University of North Carolina Asheville
Journal of Undergraduate Research
Asheville, North Carolina
May 2020

Synthesis of Indole and Triazole based CA-4 Analogs

Miriam Shechet
Department of Chemistry
The University of North Carolina at Asheville
One University Heights
Asheville, North Carolina 28804

Faculty Advisor: Dr. Holt, Herman Jr.

Abstract

The devastating effects of cancer have been documented and researched for many centuries. The first lead to the treatment of various hematological and solid tumors was the discovery of cytotoxic antitumor drugs and the use of chemotherapy. One current cytotoxic antitumor drug of interest is combretastatin A-4 (CA-4). Research has found that CA-4 and other chalcone-based molecules have shown potential as antimitotic agents for use in cancer treatment. Previous studies have shown that specific analogs containing a trimethoxy A-ring, a *cis*-conformation and a *para*-methoxy B-ring have increased CA-4 binding at the tubulin-colchicine binding site. Other studies suggest that CA-4 analogs containing a triazole indole ring system have increased water solubility as well as cytotoxicity. Based on these findings a 2-(1,2,3-triazolyl)indole analog of CA-4 was designed containing the key features discussed. A multi-step synthetic scheme was followed starting with the synthesis of ethyl azidoacetate. Production of ethyl azidoacetate was achieved by an S_N2 reaction of ethyl bromoacetate and sodium azide resulting in a 55% yield. The next step in the reaction scheme involved the use of 3-hydroxy-4-methoxybenzaldehyde. Due to the acidity of the hydroxyl group, a tetrahydropyran protecting group addition was achieved using pyridinium *p*-toluenesulfonate as a catalyst resulting in a 62 % yield. After producing the protected benzaldehyde, a reaction of protected aldehyde and ethyl azidoacetate generated a 25 % yield of crude vinyl azide. In future research the vinyl azide will undergo thermolysis via the Hemettsberger-Knittel indole synthesis involving a ring cyclization to produce an indole ring system.

1. Introduction

1.1. Antimitotic Agents

Recent studies exploring the cytotoxic effects of combretastatin A-4 (CA-4) (1a) and related structures such as chalcones (1b) have led to the development of these compounds as antimitotic agents for the use in anticancer drugs.[1,2,3]

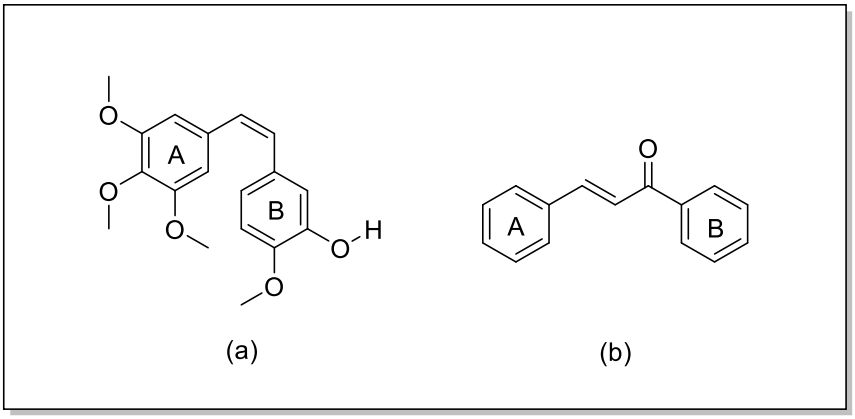


Figure 1: (a) combretastatin A-4 (CA-4), (b) Chalcone

There are four known binding sites for antimitotic agents. The four binding sites are laulimalide, taxane or epothilone, vinca alkaloid, and the colchicine site. This study will focus on the fourth type: the colchicine binding site. Antimitotic agents acting on the colchicine binding site work to combat cancer by binding to the protein tubulin, an α, β-heterodimer that forms the core of the microtubule. An antimitotic agent can bind on the β-tubulin. Colchicine inhibits microtubule assembly by binding to β-tubulin, which results in curved tubulin dimer and prevents it from adopting a straight structure, due to a steric clash between colchicine and αtubulin. Assembly into microtubules is important for various cell functions including: mitosis, cell replication, cell movement, cell maintenance, cell shape and cell segregation of chromosomes. Ideally, if the antimitotic agent can reach the cancer cells, such as those of a tumor, it can prevent these subunit functions, and the cell will die.[1] Cancer cells are more susceptible to colchicine poisoning than normal cells because they undergo mitosis at a significantly increased rate. Microtubules are important regulators of endothelial cell biology. Endothelial cells refer to cells that form an interface between circulating blood or lymph in the lumen and the rest of the vessel walls by lining the interior surface of blood vessels and lymphatic vessels. As the endothelial cells of a tumor go through mitosis at an increased rate and antimitotic agents essentially stop mitosis, antimitotic agents can target tumor vasculature. Antimitotic agents such as colchicine can prevent new blood vessels formation and are vascular disrupting agents.

One of the major limitations of using other microtubule-targeting agents is natural and acquired drug resistance however; this is not a problem with colchicine binding site inhibitors. These drugs have no multidrug resistance (MDR) issues. The most common MDR issue is the overexpression of the MDR1 gene. This gene encodes the P-glycoprotein (Pgp) drug efflux pump which is a membrane-associated ATP-binding cassette (ABC) transporter. The MDR1 gene is overexpressed in many tumor cell lines, including tissues of the liver, kidney, and gastrointestinal tract. Over-expression of Pgp decreases intracellular drug levels, consequently limiting drug cytotoxicity. In addition, over-expression of Pgp is associated with poor response to microtubule-targeted agents including taxanes and vinca alkaloids and subsequent treatment failure. These advantages of colchicine binding site inhibitors introduce therapeutically promising applications for these compounds.

Despite these many advantages, colchicine itself is not an effective anticancer drug due to its low therapeutic index. The amount of colchicine needed to induce therapeutic affects is too close to the amount that causes toxicity. Colchicine toxicity includes neutropenia, gastrointestinal upset, bone marrow damage and anemia.[4] While colchicine is not an effective anticancer agent, current research aims to clinically develop colchicine binding site agents such as CA-4 analogs.

2. Background

2.1.Combretastatin

CA-4 was originally isolated in 1984 from the willow tree *combretum caffrum*, however, it is not efficiently harvested from this source. Combretastatins, CA-4 and its analogs, are considered a class of stilbenoid phenols. Regarding tubulin binding and cytotoxicity, CA-4 is the strongest naturally occurring combretastatin known. It can also be

classified as a vascular disrupting agent (VDA). By causing the cells lining a tumors vascular network to swell, CA-4 cuts off oxygen and nutrients to the tumor. Furthermore, CA-4 has fewer side effects such as the hair loss and bone marrow damage associated with chemotherapy.[5] Some pharmaceutical companies, such as Oxigene, are developing CA-4 based cancer treatments. Currently Oxigene is testing prodrug, Zybrestat, fosbretabulin, and its salt fosbretabulin disodium (CA-4P) (Figure 2), in several phase II and phase III studies.[1] A prodrug is a pharmacologically inactive compound that can be metabolized in the body to produce a drug.[2] In vivo, CA-4P is dephosphorylated to its active metabolite CA-4. Unfortunately, clinical trials have found that use of CA-4P is associated with cardiovascular adverse events and causes pain in the area of the tumor, as VDA's work through a necrotic pathway .[5,6] Other issues with the use of CA-4 as a therapeutic drug include poor metabolic stability, poor water solubility and observed normal cell toxicity.[5] Despite these issues, research continues to synthesize CA-4 analogs and evaluate them as potential cancer drugs because of its potency as a therapeutic VDA, and it is small size relative to other microtubule interfering agents.

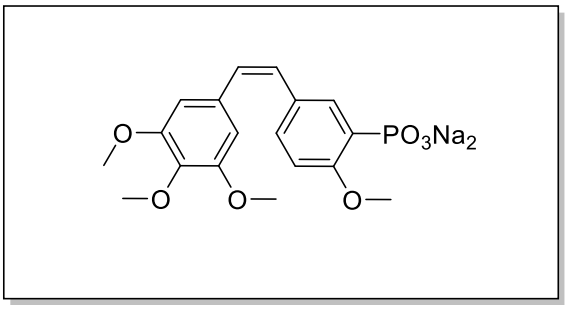


Figure 2. *Zybrestat, Oxigene*

2.2.Structure Activity Relationship

Structural activity relationship (SAR) studies are used to draw a correlation between molecular shape and biological activity. In an SAR study hypotheses about binding mechanisms are tested. Changes are made to the base structure and then biological activities of the different molecular structures are compared to determine which substituents are of importance for binding. A wide variety of analogues of CA-4 have been reported with modifications generally falling into three categories. These categories include: (I) introducing an extended side chain to the B ring; (II) replacing the alkene linker with another flexible linker such as a carbonyl, a methylene, or another suitable connection group; (III) replacing the alkene linker with a conformational rigid heterocyclic linker.[7] This research combines the discoveries made in previous research to make changes in multiple categories.

The small size of CA-4 has led to extensive SAR studies and the establishment of a pharmacophore. The 3,4,5-trimethoxy A-ring, para methoxy on the B-ring, and cis confirmation about the ethylene linker are all requisite for binding at the active site. Previous research has investigated the role of the ethylene linker which, was speculated to be largely involved with the appropriate spacing and angling of the two aromatic rings. This hypothesis was confirmed by conducting an in-silico docking experiment of CA4 and its biphenyl analogues to the tubulin heterodimer, whose crystal structure was solved in complex with a colchicine analogue. This experiment concluded that tubulin affinity correlates with the presence of an ethylene linker and cytotoxicity towards cancer cells.[7]

Further investigation of the ethylene linker or bridge revealed a disparity in activity between the *cis* and *trans* stereoisomers of CA-4. While the *trans* isomer is thermodynamically favorable, it is almost completely inactive.[8] Conformation changes can be induced by many different factors such as heat or ultraviolet light. This is a contributing factor to the poor metabolic stability of CA-4 and for this reason maintenance of the *cis* olefin bridge is a fundamental problem with CA-4 and has been a major research topic. The stabilization of the *cis* confirmation through structural modifications to the olefin bridge has been explored through the Holt laboratory research and others previously. These modifications include epoxidation or direct azirdination. Modifications to the bridge by replacement of the double bond with heterocyclic five membered rings (Figure 3a and 3b) resulted in compounds that retained both cytotoxic and antitubulin activities. Notably, these cis-locked analogues provide at least three main advantages: (a) prevention of combretastatin isomerization from cis to trans; (b) increased specificity, since the trans conformation might be recognized by other cellular targets; and (c) the possibility of using heterocyclic systems that might improve the therapeutic potential of these drugs.[9, 10] Additionally, The triazole bridge may provide opportunities for hydrogen bonding and thus increase water solubility. An increase in activity was noted when an amino group replaced the alcohol on the B ring. The resulting compound (Figure 3c) showed the best cytotoxic activities when compared to

other analogs without the amino modification. Molecular modeling supported the hypothesis that the increased activity of 3c was due to hydrogen bond interactions that occur with several amino acids in the colchicine binding sites of beta-tubulin.[11] Odlo et al. determined that a methylene group between the two rings functions as a mimic for the configuration found in CA-4 (Figure 3d). It was also indicated that the orientation of the trimethoxy substituted ring is most important for tubulin inhibition.[12] Another example of a successful bridge modification was achieved by Ducki et al. by incorporating the aryl substitution pattern of CA4 into chalcones.

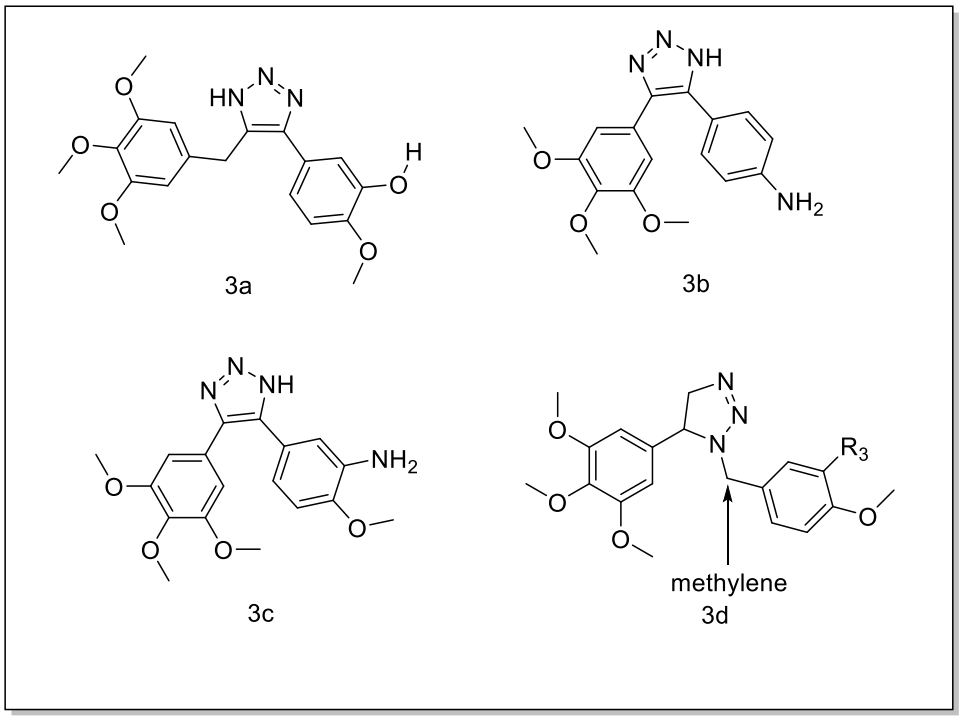


Figure 3. combretastatin analogs

2.3. Combretastatin Analogs

Chalcones are one of the few colchicine binding agents that display higher activity than CA-4 and can be synthesized in an easy one-step condensation reaction. For this reason chalcone analogs have been produced and studied for their cytotoxic effects. Research has found that the α, β-unsaturated ketone moiety of chalcones, noted in Figure 4, is important for their biological properties. However, modifications on the two aromatic rings remain an area of pharmacological interest in the screening of active chalcones.

In the research by Ducki et al., a chalcone (Figure 4) with the same A and B ring substitution pattern as CA-4 proved to have greater activity than CA-4.[13]

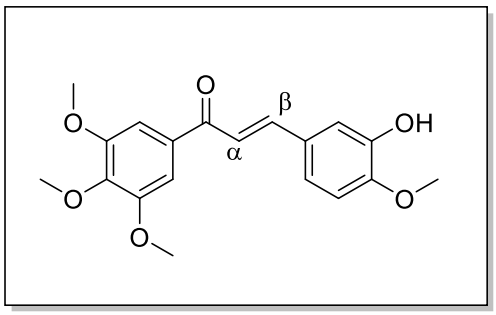


Figure 4. chalcone analog

In a comparison of over 30 different substituted chalcones, the most potent chalcones contained the CA-4 aromatic substitution pattern. In general, the activities were highly dependent on the aromatic substitution pattern and methoxylated chalcones exhibited highest activities. Chalcones containing the three methoxylated group, regardless of position, were the most active. In fact, methoxyl groups on both rings greatly increased activity.[13] These results suggest that CA-4's trimethoxy A-Ring is fundamental for colchicine domain binding. Similar conclusions can be drawn from the reoccurrence of this motif in relevant natural products such as colchicine (Figure 5a) and podophyllotoxin (Figure 5b).[3, 14] Furthermore, the loss of cytotoxic activity when the methoxy groups are substituted with bulkier groups would suggest that the steric factor plays a primary role in fitting the ring inside the active site. This was confirmed with SAR studies exploring the effects of substituting the oxidized positions for halogens.[15, 16] The trimethoxy A-Ring cannot be excluded, however major modifications to the B-Ring have resulted in higher cytotoxicity as well as increased solubility.

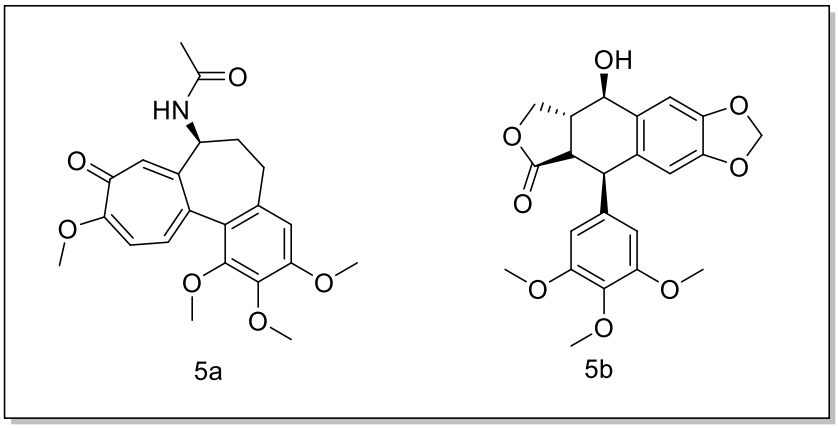


Figure 5. (a) colchicine, (b) podophyllotoxin

The B-Ring has a greater history of modifications because it was believed that it was the only moiety that could be modified and yield potent compounds. One problem with CA-4 is that it is insoluble in water, which prevents it from being transported through the bloodstream to the cancer site. Certain modifications to CA-4 may increase water solubility. CA-4's B ring is easiest to change without adverse effects to bioactivity, and for this reason past research has perused changes to this moiety in order to solve the inherent water solubility problems of CA-4. There are three categories of modifications on this ring: (i) substituted phenyl rings, (ii) heterocyclic rings, and (iii) nonsubstituted aromatic rings. SAR studies have found that the presence of a para methoxy group is fundamental while the presence of the meta hydroxyl group was not. Replacement of the meta hydroxyl group with fluorine has led to an increase in cytotoxic activity and a more metabolically stable compound. Increased metabolic stability is due to the highly electronegative fluorine preventing electrophilic substitutions. However, this approach excludes the possibility of a prodrug.[14]

Another approach to reconcile the water solubility problems of CA-4 was explored through substitution of the B-Ring with a pyridine ring. It was hypothesized that by exchanging a carbon atom for a heteroatom the pharmacodynamic and pharmacokinetic features of the molecule would be altered. The availability of a lone pair could lead to more water-soluble derivatives and to new additional interactions with biological targets. Results showed that the position to the nitrogen atom was crucial for binding. Substitution at the meta position yields products (Figure 6) that display potent cytotoxic and antitubulin action.[14]

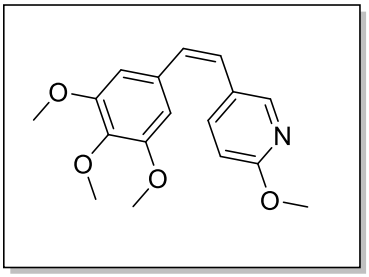


Figure 6. pyridine substitution

In a similar approach, a series of indolyl chalcones were synthesized and evaluated in vitro for their anticancer activity against three human cancer cell lines. Two compounds 7a and 7b were identified as the most potent and selective anticancer agents.[17]

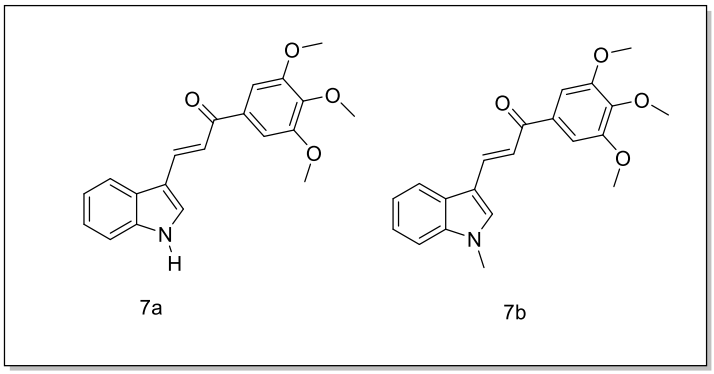


Figure 7. indole substitution

The importance of the meta position of the indole moiety was further evaluated. Twenty-nine novel indole-chalcone derivatives were synthesized and evaluated for antiproliferative activity. The most potent activity was observed in analog 8a bearing the substitution pattern of CA-4's A-Ring and a B-Ring replaced by an indole substituted with a methoxy at the Meta position. The analog displayed an 3.8−8.7-fold increase in activity when compare with compound 8b, suggesting that the indole substitution is most effective when attached at the meta position.[18] In order to determine that the antiproliferative effects of the compounds were caused by the inhibition of tubulin, the compounds were tested in a tubulin polymerization assay including podophyllotoxin and the chalcone for comparative purposes.[18] In general, research has found that heteroatom substitutions and indole ring systems increase water solubility and metabolic stability.[14,17,18,19]

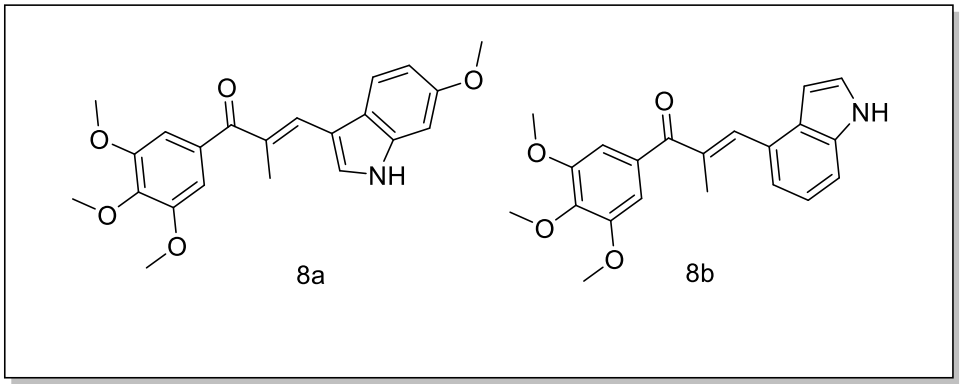


Figure 8. Indole orentation

3. Design of Indole and Triazole based CA-4 Analog

There are three key features of CA-4 that are indispensable for colchicine binding: (I) the trimethoxy A-Ring, (II) the paramethoxy on the B-Ring, and (III) The *cis*-conformation of the bridge. These key features have been incorporated into the design of the indole and triazole based CA-4 analog. Furthermore, recent studies suggest that CA-4 analogues containing both an indole and a triazole ring system may be more effective at binding to the colchicine site as well as increasing water solubility, bridge stability and metabolic stability.[14, 17,18,19,20] The incorporation of the triazolyl bridge and the indole ring system addresses the water solubility and metabolic stability issues of CA-4 through hydrogen bonding and *cis* conformation locking effects. *Cis*-locking is beneficial as previous research has demonstrated a lack of activity in *trans* CA-4 isomers. In regards to water solubility, lone pairs in the triazole bridge and indole ring system may provide opportunities for hydrogen bonding and thus increase water solubility. In a recent study by Herman Holt's research group, the synthesis of 2-(1,2,3-trazolyl)indole (Figure 4, 9b), 2-aroylindole and 2-styryl indole analogues of CA-4 was attempted however, only partial synthesis of 9b was achieved.[21]

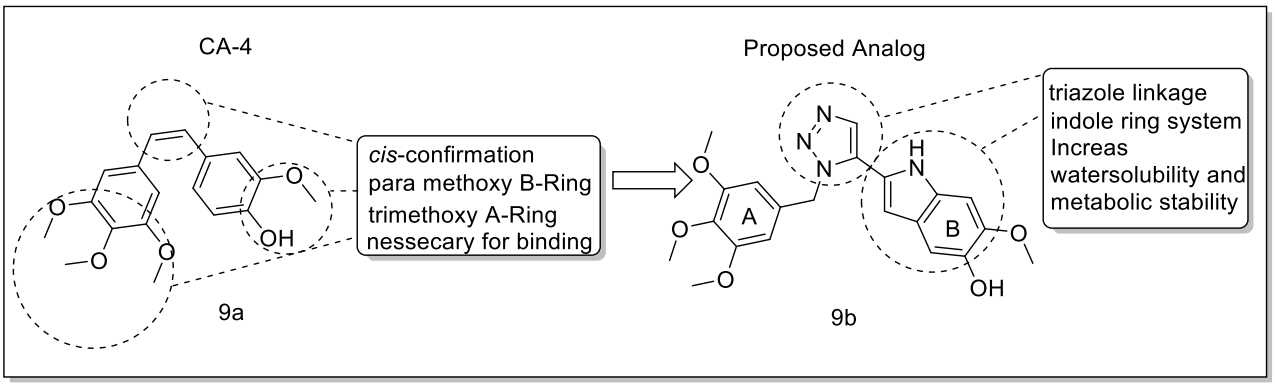


Figure 9: (9a) CA-4, (9b) 2-(1,2,3-trazolyl)indole

This project will primarily focus on achieving complete synthesis of the suggested 2-(1,2,3-trazolyl)indole (9b) analogue of CA-4. The second objective of the project will be to evaluate the growth inhibitory properties of this analogue using MTT assay against K562 leukemia. Attaining a water soluble chalcone that is successful in binding to the colchicine binding site would be a significant lead in the search for a drug to cure various hematological and solid tumor-based cancers such as leukemia.

Upon successful synthesis and evaluation of the target analog, a second phase of the study will go on to compare the 2-(1,2,3-trazolyl)indole analogue of CA-4 to *N*-phenylethyl-1*H*-indole-2-carboxamides containing a trimethoxy ring, a *cis*-conformation and a para methoxy group on one of the rings. In a previous study analyzing *N*-phenylethyl-1*H*-indole-2-carboxamides as allosteric modulators of the colchicine-binding site, it was found that the presence of the carboxamide was required for the molecule to function as an allosteric modulator. The study found that the most effective *N*-phenylethyl-1*H*-indole-2-carboxamides were 10a and 10b. Both carboxamides contain a dimethylamino or piperidinyl group (shown in red), at position 4 of the phenethyl moiety and a chlorine atom at position 5 (shown in purple) of the indole.[20] The comparison between the 2-(1,2,3-trazolyl)indole and the two carboxamides discussed will help determine a direction for future research.

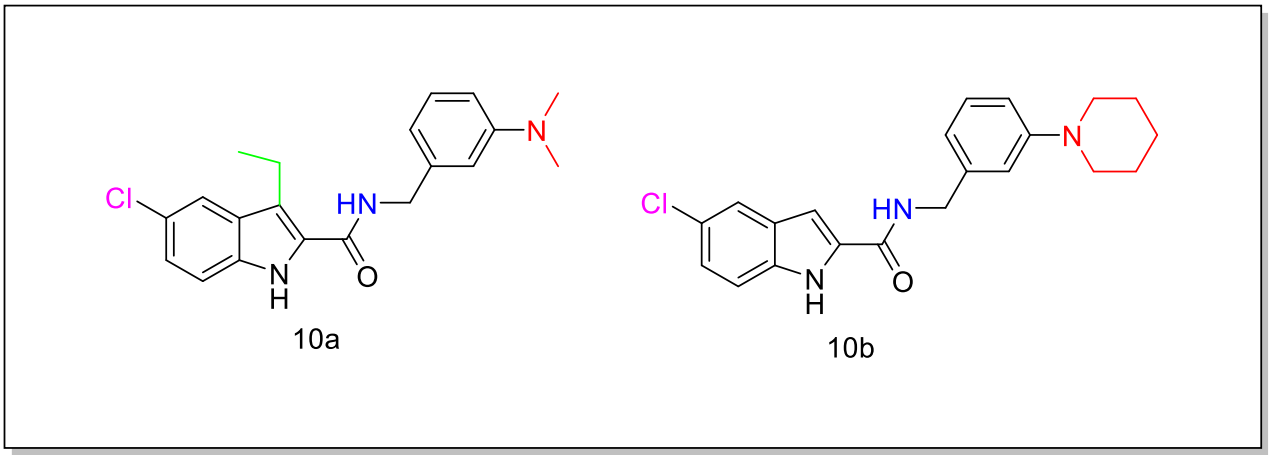
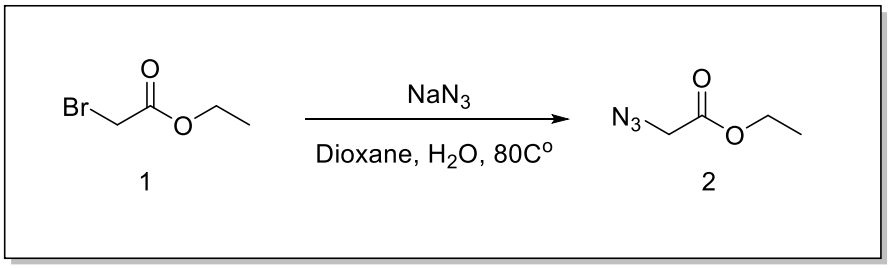


Figure 10. carboxamides

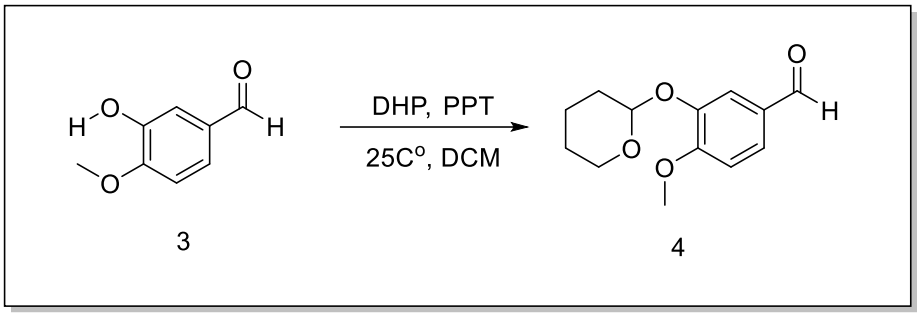
4. Synthesis and Methods

The Holt laboratory's research has primarily focused on synthesizing analogs of CA-4 as well as the synthesis of lamellarin pyrrole analogs. Lamellarin pyrrole analoges synthesized from the chalcone derivatives of CA-4 are biologically enhanced and more feasible anticancer compounds. This project follows the research of Benjamin J. Shields, who was part of the Holt research group. Shield's research approached the synthesis of 9b by using the Hemetsberger-Knittel method to produce substituted indoles (Scheme 3). This reaction required two precursors: ethyl azidoacetate (2) and 4-methoxy-3-((tetrahydro-2H-pyran-2-yl)oxy)benzaldehyde (4). The synthesis of 2 was achieved by a simple SN2 reaction of ethyl bromoacetate and sodium azide . Next the addition of a DHP protecting group to 3 to produce 4 was achieved under acidic conditions. These two precursors, 2 and 4, were then reacted to produce a vinyl azide (5). The vinyl azide (5) went through thermolysis via the Hemettsberger-Knittel indole synthesis involving a ring cyclization to produce the indole ring system 6. A tert-butoxycarbonyl (BOC) protecting group was then added to 6 to produce 7(Scheme 4). Carbon homologation using the Corey-Fuchs method produced a substituted ethynyl indole as a functional dipolarophile in 8 (Scheme 5). Finally, in a ruthenium mediated dipolar addition reaction, a 1,3-dipolar cycloaddition reaction of 8 and benzyl azide produced 9(Scheme 6).[5] While this analog is similar to the proposed analog it is missing a fundamental feature: the trimethoxy A-Ring.

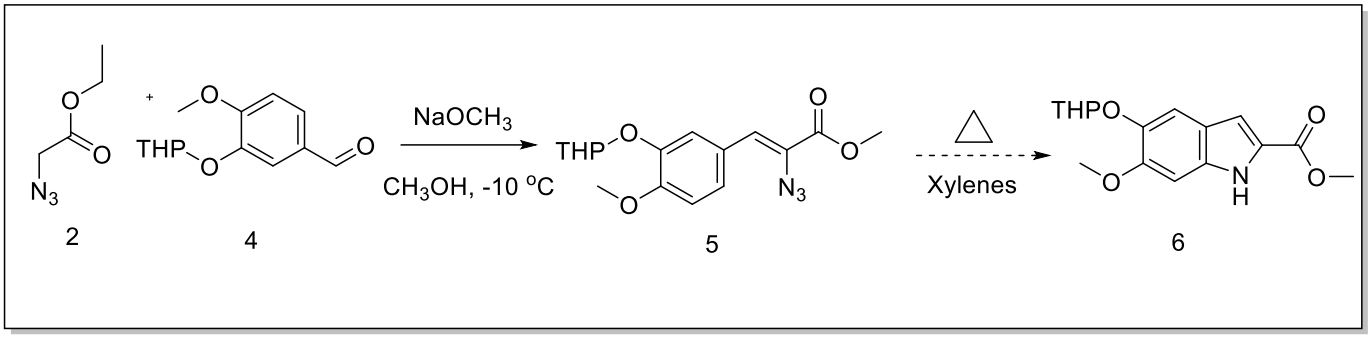
Scheme 1. Synthesis of ethyl azidoacetate



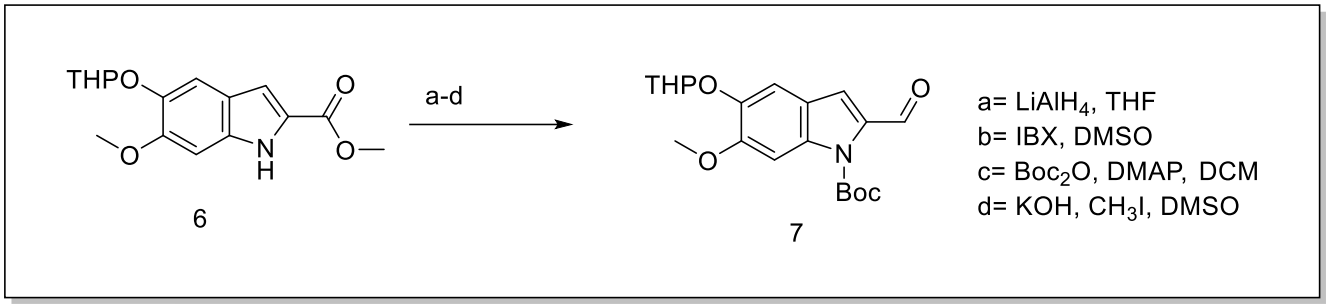
Scheme 2. Synthesis of protected aldehyde



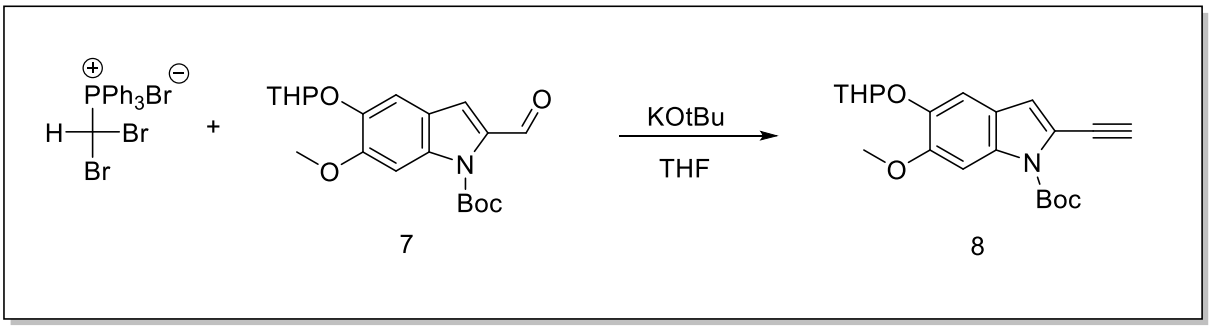
Scheme 3. Hemetsberger-Knittel indole synthesis



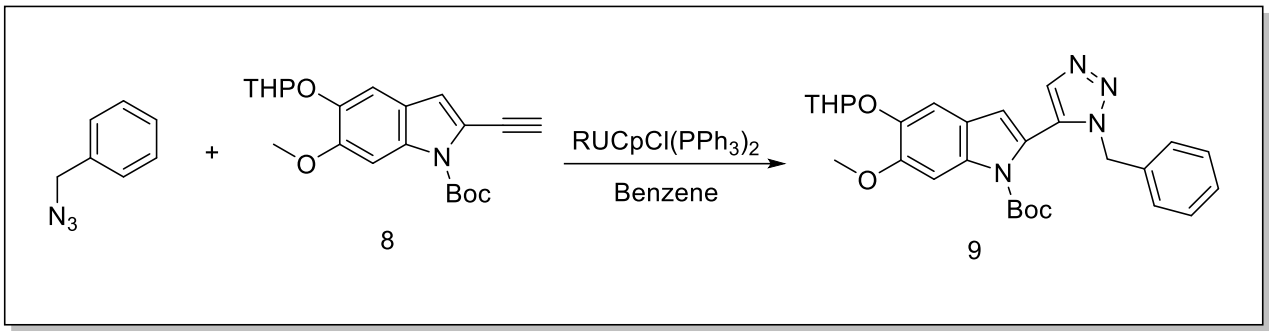
Scheme 4. Preparation of protected indole aldehyde



Scheme 5 Corey-Fuchs carbon homologation

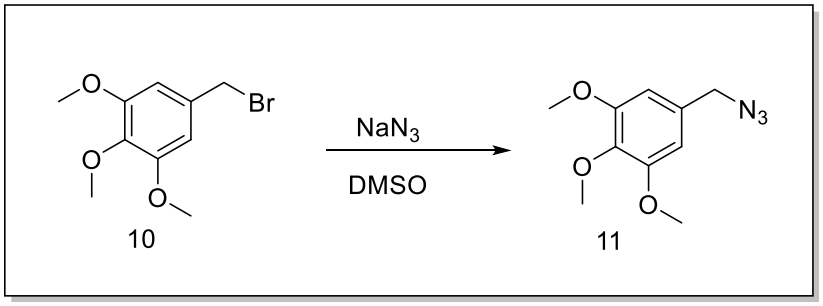


Scheme 6 Ruthenium mediated dipolar addition

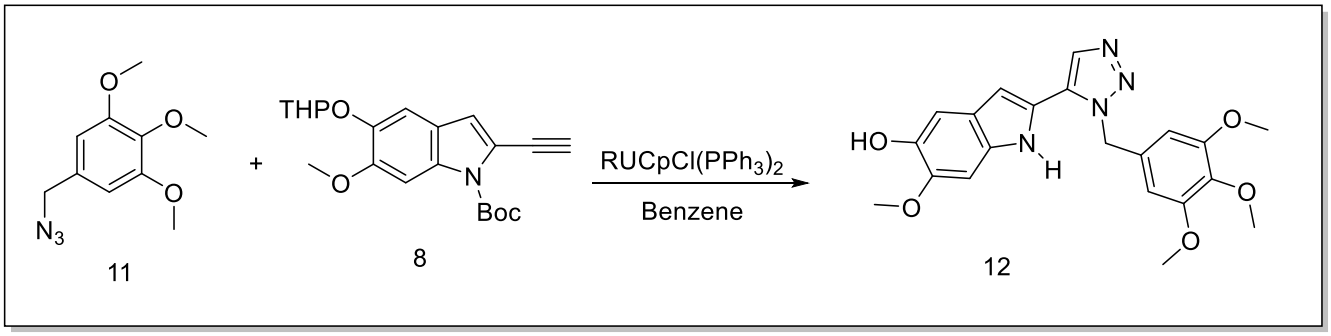


Shields research succeeded in synthesizing the pyrazoline indole ring system however, he did not successfully attach the trimethoxy ring system. Attaching the trimethoxy ring system is the next step in current research. A reaction of 3, 4, 5,-trimethoxybenzyl bromide and sodium azide (Scheme 7) will be used to generate a new azide (11) which will be reacted with the alkyne formed using the Corey-Fuchs Homologation shown above to generate the suggested 1, 2, 3-triazole analogue (Scheme 8). The methods and equipment used in the next step of the current research follows the same general format used in previous research by Shields to generate the benzyl azides from benzyl bromides except the benzyl bromides are replaced with 3,4,5,-trimethoxybenzyl bromides.

Scheme 7 New azide



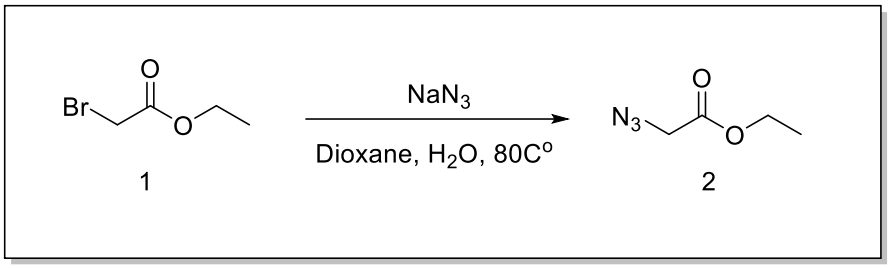
Scheme 8 Target analog



5. Results and Discussion

5.1 Synthesis of Ethyl Azidoacetate

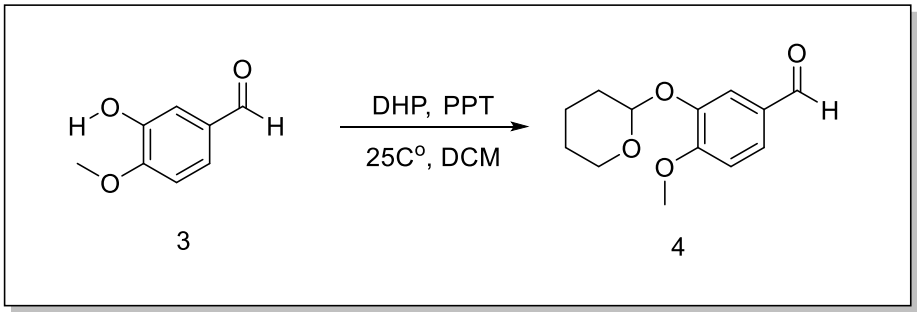
Scheme 1 Synthesis of ethyl azidoacetate



Progress towards the synthesis of 2-(1,2,3-triazolyl)indole CA-4 analog was made. The first step, synthesis of ethyl azidoacetate was successful and resulted in a 55% yield. This is significantly lower than previous research that achieved 91%. However, ^{1}H-NMR and IR data supports the conclusion that the product is relatively pure and can be used in the next synthetic step. ^{1}H-NMR data shows a signal at 1.32 ppm (3H), 3.8 ppm (2H) and 4.3 ppm (2H) which is expected for ethyl azidoacetate because it contains three unique hydrogens, two from the ethyl group and one attached to the carbonyl. Ethyl bromoacetate would contain these same peaks. To distinguish between ethyl azidoacetate and ethyl bromoacetate an IR was also produced. The IR showed a strong stretch between 2160 cm^{-1} and 2120 cm^{-1} which is characteristic of an azide. Furthermore, the resulting product was a pale yellow oil as described in previous research. During extraction, the separatory funnel leaked when inverted. This is most likely the cause of the lower yield. The efficiency of the reaction could be improved by using TLC to monitor progress. An attempt to use TLC was made involving iodine as a method of visualization, however only ethyl bromoacetate could be visualized and no conclusions were drawn from the TLC. For this reason, the reaction was allowed to reflux for more than the suggested 18 hours. Future research will employ TLC techniques to improve efficiency by using ethyl azidoacetate produced in this experiment to determine a visualization method that is effective for visualizing both ethyl azidoacetate and ethyl bromoacetate.

5.2 Synthesis of 4-methoxy-3-((tetrahydro-2H-pyran-2-yl)oxy)benzaldehyde

Scheme 2 Synthesis of protected aldehyde

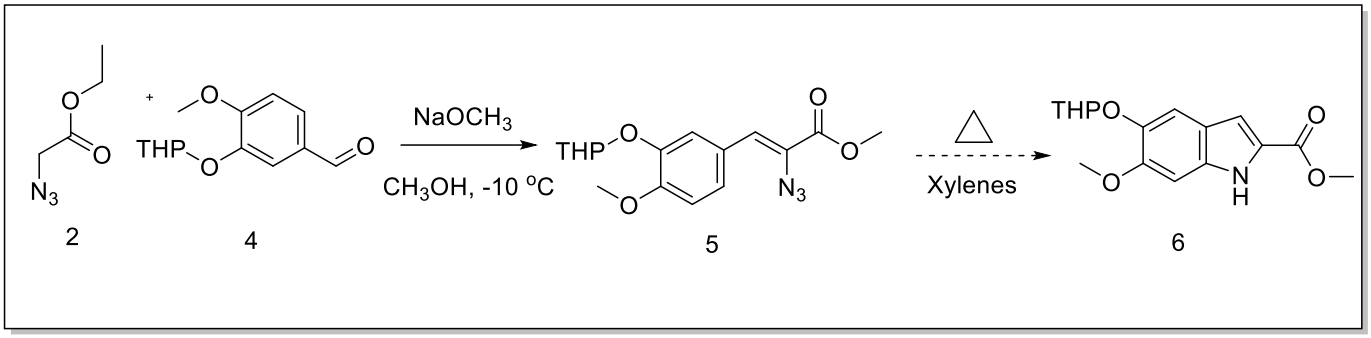


The objective of the synthesis of 4-methoxy-3-((tetrahydro-2H-pyran-2-yl)oxy)benzaldehyde (4), was to add a protecting group to the 3-hydroxy-4-methoxybenzaldehyde. The hydroxyl group needs to be protected because the pka of the hydroxyl group is 10 making it more acidic than the desired ethyl azidoacetate hydrogens which have a pka of 25. When reacted with ethyl azido acetate in a basic solution to generate a vinyl azide the hydroxyl group will become deprotonated and result in an undesired product if unprotected. The vinyl azide must undergo thermolysis via the Hemettsberger-Knittel indole synthesis. This experiment was conducted several times before it was discovered that using a plastic syringe to deposit DHP caused polymer contamination in the solution. Additionally, the PPTS used

in the earlier experiments was a slight shade of pink indicating that it was contaminated with water making it less acidic and useful as a catalyst. It should also be noted that a silica gel column cannot be used to purify this product. In previous experiments, use of a silica gel column resulted in loss of the protected aldehyde. Silica gel de-protects aldehydes protected with DHP. After these discoveries, new PPTS and only glass syringes were used in the latest attempt. These changes resulted in the successful synthesis of 4 (9.1g, 62% yield). The product was pure and no column chromatography was needed. The results of this experiment were confirmed with ^{1}H NMR. In previous experiments two peaks at 9.9 and 9.95ppm indicated that there were two aldehydes present in the final product sample. The aldehyde peak at 9.9 ppm was most likely the unprotected aldehyde while the peak at 9.95 ppm was the protected aldehyde, which would be further down field due to the oxygen on DHP. However, in the most recent experiment only one aldehyde peak at 9.95 ppm was present, indicating the absence of the starting material. The starting was further confirmed by the presence of a peak at 5.45ppm which is likely due to the lone hydrogen at the DHP aldehyde connection. This hydrogen indicates the presence of the protected product because this hydrogen is distinct as it is influenced by both the oxygen from the alcohol and the oxygen from DHP and is expected to be shifted downfield. Other observations suggest that the product is present such as color present only after ultra violet light exposure on TLC. This indicates the presence of the conjugated ring.

5.3 Synthesis of Methyl 2-azido-3-(3,4,5-trimethoxyphenyl) acrylate

Scheme 3. Hemetsberger-Knittel indole synthesis



The synthesis of 2-azido-3-(3,4,5-trimethoxyphenyl) acrylate (5) was achieved, however the yield was low (24.7 %, 0.37g) and the purity of the product is questionable. The low yield and impurity is most likely due to the imprecise measurement of the reactants. The density of 4-methoxy-3-((tetrahydro-2H-pyran-2-yl)oxy)benzaldehyde could not be found online thus it was measured by weight not volume. The protected aldehyde was weighed into a vial then transferred to glass pipet. Material was lost through container transfer and this loss was great as the protected aldehyde was extremely viscus. The aldehyde was so viscus that it could not be pulled up by a needle and had to be heated before it could be taken up by a glass pipet. ^{1}HNMR was used to characterize the product. The ^{1}HNMR showed that the product was impure. A large peak around 9.95 ppm most likely indicates the presence of the protected benzaldehyde. Past research has reported the appearance of the vinyl azide as a white crystal. The product in present research was yellow oil with some white crystals in it. The yellow oil is most likely the protected aldehyde and the crystals could be the vinyl azide. In future experiments the protected aldehyde will be diluted in order to get a more precise measurement.

6 Experimental methods

6.1 Instrumentation

NMR spectra were obtained on a Varian Unity INOVA with an Oxford Instruments 400MHz superconducting magnet using $CDCl_3$ as the solvents. A Thermo-Fischer Scientific Nicolet (Madison, WI, USA) iS10 FT-IR spectrometer was used for infrared measurements. Spectra treatment and data manipulation were carried out on Omnic (Thermo Nicolet

Corp., Madison, WI, USA) software. All glass ware was oven dried before use. All reagents were purchased from commercial sources.

6.2 Synthesis of Ethyl Azidoacetate

To a 2-neck round bottom flask (RBF) fitted with a magnetic stir bar, 5.0 g of sodium azide (NaN_3) followed by 5.5 mL of water was added and allowed to stir at room temperature for 10 minutes. The solution was then warmed to 50 ^oC. After 12.5 mL of dioxane and 4.25 mL of ethyl bromoacetate (warning: ethyl bromoacetate is a lachrymator) was added via syringe, the solution was then refluxed at 80 ^oC for 23 hours. A condenser and thermometer were used in the set up for the reaction. A dark brown/ orange color suggested that the reaction had progressed and the solution was allowed to cool to room temperature. The ethyl azioacetate was extracted with diethyl ether (3 x 20 mL). The combined organic layers were washed with brine (3 x 20 mL) then dried using sodium sulfate and concentrated under reduced pressure. The product was a pale yellow oil (2.71 g, 55%).

^{1}H-NMR ($CDCl_3$): 1.32 (3H, t), 3.8 (2H, s), 4.3 (2H, q). (Figure 11)

6.3 Synthesis of 4-methoxy-3-((tetrahydro-2H-pyran-2-yl)oxy)benzaldehyde

A 500 mL three neck RBF was oven dried, fitted with a nitrogen balloon, magnetic stir bar and rubber septum. The flask was charged with 280 mL of anhydrous DCM and 3-hydroxy-4-methoxybenzaldehyde (9.5g, 62.44 mmol) followed by a catalytic amount of pyridinium *p*-toluensulfonate (PPTS) (0.40g, 1.6mmol). The resulting mixture was set to stir until complete dissolution occurred. 3,4-dihydro-2H-pyran (DHP)(17.68 mL, 16.30g, 201.28 mmol) was dispersed in 20mL of DCM in a separate flame dried, nitrogen flushed two neck RBF and added to the mixture dropwise via syringe. The reaction mixture stirred at room temperature for 48 hours. The mixture was then concentrated under reduced pressure. The concentrate was washed with 400 mL of water and extracted with 400 mL of diethyl ether. The ethereal extracts were then washed with (12 x 50) saturated potassium carbonate. (*Note: a color change from colorless to yellow is observed when the unprotected 3-hydroxy-4-methoxybenzaldehyde is deprotonated*.) The last washing process was repeated 3 times. The water layer became colorless but the ethereal layer kept a slight yellow tint. The ethereal layer was then dried over sodium sulfate and reduced under pressure to yield the desired product: a pale yellow/orange oil. (9.1g, 62% yield).

^{1}H-NMR ($CDCl_3$): 9.85 (1H, s), 7.64 (1H, s), 7.54 (1H,d), 7.00 (1H, d), 5.47 (1H, t), 3.95 (3H, s), 3.65 (2H, m), 2-1.5 (6H, multiple peaks) (ppm). (Figure 12)

6.3 Synthesis of Methyl 2-azido-3-(3,4,5-trimethoxyphenyl) acrylate

All glassware was flame dried. A 2-neck RBF was fitted with a magnetic stir bar and was flushed with dry N_2 (g) via nitrogen balloon through a condenser and immersed in a methanol (MeOH) and ice bath at a temperature of -10 ˚C. Dry MeOH, 5 mL, was added via a syringe and allowed to cool. To the cooled MeOH, 0.47g (0.0195 mol, 4 eq.) of sodium metal was added piecewise and allowed to cool. In a separate RBF, 0.8725 g (0.0045 mol, 1 eq.) of 4-methoxy-3-((tetrahydro-2H-pyran-2-yl)oxy)benzaldehyde was added to 2.2 g (0.017 mol, 3.5 eq.) of ethyl azidoacetate and 3.8 mL of dry MeOH. The mixture was swirled then added dropwise to the sodium metal slurry. The density of 4-methoxy-3-((tetrahydro-2H-pyran-2-yl)oxy)benzaldehyde could not be found online and it was measured into a vial then added to the RBF via syringe. Due to the uncertainty in the measurements, excess sodium metal was added to the reaction. The reaction was covered in aluminum foil and left to react for one week. After one week the reaction had turned a deep orange color and a pale yellow precipitate had formed in the bottom of the flask. The contents of the flask were poured over 100 mL of ice and saturated ammonium chloride. A precipitate was expected but did not form. Liquid-liquid extraction was preformed 3 times with 100 mL ethyl acetate. The organic layer (a pale yellow) was collected, dried over magnesium sulfate and reduced under pressure. The crude product was a mixture of pale yellow oil and white crystals (0.37g, 24.7% yield).

1H-NMR ($CDCl_3$): 9.85 (1H, s), 7.8-6.8 (16 H, multiple peaks), 5.5 (1H, t), 5.4 (1H, t), 4.2-3.6 (24 H, multiple peaks), 2.1-1.3 (39 H, multiple peaks), 0.9 (3H, t). (Figure 13)

7. Conclusion

Based on previous research 2-(1,2,3-triazolyl)indole analog (compound 9b) of CA-4 was designed containing the key features for binding, water solubility and metabolic stability. The objective of this research was to synthesize 9b and test this compound for its growth inhibitory properties using MTT assay however, only partial synthesis of 9b was achieved. A multi-step synthetic scheme was followed starting with the synthesis of ethyl azidoacetate (compound 2). An SN2 reaction of ethyl bromoacetate and sodium azide was used to prepare ethyl azidoacetate in good yield. The next step in the reaction scheme involved the use of 3-hydroxy-4-methoxybenzaldehyde. Due to the acidity of the hydroxyl group, a tetrahydropyran (THP) protecting group addition was achieved using pyridinium p-toluenesulfonate as a catalyst resulting in the production of compound 4 in good yield. After preparing the protected benzaldehyde, a reaction of 2 and 4 generated a low yield of crude vinyl azide (compound 5). In future research the vinyl azide will undergo thermolysis via the Hemettsberger-Knittel indole synthesis involving a ring cyclization to produce an indole ring system. Future efforts will be focused on optimizing reaction conditions, developing alternate protecting group strategies and evaluating the compounds prepared for antitubulin activity.

8. Acknowledgments

The author would like to express her appreciation to Holt group members past and present for their efforts in laying the groundwork for this project. Additionally, she would like to give special thanks to the University of North Carolina Asheville Chemistry department faculty and staff for their patient guidance.

9. References

1. Ducki, S. Antimitotic Chalcones and Related Compounds as Inhibitors of Tubulin Assembly, Anti-Cancer Agents in Medicinal Chemistry. *Bioorganic Med.chem.,* **2009**, *9*, 336-347.
2. Onomatopoeia. (n.d.) In Merriam-Webster's collegiate dictionary. Retrieved from https://www.merriam-webster.com/medical/prodrug.
3. Ducki, S.; Mackenzie, G.; Greedy, B.; Armitage, S.; Fournier Dit Chabert, J.; Bennett, E.; Nettles, J.; Snyder, P.J.; Lawrence, J.N. Combretastatin-like chalcones as inhibitors of microtubule polymerisation. Part 2: Structure-based discovery of alpha-aryl chalcones. *Bioorganic Med. Chem.* **2009**, *17*, 7711–7722.
4. Jianjun, L.; Xiao, C.; Xiao, M.; Miller, L.; Miller, D.; An Overview of Tubulin Inhibitors That Interact with the Colchicine Binding Site. *Pharm Res*. **2012**, 11, 2943-71.
5. Tron, G. C.; Pirali, T.; Sorba, G.; Pagliai, F.; Busacca, S.; Genazzani, A. Medical Chemistry of Combretastatin A4: Present and Future Directions. *J. Med. Chem*. **2006**, 49, 3033-3044.
6. Grisham, R.; Ky, B.; Tewari, K.; Chaplin, D.; Walker, J. Clinical trial experience with CA4P anticancer therapy: focus on efficacy, cardiovascular adverse events, and hypertension management. *Gynecol Oncol Res Pract*. **2018**, 5, 1.
7. Xu, Q.; Bao, K.; Sun, M.; Jingwen, Xu, J.; Wang, Y.; Tian, H.; Zuo, D.; Guan, Q.; Wu, Y.; Zhang, W. Design, synthesis and structure activity relationship of 3,6-diaryl- 7H-[1,2,4]triazolo[3,4-b][1,3,4] thiadiazines as novel tubulin inhibitors. *Sci Rep*. **2017**, 7, 11997.
8. Pettit, R.; Singh, S. B.; Niven, H. E.; Schmidt, J. M. Isolation, Structure, and Synthesis of Combretastatins A-1 and B-1, Potent new Inhibitors of Microtubule Assembly, Derived from Combretum Caffrum. *J. Nat*. **1989**, 50, 119-131.
9. Pati, H. N.; Wicks, M.; Holt, H. L Jr.; LeBlanc, R.; Weisbrunch, P.; et al. Synthesis and biological evaluation of cis-combretastatin analogs and their novel 1,2,3-triazole derivatives. *Heterocycl. Commun.* **2005**, 11, 117-120.
10. Ohsumi, K.; Hatanaka, T.; Fujita, K.; Nakagawa, R.; Fukuda, Y.; et al. Syntheses and antitumor activity of cis-restricted combretastatins: 5-membered heterocyclic analogues. *Bioorg. Med. Chem. Lett.* **1998**, 8, 3153-3158.
11. Odlo, K.; Hentzen, J.; Fournier dit Chabert, J.; Ducki, S.; Gani, O.; Sylte, I.; Skrede, M.; Flørenesd V.A.; Hansena T.V. 1,5-Disubstituted 1,2,3-triazoles as cis-restricted analogues of combretastatin A-4: Synthesis, molecular modeling and evaluation as cytotoxic agents and inhibitors of tubulin. *Bioorganic Med.Chem.* **2008**, 16, 4829–4838.
12. Odlo, K.; Fournier-Dit-Chabert J.; Ducki, S.; A. B. S. M. Gani O.; Sylte I.; Hansen, T.V. 1,2,3-Triazole analogs of combretastatin A-4 as potential microtubule-binding agents. *Bioorganic Med.Chem.* **2010**, 18, 6874-6885.

13. Ducki, S.; Rennison, D.; Woo, M.; Kendall, A.; Fournier Dit Chabert, J.; T. McGown, A.J.; Lawrence, N. Combretastatin-like chalcones as inhibitors of microtubule polymerization. Part 1: Synthesis and biological evaluation of antivascular activity. *Bioorganic Med.Chem.* **2009**, 17, 7698-7710.
14. Tron, G. C.; Pirali, T.; Sorba, G.; Pagliai, F.; Busacca, S.; Genazzani, A.; Medical Chemistry of Combretastatin A4: Present and Future Directions. *J. Med. Chem.* **2006**, 49, 3033-3044.
15. Gaukroger, K.; Hadfield, J. A.; Lawrence, N. J.; Nolan, S.; McGown, A. T. Structural requirements for the interaction of combretastatins with tubulin: how important is the trimethoxy unit? *Org. Biomol. Chem.* **2003**, 1, 3033-3037.
16. Pettit, G. R.; Minardi, M. D.; Rosenberg, H. J.; Hamel, E.; Bibby, M. C.; et al. Antineoplastic agents. 509: synthesis of fluorcombstatin phosphate and related 3-halostilbenes(1). *J. Nat. Prod.* **2005**, 68, 1450-1458.
17. Kumar, D.; Kumar, N. M.; Akamatsu, K.; Kusaka, E.; Harada, H.; Ito, T. Synthesis and biological evaluation of indolyl chalcones as antitumor agents. *Bioorg. Med. Chem. Lett.* **2010**, 20, 3916−3919.
18. Chen J.; Zhang S.; Hu J.; Huang L.; Li X. Synthesis, Evaluation, and Mechanism Study of Novel Indole Chalcone Derivatives Exerting Effective Antitumor Activity Through Microtubule Destabilization in Vitro and in Vivo. *J. Med. Chem*. **2016**, 59 (11), 5264–5283.
19. Yu-Shan Wu et al. Synthesis and Evaluation of 3-Aroylindoles as Anticancer Agents: Metabolite Approach. *J. Med. Chem.* **2009**, 52, 4941-4945.
20. Piscitelli, F.; Ligresti, A.; La Regina, G.; Coluccia, A.; Morera, L.; Allara, A.; Novellino, E.; Di Marzo, V. Silvestri R. Indole-2-carboxamides as Allosteric Modulators of the Cannabinoid CB1 Receptor. *J. Med. Chem.* **2012**, 55 (11), 5627–5631.
21. Shields, B.; Holt, H. Design and Synthesis of Heterocyclic Combretastatin Analogues. Department of Chemistry The University of North Carolina at Asheville. **2014**

11. Supplemental Information

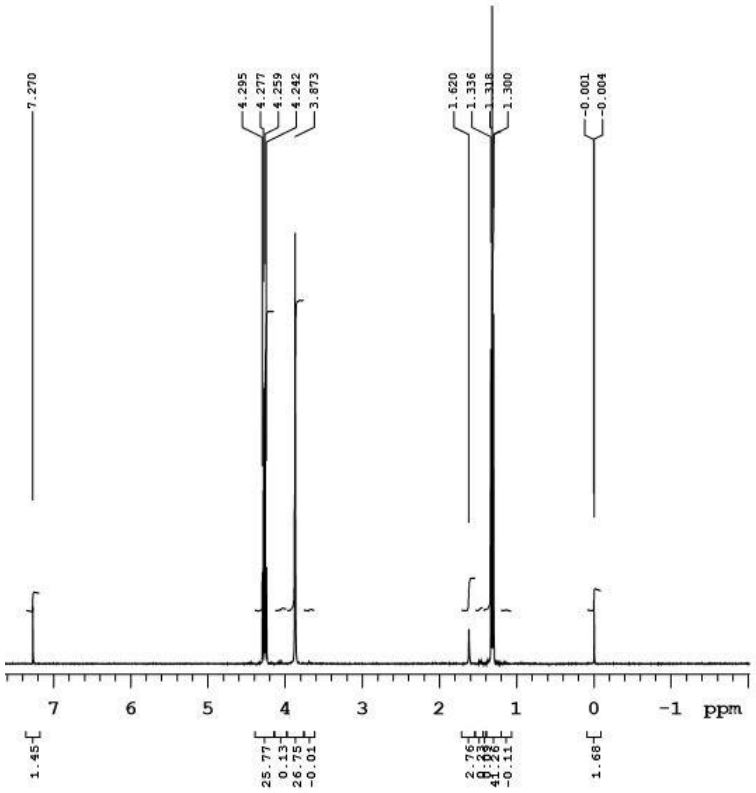


Figure 11. Ethyl azidoacetate ^{1}H NMR

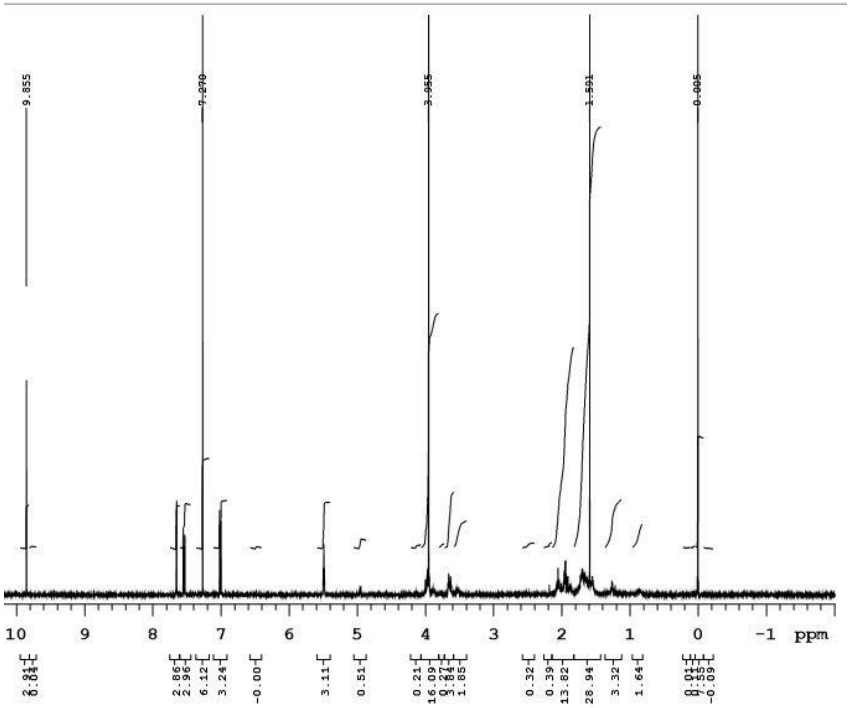


Figure 12. THP protected 3-hydroxy-4-methoxybenzaldehyde ^{1}H NMR

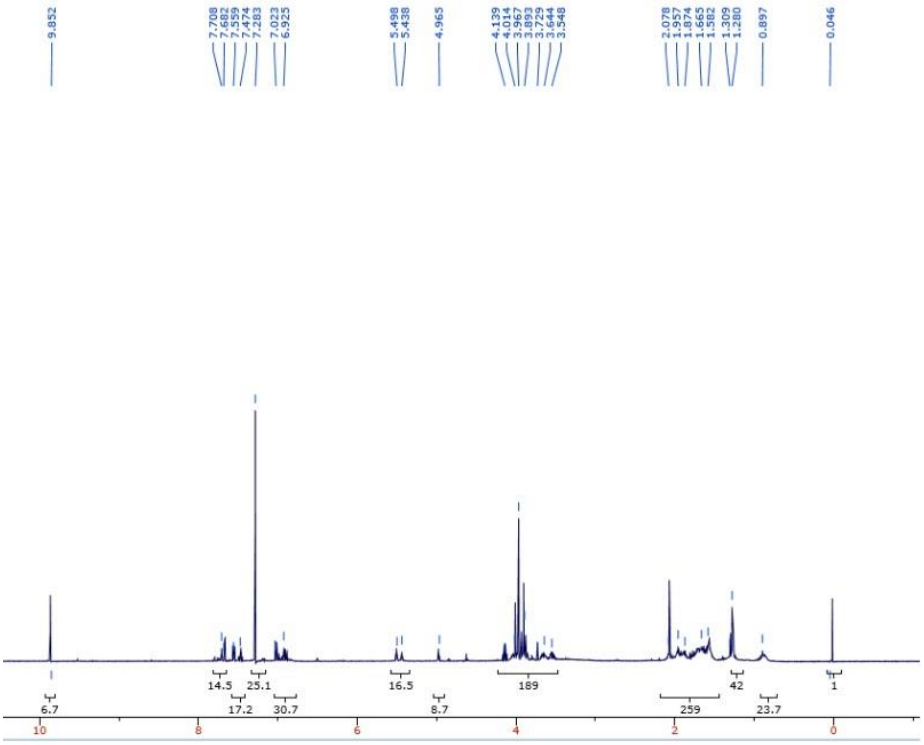


Figure 13. Methyl 2-azido-3-(3,4,5-trimethoxyphenyl) acrylate ^{1}HNMR