ABSTRACT

Design and Synthesis of Combretastatin A-1 Analogs, Small Molecule Vascular Disrupting Agents, and Bioreductive Triggers as Potential Therapeutic Agents for the Treatment of Cancer

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Cancer is the second leading cause of death in the United States with over 550,000 deaths in 2009 and a devastating 7.9 million deaths worldwide in 2007. Vascular disrupting agents (VDAs) represent a novel method developed for the treatment of cancer. VDAs, such as combretastatin A1 phosphate (CA1P, Oxi4503) and combretastatin A4 phosphate (CA4P, Zybrestat^{TM}, fosbretabulin), after undergoing phosphate cleavage by non-specific phosphatase enzymes, selectively bind to the colchicine site on tubulin, disrupting tubulin polymerization. The integrity of the microtubules that form the cytoskeleton of the endothelial cells that line the tumor vasculature is altered, causing tumor vasculature occlusion and collapse, thus preventing nutrients and oxygen from reaching the tumor. This leads to severe tumor hypoxia, tumor perfusion regression and tumor necrosis.

A series of anticancer agents were designed to incorporate a VDA or cytotoxic agent linked to a bioreductive drug to form a bioreductive prodrug conjugate. When the bioreductive portion of the molecular conjugate is reduced, the linkage between the VDA

and the bioreductive drug is broken, releasing the VDA in its active form to act upon the tumor vasculature. The reduced bioreductive drug becomes a chemotherapeutic agent that can damage the tumor cells.

CA1 is a potent inhibitor of tubulin polymerization and it has been shown that CA1 undergoes a second mechanism of action against tumors. CA1 incorporates an *ortho* diphenolic moiety that can be oxidized to form an *ortho* quinone that can damage DNA. To further elucidate the biological mechanism of action of CA1, a synthetic methodology was developed to incorporate a radioisotope at a metabolically stable position. In addition, a total synthesis was designed to prepare each of the combretastatin A1 monophosphates in regioisomeric pure form.

Design and Synthesis of Combretastatin A-1 Analogs, Small Molecule Vascular Disrupting Agents, and Bioreductive Triggers as Potential Therapeutic Agents for the Treatment of Cancer

by

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A Dissertation

Approved by the Department of Chemistry and Biochemistry

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LIST OF ABBREVATIONS

°C	Degree Celsius
Ac_2O	Acetic anhydride
AcOH	Acetic acid
ADME	Absorption, Distribution, Metabolism, and Excretion
CA1	Combretastatin A-1
CA1P	Combretastatin A-1 Phosphate
CA1-MPA	Combretastatin A-1 Monophosphate A
CA1-MPB	Combretastatin A-1 Monophosphate B
CA4	Combretastatin A-4
CA4P	Combretastatin A-4 Phosphate
conc.	Concentrated
DBP	Dibenzyl phosphate
DCM	Dichloromethane
DIPEA	*N,N*-Diisopropylethylamine
DMAP	4-(*N,N*-dimethylamino)pyridine
DMF	*N,N*-dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EtOAc	Ethyl acetate
GI_{50}	Concentration at which 50% of cell growth is inhibited
GDP	Guanosine diphosphate

GTP	Guanosine triphosphate
Hr	hour
Hrs	hours
HPLC	High Pressure Liquid Chromatography
Hz	Hertz
IC_{50}	Inhibition Constant – the concentration of the compound that inhibits tubulin assembly by 50%
IPA	Isopropyl alcohol
i-Pr	Isopropyl
LDA	Lithium diisopropylamide
MHz	Mega hertz
min	Minutes
µg/mL	Micrograms/milliliter
mL	milliliter
mM	Millimolar
µM	Micromolar
MOM	Methoxymethyl ether
MOMCl	Methoxymethyl chloride
n-BuLi	*n*-butyllithium
NCI	National Cancer Institute
NCS	*N*-chlorosuccinamide
nM	Nanomolar
NMR	Nuclear Magnetic Resonance Spectroscopy

PCC	Pyridinium chlorochromate
ppm	parts per million
p-TSA	p-Toluenesulfonic acid
RNA	Ribonucleic acid
Rotovap	Rotary evaporator
rt	Room temperature
Rf	Retention factor
Sat.	Saturated
SCID	Severe Combine Immune Deficiency
sec-BuLi	sec-Butyllithium
TBAB	tetrabutylammonium bromide
TBAF	Tetrabutylammonium fluoride
TBSCl	*tert*-Butyldimethylsilyl chloride
TFA	Trifluoroacetic acid
TLC	Thin-layer chromatography
TMEDA	Tetramethylethylenediamine
TMSBr	Trimethylsilyl bromide
TMSCl	Trimethylsilyl chloride
UV	Ultraviolet
VDA	Vascular Disrupting Agent
VEGF	Vascular Endothelial Growth factor
VTA	Vascular Targeting Agent

CHAPTER ONE

Synthesis of Combretastatin Monophosphates

Introduction

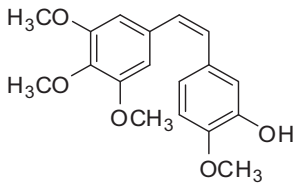
The discovery and synthesis of combretastatin A-1 (CA1) and its corresponding prodrug, combretastatin A-1 phosphate (CA1P, Figure 1), were accomplished by professer George R. Pettit and colleagues from Arizona State University.[1] CA1 and other combretastatins were extracted from *Combretum caffrum*, a South African willow tree, by the U.S. National Cancer Institute (NCI) between 1973 and 1979, and were finally isolated and identified in 1982 in collaboration with Pettit and colleagues.[2-4] The natural product CA1, showed promising biological activity against various cancer cell lines (*in vitro*). Most notably, CA1 was especially active against murine P388 lymphocytic leukemia demonstrating an effective dose (ED_{50}) of 0.99 μg/mL.[1] In addition, CA1 proved to be an effective inhibitor of tubulin polymerization (*in vitro)* demonstrating a half maximal inhibitory concentration (IC_{50}) of 2 μM.[1] Compared to its natural product counterpart, combretastatin A-4 (CA4, Figure 1) is one of the most active members in the combretastatin family that is currently in clinical trials as its corresponding prodrug salt combretastatin A-4 phosphate (CA4P, Zybrestat™, fosbretabulin).[5, 6] CA4P is currently in phase III clinical trials against anaplastic thyroid cancer and in phase II clinical trials against non-small cell lung cancer and platinum-resistant ovarian cancer.[7, 8] CA4 is a natural product also isolated from *Combretum* caffrum that differs from CA1 only in the absence of a C-2 phenolic moiety.[9] Interestingly, while CA4 is generally more potent than CA1 against human cancer cell lines (*in vitro*), the corresponding prodrug CA4P is

somewhat less active than CA1P in certain *in vivo* preclinical tumor growth delay studies carried out in SCID mice.[10-12] CA1 showed more consistent results in murine P388 leukemia *in vitro* and CA1, in preclinical trials conducted by Hill and colleagues[13], showed higher vascular disruption and antitumor activity than CA4[13-15]. The higher activity of CA1 may be due to the extra hydroxy substituent which, through oxidative metabolism, can form the exceptionally reactive *ortho*-quinone to damage DNA.[16, 17] More information concerning vascular disrupting agents is available in chapter 3.

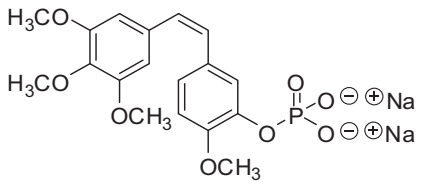
CA1P (OXi4503) is currently in phase I clinical trials.[18] *In vivo*, CA1P is metabolized by phosphatase enzymes to afford the active metabolite, and subsequently the *ortho*-quinone derivative.[16] The non-specific phosphatase enzymes commonly cleave both phosphate salts from CA1P to form CA1.[14] CA1P is a diphosphate salt and there may be some instances where the phosphatase enzymes only cleave one phosphate salt affording CA1 monophosphate salts, rather than the highly reactive diphenolic CA1. The CA1 monophosphate metabolite is a completely new molecule all together. Since the regioisomeric monophosphate salts are structurally distinct from both CA1P and CA1, it is anticipated that they may have different activity profiles in biological systems. Depending on where the phosphatase enzymes cleave on CA1P, two different regioisomers of CA1 monophosphates can be obtained: combretastatin A-1 monophosphate A (CA1-MPA) and combretastatin A-1 monophosphate B (CA1-MPB, Figure 1).

This same concept can be applied to the storage of the CA1P prodrug itself. If CA1P is stored for long periods of time prior to application, it may decompose or undergo hydrolysis to the monophosphate derivatives, due to improper storage

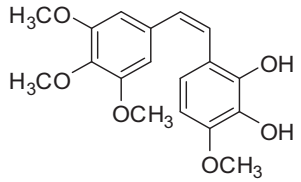
conditions, heat, or instability over time. These are causes for potential concern since little is known about the biological activity of the CA1 monophosphate derivatives. In order to facilitate absorption, distribution, metabolism, and excretion (ADME) studies of the CA1 monophosphates, it was necessary to have a synthetic route established for each CA1 monophosphate regioisomer: CA1-MPA and CA1-MPB.



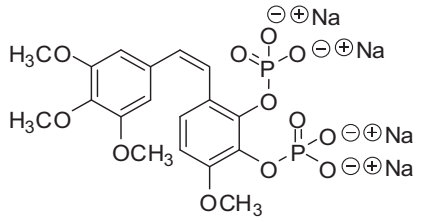
Combretastatin A-4 (CA4) **1**



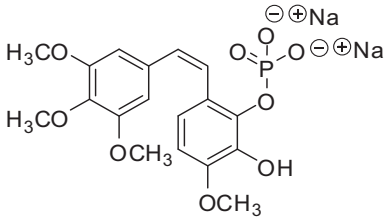
Combretastatin A-4 Phosphate (CA4P) **2**



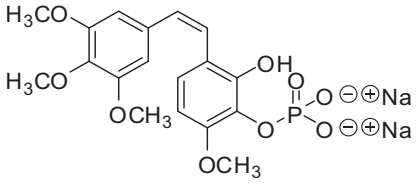
Combretastatin A-1 (CA1) **3**



Combretastatin A-1 Phosphate (CA1P) **4**



Combretastatin A-1 Monophosphate A (CA1-MPA) **5**



Combretastatin A-1 Monophosphate B (CA1-MPB) **6**

Figure 1. Combretastatin A-4 and A-1 with their corresponding phosphate prodrugs along with the regioisomeric Combretastatin A-1 monophosphates.

Materials and Methods

Reactions were performed under an inert atmosphere using nitrogen gas unless specified differently. Chemical reagents used in the synthetic procedures were obtained

from various chemical suppliers (Sigma Aldrich, Acros Chemical Co., Alfa Aesar, Fisher Scientific, EMD Chemicals, and VWR). Silica gel (200-400 mesh, 60 Å), used for column chromatography, was obtained from either Silicycle Inc or VWR. TLC plates (pre-coated glass plates with silica gel 60 F254, 0.25 mm thickness, EMD chemicals, VWR) were used to monitor reactions. A Biotage Isolera Four automated flash column chromatography system was used to separate compounds. A Biotage microwave system was used to conduct microwave reactions. Intermediates and products synthesized were characterized based on ^{1}H NMR (Bruker DPX operating at 300 MHz or Varian operating at 500 MHz), ^{13}C NMR (Bruker DPX operating at 75 MHz or Varian operating at 125 MHz), and ^{31}P NMR (Bruker DPX operating at 121 MHz or Varian operating at 202 MHz). All the chemical shifts are expressed in ppm (δ), coupling constants (*J*) presented in Hz, and peak patterns are reported as broad (br), singlets (s), doublets (d), triplets (t), quartets (q), septets (sept), and multiplets (m). NMRs were processed using Mestrec. Elemental analysis was performed by Atlantic Microlab, Norcross, GA. High-resolution mass spectra (HREIMS), unit resolution gas chromatography mass spectra (EIMS), and unit resolution mass spectra (ESIMS) were obtained on a VG Prospec Micromass spectrometer, a Thermo Scientific DSQ II, and a Thermo Finnigan LCQ Classic, respectively, in the Baylor University Mass Spectrometry Core Facility. Purity of the compounds was further analyzed using a Agilent 1200 LC System, and a Agilent Eclipse DBX-C18 4.6 x 150 mm, 5.0 mm, T = 20 °C; eluents, solvent A, 0.1 % TFA in water, solvent B, 0.08% TFA in Acetonitrile:Water (80/20 (v/v) ratio); flow rate, 1.0 mL/min; injection volume 20 μL; monitored at 264 nm wavelength.

Synthesis of combretastatin A-1 monophosphate B

*2,3-Dihydroxy-4-methoxybenzaldehyde **17***:[1] 2,3,4-Trimethoxybenzaldehyde **9** (39.2 g, 200 mmol) was dissolved in anhydrous dichloromethane (150 mL) and was cooled to 0 ^{o}C with an ice bath. Boron tricholoride (400 mL, 400 mmol) was added dropwise to the reaction due to its vigorous nature. The reaction mixture was allowed to slowly warm up to room temperature and stirred for 12 hours. The reaction was slowly and cautiously transferred over a period of time (20 minutes) into a flask containing a saturated solution of sodium bicarbonate (800 mL) to quench any excess boron trichloride. The reaction mixture was then acidified to a pH of one using hydrochloric acid. The organic phase was separated and the aqueous layer was extracted with ethyl acetate (3 x 200 mL). The organic phases were combined, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was subjected to flash column chromatography (silica gel, 30:70 EtOAc-hexanes) to yield diol **17** (29.8 g, 177 mmol, 89% yield) as a purple solid.

^{1}H NMR ($CDCl_3$, 300 MHz) δ 11.12 (s, 1H), 9.76 (s, 1H), 7.15 (d, J = 8.7 Hz, 1H), 6.62 (d, J = 8.7 Hz, 1H), 5.45 (s, 1H), 3.99 (s, 3H)

*3-[(tert-Butyldimethylsilyl)oxy]-2-hydroxy-4-methoxybenzaldehyde **16***:[1] Aldehyde **17** (1.0 g, 5.95 mmol) was dissolved in anhydrous dimethyl formamide (10 mL) at room temperature. Diisopropylethylamine (2.08 mL, 11.9 mmol) was added and the reaction was stirred for five minutes. *tert*-Butyldimethylsilyl chloride (0.89 g, 5.95 mmol) was slowly added to the reaction mixture in small portions over a 20 minute period. After the reaction mixture was stirred for 12 hours and monitored by TLC until

completion, the reaction mixture was quenched using saturated sodium bicarbonate (25 mL) and the aqueous layer extracted using diethyl ether (3 x 25 mL). The organic layers were combined and washed with copious amounts of water (6 x 50 mL) and brine solution (25 mL) to remove any excess dimethyl formamide from the organic layer. The organic layer was dried using sodium sulfate, filtered and concentrated under reduced pressure to yield the mono-protected aldehyde **16** as a tan solid, (0.900 g, 54%).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 10.90 (s, 1H), 9.57 (s, 1H), 7.01 (d, J = 9.5 Hz, 1H), 6.43 (d, J = 8.5 Hz, 1H), 3.74 (s, 3H), 0.88 (s, 9H), 0.03 (s, 6H)

*3-[(tert-Butyldimethylsilyl)oxy]-4-methoxy-2-[(para-toluenesulfonyl)oxy]-benzaldehyde **24***: Aldehyde **16** (1.40g, 4.98 mmol) was dissolved in dimethyl formamide (10 mL) at room temperature. Diisopropylethylamine (0.87 mL, 4.98 mmol) was added to the reaction mixture and stirred for five minutes. The *para*-toluenesulfonyl chloride (0.95 g, 4.98 mmol) was added slowly over a period of 20 minutes and the reaction was stirred for 12 hours, monitored by TLC to check for reaction completion. The reaction was quenched using sodium bicarbonate (200 mL) and the aqueous layer was extracted using diethyl ether (3 x 200 mL). The organic phases were combined and wash with brine (200 mL) and copious amounts of water (6 x 200 mL). The organic layer was then dried with sodium sulfate and concentrated in vacuo and subjected to flash column chromatography to yield the heteroprotected product **24** as a white solid (0.75 g, 1.72 mmol, 34% yield).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.59 (s, 1H), 7.73 (d, J = 8.4 Hz, 2H), 7.53 (d, J = 8.7 Hz, 1H), 7.33 (d, J = 7.8 Hz, 2H), 6.88 (d, J = 8.7 Hz, 1H), 3.87 (s, 3H), 2.45 (s, 3H), 0.96 (s, 9H), 0.10 (s, 6H)

3*,4,5-Trimethoxybenzyl bromide* ***35***:[19] 3,4,5-Trimethoxybenzyl alcohol **34** (50.0 g, 252 mmol) was dissolved in dry dichloromethane (300 mL) and cooled to 0 ^{o}C in an ice/water bath. To the reaction mixture was added slowly and dropwise phosphorous tribromide (17.8 mL, 189 mmol). The reaction was allowed to stir for about four to five hours. TLC was taken to make sure the reaction has gone to completion. Water was added slowly to quench the reaction. The organic layer was extracted and the aqueous layer was extracted with dichloromethane (3 x 100 mL). The organic layers were combined and dried using sodium sulfate, filtered, and concentrated using the rotary evaporator. The crude product **35** (44.8 g, crude 68%) was taken to the next step without further purification.

^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.62 (s, 2H), 4.47 (s, 2H), 3.87 (s, 6H), 3.85 (s, 3H)

3,4,5-Trimethoxybenzyl-triphenylphosphonium bromide ***20***:[1] Bromide **35** (44.8g, 171 mmol) was dissolved in dichloromethane (300 mL) and stirred under nitrogen. Triphenylphosphine (49.3g, 189 mmol) was added and the reaction was refluxed for 22 hours. The reaction mixture was concentrated using a rotary evaporator to yield the crude product as an off-white solid. Diethyl ether was added to the crude product and the solid was filtered and washed with copious amounts of diethyl ether. The white solid was collected and dried under high vacuum to afford the phosphonium salt **20** (69.8 g, 134 mmol, 78%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.70 (m, 15H), 6.47 (s, 2H), 5.44 (d, J = 14.1 Hz, 2H), 3.77 (s, 3H), 3.51 (s, 6H)

(Z)-1-[3',4',5'-Trimethoxy]-2-[2''-[(para-toluenesulfonyl)oxy]-3''-[(tert-Butyldimethylsilyl)oxy]-4''-methoxyphenyl] ethene ***25***: To an oven-dried round bottom flask was added sodium hydride (0.33 g, 13.74 mmol) and dichloromethane (20 mL) under nitrogen with good stirring and cooled to 0 ^{o}C using an ice/water bath. Phosphonium salt **20** was added to the reaction mixture and the reaction was allowed to stir for 30 to 45 minutes. Aldehyde **24** (0.75 g, 1.72 mmol) was dissolved in a small amount of dichloromethane (5 mL) and was added dropwise to the reaction mixture. The reaction was allowed to slowly warm up to room temperature and stirred for 12 hours with TLC monitoring to check for reaction completion. Water was added very slowly to quench the reaction. The organic layer was separated and the aqueous layer was washed with dichloromethane (3 x 100 mL). The organic layers were combined and dried with sodium sulfate, filtered, and concentrated to yield the crude product as a yellow, viscous oil. Boiling ethanol (150 mL) was added to the oil and the product **25** crystallized out as a white solid (0.75 g, 1.25 mmol, 73%)

^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.82 (d, *J* = 8.5 Hz, 2H), 7.25 (d, *J* = 8 Hz, 2H), 6.77 (d, *J* = 8.5 Hz, 1H), 6.69 (d, *J* = 9 Hz, 1H), 6.49 (s, 2H), 6.16 (s, 2H), 3.82 (s, 3H), 3.75 (s, 3H), 3.66 (s, 6H), 2.39 (S, 3H), 0.94 (s, 9H), 0.04 (s, 6H)

(Z)-1-[3',4',5'-Trimethoxy]-2-[2''-[(para-toluenesulfonyl)oxy]-3''-hydroxy-4''-methoxyphenyl] ethene ***26***: The hetero-protected *cis*-CA1 **25** (0.75 g, 1.23 mmol) was dissolved in acetonitrile (25 mL) and cooled to 0 ^{o}C using an ice/water bath under nitrogen. Tetrabutylammonium fluoride (0.40 mL, 1.38 mmol) was added to the reaction mixture and stirred for 10 minutes. TLC was taken every 2 minutes after the addition of

tetrabutylammonium fluoride to monitor reaction completion prior to the 10 minute mark. Once the reaction is complete, it was quenched with water and the organic phase was separated. The aqueous phase was extracted with ethyl acetate (3 x 100 mL). The organic phases were combined, dried with sodium sulfate, filtered, and concentrated using rotary evaporation to yield the crude product **26** as a yellow oil. The oil was subjected to flash column chromatography to afford the pure monoprotected CA1 **26** (0.58g, 95%).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.91 (d, J = 8.4 Hz, 2H), 7.29 (d, J = 8.0 Hz, 2H), 6.71 (d, J = 8.6 Hz, 1H), 6.62 (d, J = 8.6 Hz, 1H), 6.43 (s, 2H), 6.36 (d, J = 12.0 Hz, 1H), 6.32 (d, J = 12.0 Hz, 1H), 5.89 (bs, 1H), 3.86 (s, 3H), 3.82 (s, 3H), 3.67 (s, 6H), 2.42 (s, 3H)

*(Z)-1-[3',4',5'-Trimethoxyphenyl]-2-[2''-[(para-tolunesulfonyl)oxy]-3''-[([bis-[(benzyl)oxy]]phosphoryl)oxy]-4''-methoxyphenyl] ethene **27***: The tosyl-protected *cis*-CA1 **26** was dissolved with acetonitrile with moderate stirring under nitrogen and cooled to -20 ^{o}C using an acetone/dry ice bath. After the tosyl-protected *cis*-CA1 has completely dissolved, carbon tetrachloride was added to the reaction mixture and stirred for five minutes. Diisopropylethylamine and 4-dimethylaminopyridine were added to the reaction mixture and stirred for ten minutes. Dibenzylphosphite was added slowly to the reaction mixture and it was allowed to stir for 45 minutes with TLC monitoring at 45 minutes and every five minutes after until the reaction has gone to completion. Once the reaction has gone to completion, potassium dihydrogen phosphate was added and the reaction was allowed to warm up to room temperature. Water was added to the reaction and the organic phase was separated. The aqueous phase was extracted with ethyl acetate

(3 x 100 mL) and the organic phases were combined and dried with sodium sulfate, filtered, and concentrated using the rotary evaporator. The crude product was subject to flash column chromatography to yield a yellow oil as pure product **27** (0.44g, 0.59 mmol, 50%).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.77 (d, J = 8.5 Hz, 2H), 7.33 (m, 10H), 7.16 (d, J = 8.0 Hz, 2H), 7.00 (d, J = 8.5 Hz, 1H), 6.72 (d, J = 9.0 Hz, 1H), 6.46 (s, 2H), 6.37 (d, J = 12.0 Hz, 1H), 6.31 (d, J = 12.0 Hz, 1H), 3.81 (s, 3H), 3.74 (s, 3H), 3.68 (s, 6H), 2.32 (s, 3H)

*(Z)-1-[3',4',5'-Trimethoxyphenyl]-2-[2''-[(para-tolunesulfonyl)oxy]-3''-[(disodium)phoshphate]-4''-methoxyphenyl] ethene **28***: Prior to starting the reaction, the TMSBr was distilled over calcium hydride to give pure clear reagent. Compound **27** (0.31g, 0.41 mmol) was dissolved in acetonitrile (25 mL) and cooled down to -10 ^{o}C under nitrogen. TMSBr (0.27 mL, 2.06 mmol) was added to the reaction mixture and stirred for 3 hours at -10 ^{o}C.[20] When the time was close to the 3 hour mark for the first reaction, in a separate flask, a suspension of sodium methoxide (0.111g, 2.06 mmol) and methanol (10 mL) was made and was cooled to -10 ^{o}C. The initial reaction was slowly added dropwise to the suspension of sodium methoxide and methanol. The reaction was then allowed to stir for 2-3 hours and allowed to slowly warm up to room temperature. Once the reaction has gone to completion, the methanol was removed in vacuo, to give an off-white solid. Water (1 mL) was added to this solid and the solid was filtered and washed with more water (0.5-1 mL of water). The filtrate was collected and ethanol (5 mL) was added to the filtrate. A white solid precipitated out which was the desired product **28** (0.130 g, 52%).

^{1}H NMR (D_2O, 500 MHz) δ 7.66 (d, *J* = 8.5 Hz, 2H) 7.09 (d, *J* 8.0 Hz, 2H), 6.83 (d, *J* = 8.5 Hz, 1H), 6.74 (d, *J* = 8.5 Hz, 1H), 6.38 (s, 2H), 5.99 (d, *J* = 12.0, 1H), 5.78 (d, *J* = 12.0 Hz, 1H), 3.70 (s, 3H), 3.66 (s, 3H), 3.58 (s, 6H), 2.10 (s, 3H)

^{31}P NMR (D_2O, 122 MHz) δ 3.7

*(Z)-1-[3',4',5'-Trimethoxyphenyl]-2-[2''-[hydroxy]-3''-[(disodium)phoshphate]-4''-methoxyphenyl] ethene **6***: The white solid compound **28** (0.025 g, 0.041 mmol) was dissolved with water (1 mL) and methanol (1 mL) in a 5 mL microwave safe vial with a stir bar. To this vial was added 2 M NaOH (1 mL) and the vial was capped and placed in the microwave. The reaction was pre-stirred for 5 minutes prior to microwaving. The microwave was set to run for 30 minutes at 50 ^{o}C. Temperatures higher than 50 ^{o}C may lead to isomerization of the compound. Reverse phase TLC (30:70 acetonitrile-water) was used to monitor the reaction. After the microwave has finished, the reaction mixture should be a clear yellow-brown solution with white precipitate at the bottom. Filter the precipitate and wash with methanol (5 mL) and allowed to dry. The solid precipitate was collected and vacuum oven dried to yield pure deprotected salt product **6** (0.017 g, 0.037 mmol, 92%).

^{1}H NMR (D_2O, 300 MHz) δ 6.74 (d, *J* = 8.7 Hz, 1H), 6.60 (d, *J* = 12.0 Hz, 1H), 6.54 (s, 2H), 6.51 (d, *J* = 12.0 Hz, 1H), 6.45 (d, J = 8.7, 1H), 3.71 (s, 3H), 3.65 (s, 3H), 3.57 (s, 6H).

^{31}P NMR (D_2O, 122 MHz) δ 3.7

Synthesis of combretastatin A-1 monophosphate A

*3-Hydroxy-2-[(isopropyl)oxy]-4-methoxybenzaldehyde **19***: The dihydroxy benzaldehyde **17** (5.00g, 25 mmol) was placed in a 20 mL microwave safe vial with a stir bar. Dimethylformamide (10 mL), potassium carbonate (4.57 g, 33.1 mmol), and 2-bromopropoane were added to the microwave vial and the vial was capped. The reaction was pre-stirred for 5 minutes prior to microwaving. The microwave was set for 90 ^{o}C for 2 hours. After the reaction has gone to completion, the cap was removed and the reaction was quenched with water (10 mL) and the organic phase was separated. The aqueous phase was extracted with ethyl acetate (3 x 75 mL) and the combined organic phase was washed with water (6 x 100 mL) until the aqueous phase washed clear and not yellow. The organic phase was dried with sodium sulfate and concentrated using the rotary evaporator. The product **19** was pure by TLC and NMR and was taken to the next step without purification (5.34 g, 25.4 mmol, 85%).

1H NMR ($CDCl_3$, 500 MHz) δ 10.26 (s, 1H), 7.44 (d, J = 8.5 Hz, 1H), 6.74 (d, J = 8.5 Hz, 1H), 5.64 (s, 1H), 4.68 (sept, J = 6.0 Hz, 1H), 3.97 (s, 3H), 1.36 (d, J = 6.1 Hz, 6H)

*2-[(Isopropyl)oxy]-3-[(para-tolunesulfonyl)oxy]-4-methoxybenzaldehyde **29***: The benzaldehyde **19** (5.34 g, 25.0 mmol) was dissolved in dimethyl formamide (20 mL) at room temperature. Diisopropylethylamine (4.88 mL, 27 mmol) was added to the reaction mixture and stirred for five minutes. The *para*-toluenesulfonyl chloride (5.32 g, 27.0 mmol) was added slowly over a period of 20 minutes and the reaction was stirred for 12 hours, monitored by TLC to check for reaction completion. The reaction was quenched

using sodium bicarbonate (200 mL) and the aqueous layer was extracted using diethyl ether (3 x 200 mL). The organic phases were combined and wash with brine (200 mL) and copious amounts of water (6 x 200 mL). The organic layer was then dried with sodium sulfate and concentrated in vacuo and subjected to flash column chromatography to yield the heteroprotected product **29** as a solid (6.25 g, 17.2 mmol, 68 %).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 10.21 (s, 1H), 7.87 (d, J = 8.0, 2H), 7.78 (d, J = 8.5 Hz, 1H), 7.36 (d, J = 8.0 Hz, 2H), 6.76 (d, J = 8.5 Hz, 1H), 4.49 (sept, J = 6.0 Hz, 1H), 3.73 (s, 3H), 2.48 (s, 3H), 1.13 (d, J = 6.1 Hz, 6H)

*(Z)-1-[3',4',5'-Trimethoxy]-2-[2''-[(isopropyl)oxy]-3''-[(para-tolunesulfonyl)-oxy]-4''-methoxyphenyl] ethene **30***: To an oven-dried round bottom flask was added sodium hydride (3.29 g, 137 mmol) and dichloromethane (100 mL) under nitrogen with good stirring and cooled to 0 ^{o}C using an ice/water bath. Phosphonium salt **20** (9.87g, 18.8 mmol) was added to the reaction mixture and the reaction was allowed to stir for 30 to 45 minutes. Benzaldehyde **29** (6.25 g, 17.2 mmol) was dissolved in a small amount of dichloromethane (20 mL) and was added dropwise to the reaction mixture. The reaction was allowed to slowly warm up to room temperature and stirred for 12 hours with TLC monitoring to check for reaction completion. Water was added very slowly to quench the reaction. The organic layer was separated and the aqueous layer was washed with dichloromethane (3 x 100 mL). The organic layers were combined and dried with sodium sulfate, filtered, and concentrated to yield the crude product as a yellow, viscous oil. Boiling ethanol (150 mL) was added to the oil and the product **30** crystallized out as a off-white solid (2.52 g, 4.76 mmol, 28%)

^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.85 (d, J = 8.5 Hz, 2H), 7.32 (d, J = 8.5 Hz, 2H), 7.08 (d, J = 8.5 Hz, 1H), 6.55 (d, J = 12.5 Hz, 1H), 6.53 (d, J = 8.5 Hz, 1H), 6.47 (d, J = 12.0 Hz, 1H), 6.44 (s, 2H), 4.37 (sept, J = 6.5 Hz, 1H), 3.83 (s, 3H), 3.69 (s, 3H), 3.67 (s, 6H), 2.46 (s, 3H), 1.06 (d, J = 6.5 Hz, 6H)

(Z)-1-[3',4',5'-Trimethoxy]-2-[2''-hydroxy-3''-[(para-tolunesulfonyl)oxy]-4''-methoxyphenyl] ethene ***31***: In a dry round bottom flask was added anhydrous aluminum trichloride (5.32g, 39.9 mmol), trimethylammonium chloride (1.95 g, 20.4 mmol), and dichloromethane (50 mL). The solution formed in this flask is the ionic liquid, Al_2Cl_7. In a separate flask, the hetero protected CA1 **30** (0.563 g, 1.06 mmol) was dissolved in dichloromethane (25 mL). The ionic liquid Al_2Cl_7 (4 mL, 3.20 mmol) was added dropwise to the reaction. The reaction was monitored by TLC every five minutes until the reaction had gone to completion. The reaction was not stirred for longer than 30 minutes. Water (25 mL) was added slowly to quench the reaction. The organic layer was separated using a separatory funnel and the water layer was washed with dichloromethane (3 x 75 mL). The organic layers were combined, dried with sodium sulfate, filtered and concentrated using a rotary evaporator. The crude product was subjected to flash column chromatography to yield an off-white solid product **31** (0.357 g, 0.734 mmol, 69%).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.85(d, J = 8.3 Hz, 2H), 7.34 (d, J = 8.5 Hz, 2H), 7.08 (d, J = 8.8 Hz, 1H), 6.67 (d, J = 8.8 Hz, 1H), 6.56 (d, J = 11.8 Hz, 1H), 6.43 (s, 2H), 6.35 (d, J = 11.8 Hz, 1H), 3.81 (s, 3H), 3.66 (s, 6H), 3.66 (s, 3H), 2.46 (s, 3H)

*(Z)-1-[3',4',5'-Trimethoxy]-2-[2''-[(benzyl)oxy]]phosphoryl)oxy]-3''-[(para-tolunesulfonyl)oxy]-4''-methoxyphenyl] ethene **32***: The tosyl-protected *cis*-CA1 **31** (0.880 g, 1.80 mmol) was dissolved with acetonitrile (25 mL) with moderate stirring under nitrogen and cooled to -20 ^{o}C using an acetone/dry ice bath. After the tosyl-protected *cis*-CA1 has completely dissolved, carbon tetrachloride (0.192 mL, 1.99 mmol) was added to the reaction mixture and stirred for five minutes. Diisopropylethylamine (0.632 mL, 3.60 mmol) and 4-dimethylaminopyridine (0.022 g, 0.18 mmol) were added to the reaction mixture and stirred for ten minutes. Dibenzylphosphite (0.459 mL, 2.17 mmol) was added slowly to the reaction mixture and it was allowed to stir for 45 minutes with TLC monitoring at 45 minutes and every five minutes after until the reaction has gone to completion. Once the reaction has gone to completion, saturated potassium dihydrogen phosphate solution (50 mL) was added and the reaction was allowed to warm up to room temperature. Water (50 mL) was added to the reaction and the organic phase was separated. The aqueous phase was extracted with ethyl acetate (3 x 100 mL) and the organic phases were combined and dried with sodium sulfate, filtered, and concentrated using the rotary evaporator. The crude product was subject to flash column chromatography to yield a yellow oil as pure product **32** (0.622 g, 0.833 mmol, 46%).

1H NMR ($CDCl_3$, 500 MHz) δ 7.76 (d, J = 8 Hz, 2H), 7.30 (m, 10H), 7.21 (d, J = 7 Hz, 2H), 7.04 (d, J = 8.5 Hz, 1H), 6.64 (d, J = 12 Hz, 1H), 6.63 (d, J = 9 Hz, 1H), 6.53 (d, J = 12 Hz, 1H), 6.43 (s, 2H), 5.06 (m, 4H), 3.81 (s, 3H), 3.64 (s, 6H), 3.59 (s, 3H), 2.36 (s, 3H)

*(Z)-1-[3',4',5'-Trimethoxy]-2-[2''-[(disodium)phosphate]-3''-[(para-tolune sulfonyl)oxy]-4''-methoxyphenyl] ethene **33***: Prior to starting the reaction, the TMSBr

was distilled over calcium hydride to give pure clear reagent. Compound **32** (0.622 g, 0.833 mmol) was dissolved in acetonitrile (25 mL) and cooled down to -10 ^{o}C under nitrogen. Add the TMSBr (0.55 mL, 4.17 mmol) to the reaction mixture and stir the reaction for 3 hours at -10 ^{o}C. Prior to the 3 hour mark for the first reaction, in a separate flask, a suspension of sodium methoxide (0.225g, 4.17 mmol) and methanol (10 mL) was made and was cooled to -10 ^{o}C. The initial reaction was slowly added dropwise to the suspension of sodium methoxide and methanol. The reaction was then allowed to stir for 2-3 hours and allowed to slowly warm up to room temperature. Once the reaction has gone to completion, the methanol was removed using the rotary evaporator, to give an off-white solid residue. The temperature of the bath of the rotary evaporator should not exceed 50 ^{o}C to prevent isomeriazation. The product is only partially soluble in organic or aqueous solvents. To note, the crude product is most soluble in ethanol, but not completely. This was the crude product **33** and it was taken on to the next step without purification due to its solubility.

^{1}H NMR (D_2O, 500 MHz) δ 7.70 (d, J = 8.5 Hz, 2H), 7.39 (d, J = 8, 2H), 7.13 (d, J = 8.5 Hz, 1H), 6.66 (d, J = 12 Hz, 1H), 6.61 (d, J = 9 Hz, 1H), 6.56 (d, J = 12 Hz, 1H), 6.46 (s, 2H), 3.75 (s, 3H), 3.71 (s, 6H), 3.49 (s, 3H), 2.43 (s, 3H)

^{31}P NMR (D_2O, 200 MHz) δ -3.19

*(Z)-1-[3',4',5'-Trimethoxyphenyl]-2-[2''-[(disodium)phosphate]-3''-[hydroxy]-4''-methoxyphenyl] ethene **5***: The white solid residue **33** (0.250 g, 0.409 mmol) was dissolved with water (1 mL) and ethanol (1 mL) in a 5 mL microwave safe vial with a stir bar. To this vial was added 2 M NaOH (1 mL) and the vial was capped and placed in the microwave. The reaction was pre-stirred for 5 minutes prior to microwaving. The

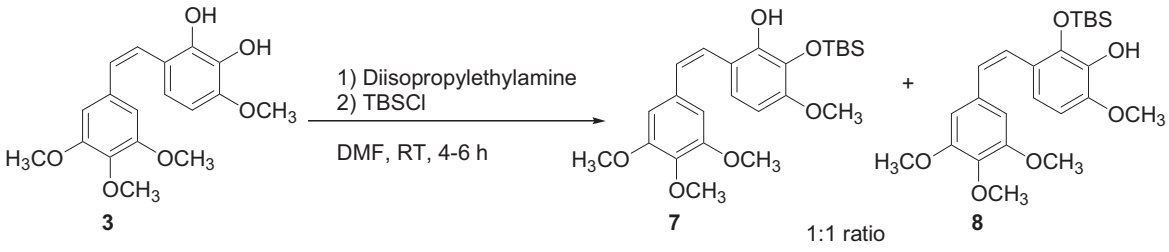
microwave was set to run for 30 minutes at 50 °C. Temperatures higher than 50 °C may lead to isomerization of the compound. Reverse phase TLC (30:70 acetonitrile-water) was used to monitor the reaction. After the microwave has finished, the reaction mixture should be a clear yellow-brown solution with white precipitate at the bottom. Filter the precipitate and wash with ethanol (5 mL) and allowed to dry. The solid precipitate was collected and vacuum oven dried to yield pure deprotected salt product **5** (0.042 g, 0.092 mmol, 11%).

^{1}H NMR (D_2O, 300 MHz) δ 6.74 (d, J = 12 Hz, 1H), 6.61 (d, J = 8.7 Hz, 1H), 6.56 (m, 3H), 6.55 (d, J = 12 Hz, 1H), 3.73 (s, 3H), 3.66 (s, 3H), 3.59 (s, 6H).

^{31}P NMR (D_2O, 122 MHz) δ 3.25

Results and Discussion

The synthesis of the regioisomeric CA1 monophosphates presented several significant challenges. It was necessary to differentiate between the two phenolic functionalities on the B ring of CA1 **3** and to separate any isomers prior to phosphorylation.



Scheme 1. Synthesis of mono-TBS-protected CA1

The synthesis proceeded initially with using the *cis* isomer of CA1 **3**. If the differentiation of the hydroxyl groups could be done on CA1, this would save multiple steps in the synthesis of the CA1 monophosphates. CA1 was treated using one molar equivalent of TBSCl and no selectivity was observed according to ^{1}H NMR which showed a 50/50 mixture of regioisomers (Scheme 1).

The di-TBS protected CA1 is known[1] and none was observed in this reaction. This mixture of monoprotected CA1, **7** and **8**, was subjected to flash column chromatography since the R_f values seemed far enough apart to separate (Table 1) but a mixture still eluted from the column. The mixture was also subjected to a gravity column chromatography as well as preparatory thin-layer chromatography (TLC). The gravity column still eluted a mixture of products as soon as the eluent spotted on the TLC plate.

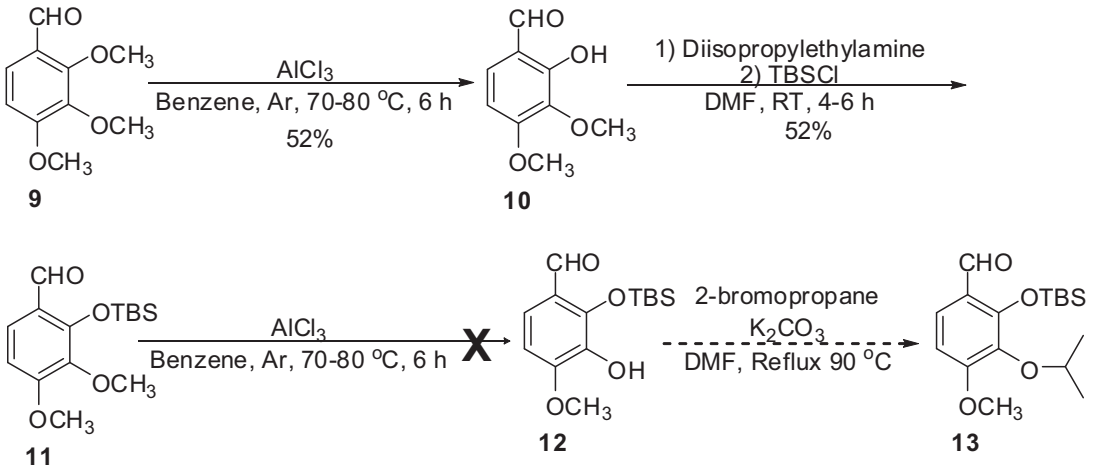
Table 1. CA1 and its monoprotected products after TBS protection and their corresponding RF values developed in 60:40, hexanes:ethyl acetate.

Spot	R_f	Product
1	0.57	CA-1 Mono TBS Protected Isomer
2	0.49	CA-1 Mono TBS Protected Isomer
3	0.17	CA-1

Preparatory TLC also showed a mixture of products, even when the compound mixture was carefully applied as a small thin layer to the baseline of the plate. Band broadening was observed and may have caused the products to not separate since the R_f values were very close. After attempting to separate the isomers using various techniques, all of which were unsuccessful, a new synthetic route was chosen. The strategy was to

determine if differentiation could occur at an earlier stage in the synthesis and if selectivity and / or the separation of isomers was more feasible.

According to Pettit and colleagues, there are known routes using BCl_3 to demethylate the 2 and 3 position methoxy functional groups from 2,3,4-trimethoxybenzaldehyde[1] or selectively demethylate the 2 or 3 position of 2,3,4-trimethoxybenzaldehyde using $AlCl_3$.[21] The goal was to selectively differentiate prior to the Wittig reaction by selectively demethylating the 2 or 3 position of 2,3,4-trimethoxybenzaldehyde, protect the resulting hydroxyl group with a TBS, and then demethylate the other methoxy functional group and protect it with an isopropyl for selective differentiation (Scheme 2).

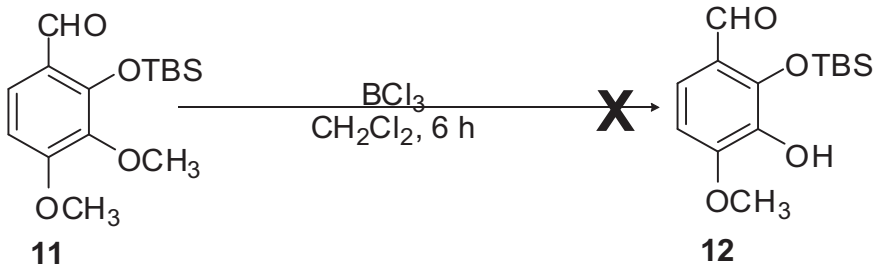


Scheme 2. Attempted synthesis of heteroprotected benzaldehyde[21]

By using $AlCl_3$, the 2,3,4-trimethoxybenzaldehyde **9** was selectively demethylated at the 2 position to give phenol **10** in a 52% yield and it was further protected using TBSCl to give the protected aldehyde **11** in a 52% yield. The $AlCl_3$ demethylation reaction was repeated again on the protected product in an attempt to

demethylate the methoxy on the 3 position. The reaction did not work as intended yielding only the deprotected aldehyde **10**.

BCl_3 is able to demethylate both the 2 and 3 position of 2,3,4-trimethoxybenzaldehyde **9** when a slight excess of 2 equivalents are used. A portion of the protected benzaldehyde **11** was dissolved in CH_2Cl_2 and subjected to 1 equivalent of BCl_3 to demethylate the 3 position (Scheme 3).



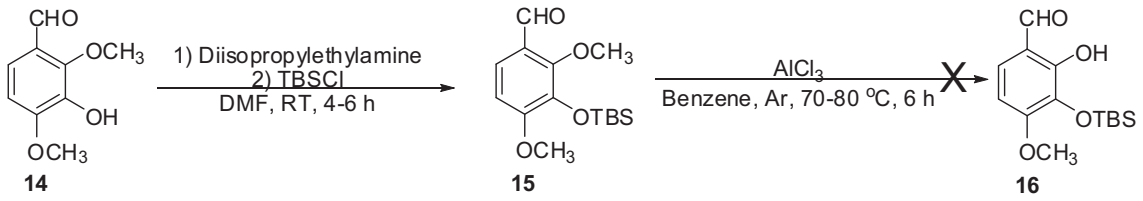
11 **12**

Scheme 3. Unsuccessful boron trichloride demethylation[1]

The reaction was very dark in color and after purification, only the starting material **11** and deprotected material **10** were recovered. An excess of over 2 equivalent may be required to demethylate the 3 position of the protected benzaldehyde **11** but another problem occurs when the TBS protecting group is removed while the 3 methoxy is demethylated. This defeats the purpose of differentiation if both hydroxy groups are free and unprotected.

2,4-Dimethoxy-3-hydroxybenzaldehyde **14** is commercially available from the Aldrich chemical company. It is known from the literature[21] that $AlCl_3$ is able to demethylate the 2 position of 2,3,4-trimethoxybenzaldehyde **9**. 2,4-dimethoxy-3-hydroxybenzaldehyde **14**, which possesses a hydroxy in the 3 position, was protected with a TBS group and further treated with $AlCl_3$ in order to demethylate the 2 position

methoxy (Scheme 4). The reaction was not successful and deprotected starting material **14** was recovered.

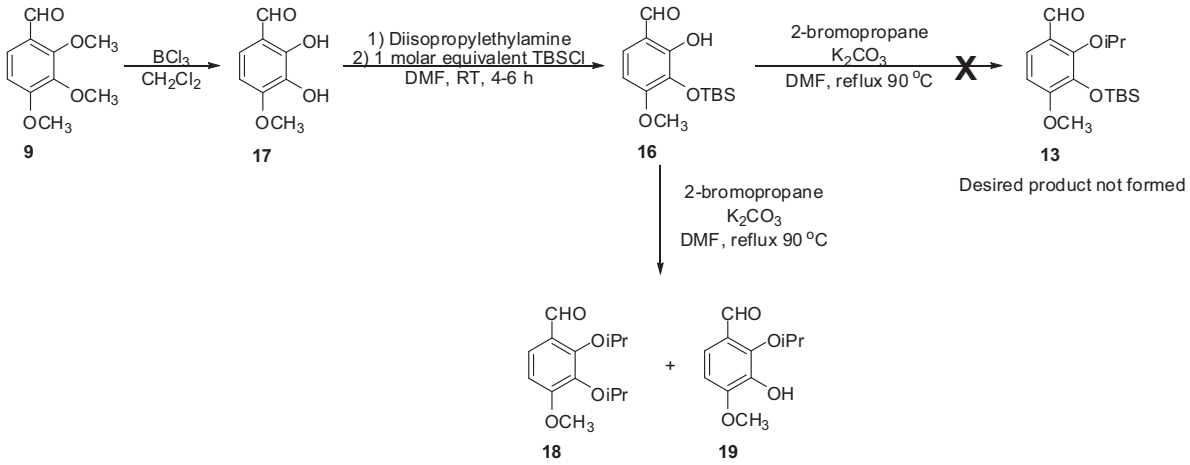


Scheme 4. Unsuccessful aluminum trichloride demethylation[21]

It is surprising to note that for 2,3,4-trimethoxybenzaldehyde **9**, the 2 position is able to be demethylated while for the 3-*tert*-butyldimethylsiloxy-2,4-dihydroxybenzaldehyde **15**, the 2 position was very resistant in demethylation. Nevertheless, even if the 2 position was able to be demethylated, the protected 3 position would still be deprotected leaving, again, another problem.

In order to solve this dilemma, a simple solution was attempted by going back to the starting reagents. The diol **17** is readily prepared through demethylation of 2,3,4-trimethoxybenzaldehyde **9** with BCl_3. The diol was subjected to treatment with only one equivalent of TBSCl to see if it would favorably protect in one position over the other (Scheme 5). This attempt proved fruitful and amazingly, the TBS only protected at the 3 position of the diol **17**. The mono TBS protected benzaldehyde **16** was treated with 2-bromopropane in order to protect the 2 position hydroxy group. The reaction did not go as anticipated to give the heteroprotected benzaldehyde **13**. Instead, the reaction gave the mono isopropyl protected product **19** and the diisopropyl protected product **18**. This may have happened because of the minimal amount of base, K_2CO_3 that was used. When 2-bromopropane is used to protect the 2 position of the monoprotected benzaldehyde, the byproduct formed is HBr. If the HBr acid is not neutralized by the base, it proceeds to

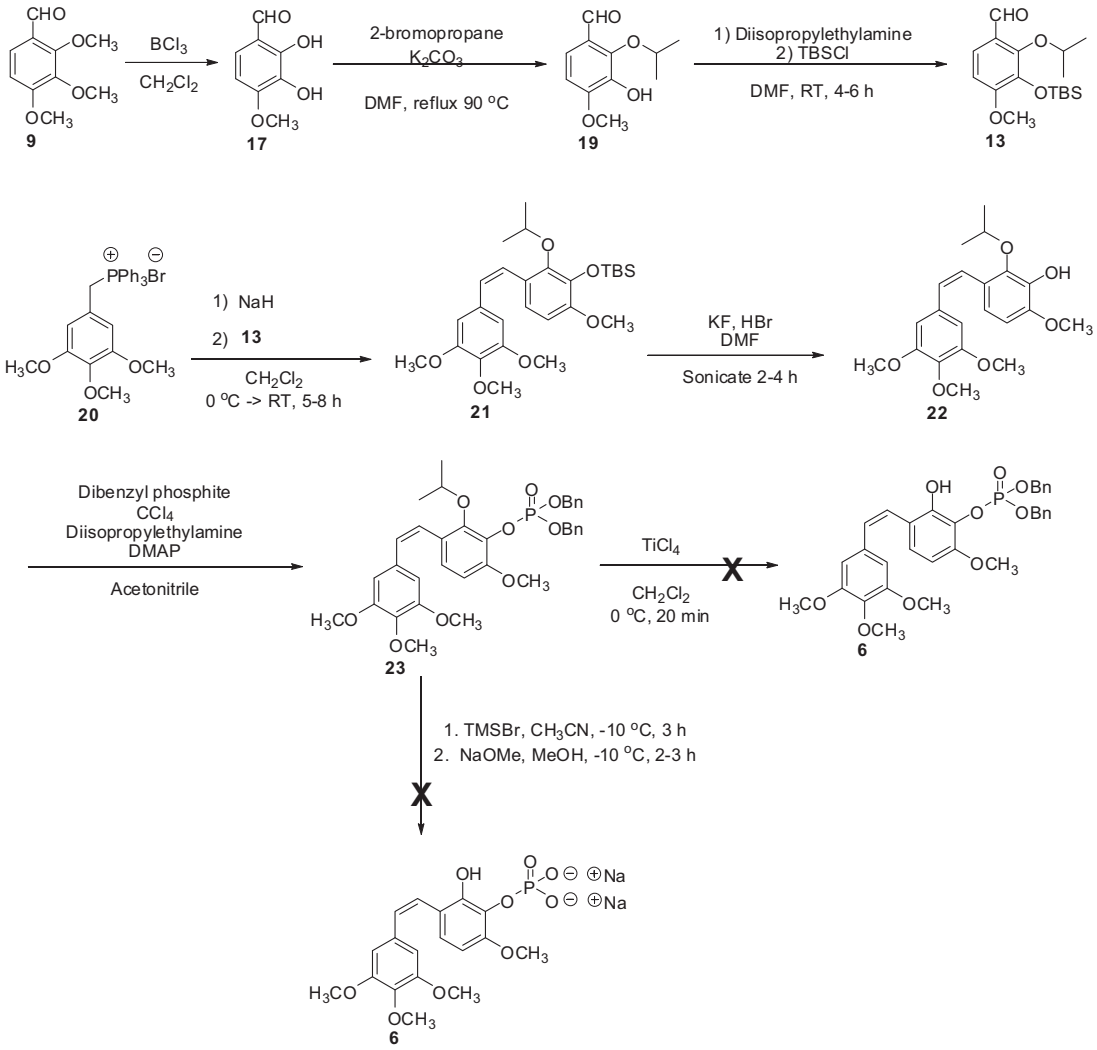
deprotect the TBS protecting group, which is very sensitive to acids. Once the TBS is deprotected, it can reprotect in the 3 position to give the diisopropyl protected product, or if all the 2-bromopropane has reacted, it will leave the mono isopropyl protected product. The reaction was carried out using one equivalent of 2-bromopropane, therefore not all of the starting material was converted to the diisopropyl protected product. Because of this mishap, a new strategy was formulated and a lesson was learned.



Scheme 5. Attempted selectivity using isopropyl and TBS protecting groups

The new strategy involves similar reactions as previously attempted (Scheme 5) but instead of carrying out the initial TBS protection, the isopropyl protection will be done first to test for selectivity (Scheme 6). Initial attempts at using 1 equivalent of 2-bromopropane gave selectivity for protection of the 2 position of the diol **17** to give the mono isopropyl protected product **19**. Following the protection of the 2 position with isopropyl, the 3 position of compound **19** was protected with TBS to give the heteroprotected aldehyde **13**. The product was isolated from flash column chromatography and recrystallized using a small amount of warm hexanes that dissolved the product and slowly allowed to cool to room temperature prior to placement into the

freezer to afford white crystals. In order to prove selectivity and differentiation, the white crystals were subjected to X-ray crystallography, which was done with the generous help of Dr. Andreas Franken at Baylor University. The white crystals defracted and a crystal structure was able to be solved for the molecule (Figure 2). Unfortunately, these white crystals are very temperature sensitive. In the freezer, the crystals are solid but at room temperature, the crystals melt into a yellow oil.



Scheme 6. Attempted synthesis of CA1 monophosphate B **6**

From this structure, a number of characteristics were able to be determined. First and foremost, selective protection of the desired positions was possible. This is important because in the later steps, selective deprotection can lead to the desired monophosphates. Since the isopropyl protecting group prefers to protect the 2 position of the aldehyde **17**, the 3 position hydroxy peak can now be derived from the proton NMR (OH hydrogen peak of compound **19**). The proton NMR of the diol **17** is known and the 2 hydroxy protons are evident. With this crystal structure (Figure 2), the chemical shifts of the hydroxy protons can now be assigned.

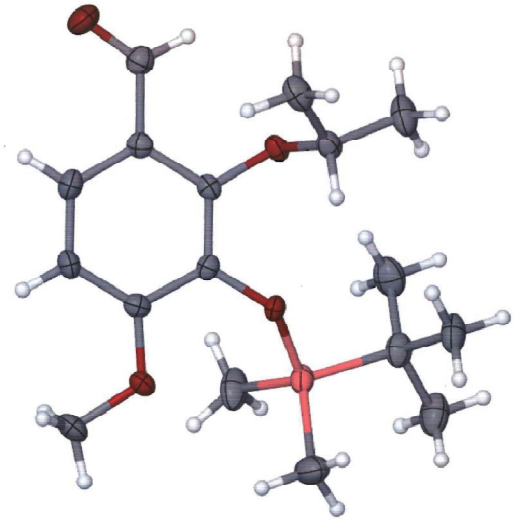
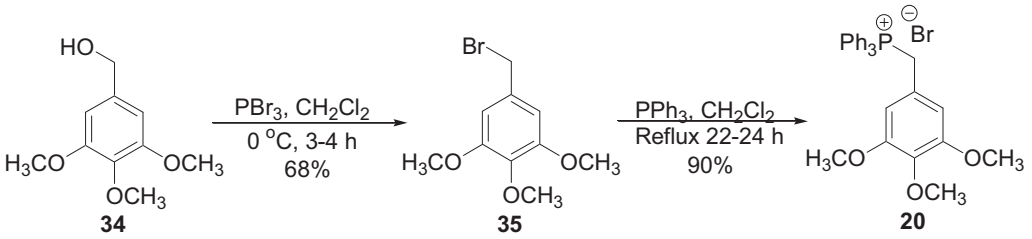


Figure 2. X-ray crystal structure of compound **13**, courtesy of Andreas Franken, Baylor University.

The diol **17** provided two hydroxy proton peaks: one at 5-6 ppm and another at 9-10 ppm. Presumably, isopropyl protection of diol **17** takes place at the 2 position. This is suggested by the disappearance in the proton NMR of the 9-10 ppm peak. Initial assignment of the 2 position OH hydrogen to the most downfield signal is based on the opportunity that this hydrogen has to participate in an intramolecular hydrogen bond to the carbonyl oxygen. If this is true then the hydroxy peak for the proton NMR of

compound **19** after isopropyl protection should be for the 3 position hydroxy functional group, which is evident between 5 and 6 ppm. It can then be deduced by going back to the proton NMR of diol **17** that the chemical shift between 9 and 10 ppm corresponds to the 2 position hydroxy functional group. This was confirmed by the X-ray crystal structure (Figure 2) which shows that the isopropyl protection took place selectively at the 2 position of diol **17** and the TBS protection took place at the 3 position to afford the heteroprotected aldehyde **13**.

Once there was evidence that the desired heteroprotected benzaldehyde **13** was synthesized, a Wittig reaction was conducted using previously prepared Wittig salt **20** (Scheme 7) and heteroprotected aldyhyde **13** to yield the heteroprotected CA1 **21** (Scheme 6). The Wittig reaction proved to be successful giving a mixture of *cis* and *trans* isomers. After separation of the isomers using flash column chromatography, the *cis* product **21** was isolated in only a 15% yield. The separation of these isomers proved to be more facile than when separating the mixture (1:1) of monoprotected TBS CA1 **7** and **8** (Scheme 1). After the Wittig reaction to install the stilbene bridge it was necessary to selectively remove the protecting groups.



Scheme 7. Synthesis of Wittig salt **20**[1, 19]

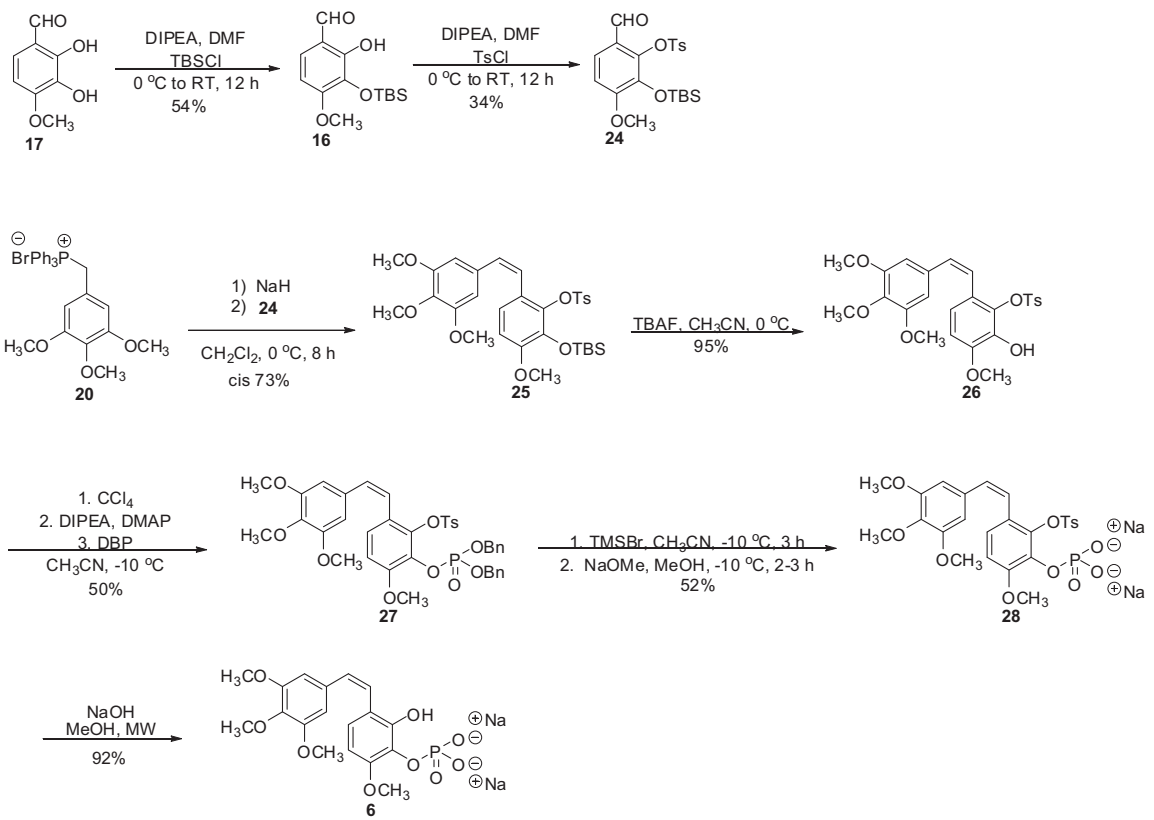
The TBS protecting group was removed using KF and sonication for 2-4 hours to give the mono isopropyl protected CA1 **22** (scheme 6).[22] This deprotection gave almost

quantitative yields and even though sonication was involved, no evidence of isomerization was observed. The mono isopropyl protected CA1 **22** was reacted with dibenzyl phosphite in DIPEA, CCl_4, and a catalytic amount of DMAP to afford the isopropyl protected CA1 dibenzyl phosphate **23** (scheme 6). The reaction went relatively well giving a 79% yield. The next step was to deprotect the benzyl groups and form the phosphate salt. TMSBr is used to deprotect the benzyl groups, which forms a siloxy ether intermediate. Titration of this intermediate into the basic solution of $NaOCH_3$ and MeOH forms the salt **6**. This route was used because there was evidence in the literature that TMSBr could also deprotect isopropyl protecting groups.[23] If this was the case, then both the isopropyl and benzyl groups could be deprotected at once, saving a step. The TMSBr was used in an attempt to deprotect both the benzyl and isopropyl groups and the reaction mixture was titrated into the NaOMe solution. The aqueous layer was tested by TLC for product and the organic layer was subjected to flash column chromatography, separated, and analyzed but the desired phosphate salt was not obtained. From the ^1H NMR, no evidence of the aromatic protons of CA1 was present for any of the products analyzed. It is a fair assumption to say that using TMSBr to deprotect both the benzyl groups and the isopropyl groups may have led to decomposition of this particular molecule. Since this may be a problem, an attempt to deprotect the isopropyl group prior to deprotection of the benzyl groups was conducted. The isopropyl protected CA1 dibenzyl phosphate **23** was reacted with $TiCl_4$ for a short period of time to deprotect the isopropyl group. After work up, the dark and black crude mixture was subjected to TLC. The initial TLC was run in 70:30 / hexanes: ethyl acetate. There appeared to be no spots that moved from the baseline. The solvent chamber's polarity was increased to 50:50 /

hexanes: ethyl acetate and a new TLC plate was spotted and developed, yet there was no movement of the spots from the baseline. Another TLC plate was spotted and this time, it was developed in 100% ethyl acetate but again, none of the spots moved. A crude ^{1}H NMR was taken and the spectrum was very messy with unrecognizable peaks. But there seemed to be some evidence that the CA1 scaffold was still present. A small amount of the dark crude mixture was subjected to flash column chromatography. The dark sludge did not move at all from the top of the flash column chromatography but one compound did elute and was isolated. Proton NMR suggested that this compound was a benzyl derivative, perhaps benzyl bromide. The $TiCl_4$ may have deprotected the isopropyl group as well as both benzyl groups giving the phosphoric acid, which may be the reason why this crude mixture is very polar. In an attempt to elute more compounds from this flash column chromatography, a solution of methanol (5-10 %) in ethyl acetate was washed through the column, but no other compounds eluted. Since the compound was very polar, a reverse phase column chromatography run with acetonitrile and water may be able to separate these compounds. Unfortunately, at this phase of the project, reverse phase flash column chromatography was not readily available in the laboratory. The final stages of the project and the final monophosphate salt was so very near, yet was not attainable by this unforeseen complication. A similar route was devised and instead of isopropyl protecting group, a tosyl protecting group was used (Scheme 8).

A tosyl group was used in place of isopropyl group due to its robust nature and unique deprotection method. The overall synthetic route (Scheme 8) will utilize the previous strategy employing different protecting groups prior to formation of the hetero protected CA1 with the tosyl group in place of the isopropyl group. TMSBr was used to

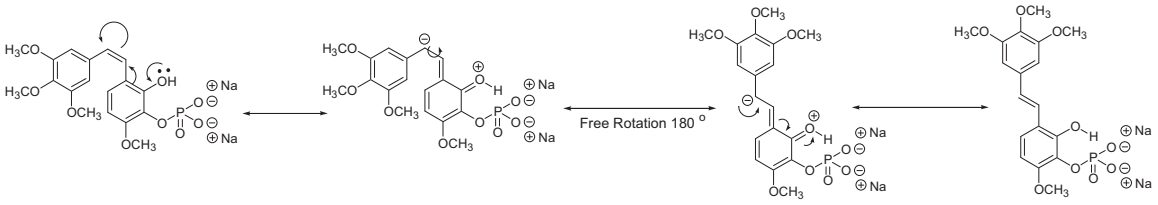
deprotect the benzyl groups and it was expected that the tosyl protecting group can remain robust under the harsh conditions of TMSBr. To deprotect the tosyl protecting group, the use of base such as NaOH is required under microwave conditions. An ample supply of the 2,3-dihydroxy-4-methoxybenzaldehyde **17** was available and it was again monoprotected, in good yield (90 %), using one equivalent of TBS chloride to give compound **16** (Scheme 8) with selective protection at the 3 position. The tosyl protection at the 2 position to give the heteroprotected aldehyde **24**, gave very low yields of only 34% even after a slight excess of 1.5 equivalents of tosyl chloride was used (Scheme 8).



Scheme 8. Synthesis of CA1 monophosphate B (CA1-MPB) **6**

After isolation of the product, the heteroprotected benzaldehyde **24** underwent a Wittig reaction with the Wittig salt **20** to form the stilbene bridge of the CA1 moiety. The Wittig reaction went surprisingly well with a relatively good isolated yield of 73 % of the *cis* isomer. The ration of *cis* to *trans* is about 7:1, respectively. At this point, the heteroprotected CA1 **25** was selectively deprotected at the 3 position of the B ring using TBAF to give the mono tosyl protected CA1 **26** in a 95% yield. The reaction itself was very clean and no purification was required. The mono tosyl protected CA1 **26** was phosphorylated using dibenzyl phosphite giving an isolated yield of 50% of the dibenzyl phosphate CA1 **27**. The benzyl groups of the dibenzyl phosphate product **27** were debenzylated using TMSBr mediated debenzylation to afford the TMS-phosphate intermediate which was then quenched with a solution of MeOH and $NaOCH_3$ to form the tosyl protected CA1 disodium monophosphate salt **28**. Since the molecule possesses both organic attributes as well as aqueous attributes, the compound is soluble in water and partially soluble in some organic solvents such as methanol and ethanol. After isolation of the product, a ^{1}H and ^{31}P NMR were taken to verify that the tosyl protecting group withstood the phosphorylation and debenzylation steps and to check if phosphorous was present within the molecule. Both the tosyl protecting group and a phosphorous atom were evident from the NMR spectra and the *cis* conformation of the molecule was verified by calculating the coupling constant of the stilbene bridge hydrogens of the ^{1}H NMR with a coupling constant of 12. Deprotection of the tosyl group was done in the microwave using 2M NaOH in MeOH to give the CA1 disodium monophosphate salt **6**. The tosyl protected CA1 disodium monophosphate salt **28** was dissolved in MeOH with a minimal amount of water along with the 2M NaOH. As the

microwave reaction proceeds, the tosyl group becomes deprotected and the salt precipitates out of the reaction as a fine white powder, which settles at the bottom of the microwave-safe vial. Care needs to be taken concerning the amount of water used. If too much water is used, then the salt will not precipitate and removal of water will be necessary. Any other organic byproducts will be soluble in the MeOH layer and remain in the filtrate layer after filtering the salt after the reaction. The actual deprotection of the tosyl group went very well giving an isolated yield after filtering of 92% of CA1-MPB **6**. Unfortunately for this CA1-MPB **6**, since the 2 position of the B ring is not protected, the actual molecule itself is unstable and may undergo isomerization to form the *E*-isomer, which was observed after one week in the freezer. The lone pair of electrons on the 2 position hydroxy can donate its electrons to the B ring, initiating a cascade of electron movement that will break the stilbene bridging of the CA1 scaffold allowing free rotation across the formerly rigid stilbene bridge (Scheme 9). Since the *E*-isomer is the less strained and more stable steroisomer, if free rotation is available, the molecule will choose to conform to the more stable isomer and reform the stilbene bridge and become the *E*-CA1-MPB. The isomerization occurs even while stored at 0 ^{o}C or below, in pure CA1-MPB **6** salt form, wrapped in foil in complete darkness.



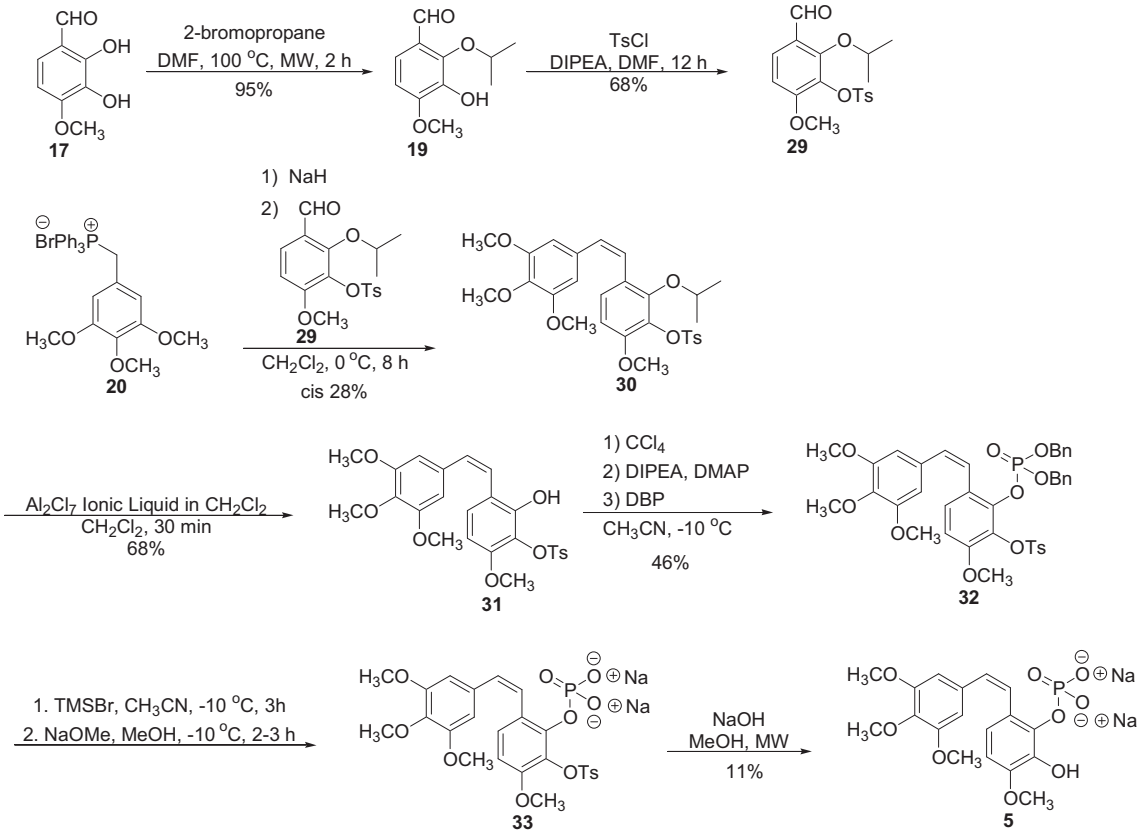
Scheme 9. Isomerization of CA1-MPB **6**

In conclusion, after various synthetic strategies, one isomer of the CA1 disodium monophosphate, CA1-MPB **6**, was successfully synthesized. One must consider that the

possibility of CA1-MPB **6** is actually a sodium salt at the 2 phenolic position. This phenylate anion would obviously fascilitate rapid isomerization. It is interesting to note that a separate synthesis of CA1-MPB **6** by Dr. Rajendra Tanpure (Pinney Group)[REF] yielded the corresponding monophosphate void of isomerization. Possibly, this second synthesis produced the phenolic moiety at the 2 position. Further study is necessary to confirm this postulation.

After successfully synthesizing CA1-MPB **6,** a modified protocol for the preparation of the other regioisomeric monophosphate salt CA1-MPA **5** was developed (Scheme 10). 2,3-Dihydroxy-4-methoxybenzaldehyde **17** was previously synthesized and available so an isopropyl protection of the diol **17** using one equivalent of 2-bromopropane selectively protected the 2 hydroxy group giving the mono isopropyl protected aldehyde **19** (Scheme 10). Instead of refluxing the isopropyl reaction for a number of hours, the reaction was conducted in the microwave for two hours which gave a 95% yield of the product and very little to no byproducts. However, a small amount of starting material was detectable. Even though problems arose when isopropyl protecting groups were used previously, this time, the strategy required the deprotection of the isopropyl group prior to phosphorylation with dibenzyl phosphite and debenzylation mediated with TMSBr. With the tosyl protecting group proving to be robust to the phosphorylation and debenzylation and with the isopropyl protecting group having been removed prior to initiating these reactions, the strategy appeared poised in successfully preparing the CA1-MPA salt **5**. Isopropyl protection followed by tosyl protection gave the heteroprotected benzaldehyde **29** in a 68% yield. A Wittig reaction was conducted to afford the *cis* heteroprotected CA1 **30** (Scheme 10), albeit in low yield. Traditionally,

$TiCl_4$ is used to deprotect isopropyl protecting groups. When it was used to deprotect the isopropyl group from compound **30**, the reaction became dark black and and the products were difficult to isolate. Instead, an ionic liquid (Al_2Cl_7) was used for the removal of the isopropyl group. The ionic liquid (Al_2Cl_7) was prepared with trimethylammonium chloride and aluminum trichloride in dichloromethane.[24]



Scheme 10. Synthesis of CA1 monophosphate A (CA1-MPA) **5**

The deprotection went very quickly (30 minutes) to give the mono tosyl protected CA1 **31** in a 68% yield (Scheme 10). The reaction was very clean and the only compounds that were present were the starting material heteroprotected CA1 **30** and the deprotected product **31** which allowed for ease of separation. Phosphorylation using dibenzyl phosphite gave the tosyl protetcted CA1 dibenzyl phosphate **32** with a 46%

yield. Debenzylation of compound **32** with TMSBr afforded the TMS phosphate ether which was quenched using $NaOCH_3$ in MeOH to generate the tosyl protected CA1 disodium monophosphate salt **33**. It appears that the tosyl protected CA1 disodium monophosphate salt **33** is the complete opposite of its counterpart, CA1-MPB **6**, where instead of being soluble in water and partially soluble in organic solvents, the tosyl protected CA1 disodium monophosphate salt **33** is not soluble in water or any organic solvents. Removal of organic solvents and water gave tosyl protected CA1 disodium monophosphate salt **33** as a sticky clear residue on the walls of the flask. Water did not dissolve it, nor did methanol or ethanol. An NMR was not taken of the intermediate due to solubility issues and the crude was taken to the next step. The insoluble residue was scrapped into a microwave safe vial and the tosyl protecting group was successfully deprotected in the microwave to give a white solid precipitate that was CA1 disodium monophosphate salt, CA1-MPA **5**. The CA1-MPA **5** was soluble in water and the structure was confirmed ^{1}H and ^{31}P NMR.

Upon the successful synthesis of both CA1-MPA **5** and CA1-MPB **6,** the compounds were characterized using high performance liquid chromatography (HPLC). The HPLC chromatograms are available in appendix B. Each CA1 monophosphate was individual characterized and were co-eluted and compared to both combretastatin A-1 (CA1) and combretastatin A-1 phosphate (CA1P).

The total synthetic methodology for both the monophosphates, CA1-MPA and CA1-MPB was finally completed and they were synthesized and characterized. Both of the monophosphates were subjected to inhibition of tubulin studies conducted by Dr. Ernest Hamel[REF] from the National Cancer Institute (NCI) and it was confirmed that both

monophosphates were not inhibitors of tubulin polymerization (IC_{50} > 40 μM). This is consistent with the diphosphate (CA1P), which is also not an inhibitor of tubulin polymerization in this pure protein assay.

In summary, the first total synthesis of each of the regioisomeric CA1 monophosphates has been achieved. The synthesis of these analogs proved especially challenging due to the need to differentiate ortho phenolic positions at an early stage in the route and by the necessary formation of the phosphate salt at a late stage. These compounds have been carefully characterized and appear quite stable. HPLC studies have shown that the isomers are robust and do not appear to equilibriate. The availability of CA1-MPA **6** and CA1-MPB **5** will fascilitate future biochemical and biological studies.

CHAPTER TWO

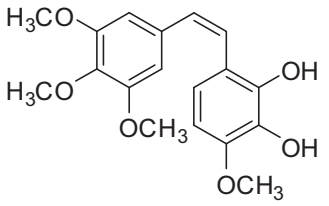
Radiosynthesis of OX16C[20]

Introduction

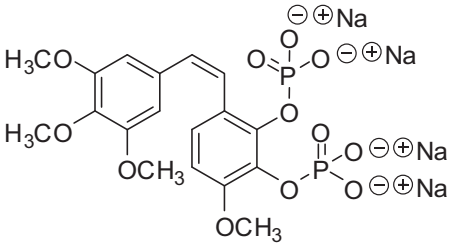
Combretastatin A-1 (CA1) and its corresponding phosphate salt, combretastatin A-1 phosphate (CA1P) are small molecule vascular disrupting agents (VDAs).[25, 26] CA1 was isolated from an African bush willow tree, *Combretum caffrum* by Pettit and coworkers and synthesized as a phosphate salt (CA1P) in order to enhance solubility in an aqueous environment (human blood serum).[1, 2, 19, 27] CA1 is a very active VDA with an effective dose (ED_{50}) of 0.99 μg/mL *in vitro* against murine P388 lymphocytic leukemia and a half maximal inhibitory concentration (IC_{50}) of 2 μM in terms of inhibition of tubulin polymerization.[1] Due to its high activity, CA1 has been studied extensively to determine its mechanism of action in the disruption of tumor vasculature but unfortunately, this mechanism is still not completely known.[28, 29] In order to facilitate pharmacological studies designed to further elucidate the biological mechanism of action of CA1, it is desirable to utilize high specific activity radiolabeled CA1. By developing a synthetic methodology to incorporate a radioisotope at a metabolically stable position, the high specific activity radiolabeled CA1P can be monitored to elucidate the biological mechanisms it uses to disrupt tumor vasculature. The radiolabeled CA1P will eventually be metabolized by non-specific phosphatase enzymes that cleave the phosphate groups leaving the active CA1 molecule to act upon the tumor and disrupt tumor vasculature.[14]

A variety of criteria were considered when selecting a radioisotope to be incorporated within CA1P. ^{32}P could be used as the potential radioisotope however,

CA1P will undergo metabolism and the phosphate groups of CA1P will be rapidly cleaved by phosphatase enzymes (*in vivo)*. Instead, ^{14}C was selected as the radiotracer for this project. The ^{14}C isotope has high sensitivity, low energy beta decay, and has a long half life (5700 years) which would be beneficial when used to monitor the radiolabeled CA1P.[30] The 4-position of the 3,4,5-trimethoxyphenyl moiety of CA1P (A ring) was chosen as the position to incorporate the ^{14}C methoxy functionality since that methoxy functionality is easily removed though demethylation and can be replaced with the radioisotope through methylation. CA1P is the tetrasodium diphosphate salt of CA1, but for this project, a specific salt of CA1P, the dipotassium diphosphate salt known as OX16C **47** was synthesized.[31, 32]



Combretastatin A-1 (CA1) **3**



Combretastatin A-1 Phosphate (CA1P) **4**

Figure 3. Combretastatin A1 (CA1) and its corresponding phosphate salt, combretastatin A1 phosphate (CA1P)

This group project was initiated by Dr. Anupama Shirali[20, 33] and it was continued and led by Dr. Madhavi Sriram.[20, 34] The group also consisted of Dr. John J. Hall, Dr. Rajsekhar Guddneppanavar, and me. This work was initially inspired by the radiolabeled synthesis of CA4P that was carried out by the Pinney Group.[35] The group worked together to synthesize starting materials and intermediates for the radiosynthesis of OX16C. Dr. Madhavi Sriram was the first to complete the total synthesis of the radiolabeled OX16C on a small scale, but various problems arose which required the

group to help troubleshoot. When the synthesis of the radiolabeled OX16C was scaled up, problems with isomerizations occurred and the yield for the (*Z)* OX16C salt formation dramatically decreased. The group was tasked to determine what, how, and where in the synthetic route, during the scale up, these isomerization problems. The group scaled up all of the starting material, enough for each person to prepare large amounts of the intermediates to be able to synthesize the radiolabeled OX16C multiple times in order to determine the problematic areas of the synthetic route. Every part of the synthetic route was conducted for this project; from scale up of starting materials, to synthesizing the intermediates, and making the final pure (*Z*) OX16C salt. For this project, ^{12}C was used as a model. Incorporation of the actual ^{14}C was carried out successfully by Almac Sciences.[31]

Materials and Methods

Reactions were performed under an inert atmosphere using nitrogen gas unless specified differently. Chemical reagents used in the synthetic procedures were obtained from various chemical suppliers (Sigma Aldrich, Acros Chemical Co., Alfa Aesar, Fisher Scientific, EMD Chemicals, and VWR). Silica gel (200-400 mesh, 60 Å), used for column chromatography, was obtained from either Silicycle Inc or VWR. TLC plates (pre-coated glass plates with silica gel 60 F254, 0.25 mm thickness, EMD chemicals, VWR) were used to monitor reactions. Intermediates and products synthesized were characterized based on ^{1}H NMR (Bruker DPX operating at 300 MHz or Varian operating at 500 MHz), ^{13}C NMR (Bruker DPX operating at 75 MHz or Varian operating at 125 MHz), and ^{31}P NMR (Bruker DPX operating at 121 MHz or Varian operating at 202 MHz). All the chemical shifts are expressed in ppm (δ), coupling constants (*J*) presented

in Hz, and peak patterns are reported as broad (br), singlets (s), doublets (d), triplets (t), quartets (q), septets (sept), and multiplets (m). NMRs were processed using Mestrec. Elemental analysis was performed by Atlantic Microlab, Norcross, GA. High-resolution mass spectra (HREIMS), unit resolution gas chromatography mass spectra (EIMS), and unit resolution mass spectra (ESIMS) were obtained on a VG Prospec Micromass spectrometer, a Thermo Scientific DSQ II, and a Thermo Finnigan LCQ Classic, respectively, in the Baylor University Mass Spectrometry Core Facility. Purity of the compounds was further analyzed using a Hewlett Packard HP Series 1050 HPLC system with UV detection, and a Supelco discovery C_{18} HPLC column 12.5 cm x 4.6 mm, 5 μm, T = 25 °C; eluents, solvent A, 25 mM tetrabutylammonium bromide (TBAB) with 0.1% trifluoroacetic acid (TFA) in water, solvent B, 25 mM TBAB with 0.08% TFA in water/acetonitrile (2/8 v/v); gradient, 80% A/20% B → 5% A/95% B over 0 to 45 min; flow rate, 0.7 mL/min; injection volume 25 μL; monitored at 264 nm wavelength.

Synthesis of Radiolabeled OX16C

Note: The methodology presented herein utilized ^{12}C as a model system for future radiolabeling.

4-Hydroxy-3,5-dimethoxybenzaldehyde ***36***:[36, 37] 3,4,5-Trimethoxybenzaldehyde (11.99 g, 61.16 mmol) was dissolved in dry CH_2Cl_2 (200 mL) at room temperature under nitrogen. Anhydrous $AlCl_3$ (16.31 g, 122.3 mmol) was added, and the reaction mixture was stirred for 12 hours. The reaction was quenched with H_2O (100 mL), the organic phase was separated, and the aqueous phase was extracted with CH_2Cl_2 (2 x 150 mL). The combined organic phases were washed with brine, dried over Na_2SO_4, filtered and concentrated to dryness under reduced pressure. Aldehyde **37** (10.58 g, 58.08 mmol,

95% yield) was obtained as a white powder, R_f 0.46 (70:30 hexanes:EtOAc); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.82 (1H, s, C-1 C*H*O), 7.16 (2H, s, H-2, H-6), 6.09 (1H, s, C-4 O*H*), 3.97 (6H, s, C-3, C-5 OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 190.7 (C, C-1 *C*HO), 147.3 (C, C-3, C-5), 140.8 (C, C-4), 128.4 (C, C-1), 106.7 (CH, C-2, C-6), 56.5 (CH_3, C-3, C-5 O*C*H_3); HRMS (EI) *m/z* 182.0577 (calcd for $C_9H_{10}O_4$, 182.0579).

4-[(tert-Butyldimethylsilyl)oxy]-3,5-dimethoxybenzaldehyde ***37***:[38] To a well stirred solution of aldehyde **37** (10.62 g, 58.29 mmol) in anhydrous CH_2Cl_2 (150 mL) at 0 °C was added Et_3N (12.15 mL, 87.12 mmol) and *N,N*-dimethylamino pyridine (DMAP) (14.0 mg, 1.20 mmol) under nitrogen. The reaction mixture was stirred for 10 minutes followed by the addition of tert-butyldimethylsilyl chloride (TBSCl) (13.18 g, 87.45 mmol) in portions. The reaction mixture was stirred for 12 hours followed by the addition of H_2O (100 mL). The organic layers were separated, and the aqueous phase was extracted with CH_2Cl_2 (2 x 400 mL). The combined organic phases were washed with brine, dried over Na_2SO_4, filtered, and evaporated *in vacuo*. The crude off-white solid was recrystallized with absolute EtOH to obtain TBS protected aldehyde **38** (13.20 g, 44.53 mmol, 77% yield) as pale yellow crystals, R_f 0.57 (70:30 hexanes:EtOAc); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.82 (1H, s, C-1 C*H*O), 7.10 (2H, s, H-2, H-6), 3.87 (6H, s, C-3, C-5 OCH_3), 1.01 (9H, s, C-4 OSi$(CH_3)_2$C(C$H_3)_3$), 0.16 (6H, s, C-4 OSi(C$H_3)_2$C$(CH_3)_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 191.0 (C, C-1 *C*HO), 151.9 (C, C-3, C-5), 140.6 (C, C-4), 129.3 (C, C-1), 106.7 (CH, C-2, C-6), 55.8 (CH_3, C-3, C-5 O*C*H_3), 25.6 (CH_3, C-4 OSi$(CH_3)_2$C(*C*$H_3)_3$), 18.8 (C, C-4 OSi$(CH_3)_2$*C*$(CH_3)_3$), -4.6 (CH_3, C-4 OSi(*C*$H_3)_2$C$(CH_3)_3$); HRMS (EI) *m/z* 296.1443 (calcd for $C_{15}H_{24}O_4Si$, 296.1444).

Note: Compound **37** is commercially available as syringaldehyde. Conversion of synthesized **37** or commercially available syringaldehyde to silyl ether **38** proceeded in analogous fashion.

*2,3-Di-[(isopropyl)oxy]-4-methoxybenzaldehyde **18***:[39] 2,3-Dihydroxy-4-methoxybenzaldehyde[1] **17** (12.12 g, 72.07 mmol) was dissolved in anhydrous DMF (100 mL). Anhydrous K_2CO_3 (21.20 g, 214.0 mmol) and 2-bromopropane (37.80 mL, 214.0 mmol) were added, and the reaction mixture was stirred and refluxed at 90 °C for 12 hours. H_2O (100 mL) was added, and the solution was extracted with CH_2Cl_2 (3 x 300 mL). The combined organic phases were washed with H_2O followed by brine, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, 5:95 EtOAc:hexanes) to afford aldehyde **18** (9.80 g, 38.8 mmol, 51% yield) as a yellow oil, R_f 0.38 (70:30 hexanes:EtOAc); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 10.28 (1H, s, C-1 C*H*O), 7.62 (1H, d, *J* = 8.8 Hz, H-6), 6.75 (1H, d, *J* = 8.8 Hz, H-5), 4.82 (1H, sep, *J* = 6.1 Hz, C-2 OC*H*$(CH_3)_2$), 4.45 (1H, sep, *J* = 6.1 Hz C-3 OC*H*$(CH_3)_2$), 3.91 (3H, s, C-4 OC*H*$_3$), 1.30 (12H, d, *J* = 6.1 Hz, C-2, C-3 OCH(C*H*$_3)_2$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 189.6 (C, C-1 *C*HO), 159.8 (C, C-4), 155.4 (C, C-2), 139.8 (C, C-3), 124.9 (CH, C-6), 123.5 (C, C-1), 107.2 (CH, C-5), 75.9 (CH, C-3, O*C*H$(CH_3)_2$), 75.4 (CH, C-2 O*C*H$(CH_3)_2$), 56.0 (CH_3, C-4 O*C*H$_3$), 22.4 (CH_3, C-3 OCH(*C*H$_3)_2$), 22.0 (CH_3, C-2 OCH(*C*H$_3)_2$); HRMS (EI) *m/z* 252.1368 (calcd for $C_{14}H_{20}O_4$, 252.1368).

*2,3-Di-[(isopropyl)oxy]-4-methoxybenzyl alcohol **38***: $NaBH_4$ (2.378 g, 62.88 mmol) was added in portions to a stirred solution of aldehyde **18** (15.81 g, 62.66 mmol) in anhydrous EtOH (100 mL) at 0 °C. The reaction mixture was stirred for 30 minutes

and quenched with H_2O (100 mL) cautiously. Organic solvent was removed *in vacuo*, and the aqueous phases were extracted with EtOAc (3 x 300 mL). The combined organic phases were washed with brine, dried over Na_2SO_4, filtered, and evaporated. The crude product was subjected to flash column chromatography (silica gel, 10:90 EtOAc:hexanes) to afford benzyl alcohol **38** (10.30 g, 40.50 mmol, 65% yield) as colorless oil, R_f 0.33 (70:30 hexanes:EtOAc); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.96 (1H, d, J = 8.5 Hz, H-6), 6.61 (1H, d, J = 8.5 Hz, H-5), 4.86 (1H, sep, J = 6.0 Hz, C-2, OC*H*$(CH_3)_2$), 4.61 (2H, d, J = 6.0 Hz, C-1 C*H*$_2$OH), 4.40 (1H, sep, J = 6.0 Hz, C-3 OC*H*$(CH_3)_2$), 3.82 (3H, s, C-4 OC*H*$_3$), 2.53 (1H, t, J = 6.5 Hz, C-1 CH_2O*H*), 1.27 (6H, d, J = 6.0 Hz, C-3 OCH(C*H*$_3)_2$), 1.26 (6H, d, J = 6.0 Hz, C-2 OCH(C*H*$_3)_2$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 154.0 (C, C-4), 149.9 (C, C-2), 139.8 (C, C-3), 127.8 (CH, C-6), 122.8 (C, C-1), 106.5 (CH, C-5), 74.9 (CH, C-3 O*C*H$(CH_3)_2$), 74.3 (CH, C-2 O*C*H$(CH_3)_2$), 62.1 (CH_2, C-1 *C*H_2OH), 55.7 (CH_3, C-4, O*C*H_3), 22.5 (CH_3, C-3 OCH(*C*$H_3)_2$), 22.4 (CH_3, C-2 OCH(*C*$H_3)_2$).

*2,3-Di-[(isopropyl)oxy]-4-methoxybenzyl bromide **39***: To a well stirred solution of alcohol **38** (10.30 g, 40.50 mmol) in dry CH_2Cl_2 (150 mL) at 0 °C, under nitrogen was added PBr_3 (3.10 mL, 40.5 mmol). The reaction mixture was stirred for 6 hours at 0 °C. H_2O (100 mL) was added, and the organic layer was separated. The aqueous phase was extracted with CH_2Cl_2 (2 x 300 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and evaporated to dryness. Benzyl bromide **39** (12.50 g, 39.40 mmol, 97% yield) was obtained as a pale yellow oil, and needed no further purification, R_f 0.70 (60:40 hexanes:EtOAc); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.10 (1H, d, J = 8.5 Hz, H-6), 6.63 (1H, d, J = 8.5 Hz, H-5), 4.87 (1H, sep, J = 6.0 Hz, C-2 OC*H*$(CH_3)_2$), 4.58 (2H, s, C-1 C*H*$_2$Br), 4.40 (1H, sep, J = 6.0 Hz, C-3 OC*H*$(CH_3)_2$), 3.83

(3H, s, C-4 OCH_3), 1.29 (6H, d, J = 6.0 Hz, C-3 OCH(CH_3)$_2$), 1.28 (6H, d, J = 6.0 Hz, C-2 OCH(CH_3)$_2$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 154.8 (C, C-4), 150.1 (C, C-2), 139.8 (C, C-3), 125.5 (CH, C-6), 124.7 (C, C-1), 107.0 (CH, C-5), 75.1 (CH, C-3 O*C*H(CH$_3$)$_2$), 74.4 (CH, C-2 O*C*H(CH$_3$)$_2$), 55.9 (CH$_3$, C-4 O*C*H$_3$), 29.7 (CH$_2$, C-1 *C*H$_2$Br), 22.6 (CH$_3$, C-3 OCH(*C*H$_3$)$_2$), 22.4 (CH$_3$, C-2 OCH(*C*H$_3$)$_2$).

2,3-Di-[(isopropyl)oxy]-4-methoxybenzyl triphenylphosphonium bromide ***40***: A mixture of bromide **39** (12.50 g, 39.40 mmol) and PPh_3 (11.40 g, 43.40 mmol) in dry CH_2Cl_2 (200 mL) was refluxed for 22 hours under N_2. Solvent was removed to afford an off-white solid. Et_2O was added and the solid was filtered and washed with Et_2O and dried under high vacuum to obtain the phosphonium salt **40** (20.01 g, 34.59 mmol, 88% yield) as a white solid. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.75 (9H, m, C-1 CH$_2$P(C$_6$*H*$_5$)$_3$), 7.64 (6H, m, CH$_2$P(C$_6$*H*$_5$)$_3$), 7.09 (1H, dd, J = 8.5 Hz, 3.0 Hz, H-6), 6.50 (1H, d, J = 8.5 Hz, H-5), 5.19 (2H, d, $J_{H\text{-}P}$ = 14.0 Hz, C-1 C*H*$_2$P(C$_6$H$_5$)$_3$), 4.72 (1H, sep, J = 6.0 Hz, C-2 O*C*H(CH$_3$)$_2$), 3.97 (1H, sep, J = 6.0 Hz, C-3 O*C*H(CH$_3$)$_2$), 3.76 (3H, s, C-4 OC*H*$_3$), 1.11 (6H, d, J = 6.0 Hz, C-3 OCH(C*H*$_3$)$_2$), 1.10 (6H, d, J = 6.5 Hz, C-2 OCH(C*H*$_3$)$_2$); ^{31}P NMR ($CDCl_3$, 202 MHz) δ 21.9; ESMS *m/z* (peak height) 499 (100, M^{+1}), 237 (15), 194 (25), 153 (25).

(*Z*)/(*E*)-1-[3′,5′-Dimethoxy-4′-[(*tert*-butyldimethylsilyl)oxy]phenyl]-2-[2″,3″-di-[(isopropyl)oxy]-4″-methoxyphenyl] ethene **41** and **42**, respectively**:** n-BuLi (2.0 M in hexanes, 19.20 mL, 38.30 mmol), was added dropwise to a well stirred solution of Wittig salt **40** (20.01 g, 34.54 mmol) in anhydrous THF (250 mL) at -10 °C. The reaction mixture was then cooled to -78 °C, and aldehyde **38** (9.30 g, 31.4 mmol) dissolved in

anhydrous THF (30 mL) was added dropwise. The reaction was stirred until the temperature gradually rose to 0 °C. The reaction was quenched by careful addition of H_2O (100 mL) followed by removal of THF *in vacuo*. The aqueous phase was extracted with Et_2O (3 x 200 mL), and the combined organic phases were washed with brine. After drying with Na_2SO_4, the solvents were removed under reduced pressure and the crude product obtained was subject to gravity column chromatography (silica gel, EtOAc:hexanes, gradient 0.5:99.5 to 2:98) to obtain the *Z*-isomer **41** (5.53 g, 10.70 mmol, 34% yield) as a off-white oil, which crystallized upon cooling to -20 ^{o}C, and the *E*-isomer **42** (10.0 g, 19.35 mmol, 62% yield) as a pale yellow oil, $R_{f(Z\text{-}isomer)}$ 0.45, $R_{f(E\text{-}isomer)}$ 0.33 (90:10 hexanes:EtOAc); *Z*-isomer **41**: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.90 (1H, d, *J* = 8.5 Hz, H-6″), 6.57 (1H, d, *J* = 12.0 Hz, H-5″), 6.45 (3H, m, H-2′, H-6′, H-2), 6.43 (1H, d, *J* = 12.0 Hz, H-1), 4.68 (1H, sep, *J* = 6.5 Hz, C-2″ OC*H*$(CH_3)_2$), 4.42 (1H, sep, *J* = 6.5 Hz, C-3″ OC*H*$(CH_3)_2$), 3.78 (3H, s, C-4″ OCH_3), 3.58 (6H, s, C-3′, C-5′ OCH_3), 1.29 (6H, d, *J* = 6.5 Hz, C-2″ OCH(C$H_3)_2$), 1.28 (6H, *J* = 6.5 Hz, C-3″ OCH(C$H_3)_2$), 0.99 (9H, s, C-4′ OSi$(CH_3)_2$C(C$H_3)_3$), 0.11 (6H, s, OSi(C$H_3)_2$C$(CH_3)_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 153.6 (C, C-4″), 151.1 (C, C-3′, C-5′), 150.4 (C, C-2″), 140.3 (C, C-3″), 133.3 (C,C-1′), 129.8 (C, C-4′), 129.2 (CH, C-1), 125.6 (CH, C-2), 125.4 (CH, C-6″), 124.8 (C, C-1″), 106.3 (CH, C-5″), 106.1 (CH, C-2′, C-6′), 75.1 (CH, C-3″ O*C*H$(CH_3)_2$), 74.8 (CH, C-2″ O*C*H$(CH_3)_2$), 55.8 (CH_3, C-4″ O*C*H_3), 55.4 (CH_3, C-3′, C-5′ O*C*H_3), 25.8 (CH_3, C-4′ OSi$(CH_3)_2$C(*C*$H_3)_3$), 22.5 (CH_3, C-2″ OCH(*C*$H_3)_2$), 22.4 (CH_3, C-3″ OCH(*C*$H_3)_2$), 18.7 (C, C-4′ OSi$(CH_3)_2$*C*$(CH_3)_3$), -4.7 (CH_3, C-4′ OSi(*C*$H_3)_2$C$(CH_3)_3$); HRMS (EI) *m/z* 516.2936 (calcd for $C_{29}H_{44}O_6Si$, 516.2907); *E*-isomer **42**: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.29 (1H, d, J = 8.6 Hz, H-6″), 7.27 (1H, d, *J* = 16.5 Hz, H-2), 6.87 (1H, d, *J* = 16.5 Hz,

H-1), 6.71 (2H, s, H-2′, H-6′), 6.68 (1H, d, J = 8.8 Hz, H-5″), 4.60 (1H, sep, J = 6.0 Hz, C-2″ OCH(CH_3)$_2$), 4.46 (1H, sep, J = 6.0 Hz, C-3″ OCH(CH_3)$_2$), 3.85 (3H, s, C-4″ OCH_3), 3.83 (6H, s, C-3′, C-5′ OCH_3), 1.30 (12H, d, J = 6.0 Hz, C-2″, C-3″ OCH(CH_3)$_2$), 1.02 (9H, s, C-4′ OSi(CH_3)$_2$C(CH_3)$_3$), 0.14 (6H s, C-4′ OSi(CH_3)$_2$C(CH_3)$_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 153.7 (C, C-4″), 151.7 (C, C-3′, C-5′), 150.0 (C, C-2″), 140.4 (C, C-3″), 134.1 (C, C-1′), 131.0 (C, C-4′), 127.3 (CH, C-1), 125.8 (CH, C-2), 122.4 (CH, C-6″), 119.8 (C, C-1″), 107.3 (C, C-5″), 103.6 (CH, C-2′, C-6′), 75.5 (CH, C-2″ O*C*H(CH_3)$_2$), 75.1 (CH, C-3″ O*C*H(CH_3)$_2$), 55.9 (CH_3, C-4″ O*C*H_3), 55.7 (CH_3, C-3′, C-5′ O*C*H_3), 25.8 (CH_3, C-4′ OSi(CH_3)$_2$C(*C*H_3)$_3$), 22.6 (CH_3, C-2″ OCH(*C*H_3)$_2$), 22.5 (CH_3, C-3″ OCH(*C*H_3)$_2$), 18.7 (C, C-4′ OSi(CH_3)$_2$*C*(CH_3)$_3$), -4.6 (CH_3, C-4′ OSi(*C*H_3)$_2$C(CH_3)$_3$); HRMS (EI) *m/z* 516.2893 (calcd for $C_{29}H_{44}O_6Si$, 516.2907).

*(Z)-1-[3',4',5'-Trimethoxyphenyl]-2-[2'',3''-di-[(isopropyl)oxy]-4''-methoxyphenyl] ethene **43***: The *Z*-isomer of cold CA1 precursor **41** (1.40 g, 2.71 mmol) was dissolved in anhydrous CH_3CN (20 mL), and the solution was cooled to 0 °C. CH_3I (0.67 mL, 10.8 mmol) was added and the reaction mixture was stirred for 10 minutes at 0 °C. To this solution was added tetrabutylammonium fluoride (TBAF) (2.98 mL, 2.98 mmol), and the resultant deep yellow colored reaction mixture was stirred for 10 minutes at 0 °C. H_2O (10 mL) was added and the product was extracted in EtOAc (3 x 50 mL). The combined organic phases were washed with brine, dried over anhydrous Na_2SO_4 and filtered. White crystals of tetrabutyl ammonium hydroxide by-product precipitated. These crystals were filtered and washed with additional ethyl acetate. The combined filtrates were then evaporated under reduced pressure to obtain the crude product, which was subjected to flash column chromatography (silica gel, 4:96 EtOAc:hexanes) to afford

product **43** (1.01 g, 2.42 mmol, 89% yield) as colorless oil, R_f 0.26 (90:10 hexanes:EtOAc); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.94 (1H, d, J = 8.6 Hz, H-6″), 6.64 (1H, d, J = 12.3 Hz, H-2), 6.49 (3H, m, H-2′, H-6′, H-5″), 6.43 (1H, d, J = 12.3 Hz, H-1), 4.70 (1H, sep, J = 6.2 Hz, C-2″ OC*H*$(CH_3)_2$), 4.43 (1H, sep, J = 6.2 Hz, C-3″ OC*H*$(CH_3)_2$), 3.82 (3H, s, C-4″ OC*H*$_3$), 3.79 (3H, s, C-4′ OC*H*$_3$), 3.64 (6H, s, C-3′, C-5′ OC*H*$_3$), 1.29 (12H, d, J = 6.2 Hz, C-2″, C-3″ OCH (C*H*$_3)_2$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 153.7 (C, C-4″), 152.6 (C, C-3′, C-5′), 150.3 (C, C-2″), 140.3 (C, C-4′), 136.8 (C, C-3″), 132.8 (C, C-1′), 128.7 (CH, C-1), 126.6 (CH, C-2), 125.1 (C, C-1″), 124.7 (CH, C-6″), 106.4 (CH, C-5″), 106.0 (C, C-2′, C-6′), 75.0 (CH, C-2″ O*C*H$(CH_3)_2$), 74.8 (CH, C-3″ O*C*H$(CH_3)_2$), 60.8 (CH_3, C-4′ O*C*H$_3$), 55.8 (CH_3, C-4″ O*C*H$_3$), 55.7(CH_3, C-3′, C-5′ O*C*H$_3$), 22.5 (CH_3, C-2″ OCH(*C*H$_3)_2$), 22.4 (CH_3, C-3″ OCH(*C*H$_3)_2$); *anal*. C 69.25%, H 7.78%, calcd for $C_{24}H_{32}O_6$, C 69.21%, H 7.74%; HPLC retention time 19.73 min.

(Z)-1-[3',4',5'-Trimethoxyphenyl]-2-[2'',3''-dihydroxy-4''-methoxyphenyl]ethene (Z-CA1) ***3***:[1, 19, 40] The *Z*-isomer **43** (1.01 g, 2.42 mmol) was dissolved in anhydrous CH_2Cl_2 (20 mL), and the solution was cooled to 0 °C. $TiCl_4$ (1.16 mL, 10.1 mmol) was added and the resultant dark brown colored reaction mixture was stirred for 40 min at 0 °C. The reaction mixture was quenched with H_2O and extracted with CH_2Cl_2 (2 x 50 mL). The combined organic phases were rinsed with brine and dried over Na_2SO_4. Removal of the solvent under reduced pressure followed by purification by flash chromatography (Silica gel capped with florisil, 40:60 EtOAc:hexanes) afforded pure *Z*-CA1 **3** as a pale yellow oil, which was crystallized with hexanes/EtOAc (50/50) yielding *Z*-CA1 **3** (0.36 g, 1.08 mmol, 45% yield) as tan colored crystals, R_f 0.15 (EtOAc:hexanes, 40:60); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.76 (1H, d, J = 8.6 Hz, H-6″), 6.59 (1H, d, J =

12.2 Hz, H-2), 6.54 (3H, m, H-1, H-2′, H-6′), 6.38 (1H, d, J = 8.8 Hz, H-5″), 5.40 (2H, s, C-2″, C-3″ O*H*), 3.86 (3H, s, C-4″ OCH_3), 3.83 (3H, s, C-4′ OCH_3), 3.67 (6H, s, C-3′, C-5′ OCH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 152.8 (C, C-3′, C-5′), 146.3 (C, C-4″), 141.6 (C, C-2″), 137.3 (C, C-4′), 132.6 (C, C-3″), 132.5 (C, C-1′), 130.3 (CH, C-1), 124.0 (CH, C-2), 120.3 (CH, C-6″), 117.8 (C, C-1″), 105.9 (CH, C-2′, C-6′), 102.9 (C, C-5′), 60.9 (CH_3, C-4′ O*C*H_3), 56.2 (CH_3, C-4″ O*C*H_3), 55.8 (CH_3, C-3′, C-5′ O*C*H_3); HPLC retention time 21.39 min.

(Z)-1-[3',4',5'-trimethoxyphenyl]-2-[2'',3''-di-[([bis-[(benzyl)oxy]] phosphoryl)oxy]-4''-methoxyphenyl] ethene ***44***:[1, 40, 41] *N*-Chlorosuccinimide (0.430 g, 3.24 mmol) was dissolved in anhydrous CH_3CN (10 mL). The reaction mixture was then heated to 40 °C and stirred at that temperature for 5 minutes. The heat source was removed and dibenzyl phosphite (DBP) (0.71 mL, 3.24 mmol) was added dropwise. The reaction mixture was then stirred for 3 h at room temperature. In a separate 100 mL dry round bottom flask, equipped with a stir bar, was charged CA1 **3** (360 mg, 1.080 mmol) followed by anhydrous CH_3CN (10 mL) and DMAP (13 mg, 0.10 mmol). The temperature of the reaction mixture was maintained between 10 to 20 °C, and anhydrous Et_3N (0.45 mL, 3.24 mmol) was added. The reaction mixture was then cooled to 0 °C, and the dibenzyl chlorophosphate solution was added slowly over a period of 5 to 10 min. The brown colored reaction mixture was then warmed to room temperature and stirred for 16 hours. The solvent was evaporated completely under reduced pressure using a rotary evaporator, followed by the addition of toluene (~15 mL). The solvent (toluene) was evaporated under reduced pressure and additional toluene (15 mL) was added. The precipitated succinimide by-product was filtered and washed with more toluene. The

combined filtrates were washed with 0.5 M KH_2PO_4 (2 x 10 mL), followed by 0.5 M NaOH (2 x 5 mL) and finally with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated to dryness. The crude reaction mixture was purified by flash column chromatography (silica gel, 40:60 EtOAc:hexanes) to obtain the tetrabenzyl phosphate derivative of CA1 **44** (0.39 g, 0.46 mmol, 42% yield) as an off-white oil. R_f 0.22 (EtOAc:hexanes, 50:50); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.24 (20H, m, C-2″, C-3″ OP(O)($OCH_2C_6H_5$)$_2$), 7.00 (1H, d, J = 8.7 Hz, H-6″), 6.67 (1H, d, J = 8.5 Hz, H-5″), 6.64 (1H, d, J = 12.0 Hz, H-2), 6.51 (1H, d, J = 12.0 Hz, H-1), 6.46 (2H, s, H-2′, H-6′), 5.09 (8H, m, C-2″, C-3″ OP(O)($OCH_2C_6H_5$)$_2$), 3.79 (3H, s, C-4″ OCH_3), 3.76 (3H, s, C-4′ OCH_3), 3.62 (6H, s, C-3′, C-5′ OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 152.8 (C, C-3′, C-5′), 151.6 (C, C-4″), 137.2 (C, C-2″), 135.9, 135.8 (C, C-2″, C-3″ OP(O)($OCH_2C_6H_5$)$_2$), 135.7 (C, C-4′), 135.6 (C, C-3″), 132.0 (C, C-1′), 131.6 (CH, C-1), 128.4, 128.3, 128.0, 127.8 (CH, C-2″, C-3″ OP(O)($OCH_2C_6H_5$)$_2$), 126.9 (CH, C-2), 124.6 (CH, C-6″), 124.4 (C, C-1″), 109.3 (CH, C-5″), 106.2 (CH, C-2′, C-6′), 70.0, 69.9, 69.8, 69.6 (CH_2, C-2″, C-3″ OP(O)($OCH_2C_6H_5$)$_2$), 60.8 (CH_3, C-4′ OCH_3), 56.4 (CH_3, C-4″ OCH_3), 55.9 (CH_3, C-3′, C-5′ OCH_3); ^{31}P NMR: ($CDCl_3$, 122 MHz) δ -5.3, -5.4; HPLC retention time 38.13 min.

(Z)-1-[3',4',5'-Trimethoxyphenyl]-2-[2'',3''-di-[(monopotassium)phosphate]-4''-methoxyphenyl] ethene (OX16C) ***47***:[41] The tetrabenzyl phosphate derivative of CA1 **44** (0.32 g, 0.38 mmol) was dissolved in anhydrous CH_3CN (10 mL), and the reaction contents were cooled to -10 °C. Trimethlysilyl bromide (TMSBr) (freshly distilled over CaH, 0.25 mL, 1.89 mmol) was added dropwise to the reaction mixture. The reaction mixture was stirred for 1.5 hours at -10 °C to afford the tetra-TMS-phosphate ester

derivative of CA1 **45**, which was added dropwise to a well-stirred solution of KOMe (260 mg, 3.78 mmol) in dry MeOH (4.5 mL) at -10 °C. The reaction mixture was gradually allowed to reach room temperature. and then stirred for additional 15 minutes at room temperature. The solvents were evaporated under reduced pressure (temperature of the H_2O bath was maintained below 35 °C) to obtain a dry off-white colored powder, which was dissolved in deionized H_2O (2 mL). The pH of the resultant solution was then carefully titrated to 4.75 to 4.85 using 1M HCl (subsequently switched to 0.1 M HCl once the pH of the reaction mixture reaches ~5.5) with good stirring. The reaction mixture was then filtered, and to the filtrate was added anhydrous EtOH (7 mL). OX16C **47** precipitated out as a white solid. The reaction mixture was stirred for 5 minutes. The solid separated was filtered, washed with anhydrous EtOH (2-4 mL), and dried (filter funnel, for about 15 to 20 minutes) to yield OX16C **47** (0.10 g, 0.16 mmol, 45% yield). ^{1}H NMR (D_2O, 300 MHz) δ 6.87 (1H, d, *J* = 8.7 Hz, H-6″), 6.74 (1H, d, *J* = 11.9 Hz, H-2), 6.68 (1H, d, *J* = 8.7 Hz, H-5″), 6.67 (2H, s, H-2′, H-6′), 6.64 (1H, d, *J* = 11.9 Hz, H-1), 3.83 (3H, s, C-4″ OC*H*$_3$), 3.74 (3H, s, C-4′ OC*H*$_3$), 3.68 (6H, s, C-3′, C-5′ OC*H*$_3$); ^{13}C NMR (D_2O, 75 MHz) δ 152.0 (C, C-3′, C-5′), 151.7 (C, C-4″), 143.5 (C, C-2″), 135.6 (C, C-4′), 134.3 (C, C-3″), 133.7 (C, C-1′), 129.9 (CH, C-1), 126.0 (CH, C-2), 125.1 (CH, C-6″), 124.0 (C, C-1″), 108.2 (CH, C-5″), 106.6 (CH, C-2′, C-6′), 60.8 (CH_3, C-4′ OCH_3), 56.1 (CH_3, C-4″ OCH_3), 55.9 (CH_3, C-3′, C-5′ OCH_3); ^{31}P-NMR (D_2O, 122 MHz) δ -3.0, -3.7; ESMS *m/z* (peak height) 491 (100, M^{-1}), 393 (40), 363 (15); HPLC retention time 17.29 min.

2,3-Di-[(tert-butyldimethylsilyl)oxy]-4-methoxybenzaldehyde 49:[1] To a well-stirred solution of 2,3-dihydroxy-4-methoxybenzaldehyde **17**[1] (4.99 g, 29.7 mmol) in

anhydrous CH_2Cl_2 (100 mL) was added successively triethylamine (12.4 mL) and DMAP (0.91 g, 7.43 mmol). The mixture was stirred for 10 min, followed by the addition of TBSCl (10.71 g, 71.32 mmol) in portions. The reaction mixture was stirred for 12 h at r.t, followed by the addition of H_2O (100 mL) and the product was extracted in CH_2Cl_2 (2 x 250 mL). The combined organic layers were washed with brine, and dried over Na_2SO_4. Filtration followed by removal of solvent under reduced pressure gave a tan colored solid. The solid was recrystallized with MeOH to afford the product **49** (7.07 g, 17.8 mmol, 60% yield) as pale yellow crystals, R_f 0.47 (EtOAc:hexanes, 15:85); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 10.22 (1H, s, C-1 C*H*O), 7.48 (1H, d, J = 9.0 Hz, H-6), 6.63 (1H, d, J = 8.5 Hz, H-5), 3.84 (3H, s, C-4 OCH_3), 1.04 (9H, s, C-2 OSi$(CH_3)_2$C(C$H_3)_3$), 1.0 (9H, s, C-3 OSi$(CH_3)_2$C(C$H_3)_3$), 0.14 (12H, s, C-2, C-3 OSi(C$H_3)_2$C$(CH_3)_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 189.2 (CH, C-1 *C*HO), 157.5 (C, C-4), 151.0 (C, C-2), 136.8 (C, C-3), 123.4 (CH, C-6), 121.4 (C, C-1), 105.4 (CH, C-5), 55.2 (CH_3, C-4 O*C*H_3), 26.2 (CH_3, C-2 OSi$(CH_3)_2$C(*C*$H_3)_3$), 26.0 (CH_3, C-3 OSi$(CH_3)_2$C(*C*$H_3)_3$), 18.7 (C, C-2 OSi$(CH_3)_2$*C*$(CH_3)_3$), 18.6 (C, C-3 OSi$(CH_3)_2$*C*$(CH_3)_3$), -3.84 (CH_3, C-2, C-3 OSi(*C*$H_3)_2$C$(CH_3)_3$).

2,3-Di-[(tert-butyldimethylsilyl)oxy]-4-methoxybenzyl alcohol ***50***: To a stirred solution of aldehyde **49** (5.30 g, 13.4 mmol) in anhydrous EtOH (50 mL) at 0 °C, was added $NaBH_4$ (0.50 g, 13.4 mmol) in portions. The reaction mixture was stirred for 30 min and terminated by cautious addition of H_2O (100 mL). Solvents were removed *in vacuo*, and the aqueous phases were extracted with EtOAc (3 x 150 mL). Combined organic phases were washed with brine, dried and filtered. The organic solvents were evaporated to dryness and the crude product crystallized with absolute EtOH to afford

product **50** as off-white crystals (5.30 g, 13.3 mmol, quantitative yield), R_f 0.49 (EtOAc:hexanes, 30:70); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.88 (1H, d, J = 8.5 Hz, H-6), 6.51 (1H, d, J = 8.0 Hz, H-5), 4.60 (2H, d, J = 5.5 Hz, C-1 CH_2OH), 3.76 (3H, s, C-4 OCH_3), 1.84 (1H, t, J = 5.5 Hz, C-1 CH$_2$O*H*), 1.04 (9H, s, C-2 OSi(CH$_3$)$_2$C(CH_3)$_3$), 0.98 (9H, s, C-3 OSi(CH$_3$)$_2$C(CH_3)$_3$), 0.14 (6H, s, C-2 OSi(CH_3)$_2$C(CH$_3$)$_3$), 0.10 (6H, s, C-3 OSi(CH_3)$_2$C(CH$_3$)$_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 151.9 (C, C-4), 145.2 (C, C-2), 136.8 (C, C-3), 126.2 (C, C-1), 120.6 (CH, C-6), 104.8 (CH, C-5), 61.6 (CH$_2$, C-1 *C*H$_2$OH), 54.9 (CH$_3$, C-4 O*C*H$_3$), 26.16 (CH$_3$, C-2 OSi(CH$_3$)$_2$C(*C*H$_3$)$_3$), 26.15 (CH$_3$, C-3 OSi(CH$_3$)$_2$C(*C*H$_3$)$_3$), 18.8 (C, C-2 OSi(CH$_3$)$_2$*C*(CH$_3$)$_3$), 18.4 (C, C-3 OSi(CH$_3$)$_2$*C*(CH$_3$)$_3$), -3.6 (CH$_3$, C-2 OSi(*C*H$_3$)$_2$C(CH$_3$)$_3$), -3.8 (CH$_3$, C-3 OSi(*C*H$_3$)$_2$C(CH$_3$)$_3$).

*2,3-Di-[(tert-butyldimethylsilyl)oxy]-4-methoxybenzyl bromide **51***: To a well-stirred solution of alcohol **50** (5.30 g, 13.4 mmol) in dry CH_2Cl_2 (100 mL) at 0 °C, was added PBr_3 (1.26 mL, 13.4 mmol). The reaction mixture was stirred for 6 h at 0 °C, followed by the addition of H_2O (100 mL). The organic layers were separated, and the aqueous phases were extracted with CH_2Cl_2 (2 x 250 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, and filtered. Benzyl bromide **51** (6.03 g, 13.06 mmol, 98% yield) was obtained as a tan colored solid, which needed no further purification, R_f 0.70 (EtOAc:hexanes, 40:60); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.94 (1H, d, J = 8.5 Hz, H-6), 6.50 (1H, d, J = 8.6 Hz, H-5), 4.53 (2H, s, C-1 CH_2Br), 3.76 (3H, s, C-4 OCH_3), 1.07 (9H, s, C-2 OSi(CH$_3$)$_2$C(CH_3)$_3$), 0.98 (9H, s, C-3 OSi(CH$_3$)$_2$C(CH_3)$_3$), 0.14 (6H, s, C-2 OSi(CH_3)$_2$C(CH$_3$)$_3$), 0.11 (6H, s, C-3 OSi(CH_3)$_2$C(CH$_3$)$_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 152.7 (C, C-4), 145.8 (C, C-2), 137.0 (C, C-3), 123.1 (CH, C-6), 122.9 (C, C-1), 105.0 (CH, C-5), 55.0 (CH$_3$, C-4 O*C*H$_3$), 30.8 (CH$_2$, C-1 *C*H$_2$Br), 26.2 (CH$_3$, C-2

$OSi(CH_3)_2C(CH_3)_3$), 26.1 (CH_3, C-3 $OSi(CH_3)_2C(CH_3)_3$), 18.7 (C, C-2 $OSi(CH_3)_2C(CH_3)_3$), 18.6 (C, C-3 $OSi(CH_3)_2C(CH_3)_3$), -3.6 (CH_3, C-2 $OSi(CH_3)_2C(CH_3)_3$), -3.8 (CH_3, C-3 $OSi(CH_3)_2C(CH_3)_3$).

*2,3-Di-[(tert-butyldimethylsilyl)oxy]-4-methoxybenzyltriphenylphosphonium bromide **52***: A mixture of bromide **51** (6.03 g, 13.1 mmol) and PPh_3 (3.77 g, 14.4 mmol) in dry CH_2Cl_2 (100 mL) was heated to reflux for 22 h. Solvent was removed under reduced pressure to afford an off-white solid. Et_2O (200 mL) was added to the solid and the resultant slurry was stirred for 1 h. The solid was filtered, and washed with Et_2O to obtain phosphonium bromide salt **52** (8.04 g, 11.1 mmol, 85% yield) as a white solid.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.75 (9H, m, C-1 $CH_2P(C_6H_5)_3$), 7.47 (6H, m, C-1 $CH_2P(C_6H_5)_3$), 6.68 (1H, dt, $J_{H\text{-}H}$ = 9.0 Hz, $J_{H\text{-}P}$ = 2.5 Hz, H-6), 6.32 (1H, d, J = 9.0 Hz, H-5), 5.21 (2H, dd, $J_{H\text{-}P}$ = 14.0 Hz, $J_{H\text{-}H}$ = 2.5 Hz, C-1 $CH_2P(C_6H_5)_3$), 3.68 (3H, s, C-4 OCH_3), 0.97 (9H, s, C-2 $OSi(CH_3)_2C(CH_3)_3$), 0.84 (9H, s, C-3 $OSi(CH_3)_2C(CH_3)_3$), 0.15 (6H, s, C-2 $OSi(CH_3)_2C(CH_3)_3$), -0.02 (6H, s, C-3 $OSi(CH_3)_2C(CH_3)_3$); ^{31}P-NMR ($CDCl_3$, 122 MHz) δ 21.3; ESMS *m/z* (peak height) 643 (100, M^{+1}).

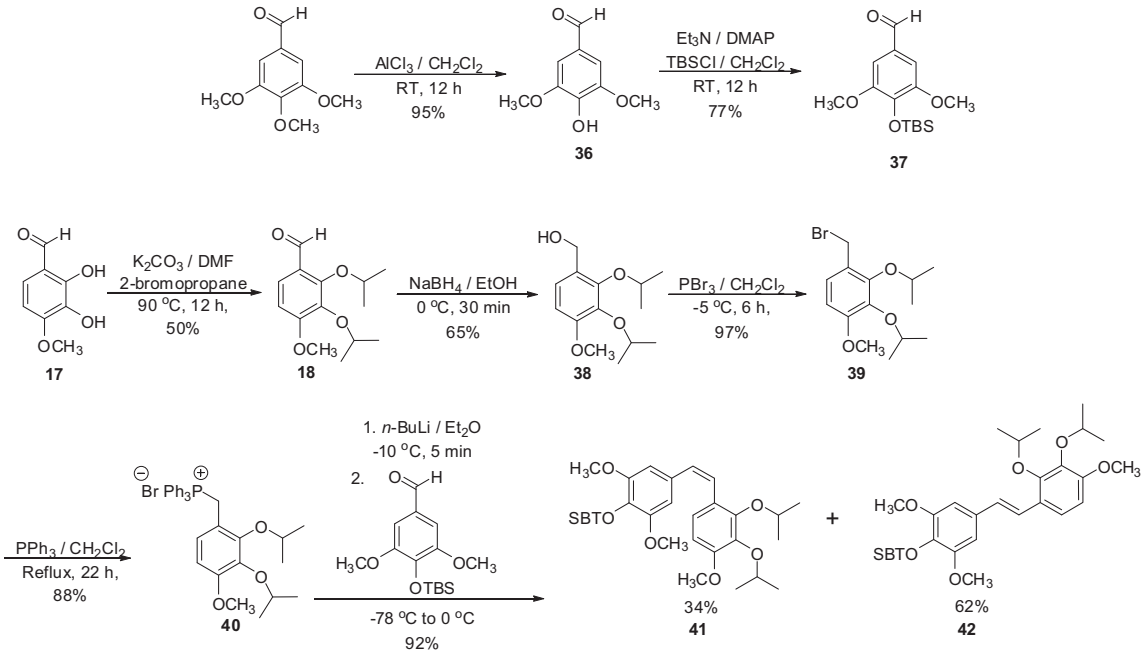
Results and Discussion

Note: The methodology presented herein utilized ^{12}C as a model system for future radiolabeling.

In order to successfully synthesize radiolabeled OX16C, the position to incorporate the ^{14}C-radioisotope needs to be determined. The position chosen was the C-4 methoxy functional group of the A-ring of OX16C. The center methoxy (4 position) of 3,4,5-trimethoxybenzaldehyde is readily demethylated easily in good yield to a phenolic

group. The starting material, 3,4,5-trimethoxybenzaldehyde contains the functionalized ring that will become the A-ring of OX16C after the Wittig reaction. Since there are two phenolic moieties present on the B-ring of OX16C, a suitable protecting group strategy was required in order to ultimately incorporate the ^{14}C-radioisotope specifically at the center methoxy (C-4) on the phenolic A-ring of OX16C. For these studies, ^{12}C was used as a model in place of the ^{14}C isotope.

The project began with the scale up of components used in the Wittig reaction: the Wittig salt and the Wittig benzaldehyde (Scheme 11). In the Wittig reactions previously reported to synthesize CA1P and OX16C,[1, 19, 42] 3,4,5-trimethoxybenzaldehyde, which is commercially available, was used as the Wittig benzaldehyde. Selective demethylation is required followed by protection of the phenolic moiety. 3,4,5-Trimethoxybenzaldehyde was demethylated using 2 equivalents of $AlCl_3$ to give 4-hydroxy-3,5-dimethoxybenzaldehyde **36** in a 95% yield.



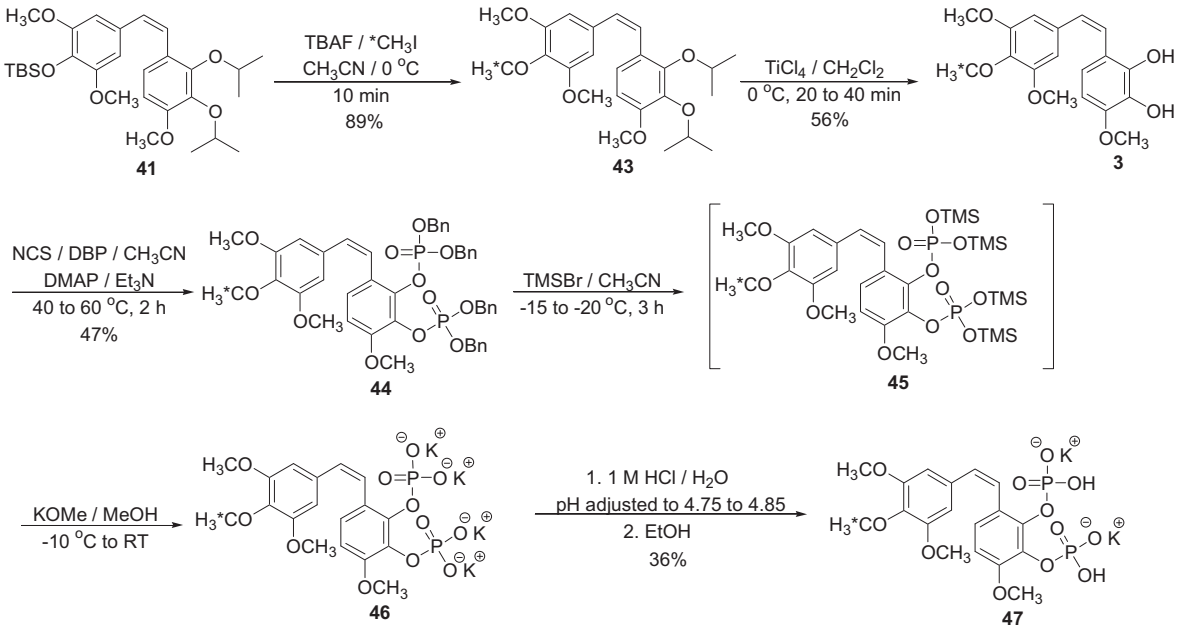
Scheme 11. Synthesis of intermediate **41**

Installation of the *tert*-butyldimethylsilyl (TBS) protecting group afforded the protected benzaldehyde **37** in a 77% yield. The TBS protecting group was chosen since its removal to allow incorporation of a radioisotope at a later stage of the synthesis was anticipated to be facile.

A scale up synthesis of the Wittig salt involved protection of 2,3-dihydroxy-4-methoxybenzaldehyde **17** using a slight excess of 2-bromopropane to give the diisopropyl protected benzaldehyde **18** in a 50% yield. Reduction of aldehyde **18** using $NaBH_4$ afforded the corresponding benzyl alcohol derivative **38** with a 65% yield. Benzyl alcohol **38** was treated with PBr_3 to yield benzyl bromide **39** in a 97% yield. The reaction was carefully monitored by TLC. If the reaction is left stirring for over six hours, polymers may form and the resulting sludge will be insoluble in aqueous and organic solvents. Benzyl bromide **39** was refluxed with PPh_3 in CH_2Cl_2 for 22 hours resulting in the phosphonium bromide salt **40** (Wittig salt).

The (*Z*)-stilbene **41** (34% yield) was prepared through a Wittig reaction utilizing phosphonium bromide salt **40** and aldehyde **38.** The isomeric (*E*)-stilbene **42** was obtained in a 62% yield and the two isomers were readily separated by flash column chromatography (Scheme 11). The (*Z*)-stilbene **41** is also known as the "(*Z*)-cold precursor" because it ultimately will be synthetically manipulated to incorporate the radioisotope. It is important to note that this synthetic methodology was completed with ^{12}C.[20] Ultimately, a ^{14}C-radiosiotope has been incorporated into the radiolabeled OX16C and has been reported as a collaborative project between the Pinney group and Almac Sciences.[31, 32] The cold precursor **41** has a TBS protecting group on the A ring, whereas the B ring has two isopropyl protecting groups. The overall strategy was to deprotect the

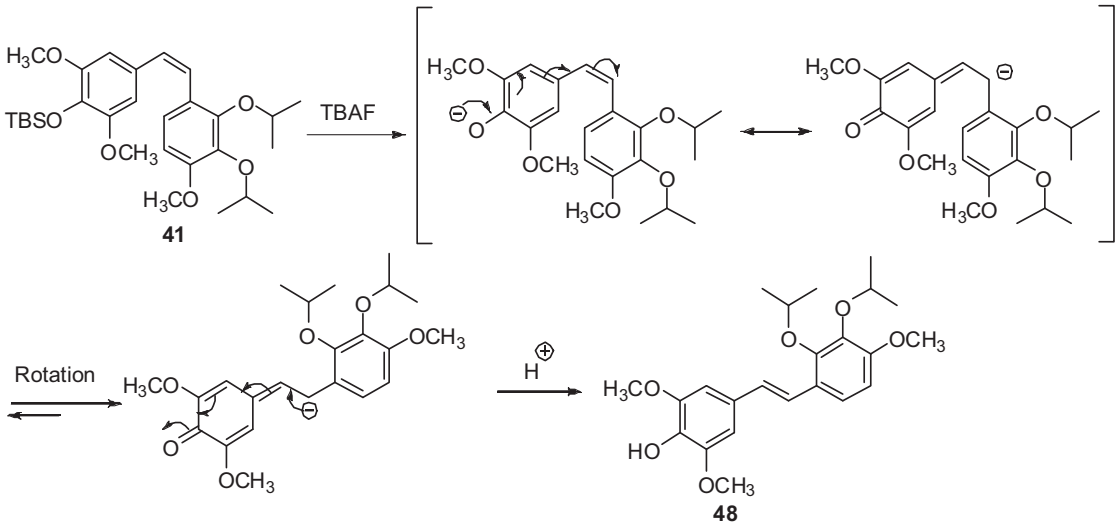
TBS group and methylate the resulting phenylate by treatment with methyl iodide (MeI). For the initial methodology development, the MeI used does not contain a ^{14}C-radiosiotope, however, this synthetic step would be the step to introduce such an isotope (Scheme 12).



Scheme 12. Synthesis of radiolabeled OX16C **47**

Interestingly, TBS deprotection of the cold precursor **41** with TBAF, followed by isolation and purification of the resulting phenol afforded the *E* isomer rather than the *Z* isomer. By deprotecting the TBS group and isolating the product, this gave the deprotected cold precursor an opportunity to isomerize into its more stable (*E*)-stilbene **48** (Scheme 13) via the phenolate anion. The solution was to deprotect and methylate the cold precursor in the presence of the methylating agent. Dr. Anupama Shirali (from the Pinney Group) was the first to develop this particular strategy to prevent isomerization during deprotection via the phenolate anion.[33] The cold precursor **41** was deprotected using TBAF in the presence of MeI to form the methylated (*Z*)-stilbene in an 89% yield.

The rate of methylation is faster than the isomerization pathway. After methylation on the A-ring, the diisopropyl groups on the B-ring of analog **43** were deprotected using $TiCl_4$ from Aldrich Chemical Co. to form (*Z*)-CA1 **3** in 56% yield. The purity of the $TiCl_4$ used in the deprotection greatly affected both the reaction time and overall yield for this step.

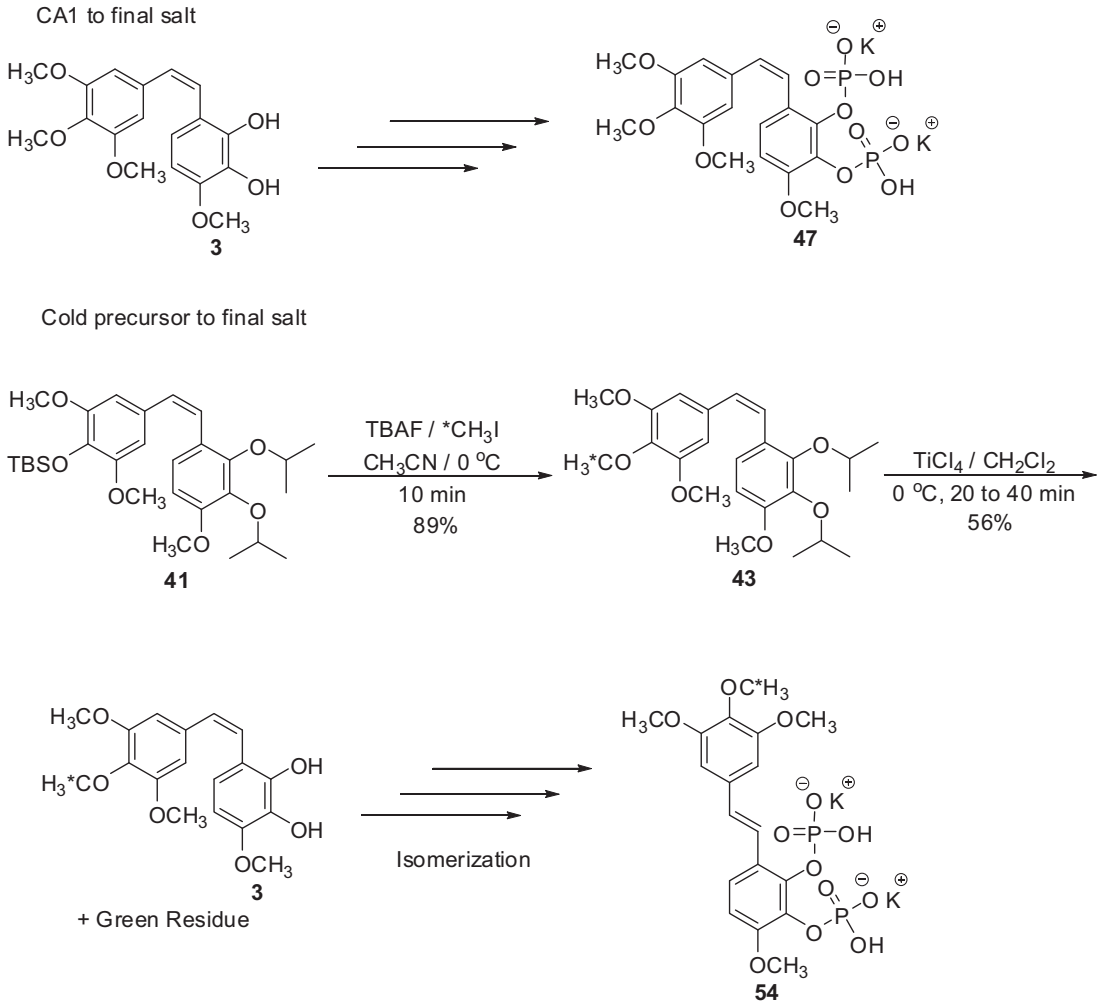


Scheme 13. Isomerization of **41** during deprotection with TBAF

A new, unopened bottle of $TiCl_4$ of 99.999% purity allowed the reaction to be complete between 20 to 40 minutes resulting in good yields with no isomerization during the reaction. Bottles of $TiCl_4$ from Acros were not as robust and low yields and isomerization was observed. It is important to note that after removal of the isopropyl protecting groups, it is imperative to remove any traces of titanium that remain during the deprotection using $TiCl_4$. It was observed that if titanium byproducts were not carefully removed, isomerization (Z to E) took place. This isomerization is attributed to complexation of the titanium to compound **41**. Due to a previous concern, Dr. Madhavi Sriram used CA1 that was prepared through traditional synthetic methodolgy[1, 41] and

converted it to its phosphate salt, CA1P, using the salt formation methodology proposed for this project. But to be absolutely sure that this methodology would be successful once the radioisotope was incorporated, the CA1 **3** derived from the cold precursor **41** was converted to the phosphate salt and surprisingly, a nearly quantitative isomerization (Z to E) of the final salt was observed. This posed a problem for the proposed synthetic methodology of the radiolabeled OX16C. The reactions leading up to the cold precursor were not problematic. The reaction protocol from the synthesis of cold precursor to the final phosphate salt was reviewed. After the isopropyl deprotection of compound **43** followed by the purification of CA1 **3**, the product had traces of green residue. This CA1 **3**, with the green residue, when taken to the final salt resulted in the fully isomerized (*E*)-isomer salt **54** (Scheme 14). In an attempt to purify the CA1 **3** after deprotection of stilbene **43** and remove any traces of this green residue, flash column chromatography was conducted using a cap of Florisil (3-4 inches) at the top of the column above the silica gel layer. The Florisil trapped the green residue and the pure, green residue free CA1 **3**, was taken to the final salt without any isomerization. It is noted that the green residue contains titanium impurities from the $TiCl_4$ used to deprotect the isopropyl groups of stilbene **43**. It is suggested that these titanium impurities complexed with the oxygen on the phenolic moieties of the B-ring of CA1 **3** or with the silbene bridge of CA1 **3** and when further reactions were conducted on CA1 **3** allowed for isomerization of the molecule (Scheme 14). For subsequent reactions, a control reaction was run to compare both the traditional synthesis of CA1P[1] against the proposed synthetic route in synthesizing OX16C from the cold precursor **41** and determine if any other problems may occur. The control reaction followed the same synthetic steps to prepare CA1P[1], but

its starting reagents, most notably the starting aldehyde prior to the Wittig reaction, did not undergo demethylation / TBS protection and deprotection / methylation with TBAF in the presence of MeI. The control reaction began with CA1 prepared using synthetic methodology previously reported (see Chapter 3).[1] After deprotection using $TiCl_4$, CA1 **3** was isolated using flash column chromatography with silica gel capped with Florasil to remove any potential titanium impurities.



Scheme 14. Control CA1 and cold precursor CA1 reactions to the final salt and evidence of isomerization

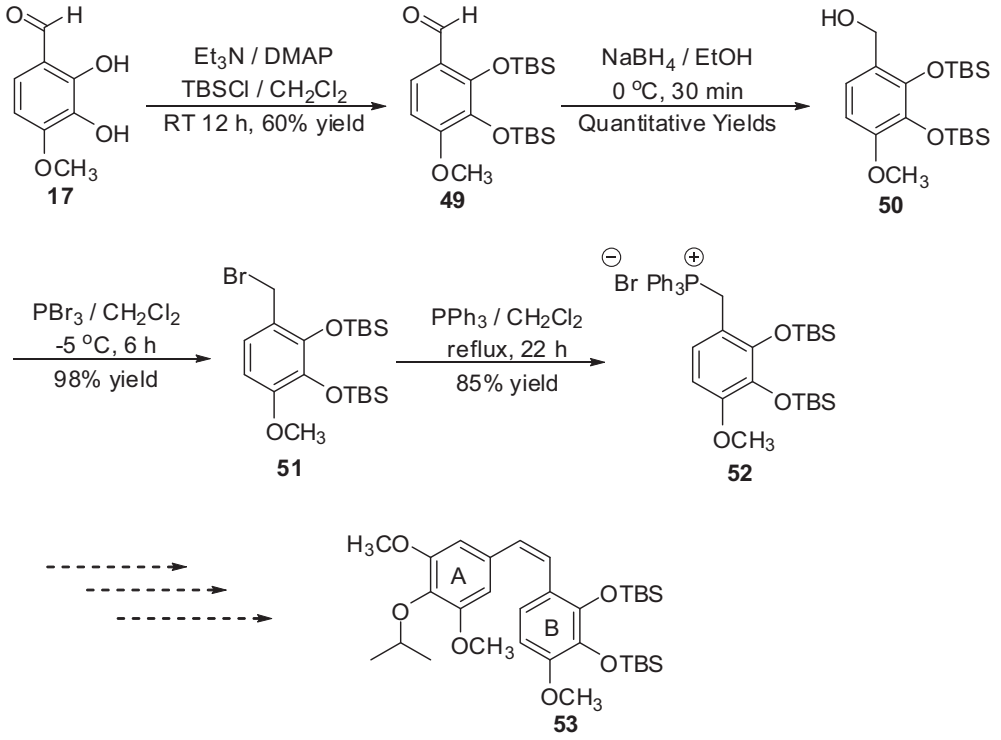
If the CA1 was not purified with the capped Florasil, a significant amount of isomerization was observed when forming the final salt **47**. CA1 **3** was converted to the tetrabenzylphosphate **44** using *n*-chlorosuccinamide (NCS) and dibenzyl phosphite with a moderate yield of 46% (Scheme 12). Normally for dibenzyl phosphorylation, carbon tetrachloride (CCl_4) is used, but if there is any chance that the final salt **47** will be used in biological studies, NCS will be used instead of CCl_4 because it is less toxic and less harmful to the environment than CCl_4 and it is desirable not to have any traces of CCl_4 in the final salt **47**. The tetrabenzylphosphate **44** was debenzylated using freshly distilled TMSBr to afford the tetra-TMS phosphate-ester **45** followed by quenching of the reaction in a solution of potassium methoxide (KOMe) and MeOH (Scheme 12). This afforded the tetrapotassium salt **46** as well as other mono-, di-, and tri-potassium salts. The reaction was concentrated under reduced pressure to give a white solid in the reaction flask. This solid was dissolved in a minimal amount of water and the aqueous solution was titrated with 1M HCl and carefully monitored using a pH meter with good stirring to achieve a pH between 4.75 to 4.85. The solution was filtered and to the filtrate was added anhydrous EtOH to precipitate out the final di-potasium salt **47** (Scheme 12).

Both the control reaction and the reaction started with cold precursor **41**, produced the desired salt **47**. When the reaction was conducted on a small scale, purification of CA1 **3** after deprotection using $TiCl_4$ removed most or all of the titanium impurities. When carried further to the final salt, no isomerization occurred. When the reaction was scaled up into multiple gram synthesis, simple flash column chromatography after the deprotection of isopropyl groups using $TiCl_4$ was not sufficient in removing all of the titanium impurities, therefore, when the reactions were carried on

further, isomerization was observed (55-95% conversion). When the CA1 **3** after isopropyl deprotection using $TiCl_4$ was subjected to flash column chromatography capped with 2-3 inches of Celite™, the titanium impurities were trapped within the Celite™ layer. This product was taken to the final salt **47** and no isomerization was observed.

For the dibenzyl phosphorylation step and further subsequent steps, ^{31}P NMR was required to check and see if the phosphorous was present after the product was purified from the reaction. On one occasion, the phosphorus peaks for the ^{31}P NMR was not detectable, especially for the final salt **47**. This was both perplexing and frustrating, since the ^{1}H NMR was correct yet not phosphorus peaks were present in the ^{31}P NMR. Ultimately, the cause was attributed to trace amounts of paramagnetic metals, mostly iron, that may have come from oven-dried reusable stainless steel needles which were used to transfer reagents. This was unanticipated. To the NMR sample of the final salt **47** that did not show any phosphorus peaks was added minute amounts of (ethylenedinitrilo)tetraacetic acid (disodium salt dihydrate) (EDTA Disodium salt) as a chelating agent to trap the trace amounts of iron.[43] The sample was filtered and reanalyzed by both ^{1}H and ^{31}P NMR which resulted in two phosphorus peaks for the ^{31}P NMR corresponding to the two phosphate salts for OX16C **47**. While attempting to discover a solution for the isomerization of OX16C in the final steps of the salt formation, various alternative routes were being tested just in case the initial synthetic methodology was not successful. One particular alternative route was to scale up Wittig salt **52,** which uses a different protecting group (TBS) instead of isopropyl protecting groups in the original methodology. Basically, for the target product **53**, the alternative

route switched the protecting groups on both the A and B rings; isopropyl protecting group on the A ring and TBS protecting groups on the B ring (Scheme 15).



Scheme 15. Alternative route to synthesize radiolabeled OX16C[1, 19, 44]

2,3-dihydroxy-4-methoxybenzaldehyde **17** was protected using TBSCl to afford the di-TBS benzaldehyde **49** in a 60% yield. The aldehyde **49** was reduced to a benzyl alcohol **50** using $NaBH_4$ in quantitative yield. The benzyl alcohol **50** was converted to the benzyl bromide **51** using PBr_3 in 98% yield and then further refluxed in the presence of PPh_3 to afford the triphenylphosphonium bromide **52** (Wittig salt) in an 85% yield. For the alternative route, another colleague, Dr. Madhavi Sriram, obtained the Wittig salt **52** and utilized the compound in the synthesis of the cold precursor **53**.

In conclusion, the strategic development of the synthesis of radiolabeled OX16C was successful. Even though an actual radiolabel was not utilized during the synthesis,

the strategy for inserting a radiolabel was developed. Future incorporation of the radiolabel will be conducted by Almac sciences.

CHAPTER THREE

Synthesis of Vascular Disrupting Agents

Introduction

Cancer may affect every person in some part of their lives, as unfortunate as that may sound; be it their friends, family, they themselves, or people they know of may have the horrendous illness. According to the World Health Organization (WHO), cancer is one of the leading causes of deaths in 2007 with over 7.9 million deaths, which is 13% of all deaths worldwide.[45] In the United States for the 2009 year, it is expected that over 550,000 people will die of cancer with about 1,500 people dying each day from this disease.[46] It is the second most common cause of death in the United States, only succeeded by the number one killer, heart disease. The death rate from this disease is so common, that about 1 in every 4 deaths is from cancer in the U.S.[47] There has been a continuous scientific investigation to understand the molecular and cellular biology of cancer.

Cancer is the uncontrolled growth of abnormal cells.[45-47] Under normal conditions, cells will replicate through the cell cycle, grow and eventually die and be replaced by new cells. Under extreme circumstances, these cells that are growing and replicating may become abnormal and may not die off as they are programmed to do but instead, continue replicating uncontrollably, making more abnormal cells which may become the start of cancer. This group of cancerous cells can grow, become a tumor, invade and destroy the surrounding cells, and eventually, metastasize, and spread cancer throughout the entire body.[46, 47] There are both internal and external

factors that may lead to cancer. Cancer may be hereditary and is an internal factor, caused from within the body. There is no solid evidence that this may be the case but it is still being debated if there is a certain cancer gene. This gene may mutate, or cause mutations, especially mutations in genes that control cell replication and cell repair and destruction that can initiate the cancer. Some external factors, that can cause cancer outside of the body, include UV radiation from the sun, which can damage the skin and DNA and cause mutations within the cell or exposure to carcinogenic chemicals and materials such as tobacco products and most notably, asbestos. Even viruses, such as the Human Papilloma virus (HPV) can cause cervical cancer in women and genital warts on men, which can also become cancerous.[45]

Leading Sites of New Cancer Cases and Deaths -2010 Estimates			
Estimated New Cases		Estimated Deaths	
Male	Female	Male	Female
Prostate	Breast	Lung And Bronchus	Lung And Bronchus
217,730 (28%)	207,090 (28%)	86,220 (29%)	71,080 (26%)
Lung And Bronchus	Lung And Bronchus	Prostate	Breast
116,750 (15%)	105,770 (14%)	32,050 (11%)	39.840 (15%)
Estimates from American Cancer Society at cancer.org			

Figure 4. Estimated leading types of new cancer cases and deaths for 2010[46]

According to the American Cancer Society, lung and bronchus cancer is highest in terms of estimated deaths in 2010 for both men and women. Tobacco products, especially cigarettes, may be the leading cause of cancer. For gender specific cases, breast cancer has risen to the top in the number of new cases for women while prostate cancer is number one in the number for new cases for men in 2009. Both gender specific

cancers surpass lung and bronchus cancer in the number of new cases for 2010.[46] Due to the increasing number of new cases of cancer each year, cancer research has become very important in cancer prevention and cancer treatment.

Cancer research has progressed throughout the years. Early cancer detection, better cancer treatments, and better understanding and knowledge of cancer have led to an improvement in the quality of life for those who have cancer. Today, there are precautions that can be taken to prevent cancer. A HPV vaccine is available to prevent cervical cancer. By knowing what can cause cancer, changes in lifestyles can be made to prevent cancer, such as not smoking, wearing proper safety equipment when working with cancer suspect agents, or reducing exposure to harmful UV light from the sun to prevent skin cancer.[45-48] The treatment of cancers has advanced tremendously. Cancers were originally excised from the body through surgery, and treatments further progressed to include chemotherapy and radiation therapy. Today, all three types of treatment, surgery, chemical, and radiation therapies are used in conjunction and are referred to as combination therapies. Unfortunately, chemotherapy and radiation therapy are not selective and target specific. These therapies can't differentiate between normal healthy cells and tumor cells. Another unfortunate drawback is the side effects due to dosage related toxicity. Chemotherapy can be accompanied by nausea and vomiting, hair loss, loss of appetite, fatigue, and anemia as some of the potent side effects.[49] Radiation therapy causes the skin in the area of treatment to burn, hair loss in the treatment area, nausea, fertility issues, fatigue and even carcinogenic effects, since radiation is one of the causes of cancer.[49] Drug resistance is also a problem for cancer treatments. The initial dosage of the cancer treatment may affect the cancer cells drastically, but over time, these

cancer cells may become resistant.[50] Increasing the dosage may work but eventually, the maximum tolerated dosage will be reached and a new cancer treatment may need to be applied.

The physiology of tumor cells may make it difficult for treatment of the cancer through chemotherapy and radiation therapy. As tumors grow to a certain size, they begin to develop their own vasculature system instead of depending on the established vasculature of healthy cells surrounding the tumor.[51, 52] The tumor requires its own vasculature to sustain itself with nutrients and oxygen. However, the development of the new tumor vasculature is neither structured, nor organized. Due to the tumor's rapid and chaotic growth of vasculature, there will be regions of the tumor that are hypoxic and deprived of both nutrient and most importantly, oxygen.[53] The poorly nourished hypoxic tumor microenvironment limits the effectiveness of both chemotherapy and radiation therapy. Hypoxic tumor cells are generally resistant to radiation therapies.[54] They tend to require a three times higher dose of radiation to inflict damage compared to well oxygenated cells.[55] Even some chemotherapies are not immune to drug resistance due to hypoxic tumor cells. A chemotherapeutic agent known as bleomycin, requires oxygen in its mechanism to damage tumors.[11] It relies on oxygen for free radical production used to damage the tumor cells. Other chemotherapeutic agents that target the cell cycle may also be affected by hypoxic tumor cells. These agents that target the cell cycle require rapidly proliferating cells.[11] Tumor cells under hypoxic conditions undergo a slow and decreased rate of cell division therefore, these chemotherapeutic agents are not highly effective.[56, 57] Due to cancer's damage resistance and resilience in surviving against chemotherapy and radiation therapy, it is imperative that alternative methods and

mechanisms to target cancer be sought and utilized in the ever growing battle against cancer.

Solid tumors make up over 90% of known human cancer types. These tumors begin as a single mutated cell, but will undergo cell division and grow rapidly until they reach the size of about 1-2 mm.[51] This is considered the critical size for a tumor. For it to grow any further, it needs to undergo neovascularization and develop its own tumor vasculature. Denekamp and coworkers, in the 1980s, observed that by blocking the blood supply to solid tumors in mice, the tumor would suffer severe regression.[58, 59] Further observations concerning the tumor vasculature led to the discovery that the tumor vasculature differs from normal vasculature. Normal vasculature is organized and provides ideal conditions for all the normal cells that surround the vasculature. On the other hand, tumor vasculature's structure and function is poor and its angiogenesis is chaotic and disorganized.[53, 60] The differences between normal and tumor vasculature have led to the development of a new concept of cancer treatment called vascular targeting agents (VTAs).[61] VTAs can be divided into two classes, anti-angiogenic agents and vascular disrupting agents (VDAs).[62, 63]

Tumors, over the size of 2 mm, require the development of a tumor vasculature in order to obtain oxygen and nutrients necessary for continued growth.[51] In order for angiogenesis to occur for the development of tumor vasculature, the tumor releases certain angiogenic factors to stimulate proliferation of endothelial cells and migration to form new tumor vasculature.

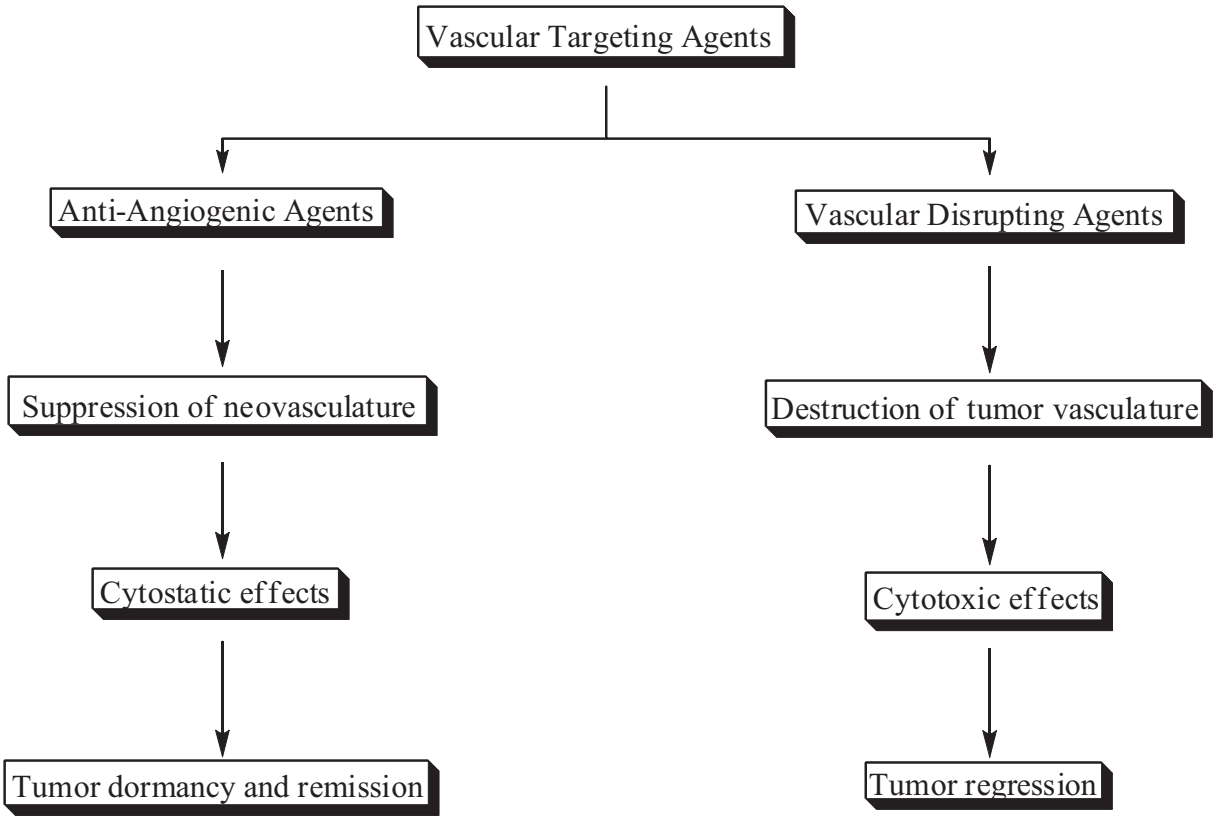


Figure 5. Two different classes of VTAs: anti-mitotic agents and vascular disrupting agents (VDAs)[62] Reprinted from *Drug Resist. Updates, 7*, Eichhorn, M. E.; Strieth, S.; Dellian, M. 125-138., Copyright 2004, with permission from Elsevier.

Some of these angiogenic factors are plasminogen activator, metalloproteinases, growth factors such as vascular endothelial growth factor (VEGF), and adhesion molecules.[52] Within the host's vasculature, there are naturally occurring anti-angiogenic factors. These anti-angiogenic factors ensure homeostasis, preventing unnecessary neovasculaturation. When the tumor angiogenic factors overcome the naturally occurring anti-angiogenic factors, angiogenesis for the tumor occurs and tumor vasculature is formed.[63, 64] Therefore, anti-angiogenic agents have been developed to target one of these several angiogenic factors in order to impede and prevent neovasculaturization from occurring. Several anti-angiogenic agents include endostatin, thalidomide, bevacizumab,

and ZD6474. These anti-angiogenic agents bind to specific angiogenic factors and inhibit a key step in angiogenesis, preventing neovasculaturization.[65, 66] Without the growth of new blood vessels for the tumor, cytostatic effects are observed. The tumor may not grow further, but it is still present within the host. The tumor may even become dormant or undergo remission, which can improve the quality of life or even extend life expectancy. Unfortunately, anti-angiogenic agents do not have many effects on existing tumor vasculature or the tumor cells themselves.

While one class of vascular targeting agent prevents the formation of new blood vessels, the other class, vascular disrupting agents (VDAs), targets the existing tumor blood vessels, and damages and destroys this tumor vasculature, eventually starving the tumor of its necessary nutrients and oxygen, leading to tumor regression and tumor cell apoptosis. In the late 1930's, colchicine, a tubulin binding agent that is also a natural product, was reportedly used to treat patients that went through consecutive biopsies. The patients treated with colchicine expressed significant tumor necrosis.[67] Unfortunately, colchicine was observed to have drastic side effects such as nausea, neuropathy, myocardio infarction, and toxicity comparable to arsenic poisoning.[67] Over the past 20 years, new vascular disrupting agents have been developed that are tubulin-binding agents that target tubulin within the endothelial cells of the tumor vasculature.

The tubulin-microtubule protein system plays an important role during the cell division process. This makes tubulin a viable target for VDAs. Microtubules are long hollow cylinders that are 25 nm in diameter and are made up of tubulin heterodimers. Tubulin is comprised of α and β subunits.[68-72] The tubulin-microtubule protein system has three binding sites, for which the different classes of tubulin-binding VDAs are

appropriately named, colchicine, vinca alkaloid, and taxane binding sites. These tubulin heterodimers attach together in a head to tail fashion to form polar protofilaments. These long strands of protofilaments, 13 in all, come together to form a hollow tube called a microtubule (Figure 6). This is the initiation of tubulin polymerization.[73]

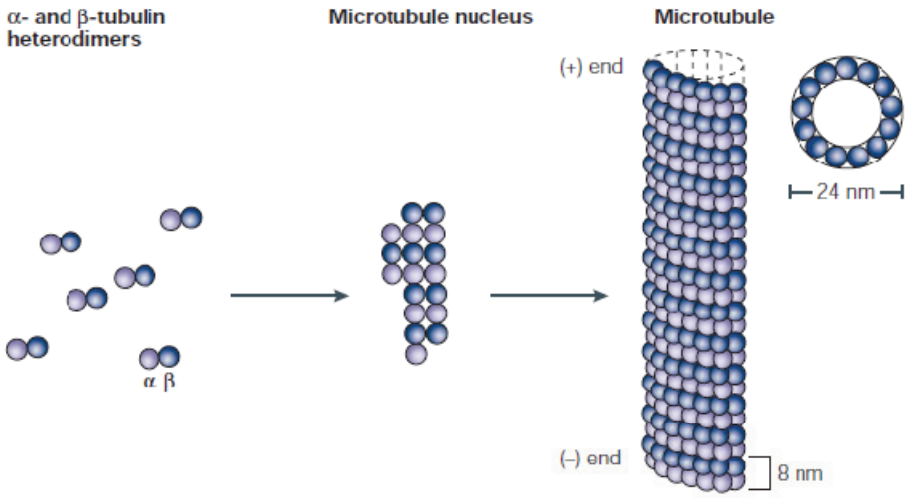


Figure 6. Tubulin polymerization[73] Reprinted by permission from Macmillan Publishers Ltd: *Nat. Rev. Cancer 4*, 253-265., copyright 2004.

Tubulin polymerization and depolymerization is a dynamic process. The first dynamic process is known as dynamic instability. The mictrotubule ends, both the positive and negative ends, switch between polymerization and depolymerization.[72, 73] During polymerization, the microtubules grow up to a certain point. Once this point is reached, the system switches to rapid disassembly known as catastrophe. After catastrophe, the rescue phase initiates and the microtubule resumes normal growth.[73] This is one of the dynamic processes that is continually occurring for tubulin polymerization and depolymerization. The other dynamic process is known as treadmilling. Treadmilling is where one end of the microtubule grows and the other end shortens at an equal rate. Through treadmilling, there is a flow of tubulin from the

positive end of the microtubule towards the negative end of the microtubule as this dynamic process occurs.[73, 74]

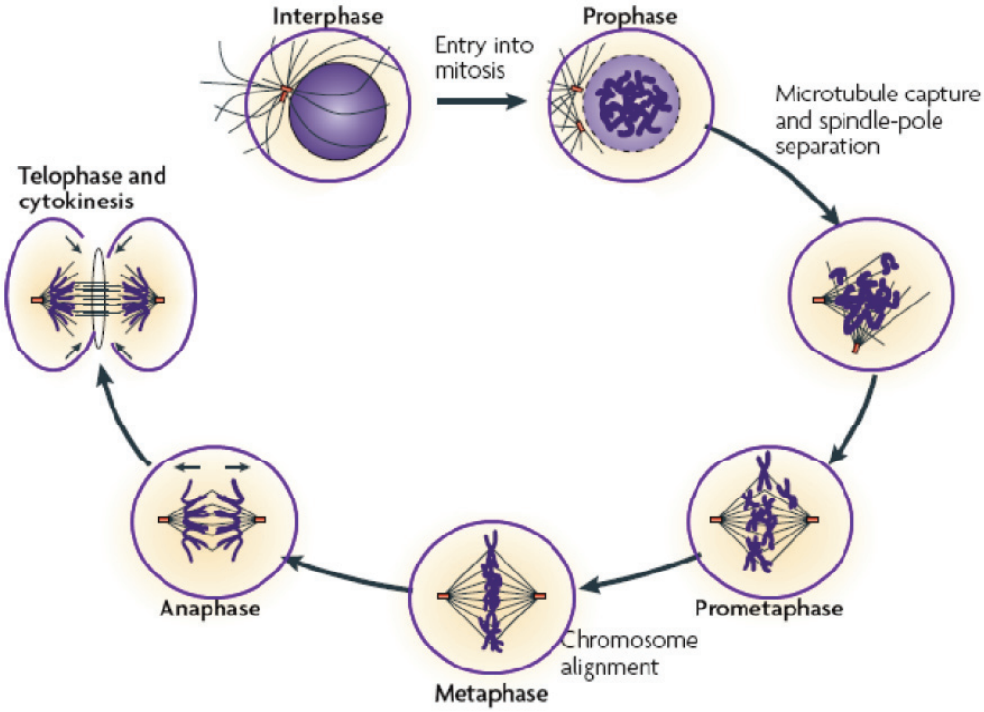


Figure 7. The phases of mitosis[75] Reprinted by permission from Macmillan Publishers Ltd: *Nat. Rev. Cancer, 7*, 107-117., copyright 2007.

Mitosis, especially for cancer cells, progresses rapidly and these highly dynamic microtubules are required as spindles during all stages of mitosis (Figure 7). In the interphase and prophase stages of the cell cycle, the microtubules restructure themselves and begin the polymerization and depolymerization process. These new microtubules that form are 4 to 100 times more dynamic than their counterpart microtubules found in the cell cytoskeleton.[73] During the phase called prometaphase, which is between prophase and metaphase, the microtubules attach themselves to the chromosomes at their kinetochores followed by alignment of the chromosomes during metaphase.[73, 75] For the microtubules to become attached to the kinetochores of the chromosome, it is necessary

for the microtubules to grow at a fast rate and then completely shorten, then grow at an even faster rate, and shorten again until it reaches the chromosome and attaches to the kinetochore.[73] For anaphase of the mitosis cycle, the microtubules undergo a unique and balanced shortening and growth process so that it can separate each half of the chromosome and pull each half to the opposite ends at the spindle poles.[75] Once the chromosomal halves are at the spindle poles, telophase and cytokinesis occurs producing two identical daughter cells.[75] It is vital to understand the role of microtubules during mitosis. If the microtubule spindles fail to attach to even one chromosome during mitosis, the cell will be trapped at the prometaphase and further phases of mitosis will not occur, therefore cell division will be halted and eventually apoptosis will occur. Tubulin-binding molecules have been developed to bind to the tubulin existing on the microtubule or bind to soluble tubulin. Binding to tubulin will disrupt the tubulin-microtubule dynamics and possibly interrupt mitosis. Tubulin-binding agents are categorized according to their binding sites: colchicine, vinca alkaloid, and taxane (Figure 8).[76]

Vincristine and vinblastine are two naturally occurring vinca alkaloids that were isolated from the leaves of *Catharanthus roseus* in the 1950s (Figure 9).[73] These vinca alkaloids exhibit anti-mitotic properties. Vinca alkaloids bind to the vinca binding domain of tubulin on the β subunit.[76] They bind to both soluble tubulin as well as the microtubules, subsequently causing the depolymerization of microtubules and destroying the mitotic spindles during mitosis.[77]

Currently, vincristine and vinblastine are in clinical use to treat a variety of cancers[75] including lymphoma, testicular cancer, leukemia and small-cell lung cancer.

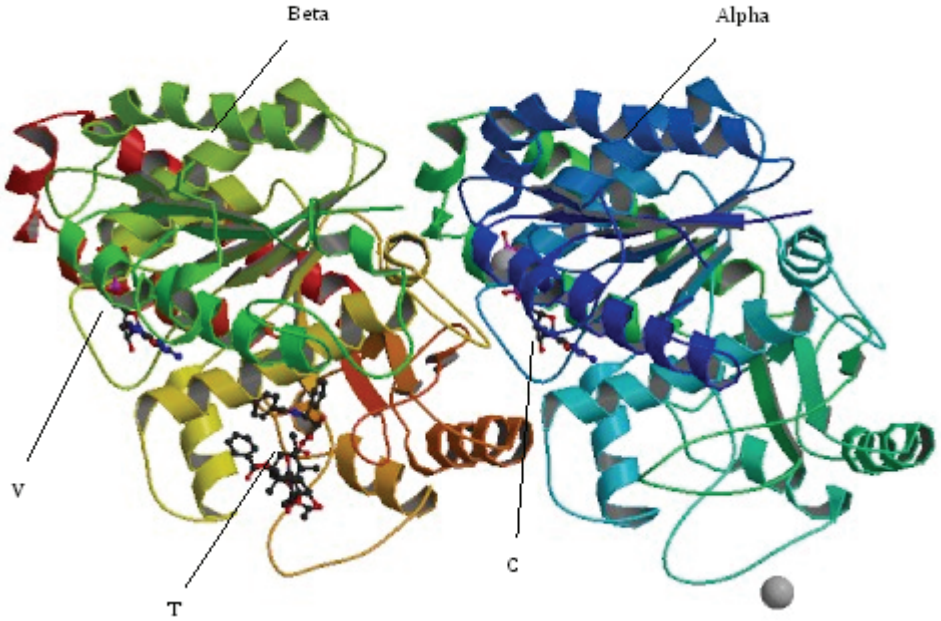


Figure 8. The tubulin binding domains. (V) = Vinca alkaloid, (T) = Taxane, (C) = Colchicine[76]

Unfortunately, like other cancer treatments, there is a maximum tolerated dose (MTD) and there can be severe side effects. Some of the most common side effects include nausea, vomiting, diarrhea, and neuropathy.[77, 78] However, an analog of vincristine and vinblastine, known as vinflunine, shows less toxicity and is effective against vinca alkaloid resistant strains of cancer (Figure 9).[78]

Paclitaxel, a taxane natural product extracted from the bark of a yew tree in 1967, expressed anti-mitotic results and became an anti-cancer drug in the 1990s (Figure 10).[73] It is currently approved for the treatment of ovarian and breast cancers. Taxanes bind very well to tubulin that is a part of the microtubule, but bind very poorly to soluble tubulin.

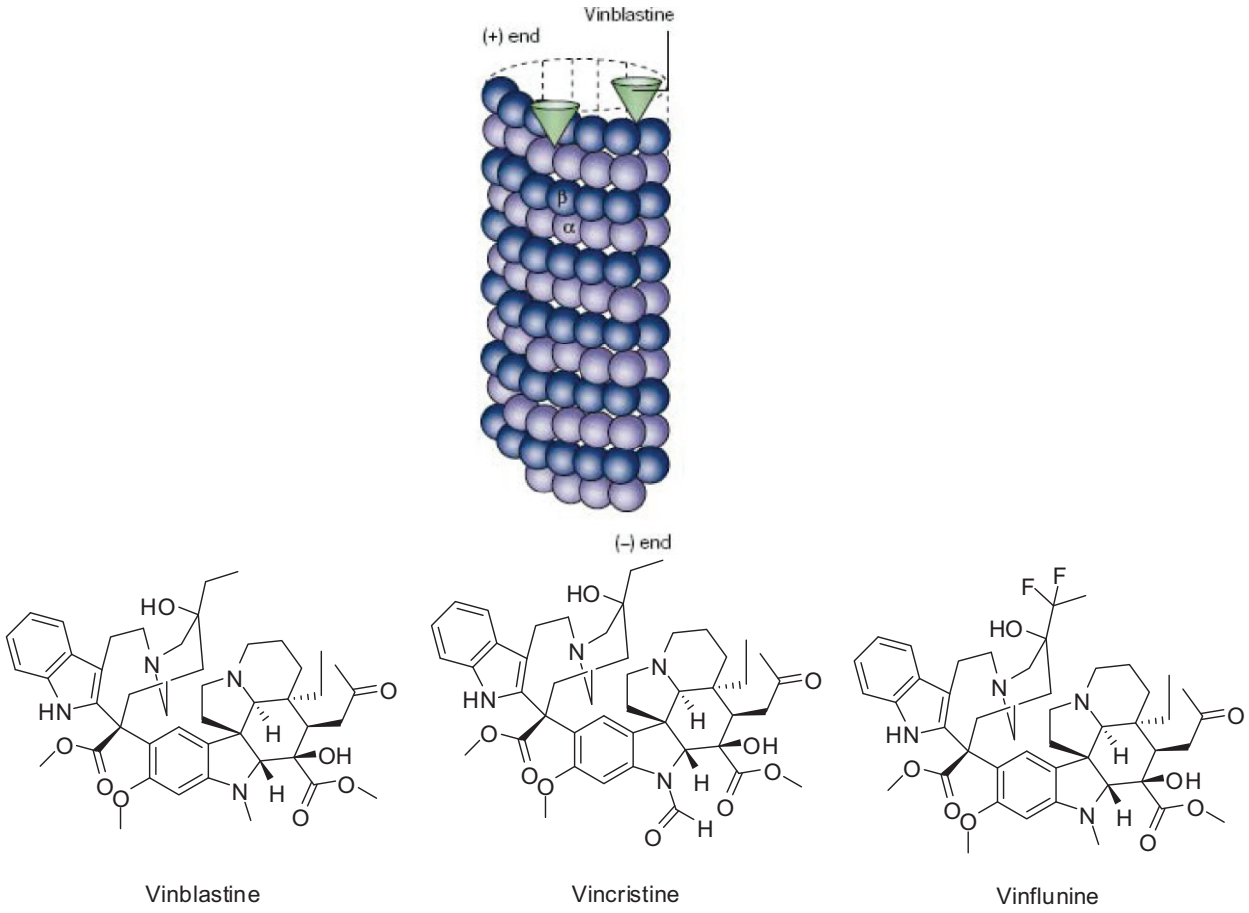


Figure 9. Vinblastine bound to microtubule; various vinca alkaloids: vinblastine, vincristine, and vinflunine[73] Reprinted by permission from Macmillan Publishers Ltd: *Nat. Rev. Cancer 4*, 253-265., copyright 2004.

When the taxane binds to tubulin on the microtubule, it stabilizes the tubulin-microtubule dynamics, preventing catastrophe and disrupting mitosis (Figure 10). Some of the side effects of paclitaxel and its semi-synthetic analog, docetaxel are neuropathy, alopecia, and gastrointestinal disorders.[79] Studies have shown that paclitaxel can work well with radiation therapy.[54] Paclitaxel was shown to dialate tumor blood vessels, which decreases the interstitial fluid pressure allowing oxygen and nutrients to readily reach the hypoxic regions of the tumor.[80] This allows for increased drug delivery to the hypoxic

regions of the tumor and increases the effects of radiation therapy due to increased oxygenation.

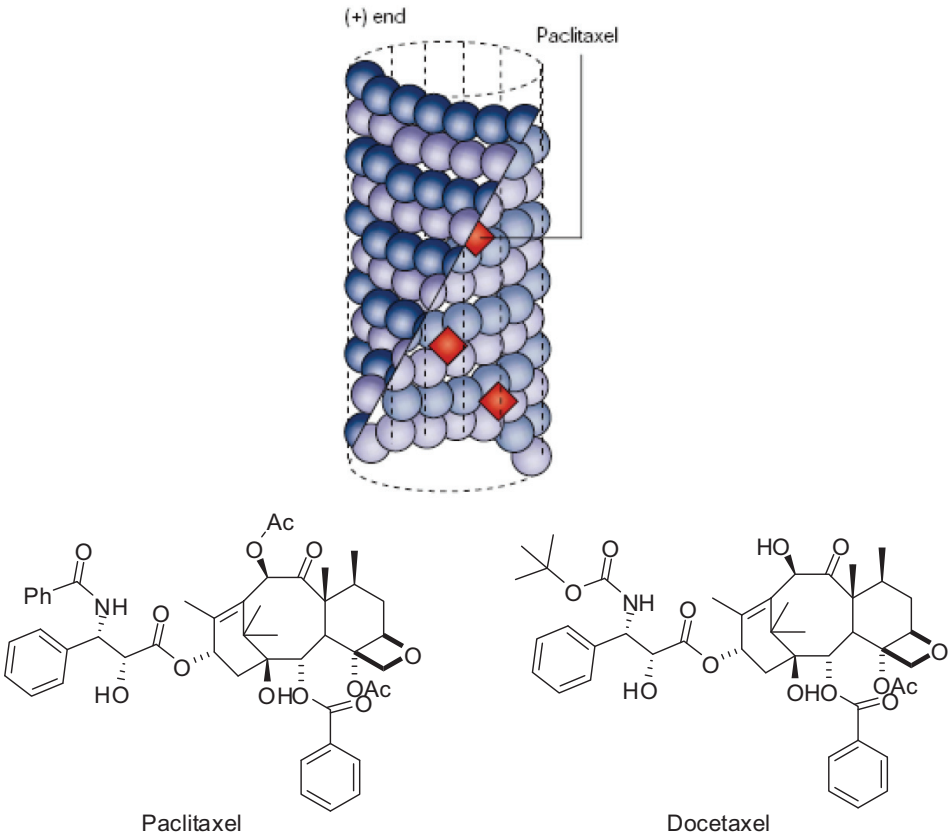


Figure 10. Paclitaxel bound to the interior of the microtubule; Two taxanes used in cancer treatment: Paclitaxel and Docetaxel[73] Reprinted by permission from Macmillan Publishers Ltd: *Nat. Rev. Cancer 4*, 253-265., copyright 2004.

Colchicine was the first known molecule to bind to tubulin. It was evident in the 1930's that the use of colchicine would cause necrosis for patients with biopsies on their tumors (Figure 11).[73] Both tubulin and colchicine were isolated to determine their mechanism of action against the tumor. Colchicine binds to the colchicine binding domain located between the α and β subunits of tubulin.[76] Colchicine binds to the soluble tubulin and forms a tubulin-colchicine complex. This tubulin-colchicine complex undergoes polymerization onto the microtubule to inhibit the tubulin dynamics (Figure

11).[73] In very high concentration, it was observed that colchicine would depolymerize microtubules and under low concentrations of colchicine, it would inhibit tubulin polymerization.[73] Studying the effects of colchicine on tumor vasculature, it was observed that colchicine would damage new tumor vasculature, causing hemorrhaging and vasculature collapse and eventually, necrosis. Unfortunately, these significant effects were observed at the maximum tolerated dosage which exhibited drastic side effects.[81]

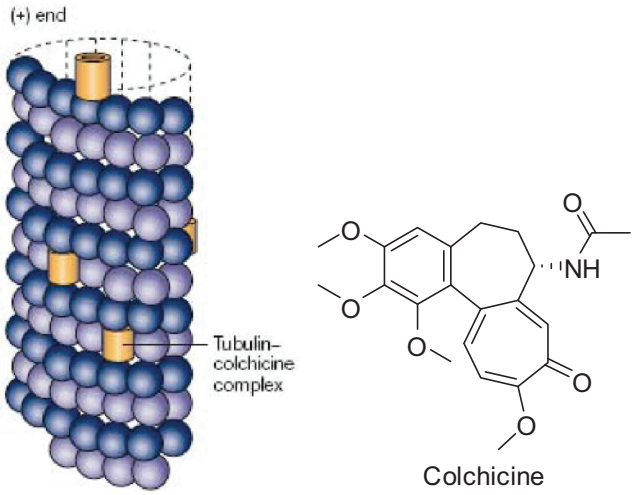


Figure 11. Colchicine bound to tubulin forming the tubulin-colchicine complex polymerized onto the microtubule[73] Reprinted by permission from Macmillan Publishers Ltd: *Nat. Rev. Cancer 4*, 253-265., copyright 2004.

Due to its side effects and high cytotoxicity, colchicine was not manufactured as a commercial anti-cancer drug to treat patients. A second generation of drugs were discovered and developed by researchers to disrupt tumor vasculature and at levels well below their maximum tolerated dose. The tumor vasculature grows and proliferates rapidly and these small molecule VDAs bind to the tubulin of these endothelial cells that line the tumor vasculature. Once bound, the VDAs disrupt tubulin polymerization and depolymerization dynamics, and the endothelial cells of the tumor vasculature lose their cytoskeleton integrity which causes morphological changes to the endothelial cells lining

the tumor vasculature. The normally flat endothelial cells will become rounded, blocking oxygen and nutrients from reaching the tumor, starving the tumor and eventually leading to tumor necrosis.[53] These changes in the cell shape of the tumor endothelial cells can lead to damage in the structural integrity of the tumor vasculature that may lead to hemorrhaging and collapse of the tumor vasculature. Two small molecule VDAs that are currently in clinical development include combretastatin A-4 phsophate (CA4P, Zybrestat™, fosbretabulin)[5] and Oxi4503 (combretastatin A-1 phosphate or CA1P).[1, 82]

CA4 and CA1 were isolated from an African bush willow tree, *Combretum caffrum* by Pettit and coworkers[3, 19, 27] and synthesized into phosphate salts (CA4P and CA1P) in order to enhance solubility in an aqueous environment (human blood serum). CA1 is very cytotoxic with an effective dose (ED_{50}) of 0.99 μg/mL *in vitro* against murine P388 lymphocytic leukemia.[1] CA4 and CA1 demonstrated potent inhibition of tubulin polymerization at a concentration of 1.1 μM *in vitro,* and when tested for *in vitro* cytotoxicity in human cancer cell lines, concentrations in the nM range proved to be highly effective.[1, 83] Mice with KHT fibrosarcoma when treated with both CA4P and CA1P exhibited reduction of about 80-90% in tumor vasculature and tumor size.[15]

The water soluble phosphate salts, CA4P and CA1P are administered into the body where they are enzymatically cleaved by endogenous phosphatases into their parent active compounds, CA4 and CA1.[14] The highly active CA4 or CA1 bind to the colchcine binding domain of the tubulin heterodimer inhibiting tubulin polymerization, damaging the integrity of the endothelial cytoskeleton of the tumor vasculature, and causing morphological changes to the endothelial cells of the tumor vasculature.[10] The change in shape of the endothelial cells from flat to round may cause occlusion to the tumor

vasculature and prevent nutrients and oxygen from reaching the tumor.[84, 85] Some of these blebbling endothelial cells may undergo apoptosis, causing void areas in the structure of the tumor vasculature.[53] These voids increase vasculature permeability and cause hemorrhaging.[61] If enough of these voids of endothelial cells are present, the high interstitial fluid pressure will be too great and cause vasoconstriction in the tumor vasculature, collapsing the tumor blood vessel and causing tumor vasculature failure (Figure 12).[53]

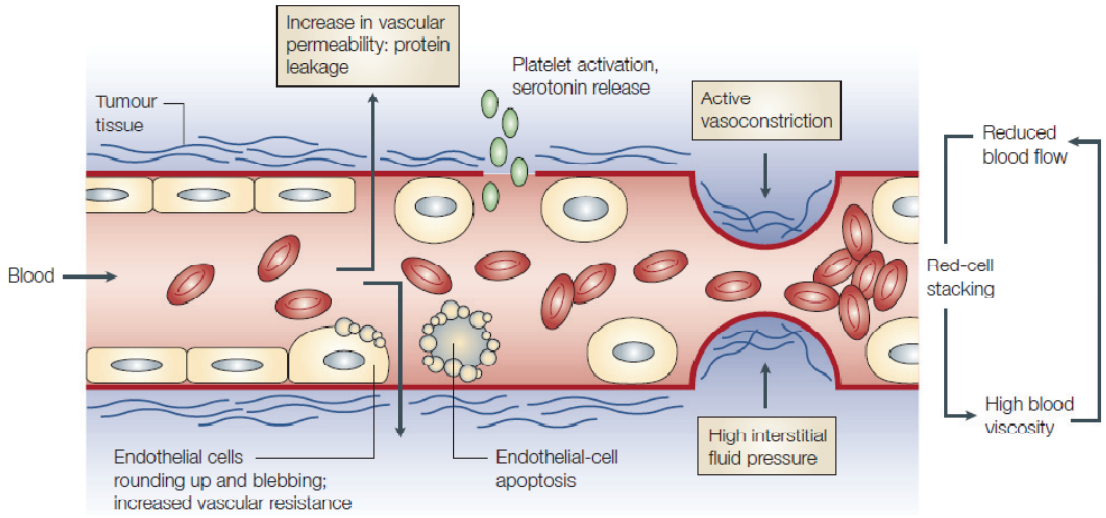


Figure 12. Collapse of tumor vasculature[53] Reprinted by permission from Macmillan Publishers Ltd: *Nat. Rev. Cancer* **2005**, *5*, 423-435, copyright 2005.

Even though both CA1 and CA4 showed promising results in tumor profusion regression, after 48 hours post-treatment with CA4 and 72 hours post-treatment with CA1, the tumor re-proliferated rapidly.[86, 87] This is due to an area called the viable rim, where the malignant cells at the edge of the tumor are supplied nutrients and oxygen from existing healthy normal cells and are spared destruction.[14] The combretastatins can be used in combination with other cancer treatments, such as chemotherapy, radiation

therapy, and recently, as bioreductive prodrugs (chapter 4) in order to destroy the tumor perfusion and the viable rim.

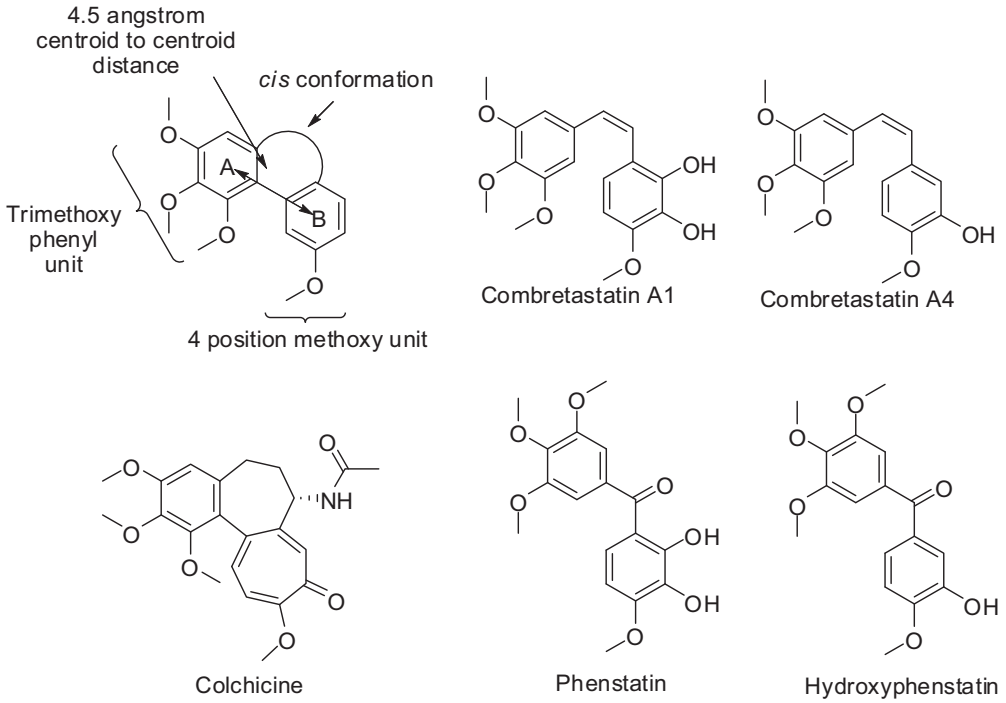


Figure 13. Pharmacophores required for VDAs tubulin binding

There are certain pharmacophores that are required for CA1 and CA4 and any of its analogs to bind to the colchicine domain of tubulin (Figure 13). First, on the A ring, a trimethoxy phenyl functional unit is required and on the B ring a methoxy group in the 4 position is highly recommended. If it is a combretastatin analog, a *cis* configuration is necessary and a centroid to centroid distance of approximately 4-5 angstoms between the aromatic rings is beneficial.[1, 9, 88-91]

For this project, a scale up synthesis of combretastatin A-1 as well as the cytotoxic agent phenstatin was conducted as practice for synthesizing VDAs and having them available for standards and biological testing. The cytotoxic agent, phenstatin, was coupled to a bioreductive agent to form a bioreductive prodrug conjugate (chapter 4).

The CA1, and its phosphate salt, CA1P, were used as a standard to compare against the combretastatin A-1 mono-phosphates on the HPLC (chapter 1). CA1 was also used in the radiolabeled OX16C project as a control reaction (chapter 2). These compounds are not commercially available and the synthesis of sufficient amounts of these compounds to have available further fascilitated the study of these compounds.

Materials and Methods

Reactions were performed under an inert atmosphere using nitrogen gas unless specified differently. Chemical reagents used in the synthetic procedures were obtained from various chemical suppliers (Sigma Aldrich, Acros Chemical Co., Alfa Aesar, Fisher Scientific, EMD Chemicals, and VWR). Silica gel (200-400 mesh, 60 Å), used for column chromatography, was obtained from either Silicycle Inc or VWR. TLC plates (pre-coated glass plates with silica gel 60 F254, 0.25 mm thickness, EMD chemicals, VWR) were used to monitor reactions. A Biotage Isolera Four automated flash column chromatography system was used to separate compounds. A Biotage microwave system was used to conduct microwave reactions. Intermediates and products synthesized were characterized based on ^{1}H NMR (Bruker DPX operating at 300 MHz or Varian operating at 500 MHz), ^{13}C NMR (Bruker DPX operating at 75 MHz or Varian operating at 125 MHz), and ^{31}P NMR (Bruker DPX operating at 121 MHz or Varian operating at 202 MHz). All the chemical shifts are expressed in ppm (δ), coupling constants (*J*) presented in Hz, and peak patterns are reported as broad (br), singlets (s), doublets (d), triplets (t), quartets (q), septets (sept), and multiplets (m). NMR processing was conducted using Mestrec. Elemental analysis was performed by Atlantic Microlab, Norcross, GA. High-resolution mass spectra (HREIMS), unit resolution gas chromatography mass spectra

(EIMS), and unit resolution mass spectra (ESIMS) were obtained on a VG Prospec Micromass spectrometer, a Thermo Scientific DSQ II, and a Thermo Finnigan LCQ Classic, respectively, in the Baylor University Mass Spectrometry Core Facility. Purity of the compounds was further analyzed using a Agilent 1200 LC System, and an Agilent Eclipse DBX-C18 4.6 x 150 mm, 5.0 mm, T = 20 °C; eluents, solvent A, 0.1 % TFA in water, solvent B, 0.08% TFA in acetonitrile:water (80/20 (v/v) ratio); flow rate, 1.0 mL/min; injection volume 20 μL; monitored at 254 nm wavelength.

Synthesis of Combretastatin A-1 **3**[1, 22, 40]

2,3-Dihydroxy-4-methoxybenzaldehyde ***17***:[1] 2,3,4-Trimethoxybenzaldehyde **9** (39.2 g, 200 mmol) was dissolved in anhydrous CH_2Cl_2 (150 mL) and was cooled to 0 ^oC with an ice bath. Boron tricholoride (400 mL, 400 mmol) was added dropwise to the reaction due to its vigorous nature. The reaction was allowed to slowly warm up to room temperature and stirred for 12 hours. The reaction was slowly and cautiously transferred over a period of time (20 minutes) into a flask containing a saturated solution of sodium bicarbonate (800 mL) to quench any excess boron trichloride. The reaction mixture is then acidified to a pH of one using hydrochloric acid. The organic phase of the reaction was separated and the aqueous layer was extracted with ethyl acetate (3 x 200 mL). The organic phases were combined, dried over sodium sulfate, filtered and concentrated. The crude product was subjected to flash column chromatography (silica gel, 30:70 EtOAc-hexanes) to yield diol **17** (29.8 g, 177 mmol, 89% yield) as a purple solid.

^{1}H NMR ($CDCl_3$, 300MHz) δ 11.12 (s, 1H), 9.76 (s, 1H), 7.15 (d, J = 8.7 Hz, 1H), 6.62 (d, J = 8.7 Hz, 1H), 5.45 (s, 1H), 3.99 (s, 3H)

2,3-Di-[(tert-butyldimethylsilyl)oxy]-4-methoxybenzaldehyde ***49***:[1] The 2,3-dihydroxy-4-methoxybenzaldehyde **17** (24.7 g, 147 mmol) was dissolved in DMF (100 mL) and diisopropylethylamine (76.9 mL, 441 mmol). *tert*-Butyldimethylsilyl chloride (49.6 g, 329 mmol) was added to the reaction mixture in several small portions and was allowed to stir for approximately 2 hours and checked by TLC until no starting material was present. The reaction was quenched and worked up with $NaHCO_3$ (100 mL), water (300 mL), and diethyl ether (200 mL). The organic phase was separated and the aqueous phase was extracted one more time with diethyl ether (100 mL). The organic phases were combined, washed with brine solution, and dried over potassium carbonate (K_2CO_3), filtered, and concentrated using the rotary evaporator. The crude mixture initially was a brown oil which solidified into a brown solid. This solid was recrystallized by first dissolving it in hot methanol (150 mL), allowing it to cool to room temperature and then placing the mixture into the freezer for several hours. The crystals were filtered as soon as they were removed from the freezer and washed with cold methanol to give pure tan product (49.6 g, 125 mmol, 85%).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 10.22 (1H, s, C-1 C*H*O), 7.48 (1H, d, *J* = 9.0 Hz, H-6), 6.63 (1H, d, *J* = 8.5 Hz, H-5), 3.84 (3H, s, C-4 OCH_3), 1.04 (9H, s, C-2 OSi$(CH_3)_2$C(C$H_3)_3$), 1.0 (9H, s, C-3 OSi$(CH_3)_2$C(C$H_3)_3$), 0.14 (12H, s, C-2, C-3 OSi(C$H_3)_2$C$(CH_3)_3$)

3,4,5-Trimethoxybenzyl bromide ***35***:[19] 3,4,5-Trimethoxybenzyl alcohol **34** (50.0 g, 252 mmol) was dissolved in dry dichloromethane (300 mL) and cooled to 0 ^{o}C in an ice/water bath. To the reaction mixture was added slowly and dropwise phosphorous tribromide (17.8 mL, 189 mmol). The reaction was allowed to stir for about four to five

hours. TLC was taken to make sure the reaction had gone to completion. Water was added slowly to quench the reaction. The organic layer was extracted and the aqueous layer was extracted with dichloromethane (3 x 100 mL). The organic layers were combined and dried using sodium sulfate, filtered, and concentrated using the rotary evaporator. The crude product (44.8g, crude 68%) was taken to the next step without further purification.

^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.62 (s, 2H), 4.47 (s, 2H), 3.87 (s, 6H), 3.85 (s, 3H)

*3,4,5-Trimethoxybenzyltriphenylphosphonium bromide **20***:[19] Bromide **35** (44.8 g, 171 mmol) was dissolved in dichloromethane (300 mL) and stirred under nitrogen. Triphenylphosphine (49.3 g, 189 mmol) was added and the reaction was refluxed for 22 hours. The reaction mixture was concentrated using a rotary evaporator to yield the crude product as an off-white solid. Diethyl ether was added to the crude product and the solid was filtered and washed with copious amounts of diethyl ether. The white solid was collected and dried under high vacuum to afford the phosphonium salt **20** (69.8 g, 134 mmol, 78%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.70 (m, 15H), 6.47 (s, 2H), 5.44 (d, J = 14.1 Hz, 2H), 3.77 (s, 3H), 3.51 (s, 6H)

*(Z)-2-[2''-3''-di-[(tert-Butyldimethylsilyl)oxy]-1-[3',4',5'-Trimethoxyphenyl]-4''-methoxyphenyl] ethene **55***:[1] In an oven dried round bottom flask was added phosphonium bromide **20** (26.8 g, 51.2 mmol) with a large stir bar, capped with a septum and placed under inert atmosphere. The bromide **20** was dissolved with anhydrous

tetrahydrofuran (300 mL), and cooled to -10 ^{o}C with good stirring. *n*-Butyllithium (25.5 mL, 63.7 mmol) was added dropwise to the reaction mixture, which immediately turned red-orange in color due to the formation of the ylide. After the *n*-butyllithium addition was complete, the reaction was allowed to stir for 10 minutes prior to cooling to -78 ^{o}C with an acetone/dry ice bath. Di-TBS-aldehyde **49** (20.7 g, 52.2 mmol) was dissolved in THF (80 mL) and added slowly to the ylide reaction mixture. The reaction mixture was allowed to stir and slowly warm up to room temperature over a 2-3 hour period. The reaction mixture was slowly and cautiously quenched using a few drops of water at first due to it highly reactive effervescent nature. Once the reaction is quenched, when addition of water does not cause explosive effervescence, the reaction mixture was transferred to a separatory funnel with water (200 mL), brine (50 mL), diethyl ether (200 mL), and hexanes (200 mL). The organic phase was separated and the aqueous phase was worked up with diethyl ether (2 x 200 mL). The organic phases were combined, dried with sodium sulfate, filtered, and concentrated using the rotary evaporator to give a dark greenish solid as the crude product. The crude product was dissolved in boiling ethanol (125 mL) and allowed to cool to room temperature before placing it into the freezer. If a small sample is available, it is recommended to seed to quicken the recrystallization process. After recrystallization, the solid was filtered and the crystals were washed with ice cold ethanol to yield pure di-TBS- CA1 **55** (11.7 g, 20.9 mmol, 40%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.91 (d, J = 8.5 Hz, 1H), 6.62 (s, 2H), 6.59 (d, J = 12.0 Hz, 1H), 6.36 (d, J = 8.9 Hz, 1H), 6.36 (d, J = 12.0 Hz, 1H), 3.83 (s, 3H), 3.74 (s, 3H), 3.67 (s, 6H), 1.04 (s, 9H), 0.99 (s, 9H), 0.19, (s, 6H), 0.10 (s, 6H)

*(Z)-2-[2''-3''-dihydroxy-4''-methoxyphenyl]-1-[3',4',5'-Trimethoxyphenyl] ethene **3***:[1] The di-TBS CA1 **55** (0.764 g, 1.36 mmol) was dissolved in acetonitrile (5 mL) and cooled to 0 ^{o}C using an ice/water bath under nitrogen. Tetrabutylammonium fluoride (0.867 ml, 2.99 mmol) was added to the reaction mixture and stirred for 10 minutes. TLC was taken every 2 minutes after the addition of tetrabutylammonium fluoride to monitor reaction completion prior to the 10 minute mark. Once the reaction is complete, it was quenched with water and the organic phase was separated. The aqueous phase was extracted with ethyl acetate (3 x 20 mL). The organic phases were combined, dried with sodium sulfate, filtered, and concentrated using rotary evaporation to yield the crude product as a slightly yellow oil. This oil was dissolved in hot ethyl acetate (1 mL) followed by hexanes (5 mL) which initiated crystallization. The crystals formed tiny tan pebbles which were filtered to yield pure tan solid CA1 product **3** (0.215 g, 0.647 mmol, 49%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.77 (d, J = 8.6 Hz, 1H), 6.59 (d, J = 12.1 Hz, 1H), 6.53 (d, J = 12.1 Hz, 1H), 6.52 (s, 2H), 6.39 (d, J = 8.6 Hz, 1H), 5.38 (s, 2H), 3.86 (s, 3H), 3.83 (s, 3 H), 3.67 (s, 6H)

Synthesis of Phenstatin[90, 91]

*3-[(tert-Butyldimethylsilyl)oxy]-4-methoxybenzaldehyde **57***: 3-Hydroxy-4-methoxybenzaldehyde **56** (isovanillin) is commercially available. The 3-hydroxy-4-methoxybenzaldehyde (1.00 g, 6.57 mmol) was dissolved in anhydrous dimethyl formamide (5 mL) at room temperature. Diisopropylethylamine (2.29 mL, 13.1 mmol) was added to the reaction and stirred for five minutes. *Tert*-butyldimethylsilyl chloride (1.08 g, 7.23 mmol) was slowly added to the reaction mixture in small portions over a 20

minute period. The reaction was stirred for 12 hours and monitored by TLC. The reaction mixture was quenched using saturated sodium bicarbonate (50 mL) and the aqueous layer extracted using diethyl ether (3 x 100 mL). The organic layers were combined and washed with brine solution (100 mL) and copious amounts of water (6 x 100 mL) to remove any excess dimethyl formamide from the organic layer. The organic layer was dried using sodium sulfate, filtered and concentrated to yield the mono-protected aldehyde 57 as a tan solid (1.61 g, 60.4 mmol, 92%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.72 (s, 1H, CHO), 7.39 (dd, 1 H, J = 2 and 8.5 Hz, Ar-5H), 7.27 (d, 1H, J = 2 Hz, Ar-1H), 6.87 (d, 1H, J = 8.5, Ar-4H), 3.80 (s, 1H, OCH3), 0.91 (s, 9H, C-CH3), 0.08 (s, 6H, Si-CH3).

1-[3''-[(tert-Butyldimethylsilyl)oxy]-1-[3',4',5'-Trimethoxyphenyl]-4''-methoxy-phenyl] methanol ***59***: To an oven dried flask was added commercially available 3,4,5-trimethoxybromobenzene **58** (7.63 g, 30.9 mmol) which was dissolved in diethyl ether and cooled to -78 ^{o}C using an acetone/dry ice bath with good stirring. The reaction flask was evacuated and refilled with inert atmosphere and cycled at least five times. To this solution was added *n*-butyllithium (11.7 mL, 29.4 mmol) dropwise and the reaction was allowed to stir for at least 45 minutes. In another over dried round bottom flask was added the TBS-protected benzaldehyde (4.19 g, 15.4 mmol), dissolved with diethyl ether, and cooled to -78 ^{o}C using an acetone/dry ice bath. The phenyllithium that was formed in the first reaction flask was slowly transferred to the TBS aldehyde mixture through the use of a cannula. The reaction was allowed to stir at -78 ^{o}C for 4 hours followed by slow warming up to room temperature. The reaction was monitored by TLC and the reaction was deemed completed when the TBS aldehyde was no longer evident. The reaction was

cautiously quenched with water (150 mL) and slightly acidified with 0.5 M HCl solution (25 mL). The organic layer was separated and the aqueous layer was extracted with diethyl ether (3 x 150 mL). The organic phases were combined, dried with sodium carbonate, filtered, and concentrated using the rotary evaporator to yield the crude product as a dark yellow oil. The crude product (9.54 g, 77% crude) was not purified but taken to the next step.

*[3''-[(tert-Butyldimethylsilyl)oxy][3',4',5'-Trimethoxy]-4''-methoxy] benzophenone **60***: The crude product of TBS protected secondary alcohol **59** (9.54 g, 22.0 mmol) was dissolved in dry CH_2Cl_2 (250 mL). Celite (7 g) and anhydrous K_2CO_3 (7 g) were added to the solution and the solution was cooled to 0 ^oC in an ice bath under nitrogen. Once the reaction mixture was cool, PCC (5.21 g, 21.9 mmol) was added in small increments; one increment every 10-15 minutes until all the PCC was added. The reaction mixture was allowed to stir overnight (12 hrs). Once the reaction had gone to completion, the reaction mixture was filtered through a 50/50 mixture of silica gel/celite using a filter funnel and the resulting solution was concentrated using rotary evaporation. The crude benzophenone was subjected to flash column chromatography to yield (2.8 g, 29%) pure TBS protected phenstatin **60** as a tan solid.

^1H NMR ($CDCl_3$, 300 MHz) δ 7.46 (dd, 1 H, J = 8.5 and 2 Hz, Ar-5H), 7.38 (d, 1H, J = 2, Ar-4H), 7.03 (s, 2H, Ar-1',5'H), 6.92 (d, 1H, J = 8.5 Hz, Ar-1H), 3.94 (s, 3H, 3-OCH3), 3.90 (s, 3H, 3'-OCH3), 3.88 (s, 6H, 2',4'-OCH3), 1.00 (s, 9H, C-CH3), 0.19 (s, 6H, Si-CH3)

[3''-hydroxy-4''-methoxy][3',4',5'-Trimethoxy] benzophenone ***61***: The TBS protected phenstatin (2.70 g, 6.35 mmol) was placed in a round bottom flask along with KF (1.45 g, 25.1 mmol) and DMF (10 mL). The reaction was stoppered with a septum and a catalytic amount of HBr (0.75 mL) was added and the reaction was sonicated for 2-4 hours with monitoring by TLC until the TBS phenstatin was no longer evident. The reaction was worked up with ice cold HCl (5 mL), ethyl acetate (20 mL), brine (40 mL), and water (5 mL). The organic layer was separated and the aqueous layer was worked up with ethyl acetate (3 x 75 mL). The organic phases were combined, dried with magnesium sulfate, filtered, and concentrated with a rotary evaporator. The crude product was subjected to flash column chromatography (75:25 / hexanes:ethyl Acetate) and recrystallized in hexanes to give clear colorless crystals (1.58g, 80% yield) as pure phenstatin **61**.

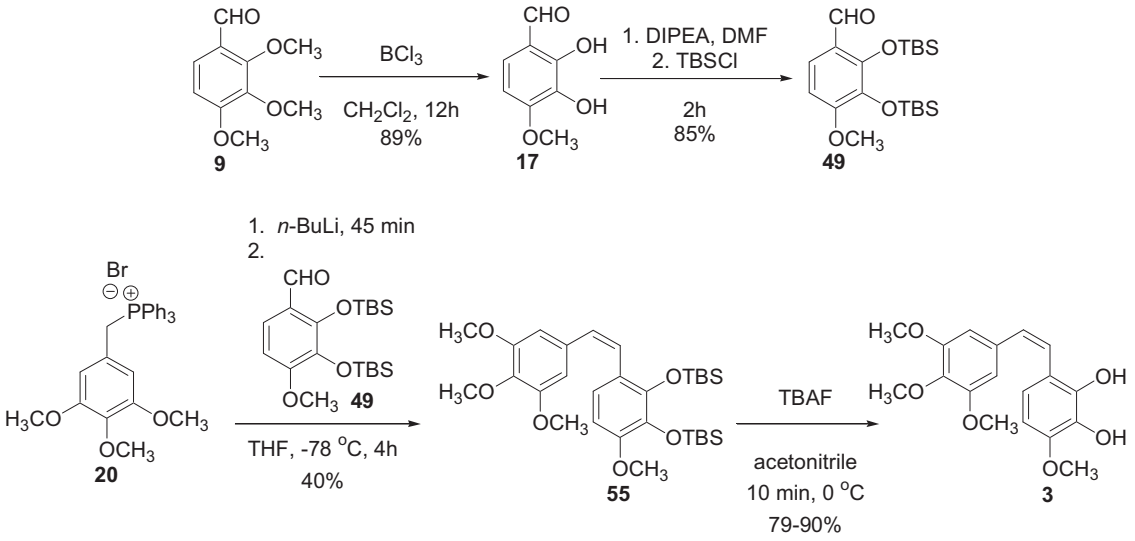
^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.45 (d, 1H, J = 2 Hz, Ar-1H), 7.41 (dd, 1 H, J = 8.5 Hz, Ar-5H), 7.04 (s, 2H, Ar-1',5'H), 6.93 (d, 1H, J = 8.5, Ar-4H), 5.79 (s, 1H, OH), 3.98 (s, 3H, 3-OCH3), 3.93 (s, 3H, 3'-OCH3), 3.88 (s, 6H, 2',4'-OCH3)

Results and Discussion

Synthesis of Combretastatin A-1 **3**

When working in the area of vascular targeting and vascular disruption, it is often helpful to have experience synthesizing known VDAs prior to developing new VDAs. It is also important to have a library of known VDAs available for comparison standards. The synthesis of combretastatin A-1 (Scheme 16) was required in order to have sufficient material to study the VDA as well as for comparison standards. The strategy in preparing

CA1 was to first synthesize an aldehyde portion and then a phosphonium bromide portion in order to couple the two pieces together using a Wittig reaction. Commercially available 2,3,4-trimethoxybenzaldehyde **9** was selectively demethylated using BCl_3.[1] The aldehyde **9** was dissolved in CH_2Cl_2 and cooled to 0 ^oC using an ice bath. Depending on the size of the reaction, a 1-2 liter round bottom flask may be used for large scale reactions.



Scheme 16. Synthesis of CA1 **3**[1, 40]

After the reaction flask was cooled to 0 ^oC, BCl_3 was added dropwise to the reaction. This could be done in two ways. The first method involved using a large, oven-dried graduated cylinder with a ground glass joint (size 24/40 at the mouth) to which a septum was placed over the mouth and tightened down with a wire. The graduated cylinder was then evacuated and filled with inert atmosphere. The reason for the graduated cylinder was to measure out the exact amount of BCl_3 prior to addition to the reaction. If the reaction was microscale, then a syringe would suffice for addition of less than 20 mL of BCl_3. Since this reaction was large scale and in quantities of over 20

grams, the addition of large quantities of BCl_3 using a syringe did not seem adequate nor safe. A cannula was used to transfer the BCl_3 to the graduated cylinder to the desired volume. The BCl_3 was then transferred to the reaction flask dropwise via cannula. The second method of transferring BCl_3 involved the use of an oven-dried dropping funnel attached via ground glass joint to the round bottom reaction flask. Once the dropping funnel was connected to the reaction flask, a septum was placed over the top mouth of the dropping funnel. The dropping funnel was evacuated and filled with inert atmosphere. The valve should be closed on the dropping funnel. BCl_3 was transferred to the dropping funnel via cannula to the desired volume. The valve was opened until only a slow drip was evident. As the BCl_3 was added to the reaction, the reaction mixture turned dark brown in color and eventually became black. After addition of BCl_3, which may take hours due to the necessity of slow addition, the reaction mixture was allowed to stir for approximately 12 hours. Stirring for more than 2h hours is not advised because the desired product diminishes over time. The reaction was cautiously and slowly transferred to a large flask containing saturated $NaHCO_3$ due to its effervescent nature. Be cautious to avoid contact as the solution will stain skin yellow. The solution was then acidified with HCl until the pH reached 1 and further extracted with CH_2Cl_2, dried, concentrated in vacuo, and isolated to yield 2,3-dihydroxy-4-methoxybenzaldehyde **17** in an 89% yield. The diol **17** was protected using TBSCl and the crude product was recrystallized to afford the 2,3-di-[(*tert*-butyldimethylsilyl)oxy]-4 methoxybenzaldehyde **49** in an 85% yield (Scheme 16). The phosphonium bromide **20** was synthesized next starting with commercially available 3,4,5-trimethoxybenzyl alcohol **34**, which was brominated using PBr_3 to afford the benzyl bromide **35** in 68% crude yield. The benzyl bromide was

refluxed with PPh_3 for 22 hours to give the triphenylphosphonium bromide **20**. After refluxing for 22 hours, the solvent was removed and a crude off-white solid was present. Diethyl ether was added to the crude product and this resulting slurry was filtered and washed with copious amounts of diethyl ether. The triphenylphosphonium bromide **20** was insoluble in diethyl ether whereas any excess triphenylphosphine was soluble in diethyl ether could be washed away from the product. Both the triphenylphosphonium bromide **20** and the protected benzaldehyde **49** underwent a Wittig reaction to couple the compounds together through the formation of a stilbene bridge to give the (Z) di-TBS-protected CA1 **55** in a 50% yield. It is important to note that a small amount of the (*E*) di-TBS-protected CA1 was formed but in very low yield. By ^{1}H NMR, the E:Z isomer ratio was estimated to be a 1:5. After the Wittig reaction, the protected CA1 **55** was deprotected using TBAF and recrystallized to afford pure (*Z*) CA1 **3** as a tan solid (Scheme 16).

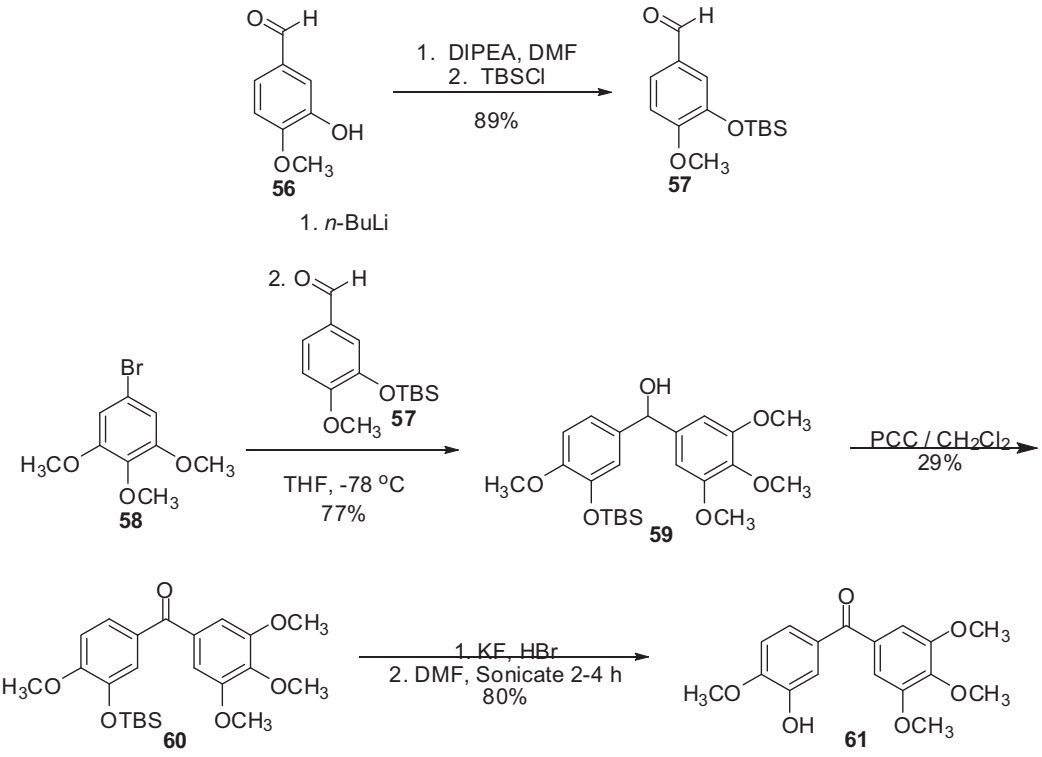
Synthesis of Phenstatin[90, 91]

Commercially available 3-hydroxy-4-methoxybenzaldehyde **56** was protected using TBSCl in dimethylformamide and diisopropylamine to afford the TBS-protected benzaldehyde **57**. 3,4,5-trimethoxyphenyllithium was formed through a halogen metal exchange when 3,4,5-trimethoxybromobenzene **58** was subjected to *n*-butyllithium. The aryllithium was slowly added to a pre-cooled solution of the TBS-protected benzaldehyde **57,** at which the aryllithium underwent addition to the protected aldehyde **57,** to yield the secondary alcohol **59** upon acidification. The TBS-protected secondary alcohol **59** was oxidized using pyridinium chlorochromate to the TBS-protected benzophenone **60** in a 29% yield. The TBS-protected benzenephenone **60** was subjected to potassium fluoride

with trace amounts of HBr under sonication to deprotect the TBS protecting group. Due to silicon's high affinity for fluorine, the deprotection occurred cleanly and rapidly to afford phenstatin **61** (Scheme 17).

The TBS protection step and isolation was completed without any problems. The halogen metal exchange required practice prior to achieving the correct reaction conditions to produce the desired product and further experimentation to achieve higher yields. The halogen metal exchange reaction was very meticulous in that it mandated a zero tolerance for water. The glassware needed to be oven-dried, the reagents needed to be dry, and solvents needed to be anhydrous. On several occasions, when practicing the halogen metal exchange, the bromide would not react at all and the starting TBS-protected aldehyde **57** would be recovered. Another compound that was recovered was later identified as 3,4,5-trimethoxybenzene. Due to a miniscule amount of water, the aryllithium that was formed was immediately quenched into 3,4,5-trimethoxybenzene before it had the opportunity to undergo addition to the TBS-protected benzaldehyde. Since *n*-butyllithium was used, both the molar concentration and the stoichiometric ratio needed careful consideration. Even when the *n*-butyllithium was from a new bottle, the molar concentration was not always correct. Titration of *n*-butyllithium with *n*-benzylbenzamide was one of the many ways of determining the molar concentration of the *n*-butyllithium. The exact stoichiometric ratio was also necessary in order to avoid having excess of *n*-butyllithium for the halogen metal exchange. Any excess, whether it is due to the incorrect stoichiometric ratio or higher molar concentrations of *n*-butyllithium, could allow more byproduct formation through alkyl addition.

After the lithium from *n*-butyllithium had undergone a halogen metal exchange with the 3,4,5-trimethoxybromobenzene to form the aryllithium reagent, *n*-butyllithium can compete with the aryllithium reagent in terms of nucleophilic attack toward the TBS-protected benzaldehyde **57**.



Scheme 17. Synthesis of phenstatin **61**[90, 91]

The butyl addition byproduct was observed during the halogen metal exchange reaction. To reduce the amount of byproduct formed, a sterically hindered, bulky organolithium could be used, such as *tert*-butyllithium. When the addition of the aryllithium reagent was successful and the alkoxide was formed, most of the alkoxide was located in the aqueous layer during the liquid-liquid extraction probably due to forming a salt with the lithium cation. The organic layer was separated and collected. Since the aqueous layer was not acidified, the product remained in the aqueous layer.

The organic layer was reduced *in vacuo* and very low yields of the secondary alcohol product **59** were observed. During liquid-liquid extraction, the pH of the aqueous layer is sometimes low enough to protonate the alkoxide into its corresponding alcohol. For this instance, water was not acidic enough. However, the aqueous layer was not discarded and was later acidified and re-extracted with organic solvents to recover much of the desired product. A pyridinium chlorochromate oxidation converted alcohol **59** into ketne **60**. It is advisable to use excessive amounts of solvent in a suspension of Celite prior to addition of the pyridinium chlorochromate. This prevents the pyridinium chlorochromate from clumping together and forming a large solid within the reaction mixture, which hinders stirring. To also help control clumping, small amounts of pyridinium chlorochromate are added in very small increments over a long period of time (about 1-2 hours). The TBS-protected benzophenone **60** was deprotected using KF with trace amounts of HBr under sonication. Since fluorine has a high affinity towards silicon, deprotection occurred rapidly. Other reagents, such as TBAF, can be used in place of KF as a deprotecting agent.

Successful completion of the scale-up of both combretastatin A-1 and phenstatin afforded large quantities of these materials in high yield. With the the library of these compounds, they were used as successful standards in various projects such as comparing CA1 to CA1 monophosphates mentioned in chapter 1 as well as using the synthetic route in synthesizing CA1 to develop the radiolabeled synthesis of OX16C in chapter 2. The synthesized phenstatin was also used in developing novel bioeductive prodrug conjugates in chapter 4.

CHAPTER FOUR

Synthesis of Bioreductive Triggers and Their Corresponding Bioreductive Prodrug Conjugates

Introduction

Cancer is a malignant disease and is considered to be the second most common cause of death after heart disease.[45] Twenty-five percent of all recorded deaths in the United States are attributed to cancer. Cancer has been very prolific and as time turns, it is also progressing and may eventually surpass heart disease as the leading killer in the United States.[47] Worldwide, significant efforts continue to be directed towards improved treatment methods for cancer, however in order to be successful in this endeavor, a deeper understanding of cancer, at the molecular level, is imperative.

Cancer is the uncontrolled growth of cells. [45-47] Normal cells go through a cell cycle for cell replication. During this process, due to genetic mutation or through external factors such as smoking and certain types of viruses such as human papillomavirus (HPV), the cells may become damaged.[47] In many instances, these damaged cells will be programmed to die through a process known as apoptosis. However, on certain occasions and for unknown reasons, these damaged cells may begin to replicate, making more copies of the abnormal cells. A vicious cycle occurs where all of these abnormal cells uncontrollably replicate, become cancerous, and eventually become large enough to be classified as a tumor. These cells will grow, invade, and destroy the surrounding cells, and eventually metastasize and spread throughout the body if left unchecked.[92]

The tumor microenvironment differs drastically when compared to a normal healthy cellular environment. The tumor microenvironment is often hypoxic (oxygen deprived).[93-95] As the tumor grows, it requires blood vessels to form in order to provide oxygen and nutrients to these rapidly proliferating abnormal cells. The tumor releases certain growth factors, which allow angiogenesis to occur.[52] Even though angiogenesis is occurring within the tumor, the rate of growth of the tumor is much greater than the rate at which the new tumor vessels are formed.[53, 64] The slow angiogenic rate is unable to meet the demand for oxygen and nutrients required by the tumor thus rendering the tumor microenvironment hypoxic, nutrient deprived, and slightly acidic.[96]

These newly formed blood vessels from tumor angiogenesis differ greatly from normal blood vessels.[60, 61, 97] Since these tumor blood vessels form quickly in an attempt to meet the demands of the tumor, the growth of these vessels is chaotic and erratic, with random branching within the tumor, including irregular sizes, both in diameter and length.[93] This results in a weak and disorganized vasculature. The vasculature is so disorganized that in some areas, reversible blood flow and arteriovenous shunts (AV shunts) are observed along with areas where the vasculature actually leads nowhere and has no apparent purpose (Figure 14).[93]

Due to the immature and weak nature of the tumor vasculature, the tumor vessel walls are poorly developed with discontinued sections of endothelial cells that have little or no basement membrane (Figure 14).[53] This allows the tumor vasculature to have increased permeability which may be a double-edged sword. The increased permeability allows for enhanced diffusion of anti-tumor drugs to the tumor itself, yet it also allows the tumor cells to seep into the tumor vasculature and deposit at a distant site.

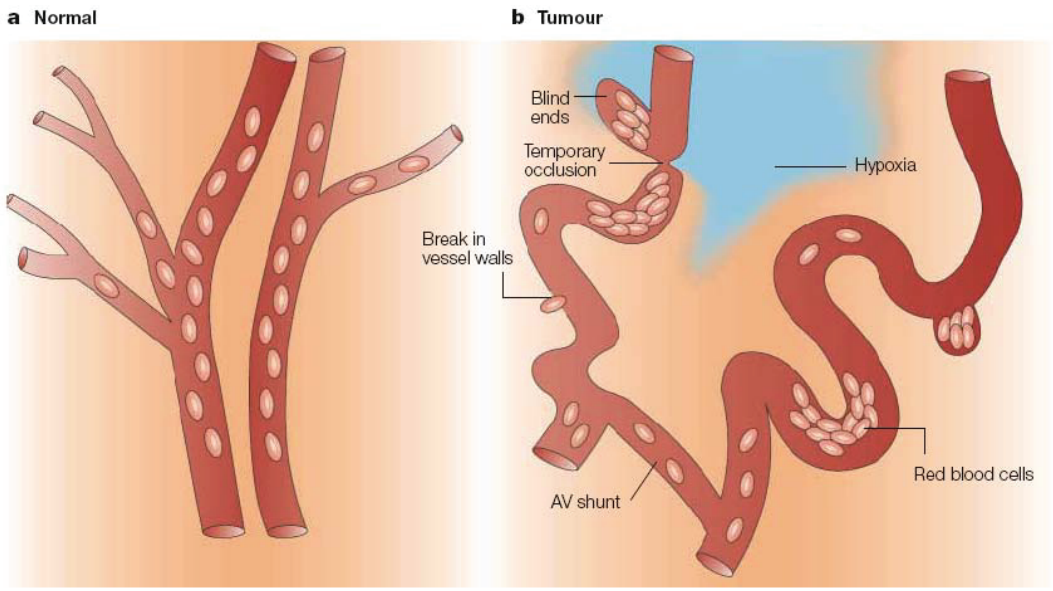


Figure 14. Normal vasculature and tumor vasculature[93] Reprinted by permission from Macmillan Publishers Ltd: *Nat. Rev. Cancer, 4*, 437-447., copyright 2004.

The ultimate goal of any type of cancer treatment is to completely destroy all of the tumor cells, with no toxicity to any non-malignant cells. Unfortunately, this is nearly always not the case. Chemotherapy, a toxic and non-specific type of therapy, requires varying doses to exhibit remission for the cancer. Due to its non-specific attribute, normal healthy cells are affected which leads to dose limiting toxicities (DLT). There will be a maximum effective dose that the body can take, once this maximum has been reached, any further chemotherapy would result in harming the person more than treating the actual cancer itself. Therefore, novel approaches toward the treatment of cancer have been developed to be used as stand-alone therapy and/or in conjunction with traditional therapies in order to increase the chances of successful treatment.

A novel method of treating cancer is through vascular targeting.[65] Vascular targeting is a strategy that targets the blood flow associated with the tumor by either selectively damaging the tumor vasculature or by inhibiting the growth of tumor

vasculature, thus preventing oxygen and nutrients from reaching the tumor cells, eventually starving the tumor.[28, 51, 63, 85, 96] Since the tumor microenvironment is often hypoxic and tumor vasculature is unorganized and spread far apart, shutting down even one tumor vessel can lead to substantial death of malignant cells.[11] Because the tumor vasculature is proliferating rapidly to meet the needs of the tumor, the properties of the endothelial cells lining the tumor vasculature differ from that of normal vasculature, allowing for selectivity. Vascular targeting can be categorized into two groups: anti-angiogenic agents and vascular disrupting agents (VDAs). [62, 63]

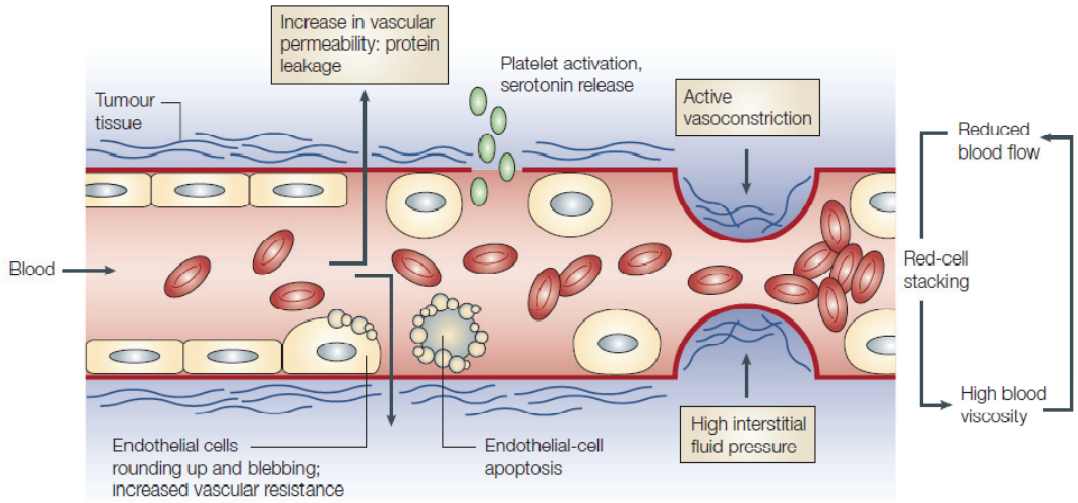


Figure 12. Increased permeability due to loss of endothelial cells[53] Reprinted by permission from Macmillan Publishers Ltd: *Nat. Rev. Cancer* **2005**, *5*, 423-435, copyright 2005.

Anti-angiogenic agents are molecules that are used to prevent new vasculature from forming within the tumor microenvironment.[52, 64, 98] These anti-angiogenic agents act as inhibitors of certain angiogenic growth factors or certain growth factor receptors. There are even some agents that work as transport inhibitors, thus intercepting and blocking the transport of angiogenic growth factors from reaching their target

destination.[66] Anti-angiogenic agents, such as angiostatin and endostatin, have been a part of experimental cancer therapy for a long time and have proven to prevent the further growth of the tumor vasculature.[63, 64] Unfortunately, anti-angiogenic agents have no effect on established tumor vasculature.

Vascular disrupting agents (VDAs), instead of preventing the formation of new tumor vasculature, as in the case of anti-angiogenic agents, target the existing tumor vasculature to shutdown blood flow to the tumor, starving the tumor of oxygen and nutrients, leading to further hypoxia and eventually, necrosis.[10, 11, 25, 26] A selective yet limited grouping of the currently known VDAs includes combretastatin A-4 phosphate (CA4P, Zybrestat™, fosbretabulin),[5, 9] combretastatin A-1 phosphate (CA1P, OXi4503),[1, 40] along with the non-clinical entities, phenstatin phosphate,[91] and Oxi8007 (Figure 15).[39]

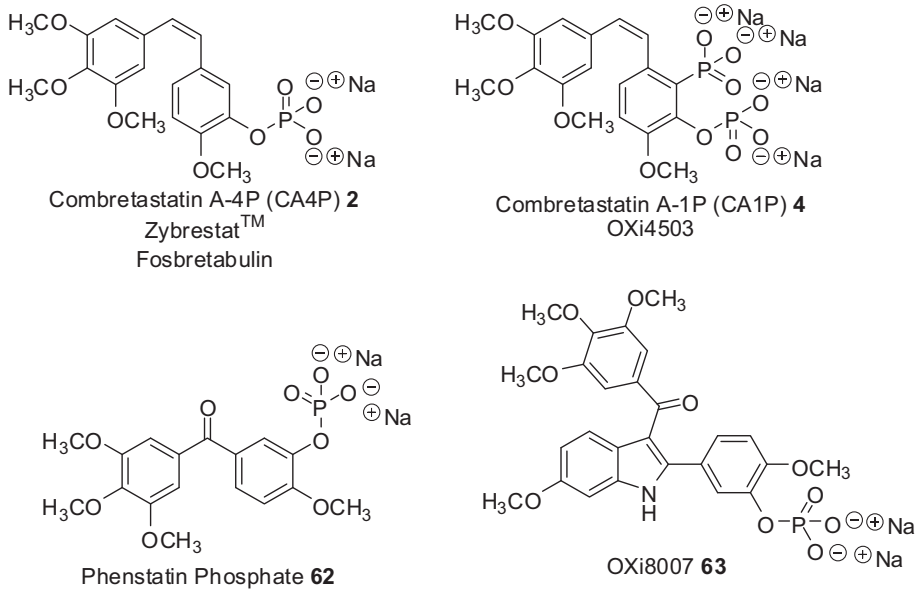


Figure 15. VDAs and cytotoxic agents

These VDAs, once cleaved enzymatically to their non-prodrug parent compounds, bind to the α,β tubulin heterodimer, and are potent inhibitors of tubulin polymerization

(assembly). The tubulin-microtubule protein system is also a key component of the cell cytoskeleton, which gives the cell its shape and supports its structure.[84] The α and β tubulin heterodimer within the cell is constantly undergoing tubulin polymerization, which is a dynamic process. This means that the microtubules are constantly being depolymerized (catastrophe) into its subunit tubulin and being polymerized (recovery).[53] If the VDAs were to bind and inhibit tubulin within the cell, then the recovery phase of the dynamic process of microtubule formation will not take place. As more of the microtubules undergo catastrophe and more tubulin is released and inhibited, eventually the concentration of microtubules within the cell will be greatly diminished. From a tumor vasculature stand point, the VDA disrupts microtubule formation binding to tubulin and inhibiting the assembly of tubulin inot microtubules. Since the concentration of microtubules is reduced, the cell cytoskeleton and substructures are destroyed. The endothelial cells of the tumor vasculature change their shape, from flat to rounded. The rounding of the tumor vasculature endothelial cells occlude the blood vessels, thus blocking blood flow from reaching the tumor. If enough blood flow is shutdown to the tumor, the tumor will starve and eventually die.[53, 96] The tubulin-microtubule protein system is an essential component of vasculature endothelial cells and plays an important role in cell division. For cell division, microtubule spindles are formed to pull apart DNA.[73] These spindles are microtubules and are made from the tubulin within the endothelial cell. From a cell division standpoint, once microtubule formation from tubulin is inhibited by a VDA, then spindle formation is negatively impacted.[73] If spindles can not form to separate the DNA for cell division, then cell division will not take place, which is one concept in treating cancer, since it is the uncontrollable growth

of cells.[75] A more detailed biological mechanism of action of vascular disrupting agents is provided in the introduction of Chapter 3.

Bioreductive drugs represent a novel strategy that has potential application in cancer therapy.[99, 99] Initially bioreductive agents were known as radiosensitizers and were developed to be used in combination with radiation therapy.[56] For solid tumors, some of the hypoxic tumor cells are resistant to radiation therapy due to the lack of oxygen.[54] In order to amplify the response of such tumors to radiation therapy, radiosensitizing agents are introduced prior to external radiation to enhance radiation induced radical formation. The bioreductive drug strategy takes advantage of the overexpression[100] of certain reductase enzymes present in hypoxic regions of tumors. Ideally, the bioreductive prodrug itself is not cytotoxic to healthy cells. However, in the presence of certain enzymes in hypoxic regions of the tumor, the bioreductive prodrug undergoes either a one or two electron reduction, releasing the active chemotherapeutic agent (Figure 18).[99, 101, 102] Depending on the bioreductive drug used, the chemotherapeutic portion, once released, can alkylate DNA, intercalate DNA, or cause strand breaks in the DNA which will prevent tumor cell proliferation and eventually lead to apoptosis.[99]

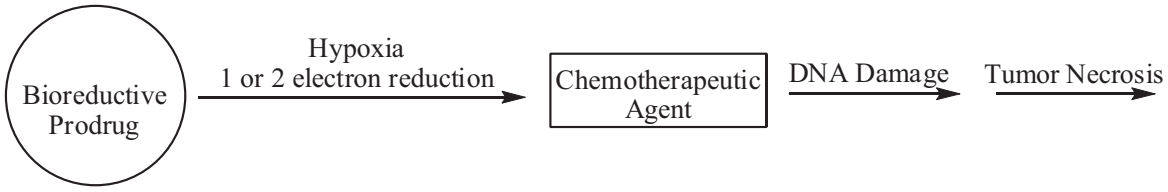


Figure 16. Bioreductive prodrug reduction[99]

Even though under hypoxic conditions the bioreductive drug can be quite potent in damaging DNA, it can be quenched into its inactive form in if low levels of oxygen are present. These bioreductive drugs are ineffective against tumor cells in aerobic areas and

even if these bioreductive drugs are active in aerobic environment, they require the reductase enzymes expressed in the hypoxic regions of the tumor to reduce them to their active form. Recently, there have been studies concerning a new enzyme called DT-diaphorase (DTD) which is present in aerobic tumor cells and is not oxygen dependent in its mechanism of reduction (Figure 17).[100, 102, 103]

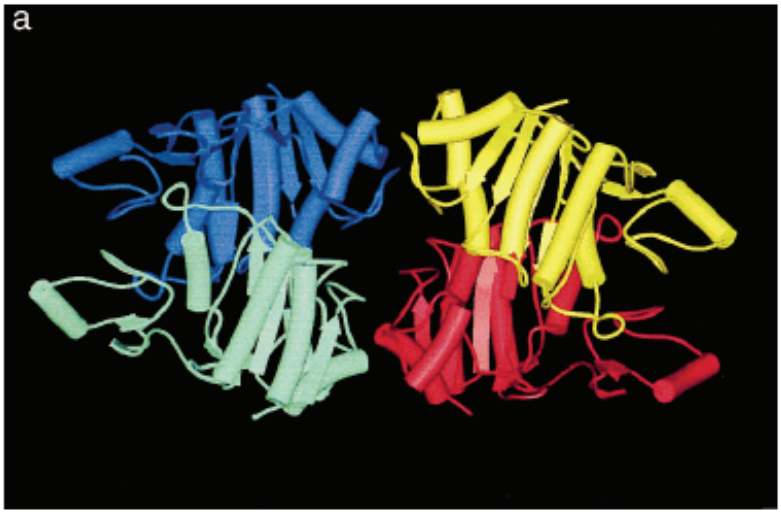


Figure 17. Three-dimensional structure of DT diaphorase (DTD)[103] Reproduced with permission from *J. Med. Chem.* **2005**, *48*, 7917. Copyright 2005 American Chemical Society.

A new family of bioreductive quinones has been developed (Figure 18) as a target for DTD as a reductase enzyme for selective bioreduction.[99, 101, 104]

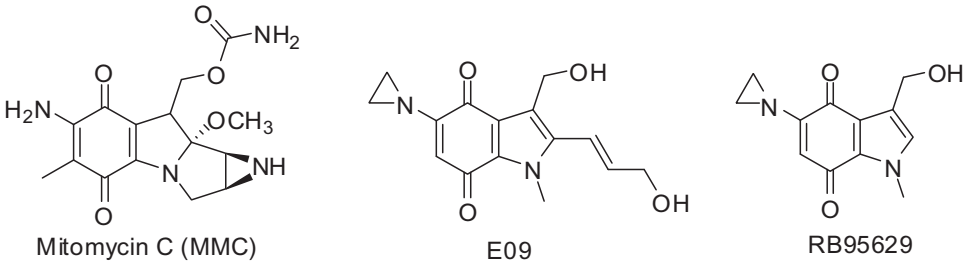


Figure 18. Bioreductive quinones

DTD is an enzyme that cells produce as protection from one electron reductions that give rise to free radicals and reactive oxygen species.[100] Ineffectiveness of first generation bioreductive drugs can be attributed, in part, to inactivation in aerobic environments due to the presence of O_2 and DTD. Ironically, the same mechanism used by DTD to deactivate these first generation bioreductive drugs, in turn, reduces the new family of quinones through a two electron process into a more reactive species that is capable of damaging DNA.[99] One of the early known substrates for this quinone family was mitomycin C (MMC). The mechanism of action of MMC after being reduced by DTD is to cross link adjacent guanine nucleobases in DNA.[99, 101, 105] Studies have shown that there is a six fold increase in activity when MMC is subjected to cell lines expressing DTD compared to cell lines that do not have DTD expressed.[99] Further development led to a new substrate referred to as E09 (Figure 18). E09 is an indolequinone that showed promising results, with a 49 fold activity compared to MMC in similar cell line studies.[99]

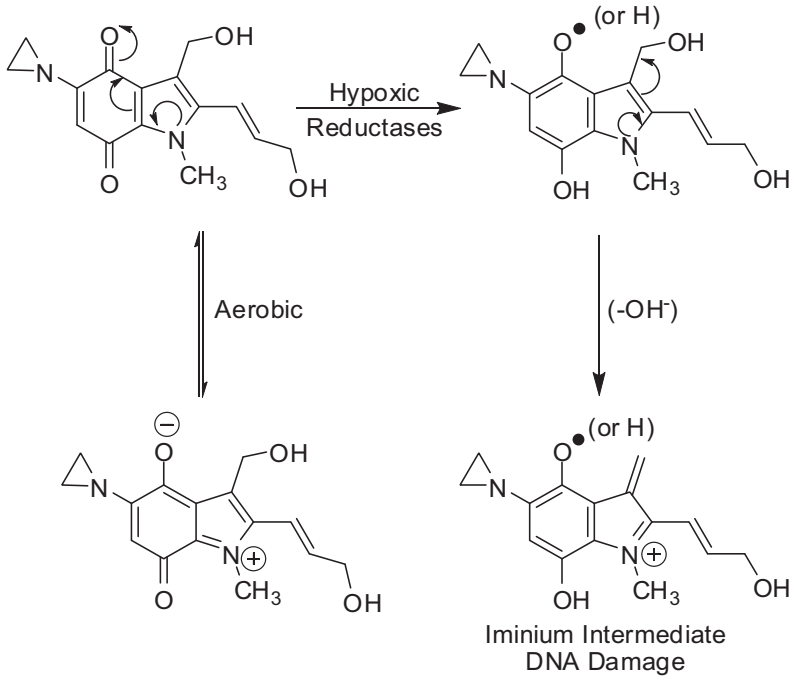


Figure 19. Reduction of E09 under aerobic and anaerobic conditions[99]

It is interesting to note that under aerobic conditions, DTD reduces E09 into its active bioreductive form, yet in hypoxic regions of the tumor that lack DTD, it was observed that E09 also damages anaerobic tumor cells (Figure 19).[99] It was discovered that cytochrome P-450 reductase, found in the hypoxic regions of the tumor, is able to reduce E09 into another active form that is also capable of damaging the tumor cells in anaerobic conditions.[99] This is a very important discovery that a single bioreductive drug can be reduced under both aerobic and anaerobic environments into its active drug form. Unfortunately, E09 did not pass phase II clinical trials which led researchers to search for a more potent analog of E09.[99]

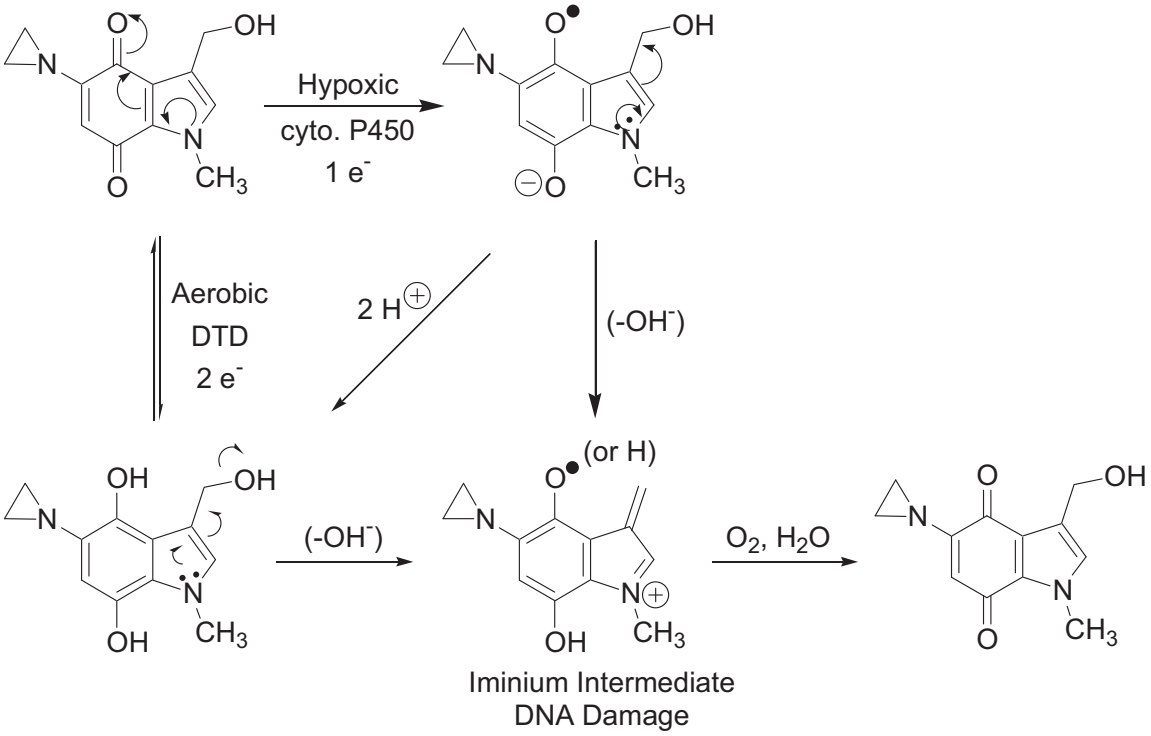


Figure 20. Reduction of RB95629

One such potent analog of E09 is indolequinone known as RB95629 (Figure 18).[99, 104] RB95629 has shown increased substrate reactivity with DTD as well as

increased cytotoxic potency compared to both MMC and E09 in both aerobic and anaerobic conditions. The potency of RB95629 and E09 can be attributed to the aziridine functional group and to the iminium intermediate formed through reduction under hypoxic conditions.[99] The aziridine functionality can alkylate DNA whereas the iminium intermediate can damage DNA. Both the aziridine functionality and the iminium intermediate enable increased alkylation of DNA. Another benefit attributed to RB95629 is that it consists of a primary alcohol and when RB95629 is reduced to form the iminium intermediate (Figure 20), it is able to cleave this alcohol functional group, which is a good strategy when coupling bioreductive triggers to VDAs as will be described later in this chapter.

The goal of this project was to create a bioreductive prodrug where a VDA is coupled to a bioreductive agent.[95] The idea was that the VDA-bioreductive prodrug conjugate would be inactive in this form however, in the presence of certain reductases in tumors, the bioreductive agent attached to the VDA would be reduced, releasing both the active form of the bioreductive agent and the active form of the VDA. This allows the VDA to act upon the tumor vasculature while the bioreductive agent damages DNA. It enables for one method of delivery to yield two different agents to damage the tumor cells (Figure 21).

Phenstatin[91] (referenced in chapter 3) was chosen as the cytotoxic portion of the bioconjugate molecule and an analog of RB95629 itself was chosen as the bioreductive agent (Figure 22). RB95629 was not chosen since it would be necessary to incorporate an aziridine moiety to the bioreductive prodrug. The analog of RB95629 **64** differs only from RB95629 by the incorporation of a methoxy group, instead of an aziridine moiety.

If the RB95629 analog could be successfully coupled to the VDA to form the bioreductive prodrug, then the methoxy functionality could be converted to the aziridine moiety at a later stage in the synthesis. Initially, the aziridine moiety was avoided due to its hazardous alkylating DNA effects and the added complexity that it contributes to the overall synthetic strategy. The aziridine moiety is also highly reactive, which makes it very sensitive to reaction conditions. If the aziridine moiety is incorporated at an early stage, it may react in the subsequent stages and form undesired byproducts. Therefore, it is ideal to introduce the aziridine moiety in the last stages of the synthetic methodology.

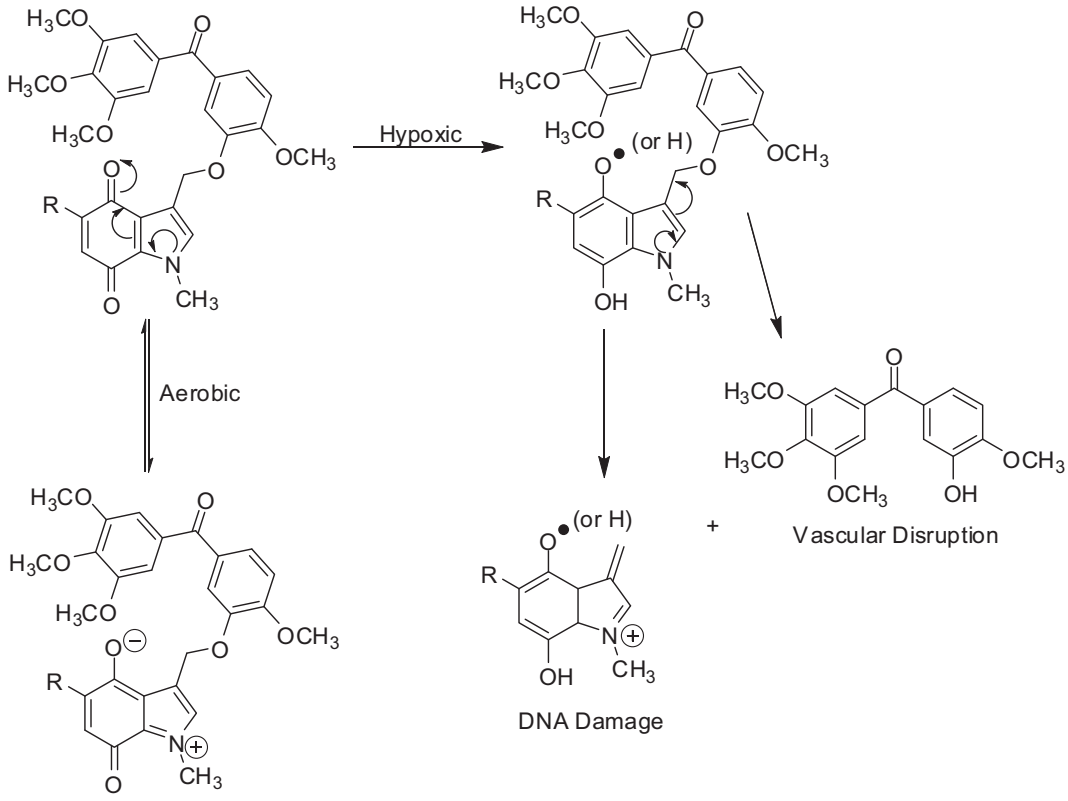


Figure 21. Reduction of bioreductive prodrug conjugate

The ultimate goal with the phenstatin-RB95629 analog bioreductive prodrug is to achieve selective enzyme mediated cleavage within the hypoxic region of the tumor

microenvironment. As previously stated, the reduction of molecular conjugate **65** in the hypoxic environment should facilitate conversion of the RB95629 analog to form the corresponding iminium intermediate which will subsequently cleave the bridging oxygen of the linked phenstatin, releasing the cytotoxic agent in its active form (Figure 23).

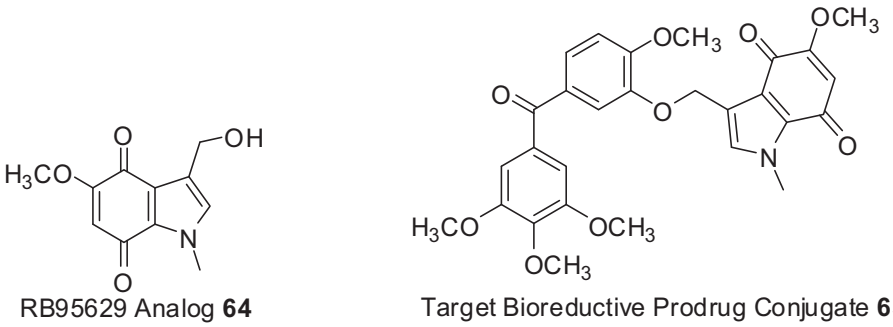


Figure 22. Target bioreductive trigger, RB95629 analog **64** and target bioreductive prodrug conjugate **65**

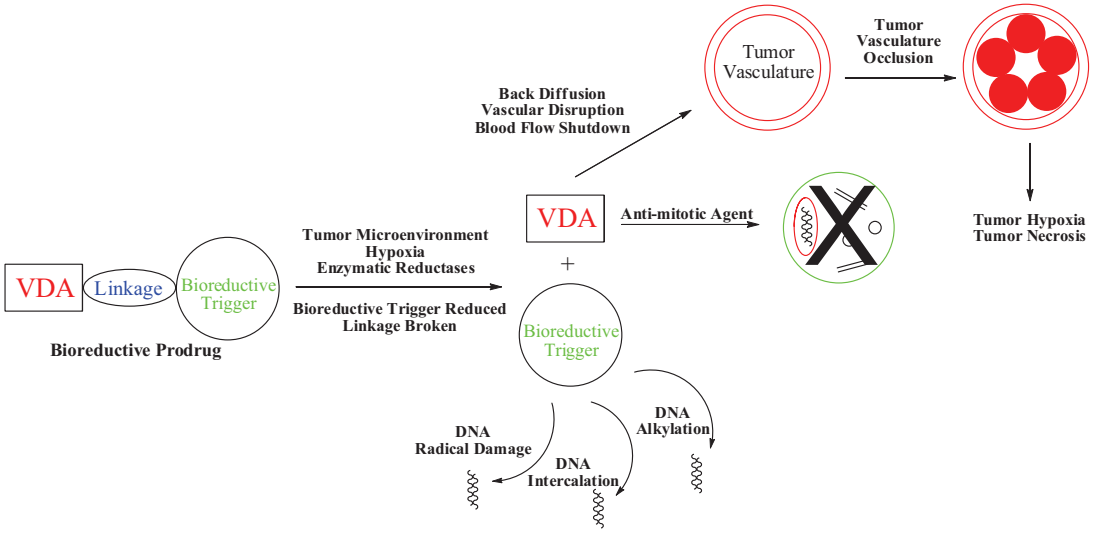


Figure 23. Bioreductive prodrug conjugate reduction to release chemotherapeutic drugs

Once the RB95629 analog is released, it can alkylate DNA in both aerobic and anaerobic tumor microenvironments.[99, 106] Phenstatin can back diffuse into the tumor vasculature and disrupt blood flow to the tumor or it can act as an anti-mitotic agent.

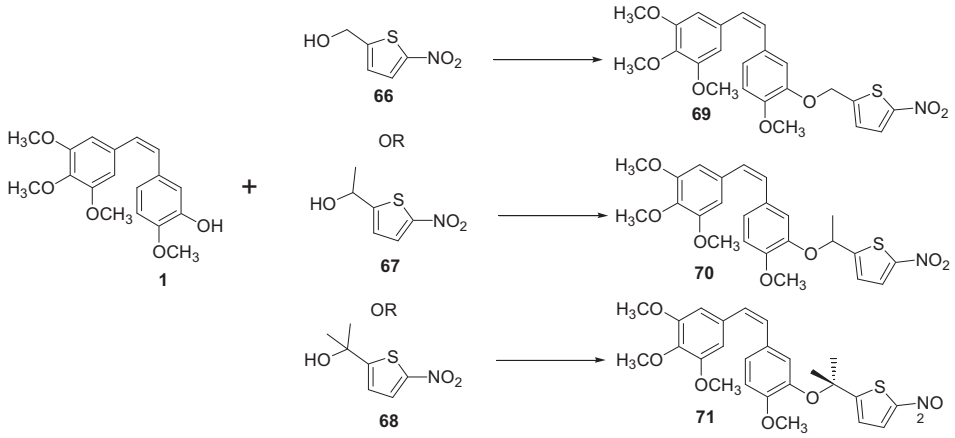


Figure 24. CA1-nitrothiophene bioreductive prodrug conjugate analogs[106]

Peter Davis and a group at the University of Oxford coupled 2-(5-nitrothien-2-yl)propan-2-ol to a known VDA, combretastatin A-4 (CA4), to form the bioreductive prodrug **71 (**figure 24).[106] Davis and coworkers carried out a comparison of various bioreductive prodrugs of CA4 coupled to different nitrothiophene analogs. The VDA, in parent drug form, employed in this study is CA4. The bioreductive prodrug triggers included normethyl, monomethyl, and *gem*-dimethyl nitrothiophene derivatives. Coupling of CA4 to the bioreductive triggers afforded bioreductive prodrugs **69, 70,** and **71 (**Figure 24).[106]

In figure 25, graph A shows the release of CA4 from the various bioreductive prodrugs in the presence of P450 supersomes enzyme reductases in aerobic conditions. In aerobic conditions, the nitrothiophenes are not able to be reduced, hence little to no CA4 is released. On the other hand, under anaerobic conditions as shown in graph B of

Figure 25, bioreductive prodrugs **69** and **70** are not reduced, but the *gem*-dimethyl nitrothiophene prodrug **71** is reduced, thus releasing CA4 **1** (Figure 25).[106]

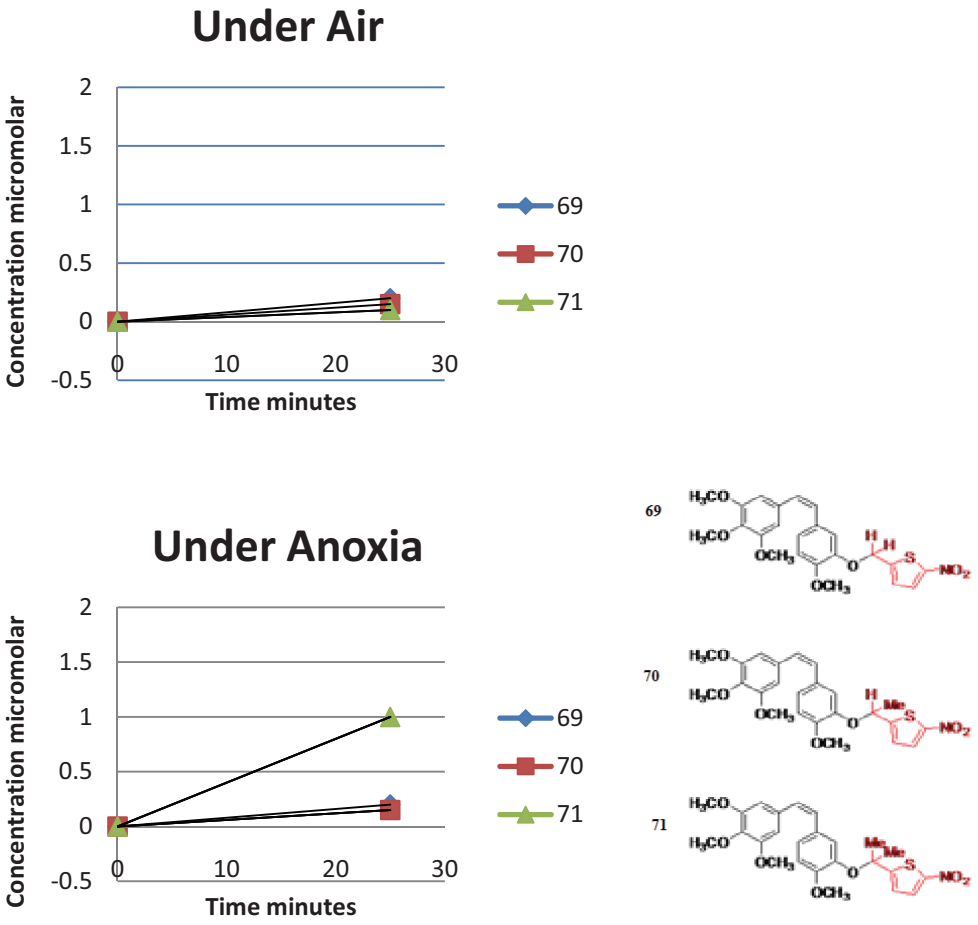


Figure 25. Reduction of the *gem*-dimethyl nitrothiophene analog **71** in anoxic conditions[106]

In order to evaluate the durability and metabolic stability of the bioreductive drugs, Davis and coworkers studied the metabolism of these bioreductive prodrugs within mouse liver. The *gem*-dimethyl bioreductive prodrug **71** is resistant to metabolism by the liver, which enhances its availability within the body and allows it time to reach the tumor cells (Figure 26). Both the monomethyl and normethyl bioreductive prodrug analogs were metabolized within the first few minutes. If bioreductive prodrugs of these

types are readily metabolized by the body, their chances of reaching the tumor are decreased dramatically.[106]

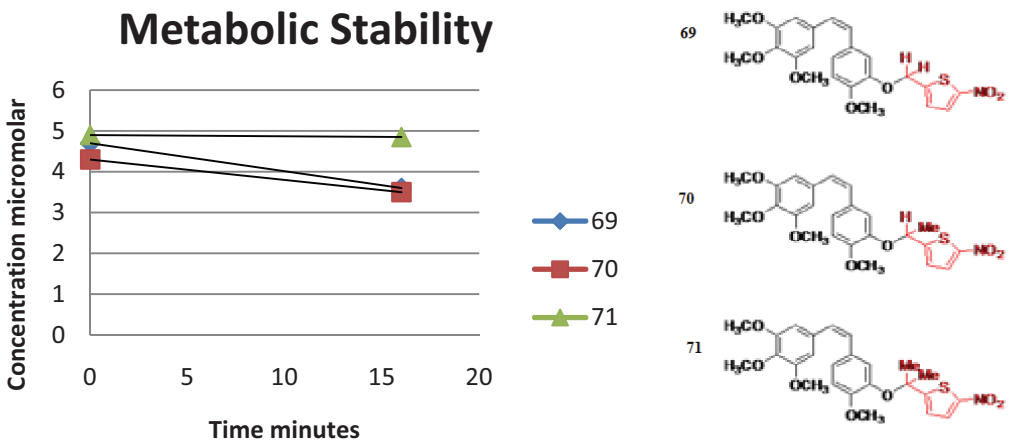


Figure 26. Metabolic stability of the bioreductive prodrug conjugates within mouse liver[106]

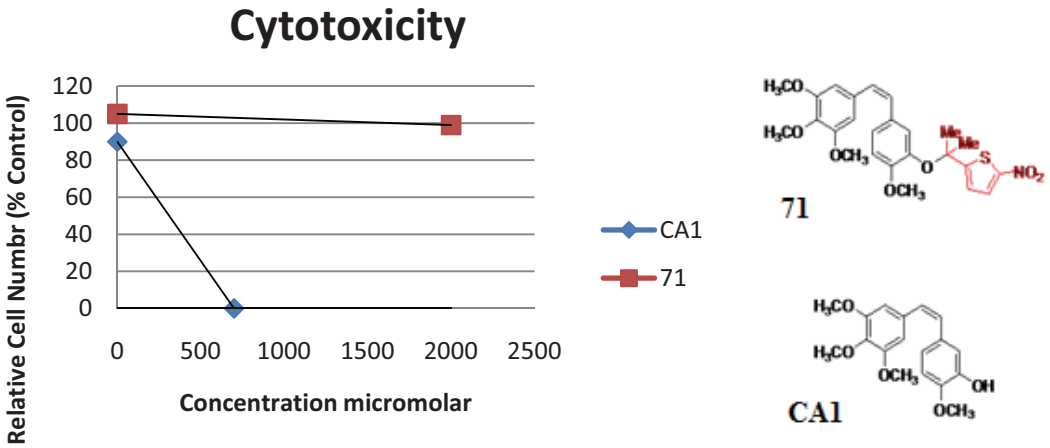


Figure 27. Cytotoxicity of bioconjugate **71** compared to CA4[106]

New drug candidates must be evaluated for their cytotoxicity. A comparison of the cytotoxicity of bioreductive prodrug **71** along with CA4 is illustrated in Figure 27. With increasing concentrations of bioreductive prodrug **71**, there is relatively no

associated increase in cytotoxicity. However, as the concentration increases for CA4, the relative cell number in the presence of CA4 decreases to zero very rapidly. The conclusion for this study is that a high concentration of the bioreductive prodrug causes only minimal cytotoxicity compared to the analogous study of CA4, *in vitro*.[106]

It is encouraging to consider whether these results obtained *in vitro* will correlate to observations made *in vivo*. If so, then one would be able to achieve a high dose of the bioreductive prodrug with limited cytotoxicity. Selective cytotoxicity for the tumor microenvironment would be achieved upon cleavage of the prodrug within the hypoxic environment.

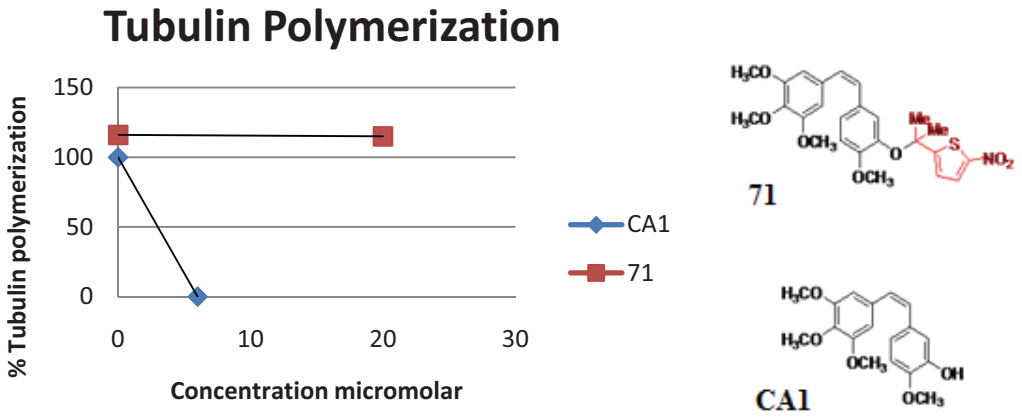


Figure 28. Tubulin polymerization study of bioconjugate **71**[106]

The ability of these bioreductive prodrugs to inhibit tubulin assembly needs to be examined. Theoretically, the bioreductive prodrug should be inactive since the parent VDA is linked to the bioreductive trigger preventing the VDA from binding to its desired substrate and the bioreductive drug from damaging DNA. It could be considered that the bioreductive drug is a "protected" form of both parent drugs, preventing inhibition of tubulin assembly. Davis and his group tested their bioreductive prodrug with CA4 linked

to the *gem*-dimethyl nitrothiophene to see it would have any effect on tubulin polymerization. They also did a comparative study of their bioreductive prodrug with the parent VDA, CA4. From their study, increasing the concentration of the bioreductive prodrug led only to a minimal change in tubulin polymerization whereas increasing the concentration of CA4 lead to a drastic reduction in tubulin polymerization with it almost ceasing at a concentration of 5 μM of CA4 (Figure 28).[106]

Due to the low cytotoxicity, resistance to liver metabolism, and ease of reduction in hypoxic regions of the tumor microenvironment, the 2-(5-nitrothien-2-yl)propan-2-ol[106] (*gem*dimethyl nitrothiophene) was chosen as the bioreductive trigger to couple with the selected VDAs, phenstatin and Oxi8006, to synthesize the novel bioreductive prodrugs.

Materials and Methods

Reactions were performed under an inert atmosphere using nitrogen gas unless specified differently. Chemical reagents used in the synthetic procedures were obtained from various chemical suppliers (Sigma Aldrich, Acros Chemical Co., Alfa Aesar, Fisher Scientific, EMD Chemicals, and VWR). Silica gel (200-400 mesh, 60 Å), used for column chromatography, was obtained from either Silicycle Inc or VWR. TLC plates (pre-coated glass plates with silica gel 60 F254, 0.25 mm thickness, EMD chemicals, VWR) were used to monitor reactions. A Biotage Isolera Four automated flash column chromatography system was used to separate compounds. A Biotage microwave system was used to conduct microwave reactions. Intermediates and products synthesized were characterized based on ^{1}H NMR (Bruker DPX operating at 300 MHz or Varian operating at 500 MHz), ^{13}C NMR (Bruker DPX operating at 75 MHz or Varian operating at 125

MHz), and ^{31}P NMR (Bruker DPX operating at 121 MHz or Varian operating at 202 MHz). All the chemical shifts are expressed in ppm (δ), coupling constants (*J*) presented in Hz, and peak patterns are reported as broad (br), singlets (s), doublets (d), triplets (t), quartets (q), septets (sept), and multiplets (m). Elemental analysis was performed by Atlantic Microlab, Norcross, GA. High-resolution mass spectra (HREIMS), unit resolution gas chromatography mass spectra (EIMS), and unit resolution mass spectra (ESIMS) were obtained on a VG Prospec Micromass spectrometer, a Thermo Scientific DSQ II, and a Thermo Finnigan LCQ Classic, respectively, in the Baylor University Mass Spectrometry Core Facility.

Synthesis of 3-(Hydroxymethyl)-5-methoxy-1-methylindole-4,7-dione **64**[104]

5-Methoxy-1-methylindole-3-carboxaldehyde ***73***:[104] In a 250 mL round bottom flask, 5-methoxy-1-methylindole-3-carboxaldehyde **72** (2.0 g, 11.4 mmol) was added gradually to a suspension of NaH (0.55 g, 13.7 mmol) in DMF (50 mL) while stirring. The suspension was stirred for 0.5 h, MeI (1.94 g, 13.7 mmol) was added, and the mixture was stirred for 1 h at room temperature. The reaction mixture was then poured into $NaHCO_3$ (10%, 300 mL), extracted with EtOAc (4 x 75 mL), washed with $NaHCO_3$ (10%, 3 x 500mL), saturated NaCl (3 x 100mL), and dried. The excess solvent was removed *in vacuo* to afford compound **73** (1.64 g, 8.67 mmol, 76%).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.85 (s, 3 H, C*H*$_3$N), 3.90 (s, 3 H, C*H*$_3$O), 7.00 (dd, 1 H, *J* = 2 and 9 Hz, Ar-6*H*), 7.26 (d, 1H, *J* = 9 Hz, Ar-7*H*), 7.63 (s, 1 H, 2-H), 7.80 (d, 1 H, *J* = 2 Hz, Ar-4*H*), and 9.96 (s, 1H, C*H*O).

5-Methoxy-1-methyl-4-nitroindole-3-carboxaldehyde ***74***:[104] Compound **73** (1.50 g, 7.94 mmol) was dissolved in AcOH (150 mL). A mixture of concentrated HNO_3 (4.5 mL) in AcOH (25 mL) was added dropwise to the solution over 3 h at 0°C. The mixture was then stirred for 16 h at room temperature, quenched with crushed ice, filtered through a filter funnel, washed with distilled water (5 x 100 mL), and dried. The excess AcOH was removed *in vacuo* to yield compound **74** (1.46 g, 6.23 mmol, 78%).

^{1}H NMR ($(CD_3)_2SO$, 500 MHz) δ 3.90 (s, 3 H, C*H*$_3$N), 3.96 (s, 3 H, C*H*$_3$O), 7.12 (d, 1 H, *J* = 9 Hz, Ar-7*H*), 7.46 (d, 1H, *J* = 9 Hz, Ar-6*H*), 7.84 (s, 1 H, 2-*H*), and 9.86 (s, 1 H, C*H*O).

4-Amino-5-methoxy-1-methylindole-3-carboxaldehyde ***75***:[104] The nitroindole **74** (1.0 g, 4.27 mmol) was dissolved in anhydrous ethanol (150 mL) and tin (4.43 g, 37 mmol) was added followed by HCl (12 M, 15 mL). The reaction was allowed to stir for 2 h and was decanted. The solution was then gradually added to saturated $NaHCO_3$ (300 mL) and extracted with EtOAc (3 x 100 mL). The organic layer was separated and washed with saturated $NaHCO_3$ (2 x 175 mL) and an NaCl solution (3 x 75 mL). The solution was dried and the solvent removed *in vacuo* to give compound **75** (0.42 g, 2.06 mmol, 48%).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.75 (s, 3 H, C*H*$_3$N), 3.90 (s, 3 H, C*H*$_3$O), 5.80 (br s, 2 H, N*H*$_2$), 6.55 (d, 1H, *J* = 9 Hz, Ar-7*H*), 6.95 (d, 1H, *J* = 9 Hz, Ar-6*H*), 7.53 (s, 1 H, 2-H), and 9.59 (s, 1H, C*H*O).

3-Formyl-5-methoxy-1-methylindole-4,7-dione ***76***:[104] Compound **75** (0.42 g, 2.06 mmol) was dissolved in acetone. Fremy's salt (potassium nitroso disulfonate) (2.24 g,

8.35 mmol) was dissolved in water and added to the solution followed by a Na_2HPO_4/NaH_2PO_4 buffer system. The mixture was stirred for 0.75 h. Rotary evaporation was used to remove the excess acetone and the remaining solution was filtered, washed with water, dried, and recrystalized from EtOAc to afford compound **76** (0.26 g, 1.19 mmol, 57%).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.87 (s, 3 H, C*H*$_3$N), 4.03 (s, 3 H, C*H*$_3$O), 5.77 (s, 1 H, 6-*H*), 7.45 (s, 1 H, 2-*H*), and 10.41 (s, 1 H, C*H*O).

3-(Hydroxymethyl)-5-methoxy-1-methylindole-4,7-dione ***64***:[104] Anhydrous MeOH (300 mL) was degassed under argon and compound **76** was added followed by $NaBH_4$. The resulting solution was degassed with argon and stirred for two hours. The solvent was evaporated using rotary evaporation and the solid was then diluted with CH_2Cl_2, washed with water (2 x 100 mL), saturated with NaCl (100 mL), condensed, and recrystalized from EtOAc to give compound **64** (0.13 g, 0.59 mmol, 50%).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.84 (s, 3 H, C*H*$_3$N), 3.93 (s, 3 H, C*H*$_3$O), 4.65 (d, 2 H, *J* = 7, C*H*$_2$OH), 5.69 (s, 1 H, 6-*H*), and 6.70 (s, 1 H, 2-*H*).

Synthesis of 5-Methoxy-3-[2-methoxy-6-(3,4,5-trimethoxy-benzoyl)-phenoxymethyl]-1-methyl-1H-indole-4,7-dione ***65***

5-Methoxy-3-[2-methoxy-6-(3,4,5-trimethoxy-benzoyl)-phenoxymethyl]-1-methyl-1H-indole-4,7-dione ***65***: In a round bottom flask compound **64** (0.15 g, 0.69 mmol) was weighed and dissolved in approximately 10 mL of anhydrous benzene. Azodicarboxylic dipiperidide (ADDP, 0.24 g, 0.94 mmol) and compound **61** (0.20 g, 0.63 mmol) were added to the reaction flask. Either Tributylphosphine **A** (0.23 mL, 0.95 mmol) or

triphenylphosphine **B** (0.25 g, 0.95 mmol) (Scheme 18) was added to the reaction flask and was allowed to stir for 24 to 48 hours or until no starting material was present. The mixture was then transferred into a separatory funnel. The organic layer was extracted from the water layer using ethyl acetate (3 x 200 mL). A brine solution was used to wash the organic layer. The organic layer was extracted and dried with sodium sulfate. The solution was filtered and then concentrated using rotary evaporation. This reaction was not successful.

Synthesis of 2-(5-Nitrothien-2-yl)propan-2-ol ***81***[106]

2-Thien-2-yl-propan-2-ol ***80*****:** In an oven-dried round bottom flask was added 2-acetylthiophene **79** (10.0 g, 79.2 mmol) under nitrogen. Vacuum was applied to the flask in order to evacuate it. The flask was refilled with nitrogen gas. This was repeated three times. Anhydrous diethyl ether (150 mL) was added to the flask to dissolve the 2-acetylthiophene and it was cooled to -10 ^{o}C using an acetone/ice bath. The methylmagnesium bromide was slowly added to the reaction and it was stirred for three and a half hours and returned to room temperature. It is important that there is enough solvent along with vigorous stirring to prevent the solids formed from impeding the stirring. Upon completion, the reaction mixture was poured into an Erlenmeyer flask with ice (10-15 g). HCl (0.1 M) was added to the Erlenmeyer flask (200 mL). The acidified reaction mixture was extracted using a separatory funnel and the organic layer was collected. The water layer was washed with diethyl ether (3 x 100 mL). The combined organic layers were washed once with brine (200 mL), dried using sodium sulfate, and concentrated using a rotary evaporator. The crude product was subjected to

flash column chromatography to yield the pure product as a yellow oil (3.3 g, 23.2 mmol, 29%).

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.67 (s, 1H), 2.06 (s, 1H), 6.95 (m, 2H), 7.19 (dd, *J* = 1.5, 4.8 Hz, 1H)

2-(5-Nitrothien-2-yl)propan-2-ol 81**:** 2-Thien-2-yl-propan-2-ol **80** (0.500 g, 3.5 mmol) was dissolved in acetic anhydride (15 mL). If stirring proved difficult additional aliquots of acetic anhydride were added until stirring was sufficient. The reaction mixture was cooled to -70 ^{o}C using a dry ice/acetone bath. Under vigorous stirring, slowly add dropwise the fuming nitric acid (0.16 mL, 3.5 mmol) and stir for 1 hour allowing the reaction mixture to warm up to -40 ^{o}C. After the first hour, stir an additional hour at 40 ^{o}C. The reaction was quenched by the slow addition of ice. The reaction turned red, and after additional stirring changed to purple, green and then yellow after a total of 45 minutes. The reaction mixture was transferred to a separatory funnel, ethyl acetate (100 mL) was added and initially, there were no layers. Saturated sodium bicarbonate (100 mL) was added and two layers formed. The organic layer was separated and the aqueous layer was extracted with ethyl acetate (3 x 75 mL). The organic phases were combined and washed with saturated sodium bicarbonate, dried with sodium sulfate, filtered, and concentrated under reduced pressure. The black crude product was subjected to flash column chromatography to yield a dark red/black oil as pure product (0.456 g, 2.44 mmol, 30% yield).

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.67 (s, 6H), 6.87 (d, *J* = 4.2 Hz, 1H), 7.77 (d, *J* = 4.2 Hz, 1H)

*Synthesis of 2-(3'-[1-methyl-1-(5-nitro-thien-2-yl)ethoxy]-4'-methoxyphenyl)-3-(3",4",5"-trimethoxybenzoyl)-6-methoxyindole **84***

*2-(3'-[1-Methyl-1-(5-nitro-thien-2-yl)ethoxy]-4'-methoxyphenyl)-3-(3",4",5"-trimethoxybenzoyl)-6-methoxyindole **84***: The VDA 2-(3′-hydroxy-4′-methoxyphenyl)-3-(3″,4″,5″-trimethoxybenzoyl)-6-methoxyindole **83** (Oxi8006, 0.097 g, 0.21 mmol), triphenylphosphine (0.055 g, 0.21 mmol), and 2-(5-nitrothien-2-yl)propan-2-ol **81** (0.020 g, 0.10 mmol) were dissolved in THF (1.2 mL) and stirred for 10 minutes under N_2. The reagent DEAD (0.033 mL, 0.21 mmol) was added slowly over a 15 minute period to the reaction mixture. The reaction mixture was stirred for 24-72 hours and monitored by TLC to check for product formation. Close monitoring of solvent is necessary to ensure not too much solvent has evaporated. When stirring overn nitrogen for long periods, the solvent may evaporate. If so, then add more THF as necessary. This reaction was not successful and some starting material was recovered.

*Synthesis of {4-methoxy-3-[1-methyl-1-(5-nitro-thien-2-yl)ethoxy]phenyl}-(3,4,5-trimethoxy-phenyl)methanone **82***

*4-Methoxy-3-[1-methyl-1-(5-nitro-thien-2-yl)-ethoxy]-phenyl}-(3,4,5-trimethoxy-phenyl)methanone **82***: Phenstatin **61** (0.200 g, 0.63 mmol), ADDP (0.148 g, 0.59 mmol), and 2-(5-nitrothien-2-yl)propan-2-ol **81** (0.110 g, 0.59 mmol) were dissolved in benzene (2.5 mL) and allowed to stir for 10 minutes under N_2. Tributylphosphine (0.145 mL, 0.59 mmol) was dissolved in benzene (0.5 mL) and added to the reaction mixture. The reaction mixture was stirred for 72 hours with close monitoring to ensure the solvent has not all evaporated and by TLC to ensure product formation. When the reaction was complete, benzene was removed under reduced pressure using a rotary evaporator. The

resulting crude product was subjected to column chromatography to yield a grey solid (0.025 g, 0.049 mmol, 8%)

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.76 (s, 3H), 3.87 (s, 3H), 3.90 (s, 3H), 3.94 (s, 3H), 6.93 (d, J = 2.4 Hz, 1H), 6.97 (s, 2H), 7.05 (d, J = 5.1 Hz, 1H), 7.37 (s, 1H), 7.59 (d, J = 5.1 Hz, 1H) 7.78 (d, J = 2.4 Hz, 1H)

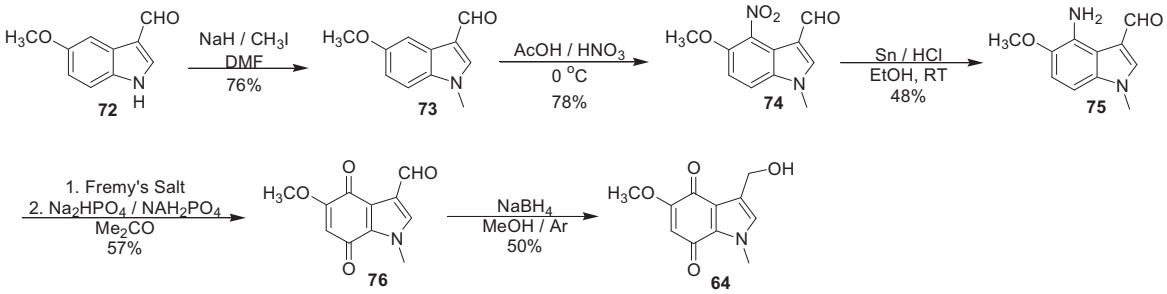
Results and Discussion

The synthesis of these bioreductive triggers and their related analogs are known in the literature.[104, 106] Modification of these procedures was required to form the bioreductive prodrug conjugate; where the bioreductive trigger is linked to the VDA. The design rationale is to synthesize the bioreductive trigger while being aware of any sensitive functional groups as well as synthesizing the VDA. Once both parts of the bioreductive prodrug conjugate have been synthesized, the two compounds will be coupled together depending on the available functional groups.

The final alcohol product **64** (Scheme 18), obtained by the reduction of indolequinone **76** using $NaBH_4$, is a derivative of E09, and differs only by incorporating a methoxy functional group instead of E09's highly reactive aziridine functional group. The strategy was to couple this E09 derivative **64** to the previously synthesized phenstatin **61** (chapter 3) and then convert the methoxy group on the E09 analog portion to an aziridine functional group in the final step. The addition of the aziridine functional group increases the bioreductive drug's reactivity by making the bioreductive drug a more potent DNA alkylating agent.

Synthesis of 3-(hydroxymethyl)-5-methoxy-1-methylindole-4,7-dione ***64*** [104]

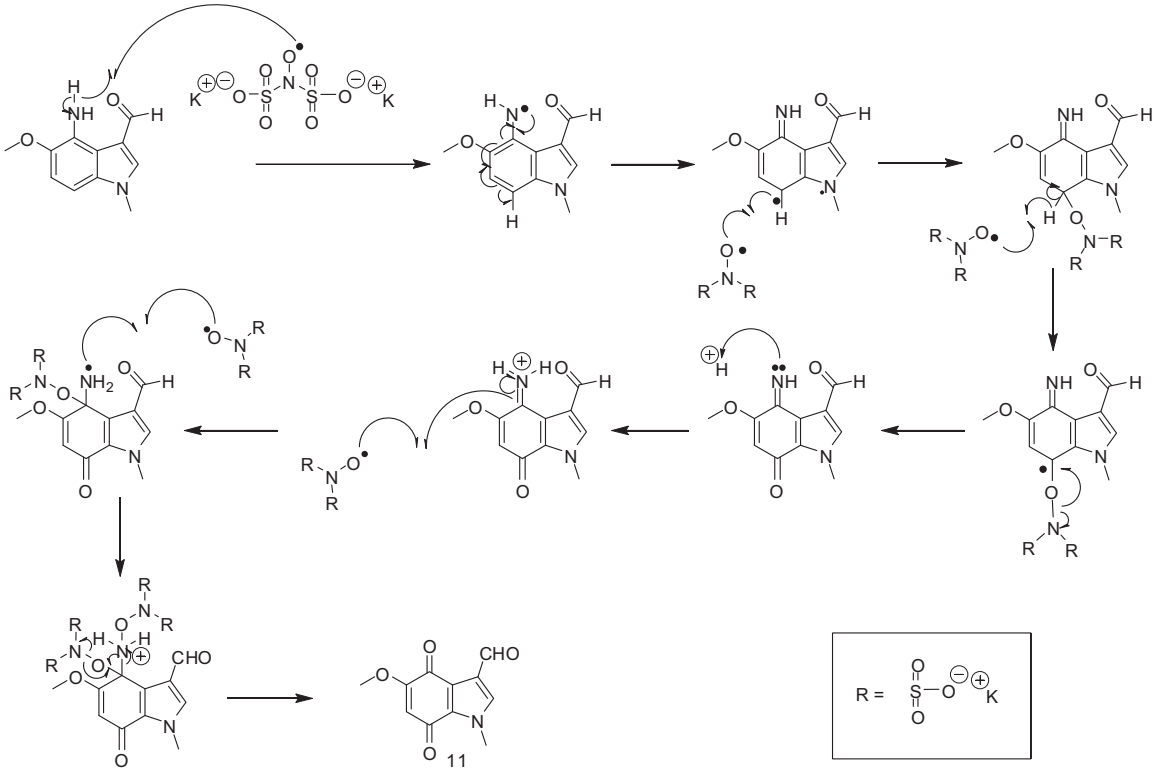
The starting material, 5-methoxy-1-methylindole-3-carboxaldehyde **72**, was purchased from the Aldrich Chemical Company. This indole **72** was slowly added to a suspension of NaH in DMF and stirred for a short period of time to allow the strong base, NaH, to remove the acidic hydrogen from the nitrogen of the indole.



Scheme 18. Synthesis of RB96529 analog **64**[104]

MeI was slowly added to the reaction mixture to methylate the nitrogen of the indole thus affording the methylindole **73** in 76% yield (Scheme 18). The methylindole **73** underwent nitration when it was dissolved in AcOH and a mixture of AcOH and concentrated HNO_3 was slowly added over a three hour period at 0 ^oC. The nitronium ion cation (formed *in situ* from AcOH and HNO_3) selectively reacted at the 4 position of the methyl indole to yield the nitro indole **74** in 78%. The nitro functional group of the nitroindole **74** was reduced to its corrsponding amine using the metal catalyst, tin in the presence of HCl to afford the desired aminated product **75** in a 48% yield (Scheme 18). The aminated indole **75** formed the indolequinone **76** through a radical mechanism with Fremy's salt (potassium nitroso disulfonate) in a 57% yield. The actual Fremy's salt mechanism is not totally understood but a hypothesized mechanism is presented (Scheme 19). Fremy's salt can donate an electron to the amine hydrogen and through a cascade of electron movement, place a radical on the 7th carbon of the indole. At this position, 7, of

the indole, another molecule of Fremy's salt will form a bond by donating an electron to the radical at position 7.

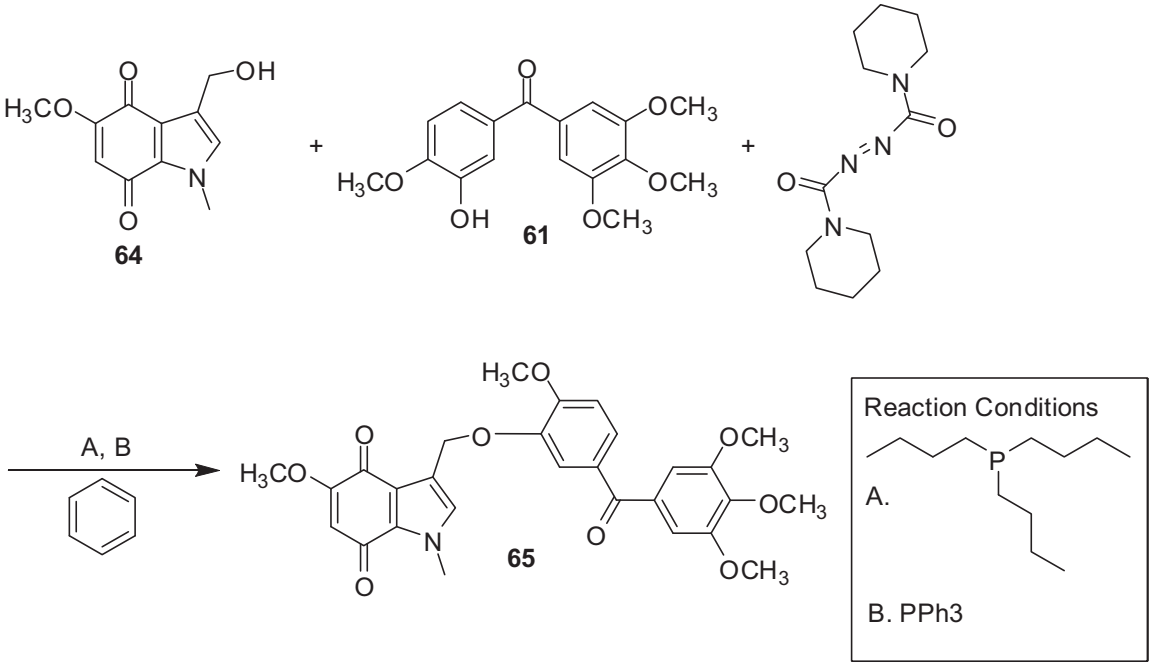


Scheme 19. Indolequinone formation of **76** through Fremy's salt radical mechanism

A third molecule of Fremy's salt will use its single electron to deprotonate the hydrogen at the 7 position and leave a single radical behind. The remaining Fremy's salt moiety attached to position 7 will donate an electron from the oxygen and nitrogen bond to the radical at position 7 to form a double bond between the carbon at position 7 and the oxygen. This completes the first part of the indolequinone. Protonation occurs at the 4 position of the indole followed by addition of a Fremy's salt molecule at the 4 position carbon, breaking the double bond to the nitrogen and giving a radical to the nitrogen. The radical located on the nitrogen of the amine will form a bond with another molecule of Fremy's salt causing the nitrogen on this amine to have four bonds and be cationic. To

alleviate this positive charge on the nitrogen, the bond between the nitrogen and carbon at the 4 position undergoes homolytic cleavage, with one electron going to the nitrogen and another electron going to the carbon at the 4 position. The remaining Fremy's salt moiety attached to the 4 position of the indole will donate an electron from its N-O bond to form a double bond with the oxygen and carbon, completing the formation of the indolequinone. After the indolequinone **76** was formed, the aldehyde functional group was reduced using $NaBH_4$ in methanol to give the final alcohol product **64** in 50% yield (Scheme 18).

A Mitsunobu reaction was attempted as a strategy to successfully couple the desired bioreductive trigger **64** to the VDA. Two different Mitsunobu phosphine reagents, tributylphosphine and triphenylphosphine, were employed. These phosphine reagents were chosen since they vary in their size of their substituents on the phosphorus atom (Scheme 20).



Scheme 20. Varying conditions of Mitsunobu coupling

Synthesis of 5-methoxy-3-[2-methoxy-6-(3,4,5-trimethoxy-benzoyl)-phenoxymethyl]-1-methyl-1H-indole-4,7-dione ***65***

The E09 derivative **64** was dissolved in benzene and azodicarbonyl dipiperidine (ADDP) along with phenstatin **61** were added with either tributylphosphine **A** or triphenylphosphine **B** (Scheme 20). Initially, the phosphine reagent, either tributylphosphine **A** or triphenylphosphine **B**, complexes with the ADDP. The lone pair of electrons on the alcohol on the E09 derivative will attack the positively charged phosphorous on the phosphine-ADDP complex **77** which leads the ADDP to leave as a leaving group forming a new complex between the E09 derivative **64** and the phosphine (Figure 29). The oxygen bridging the E09 derivative and the phosphine is positively charged which provides a driving force for the phenolic functional group on the CA4 or phenstatin to undergo an S_N2 addition, displacing phosphine oxide as a leaving group to form the VDA-E09 derivative bioreductive prodrug (Figure 29).

Unfortunately, the desired bioreductive prodrugs were not obtained through this synthetic strategy. Several attempts were made to synthesize these compounds. A variety of modifications were made to the procedure. For instance, the reagents were changed. Tributyl and triphenyl phosphines as well as DIAD and ADDP were substituted for the Mitsunobu reactions. Changing the order of addition did not give any further success in coupling the VDA and bioreductive drug. Both the VDA and bioreductive drug were recovered to some degree and the phosphine oxide byproduct was observed.

For the Mitsunobu mechanism, there are two areas for concern that may prevent the coupling from occurring due to steric hinderance. The first area is after the ADDP-phosphine complex **77** is formed and the lone pair of electrons on the alcohol of the E09 derivative **64** attacks the positively charged phosphorous of the ADDP-Phosphine

complex **77**, displacing the ADDP (Figure 29). From observing the ADDP-phosphine complex **77**, it is a very bulky molecule with the ADDP blocking one side of the phosphorus area required for attack and three butyl are blocking the other side of the phosphorous. The E09 derivative **64** itself, is a moderately sized molecule, and the alcohol of the E09 derivative molecule **64** is a primary alcohol protruding from an alkyl (CH_2) chain, thus the chance of a successful attack on the positively charged phosphorous is increased slightly. Due to these steric hinderance issues of the ADDP-phosphine complex **77**, either a low yield or no yield of the E09 derivative-phosphine **78** complex may be formed (Figure 29).

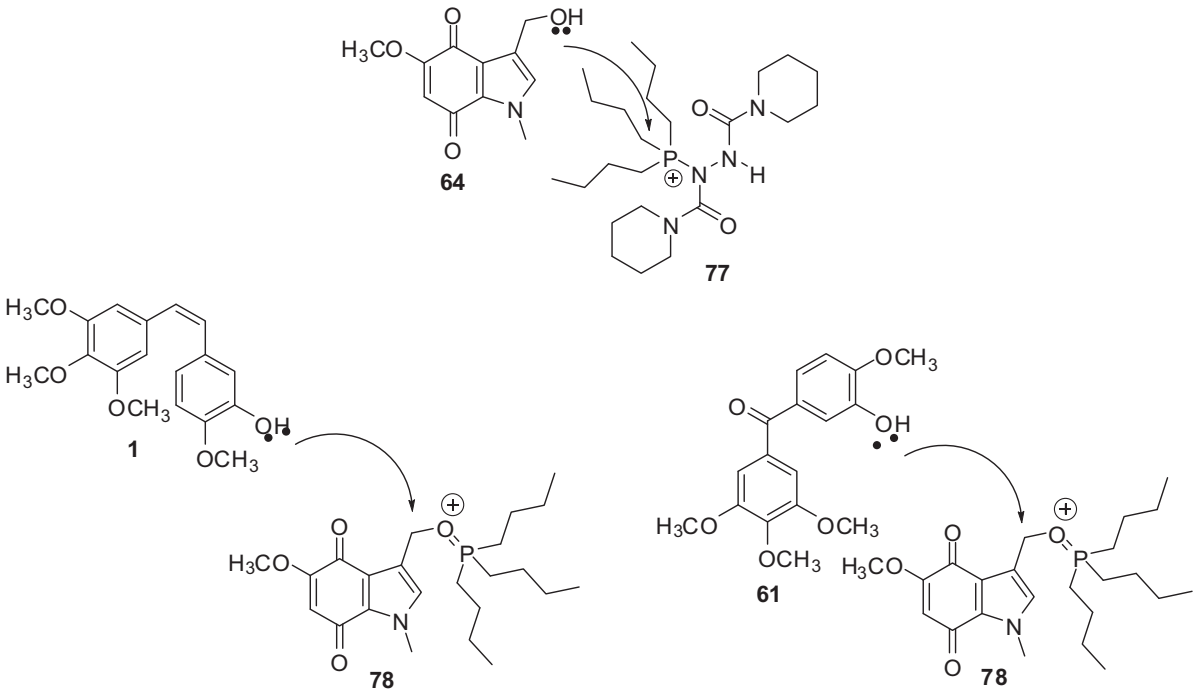


Figure 29. Formation of the Mitsunobu intermediate **78** and addition by CA4 **1** and phenstatin **61**

If the E09 derivative-phosphine complex **78** is successfully formed, it still must overcome one additional obstacle before completing the coupling of the VDA and the E09 derivative **64**. The E09 derivative-phosphine complex **78** has a positively charged

oxygen bridging the two compounds. The oxygen does not favor this, which drives a nucleophile, the lone pair of electrons on the phenolic oxygen of the VDA, to attack the carbon attached to the positively charged oxygen, giving electrons back to the oxygen and displacing the phosphine oxide as a by-product, coupling the VDA and E09 derivative **64**. Again, as can be observed, one side of the positively charged oxygen is blocked by long butyl groups and on the other is the E09 derivative **64**, which hinders the attack on the alpha carbon to the positively charged oxygen. The VDA has to overcome these steric issues in order to attack and couple with the bioreductive drug. Overall, for this reaction and its mechanism, there are two areas that need to be triumphed in order to couple the VDA and bioreductive drug and if at either stage, the process is hindered, then the overall reaction will not be successful.

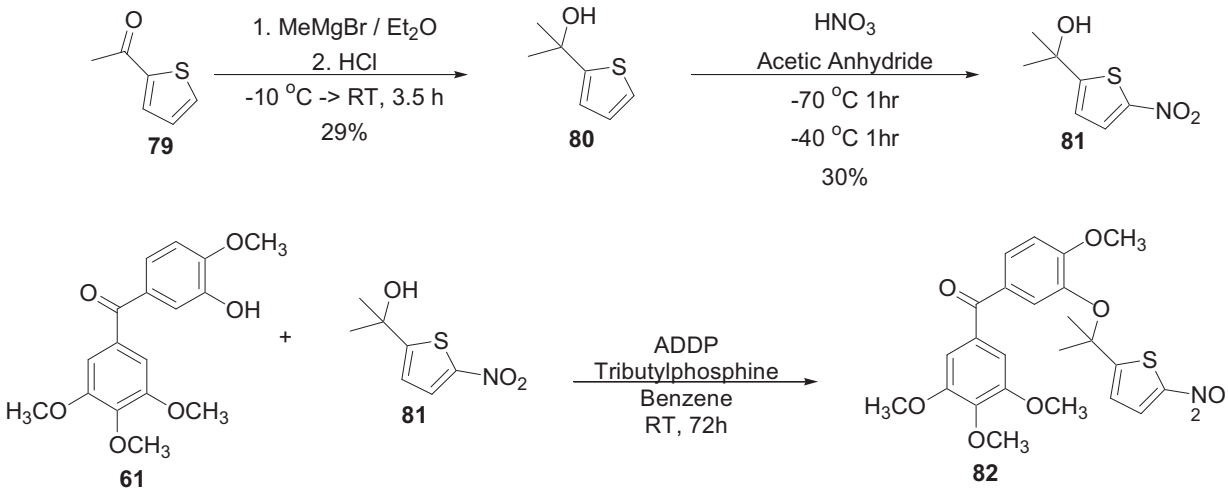
As stated earlier several attempts to couple the VDA and bioreductive drug were unsuccessful. Both the VDA and bioreductive drug were recovered as well as some triphenylphosphonium oxide. The conclusion is that an important intermediate in which the bioreductive drug is complexed to the phosphine does form, however the VDA is unable to attack and displace the phosphine to yield the coupled desired product and phosphine oxide. Instead, the complexed VDA to the phosphine remains in the reaction mixture until it is quenched and worked up in aqueous solution where water hydrolyzes the bioreductive drug and the phosphine complex releasing the E09 derivative **64** and triphenylphosphine oxide.

The order of addition has also been considered and changes were made accordingly such that the E09 derivative **64** was dissolved in benzene first along with ADDP and either tributylphosphine or triphenylphospine, and allowed to stir and react

for a certain period of time. There was some concern that the phenolic functionality of the VDA may react with the ADDP-phosphine complex **77** first as a competing reaction, hence the addition of the VDA in benzene last to the reaction mixture. Even so, the reaction did not go to completion and the VDA and bioreductive drug were recovered.

*Synthesis of {4-methoxy-3-[1-methyl-1-(5-nitro-thien-2-yl)ethoxy]phenyl}-(3,4,5-trimethoxy-phenyl)methanone **82***[106]

The starting material, 2-acetylthiophene **79**, was purchased from Aldrich chemical company. The 2-acetylthiophene **79** was subjected to a Grignard reaction with methylmagnesium bromide resulting in the incorporation of a methyl group to form the tertiary alchohol **80** in 29% yield (Scheme 21).



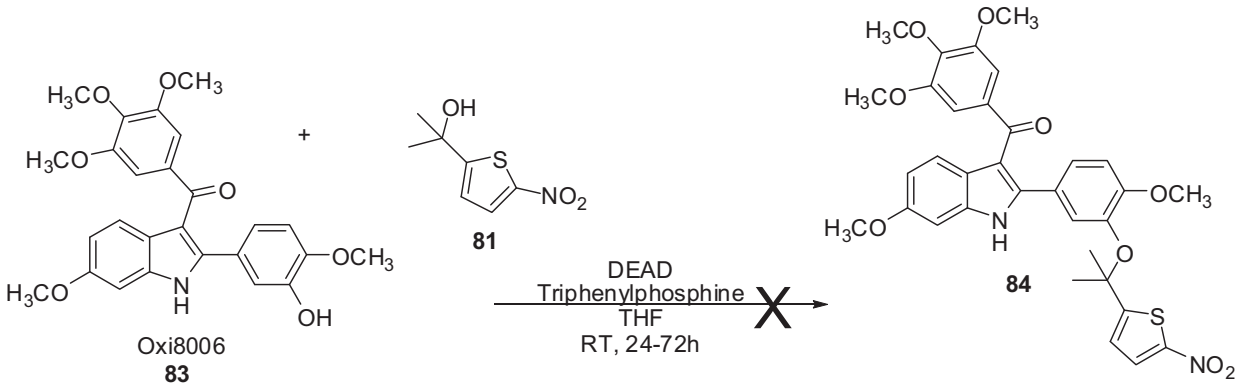
Scheme 21. Synthesis of bioreductive prodrug conjugate **82**[106]

Once the tertiary alcohol **80** was isolated and purified, it underwent nitration at the 5 position of the thiophene ring using nitric acid and acetic anhydride to give the nitrothiophene **81** in a 30% yield (Scheme 21). The nitrothiophene was then subjected to a Mitsunobu reaction to couple with cytotoxic agent phenstatin **61**. First the ADDP and tributylphosphine form a complex together which results in a positive charge on the

phosphorus of the tributylphosphine-ADDP complex **77**. This allows the tertiary alcohol of the nitrothiophene **81** to attack that positively charged phosphorus displacing the ADDP to form the nitrothiophene-tributylphosphine intermediate **86** (Figure 32). In this new nitrothiophene-tributylphosphine intermediate **86**, there is a positively charged oxygen bridging the two molecules together (Figure 32). The oxygen does not want a positive charge thus providing the driving force for the phenolic group on phenstatin **61** to undergo an S_N2-like addition to the carbon directly attached to the positively charged oxygen with displacement of triphenylphosphonium oxide. The end result is the coupling of the phenstatin, and the bioreductive drug, 2-(5-nitrothien-2-yl)propan-2-ol to form the bioreductive prodrug conjugate **82** in an 8% yield (Scheme 21).

*Synthesis of 2-(3'-[1-methyl-1-(5-nitro-thien-2-yl)ethoxy]-4'-methoxyphenyl)-3-(3",4",5"-trimethoxybenzoyl)-6-methoxyindole **84***

The synthesis of the bioreductive trigger, 2-(5-nitrothien-2-yl)propan-2-ol **81**, was discussed in the previous reaction. The nitrothiophene **81** attempted an ether linkage coupling with another known VDA, 2-(3'-hydroxy-4'-methoxyphenyl)-3-(3",4",5"-trimethoxybenzoyl)-6-methoxyindole or also known as Oxi8006 **83**. The Mitsunobu reaction between the nitrothiophene **81** and Oxi8006 **83** was not successful.



Scheme 22. Unsuccessful coupling of Oxi8006 **63** and nitrothiophene **81**

The reaction conditions for this Mitsunobou reaction was adapted from a fellow colleague's procedure, Dr. Rajsekhar Guddneppanavar (Pinney Reseach Group) developed for a separate compound. Since the reaction (Scheme 22) was not successful, another colleague, Matthew MacDonough, a new graduate student collaborating on this project, adapted ADDP and tributylphosphine as reagents for the Mitsunobu reaction and applied them to this reaction between nitrothiophene **81** and Oxi8006 **83**. The procedure Mr. MacDonough carried out was exactly the same as the successful coupling of phenstatin **61** and nitrothiophene **81**. The reaction also did not go to completion and for both reactions, a decent amount of the nitrothiophene **81** was recovered and little to no Oxi8006 **83** was recovered. As discussed previously, steric hinderance may play a part in preventing the successful coupling of the VDA and bioreductive drug (Figure 30).

The tertiary alcohol of the nitrothiophene bioreductive drug has to overcome the sterics of the tributyl or triphenyl functional groups when attempting to attack the positively charged phosphorous in the first part of the Mitsunobou reaction. For the second part of the Mitsunobu reaction, an S_N2 attack by the VDA, in this case, Oxi8006, at the tertiary carbon directly attached to the positively charged oxygen seems very hindered considering the tertiary carbon has two methyl groups protruding from the area of attack and since Oxi8006 is quite a large molecule. Another possible reason why the reaction was not successful may be may be attributed to the indole nitrogen of Oxi8006 functioning as a nucleophile.

Typically, the phenolic functionality of Oxi8006 will undergo the S_N2 attack to couple with the bioreductive drug. However, the nitrogen atom on the indole ring of

OXi8006, could also act as a nucleophile and compete in the final S_N2 reaction step of the Mitsunobu reaction.

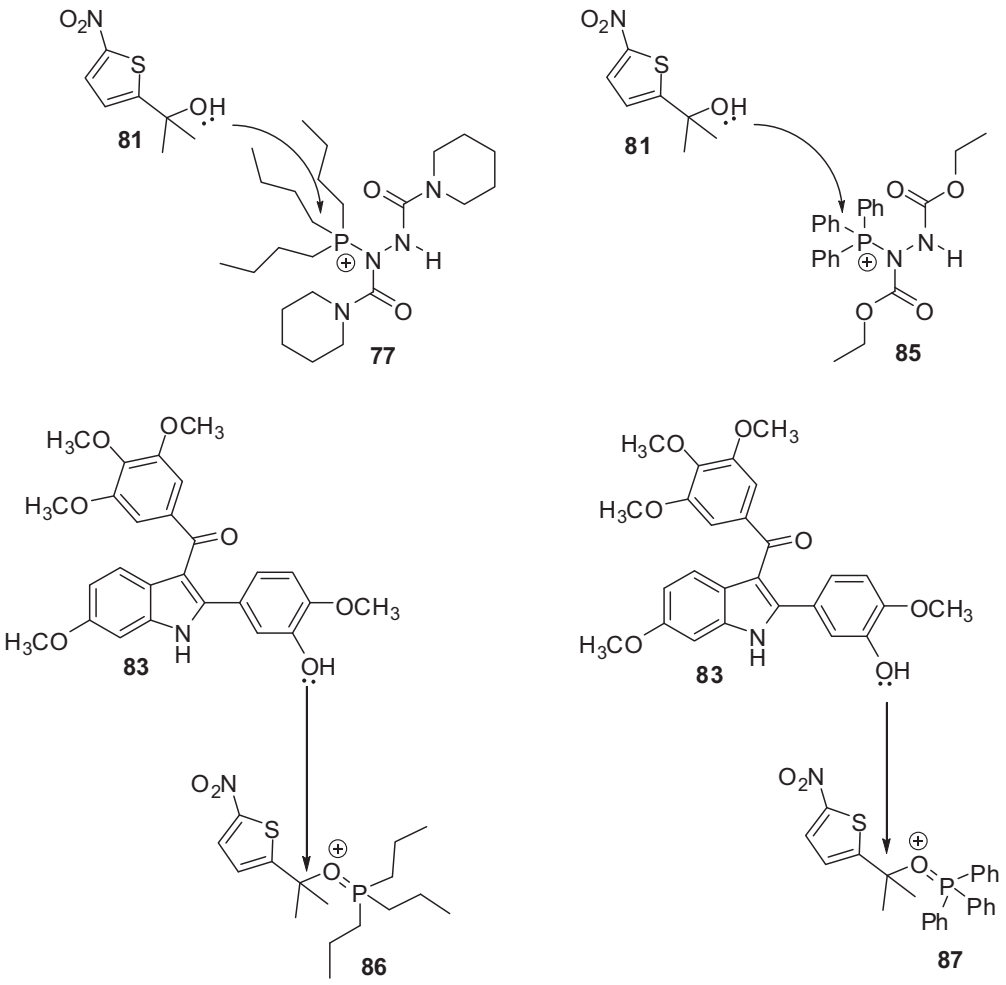


Figure 30. Steric hinderance preventing Mitsunobu coupling

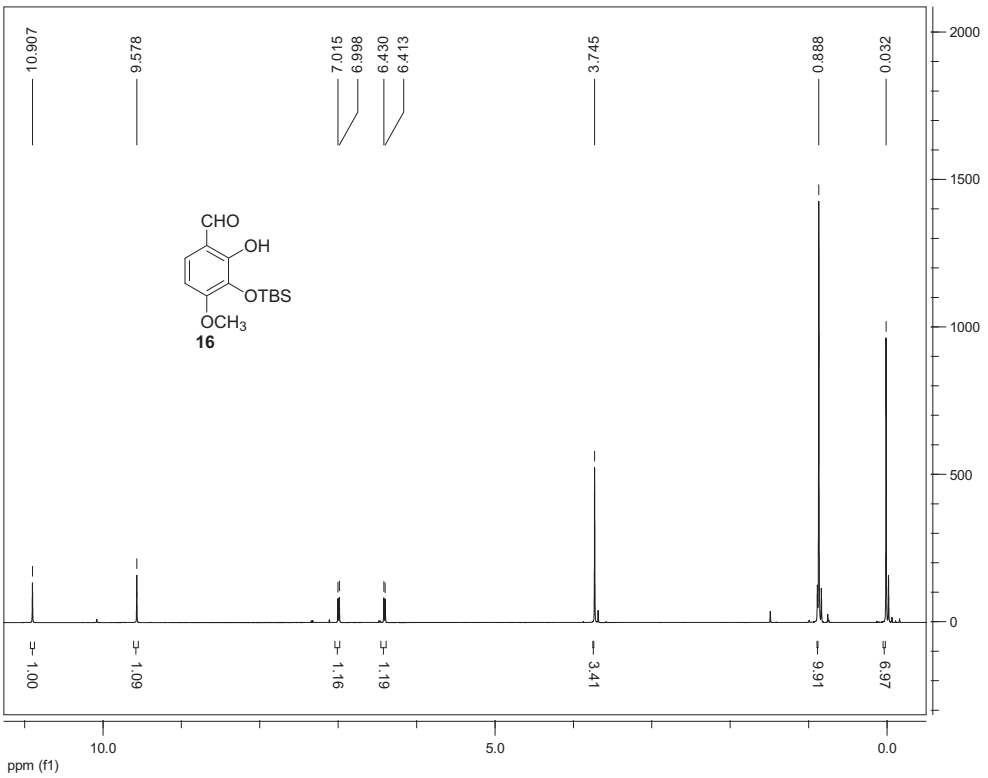
This project proved to be challenging. It required the synthesis of the VDA or cytotoxic agent (chapter 3) and then the synthesis of the bioreductive trigger. Learning and adjusting the reactions conditions for the Mitsunobu proved difficult but perseverance led to a successful coupling between phenstatin and **61** and nitrothiophene **81** to form the bioreductive prodrug conjugate **82**. Further studies on this molecular

conjugate **81** can be done to test for its applicable reduction and release of the chemotherapeutic agents.

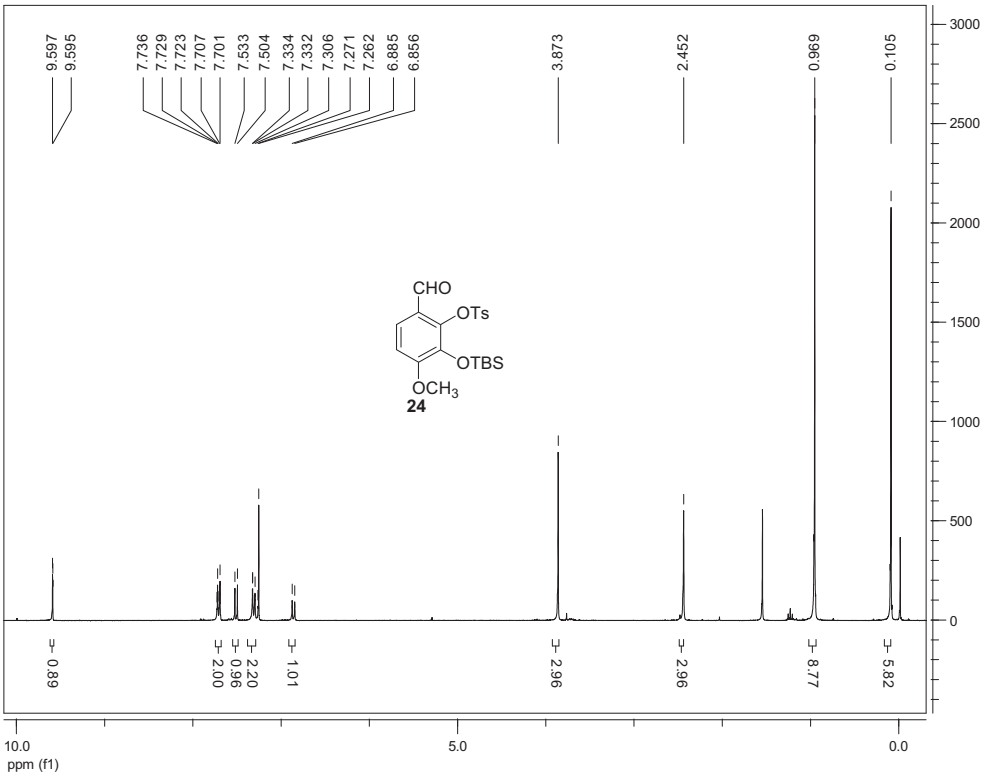
APPENDICES

APPENDIX A

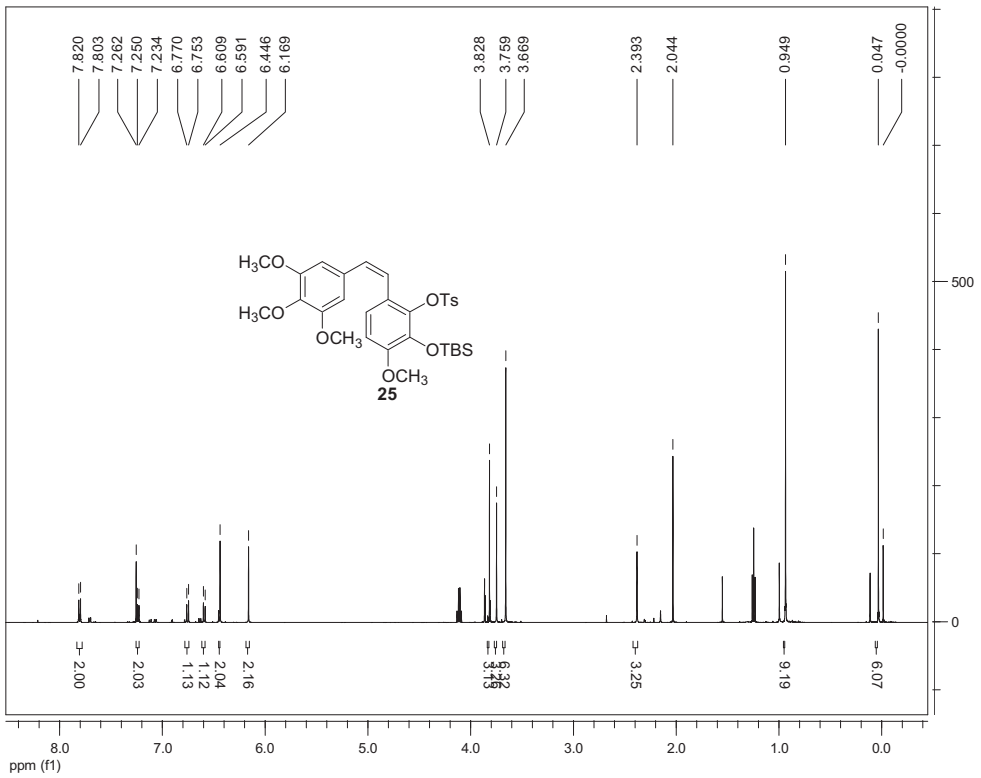
Nuclear Magnetic Resonance (NMR) Spectra



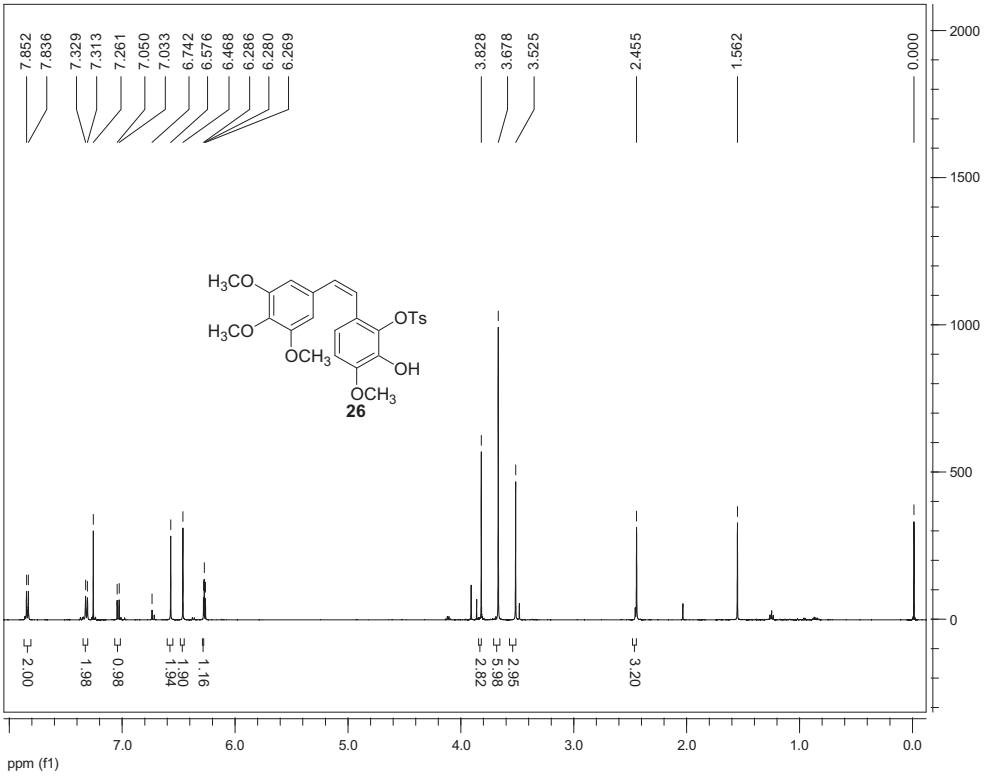
^{1}H NMR (500 MHz, $CDCl_3$) of compound **16**



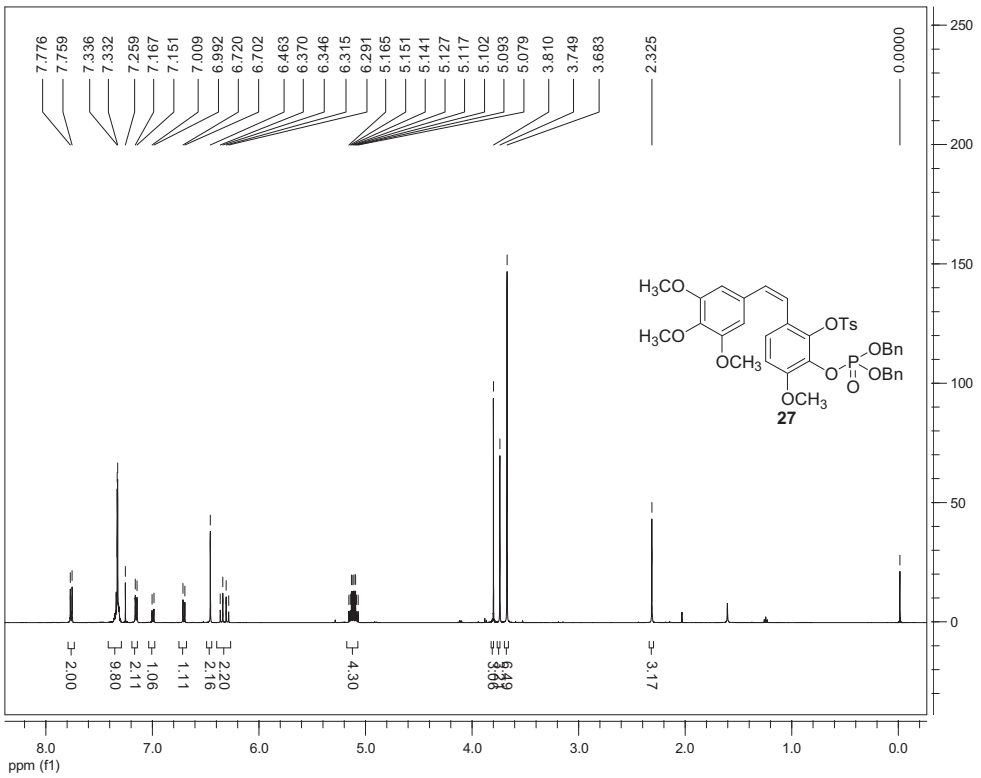
^{1}H NMR (300 MHz, $CDCl_3$) of compound **24**



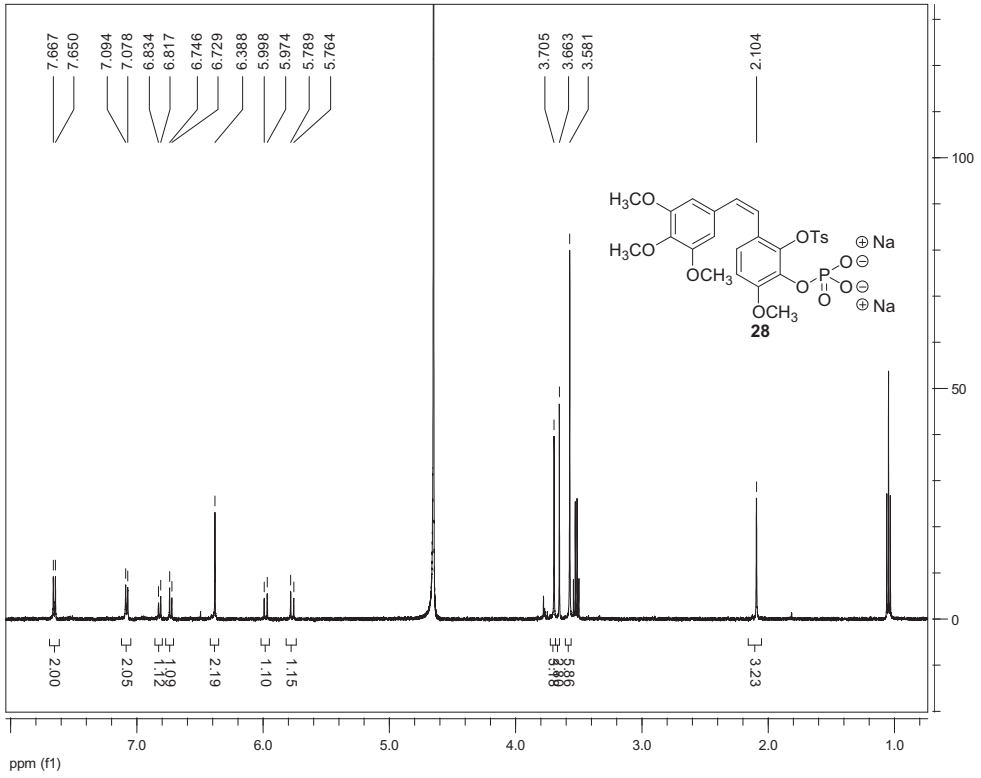
^{1}H NMR (500 MHz, $CDCl_3$) of compound **25**



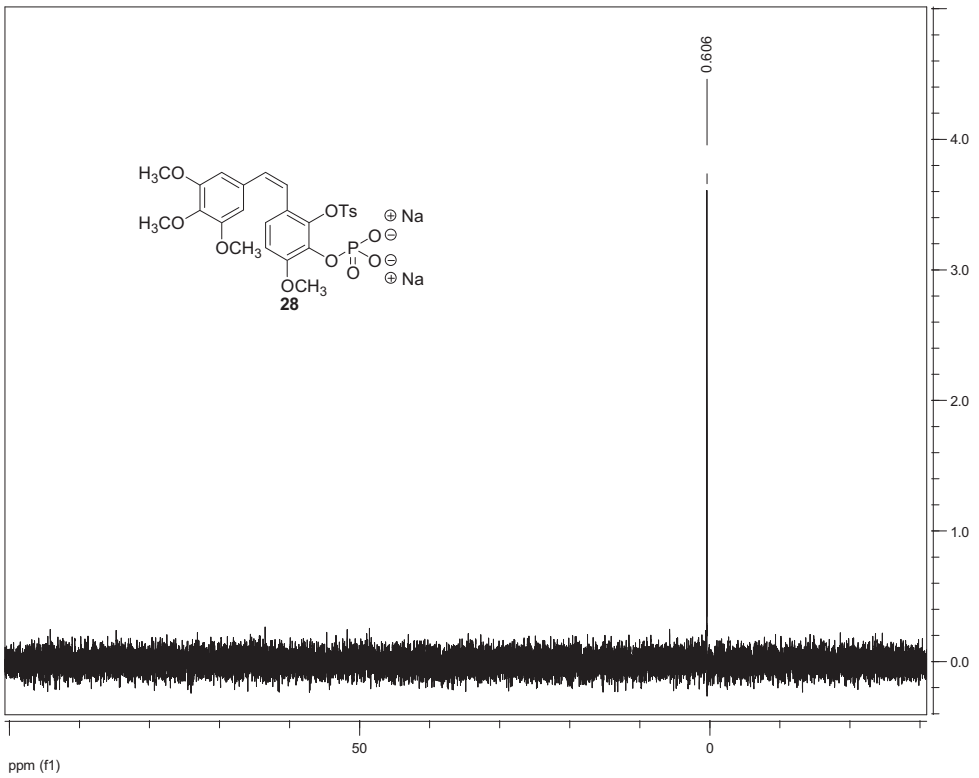
^{1}H NMR (500 MHz, $CDCl_3$) of compound **26**



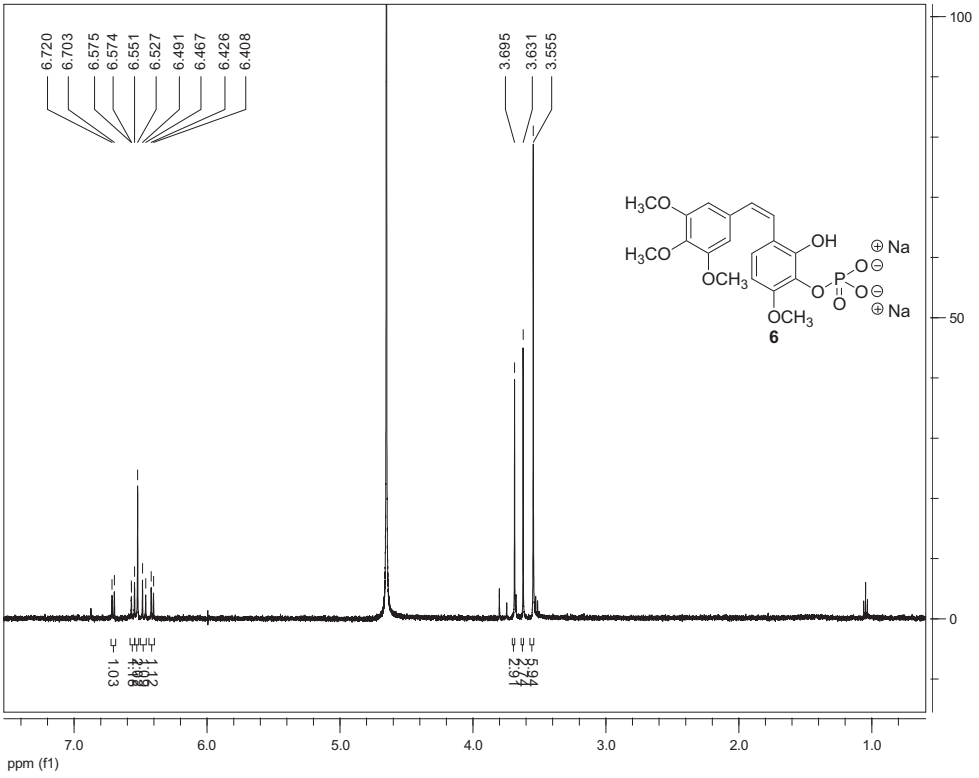
^{1}H NMR (500 MHz, $CDCl_3$) of compound **27**



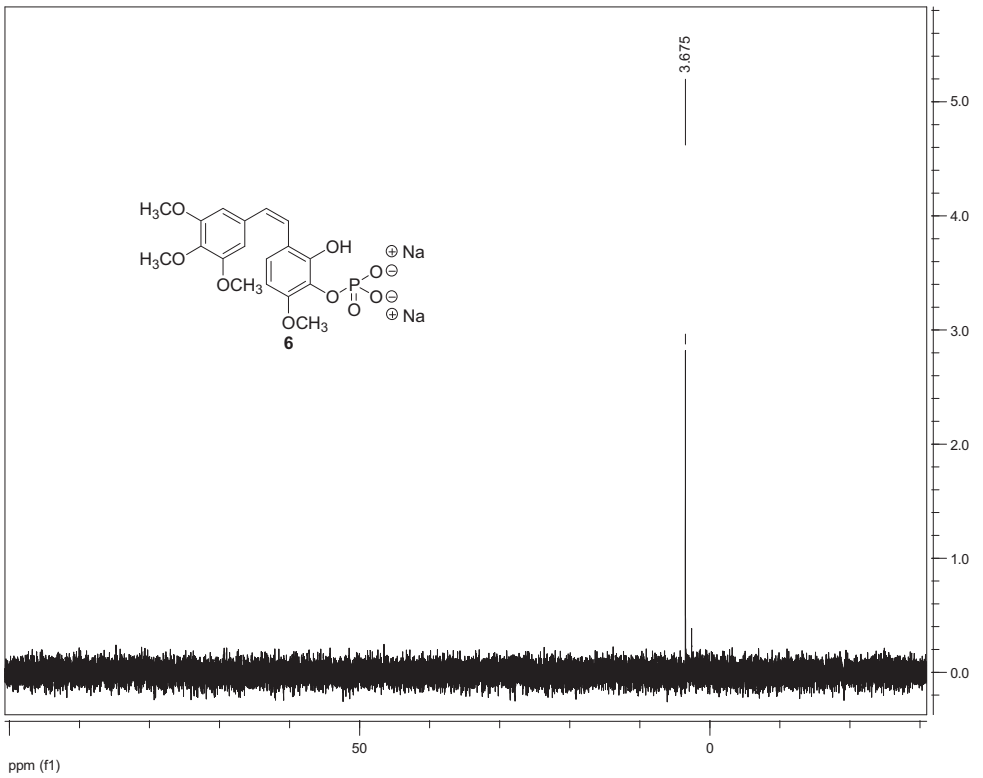
^{1}H NMR (500 MHz, D_2O) of compound **28**



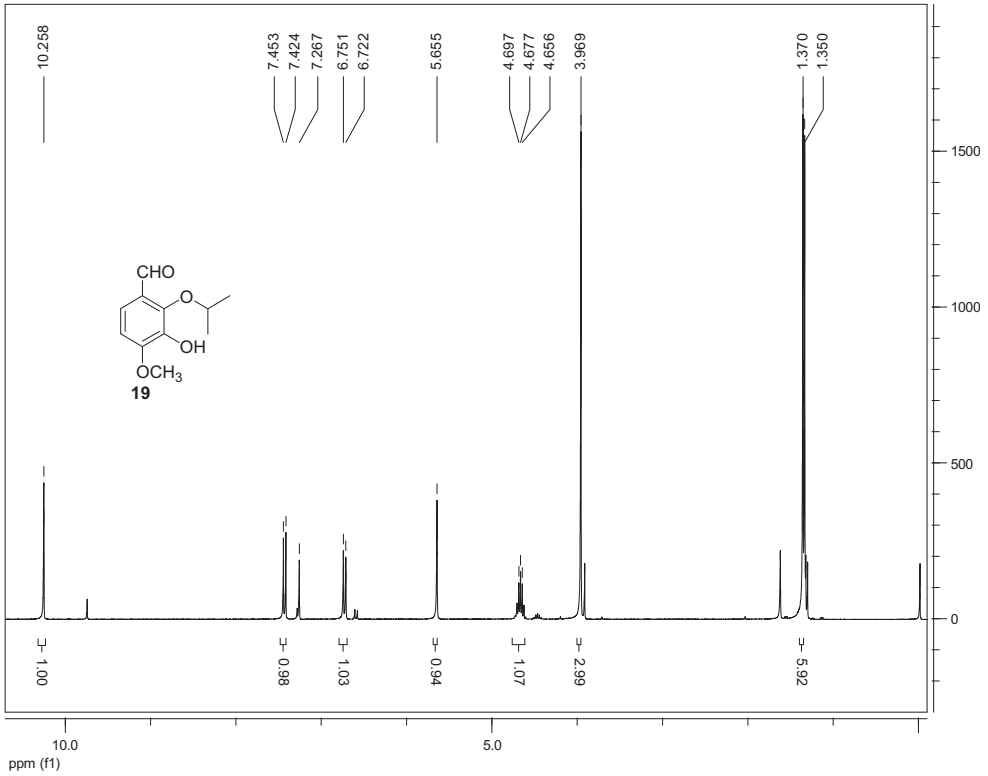
^{31}P NMR (200 MHz, D_2O) of compound **28**



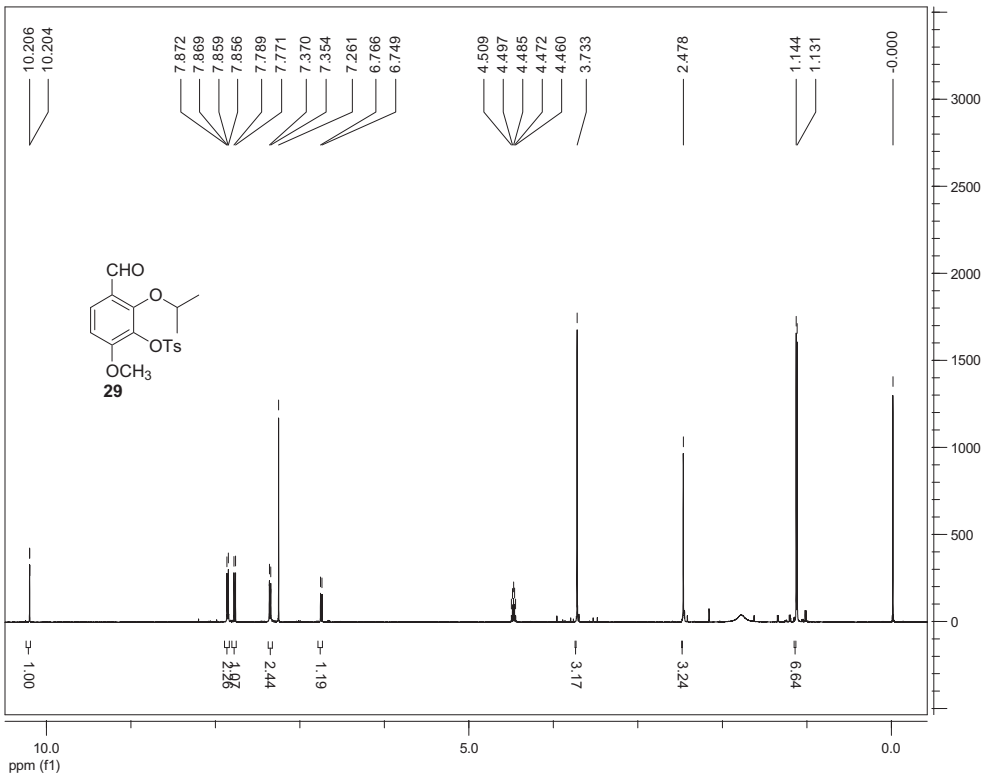
^{1}H NMR (500 MHz, D_2O) of compound **6**



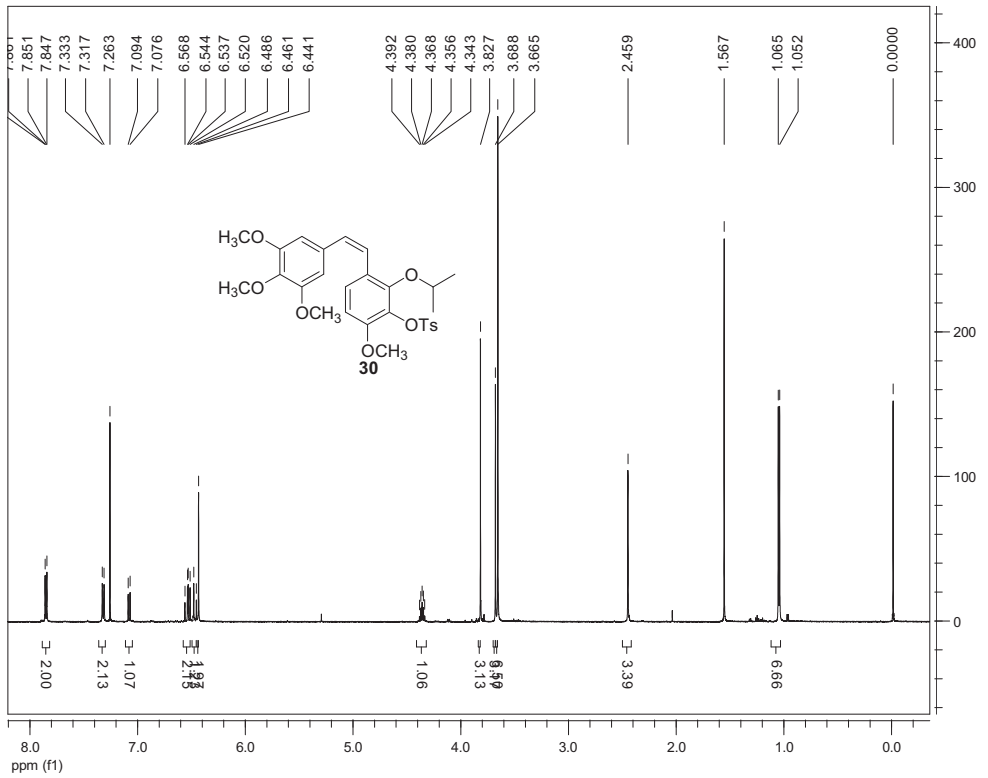
^{31}P NMR (200 MHz, D_2O) of compound **6**



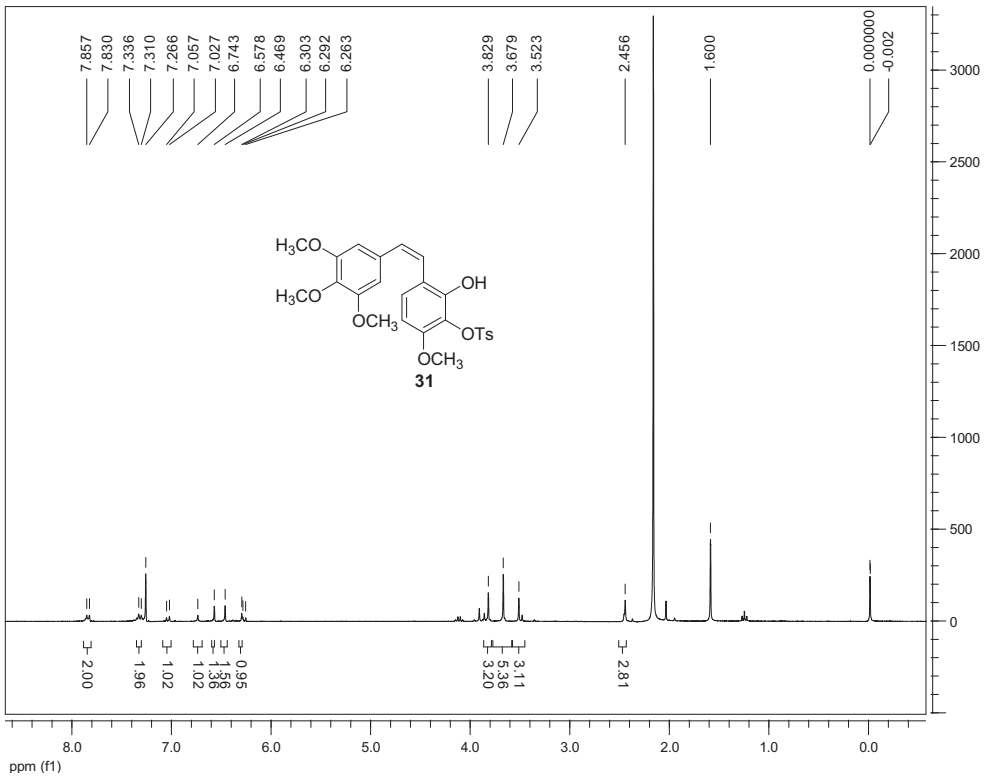
^{1}H NMR (300 MHz, $CDCl_3$) of compound **19**



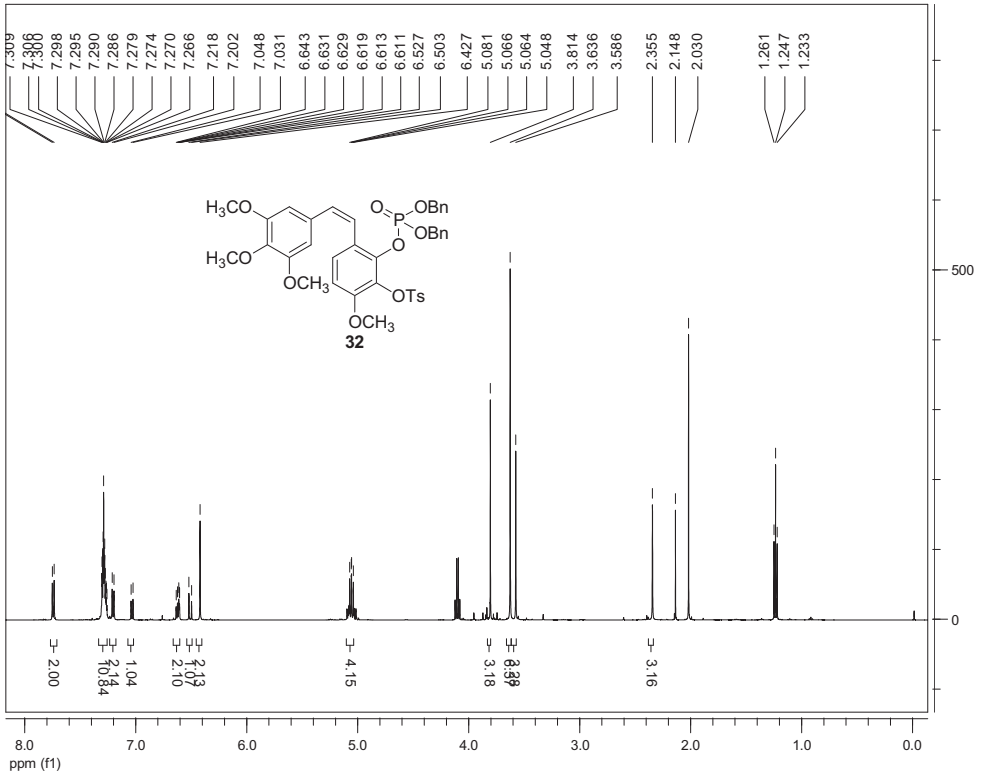
^{1}H NMR (500 MHz, $CDCl_3$) of compound **29**



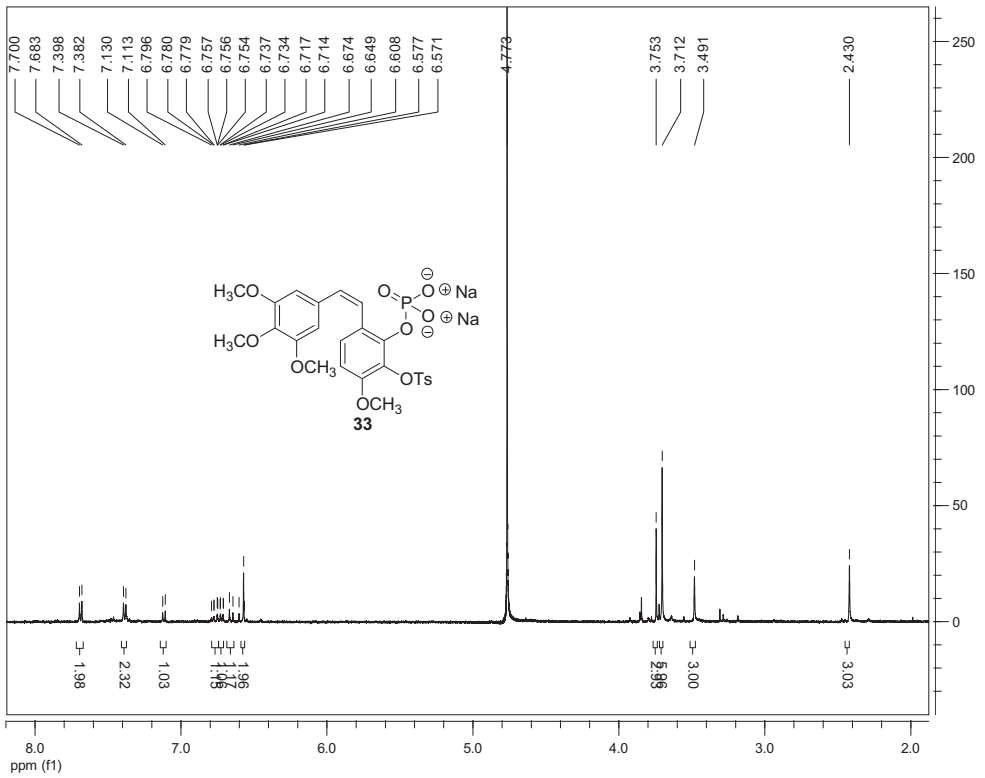
^{1}H NMR (500 MHz, $CDCl_3$) of compound **30**



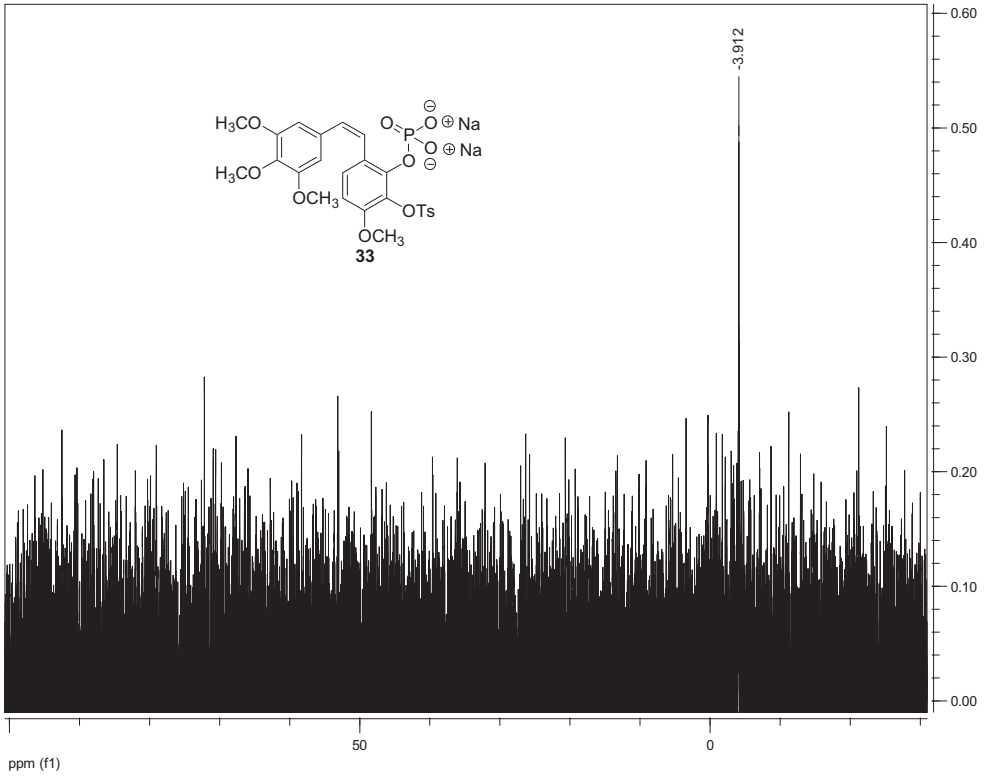
^{1}H NMR (500 MHz, $CDCl_3$) of compound **31**



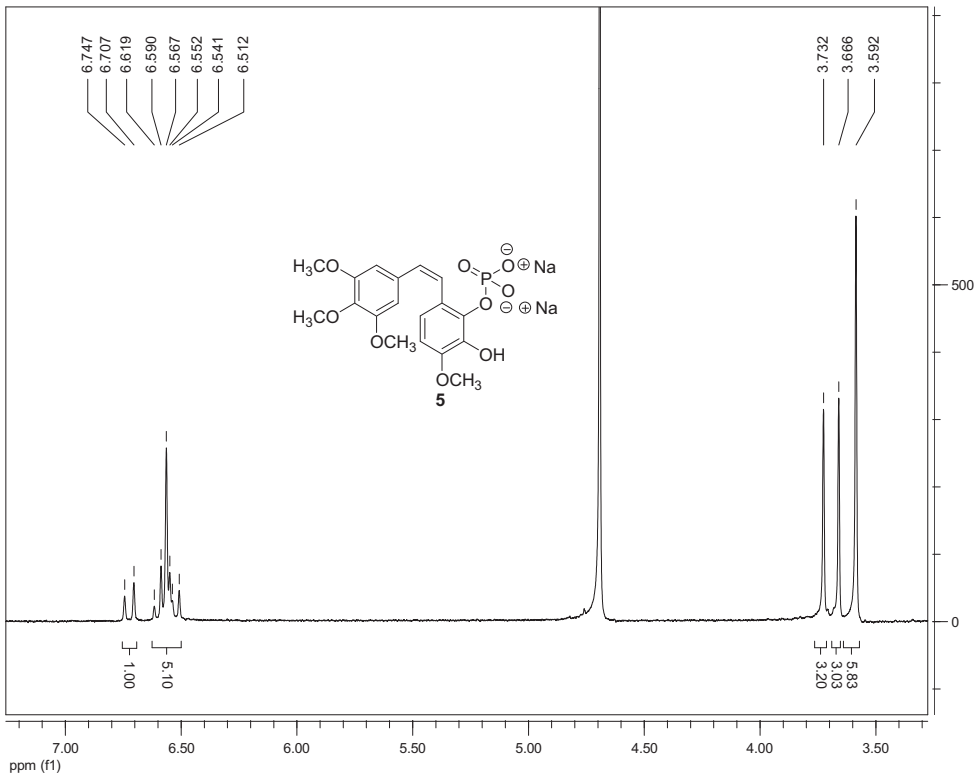
^{1}H NMR (500 MHz, $CDCl_3$) of compound **32**



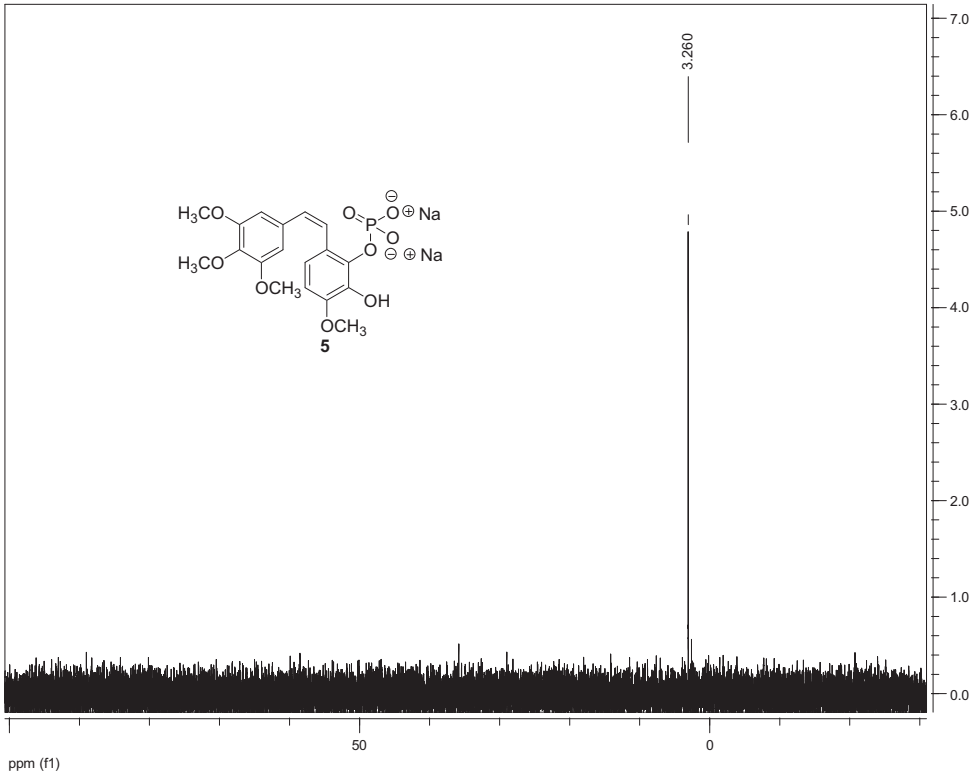
^{1}H NMR (500 MHz, D_2O) of compound **33**



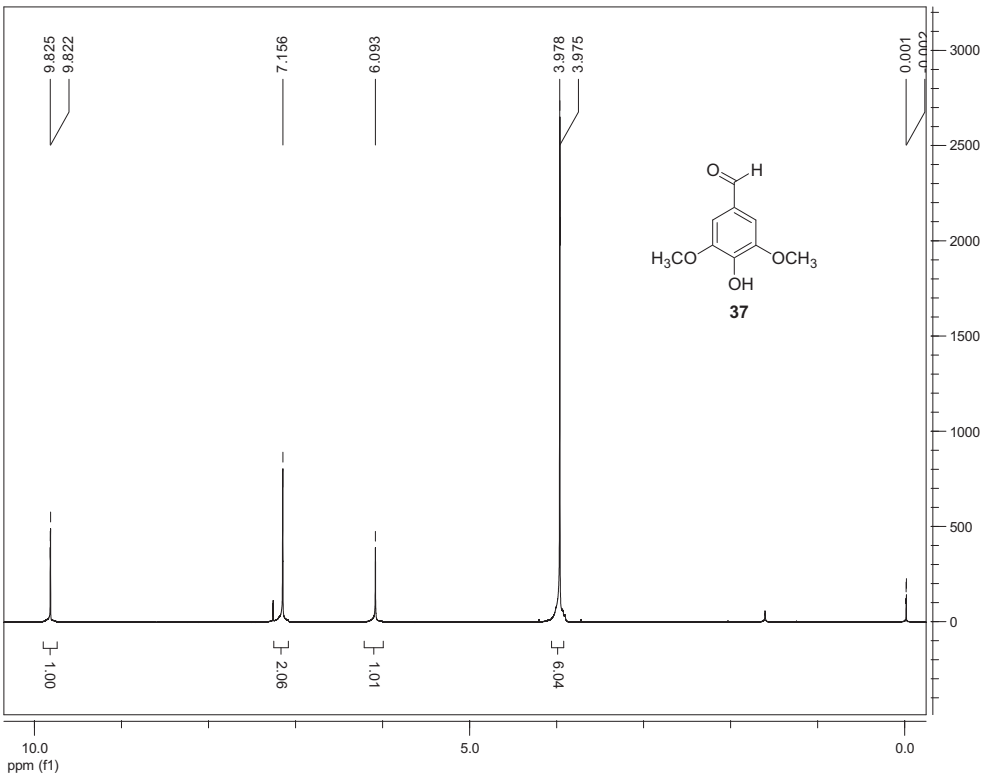
^{31}P NMR (200 MHz, D_2O) of compound **33**



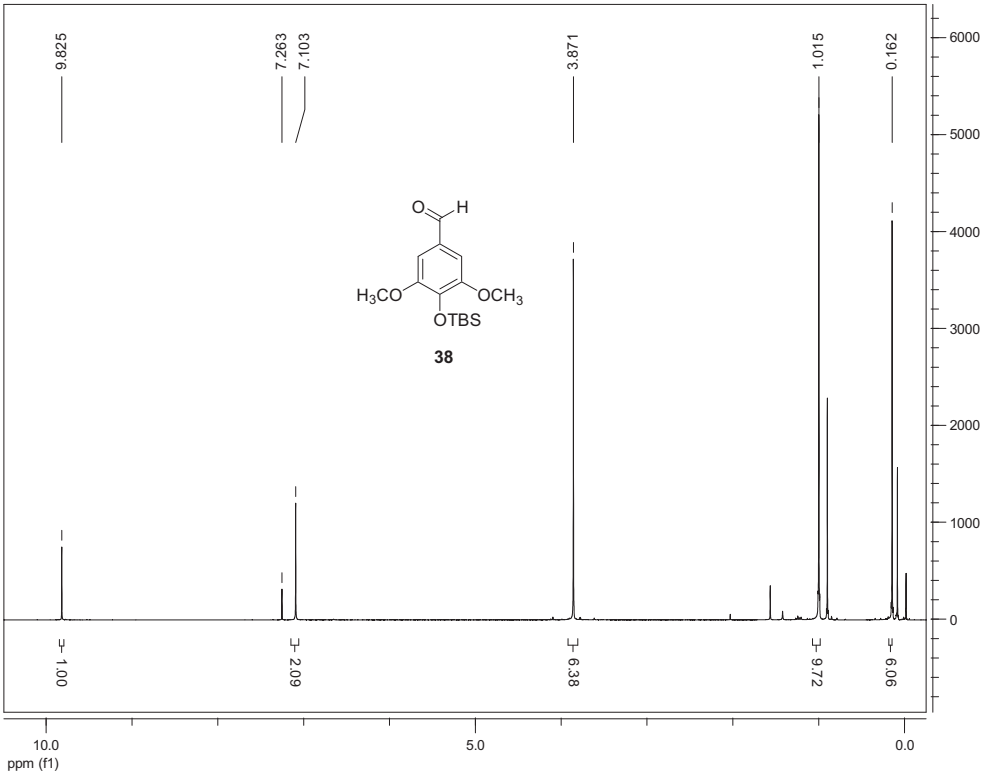
^{1}H NMR (500 MHz, D_2O) of compound **5**



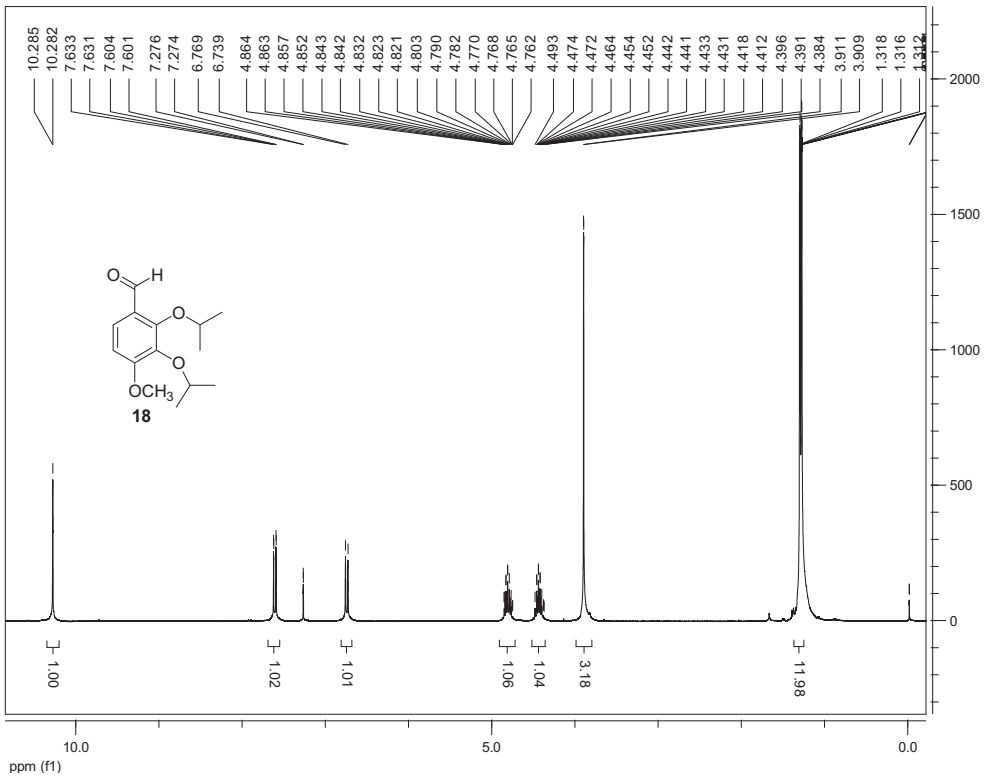
^{31}P NMR (200 MHz, D_2O) of compound **5**



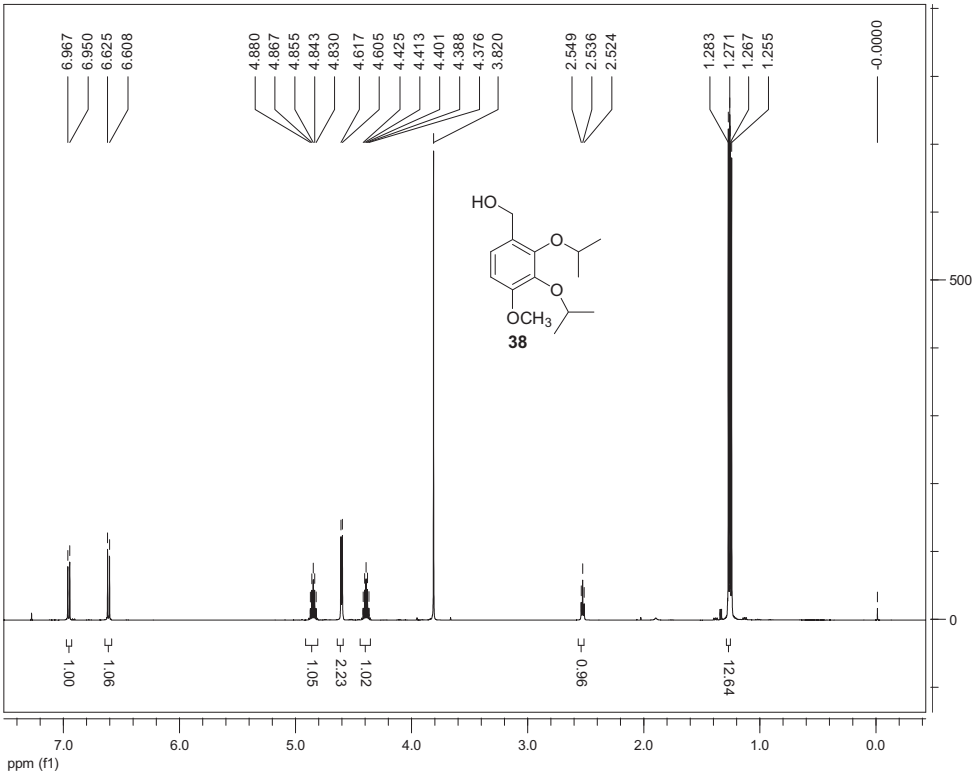
^{1}H NMR (300 MHz, $CDCl_3$) of compound **37**



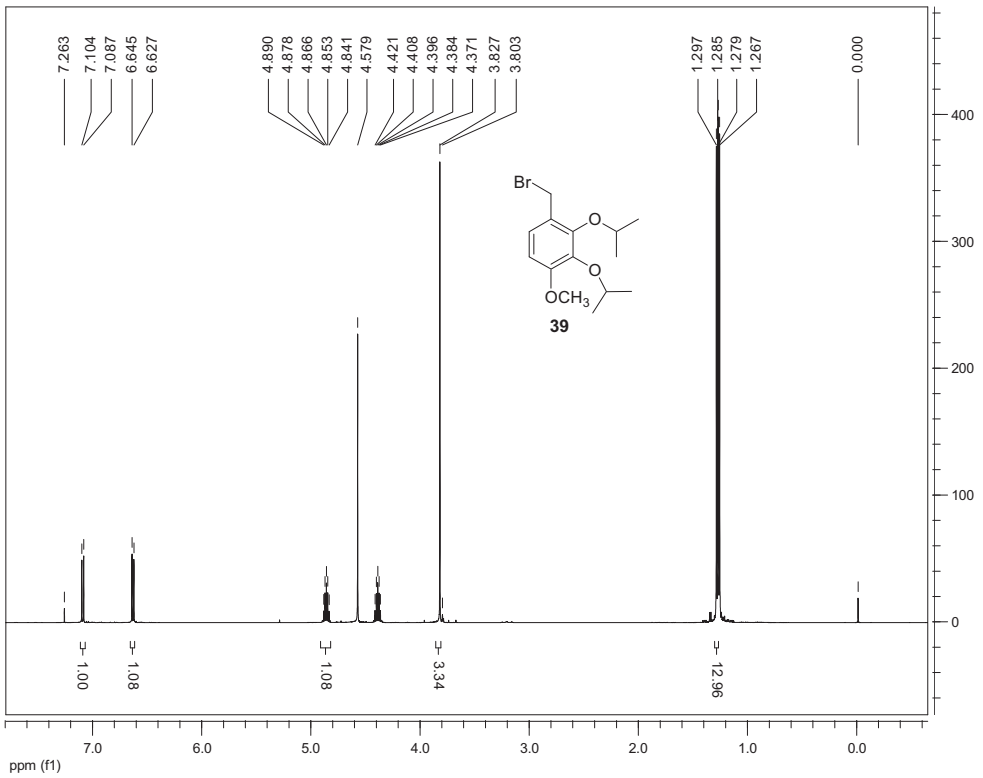
^{1}H NMR (300 MHz, $CDCl_3$) of compound **38**



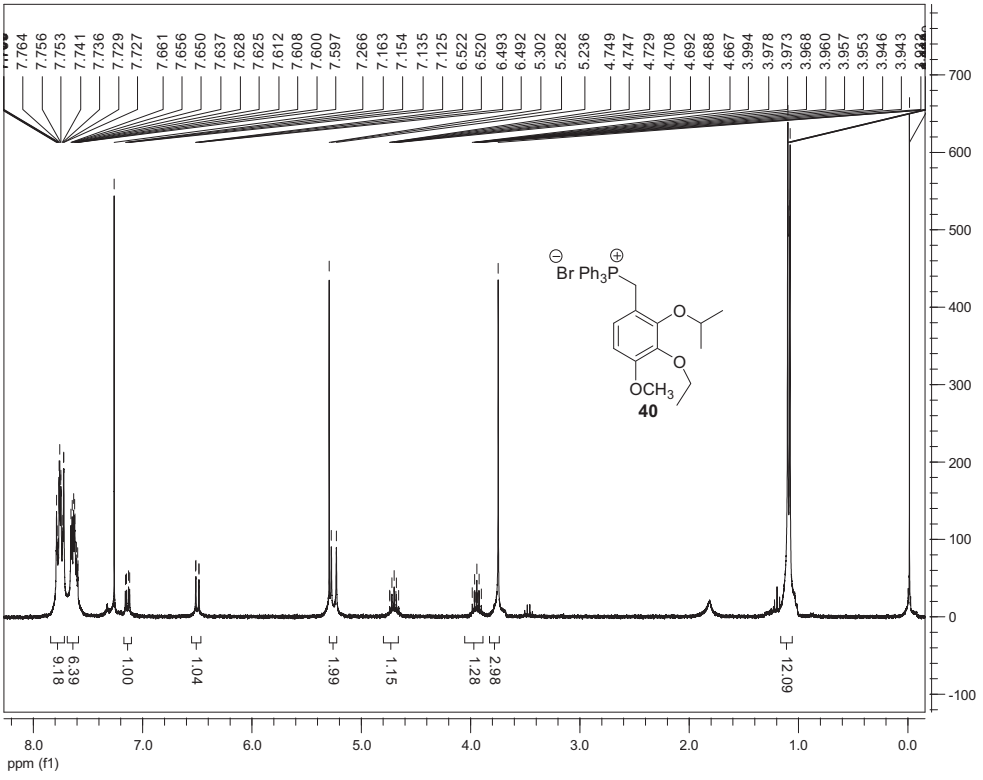
^{1}H NMR (300 MHz, $CDCl_3$) of compound **18**



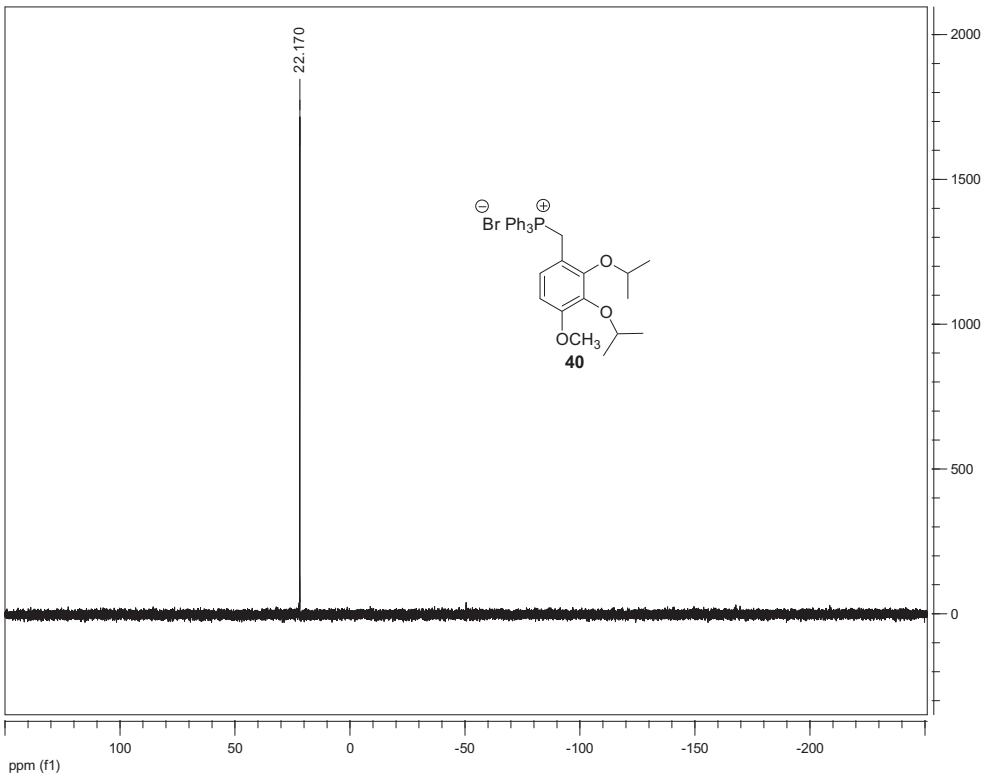
^{1}H NMR (500 MHz, $CDCl_3$) of compound **38**



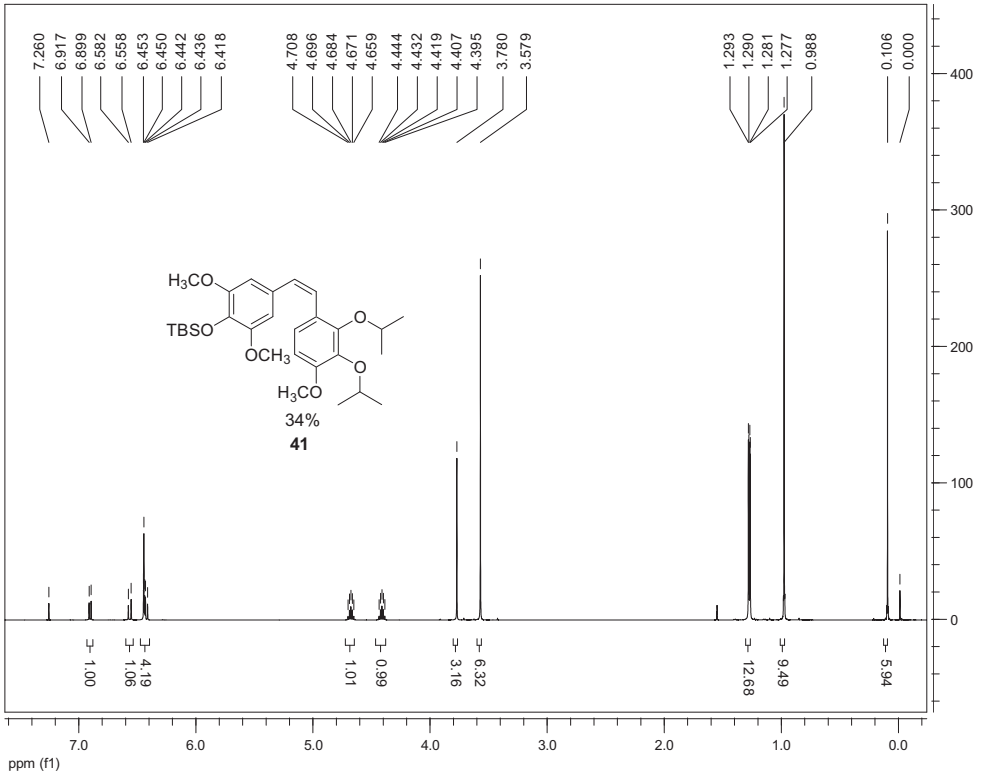
^{1}H NMR (500 MHz, $CDCl_3$) of compound **39**



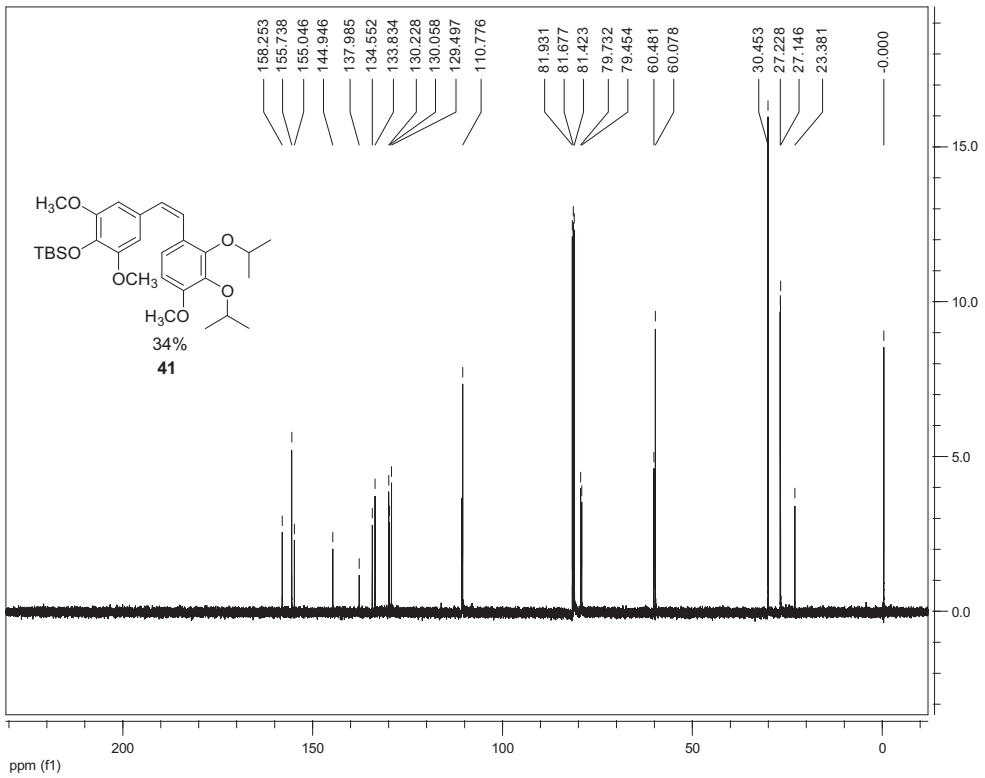
^{1}H NMR (300 MHz, $CDCl_3$) of compound **40**



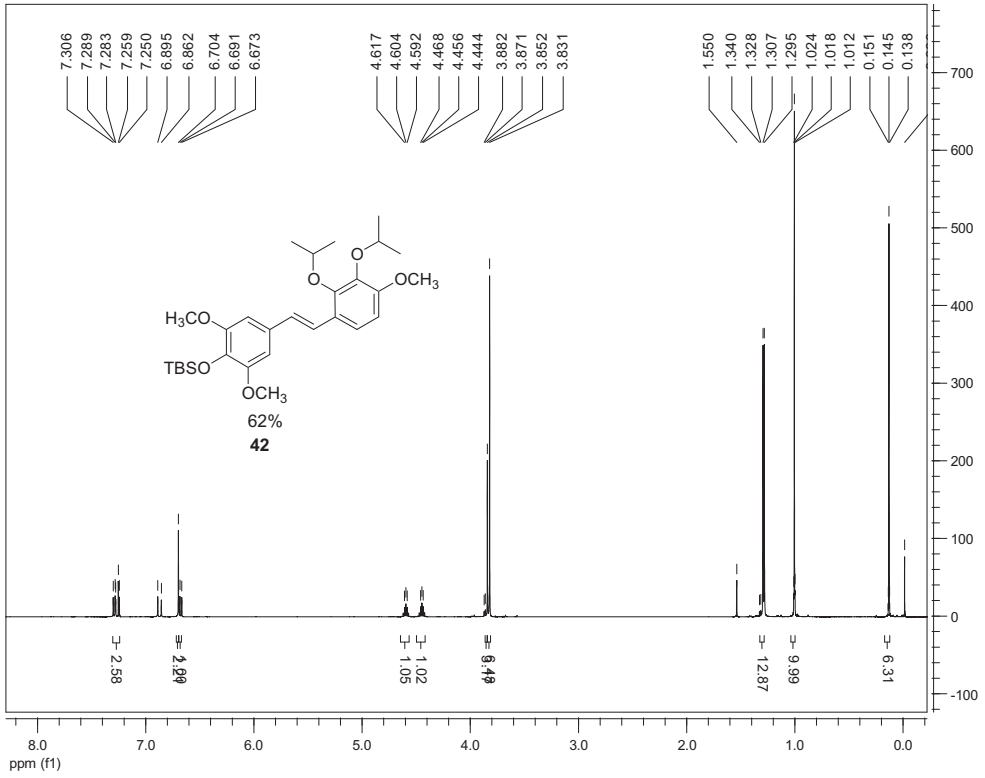
^{31}P NMR (90 MHz, $CDCl_3$) of compound **40**



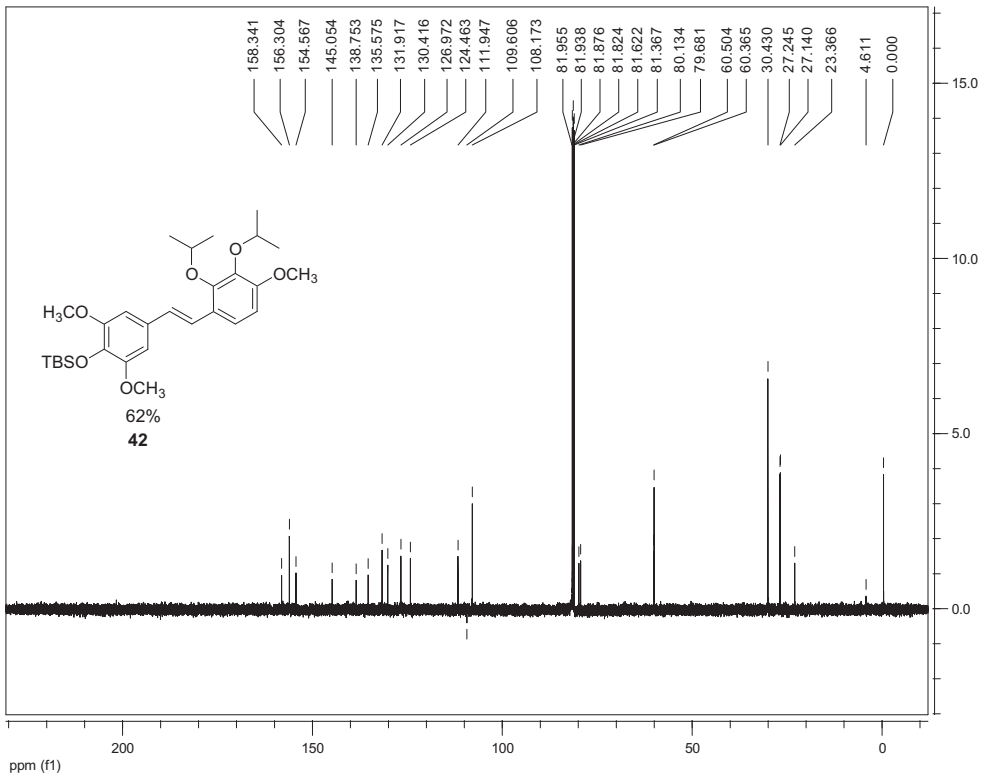
^{1}H NMR (500 MHz, $CDCl_3$) of compound **41**



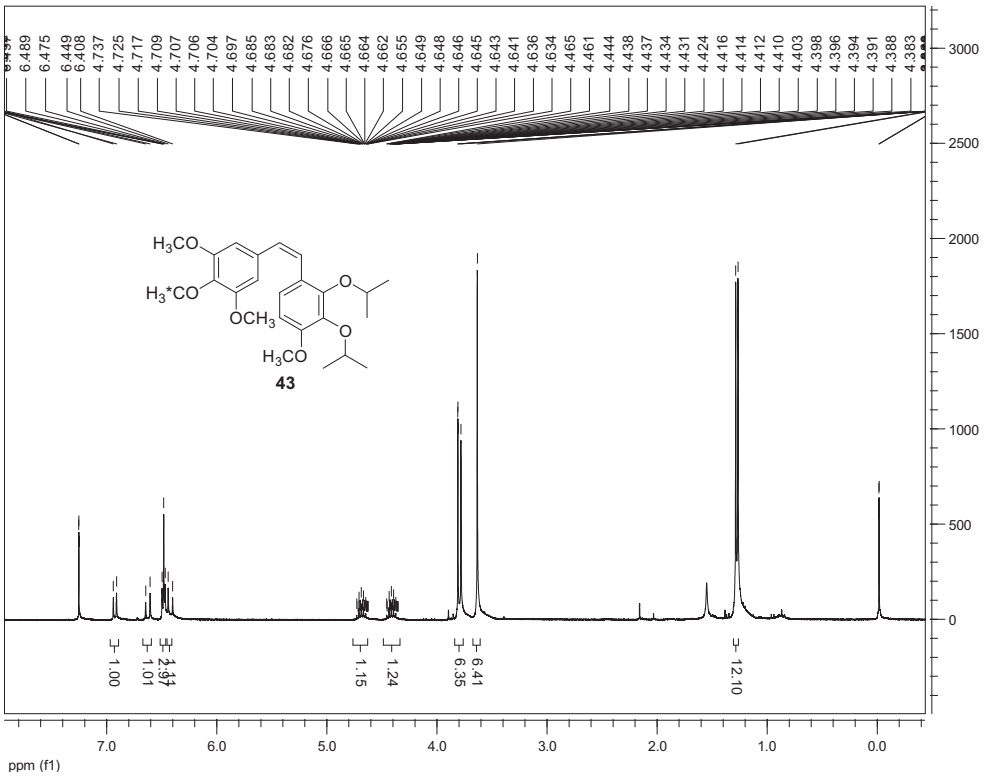
^{13}C NMR (125 MHz, $CDCl_3$) of compound **41**



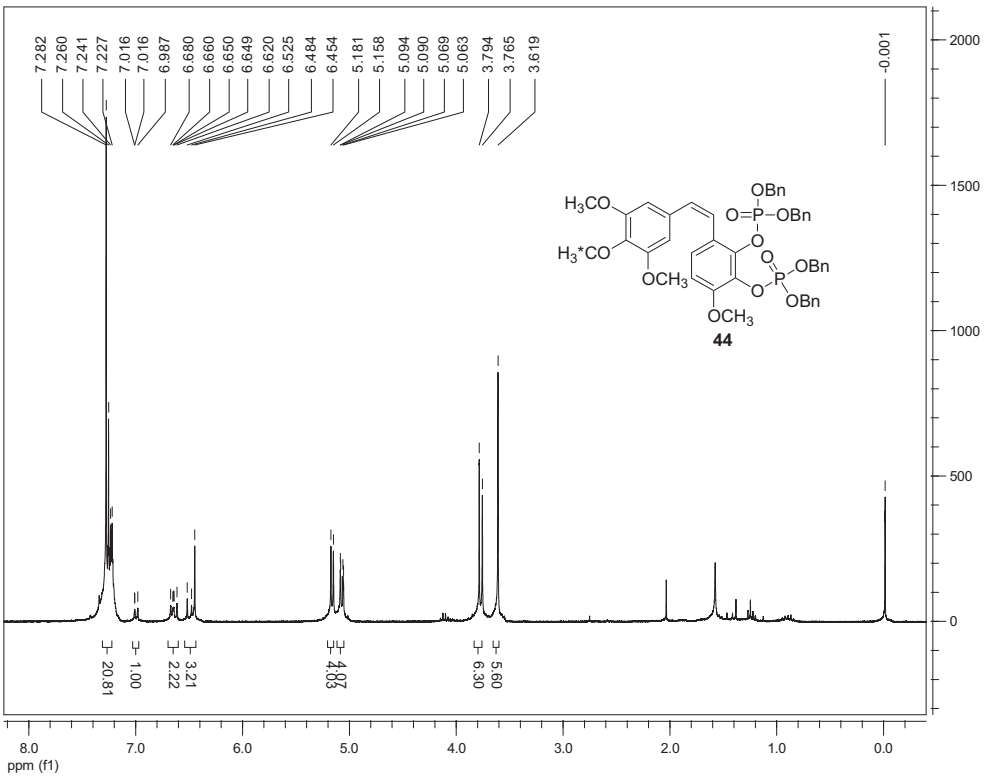
^{1}H NMR (500 MHz, $CDCl_3$) of compound **42**



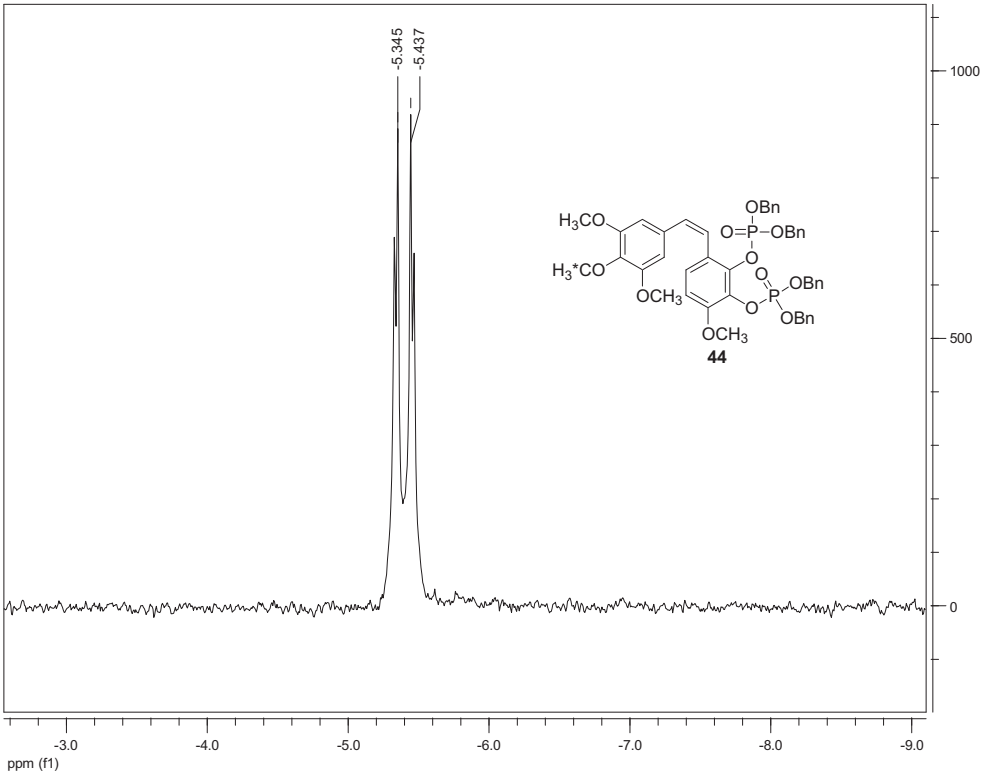
^{13}C NMR (125 MHz, $CDCl_3$) of compound **41**



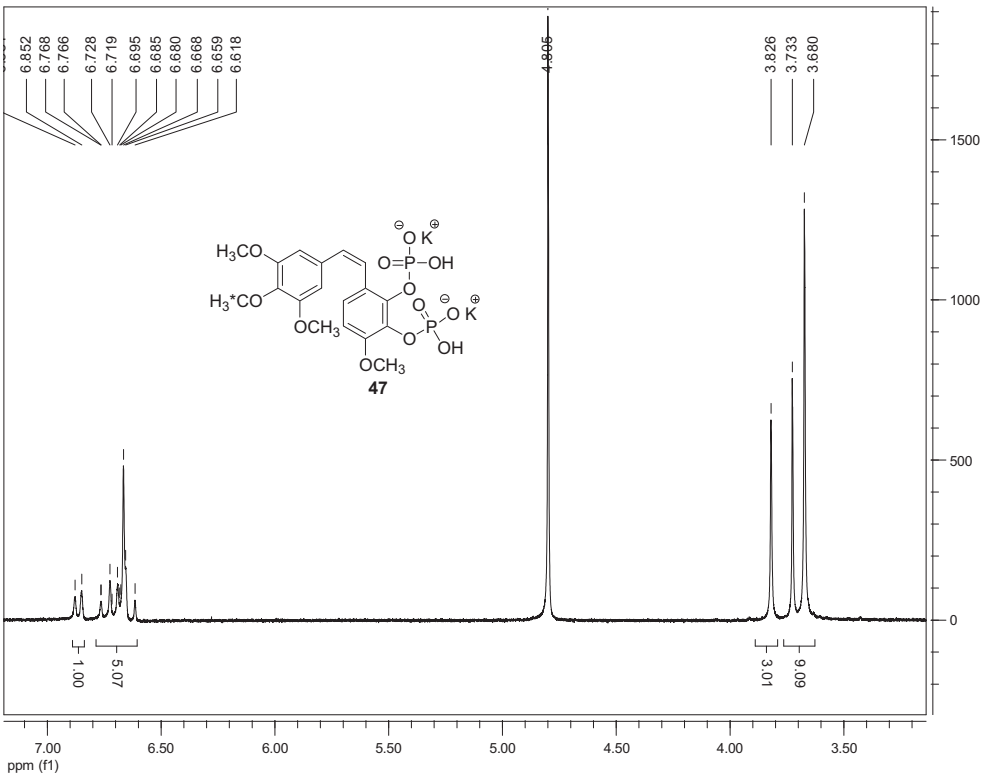
^{1}H NMR (300 MHz, $CDCl_3$) of compound **43**



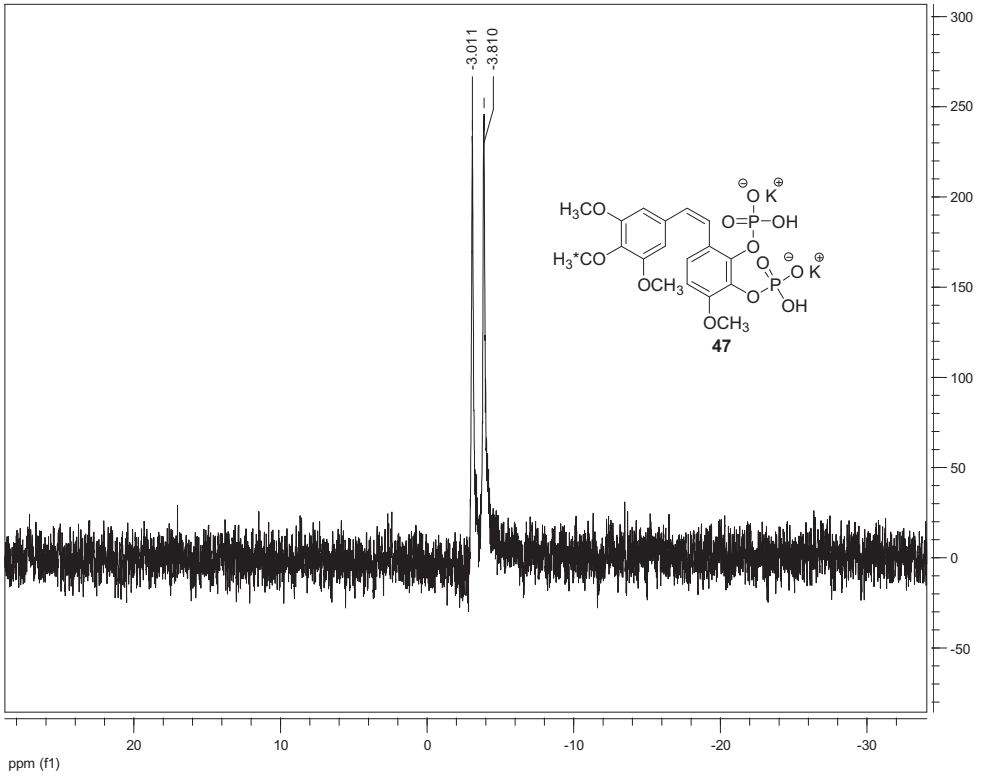
^{1}H NMR (300 MHz, $CDCl_3$) of compound **44**



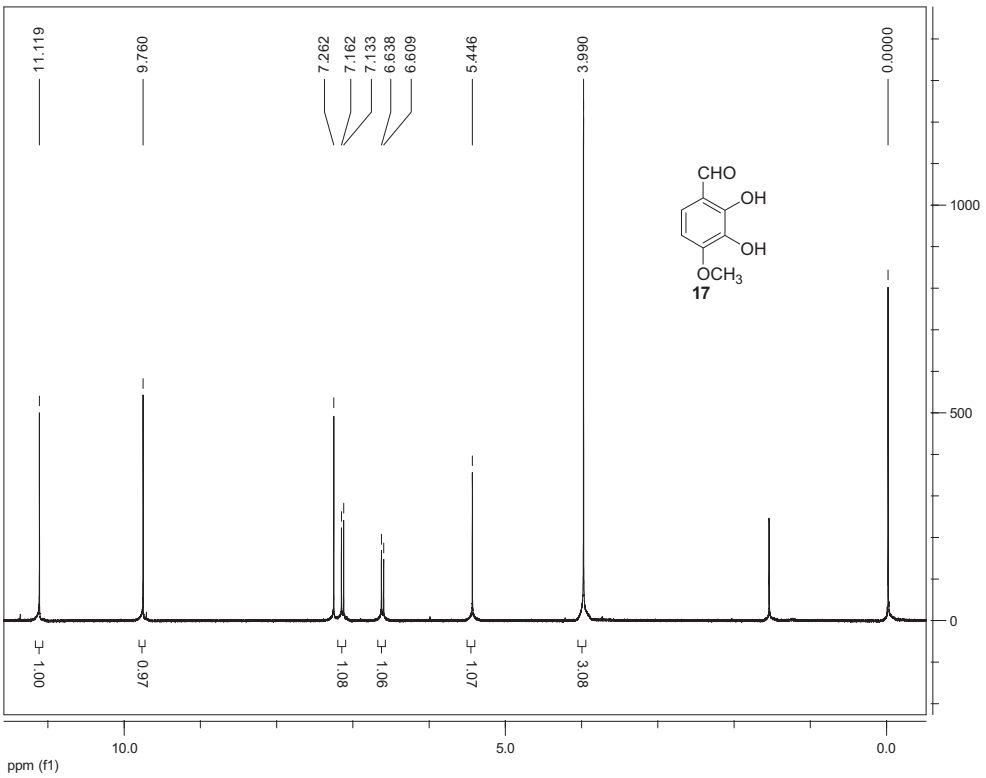
^{31}P NMR (90 MHz, $CDCl_3$) of compound **44**



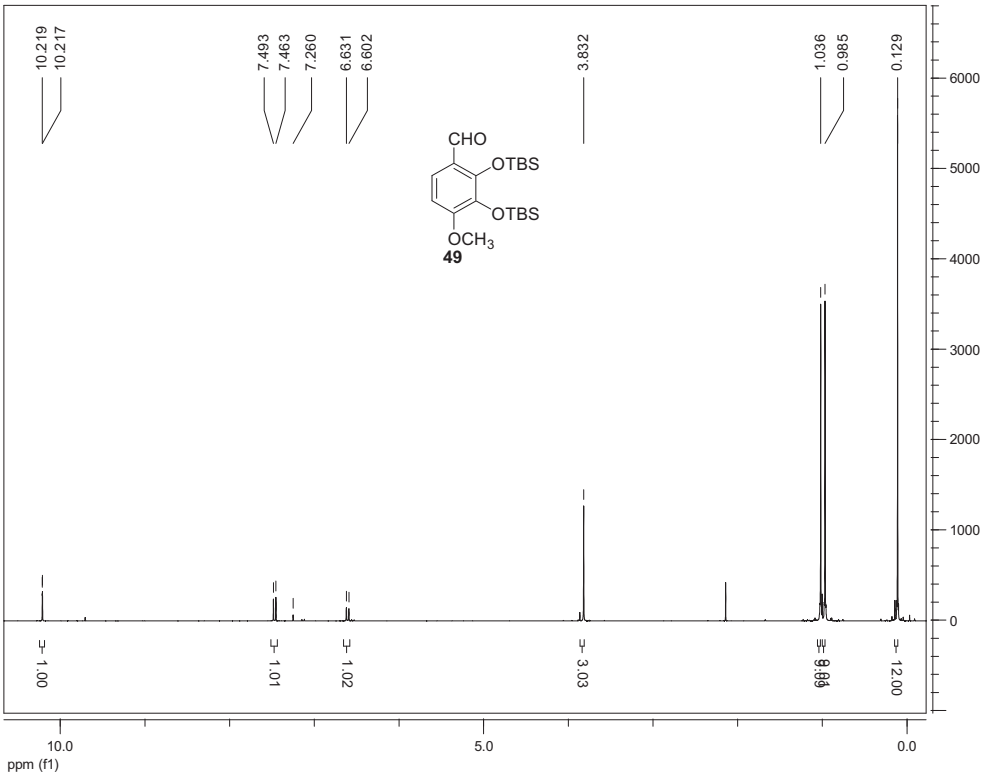
^{1}H NMR (300 MHz, D_2O) of compound **47**



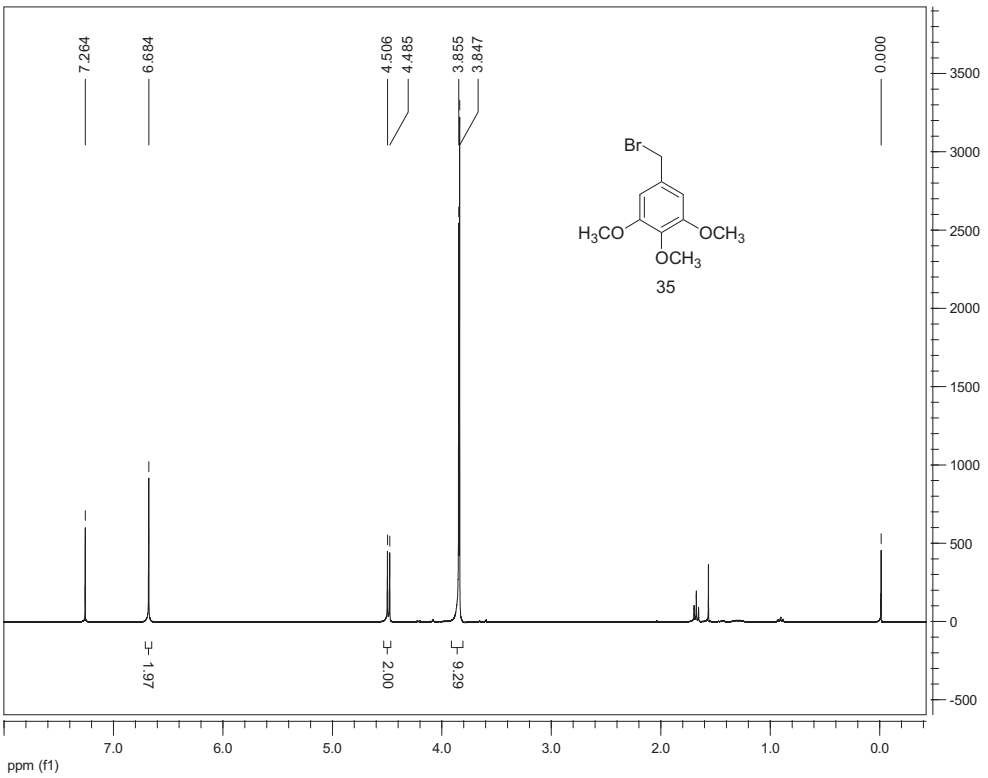
^{31}P NMR (90 MHz, D_2O) of compound **47**



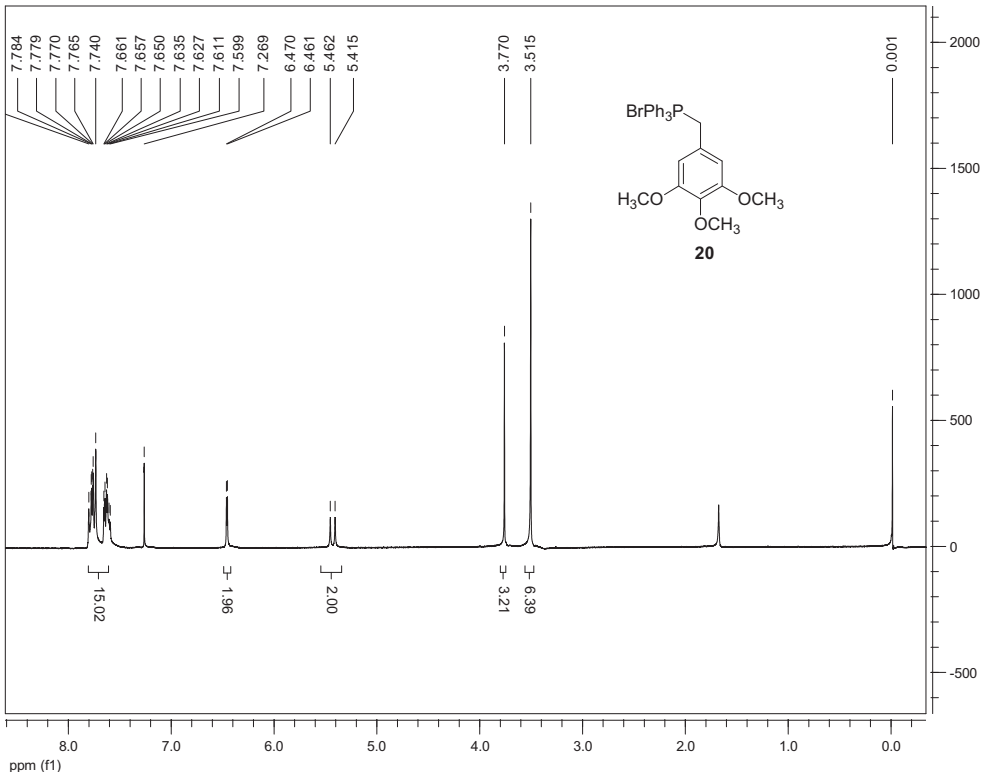
^{1}H NMR (300 MHz, $CDCl_3$) of compound **17**



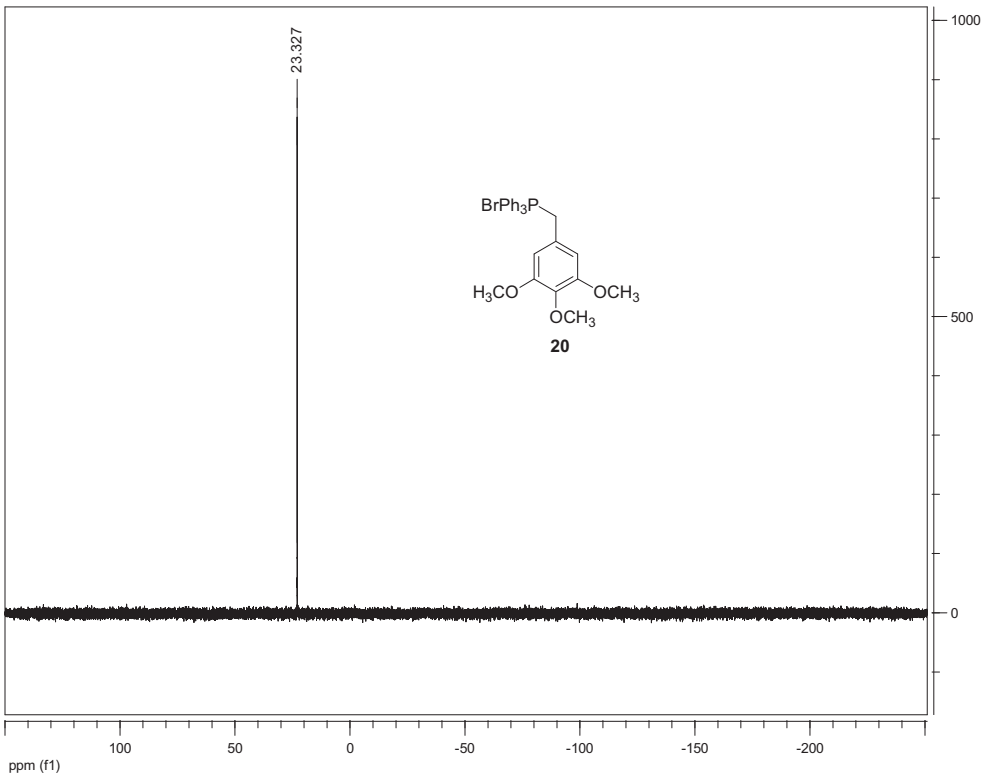
^{1}H NMR (300 MHz, $CDCl_3$) of compound **49**



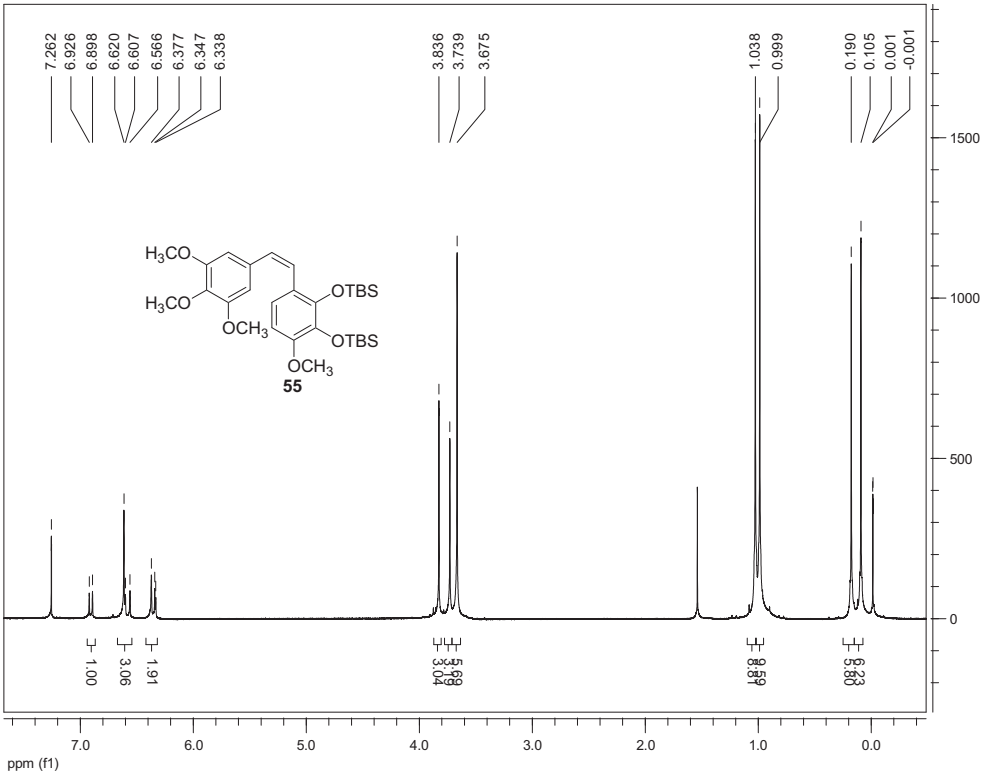
^{1}H NMR (300 MHz, $CDCl_3$) of compound **35**



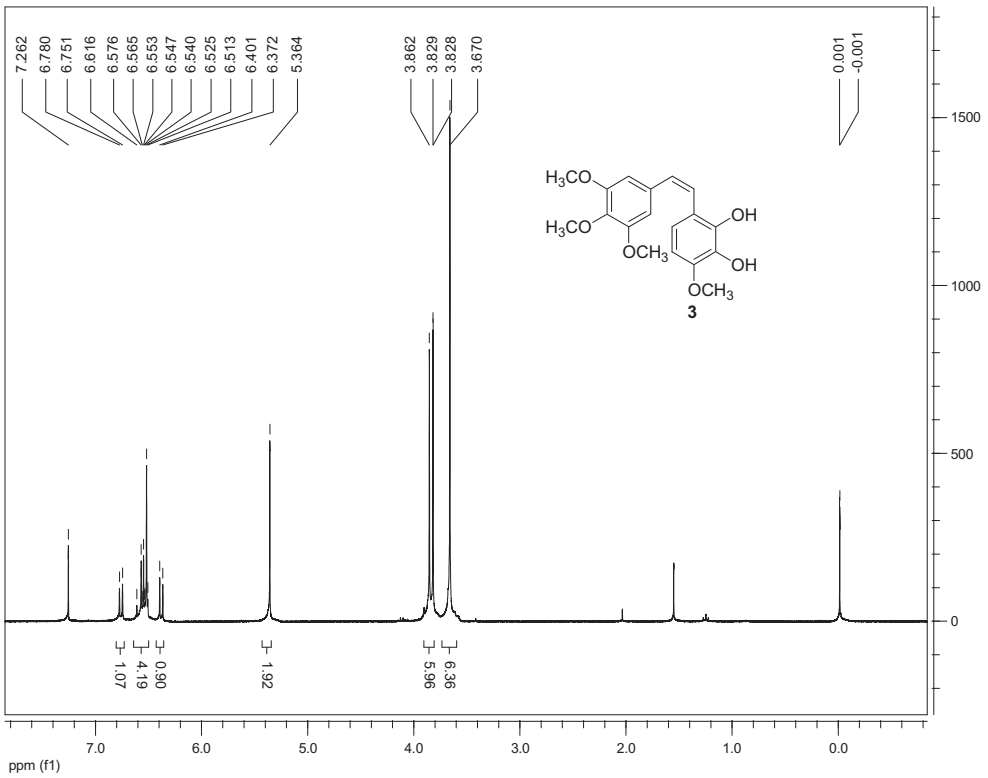
^{1}H NMR (300 MHz, $CDCl_3$) of compound **20**



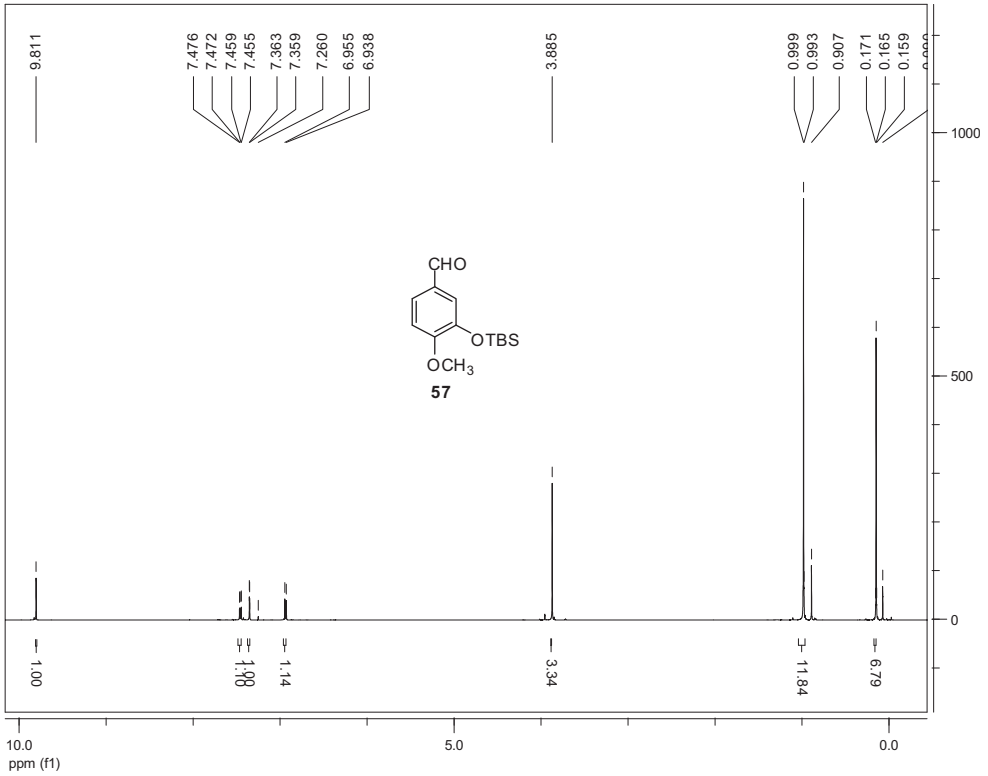
^{31}P NMR (90 MHz, $CDCl_3$) of compound **20**



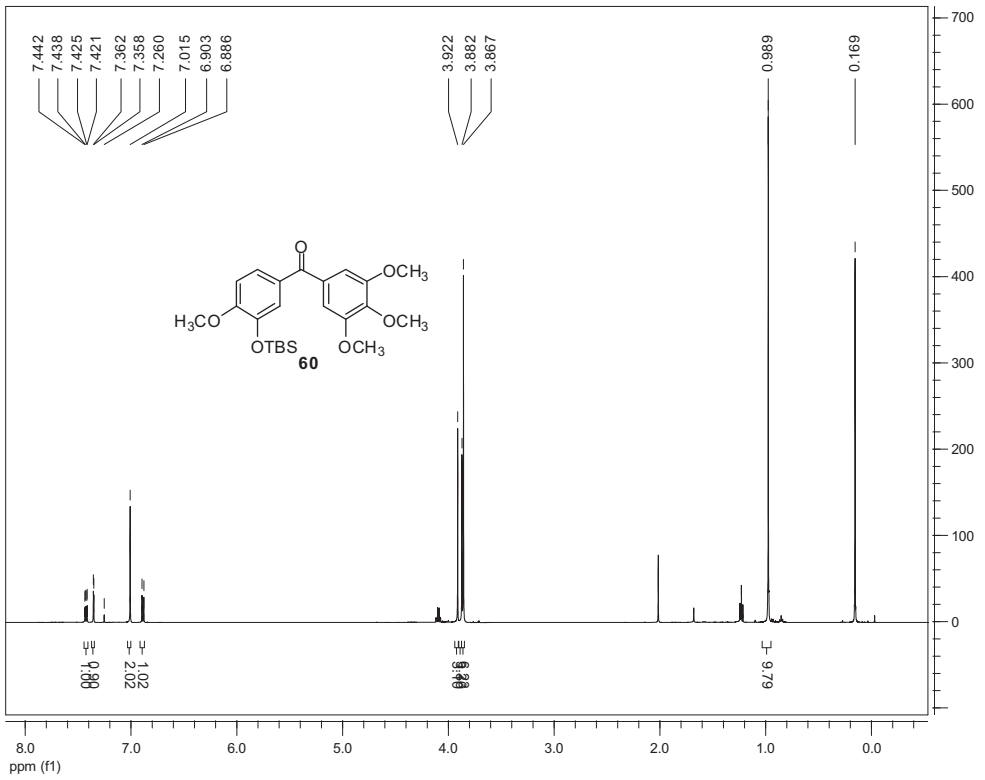
^{1}H NMR (300 MHz, $CDCl_3$) of compound **55**



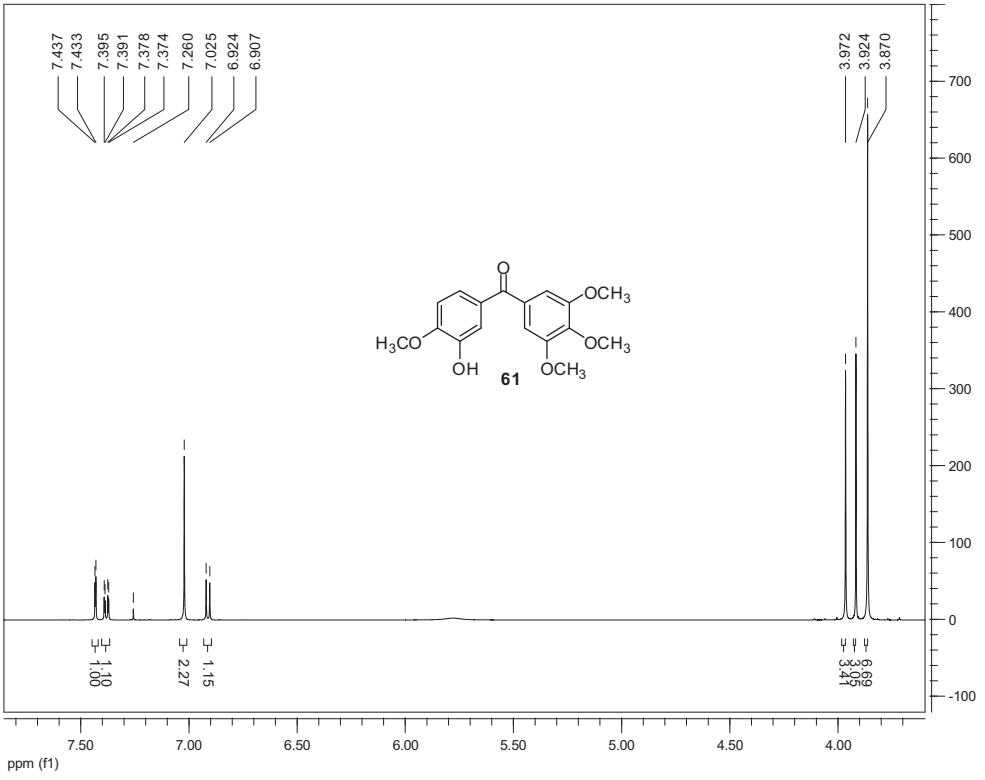
^{1}H NMR (300 MHz, $CDCl_3$) of compound **3**



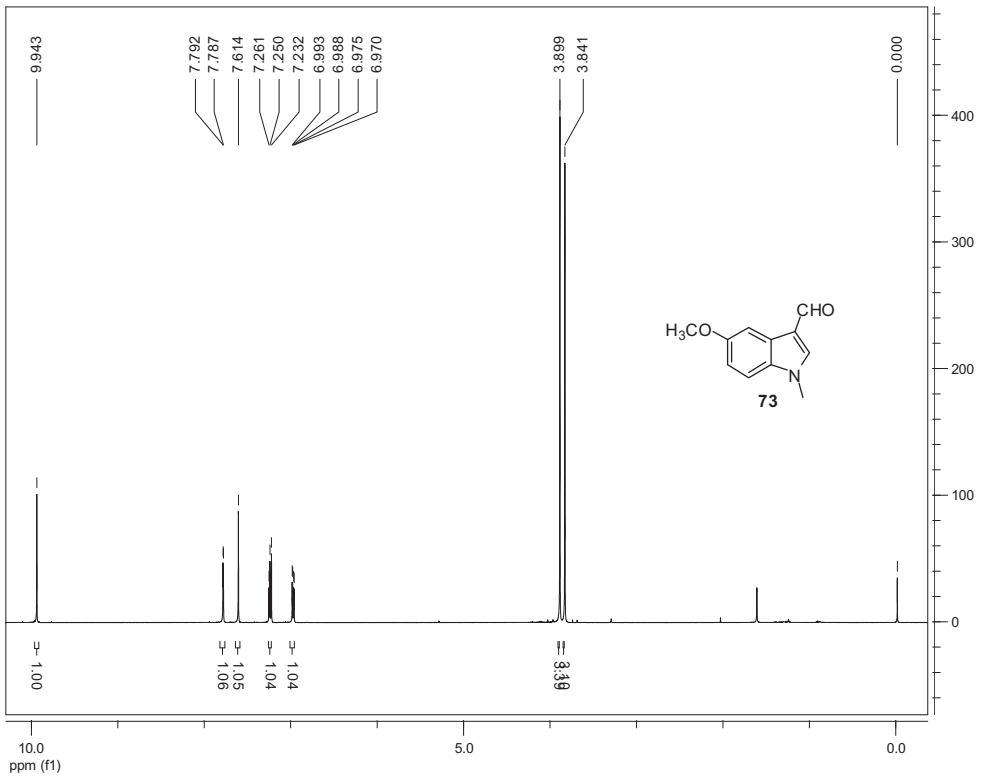
^{1}H NMR (500 MHz, $CDCl_3$) of compound **57**



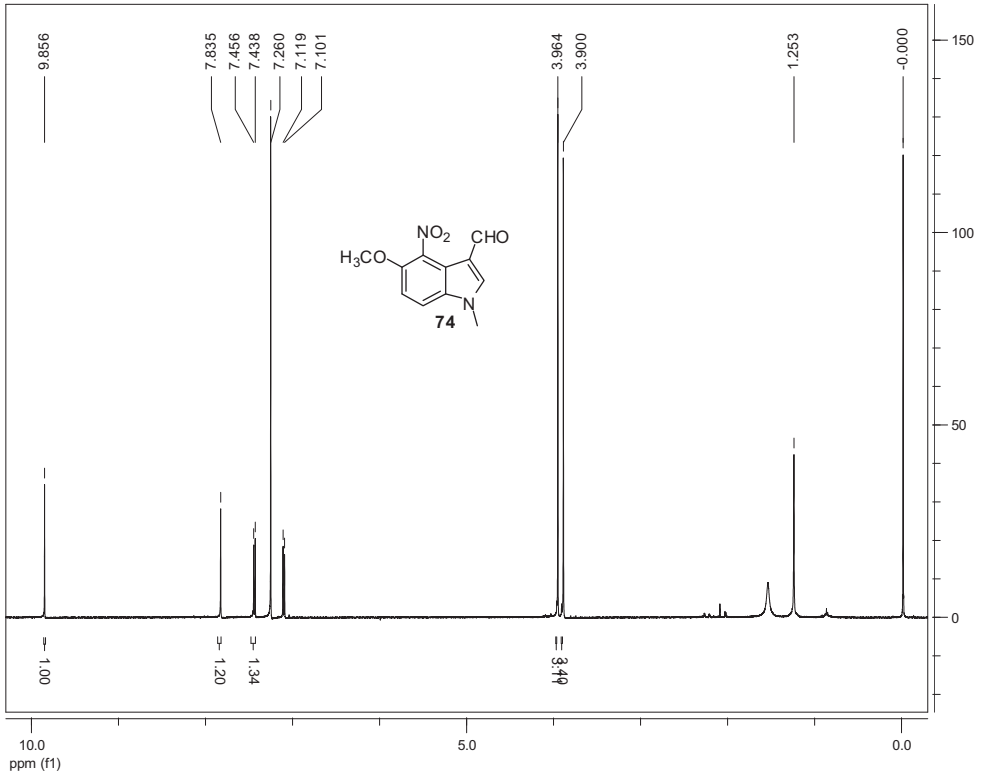
^{1}H NMR (500 MHz, $CDCl_3$) of compound **60**



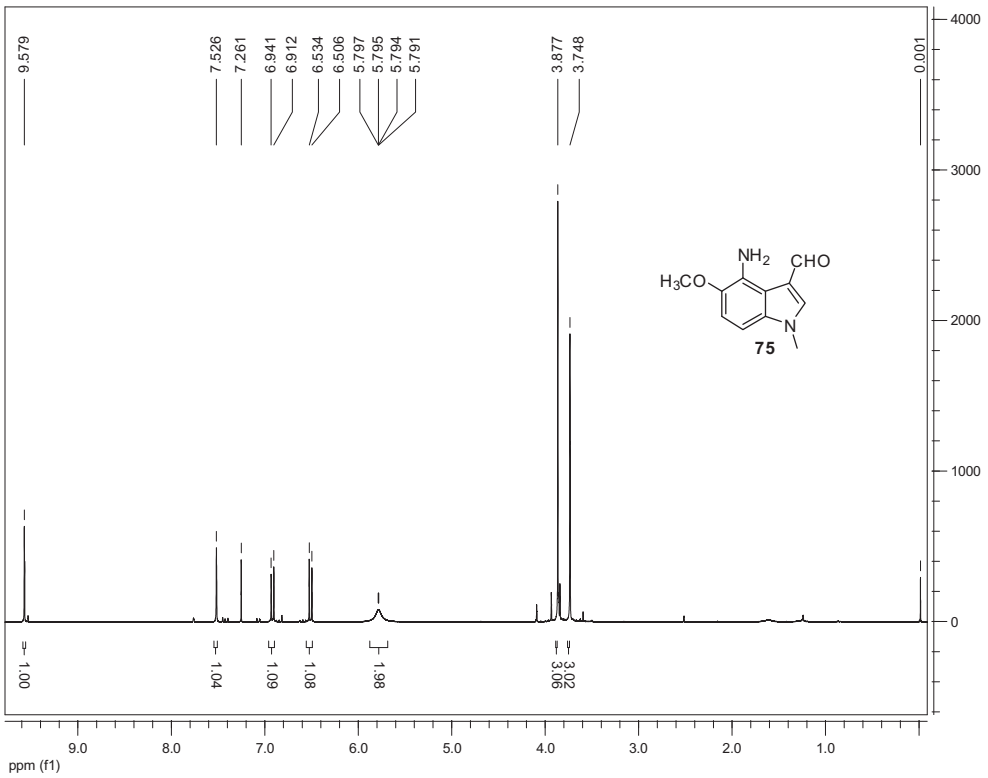
^{1}H NMR (500 MHz, $CDCl_3$) of compound **61**



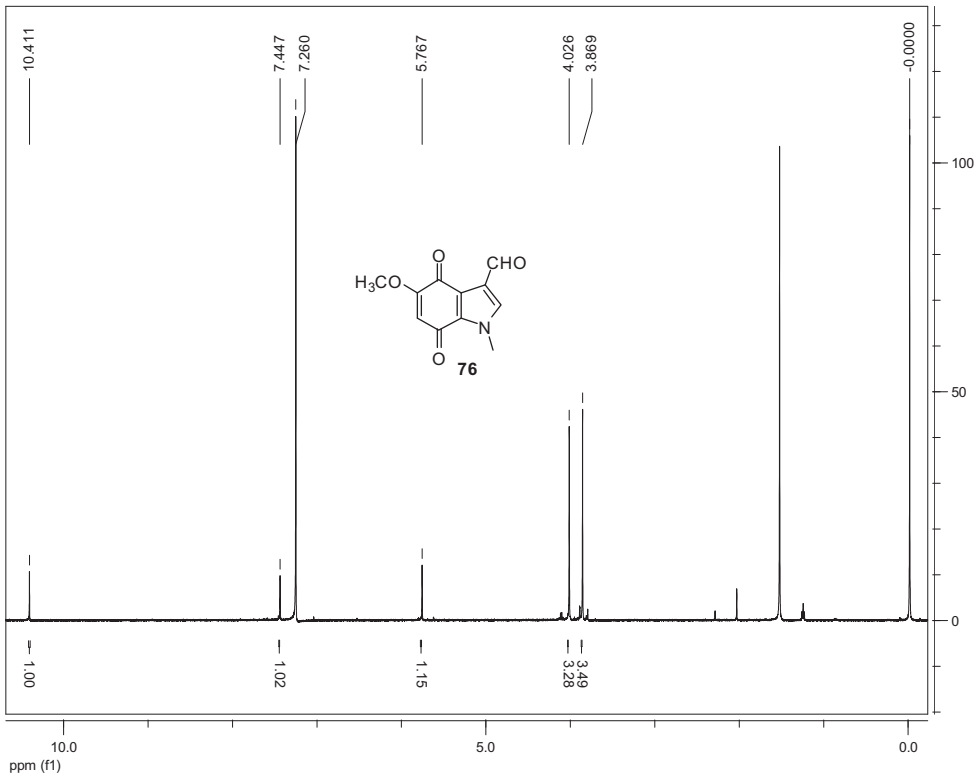
^{1}H NMR (500 MHz, $CDCl_3$) of compound **73**



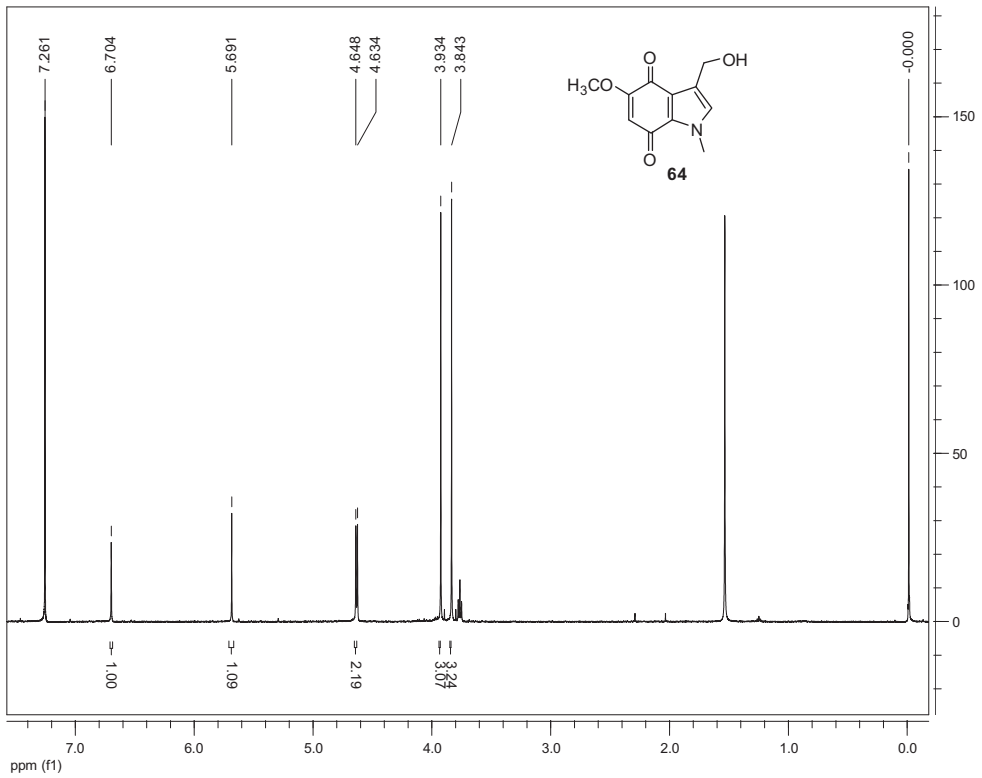
^{1}H NMR (500 MHz, $(CD_3)_2SO$) of compound **74**



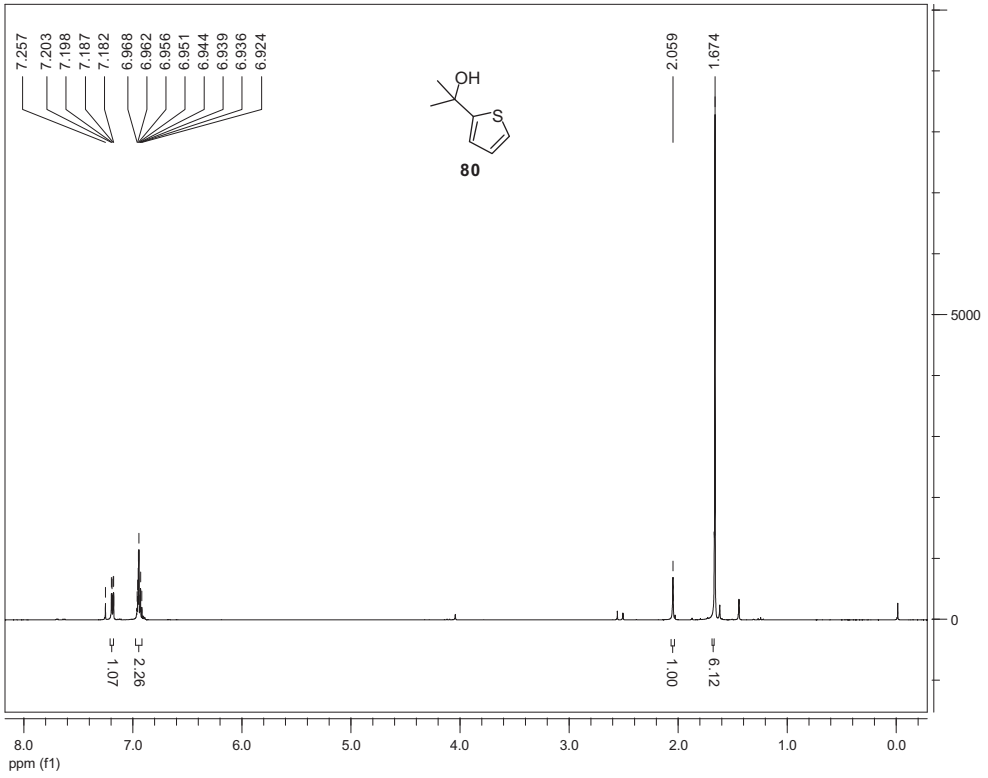
^{1}H NMR (300 MHz, $CDCl_3$) of compound **75**



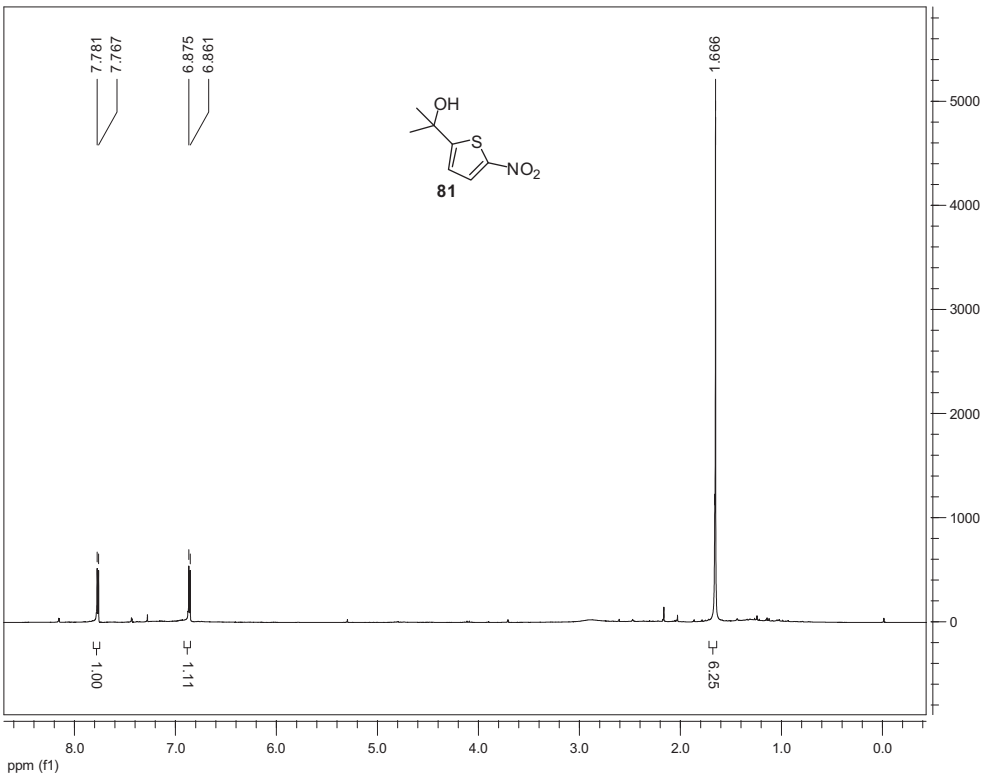
^{1}H NMR (500 MHz, $CDCl_3$) of compound **76**



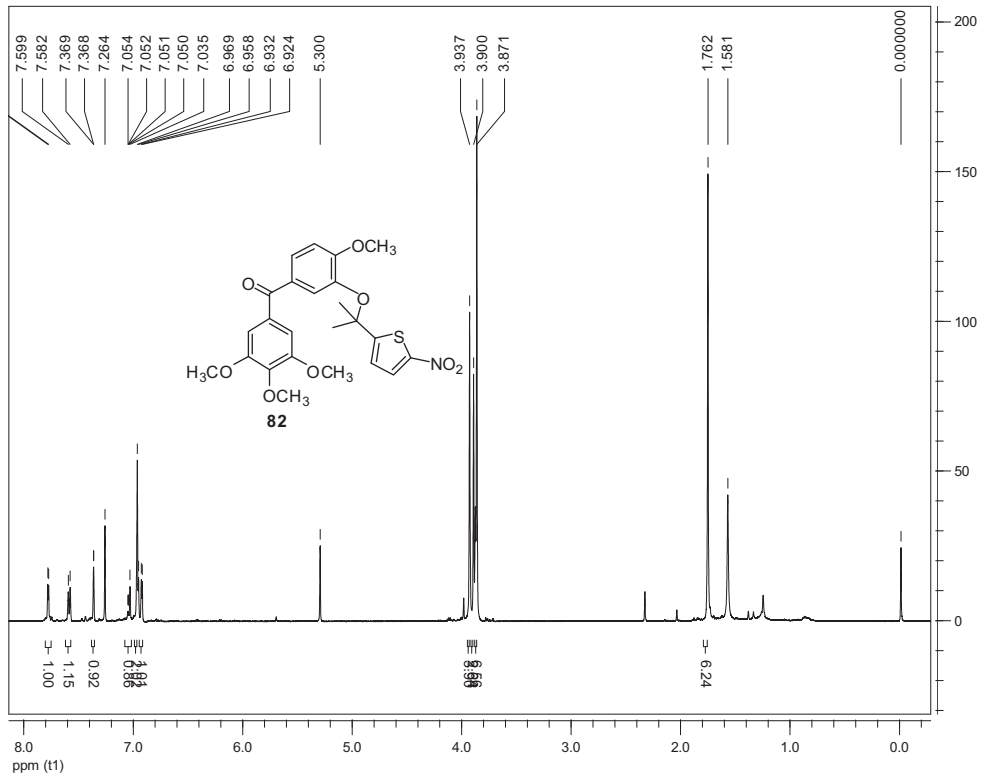
^{1}H NMR (500 MHz, $CDCl_3$) of compound **64**



^{1}H NMR (300 MHz, $CDCl_3$) of compound **80**



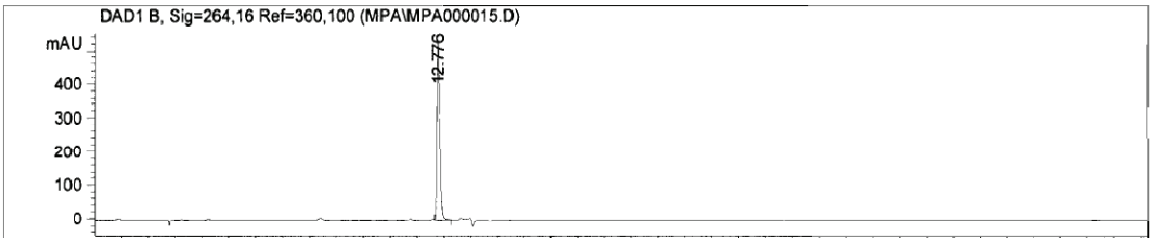
^{1}H NMR (300 MHz, $CDCl_3$) of compound **81**



^{1}H NMR (500 MHz, $CDCl_3$) of compound

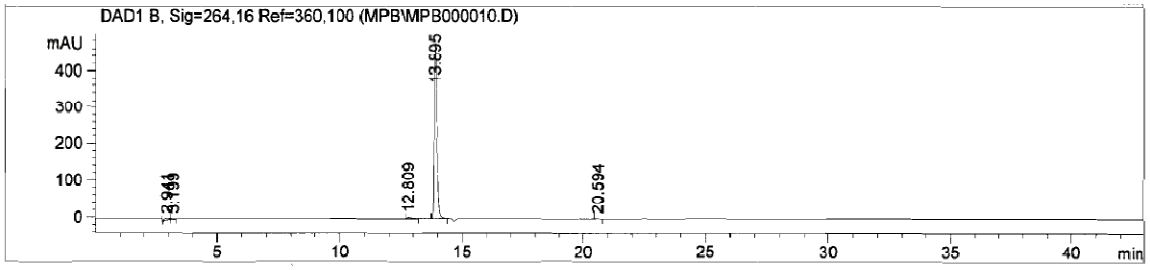
APPENDIX B

High Performance Liquid Chromatography (HPLC) Chromatograms of Combretastatin A-1 Monophosphate A (CA1-MPA), Combretastatin Monophosphate B (CA1-MPB), Combretastatin A-1 (CA1), and Combretastatin A-1 Phosphate (CA1P).



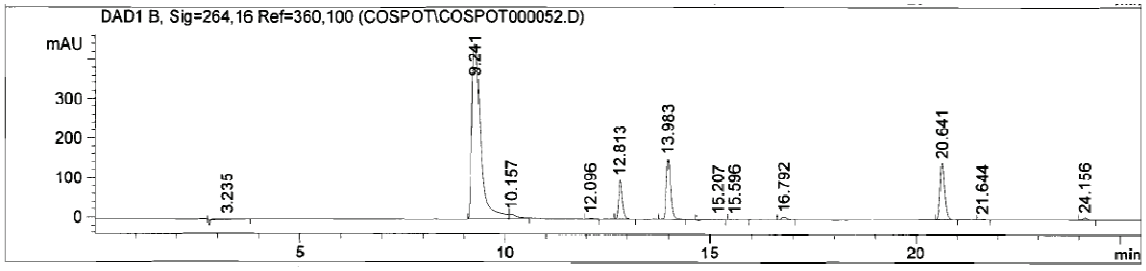
Retention Time = 12.776

HPLC Chromatogram of Combretastatin A-1 Monophosphate A (CA1-MPA)



Retention time = 13.895

HPLC Chromatogram of Combretastatin A-1 Monophosphate B (CA1-MPB)



Retention Time of Combretastatin A-1 Phosphate (CA1P) = 9.241
Retention Time of Combretastatin A-1 Monophosphate A (CA1-MPA) = 12.813
Retention Time of Combretastatin A-1 Monophosphate A (CA1-MPB) = 13.983
Retention Time of Combretastatin A-1 (CA1) = 20.641

HPLC Chromatogram Co-elution of Combretastatin A-1 Monophosphate A (CA1-MPA), Combretastatin Monophosphate B (CA1-MPB), Combretastatin A-1 (CA1), and Combretastatin A-1 Phosphate (CA1P)

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