this finding is that there is primarily one HSA binding site that PRODAN will preferentially bind to: subdomain IIA.

HSA, unlike a membrane, contains some specificity regarding orientation of PRODAN derivatives. While membranes allow for an upside-down cholesterol orientation which can rotate to a rightside-up orientation, and vice versa, HSA will bind to PRODAN such that the polar features, such as the carbonyl group, will be found at the mouth of the binding cavity.

An important note about HSA is that it has a single tryptophan residue which emits at 445nm when absorbing at 295nm. Fortunately, this fluorescence of free HSA is quenched by
the addition of PRODAN. The absorption of PRODAN is far enough apart from HSA’s tryptophan residue’s absorption that we can selectively analyze PRODAN’s absorption.

PRODAN is an amphiphilic molecule containing two rings, and like cholesterol, it is relatively hydrophobic. The carbonyl group is capable of accepting electrons and the alkylamino group is capable of donating electrons, which make PRODAN derivatives sensitive to their micropolarity and microacidity.  

The challenge is discerning the difference between solvent polarity and solvent acidity effects. Shifts in fluorescence peak maxima are a result of sensitivity to micropolarity of the probe’s environment. According to Park, Park, and Hamilton, a red shift is due to fluorescence of the probe after binding to the nonpolar cavity of HSA. Changes in maximum peak intensity are a result of changes in solvent acidity.

Catalan developed a method of analyzing solvents by four characteristics: solvent dipolarity, solvent polarizability, solvent acidity, and solvent basicity. They determined that, of the four characteristics, solvent acidity has the greatest influence on the electronic transitions observed by the probes. Solvent acidity is the ability of the solvent to donate protons.
Experimental

NMR spectra were obtained using a Varian Mercury VX-400 spectrometer. Absorption and fluorescence emission measurements were obtained using an Ocean Optics HR2000 spectrometer. Fluorescence excitations were done by an SLM-AMinco SPF-500 spectrometer. Solvents used for quantum yield determinations were spectrophotochemical grade from Acros.

Synthesis of New Prodan Derivative

1-[6-(dimethylamino)-2-naphthalenyl]-2,2-dimethyl-1-propanone. 6-Bromo-N,N-dimethyl-2-naphthylamine (2.00g, 0.008mol) was dissolved in dry THF (30mL) under N\textsubscript{2} and cooled to -78°C. BuLi (5.3mL, 1.6M in hexanes) was added slowly dropwise and the reaction was left to stir for 30 minutes. 2,2-Dimethyl-1-(1\textit{H}-pyrrol-1-yl)-1-propanone (1.13mL) was added dropwise and the reaction was left to stir for 1.5 hrs, allowing the temperature to rise to -40°C. The reaction was quenched with water (440mL) and left to stir overnight. NaCl (44.45g) was added to the quenched reaction. Acetic acid (44mL) was added to the reaction. The product was extracted with ether (2 x 150mL), then washed with water (2 x 74mL), and finally washed with a 2% NaHCO\textsubscript{3} solution (3 x 74mL). The product was dried over Na\textsubscript{2}SO\textsubscript{4} and concentrated \textit{in vacuo}. The crude product was purified with column chromatography. The fractions collected from the column were sublimed and recrystallized giving purified product (0.13g, 0.0005mol, 6.4% yield). \textsuperscript{1}HNMR (CDCl\textsubscript{3}) δ 8.15 (d, J= 1.6 Hz, 1H), δ 7.73 (dd, J= 2.0, 1.6 Hz, 1H), δ 7.67 (d, J= 9.0Hz, 1H), δ 7.53 (d, J= 9.0 Hz, 1H), δ 7.07 (dd, J= 2.7, 2.3 Hz, 1H), δ 6.78 (d, J= 2.7 Hz, 1H), δ 3.0 (s, 6H), δ 1.35 (s, 9H); \textsuperscript{13}C NMR (CDCl\textsubscript{3}) δ207.65, 150.13, 136.75, 131.01, 130.63, 129.91, 126.19, 125.21, 116.57, 105.54, 44.31, 40.77, 28.83.
Quantum Yield Determinations

A 20mM phosphate buffer solution was made by dissolving 0.24g NaH$_2$PO$_4$·2H$_2$O and 0.35g NaHPO$_4$ in 100mL H$_2$O. A 100μM HSA stock solution was made by dissolving 0.17g of Sigma-Aldrich’s essentially fatty acid free HSA in 25mL of the 22mM phosphate buffer solution. A stock 2.1mM PRODAN solution was made by dissolving 5.1mg PRODAN in 10mL of MeOH. A stock 2.3mM solution of Compound 1 was made by dissolving 5.9mg in 10mL MeOH. A stock 2.6mM solution of Compound 2 was made by dissolving 6.6mg in 10mL of MeOH. 13 test tubes were prepared for the quantum yield determination of each probe: 11 test tubes containing 1mL of 20mM phosphate buffer and a 50μM injection of probe, one test tube containing only 1mL of buffer solution, and one containing 1mL of buffer solution and a 5μL injection of a the probe’s stock solution. The 50μM injections of stock solution were 25μL for PRODAN, 21.5μL for Compound 1, and 19.5μL for Compound 2. Injections of 0μM, 3μM, 6μM, 9μM, 12μM, 15μM, 18μM, 21μM, 24μM, 27μM, and 30μM HSA were added to labeled test tubes and the tubes were left over 2 nights to equilibrate with the probe.
Results and Discussion

The synthesis of Compound 2 first involves the exchange of bromine with lithium. The aryl-lithium compound places most of the electron density on the aromatic carbon atoms since lithium is highly electropositive. This allows the electrophilic attack of the aryl ring to the carbonyl of 2,2-dimethyl-1-(1H-pyrrol-1-yl)-1-propanone. The pyrrole is the better leaving group since a pyrrole anion is more stable than tert-butyl anion. Using a pyrrole reduces the probability of dimerization to produce a highly polar byproduct. However, dimerization was problematic in this synthesis due to excess of starting material with respect to the pyrrole compound.

Figure 7. Schematic of Synthesis for Compound 2

Figure 8. Structure of 1,1-bis(6-(dimethylamino)naphthalen-2-yl)-2,2-dimethylpropan-1-ol (Byproduct)
The increase in quantum yields of probe-HSA complexes relative to free probe in water was determined via fluorescence titrations. The titrations were done by maintaining a constant probe concentration and increasing the concentration of HSA by about 3uM, initially ignoring dilution effects. Dilution effects were accounted for later in the analysis. A buffer solution was necessary instead of purely spectroscopic grade water to prevent denaturing of HSA. Plots of absorption and fluorescence data for each probe are shown in Figures 9-14.

![Figure 9. Titration of PRODAN with HSA (Absorption)](https://example.com/fig9.png)
Figure 10. Titration of PRODAN with HSA (Fluorescence)
Figure 11. Titration of Compound 1 with HSA (Absorption)
Figure 12. Titration of Compound 1 with HSA (Fluorescence)
Figure 13. Titration of Compound 2 with HSA (Absorption)
As the concentration of HSA was increased, the fluorescence peak exhibited a blue shift from about 530nm to 460nm for both PRODAN and Compound 1. This shift is indicative of probe-HSA bonding since HSA has a relatively nonpolar cavity. A red shift from about 430nm to 460nm was observed for increased HSA concentration to Compound 2, indicative of probe aggregation. Aggregation resulted in less precise absorption values since the probe was not dissolved in buffer solution as PRODAN and Compound 1 had been.

The red shifts of PRODAN and Compound 1 were greater than the blue shift of Compound 2. This indicates that the polarity of the aggregate is similar to the polarity of the microenvironment in HSA. However, this also results in poorer absorption data.
All three probes exhibited significant sensitivity to solvent acidity based on increases in fluorescence intensities. The integrated fluorescence intensities are related to an increase in relative quantum yield.

The relative quantum yields were calculated by first determining the fraction, \( f \), of bound probe. This was done by using Equation 1 from Park, Park, and Hamilton. \(^9\)

\[
f = \frac{\sum_i [F - HSA]_i}{[F]_i}
\]  

(1)

In this equation, \([F - HSA]\) is the concentration of probe bound to HSA at site \( i \). The assumption is made that PRODAN only binds to subdomain IIA. \([F]_o\) is the initial concentration of probe. A second assumption made is that the concentration of free HSA is zero since the concentration of probe is so much greater than the concentration of HSA being added and the binding constant is relatively large. This results in essentially complete binding of HAS, meaning that the fraction of bound probe is dependent largely on dilution effects. The ratio of bound probe to free probe is then multiplied by the absorption and integrated fluorescence to yield adjusted absorption and adjusted fluorescence intensities of bound probe, as shown in Equation 2 and Equation 3, respectively.

\[
A_{adj} = fA
\]  

(2)

\[
F_{adj} = \sum_i f F_i
\]  

(3)

The change in adjusted absorbance and fluorescence was plotted against increasing initial HSA concentration, accounting for dilution effects, as shown in Figures 15-20. The best fit line was determined for each plot.
Figure 15. Change in Absorption of PRODAN + HSA vs. Concentration HSA

\[
y = 1.14 \times 10^4 x + 6.16 \times 10^{-3}
\]

\[
R^2 = 9.96 \times 10^{-1}
\]

Figure 16. Change in Integrated Fluorescence of PRODAN + HSA vs. Concentration HSA

\[
y = 4.56 \times 10^{11} x - 6.90 \times 10^5
\]

\[
R^2 = 9.96 \times 10^{-1}
\]
Figure 17. Change in Absorption of Compound 1 + HSA vs. Concentration HSA

\[ y = 27.719x + 0.034 \]

\[ R^2 = 0.9883 \]

Figure 18. Change in Integrated Fluorescence of Compound 1 + HSA vs. Concentration HSA

\[ y = 6E+11x + 1E+06 \]

\[ R^2 = 0.9882 \]
Figure 19. Change in Absorption of Compound 2 + HSA vs. Concentration HSA

\[ y = 6239.5x + 0.0128 \]
\[ R^2 = 0.8351 \]

Figure 20. Change in Integrated Fluorescence of Compound 2 + HSA vs. Concentration HSA

\[ y = 5E+11x - 314659 \]
\[ R^2 = 0.9949 \]
By definition, quantum yield is a measure of the ratio of photons emitted to photons absorbed. Relative quantum yields of probe-HSA complex compared to free probe were determined using Equation 4.

\[ \Phi_{\text{complex, relative to free probe}} = \frac{F_{\text{adj}}/[HSA]}{A_{\text{adj}}/[HSA]} \times \frac{A_{\text{free}}}{F_{\text{free}}} \]  \hspace{1cm} (4)

\( F_{\text{adj}}/[HSA] \) and \( A_{\text{adj}}/[HSA] \) were obtained from the slopes of the plots determining relative increases in absorption and integrated fluorescence.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Relative Quantum Yield Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRODAN</td>
<td>0.80</td>
</tr>
<tr>
<td>Compound 1</td>
<td>3.0</td>
</tr>
<tr>
<td>Compound 2</td>
<td>2.7</td>
</tr>
</tbody>
</table>

**Figure 21. Relative Increases in Quantum Yields**

The relative quantum yield can be used to determine solvent acidity using the calibration curve shown in Figure 22 which was developed in the Abelt lab.

**Figure 22. Relative Fluorescence of Probe with Changing Solvent Acidity**

The linear relationships in Figure 22 can be represented by Equation 5, where \( I \) is the integrated fluorescence of probe and SA is solvent acidity.
\[ \text{slope} = \frac{\log(I_{\text{max}}/I)}{SA} \]  

(5)

The \(I_{\text{max}}\) value falls out of the equation when solving for the acidity of an unknown solvent, as shown in Equation 6. \(I_{\text{water}}\) is the fluorescence of probe in water. \(I\)

\[ \log\left(\frac{I_{\text{max}}}{I_{\text{water}}}\right) - \log\left(\frac{I_{\text{max}}}{I_{\text{HSA}}}\right) = \text{slope} \times (SA_{\text{water}} - SA_{\text{HSA}}) \]  

(6)

Equation 6 can be rearranged to yield Equation 7, the solvent acidity of an unknown, in this case the solvent acidity inside HSA. The solvent acidity of water, as reported by Catalan is 1.062.\(^{10}\)

\[ SA_{\text{unknown}} = SA_{\text{water}} - \frac{\log I_{\text{HSA}}}{\text{slope}} = SA_{\text{water}} - \frac{\log(\text{relative quantum yield})}{\text{slope}} \]  

(7)

<table>
<thead>
<tr>
<th>Probe</th>
<th>Solvent Acidity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRODAN</td>
<td>1.20</td>
</tr>
<tr>
<td>Compound 1</td>
<td>0.81</td>
</tr>
<tr>
<td>Compound 2</td>
<td>0.86</td>
</tr>
</tbody>
</table>

Figure 23. Summary of Solvent Acidity in HSA by Probe

PRODAN is not nearly as accurate a probe of solvent acidity as the other two PRODAN derivatives, based on relative quantum yields since there is greater overlap of free probe and probe-HSA peak fluorescences. A decreased relative fluorescence of the free probe results in less overlap of free probe and probe-HSA fluorescence peaks. However, it is difficult to determine which of the derivatives is better since the absorption data for Compound 2 is not as precise as for the other probes due to aggregation.

The t-butyl group on the synthesized probe allows for increased hydrogen bond accepting ability of the carbonyl relative to the strained carbonyl of Compound 1. However, steric strain of the bulky t-butyl group limits movement of the carbonyl at the mouth of the HSA binding cavity which decreases hydrogen bonding ability relative to PRODAN.
Conclusion

The synthesis of 1-[6-(dimethylamino)-2-naphthalenyl]-2,2-dimethyl-1-propanone has a relatively low yield of 6.4%. Despite using a pyrrole to add the t-butyl and carbonyl substituent, the majority of starting material dimerized to form a highly polar byproduct. Research in determining a better method of reducing dimerization would make the reaction much more efficient.

**Compound 1**, 3-(dimethylamino)-8,9,10,11-tetrahydro-7H-cyclohepta[a] naphthalen-7-one, was the best probe for measuring solvent acidity since the fluorescence of the free probe was almost entirely quenched and there was great sensitivity in fluorescence due to hydrogen bonding effects. The formation of an aggregate of **Compound 2** makes it a less practical probe of solvent acidity. The true acidity in HSA due to presence of water molecules is likely around 0.81.

Cholesterol is a four ring system. To have a better understanding of cholesterol transport, it is best if the models also contain four rings. The next step for this research would be to develop a four ring PRODAN model of cholesterol for each of the probes and determine solvent acidity within HSA of the modified probe-HSA complex.

Another possible route of investigation would be to crystallize the probe-HSA complexes and determine exactly how the probe is interacting with HSA, and to what extent. This will give information regarding the availability of the carbonyl to hydrogen bond with solvent molecules trapped within the HSA molecule. It would also be important to determine a binding constant.

Lastly, it would be interesting to develop a computational model to understand the role of solvent particles within HSA and monitor how changes in microacidity within HSA affect the molecule’s ability to bind to and transport cholesterol.
Appendix A

$^1$HNMR and $^{13}$CNMR for

1-[(6-(dimethylamino)-2-naphthalenyl]-2,2-dimethyl-1-propanone
Figure A1. $^1$HNMR of 1-[6-(dimethylamino)-2-naphthalenyl]-2,2-dimethyl-1-propanone

Figure A2. $^{13}$CNMR of 1-[6-(dimethylamino)-2-naphthalenyl]-2,2-dimethyl-1-propanone
References

(1) Zhao, Y.; Marcel, Y. L. Biochemistry (N. Y. ) 1996, 35, 7174-7180.


