TAN B: A LONG WAVELENGTH FLUOROGENIC PROBE FOR THIAMINASE I ACTIVITY

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

**TAN B: A LONG WAVELENGTH FLUOROGENIC PROBE FOR THIAMINASE I ACTIVITY**

presented by Kang Han,

a candidate for the degree of master of science,

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

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Abstract

Thiamine, known as Vitamin B₁, is one of the 9 essential Vitamins of the B-complex. It is water-soluble and involved in many cellular processes, such as in the biosynthesis of neurotransmitter. Thiamine deficiency may cause fatal outcomes such as Wernicke’s encephalopathy in human, idiopathic paralytic disease in wild birds and EMS (Early Mortality Syndrome) in the predatory fish salmon in the Great Lakes. Deficiency of thiamine 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 cleave thiamine. However, the occurrence of EMS in the Great Lakes is still not clear. In order to understand the pattern of EMS occurrence, methods for thiaminase I activity measurement are needed.

A radiometric assay was developed and has been dominant for nearly 30 years. However this assay is only available to laboratories equipped for radioactive material measurement and it is very expensive. Another assay is the 4-NTP colorimetric assays, but it suffers other limitations.

Due to the limitation of above two methods, it is necessary to measure thiaminase I by using a novel fluorescence method. TanA has proved to be an effective fluorescent probe. Based on this fact, we decided to improve the structure of the probe to obtain a better probe for thiaminase I activity with a longer wavelength in both UV absorption and fluorescence emission.
Chapter 1 Introduction to Thiaminase
1.1 Thiamine and its deficiency

Thiamine (Figure 1.1), known as Vitamin B1, is one of the 9 essential Vitamins of the B-complex. It is water-soluble and involved in many cellular processes, such as in the biosynthesis of neurotransmitter. In addition, it helps the body convert carbohydrates into glucose.

![Figure 1.1 Structure of thiamine](image)

Although it can only be synthesized in bacteria, fungi and plants, thiamine is needed in all living organisms. Thus thiamine is an essential nutrient for animals that must obtain it from diet.

Thiamine deficiency may cause fatal outcomes\(^1\). For example, it is one of the many possible causes of peripheral neuropathy\(^2\). Other syndromes include beriberi\(^3\) and Wernicke’s encephalopathy\(^4\) in human, polioencephalomalacia in ruminant and idiopathic paralytic disease in wild birds\(^5\). In less fatal cases, outcomes include malaise, weight loss, irritability and confusion\(^6\).

In 2004, researchers showed that early mortality syndrome (EMS) and avitaminos syndrome are caused by thiamine deficiency in the salmonidae\(^7\) family in The Great Lakes\(^8\)\(^,\)\(^9\). Signs of EMS include low egg thiamine level, low survival rate between hatch and fry and symptoms of avitaminos include hemorrhage, loss of
equilibrium, and hyper-excitability. Thiamine deficiency not only prohibited the growth of salmons, specifically in the Lake Michigan Area, but also threatened their population. Moreover, syndromes similar to EMS has been found in Atlantic salmons from The Finger Lakes\textsuperscript{10}.

Thiamine deficiency has many causes, all of which can be sorted into two categories: insufficient intake of thiamine or excessive intake of thiaminase. Foods that are low in thiamine or high in anti-thiamine factors\textsuperscript{11} can cause the lack of thiamine, such as tea and coffee. Also, it could be true that consuming forage fish, which are high in thiaminase activity\textsuperscript{8,9} may cause the thiamine deficiency.

In Lake Michigan, Zajicek and coworkers examined the major forage fish species for their content of thiamine activity and found that the main cause of thiamine deficiency is alewife and rainbow smelt\textsuperscript{12}. Researchers also found that the forage fish Baltic herring is the factor for thiamine deficiency among Atlantic salmon and the Baltic Sea brown trout\textsuperscript{13}. Wolgamood and coworkers found that the incidence of EMS in salmonids from three Lake Michigan tributaries (Platte River, Thompson Creek, and Root River) varies by species, location, and year\textsuperscript{14}.

1.2 Thiaminase

Thiaminase is an enzyme that cleaves thiamine molecules to two parts. Two types of thiaminase have been found: thiaminase I (enzyme number 2.5.1.2) and thiaminase II (enzyme number 3.5.99.2). Basically, they are the same in terms of function. They replace the thiazole moiety with a nucleophile. The only difference is
that thiaminase I can use a wide range of nucleophiles\textsuperscript{15} to cleave thiamine structure, whereas thiaminase II can only use water as nucleophile specifically.

\section*{1.3 Thiamine cleavage mechanism}

Thiaminase I is thought to be solely responsible for thiamine deficiency, because thiaminase II is more likely to be participate in thiamine salvage\textsuperscript{16}. In the process of thiamine decomposition, it is known that a thiol group adds to C6 of the pyrimidine, followed by loss of the thiazole (Scheme 1.1). Then addition of a nucleophile and lost of the enzyme results in the final product\textsuperscript{17,18}.

![Scheme 1.1 Mechanism of thiamine decomposition](image_url)

\subsection*{1.4 Thiaminase I activity measurement}

After its discovery, activity measurement of thiaminase I caught people’s attention. Two methods came out by using bacteria growth with soft-agar overlay\textsuperscript{19} and measuring thiamine disappearance\textsuperscript{20}. Later a radiometric assay was developed, which has been dominant for nearly 30 years. By measuring the release of \textsuperscript{14}C-thiazole from labeled thiamine\textsuperscript{21-23}, one can tell activity of the thiamine (Scheme
1.2). This radiometric assay is great, but there are some limitations. First, this assay is only available to laboratories equipped for radioactive material measurement. Second, $^{14}$C-thiamin is very expensive.

![Scheme 1.2 Radiometric assay](image)

Recently a novel colorimetric assay has been published (Scheme 1.3). It is based on measuring the consumption of the highly chromophoric 1-nitrothiophenolate, which has an absorbance at 411mm. It is hard to make sure whether the better performance, compared to radiometric assay, came from more effective 4-NTP than nicotinic acid in the radiometric assay. In addition, another one of the defects for colormetric assay was that this assay didn’t apply to goby and forage fish.

![Scheme 1.3 Colormetric assay](image)

Due to the limitation of above two methods, it is necessary to measure thiaminase I by using a novel fluorescence method. TanA has proved to be an
effective fluorescent probe\textsuperscript{24}. Based on this fact, we decided to improve the structure of the probe to obtain a better probe for thiaminase I activity with a longer wavelength in both UV absorption and fluorescence emission.
Chapter 2 Introduction to Fluorescent Probes
2.1 Definition of fluorescence and Jablonski diagram

Fluorescence is the emission, in form of light, of a substance that absorbs electromagnetic radiation. The term *Luminescence* was first introduced in 1888 by Eilhard Wiedemann. Luminescence is a cold-body radiation with many causes such as chemical reactions, electronic excitation and so on. It is an emission in form of light, not resulting from heat. There are many types of luminescence, one of which is called photoluminescence. It is a result of absorption of photons. Photoluminescence has two sub types: fluorescence and phosphorescence.

Generally fluorescence has a longer wavelength compared to the absorbed electromagnetic radiation due to the energy loss in the electronic relaxation process. However, when the absorbed radiation is intensive enough, it is possible that the substance absorbs two photons at the same time. Then the fluorescence wavelength can be shorter than that of the photon.

At the molecule level, fluorescence occurs when one electron is excited to a higher energy state and relaxes to its ground state. In this progress energy would be released in the form of an emitting a photon of light. In order to illustrate the electronic states of a molecule between two states, a diagram, called Jablonski Diagram, is introduced. It describes most of the relaxation of excited molecules.
In Figure 2.1, each electronic state is illustrated using thick lines. Electrons in molecular orbital ($\sigma, \pi, \theta$) are able to be promoted to anti-bonding orbitals ($\sigma^*, \pi^*$) by absorbing photons with proper energy to form the excited states ($S_1, S_2$) of the molecule. After getting excited, the molecule can go through de-excitation processes to get back to ground state ($S_0$) $^{27}$. The Energy gap of fluorescence is smaller than that of absorption. This is the reason that fluorescence emission has a longer wavelength than the corresponding absorption.

### 2.2 Fluorophores
Fluorophores are those chemicals which can go through excitation and de-exitation processes with light absorption and emission respectively. Normally they have aromatic groups and/or multiple π bonds. In terms of complexity of molecular structure and synthetic method, fluorophore molecules can be classified into four categories: proteins and peptides, small organic compounds, synthetic oligomers and polymers, and multi-component systems\(^2\).

There are some characteristics of fluorophores used to describe performance of fluorophores: maximum excitation and emission wavelength, molar absorption, quantum yield, lifetime and stokes shift. Among all those characteristics, maximum emission and quantum yield are the most important ones.

Maximum emission of different wavelength puts fluorophores into 3 categories: ultraviolet region (200 nm – 380 nm), visible region (380 nm – 750 nm) and near IR region (750 nm – 1000 nm). Fluorophores in different categories have different range of applications. Quantum yield represents the efficiency of transferring energy from absorbed light to emitted fluorescence. It is defined as a ratio of number of photons emitted over the number of photons absorbed. Generally speaking, extending π-structure leads to a larger wavelength, which makes a fluorophore more useful.

### 2.3 Fluorescent probes and their classification

Fluorescent probes are made from fluorophores alone or covalently bonded with macromolecules. They are used as tracers in fluids, dyes for staining cell structures or as substrates of enzymes. When bonded to macromolecules, they are more
frequently used as markers for antibodies, peptides and nucleic acids. In addition, since fluorescent probes have switchable states between “on” and “off” along with their emission wavelengths adjustable, they are widely used as indicators for solvent polarity\textsuperscript{29}, pH value\textsuperscript{30} and concentration changes\textsuperscript{31,32}.

In terms of fluorescence mechanism, fluorescent probes can be sorted into three important classes: Intramolecular Charge Transfer (ICT), Photoinduced Electron Transfer (PET) and Forster Resonance Energy Transfer (FRET).

2.3.1 **Intramolecular Charge Transfer**

Charge transfer (CT) is a ubiquitous phenomenon. A charge transfer system typically consists of a donor (D) and acceptor (A) complex, which can be molecules or ions, leading to intermolecular CT, or they can be separate parts within a large molecule, leading to intramolecular charge transfer (ICT)\textsuperscript{33}. When locally excited, fluorophores transfer one electron from HOMO to LUMO. Consequently, there will be increase in dipole moments. Thus, the fluorophore with increased dipole moment will reach a lower energy state via ICT because of the solvation effect by solvent. That means surrounded solvent can sense the dipole moment change of fluorophore and make its energy lower by going through ICT. This results in a red shift of the fluorescence spectrum.
ICT can be used in measurement of ion concentration, pH value, solvent polarity and so on. Take solvent polarity measurement as an example. In nonpolar solvents, the emission of the conjugated fluorescent compound shows narrow and well-resolved features, suggesting that the emission comes from a local excited state (LE). However, in polar solvents, only a featureless broad emission is observed at longer wavelengths (ICT emission)\textsuperscript{34}.

![Figure 2.2 Illustration of ICT process](image1)

**Figure 2.2** illustration of ICT process

![Figure 2.3 ICT process makes fluorescence red shift](image2)

**Figure 2.3** ICT process makes fluorescence red shift
2.3.2 Photoinduced Electron Transfer

Photoinduced Electron Transfer (PET) is one of the most widely used fluorescent sensing mechanism. It can go through two pathways: sensor with a receptor (Reductive Electron Transfer) and with a donor (Oxidative Electron Transfer) respectively\textsuperscript{35}. Shown in Figure 2.4, an electron transfer to the LUMO of analyte-free acceptor from the photo-excited fluorophore creates the ‘off’ state of the sensor. When the acceptor binds analyte, the PET process will be prohibited. Thus fluorophore is turned ‘on’.

![PET fluorescent probe with acceptor](image)

Figure 2.4 PET fluorescent probe with acceptor

Similarly, shown in Figure 2.5, en electron transfer from the HOMO of analyte-free donor to the photo-excited fluorophore creates the ‘off’ state of the sensor. Also when donor binds analyte the PET process will be prohibited. Thus fluorophore is turned ‘on’.
A turn-on PET fluorescence sensor for imaging Cu$^{2+}$ in living cell was developed by Guangjie He and co-workers. The sensor is not fluorescent due to the electron transfer from the N atom to the fluorophore. However, the sensor is turned on with the presence of Cu$^{2+}$ because the lone pair electrons of the N atoms are trapped by Cu$^{2+}$ as shown in Figure 2.6.
2.3.3 Forster Resonance Energy Transfer

Forster Resonance Energy Transfer is a fluorescence mechanism describing energy transfer between two fluorophores, a donor and an acceptor\textsuperscript{37}. A donor fluorophore in its excited state may transfer energy instantly, in form of virtual photons, to an acceptor (Figure 2.7)\textsuperscript{38}. The efficiency of this mechanism is inversely proportional to the sixth power of the distance between donor and acceptor\textsuperscript{39}. This feature makes FRET much useful in measuring the distance between two fluorophores.

![Jablonski Diagram of FRET fluorescence](image)

**Figure 2.7** Jablonski Diagram of FRET fluorescence

FRET has been used to measure distance and detect molecular interactions in a number of systems and has applications in biology and chemistry\textsuperscript{40}. One of the most commonly used FRET fluorophore pair in biology is CFP-YFP (cyan fluorescent protein - yellow fluorescent protein) pair\textsuperscript{41}. 

15
When the distance between CFP and YFP are larger than 5 nm, the energy emitted from excited CFP cannot be absorbed by YFP leading to a 500 nm wavelength fluorescence emission from CFP directly. When the distance is less than 5 nm, FRET happens leading to a 530 nm wavelength fluorescence from YFP\textsuperscript{42}. 

\textbf{Figure 2.8} illustrations of CFP-YFP pair (far away and close)
Chapter 3 Tan B: A Long Wavelength Fluorogenic Probe for Thiaminase I Activity
3.1 Completed work

Currently, the activity of thiaminase I is measured by a quantitative radiometric method\textsuperscript{13}, which is not available to many labs. In addition, there is a colorimetric method\textsuperscript{14}, which is not accurate\textsuperscript{45}. Due to the limitations of above two methods, it is necessary to measure thiaminase I by using a novel fluorescence method. *TanA* has proved to be an effective fluorescent probe\textsuperscript{24}. Though a significant fluorescence (at 575 nm) intensity increase was seen with presence of thiaminase I, there were still problems with the assay. Since the ultimate goal of the project is to measure activity of thiaminase I *in vivo*, the problem of background autofluorescence has to be dealt with. Autofluorescence is the natural emission of light by biological media. It comes from both instrument components and biomolecules including NADPH, flavin, collagen, elastin etc.\textsuperscript{46}. These fluorophores in general have an absorption range from UV to blue and emit from visible region to near-infrared spectrum, which overlaps somewhat with our previous probe *TanA*. This overlap may limit the use of *TanA* dramatically. So, we decided to modify the structure of the probe to obtain a better one with longer wavelength in both absorption and fluorescence emission.

\begin{center}
\includegraphics[width=\textwidth]{scheme3.png}
\end{center}

\textbf{Scheme 3.1} potential fluorophores for *TanB* probe
When we began looking for alternative fluorophores to build up our probe, we had two criteria: 1, the fluorescence emission must be as close to near-infrared as possible; 2, the fluorophore must be an electron-rich type so that it could be quenched by pyridinium cation. 9,10-anthraquinone (1) is a well-known building block for many electron-rich fluorophores. Its derivatives have varied absorption and fluorescence emission ranges. Perpete and co-workers introduced two potential derivatives of 9,10-anthraquinone (1), 1,5-diamino-4,8-dihydroxyanthracene-9,10-dione (2) and 1,4,5,8-tetraaminoanthracene-9,10-dione (3). Compound 2 has an absorption at 590 nm and compound 3 at 610 nm. Although compound 3 has a longer absorption wavelength, it has 4 amino groups, which may cause synthetic selectivity problems when coupling it with pyridine to build probes. So we chose 1,5-diamino-4,8-dihydroxyanthracene-9,10-dione (2) as the fluorophore for our probe.

![Scheme 3.2 Decomposition of TanB by thiaminase I](image)

**Scheme 3.2** Decomposition of TanB by thiaminase I

The probe, TanB, is originally non-fluorescent because the fluorophore is quenched by the pyridinium moiety due to Donor-Photoinduced Electron Transfer
(D-PET) quenching. As illustrated in Scheme 3.2 when TanB reacts with thiaminase I, free pyridine, which is not a quencher, is released. Consequently fluorescence is restored.

Initially, TanB was planned to be prepared as shown in Scheme 3.3. 1,5-dichloroanthracene-9,10-dione (6) was reacted with 4-(aminomethyl)pyridine to give compound 7 followed by a reaction with n-butylamine to give compound 8. Then oxidation of 8 should give the free fluorophore 4. The last step was alkylation of 4 to yield full probe TanB.

Compound 7 could be prepared in low yield, however, conversion of compound 7 to compound 8 failed under many different conditions. Interestingly, all the isolated products lacked a pyridine group. So, we changed to synthetic route 2 as shown below.
This time compound 8 was successfully synthesized. We decided to test the ability of a pyridinium ion to quench the fluorescence of compound 8 by performing a pH titration. (Figure 3.1)

**Figure 3.1** Absorption (a) and fluorescence titration (b) of compound 8 at the range of pH values from 3.0 to 8.5 ([8] = 10 μM in 20 mM citrate buffer)
Compound 8 has absorption of maximum wavelength at 550 nm and a fluorescence emission at around 640 nm. As shown in figure 3.1b, when the pH decreased from 8.5 to 3.0, the fluorescence intensity of 8 increased. This result indicated that protonated pyridine was not a quencher for the fluorophore. Apparently, compound 8 is not electron-rich enough to donate to the pyridinium, so we decided to complete the synthesis by oxidation compound 8.

\[
\text{Scheme 3.5 result of oxidation of compound 8}
\]

Methods to oxidize compound 8 could only be found in patent! These procedures were conducted on totally different scales (tens of grams). Adapting these procedures to compound 8 gave poor results. Though we were able to isolate some compounds for mass spectroscopy tests, the results indicated that compound 11 (scheme 3.5) was the most likely product. Therefore a different scheme was proposed based on literature precedent (scheme 3.6)\textsuperscript{48}. 
We started with 1,8-diamino-4,5-dihydroxy-9,10-anthraquinone (2) reacting with n-butyl iodine and then 4-(chloromethyl)pyridine to give the free fluorophore 4. And then the pyridine will be alkylated to pyridinium cation yielding full probe as an HBr salt.

In conversion of compound 2 to compound 11, problems on solubility issue came. The crude after the reaction was not dissolved well in neither commonly used solvent such as dichloromethane, acetone, methanol or ethyl acetate. We could not isolate compound by running silica gel chromatography column. So we tried HPLC to isolate compounds. The running method was shown in Table 3.1.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Percentage of Acetonitrile</th>
<th>Percentage of Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>30%</td>
<td>70%</td>
</tr>
<tr>
<td>1</td>
<td>30%</td>
<td>70%</td>
</tr>
<tr>
<td>31</td>
<td>90%</td>
<td>10%</td>
</tr>
</tbody>
</table>

**Table 3.1** HPLC running method
Figure 3.2 HPLC results for compound 2 (a) and crude of compound 11 (b)

The HPLC results were shown in Figure 3.2. Compound 2 had a retention time of 13.3 min. Other three compounds showing up at 9.5 min, 12.5 min, 15 min respectively had large absorption at 610 nm, the same wavelength as the starting material. This made them potentially to be expected product, compound 11. Further isolation and characterization are being conducted.

3.2 Future work

Compound 4 will be completed as per scheme 3.8. Then in order to confirm that the pyridinium group can quench the anthraquinone fluorophore, a pH titration will be conducted. After completion of TanB, a kinetic release study of compound 4 from TanB has to be performed before actually sending TanB for in vivo activity measurement.
Supplementary Information

Preparation of compound 10:

1,5-dichloroanthracene-9,10-dione (554.2 mg, 2 mmol) was added into a solution of n-butylamine (0.8 ml, 4 mmol) in DMSO (10 mL). The mixture was stirred for 12 hours at room temperature, followed by dilution with water. The mixture was extracted with CH$_2$Cl$_2$ (3 x 10 ml) and the organic layers were dried over anhydrous Na$_2$SO$_4$ and concentrated. The crude product was purified by chromatography on silica gel with DCM: hexane (1:1) giving compound 10 (401.8 mg, 64% yield) as an orange solid. (m.w. 313.18 g/mol)

Figure S1 mass spectra for compound 10
Preparation of compound 8:

Compound 10 (50.0 mg, 0.16 mmol) was added into a solution of 4-(aminomethyl)pyridine (70.0 mg, 0.64 mmol) in toluene (5 mL). The mixture was stirred in sealed tube for 24 hours at 120 °C, followed by dilution with water. The mixture was extracted with CH₂Cl₂ (3 x 10 ml) and the organic layers were dried over anhydrous Na₂SO₄ and concentrated. The crude product was purified by chromatography on silica gel with DCM: hexane (1:1) giving compound 8 (14.1 mg, 23% yield) as a red solid. (m.w. 385.46 g/mol)
Figure S3 mass spectra for compound 8

Figure S4 $^1$H-NMR for compound 8
References


(22) McCleary, B. V.; Chick, B. F. *Phytochemistry* 1977, 16, 207.


