

Polymethine Cyanine Dyes: A Gateway to Near Infrared Fluorescence Syntheses

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ABSTRACT

Polymethine cyanine near-infrared dyes are important tools for optical imaging of deep tissues. However, these dyes often aggregate in aqueous solutions *via* π - π stacking, suffer from poor photostability, and frequently exhibit structural instability when utilizing simple heptamethine linkers.

The goal of this experiment was to synthesize a pentamethine cyanine dye using an indolenium salt and a pentamethine linker, under mild conditions, specifically 1,3,3-trimethyl-2-((1E,3E,5E)-5-(1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3H-indol-1-ium iodide. Utilizing malonaldehyde bis(phenylimine) hydrochloride instead of free malonaldehyde provided greater reactant stability. The mixture was refluxed in acetic anhydride using sodium acetate as a base, with reaction progress monitored by thin-layer chromatography

^1H NMR, ^{13}C NMR, and UV-VIS spectra of the product supported the formation of the target compound. The synthesis demonstrated polymethine cyanine dyes can be prepared using this protocol. This methodology provides a framework for varying structural elements of the molecule and tuning its properties for different applications. Modifications to the methodology can be used to increase percent yield and reduce the use of less environmentally favorable or hazardous materials.

Keywords: Near infrared fluorescence dyes; pentamethine cyanine; indolium salts; single electron transfer; quaternary ammonium salts.

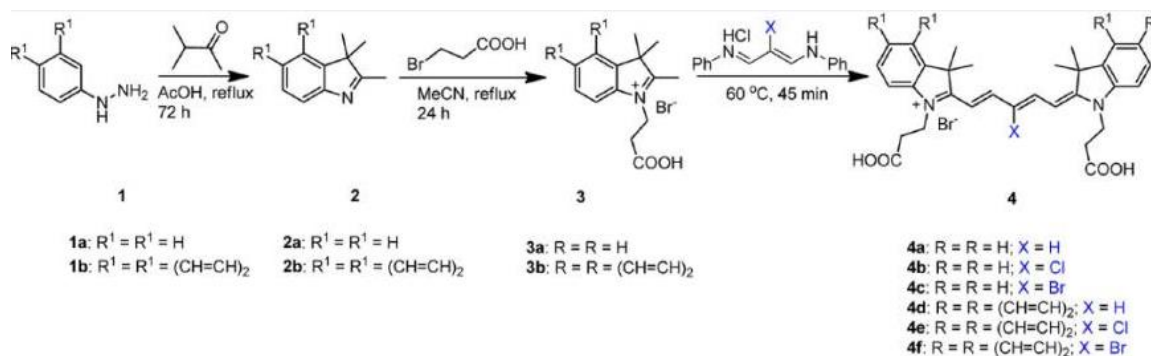
1.0 INTRODUCTION

Near-infrared (NIR) fluorescent dyes are valuable tools in biomedical imaging and biological chemistry. The NIR region is generally divided into NIR-I (700–1000 nm) and NIR-II (1000–

2300 nm). Light at these wavelengths penetrates tissue more deeply than visible light and does not involve the ionizing radiation associated with X-rays and gamma rays.¹ Polymethine cyanine dyes are a class of NIR fluorescent dyes containing two nitrogen-containing heterocyclic rings connected by an odd-numbered, conjugated carbon chain (polymethine). These dyes possess tunable emission wavelengths, structural versatility, and high molar extinction coefficients.²

Applications of cyanine dyes are constrained by several factors, including poor aqueous solubility, susceptibility to photobleaching, and synthetic limitations. Modifying the heterocyclic rings and the conjugated polymethine chain provides strategies for overcoming these limitations and optimizing the properties of cyanine dyes.^{3,4}

This report describes the design, synthesis, and characterization of a NIR-I pentamethine cyanine dye. The synthetic protocol and characterization of the product are presented, with Pentamethine cyanine dyes can be synthesized by reacting nitrogen-containing aromatic heterocycles with malonaldehyde bis(phenylimine) hydrochloride to form the polymethine bridge. A generalized reaction scheme is shown in Scheme 1.



Scheme 1. Generalized synthesis of pentamethine cyanine dyes. Reproduced from Dost et al., “Synthesis and Optical Properties of Pentamethine Cyanine Dyes with Carboxylic Acid Moieties,” *Analytical Chemistry Insights*.

The formation of pentamethine cyanine dyes involves condensation reactions that generate the conjugated polymethine bridge. Malonaldehyde bis(phenylimine) hydrochloride serves as

a stabilized precursor for formation of this bridge and enables synthesis under relatively mild reaction conditions.^{5,6}

2.0 RESULTS AND DISCUSSION

The stoichiometric ratio of malonaldehyde bis(phenylimine) hydrochloride to 1,2,3,3-tetramethyl-3H-indolium iodide is 1:2. As shown in Table 1, the two reactants were used in an approximately 1:2 molar ratio, corresponding closely to the stoichiometric ratio. Therefore, neither reactant was present in a substantial excess.

Table 1 Reagents used in this synthesis

Reactants	RMF = relative mole factor - integer					
	RMF	MW	Molarity (mols)	mass (g)	volume (mL)	density (g/mL)
malonaldehyde bis (phenylimine) hydrochloride	1	258.8	0.00083	0.215		
1,2,3,3-tetramethyl-3H-indolium iodide	2	301.2	0.00166	0.500		
sodium acetate		82.03	0.00170	0.139		
Acetic anhydride				6.5	7	1.082

The synthesis proceeded normally. Initial purification was done by precipitation in diethyl ether. TLC was used to evaluate potential eluents for chromatographic purification. Dichloromethane and dichloromethane containing 1–3% methanol were evaluated, with increasing methanol concentrations used to determine suitable separation conditions. Flash chromatography separated the crude mixture, producing a blue fraction believed to contain the desired product. The product was eluted into nineteen fractions of 100 mL each. TLC analysis indicated that fractions 15–19 contained the pure product. These fractions were combined, and the solvent was removed using a rotary evaporator.

The labeled ^1H NMR spectrum is provided in Figure S1 of the Supporting Information, and the corresponding proton assignments are shown in Figure 1. The ^1H NMR spectrum contained signals consistent with the proposed structure. The major signals were assigned as follows: a singlet, 'A', on the spectrum, representing the four equivalent

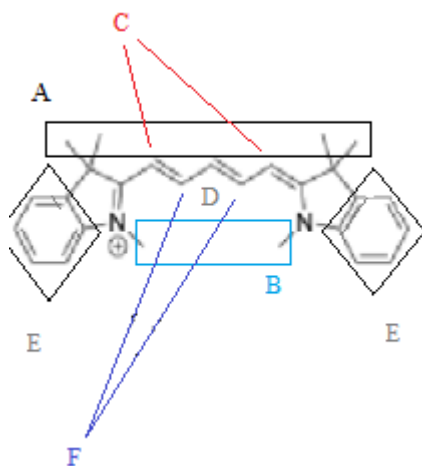


Figure 1 Hydrogen designations used in the ^1H NMR

methoxy groups, at 1.75 ppm integrated to 12 hydrogens; a singlet, 'B', at 3.70 ppm integrated to 6 hydrogens, consistent with the two equivalent N- methoxy groups; a doublet, 'C', integrated to 2 hydrogens corresponding to the two identical α polymethine hydrogens is at 6.34 ppm (J coupling constant of 13.2 Hz); a triplet signal, 'F', integrating to 2 hydrogens and corresponding to the two identical β polymethine hydrogens, present at 8.15 ($J=13.0$ Hz); a triplet, 'D', integrating to 1 hydrogen which corresponds to the central γ polymethine hydrogen at ~ 7.8 ppm (estimated $J=13$ Hz) [the 'D' signal had no ppm shown on the spectrum by the NMR software, so the ppm and J had to be estimated]; and a multiplet signal, 'E', integrating to 8 hydrogens between 7.11 and 7.41 ppm, corresponding to the 8 indolium aromatic hydrogens.

Although the ^1H NMR spectrum was consistent with the proposed pentamethine cyanine structure, additional signals indicated the presence of impurities. Figure S1 shows signals below 1.75 ppm, between 1.76-2.60, some small signals between 3.70 and 4.30 ppm, and a sharp singlet at about 5.35 ppm all of which do not match the desired product, indicating contamination. Possible sources of these signals include residual solvents, unreacted starting materials, or reaction byproducts. If there was significant half-dye present, of the polymethine iodolium, some of the proton integrations would have been skewed.⁷ A large singlet peak was observed at 7.28 ppm. A large singlet was observed at approximately 7.28 ppm. Residual CHCl_3 in CDCl_3 typically appears at approximately 7.26 ppm.⁸ Although the observed signal is close to this value, its unusually large intensity suggests that additional material may

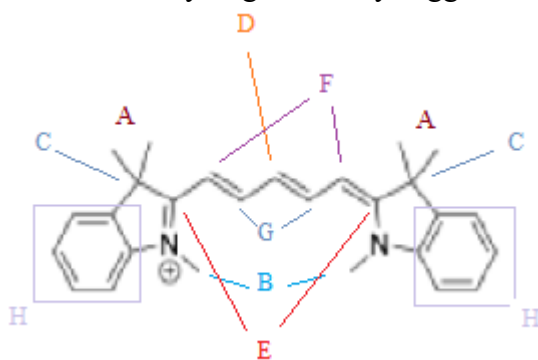


Figure 2 Carbon designations used in the ^{13}C NMR

contribute to the signal. Because it overlaps the aromatic proton region, its assignment cannot be made with certainty. Being the signal is in the middle of the signals of 8 aromatic hydrogens; a clear attribution of the peak isn't possible.

All but one of the ^{13}C signals can be attributed to the product or the solvent. The ^{13}C spectrum for the product is in figure S2, in Supplemental information, section 6.4 specifically, with the labeled carbons in figure 2. The 'A' signal presents at 28.04 ppm, in the alkane region, which is where the two gem dimethyl groups (4 C) should appear. These would have the greatest shielding and be the most upfield of all the carbons. The 'B' signal appears at approximately 33 ppm (too small a signal to automatically register the ppm, by the Bucker software) and

corresponds to the expected chemical shift of the two N-methyl groups. This is somewhat upfield, but less shielded than the gem dimethyls groups. The 'C' signal reads at 49.34 ppm corresponding to the two quaternary sp^3 carbons of the indolium groups. The $CDCl_3$ solvent signal appeared as a triplet at 77.11 ppm, at the expected signal of 77.11 ppm (average of two sources) and had a ^{13}C - ^2H J coupling constant of 32 Hz, at the upper range of the expected value.^{9,10} The 'G' signal at 103.91 ppm corresponds to the 2 β polymethine carbons would be expected. The 'F' signal shows at 110.48 ppm, corresponding to the α carbons, closest to the indolium groups and less shielded than the 'G' carbons. The 'H' group is a collection of signals that seem to belong to the aromatic indolium carbons. They show 7 signals clearly, ranging from 120.81 to 142.49 ppm, with a possible 8th that isn't clear and was not automatically registered by the Bruker software. They represent the 16 aromatic carbons on the two identical indolium groups. The 'D' signal at 153.76 ppm was assigned to the central γ -polymethine carbon. Its downfield chemical shift is consistent with the significant electronic delocalization associated with the central region of the polymethine chain. The 'E' signal, at 173.44 ppm, represents the two quaternary carbons connecting the two indolium groups with the polymethine linker. The 'E' carbons act as a bridge between the polymethine linker and the nitrogens and show the greatest deshielding.

The UV–Vis spectrum of the product in methanol (Figure S3) exhibited a maximum absorbance (λ_{max}) at 640 nm. The reported literature value in ethanol is 638 nm.¹¹ The close agreement with the reported λ_{max} provides additional evidence consistent with formation of the target compound.

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π stacking or aggregation of the cyanine molecules in the solid state, which has been reported for cyanine dyes.¹²

The product targeted, 1,3,3-trimethyl-2-((1E,3E,5E)-5-(1,3,3-trimethylindolin-2-ylidene) penta-1,3-dien-1-yl)-3H-indol-1-ium iodide, was produced, with a high degree of confidence. Taken together, the ¹H NMR, ¹³C NMR, and UV–Vis data provide strong evidence that the targeted pentamethine cyanine dye was formed. Although the ¹H NMR spectrum indicated the presence of impurities, the major resonances were consistent with the proposed structure. Because the purified product was pooled with material from other groups, a final isolated yield could not be determined.

3.0 EXPERIMENTAL

3.1 Chemicals

The following chemicals were used for the experiments described in this paper. ethyl ether, made by BDH, a distributor of VWR Industries, Radner, PA, CAS # 60-29-7; ethyl acetate, by BDH, a distributor of VWR Industries, Radner, PA, CAS # 141-78-6; methanol, 99.8 %, Celedon Labs Ltd., Georgetown, Ontario, Canada, CAS # 67-56-1; hexane – mixed isomers, > 98.5%, Sigma Aldrich, St Louis, MO, CAS# 107-83-5; 1,2,3,3-tetramethyl-3H-indolium iodide, 98%, Sigma Aldrich, St Louis, MO, CAS# 5418-63-3; malonaldehyde bis(phenylimine) monohydrochloride, 98%, Alfa Aesar, Shore Rd, Heysham, Morecambe, UK, CAS# 12307-42-1; acetic anhydride – 98%, ACS reagent grade, Aldrich Chemicals Co., Milwaukee, WI, CAS# 108-24-1; sodium acetate – anhydrous, ACS grade, ALFA Aesar, Ward Hill, MA, CAS# 127-09-3; and dichloromethane, 99.9%, ACS certified, Fisher Scientific, Fairlawn, NJ, CAS# 75-09-2; and silica gel, ultrapure, 60-200 μ m, Acros Organics, Janssen Pharmaceuticaaan 3A, 2440 Geel, Belgium, CAS# 7631-86-9. Also used were TLC glass plates – Silica Gel 60 F₂₅₄, Merck KGaD, Darmstad, Germany.

3.2 Equipment

The equipment used included rotary evaporator, Rotavap R-114, manufactured by Büchi Labortechnik AG, Meierseggstrasse 40, 9230 Flawil, Switzerland; PerkinElmer Spectrum IR, 710 Bridgeport Avenue, Shelton, CT 06484; Bruker NMR, Bruker Avance 400 MHz, manufactured by Bruker BioSpin, 40 Manning Road, Billerica, MA 01821; the Nicolet iS10 FTIR spectrometer, manufactured by Thermo Fisher Scientific, 2797 Peterson Pt NW, Norcross, Ga 30071; and a Cary 50 Scan UV-Vis spectrophotometer, Agilent Cary, 13000 Weston Pkwy, Cary, NC 27513.

3.3 Procedures

Malonaldehyde-bis (phenyl imine) monohydrochloride (0.215 g), sodium acetate (0.139 g), 1,2,3,3 tetramethyl-3H-indolium (0.500 g) and acetic anhydride (7 mL) were added to a round-bottom flask, and the mixture was refluxed for one hour. After one hour of reflux, thin-layer chromatography (TLC) was performed to monitor the reaction while the mixture continued to reflux. The TLC run had a precursor lane (S), a precursor plus product lane (S+P), and a product only lane (P). The product lane produced an R_f range of 0.37-0.40, the S+R lane R_f range of 0.33-0.37 and 0.47-0.60, and the precursor lane an R_f range of 0.46-0.58. The

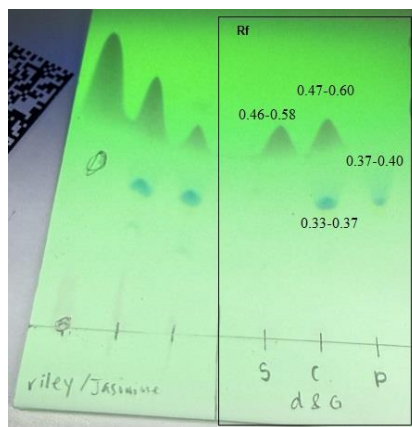


Figure 3 – TLC with R_f values

figure above, Figure 3, shows the R_f values measured (right side) on the TLC plate. After a total reflux time of two hours, most of the acetic anhydride was removed using a Rotavap R-114 rotary evaporator (rotovap).

Diethyl ether was used to precipitate the indolium pentamethine cyanine dye product. Vacuum filtering produced a grey-black solid residue and dark blue filtrate. The solid exhibited signs of water absorption and was therefore dissolved in dichloromethane, then filtered to remove any remaining sodium acetate. The dichloromethane was removed using a rotary evaporator, and the solid was left open to dry over one week.

The crude product was sticky, and the weight exceeded the predicted theoretical yield by approximately 15%, indicating some contaminants remained. Thin layer chromatography (TLC) was run to check purity and determine best eluent. A mixture of 1–2% methanol in dichloromethane was identified as a suitable eluent for column chromatography. Flash chromatography was used to purify the product, with that apparent product separated into several fractions. These were checked with TLC to determine the fractions with pure product. The fractions containing the pure product were combined, and the solvent was removed using a rotary evaporator. The result was sent for analysis.

3.4 Product Characterization

1. *1,3,3-trimethyl-2-((1E,3E,5E)-5-(1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3H-indol-1-ium iodide*: A dark blue solid; ^1H NMR (400 MHz, CDCl_3) δ /ppm 1.753 (s, 12H), 3.695 (s, 6H), 6.325-6.358 (2H, d), 6.8 (1H, t), 7.113-7.409 (8H, m), 8.118-8.183 (2H, t); ^{13}C $\{^2\text{H}\}$ -NMR 100 MHz, CDCl_3) δ /ppm 28.04, 33, 49.34, 103.91, 110.48, 120.01, 122.22, 125.19, 126.31, 128.63, 140.98, 142.69 153.73, 173.46; UV-Vis (MeOH) λ_{max} 640 nm.

4.0 CONCLUSIONS

The pentamethine cyanine dye was synthesized through the condensation of an indolenium salt with a malonaldehyde bis(phenylimine) salt. The compound synthesized was 1,3,3-trimethyl-2-((1E,3E,5E)-5-(1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3H-indol-1-ium iodide.

The ^1H NMR spectrum of the product showed signals consistent with the targeted compound, although some contamination was evident. The ^{13}C NMR spectrum showed signals at chemical shifts consistent with the reported literature values, further supporting formation of the target compound. The UV-Vis spectrum exhibited a λ_{max} at 640 nm, within 2 nm of the reported literature value, providing additional evidence for formation of the target dye. The isolated product was an intense dark blue solid that exhibited a slight golden sheen when dried against the glass wall of the flask.

The product belongs to the polymethine cyanine class of dyes, which has applications in biological and medical imaging. This protocol provides a framework for modifying the structure of this class of fluorophores, potentially allowing their photophysical and biological properties to be tuned for different imaging applications.

5.0 REFERENCES

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6.0 SUPPLEMENTAL INFORMATION

6.1 Formulas

6.2 Significant Figures

Significant figures used were the significant figures of the raw data plus one, for all calculation results.

6.3 Raw Data

Available on request.

6.4 Tables, Graphs, and Plots

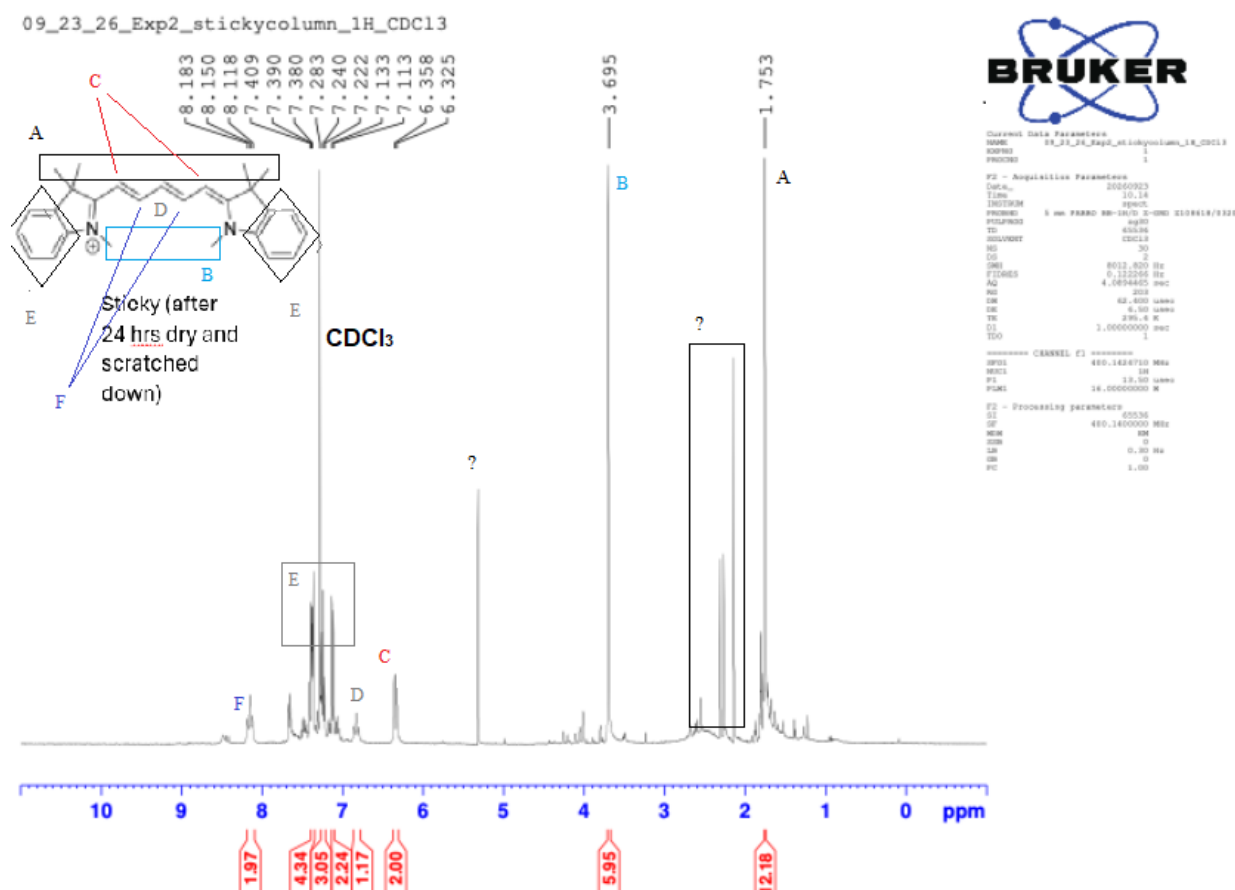
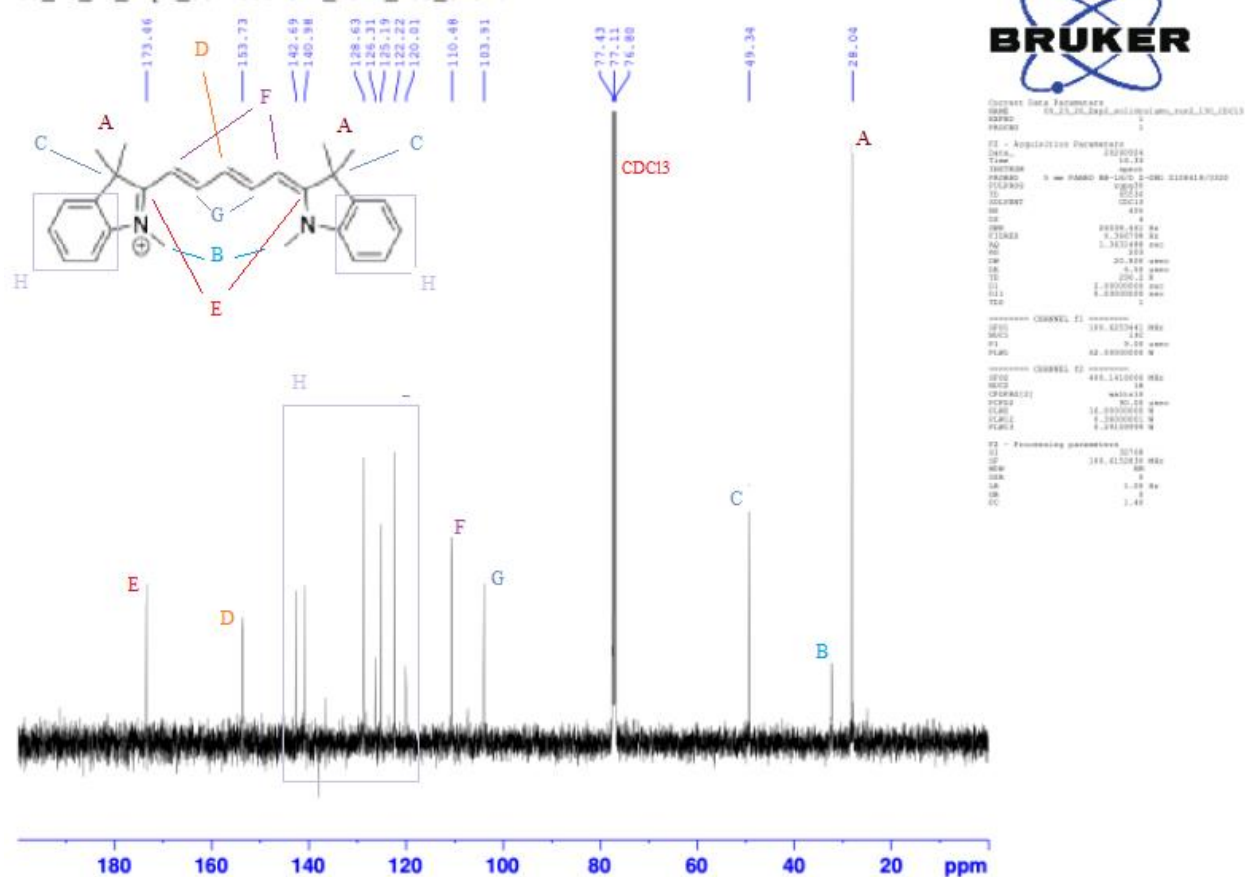


Figure S1 ¹H NMR of the pentamethine cyanine product

Figure S2 ^{13}C NMR of the pentamethine cyanine product

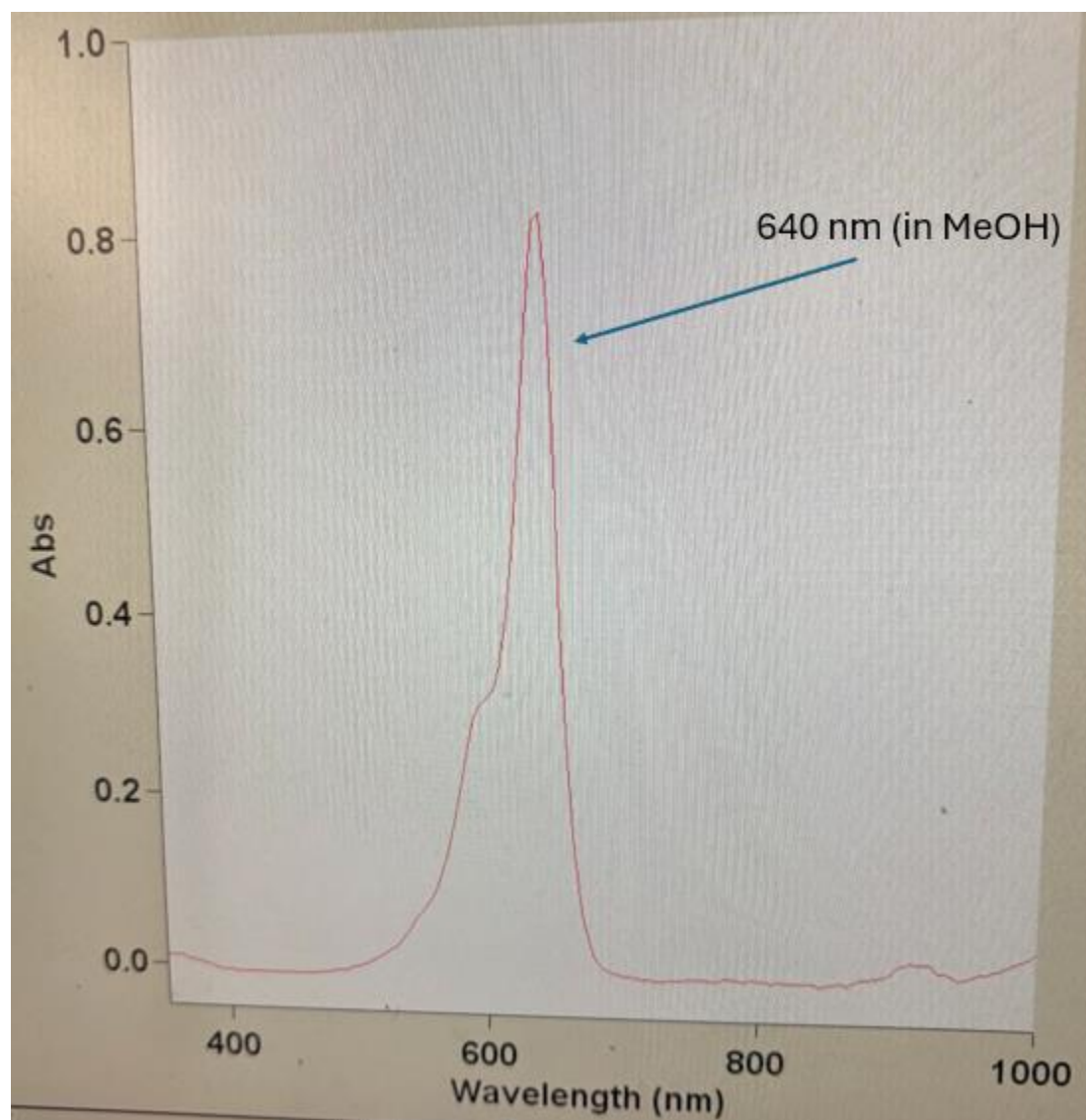


Figure S3 UV-Vis of the pentamethine cyanine product, λ_{max} at 640 nm