11-23-2015

Synthesis of Agents for the Treatment and Analysis of Tropical Diseases

Jeanine Yacoub
University of South Florida, jyacoub@mail.usf.edu

Synthesis of Agents for the Treatment and Analysis of Tropical Diseases

by

Jeanine Yacoub

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
Department of Chemistry
College of Arts and Sciences
University of South Florida

Major Professor: James W. Leahy, Ph.D.
Roman Manetsch, Ph.D.
Bill Baker, Ph.D.
Michael White, Ph.D.
Juan Del Valle, Ph.D.

Date of Approval:
March 25, 2016

Keywords: malaria, *Plasmodium*, toxoplasmosis, *Toxoplasma gondii*, kinase inhibitors, anti-malarials, Shield-1, SB-505124

Dedication

I'd like to dedicate this manuscript to my sister, Palestine Yacoub – her strength and compassion is representative of the country she was named after.

Acknowledgments

My sincerest gratitudes go to my research advisors, Dr. James Leahy and Dr. Roman Manetsch, for their continuous guidance and support. I'd also like to thank the members of my committee: Dr. Bill Baker, Dr. Michael White, and Dr. Juan Del Valle.

I'd like to especially thank Travis Bland, the undergraduate researcher that worked with me on the synthesis of Shield-1. All of the hours he put in moved the project forward and he has been a big help in many ways.

Thanks to my lab mates, Linda Barbeto, Andrea Lemus, Benjamin Eduful, Ankush Kanwar, and Zachary Schultz, for all of their support. Kurt Van Horn was a great mentor when I first started graduate school, and I appreciate all that he's taught me about synthetic organic chemistry. Michael Kemp has been a wonderful friend. I'd also like to recognize Susana Lopez for her help and support.

I'd like to give thanks to my parents, Mustafa and Wedad Yacoub; they came to America to give their children a better life, and I hope to make them proud. Finally, I want to thank my sisters, for their patience and support through the long hours I put in during graduate school. They are my best friends and my biggest critics.

Table of Contents

List of Tables

List of Figures

Abstract

Toxoplasmosis is an opportunistic disease caused by the protozoan parasite *Toxoplasma gondii*. The parasite is usually staved off by a healthy immune system and remains dormant in the body. In immunocompromised patients, the parasite can become active and spread throughout the body causing symptoms such as encephalitis, cognitive disorders, seizures, and death. Combination drug therapy is the usual treatment for toxoplasmosis; however, patients suffer from problems of intolerance, allergic reactions, and cytotoxicity. In an effort to identify new drug targets for toxoplasmosis, a series of compounds have been synthesized that can be used as tools to probe the unique pathways used by *T. gondii* to survive in the human host. One class of these compounds is pyridinyl imidazoles, which have been shown to be active against *T. gondii* MAP kinases. To set up a protein pull down assay, a biotinylated linker was synthesized. We have also synthesized a compound that's being used to study the pathways involved in the most active and proliferative form of *T. gondii*.

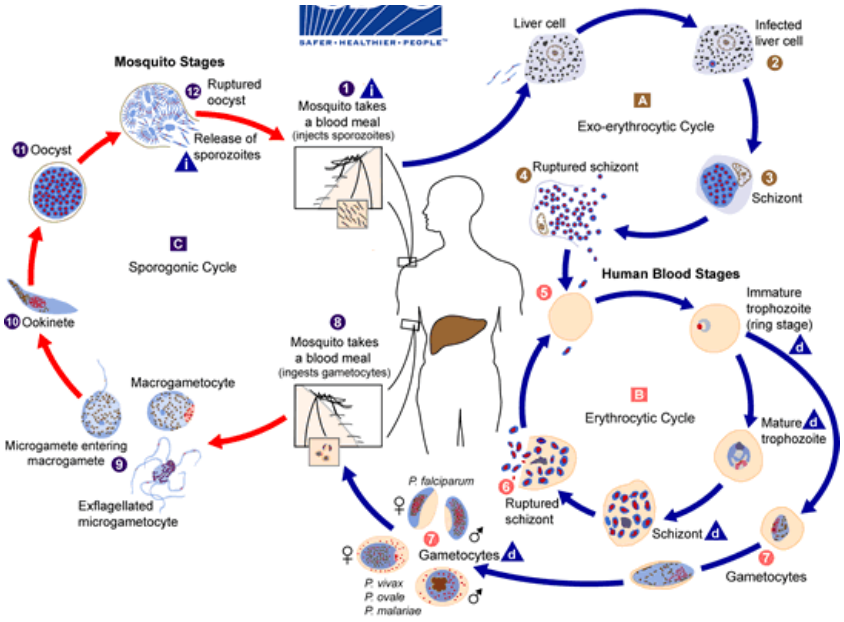
Chapter 1: Synthesis of Dihydropyridines as Anti-Malarials

1.1 Introduction

Malaria is a mammalian disease caused by parasites of the *Plasmodium spp*: *P. falciparum, P. vivax, P. ovale,* and *P. malariae.*[1] Although cases in the United States have been nearly eradicated, malaria is still prevalent in over 109 countries, mostly in the tropics and the subtropics. According to the World Health Organization, 198 million cases of malaria were reported in 2013 worldwide,[2] and approximately 580,000 deaths were reported (mostly among young African children). The most severe cases of malaria are caused by *P. falciparum* and *P. malariae*, which can sometimes be fatal.[1]

The life cycle of the parasite (Figure 1)[3] can be divided into three phases: the exoerythrocytic phase, the erythrocytic cycle, and the sporogonic phase.[1] The exoerythrocytic stage allows for the parasite to enter the bloodstream of the human and reaches the liver, where it proceeds to develop until it ruptures the liver cells and enters the bloodstream once more. After entering the erythrocytic cycle, the parasite burrows into red blood cells, thereby evading the immune system. At this point of the cycle, the parasite continues to multiply until the red blood cell ruptures, and then moves on to infect more red blood cells, thereby continuing to replicate and spread throughout the body.

Figure 1. Life Cycle of the *Plasmodium* parasite[3]



In the sporogonic phase, which takes place in the digestive track of the mosquito, the parasite reproduces sexually and then proceeds to the gut of the infected mosquito. It is here where the zygotes differentiate into ookinetes and develop into cysts which eventually rupture, spreading the sporozoites into the salivary glands of the mosquito and passed along duriing its next blood meal.

The symptoms presented by the patient are usually associated with a specific point in the parasite's erythrocytic life cycle.[1] For instance, when the parasite ruptures the red blood cells, the increased number of parasites in the blood elicits an inflammatory response, and the patient develops feverish symptoms such as headaches, chills, and diarrhea.[1] The dying red blood cells release an excess amount of hemoglobin, and when the liver cannot process the large amount of hemoglobin, causing jaundice in the patient.[1] Red blood cells become more rigid from the parasite's proteins that get embedded on the surface. This causes blockage of the capillaries and eventually tissue death. Cardiac and pulmonary complications can arise that can lead to death.

Malaria is typically treated with a combination of drugs that have been historically used including: quinine, chloroquine, mefloquine, atovaquone, sulfadoxine, and pyrimethamine (Figure 2).[4]

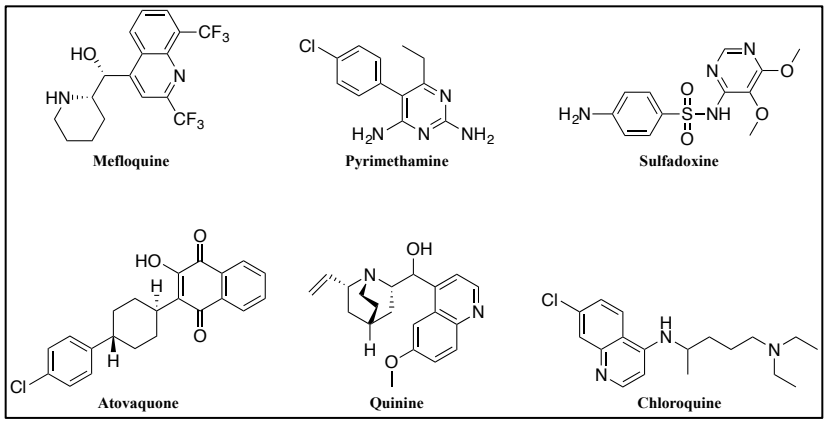


Figure 2. Anti-malarial pharmaceutical agents

Artemisinin, an anti-malarial, is a natural product found in the Chinese wormwood plant, *Artemisia annua;*[5] the 2015 Nobel Prize in Physiology/Medicine was awarded to Youyou Tu, a pharmacologist, for the discovery of artemisinin. Derivatives of artemisinin are also used to treat the disease (Figure 3). It is recommended by the World Health Organization to combine artemisinin, or one of its derivatives, with a historical anti-malarial drug, such as those in Figure 2.

Preventative measures are also used in areas with high malaria endemics to reduce the spread of the infection due to limited access to pharmaceuticals. These initiatives are not always successful.[6] One example of this is the misuse of nets provided to keep mosquitoes out. Nets lined with pesticides are provided to local populations in endemic areas. However, the locals use them as

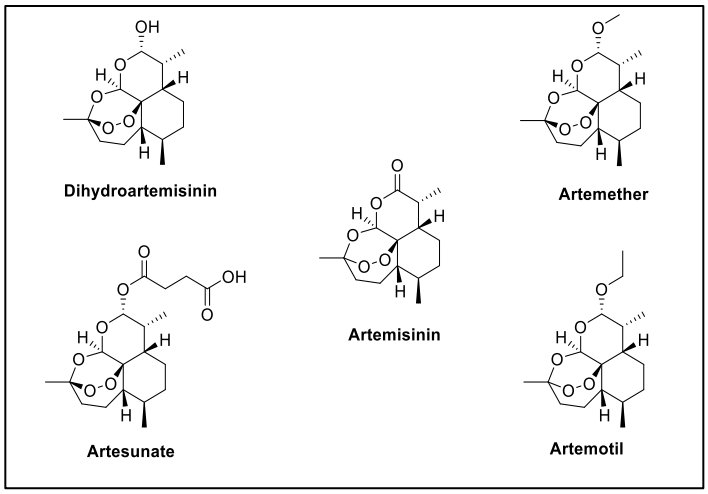


Figure 3. Artemisinin and derivatives

a means of getting food by fishing with these nets because they are cheaper than buying fishing nets. The nets are not used for their intended purposes, and the pesticides get into the local bodies of water, reducing the population of fish at an alarming rate.[6]

Toxicity of some of these drugs and development of drug resistance have prompted a worldwide research and development initiative toward novel treatments for malaria.[7]

In a high throughput screening campaign conducted by St. Jude Children's Research Hospital, GlaxoSmithKline, Novartis, and many other academic institutions, 309,474 compounds were tested against the 3D7 strain of *P. falciparum*.[8] Of those compounds, 1,300 showed >80% activity, 561 of which had EC_{50} values less than 2 μM and a selectivity index of >10 when compared to two mammalian cell lines. Out of the 561 compounds, 172 were reconfirmed by three separate labs to have anti-malarial activity. A review of the results reported the different classes of compounds that could be potential anti-malarial drugs.[8] Dihydropyridines represent one of the classes reported.

1.2 DHPs: Synthesis, Separation of Stereoisomers, and *in vitro* Testing

Dihydropyridines (DHPs) can be synthesized via the Hantszch reaction (Figure 4).[9] The mechanism begins with a Knoevenegal condensation between the diketone and the aldehyde. The enamine, formed from the β-ketoester and ammonia, goes through a condensation reaction with the Knoevenegal intermediate to the cyclized product. The DHPs in this study were synthesized according to the reaction scheme in Figure 5.

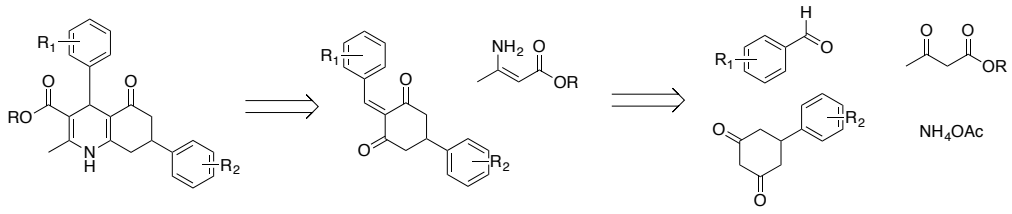


Figure 4. Retrosynthetic scheme of asymmetric 1,4-DHPs

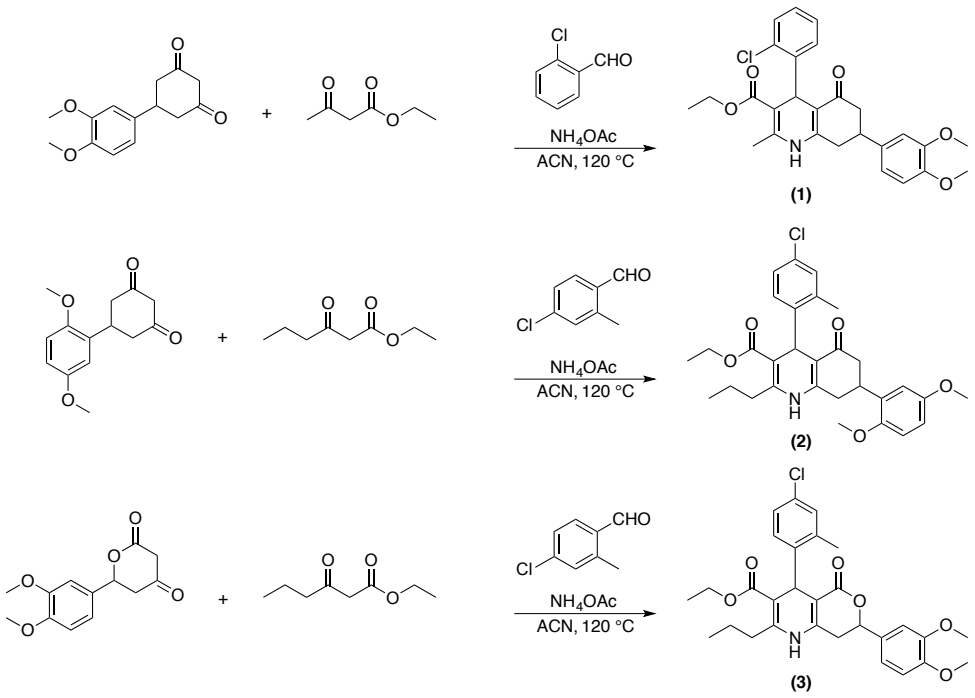


Figure 5. Synthesis of 1,4-DHP analogs for *in vitro* studies

The asymmetric reaction produces two potential racemic diastereomers. For the in vitro studies, the mixture of isomers for each compound were separated by reverse phase preparative HPLC. All four isomers were tested against the following strains of *P. falciparum*: 3D7 (chloroquine sensitive), K1 (chloroquine resistant), W2 (chloroquine and pyrimethamine resistant) and TM90-C2B (chloroquine, mefloquine, pyrimethamine, and atovaquone resistant).

Three analogs were synthesized to add to the library of DHP's from previous work (Figure 5).[10] Compounds **1**-**3** were tested in vitro, and the IC_{50} values are reported in Table 1. The (4,7)–cis and (4,7)–trans isomers for compounds **2** and **3** were separated by reverse phase preparative HPLC and tested individually in vitro.

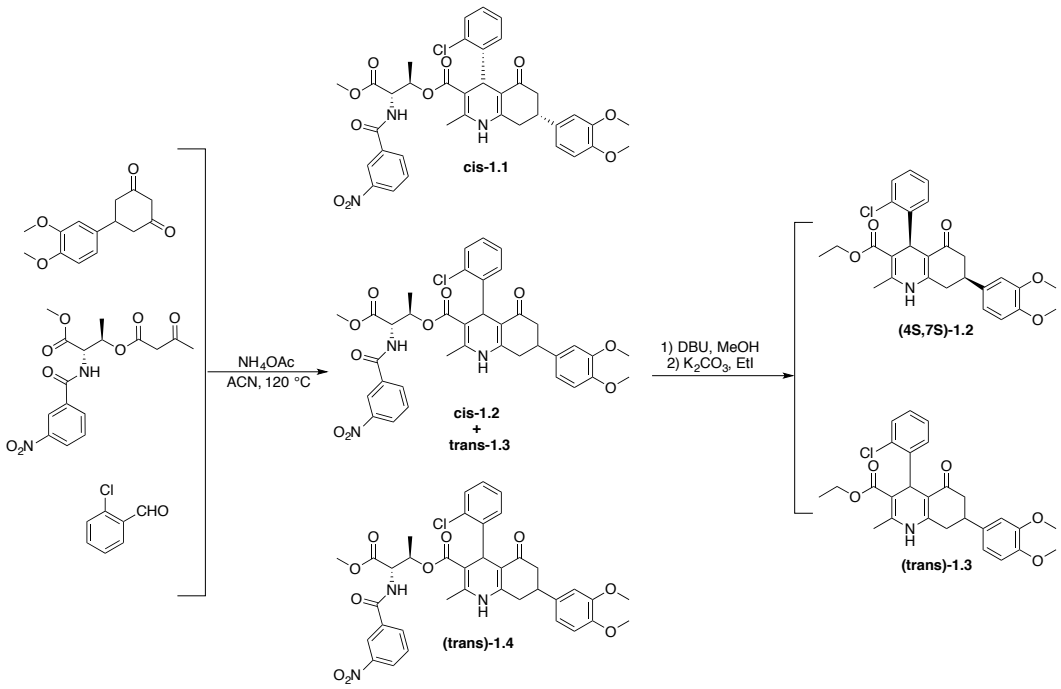


Figure 6. Synthesis of compound 1 with threonine chiral auxiliary

Another aspect of this synthetic study was to determine the absolute stereochemistry of the 4 and 7 position for compound **1**, and then test each of the four isomers for in vitro activity against the *P. falciparum* parasite. From previous work, using the Evans oxazolidinone as a chiral auxiliary, x-ray crystal structures confirmed the absolute stereochemistry for the 4,7-cis conformations for compound **1**. With that as a standard, the synthesis of compound **1** (Figure 6) using a different chiral auxiliary at the 3 position was done to grow crystals for x-ray crystallography, since this was unsuccessful with the Evans oxazolidinone.

The order in which the compounds are shown in Figure 6 represents how the order in which the isomers eluted off of the reverse phase HPLC column. Cis-DHP **1.1** eluted first off of the column, a mixture of cis **1.2** and trans **1.3** followed, and trans **1.4** eluted at the end. The chiral auxiliary was removed from all three fractions with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), followed by an alkylation with ethyl iodide to

obtain the corresponding ethyl esters (shown in Figure 6 with the cis-**1.2** and trans-**1.2**). Diastereomers **1.2** and **1.3** were then separated once more using preparative HPLC.

An attempt was made at growing crystals of trans-**1.4** (with the chiral auxiliary) in ethanol, methanol, dimethylsulfoxide, dimethylformamide, toluene, chloroform, and a combination of solvent systems, but high quality crystals were not attained. The absolute stereochemistry of trans-**1.4** was subsequently determined from an undesired side product. In an attempt to derivatize the C2 position, a reaction was performed to

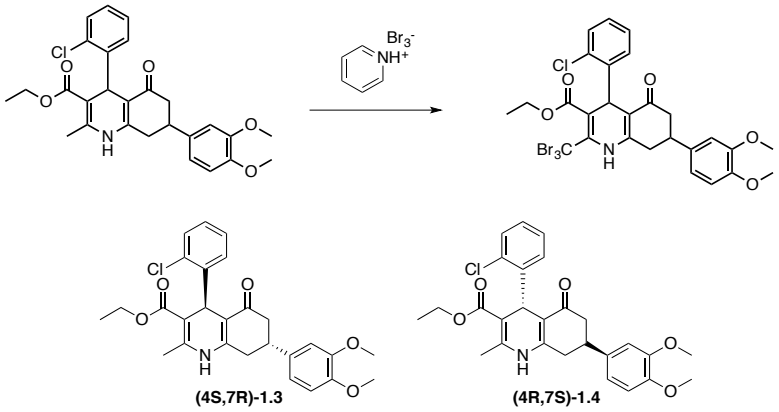


Figure 7. Tribrominated side product, and absolute stereochemistry for trans isomers

brominate the methyl group, but an undesired tribrominated product formed (Figure 7). X-ray crystallography of this side product revealed the absolute stereochemistry of the trans isomers (Figure 7). All of the pure isomers for compound **1** were tested in vitro along with the racemic diastereomers of compounds **2** and **3** (Table 1).

Table 1. IC_{50} values for DHPs 1-3

DHP	3D7 (nM)	K1 (nM)	W2 (nM)	TM90C2B (nM)
(1.1) (4R,7R)	16.7	7.5	200	514
(1.2) (4S,7S)	>6033	>6033	2132	2110
(1.3) (4S,7R)	>5833	>5833	>5000	>5000
(1.4) (4R,7S)	1.6	32.8	18.12	49.08
(2) Mix (cis and trans)	28.7	66.2	55.1	43.7
(2) 4,7-cis	205.7	869	542.1	191.5
(2) 4,7-trans	22.4	55.4	37.5	28
(3) Mix (cis and trans)	20.4	39.7	30	25.4
(3) 4,7-cis	134	70.3	147.5	108.9
(3) 4,7-trans	3	6.8	5.1	4

The 4,7-trans compounds were more active than the 4,7-cis analogs. Compound **1** revealed the importance of the stereochemistry at position 4. The IC_{50} values for the DHP's with a 4S stereocenter (compounds **1.2** and **1.3**) were in the micromolar range, whereas the 4R DHP's (compounds **1.1** and **1.4**) had single or double-digit nanomolar activity.

1.3 Conclusion

As part of a structure activity relationship study for 1,4-dihydropyridines as anti-malarials, three DHP analogs were synthesized and tested in vitro. The absolute stereochemistry for the four isomers of one particular analog were separated with the use of a chiral auxiliary. Crystallization of this compound was attempted for x-ray crystallography, however, this was unsuccessful. An undesired product for the bromination of the pure trans enantiomer produced quality crystals that gave x-ray crystallographic data and determined the absolute stereochemistry of the 4,7-trans products of compound **1**. In vitro tests of the compounds **1.1**-**1.4** revealed the significance of the stereochemistry of position 4 for activity. The 7 position also seems to improve the activity 10-fold (Table 1, compound **1.1** vs. **1.4**). The compounds

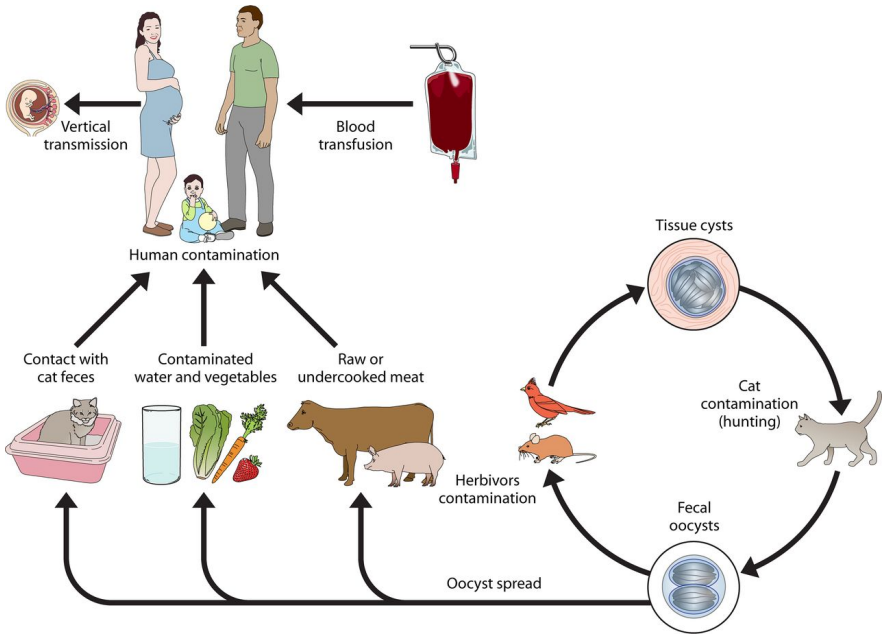
presented here are part of a large library of compounds in a structure activity relationship study of 1,4-dihydropyridines as anti-malarials and a manuscript of this work is being compiled for publication.[10]

Chapter 2: Synthetic Agents for the Treatment and Analysis of Toxoplasmosis

2.1 *Toxoplasma gondii*

Toxoplasma gondii is an apicomplexan protozoan parasite that causes the disease toxoplasmosis. The definitive host for the parasite is the feline.[4] After the cat ingests tissue cysts from infected small rodents, the parasite sexually reproduces and develops into oocysts. The infective oocysts are shed in the cat's feces and transmitted back to small rodents or to humans from the consumption of contaminated soil, plant material, or undercooked meat.[4] The parasite can also be transmitted to the fetus if the mother is infected with the parasite.[11]

The oocysts develop into rapidly dividing tachyzoites in the intermediate host (Figure 8).[12,13]



As a tachyzoite in the gut, the parasite continues to proliferate and elicits a number of inflammatory response cells, which can also get infected and spread throughout the body like a Trojan horse.[14] The tachyzoite also responds to IFNɣ which causes it to develop into the slow growing bradyzoite form as tissue cysts – a mechanism by which the parasite can evade the immune system.

2.2 Toxoplasmosis: Epidemiology, Symptoms, and Treatments

According to the Centers for Disease Control and Prevention, toxoplasmosis is one of five neglected parasitic infections in the United States.[15] Approximately 22.5% of the U.S. population has been infected with *T. gondii*.[11] In some areas of the world, usually in hotter, more humid climates, up to 95% of the population has been infected.

Figure 8. Life cycle of *Toxoplasma gondii*[12]

Toxoplasmosis is an opportunistic infection that can usually be staved off by a healthy immune system, although the parasite remains dormant. Toxoplasmosis causes flu-like symptoms (body aches, swollen lymph nodes, headache, fatigue, etc.).[11] In patients with compromised immune systems, symptoms include dizziness, confusion, retinitis, and seizures. AIDS patients often contract an opportunistic infection in the lungs, such as tuberculosis, or pneumonia.[11, 16] If a woman acquires the infection during pregnancy, the parasite can be transmitted to the fetus, which can lead to miscarriage or stillbirth in the early stages of pregnancy. Fetal toxoplasmosis can cause jaundice and retinitis in the baby. In later years, the child often develops hearing loss or mental disability. Recently, studies have shown that toxoplasmosis can cause behavioral changes in rats, chimpanzees, and humans.[17] There is a link between toxoplasmosis and severe mental disorders, such as schizophrenia and bipolar disorder.[18]

Toxoplasmosis is commonly treated with a combination drug therapy of pyrimethamine and sulfadiazine (Figure 9).[12] In 2015, pyrimethamine (trade name Daraprim®) received national media coverage due to a significant increase in its price by the CEO of Turing Pharmaceuticals, Martin Shkreli.[19] It can be cytotoxic because it acts as an anti-folate and affects rapidly dividing cells. Leucovorin (folinic acid) is also administered to alleviate the toxicity.[12] Clindamycin or atovaquone is substituted in for sulfadiazine for patients that are allergic to sulfa drugs.[20] Mechanisms of drug resistance have also been shown to be an issue in Toxoplasmosis.[21] *T. gondii* is known for being a good model organism to study for other parasitic infections.[22] The search for novel drug therapies and studying the mechanisms of action for *T. gondii* are significant.

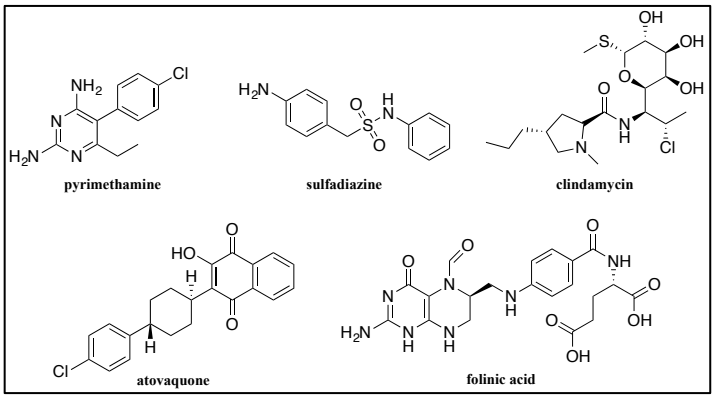


Figure 9. Pharmaceutical treatments for toxoplasmosis

2.3 HIF-1, *Tg*MAPK, and SB-505124

Protein kinases (PKs) are involved in many cellular processes and they function by transferring a phosphate group to other substrates. These phosphate transfers are involved in signal transduction pathways that regulate cellular growth, division, and apoptosis, among other homeostatic processes.[23] Protein kinases are a way for the parasite to coordinate its infectious cycle in the body. If this signaling is disrupted, the parasite can be more easily eradicated.

Toxoplasma gondii encodes for 190 kinases;[14] some drug discovery efforts for toxoplasmosis include targeting of parasitic CAMK (Calcium Dependent Protein Kinases),[24] MAPKs (Mitogen Activated Protein Kinases),[25] and ROPK (Rhoptry Kinases).[26] Each set of kinases is involved in invasion, host immune manipulation, proliferation, and egress.[27]

T.gondii manipulates the molecular processes of the host in order to survive in the intermediate host. One such way is by recruiting HIF-1 (Hypoxia Inducible Factor), a set of transcription factors that regulate the cell under hypoxic (low oxygen) conditions.[28] *T. gondii*, under physiological oxygen levels, stabilizes the HIF-1α subunit, which is achieved by downregulating PHD2 (Prolyl Hydroxylase Domain 2).[25] *Toxoplasma* requires the use of activin receptor-like kinases (ALK4, ALK5, and ALK7) to downregulate PHD2.

Pyridinyl imidazole SB505124 (Figure 10), an inhibitor of ALK4, ALK5, and ALK7,[29] was used to study the role of ALK's in HIF-1α activation.[25] In this study, an unexpected result showed an inhibition of parasite growth in a HIF-1α knockout cell line. This led to the discovery that SB-505124 (Figure 10) reduced parasite growth by simultaneously inhibiting both host HIF-1 and *Tg*MAPK (*Toxoplasma gondii* MAPK).

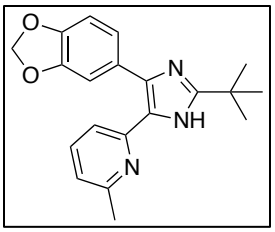


Figure 10. SB-505124 - a known ALK inhibitor

In an effort to identify new pharmaceuticals to treat this disease, the synthesis of a series of compounds was undertaken to set up a protein pull down assay to test against *Toxoplasma gondii* kinases (Figure 11).[30] One class of these compounds are pyridinyl imidazoles, such as SB-505124. In addition, the synthesis of a biotinylated linker is in

progress that can have a series of kinase inhibitors coupled at the end of it. Staurosporine, a known inhibitor of most kinases,[31] has been coupled to the linker and will be used as a starting point for the assay. To make this more specific for *Tg*MAPK, an analog of SB505124 has been synthesized to attach to the linker.

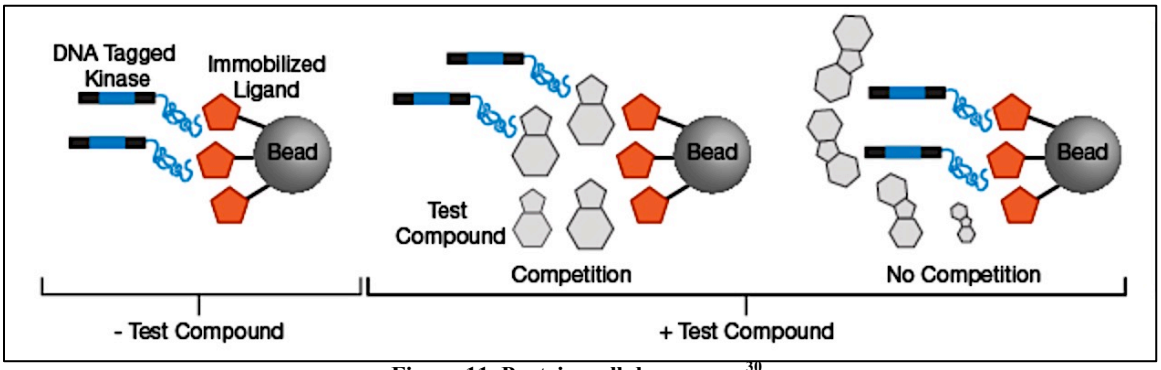


Figure 11. Protein pull down assay[30]

As shown in the Figure 11, the setup for the protein pull down assay is going to be developed so that the kinases of *T.gondii* can be isolated. The immobilized ligand is composed of a biocompatible, biotinylated linker with an immobilized ligand attached to it. The ligand has a high affinity for the target kinase. In this case, the initial development of the assay will use staurosporine, a promiscuous kinase inhibitor, as the active site directed ligand.[31] It is known that *T.gondii* kinases cannot be transfected into other organisms for the purposes of purifying the protein, so these compounds will provide a tool to isolate the kinases and screen any potential inhibitors against *T. gondii* kinases.[29]

2.4 Synthesis of Pyridinyl Imidazole SB-505124

The synthesis of pyridinyl imidazoles can be achieved from a condensation reaction of a 1,2-diketone with an aldehyde,[32] as shown in Figure 12.

SB-505124 can be synthesized from the diketone, which is prepared via alpha oxidation of the monoketone. The ketone can be made from a nucleophilic acyl substitution of a piperonylic acid derivative with 2,6-lutidine.

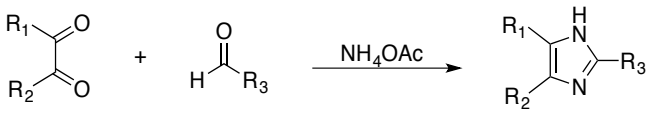


Figure 12. General synthesis for pyridinyl imidazoles

In the first step of the synthesis, piperonylic acid is converted to methyl ester **2.1**, which is then used for a nucleophilic acyl substitution with 2,6-lutidine (Figure 13) to obtain ketone **2.2**. However, this synthetic step provided low yields, possibly due further reaction with the product ketone.

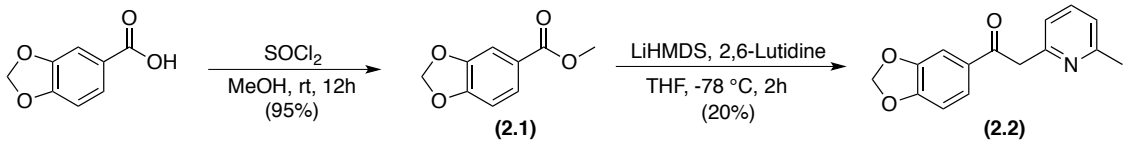


Figure 11. Synthesis of ketone 2.2 from methyl ester 2.1

A Weinreb amide was used instead of the ester to take advantage of the chelation effects of the N-methoxy group in the transition state, until the reaction is quenched with water (Figure 14).[33] Amide **2.1a** was synthesized from piperonylic acid through the acid chloride.[34] Nucleophilic substitution with 2,6-lutidine provided ketone **2.2**. The

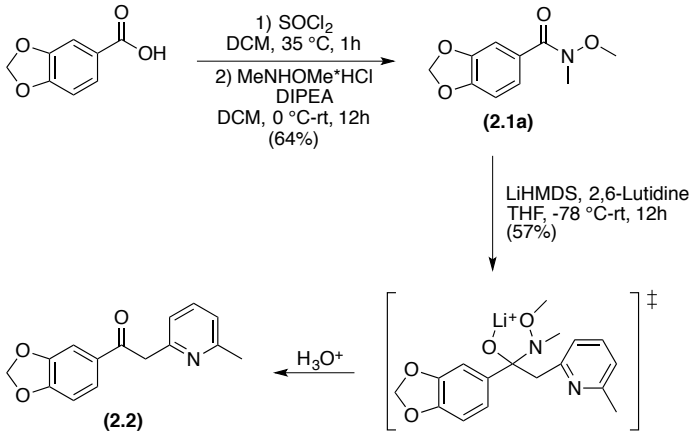


Figure 12. Synthesis of ketone 2.2 fromWeinreb amide 2.1a

chelation effects of the methoxy group on the amide holds the tetrahedral intermediate in place after one addition of the nucleophile until the reaction is quenched. This method provided much better yields for this synthetic transformation.

Diketone **2.3** was prepared by α-oxidation of monoketone **2.2** with aqueous hydrobromic acid in the presence of dimethyl sulfoxide (Figure 15).[35]

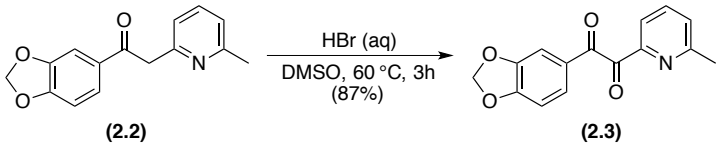


Figure 13. Alpha oxidation to diketone 2.3

A condensation of diketone **2.3** with pivaldehyde provided SB-505124 (Figure 16; Compound **2.4**).[36] Compound **2.5** was synthesized initially with an eye to coupling the test compound and the biotinylated linker via a Mitsunobu reaction (Figure 16). Compound **2.6** was synthesized and tested in vitro along with compounds **2.4** and **2.5** to confirm there was not a significant change in activity against *T.gondii*.

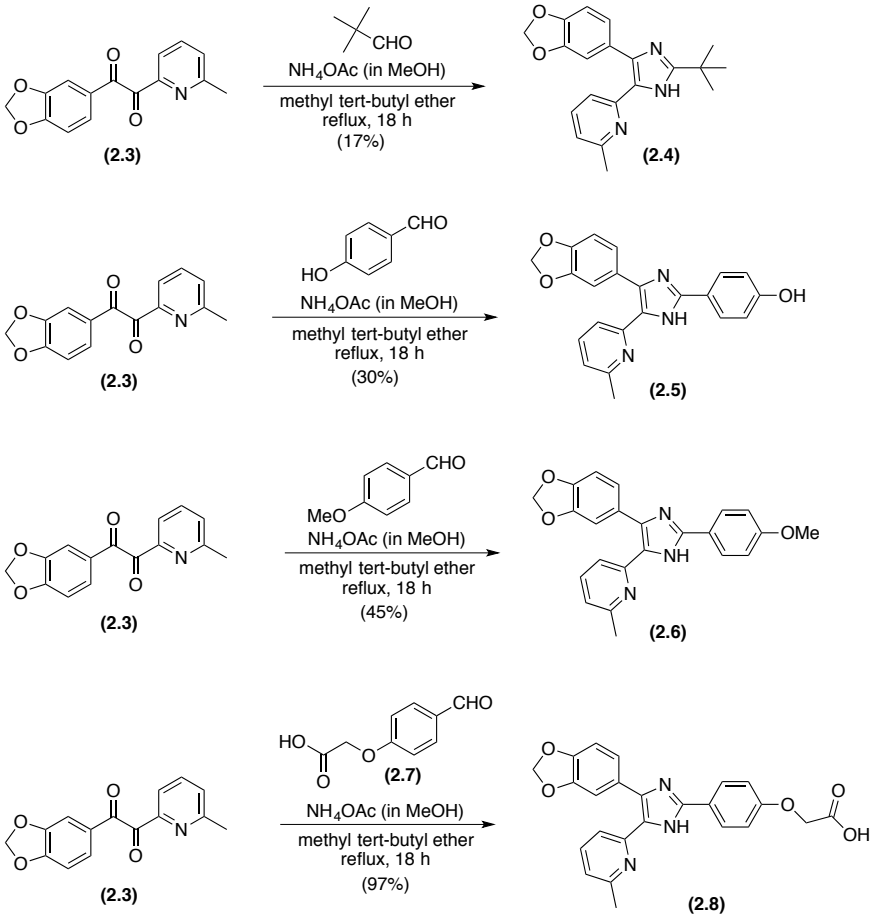
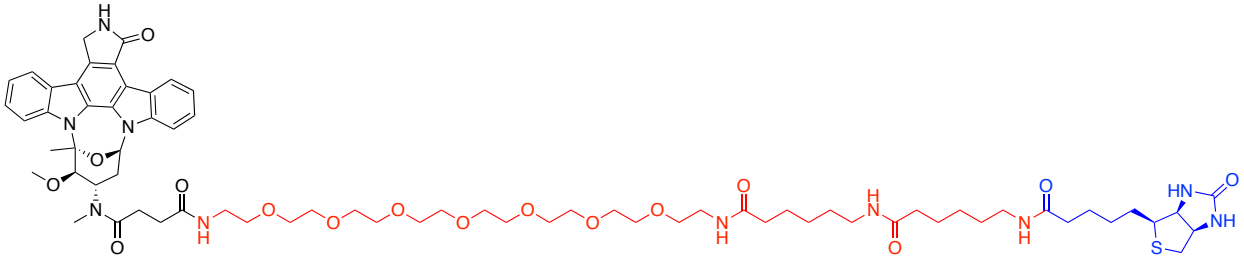


Figure 14 Pyridinyl imidazole analogs

Due to a change in the synthetic route for the biotinylated linker, compound **2.5** could not be used for coupling with the biotinylated linker, so compound **2.8** (shown above) was synthesized to attach the linker using a peptide coupling reagent.

2.5 Synthesis of Biotinylated Linker

The biotin conjugated flexible linker (Figure 17) is composed of four parts. The first and second part come from the coupling of the dipeptide portion with the octaethylene glycol chain (both shown in red). Biotin (blue) is then coupled to the linker as a way to immobilize the linker with streptavidin beads. The final part of the synthesis



(2.22)

Figure 15 Biotinylated linker

is the attachment of different small molecules, in this case, staurosporine (black), which will serve as active site directed ligands for the kinases in the assay (Figure 17).

The synthesis of the dipeptide (Figure 18) began with Boc protection of 6-aminocaproic acid, followed by an esterification with N-hydroxysuccinimide in the presence of EDC to afford activated ester **2.10**. The activated ester was coupled with another 6-aminocaproic acid followed by another esterification to obtain dipeptide **2.12**.

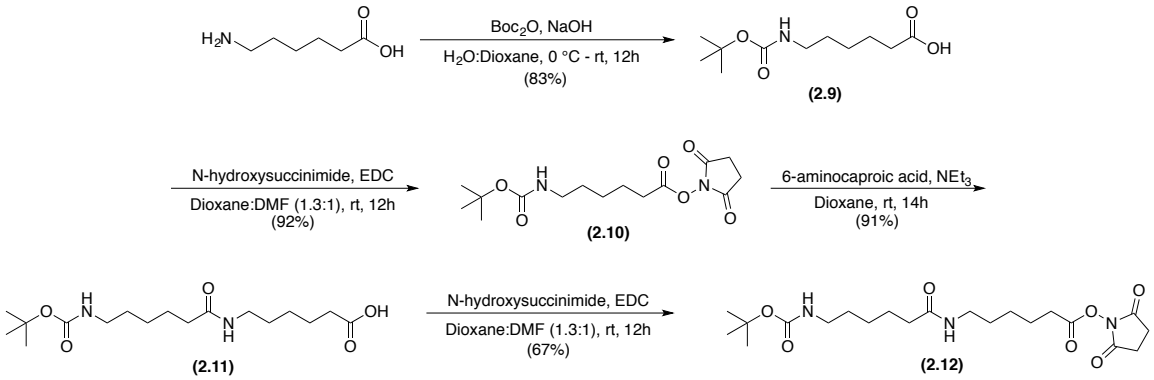


Figure 16 Synthesis of dipeptide 2.12

The octaethylene glycol chain was originally going to be made from two tetraethylene glycol units with protected amines (Figure 19). Several attempts at this reaction were made using different bases such as potassium carbonate, sodium tert-

butoxide, and sodium hydride. This reaction did not work, so the ethylene glycol chain was made from octaethylene glycol (OEG).

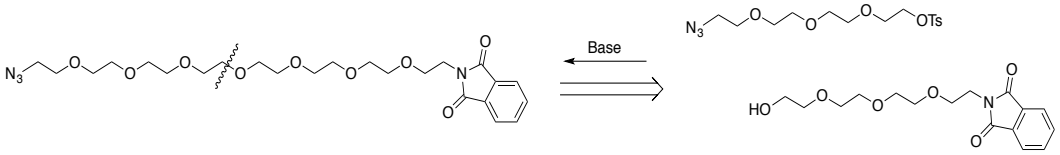


Figure 17 First proposed route to synthesize protected OEG

Starting from octaethylene glycol, the free alcohols on each end were mesylated and displaced with sodium azide (Figure 20).[37] A mono-Staudinger reduction was performed with triphenylphosphine to reduce one of the azides to monoamine **2.14**.[38] Monoamine **2.14** was coupled with dipeptide **2.12** to obtain compound **2.15**, the flexible portion of the linker.

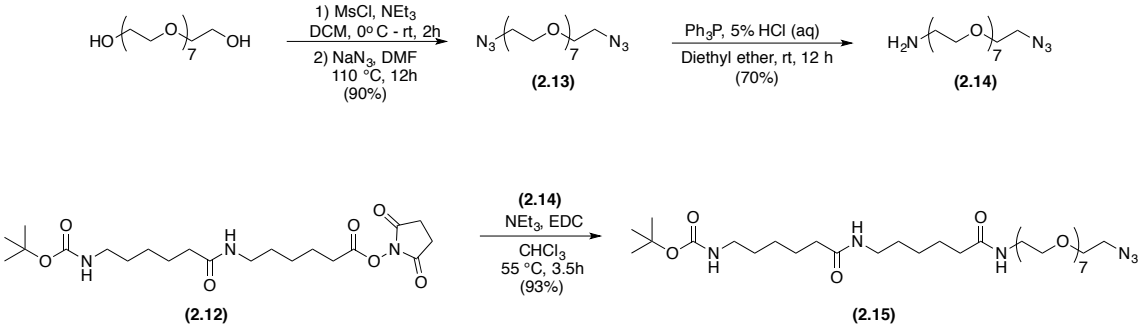


Figure 20. Synthetic sheme for linker 2.15

The activated ester of biotin was synthesized with N-hydroxysuccinimide and EDC (Figure 21). After Boc deprotection of **2.15** using trifluoroacetic acid, the flexible linker was coupled to the activated ester of biotin (Figure 21). Subsequent reduction of the azide using hydrogen over palladium catalyst afforded biotinylated linker **2.19**, which can be coupled potentially with any carboxylic acid.

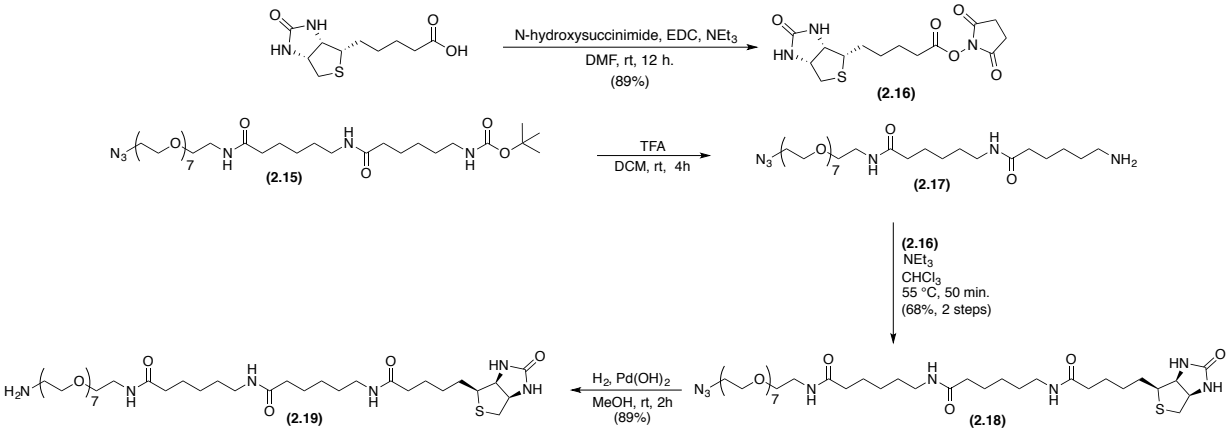


Figure 21. Synthetic scheme to biotinylated linker

Our next goal was to couple staurosporine with linker **2.19**. Fluorescein was first coupled to the linker to give compound **2.20**. This will be used to ensure that the linker loads onto the streptavidin beads and to test the solubility of the linker in the media used for the *T.gondii* assay (Figure 22).

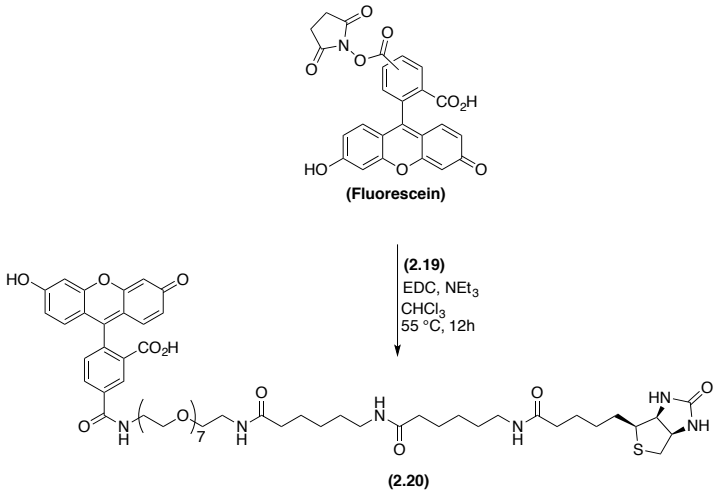


Figure 22. Fluorescein - biotinylated inker

Amidation of Staurosporine was achieved with succinic anhydride in dimethyl sulfoxide and then coupled to linker **2.19** (Figure 23).[39]

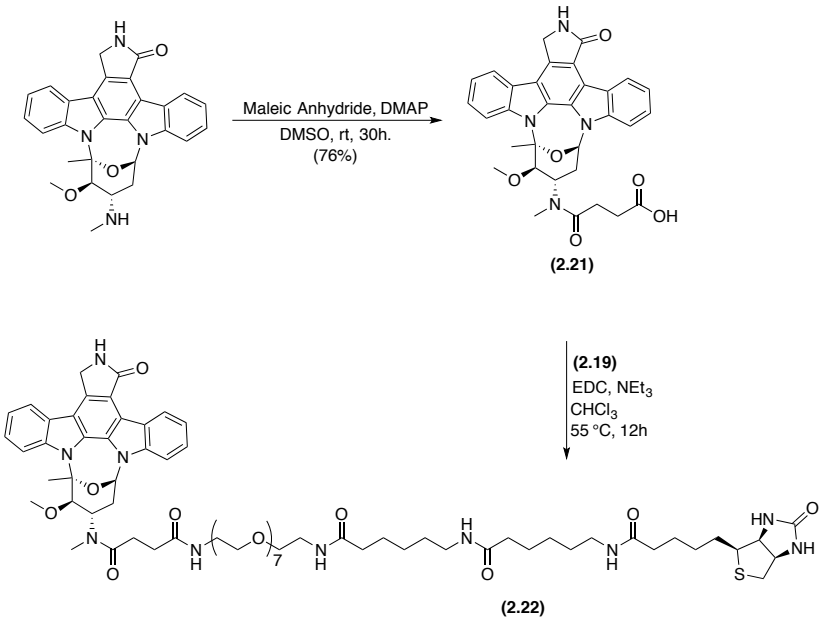


Figure 23. Staurosporine-biotinylated linker

F inally, pyridinyl imidazole **2.8** was coupled to linker **2.19** to make compound **2.23** (Figure 24). Reverse phase preparative-HPLC was used for purification of compounds **2.20**, **2.22**, and **2.23**.

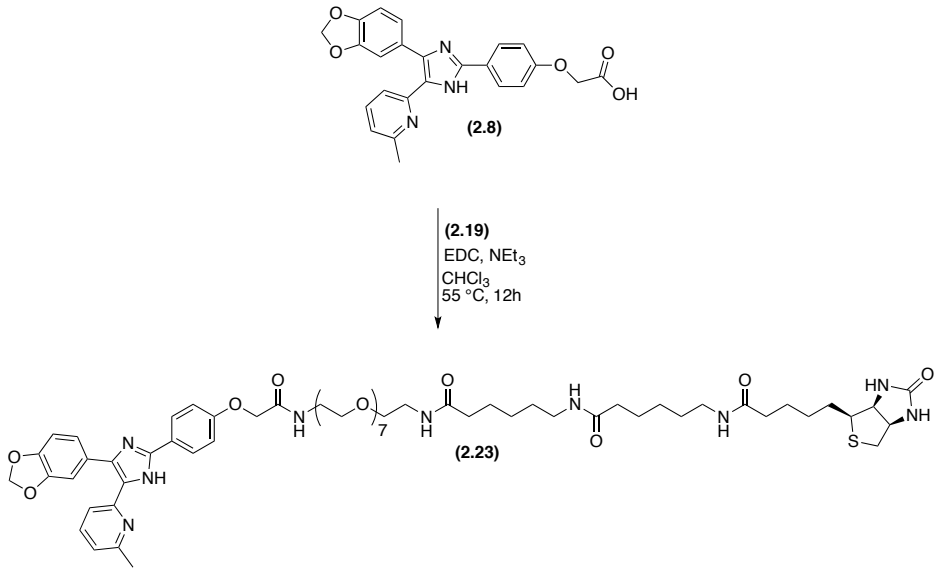


Figure 24. Test compound 2.8-biotinylated linker

2.6 Summary and Future Plans

Pyridinyl imidazole SB505124 is a known ALK4, 5, and 7 inhibitor, and has been shown to significantly reduce the growth of *Toxoplasma gondii* in vitro by targeting host HIF-1 and *Tg*MAPK. The purificaiton of *T. gondii* kinases is not ideal, so a protein pull down assay will be set up to search for novel drug therapies for toxoplasmosis.

Several compounds were synthesized for this assay including: SB-505124 to be used as a standard, a set of analogs, and a biotinylated linker as a way to potentially isolate *T. gondii* MAP kinases.

Initially, fluorescein linker **2.20** will be used to show that the linker can be loaded onto the streptavidin beads, and that it is compatible with the assay media. Staurosporine linker **2.22** will be used in the assay to test overall kinase activity, and attempt to isolate *T. gondii* kinases. Linker **2.23** can then be used for the same purposes but more specifically targeted to *Tg*MAPKs.

2.7 Synthesis of Shield-1 for the Molecular Studies of *T. gondii*

The ability to probe molecular pathways in biological systems is extremely useful in biological research. Dr. Thomas J. Wandless and his group at Stanford University developed a method to control the perturbation of protein function for studying biological systems. Shield-1 is a cell permeable small molecule that binds tightly to a mutant of FK binding protein 12 (FKBP12).[40] FKBP12 is a 12 kD protein that forms a tightly bound complex with immunosuppressant drugs such as FK506 and Rapamycin.[41] Figure 25 shows the structural similary between FK506, rapamycin, and Shield-1. FKBP12 is genetically fused to a protein of interest so that the activity of the protein is directly controlled by dosing with Shield-1.[41]

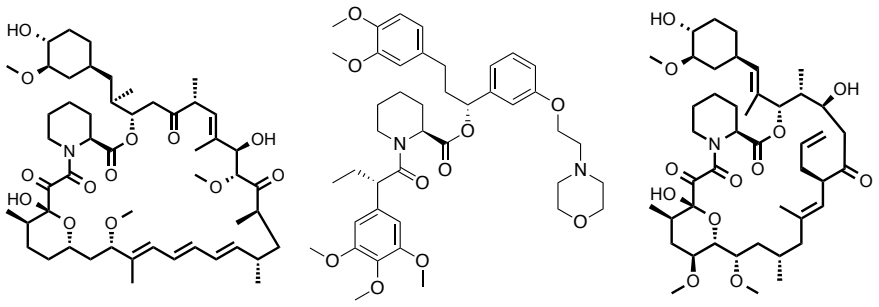


Figure 25. Structures for rapamycin, Shield-1, and FK506

Toxoplasma gondii, in the human host, rapidly divides and spreads in the body as a tachyzoite. When the parasite is overwhelmed by the immune system, it forms tissue cysts called bradyzoites (Figure 26).[42] In a previous study conducted by Dr. Michael

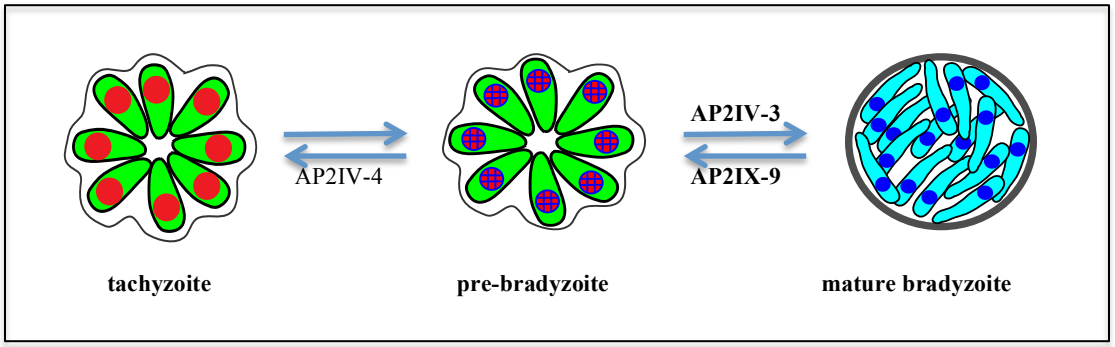


Figure 26. Transcription factors Involved in *T. gondii* bradyzoite formation[43]

White's group, Shield-1 was used to study the ApiAP2 transcription factors involved in the change from the highly motile, rapidly proliferating tachyzoite to the slow growing bradyzoite (dormant form of the parasite) (Figure 26).[43]

From previous studies, it was shown that transcription factor AP2IX-9, when over expressed in *T. gondii* with the destabilizing domain, decreases the amount of bradyzoite formation in the presence of Shield1.[43-44] Our goal is to use this compound to study the opposite effects of AP2IV-3 on tissue cyst formation in vitro and in vivo, so a reliable source of Shield-1 is required. The synthesis of Shield-1 (compound **2.32**) is needed for

these studies because, although Shield-1 is commercially available, it is expensive (5 mg for $1200), and for in vivo experiments, more of the compound is needed.

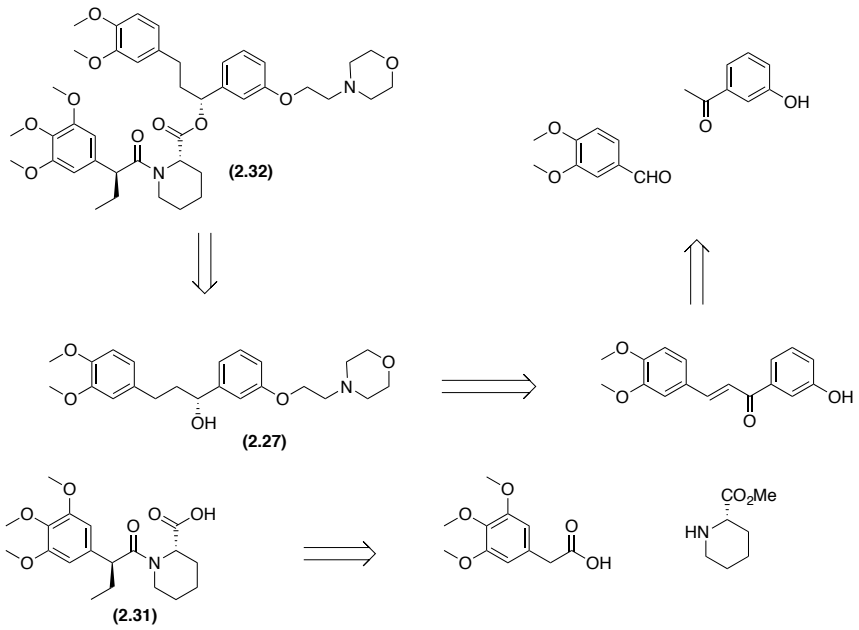


Figure 27. Retrosynthetic scheme for Shield-1

The synthesis of **2.32** was envisioned from a Steglich esterification of alcohol **2.27** and carboxylic acid **2.31** (Figure 27). Alcohol **2.27** can be obtained from the corresponding α,β-unsaturated ketone which can be made from an aldol condensation. Carboxylic acid **2.31** can be synthesized from an alkylation of trimethoxyphenyl acetic acid and amidation with homoproline.

The synthesis of Shield-1 began with an aldol condensation of 3,4-dimethoxybenzaldehyde and 3-hydroxyacetophenone followed by a reduction of the alkene **2.24** with ammonium formate in the presence of palladium catalyst to get ketone **2.25** (Figure 28). Alkylation of the phenol with 4-(2-chloroethyl)morpholine followed by an asymmetric reduction of the ketone with (+)-chlorodiisopinocampheylborane (DIP-Cl)

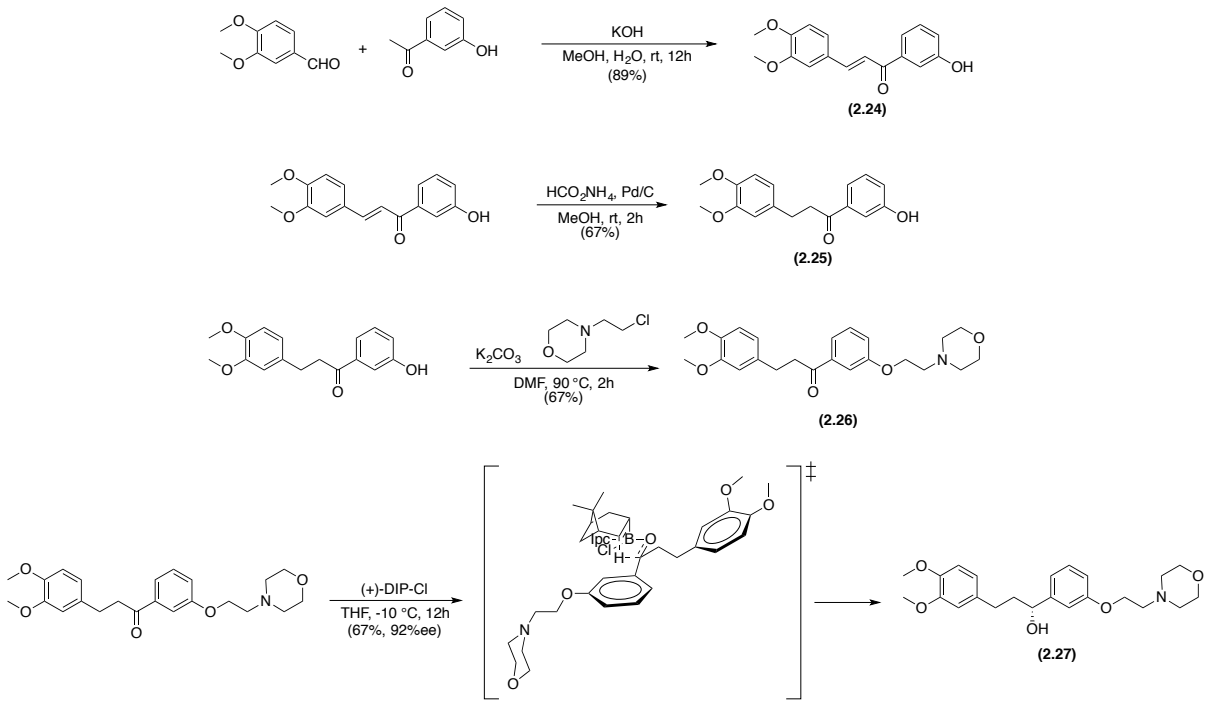


Figure 28. Synthetic scheme for alcohol 2.27

gave alcohol **2.27** (Figure 28). The enantiomeric excess for alcohol **2.27** was determined using analytical chiral HPLC.

Alkylation of 3,4,5-trimethoxyphenyl acetic acid was carried out with sodium bis(trimethylsilyl)amide and ethyl iodide (Figure 30). Recrystallization with (-)-cinchonidine (Figure 29), a chiral base, was done in acetonitrile to obtain carboxylic acid **2.29**. Amidation with pipecolic acid methyl ester using Mukaiyama's reagent, and saponification of the methyl ester gave carboxylic acid **2.31**. Finally, esterification of the two intermediates using dicyclohexylcarbodiimide and dimethylaminopyridine gave the final product (Figure 30; Compound **2.32**).

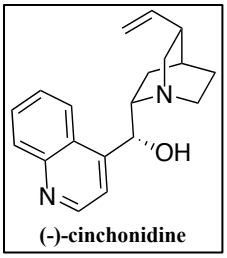


Figure 29. Structure of chiral base used for recrystallization

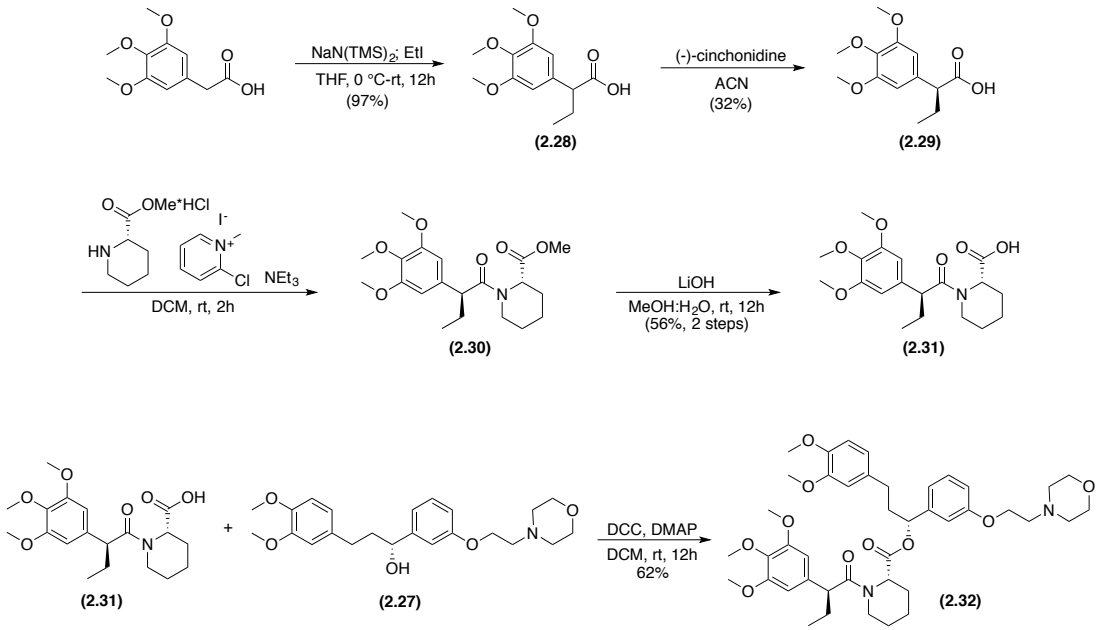


Figure 30. Synthetic scheme for carboxylic acid 2.31 and Shield-1 (2.32)

The AP2IV-3 transcription factor is currently being studied in vitro to determine the effects on AP2IV-3 on bradyzoite formation (Figure 31). At pH 7.8, the parasite with overexpressed AP2IV-3 shows a high level of bradyzoite formation (purple) in the presence of Shield-1 (S+), and less bradyzoite formation in the absence of Shield-1 (S-) due to the degradation of the transcription factor.

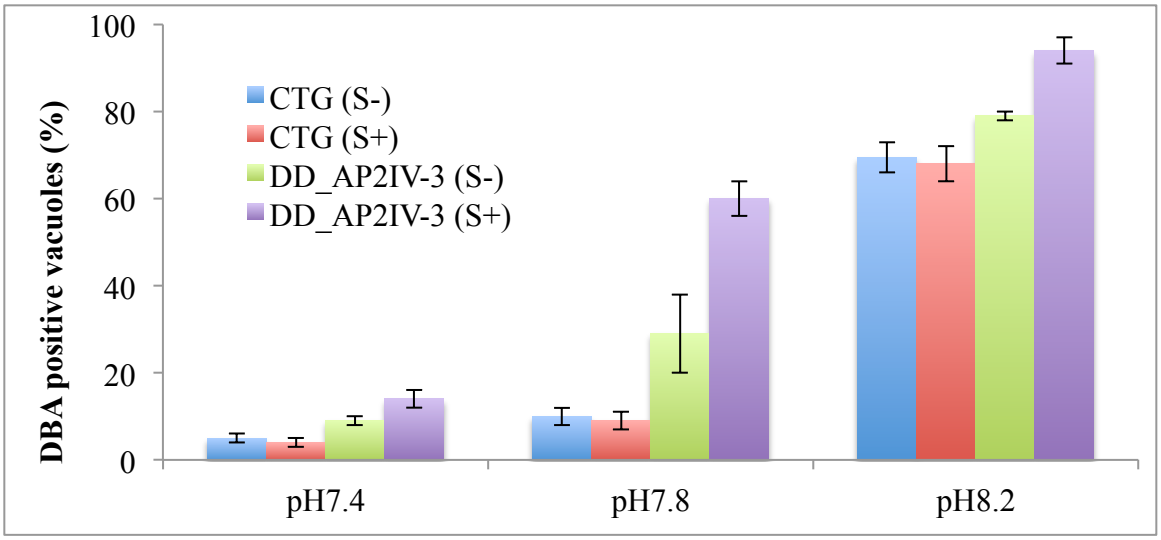


Figure 31. Graph showing the effects of AP2IV-3 on bradyzoite formation

2.8 Conclusion for Shield-1 Synthesis and Data

Shield-1 was successfully synthesized and tested, and showed comparable activity to the commercially available compound. In addition, synthetic Shield-1 was used to test the effects of AP2IV-3 transcription factor and bradyzoite formation in *T.gondii.* Shield-1 was also sent to Dr. Emma Wilson at University of California, Riverside to conduct in vivo studies for the first time. The synthesis of analogs of Shield-1 using the described methodology could allow for more optimal binding to FKBP.

References

1. Bauman, R. W., *Microbiology With Diseases by Taxonomy*. Fourth ed.; Pearson Education, Inc.: Glenview, IL, 2014.

2. WHO Malaria. http://www.who.int/mediacentre/factsheets/fs094/en/ (accessed 28 February 2015).

3. CDC Malaria – Biology. http://www.cdc.gov/malaria/about/biology/index.htmL.

4. Willey, J. M.; Sherwood, L. M.; Woolverton, C. J., *Microbiology*. 8th ed.; McGraw-Hill: New York, NY, 2011.

5. Callaway, E.; Cyranoski, D., China celebrates first Nobel. *Nature,* **2016**, *526*, p 174.

6. Gettleman, J., Meant to Keep Malaria Out, Mosquito Nets Are Used to Haul Fish In. *The New York Times* 2015.

7. (a) Novartis Malaria Initiative. http://www.malaria.novartis.com; (b) Medicines for Malaria Venture: Our Mission. http://www.mmv.org/about-us; (c) Drugs for Neglected Diseases initiative – About Malaria. http://www.dndi.org/diseases-projects/malaria/.

8. Guiguemde, W. A.; Shelat, A. A.; Bouck, D.; Duffy, S.; Crowther, G. J.; Davis, P. H.; Smithson, D. C.; Connelly, M.; Clark, J.; Zhu, F.; Jimenez-Diaz, M. B.; Martinez, M. S.; Wilson, E. B.; Tripathi, A. K.; Gut, J.; Sharlow, E. R.; Bathurst, I.; El Mazouni, F.; Fowble, J. W.; Forquer, I.; McGinley, P. L.; Castro, S.; Angulo-Barturen, I.; Ferrer, S.; Rosenthal, P. J.; Derisi, J. L.; Sullivan, D. J.; Lazo, J. S.; Roos, D. S.; Riscoe, M. K.; Phillips, M. A.; Rathod, P. K.; Van Voorhis, W. C.; Avery, V. M.; Guy, R. K., Chemical genetics of Plasmodium falciparum. *Nature* **2010,** *465* (7296), 311-5.

9. Phillips, A. P., Hantzsch's Pyridine Synthesis. *Journal of the American Chemical Society* **1949,** *71* (12), 4003-4007.

10. Horn, K. S. V., Anti-Parasitic and anti-bacterial agents: Studies on 1,4-dihydropyridines and 2,4-diaminoquinazolines. **2013**.

11. CDC Toxoplasmosis - Epidemiology & Risk Factors. http://www.cdc.gov/parasites/toxoplasmosis/epi.htmL.

12. Montoya, J. G.; Liesenfeld, O., Toxoplasmosis. *The Lancet* **2004,** *363* (9425), 1965-1976.

13. Esch, K. J.; Petersen, C. A., Transmission and epidemiology of zoonotic protozoal diseases of companion animals. *Clin Microbiol Rev* **2013,** *26* (1), 58-85.

14. Blader, I. J.; Arrizabalaga, G.; Jr., W. J. S., The Role of Host- and Parasite-Encoded Kinases in Toxoplasma-Host Interactions. In *Protein Phosphorylation in Parasites Novel Targets for Antiparasitic Intervention*, Doerig, C.; Spath, G.; Wiese, M., Eds. 2014; pp 199-216.

15. CDC Parasites - Neglected Parasitic Infections (NPIs) in the United States. http://www.cdc.gov/parasites/npi/.

16. Toxoplasmosis: Disease and Conditions. http://www.mayoclinic.org/diseases-conditions/toxoplasmosis/basics/symptoms/con-20025859.

17. Poirotte, C.; Kappeler, P. M.; Ngoubangoye, B.; Bourgeois, S.; Moussodji, M.; Charpentier, M. J., Morbid attraction to leopard urine in Toxoplasma-infected chimpanzees. *Curr Biol* **2016,** *26* (3), R98-9.

18. Yolken, R. H.; Torrey, E. F., Are some cases of psychosis caused by microbial agents? A review of the evidence. *Molecular psychiatry* **2008,** *13* (5), 470-9.

19. Pollack;, A.; Creswell, J., Martin Shkreli, the Mercurial Man Behind the Drug Price Increase That Went Viral. *The New York Times* 2015.

20. Katlama, C.; Wit, S. D.; O'Doherty, E.; Glabeke, M. V.; Clumeck, N., Pyrimethamine-Clindamycin vs. Pyrimethamine-Sulfadiazine as Acute and Long-Term Therapy for Toxoplasmic Encephalitis in Patients with AIDS. *Clin Infect Dis* **1996,** *75*, 268-275.

21. (a) Aspinwall, T. V.; Joynson, D. H. M.; Guy, E.; Hyde, J. E.; Sims, P. F. G., The Molecular Basis of Sulfonamide Resistance in Toxoplasma gondii and Implications for the Clincal Management of Toxoplasmosis. *The Journal of Infectious Diseases* **2002,** *185* (11), 1637-1643; (b) Baatz, H.; Mirshahi, A.; Puchta, J.; Gumbel, H.; Hattenbach, L. O., Reactivation of toxoplasma retinochoroiditis under atovaquone therapy in an immunocompetent patient. *Ocul Immunol Inflamm* **2006,** *14* (3), 185-7; (c) Sims, P. F. G., Drug Resistance in *Toxoplasma gondii*. In *Antimicrobial Drug Resistance*, Mayers, D. L., Ed. Humana Press: 2009; pp 1121-1126.

22. Kim, K.; Weiss, L. M., Toxoplasma gondii: the model apicomplexan. *Int J Parasitol* **2004,** *34* (3), 423-32.

23. Nelson, D. L.; Cox, M. M., *Principles of Biochemistry*. 6th ed.; W. H. Freeman and Company: New York, NY, 2012.

24. (a) Johnson, S. M.; Murphy, R. C.; Geiger, J. A.; DeRocher, A. E.; Zhang, Z.; Ojo, K. K.; Larson, E. T.; Perera, B. G.; Dale, E. J.; He, P.; Reid, M. C.; Fox, A. M.; Mueller, N. R.; Merritt, E. A.; Fan, E.; Parsons, M.; Van Voorhis, W. C.; Maly, D. J., Development of Toxoplasma gondii calcium-dependent protein kinase 1 (TgCDPK1)

inhibitors with potent anti-toxoplasma activity. *J Med Chem* **2012,** *55* (5), 2416-26; (b) Lourido, S.; Jeschke, G. R.; Turk, B. E.; Sibley, L. D., Exploiting the unique ATP-binding pocket of toxoplasma calcium-dependent protein kinase 1 to identify its substrates. *ACS Chem Biol* **2013,** *8* (6), 1155-62; (c) Lourido, S.; Zhang, C.; Lopez, M. S.; Tang, K.; Barks, J.; Wang, Q.; Wildman, S. A.; Shokat, K. M.; Sibley, L. D., Optimizing small molecule inhibitors of calcium-dependent protein kinase 1 to prevent infection by Toxoplasma gondii. *J Med Chem* **2013,** *56* (7), 3068-77.

25. Brown, K. M.; Suvorova, E.; Farrell, A.; McLain, A.; Dittmar, A.; Wiley, G. B.; Marth, G.; Gaffney, P. M.; Gubbels, M. J.; White, M.; Blader, I. J., Forward genetic screening identifies a small molecule that blocks Toxoplasma gondii growth by inhibiting both host- and parasite-encoded kinases. *PLoS pathogens* **2014,** *10* (6), e1004180.

26. Simpson, C.; Jones, N. G.; Hull-Ryde, E. A.; Kireev, D.; Stashko, M.; Tang, K.; Janetka, J. W.; Wildman, S. A.; Zuercher, W. J.; Schapira, M.; Hui, R.; Janzen, W.; Sibley, L. D., Identification of Small Molecule Inhibitors That Block theToxoplasma gondiiRhoptry Kinase ROP18. *ACS Infectious Diseases* **2016**.

27. Wei, F.; Wang, W.; Liu, Q., Protein kinases of Toxoplasma gondii: functions and drug targets. *Parasitol Res* **2013,** *112* (6), 2121-9.

28. Boron, W. F.; Boulpaup, E. L., *Medical Physiology: A Cellular and Molecular Approach*. 2nd ed.; Elsevier, Inc.: Philadelphia, PA, 2009.

29. Byfield, S. D.; Major, C.; Laping, N. J.; Roberts, A. B., SB-505124 is a Selective Inhibitor of Transforming Growth Factor-B Type 1 Receptors ALK4, ALK5, and ALK7. *Molecular Pharmacology* **2004,** *65* (3), 744-752.

30. DiscoverX KINOMEscan Assay Platform. https://www.discoverx.com/technologies-platforms/competitive-binding-technology/kinomescan-technology-platform.

31. Patrick, G. L., *An Introduction to Medicinal Chemistry*. Fifth ed.; Oxford University Press: Oxford, UK, 2013.

32. Ebel, K.; Koehler, H.; Gamer, A. O.; Jackh, R., Imidazoles and Derivatives. In *Ullman's Encyclopedia of Industrial Chemistry*, Wiley: 2000.

33. (a) Carey, F. A.; Sundberg, R. J., *Advanced Organic Chemistry Part B: Reactions and Synthesis*. Fifth ed.; Springer: Charlottesville, 2007; (b) Turner, J. A.; Jacks, W. S., Acylation of Ester Enolates by N-methoxy-N-methylamides: An Effective Synthesis of B-Keto Esters. *J. Org. Chem* **1989,** *54*, 4229-4231.

34. Hintermann, S.; Chiesi, M.; von Krosigk, U.; Mathe, D.; Felber, R.; Hengerer, B., Identification of a series of highly potent activators of the Nurr1 signaling pathway. *Bioorg Med Chem Lett* **2007,** *17* (1), 193-6.

35. Cao, Z.; Shi, D.; Qu, Y.; Tao, C.; Liu, W.; Yao, G., Synthesis of dimethyl aryl acylsulfonium bromides from aryl methyl ketones in a DMSO-HBr system. *Molecules* **2013,** *18* (12), 15717-23.

36. Laramie, M. Pyridinylimidazoles. WO 01/62756 A1, Feb. 21 2001, 2001.

37. Goswami, L. N.; Houston, Z. H.; Sarma, S. J.; Jalisatgi, S. S.; Hawthorne, M. F., Efficient synthesis of diverse heterobifunctionalized clickable oligo(ethylene glycol) linkers: potential applications in bioconjugation and targeted drug delivery. *Org Biomol Chem* **2013,** *11* (7), 1116-26.

38. Grotzfeld, R. M.; Milanov, Z. V.; Patel, H. K.; Lai, A. G.; Mehta, S. A.; Lockhart, D. J. Conjugated Small Molecules. US2005/0153371A1, 2005.

39. Shi, H.; Cheng, X.; Sze, S. K.; Yao, S. Q., Proteome Profiling Reveals Potential Cellular Targets of Staurosporine Using a Clickable Cell-Permeable Probe. *Chem. Commun.* **2011,** *47*, 11306-11308.

40. Banaszynski, L. A.; Chen, L. C.; Maynard-Smith, L. A.; Ooi, A. G.; Wandless, T. J., A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. *Cell* **2006,** *126* (5), 995-1004.

41. GrimLey, J. S.; Chen, D. A.; Banaszynski, L. A.; Wandless, T. J., Synthesis and analysis of stabilizing ligands for FKBP-derived destabilizing domains. *Bioorg Med Chem Lett* **2008,** *18* (2), 759-61.

42. Tenter, A. M.; Heckeroth, A. R.; Weiss, L. M., Toxoplasma gondii: from animals to humans. *International Journal of Parasitology* **2000,** *30*.

43. Behnke, M. S.; Wootton, J. C.; Lehmann, M. M.; Radke, J. B.; Lucas, O.; Nawas, J.; Sibley, L. D.; White, M. W., Coordinated progression through two subtranscriptomes underlies the tachyzoite cycle of Toxoplasma gondii. *PloS one* **2010,** *5* (8), e12354.

44. Radke, J. B.; Lucas, O.; Silva, E. K. D.; Ma, Y.; Sullivan, W. J.; Weiss, L. M.; Llnas, M.; White, M. W., ApiAP2 Transcription Factor Restricts Development of the *Toxoplasma* Tissue Cyst. *PNAS* **2013,** *110* (17), 6871-6876.

45. Kiselgof, E.; Tulshian, D. B.; Arik, L.; Zhang, H.; Fawzi, A., 6-(2-Furanyl)-9H-purin-2-amine derivatives as A2A adenosine antagonists. *Bioorg Med Chem Lett* **2005,** *15* (8), 2119-22.

46. Kumar, Y. Rosiglitazone Derivatives and Putative Metabolites as Antidiabetic Agents and Process for Their Preparation. 2004089945, 2004.

47. Becker, S.; Hobenreich, H.; Vogel, A.; Knorr, J.; Wilhelm, S.; Rosenau, F.; Jaeger, K. E.; Reetz, M. T.; Kolmar, H., Single-cell high-throughput screening to identify enantioselective hydrolytic enzymes. *Angew Chem Int Ed Engl* **2008,** *47* (27), 5085-8.

48. Gent, L. Methods and Compositions for the Synthesis of Multimerizing Agents. WO 2012/103279 A2, August 2, 2012.

49. Gopalakrishnan, R. Pipecolate-Diketoamides For Treatment of Psychiatric Disorders. WO 2013/091900 A1, June 27, 2013, 2013.

Appendix A – Experimental Procedures

All NMR experiments were done on Varian UnityInova 400MHz spectrometer or Direct Drive 500 MHz Spectrometer. High Resolution Mass Spectrometry data was obtained from an Agilent 6540 LC/QTOF Spectrometer. Reverse phase preparative HPLC was done on an Agilent preparative 1200 LC/6120B single quadrupole mass spectrometer.

Synthesis of *N*-methoxy-*N*-methylbenzo[*d*][1,3]dioxole-5-carboxamide (2.1a)[34]
Piperonylic acid (6 g, 36.1 mmol) was dissolved in dichloromethane (360 mL) followed by a catalytic amount of dimethylformamide (2 mL). Thionyl chloride (2.88 mL, 39.7 mmol) was added dropwise and the reaction was stirred under reflux for 4 hours. The reaction was cooled to room temperature, and N,O-dimethylhydroxylamine (3.88 g, 39.7 mmol) was added. At 0 °C, N,N-diisopropylethylamine (12.56 mL, 72.2 mmol) was added dropwise, and the reaction was left to stir overnight. The reaction was quenched with 250 mL of 2 N sodium hydroxide. The organic layer was collected and the product was extracted with dichloromethane (2 x 100 mL). The combined organic layers were dried over magnesium sulfate and concentrated down under reduced pressure to give compound **2.1a** (4.53 g, 63 %) as a pale oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.10 (br d, *J*=8.10 Hz, 1 H), 7.01 (s, 1 H), 6.62 (br d, *J*=7.81 Hz, 1 H), 5.81 (s, 2 H), 3.38 (s, 3 H), 3.13 (s, 3 H),

Synthesis of 1-(benzo[*d*][1,3]dioxol-5-yl)-2-(6-methylpyridin-2-yl)ethan-1-one (2.2)[45]
Compound **2.1a** (2.4 g, 10.9 mmol) was dissolved in anhydrous tetrahydrofuran (60 mL) and cooled to -60 °C. In a separate pear shaped flask, 2,6-lutidine (2.6 mL, 2.6 mmol) was added to anhydrous tetrahydrofuran (60 mL). At -60 °C, lithium bis(trimethylsilyl)amide (46 mL, 1 M sol'n in THF, 44 mmol) was added via cannula to this solution stirred for 30 minutes. The solution of 2,6-lutidine in THF was added dropwise via cannula to the first flask, and the reaction was left to stir overnight. The reaction was quenched with 150 mL of water and extracted with ethyl acetate (3 x 100 mL). The organic layer was dried over magnesium sulfate and concentrated down under reduced pressure. The crude orange oil was purified by flash chromatography on silica gel (3:1 – 2:1 hexanes:ethyl acetate) to give compound **2.2** (1.59 g, 57 %) as a yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) [Ketone] = 7.71 (dd, *J*=8.19, 1.71 Hz, 1 H), 7.38 - 7.55 (m, 2 H), 7.11 (d, *J*=7.71 Hz, 1 H), 7.06 (d, *J*=7.72 Hz, 1 H), 6.73 - 6.91 (m, 1 H), 6.03 (s, 2 H), 4.44 (s, 2 H), 2.57 (s, 3 H); [Enol] = 7.56 (t, *J*=7.67 Hz, 1 H), 7.52 (d, *J*=1.65 Hz, 1 H), 7.41 (dd, *J*=8.16, 1.74 Hz, 1 H), 7.33 (d, *J*=1.65 Hz, 1 H), 6.85 - 6.86 (m, 1 H), 6.82 - 6.84 (m, 1 H), 6.00 (s, 2 H), 5.92 (s, 1 H), 2.51 (s, 3 H)

Synthesis of 1-(benzo[*d*][1,3]dioxol-5-yl)-2-(6-methylpyridin-2-yl)ethane-1,2-dione (2.3)[36]

Compound **2.2** (1 g, 3.92 mmol) was dissolved in dimethyl sulfoxide (17 mL) and heated to 60° C. Hydrobromic acid (4 mL, 48% solution in water) was added dropwise and the reaction stirred for three hours at 60° C. The cooled reaction mixture was quenched with 50 mL of water. The pH was adjusted to 8 with a saturated sodium bicarbonate solution. The product was extracted with ethyl acetate (3 x 50 mL). The combined organic layers were dried over magnesium sulfate and concentrated down under reduced pressure. The yellow residue was purified by flash chromatography on silica gel (2:1 hexanes:EtOAc) to give compound **2.3** (0.91 g, 87 %) as an orange solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) = 7.97 (d, J=7.70 Hz, 1H), 7.78 (t, J=7.73 Hz, 1H), 7.42 (dd, J=8.13, 1.53 Hz, 1H), 7.36 (d, J=7.83 Hz, 1H), 6.86 (d, J=8.13 Hz, 1H), 6.08 (s, 2H), 2.51, (s, 3H).

Synthesis of 2-(4-(benzo[*d*][1,3]dioxol-5-yl)-2-(*tert*-butyl)-1*H*-imidazol-5-yl)-6-methylpyridine (2.4)[36]

Diketone **2.3** (0.7 g, 2.60 mmol) was dissolved in tert-butyl methyl ether (28 mL). Pivaldehyde (430 µl) was added. A solution of ammonium acetate (1.978 g in 28 mL methanol) was added in portions. The reaction was heated to reflux overnight. After the reaction was cooled to room temperature, 2M sodium hydroxide (25 mL) was added. The yellow precipitate was extracted with dichloromethane (3 x 50 mL), dried over magnesium sulfate, and concentrated down under reduced pressure. The yellow residue was purified by flash chromatography on silica gel (1:90:190 ammonia:methanol:DCM) to give compound **2.4** (154.9 mg, 17 %) as a yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) = 7.42 (t, 1H), 7.26 (d, 1H), 7.10 (d, 1H), 7.09 (s, 1H), 6.92 (d, 1H), 6.83 (d, 1H), 5.97 (s, 2H), 2.41 (s, 3H), 1.39 (s, 9H). HRMS (ESI) calc'd for $[C_{20}H_{21}N_3O_2]^+$: *m/z* $[M+H]^+$ 336.1704, found 336.1634.

Synthesis of 4-(4-(benzo[*d*][1,3]dioxol-5-yl)-5-(6-methylpyridin-2-yl)-1*H*-imidazol-2-yl)phenol (2.5)[36]

Diketone **2.3** (1 g, 3.72 mmol) was dissolved in tert-butyl methyl ether (37 mL). p-anisaldehyde (678 µl, 5.57 mmol) was added. A solution of ammonium acetate (2.87 g in 41 mL of methanol) was added in portions. The reaction was heated to reflux overnight. After the reaction cooled to room temperature, water (35 mL) was added. The reaction mixture was acidified to pH 4 with 2M hydrochloric acid. The brown precipitate was extracted with dichloromethane (3 x 100 mL), dried over magnesium sulfate, and concentrated down. The brown residue was purified by flash chromatography on silica gel (4:96-6:94 methanol:dichloromethane) to give compound **2.5** (420 mg, 30 %) as a light brown solid. ^{1}H NMR (500 MHz, CD_3OD): δ (ppm) = 7.84 (m, 2 H), 7.59 (t, J=7.76 Hz, 1H), 7.25 (d, J=7.82 Hz, 1H), 7.14 (d, J=7.64, 1H), 6.98-7.01 (m, 2H), 6.89 (d, J=8.86 Hz, 2H), 6.84 (d, J=7.89 Hz, 1H), 5.96 (s, 2H), 2.25 (s, 1H). HRMS (ESI) calc'd for $[C_{22}H_{17}N_3O_3]^+$: *m/z* $[M+H]^+$ 372.1340, found 372.1349.

Synthesis of 2-(4-(benzo[*d*][1,3]dioxol-5-yl)-2-(4-methoxyphenyl)-1*H*-imidazol-5-yl)-6-methylpyridine (2.6)[36]

Diketone **2.3** (1 g, 2.60 mmol) was dissolved in tert-butyl methyl ether (28 mL). Pivaldehyde (430 µl) was added. A solution of ammonium acetate (1.978 g in 28 mL methanol) was added in portions. The reaction was heated to reflux overnight. After the reaction was cooled to room temperature, 2M sodium hydroxide (25 mL) was added.

The yellow precipitate was extracted with dichloromethane (3 x 50 mL), dried over magnesium sulfate, and concentrated down under reduced pressure. The yellow residue was purified by flash chromatography on silica gel (1:70:130 ammonia:methanol:DCM) to give compound **2.4** (643.2, 45 %) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.89 (br d, J=8.64 Hz, 2H), 7.59 (d, J=8.59 Hz, 1H), 7.39-7.44 (m, 1H), 7.13-7.17 (m, 2H), 6.91-6.98 (m, 2H), 6.85 (d, J=8.40 Hz, 1H), 5.99 (s, 2H), 2.53 (s, 3H). HRMS (ESI) calc'd for $[C_{23}H_{19}N_3O_3]^+$: *m/z* $[M+H]^+$ 386.1496, found 386.1500.

Synthesis of 2-(4-formylphenoxy)acetic acid (2.7)[46]

4-hydroxybenzaldehyde (0.3 g, 2.46 mmol) and potassium carbonate (1.358 g, 9.83 mmol) was dissolved in acetone (4.9 mL). The reaction was heated to 40^o C for 15 minutes. Bromoacetic acid (0.34 g, 2.46 mmol) was added in portions. The reaction was left to stir under reflux overnight. After the reaction was cooled down to room temperature, water (10 mL) was added. The reaction mixture was washed with ethyl acetate (2 x 10 mL). The aqueous layer was acidified to pH 2 with 6N HCl. The fine precipitate was extracted with ethyl acetate (3 x 20 mL), dried over magnesium sulfate, and concentrated down to give compound **2.7** (415.5 mg, 94 %) as an off-white solid. ^{1}H NMR (CD_3OD): δ (ppm) = 7.34 (d, J=8.62 Hz, 2H), 6.93 (d, J=8.80 Hz, 2H), 4.66 (s, 2H).

Synthesis of 2-(4-(4-(benzo[*d*][1,3]dioxol-5-yl)-5-(6-methylpyridin-2-yl)-1*H*-imidazol-2-yl)phenoxy)acetic acid (2.8)[36]

Diketone **2.3** (1 g, 3.72 mmol) was dissolved in tert-butyl methyl ether (37 mL). p-anisaldehyde (678 µl, 5.57 mmol) was added. A solution of ammonium acetate (2.87 g in 41 mL of methanol) was added in portions. The reaction was heated to reflux overnight. After the reaction cooled to room temperature, water (35 mL) was added. The reaction mixture was acidified to pH 4 with 2M hydrochloric acid. The yellow precipitate was filtered to give compound **2.5** (309 mg, 97 %) as a yellow solid. ^{1}H NMR (400 MHz, CD_3OD): δ (ppm) = 8.11 (t, *J*=7.96 Hz, 1H), 8.05 (d, *J*=8.79 Hz, 2H), 7.65 (d, *J*=7.91 Hz, 1H), 7.61 (d, *J*=7.91 Hz, 1H), 7.18 - 7.23 (m, 2H), 7.09 - 7.13 (m, 2H), 6.99 (d, *J*=8.54 Hz, 1H), 6.07 (s, 2H), 4.86 (s, 2H), 2.79 (s, 3H). HRMS (ESI) calc'd for $[C_{24}H_{19}N_3O_5]^+$: *m/z* $[M+H]^+$ 430.1395, found 430.1394.

Synthesis of 6-((*tert*-butoxycarbonyl)amino)hexanoic acid (2.9)[47]

6-aminocaproic acid (20 g, 152 mmol) was dissolved in H_2O:Dioxane (488 mL, 1:2). The reaction mixture was adjusted to pH 10 with sodium hydroxide. A solution of di-tert-butyl dicarbonate (37.3 g, 85 mmol) in H_2O:dioxane (250 mL, 1:2) was added dropwise while maintaining a basic pH. The reaction was left to stir overnight. The solvent was removed under reduced pressure, and the reaction mixture was acidified to pH 3 with 1M citric acid. The product was extracted with ethyl acetate (4 x 300 mL), dried over magnesium sulfate, and then concentrated down to give compound **2.9** as a colorless oil (29.2 g, 83 %). ^{1}H NMR 400 MHz, $CDCl_3$): δ (ppm) = 2.31 (t, *J*=7.40 Hz, 2H), 1.61 (dt, *J*=15.11, 7.48 Hz, 2H), 1.43 - 1.51 (m, 4H), 1.41 (br s, 9H).

Synthesis of 2,5-dioxopyrrolidin-1-yl 6-((*tert*-butoxycarbonyl)amino)hexanoate (2.10)[47]

Carboxylic acid **2.9** (28 g, 121 mmol) and N-hydroxysuccinimide (15.74 g, 137 mmol) were dissolved in dioxane (138 mL). A solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (18.79 g, 121 mmol) dissolved in dimethylformamide (104 mL) was added dropwise to the reaction mixture at 0° C and the reaction was left to stir overnight. The reaction was quenched with water (75 mL) and the product was extracted with ethyl acetate (3 x 150 mL), dried over magnesium sulfate and concentrated down under reduced pressure to give compound **2.10** (36.4 g, 92 %) as a dirty white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 2.78 - 2.83 (m, 4H), 2.58 (t, *J*=7.35 Hz, 2H), 1.74 (dt, *J*=14.88, 7.38 Hz, 3H), 1.53 (br s, 1H), 1.44 - 1.54 (m, 3H), 1.41 (s, 9H).

Synthesis of 6-(6-((*tert*-butoxycarbonyl)amino)hexanamido)hexanoic acid (2.11)[47]
Activated ester **2.10** (36.4 g, 111 mmol) and triethylamine (19 mL, 136 mmol) were dissolved in dioxane (231 mL). 6-aminocaproic acid (14.83 g, 113 mmol) was added and the reaction was left to stir overnight. The reaction was then quenched with water (150 mL) and extracted with ethyl acetate (3 x 100 mL), dried over magnesium sulfate and concentrated down under reduced pressure to give compound **2.11** (34.7 g, 91 %) as a waxy solid. LRMS: *m/z* $[M+H]^+$ 345.5.

Synthesis of 2,5-dioxopyrrolidin-1-yl 6-(6-((*tert*-butoxycarbonyl) amino) hexanamido) hexanoate (2.12)[47]
Carboxylic acid **2.11** (34.7 g, 101 mmol) and N-hydroxysuccinimide (14.14 g, 123 mmol) were dissolved in dioxane (174 mL). A solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (17.20 g, 111 mmol) dissolved in dimethylformamide (116 mL) was added dropwise to the reaction mixture at 0 °C and the reaction was left to stir overnight. The solvent was removed under reduced pressure and the product was extracted with ethyl acetate (3 x 150 mL). The organic layer was dried over magnesium sulfate and reduced down to a waxy solid. The crude product was purified by flash chromatography on silica gel (3:1 – 2:3 hexanes:ethyl acetate) to give compound **2.12** (30 g, 67 %) as a white waxy solid. ^{1}H NMR (400 MHz, CD_3OD): δ (ppm) = 3.25 (q, *J*=6.43 Hz, 2H), 3.08 (br d, *J*=5.96 Hz, 2H), 2.80 - 2.86 (m, 4H), 2.61 (t, *J*=7.08 Hz, 2H), 2.16 (br t, *J*=7.49 Hz, 2H), 1.73 - 1.80 (m, 2 H), 1.58 - 1.67 (m, 5 H), 1.45 - 1.50 (m, 3 H), 1.42 (s, 9H), 1.25 - 1.39 (m, 3H).

Synthesis of 1,23-diazido-3,6,9,12,15,18,21-heptaoxatricosane (2.13)[37]
Octaethylene glycol (10 g, 27 mmol) and triethylamine (11.29 mL, 81 mmol) was added to dichloromethane (33 mL). A solution of mesyl chloride (6.31 mL, 81 mmol) in dichloromethane (33 mL) was added to the reaction mixture dropwise at 0 °C and the reaction mixture was left to stir for two hours, and then two hours at room temperature. The reaction mixture was washed with 1 N hydrochloric acid (100 mL) and brine (100 mL). The organic layer was dried over sodium sulfate and concentrated down under reduced pressure. The colorless oil was redissolved in dimethylformamide (67 mL), sodium azide (8.77 g, 135 mmol) was added and the reaction mixture was left to stir at 65 °C overnight. The solvent was removed under reduced pressure. The residue was redissolved in 100 mL of ether. The precipitate was filtered and the filtrate was washed with 5% lithium chloride (2 x 50 mL). The organic layer was dried over magnesium

sulfate and concentrated down. The crude product was purified by flash chromatography on silica gel (0:100 – 2:98 methanol:DCM). Compound **2.13** (10.3 g, 91 %) was collected as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 3.62 - 3.66 (m, 28 H), 3.35 - 3.39 (m, 4 H).

Synthesis of 23-azido-3,6,9,12,15,18,21-heptaoxatricosan-1-amine (2.14)[37]
Compound **2.3** (10.3 g, 24.5 mmol) was dissolved in 1 N hydrochloric acid (122 mL). A solution of triphenylphosphine (5.78 g, 22.05 mmol) in ether (122 mL) was added in portions to the reaction mixture. The reaction was left to stir for 24 hours at room temperature. The reaction mixture was diluted with 50 mL of ethyl acetate. The organic layer was collected, and the aqueous layer was extracted with ethyl acetate (2 x 50 mL). The pH of the aqueous layer was adjusted to 12 with concentrated potassium hydroxide and extracted with dichloromethane (3 x 75 mL). The organic layer was dried over sodium sulfate and concentrated down. The crude product was purified by flash chromatography on silica gel (4:96 methanol:DCM) to give compound **2.14** (7.6 g, 79 %) as a colorless oil. IR_{max} 3350, 2200 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) = 3.72 (br d, *J*=3.97 Hz, 2 H), 3.63 - 3.68 (m, 26 H), 3.59 - 3.63 (m, 2 H), 3.37 - 3.41 (m, 1 H), 3.37 - 3.41 (m, 1 H).

Synthesis of *tert*-butyl (1-azido-25,32-dioxo-3,6,9,12,15,18,21-heptaoxa-24,31-diazaheptatriacontan-37-yl)carbamate (2.15)[37]
Compound **2.14** (7.6 g, 19.27 mmol) and triethylamine (3.41 mL, 24.47 mmol) were dissolved in chloroform (96 mL). 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (2.99 g, 19.27 mmol) was added. The reaction was left to stir at 55 °C for 3.5 hours. The reaction was cooled to room temperature, diluted with 150 mL of chloroform, and washed with 50 mL of water. After the emulsion slowly separated, the organic layer was collected, dried over sodium sulfate, and concentrated down to give compound **2.15** (8.1 g, 58.3 %) as a waxy solid. ^{1}H NMR (500 MHz, CD_3OD): δ (ppm) = 3.57 - 3.70 (m, 22 H), 3.53 (t, *J*=5.44 Hz, 2 H), 3.36 (dt, *J*=10.04, 5.04 Hz, 4 H), 3.13 - 3.19 (m, 2 H), 2.98 - 3.06 (m, 3 H), 2.13 - 2.23 (m, 4 H), 1.61 (dq, *J*=15.22, 7.68 Hz, 4 H), 1.45 - 1.56 (m, 5 H), 1.42 (s, 9 H), 1.27 - 1.39 (m, 5 H).

Synthesis of 2,5-dioxopyrrolidin-1-yl 5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanoate (2.16)[37]
D-Biotin (8 g, 32.7 mmol) and N-hydroxysuccinimide (5.65 g, 49.1 mmol) were dissolved in dimethylformamide (65 mL). 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (6.10 g, 39.3 mmol) was added to the reaction and stirred at room temperature for 18 hours. The reaction was diluted with 50 mL of water. The precipitate was collected by vacuum filtration through a fine glass filter to give compound **2.16** (9.8 g, 88 %) as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) = 6.40 (br s, 1 H), 6.39 - 6.42 (m, 1 H), 6.34 (br s, 1 H), 4.26 - 4.32 (m, 1 H), 4.09 - 4.17 (m, 1 H), 3.06 - 3.13 (m, 1 H), 2.76 - 2.85 (m, 5 H), 2.66 (t, *J*=7.40 Hz, 2 H), 2.54 - 2.59 (m, 1 H), 2.19 (t, *J*=7.43 Hz, 1 H), 1.50 - 1.67 (m, 3 H), 1.28 - 1.49 (m, 3 H).

Synthesis of 6-amino-*N*-(1-azido-25-oxo-3,6,9,12,15,18,21-heptaoxa-24-azatriacontan-30-yl)hexanamide (2.17)[37]

Compound **2.15** (8.1 g, 11.24 mmol) was dissolved in dichloromethane (42 mL) and trifluoroacetic acid (14.72 mL). The reaction was left to stir at room temperature for 4 hours. The reaction mixture was concentrated down under reduced pressure and the glassy solid was redissolved in 2 mL of water. Potassium carbonate was added until all of the acid was neutralized, and the product was extracted with chloroform (3 x 50 mL). The organic layer was dried over sodium sulfate and concentrated down to a waxy solid to give the product (5.2 g, 74 %) as a waxy solid (used crude in the next step). LRMS: *m/z* $[M+H]^+$ 621.7.

Synthesis of *N*-(1-amino-25-oxo-3,6,9,12,15,18,21-heptaoxa-24-azatriacontan-30-yl)-6-(5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)hexanamide (2.18)

Compound **2.16** (3.8 g, 6.12 mmol) and triethylamine (4.07 mL, 29.2 mmol) were added to chloroform (27 mL). Compound **2.17** (g, __ mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (948 mg, 6.12 mmol) were added and the reaction was heated to 55 °C for 50 minutes. The reaction was cooled to room temperature and diluted with 30 mL of chloroform. The product was washed with water (2 x 50 mL). The organic layer was dried over sodium sulfate and concentrated down to give compound **2.18** (3.8 g, 74%) as a waxy solid. HRMS (ESI) calc'd for $[C_{38}H_{70}N_8O_{11}S]^+$: *m/z* $[M+H]^+$ 847.4955, found 847.5005.

Synthesis of *N*-(1-amino-25-oxo-3,6,9,12,15,18,21-heptaoxa-24-azatriacontan-30-yl)-6-(5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)hexanamide (2.19)

Compound **2.18** (2.3 g, 2.72 mmol) was dissolved in methanol (22 mL). Palladium (II) hydroxide (0.3 g) was added and the round bottom flask was put under vacuum. Hydrogen gas was introduced into the system and the reaction was monitored by LCMS. After no more azide was detected by LCMS, the reaction mixture was filtered through celite and the filtrate was concentrated down. The crude product was purified by flash chromatography on silica gel (15:85 - 45:55 methanol:DCM) to give compound **2.19** (1.9 g, 89 %) as a waxy solid. ^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) = 6.31-6.44 (m, 1H), 4.30 (br dd, *J*=7.52, 5.01 Hz, 1 H), 4.12 (dd, *J*=7.70, 4.40 Hz, 1 H), 3.62 (br s, 22 H), 3.58 (br t, *J*=5.17 Hz, 2 H), 3.54 (br d, *J*=3.24 Hz, 3 H), 3.52 - 3.56 (m, 1 H), 3.37 (t, *J*=5.87 Hz, 2 H), 3.13 - 3.17 (m, 2 H), 2.93 - 2.99 (m, 5 H), 2.79 (dd, *J*=12.53, 5.01 Hz, 1 H), 2.56 (br d, *J*=12.47 Hz, 1 H), 1.98 - 2.05 (m, 5 H), 1.40 - 1.50 (m, 6 H), 1.23 - 1.38 (m, 5 H), 1.13 - 1.21 (m, 4 H). HRMS (ESI) calc'd for $[C_{38}H_{72}N_6O_{11}S]^+$: *m/z* $[M+H]^+$ 821.5050, found 821.5066.

Synthesis of 2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-5-((25,32,39-trioxo-43-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)-3,6,9,12,15,18,21-heptaoxa-24,31,38-triazatritetracontyl)carbamoyl)benzoic acid (2.20)

Compound **2.19** (34.7 mg, 0.024 mmol) and triethylamine (28 µl, 0.116 mmol) was dissolved in chloroform (500 µl). A solution of fluorescein (20 mg, 0.024 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (7.2 mg, 0.027 mmol) in 1 mL of chloroform was added to the reaction mixture and left to stir at 60 °C overnight. The

reaction mixture was concentrated down and purified by preparative HPLC to give compound **2.20** (40 mg, 81%) as an orange oil. HRMS (ESI) calc'd for $[C_{59}H_{82}N_6O_{17}S]^+$: *m/z* $[M+H]^+$ 1179.5527, found 1179.5518.

Synthesis of 4-(((5*S*,6*R*,7*S*,9*R*)-6-methoxy-5-methyl-14-oxo-6,7,8,9,15,16-hexahydro-5*H*,14*H*-17-oxa-4b,9a,15-triaza-5,9-methanodibenzo[*b*,*h*]cyclonona[*jkl*]cyclopenta[*e*]-*as*-indacen-7-yl)(methyl)amino)-4-oxobutanoic acid (2.21)

Staurosporine (40 mg, 0.064 mmol), succinic anhydride (12 mg, 0.090 mmol), and dimethylaminopyridine (20 mg, 0.129 mmol) were dissolved in dimethylsulfoxide (1 mL). The round bottom flask was covered in aluminum foil to stir in the dark for 30 hours. The reaction was acidified with 0.1% trifluoroacetic acid. The fine precipitate was filtered and collected as a yellow solid to give compound **2.21** (36 mg, 76%). LRMS: *m/z* $[M+H]^+$ 567.6.

Synthesis of N^1-((5*S*,6*R*,7*S*,9*R*)-6-methoxy-5-methyl-14-oxo-6,7,8,9,15,16-hexahydro-5*H*,14*H*-17-oxa-4b,9a,15-triaza-5,9-methanodibenzo[*b*,*h*]cyclonona[*jkl*]cyclopenta[*e*]-*as*-indacen-7-yl)-N^1-methyl-N^4-(25,32,39-trioxo-43-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)-3,6,9,12,15,18,21-heptaoxa-24,31,38-triazatritetracontyl)succinamide (2.22)

Compound **2.19** (14.49 mg, 0.018 mmol) and triethylamine (92 µl, 0.018 mmol) were dissolved in chloroform (500 µl). A solution of compound **2.21** (10 mg, 0.018 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (2.7 mg, 0.018 mmol) in 1 mL of chloroform was added to the reaction mixture and left to stir at 60 °C overnight. The reaction mixture was concentrated down and purified preparative HPLC to give compound **2.22** (0.3 mg, 1.25%) as a yellow solid. HRMS (ESI) calc'd for $[C_{70}H_{100}N_{10}O_{16}S]^+$: *m/z* $[M+H]^+$ 1369.7109, found 1369.7104.

Synthesis of *N*-(1-(4-(4-(benzo[*d*][1,3]dioxol-5-yl)-5-(6-methylpyridin-2-yl)-1*H*-imidazol-2-yl)phenoxy)-2,28-dioxo-6,9,12,15,18,21,24-heptaoxa-3,27-diazatritriacontan-33-yl)-6-(5-((3a*R*,4*R*,6a*S*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)hexanamide (2.23)

Compound **2.19** (60 mg, 0.073 mmol) and triethylamine (46.4 µl, 0.33 mmol) were dissolved in chloroform (500 µl). A solution of compound **2.8** (34 mg, 0.079 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (12.2 mg, 0.079 mmol) in 1 mL of chloroform was added to the reaction mixture and left to stir at 60 °C overnight. The reaction mixture was concentrated down and purified preparative HPLC to give compound **2.23** (4 mg, 4.6 %). HRMS (ESI) calc'd for $[C_{62}H_{89}N_9O_{15}S]^+$: *m/z* $[M+H]^+$ 1232.6269, found 1232.6263.

Synthesis of (*E*)-3-(3,4-dimethoxyphenyl)-1-(3-hydroxyphenyl)prop-2-en-1-one (2.24)[48]

3,4-dimethoxybenzaldehyde (15 g, 90 mmol) and 3-hydroxyacetophenone (12.29 g, 90 mmol) were dissolved in methanol (903 mL). A solution of potassium hydroxide (28.7 g, 512 mmol) in 15 mL of water was added to the reaction and left to stir overnight. Half of the solvent was removed under reduced pressure, and 500 mL of water was added to the

reaction mixture. The reaction mixture was acidified to pH 6 using concentrated hydrochloric acid. The precipitate was filtered to give compound **2.24** (23 g, 89 %) as a yellow solid. ^{1}H NMR (500 MHz, CD_3OD): δ (ppm) = 7.72 (br s, 1 H), 7.72 (d, *J*=15.59 Hz, 1 H), 7.53 - 7.58 (m, 2 H), 7.42 - 7.44 (m,1 H), 7.32 - 7.37 (m, 2 H), 7.29 (dd, *J*=8.31, 1.90 Hz, 1 H), 7.02 - 7.05 (m, 1 H), 7.00 (d, *J*=8.31 Hz, 1 H), 3.89 (d, *J*=13.57 Hz, 6 H).

Synthesis of 3-(3,4-dimethoxyphenyl)-1-(3-hydroxyphenyl)propan-1-one (2.25)[48]
Ammonium formate (6.85 g, 109 mmol) and palladium catalyst on carbon (0.5 g) were put into methanol (40 mL). A solution of compound **2.24** (20.6 g, 72.5 mmol) in 50 mL of methanol was added to the reaction mixture and stirred under reflux for 3.5 hours. The reaction was cooled to room temperature, filtered over celite and the filtrate was concentrated down under reduced pressure. 50 mL of water was added and the resulting solid was extracted with ethyl acetate (3 x 50 mL). The organic layer was dried over magnesium sulfate, and concentrated down under reduced pressure to give compound **2.25** (18.2 g, 88 %) as a light brown solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.49 (d, *J*=7.76 Hz, 1 H), 7.44 - 7.46 (m, 1 H), 7.30 (t, *J*=7.86 Hz, 1 H), 7.04 (dd, *J*=8.05, 1.90 Hz, 1 H), 6.73 - 6.79 (m, 3 H), 3.84 (s, 3 H), 3.83 (s, 3 H), 3.21 - 3.27 (m, 2 H), 2.96 - 3.02 (m, 2 H).

Synthesis of 3-(3,4-dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)propan-1-one (2.26)[49]
Compound **2.25** (5 g, 17.46 mmol), potassium carbonate (9.85 g, 69.9 mmol), and 4-(2-chloroethyl)morpholine hydrochloride (3.25 g, 17.46 mmol) were dissolved in dimethylformamide (88 mL). The reaction was left to stir at 90 °C for 2 hours. After the reaction cooled to room temperature, the mixture was poured over 100 mL of ice water. The precipitate was extracted with ethyl acetate (3 x 75 mL), dried over sodium sulfate, and concentrated down under reduced pressure. The crude product was purified by flash chromatography on silica gel (4:1-1:1 hexanes:ethyl acetate) to give compound **2.26** (4.7 g, 67 %) as a light brown solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) = 7.53 (d, *J*=7.70 Hz, 1 H), 7.49 (d, *J*=2.08 Hz, 1 H), 7.33 - 7.37 (m, 1 H), 7.35 (t, *J*=7.92 Hz, 1 H), 7.09 - 7.12 (m, 1 H), 7.10 (dd, *J*=8.04, 2.23 Hz, 1 H), 6.76 - 6.81 (m, 3 H), 3.86 - 3.87 (m, 1 H), 3.87 (s, 2 H), 3.85 (s, 3 H), 3.76 (br s, 4 H), 3.23 - 3.29 (m, 1 H), 3.25 (br s, 1 H), 3.26 (t, *J*=7.64 Hz, 1 H), 3.00 (t, *J*=7.64 Hz, 2 H), 2.55 - 2.69 (m, 4 H).

Synthesis of (*R*)-3-(3,4-dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)propan-1-ol (2.27)[49]
Compound **2.26** (4 g, 10.01 mmol) was dissolved in anhydrous tetrahydrofuran (36 mL) under argon atmosphere. A solution of 1.8 M (+)-B-chlorodiisopinocampheylborane (8.34 mL, 15.02 mmol) in hexane was added dropwise via cannula to the reaction mixture at -10 °C. The reaction stirred at -10 °C for three hours, after which another 0.2 equivalents of (+)-DIP-Cl was added and the reaction was left in the refrigerator overnight. 50 mL of ether was added to the reaction mixture and cooled to 0 °C. Diethanolamine (50 mL) was added and the reaction stirred for 1 hour, after which the precipitate was filtered and washed with ether. The filtrate was reduced down and the crude product was purified by flash chromatography on silica gel (0:100 – 6:94

methanol:dichloromethane) to give compound **2.27** (2.7 g, 67 %, 92 %ee) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.21 - 7.27 (m, 1 H), 6.88 - 6.93 (m, 2 H), 6.68 - 6.82 (m, 4 H), 4.64 (br t, *J*=6.27 Hz, 1 H), 4.09 (t, *J*=5.69 Hz, 2 H), 3.84 (d, *J*=2.10 Hz, 6 H), 3.67 - 3.75 (m, 4 H), 2.78 (t, *J*=5.66 Hz, 2 H), 2.52 - 2.68 (m, 6 H), 1.92 - 2.12 (m, 3 H).

Synthesis of 2-(3,4,5-trimethoxyphenyl)butanoic acid (2.28)[48]

3,4,5-trimethoxyphenyl acetic acid (10 g, 44.2 mmol) was dissolved in anhydrous tetrahydrofuran under argon atmosphere. The solution was cooled to -25 °C and a 2M solution of sodium bis(trimethylsilyl)amide in tetrahydrofuran (50 mL, 100 mmol) was added via cannula. The mixture was allowed to stir for 30 minutes. Ethyl iodide (4.29 mL, 53 mmol) was added and left to stir at °C for two hours. The reaction was allowed to warm to room temperature and stirred overnight. The solution was washed with water (3 x 20 mL) and the combined aqueous layers were acidified to pH 2 with 2 N hydrochloric acid. The product was extracted with ethyl acetate (3 x 100 mL). The organic layer was dried over magnesium sulfate and concentrated down under reduced pressure to give compound **2.28** (10.9 g, 97 %) as a light brown solid.

Synthesis of (*S*)-2-(3,4,5-trimethoxyphenyl)butanoic acid (2.29)[48]

Compound **2.28** (10 g, 44.2 mmol) and (-)-cinchonidine (12.6 g, 44.2 mmol) were dissolved in 165 mL of acetonitrile. The mixture was heated to reflux for thirty minutes and removed from heat to cool to room temperature. The solid was collected by filtration and the filtrate was heated to reflux for thirty minutes. This process was repeated once more. The combined precipitates were dissolved in a 2.3:1 mixture of water and dichloromethane (200 mL). The mixture was adjusted to pH 2 with concentrated hydrochloric acid. The organic layer was collected and the aqueous layer was extracted with dichloromethane (2 x 150 mL). The combined organic layers were washed with 1 N hydrochloric acid (2 x 150 mL) and then 10 % saturated sodium bicarbonate solution (150 mL). The organic layer was dried over magnesium sulfate and then concentrated down under reduced pressure to give compound **2.28** (1.9 g, 38 %) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) = 6.53 (s, 2 H) 3.84 (s, 6 H) 3.82 (s, 3 H) 3.37 (t, *J*=7.70 Hz, 1 H) 2.03 - 2.13 (m, 1 H) 1.79 (dquin, *J*=14.14, 7.29, 7.29, 7.29, 7.29 Hz, 1 H) 0.92 (t, *J*=7.37 Hz, 3 H).

Synthesis of (*S*)-1-((*S*)-2-(3,4,5-trimethoxyphenyl)butanoyl)piperidine-2-carboxylic acid (2.31)

Compound **2.29** (608 mg, 2.391 mmol), 2-chloro-1-methylpyridinium iodide (794 mg, 3.11 mmol), and (S)-methyl piperadine-2-carboxylate hydrochloride (430 mg, 2.391 mmol) were dissolved in dichloromethane (10 mL). Triethylamine (333 µl, 2.391 mmol) was added and the reaction was left to stir at room temperature for 2 hours. The reaction mixture was washed with saturated sodium bicarbonate (3 x 20 mL). The organic layer was collected, dried over magnesium sulfate, and concentrated down. The yellow residue was redissolved in 8 mL of methanol and 4 mL of water. Lithium hydroxide monohydrate (502 mg, 11.96 mmol) was added and the reaction stirred overnight. 5 mL of water was added and the methanol was removed by rotary evaporation. The aqueous solution was treated with 10 mL of ethyl acetate, and then washed with 10 mL of

saturated sodium bicarbonate. The combined aqueous layers were acidifed to pH 4 with 2 N hydrochloric acid. The precipitate was collected by extraction with ethyl acetate (3 x 15 mL), dried over magnesium sulfate, and concentrated down to give compound **2.31** (700 mg, 80 %) as a white solid. ^{1}H NMR (400 MHz, CD_3OD): δ (ppm) = 6.59 (s, 1 H), 6.55 (s, 1 H), 3.77 - 3.84 (m, 6 H), 3.69 - 3.75 (m, 3 H), 2.15 - 2.32 (m, 1 H), 1.90 - 2.08 (m, 2 H), 1.13 - 1.77 (m, 8 H), 0.81 - 0.94 (m, 3 H).

Synthesis of (*R*)-3-(3,4-dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)propyl (*S*)-1-((*S*)-2-(3,4,5-trimethoxyphenyl)butanoyl)piperidine-2-carboxylate (2.32)
Compound **2.31** (900 mg, 2.46 mmol), dimethylaminopyridine (301 mg, 2.46 mmol), and N,N'-dicyclohexylcarbodiimide (508 mg, 2.46 mmol) were dissolved in dichloromethane (3 mL). Compound **2.27** (989 mg, 2.46 mmol) was added to the reaction mixture and left to stir at room temperature overnight. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography on silica gel (4:96 methanol:dichloromethane). The resulting oil was further purified by reverse phase preparative HPLC to give compound **2.32** (1.5 g, 81%) as an off white solid. ^{1}H NMR (500 MHz, $CDCl_3$, mixture of rotamers): δ (ppm) = 7.25 (m, 1 H,) 6.76 - 6.80 (m, 1 H), 6.76 – 6.78 (m, 2 H), 6.72 - 6.94 (m, 2 H), 6.62 - 6.70 (m, 1 H), 6.46 (s, 1 H) 6.39 (s, 1 H), 4.07 - 4.12 (m, 2 H), 3.85 (d, *J*=4.10 Hz, 9 H), 3.82 - 3.84 (m, 6 H), 3.70 - 3.75 (m, 4 H), 3.57 (br t, *J*=7.06 Hz, 1 H), 2.79 (dt, *J*=8.53, 5.73 Hz, 2 H), 2.30 - 2.63 (m, 8 H), 2.02 - 2.13 (m, 4 H), 1.23 - 1.80 (m, 9 H), 0.86 - 0.92 (m, 3 H). ^{1}H NMR HRMS (ESI) calc'd for $[C_{42}H_{56}N_2O_{10}]^+$: *m/z* $[M+H]^+$ 749.4005, found 749.4033.

Appendix B – Proton NMR for Selected Compounds

All NMR experiments were done on Varian UnityInova 400MHz spectrometer or Direct Drive 500 MHz Spectrometer.