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Synthesis of Cyclic Enolphosphonates and Enolphosphates as Inhibitors of Serine Hydrolases

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Synthesis of Cyclic Enolphosphonates and Enolphosphates as Inhibitors of Serine Hydrolases

by

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A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Chemistry)

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Abstract

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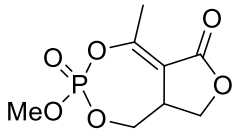
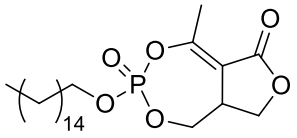
Prof. Christopher D. Spilling, Advisor

Cyclophostin (**1)** is a potent naturally occurring inhibitor of acetylcholinesterase (AChE) with a novel bicyclic enolphosphate structure. The cyclipostins (e.g. **2**) are a family of potent hormone-sensitive lipase inhibitors (HSL) which share the same bicyclic enolphosphate core structure with cyclophostin which but differ primarily in that they are long-chain alkyl phosphate esters. Due to the novelty of the inhibitor structure, potency, and ability to select between two serine hydrolase enzymes with a simple change in the ester substituent, the ongoing structure activity relationship investigation of this family of inhibitors was continued.

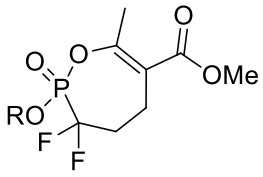


1
cyclophostin

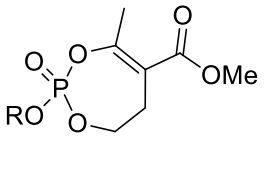


2
cyclipostin P

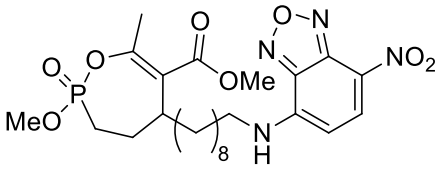
An *α*,*α*-difluorophosphonate monocyclic analog (**3**) was prepared to investigate the possibility of enhancing the potency of these inhibitors. A monocyclic phosphate analog (**4**) was prepared in order to provide, in conjunction with a previously synthesized phosphonate analog, a first recorded direct comparison of *α*,*α*-difluorophosphonate esters, phosphate esters, and phosphonate esters as inhibitors of serine hydrolases. The results indicated that phosphates are most active against AChE and that *α*,*α*-difluorophosphonates show very little inhibition. Methods of transesterification were investigated in order to prepare new analogs of the cyclipostins for testing against HSL. Direct comparison of compounds **5** and **6** and a phosphonate analog followed the same trend as was observed for AChE inhibition. Lipase inhibitors synthesized for inhibition of HSL were submitted for activity against *Mycobacterium tuberculosis* (*M*. *tb*.). Inhibitor **6** was found to be active against the bacterium, but unable to inhibit growth in infected macrophages. Finally, approaches to the synthesis of a fluorescent analog (**7**) of an existing phosphonate inhibitor were investigated in order to probe the mechanism by which these compounds inhibit the growth of *M*. *tb*.



3, R = Et
5, R = $C_{16}H_{31}$



4, R = Me
6, R = $C_{16}H_{31}$



7

Acknowledgment

Foremost, I must acknowledge my advisor Dr. Christopher D. Spilling. His particular style of timely guidance effectively fosters an invaluable self-reliant strength in his students while never leaving them to wander aimlessly.

Next, I acknowledge my committee, whose admirable willingness to correct this document must have taxed each one's patience to an extreme degree. I owe any excellence found here to their freely given expert advice.

The faculty and staff of the University of Missouri-St. Louis in general has always been warm and inviting. The skilled professionals here have been willing to answer any question I have asked and assist with any problem I have encountered.

I must acknowledge past and present colleagues. The education received from one's fellow students often goes unrecognized, and my lab partners probably don't suspect how much they have taught me.

Finally, I acknowledge my beautiful wife. Her unrelenting support has been a constant source of inspiration. When a man sees that he is loved unconditionally, he can be convinced that he has to become lovable. If I have achieved anything, it is because I strive to become a husband who deserves her and the husband she deserves.

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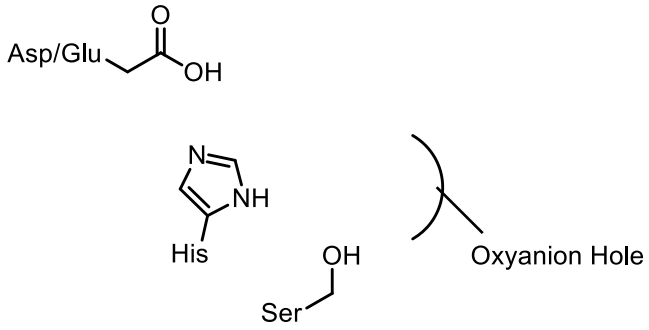
Chapter 1 : Introduction

I. Serine Hydrolases

1. Background

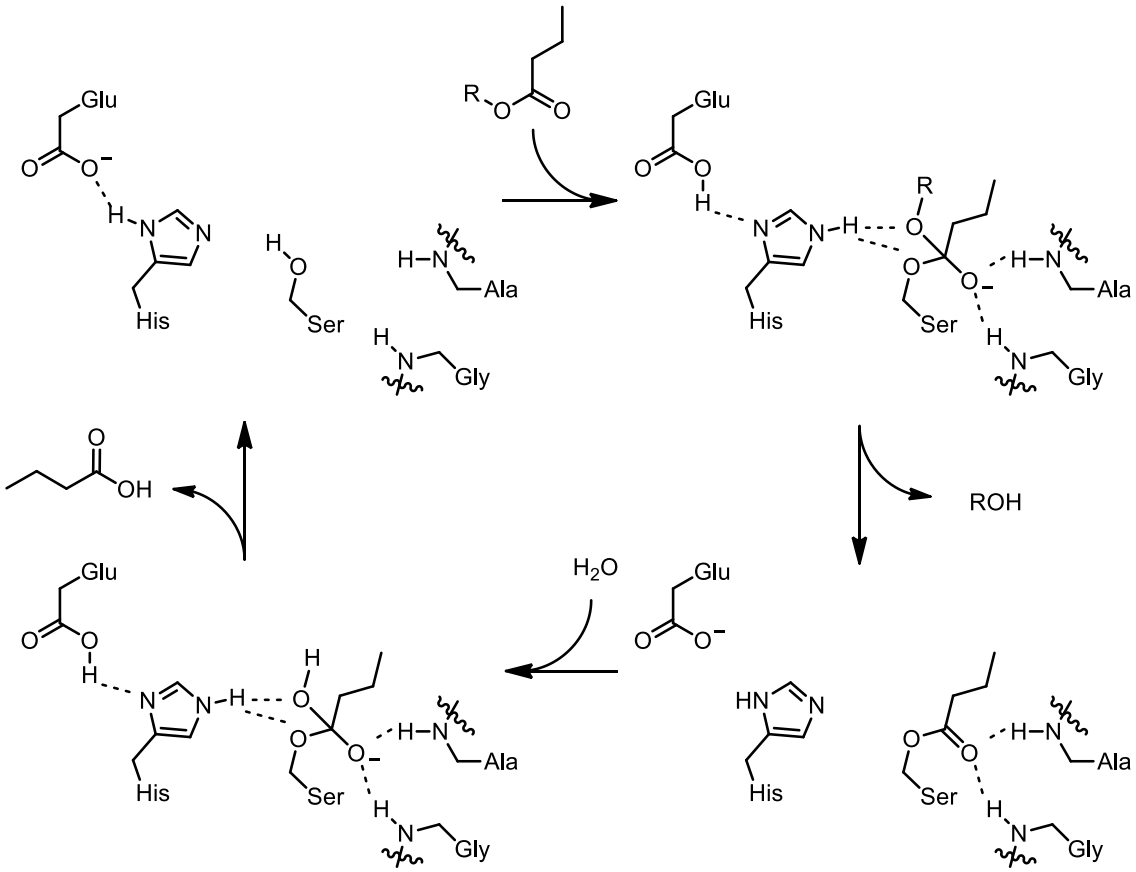
The serine hydrolase superfamily is one of the largest and most diverse classes of enzymes and includes lipases, esterases, proteases, amidases, and peptidases. While their roles in biology are extensive, the function of many is still unknown. The active sites within this family may vary extensively with respect to amino acid sequence, but the morphology of the active site, described as an α/β hydrolase fold, and a catalytic triad, consisting of Ser, His, and Asp or Glu, is common to the family as a whole.[1.1] Also adjacent to the catalytic serine is a binding domain called the oxyanion hole, composed of hydrogen-bonding residues oriented toward the binding site (**Figure 1.1**). This domain stabilizes the tetrahedral intermediate enzyme-substrate complex through hydrogen bonding to the oxyanion.

Figure 1.1: Serine hydrolase active site



By reducing the energy of the intermediate, the activation energy of the hydrolysis reaction is reduced, accounting for the catalytic properties of these enzymes. A full catalytic cycle is depicted in **Scheme 1.1** of hydrolysis of an ester by *Candida rugosa* lipase.[1.2]

Scheme 1.1



Other aspects of enzyme sequence and morphology, particularly within the active site, are responsible for diversity of enzyme specificity toward esters, lipids, proteins,

etc., but also for selectivities within these classes. Esterases catalytically hydrolyze ester bonds of solution-phase substrates, and the active site morphology influences the selectivity of an enzyme by having particular amino acid sequences which accommodate the moieties present in its natural substrate. The stereomorphology of the active site largely influences any enzyme stereopreference.[1.2] Acetylcholinesterase (AChE), in particular, possesses a gorge lined with aromatic residues which is believed to interact with the quaternary ammonium moiety of acetylcholine through extensive π-cation interaction.[1.3] Lipases differ from esterases in that they hydrolyze the ester bonds of water-insoluble substrates at an oil/water interface[1.4] and possess a surface in the catalytic site composed of hydrophobic amino acid residues which accommodate longer alkyl substituents than the corresponding domains in esterases.[1.5] Proteases catalyze the hydrolysis of amide bonds in peptide sequences and have active sites bearing residues which interact with the substrate's peptide sequence.

2. Enzyme Inhibition

The central role of serine hydrolase enzymes in biological functions makes them key targets in the treatment of numerous physiological disorders such as Alzheimer's disease,[1.6] chronic obstructive pulmonary disease (COPD),[1.7] tuberculosis,[1.8,1.9] and other diseases. Inhibition of hormone-sensitive lipase (HSL) reduces free fatty acid (FFA) plasma levels but also has been shown to reduce blood glucose levels, making it a desirable target for diabetes treatment.[1.10] Because of the ubiquity of serine

hydrolase enzymes, it is important that any medicinal inhibitor be selective for the enzyme or enzymes targeted in order to achieve a useful therapeutic index.

While there are known cases of inhibitors non-covalently binding to positions other than the catalytic site[1.6] (e.g. allosteric and noncovalent competitive inhibition) the mode of inhibition which most typifies serine hydrolase research is covalent bond formation to the catalytic serine (covalent competitive inhibition). This mode can be divided into two classes: reversible inhibition and irreversible inhibition. Reversible inhibitors take advantage of the oxyanion stabilizing features of the enzyme by using moieties which form very stable, slow-hydrolyzing charged tetrahedral complexes with the catalytic serine. Enzymes inhibited in this way can be reactivated by reducing the inhibitor concentration or by increasing substrate concentration. Irreversible inhibitors employ materials which alkylate, phosphorylate, or acylate the catalytic machinery such that the stable, uncharged complexes are not hydrolyzed readily, if at all ($k_{-2} \approx 0$, **Equation 1.1**).[1.11]

Equation 1.1

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} E.I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{-}I$$

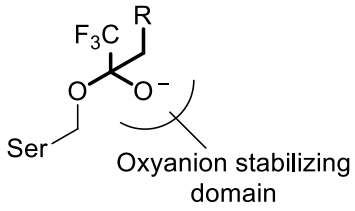
E: Enzyme; I: Inhibitor; E.I.: noncovalent enzyme inhibitor binding; E-I: covalent binding[1.11]

II. Reversible Inhibitors

1. α-Halomethylketones

Activated carbonyl moieties such as trifluoromethyl ketones (TFKs) inhibit serine hydrolase enzymes reversibly by forming a tetrahedral adduct with the serine which is stabilized by the effect of adjacent electron withdrawing substituents (**Figure 1.2**). This strategy of inhibitor design is understandably diverse, reflective of the potential number of possible electron-withdrawing substituents.

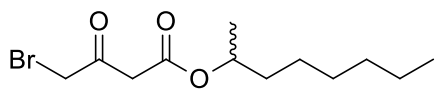
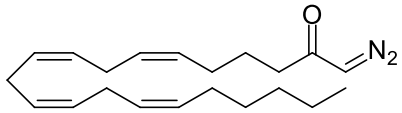
Figure 1.2: Reversible covalent serine hydrolase inhibition



Patricelli, *et al.*[1.12] discovered that 2-octyl-γ-bromoacetoacetate (**1.1**, **Table 1.1**), a naturally-occurring compound known to affect sleep, [1.13] is an inhibitor of fatty acid amide hydrolase (FAAH) with an IC_{50} value of 2.6 µM. Previous studies led them to anticipate that the α-haloketone would be an irreversible inhibitor (see **1.III.4. *α*-Halomethylketones**). However, inhibition kinetics suggested competitive inhibition, and enzyme activity could be restored by anion exchange chromatography purification, suggesting that the enzyme-inhibitor adduct is a stabilized tetrahedral

anion rather than a S_N2 alkylation product. De Petrocellis compared the activity of a collection of inhibitors and found arachidonoyl-diazo-methyl-ketone (**1.2**, **Table 1.1**) to be a mildly potent inhibitor against anandamide amidohydrolase (later identified as FAAH) from RBL-1 and $N_{18}TG_2$ cells and porcine brain. [1.14]

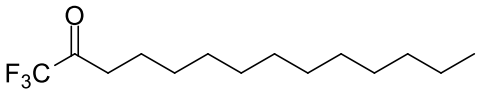
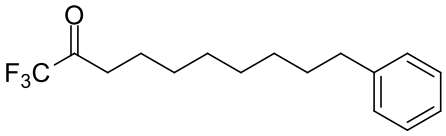
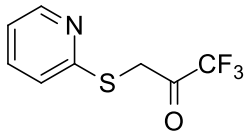
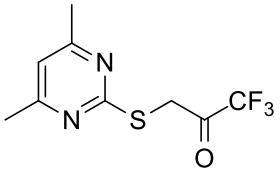
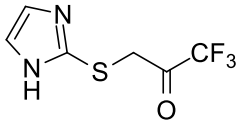
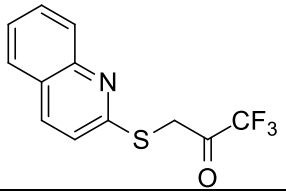
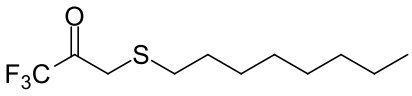
Table 1.1: Inhibitors of fatty acid amide hydrolase

Inhibitor	FAAH Inhibition IC_{50} (μM)
1.1 	2.6[1.12]
1.2 	2, 3, & 6[1.14]

Within this class of inhibitors, the TFKs are the most represented if not always the most potent. Hammock, *et al.* found 1,1,1-trifluorotetradecan-2-one (**1.3**, **Table 1.2**) to be a potent (IC_{50} = 100 nM) inhibitor of crude juvenile hormone esterase (JHE).[1.15] 10-Phenyl-1,1,1-trifluoro-2-decanone (**1.4**, **Table 1.2**) was found by Boger, *et al.*[1.16] to be the most potent (IC_{50} = 120 nM) of numerous TFKs screened against FAAH including those previously reported by Koutek, *et al.*[1.17] and Patterson, *et al.*[1.18] A series of seventeen 3-heteroarylthio-l,l,l-trifluoro-2-propanones (e.g. **1.5a**–**d**, **Table 1.2**) were tested against JHE, acetylcholinesterase (AChE), and yeast lipase (LP) by Szèkács, *et al.*[1.19] Ring substitutions such that hydrogen bonding could stabilize the

serine adduct were found to be more potent inhibitors of AChE. However, 3-octylthio-1,1,1-trifluoro-2-propanone (**1.6**, **Table 1.2**) (as reported in a previous study[1.20]) was found to be superior to the aryl-substituted inhibitors against JHE and LP.

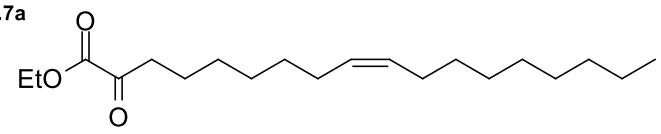
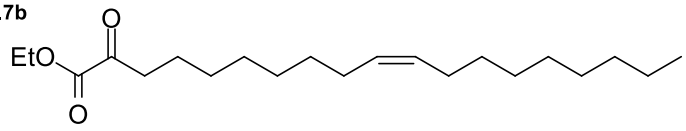
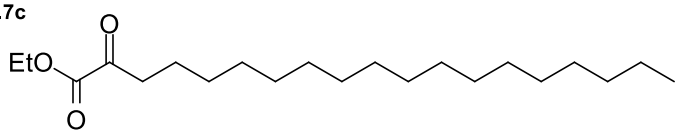
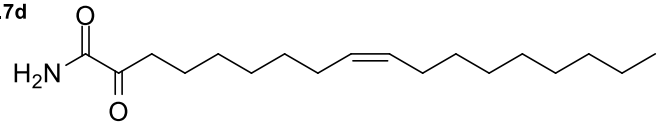
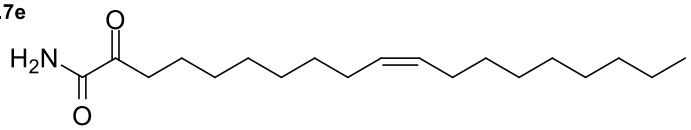
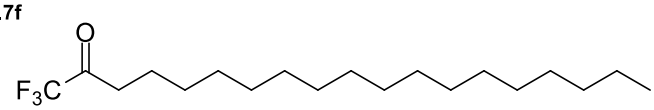
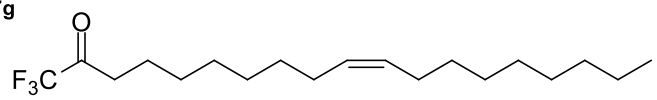
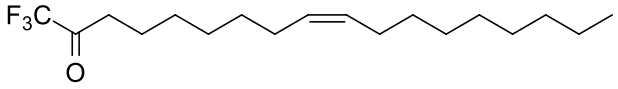
Table 1.2: Inhibitors of juvenile hormone esterase (JHE), fatty acid amide Hydrolase (FAAH), acetylcholinesterase (AChE), and yeast lipase (LP)

Inhibitor	JHE Inhibition IC_{50} (μM)	FAAH Inhibition IC_{50} (μM)	AChE Inhibition IC_{50} (μM)	LP Inhibition IC_{50} (μM)
1.3 	0.1[1.15]			
1.4 		0.12[1.16]		
1.5a 	0.0978[1.19]		8.01[1.19]	106[1.19]
1.5b 	385[1.19]		13.8[1.19]	135[1.19]
1.5c 	324[1.19]		0.627[1.19]	>1000[1.19]
1.5d 	0.631[1.19]		14.8[1.19]	175[1.19]
1.6 	0.00181[1.20]		5.21[1.20]	0.325[1.20]

2. α-Ketocarbonyls

Like α-haloketones, α-dicarbonyls form stable hemiketals with the reactive serine in hydrolase enzymes. Patterson, *et al.* compared inhibiton of a number of inhibitors against oleamide hydrolase (later, this enzyme came to be identified as FAAH) and found very low K_i values associated with α-keto esters (**1.7a**–**c**, **Table 1.3**) and amides (**1.7d** and **1.7e**, **Table 1.3**) which compare well with the analogous TFKs (**1.7f**–**h**, **Table 1.3**).[1.18] Similar comparisons of peptidyl TFKs and α-keto esters as peptidase inhibitors had been performed in the past with comparable results.[1.21]

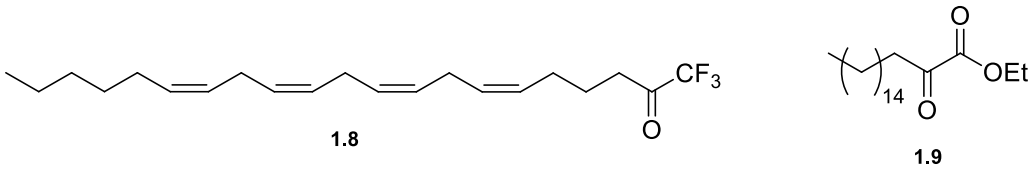
Table 1.3: Fatty acid amide hydrolase (FAAH) inhibitors tested by Patterson, et al.[1.18]

Inhibitor	FAAH Inhibition IC_{50} (nM)
1.7a 	1.4
1.7b 	9
1.7c 	12
1.7d 	16
1.7e 	17
1.7f 	23
1.7g 	1.2
1.7h 	2

Koutek, *et al.* found that trifluoromethyl ketones and α-ketoesters are effective inhibitors of anadamide amidase (also later identified as FAAH), particularly **1.8** and

1.9 (**Figure 1.3**).[1.17] The arachidonyl ketonic structure of **1.8** demonstrates a common theme in rational inhibitor design in which potential inhibitors are functionalized to mimic the enzyme's natural substrate.

Figure 1.3: Fatty acid amide hydrolase inhibitors investigated by Koutek, et al.[1.17]

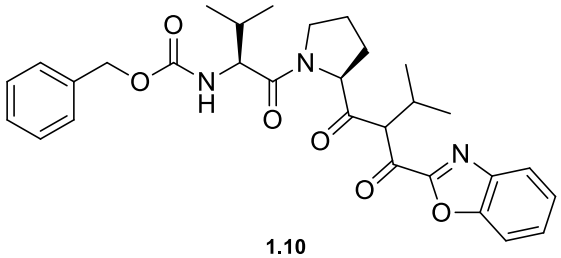


3. α-Heterocyclic Ketones

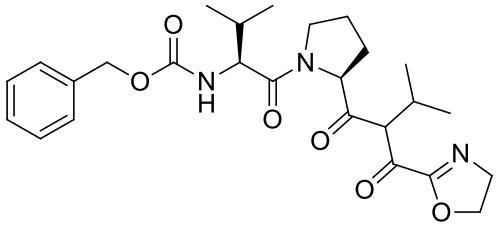
Advancing the concept explored by Szèkács, *et al.*,[1.19] Edwards, *et al.* discovered that α-heterocyclic ketones can be equally effective moieties for the inhibition of serine proteases as TFKs.[1.22] Benzoxazole **1.10** (**Figure 1.4**) was found to inhibit human leukocyte elastase (HLE) with the same order of magnitude as the analogous TFK. Crystallographic data of the inhibitor bound to porcine pancreatic elastase (PPE) demonstrated a hydrogen bonding interaction between the benzoxazole nitrogen and the catalytic histidine. Comparing the activity of **1.10** with that of oxazoline **1.11** (**Figure 1.4**) further demonstrated the inhibitory advantage of hydrogen bonding within the active site. A benzoxazole substituent is more electron withdrawing than oxazoline, so if activation of the ketone toward the serine residue is considered alone, **1.10** should be more active toward the enzyme than **1.11**. Oxazoline **1.11** was found to be five times more active than benzoxazole **1.10**, however. Because

oxazolines are stronger hydrogen bond acceptors than benzoxazoles while being weaker electron withdrawing substituents in addition to the visible interaction in the X-ray crystallographic data, it was reasoned by Edwards, *et al.* that the hydrogen bonding interaction contributed significantly to the stability of the enzyme-inhibitor adduct.

Figure 1.4: α-Heterocyclic ketone porcine pancreatic elastase inhibitors of Edwards, et al.[1.22]



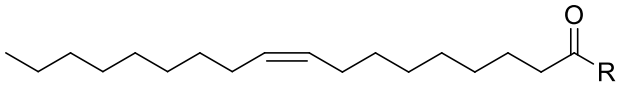
1.10

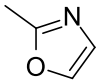
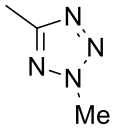
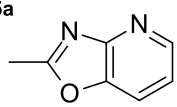
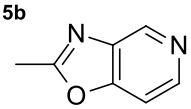
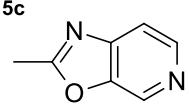
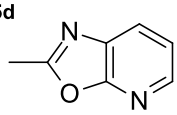


1.11

The role of a hydrogen bonding nitrogen atom was further demonstrated by Tsutsumi, *et al.* who found that the presence or absence of such a *β*-nitrogen makes a three orders of magnitude difference in IC_{50} in prolyl endopeptidase inhibition.[1.23] In a subsequent study by Boger, *et al.* inhibitory activities were achieved with *α*-heterocyclic ketones (**1.13**–**1.15**, **Table 1.4**) superior to that of analogous oleyl TFK **1.12**.[1.24]

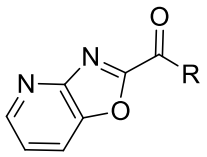
Table 1.4: Low nanomolar inhibitors of fatty acid amide hydrolase (FAAH)[1.24]

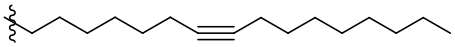


R=	FAAH Inhibition K_i (nM)
1.12 	82
1.13 	17
1.14 	65
1.15a 	2.3
1.15b 	7.2
1.15c 	3.7
1.15d 	11

Subnanomolar K_i values were ultimately achieved by substitution of a short aliphatic chain (**1.16a**–**c**), a short aliphatic chain terminating in a phenyl group (**1.16d**–**h**), or by introducing unsaturation at $\Delta^{9,10}$ (**1.16i**–**k**, **Table 1.5**).

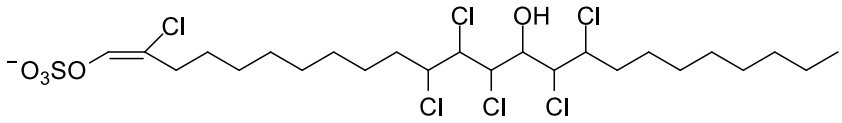
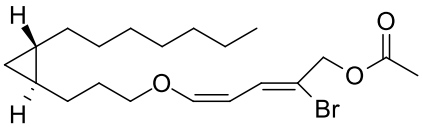
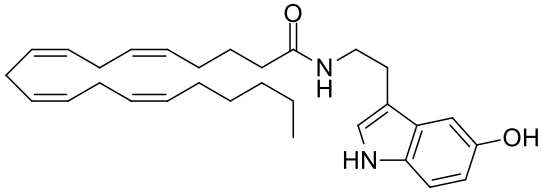
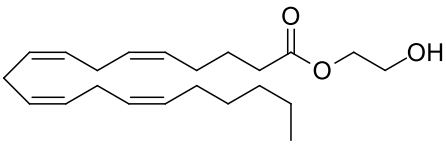
Table 1.5: Subnanomolar inhibitors of fatty acid amide hydrolase (FAAH)[1.24]



R=	FAAH Inhibition K_i (nM)
1.16a $CH_3(CH_2)_{10}$	0.57
1.16b $CH_3(CH_2)_8$	0.75
1.16c $CH_3(CH_2)_6$	0.69
1.16d $Ph(CH_2)_4$	0.30
1.16e $Ph(CH_2)_5$	0.20
1.16f $Ph(CH_2)_6$	0.28
1.16g $Ph(CH_2)_7$	0.39
1.16h $Ph(CH_2)_8$	0.52
1.16i $CH_2{=}CH(CH_2)_7$	0.19
1.16j $HC{\equiv}C(CH_2)_7$	0.18
1.16k 	0.14

Bisogno, *et al.*[1.25] described the inhibitory effects of malhamensilipin A (**1.17**),[1.26] grenadadiene (**1.18**),[1.27] arachidonylserotonin (**1.19**), and arachidonylethylene glycol (**1.19**, **Table 1.6**) toward FAAH. Due to limited availability, the mechanisms of natural products **1.17** and **1.18** were not investigated. Glycol ester **1.19** was found to be a competitive inhibitor which is slowly hydrolyzed to arachidonic acid by the enzyme. Further study showed the possibility of reactivation of enzyme inhibited with inhibitor **1.19** by protein purification and that serotonin and arachidonic acid are not generated in the inhibition studies with **1.19**, demonstrating that the inhibition by the amide is reversible. Though not a ketone, the carbonyl of **1.19** may be activated toward inhibition by a nearby hydrogen bonding moiety.

Table 1.6: Fatty acid amide hydrolase (FAAH) inhibitors investigated by Bisogno, *et al.*[1.25]

Inhibitor	FAAH Inhibition IC_{50} (μM)
1.17 	21
1.18 	22.5
1.19 	12
1.20 	23.5

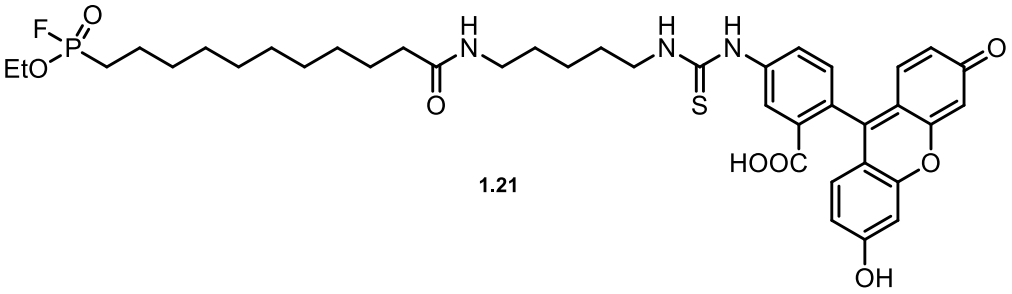
III. Irreversible Inhibitors

1. Fluorophosphates and -Phosphonates

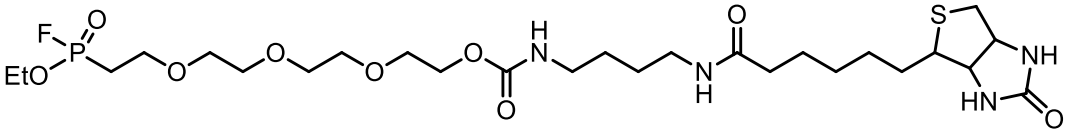
Irreversible inhibitors form a stable covalent complex with the catalytic serine which is not hydrolyzed under normal conditions. Perhaps the most well-known class of irreversible inhibitors is the fluorophosphonates, which phosphorylate the catalytic serine, notoriously those of cholinesterases such as AChE. They have been studied

since the discovery of diisopropyl fluorophosphate's activity in 1942,[1.28] and have since been found to be highly unselective inhibitors.[1.29] Pan-proteomic enzyme inhibitors have found a niche in enzymology. Activity-based protein profiling (ABPP) is a technique for the classification of enzyme sub-families by use of structurally-tuned, labeled inhibitors, but it also has uses in drug discovery. [1.30] If a promising inhibitor candidate is tested against a mixture of enzymes, treatment with a labeled unselective inhibitor can allow the detection of selectivity of the former inhibitor.[1.31] Those enzymes which are completely inactivated by the first enzyme will be blocked from reaction with the second labeled one. Testing against a suitable control experiment would give strong evidence of the investigated inhibitor's selectivity. Furthermore, the technique can be used to detect enzyme dysregulation during the progression of diseases such as cancer.[1.32] By labeling every enzyme of a particular class with a labeled inhibitor, enzymes can be identified, quantified, or even discovered. For such purposes, the fluorophosphonates have a primacy of place. Liu, *et al.* synthesized the fluorescein labeled fluorophosphonate **1.21** (**Figure 1.5**) for ABPP.[1.33] Out of concern that it may exhibit bias toward hydrophobic enzymes, the PEG analog **1.22** (**Figure 1.5**) was prepared by Kidd, *et al.*[1.34] More recently, the tetramethylrhodamine labeled fluorophosphonate **1.23** was synthesized (**Figure 1.5**).[1.35]

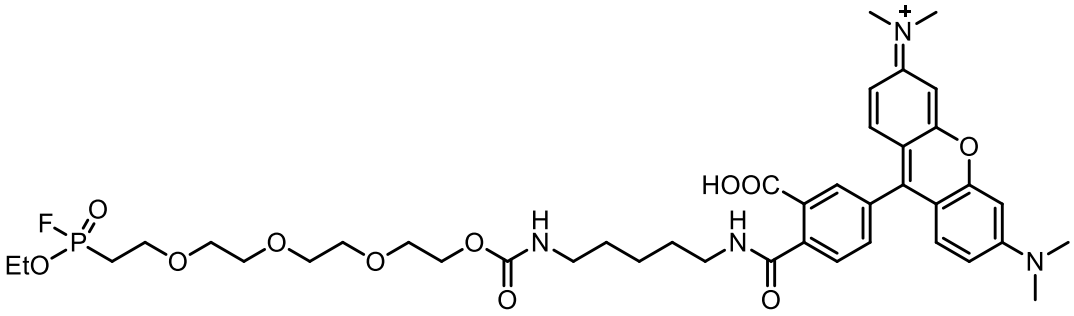
Figure 1.5: Fluorophosphonate inhibitors[1.14,1.33–1.37]



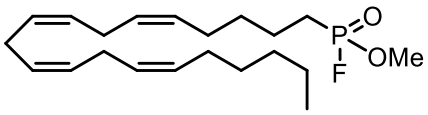
1.21



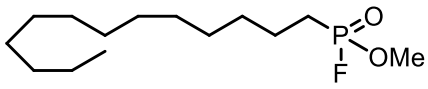
1.22



1.23



1.24



1.25

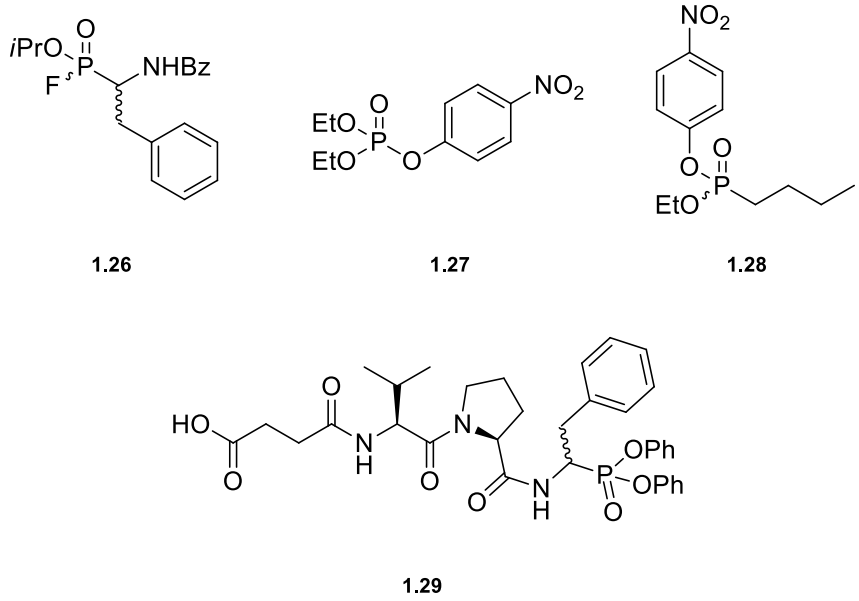
In spite of their general nonspecificity, fluorophosphonates have been investigated as targeted inhibitors. Methylarachidonylfluorophosphonate (**1.24**, **Figure 1.5**) had been demonstrated to be rather selective for FAAH over chymotrypsin, trypsin,

sheep cycloxygenase-1, porcine leukocyte-type 12-lipoxygenase, and rabbit reticulocyte 15-lipoxygenase.[1.14,1.36] Martin, *et al.* tested **1.24** and variously saturated analogs such as **1.25** (**Figure 1.5**) in mice.[1.37] While the resulting symptoms resemble the expected outcome of FAAH inhibition, the mode of action remained ambiguous. Later study by Quistad, *et al.* demonstrated that inhibition of monoacylglycerol lipases by similar fluorophosphonates can lead to the same results.[1.38] The peptidyl fluorophosphonate **1.26** (**Figure 1.6**) synthesized by Lamden and Bartlett, however, shows preference for chymotrypsin over PPE,[1.39] and a peptidyl inhibitor is unlikely to show much reactivity with lipases or esterases.

2. Aryl Phosphate and Phosphonate Esters

Aryl phosphate and phosphonate esters have been investigated as more selective and water-stable alternatives to fluorophosphates and -phosphonates. Hartley and Kilby used diethyl *p*-nitrophenyl phosphate (**1.27**, **Figure 1.6**) to inhibit chymotrypsin, finding inhibition to be irreversible by tracking the release of nitrophenol, providing early evidence that chymotrypsin has only one active site.[1.40] Pentyl phosphonate **1.28** (**Figure 1.6**) was shown by Nayak and Bender to inhibit PPE.[1.41] Oleksyszyn and Powers synthesized peptidyl phosphonates such as **1.29** (**Figure 1.6**), which shows selectivity for chymotrypsin over porcine pancreatic elastase (PPE) and human leukocyte elastase (HLE).[1.42] This strategy exploits both the selectivity of peptidyl inhibitors for peptidases over other enzymes, most notably AChE, observed by Lamden and Bartlett.[1.39] In addition, the stability of arylphosphonates was observed by Nayak and Bender.[1.41]

Figure 1.6: Activated phosphonates for targeted enzyme inhibition[1.39–1.42]



1.26 **1.27** **1.28**

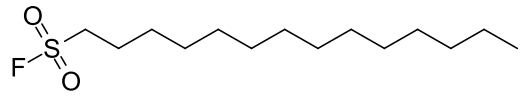
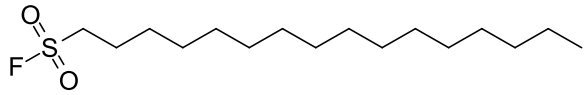
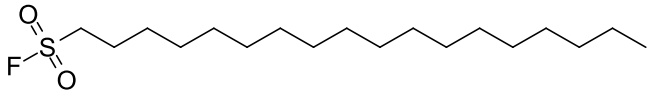
1.29

3. Fluorosulfonates

A moiety similar to the fluorophosphonates is the fluorosulfonates which were first determined to inhibit serine hydrolases, cholinesterases in particular, by Myers and Kemp.[1.43] While no large degree of specificity was observed, Lively and Powers reported that some small degree of selectivity between proteases could be achieved by incorporating a peptide sequence onto the inhibitor.[1.44] Deutsch found that long chain sulfonyl fluorides are more potent inhibitors of FAAH than the previously investigated arachidonyltrifluoromethyl ketone (**1.8**, **Figure 1.3**).[1.45,1.17] The

myristyl-, palmityl-, and stearylsulfonyl fluorides (**1.30**–**1.32**, **Table 1.7**) showed potent inhibition of the enzyme and 100–10,000-fold lower affinity to cannabinoid receptor CB1, an appreciable degree of selectivity for such reactive inhibitors.

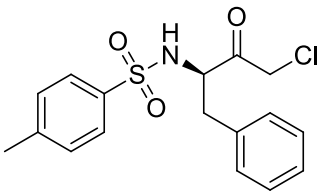
Table 1.7: Sulfonyl fluoride inhibitors of fatty acid amide hydrolase (FAAH)[1.45]

Inhibitor	FAAH Inhibition IC_{50} (nM)
1.30 	6
1.31 	7
1.32 	4

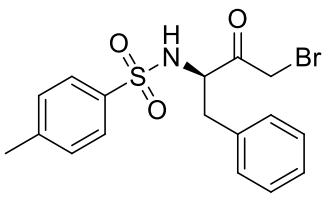
4. α-Halomethylketones

Chloro- and bromomethyl ketones have been shown to alkylate serine hydrolase enzymes with some degree of selectivity. L-l-tosylamido-2-phenylethyl chloromethyl ketone (**1.33**, **Figure 1.7**) and its analogous bromomethyl ketone **1.34** (**Figure 1.7**) were shown to inhibit chymotrypsin by alkylation of the catalytic histidine, which was lost according to amino acid analysis of the acid hydrolyzed inhibited enzyme, giving some early evidence for histidine's presence at the catalytic site.[1.46]

Figure 1.7: Schoellman and Shaw's chloro- and bromomethyl ketone inhibitors[1.46]



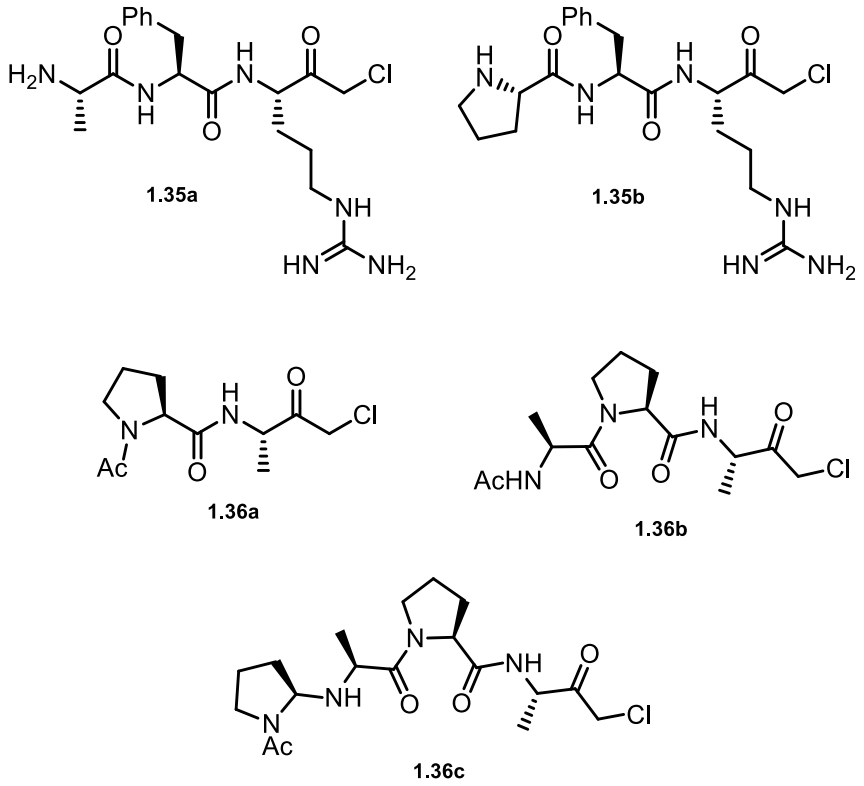
1.33



1.34

This style of inhibitor has demonstrated a degree of selectivity by exploiting mimicry of the target protease's natural substrate, for instance, Ala-Phe-ArgCH_2Cl and Pro-Phe-ArgCH_2Cl (**1.35a** and **135b**, **Figure 1.8**) both showed nanomolar (IC_{50} = 20 nM) inhibition of kallikrein, a serine protease, with the loss of two or more orders of magnitude inhibition against similar proteases plasmin, factor X_a, thrombin, and urokinase.[1.47] Likewise, Ac-Pro-Ala-CH_2Cl, Ac-Ala-Pro-Ala-CH_2Cl, and Ac-Pro-Ala-Pro-Ala-CH_2Cl (**1.36a–c**, **Figure 1.8**) were shown to inhibit elastase more rapidly with increasing peptide length.[1.48]

Figure 1.8: Peptidyl chloromethyl ketone protease inhibitors[1.47,1.48]

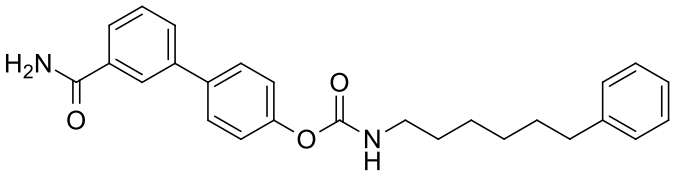
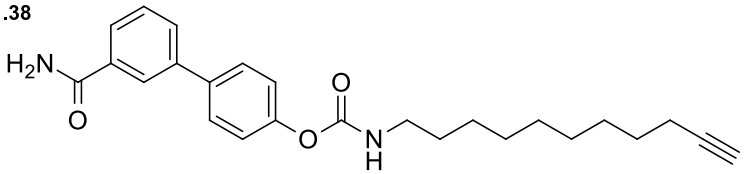


5. Carbamates

Wilson, *et al.* demonstrated that the same reactivation kinetics are observed for AChE inactivated with dimethylcarbamoyl choline as are observed when it is inactivated with dimethylcarbamoyl fluoride. This suggests that both form the same carbamyl serine complex and demonstrates that carbamate inhibitors react irreversibly with the catalytic serine.[1.49] Irreversible carbamate inhibitors, then, form a carbamate with the catalytic serine of the target enzyme which is stable to hydrolysis, freeing a usually-activated alcohol (often, a phenol). Inhibitors **1.37** and

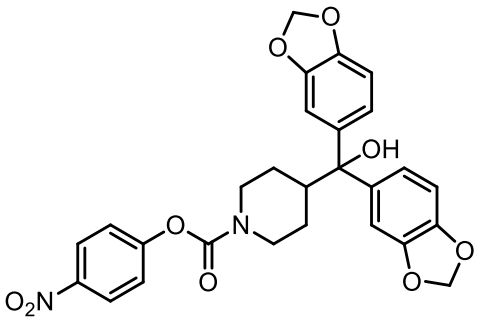
1.38 (**Table 1.8**), among numerous similar inhibitors tested by Kathuria, *et al.* and Alexander and Cravatt, were shown to be active toward FAAH. [1.50,1.51]

Table 1.8: Carbamate fatty acid amide hydrolase inhibitors (FAAH)[1.50]

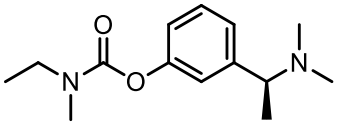
Inhibitor	FAAH Inhibition IC_{50} (nM)
1.37 	1.6
1.38 	1.6

Carbamate **1.39** (**Figure 1.9**) is a potent (IC_{50} = 8 nM) inhibitor of MAGL and is selective *in vivo*,[1.52] and Rivastigmine (**1.40**, **Figure 1.9**) is an irreversible carbamate AChE inhibitor and an approved drug for Alzheimer's and Parkinson's related dementia marketed as Exelon.[1.53] Cholinesterase enzymes show very slow reactivation (e.g. >10% after 48 hours for AChE) after inhibiton with **1.40**,[1.53] so it has been classified in this work as irreversible.

Figure 1.9: *In vivo* Carbamate inhibitors[1.52,1.53]



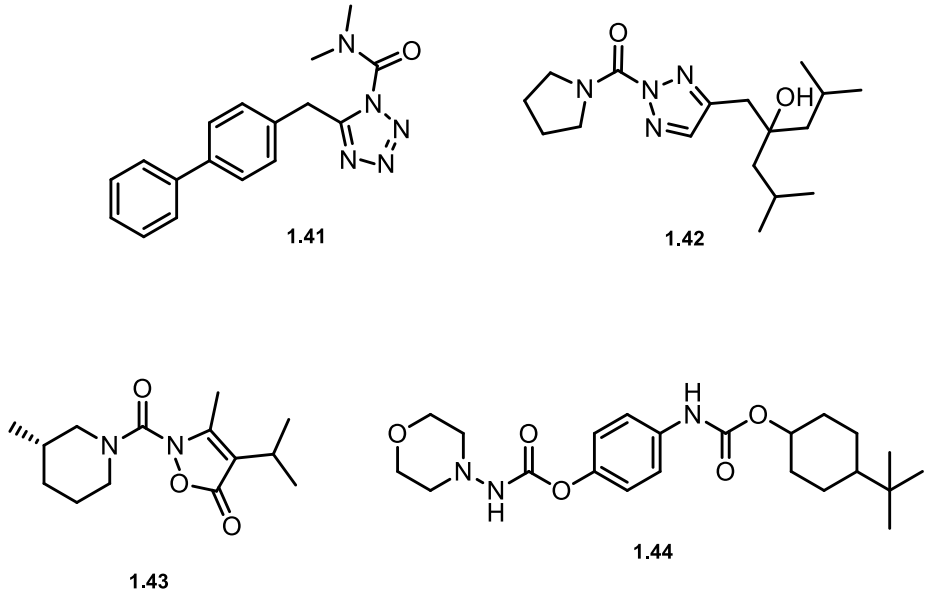
1.39



1.40
Rivastigmine
(Exelon)

Tetrazole **1.41** (**Figure 1.10**) was found to be a potent, albeit unselective, inhibitor of FAAH which carbamylates the enzyme's catalytic serine.[1.54] Pursuit of the emerging concept of heterocyclic ureas as carbamylating inhibitors led to triazole **1.42** (**Figure 1.10**), a potent inhibitor of acylaminoacyl-peptide hydrolase (APEH) (IC_{50} = 5 nM), which is both active and selective in vivo.[1.55] Likewise, Lowe, et al. found urea **1.43** (**Figure 1.10**), known as BAY, to be a potent inhibitor of HSL with very slow reactivation kinetics.[1.56,1.10] Carbazates were investigated by de Jong, *et al.*, and carbazate **1.44** (**Figure 1.10**) was found to inhibit HSL with an IC_{50} value of 1 nM.[1.57]

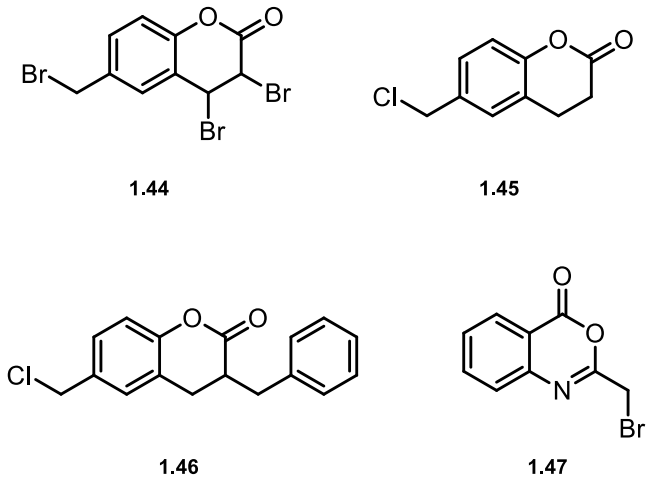
Figure 1.10: Other carbamylating or carbazylating inhibitors[1.54–1.57]



6. Enzyme-activated Inhibitors

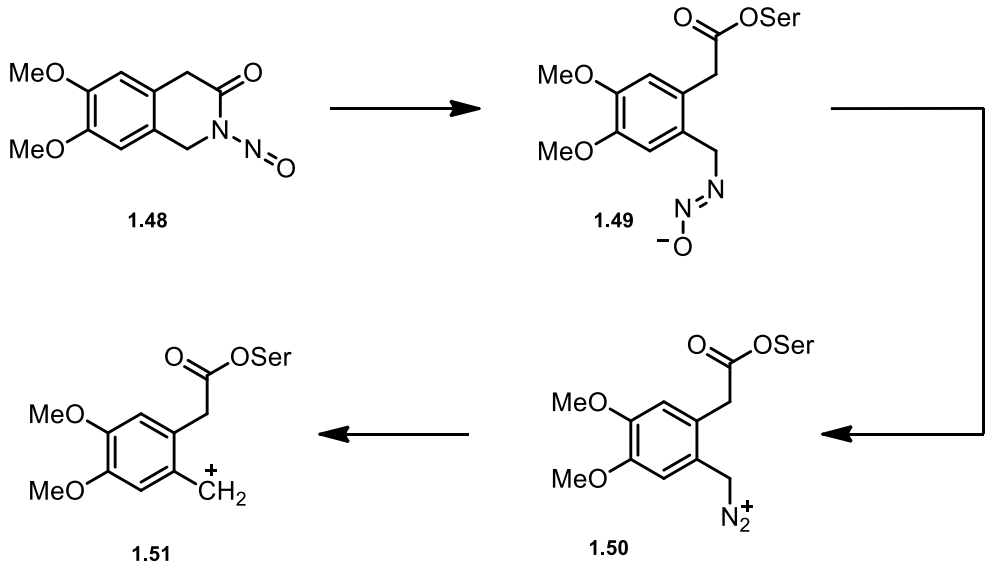
Bechet, *et al.* found that halomethylcoumarins such as **1.45–1.46** and benzoxazin-4-one **1.47** (**Figure 1.11**) effectively inactivate proteases at neutral pH with low specificity and suggested that the inhibition is due to alkylation of the catalytic histidine.[1.58] The cyclic ester acts as a substrate for the enzyme, and the histidine residue is alkylated when the inhibitor occupies the active site.

Figure 1.11: Coumarin and benzoxazin-4-one inhibitors tested by Bechet, *et al*.[1.58]



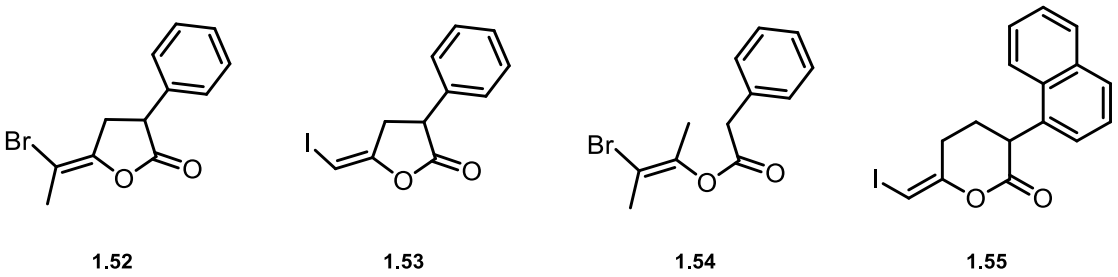
An analogous style of inhibitor was devised by White, *et al.* in which the hydrolysis of the nitrosolactam **1.48** forms the carbenium **1.51** (**Scheme 1.2**) which can then alkylate nearby basic residues. Inhibitors which form reactive species upon hydrolysis by the enzyme have come to be known as suicide reagents,[1.59] mechanism-based inhibitors, or enzyme-activated inhibitors.

Scheme 1.2



Chakravarty, *et al*. found haloenol lactones **1.52** and **1.53** (**Figure 1.12**) to be irreversible inhibitors of α-chymotrypsin and noted the importance of having the electrophile tethered to the ester. Acyclic ester **1.54** (**Figure 1.12**) was found to be an ineffective inhibitor.[1.60a] Further study determined that six-membered lactones are superior inhibitors and that a naphthyl substituent is superior to a phenyl group. Haloenol lactone **1.55** (**Figure 1.12**) was found to inhibit α-chymotrypsin with a K_i value of 0.339 μM, a 100-fold improvement over **1.53**.[1.60b]

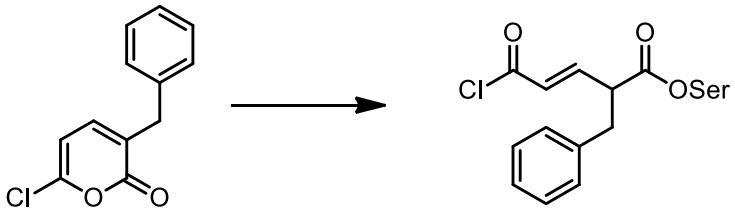
Figure 1.12: Haloenol lactone inhibitors of α-chymotrypsin[1.60]



1.52 **1.53** **1.54** **1.55**

Chloropyrones were investigated by Westkaemper and Abeles as mechanism-based inhibitors of *α*-chymotrypsin, and pyrone **1.56** was found to form acid chloride **1.57** upon reaction with the enzyme (**Scheme 1.3**).[1.61]

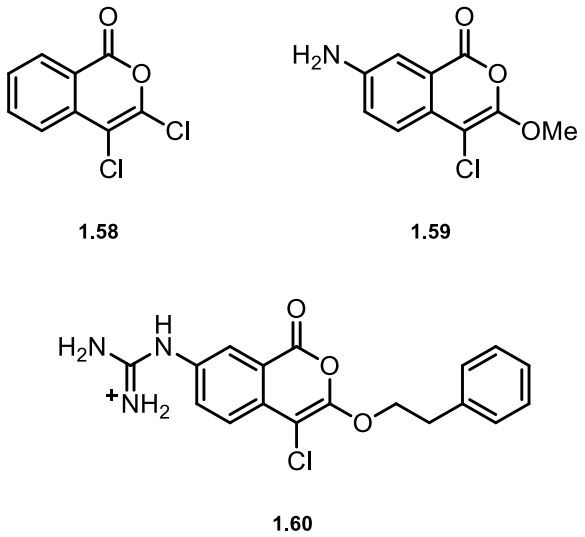
Scheme 1.3



1.56 **1.57**

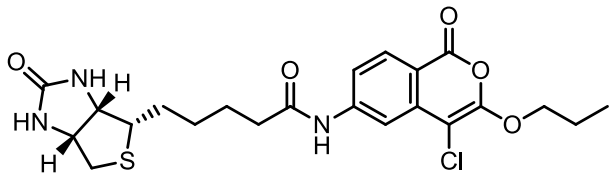
Harper, *et al.* employed this concept with chloroisocoumarin **1.58** (**Figure 1.13**) which was found to be a general protease inhibitor with low micromolar activity against HLE and PPE.[1.62a] Inhibition of proteases was found to be effective using 3-alkoxy isocoumarins, particularly those with an amino or guanidyl group at the 7-position (e.g. **1.59** and **1.60**, **Figure 1.13**).[1.62b,c]

Figure 1.13: Isocoumarin inhibitors[1.62]

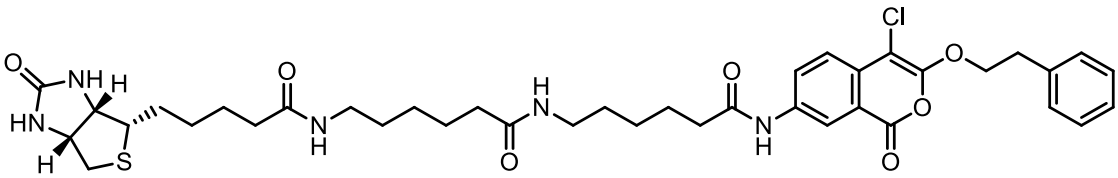


Because of their general activity toward serine proteases, biotinylated isocoumarins such as **1.61** and **1.62** (**Figure 1.14**) have been investigated as a means of detecting serine proteases in mixtures by ABPP.[1.63]

Figure 1.14: Biotinylated 4-chloro-3-alkoxy isocoumarins[1.63]



1.61



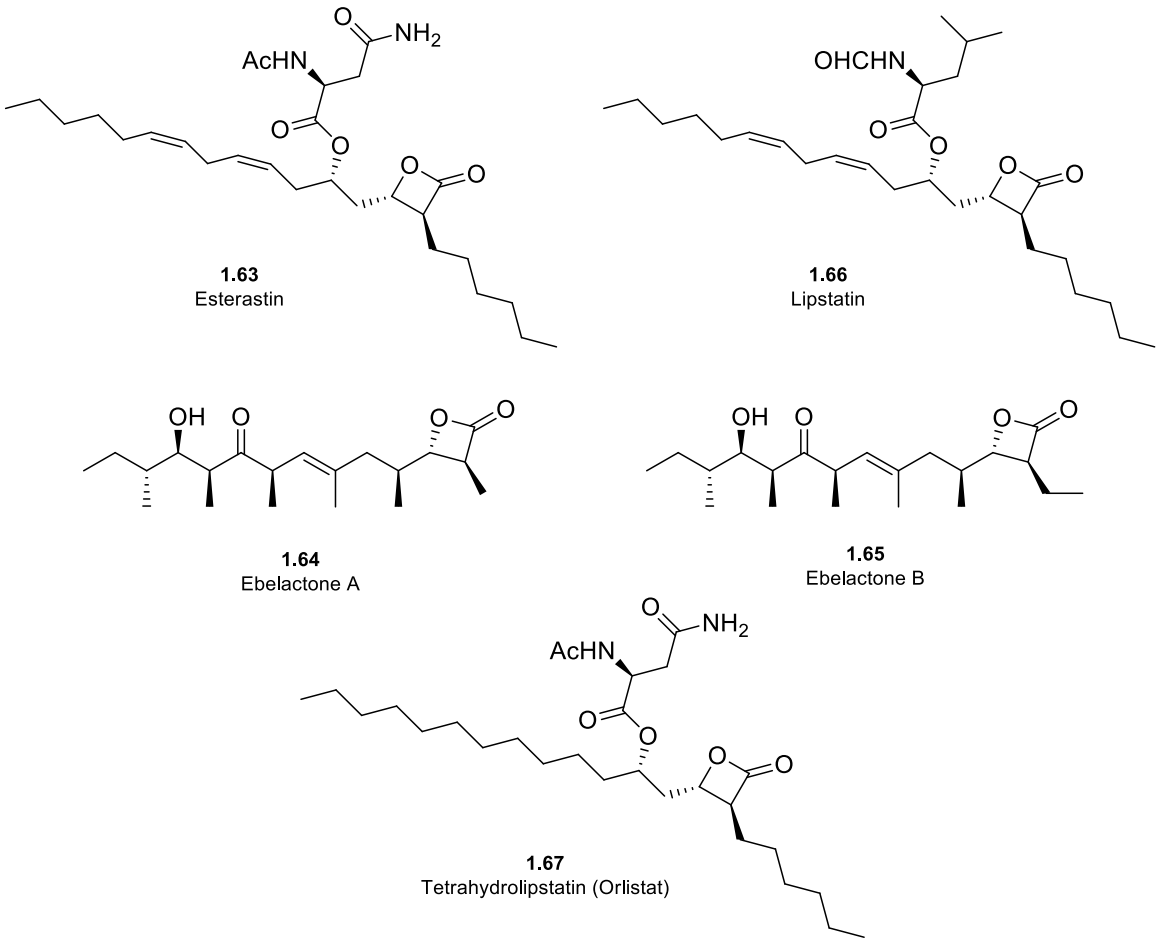
1.62

7. β-Lactones and β-Lactams

Esterastin (**1.63**, **Figure 1.15**) is a natural product isolated from fermentation of *Streptomyces levendulae* species MD4-C1 which was found to strongly inhibit hog pancreatic lipase (HPL, IC_{50} = 0.4 nM).[1.64] Later study found that esterastin (**1.63**) is a rather selective inhibitor of acid lipase, showing nearly tenfold greater inhibition than against pancreatic lipase.[1.65] A similar pair of compounds, ebelactone A and B (**1.64** and **1.65**, **Figure 1.15**), were isolated from *Streptomyces aburaviensis* related strain MG7-G1 which showed similar activity against HPL and superior inhibition of pig liver esterase (PLE, IC_{50} = 17 and 0.99 nM vs. 9.9 μM).[1.66] Lipstatin (**1.66**, **Figure 1.15**), isolated from *Streptomyces toxytricini*, is highly structurally related to esterastin (**1.63**) and is also a potent inhibitor of pancreatic lipase, along with the analogous tetrahydrolipstatin (**1.67**, **Figure 1.15**) which showed only a moderate decrease in inhibition from the parent lipstatin (**1.66**).[1.67] Lipstatin (**1.66**) was found to effectively reduce triolein absorption in mice without effecting the oleic acid

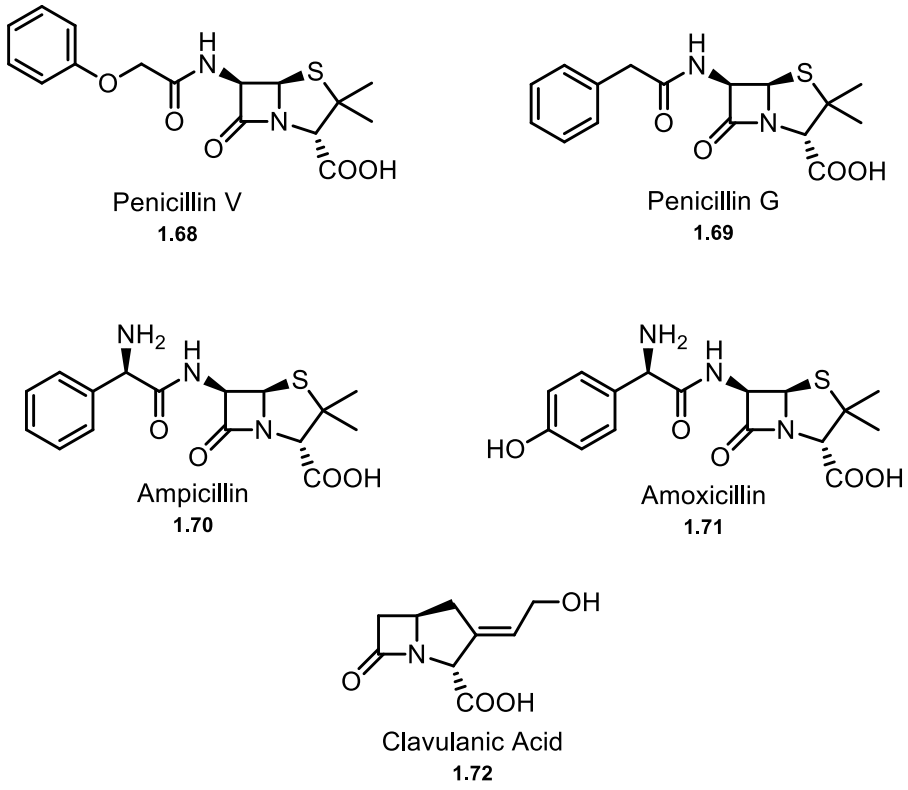
absorption, demonstrating the effective inhibition of hydrolytic lipid digestion. The saturated tetrahydrolipstatin (**1.67**), under the name Orlistat, has been found to be safe for use as an antiobesity medication[1.68] and is marketed under the names Xenical and Alli.

Figure 1.15: β-Lactone lipase inhibitors[1.64–1.68]



β-Lacatams are a very common motif among antibiotics (e.g. **1.68-1.71**, **Figure 1.16**) and are known to bind the reactive serine of penicillin binding proteins (PBPs) irreversibly, disrupting bacterial cell wall synthesis.[1.69]

Figure 1.16: *β*-Lactam bactericidal inhibitors[1.69,1.70]



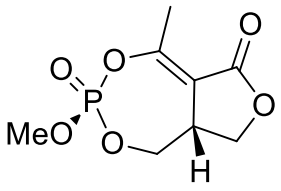
Multiple drug resistant infections, particularly *Mycobacterium tuberculosis* (TB), are the result of the presence of bacterial *β*-lactamase enzymes. *β*-Lactamases have a water molecule conserved in the active site of the enzyme which can hydrolyze an acylserine complex when formed by a *β*-lactam antibiotic. Clavulanic acid (**1.72**, **Figure 1.16**) has been found to be a potent inhibitor of *β*-lactamase and is FDA

approved for use in conjunction with traditional antibiotics.[1.9,1.70] Augmentin, for example, is a combination of Amoxicillin (**1.71**) and clavulanic acid (**1.72**).

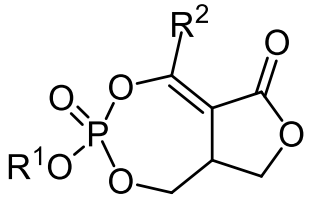
8. Cyclic Enolphosphates and -Phosphonates

Kurokawa, *et al.* isolated cyclophostin (**1.73**, **Table 1.9**), a low-nanomolar inhibitor of insect AChE, from *Streptomyces lavendulae* strain NK901093.[1.71] A series of natural products were later isolated from *Streptomyces* species DSM 13381, called the cyclipostins (e.g. **1.74a**–**l**, **Table 1.9**), which exhibited low-nanomolar inhibition of HSL.[1.72]

Table 1.9: Cyclophostin and cyclipostins[1.71,1.72]

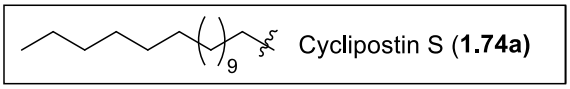


1.73



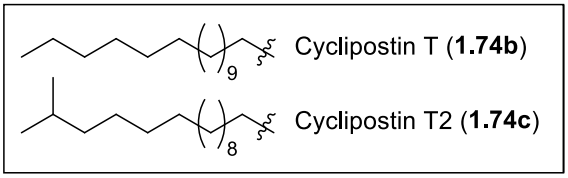
1.74

R^1 =



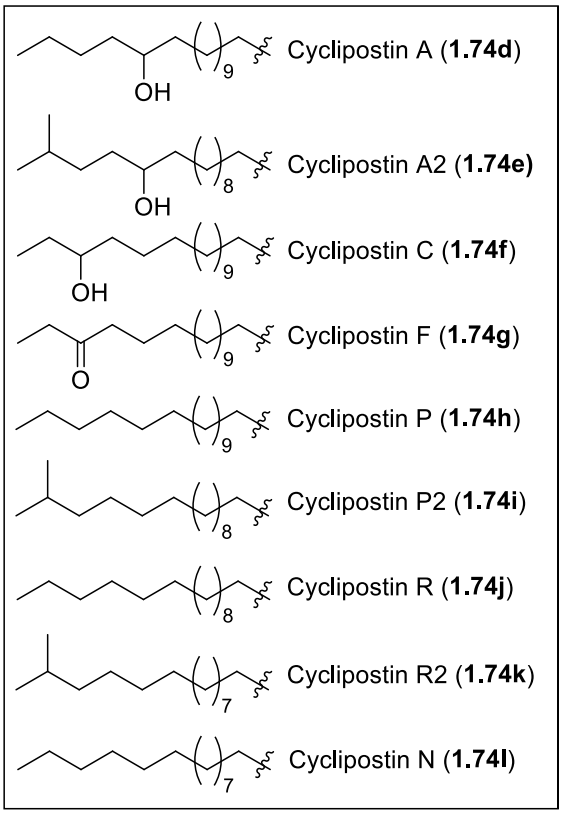
R^2 = Et

R^1 =



R^2 = *n*Pr

R^1 =

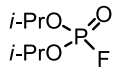
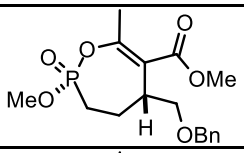
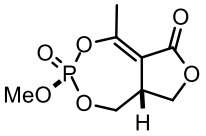
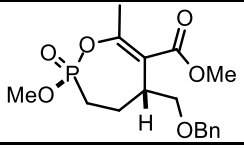
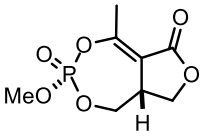
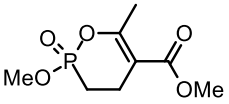
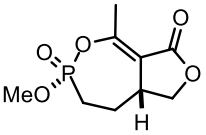
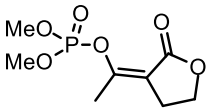
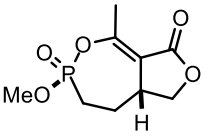
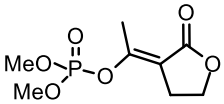
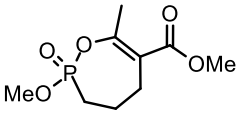
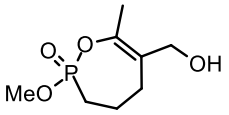


R^2 = Me

These natural products, sharing a novel bicyclic phosphate core structure, demonstrate absolute tunability from AChE to HSL by the simple substitution of a long alkyl ester for a methyl ester, while already displaying the potency of inhibition of some of the most reactive and unstable inhibitors. This new class of inhibitor was

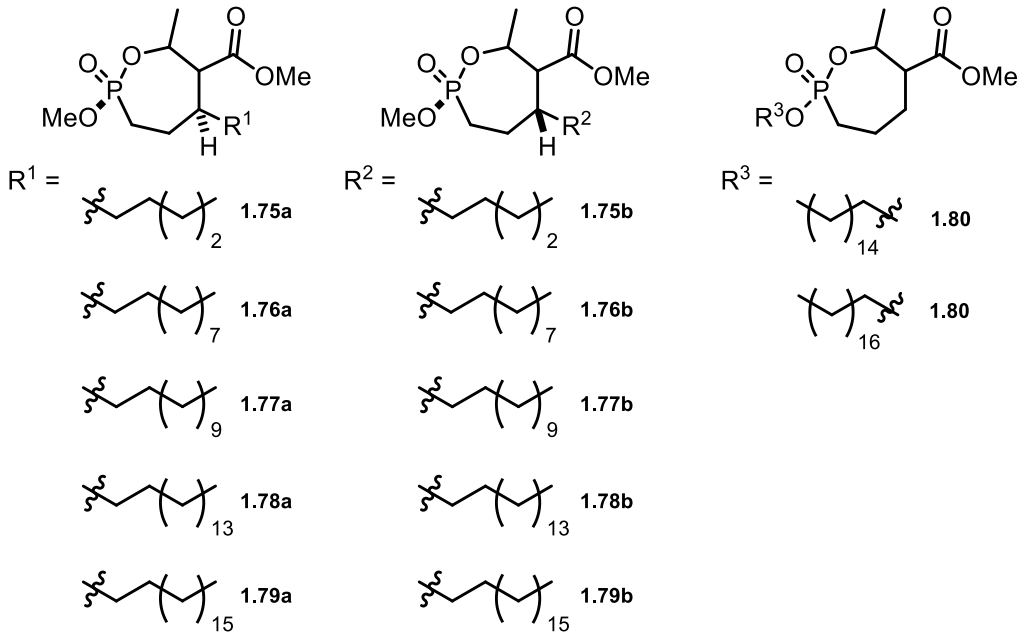
investigated by the Spilling group for the sake of this potential. Racemic cyclophostin (**1.73**) and cyclipostin P (**1.74h**) were synthesized, and synthetic **1.73** proved to be a low-nanomolar inhibitor of human AChE.[1.73] Additionally, various phosphonate analogs were prepared for structure activity relationship (SAR) comparison, demonstrating that while phosphonates are weaker inhibitors of AChE, the lactone ring-opened phosphonate analogs showed inhibition within the same order of magnitude as the bicyclic phosphonate analog (**Table 1.10**).[1.11,1.73,1.74] Also important for inhibition is the *E* geometry of the enolphosphate moiety and the conjugated ester.

Table 1.10: Analogs of cyclophostin as inhibitors of acetylcholinesterase[1.11,1.73,1.74]

Inhibitor	IC_{50} (μM)	Inhibitor	IC_{50} (μM)
 DIFP	0.12		35
	0.045		6
	0.040		5
	3		>1000
	30		70
	7		600

A series of phosphonate analogs of the cyclipostins (**1.75-1.81**, **Figure 1.17**) were found to be inhibitors of microbial lipases cutinase, Rv0183, and LipY, the latter two being associated with the persistence of *Mycobacterium tuberculosis*, and inactive toward mammalian digestive enzymes dog gastric lipase (DGL), human pancreatic lipase (HPL), and guinea pig pancreatic lipase related protein 2 (GPLRP2), suggesting a particular preference for microbial enzymes.[1.8]

Figure 1.17: Microbial lipase inhibitors[1.8]



Rv0183 was chosen as a target because it is a monoacylglycerol lipase implicated in the degradation of host cell lipids and could play a key role in the life cycle of *M. tuberculosis*.[1.75] LipY is a triacylglycerol lipase which may be key to the bacterium's survival during dormancy.[1.11,1.76] Lipase inhibitors selective for these enzymes could therefore be effective drug targets which act through a new bactericidal mechanism, representing a new approach to battling drug-resistant *M. tuberculosis* infections.

IV. Conclusion

Cyclic enolphosphates and -phosphonates are novel and potent inhibitors which already display tunable selectivity toward serine lipase enzymes after SAR study, and the structural motifs which govern inhibition have been investigated to an

extent.[1.8,1.73,1.74] In this thesis, further SAR has been performed to determine other ways the potency and selectivity of these inhibitors might be improved or directed.

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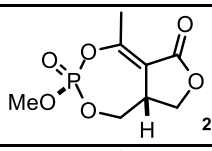
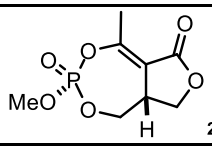
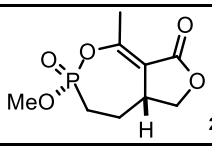
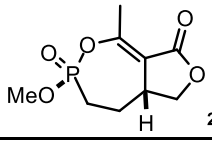
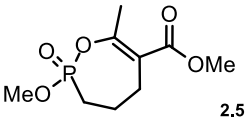
Chapter 2 : α,α-Difluorophosphonate Analogs of Cyclophostin and Analogs of Cyclipostins

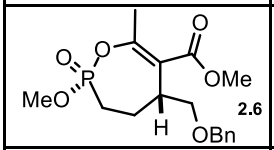
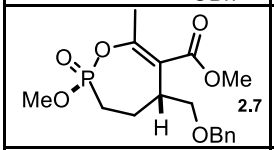
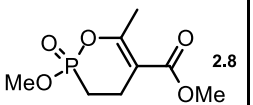
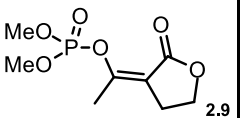
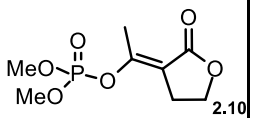
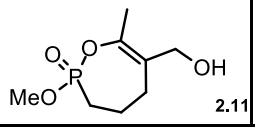
I. Introduction

1. Background

Cyclophostin (**2.1**, **Table 2.1**) is a natural bicyclic enolphosphate inhibitor of acetylcholinesterase (AChE) isolated from *Streptomyces levendulae*.[2.1] Racemates of the novel natural inhibitors and analogs were synthesized by the Spilling lab to probe the structure activity relationship (SAR) toward AChE.[2.2] The non-natural diastereomer **2.2** was found to have similar inhibition to natural *cis* diastereomer **2.1**. Phosphonate analogs **2.3** and **2.4** are 100- and 1000-fold less potent, respectively, with a preference for the *trans* isomer. Monocyclic phosphonate analog **2.5** is only marginally less potent than the *trans* bicyclic phosphonate **2.4**, and the ring opened synthetic precursors to **2.3** and **2.4**, **2.6** and **2.7**, show insignificant loss of activity as well but with preference for the *cis* diastereomer. Six-membered monocycle **2.8** shows comparable inhibition to the seven-membered **2.5**, though it was determined that the inhibitor had slower initial binding to AChE and a faster covalent bond forming reaction. Greater inhibition of **2.10** than **2.9** demonstrates the importance of *E*-geometry, and the loss of activity upon reduction of the ester of **2.5** to an alcohol (**2.11**) shows the importance of the conjugated ester moiety in activating the enolphosphonate toward phosphorylation.

Table 2.1: Structure activity relationship comparison of Cyclophostin and analogs as inhibitors of acetylcholinesterase[2.2a,b,c]

Inhibitor	IC_{50} (μM)
 **2.1**	0.045[a]
 **2.2**	0.040[a]
 **2.3**	3[b]
 **2.4**	30[b]
 **2.5**	7[c]

Inhibitor	IC_{50} (μM)
 **2.6**	35[b]
 **2.7**	6[b]
 **2.8**	5[c]
 **2.9**	>1000[c]
 **2.10**	70[c]
 **2.11**	600[c]

The cyclipostins (**2.12**, **Table 2.2**) are natural inhibitors of hormone-sensitive lipase (HSL) which share the core bicyclic phosphate structure of cyclophostin (**2.1**), and were isolated from *Streptomyces* species DSM 13381.[2.3] Cyclipostin P, **2.12h**,[2.2a] and a number of phosphonate analogs of the cyclipostins[2.4] have been synthesized and submitted for testing for HSL inibition (**Table 2.3**) by tritiated triolein assay.[2.5] Cyclophostin (**2.1**, **2.2**) and the phosphonate analog thereof (**2.3**) are largely inactive ($IC_{50} \geq 100$ μM), while the natural HSL inhibitor cyclipostin P (**2.12h**) is very active with a seventeen-fold preference for the *trans* diastereomer. The monocyclic phosphonates **2.13**–**2.17**, which have long alkyl

substituents attached to the ring rather than the phosphonate ester, were weak to very weak inhibitors with IC_{50} values ranging from ~10 to 100 μM.

Table 2.2: Cyclipostins[2.3]

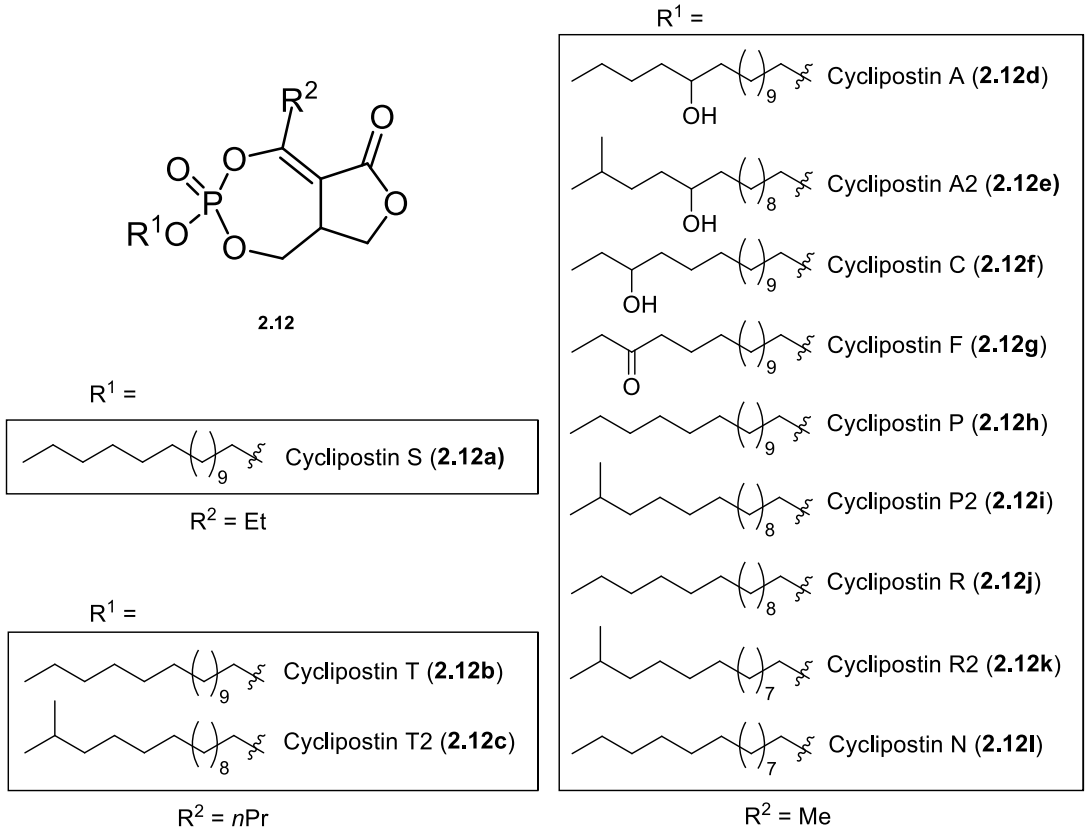
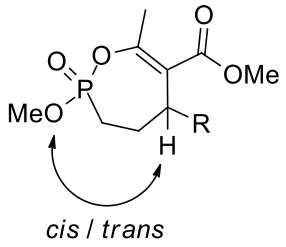
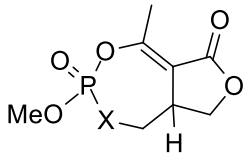
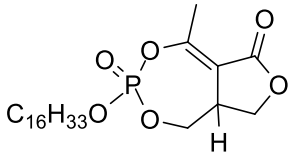


Table 2.3: Hormone-sensitive lipase inhibition results[2.5a,b,c]

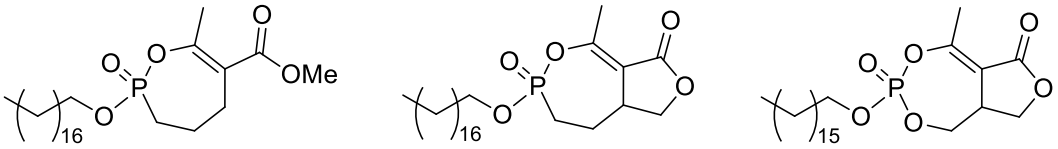
Inhibitor		IC_{50} (μM)
	**2.13a,b** R = C_5H_{11} **2.14a,b** R = $C_{10}H_{21}$ **2.15a,b** R = $C_{12}H_{25}$ **2.16a,b** R = $C_{16}H_{33}$ **2.17a,b** R = $C_{18}H_{37}$	*cis* *trans* 100[b] 100[b] 40[c] 100[b] 35[c] 13.5[c] ~60[a] ~10[a] >100[a] ~27[a]
	**2.1**, **2.2** X = O **2.4**, **2.3** X = CH_2	*cis* *trans* >100[b] >100[b] 100[b]
	**2.12h**	*cis* *trans* 0.42[a] 0.025[a]

2. Rationale for Synthesis of New HSL Inhibitors

The most immediate work to be done was to complete the readily accessible cyclipostin analogs which could be synthesized through the existing synthetic routes. Compounds **2.18** and **2.19** (**Figure 2.1**) could be prepared through transesterification of cyclophostin analogs **2.3** – **2.5**, so a method for this transformation had to be developed. Comparison of their activities would determine the significance of the lactone in enzyme inhibition and comparison of **2.19** to the natural product **2.12h** would establish the importance of the

phosphate oxygen. The natural product cyclipostin R (**2.12j**) could also be synthesized from **2.1**/**2.2** by transesterification.

Figure 2.1: Phosphonate analogs of cyclipostin P and cyclipostin R

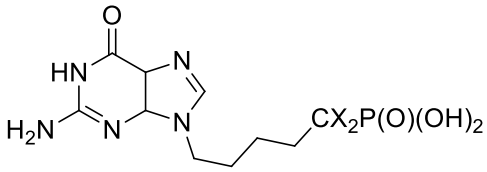


2.18 **2.19** **2.12j**

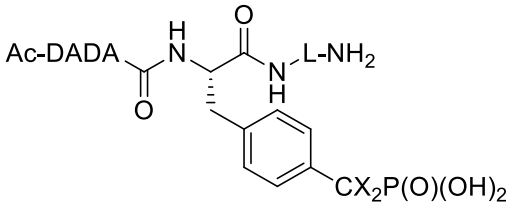
3. *α*,*α*-Difluorophosphonate analogs

Blackburn and McKenna predicted that *α*-fluorophosphonates would be better analogs of phosphates than unfunctionalized phosphonate analogs,[2.6] and numerous examples of *α*-fluorophosphonate inhibitors have been discovered to have greater potency than their phosphonate analogs.[2.7] **2.20b**, for example is a potent inhibitor of purine nucleoside phosphorylase with K_i values 10- to nearly 100-fold more potent than its phosphonate analog **2.20a** (**Figure 2.2**).[2.8] Likewise, *α*,*α*-difluorophosphonate **2.21b** inhibits PTP 1B reversibly with an IC_{50} value of 100 nM, 1000-fold more potent than the phosphonate analog **2.21b** (**Figure 2.2**).[2.9]

Figure 2.2: *α,α*-Difluorophosphonate inhibitors[2.8,2.9]



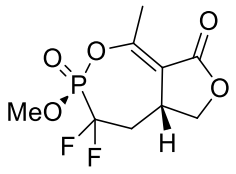
X = H, **2.20a**
X = F, **2.20b**



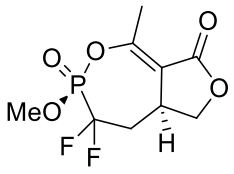
X = H, **2.21a**
X = F, **2.21b**

Not represented in the literature, however, was an *α*-fluorophosphonate diester inhibitor, particularly as an inhibitor of a serine hydrolase enzyme. An *α,α*-difluorophosphonate analog was chosen as the first synthetic target rather than a monofluorinated analog because it maximizes the electron-withdrawing effects and hydrogen bonding potential without introducing a new chiral center. *α,α*-difluorophosphonate cyclophostin analog **2.22** (**Figure 2.3**) could not only improve upon the AChE inhibition of phosphonate analogs **2.3** and **2.4**, but could also pioneer an new class of phosphorylating inhibitors.

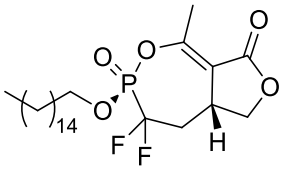
Figure 2.3: Proposed α,α-difluorophosphonate analogs of cyclophostin, its diastereomer, and cyclipostin P



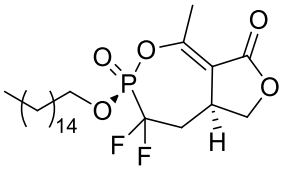
2.22a



2.22b



2.23a



2.23b

Transesterification of the cyclophostin analogs would then be performed in order to synthesize *α,α*-difluorophosphonate analogs of cyclipostin P **2.23a** and **2.23b** (**Figure 2.3**) for testing against HSL.

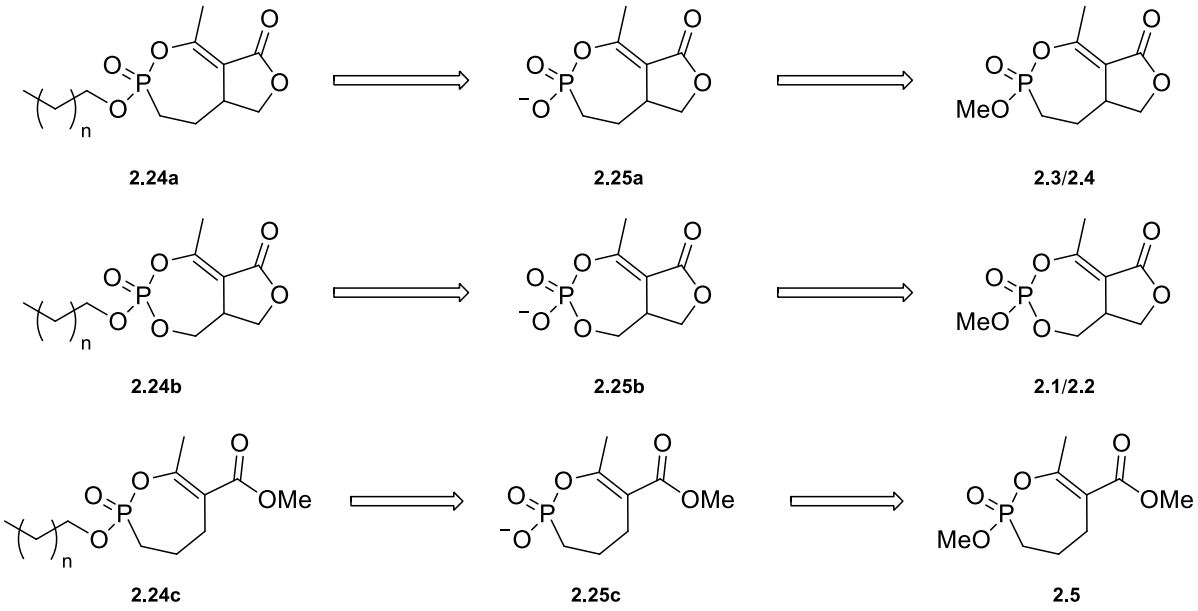
II. Synthesis

1. Retrosynthesis of Cyclipostin Analogs

The synthesis of cyclipostin analogs follows the synthesis of cyclophostin analogs with the addition of a final transesterification step. The methyl phosphonate ester substituent must be removed to form a phosphonate anion without opening the cyclic enol phosphonate or disturbing the methyl ester or lactone, and an electrophilic alkyl cation synthon must be supplied for nucleophilic attack (**Scheme 2.1**). The more reactive methods such as trimethylsilyl bromide or strong acids were therefore ruled out, particularly because they

favor complete dealkylation rather than removal of one methyl group. Amines have been used for these purposes[2.10] but were judged too reactive for the sensitive cyclic enol phosphonate moiety which has proven to be unstable in basic solution. Sodium iodide was chosen because of the unlikelihood of reaction with other substituents and because of its extensive use in previous works,[2.11] particularly from the Spilling laboratory.[2.2,2.4]

Scheme 2.1

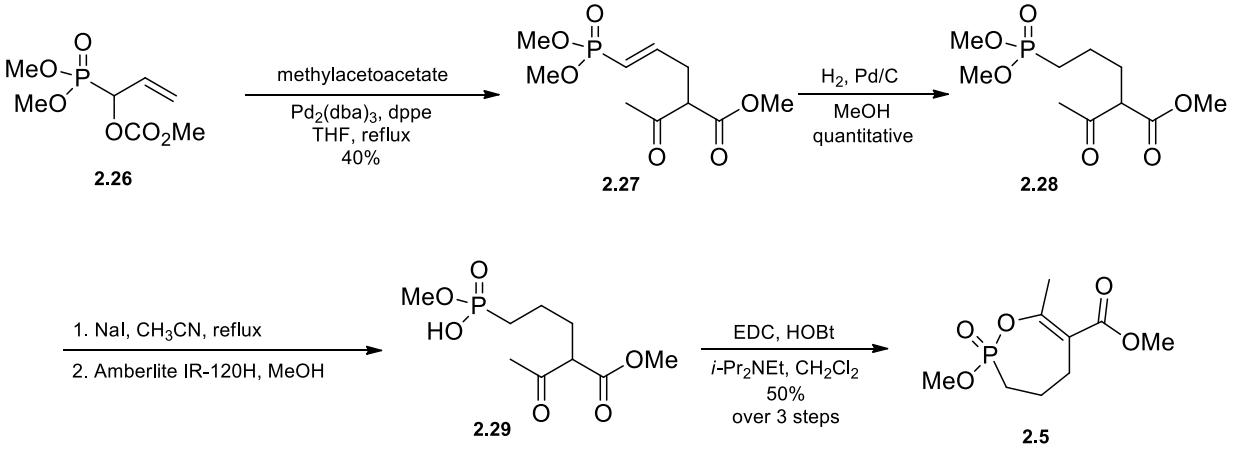


Because this technique requires the formation of a phosphonate anion (**2.25a**–**b**, **Scheme 2.1**), stereochemistry at phosphorus is scrambled, so diastereomers of general structures **2.24a** and **2.24b** must be separated regardless of the diastereopurity of any starting material.

2. Synthesis of Cyclipostin Analogs

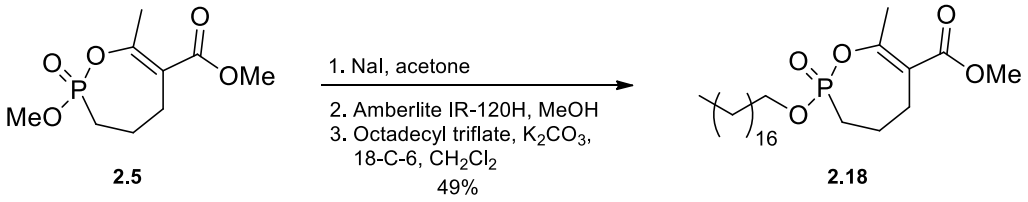
Initial studies of the synthesis of cyclipostin analogs began with the attempt to synthesize general structure **2.24c**. Compound **2.5** was synthesized according to the published route (**Scheme 2.2**).[2.2c]

Scheme 2.2



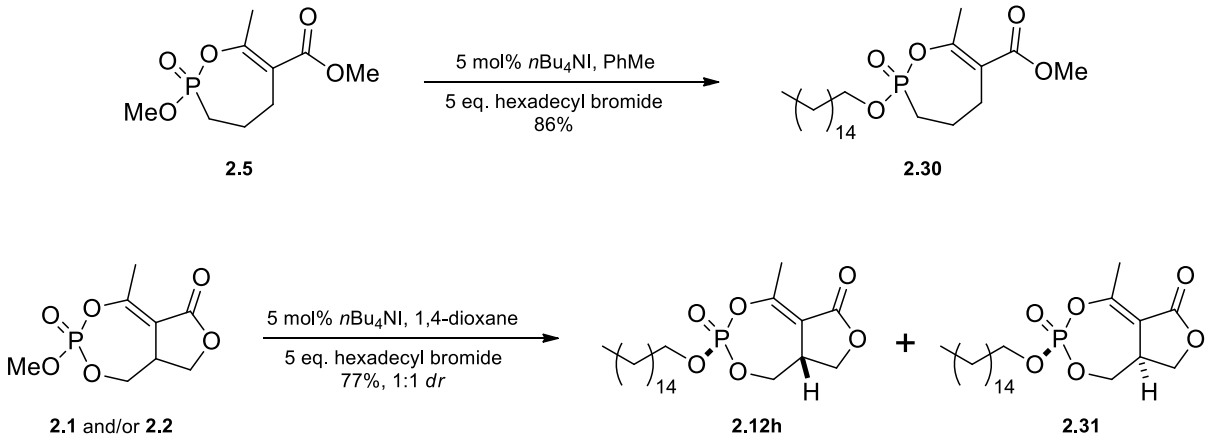
A technique was devised by which the methyl substituent of the cyclic enol phosphonate **2.5** was removed by treatment with sodium iodide in refluxing acetone, as demonstrated in the conversion of **2.28** to **2.29** and likewise treated with sulfonic acid resin. Treating the resulting phosphonic acid with potassium carbonate, octadecyl triflate, and phase-transfer catalyst gave phosphonate ester **2.18** in 49% yield (**Scheme 2.3**).[2.4]

Scheme 2.3



A more efficient method was developed by another graduate student, Raj Malla, allowing for one-pot transesterification and higher yields. By treating **2.5** with five mole percent tetra-*n*-butylammonium iodide and five equivalents of hexadecyl bromide, cyclipostin analog **2.30** was synthesized in 86% yield (**Scheme 2.4**).[2.4]

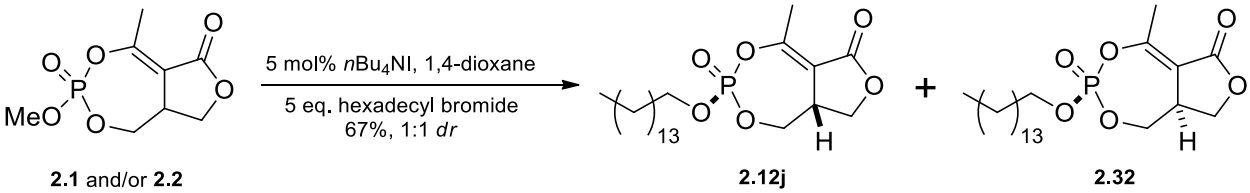
Scheme 2.4



It is believed that the anion **2.25c** is formed with a tetra-*n*-butylammonium counter cation which is alkylated by the bromide, releasing the halide which carries forward further

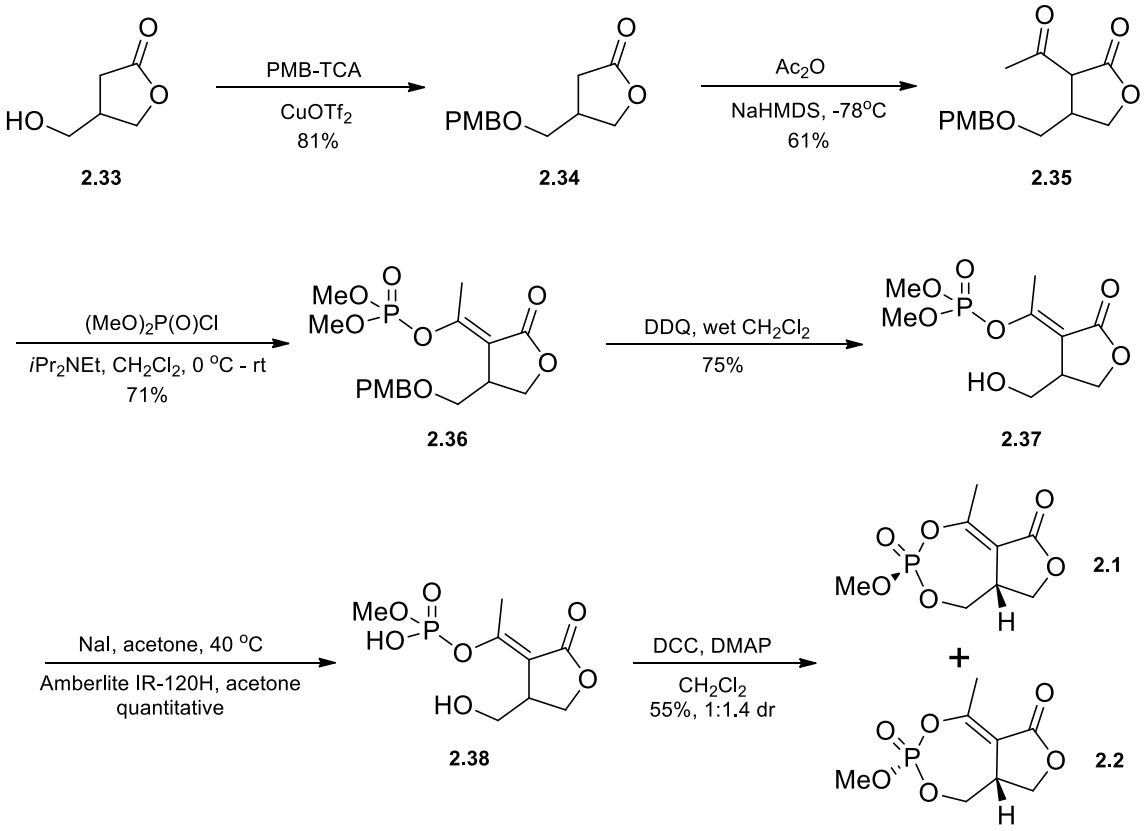
reaction.[2.2a] The technique had also been employed to synthesize cyclipostin P (**2.12h**) and its diastereomer **2.31** (**Scheme 2.4**).[2.2a]

Scheme 2.5



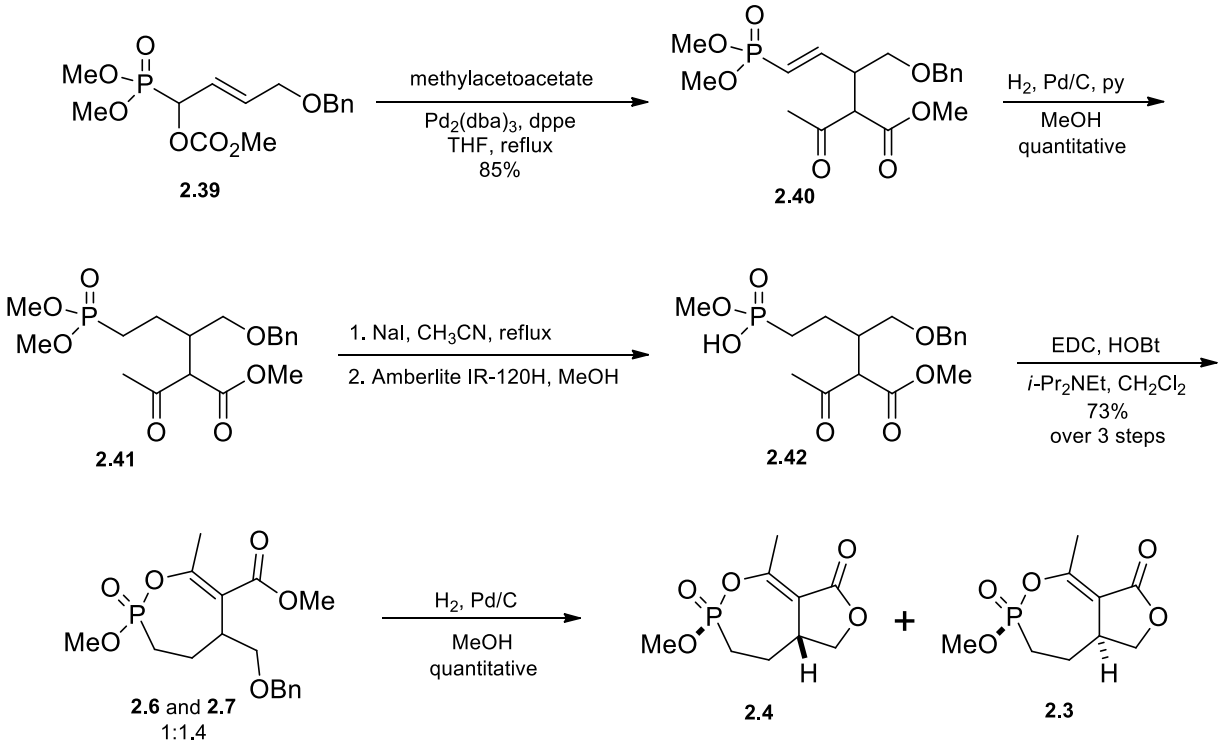
Using this technique, cyclipostin R (**2.12j**) and its diastereomer **2.32** were synthesized in 67% yield (**Scheme 2.5**) after completion of the synthesis of cyclophostin (**2.1**) following the reported procedure (**Scheme 2.6**).[2.2a]

Scheme 2.6



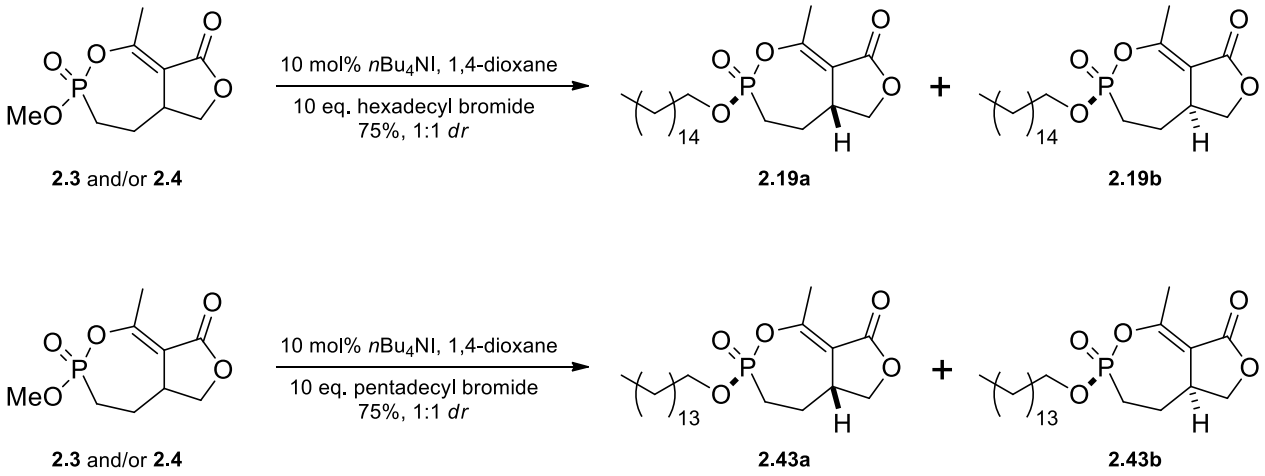
Synthesis of compounds of the general structure **2.24a** began with the published synthesis of cyclophostin phosphonate analogs **2.3** and **2.4** (**Scheme 2.7**).[2.2b]

Scheme 2.7



It was found that phosphonates react sluggishly in the established transesterification conditions, so compounds **2.3** and/or **2.4** were treated with 10 mole percent of tetra-*n*-butylammonium iodide and 10 equivalents of hexadecyl or pentadecyl bromide to give cyclipostin P phosphonate analogs **2.19a** and **2.19b** in 75% yield and cyclipostin R analogs **2.43a** and **2.43b** in 75% yield as well (**Scheme 2.8**).

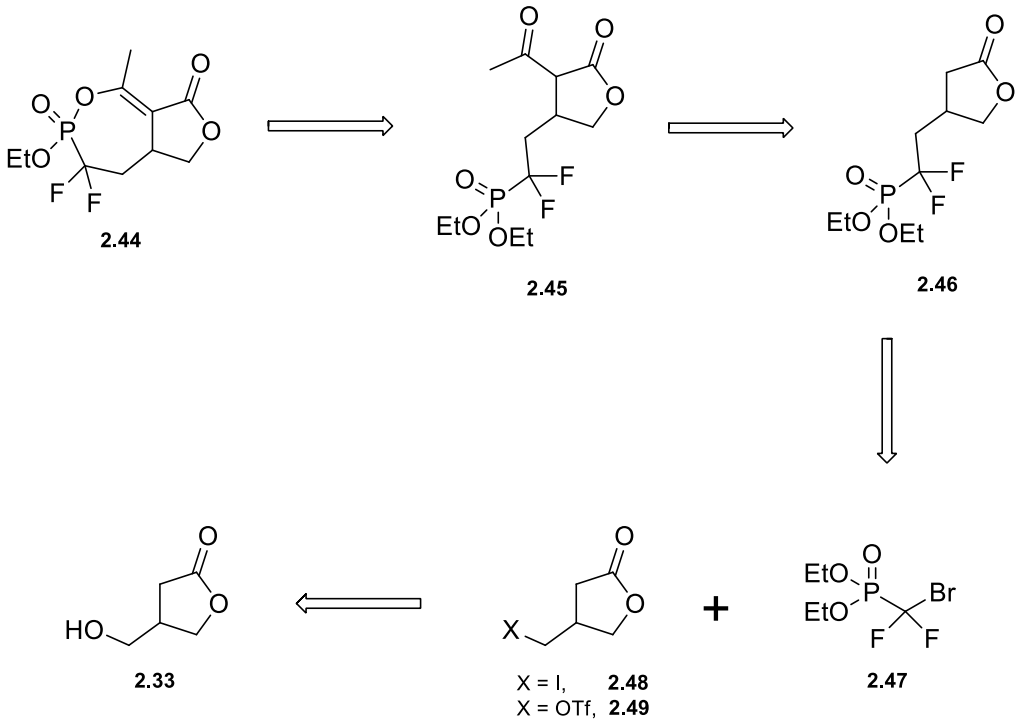
Scheme 2.8



3. Retrosynthesis of *α,α*-difluorophosphonates

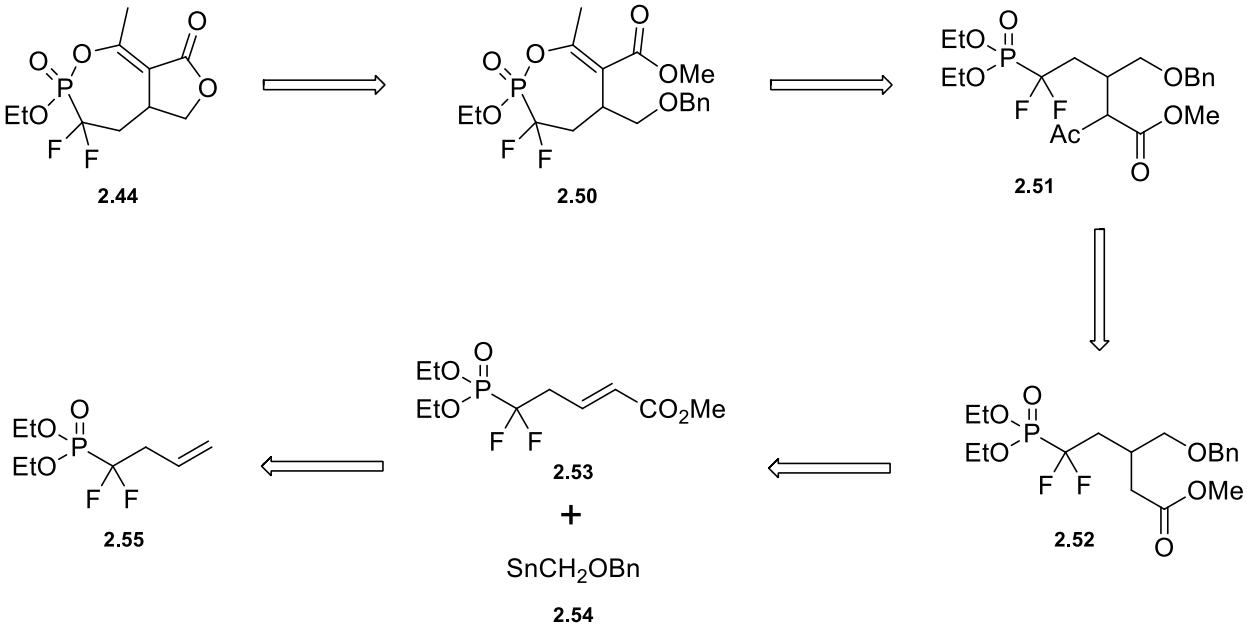
The simplest route to the *α,α*-difluorophosphonate analog of cyclophostin (**2.22a**) is to substitute a leaving group onto the hydroxymethyl lactone **2.33**, which began the cyclophostin synthesis (**Scheme 2.9**),[2.2a] and install a diethyl difluoromethylphosphonate group by substitution. Diethyl bromodifluoromethylphosphonate **2.47** is commercially available and the corresponding lithiate has been utilized for S_N2 chemistry.[2.12] The iodide **2.48** is a convenient electrophile because it is stable and known in the literature.[2.13] Berkowitz, *et al.* noted, however, that the lithiate does not readily undergo substitution with halides, so the triflate **2.49** may be necessary. [2.12,2.14]

Scheme 2.9



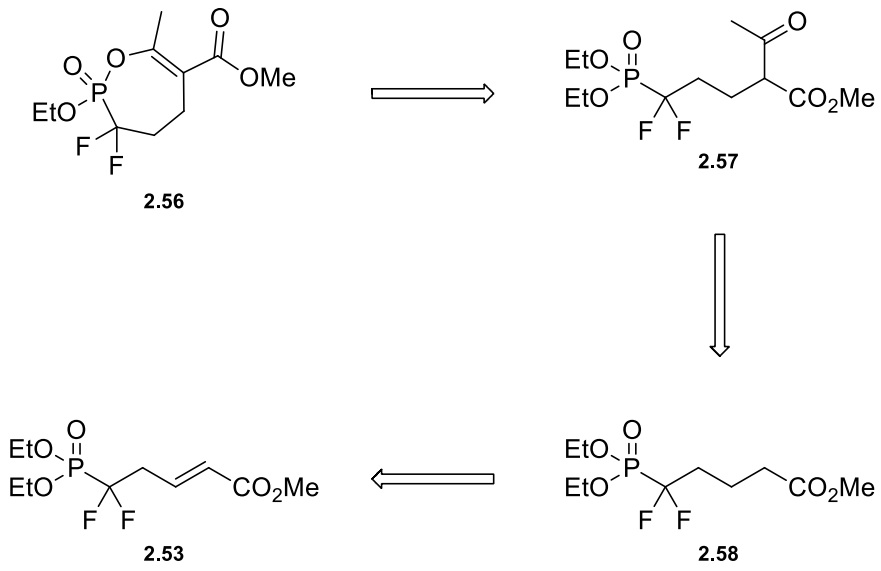
Another approach beginning from a more established addition of the difluorophosphonate moiety to allyl bromide is depicted in **Scheme 2.10**. This route was to be employed should addition to **2.48** or **2.49** fail.

Scheme 2.10



Allyldifluorophosphonate **2.55** has been synthesized from bromodimethylphosphonate **2.47** by preparation of a zinc cuprate or by insertion of cadmium into the carbon-bromine bond followed by treatment with allyl bromide, chloride, or iodide.[2.15] Cross metathesis with methyl acrylate followed by conjugate addition of a cuprate prepared from stannane **2.54** could give **2.52**. Acetylation following the preparation of **2.35** (**Scheme 2.6**)[2.2a] would give *β*-ketoester **2.51** which is a difluorophosphonate analog of synthetic intermediate **2.41** (**Scheme 2.7**)[2.2b] and could be cyclized likewise.

Scheme 2.11

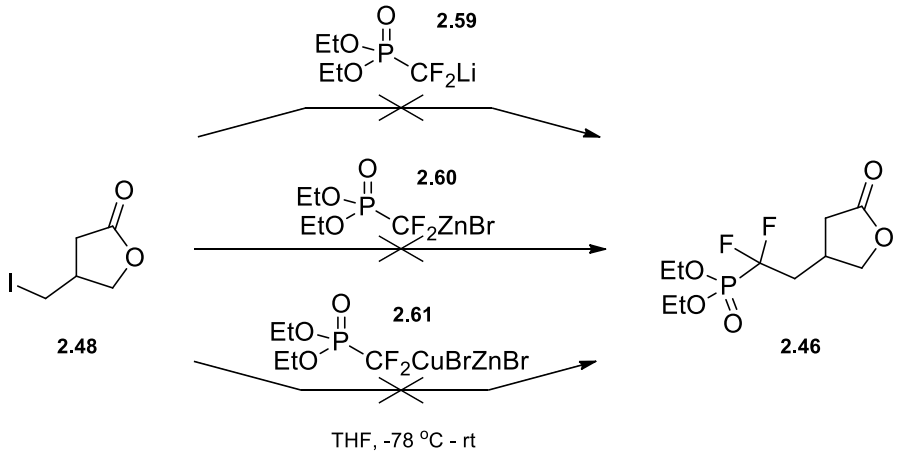


Should the conjugate addition fail, the unsaturated ester could be hydrogenated to aliphatic ester **2.58**. Acetylation to form *β*-ketoester **2.57** (**Scheme 2.11**) could be performed according to the conditions[2.2a] used to prepare **2.35** (**Scheme 2.6**), and cyclized to monocyclic cyclophostin analog **2.56** (**Scheme 2.11**) using the conditions[2.2b] by which phosphonate analog precursors **2.6** and **2.7** were prepared (**Scheme 2.7**).

4. Synthesis of *α*,*α*-Difluorophosphonates

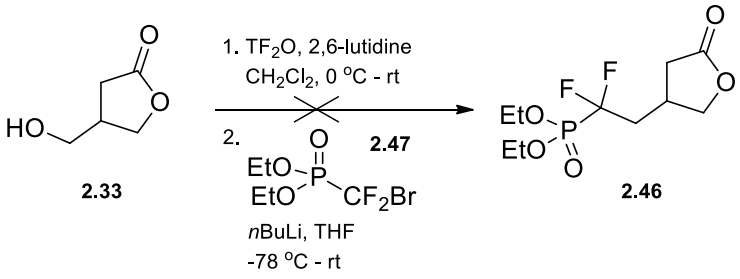
The lithiate **2.59** formed from treatment of bromodifluorophosphonate **2.47** with *n*BuLi was found to be unreactive toward the iodide **2.48** (**Scheme 2.12**). This was found to be true of the zincate **2.60** and cuprate **2.61**, as well.

Scheme 2.12



Following the suggestion of Berkowitz *et al.*,[2.12] the triflate **2.49** was prepared from hydroxymethyl lactone **2.33** and treated with the lithiate **2.59** prepared from **2.47**, but no product was generated (**Scheme 2.13**). The route depicted in **Scheme 2.9** was therefore abandoned.

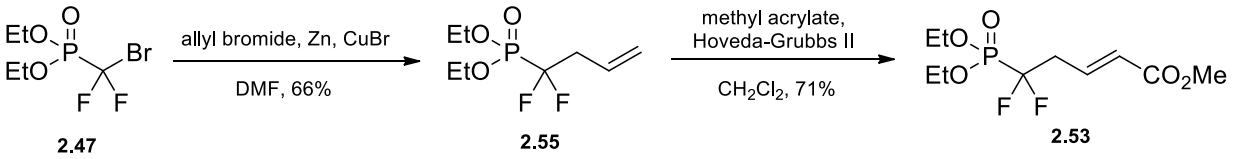
Scheme 2.13



Due to cadmium's toxicity, allyldifluorophosphonate **2.55** was prepared from the zinc cuprate of **2.47** with modest improvement of the reported yields,[2.15] likely due to the choice

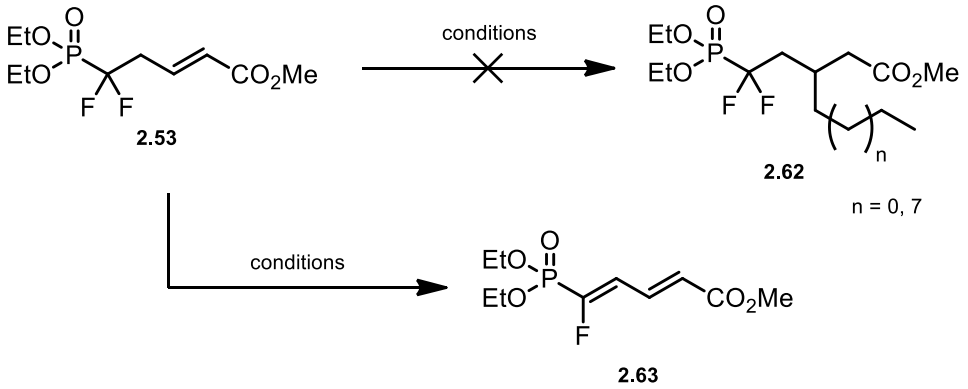
of DMF as a solvent. Unsaturated ester **2.53** was then synthesized in good yield by cross metathesis using Hoveyda-Grubbs II catalyst (**Scheme 2.14**).

Scheme 2.14



Attempts to perform conjugate addition with model cuprates such as those prepared from dodecylmagnesium bromide and *n*BuLi were unsuccessful. Even at room temperature in the presence of catalytic cuprous bromide, the Grignard reagent was unreactive toward unsaturated ester **2.53** as a nucleophile (**Scheme 2.15**). As the temperature of the reaction mixtures approached room temperature, however, detectable quantities of dehydrohalogenation product **2.63** was generated, but the material could not be separated from the starting material **2.53**. The synthetic route depicted in **Scheme 2.10** was abandoned.

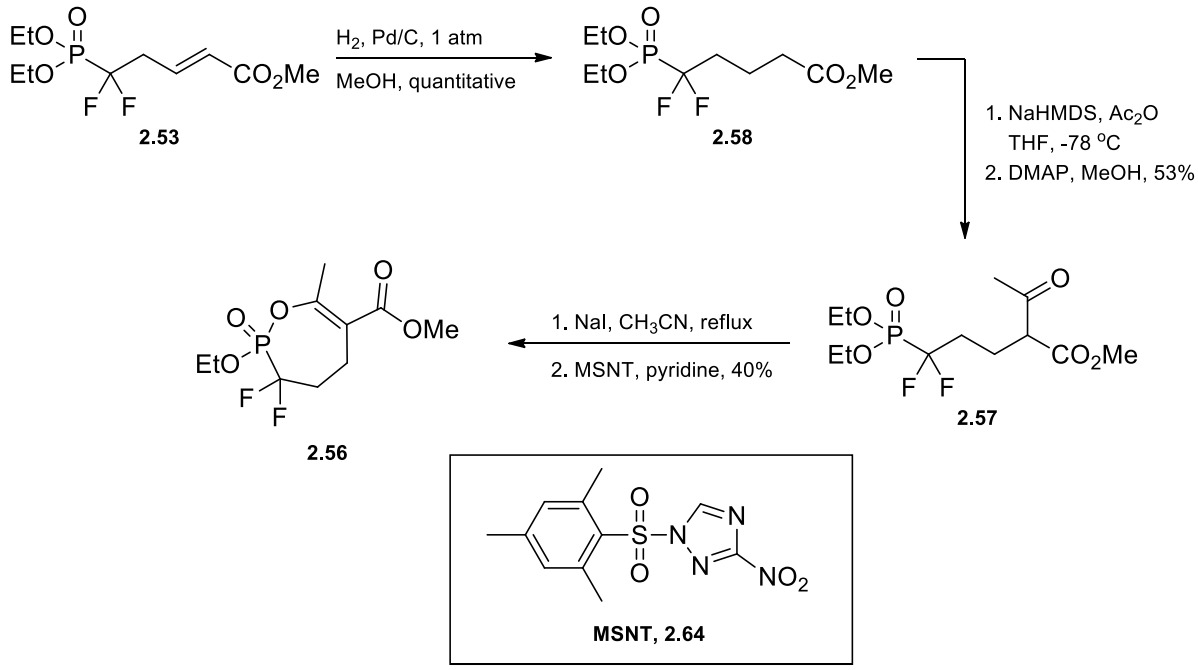
Scheme 2.15



Conditions: dodecylmagnesium bromide, CuBr, -78 °C – rt; dodecylmagnesium bromide, 10 mol % CuBr, rt; n*BuLi, CuBr, THF-Me*$_2$*S (2:1), -78 °C – rt*

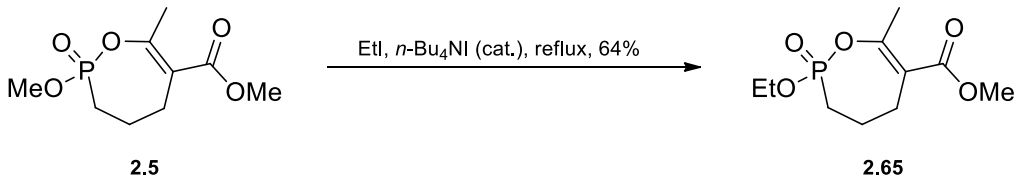
Hydrogenation of the unsaturated ester **2.53** to saturated ester **2.58** was clean and complete if halted at 30 minutes to prevent hydrogenolysis of the carbon-fluorine bond (**Scheme 2.16**). Acetylation following the procedure to prepare cyclophostin synthetic intermediate **2.35** (**Scheme 2.6**)[2.2a] resulted in overacetylation, so treatment of the product mixture with DMAP in methanol gave *β*-ketoester **2.57** in modest yield. Cleavage of the ethyl phosphonate ester with sodium iodide in refluxing acetonitrile was successful, but coupling of the phosphonic acid, formed by treatment of the sodium salt with acid resin, was unsuccessful with conventional coupling reagents DCC and EDC. There is precedence for the use of MSNT (**2.64**) in the coupling of an *α*,*α*-difluorophosphonate salt to an intramolecular alcohol in pyridine at 90 °C.[2.16] These conditions were found to be sufficient at room temperature to cyclize the sodium salt with complete conversion in one hour, however the marked instability of monocycle **2.56** resulted in low to modest yields upon isolation.

Scheme 2.16



Attempts to exchange the ethyl phosphonate ester for a methyl ester were unsuccessful, so the effect of the ethyl group in SAR must be investigated by preparation of another analog. Monocyclic analog **2.5** was chosen as the most rational candidate for transesterification due to its greater relative stability and the fact that the resulting ethyl phosphonate ester **2.65** would share all the structural features of difluorophosphonate analog **2.56** with the exception of the fluorine substituents. By treating monocyclic methyl ester **2.5** with ten mole percent tetra-*n*-butylammonium iodide in neat refluxing ethyl iodide, ethyl ester **2.65** was prepared in fair yield (**Scheme 2.17**).

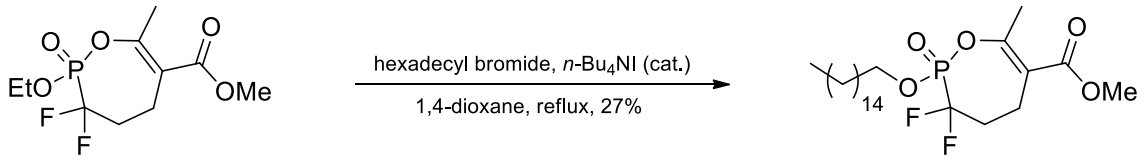
Scheme 2.17



2.5 **2.65**

Transesterification to the monocyclic cyclipostin analog **2.66** was effective using the established conditions, but in low isolated yield due to instability during reaction and isolation (**Scheme 2.18**).

Scheme 2.18



2.56 **2.66**

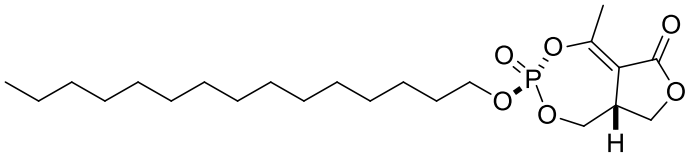
III. Conclusion

An effective method for transesterification of phosphate and phosphonate esters was found, and it was utilized in the synthesis of natural cyclipostins and analogs thereof. Though synthesis of the α,α-difluorophosphonate analog of cyclophostin was unsuccessful, a monocyclic analog was completed which could contribute to ongoing SAR investigation. The enzyme assay results will be reported in Chapter 4.

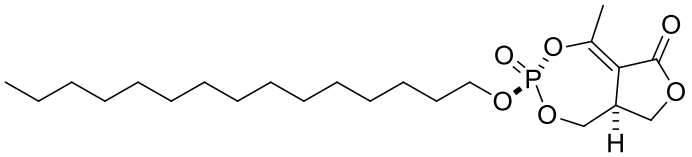
IV. Experimental Section

General Experimental. All reactions were carried out in oven dried glassware (150 °C) under an atmosphere of argon unless otherwise noted. Dry THF was distilled from sodium and benzophenone. Dry CH_2Cl_2 and CH_3CN were distilled from CaH_2. Dry MeOH was distilled from magnesium. Dry 1,4-dioxane was distilled freshly from sodium metal. Dry pyridine was distilled freshly from KOH. All chemicals and reagents were purchased from commercial suppliers unless otherwise noted. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded at 300, 75 and 121 MHz, respectively. ^{1}H NMR spectra are referenced to $CDCl_3$ (7.27 ppm), ^{13}C NMR spectra are referenced to $CDCl_3$ (77.23 ppm), and ^{31}P NMR spectra are referenced to external H_3PO_4.

Cyclipostin R (2.12j) and its diastereomer (2.32). To a solution cyclophostin diastereomer **2.2** (28.5 mg, 0.122 mmol) and *n*-bromopentadecane (350 μL, 1.21 mmol) in dry 1,4-dioxane (0.5 mL) was added *n*-Bu_4NI (2 mg, 0.005 mmol) at room temperature. The flask was immersed in a, oil bath preheated at 110 °C until the reaction was complete (TLC and ^{31}P NMR analysis). The solvent was removed under vacuum and the crude product was purified by column chromatography (SiO_2, EtOAc/ hexanes) giving cyclipostin R (**2.12j**) (16.6 mg) and its unnatural diastereomer (**2.32**) (9.5 mg) as white solids (67% overall).



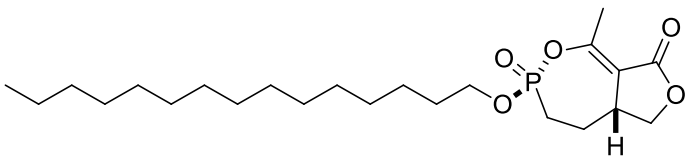
Cyclipostin R. (±)-(3R(S),8aR(S))-5-Methyl-3-(pentadecyloxy)-8,8a-dihydrofuro[3,4-e][1,3,2]dioxaphosphepin-6(1H)-one 3-oxide (2.12j). IR (neat, ATR): 2916, 2850, 1749, 1671 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.45 (1H, m), 4.35 (1H, td, J_{HH} = 10.8 Hz, J_{HP} = 6.2 Hz), 4.24 (2H, dt, J_{HP} = J_{HH} = 6.8 Hz), 4.15 (1H, ddd, J_{HP} = 25.6 Hz, J_{HH} = 11.2, 3.5 Hz), 3.80 (2H, m), 2.47 (3H, d, J_{HP} = 1.9 Hz), 1.72 (2H, p, J_{HH} = 6.8), 1.39 (2H, m), 1.26 (22H, br s), 0.88 (3H, t, J_{HH} = 6.7 Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 169.1, 161.8 (d, J_{CP} = 7.7 Hz), 112.1 (d, J_{CP} = 3.2 Hz), 70.4 (d, J_{CP} = 6.3 Hz), 67.6 (d J_{CP} =, 5.7 Hz), 64.4, 39.9, 32.1, 30.3 (d, J_{CP} = 6.6 Hz), 29.90, 29.88, 29.85, 29.76, 29.68, 29.58, 29.26, 25.5, 22.9, 18.4 (d, J_{CP} = 4.7 Hz), 14.3; ^{31}P NMR (121.4 MHz, $CDCl_3$): δ -8.55; HRMS (FAB, NBA, MH^+) calcd for $C_{22}H_{40}O_6P$: 431.2563, found: 431.2563



(±)-(3R(S),8aS(R))-5-Methyl-3-(pentadecyloxy)-8,8a-dihydrofuro[3,4-e][1,3,2]dioxaphosphepin-6(1H)-one 3-oxide (2.32). IR (neat, ATR): 2914, 2847, 1745, 1666 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.46 (1H, t, J_{HH} = 9.3 Hz), 4.29 (1H, m), 4.18 (2H, m), 4.04 (2H, m), 3.79 (1H, dd, J_{HH} = 9.6, 5.7 HZ), 2.44 (3H, d, J_{HP} = 1.8 Hz), 1.73 (2H, p, J_{HH} = 6.7 Hz), 1.26 (24H, br s), 0.89 (3H, t, J_{HH} = 6.6 Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 169.2, 161.6 (d, J_{CP} = 10.5 Hz), 111.0 (d, J_{CP} = 2.6 Hz), 69.9 (d, J_{CP} = 6.3 Hz),

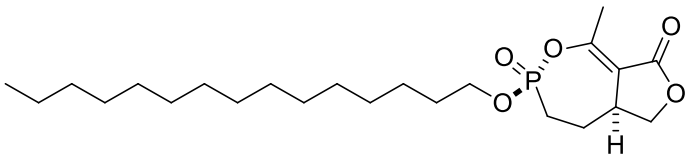
69.5 (d, J_{CP} = 7.6 Hz), 64.7, 38.6, 32.1, 30.4 (d, J_{CP} = 6.5 Hz), 29.90, 29.88, 29.84, 29.76, 29.67, 29.58, 29.3, 25.5, 18.1 (d, J_{CP} = 5.9 Hz), 14.3. ^{31}P NMR (121.4 MHz, $CDCl_3$): δ -12.4; HRMS (FAB, NBA, MH^+) calcd for $C_{22}H_{40}O_6P$: 431.2563, found: 431.2570

Phosphonate analog of cyclipostin R and its diastereomer (2.43). To a solution of phosphonate analogs **2.3** and **2.4** (17.8 mg, 0.077 mmol) and *n*-bromopentadecane (220 μL, 0.76 mmol) in dry 1,4-dioxane (390 µL) was added *n*-Bu_4NI (3 mg, 0.008 mmol). The flask was immersed in an oil bath preheated to 110 °C until the reaction was complete (TLC, ^{31}P NMR analysis). The solvent was removed under vacuum and the crude product was purified by column chromatography (SiO_2, EtOAc/ hexanes) giving the phosphonate analog of cyclipostin R (**2.43a**) (13.4 mg) and its diastereomer (**2.43b**) (8.9 mg) as a white solid and white semisolid, respectively (75% overall).



(±)-(3R(S),5aR(S))-1-Methyl-3-(pentadecyloxy)-4,5,5a,6-tetrahydrofuro[3,4-e][1,2]oxaphosphepin-8(3H)-one 3-oxide (2.43a). IR (neat, ATR): 2915, 2849, 1742, 1662 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.50 (1H, t, J_{HH} = 9.3 Hz), 4.19 (2H, m), 3.85 (1H, dd, J_{HH} = 9.2, 6.6 Hz), 3.32 (1H, m), 2.45 (3H, s), 2.32 (1H, m), 1.89–2.13 (3H, m), 1.72 (2H, p, J_{HH} = 6.9), 1.26 (24H, br s), 0.88 (3H, t, J_{HH} = 6.6 Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 170.1 (d, J_{CP} = 1.6 Hz), 161.0 (d, J_{CP} = 7.2 Hz), 114.5 (d, J_{CP} = 3.8), 69.9, 66.8 (d, J_{CP} = 7.3

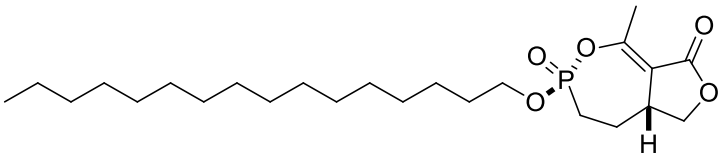
Hz), 39.1 (d, J_{CP} = 1.0 Hz), 32.1, 30.5 (d, J_{CP} = 6.1 Hz), 29.89, 29.88, 29.86, 29.85, 29.83, 29.75, 29.7, 29.6, 29.3, 26.8 (d, J_{CP} = 136.4 Hz), 26.6 (d, J_{CP} = 6.9 Hz), 25.6, 22.9, 19.0 (d, J_{CP} = 2.3 Hz), 14.3; ^{31}P NMR (121.4 MHz, $CDCl_3$): δ 23.4; HRMS (FAB, NBA, MH^+) calcd for $C_{23}H_{42}O_5P$: 429.2770, found: 429.2777



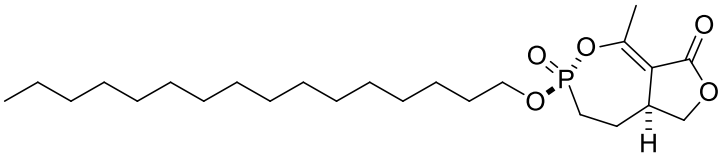
(±)-(3R(S),5aS(R))-1-Methyl-3-(pentadecyloxy)-4,5,5a,6-tetrahydrofuro[3,4-e][1,2]oxaphosphepin-8(3H)-one 3-oxide (2.43b). IR (neat, ATR): 2915, 2848, 1742, 1672 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.50 (1H, t, J_{HH} = 9.3 Hz), 4.24 (1H, ddt, J_{HH} = 8.3 Hz, J_{HP} = J_{HH} = 7.7 Hz), 4.06 (1H, ddt, J_{HH} = 9.9 Hz, J_{HP} = J_{HH} = 6.9 Hz), 3.84 (1H, dd, J_{HH} = 9.2, 6.5 Hz), 3.40 (1H, m), 2.43 (3H, d, J_{HP} = 1.7 Hz), 1.80–2.40 (4H, m), 1.70 (2H, p, J_{HH} = 6.9 Hz), 1.26 (24H, br s), 0.88 (3H, t, J_{HH} = 6.7 Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 170.3, 160.5 (d, J_{CP} = 9.6 Hz), 113.8 (d, J_{CP} = 3.0 Hz), 69.9, 67.0 (d, J_{CP} = 7.0 Hz), 38.6 (d, J_{CP} = 2.0 Hz), 32.1, 29.89, 29.87, 29.83, 29.76, 29.68, 29.57, 29.3, 27.0 (d, J_{CP} = 7.5 Hz), 26.6 (d, J_{CP} = 134.5 Hz), 25.6, 22.9, 18.7 (d, J_{CP} = 3.5 Hz), 14.3; ^{31}P NMR (121.4 MHz, $CDCl_3$): δ 19.9. HRMS (FAB, NBA, MNa^+) calcd for $C_{23}H_{41}O_5PNa$: 451.2589, found: 451.2601

Phosphonate analog of cyclipostin P and its diastereomer (2.19). To a solution of phosphonate analog **2.4** (10.7 mg, 0.046 mmol) and *n*-bromohexadecane (140 μL, 0.46

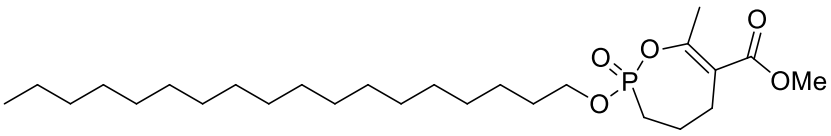
mmol) in dry 1,4-dioxane (230 µL) was added *n*-Bu_4NI (1.7 mg, 0.0046 mmol). The flask was immersed in an oil bath preheated to 110 °C until the reaction was complete (TLC, ^{31}P NMR analysis). The solvent was removed under vacuum and the crude product was purified by column chromatography (SiO_2, EtOAc/hexanes) giving the phosphonate analog of cyclipostin P (**2.19a**) (7.8 mg) and its diastereomer (**2.19b**) (5.5 mg) as a white solid and white semisolid, respectively (75% overall).



(±)-(3R(S),5aR(S))-3-(Hexadecyloxy)-1-methyl-4,5,5a,6-tetrahydrofuro[3,4-e][1,2]oxaphosphepin-8(3H)-one 3-oxide (2.19a). IR (neat, ATR): 2918, 2852, 1742, 1661 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 4.50 (1H, t, J_{HH} = 9.3 Hz), 4.20 (2H, m), 3.86 (1H, dd, J_{HH} = 9.2, 6.6 Hz), 3.34 (1H, m), 2.46 (3H, s), 2.26–2.38 (1H, m), 1.89–2.13 (3H, m), 1.72 (2H, p, J_{HH} = 6.9 Hz), 1.26 (26H, br s), 0.88 (3H, t, J_{HH} = 6.7 Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 170.1, 161.0 (d, J_{CP} = 7.0 Hz), 114.5 (d, J_{CP} = 4.0 Hz), 69.9, 66.8 (d, J_{CP} = 7.1 Hz), 39.1, 32.1, 30.5, 29.89, 29.87, 29.84, 29.77, 29.70, 29.6, 29.3, 26.8 (d, J_{CP} = 136.0 Hz), 26.6 (d, J_{CP} = 7.0 Hz), 25.6, 22.9, 19.0 (d, J_{CP} = 2.6 Hz), 14.3; ^{31}P NMR (121.4 MHz, $CDCl_3$): δ 23.4; HRMS (FAB, NBA, MH^+) calcd for $C_{24}H_{44}O_5P$: 443.2926, found: 443.2935

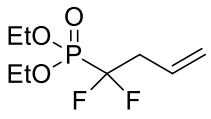


(±)-(3R(S),5aS(R))-3-(Hexadecyloxy)-1-methyl-4,5,5a,6-tetrahydrofuro[3,4-e][1,2]oxaphosphepin-8(3H)-one 3-oxide (2.19b). IR (neat, ATR): 2916, 2848, 1749, 1669 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.51 (1H, t, J_{HH} = 9.1 Hz), 4.24 (1H, ddt, J_{HH} = 9.8 Hz, J_{HH} = J_{HP} = 7.2 Hz), 4.06 (1H, ddt, J_{HH} = 10.0 Hz, J_{HH} = J_{HP} = 7.0 Hz), 3.84 (1H, dd, J_{HH} = 9.1, 6.4 Hz), 3.41 (1H, m), 2.43 (3H, s), 1.80–2.49 (4H, m), 1.70 (2H, p, J_{HH} = 6.8 Hz), 1.32 (2H, m), 1.26 (24H, br s), 0.88 (3H, t, J_{HH} = 6.6 Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 170.3, 160.5 (d, J_{CP} = 9.8 Hz), 113.8 (d, J_{CP} = 3.0 Hz), 70.0, 67.1 (d, J_{CP} = 7.1 Hz), 38.6 (d, J_{CP} = 2.2 Hz), 32.1, 30.6 (d, J_{CP} = 5.9 Hz), 29.92, 29.90, 29.88, 29.87, 29.84, 29.76, 29.68, 29.58, 29.3, 27.0 (d, J_{CP} = 7.9 Hz), 26.6 (d, J_{CP} = 134.7 Hz), 25.6, 22.9, 18.7 (d, J_{CP} = 3.2 Hz), 14.3; ^{31}P NMR (121.4 MHz, $CDCl_3$): δ 20.0; HRMS (FAB, NBA, MH^+) calcd for $C_{24}H_{44}O_5P$: 443.2926, found: 443.2919



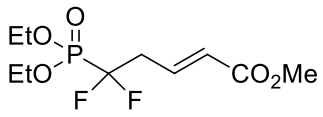
(±)-Methyl 7-methyl-2-(octadecyloxy)-2,3,4,5-tetrahydro-1,2-oxaphosphepine-6-carboxylate 2-oxide (2.18) . The monocyclic phosphonate **2.5** (0.064 g, 0.26 mmol) was dissolved in dry acetone (0.5 mL) and NaI (0.044 g, 0.29 mmol) was added. The yellow solution was heated at reflux overnight. After completion of the reaction (^{31}P NMR analysis) the solvent was removed under reduced pressure. The dark orange residue was dissolved in

dry methanol (10 mL) and pre-rinsed Amberlite IR-120H resin (0.320 g) was added. The mixture was shaken on an orbit shaker for 45 min, filtered and concentrated under reduced pressure to yield a light orange oil. The oil was dissolved in dry CH_2Cl_2 (0.20 mL) and then potassium carbonate (0.040 g, 0.29 mmol) and 18-crown-6 (0.002 g, 0.08 mmol) were added, followed by the addition of *n*-octadecyl triflate (0.149 g, 0.369 mmol) 40 in dry CH_2Cl_2 (0.30 mL) dropwise. After 48 h, the reaction was quenched with deionized water and extracted three times with CH_2Cl_2. The combined organic extracts was dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by column chromatography (SiO_2, 7–15% EtOAc in hexanes) to give **2.18** (0.061 g, 49%) as a white waxy solid. IR (neat, ATR): 1712, 1646 cm^{-1}; ^1H NMR ($CDCl_3$) δ 4.13 (2H, m), 3.72 (3H, s), 2.65 (1H, m), 2.47 (1H, m), 2.30 (3H, s), 2.20 – 1.83 (4H, m), 1.67 (2H, p, J_{HH} = 6.8 Hz), 1.30 – 1.16 (28H, br s), 0.85 (3H, t, J_{HH} = 6.6 Hz); ^{13}C NMR ($CDCl_3$) δ 168.19, 159.414 (d, J_{CP} = 7.8 Hz), 119.18 (d, J_{CP} = 4.6 Hz), 66.22 (d, J_{CP} = 7.2 Hz), 51.98, 32.07, 30.56 (d, J_{CP} = 6.2), 29.84, 29.81, 29.78, 29.70, 29.65, 29.51, 29.28, 26.78 (d, J_{CP} = 134.3 Hz), 26.39 (d, J_{CP} = 2.7 Hz), 25.61, 22.84, 21.33, 21.12, 14.27; ^{31}P NMR ($CDCl_3$) δ 23.7; HRMS (FAB, NBA, MH^+) calcd. For $C_{26}H_{49}O_5P$: 473.3396, found 473.3385.



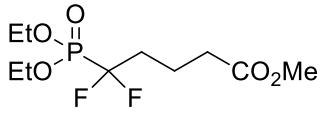
Diethyl (1,1-difluorobut-3-en-1-yl)phosphonate (2.55). Activated Zn powder (1.22 g, 18.7 mmol) was weighed into an oven dried Schlenk flask equipped with a Teflon-coated stir bar. The system was flame dried under vacuum and flushed with Ar. After cooling to room temperature, dry DMF (9.3 mL) was added by syringe followed by slow dropwise

addition of diethyl (bromodifluoromethyl)phosphonate (**2.47**, 3.3 mL, 18.6 mmol). After stirring 3 h at room temperature, CuBr (2.66 g, 18.5 mmol) was added in one part followed by allyl bromide (1.6 mL, 18.9 mmol) by slow dropwise addition. After stirring 40 h, the mixture was partitioned between CH_2Cl_2 and 10% aqueous NH_4Cl. The aqueous phase was extracted four times with CH_2Cl_2. The combined organic phases were dried over anhydrous Na_2SO_4 and concentrated by rotary evaporation. The residue was purified by column chromatography (SiO_2, 16–18% EtOAc in hexanes) to give **2.55** (2.80 g, 66%) as a pale yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.74 – 5.88 (1H, m), 5.26 (1H, s), 5.21 (1H, m), 4.23 (4H, dq $J_{HH} = J_{HP} = 7.3$ Hz), 2.80 (2H, m), 1.34 (6H, t, $J_{HH} = 7.1$ Hz).

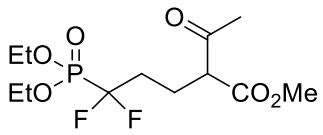


(E)-Methyl 5-(diethoxyphosphoryl)-5,5-difluoropent-2-enoate (2.53). To a solution of diethyl 1,1-difluoro-3-butenephosphonate (**2.55**, 108 mg, 0.473 mmol) in CH_2Cl_2, 250 μL, was added methyl acrylate (86 µL, 0.95 mmol) followed by Hoveyda-Grubbs II catalyst (15 mg, 0.024 mmol). The green solution was heated in an oil bath at 40 °C for 1 h. The solution was cooled to room temperature and concentrated by rotary evaporation. The resulting residue was purified by column chromatography (20% EtOAc in hexanes) to give **2.53** (96 mg, 71%) as a yellow oil. IR (neat, NaCl) 2986, 2950, 2910, 1727 cm^{-1}, 1663 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.93 (1H, dt, $J_{HH} = 15.7, 7.5$ Hz), 6.02 (1H, dt, $J_{HH} = 15.7, 1.35$ Hz), 4.28 (4H, dq, $J_{HP} = J_{HH} = 7.3$ Hz), 3.75 (3H, s), 2.98 (2H, m), 1.38 (6H, t, $J_{HH} = 7.1$ Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 166.1, 137.0 (dt, $J_{CF} = J_{CP} = 5.7$), 126.9, 119.0, (td, $J_{C\,F} = 261.7$ Hz, $J_{CP} = 216.6$ Hz), 64.9 (d, $J_{CP} = 6.6$ Hz), 51.9, 37.3 (td, $J_{CF} = 21.8$ Hz,

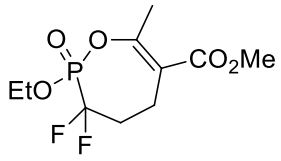
J_{CP} = 15.6 Hz), 16.5 (d, J_{CP} = 5.0 Hz); ^{31}P NMR (121.4 MHz, $CDCl_3$): δ 6.14 (t, J_{PF} = 105.4 Hz); ^{19}F NMR (282.2 MHz, $CDCl_3$): δ -110.9 (d, J_{FP} = 105.7 Hz); HRMS (FAB, NBA, MH^+) calcd for $C_{10}H_{18}F_2O_5P$: 287.0860, found: 287.0866.



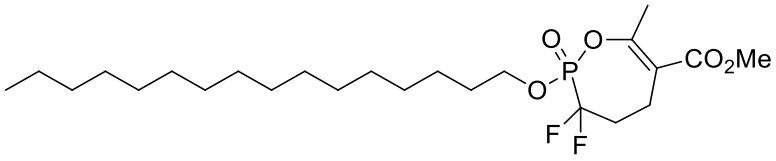
Methyl 5-(diethoxyphosphoryl)-5,5-difluoropentanoate (2.58). To a solution of phosphonate **2.53** (101 mg, 0.353 mmol) in MeOH, 900 μL, was added 10% Pd on carbon (38 mg, 0.036 mmol). The mixture was stirred under H_2 for 30 min, flushed with argon, and filtered through a pad of Celite with ethanol to give **2.58** (100 mg, 99%) as a pale yellow oil. IR (neat, NaCl) 2986, 2955, 2910, 1740 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.23 (4H, dq, J_{HP} = J_{HH} = 7.3 Hz), 3.63 (3H, s), 2.36 (2H, t, J_{HH} = 7.3 Hz), 1.97 – 2.17 (2H, m), 1.83 – 1.93 (2H, m), 1.34 (6H, t, J_{HH} = 7.1 Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 173.2, 120.6 (td, J_{CF} = 259.6 Hz, J_{CP} = 215.7 Hz), 64.6 (d, J_{CP} = 6.8 Hz), 51.7, 33.3, 33.1 (td, J_{CF} = 20.9 Hz, J_{CP} = 14.7 Hz), 16.5 (d, J_{CP} = 5.5 Hz), 16.5 (J_{CF} = J_{CP} = 5.4 Hz); ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 7.05 (t, J_{PF} = 108.3 Hz). ^{19}F NMR (282.2 MHz, $CDCl_3$) δ -112.1 (d, $J_{F,H}$ = 108.3 Hz). HRMS (FAB, NBA, MH^+) calcd for $C_{10}H_{20}F_2O_5P$: 289.1016, found 289.1017.



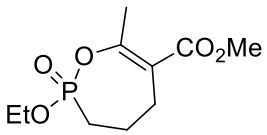
(±)-Methyl 2-acetyl-5-(diethoxyphosphoryl)-5,5-difluoropentanoate (2.57). A solution of phosphonate **2.58** (454 mg, 1.58 mmol) in dry THF, 9.5mL, was cooled to –78 °C in a dry ice acetone bath. 1 M solution of NaHMDS (3.2 mL) in THF was added dropwise. After 10 min, Ac_2O (450 µL, 4.76 mmol) was added dropwise. After 3 h, the solution was diluted with CH_2Cl_2 and treated with 10% aq. NH_4Cl. The mixture was extracted three times with CH_2Cl_2, and the organic phases were combined, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was dissolved in 16 mL dry MeOH. DMAP (39 mg, 0.32 mmol) was added, and the solution was stirred overnight. The solution was concentrated by rotary evaporation, partitioned between CH_2Cl_2 and 0.25 N aq. HCl, and extracted three times with CH_2Cl_2. The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated by rotary evaporation. The residue was purified by silica gel chromatography (30% EtOAc in hexanes) to give **2.57** (278 mg, 53%) as a yellow oil. IR (neat, NaCl) 2988, 2957, 1739, 1717 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 4.27 (4H, dq, $J_{HH} = J_{HP} = 7.3$ Hz), 3.76 (3H, s), 3.55 (1H, t, $J_{HH} = 6.9$ Hz), 2.26 (3H, s), 1.95 – 2.19 (4H, m), 1.38 (6H, t, $J_{HH} = 7.1$ Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 202.2, 169.7, 120.5 (td, $J_{CF} = 260.1$ Hz, $J_{CP} = 215.6$ Hz), 64.7 (d, $J_{CP} = 7.1$ Hz), 58.5, 52.74, 31.6 (dt, $J_{CF} = 21.0$ Hz, $J_{CP} = 15.3$ Hz), 29.3, 19.6 (q, $J_{CF} = J_{CP} = 5.4$ Hz), 16.5 (d, $J_{CP} = 5.1$ Hz); ^{31}P NMR (121.4 MHz, $CDCl_3$): δ 6.72 (t, $J_{PF} = 107.5$ Hz); ^{19}F NMR (282.2 MHz, $CDCl_3$): δ -112.6 (d, $J_{FP} = 107.6$ Hz); HRMS (FAB, NBA, MH^+) calcd for $C_{12}H_{22}F_2O_6P$: 331.1122, found 331.1126.



(±)-Methyl 2-ethoxy-3,3-difluoro-7-methyl-2,3,4,5-tetrahydro-1,2-oxaphosphepine-6-carboxylate 2-oxide (2.56). To a solution of phosphonate **2.57** (381 mg, 1.15 mmol) in dry CH_3CN, 0.2 mL, was added NaI (190 mg, 1.27 mmol). The mixture was heated at reflux for 4 h and concentrated *in vacuo*. The residue was dissolved in dry distilled pyridine, 5.8 mL. To the resulting solution was added 1-mesitylene-sulfonyl-3-nitrotriazole (682 mg, 2.34 mmol). After 1 h, the mixture was partitioned between 5 % aqueous NH_4Cl and CH_2Cl_2. The aqueous layer was extracted three times with CH_2Cl_2, and the combined organic phase was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude material was purified by C_{18} reverse phase silica gel chromatography (60% CH_3OH) to give **2.56** (131 mg, 40%) as a yellow oil. IR (neat, NaCl) 2988, 2956, 2925, 1719, 1655 cm^{-1}; ^1H MHz, NMR (300 MHz, $CDCl_3$) δ 4.43 (2H, dq, J_{HP} = 8.4 Hz, J_{HF} = 7.1 Hz), 3.78 (3H, s), 2.65–2.77 (1H, m), 2.46–2.58 (1H, m), 2.40 (3H, s), 2.14–2.42 (2H, m), 1.45 (3H, t, J_{HH} = 7.1 Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 167.3 (d, J_{CP} = 2.0 Hz), 159.2 (d, J_{CP} = 8.0 Hz), 120.3 (td, J_{CF} = 261.4 Hz, J_{CP} = 205.0 Hz), 120.2 (d, J_{CP} = 6.0 Hz), 65.9 (d, J_{CP} = 7.1 Hz), 52.3, 35.3 (td, J_{CF} = 20.6 Hz, J_{CP} = 11.6 Hz), 21.4, 20.1 (dd, J_{CF} = 8.8 Hz, 3.8 Hz), 16.5 (d, J_{CP} = 5.5 Hz); ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 1.4 (dd, J_{PF1} = 111.4 Hz, J_{PF2} = 100.9 Hz). ^{19}F NMR (282.2 MHz, $CDCl_3$) δ -109.6 (dd, J_{FF} = 290.5 Hz, J_{FP} = 100.8 Hz), -106.3 (dd, J_{FF} = 290.5 Hz, J_{FP} = 111.5 Hz); HRMS (FAB, NBA, MH^+) calcd for $C_{10}H_{15}F_2O_5P$: 285.0703, found 285.0708.



(±)-Methyl 3,3-difluoro-2-(hexadecyloxy)-7-methyl-2,3,4,5-tetrahydro-1,2-oxaphosphepine-6-carboxylate 2-oxide (2.66). To a solution of cyclic phosphonate **2.56** (26 mg, 0.091 mmol) in dry 1,4-dioxane (0.45 mL) was added *n*-bromohexadecane (280 μL, 0.92 mmol) and tetra-*n*-butylammonium iodide (1.7 mg, 0.0046 mmol). The mixture was heated to reflux. After 30 min, tetra-n-butylammonium iodide (1.7 mg, 0.0046 mmol) was added. After 4 h, the mixture was cooled to room temperature and concentrated *in vacuo*. The residue was purified by C_{18} reverse phase silica gel chromatography (95% CH_3OH) to give **2.66** (12 mg, 27%) as a white solid. IR (Neat, ATR) 2963, 2917, 2850, 1717, 1661 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 4.34 (2H, dt, $J_{HH} = J_{HP} = 7.0$ Hz), 3.79 (3H, s), 2.65–2.79 (1H, m), 2.45–2.59 (1H, m), 2.40 (3H, s), 2.17–2.39 (2H, m), 1.78 (2H, p, $J_{HH} = 6.9$ Hz), 1.26 (26H, br s), 0.89 (3H, t, $J_{HH} = 6.8$ Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 167.4, 159.2 (d, $J_{CP} = 8.1$ Hz), 120.4 (td, $J_{CF} = 261.7$ Hz, $J_{CP} = 205.1$ Hz), 120.1 (d, $J_{CP} = 5.6$ Hz), 69.9 (d, $J_{CP} = 7.0$ Hz), 52.3, 35.3 (td, $J_{CF} = 20.5$ Hz, $J_{CP} = 11.4$ Hz), 32.152, 30.6 (d, $J_{CP} = 5.6$ Hz), 29.9 (br s), 29.7, 29.7, 29.6, 25.4, 22.9, 21.4, 20.2 (dd, $J_{CF} = 9.1$ Hz, 3.5 Hz), 14.3; ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 1.4 (dd, $J_{PF} = 111.2$ Hz, 100.6 Hz); ^{19}F NMR (282.2 MHz, $CDCl_3$) δ -109.2 (dd, $J_{FF} = 290.1$ Hz, $J_{FP} = 111.2$ Hz), -106.1 (dd, $J_{FF} = 290.1$ Hz, $J_{FP} = 100.3$ Hz); HRMS (FAB, NBA, MH^+) calcd for $C_{24}H_{44}F_2O_5P$: 481.2894, found 481.2900.



(±)-Methyl 2-ethoxy-7-methyl-2,3,4,5-tetrahydro-1,2-oxaphosphepine-6-carboxylate 2-oxide (2.65): Methyl phosphonate ester **2.5** (31 mg, 0.13 mmol) was dissolved in iodoethane (210 μL, 2.6 mmol), and to the resulting solution was added tetra-*n*-butylammonium iodide (5.0 mg, 0.014 mmol). The yellow solution was heated to reflux until completion of the reaction, monitoring by ^{31}P NMR. The crude mixture was concentrated *in vacuo* and purified by flash chromatography on silica gel (15% EtOAc/hexanes) giving **2.65** (21 mg, 64%) as a yellow oil. IR (neat, NaCl) 2985, 2955, 2927, 2866, 1717, 1653 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.23 (2H, m), 3.76 (3H, s), 2.68 (1H, m), 2.50 (1H, m), 2.34 (s, 3H), 1.85-2.34 (4H, m), 1.38 (3H, t, J_{HH} = 7.1 Hz). ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 168.3, 159.4 (d, J_{CP} = 7.7 Hz), 119.3, 62.3 (d, J_{CP} = 6.8 Hz), 52.1, 26.9 (d, J_{CP} = 134 Hz), 26.5, 21.3 (d, J_{CP} = 7.5 Hz), 21.2, 16.6 (d, J_{CP} = 5.9 Hz); ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 23.15; HRMS (FAB, NBA, MH^+) calcd for $C_{10}H_{18}O_5P$: 249.0892, found: 249.0896

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Chapter 3 : Monocyclic Phosphate Analogs of Cyclophostin and Cyclipostins

I. Introduction

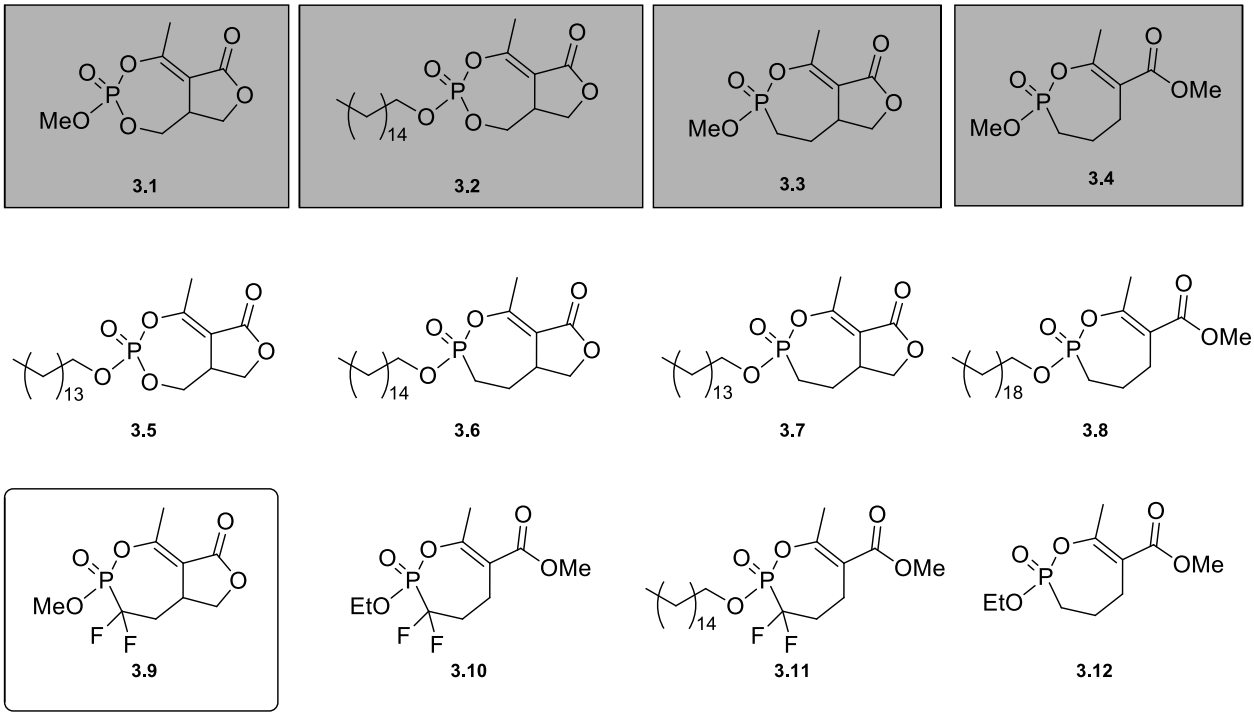
1. Background

In previous work, the Spilling group synthesized cyclophostin (**3.1**, **Figure 3.1**), [3.1] a low-nanomolar inhibitor of acetylcholinesterase (AChE) isolated from broths of *Streptomyces levindulae*,[3.2] and Cyclipostin P (**3.2**),[3.1] a low-nanomolar inhibitor of hormone-sensitive lipase (HSL) isolated from *Streptomyces* species DSM 13381.[3.3] In other work, the phosphonate analog **3.3**[3.4] and monocyclic phosphonate analog **3.4**[3.5] were synthesized for structure activity relationship (SAR) comparison with natural cyclophostin **3.1**. The goal of this work is to continue the SAR investigation of this novel class of inhibitors.

In the previous chapter, the syntheses of cyclipostin R (**3.5**), phosphonate analogs of cyclipostins P and R (**3.6** and **3.7**), and octadecyl monocyclic phosphonate ester **3.8** were described. The prominence of fluorine in medicinal chemistry and the absence of any reported α,α-difluorophosphonate ester inhibitors in the literature prompted the unsuccessful attempt to synthesize cyclophostin analog **3.9**. The syntheses of monocyclic analogs **3.10** and **3.11** (**Figure 3.1**) were completed, but ethyl phosphonate ester **3.10** could not be effectively converted into the corresponding methyl phosphonate ester. This matter was resolved by

preparing ethyl phosphonate **3.12**, which provided a means of comparing the SAR of methyl and ethyl phosphonate esters. Likewise, comparison of monocyclic α,α-difluorophosphonate **3.10** to cyclophostin (**3.1**), the only synthesized phosphate inhibitor of AChE, requires one to account for the missing lactone moiety, potentially by way of comparing the relative inhibitions of phosphonate **3.3**, and monocyclic phosphonate **3.4**. The integrity of this study would be greatly served, however, by the synthesis of monocyclic phosphate inhibitors.

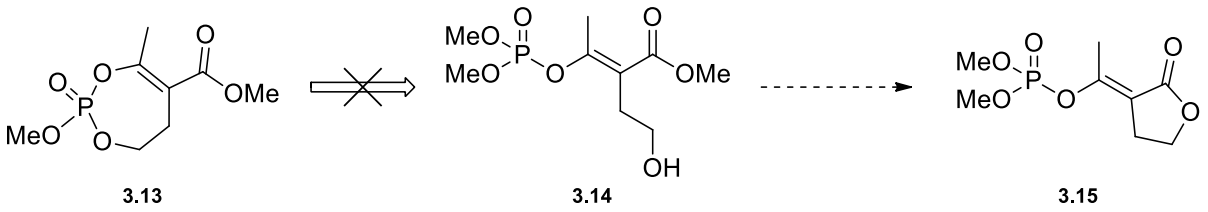
Figure 3.1: Cyclophostin, cyclipostins P and R, and analogs



2. Retrosynthesis of Monocyclic Phosphates

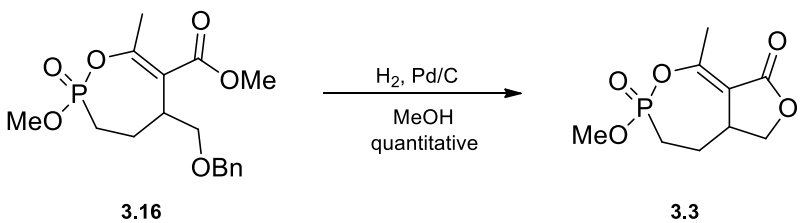
Phosphate monocycle **3.13** (**Scheme 3.1**) is a simple structure, but the retrosynthesis poses a number of challenges. Unprotected alcohol **3.14** is the first most rational synthetic precursor to **3.13**, as it could be cyclized by coupling after demethylation of the phosphate moiety. An unprotected alcohol in the presence of such a methyl ester would certainly form lactone **3.15** spontaneously, however.

Scheme 3.1



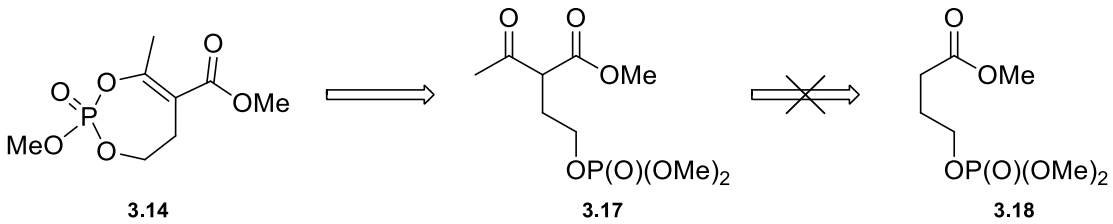
This concept was employed in the synthesis of cyclophostin phosphonate analog **3.3** (**Scheme 3.2**).[3.4] By removing the benzyl protecting group of compound **3.16** in hydrogenolysis conditions, phosphonate analog **3.3** was formed spontaneously upon formation of the intermediate unprotected alcohol.

Scheme 3.2



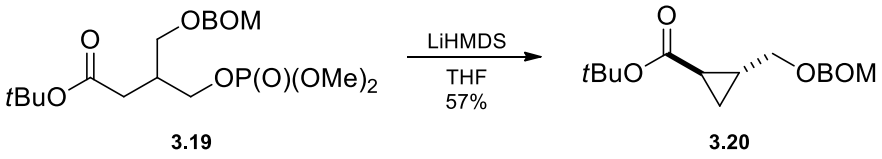
Another potential approach is to cyclize from phosphate **3.17** onto the enol oxygen (**Scheme 3.3**). Compound **3.17** would be formed by carboacetylation of ester **3.18** by way of an enolate intermediate.

Scheme 3.3



Such enolates have been determined to form cyclopropanes. During the investigation toward the synthesis of cyclophostin **3.1**, ester **3.19** formed cyclopropane **3.20** as the only isolable product (**Scheme 3.4**).[3.1]

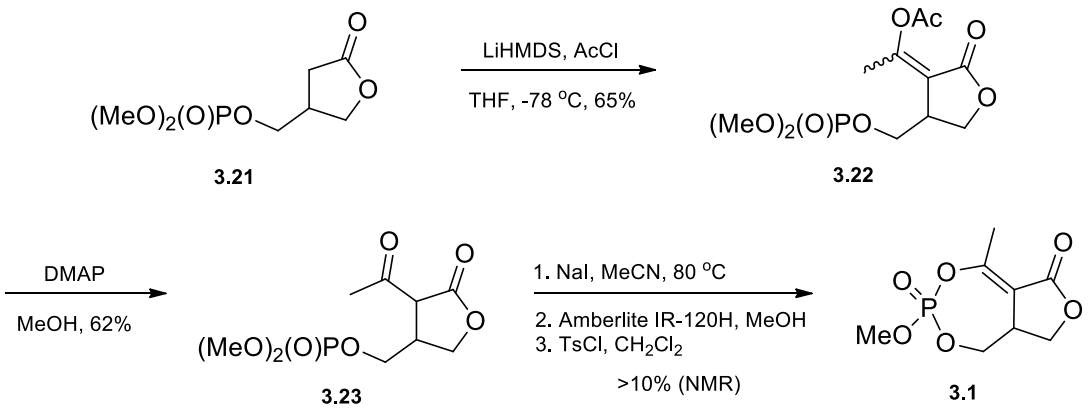
Scheme 3.4



Acetylation of lactone **3.21** was successful,[3.1] however, though this is likely attributable to the unfavorable sterics and strain associated with forming a fused lactone cyclopropane (**Scheme**

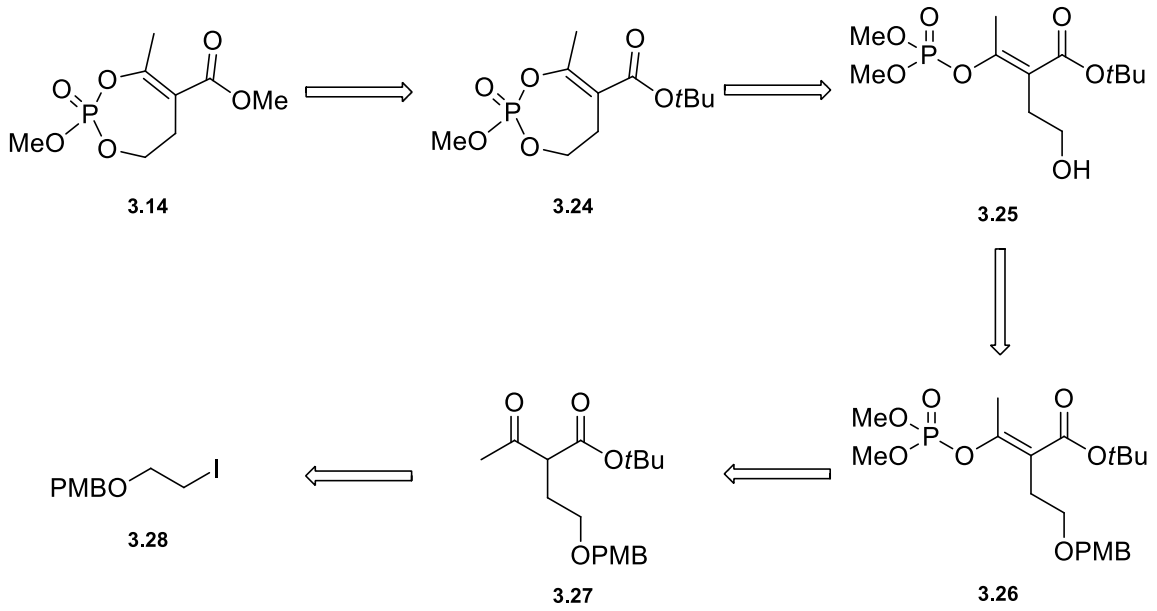
3.5). Regardless, the final synthetic intermediate **3.23** could not be cyclized onto the enol oxygen upon treatment with a carbodiimide reagent, and tosyl chloride generated trace amounts of product **3.1**.[3.1] Because of these problems, the sequence depicted in **Scheme 3.3** was not pursued.

Scheme 3.5



The sequence in **Scheme 3.6** circumvents the problems delineated above. The final product could be generated from *t*-butyl ester **3.24** by trifluoroacetic acid (TFA) hydrolysis of the *t*-butyl group followed by methyl ester formation. This is the weakest step in the proposed synthesis because harsh acidic conditions may hydrolyze the potentially sensitive enolphosphate moiety. Cyclization to **3.24** from alcohol **3.25** should not be complicated by formation of lactone **3.15** (**Scheme 3.6**) because the steric bulk of the *t*-butyl ester retards this reaction.

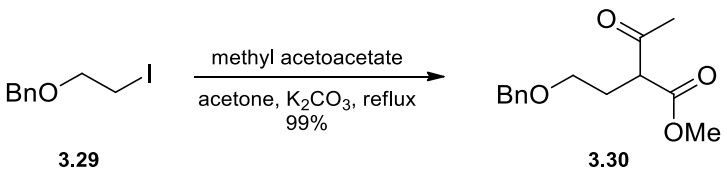
Scheme 3.6



3.14 3.24 3.25 3.28 3.27 3.26

Deprotection of **3.26** (**Scheme 3.6**) is analogous to a high yielding step from the synthesis of cyclophostin **3.1**. Phosphorylation of *β*-ketoester **3.27** follows directly from the natural product synthesis, as well.[3.1] Addition of methyl acetoacetate to analogous benzyl protected iodide **3.29** (**Scheme 3.7**) was demonstrated by Mulholland, *et al.* to be a very high yielding reaction,[3.6] so the sequence depicted in **Scheme 3.6** appears to be the most viable.

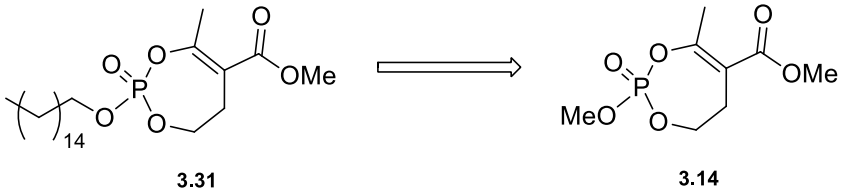
Scheme 3.7[3.6]



3.29 3.30

Transesterification of cyclophostin analog **3.14** to monocyclic cyclipostin analog **3.31** (**Scheme 3.8**) can be accomplished using the conditions established in the synthesis of cyclipostin P (**3.2**),[3.1] employed in the transesterification of analogous phosphonates,[3.7] and in the preceding chapter of this work.

Scheme 3.8

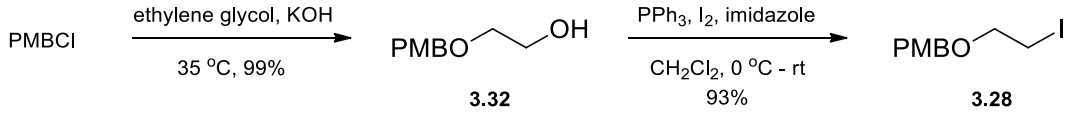


II. Synthesis of Monocyclic Phosphates

1. *β*-Ketoester Synthesis

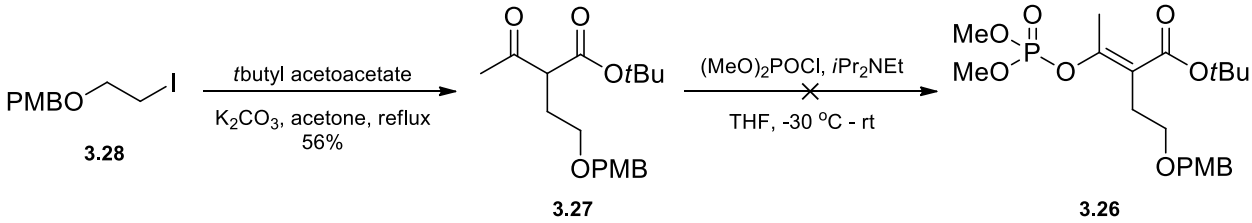
Reaction of ethylene glycol with *p*-methoxybenzyl chloride followed the conditions used by Chehade, *et al.* to give alcohol **3.32** (**Scheme 3.9**), improving significantly on the reported isolated yield by purifying by silica gel chromatography rather than distillation. [3.8] Iodination to **3.28** followed standard triphenylphosphine and iodine conditions.

Scheme 3.9



Following the procedure of Mulholland, *et al.*,[3.6] addition of *t*-butyl acetoacetate to iodide **3.32** was effective, but gave *β*-ketoester **3.27** in modest yield (**Scheme 3.10**). Their reaction of methyl acetoacetate with iodide **3.29** formed *β*-ketoester **3.30** in 99% yield (**Scheme 3.7**). The hindrance of the reaction may be attributable to the steric effects of the *t*-butyl substituent.

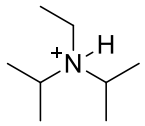
Scheme 3.10



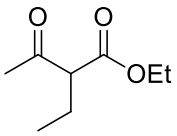
2. Phosphorylation

Phosphorylation of *β*-ketoester **3.27** (**Scheme 3.10**) was attempted following the conditions used in the synthesis of cyclophostin (**3.1**),[3.1] but product **3.26** was not generated. One approach to solving such a problem would be to employ a stronger base, forming a more nucleophilic formal anion. The pKa of protonated Hünig's base (**3.32**, **Figure 3.2**) is 10.5[3.9] whereas that of model *β*-ketoester **3.33** is 12.7.[3.10] If mixed in a 1:1 ratio, deprotonation of *β*-ketoester **3.33** by Hünig's base would only be approximately 8%.

Figure 3.2: Literature pKa values



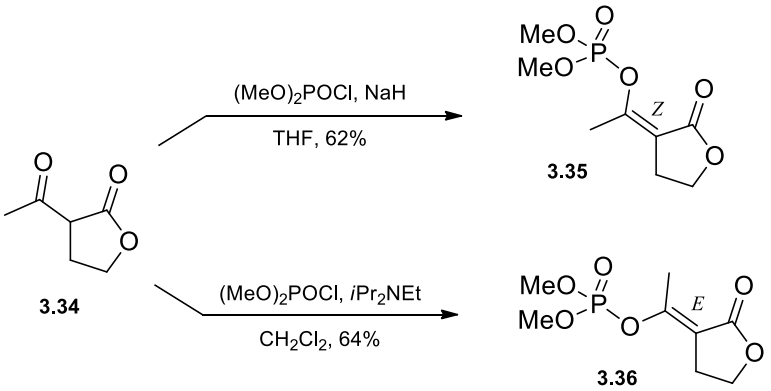
3.32
pKa = 10.5[3.9]



3.33
pKa = 12.7[3.10]

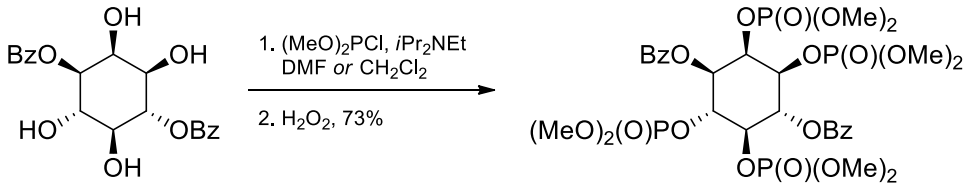
If the rate of the reaction is dependent on the concentration of enolate anion, the reaction could be accelerated by a stronger base. Unfortunately, stronger bases often employ a metal counter cation which would form the wrong geometric isomer via chelating effects. This principle was employed for a previous SAR study of geometric isomers. *β*-ketolactone **3.34** was phosphorylated using NaH to give *Z*-enolphosphate **3.35**, while Hünig's base was used to prepare *E*-enolphosphate **3.36** (**Scheme 3.11**).[3.5] The *E*-isomer was found to be more than fourteen-fold more potent an inhibitor than the *Z*-isomer.[3.5] Furthermore, for the preparation of cyclic phosphates such as the target **3.14**, *E* geometry is crucial for the success of the cyclization step.

Scheme 3.11[3.5]



The alternative is to use a more reactive electrophile. A method often employed when phosphorylation with P(V) reagents is ineffective is to use an analogous P(III) reagent. Meek, *et al.* used dimethylchlorophosphite followed by oxidation with hydrogen peroxide to install dimethyl phosphate moieties onto various congested adjacent alcohols of inositol **3.37** (**Scheme 3.12**).[3.11]

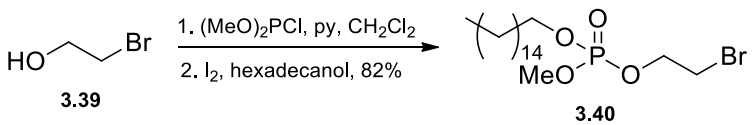
Scheme 3.12[3.11]



3.37 **3.38**

Hendrickson and Hendrickson developed an alternate P(III) to P(V) *in situ* oxidation technique for the preparation of hexadecyl phosphate **3.40** employing elemental iodine and hexadecanol (**Scheme 3.13**).[3.12]

Scheme 3.13[3.12]

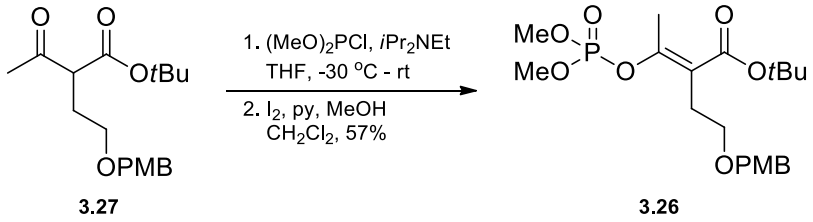


3.39 **3.40**

Dialkylchlorophosphites have been used to phosphorylate β-diketones,[3.13] and dimethylchlorophosphite, though not available commercially, can be readily prepared from phosphorus trichloride and dimethylphosphite.[3.14]

Phosphorylation with dimethylchlorophosphite and subsequent oxidation with iodine and methanol was effective, giving phosphate **3.26** in modest yield (**Scheme 3.14**). The conversion of the initial phosphonylation reaction appears complete by crude ^{1}H and ^{31}P NMR, but hydrolysis of the unstable enolphosphite moiety occurs during the oxidation step. Starting ketoester **3.27** is recoverable.

Scheme 3.14

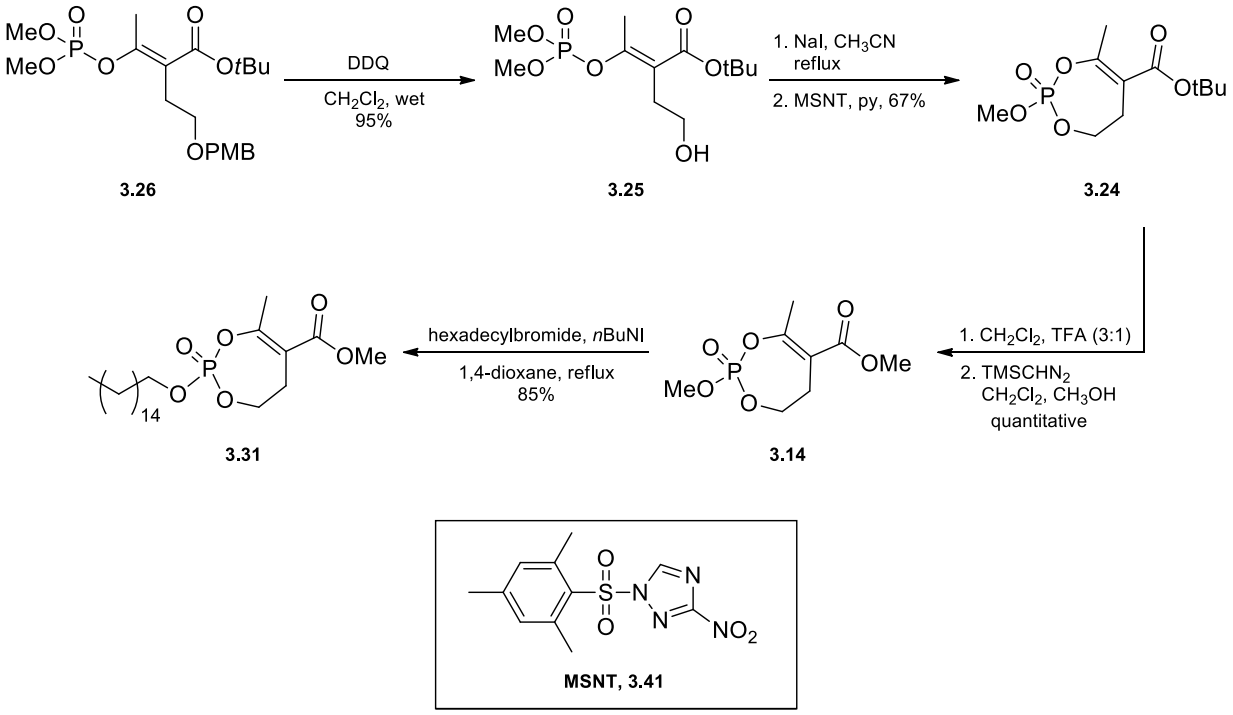


3.27 **3.26**

3. Cyclization and Transesterification

Cleavage of the PMB ether (**3.26**) under standard oxidative conditions gave free alcohol **3.25** in very good yield (**Scheme 3.15**). Gratifyingly, the *t*-butyl ester is stable at room temperature to lactonization. Demethylation with sodium iodide followed by cyclization of the resulting sodium salt with MSNT (**3.41**) gave cyclic phosphate **3.24** in moderate yield.

Scheme 3.15



Treatment of *t*-butyl ester **3.24** with trifluoroacetic acid under anhydrous Schlenk conditions cleanly and effectively hydrolyzed the ester leaving the cyclic phosphate intact. Treatment of the resulting carboxylic acid with trimethylsilyldiazomethane gave methyl ester **3.14** in quantitative yield (**Scheme 3.15**). Transesterification using the established conditions[3.1,3.7] gave cyclipostin analog **3.31** in good yield.

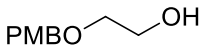
III. Conclusion

A monocyclic phosphate analog of cyclophostin and cyclipostin P was synthesized in order to give a complete SAR comparison of phosphates, phosphonates, and α,α-difluorophosphonates as inhibitors of serine hydrolases AChE and HSL. The compounds

synthesized in this chapter and Chapter 2 were submitted to collaborators in the laboratory of Prof. Cynthia Dupureur for assay against AChE and HSL. The results of that study are reported in the following chapter.

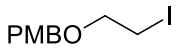
IV. Experimental Section

General Experimental. All reactions were carried out in oven dried glassware (150 °C) under an atmosphere of argon unless otherwise noted. Dry THF was distilled from sodium and benzophenone. Dry CH_2Cl_2 and CH_3CN were distilled from CaH_2. Dry MeOH was distilled from magnesium. Dry pyridine was distilled from KOH. All chemicals and reagents were purchased from commercial suppliers unless otherwise noted. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded at 300, 75 and 121 MHz, respectively. ^{1}H NMR spectra are referenced to $CDCl_3$ (7.27 ppm), ^{13}C NMR spectra are referenced to $CDCl_3$ (77.23 ppm), and ^{31}P NMR spectra are referenced to external H_3PO_4.

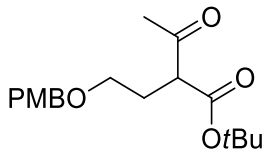


2-((4-Methoxybenzyl)oxy)ethanol (3.32): Following published procuedures,[3.8] potassium hydroxide (85%, 1.46 g, 22.1 mmol) was dissolved in ethylene glycol (12.3 mL) and heated at 130 °C for 4 h. The solution was cooled to rt, and PMBCl (3 mL, 22.1 mmol) was added. The solution was stirred at 30 °C for 14 h. The mixture was diluted with water and extracted three times with Et_2O. The Et_2O layers were dried over $MgSO_4$ and concentrated *in vacuo*. The crude mixture was purified by silica gel chromatography (40-50% EtOAc/hexanes) to give **3.32** (3.99 g, 99%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.30 (2H, d, J_{HH} =

8.1 Hz), 6.92 (2H, d, J_{HH} = 8.7 Hz), 4.53 (2H, s), 3.84 (3H, s), 3.78 (2H, t, J_{HH} = 4.5 Hz), 3.61 (2H, t, J_{HH} = 4.7 Hz).

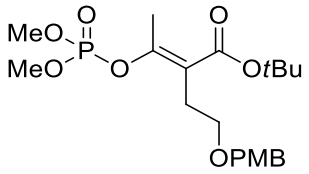


1-((2-Iodoethoxy)methyl)-4-methoxybenzene (3.28): Triphenyl phosphine (1.48 g, 5.64 mmol) and I_2 (1.43 g, 5.63 mmol) were combined in dry THF, 5 mL, and stirred 10 min. The mixture was cooled to 0 °C, and solution of **3.32** (1.02 g, 5.60 mmol) and imidazole (762 mg, 11.19 mmol) in dry THF, 2 mL, was added by cannula, rinsing further with dry THF, 3 mL. The ice bath was removed, and the mixture was stirred 1 h. The red-brown solution was poured onto silica gel and concentrated *in vacuo*. The mixture purified by silica gel chromatography (30% EtOAc/hexanes) to give **3.28** (1.515 g, 93%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.29 (2H, d, J_{HH} = 8.7 Hz), 6.90 (2H, d, J_{HH} = 8.7 Hz), 4.52 (2H, s), 3.82 (3H, s), 3.72 (2H, t, J_{HH} = 6.8 Hz), 3.27 (2H, t, J_{HH} = 6.9 Hz).



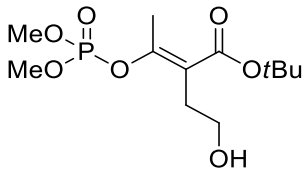
***tert*-Butyl 2-acetyl-4-((4-methoxybenzyl)oxy)butanoate (3.27)**: To a solution of **3.28** (1.51 g, 5.17 mmol) in dry acetone, 26 mL, was added *tert*-butyl acetoacetate (1.3 mL, 7.8 mmol) followed by K_2CO_3 (1.8 g, 13 mmol). The mixture was heated to reflux 36 h, partitioned between water and Et_2O, and extracted three times with Et_2O. The organic extracts were dried over Na_2SO_4 and concentrated *in vacuo*. The crude mixture was purified

by silica gel chromatography (5-10% EtOAc/hexanes) to give **3.27** (927 mg, 56%) as a pale yellow oil. IR (neat, NaCl) 2976, 2935, 2856, 1736, 1714 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.22 (2H, d, J_{HH} = 8.5 Hz), 6.86 (2H, d, J_{HH} = 8.4 Hz), 4.37 (2H, s), 3.78 (3H, s), 3.58 (1H, t, J_{HH} = 7.2 Hz), 3.43 (2H, t, J_{HH} = 6.0 Hz), 2.20 (3H, s), 2.11 (2H, m), 1.42 (9H, s); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 203.3, 168.9, 159.4, 130.5, 129.4, 114.0, 81.9, 72.7, 67.4, 57.8, 55.4, 29.2, 28.3, 28.0; HRMS (FAB, NBA, MNa^+) calcd for $C_{18}H_{26}O_5Na$: 345.16779, found: 345.1673.

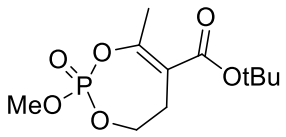


(*E*)-*tert*-Butyl 3-((dimethoxyphosphoryl)oxy)-2-(2-((4-methoxybenzyl)oxy)ethyl)but-2-enoate (3.26): Solution of acetoacetate **3.27** (206 mg, 0.639 mmol) and Hünig's base (560 µL, 3.20 mmol) in dry CH_2Cl_2, 650 µL, was cooled to -30 °C. $(CH_3O)_2PCl$ (82%, 137 µL, 0.96 mmol) was added dropwise, and the solution was allowed to warm to room temperature after stirring 10 min. After 1 h, the solution was concentrated *in vacuo* and dissolved in dry CH_2Cl_2. Pyridine (257 µL, 3.19 mmol) was added followed by I_2 (89 mg, 0.70 mmol). After stirring 5 min, dry MeOH, 6.4 mL, was added, and saturated solution of I_2 in CH_2Cl_2 was added dropwise until color persisted. The solution was washed with 10% aqueous $Na_2S_2O_3$, extracting 3 times with CH_2Cl_2. The organic extracts were dried over Na_2SO_4 and concentrated *in vacuo*. The crude material was purified by silica gel chromatography (40% EtOAc/hexanes) to give **3.26** (156 mg, 57%) as a pale yellow oil. IR (neat, NaCl) 2959, 2857, 1709, 1514 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.20 (2H, d, J_{HH} = 8.4 Hz), 6.81 (2H,

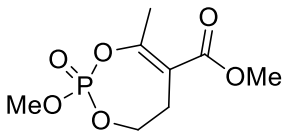
d, J_{HH} = 8.6 Hz), 4.39 (2H, s), 3.81 (6H, d, J_{HP} = 11.4 Hz), 3.80 (3H, s), 3.50 (2H, t, J_{HH} = 7.4 Hz), 2.68 (2H, t, J_{HH} = 7.3 Hz), 2.39 (3H, s), 1.46 (9H, s); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 167.0, 159.3, 154.8 (d, J_{CP} = 7.0 Hz), 130.8, 129.3, 118.3 (d, J_{CP} = 9.0 Hz), 113.8, 81.1, 72.4, 68.5, 55.3, 54.7 (d, J_{CP} = 6.0 Hz), 28.2, 30.0, 19.0; ^{31}P NMR (121.4 MHz, $CDCl_3$) δ -5.25; HRMS (FAB, NBA, MH^+) calcd for $C_{20}H_{32}O_8P$: 431.18347, found: 431.1834.



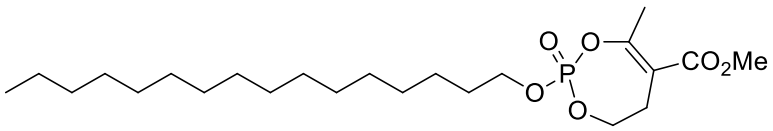
(*E*)-*tert*-Butyl 3-((dimethoxyphosphoryl)oxy)-2-(2-hydroxyethyl)but-2-enoate (3.25): Solution of phosphate **3.26** (93.4 mg, 0.217 mmol) in CH_2Cl_2, 4 mL, containing 4 drops H_2O was cooled to 0 °C, and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (74 mg, 0.33 mmol) was added. The solution was stirred 2 h and partitioned between CH_2Cl_2 and 10% aqueous Na_2SO_3, adding saturated aqueous $NaHCO_3$ to complete dissolution and extracting three times with CH_2Cl_2. The organic extracts were dried over Na_2SO_4 and concentrated *in vacuo*. The crude material was purified by silica gel chromatography (60% EtOAc/hexanes) to give **3.25** (57 mg, 85%) as a pale yellow oil. IR (neat, NaCl) 3439, 2976, 2880, 1709, 1647 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.86 (6H, d, J_{HP} = 11.5 Hz), 3.73 (2H, t, J_{HH} = 6.2 Hz), 2.64 (2H, t, J_{HH} = 6.2 Hz), 2.38 (3H, s), 1.51 (9H, s); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 167.6, 154.9 (d, J_{CP} = 7.6 Hz), 119.4 (d, J_{CP} = 8.6 Hz), 81.8, 61.7, 55.1 (d, J_{CP} = 6.1 Hz), 31.1, 28.3, 19.4; ^{31}P NMR (121.4 MHz, $CDCl_3$) δ -4.83; HRMS (FAB, NBA, MH^+) calcd for $C_{12}H_{24}O_7P$: 311.12598, found: 311.1253.



***tert*-butyl 2-Methoxy-4-methyl-6,7-dihydro-1,3,2-dioxaphosphepine-5-carboxylate 2-oxide (3.24):** To phosphate **3.25** (57 mg, 0.18 mmol) in dry CH_3CN, 100 µL, was added NaI (30 mg, 0.20 mmol), and the mixture was heated to reflux 30 min and concentrated *in vacuo*. The sodium salt was dissolved in dry distilled pyridine, 900 µL, and treated with 1-mesitylene-sulfonyl-3-nitrotriazole (107 mg, 0.36 mmol). After stirring 1.5 h, the mixture was partitioned between H_2O and EtOAc, extracting four times with EtOAc. The organic extracts were dried over Na_2SO_4 and concentrated *in vacuo*. The crude material was purified by C_{18} reverse phase silica gel chromatography (60% MeOH) to give **3.24** (36 mg, 71%) as a pale yellow oil. IR (neat, NaCl) 2977, 2930, 2853, 1711, 1653 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 4.36 (1H, m), 4.16 (1H, m), 3.87 (3H, J_{HP} = 11.5 Hz), 2.93 (1H, ddd, J_{HH} = 15.8, 8.8, 3.7 Hz), 2.77 (1H, ddd, J_{HH} = 15.8, 6.3, 3.3 Hz), 2.32 (3H, s), 1.50 (9H, s); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 166.1 (d, J_{CP} = 2.0 Hz), 159.2 (d, J_{CP} = 9.5 Hz), 117.3 (d, J_{CP} = 4.0 Hz), 82.1, 68.5 (d, J_{CP} = 6.6 Hz), 55.1 (d, J_{CP} = 6.0 Hz), 28.7, 28.4, 20.3 (d, J_{CP} = 3.5 Hz); ^{31}P NMR (121.4 MHz, $CDCl_3$) δ -9.70; HRMS (FAB, NBA, MH^+) calcd for $C_{11}H_{20}O_6P$: 279.0998, found 279.0997.



Methyl 2-methoxy-4-methyl-6,7-dihydro-1,3,2-dioxaphosphepine-5-carboxylate 2-oxide (3.13): To a Schlenk flask containing a solution phosphate **3.24** (93 mg, 0.13 mmol) in dry CH_2Cl_2, 3.3 mL, was added TFA, 1.1 mL, dropwise. After 1 h, the solution was concentrated to dryness. The carboxylic acid was dissolved in CH_2Cl_2, 3.3 mL, and MeOH, 3.3 mL, and $TMSCHN_2$ solution (2 M in hexane) was added dropwise until bubbling ceased and yellow color persisted. The solution was concentrated *in vacuo*, and the crude material was filtered in EtOAc through a pad of Celite to give **3.13** (78 mg, 99%) as a pale yellow oil. IR (neat, NaCl) 2997, 2954, 2911, 2852, 1717, 1652 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 4.39 (1H, m), 4.18 (1H, m), 3.88 (3H, d, J_{HP} = 11.5 Hz), 3.78 (3H, s), 3.00 (1H, ddd, J_{HH} = 15.7, 9.4, 3.7 Hz), 2.85 (1H, ddd, J_{HH} = 15.8, 6.4, 3.4 Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 167.2 (d, J_{CP} = 2.0 Hz), 161.0 (d, J_{CP} = 9.6 Hz), 115.7 (d, J_{CP} = 3.5 Hz), 68.5 (d, J_{CP} = 6.6 Hz), 55.1 (d, J_{CP} = 5.5 Hz), 52.2, 28.4, 20.4 (d, J_{CP} = 4.1 Hz); ^{31}P NMR (121.4 MHz, $CDCl_3$) δ -9.94; HRMS (FAB, NBA, MH^+) calcd for $C_8H_{14}O_6P$: 237.0528, found 237.0528.



Methyl 2-(hexadecyloxy)-4-methyl-6,7-dihydro-1,3,2-dioxaphosphepine-5-carboxylate 2-oxide (3.31): To a solution of phosphate **3.13** (11 mg, 0.047 mmol) and 1-bromohexadecane (175 µL, 0.473 mmol) in dry 1,4-dioxane, 235 µL, was added *n*-Bu_4NI (1 mg, 0.003 mmol). The flask was immersed at 110 °C in an oil bath until reaction was

complete (TLC, ^{31}P NMR analysis). The solvent was removed under vacuum, and the crude product was purified by silica gel chromatography (10–20% EtOAc/ hexanes) giving **3.31** (18 mg, 85%) as a colorless oil. IR (neat, NaCl) 2924, 2854, 1723, 1645 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.37 (1H, m), 4.17 (3H, m), 3.77 (3H, s), 3.00 (1H, ddd, J_{HH} = 15.5, 8.8, 3.2 Hz), 2.84 (1H, ddd, J_{HH} = 15.6, 5.9, 3.6 Hz), 1.71 (2H, p, J_{HH} = 6.7 Hz), 1.26 (26H, br s), 0.88 (3H, t, J_{HH} = 6.7 Hz); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 167.3 (d, J_{CP} = 2.0 Hz), 161.1 (d, J_{CP} = 9.6 Hz), 115.6 (d, J_{CP} = 4.1 Hz), 69.3 (d, J_{CP} = 6.6 Hz), 68.3 (d, J_{CP} = 6.6 Hz), 52.2, 32.1, 30.5 (d, J_{CP} = 6.6 Hz), 29.90, 29.87, 29.84, 29.76, 29.69, 29.55, 29.3, 28.5, 25.6, 22.9, 20.5 (d, J_{CP} = 4.0 Hz), 14.3; ^{31}P NMR (121.4 MHz, $CDCl_3$) δ -10.8; HRMS (FAB, NBA, MH^+) calcd for $C_{23}H_{44}O_6P$: 447.2876, found 447.2864.

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Chapter 4 : AChE and HSL Inhibition Results

I. Introduction

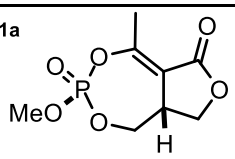
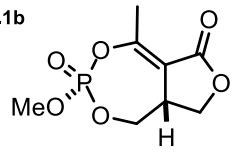
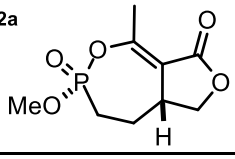
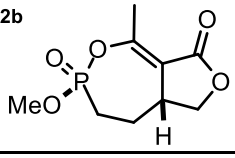
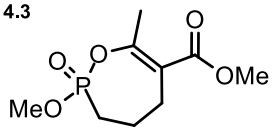
The materials synthesized in Chapters 2 and 3 were submitted to the laboratory of Prof. Cynthia Dupureur for assay against acetylcholinesterase (AChE) and hormone-sensitive lipase (HSL). The assay for AChE inhibition is a modified Ellman assay,[4.1,4.2] and the assay for HSL inhibition is a tritiated triolein assay.[4.3]

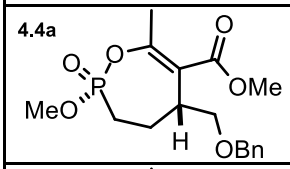
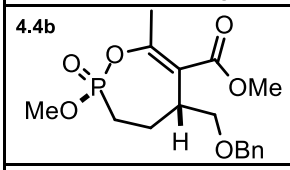
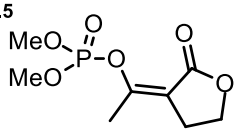
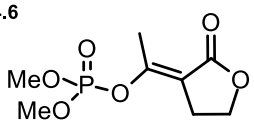
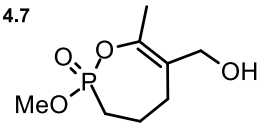
II. AChE Inhibition Results and Discussion

1. Background

SAR studies in previous works found that the diastereomers of cyclophostin (**4.1a** and **4.1b**, **Table 4.1**) are both nanomolar inhibitors of human AChE and that corresponding phosphonates are 100- to 1000-fold less potent.[4.2] Cyclophostin analogs **4.2a** and **4.2b** show a tenfold preference for the *trans* diastereomer (**4.2a**), whereas the preference for synthetic ring-opened intermediates **4.4a** and **4.4b** favors the *cis* isomer (**4.4b**). For consistency between mono- and bicyclic inhibitors, *cis* is reasoned as being the relationship between the methyl phosphonate ester and the hydrogen at the chiral carbon.

Table 4.1: Results from previous studies for acetylcholinesterase inhibition

Inhibitor	IC_{50} (μM)
4.1a 	0.045[4.2a]
4.1b 	0.040[4.2a]
4.2a 	3[4.2b]
4.2b 	30[4.2b]
4.3 	7[4.2c]

Inhibitor	IC_{50} (μM)
4.4a 	35[4.2b]
4.4b 	6[4.2b]
4.5 	>1000[4.2c]
4.6 	70[4.2c]
4.7 	600[4.2c]

Monocyclic analog **4.3** showed inhibition of the same order of magnitude as the more active phosphonate diastereomers **4.2a** and **4.4b**, which suggested that the lactone ring was unimportant (**Table 4.1**). *Z* (**4.5**) and *E* (**4.6**) ring opened phosphates showed clear preference for the *Z* isomer (**4.5**), but the high IC_{50} demonstrates that cyclic phosphates are preferred inhibitors. The reduced phosphonate (**4.7**), is likewise a poor inhibitor, probably as a result of the loss of charge dissociation of the acetoacetate moiety which forms upon binding the catalytic serine residue.

2. AChE Inhibiton of Synthesized Inhibitors

The compounds reported in this work were submitted to Elena Vasilieva in the laboratory of Cynthia M. Dupureur for enzymatic assay. The α,α-difluorophosphonate **4.8** showed no significant inhibition of AChE (**Table 4.2**).[4.3a] Ethyl phosphonate ester **4.9** shows little difference in activity from the analogous methyl ester **4.3** (**Table 4.1**), so the loss of activity is not attributable to the ethyl ester.[4.3a] ^{31}P NMR analysis of difluorophosphonate **4.8** in 0.1 M phosphate pH 8 buffered aqueous solution showed significant hydrolysis (24% hydrolysis in 1 h), indicating that the inhibitor was unstable in the assay conditions (**Table 4.3**). A similar study of difluorophosphonate **4.8** in 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) pH = 6 buffered aqueous solution showed reasonable stability (4% hydrolysis in 1 h, **Table 4.3**), so the enzyme inhibition assay was repeated at a lower pH.[4.3a] AchE retains some of its activity at this pH.[4.4] Difluorophosphonate **4.8** showed no improvement in activity, confirming that the fluorine substituents are responsible for the negligible inhibition. Phosphate monocycle **4.10** (**Table 4.2**) was a high-nanomolar inhibitor, showing a 15-fold loss of activity from bicyclic *trans* cyclophostin **4.1b** (**Table 4.1**). This loss of activity is enigmatic in light of the comparable inhibition of monocyclic phosphonate **4.3** and the bicyclic phosphonate **4.2a** (**Table 4.1**).

Monocycle **4.10** was tenfold more active than analogous *t*-butyl ester **4.11**, indicating a steric influence at the ester position (**Table 4.2**).

Table 4.2: Acetylcholinesterase inhibition by compounds reported in this work[4.3a]

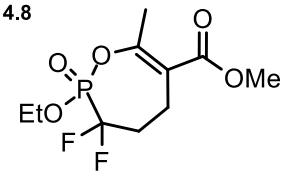
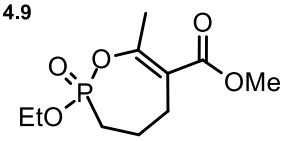
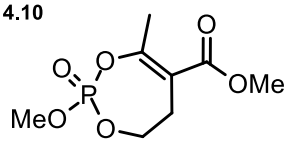
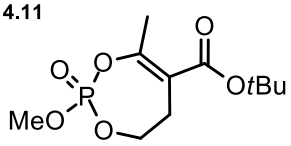
Inhibitor	IC_{50} (μM)
4.8 	>100
4.9 	7.5
4.10 	0.6
4.11 	5.7

Table 4.3: Stability of inhibitors in aqueous media as a function of pH

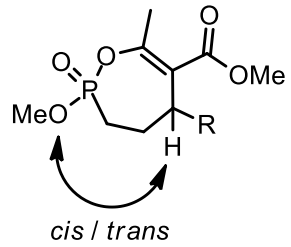
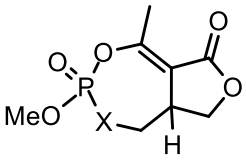
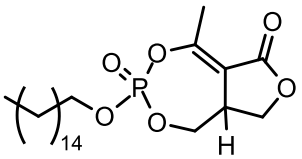
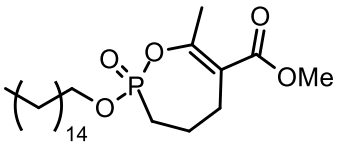
Inhibitor	pH	Percent Decomposition (30 min.)	Percent Decomposition (1 h)	Percent Decomposition (24 h)
4.8	8	~5%	~50%	100%
	7	20%	34%	100%
	6.5	2.8%	7.2%	
	6	0.5%	4.1%	65%
4.10	8	0%	4%	57%
4.3	8	2%	2%	9.6%

III. HSL Inhibition Results and Discussion

1. Background

Previously synthesized inhibitors **4.12**–**4.16**,[4.5] **4.1**,[4.2a] **4.17,**[4.2a] **4.2**,[4.2b] and **4.18**[4.5] were tested for inhibition of HSL by Supratik Dutta and Elena Vasilieva in the laboratory of Cynthia M. Dupureur (**Table 4.4**).[4.3] C-alkylated phosphonates **4.12**–**4.16** were found to be rather poor inhibitors with varying preference for relative stereochemistry.[4.3a] Optimal activity occurs at the C_{16} chain length (**4.16**).[4.3] Cyclophostin (**4.1**) and its phosphonate analog (**4.2b**) were found to be ineffective inhibitors, likely for lack of a lipophilic substituent.[4.3b] Cyclipostin P (**4.17**) is a nanomolar inhibitor with a nearly 17-fold preference for *trans* diastereomer **4.17b**.[4.3a] Monocyclic phosphonate **4.18** was found to be a rather good inhibitor.[4.3a] The activity of phosphonate inhibitors was found to be sensitive to the ratio of PC and PI in the assay conditions,[4.3a] so the lowest observed IC_{50} are be cited for the purposes of comparison.

Table 4.4: Inhibition of hormone-sensitive lipase by inhibitors from previous work[4.3a,b,c]

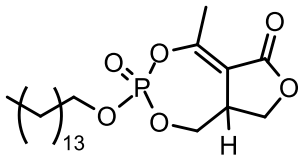
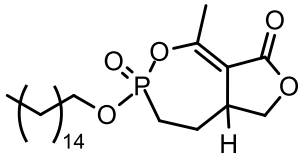
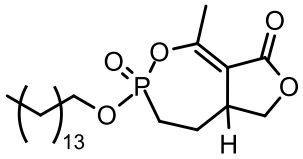
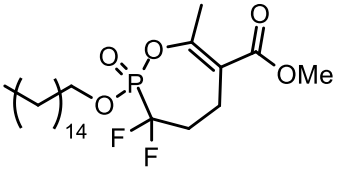
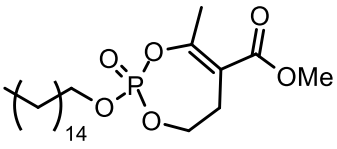
Inhibitor		IC_{50} (μM)
	**4.12a,b** R = C_5H_{11} **4.13a,b** R = $C_{10}H_{21}$ **4.14a,b** R = $C_{12}H_{25}$ **4.15a,b** R = $C_{16}H_{33}$ **4.16a,b** R = $C_{18}H_{37}$	*cis* *trans* 100[b] 100[b] 40[c] 100[b] 35[c] 13.5[c] ~60[a] ~10[a] >100[a] ~27[a]
	**4.1a,b** X = O **4.2b,a** X = CH_2	*cis* *trans* >100[b] >100[b] 100[b]
	**4.17a,b**	*cis* *trans* 0.42[a] 0.025[a]
	**4.18**	0.54[a]

2. HSL Inhibition of Synthesized Inhibitors

The cyclipostin analogs completed in this work were submitted for HSL inhibition assay to Elena Vasilieva in Cynthia M. Dupureur's laboratory. Cyclipostin R (**4.19**) was found to be a nanomolar inhibitor of HSL with 12-fold preference for the *cis* diastereomer (**4.19a**, **Table 4.5**), displaying a shift in diastereoselectivity resulting from a change in chain length of only

one methylene (compare cyclipostin P, **4.17**, **Table 4.4**). High nanomolar inhibition was observed for the phosphonate analogs **4.20** and **4.21**, indicating that phosphates are more effective inhibitors but by a smaller margin than is observed for AChE.

Table 4.5: Hormone-sensitive lipase inhibition by compounds reported in this work[4.3a]

Inhibitor		IC_{50} (μM)
	**4.19a,b**	*cis* 0.075 *trans* 0.9
	**4.20a,b**	*cis* 0.36 *trans* 6.9
	**4.21a,b**	*cis* 0.47 *trans* 0.45
	**4.22**	>100
	**4.23**	0.06

The analog of cyclipostin P **4.20** showed an appreciable preference for the *trans* diastereomer (**4.20b**), while cyclipostin R analog **4.21** showed no significant distereoselectivity at all (**Table 4.5**). These values, when compared to the activity of monocyclic analog **4.18**, would suggest little influence by the lactone moiety. Difluorophosphonate **4.22** showed no appreciable inhibition, and monocyclic phosphate **4.23** was a low nanomolar inhibitor on the order of the bicyclic natural products **4.17** and **4.19**. The latter result confirms the negligible influence by the lactone in which was observed for mono- and bicyclic phosphonates.

IV. Conclusion

α,*α*-Difluorophosphonates show little detectable activity as inhibitors of AChE and HSL. Fluorination of cyclic phosphonates clearly activates the phosphorus atom by increasing its electrophilicity (**Table 4.3**). Difluorophosphonate **4.8** is significantly less stable to hydrolysis in aqueous solution than analogous phosphate **4.10** and phosphonate **4.3**. The loss of activity must be explained as a reduced affinity of the inhibitor toward the enzyme, likely due to steric effects or, more probably, the unusual hydrogen bonding characteristics of fluorine. While the importance of diastereoisomerism is enigmatic, the superiority of phosphates to phosphonates as inhibitors of both HSL and AChE is ubiquitous.

V. Experimental Section

General NMR Stability Experimental: Inhibitor (~0.01 mmol) was dissolved in isopropanol, 25 µL. The solution was diluted with D_2O, 35 µL, followed by 0.1 M buffer

solution (phosphate, pH 8 and 7.5; 2-morpholinoethanesulfonic acid, pH 6.5 and 6), 700 µL. The solution was analyzed by ^{31}P NMR (600 MHz, inverse-gated proton decoupled, D1 = 5 s) at time points 0 minutes, 30 minutes, 1 hour, and 24 hours.

4.1. Ellman, G.J.; Courtney, K.D.Jr.; Featherstone, R.M. *Biochem. Pharmacol.* **1961**, *7*, 88–95.

4.2. (a) Malla, R. K.; Bandyopadhyay, S.; Spilling, C. D.; Dutta, S. & Dupureur, C. M. *Org. Lett.* **2011**, *13*, 3094–3097. (b) Bandyopadhyay, S.; Dutta, S.; Spilling, C.D.; Dupureur, C.M. & Rath, N.P. *J. Org. Chem.* **2008**, *73*, 8386–8391. (c) Dutta, S.; Malla, R.K.; Bandyopadhyay, S.; Spilling C.D.; Dupureur, C.M. *Bioorg. Med. Chem.* **2010**, *18*, 2265–2274.

4.3. (a) Vasilieva, E.; *Binding Properties of Large Antiviral Polyamides (PA1 and PA25) and Natural Product Derivatives as Inhibitors of Serine Hydrolases,* **2014** Ph.D. Dissertation. (b) Dutta, S. *Inhibition studies of serine hydrolases by cyclic phosphates and phosphonates,* **2010** Ph.D. Dissertation. (c) Vasilieva, E. *Quarterly Report* **June 6, 2014**.

4.4. Reiner, E.; Aldridge, W.N. *Biochem. J.* **1967**, *105*, 171–179.

4.5. Point, V.; Malla, R.; Diomande, S.; Martin, B.P.; Delorme, V.; Carriere, F.; Canaan, S.; Rath, N.; Spilling, C.; Cavalier, J. *J. Med. Chem.* **2012**, *55*, 10204–10219.

Chapter 5 : Bactericidal Lipase Inhibitors and Fluorescent Analog

I. Introduction

1. Background

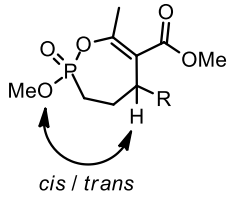
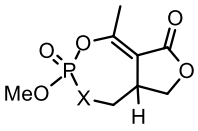
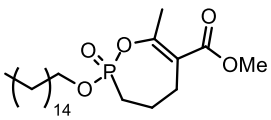
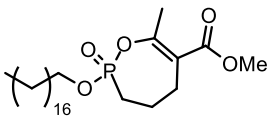
Mycobacterium tuberculosis is a notoriously drug resistant disease. The bacterium is resistant to traditional antibiotics due to its production of β-lactamase enzymes[5.1] and its ability to lie dormant in the host's macrophages indefinitely, reactivating once the immune system is weakened. Research has shown that the bacterium may be dependent on fatty acids for survival during its inactive state.[5.2] For this reason, microbial lipases have been the target of recent research as targets for future *M. tuberculosis* treatment. In particular, Rv0183, a monoacylglycerol (MAG) lipase, is believed to play a role in host cell lipid degradation,[5.3,5.4] and LipY, a triacylglycerol (TAG) lipase, is believed to be a key enzyme for the bacterium's survival during dormancy.[5.4,5.5]

2. Previous Work

A series of lipase inhibitor analogs of cyclipostins (**5.1-5.9**, **Table 5.1**) were submitted to the laboratory of Jean-François Cavalier at the laboratory of Enzymology at Interfaces and Physiology of Lipolysis (EIPL UMR7282 CNRS - Marseille, France) for testing against three microbial lipases and three mammalian lipases.[4.6] Cutinase, a microbial enzyme with a diverse array of substrates, Rv0183, and LipY were the microbial lipases chosen, and dog

gastric lipase (DGL), human pancreatic lipase (HPL), and guinea pig pancreatic lipase related protein 2 (GPLRP2) were chosen as a diverse representative sample of mammalian lipases. Activity was assayed using a pH-stat technique which employs surfactants in the pre-incubation stage to compensate for the insolubility of the substrate and enzymes. Because the assay does not take place in a solution phase, IC_{50} cannot be employed, and the results were reported as the inhibitor molar excess resulting in inhibition of 50% enzyme activity (xI_{50}).

Table 5.1: Inhibition of microbial lipases by phosphonate analogs of cyclipostin[5.6]

Inhibitor		Cutinase (xI_{50})		Rv0183 (xI_{50})		LipY (xI_{50})	
		*cis*	*trans*	*cis*	*trans*	*cis*	*trans*
	5.1a,b R = C_5H_{11}	1.42	26.2	2.70	3.79	>100	>100
	5.2a,b R = $C_{10}H_{21}$	1.98	14.6	1.13	3.57	2.81	25.8
	5.3a,b R = $C_{12}H_{25}$	2.00	3.63	5.23	2.44	3.46	6.14
	5.4a,b R = $C_{16}H_{33}$	2.54	4.43	1.32	>100	0.60	3.47
	5.5a,b R = $C_{18}H_{37}$	1.18	7.26	3.15	>100	0.77	7.47
		*cis*	*trans*	*cis*	*trans*	*cis*	*trans*
	5.6 X = O	0.96		5.22		>100	
	5.7a,b X = CH_2	8.77	4.49	43.2	41.6	>100	>100
	**5.8**	0.56		NI		0.83	
	**5.9**	0.95		NI		0.59	

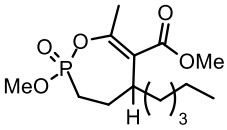
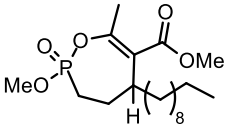
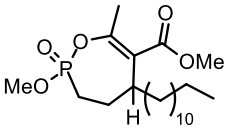
None of the inhibitors displayed any inhibition of the mammalian lipases, which in conjunction with their being weak to very weak inhibitors of hormone-sensitive lipase (HSL) as described in Chapter 4, is a potential sign of inactivity toward human lipases. The C-alkylated phosphonate analogs of cyclipostins (**5.1-5.5**, **Table 5.1**) were found to be good inhibitors of cutinase, with diastereopreference for the *cis* isomers. This trend carried to their Rv0183 results, with an inverted preference for *trans* C_{12} **5.3b** over *cis* **5.3a**, however. Inhibition of LipY was greatest with *cis* isomers **5.4a** and **5.5a**. The increased potency of inhibitors with increasing chain length comports with LipY being a TAG lipase favoring lipophilic substrates. While cyclophostin (**5.6**) was shown to be a good inhibitor of cutinase, it was a less potent inhibitor of Rv0183, and a very weak inhibitor of LipY, as would be suspected given its lack of long aliphatic substituents. The phosphonate analog **5.7** was significantly less potent in all respects. Long-chain phosphonate esters **5.8** and **5.9** were very potent inhibitors of cutinase and LipY, but showed no inhibition of Rv0183.

3. Activity against Live Cultures

A number of the inhibitors were chosen for antibacterial evaluation. Collaborators evaluated the activities of inhibitors **5.1b**, **5.2**(**a**,**b**), and **5.3**(**a**,**b**) against the green fluorescent protein expressing strain *M. tb* H37Rv-GFP and against infected murine macrophage cells.[5.7] The results were reported as concentration of compound resulting in 50% reduction of growth rate (IC_{50}) and compound concentration resulting in 50% macrophage toxicity (CC_{50}). The compounds tested displayed IC_{50}s against intracellular *Mycobacterium tuberculosis* comparable to reported[5.8] values for first-line medications isoniazid, ethionamide, and rifampin (**Table 5.2**). Of additional interest are the low cell toxicities. These preliminary

results suggest the potential of these analogs as drug candidates, especially C_{10} phosphonate **5.2(a,b)**.

Table 5.2: Antibiotic activities on *Mycobacterium tuberculosis*[5.7]

Compounds		Extracellular Growth IC_{50} (μM)	Infected Macrophage Growth IC_{50} (μM)	Macrophage Toxicity CC_{50} (μM)
	**5.1b**	*cis* *trans* *no effect*	*cis* *trans* 6.0	*cis* *trans* >100
	**5.2a,b**	*cis* *trans* 15-20 >100	*cis* *trans* 3.0 4.0	*cis* *trans* >100 >100
	**5.3a,b**	*cis* *trans* >100 30-50	*cis* *trans* 10.0 4.0	*cis* *trans* >20 >100
Isoniazid[a]		1.2	1.2	>150
Ethionamide[a]		6.0	6.0	120
Rifampin[a]		0.01	2.9	24

[a] *Referenced results*[5.8]

II. Microbial Lipase Inhibition of Additional Analogs

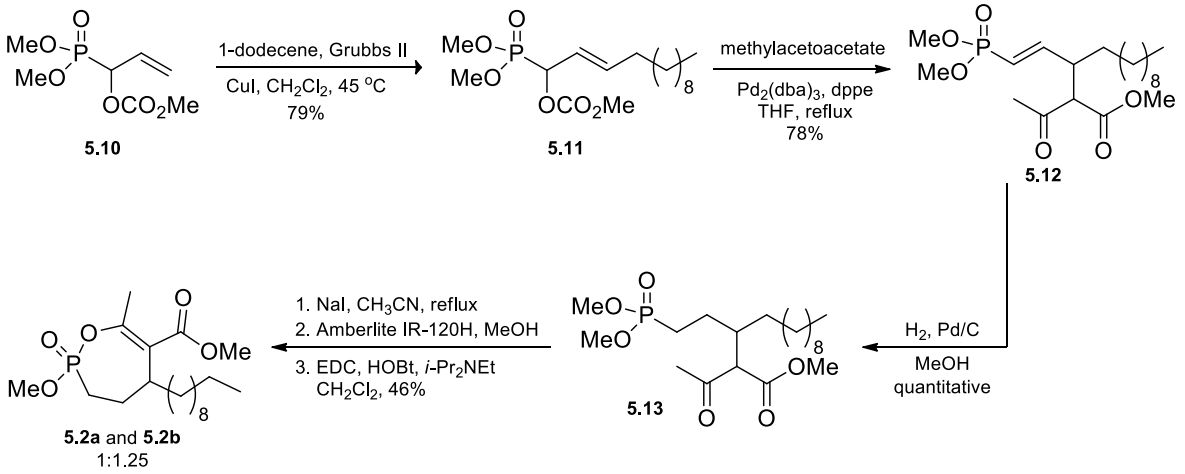
1. Background

In order to further the structure activity relationship (SAR) investigation, compounds reported in this work were submitted for similar analysis. Furthermore, because of the marked activity of C-alkyl monocycles **5.1**-**5.5**, as well as the long chain phosphonate esters **5.8** and **5.9**, an additional analog bearing two long alkyl chains was synthesized by transesterification of **5.2** and submitted for assay.

2. Synthesis of New Analog

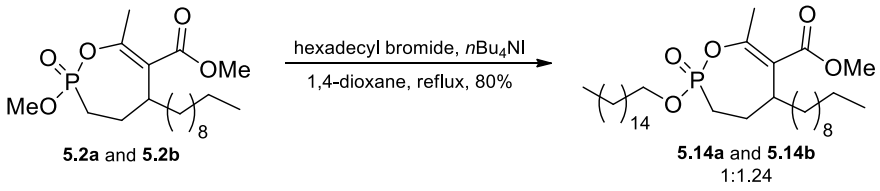
Monocyclic cyclipostin analog **5.2** was synthesized following the published procedure (**Scheme 5.1**).[5.6] The synthesis employs cross metathesis of carbonate **5.10** and Pd π-allyl coupling of the resulting carbonate **5.11** with methylacetoacetate, techniques extensively developed and employed by the Spilling research group.[5.9] Hydrogenation and ultimate cyclization gave phosphonate **5.2** as a mixture of diastereomers.

Scheme 5.1[5.6]



Transesterification of **5.2** (**Scheme 5.2**) was performed by the established technique,[5.10,5.6] giving phosphonate **5.14** in 80% yield as a mixture of diastereomers.

Scheme 5.2

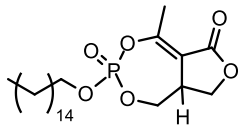
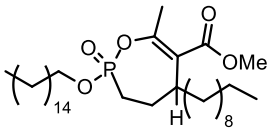
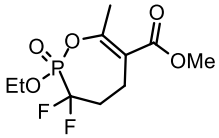
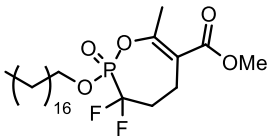
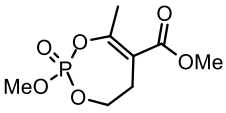
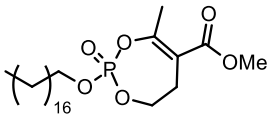


3. Biological Activities of Synthesized Materials

Cyclipostin P (**5.15**) and the compounds completed in this work were submitted for evaluation by collaborators in the laboratory of Jean-François Cavalier at the laboratory of Enzymology at Interfaces and Physiology of Lipolysis (EIPL UMR7282 CNRS - Marseille, France). Cyclipostin P (**5.15**) was found to be a very potent inhibitor of both cutinase and

LipY with xI_{50}s approaching 0.50, the lowest obtainable value (**Table 5.3**).[5.7] The *cis* lipophilic phosphonate **5.14a** was found to inhibit cutinase nearly as potently as the parent *cis* methyl ester (**5.2a**) while the *trans* isomer (**5.14b**) showed tenfold improvement compared to *trans* methyl ester **5.2b**.[5.7] *cis* Isomer **5.14a** displayed only a subtle gain of activity against LipY while the *trans* isomer **5.14b** displayed nearly a twofold loss.[5.7] *α*,*α*-Difluorophosphonate ethyl ester **5.16** was a good inhibitor of cutinase and a very poor inhibitor of LipY.[5.7] The corresponding hexadecyl ester (**5.17**) showed a very low xI_{50} against cutinase and moderate inhibition of LipY.[5.7] Phosphate methyl ester **5.18** was a strong inhibitor of both enzymes, and the hexadecyl phosphate ester (**5.19**) was a stoichiometric inhibitor.[5.7] All of the phosphates in **Table 5.3** displayed >90% inhibition when lipases were treated with four molar equivalents, but the phosphonates and difluorophosphonates only achieved 60–75% inhibition at one hundredfold excess.[5.7]

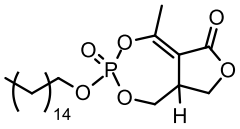
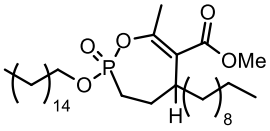
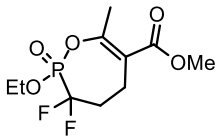
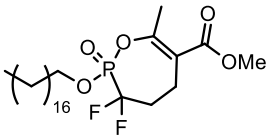
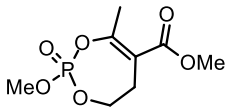
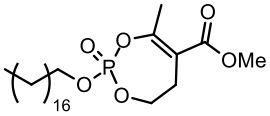
Table 5.3: Lipase inhibition by cylipostin P and compounds reported in this work[5.7]

Inhibitor		Cutinase (xI_{50})	LipY (xI_{50})
	**5.15a,b**	*cis* *trans* 0.55 0.52	*cis* *trans* 0.51 0.50
	**5.14a,b**	*cis* *trans* 2.04 1.56	*cis* *trans* 1.17 >40
	**5.16**	1.18	>40
	**5.17**	0.56	3.42
	**5.18**	0.61	0.87
	**5.19**	0.50	0.50

Collaborators then evaluated these compounds' activities against *Mycobacterium tuberculosis* and infected macrophages in the manner previously described. Cyclipostin P (**5.15**) and long-chain phosphate **5.19** alone showed activity against the bacterium (**Table 5.4**).[5.7] The *cis* diastereomer **5.15a** and monocyclic **5.19** in particular were potent, with low micromolar and high nanomolar IC_{50}s, respectively.[5.7] In spite of some of the excellent

enzymatic inhibitions, none of this set of compounds showed activity against *Mycobacterium tuberculosis* in infected macrophages, however.[5.7]

Table 5.4: Activity of cyclipostin P and synthesized compounds against *Mycobacterium tuberculosis*[5.7]

Compounds		Extracellular Growth (IC_{50})	Infected Macrophage Growth (IC_{50})
	**5.15a,b**	*cis* 1.6 *trans* 24.4	*cis* NE *trans* NE
	**5.14a,b**	*cis* NE *trans* NE	*cis* NE *trans* NE
	**5.16**	NE	NE
	**5.17**	NE	NE
	**5.18**	NE	NE
	**5.19**	0.50	NE

III. Fluorescent Analog

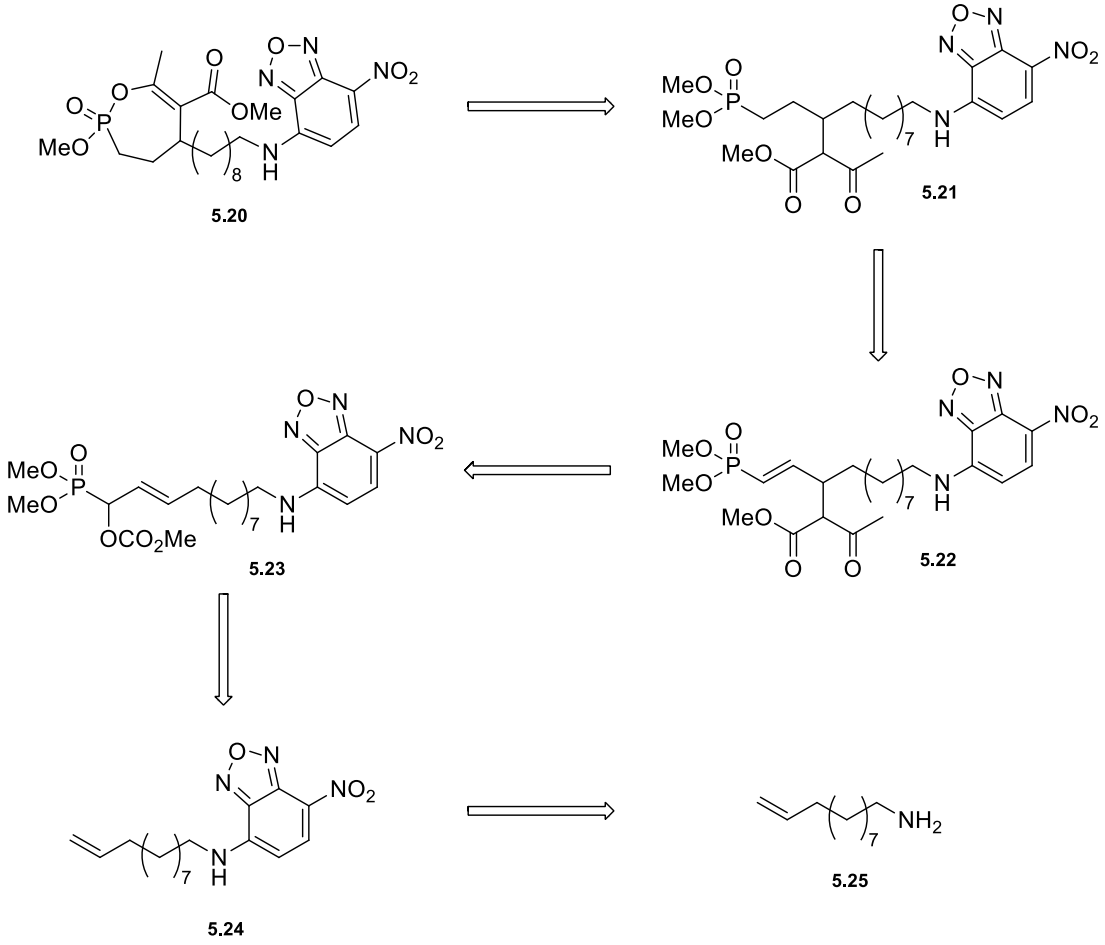
1. Introduction

Compounds **5.1**–**5.3** (**Table 5.1**, **Table 5.2**) were potent inhibitors of *Mycobacterium tuberculosis* lipases Rv0183 and LipY and were active against the bacterium inside infected macrophages. In order confirm the proposed mechanism of action, a fluorescently labeled analog of the inhibitor **5.2** was chosen for synthesis labeled with nitrobenzo-2-oxa-1,3-diazole (NBD). Inhibited enzymes in a complex mixture can then be identified by their fluorescence in gel electrophoresis.[5.11] By placing the fluorophore at the end of the alkyl chain, synthesis of the analog would closely follow **Scheme 5.1**.

2. Retrosynthesis

The initial approach to synthesize fluorescent analog **5.20** is described in **Scheme 5.3**. Dimethyl phosphonate **5.21** could be cyclized to **5.20** in the same way as described in **Scheme 5.1**. Hydrogenation of **5.22** may require special conditions such as potassium azodicarboxylate rather than palladium on carbon conditions due to possible reaction at the NBD moiety. Methyl acetoacetate addition to a Pd π-allyl formed from **5.23** could give **5.22**, and **5.23** could be formed from cross metathesis between NBD-functionalized undecenamine **5.24** and carbonate **5.10** (**Scheme 5.1**). Cross-metathesis with **5.24**[5.12] and synthesis of undecenamine **5.24**[5.13] are preceded in the literature.

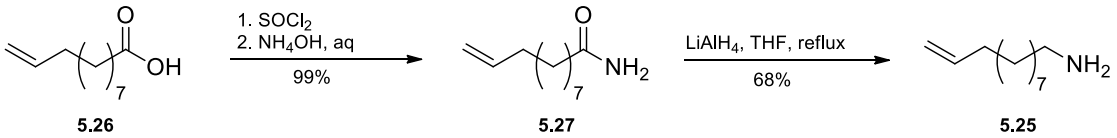
Scheme 5.3



3. Synthesis

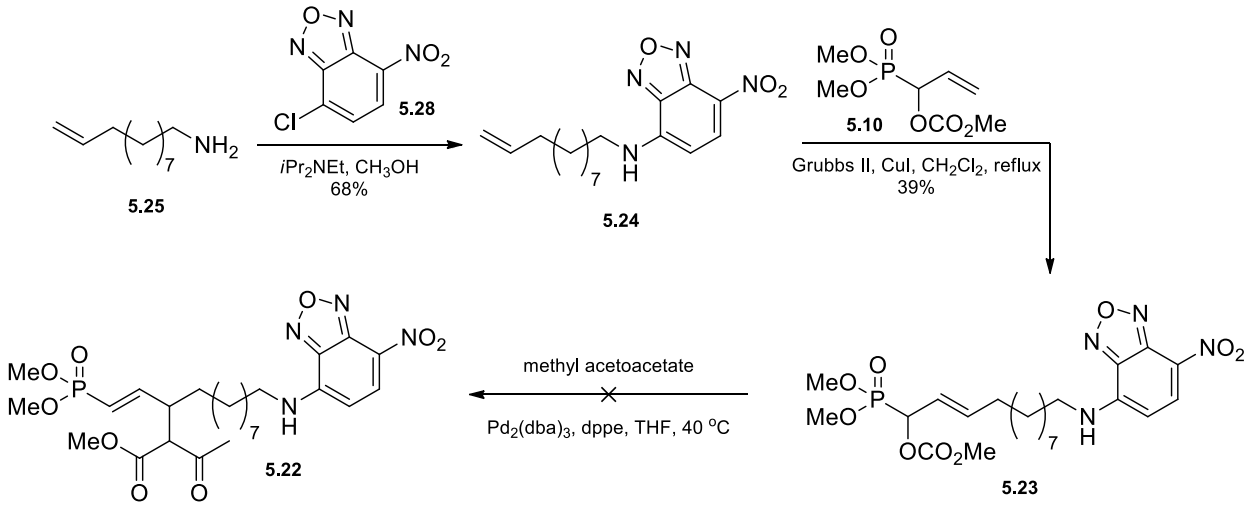
Undecenamine **5.25** was synthesized in two steps (**Scheme 5.4**). Commercially available 10-undecenoic acid (**5.26**) was converted to 10-undecenamide (**5.27**) by reaction with thionyl chloride followed by concentrated aqueous ammonium hydroxide. The reduction of amide **5.27** to amine **5.25** followed the procedure of Cheng and Landry.[5.13a]

Scheme 5.4



S_NAr reaction of amine **5.25** with NBDCl (**5.28**) followed literature precedent,[5.13b] and cross-metathesis of fluorescent **5.24** employed procedures established for reaction with phosphonoallylic carbonate **5.10**[5.9a,5.14] gave carbonate **5.23** in low yield (**Scheme 5.5**). The subsequent Pd π-allyl reaction with methyl acetoacetate, however, generated no product.

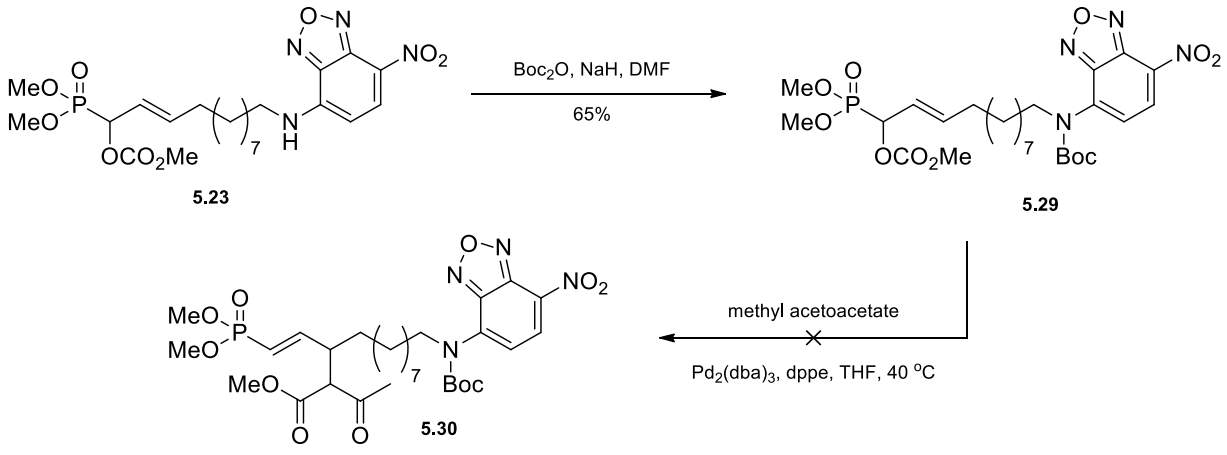
Scheme 5.5



Lewis basic nitrogen moieties have been known to interfere with Pd(0) catalyzed reactions,[5.15] so the secondary aniline (**5.23**) was protected with a *t*-butyloxy-carbonyl (Boc)

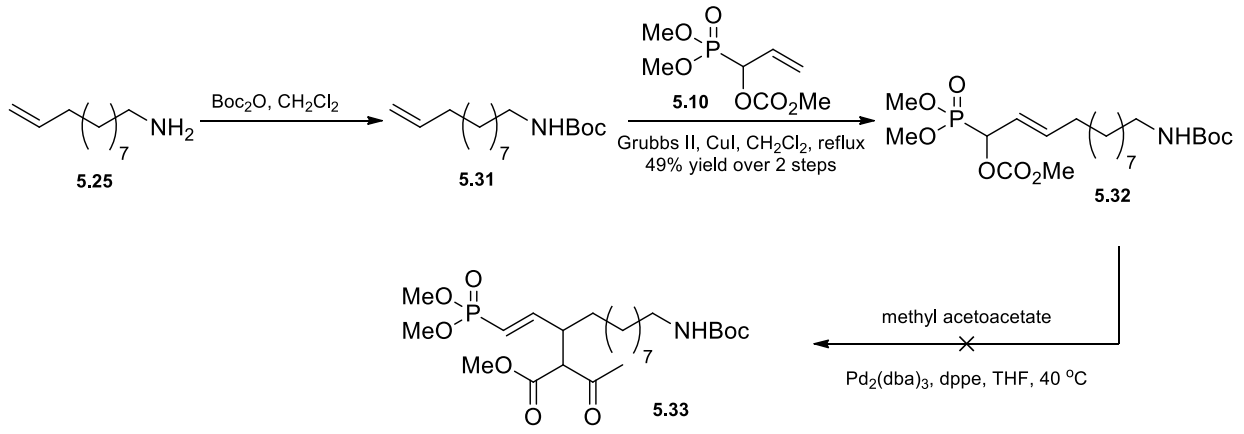
group, giving **5.29** in 65% yield (**Scheme 5.6**). However, Pd π-allyl reaction product **5.30** was not generated. This result suggests that the NBD moiety may be incompatible with the reaction conditions, and must be installed at a later step.

Scheme 5.6



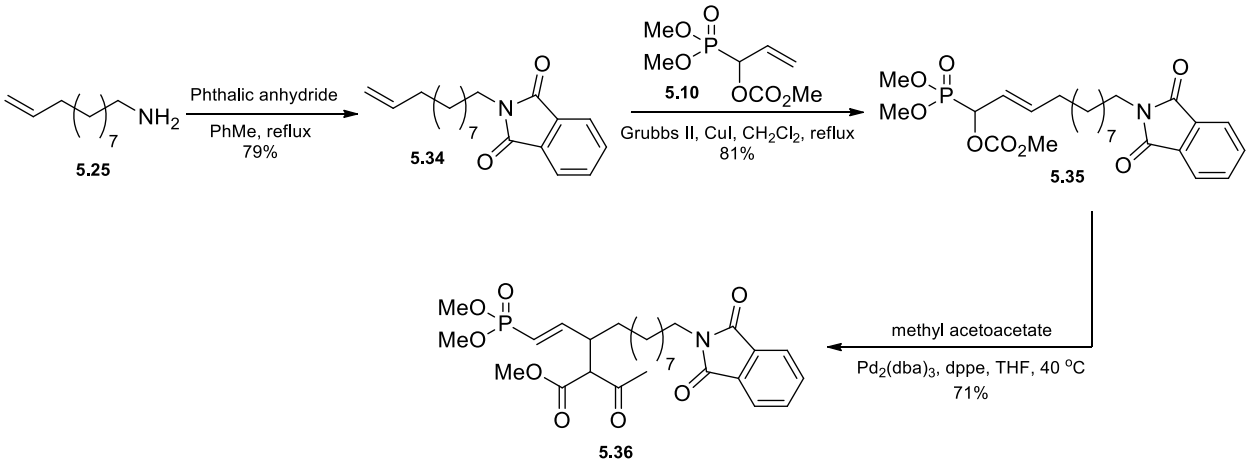
Amine **5.25** was protected as carbonate **5.31** and introduced crude to cross metathesis reaction with phosphonate **5.10** giving carbonate **5.32** in fair yield (**Scheme 5.7**). Surprisingly, the Boc-protected amine (**5.32**) was found to be unreactive to the Pd π-allyl reaction conditions, as well.

Scheme 5.7



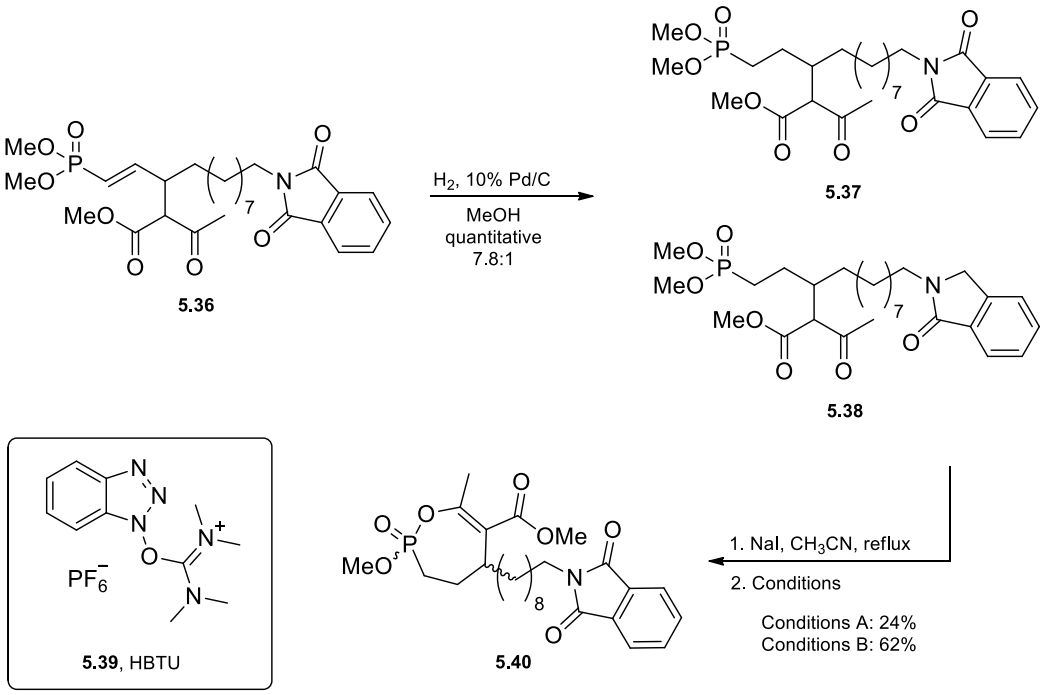
Amine **5.25** was protected by refluxing in toluene with phthalic anhydride, giving phthalimide **5.34** in good yield (**Scheme 5.8**). Cross-metathesis with phosphonate **5.10** generated carbonate **5.35** in only moderate yield, but subsequent Pd π-allyl reaction with methyl acetoacetate successfully gave β-ketoester **5.36** in good yield.

Scheme 5.8



Hydrogenation of the olefin to aliphatic phosphonate **5.37** was effective, but a side product (**5.38**) was generated due to reduction of the phthalimide (**Scheme 5.9**). The mixture was carried forward and cyclized using the techniques detailed in **Scheme 5.1**, but the yield was low. Cyclization with HBTU (**5.39**) after demethylation with sodium iodide gave cyclic phosphonate **5.40** in 64% yield over three steps.

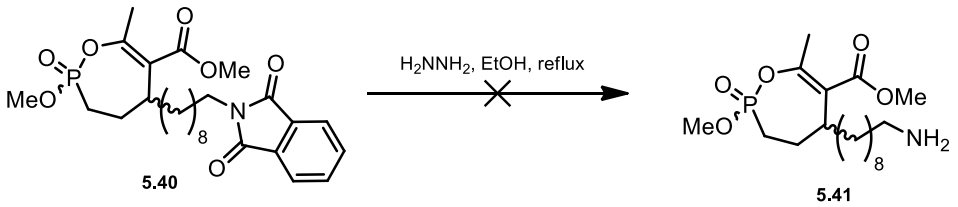
Scheme 5.9



Conditions A: a.) Amberlite IR-120H, MeOH b.) EDC, HOBt, i*Pr*$_2$*NEt, CH*$_2$*Cl*$_2$
Conditions B: HBTU, DMF, i*Pr*$_2$*NEt*

Attempted phthalimide removal using hydrazine generated a complex mixture of products (**Scheme 5.10**). Reaction is suspected to have occurred at the methyl ester and the methyl phosphonate ester in addition to removal of the protecting group.

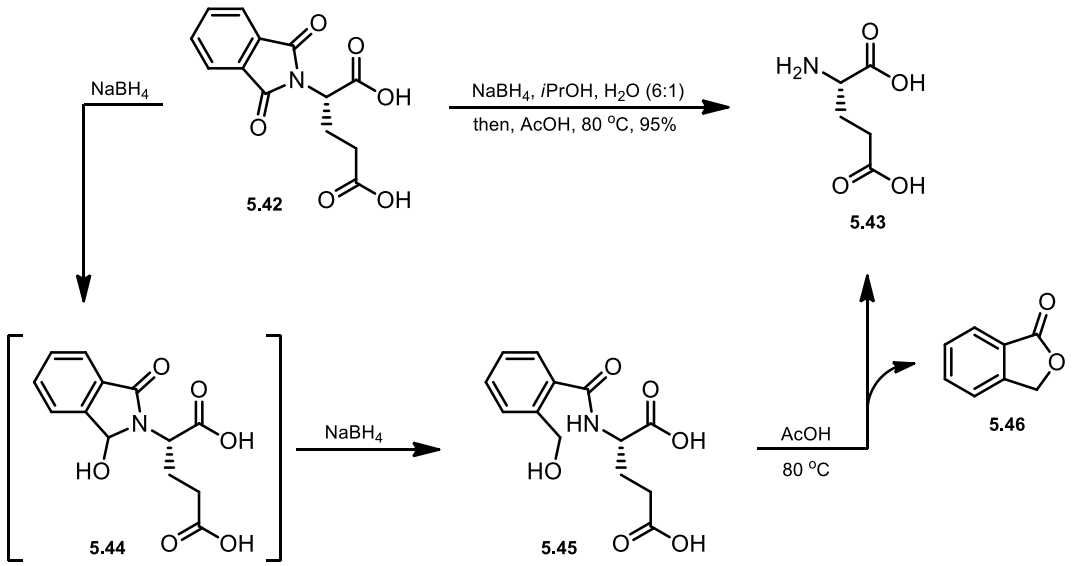
Scheme 5.10



5.40 **5.41**

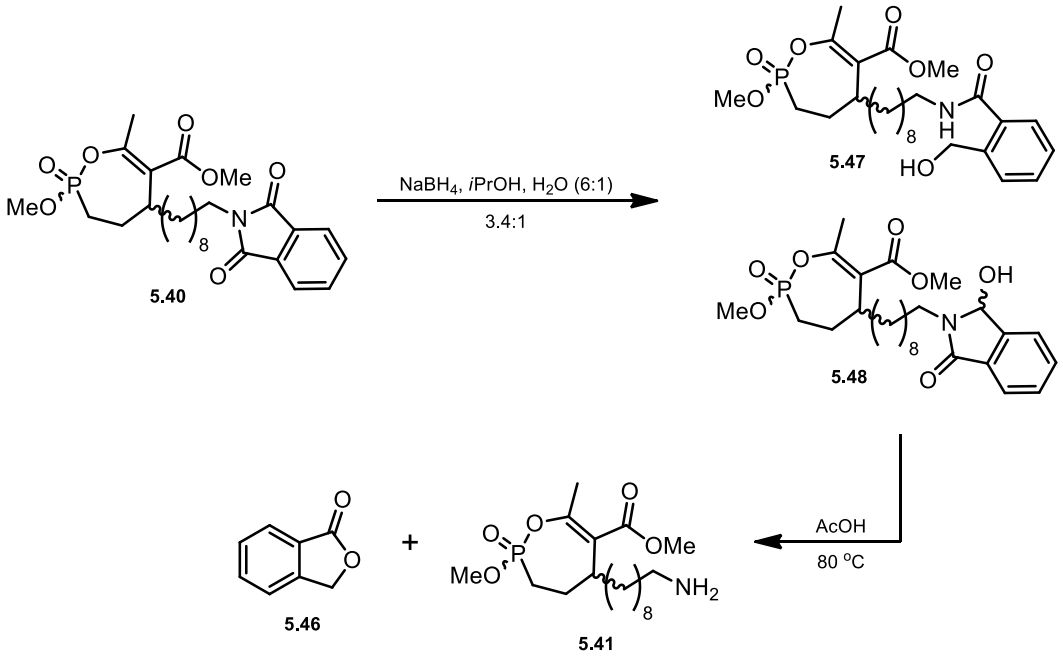
A milder alternative to hydrazine deprotection is reduction of phthalimide with sodium borohydride. The technique was developed by Ganem, *et al.* and is exemplified in their work depicted in **Scheme 5.11**.[5.16] Phthalimide protected glutamic acid (**5.42**) was deprotected by reduction of the phthalimide to amide **5.45** which was cyclized by addition of a large excess of acetic acid and heating to give glutamic acid (**5.43**) in excellent yield and phthalide (**5.46**, **Scheme 5.11**).

Scheme 5.11[5.16]



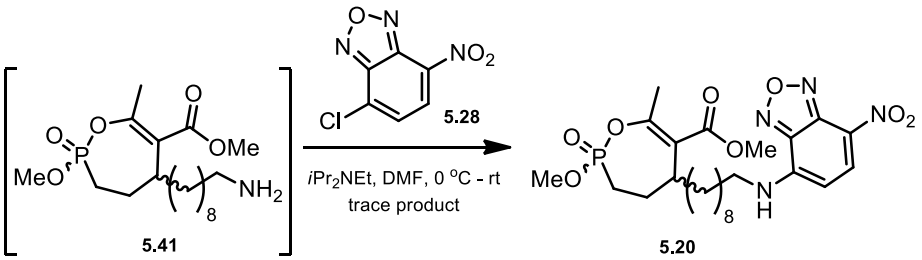
The reaction of phthalimide protected cyclic phosphonate **5.40** with sodium borohydride is effective, but has to be halted before reduction is complete due to the instability of the enol phosphonate to aqueous base (**Scheme 5.12**). The resulting mixture of amide (**5.47**) and amidol (**5.48**) was treated with a large excess acetic acid and heated to 80 °C. Phthalide (**5.46**) was visible in the crude ^{1}H NMR spectrum and the phosphonate ring was intact according to crude ^{31}P NMR, suggesting successful reaction. However, free amine **5.41** was unstable upon concentration of the crude mixture after basic extraction.

Scheme 5.12



Attempting to concentrate the acidic mixture in order to isolate the acetic acid salt of **5.41** also resulted in decomposition. DMF was added to the crude solution of amine **5.41** and the remaining isopropanol was evaporated. The solution was added to a cold basic solution of NBD-Cl (**5.28**), and trace quantities of target product **5.20** were isolated (**Scheme 5.13**).

Scheme 5.13



IV. Conclusion

1. Inhibitors

The inhibitors synthesized in Chapters 2 and 3 displayed impressive inhibition against the lipases cutinase and LipY, excepting the low activity of *α,α*-diflurophosphonates **5.16** and **5.17** toward the latter. Cyclipostin P (**5.15**) and monocyclic phosphate **5.19** were both impressive inhibitors of LipY (**Table 5.3**), but neither was as effective against *M. tuberculosis* inside human macrophages (**Table 5.4**) as phosphonate monocycle **5.2** (**Table 5.2**). This is a strong indicator from a SAR perspective of the importance of the C-alkyl substituents of compounds **5.1**–**5.5**. Transesterification of C-alkylated phosphonate **5.2** with hexadecyl bromide to compound **5.14** (**Scheme 5.2**) was expected to increase inhibition of triacyglycerol lipase LipY. A mild improvement in inhibition was observed comparing the *cis* isomers **5.2a** and **5.14a** (**Table 5.2** and **Table 5.3**), but the low inhibition of *trans* isomer **5.2b** was worsened rather than improved by the substitution. Furthermore, the methyl phosphonate esters (e.g. **5.2**) are active against *M. tuberculosis* inside human macrophages (**Table 5.2**), but analogous hexadecyl phosphonate ester **5.14** is not (**Table 5.4**). From a SAR perspective, this casts doubt on the utility of long alkyl phosphonate ester substituents for the purposes of this project.

2. Fluorescent Analog

Crude NMR gives reasonable confidence that free amine **5.41** (**Scheme 5.12**) has been successfully synthesized, but S_NAr chemistry with the crude material has thus far been largely unsuccessful (**Scheme 5.13**). Use of a more traditional solvent such as methanol has

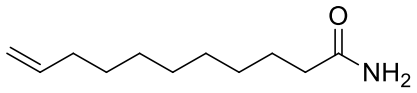
yet to be investigated. Methanol, however, having a lower boiling point than isopropanol, would not provide the opportunity to remove the solvent from the previous step without concentrating the mixture to dryness. This means the S_NAr chemistry would have to be performed in a mixture of isopropanol and methanol in order to avoid the decomposition of the unisolable amine (**5.41**). Another option might be to use a more easily installed fluorescent moiety such as a dansyl group or NBD-glycine activated for coupling to a free amine.

V. Experimental Section

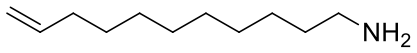
General Experimental. All reactions were carried out in oven dried glassware (150 °C) under an atmosphere of argon unless otherwise noted. Dry THF was distilled from sodium and benzophenone. Dry CH_2Cl_2 and CH_3CN were distilled from CaH_2. Dry MeOH and *i*-PrOH were distilled from magnesium. All chemicals and reagents were purchased from commercial suppliers unless otherwise noted. ^1H, ^{13}C and ^{31}P NMR spectra were recorded at 300, 75 and 121 MHz, respectively. ^1H NMR spectra are referenced to $CDCl_3$ (7.27 ppm), ^{13}C NMR spectra are referenced to $CDCl_3$ (77.23 ppm), and ^{31}P NMR spectra are referenced to external H_3PO_4.

Preparative HPLC Specifications and Conditions: Manual Preparative injector: Rheodyne 1700 (3725i-119) with 20 mL loop; Solvent A – MeOH; Solvent B – H_2O; Varian ProStar Model 210 pumps equipped with 25 mL/min Rainin/Gilson type pump heads. Kromasil 100-10C18-2025 column; 10 um particle diameter; 250 mm x 20 mm i.d.

Spectra-Physics Spectra 100 UV detector with prep cell. LKB 2211 Superac fraction collector. 50% MeOH/50% H_2O to 100% MeOH from 12 min to 60 min at a flow rate of 10 mL/min.

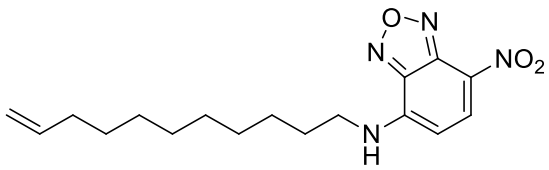


Undec-10-enamide (5.27): Undec-10-enoic acid (20.25 g, 109.9 mmol) was dissolved in thionyl chloride, 136 mL. The solution was heated at reflux 1 h, and thionyl chloride was distilled away. The resulting oil was dissolved in CH_2Cl_2 and added dropwise to concentrated aqueous NH_4OH at 0 °C. The mixture was diluted with water and CH_2Cl_2 until two clear layers formed. The organic phase was washed with 1 N HCl (aq.), and brine was added to aid separation. The organic phase was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* giving **5.27** (19.91 g, 99%) as a pale pink solid. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.82 (1H, ddt, J_{HH} = 17.1, 10.2, 6.8 Hz), 5.38 (2H, br s), 4.98 (2H, m), 2.23 (2H, t, J_{HH} = 7.5 Hz), 2.04 (2H, q, J_{HH} = 6.9 Hz), 1.64 (2H, p, J_{HH} = 7.5 Hz), 1.31 (m, 12H).

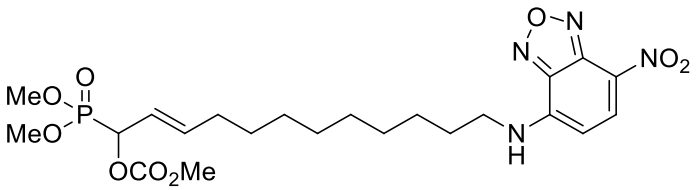


Undec-10-en-1-amine (5.25): Following the procedure of Cheng and Landry,[5.13a] THF, 50 mL, was charged to a 250 mL RBF. $LiAlH_4$ (2.3 g, 60.6 mmol) was weighed and slowly added to the flask in two parts. The suspension was heated at reflux for 30 min. The mixture was cooled to room temperature, and a solution of **5.27** (5.0 g, 27.3 mmol) in THF, 100 mL, was added dropwise. The mixture was refluxed for 24 h. EtOAc, 20 mL, was

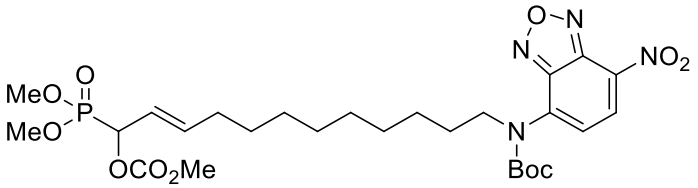
added dropwise, and the mixture was cooled to 0 °C in an ice water bath. Saturated aqueous Na_2SO_4, 40 mL, was added slowly, and the resulting white suspension was filtered, rinsing 5 times with EtOAc, 20 mL each. The organic phase was separated, dried, and concentrated *in vacuo*. The resulting oil was distilled by kugelrohr, giving **5.25** (3.12 g, 68%) as a green oil. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.82 (1H, ddt, J_{HH} = 17.1, 10.2, 6.8 Hz), 4.97 (2H, m), 2.69 (2H, t, J_{HH} = 6.8 Hz), 2.05 (2H, q, J_{HH} = 7.0 Hz), 1.29 (14H, m).



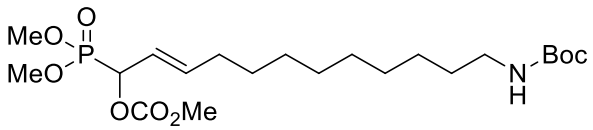
7-Nitro-N-(undec-10-en-1-yl)benzo[c][1,2,5]oxadiazol-4-amine (5.24): Following the procedure of Bhabak, *et al.*,[4.13b] NBDCl (1.08 g, 5.43 mmol) and Hünig's base (4.7 mL, 27 mmol) were dissolved in MeOH, 55 mL, and cooled to 0 °C. A solution of **5.25** in MeOH, 55 mL, was added dropwise, and the mixture was stirred 15 h at rt. The mixture was concentrated *in vacuo*, and purified by silica gel chromatography (10% EtOAc/hexanes), giving **5.24** (68%) as a red solid. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.52 (1H, d, J_{HH} = 8.6 Hz), 6.19 (2H, m), 5.82 (1H, ddt, J_{HH} = 17.0, 10.3, 6.7 Hz), 4.98 (2H, m), 3.49 (2H, q, J_{HH} = 6.7 Hz), 2.05 (2H, q, J_{HH} = 7.0 Hz), 1.82 (2H, p, J_{HH} = 7.3 Hz), 1.32 (12H, m).



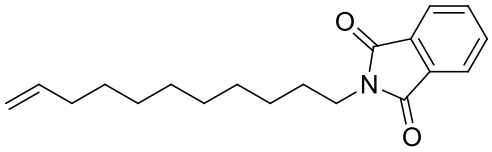
(±)-(*E*)-1-(Dimethoxyphosphoryl)-12-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)dodec-2-en-1-yl methyl carbonate (5.23): **4.24** (111 mg, 0.334 mmol) and **4.10** (75 mg, 0.33) were dissolved in CH_2Cl_2, 1.2 mL, and CuI (3 mg, 0.02 mmol) was added followed by Grubbs 2nd generation catalyst (14 mg, 0.017 mmol). The mixture was stirred in a 40 °C oil bath overnight. The mixture was concentrated *in vacuo* and purified by silica gel chromatography (60–75% EtOAc/hexanes) giving **5.23** (69 mg, 39%) as a red sticky oil. IR (neat, NaCl) 3236, 3072, 2927, 2854, 1753, 1618 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.46 (1H, d, J_{HH} = 8.7 Hz), 6.89 (1H, br s), 6.16 (1H, d, J_{HH} = 8.8 Hz), 5.93 (1H, m), 5.49 (2H, m), 3.80 (9H, m), 3.50 (2H, m), 2.06 (2H, m), 1.80 (2H, p, J_{HH} = 7.3 Hz), 1.25 (12H, m); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 155.0 (d, J_{CP} = 10.0 Hz), 144.4 (d, J_{CP} = 5.5 Hz), 144.2, 139.0 (d, J_{CP} = 12.6 Hz), 136.7, 123.7, 120.5, 98.7, 72.3 (d, J_{CP} = 171.2 Hz), 55.4, 54.0 (d, J_{CP} = 7.1 Hz), 53.8 (d, J_{CP} = 6.6 Hz), 44.3, 32.4, 29.4, 29.2, 28.7, 28.6, 27.0; ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 20.0, 19.7 (*Z*:*E* = 1:14); HRMS (FAB, NBA, MNa^+) calcd for $C_{22}H_{36}N_4O_9PNa$: 551.1883, found 551.1879.



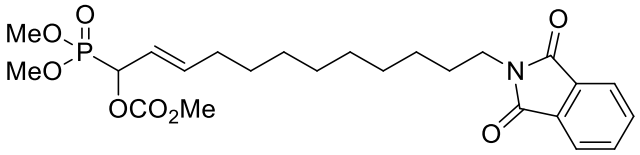
(±)-(E)-tert-Butyl (12-(dimethoxyphosphoryl)-12-((methoxycarbonyl)oxy)dodec-10-en-1-yl)(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)carbamate (5.29): To a stirred solution of NaH (60% w/w, 5 mg, 0.13 mmol) in dry DMF, 0.5 mL, was added a solution of **5.23** (44 mg, 0.083 mmol) in dry DMF, 0.5 mL, followed by Boc_2O (27 mg, 0.12 mmol). The mixture was stirred overnight and partitioned between water and EtOAc. The organic phase was extracted twice more with water, dried over Na_2SO_4, and concentrated *in vacuo*. The crude mixture was purified by silica gel chromatography (70–100% EtOAc/hexanes) giving **5.29** (32 mg, 65%) as a red sticky oil. IR (neat, NaCl) 3096, 2929, 2855, 1752, 1719, 1632 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 8.51 (1H, d, J_{HH} = 8.1 Hz), 7.46 (1H, d, J_{HH} = 8.1 Hz), 5.95 (1H, m), 5.49 (2H, m), 4.01 (2H, 7, J_{HH} = 7.5 Hz), 3.80 (9H, m), 2.07 (2H, m), 1.60 (2H, p, J_{HH} = 7.0 Hz), 1.46 (9H, s), 1.25 (12H, m); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 154.9 (d, J_{CP} = 9.6 Hz), 153.1, 147.8, 143.8, 139.5, 138.9 (d, J_{CP} = 12.1 Hz), 133.4. 131.6, 123.2, 120.5 (d, J_{CP} = 4.0 Hz), 83.2, 73.3 (d, J_{CP} = 170.8 Hz), 55.4, 54.0 (d, J_{CP} = 7.1 Hz), 53.8 (d, J_{CP} = 6.6 Hz), 50.4, 32.5, 29.5, 29.4, 29.1, 28.7 (d, J_{CP} = 2.5 Hz), 28.2, 27.6, 26.8; ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 20.1, 19.8 (*Z*:*E* = 1:14).



(±)-(*E*)-*tert*-Butyl (12-(dimethoxyphosphoryl)-12-((methoxycarbonyl)oxy)dodec-10-en-1-yl)carbamate (5.31): To a solution of **5.25** (200 mg, 1.18 mmol) in dry CH_2Cl_2, 3 mL, was added di-*tert*-butyl dicarbonate (258 mg, 1.18 mmol), and the resulting solution was stirred overnight at room temperature. The mixture was extracted with saturated aqueous $NaHCO_3$, and the aqueous layer was extracted twice with CH_2Cl_2. The organic phases were dried over Na_2SO_4 and concentrated *in vacuo*. The crude material was filtered through silica gel (10% EtOAc/hexanes). To a solution of the resulting crude carbamate and **5.10** in dry CH_2Cl_2, 1 mL, was added CuI (4 mg, 0.02 mmol) and Grubbs 2nd generation catalyst (16 mg, 0.019 mmol). The mixture was stirred in a 40 °C oil bath overnight. The mixture was concentrated *in vacuo* and purified by repeated silica gel chromatography (60–75% EtOAc/hexanes) giving **5.31** (86 mg, 16% over two steps) as a pale yellow oil. IR (neat, NaCl) 3342, 3003, 2930, 2856, 1753, 1699 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.92 (1H, m), 5.47 (2H, m), 3.78 (9H, m), 3.07 (2H, q, J_{HH} = 6.5 Hz), 2.06 (2H, m), 1.41 (13H, m), 1.23 (10H, br s); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 156.2, 155.0 (d, J_{CP} = 9.6 Hz), 139.0 (d, J_{CP} = 12.1 Hz), 120.5 (d, J_{CP} = 3.5 Hz), 79.1, 73.3 (d, J_{CP} = 171.2 Hz), 55.4, 53.9 (d, J_{CP} = 10.5 Hz), 53.8 (d, J_{CP} = 10.0 Hz), 40.9, 32.5, 30.2, 29.6, 29.43, 29.38, 29.2, 28.7, 28.7, 28.6, 26.9; ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 20.2, 19.8 (*Z*:*E* = 1:12.4); HRMS (FAB, NBA, MH$^+$) calcd for $C_{21}H_{41}NO_8P$: 466.2570, found 466.2581.

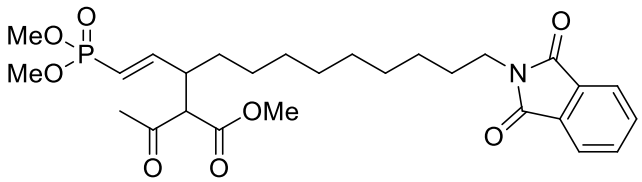


2-(Undec-10-en-1-yl)isoindoline-1,3-dione (5.34): To a solution of **5.25** (333 mg, 1.97 mmol) in toluene, 5 mL, was added phthalic anhydride (291 mg, 1.96 mmol), and the solution was heated at reflux for 16 h. The mixture was concentrated *in vacuo*, and the crude material was purified by silica gel chromatography (5% EtOAc/ hexane), giving **5.34** (464 mg, 79%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.83 (2H, m), 7.70 (2H, m), 5.79 (1H, ddt, J_{HH} = 17.0, 10.2, 6.7 Hz), 4.95 (2H, m), 3.67 (2H, t, J_{HH} = 7.3 Hz), 2.02 (2H, q, J_{HH} = 7.0 Hz), 1.66 (2H, p, J_{HH} = 7.2 Hz), 1.30 (12H, m).



(±)-(*E*)-1-(Dimethoxyphosphoryl)-12-(1,3-dioxoisoindolin-2-yl)dodec-2-en-1-yl methyl carbonate (5.35): To a solution of **5.34** (2.06 g, 6.88 mmol) and **5.10** (2.00 g, 8.92 mmol) in CH_2Cl_2, 17.2 mL, was added CuI (66 mg, 0.35 mmol) and Grubbs 2nd generation catalyst (292 mg, 0.344 mmol). The mixture was heated to reflux for 4 h and concentrated *in vacuo*. The crude material was eluted through silica gel (30–50% EtOAc/hexanes) to remove nonpolar impurities and purified by preparative HPLC to give **5.35** (2.75 g, 81%) as a yellow oil. IR (neat, NaCl) 3006, 2928, 2855, 1755, 1713, 1615 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.76 (2H, m), 7.65 (2H, m), 5.88 (1H, m), 5.44 (2H, m), 3.75 (9H, m), 3.60 (2H, t, t, J_{HH} = 7.3), 2.01 (2H, m), 1.60 (2H, p, J_{HH} = 7.0 Hz), 1.24 (12H, m); ^{13}C NMR (75.4 MHz,

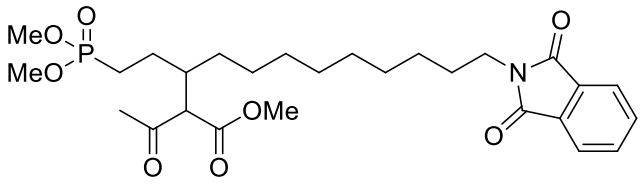
$CDCl_3$) δ 168.4, 155.9 (d, J_{CP} = 9.6 Hz), 138.9 (d, J_{CP} = 12.6 Hz), 133.9, 132.3, 123.15, 120.4 (d, J_{CP} = 3.5 Hz), 73.2 (d, J_{CP} = 171.2 Hz), 55.3, 53.8 (d, J_{CP} = 10.6 Hz), 53.7 (d, J_{CP} = 9.5 Hz), 38.0, 32.4, 29.4, 29.3, 29.2, 29.1, 29.0, 28.6, 26.9; ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 20.1, 19.8 (*Z*:*E* = 1:11); HRMS (FAB, NBA, MH^+) calcd for $C_{24}H_{35}NO_8P$: 496.2100, found 496.2117.



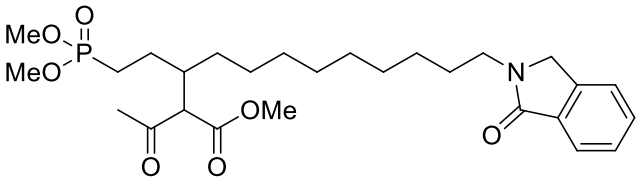
(±)-(*E*)-Methyl 2-acetyl-3-(2-(dimethoxyphosphoryl)vinyl)-12-(1,3-dioxoisoindolin-2-yl)dodecanoate (5.36): Solution of $Pd_2(dba)_3$ (11 mg, 0.012 mmol) and dppe (12 mg, 0.030 mmol) in dry THF, 500 µL, was transferred by cannula to a solution of **5.35** (300 mg, 0.605 mmol) and methyl acetoacetate (200 µL, 1.8 mmol) in dry THF, 750 µL, rinsing with dry THF, 250 µL. The resulting green solution was heated in a 40 °C oil bath for 2 h and concentrated *in vacuo*. The crude mixture was purified by silica gel chromatography (70–85% EtOAc/ hexane), giving **5.36** (229 mg, 71%) in a 1:1 mixture of diastereomers as a pale yellow oil. IR (neat, NaCl) 2930, 2855, 1772, 1713, 1633 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.76 (2H, m), 7.65 (2H, m), ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.79 (2H, m), 7.68 (2H, m), 6.49 (1H, m), 5.64 (1H, m), 3.66 (11H, m), 3.51 (1H, m), 2.93 (1H, m), 2.21 (1.5H, s), 2.15 (1.5H, s) 1.62 (2H, p, J_{HH} = 6.7 Hz), 1.25 (14H, m); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 201.3, 168.6, 153.3, 134.0, 132.3, 123.2, 118.8 (118.6) (d, J_{CP} = 185.7 Hz), 63.8 (63.7), 52.7 (52.5), 52.4 (d, J_{CP} = 5.6 Hz), 44.2 (43.9), 38.1, 31.8 (31.7), 30.2, 30.0, 29.8, 29.5, 29.4,

29.3, 29.2, 28.7, 27.2, 27.0, 26.9; ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 20.0, 19.9; HRMS (FAB, NBA, MH^+) calcd for $C_{27}H_{39}NO_8P$: 536.24133, found 536.2428.

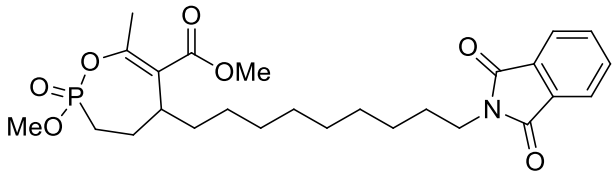
Hydrogenation of 5.36: To a solution of **5.36** (1.65 g, 3.08 mmol) in MeOH, 7.7 mL, was added palladium on carbon (10%, 328 mg), and the mixture was stirred under hydrogen atmosphere, filtered, and concentrated *in vacuo* to give **5.37** and **5.38** (1.65 g, 100%) in a 7.8:1 mixture as a colorless oil. The components were separated by preparative HPLC for characterization.



(±)-Methyl 2-acetyl-3-(2-(dimethoxyphosphoryl)ethyl)-12-(1,3-dioxoisoindolin-2-yl)dodecanoate (5.37): 1:1 mixture of diastereomers. IR (neat, NaCl) 2929, 2855, 1772, 1713 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.79 (2H, m), 7.67 (2H, m), 3.68 (9H, m), 3.62 (2H, t, J_{HH} = 7.3 Hz), 3.42 (1H, d, J_{HH} = 8.3 Hz), 2.18 (4H, m), 1.62 (6H, m), 1.23 (14H, m); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 202.90 (202.87), 169.62 (169.60), 168.5, 134.0, 132.2, 123.2, 63.0, 52.5 (d, J_{CP} = 5.7 Hz), 38.1, 37.9, 30.5 (30.4), 29.8 (29.7), 29.6, 29.5, 29.2, 28.7, 26.9 (26.3), 23.5 (23.4) (d, J_{CP} = 5.9 Hz), 21.7 (21.5) (d, J_{CP} = 141.2 Hz); ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 34.4, 34.2; HRMS (FAB, NBA, MNa^+) calcd for $C_{27}H_{40}NO_8PNa$: 560.2389, found 560.2394.



(±)-Methyl 2-acetyl-3-(2-(dimethoxyphosphoryl)ethyl)-12-(1-oxoisoindolin-2-yl)dodecanoate (5.38): 1:1 mixture of diastereomers. IR (neat, NaCl) 2928, 2854, 1712, 1678 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.85 (1H, m), 7.49 (3H, m), 4.38 (2H, s), 3.73 (9H, m), 3.61 (2H, t, J_{HH} = 7.3 Hz), 3.46 (1H, d, 8.2 Hz), 2.23 (4H, m), 1.62 (6H, m), 1.29 (14H, m); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 203.03 (203.01), 169.74 (169.71), 168.6, 141.3, 133.3, 131.3, 128.2, 123.8, 122.8, 63.14 (63.11), 52.62, 52.58, 52.5, 50.1, 42.6, 38.2 (38.0), 30.6 (30.5), 29.9 (29.8), 29.7, 29.6, 29.5, 28.7, 27.0, 26.5, 23.62 (23.55), 21.8 (21.7) (d, J_{CP} = 141.4 Hz), 9.8; ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 34.4, 34.2; HRMS (FAB, NBA, MH^+) calcd for $C_{27}H_{43}NO_7P$: 524.2777, found 524.2786.



(±)-Methyl 5-(9-(1,3-dioxoisoindolin-2-yl)nonyl)-2-methoxy-7-methyl-2,3,4,5-tetrahydro-1,2-oxaphosphepine-6-carboxylate 2-oxide (5.40a & 5.40b): To a solution of **5.37** and **5.38** (7.8:1, 528 mg, 0.98 mmol) in dry CH_3CN, 500 µL, was added NaI (162 mg, 1.08 mmol). The solution was heated to reflux and stirred until completion of reaction (^{31}P NMR). The mixture was concentrated *in vacuo*. The resulting solid was dissolved in dry DMF, 4 mL, and Hünig's base (260 µL, 1.5 mmol) was added followed by HBTU (559 mg,

1.47 mmol). After 2 h, the red solution was partitioned between 1 N HCl and CH_2Cl_2, extracted twice more with CH_2Cl_2. The organic layers were extracted with saturated aqueous $NaHCO_3$ followed by brine and concentrated *in vacuo*. The crude material was purified by preparative HPLC, giving **5.40** (310 mg, 62%) in a 1:1.8 mixture of diastereomers as a yellow oil. Further chromatographic separation of the diastereomers gave **5.40a** as a yellow oil. IR (neat, NaCl) 2928, 2855, 1773, 1718 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.77 (2H, m), 7.66 (2H, m), 3.77 (3H, d, J_{HP} = 11.2 Hz), 3.69 (3H, s), 3.61 (2H, t, J_{HH} = 7.3 Hz), 2.92 (1H, m), 2.17 (3H, d, J_{HP} = 1.6 Hz), 2.15–1.78 (4H, m), 1.58 (3H, m), 1.40 (1H, m), 1.57 (12H, m); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 169.0 (d, J_{CP} = 1.7 Hz), 168.5, 155.7 (d, J_{CP} = 7.3 Hz), 133.9, 132.2 123.2, 123.1, 52.1 (d, J_{CP} = 7.1 Hz), 38.1, 37.2 (d, J_{CP} = 1.5 Hz), 30.7, 29.6, 29.4, 29.2, 28.6, 27.7, 26.9, 24.9 (d, J_{CP} = 6.9 Hz), 21.9 (d, J_{CP} = 135.1 Hz), 21.4 (d, J_{CP} = 1.2 Hz); ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 26.1; HRMS (FAB, NBA, MNa^+) calcd for $C_{26}H_{36}NO_7P$: 528.2128, found 528.2139.

Also isolated was **5.40b** as a yellow oil. IR (neat, NaCl) 2928, 2855, 1771, 1712 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.73 (1H, m), 7.62 (3H, m), 3.73 (3H, d, J_{HP} = 11.0 Hz), 3.65 (3H, s), 3.56 (2H, t, J_{HH} = 7.3 Hz), 2.78 (1H, m), 2.13–1.76 (4H, m), 2.08 (3H, d, J_{HP} = 0.8 Hz), 1.56 (3H, m), 1.40 (1H, m), 1.17 (12H, m); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 169.0 (d, J_{CP} = 1.6 Hz), 168.3, 154.8 (d, J_{CP} = 9.4 Hz), 133.8, 132.1, 123.1, 123.0, 52.5 (d, J_{CP} = 6.8 Hz), 51.9, 37.9, 37.0, 30.8, 29.4, 29.3, 29.1, 28.5, 27.5, 26.8, 25.1 (d, J_{CP} = 7.9 Hz), 25.1 (d, J_{CP} = 134.0 Hz), 21.0 (d, J_{CP} = 2.0 Hz); ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 23.4; HRMS (FAB, NBA, MNa^+) calcd for $C_{26}H_{36}NO_7P$: 528.2128, found 528.2126.

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