TAN A: A FLUOROGENIC PROBE FOR THIAMINASE I ACTIVITY

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 2012

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

TAN A: A FLUOROGENIC PROBE FOR

THIAMINASE I ACTIVITY

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

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ACKNOWLEDGEMENT

Above all things, I am extremely thankful for my advisor Dr. Timothy E. Glass for being understanding and supportive throughout my master's study. I am also grateful that Dr. Glass gave me such a great project and an opportunity to collaborate with USGS (U.S. Geological Survey), not to mention his patience and guidance for my research and thesis writing. No words would be enough to express my gratitude for my advisor.

Besides my advisor, I would like to thank the rest of my thesis committee: Dr. Susan Z. Lever and Dr. Peter A. Tipton for their time, great suggestions and their support and encouragement for all times.

My sincere thanks also go to Dr. James L. Zajicek, our collaborator in USGS, for being informative and devoted to our project and being an exceptionally good collaborator.

I would like to thank Dr. Nathan D. Leigh for his instructions and patience with discussing Mass Spectra with me.

I also thank my lab-mates for creating a pleasant environment to work in. They have always been kind and helpful.

Last but not the least, my special thanks go to my dear friends Xiaole Shao, Rui Sun, Chun Ren, Xutao Lv and Jin Zhang for their great support for me and the great times they spend with me.

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ABSTRACT

Vitamin B_1 plays important role in cell metabolism. Deficiency of vitamin B_1 can cause ailments in living organism, including EMS (Early Mortality Syndrom) in the predatory fish salmon in the Great Lakes. Deficiency of vitamin B_1 can be caused by excessive intake of thiaminase I, which is contained excessively in major forage fishes in the Great Lakes, is a transferase-type enzyme that can degrade vitamin B_1 by S_N(AE) mechanism. However, the occurrence of EMS in the Great Lakes has a year to year variation as large as 90%. In order to understand the pattern of EMS occurrence, a method for thiaminase I activity measurement is needed.

A radiometric assay is accurate and has been used for over 30 years, however it is expensive and requires labs to be permit for radioactive material. Another assay is the 4-NTP colorimetric assay, but it suffers from accuracy problems.

For these reasons, a fluorophorogenic thaimine analogue is presented as a fluorescent probe for Thiaminase activity. The emission of the fluorophore is quenched by photoinduced electron transfer (PET) to the N-substituted pyridinium portion of the probe. Action of the enzyme releases the free pyridine group causing a substantial increase in fluorescence and the measured thiaminase I activity (based on initial rates) was proportional to enzyme concentration up to 60 ng/assay.

Chapter 1 Introduction of Thiaminase

1.1 Vitamin B_1 and Vitamin B_1 deficiency

Vitamin B_1 (Figure 1.1), also known as thiamin or thiamine, is water-soluble vitamin that plays important roles in cell metabolism and is used in the biosynthesis of the neurotransmitter acetylcholine and gamma-aminobutyric acid (GABA).

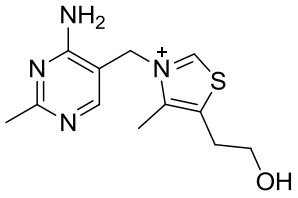


Figure 1.1 Structure of vitamin B_1 (thiamin)

All living organisms use thiamin in their biochemistry, but it is only synthesized in bacteria, fungi, and plants thus animals must obtain it from their diet. Lack of thiamin consumption leads to many syndromes include beriberi[1] and Wernicke's encephalopathy[2] in human, polioencephalomalacia in ruminant and idiopathic paralytic disease in wild birds[3]. Moreover, it is recently found that early mortality syndrome (EMS) in the offspring of the predatory fish *salmonidae* family (mainly salmon, trout) and *avitaminos* syndrome in their adults[4] in the Great Lakes are caused by vitamin B_1 deficiency [5,6]. The characteristics of EMS include low egg thiamin levels, and death between hatch and fry[7]. The clinical symptoms of *avitaminos* include loss of equilibrium, a spiral swimming pattern, lethargy, hyper-excitability and hemorrhage. Thiamin deficiency has impeded the recruitment of those salmonines, especially of coho salmon and lake trout from Lake Michigan, to

the point of threatening their population. Furthermore, stocks of Atlantic salmon from the Finger Lakes and the Baltic Sea also exhibit syndrome similar to EMS, called Cayuga syndrome[8] and M74[9], respectively.

It is widely known that not only insufficient intake of thiamin but also excessive intake of thiaminase (a series of enzymes contained in bacteria, some freshwater fish, plants, shellfish and some forages fish which catalyzes the degradation of vitamin B_1) can cause thiamin deficiency and it is suspected that consuming forage fish high in thiaminase activity[6] caused thiamin deficiency of salmonines.

Zajicek and coworkers examined the major forage fish species (alewife, bloater, spot tail shiner, deepwater sculpin, yellow perch, nine spine stickleback and round goby) in Lake Michigan for their content of thiamin, thiamin vitamers, and thiaminase activity.[10] They found that, among most forage fishes, the concentrations of total thiamin were similar and above dietary requirements, suggesting that the thiamin content of forage fish was not the critical factor in the development of thiamin deficiency in Lake Michigan salmonines. However, thiaminase activity varies among those species. It is highest in exotic species gizzard shad, spottail shiner, alewife and rainbow smelt, especially the last two species, suggesting that the main cause of thiamin deficiency in Lake Michigan is alewife and rainbow smelt. Researchers also found that the forage fish Baltic herring is the causative factor for thiamin deficiency among Atlantic salmon and the Baltic Sea brown trout.[11]

The occurrence of thiamin deficiency and subsequent EMS have a year to year variation in Great Lakes salmonines.[12] The amount of EMS observed in coho salmon

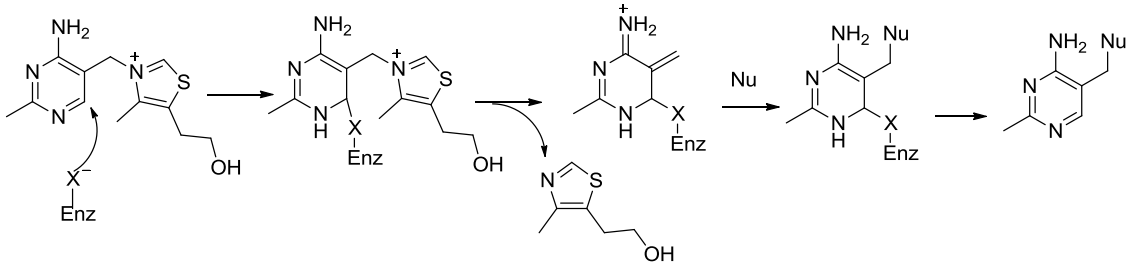
in the fall of 1999 at the Platte River was approximately 5%, while the following year, it was 90%. In order to shed some light to this phenomenon, investigations towards the thiaminase activity were carried out. And it is already known that thiaminase activity varies among species, locations, season, and size of the fish. In order to understand the pattern and cause of this variation and further develop predictive models for the onset of thiamin deficiency in Great Lakes salmonines, measurement of the thiaminase activity of forage fish in the Great Lakes area is strongly in demand.

1.2 Introduction of Thiaminase And Mechanism of Thiamin Cleavage

After years of investigation, the mechanism of thiaminase degrading thiamin is well understood. Two types of thiaminase have been found: thiaminase I (enzyme number 2.5.1.2; IUBMB 1992) and thiaminase II (enzyme number 3.5.99.2). Both types of thiaminase catalyze the cleavage of thiamin by replacing the thiazole moiety with a variety of nucleophiles. The mechanism for both are the same except that Thiaminase I can utilize a wide range of nucleophiles, including sulfite ion, azide ion, amines, thiols, heterocyclic compounds,[13] whereas thiaminase II is specific for the use of water as the nucleophile. Although they share similar mechanisms of thiamin degradation, thiaminase I is thought to be solely responsible for thiamin deficiencies. And increasing evidence suggests that thiaminase II is likely to play a role in thiamin

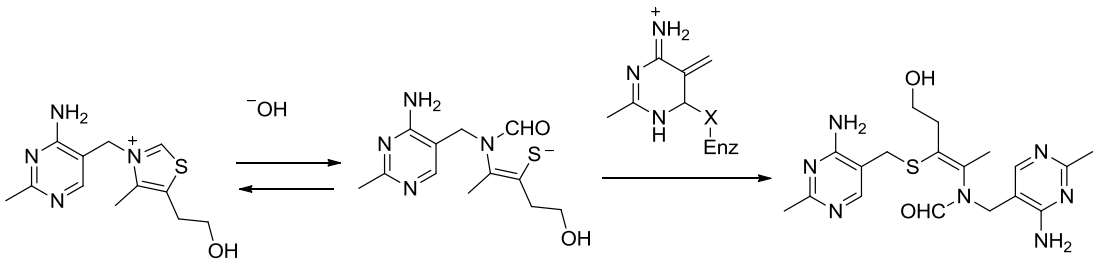
salvage.[14]

While taking a close look at the mechanism for thiamin degradation, it is generally acknowledged that the active site of thiaminase (X), perhaps a thiol group on cysteine, adds to the C6 of the pyrimidine. This addition results in the expulsion of thiazole group, allowing the addition of the nucleophile to the resulting methylene (scheme 1.1).[15,16] Finally, the nucleophilic group on the enzyme is lost and the product leaves the active site.



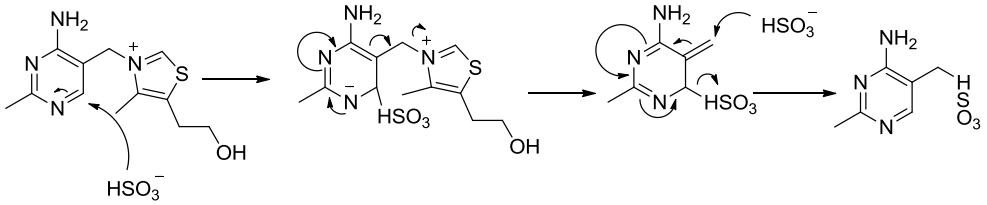
Scheme 1.1 Mechanism of Thiamin degradation by thiaminase.

Under basic conditions, the thiazole rings can be opened to produce a thiol nucleophile which an act as a co-substrate for thiaminase.[17] (Scheme 1.2) For this reason, thiaminase activity is often measured at neutral or acidic pH.



Scheme 1.2 Mechanism of degradation of thiamin in the absence of nucleophilic cosubstrate

It's worth noting that sulfite ion catalyzes the breakdown of thiamin in a similar mechanism to thiaminase. It also works through an addition-elimination mechanism S_N(AE): Sulfite ion adds to the C6 of pyrimidine and expells the thiazole, followed by addition of a nucleophile (nucleophile could again be sulfite ion)[18] [19,20], along with final loss of the original sulfite ion (Scheme 1.3). The similar mechanism can be put into good use. Since thiaminase purification is complicated and purchasing pure thiaminase is expensive, testing the degradation possibility of a thiamin-like molecule by thiaminase is time consuming or expensive. The similarity of the sulfite ion and thiaminase catalytic mechanism allows us to pre-test the degradation of thiamin-like molecules with sulfite ion before testing with thiaminase thus reduce time and expense.



Scheme 1.2 Mechanism of Thiamin degradation by sulfite ion

1.3 Measurement of Thiaminase I Activity

Over the years after discovery of thiaminase I, there have been several ways of measuring thiaminase I activity. Two early ones are detection in bacteria growing on

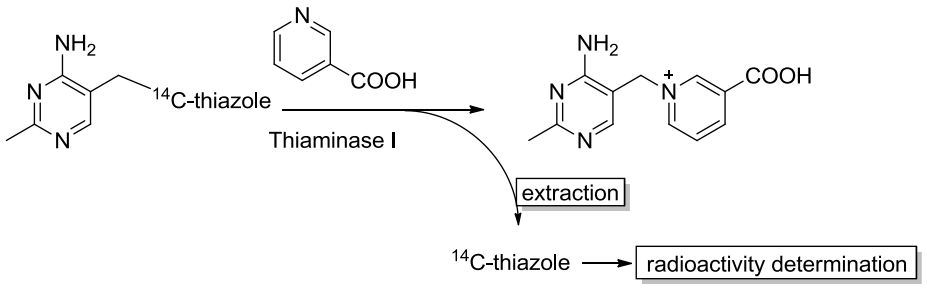
Petriplates with a soft-agar overlay[21] and measuring thiamin disappearance[22]. The dominant assay for the past 30 years was by measuring the liberation of ^{14}C-thiazole from radioactive labeled thiamin[23-25]. The radiometric assay is preferred but its use is limited to laboratories licensed for radioactive material. Furthermore, ^{14}C-thiamin is only commercially available through costly custom synthesis. Thus, a recent novel colorimetric thiaminase I assay that has good sensitivity and can be used in more laboratories was discovered and widely used. It is based on the selective consumption of the highly chromophoric 1-nitrothiophenolate by thiaminase I, resulting in a large decrease in absorbance at 411nm.

1.3.1 Radiometric Assay

The radiometric assay that measures the liberation of ^{14}C-thiazole from radioactive labeled thiamin is preferred for its accuracy and sensitivity. It was recently optimized by Zajicek and co-workers[25]. (Scheme 1.3) The biggest difference of the optimized assay with the traditional radiometric assay is the use of co-substrate (nicotinic acid) to insure that the measured activity is only dependent on the amount of thiaminase I enzyme present, rather than both the amount of thiaminase I and the ambient concentration(s) of nucleophilic co-substrates. The previously used measurements[26] were not optimal since they presumed that all nucleophilic co-substrates were present in the crude sample. However, the

concentrations that actually occur *in situ* at the active site of the enzyme are unknown. And it was shown by Zajicek that the measured activities of thiaminase I were higher than the ones without co-substrate by 2 to 9 times.

The procedure of their assay is as following: fish samples were collected, frozen and then pulverized. The frozen powdered tissue was then homogenized with cold phosphate buffer (pH=6.5), centrifuged to get supernatants which is designated as the crude extract. The crude extract was diluted and mixed with nicotinic acid and a ^{12,14}C–thiamin mixture (half ^{12}C-thiamin and half ^{14}C-thiamin). The reaction was incubated for 10min at 37°C then cooled. The ^{12,14}C-thiazole was extracted with ethyl acetate and the ^{14}C measured with scintillation counter. (Zero-time assay blanks are prepared the same way except that it is kept in ice bath for the entire time to prevent degradation of thiamin.)

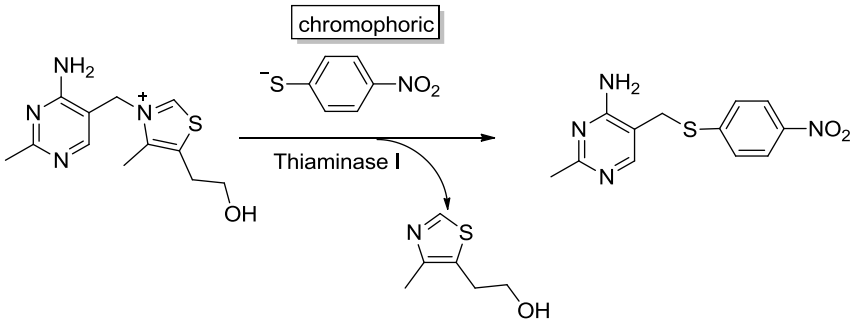


Scheme 1.3 Radiometric assay of thiaminase I activity measurement

1.3.2 4-nitrothiophenol (4-NTP) colorimetric method[27]

Because of the limitations of radiometric assay, an alternative assay using 4-nitrothiophenol was discovered by Hanes and co-workers. It is based on the

selective consumption of the highly chromophoric 1-nitrothiophenolate by thiaminase I, resulting in a large decrease in absorbance at 411nm. (Scheme 1.4) Preparation of sample is performed in the similar manner as the radiometric assay, except that a TCEP buffer (50mM phosphate, 100mM NaCl and 10mM TCEP, pH=7.2) is used instead of phosphate buffer. The inclusion of the nonnucleophilic reducing agent TCEP is for prevention of the nonenzymatic oxidation of the 4-nitrothiophenolate. A control group without thiamin is also essential for correction purpose because it was found that the 4-nitrothiophenolate was consumed even without addition of thiamin (although with a slower consumption rate). The reason for 4-nitrothiophenolate consumption was unknown. Further experiments in which purified thiaminase I was added to the control group was performed and there was no change in the consumption rate compared to the control group. The experiment excluded the possibility of presence of endogenous thiamin or other potential thiaminase I substrates in the sample.



Scheme 1.4 4-NTP assay of thiaminase I activity measurement

Honeyfield and Hanes compared the radiometric and 4-nitrothiophenol method

in a later paper[28]. They found a strong linear relationship between the two assays for alewife (R^2=0.85), smelt (R^2=0.87) and sculpin (R^2=0.82). In addition, the Thiaminase I activity in the colorimetric assay was about 1000 times higher than the activity measured by the radioactive method. However, it is unknown whether the better sensitivity came from the more effective co-substrate 4-NTP than nicotinic acid that was used in the radiometric assay. Furthermore, the 4-NTP method suffers from several defects. One is that, even though on any given day the relative activity among the samples produced the same relative relationship with radiometric assay, it still suffers from a day to day variance of measured value for thiaminase I activity. Another one is that the strong linear relationship between the two assays didn't apply to round goby and some other forage fish.

Because of the importance of measurement of thiaminase I activity and limitations of current assay, a new assay for thiaminase I activity is needed. The new assay should be accurate, sensitive, less expensive and allow in situ measurement. All those requirements prompted us to develop a fluorescent assay for thiaminase I activity measurement.

Chapter 2 Introduction of Fluorescent Probes

2.1 Definition of Fluorescence and Perrin-Jablonski Diagram

Some organic species can be brought to electronic excited states after their absorption of light. The phenomenon that organic species, after their absorption of certain wavelength of light, emit longer wavelength light is called *photoluminescence*. This phenomenon includes *fluorescence, phosphorescence* and *delayed fluorescence*. Unlike fluorescence which occurs very quickly after light absorption, phosphorescence and delayed fluorescence occur on a much slower time-scale. Many examples of photoluminescence can be found in real life. For phosphorescence, an application is “glow in the dark” toys. They first absorb light and then later emit light in the dark and the light emission could last for several minutes or up to hours. A practical example of fluorescence is the fluorescent lamp, the inside walls of whose glass tubes are coated with fluorescent material (*fluorophores*). The fluorescent material is excited by UV light produced by mercury vapor inside the tube the fluorophores fluorescence in the visible region.

Excitation of fluorophores means the promotion of an electron from an orbital in the ground state to an unoccupied orbital by absorption of a photon (sometimes two photons). It could be explained with the diagram of energy levels of molecular orbital. (Fig 2.1)

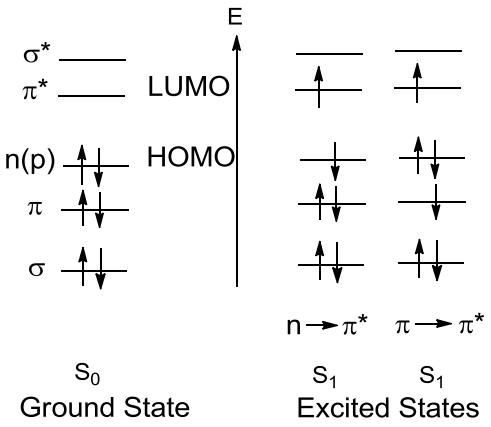


Figure 2.1. Energy levels of molecular orbitals (showing only n → π*and π → π*)

As shown in Figure 2.1, the electrons on the molecular orbital (σ, π, n) may be promoted to antibonding orbital (σ* or π*) by absorbing photons of the right energy. This process gives the excited state of the molecule. After the molecule is excited, it can go through several de-excitation processes, including non-radiative transitions (*internal conversion, intersystem crossing and vibrational relaxation*) or radiative transition (*phosphorescence, delayed fluorescence and fluorescence*). These excitation and de-excitation processes can be illustrated by a Perrin-Jablonski diagram. (Figure 2.2)

Non-radiative transitions: *Internal conversion (IC)* is a transition between two electronic states of the same spin multiplicity and *Intersystem crossing (ISC)* is a transition between two electronic states of different multiplicities. Those two processes are often followed by a *vibrational relaxation* towards the lowest vibrational level of the destined electronic state or energy transfer to the solvent during molecular collision.

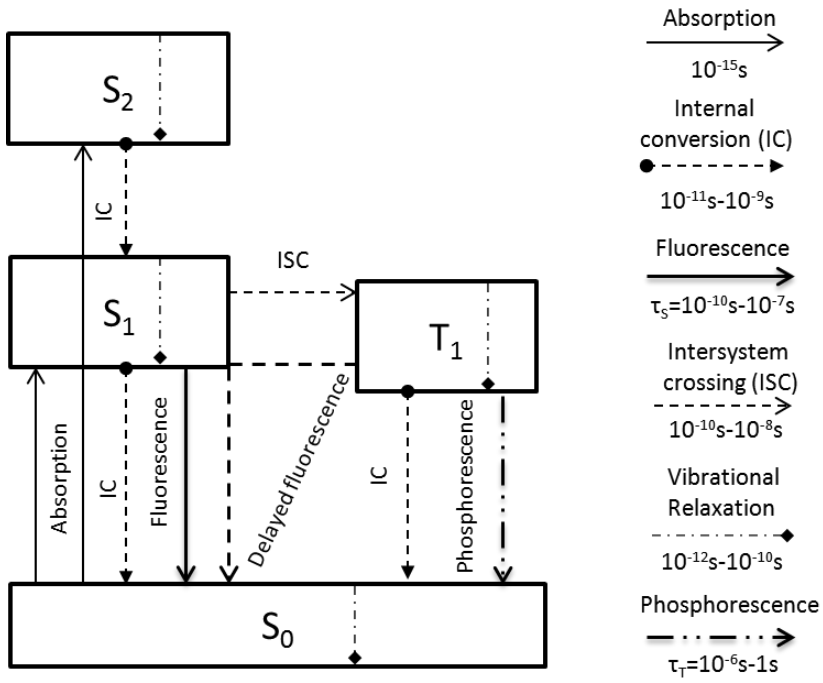


Figure 2.2 Perrin-Jablonski diagram

Radiative transition*: Fluorescence* is the transition from S_1 singlet state to ground state, *phosphorescence* is the transition from triplet state T_1 to ground state. Those two processes are always accompanied with light emission. However, for the same molecule, fluorescence process gives light with shorter wavelength compared to phosphorescence process. This is because the lowest vibrational level of T_1 has lower energy than that of S_1. *Delayed fluorescence* occurs when the energy difference between S_1 and T_1 is small and lifetime of T_1 is long enough. Under those circumstances, reverse intersystem crossing from T_1 to S_1 can occur and it is followed by radiative emission from S_1 to ground state. The light given by delayed fluorescence has the same wavelength with normal fluorescence but with a longer decay time constant since the excited molecule stayed at T_1 before emission.

Fluorescence, phosphorescence and delayed fluorescence have different

life-times. The *fluorescence life-time* refers to the average time the molecule stays in its singlet excited state before emitting a photon and denoted as τ_S. Similarly, the phosphorescence lifetime is denoted as τ_T. Some characteristic times involved in the excitation and de-excitation process are listed in Figure 2.2.

2.2 Fluorophores

2.2.1 Common Fluorophores

Fluorophores are usually fluorescent proteins or small organic molecules. The former include GFP (green fluorescent protein), YFP (yellow fluorescent protein), RFP (red fluorescent protein). The latter are generally aromatic or highly unsaturated compounds. As of wavelengths for small organic molecules, the excitation wavelength ranges from part of ultraviolet region (200nm-380nm) to the visible region (380nm-750nm), and the emission wavelength ranges from ultraviolet region (200nm-380nm) through the visible region to part of the near infrared region (750nm-1000nm).

Commonly used small molecule fluorophores fall into the following categories: Xanthene derivatives, Cyanine derivatives, merocyanine - naphthalene derivatives, coumarin derivatives, oxadiazole derivatives, benzoxadiazole - pyrene derivatives, oxazine derivatives, acridine derivatives, arylmethine derivatives and tetrapyrrole derivatives. Table 2.1 lists examples of fluorophores in some of these categories.

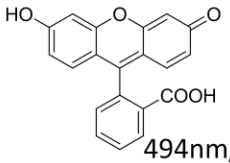
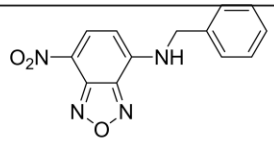
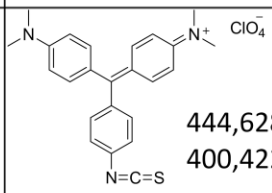
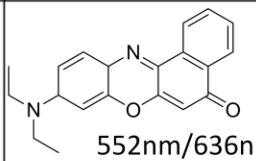
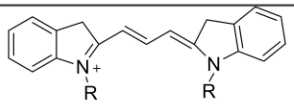
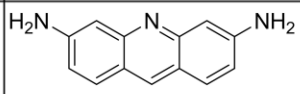
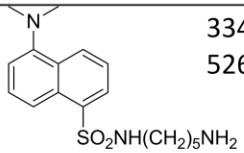
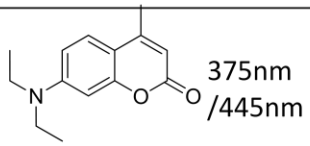
Example (Category)	Structure (Absorption/emission wavelength)	Example (Category)	Structure (Absorption/emission wavelength)
Fluorescein (Xanthene derivative)	 494nm/521nm	Nitrobenzoxadiazole (Oxadiazole derivative)	 459nm/529nm
Malachite Green Isothiocyanate (Arylmethine derivative)	 444,628nm/ 400,422nm	Nile Red (Oxazine derivative)	 552nm/636nm
Cyanine 3 (Cyanine derivative)	 550nm/570nm	Proflavine (Acridine derivative)	 444nm/511nm
Dansyl Cadaverine (Naphthalene derivative)	 334nm/ 526nm	Coumarin 1 (Coumarin derivative)	 375nm /445nm

Table 2.1.Examples of fluorophores and their wavelengths

2.2.2 Quantum yields and the factors that influence quantum yields and wavelengths

The *fluorescence quantum yield* ϕ represents the efficiency of the fluorescence process. It is defined as the ratio of the number of photons emitted to the number of photons absorbed. The maximum fluorescence quantum yield is 1.0 (100%)

$$\phi = \frac{number\ of\ photons\ emitted}{number\ of\ photons\ absorbed}$$

Factors which influence the quantum yields and wavelengths have been elucidated. To explain the factors for the wavelengths and quantum yields of fluorophores, we can refer to the energy levels of the molecular orbital diagram (Figure 2.1). Generally speaking, an increase in the extent of π-electron system leads to a shift of absorption and fluorescence spectra to longer wavelengths and to an increase in the fluorescence quantum yield.

Some substitutents can have great effects on the fluorescence characteristics of aromatic hydrocarbons. In general, electron-donating substituents such as –OH,-OR, $-NH_2$, -NHR, NR_2 induce an increase in the molar absorption coefficient and a shift in both absorption and fluorescence spectra.

For electron-withdrawing substituents, the situation is more complicated. For nitro groups, the fluorescence of the attached aromatic hydrocarbons is generally not detectable. For electron-withdrawing carbonyl groups, the situations depend on the type of low-lying excited states (n → π*or π → π*) in the specific molecules. For heterocycles containing nitrogen, oxygen and sulfur, if the heteroatom is singly bonded to carbon atoms in a heterocycle, which means non-bonding orbitals on the heteroatoms are perpendicular to the π-system, fluorescence of those molecules are still relatively high. Examples include indole, carbazole and coumarin. Otherwise, for molecules like pyridine and acridine, the quantum yields are relatively low. This can be explained by low-lying transition type. For aromatic hydrocarbons which are

devoid of *n* electrons, the lowest-lying transitions are π → π* type, which is characterized by high molar absorption coefficients and relatively high fluorescence quantum yields. When a heteroatom is involved in the π-system, an n → π* transition may be the lowest-lying transition. Such transitions are characterized by molar absorption coefficients that are at least a factor of 10^2 smaller than π → π*. Moreover, the resulting radiative lifetime for n → π* transition is too long to compete with non-radiative processes and thus leads to low fluorescence quantum yield.

2.3 Fluorescent Probes And Their Classification

Fluorophores are sometimes used alone as a tracer in fluids, such as a dye for staining certain cell structures, and sometimes they are used as a substrate of enzymes. More importantly, fluorophores can be used as markers for bioactive reagents (antibodies, peptides, nucleic acids) when covalently bonded to a macromolecule. Apart from these applications, they are also widely used as probes or indicators for solvent polarity[29], pH value[30], concentration of many substances[31,32], whose change can result in fluorescence "on/off", or fluorescence wavelength change. The four most important mechanisms correlated to these changes are intramolecular charge transfer (ICT), photoinduced electron transfer (PET), Förster Resonance Energy Transfer (FRET) and excimer formation.

2.3.1 Intramolecular Charge Transfer (ICT)

Excitation of a fluorophore induces the transition of an electron from one orbital to another hence gives a change in the dipole moment. When a fluorophore possesses an electron-donating group conjugated to an electron-withdrawing group, the increase in dipole moment can be quite large. Consequently, the fluorophore on locally excited (LE) state is no longer in equilibrium with the surrounding solvent molecules. Upon excitation, the solvent will reorganize so that the solvent environment reaches equilibrium with the fluorophore. A relaxed intramolecular chage transfer (ICT) state at a lower energy level is then reached. As a result, a red-shift of the fluorescence spectrum is observed. (Figure 2.3)

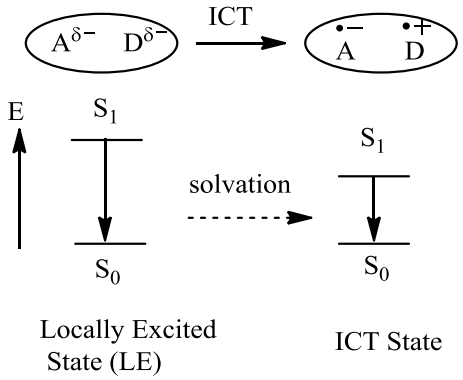
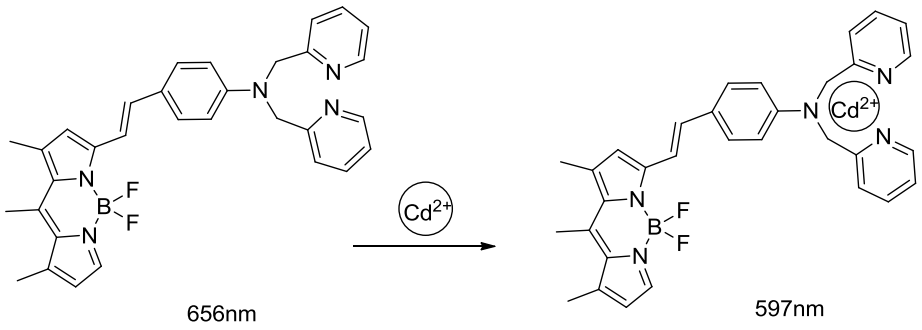


Figure2.3 Scheme of ICT process

ICT is widely used to measure concentrations of ions, along with PH value,

polarity and fluidity of solvent. Take ion measurement for example, when an ion interacts with either the donor group or the acceptor group, the transition from an orbital of the electron donating group to an orbital of the electron withdrawing group, *i.e.* ICT process, is diminished. This process leads to a blue shift. As can be illustrated by the following example of Cd^{2+} sensing[33], the free probe exhibits fluorescence at 656 nm. Upon addition of $CdCl_2$, the fluorescence undergoes a blue shift to 597 nm. The reason for the blue shift is that Cd^{2+} weakens the donating ability of nitrogen which reduces ICT process. As a result, fluorescence from an LE state with a shorter wavelength is produced. (Scheme 2.1)



Scheme 2.1 Fluorescent probe for Cd^{2+} based on ICT

2.3.2 Förster Resonance Energy Transfer (FRET)

When two fluorophores are close in space (usually less than 10nm) and one of them is in excited state, the phenomenon that the un-excited fluorophore absorbs the light that is emitted by the excited fluorophore is called "Förster resonance

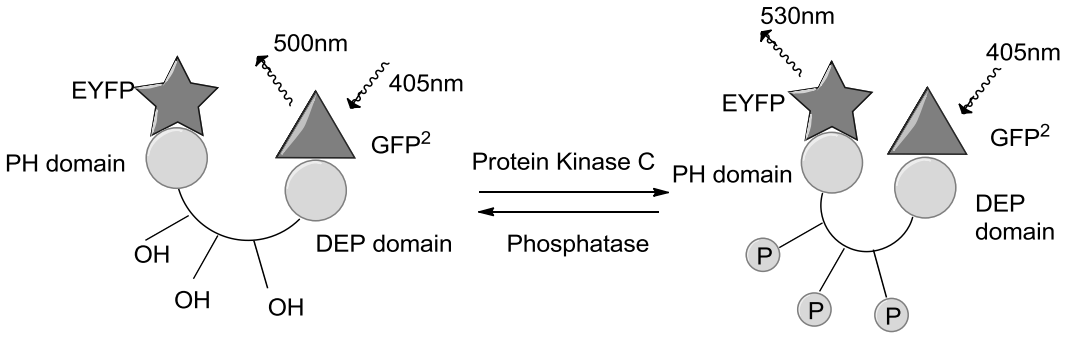
energy transfer" (in honor of German scientist Theodor Förster). The *FRET (Forster resonance energy transfer) efficiency E* describes the quantum yield of energy transfer transition. It mainly depends on the distance of fluorophores and is given by $E = \frac{1}{1+\left(\frac{r}{R}\right)^6}$, with r being the real distance of donor and acceptor fluorophores, R being the Förster distance (*i.e.* Förster critical radius) of them. R represents the distance at which the energy transfer efficiency is 50% and depends on many factors such as the overlap integral of the donor emission spectrum with the acceptor absorption spectra, the orientational factor which can take values from 0 (perpendicular transition moments) to 4 (collinear transition moments), quantum yield of donor, average refractive index of the medium in the wavelength range where spectral overlap is significant. Examples of Förster distance are listed in Table 2.2. As can be derived from efficiency equation, when r<0.5R, E is close to 1; when r<1.5R, efficient energy transfer is considered efficient; when r>2R, E approaches 0.

Donor	**Acceptor**	**R(Å)**
Naphthalene	Dansyl	~22
Anthracene	Perylene	~31
Pyrene	Perylene	~36
Phenanthrene	Rhodamine B	~47
Fluorescein	Tetramethylrhodamine	~55
Rhodamine 6G	Malachite green	~61

Table 2.2 Some FRET pairs and their Förster distance

A good example of FRET is the fluorescent Probe for PKC (Protein Kinase C) activity, developed by Carsten Schultz and co-workers[34]. (Scheme 2.2) It consists of a

DEP (Dishevelled, Egl-10, pleckstrin) domain and two PH (pleckstrin homology) domains, connected with a PKC recognition amino acid loop. Yellow (EYFP) and green (GFP^2) fluorescent protein were attached to the PH domain and DEP domain respectively.



Scheme 2.2 Protein Kinase C probe based on FRET

Before phosphorylation, GFP^2 can be excited with 405 nm light and gives off 500nm light without excitation of EYFP. However, when the hydroxyl groups on the loop are phosphorylated by PKC, the probe undergoes a conformational change that alters the orientation of two protein domains, thus increase the FRET efficiency. Since the emission spectrum of GFP^2 largely overlaps with the excitation spectrum of EYFP, EYFP absorbs light emitted by GFP^2 and emits 530nm light.

2.3.3 Photoinduced Electron Transfer (PET)

PET is widely used in fluorescent sensing with a “turn on/off” mechanism and is often responsible for fluorescence quenching. It works through two ways: reductive electron transfer and oxidative electron transfer. The mechanism is that the

quencher group transfers an electron to the excited fluorophore, which makes an elevated electron stay on higher energy level and leaves an electron vacancy. (Figure 2.4)

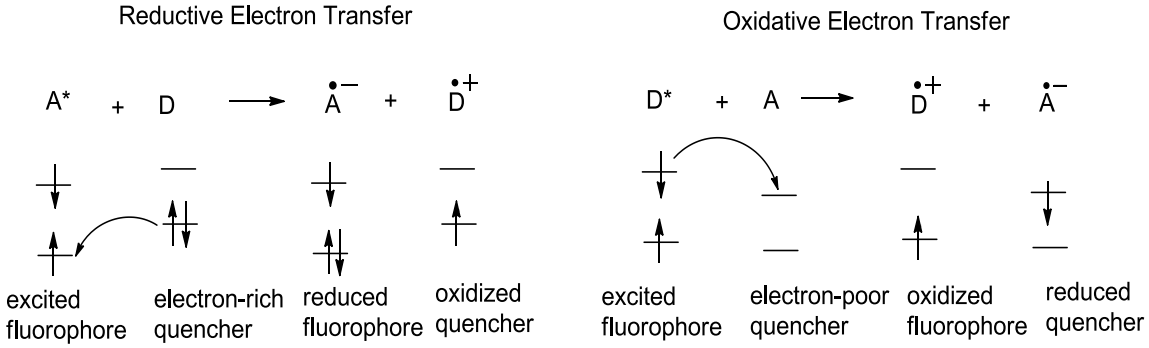
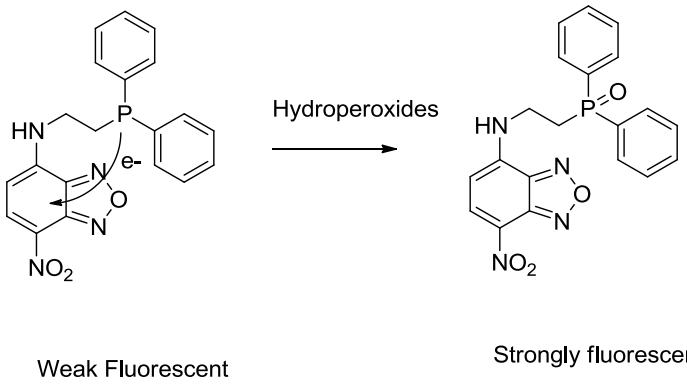


Figure 2.4 Illustration of PET theory

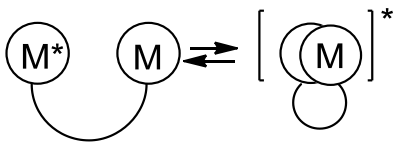
An example of PET is the hydroperoxide probe developed by Maki Onoda and co-workers.[35] The probe was not fluorescent because the phosphine can donate an electron to the fluorophore 4-methylamino-7-nitro-2,1,3-benzoxadiazole (NBD-NHMe) and thus quench its fluorescence. However, after the probe is oxidized by hydroperoxides, the corresponding oxides cannot donate to the NBD-NHMe and it becomes fluorescent. (Scheme 2.3)



Scheme 2.3 Hydroperoxides probe based on PET

2.3.4 Formation of Excimers

Excimers are dimers in the excited state (the term excimer is from 'excited dimer'). They are formed by collision between an excited moiety and an identical unexcited moiety, often in the same molecule. The excitation energy is then delocalized over the two moieties. As a result, the fluorescence corresponding to an excimer is located at wavelengths higher than the monomer. Many aromatic hydrocarbons such as naphthalene or pyrene can form excimers. The process can be illustrated by Scheme 2.4.

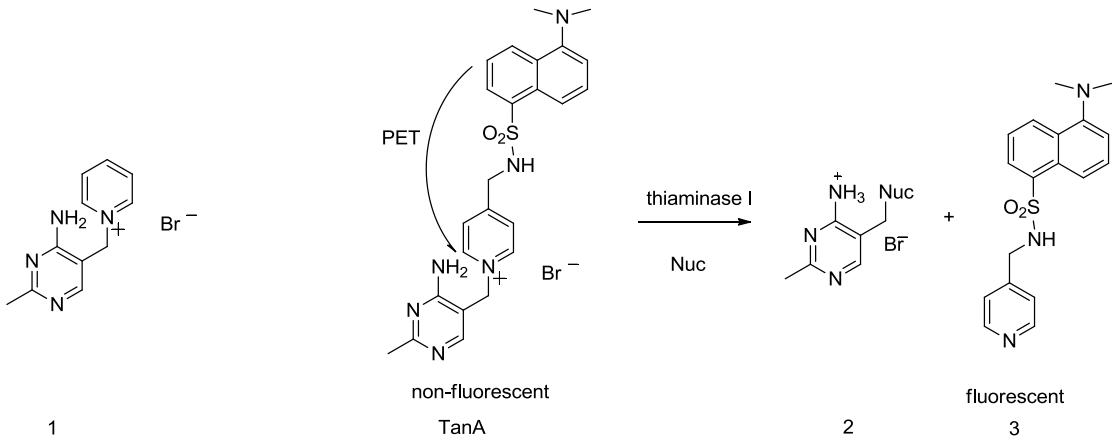


Scheme 2.4 Formation of excimer

Chapter 3 TanA: A fluorogenic probe for Thiaminase activity

3.1 Completed Work

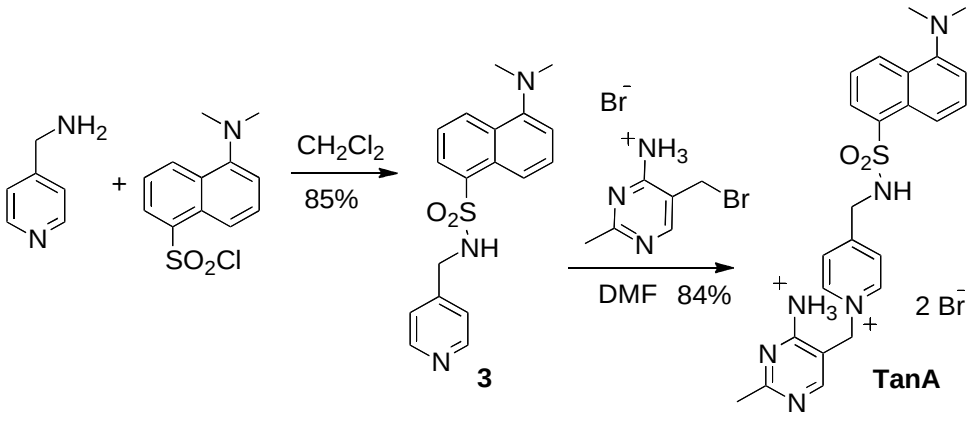
Currently, the activity of thiaminase I in a sample is mainly measured by a quantitative radiometric assay following the release of ^{14}C-thiazole from the radio-labeled thiamine. Because radiometric assays are not available to many laboratories, a colorimetric assay was developed using 4-nitrothiophenol as the nucleophile for the enzymatic reaction. This assay relies on co-substrate absorption changes that may also result from non-thiaminase reactions, which could positively bias measured thiaminase activity in selected samples.



Scheme 3.1 Decomposition of TanA by thiaminase I

Limitations of current assays prompted us to propose a novel fluorescence assay for measurement of thiaminase activity. The assay design is predicated on the known substrate promiscuity of thiaminase I. For example, compound 1 (Scheme 3.1) is a pyridinium derivative that is cleaved by thiaminase

I.[36] Importantly, pyridinium ions are excellent quenchers of certain electron rich fluorophores, due to photoinduced electron transfer (PET) quenching. Example include alkylpyridinium halides fluorescence quenching of aromatic hydrocarbon by [37,38] and of BSA intrinsic fluorescence[39]. Examination of the X-ray crystal structure of a thiaminase I enzyme-inhibitor complex indicates that the pyrimidine portion of the substrate is buried deep in the active site cleft, but the pyridinium portion of substrates such as compound **1** would be accessible.[40] Thus, we designed the probe TanA in which a dansyl group was appended to a C4 aminomethyl group of the pyridinium of **1** as this position should be well tolerated by the enzyme based on the X-ray data. The fluorescence of the dansyl group in TanA should be quenched by the N-substituted pyridinium group. But upon reaction of thiaminase I, the liberated pyridine adduct **3** should become fluorescent because the free pyridine is not sufficiently electron poor to quench the emission of the dansyl group.



Scheme 3.2 Synthesis of TanA

TanA was prepared as shown in Scheme 3.2. Aminomethylpyridine was

reacted with dansyl chloride to give the free fluorophore **3** and the pyridine was then alkylated to give the full probe construct as the HBr salt.

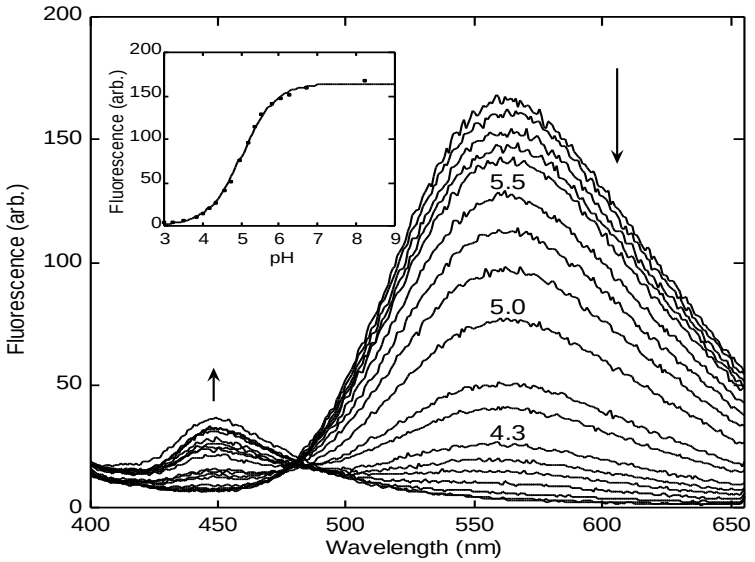


Figure 3.1 Fluorescence emission of compound **3** (λ_{ex} = 335 nm) at various pH values ranging from 3.0 to 8.2 ([**3**] = 10 μM in 20 mM citrate buffer). Inset is a plot of fluorescence (λ_{em} = 575 nm) vs. pH, which was fit to a curve with a pKa of 5.0.

In order to confirm that a pyridinium group can quench the dansyl in this configuration, compound **3** was taken up in buffer at a pH of 3.0, which was expected to fully protonate the pyridine. Indeed, the typical dansyl emission at 560 nm was nearly absent (Figure 3.1). It can be seen that the fluorescence at 560 nm increases 42 fold as the pH was raised from 3 to 8.2. The pKa of the pyridine was measured to pKa 5.0. Importantly, because thiaminase assays are often conducted at low pH, this experiment also allowed us to set a lower limit of pH = 5.5 for which enzymatic assays can be carried out and still observe large

changes in fluorescence.

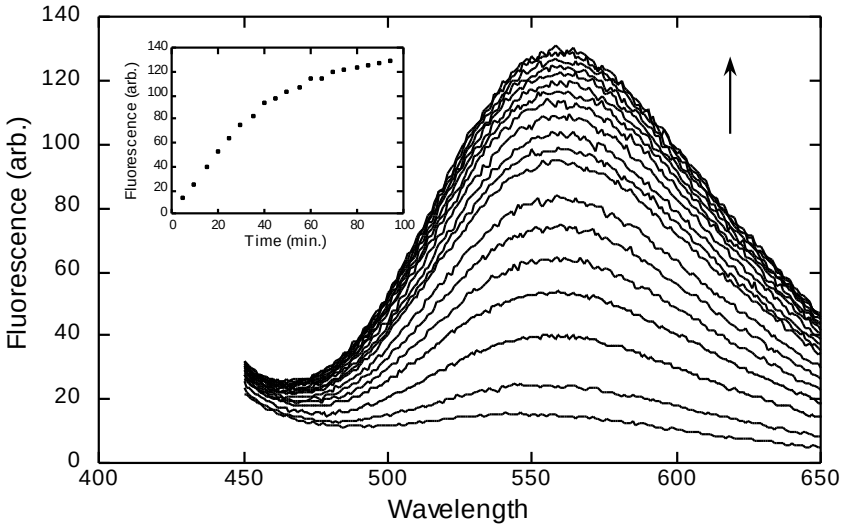


Figure 3.2 Increased fluorescence emission (λ_{ex} = 335 nm) over time resulting from the bisulfite mediated release of compound **3** from TanA at pH 6.4 ([TanA] = 8.8 µM, [bisulfite] = 0.1 mM in 0.8 mM citrate buffer). Inset is a plot of fluorescence (λ_{em} = 575 nm) vs. time for the reaction.

Thiaminase I acts via an unusual multi-step addition-elimination reaction (S_NAE). It has been shown that treatment of thiamine and its derivatives with bisulfite results in a reaction with a similar mechanism where the nucleophile is sulfite ion. Thus, TanA was treated with bisulfite at pH = 6.4 (Figure 3.2). As expected, the fluorescence intensity at 560 nm increases as a function of time due to dealkylation of the pyridinium moiety. This result confirmed our hypothesis that TanA could function as a 'turn-on' fluorescent sensor for the thiaminase reaction.

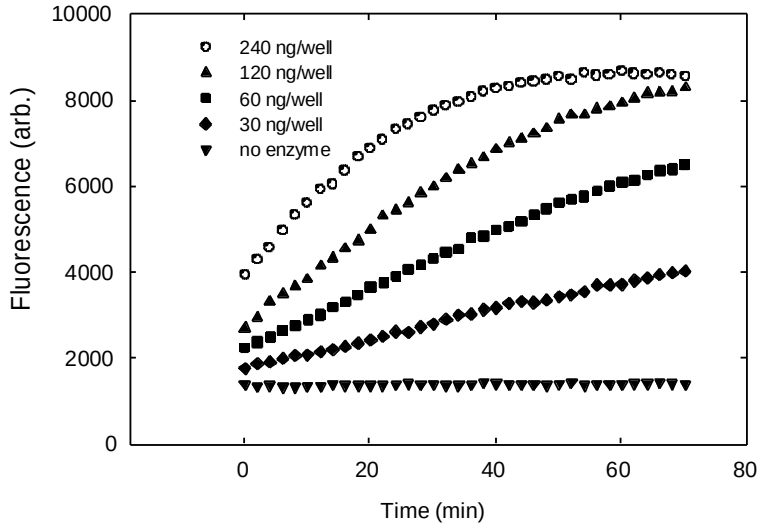


Figure 3.3 Kinetic release of product **3** fluorescence from TanA catalyzed by purified, recombinant thiaminase I. Two-fold dilution series of enzyme from 240 to 0 ng/well incubated at 28°C and pH 6.5; 0.1 M potassium phosphate buffer (0.1 M NaCl), 7.14 µM TanA, and 17.1 mM nicotinic acid (λ_{ex} = 350 nm, λ_{em} = 575 nm).

Next, the ability of purified recombinant thiaminase I to catalyze the release of fluorescent product **3** was also demonstrated (Figure 3.3). Nicotinic acid was used as the co-substrate (nucleophile). At the lower concentrations of the enzyme (30 and 60 ng/assay), the concentrations of probe substrate, TanA, and nicotinic acid were not limiting, and the enzyme-catalyzed release of product **3** fluorescence was linear for over 30 minutes consistent with a pseudo-first order conditions. At the highest concentrations of enzyme (120 and 240 ng/assay) the probe and nucleophile concentrations became limiting, such that the release of fluorescence quickly became non-linear, and estimates of initial rates were no longer directly proportional to enzyme concentrations (Figure 3.4).

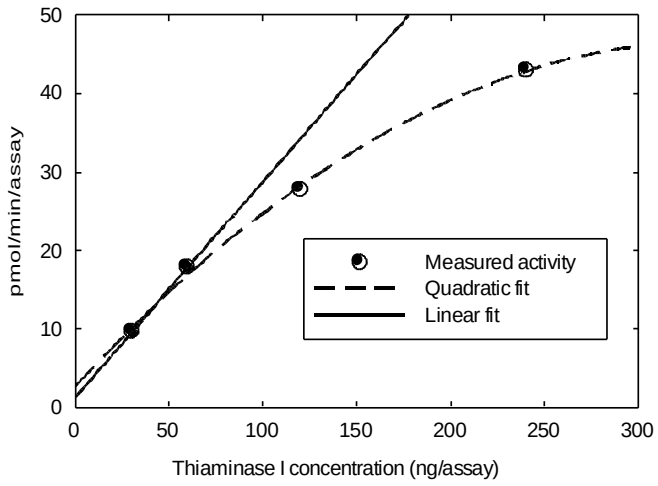


Figure 3.4 Measured activities for serially diluted recombinant thiaminase I enzyme. Catalytic rates were estimated from the initial linear portions of fluorescence/assay/min plots in Fig.3.3 A calibration curve of product **3** (Pr3) fluorescence vs concentration was used to convert measured fluorescence/ min/assay to pmol Pr3/min/assay.

Assay concentrations of probe TanA and nucleophile, nicotinic acid were selected to emulate the thiaminase and nicotinic acid concentrations used in the radiometric assay[10] and were not further optimized. Under these assay conditions, the measured thiaminase I activity (based on initial rates) was proportional to enzyme concentration up to 60 ng/assay (Figure 3.4).

In conclusion, thiamine analog TanA was developed as a fluorogenic probe for thiaminase I activity. The probe is non-emissive due to internal quenching of the fluorophore by the attached pyridinium group, however action of the enzyme liberates the free pyridine, which results in substantial (up to 40 fold) increase in fluorescence.

3.2 Future Work

Although significant fluorescence increase was seen upon detection of thiaminase I, there are still improvements to make. Since *in vivo* measurement of thiaminase is the ultimate goal of this project, looking for ways to avoid *autofluorescence i.e. background fluorescence* is the first problem to solve in this project. Autofluorescence is a fluorescent signal originating from substances other than the fluorophore of interest. Sources of autofluorescence include both an instrument component due to the optics and filters of the imaging system and a biological component from the animal tissue itself[41], including flavins, NAD(P)H, collagen, AGEs, lipofuscins , etc. These chromophores have absorption bands centered from the ultraviolet to blue regions of the spectrum and emission bands throughout the visible to near-infrared spectrum. Tissue autofluorescence is typically much higher than instrument autofluorescence and the level of autofluorescence ultimately determines the limit of detection for fluorescent probes. Thus, in future work, analogues with longer wavelength fluorophores will be developed such that the assay can be carried out in cellular or tissue samples.

Since different species possess different endogenous chromophores, In order to find out the range the autofluorescence in fish, Jim L. Zajicek measured the autofluorescence in American shad whole tissue homogenate. And it was found that all three fluorescence spectra with excitation set successively at 360 nm (blue), 495 nm (red), and 645 nm (green) appear to have background

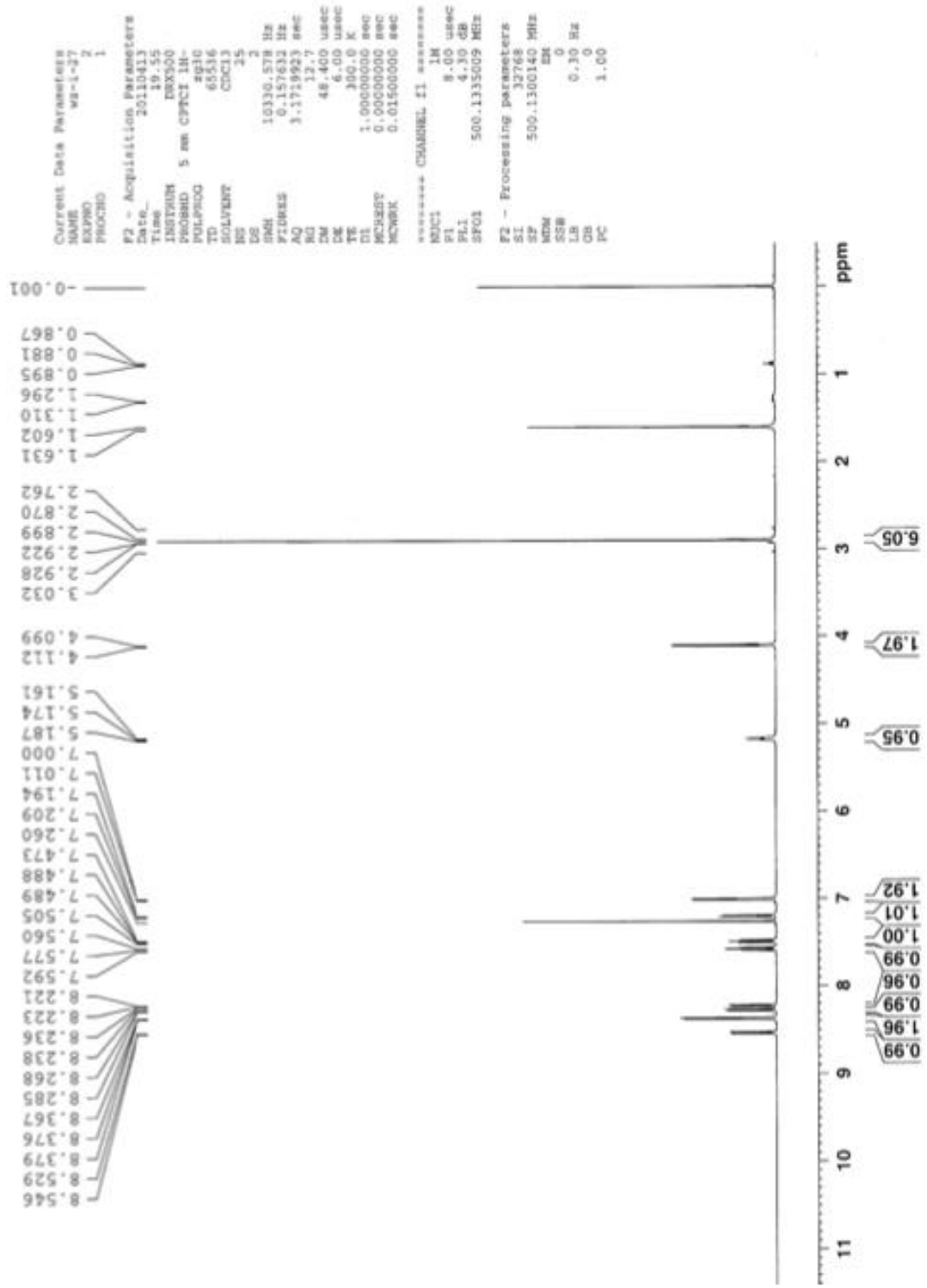
emission ranging from 350nm to 600nm. Therefore, it is necessary to find a fluorophore with an emission wavelength longer than 600nm to obtain high sensitivity.

Candidate fluorophore should have following properties: 1. Emission wavelength longer than 600nm. 2. Reactive group to attach 4-methylpyridine group. 3. Electron rich in order to be quenched by pyridinium ion.

Supplemental Information

Preparation of compound 3:

4-aminomethylpyridine (112 ul, 1.11 mmol) was added into a solution of dansyl chloride (100 mg, 0.37 mmol) in dry CH_2Cl_2 (3 mL). The mixture was stirred overnight at room temperature, followed by dilution with water. The mixture was extracted with CH_2Cl_2 (3 x 10 ml) and the organic layers were dried over Na_2SO_4 and concentrated. The crude product was purified by chromatography on silica gel with methanol-methylene chloride (2:98) giving compound **3** (101 mg, 85%yield) as a white solid; mp 90-92°C; IR (film, cm^{-1}) ν 3431, 3078, 2939, 2828, 2780, 1314, 1139, 790; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.90 (s, 6H), 4.10 (d, *J*=6.5Hz, 2H), 5.17 (t, *J*= 6.5 Hz, 1H), 7.00 (d, *J*=5.5Hz, 2H), 7.20 (d, *J*=7.5Hz, 1H), 7.49 (t, *J*=8.0 Hz, 1H), 7.58 (t, *J*=8.5 Hz, 1H), 8.23 (dd, *J*=1.0, 7.5 Hz, 1H), 8.28 (d, *J*= 7.5 Hz, 1H), 8.37 (d, *J*=6Hz, 2H), 8.5 (d, *J*=8.5 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 45.3, 45.8, 115.2, 118.4, 122.2, 123.0, 128.5, 129.4, 129.7, 129.8, 130.7, 134.3, 145.7, 149.5 and 152.0; HRMS $[M+Na]^+$ calcd for $(C_{18}H_{19}N_3O_2SNa)^+$: 364.1090, found 364.1088.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GB 0
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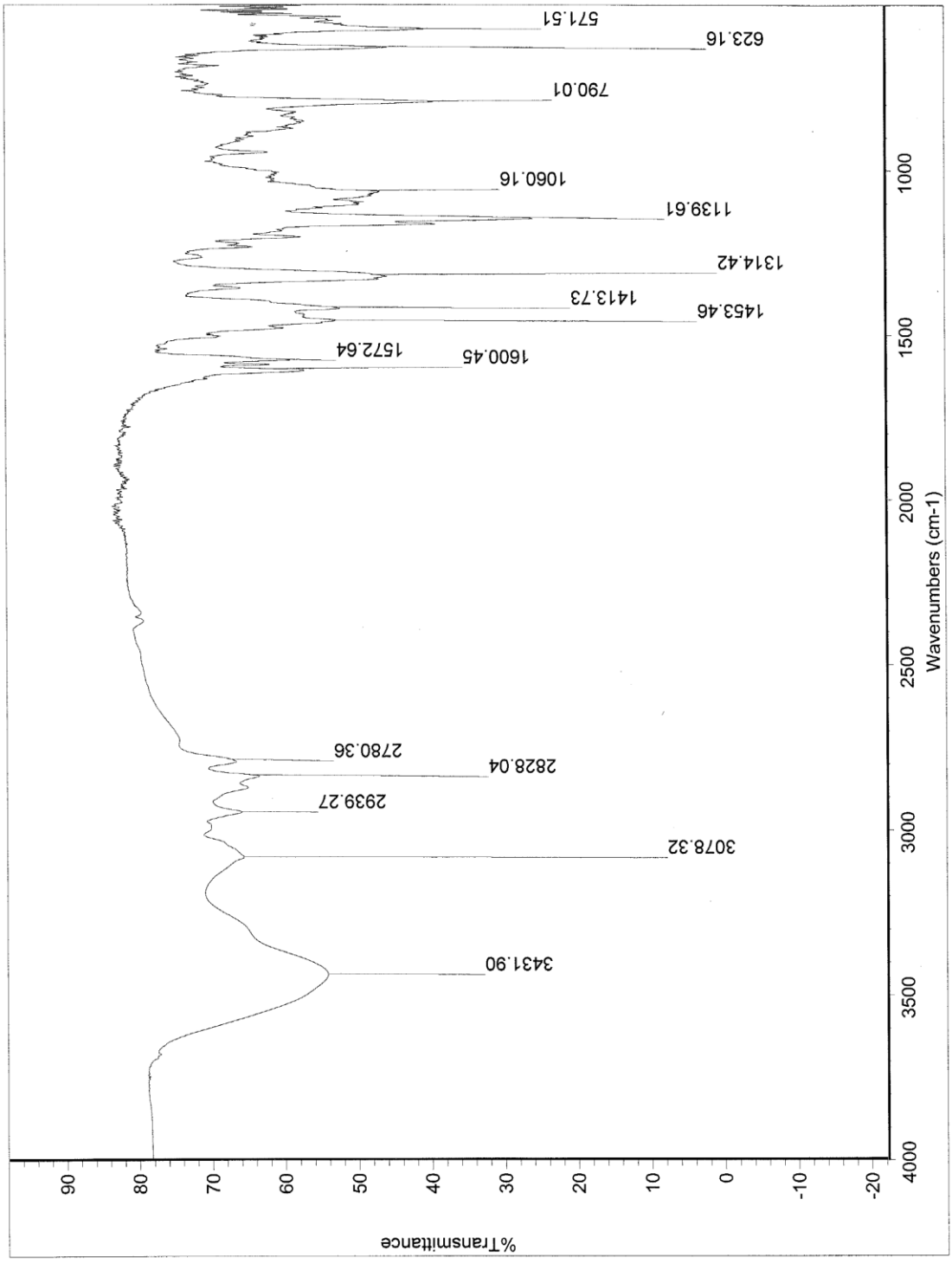
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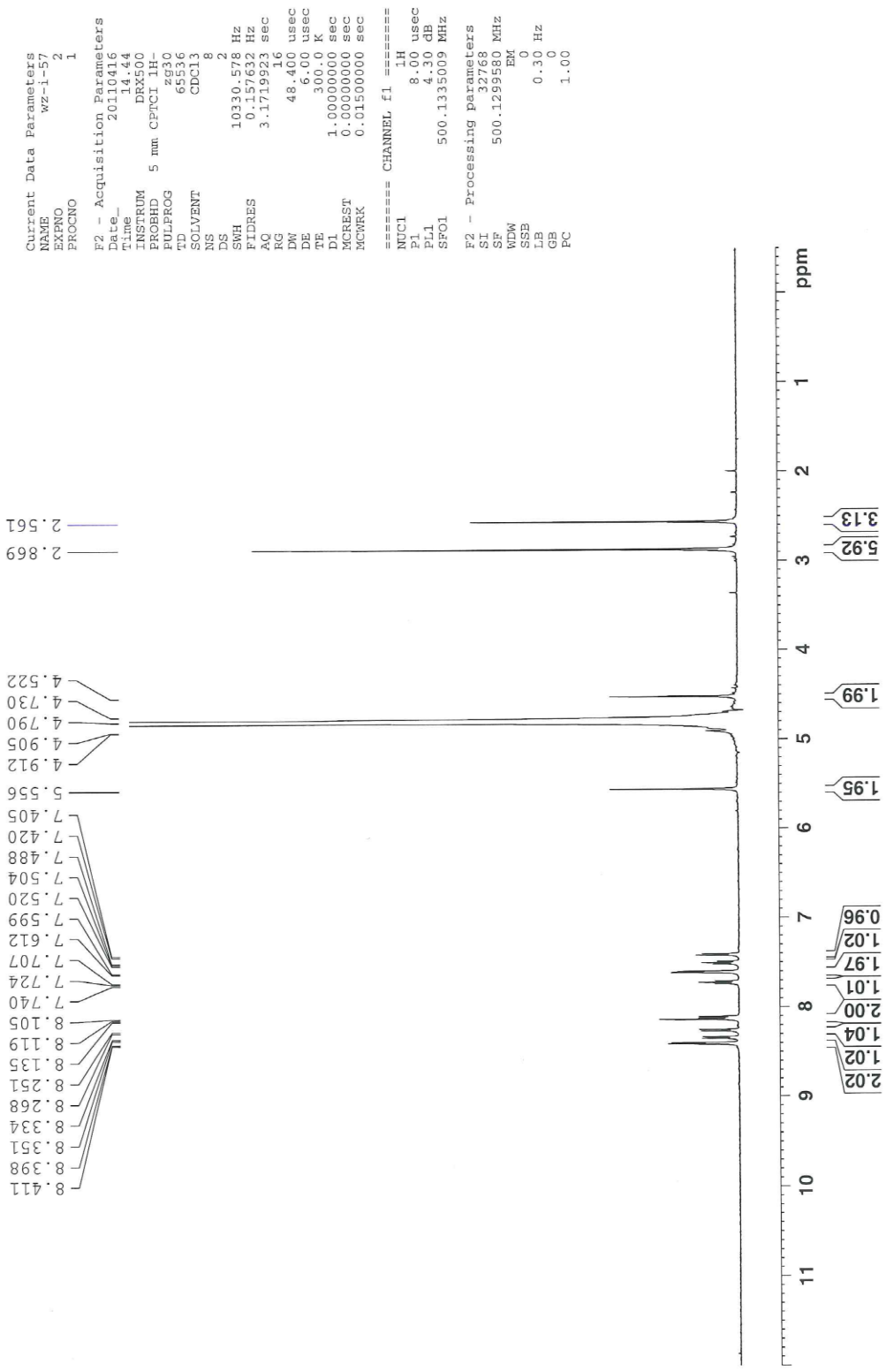
1413.73
1314.42
1139.61
1060.16
790.01
623.16
571.51

Preparation of TanA:

Compound 3 (72mg, 0.21mmol) was added to solution of 5-(bromomethyl)-2-methylpyrimidin-4-amonium bromide (20mg, 0.07mmol) in DMF (0.21 mL). The mixture was stirred under nitrogen overnight. CH_2Cl_2 was added to the DMF solution to give a yellow precipitate that was collected and washed with CH2Cl2 several times. The precipitate was dissolved in MeOH and allowed to evaporate slowly overnight. Crystals of pure TanA, as the HBr salt, were collected (37 mg, 84% yield) as a white solid; mp 208-210°C; IR (film, cm-1) ν 3427, 1652, 1469, 1139, 1087; 1H NMR (500MHz, D2O) δ 2.56 (s, 3H), 2.87 (s, 6H), 4.52 (s, 2H), 5.56 (s, 2H), 7.41 (d, J=7.5 Hz, 1H), 7.50 (dd, J=8 Hz, 1H), 7.61 (d, J=6.5 Hz, 2H), 7.72 (dd, J=8 Hz, 1H), 8.11 (d, J=7 Hz, 1H), 8.14 (s, 1H), 8.26 (d, J=8.5 Hz, 1H), 8.34 (d, J=8.5 Hz, 1H), 8.4 (d, J=6.5 Hz, 2H); 13C NMR (125 MHz, D2O) δ 24.0, 47.4, 48.3, 58.9, 109.2, 120.1, 124.3, 127.7, 130.2, 131.3, 131.4, 131.5, 133.0, 136.5, 146.0, 148.8, 149.4, 162.2, 165.7, 166.7; HRMS [M-HBr-Br-]+ calcd for (C24H27N6O2S)+: 463.1910, found 463.1911.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7.724
7.740
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8.119
8.135
8.251
8.268
8.334
8.351
8.398
8.411
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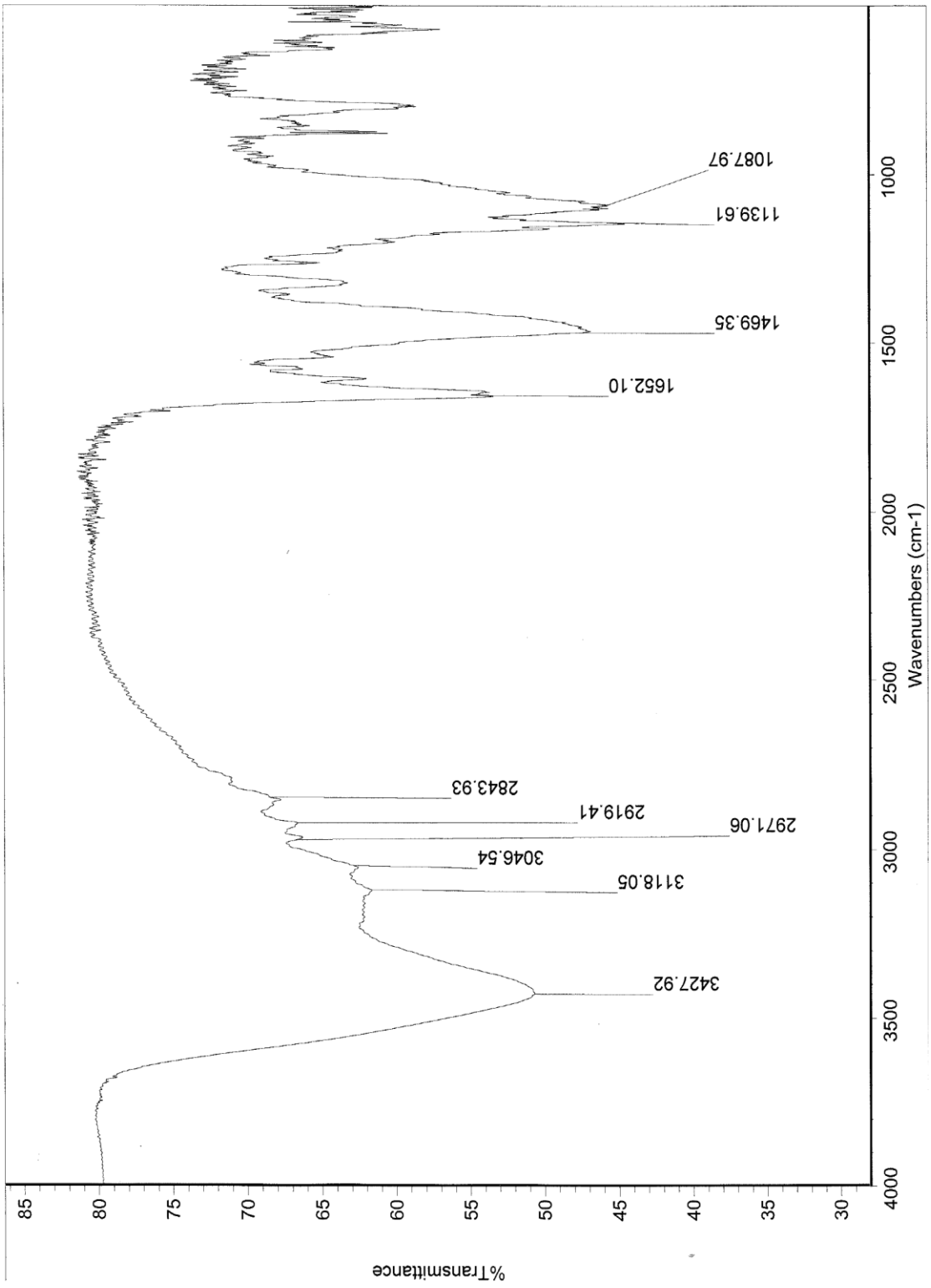
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1652.10
1469.35
1139.61
1087.97

pKa determination

Compound 3 (10 µM in 20 mM citrate buffer) was placed in a 1.4 ml cuvette and the pH adjusted to 3.0. The fluorescence (λex = 335 nm) was recorded on a Shimadzu RF-5301. The pH was adjusted by addition of a concentrated solution of NaOH and the fluorescence and pH recorded. The fluorescence at 575 nm was plotted vs. pH and fit to a standard pKa curve.

Bisulfite kinetics

TanA (8.8 µM in 0.8 mM citrate buffer) was placed in a 1.4 ml cuvette and the pH adjusted to 6.4. Sodium bisulfite was added to bring the final concentration of bisulfite up to 0.1mM and the fluorescence (λex = 335 nm) was recorded at 5 minute intervals.

Thiaminase activity

Thiaminase activity was determined kinetically in dilutions of partially purified his(10)-tagged recombinant thiaminase I derived from P. thiaminolyticus strain 8118 (Honeyfield)1 using probe TanA as substrate (in place of thiamine) and nicotinic acid as co-substrate at concentrations and pH employed in the standard radiometric

assay2. Partially purified thiaminase I protein was diluted 1000, 2000, 4000 and 8000-fold in 0.1 M, pH 6.5, potassium phosphate buffer containing 0.1 M NaCl and 0.1 % (w/v) BSA (bovine serum albumin, globulin and protease free; Sigma-Aldrich, St. Louis, MO) to provide enzyme solutions containing 30, 60, 120, and 240 ng protein/assay. Aliquots of diluted enzyme (120 µL) were added to triplicate microwells each containing 108 µL of co-substrate, nicotinic acid (40 mM, pyridine-3-carboxylic acid, Sigma-Aldrich Corp., St. Louis, MO). The plate was covered with a lid, moved into the sample chamber of a Synergy 4 multi-mode plate reader (Millipore), shaken for 10 s and allowed to equilibrate at 28°C for 5 min. Following pre-incubation, the plate was ejected and 24 µL of probe TanA (75 µM) or buffer was added to appropriate wells, the plate returned to the sample chamber, and was shaken for 10 seconds to mix the contents. The emission of released compound 3 at 575 nm was measured at 2 minute intervals using xenon-flash lamp excitation at 350 nm. A dilution series of fluorescent compound 3 contained in separate microwells of the thiaminase assay plate was used to show that the emission was linearly related to solution concentration as pmol/assay (Figure S1); where the volume of solution/assay was 252 µL.

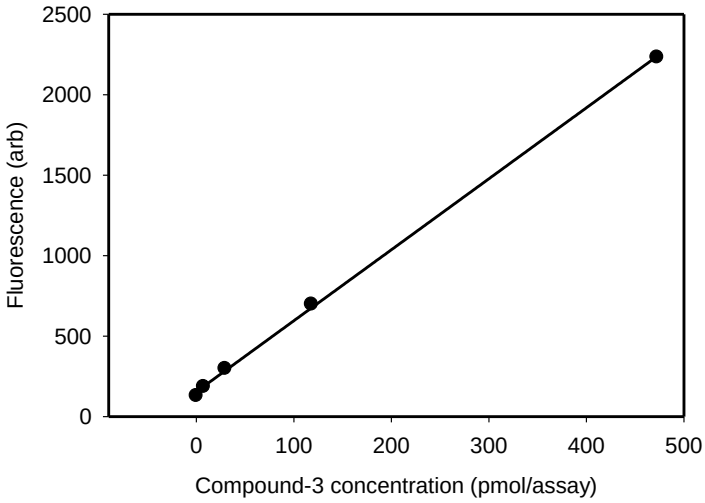

Figure S1. Fluorescent compound **3** emission was calibrated using a series of dilutions. The mean (n = 3) fluorescence emission at 575 nm, excited at 350 nm, for dilutions of free compound **3** in 0.1 M, pH 6.5, potassium phosphate buffer containing 0.1 M NaCl and 0.1 % (w/v) BSA was measured at 28 °C using a Synergy 4 multimode plate reader. Assay volumes were 252 µL/well. Fluorescence was linearly related to concentration, with F = 4.4102*[pmol compound-3/assay] + 154.58, R^2 = 0.9994.

Autofluorescence Measurement

Samples were prepared with following procedures: American shad whole tissue homogenate (0.5 g in 8 mL of pH 6.5 PO4 buffer), centrifuged at 17,000 rcf (13,500 rpm, Thermo Scientific refrigerated microcentrifuge), 4 C, upper lipid layer removed using a cotton swab.

Measurements were made at ambient temperature using a 1-cm pathlength rectangular quartz cuvette and LS50B spectrofluorometer (Perkin-Elmer), and

excitation slits at 10 nm and emission slits at 5 nm. Excitation wavelengths were set successively at 360 nm (blue), 495 nm (red), and 645 nm (green).

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