Fluorescence Spectra Study of Sterically Strained Isomers of C24H14 polycyclic aromatic (PAHs) & Characterization of a Bio-sourced, Fluorescent, Ratiometric pH Indicator with Alkaline pKa

Samar Abd Alelah
University of Central Florida

Part of the Chemistry Commons
Find similar works at: https://stars.library.ucf.edu/etd2020
University of Central Florida Libraries http://library.ucf.edu

This Doctoral Dissertation (Open Access) is brought to you for free and open access by STARS. It has been accepted for inclusion in Electronic Theses and Dissertations, 2020- by an authorized administrator of STARS. For more information, please contact STARS@ucf.edu.

STARS Citation
https://stars.library.ucf.edu/etd2020/320
FLUORESCENCE SPECTRA STUDY OF STERICALLY STRAINED ISOMERS OF C24H14 POLYCYCLIC AROMATIC (PAHs) & CHARACTERIZATION OF A BIO-SOURCED, FLUORESENT, RATIOOMETRIC pH INDICATOR WITH ALKALINE pK_a

by

SAMAR MOHAMMED ABD ALELAH
B.S. Chemistry Anbar University, Iraq, 20005
M.S. Chemistry Anbar University, Iraq, 2009
M.S. Chemistry University of Central Florida, 2019

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry in the College of Sciences at the University of Central Florida Orlando, Florida

Fall Term
2020

Major Professor: James K. Harper, Andres Campiglia
©2020 Samar Mohammed
ABSTRACT

This thesis is composed to two studies, each of which relies on recent advances in time-dependent density functional theory (TD-DFT) to accurately model fluorescence spectra. The first study focuses on evaluating the fluorescence spectrum of polycyclic aromatic hydrocarbons (PAHs) having a molecular weight of 302. Three isomers are analyzed including dibenzo[a,j]fluoranthene, dibenzo[a,k]fluoranthene and dibenzo[a,l]fluoranthene. These isomers differ in the placement of one of six aromatic rings and in the amount that each molecule deviates from planarity. The TD-DFT methods are used to compute spectra in an environment that simulates solvent (n-hexane). These computed spectra are compared to vibronically resolved experimental data obtained at cryogenic temperatures. Computed spectra for dibenzo[a,j]fluoranthene and dibenzo[a,k]fluoranthene are blue shifted versus experimental data. Conversely, the computed spectra of dibenzo[a,l]fluoranthene is red shifted. This difference is attributed to steric interactions in dibenzo[a,l]fluoranthene that creates a deviation from planarity.

The second project describes the characterization of a pH-responsive fluorophore from an endophytic fungus isolated from sand pine. The endogenous fluorescence of the live organism was measured using fluorescence microscopy. Computational interpretation of the spectra was accomplished with the TD-DFT
methods and include solvent effects. The combined use of experimental and theoretically predicted spectra revealed the pH dependent equilibria and photo-excited tautomerization of the natural product, 5-methylmellein. This product shows promise both as a stand-alone pH-indicating fluorophore with alkaline pK\textsubscript{a}, and as "green" feedstock for synthesis of custom fluorophores.
In loving the memory of my parents
Thank you for your constant source of encouragement and making me the person
I am today.

To my husband, Dr. Muthanna
Without your endless support, my achievement would not have been possible.
I love you!

To my dear children, Yaqeen, Lujain, Wafaa, and Husam
For putting a smile on my face and being the bright light in my heart.

To my lovely brothers and sisters
ACKNOWLEDGMENTS

Foremost, I would like to express special appreciation and thanks to my research advisors Dr. James Harper and Dr. Andres Campiglia for their enormous assistance and continuous support towards the fulfillment of my Ph.D. degree. I will be forever grateful for your excellent guidance, contribution continued support, encouragement during this journey, and your advice helped me in all the time of research and writing of this thesis.

Besides my advisor, I am most grateful to Dr. Emily Heider for imparting her knowledge, patience, enthusiasm, and time towards our research project. I owe much gratitude to her for the valuable help.

I would also like to thank my dissertation committee members: Dr. Andres Campiglia, Dr. Shengli Zou, and Dr. Andrew Frazer, thanks for taking the time to assist and advise me in this effort. It was such a privilege to have you on my dissertation committee.

I also thank the University of Central Florida, College of Sciences, and the Department of Chemistry for giving me the opportunity to further pursue my professional goals.
I would especially like to thank the Iraqi government and the Iraqi Ministry of Higher Education and Scientific Research (MOHESR) for providing this opportunity and their continuous support to complete the doctoral journey.

I would like to present sincere thankfulness to my mother and the soul of my dear father, who died in August 2017 for their numerous engorgements. I dedicate my dissertation work to them.

My deepest gratitude goes to my husband Muthanna Owaid for being the first person to encourage me to pursue my interests. You have given me endless support at the very start, and motivation to the very end.

Above all, I thank Allah for everything you have given to me, Alhamdulillah.
# TABLE OF CONTENTS

LIST OF FIGURES........................................................................................................... x

LIST OF ACRONYMS/ABBREVIATIONS........................................................................... xiv

CHAPTER ONE: GENERAL INTRODUCTION................................................................. 1

Overview of Polycyclic Aromatic Hydrocarbons......................................................... 1

PAHs Structure................................................................................................................. 1

PAHs Characteristics......................................................................................................... 2

Sources of PAHs................................................................................................................ 6

Toxicity & de-Toxicity of PAHs....................................................................................... 8

Fluorescence Spectroscopy............................................................................................. 16

References......................................................................................................................... 24

CHAPTER TWO: THEORETICAL PREDICTION OF FLUORESCENCE SPECTRA FOR STERICALLY STRAINED ISOMERS OF C\textsubscript{24}H\textsubscript{14}: A COMPARISON WITH VIBRONICALLY-RESOLVED EXPERIMENTAL DATA......................................................................................................................... 37

Abstract............................................................................................................................ 37

Introduction......................................................................................................................... 39

Material and Methods..................................................................................................... 44

Results and Discussion.................................................................................................... 47

Acquiring High Resolution Experimental Fluorescence Spectra................................. 47

Comparing Computed and Experimental Fluorescence Spectra................................. 50
CHAPTER THREE: CHARACTERIZATION OF A BIO-SOURCED, FLUORESCENT, RATIO METRIC PH INDICATOR WITH ALKALINE $PK_a$. 75

Abstract .................................................................................................................. 75

Introduction ............................................................................................................. 76

Materials and Methods............................................................................................ 78

Reagents and Preparation ....................................................................................... 78

Fungal Growth and Isolation .................................................................................... 79

Instrumentation ....................................................................................................... 80

Fluorescence Microscopy ....................................................................................... 80

UV-Vis Spectroscopy ............................................................................................... 81

Quantum Yield Measurements ............................................................................... 81

Computational Methods ......................................................................................... 82

Results and Discussion ........................................................................................... 84

References ............................................................................................................... 99
LIST OF FIGURES

Figure 1. Structures of the three PAHs isomers studied herein ....................... 2
Figure 2. Molecular structures of 16 pollutants EPA-PAHs (12) ...................... 4
Figure 3. Chemical and physical characteristics of 16 EPA-PAHs (13) ............... 5
Figure 4. Sector share of PAH emission, EEA member countries (EEA, 2012) (17) .................................................................................................................. 7
Figure 5. Mechanism of DNA damage motivate by ROS (28) ......................... 10
Figure 6. Mechanism of forming DNA adducts by electrophilic metabolites of B[a]P (28) .................................................................................................................. 11
Figure 7. Damaging of DNA process by creating toxic products from PAHs (30) ............................................................ .............................................................. 12
Figure 8. The main pathways controlling the metabolization and toxicity of PAHs (30) .................................................................................................................. 13
Figure 9. Induction of the aryl hydrocarbon receptor (AhR) pathway by a model PAH, and expression of the CYP450 system (36) ...................................................... 15
Figure 10. Jablonski diagram. A is the absorption, F is the Fluorescence, P is the phosphorescence, IC is internal conversion, ISC is the intersystem crossing, and VR is the vibrational relaxation ...................................................................................... 17
Figure 11. Structures of the three isomers of C_{24}H_{14} studied herein ............ 44
Figure 12. The experimental fluorescence spectra of the three PAHs evaluated herein. All spectra are acquired in n-octane. The wavelength of the S_{1,0}→S_{0,0} transition is included on each of the spectra acquired at 4.2K ............... 49
Figure 13. The correction between computed and theoretical emission data. The best fit line is linear and is described by $\lambda_{cm} - 1_{Calculated} = -51.8 \lambda_{nm_{Experimental}} + 46,539$ cm$^{-1}$ ($R^2 = 0.925$).

Figure 14. A comparison of the computed and experimental peak positions for major peaks in the emission spectra of benzo[a]pyrene, dibenzo[a,e]pyrene, dibenzo[a,l]pyrene, dibenzo[a,i]pyrene and benzo[a]pyrene-7,8,9,10-tetrol (green) and the new data for DB[a,j]F and DB[a,k]F (yellow).

Figure 15. Experimental (top) and theoretical (bottom) fluorescence spectra for DB[a,j]F (left) and DB[a,k]F (right). Theoretical spectra were computed at the CAM-B3LYP/cc-pVDZ level of theory and included n-hexane as a solvent using the polarizable continuum model.

Figure 16. Experimental (top) and theoretical (bottom) fluorescence spectra for DB[a,j]F (left) and DB[a,k]F (right). Theoretical spectra shown were empirically corrected using the relationship $\lambda_{cm} - 1_{Calculated} = -46.5 \lambda_{nm_{Experimental}} + 44,262$ cm$^{-1}$ ($R^2 = 0.98$). Computed at the CAM-B3LYP/cc-pVDZ level of theory and included n-hexane as a solvent using the polarizable continuum model.

Figure 17. A comparison of the experimental and theoretical fluorescence spectrum of DB[a,l]F. The large overestimation of the $S_{1,0} \rightarrow S_{0,0}$ transition in the theoretical spectrum is unexpected and deviate by roughly +65 nm, on average, from prior computed spectra where an underestimation of this transition is consistently observed.

Figure 18. The computed peak positions of DB[a,l]F (blue circles) systematically differs from those predicted for other PAHs studied herein.

Figure 19. The correction between the deviation planarity in the PAH evaluated and the difference between the computed and experimental $S_{1,0} \rightarrow S_{0,0}$ transition. A least-
squares fit to the data gives \( y = -0.37x + 16.32 \) \((R^2 = 0.9996)\) where \( x \) and \( y \) are as defined in the plot.

Figure 20. An illustration of the deviation from planarity that is observed in the energy minimized structure of DB[a,l]F. The ring twisting alleviates some of the close contact between two protons but is postulated to alter \( \pi \)-delocalization and thereby diminish the accuracy of the theoretical spectrum.

Figure 21. The endophytic fungus was isolated from a small twig of a sand pine (Pinus clausa). Emergent hyphae were imaged using a light microscope (image A). The emission spectrum (B) obtained from the hyphae were recorded using a fluorimeter optically coupled to a microscope.

Figure 22. Structures of the two tautomeric forms of fluorescent product 5-methylmellein. As determined using carbon-13 chemical shifts and \( ^1J_{CC} \) couplings (Hz), 5-methylmellein in CDCl3 is exclusively form 1.

Figure 23. A) Example of pH-dependent absorbance spectra of 0.26 \( \mu \)M 5-methylmellein in various solutions ranging from pH 7.0 to 12.3 (0.010 M phosphate buffer). A single isosbestic point, \( \lambda = 332 \) nm, is present in the absorbance spectra and indicates the presence of only two forms of 5-MM in equilibrium. B) Emission spectra of 1.24x10\(^{-4}\) M 5-methylmellein with \( \lambda_{ex} = 390 \) nm show two isosbestic points (401 nm and 478 nm) indicating the presence of three species.

Figure 24. Measured emission spectra for the protonated (pH 5.8) and deprotonated (pH 11.2) forms of 5-MM. Also plotted are the computed spectra for deprotonated base and the keto and enol tautomers of 5-MM.

Figure 25. Structure of the dihydronaphthalenones that show some features analogous to 5-methylmellein. In the figure, \( R \) and \( R' \) represent functional groups.
varied over 11 different structures as reported elsewhere, with indications from spectral data the keto form was the dominant form in solution………………… 91

Figure 26. Emission intensities of the 5-MM keto tautomer (emission measured at \( \lambda = 390 \) nm) and enol tautomer (\( \lambda = 520 \) nm) as a function of pH. The sigmoidal fit to the data indicated the pK\(_a\) of both forms of 5-MM. The R\(^2\) for the fit of the data at 520 nm is 0.997. The R\(^2\) for the fit of the data at 390 nm is 0.985……………… 93

Figure 27. The 5-MM spectrum in pH 10.05 buffered solution (black line) and the linear-least squares fit to the spectrum (red line) show how the pH of an unknown solution might be ascertained using spectra of 5-MM in standard acidic and basic solutions………………… 96
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Absorption</td>
</tr>
<tr>
<td>DB[a,j]F</td>
<td>dibenzo[a,j]fluoranthene</td>
</tr>
<tr>
<td>DB[a,k]F</td>
<td>dibenzo[a,k]fluoranthene</td>
</tr>
<tr>
<td>DB[a,l]F</td>
<td>dibenzo[a,l]fluoranthene</td>
</tr>
<tr>
<td>ELCR</td>
<td>excess lifetime cancer risks</td>
</tr>
<tr>
<td>F</td>
<td>Fluorescence</td>
</tr>
<tr>
<td>FOP</td>
<td>fiber optic probe</td>
</tr>
<tr>
<td>HMWPAHs</td>
<td>High molecular weight Polycyclic aromatic hydrocarbons</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>IC</td>
<td>internal conversion</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>ISC</td>
<td>Inter-system crossing</td>
</tr>
<tr>
<td>IUPAC</td>
<td>International Union of Pure and Applied Chemistry</td>
</tr>
<tr>
<td>LAM</td>
<td>large amplitude motion</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>P</td>
<td>Phosphorescence</td>
</tr>
<tr>
<td>PAHs</td>
<td>Polycyclic aromatic hydrocarbons</td>
</tr>
<tr>
<td>PCM</td>
<td>Polarizable Continuum Model</td>
</tr>
<tr>
<td>PDA</td>
<td>Potato dextrose agar</td>
</tr>
<tr>
<td>QSAR</td>
<td>Quantitative structure-activity relationship</td>
</tr>
<tr>
<td>RMSD</td>
<td>Root-mean squared deviation</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>TD-DFT</td>
<td>Time-dependent density functional theory</td>
</tr>
<tr>
<td>USEPA</td>
<td>US Environmental Protection Agency</td>
</tr>
<tr>
<td>VR</td>
<td>Vibrational relaxation</td>
</tr>
<tr>
<td>5-MM</td>
<td>5-methylmellein</td>
</tr>
</tbody>
</table>
CHAPTER ONE: GENERAL INTRODUCTION

Overview of Polycyclic Aromatic Hydrocarbons

PAHs Structure

High molecular weight polycyclic aromatic hydrocarbons (HMWPAHs) are organic compounds which consist mainly of carbon and hydrogen, fused aromatic rings and a sparse representation of heteroatoms (i.e. N, S, and O) sometimes substituted for carbons.(1,2,3) The PAHs are produced from the partial combustion of organic substances and, because many are toxic to humans, they are classified as pollutants. In practice, most studies on PAHs focus on compounds having less than 8 rings because such materials exhibit the most significant bioactivity.(4) In this thesis the first study focuses on HMWPAHs shown in figure 1 and having a molecular weight of 302.
Figure 1. Structures of the three PAHs isomers studied herein.

PAHs Characteristics

Nowadays, due to the significant development in industry and transportation, PAHs have become a critical environmental threat that is recognized worldwide. This toxicity justifies the considerable research on the properties of these compounds. PAHs have biological activity that depends somewhat on molecular weight. Accordingly, PAHs can be conveniently classified into high molecular weight range having 4-7 aromatic rings and a low molecular weight range with 2-3 aromatic rings. Although the HMWPAHs have limited solubility in water, long term exposure results in high mutagenic and carcinogenic activity in mammals.(7,8) In some biological systems PAHs can behave as ligands that interact with certain
enzyme receptors. For example, Cytochrome P450 is an essential detoxification enzyme that has an aryl hydrocarbon receptor which binds some PAHs to enable their degradation. (10)

Among the myriad PAHs that exist in the environment, the US Environmental Protection Agency (EPA) has listed 16 compounds as “Consent Decree” priority pollutants which are notable due to their toxicological influence on the environment. According to the EPA list, benzo[a]pyrene (BaP) is considered as the most carcinogenic PAH due to its high toxicity. The EPA recommends routine monitoring of these 16 pollutants to reduce human exposure. Figure 2 and Figure 3 display the molecular structure of the 16 EPA PAH priority pollutants and their properties, respectively. (11,12)
Figure 2. Molecular structures of 16 pollutants EPA-PAHs (12)
<table>
<thead>
<tr>
<th>PAHs Name</th>
<th>Formula</th>
<th>Molecular weight (g/mol)</th>
<th>Solubility in water (mg/L)</th>
<th>B.P. (°C)</th>
<th>M.P. (°C)</th>
<th>Vapor Pressure (mmHg at 25 °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>C_{10}H_{8}</td>
<td>128.17</td>
<td>31.8</td>
<td>218</td>
<td>80.2</td>
<td>8.52 × 10^{-2}</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>C_{12}H_{10}</td>
<td>154.21</td>
<td>3.8</td>
<td>279</td>
<td>93.4</td>
<td>2.5 × 10^{-3}</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>C_{12}H_{8}</td>
<td>152.2</td>
<td>16.1</td>
<td>280</td>
<td>91.8</td>
<td>6.68 × 10^{-3}</td>
</tr>
<tr>
<td>Anthracene</td>
<td>C_{14}H_{10}</td>
<td>178.23</td>
<td>0.045</td>
<td>342</td>
<td>216.4</td>
<td>6.53 × 10^{-6}</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>C_{14}H_{10}</td>
<td>178.23</td>
<td>1.1</td>
<td>340</td>
<td>100.5</td>
<td>1.2 × 10^{-4}</td>
</tr>
<tr>
<td>Fluorene</td>
<td>C_{13}H_{10}</td>
<td>166.22</td>
<td>1.9</td>
<td>295</td>
<td>216-7</td>
<td>6 × 10^{-4}</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>C_{16}H_{10}</td>
<td>202.26</td>
<td>0.26</td>
<td>375</td>
<td>108.8</td>
<td>9.22 × 10^{-6}</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>C_{18}H_{12}</td>
<td>228.29</td>
<td>0.011</td>
<td>438</td>
<td>158</td>
<td>4.11 × 10^{-3}</td>
</tr>
<tr>
<td>Chrysene</td>
<td>C_{18}H_{12}</td>
<td>228.29</td>
<td>0.0015</td>
<td>448</td>
<td>254</td>
<td>6.23 × 10^{-9}</td>
</tr>
<tr>
<td>Pyrene</td>
<td>C_{16}H_{10}</td>
<td>202.26</td>
<td>0.132</td>
<td>150.4</td>
<td>393</td>
<td>4.5 × 10^{-6}</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>C_{20}H_{12}</td>
<td>252.32</td>
<td>0.0038</td>
<td>495</td>
<td>179</td>
<td>5.49 × 10^{-9}</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>C_{20}H_{12}</td>
<td>252.32</td>
<td>0.0015</td>
<td>481</td>
<td>168.3</td>
<td>5 × 10^{-7}</td>
</tr>
<tr>
<td>Benzo[k]fluoranthene</td>
<td>C_{20}H_{12}</td>
<td>252.32</td>
<td>0.0008</td>
<td>480</td>
<td>215.7</td>
<td>9.7 × 10^{-10}</td>
</tr>
<tr>
<td>Dibenzo[a,h] anthracene</td>
<td>C_{22}H_{14}</td>
<td>278.35</td>
<td>0.0005</td>
<td>524</td>
<td>262</td>
<td>9.55 × 10^{-10}</td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene</td>
<td>C_{22}H_{12}</td>
<td>276.34</td>
<td>0.00026</td>
<td>500</td>
<td>277</td>
<td>1 × 10^{-10}</td>
</tr>
<tr>
<td>Indenol[1,2,3-cd] pyrene</td>
<td>C_{22}H_{12}</td>
<td>276.34</td>
<td>0.062</td>
<td>536</td>
<td>161-3</td>
<td>1.25 × 10^{-3}</td>
</tr>
</tbody>
</table>

Figure 3. Chemical and physical characteristics of 16 EPA-PAHs (13).
Sources of PAHs

PAHs are ubiquitous environmental pollutants that originate from natural and anthropogenic sources through various processes (14). Natural PAH sources include volcanoes, forest fires, oil seeps, and coal deposits. Furthermore, PAH environmental pollution is caused by microorganisms & plant metabolisms (15). Generally, humans are responsible for the greatest amount of PAHs pollution due to the burning of oil, wood, coal, and gas as organic fuels. Such activities can produce huge PAH emissions. The creation of PAHs depends on the condition under which fuels are combusted including moisture content, temperature of combustion, and the presence of sufficient oxygen. (16) Figure 4 depicts the rate of PAHs emission from various sources. (17)
Figure 4. Sector share of PAH emission, EEA member countries (EEA,2012) (17).
Toxicity & de-Toxicity of PAHs

PAHs are considered to be a significant health concern due to their carcinogenic and mutagenic characteristics. Accordingly, the possible risks of these compounds have been widely studied in different environments. These studies have revealed their toxic influence to human organs like kidney, lung, and liver. Moreover, frequent exposure to PAHs might raise the risk of bladder, skin and lung cancers (18). The toxicity of PAHs arises from the chemical structure of PAHs and their metabolic transformation into reactive intermediates having the potential to bind to DNA and cellular proteins. There are two basic mechanisms to express the toxicity of PAHs: genotoxicity and phototoxicity. Because PAHs are generally biologically inert compounds, metabolic activation is required for their elimination. This activation requires enzymatic modification to convert them to high water-soluble substances that are more easily excreted.(19). Metabolic modification occurs by addition of a polar group in the PAH via oxidation, reduction, and hydrolysis. The product created is more soluble in water and can therefore be excreted. For PAH metabolism, cytochrome-P450 (CPY) enzymes perform the most common metabolic biological activation.(20,21) The metabolically modified PAHs are often highly reactive metabolites capable of covalently binding to DNA. This binding creates the carcinogenic activity of PAHs (22).
In 2013, the International Agency for Research on Cancer (IARC) reported that air pollution, designated as group 1 pollution, causes cancer in humans. Moreover, a complex mixture of PAHs was found to occur in group 1. This finding helps explain the 6.4 million deaths from cancer in 2015 caused by exposure to air pollution.(23)

The mutagenicity and carcinogenicity caused by the 16 priority pollutants (Figure 1 and 2) has been assessed, using benzo(a)pyrene as an indicator. This assessment provides an estimate of the “excess lifetime cancer risks” (ELCR).(23) PAHs containing four or more aromatic rings adsorb onto surfaces of particles suspended in the air, soil, or water, are a key environmental concern because of their mutagenic and carcinogenic properties. In air, PAHs are particularly reactive with other chemicals and can create PAHs that are more toxic than the original compounds.(24,25)

PAHs that are activated by metabolization can react with macromolecules to cause damage and to form free radicals such as Reactive Oxygen Species (ROS). These ROS are particularly destructive because they can form DNA adducts as shown in figure 5 (26,27,28)
Figure 5. Mechanism of DNA damage motivated by ROS (28).
In DNA, certain PAHs can bind at two locations with 2-deoxyguanosine residues. The first binding is (N2-dG) from DNA strands. The activated PAHs bond with the exocyclic amino group. The second potential bonding is at the N7-deoxyguanosine location to yield cation radicals. In cells, the final PAHs attach to DNA strands, as a result, genotoxic impacts of their mother PAHs hydrocarbons shown as in figure 6 (28).

Figure 6. Mechanism of forming DNA adducts by electrophilic metabolites of B[a]P (28).
During the exposure to polluted air, PAHs are absorbed, spread, and accumulated mostly in lipophilic tissues of the body, and by a passive diffusion process, they pass cell membranes (29). The biotransformation of PAHs described above, creates toxic products that can cause genetic defect (30,31) as depicted in figure 7.

Figure 7. Damaging of DNA process by creating toxic products from PAHs (30).
The mechanism of the metabolization of PAHs involves three main pathways of the enzymatic activation of PAHs: CYP (peroxidase pathway), CYP/EH and CYP1A1/1B1 epoxide hydrolase pathway, and AKR (aldo-keto reductase) pathway (30) as shown in figure 8.

Figure 8. The main pathways controlling the metabolization and toxicity of PAHs (30).
Xenobiotics (including PAHs, drugs, flavors, etc.) that humans are exposed to daily can be metabolized by enzymes in reactions the typically oxidize or reduce these materials. Cytochromes, such as, CYP1A1, CYP1A2, and CYP1B1, play a vital role in this PAHs activation and it has been demonstrated that CYP1A1 has a higher turnover rate than other isoforms such as CYP1A2, CYP1B1(32).

Generally, the biotransformation of PAHs can be accomplished by the activation of CYP450 system that results in the regulation of enzymes which oxidize PAHs and lead to the creation of PAHs metabolites (33). The aryl hydrocarbon receptor (AhR) is responsible for the binding with the aromatic ring of the PAHs compounds to start PAHs metabolism(34). In the cytosol of the cell, PAHs bind to aryl hydrocarbon receptor which induces a transcription mRNA to synthesize CYP450 enzymes in the cytosol. These enzymes target PAHs and complete their biotransformation (35) as shown in figure 9.
Figure 9. Induction of the aryl hydrocarbon receptor (AhR) pathway by a model PAH, and expression of the CYP450 system (36).
Fluorescence Spectroscopy

Photoluminescence spectroscopy involves the observation of emitted light radiation of 200-700nm through the deactivation of electronically excited substances. At normal conditions, most organic molecules have pairs of electrons (mostly even number of valence electrons) with spin in opposite directions occupied in the orbitals of the lowest energy and this results in electron spin is zero. A state with net spin equal to zero is termed a singlet state (37). The singlet state of lowest energy is called as the ground state, and it is symbolized (S\(_0\)) in the Jablonski diagram figure 10.
Figure 10. Jablonski diagram. A is the absorption, F is the Fluorescence, P is the phosphorescence, IC is internal conversion, ISC is the intersystem crossing, and VR is the vibrational relaxation.
During the absorption of the light (A), an atom or a molecule can move from the ground state to an excited state of higher energy with about $10^{-15}$ seconds. If the movement of the electron happens without changing in the spin, the excited state will be occupied with two unpaired electrons with anti-parallel spins and, consequently, net spin equivalent to zero. This state with these features is known as a singlet excited state. A singlet excited states are called first singlet-excited states ($S_1$) and second singlet excited states ($S_2$) as shown in figure 10. If the electronic spin changes through the transition , the excited state will be occupied by two unpaired electrons with parallel spin and ,therefore, the net spin is equal to one, and the excited state is termed triplet state with a symbol ($T_1$) with the lowest energy whereas the triplet state of higher energy is denoted by $T_n$ as shown in figure 1-10. Following the excitation, the molecule usually experiences a radiationless process that is called a vibrational relaxation (VR). VR occurs typically on the order of $10^{-11}$ to $10^{-10}$ s. This mechanism happens when the excited molecule releases the extra vibrational energy to get to the lowest vibrational level of the state energy, and it is resulting in the excess of vibrational energy is transferred to thermal energy. Another radiationless phenomenon is known as an internal conversion (IC). The internal conversion process happens on order of $10^{-12}$ s. This mechanism occurs when the excited molecule stays in an upper excited singlet state ($S_2$). The molecule moves
from the ground vibrational energy level of $S_2$ to the upper excited singlet state ($S_1$). This process results in the conversion of excitation energy into vibrational-rotational energy. After the internal conversion process, vibrational relaxation takes place into the ground vibrational level of the $S_1$ state. From the $S_1$ state, deactivation of the molecule may occur throughout different mechanisms, for example, a non-radiative deactivation method which is called the External conversion (EC). In this process, the excess electronic energy from the excited molecule passes on to the surrounding or solvent molecules via collisions.(38,39,40) Also, The excited molecule from the lowest vibrational level of $S_1$ may return to the ground state by emitting a photon with no change in the electronic spin through a process that is known as fluorescence (F), and it happens in a time of $10^{10} - 10^{-7}$ s. Fluorescence typically happens from the lowest vibrational level of $S_1$, and it emits spectra with longer wavelength than absorption process. It occurs if the energy gap between the ground state and the lowest vibrational level of singlet $S_1$ state equal to the energy of the emitted photon. Fluorescence from second singlet state $S_2$ is unusual, and it only happens if the energy gap between the first singlet $S_1$ state and second singlet $S_2$ state is large enough that internal conversion is not preferred. The emission of fluorescence does not take place if the lowest vibrational level of the $S_1$ state overlaps with the ground state, the excited state is deactivated by non-radiative relaxation process (41).
The power of fluorescence emission, $F$, is proportional to the radiant power of the excitation beam that is absorbed by the system

$$F = K'(P_0 - P)$$

$P_0$ is the power of the beam incident upon the solution and $P$ is its power after traversing a length $b$ in the solution. The constant $K'$ depends upon the quantum efficiency of the fluorescence process.

Use Beer’s Law in the form:

$$\frac{P}{P_0} = 10^{-\varepsilon bc}$$

$\varepsilon$ is the molar absorptivity, $\varepsilon bc$ is the absorbance

$$F = K'P_0(1 - 10^{-\varepsilon bc})$$

The exponential term can be expanded as a Maclaurin series to

$$F = K'P_0(2.303\varepsilon bc - \frac{(2.303\varepsilon bc)^2}{2!} \frac{(2.303\varepsilon bc)^3}{3!} \ldots)$$

All of the subsequent terms are small with respect to the first

$$F = K'P_0(2.303\varepsilon bc)$$

$$F = Kc$$

For small absorbance values $\varepsilon bc<0.05$, fluorescence intensity is proportional to concentration.

Inter-system crossing ISC is another process may occur from $S1$ to ground state. ISC is a non-radiative process involving a crossover between two electronic
states with various spin multiplicities, and it occurs on the time \((10^{-10} – 10^{-7} \text{ s})\) that is like to the one for fluorescence. The molecule can move from \(S_1\) to the excited triplet state manifold \(T_n\), and by series of IC and VR mechanisms, the molecule can get to the lowest vibrational level of \(T_1\), and via ISC process the molecule can get back to the manifold of the excited singlet state. From \(T_1\), the molecule can pass to the ground state, if reverse ISC does not happen, through two possible ways either via ISC followed by VR or via the emission of a photon in a mechanism known as phosphorescence (P). The emission of phosphorescence happens on the order between \(10^{-3}\) and \(10^{3}\) s, and it occurs in the area with a long wavelength due to the smaller energy gap between the first triplet state and the ground state comparing to the fluorescence. Usually, the radiative deactivation mechanisms like fluorescence and phosphorescence are in competition with the different non-radiative deactivation mechanisms. The intensity of fluorescence and phosphorescence essentially rely on the relative efficiencies of all competing mechanisms.\(^{38,42}\)

The efficiencies of fluorescence and phosphorescence are frequently expressed in terms of quantum yield \(\phi\). The quantum yield for fluorescence, \(\phi_F\), is equal to, \(\Phi_F/\Phi_A\), the ratio between the rate of fluorescence to the rate of absorption where \(\Phi_F = k_F n_{s1} V\) and \(\Phi_A = k_A n_{s0} V\). \(k_F\) and \(k_A\) are the rate of fluorescence and
absorption, respectively, \( V \) is the volume of sample illuminated, \( n_{sx} \) is the number of molecules dominating the given electronic state \( x \).

We can presume under the steady state that 
\[
    n_{S1} = n_{S0} k_A / (k_F + k_{nr}),
\]
where \( k_{nr} \) is the total of rates for the radiationless mechanisms including internal conversion, \( k_{ic} \), external conversion, \( k_{ec} \), and intersystem crossing, \( k_{isc} \). The fluorescence quantum yield in terms of the rate constants of the different activation and deactivation mechanisms \((43,44)\) is shown by equation (1):
\[
    \phi_F = \frac{K_F}{K_F + K_{nr}} \quad (1 - 1)
\]

This explains that to enhance the fluorescence quantum yield, and consequently the fluorescence intensity, one needs to minimize the rate influences from radiationless processes. A similar statue occurs for phosphorescence. The quantum yield of phosphorescence can be expressed by the equation (2)
\[
    \phi_P = \frac{K_{isc}}{K_{isc} + K_F + K_{nr}} \times \frac{K_P}{K_P + K_{nr}} \quad (1 - 2)
\]

We can notice that the equation (1.2) takes into consideration the efficiency of ISC process, Consequently, to maximize the phosphorescence intensity and the
phosphorescence efficiency, minimization of both radiationless mechanisms and fluorescence relative to ISC becomes obvious.

The decay of both processes (fluorescence and phosphorescence) can illustrate by the equation (3) because both excited states including $S_1$ and $T_1$ are typically deactivated by first-order mechanisms

$$I_t = I_0 e^{-t/\tau_L} \quad (1-3)$$

Where $I_0$ is the luminescence intensity of fluorescence or phosphorescence at time zero and $\tau_L$ is the luminescence lifetime. Generally, the definition of the lifetime decay is the time it requires for the luminescence signal to decay to 1/e of its initial value. Equations (4) and (5) describe the lifetimes for both fluorescence and phosphorescence.

$$\tau_F = (K_F + K_{nr})^{-1} \quad (1-4)$$

$$\tau_P = (K_P + K_{nr})^{-1} \quad (1-5)$$

Equations (1), (2), (4) and (5) clarify that radiationless decay mechanisms lower the fluorescence and phosphorescence intensities and lifetimes by the same factor. In another meaning, both the quantum efficiency and the lifetime are relative to $(k_L + k_{nr})^{-1} (43)$. 

23
References


(17) Siopi, A. Substance Flow Analysis (SFA) of Polycyclic Aromatic Hydrocarbons (PAHs) in Road Runoff.


CHAPTER TWO: THEORETICAL PREDICTION OF FLUORESCENCE SPECTRA FOR STERICALLY STRAINED ISOMERS OF C_{24}H_{14}: A COMPARISON WITH VIBRONICALLY-RESOLVED EXPERIMENTAL DATA

Samar Alheety,^a James K. Harper,^b Andres Campiglia^a, Emily C. Heider^c^,*

^aDepartment of Chemistry, University of Central Florida, 4111 Libra Drive, Orlando, FL 32816, USA, ^bDepartment of Chemistry and Biochemistry, Brigham Young University, Provo, UT 84602, USA., ^cDepartment of Chemistry, 800 West, University Parkway, Utah Valley University, Orem, UT 84058, USA.

Abstract

This study evaluates the ability of time-dependent density functional theory to predict fluorescence spectra for polycyclic aromatic hydrocarbons (PAHs) isomers of C_{24}H_{14}. The PAHs studied including dibenzo[a,j]fluoranthene, dibenzo[a,k]fluoranthene and dibenzo[a,l]fluoranthene. These isomers differ in the placement of one of six aromatic rings and in the amount each molecule deviates from planarity. Spectra are calculated at the CAM-B3LYP/cc-pVDZ level and include solvent. Theoretical spectra are compared to vibronically resolved experimental data obtained at cryogenic temperatures. Computed spectra for dibenzo[a,j]fluoranthene and dibenzo[a,k]fluoranthene are blue shifted versus
experiment by 24.5 nm in the $S_{1,0} \rightarrow S_{0,0}$ transition and exhibit a wavelength range that is overestimated by 30.8 %. Conversely, the computed $S_{1,0} \rightarrow S_{0,0}$ transition of dibenzo[a,l]fluoranthene is red shifted by 51 nm. This difference is attributed to steric interactions in dibenzo[a,l]fluoranthene that creates a deviation from planarity of 35.2°. Deviations from planarity are linearly correlated with error in computed $S_{1,0} \rightarrow S_{0,0}$.

**Graphical abstract.**

**Keywords:** Polycyclic aromatic hydrocarbon, Shpol’skii spectroscopy, Fluorescence spectroscopy, time-dependent density functional theory, CAM-B3LYP

*Department of Chemistry, Utah Valley University, Orem, UT 84058, USA, Phone: 801-863-6080, eheider@uvu.edu
Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of compound that are ubiquitous worldwide because they are produced when coal, oil, gasoline and other organic materials are burned. While it has long been known that many PAHs are toxic, hundreds of millions of kilograms of PAHs are still released worldwide each year [1]. As a result, measurable concentrations of PAHs are found in nearly all individuals [2]. The toxicity of different PAHs varies significantly [3] and the U.S. environmental protection agency (EPA) recognizes 16 compounds as “priority pollutants” having at least some level of toxicity [4]. Accordingly, monitoring of environmental PAH often focuses on these 16 compounds. Despite this limited focus, other PAHs have also been found to be toxic and the European Union recognizes three additional PAHs as having significant toxicity. These compounds are dibenzo[a,e]pyrene, dibenzo[a,i]pyrene and dibenzo[a,l]pyrene and each of these has toxicity equal to or greater than benzo[a]pyrene [3], the most toxic PAH on the EPA list.

A wide variety of analytical procedures have been employed to detect and quantify PAHs as summarized elsewhere [5]. In this manuscript, the focus is on the use of experimental and computed fluorescence spectra to analyze PAHs.
Monitoring of PAHs using fluorescence emission spectroscopy has certain advantages including high sensitivity, with detection limits typically in the parts-per-trillion range. It is also possible to obtain unusually high resolution fluorescence spectra if PAHs are prepared as dilute solutions in a weakly interacting solvent (e.g. $n$-alkanes) and then frozen at liquid nitrogen (77 K) or liquid helium (4.2 K) temperatures. Analysis of these low temperature solids is known as Shpol’skii spectroscopy [6] and can produce vibrationally resolved fluorescence spectra with tens of lines observable in some cases. Shpol’skii methods provide sufficiently high resolution that mixtures of PAHs can be accurately analyzed without prior chromatographic separation in analyses taking only a few minutes [7].

A complication to these Shpol’skii analyses on environmental samples is that emission peaks purported to represent PAHs are often observed at frequencies not corresponding to any of the peaks from the 16 EPA-PAH standards. Although the presence of unknown PAHs in these samples is not unexpected, identifying these PAHs is challenging and unknown peaks are typically neglected. Nevertheless, focusing on these unknowns is of value because they may have significant toxicity. Unfortunately, a very limited number of PAHs are commercially available beyond the 16 EPA standards, preventing identification of unknowns by simply purchasing
standards and analyzing each to find matching peaks. Thus, alternative approaches for identifying unknown PAHs are needed.

One approach for potentially identifying unknown PAHs in environmental samples is to generate possible candidate structures using theoretical methods. By calculating emission spectra for each candidate, a best-fit to experimental data can be identified. This approach has been described as a “virtual spectrometer” [8] because it provides a spectrum for a molecule of interest even when a standard is not available for experimental study. This approach can potentially explore dozens of structures but have only recently become feasible for larger molecules. At the present time, very few PAHs have been studied by these computational methods. For example, a 2016 study [9] found that only 15 PAHs had ever been studied by these theoretical methods and only eight of these were PAHs found on the EPA list. These calculations of emission spectra are now tractable due to a computational process that identifies vibronic transitions that do not contribute to a spectrum and excluding them from further calculations [10,11]. Such transitions make up the majority of possible transitions and omitting them greatly reduces computational cost and expands the size of structures that can be evaluated since calculations are restricted to only those transitions that contribute to the spectrum. At present, most computations of emission spectra use time-dependent density functional theory (TD-
DFT) because it extends conventional DFT methods to excited states [12,13,14], providing the ability to compute emission spectra. The influence of solvent can also be included in DFT calculations allowing solution phase spectra to be accurately predicted.

When using theoretical TD-DFT methods to select feasible candidate PAH structures, prior studies can provide valuable guidance. For example, one such study of PAHs reported high toxicity from components having a molecular mass of 302.4 Da (i.e. C_{24}H_{14}) [15]. Unfortunately, most of these compounds were not identified beyond their mass and structures remain largely unknown. Generating possible candidate isomers of C_{24}H_{14} is not particularly difficult, but before this approach is adopted, the accuracy of the computed spectra must be verified against experimental data. The aim of this manuscript is to verify that DFT computed spectra can accurately reproduce experimental information for a group of closely related isomers of C_{24}H_{14}. Prior related work has calculated emission spectra for three isomers of C_{24}H_{14} [9]. The present study expands upon this work [9] by including three additional isomers of C_{24}H_{14} that differ due to the presence of significant steric strain arising from two non-bonded hydrogens being forced into close proximity (< 2.0 Å) because of ring fusions. Comparison of such data with experimental spectra is now possible because the synthesis of the three isomers of C_{24}H_{14} has recently been
achieved [16], making them available for the first time. Fluorescence spectra of these compounds at cryogenic temperatures are reported here. These spectra exhibit extraordinarily high resolution and provide ideal data for comparison. The compounds evaluated are dibenzo[a,j]fluoranthene (DB[a,j]F), dibenzo[a,k]fluoranthene (DB[a,k]F) and dibenzo[a,l]fluoranthene (DB[a,l]F). Molecular structures for each PAH are shown in Figure 1. Dibenzo[a,j]fluoranthene and Dibenzo[a,l]fluoranthene represent compounds not available from commercial sources.

Several theoretical studies involving DFT predictions of PAHs spectra have previously been published [10,17,18,19,20,21,22]. The present study differs from these in two respects. Perhaps most significantly, the experimental data employed herein exhibit usually high resolution with as many as 25 vibronic transitions observable in a given spectrum. In all cases described herein, at least 10 lines are observed and available for comparison. In contrast, most prior comparisons have employed experimental spectra exhibiting fewer than five lines. Indeed, in many comparisons, the experimental spectra display only a single broad emission band. This inability to observe individual vibronic transitions in these spectra represents a serious limitation to analysis. The methods described herein partially overcome this challenge.
A second difference in the present study is that the compounds studied have a higher molecular weight than those typically studied by TD-DFT methods. This larger size limits the level of theory that can be utilized. It has been previously demonstrated that a double zeta basis sets provide accurate line shapes for coumarin dyes [8]. Here it is demonstrated that such basis sets can also accurately predicts PAH spectra.

![Figure 11. Structures of the three isomers of C24H14 studied herein.](image)

**Material and Methods**

### 2.1 Computations of all theoretical emission spectra included geometry optimizations and frequency calculations in both the ground and excited states. These computations were performed at the CAM-B3LYP/cc-pVDZ level of theory. Fluorescence spectra were computed using the Franck-Condon approximation
Solvent effects were simulated using the polarizable continuum model [25]. The computations were performed with the Gaussian 16 software package [26].

2.2 Room-temperature fluorescence spectra were recorded with a FluoroMax-P spectrofluorimeter (Horiba Jobin-Yvon) equipped with a continuous 100 W pulsed xenon lamp with broadband illumination in the ultraviolet and visible spectral regions. The excitation and emission monochromators had the same reciprocal linear dispersion (4.2 nm·mm\(^{-1}\)) and accuracy (±0.5 nm). Both monochromators were equipped with diffraction gratings containing the same number of grooves per unit length (1200 grooves·mm\(^{-1}\)). The excitation grating was blazed at 330 nm and emission grating at 500 nm. The photomultiplier tube (Hamamatsu, model R928) had a spectral response ranging from 185 to 650 nm. It was operated at room temperature operated in the photon-counting mode. Commercial software (DataMax version 2.20, Hriba-Jobin-Yvon) was used to computer-control the spectrofluorimeter.

Room-temperature fluorescence measurements were made by pouring un-degassed liquid solutions into micro-quartz cuvettes (1 cm path length x 2 mm width) that held a maximum volume of 400 µL. Fluorescence emission was collected at 90° from excitation using appropriate cutoff filters to reject straight-light and
second order emission.

2.3 Low-temperature fluorescence measurements were performed with the aid of a fiber optic probe (FOP) consisting of one excitation and six emission fibers [27]. All fibers were 2 m long and 500 μm core diameter, silica-clad silica with polyimide buffer coating (Polymicro Technologies, Inc.). All fibers were fed into a 1.2 m long section of copper tubing that provided mechanical support for lowering the probe into the liquid cryogen. At the sample end, the fibers were arranged in a six-around-one configuration with the excitation fiber(s) in the center. At the instrument end, the six emission fibers were position in a “slit” (vertical line) configuration. Vacuum epoxy was used to hold the fibers in place, which were then fed into metal sleeves for mechanical support. At the sample end, the copper tubing section was flared, stopping at a phenolic screw cap threaded for a 0.75 mL propylene sample vial.

Shpol’skii spectra were recorded with an instrumental set-up built in-house [28]. A Radiant 355 LD UV pulsed tunable laser system (OPOTek Inc.) provided sample excitation from 210 to 2500 nm. A half-wave plate and a polarizer cube controlled the intensity of the laser radiation sent to a neutral density filter that blocked the residuals of the laser output at 1064 nm, 532 nm and 355 nm. After passing through a digitally controlled shutter, the portion of laser light transmitted
by the density filter entered the excitation fiber of the laser FOP placed on a translation stage that allowed fine movements to achieve horizontal (X) and vertical (Y) alignment with the laser beam. A focusing lens reduced the spot size of the laser beam to the inner diameter of the fiber and helped to maximize optical throughput into the sample. The six-emission bundle of the laser FOP was mounted in a XY translational stage that had an additional mode of rotation to allow for alignment into the entrance slit of the spectrograph (Shamrock; Andor). The spectrograph was equipped with a diffractive grating (1200 grooves.mm\(^{-1}\)) blazed at 500 nm and attached to an iStar ICCD (Andor) with an active area of 690 x 256 pixels, pixel size of 26 microns, and a well depth of 500,000 electrons. Data acquisition and instrument control was made possible with the aid of LABVIEW software developed in-house. Site-selective excitation was not attempted.

Results and Discussion

*Acquiring High Resolution Experimental Fluorescence Spectra*

The computed emission spectra of PAHs identify all vibronic transitions and provide “stick spectra” with a linewidth for each transition that is unachievable experimentally. By using line broadening methods, an improved match to experimental data is achieved. Naturally, higher resolution experimental data
enhance this process by providing more detailed and accurate comparisons. In an effort to obtain the highest resolution experimental data possible with the available instrumentation, experimental data for DB[a,j]F, DB[a,k]F and DB[a,l]F were obtained using Shpol’skii methods at 77 K and 4.2 K. These spectra all have well resolved $S_{1,0} \rightarrow S_{0,0}$ transitions and include a large number of other well resolved peaks that correspond to individual vibronic transitions. These spectra are shown in Figure 2 and provide the high-resolution datasets needed to evaluate theoretical predictions. All spectra were acquired as dilute solutions of approximately 50 ppb in $n$-octane.
Figure 12. The experimental fluorescence spectra of the three PAHs evaluated herein. All spectra are acquired in n-octane. The wavelength of the $S_{1,0} \rightarrow S_{0,0}$ transition is included on each of the spectra acquired at 4.2K.
Comparing Computed and Experimental Fluorescence Spectra

Prior theoretical calculations of higher molecular weight PAHs in our laboratory [9] and others [8,29,30] have demonstrated that CAM-B3LYP [31] provides reasonably accurate emission spectra. These computations accurately reproduce the majority of the lines observed experimentally using only the double zeta basis set cc-pVDZ [32]. The predicted spectra also closely match the relative peak intensities observed in experimental data. Nevertheless, these theoretical spectra exhibit two types of systematic errors. First, the wavelengths of predicted peaks are consistently underestimated by 14.5 ± 7.6 nm based on the $S_{1,0} \rightarrow S_{0,0}$ transition. Inclusion of the solvent effects in computed spectra shifted all peaks by +11 nm on average, and largely corrects this error. The second error observed was that the wavelength range over which vibronic transitions are predicted to occur was too large by roughly 16% compared to experimental spectra.

This theoretical overestimation of the wavelength range has been observed by others and various explanations have been proposed for its origin. For example, in a tutorial review describing methodologies for the prediction of vibrational and electronic spectra, Bloino [29] et al. studied the accuracy of computationally reproducing the vibrationally resolved spectrum of trans-2,2'-biothiophene. This molecule, predicted to be planar in the first electronic excited state, is subject to
torsional rotation of the two rings, and is predicted to have large amplitude motion (LAM) coupled with vibrational motion. Their computations of vibrational-resolved emission spectra compare favorably in terms of the intensities of the bands, but predict frequencies that cover a broader range than is observed experimentally. They attribute this inaccuracy to the computational uncoupling of the LAM from the normal modes, resulting in inaccurate reproduction of the vibrational energies.

An alternative explanation for the computational broadening of the wavelength range of spectral lines is offered by Cerezo et al. [33] who examined two models for including the influence of solvent in computed emission spectra of coumarin dyes. Their work compared the implicit solvent model, which accounts for mean-field solvent effects on the predicted vibronic structure, with explicit inclusion of the solvent in quantum mechanical calculations. Both solvent models result in spectra that overestimate the range of wavelengths, but the implicit model results in larger band broadening than does the explicit model. They also note that qualitative trend of predicted spectral width increasing with the size of the fluorophore, which is associated with larger change in polarity during the transition. Both the implicit PCM solvent model (utilized herein), and the relatively large size of the PAH fluorophores, are likely to play a role in the broadening of the frequency range over which the spectra are predicted to occur.
Regardless of the origin of the error in the wavelength range, it has been previously demonstrated in other types of spectra [34,35,36,37,38,39,40] that it is possible to simultaneously reduce both types of errors by finding the relationship between experimental and computed data. Such an analysis is illustrated in Figure 3 using previously reported data [9] from five PAHs. A least-squares fit to these data gives the relationship

\[ \lambda_{\text{cm}^{-1}}^{\text{Calculated}} = -51.8 \lambda_{\text{nm}}^{\text{Experimental}} + 46,539 \text{ cm}^{-1} \left( R^2 = 0.925 \right). \]

Using this relationship to treat computed spectra, both kinds of systematic errors are reduced and the uncertainty decreases to 5.7 ± 5.1 nm based on the S_{1,0} → S_{0,0} transition. It is notable that although it is common to correct for the S_{1,0} → S_{0,0} transition (related to the intercept of the least-squares line) by moving the entire spectrum to make these peaks align, the correction for error in the wavelength range (related to the slope of the least-squares line) has seldom been performed. This omission may be due to the paucity of highly resolved experimental spectra for comparison in most studies. In the present study, the CAM-B3LYP/cc-pVDZ approach was employed to compute fluorescence spectra for DB[a,j]F, DB[a,k]F and DB[a,l]F. This choice provides a direct comparison to prior studies [9] of PAHs. Results for each PAH are described below.
Figure 13. The correction between computed and theoretical emission data. The best fit line is linear and is described by 
\[ \lambda_{cm^{-1}}^{Calculated} = -51.8 \lambda_{nm}^{Experimental} + 46,539 \text{ cm}^{-1} \ (R^2 = 0.925). \]

One way to assess the accuracy of the CAM-B3LYP computations for the three PAHs studied herein is to compare these data with the previous results illustrated in Figure 3. Such a comparison of previous data from five PAHs [9] with the new values from DB[a,j]F and DB[a,k]F yields the plot shown in Figure 4. The new data are statistically indistinguishable from those previously acquired. However, the DB[a,j]F and DB[a,k]F data populate a different region of the plot and thus slightly modify the equation describing the correlation. A least-squares fit gives the equation 
\[ \lambda_{cm^{-1}}^{Calculated} = -46.5 \lambda_{nm}^{Experimental} + 44,262 \text{ cm}^{-1} \ (R^2 = 0.98). \] This equation is preferable to that previously reported due to the superior correlation.
Data for DB[a,l]F differ from those illustrated in Figure 4 and thus are described separately hereinafter.

Figure 14. A comparison of the computed and experimental peak positions for major peaks in the emission spectra of benzo[a]pyrene, dibenzo[a,e]pyrene, dibenzo[a,l]pyrene, dibenzo[a,i]pyrene and benzo[a]pyrene-7,8,9,10-tetrol (green) and the new data for DB[a,j]F and DB[a,k]F (yellow).
Visual comparisons between theoretical and experimental spectra for DB[a,j]F and DB[a,k]F are shown in Figure 5. This plot shows that CAM-B3LYP accurately reproduces the number of peaks and the relative peak intensities of the experimental spectra. As anticipated from previous work [9], the CAM-B3LYP spectra underestimate the $S_{1,0} \rightarrow S_{0,0}$ transition and also predict a wavelength range that is too large by 30.8 % on average. The computed spectra also accurately reproduces one of the most significant differences between the experimental spectra of the two PAHs, namely that the entire DB[a,k]F spectrum is significantly blue shifted compared to the DB[a,j]F spectrum.
Figure 15. Experimental (top) and theoretical (bottom) fluorescence spectra for DB[a,j]F (left) and DB[a,k]F (right). Theoretical spectra were computed at the CAM-B3LYP/cc-pVDZ level of theory and included n-hexane as a solvent using the polarizable continuum model.

It is desirable to minimize the systematic error in the computed spectra before comparing computed and experimental spectra. Accordingly, the empirical correction described above was employed to adjust the spectra of DB[a,j]F and DB[a,k]F (Figure 6). The quality of this correction can be evaluated by computing the error in the fit, expressed here as the root-mean squared deviation (RMSD = $\sqrt{\frac{\sum (X_{\text{theory}} - X_{\text{exp.}})^2}{N}}$) between corresponding points in the spectra before and after the adjustment. In the case of DB[a,j]F, the RMSD is smaller after adjusting the
spectrum indicating improvement. An F-test comparing the squared RMSD before and after the adjustment indicates that the adjusted spectrum differs from the uncorrected spectrum at the $p = 0.04$ level, indicating a statistically significant improvement. In contrast, the RMSD of the adjusted DB[a,k]F spectrum is found to be statistically indistinguishable from the RMSD of the uncorrected spectrum. This outcome is surprising because a prior evaluation of four closely related PAHs all were significantly improved by this correction. It is interesting to postulate an origin for the failure of the empirical correction to improve DB[a,k]F and a more complete discussion is provided below in the discussion of DB[a,l]F where deviations from planarity are postulated to contribute to this failure.
Figure 16. Experimental (top) and theoretical (bottom) fluorescence spectra for DB[a,j]F (left) and DB[a,k]F (right). Theoretical spectra shown were empirically corrected using the relationship $\lambda_{\text{cm}^{-1}}^{\text{calculated}} = -46.5 \lambda_{\text{nm}}^{\text{experimental}} + 44,262 \text{ cm}^{-1}$ ($R^2 = 0.98$). Computed at the CAM-B3LYP/cc-pVDZ level of theory and included n-hexane as a solvent using the polarizable continuum model.

Although the theoretical spectra of DB[a,j]F and DB[a,k]F (described above) are predicted with reasonable accuracy by CAM-B3LYP, a much less accurate prediction of the $S_{1,0} \rightarrow S_{0,0}$ transition is obtained by CAM-B3LYP for DB[a,l]F. All other transitions are offset by an amount similar to the $S_{1,0} \rightarrow S_{0,0}$ transition. The difference between the computed spectrum and experimental data are illustrated in Figure 7. A second kind of comparison can be made involving the peak positions of
DB[a,l]F against a larger dataset of seven PAHs as shown in Figure 4. This comparison is illustrated in Figure 8 and shown that the computed peaks of DB[a,l]F deviate significantly from all prior data shown in Figure 4 and are not part of the statistical population of all other PAHs studied in our laboratory.

Figure 17. A comparison of the experimental and theoretical fluorescence spectrum of DB[a,l]F. The large overestimation of the $S_{1,0} \rightarrow S_{0,0}$ transition in the theoretical spectrum is unexpected and deviate by roughly +65 nm, on average, from prior computed spectra where an underestimation of this transition is consistently observed.
Figure 18. The computed peak positions of DB[a,l]F (blue circles) systematically differs from those predicted for other PAHs studied herein.

It is interesting to speculate on why the predicted spectrum of DB[a,l]F differs from the experimental data so significantly. One structural difference that can be observed between DB[a,l]F and the other PAHs is that DB[a,l]F contains a region with four consecutive non-protonated carbons due to ring fusion (Figure 1). This structural feature results in two hydrogen being forced into very close proximity with one another. Specifically, the H/H separation in the geometry optimized structure is
1.9 Å, a value much less than the sum of their van der Waals radii of 2.4 Å. To diminish this unfavorable interaction, the entire ring system twists out of the expected planar arrangement into a shape that is helix-like (Figure 10). In this shape, the two hydrogens have a more favorable interaction but the conflicting rings diverge from one another by 35.2°. In contrast, DB[a,j]F and DB[a,k]F each include regions having three consecutive non-protonated carbons, an arrangement known as a “fjord”. This arrangement causes similar close contacts between two hydrogens of 2.1 Å in each structure. The ring twist needed to alleviate this conflict in DB[a,k]F is 10.8°. A much smaller deviation of 3.7° is observed in DB[a,j]F. This deviation from planarity is strongly correlated with the error in computed $S_{1,0} \rightarrow S_{0,0}$ wavelengths as illustrated in Figure 9. This plot demonstrates a linear relationship between the deviation from planarity and the predicted wavelength of the $S_{1,0} \rightarrow S_{0,0}$ transition. While this comparison is limited to three points, thus warranting further evaluation, the strong correlation ($R^2 = 0.9996$) suggests that the failure of the empirical correction in the cases of DB[a,k]F and DB[a,l]F is related to their deviation from planarity. This deviation is postulated to alter the $\pi$-delocalization throughout the molecule and to account for difference in the fluorescence spectra. No other structural differences are observed.
Figure 19. The correction between the deviation planarity in the PAH evaluated and the difference between the computed and experimental $S_{1,0} \rightarrow S_{0,0}$ transition. A least-squares fit to the data gives $y = -0.37x + 16.32$ ($R^2 = 0.9996$) where $x$ and $y$ are as defined in the plot.
Figure 20. An illustration of the deviation from planarity that is observed in the energy minimized structure of DB[a,l]F. The ring twisting alleviates some of the close contact between two protons but is postulated to alter \( \pi \)-delocalization and thereby diminish the accuracy of the theoretical spectrum.
Conclusions

This study demonstrates the ability of TD-DFT methods to accurately compute fluorescence spectra for PAH isomers having very closely related structures. As expected from previous work [9], the calculated spectrum for DB[a,j]F produces peaks that are systematically shifted to lower wavelengths and having a wavelength range that is expanded relative to experimental data. The previously proposed empirical correction partially alleviates these errors and a more accurate empirical correction is proposed here to further reduce uncertainty. In the case of DB[a,l]F and, to a lesser extent, DB[a,k]F, the expected TD-DFT results were not obtained, rather the peaks were shifted to longer wavelengths. Inspection of the energy minimized structure revealed a structural difference between DB[a,j]F and the other two PAHs arising from steric interactions in DB[a,l]F and DB[a,k]F that create non-planar structures. Thus, PAHs that deviate significantly from planarity appear to be less accurately predicted by CAM-B3LYP/cc-pVDZ.

5. Acknowledgements. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sector.
References


https://doi.org/10.1080/10406638.2014.892886.

(5) S.A. Wise, L. Sander, M. Schantz Analytical Methods for Determination of Polycyclic Aromatic Hydrocarbons (PAHs) – A Historical Perspective on the


(20) M. D’Alessandro, M. Aschi, C. Mazzuca, A. Palleschi, A. Amadei, Theoretial modeling of UV Vis absorption and emission spectra in liquid state systems including vibrational and conformational effects: The vertical transition


CHAPTER THREE: CHARACTERIZATION OF A BIO-SOURCED, FLUORESCENT, RATIOMETRIC PH INDICATOR WITH ALKALINE PKₐ

Adapted with permission from: Samar Alheety, Domenic Valenti, Nirvani Mujumdar, Nakita Ellis, Andres D. Campiglia, James K. Harper, Emily C. Heider


Abstract

Utilizing organisms as sources of fluorophores relieves the demand for petroleum feedstock in organic synthesis of fluorescent products, and endophytic fungi provide a promising vein for natural fluorescent products. We report the characterization of a pH-responsive fluorophore from an endophytic fungus isolated from sand pine. The endogenous fluorescence of the live organism was measured using fluorescence microscopy. Computational interpretation of the spectra was accomplished with time-dependent density functional-theory methods. The combined use of experimental and theoretically predicted spectra revealed the pH equilibria and photo-excited tautomerization of the natural product, 5-methylmellein. This product shows promise both as a stand-alone pH-indicating fluorophore, with alkaline pKₐ, and as "green" feedstock for synthesis of custom fluorophores.
Introduction

The intentional use of fluorophores from biological sources may be a way to comply with Anastas’ and Warner’s 7th Principle of Green Chemistry: use of renewable feedstock. (1) Fluorophore design based on green principles is a rapidly developing field of research; for example, Woodward et al. investigated the derivatization of guaiazulene, a halochromic natural product isolated from fungi, for potential use as switchable optical electronics. (2) Using organisms as sources of fluorophores has the potential to relieve the demand for petroleum feedstock in organic synthesis of fluorescent products. One rich source of useful natural products are endophytic microorganisms. These microbes live inside higher plant tissue without causing disease symptoms in the host. Endophytic fungi provide a promising vein for identification and isolation of natural fluorescent products.

For this research, the endophytic hypoxylon sp. was isolated from sand pine (Pinus clausa) and exhibited endogenous fluorescence. The emission spectrum was measured in the hyphae of the live fungal organism. Extraction and analysis of the fungi resulted in the isolation of 5-methylmellein (hereafter, 5-MM), a fluorophore with pH-dependent spectral properties. This dihydroisocoumarin natural product, which was first isolated and identified in 1966 from Fusicoccum amygdali Del. (3),
has recently been more fully characterized by NMR using one-bond $^{13}\text{C}-%^{13}\text{C}$ scalar couplings (4).

Herein, emission spectra from the luminescent fungal hyphae were measured using fluorescence microscopy, and experimental and in silico methods applied to the characterization of the pH-responsive fluorophore present in the fungus. Interpretation of the emission spectra from biological sources can be a challenging task, particularly when fluorophores are subject to equilibria conditions or are photoreactive. This task was partially accomplished using time-dependent density functional-theory methods. Until recently, conventional density functional theory (DFT) was limited to evaluation of ground states, limiting possibility of accurate calculations of emission spectra (5). Recent extension of DFT, to include time-dependent excited states, allows for relatively rapid calculation of emission spectra. Additional obstacles to facile computational prediction of emission spectra arose from the large number of excited states present in large molecules – calculation of over-lap integrals between these vibrational excited states and the ground states was computationally costly. Recent development of an algorithm to assess only the spectrum’s non-negligible excited states has made theoretical comparisons to experiment a reality (6, 7). Furthermore, the influence on solvent effects on the computation of luminescent emission can be included in the modelling using the
Polarizable Continuum Model (PCM) (8). Incorporation of these advances in the commercially-available software, Gaussian 09 (9), provides access to computational prediction of emission spectra for large (10) and small molecules. The combined use of experimental and theoretically predicted spectra revealed photo-induced tautomerationization properties of the natural product, 5-MM. To our knowledge, this is the first report of an alkaline-responsive fluorescent ratiometric pH indicator. We also provide the first complete description of the spectroscopic properties of 5-methylmelllein.

Materials and Methods

Reagents and Preparation

Solvents and reagents including methanol, N,N-dimethylformamide, magnesium sulfate, monobasic potassium phosphate (KH$_2$PO$_4$), concentrated sulfuric acid, and sodium chloride were acquired from Fisher Chemical (Fair Lawn, New Jersey, U.S.A.). Zinc sulfate, quinine sulfate, and potassium chloride were purchased from Acros Organics (New Jersey, USA) and chloroform acquired from Millipore Corporation (U.S.A.). Buffers and solutions were prepared in deionized water. Solutions of 5-MM were prepared by first dissolving the crystalline solid in methanol (1.00 mg/mL) and then diluting into buffer with the desired pH.
Fungal Growth and Isolation

The surface of a small twig of sand pine from central Florida, U.S.A. was sterilized using ethanol and then plated on water agar. Emergent hyphae were observed, transferred to potato dextrose agar (PDA), and later identified as endophytic fungus belonging to the *Biscogniauxia* genus based on phylogenetic analysis, described elsewhere (4). An 8-liter solution of potato dextrose broth was inoculated with the fungus and cultured at 25°C without aeration for 1-month. Cheesecloth was used to filter the flocculent solution, which was then extracted with 1:1 (v:v) ethyl acetate (3x). The ethyl acetate mixture was subjected to rotary evaporation, yielding 1.054 g of solid material. Separation of the components of the crude extract was accomplished with column chromatography on silica gel (Fisher, 40–63 μm particles) with 100% CH$_2$Cl$_2$ mobile phase. Once dried, 100.6 mg of 5-methylmellein were recrystallized in hexane. The purified white solid was analyzed thoroughly with electron ionization mass spectrometry and characterized using NMR methods. A complete description of both characterization techniques can be found elsewhere.(4) Briefly, analysis of the material with liquid chromatography-mass spectroscopic analysis (LC-MS, Agilent 6230) revealed a single peak with a M/Z 193.0866, corresponding to ([C11H12O3]+H)+.

To fully characterize the structure of 5-MM with liquid NMR, $^1$J$_{CC}$ coupling experiments were conducted using a Varian 500 spectrometer operating at 125.694
MHz. All spectra were measured at a temperature of 25 °C in CDCl₃ using a 5-mm probe, a 9.2-μs ¹³C 90° pulse width, and a 2-s recycle time. The spectra were referenced with the central line of CDCl₃ assigned to 77.2 ppm. The analyses were performed using natural abundance samples and the ¹J_CC coupling constants were determined by processing the experimental data with the NMR Analyst software acquired from ScienceSoft LLC. The INADEQUATE spectra of 5-methylmellein provided ¹J_CC values for all ¹³C—¹³C bonds, along with unambiguous ¹³C chemical shift assignments. For a complete description of the structural characterization of 5-MM in CDCl₃, see reference 4. The structure determined through these methods is depicted in Figure 2.

**Instrumentation**

*Fluorescence Microscopy*

A FluoroMax-P spectrofluorimeter (Horiba Jobin Yvon) was coupled via commercially available fiber-optic bundles to an Olympus (BX-51) fluorescence microscope with epi-configuration. An illumination source was provided by a continuous 100 W pulsed xenon lamp with 200-2000 nm illumination. Emission spectra were recorded with spectrometers containing 1200 grooves-mm⁻¹ resulting in 4.25 nm-mm⁻¹ reciprocal linear dispersion and 0.3 nm resolution (slit-width = 20 nm). A photomultiplier tube in the photon counting mode (Hamamatsu, model R928) with 185-
850 nm spectral response was operated at room temperature. Commercial software (DataMax) was utilized to control the instrument.

The microscope was equipped with two 50/50 beam splitters for the ultraviolet and visible spectral regions. A 40x UPlanSAPO Olympus objective collected light from the sample. A rotating pinhole wheel, with diameters ranging from 0 - 1000 μm, was placed between the beam splitter and the mirror directed emission either to the CCD camera (iDS UI-1450SE-C-HQ USB camera) or to the spectrofluorometer. capitalize Text follows immediately on same line.

UV-Vis Spectroscopy

A Cary 50 dual beam spectrophotometer with reference beam was used to collect absorbance spectra. A xenon flash lamp illuminated the sample and absorbance measurements were collected every 1-nm increment with a scan rate of 600 nm per minute.

Quantum Yield Measurements

Reference quantum yield data (11) for quinine sulfate were reported previously in 0.10 M sulfuric acid. Solutions of the same composition were utilized to measure the spectra of 5-MM. The absorbance spectrum for both quinine and 5-MM were recorded at various concentrations (4-6 μM for quinine, 25-90 μM for 5-
MM). The fluorescence emission spectra were also recorded for the same solutions and plots were constructed showing integrated fluorescence intensities (I) versus the integrated absorbance values (Abs). The plots were fit using a linear-least squares process and the slopes of those plots were used to find the I/Abs ratio. The ratio of the quantum yield for 5-MM relative to quinine was quantified using equation 1.

$$\frac{\phi_{MM}}{\phi_{Quinine}} = \frac{Abs_{Quinine}I_{MM}}{Abs_{MM}I_{Quinine}} \left( \frac{\eta_{MM}^2}{\eta_{Quinine}^2} \right)$$  \hspace{1cm} (3-1)

Where $\eta_{MM}$ and $\eta_{Quinine}$ refer to the refractive indexes of MM and quinine solutions, respectively. Equation 1 features the fluorescence quantum yield ($\phi$), absorbance (Abs) of the quinine and 5-MM solutions, and integrated fluorescence intensity (I) of the quinine and 5-MM solutions.

**Computational Methods**

Two isosbestic points were observed in the emission spectra, but only one was present in the absorbance spectra of 5-methylmellein. This outcome indicates possible photo-induced equilibria between the acidic forms. In an effort to elucidate the structures of the acidic forms of 5-methylmellein, models of the keto-enol tautomers of 5-methylmellein constructed and geometry optimized at both the ground and excited states using Gaussian 09 software. Time-dependent density
functional theory methodology, with CAM-B3LYP functionals cc-pVDZ basis set, was used to calculate emission spectra of the protonated keto- and enol-tautomers of 5-methylmellein and of the basic form. The Franck-Condon approximation was used to calculate vibrationally-resolved emission spectra, and the polarizable continuum model used to simulate solvent effects. The vibrationally-resolved calculated spectra were convoluted with a Gaussian function to achieve the shape most consistent with the spectra show in this work. Previous reports (10) comparing experimental and theoretically predicted spectra have shown that use of the CAM-B3LYP functional systematically underestimates the wavelength of $S_0 \rightarrow S_1$ transitions and this limitation was also found here. The computed spectrum of the deprotonated form was used to empirically determine the extent to which the predicted spectra of the protonated form required adjustment – in that case a hypsochromic shift of 55 nm was observed in the calculated spectrum relative to the experimentally measured spectrum. Hence, all computationally predicted spectra were red-shifted 55 nm.
Results and Discussion

An endophytic fungus showing biofluorescent properties was grown from the interior of sand pine twigs. The hyphae that were grown on agar plates were transferred to a microscope slide and imaged using a bright field microscope. A microscope image of the fungal hyphae is shown in Figure 1, along with the fluorescence spectrum of the hyphae obtained using a fluorescence microscope with 322 nm excitation. The emission from the bulk fungus indicated the presence of at least one natural product with fluorescent properties.
Figure 21. The endophytic fungus was isolated from a small twig of a sand pine (Pinus clausa). Emergent hyphae were imaged using a light microscope (image A). The emission spectrum (B) obtained from the hyphae were recorded using a fluorimeter optically coupled to a microscope.
To further explore the fluorescent behavior of the fungus, the compound 5-MM was extracted and its chemical structure characterized, as described elsewhere, using mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy. Although two tauotomeric forms of 5-MM are possible (Figure 2), $^{13}$C-$^{13}$C scalar couplings show the form found in CDCl$_3$ is exclusively that of form 1. Characterization of the spectroscopic properties of 5-MM was undertaken, including measurement of absorbance and emission spectra, and quantum yield. Quinine sulfate was selected as a reference standard, and the quantum yield of 5-MM relative to quinine sulfate and was found to be 0.25. Previous studies report the absolute quantum yield of quinine to be 0.546 (11).

![Figure 22. Structures of the two tautomeric forms of fluorescent product 5-methylmellein. As determined using carbon-13 chemical shifts and $^1$J$_{CC}$ couplings (Hz), 5-methylmellein in CDCl3 is exclusively form 1.](image)
Because 5-MM contains an acidic hydrogen, the luminescent properties of the 5-MM were further investigated by measuring the absorbance and emission spectra of solutions ranging from pH 7.0 to 12.5 (0.10 M phosphate buffer). The pH-responsive absorption spectra are shown in Figure 3. The isosbestic point in the absorbance spectra is consistent with a simple acid-base equilibrium model for which the acidic form has \( \lambda_{\text{max}} = 322 \text{ nm} \) with molar absorptivity \( 1.1 \pm 0.2 \times 10^6 \text{ M}^{-1}\text{cm}^{-1} \), determined from the linear-least-squares fit to a Beer-Lambert plot at pH 7.0. The basic form has \( \lambda_{\text{max}} = 356 \text{ nm} \) with molar absorptivity \( 1.03 \pm 0.06 \times 10^6 \text{ M}^{-1}\text{cm}^{-1} \) measured at pH 11.0.

Fluorescence emission spectra of 5-MM were also recorded under a variety of pH conditions. As observed in Figure 3b, the pH of the solution also influences the features of the emission spectra. The spectra under lower pH conditions show emission maximum 475 nm and a peak “shoulder” at 390 nm. The basic solutions produce a single peak with \( \lambda_{\text{max}} = 456 \text{ nm} \). The curious appearance of two isosbestic points in the emission spectra at 401 and 478 nm indicates that a two-component acid-base equilibrium model, suggested by the absorbance spectra, does not necessarily apply to the excited state species. One rationale for the presence of two excited state protonated forms 5-MM would be the tautomerization of 5-MM with intramolecular proton transfer. Although the previously cited NMR J-coupling data
(4) indicated that 5-MM was present as the keto tautomer for the ground state species in CDCl₃, the excited state aqueous species is hypothesized to have a different ratio of the keto/enol tautomer in aqueous buffer.

Figure 23. A) Example of pH-dependent absorbance spectra of 0.26 μM 5-methylmellein in various solutions ranging from pH 7.0 to 12.3 (0.010 M phosphate buffer). A single isosbestic point, \( \lambda = 332 \) nm, is present in the absorbance spectra and indicates the presence of only two forms of 5-MM in equilibrium. B) Emission spectra of 1.24x10⁻⁴ M 5-methylmellein with \( \lambda_e = 390 \) nm show two isosbestic points (401 nm and 478 nm) indicating the presence of three species.
To explore the possibility of photo-induced tautomerization of 5-MM, computer models of deprotonated 5-MM and the protonated keto and enol form were built and geometry-optimized using DFT calculations with Gaussian software. Then TD-DFT calculations, along with aqueous solvent modelling using the polarizable continuum model, were performed to optimize the geometry of the excited state and calculate the excited state frequencies. The excited state geometry optimized structure of tautomer 1 (Figure 2) showed transfer of the proton to the oxygen of the anomeric carbon, indicating tautomer 2 is present in the excited state. To continue the spectral calculations with both forms 1 and 2, an excited state structure of tautomer 1 was recalculated while fixing the hydrogen to the oxygen of the aromatic ring. The resulting vibrationally resolved emission energies were plotted as emission spectra. As the computed emission frequencies are delta functions, the spectra were convoluted with a Gaussian function until broadening matching the experimental data were achieved. Spectra for the deprotonated, and for the protonated tautomeric forms of 5-MM, are shown in Figure 4 along with the experimentally measured spectra. The computed spectra of the two tautomers model the measured emission spectra of 5-MM at low pH. The existence of both tautomers in the excited state provides a rationale for the presence of the peak shoulder at 401 nm. The absence of any indication of a second tautomer in the absorbance spectrum indicates that
structure 2 (Figure 1) is only present in the excited state, and that intramolecular proton transfer is induced by photoexcitation.

Figure 24. Measured emission spectra for the protonated (pH 5.8) and deprotonated (pH 11.2) forms of 5-MM. Also plotted are the computed spectra for deprotonated base and the keto and enol tautomers of 5-MM.
The observation of a photo-induced tautomerization is consistent with the findings of Bakalova et al. (12), who studied the keto-enol tautomers of dihydronaphthalenones (see Figure 5). Using absorbance, fluorescence, infrared and computational chemistry, their work indicated that the keto tautomeric form of the studied dihydronaphthalenone predominates in solution. The IR spectra indicated the presence of an intramolecular hydrogen bond to the ketone, and PPP-SCF-CI computations indicated that the H-bond was strengthened in the first excited singlet $\pi$-$\pi^*$ state.

Figure 25. Structure of the dihydronaphthalenones that show some features analogous to 5-methylmellein. In the figure, R and R’ represent functional groups varied over 11 different structures as reported elsewhere, with indications from spectral data the keto form was the dominant form in solution.
The pK\textsubscript{a} of both protonated tautomers of 5-MM was evaluated from the emission spectra by plotting the intensity of the emission maxima at 390 nm and 500 nm as a function of pH. The plots were fit to sigmoidal functions and the inflection points determined from the fit. The resulting data and sigmoidal fits are shown in Figure 6. The pK\textsubscript{a} as determined from the fit to the data were determined to be 10.41 ± 0.07 for the keto tautomer (the form that is present without photoexcitation), and the pK\textsubscript{a} for the enol (photoexcited tautomer) was found to be 10.88 ± 0.02. The increasing pK\textsubscript{a} of the photoexcited form is also consistent with the analogous compound reported by Bakalova (12). In their studies, photoexcitation of the dihydronaphthalenone resulted in a strengthening of the intramolecular hydrogen bond.
Figure 26. Emission intensities of the 5-MM keto tautomer (emission measured at λ= 390 nm) and enol tautomer (λ=520 nm) as a function of pH. The sigmoidal fit to the data indicated the pKₐ of both forms of 5-MM. The R² for the fit of the data at 520 nm is 0.997. The R² for the fit of the data at 390 nm is 0.985.
The strongly alkaline pK\textsubscript{a} of this ratiometric fluorophore is striking. Fluorescence imaging with pH-sensitive probes have proven to be a sensitive, rapid, specific, and noninvasive method to determine intracellular pH (13), liposomal pH for artificial organelles (14), and bioprocess monitoring (15). Demand for fluorescent pH sensitive-probes has stimulated productive research in the development of molecular dyes (16), proteins (17), and particle-based (18) fluorophores that respond to pH. Such probes are typically designed to either alter intensity with pH or to ratiometrically shift the wavelength at which they emit light as a function of pH. Of the two, ratiometric approaches offer advantages over intensity-based probes because parameters such as local concentration of the probe, optical pathlength, cellular leakage, and photobleaching are not problematic when the reported pH depends on the fraction of basic/acidic forms of the ratiometric probe (13). Recent innovation in the development of such ratiometric fluorophores include fluorescently-labeled i-motif gold nanoparticles for physiological pH range (19) synthesis of a small molecule probe with ethylene bridging of benzoindole and quinoline for extremely acidic pH conditions (20) and lysosome-targeting merocyanine-based probe with dual colorimetric functionality (21).

To date, the bulk of ratiometric fluorophore design and synthesis has naturally focused on probes with neutral to acidic pK\textsubscript{a}, often motivated by intracellular pH
monitoring. Very few such probes (such as carboxy-SNARF, \( pK_a \sim 7.5 \)) can quantify pH in biological systems above the neutral range, such as in mitochondria (pH 8.1) and peroxisomes (pH 8.4) (22), although there have been recent reports of pioneering efforts to synthetically produce fluorescent probes to measure mitochondrial pH (23, 24). A table summarizing a review of pH probes in the recent literature can be found in the supporting materials file.

To demonstrate the feasibility of using 5-MM as a ratiometric pH indicator, a buffered solution of 5-MM (52 \( \mu \text{M} \)) was prepared at pH 10.05. The least-squares fit to the pH 10.05 emission spectrum was determined using the linear combination of the acidic and basic spectra, as described by Frans and Harris, (25) using the model equation:

\[
I_{\lambda}^{\text{total}} = A I_{\lambda}^{\text{acid}} + B I_{\lambda}^{\text{base}}
\] (3-2)

In the above equation, \( A \) and \( B \) are the fraction of the acidic and basic forms of 5-MM, respectively, where

\[
A = \frac{1}{1 + 10^{(pH-pK_a)}}
\] (3-3)

\[
B = \frac{1}{1 + 10^{(pK_a-pH)}}
\] (3-4)
The spectra for the $I^\text{acid}_A$ 5-MM and $I^\text{base}_A$ are the background-corrected 5-MM spectra at pH 9.11 and 11.49, and a model spectrum was calculated using the pKa of 10.88. The linear least-squares fit of the model to the buffered spectrum was determined by varying the estimated pH to minimize the residual sum of squares between model and the measured spectrum (using Microsoft Excel’s Solver function). The pH from the fitted spectrum was determined to be 10.17 (for the pH 10.05 buffer solution). This measurement represents a 1.25% error in the pH. The measured and fitted model spectra are shown in Figure 7.

![Figure 27. The 5-MM spectrum in pH 10.05 buffered solution (black line) and the linear-least squares fit to the spectrum (red line) show how the pH of an unknown solution might be ascertained using spectra of 5-MM in standard acidic and basic solutions.](image-url)
Ideally, one could use the endogenous 5-MM spectrum to determine pH in the microorganism from which it is produced. The endogenous hyphae spectrum (Figure 1) was therefore analyzed in an effort to quantify internal pH of the organism. By fitting the spectra to a linear combination of the acidic and basic forms of 5-MM and with knowledge of their pKa, a pH of 7.4 was determined from the linear least-squares fit. This is likely a large overestimate of the pH of the organism since the ideal reporting range of the 5-MM indicator is only 9.88-11.88 (±1 pH unit of the pKa). As a comparison to the interior pH of other fungi, Bagar et al. (26) described the use of a genetically-encoded pH probe to measure the pH in filamentous fungi. In that case, their pH measurement for the intracellular region was 6.2-6.5.26 If the fungus that produced 5-MM has a similar pH, the 14-19% error in measured pH shows that the 5-MM pH measurement is optimal for alkaline pH measurement, but should be limited to pH range to which it is most sensitive.

To our knowledge, no ratiometric fluorescence probes have been identified for extreme alkaline environments. The identification of an alkaline ratiometric probe shows potential as an asset to the pH imaging field. For example, studies of alkaline pH in the midgut of mosquito larvae using pH-sensitive microelectrodes were able to discern gradients in pH across midgut membranes ranging from 0.02-
0.15 pH units (27); however, the spatial resolution of their measurements were limited to 2 μm, an order of magnitude larger than the resolution achieved by fluorescence microscopic measurements. Tran et al. (28) relied on light scattering to analyze the pH-dependent, pore-forming ability of insecticidal toxins on brush border membrane vesicles isolated from tobacco hornworms. The highly alkaline environment (pH 10-11) of lepidopteran midgut renders them vulnerable to pH-activated toxins, such as *Bacillus thuringiensis* Cry1E and Cry1A crystal toxins. Absorbance pH probes, such as *m*-cresol purple, are well-suited to spectroscopic measurements of such systems, but fluorescence measurements are many orders of magnitude more sensitive than absorbance measurements are. Ratiometric, membrane-permeable fluorescent probes, with alkaline pK<sub>a</sub>, could be invaluable in such research scenarios.

**ACKNOWLEDGMENTS:** We thank Dr. Angelina Georgieva for productive insight and conversations. This work was supported in part by the National Science Foundation under CHE-2016185.
References


