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1. Introduction

N	Absorbance (nm)	Emission (nm)
1	540-560	560-580
2	650-690	670-710
3	760-980	780-1000



Microwave assisted organic synthesis (MAOS) has been used in the development of eco-friendly syntheses of biological and chemical sensor substrates. Often, sensor substrates designed to monitor and detect environmental pollutants are synthesized using reaction conditions and reagents that are not environmentally friendly and contribute to the problem they are designed to detect and or mitigate. Consequently, there is a need for a change in reaction conditions; increased atom efficiency, catalytic processes, and a decrease in solvent use in reactions, extractions, and purifications (Anastas 1998) are strategies used to improve reaction conditions. MAOS is widely accepted as a “green” technology; synthetic strategies

enable solvent-free or minimal solvent use and increase atom efficiency in reactions (Hayes 2002). Further, because of the nature of MAOS, a variety of compounds can be synthesized that would not be feasible under conventional synthetic approaches (Lew, Krutzik et al. 2002).

The field of MAOS has changed dramatically from its first introduction in 1986 (Gedye, Smith et al. 1986; Giguere, Bray et al. 1986). In early work, microwave chemistry was often a last resource for an unsuccessful reaction or used to shorten reaction times from hours and days to minutes and seconds. The experiments were conducted in inexpensive domestic microwave ovens with time and power as the reaction conditions. Today, commercial microwave reactors control time, temperature, power, stirring and simultaneous cooling with real time monitoring of temperature, pressure, and power. Cutting edge instruments equipped with in situ analysis via raman spectroscopy (Leadbeater and Smith 2007), UV/VIS spectroscopy (Getvoldsen, Elander et al. 2002), and digital cameras and video that enable visual monitoring (Bowman, Leadbeater et al. 2008) have been developed capable of addressing these needs.

Our focus is the development of advanced microwave synthetic techniques that will aid in the synthesis of cyanine fluorophores and coupling agents needed in the development of biosensors with increased reaction efficiency, are environmentally benign, and cost effective.

1.1 MAOS of sensor probes

The rapid, cost-effective manner in which MAOS operates allows scientists access to a wide range and quantity of molecules that can be screened as potential biosensors. This section describes the application of MAOS to the synthesis of some fluorescent components in sensors. Perumal et al reported the microwave synthesis of an array of pyridinyl-1,2,4-triazine derivatives used as fluorescent sensors for ferric salts with reduced reaction times and comparable yields (66-85%) (Figure 2) (Perumal 2011). Bisaryl-3-pyrazine-1,2,4-triazine derivatives displayed good sensor property with Fe(III) ions in micro level concentrations.

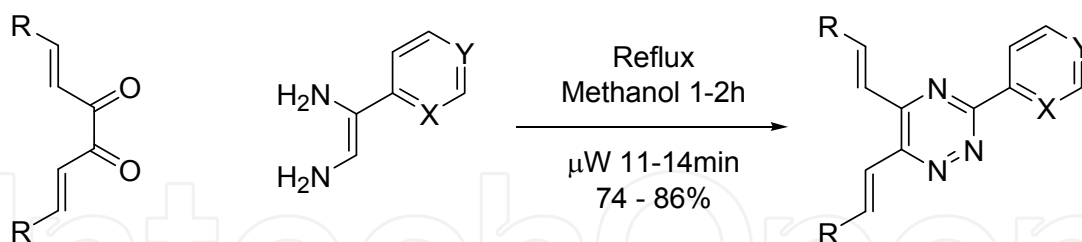


Fig. 2. Microwave synthesis of pyrazine derivatives

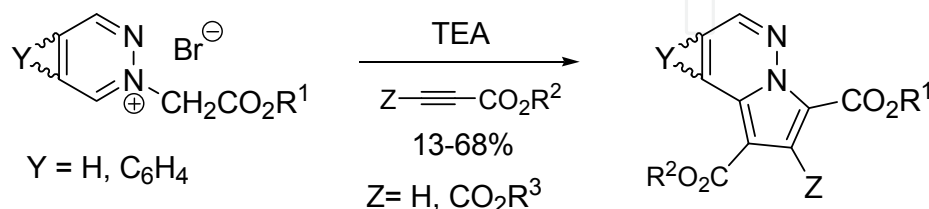


Fig. 3. Microwave synthesis of pyrrolodiazine derivatives

Pyrrolodiazine derivatives are highly fluorescent molecules that have potential applications in sensors and biosensors. The absorption and fluorescence of these N-heterocycles can be influenced by both solvent and substituent effects. MAOS has been used to rapidly and

efficiently synthesize an array of pyrrolodiazine derivatives to study the relationship between structure and optical properties. The reactions were carried out using a monomode reactor varying temperature (by manipulating the power) and time with yields ranging from 13-68% (Figure 3).

Lamberto et al, studied the synthesis of symmetric and asymmetric viologen as well as a bis-viologen using microwave technology. Viologen derivatives are building blocks in biosensors for nitrite detection. A variety of solvents, reaction times, and molar ratios were manipulated. Seventeen viologen derivatives were successfully synthesized using microwave irradiation using a CEM LabMate microwave synthesizer (Figure 4).

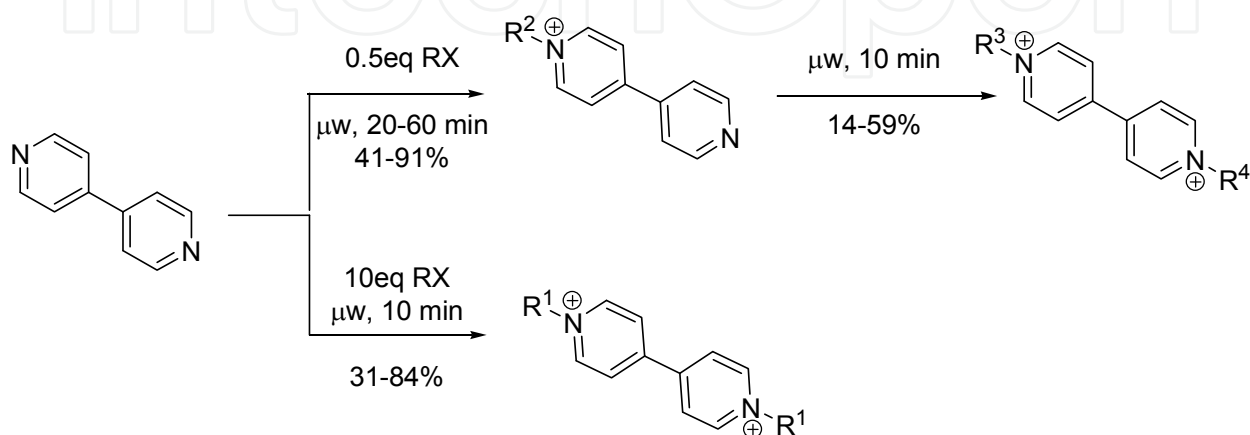


Fig. 4. Microwave synthesis of viologen derivatives

1.2 NIR cyanines

Applications that utilize NIR fluorescence technology are rapidly expanding. Heptamethine cyanines are an important class of NIR fluorescent molecules (Figure 5). Currently less than 1% of cyanine dye literature is concentrated on heptamethine derivatives. Consequently, progress in this area is limited by the lack of availability of suitable cyanine compounds that can be utilized as NIR labels and/or probes. More efficient ways of synthesizing these dyes are needed so that a variety of dyes with various spectral and chemical properties can be made available to thoroughly investigate their potential use as label and probes in analytical applications of NIR fluorescence.

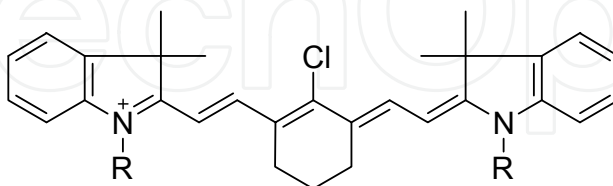


Fig. 5. Structure of NIR-1

Detection in the near-IR region demonstrates improved sensitivity over ultraviolet and visible (uv-vis) region detection because of the absence of background interference from competing analytes. This translates to a detection sensitivity based on the limitations of the detection instrument and not upon background interference. Based on this principle, shifting the fluorescence detection to longer wavelengths results in improved detection sensitivity. The increase in the polymethine chain increases the absorbance to the desired

longer wavelengths and the inclusion of a six membered cyclic ring into the polymethine chain increases the photostability of the NIR Cy7 dye (Stoyanov). Near-IR fluorescent molecules also have other advantages including:

- higher molar absorptivities
- compatible with solid state excitation sources
- lower excitation energy requirements associated with the near-infrared region

1.2.1 NIR-cyanine synthesis

Cyanines can be described as either symmetrical (identical heterocyclic salts) or unsymmetrical (different heterocyclic salts). The general synthetic strategy used in the preparation of cyanine dyes is a multistep process (Figure 6). First, the nitrogen-containing heterocycle is quaternized with an alkyl halide to form a *N*-alkyl heterocyclic quaternary salt. The symmetrical dye is prepared through the addition of 2eq of the same heterocyclic salt to the an electrophile such as an imine, bisimine, or bisaldehyde and subjected to the reaction conditions.

Two published methods of synthesizing symmetrical cyanine dyes requires heating a mixture of a substituted quaternary salt (I) and either a bisimine or bisaldehyde to reflux in 1-butanol and benzene (Narayanan and Patonay 1995) or sodium acetate and ethanol (Jung and Kim 2006).

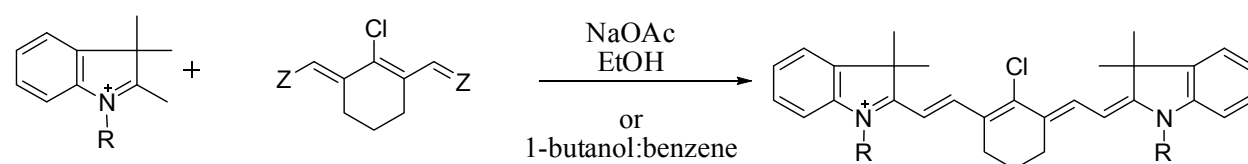


Fig. 6. Conventional synthesis of heptamethine cyanine dyes

The synthesis of unsymmetrical cyanines involves treatment of heterocyclic salt the desired electrophile to form the hemicyanine. The hemicyanine is reacted with a second heterocyclic quaternary salt to form an unsymmetrical cyanine dye. The major problem in the synthesis is the formation of the symmetric dye as a byproduct. A common solution is the isolation and rigorous purification of the hemicyanine, even though they are a challenge to isolate and purify (Mujumdar, Ernst et al. 1993; Mank, Van der Laan et al. 1995). This is a problem regardless of the methine chain length (Cy₃, Cy₅, Cy₇), heterocyclic salt precursors, or water-solubility. The solid phase technique was applied to unsymmetrical cyanine synthesis by Mason et al (Mason, Hake et al. 2005) Solid phase synthesis of Cy₃ and Cy₅ unsymmetrical dyes is five and three steps respectively, in overall crude yields ranging from 3-60% with 50->95% purity (Figure 7). The synthesis requires extensive use of solvents as reaction media, and multiple purification techniques after each step with the amount of desired product only 2 - 11mg.

A second multi-step protocol that was developed by Caputo (Caputo 2002): 1) synthesized the acetylated hemicyanine with the less reactive heterocyclic salt 2) purified the hemicyanine with continuous extraction with ethyl acetate 3) reacted the pure hemicyanine with the more reactive heterocyclic salt. This strategy requires a large amount of solvent in the purification of the hemicyanine and the unsymmetrical dye is purified by column chromatography. The purity was not reported and the dye was not characterized or quantified prior to derivatization for several examples.

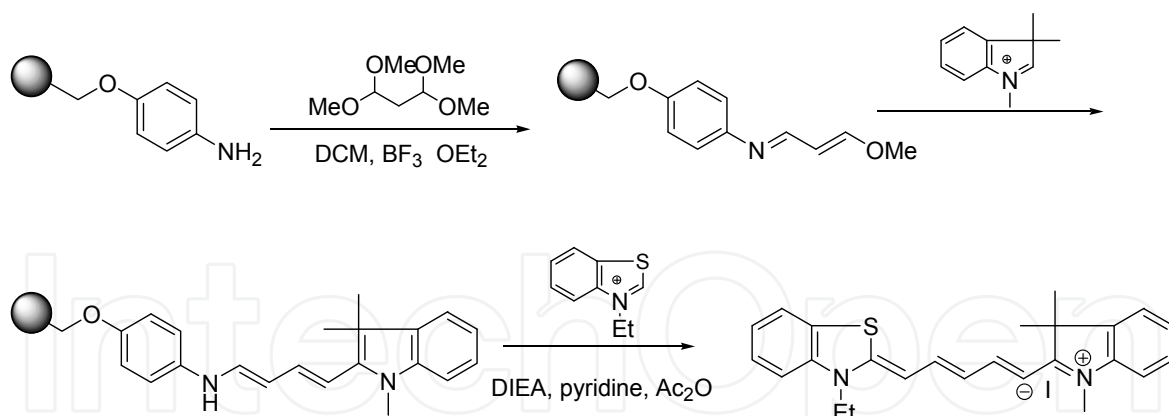


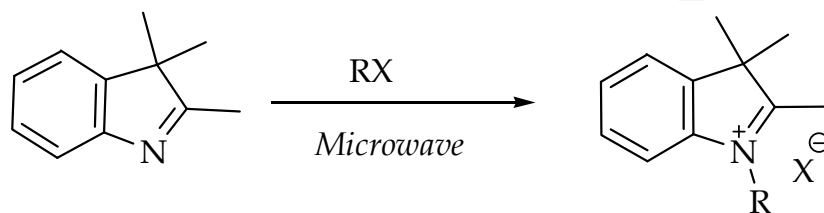
Fig. 7. Solid phase synthesis of cyanine dyes

2. Salt synthesis

N-Alkyl quaternary ammonium salts are used extensively as precursors of various cyanine dyes (Narayanan and Patonay 1995) and near-IR spiropyrans (Hirano, Osakada et al. 2002). The salts are synthesized by refluxing reagents with solvents such as chloroform, *o*-dichlorobenzene, acetonitrile and ethanol for 6 – 48h. One example requires refluxing in acetonitrile for 24 h, then treatment with diethyl ether followed by filtration. The combined filtrates were concentrated and refluxed for an additional 24 h, treated with diethyl ether and filtered (Pardal, Ramos et al. 2002). This process was repeated 1-3 times to achieve the published yields (25 – 78%). Another method heats the reagents at 80 °C for 21 h in an ampule tube sealed with a torch (Hirano, Osakada et al. 2002). Purification of the salts range from Soxhlet extraction with benzene for 24 h (Elizalde, Ledezma et al. 2005) to filtration with cold ether (Pardal, Ramos et al. 2002).

2.1 2,3,3-trimethylindolenine derivatives

The reaction of 2,3,3-trimethylindolenine with an array of alkyl halides with varied functionality were studied (Figure 8). The reactions were performed by charging each microwave reaction vial with of 2,3,3-trimethylindolenine and an alkyl halide. Our previously studied reaction of ethyl iodide with 2,3,3-trimethylindolenine served as the model system (Winstead 2008a). The microwave reaction conditions were determined using a single-mode microwave system. The temperature was monitored throughout each reaction. The optimized reaction condition was 130 °C, ramp time: 2:50 min, reaction time: 5:00 min giving a 95% yield.



1 R = Me, 2 R = Et, 3 R = Pr, 4 R = -CH₂CH₂OH, 5 R = -(CH₂)₅CO₂H

Fig. 8. Synthesis of 2,3,3-trimethylindolenine quaternary salts

The scope of the reaction was examined with the coupling of 2,3,3-trimethylindolenine with iodomethane, iodopropane, bromoethanol and 6-bromohexanoic acid (Winstead 2008b). The hold time, ramp time, and temperature for each electrophile was studied. The optimized reaction conditions are presented in Table 1. In most cases, the yields were comparable or exceeded the published yields. Most significant is the substantially decreased reaction time and simplicity of the reaction procedure. The yields presented are the yields without resubjection of the filtrates.

Alkyl salts **1-3** were simply filtered and washed with cold ether. The products were pure by NMR analysis and no further purification was necessary. Salts **4** and **5** which all contain hydroxyl groups, did not crystallize right away. The reaction solution containing **4** was concentrated followed by the addition of hexanes. The solution was heated until crystals formed and then filtered. Similarly, salt **5** was recrystallized from acetone.

Salt	T (°C)	Time (min)	Yield (%)	Lit. Yield (%)	Lit Time (h)
1	130	5:00	95	59	48
2	110	2:30	93	75	21
3	110	7:00	83	44	24
4	110	7:00	73	69	24
5	110	7:00	59	67	12

Table 1. Reaction conditions for the synthesis of 2,3,3-trimethylindolenine salts

X ray chrystallography was taken of carboxylic acid derivative **5** (Figure 9) (Winstead 2010). The data revealed the hydrogen bonding of a water molecule to CO₂H moiety and the bromide ion is associated with water molecule.

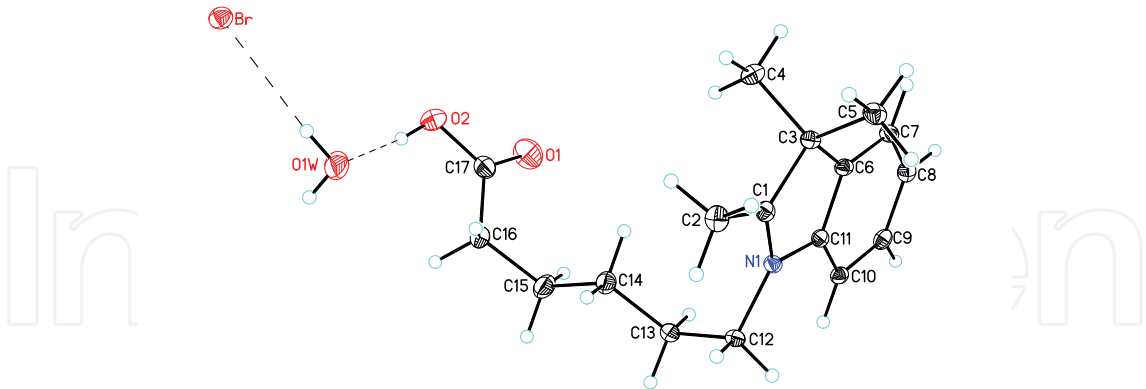
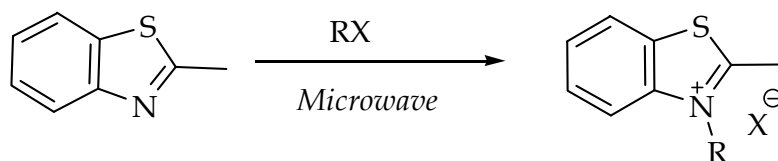


Fig. 9. X ray chrystallography

2.2 Benzothiazole derivatives

Benzothiazole derivatives were synthesized by reacting benzothiazole with various alkyl halides under microwave irradiation conditions to produce compounds 7-11 in good yields (Figure 10). Methyl indolenine salt **7** was synthesized in excellent yield without solvent. The reaction was complete in only 20 min (85%) compared to 7h and 60% yield under reflux conditions.



6 R = Me, 7 R = Et, 8 R = Pr, 9 R = $-\text{CH}_2\text{CH}_2\text{OH}$, 10 R = $-(\text{CH}_2)_5\text{CO}_2\text{H}$

Fig. 10. Synthesis of benzothiazole quaternary salts

The reaction ethyl indolenine **3** was synthesized in yields ranging from 4-80% (Table 2). The reaction conditions used for the methyl indolenine synthesis resulted in a 17% yield. The temperature was increased in 10 °C increments. The reaction was placed in a microwave vial and subjected to microwave irradiation at the set temperature (20 min). The vial was removed and checked for the presence of a solid. If there was small amount of solid, the reaction was placed back into the microwave oven for an addition 10 min. The yields reported are after the second reaction time and filtration. The reaction yield increased as the temperature increased to 170 °C. The reaction conditions for the synthesis of propyl indolenine **8** are the same as **7**.

T (°C)	Time (min)	Yield (%)
120	15	4
120	20	17
130	20 + 10	31
140	20 + 10	45
150	20 + 10	47
160	20 + 10	69
170	20	80

Table 2. Reaction conditions for ethyl indolenine **8**

2-ethanol indolenine **9** was synthesized in yield in 56% (Table 3). The temperatures studied were between 150 °C – 100 °C. 150 °C was selected as the starting point because it was in between the optimum reaction temperature for the methyl and the ethyl derivatives. The reaction time was 20 min. These initial conditions led to decomposition. A temperature reduction of 20 °C also provided decomposition (Entry 2), however a 30 °C decrease yielded starting material (Entry 3). When an increase in time produced decomposition (Entry 4), the temperature was reduced to 100 °C and held for 35 min. The reaction vial sat at room temperature for 2-4 h, after which time crystals formed and were filtered.

Entry	T (°C)	Time (min)	Yield (%)
1	150	20	Decomp
2	130	20	Decomp
3	120	20	SM
4	120	25	Decomp
5	100	35	56

Table 3. Reaction conditions for 2-ethanol indolenine **9**

It has been reported that the longer the alkyl chain length, the more difficult it is to perform the alkylation. Therefore, the higher 170 °C and 20 min was selected as the starting reaction conditions and yielded a low 30% yield (Table 4). The time was increased in 5 min increments to 35 min. Reaction times longer than 35 min lead to inconsistent results. The reaction vial sat at room temperature for 2-4 h, after which time crystals formed and were filtered.

T (°C)	Time (min)	Yield (%)
170	20	30
170	25	43
170	30	66
170	35	75

Table 4. Reaction conditions for 6-hexanoic acid indolenine **10**

The optimized reaction conditions for the synthesis of benzothiazole derivatives under microwave conditions and conventional heating methods in the literature are presented in Table 5. The reaction times are significantly shorter and the percent yields are higher in every example of the synthesis of these derivatives.

Salt	R	T (°C)	Time (min)	Yield (%)	Lit. Yield (%)	Lit Time (h)
6	Et	170	20	83	48	48
7	Me	120	20	85	60	7
8	Pr	170	20	65	5	7
9	-(CH ₂) ₂ OH	100	35	58	N/A	6
10	-(CH ₂) ₅ CO ₂ H	170	35	75	61	48

Table 5. Reaction conditions for benzothiazole quaternary salts

2.3 Other salt derivatives

2.3.1 Lepidine derviative

An interesting RNA antagonist precursor, 1-(6-ethoxy-6-oxohexyl)-4-methylquinolinium iodide quaternary ammonium salt has been synthesized. 1-(6-methoxy-6-oxohexyl)-4-methylquinolinium chloride quaternary salt has been synthesized in two steps in 37% overall yield (Figure 11) (Carreon, Stewart et al. 2007). The rapid solvent-free synthesis of the *N*-ethoxycarbonylhexyl quaternary salt from commercially available lepidine in one step in 56% yield with minimal purification is described.

Commercially available 6-bromohexanoic acid was converted to the iodide using Finkelstein reaction conditions. In these studies, the typically more reactive iodide alkyl halide yielded yields comparable to the commercially available bromide suggesting the extra step of halogen exchange is not necessary. The percent yields for the iodo and bromo salts were comparable (Table 6).

During the reaction time studies an increase in yield was observed when the reaction stopped midway, cooled to room temperature and resubjected to microwave irradiation for additional time. The difference was 10 points for both 9 and 7 min total reaction time (Table 7). This could be attributed to the additional time exposed to microwave irradiation due to a second 4 min ramp time during the resubjections.

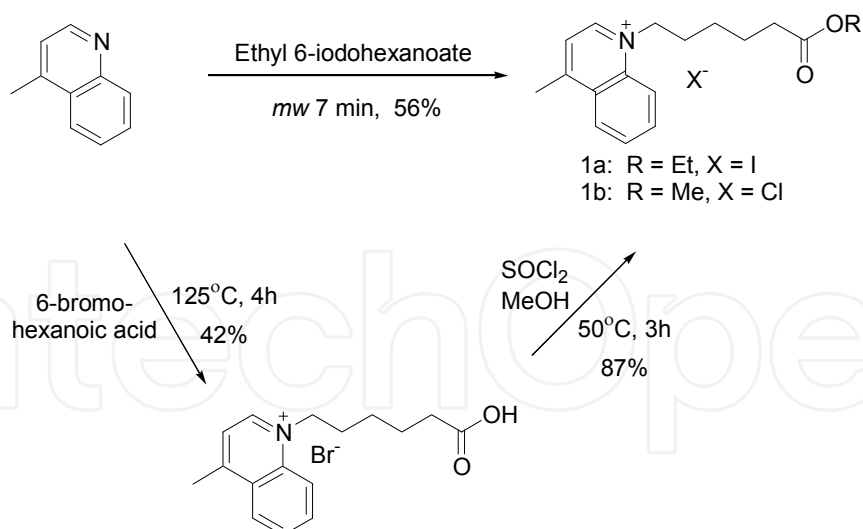


Fig. 11. Synthesis of lepidine quaternary salt

X	Temp	Time	Yield
Br	120	7	49
Br	120	7	54
I	120	7	52
I	120	7	56

Table 6. Reaction conditions for lepidine synthesis

Trial	Time	Yield
1	9	49
2	9	46
3	5 + 4	56
4	5 + 4	60
5	7	44
6	3 + 4	54

Table 7. Lepidine derivative time studies

2.3.2 Methylbenzothiazole acetonitrile

In some cases, cyanine dyes have a tendency to photobleach. The incorporation of α -cyano group has been shown to increase the photostability of the dye. Initial studies focus on the improvement of the synthesis of the α -cyano heterocyclic salt. Previous studies utilized conventional heating to synthesize α -cyano cyanine dyes.

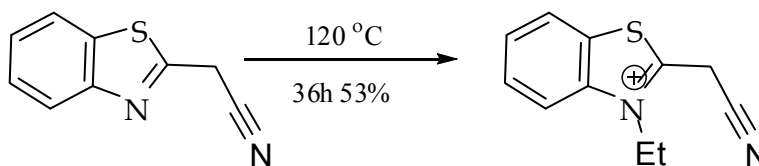


Fig. 12. Synthesis of methylbenzothiazole acetonitrile quaternary salt

Methylbenzothiazole acetonitrile was treated with iodoethane; temperature, hold time, and mole ratio were manipulated. Based on NMR data, the reaction yielded a mixture of starting material and product. The reaction required heating at 120 °C for 36h to achieve the cyano salt in 53%. The sluggish nature of this reaction under conventional heating conditions was mimicked in our preliminary studies (Table 8). Higher temperatures lead to decomposition, while lower temperatures provide a mixture of starting material and product.

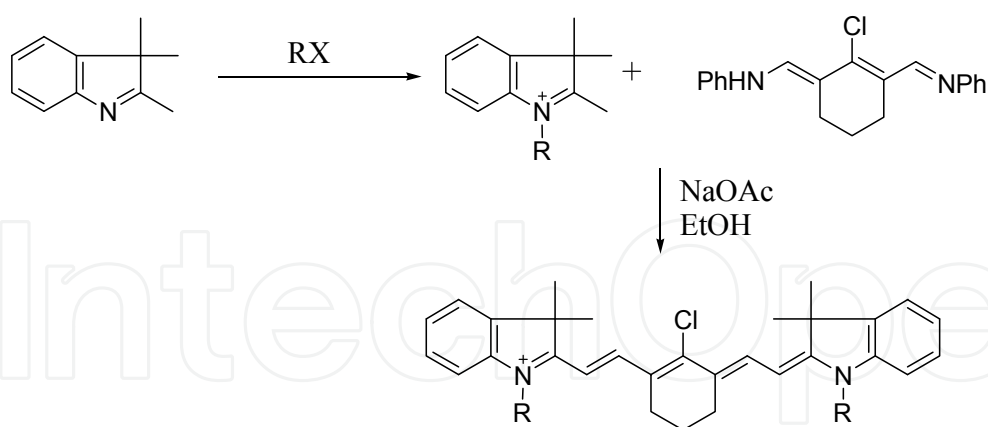
Entry	Temp (°C)	Time (min)	Outcome	Yield (%)
1	130 °C	40	Product	34%
2	130 °C	40	Product/ SM	36%
3	130 °C	50	Product/ SM	41%
5	140 °C	40	Product	21%
6	170 °C	10	decomposed	-
7	170 °C	20	decomposed	-

Table 8. Reaction conditions for methylbenzothiazole acetonitrile quaternary salts

3. Cyanine dye synthesis

3.1 Symmetrical cyanine dye synthesis

Herein, we report the microwave synthesis of five cyanine dyes derived from the aforementioned heterocyclic salts. The microwave reaction conditions were determined using the Biotage single-mode microwave system. The reaction of the N-((E)-(2-chloro-3-((E)-(phenylimino)methyl)cyclohex-2-enylidene)methyl)aniline (1eq) and 2,3,3-trimethyl-1-ethyl-3*H*-indolium iodide (2eq) served as the model system (Figure 13).



NIR-1: R = Me; **NIR-2:** R = Et; **NIR-3:** R = Pr; **NIR-4:** R = CH₂CH₂OH; **NIR-5:** R = (CH₂)₅CO₂H

Fig. 13. Synthesis of NIR dyes

The reaction was examined using a temperature range from 100 °C to 160 °C in 10 °C increments (Table 9). The vials were cooled to 0 °C, filtered and washed with diethyl ether to yield greenish-gold crystals in yields from 53 – 79%. The optimum temperature proved to be 120 °C (79%). The yield decreased by around 13% for each 10 °C increase from 140 °C to 160 °C. An increase in time from 20 min to 30 min did not lead to an improved percent yield. The purity of each sample produced from each trial was the same by ¹H and ¹³C NMR.

Trial	Temp. (°C)	Time (min)	Yield (%)
1	100	20	61
2	110	20	63
3	120	20	79
4	130	20	70
5	140	20	72
6	150	20	61
7	160	20	53
8	120	30	77

Table 9. Temperature studies for NIR-2

The optimized reaction conditions for NIR-2 were examined using a variety of heterocyclic salts. The hold time, and temperature for each heterocyclic salt was studied. The optimized reaction conditions are presented in Table 2. NIR-5 provides two sites for protein labeling in biosensor applications. In most cases, the yield was comparable or exceeded the published yields (Table 10). Most significant is the substantially decreased reaction time and simplicity of reaction procedure. The yields presented are the yields without resubjection of the filtrates.

Dye	R	Yield (%)	Lit. Yield (%)
NIR-1	Me	72	66
NIR-2	Et	79	81
NIR-3	Pr	81	NR
NIR-4	(CH ₂) ₂ OH	64	NR
NIR-5	(CH ₂) ₅ CO ₂ H	83	NR

Table 10. Structure and yields for synthesis of symmetrical dyes

3.2 Unsymmetrical cyanine dye synthesis

Unsymmetrical cyanine dyes have been widely used in probes for bio-analytical applications. The growing range of applications has warranted the need for a convenient, reliable synthesis of these molecules. The synthesis of these compounds has been a challenge due to the production of the symmetrical dye alongside the desired unsymmetrical dye (Lin, Weissleder et al. 2002; Touthkine, Nalbant et al. 2002; Kim, Kodagahally et al. 2005; Jiang, Dou et al. 2007). The purification of this mixture is nontrivial requiring multiple chromatographic steps that often lead to low yields. The reaction is a two step process that involves the synthesis of the hemicyanine intermediate followed by conversion to the unsymmetrical dye.

Our reaction protocol is: HET₁, bisimine, and NaOAc in ethanol are combined and irradiated for 15 min at 100 °C. Then HET₂ and NaOAc are added to the mixture and irradiated for 15 min at 100 °C. The reaction time is reduced from 2-4 hours at 120 °C to 15 min at 100 °C for

initial step **A** (Figure 15). Isolation of the hemicyanine has been eliminated from the process, completely removing the opportunity for the hemicyanine to form symmetric dye during isolation. The absence of this also eliminates the use of large amounts of solvents during purification. The reduced reaction time substantially minimizes hemicyanine exposure to conditions that facilitate its reversibility. The same line of reasoning holds true for the second condensation reaction **C** between the hemicyanine and HET₂ to form the unsymmetrical dye. Although symmetric dye was not detected in the ¹H NMR, it cannot be stated that none was formed during the microwave process. The actual amount of symmetrical dye formed at each step must be quantified and will be the focus of future studies.

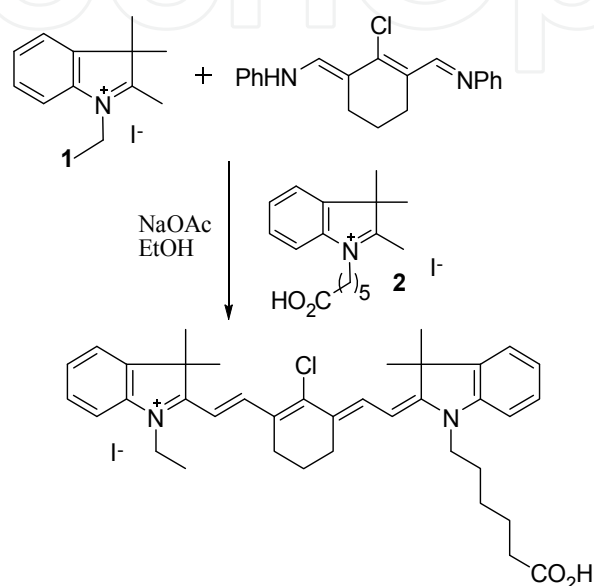


Fig. 14. Synthesis of unsymmetric dye

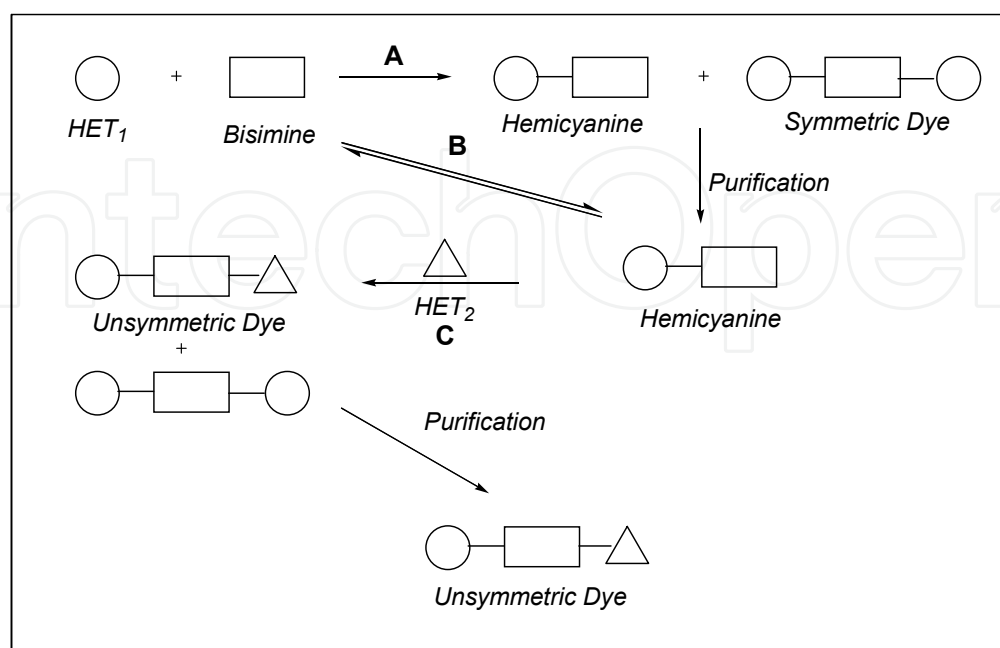


Fig. 15. Pathways of symmetrical dye formation

The treatment of ethyl salt **1** with bisimine and sodium acetate in ethanol for 10 min at 120 °C in a microwave oven affords the hemicyanine intermediate on literature precedence. The solution was allowed to cool to rt and salt **2** and 1 eq. of sodium acetate based was added to the vial and irradiated for an additional 10 min at 120 °C. The product was filtered and analyzed using ¹H NMR. These conditions were applied to the synthesis of three additional unsymmetrical cyanine dyes (Table 11).

Dye	R ₁	R ₂	Wavelength	Percent Yield
NIR-6	Et	Pr	780	58%
NIR-7	Et	Me	780	44%
NIR-8	(CH ₂) ₂ OH	Et	782	75%
NIR-9	(CH ₂) ₅ CO ₂ H	Me	780	69%

Table 11. Structure and yields for synthesis of unsymmetrical dyes

4. Properties

Fluorescence spectroscopy has become a key technique for the detection and elucidation of biological processes. Most fluorescence sensors for bio-analytical applications fluoresce in the visible region (400-650 nm) (Wehry 1976). A disadvantage of this technique is that most biomolecules also fluoresce in this region, causing significant background interference. Near infrared (NIR) dyes, however, fluoresce in the 650-1000 nm range with minimal background interference from biomolecules and high sensitivity (Hammer, Owens et al. 2002). Some of the advantages of NIR fluorescence based detection techniques include: increased detection sensitivity and selectivity due to the absence of background interference; increased photo stability and less photo bleaching effects because of lower excitation energies associated with the NIR region; good compatibility with cost effective light excitation sources and solid state detectors; and the easy adaptation to valuable visible fluorescence analytical techniques such as fluorescence energy transfer (FRET), two-photon excitation, and metal enhanced fluorescence (MEF).

In particular, cyanine dyes have widespread application as fluorescent probes. The spectral properties of fluorescent probes assist in the determination of how they are applied in a variety of analytical techniques. Properties such as wavelength absorbance and emission ranges, Stokes shifts, and spectral bandwidths are used to address the various requirements associated with different analytical techniques. One emerging area of interest for promising analysis applications that use cyanine dyes is metal enhanced fluorescence. The characteristic low quantum yields of these dyes suggest they have potential applications in metal enhanced fluorescence related techniques. Metal enhanced fluorescence is a form of fluorescence where plasmon waves generated by metals deposited on a glass substrate can increase the fluorescence of fluorophores within the influencing range of the wave. The phenomenon is demonstrated most effectively in fluorophores that have low quantum yields. Because of the energy associated with the longer wavelengths of the NIR, and the moderately low quantum yields associated with this class of compounds, the potential application for these compounds as MEF probes or labels are promising (Lakowicz, Parfenov et al. 2003; Lakowicz, Geddes et al. 2004). While these dyes are well-known, they are costly with limited commercial availability.

Figure 16 shows the absorbance spectrum for microwave synthesized NIR-2 along with its corresponding fluorescence spectrum. The fluorescence intensity was scaled to the absorbance scale for comparison purposes. The absorbance maximum was absorbed at 779 nm. A fluorescence maximum intensity was observed at 795 nm, demonstrating a Stokes shift of 16 nm. While the observed Stoke's shift is considered moderate, it is typical for the heptamethine cyanine class of NIR dyes.

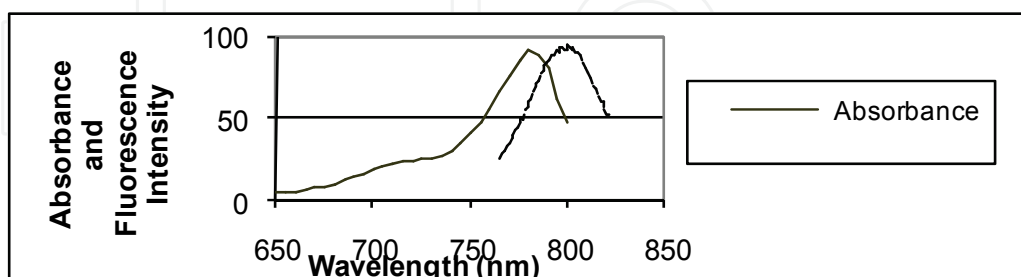


Fig. 16. A graph of the absorbance spectra along with the relative fluorescence intensity of the NIR dye, NIR-2 (in ethanol) demonstrates a Stokes shift of 16 nm

The spectral analysis of the five synthesized compounds demonstrates characteristics that are similar to what has been reported previously for this class of compounds (Table 12). They typically have large molar absorption extinction coefficients (molar absorptivities), moderate Stoke's shifts, and small quantum yields. The overall detection sensitivities are a reflection of the moderate Stoke's shifts observed for the compounds as well as the limitation of the Hamatsu 928-PMT red sensitive photomultiplier tube used in the Cary Eclipse fluorescence spectrophotometer. This is expected as fluorescence detection moves to longer wavelengths because background interference from molecules with similar fluorescence properties is minimized. In the visible part of the spectrum, fluorescence detection sensitivity depends on both the responsivity of the detector and on background interference from molecules found in the detection medium with similar spectral properties. In the NIR spectral region, only the responsivity of the detector and the optical limitations of the instrument limit detection sensitivity. Because of the size of the Stoke's shift, detection sensitivity is influenced by scattered light effects that occur when light from the excitations source of the fluorescence spectrophotometer is scattered in the solvent medium and bleeds over into the instrument's detector. This reduces the signal-to-noise ratio (S/N) which limits the detection sensitivity. As the size of the Stoke's shift increases, the S/N increases and this improves the detection sensitivity. Increasing the size of the Stoke's shift is an important priority in future synthetic work as a way to directly improve detection sensitivity and create compounds that are effective labels and probes. Some promising work on increasing the Stoke's Shift of NIR heptamethine cyanine dyes has been reported (Peng, Song et al. 2005). Large Stoke's shifts greater than 100 nm can be obtained by substituting the chlorine with an alkyl amino group at the central position of the heptamethine cyanine dyes. This substitution process is very compatible with microwave assisted synthesis techniques and is expected to contribute greatly to future generations of NIR fluorescent probes. Increasing the Stoke's shifts of these dyes will translate into photo stable NIR fluorescent probes with significant increases in sensitivity. When used as the probe part of fluorescence based biosensors, novel NIR fluorescent dyes are expected to improve the sensitivity and dynamic range of current fluorescence biosensor analytical detection techniques.

Dye	Absorption λ_{abs} (nm)	Emission λ_{ems} (nm)	Stokes shift (nm)	Quantum yield	Detection sensitivity (M)	Molar absorptivity	E'_{ox} (V)
NIR-1	779	795	16	0.041	1E-08	2.8E+05	0.42
NIR-2	780	798	18	0.024	7.2E-09	1.6E+05	0.40
NIR-3	785	802	17	0.025	1.7E-08	1.2E+05	0.40
NIR-4	785	802	17	0.022	4E-09	4.3E+05	N/A
NIR-5	785	805	20	0.022	1.8E-08	2.4E+05	N/A

Table 12. Spectral characteristics

While the microwave assisted syntheses of unsymmetrical heptamethine cyanines remain a work in progress, preliminary spectral analyses of unsymmetrical compounds indicate no significant difference in spectral properties from their symmetrical counterparts (Table 13). While shifts were detected in absorption and emission wavelengths, the absorption wavelengths remained in the 780 nm range and the Stoke’s shifts observed were considered insignificant. The quantum yields observed remained moderately low and very compatible for use in FRET and MEF applications. The slight increases observed for detection sensitivity were not significant, and the overall molar absorptivities remained in the range of symmetrical dyes with similar peripheral functional groups. The effect of substituting functional groups on the outer edges of the compounds in order to change their symmetry has no significant overall effect on the spectral properties of this class of dyes. This demonstrates the flexibility of these compounds in their ability to be used in fluorescence analytical applications. It is also a further indication of the potential of this class of compounds as probes for NIR fluorescence biosensor applications.

Dye	Absorption λ_{abs} (nm)	Emission λ_{ems} (nm)	Stokes shift (nm)	Quantum yield	Detection sensitivity (M)	Molar absorptivity
NIR-6	785	801	16	0.021	5.2E-09	1.9E+05
NIR-7	780	796	16	0.021	4E-09	3.1E+05
NIR-8	780	800	20	0.020	2.2E-09	2.1E+05
NIR-9	780	796	16	0.021	7.7E-09	2.3E+05

Table 13. Spectral characteristics of unsymmetrical dyes

In addition to Stoke’s shift properties, the high molar absorptivity and molar extinction coefficients that characterize this class of dye are additional spectral properties that can be successfully utilized in the improvement of current fluorescence biosensor analytical techniques. The size of the molar extinction coefficients coupled with the relatively low quantum yields suggest several potentially useful application possibilities. A main concern for this class of NIR dyes are chemical photostability and photobleaching effects. Because the molar extinction coefficients are high compared to conventional visible fluorophores, larger amounts of energy can be pumped into these dyes and at longer wavelengths. When this effect is coupled with the presence of a stabilizing ring system in the center of the molecule, chemical photo stability is maximized. Typically, photostability is limited by the number of cycles that a single dye molecule can achieve before it decomposes. A cycle is defined as the process where a fluorophore is excited and then fluoresces as it returns to its

ground state. More cycles suggest greater detection sensitivity because more photons are produced as photo stability increases. The combination of high molar absorptivity at longer wavelengths for the NIR cyanine class of fluorophores, coupled with the low excitation energy from NIR excitation light sources, insures maximized photo stability for dyes in this region.

Another useful application is related to the property of moderately low quantum yields that typically characterize cyanine dyes. Metal enhanced fluorescence is a form of fluorescence where plasmon waves generated by metals deposited on a glass substrate can increase the fluorescence of fluorophores within the influencing range of the wave. The phenomenon is dependent on the fraction of nonradiative decay being much greater than radiative decay. The lower the quantum yield, the greater the potential for the MEF effect to occur. Again, because of the energy associated with the longer wavelengths of the NIR, and the low quantum yields associated with this class of compounds, the potential application for these compounds as MEF probes or labels are promising.

5. Conclusions

MAOS has provided substantial decreased reaction times, simplicity of reaction procedure, and comparable or increased reaction yields for a variety of *N*-alkylheterocyclic salts and symmetrical and unsymmetrical cyanines. The microwave synthesis is a straightforward synthesis that yields cyanine dyes that do not need rigorous purification via column chromatography. The percent yields are reflective of single runs without subjecting unreacted starting materials to the reaction conditions multiple times to achieve high percent yields.

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