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**A Collaborative Study of Vitamin D<sub>3</sub> Uptake by Microcrustaceans (*Daphnia* spp.): Studies  
Towards Linking Vitamin D<sub>3</sub> and Nile Blue A**

**Pamela Meehl**

July 2011

Submitted in partial fulfillment of the requirements for the degree of

**Masters of Science in Chemistry**

Approved:

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Dr. Jeremy Alan Cody

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Dr. L. Paul Rosenberg

Department of Chemistry  
Rochester Institute of Technology  
Rochester, NY 14623-5603

**Copyright Release Form**

**A Collaborative Study of Vitamin D<sub>3</sub> Uptake by Microcrustaceans (*Daphnia* spp.): Studies  
Towards Linking Vitamin D<sub>3</sub> and Nile Blue A**

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### **Abstract**

A collaborative study to determine what role, if any, vitamin D<sub>3</sub> plays in the survival of a zooplankton genus, *Daphnia* (spp.), under the stress of UV radiation is currently ongoing. The portion of the study described here is a method towards linking vitamin D<sub>3</sub> to a fluorophore (Nile Blue A). Functionalization and linking of Nile Blue A and vitamin D<sub>3</sub> is explored utilizing "click" chemistry, carbonyldiimizaole (CDI) coupling and diisopropylcarbodiimide (DIC) coupling.

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### List of Abbreviations

Boc	<i>tert</i> -Butyloxycarbonyl
Brine	Saturated sodium chloride solution
C	Celsius
CDI	Carbonyldiimidazole
DCM	Dichloromethane
DIC	Diisopropylcarbodiimide
DIPEA	N,N-diisopropylethylamine
DMAP	Dimethylamino pyridine
DMF	N,N-dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
eq	Equivalence
Et <sub>2</sub> O	Diethyl ether
EtOAc	Ethyl acetate
g	Gram
HOBt	N-hydroxybenzotriazole
HPLC	High performance liquid chromatography
<sup>1</sup> H NMR	Proton nuclear magnetic resonance
h	Hour
LRMS	Low resolution mass spectroscopy
m/z	Mass to charge ratio
NaOAc	Sodium acetate
nm	Nanometer
ppm	Parts per million

rt	Room temperature
Soln	Solution
spp	Multiple species
t-BuOH	<i>tert</i> -butoxide
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TRSC	Texas Red sulfonyl chloride
UV	Ultraviolet
UVR	Ultraviolet radiation
Vit D <sub>3</sub>	Vitamin D <sub>3</sub>
wt	Weight



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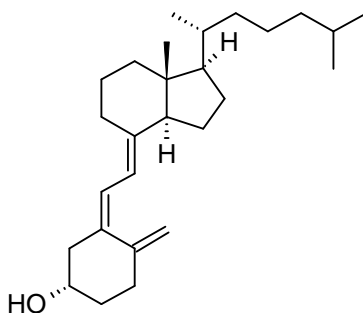
Lastly I want to thank my family, especially my husband Jordan, for their support throughout my education, I love you all.

## 1. Introduction

Over the past few decades there has been growing concern due to the presence of two holes in the ozone layer over each of the Arctic and Antarctic regions<sup>1</sup>. The destruction of ozone in the stratosphere is dominated by the catalytic reaction of nitrogen, hydrogen, chlorine, and bromine oxides by green house gases<sup>2</sup>.

The effects of decreased ozone include increased concentrations of ultraviolet radiation (UVR) reaching the Earth's surface. The UVR can be broken down into three different types, based on wavelength of radiation: UVA, 400-320nm; UVB, 320-290nm; and UVC, 290-200nm.<sup>3</sup> Longer wavelengths are required for vision in animals and photosynthesis in plants, while shorter wavelength UVB radiation is damaging to living organisms (through DNA damage), and even shorter wavelength UVC radiation is instant cell death. Ozone absorbs most of the solar UVB radiation, in addition to UVC and some UVA. However with the thinning of the stratospheric ozone an increasing percentage of UVB radiation at Earth's surface has been detected<sup>4</sup>.

In humans, UV radiation damages the DNA, and vitamin D<sub>3</sub> (**1**) is utilized in the repair mechanism for that UV damage<sup>5</sup>. Vitamin D<sub>3</sub> (**1**) can act as a secondary target for UVR rather than DNA undergoing more damage<sup>6</sup>. There are also thoughts that vitamin D<sub>3</sub> (**1**) has the potential to increase repair rates. The purpose of this collaborative research is to determine how freshwater systems are affected by the increased UVR. In order to do that daphnia are studied, in particular how they are utilizing vitamin D<sub>3</sub> (**1**) in UVR protection.



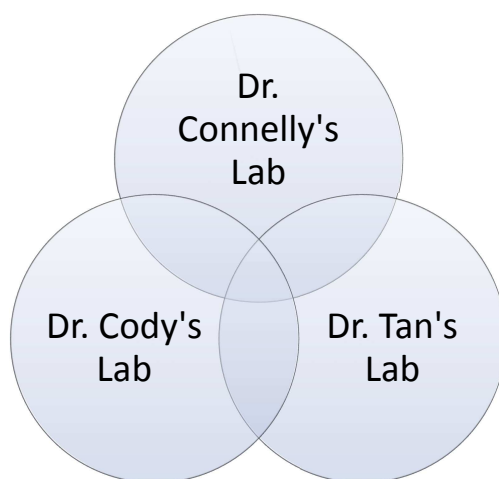
**Figure 1:** Molecular structure of vitamin D<sub>3</sub> (**1**)

In order to study the effects of the increased UVR, freshwater zooplankton, specifically daphnia (Figure 2), were chosen. Daphnia are an excellent model because they are primary consumers and they have a significant effect as a food source on their environment<sup>4</sup>. They are the base of many freshwater food webs, so scientists can extrapolate the survival and reproductive effects of the daphnia to the rest of the food web. Daphnia are an excellent organism to study for multiple reasons. Daphnia are highly sensitive to environmental stressors such as UV radiation<sup>7</sup>. Daphnia are readily available in freshwater environments, they are common in clear lake systems, and easily maintained in a laboratory setting. Also, proper assessment of their reproduction and survival is well known, they are sometimes transparent, and they are parthenogenic (clonal organisms with females giving rise to females). Without sexual reproduction, the genome of the daphnia changes very little from generation to generation. This allows for the study of essentially the same organism over many months without having to consider individual variations of any adaptive mechanisms.



**Figure 2:** Picture of *Daphnia pulicaria*<sup>8</sup>

This research is the result of a collaboration between Dr. Sandra Connelly, Dr. Loraine Tan, and Dr. Jeremy Cody. The goal of the collaboration is to study the uptake of vitamin D<sub>3</sub> (**1**) in daphnia. For each aspect of these studies it takes some overlap between the different specialties of organic chemistry, biology and analytical chemistry (Figure 3).



**Figure 3:** Diagram of overlapping skill sets

### 1.1 Overall goals

The goal of the collaboration is to investigate the potential benefits of vitamin D<sub>3</sub> (**1**) metabolites as a protective mechanism against ultraviolet radiation exposure. This is accomplished by exposing the daphnia to vitamin D<sub>3</sub> (**1**), allowing them to metabolize the compound, and then isolating the organic metabolites. Also, the survival rates are studied by exposing daphnia to vitamin D<sub>3</sub> (**1**) and exposing them to UVR and monitoring their survival over time. Another goal of the collaboration is to determine where in the body of the daphnia vitamin D<sub>3</sub> (**1**) is being sequestered. In order for this last goal to be accomplished, the Cody group in addition to the Tan group is utilized by linking a fluorophore to vitamin D<sub>3</sub> (**1**) and determining where it is located in the body of the daphnia using a fluorescent microscope. Distinguishing the location of vitamin D<sub>3</sub> (**1**) in the daphnia is important for identifying which organs or tissues may be absorbing the vitamin D<sub>3</sub> (**1**).

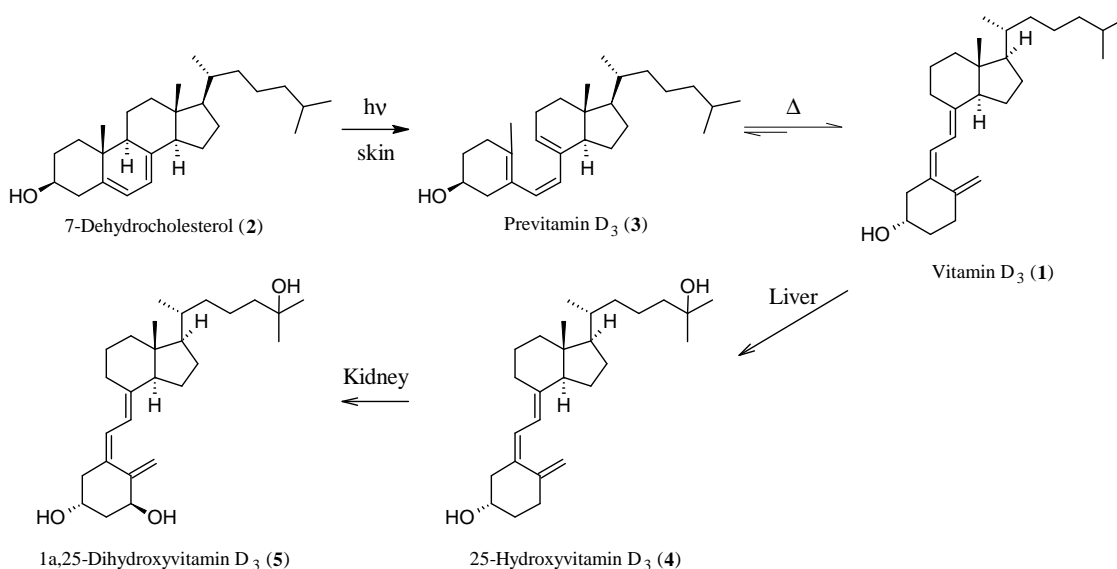


## 1.2 Cody group goals

The goal of the Cody group is to design and prepare a compound that allows for the visualization of vitamin D<sub>3</sub> (**1**) while it is in the body of the daphnia. Vitamin D<sub>3</sub> (**1**) is not colored nor does it fluoresce, so in order to see it, one approach is to add on a fluorescent group to vitamin D<sub>3</sub> (**1**). Another goal of the Cody group is to isolate the organic metabolites of vitamin D<sub>3</sub> (**1**) (along with the Tan lab) and analyze them *via* various instrumentation techniques.

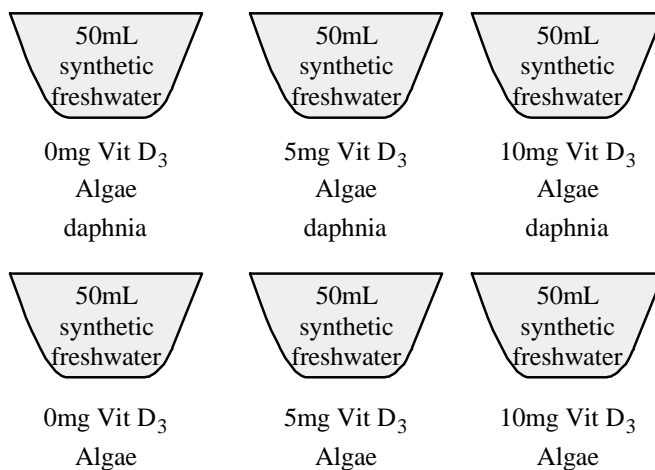
## 2. Isolation of organic metabolites of vitamin D<sub>3</sub> (**1**)

In the human body there is a pathway in which vitamin D<sub>3</sub> (**1**) is made and metabolized. As seen in Scheme 1, the body contains 7-dehydrocholesterol (**2**) in the dermis layer of the skin. There, the 7-dehydrocholesterol (**2**) is photolyzed by ultraviolet irradiation. The photolysis leads to previtamin D<sub>3</sub> (**3**) which then spontaneously isomerizes to vitamin D<sub>3</sub> (**1**). Vitamin D<sub>3</sub> (**1**) is then transported to the liver *via* the blood where it is hydroxylated to 25-hydroxyvitamin D<sub>3</sub> (**4**). 25-Hydroxyvitamin D<sub>3</sub> (**4**) is further metabolized in the kidneys to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (**5**). The final metabolite is then transported *via* the plasma to target cells located in the intestines, kidneys and bones where it is put into use aiding in the repair of DNA damage<sup>9</sup> (Scheme 1).



**Scheme 1** : Metabolic pathway of vitamin D<sub>3</sub> (**1**) in humans

Part of the research involves determining which metabolites of vitamin D<sub>3</sub> (**1**) are made by daphnia. The goal of this part of the research involves determining if the daphnia are ingesting and metabolizing the vitamin D<sub>3</sub> (**1**) into any recognizable metabolites. In order to accomplish this goal, Dr. Connelly's group set up experiments where daphnia were exposed to vitamin D<sub>3</sub> (**1**) (Figure 4).



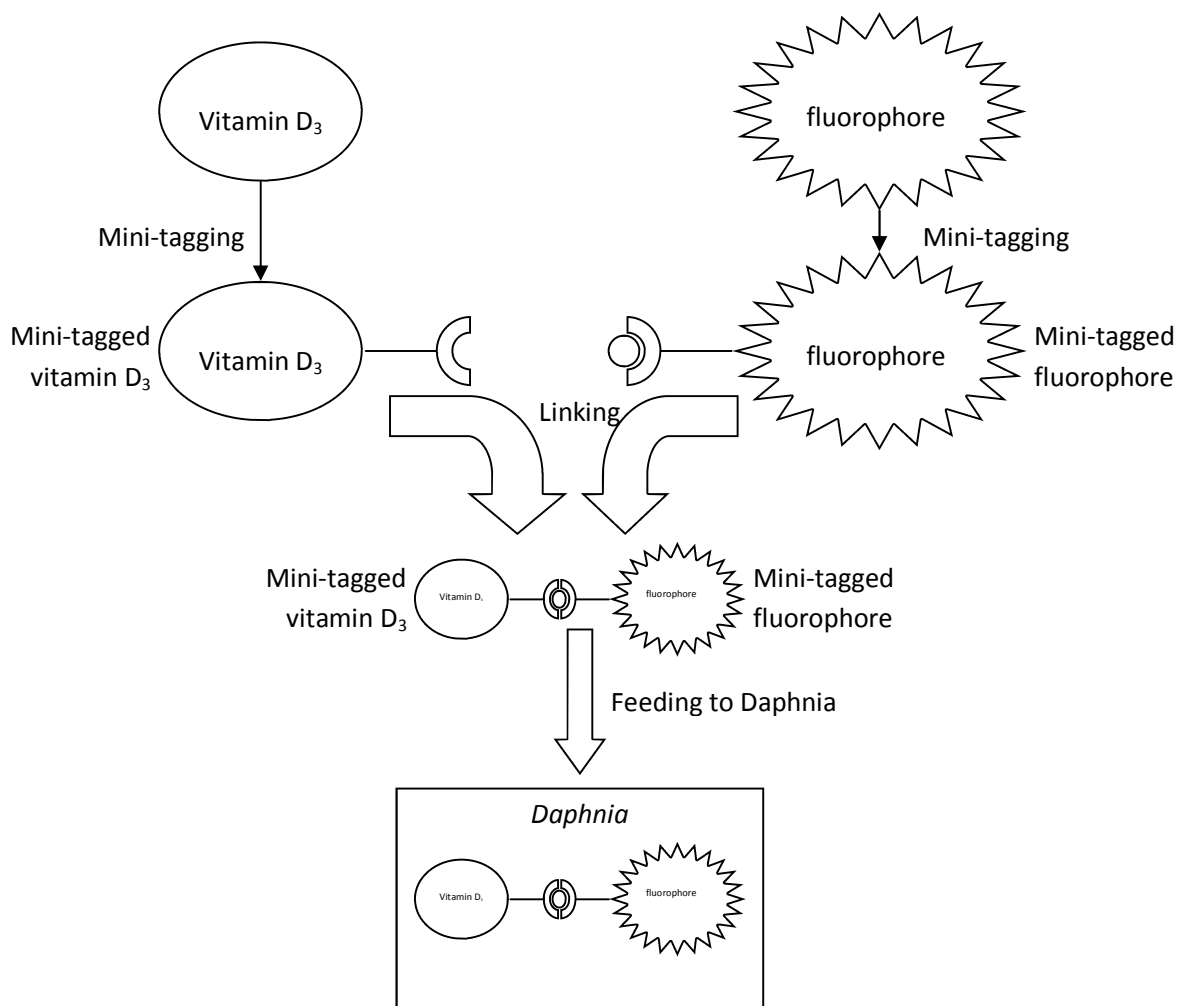
**Figure 4:** Set up of biological experiments<sup>10</sup>

Six 50mL centrifuge tubes were set up with varying amounts of vitamin D<sub>3</sub> (**1**), a constant concentration of green algae food, and daphnia or no daphnia. The setup (both daphnia and no daphnia) were incubated for 72 hours at 20°C on a light cycle of 16:8, (light: dark, UVA: dark; 103 kJ/m<sup>2</sup> total UVA). After 72 hours, the daphnia, algae and synthetic freshwater were separated<sup>10</sup>. The organic metabolites were then extracted using ethyl acetate. The resulting solutions were analyzed using HPLC to determine the amount of vitamin D<sub>3</sub> (**1**) and its metabolites that were isolated. Both vitamin D<sub>3</sub> (**1**) and 25-hydroxyvitamin D<sub>3</sub> (**4**) were then quantified by Dr. Tan's group<sup>11</sup>.

### 3. Thesis goals

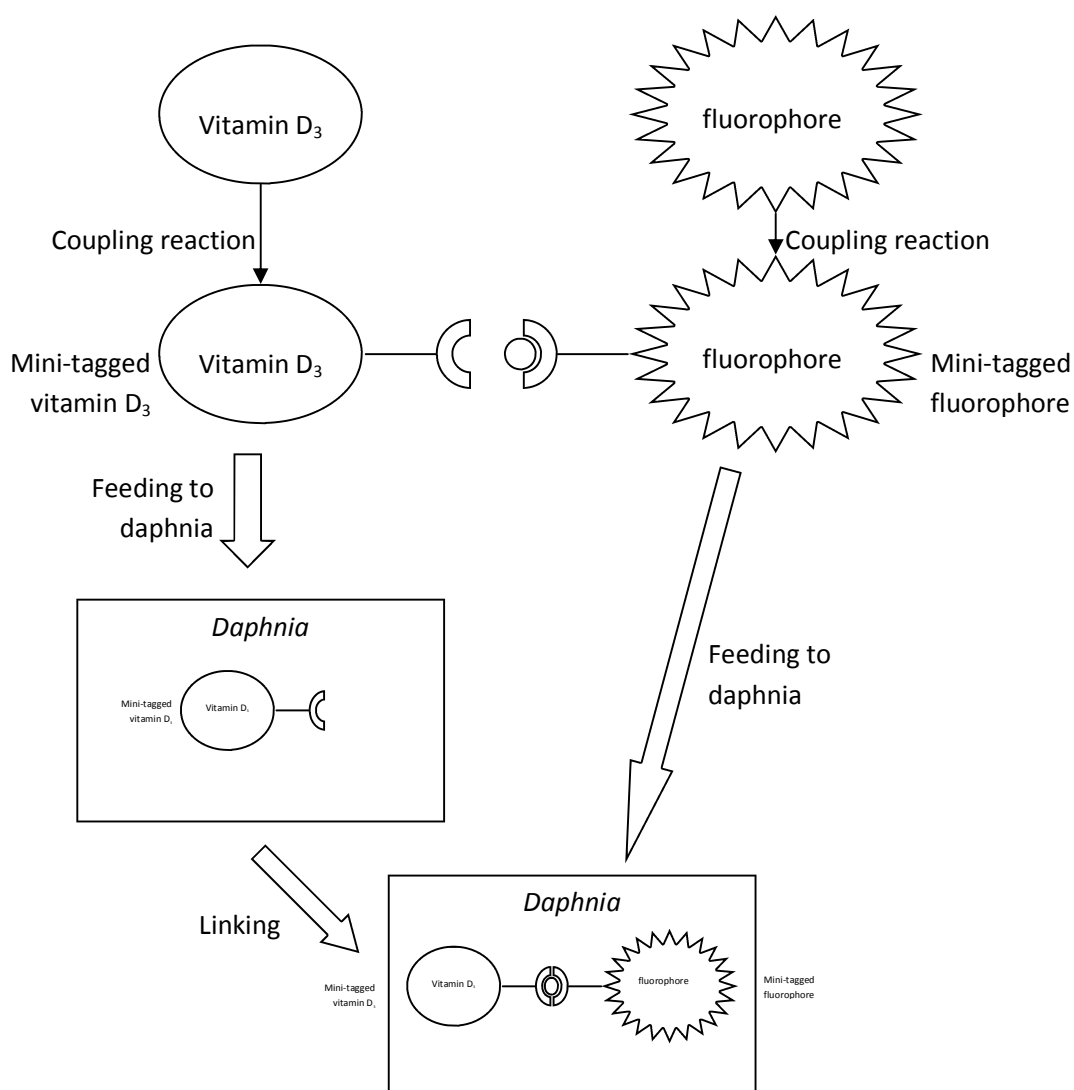
The goal of this thesis is to link a fluorophore to vitamin D<sub>3</sub> (**1**). This is accomplished in two ways. The first approach includes coupling a fluorophore onto vitamin D<sub>3</sub> (**1**), exposing

daphnia to the compound, then using a fluorescent microscope to visualize where in the daphnia's body vitamin D<sub>3</sub> (**1**) is sequestered (Figure 5).



**Figure 5:** Pictorial representation of approach A (linking before ingestion)

The second approach involved creating a minitag on vitamin D<sub>3</sub> (**1**), exposing the compound to daphnia then saturating the daphnia with the corresponding minitagged fluorophore (Figure 6). The theory is that the two compounds would link in the body where vitamin D<sub>3</sub> (**1**) had been previously sequestered allowing visualization of the fluorophore under a fluorescent microscope<sup>12</sup>.

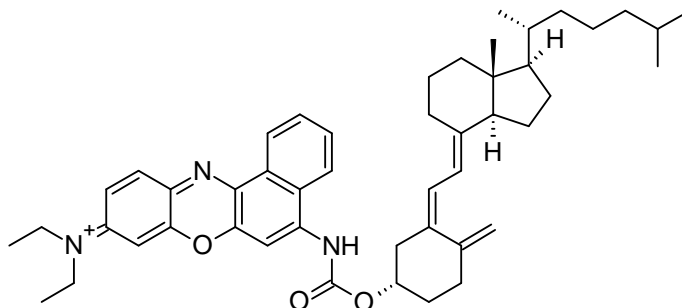


**Figure 6:** Pictorial representation of approach B (linking after ingestion)

### 3.1 Linking vitamin D<sub>3</sub> (1) to the fluorophore before ingestion (approach A)

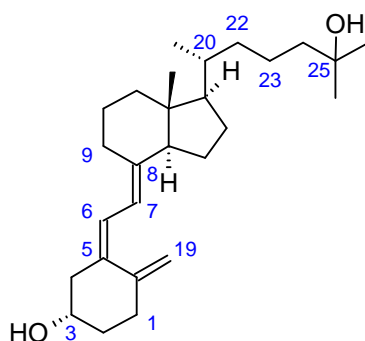
Approach A (linking before ingestion) of this project is the first approach investigated. There are both positive and negative aspects to this approach. One positive aspect is the ability to synthesize the linked species of vitamin D<sub>3</sub> (1) and a fluorophore, in a laboratory setting under controlled situations. A negative aspect is that we do not know whether or not the daphnia are going to metabolize the large molecule the same way as they would to a normal vitamin D<sub>3</sub> (1) molecule.

When linked, as in Figure 7, the fluorophore may interfere with the active sites on vitamin D<sub>3</sub> (**1**).



**Figure 7:** Molecular structure of Nile Blue A (**12**) linked to vitamin D<sub>3</sub> (**1**) (**6**)

The first hydroxylation occurs at C-25 which is on the opposite side of the molecule from where the fluorophore is being linked (Figure 8). So the formation of 25-hydroxyvitamin D<sub>3</sub> (**4**) is not the concern; the second hydroxylation occurs at C-1 which is 2 carbons away from where the fluorophore is to be linked. This means if daphnia are converting to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (**5**) the fluorophore might get in the way of the metabolism. The overall size of the linked species (**6**) is twice the size of vitamin D<sub>3</sub> (**1**) which can interfere with the absorption capabilities of daphnia. Also, the linking adjusted the polarity of vitamin D<sub>3</sub> (**1**). Adding in a fluorophore increases the polarity of the molecule which affects the absorption and sequestering ability.

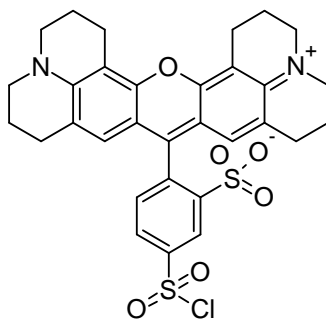


**Figure 8:** Molecular structure of 25-hydroxyvitamin D<sub>3</sub> (**4**)

### 3.1.1 Initial fluorophore selection

Initially Texas Red sulfonyl chloride (TRSC) (**7**) (Figure 9) was chosen as the fluorophore. It is a fluorescent dye with an excitation wavelength of 594nm and an emission

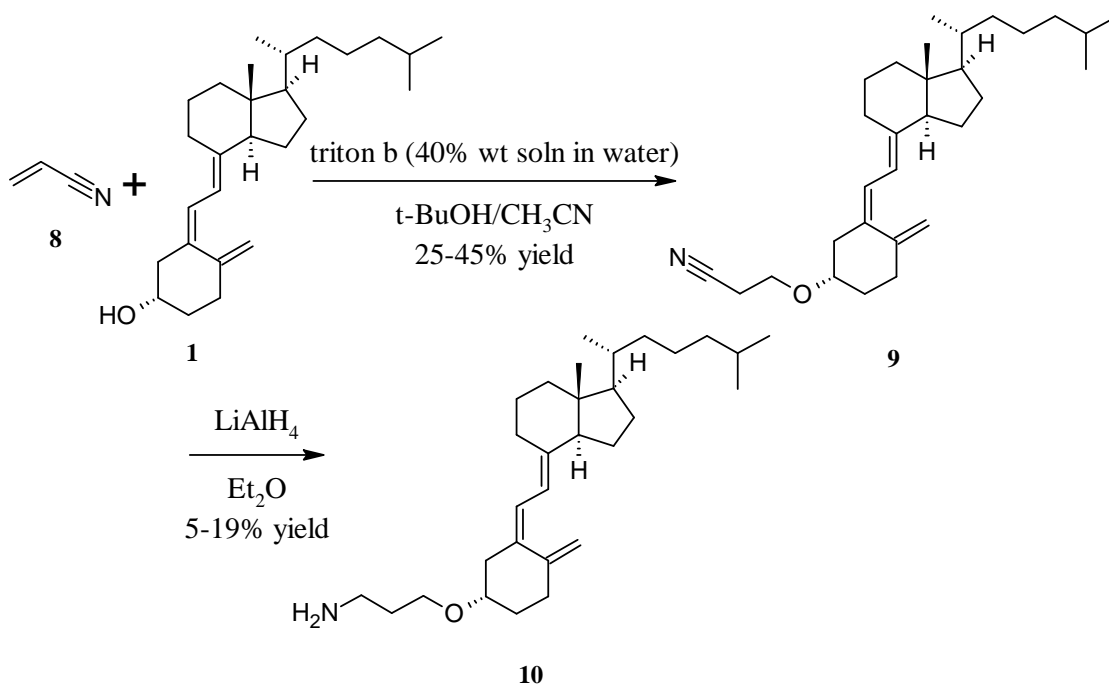
wavelength of 623nm while in ethanol<sup>13</sup>. The wavelengths fall into the wavelength range needed for the fluorescent microscope that is available to us. Also, the emission wavelength does not overlap with the fluorescence wavelength of the algae that are fed to the daphnia. TRSC (**7**) also easily forms conjugates *via* the sulfonyl chloride group.



**Figure 9:** Molecular structure of Texas Red sulfonyl chloride (**7**)

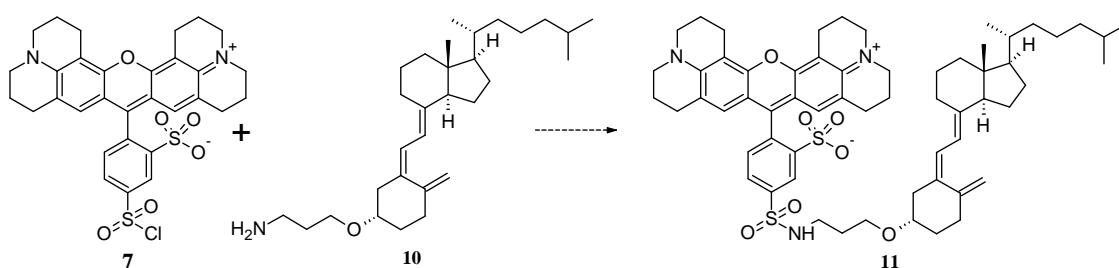
### 3.1.2 Sulfonamide bond formation with vitamin D<sub>3</sub> (**1**)

Vitamin D<sub>3</sub> (**1**) must first be functionalized to amine **10** in order to link to TRSC (**7**). This functionalization was done using a known procedure<sup>14</sup>. First a 1,4-addition was performed to add acrylonitrile (**9**) to vitamin D<sub>3</sub> (**1**) to form nitrile **9**. Nitrile **9** was then reduced to amine **10** using lithium aluminum hydride in diethyl ether (Scheme 2). There was a very poor isolation of amine **10** *via* column chromatography; due to the high polar nature of the material.



**Scheme 2:** Preparation of vitamin D<sub>3</sub> amine **10**

If amine **10** was readily available in our hands, then it would have been linked to TRSC (**7**) as seen in Scheme 3.



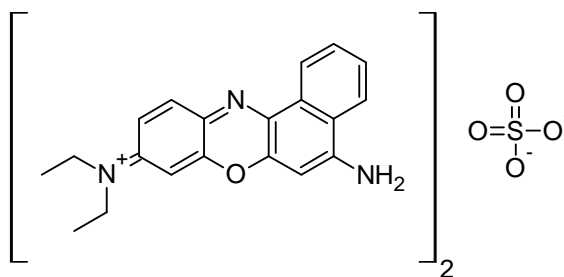
**Scheme 3:** Proposed coupling of vitamin D<sub>3</sub> amine **10** to TRSC (**7**)

The synthesis was abandoned for multiple reasons. One reason was the inability to isolate amine **10** effectively. Another reason is, TRSC (**7**) is a very expensive fluorophore. Also, the method described in Schemes 2 and 3 cannot be utilized in satisfying approach B (linking after ingestion). Lastly commercial TRSC (**7**) has various isomers, resulting in the undesired generation of disubstituted products. These disubstituted products have been seen to form from TRSC (**7**) having two sulfonyl chloride groups. After coupling TRSC (**7**) with a model amine,

diadditions were observed as a significant by-product. Therefore an alternate fluorophore was investigated.

### 3.1.3 Final fluorophore selection

The next fluorophore that was studied was Nile Blue A (**12**) (Figure 10). Nile Blue A (**12**) is a fluorescent dye with an absorption wavelength at 626nm and an emission wavelength of 668nm (in methanol)<sup>15</sup>. Once again the emission wavelength does not overlap with the fluorescence wavelength of the algae. Nile Blue A (**12**) has an amine rather than a sulfonyl chloride functionality necessitating a different type of chemistry to allow for coupling. An added benefit to Nile Blue A (**12**) is it is less expensive (\$62.70/25g)<sup>16</sup> than TRSC (**7**) (\$103/5mg)<sup>17</sup>. Also, Nile Blue A (**12**) only has one isomer as it comes commercially which is more desirable.



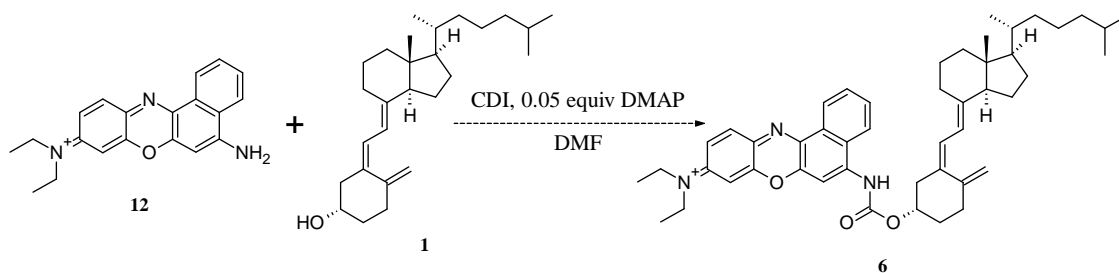
**Figure 10:** Molecular structure of Nile Blue A (**12**)

In order to link the new fluorophore to vitamin D<sub>3</sub> (**1**) different coupling strategies were utilized.

### 3.1.4 Carbonyl diimidazole coupling

In order to link vitamin D<sub>3</sub> (**1**) and Nile Blue A (**12**), carbonyldiimidazole (CDI) coupling was explored (Scheme 4).



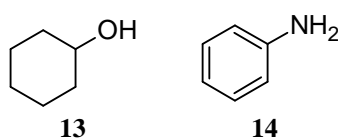


**Scheme 4:** CDI coupling between vitamin D<sub>3</sub> (**1**) and Nile Blue A (**12**)

The CDI reaction forms a carbamate bond between an amine and an alcohol in two steps. The first step is adding the imidazole ring to the alcohol, then to link that isolatable intermediate with the amine to form the carbamate bond.

#### 3.1.4.1 Model system to develop CDI coupling

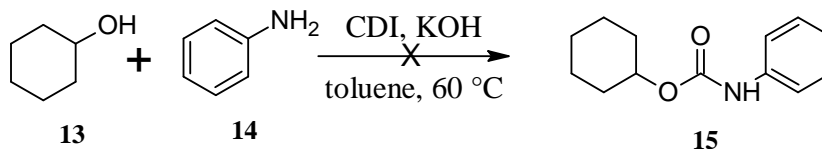
Prior to starting to work with vitamin D<sub>3</sub> (**1**) and Nile Blue A (**12**), model systems were studied. A model system allows for optimization of reaction conditions without using expensive and unstable materials. Nile Blue A (**12**) and vitamin D<sub>3</sub> (**1**) are unstable due to the light and heat sensitive nature of the compounds. The models utilized are seen in Figure 11. Cyclohexanol (**13**) was chosen as the model for vitamin D<sub>3</sub> (**1**) due to both being a secondary alcohol and on a cyclohexane ring. Aniline (**14**) was chosen to model Nile Blue A (**12**) since it bears both an aromatic ring and an amine.



**Figure 11:** Cyclohexanol (**13**) and aniline (**14**) as models for vitamin D<sub>3</sub> (**1**) and Nile Blue A (**12**), respectively

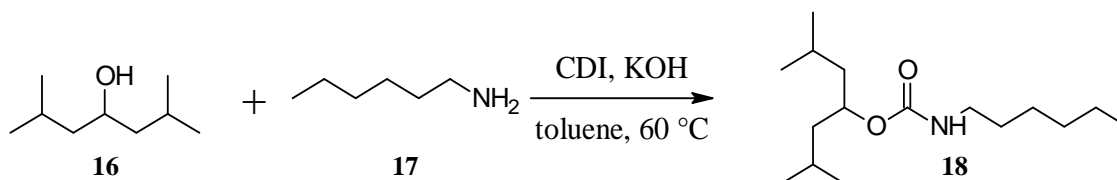
The first attempt at this reaction was completed following the one-pot two-step literature conditions from Rannard and Davis<sup>18</sup> (Scheme 5). One equivalent of cyclohexanol (**13**) was dissolved in toluene under argon at 60°C. One equivalent of aniline was then added along with

0.05 eq. KOH. The solution was allowed to react for 2 hours, and then 1.5 eq. aniline was added to the reaction. The solution was allowed to stir for 24 h.



**Scheme 5:** CDI coupling of **13** and **14** to form carbamate **15**

The reaction performed by Rannard and Davis had a secondary alcohol interacting with an aliphatic primary amine (Scheme 6). Amine **17** produces high yields<sup>18</sup> under these conditions whereas amine **14** does not produce any of carbamate **15**. Our model reaction may not have worked since our model amine was attached to an aromatic ring making it less nucleophilic.

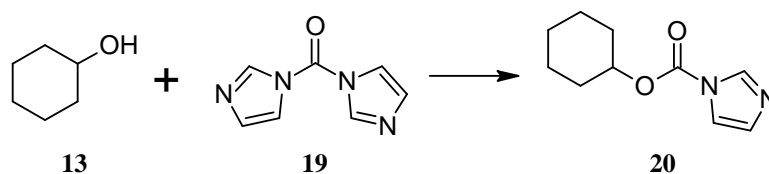


**Scheme 6:** Literature model reaction

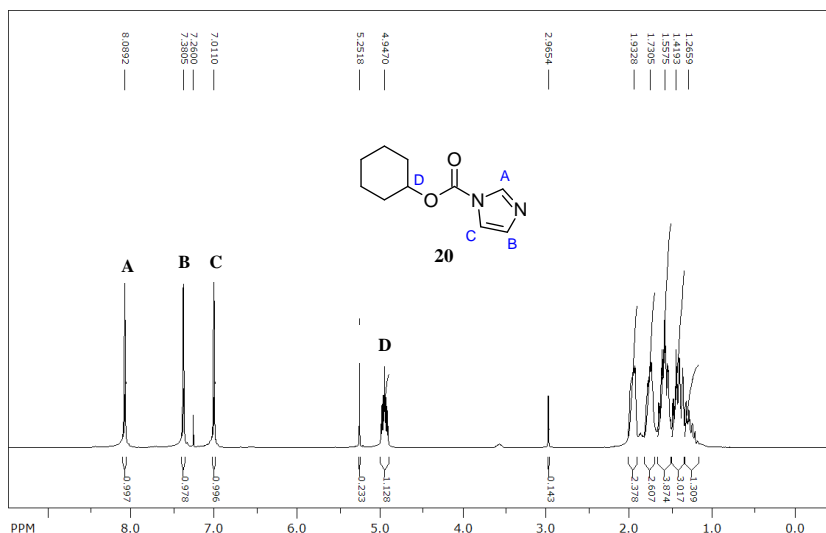
Since the one-pot two-step procedure did not form any of carbamate **15**, both steps were explored individually. The first step, formation of the imidazole intermediate, was optimized using the conditions described in Table 1<sup>19</sup>.

None of the reactions required purification because the crude product was deemed pure enough to continue *via* <sup>1</sup>H NMR (Figure 12). The run using DCM and DMAP (Table 1, Entry 3), was chosen as the optimal conditions for the first half of the reaction due to the conditions provided the highest yield (90%) of clean product.

**Table 1:** Optimizing conditions for formation of cyclohexanol imidazole **20**

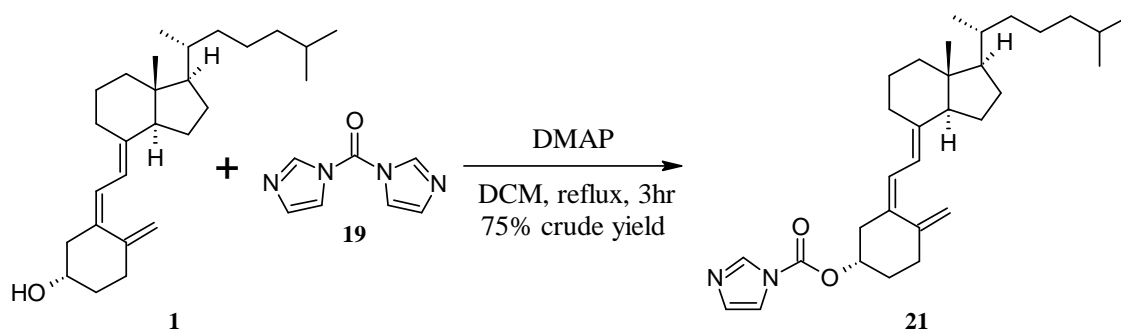


Entry	Conditions	Yield (crude)
1	1 eq. CDI, DCM, KOH, 40°C, 19hr	33%
2	1 eq. CDI, THF, DMAP, 48°C, 4hr	84%
3	1 eq. CDI, DCM, DMAP, 40°C, 4hr	90%



**Figure 12:** <sup>1</sup>H NMR (CDCl<sub>3</sub>) of cyclohexanol imidazole **20**

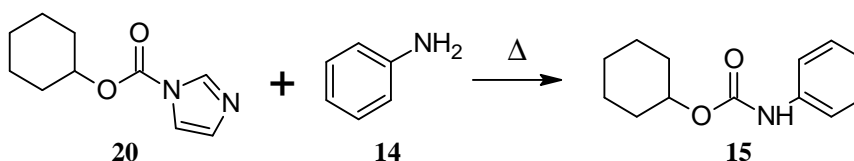
The optimized reaction conditions were then utilized to prepare imidazole intermediate **21** from vitamin D<sub>3</sub> (**1**), as seen in Scheme 7, in a 75% crude yield. Vitamin D<sub>3</sub> imidazole **21** has been seen to degrade over time necessitating purification using silica gel column chromatography if stored.



**Scheme 7:** Formation of vitamin D<sub>3</sub> imidazole **21**

The second half of the CDI coupling reaction involved reacting cyclohexanol imidazole **20** with aniline (**14**). As seen in Table 2, multiple conditions were investigated until the optimal conditions, with the greatest percent conversion, were found. In the first five reactions no product was detected *via* <sup>1</sup>H NMR or LRMS (Table 2, entries 1-5). When aniline was used as the solvent the desired product was observed (Table 2, entry 6)<sup>19</sup>.

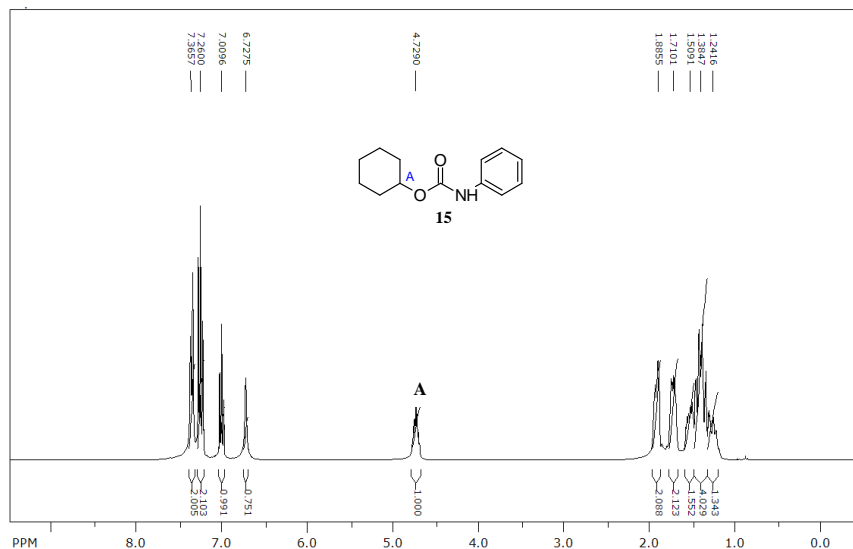
**Table 2:** Conditions for formation of carbamate **15**



Entry	Conditions	Crude Mass Recovery	Percent Conversion (Isolated Yield)
1	Toluene, 60°C, 5 hr	>100%	0%
2	Toluene, 60°C, 1.5 eq aniline, 71 hr	55%	0%
3	Toluene, 60°C, 0.1 eq DMAP, 28 hr	46%	0%
4	DCM, 40°C, 0.1 eq DMAP, 28 hr	76%	0%
5	DCM, 60°C, 0.1 eq DMAP, high pressure, 6 hr	36%	0%
6	DMAP, aniline as solvent, 40-103.3°C, 98.5 hr	41%	>95% (32%)
7	DMAP, 60°C, aniline as solvent, 73 hr	>100%	>95%
8	DMAP, 80°C, aniline as solvent, 73 hr	>100%	>95%
9	DMAP, DMF, 60°C, 72 hr	>100%	40%
10	DMAP, DMSO, 60°C, 72 hr	>100%	49%

To access the effectiveness of the reaction conditions the percent conversion was calculated *via* <sup>1</sup>H NMR by monitoring at the peak of the tertiary proton on cyclohexanol (letter A

in Figure 13). When the reaction did not go to completion there were two tertiary proton peaks allowing for the calculation of a conversion percentage.



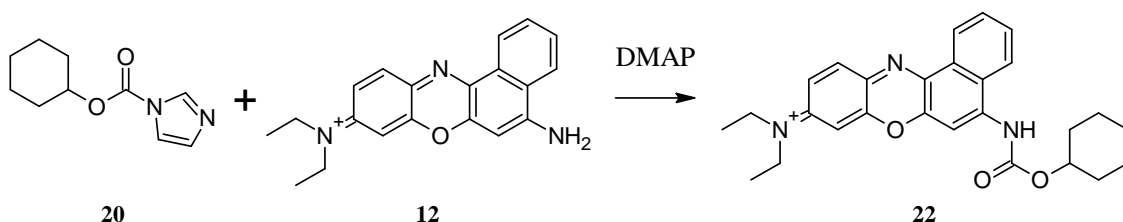
**Figure 13:**  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) of carbamate **15**

Once the reaction proceeded, the temperature was optimized<sup>19</sup>. Both reactions (Table 2, entries 7 & 8) worked affording the same percent conversion. The lower temperature (60°C vs. 80°C) was used (Table 2, Entry 7 and 8), since vitamin D<sub>3</sub> (**1**) degrades in heat. Lastly, the solvent was optimized<sup>19</sup>. Nile Blue A (**12**) is a solid which means, unlike aniline, it cannot act as the solvent of a reaction, so DMSO and DMF were tried. Both solvents dissolve Nile Blue A (**12**) and were shown to be effective for the reaction (see entries 9 and 10).

Once the optimal conditions were identified, the coupling of Nile Blue A (**12**) to cyclohexanol imidazole **20** was explored. The reaction was taken in a stepwise fashion as with the model system. The reaction in Scheme 8 was performed once in DMF and once in DMSO both with 0.01 eq DMAP, and 5 eq. of Nile Blue A (**12**). The reaction stirred at 60°C for 288 h under argon, meanwhile being monitored by LRMS. The product mass was observed during the reaction. When running with DMF there was a >100% mass recovery after extracting with dichloromethane. The product appeared to contain residual DMF, so a second extraction with diethyl ether was performed. It was very difficult to separate the organic and aqueous layers

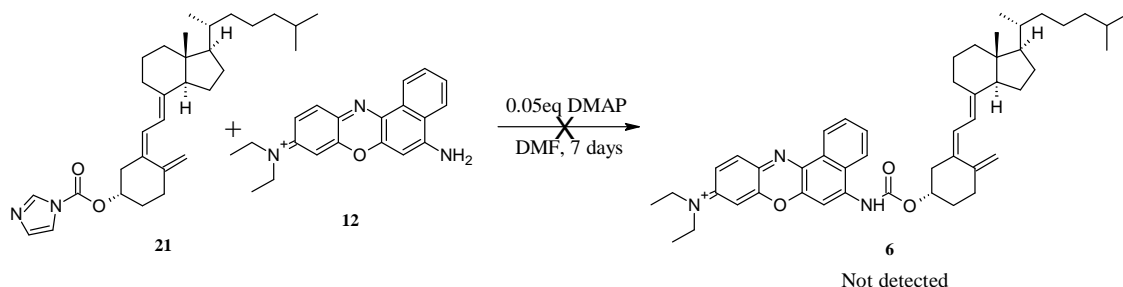
during the work up. A UV light (short wavelength) was shown on the layers and the organic layer fluoresced whereas the aqueous layer did not. After the second work up, the mass recovery was 8%. The product was not observed *via*  $^1\text{H}$  NMR.

The same reaction was repeated using DMSO and extracted with dichloromethane. The product was observed with LRMS but was not observed with  $^1\text{H}$  NMR. The lack of detection in  $^1\text{H}$  NMR could be because there was not an appreciable yield of product. The LRMS is extremely sensitive and can detect very small concentrations of product.



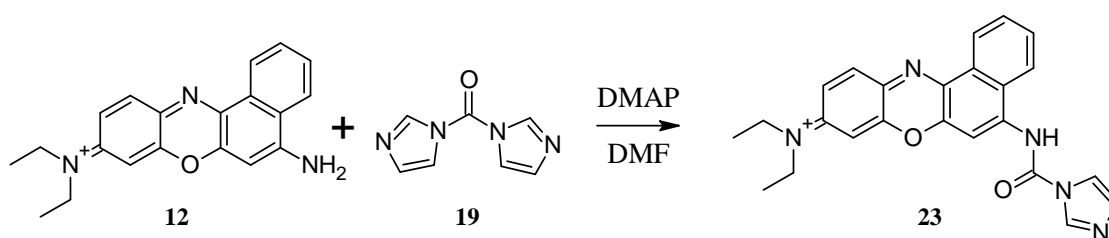
**Scheme 8:** Coupling of Nile Blue A (**12**) with cyclohexanol imidazole **20**

Finally, CDI coupling was applied to the Nile Blue A (**12**), vitamin D<sub>3</sub> (**1**) system. The LRMS for Scheme 8 data did show the MW product peak making us optimistic that the real system would work. There are two routes to couple vitamin D<sub>3</sub> (**1**) to Nile Blue A (**12**) using CDI coupling. First CDI can be reacted with vitamin D<sub>3</sub> (**1**) to form vitamin D<sub>3</sub> imidazole **21** then the resulting compound can be coupled to Nile Blue A (**12**). The other route is to add CDI into Nile Blue A (**12**), then to couple it with vitamin D<sub>3</sub> (**1**).



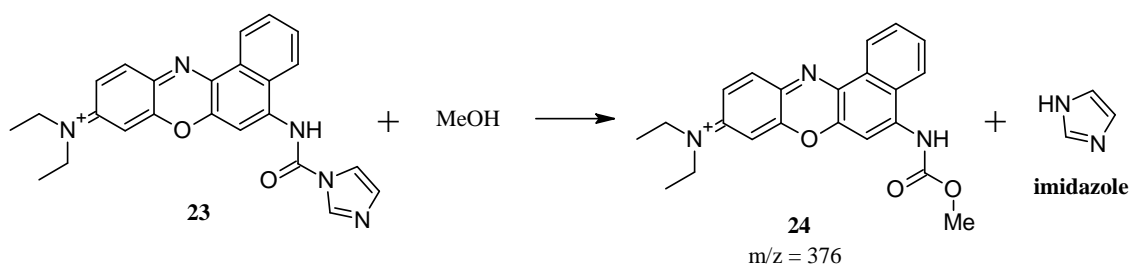
**Scheme 9:** Formation of Nile Blue A/vitamin D<sub>3</sub> carbamate **6** *via* vitamin D<sub>3</sub> imidazole **21**

It was predicted Nile Blue A (**12**) would link with vitamin D<sub>3</sub> imidazole **21** as seen in Scheme 9. Initially, **21** was reacted with Nile Blue A (**12**) in the presence of DMF and 0.05eq DMAP. Only starting material **21** was observed after 168 h of monitoring *via* LRMS. Since the sequence of adding CDI to vitamin D<sub>3</sub> (**1**) first did not work, CDI was reacted with Nile Blue A (**12**) prior to coupling to vitamin D<sub>3</sub> (**1**). It was thought that vitamin D<sub>3</sub> (**1**) is a better nucleophile than Nile Blue A (**12**) and the addition would be more effective with the better nucleophile added last.



**Scheme 10:** Formation of Nile Blue A imidazole **23**

When Nile Blue A (**12**) was stirred with 1.1 equivalents CDI (**19**) in the presence of DMAP (0.05 eq.) in DMF at 40°C for 4 days (Scheme 10), formation of **23** was observed *via* LRMS. A *m/z* peak was present at 376 for Nile Blue A/ methanol adduct **24**. The 376 *m/z* may be a result of methanol acting as the nucleophile and displacing the imidazole (Scheme 14).



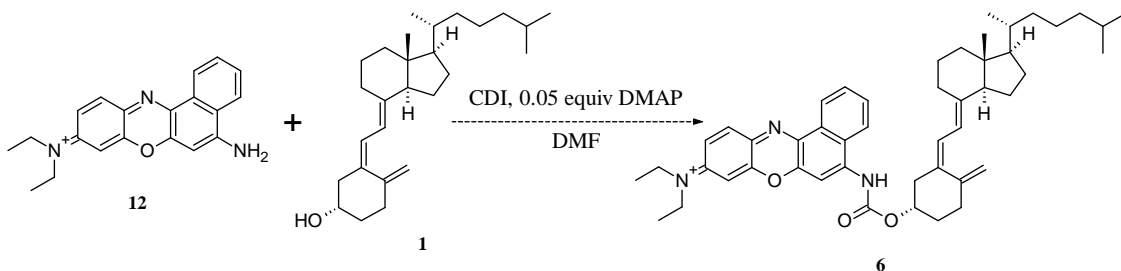
**Scheme 11:** Methanol adding into Nile Blue A imidazole **23** to form Nile Blue A/methanol adduct **24**

Isolation of imidazole **23** is very difficult. To work up the reaction an extraction was performed using dichloromethane, and similar to the coupling in Scheme 8, there was poor

separation between the aqueous layer and organic layer. Attempts to work up this reaction lead to entire loss of Nile Blue A imidazole **23**. Therefore, the CDI coupling with Nile Blue A (**12**) was completed as a one-pot two-step reaction.

The one-pot two-step coupling of Nile Blue A (**12**) and vitamin D<sub>3</sub> (**1**) was performed as seen in Scheme 4 (and Table 3). The reaction was performed with CDI, 0.05eq DMAP, and DMF. The temperature varied from 40°C to 85°C and the length of the reaction varied from 25 h to 216 h as seen in Table 3. When monitoring *via* LRMS Nile Blue A imidazole **23** is assumed to be forming due to the presence of the 376 m/z peak (Nile Blue A/methanol adduct **24**, Scheme 11), however Nile Blue A imidazole **23** was not observed *via* <sup>1</sup>H NMR. The final product **6** was visualized also *via* LRMS but again was not visible *via* <sup>1</sup>H NMR. Therefore it was assumed that the reaction did occur, just not in an appreciable yield.

**Table 3:** Conditions for formation of Nile Blue A/vitamin D<sub>3</sub> carbamate **6**



Entry	Eq. of <b>1</b>	Temperature	Duration
1	1	40°C	191hr
2	1	80°C	96hr
3	1	85°C	25hr
4	3	60°C	146hr
5	3	40°C	216hr

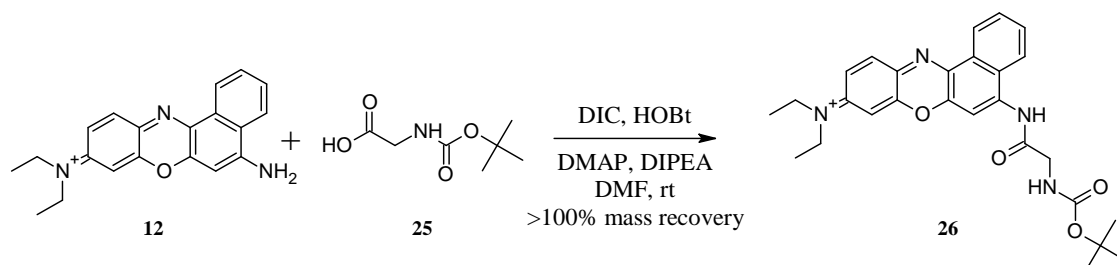


A novel work up for this reaction was discovered that may be effective for other Nile Blue A (**12**) coupling reactions. Rather than attempting to extract with an organic solvent (which leads to an inability to distinguish between organic and aqueous layers and the development of rag layers), adding in an aqueous wash precipitated the product out. The reaction was diluted in 20mL 50% brine and then suction filtered. Afterwards it was observed that an abundance of unreacted Nile Blue A (**12**) was left in the solid, so the product was then triturated with EtOAc overnight. The solution was then filtered and concentrated resulting in a sticky blue/purple material.

After failure to obtain any product from experiments described in Table 3, the CDI coupling approach was abandoned. The coupling reaction ran for over 168 h at an elevated temperature yielding no appreciable amount. The failure of the coupling reaction was thought to be due to Nile Blue A (**12**) being a better leaving group than imidazole which was observed by LRMS. Therefore, when vitamin D<sub>3</sub> (**1**) added in to form the carbamate, Nile Blue A (**12**) would be displaced rather than the imidazole ring. The CDI coupling was abandoned and alternate coupling reactions were investigated.

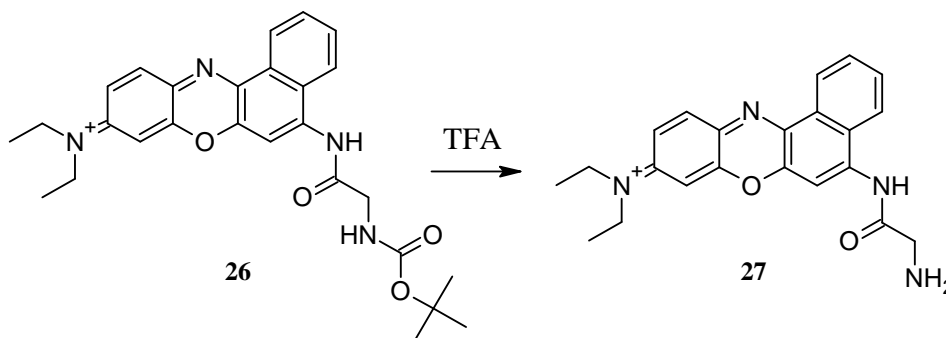
### 3.1.5 Diisopropylcarbodiimide coupling

Diisopropylcarbodiimide (DIC) is a peptide coupling reagent, and is used in conjunction with *N*-hydroxybenzotriazole to form amide bonds<sup>20</sup>. The reaction conditions from Zhang and his associates<sup>21</sup> were combined with the reaction conditions from Ho and his associates<sup>22</sup> to develop conditions that work with the system at hand (Scheme 12).



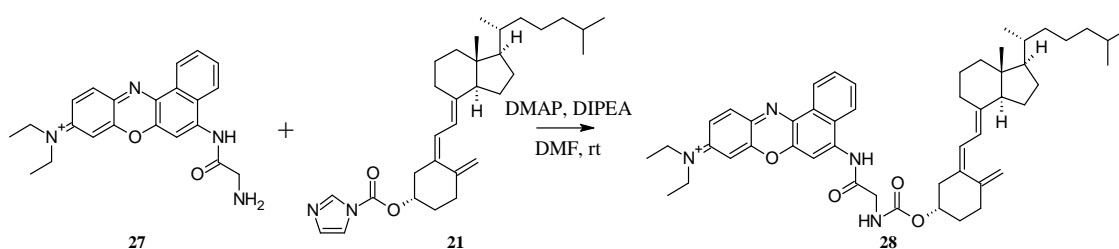
**Scheme 12:** DIC coupling between Nile Blue A (**12**) and Boc-Gly-OH (**25**)

Nile Blue A (**12**) was coupled with Boc-protected glycine (**25**) to form amide **26** (Scheme 12). Coupling to the glycine molecule allows the amine that is to be reacted with to be changed from an aromatic amine to an aliphatic amine. This change allows for easier coupling of the amine. Also, the DIC coupling reaction eliminates the flaws that the CDI coupling has such as having imidazole as a worse leaving group than Nile Blue A (**12**). The formation of amide **26** was a relatively easy reaction, taking from 1 - 4 h and proceeding at room temperature. Amide **26** was readily precipitated out with saturated sodium bicarbonate and suction filtered. The Boc protecting group must be removed from amide **26** using TFA prior to additional couplings (Scheme 13)<sup>22</sup>.



**Scheme 13:** Deprotection of Nile Blue A Boc-gly **26** by TFA

The isolation of the deprotected Nile Blue A/glycine **27** has proven to be difficult. The product was precipitated out of solution by diethyl ether but it only afforded an 8-9% yield. The mass peak was observed *via* LRMS. After Nile Blue A/glycine **27** was deprotected it was reacted with vitamin D<sub>3</sub> imidazole **21** (3.0 eq.), and **27** (1.0 eq.) were dissolved in DMF (Scheme 14). To that DMAP (1.0 eq.) and DIPEA (3.0 eq.) were added and the reaction was allowed to stir at rt for 72 h. Once **27** was seen to disappear by LRMS the reaction was diluted in saturated NaHCO<sub>3</sub> and suction filtered to yield carbamate **28**. The mass peak was never seen for carbamate **28** which may be due to the molecule's size. More work needs to be done with this system to fully synthesize carbamate **28** such as optimizing the formation of the deprotected amide **27**, then reacting amide **27** with vitamin D<sub>3</sub> imidazole **21** again.



**Scheme 14:** Formation of carbamate **28**

With all of the problems that have been occurring for the coupling reactions, a different avenue needed to be investigated. “Click” chemistry was studied as an alternate to the failing coupling reactions.

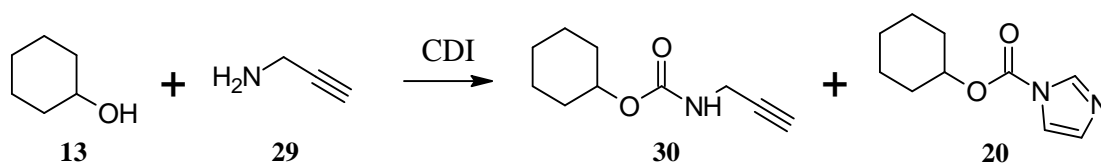
### 3.1.6 “Click” chemistry coupling

“Click” chemistry was first discovered by Ralph Huisgen as a reaction between a terminal alkyne and an azide. These two functional groups react together in the presence of copper (I) to form a 1,2,3-triazole ring<sup>23</sup>. The name “click” chemistry was then coined by Barry Sharpless due to the reaction’s progression at room temperature under aqueous conditions while being open to atmosphere, thus increasing the ease of the chemistry<sup>24</sup>.

Before starting the “click” chemistry the linking functionality (minitag –alkyne and azide) must be coupled to our species. An effective strategy was to use CDI coupling reactions from the previous section. A model system was used again to optimize the conditions prior to testing the target system of vitamin D<sub>3</sub> (**1**) and Nile Blue A (**12**).

#### 3.1.6.1 Model study of “click” chemistry

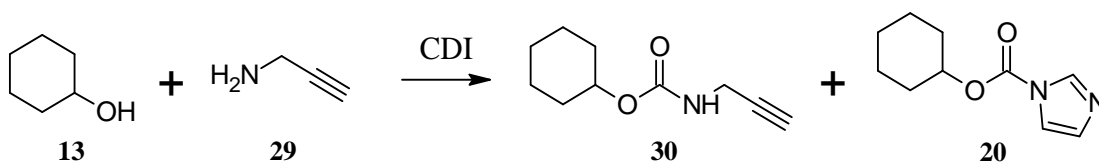
The first step was to synthesize the terminal alkyne minitag on the model cyclohexanol as seen in Scheme 15.



**Scheme 15:** CDI coupling to form model alkyne

This reaction was performed under the original conditions of KOH and toluene before being optimized with DMAP and DCM (Table 4). The optimization involved fully converting cyclohexanol imidazole **20** to alkyne **30** resulting in increased yields of cyclohexanol alkyne **30**. In entries 1-3 (Table 4) the ratio of cyclohexanol (**13**) to propargyl amine (**29**) was optimized. It was found that 1 equivalent of **13** to every 1.5 equivalents of **29** was optimal. The final conditions of 1.0 eq. CDI, DCM and 0.05 eq. DMAP were carried over from the optimization of the formation of cyclohexanol imidazole **20** (Table 4, Entry 4).

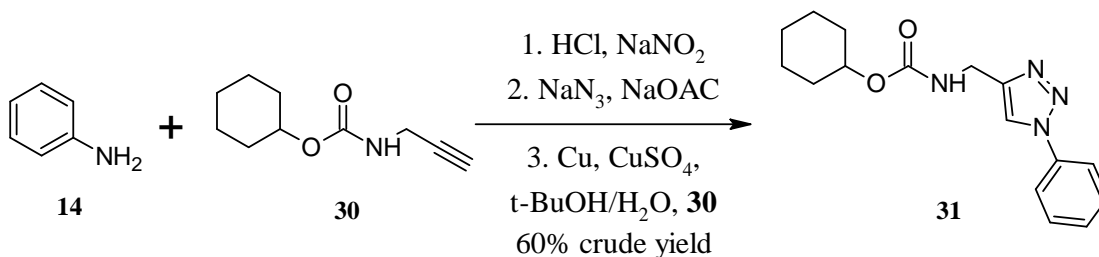
**Table 4:** Conditions for formation of cyclohexanol alkyne **30**



Entry	Conditions	Ratio (alcohol <b>13</b> :amine <b>29</b> )	% Yield <sup>a</sup>	% <b>20</b> <sup>a</sup>
1	CDI, toluene, KOH	2:1	19%	50%
2	CDI, toluene, KOH	1:1	32%	22%
3	CDI, toluene, KOH	1:1.5	30%	28%
4	CDI, DCM, DMAP	1:1.5	54%	15%

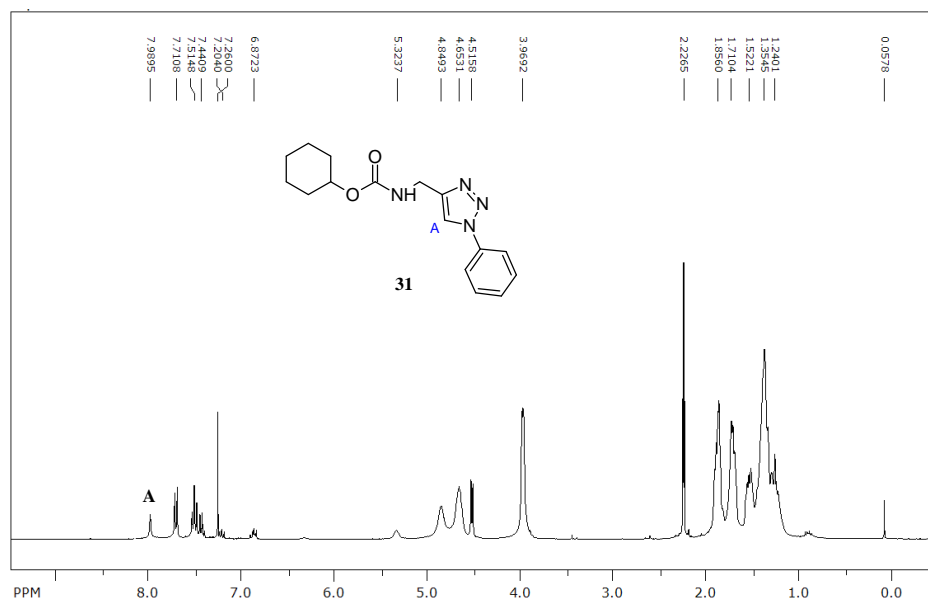
<sup>a</sup> Isolated yields

Once cyclohexanol alkyne **30** was made, a one pot synthesis was carried out that formed the azide on aniline (**14**) and then clicking *in situ* (Scheme 16).



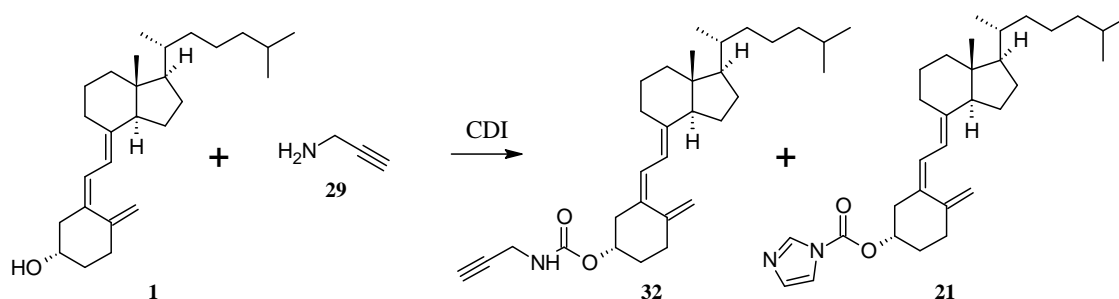
**Scheme 16:** Model system of one pot click reaction

This reaction yielded a 60% crude mass recovery. The 1,2,3-triazole ring was observed using  $^1\text{H}$  NMR (Figure 14) and the mass peak was visualized with LRMS.



**Figure 14:**  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) of cyclohexanol click product **31** with indicative 1,2,3-triazole peak

Triazole **31** was never purified as confirmation of the reaction was the goal. The CDI coupling to prepare vitamin D<sub>3</sub> alkyne **32** was undertaken by using the previously optimized conditions (Table 4, entry 4) for the model system (Table 5, entry 3).

**Table 5:** Conditions for CDI coupling of propargyl amine (**29**) and vitamin D<sub>3</sub> (**1**)

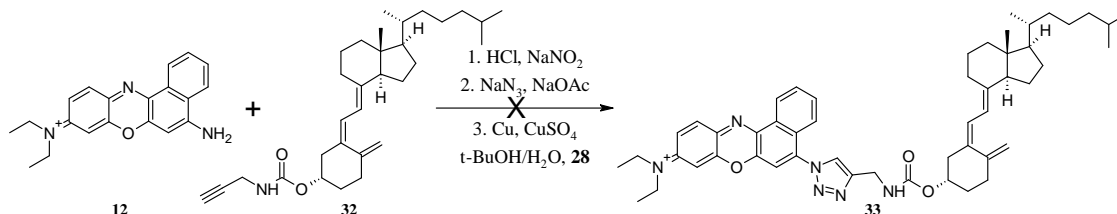
Entry	Conditions	Ratio (alcohol <b>1</b> :amine <b>29</b> )	% Crude Yield <sup>a</sup>	% <b>21</b>
1	CDI, toluene, KOH	1:1	80% (17%)	46%
2	CDI, toluene, KOH	1:1.5	71-85% (32%)	41%
3	CDI, DCM, DMAP	1:1.5	57-85% (40-51%)	<1%

<sup>a</sup> Isolated yield in ( )

As seen in Table 5, vitamin D<sub>3</sub> imidazole intermediate **21** was formed along with vitamin D<sub>3</sub> alkyne **32**. These reactions were performed to optimize the CDI coupling (Table 5). The standard CDI coupling conditions of DCM and 0.05eq DMAP proved to be optimal based on isolated yields.

Nile Blue A (**12**) and vitamin D<sub>3</sub> alkyne **32** were attempted to be clicked together (Scheme 17). To accomplish this linking, an azide needed to be functionalized onto Nile Blue A (**12**); in order to accomplish this 2.0 eq. Nile Blue A (**12**) was dissolved in 50% HCl (0.48mL) at 0°C and 2.4 eq. of NaNO<sub>2</sub> was added. The resulting solution was stirred for 4 h. Then a solution of 4 eq. NaN<sub>3</sub> and 40 eq. NaOAc in 4.3mL of water was added dropwise. The solution was stirred for an additional 15 minutes before adding 0.41mL 7N NaOH, then 5.2mL of t-BuOH, 5.25 eq. Cu<sub>(s)</sub>, 1.3 eq. of CuSO<sub>4</sub> and 1 eq. vitamin D<sub>3</sub> alkyne **32**. The mixture was allowed to stir at rt for 144 h. During the reaction, the disappearance of starting materials was observed by LRMS however no triazole **33** was seen. The indicative 1,2,3-triazole peak (as in Figure 14) was not observed in the crude <sup>1</sup>H NMR, therefore the one-pot click reaction was abandoned for approach A (linking

before ingestion). Further discussions regarding the formation of the azide on Nile Blue A can be found in section 3.2.2.



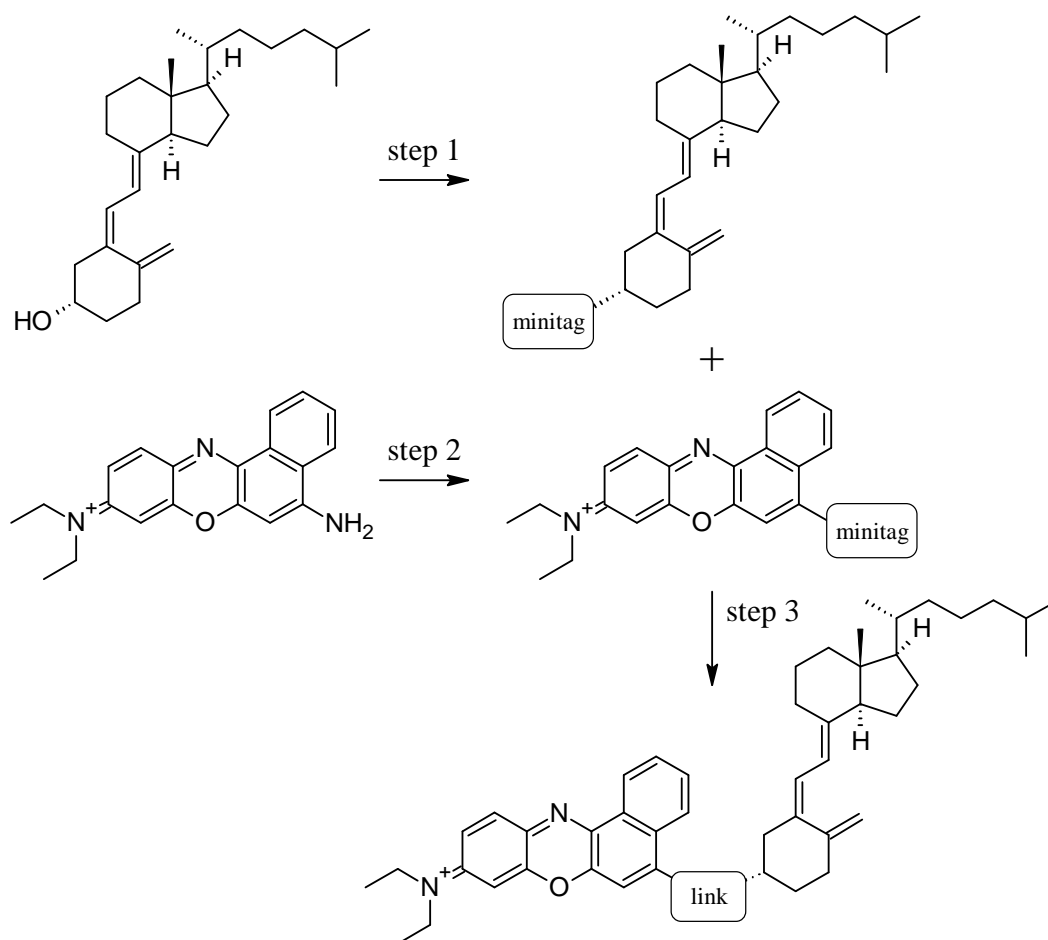
**Scheme 17:** One pot click coupling of **12** and **32**

A future direction for this reaction could be to run this again, but use 10 equivalents of Nile Blue A (**12**) to assure that enough of the azide is formed.

### 3.2 Linking to the fluorophore after ingestion (approach B)

The benefit of linking the fluorophore to vitamin D<sub>3</sub> (**1**) after ingestion to the daphnia is the smaller size of the molecule being ingested by daphnia and polarity. The fluorophore attached to the vitamin D<sub>3</sub> (**1**) may obstruct the second active site on vitamin D<sub>3</sub> (**1**) which then would hinder the daphnia's ability to utilize the compound the same as vitamin D<sub>3</sub> (**1**). In approach B (linking after ingestion) a minitag on vitamin D<sub>3</sub> (**1**) and Nile Blue A (**12**) are created then the two are linked after ingestion of the daphnia. The minitags are either a terminal alkyne or an azide, which will be used in subsequent click reactions

There are three steps to approach B (linking after ingestion): forming minitagged vitamin D<sub>3</sub> (**1**), forming minitagged Nile Blue A (**12**), then clicking the two compounds together (Scheme 18).



**Scheme 18:** Three steps for approach B (linking after ingestion)

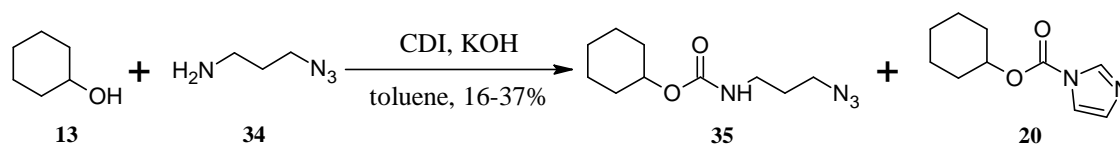
### 3.2.1 Synthesis of minitagged vitamin D<sub>3</sub> (**1**)

In click chemistry there are two functional groups that are necessary to form the 1,2,3-triazole ring. Both of these functional groups are going to function as minitags for the proposed approach B (linking after ingestion), as they are small additions to the larger molecule of vitamin D<sub>3</sub> (**1**) or Nile Blue A (**12**). The two minitags that need to be present are a terminal alkyne and an azide group.

Propargyl amine (**29**), was linked onto vitamin D<sub>3</sub> (**1**) to form vitamin D<sub>3</sub> alkyne **32** using the optimized CDI reaction from approach A (linking before ingestion) (Table 5). Then the azide minitag was synthesized on vitamin D<sub>3</sub> (**1**), first with the model system. These reactions were done so as to have vitamin D<sub>3</sub> (**1**) linked with both possible minitags.

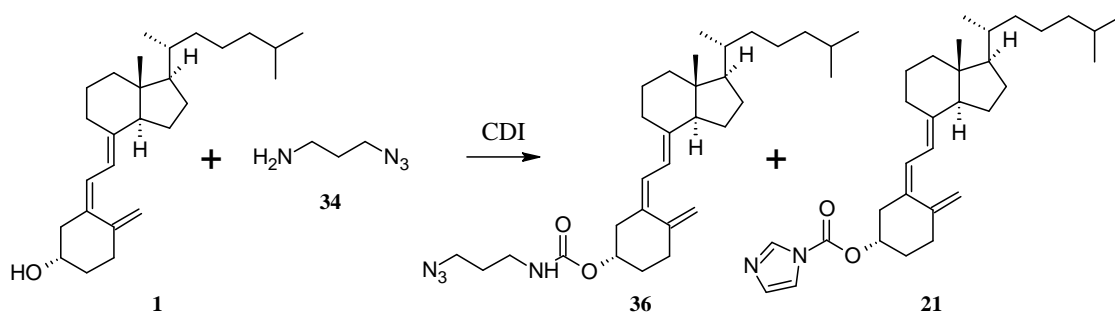


Azide **34** was synthesized using the conditions from Carboni, Benalil and Vaultier<sup>25</sup> by stirring 9.75g of 3-chloropropyl amine hydrochloride (75 mmol, 1 eq.) with 14.64g of sodium azide (225 mmol, 3 eq.) in 225mL water for 18h at 80°C. At the end of the reaction most of the water was removed by vacuum distillation, followed by addition of Et<sub>2</sub>O (300mL) and KOH (24g) pellets. The aqueous layer was extracted with Et<sub>2</sub>O. The organics were washed with brine (120mL), dried over magnesium sulfate and concentrated to yield 6.46g of 3-azidopropan-1-amine (**34**) (86% yield), which was used without purification. The reaction in Scheme 19 was run prior condition optimization with DCM and DMAP. The reaction yielded 24-37% of cyclohexanol imidazole **20** in addition to cyclohexanol azide **35** being formed.



**Scheme 19:** Formation of azide minitagged cyclohexanol **35**

When the KOH reaction conditions were extrapolated to the vitamin D<sub>3</sub> (**1**) system a significant amount of vitamin D<sub>3</sub> imidazole **21** was obtained (Table 6, Entry 1). As seen in Table 6, the yields of formation of azide **36** are less than that of the formation of vitamin D<sub>3</sub> alkyne **32** (Table 5). This may be a result of the instability of azide **36** compared to alkyne **32**. It was predicted that alkyne **32** would be an efficient alkyne coupling partner for the click chemistry. Vitamin D<sub>3</sub> (**1**) minitag was successful with both the azide and the alkyne functionality.

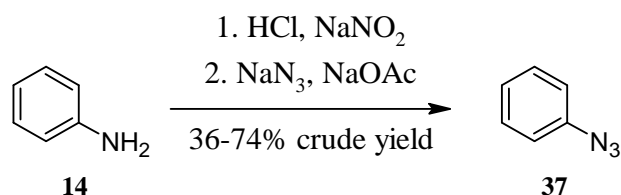
**Table 6:** Conditions for formation of compound **36**

Entry	Conditions	% yield <b>36</b>	% <b>21</b>
1	CDI, toluene, KOH	51%	62%
2	CDI, DCM, DMAP	63% (crude)	ND <sup>a</sup>

<sup>a</sup> Not detected by <sup>1</sup>H NMR of crude product

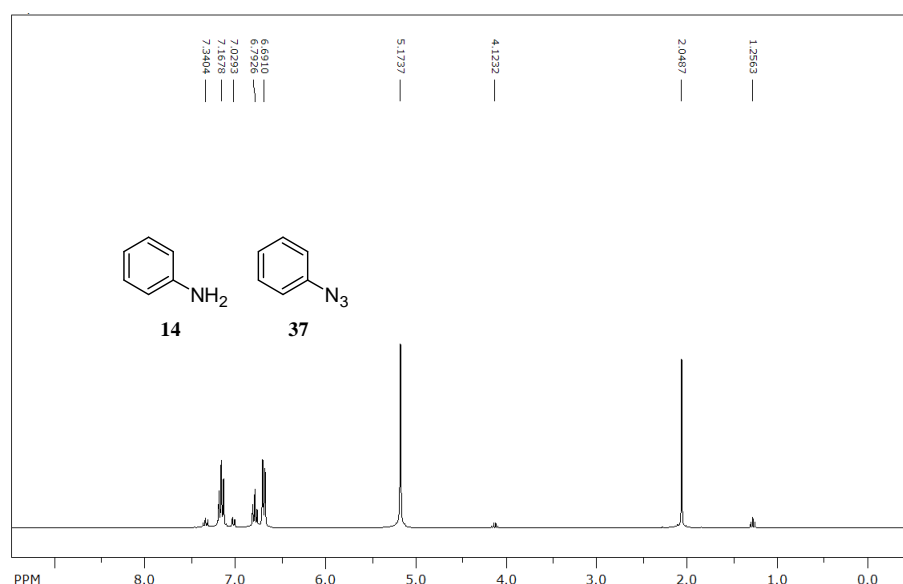
### 3.2.2 Synthesis of minitagged Nile Blue A (**12**)

For “click” chemistry whichever minitag vitamin D<sub>3</sub> (**1**) has linked to it, Nile Blue A (**12**) needs the corresponding minitag. Seeing as the alkyne had higher yields, putting the azide on Nile Blue A (**12**) was focused on. Nile Blue A (**12**) has an amine functionality and utilizing Hu and his associates’ reaction conditions it is possible to convert the amine group into an azide group<sup>26</sup>. Using aniline as an example the standard conditions are as follows: to 560mg of aniline (6 mmol, 1.0 eq.) in a solution with 20mL 50% HCl at 0°C was added 497mg of NaNO<sub>3</sub> (7.2 mmol, 1.2 eq.). The solution stirred for 3 h then 780mg of NaN<sub>3</sub> (12 mmol, 2.0 eq.) and 9.84g NaOAc (120 mmol, 20 eq.) in 30mL of water was added dropwise over 15 minutes. The reaction was extracted with EtOAc (3 x 25mL). Combined organics were washed with brine (25mL), dried over magnesium sulfate and concentrated to yield 526mg of azidobenzene in a 74% yield (Scheme 20). Using aniline was done to practice forming the azide and to use it as a model system in click chemistry.



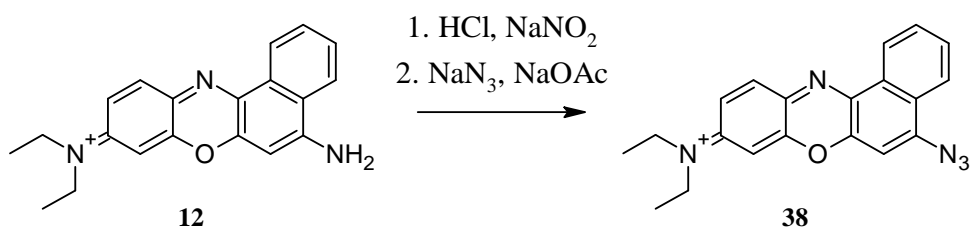
**Scheme 20:** Formation of azide **37**

With the use of  $^1\text{H}$  NMR, azide **37** was deemed pure enough to use in further reactions. Azide **37** was mixed with 1 equivalent of aniline (**14**) to prove that azide **37** was not contaminated with aniline (Figure 15). In the aromatic region there are two distinct groups of aromatic peaks showing aniline (**14**) and the shifted aromatic peaks of azide **37**. The reaction conditions above gave reasonable yields so it was then applied to Nile Blue A (**12**) (Scheme 21).



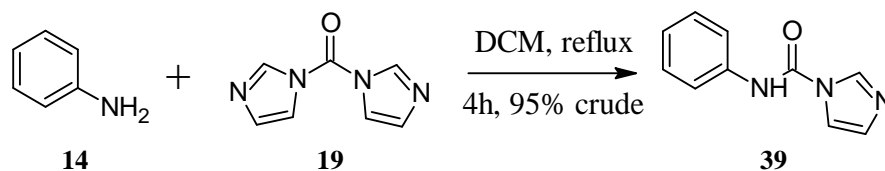
**Figure 15:**  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) of a mixture of aniline (**14**) and azide **37**

The same reaction conditions as in Scheme 20 were used on the Nile Blue A (**12**) system. Nile Blue A azide **38** was never successfully isolated. It was seen to exist *via* LRMS, however the  $^1\text{H}$  NMR was a complex mixture. Aromatic azides are known to be unstable and isolation has not been achieved thus far. It was then thought that if Nile Blue A azide **38** was prepared it would be able to undergo the click reaction without being isolated in a one-pot two-step reaction which was attempted in Scheme 17.



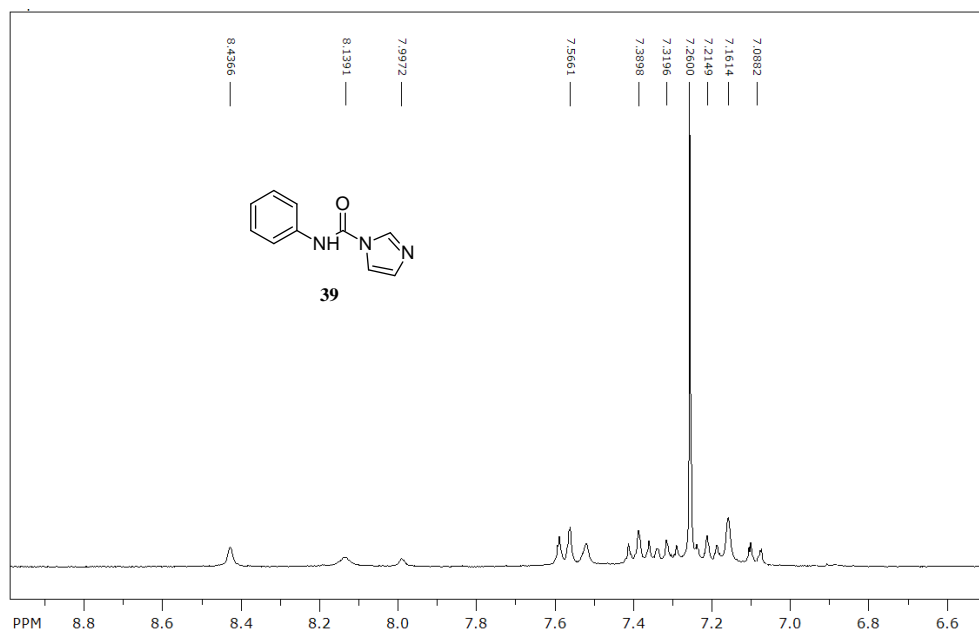
**Scheme 21:** Formation of Nile Blue A azide **38**

In order to circumvent the problem of the aromatic azides decomposing a coupling between Nile Blue A (**12**) and azide **34** was undertaken. Before this was done it was attempted using the model system (Scheme 22).



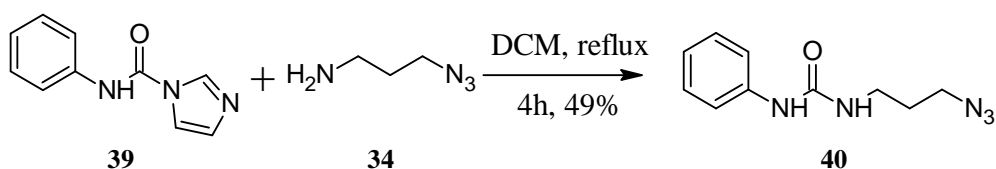
**Scheme 22:** CDI addition onto aniline (**14**)

Aniline (**14**) (500mg, 5.4mmol, 1eq.) and 960mg of CDI (5.9mmol, 1.1eq.) were refluxed in 2.2mL DCM for 4 h. The solution was diluted in 30mL DCM, washed with distilled water (3x12mL), brine (12mL), dried over magnesium sulfate and concentrated to yield 962mg of aniline imidazole **39** (5.1 mmol, 95% yield)<sup>19</sup>. The synthesis of aniline imidazole **39** was successful with a 95% crude yield.



**Figure 16:** <sup>1</sup>H NMR of aniline imidazole **39**

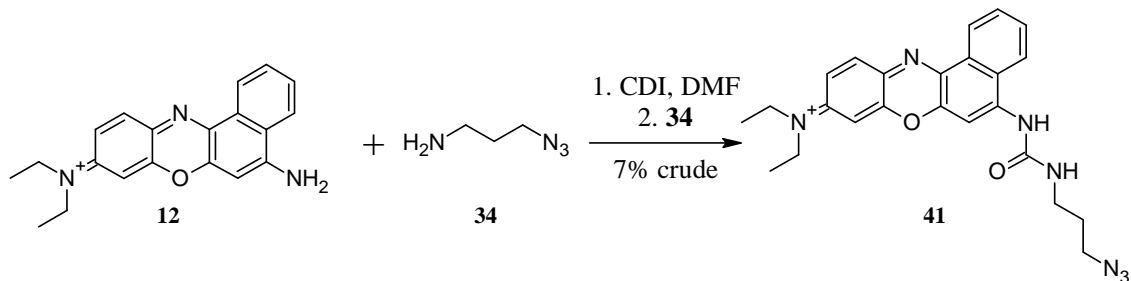
The aniline imidazole **39** was observed *via* <sup>1</sup>H NMR (Figure 16). The imidazole peaks are hidden by the aromatic peaks, however one is observed at 7.16 ppm. One of the peaks downfield can be assigned as the amide proton on the structure. Aniline imidazole **39** was utilized without purification in the next step (Scheme 23).



**Scheme 23:** Formation of azide linked aniline **40**

Azide **40** was synthesized by refluxing 500mg of aniline imidazole **39** (2.7mmol, 1.0 eq.) and 267mg of azide **34** (2.7mmol, 1.0 eq.) in 1.0mL DCM for 4 h. At the end of the reaction the mixture was diluted in 80mL Et<sub>2</sub>O, washed with 0.5N HCl (3x20mL), saturated sodium bicarbonate (20mL), brine (20mL), dried over magnesium sulfate, and concentrated. The product was purified using silica gel column chromatography (40% EtOAc/hexanes → 70%

EtOAc/hexanes) to yield 290mg of azide **40** (1.3 mmol, 49% yield). Seeing as this reaction was successful it was then extrapolated to the real system of Nile Blue A (**12**) (Scheme 28).

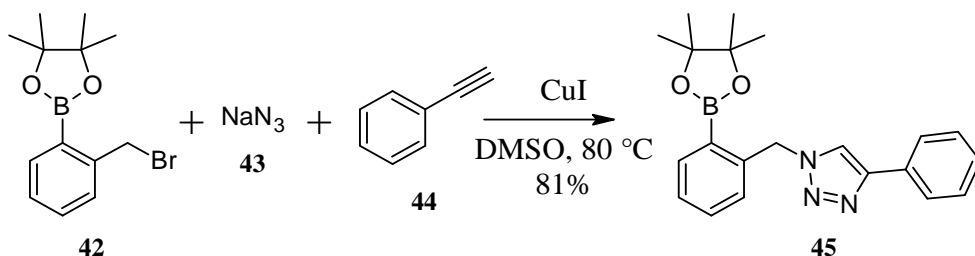


**Scheme 24:** Formation of Nile Blue A azide **41**

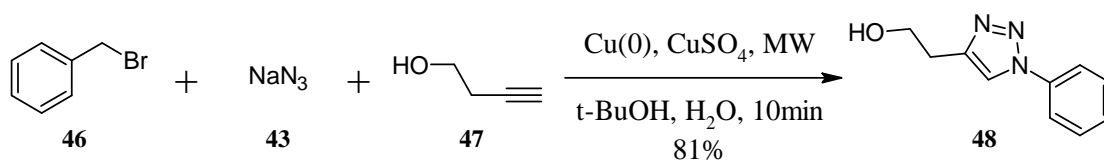
Nile Blue A azide **41** was visualized *via* LRMS however the  $^1\text{H}$  NMR is a complex mixture, 7% mass recovery. As has been previously stated (Section 3.1.4.1) CDI coupling with Nile Blue A (**12**) does not work, and this route was abandoned.

### 3.2.3 “Click” chemistry

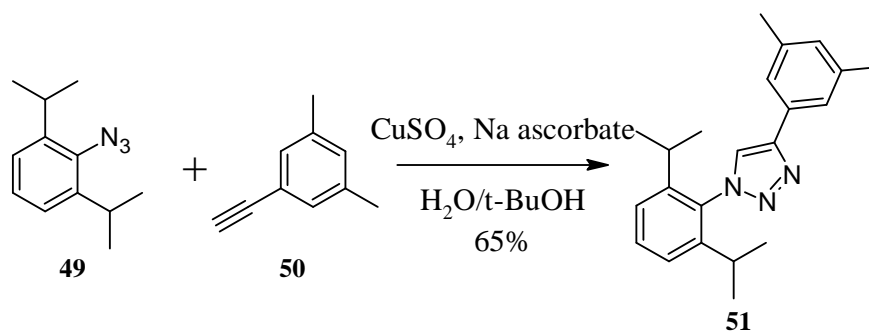
Using  $\text{Cu}^{(I)}$  catalyzed “click” chemistry will be an essential component to approach B (linking after ingestion). Therefore, preparation and utilization of azides and alkyne compounds in developing a robust method to link the fluorophore and vitamin D<sub>3</sub> (**1**) has been undertaken. “Click” reaction conditions vary based on which  $\text{Cu}^{(I)}$  source they used, which can be seen by the examples of Scrafton and coworkers who uses  $\text{CuI}^{12}$  (Scheme 25) and Appukkuttan and coworkers who uses  $\text{Cu}_2\text{S}/\text{CuSO}_4^{27}$  (Scheme 26). Nakamura and coworkers use  $\text{CuSO}_4$  and sodium ascorbate to perform “click” chemistry<sup>28</sup> (Scheme 27).



**Scheme 25:** Scrafton et. al.  $\text{CuI}$  reaction conditions<sup>12</sup>



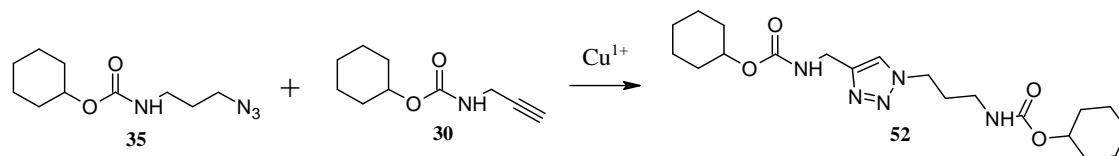
**Scheme 26:** Appukkuttan et. al. Cu<sub>(s)</sub>/CuSO<sub>4</sub> reaction conditions<sup>26</sup>



**Scheme 27:** Nakamura et. al. CuSO<sub>4</sub>/Na ascorbate reaction conditions<sup>28</sup>

A comparison of the three conditions is summarized in Table 7 using the model system azide **35** and alkyne **30**.

**Table 7:** Click chemistry conditions for the formation of triazole **52**



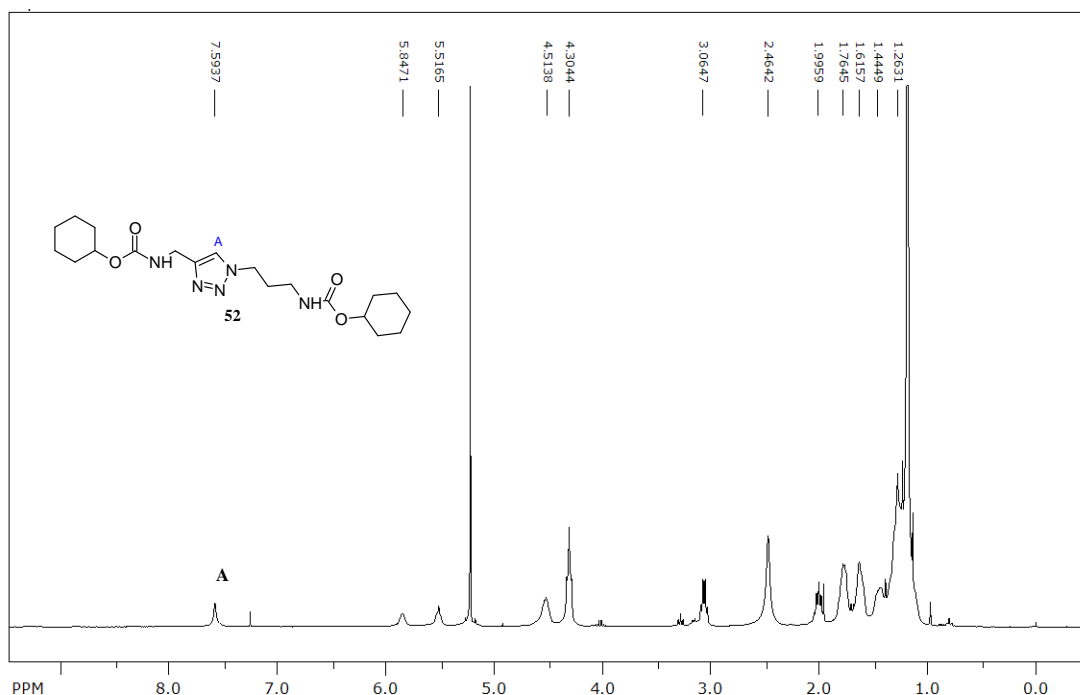
Entry	Conditions	Yield of <b>52</b> <sup>a</sup>
1	Cu, CuSO <sub>4</sub> , H <sub>2</sub> O/t-BuOH, rt, 18hr	68%
2	CuI, DMSO, 80°C, 5hr	32%
3	CuSO <sub>4</sub> , Na ascorbate, H <sub>2</sub> O/t-BuOH, rt, 18hr	9%

<sup>a</sup> Isolated yield

All sets of conditions yielded product. When utilizing Cu<sub>(s)</sub> and CuSO<sub>4</sub> (Table 7, entry 1) a higher yield was obtained. Therefore these reaction conditions were used in the majority of reactions. It was not until later that the third condition was attempted (Table 7, entry 3). It has a low yield however the product was mostly lost during purification and the reaction was never

repeated. When running the click reaction using the Na ascorbate procedure, there was a very high crude yield with a very clear looking  $^1\text{H}$  NMR. Crude azide **52** was then purified where the majority of the product degraded during column chromatography. Due to the high purity of the crude product and the high yield of it, the Na ascorbate procedure was deemed the preferred procedure for future click reactions.

A 1,2,3-triazole ring is easy to distinguish when looking at  $^1\text{H}$  NMR due to the singlet it produces at 7.6-8.0ppm from the single proton in the triazole ring (i.e. triazole **52** (Figure 17)). The 1,2,3-triazole peak was seen for triazole **52** so the reaction conditions (Table 7, entry 1) were applied to the vitamin D<sub>3</sub> alkyne **32**.

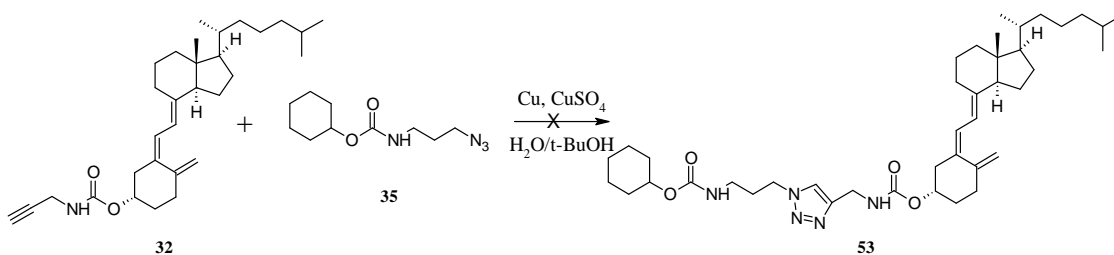


**Figure 17:**  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) of triazole **52** using entry 3 conditions of Table 7

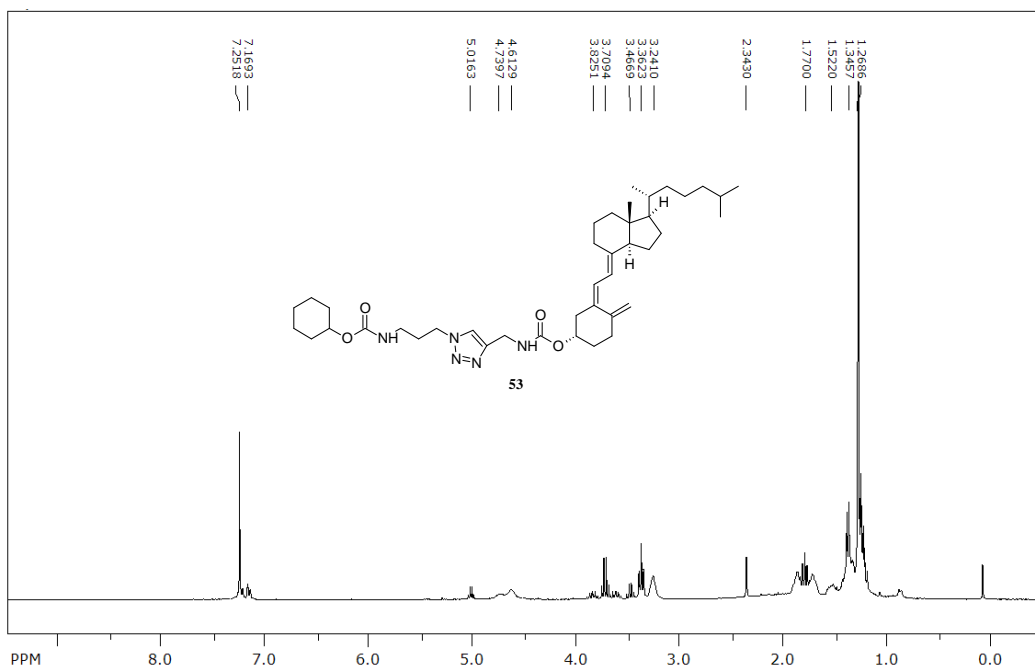
The formation of vitamin D<sub>3</sub> triazole **53** was attempted by dissolving 100mg of alkyne **32** (0.2mmol, 1.0 eq.) in 0.6mL 1:1 t-BuOH:H<sub>2</sub>O, adding 50mg of azide **35** (0.2mmol, 1.0 eq.), 11mg Cu<sub>(s)</sub> (0.17mmol, 0.8 eq.), 7mg CuSO<sub>4</sub> (0.04mmol, 0.2 eq.), and stirring at rt for 20h (Scheme 28). At the end of the reaction, the solution was diluted in 12mL Et<sub>2</sub>O and 12mL water,



filtered and separated. The aqueous layer was extracted with Et<sub>2</sub>O (2x10mL). The combined organic layers were washed with brine (6mL), dried over magnesium sulfate, and concentrated. Due to the lack of the 1,2,3-triazole peak around 8.0ppm in the <sup>1</sup>H NMR (Figure 18), it was surmised that the reaction did not produce any triazole **53**.

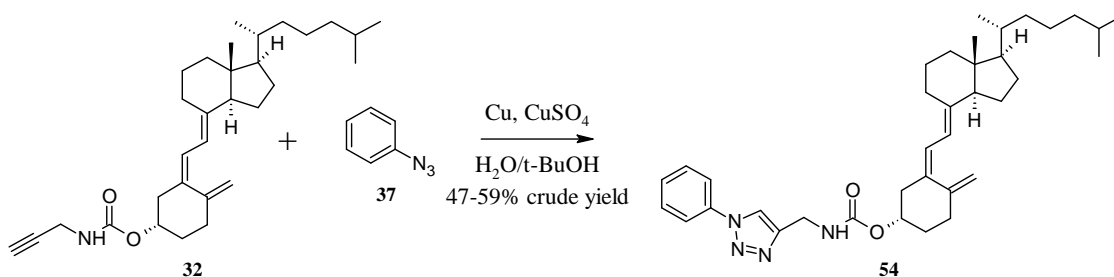


**Scheme 28:** Click chemistry formation of vitamin D<sub>3</sub> triazole **53**



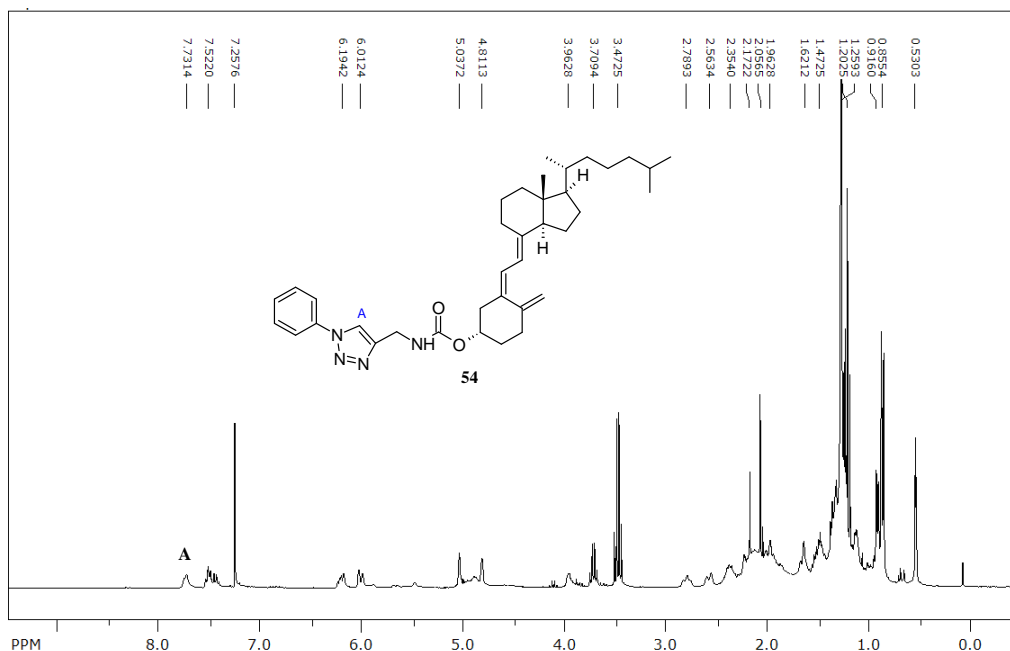
**Figure 18:** <sup>1</sup>H NMR (CDCl<sub>3</sub>) of crude vitamin D<sub>3</sub> triazole **53**

Another click reaction was investigated using vitamin D<sub>3</sub> alkyne **32** and azide **37** (Scheme 29).



**Scheme 29:** Click chemistry formation of aniline triazole **54**

The click reaction was conducted under the same conditions as the attempted synthesis of **53** (Scheme 28), however it had higher crude yields of 47-59%. The 1,2,3-triazole peak observed was indicative of aniline triazole **54** being produced. There was no mass peak observed by LRMS, so the assumption that the peak at 7.73 was the 1,2,3-triazole peak could be wrong. Peak A (Figure 19) also falls into the range of the exchangeable proton of a carbamate group. Therefore, it is inconclusive if aniline triazole **54** was synthesized. The click reaction should be repeated using the Na ascorbate conditions for the formation of triazole **54** (Table 7, Entry 3).



**Figure 19:** <sup>1</sup>H NMR (CDCl<sub>3</sub>) of aniline triazole **54**

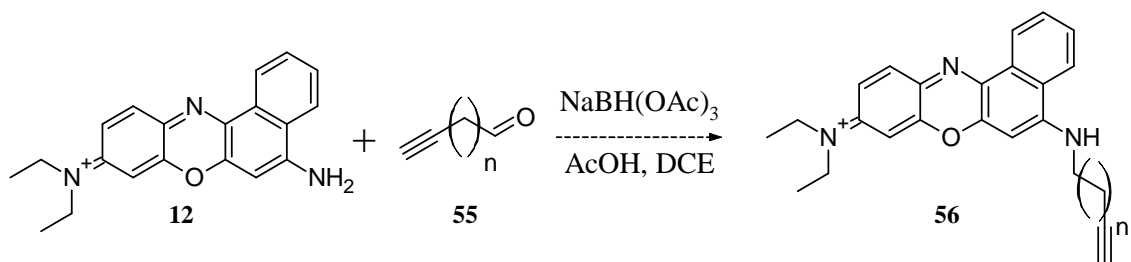
#### 4. Future Directions

Currently there are still aspects of this research that need to be further investigated. The following sections will discuss how to further this work.

##### 4.1 Possible routes for approach A (linking before ingestion)

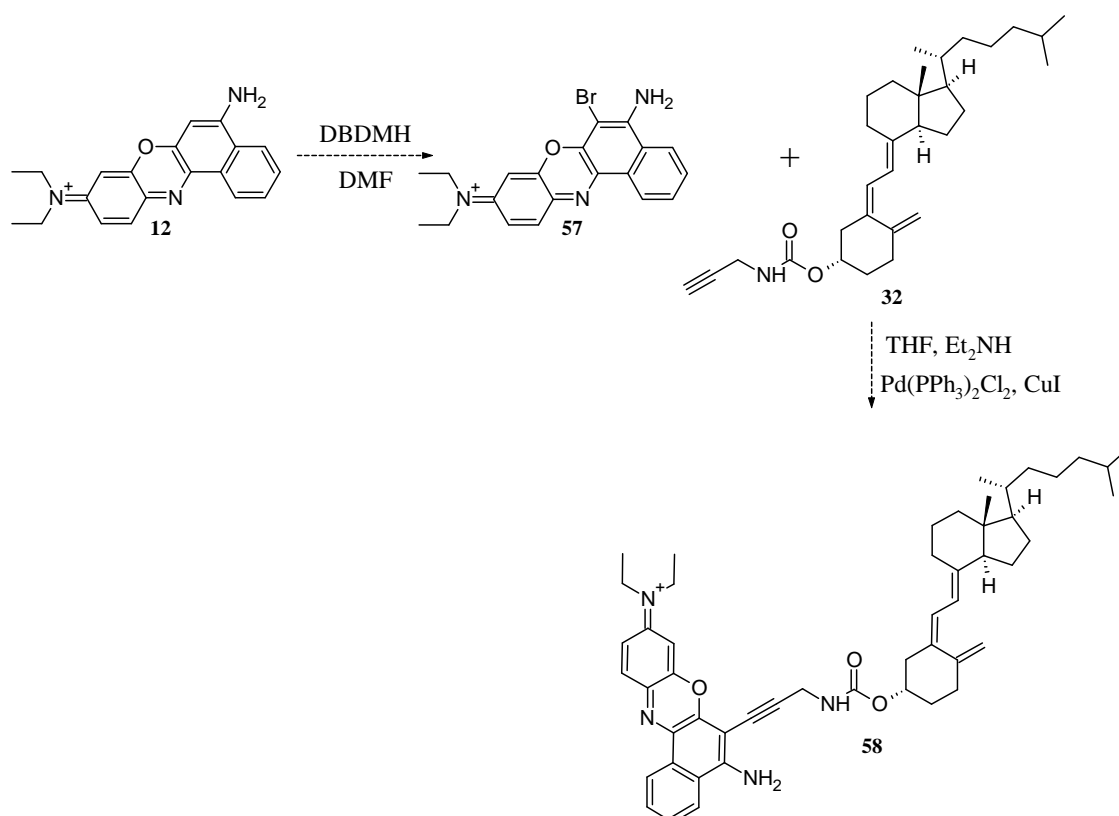
There were difficulties in synthesizing a carbamate bond on Nile Blue A (**12**) using the CDI coupling method. Other methods are left to be explored such as using reductive amination and Sonogashira coupling.

The reductive amination can be used to link on an aldehyde that has an alkyne (or azide) on it to then have the product be clicked with the partnering vitamin D<sub>3</sub> (**1**) minitag (Scheme 30). We have initiated studies on the reductive amination and it looks like a promising avenue.



**Scheme 30:** Proposed reductive amination method

Another option is to brominate Nile Blue A (**12**), then perform a Sonogashira coupling to link Nile Blue A (**12**) and vitamin D<sub>3</sub> (**1**) (Scheme 31).



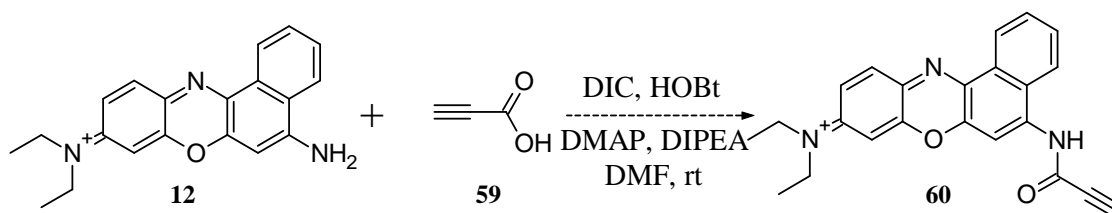
**Scheme 31:** Proposed Sonogashira coupling method

Lastly, in terms of the DIC coupling (Section 3.1.5) further work needs to be done to optimize the conditions of TFA deprotection reactions (Scheme 13). Higher yields need to be obtained to keep DIC coupling a viable option.

#### 4.2 Possible routes for approach B (linking after ingestion)

In addition to developing approach B (linking after ingestion) to address polarity and binding issues it is also important to have the wavelength change once the vitamin D<sub>3</sub> (**1**) has been linked *in vivo*. This change in wavelength is important so that it is possible to distinguish between linked fluorophore and unlinked fluorophore. Both minitags were synthesized on vitamin D<sub>3</sub> (**1**), however an effective minitag on Nile Blue A (**12**) that is compatible with the aromatic ring has yet to be produced.

The DIC coupling can be used to fulfill the requirements for approach B (linking after ingestion). If propiolic acid (**59**) was coupled to Nile Blue A (**12**) the conjugation can be extended therefore shifting the wavelength of the absorbance once it is clicked (Scheme 32).



**Scheme 32:** Proposed DIC coupling for approach B (linking after ingestion)

## Conclusions

A method of minitagging vitamin D<sub>3</sub> (**1**) was described above. Vitamin D<sub>3</sub> (**1**) was functionalized with both an alkyne and an azide successfully while limiting the formation of intermediate imidazole compounds. The functionalization of Nile Blue A (**12**) was described utilizing DIC coupling. High percent recoveries were obtained with more work needed to be done on the deprotection of the Boc group. “Click” chemistry was studied as is still a viable option for the *in vivo* coupling of vitamin D<sub>3</sub> (**1**) and Nile Blue A (**12**). Multiple avenues were discussed for future directions for this project such as Sonogashira coupling, reductive amination and further use of DIC coupling.

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### **General Experimental Procedure**

All reactions were stirred magnetically. Reactions were heated in a sand bath encased in a heating mantel, controlling the temperature with a variable transformer. Temperatures reported refer to bath temperatures unless otherwise noted. Distillations were performed under argon atmosphere. Vacuum distillations utilized a water aspirator ( $> 15$  mmHg).

The phrase “concentrated” or “concentrated in vacuo” refers to removal of solvents *via* a rotary-evaporator using a water aspirator.

### **Reagents and Solvents**

All reagents and solvents were of commercial grade and were used as obtained from the suppliers except for the ones listed below that were purified as follows:

Recrystallized copper iodide using the following procedure: 16g CuI was added to a boiling saturated solution of 50mL distilled H<sub>2</sub>O and 50g NaI. The solution was boiled for 15 minutes. Then added 1g of decolorizing charcoal and boiled the solution for an additional 15 minutes. Filtered and then diluted the cooled filtrate with 50mL H<sub>2</sub>O. The resulting crystals were then filtered and washed with the following: water (10mL), ethanol (10mL), ethyl acetate (10mL), diethyl ether (10mL), and pentane (10mL). The crystals were then dried overnight under vacuum from an oil pump and stored in a desiccator.

Cyclohexanol was distilled under vacuum from CaO.

Aniline was distilled under vacuum from CaH<sub>2</sub>.

### **Chromatography**

“Chromatography” refers to flash column chromatography using 230-400 mesh silica gel (EMD Reagents). Analytical and preparative thin layer chromatography (TLC) was performed on EMD pre-coated silica gel 60 F-254 plastic plates (0.25mm). Visualization was performed by short wave UV light and/or staining the plate followed by heating with a heat gun for approximately 15 seconds when appropriate. *P*-anisaldehyde stain was prepared by mixing 15mL of *p*-



anisaldehyde, 3mL glacial acetic acid, 10mL concentrated sulfuric acid, and 260mL of 95% ethanol.

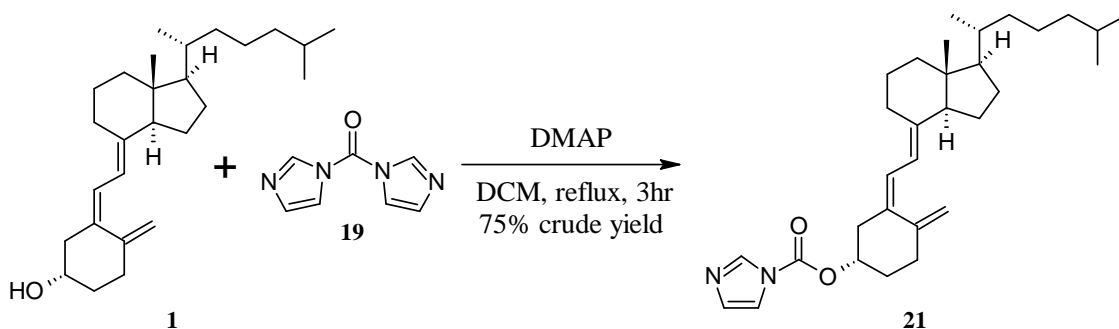
### **Physical Data and Instrumentation**

Proton Nuclear Magnetic Resonance ( $^1\text{H}$  NMR) spectra were obtained on a Bruker DRX-300 (300MHz) instrument. Chemical shifts are reported in ppm downfield and are internally referenced using  $\text{CDCl}_3$  (7.26 ppm) or DMSO (2.50 ppm) as solvent. Proton NMR data are reported as follows: chemical shift (multiplicity, coupling constants in Hz, number of protons). Multiplicity was abbreviated as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of a doublet), dt (doublet of a triplet), s (br) (broad singlet).

Low-resolution mass spectra were obtained using an Applied Biosystems – Q Trap 3200 – LC-MS/MS.

## **Experimentals**

The following pages contain experimental procedures for several reactions portrayed in this thesis.



**Imidazole-1-carboxylic acid 3-{2-[1-(1,5-dimethylhexyl)-7a-methyloctahydroinden-4-ylidene]ethylidene}-4-methylenecyclohexyl ester (21)**

To 211mg of CDI (1.3 mmol, 1.0 eq.) in 5.2 mL of DCM at 40°C under argon atmosphere and in the absence of light was added 8mg of DMAP (0.06 mmol, 0.05 eq.) and 500mg of vitamin D<sub>3</sub> (**1**). The solution was stirred for 3 h. The reaction was monitored by silica gel TLC ( $R_f$ =0.49 ethyl acetate/hexanes 1:1). At the end of the reaction, the solution was allowed to cool to room temperature. The organics were washed with water (2 x 5mL), and brine (5mL), dried over magnesium sulfate, filtered, and concentrated *in vacuo* to yield 493mg (1.0 mmol, 75% crude yield). At times the crude product does require purification. The crude product may be purified using silica gel column chromatography (5% EtOAc/hexanes  $\rightarrow$  40% EtOAc/hexanes) 58% recovery of imidazole-1-carboxylic acid 3-{2-[1-(1,5-dimethylhexyl)-7a-methyloctahydroinden-4-ylidene]ethylidene}-4-methylenecyclohexyl ester (**21**) as a yellow solid product.

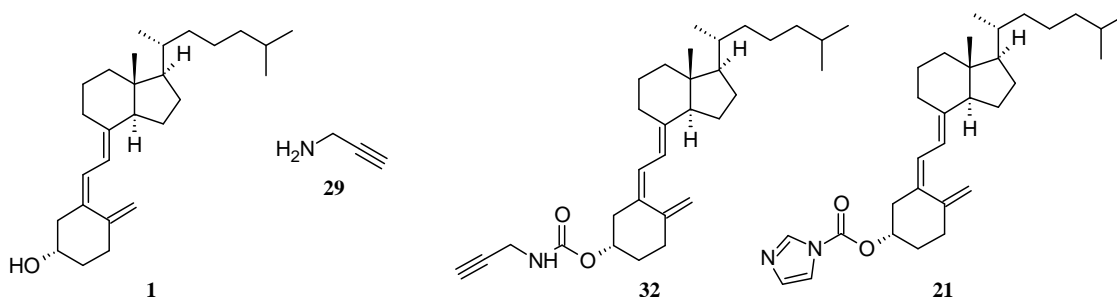
**Compound 21**

$R_f$ =0.49 (50% EtOAc/Hex)

IR  $\nu_{\max}$  (CHCl<sub>3</sub>)/cm<sup>-1</sup>: 2949, 1751, 1394, 1278, 1238, 1001, 906, 729, 648

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  8.12 (s, 1H), 7.40 (s, 1H), 7.04 (s, 1H), 6.25 (d,  $J$  = 11.25 Hz, 1H), 6.01 (d,  $J$  = 11.43, 1H), 5.19 (m, 1H), 5.10 (s, 1H), 4.88 (s, 1H), 2.81-2.65 (m, 2H), 2.58-2.38 (m, 2H), 2.33-2.21 (m, 1H), 2.15-1.80 (m, 4H), 1.69-1.60 (m, 2H), 1.56-1.42 (m, 4H), 1.37-1.22 (m, 4H), 1.14-1.08 (m, 2H), 0.90 (d,  $J$  = 6.06Hz, 3H), 0.84 (d,  $J$  = 6.57, 6H), 0.53 (s, 3H)

LRMS (ESI+): Exact mass calculated for  $C_{31}H_{46}N_2O_2[M+Na^+] = 501.35$ , found = 501.6



**Prop-2-ynylcarbamic acid 3-{2-[1-(1,5-dimethylhexyl)-7a-methyloctahydroinden-4-ylidene]ethylidene}-4-methylenecyclohexyl ester (32)**

To 211mg of CDI (1.3 mmol, 1.0 eq.) in 5.2mL of DCM under argon atmosphere and in the absence of light was added 8mg of DMAP (0.06 mmol, 0.05 eq.) and 500mg of vitamin D<sub>3</sub> (**1**). The solution was heated to 40°C and stirred for 4 h. The reaction was monitored *via* silica gel TLC, when the reaction wasn't seen to progress further added an additional 62mg of CDI (0.4 mmol 0.3 eq.) was added. The solution was stirred for 2 h more at 40°C. Added 72mg of propargyl amine (**29**) (1.3 mmol, 1.0 eq.). The solution was stirred for 18 h at 40°C. At the end of the reaction the solution was allowed to cool to room temperature. The reaction was diluted with DCM (15mL), washed with water (3 x 5mL), brine (5mL), dried over magnesium sulfate, filtered, and concentrated *in vacuo*. The crude product was purified using silica gel column chromatography (5% EtOAc/hexanes → 40% EtOAc/hexanes) to give 311mg (0.7mmol, 51% yield) of prop-2-ynyl-carbamic acid 3-{2-[1-(1,5-dimethylhexyl)-7a-methyloctahydroinden-4-ylidene]ethylidene}-4-methylenecyclohexyl ester (**32**) as a yellow viscous product.

**Compound 32**

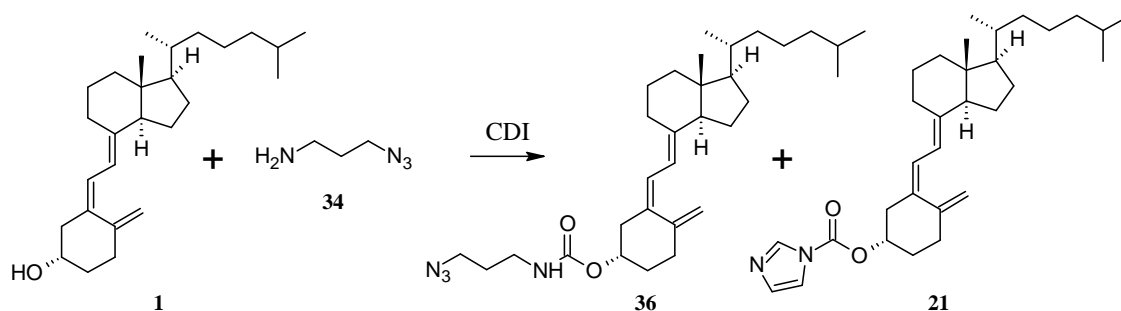
R<sub>f</sub> = 0.88 (50% EtOAc/Hex)

IR  $\nu_{\text{max}}$  (neat)/cm<sup>-1</sup>: 3350, 2947, 2866, 1701, 1508, 1240, 1041, 906, 732, 655

<sup>1</sup>H NMR (300MHz, CDCl<sub>3</sub>):  $\delta$  6.21 (d, *J* = 11.43, 1H), 6.02 (d, *J* = 10.95, 1H), 5.04, (s, 1H), 4.91 (m, 1H), 4.83 (s, 1H), 3.97 (s, 2H), 2.84-2.75 (m, 1H), 2.62-2.53 (m, 1H), 2.42-2.31 (m, 2H), 2.25-2.17 (m, 2H), 2.03 (s, 1H), 2.02-1.91 (m, 4H), 1.89-1.6s8 (m, 4H), 1.68-1.61 (m, 2H), 1.55-

1.43 (m, 4H), 1.38-1.21 (m, 4H), 1.15-1.08 (m, 2H), 0.91 (d,  $J = 6.42\text{Hz}$ , 3H), 0.86 (d,  $J = 6.61$ , 6H), 0.53 (s, 3H)

LRMS (ESI+): Exact mass calculated for  $\text{C}_{31}\text{H}_{47}\text{NO}_2[\text{M}+\text{Na}^+]$  = 488.35, found = 488.2



**(3-Azidopropyl)carbamic acid 3-{2-[1-(1,5-dimethylhexyl)-7a-methyloctahydroinden-4-ylidene]ethylidene}-4-methylenecyclohexyl ester (36)**

To 250mg of **21** (0.52 mmol, 1.0 eq.) in 0.5mL DCM under argon atmosphere was added 78mg of **34** (0.78mmol, 1.5 eq.). The solution was stirred for 4 h at 40°C. The reaction was monitored *via* silica gel TLC. Upon the completion of the reaction, the solution was cooled to rt, diluted in diethyl ether (80mL), washed with 0.5N HCl (3x 20mL), saturated sodium bicarbonate solution (20mL), brine (20mL), dried over magnesium sulfate, filtered, and concentrated *in vacuo*. The crude product was purified using silica gel column chromatography (5% EtOAc/hexanes → 30% EtOAc/hexanes) to give 96mg (0.18mmol, 36% yield) of (3-azidopropyl)-carbamic acid 3-{2-[1-(1,5-dimethylhexyl)-7a-methyloctahydroinden-4-ylidene]ethylidene}-4-methylenecyclohexyl ester (**36**) as a yellow viscous product.

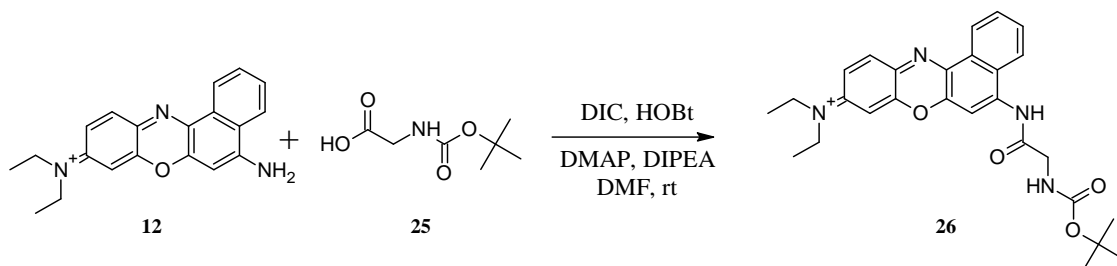
**Compound 36**

$R_f = 0.92$  (50% EtOAc/Hex)

IR  $\nu_{\max}$  (neat)/ $\text{cm}^{-1}$ : 3490, 2645, 2866, 2094, 1701, 1516, 1238, 906, 731

$^1\text{H}$  NMR (300MHz,  $\text{CDCl}_3$ ):  $\delta$  6.22 (d,  $J = 10.98$ , 1H), 6.03 (d,  $J = 10.98$ , 1H), 5.05 (s, 1H), 4.86 (m, 1H), 4.83 (s, 1H), 3.36 (t,  $J = 6.18$ , 2H), 3.26 (q,  $J = 6.18$ , 2H), 2.85-2.76 (m, 1H), 2.61-2.53 (m, 1H), 2.43-2.31 (m, 2H), 2.26-2.12 (m, 2H), 2.00-1.57 (m, 2H), 1.57-1.41 (m, 1H), 1.38-1.25 (m, 15H), 1.17-1.07 (m, 2H), 0.96 (d,  $J = 6.54$ , 2H), 0.91 (d,  $J = 12.78$ , 3H), 0.86 (d,  $J = 7.32$ , 6H), 0.54 (s, 3H)

LRMS (ESI<sup>+</sup>): Exact mass calculated for  $\text{C}_{31}\text{H}_{50}\text{N}_4\text{O}_2[\text{M}+\text{Na}^+]$  = 533.38, found = 533.6



**[5-(2-*tert*-Butoxycarbonylaminoacetylamino)benzo[*a*]phenoxazin-0-ylidene]diethylammonium (26)**

To 294mg of Nile Blue A (**12**) (0.8 mmol, 1 eq.) in 6mL DMF was added 198mg DIC (1.6 mmol, 2 eq.), 240mg HOBt\*H<sub>2</sub>O (1.6 mmol, 2 eq.), 96mg DMAP (0.8 mmol, 1 eq.), 304mg DIPEA (2.4 mmol, 3 eq.), 413 mg Boc-Gly-OH (**25**) (2.4 mmol, 3 eq.). The solution stirred for 2 h at rt. The reaction was monitored *via* LRMS. After 2 h added 198mg DIC (1.6 mmol, 2 eq.), 240 mg HOBt\*H<sub>2</sub>O (1.6 mmol, 2 eq.), 202mg DIPEA (1.6 mmol, 2 eq.), 275mg Boc-Gly-OH (1.6 mmol, 2 eq.). The solution stirred for an additional 1 h at which point the reaction had been deemed completed *via* LRMS. The product was precipitated out from 12mL saturated sodium bicarbonate and filtered. Rinsed solid with 10mL saturated sodium bicarbonate to yield a wet blue solid. It was dried under hi-vac for 1 hour to yield 320 mg (0.6mmol, 86% yield) of [5-(2-*tert*-butoxycarbonylaminoacetylamino)benzo[*a*]phenoxazin-0-ylidene]diethylammonium (**26**) as a dark blue powdery solid. The compound was deemed pure enough to continue onto the next step.

**Compound 26**

IR  $\nu_{\text{max}}$  (neat)/cm<sup>-1</sup>: 3338, 2966, 1614, 1246, 1165, 831, 694

<sup>1</sup>H NMR (300MHz, CDCl<sub>3</sub>):  $\delta$  8.55 (d, *J* = 6.96, 1H), 8.26 (d, *J* = 8.1, 1H), 7.79 (t, *J* = 7.59, 1H), 7.70 (t, *J* = 7.65, 1H), 7.58 (d, *J* = 8.64, 1H), 7.19 (t, *J* = 5.79, 1H), 6.80 (dd, *J* = 8.94, *J* = 2.25, 1H), 6.63 (ds, *J* = 2.61, 1H), 6.38 (s, 1H), 5.48 (d, *J* = 7.65, 18H), 3.85 (d, *J* = 6.12, 2H), 3.63 (m, 22H), 3.50 (q, *J* = 6.45, 6H), 3.31 (s, 40H), 1.37 (s, 8H), 1.14 (d, *J* = 6.36, 11H),

LRMS (ESI<sup>+</sup>): Exact mass calculated for C<sub>31</sub>H<sub>50</sub>N<sub>4</sub>O<sub>2</sub> = 475.23, found = 475.3

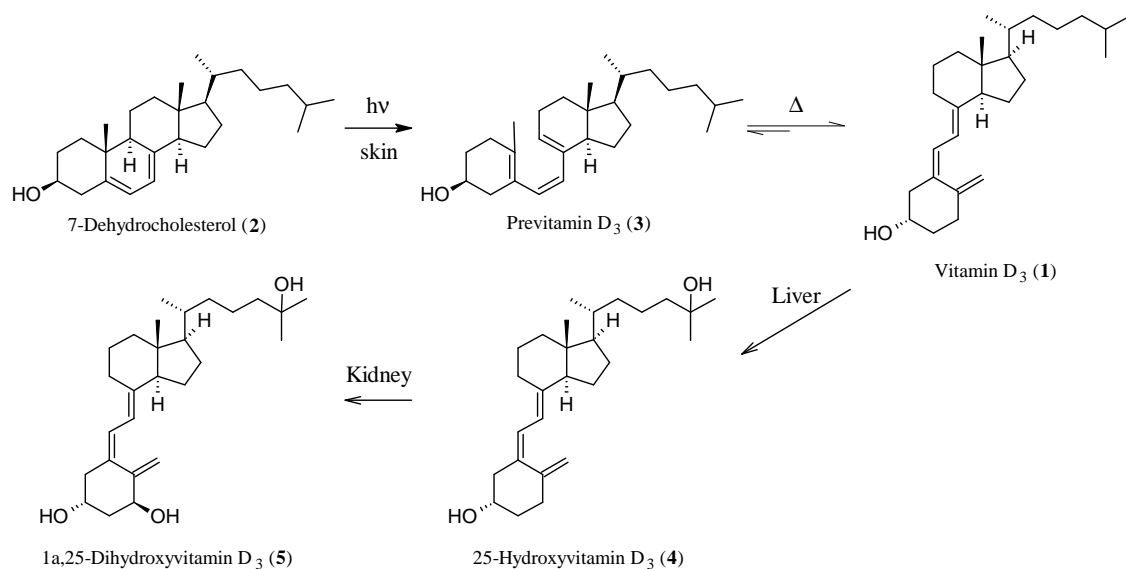


## **Appendix**

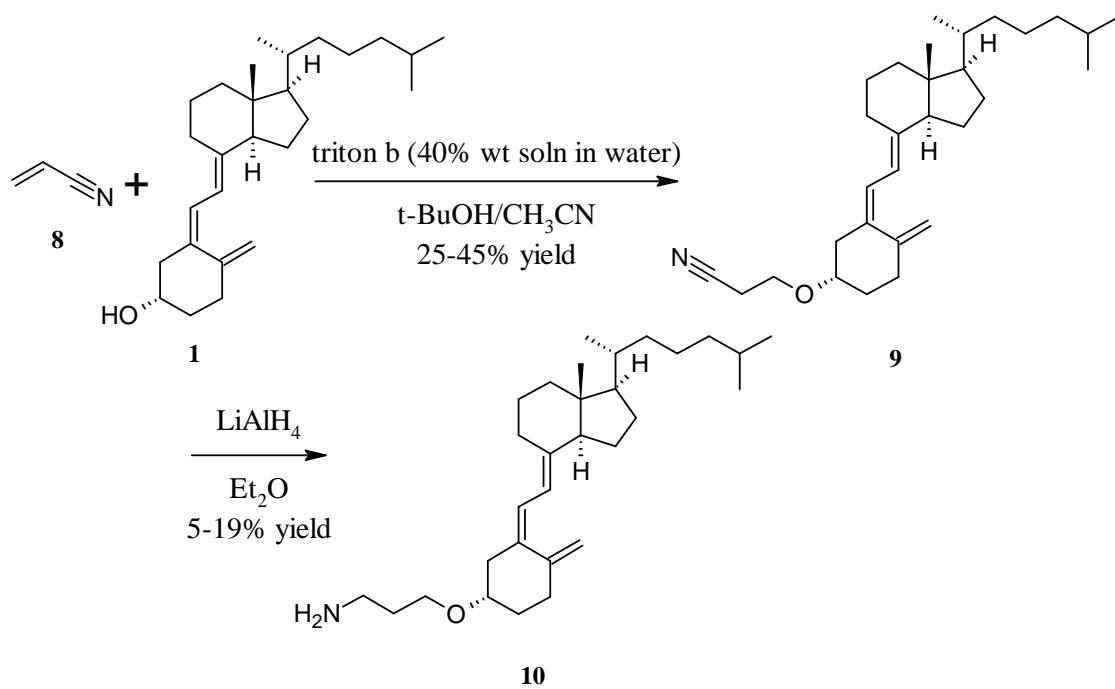
The following pages contain  $^1\text{H}$  NMR, and IR spectra for certain compounds. The structures and numbers of the structures are located with each spectrum.

There is provided a list of all of the schemes prior to the spectra.

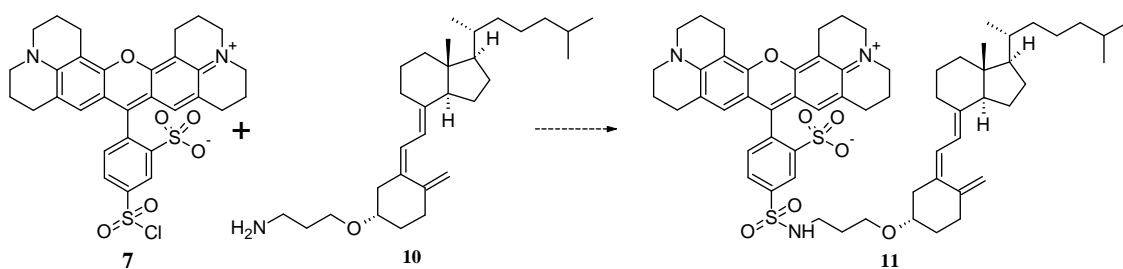
## List of Schemes



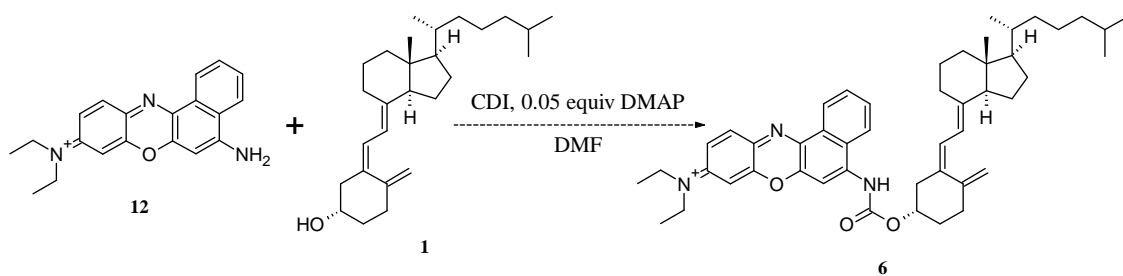
**Scheme 1:** Metabolic pathway of vitamin D<sub>3</sub> (1) in humans



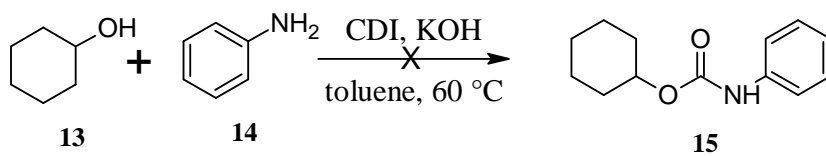
**Scheme 2:** Preparation of vitamin D<sub>3</sub> amine 10



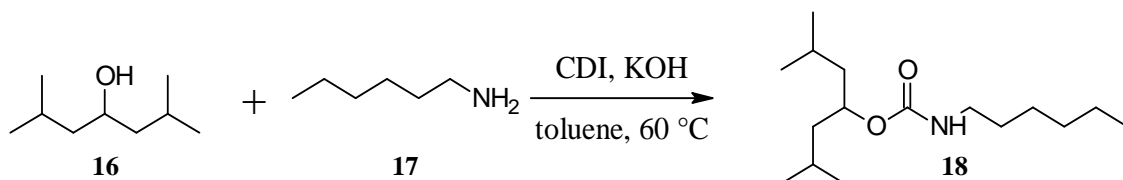
**Scheme 3:** Proposed coupling of vitamin D<sub>3</sub> amine **10** to TRSC (**7**)



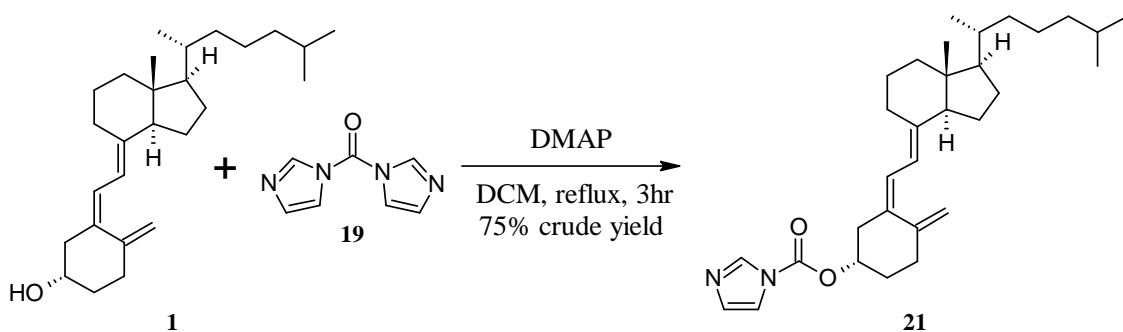
**Scheme 4:** CDI coupling between vitamin D<sub>3</sub> (**1**) and Nile Blue A (**12**)



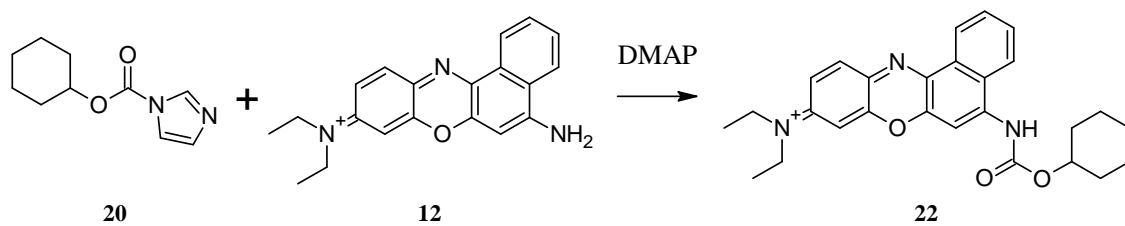
**Scheme 5:** CDI coupling of **13** and **14** to form carbamate **15**



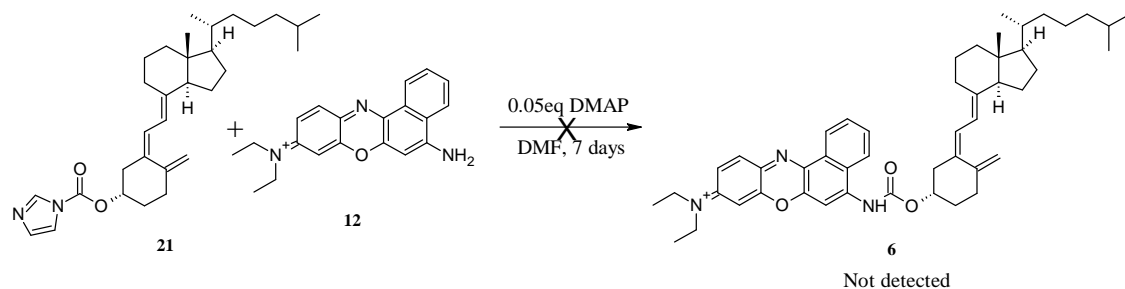
**Scheme 6:** Literature model reaction



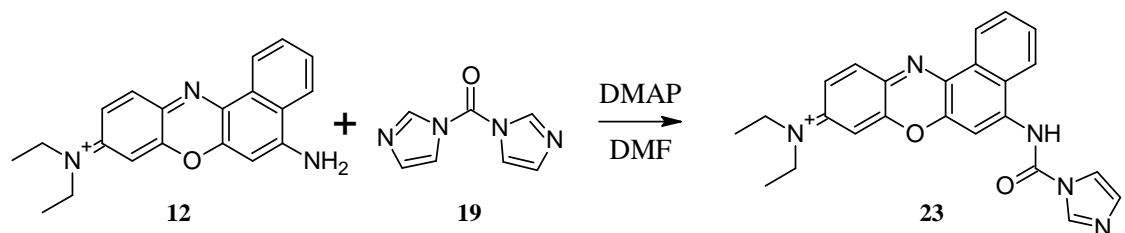
**Scheme 7:** Formation of vitamin D<sub>3</sub> imidazole **21**



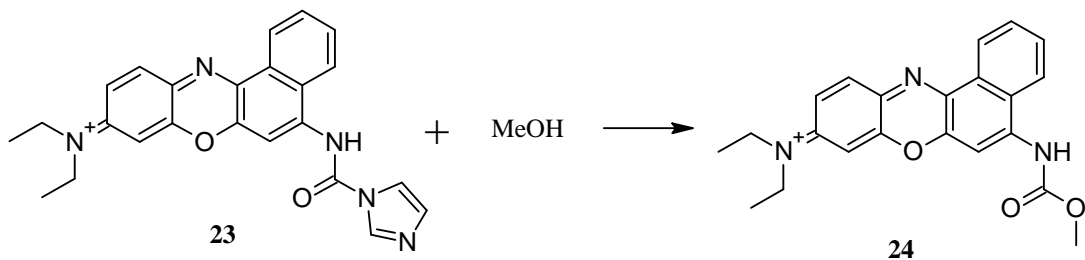
**Scheme 8:** Coupling of Nile Blue A (12) with cyclohexanol imidazole **20**



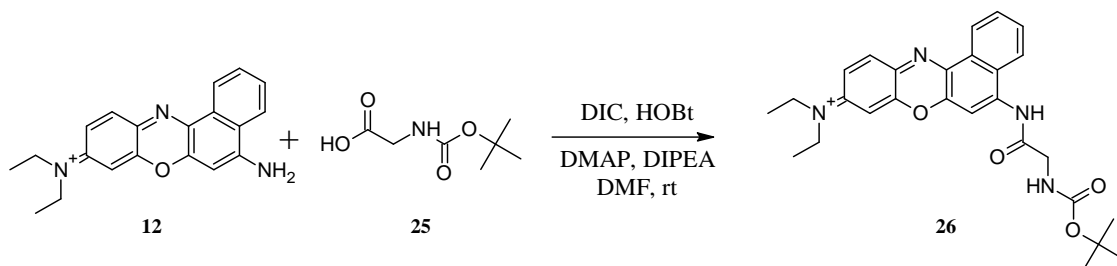
**Scheme 9:** Formation of Nile Blue A/vitamin D<sub>3</sub> carbamate **6** via vitamin D<sub>3</sub> imidazole **21**



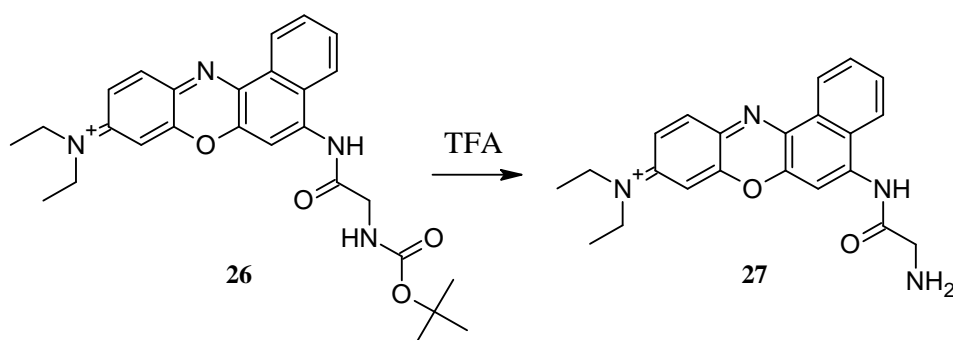
**Scheme 10:** Formation of Nile Blue A imidazole **23**



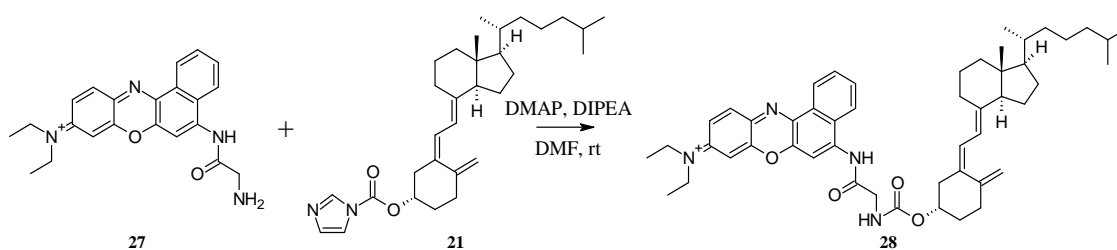
**Scheme 11:** Methanol adding into Nile Blue A imidazole **23** to form Nile Blue A/methanol adduct **24**



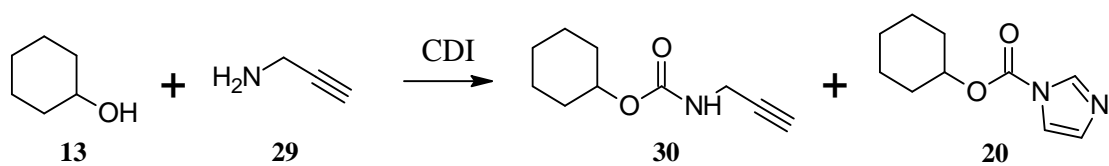
**Scheme 12:** DIC coupling between Nile Blue A (**12**) and Boc-Gly-OH (**25**)



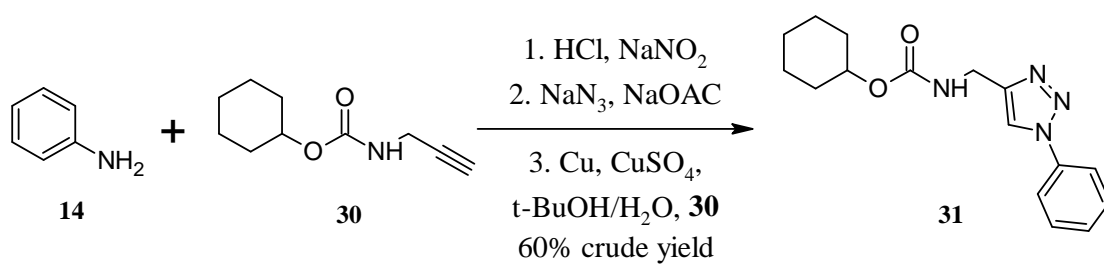
**Scheme 13:** Deprotection of Nile Blue A Boc-gly **26** by TFA



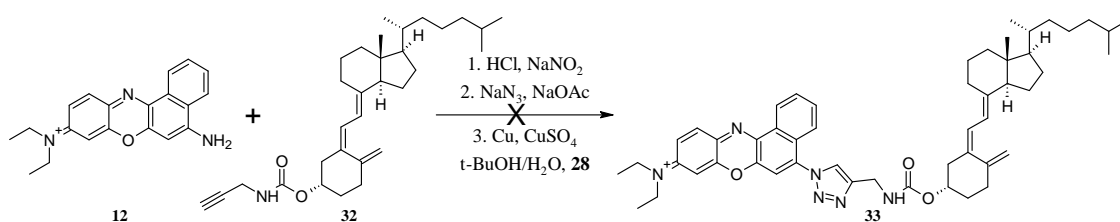
**Scheme 14:** Formation of carbamate **28**



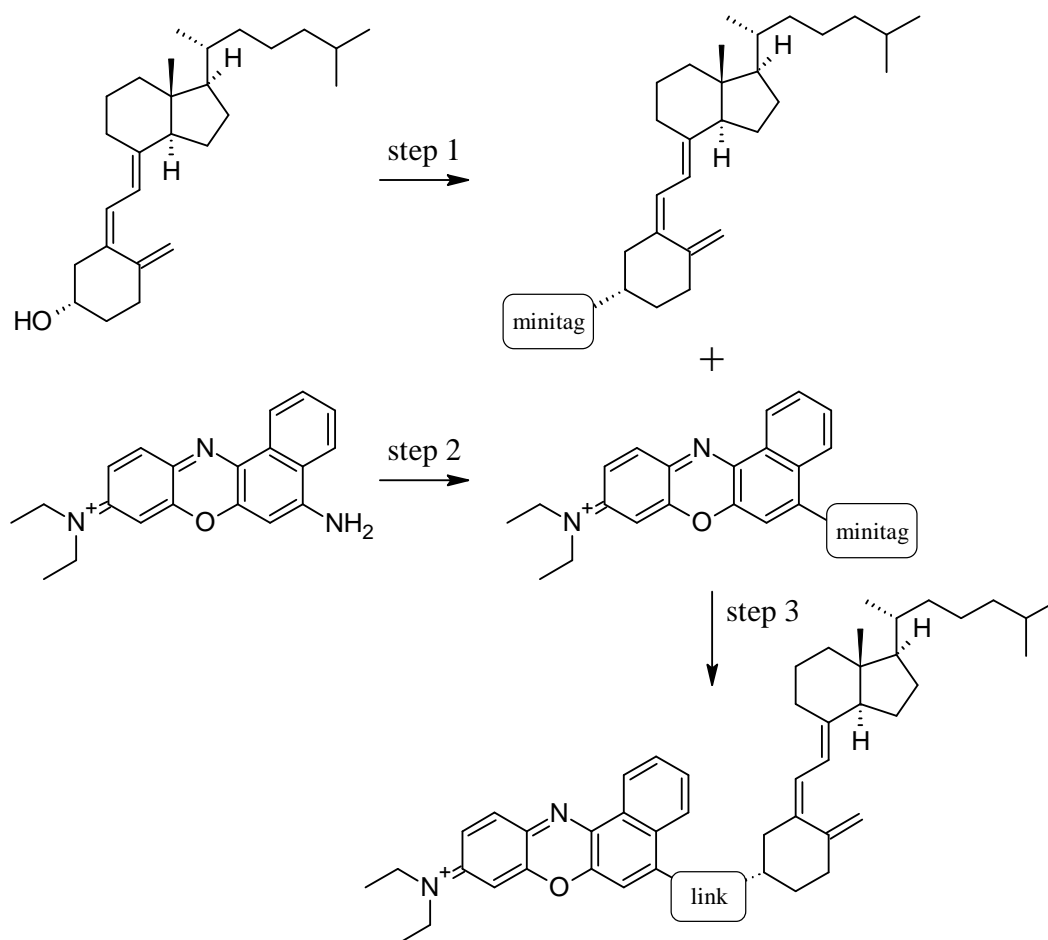
**Scheme 15:** CDI coupling to form model alkyne



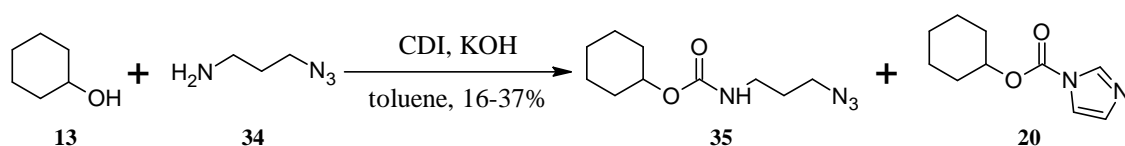
**Scheme 16:** Model system of one pot click reaction



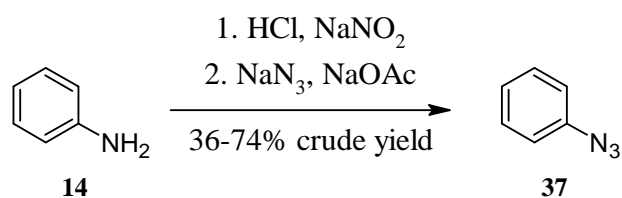
**Scheme 17:** One pot click coupling of **12** and **32**



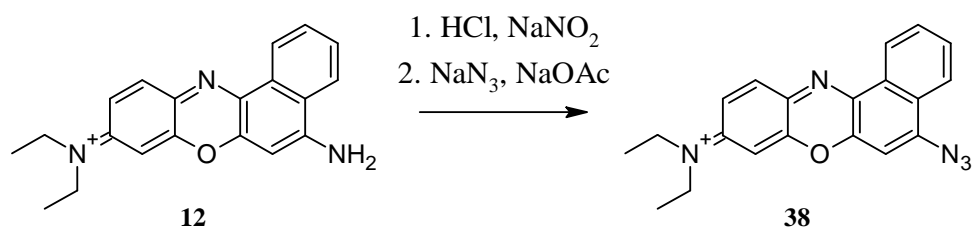
**Scheme 18:** Three steps for approach B (linking after ingestion)



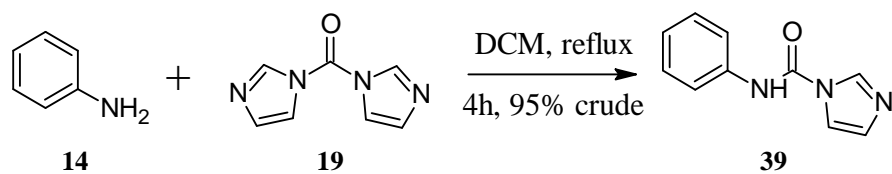
**Scheme 19:** Formation of azide minitagged cyclohexanol **35**



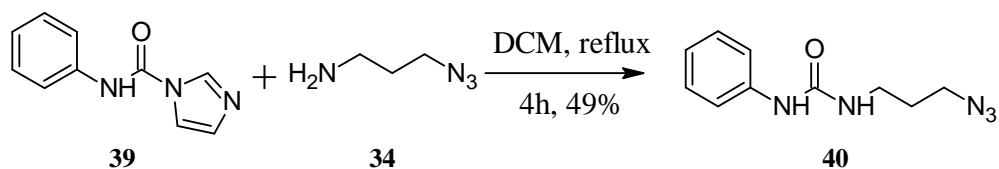
**Scheme 20:** Formation of azide **37**



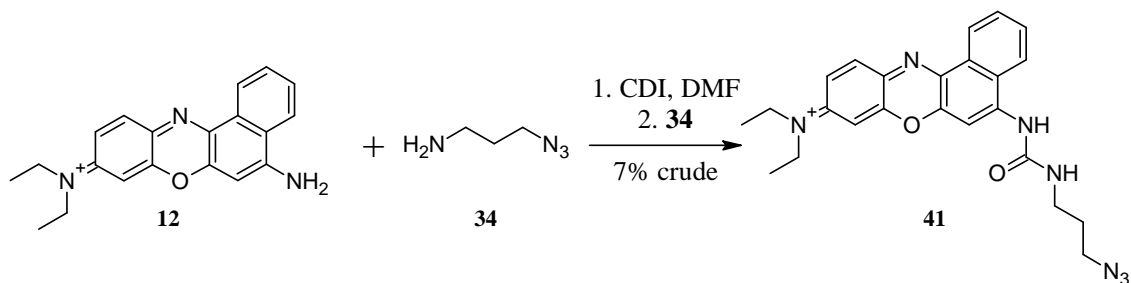
**Scheme 21:** Formation of Nile Blue A azide **38**



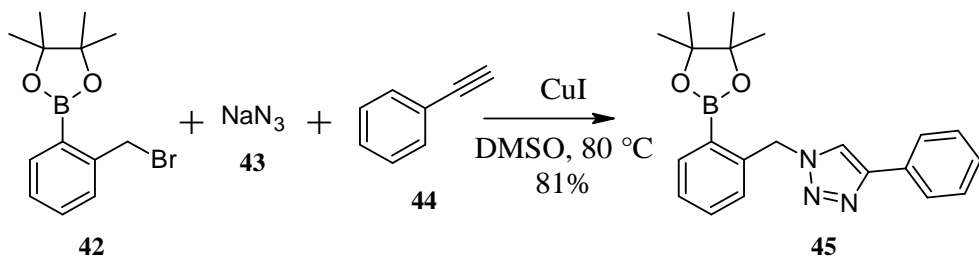
**Scheme 22:** CDI addition onto aniline (**14**)



**Scheme 23:** Formation of azide linked aniline **40**

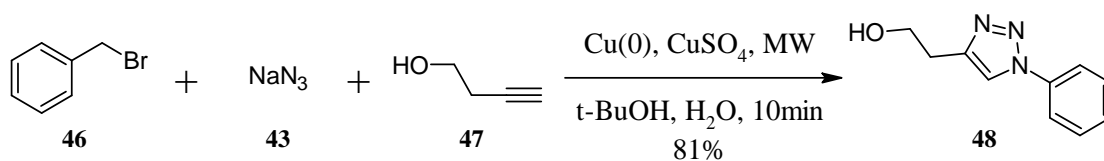


**Scheme 24:** Formation of Nile Blue azide **41**

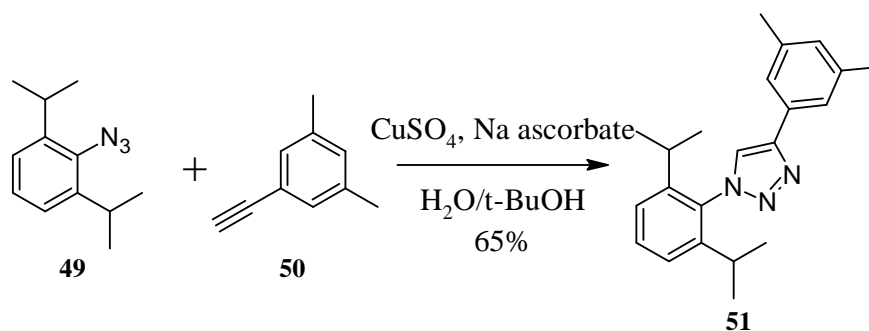


**Scheme 25:** Scafton et. al. CuI reaction conditions

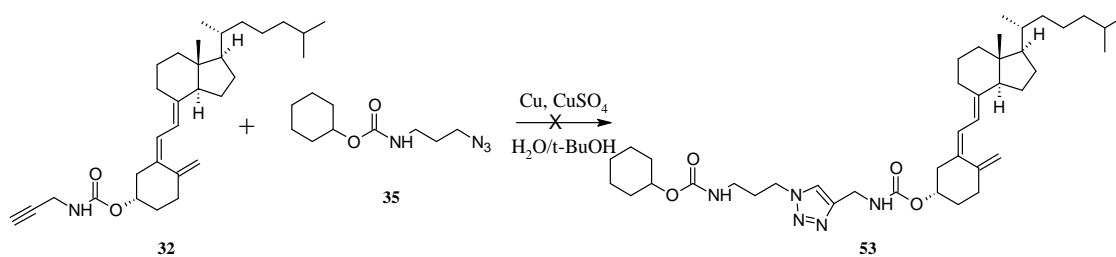




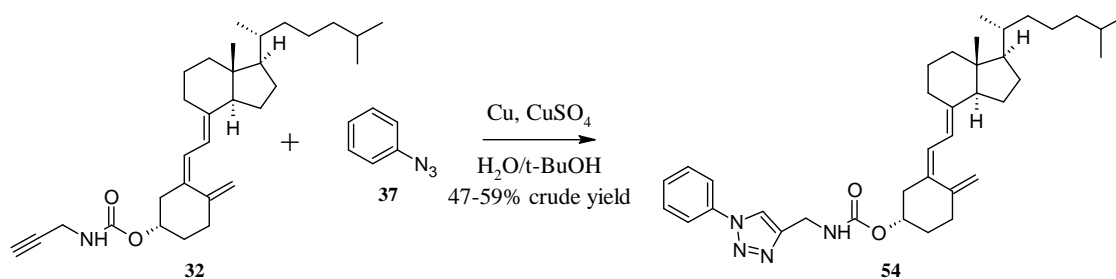
**Scheme 26:** Appukkuttan et. al.  $\text{Cu}_{(s)}/\text{CuSO}_4$  reaction conditions



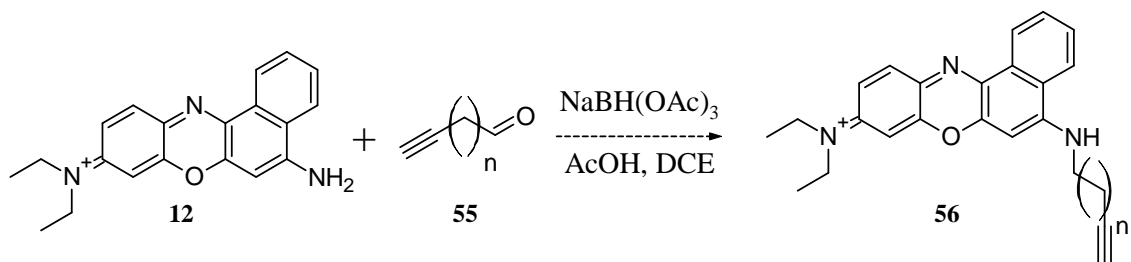
**Scheme 27:** Nakamura et. al.  $\text{CuSO}_4/\text{Na ascorbate}$  reaction conditions



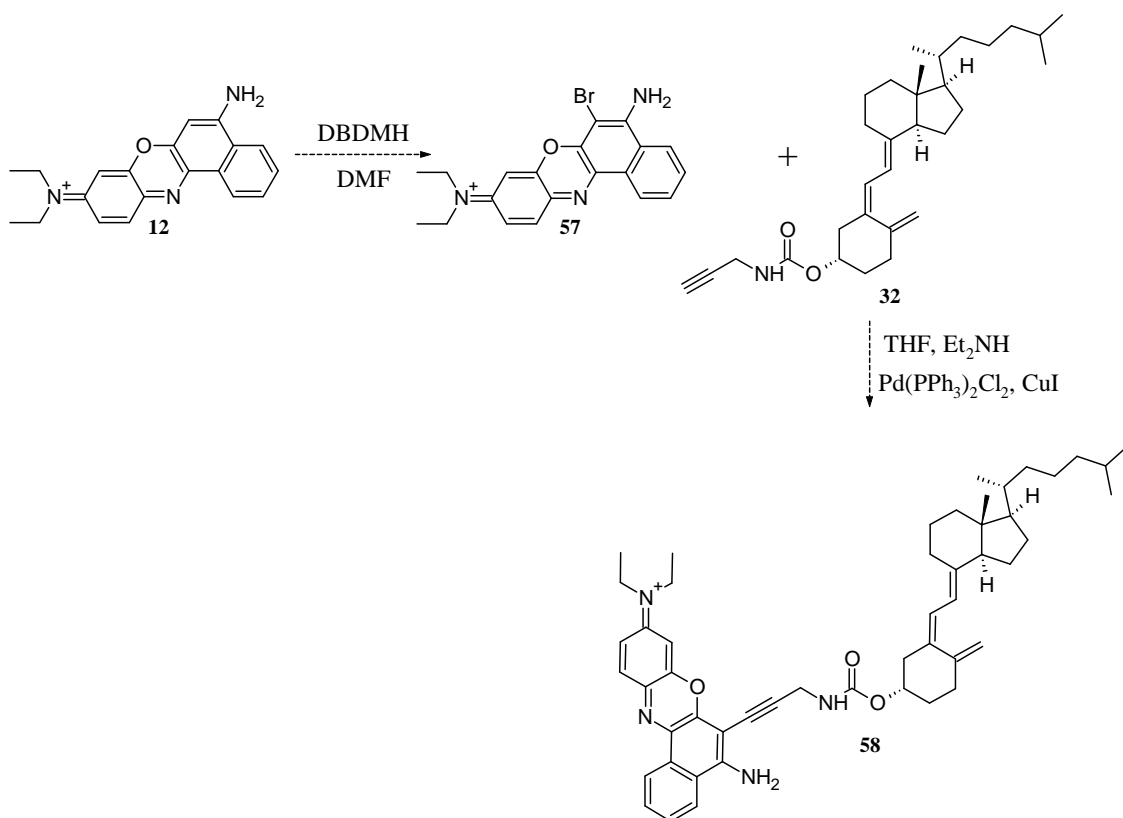
**Scheme 28:** Click chemistry formation of vitamin D<sub>3</sub> triazole **53**



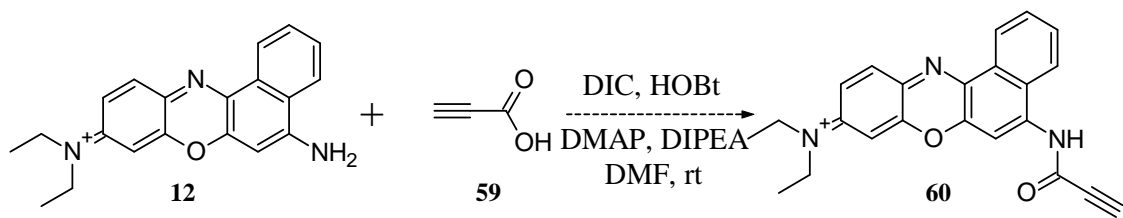
**Scheme 29:** Click chemistry formation of aniline triazole **54**



**Scheme 30:** Proposed reductive amination method



**Scheme 31:** Proposed Sonogashira coupling method



**Scheme 32:** Proposed DIC coupling for approach B (linking after ingestion)

