Synthesis and Optical Properties of Pentamethine Cyanine Dyes With Carboxylic Acid Moieties

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ABSTRACT: Cyanine dyes possessing carboxylic acid groups have been used in many different fields of study. The acid groups can act as handles for bioconjugation or as metal chelators. Several pentamethine cyanine dyes with propionic acid handles were synthesized and their optical properties were studied to determine their usefulness as fluorescent probes. The optical properties studies performed include the absorbance and emission maxima values as well as the calculation of quantum yield and molecular brightness levels. Molecular models were also calculated to help analyze the dyes' behavior and were compared with similar dyes with varying alkyl chain lengths replacing the acid moieties.

KEYWORDS: Carboxylic acid, pentamethine, cyanine, optical properties

Introduction

Since the discovery of a blue carbocyanine compound in 1856,[1] the cyanine class has been studied extensively for various applications, such as in vivo imaging,[2–6] ex vivo imaging (pH sensing and DNA stains),[7,8] blood spatter analysis,[9] and solar energy conversion.[10,11] The interest in the use of these dyes for the treatment and imaging of cancer and other diseases has increased in the past few decades. Dyes, and in particular polymethine cyanine dyes, can be relied on during tumor detection of many cancers with techniques such as magnetic resonance imaging (MRI) and positron emission tomography (PET), as well as intraoperatively in real time.[12] Increasingly, there is an interest in using near-infrared (NIR) dyes for imaging carcinomas due to the many distinctive properties of the cyanine dye family. A cyanine dye molecule consists of 2 nitrogen-containing heterocycles connected by an elongated, unsaturated carbon chain. The carbon chain contributes to the overall shape of the molecule being linear.[13] The length of this chain determines the wavelength at which the dyes absorb and fluoresce light. If the length of the polymethine bridge is extended by 1 double bond, the absorbance and fluorescence maxima red shift by about 100 nm.[5] This places the wavelengths of the dyes in what is called the NIR window. The NIR window lies between about 650 and 900 nm, and it is an important criterion to design dyes within this wavelength range for in vivo imaging. Body tissue will autofluoresce light at wavelengths lower than 650 nm, whereas water absorbs light above 1000 nm; the region between these 2 extremes is very valuable for tissue imaging. The cyanine dye class is also highly modifiable, with examples of substitution on every position existing in the literature.[2] Another beneficial property of cyanine dyes is their relatively narrow excitation band from S_0 to S_1.[14]

Cyanine dyes featuring alkyl carboxylic acids have been previously explored extensively for both dye-sensitized solar cells and conjugation of other groups onto the dye.[15–19] Researchers have taken advantage of several key properties of the acid group(s) incorporated onto the chromophore: (1) the water-soluble nature of the acid groups, as this allows for the exploration of larger, bulkier groups elsewhere on the dye and for aqueous in vivo studies[20]; (2) the pH sensitivity of the acid groups[21]; (3) the metal-binding ability of the acid groups, which is utilized in dye-sensitized solar cells to anchor the dye onto a metal surface[22]; and (4) the ability of the acid groups to be functionalized as a link to other targeting ligands.[23] Even though dyes containing alkyl acids exist and have been used for varying applications, there is an extreme lack of published material available when it comes to the optical efficiency of these dyes. The current literature has not yet answered whether the carboxylic acid groups affect the shape of the dye or the overall optical properties of the dyes if they are outside the conjugated system. Furthermore, lead chromophores that utilize the COOH handle for further ligand conjugation have been completely ignored when studying their optical properties. Herein, a set of 6 cyanine probes was synthesized and their optical efficiency was studied. To achieve this task, the absorbance and fluorescence spectra were obtained. From these data sets, the molar absorptivity (ε), quantum yield values (φ), and molecular brightness values are derived. These values are crucial for the implementation of the dyes as they show the overall efficiency and usefulness of the lead probes. The molecular brightness is, in this case, the product of the 2. By setting a lower-end acceptable threshold for these 3 optical properties, potential compound candidates for in vivo studies can be effectively filtered from unacceptable molecules. Molecular

brightness, as the product of molar absorptivity and quantum yield, allows quick comparison of the relative fluorescence of 2 or more dyes. After the analysis of the probes, a decision to utilize the probes in vivo or other applications can be made based on the outcomes of these experiments. The goal when designing fluorescent dyes is to achieve high molar absorptivity as well as a high quantum yield. However, the acceptable window of quantum yield values is quite large and can tolerate small values if the molar absorptivity is large enough. One factor that affects the optical properties of the dyes is functional groups. Synthesizing dyes that have electron-withdrawing or electron-donating effects on the conjugated system can lead to the overall stabilization of the molecule.[13] This study utilizes propionic acid groups stemming from the nitrogen on the indolenine end units of the pentamethine cyanine dyes to collect their optical property data that could influence further research on NIR applications. Although the carboxylic acid groups are not directly involved in the excitation-relaxation process of the fluorescent dye, their presence may have an effect on the optical efficiency of the dye. To analyze this further, Spartan '14 molecular modeling was used to determine the role of the carboxylic acid group in the dye geometry.

Experimental

Instrumentation

Absorbance spectra were measured using a Cary 50 spectrophotometer (Varian, Palo Alto, CA, USA) interfaced to a PC, with a spectral bandwidth of 2 nm. Fluorescence spectra for the pentamethine cyanine dyes were obtained using a Shimadzu RF-1501 Spectrofluorophotometer (Shimadzu Scientific Instruments, Columbia, MD, USA) interfaced to a PC, with the spectral bandwidths for both excitation and emission set to 10 nm and the sensitivity set to high.

Stock solutions

Stock solutions of the dyes were prepared by weighing the solid on a 5-digit analytical balance directly into a brown glass vial and adding dimethyl sulfoxide (DMSO) (99.9% for spectroscopy; Acros Organics, Bridgewater, NJ, USA) *via* a class A volumetric pipette (Kimble/Kontes, Vineland, NJ, USA). The contents of the vial were vortexed for 20 seconds and then sonicated for 5 minutes to ensure complete dissolution. The stock solutions were protected from light and stored in the freezer when not in use.

Method for determining molar absorptivity

Stock solutions were used to prepare serial dilutions in ethanol or phosphate-buffered saline (PBS) ranging from 0.8 to 16 μM. Samples were prepared in 5.00 (±0.02) and 10.00 (±0.02) mL volumetric flasks using a 5- to 50-μL Micropipette 821 and a 200- to 1000-μL Pipetman (P1000) micropipette (Gilson,

Inc., Middleton, WI, USA). The absorbance spectrum of each sample was measured using the Cary 50 spectrophotometer (Varian, Palo Alto, CA, USA), and the absorbance at the wavelength of maximum absorbance (λ_{max} AB) was determined. The absorbance values (A) of each sample at λ_{max} AB were plotted as a function of dye concentration (C), and the linear regression equation using Microsoft Excel was computed.

Computational methods

The structure of each compound was first optimized using the Hartree-Fock Density Functional Theory (HF-DFT) method with the hybrid exchange-correlation functional B3LYP/6-31G* basis set using Spartan '14 (Irvine, CA, USA). The torsional angles from the quaternary nitrogen to the α-carbon on the alternate heterocycle were restricted to obtain the calculated absorbance values and highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO). The electrostatic potential maps and the calculated HOMO and LUMO orbitals were obtained using a restricted hybrid HF-DFT self-consistent field in vacuum performed with B3LYP/6-31G* basis set.

Method for determining quantum yield

Standards were chosen with wavelengths of maximum emission within 10 nm of those of the unknowns to prevent errors resulting from wavelength-dependent variation in fluorimeter response. Samples of the dyes and their respective standards were prepared from stock solutions such that their absorbance at λ_{max} AB was less than 0.1 (to prevent the inner filter effect in fluorescence measurements). The absorbance and fluorescence spectra of each sample were obtained concurrently to minimize experimental error from photobleaching and potential solubility issues, and for all scans, the standard was run both prior to and following the unknowns (to ensure no change in instrumental response over the course of the runs). For the pentamethine dyes, duplicate absorbance scans were obtained and the absorbance values at both λ_{max} AB and λ_{ex} were averaged. The emission spectra of the pentamethine dyes were measured in triplicate using the RF-1501 fluorimeter with the excitation wavelength set to 620 nm. The area under each fluorescence curve was calculated and corrected for the Rayleigh peak area (if necessary). The average fluorescence peak areas were then calculated for each sample.

General

All chemicals and solvents were of American Chemical Society grade or HPLC (high-performance liquid chromatography) purity and were used as received. All chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA), Sigma-Aldrich (Saint Louis, MO) and Acros Organics. Melting points (mp, open Pyrex capillary) were measured on a Thomas Hoover
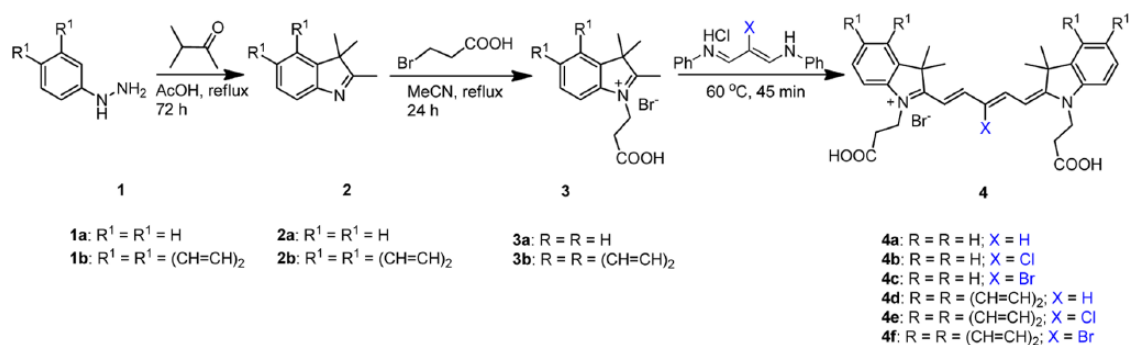
Scheme 1. The synthetic route used to obtain compounds **4a-f** from their corresponding phenylhydrazines 1.

apparatus. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on Bruker Avance (400 MHz) spectrometer.

Synthesis

The aim of this study was to evaluate the optical properties of 6 carboxylated cyanine dyes. A brief synthesis is provided; however, a much more comprehensive set of compounds that include the current 6 compounds with complete characterizations will be published elsewhere. As outlined in Scheme 1, all dyes underwent the same synthetic route which began with the corresponding phenyl hydrazine salts being refluxed in the presence of 3-methyl-2-butanone under acidic conditions to give indolenines **2a-b** in an excellent yield. Then, indolenines **2a-b** were alkylated using 3-bromopropionic acid in acetonitrile to yield the corresponding cationic salts **3a-b**. These salts are individually combined in the presence of unsubstituted or halogenated malonaldehyde bisphenylimines in the presence of acetic anhydride and sodium acetate and heated under reflux to afford the pentamethine cyanine fluorophores **4a-f**. During the synthesis, the formation of the fluorophores was monitored using regular-phase thin layer chromatography with a mobile phase of 95% dichloromethane (DCM) and 5% methanol. The purification of the final dyes was attempted through several methods. Column chromatography was used and mobile phase composition began at 100% DCM. The compound band did not move in this mobile phase, so an increased amount of methanol up to 10% was used in DCM. Three bands of similar color appeared, and it was determined through ^1H NMR that the isolated compound had formed a mixture of 3 compounds—di-acid, mono-acid, and di-methanolic ester—and the isolation of each compound was difficult. Another method of purification had to be used to obtain the dicarboxylic acid as the sole compound. Regular crystallization did not show improved purity; therefore, vapor exchange crystallization method was used. The compound was dissolved in DCM and placed in a small vial. The vial was then placed in a larger sample container of diethyl ether and the lid was secured. The container was left untouched for several days while the vapors exchanged, and the crystals were allowed to grow slowly over time as opposed to quickly. After isolating each compound as

blue crystals, the ^1H NMR confirmed the synthesis of dicarboxylic acid version and showed that there was a COOH peak around 11.5 ppm, and it was exchangeable with D_2O.

Results and Discussion

*Optical properties and molecular modeling*

The optical properties of compounds **4a-f** (Scheme 1) were measured separately in either an ethanol solution or a phosphate buffer, pH 7.4, and the collected data can be seen in Table 1. A stock solution of 1 mM was made by weighing 2.0 mg of each dye which then was dissolved with the necessary amount of DMSO depending on the molecular weight of the compound. From the stock solution, dilutions with either ethanol or PBS to achieve working samples of 16, 14, 12, 10, 8 and 6 µM were tested. The absorption values and excitation and emission profiles from 400 to 800 nm of each sample were obtained. To obtain the fluorescence spectra, a 10-times dilution from the absorption sample was performed so as to not flood the detector. A representative absorbance spectrum of compound **4a** can be seen in Figure 1. The molar absorptivities of compounds **4a-f** in PBS are below 100 000 $M^{-1} cm^{-1}$, with higher values in organic solvent. As a reference, Cy5.5[21] is known to have a molar absorptivity of around 200 000 $M^- cm^{-1}$, more than double the highest absorbing dye featured here. Based on analysis of the optical efficiency obtained through absorbance and fluorescence, it would be difficult to determine the reason for the low molar absorptivity values. However, molecular modeling can provide more structural insight and lend to the formation of hypotheses in this case. Although performing computational work without experimental proof has its limitations, molecular modeling through Spartan '14 can still help to explain the properties exhibited by these molecular probes in particular. One observation relates to an intramolecular dimerization through hydrogen bonding, which causes the carboxylic acid groups to stretch and the methine bridge to bend. This must be confirmed through other experiments such as 2-dimensional NMR spectra or x-ray crystallography.

A high Stokes shift value can allow for a clear distinction between excitation light and fluoresced light. A Stokes shift value of about 10 to 12 nm is considered adequate for in vivo

Table 1. The optical property data collected from compounds **4a-f** in 2 solvents.

ID	λ_{ABS} (NM)		MOLAR ABSORPTIVITY (ε), $M^{-1}CM^{-1}$)		λ_{EM}, NM		STOKES SHIFT, NM		QY (Φ), %		MB ($\varepsilon \times \Phi$)	
	ETOH	PBS	ETOH	PBS	ETOH	PBS	ETOH	PBS	ETOH	PBS	ETOH	PBS
4a	646	643	60 700	56 300	668	659	22	16	23	72.3	13 961	40 705
4b	645	644	32 200	30 300	663	657	18	13	67.5	42.2	21 735	12 787
4c	646	644	24 500	18 000	662	655	18	11	78.5	22.5	19 233	4050
4d	685	680	78 400	50 700	708	697	23	17	42.3	43.4	33 163	22 004
4e	684	680	93 100	79 500	702	695	18	15	21	20.7	19 551	16 457
4f	684	681	95 400	81 000	702	695	18	14	19.9	16.2	19 985	13 122

Abbreviations: EtOH, ethanol; PBS, phosphate-buffered saline.
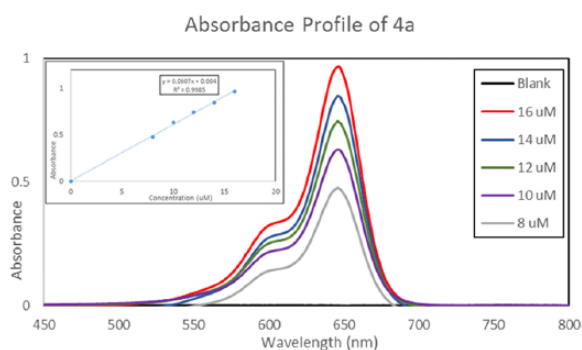
Figure 1. The absorbance spectra and r² spectra and value for each dilution of compound **4a**.

applications. In ethanol, the studied dyes approached a 20-nm Stokes shift, leading to a clear distinction in the absorbed light and the emitted energy. Although the values in buffer are lower than in organic solvent, they still remain above the standard for fluorescent in vivo probes. The quantum yield values show a few unique trends. Compounds **4a-c** show an increase in quantum yield values in ethanol as it relates to substituents at the meso-position on the pentamethine bridge. As the halogen at this position increases in size from unsubstituted to chlorine to bromine, the quantum yield values improve and the dyes become more optically efficient. However, the opposite trend occurs when looking at the quantum yield in aqueous medium. It seems that the hydrophobicity of the molecules increases when a larger halogen is added to the polymethine bridge of the molecule. Fluorophores **4d-f** show a consistent decrease in quantum yield values over the 2 solvents as the substituent size at the meso-position increased. As this set of molecules is more hydrophobic due to the larger benz[e] indole end units compared with the unsubstituted indolenine heterocycle, the hydrophobicity added when substituting atoms is not tolerated as was the case with the indole compounds **4a-c**. The quantum yield values for all 6 compounds, when looked at alongside compounds not featuring the propionic acid groups, yield comparable results in organic solvent or aqueous medium.[11]

Another unique observation from the molecular modeling is the conformation of each atom on the pentamethine bridge. Usually, as is the case with pentamethine cyanine dyes not featuring acid groups stemming from the indole nitrogens, the gamma hydrogen points down, away from the dimethyl groups, the beta hydrogens point up, and the alpha protons point down. However, after minimization using a Hartree-Fock force field, the hydrogens at each position on the bridge can be seen to be pointing in the opposite direction from what is expected. This phenomenon may be another consequence of the COOH groups dimerizing; because the propionic acid groups dimerize "under" the bridge, there is less room "under" the molecule. This is especially apparent when looking at the minimized calculation of compound **4c**, featuring a bromine molecule substituted at the meso-position. It can be seen that the bromine molecule is pointing up, as shown in Figure 2. Figure 3 points out the shape difference between several pentamethine cyanine dyes. The structural difference between the dyes in Figure 3 is the R-group bonded to the nitrogen on the indolenine salts. With the carboxylic acid dye, a clear conformation difference compared with the other 2 compounds (methyl or butyl groups) exists; there is a definite puckering out in comparison with the other dyes featuring methyl or butyl groups This shape difference would occur if intramolecular dimerization of the acid groups arises in these compounds, whereas it is not possible with the alkyl chains (Figure 4).

Conclusions

The optical properties of cyanine dyes featuring carboxylic acids remain unstudied and unpublished. In this study, 6 cyanine compounds, **4a-f**, were synthesized and their optical properties were analyzed to learn more about the effects that carboxylic acid groups would have on the overall molecule structure. The efficiency of the dyes was analyzed in 2 different solvents, one organic and one aqueous, to observe the solvent effects. When looking at the trends for the indolenine dyes, the molar absorptivities of the dyes **4a-c** decreased with increasing hydrophobicity afforded from increasing halogen size on the bridge. The
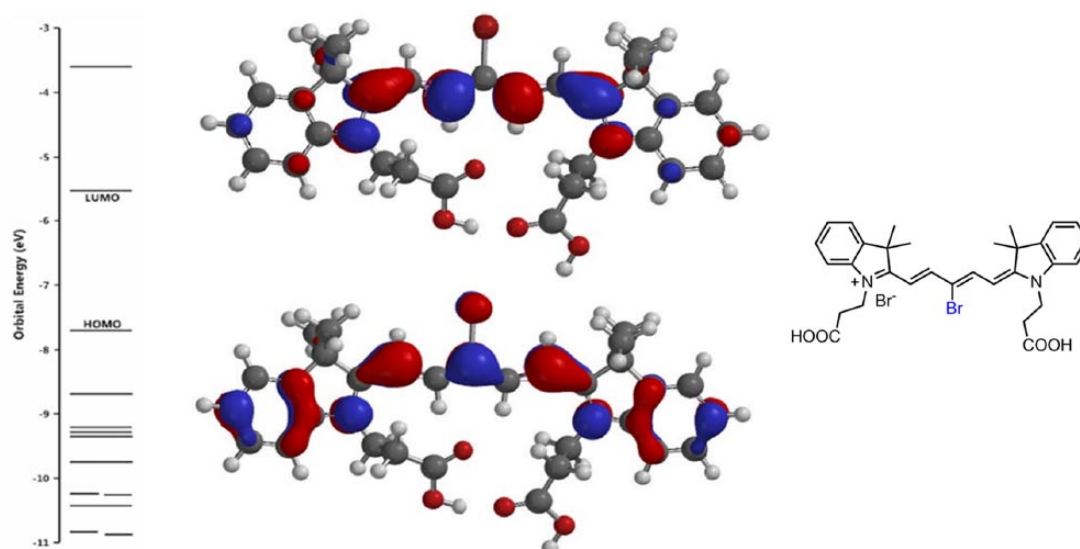
Figure 2. The highest occupied molecular orbital (HOMO)/lowest unoccupied molecular orbital (LUMO) electron cloud distribution for compound **4c**. The bromine atom at the meso-position points up instead of down.
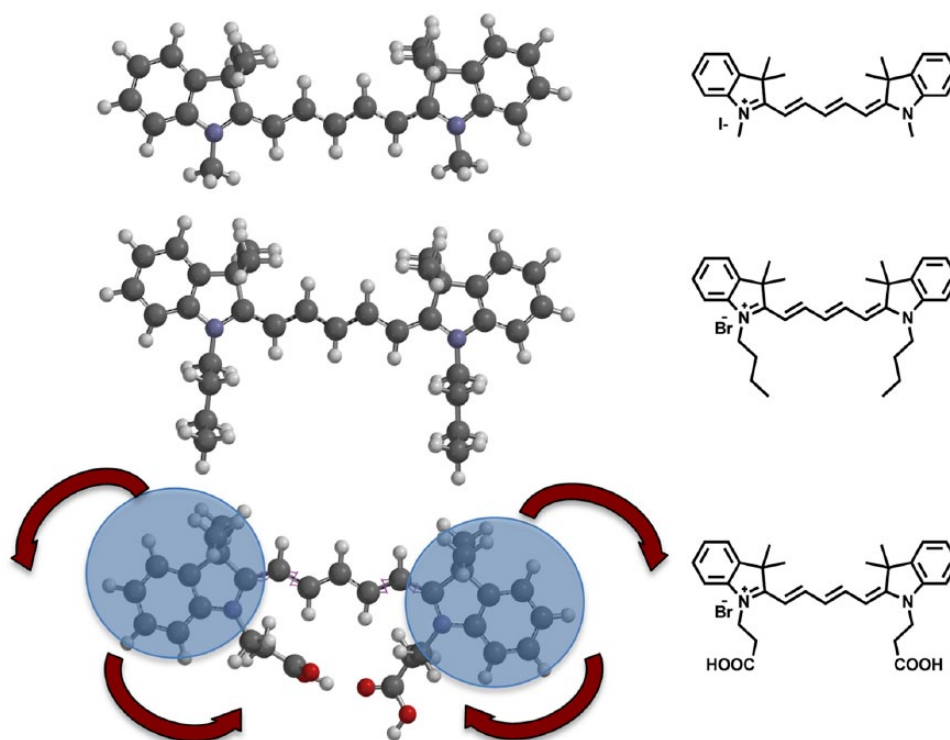
Figure 3. The minimized structures of compounds with varying *N*-groups: methyl (top), butyl (middle), and propionic acid (bottom) to visualize the puckering displayed by the acid compounds.

opposite trend was seen in dyes **4d-f** in both solvents, suggesting more stabilization across the molecule due to the larger amount of conjugation. Overall, the molecular brightness values of the dyes do not necessarily make these dyes suitable for in vivo visualization of tissues. However, future in vivo studies could be performed to determine the inherent uptake and biodistribution of these dyes. Other observations through molecular modeling were seen and used to come up with a hypothesis of how the structure affects the optical properties of the dyes. In comparison with alkyl chain (methyl or butyl) substituted

indolenine compounds, there was significant puckering of the molecule that alters the direction in which the hydrogens and substituents on the unsaturated bridge point. The puckering may be due to the intramolecular dimerization of the carboxylic acid groups. Further studies on bond length throughout the bridge of the compounds could confirm that due to the puckering and length between the carbons in the chain, the molar absorptivity values of the dyes are low. Other studies, such as x-ray crystallography, and various other theoretical conformational calculations to expand the scope of knowledge on this
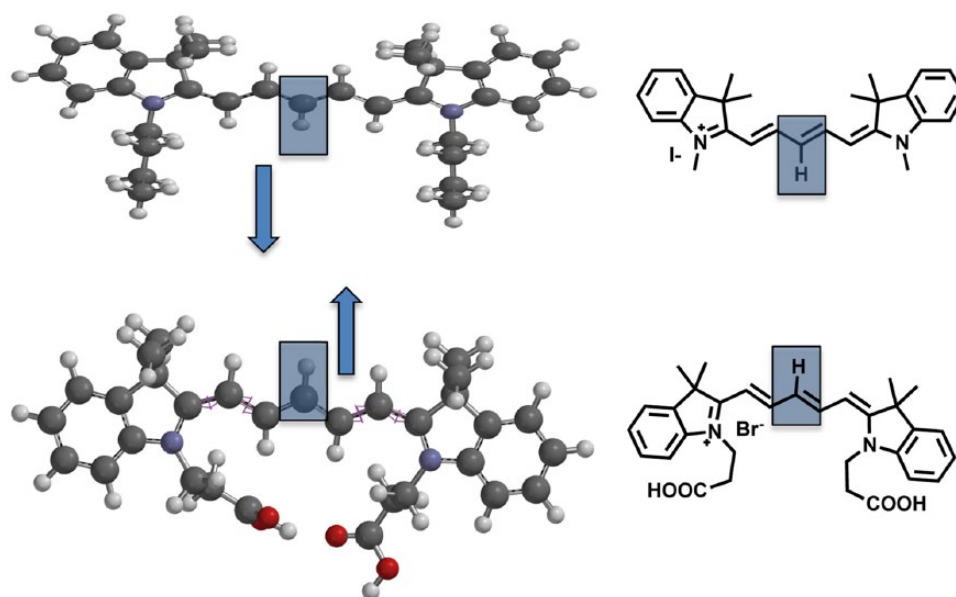
Figure 4. A minimized calculated structure comparison of 2 pentamethine cyanine dyes that show the difference in the alpha, beta, and gamma substituents on the polymethine bridge.

subset of cyanine probes could be conducted. However, because of this study, a new outlook has arisen on the optical properties of cyanine probes featuring propionic acid groups.

Author Contributions

MH conceived and designed the experiments. MH, TLD, and MTG analyzed the data. MH agrees with manuscript results and conclusions. TLD and MH wrote the manuscript, jointly developed the structure and arguments for the paper, and made critical revisions and approved the final version. All authors reviewed and approved the final manuscript.

Disclosures and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including, but not limited to, the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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