PH SENSITIVE FLUORESCENT SENSORS

A Thesis

presented to

the Faculty of the Graduate School

at the University of Missouri-Columbia

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

By

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May 2007

The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

pH sensitive fluorescent sensors

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A candidate for the degree of Master of Science

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Acknowledgements

First of all, I would like to give thank to my parents, Santosh and Japamala Barman in India. I would not be here today without their encouragement and blessing. Additionally, I thank my elder sister, Indu Prava Barman, for continual support throughout my education as well as every other facet of life.

I would like to extend my gratitude to my research advisor, Dr. Timothy E. Glass for spending his valuable time in teaching me. He has provided me hands on experiment in the laboratory. He helped me to develop my abilities with oral presentation and the basics of science writing.

I also thank all the past and present group members for their friendship and for sharing their experience during the early and present days in lab. I would especially like to show my gratitude to my friends Dr. Nripen Chanda, Dr. Shana Garrison and Ms. Nicole Nelson for the support and the inspiration on my tough time.

Finally, I would like to thank Dr. Rabindranath Dhara of Ramakrishna Mission Vidyamandira (Belur Math), Howrah, India; who first inspired me to pursue a higher education in chemistry. His teaching skill and guidance in my bachelor's degree made organic chemistry more enjoyable in my undergraduate days.

Abstract

Pheochromocytoma is a cancer of the adrenal gland. Pheochromoocytomas produce a variety of catecholamines, including norepinephrine. The result is high blood pressure which is caused by the release of catecholamines into the bloodstream (i.e. elevated concentration of plasma or urinary catecholamines). Detecting this cancer by measurement of catecholamine concentrations in serum can be challenging for the clinician.

Fluorescence probes are becoming useful tools for investigating cellular events by fluorescence microscopy. Our group has developed a dopamine/norepinephrine sensor which binds norepinephrine in the vesicles of chromaffin cells. We have designed and synthesized both coumarin and quinolone based sensors that reversibly covalently bind with both the amine and catechol moieties of catecholamines via the formation of an iminium ion and boronate ester respectively. With the fluorescence response of the binding process we can then accurately determine the concentration of catecholamines, thereby facilitating diagnosis. However the binding rate is slow; making synaptic measurements difficult (synapse firing takes 10 milliseconds). My goal is to modify these sensors to be pH sensitive. Then the probe can bind neurotransmitter in the vesicle and remain off (pH=5.5). When stimulated the cell will exocytose the sensor and at pH=7.4, the sensor will fluoresce. The synthesis of pH sensitive fluorescent chemosensors for amines and their properties is described.

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Chapter 1
Pheochromocytoma

Introduction

Pheochromocytoma, a rare neoplasm of chromaffin cells, is derived from embryonic neural crest that can take up, store or metabolize catecholamines. Every year at least 800 cases are diagnosed in the United States. According to the statistics, people are affected by this disease in their thirties to fifties. For the patients who are already affected by multiple endocrine neoplasia (MEN) type 2 syndromes; there is a risk of 50% chance for developing a contralateral tumor following by a unilateral adrenalectomy. Other syndromes included with pheochromocytoma are neurofibromatosis, von Hippel-Lindau disease, cerebellar hemangioblastoma, Sturge-Weber's syndrome, and tuberous sclerosis.[1,2(a, b)] Most pheochromocytoma begins with surgically curable benign adrenal tumors which result in sudden, unexpected and potentially lethal complications. The diagnosis and the treatment of pheochromocytoma are depending on the increase of the production of catecholamines and the identification of the tumor location. The metanephrines are produced continuously from leakage of catecholamines from storage vesicles into the cytoplasm of pheochromocytoma tumor cells. [3,4] The free metanephrines can be determined[5] by monoamine oxidase or sulphate that is conjugated with sulfotransferase isoenzymes. Because of the production and release of catecholamines; pheochromocytomas often cause hypertension. Clinical tests often rely on hypertension as a sign that suggests excess catecholamine. About 0.1% to 0.5% of all hypertension patients were found to have pheochromocytoma. Of the pheochromocytoma patients, approximately 90% can be treated if the disease is properly diagnosed. [6 (a, b), 7]

Detection of this disease can be done by computed tomography or by MRI but these methods can not differentiate between the patients who have incidental adrenal mass and those who are suffering from Pheochromocytoma. The uptake of catecholamines and sympathomimetic amines in chromaffin cells can be

monitored by ^{131}I-metaiodobenzylguanidine scintigraphy [8(a, b)] either by specific cell membrane norepinephrine transporter and vesicular sequestration of these compounds or via a vesicular monoamine transporter. With consistent results from all three tests, pheochromocytoma can be diagnosed[9]. The detection of pheochromocytoma is very challenging to diagnose and to treat.

Figure 1 depicts an algorithm [10] for diagnosing norepinephrine- producing pheochromocytoma. The combination of positron emission tomography and CT scanning has some promising results for diagnosis of pheochromocytoma.

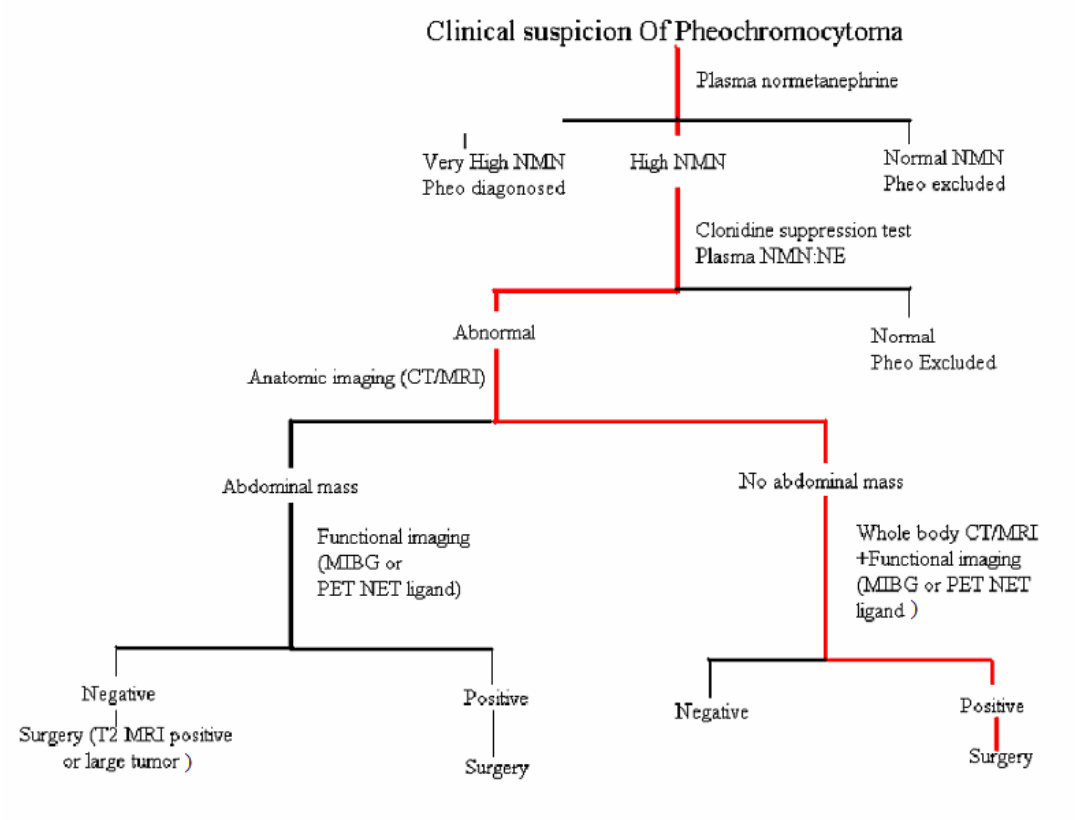
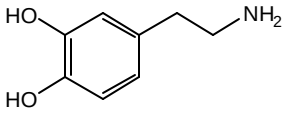


Figure 1

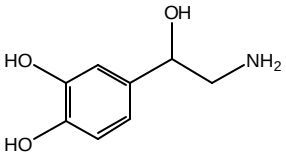
NMN= Normetanephrine; NE= Norepinephrine

The initial diagnostic test is to measure plasma levels of free metanephrines. If this test is negative, then pheochromocytoma can be excluded. [11] If plasma levels of free normetanephrine are four times more than the upper reference limit then confirmatory biochemical testing is indicated. With a clonidine suppression test, the plasma norepinephrine and normetanephrine can be measured.[12] If the results are consistent with pheochromocytoma, then conventional anatomic tests with other expensive and time-consuming imaging tests, can be performed. It is thereby imperative that researchers develop a sensor that can detect these increase catecholamines that can be coupled with other diagnosis technologies to better screen for this pheochromocytoma.

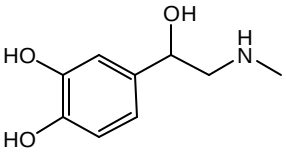
Structures of Catecholamines



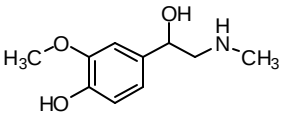
Dopamine



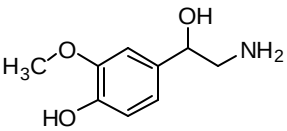
Norepinephrine



Epinephrine



Metanephrine



Normetanephrine

Figure 2

The Basics of Molecular recognition

Molecular recognition is an event where a host molecule is able to form a complex with a guest molecule. The compounds may bind together through non-covalent bonds such as hydrogen bonding, hydrophobic interactions, hydrophilic interactions, p-stacking interactions and ionic interactions or by the formation of metal coordination complexes. For these forces to operate effectively, the receptor must provide a cavity (a pocket) that matches the size and the shape of the guest molecule. The component molecules recognize each other through the interplay of non-covalent forces, so this event is termed as "molecular recognition".

To determine that binding has occurred between the guest and host molecules (Figure 2), an output physical signal must be observed that is tied to the molecular recognition process. Most often NMR, fluorescence absorption (UV/Vis), and circular dichroism (ORD/CD) are used. For an optical sensor, fluorescence and absorption are most useful.

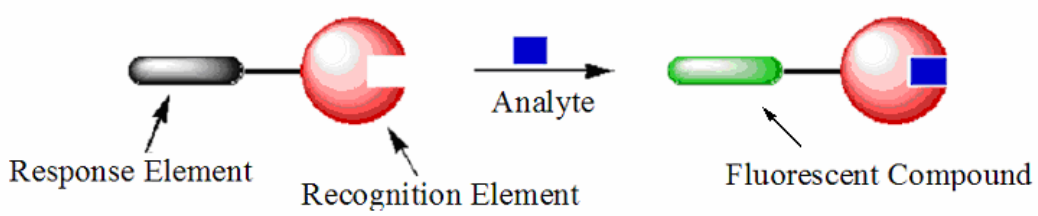


Figure 3

The Basics of Fluorescence

Fluorescence is a member of the ubiquitous luminescence processes where molecules emit light from electronically excited states which are created by either a physical, mechanical or chemical mechanism.[13] When a molecule is excited by UV or visible photons it creates an excited state (S_1). The excited singlet state of the molecule can decay through various radiative or non-radiative methods. Radiative methods may include fluorescence, a spin allowed process in which the molecule returns to the ground state by the emission of a photon of light. Intersystem crossing related to the non-radiative spin inversion of a singlet state (S_1) into a triplet state (T_1). Emission from the triplet state is termed phosphorescence. Non radiative processes include quenching.[14] When an appropriate molecule (quencher) is present, the excited molecule may participate in energy transfer or electron transfer processes that decrease the fluorescence emission. When a fluorophore is quenched upon contact with some other molecule in the solution, it is called as collision quenching.[15] There are two types of fluorescence quenching, dynamic quenching, and static quenching. In dynamic quenching, the collision process is usually controlled by diffusion. In static quenching, the energy transfer is immediate if a quenching molecule is within a certain distance of the donor. Generally in collision quenching molecules are not chemically altered.

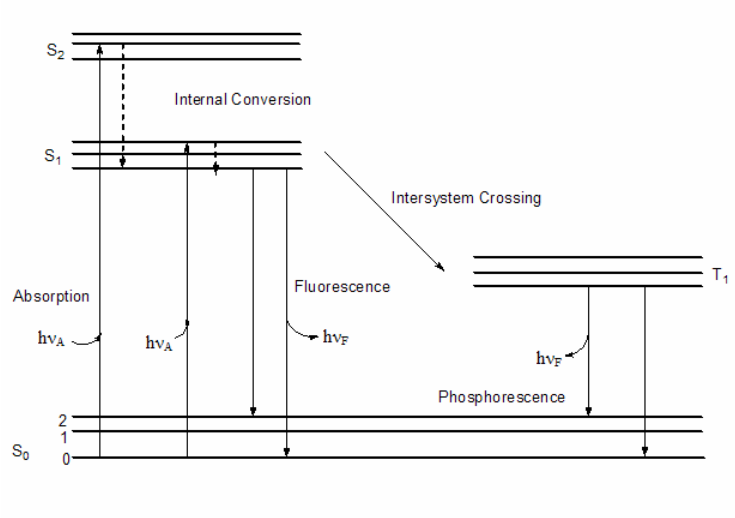


Figure 4

The fluorescence quantum yield and the lifetime are important fluorophore characteristics. The classical definition of quantum yield (Q) is the ratio of the number of emitted photons to number of absorbed photons.

$$Q = \frac{\text{Photon}_{\text{emission}}}{\text{Photon}_{\text{absorption}}}$$

The quantum yield can also been written as in the below equation below. [14] Here K_r is the rate of fluorescence emission and K_{nr} are the rates of the non radiative process.

$$Q = \frac{K_r}{K_r + \sum K_{nr}}$$

Fluorophore

Fluorescent probes are very important for many biological applications. Most fluorophores have short lifetime (1-10 ns). In general, fluorophores are divided into two classes, intrinsic and extrinsic. [16-17] Biological samples often contain fluorescent compounds referred to intrinsic fluorophores. Aromatic amino acids, NADH, flavins, neurotransmitters, porphyrins, chlorophyll and derivative of pyridoxal are among these intrinsic fluorophores. When a synthetic dye or modified biomolecule with a specific spectral property is added to a system, it is called an extrinsic fluorophore. Fluorescence from the intrinsic fluorophores is referred to as autofluorescence or background fluorescence. The extrinsic fluorophore should have longer wavelengths of excitation and emission to prevent overlap with any background emission. One type of fluorophore which is useful in this regard absorb in the near infrared (NIR). Here the HOMO-LUMO gap is small, due to an extended p-conjugation or a heavy element in the backbone.[18] These long wave length dyes can be simply excited by the common laser source. One type of such probes is called fluorogenic probes. These are non fluorescent dyes which are activated when enzymatic cleavage occurs. For example 7-umbelliferyl phosphate (7-UmP) is non fluorescent but in presence of alkaline phosphatase it becomes highly fluorescent.[19] Lanthanides also display emission in aqueous solution. Lanthanides can substitute chemically for calcium in many calcium dependent proteins. [20-22]

Dye Displacement Mechanism

One common method for fluorescent sensing is called dye displacement (Figure 5). This approach involves the use of binding and signaling subunits. Here the subunits are not covalently attached but form a complex which is non fluorescent. Then, when a target analyte is added to the solution containing the sensing ensemble, there is a displacement reaction. The free dye is fluorescent and produces the output signal. Often this sensing method is not useful in biological system since it is difficult to produce the initial sensing ensemble in a biological sample.

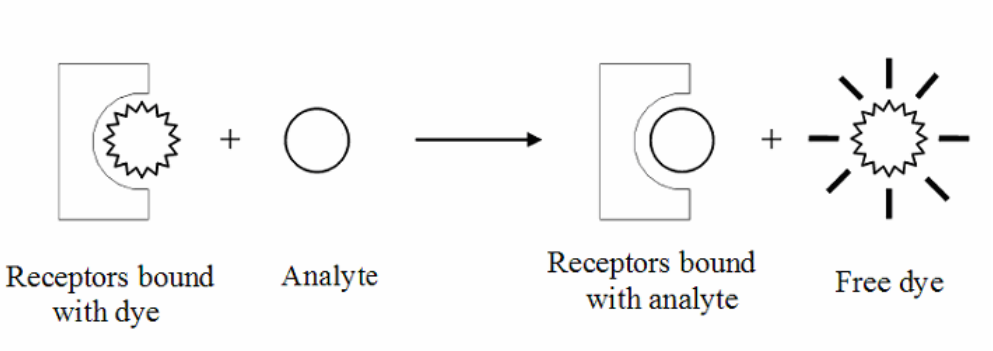


Figure 5

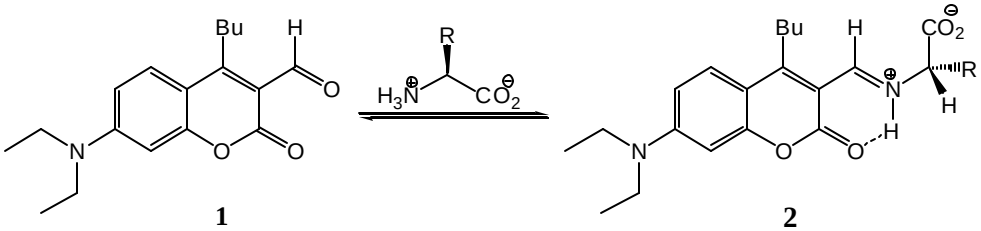
Chapter 2

Coumarin and the Quinolone based chemosensors

Overview

The Glass group has been interested in preparing fluorescent chemosensors to recognize amines in high salt condition. As shown in Scheme 1, aldehyde containing chromophores detect amines and amino acids via the reversible formation of imines. [23] Both coumarin and quinolone based fluorescent chemosensors have been designed to generate a fluorescent response upon binding with amines. The imines of amino acids are acidic and are largely protonated at pH 7. [24] Thus, the aldehyde substituted coumarins can make an iminium ion by reaction with a primary amine group which forms a hydrogen bond to the carbonyl of the coumarin, changing its fluorescence.

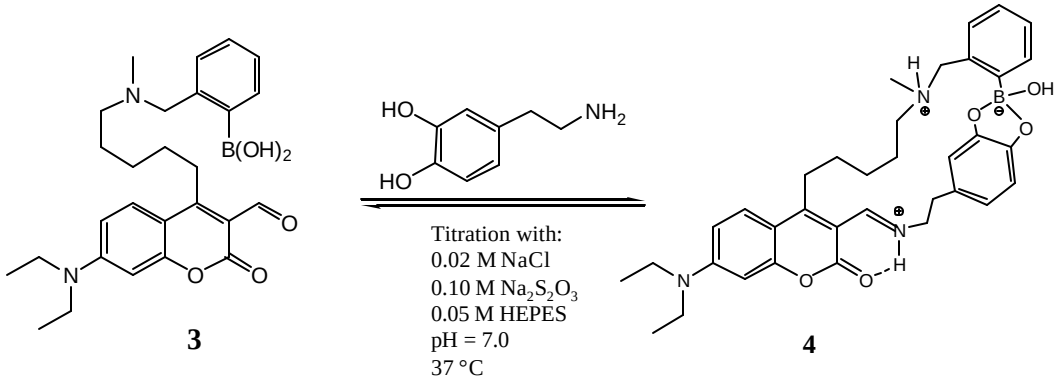
Scheme 1



1 **2**

For the example shown in Scheme 1, the fluorescence of the sensor increases by 26-fold [25] upon the addition of glycine. The next goal was to make a fluorescence sensor that will effectively detect catecholamines. As shown in Scheme 2 a sensor was designed which made an iminium ion with the amine part of catecholamine and a boronate ester with the catechol part. [26]

Scheme 2



3 **4**

Based on their results, the sensor binds to catecholamines with good affinity with selectivity for dopamine and norepinephrine over epinephrine or amino acids. The main core of the coumarin could be replaced with more electron rich quinolone moiety for a better fluorescence response. But ultimately we need to get a sensor that is pH sensitive as well as work with catecholamines. My project's primary goal was to make pH sensitive sensors that work effectively with amine containing analytes.

Significance of Detecting Amino Acids and Neuro Transmitters

Amino acids are the building blocks of proteins. In the late 50's the research focus shifted to determine their role as neurotransmitters. Among the many amino acids glutamate, aspartic acids, glycine and ?-aminobutyric acids act as either excitatory or inhibitory neurotransmitters. For example glycine binds to a receptor which makes the post-synaptic membrane more permeable to chloride ion. [27] Glutamate receptors play a vital role in excitatory synaptic transmission by which brain cells can communicate with each other. [28] Gamma amino butyric acid (GABA) plays a major inhibitory role in neurotransmition. [29] Like glycine, the GABA receptor is connected to a chloride ion channel, which allows more chloride ion to enter the neuron and makes the membrane less likely to depolarize. [30]

A simple cartoon of synapses is shown in Figure 6. The electrical impulse of one cell causes an influx of calcium ions and subsequently the neurotransmitter is released. This neurotransmitter diffuses through the synaptic cleft and stimulates the next cell in the chain by interacting with receptor proteins. Ions such as Na^{+} and Ca^{2+} pass through ligand gated ion channels in the center of the receptor complex. [31-32] This flow of ions results in depolarization of the plasma membrane of the target neuron.

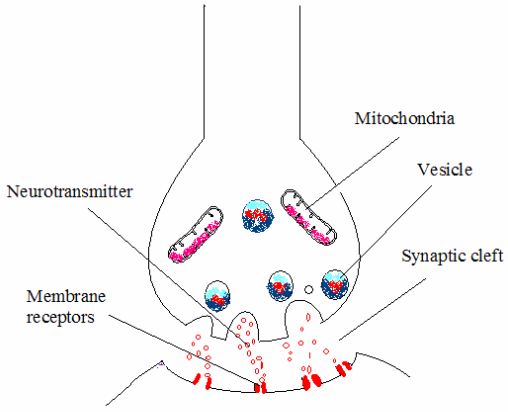
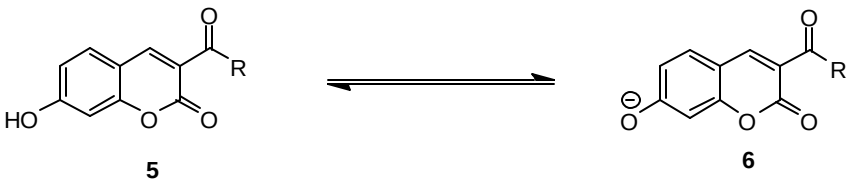


Figure 6

The Significance of pH Sensitive Sensors

In order to design useful chemosensors for cells, one needs to consider factors such as selectivity, good binding constant, high quantum efficiency, reversibility, and excitation wavelength. As our goal of this project is to make pH sensitive sensors which are non-fluorescent at low pH, but highly fluorescent at neutral pH or basic pH. This method will take advantage of the fact that vesicles are acidic (pH=5.5) but the synapse is neutral (pH=7.4). As the intracellular pH is generally varies between ~6.8-7.4 in cytosol and in the cell's acidic organelles it varies in between ~4.5-6.0. The sensor will be "off" in the vesicle, but "on" in the synapse.

Coumarin's can be made pH sensitive by the use of a 7-hydroxy [33] group (Figure 7). The hydroxyl group can be deprotonation at basic pH. In its protonated form (5), the major absorption is in the UV due to a p-p* transition. When deprotonated (6) an n-p* transition is possible. That's giving rise to a visible absorbance. Thus when exciting with visible light, only the deprotonated form fluoresces.



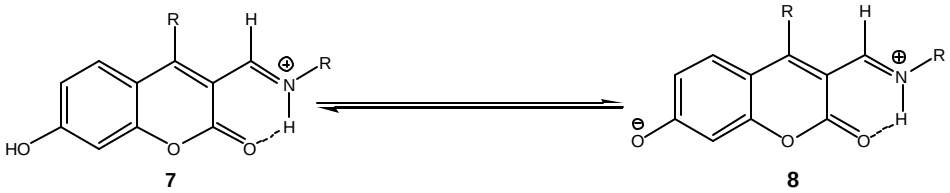
p-p* n-p*

Figure 7

In order to take advantage of changes in pH, sensors such as shown in scheme 3 will be prepared. The prior sensor will be modified with a hydroxyl group which will make the sensor non-fluorescent at

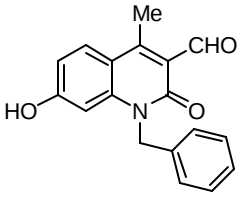
acidic pH (protonated) and fluorescent at basic pH (deprotonated). So our goal is to make compound that has pK_a around 6.

Scheme 3

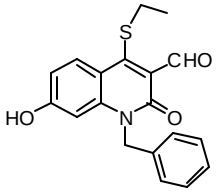


Strategy 1

Based on the above principle we designed the two sensors; compound **9** and compound **10**. The sensor **9** and **10** bind with the glutamate to make imines as shown in space filling model (Figure 8).



9



10

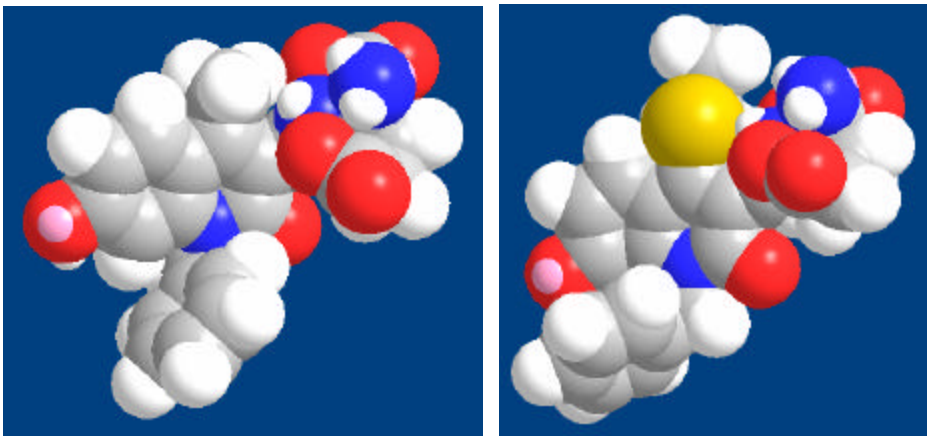
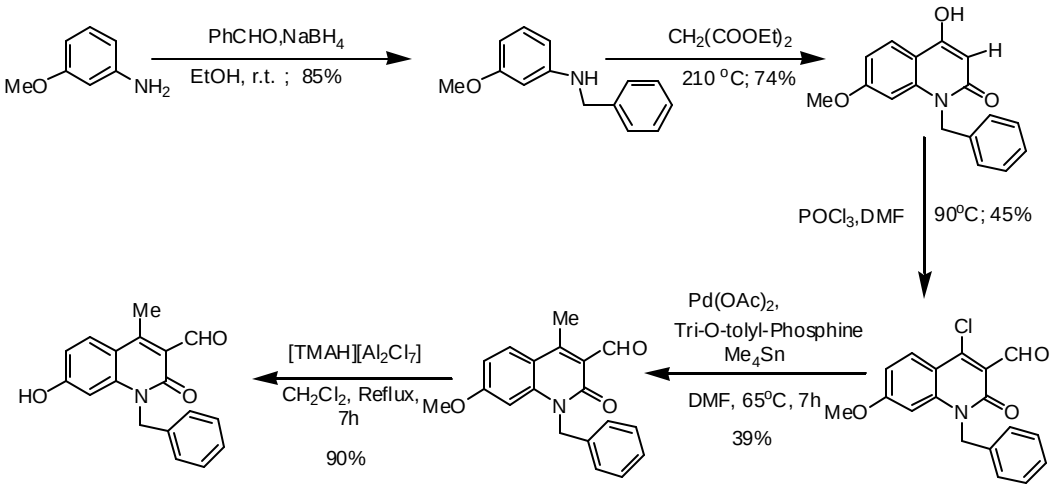


Figure 8

Synthesis of Compound 9

Compound **9** was prepared as shown in scheme 4. Compound **12** was prepared from compound **11** by imine formation followed by reductive amination. Compound **12** was mixed with diethyl malonate and heated to 210?C to provide compound **13**. Compound **14** was performed by formylation under Vilsmeier conditions. Stille coupling of compound **14** was done with palladium acetate, tri-*O*-tolylphosphine and tetramethyltin to give compound **15**. Demethylation of compound **15** was achieved using a reagent prepared from trimethylamine hydrochloride and aluminum chloride.

Scheme 4



Binding studies of compound 9

The UV/Vis pH titration of the compound 1 (3.18 µM) was done in ammonium formate buffer (50 mM) and with glutamate (1 M) and sodium chloride (20 mM). The absorption of 403 nm was plotted against pH (Figure 9) and the curve fitted using prism to give pK_a of 4.3.

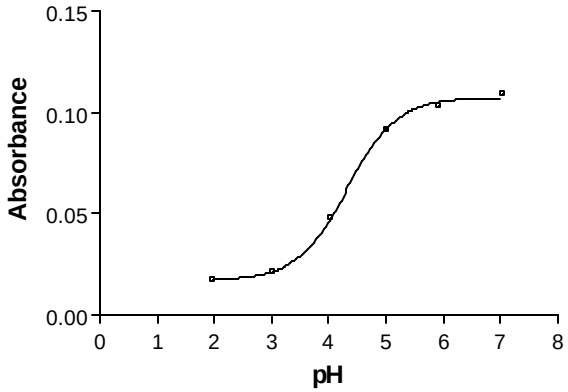
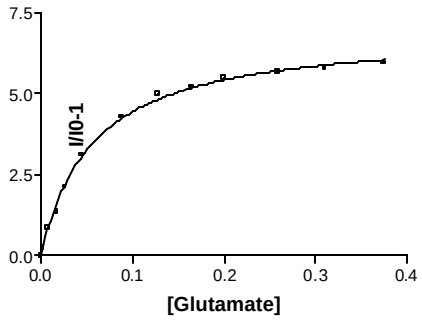


Figure 9

The fluorescent titration of the compound **9** was performed at pH 7.4 and pH 5.5 and sodium chloride (100 mM), HEPES (50 mM) with glutamate (1 M) as guest and the results are shown below (Figure 10). The K_d was calculated for pH 7.4 was 0.056 M and for pH 5.5 was 0.16 M

pH 7.4



pH 5.5

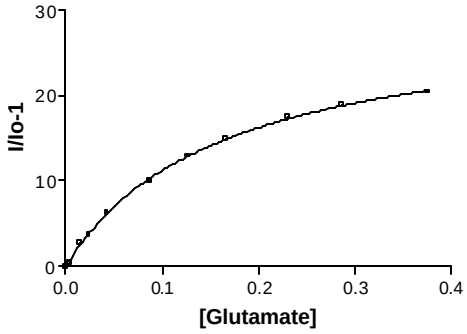
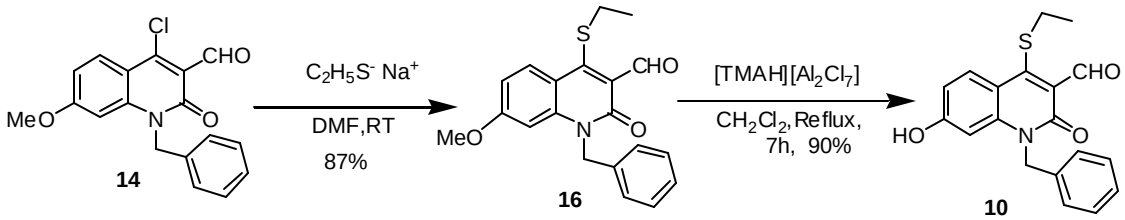


Figure 10

Synthesis of compound 10

Compound **10** was prepared as shown in scheme 5. To obtain thioether **16,** compound **14** was treated with ethane thiolate in DMF at room temperature. Demethylation of compound **10** was achieved by using a reagent prepared from trimethylamine hydro chloride and $AlCl_3$.

Scheme 5



Binding Studies of compound 10

The UV/Vis pH titration of the compound **10** (3.18 µM) was performed in ammonium formate buffer (50 mM) and with glutamate (1 M) and sodium chloride (20 mM). The absorption at 421 nm was plotted against pH (Figure 11) and the curve fitted using prism to give pK_a of 3.9.

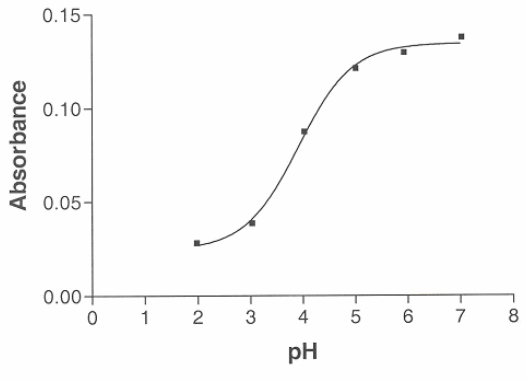


Figure 11

The fluorescent titration of the compound was performed at pH 7.4 and pH 5.5 and sodium chloride (100 mM), HEPES (50 mM) with glutamate (1 M) as guest and the results are shown below (Figure 12). The K_d was calculated for pH 7.4 was 0.90 M and for pH 5.5 was 0.17 M.

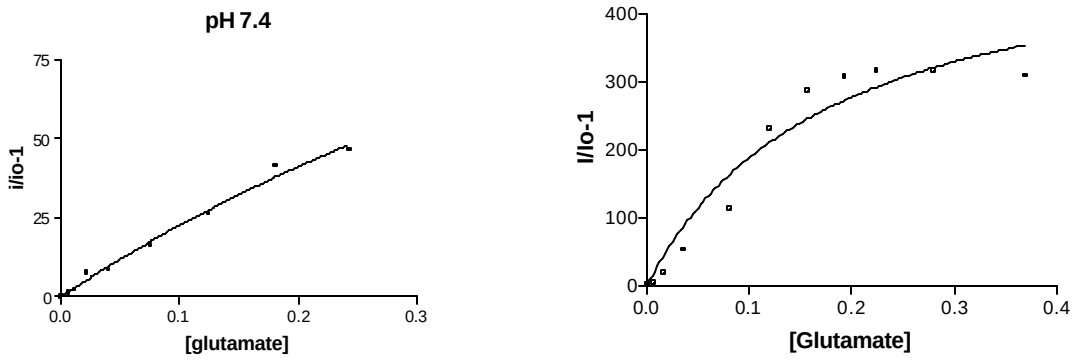
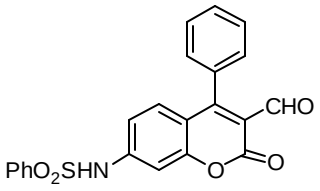


Figure 12

Strategy 2

Because the pK_a of compounds **9** and **10** were not optimal, we wanted to prepare a similar sensor which has a more appropriate pK_a. Thus compound **17** (Figure 13) was designed to bind amines but have high pK_a than compound **9** and **10**.



17

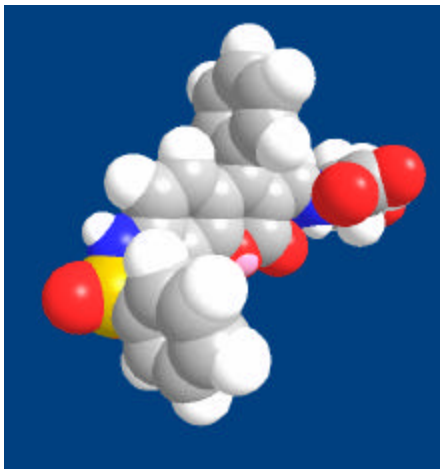
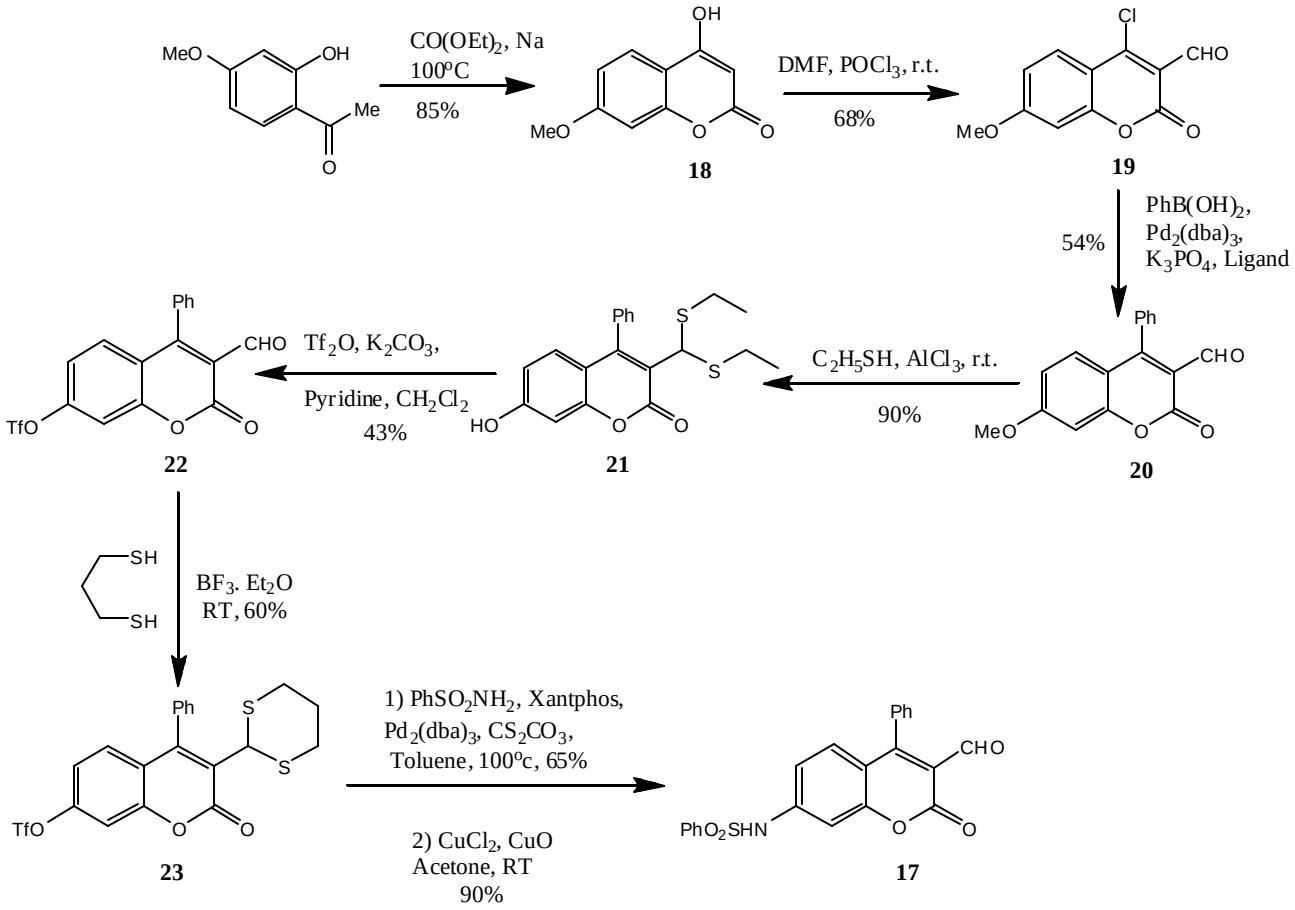


Figure 13

Synthesis of compound 17

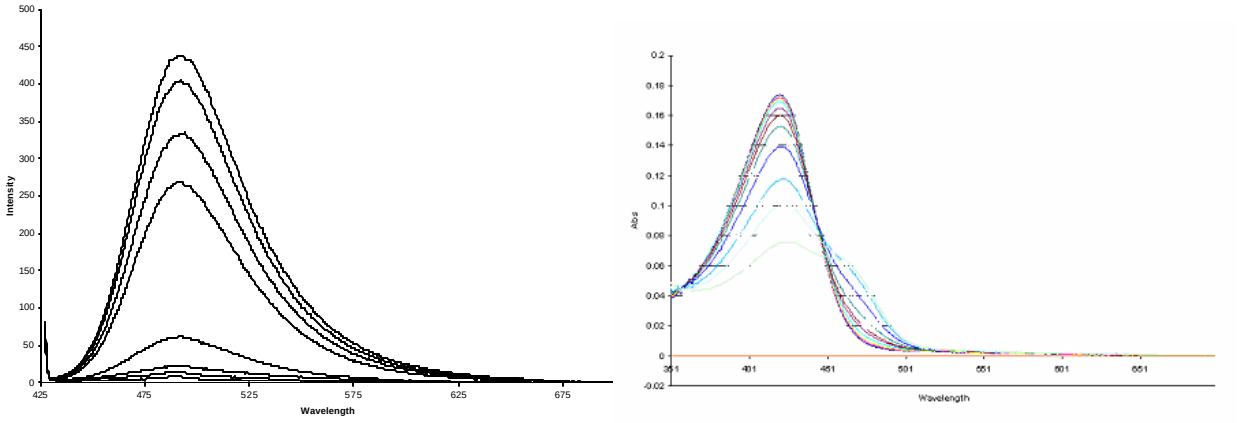
Compound **17** was prepared as shown in Scheme 6. Started with compound **19,** compound **20** was demethylated with aluminum chloride and ethane thiol, and the phenol converted to the corresponding triflate. Compound **22** was protected by 1,3-propane dithiol and boron trifluoride etherate. Compound **17** was made by Buchwald's procedure with xantphos catalyst, followed by treatment with copper (II) chloride in the presence of copper oxide to hydrolyze the protective group.

Scheme 6



Binding Studies of compound 17

The UV/Vis and fluorescent pH titration (Figure 14) of the compound 9 (3.18 µM) was done in Bis-tris propane (50 mM) buffer and with glutamate (1 M) and sodium chloride (120 mM). The absorption of 467 nm was plotted against pH (Figure 15) and the curve fitted using prism to give pK_a of 5.3. The K_d calculated for compound 17 and glutamate was 0.12 M.



Fluorescent titration UV/Vis titration

Figure 14

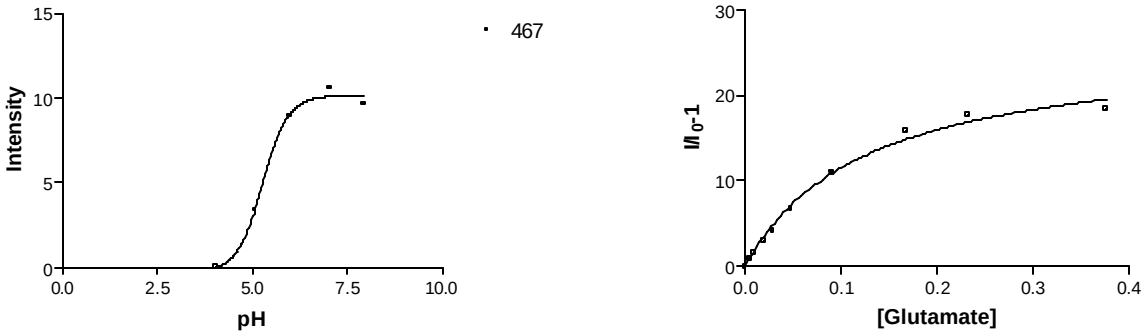


Figure 15

Conclusions

None of the sensors stated above compound **9**, **10** and **17**; shows the expected pK_a (6). The synthetic route for compound **17** is very tough so in future our goal will be making some pH sensitive sensors that has more appropriate pK_a's and try to develop easy synthesis route for making sulphonamide substituted quinolone system.

Appendix

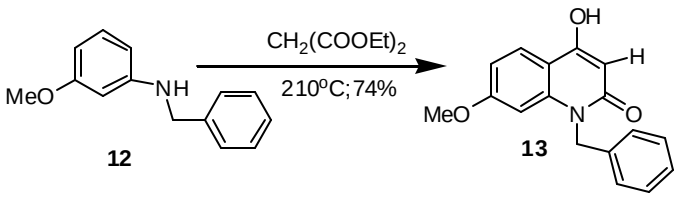
Experimental procedure

UV/visible and Fluorescence procedures. Absorption spectra experiments were carried out on a Carey 1E spectrophotometer at 37°C. Fluorescence experiments were carried out on a Shimadzu FR-5301 PC spectrofluorometer at 37°C. Prism 3.0 software was used to calculate K_d and maximum fluorescence intensities after titration to fit the data.

Detail procedure for pH titration. All the titrations were done at 37°C. To determine the pK_a of the imine, a solution containing Bis-tris propane (50 mM), NaCl (120 mM), Glutamate (1 M) and compound 17 (10^{-3} mM) was prepared. Aliquots (1 ml) of this solution were placed in seven vials and the pH was adjusted to specific values (e.g. pH 5, 6, 7, 8, 9, 10 and 11). The UV/visible spectra were recorded for each pH value. The absorption at 467 nm was plotted vs. pH and fitted to a pK_a isotherm using prism 3.0 software. The experiment was repeated without glutamate to determine the pK_a of the aldehyde.

Detail procedure for fluorescent titration. All the titrations were done at 37°C. A solution containing ammonium formate (50 mM), NaCl (120 mM) and compound 9 (10^{-3} mM) was prepared. Aliquots of this solution were placed in two vials and the pH was adjusted to specific values (5.5 and 7.4). The analyte solution was prepared by taking aliquot of each of the above solution and added and enough solid sodium glutamate to produce a solution which was 1 M in glutamate. To fluorescence cuvette was added the sensor solution and the emission spectra was recorded (? $_{ex}$ = 450 nm). This solution was titrated with analyte solution and the emission spectrum was at each point. The emission of 450 nm was plotted vs. concentration of glutamate and fitted to a binding isotherm using prism 3.0 software and the K_d value was calculated.

General Synthetic procedures. Tetrahydrofuran (THF) and diethyl ether (Et_2O) were distilled from sodium benzophenone under N_2 atmosphere. The benzene (PhH) and toluene (PhMe) were distilled from 9-fluorenone and sodium under N_2 atmosphere. Dichloromethane (CH_2Cl_2) and triethylamine (NEt_3) were distilled from CaH_2 under N_2 atmosphere. NMR spectra were recorded on a Bruker DRX-250, DRX-300, and DRX-500 as noted.



Compound 13—Compound 12 (13.3 g, 66.7 mM) and diethyl malonate (17 ml, 100.1 mM) were placed in a flame dried flask and then heated to 210 ^{o}C under N_2 atmosphere. The ethanol by-product was collected in a Dean-Stark apparatus. After 7 hours, the reaction mixture was cooled and crystals were filtered. The crystals were washed with methanol to yield the title compound as yellow crystals (12.77 g, 68%).

Data from compound 13:

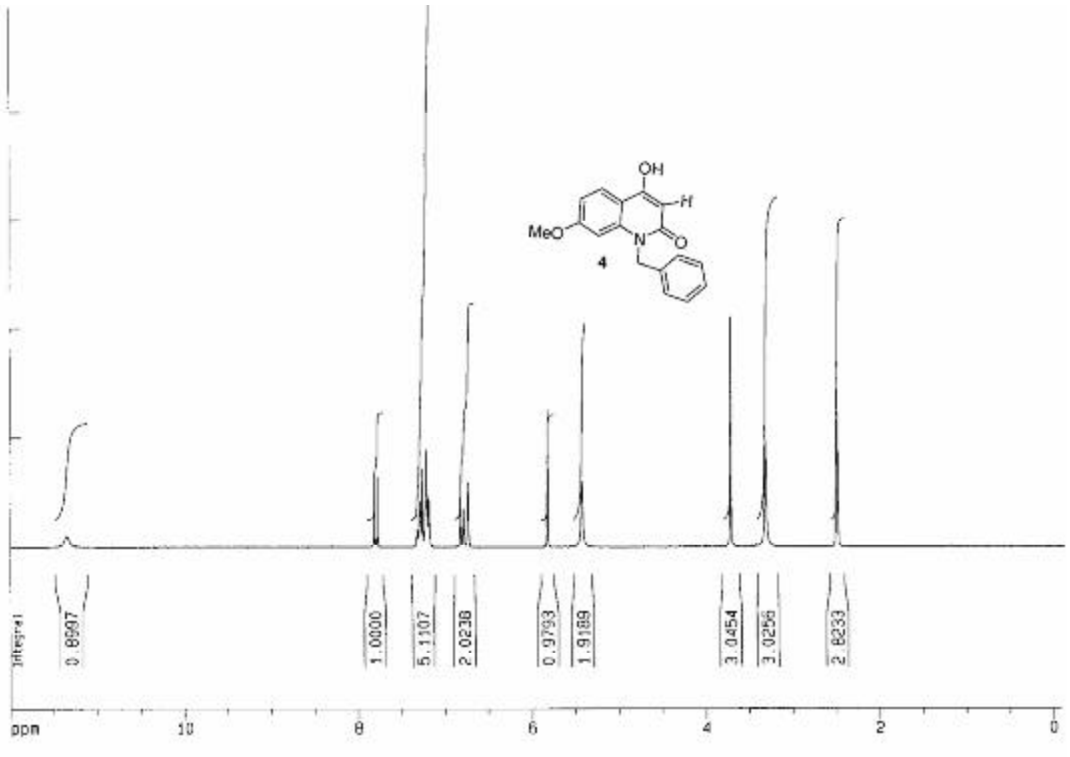
mp: 290 ^{o}C

^{1}H NMR (250 MHz, DMSO, d) 7.79 (d, 1H, *J*=7.32Hz), 7.32-7.18 (m, 5H), 6.82-6.73 (m, 2H), 5.82 (s, 1H) 5.42 (s, 1H), 3.71 (s, 3H);

^{13}C NMR (75 MHz, DMSO, d) 163.6, 162.0, 161.9, 141.2, 137.9, 128.9, 127.3, 126.9, 125.3, 110.5, 109.1, 99.9, 95.8, 55.7, 44.3;

FTIR (neat, cm^{-1}): 3083, 3021, 2953, 1596, 1499.

HRMS (m/z): ESI of $C_{17}H_{15}NO_3$ (M+H) calculated are 282.1130 found 282.1117.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MeO
N
O
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1.9189
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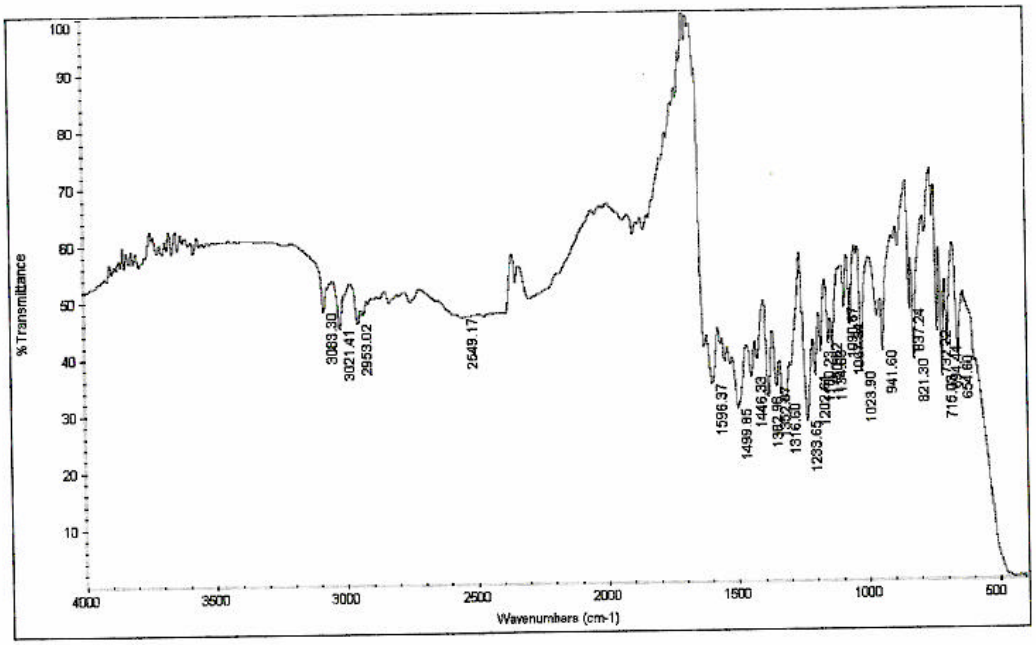
3.0256
2.8233
ppm
10
8
6
4
2
0


% Transmittance
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80
70
60
50
40
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20
10
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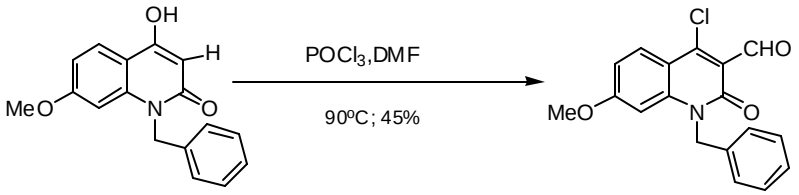
3021.41
2953.02
2649.17
1596.37
1499.85
1446.39
1316.60
1293.65
1023.90
941.60
821.30
837.24
716.00
654.60
4000
3500
3000
2500
2000
1500
1000
500
Wavenumbers (cm-1)



13 **14**

Compound 14- In a dry flask, dimethyl formamide (4.69 mL, 62.83 mM) was mixed with phosphorous oxychloride (7.92 mL, 31.4 mM). The solution was stirred at 0 ^{o}C for 15 minutes and at ambient temperature for two hours. In another flame dried flask, compound 13 (2.52 g, 8.98 mM) was added to dimethyl formamide (10 mL) and the above prepared solution was added slowly into it. The reaction mixture was heated to 90 $^{?}$C for 48 hours. The mixture was cooled to room temperature and then slowly poured onto ice water and the solids were filtered. The solids were dissolved in dichloromethane and dried over anhydrous sodium sulfate. The product was purified via flash chromatography (25% ethyl acetate in hexane). The resulting yellow solid was isolated (1.65 g, 56%).

Data from compound 14:

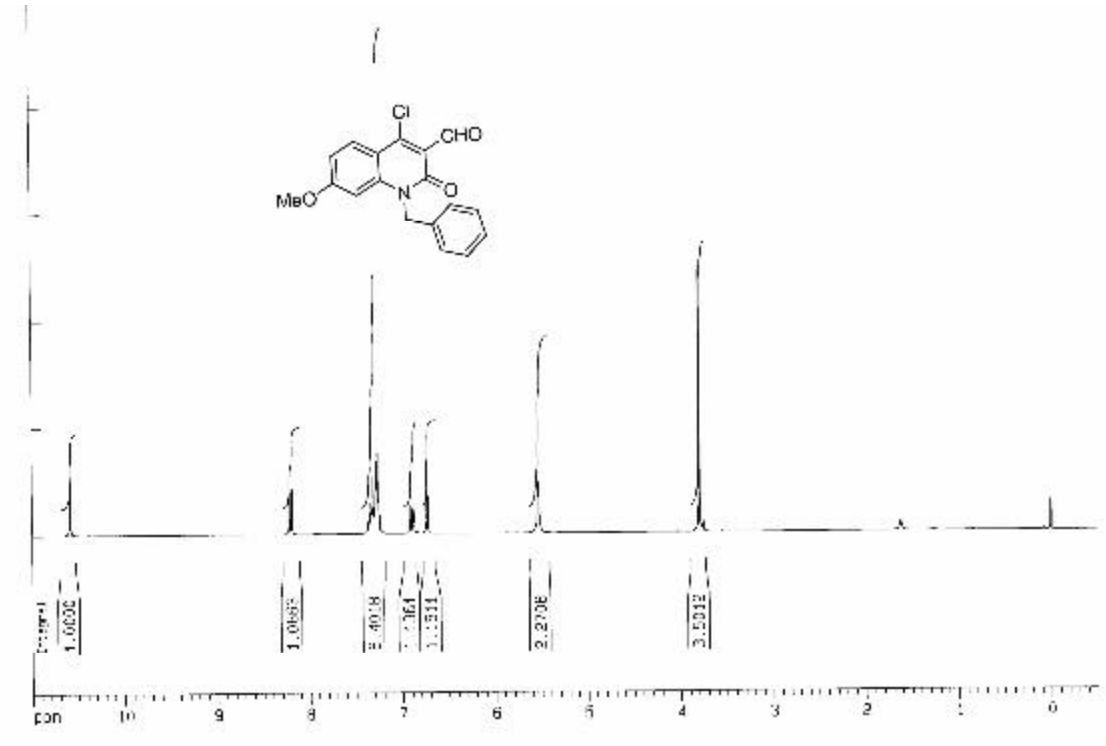
mp: 185- 186 ^{o}C

^{1}H NMR (300 MHz, $CDCl_3$, d) 10.59 (s, 1H, aldehyde CHO), 8.21 (d, 1H, *J*= 9.2 Hz), 7.37-7.26 (m, 5H), 6.89 (dd, 1H, *J*=2.3 Hz, *J*=9.2 Hz), 6.73 (d, 1H, *J*= 2.28 Hz), 5.54 (s, 2H), 3.79 (s, 3H).

^{13}C NMR (75 MHz, $CDCl_3$, d) 189.3, 164.5, 161.5, 148.1, 142.2, 135.3, 130.3, 128.9, 127.6, 126.5, 119.2, 113.2, 111.9, 99.2, 55.7, 46.3.

FTIR (neat, cm^{-1}): 2924, 2853, 1689, 1637, 1605, 1536.

HRMS (m/z): calc. for $C_{18}H_{14}ClNO_3$, (M+H), 328.014 found 328.1123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N
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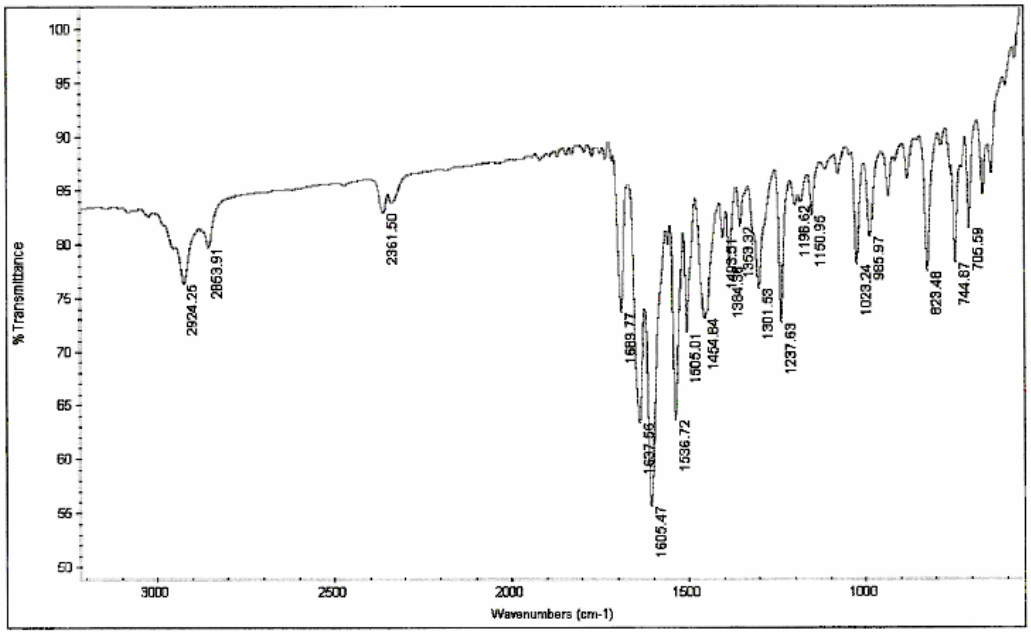
1.0063
2.2708
3.5013
ppm


% Transmittance
Wavenumbers (cm-1)
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1605.47
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1505.01
1454.84
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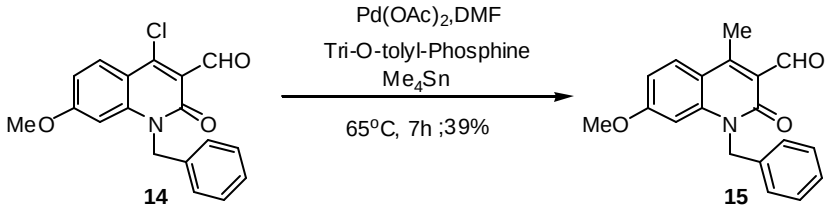
1150.96
1023.24
985.97
828.48
744.87
705.59



Compound 15 - In a flame dry N_2-filled flask, compound 14 (1.08 g, 2.87 mM) was mixed with palladium acetate (20 mg, 0.0632 mM), tri-*O*-tolylphosphine (90 mg, 0.29 mM) in of dimethyl formamide (5 mL) solution. The triethyl amine (870 µL, 8.639 mM) and tetramethyltin (1.33 mL, 5.75 mM) were added subsequently into it. The reaction mixture was heated to 68°C for 7 hours. The dimethyl formamide was removed in vacuo make soluble in ethyl acetate and washed with brine and dried over anhydrous sodium sulfate. The residue was purified via flash chromatography (25% ethyl acetate in hexane). The product was isolated as a yellow solid (344.8 mg, 39%).

Data from compound 15:

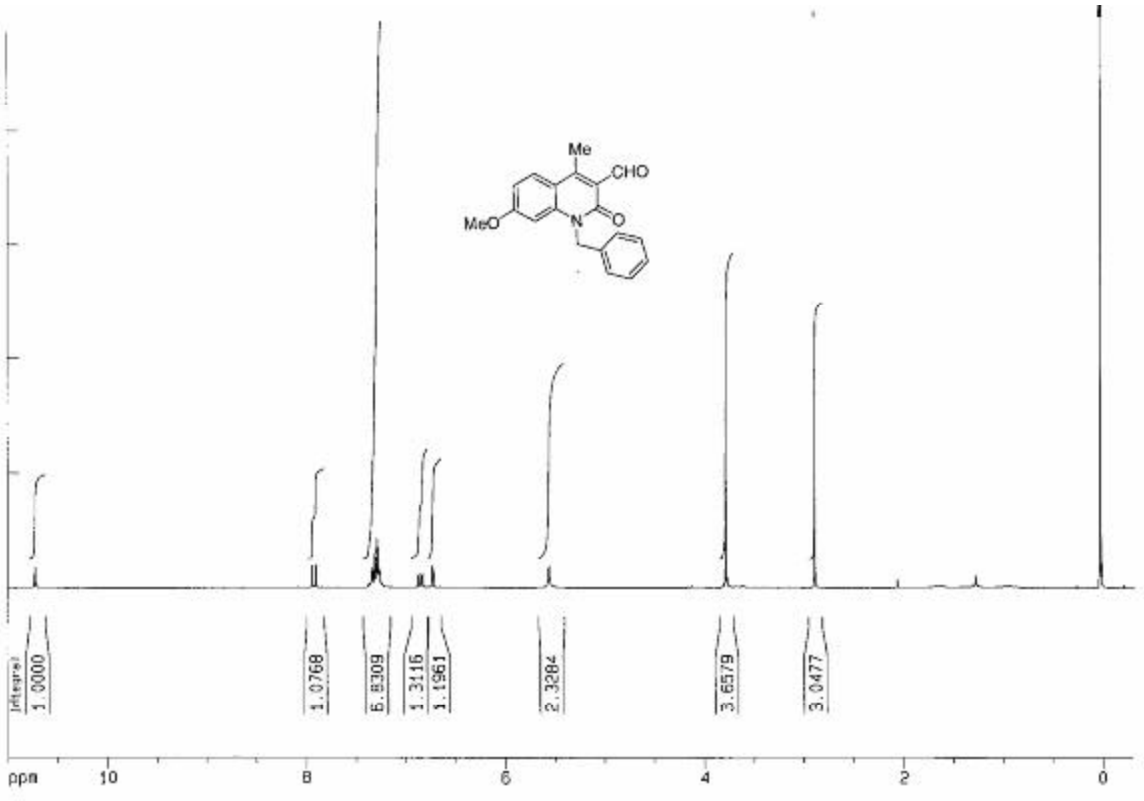
mp: 169-170 °C

^{1}H NMR (250 MHz, $CDCl_3$, d) 10.72 (s, 1H, Aldehyde –CHO), 7.92 (d, 1H, *J*= 9.13 Hz), 7.34- 7.26 (m, 5H), 6.73 (dd, 1H *J*= 2.7 Hz, *J*= 10.9 Hz), 6.70 (d, 1H, *J*=3.55 Hz), 5.53 (s, 2H), 3.75 (s, 3H), 2.85 (s, 3H);

^{13}C NMR (125 MHz, $CDCl_3$, d) 193.2, 162.7, 162.0, 152.8, 142.7, 136.6, 130.0, 128.6, 127.0, 126.6, 126.5, 120.2, 114.1, 112.2, 100.9, 44.9, 13.6.

FTIR (neat, cm^{-1}): 1733, 1675, 1636, 1607, 1539.

HRMS (m/z): calc. for $C_{19}H_{17}NO_3$ $(M+ Na)^+$; 330.1106 found 330.1113.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N
O
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1.1961
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3.6579
3.0477
ppm
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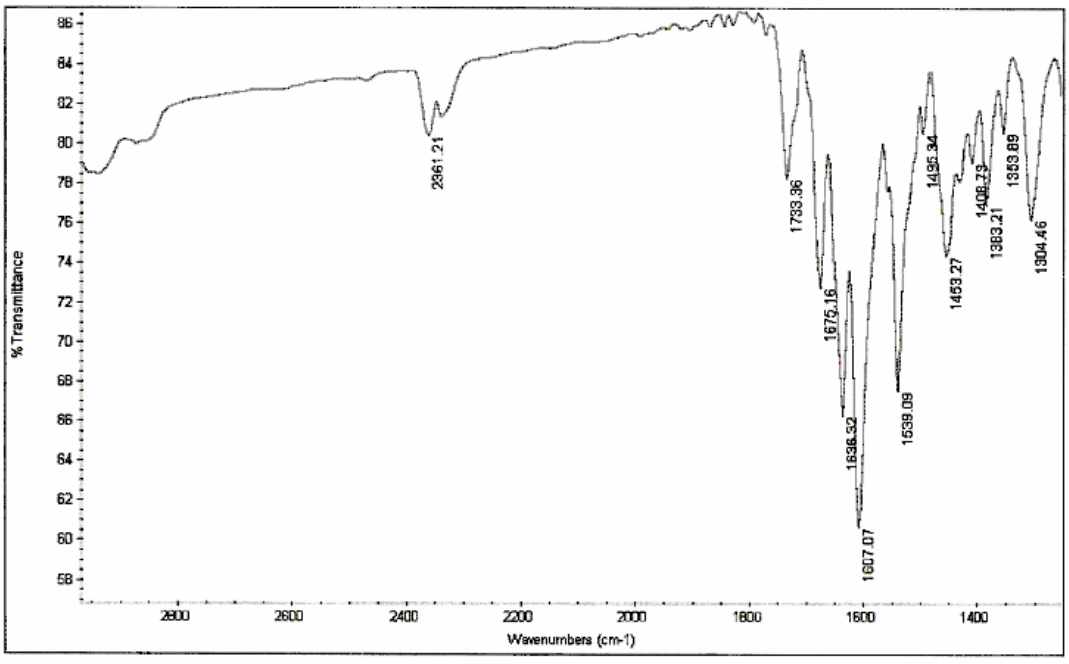
8
6
4
2
0


% Transmittance
Wavenumbers (cm-1)
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1539.09
1495.34
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1408.73
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1353.89
1304.46
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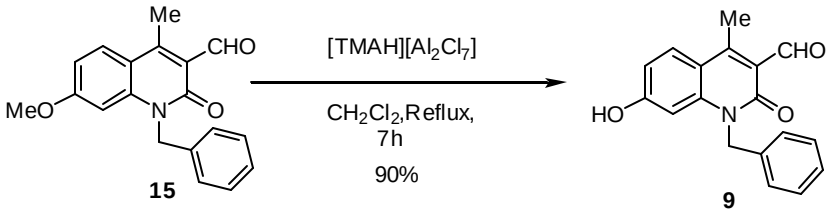
2000
1800
1600
1400



Compound 9-- In a flame dry flask, anhydrous aluminum chloride (1.73 g, 1.3 mM) was dissolved in dry dichloromethane (CH_2Cl_2) and put into an ice-bath. When the mixture was cooled the trimethylamine hydrochloride (0.62 g, 0.65 mM) was added slowly. That reaction mixture was stirred for 2 hours at room temperature. The dichloromethane was removed in vacuo. This ionic liquid ([TMAH] Al_2Cl_7) was used for the demethylation reaction.

In a very dry flask, compound 15 (1.52 g, 4.63 mM) was mixed with dry dichloromethane (CH_2Cl_2), and then above prepared ionic liquid, [TMAH] Al_2Cl_7 (6.2 mL, 13.9 mM) was added into it. The reaction mixture was refluxed in 50 ?C and monitored. After 7 hours, the reaction mixture was cooled to room temperature and treated with dilute hydrochloric acid and extracted with dichloromethane (CH_2Cl_2) and dried over anhydrous sodium sulfate. The resulted product was purified via flash chromatography (40% ethyl acetate in hexane). The product was isolated (1.196 g, 88%).

Data from compound 9:

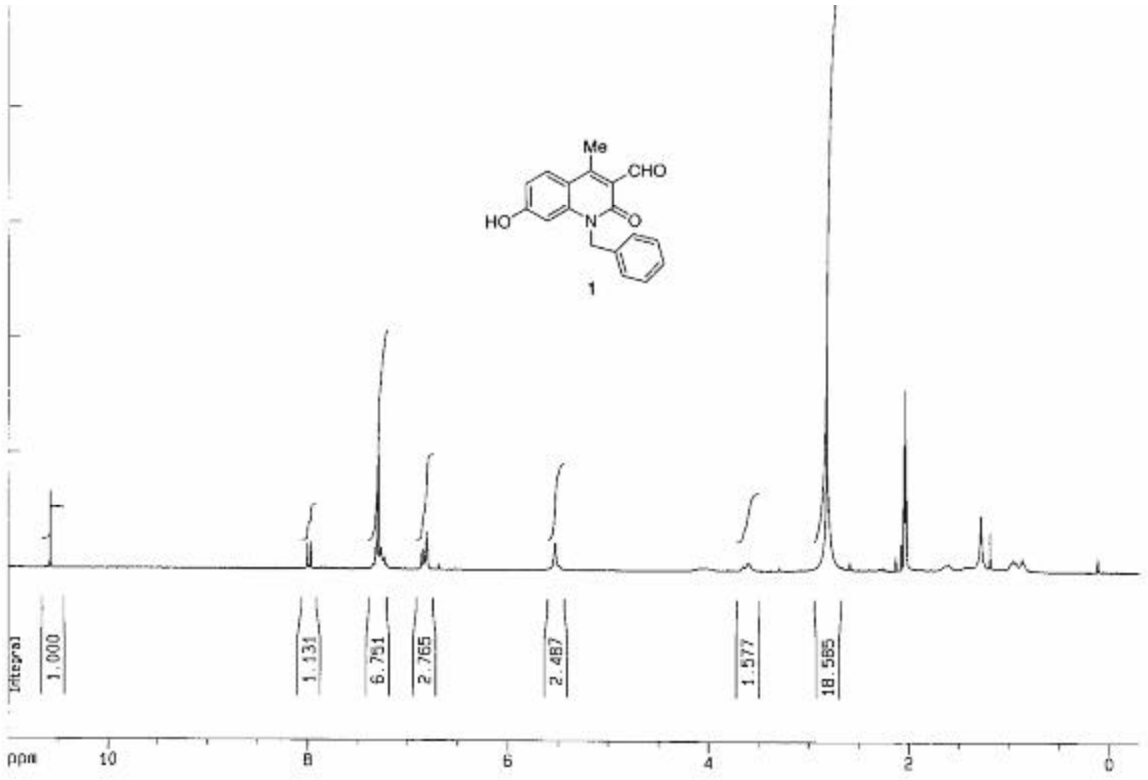
mp: 142- 143 ^{o}C

^{1}H NMR (500 MHz, CD_3COCD_3, d) 10.59 (s, 1H, aldehyde CHO), 7.99 (d, 1H, J=8.85 Hz), 7.33-7.28 (m, 5H), 6.87 (d, 1H, *J*= 2.3 Hz), 6.82 (dd, 1H, *J*= 2.5 Hz, *J*= 5.8 Hz), 5.54 (s, 2H).

^{13}C NMR (125 MHz, CD_3COCD_3, d) 193.3, 162.7, 161.9, 152.8, 142.7, 136.6, 130.0, 128.6, 127.0, 126.6, 126.5, 120.2, 114.1, 112.2, 100.9, 44.9.

FTIR (neat, cm^{-1}): 2926, 1733, 1685, 1604, 1543.

HRMS (m/z): calc. for $C_{18}H_{15}NO_3$ $(M+Na)^+$ 316.095 observed 316.095.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ppm
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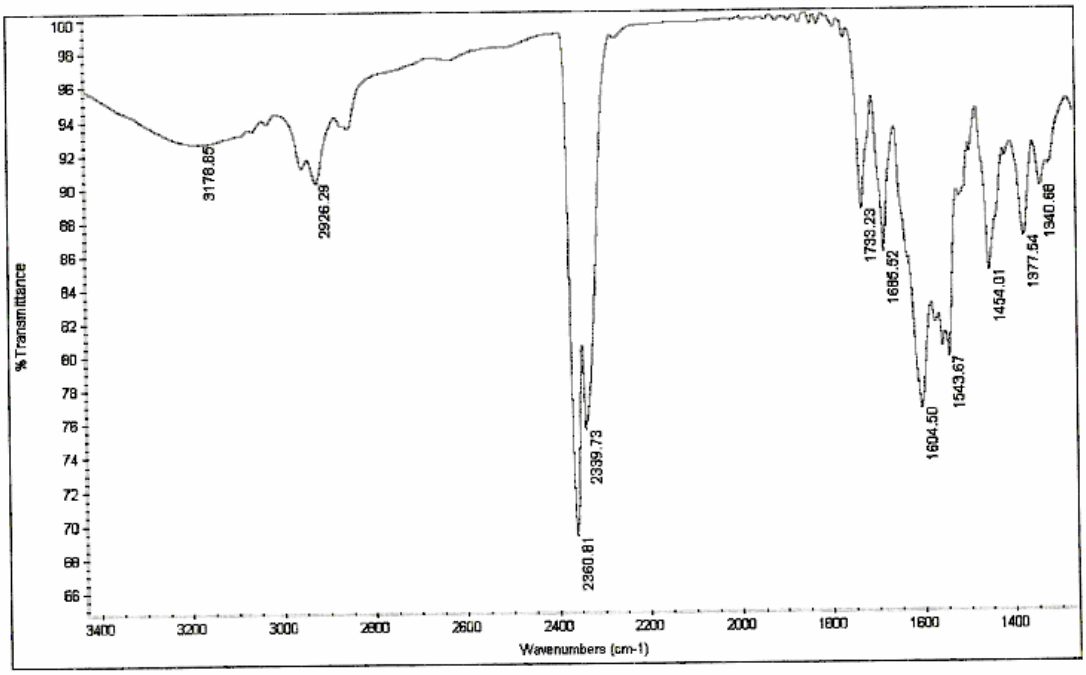
% Transmittance
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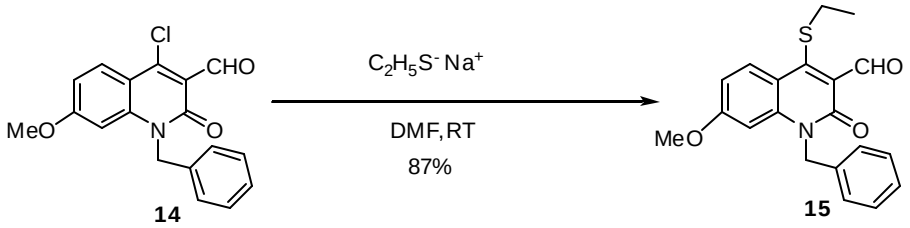
96
94
92
90
88
86
84
82
80
78
76
74
72
70
68
66
3178.85
2926.29
2360.81
2339.73
1738.23
1685.92
1604.50
1543.67
1454.01
1377.54
1340.66
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
Wavenumbers (cm-1)



Compound 15- In a flame dried, N_2-filled flask, compound 14 (3.56 g, 10.86 mM) was mixed with sodium ethane thiolate (1.83 g, 21.72 mM) then dry dimethyl formamide was added carefully. The reaction mixture was stirred overnight at room temperature. Dimethyl formamide was removed in vacuo and the reaction mixture was dissolved at ethyl acetate and washed with brine and dried over anhydrous sodium sulfate. The resulted yellow solid crystals were purified via flash chromatography (20% ethyl acetate in hexane). The isolated product was a yellow solid (3.22 g, 84%).

Data from compound 15:

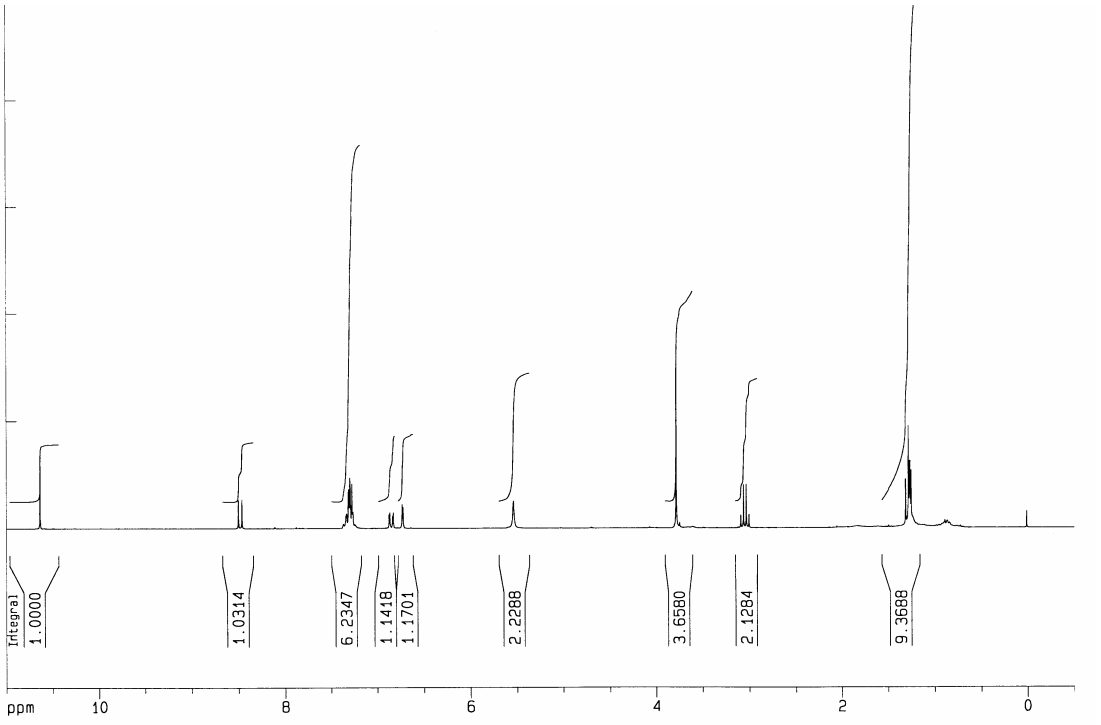
mp: 148-149 ^oC

^{1}H NMR (250 MHz, $CDCl_3$, d) 10.64 (s, 1H, Aldehyde –CHO), 8.48 (d, 1H, *J*= 9.2 Hz), 7.31-7.26 (m, 5H), 6.73 (d, 1H, *J*= 2.4 Hz), 6.71 (s, 1H), 5.54 (s, 2H), 3.79 (s, 3H), 3.05 (q, 2H, *J*= 7.4 Hz), 1.32-1.26 (m, 3H);

^{13}C NMR (75 MHz, $CDCl_3$, d) 163.8, 161.3, 154.8, 141.6, 135.7, 130.9, 128.9, 127.5, 126.7, 123.9, 115.8, 111.0, 99.3, 55.5, 46.3, 32.6, 29.6, 14.8.

FTIR (neat, cm^{-1}): 2958, 2925, 2847, 1701, 1604.

HRMS (m/z): calc. for $C_{20}H_{19}NO_3S$, (M+H) 354.1164 observed 354.1163.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ppm
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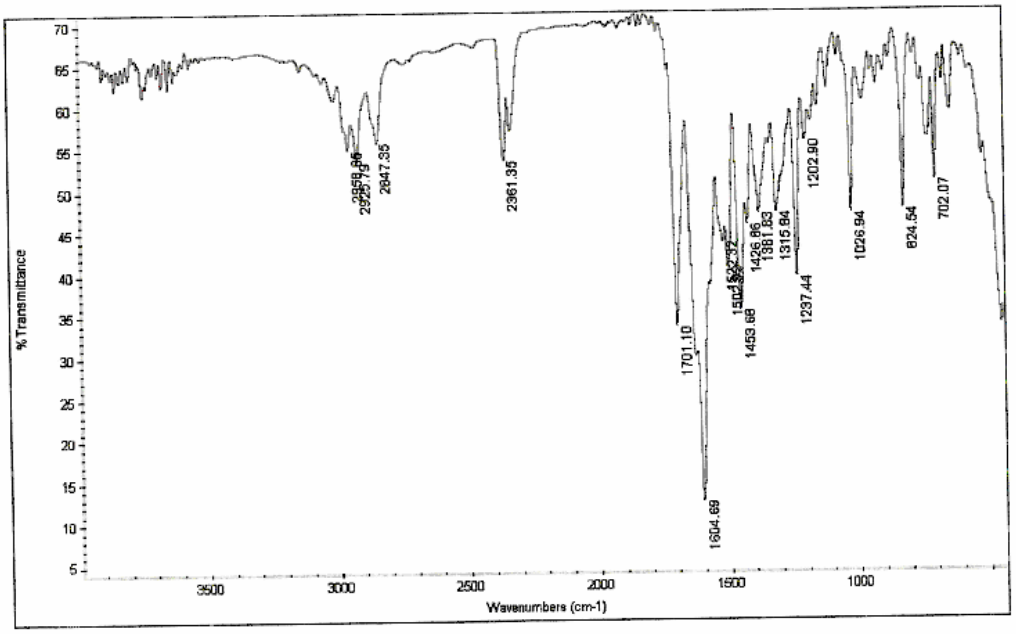
8
6
4
2
0


% Transmittance
Wavenumbers (cm-1)
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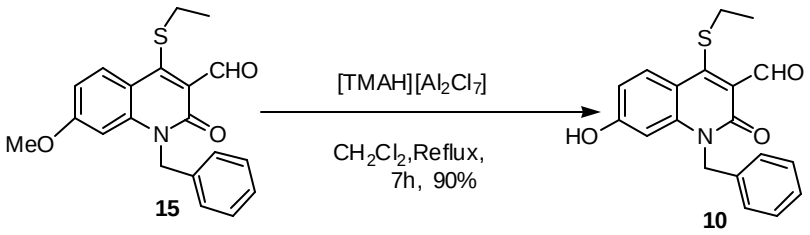
1381.83
1315.84
1237.44
1202.90
1026.94
824.54
702.07



Compound 10- In a flame dried N_2 filled flask, compound 15 (3.27 g, 9.25 mM) was mixed with dry dichloromethane (CH_2Cl_2), then previously mentioned ionic liquid [TMAH] Al_2Cl_7 (12.4 mL, 27.756 mM) was added slowly into it. The reaction mixture was refluxed in 50$^?$C and monitored carefully. After 7 hours the reaction mixture was cooled and hydrochloric acid (1M) was added with it and extracted with dichloromethane (CH_2Cl_2) and dried over anhydrous sodium sulfate. The resulted yellow residue was purified via flash chromatography (40% ethyl acetate in hexane). The isolated product was isolated a yellow solid (2.76 g, 88%).

Data from compound 10:

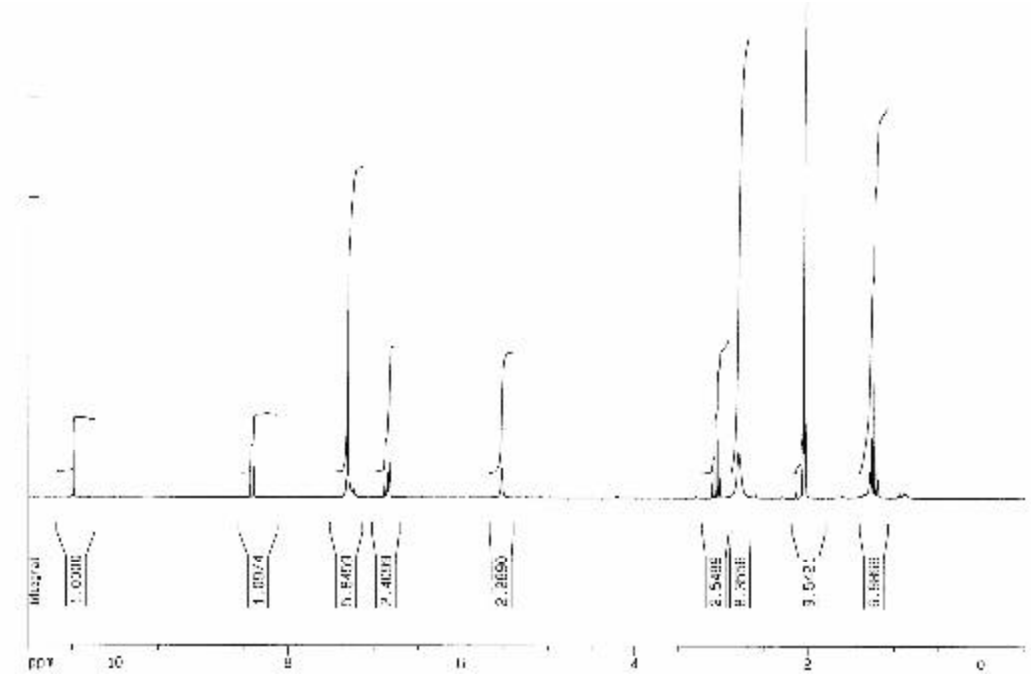
mp: 198-199 ^{o}C

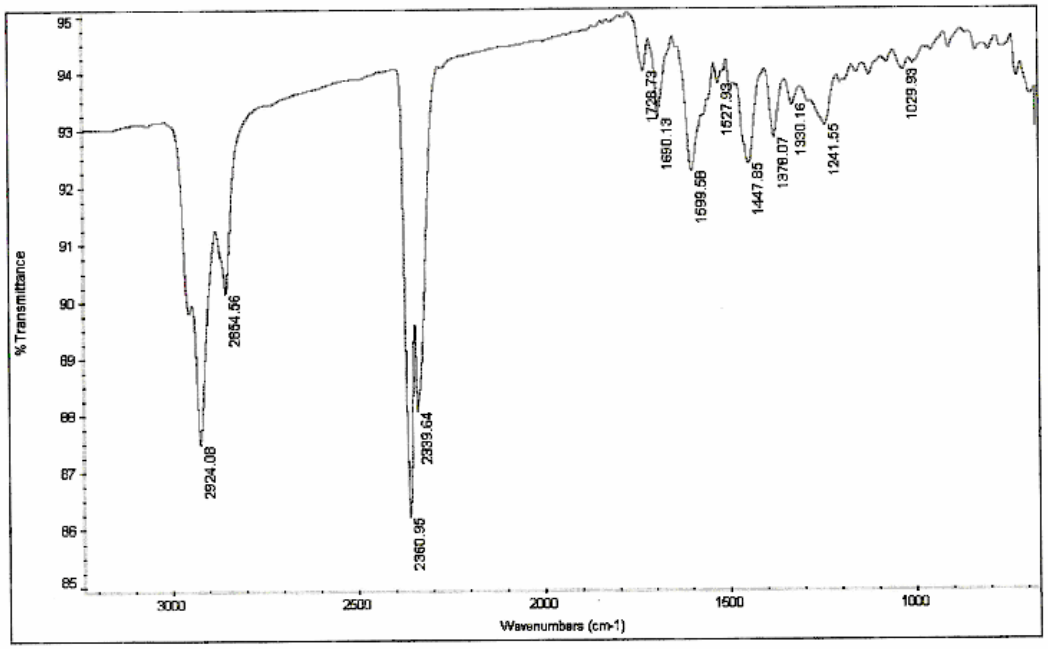
^{1}H NMR (250 MHz, $CDCl_3$, d) 10.48 (s, 1H, Aldehyde –CHO), 8.41 (d, 1H, *J*= 8.9 Hz), 7.33-7.28 (m, 5H), 6.88 (d, 1H, *J*= 2.3 Hz), 6.84-6.82 (m, 1H), 5.53 (s, 2H), 3.10-3.01 (m, 2 H), 1.26-1.19 (m, 3H).

^{13}C NMR (75 MHz, $CDCl_3$, d) 189.8, 162.2, 161.0, 152.5, 141.9, 136.4, 131.2, 128.6, 127.1, 126.6, 124.4, 114.7, 112.2, 101.0, 45.3, 31.8, 14.1.

FTIR (neat, cm^{-1}): 2924, 2854, 1690, 1599, 1447.

HRMS (m/z): calc. for $C_{19}H_{17}NO_3S$, (M+H) 340.1006, observed 340.1007.




% Transmittance
Wavenumbers (cm-1)
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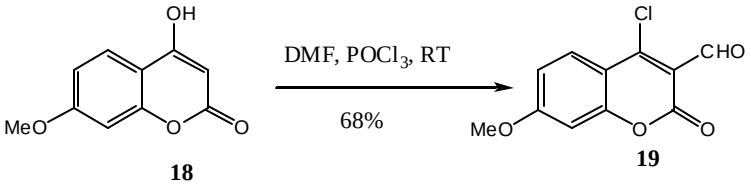
1330.16
1241.55
1029.93


OH
MeO
O
O
DMF, POCl3, RT
68%
Cl
CHO
MeO
O
O

18 **19**

Compound 19- In a dry flask dimethyl formamide (11.33 mL, 146.32 mM) was mixed with of phosphorous oxychloride (6.82 mL, 73.16 mM). The solution was stirred at 0 ^{o}C for 15 minutes and at ambient temperature for two hours. In another flame dried flask, compound 18 (4.13 g, 20.9 mM) was added to dimethyl formamide (20 mL) and the above prepared solution was added slowly into it. The reaction mixture was stirred in ambient temperature for overnight. The mixture was slowly poured onto ice water and the solids were filtered. The solids were dissolved in dichloromethane and dried over anhydrous sodium sulfate. The product was purified via flash chromatography (50% ethyl acetate in hexane). The resulting yellow solids were isolated (2.37 g, 68%).

Data from compound 19:

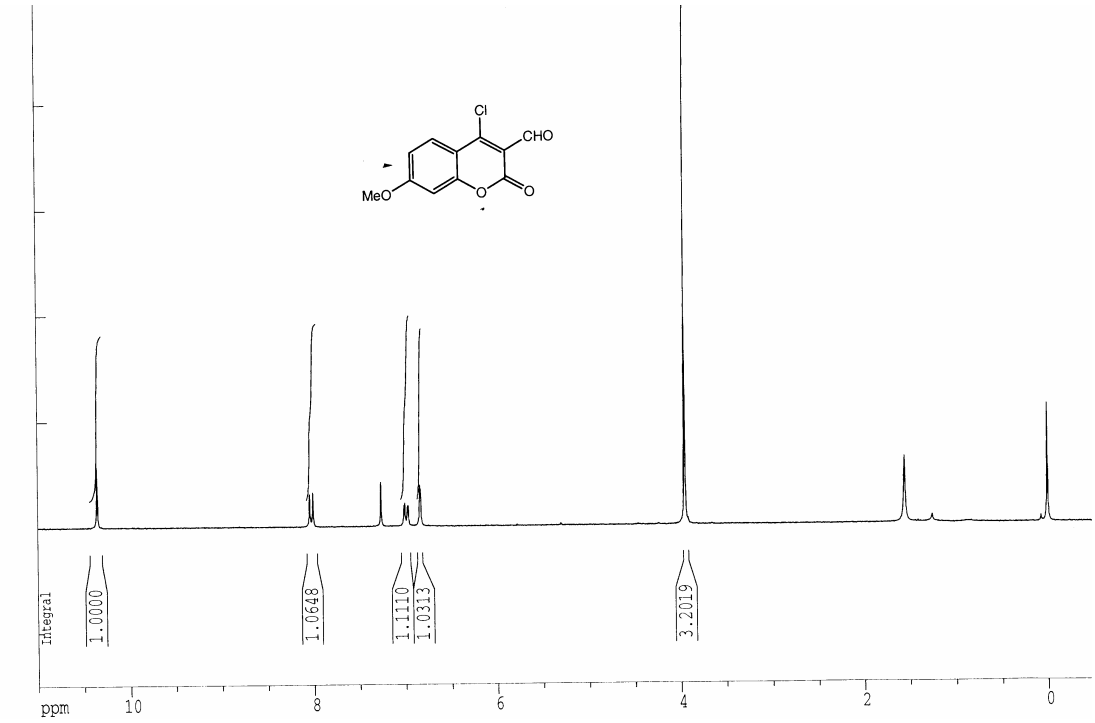
mp: 168-169 ^{o}C

^{1}H NMR (250 MHz, $CDCl_3$, d) 10.35 (s, 1H, Aldehyde –CHO), 8.02 (d, 1H, J= 9.0 Hz), 6.98 (dd, 1H, *J*=2.4 Hz, J= 9.1 Hz), 6.84 (d, 1H, *J*= 2.3 Hz), 3.95 (s, 3H).

^{13}C NMR (75 MHz, $CDCl_3$, d) 186.3, 166.2, 158.8, 155.8, 154.0, 129.0, 114.9, 114.5, 111.9, 100.6, 56.2.

FTIR (neat, cm^{-1}): 2970.1, 1697.2, 1614.4.

HRMS (m/z): calc. for $C_{11}H_7ClO_4$, (M+H) 239.0105 observed 239.0111.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1.1110
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ppm
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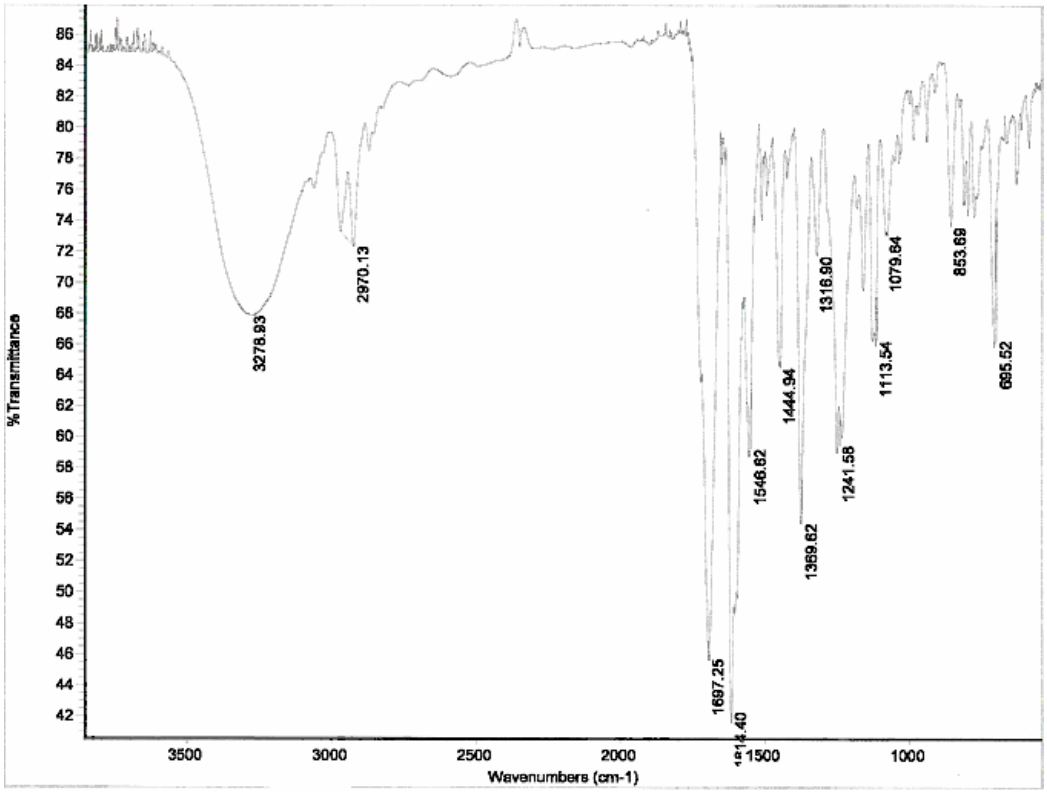
4
2
0


%Transmittance
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84
82
80
78
76
74
72
70
68
66
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62
60
58
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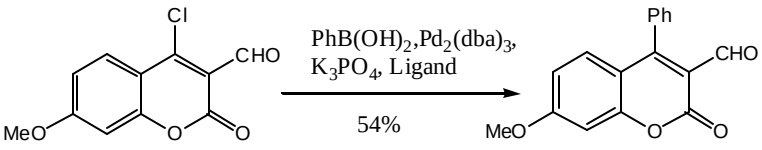
52
50
48
46
44
42
3278.93
2970.13
1697.25
1546.62
1444.94
1369.62
1318.90
1241.58
1113.54
1079.84
853.68
695.52
3500
3000
2500
2000
1500
1000
Wavenumbers (cm-1)



19 **20**

Compound 20- In a flame dried flask, compound 19 (1.6 g, 6.7 mM) was combined with phenyl boronic acid (981 mg, 8.04 mM), tris (dibenzyledeneacetone) dipalladium (491 mg, 0.54 mM), 2-dicyclohexylphosphino-2`, 6`-dimethoxy-1,1` biphenyl (56 mg, 0.134 mM) and potassium phosphate (3.98 g, 18.76 mM). Then this mixture was put under vacuum for 30 min. The flask was filled with nitrogen gas slowly. Then dry toluene (20 mL) was slowly added into it. The reaction mixture was heated to 65 ^{o}C in oil bath for overnight. The solvent was removed in-vacuo. Then reaction mixture was dissolved in dichloromethane and washed with brine and finally dried over anhydrous sodium sulfate. The residue was purified via flash chromatography (2.5% ethyl acetate in dichloromethane). The isolated product was a yellow solid (1.013 g, 54%).

Data from compound 20:

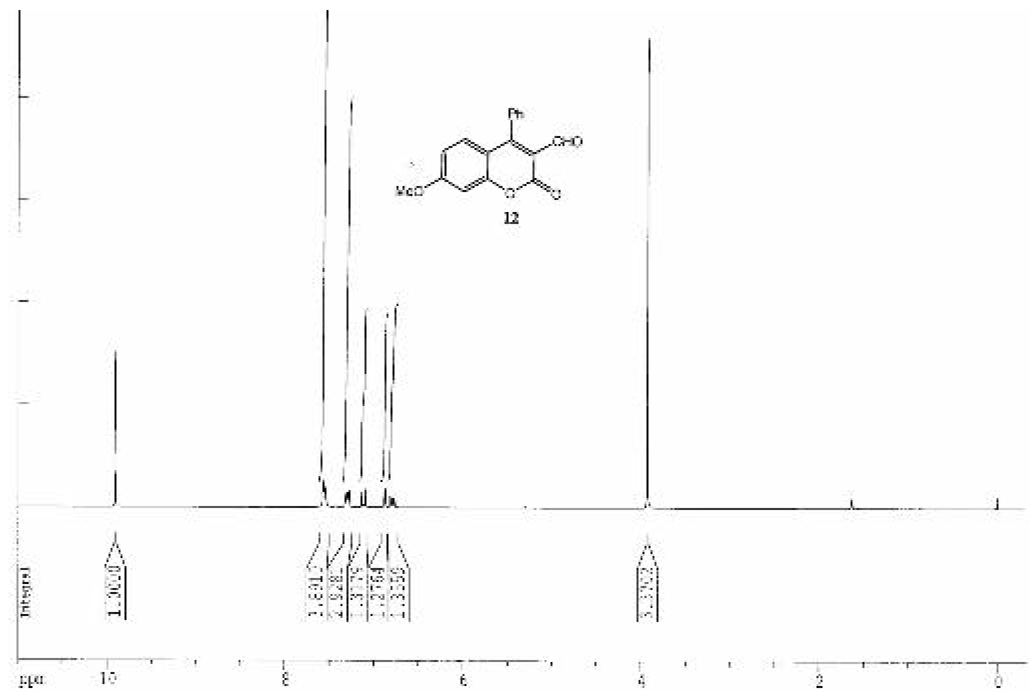
mp: 164-165 ^{o}C

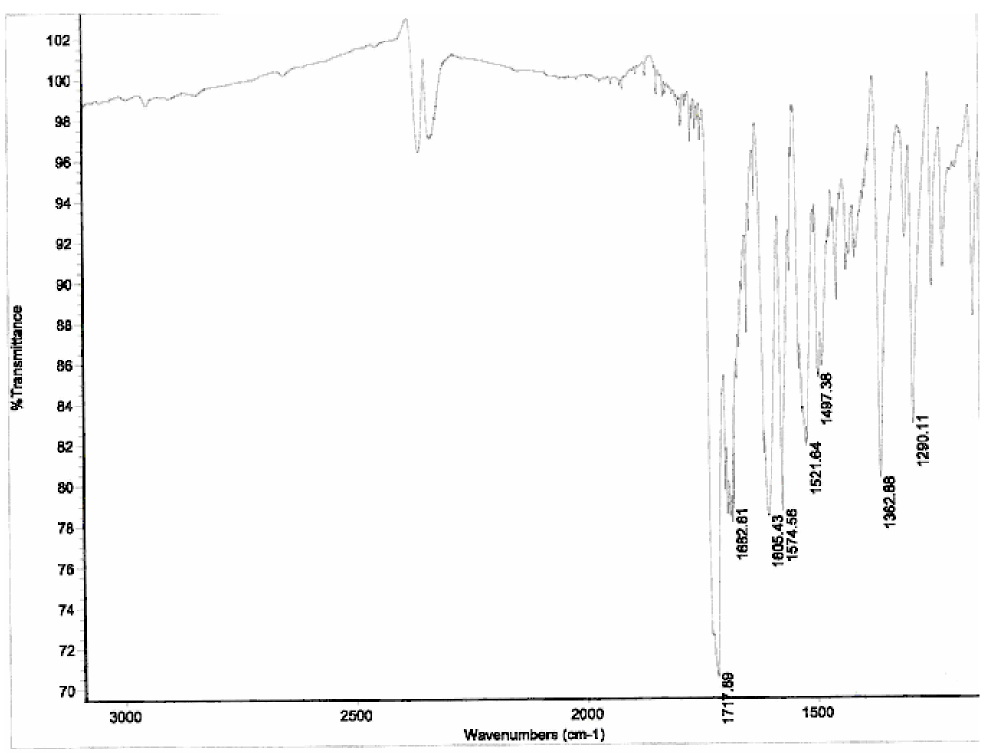
^{1}H NMR (250 MHz, $CDCl_3$, d) 9.92 (s, 1H, Aldehyde –CHO), 7.60-7.55 (m, 3H), 7.34-7.29 (m 2H), 7.13 (d, 1H, *J*= 9.0 Hz), 6.89 (d, 1H, *J*= 2.4 Hz), 6.87 (d, 1H, *J*= 2.4 Hz), 3.94 (s, 3H).

^{13}C NMR (75 MHz, $CDCl_3$, d) 188.2, 162.4, 161.8, 158.5, 156.8, 131.9, 130.6, 129.6, 128.6, 128.3, 115.8, 113.5, 113.2, 100.6, 56.1.

FTIR (neat, cm^{-1}): 1717, 1652,

HRMS (m/z): calc. for $C_{17}H_{12}O_4$, (M+H) 281.0816 observed 281.0814.




%Transmittance
Wavenumbers (cm-1)
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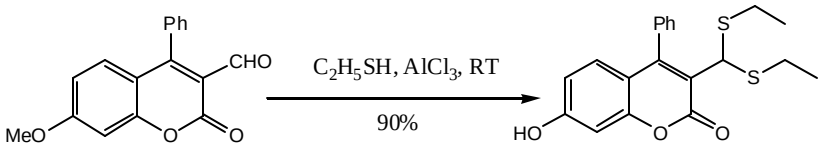
1362.88
1290.11



20 **21**

Compound 21- In a dried N_2 filled flask compound 20 (823.7 mg, 2.94 mM) was mixed with anhydrous aluminum chloride (1.18 g, 8.83 mM). Then ethane thiol (5 mL) was added into it. The reaction mixture was stirred in room temperature for overnight. Then 10% hydrochloric acid solution was added into the reaction mixture. The reaction mixture was extracted with dichloromethane (CH_2Cl_2) and finally dried over anhydrous sodium sulfate. The residue was purified via flash chromatography (5% ethyl acetate in dichloromethane). The product was isolated as yellow solid (986.3 mg, 96%).

Data from compound 21:

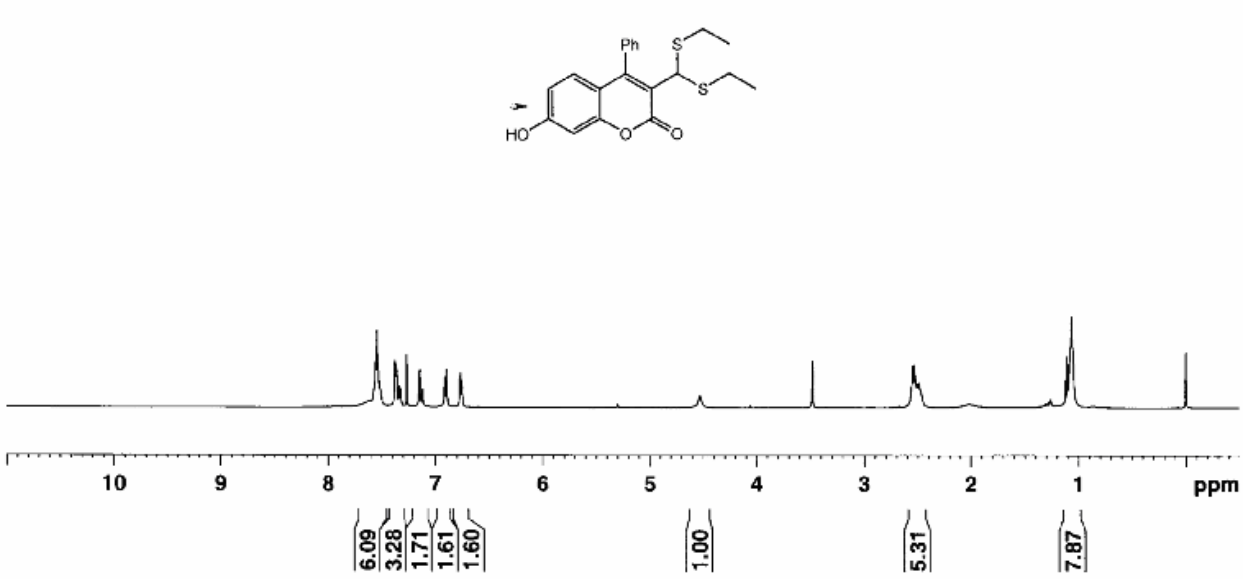
mp: 198-200 ^oC

^{1}H NMR (500 MHz, $CDCl_3$, d) 7.55-7.53 (m, 2H), 7.36 (d, 1H, *J*= 6.0 Hz), 7.0 (s, 1H), 6.89 (d, 1H, *J*= 8.5 Hz), 6.77-6.75 (m, 1H), 2.58-2.53 (q, 2H), 1.13-1.07 (q, 3H).

^{13}C NMR (125 MHz, $CDCl_3$, d) 159.2, 154.1, 133.8, 129.4, 129.2, 128.9, 128.6, 128.5, 128.4, 113.3, 113.1, 102.9, 96.0, 48.2, 27.3, 14.6.

FTIR (neat, cm^{-1}): 2964, 2921, 1692, 1612, 1551.

HRMS (m/z): calc. for $C_{20}H_{20}O_3S_2$ $(M+Na)^+$ 395.0777, observed 395.0742.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O
O
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9
8
7
6
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4
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2
1
ppm
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1.61
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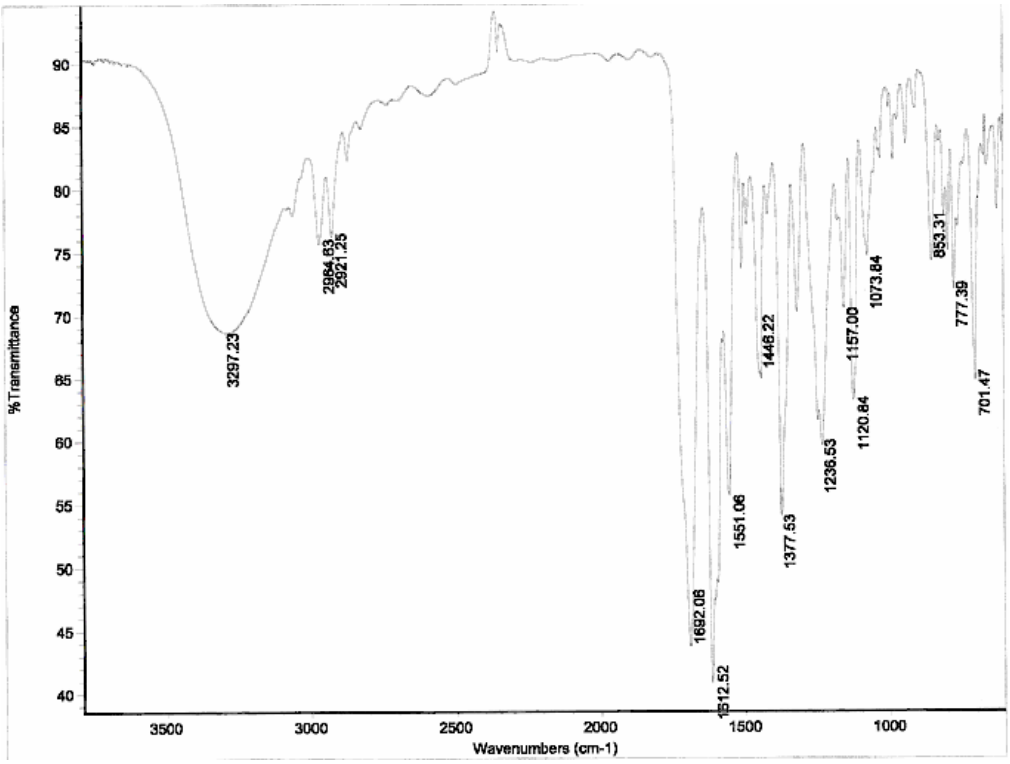
1.00
5.31
7.87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1377.53
1236.53
1157.00
1120.84
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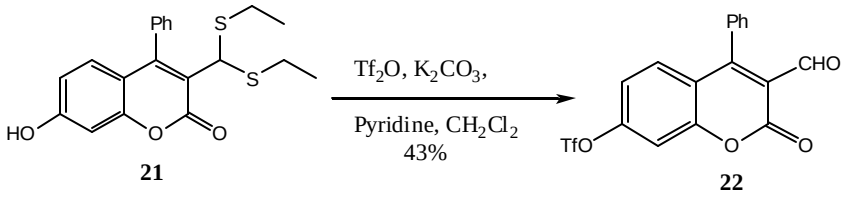
1500
1000
Wavenumbers (cm-1)



Compound 22- In a dried flask compound 21 (210 mg, 0.56 mM) was mixed with potassium carbonate (78 mg, 0.56 mM). The flask was put under vacuum for 30 min and slowly purged by nitrogen gas. Then dichloromethane (5 mL) was added. After 30 min, pyridine (125 µL, 1.58 mM) was added to the reaction mixture. Then the reaction mixture was stirred in the room temperature for one hour and then slowly trifluoro methane sulfonyl anhydride (397 µL, 1.41 mM) was added. The reaction mixture was stirred in the room temperature for 7 hours. The reaction mixture was extracted with sodium bicarbonate solution and dichloromethane and finally dried over anhydrous sodium sulfate. The residue was purified via flash chromatography (10% ethyl acetate in dichloromethane). The product was isolated as yellow solid (89.3 g, 43%).

Data from compound 22:

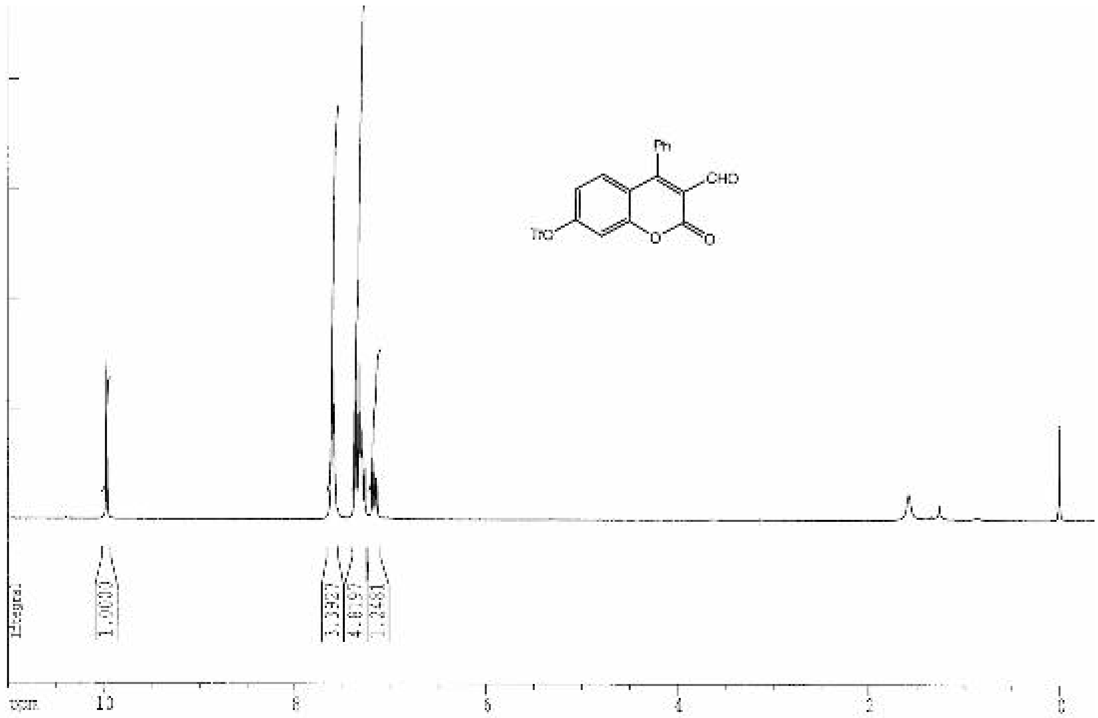
mp: 198-200 ^{o}C

^{1}H NMR (300 MHz, $CDCl_3$, d): 9.96 (s, 1H, Aldehyde –CHO), 7.64-7.55 (m, 3H), 7.33-7.26 (m, 3 H), 7.18-7.14 (dd, 1H, *J*= 2.3 Hz, *J*= 8.9 Hz).

^{13}C NMR (125 MHz, $CDCl_3$, d): 187.6, 159.3, 157.1, 155.0, 152.4, 131.3, 130.8, 130.3, 129.0, 128.3, 120.7, 119.8, 118.7(q, J_{CF} =189.1 Hz), 116.5, 110.5.

FTIR (neat, cm^{-1}): 3080, 2921, 2852, 1760, 1728, 1608, 1547, 1420.

HRMS (m/z): calc. for $C_{17}H_9F_3O_6S$ (M+H) 399.0132, observed 399.0116.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Integral
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6
4
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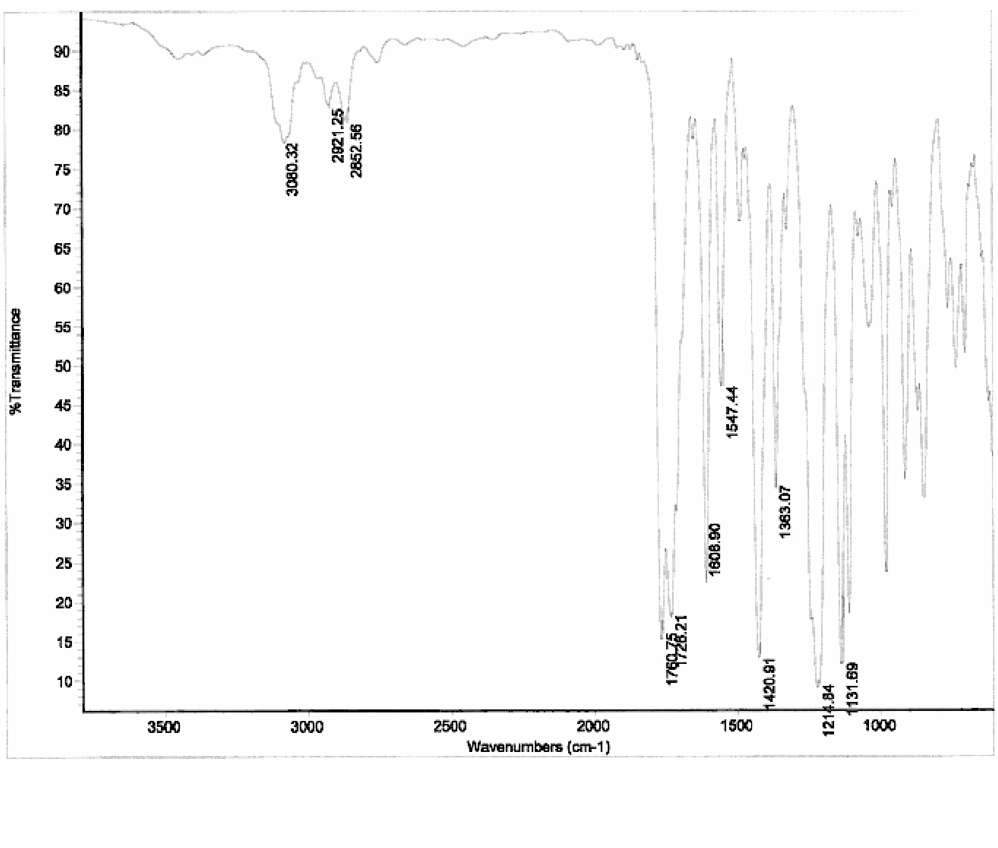
%Transmittance
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1131.69
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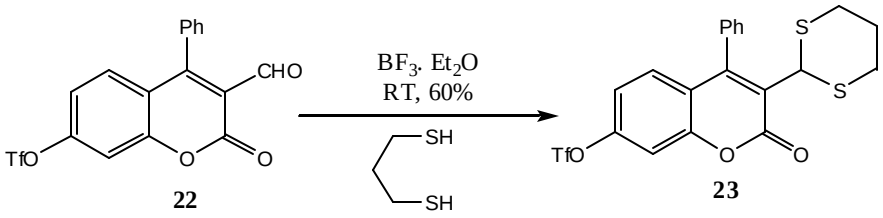
3000
2500
2000
1500
1000
Wavenumbers (cm-1)



Compound 23- Compound 22 (48 mg, 0.121 mM) was placed in a flame dry flask and put under vacuum for 30 min and slowly purged by nitrogen gas. With this starting material dry dichloromethane (2 mL) was added. Then boron trifluoride etherate (0.5 mL) was added with it. After 30 min 1, 3 propane dithiol (43 µL, 0.43 mM) was added in the reaction mixture. The reaction mixture was stirred for 12 hours at room temperature. The dichloromethane was removed in vacuo. The residue was purified via flash chromatography (8% ethyl acetate in hexane). The product was isolated as pale yellow solid (35.5 g, 60%). Data from compound 23:

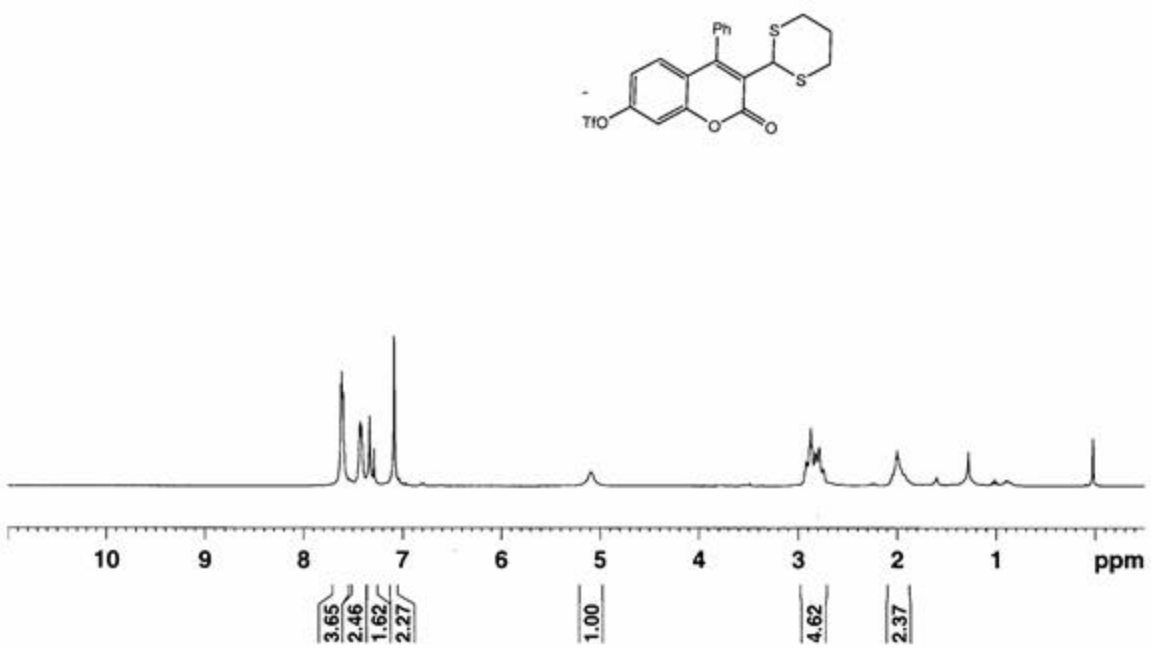
mp: cannot be determined

^{1}H NMR (300 MHz, $CDCl_3$, d) 7.62-7.60 (m, 3H), 7.53 (d, 2H, *J*=3.9 Hz), 7.43-7.42 (m, 2H), 7.33 (s, 1H), 7.06 (d, 2H, *J*= 1.2 Hz), 5.09 (s, 1H), 2.92-2.75 (m, 4H), 2.04-1.92 (m, 2H).

^{13}C NMR (75 MHz, $CDCl_3$, d) 158. 3, 153.5, 151.5, 150.7, 132.6, 129.9, 129.8, 128.8, 128.2, 125.2, 118.6, (q, J_{CF} =318.8 Hz), 120.5, 117.4, 110.0, 47.2, 32.1, 24.9.

FTIR (neat, cm^{-1}): 1721, 1604, 1558.

HRMS (m/z): calc. for $C_{20}H_{15}F_3O_5S_3$ $(M+H)^+$ 489.0128, observed 489.0112.


Ph
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TfO
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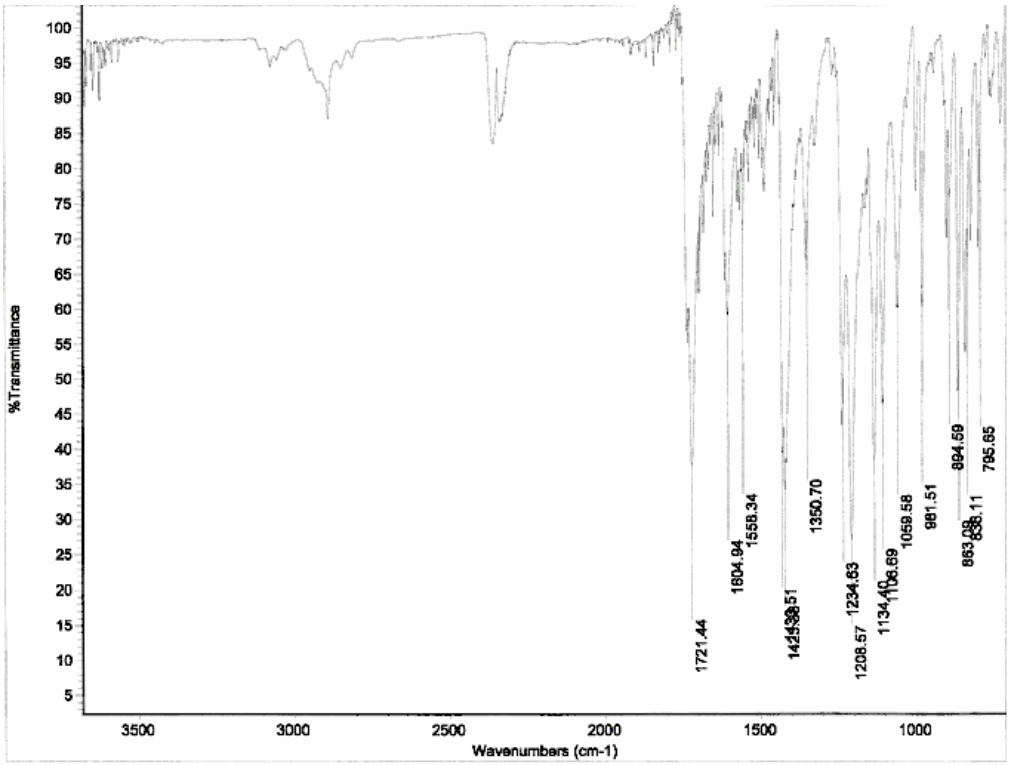
1.00
4.62
2.37


%Transmittance
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10
5
3500
3000
2500
2000
1500
1000
Wavenumbers (cm-1)
1721.44
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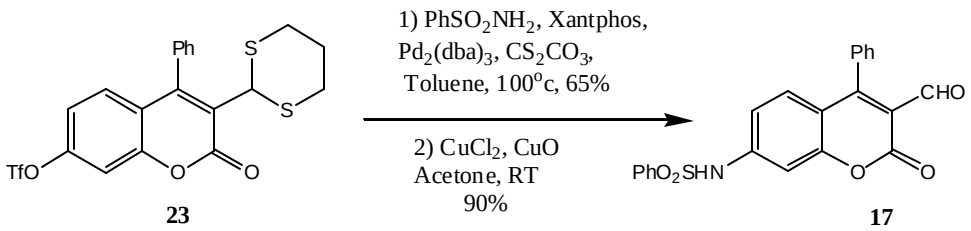
795.65



Compound 17- In a flame dried flask compound 23 (230.6 mg, 0.472 mM) was mixed with benzene sulphonamide (90 mg, 0.566 mM), cesium carbonate (385 mg, 1.18 mM), tris (dibenzyledeneacetone) dipalladium (11 mg, 0.0118 mM) and 4, 5-bis (diphenyl-phosphino)-9, 9-dimethyl xanthene (21 mg, 0.0354 mM). The flask was put under vacuum for 30 min and purge with N_2 gas slowly. Then dry distilled toluene (8 mL) was added with the residue. The reaction mixture was heated on 65 ^oC overnight. The toluene was removed in vacuo and dissolved in dichloromethane and washed with brine and finally dried over anhydrous sodium sulfate. This reaction mixture was purified by column chromatography (15 % ether in dichloromethane). The product was isolated as (152.1 mg) 65% and used immediately for the next step.

In a flame dried flask, the protected derivative coumarin (152.1 mg, 0.306 mM) was mixed with copper (II) chloride (4.95 mg, 0.0368 mM) and catalytic amount of copper oxide. Dry acetone (3 mL) was added to catalytic amount of dimethyl formamide was added slowly into it. The reaction was monitored in every 30 min with TLC. After 7 hours the reaction mixture was filtered and the solvent was removed in vacuo. The residue was purified via flash chromatography (15% ether dichloromethane). The product was isolated as amorphous solid pale yellow (111.6 mg, 90%).

Data from compound 17:

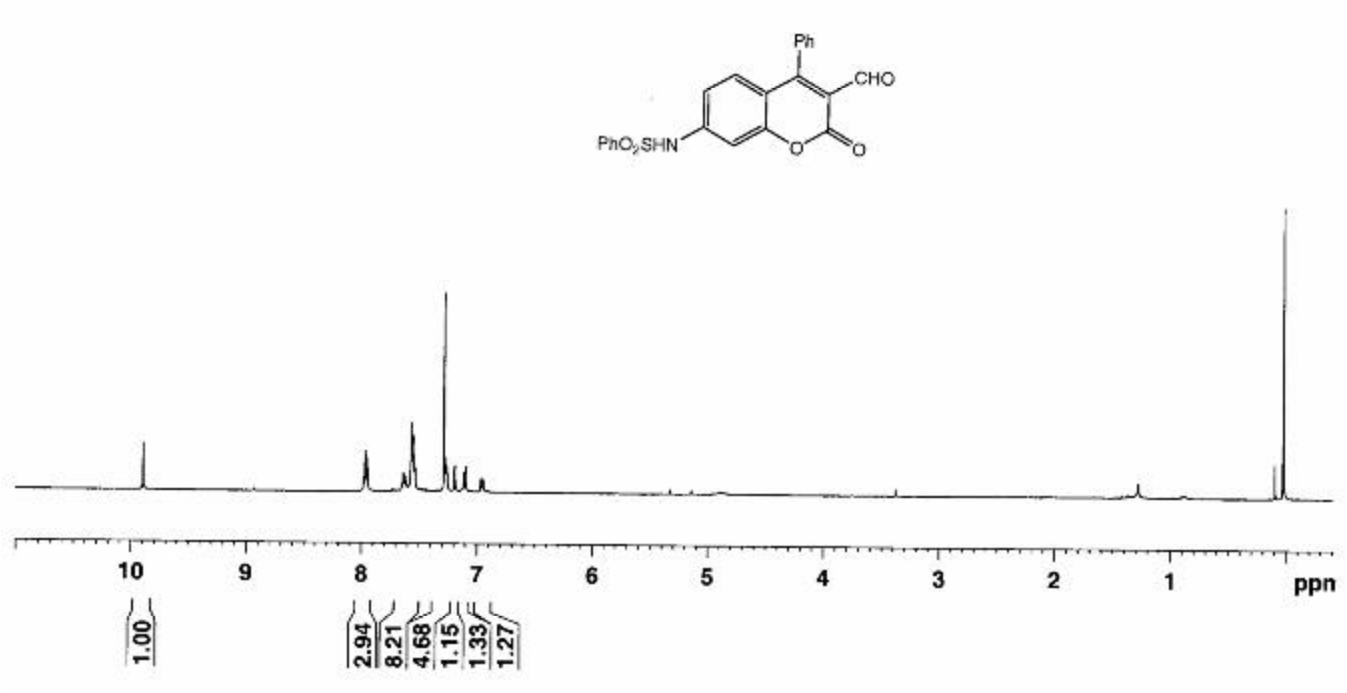
mp: cannot be determined

^{1}H NMR (500 MHz, $CDCl_3$, d) 9.92 (s, 1H, Aldehyde –CHO), 7.98-7.95 (m, 1H), 7.63-7.54 (m, 4H) 7.25 (m, 6H) 7.19 (d, 1H, *J*= 2.0 Hz), 7.16 (d, 1H, *J*=3.0 Hz) 6.96 (dd, 1H, *J*= 2.0 Hz, *J*= 9.0 Hz).

13C NMR (125 MHz, CD_6CO, d) 187.3, 159.7, 157.98, 155.6, 144.6, 139.7, 133.3, 132.5, 131.7, 130.5, 129.3, 128.7, 128.3, 127.1, 125.9, 117.1, 115.2, 104.6.

FTIR (neat, cm^{-1}): 3248 2928, 2853, 1735, 1615, 1532, 1321.

HRMS (m/z): calc. for $C_{22}H_{15}NO_5S$ (M+H) 406.0760 and observed was 406.0749.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9
8
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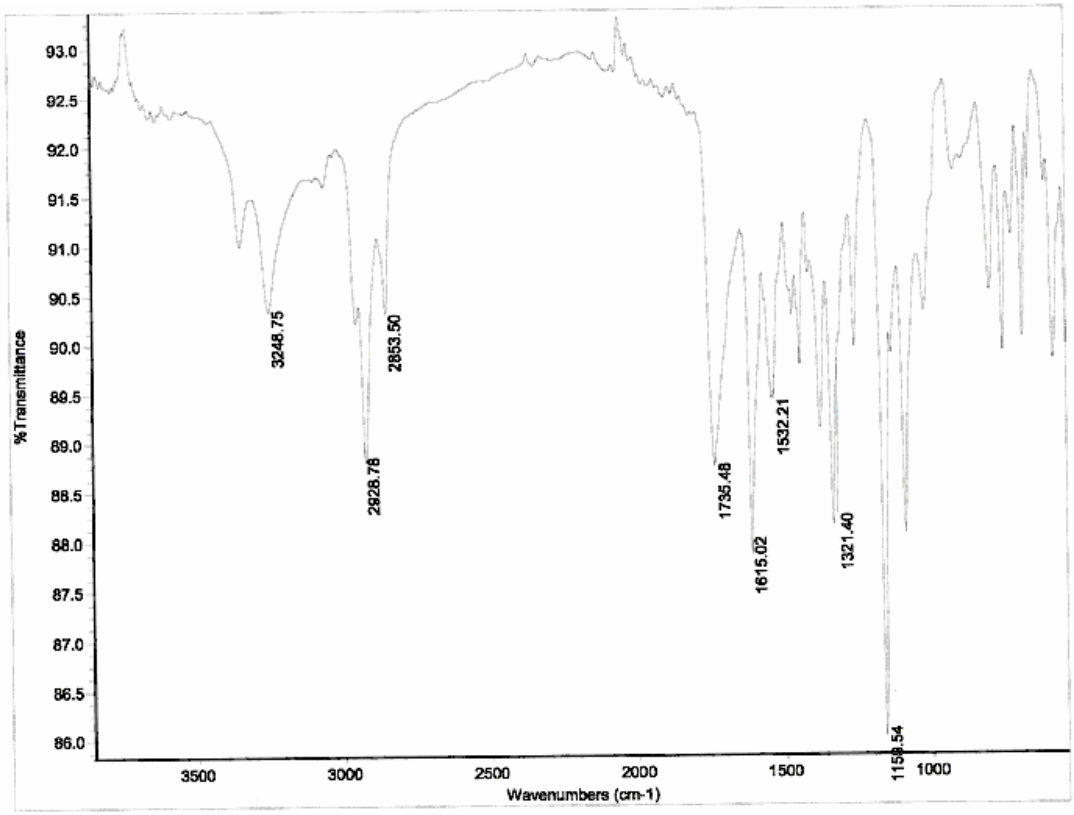
1.15
1.33
1.27


%Transmittance
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86.0
3248.75
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1615.02
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1321.40
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1500
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Wavenumbers (cm-1)

Reference:

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