Development of a pH sensitive coumarin based chemosensor

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By

DANIEL MUBIMA MILANG

Advisor

Dr. TIMOTHY E. GLASS

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

Development of a pH sensitive coumarin based chemosensor

Presented by Daniel Milang Mubima,

A candidate for the degree of Master of Science,

and hereby certify that, in their opinion, it is worthy of acceptance.

Professor Timothy E. Glass
Associate Professor of Chemistry
Department of Chemistry
Thesis Advisor

Professor Kent S. Gates
Professor of Chemistry
Department of Chemistry
Committee member

Professor Susan Z. Lever
Associate Professor of Chemistry
Department of Chemistry
Committee member

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It would be unthinkable to for me to have done this work without acknowledging the constellation of individuals who have made it possible.

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ABSTRACT

Fluorescent probes have found several uses in monitoring the physiological state of cells. Recently, the Glass lab has developed a fluorescent sensor capable of binding norepinephrine in chromaffin cell vesicles. This sensor is based on coumarin aldehyde compounds which have been shown to reversibly bind primary amines such as those found in neurotransmitters. The synthesis and evaluation of a coumarin based pH sensitive fluorescent chemosensor is described. The sensor would overcome two major challenges caused by the previous catecholamine sensor. The first is in the rate of binding of the sensor to the catecholamine; a slow rate of binding makes synaptic measurements difficult. To circumvent this impediment, a pH sensitive sensor is proposed since, it remains off upon binding to the neurotransmitter in the vesicle (pH=5.5). When the cell is stimulated and the neurotransmitters released into the synapse (pH=7.4) the sensor will fluoresce. The second challenge that is overcome is the lengthy synthesis of the coumain sensor. A short synthetic route is designed.

CHAPTER 1

FLUORESCENT CHEMOSENSORS

INTRODUCTION

1.1 OVERVIEW

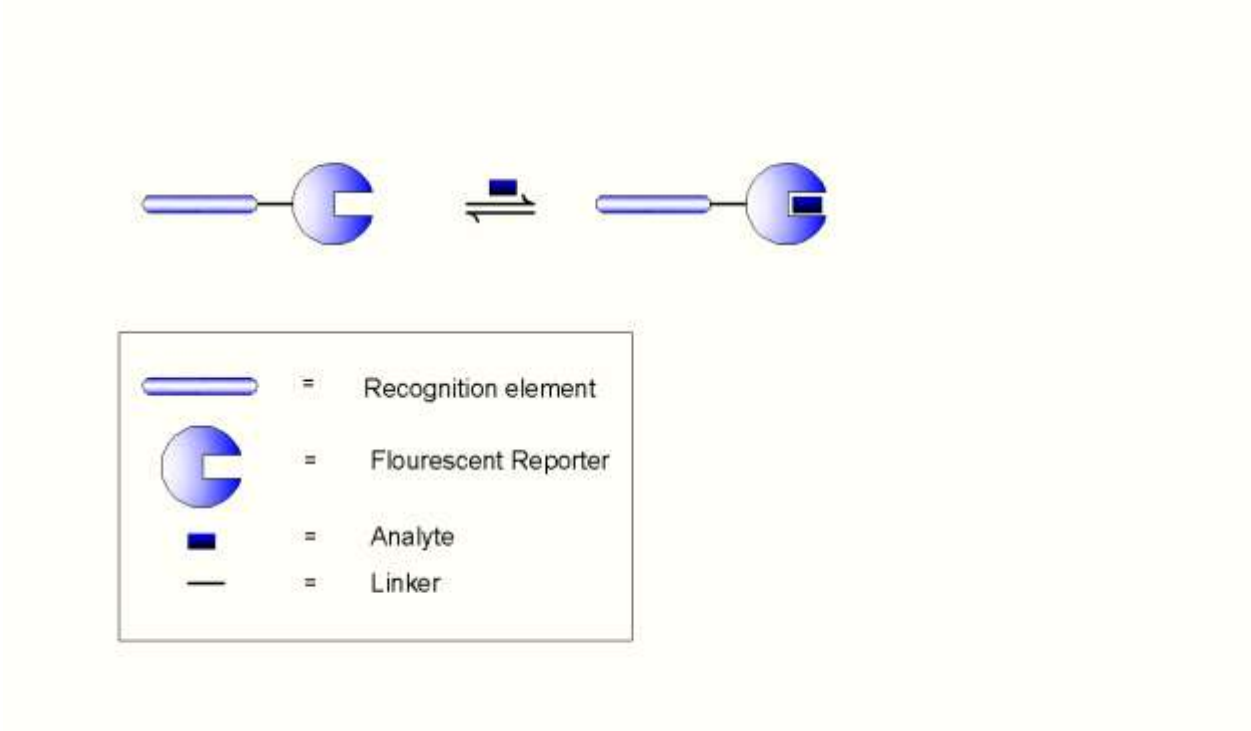
The study of molecular interactions can provide useful understanding of biological systems. This fact has led to an interest in chemical sensing and the development of small molecules that allow the probing of cell activity. Molecular recognition is a focal point in chemical sensing. Molecular recognition is described as a noncovalent interaction between a host molecule and a guest molecule. The noncovalent interactions could be Van der Waals forces, hydrogen bonding, dipole-dipole interactions, π-stacking, the hydrophobic effect, or metal coordination. These interactions govern many vital processes; from the self-associations of phospholipids into cellular membranes which creates the barrier between the cell and the external environment, to the complimentary base pairing of DNA, which stores genetic information necessary for life. The principle of molecular recognition is also applied in the design of chemical sensors.

A chemical sensor is a chemical entity that gives a visible response to the presence of a target substrate (analyte). Generally, sensors can be made up from different materials, ranging from proteins and enzymes to polymers to small organic molecules. Sensors derived from small organic molecules are referred to as chemosensors. A chemosensor can be defined as a molecule of abiotic origin that is able to bind selectively and reversibly, to a target analyte resulting in change in one of the properties of the system.[1]

A fluorescent chemosensor usually consists of a series of components: recognition element, a fluorescent reporter, and a linker betweent the two. The basic design of a chemosensor is outlined in Figure 1. A recognition element is directly connected to the recognition reporter via a linker, and when the target analyte is bound , a change in fluorescence is observed. The binding event is reversible, which allows continuous

monitoring of the analyte concentration.There are three factors to consider in the design of a chemosensor for a particular analyte, which are affinity, selectivity and readout. A chemosensor must have a dissociation constant K_d that matches the concentration of the analyte being detected; this allows minor changes in analyte concentration to be detected. The isssue of selectivity is crucial in that the chemosensor must be designed so as to bind only with the desired analyte and not other competitors. The choice of readout is contigent on both the application of the chemosensor and the availability of instrumentation. Possible signaling methods include fluorescent spectroscopy,which is preferred due to its sensitivity, allowing low concentrations of the analyte to produce large changes in output.

Figure 1: Schematic depiction of the components of a chemical sensing system

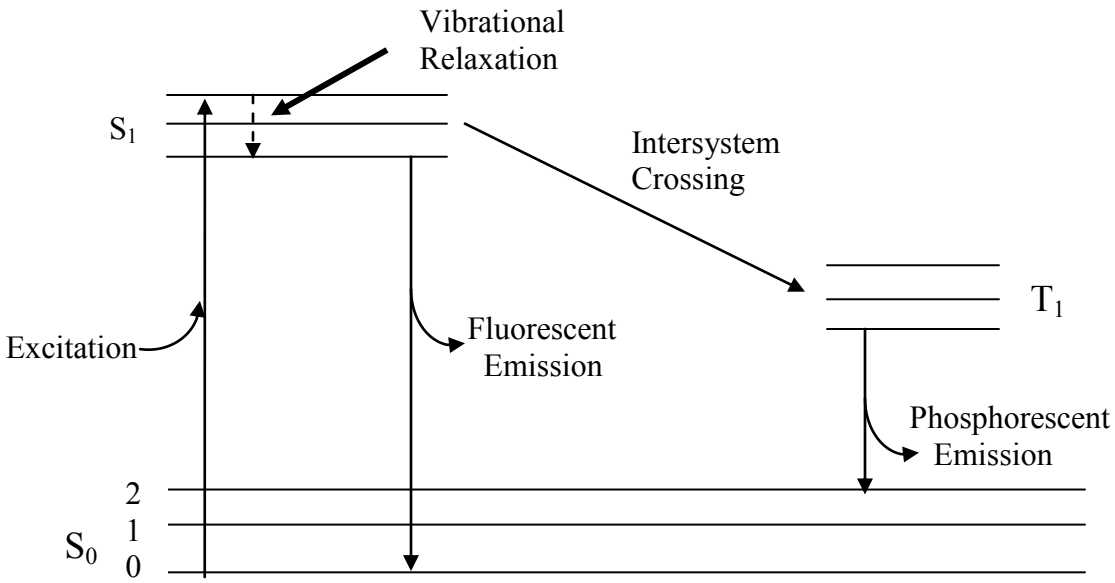


1.2 FLUORESCENT SIGNALING FOR CHEMOSENSORS

1.2.1 BASICS OF FLUORESCENCE

Fluorescence is a process whereby a molecule absorbs a photon of light at a particular wavelength and emits a photon of a different, longer wavlength. The difference between the wavelengths is termed Stokes shift.

Figure 2: Jablonski Diagram.

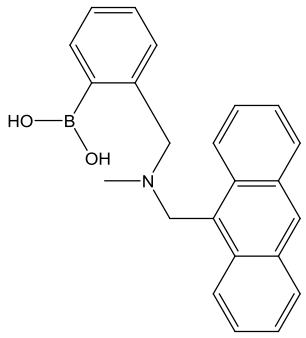


When a fluorophore is irradiated, a photon of light promotes an electron from the electronic ground state (S_o) to an excited state (S_1).Overlaying the excited states (S_o) and (S_1) are vibrational states 0,1,2, etc. When an electron absorbs a photon of energy, it is excited from its ground electronic state to an excited vibrational energy level. From this states the electron can decay thermally to the ground vibrational state (S_1) resulting in the emission of fluorescent radiation, this in turn has half life of about 10^{-8}s. An electron can also decay to a triplet energy state, T_1, by the process of intersystem crossing, the decay from the triplet state T_1 is termed phosphorescence this occurs at around 10^{-3}s.

The wavelength of radiation, and the intensity of the fluorescent emission are highly dependent on the structure of the fluorophore. Compounds that are conjugated tend to have higher excitation and emission wavelengths.

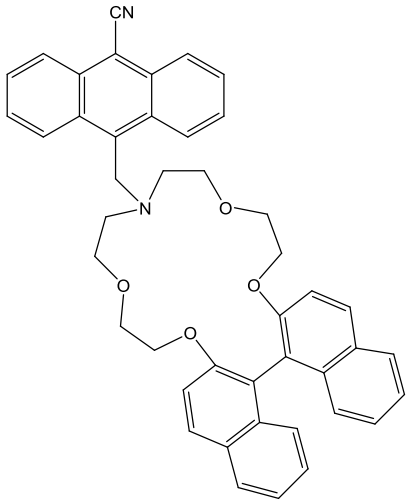
1.2.2 FLUORESCENT MODULATION

They are several mechanisms by which fluorescent radiation is modulated. This modulation can lead to events whereby the intensity of the fluorescence can either decrease or increase.



1

CHEQ (Chelation Enhanced Quenching) is a mechanism by which some chemosensors exhibit fluorescence quenching upon binding their target analyte. Compound **1**[2] shows a a large fluorescent decrease upon complexation with dopamine, epinephrine and catechol. The boronic acid functional group reacts with the 1,2 diols of the catecholamine guests, and forms a boronic acid ester. Although the interaction formed is covalent, the process is highly reversible in aqueous solution, allowing for real time detection of catechol guests. CHEQ is not optimal in the design of chemosensor due to the presence of several non-analyte based mechanisms by which fluorescence could decrease.



2

CHEF (Chelation Enhanced Flouresence) is the converse of CHEQ, it is a mechanism by which a chemosensor exhibits an increase in fluorescence upon binding to an analyte. Compound **2**[3] forms a complex with Hg^{+2} and Zn^{+2} that lead to increase in fluorescence. This increase is attributed to the blocking of the PET (Photoinduced Electron Transfer) mechanism from the nitrogen lone pair to the anthracene fluorophore.

1.2.3 CHEMOSENSOR FOR CATECHOLAMINES

The development and use of fluorescent chemosensors has increased over the recent years. Chemosensors play an important role in not only the elucidation of cellular mechanisms through molecular recognition but also in the diagnosis of several degenerate diseases that are linked to the deficiency of certain biomolecules.[4] The ability of chemosensors to bind to neurotransmitters, in particular catecholamines, have been of interest. Catecholamines, including dopamine are an important class of neurotransmitters that are involved in a variety of central nervous system functions. Malfunction of dopamine responsive neurons has been implicated in a number of disease states including Parkinson's disease, sparking the development of diagnostic systems.[5] Currently, there are no blood or lab test that have been used to help diagnose Parkinson's disease, therefore, diagnosis is based on medical history and neurological examination. The disease can be difficult to diagnose directly, with 75% of clinical diagnoses of Parkinson's disease confirmed after autopsy.[6] Early symptoms are sometimes dismissed as effects of normal aging. Usually, doctors look for shuffling of feet and lack of swinging of the arms.

Direct dopamine detection has been accomplished using electrochemical techniques due the favorable redox properties of the catechol.[7] This method has proven to be destructive. However, to complement this method, a fluorescent chemosensor for dopamine could contribute to the enhanced understanding of the role of dopamine in neurons and could potentially aid in understanding Parkinson's disease.

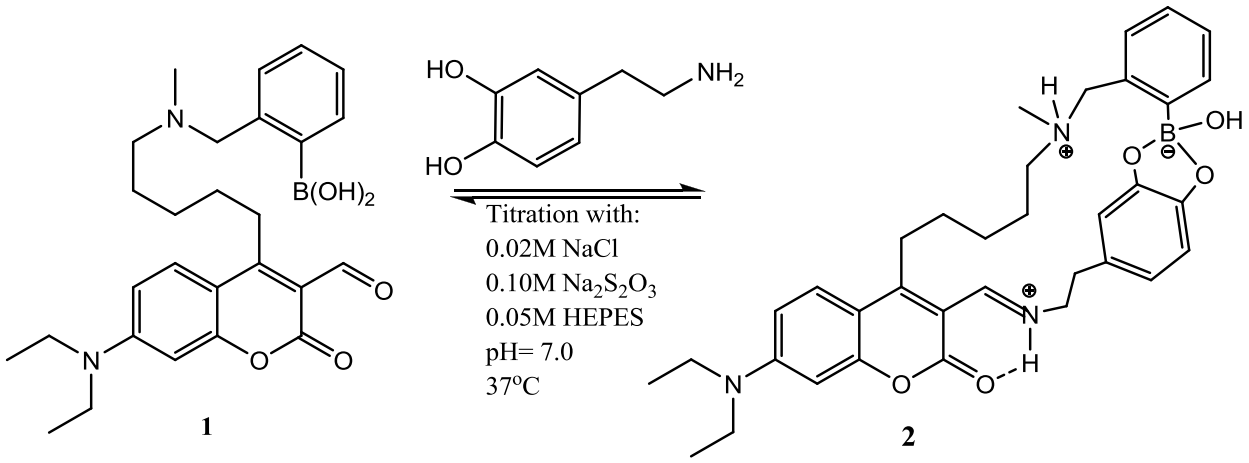
CHAPTER 2

COUMARIN- BASED CHEMOSENSORS

2.1 USE OF COUMARINS AS CHEMOSENSORS

The Glass group has developed sensor **2**[8] that binds to primary catecholamines and has good affinity and selectivity for dopamine.

Scheme 1: Binding of dopamine by Coumarin Sensor **1**



As shown Scheme 1, the sensor consists of a coumarin fluorophore, a tertiary amine linker and a boronic acid group. Upon binding to a catecholamine in this case dopamine, the sensor forms an iminium ion with the amine part of the dopamine, as well a boronate ester with catechol part. One other salient feature is the presence of an internal H-bond which produces a colorimetric response to the analyte binding and gives suitable selectivity for the required catecholamine. The fluorescence of sensor **2** was quenched upon binding dopamine, giving an overall decrease in emission when it was excited at 484nm. The fluorescent quenching effect was found to be directly related to the catechol group. This is due to the fact that the electronrich catechol is likely to act as a photoinduced electron transfer (PET) quencher of the coumarin under the given conditions.

2.2 SIGNIFICANCE OF DETECTING NEUROTRANSMITTERS

Figure 3: Schematic depiction of synapse

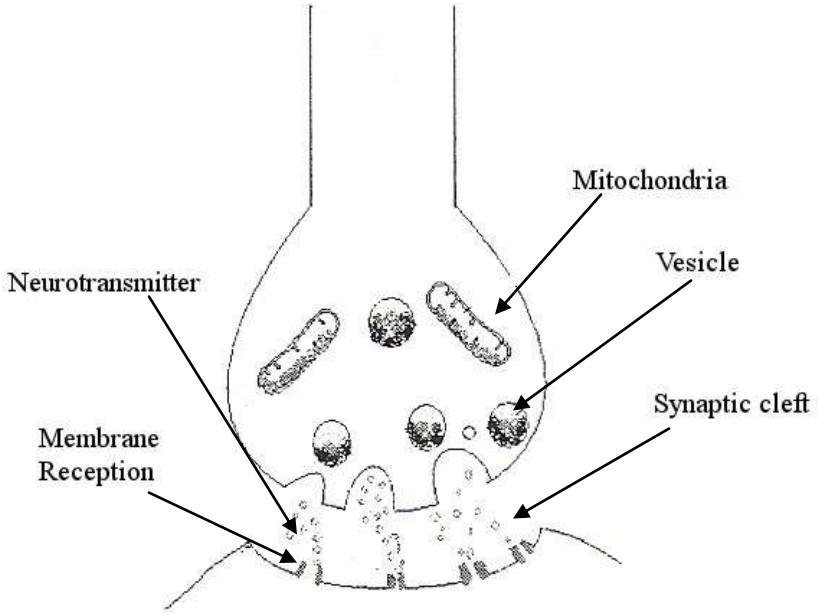
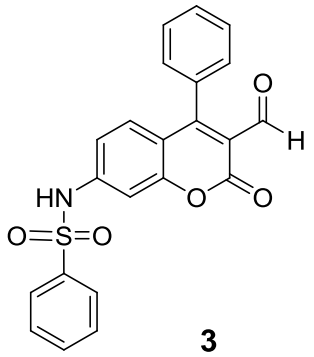


Figure 3 depicts the basic function and contents of a nerve cell. The release of a neurotransmitter is triggered by the arrival of a nerve impulse or action potential. This action potential produces an influx of calcium ions through the voltage-dependent, calcium-selective ion channels at the down stroke of the action potential.[9] The calcium ions then trigger a biochemical cascade which result in the vesicles fusing with the presynaptic membrane and releasing their contents to the synaptic cleft within 180 microseconds of calcium.[10] The neurotransmitter is either reabsorbed by the presynaptic cell, and then repackaged for future release, or else it is broken down metabolically.

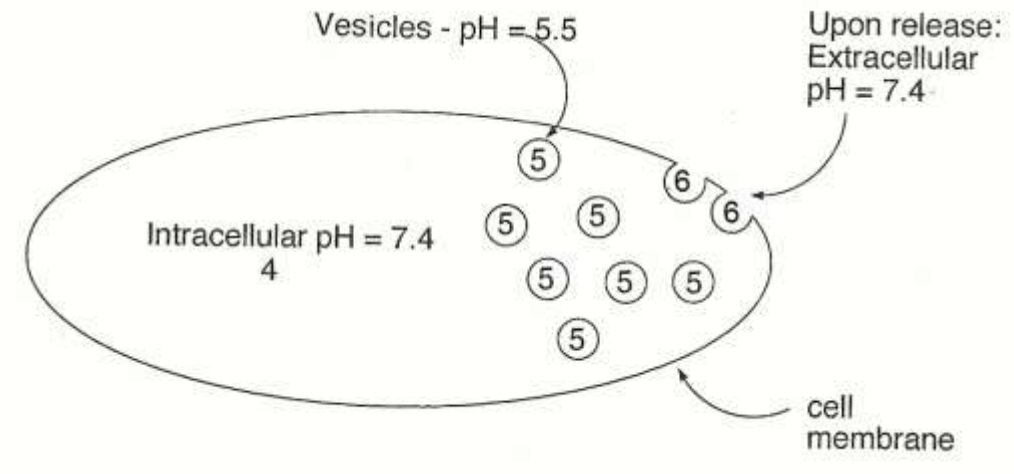
2.3 DESIGN AND MODE OF ACTION OF A pH SENSITIVE CHEMOSENSOR



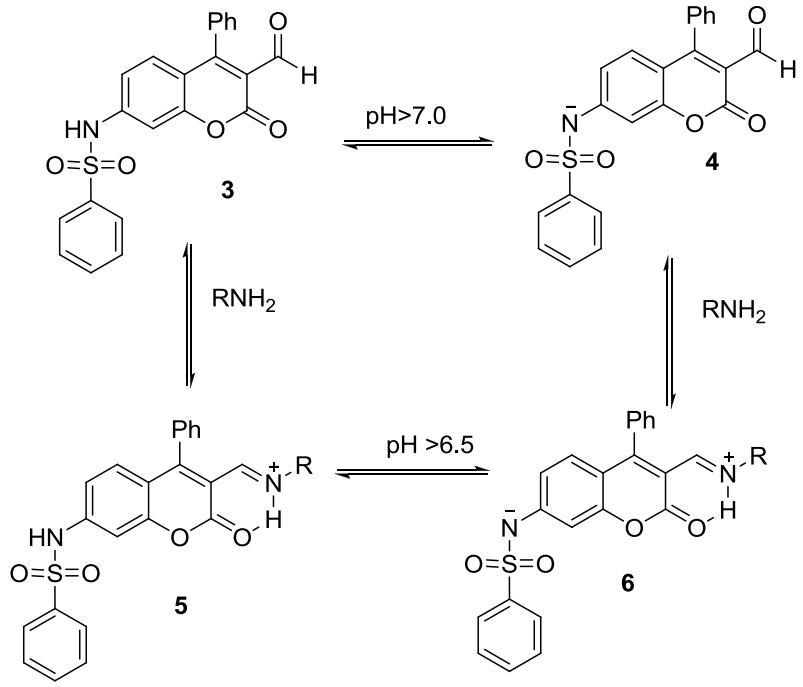
3

Fluorophore **3** is proposed for use as a pH sensitive sensor. It consists of a coumarin aldehyde attached to a phenyl ring and a sulfanilamide group as the pH dependant portion of the molecule. The mode of action of the molecule is as shown in Figure 4 and Scheme 2:

Figure 4: States of compound **3** in a cell

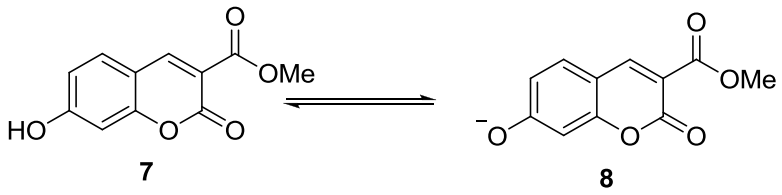


Scheme 2: Four different states of fluorophore **3**



Our goal 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 the vesicles are acidic (pH=5.5), but the synapse is neutral (pH=7.4). As the intracellular pH generally varies between 6.8-7.4 in the cytosol, and between 4.5-6.0 in the cell's acidic organelles, the sensor will be "off" in the vesicle, but "on" in the synapse (see Figure 4). The mode of action of the fluorophore is based on the fact that as the cell permeable sensor enters the cell cytosol where the pH is near neutral, it becomes deprotonated at the sulfanilamide position and the molecule exists as form **4.** As this fluorophore makes it entrance in the acidic cell vesicles pH= 5.5, it gets protonated and binds with the amine part of the catecholamine and exists as form 5**.** As the fluorophore is released into the synapse, where the pH is around 7.4 it gets deprotonated and exists as the fluorescent

fluorophore **6.** This deprotonated form is highly fluorescent due to the various absorption changes it undergoes. Coumarins can be made pH sensitive by the use of a 7- hydroxyl group.[11] The hydroxyl group can be deprotonated at basic pH. In the protonated form of molecule **7**, the major absorption is in the UV region due to a $\pi \rightarrow \pi^*$ transition. Conversely, in the deprotonated form of molecule **8** an $n \rightarrow \pi^*$ transition is possible; this gives rise to visible absorbance. Thus when exciting with visible light, only the deprotonated form will fluoresce.

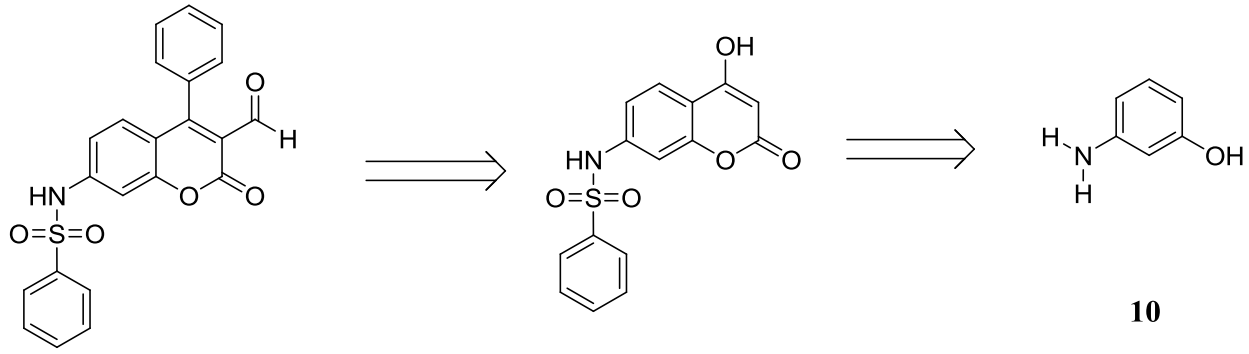


$\pi \rightarrow \pi^*$ $n \rightarrow \pi^*$

In order to take advantage of the changes in pH, we designed fluorophore **3** based on this principle. The only difference is the replacement of the hydroxyl group by a sulfanilamide group, which will similarly undergo deprotonation at the synapse and will be protonated inside the acidic vesicles but with the proper pKa.

2.4 SYNTHESIS OF COUMARIN CHEMOSENSOR

Scheme 3: Retrosynthetic approach for Coumarin **3**

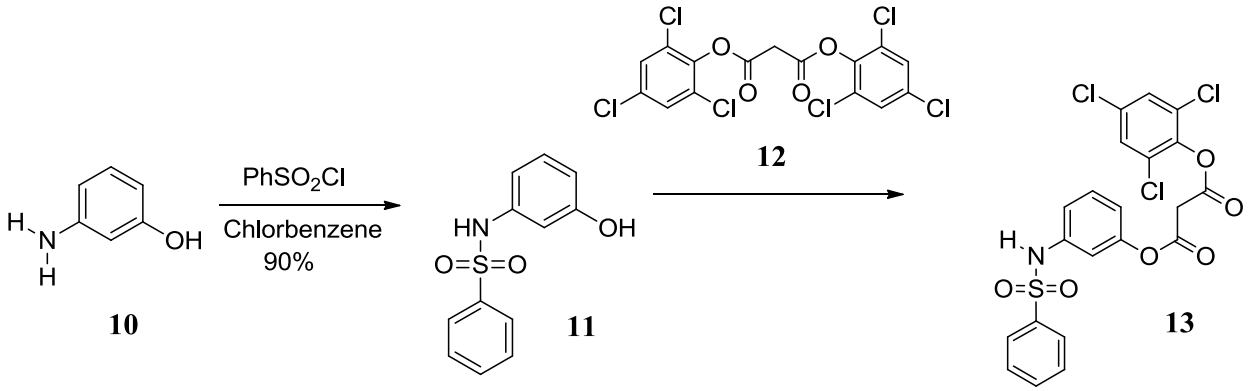


3 **9** **10**

The synthetic approach for Chemosensor **3** is described under (Scheme 3). Compound **3** can be made from **9** by formylation under Vilsmeyer conditions and by Palladium catalyzed cross-coupling reaction. Compound **9** could be made from commercially available compound **10** by formation of the sulfonamide followed by a Friedel-Crafts type cyclization reaction.

With this in mind, we set forth to accomplish the synthesis of our pH sensitive chemosensor.

Scheme 4: Attempt to Synthesize Coumarin **11**



Reaction of compound **10** with benzenesulfonylchloride gave compound **11** in high yield (Scheme 4). Cyclization of the alcohol **11** to give the key intermediate for the synthesis, coumarin **9**, was unsuccessful upon treatment with tricholorphenyl malonate **12**, in refluxing toulene. This reaction yielded compound **13** which suggests that the Friedel Crafts-type cylization that we expected did not occur. Similar results were observed with xylene, a similar yet higher boiling solvent. This reaction works well with an amine substituent , thus the sulfonamide group mush be too electron poor.

Spured by these results, we then sought to investigate various other ways of synthesizing coumarins. Cyclization reaction using a propiolic derivative was particularly appealing. This is due to the fact that upon cyclization, the phenyl groups ends up in the 4-position of the coumarin. This reduces the step count of the synthesis.

Scheme 5: Attempts at cylicization

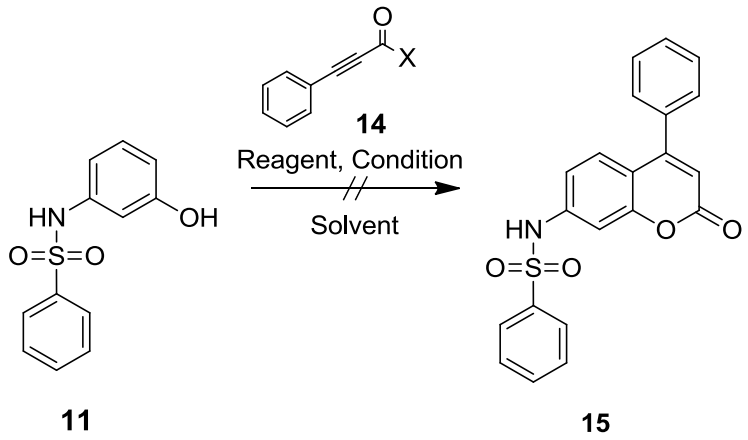
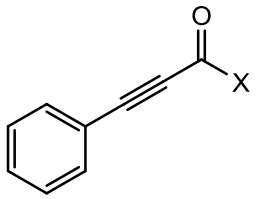


Table 1: Results on the attempts for the synthesis of Coumarin **15**

Reagents	**Conditions**	**Results**
16	$ZnCl_2$,Chlorobenzene	No Reaction
16	K-10, Nitrobenzene, MW	SM Decomposed
17	K-10, DCM, MW	No Reaction
18	K-10, Nitrobenzene, MW	SM Decomposed



16 X = OEt; **17** X =OH

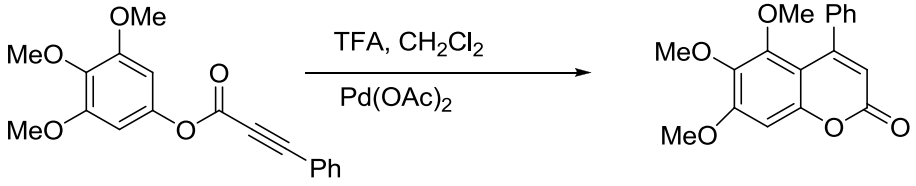
18 X = Cl

The first attempt to synthesize coumarin **15** was the condensation reaction [12] of phenol **11** with the ester of phenypropiolic acid **16** in the presence of anhydrous zinc chloride. This procedure failed to produce the desired coumarin, and the starting material was recovered. The use of the ecofriendly solid-acid catalyst montmorillonite K-10 was then explored.[13] Different derivatives of phenylpropiolic acid, including the ester **16** and the acid chloride **18** were used in microwave conditions. In the presence of

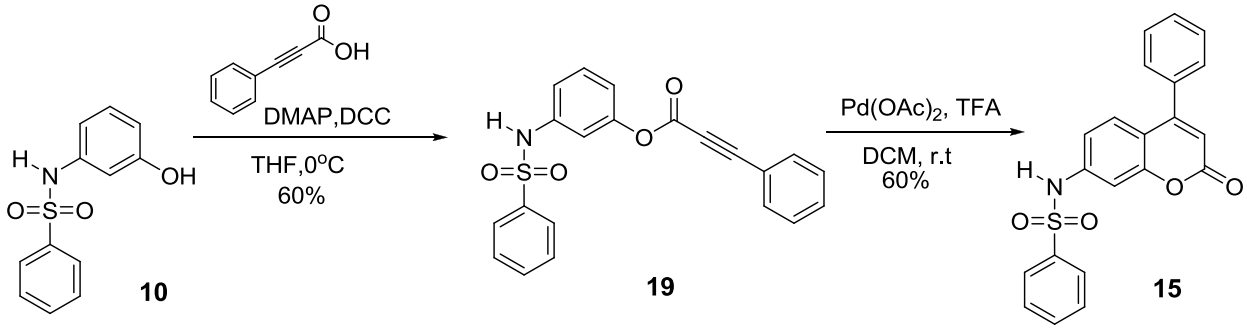
dichloromethane, a lower boiling point solvent, no reaction was observed, whilst in the presence of nitrobenzene the starting material decomposed and product characterization was not not possible.

The synthesis of the key intermediate coumarin **15** proved very elusive. We then shifted our attention to recently published studies involving palladium-catalyzed reactions of phenols with propiolic esters.[14,15] These involve an intramolecular hydroarylation of the C-C triple bond in the presence of TFA as highlighted in Scheme 6. In light of this, we sought to apply this method to synthesize coumarin **15.**

Scheme 6: Intramolecular palladium-catalyzed formation of a coumarin



Scheme 7: Synthesis of Coumarin **15**

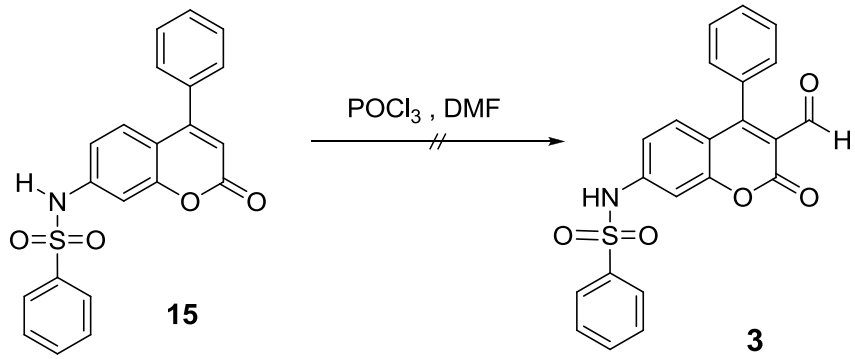


The DCC coupling reaction involving phenol **10**, phenylpropiolic acid, and DMAP was successful and afforded the phenyl propiolic ester **19** in modest yield.[16] Intramolecular

palladium catalyzed cyclization of this ester yielded the key intermediate for the synthesis, coumarin **15.**

With the key intermediate of the synthesis made, our attention turned to formylating the molecule. Different Vilsmeyer conditions (Scheme 8) were used, 0^{o}C, room temperature and 50^{o}C. The reason for the unsuccessful formylation of compound **15**, is due to the weak electron donating ability of the sulfanilamide, which renders the coumarin system into a weak nucleophile, unable to initiate an attack on the Vilsmeyer reagent.

Scheme 8: Formylation under Vilsmeyer conditions



Another interesting way of formylating was the use a lithium halogen exchange and subsequent trapping with DMF . Bromination of the compound **15** gave the brominated compound **20** in high yield. (Scheme 9)

Scheme 9: Lithium halogen exchange and trapping with DMF

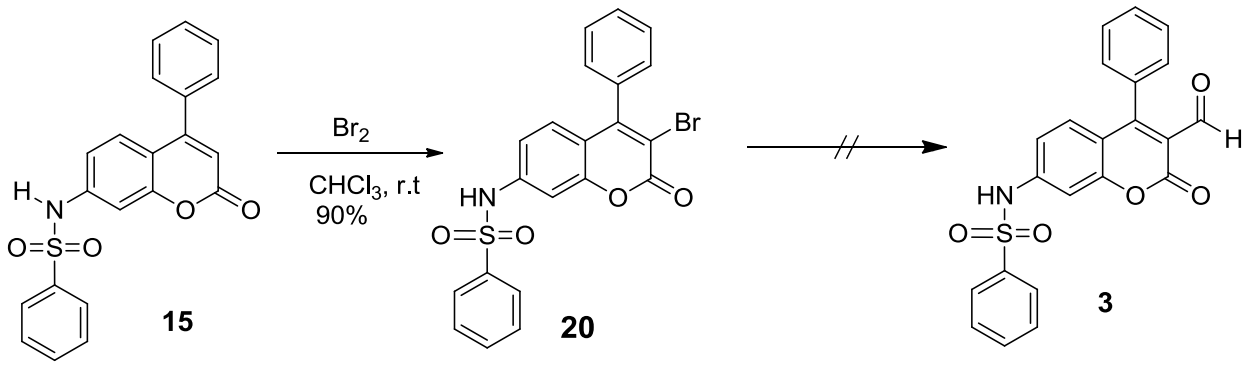


Table 2: Conditions and Results on formylations attempts on compound **20**

Reagents, conditions	**Results**
n-BuLi, DMF	No reaction
NaH, TMEDA, n-BuLi, DMF	No reaction
t-BuLi, DMF	Starting material decomposed

Attempts to formylate compound **20**, are shown in (Table 2). The use of n-BuLi and subsequent trapping with DMF, failed to give the desired results. Pretreatment with NaH, TMEDA, deprotonation with n-BuLi and trapping with DMF yielded no reaction. Decomposition of the starting material was observed with the use of t-BuLi and DMF. The use of palladium carbonylation reaction was then explored, involving the use of both mondentate and bidentate ligands. However, no reaction was observed in any of these attempts.

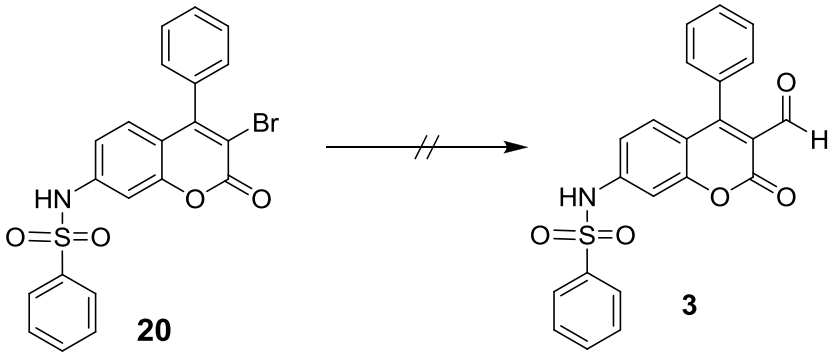


Table 3: Conditions and Results on palladium carbonylation on compound **20**

Reagents, Conditions	**Results**
$Pd(OAc)_2$, PPh_3, K_2CO_3, 50 psi CO, 50°C, THF	No reaction
$Pd(PPh_3)_4$, CO, Bu_3SnH, 50 psi CO, 50°C, THF	No reaction
Pd(dppf), mesitylene, Et_3SiH, Na_2CO_3, 50 psi CO, 90°C , DMF	No reaction

2.5 CONCLUSIONS

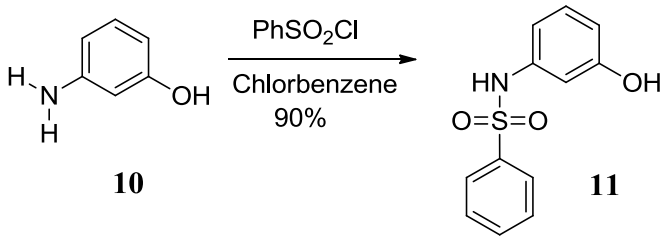
In conclusion, we were unable to the synthesis of the target coumarin **3**. Despite the fact that we explored several ways of formylating the 3-position. The presence of the sulfanilamide group, which is weakly deactivating, disfavors the molecule from being nucleophilic and initiating attack on the Vilsmeyer reagent. The use of metal-halogen exchange and trapping with DMF was also explored; we believe that deprotonation of the sulfanilamide group and exchange of halogen and metal were successful but the trapping of DMF was not successful due to the formation of the dianion. In the same light, palladium carbonylation was unsuccessful due the steric problem at the 4 position of the coumarin.

However, we were able to synthesize a key intermediate, (compound **15**) in 3 steps with moderately high yields, this compound could serve as a key intermediate for several coumarins.

APPENDIX

EXPERIMENTAL PROCEDURES

General Synthetic Procedures. All reactions were carried out in dried glassware under nitrogen atmosphere unless otherwise noted. Tetrahydrofuran (THF) and diethyl ether (Et_2O) were distilled from sodium benzophenone under N_2 atmosphere before use. Benzene (PhH) and toluene (PhMe) were distilled from 9-fluorenone and sodium under N_2 atmosphere. Methylene Chloride (CH_2Cl_2) was distilled from CaH_2 under N_2 atmosphere. NMR spectra were recorded on a Brunker DRX-250, DRX-300, and DRX-500 using TMS as a reference unless otherwise noted.



10 **11**

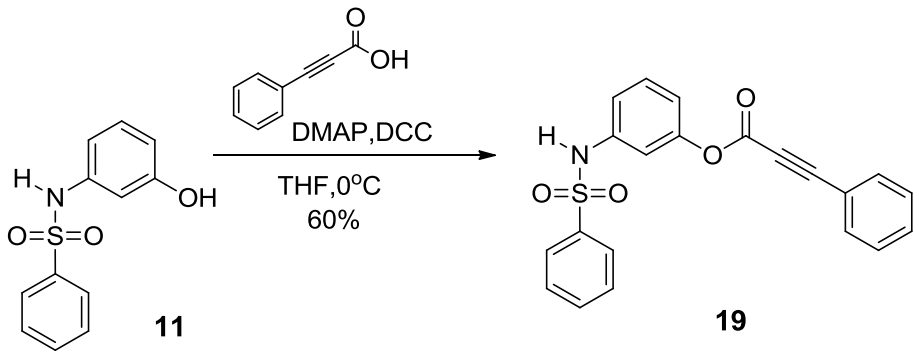
Compound 10:

In a flame dried N_2-filled flask**,** 3-aminophenol (3g, 27.6 mmol) was dissolved in chlorobenzene (100 ml) and heated at 130^{o}C. Upon dissolution, benzene sulfonyl chloride (52 ml, 41.2 mmol), was added dropwise to the heated solution. The reaction mixture was heated for 3 hours, and allowed to cool. The solution was filtered; the resultant filtrate was allowed to sit at room temperature overnight. Compound **10** crystallized from this solution and was filtered. The resultant crystals were dried. The title compound **10** was obtained as a light-grey solid (2.71g, 10.8 mmol, 90% yield).

Data for compound **10:**

^{1}H NMR (300 MHz, DMSO): δ 10.2 (s, 1H), 9.5 (s, 1H), 7.73 (dt, *J* = 6.7, 1.7 Hz, 2H), 7.52 – 7.64 (m, 3H), 6.97 (t, *J* = 8.1Hz, 1H), 6.57 (t, *J* = 2.1 Hz, 1H), 6.51 (dd, *J* = 8.0, 2.1Hz, 1H), 6.39 (dd, *J* = 7.8, 1.9 Hz, 1H).

LRMS: Calculated for C_{12} $H_{11}NO_3S$ ($M+H^+$) 249, found 249



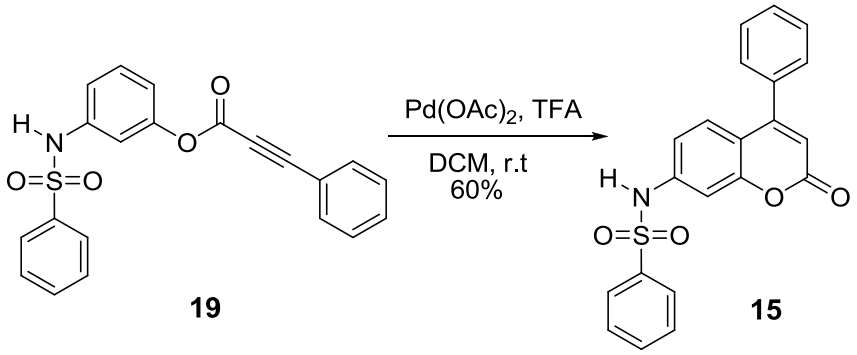
Compound 19:

In a flame dried N_2-filled flask, compound **10** (2.00g, 8.03 mmol) was dissolved in 2ml THF. Phenylpropiolic acid (1.168g, 7.99 mmol), DMAP (0.096g, 0.78 mmol), and a solution of (DCC) dicyclohexyl-carbodiimide (1.652g, 8.01mmol) dissolved in 1ml THF, were added and stirred at 0°C for 3 hours. The contents of the flask were then poured in 20ml water and were extracted with 3 x 25ml ethyl acetate. The organic layer was dried over anhydrous sodium sulfate. The solvent was removed *in vacuo* and the resulting residue was purified via flash chromatography (Hex: EtOAc, 60:40) to afford the title compound **19** as a pale yellow solid (1.198g, 3.17mmol, 60% yield).

Data for compound **19:**

NMR (300MHz, $CDCl_3$): δ 7.79 (d, *J* = 7.3 Hz, 2H), 7.62 (d, *J* = 7.3 Hz, 2H), 7.37 – 7.59 (m, 6H), 7.28 (t, *J* =8.1Hz, 1H), 7.02(t, *J* = 1.89 Hz, 3H), 6.96 (dt, *J* = 8.1, 2.1 Hz, 1H).

LRMS: Calculated for $C_{21}H_{15}NO_4S$ ($M+H^+$) 377, found 377



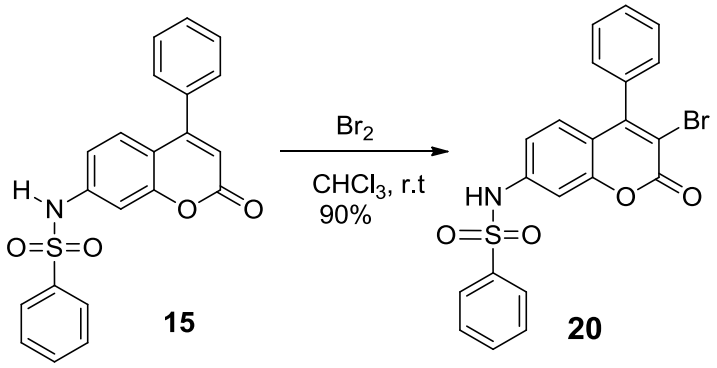
Compound 15:

In a flame died N_2-filled flask, compound **19** (1.00g, 2.65mmol), trifluoracetic acid (2 ml), methylene chloride (3ml), $Pd(OAc)_2$ (0.006g, 0.0265mmol) were stirred at room temperature for 3 hours. The contents of the flask were then passed through a silica plug and eluted with methylene chloride and ether. The solvent was then removed *in vacuo* and the resultant residue was purified via flash chromatography (CH_2Cl_2: Ether, 90:10) to afford the title compound **15** as a white solid (0.604g, 1.60mmol, 60% yield).

Data for compound **15:**

^{1}H NMR (300MHz, $CDCl_3$): δ7.89 (d, *J* = 7.5 Hz, 2H), 7.44 –7.61 (m, 6H), 7.39 – 7.32 (m, 3H), 7.12 (d, *J* =2.1 Hz, 1 H), 6.98 (s, 1H), 6.94 (dd, *J* = 2.0, 8.8 Hz, 1H), 6.29 (s, 1H).

LRMS: Calculated for $C_{21}H_{15}NO_4S$ ($M+H^+$) 377, found 377



Compound **20:**

In a flame dried N_2 filled flask, compound **15** (0.25g, 0.66mmol) was dissolved in 1.5ml $CHCl_3$. In a separate flask, (1ml) of Bromine was dissolved in 10ml $CHCl_3$. From this mixture 800µl was removed and added drop wise to the flask which was stirred overnight at room temperature. The contents of the flask were then poured into 10ml of ice water and stirred with a solution of sodium bisulfate until the yellow color disappeared. The resulting solution was then filtered; the residue was then allowed to dry to afford the title compound **20** as a white solid (0.227g, 0.498mmol, 90% yield).

Data for compound **20:**

^{1}H NMR (300MHz, $CDCl_3$): δ 7.88 (d, *J* =8.1 Hz, 2H), 7.47 –7.63 (m, 6H), 7.21 – 7.25 (m, 2H), 7.14 (d, *J*= 2.2 Hz, 1H), 6.96 (d, *J*= 8.1Hz, 1H) 6.89 (s, 1 H), 6.86 (dd, *J* = 8.1, 2.1 Hz, 1H).

LRMS: Calculated for $C_{21}H_{14}NO_4SBr$ ($M+H^+$) 454 found 454

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