University of Alberta

Design and Synthesis of α-Bromo Phosphonates as Analogues of Glucose-6-Phosphate

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

Department of Chemistry

Fall 2012
Edmonton, Alberta

Dedicated to Quarters at Dukes with Mitch Huot.

ABSTRACT

Protein phosphorylation is a crucial component in physiological signal transduction pathways. It is estimated that one-third of all cellular proteins are modified through phosphorylation, and these pathways are regulated by kinase and phosphatase enzymes. Glucose-6-phosphatase (G6Pase) is an essential enzyme that catalyzes the last step in both glycogenolysis and gluconeogenesis by converting glucose-6-phosphate (G6P) into glucose. As a result, aberrant G6Pase signaling has been implicated in diabetes. The active site of G6Pase contains a nucleophilic histidine residue, and two arginine residues that stabilize binding through hydrogen bonding to the phosphate moiety. In this thesis we present novel synthetic methodology to install α-bromophosphonate moieties on G6P analogues to test as irreversible inhibitors of G6Pase, which could serve as a valuable tool in the study of glucose metabolism. We describe our efforts towards the synthesis of a panel of phosphonate-based G6P analogues which were tested for in vitro activity against the enzyme.

Acknowledgements

I have way too many people to thank for all the support and guidance I have received over the last 3.5 years in the Cairo lab. Firstly, I would like to thank my supervisor, Professor Chris Cairo for his leadership and assistance with my projects. I am also indebted to Naresh Tulsi for getting me started and helping me out in the lab as a fledgling undergraduate researcher. I also thank all of the other members of Cairo lab past and present for their advice, and, more importantly, the good times we have shared together. I am also very grateful for the care and provision of my parents and David and Jeffrey over the course of my degree.

Outside of the lab I thank my great friends that have both inspired me and supported me throughout my degree. They include Jim-ski and Drew-ski, C-Bones and G-Bones, Nilsy, G-Unit, K-Tom and D-Reaugh, Bearjeh, J-Thuss, JC, JT, KK, KP and the Jan. I must also thank Shaun McKinnie, Thomas Janiszewski, Jeannie Dorran, Deana Trites, Chris Sadek, James Gibeault, Gord Brasnett, Katie Gamble, Dr. Ben and Lindsay Adam, Dr. Simmon Hofstetter, Dr. Jen Zwicker, Dr. Sarah Wozney and Lu Deng who are far more mature than I am so I cite their names in full out of respect. I also thank Professor Todd Lowary for his mentorship and kind letters of reference as well as Dr. Mickey Richards for helping me run and analyze my “salty” compounds. Anushka’s place will always be the largest rock in my foundation.

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LIST OF ABBREVIATIONS

α–BBP	α-Bromobenzylphosphonate
ABP	Activity-based probe
ABPP	Activity-based protein probes
AIBN	Azobisisobutyronitrile
Al_2O_3	Aluminum dioxide
AlF_4^-	Aluminum tetrafluoride ion
$BF_3{\cdot}Et_2O$	Boron trifluoride etherate
BrPmp	L-Bromophosphonomethylphenylalanine
CDI	*N*,*N*-Carbonyldiimidazole
CrO_3	Chromium trioxide
DAST	(Diethylamino)sulfur trifluoride
DCC	*N*,*N*′-Dicyclohexylcarbodiimide
DDQ	2,3-Dichloro-5,6-dicyano-1,4-benzoquinone
DECP	Diethylpyrocarbonate
DHAP	Dihydroxyacetone phosphate
DIBAL-H	Diisobutylaluminum hydride
DiF4-MU	4-Methylumbelliferone (DiF4-MU)
DiFMUP	6,8-Difluoro-4-methylumbelliferyl phosphate
DMMP	Dimethylmethyl phosphonate

DMP	Dess-Martin periodinane
DOIS	2-Deoxy-*scyllo*-inososе synthase
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
F_2Pmp	Phosphonodifluoromethylphenylalanine
Fmoc	Fluorenylmethoxycarbonyl
G3P	Glycerol-3-phosphate
G6P	Glucose-6-phosphate
G6Pase	Glucose-6-phosphatase
G6PDH	6-Phosphonoglucono-δ-lactone by glucose 6-phosphate dehydrogenase
GPCR	G-protein coupled receptor
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)
HWE	Horner-Wadsworth-Emmons
IBX	2-Iodoxybenzoic acid
$KMnO_4$	Potassium permanganate
LDA	Lithium diisopropylamide
LiHMDS	Lithium hexamethyldisialazide
LPA	Lysophosphatidic acid
M6P	Mannose 6-phosphate (M6P)
MOPS	3-(*N*-Morpholino)propanesulfonic acid

MS	Mass spectrometry
$NaBH_4$	Sodium borohydride
NADPH	Nicotinamide adenine dinucleotide phosphate co-enzyme
NaH	Sodium hydride
NaHMDS	Sodium hexamethyldisialazide
NBS	*N*-Bromosuccinimide
n-BuLi	*n*-Butyl lithium
NEt_3	Triethylamine
NFSI	*N*-Fluorobenzenesulfonimide
NMR	Nuclear magnetic resonance
PEP	Phosphoenolpyruvate
PMB	*p*-Methoxybenyl
Pmp	Phosphonomethylphenylalanine
PPh_3	Triphenylphosphine
PPh_3Br_2	Dibromotriphenylphosphorane
PTP	Protein tyrosine phosphatase
pTyr	Phosphotyrosine
$SOBr_2$	Thionyl bromide
SPPS	Solid phase peptide synthesis (SPPS)
TBDMSCl	*tert*-Butyldimethylsilyl chloride
TEMDP	Tetraethylmethylene diphosphonate

TEMPO	2,2,6,6-Tetramethylpiperidin-1-yl)oxyl
TMSBr	Trimethylsilyl bromide
TMSCl	Trimethylsilyl chloride
TMSOTf	Trimethylsilyl trifluoromethansulfonate
TSR	Taussky-Shorr reagent

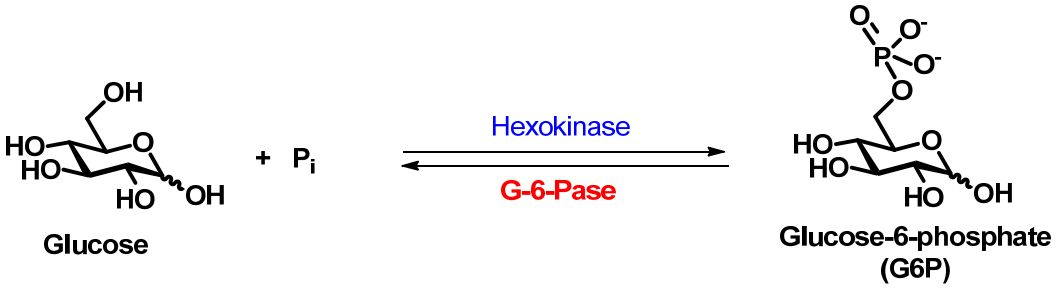
Chapter 1: Glucose-6-phosphatase and synthetic analogues of the phosphate group

1.1 Introduction to phosphorylation and glucose-6-phosphatase

Protein phosphorylation is a pivotal modulator of cellular behaviour. It is estimated that one-third of all cellular proteins are modified through phosphorylation making it the second most prevalent post-translational modification behind glycosylation.[1] Kinases phosphorylate target molecules and act in contrast to phosphatases, which dephosphorylate these same targets.[2] Protein phosphorylation is implicated in many important physiological processes including cellular differentiation,[2] metabolism[2] and apoptosis.[3] Aberrant phosphorylation pathways can have devastating physiological consequences and play a role in pathologies including cancer,[3] diabetes[4] and Alzheimer's disease.[5] The majority of human phosphatases hydrolyze phospho-serine, threonine, tyrosine, or histidine residues in proteins and are termed protein phosphatases.[2, 6] However, phosphatases that dephosphorylate lipid- and carbohydrate-based substrates also play fundamental biochemical roles.[7] Glucose-6-phosphatase (G6Pase) is one example of a phosphatase with a carbohydrate substrate, which plays a central role in the regulation of glucose metabolism.

G6Pase is primarily localized to the liver, where it catalyzes the last step in both glycogenolysis and gluconeogenesis by converting glucose-6-phosphate (G6P) to glucose; thus allowing for its release into the bloodstream (**Scheme 1.1**).[1] Glycogenolysis is the breakdown of glycogen into glucose monomers and

gluconeogenesis is the generation of glucose from non-carbohydrate sources such as glycerol and lactic acid.[1] In Type II diabetic patients these processes are augmented due to the upregulated activity of G6Pase resulting in glucose release from hepatic cells when blood-glucose levels are already elevated.[4, 8] Inhibition of G6Pase is therefore a potential therapeutic target. Reduction of G6Pase activity could slow the release of glucose back into the bloodstream, facilitating glycogen storage.[4]



Scheme 1.1: The last step in both glycogenolysis and gluconeogenesis catalyzed by G6Pase.[1]

G6Pase exists as two isoforms in humans: G6Pase-α and G6Pase-β.[9] G6Pase-α consists of 357 amino acids with nine transmembrane domains, which are anchored within the membrane of the endoplasmic reticulum (ER).[9] It is uncertain how G6Pase-α is localized to the ER or what its orientation within the membrane is.[9] G6Pase-α is expressed primarily in the kidney, liver, and intestines. G6Pase-β contains 346 amino acids and shares only 36% homology to G6Pase-α. In contrast

to G6Pase-α, G6Pase-β is expressed ubiquitiously; however, both isoforms are coupled to the G6P transporter.[9] At present it is believed that only G6Pase-α is implicated in Type II diabetes.[9] The remainder of this chapter will discuss human G6Pase-α, which we will refer to hereafter as G6Pase.

Due to its tight association with the ER membrane, the structure of G6Pase remains to be fully elucidated;[10] however, insight into the structure of the active site of G6Pase has been obtained using biochemical methods.[11] Seminal work done by Chou and co-workers[11] has demonstrated that the G6Pase active site, which is oriented towards the lumen of the ER,[12] includes five key amino acid residues, Lys76, Arg83, Arg170, His119 and His176. The proposed mechanism of enzymatic cleavage of G6P is shown in **Figure 1.1**. The Arg83 and Arg170 residues are proposed to stabilize the anionic oxygens of the G6P phosphate moiety preparing His176 for nucleophilic attack on the phosphorus to provide a pentahedral intermediate. Subsequently, collapse of the pentahedral intermediate after protonation by His119 liberates glucose as the product.[11] Although not discussed by Chou and co-workers directly in their elucidation of this mechanism, we postulate the active site is likely regenerated by nucleophilic attack on phospho-His176 by water to liberate P_i as shown in the final step of **Figure 1.1**. This is consistent with evidence from other phosphorylated active site intermediates.[13] G6Pase has broad tissue distribution and it is assumed that this

process occurs in any cell containing G6Pase. However, the physiological significance of this finding is unclear outside of hepatocytes.[14] As a result, side effects of non-selective G6Pase inhibition are of therapeutic concern.[14] Nonetheless, it is thought that selective inhibitors could be important treatments for Type II diabetes.[4] Furthermore, we propose that because the crystal structure of G6Pase has not been elucidated, inhibitors of the enzyme could be beneficial in obtaining the structure of the active site generating a crystal structure, or in the development of cell labelling and activity-based probe (ABP) strategies.

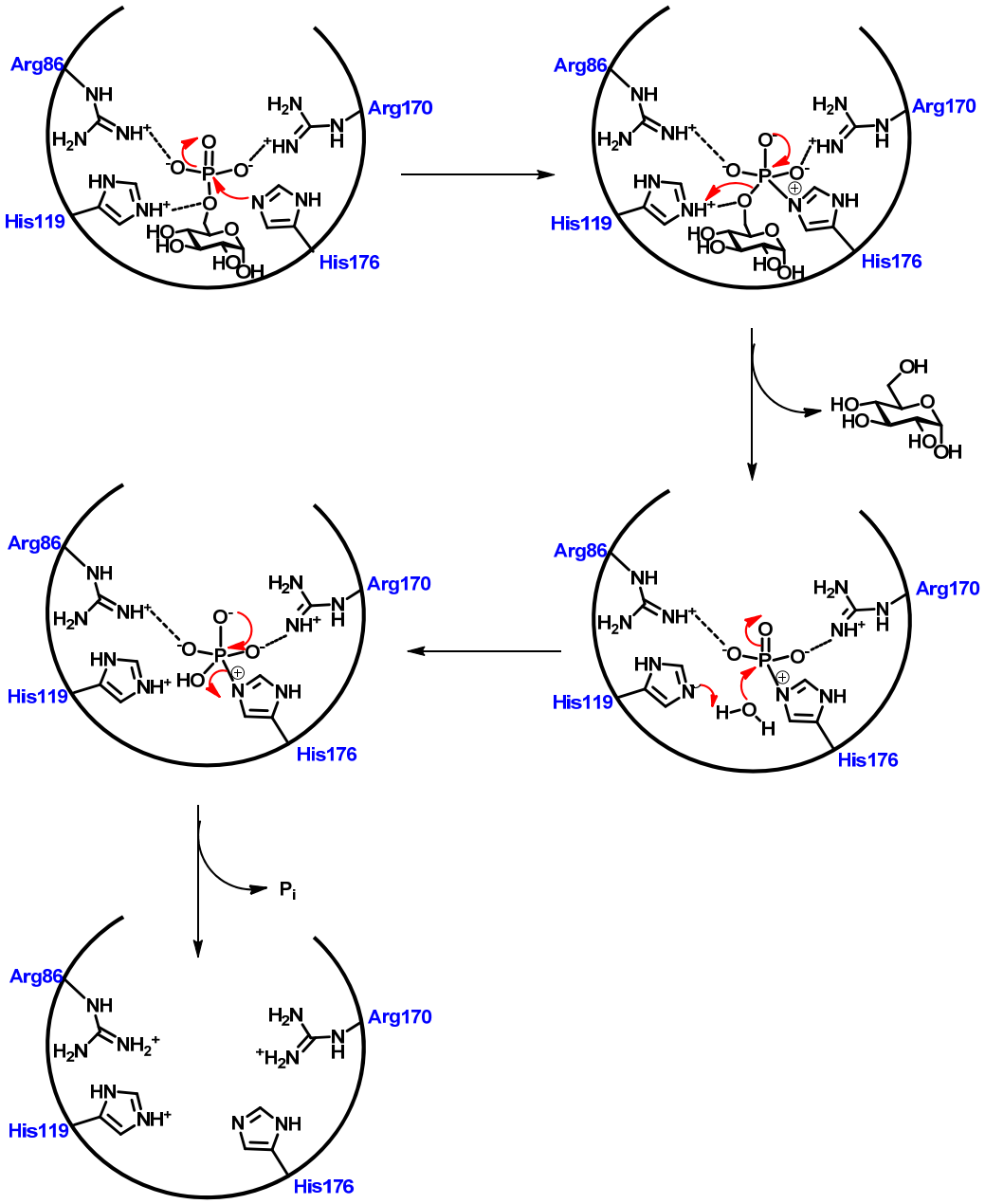


Figure 1.1: The proposed catalytic mechanism of G6Pase (modified from Ghosh, et al., 2002).[11]

It is also important to note that because the active site of G6Pase is located on the luminal side of the ER, a transporter, termed the T_1 translocase, is required to transport G6P from the cytoplasm into the luminal compartment where it can access the active site of G6Pase.[12]

1.2 Inhibitors of G6Pase

Since selective inhibitors of G6Pase have been considered for the therapy of Type II diabetes, it is not surprising that direct G6Pase inhibitors or modulators of G6Pase expression are known. To date all known compounds have drawbacks that limit their potential as therapeutics. We provide a brief survey of the known inhibitors of G6Pase starting with non-selective, metal-based inhibitors followed by carbohydrate-based and irreversible inhibitors of the enzyme. This discussion focuses only on inhibitors that target G6Pase directly. Alternative strategies have been explored, which target the T_1 translocase protein as a means for affecting glucose metabolism; however, these inhibitors are not discussed here since it is only G6Pase that is upregulated in Type II diabetes.[4, 12] In vitro assay of G6Pase activity is done directly by disrupting the microsomal membrane with a surfactant, allowing free access of G6P to the G6Pase active site thus avoiding the necessity of the translocase.[12]

1.2.1 Metal-based inhibitors

Metal-based G6Pase inhibitors containing tungsten,[15-16] vanadium[17-19] and molybdenum[12] have been shown to inhibit G6Pase contained in rat microsomes with K_i values in the 10-20 μM range. It has also been shown that increasing the oxidation state of the vanadium species can have a ten-fold increase in inhibitory potency (**Figure 1.2**).[12] Unfortunately, it is unknown what effect the counter ion has on inhibitory potency of G6Pase or why analogue **1.1** is more than twice as active as **1.2**. Also, the analogous tungstate and molybdate high oxidation complexes were tested but were found not to be of lower potency.[12]

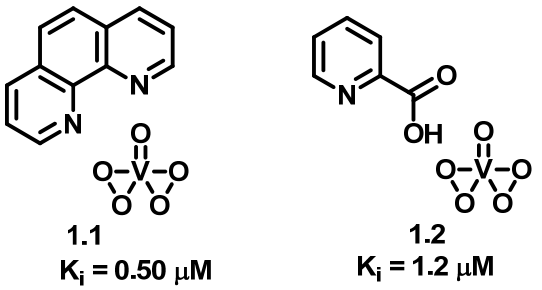


Figure 1.2: High oxidation state-vanadium complexes.

Basic tungstates and vanadate **1.1** and have been shown to lower blood glucose in mice.[12] However, it has not yet been confirmed if the blood glucose levels are lowered due to direct inhibition of G6Pase or as a result of an indirect effect due to non-specific binding to random protein tyrosine phosphatases (PTPs) within

hepatic cells.[19] Aluminum tetrafluoride ion (AlF_4^-) has also demonstrated inhibition of G6Pase.[20] Although these inhibitors do show some promise, a lack of specificity for their targets resulting in toxicity concerns have always limited the utility of these metal-based inhibitors.

1.2.2 Organic competitive inhibitors

Shown in **Figure 1.3** are the known organic competitive inhibitors of G6Pase. The species of G6Pase is mentioned in the discussion of each inhibitor.

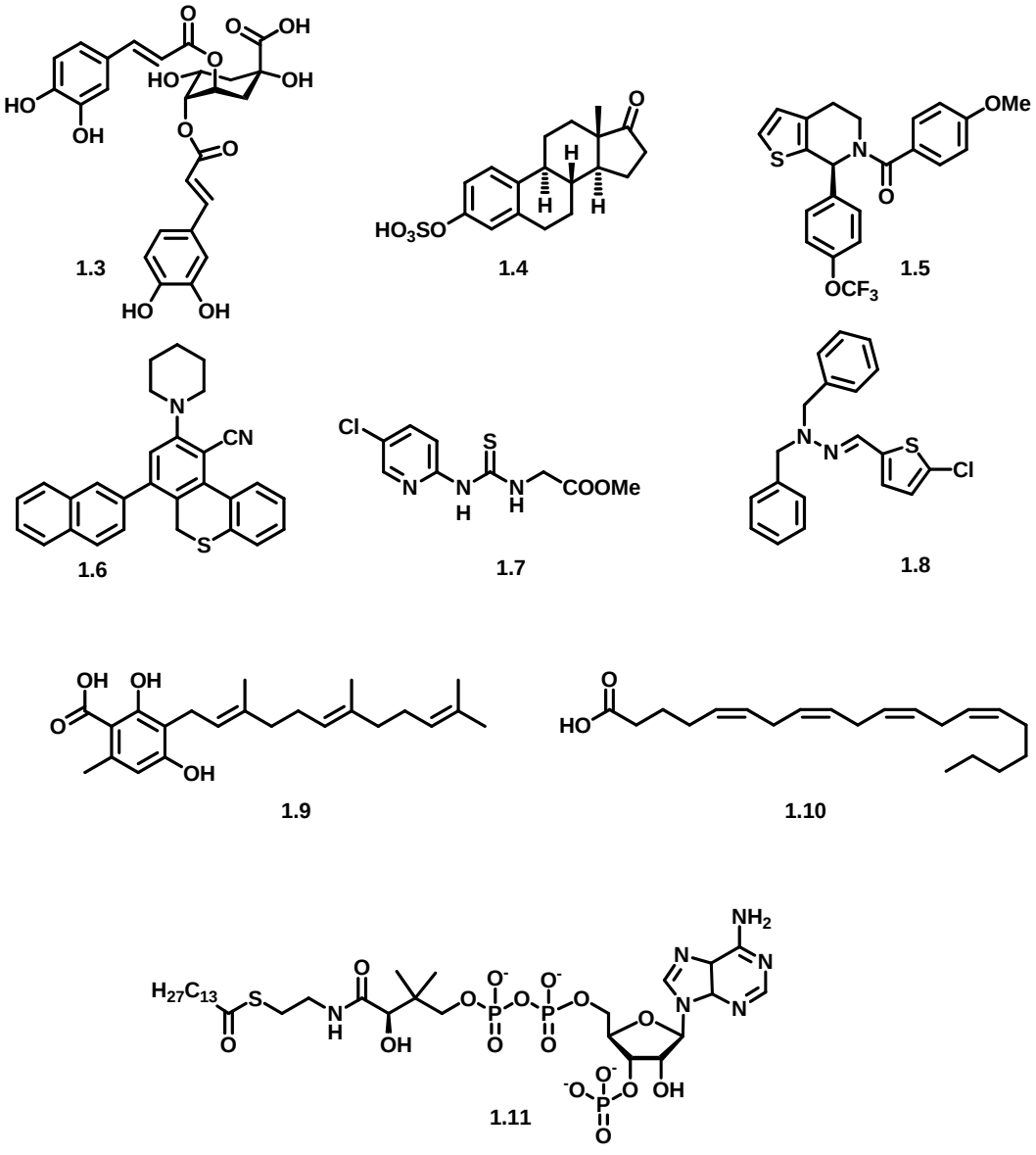


Figure 1.3: Organic competitive inhibitors of G6Pase or G6Pase expression modulators.

Daily coffee use has been linked to the prevention of type II diabetes.[21] Extensive research into the reasons for this phenomenon has revealed that chlorogenic acids

found in coffee may be responsible.[22] It has been postulated that these chlorogenic acids, including **1.3**, could treat type II diabetes by inhibition of G6Pase.[23] Svetol is a decaffeinated green coffee extract that contains high levels of these chlorogenic acids. Henry-Vitrac et al.[23] isolated a number of chlorogenic acids from Svetol and were able to show that these chlorogenic acids did, in fact, inhibit human G6Pase and were responsible for 96% of Svetol's inhibitory potency against the enzyme.[23] Derivative **1.3** was shown to be the most active of the chlorogenic acids.

The estrone sulfate compound **1.4** reduced G6Pase expression in diabetic mice and successfully lowered blood glucose levels to match those of healthy mice,[24] however, the estrogenic effects caused by **1.4** made it an ineffective therapeutic strategy for diabetes.[14]

Compounds **1.5**,[25-26] **1.6**,[27] **1.7**,[28] and **1.8**[29] were identified by high throughput screening. In all four instances several analogues were synthesized. Shown in **Figure 1.3** are the best inhibitors found in the individual screens. Compound **1.5** is a member of a class of 4,5,6,7-tetrahydrothienopyridines and displayed an IC_{50} of 360 nM in rat microsomes, placing it among the best G6Pase inhibitors. Furthermore, it was determined experimentally that the *S* configuration of compound **1.5** was crucial to activity.[12] Based on promising in vitro results, compound **1.5** was tested for activity in cultured rat heptocytes and was

demonstrated to lower basal glucose levels in these cells with concomitant increase in G6P levels indicating a blockage of G6Pase activity.[26] The thienopyridine class may show promise in vivo. It is uncertain whether they could be a viable diabetic agent due to poor water solubility.

Compounds **1.6** and **1.7** both inhibited rat G6Pase activity by 75% at a concentration of 100 μM, however, no further biological screening was reported.[27-28] The final compound derived from screening was compound **1.8**, a member of a novel *N*,*N*-dibenzyl-*N*-benzylidenehydrazine class of compounds. It is a competitive inhibitor with an IC_{50} of 170 nM. To date, this is the most potent inhibitor of rat G6Pase known, and it also demonstrated selectivity for G6Pase over alkaline phosphatase and fructose-1,6-bisphosphatase. No cellular tests have been reported.

The final three compounds in **Figure 1.3** are fatty acid derivatives **1.9**, **1.10** and **1.11**. Ilicicolinic acid derivative **1.9**, has been shown to inhibit rat G6Pase with IC_{50} values ranging from 40 to 50 μM.[12] Arachadonic acid (**1.10**) inhibited rat G6Pase in the lower μM range (IC_{50} = 25.0 – 28.0 μM).[30] Myristoyl CoA was the best of the fatty acids tested, providing an IC_{50} of 1.2 μM. This activity is comparable to many of the synthetic derivatives in this discussion.[31] Unfortunately, no hepatic cell studies were conducted using any of these fatty acid derivatives.

1.2.3 Carbohydrate-based inhibitors

Since the substrate of G6Pase is a glucose-based residue, several carbohydrate-based inhibitors have been investigated. **Figure 1.4** summarizes the structures of known carbohydrate-based inhibitors of G6Pase.

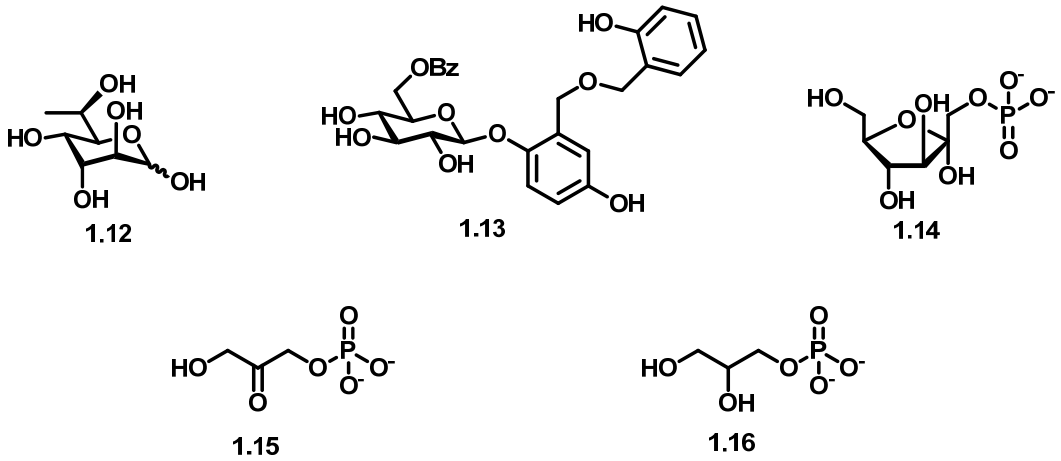


Figure 1.4: Known carbohydrate-based inhibitors of G6Pase.

D-Homoaltrose (compound **1.12**) is a weak competitive inhibitor with an IC_{50} exceeding 6 mM.[32] The Maurya group studied phenolic glycoside **1.13**, which was isolated from *Dodecadenia grandiflora*, a flowering plant found in India, Nepal, Myanmar, and Bhutan. Interestingly, *D. grandiflora* is used in traditional medicine to regulate blood glucose levels.[33] A number of phenolic glycosides were isolated, but catecholic derivative **1.13** was found to be the most active against G6Pase (IC_{50} = 50 μM). The authors determined that the catecholic

aglycone was required for inhibition.[33] It can be postulated that these catecholic glycosides are some of the active compounds in *D. grandiflora* responsible for the blood glucose lowering effect of the plant; to our knowledge this proposal has not been tested directly.

Fructose-1-phosphate (**1.14**) was discovered as an inhibitor G6Pase by Robbins et al.[34] as part of a screen of metabolites present in liver. The only active compound identified from the screen was fructose-1-phosphate with a K_i of 3.8 mM via a competitive mode of action. Dihydroxyacetone phosphate (DHAP; **1.15**) and glycerol-3-phosphate (G3P; **1.16**) were examined as inhibitors of G6Pase in rat microsomes. DHAP was found to be a weak inhibitor of G6Pase with an IC_{50} value of 9 mM. G3P had no effect on G6Pase activity in microsomes. Intriguingly, in vivo assays using both well- and malnourished rats found that a G3P concentration of 2 g/kg significantly reduced blood glucose levels when followed by a 1 g/kg alanine injection. DHAP was not assayed in vivo.

1.2.4 Irreversible inhibitors

To our knowledge only one irreversible inhibitor of G6Pase has been reported, diethylpyrocarbonate (DECP; **1.17**, **Figure 1.5a**).

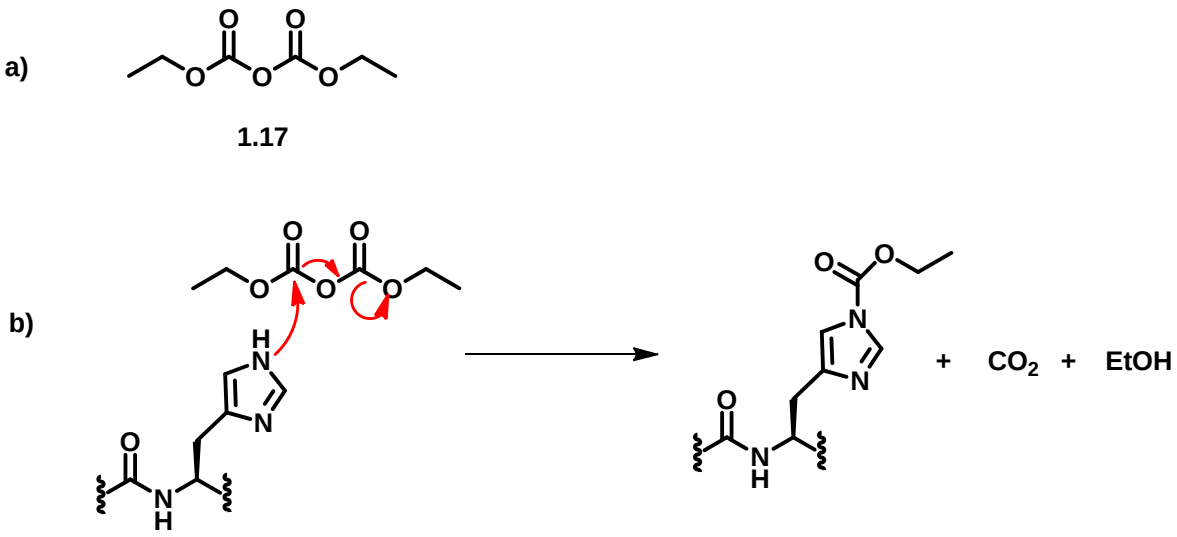


Figure 1.5: a) The structure of DECP. b) The proposed mechanism of acylation by active site His176 to form a mixed carbamate resulting in covalent inhibition.[35]

Using a histochemical study of human hepatocytes, Benkoël and co-workers determined that DECP irreversibly knocked out all G6Pase activity at a concentration of 12.6 mM after a ten minute incubation time.[35] We assume that DECP could inhibit a variety of enzymes that exploit histidine residues as an active site nucleophile, including phosphatases such as G6Pase. However, it seems unlikely that such a potent acylating agent would provide enough specificity for use as a therapeutic.

1.2.5 Summary

We have presented a number of G6Pase inhibitors in the sections above. To our knowledge none of these compounds are in clinical use, however, in vivo examination of the 4,5,6,7-tetrahydrothienopyridines (**1.5**) did show promise by lowering blood glucose levels in rats.[25-26] An obvious shortcoming of this class of molecules is their limited solubility in water. A barrier to the use of chlorogenic acid analogues (**1.3**) is the heterogenous composition of Svetol. Svetol is a collection of more than 10 chlorogenic analogues, each displaying low individual activity.[23] Interestingly, as discussed in **Section 1.2.3**, very few carbohydrate derivatives have been explored as inhibitors of G6Pase. Of these, few are reported with potent activity. This observation may be due to the weak resemblance of compounds to the enzyme substrate, G6P. To our knowledge, no in vivo analysis of the catecholic glycosides (**1.13**) has been conducted. Finally, only one irreversible inhibitor of G6Pase protein (**1.17**) has been identified and it is unlikely to be selective.

It is clear from the discussion thus far that an appreciable number of compounds have been explored as inhibitors of G6Pase. Furthermore, no crystal structure of G6Pase has been elucidated yet, nor were any of these compounds examined for potential in cell labelling strategies. For these reasons, there is a need for improved inhibitors. We hypothesize that through the rational design of new

G6Pase inhibitors, a selective irreversible inhibitor can be developed which targets G6Pase. An irreversible inhibitor that closely resembles the G6P substrate could be of potential use for crystalization studies of G6Pase, or could be used to deliver a specific label to the enzyme in live hepatocytes. Finally, potent inhibitors would expand the limited examples of analogues that target this essential metabolic enzyme. We postulate that by designing a G6P mimetic with an electrophilic site installed in close proximity to the phosphate moiety, we could trap the phosphohistidine intermediate covalently, in an analogous fashion to the known DECP inhibitor. This strategy would presumably be more selective if the inhibitor design includes specific recognition elements of the substrate. We now address general strategies that could be used in the rational design of specific inhibitors for G6Pase.

1.3 Non-hydrolyzable phosphonate analogues of G6P

One general strategy for producing competitive inhibitors of phosphatases is to create substrate mimics that replace the hydrolyzable C-O-P bond of the phosphate moiety with a non-hydrolyzable C-C-P bond to yield a phosphonate moiety (**Figure 1.6a**).[36]

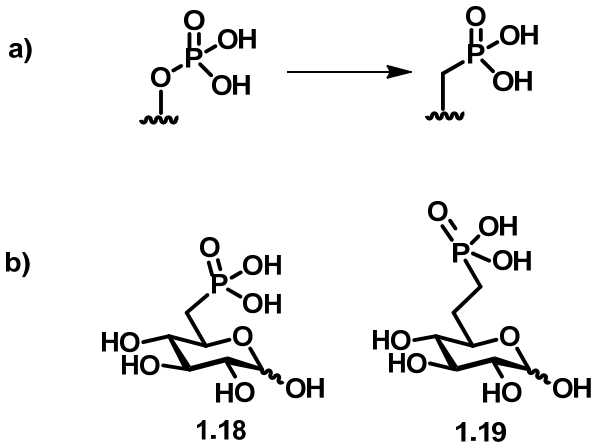


Figure 1.6: a) Conceptual depiction of a phosphonate moiety. b) Previously synthesized G6P-based phosphonate analogues.

This strategy has been employed extensively over the last 60 years in phosphatase inhibitor design; however, while phosphonates are hydrolytically stable they are unlikely to act as irreversible inhibitors without further modification. Two phosphonate-based G6P mimetics have been synthesized previously, **1.18**[37-42] and **1.19**,[37-38, 42-49] however, neither has been tested for inhibition of G6Pase (**Figure 1.6b**).

1.3.1 α-Fluorophosphonates

A more recent strategy builds on the use of non-hydrolyzable phosphonates by installing fluorine atoms on the phosphonate carbon α to the phosphorus. Fluorine is proposed to modify the phosphonate make this group a better isosteric

replacement of the phosphate oxygen in these analogues (**Figure 1.7a**).[50-51]

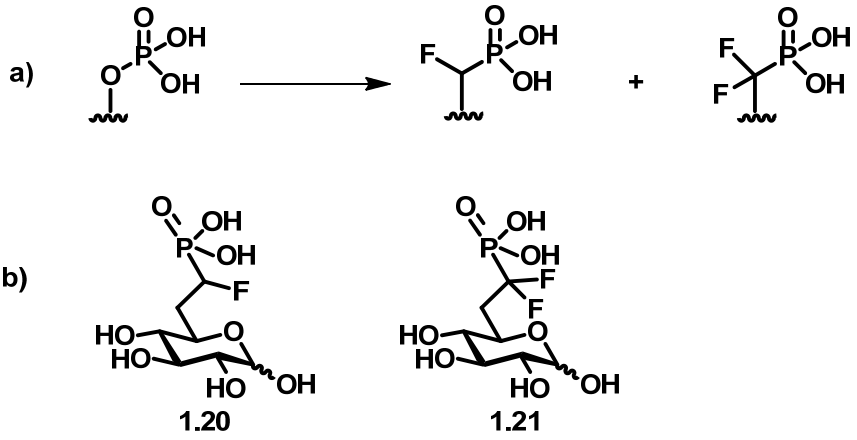


Figure 1.7: a) Conceptual depiction of α-fluorophosphonate moieties. b) Previously synthesized G6P-based α-fluorophosphonate analogues.

This strategy was first utilized by Blackburn in 1981[51] and has expanded in popularity in the last 30 years.[52] Two G6P analogues incorporating this functionality have been synthesized, compounds **1.20**[45, 53] and **1.21** (**Figure 1.7b**).[46, 54-56] Interestingly, as in the case of the saturated phosphonates, neither analogue has been assayed for G6Pase inhibition. Because fluoride is a poor leaving group these compounds are most likely to act as competitive inhibitors of G6Pase.

1.4 Project hypothesis

With the knowledge that both the fully saturated phosphonate and the α-fluorophosphonate G6P analogues have been synthesized previously, we considered whether a phosphonate analogue could be designed to include an electrophilic functionality at the α-position such that the compound would act as an irreversible inhibitor of G6Pase. Such a strategy would require methodology for the synthesis of phosphonate analogues compatible with the carbohydrate scaffold, and with any required protecting group manipulations. If successful, this approach would generate the first rationally-designed irreversible inhibitor known for G6Pase, and could provide a valuable research tool for the development of human therapeutics.

Previously, our group devised a strategy to irreversibly inhibit the immune protein tyrosine phosphatase CD45 by synthetically installing a bromine atom at the α position of phosphonotyrosine.[57] In 13 steps and 18% overall yield we arrived at L-bromophosphonomethylphenylalanine (**BrPmp**).[57] Installation of bromine at the α-position of benzylic phosphonates is a known function group termed bromobenzylphosphonate (BBPs). This functional group was first identified by Widlanski and coworkers in 1996.[58]

CD45 contains a nucleophilic cysteine in the active site. Previous work by the

Zhang group has demonstrated that an α-bromophosphonotyrosine[59] probe could irreversibly inhibit PTPs non-specifically. Zhang postulated that one of two mechanisms could be responsible for the irreversible inhibition of PTPs by BBP (**Figure 1.8**).[59] In the first mechanism the thiol from the cysteine in the active site does a direct S_N2 displacement of the α-bromide to create a covalent C-P bond that inactivates the enzyme. In the second proposed mechanism, the nucleophilic cysteine attacks the phosphorus to provide a pentahedral intermediate as in G6Pase. This intermediate then collapses and concomitantly displaces the α-bromide to produce an epoxide-based pentahedral structure. Another pentahedral collapse affords the covalent phosphorus-cysteine bond, and the inactivated enzyme.

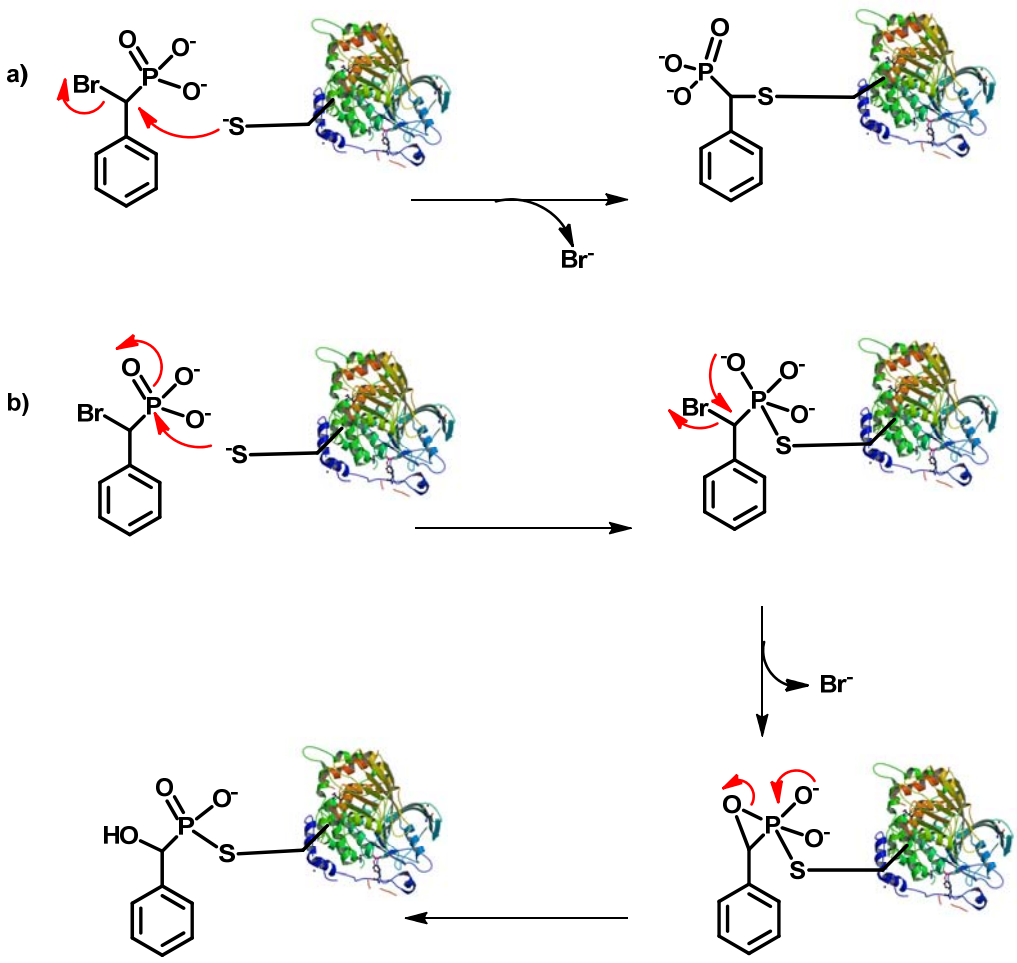


Figure 1.8: Two proposed mechanisms for covalent inhibition of a PTP by BBP.

We hypothesized that an α-BBP, or analogous electrophilic phosphonate moiety, could be used to construct G6P derivatives similar to **1.22** and **1.23**. Compounds of this form could then act as irreversible inhibitors of G6Pase via a similar mechanism. Shown in **Figure 1.9** are the general forms of our proposed

analogues. Compounds of the form of **1.22** replace the *C*6 oxygen atom of G6P with the phosphonate moiety, thus placing an electrophilic site at *C*6, while analogues of the form of **1.23** replace *O*6 of G6P with the electrophilic site. Any differences observed between these analogues would likely be due to advantageous placement of the electrophilic site in proximity to the active site nucleophile. Based on the precedented α-F phosphonate chemistry, and the identification of irreversible α-Br inhibitors of PTP enzymes, we considered that α-halo analogues of G6P would be a good starting point for the identification of specific irreversible inhibitors of G6Pase. In the following sections of this chapter, we provide a summary of known α-bromo phosphonates, and synthetic strategies for their generation. In Chapter 2 we will summarize enzymatic studies of α-bromophosphonate inhibition of CD45 using peptide analogues. Finally, in Chapter 3 we will present work towards α-functionalized analogues of G6P and their activity as inhibitors of G6Pase.

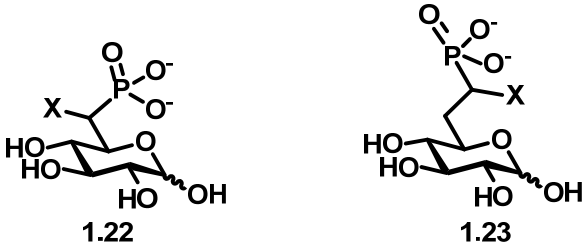


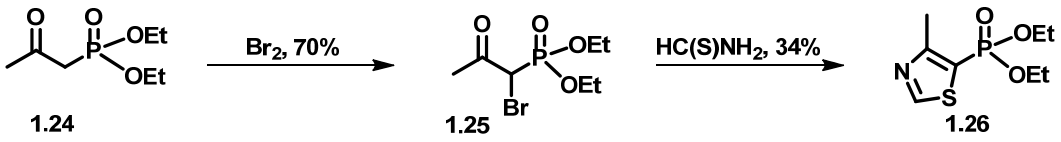
Figure 1.9: Postulated irreversible inhibitors of G6Pase.

1.5 α-Bromophosphonates

The α-bromophosphonate moiety has been known in the literature for over 60 years.[60] The earliest examples of α-bromophosphonates were reported as intermediates in the synthesis of α-functionalized phosphonates. However, as phosphonates were explored in medicinal chemistry, reports of the syntheses of α-bromophosphonates as a target emerged. Examples of α,α-disubstituted phosphonates where one substituent is a bromine atom are also known. Benzylic and aliphatic α-bromophosphonates are well precedented. Herein we review the chemistry of α- and α,α-halo substituted phosphonates.

1.5.1 α-Bromophosphonates as synthetic intermediates

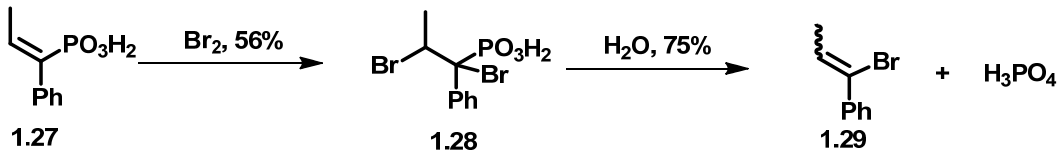
To the best of our knowledge, the first example of an isolated α-bromophosphonate occurred in 1952 in the form of α-bromo-β-ketophosphonates as an intermediate in the synthesis of thiazolephosphonates (**Scheme 1.2**).[60]



1.24 1.25 1.26

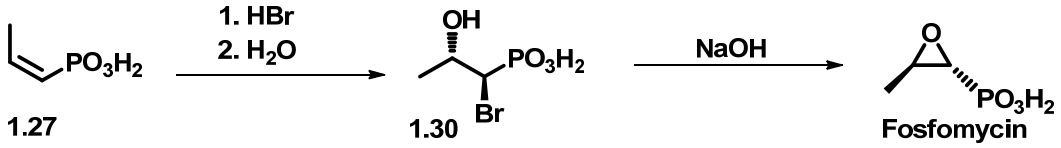
Scheme 1.2: α-Bromophosphonate as an intermediate in the synthesis of thiazolephosphonates.[59]

The second unique example of the α-bromophosphonate occurred in 1966. In this instance bromination across an α,β-unsaturated phosphonate furnished dibromo adduct **1.28** as shown in **Scheme 1.3**.[61] Alkene **1.27** was treated with Br_2 and carefully isolated via recrystallization in moderate yield. The dibromo adduct, **1.28,** was then stirred in the presence of H_2O, resulting in rapid elimination of phosphoric acid to generate olefin **1.29** in good yield.[61]



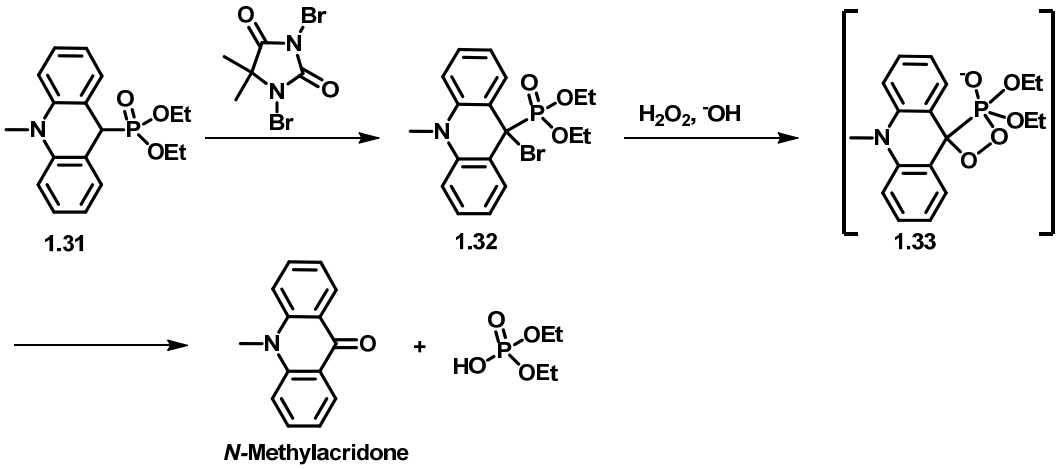
Scheme 1.3: α-Bromophosphorylation via olefinic bromination.[61]

The first report of an α-bromo-β-hydroxyphosphonate was in 1969, where the functional group was used as an early strategy for synthesis of the antibiotic fosfomycin (**Scheme 1.4**).[62] Similar to the bromination strategy used to generate alkene **1.26**, compound **1.27** was hydrobrominated, followed by displacement of bromine under basic conditions to furnish fosfomycin. The yields for these reactions were not reported.



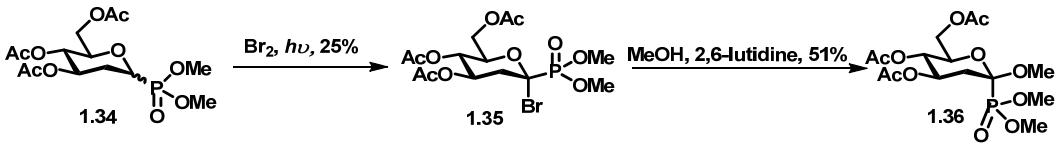
Scheme 1.4: The second example of an α-bromophosphonate used in the synthesis of fosfomycin.[62]

α-Bromophosphonates have been used as intermediates to generate chemiluminscence through an oxidative mechanism.[63] Shown in **Scheme 1.5** is the synthesis of the highly reactive phospha-1,2-dioxetane, **1.33**. Phosphonate **1.31** was brominated using 1,3-dibromo-5,5-dimethylhydantoin in refluxing CCl_4 to furnish α-bromophosphonate intermediate **1.32**. Compound **1.32** was then subjected to alkaline H_2O_2 resulting in the reactive phosphatane intermediate **1.33**. Compound **1.33** decomposes to form the chemiluminescent product *N*-methylacridone and diethyl phosphate, likely through collapse of the phosphatane.[63] The authors did not present any data regarding the yield of these transformations.



Scheme 1.5: Utilization of an α-bromophosphonate to obtain the reactive phospha-1,2-dioxetane intermediate 1.33.[63]

α-Bromophosphonates have been used in carbohydrate synthesis as intermediates. Barnes et al. utilized an α-bromophosphonate intermediate as a method to install an α-methoxy substituent in 1996.[64] As shown in **Scheme 1.6**, compound **1.34** was photobrominated[65] to exclusively yield the α-stereochemistry at *C*1 of the α-bromophosphonate **1.35**, albeit in poor yield. An S_N2 displacement of bromine using MeOH under mildly basic conditions resulted in inversion of the anomeric stereochemistry, to provide α-methoxy compound **1.36**.[64]



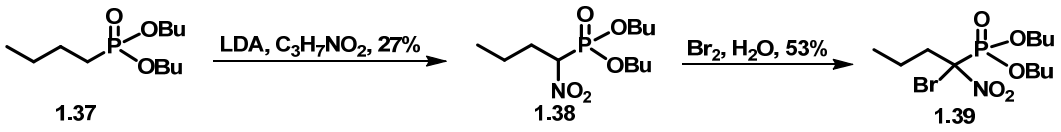
Scheme 1.6: Carbohydrate-based α-bromination as an intermediate in the synthesis of an α-methoxy analogue.[64]

The most recent examples of α-bromophosphonates as intermediates in organic synthesis have exploited these functional groups as precursors for a Horner-Wadsworth-Emmons (HWE; 2011)[66] reaction and radical cyclization (2002).[67] However, the methods by which these compounds are accessed provide useful illustrations of the preparation of α-halo phosphonates so we present them later in the chapter.

1.5.2 α,α-Disubstituted phosphonates containing bromine

α,α-Disubstituted phosphonates containing bromine have appeared in synthetic routes dating over 30 years. In 1978, Feuer et al. demonstrated the synthesis of α-bromination of a phosphoenolate. Shown in **Scheme 1.7** is an example of one such compound, **1.39**. The nitration is performed first: presumably via the quenching of a lithium phosphoenolate with propyl nitrate. Subsequent bromination is carried out in water to furnish compound **1.39**.[68] Although this mechanism is not discussed directly by the authors, we postulate that with the

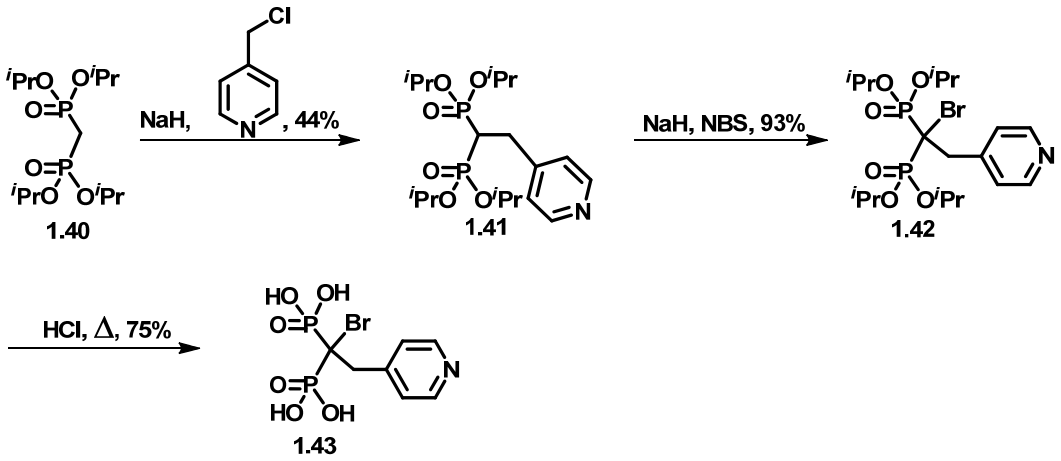
nitro group installed at the α-position, the remaining proton is acidic enough to be deprotonated by water prior to quenching of the phosphoenolate with bromine.



1.37 1.38 1.39

Scheme 1.7: The synthesis of α-bromo-α-nitrophosphonate 1.30.[68]

Examples of α,α-disubstituted phosphonates in the literature are rare until 2007, after the biological potential of α-bromophosphonates had been established. Marma et al. explored the synthesis and biological activity of α-bromobisphosphonate and α-bromophosphonocarboxylate derivatives.[69] Synthetic strategies for α-bromobisphonate derivative **1.43** are shown in **Scheme 1.8**. Deprotonated tetraisopropyl methylenediphosphonate (**1.40**) was quenched with picolyl chloride to furnish compound **1.41** in moderate yield.[69] In a step analogous to the conversion of **1.38** to **1.39**, phosphonate **1.41** was deprotonated using NaH and the phosphoenolate was quenched using *N*-bromosuccinimide (NBS) to furnish **1.42** in high yield. Final cleavage of the phosphonate esters in refluxing HCl afforded α-bromophosphonic acid derivative **1.43**.[69]

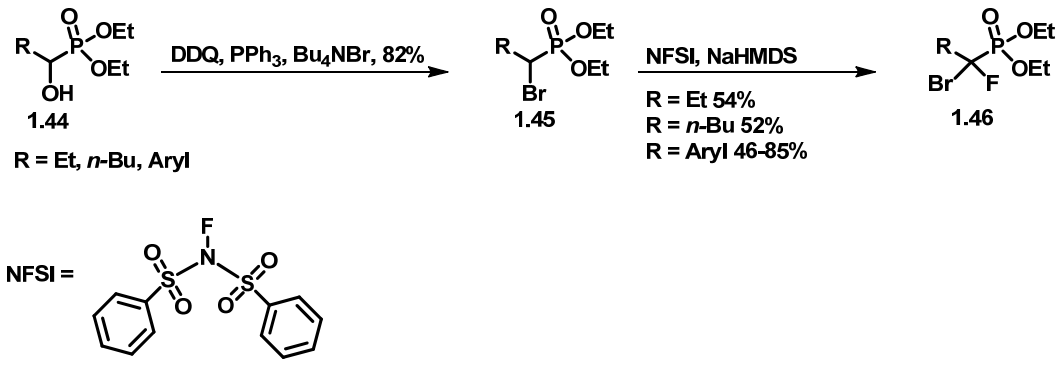


Scheme 1.8: The synthesis of α-bromobisphosphonate derivative 1.43 by Marma et al.[69]

Importantly, biological testing indicated that analogue **1.43** was a sub-micromolar inhibitor of the mevalonate pathway (IC_{50} = 340 nM), which is implicated in osteoporosis.[69]

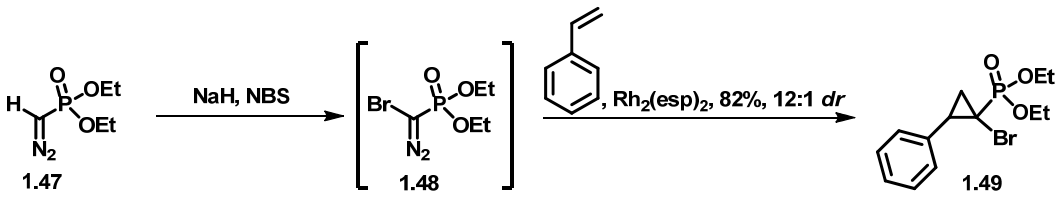
Guan and coworkers[70] have reported access to α-bromo- α-fluorophosphonates. Several compounds were synthesized in this study; however we highlight a representative aliphatic example in **Scheme 1.9**. α-Hydroxyphosphonate **1.44** was prepared using triphenylphosphine (PPh_3), 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), and tetrabutylammonium bromide under standard conditions in good yield.[71] Mechanistic details of the method were studied by Firouzabadi et al. and they concluded that the bromination process did not involve

a radical intermediate. They propose instead a two electron, neutral process.[71] With α-bromophosphonate derivative, **1.45**, in hand the Guan group used sodium hexamethyldisialazide (NaHMDS) to generate a sodium phosphoenolate. The phosphoenolate was quenched using *N*-fluorobenzenesulfonimide (NFSI) in moderate to high yield to furnish α-bromo-α-fluorophosphonate **1.46**. Electron withdrawing groups *para* to the phosphonate were found to increase the yield of the fluorination dramatically (~80%). This is presumably due to stabilization of the intermediate phosphoenolate generated by NaHMDS. Aliphatic or electron donating groups at the α-position resulted in substantially lower yields (~50%). The bromination step, however, was not affected by the presence of an electron withdrawing group. We propose this is because the bromination step is alleged to be neutral,[71] however the Guan group does not comment on this aspect.[70] The authors do not discuss the stereochemistry at the α-carbon in the final products



Scheme 1.9: The synthesis of α-bromo-α-fluorophosphonate 1.46.

The most recent example of an α,α-disubstituted phosphonate used rhodium-catalyzed conditions to install an α-bromocyclopropylphosphonate.[72] It has been shown previously that cyclopropylphosphonates have biological activity.[73] This finding inspired Schnaars and Hansen to undertake the synthesis of α-bromocyclopropylphosphonates for future use in biological assays. **Scheme 1.10** depicts the optimized conditions reported for this transformation. Diazophosphonate **1.47** was dissolved in toluene/CH_2Cl_2 with sodium hydride (NaH), NBS, styrene and $Rh_2(esp)_2$ to yield α-bromo analogue **1.49** in a 12:1 *dr*. It is important to note that based on previous work, the authors were able to confirm this reaction proceeded through an α-bromodiazophosphonate intermediate (**1.48**) prior to the cyclopropanation of styrene.[72]

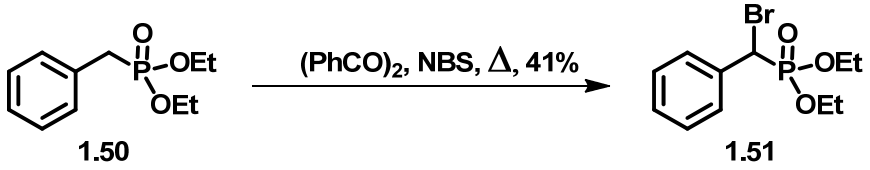


Scheme 1.10: One pot synthesis of α-bromocyclopropylphosphonate 1.46.

1.5.3 Benzylic α-bromophosphonates

Synthetic strategies to access benzylic α-bromophosphonates have been known for over 25 years. Collins et al. were the first to report a benzylic α-

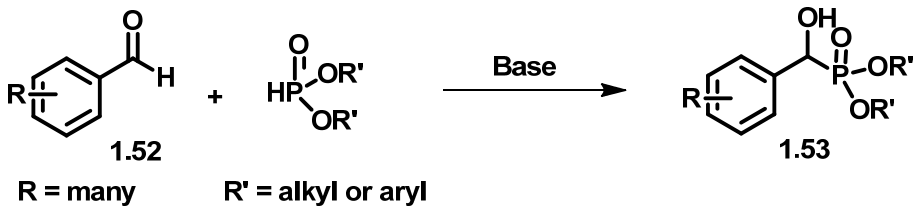
bromophosphonate, which was arrived at serendipitously.[74] The authors observed that treatment of phosphonate **1.50** with benzoyl peroxide and NBS under reflux yielded α-bromo analogue **1.51** in moderate yield (**Scheme 1.11**).[74]



1.50 1.51

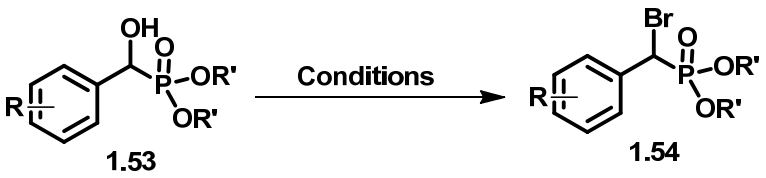
Scheme 1.11: An early synthetic example of a benzylic α-bromophosphonate.

The example in **Scheme 1.11** was unique, as the α-phosphonate position of **1.50** was fully saturated, suggestive of a radical bromination mechanism. Most subsequent literature examples of phosphonate α-bromination have been executed via an α-hydroxyphosphonate intermediate. Shown in **Scheme 1.12** is a general approach to the synthesis of the parent α-hydroxy adduct via a Pudovik reaction.[75] In this transformation, a benzyaldehyde derivate (**1.52**) is subjected to treatment with a deprotonated dialkyl- or diarylphosphite to furnish a diastereomeric mixture of the α-hydroxy analogue **1.53**.



Scheme 1.12: A generic Pudovik reaction to form an α-hydroxyphosphonate.

After obtaining the α-hydroxy precursor (**1.53**), the subsequent bromination reaction can be performed using a variety of conditions. Several conditions used to achieve this transformation are summarized in **Table 1.1**.



Entry	R	R'	Conditions	Yield	Reference
a	H	Et	CBr_4, PPh_3	42%	Gajda, 1990[76]
b	H	Et	PPh_3Br_2*, C_5H_5N	42%	Gajda, 1990[76]
c	2-OBn	Et	$SOBr_2$, C_5H_5N	88%	Gross et al., 1993[77]
d	4-OEt	Et	CDI#, allyl bromide	100%	Green et al., 1996[78]
e	H	Et	NBS, C_5H_5N	N/A	Taylor et al., 1996[58]
f	4-Cl	Et	*n*-BuNBr, PPh_3, DDQ	98%	Firouzabadi et al., 2004[79]
g	4-CH_2NBoc	Et	PPh_3Br_2, C_5H_5N	51%	Kumar et al., 2004[59]
h	4-OMe	Et	**1.55**, Br_2	100%	Firouzabadi et al., 2006[80]
i	4-Tyr	Me	$SOBr_2$, C_5H_5N	88%	Tulsi et al., 2010[57]
j	4-$C_{15}H_{31}$	Me	PBr_3	N/A	Gupte et al., 2011[81]
k	4-I	Bn	*n*-BuNBr, PPh_3, DDQ	84%	Stephenson, 2011[66]
l	4-N(O)$C_{13}H_{27}$	Et	CBr_4, PPh_3	81%	Jiang et al., 2011[82]

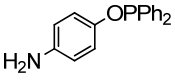
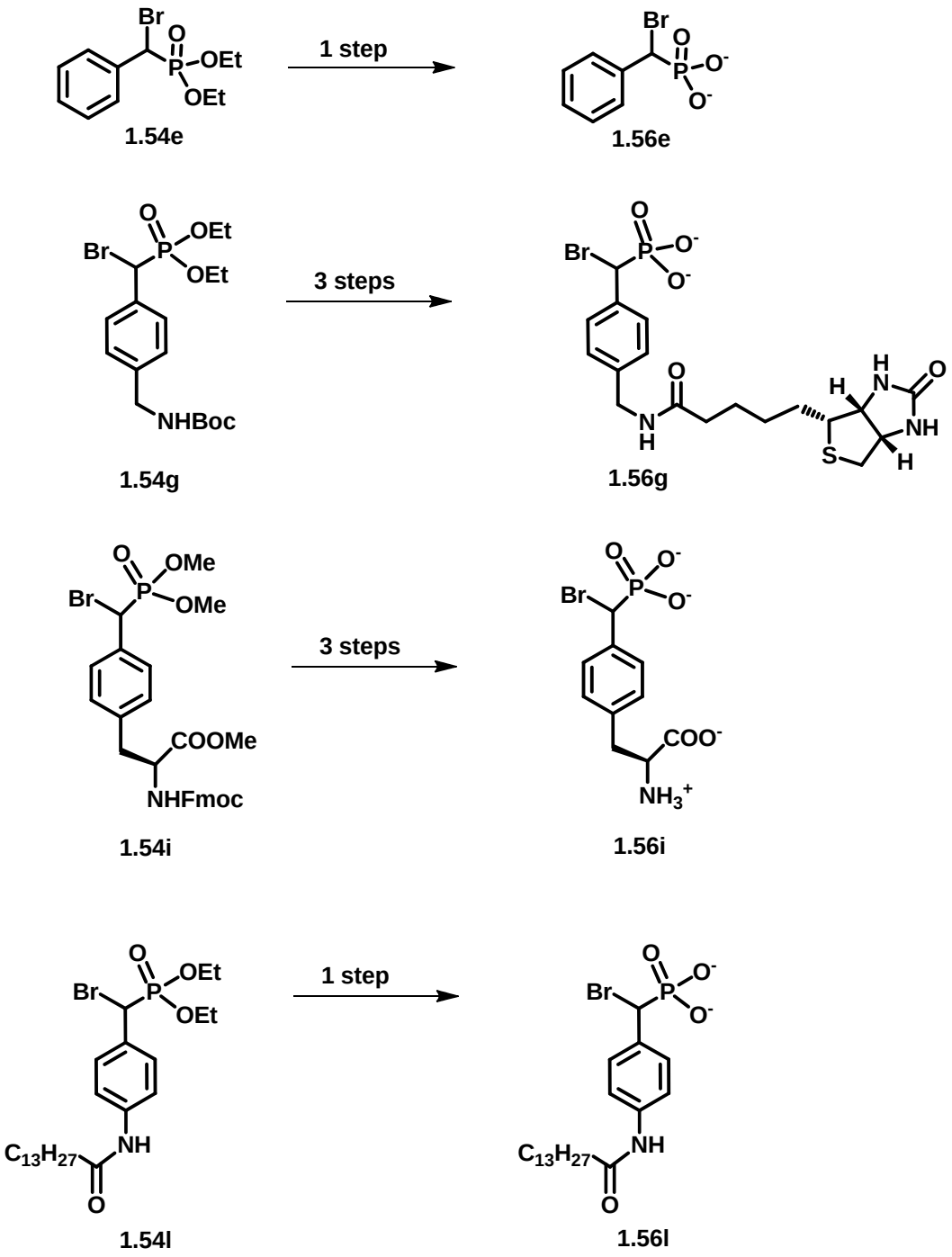
*: dibromotriphenylphosphorane; #: *N*,*N*-carbonyldiimidazole; **1.55**: 

Table 1.1: Precedented benzylic α-bromination strategies.

As discussed previously, α-bromophosphonates are of increasing biological interest as isosteres of phosphate groups. Several of the examples included in **Table 1.1** were generated for the purpose of biological assay, including entries e, g, i, and l. In all instances, no more than three chemical steps were required to obtain the molecule for assay and the chemistry was straightforward. Shown in **Scheme 1.13** are the final analogues tested for activity from the references included in **Table 1.1**.



Scheme 1.13: Biologically active benzylic α-bromophosphonates..

To the best of our knowledge, analogue **1.56e** was the first α-bromophosphonate assayed for biological activity, in this case against the bacterial phosphatase enzyme Yop51. Widlanski and co-workers screened **1.56e** against a truncated recombinant form of the Yop51 PTP from *Yersinia enterocolitica.*[58] The authors discovered that **1.56e** inhibited Yop51 in a time dependent manner in the low mM range. Furthermore, they tested for specificity for PTPs by screening **1.56e** against a non-specific phosphatase, alkaline phosphatase. They did not find any inhibition of alkaline phosphatase, indicating **1.56e** was specific for PTPs.[58]

Eight years later Zhang and coworkers expanded on this finding by constructing compound **1.56g**, which was proposed as an activity-based probe (ABP) to covalently tag phosphatase enzymes. Zhang's study modified Widlanski's original design by biotinylating the *para*-position of the aromatic ring.[59] This modification enabled streptavidin to be used for sensitive detection of any bioconjugates formed in the reaction of **1.56g** with its targets by Western blot. When Zhang and coworkers introduced probe **1.56g** to a mixture PTPs (YopH, PTP1b, HePTP, SHP2, FAP-1, PTPα, DEP-1, VHR, Cdc14 and PRL-3) at a 1 mM concentration, they observed that the compound reacted with all of these enzymes to form covalent adducts.[59] To test the specificity of this reagent toward PTPs over other phosphatase enzymes, **1.56g** was tested for labelling of alkaline, potato, prostatic acid, DSP and λ phosphatases. Compound **1.56g** displayed no reactivity toward any non-PTP enzymes, or against other proteins found in the cell lysate of

E. coli.[59] Thus, these results confirmed that α-bromobenzylphosphonate derivatives could be used for irreversible inhibition of PTPs – but also raised the concern that the α-BBP functional group alone confers no specificity.

Tulsi et al. developed an improved strategy for the elaboration of α-BBP into peptide analogues, offering the possibility of greatly improved specificity for individual PTP enzymes. Tulsi et al. were the first to develop a synthetic route to a protected phosphotyrosine analogue including the α-BBP functional group. The L-bromophophonomethylphenylalanine derivative, which they termed **BrPmp** (**1.56i**), was tested as an individual amino acid and as a tripeptide analogue for inhibition of a human PTP enzyme, CD45. Tulsi et al. confirmed that **BrPmp** was indeed a covalent inhibitor of CD45 with a K_i in the low-μM range.[57] Importantly, these authors found that when BrPmp was tested in the context of a tripeptide derivative, its specificity for the enzyme was increased by 4-fold. These results strongly suggest that if the appropriate BrPmp-peptide sequence were used it could target an individual PTP enzyme with high potency. Details of this project are provided in Chapter 2.

More recently, the Prestwich group has elucidated an α-bromobenzylphosphocholine derivative as an inhibitor of autotaxin, an enzyme that hydrolyzes lysophosphatidylcholine to form the bioactive lipid lysophosphatidic acid (LPA).[83] This pathway is implicated in angiogenesis,

making autotaxin a potential anti-cancer target.[84] Improving upon previous work,[85] Prestwich and coworkers synthesized α-bromophosphocholine adduct **1.56l**, which was found to inhibit autotaxin with a K_i of 9 nM.[82]

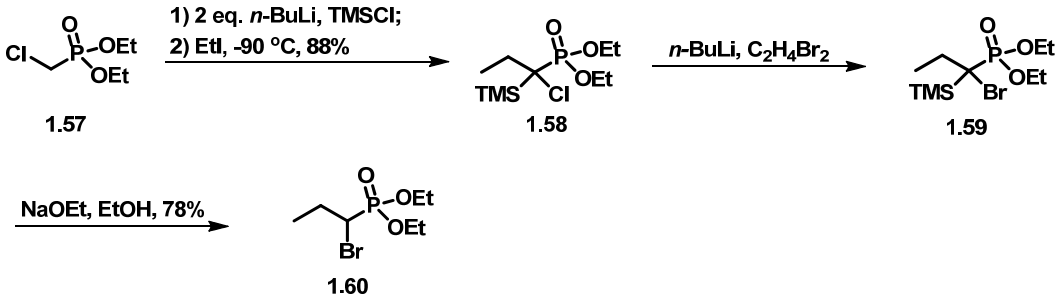
As discussed above, there are an appreciable number of examples of the synthesis of α-BBP containing analogues. However, there are far fewer examples of aliphatic α-bromophosphonate analogues and we summarize these in detail below.

1.5.4 Aliphatic α-bromophosphonates

Synthesis of aliphatic α-bromophosphonates and α-halophosphonates are generally more difficult than that of α-BBP. The synthesis of α-BBP analogues is substantially simplified by the benzylic character of the α-phosphono position. Interestingly, apart from the α-bromo- α-fluorophosphonate, compound **1.51,** only two reports have utilized an α-hydroxy precursor, which is the most commonly used parent compound for synthesis of benzylic α-bromophosphonates.

An early example of an aliphatic α-bromophosphonate synthesis was reported by Teulade and Savignac in 1988.[86] They synthesized a series of five aliphatic α-bromophosphonates. The synthesis of propyl phosphonate derivative **1.60** is

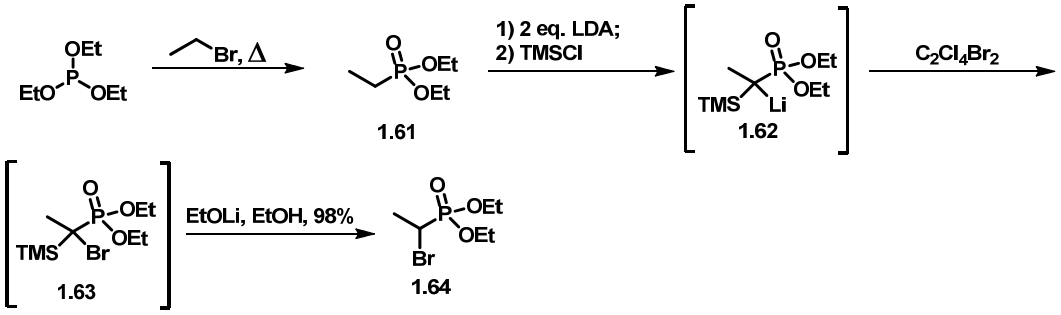
shown in **Scheme 1.14**. Teulade and Savignac began their synthesis from compound **1.57**, which was silyated and subsequently alkylated in one pot using 2 equivalents of *n*-butyl lithium (*n*-BuLi), trimethylsilyl chloride (TMSCl), and ethyl iodide in good yield.[86] Subsequent treatment of analogue **1.58** with *n*-BuLi in the presence of a source of bromine resulted in halogen-lithium exchange with the chlorine atom. The α-bromo- α-silylphosphonate was converted to the final α-bromo product by cleavage of the TMS group with sodium ethoxide to furnish **1.60** in 78% yield over the final two steps.[86]



Scheme 1.14: The synthesis of an aliphatic α-bromophosphonate derivative.

In 2000, Savignac and coworkers reported an improvement to their aliphatic bromination strategy that obviates the need for starting α-chlorophosphonate **1.57**.[87] Instead, they utilize the Michaelis-Arbuzov reaction by treatment of triethylphosphite with an alkyl halide under reflux[88] to obtain the starting alkyl phosphonate. Savignac and coworkers synthesized a number alkylphosphonates using this method; however, **Scheme 1.15** only depicts the synthesis of a single

representative ethyl adduct (**1.61**). Treatment of compound **1.61** with 2 equivalents of lithium diisopropyl amide (LDA) followed by dropwise addition of TMSCl[87] produced intermediate **1.62** which was confirmed by ^{31}P NMR studies. Lithium-bromine exchange was performed using hexachloroethane without issue. Finally, compound **1.63** was desilylated using lithium ethoxide in ethanol to furnish α-bromo product **1.64** in nearly quantitative yield.[87]

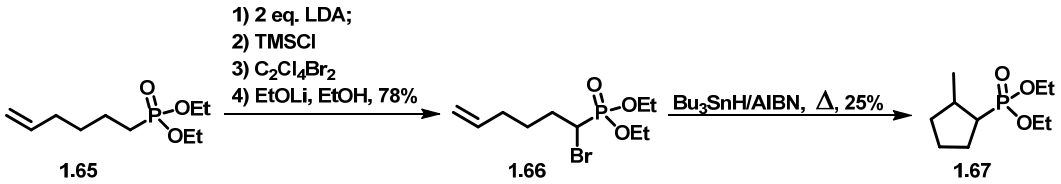


Scheme 1.15: An improved synthetic strategy to obtain alkyl α-bromophosphonates.

The Savignac group developed this methodology as proof of concept without consideration to its application in biological systems. To date, there are no reports of biological testing of these analogues.

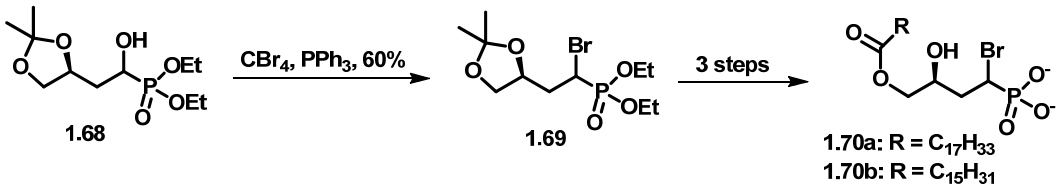
To the best of our knowledge, the only other application of this methodology for the synthesis of α-bromophosphonate analogues has been reported by Wnuk and coworkers.[67] In 2002, the Wnuk group applied Savignac's strategy to synthesize

analogue **1.66** as a precursor to radical cyclization (**Scheme 1.16**). Compound **1.66** was cyclized in the presence of tributyltin hydride and azobisisobutyronitrile (AIBN) in very moderate yield to produce cyclopentyl analogue **1.67**.[67]



Scheme 1.16: An α-bromophosphonate as a precursor for radical cyclization.

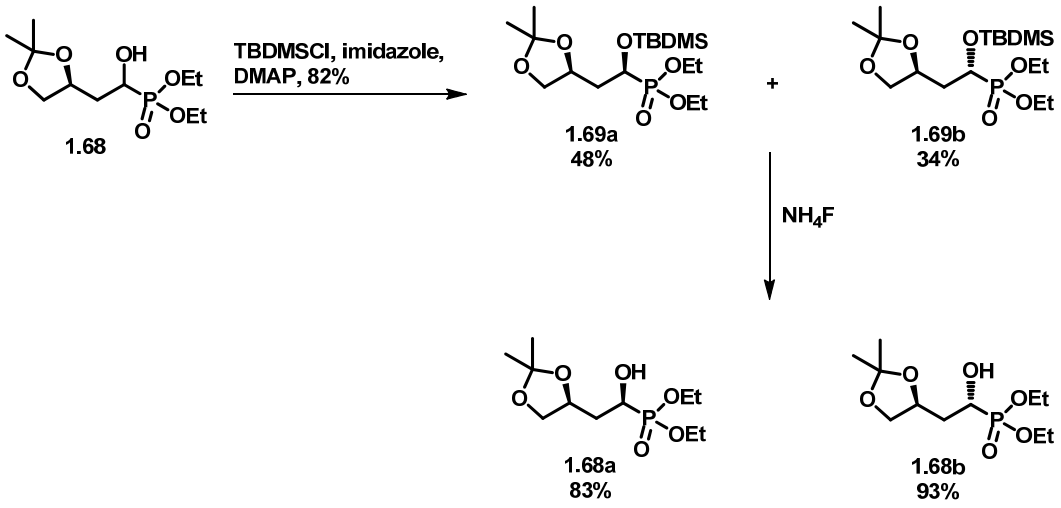
Interestingly, the two most recent syntheses of alkyl α-bromophosphonates make use of an α-hydroxy intermediate as discussed in **Section 1.5.3**. In 2007, Prestwich and coworkers synthesized a number of aliphatic derivatives of lysophatidic acid (LPA). LPA is an initiator for a number of signal transduction pathways implicated in cancer.[89] They designed their synthesis to create analogues of LPA to target the G-protein coupled receptors (GPCRs) of LPA, which regulate cancer cell proliferation, invasion, angiogenesis, as well as biochemical resistance to chemotherapy- and radiotherapy-induced apoptosis.[89] **Scheme 1.17** depicts the synthesis of α-bromophosphonates **1.70a** and **1.70b**. The parent α-hydroxy analogue, **1.68**, was treated with CBr_4 and PPh_3 to furnish alkyl α-bromo analogue **1.69** in moderate yield. Biologically active phosphonates **1.70a** and **1.70b** were then obtained through deprotection and acylation.[89]



Scheme 1.17: The abbreviated synthesis of biologically active α-bromo analogues 1.70a and 1.70b via the key brominated intermediate 1.69.

Compounds **1.70a** and **1.70b** were found to inhibit the four GPCRs screened, LPA_1, LPA_2, LPA_3 and LPA_4 with K_i values ranging from 0.17 μM to 2.5 μM.[89]

Importantly, the Prestwich group was the first to separate the two α-hydroxy diastereomers and assign their stereochemistry. **Scheme 1.18** depicts their method for separation. By treating analogue **1.68** with *tert*-butyldimethylsilyl chloride (TBDMSCl) under basic conditions the authors obtained enough steric bulk on the hydroxyl group to achieve separation using only flash column chromatography. The TBDMS group was then cleaved under mildly acidic conditions to furnish the isolated α-hydroxy diastereomers.[89] A discussion of the stereochemical assignments is provided in the text.[89] Although Prestwich and coworkers demonstrated the isolation of each diastereomer of the α-hydroxyphosphonates, the biological activity of the individual α-bromophosphonate diastereomers was not reported.[89]



Scheme 1.18: The separation of α-hydroxy derivative 1.68 into its two stereoisomers.

In 2009 the Prestwich group opted to selectively brominate each of their phosphocholine-based diastereomers to complete the most thorough biological analysis of any α-bromophosphonate to date.[90] The chemistry used to obtain these analogues was nearly identical to the strategy shown in **Scheme 1.18** and discussed above. The two analogues tested are shown in **Figure 1.10**. They termed analogues **1.71a** and **1.71b** collectively as **Br-LPA**. Importantly, neither diastereomeric analogue was found to have improved activity after testing,[90] indicating that the biological activity of the α–bromophosphonate may not depend on the stereochemistry of the α-bromo group.

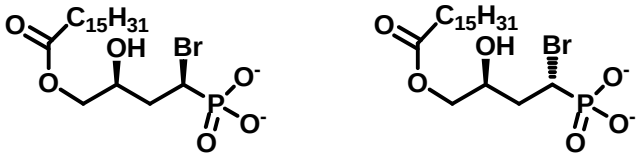


Figure 1.10: The structures of the two α-bromo diastereomers tested by the Prestwich group.

Br-LPA was found to inhibit all four LPA GPCRs, as well as the autotaxin receptor discussed in **Section 1.5.3**. As a result of these findings **Br-LPA** was tested as an inhibitor of LPA-dependent cell activation. The compound impeded migration of MDA-MB-231 breast cancer cells by over 50% at a concentration of 40 μM using a scratch wound assay. **Br-LPA** caused a reduction in orthotopic breast tumor size and vascularity at an administration concentration of 3 mg/kg.[90] These data are extremely encouraging and indicate that α-bromophosphonates may be useful not only as enzyme inhibitors and ABPs, but also as potential cancer therapeutics.

1.6 Conclusion

We have provided a chronological discussion of the precedented syntheses of α-bromo phosphonates. These reports provide a starting point for synthetic strategies towards these important isosteres of the phosphate moiety. Additionally,

many of these compounds have shown promising biological activity in a variety of systems. The biological data summarized above also provides strong support for our hypothesis that α-bromophosphonate analogues **1.22** and **1.23** could act as covalent inhibitors of the G6Pase catalytic site. Further inspiration can be drawn from the work of the Prestwich laboratory in their design of phosphocholine derivatives **1.56l** and **Br-LPA**, which show excellent selectivity for the LPA GPCRs targets.

This thesis will discuss our initial endeavors to measure the biological activity of BrPmp analogues synthesized by Tulsi et al. These studies explore the activity of BrPmp analogues to act as irreversible inhibitors of the PTP CD45 (**Chapter 2**). We will then present our efforts towards the synthesis of a library of G6P-based phosphonates containing α-halo and α,α-dihalo phosphonate derivatives. We also report the results of biological testing of these novel carbohydrate analogues as inhibitors of G6Pase (**Chapter 3**). The studies reported in Chapter 3 identify the first rationally designed α–halo phosphonate inhibitors of the G6Pase active site.

1.7 References

(1) Nordlie, R. C.; Foster, J. D.; Lange, A. J. Regulation of glucose production by the liver *Annu Rev Nutr* **1999,** *19,* 379.

(2) Virshup, D. M.; Shenolikar, S. From Promiscuity to Precision: Protein Phosphatases Get a Makeover *Mol Cell* **2009,** *33,* 537.

(3) Chan, G.; Kalaitzidis, D.; Neel, B. G. The tyrosine phosphatase Shp2 (PTPN11) in cancer *Cancer Metast Rev* **2008,** *27,* 179.

(4) Kim-Muller, J. Y.; Accili, D. Selective Insulin Sensitizers *Science* **2011,** *331,* 1529.

(5) Martin, L.; Latypova, X.; Terro, F. Post-translational modifications of tau protein: Implications for Alzheimer's disease *Neurochem Int* **2011,** *58,* 458.

(6) Besant, P. G.; Attwood, P. V. Histone H4 histidine phosphorylation: kinases, phosphatases, liver regeneration and cancer *Biochem Soc Trans* **2012,** *40,* 290.

(7) Maehama, T.; Okahara, F.; Kanaho, Y. The tumour suppressor PTEN: involvement of a tumour suppressor candidate protein in PTEN turnover *Biochem Soc Trans* **2004,** *32,* 343.

(8) Ashmore, J.; Hastings, A. B.; Nesbett, F. B. The Effect of Diabetes and Fasting on Liver Glucose-6-Phosphatase *Proc Natl Acad Sci USA* **1954,** *40,* 673.

(9) Ghosh, A.; Shieh, J. J.; Pan, C. J.; Chou, J. Y. Histidine 167 is the phosphate acceptor in glucose-6-phosphatase-beta forming a phosphohistidine enzyme intermediate during catalysis *J Biol Chem* **2004,** *279,* 12479.

(10) Pan, C. J.; Lei, K. J.; Annabi, B.; Hemrika, W.; Chou, J. Y. Transmembrane topology of glucose-6-phosphatase *J Biol Chem* **1998,** *273,* 6144.

(11) Ghosh, A.; Shieh, J. J.; Pan, C. J.; Sun, M. S.; Chou, J. Y. The catalytic center of glucose-6-phosphatase. HIS176 is the nucleophile forming the phosphohistidine-enzyme intermediate during catalysis *J Biol Chem* **2002,** *277,* 32837.

(12) Madsen, P.; Westergaard, N. Glucose-6-phosphatase inhibitors for the treatment of Type 2 diabetes *Expert Opin Ther Pat* **2001,** *11,* 1429.

(13) Feldman, F.; Butler, L. G. Detection and characterization of the phosphorylated form of microsomal glucose-6-phosphatase *Biochem Biophys Res Commun* **1969,** *36,* 119.

(14) Kurukulasuriya, R.; Link, J. T.; Madar, D. J.; Pei, Z.; Richards, S. J.; Rohde, J. J.; Souers, A. J.; Szczepankiewicz, B. G. Potential drug targets and progress towards pharmacologic inhibition of hepatic glucose production *Curr Med Chem* **2003,** *10,* 123.

(15) Foster, J. D.; Young, S. E.; Brandt, T. D.; Nordlie, R. C. Tungstate: A potent inhibitor of multifunctional glucose-6-phosphatase *Arch Biochem Biophys* **1998,** *354,* 125.

(16) Foster, J. D.; Young, S. E.; Brandt, T. D.; Nordlie, R. C. Tungstate: A potent inhibitor of multifunctional glucose-6-phosphatase *Faseb J* **1998,** *12,* A1360.

(17) Singh, J.; Nordlie, R. C.; Jorgenson, R. A. Vanadate - a Potent Inhibitor of

Multifunctional Glucose-6-Phosphatase *Biochim Biophys Acta* **1981,** *678,* 477.

(18) Westergaard, N.; Brand, C. L.; Lewinsky, R. H.; Andersen, H. S.; Carr, R. D.; Lundgren, K. Peroxyvanadium compounds inhibit glucose-6-phosphatase activity and glucagon-stimulated hepatic glucose output in the rat in vivo *Diabetes* **1999,** *48,* A277.

(19) Westergaard, N.; Brand, C. L.; Lewinsky, R. H.; Andersen, H. S.; Carr, R. D.; Burchell, A.; Lundgren, K. Peroxyvanadium compounds inhibit glucose-6-phosphatase activity and glucagon-stimulated hepatic glucose output in the rat in vivo *Arch Biochem Biophys* **1999,** *366,* 55.

(20) Lange, A. J.; Arion, W. J.; Burchell, A.; Burchell, B. Aluminum ions are required for stabilization and inhibition of hepatic microsomal glucose-6-phosphatase by sodium fluoride *J Biol Chem* **1986,** *261,* 101.

(21) Salazar-Martinez, E.; Willett, W. C.; Ascherio, A.; Manson, J. E.; Leitzmann, M. F.; Stampfer, M. J.; Hu, F. B. Coffee consumption and risk for type 2 diabetes mellitus *Ann Intern Med* **2004,** *140,* 1.

(22) van Dijk, A. E.; Olthof, M. R.; Meeuse, J. C.; Seebus, E.; Heine, R. J.; van Dam, R. M. Acute effects of decaffeinated coffee and the major coffee components chlorogenic acid and trigonelline on glucose tolerance *Diabetes Care* **2009,** *32,* 1023.

(23) Henry-Vitrac, C.; Ibarra, A.; Roller, M.; Merillon, J. M.; Vitrac, X. Contribution of Chlorogenic Acids to the Inhibition of Human Hepatic Glucose-6-

phosphatase Activity in Vitro by Svetol, a Standardized Decaffeinated Green Coffee Extract *J Agr Food Chem* **2010,** *58,* 4141.

(24) Borthwick, E. B.; Houston, M. P.; Coughtrie, M. W.; Burchell, A. The antihyperglycemic effect of estrone sulfate in genetically obese-diabetic (ob/ob) mice is associated with reduced hepatic glucose-6-phosphatase *Horm Metab Res* **2001,** *33,* 721.

(25) Madsen, P.; Lundbeck, J. M.; Jakobsen, P.; Varming, A. R.; Westergaard, N. Glucose-6-phosphatase catalytic enzyme inhibitors: synthesis and in vitro evaluation of novel 4,5,6,7-tetrahydrothieno[3,2-c]- and -[2,3-c]pyridines *Bioorg Med Chem* **2000,** *8,* 2277.

(26) Westergaard, N.; Madsen, P.; Lundbeck, J. M.; Jakobsen, P.; Varming, A.; Andersen, B. Identification of two novel and potent competitive inhibitors of the glucose-6-phosphatase catalytic protein *Diabetes Obes Metab* **2002,** *4,* 96.

(27) Farhanullah; Tripathi, B. K.; Srivastava, A. K.; Ram, V. J. Synthesis of bicyclic biaryls as glucose-6-phosphatase inhibitors *Bioorg Med Chem* **2004,** *12,* 1543.

(28) Farhanullah; Sil, D.; Tripathi, B. K.; Srivastava, A. K.; Ram, V. J. Synthesis and glucose-6-phosphatase inhibitory activity of (thiouriedo)alkanoic acid esters *Bioorg Med Chem Lett* **2004,** *14,* 2571.

(29) Madsen, P.; Jakobsen, P.; Westergaard, N. N,N-dibenzyl-N'-benzylidenehydrazines: potent competitive glucose-6-phosphatase catalytic

enzyme inhibitors *Bioorg Med Chem Lett* **2001,** *11,* 2165.

(30) Mithieux, G.; Bordeto, J. C.; Minassian, C.; Ajzannay, A.; Mercier, I.; Riou, J. P. Characteristics and specificity of the inhibition of liver glucose-6-phosphatase by arachidonic acid. Lesser inhibitability of the enzyme of diabetic rats *Eur J Biochem* **1993,** *213,* 461.

(31) Mithieux, G.; Zitoun, C. Mechanisms by which fatty-acyl-CoA esters inhibit or activate glucose-6-phosphatase in intact and detergent-treated rat liver microsomes *Eur J Biochem* **1996,** *235,* 799.

(32) Bleriot, Y.; Smelt, K. H.; Cadefau, J.; Bollen, M.; Stalmans, W.; Biggadike, K.; Johnson, L. N.; Oikonomakos, N. G.; Lane, A. L.; Crook, S.; Watkin, D. J.; Fleet, G. W. J. 7-carbon mimics of D-glucose and L-fucose: Activation by 6R-, and inactivation by 6S, -6C-methylglucose of glycogen synthase: Inhibition of glucokinase and/or glucose-6-phosphatase *Tetrahedron Lett* **1996,** *37,* 7155.

(33) Kumar, M.; Rawat, P.; Khan, M. F.; Tamarkar, A. K.; Srivastava, A. K.; Arya, K. R.; Maurya, R. Phenolic glycosides from Dodecadenia grandiflora and their glucose-6-phosphatase inhibitory activity *Fitoterapia* **2010,** *81,* 475.

(34) Robbins, B. L.; Foster, J. D.; Nordlie, R. C. Metabolic intermediates as potential regulators of glucose-6-phosphatase *Life Sci* **1991,** *48,* 1075.

(35) Benkoel, L.; Gulian, J. M.; Bongrand, P.; Dalmasso, C.; Xerri, L.; Brisse, J.; Sastre, B.; Chamlian, A. Image analysis for histochemical study of glucose-6-phosphatase inactivation by diethyl pyrocarbonate in normal human liver *J*

Histochem Cytochem **1990,** *38,* 1565.

(36) Engel, R. Phosphonates as Analogs of Natural Phosphates *Chem Rev* **1977,** *77,* 349.

(37) Lemarechal, P.; Froussios, C.; Level, M.; Azerad, R. Synthesis of Phosphono Analogs of 3-Deoxy-D-Arabino-Hept-2-Ulosonic Acid 7-Phosphate *Carbohydr Res* **1981,** *94,* 1.

(38) Huang, Z.; Kakinuma, K.; Eguchi, T. Stereo specificity of hydride transfer in NAD(+)-catalyzed 2-deoxy-scyllo-inosose synthase, the key enzyme in the biosynthesis of 2-deoxystreptamine-containing aminocycl itol antibiotics *Bioorg Chem* **2005,** *33,* 82.

(39) Padyukova, N. S.; Afsar, S.; Dixon, H. B. F.; Karpeiskii, M. Y. Synthesis and Properties of Glucose and Galactose Phosphonate Derivatives *Bioorg Khim* **1995,** *21,* 382.

(40) Smith-Griffin, B.; Burger, A. D-Glucopyranose 6-Deoxy-6-Phosphonic Acid *J Am Chem Soc* **1956,** *78,* 2336.

(41) Nifantev, E. E.; Gudkova, I. P.; Kochetko.Nk Singular of 1,6-Anhydrohexose with Hypophosphorous Acid *Zh Obshch Khim* **1970,** *40,* 460.

(42) Hirayama, T.; Kudo, F.; Huang, Z.; Eguchi, T. Role of glutamate 243 in the active site of 2-deoxy-scyllo-inosose synthase from Bacillus circulans *Bioorg Med Chem* **2007,** *15,* 418.

(43) Adams, P. R.; Harrison, R.; Inch, T. D.; Rich, P. Dehydrogenation of

Phosphonate Analog of Glucose-6-Phosphate by Glucose-6-Phosphate Dehydrogenase *Biochem J* **1976,** *155,* 1.

(44) Roach, D. J. W.; Harrison, R.; Hall, C. R.; Inch, T. D. An Improved Synthesis of 6,7-Dideoxy-Alpha-D-Gluco-Heptose 7-Phosphonic Acid - Appendix *Biochem J* **1981,** *197,* 735.

(45) Berkowitz, D. B.; Bose, M.; Pfannenstiel, T. J.; Doukov, T. alpha-fluorinated phosphonates as substrate mimics for glucose 6-phosphate dehydrogenase: the CHF stereochemistry matters *J Org Chem* **2000,** *65,* 4498.

(46) Berkowitz, D. B.; Bhuniya, D.; Peris, G. Facile installation of the phosphonate and (alpha,alpha-difluoromethyl)phosphonate functionalities equipped with benzyl protection *Tetrahedron Lett* **1999,** *40,* 1869.

(47) Benyoseph, O.; Sparkes, M. J.; Dixon, H. B. F. A Rapid Spectrophotometric Estimation of Individual Phosphates and Phosphonates - Its Application to Chromatography of Sugar Phosphates and Their Phosphonate Analogs *Anal Biochem* **1993,** *210,* 195.

(48) Benyoseph, O.; Sparkes, M. J.; Dixon, H. B. F. A Rapid Spectrophotometric Estimation of Individual Phosphates and Phosphonates - Its Application to Chromatography of Sugar Phosphates and Their Phosphonate Analogs, (Vol 210, Pg 195, 1994) *Anal Biochem* **1994,** *219,* 389.

(49) Berkowitz, D. B.; Bose, M. (alpha-monofluoroalkyl)phosphonates: a class of isoacidic and "tunable" mimics of biological phosphates *J Fluorine Chem*

2001, *112,* 13.

(50) Romanenko, V. D.; Kukhar, V. P. Fluorinated phosphonates: synthesis and biomedical application *Chem Rev* **2006,** *106,* 3868.

(51) Blackburn, G. M. Phosphonates as Analogs of Biological Phosphates *Chem Ind (London)* **1981,** 134.

(52) Romanenko, V. D.; Kukhar, V. P. Fluorinated phosphonates: Synthesis and biomedical application *Chem Rev* **2006,** *106,* 3868.

(53) Berkowitz, D. B.; Bose, M.; Asher, N. G. A convergent triflate displacement approach to (alpha-monofluoroalkyl)phosphonates *Org Lett* **2001,** *3,* 2009.

(54) Berkowitz, D. B.; Sloss, D. G. Diallyl (Lithiodifluoromethyl)Phosphonate - a New Reagent for the Introduction of the (Difluoromethylene)Phosphonate Functionality *J Org Chem* **1995,** *60,* 7047.

(55) Berkowitz, D. B.; Eggen, M.; Shen, Q.; Sloss, D. G. Synthesis of (Alpha,Alpha-Difluoroalkyl)Phosphonates by Displacement of Primary Triflates *J Org Chem* **1993,** *58,* 6174.

(56) Panigrahi, K.; Eggen, M.; Maeng, J. H.; Shen, Q. R.; Berkowitz, D. B. The alpha,alpha-Difluorinated Phosphonate L-pSer-Analogue: An Accessible Chemical Tool for Studying Kinase-Dependent Signal Transduction *Chem Biol* **2009,** *16,* 928.

(57) Tulsi, N. S.; Downey, A. M.; Cairo, C. W. A protected l-

bromophosphonomethylphenylalanine amino acid derivative (BrPmp) for synthesis of irreversible protein tyrosine phosphatase inhibitors *Bioorg Med Chem* **2010,** *18,* 8679.

(58) Taylor, W. P.; Zhang, Z. Y.; Widlanski, T. S. Quiescent affinity inactivators of protein tyrosine phosphatases *Bioorg Med Chem* **1996,** *4,* 1515.

(59) Kumar, S.; Zhou, B.; Liang, F. B.; Wang, W. Q.; Huang, Z. H.; Zhang, Z. Y. Activity-based probes for protein tyrosine phosphatases *Proc Natl Acad Sci USA* **2004,** *101,* 7943.

(60) Dawson, N. D.; Burger, A. Some Alkyl Thiazolephosphonates *J Am Chem Soc* **1952,** *74,* 5312.

(61) Kenyon, G. L.; Westheim.Fh Sterochemical Course of Fragmentation of Beta-Halophosphonates *J Am Chem Soc* **1966,** *88,* 3561.

(62) Girotra, N. N.; Wendler, N. L. Synthesis and Transformations in Phosphonomycin Series *Tetrahedron Lett* **1969,** 4647.

(63) Motoyoshiya, J.; Isono, Y.; Hayashi, S.; Kanzaki, Y.; Hayashi, S. Chemiluminescent Oxidation of Phosphonates - Phospha-1,2-Dioxetanes as Possible Intermediates *Tetrahedron Lett* **1994,** *35,* 5875.

(64) Barnes, N. J.; Probert, M. A.; Wightman, R. H. Synthesis of 2-deoxy-alpha and -beta-D-arabino-hexopyranosyl phosphonic acids and related compounds; analogues of early intermediates in the shikimate pathway *J Chem Soc, Perkin Trans 1* **1996,** 431.

(65) Somsak, L.; Ferrier, R. J. Radical-Mediated Brominations at Ring Positions of Carbohydrates *Adv Carbohydr Chem Biochem* **1991,** *49,* 37.

(66) Stephenson, A. W. I.; Partridge, A. C.; Filichev, V. V. Synthesis of beta-Pyrrolic-Modified Porphyrins and Their Incorporation into DNA *Chem-Eur J* **2011,** *17,* 6227.

(67) Wnuk, S. F.; Bergolla, L. A.; Garcia, P. I. Studies toward the synthesis of alpha-fluorinated phosphonates via tin-mediated cleavage of alpha-fluoro-alpha-(pyrimidin-2-ylsulfonyl)alkylphosphonates. Intramolecular cyclization of the alpha-phosphonyl radicals *J Org Chem* **2002,** *67,* 3065.

(68) Feuer, H.; Vanburen, W. D.; Grutzner, J. B. Alkyl Nitrate Nitration of Active Methylene-Compounds .14. Nitration of Alkylphosphonate Esters *J Org Chem* **1978,** *43,* 4676.

(69) Marma, M. S.; Xia, Z. D.; Stewart, C.; Coxon, F.; Dunford, J. E.; Baron, R.; Kashemirovli, B. A.; Ebetino, F. H.; Triffitt, J. T.; Russell, R. G. G.; McKenna, C. E. Synthesis and biological evaluation of alpha-halogenated bisphosphonate and phosphonocarboxylate analogues of risedronate *J Med Chem* **2007,** *50,* 5967.

(70) Fu, J. P.; He, Y. H.; Zhong, J.; Yang, Y.; Deng, X.; Guan, Z. An efficient and general route to the synthesis of diethyl alpha,alpha-bromofluorophosphonates *J Fluorine Chem* **2011,** *132,* 636.

(71) Firouzabadi, H.; Iranpoor, N.; Sobhani, S. PPh3/DDQ as a neutral system for the facile preparation of diethyl alpha-bromo, alpha-iodo and alpha-

azidophosphonates from diethyl alpha-hydroxyphosphonates *Tetrahedron* **2004,** *60,* 203.

(72) Schnaars, C.; Hansen, T. Halodiazophosphonates, a New Class of Diazo Compounds for the Diastereoselective Intermolecular Rh(II) Catalyzed Cyclopropanation *Org Lett* **2012,** *14,* 2794.

(73) Wessjohann, L. A.; Brandt, W.; Thiemann, T. Biosynthesis and metabolism of cyclopropane rings in natural compounds *Chem Rev* **2003,** *103,* 1625.

(74) Collins, D. J.; Drygala, P. F.; Swan, J. M. Organo-Phosphorus Compounds .20. Approaches to the Synthesis of 2,3-Dihydro-1h-1,2-Benzazaphospholes Involving C-C and C-P Ring-Closure *Aust J Chem* **1984,** *37,* 1009.

(75) Pudovik, A. N.; Konovalova, I. V. Addition-Reactions of Esters of Phosphorus(Iii) Acids with Unsaturated Systems *Synthesis* **1979,** 81.

(76) Gajda, T. Preparation of Diethyl 1-Bromoalkylphosphonates *Phosphorus, Sulfur Silicon Relat Elem* **1990,** *53,* 327.

(77) Gross, H.; Costisella, B.; Ozegowski, S.; Keitel, I.; Forner, K. Alpha-Substituted Phosphonates .67. Reaction of Salicylaldehyde with Triethylphosphite - New C-O Phosphoryl Group Rearrangement *Phosphorus, Sulfur Silicon Relat Elem* **1993,** *84,* 121.

(78) Green, D.; Elgendy, S.; Patel, G.; Baban, J. A.; Skordalakes, E.; Husman, W.; Kakkar, V. V.; Deadman, J. The facile synthesis of O,O-dialkyl alpha-halobenzylphosphonates from O,O-dialkyl alpha-hydroxybenzylphosphonates

Tetrahedron **1996,** *52,* 10215.

(79) Firouzabadi, H.; Iranpoor, N.; Sobhani, S. Ph3P/DDQ/NH4SCN as a new and neutral system for direct preparation of diethyl alpha-thiocyanatophosphonates from diethyl alpha-hydroxyphosphonates *Synthesis* **2004,** 290.

(80) Iranpoor, N.; Firouzabadi, H.; Gholinejad, M. 4-aminophenyldiphenylphosphinite (APDPP), a new heterogeneous and acid scavenger phosphinite - Conversion of alcohols, trimethylsilyl, and tetrahydropyranyl ethers to alkyl halides with halogens or N-halosuccinimides *Can J Chem* **2006,** *84,* 1006.

(81) Gupte, R.; Patil, R.; Liu, J. X.; Wang, Y. H.; Lee, S. C.; Fujiwara, Y.; Fells, J.; Bolen, A. L.; Emmons-Thompson, K.; Yates, C. R.; Siddam, A.; Panupinthu, N.; Pham, T. C. T.; Baker, D. L.; Parrill, A. L.; Mills, G. B.; Tigyi, G.; Miller, D. D. Benzyl and Naphthalene Methylphosphonic Acid Inhibitors of Autotaxin with Anti-invasive and Anti-metastatic Activity *Chemmedchem* **2011,** *6,* 922.

(82) Jiang, G. W.; Madan, D.; Prestwich, G. D. Aromatic phosphonates inhibit the lysophospholipase D activity of autotaxin *Bioorg Med Chem Lett* **2011,** *21,* 5098.

(83) Umezu-Goto, M.; Kishi, Y.; Taira, A.; Hama, K.; Dohmae, N.; Takio, K.; Yamori, T.; Mills, G. B.; Inoue, K.; Aoki, J.; Arai, H. Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by

lysophosphatidic acid production *J Cell Biol* **2002,** *158,* 227.

(84) Mills, G. B.; Moolenaar, W. H. The emerging role of lysophosphatidic acid in cancer *Nat Rev Cancer* **2003,** *3,* 582.

(85) Ferry, G.; Moulharat, N.; Pradere, J. P.; Desos, P.; Try, A.; Genton, A.; Giganti, A.; Beucher-Gaudin, M.; Lonchampt, M.; Bertrand, M.; Saulnier-Blache, J. S.; Tucker, G. C.; Cordi, A.; Boutin, J. A. S32826, A Nanomolar Inhibitor of Autotaxin: Discovery, Synthesis and Applications as a Pharmacological Tool *J Pharmacol Exp Ther* **2008,** *327,* 809.

(86) Teulade, M. P.; Savignac, P. Alpha-Lithiated O,O-Diethyltrimethylsilylchloromethylphosphonate (C2h5o)2p(O)C(Cl)Si(Ch3)3li .1. Preparations and Properties *J Organomet Chem* **1988,** *338,* 295.

(87) Iorga, B.; Eymery, F.; Savignac, P. Controlled monohalogenation of phosphonates, Part II: Preparation of pure diethyl alpha-monohalogenated alkylphosphonates *Synthesis* **2000,** 576.

(88) Bhattacharya, A. K.; Thyagarajan, G. The Michaelis-Arbuzov Rearrangement *Chem Rev* **1981,** *81,* 415.

(89) Jiang, G. W.; Xu, Y.; Fujiwara, Y.; Tsukahara, T.; Tsukahara, R.; Gajewiak, J.; Tigyi, G.; Prestwich, G. D. alpha-substituted phosphonate analogues of lysophosphatidic acid (LPA) selectively inhibit production and action of LPA *Chemmedchem* **2007,** *2,* 679.

(90) Zhang, H. L.; Xu, X. Y.; Gajewiak, J.; Tsukahara, R.; Fujiwara, Y.; Liu, J.

X.; Fells, J. I.; Perygin, D.; Parrill, A. L.; Tigyi, G.; Prestwich, G. D. Dual Activity Lysophosphatidic Acid Receptor Pan-Antagonist/Autotaxin Inhibitor Reduces Breast Cancer Cell Migration In vitro and Causes Tumor Regression In vivo *Cancer Res* **2009,** *69,* 5441.

Chapter 2: Assaying synthetic substrate analogues of CD45 as irreversible inhibitors[a,b]

[a] Portions of the work described in this chapter have been published in N.S. Tulsi, A.M. Downey, and C.W. Cairo, *Bioorg. Med. Chem.*, **2010**, *18*, 8679.

[b]Compounds described in this chapter were synthesized by Naresh Tulsi (University of Alberta).

2.1 Introduction

CD45 is a protein tyrosine phosphatase (PTP) that is expressed solely on the cellular membrane of hematopoietic cells and may occupy in excess of 10% of the T-cell membrane. CD45 is important to immunoregulation through its dephosphorylation of tyrosine residues found on Lck, CD3ζ and Src proteins.[1] Misregulation of CD45 results in severe combined immunodeficiency (SCID), and the receptor is implicated in autoimmune diseases such as lupus and arthritis. Currently, the primary strategies to examine CD45 specificity rely on the use of phosphotyrosine-specific antibodies, or the synthesis of phosphopeptide substrates.[2] Methods that allow for detection, labelling, or inhibition of active PTP enzymes could complement or improve these strategies to expand our understanding of PTP signaling.[1]

The design of specific PTP inhibitors remains a challenge, and new strategies that provide enhanced activity or reduce development time are of continued interest.[3] A classic strategy for designing PTP inhibitors has exploited non-hydrolyzable phosphotyrosine (pTyr) mimics, such as phosphonomethylphenylalanine (Pmp) which act as competitive inhibitors of the enzyme.[4] Modification of Pmp to a phosphonodifluoromethylphenylalanine (F_2Pmp) improves the potency of these derivatives (**Figure 2.1**).[5] A key advantage of these compounds is their adaptability for solid phase peptide synthesis (SPPS) when prepared with the

appropriate α-amino and phosphonate protection. Reported derivatives of Pmp include fluoro, difluoro, chloro, and dichloro derivatives.[5-9] These strategies have been successfully applied to develop competitive inhibitors of a variety of PTPs.[10]

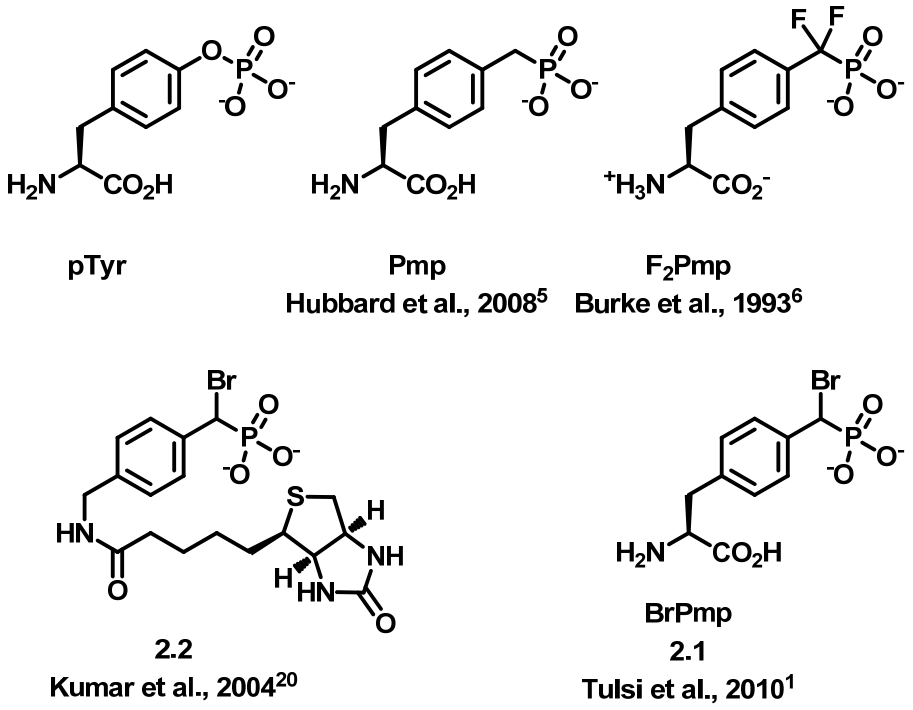


Figure 2.1: Synthetic phosphotyrosine mimics

There has been sustained interest in identifying covalent inhibitors of PTPs. In addition to improved potency, covalent inhibitors (sometimes referred to as suicide substrates) can be of interest for developing enzyme labelling strategies. For example, covalent inhibitors when attached to fluorophores or affinity tags have been employed as activity-based protein probes (ABPP).[11,12] Some of the

known covalent inhibitors for PTPs include quinone methides,[13-16] aryl vinyl sulfonates,[17] nitrostyrene,[18] and α-bromobenzylphosphonate (α-BBP) derivatives.[19] Other notable strategies have included fluorogenic substrates of PTP enzymes, which allow improved assay, detection, and imaging applications.[20]

Taylor et al. were the first to test the activity of α-BBP analogues as inhibitors of PTPs,[21] and this was later used as a labelling strategy with biotin-tagged derivative **2.2**.[19] In contrast to the fluoro, difluoro, and chloro-Pmp derivatives, the BBP analogues were found to form covalent adducts with PTPs, forming the basis of proteomic strategies for PTP identification.[20] Compound **2.2** was shown to covalently label a variety of PTPs, limiting its application as an inhibitor of specific enzymes.[20] We considered that PTPs often recognize specific amino acid sequences,[23] and that additional functional groups could potentially impart specificity to the α-bromophosphonate group. Additionally, we desired an efficient route to an α-bromophoshonate that could be used for both labelling and inhibitor studies.

As result, we synthesized three analogues for screening as inhibitors against CD45 (**Figure 2.2**). The fluorenylmethoxycarbonyl (Fmoc)-protected form of L-bromophosphonomethylphenylalanine (**BrPmp**; **2.1**) was synthesized in 11 steps in 30% overall yield. We then opted to incorporate the Fmoc-protected form of **2.1** into a short peptide sequence, Asp-**BrPmp**-Leu (**2.3**) using solid phase peptide

synthesis (SPPS). This sequence was selected as an example of a short sequence that contained an acidic residue *N*-terminal to the phosphotyrosine site, and a hydrophobic residue at the *C*-terminal side – both of which are features commonly found in phosphopeptide substrates of CD45.[23] The peptide was generated using standard solid phase methods, based on the Fmoc protecting group strategy. Analogue **2.4** was originally synthesized by the Widlanski group,[21] and was used in our studies as a model compound.

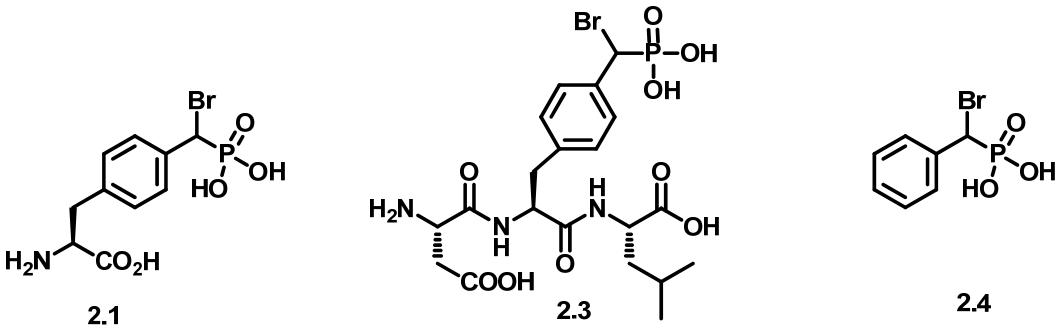
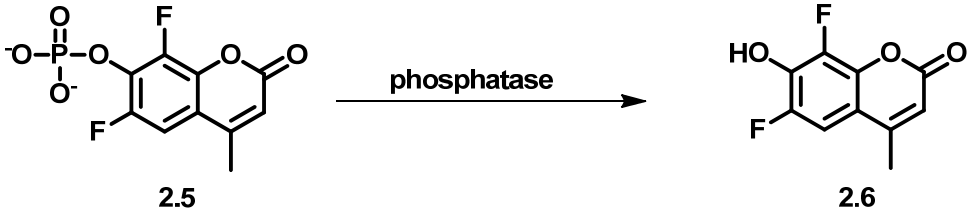


Figure 2.2: Synthetic analogues screened for inhibition against CD45.

2.2 Inhibition of CD45

Following the synthesis of analogues **2.1**, **2.3**, and **2.4**, we endeavored to test their activity inhibitors of CD45. To do so, we modified a standard kinetic assay[25] for measuring phosphatase activity by quantifying the rate of conversion for the fluorogenic substrate, 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP;

2.5) into 4-methylumbelliferone (DiF4-MU; **2.6**; **Scheme 2.1**). In order to reduce the amount of requisite material for the assay we utilized a 96-well plate and generated the raw data using a plate reader that measured the rate of conversion of **2.5** into **2.6** for 30 minutes (details in **Section 2.4**). The rate was measured as relative fluorescent units^{-1} (RFU^{-1}) at various concentrations of inhibitors **2.1**, **2.3** and **2.4** and a control. **Figure 2.3** displays these data graphically.



2.5 **2.6**

Scheme 2.1: Phosphatase conversion of DiFMUP into DiF4-MU.

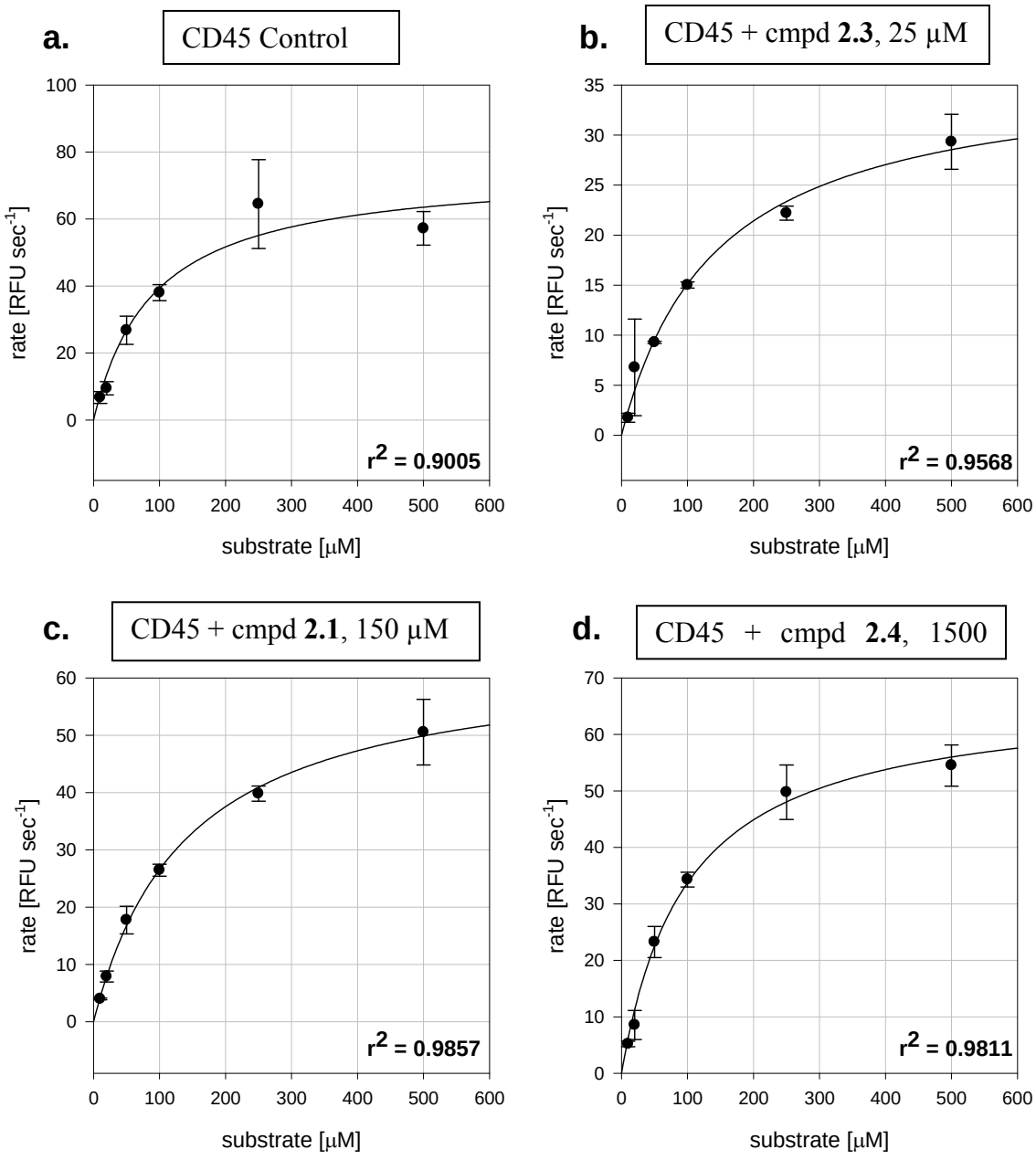


Figure 2.3: CD45 enzyme activity in the presence of inhibitors 2.1, 2.3 and 2.4.

Cmpd	inhib. [μM]	$K_{m,\,obs}$ ± [μM][a]	K_i ± [μM][b]	k_3 ± [min^{-1}]
-	0	90 ± 20	na	-
2.4	1500	99 ± 14	na	-
2.1	150	141 ± 18	40 ± 8	0.041 ± 0.001
2.3	35	141 ± 34	16 ± 4	0.048 ± 0.003

a. Values were determined by non-linear regression of the observed rate of reaction in the presence of inhibitor using the Michaelis-Menten equation.[26] Error is reported as the relative error from the fit. b. For compounds **1** and **17**, K_i was determined by Kitz-Wilson analysis. The rate of enzyme inactivation, k_3, was also determined.[27]

Table 2.1: A summary of the kinetic data for CD45 activity in the presence of compounds 2.1, 2.3, and 2.4.

To determine the inhibitory potency of the α-BBP analogues, the K_m for DiFMUP with CD45 was first determined, and the experiment was then repeated in the presence of compounds **2.1**, **2.3**, and **2.4** to provide an apparent K_m ($K_{m,\,obs}$). We found significant inhibition of CD45 for compounds **2.1** and **2.3** at micromolar concentrations; however, compound **2.4** did not have a significant effect on enzyme kinetics even at millimolar concentration. Previous reports of PTP inhibition using α-BBP analogues had observed irreversible enzyme inhibition.[19] Our Michaelis-Menten plots are shown in **Figure 2.4**. Evaluation of our kinetic data using linear transforms (Michaelis-Menten double-reciprocal plots) was not consistent with pure competitive inhibition of CD45 by **2.1** or **2.3**. While this

finding is consistent with irreversible inhibition this analysis is not conclusive on its own. As a result, further experimentation and analysis was required.

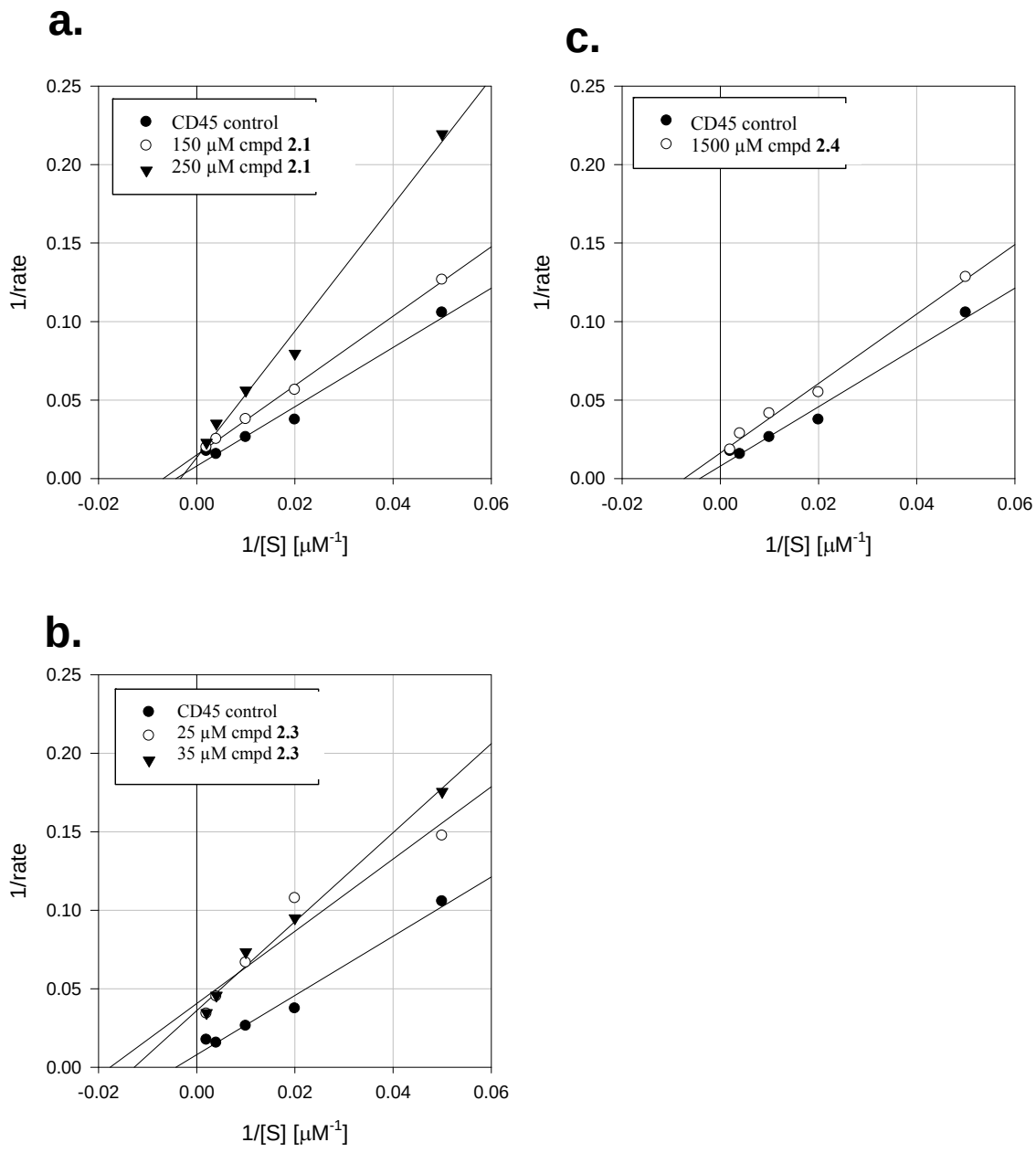


Figure 2.4: Michaelis-Menten plots of CD45 activity with compounds 2.1, 2.3 and 2.4.

To provide additional insight into the inhibition of these compounds, we obtained K_i values using a Kitz-Wilson analysis.[24,25] Compound **2.4** did not give a line with positive slope in this analysis, and therefore could not be analyzed by this method, consistent with its failure to alter the rate of reaction (*vide supra*). Compound **2.4** has been previously tested as an inhibitor of the PTP Yop51, and exact kinetic constants were difficult to obtain, and we found similar difficulties for this determination with CD45.[21] This result may be due to the more hydrophobic nature of the compound. Using the Kitz-Wilson analysis, both compound **2.1** and **2.3** were found to have K_i values of 40 ± 8 μM and 16 ± 4 μM, respectively (**Figure 2.5**). These results indicate that the tripeptide was approximately 4-fold more potent than **BrPmp** alone, suggesting that the adjacent amino acid side chains contribute additional specificity to the inhibitor. The Kitz-Wilson analysis estimated the rate of irreversible inhibition (k_3) of CD45 at 0.05 min^{-1} for both compounds.

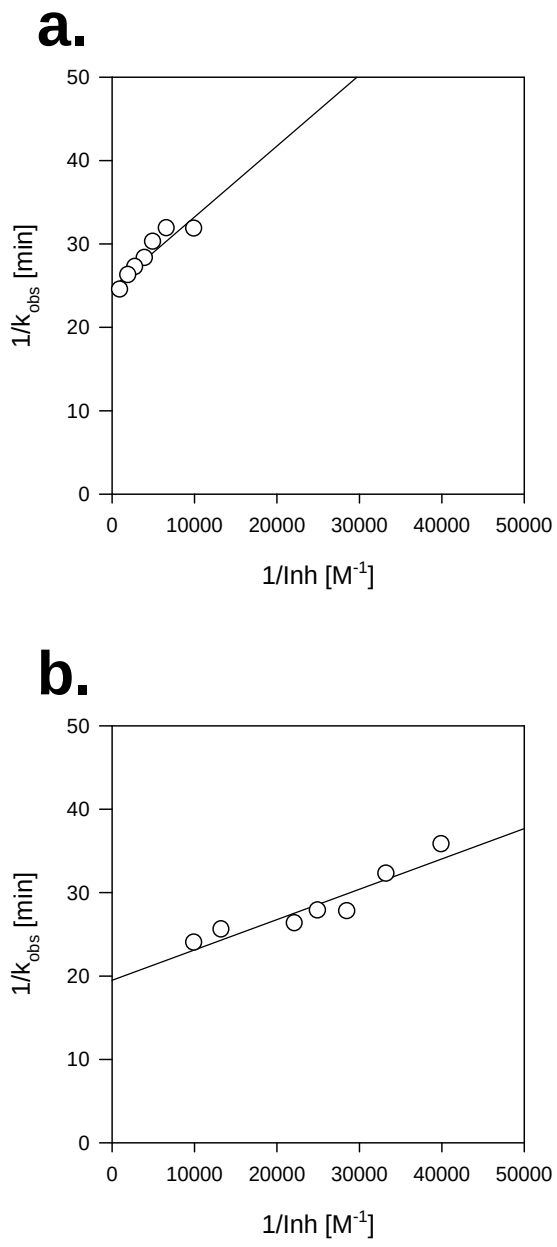


Figure 2.5: Kitz-Wilson analysis of compounds 2.1 and 2.3. Compounds (**a.**) **2.1** and (**b.**) **2.3** were examined using a Kitz-Wilson analysis.[25] The apparent rate constant (k_{obs}) was determined for a series of concentrations of the inhibitor.[26] The

data are plotted as the linear transform based on eq. 1 (**Section 2.4**). Values from the non-linear regression were used for **Table 2.1**.

To provide additional support for the expected mechanism of inhibition, we measured CD45 activity for an enzyme sample which was pre-incubated with inhibitor **2.1** and compared it to an enzyme sample that was only incubated with inhibitor **2.1** for a short period. We found that pre-incubation of the enzyme reduced activity, and at long incubation times completely inactivated the enzyme (**Figure 2.6**), confirming that the inhibitors act irreversibly at long incubation times.

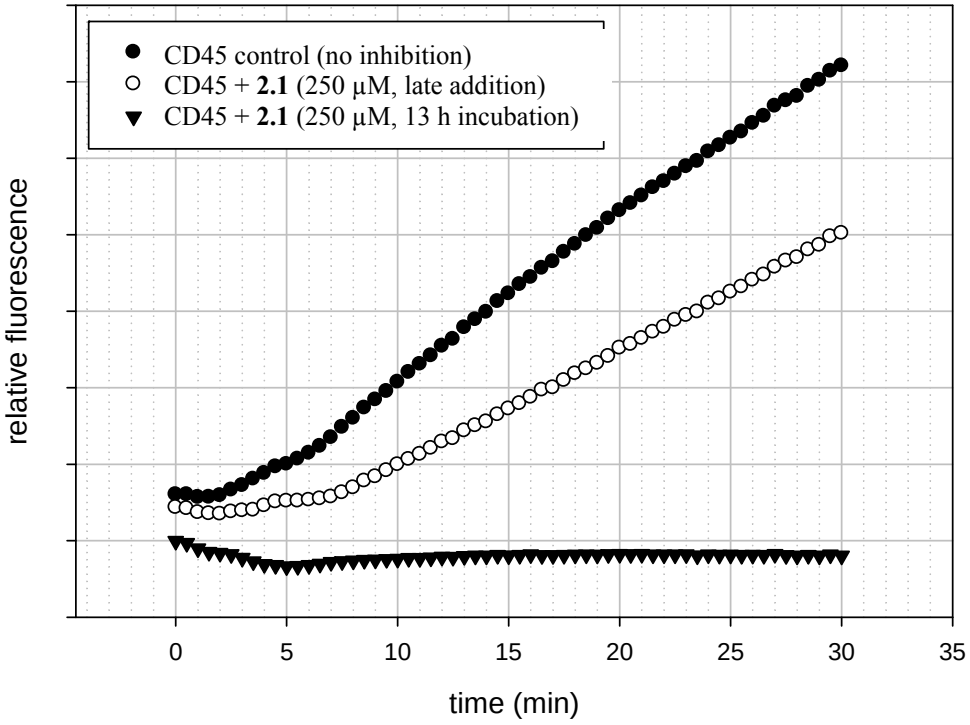


Figure 2.6: Long term inhibition experiment of CD45 using inhibitor 2.1.

2.3 Conclusions and future directions

We synthesized three inhibitors of CD45, compounds **2.1**, **2.3,** and **2.4**. **BrPmp 2.1** and tripeptide **2.3** were shown to be irreversible inhibitors of the enzyme. Interestingly, **2.3** was shown to be a four-fold better inhibitor of CD45 than **2.3** indicating that incorporating **BrPmp** into peptides aides in engineering the specificity of the compound. It is also important to note that Widlanski's analogue **2.4** was a poor inhibitor of CD45 showing that the amino acid moiety of the

synthetic analogue is crucial for potency against the enzyme.

In the future we plan to incorporate **2.1** into longer peptide sequences that are known to be specific for CD45 in order to improve specificity for the enzyme with the hopes of creating a nanomolar inhibitor. As well, confirming selectivity for PTPs necessitates screening the three analogues as inhibitors against non-specific phosphatases such as alkaline phosphatase. Since we have created such a good inhibitor of CD45, we plan to continue screening compound **2.1** as an inhibitor of other crucial endogenous human PTPs such as SHP2, PTP1b and Src. Incorporation into peptide sequences specific for these three enzymes will provide critical evidence that **BrPmp** can be used to selectively target a desired PTP enzyme. As well, adding a fluorescent or biotin handle to **2.1** would enable its use an ABPP as well as functionalize it for cell labelling strategies. One final synthetic challenge may also remain, as we were unable to separate diastereomers of compound **2.1** for separate biological testing.

2.4 Experimental Procedures

2.4.1 Phosphatase inhibition assays

Enzyme assays were conducted using human CD45-cytoplasmic domain (Enzo Life Science; diluted to 4 mU/μL in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.2, 1 mM

Ethylenediaminetetraacetic acid (EDTA), and 0.1% nonidet P-40). Enzyme activity was detected with the fluorogenic substrate, DiFMUP (Invitrogen). Assays were performed in black 96-well plates and read in a Spectra Max M2 plate reader (Molecular Devices). Stock solutions of inhibitors were prepared and stored at -20 °C. Final solutions in microplate wells contained a total volume of 100 μL consisting of 2 μL of diluted enzyme, inhibitor, and DiFMUP substrate. All wells were incubated for 10 min at 37 °C in the plate reader prior to the addition of DiFMUP. After addition of the substrate, the plate was read at an excitation maximum of 358 nm and an emission maximum of 450 nm. Competitive inhibition was determined by observing the initial velocity of CD45 over a range of substrate concentrations to determine K_m, or, in the presence of the inhibitor, $K_{m,\ obs}$. To determine the activity of irreversible inhibitors, the enzyme activity was monitored over 60 min in the presence of inhibitor, and the curve was fit to obtain k_{obs} as described elsewhere.[26] Values of k_{obs} were then fit to

$$k_{obs} = \frac{k_3}{1 + K_I/(I)} \qquad \text{(eq. 1)}$$

by non-linear regression; where K_i was the inhibition constant, I was the concentration of inhibitor, and k_3 was the rate of inactivation of the enzyme.[25] Inhibitors which did not show a positive slope by Kitz-Wilson analysis were treated as competitive inhibitors for the determination of K_i.

2.4.2 Time dependent inhibition study

To test the mechanism of CD45 inhibition, three solutions of CD45 in assay buffer were prepared, with one containing inhibitor (**2.1**). The solutions were incubated for 13 h at 4 °C. At the end of the incubation period, inhibitor was added to the second enzyme sample, and the third had only buffer added. After 10 min, all three samples were observed for turnover of DiFMUP substrate (1 mM) as above. Fit values were determined for the region of the curve between 10 – 30 min.

2.5 References

(1) Earl, L. A.; Baum, L. G. *Immunology and cell biology* **2008**, *86*, 608.

(2) Hegedus, Z.; Chitu, V.; Toth, G. K.; Finta, C.; Varadi, G.; Ando, I.; Monostori, E. *Immunol Lett* **1999**, *67*, 31.

(3) Burke Jr, T. R.; Zhang, Z. Y. *Biopolymers* **1998**, *47*, 225.

(4) Hubbard, C. E.; Barrios, A. M. *Bioorg Med Chem Lett* **2008**, *18*, 679.

(5) Burke Jr, T. R.; Smyth, M. S.; Nomizu, M.; Otaka, A.; Roller, P. P. *J Org Chem* **1993**, *58*, 1336.

(6) Leung, C.; Grzyb, J.; Lee, J.; Meyer, N.; Hum, G.; Jia, C.; Liu, S.; Taylor, S. D. *Bioorg Med Chem* **2002**, *10*, 2309.

(7) Li, P.; Zhang, M.; Peach, M. L.; Liu, H.; Yang, D.; Roller, P. P. *Org Lett* **2003**, *5*, 3095.

(8) Qabar, M. N.; Urban, J.; Kahn, M. *Tetrahedron* **1997**, *53*, 11171.

(9) Yokomatsu, T.; Yamagishi, T.; Matsumoto, K.; Shibuya, S. *Tetrahedron* **1996**, *52*, 11725.

(10) Lee, K.; Gao, Y.; Yao, Z. J.; Phan, J.; Wu, L.; Liang, J.; Waugh, D. S.; Zhang, Z. Y.; Burke Jr, T. R. *Bioorg Med Chem Lett* **2003**, *13*, 2577.

(11) Saghatelian, A.; Cravatt, B. F. *Nat Chem Biol* **2005**, *1*, 130.

(12) Sadaghiani, A. M.; Verhelst, S. H. L.; Bogyo, M. *Curr Opin Chem Biol* **2007**, *11*, 20.

(13) Huang, Y. Y.; Kuo, C. C.; Chu, C. Y.; Huang, Y. H.; Hu, Y. L.; Lin, J. J.; Lo,

L. C. *Tetrahedron* **2010**, *66*, 4521.

(14) Lo, L. C.; Chiang, Y. L.; Kuo, C. H.; Liao, H. K.; Chen, Y. J.; Lin, J. J. *Biochem Biophys Res Commun* **2004**, *326*, 30.

(15) Shen, K.; Qi, L.; Ravula, M.; Klimaszewski, K. *Bioorg Med Chem Lett* **2009**, *19*, 3264.

(16) Shen, K.; Qi, L. X.; Ravula, M. *Synthesis* **2009**, 3765.

(17) Liu, S.; Zhou, B.; Yang, H.; He, Y.; Jiang, Z. X.; Kumar, S.; Wu, L.; Zhang, Z. Y. *J Am Chem Soc* **2008**, *130*, 8251.

(18) Park, J.; Pei, D. *Biochemistry* **2004**, *43*, 15014.

(19) Kumar, S.; Zhou, B.; Liang, F.; Wang, W. Q.; Huang, Z.; Zhang, Z. Y. *Proc Natl Acad Sci U. S. A.* **2004**, *101*, 7943.

(20) Mitra, S.; Barrios, A. M. *ChemBioChem* **2008**, *9*, 1216.

(21) Taylor, W. P.; Zhang, Z. Y.; Widlanski, T. S. *Bioorg Med Chem* **1996**, *4*, 1515.

(22) Barr, A. J.; Ugochukwu, E.; Lee, W. H.; King, O. N. F.; Filippakopoulos, P.; Alfano, I.; Savitsky, P.; Burgess-Brown, N. A.; Muller, S.; Knapp, S. *Cell* **2009**, *136*, 352.

(23) Fernley, H. N.; Walker, P. G. *Biochem J* **1965**, *97*, 95.

(24) Copeland, R. A. *Enzymes: a practical introduction to structure, mechanism, and data analysis*; 2nd ed.; John Wiley & Sons, Inc.: New York, NY, **2000**.

(25) Kitz, R.; Wilson, I. B. *J Biol Chem* **1962**, *237*, 3245.

(26) Skorey, K.; Ly, H. D.; Kelly, J.; Hammond, M.; Ramachandran, C.; Huang, Z.; Gresser, M. J.; Wang, Q. P. *J Biol Chem* **1997**, *272*, 22472.

Chapter 3: Design, synthesis and assay of phosphonate analogues of G6P

3.1 Introduction

Glucose-6-phosphatase (G6Pase) is a pivotal regulator of glucose metabolism, catalyzing the last step of gluconeogenesis and glycogenolysis by converting glucose-6-phosphate (G6P) into glucose.[1] It has also been shown that G6Pase activity is upregulated in the livers of Type II diabetic patients.[2-3] Furthermore, due to its tight association with the endoplasmic reticulum (ER), the exact structure of G6Pase remains to be fully elucidated.[4] As a result, inhibitors of G6Pase could serve as therapeutics, research tools for understanding glucose metabolism, and in structural studies of the enzyme. As discussed in Chapters 1 and 2, a general strategy for producing competitive inhibitors of protein phosphatase enzymes has been to create substrate mimics that replace the hydrolyzable C-O-P bond of a phosphate with a non-hydrolyzable C-C-P bond to yield a phosphonate moiety.[5] Improvements on this strategy have installed fluorine atoms on the phosphonate α-carbon to exploit fluorine as an isosteric replacement for the phosphate oxygen.[6-7] As a result, many specific G6P analogues that incorporate these functional groups (CH_2-P,[8-13] **3.1**; CH_2-CH_2-P,[8-9, 13-20] **3.2**; CH_2-CHF-P,[16, 21] **3.3**; and CH_2-CF_2-P,[17, 22-24] **3.4**) have been explored and are summarized in **Figure 3.1**.

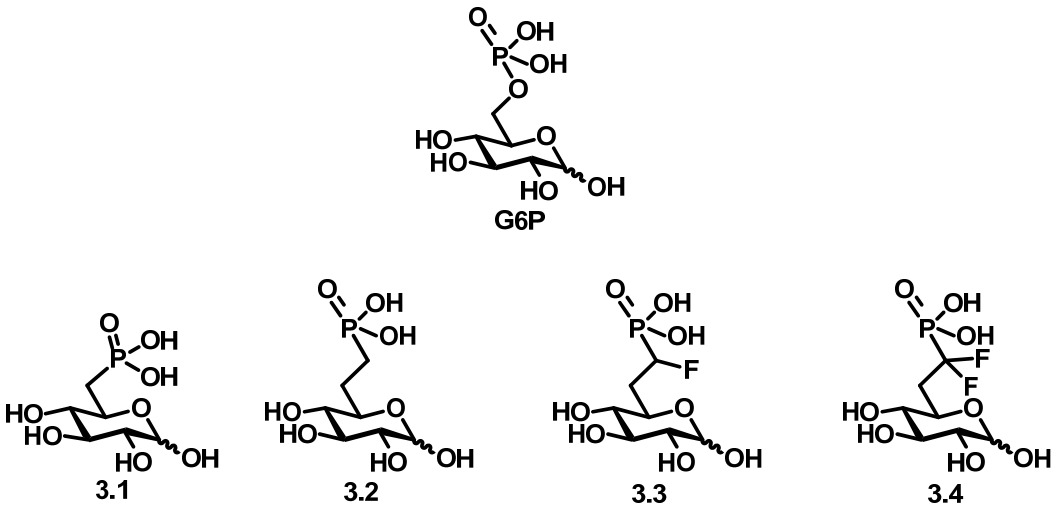


Figure 3.1: G6P and known phosphonate-based analogues. References to these compounds appear in the text.

Although none of the G6P analogues in **Figure 3.1** have been tested against G6Pase, several have been tested for biological activity with other enzymes. Analogue **3.1** was first screened as an inhibitor of choline acetylase, histidine decarboxylase, xanthine oxidase, and hyaluronidase but did not demonstrate any activity toward these enzymes at a concentration of 1 mg/mL.[11] Unfortunately, the authors provided no rationale for the selection of these enzymes.

G6P is a substrate for the enzyme 2-deoxy-*scyllo*-inosose synthase (DOIS), which converts G6P into 2-deoxy-*scyllo*-inosose through a series of hydride transfers from the nicotinamide adenine dinucleotide phosphate co-enzyme (NADPH). In 2005, Eguchi and co-workers[9] tested analogues **3.1** and **3.2** as inhibitors of DOIS.

They discovered that phosphonate **3.1** (K_i = 1.3 mM) inhibited DOIS twice as well as phosphonate **3.2** (K_i = 2.8 mM); however, neither of these compounds exceeded the K_m of G6P (0.21 mM). The authors proposed that the increase in affinity for G6P analogue **3.1** was due to its smaller size as compared to **3.2**.[9]

Interestingly, analogues **3.2**, **3.3**, and **3.4** have been tested as substrates in the pentose phosphate pathway. The rate limiting step in the pentose phosphate pathway is conversion of G6P into 6-phosphonoglucono-δ-lactone by glucose 6-phosphate dehydrogenase (G6PDH).[25] G6P phosphonate analogue **3.2** was tested as a substrate for G6PDH in yeast, and was found to be a substrate for the enzyme; however, the substrate had an 8-fold increase in K_m and a 50% reduction in reaction rate over the native G6P substrate.[14] More recently Berkowitz and co-workers[16] tested compounds **3.2**, **3.3**, and **3.4** on G6PDH in *L. mesenteroides*. They found that all three analogues were substrates for the enzyme with similar k_{cat} values; however, the stereochemistry at *C*7 of **3.3** was shown to be an important determinant of affinity. The K_m value for the *S* isomer was ten times lower than that of the *R* isomer (0.23 mM vs. 2.26 mM), while the K_m values of the isosteres **3.2** and **3.4** were 0.49 mM and 1.35 mM, respectively.[16]

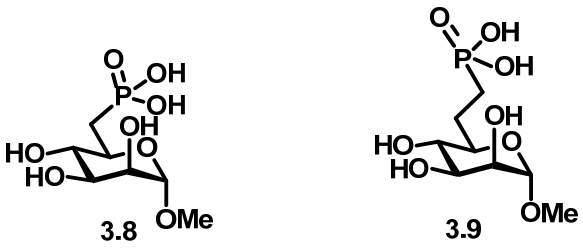


Figure 3.2: Known mannose 6-phosphate (M6P) derivatives.[26-27]

Glycosyl phosphonate derivatives incorporating the *C*1 α-OMe functionality were first synthesized by the Montero group in their studies of M6P (**Figure 3.2**).[26] Interestingly, analogue **3.9** was shown to have the same affinity for M6P-insulin-like growth factor II as native M6P, which is implicated in lysosomal signaling and apoptosis.[27] The *C*6-phosphono analogue **3.8** was not active against the growth factor. These two studies confirm the potential of α-OMe mannophosphonates to interrupt lysosomal signalling, and provided support for the use of phosphonate analogues to target glycosyl phosphatase enzymes. Evidently, in the systems the G6P analogues have been tested in the *C*6 and *C*7 phosphonates were both active. However, only the *C*7 M6P analogue (**3.9**) were found to be active in the lysosomal signalling system.

It is clear from these examples that phosphonate analogues of G6P have potential as substrates and inhibitors for a range of enzymatic pathways. Based on these reports, we hypothesized that phosphonate analogues of G6P could be used as

inhibitors of G6Pase. Although no crystal structure of G6Pase is available, the active site is known to contain a nucleophilic histidine residue.[28] This observation led us to consider that an electrophilic phosphonate moiety could be used to generate an irreversible inhibitor of G6Pase. Based on previous successes with benzylic α–bromophosphonates, we first considered the installation of a bromine atom at the aliphatic phosphonate α-carbon,[29] as shown in **Figure 3.3**.

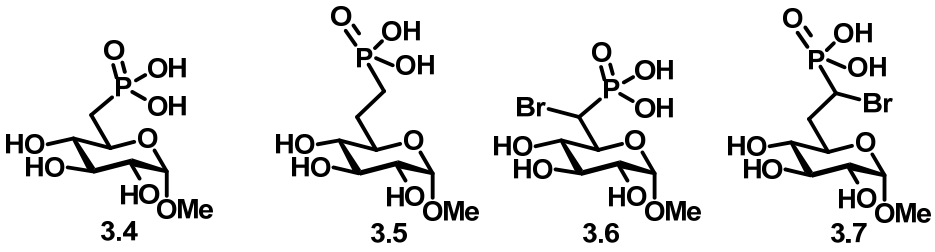


Figure 3.3: Initial G6P-based targets devised.

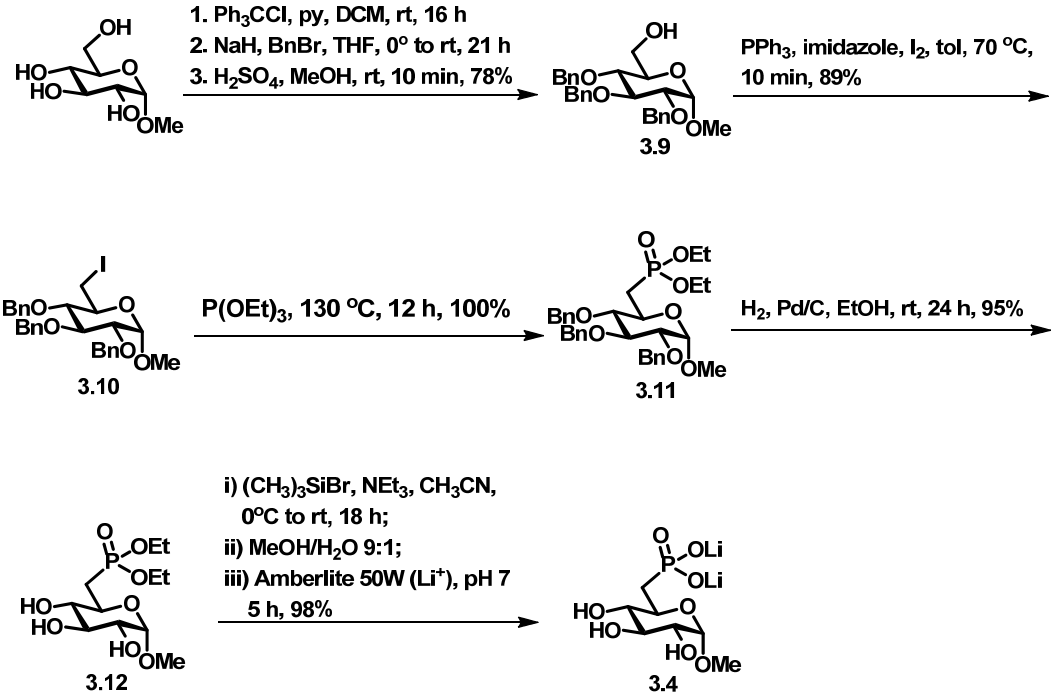
In the design of these targets, we considered that the target enzyme, G6Pase, catalyzes the last step in glycogenolysis where the glycogen substrate contains α-(1-4)- and α-(1-6)-linkages.[30] Thus, we planned to synthesize these analogues as α-methyl glucosides, which has the benefit of preventing mutarotation at the anomeric position. To provide control compounds for any biological testing, we planned to first synthesize phosphonate analogues **3.4** and **3.5**.

Our discussion to this point has focused on previously synthesized G6P and M6P

analogues. It is important to realize there are no reports of α-bromophosphonate analogues in carbohydrate-based molecules. The α-bromo analogue functional group in **3.6** and **3.7** has not previously been installed in an exocyclic position of a carbohydrate. We describe in the forthcoming sections, our efforts into synthesizing these novel α-bromo analogues.

3.2 Synthesis of G6P analogues 3.4 and 3.5

Analogue **3.4** has been synthesized as an anomeric mixture previously.[11] As a result, chemistry to synthesize this analogue was already available to us. We followed the route reported by Montero and co-workers in the synthesis of M6P derivative **3.8** (**Scheme 3.1**).[26]



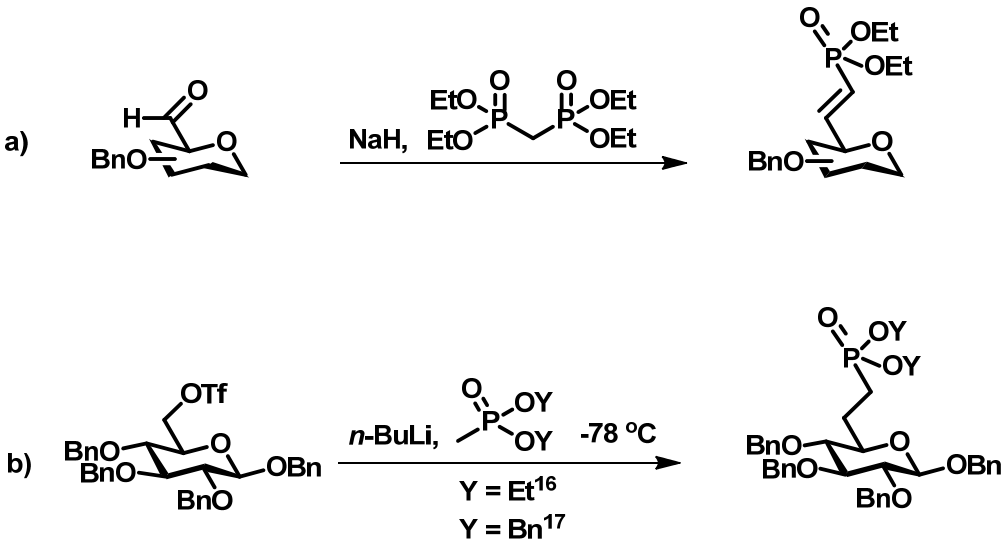
Scheme 3.1: Synthesis of G6P analogue 3.4.

We began our synthesis with commercially available α-methyl glucoside, which was converted in three steps to the 2,3,4 tri-benzyl α-methyl glycoside, **3.9** in 78% overall yield.[28, 31-32] Although Montero and coworkers used the *C*6-bromide intermediate, we chose to employ the *C*6-iodo derivative **3.10** instead, which was obtained in good yield using standard conditions.[26, 33] With compound **3.10** in hand, we employed a Michaelis-Arbuzov reaction, which converted the *C*6 iodide to phosphonate **3.11** in quantitative yield.[34] Removal of the benzyl groups from **3.11** was achieved with standard hydrogenation conditions to afford **3.12** in high yield. Deprotection of the phosphonate diester required modification from

standard conditions, which typically involve trimethylsilyl bromide (TMSBr) in MeCN or CH_2Cl_2 at room temperature for 12 h.[35] We found TMSBr to be acidic enough to cleave the glycosidic linkage, resulting in a mixture of anomers.[36] Montero had encountered the same problem, which they solved by buffering TMSBr with anhydrous pyridine.[26] This protocol results in a NaBr salt, which we found difficult to separate from the desired product. Instead, we used anhydrous triethylamine (NEt_3)[37] followed by ion exchange with cationic resin to the Li^+ salt. Excess LiBr was removed by soaking the final compound in ether, followed by addition of ethanol dropwise to dissolve the salt, followed by filtration of the compound to afford pure **3.4** in 92% yield.[38] The overall yield of compound 3.4 over 7 steps from α-methyl glucoside was 61%, a reasonable improvement previous reports for compounds **3.1** (26%)[11] and **3.8** (31%).[26]

We then turned our attention to the synthesis of the heptose phosphonate analogue of G6P, compound **3.5**. The key step in the synthesis of this analogue was the homologation of *C*6 to form the saturated deoxy-heptose. Two general strategies have been reported to solve this problem. The groups of Montero,[26] Padyukova,[10] and Roach[14-15] used oxidation of *O*6, followed by a Horner-Wadsworth-Emmons (HWE) condensation to arrive at a homologated and unsaturated intermediate that could be reduced concomitantly with hydrogenolysis of the benzyl groups (**Scheme 3.2a**). Berkowitz and co-workers[17, 20-21] addressed the *C*6 homologation via installation of a triflate at *O*6 and a subsequent S_N2 displacement with a

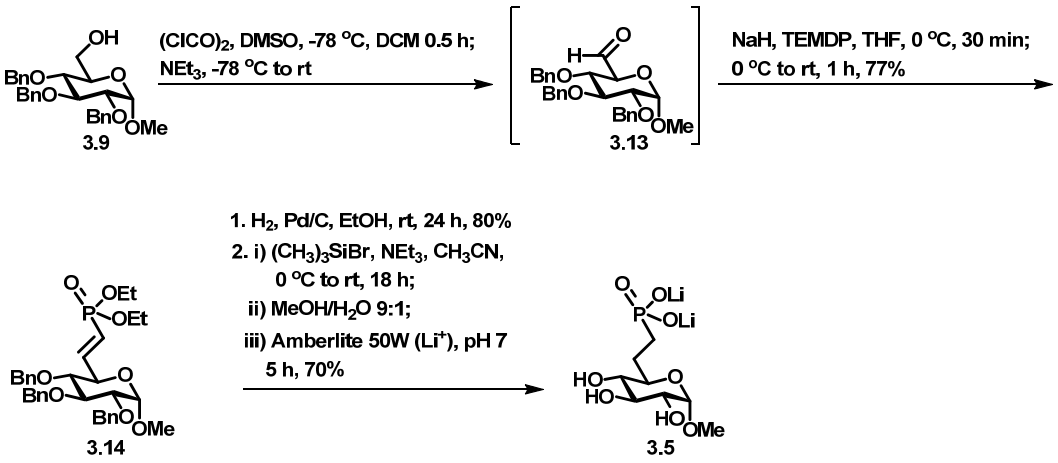
lithium phosphoenolate generated *in situ*, providing the target deoxy-heptose (**Scheme 3.2b**).



Scheme 3.2: a) The key step of the Montero,[26] Padyukova[10] and Roach[14-15] synthesis, an HWE reaction. b) The key step in the Berkowitz syntheses, an S_N2 displacement with a lithium phosphorus-stablilized anion.[16-17]

Although we tested both strategies, we found that the HWE strategy gave higher yield. Our route to compound **3.5** is illustrated in **Scheme 3.3**. Starting from glucose analogue **3.9**, Swern oxidation of *O*6 to the aldehyde was followed by addition of tetraethylmethylene diphosphonate (TEMDP) as the phosphorus-stablilized anion. This furnished the *trans* alkene in good yield over two steps. Although we attempted to oxidize *O*6 using both 2-iodoxybenzoic acid (IBX)[39]

and Dess-Martin Periodinane (DMP),[40] the yields in both cases were much lower than under Swern conditions. The olefin of **3.14** was reduced concomitantly with benzyl deprotection using standard hydrogenation conditions in moderate yield. The phosphonate was then deprotected using TMSBr/NEt_3 conditions as described for **3.4**, to afford **3.5** after seven steps and 34% overall yield. Our yields were comparable to those reported by the Montero (36%)[26] and the Berkowitz groups (38%).[16]

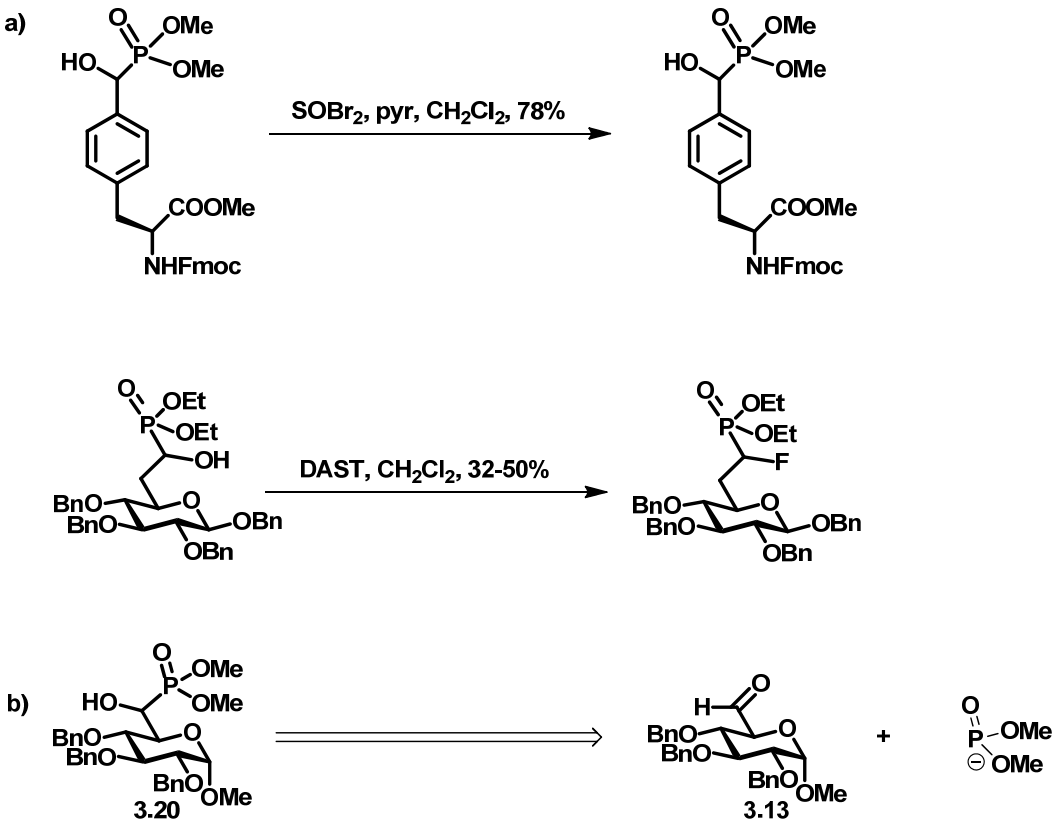


Scheme 3.3: The synthesis of G6P analogue 3.5 via HWE reaction.

3.3 Initial design and retrosynthesis of α-bromo analogue 3.6

With G6P analogues **3.4** and **3.5** in hand, we focused our attention on designing a route to the novel α-bromo analogue, **3.6**. In our intial retrosynthesis, we drew inspiration from the Berkowitz lab[16] as well as previous work in our lab in the

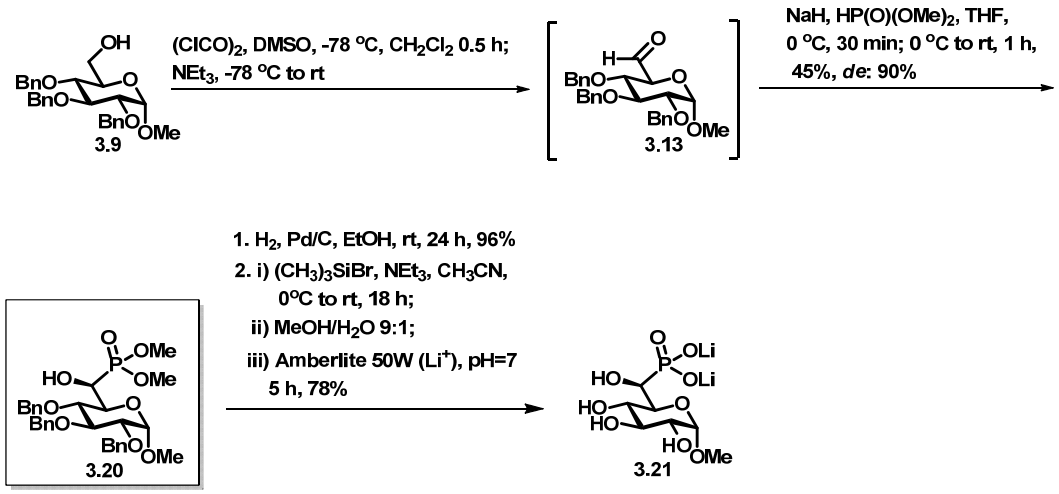
synthesis of **BrPmP**.[29] In both the Berkowitz lab and our group the pivotal halogenation step was carried out via an α-hydroxy phosponate intermediate as depicted in Scheme **3.4a**. This was the first route we chose to test, which would require access to the precursor α-hydroxy derivatives, compounds **3.17** and **3.19**. We were able to generate the hexose α-hydroxy derivative, **3.17**, in one new step using the Pudovik reaction.[41] The reaction proceeds by nucleophilic addition of the dimethyl phosphite anion into *C*6 aldehyde **3.13** (**Scheme 3.4b**).



Scheme 3.4: a) Precedented halogenations from an α-hydroxy precursor. The benzylic bromination is previous work from the Cairo group.[29] The fluorination of the G6P moiety is work from Berkowitz et al.[16] b) Access to the pyranoside α-hydroxy moiety from the *C*6 aldehyde.

In an analogous strategy, we generated compound **3.20** by a three step synthesis summarized in **Scheme 3.4b**. Aldehyde **3.13** was cannulated into a stirring solution of deprotonated dimethylphosphite affording bromination precursor **3.20** in only modest yield. We attempted to improve the yield using diethylphosphite as

the phosphonate donor (36%), as well as CsF[29, 42] (inseparable side products) or NEt_3 (28%)[43] as the base for the deprotonation; however neither of these conditions improved the yield. We were pleased to observe that the transformation of compound **3.13** to **3.20** was stereoselective, and furnished the *S* isomer in >90% *de*. Satisfyingly, the observed stereochemistry was consistent with the Cram model.[44] Deprotection of compound **3.20** could be executed by hydrogenolysis (96%) and phosphonate ester cleavage (78%) in good yield, providing the L-*glycero* G6P analogue **3.21** in seven steps and 26% overall yield.



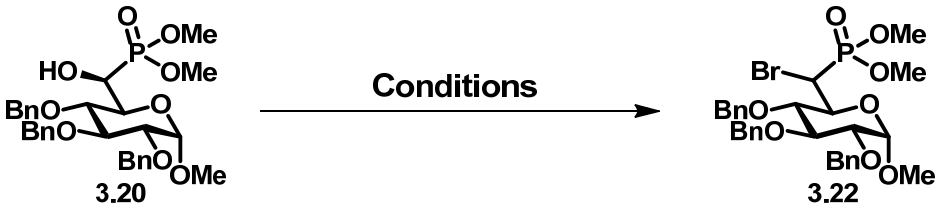
Scheme 3.5: Synthesis of bromination precursor 3.20 and G6P analogue 3.21.

With compound **3.20** in hand we set out to identify suitable α-bromination conditions. We first tested the thionyl bromide ($SOBr_2$)-pyridine conditions our group has previously employed in the synthesis of BrPmp.[29, 45] Unfortunately, no

reaction was observed, despite a number of attempts. Further examination of the literature provided a number of alternative bromination conditions, which were attempted with compound **3.20**, summarized in **Table 3.1** below. It is worth mentioning that entry 2 in **Table 3.1** offers the only example in the literature of an α-halogenation via an α-hydroxy intermediate of an aliphatic phosphonate.[46] Unfortunately, all of these conditions resulted in either no reaction or degradation of the starting hydroxy-phosphonate. Attempts to buffer the reaction with pyridine (entry 2, **Table 3.1**), to manage any formation of HBr in these conditions did not prevent degradation of the starting material. We postulate that this may be a result of CBr_4 acting as a radical initiator, which would result in benzyl group deprotection. It should be noted that only the second example of a successful aliphatic α-bromination employing PPh_3Br_2/pyridine (~50% yield) was reported in 2011[47] while our studies were underway. However, all previous electrophilic bromination attempts had failed in our hands, we do not expect these conditions to be compatible with our substrate (entry 9, **Table 3.1**).

After trying all of these conditions without success we thought it was time to turn our attention from the *C*6-homologated target **3.6**, and focus on the heptose α-hydroxy derivative in the event that steric congestion caused by the close proximity of the hydroxyl group to the carbohydrate ring was interfering with the reactivity of compound **3.20**. We also confirmed that the deprotection of

compound **3.20** was achieved via hydrogenolysis (96%) and the phosphonate ester cleavage (78%) as shown in **Scheme 3.5**. We obtained the L-*glycero* G6P analogue **3.21** seven steps and 26% overall yield.

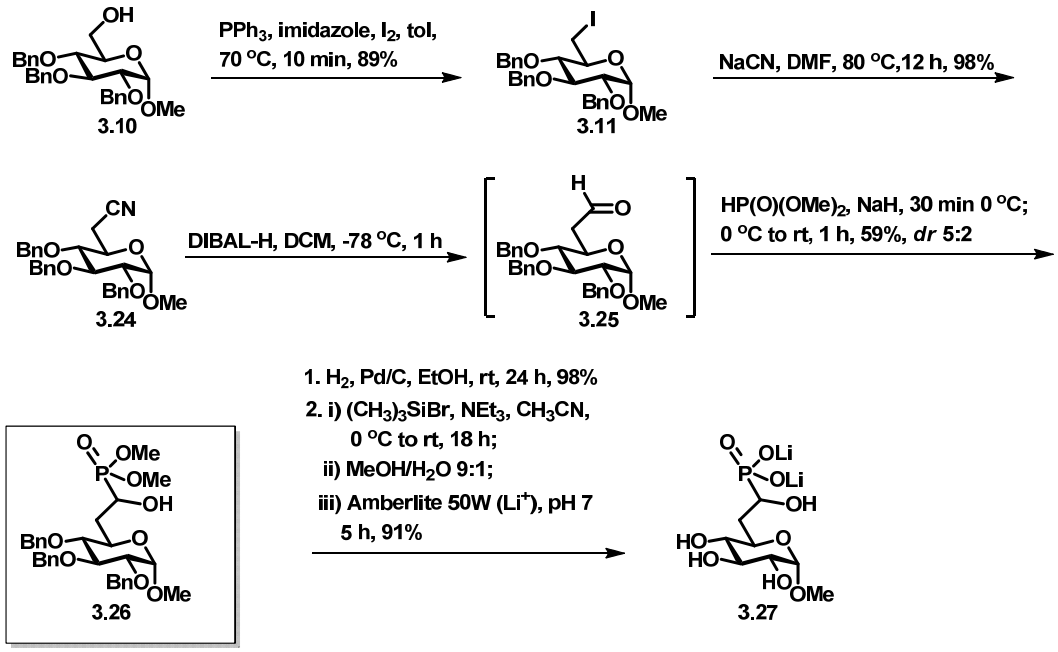


Entry	Reaction conditions	Outcome	Reference
1	$SOBr_2$, pyridine	No reaction	Tulsi et al., 2010[29] Gross et al., 1993[45]
2	CBr_4, PPh_3	Degradation	Jiang et al., 2007[46]
3	CBr_4, PPh_3, pyridine	Degradation	Gajda, 1990[48]
4	DDQ, $^tBuNH_4Br$, PPh_3	Degradation	Firouzabadi et al., 2004[49]
5	NEt_3,MesCl; LiBr	No reaction	Bernotas et al., 1987[31]
6	$SOBr_2$	No reaction	Kumaraswamy et al., 1997[50]
7	DMS, NBS	No reaction	Raghavan et al., 2006[51]
8	PBr_3	Degradation	Kumara et al., 2007[52]
9	PPh_3Br_2, pyr	No reaction	Kumar et al., 2004[53]

Table 3.1: Attempts at α-halogenation of precursor 3.20.

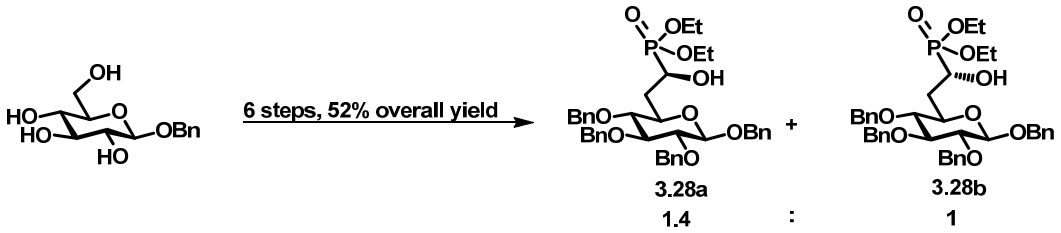
Analogous to the synthesis of the fully saturated G6P analogue **3.5**, the key step in

accessing the α-hydroxy precursor to compound **3.7** was the homologation at *C*6. We still envisioned utilizing the Pudovik reaction; however, in this case the dimethyl phosphite anion would react with a *C*7 aldehyde, such as compound **3.25**, to furnish the desired α-hydroxy precursor. **Scheme 3.6** summarizes the route we used to obtain compound **3.27**. The free alcohol of **3.10** was converted to the *C*6-iodo derivative, subsequent nucleophilic displacement by cyanate anion was performed followed by reduction to the corresponding aldehyde **3.25** using diisobutylaluminum hydride (DIBAL-H).[54] Pudovik reaction of the crude material furnished α-hydroxy analogue **3.26** in moderate yield as a 5:2 *S*:*R* mixture of diastereomers. We can explain the partial selectivity for the *S* isomer using the Cram model.[44] Unfortunately, we were unsuccessful in our attempts to separate the diastereomers of **3.27**, or in crystallization of compound **3.26**.



Scheme 3.6: Synthesis of the homologated bromination precursor 3.26

As mentioned, the Berkowitz group set up their α-phosphonofluorination reaction by utilizing an α-phosphonohydroxy precursor as part of their synthesis of **3.3** (**Scheme 3.4a**).[16] Berkowitz and coworkers arrived at the analogous β-benzyl derivative **3.28** in 7 steps and 52% overall yield from β-benzyl glucose (**Scheme 3.7**).



Scheme 3.7: Synthesis of the b-benzyl G6P analogues by the Berkowitz group.16

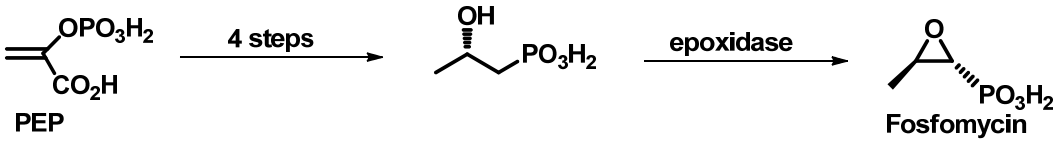
Berkowitz was able to separate the *R* and *S* isomers of **3.28** using column chromatography, which may be attributed to the differing stereochemistry and the bulkier aglycone (**Scheme 3.7**). We used sodium hydride (NaH) as the base for the Pudovik reaction and can explain our selectivity using the Cram model. In contrast, the Berkowitz group used lithium hexamethyldisialazide (LiHMDS), and obtained partial selectivity for the *R* isomer which may be caused by the increased bulkiness of the anomeric protecting group as a result of the phosphite anion adding into the opposite side of the aldehyde. It is important to mention that our stereochemical assignments for both **3.20** and **3.26** were based on the reports from Berkowitz et al.[16]

Armed with precursor **3.26**, we again attempted bromination of the α-hydroxyphosphono substrate with $SOBr_2$[29, 45] on its own, and $SOBr_2$[50] buffered with pyridine; both to no avail. Therefore, we concluded that the lack of reactivity in **3.20** was not due strictly to steric factors. Deprotection of compound **3.26** to

generate **3.27** gave an overall yield of 37% over 9 steps. At this point, we considered alternative strategies to obtain α-electrophilic phosphonate functionality.

3.4 Design and efforts into α,β-epoxy- and α-keto-phosphonates

A review of the literature identified two known electrophilic phosphonate functional groups that might be reasonably obtained from starting materials we already had available. Both α,β-epoxy- and α-keto-phosphonates are known. Fosfomycin is a unique clinically utilized antibiotic that inhibits cell wall biosynthesis in *E. coli*, and features an α,β-epoxyphosphonate moiety.[55] The compound is biosynthesized in five steps from phosphoenolpyruvate (PEP) by *Streptomyces fradiae* with epoxidation occurring in the terminal step by an epoxidase enzyme (**Scheme 3.8**).[55-56]

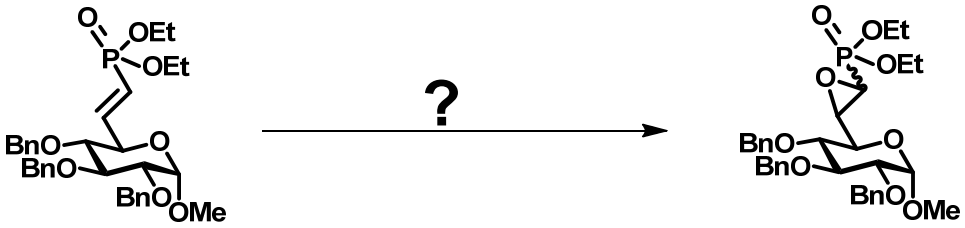


Scheme 3.8: Biosynthetic pathway from PEP in 5 steps to fosfomycin.[56]

Previous approaches to epoxidation were carried out via an α-bromo-β-hydroxyphosphonate, followed by displacement of the bromide and closure of the

expoxide ring in the presence of strong base,[57-58] or via direct epoxidation of an α,β-unsaturated phosphonate using an oxidizing agent (references in **Table 3.2**). Both of these strategies were attempted using olefin **3.14**.

We attempted the first oxidation strategy by stirring unsaturated analogue **3.14** in H_2O and *N*-bromosuccinimide (NBS) to install the α-bromo-β-hydroxyphosphonate moiety.[59-60] Unfortunately, after three days no reaction with the olefin had occurred so we evaluated alternative approaches. Since alkene **3.14** was unresponsive to treatment with H_2O/NBS, we envisioned a direct olefinic epoxidation strategy could be more feasible. We explored a number of conditions which are summarized in **Table 3.2**. It should be noted that we tested both the ester-protected and the free acid phosphonate substrates as we expected the phosphonate deprotection conditions would also open the epoxide.



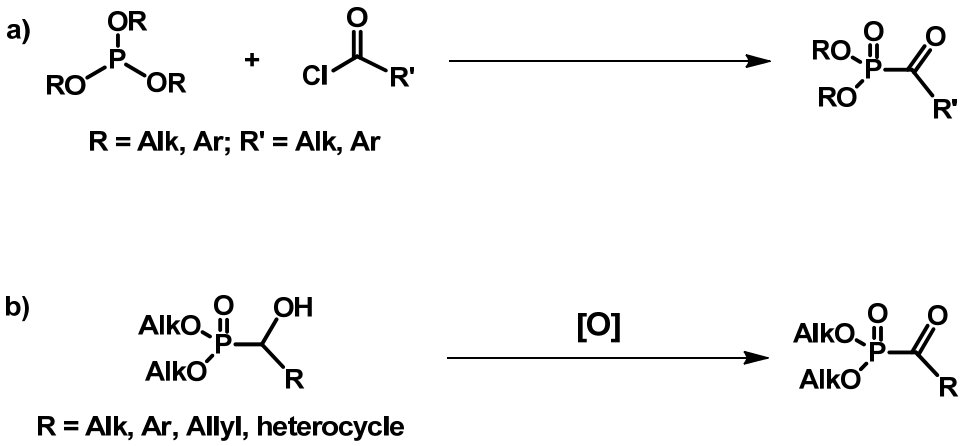
Attempt	Starting material	Outcome	Reference
1	$Na_2WO_4 \cdot 2H_2O$, NEt_3, H_2O_2	No Reaction	Ono et al., 2006[61] McGrath et al., 2009[62]
2	DMDO, acetone	No Reaction	Adam et al., 1991[63]
3	NaOCl, pyr., 65˚C	No Reaction	Marmor, 1963[64]
4	H_2O_2, 6M NaOH, MeOH	No Reaction	Katsumara et al., 1989[65]
5	Oxone©, $NaHSO_4$, butanone, MeOH	Trace	Cristau et al., 1998[66]
6	m-CPBA	No reaction	Coutrot et al., 1998[67]

Table 3.2: Attempts at the epoxidation of unsaturated analogue 3.14

None of the six conditions shown in Table **3.2** were successful in oxidation of the olefinic phosphonate. Entries 1, 5 and 6 have previously been used to successfully epoxidize α,β-unsaturated phosphonates, albeit in simple alkyl phosphonates with less functionality than compound **3.14**. Thus, we decided to cease our pursuit of α,β-epoxyphosphonates at this point.

Another well studied[68] electrophilic phosphonate functional group we postulated

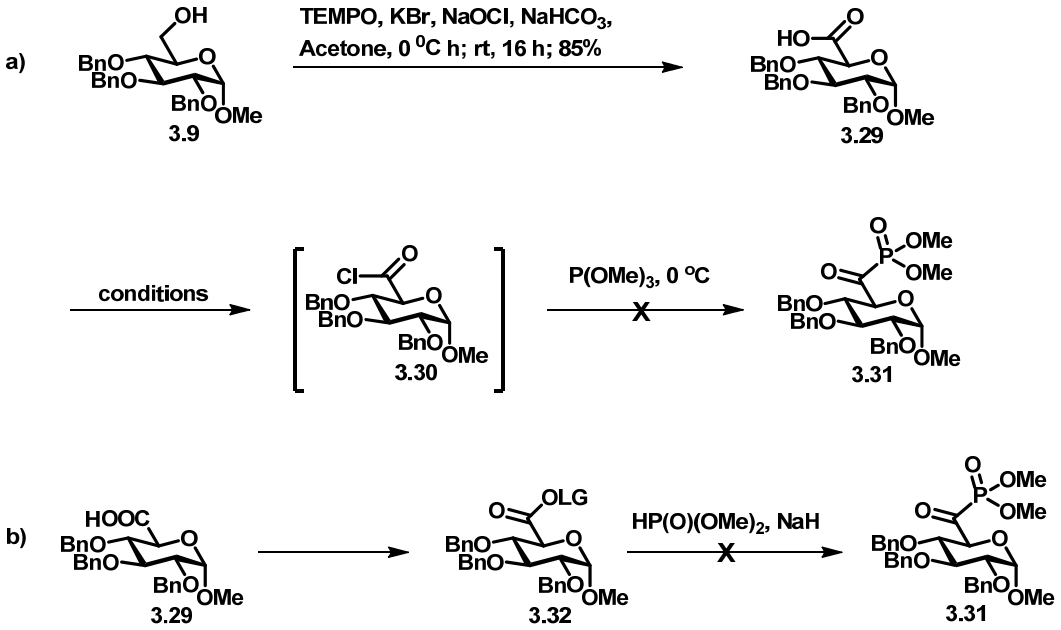
could be viable was an α-ketophosphonate. There are many examples in the literature with two basic approaches to their synthesis, which are outlined in Scheme **3.8**. The first approach utilizes a Michaelis-Arbuzov reaction on an acid chloride (**Scheme 3.9a**),[69-71] the second approach requires oxidation of an α-hydroxy phosphonate to the α-keto moiety (**Scheme 3.9b**).[68]



Scheme 3.9: Two general approaches to access α-keto phosphonates.

Both routes made use of substrates to which we already had access. The route shown in **Scheme 3.9a** could be accessed by converting 6-hydroxyl compound **3.9** into α-methyl glucuronic acid using a 2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) oxidation[72] followed by conversion into the acid chloride setting up the crucial Michaelis-Arbuzov reaction. The oxidation shown in **Scheme 3.9b** could be attempted from either α-hydroxy precursor **3.20** or **3.26**. We chose first to pursue route **3.9a** as this approach has been better studied and more literature is

available.[68] **Scheme 3.10** summarizes our results.



Scheme 3.10: Attempts at α-ketophosphonate 3.31 via a) Michaelis-Arbuzov reaction and b) Pudovik reaction.

The α-methyl glucuronic acid was easily accessed from precursor **3.9** using the standard TEMPO oxidation;[73-74] however, the two subsequent steps did not yield our desired target. Acid chloride **3.30** was too unstable to work up, so the Arubuzov reaction was carried out immediately after the acid conversion. Unfortunately, through pathway **3.10a** we were never able to isolate the α-ketophosphonate **3.31**. Furthermore, we needed the methyl glycoside to remain intact, we had to explore a number of acid chloride conditions that would not also

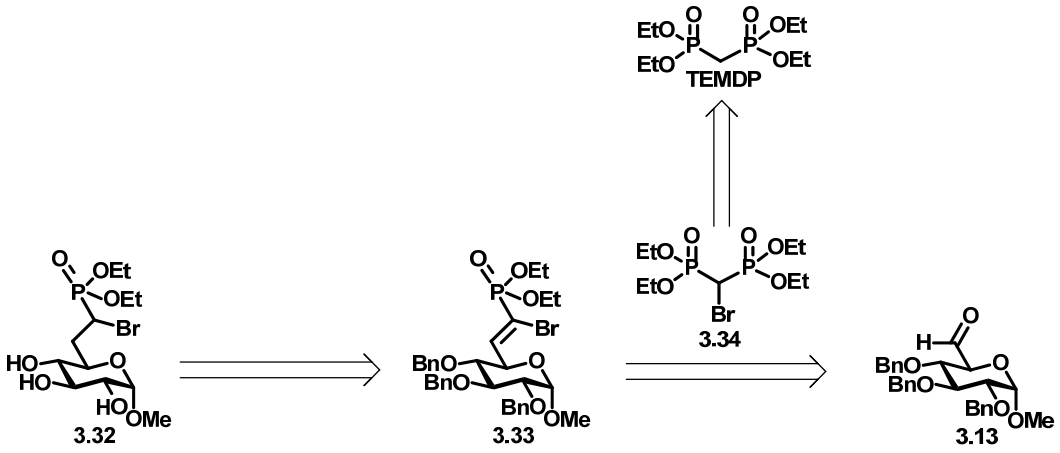
destroy the glycosidic linkage. We tried oxalyl chloride,[69-71] phosphoryl chloride[75] and phosphorus trichloride;[76] however, no product was observed.

At this point we turned our attention to pathway **3.10b**, which was to convert glucuronic acid derivative **3.29** into an activated ester followed by Pudovic reaction. We attempted to activate **3.29** using *N*,*N*′-dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*,*N*-carbonyldiimidazole (CDI)[77] followed by the Pudovic reaction; however, we did not observe any formation of **3.31**. We then turned our attention to the α-hydroxy oxidation of analogues **3.20** or **3.26** (**Scheme 3.9b**). We tried a number of literature procedures for this type of oxidation exist them; however, once again we met resistance with this route. Attempted strategies included: the Swern oxidation (degradation),[78] potassium permanganate ($KMnO_4$) (no reaction)[79] and chromium trioxide/aluminum dioxide (CrO_3/Al_2O_3) (no reaction).[80] We concluded that the reactivity at α-phosphono position of carbohydrate analogues is greatly reduced, complicating the use of these methods.

3.5 Indirect α-bromination strategies using HWE reaction

We have shown that installation of electrophiles at the α-phosphonate position of carbohydrates cannot be approached by conventional means. At this point, we considered that installation of an electrophilic site would require a more indirect

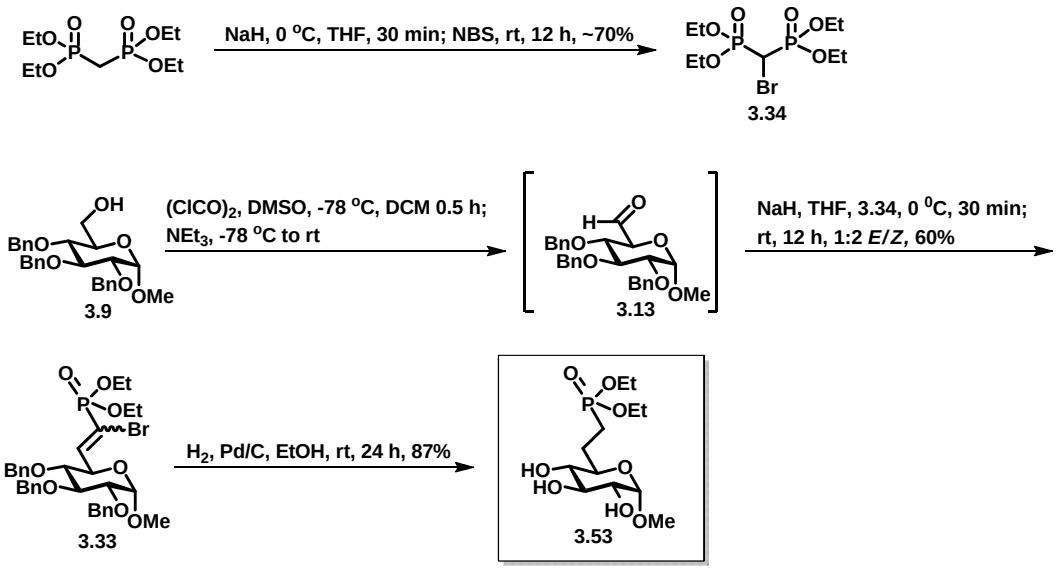
approach. We considered bromination of the α-phosphono position through generation of the corresponding phosphorus-stablilized anion. This approach would be analogous to the use of TEMDP in the synthesis of G6P analogue **3.5** (**Scheme 3.3**), and HWE reaction using aldehyde **3.13.** By employing a brominated form of the reagent we would have access to an unsaturated α-bromophosphonate. Subsequent reduction of the double bond via hydrogenation would provide access to the target α-bromophosphonate. **Scheme 3.11** depicts this strategy retrosynthetically.



Scheme 3.11: Retrosynthesis of 3.32 heptose α-bromo moiety via an HWE reaction.

Reagent **3.34** had been previously reported as a substrate for a HWE reaction.[81] Treatement of TEMDP with base and quenching with an electrophilic bromine source should furnish **3.34**.[82-83] In our hands, deprotonation of TEMDP using NaH

followed by addition of NBS yielded ~70% conversion to analogue **3.34**, ~15% of the analogous dibromo compound and ~15% unreacted TEMDP. A second treatment of **3.34** with NaH followed by addition of aldehyde **3.13** furnished compound **3.33** as a 1:1.5 *E*-*Z* ratio (based on the assignment reported by Kobayashi and William, 2002)[84] that was easily separable using column chromatography in good overall yield in three steps (14% *E* isomer, 27% *Z* isomer) (**Scheme 3.12**).

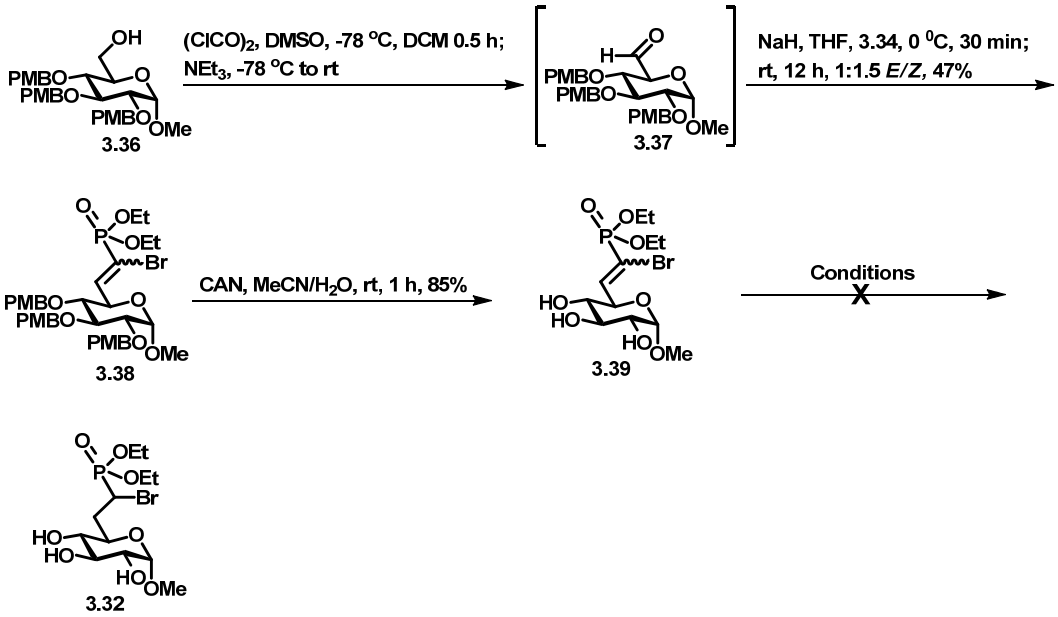


Scheme 3.12: Synthesis of α-bromo unsaturated analogue 3.33

With **3.33** in hand we were faced with the problem of debenzylation and reduction in the presence of bromine. Standard hydrogenation conditions using palladium

on charcoal in ethanol resulted in complete cleavage of the bromide to generate **3.53**.

We employed an alternative protecting group strategy for the 2,3,4-hydroxyl groups through the use of *para*-methoxybenyl (PMB) groups instead of benzyl protection. We synthesized the free *O*6 compound, **3.36**, according to the synthetic procedure of Tennent-Elyes et al. with 42% overall yield in three steps.[85] Following the same HWE sequence as in the synthesis of **3.33**, we arrived at **3.38** in 47% yield in a 1:1.5 *E*-*Z* ratio (**Scheme 3.13**).

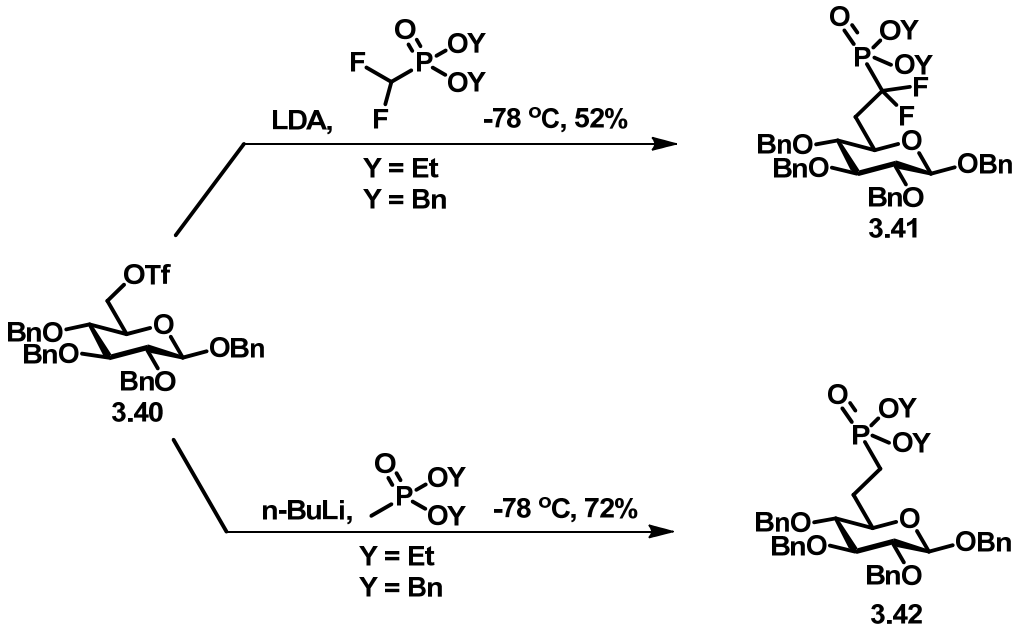


Scheme 3.13: Attempts at accessing analogue 3.32 using PMB protection/deprotection strategy

We then utilized the standard ceric ammonium nitrate deprotection strategy[86] to arrive at **3.39** in 85% yield for both the *E* and *Z* isomers. Attempts at hydrogenation conditions that would spare the bromine atom were unsuccessful. Conditions attempted included Crabtree[87] and Wilkinson's catalysts;[88] however, we were unable to observe any reduction of the double bond using either catalyst. This transformation is clearly challenging and to our knowledge would be an unprecedented manipulation. However, due to the failure of any conditions we attempted, we did not fully characterize these compounds, but instead decided to revaluate our strategy.

3.6 Revisiting direct bromination strategies

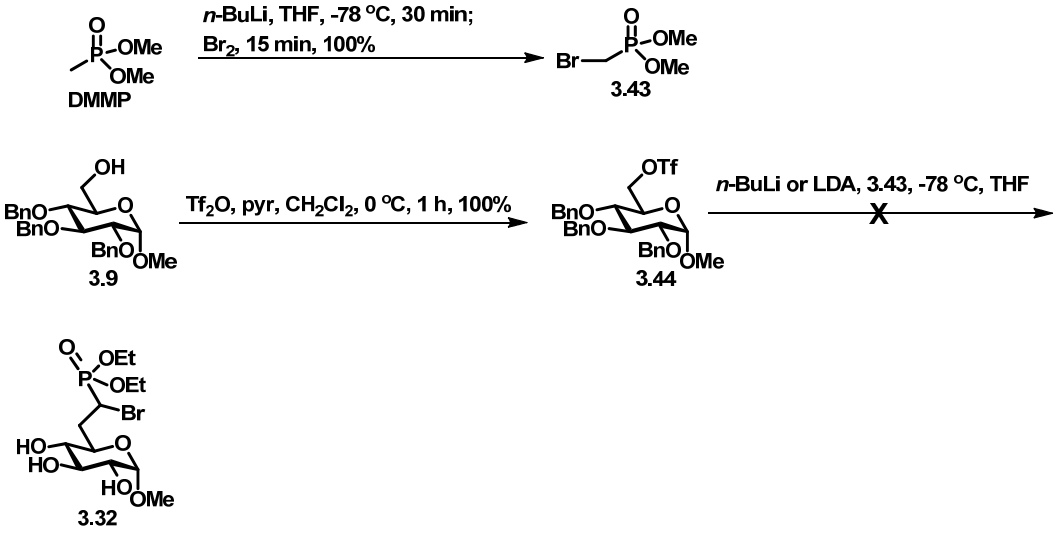
We decided to re-visit α-halogenation strategies before devising another route to our desired α-brominated product. We had already tested Berkowitz's approach to mono-fluorination[16] and found it to be incompatible with our α-bromination strategy. However, what we had not tested was whether we could utilize their difluorination pathways to access our brominated targets. Previous work by the Berkowitz group is summarized in Scheme **3.14**.[17, 22-23]



Scheme 3.14: Previous synthetetic routes by the Berkowitz group to access the α,α-difluorophosphonate 3.41[17, 22-23] and the fully saturated 3.42.[16-17]

The strategy shown in **Scheme 3.14a** seemed feasible, as long as we could first brominate a phosphonate precursor setting up an S_N2 displacement to provide the α-bromo target. We thought the most logical and economical way to the α-bromo dimethyl phosphonate **3.43** would be to treat dimethylmethyl phosphonate (DMMP) with *n*-butyl lithium (*n*-BuLi) at low temperature, followed by quenching with bromine. We were delighted to find this reaction yielded the mono brominated product, **3.43**, in quantitative yield (**Scheme 3.15**). To the best of our knowledge, this transformation is unprecedented. Although the triflate compound, **3.9**, was generated in quantitative yield (following a similar protocol to that

reported by the Berkowitz group);[16] treatment of **3.43** with both lithium diisopropyl amide (LDA) and *n*-BuLi did not result in a reaction with triflate **3.44**.

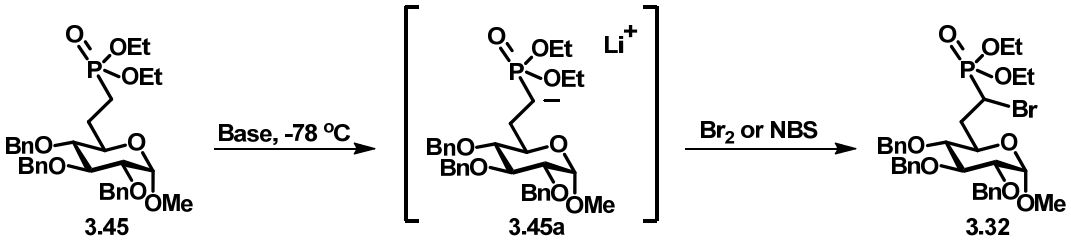


Scheme 3.15: Attempts to prepare α-bromo adduct 3.32 by nucleophilic triflate displacement.

Although disappointing, these results may not be terribly surprising. α-Bromo derivitives such as **3.43** have a tendency to form reactive carbenes competitively with carbanions in the presence of strong base even at temperatures as low as -78 ^{o}C.[89]

Although this pathway was unsuccessful overall, we were able to recognize two key points from schemes **3.13** and **3.14** that allowed us to formulate a new synthetic approach to compound **3.32**. The first was that unsaturated analogue

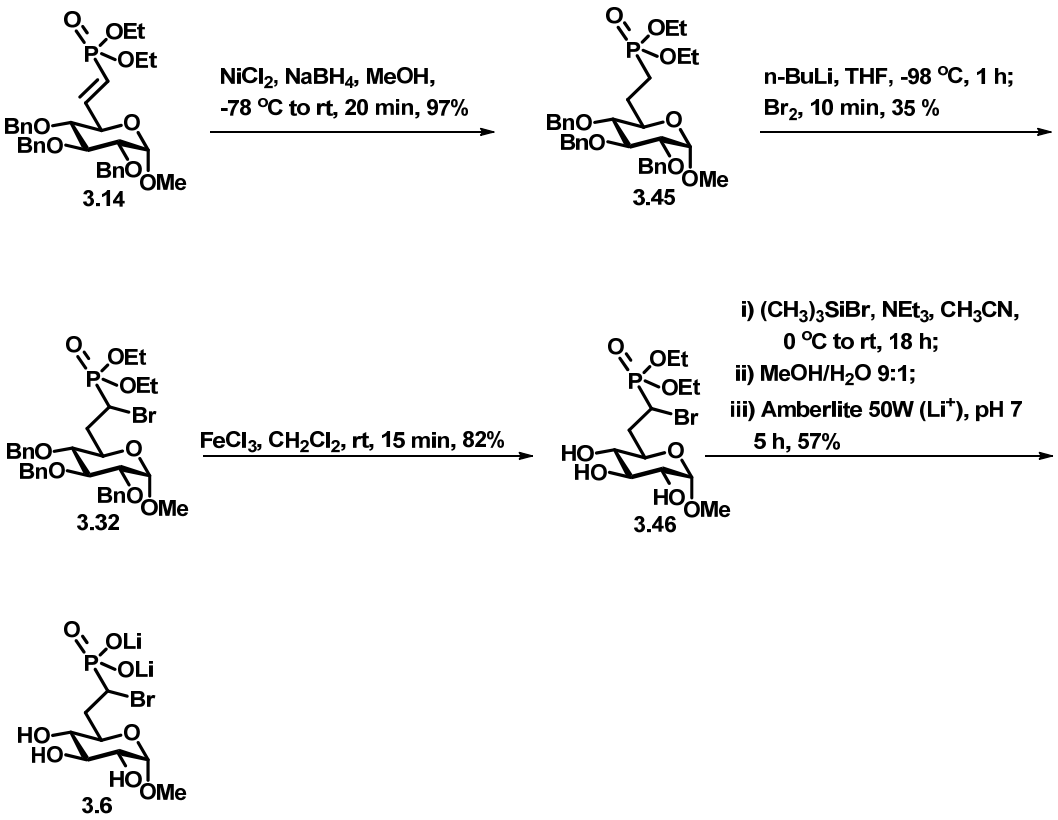
3.42 (**Scheme 3.14**) can be readily synthesized and secondly that DMMP can be quantitatively brominated. This led us to postulate that if we first synthesized the unsaturated phosphonate analogue, **3.45**, we could then perform an α-bromination by quenching of intermediate, **3.45a**, with an electrophilic bromine source to furnish compound **3.32** directly (**Scheme 3.16**). Structural studies on species such as **3.45a** have been published.[90] Cantat et al. confirmed that anionic C-P bonds are shorter than in saturated P-C bonds through crystallography. Computational analysis by the authors suggest there is stabilization by hyperconjugation resulting from p(C)→σ*(P–O) donation.[90] We will refer to species of the form **3.45a** as a phosphorus-stabilized anion. Importantly, Cantat et al. also suggest that the Li cation is shared between the O=P–C atoms.[90]



Scheme 3.16: Proposed route to α-bromination by forming a phosphorus-stablilized anion intermediate.

With this hypothesis in mind we set about to synthesize precursor **3.45**. In the synthesis of G6P analogue **3.5** (**Schemes 3.2** and **3.3**) we had two routes available

to us that could result in compound **3.45**, via either a HWE reaction[10, 14, 26] or an S_N2 reaction.[16-17] Although Berkowitz reported good yields in his key S_N2 displacement step on the β-benzyl glycoside, we found the yields on our α-methyl glycoside to be less reliable due to difficulties in the purification. Being cognizant of this, we opted to approach **3.45** via the HWE reaction as we had done previously. Furthermore, we were aware of conditions that could selectively reduce a conjugated olefin substituent while leaving the benzyl protecting groups in tact using sodium borohydride ($NaBH_4$) and $NiCl_2{\cdot}6H_2O$.[91-92] Scheme **3.17** summarizes our approach in this route.



Scheme 3.17: Synthesis of α-bromo G6P analogue 3.6

With compound **3.14** in hand we were ready to attempt the olefin reduction to the fully unsaturated derivative, **3.45**, leaving the benzyl groups intact. We were satisfied to observe that stirring compound **3.14** with $NaBH_4$ and $NiCl_2{\cdot}6H_2O$ in MeOH at -78 °C[91] provides precursor **3.45** in near quantitative yield without purification. This reaction is thought to proceed through a nickel boride intermediate that catalyzes the hydrogenation.[92] We duplicated the conditions used to brominate DMMP (**Scheme 3.15**) by adding *n*-BuLi dropwise to a cooled

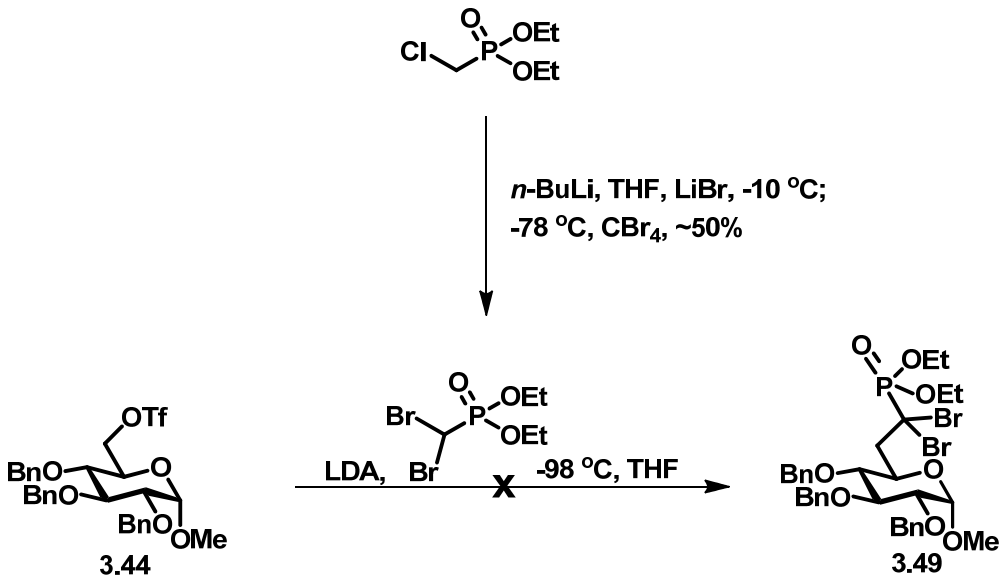
solution of **3.45** and the reaction was left to stir for 30 minutes. After 30 minutes the phosphorus-stablilized anion was quenched by addition of Br_2 followed by warming to room temperature. We observed formation of the protected α-bromo analogue, **3.32**, as a 1:1 mixture of diastereomers after purification on silica in moderate yield (35%). Although this strategy is not wholly unprecedented on simple organic substrates[93-94] as discussed in **Chapter 1**, we believe this to be the first report of this chemistry on a carbohydrate analogue. Although we had obtained the desired intermediate compound **3.32**, there remained three major issues to address. The first was optimization and reproducibility of the reaction. After our initial attempts to generate compound **3.46,** we were unable to reproduce our initial yield. Furthermore, we sometimes obtained no product at all, and instead obtained numerous uncharacterized side products. We attempted the same procedure using alternative bases, including LDA, LiHMDS, *s*-BuLi and *t*-BuLi. LDA and LiHMDS offered very poor yields, and *s*-BuLi and *t*-BuLi increased the presence of side products. We also tried to utilize NBS and dibromoethane in lieu of bromine, however, the yields again were very poor. We considered that the propensity of alkyl bromides to form carbenes in the presence of very strong base could be responsible for these issues. To explore this possibility, we attempted the reaction using more controlled conditions at lower temperature. Using a frozen methanol bath (-98 ^{o}C) and adding substrate **3.45** at a rate of 100 μL/min, we found the bromination reaction proceeded with a reliable

35% yield over multiple attempts.

With molecule **3.45** in hand we were left with the challenge of having to cleave the benzyl groups without using standard hydrogenation conditions. Several harsh alternative debenzylation conditions have been used in carbohydrate synthesis, including the Birch reduction[95] and tin(IV) chloride;[96] however, we were certain neither condition would be compatible with our substrate. Thus, we turned our attention toward milder Lewis acids that had been successful in cleaving benzyl ethers on carbohydrate substrates. We first tried boron trifluoride etherate ($BF_3{\cdot}Et_2O$) in ethanethiol;[97] however, no reaction took place. We then tried indirect debenzylation by conversion of the benzyl groups to acetyl groups followed by basic cleavage.[97] To achieve this we stirred **3.47** and trimethylsilyl trifluoromethansulfonate (TMSOTf) in acetic anhydride, once again no product was observed. Attempts to oxidize the benzyl ethers to the benzoyl ester using chromium trioxide[98] did not provide quantitative conversion to the product, making purification very difficult. The best condition we identified was the use of anhydrous iron(III) chloride in CH_2Cl_2[98-99] (**Scheme 3.17**). These conditions did not cleave the bromide or the glycosidic linkage, but did offer a difficult work up to remove the iron salts. We found that filtration of the crude black reaction mixture through Celite using methanol with 1% to 5% NEt_3 improved the overall yield of **3.48** from 40% to >80%. With **3.48** in hand our standard phosphonate ester deprotection steps furnished G6P analogue **3.7** without issue (9 steps, 9.5%

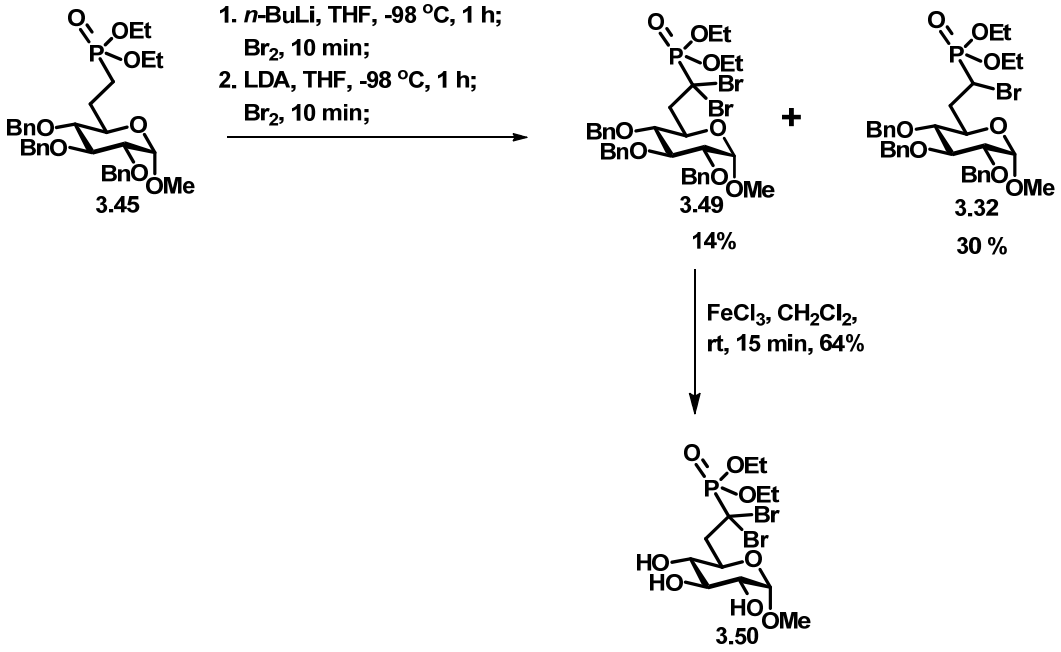
overall yield).

During the optimization process of compound **3.47**, we noticed something surprising in the ^{31}P nuclear magnetic resonance (NMR) of the crude product, a small peak at ~11 ppm was observed in several samples. Furthermore, mass spectral data indicated that a molecule with two bromine atoms was present in the sample. Unfortunately, the dibromo side product was only present in ~8% based on the crude NMR, and we were unable to isolate it on silica gel since the reaction conditions were not yet amenable to large scale syntheses. We hypothesized that the NMR and mass spectrometry (MS) data were consistent with dibromine analogue **3.49**, a functional group that has been reported in other simple molecules in the literature twice as an unwanted by product.[58, 100] We decided to pursue this novel carbohydrate analogue as a potentially interesting electrophilic phosphonate. Our initial tactic was to repeat Berkowitz's difluorination strategy from scheme **3.13** using the analogous dibrominated starting material (**Scheme 3.18**).[101] Furthermore, similar chemistry has been used to alkylate dibromo substrates previously,[101] so we postulated this route would be feasible. We were able to dibrominate diethyl chloromethylphosphonate in approximately 50% yield following precedent[102] which set us up for the low temperature triflate displacement. However, no displacement occurred, so we abandoned this route. We hypothesize that at such low temperature, there is not enough energy for such a sterically crowded carbanion to perform the displacement.



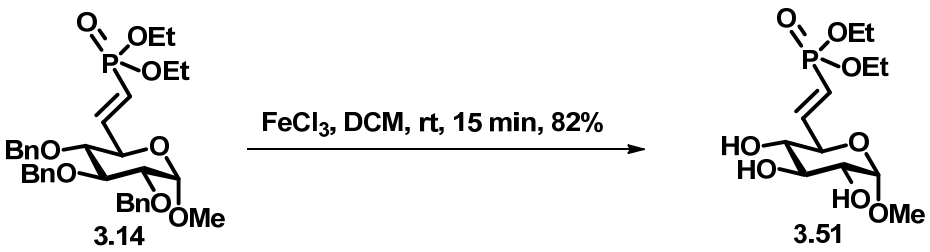
Scheme 3.18: Initial dibromination attempt to analogue 3.49.

We decided to take advantage of our observation of the dibromo side product in the bromination of the phosphoenolate of **3.45**. We re-subjected the crude product of the reaction with LDA at -98 ^{o}C, and once again quenched the reaction with Br_2 (**Scheme 3.19**). As we expected, we were able to isolate an improved yield of the dibromo compound, **3.49**, although in only modest yield (14%), along with the monobromo compound **3.47** in 30% yield. To complete the synthesis, we were able to obtain compound **3.50** without issue using $FeCl_3$ conditions for deprotection of the benzyl groups in moderate yield. We obtained **3.50** in ten steps and 5.2% overall yield.



Scheme 3.19: Synthesis of dibromo adduct 3.50.

Finally, as a demonstration of the optimized deprotection chemistry, we were able to deprotect the α,β-unsaturated compound, **3.14**, without disruption of the double bond. We considered that the resulting α,β-unsaturated phosphonate could act as an inhibitor of G6Pase by acting as a Michael acceptor for the nucleophilic His176 residue. This chemistry proceeded without any issue in 82% yield (**Scheme 3.20**).



Scheme 3.20: The deprotection of unsaturated analogue 3.14.

With a number of glycosyl phosphate analogues in hand, we turned our attention to biological testing of our compounds for inhibition of G6Pase.

3.7 Assaying deprotected phosphonate analogues of G6P against G6Pase

With five fully deprotected G6P analogues in hand (**Figure 3.4**), we turned our attention to biological assay of the compounds as inhibitors of G6Pase. G6Pase is difficult to purify due its instability when separated from the membrane of the ER;[103] however the enzyme is typically tested using microsomes isolated from hepatocytes.

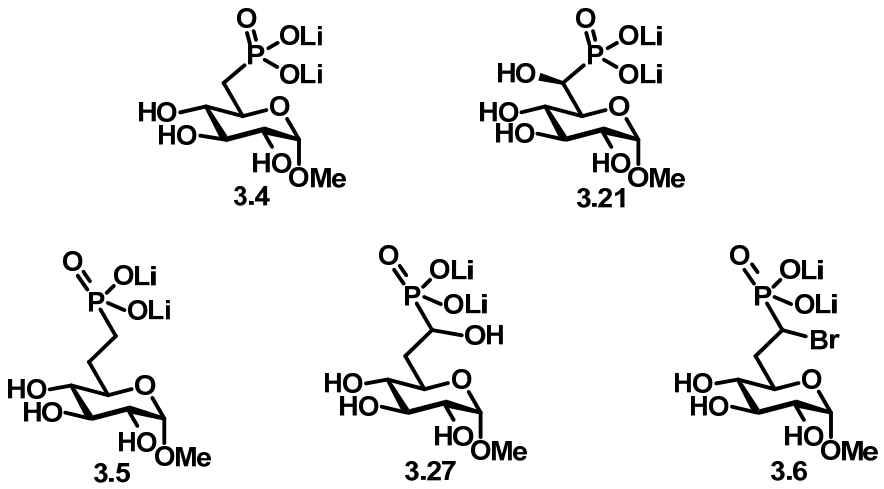


Figure 3.4: The five fully deprotected phosphonic acid G6P derivatives used in our initial microsome assays.

We opted to use male wister han rat microsomes for our studies, which are available commercially. We designed our assays using a variation of two procedures available in the literature (Sigma-Aldrich®[103] and Nordlie and Arion, 1966[105]) Assays were conducted in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer at pH 6.5, the pH optimum of G6Pase.[106] We also considered that the charge of our derivatives could present an issue for microsomal permeability. Therefore, we included Triton X-100 surfactant to disrupt the microsomal membrane according to previous reports.[107] Since G6Pase converts G6P to glucose and inorganic phosphate (P_i) in the ultimate step of gluconeogenesis and glycogenolysis, we envisioned the easiest way to assay this conversion would be to utilize a method that can monitor P_i or glucose production. Both such

techniques are known,[108-110] but the literature suggests that the best technique for the study of G6Pase activity in microsomes is to follow the formation of P_i. Three common methods for quantifying P_i production are through the use of radiolabelled ^{32}P,[111] the Fiske-Subbarow method,[108] and the Taussky-Shorr method.[109] We chose the Taussky-Shorr procedure, which determines P_i by utilizing a ferrous sulfate-molybdate reagent (the Taussky-Shorr reagent; TSR) in an acidic medium to reduce P_i and produce a clear blue colour that can be quantified using colorimetry.[109] We adapted this procedure to be compatible with a 96 well plate end-point assay.

With this assay in hand we were ready to analyze our compounds for inhibition of G6P hydrolysis activity. We screened all five phosphonic acids we had synthesized (**Figure 3.4**) at a concentration of 2.5 mM after an incubation period of 15 minutes at 37 ^oC and discovered that none of the synthetic analogues inhibited P_i production significantly (**Table 3.3**). We considered that this result could be due to the charged phosphonate compounds being unable cross the microsomal membrane to reach the enzyme active site.[103] We decided to incubate our analogues for an extended period of time with the microsome preparation at the same concentration, 2.5 mM. We first confirmed that the microsomes remained active at 4 ^oC after as long as 60 hours.

Compound		G6P hydrolysis (µmol/mg protein)	% activity
3.4	**Control**	0.98 ± 0.09	>95 %
	Inhibitor	1.0 ± 0.01	
3.21	**Control**	1.1 ± 0.02	>95%
	Inhibitor	1.1 ± 0.08	
3.5	**Control**	1.0 ± 0.07	92 ± 9.
	Inhibitor	0.94 ± 0.09	
3.6	**Control**	0.84 ± 0.1	88 ± 9
	Inhibitor	0.73 ± 0.07	
3.27	**Control**	0.92 ± 0.06	>95%
	Inhibitor	0.87 ± 0.07	

Table 3.3: Hydrolysis and % activity ± S.E. by the five deprotected phosphonic acids after short term incubation. Data were obtained after incubating disrupted microsome in MOPS buffer at pH 7.4 in the presence of inhibitor at 2.5 mM for 15 minutes at 37 ^{o}C. After the incubation the Taussky-Shorr method was employed as explained in **Section 3.9.3.1**. Pertinent calculations are explained in **Section 3.9.3.3** (n = 4).

The results of the long-term incubations are summarized in **Table 3.4**. We were pleased to find that the α-bromo analogue, **3.6**, was active as an inhibitor of G6P

hydrolysis. Compound **3.6** reduced the enzyme activity by approximately 70% under these conditions, and was the most potent compound tested in the experiment. Furthermore, the two other heptose derivatives, **3.5** and **3.27**, showed weak inhibition of G6Pase, indicating that homologation of the glycoside affects recognition by the active site (**Table 3.4**). The data provided in **Table 3.4** suggest that inhibition by α-bromophosphonate, **3.6**, may be time-dependent. This observation could be due to several factors, including permeability of the microsomal membrane, irreversible inhibition, or slow binding of the compound.

Compound	G6P hydrolysis (μmol/mg protein)	% G6Pase activity
control	1.065 ± 0.042	100
3.4	1.085 ± 0.057	>95
3.21	1.055 ± 0.062	>95
3.5	0.905 ± 0.053	85 ± 5
3.6	0.278 ± 0.059	26 ± 5
3.27	0.860 ± 0.038	81 ± 4

Table 3.4 Hydrolysis and % activity ± S.E. by the five deprotected phosphonic acids after long term incubation. Data were obtained after incubating disrupted microsome in MOPS buffer at pH 7.4 in the presence of inhibitor at 2.5 mM for 60 hours at 4^{o}C. After the incubation the Taussky-Shorr method was employed as explained in **Section 3.9.3.1**. Pertinent calculations are explained in **Section 3.9.3.3** (n = 4).

We first considered that an explanation for the slow time-dependent deactivation of G6Pase by α-bromo analogue **3.6** could result from its reduced permeability through the microsomal membrane**.** Although our assays had included a surfactant to avoid this type of artifact, we considered that it would be worthwhile to screen more hydrophobic analogues of the phosphonate analogues. We decided to test the available phosphonate ester analogues from our synthetic study for inhibition of

G6Pase. Depicted in **Figure 3.5** are the seven G6P analogues that were tested.

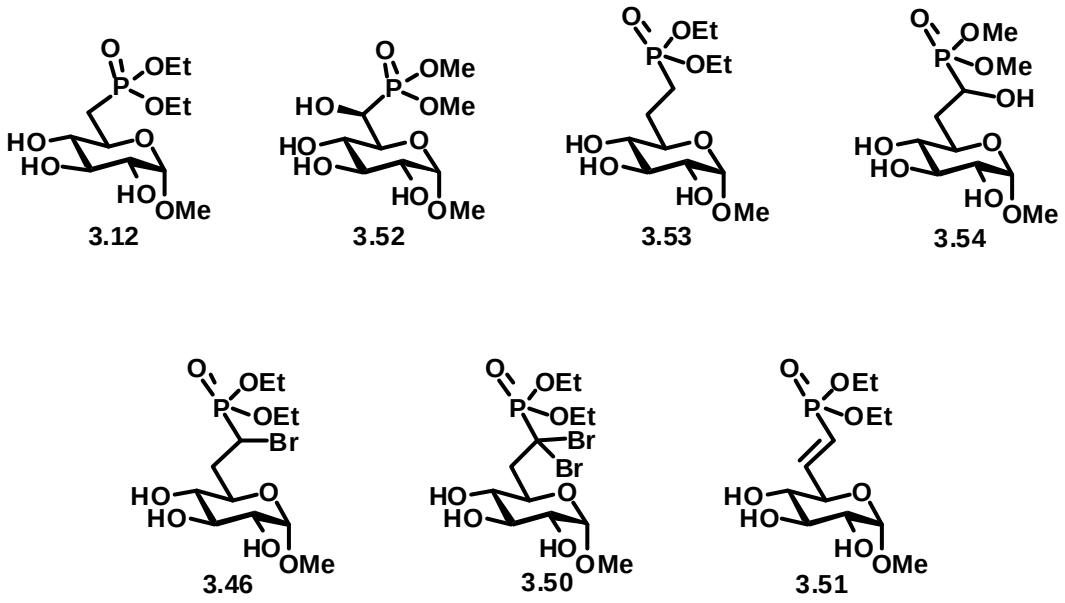


Figure 3.5: The seven G6P analogues screened for inhibition of G6Pase from rat microsomes.

The initial assay we used for these compounds was very similar to the conditions we used to test the phosphonic acid derivatives, except that we omitted the Triton X-100 surfactant from the assay medium. Thus, active compounds would need to have increased membrane permeability or be selective for the T_1 translocase as described in **Chapter 1**. Initial experiments with a 15 minute incubation time at 2.5 mM concentration of the compounds gave inconclusive results. Extending the incubation time from 15 to 60 minutes showed that several of the compounds

tested were active. All compounds except **3.53** displayed detectable inhibition as summarized in **Table 3.5**. It is also worth noting, that since no disrupting surfactant was used, these data offer strong evidence that these molecules can diffuse freely across the endosomal membrane to access G6Pase or were selective for the T_1 transport protein.

Compound	Rate of G6P hydrolysis (µmol/mg protein)	% activity
control	0.826 ± 0.019	100
3.12	0.318 ± 0.024	38 ± 3
3.52	0.335 ± 0.0573	41 ± 7
3.53	0.824 ± 0.022	>95
3.54	0.410 ± 0.017	50 ± 2
3.46	0.422 ± 0.025	51 ± 3
3.50	0.171 ± 0.016	21 ± 2
3.51	0.603 ± 0.058	73 ± 7

Table 3.5: Hydrolysis and % activity ± S.E. by the seven G6P phosphonate esters after 1 h incubation. Data were obtained for each entry in triplicate after incubating undisrupted microsome in MOPS buffer at pH 7.4 in the presence of inhibitor at 2.5 mM for 60 minutes at 4 ^{o}C. After the 60 minutes the Taussky-Shorr method was employed as explained in **Section 3.9.3.1**. Pertinent

calculations are explained in **Section 3.9.3.3**.

The most active compound tested was dibromo analogue **3.50**, which inhibited G6Pase at close to 80%. The two hexose derivatives, **3.12** and **3.52**, inhibit G6Pase by over 60%. This was a surprising result because it is in direct contrast to our results for the analogous deprotected phosphonic acid derivatives **3.4** and **3.21**, which did not inhibit G6Pase in any capacity (**Table 3.3** and **3.4**). Phosphonate ester **3.46**, which contains the monobromo phosphonate ester showed appreciable inhibition (51 ± 3%) although the corresponding phosphonic acid **3.6** was more potent at longer incubation time (26 ± 5%). The homologated α-hydroxy derivative **3.54** was also a weaker inhibitor (81 ± 4%) than its 6-carbon analogue, which showed significant inhibition (compound **3.27**, 50 ± 2%). Finally, the α,β-unsaturated moiety **3.51** showed moderate inhibition (73 ± 7). The saturated analogue, **3.53**, did not show detectable inhibition (>95%).

We postulate that in the case of the heptose ring, functionality α to the phosphonate is important in providing specificity for the active site of G6Pase. Unfunctionalized heptose derivatives (**3.12** and **3.53**) did not show significant activity. It also appears that di-substitution offers improvement over mono-substitution at the α-position. However, what was most puzzling was the drastic improvement in the inhibitory capacity of the two protected hexose derivatives **3.12** and **3.52** over the deprotected hexose derivatives **3.4** and **3.21**. These

compounds may be able to diffuse more quickly across the endosomal membrane, or may be more easily accommodated by the G6Pase active site once the phosphonate carries more steric bulk.

Since both the mono-bromo **3.46** and di-bromo analogue **3.50 o**ffered measurable inhibition of G6Pase we decided to assay whether or not they function as competitive or irreversible inhibitors of the G6Pase active site. We also assayed both hexose derivatives (**3.12** and **3.52**) since they inhibited the enzyme by more than 50% (**Table 3.6**). We assayed for irreversible inhibition by repeating the same experiment discussed above; however, the assay buffer volume was decreased by three-fold, and at the end of the assay the quantity of enzyme and inhibitor was scaled down by three-fold as well to leave their concentrations the same as in the previous assays. After incubating for 60 min, the buffer volume was restored to its original dilution which offers the inhibitor the opportunity to diffuse out of the active site proportionally if it is not covalently bound. The inhibitory capacity is then compared to a control value where the inhibitor is added to the enzyme after the dilution has occurred. If the inhibitory capacity of the analogue that was incubated with the endosome for 60 minutes exceeds the inhibitory capacity of the analogue that was not incubated with the enzyme, this provides evidence of irreversible inhibition. Analogues **3.46**, **3.50**, **3.12** and **3.20** were tested using the dilution assay; however, no appreciable differences between the incubated and unincubated samples were observed, which is inconsistent with

irreversible inhibition. Although the data are not conclusive, they do suggest that the unincubated analogues may provide better inhibition than the incubated ones although this correlation is weak. In light of these data, we hypothesize that bromo analogues **3.46** and **3.50** act as competitive or slow-binding inhibitors. Importantly, using ^{31}P NMR we did determine that 30% phosphonate ester cleavage into the free acid form does occur when incubating monobromo analogue **3.46** in the presence of microsome and buffer in an NMR tube for 27 h. Future work will explore the role of the phosphonate ester in the increased activity of derivatives **3.52** and **3.54**.

Compound		G6P hydrolysis (µmol/mg protein)	% activity
3.12	**Control**	0.94 ± 0.03	
	Unincubated	0.78 ± 0.04	83 ± 4
	Incubated	0.95 ± 0.09	> 95 %
3.52	**Control**	0.61 ± 0.01	
	Unincubated	0.44 ± 0.03	71 ± 4
	Incubated	0.70 ± 0.07	>95
3.52	**Control**	0.44 ± 0.04	
	Unincubated	0.31 ± 0.04	71 ± 10
	Incubated	0.39 ± 0.04	89 ± 8
3.50	**Control**	0.62 ± 0.05	
	Unincubated	0.54 ± 0.09	87 ± 15
	Incubated	0.67 ± 0.03	>95

Table 3.6: Hydrolysis and % inhibition ± S.E. by the seven G6P phosphonate esters. Data were obtained for each entry in triplicate according to the experiment described in the text and in **Section 3.9.3.2**. After the 60 minutes the Taussky-Shorr method was employed as explained in **Section 3.9.3.1**. Pertinent calculations are explained in **Section 3.9.3.3**.

3.8 Conclusions and future directions

This thesis has offered a discussion on the synthesis of a library of nine G6P derivatives that were assayed as inhibitors of the G6Pase protein. Completing these derivatives required utilizing a novel bromination strategy that lead us to synthesize an α,α-dibromophosphonate G6P analogue. To our knowledge, such a functional group has only been isolated on one other occasion and no such compound has ever been tested for biological activity. We were pleased to find that dibromo compound **3.50** was the most active analogue tested against G6Pase. Perhaps most surprising was the fact that the protected phosphonate esters of the G6P analogues were far more active than their phosphonic acid counterparts, which we attribute to their increased lipophilicity.

Future studies will optimize the synthesis of the dibromo analogue in order to explore its activity and mechanism of action. A dilution experiment performed at a longer time course should test this theory. A dilution experiment on phosphonic acid α-bromo analogue **3.6** would also clarify if this compound acts by a non-competitive mechanism. As well, we expect that the free acid form of dibromo analogue **3.50** will inhibit G6Pase in a time dependent manner like its monobromo counterpart **3.6** over a similar time period. To test this hypothesis the final phosphonate ester cleavage would need to be completed. We would also like to

complete the synthesis of *E/Z* analogue **3.39** as a newly functionalized carbohydrate and explore its activity as an inhibitor in the G6Pase system.

We also would like to confirm that the phosphonate esters are targeting the active site of G6Pase directly. To do this, the inhibition studies we have completed must be repeated on disrupted microsomes and compared to the data obtained from intact microsomes. An analysis of the G6P-based α–fluorophosphonates and α,α-difluorophosphonates popularized by Berkowitz as competitive inhibitors of G6Pase would provide an important point of comparison.

In conclusion, we have devised a general synthetic strategy to monobromo and dibromophosphonates that we postulate is amenable to many types of glycosyl phosphates. We aim to confirm this by applying these methods for study of G6Pase inhibition as well as other lectins. Future directions will include exploring other α-electrophiles, expanding these syntheses to include oligosaccharides, and incorporating these moieties at positions other than *C*6.

3.9 Experimental Methods

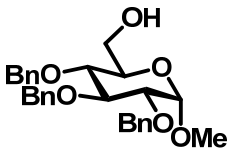
3.9.1 General

All reagents were purchased from commercial sources and used without purification unless otherwise stated. Anhydrous solvents used in the reaction were

purified by successive passage through columns of alumina and copper under argon. If reactions were run under an inert environment using anhydrous solvents, it is stated. The reactions were monitored by analytical thin layer chromatography (TLC) using silica gel (60-F_{254}, 0.25 mm, Silicycle, Quebec, Canada) as a medium and the spots were visualized under ultraviolet light (254 nm) or stained by charring with a ceric ammonium molybdate (CAM) solution. The products purified by column chromatography are indicated using silica gel (230-400 mesh, Silicycle, Quebec, Canada). All NMR spectra were obtained using Varian instruments. ^{1}H NMR spectra were performed at 400, 500 or 700 MHz as indicated. ^{13}C NMR spectra were performed at 125 MHz. ^{31}P NMR spectra were recorded at 160 or 202 MHz as indicated. ^{1}H NMR data are reported as first order, and the peak assignments were made on the basis of 2D-NMR (^{1}H–^{1}H COSY and HSQC) experiments. Electrospray mass spectra were recorded on Agilent Technologies 6220 TOF using CH_2Cl_2, MeOH or H_2O as solvent with added NaCl. Optical rotations were measured at 22 ± 2 °C at the sodium D line (589 nm) and are in units of deg·mL(dm·g)$^{-1}$.

3.9.2 Synthetic Methods

Methyl 2,3,4-Tri-*O*-benzyl-α-D-glucopyranoside (3.9)

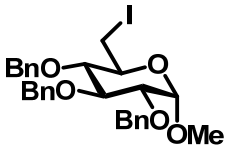


This procedure was developed as a variation of the methods published by Bernotas et al., 1987[31]and Julina and Vasella, 1985.[32] To a solution of α-methyl glucose (10.0 g, 51.5 mmol, 1.0 equiv.) in 1:1 anhydrous pyridine-CH_2Cl_2 (240 mL) was added trityl chloride (15.8 g, 56.7 mmol, 1.1 equiv) and catalytic DMAP (318 mg). After stirring under an inert atmosphere for 12 h at rt, the reaction was diluted with CH_2Cl_2 (400 mL) and the organic layer was washed with saturated ammonium chloride (400 mL) and brine (400 mL), dried over Na_2SO_4 and concentrated *in vacuo* to yield a light yellow foam (crude wt. 26.6 g). This foam was then dissolved in anhydrous DMF (200 mL) and the flask was cooled to 0 ^oC under an inert atmosphere. With vigorous stirring, NaH (60% in mineral oil, 12.35 g, 0.309 mol, 6.0 equiv.) was added. Stirring was continued at 0 ^oC for 1 h before benzyl bromide (45.9 mL, 0.386 mol, 7.5 equiv.) was added dropwise over 1 hr using an addition funnel, followed by tetrabutylammonium iodide (1.32 g). The reaction was slowly warmed to rt and stirring was continued for 24 h before excess NaH was quenched with anhydrous MeOH (10 mL). DMF was partially

removed prior to work-up by use of a high vacuum rotary evaporator in a water bath warmed to 60 ^{o}C. The residue was then dissolved in CH_2Cl_2 (750 mL) and washed with 1 M HCl (400 mL). The HCl solution was rinsed with CH_2Cl_2 (2x 200 mL). The combined organic layers were then washed with H_2O (400 mL) and brine (400 mL), dried over Na_2SO_4 and concentrated *in vacuo* to yield a dark red oil. This oil was subsequently dissolved in MeOH (300 mL) and concentrated H_2SO_4 (14 mL) was added slowly. Stirring was continued for 30 min at rt. After 30 min, the flask was cooled to 0 ^{o}C and saturated $NaHCO_3$ solution (200 mL) was added carefully to neutralize the excess H_2SO_4. The organic layer was then extracted using EtOAc (3 x 300 mL) and rinsed with 1 M HCl (400 mL) and brine (300 mL), dried over Na_2SO_4 and concentrated *in vacuo* to furnish a dark red oil. The residue was purified using flash chromatography (1:4 EtOAc-hexane to 1:1 EtOAc-hexane) to provide **3.9** (18.17 g, 39.11 mmol, 76%) as an orange-yellow oil. R_f = 0.64 (1:1 EtOAc-hexane); $[\alpha]_D$ +46.95 (*c* 4.57, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.39-7.30 (m, 15H, *Ph*CH_2) 5.00 (d, 1H, *J* = 11.0 Hz, C*H*$_2$Ph) 4.90 (d, 1H, *J* = 11.1 Hz, C*H*$_2$Ph) 4.86 (d, 1H, *J* = 10.9 Hz, C*H*$_2$Ph) 4.81 (d, 1H, *J* = 12.1 Hz, C*H*$_2$Ph) 4.68 (d, 1H, *J* = 9.5 Hz, C*H*$_2$Ph) 4.66 (d, 1H, *J* = 8.5 Hz, C*H*$_2$Ph) 4.59 (d, 1H, *J* = 3.5 Hz, H-1) 4.02 (app t, 1H, *J* = 9.5 Hz, H-3) 3.65 (dd, 1H, *J* = 11.8, 2.6 Hz, H-6a) 3.71 (dd, 1H, *J* = 11.8, 4.0 Hz, H-6b) 3.67 (app dt 1H, *J* = 9.5, 3.3 Hz, H-5) 3.54 (app t, 1H, *J* = 9.5 Hz, H-4) 3.52 (dd, 1H, *J* = 9.5, 3.6 Hz, H-2) 3.38 (s, 3H, OC*H*$_3$); ^{13}C NMR (125 MHz, $CDCl_3$): δ 138.8, 138.1(7),

138.1(4), 128.4(9), 128.4(1), 128.1(3), 128.0(3), 127.9(7), 127.9(5), 127.8(7), 127.6, 98.2 (C-1), 82.0 (C-3), 80.0 (C-2) 77.4(4) (C-4), 75.7 (CH$_2$Ph), 75.0 (CH$_2$Ph), 73.4 (CH$_2$Ph), 70.7 (C-5), 61.9 (C-6), 55.2 (OCH$_3$). HR ESIMS: *m/z* [M+Na$^+$] calcd for $C_{28}H_{32}O_6Na$: 487.20911. Found: 487.20893.

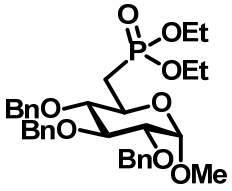
Methyl 2,3,4-Tri-*O*-benzyl-6-deoxy-6-iodo-α-D-glucopyranoside (3.10)



This procedure is similar to that of Ko et al., 2004.[112] To a solution of **3.10** (187 mg, 0.403 mmol, 1.0 equiv) in toluene (4 mL) was added triphenylphosphine (158 mg, 0.604 mmol, 1.5 equiv), imidazole (69 mg, 1.01 mmol, 2.5 equiv) and iodine (153 mg, 0.604 mmol, 1.5 equiv) with stirring and the reaction was heated to 80 ^{o}C for 15 min. After 15 min an oily brown precipitate persisted that indicated the reaction had gone to completion. The toluene was then removed *in vacuo* and the residue was dissolved in EtOAc (20 mL). The organic layer was washed with concentrated $Na_2S_2O_3$ solution (10 mL), a concentrated $NaHCO_3$ solution (10 mL) and brine (10 mL), dried over Na_2SO_4 and concentrated *in vacuo* to furnish a crude yellow oil. The residue was purified using flash chromatography (1:9 EtOAc-hexane) to provide **3.10** (205 mg, 0.357 mmol, 89%) as a colourless oil. R_f = 0.32 (1:9 EtOAc-hexane); $[\alpha]_D$ +32.58 (*c* 0.34, CH_2Cl_2); ^{1}H NMR (500 MHz,

$CDCl_3$): δ 7.40-7.29 (m, 15H, *Ph*CH_2) 5.02 (d, 1H, *J* = 10.8 Hz, C*H*$_2$Ph) 4.97 (d, 1H, *J* = 10.9 Hz, C*H*$_2$Ph), 4.83(1) (d, 1H, *J* = 10.8 Hz, C*H*$_2$Ph), 4.82(6) (d, 1H, *J* = 12.1 Hz, C*H*$_2$Ph), 4.70 (app t, 2H, *J* = 11.5 Hz, C*H*$_2$Ph), 4.64 (d, 1H, *J* = 3.6 Hz, H-1), 4.04 (app t, 1H, *J* = 9.4 Hz, H-3), 3.57 (dd, 1H, *J* = 9.4, 3.6 Hz, H-2), 3.50 (dd, 1H, *J* = 10.4, 2.3 Hz, H-6a), 3.48 (app dt, 1H, *J* = 9.4, 3.2 Hz, H-5), 3.45 (s, 3H, OC*H*$_3$), 3.37 (app t, *J* = 9.4, 1H, H-4), 3.32 (dd, *J* = 10.4, 6.4 Hz, H-6b); ^{13}C NMR (125 MHz, $CDCl_3$): δ 138.6, 138.0(5), 138.0(2), 128.5(4), 128.5(3), 128.4(7), 128.1(1), 128.0(2), 128.0(0), 127.9(6), 127.7(3), 98.1 (C-1), 81.6 (C-3), 81.5 (C-4), 80.1, (C-2), 75.8 (*C*H_2Ph), 75.4 (*C*H_2Ph), 73.5 (*C*H_2Ph), 69.3 (C-5), 55.5 (O*C*H_3), 7.7 (C-6). HR ESIMS: *m/z* [M+Na^+] calcd for $C_{28}H_{31}O_5INa$: 597.11084. Found: 597.11016.

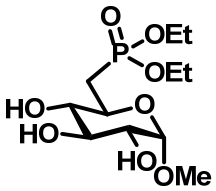
Methyl 2,3,4-Tri-*O*-benzyl-6-deoxy-6-diethoxyphosphoryl-α-D-glucopyranoside (3.11)



To compound **3.10** (190 mg, 0.331 mmol, 1.0 equiv) was added triethyl phosphite neat (2.0 mL, 11.5 mmol, ~35 equiv) and the solution was heated at 130 ^{o}C for 6 h. After 6 h, the reflux condenser was removed and the flask was placed under

vacuum to remove excess triethyl phosphite. After 8 h a faint yellow oil **3.11** (196 mg, 0.331 mmol, 100%) remained that required no purification. R_f = 0.34 (4:1 EtOAc-hexane); $[\alpha]_D$ +19.6 (*c* 1.53, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40-7.29 (m, 15H, *Ph*CH_2), 5.01 (d, 1H, *J* = 10.8 Hz, C*H*$_2$Ph), 4.95 (d, 1H, *J* = 11.3, C*H*$_2$Ph), 4.83 (d, 1H, *J* = 10.8 Hz, C*H*$_2$Ph), 4.81 (d, 1H, *J* = 12.1 Hz, C*H*$_2$Ph), 4.69 (d, 1H, *J* = 12.1, C*H*$_2$Ph), 4.63 (d, 1H, *J* = 11.3 Hz, C*H*$_2$Ph), 4.60 (d, 1H, *J* = 3.6 Hz, H-1), 4.13-3.99 (m, 6H, H-3, H-5, 2 x CH_3C*H*$_2$OP), 3.55 (dd, 1H, *J* = 9.5, 3.6 Hz, H-2), 3.50 (s, 3H, OC*H*$_3$), 3.21 (app t, 1H, *J* = 9.5 Hz, H-4), 2.29 (ddd, 1H, *J* = 17.5, 15.5, 2.0 Hz, H-6a), 1.78 (ddd, 1H, *J* = 15.5, 10.6, 10.3 Hz, H-6b), 1.31(9) (t, 3H, *J* = 7.0 Hz, C*H*$_3$$CH_2$OP), 1.31(5) (t, 3H, *J* = 7.0 Hz, C*H*$_3$$CH_2$OP); ^{13}C NMR (125 MHz, $CDCl_3$): δ 136.1, 135.6(1), 135.5(9), 126.0, 125.9(4), 125.9(3), 125.6, 125.5, 125.4(3), 125.3(8), 125.3, 125.2, 95.5 (C-1), 79.6 (C-4), 79.5 (C-3), 79.4 (C-2) 73.3 (*C*H_2Ph), 72.6 (*C*H_2Ph), 70.8 (*C*H_2Ph), 63.3 (d, $J_{C\text{-}P}$ = 6.7 Hz, C-5), 59.2 (d, $J_{C\text{-}P}$ = 6.2 Hz, CH_3*C*H_3OP), 58.9 (d, $J_{C\text{-}P}$ = 6.2 Hz, CH_3*C*H_3OP), 53.0 (O*C*H_3), 25.9 (d, $J_{C\text{-}P}$ = 143.3 Hz, C-6), 13.9 (m, 2 x *C*$H_3$$CH_2$OP); ^{31}P NMR (160 MHz, $CDCl_3$) δ 24.78. HR ESIMS: *m/z* [M+Na^+] calcd for $C_{32}H_{41}O_8PNa$: 607.2431. Found: 607.2422.

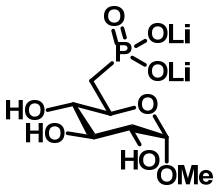
Methyl 6-Deoxy-6-diethoxyphosphoryl-α-D-glucopyranoside (3.12)



General hydrogenation procedure: To a solution of **3.11** (89 mg, 0.152 mmol) in 100 % EtOH (3 mL) was added 10% Pd/C (9.0 mg) at rt. A balloon filled with H_2 was subsequently fitted on top of the flask and the reaction was stirred for 18 hours. After 18 hours, the balloon was removed and the reaction mixture was filtered through celite and concentrated *in vacuo* to yield a colourless oil **3.12** (45 mg, 0.144 mmol, 95%) that required no further purification. R_f = 0.19 (9:1 CH_2Cl_2-MeOH); $[\alpha]_D$ +63.8 (*c* 0.40, H_2O); ^{1}H NMR (500 MHz, CD_3OD) δ 4.64 (d, 1H, *J* = 3.8 Hz, H-1), 4.16-4.06 (m, 4H, 2 x CH_3CH_2OP), 3.85 (ddd, 1H, *J* = 19.4, 10.6, 2.0 Hz, H-5), 3.58 (app t, 1H, *J* = 9.4 Hz, H-3), 3.47 (s, 3H, OCH_3), 3.40 (dd, 1H, *J* = 9.4, 3.8 Hz, H-2) 3.05 (app t, 1H, *J* = 9.4 Hz, H-4) 2.42 (ddd, 1H, *J* = 19.9, 15.7, 2.0 Hz, H-6a), 1.95 (app ddd, 1H, *J* = 15.7, 10.6, 10.4 Hz, H-6b), 1.33 (t, 6H, *J* = 7.1 Hz, CH_3CH_2OP); ^{13}C NMR (125 MHz, CD_3OD): δ 101.4 (C-1), 76.2 (d, *J* = 15.7 Hz, C-4), 74.8 (d, *J* = 2.8 Hz, C-3), 73.6 (C-2), 68.3 (d, *J* = 6.7 Hz, C-5), 63.4 (d, $J_{C\text{-}P}$ = 6.2 Hz, CH_3CH_3OP), 63.0 (d, $J_{C\text{-}P}$ = 6.2 Hz, CH_3CH_3OP), 56.1 (OCH_3), 29.0 (d, *J* = 143.3 Hz, C-6), 16.7(2) (CH_3CH_2OP), 16.6(7) (CH_3CH_2OP); ^{31}P NMR (160 MHz, CD_3OD) δ 30.38. HR ESIMS: *m/z*

[M+Na^+] calcd for $C_{11}H_{23}O_8PNa$: 337.1023. Found: 337.1020.

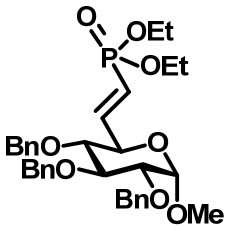
Methyl 6-Deoxy-6-phosphoryl-α-D-glucopyranoside, Bis-Lithium Salt (3.4)



General procedure for phosphonate ester cleavage: To a solution of **3.12** (20.8 mg, 0.0668 mmol, 1.0 equiv) in anhydrous CH_3CN under an N_2 environment was added trimethylsilyl bromide (87 μL, 0.661 mmol, 10.0 equiv) dropwise at 0 ^oC with rigorous stirring. 5 minutes later, anhydrous NEt_3 (98 μl, 0.701 mmol, 10.5 equiv.) was added dropwise and the reaction was allowed to slowly warm to rt. After stirring for 12 h the reaction was concentrated *in vacuo* to leave a dark red oil. The residue was then washed with 3 x H_2O-MeOH (1:9) which was re-concentrated *in vacuo* after each dissolution. The residue was then dissolved in H_2O and the pH was increased to 7.0 using triethylamine. To this solution Amberlite 120 resin (Li^+ form) was added and stirring continued for 5 h. After 5 h the resin was filtered off and the H_2O was removed via lyophilisation to yield a pale yellow, amorphous solid. Et_2O was added to the crude solid to create a suspension which was stirred vigorously as a minimum volume of 100% EtOH was carefully added dropwise to dissolve the excess LiBr salt. The suspension

was filtered and the remaining solid was dissolved in H_2O. Removal of the H_2O via lyophilisation afforded pure **3.4** (96.9:3.1 bis-lithium salt- lithium/$HNEt_3^+$ salt mixture, 17.7 mg, 0.0648 mmol, 98%) as a colourless foam. $[\alpha]_D$ +64.0 (*c* 0.66, H_2O); ^{1}H NMR (400 MHz, D_2O): δ 4.73 (d, 1H, *J* = 3.5 Hz, H-1), 3.85 (ddd, 1H, *J* = 16.2, 9.7, 3.3 Hz, H-5), 3.63 (dd, 1H, *J* = 9.8, 8.9 Hz, H-3), 3.55 (dd, 1H, *J* = 9.8, 3.7 Hz, H-2), 3.45 (s, 3H, OC*H*$_3$), 3.20 (app t, 1H, *J* = 9.2 Hz, H-4), 2.13 (ddd, 1H, *J* = 18.8, 15.5, 3.3 Hz, H-6a) 1.79 (app td, 1H, *J* = 15.5, 10.9, 9.5 Hz, H-6b); ^{13}C NMR (125 MHz, D_2O): δ 99.9 (C-1), 75.4 (d, $J_{C\text{-}P}$ = 12.4 Hz, C-4), 73.7 (d, $J_{C\text{-}P}$ = 2.0 Hz) (C-3), 72.2 (C-2), 68.7(d, $J_{C\text{-}P}$ = 4.6 Hz, C-5), 56.5 (O*C*H$_3$), 31.5 (d, $J_{C\text{-}P}$ = 134.7 Hz, C-6); ^{31}P NMR (160 MHz, D_2O): δ 21.02. HR ESIMS: *m/z* [M-H$^-$] calcd for $C_7H_{14}O_8P$: 257.0432. Found: 257.0431.

Methyl 2,3,4-Tri-*O*-benzyl-6-Deoxy-6-(diethylphosphonomethylene)-α-D-glucopyranoside (3.14):

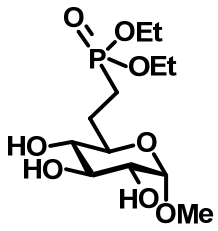


A solution of oxalyl chloride (1.4 mL, 15.9 mmol, 4.0 equiv) in anhydrous CH_2Cl_2 under an N_2 atmosphere was cooled to -78 ^{o}C and to it anhydrous DMSO (2.3 mL, 31.8 mmol, 8.0 equiv) was added dropwise with rigorous stirring. After

stirring at -78 °C for 20 min, a pre-dissolved solution of **3.9** (1.85 g, 3.98 mmol, 1.0 equiv) in anhydrous CH_2Cl_2 (30 mL) was added dropwise via syringe. Stirring was continued for 1 h before the reaction was quenched with NEt_3 (8.9 mL, 63.7 mmol, 16.0 equiv). Stirring was continued at -78 °C for 15 min before the reaction was warmed to room temperature. The reaction was diluted with 60 mL of CH_2Cl_2 and the organic layer was washed with concentrated NH_4Cl (100 mL). The aqueous layer was rinsed with CH_2Cl_2 (50 mL) and the combined organic layers were washed with brine (2 x 100 mL), dried over Na_2SO_4 and concentrated. The crude aldehyde product **3.13** was isolated as a dark orange oil that was used immediately without purification. To a solution of TEMDP (2.5 mL, 9.95 mmol, 2.5 equiv) in anhydrous THF (30 mL) under an N_2 atmosphere was added NaH (60% in mineral oil, 318 mg, 7.96 mmol, 2.0 equiv) with vigorous stirring at 0 °C. After 30 min, a pre-dissolved solution of the crude aldehyde in anhydrous THF (20 mL) was added dropwise via syringe over 30 min. After the addition was complete, the reaction was warmed to rt and stirring continued for 10 min. The THF was then removed *in vacuo* and the crude residue was dissolved in EtOAc (100 mL) and washed with 1 M HCl (60 mL) and brine (60 mL), dried over Na_2SO_4 and concentrated. The residue was purified using flash chromatography (3:2 EtOAc-hexane) to provide **3.14** (1.68 g, 2.83 mmol, 71%) as a pale yellow oil. R_f = 0.34 (3:2 EtOAc-hexane); $[\alpha]_D$ +57.51 (*c* 3.00, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.35-7.26 (m, 15H, *Ph*CH_2), 6.88 (ddd, 1H, J = 22.5, 17.3, 4.3

Hz, H-6), 6.04 (ddd, 1H, J = 20.9, 17.3, 1.7 Hz, H-7), 4.96 (d, 1H, J = 10.9 Hz, CH_2Ph), 4.83 (d, 1H, 10.6 Hz, CH_2Ph), 4.82 (d, 1H, J = 11.9 Hz, CH_2Ph), 4.80 (d, 1H, J = 12.2 Hz, CH_2Ph), 4.66 (d, 1H, J = 12.2 Hz, CH_2Ph), 4.61 (d, 1H, J = 3.6 Hz, H-1), 4.57 (d, 1H, J = 10.6 Hz, CH_2Ph), 4.25-4.21 (m, 1H, H-5), 4.09-4.02 (m, 4H, CH_3CH_2OP), 4.01 (app t, 1H, J = 9.6 Hz, H-3), 3.51 (dd, 1H, J = 9.6, 3.6 Hz, H-2), 3.36 (s, 3H, CH_3O), 3.23 (dd, 1H, J = 9.6, 8.9 Hz, H-4), 1.31-1.27 (m, 6H, CH_3CH_2OP); ^{13}C NMR (125 MHz, $CDCl_3$): δ 145.3 (d, $J_{C\text{-}P}$ = 5.9 Hz, C-6), 136.1, 135.6, 135.2, 126.0, 125.9(7), 125.9(3), 125.6, 125.5, 124.4(9), 125.4(5), 125.3(8), 125.2, 115.6 (d, $J_{C\text{-}P}$ = 188.4 Hz, C-7), 95.7 (C-1), 79.3 (C-3), 79.2 (C-4), 77.3 (C-2), 73.3 (CH_2Ph), 72.9 (CH_2Ph), 71.0 (CH_2Ph), 67.4 (d, $J_{C\text{-}P}$ = 21.1 Hz, C-5), 59.3-59.2, (m, 2 x CH_3CH_2OP), 52.9 (CH_3O), 13.9 (CH_3CH_2OP), 13.8 (CH_3CH_2OP); ^{31}P NMR (202 MHz, $CDCl_3$) δ 17.91. HR ESIMS: *m/z* [M+Na^+] calcd for $C_{33}H_{41}O_8PNa$: 619.2431. Found: 619.2426.

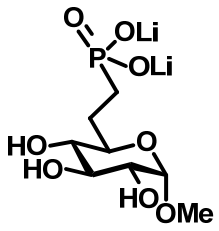
Methyl-6,7-dideoxy-7-diethoxyphosphoryl-α-D-*gluco*-heptopyranoside (3.53)



A solution of **3.14** (247 mg, 0.424 mmol) in 100% EtOH (15 mL) using 10% Pd/C (25 mg) was hydrogenated according to the procedure for compound **3.11**, except

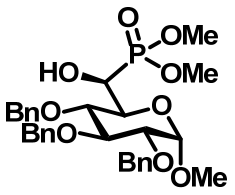
that the resulting residue required further purification using flash chromatography (9:1 DCM:MeOH), which provided **3.53** (107 mg, 0.326 mmol, 73%) as a colourless oil. $[\alpha]_D$ +26.54 (*c* 0.37, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ 4.64 (d, 1H, *J* = 3.8 Hz, H-1) 4.16-4.05 (m, 4H, 2 x CH_3CH_2OP), 3.56 (app t, 1H, *J* = 9.5 Hz, H-3), 3.48 (dt, 9.5, 2.6 Hz, H-5), 3.39 (s, 3H, CH_3O), 3.38 (dd, 1H, *J* = 9.5, 3.8 Hz, H-2), 3.05 (dd, 1H, *J* = 9.5, 9.2 Hz, H-4), 2.18-2.10 (m, 1H, H-6a), 2.09-1.99 (m, 1H, H-7a), 1.82 (dddd, 1H, *J* = 22.8, 15.8, 10.6, 3.3 Hz, H-7b), 1.71-1.61 (m, 1H, H-6b), 1.33(4), (t, 3H, *J* = 7.0 Hz, CH_3CH_2OP), 1.33(3) (t, 3H, *J* = 7.0 Hz, CH_3CH_2OP); ^{13}C NMR (125 MHz, CD_3OD): δ 99.9 (C-1), 73.9 (C-4), 73.6 (C-3), 72.2 (C-2), 70.5 (d, $J_{C\text{-}P}$ = 17.0, C-5), 61.8(2) (d, $J_{C\text{-}P}$ = 7.0 Hz, CH_3CH_2OP), 61.8(0) (d, $J_{C\text{-}P}$ = 7.0 Hz, CH_3CH_2OP), 54.2 (CH_3O), 24.3 (d, $J_{C\text{-}P}$ = 4.4 Hz, C-6), 20.5 (d, $J_{C\text{-}P}$ = 142.5 Hz, C-7), 15.3(1) (CH_3CH_2OP), 15.2(7) CH_3CH_2OP); ^{31}P NMR (160 MHz, CD_3OD): δ 33.49. HR ESIMS: *m/z* [$M+Na^+$] calcd for $C_{12}H_{25}O_8PNa$: 351.1179. Found: 351.1173.

Methyl-6,7-dideoxy-7-phosphoryl-α-D-*gluco*-pyranoside, Bis-Lithium Salt (3.5)



According to the typical phosphono-ester cleavage procedure for compound **3.12**: **3.53** (28.7 mg, 0.0849 mmol, 1.0 equiv), trimethylsilyl bromide (112 µl, 0.849 mmol, 10.0 equiv) and anhydrous triethylamine (124 µl, 0.891 mmol, 10.5 equiv.) in anhydrous CH_3CN under an N_2 environment was stirred for 12 h at 0 ^{0}C to rt to furnish **3.5** as a pale yellow foam (83.3:16.7 bis-lithium salt-lithium/HEt_3^+ salt, 17.2 mg, 0.0573 mmol, 68%). $[\alpha]_D$ +104.0 (*c* 1.03, H_2O); ^{1}H NMR (500 MHz, D_2O): δ 4.77 (H-1),* 3.61 (app t, 1H, *J* = 9.3 Hz, H-3), 3.58-3.55 (m, 2H, H-2, H-5), 3.41 (s, 3H, C*H*$_3$O), 3.26 (app t, 1H, *J* = 9.3 Hz, H-4), 2.08-2.02 (m, 1H, H-6a), 1.76-1.70 (m, 1H, H-7a), 1.70-1.63 (m, 1H, H-6b), 1.50-1.43 (m, 1H, H-7b); ^{13}C NMR (125 MHz, D_2O): δ 99.1 (C-1), 73.0(3) (C-4), 72.9(6), (C-3), 71.8 (d, $J_{C\text{-}P}$ = 16.6 Hz, C-5), 71.5 (C-2), 55.0 (*C*H$_3$O), 25.5 (d, $J_{C\text{-}P}$ = 2.8 Hz, C-6), 24.3 (d, $J_{C\text{-}P}$ = 132.4 Hz, C-7); ^{31}P NMR (160 MHz, D_2O) δ 25.92. HR ESIMS: *m/z* [M-H$^-$] calcd for $C_8H_{16}O_8P$: 271.0588. Found: 271.0590. *Note coupling for H-1 is not available as it is distorted by the solvent peak.

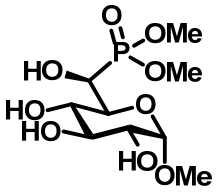
Methyl 2,3,4-Tri-*O*-benzyl-6-dimethoxyphosphoryl-L-*glycero*-α-D-*gluco*-pyranoside (3.20):



Dimethyl phosphite (73 µl, 0.796 mmol, 2.0 equiv) in anhydrous THF (2 mL) under an N_2 atmosphere was added NaH (60% in mineral oil, 24 mg, 0.597 mmol, 1.5 equiv) with vigorous stirring for 30 min. After 30 min a pre-dissolved solution of crude aldehyde **3.13** (184 mg, 0.398 mmol, 1.0 equiv) as prepared for compound **3.14** was cannulated into the reaction. Stirring was continued for 12 h after which time the THF was removed *in vacuo.* The crude residue was diluted with EtOAc (25 mL) and the organic layer was rinsed with 1 M HCl (20 mL) and brine (20 mL), dried over Na_2SO_4 and concentrated *in vacuo* to afford a pale brown oil. The residue was purified using flash chromatography (4:1 EtOAc-hexane to EtOAc) to furnish **3.20** (102 mg, 0.178 mmol, 90% *de*, 45%) as a colourless oil. Spectroscopic data provided are for the 6(*S*) isomer: R_f = 0.18 (4:1 EtOAc-hexane); $[\alpha]_D$ +10.60 (*c* 1.20, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.40-7.29 (m, 15H, *Ph*CH_2), 5.02 (d, 1H, *J* = 11.8 Hz, C*H*$_2$Ph), 4.95 (d, 1H, *J* = 11.2 Hz, C*H*$_2$Ph), 4.86 (d, 1H, *J* = 10.9 Hz, C*H*$_2$Ph), 4.83 (d, 1H, *J* = 12.3 Hz, C*H*$_2$Ph), 4.68 (d, 1H, *J* = 12.0 Hz, C*H*$_2$Ph), 4.65 (d, 1H, *J* = 3.5 Hz, H-1), 4.20

(dd, 1H, J = 17.3, 1.1 Hz, H-6), 4.06 (app t, 1H, J = 9.6 Hz, H-3), 4.02 (ddd, 1H, J = 9.6, 3.3, 1.1 Hz, H-5), 3.82 (d, 3H, $J_{H\text{-}P}$ = 10.5 Hz, CH_3OP), 3.77 (d, 3H, $J_{H\text{-}P}$ = 10.6 Hz, CH_3OP), 3.71 (app dt, 1H, J = 9.6, 1.1, H-4), 3.51 (dd, 1H, J = 9.6, 3.5 Hz, H-2), 3.49 (s, 3H, CH_3O); ^{13}C NMR (125 MHz, $CDCl_3$): δ 134.7, 134.3, 134.1, 124.5(6), 124.5(2), 124.4(7), 124.1, 124.0(3), 124.0(2), 123.9, 123.7, 94.6 (C-1), 77.9 (C-3), 75.7 (C-2), 72.3 (d, J = 10.3$_{C4\text{-}P}$ Hz, C-4), 71.8 (CH$_2$Ph), 71.1 (CH$_2$Ph), 69.5 (CH$_2$Ph), 65.7 (C-5), 62.0 (d, $J_{C\text{-}P}$ = 162.4 Hz, C-6), 51.8 (CH$_3$O), 49.6 (d, $J_{C\text{-}P}$ = 6.4, Hz, CH$_3$OP), 48.6 (d, $J_{C\text{-}P}$ = 7.2 Hz, CH$_3$OP); ^{31}P NMR (160 MHz, $CDCl_3$): δ 24.78. HR ESIMS: *m/z* [M+Na$^+$] calcd for $C_{30}H_{37}O_9PNa$: 595.2067. Found: 595.2060.

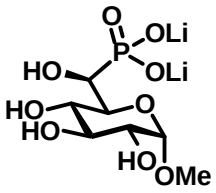
Methyl 6-dimethoxyphosphoryl-L-*glycero*-α-D-*gluco*-pyranoside (3.52):



Compound **3.20** (90 mg, 0.157 mmol) in 100% EtOH (3 mL) using 10% Pd/C (9.0 mg) was hydrogenated according to the typical procedure of compound **3.11**, which furnished **3.52** (44 mg, 0.145 mmol, 92%) as a colourless oil. Spectroscopic data are for the 6(*R*) isomer: R_f = 0.08 (9:1 CH_2Cl_2-MeOH). $[\alpha]_D$ +88.63 (*c* 1.20, MeOH); ^{1}H NMR (500 MHz, CD_3OD): δ 4.72 (d, 1H, J = 3.6 Hz,

H-1), 4.37 (app d, 1H, $J_{H\text{-}P}$ = 12.0 Hz, H-6), 3.83 (m, 4H, C*H*$_3$OP, H-5), 3.78 (d, 3H, $J_{H\text{-}P}$ = 10. 6 Hz, C*H*$_3$OP), 3.64 (app t, 1H, *J* = 9.5 Hz, H-3), 3.53 (app t, 1H, *J* = 9.5 Hz, H-4), 3.48 (s, 3H, C*H*$_3$O), 3.40 (dd, 1H, *J* = 9.5, 3.6 Hz, H-2); ^{13}C NMR (125 MHz, CD_3OD): δ 100.4 (C-1), 73.6 (C-3), 71.9 (C-2), 70.9 (C-5), 68.6 (d, $J_{C\text{-}P}$ = 10.6 Hz, C-4), 65.3 (d, $J_{C\text{-}P}$ = 165.5 Hz, C-6), 54.8 (*C*H$_3$O), 53.0 (d, $J_{C\text{-}P}$ = 6.7, *C*H$_3$OP), 51.8 (d, $J_{C\text{-}P}$ = 7.2, *C*H$_3$OP); ^{31}P NMR (202 MHz, CD_3OD) δ 28.02. HR ESIMS: *m/z* [M+Na$^+$] calcd for $C_9H_{19}O_9PNa$: 325.0659. Found: 325.0654.

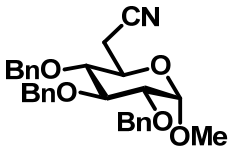
Methyl 6-phosphoryl-L-*glycero*-α-D-*gluco*-pyranoside, Bis-Lithium Salt (3.21):



According to our phosphinyl cleavage procedure: **3.52** (40.0 mg, 0.132 mmol, 1.0 equiv), trimethylsilyl bromide (175 µl, 1.32 mmol, 10.0 equiv) and anhydrous pyridine (112 µl, 1.39 mmol, 10.5 equiv.) in anhydrous CH_3CN (3 mL) under an N_2 environment was stirred for 12 h at 0 ^{0}C to rt to furnish **3.21** as a colourless foam (~100% bis-lithium salt, 29.3 mg, 0.102, 78%). Data are for the 6(*S*) isomer: $[\alpha]_D$ +47.7 (*c* 1.16, H_2O); ^{1}H NMR (400 MHz, D_2O): δ 4.79 (d, 1H, *J* = 3.8 Hz, H-1), 4.08 (dd, 1H, *J* = 13.3, 1.5 Hz, H-6), 3.89 (ddd, 1H, *J* = 9.6, 3.0, 1.5 Hz, H-5), 3.69 (app t, 1H, 9.6 Hz, H-3), 3.55 (td, 1H, *J* = 9.6, 1.0 Hz, H-4), 3.53 (dd, 1H,

J = 9.6, 3.8 Hz, H-2), 3.44 (s, 2.8H, OCH_3); ^{13}C NMR (125 MHz, D_2O): δ 99.3 (C-1) 73.2 (C-3) 71.2 (C-5), 70.8 (C-4), 68.9 (C-2), 66.0 (d, $J_{C\text{-}P}$ = 158.5 Hz, C-6) 55.4 (OCH_3); ^{31}P NMR (160 MHz, D_2O): δ 17.87. HR ESIMS: *m/z* [M-H$^-$] calcd for $C_7H_{14}O_9P$: 273.0381. Found: 273.0388.

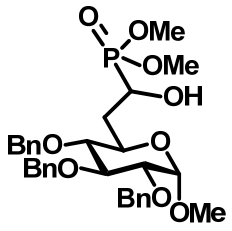
Methyl 2,3,4-Tri-*O*-benzyl-6-cyano-6-deoxy-α-D-glucopyranoside (3.24)



To a solution of **3.10** (583 mg, 1.01 mmol, 1.0 equiv) in DMF (7.5 mL) was added NaCN (100 mg, 2.03 mmol, 2.0 equiv) with stirring and the reaction was heated at 70 ^{o}C for 12 h. After 12 h the DMF was removed *in vacuo* using a high vacuum rotary evaporator in a water bath at 60 ^{o}C. The residue was dissolved in 30 mL of CH_2Cl_2 and the organic layer was washed with H_2O (2 x 15 mL) and brine (15 mL), dried over Na_2SO_4 and concentrated to yield a colorless oil **3.24** (469 mg, 0.990 mmol, 98%) that required no further purification. R_f = 0.31 (1:4 EtOAc-hexane); $[\alpha]_D$ +58.23 (*c* 2.52, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.37-7.30 (m, 15H, *Ph*CH_2), 5.01 (d, 1H, J = 11.0, CH_2Ph), 4.94 (d, 1H, J = 11.2 Hz, CH_2Ph), 4.81 (d, 1H, 11.0 Hz, CH_2Ph), 4.79 (d, 1H, J = 12.1 Hz, CH_2Ph), 4.65 (d, 1H, 12.1 Hz, CH_2Ph), 4.61 (d, 1H, J = 11.2 Hz, CH_2Ph), 4.58 (d, 1H, 3.6 Hz, H-1), 3.98 (app t, 1H, J = 9.2 Hz, H-3) 3.80 (ddd, 1H, J = 9.9, 7.1, 3.4 Hz, H-5) 3.56

(dd, 1H, *J* = 9.2, 3.6 Hz, H-2), 3.40 (s, 3H, OCH_3), 3.33 (dd, 1H, *J* = 9.9, 9.2 Hz, H-4) 2.63 (dd, 1H, *J* = 17.0, 3.4 Hz, H-6a) 2.43 (dd, 1H, *J* = 17.0, 7.1 Hz, H-6b); ^{13}C NMR (125 MHz, $CDCl_3$): δ 138.4, 137.9, 137.6(3), 128.6(1), 128.5(4), 128.4(5), 128.1(2), 128.1(0), 128.0(7), 127.9, 127.7, 116.7 (*C*≡N), 98.1 (C-1), 81.6 (C-3), 79.9(5) (C-4), 79.9(2) (C-2), 75.7 (*C*H_2Ph), 75.2(*C*H_2Ph), 73.5(*C*H_2Ph), 66.3 (C-5) 55.5 (O*C*H_3), 20.8 (C-6). HR ESIMS: *m/z* [M+Na^+] calcd for $C_{29}H_{31}NO_5Na$: 496.2094. Found: 496.2098.

Methyl 2,3,4-Tri-*O*-benzyl-6-Deoxy-7-dimethoxyphosphoryl-D/L-*glycero*-α-D-*gluco*-heptopyranoside (3.26)



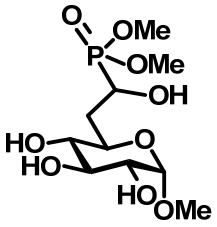
A solution of **3.24** (390 mg, 0.824 mmol, 1.0 equiv) in anhydrous CH_2Cl_2 (15 mL) under N_2 was cooled to -78 ^oC and to it diisobutylaluminum hydride (1.0 M in hexanes, 2.47 mL, 2.47 mmol, 3.0 equiv) was added dropwise over 15 min. Stirring was continued at -78 ^oC for 1 h at which point the reaction was quenched using 1 M HCl (7.5 mL, 9.0 equiv) and the reaction was allowed to warm to rt. After stirring at rt for 15 min the reaction was diluted further with CH_2Cl_2 (15 mL) and the organic layer was washed with 1 M HCl (2 x 15 mL). The HCl

solution was rinsed with CH_2Cl_2 (2 x 10 mL) and the combined organic layers were washed with brine (15 mL), dried over Na_2SO_4 and concentrated *in vacuo* to yield the aldehyde **3.25** as a colourless oil that was used immediately without further purification. Dimethyl phosphite (151 µl, 1.65 mmol, 2.0 equiv) was dissolved in anhydrous THF (5 mL) under an N_2 atmosphere and the flask was cooled to 0 ^oC before NaH (60% in mineral oil, 49 mg, 1.24 mmol, 1.5 equiv) was added in one portion with vigorous stirring. After 30 min a pre-dissolved solution of the crude aldehyde (0.824 mmol, 1.0 equiv) in anhydrous THF (5 mL) was added dropwise via syringe over 15 min. The reaction was warmed to rt and stirring was continued for 1 h. After 1 h the THF was removed *in vacuo* and the residue was re-dissolved in CH_2Cl_2 (30 mL) and the organic layer was washed with 1 M HCl (15 mL) and brine (15 mL), dried over Na_2SO_4 and concentrated. The residue was purified using flash chromatography (1:1 EtOAc-hexane then 50:50:2 EtOAc-hexane-AcOH) to afford a faint brown oil **3.26** (285 mg, 0.486 mmol, 59%) as an inseparable 5:2 *S*-*R* mixture of diastereomers that co-eluted. R_f = 0.17 (4:1 EtOAc-hexane); $[\alpha]_D$ +43.96 (*c* 1.00, CH_2Cl_2). HR ESIMS: *m/z* [M+Na^+] calcd for $C_{31}H_{43}O_9PNa$: 609.2224. Found: 609.2216. Spectroscopic data for the major *S* isomer: ^1H NMR (500 MHz, $CDCl_3$): δ 7.37-7.25 (m, 15H, *Ph*CH_2), 4.98 (d, 1H, *J* = 10.8 Hz, C*H*$_2$Ph), 4.90 (d, 1H, *J* = 11.0 Hz, C*H*$_2$Ph), 4.80 (d, 1H, *J* = 10.8 Hz, C*H*$_2$Ph), 4.79 (d, 1H, *J* = 12.2 Hz, C*H*$_2$Ph), 4.66 (d, 1H, *J* = 12.1 Hz, C*H*$_2$Ph), 4.60 (d, 1H, *J* = 11.0 Hz, C*H*$_2$Ph), 4.53 (d, 1H, *J* = 3.6 Hz,

H-1), 4.17 (ddd, 1H, *J* = 11.1, 6.6, 2.2 Hz, H-7), 4.02-3.97 (m, 2H, H3, H5), 3.80-3.75 (m, 6H, 2 x C*H*$_3$OP), 3.50 (dd, 1H, *J* = 9.5, 3.6 Hz, H-2), 3.39 (s, 3H, C*H*$_3$O), 3.22 (app t, 1H, *J* = 9.5 Hz, H-4), 2.17 (dddd, 1H, 15.3, 9.4, 6.6, 2.6 Hz, H-6a), 1.78 (dddd, 1H, *J* = 15.3, 8.4, 7.4, 2.2 Hz, H-6b); ^{13}C NMR (125 MHz, $CDCl_3$): δ 136.2, 135.6(2), 135.6(1), 126.0, 125.9(9), 125.9(7), 125.9(6), 125.9(3), 125.9(1), 125.6, 125.5(4), 125.5(2), 125.4(6), 125.4(3), 125.3(7), 125.2(5), 125.2(2), 125.1(7), 95.4 (C-1), 79.4 (C-3), 79.0 (C-4), 77.6 (C-2), 73.3 (*C*H$_2$Ph), 72.6 (*C*H$_2$Ph), 70.9 (*C*H$_2$Ph), 63.4 (d, $J_{C\text{-}P}$ = 14.2 Hz, C-5), 61.8 (d, $J_{C\text{-}P}$ = 164.2 Hz, C-7), 52.8 (*C*H$_3$O), 50.9 (d, $J_{C\text{-}P}$ = 7.0 Hz, *C*H$_3$OP), 50.7 (d, $J_{C\text{-}P}$ = 7.0 Hz, *C*H$_3$OP), 30.7 (d, $J_{C\text{-}P}$ = 2.1 Hz, C-6); ^{31}P NMR (202 MHz, $CDCl_3$) δ 24.37. Spectroscopic data for the minor *R* isomer: ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.37-7.25 (m, 15H, *Ph*CH$_2$), 4.98 (d, 1H, *J* = 10.8 Hz, C*H*$_2$Ph), 4.90 (d, 1H, *J* = 11.0 Hz, C*H*$_2$Ph), 4.80 (d, 1H, *J* = 10.8 Hz, C*H*$_2$Ph), 4.79 (d, 1H, *J* = 12.2 Hz, C*H*$_2$Ph), 4.64 (d, 1H, *J* = 11.8 Hz, C*H*$_2$Ph), 4.60 (d, 1H, *J* = 11.0 Hz, C*H*$_2$Ph), 4.53 (d, 1H, *J* = 3.6 Hz, H-1), 4.14 (ddd, 1H, *J* = 10.9, 7.1, 2.6 Hz, H-7), 3.95 (s, 1H, *J* = 9.2 Hz, H-3), 3.90-3.82 (m, 1H, H-5), 3.80-3.75 (m, 6H, 2 x C*H*$_3$OP), 3.50 (dd, 1H, *J* = 9.5, 3.6 Hz, H-2), 3.38 (s, 3H, C*H*$_3$O), 3.27 (app t, 1H, *J* = 9.2 Hz, H-4), 2.34 (dddd, 1H, 14.8, 7.1, 2.8, 2.8 Hz, H-6a), 1.84 (m, 1H, H-6b); ^{13}C NMR (125 MHz, $CDCl_3$): δ 136.2, 135.6(2), 135.6(1), 126.0, 125.9(9), 125.9(7), 125.9(6), 125.9(3), 125.9(1), 125.6, 125.5(4), 125.5(2), 125.4(6), 125.4(3), 125.3(7), 125.2(5), 125.2(2), 125.1(7), 95.7 (C-1), 79.2 (C-3), 79.1 (C-4), 77.3 (C-2), 73.3 (*C*H$_2$Ph), 72.8

(CH_2Ph), 71.0 (CH_2Ph), 68.9 (d, $J_{C\text{-}P}$ = 17.3 Hz, C-5), 65.4 (d, $J_{C\text{-}P}$ = 170.0 Hz, C-7), 53.0 (CH_3O), 51.0 (d, $J_{C\text{-}P}$ = 7.0 Hz, CH_3OP), 50.7 (d, $J_{C\text{-}P}$ = 7.5 Hz, CH_3OP), 30.7 (C-6); ^{31}P NMR (202 MHz, $CDCl_3$) δ 22.69.

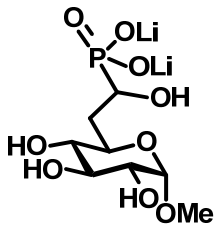
Methyl 6-Deoxy-7-dimethoxyphosphoryl-D/L-*glycero*-α-D-*gluco*-heptopyranoside (3.54)



Compound **7** (140 mg, 0.239 mmol) in 100% EtOH (8 mL) using 10% Pd/C (15 mg) was hydrogenated according to the procedure for compound **3.11**, to furnish **3.54** (74 mg, 0.234 mmol, 98%) as a colourless oil. R_f = 0.11 (9:1 CH_2Cl_2-MeOH); $[\alpha]_D$ +43.11 (*c* 0.34, CH_3OH); HR ESIMS: *m/z* [M+Na^+] calcd for: $C_{10}H_{21}O_9PNa$: 339.0815. Found: 339.0812. Spectroscopic data for the major *S* isomer: ^{1}H NMR (500 MHz, CD_3OD): δ 4.62 (d, 1H, *J* = 3.9 Hz, H-1), 4.18 (ddd, 1H, *J* = 12.0, 4.6, 1.9 Hz, H-7), 3.81 (d, 3H, *J* = 10.3 Hz, C*H*$_3$OP), 3.79 (d, 3H, *J* = 10.3 Hz, C*H*$_3$OP) 3.76-3.71 (m, 1H, H-5) 3.58 (app t, *J* = 9.4 Hz, H-3), 3.39 (m, 4H, C*H*$_3$O, H-2), 3.04 (app t, 1H, *J* = 9.4 Hz, H-4), 2.23 (dddd, 1H, 14.3, 9.4, 7.6, 2.2 Hz, H-6a), 1.78 (dddd, 1H, J = 14.3, 11.5, 3.9, 1.9 Hz, H-6b). ^{13}C NMR (125 MHz, CD_3OD): δ 99.7 (C-1), 74.5 (C-4), 73.6 (C-3), 72.3 (C-2), 66.0 (d, $J_{C\text{-}P}$ =

15.0 Hz, C-5), 62.6 (d, $J_{C\text{-}P}$ = 165.5 Hz, C-7) 54.1 (CH$_3$O),), 52.8 (d, $J_{C\text{-}P}$ = 7.5 Hz, CH$_3$OP), 52.4 (d, $J_{C\text{-}P}$ = 7.5 Hz, CH$_3$OP), 33.3 (d, $J_{C\text{-}P}$ = 3.9 Hz, C-6); ^{31}P NMR (202 MHz, CDCl$_3$) δ 28.37. Spectroscopic data for the minor *R* isomer: ^{1}H NMR (500 MHz, CD$_3$OD): δ 4.62 (d, 1H, *J* = 3.9 Hz, H-1), 4.22 (ddd, 1H, *J* = 8.4, 4.2, 4.2 Hz, H-7), 3.81 (d, 3H, *J* = 10.3 Hz, C*H*$_3$OP), 3.79 (d, 3H, *J* = 10.3 Hz, C*H*$_3$OP) 3.74-3.70 (m, 1H, H-5) 3.58 (app t, *J* = 9.3 Hz, H-3), 3.39 (m, 4H, C*H*$_3$O, H-2), 3.04 (app t, 1H, *J* = 9.3 Hz, H-4), 2.30 (dddd, 1H, 15.0, 10.5, 5.5, 4.0 Hz, H-6a), 1.84 (m, 1H, J = 14.3, 11.5, 3.9, 1.9 Hz, H-6b). ^{13}C NMR (125 MHz, CD$_3$OD): δ 100.0 (C-1), 74.7 (C-4), 73.5 (C-3), 72.2 (C-2), 68.9 (d, $J_{C\text{-}P}$ = 12.1 Hz, C-5), 64.7 (d, $J_{C\text{-}P}$ = 165.7 Hz, C-7) 54.4 (CH$_3$O), 52.9 (d, $J_{C\text{-}P}$ = 7.5 Hz, CH$_3$OP), 52.4 (d, $J_{C\text{-}P}$ = 7.0 Hz, CH$_3$OP), 34.2 (d, $J_{C\text{-}P}$ = 2.8 Hz, C-6); ^{31}P NMR (202 MHz, CDCl$_3$) δ 27.43.

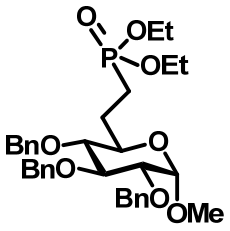
Methyl 6-Deoxy-7-phosphoryl-D/L-*glycero*-α-D-*gluco*-heptopyranoside, Bis-Lithium Salt (3.27)



According to the typical phosphono-ester cleavage procedure of compound **3.12**: **3.54** (39.6 mg, 0.125 mmol, 1.0 equiv), trimethylsilyl bromide (112 µl, 0.849

mmol, 10.0 equiv) and anhydrous triethylamine (124 µl, 0.891 mmol, 10.5 equiv.) in anhydrous CH_3CN under an N_2 environment was stirred for 12 h at 0 ^{0}C to rt to furnish **3.27** as a pale yellow foam (84:16 bis-lithium salt- lithium/$HNEt_3^+$ salt mixture, 36.0 mg, 0.0632 mmol, 91%). The product was isolated as a 5:2 mixture of diastereomers: $[\alpha]_D$ +46.7 (*c* 3.00, H_2O). HR ESIMS: calcd for: $C_8H_{16}O_9P$: 287.0537. Found: 287.0537. NMR data for the major (*S)* isomer: ^{1}H NMR (700 MHz, D_2O): δ 4.80 (d, 1H, *J* = 3.8 Hz, H-1), 3.96-3.92 (m, 1H, H-7), 3.85-3.80 (m, 1H, H-5), 3.65 (app t, 1H, *J* = 9.7 Hz, H-3), 3.60 (dd, 1H, *J* = 9.7, 3.8 Hz, H-2), 3.43 (s, 3H, C*H*$_3$O), 3.27 (app t, 1H, *J* = 9.7 Hz, H-4), 2.13 (dddd, 1H, *J* = 12.5, 10.1, 7.0, 1.7 Hz, H-6a), 1.84-1.76 (m, 1H, H-6b); ^{13}C NMR (125 MHz, D_2O): δ 99.1 (C-1), 73.5 (C-4), 73.1 (C-3), 71.5 (C-2), 67.0 (d, $J_{C\text{-}P}$ = 13.7 Hz, C-5), 64.4 (d, $J_{C\text{-}P}$ = 158.5 Hz, C-7), 55.0 (*C*H_3O), 33.2 (d, $J_{C\text{-}P}$ = 3.9 Hz, C-6); ^{31}P NMR (160 MHz, D_2O) δ 20.13. NMR data for the minor (*R*) isomer: ^{1}H NMR (700 MHz, D_2O): 4.79 (d, 1H, *J* = 3.8 Hz, H-1), 3.96-3.92 (m, 1H, H-7), 3.85-3.80 (m, 1H, H-5), 3.63 (app t, 1H, *J* = 8.9 Hz, H-3), 3.58 (dd, 1H, *J* = 8.9, 3.8 Hz, H-2), 3.44 (s, 1H, C*H*$_3$O), 3.32 (app t, 1H, *J* = 8.9 Hz, H-4), 2.32 (dddd, 1H, *J* = 11.2, 7.6, 3.9, 3.2 Hz, H-6a); 1.84-1.76 (m, 1H, H-6b); ^{13}C NMR (125 MHz, D_2O): δ 99.2 (C-1), 73.9 (C-4), 73.0 (C-3), 71.3 (C-2), 70.7 (d, $J_{C\text{-}P}$ = 14.2 Hz, C-5), 67.6 (d, $J_{C\text{-}P}$ = 156.7 Hz, C-7), 55.2 (*C*H_3O), 33.8 (d, $J_{C\text{-}P}$ = 2.3 Hz, C-6); ^{31}P NMR (160 MHz, D_2O) δ 19.38.

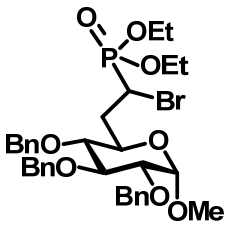
Methyl 2,3,4-Tri-*O*-benzyl-6,7-dideoxy-7-diethylphosphoryl-α-D-*gluco*-heptopyranoside (3.45):



A solution of **3.14** (1.293 g, 2.17 mmol, 1.0 equiv) in MeOH (100 mL) was cooled to -78 °C. With stirring, $NiCl_2 \cdot 6H_2O$ (2.579 g, 10.85 mmol, 5.0 equiv) was added followed by $NaBH_4$ (763 mg, 20.17 mmol, 10.0 equiv) in 3 portions every 5 min. Stirring was continued at -78 °C for 15 min before the cooling bath was removed and the reaction was allowed to warm rt. While warming the reaction colour changed from a homogeneous green solution to a heterogeneous black mixture. The reaction mixture was then partially concentrated *in vacuo* to reduce the volume before being diluted with CH_2Cl_2 (200 mL). 1 M HCl (100 mL) was added and the flask was stirred vigorously for 15 min. The mixture was then extracted with CH_2Cl_2 (3 x 100) and the combined organic layers were washed with brine (2 x 100 mL), dried over Na_2SO_4 and concentrated *in vacuo* to afford a colourless oil **3.45** (1.251 g, 2.09 mmol, 97%) that was spectroscopically pure. If further purification of the compound was performed it was passed through a silica plug (hexane then EtOAc) but the yield was reduced to 85%. R_f = 0.43 (EtOAc);

$[\alpha]_D$ +63.11 (*c* 0.50, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.38-7.28 (m, 15H, *Ph*CH_2), 4.98 (d, 1H, *J* = 10.8 Hz, C*H*$_2$Ph), 4.90 (d, 1H, 10.8 Hz, C*H*$_2$Ph), 4.81 (d, 1H, *J* = 10.8 Hz, C*H*$_2$Ph), 4.79 (d, 1H, *J* = 12.2 Hz, C*H*$_2$Ph), 4.66 (d, 1H, *J* = 12.1 Hz, C*H*$_2$Ph), 4.61 (d, 1H, *J* = 10.8 Hz, C*H*$_2$Ph), 4.54 (d, 1H, *J* = 3.6 Hz, H-1), 4.12-4.02 (m, 4H, 2 x CH_3C*H*$_2$OP), 3.96 (app t, 1H, *J* = 9.4 Hz, H-3), 3.57 (app td, 1H, *J* = 9.2, 2.7 Hz, H-5), 3.50 (dd, 1H, *J* = 9.4, 3.6 Hz, H-2), 3.35 (s, 3H, C*H*$_3$O), 3.18 (app t, 1H, *J* = 9.4 Hz, H-4), 2.19-2.09 (m, 1H, H-6a), 2.03-1.92 (m, 1H, H-7a), 1.75-1.57 (m, 2H, H-6b, H-7b), 1.30 (t, 6H, *J* = 7.1 Hz, 2 x C*H*$_3$$CH_2$OP); ^{13}C NMR (125 MHz, $CDCl_3$): δ 138.7, 138.2, 138.1, 128.5, 128.4, 128.1, 128.0(2), 128.0(0), 127.9(6), 127.8, 127.7, 97.9 (C-1), 82.0 (C-3), 81.7 (C-4), 80.1 (C-2), 75.8 (*C*H_2Ph), 75.3 (*C*H_2Ph), 73.4 (*C*H_2Ph), 70.0 (d, $J_{C\text{-}P}$ = 17.5 Hz, C-5), 61.6-61.5, (m, 2 x CH_3*C*H_2OP), 55.1 (*C*H_3O), 24.8 (d, $J_{C\text{-}P}$ = 4.1 Hz, C-6), 21.8 (d, $J_{C\text{-}P}$ = 142.8 Hz, C-7) 16.5 (d, $J_{C\text{-}P}$ = 1.3 Hz, *C*$H_3$$CH_2$OP), 16.4 (d, $J_{C\text{-}P}$ = 1.5 Hz, *C*$H_3$$CH_2$OP); ^{31}P NMR (160 MHz, $CDCl_3$) δ 32.00. HR ESIMS: *m/z* [M+Na^+] calcd for $C_{33}H_{43}O_8PNa$: 621.2588. Found: 621.2579.

Methyl 2,3,4-Tri-*O*-benzyl-6-deoxy-6-[(diethoxyphosphoryl)bromomethyl]-α-D-glucopyranoside (3.32)

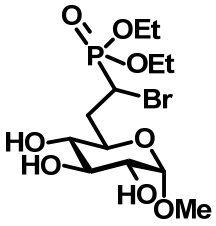


A solution of n-butyl lithium (2.5 M in hexanes, 163 μL, 0.407 mmol, 3.0 equiv) in anhydrous THF (1 mL) under N_2 was cooled to -97 ^oC in a liquid N_2/MeOH bath. To this solution **3.45** (81.1 mg, 0.136 mmol, 1.0 equiv) in anhydrous THF (2 mL) was added dropwise at a rate of 100 μL/min. The rate of addition was maintained by use of a syringe pump. During the addition of **3.45** it is essential the temperature does not rise above -90 ^oC. After complete addition, stirring was continued at -97 ^oC for an additional 5 min before the reaction was quenched with bromine (21 μL, 0.407 mmol, 3.0 equiv). The reaction was kept at -97 ^oC for 5 min longer before H_2O (0.5 mL) was added and the cooling bath was removed. The reaction was partially concentrated *in vacuo* to remove the majority of the THF before diluting with EtOAc (15 mL) and washing with H_2O (10 mL). The aqueous layer was then extracted with EtOAc (2 x 15 mL) and the combined organic layers were washed with brine (10 mL), dried over Na_2SO_4 and concentrated. Purification using column chromatography (1:2 to 1:1 EtOAc-

hexane) produced **3.32** (32.2 mg, 0.0476 mmol, 35%) as a colourless oil and a ~1:1 mixture of diastereomers that co-eluted on silica gel. R_f = 0.28 (1:1 EtOAc-hexane); $[\alpha]_D$ +35.27 (*c* 2.70, CH_2Cl_2); HR ESIMS: *m/z* [M+Na^+] calcd for: $C_{33}H_{46}O_8PBrNa$: 699.1693. Found: 699.1701. NMR data for the major isomer: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37-7.25 (m, 15H, *Ph*CH_2), 5.02 (d, 1H, *J* = 10.8 Hz, C*H*$_2$Ph), 4.92 (d, 1H, *J* = 11.2 Hz, C*H*$_2$Ph), 4.83 (d, 1H, *J* = 12.1 Hz, C*H*$_2$Ph), 4.82 (d, 1H, *J* = 11.7 Hz, C*H*$_2$Ph), 4.69 (d, 1H, *J* = 12.2 Hz, C*H*$_2$Ph), 4.64 (d, 1H, *J* = 11.2 Hz, C*H*$_2$Ph), 4.57 (d, 1 H, *J* = 3.6 Hz, H-1), 4.26-3.92 (m, 7H, 2 x CH_3C*H*$_2$OP, H-5, H-3, H-7), 3.52 (dd, 1H, *J* = 9.5, 3.5 Hz, H-2b), 3.48 (s, 2.4H, C*H*$_3$O), 3.25 (app t, 1H, *J* = 9.5 Hz, H-4), 2.37 (dddd, 1H, *J* = 14.7, 10.8, 5.6, 2.2 Hz, H-6a), 2.00 (dddd, 1H, *J* =14.7, 10.6, 5.1, 2.0 Hz, H-6), 1.39-1.32 (m, 6H, 2 x C*H*$_3$$CH_2$OP); ^{13}C NMR (125 MHz, $CDCl_3$): δ 136.2, 136.1, 135.7, 135.6, 135.5, 126.0, 125.9(3), 125.9(2), 125.8(9), 125.6(3), 125.6(2), 125.5, 125.4(9), 125.4(7), 125.4(1), 125.3, 125.2(8), 125.2(4), 125.1(7), 95.5 (C-1), 79.5 (C-4), 79.4 (C-3), 77.5 (C-2), 73.3 (*C*H_2Ph), 72.6 (*C*H_2Ph), 70.9 (*C*H_2Ph), 64.2 (d, $J_{C\text{-}P}$ = 12.3 Hz, C-5b), 61.3-61.1 (m, 2 x CH_3*C*H_2OP), 53.2 (*C*H_3O), 35.2 (d, $J_{C\text{-}P}$ = 160.3 Hz, C-7), 32.5 (C-6), 13.9(2)-13.8(7) (m, 2 x *C*$H_3$$CH_2$OP); ^{31}P NMR (160 MHz, $CDCl_3$): δ 17.80. NMR data for the minor isomer: 7.37-7.25 (m, 15H, *Ph*CH_2), 5.02 (d, 1H, *J* = 10.8 Hz, C*H*$_2$Ph), 4.98 (d, 1H, *J* = 10.9 Hz, C*H*$_2$Ph), 4.84 (d, 1H, *J* = 9.9 Hz, C*H*$_2$Ph), 4.82 (d, 1H, *J* = 11.0 Hz, C*H*$_2$Ph), 4.69 (d, 1H, *J* = 12.2 Hz, C*H*$_2$Ph), 4.67 (d, 1H, *J* = 12.3 Hz, C*H*$_2$Ph), 4.58 (d, 1H, *J* = 3.6 Hz, H-1), 4.26-3.92 (m,

7H, 2 x CH_3CH_2OP, H-5, H-3, H-7), 3.54 (dd, 1H, J = 9.5, 3.6 Hz, H-2), 3.45 (s, 3H, CH_3O), 3.31 (app t, 1H, J = 9.5 Hz, H-4), 2.75 (dddd, 1H, J = 15.1, 9.9, 6.2, 4.4 Hz, H-6a), 2.19-2.10 (m, 1H, H-6b), 1.39-1.32 (m, 6H, 2 x CH_3CH_2OP); ^{13}C NMR (125 MHz, $CDCl_3$): δ 136.2, 136.1, 135.7, 135.6, 135.5, 126.0, 125.9(3), 125.9(2), 125.8(9), 125.6(3), 125.6(2), 125.5, 125.4(9), 125.4(7), 125.4(1), 125.3, 125.2(8), 125.2(4), 125.1(7), 95.6 (C-1), 79.9 (C-4), 79.0 (C-3), 77.6 (C-2), 73.3 (CH_2Ph), 72.6 (CH_2Ph), 70.9 (CH_2Ph), 66.1 (d, $J_{C\text{-}P}$ = 9.8 Hz, C-5), 61.3-61.1 (m, 2 x CH_3CH_2OP), 53.2 (CH_3O), 34.8 (d, $J_{C\text{-}P}$ = 157.5 Hz, C-7), 34.5 (C-6), 13.9(2)-13.8(7) (m, 2 x CH_3CH_2OP); ^{31}P NMR (160 MHz, $CDCl_3$): δ 17.59.

Methyl 6-Deoxy-6-[(diethoxyphosphoryl)bromomethyl]-α-D-glucopyranoside (3.46)

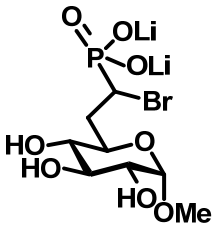


To a solution of **3.32** (65 mg, 0.0961 mmol, 1.0 equiv) in anhydrous DCM under N_2 was added anhydrous $FeCl_3$ (140 mg, 0.865 mmol, 9.0 equiv) with stirring in one batch at rt. This dark brown heterogeneous solution was stirred for 10 min before it was quenched with H_2O (0.5 mL). Stirring for 15 minutes longer resulted in a light brown heterogeneous solution that was filtered through a bed of Celite

eluted with 90:9:1 CH_2Cl_2-MeOH-NEt_3. The solvent was then removed *in vacuo* and dissolved in MeOH. To this solution Amberlite 120 resin (H^+ form) was added to remove some of the $NEt_3 \cdot HCl$ salt. After stirring for 2 h the resin was filtered off and the eluent was purified using column chromatography (9:1 CH_2Cl_2-MeOH) to furnish a light brown oil. This oil was dissolved in MeOH and Amberlite 120 resin (H^+ form) was added to remove the remaining $NEt_3 \cdot HCl$ salt. After 2 hours the resin was filtered off and the solvent was removed *in vacuo* to produce a light brown oil **3.46** (32 mg, 0.0788 mmol, 82%). R_f = 0.32 (9:1 CH_2Cl_2-MeOH); $[\alpha]_D$ +66.65 (*c* 0.39, CH_3OH); HR ESIMS: *m/z* [M+Na^+] calcd for: $C_{12}H_{24}O_8PBrNa$: 429.0284. Found: 429.0273. NMR data for the *S* isomer: ^1H NMR (500 MHz, CD_3OD): δ 4.62 (d, 1H, *J* = 3.9 Hz, H-1), 4.25-4.18 (m, 5H, H-7, 2 x CH_3CH_2OP), 3.83-3.76 (m, 1H, H-5), 3.61-3.50 (m, 1H, H-3), 3.42 (s, CH_3O), 3.36 (dd, 1H, *J* = 5.1, 3.9 Hz, H-2), 3.12-3.06 (m, 1H, H-4), 2.39 (dddd, 1H, *J* = 14.8, 10.9, 5.6, 2.1 Hz, H-6b), 2.12-1.99 (m, 1H, H-6b), 1.36-1.33 (m, 6H, 2 x CH_3CH_2OP); ^{13}C NMR (125 MHz, CD_3OD): δ 101.5 (C-1), 76.6 (C-4), 74.9 (C-3), 73.6 (C-2), 70.5 (d, $J_{C\text{-}P}$ = 9.5 Hz, C-5), 65.5-65.4 (m, CH_3CH_2OP), 65.3-65.2 (m, CH_3CH_2OP), 56.1 (CH_3O), 38.6 (C-6), 38.5 (d, $J_{C\text{-}P}$ = 161.6 Hz, C-7), 16.8 (m, CH_3CH_2OP), 16.7 (m, CH_3CH_2OP); ^{31}P NMR (202 MHz, CD_3OD): δ 20.93. NMR data for the *R* isomer: ^1H NMR (500 MHz, CD_3OD): δ 4.65 (d, 1H, *J* = 3.9 Hz, H-1), 4.28 (m, 1H, H-7a), 4.25-4.18 (m, 4H, 2 x CH_3CH_2OP), 3.83-3.76 (m, 1H, H-5), 3.61-3.50 (m, 1H, H-3), 3.46 (s, CH_3O), 3.38 (dd, 1H, *J* = 5.0, 3.9

Hz, H-2), 3.12-3.06 (m, 1H, H-4), 2.68 (dddd, 1H, *J* = 15.0, 9.9, 6.6, 4.6 Hz, H-6a), 2.12-1.99 (m, 1H, H-6b), 1.36-1.33 (m, 6H, 2 x CH_3CH_2OP); ^{13}C NMR (125 MHz, CD_3OD): δ 101.4 (C-1), 75.6 (C-4), 75.0 (C-3), 73.7 (C-2), 68.9 (d, J_{C-P} = 13.7 Hz, C-5), 65.5-65.4 (m, CH_3CH_2OP), 65.3-65.2 (m, CH_3CH_2OP), 56.2 (CH_3O), 37.9 (d, J_{C-P} = 158.8 Hz, C-7), 36.3 (C-6), 16.8 (m, CH_3CH_2OP), 16.7 (m, CH_3CH_2OP); ^{31}P NMR (202 MHz, CD_3OD): δ 20.92.

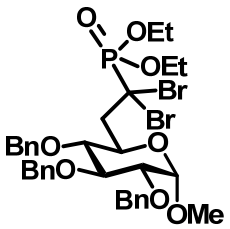
Methyl 6-Deoxy-6-[monobromophosphorylmethyl]-α-D-glucopyranoside, Bis-Lithium Salt (3.6)



According to our phoshonoester cleavage procedure: **1a** (27.2 mg, 0.0670 mmol, 1.0 equiv), trimethylsilyl bromide (88 µl, 0.670 mmol, 10.0 equiv) and anhydrous NEt_3 (65 µl, 0.804 mmol, 12.0 equiv.) in anhydrous CH_3CN (3 mL) under an N_2 environment was stirred for 12 h at 0 ^oC to rt to furnish **3.6** as a colourless foam (73:27 bis-lithium salt- lithium/$HNEt_3^+$ salt mixture: 29.3 mg, 0.102, 78%). $[\alpha]_D$ +66.69 (*c* 0.34, H_2O); Data for the *S* isomer: ^1H NMR (500 MHz, D_2O): δ 4.79 (d, 1H, *J* = 3.8 Hz, H-1), 4.05-3.95 (m, 1H, H-7), 3.92-3.82 (m, 1H, H-5), 3.64-3.54 (m, 2H, H-3, H-2), 3.49 (s, 3H, CH_3O), 3.28 (app t, 1H, *J* = 9.4 Hz, H-4), 2.34-

2.26 (m, 1H, H-6a), 2.14-2.03 (m, 1H, H-6b); ^{13}C NMR (125 MHz, D_2O): δ 99.2 (C-1), 74.3 (C-4), 73.1 (C-3), 71.4 (C-2), 68.8 (d, $J_{C\text{-}P}$ = 12.6 Hz, C-5), 55.6 (*C*H_3O), 43.3 (d, $J_{C\text{-}P}$ = 143.1 Hz, C-7), 35.2 (C-6); ^{31}P NMR (160 MHz, D_2O) δ) δ 17.18. Data for the *R* isomer: ^{1}H NMR (500 MHz, D_2O): δ 4.79 (d, 1H, *J* = 3.8 Hz, H-1), 4.05-3.95 (m, 1H, H-7), 3.92-3.82 (m, 1H, H-5), 3.64-3.54 (m, 2H, H-3, H-2), 3.46 (s, 3H, C*H*$_3$O), 3.32 (app t, 1H, *J* = 9.4 Hz, H-4), 2.73-2.66 (m, 1H, H-6a), 2.14-2.03 (m, 1H, H-6b); ^{13}C NMR (125 MHz, D_2O): δ 99.3 (C-1), 74.3 (C-4), 73.2 (C-3), 71.3 (C-2), 70.7 (d, $J_{C\text{-}P}$ = 12.4 Hz, C-5), 55.6 (*C*H_3O), 43.8 (d, $J_{C\text{-}P}$ = 142.5 Hz, C-7), 36.9 (C-6); ^{31}P NMR (160 MHz, D_2O) δ 17.02. HR ESIMS: *m/z* [M-H$^-$] calcd for: $C_8H_{15}O_8PBrNa$: 348.9693 Found: 348.9692.

Methyl 2,3,4-Tri-*O*-benzyl-6-deoxy-6-[(diethoxyphosphoryl)dibromomethyl]-α-D-glucopyranoside (3.49)



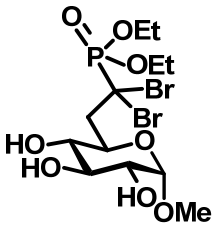
A solution of *n*-butyl lithium (2.5 M in hexanes, 373 μL, 0.933 mmol, 3.0 equiv) in anhydrous THF (2 mL) under N_2 was cooled to -97 ^{o}C in a liquid N_2/MeOH bath. To this solution **3.45** (186 mg, 0.311 mmol, 1.0 equiv) in anhydrous THF (5 mL) was added dropwise at a rate of 100 μL/min. The rate of addition was

maintained by use of a syringe pump. During the addition of **3.45** it is essential the temperature does not rise above -90 ^{o}C. After complete addition, stirring was continued at -97 ^{o}C for an additional 5 min before the reaction was quenched with bromine (48 μL, 0.933 mmol, 3.0 equiv). The reaction was kept at -97^{o}C for 5 min longer before H_2O (1 mL) was added and the cooling bath was removed. The reaction was partially concentrated *in vacuo* to remove the majority of the THF before diluting with EtOAc (30 mL) and washed with H_2O (20 mL). The aqueous layer was then extracted with EtOAc (2 x 30 mL) and the combined organic layers were washed with brine (20 mL), dried over Na_2SO_4 and concentrated *in vacuo* to produce a very crude product of **3.32** as brown oil (200 mg) that was not purified before use in the next step. This oil was dried on a high vacuum for a minimum of 12 hours prior to use. To a solution of anhydrous diisopropylamine (125 μL, 0.887 mmol, 3.0 mmol) in anhydrous THF cooled to 0 ^{o}C under an N_2 environment was added *n*-butyl lithium (2.5 M solution in hexanes, 350 μL, 0.887 mmol, 3.0 equiv) dropwise with stirring. Stirring was continued at 0 ^{o}C for 15 min before the flask was cooled to -97 ^{o}C in a liquid N_2/MeOH bath. To this solution, crude **3.32** (200 mg, 0.296 mmol, 1.0 equiv) in anhydrous THF (5 mL) was added dropwise at a rate of 100 μL/min. The rate of addition was maintained by use of a syringe pump. Once again the temperature of the bath did not rise above -90 ^{o}C. After complete addition, stirring was continued at -97 ^{o}C for an additional 5 min before the reaction was quenched with bromine (46 μL, 0.887 mmol, 3.0 equiv). The

reaction was kept at -97 °C for 5 min longer before H_2O (1 mL) was added and the cooling bath was removed. The reaction was partially concentrated *in vacuo* to remove the majority of the THF before diluting with EtOAc (30 mL) and washed with H_2O (20 mL). The aqueous layer was then extracted with EtOAc (2 x 30 mL) and the combined organic layers were washed with brine (20 mL), dried over Na_2SO_4 and concentrated *in vacuo*. Purification using column chromatography (1:2 to 1:1 EtOAc-hexane furnished **3.49** (33.2 mg, 0.0440 mmol, 14% from **3.32**) as a colourless oil followed by recovered **3.32** (63.2 mg, 0.0935 mmol, 30% from **3.45**) also as a colourless oil and as a ~1:1 mixture of diastereomers that co-eluted. R_f = 0.50 (1:1 EtOAc-hexane); $[\alpha]_D$ +25.31 (*c* 1.00, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.38-7.26 (m, 15H, *Ph*CH_2), 5.01 (d, 1H, *J* = 10.7 Hz, C*H*$_2$Ph), 4.93 (d, 1H, 11.0 Hz, C*H*$_2$Ph), 4.82-4.79 (m, 2H, 2 x C*H*$_2$Ph), 4.67 (d, 1H, *J* = 12.2 Hz, C*H*$_2$Ph), 4.66 (d, 1H, *J* = 12.1 Hz, C*H*$_2$Ph), 4.61 (d, 1H, *J* = 3.6 Hz, H-1), 4.58 (d, 1H, *J* = 11.1 Hz, C*H*$_2$Ph), 4.38-4.25 (m, 5H, 2 x CH_3C*H*$_2$OP, H-5), 4.07 (app t, 1H, *J* = 9.4 Hz, H-3), 3.57 (s, 3H, C*H*$_3$O), 3.52 (dd, 1H, *J* = 9.4, 3.6 Hz, H-2), 3.21 (app t, 1H, *J* = 9.4 Hz, H-4), 3.06 (dd, 1H, *J* = 15.2, 6.1 Hz, H-6a), 2.52 (ddd, 1H, 15.2, 8.9, 5.0 Hz, H-6b), 1.35-1.31 (m, 6H, 2 x C*H*$_3$$CH_2$OP); ^{13}C NMR (125 MHz, $CDCl_3$): δ 138.6, 138.2, 137.9, 128.5, 128.4(8), 128.3(8), 128.1, 128.0(6), 128.0(0), 127.9, 127.8, 127.7, 98.2 (C-1), 81.9 (C-3), 80.3 (C-4), 79.8 (C-2), 75.8 (*C*H_2Ph), 75.2 (*C*H_2Ph), 73.4 (*C*H_2Ph), 69.2 (d, $J_{C\text{-}P}$ = 10.8 Hz, C-5), 65.9, (d, $J_{C\text{-}P}$ = 7.2 Hz, CH_3*C*H_2OP), 65.8 (d, $J_{C\text{-}P}$ = 7.5 Hz, CH_3*C*H_2OP),

56.7 (CH_3O), 55.9 (d, $J_{C\text{-}P}$ = 170.4 Hz, C-7), 44.5 (C-6), 16.5 (CH_3CH_2OP), 16.4 (CH_3CH_2OP); ^{31}P NMR (202 MHz, $CDCl_3$) δ 11.63. HR ESIMS: *m/z* [M+Na^+] calcd for: $C_{33}H_{41}O_8PBr_2Na$: 777.0798. Found: 777.0803.

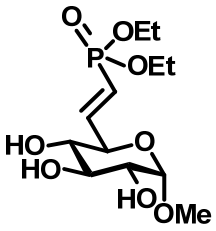
Methyl 6-Deoxy-6-[(diethoxyphosphoryl)dibromomethyl]-α-D-glucopyranoside (3.50)



Compound **3.50** was prepared according to the $FeCl_3$ debenzylation conditions used in the synthesis of **3.46**: Compound **3.49** (19.0 mg, 0.0252 mmol, 1.0 equiv) was reacted with anhydrous $FeCl_3$ (37.0 mg, 0.227 mmol, 9.0 equiv) in anhydrous CH_2Cl_2 at rt for 10 min to furnish **3.50** (8.2 mg, 0.0162 mmol, 64%). R_f = 0.35 (9:1 CH_2Cl_2-MeOH); $[\alpha]_D$ +27.32 (*c* 0.32, CH_3OH); ^1H NMR (500 MHz, CD_3OD): δ 4.65 (d, 1H, *J* = 3.8 Hz, H-1) 4.16-4.05 (m, 4H, 2 x CH_3CH_2OP), 4.16 (app t, 1H, *J* = 9.0 Hz, H-5), 3.63 (app t, 9.4 Hz, H-3), 3.56 (s, 3H, CH_3O), 3.38 (dd, 1H, *J* = 9.4, 3.8 Hz, H-2), 3.14 (ddd, 1H, *J* = 15.4, 6.4, 1.0 Hz, H-6a), 3.04 (app t, 1H, *J* = 9.4 Hz, H-4), 2.52 (ddd, 1H, *J* = 15.4, 8.4, 6.0 Hz, H-6b), 1.40-1.36 (m, 6H, 2 x CH_3CH_2OP); ^{13}C NMR (125 MHz, CD_3OD): δ 100.1 (C-1), 73.6 (C-4), 73.4 (C-3), 72.0 (C-2), 69.9 (d, $J_{C\text{-}P}$ = 10.3, C-5), 66.0 (d, $J_{C\text{-}P}$ = 7.7 Hz,

CH_3CH_2OP), 66.71 (d, $J_{C\text{-}P}$ = 7.5 Hz, CH_3CH_2OP), 55.7 (CH_3O), 54.7 (d, $J_{C\text{-}P}$ = 173.0 Hz, C-7), 44.7 (C-6), 15.3(3) (CH_3CH_2OP), 15.2(9) (CH_3CH_2OP); ^{31}P NMR (202 MHz, CD_3OD): δ 11.71. HR ESIMS: *m/z* [M+Na^+] calcd for: $C_{12}H_{23}O_8PBr_2Na$: 506.939 Found: 506.9385.

Methyl 6-Deoxy-6-(diethoxyphosphorylmethylene)-α-D-glucopyranoside (3.51)



Compound **3.51** was prepared according to the $FeCl_3$ debenzylation conditions used in the synthesis of **3.46**: Compound **15** (37.0 mg, 0.0620 mmol, 1.0 equiv) was reacted with anhydrous $FeCl_3$ (90.5 mg, 0.558 mmol, 9.0 equiv) in anhydrous CH_2Cl_2 at rt for 10 min to furnish **3.51** (16.6 mg, 0.0508 mmol, 82%). R_f = 0.43 (9:1 CH_2Cl_2-MeOH); $[\alpha]_D$ +85.16 (*c* 0.58, CH_3OH); ^{1}H NMR (500 MHz, CD_3OD) δ 6.97 (ddd, 1H, *J* = 22.0, 17.2, 3.8 Hz, H-6), 6.06 (ddd, 1H, *J* = 21.9, 17.2, 2.0 Hz, H-7), 4.76 (d, 1H, *J* = 3.8 Hz, H-1), 4.16-4.06 (m, 5H, CH_3CH_2OP, H-5), 3.65 (app t, 1H, *J* = 9.4 Hz, H-3), 3.42 (dd, 1H, *J* = 9.4, 3.8 Hz, H-2), 3.41 (s, 3H, CH_3O), 3.09 (app t, 1H, *J* = 9.4 Hz, H-4), 1.33 (t, 6H, *J* = 7.1 Hz, CH_3CH_2OP); ^{13}C NMR (125 MHz, CD_3OD): δ 149.8 (d, $J_{C\text{-}P}$ = 6.2 Hz, C-6),

115.7 (d, $J_{C\text{-}P}$ = 188.9 Hz, C-7), 100.2 (C-1), 74.0 (d, $J_{C\text{-}P}$ = 1.5 Hz, C-4), 73.7 (C-4), 71.9 (C-2), 70.5 (d, $J_{C\text{-}P}$ = 20.9 Hz, C-5), 62.1-62.0, (m, 2 x CH_3CH_2OP), 54.5 (CH_3O), 15.2(2) (CH_3CH_2OP), 15.1(8) (CH_3CH_2OP); ^{31}P NMR (202 MHz, $CDCl_3$) δ 19.20.

3.9.3 G6Pase inhibition studies

These assays were developed using a variation of two procedures (Sigma-Aldrich®[103] and Nordlie and Arion, 1966[105]). Assays were conducted using male wister rat microsomes (Invitrogen, 20 mg/mL protein) which were purchased and stored at -80 ^oC in 25 µL aliquots. Assay protein concentration varied depending on the length of incubation time. All inhibitors were diluted to 75 mM, 100 mM or 250 mM solutions in H_2O and stored at -20 ^oC. The G6P substrate was diluted to 200 mM in H_2O and stored at 4 ^oC. All assays consisted of a total volume of 150 µL in a microfuge tube. All other reagents discussed herein were prepared as an aqueous solution and stored at 4 ^oC. All experiments were completed in triplicate or quadruplicate as indicated.

3.9.3.1 Phosphonic acid assays

For long term incubations, inhibitor (2.5 mM) and microsome (2.5 µL, 0.33 mg/mL protein) were incubated in assay buffer (3-(*N*-morpholino)propanesulfonic acid (MOPS), 100 mM, pH 6.5) and Triton X-100 surfactant (0.0067%) at 4 ^oC

for 60 h. After 60 h the tubes were warmed to 37 ^{o}C. G6P (3 μL, 4 mM) was then added and the tubes were then reacted for 15 min at 37 ^{o}C. The reaction was subsequently quenched with 40% trichloroacetic acid (TCA) (10 μL) and the tubes were centrifuged at 15 000 RPM for 5 min. After 5 min, a 100 μL aliquot was removed and placed in a well of a clear-bottomed 96 well plate (FD Falcon clear). To this, 100 μL of Taussky-Shorr reagent (TSR) (1% w/v ammonium molybdate, 2.7% v/v H_2SO_4 and 5% w/v ferrous sulfate hexahydrate)[109, 113] was added and the wells were reacted at room temperature for 5 min before A_{660} was read by a Spectra Max M2 plate reader (n = 4).

For short term incubations inhibitor (2.5 mM) and microsome (1.5 μL, 0.2 mg/mL protein) were incubated in MOPS buffer and Triton X-100 surfactant (0.0067%) at 37 ^{o}C for 15 min. G6P (2 μL, 2.67 mM) was then added and the tubes were then reacted for 10 min at 37 ^{o}C. The reaction was subsequently quenched with 40% TCA (10 μL) and the tubes were centrifuged at 15 000 RPM for 5 min. After 5 min, a 100 μL aliquot was removed and placed in a well of a clear-bottomed 96 well plate (FD Falcon). To this, 100 μL of TSR was added and the wells were reacted at room temperature for 5 min before A_{660} was read by Spectra Max M2 plate reader (n = 3).

3.9.3.2 Phosphonate ester assays

Inhibitor (2.5 mM) and microsome (2.0 μL, 0.27 mg/mL protein) were incubated in MOPS buffer at 4 ^{o}C for 60 min. G6P (2.5 μL, 3.33 mM) was then added and the tubes were reacted for 15 min at 37 ^{o}C. The reaction was subsequently quenched with 40% TCA (10 μL) and the tubes were centrifuged at 15 000 RPM for 5 min. After 5 min, a 100 μL aliquot was removed and placed in a well of a clear-bottomed 96 well plate (FD Falcon). To this, 100 μL of TSR was added and the wells were reacted at room temperature for 5 min before A_{660} was read by the Spectra Max M2 plate reader (n = 3).

3.9.3.3 Dilution experiment

This experiment is similar to the experiment described by Nordlie et al (1996).[114] Three microfuge tubes were used. In one tube, inhibitor (2.5 mM) and microsome (1.0 μL, 0.4 mg/mL protein) were incubated. In the other two tubes microsome (1.0 μL, 0.4 mg/mL protein) was incubated in buffer alone. All incubations occurred in MOPS buffer at 4 ^{o}C for 60 min in total assay volume of 50 μL (1/3 the volume of the above assays). After 60 min, 100 μL of MOPS buffer was added to all three tubes to restore the volume to 150 μL as in all the other assays. In the second tube, inhibitor (2.5 mM) was added. All three tubes were incubated on the bench top at 20 ^{o}C for ten minutes at this point. After ten minutes, G6P substrate

(9 μL, 12 mM) was added and the tubes were reacted at 37 ^{o}C for 15 minutes. The reaction was subsequently quenched with 40% TCA (10 μL) and the tubes were centrifuged at 15 000 RPM for 5 min. After 5 min, a 100 μL aliquot was removed and placed in a well of a clear-bottomed 96 well plate (FD Falcon). To this, 100 μL of TSR was added and the wells were reacted at room temperature for 5 min before A_{660} was read by Spectra Max M2 plate reader.

3.9.3.4 Rate of G6P hydrolysis by Taussky-Shorr assay

Colorimetric data was converted to a rate of G6P hydrolysis by use of the equation y = 0.0068x + 0.0174 from the line of best fit in **Figure 3.6** to provide a rate in μmol G6P/mg protein. **Figure 3.6** was generated by reacting a known concentration of phosphorus with the TSR for 5 minutes in a well of a clear-bottomed 96 well plate. The data were then plotted in Microsoft Excel (n = 3).

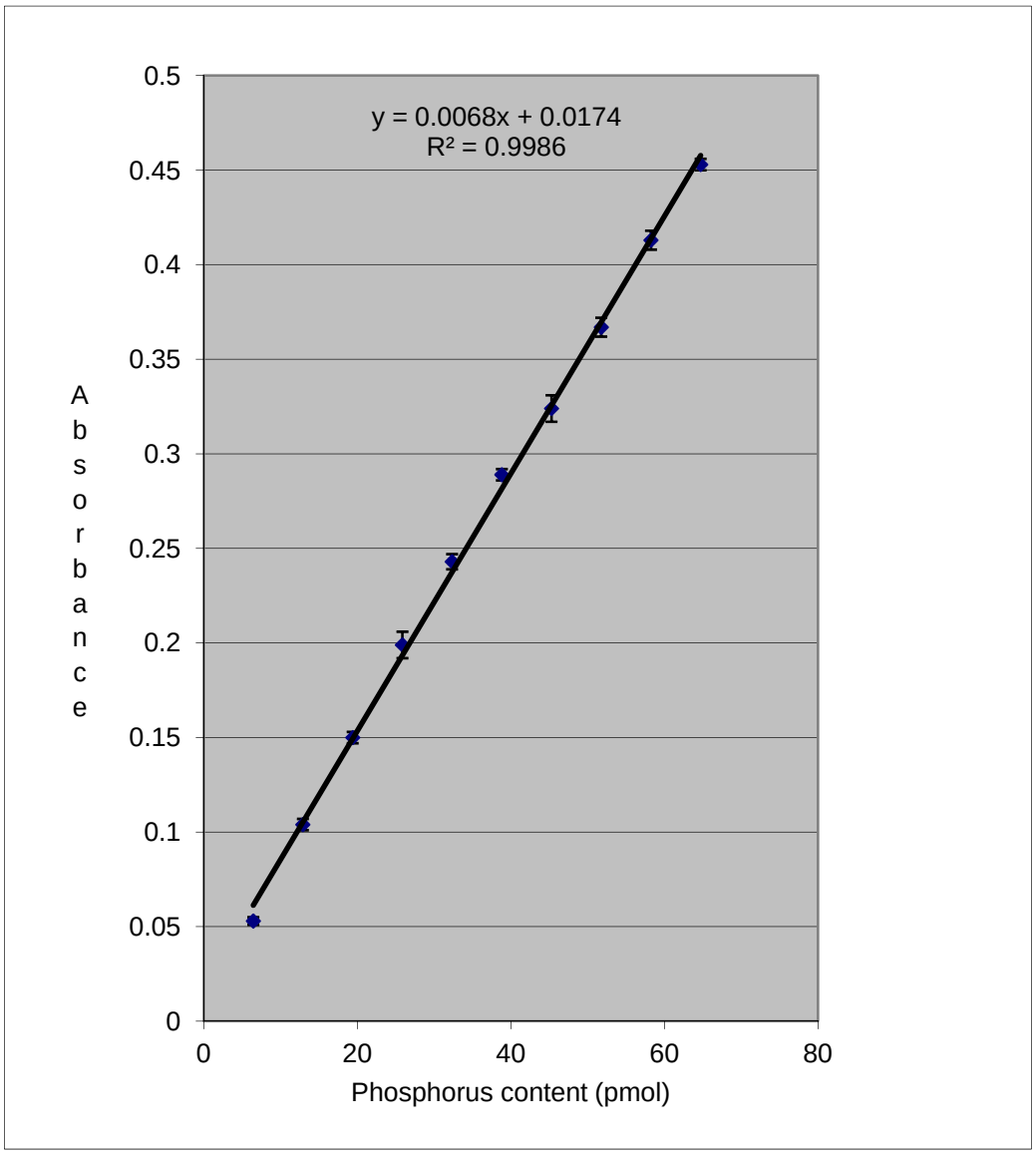


Figure 3.6: Quantification of phosphorus content from absorbance.

3.10 References

(1) Nordlie, R. C.; Foster, J. D.; Lange, A. J. Regulation of glucose production by the liver *Annu Rev Nutr* **1999,** *19,* 379.

(2) Ashmore, J.; Hastings, A. B.; Nesbett, F. B. The Effect of Diabetes and Fasting on Liver Glucose-6-Phosphatase *Proc Natl Acad Sci USA* **1954,** *40,* 673.

(3) Kim-Muller, J. Y.; Accili, D. Cell biology. Selective insulin sensitizers *Science* **2011,** *331,* 1529.

(4) Pan, C. J.; Lei, K. J.; Annabi, B.; Hemrika, W.; Chou, J. Y. Transmembrane topology of glucose-6-phosphatase *J Biol Chem* **1998,** *273,* 6144.

(5) Engel, R. Phosphonates as Analogs of Natural Phosphates *Chem Rev* **1977,** *77,* 349.

(6) Romanenko, V. D.; Kukhar, V. P. Fluorinated phosphonates: Synthesis and biomedical application *Chem Rev* **2006,** *106,* 3868.

(7) Blackburn, G. M. Phosphonates as Analogs of Biological Phosphates *Chem Ind (London)* **1981,** 134.

(8) Lemarechal, P.; Froussios, C.; Level, M.; Azerad, R. Synthesis of Phosphono Analogs of 3-Deoxy-D-Arabino-Hept-2-Ulosonic Acid 7-Phosphate *Carbohydr Res* **1981,** *94,* 1.

(9) Huang, Z.; Kakinuma, K.; Eguchi, T. Stereo specificity of hydride transfer in NAD(+)-catalyzed 2-deoxy-scyllo-inososе synthase, the key enzyme in the biosynthesis of 2-deoxystreptamine-containing aminocycl itol antibiotics *Bioorg*

Chem **2005,** *33,* 82.

(10) Padyukova, N. S.; Afsar, S.; Dixon, H. B. F.; Karpeiskii, M. Y. Synthesis and Properties of Glucose and Galactose Phosphonate Derivatives *Bioorg Khim* **1995,** *21,* 382.

(11) Smith-Griffin, B.; Burger, A. D-Glucopyranose 6-Deoxy-6-Phosphonic Acid *J Am Chem Soc* **1956,** *78,* 2336.

(12) Nifantev, E. E.; Gudkova, I. P.; Kochetko.Nk Singular of 1,6-Anhydrohexose with Hypophosphorous Acid *Zh Obshch Khim* **1970,** *40,* 460.

(13) Hirayama, T.; Kudo, F.; Huang, Z.; Eguchi, T. Role of glutamate 243 in the active site of 2-deoxy-scyllo-inosose synthase from Bacillus circulans *Bioorg Med Chem* **2007,** *15,* 418.

(14) Adams, P. R.; Harrison, R.; Inch, T. D.; Rich, P. Dehydrogenation of Phosphonate Analog of Glucose-6-Phosphate by Glucose-6-Phosphate Dehydrogenase *Biochem J* **1976,** *155,* 1.

(15) Roach, D. J. W.; Harrison, R.; Hall, C. R.; Inch, T. D. An Improved Synthesis of 6,7-Dideoxy-Alpha-D-Gluco-Heptose 7-Phosphonic Acid - Appendix *Biochem J* **1981,** *197,* 735.

(16) Berkowitz, D. B.; Bose, M.; Pfannenstiel, T. J.; Doukov, T. alpha-fluorinated phosphonates as substrate mimics for glucose 6-phosphate dehydrogenase: the CHF stereochemistry matters *J Org Chem* **2000,** *65,* 4498.

(17) Berkowitz, D. B.; Bhuniya, D.; Peris, G. Facile installation of the

phosphonate and (alpha,alpha-difluoromethyl)phosphonate functionalities equipped with benzyl protection *Tetrahedron Lett* **1999,** *40,* 1869.

(18) Benyoseph, O.; Sparkes, M. J.; Dixon, H. B. F. A Rapid Spectrophotometric Estimation of Individual Phosphates and Phosphonates - Its Application to Chromatography of Sugar Phosphates and Their Phosphonate Analogs *Anal Biochem* **1993,** *210,* 195.

(19) Benyoseph, O.; Sparkes, M. J.; Dixon, H. B. F. A Rapid Spectrophotometric Estimation of Individual Phosphates and Phosphonates - Its Application to Chromatography of Sugar Phosphates and Their Phosphonate Analogs, (Vol 210, Pg 195, 1994) *Anal Biochem* **1994,** *219,* 389.

(20) Berkowitz, D. B.; Bose, M. (alpha-monofluoroalkyl)phosphonates: a class of isoacidic and "tunable" mimics of biological phosphates *J Fluorine Chem* **2001,** *112,* 13.

(21) Berkowitz, D. B.; Bose, M.; Asher, N. G. A convergent triflate displacement approach to (alpha-monofluoroalkyl)phosphonates *Org Lett* **2001,** *3,* 2009.

(22) Berkowitz, D. B.; Sloss, D. G. Diallyl (Lithiodifluoromethyl)Phosphonate - a New Reagent for the Introduction of the (Difluoromethylene)Phosphonate Functionality *J Org Chem* **1995,** *60,* 7047.

(23) Berkowitz, D. B.; Eggen, M.; Shen, Q.; Sloss, D. G. Synthesis of (Alpha,Alpha-Difluoroalkyl)Phosphonates by Displacement of Primary Triflates *J*

Org Chem **1993,** *58,* 6174.

(24) Panigrahi, K.; Eggen, M.; Maeng, J. H.; Shen, Q. R.; Berkowitz, D. B. The alpha,alpha-Difluorinated Phosphonate L-pSer-Analogue: An Accessible Chemical Tool for Studying Kinase-Dependent Signal Transduction *Chem Biol* **2009,** *16,* 928.

(25) Cappellini, M. D.; Fiorelli, G. Gluclose-6-phosphate dehydrogenase deficiency *Lancet* **2008,** *371,* 64.

(26) Vidil, C.; Morere, A.; Garcia, M.; Barragan, V.; Hamdaoui, B.; Rochefort, H.; Montero, J. L. Synthesis and biological activity of phosphonate analogs of mannose 6-phosphate (M6P) *Eur J Org Chem* **1999,** 447.

(27) Clavel, C.; Barragan-Montero, V.; Garric, X.; Moles, J. P.; Montero, J. L. Synthesis and biological activity of M6-P and M6-P analogs on fibroblast and keratinocyte proliferation *Farmaco* **2005,** *60,* 721.

(28) Ghosh, A.; Shieh, J. J.; Pan, C. J.; Sun, M. S.; Chou, J. Y. The catalytic center of glucose-6-phosphatase - HIS176 is the nucleophile forming the phosphohistidine-enzyme intermediate during catalysis *J Biol Chem* **2002,** *277,* 32837.

(29) Tulsi, N. S.; Downey, A. M.; Cairo, C. W. A protected l-bromophosphonomethylphenylalanine amino acid derivative (BrPmp) for synthesis of irreversible protein tyrosine phosphatase inhibitors *Bioorg Med Chem* **2010,** *18,* 8679.

(30) Roach, P. J.; Depaoli-Roach, A. A.; Hurley, T. D.; Tagliabracci, V. S. Glycogen and its metabolism: some new developments and old themes *Biochem J* **2012,** *441,* 763.

(31) Bernotas, R. C.; Pezzone, M. A.; Ganem, B. Synthesis of (+)-1,5-Dideoxy-1,5-Imino-D-Galactitol, a Potent Alpha-D-Galactosidase Inhibitor *Carbohydr Res* **1987,** *167,* 305.

(32) Julina, R.; Vasella, A. Deoxy-Nitrosugars .10. Synthesis of Isosteric Phosphonate Analogs of Ulose-1-Phosphates *Helv Chim Acta* **1985,** *68,* 819.

(33) Mukherjee, C.; Ghosh, S.; Nandi, P.; Sen, P. C.; Misra, A. K. Efficient synthesis of (6-deoxy-glycopyranosid-6-yl) sulfone derivatives and their effect on Ca2+-ATPase *Eur J Med Chem* **2010,** *45,* 6012.

(34) Bhattacharya, A. K.; Thyagarajan, G. The Michaelis-Arbuzov Rearrangement *Chem Rev* **1981,** *81,* 415.

(35) Mckenna, C. E.; Higa, M. T.; Cheung, N. H.; Mckenna, M. C. Facile Dealkylation of Phosphonic Acid Dialkyl Esters by Bromotrimethylsilane *Tetrahedron Lett* **1977,** 155.

(36) Mckenna, C. E.; Schmidhauser, J. Functional Selectivity in Phosphonate Ester Dealkylation with Bromotrimethylsilane *J Chem Soc Chem Comm* **1979,** 739.

(37) Dinev, Z.; Gannon, C. T.; Egan, C.; Watt, J. A.; McConville, M. J.; Williams, S. J. Galactose-derived phosphonate analogues as potential inhibitors of

phosphatidylinositol biosynthesis in mycobacteria *Org Biomol Chem* **2007,** *5,* 952.

(38) Li, M. Y.; Wang, L. S.; Wang, K. P.; Jiang, B.; Gmehling, J. Experimental measurement and modeling of solubility of LiBr and LiNO3 in methanol, ethanol, 1-propanol, 2-propanol and 1-butanol *Fluid Phase Equilib* **2011,** *307,* 104.

(39) Yonekawa, S.; Lingwood, C. A.; Mylvaganam, M. Oxidation of the primary hydroxyl group of galactose of galactaosyl ceramide analogue by chemical method-precursors for the synthesis of labeled conjugates *Carbohydr Res* **2009,** *344,* 501.

(40) Dess, D. B.; Martin, J. C. A Useful 12-I-5 Triacetoxyperiodinane (the Dess-Martin Periodinane) for the Selective Oxidation of Primary or Secondary Alcohols and a Variety of Related 12-I-5 Species *J Am Chem Soc* **1991,** *113,* 7277.

(41) Pudovik, A. N.; Konovalova, I. V. Addition-Reactions of Esters of Phosphorus(Iii) Acids with Unsaturated Systems *Synthesis* **1979,** 81.

(42) Texierboullet, F.; Foucaud, A. A Convenient Synthesis of Dialkyl 1-Hydroxyalkanephosphonates Using Potassium or Cesium Fluoride without Solvent *Synthesis* **1982,** 165.

(43) Schaub, C.; Muller, B.; Schmidt, R. R. Sialyltransferase inhibitors based on CMP-quinic acid *Eur J Org Chem* **2000,** 1745.

(44) Cram, D. J.; Elhafez, F. A. A. Studies in Stereochemistry .10. The Rule of Steric Control of Asymmetric Induction in the Syntheses of Acyclic Systems *J Am*

Chem Soc **1952,** *74,* 5828.

(45) Gross, H.; Costisella, B.; Ozegowski, S.; Keitel, I.; Forner, K. Alpha-Substituted Phosphonates .67. Reaction of Salicylaldehyde with Triethylphosphite - New C-O Phosphoryl Group Rearrangement *Phosphorus, Sulfur Silicon Relat Elem* **1993,** *84,* 121.

(46) Jiang, G. W.; Xu, Y.; Fujiwara, Y.; Tsukahara, T.; Tsukahara, R.; Gajewiak, J.; Tigyi, G.; Prestwich, G. D. alpha-substituted phosphonate analogues of lysophosphatidic acid (LPA) selectively inhibit production and action of LPA *Chemmedchem* **2007,** *2,* 679.

(47) Fu, J. P.; He, Y. H.; Zhong, J.; Yang, Y.; Deng, X.; Guan, Z. An efficient and general route to the synthesis of diethyl alpha,alpha-bromofluorophosphonates *J Fluorine Chem* **2011,** *132,* 636.

(48) Gajda, T. A Convenient Synthesis of Diethyl 1-Chloroalkylphosphonates *Synthesis* **1990,** 717.

(49) Firouzabadi, H.; Iranpoor, N.; Sobhani, S. Ph3P/DDQ/NH4SCN as a new and neutral system for direct preparation of diethyl alpha-thiocyanatophosphonates from diethyl alpha-hydroxyphosphonates *Synthesis* **2004,** 290.

(50) Kumaraswamy, S.; Selvi, R. S.; Swamy, K. C. K. Synthesis of new alpha-hydroxy-, alpha-halogeno- and vinylphosphonates derived from 5,5-dimethyl-1,3,2-dioxaphosphinan-2-one *Synthesis* **1997,** 207.

(51) Raghavan, S.; Krishnaiah, V. A short stereoselective synthesis of (+)-boronolide *Tetrahedron Lett* **2006,** *47,* 7611.

(52) Swamy, K. C. K.; Kumar, K. V. P. P.; Suresh, R. R.; Kumar, N. S. Easy and stereoselective synthesis of cyclopropyl-substituted phosphonates via alpha-chlorophosphonates *Synthesis* **2007,** 1485.

(53) Kumar, S.; Zhou, B.; Liang, F. B.; Wang, W. Q.; Huang, Z. H.; Zhang, Z. Y. Activity-based probes for protein tyrosine phosphatases *Proc Natl Acad Sci USA* **2004,** *101,* 7943.

(54) Nicolaou, K. C.; Duggan, M. E.; Hwang, C. K. Synthesis of the Abc Ring-System of Brevetoxin-B *J Am Chem Soc* **1989,** *111,* 6666.

(55) Schweifer, A.; Hammerschmidt, F. On the conversion of structural analogues of (S)-2-hydroxypropylphosphonic acid to epoxides by the final enzyme of fosfomycin biosynthesis in S-fradiae *Bioorg Med Chem Lett* **2008,** *18,* 3056.

(56) Seidel, H. M.; Freeman, S.; Schwalbe, C. H.; Knowles, J. R. Phosphonate Biosynthesis - the Stereochemical Course of Phosphoenolpyruvate Mutase *J Am Chem Soc* **1990,** *112,* 8149.

(57) Giordano, C.; Castaldi, G. 1st Asymmetric-Synthesis of Enantiomerically Pure (1r,2s)-(-)-(1,2-Epoxypropyl)Phosphonic Acid (Fosfomycin) *J Org Chem* **1989,** *54,* 1470.

(58) Kitamura, M.; Tokunaga, M.; Noyori, R. Asymmetric Hydrogenation of

Beta-Keto Phosphonates - a Practical Way to Fosfomycin *J Am Chem Soc* **1995,** *117,* 2931.

(59) Raghavan, S.; Krishnaiah, V. An Efficient Stereoselective Synthesis of Penaresidin A from (E)-2-Protected Amino-3,4-unsaturated Sulfoxide *J Org Chem* **2010,** *75,* 748.

(60) Batchelor, R.; Harvey, J. E.; Northcote, P. T.; Teesdale-Spittle, P.; Hoberg, J. O. Heptanosides from Galactose-Derived Oxepenes via Stereoselective Addition Reactions *J Org Chem* **2009,** *74,* 7627.

(61) Ono, Y.; Han, L. B. Epoxidation of phosphinoyl alkenes with hydrogen peroxide *Tetrahedron Lett* **2006,** *47,* 421.

(62) McGrath, J. W.; Hammerschmidt, F.; Preusser, W.; Quinn, J. P.; Schweifer, A. Studies on the biodegradation of fosfomycin: Growth of Rhizobium huakuii PMY1 on possible intermediates synthesised chemically *Org Biomol Chem* **2009,** *7,* 1944.

(63) Adam, W.; Hadjiarapoglou, L.; Smerz, A. Dioxirane Epoxidation of Alpha,Beta-Unsaturated Ketones *Chem Ber* **1991,** *124,* 227.

(64) Marmor, S. Epoxidation of Certain Alph,Beta-Unsaturated Ketones with Sodium Hypochlorite *J Org Chem* **1963,** *28,* 250.

(65) Katsumura, S.; Kimura, A.; Isoe, S. Total Synthesis of (+/-)-Jolkinolide a, B, and E Utilizing a New Mild Esterification Followed by Intramolecular Wittig Horner Reaction *Tetrahedron* **1989,** *45,* 1337.

(66) Cristau, H. J.; Mbianda, X. Y.; Geze, A.; Beziat, Y.; Gasc, M. B. Dioxirane oxidation of substituted vinylphosphonates: a novel efficient route to 1,2-epoxyalkylphosphonates *J Organomet Chem* **1998,** *571,* 189.

(67) Coutrot, P.; Grison, C.; Lecouvey, M.; Kribii, A.; El Gadi, A. New highly functionalized molecules: Preparation of alpha-chlorinated epoxyphosphonates. Their transformation to beta-halogenated alpha-ketophosphonates *Phosphorus, Sulfur Silicon Relat Elem* **1998,** *133,* 167.

(68) McKenna, C. E.; Kashemirov, B. A. Recent progress in carbonylphosphonate chemistry *Top Curr Chem* **2002,** *220,* 201.

(69) Kedrowski, S. M. A.; Dougherty, D. A. Room-Temperature Alternative to the Arbuzov Reaction: The Reductive Deoxygenation of Acyl Phosphonates *Org Lett* **2010,** *12,* 3990.

(70) Fang, M. H.; Toogood, R. D.; Macova, A.; Ho, K.; Franzblau, S. G.; McNeil, M. R.; Sanders, D. A. R.; Palmer, D. R. J. Succinylphosphonate Esters Are Competitive Inhibitors of MenD That Show Active-Site Discrimination between Homologous alpha-Ketoglutarate-Decarboxylating Enzymes *Biochemistry* **2010,** *49,* 2672.

(71) Glabe, A. R.; Sturgeon, K. L.; Ghizzoni, S. B.; Musker, W. K.; Takahashi, J. N. Novel functionalized acylphosphonates as phosphonoformate analogs *J Org Chem* **1996,** *61,* 7212.

(72) Barriga, S. 2,2,6,6-Tetramethylpiperidin-1-oxyl (TEMPO) *Synlett* **2001,**

563.

(73) Cheng, K. G.; Liu, J.; Liu, X. F.; Li, H. L.; Sun, H. B.; Xie, J. Synthesis of glucoconjugates of oleanolic acid as inhibitors of glycogen phosphorylase *Carbohydr Res* **2009,** *344,* 841.

(74) Pravdic, N.; Keglevic, D. Glucuronic Esters .3. Synthesis of Fully Benzylated C-1 Hydroxyl Free Glucuronic Acid *Tetrahedron* **1965,** *21,* 1897.

(75) Haworth, W. N.; Gregory, H.; Wiggins, L. F. Some Derivatives of Simple Carbohydrates Containing Unsaturated Substituents *J Chem Soc* **1946,** 488.

(76) Mamedova, V. L.; Nikitina, K. A.; Al'fonsov, V. A. Isosteviol acylphosphonates *Russ Chem Bull* **2009,** *58,* 244.

(77) Ibarra, C. A.; Rodriguez, R. C.; Monreal, M. C. F.; Navarro, F. J. G.; Tesorero, J. M. One-Pot Synthesis of Beta-Keto Sulfones and Beta-Keto Sulfoxides from Carboxylic-Acids *J Org Chem* **1989,** *54,* 5620.

(78) Kolodyazhnaya, A. O.; Kolodyazhnaya, O. O.; Kolodyazhnyi, O. I. An Efficient Method for the Phosphonation of C=X Compounds *Russ J Gen Chem* **2010,** *80,* 709.

(79) Firouzabadi, H.; Iranpoor, N.; Sobhani, S. Preparation of alpha-ketophosphonates by oxidation of alpha-hydroxyphosphonates with neutral alumina supported potassium permanganate (NASPP) under solvent-free conditions and potassium permanganate in dry benzene *Tetrahedron Lett* **2002,** *43,* 477.

(80) Kaboudin, B. Surface-mediated solid-phase reactions: the preparation of acyl phosphonates by oxidation of 1-hydroxyphosphonates on the solid surface *Tetrahedron Lett* **2000,** *41,* 3169.

(81) Yuan, C. Y.; Li, C. Z.; Ding, Y. X. Studies on Organophosphorus Compounds .51. A New and Facile Route to 2-Substituted 1,1-Cyclopropanediylbis(Phosphonic Acids) *Synthesis* **1991,** 854.

(82) Nuretdinov, I. A.; Yanilkin, V. V.; Morozov, V. I.; Gabskaya, V. P.; Zverev, V. V.; Nastapova, N. V.; Fazleeva, G. M. Electrochemical reduction and oxidation of fullerenopyrrolidines and the ESR spectra of paramagnetic intermediates *Russ Chem Bull* **2002,** *51,* 263.

(83) Iorga, B.; Savignac, P. Controlled monohalogenation of phosphonates Part IV. Selective synthesis of monohalogenomethylenediphosphonates *J Organomet Chem* **2001,** *624,* 203.

(84) Kobayashi, Y.; William, A. D. Coupling reactions of alpha-bromoalkenyl phosphonates with aryl boronic acids and alkenyl borates *Org Lett* **2002,** *4,* 4241.

(85) Tennant-Eyles, R. J.; Davis, B. G.; Fairbanks, A. J. Peptide templated glycosylation reactions *Tetrahedron: Asymmetry* **2000,** *11,* 231.

(86) Johansson, R.; Samuelsson, B. Regioselective Reductive Ring-Opening of 4-Methoxybenzylidene Acetals of Hexopyranosides - Access to a Novel Protecting-Group Strategy .1. *J Chem Soc, Perkin Trans 1* **1984,** 2371.

(87) Brown, J. M. Directed Homogeneous Hydrogenation *Angew Chem, Int Ed*

Engl **1987,** *26,* 190.

(88) Ojima, I.; Nagai, Y.; Kogure, T. Selective Reduction of Alpha,Beta-Unsaturated Terpene Carbonyl-Compounds Using Hydrosilane-Rhodium(I) Complex Combinations *Tetrahedron Lett* **1972,** 5035.

(89) Brahms, D. L. S.; Dailey, W. P. Fluorinated carbenes *Chem Rev* **1996,** *96,* 1585.

(90) Cantat, T.; Ricard, L.; Le Floch, P.; Mezailles, N. Phosphorus-stabilized geminal dianions *Organometallics* **2006,** *25,* 4965.

(91) Molander, G. A.; Quirmbach, M. S.; Silva, L. F.; Spencer, K. C.; Balsells, J. Toward the total synthesis of variecolin *Org Lett* **2001,** *3,* 2257.

(92) Khurana, J. M.; Gogia, A. Synthetically useful reactions with nickel boride. *Org Prep Proced Int* **1997,** *29,* 1.

(93) Iorga, B.; Eymery, F.; Savignac, P. Controlled monohalogenation of phosphonates, Part II: Preparation of pure diethyl alpha-monohalogenated alkylphosphonates *Synthesis* **2000,** 576.

(94) Teulade, M. P.; Savignac, P. Alpha-Lithiated O,O-Diethyltrimethylsilylchloromethylphosphonate (C2h5o)2p(O)C(Cl)Si(Ch3)3li .1. Preparations and Properties *J Organomet Chem* **1988,** *338,* 295.

(95) Philips, K. D.; Zemlicka, J.; Horwitz, J. P. Unsaturated Sugars .1. Decarboxylative Elimination of Methyl 2,3-Di-O-Benzyl-Alpha-D-Glucopyranosiduronic Acid to Methyl 2,3-Di-O-Benzyl-4-Deoxy-Beta-L-Threo-

Pent-4-Enopyranoside *Carbohydr Res* **1973,** *30,* 281.

(96) Hori, H.; Nishida, Y.; Ohrui, H.; Meguro, H. Regioselective De-O-Benzylation with Lewis-Acids *J Org Chem* **1989,** *54,* 1346.

(97) Alzeer, J.; Vasella, A. Oligosaccharide Analogs of Polysaccharides .2. Regioselective Deprotection of Monosaccharide-Derived Monomers and Dimers *Helv Chim Acta* **1995,** *78,* 177.

(98) Rodebaugh, R.; Debenham, J. S.; FraserReid, B. Debenzylation of complex oligosaccharides using ferric chloride *Tetrahedron Lett* **1996,** *37,* 5477.

(99) Park, M. H.; Takeda, R.; Nakanishi, K. Microscale Cleavage Reaction of (Phenyl)Benzyl Ethers by Ferric-Chloride *Tetrahedron Lett* **1987,** *28,* 3823.

(100) Polozov, A. M.; Cremer, S. E. Synthesis of 2H-1,2-oxaphosphorin 2-oxides *J Organomet Chem* **2002,** *646,* 153.

(101) Villieras, J.; Bacquet, C.; Normant, J. F. Formation and Reactivity of Alpha,Alpha-Dibromoalkyllithium Reagents *Bull Soc Chim Fr* **1975,** 1797.

(102) Savignac, P.; Coutrot, P. Preparation of 1,1-Dibromoalkenes by Halogen Exchange *Synthesis* **1976,** 197.

(103) Speth, M.; Schulze, H. U. The Purification of a Detergent-Soluble Glucose-6-Phosphatase from Rat-Liver *Eur J Biochem* **1992,** *208,* 643.

(104) Enzymatic Activity of Glucose-6-Phosphatase. http://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-glucose-6-phosphatase.html (accessed March 16, 2012).

(105) Nordlie, R. C.; Arion, W. J. Glucose-6-Phosphatase *Methods Enzymol* **1966,** *9,* 619.

(106) Foster, J. D.; Young, S. E.; Brandt, T. D.; Nordlie, R. C. Tungstate: A potent inhibitor of multifunctional glucose-6-phosphatase *Arch Biochem Biophys* **1998,** *354,* 125.

(107) Foster, J. D.; Nelson, K. L.; Sukalski, K. A.; Lucius, R. W.; Nordlie, R. C. Hysteretic Behavior of the Hepatic-Microsomal Glucose-6-Phosphatase System *Biochim Biophys Acta* **1991,** *1118,* 91.

(108) Fiske, C. H.; Subbarow, Y. The colorimetric determination of phosphorus *J Biol Chem* **1925,** *66,* 375.

(109) Taussky, H. H.; Shorr, E. A Microcolorimetric Method for the Determination of Inorganic Phosphorus *J Biol Chem* **1953,** *202,* 675.

(110) Steiner, M. S.; Duerkop, A.; Wolfbeis, O. S. Optical methods for sensing glucose *Chem Soc Rev* **2011,** *40,* 4805.

(111) Arion, W. J.; Ballas, L. M.; Lange, A. J.; Wallin, B. K. Microsomal Membrane-Permeability and Hepatic Glucose-6-Phosphatase System - Interactions of System with D-Mannose 6-Phosphate and D-Mannose *J Biol Chem* **1976,** *251,* 4901.

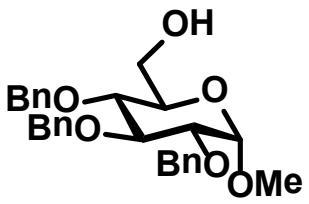
(112) Ko, K. S.; Zea, C. J.; Pohl, N. L. Surprising bacterial nucleotidyltransferase selectivity in the conversion of carbaglucose-1-phosphate *J Am Chem Soc* **2004,** *126,* 13188.

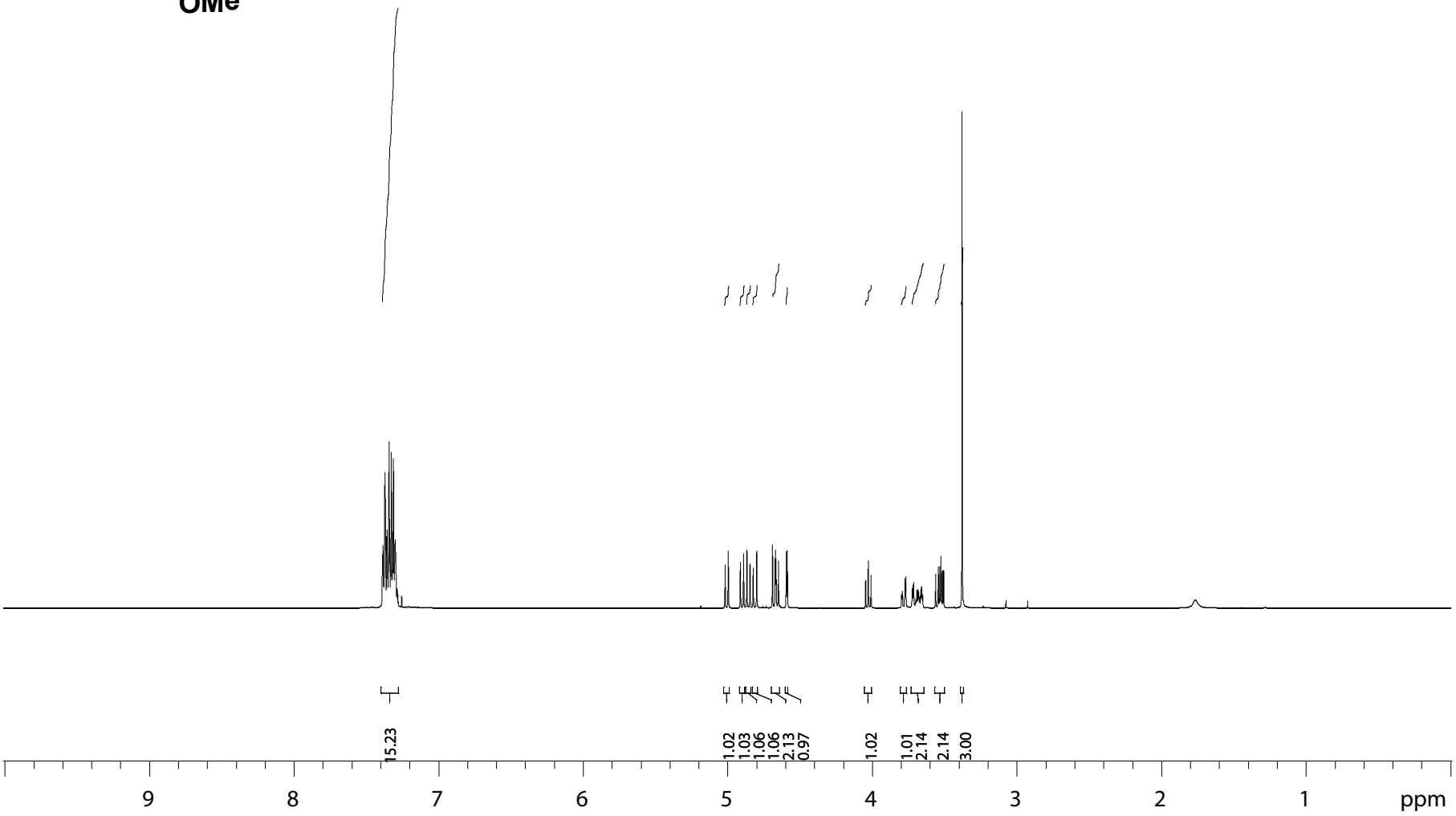
(113) Chen, Y.; Schindler, M.; Simon, S. M. A mechanism for tamoxifen-mediated inhibition of acidification *J Biol Chem* **1999,** *274,* 18364.

(114) Foster, J. D.; Bode, A. M.; Nordlie, R. C. Time-Dependent Inhibition of Glucose 6-Phosphatase by 3-Mercaptopicolinic Acid *Biochim Biophys Acta* **1994,** *1208,* 222.

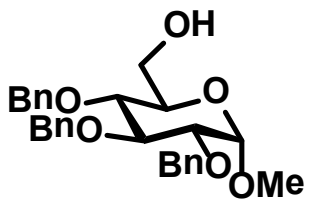
Appendix: Spectra

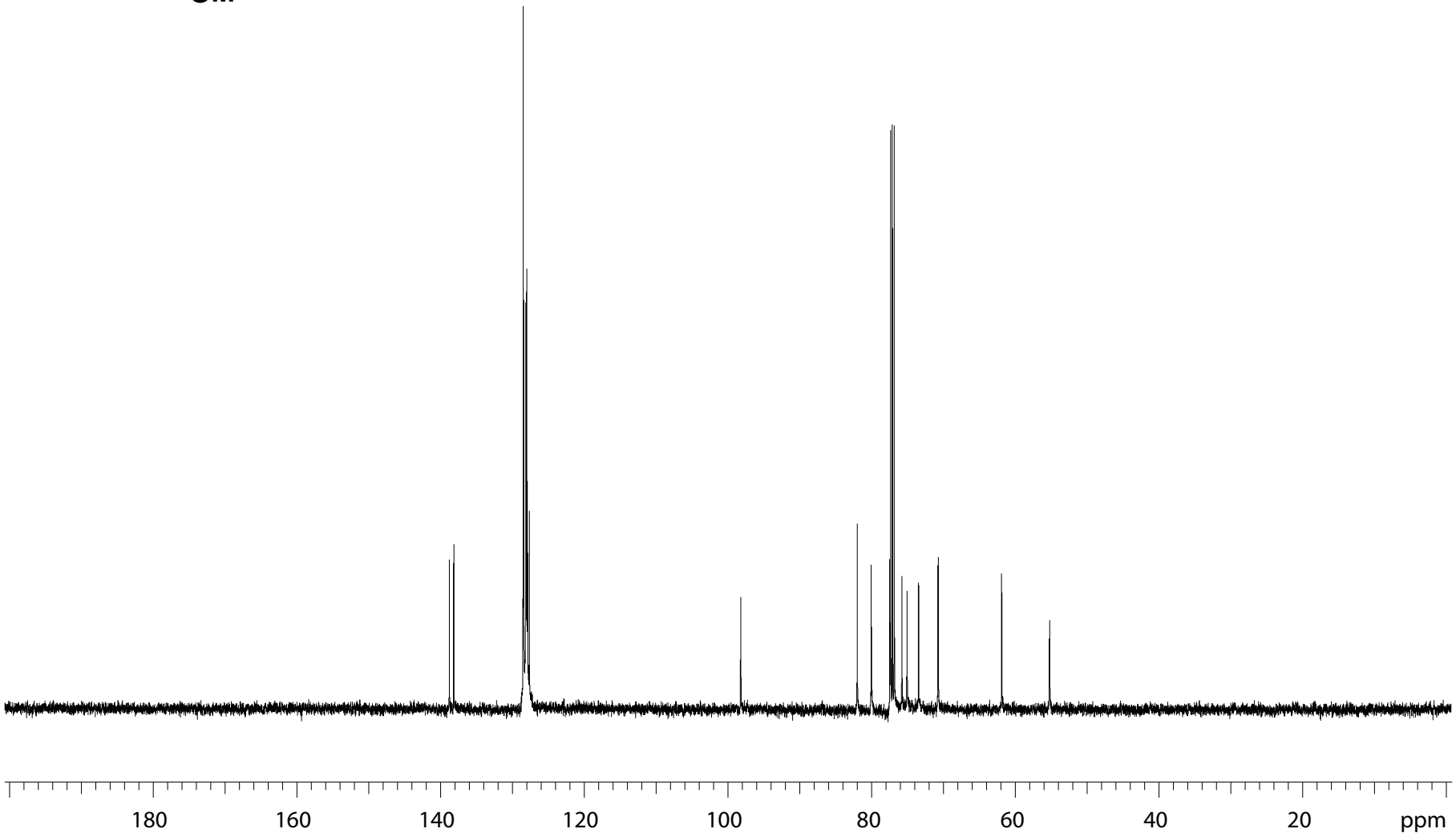
498.122 MHz H1 1D in CDCl3



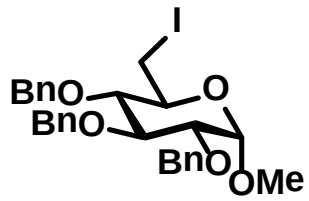


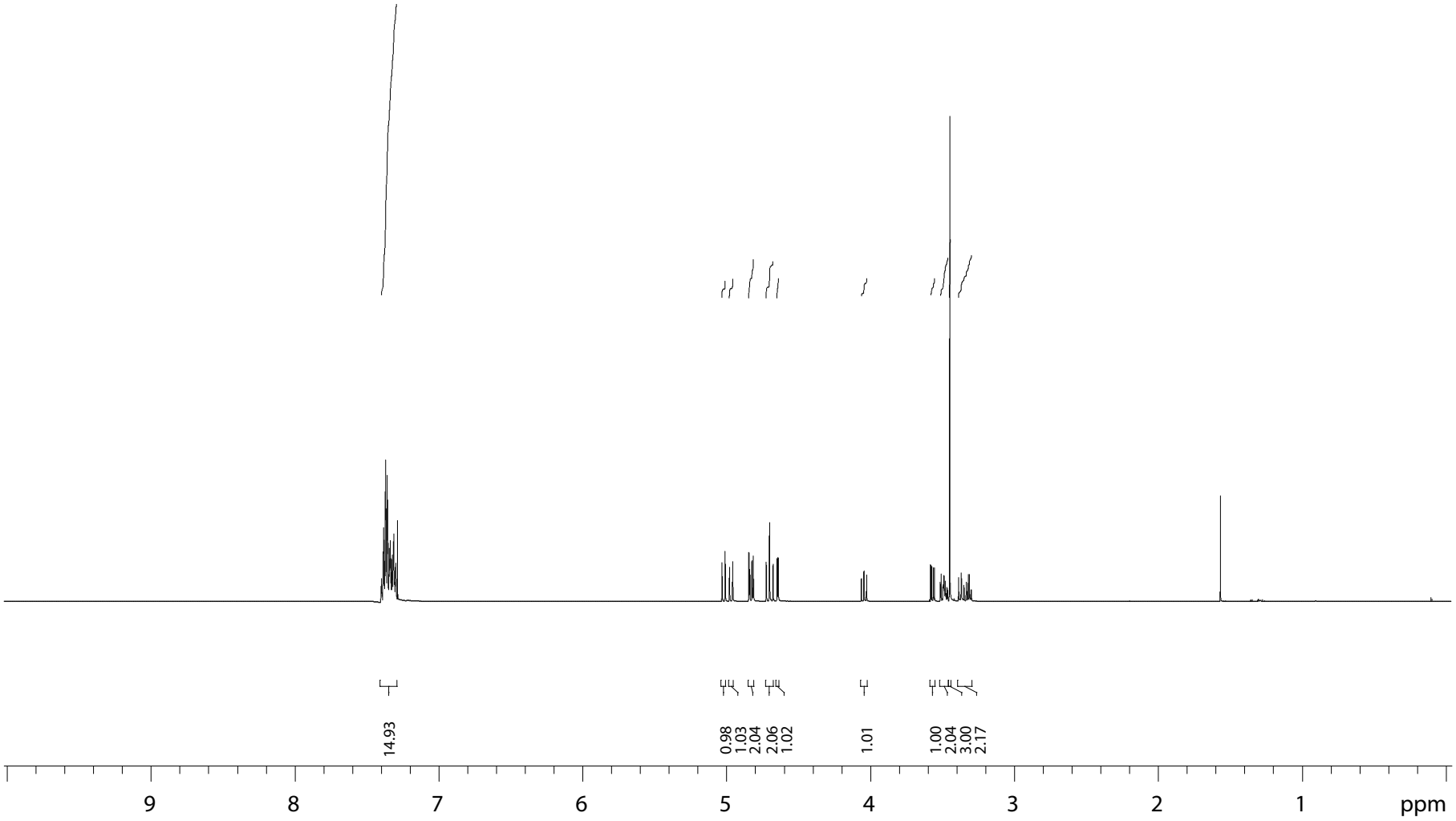
125.264 MHz C13[H1] 1D in cdcl3





499.815 MHz H1 1D in CDCl3





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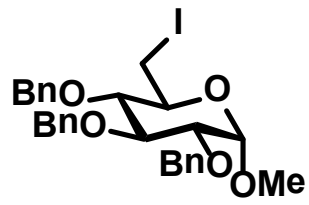

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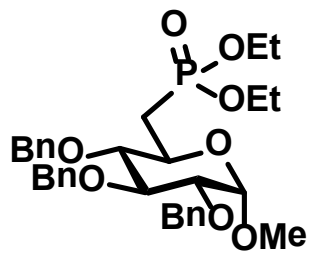
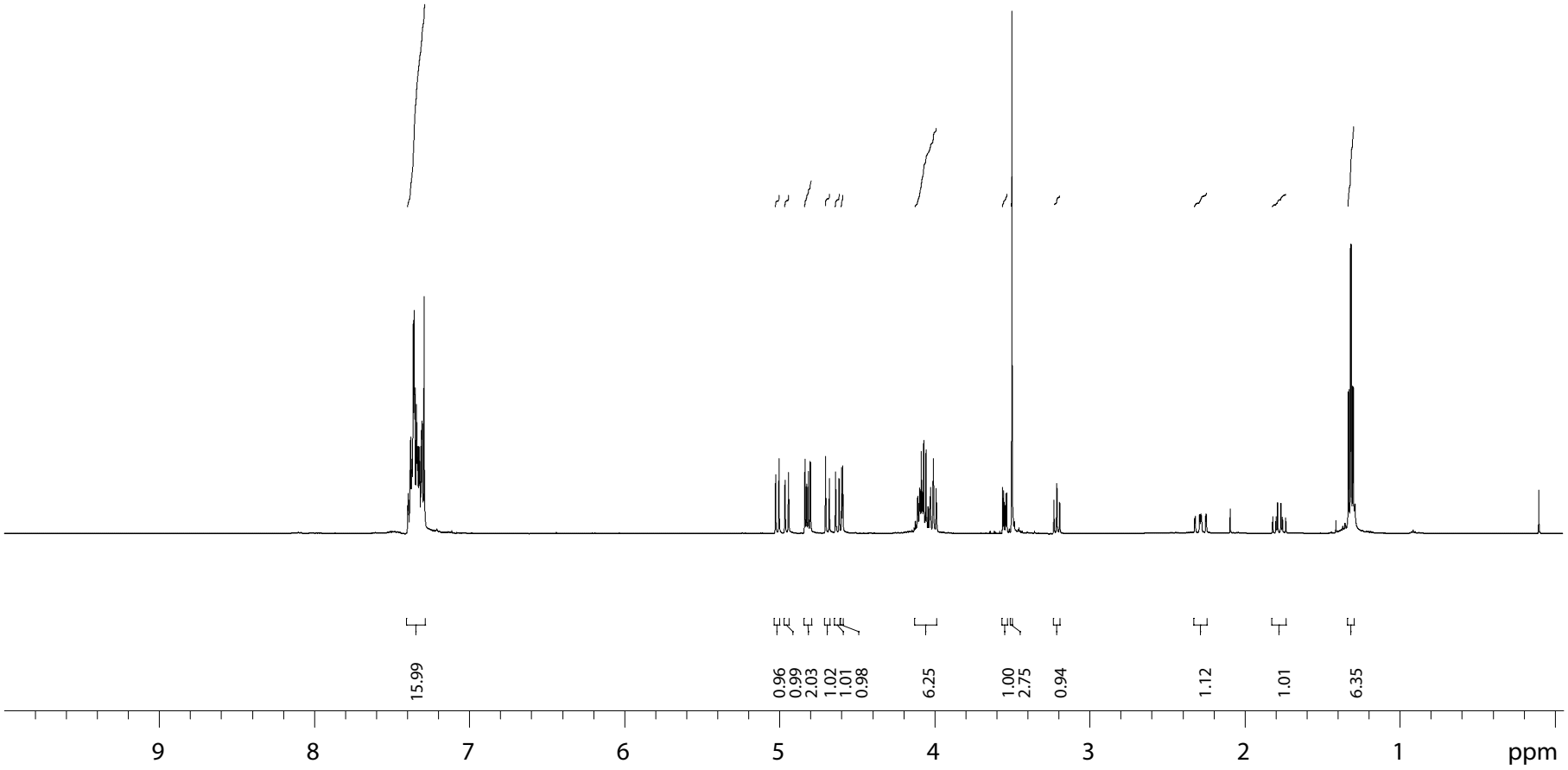
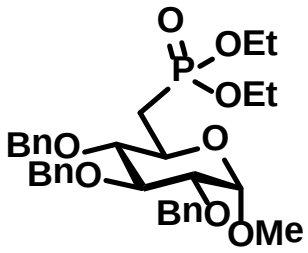




125.693 MHz C13[H1] DEPT_chempack in cdcl3



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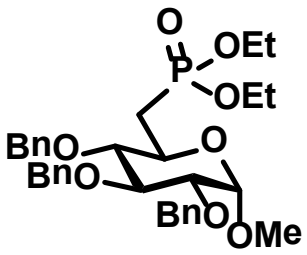

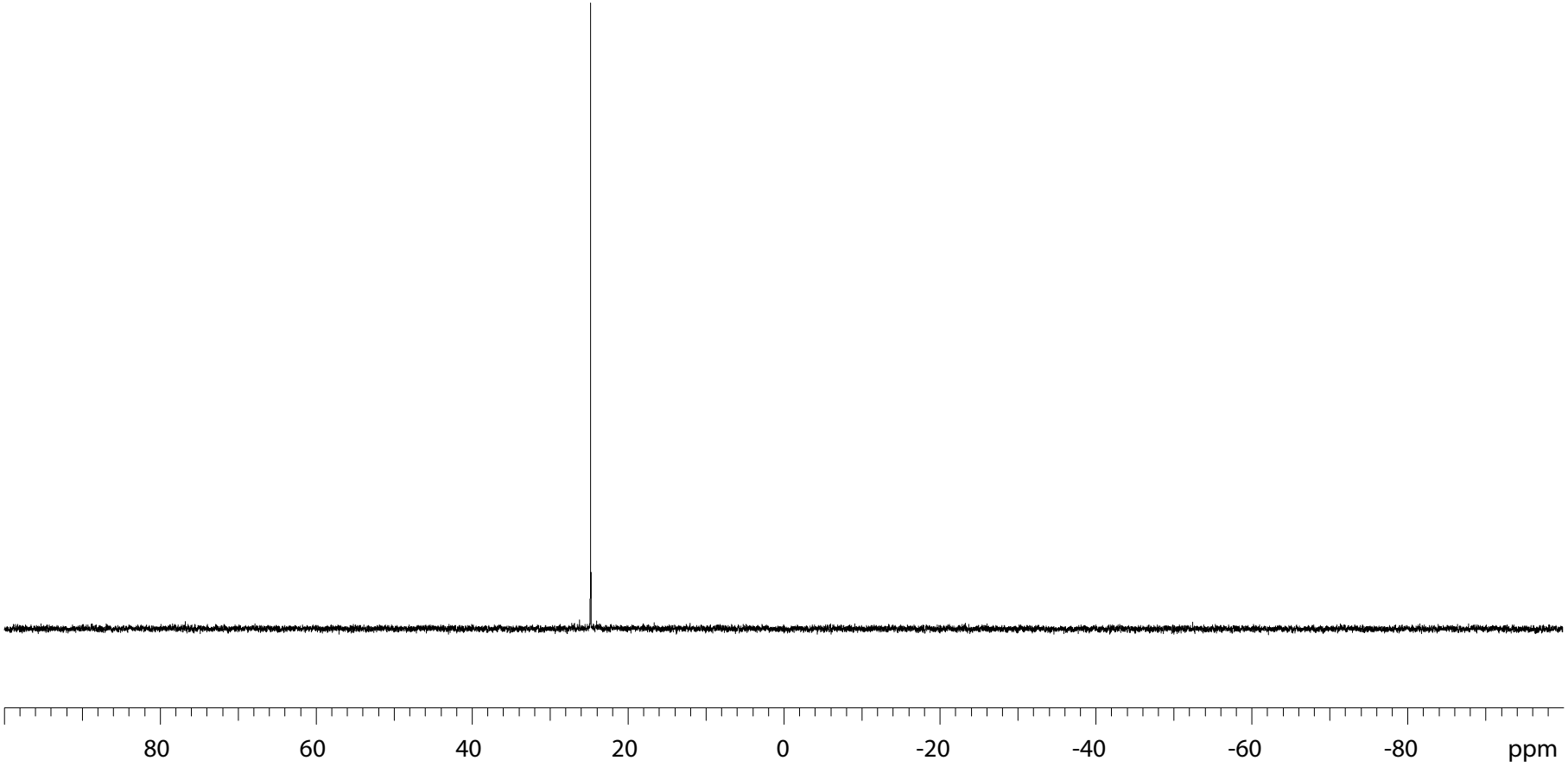

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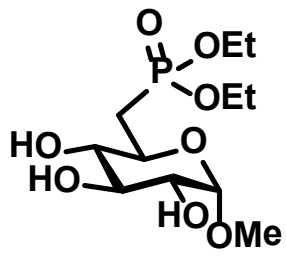

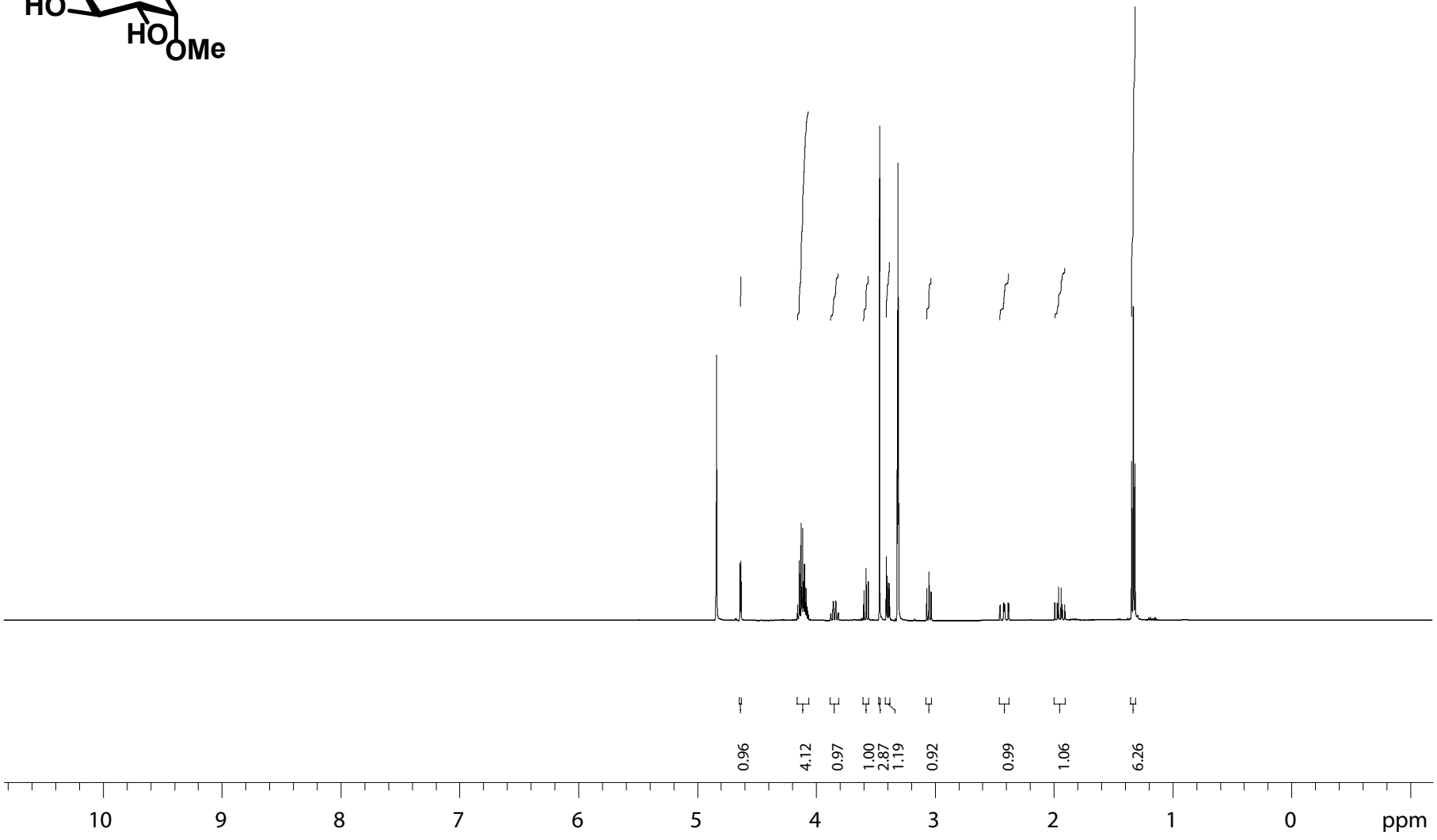

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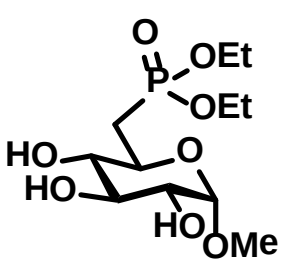

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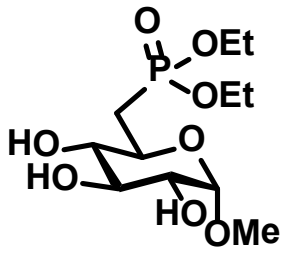

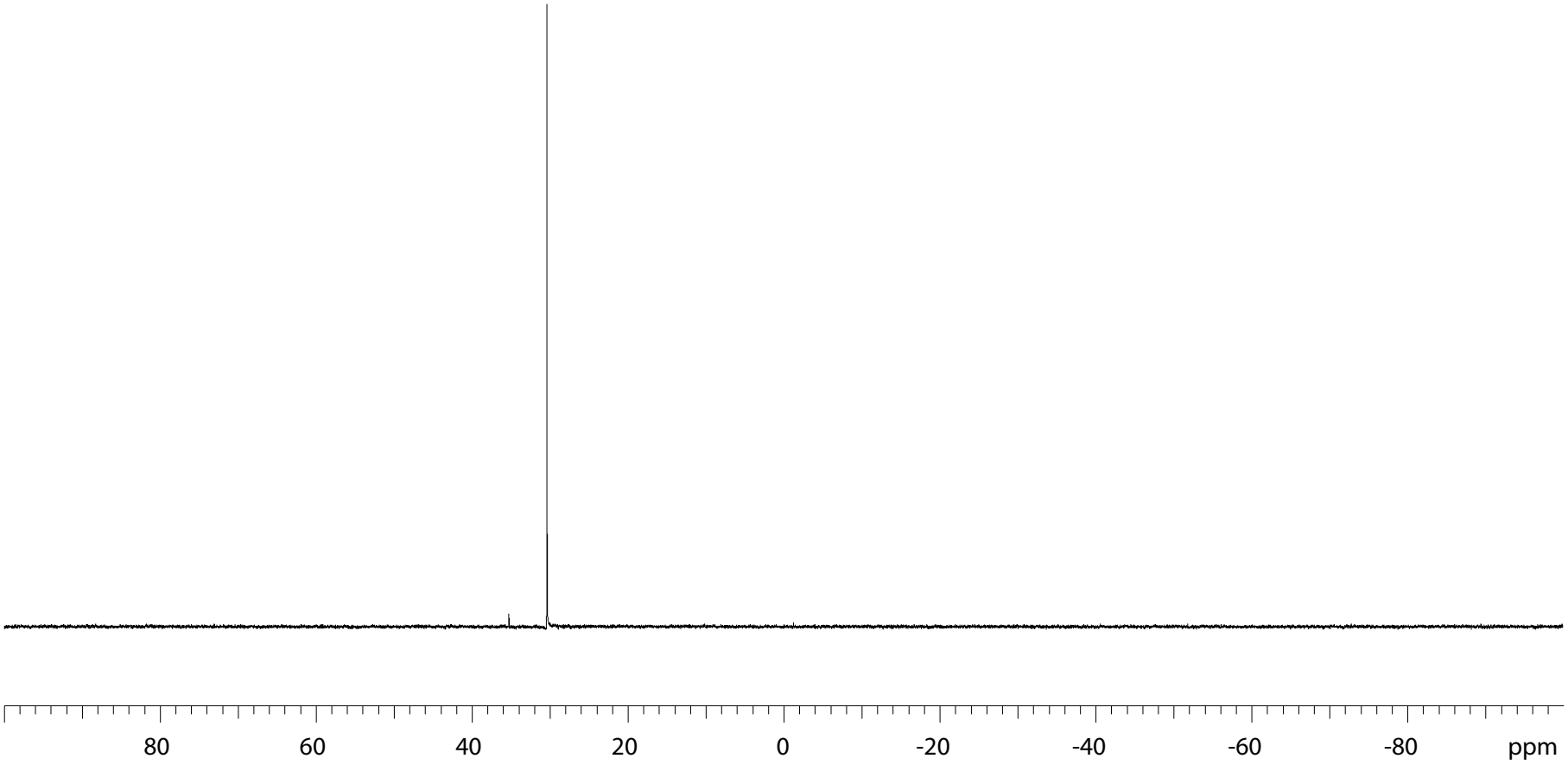

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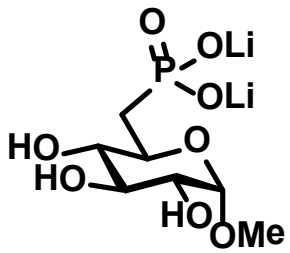
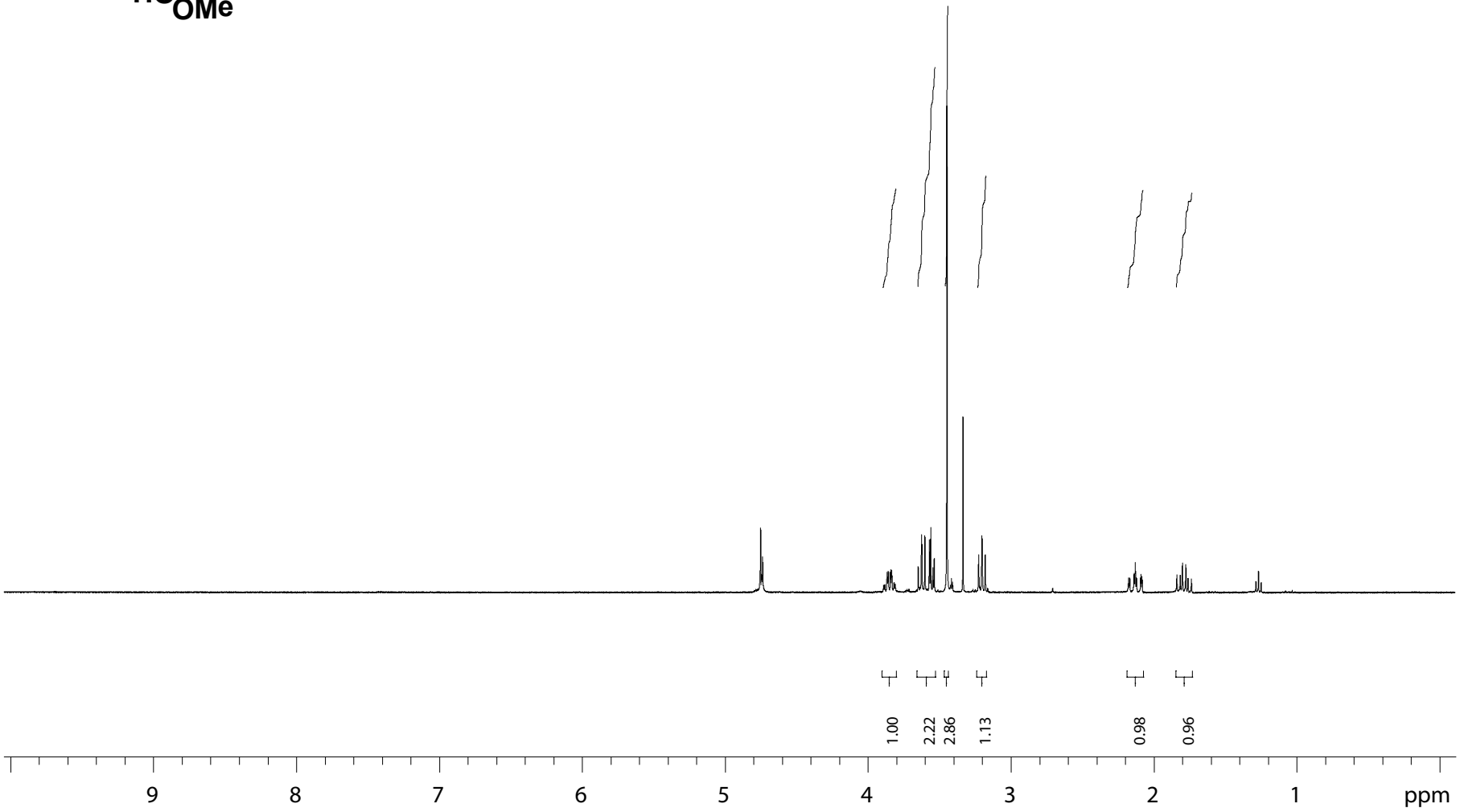
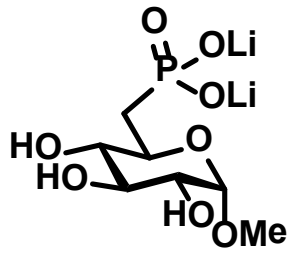
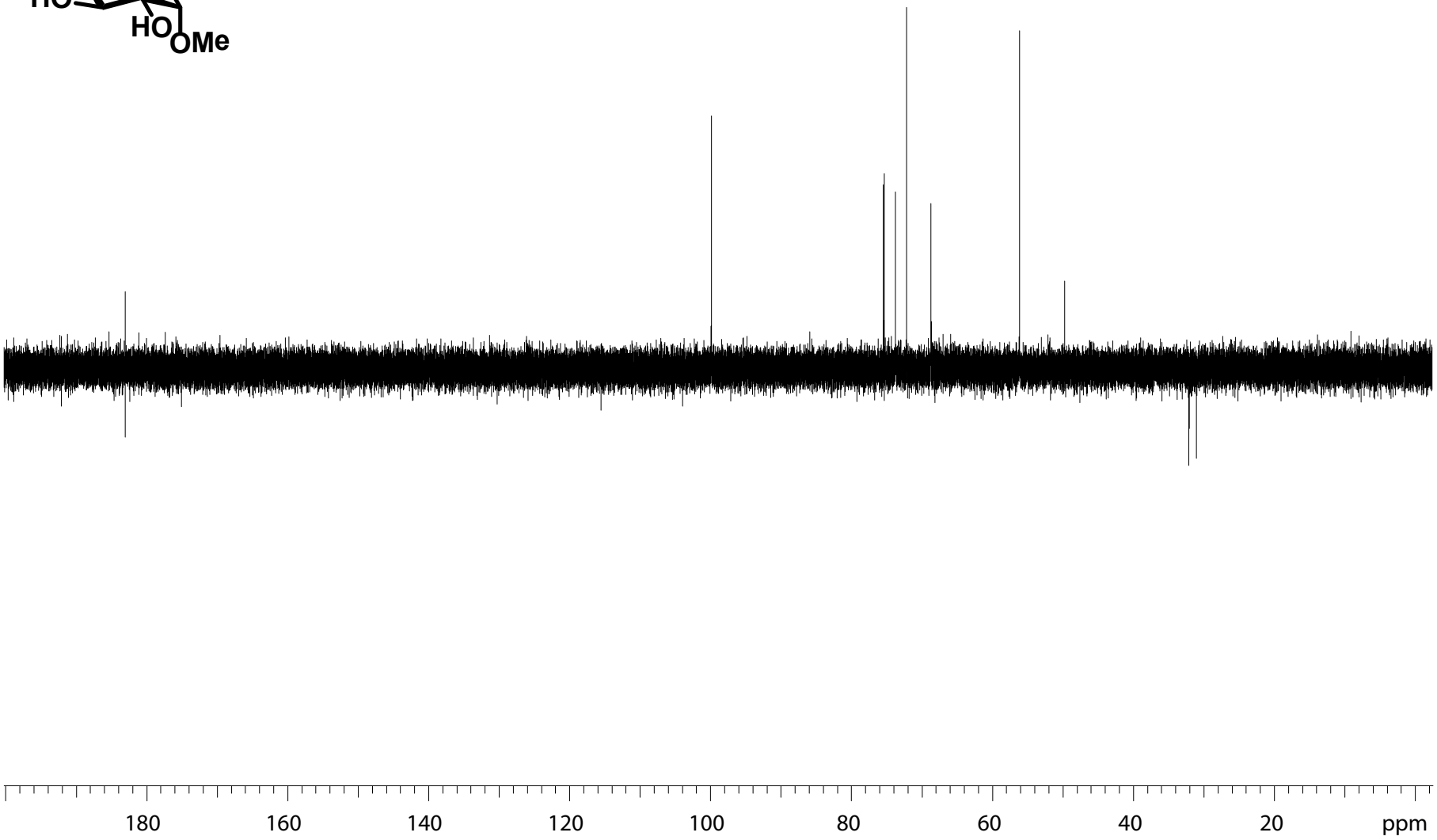


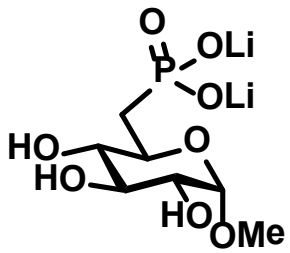


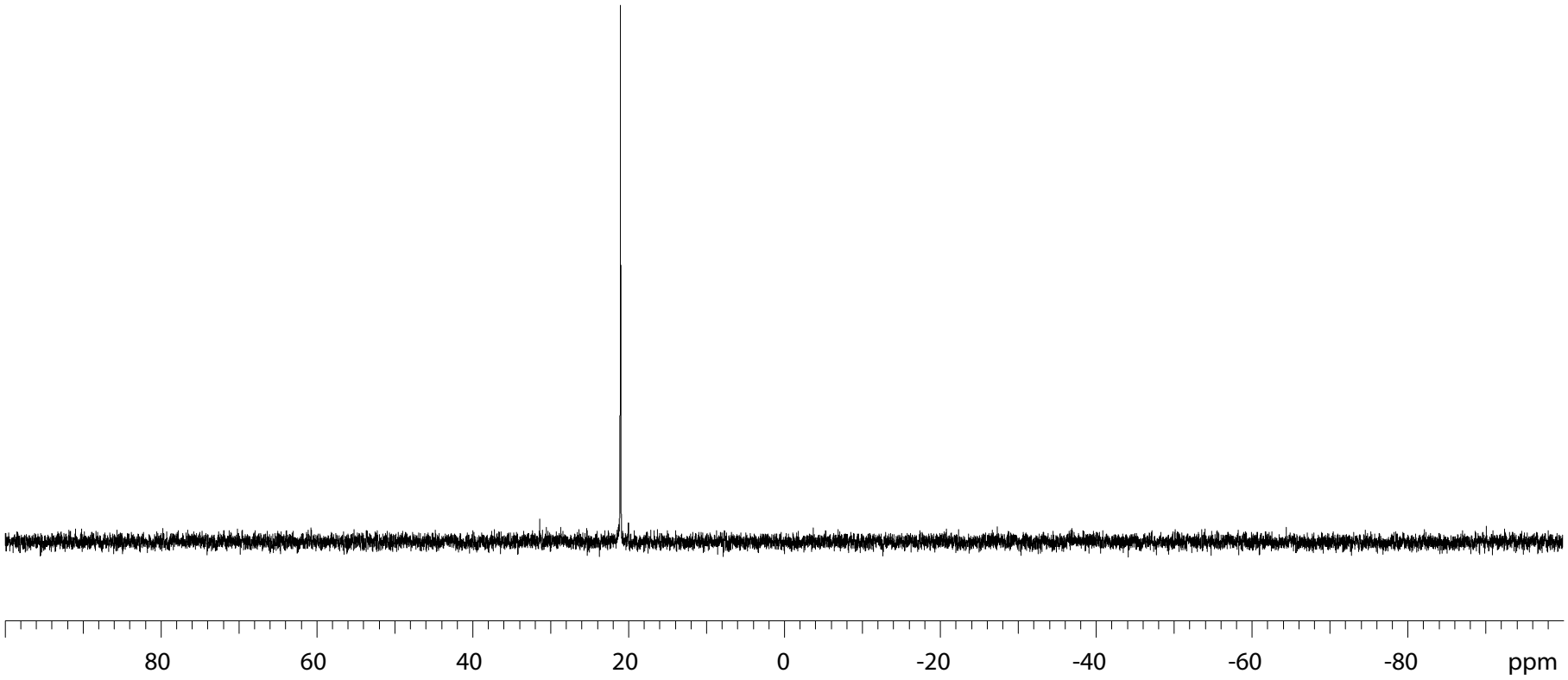
125.267 MHz C13[H1] APT_ad in d2o



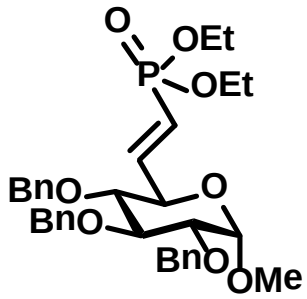


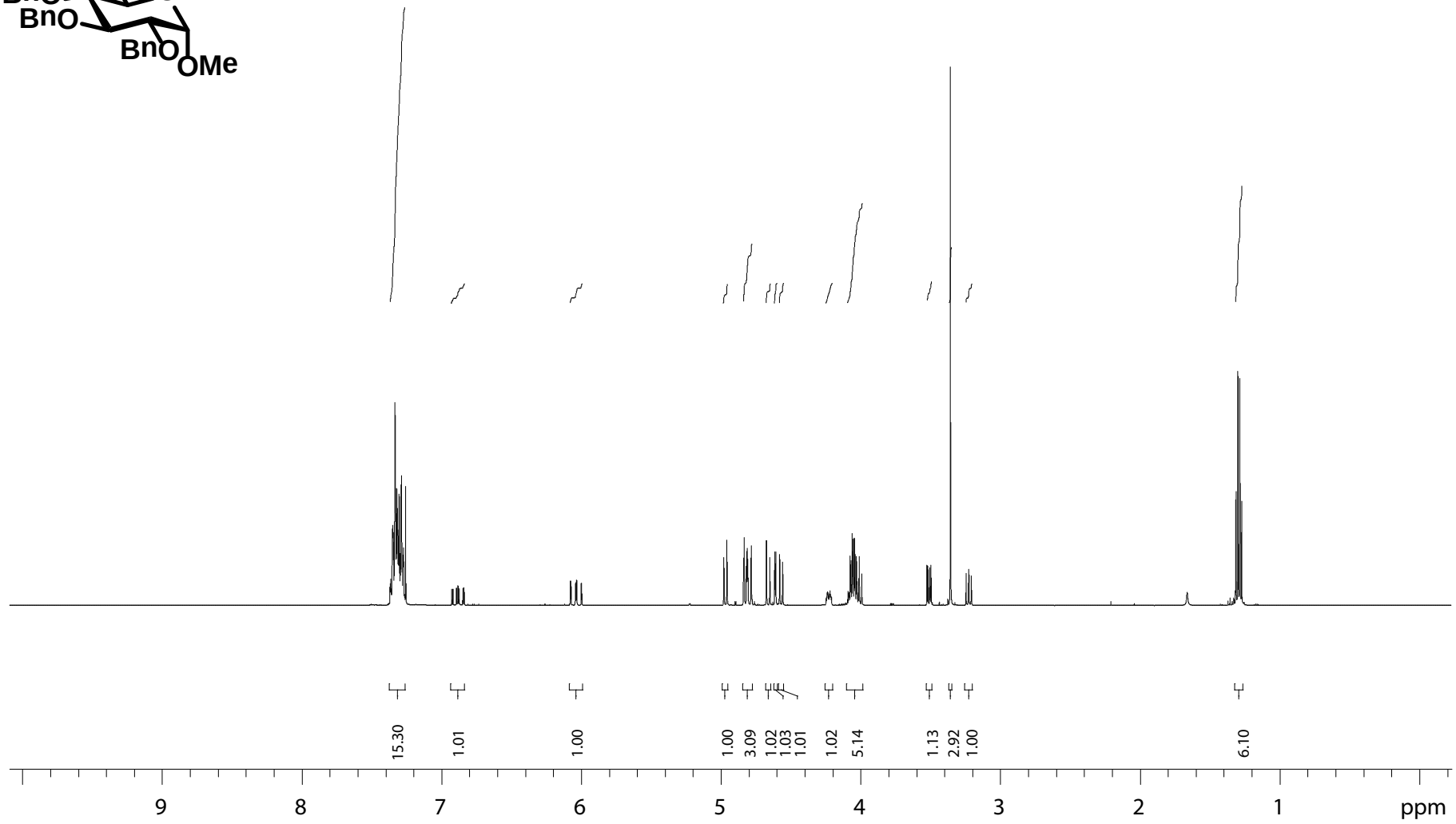
161.839 MHz P31[H1] 1D in d2o



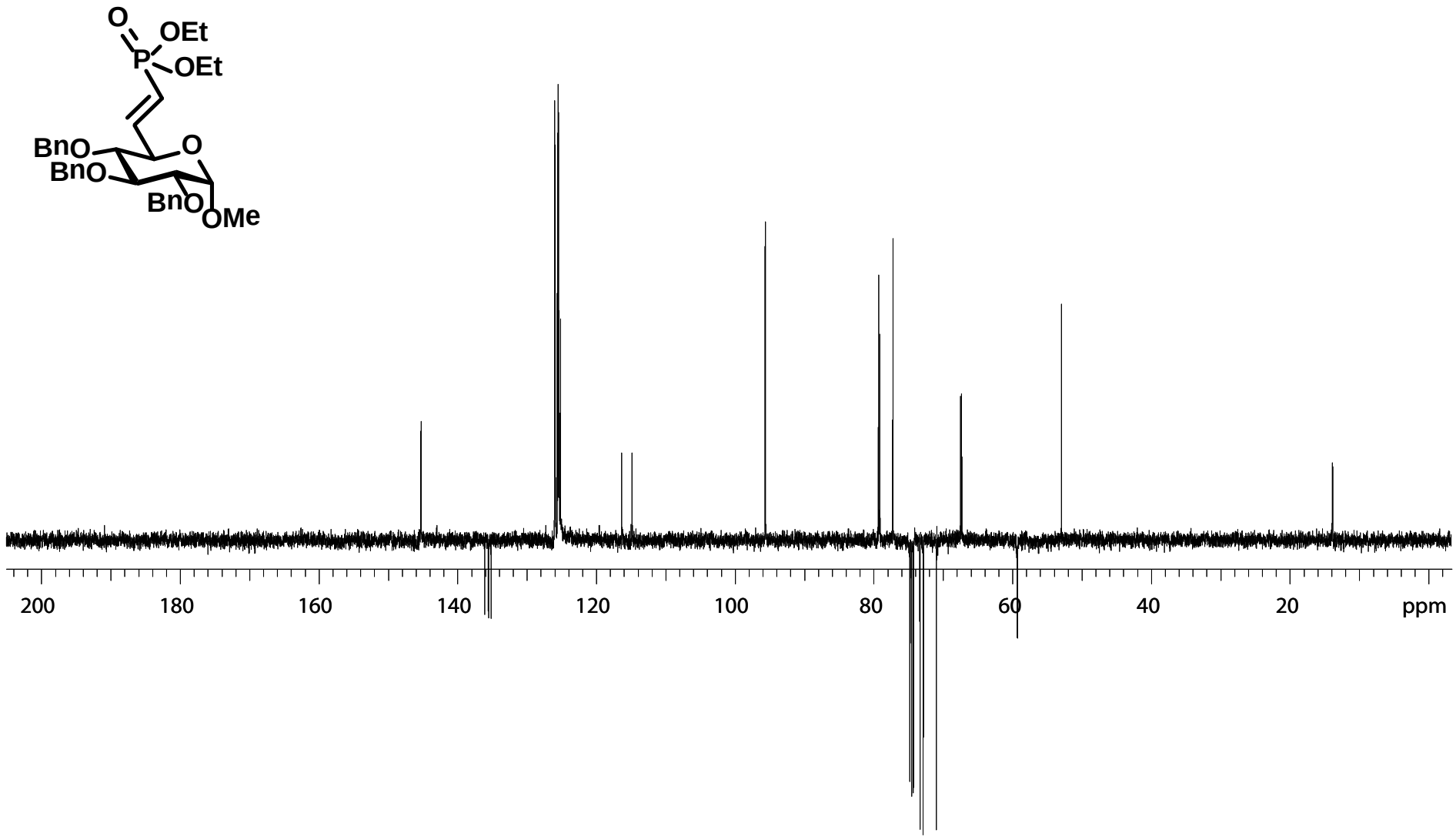


498.122 MHz H1 1D in cdcl3

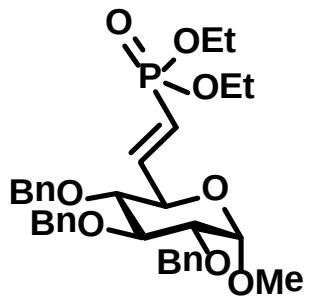


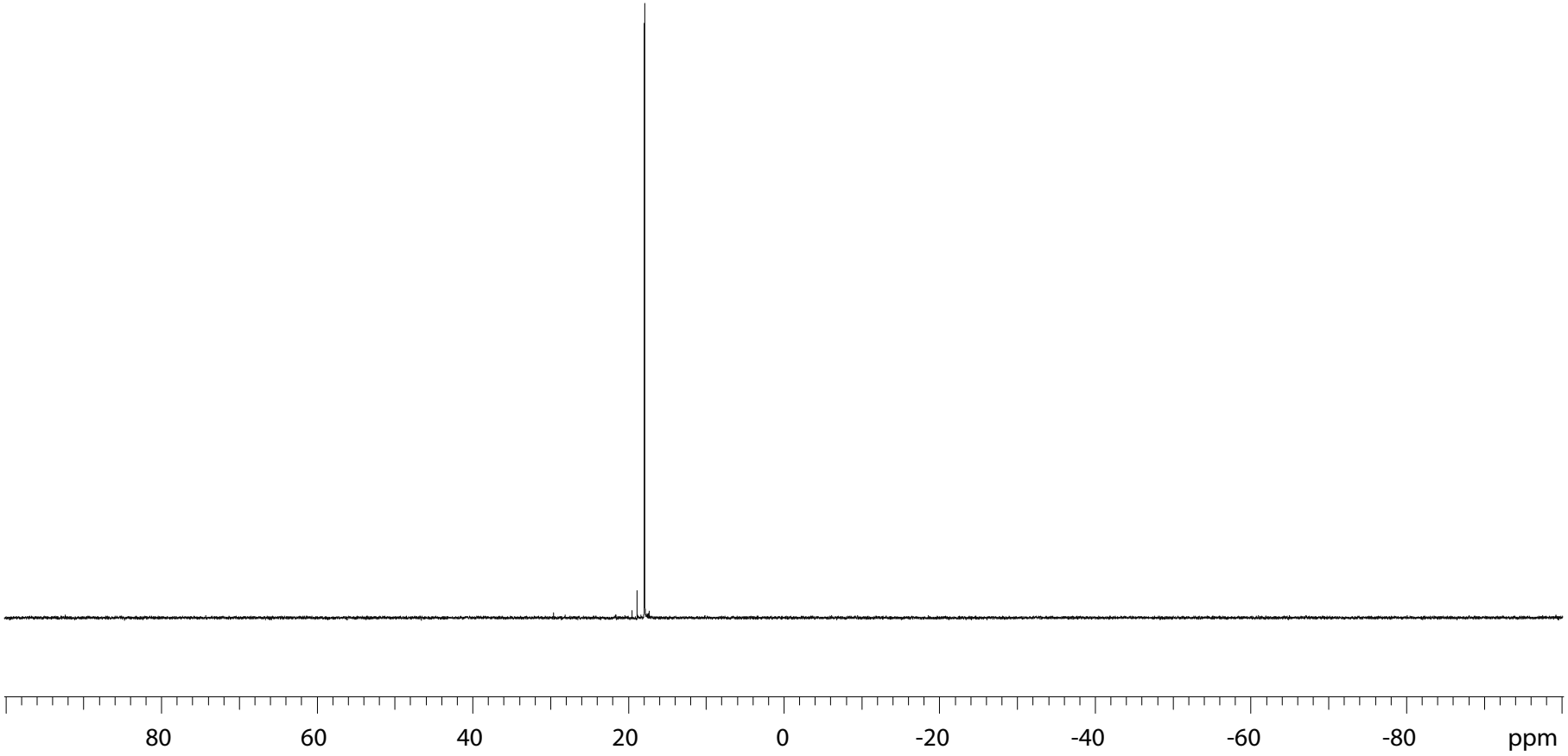


125.693 MHz C13[H1] DEPT_chempack in cdcl3

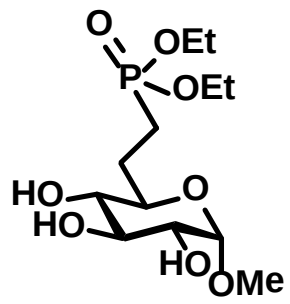


201.642 MHz P31[H1] 1D in cdcl3

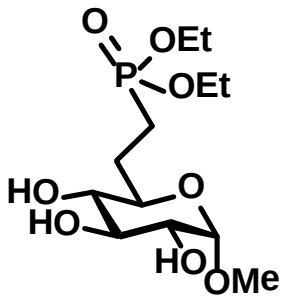




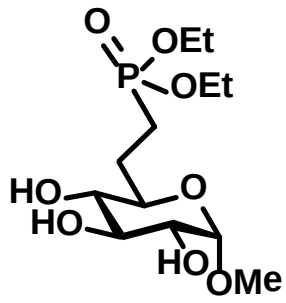
499.817 MHz H1 1D in cd3od

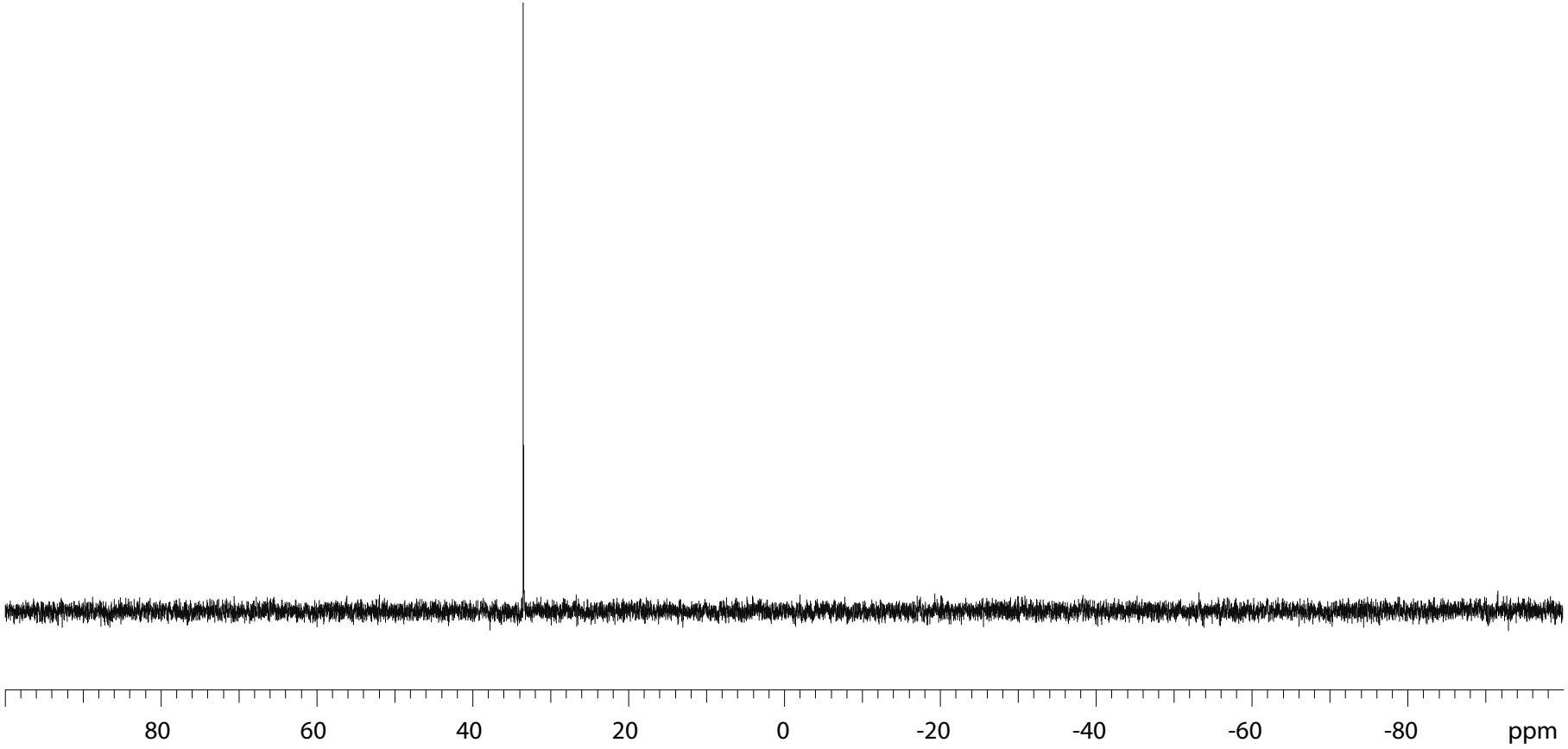


125.693 MHz C13[H1] DEPT_chempack in cd3od

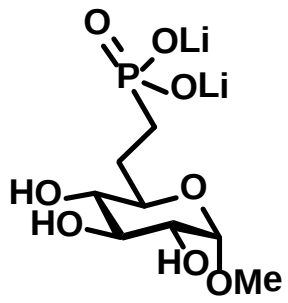


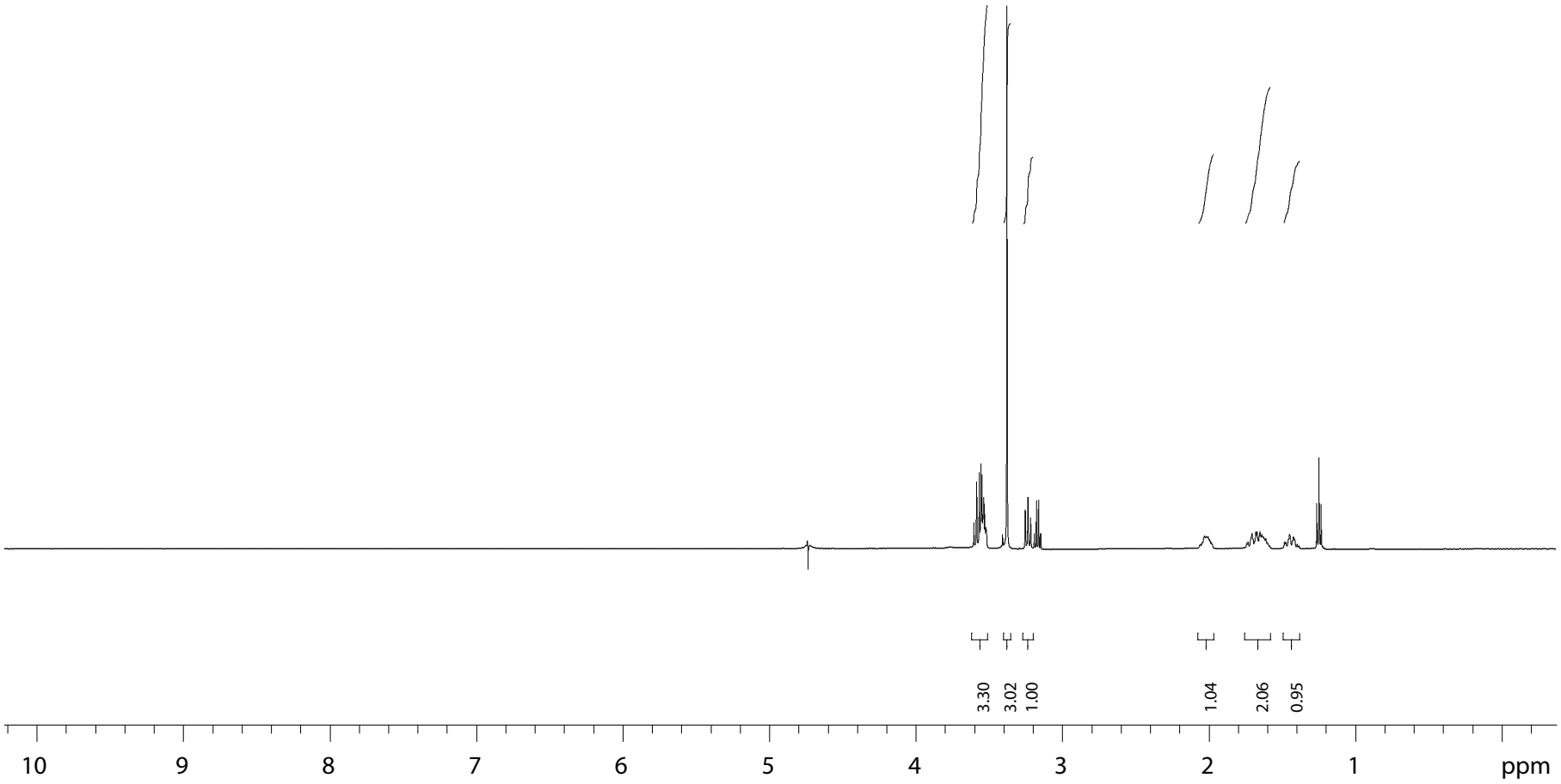
161.840 MHz P31[H1] 1D in cd3od



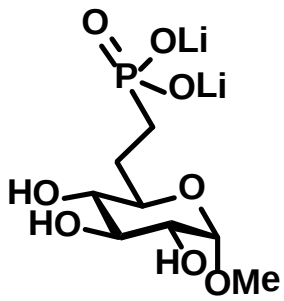


499.816 MHz H1 1D in d2o

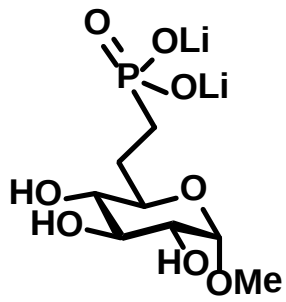


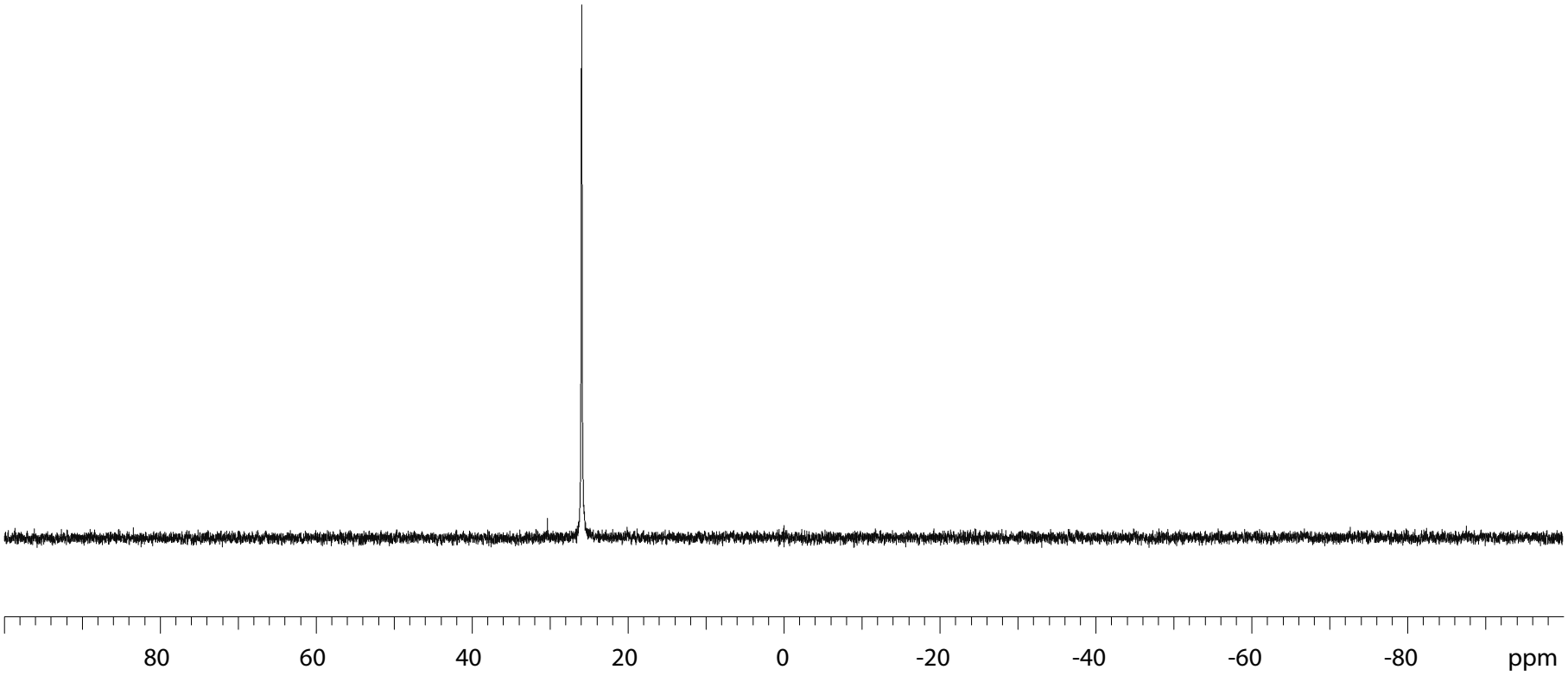


125.693 MHz C13[H1] DEPT_chempack in d2o

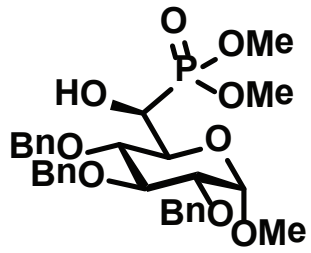


161.839 MHz P31[H1] 1D in d2o

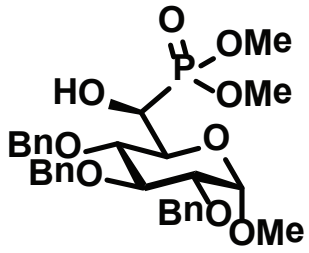




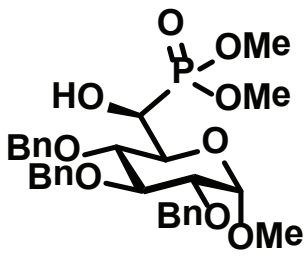
499.815 MHz H1 1D in cdcl3

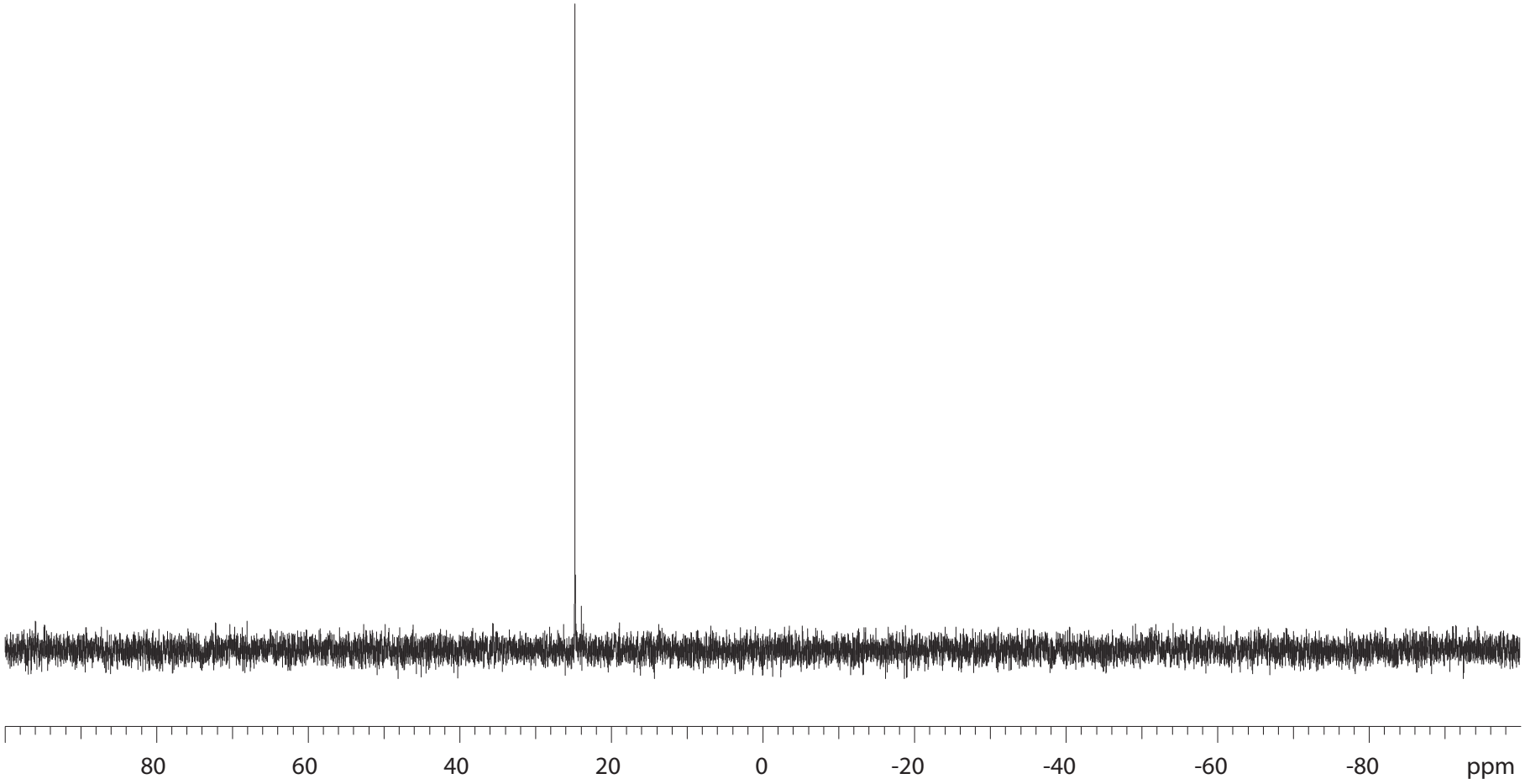


125.693 MHz C13[H1] DEPT_chempack in cdcl3

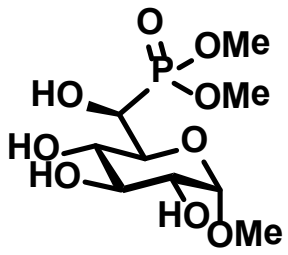


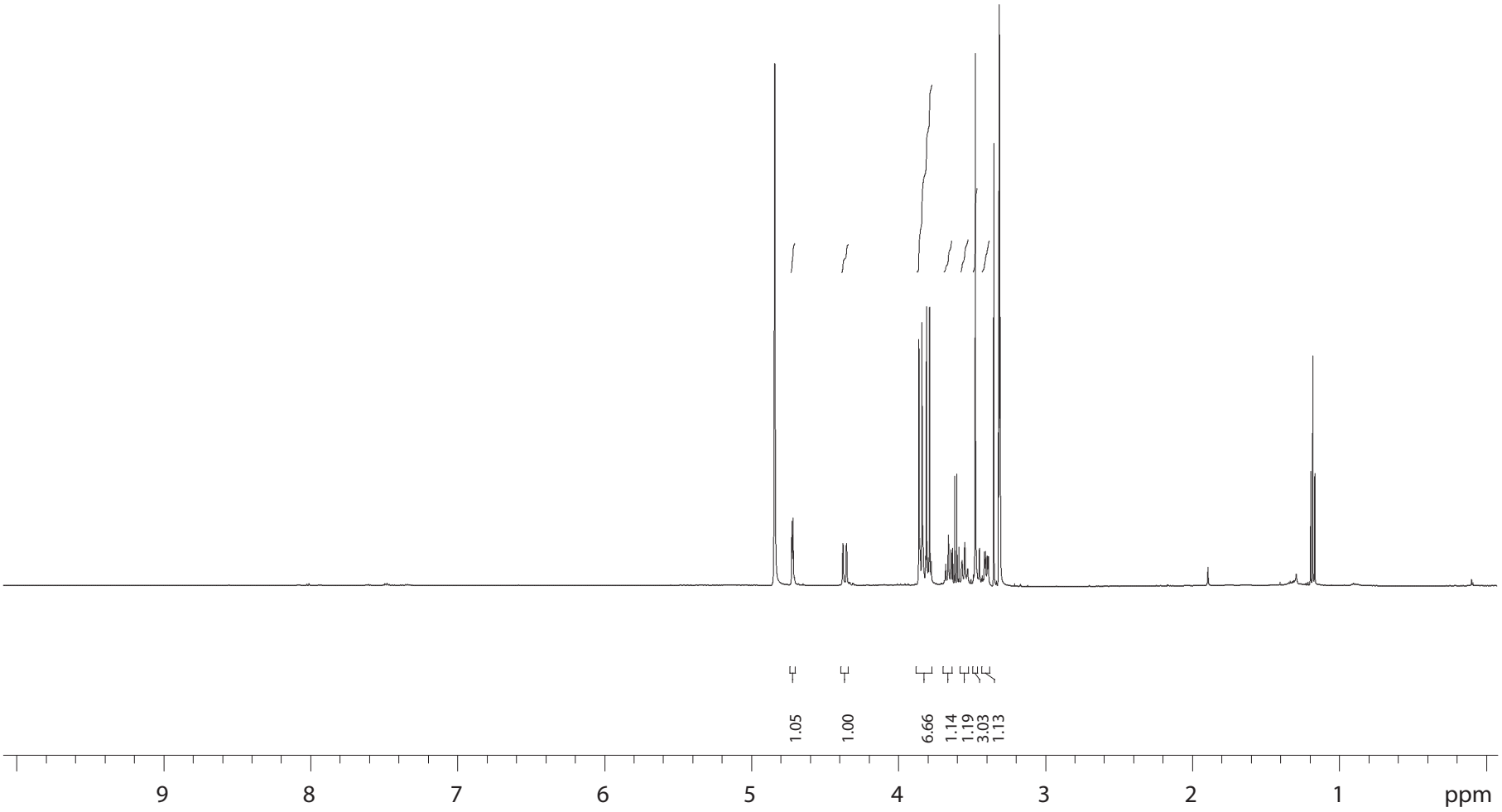
161.839 MHz P31[H1] 1D in cdcl3



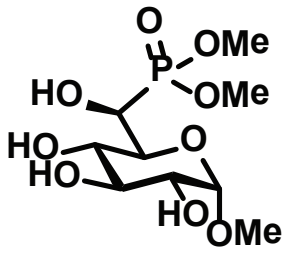


499.817 MHz H1 1D in cd3od

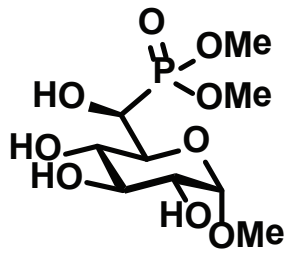


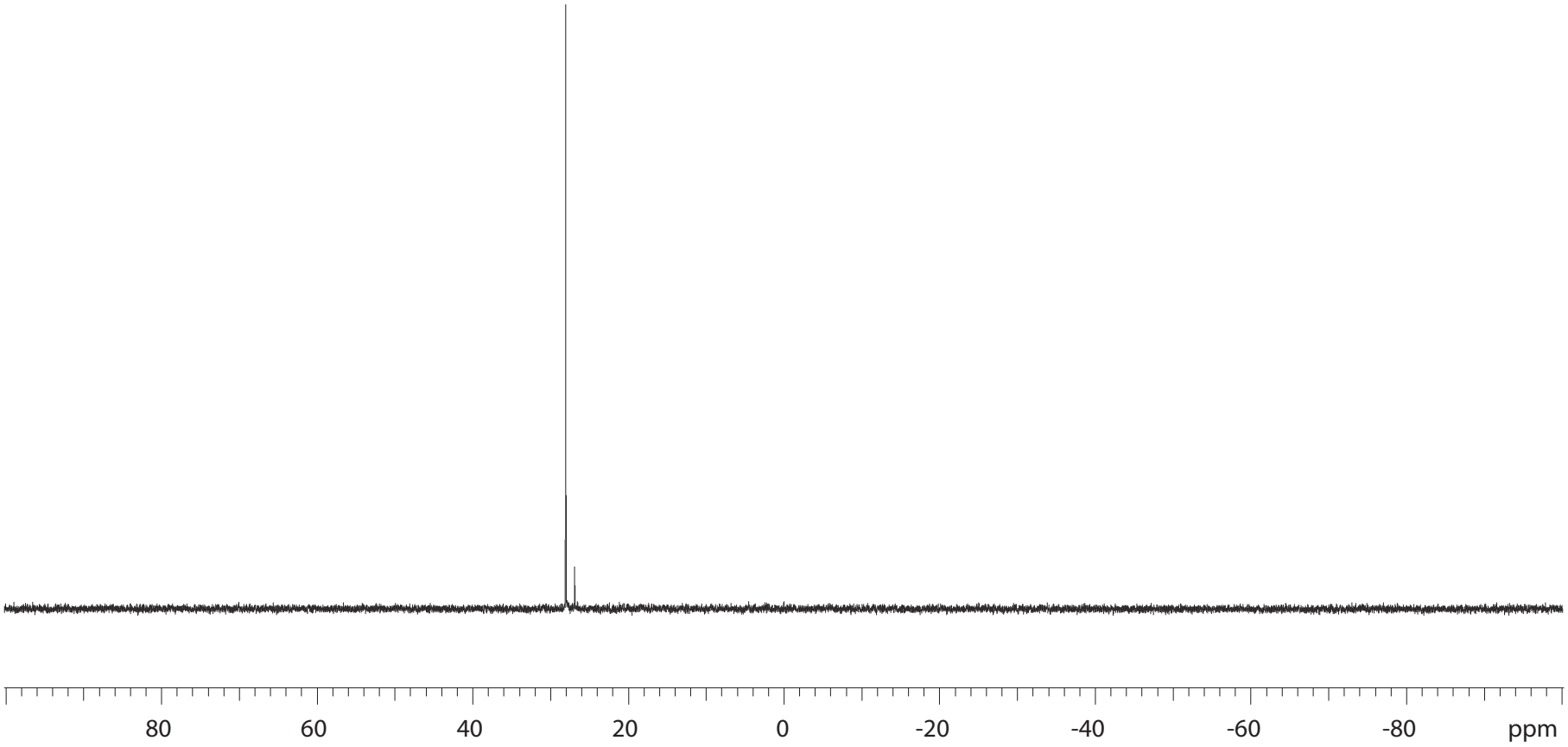


125.693 MHz C13[H1] DEPT_chempack in cd3od

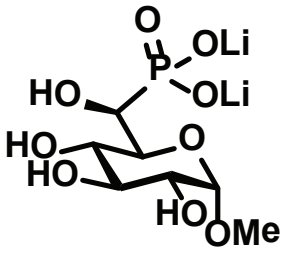


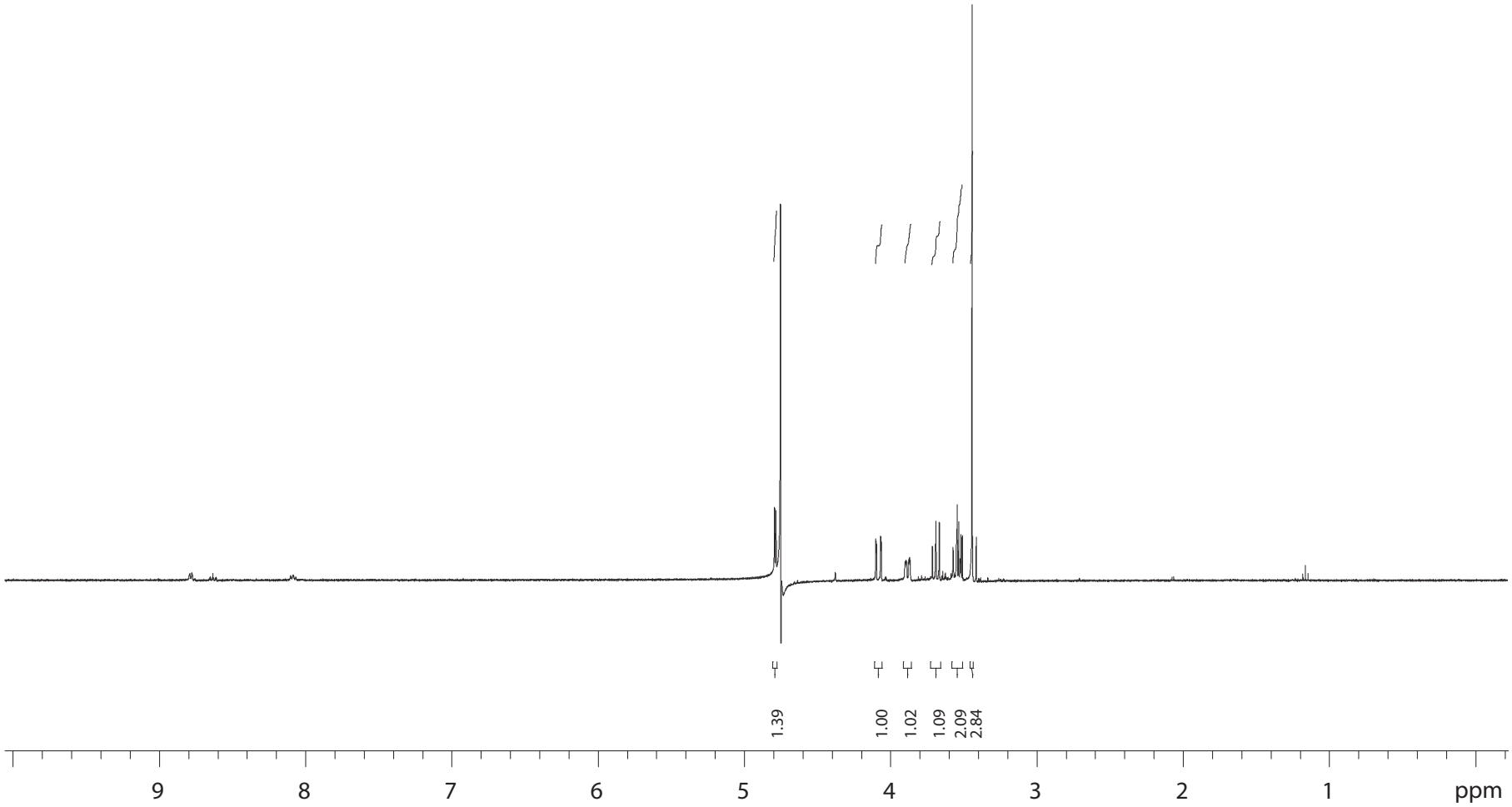
201.643 MHz P31[H1] 1D in cd3od



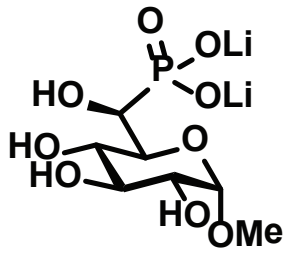


399.795 MHz H1 1D in d2o

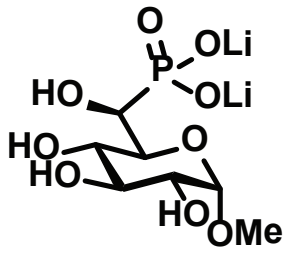


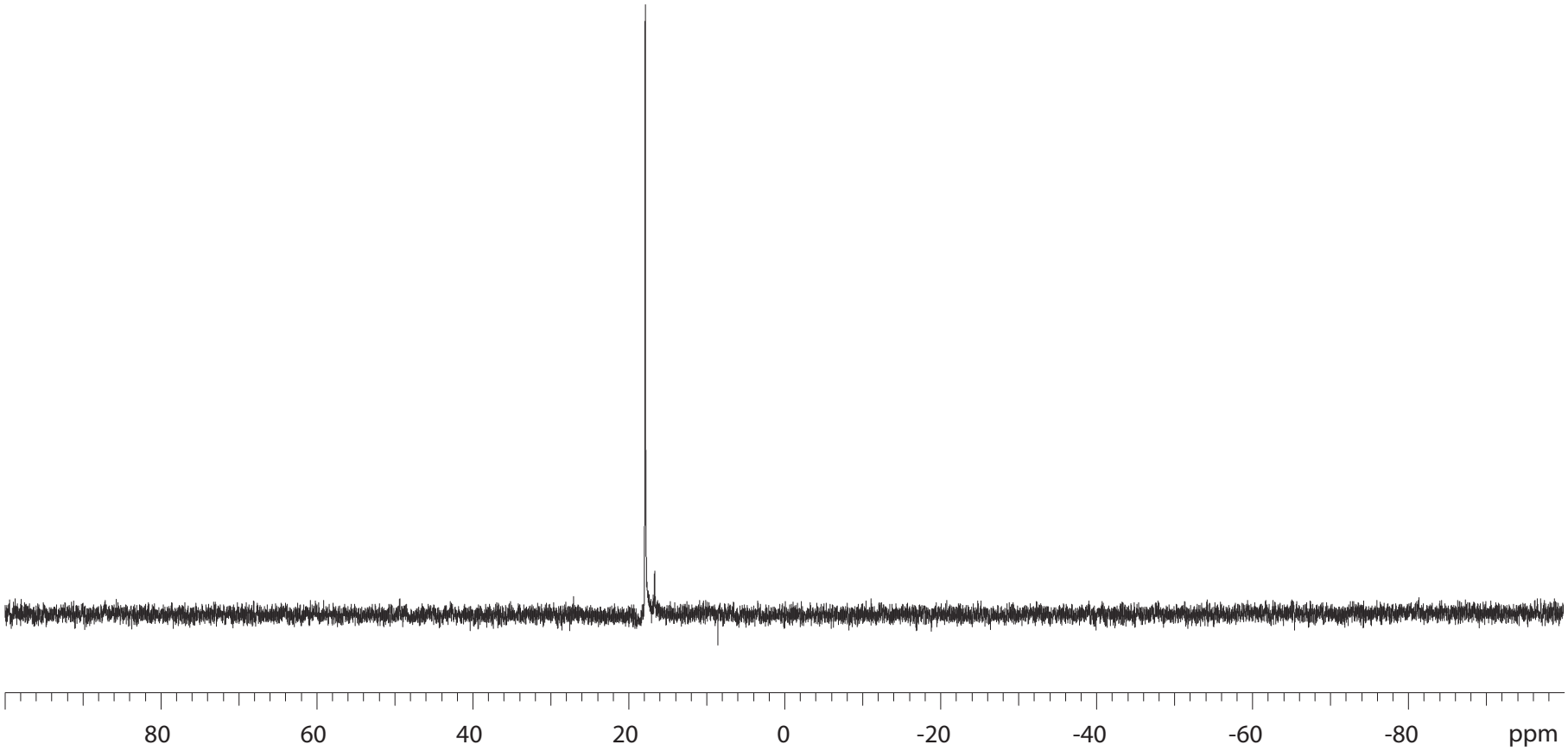


125.693 MHz C13[H1] DEPT_chempack in d2o

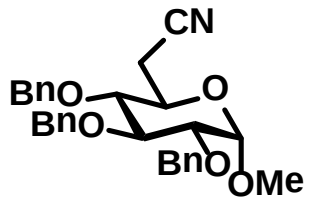


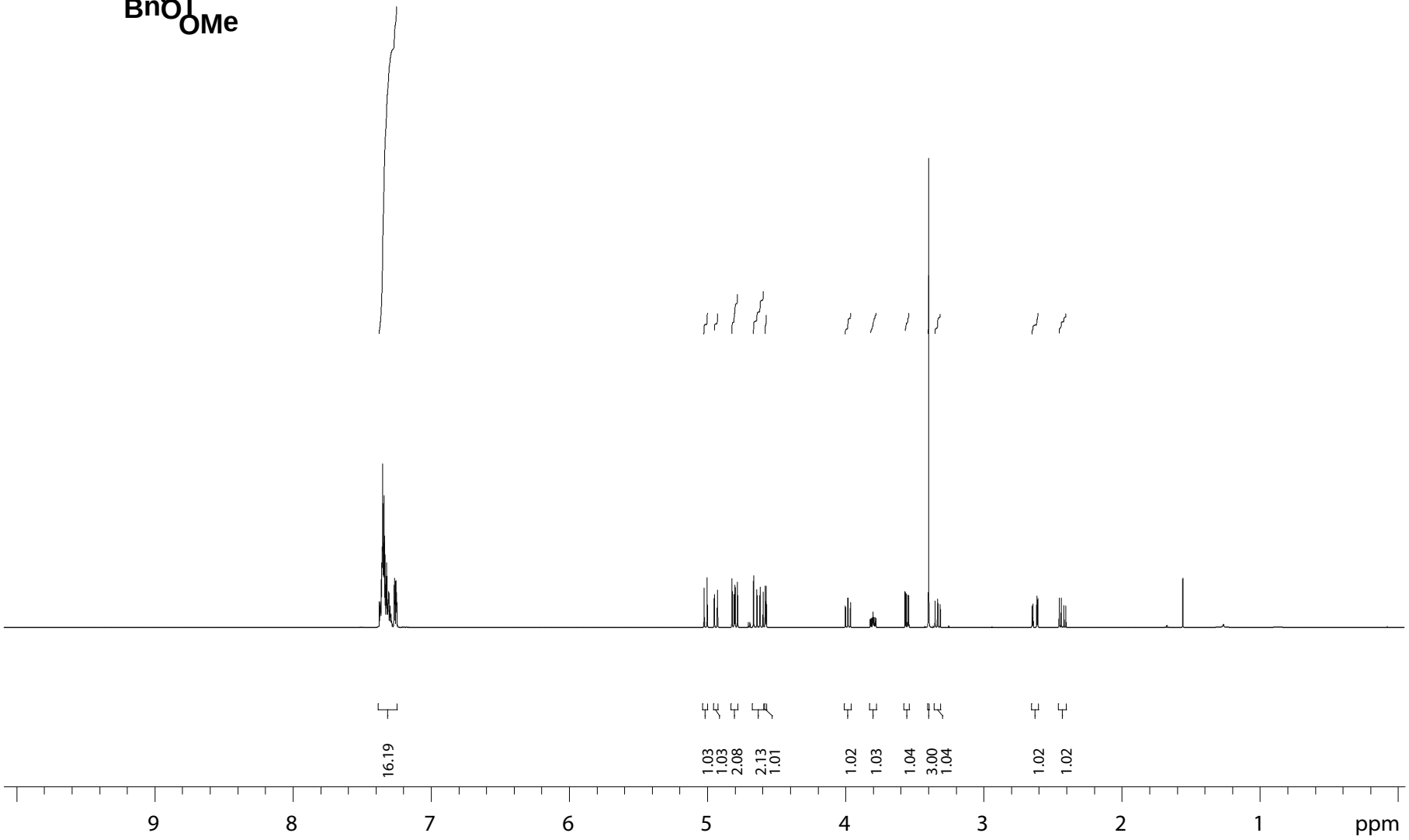
161.839 MHz P31[H1] 1D in d2o



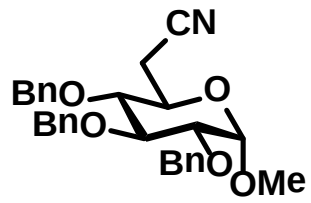


498.122 MHz H1 1D in cdcl3

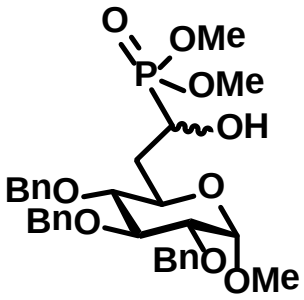




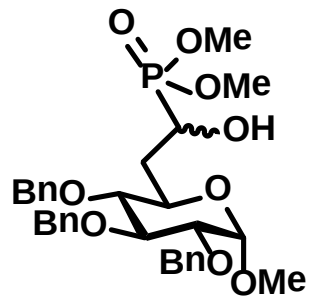
125.264 MHz C13[H1] 1D in cdcl3



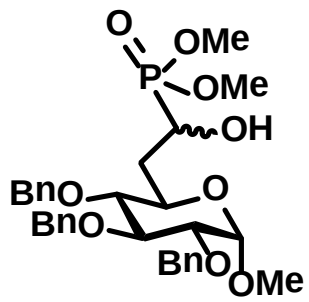
499.815 MHz H1 1D in cdcl3

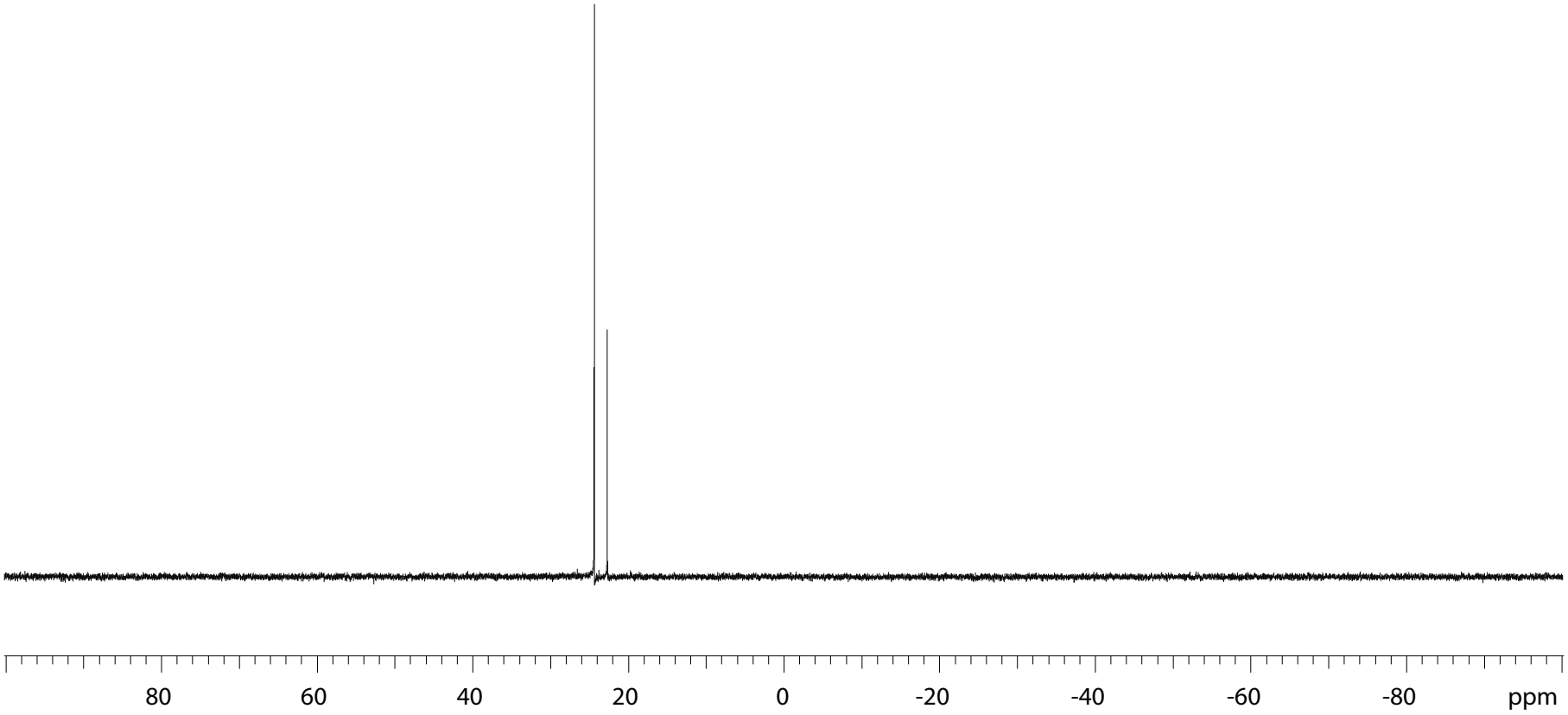


125.693 MHz C13[H1] DEPT_chempack in cdcl3

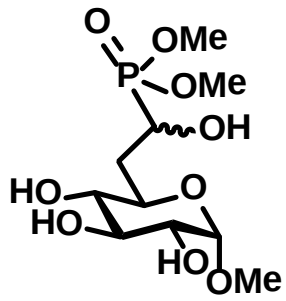


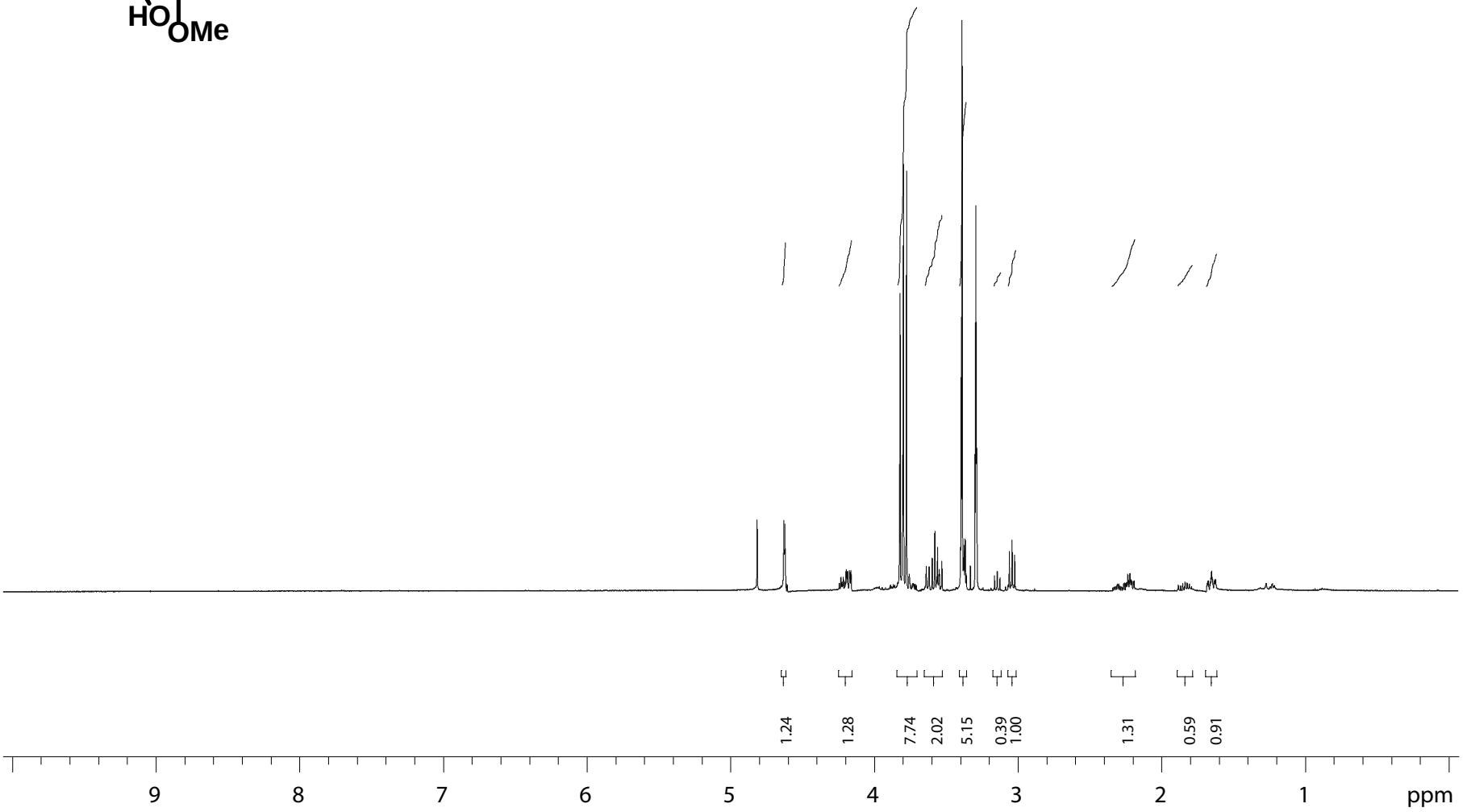
201.643 MHz P31[H1] 1D in cdcl3



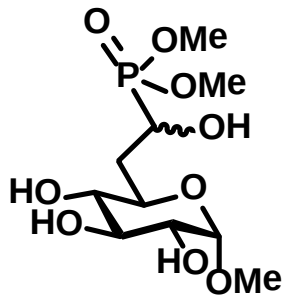


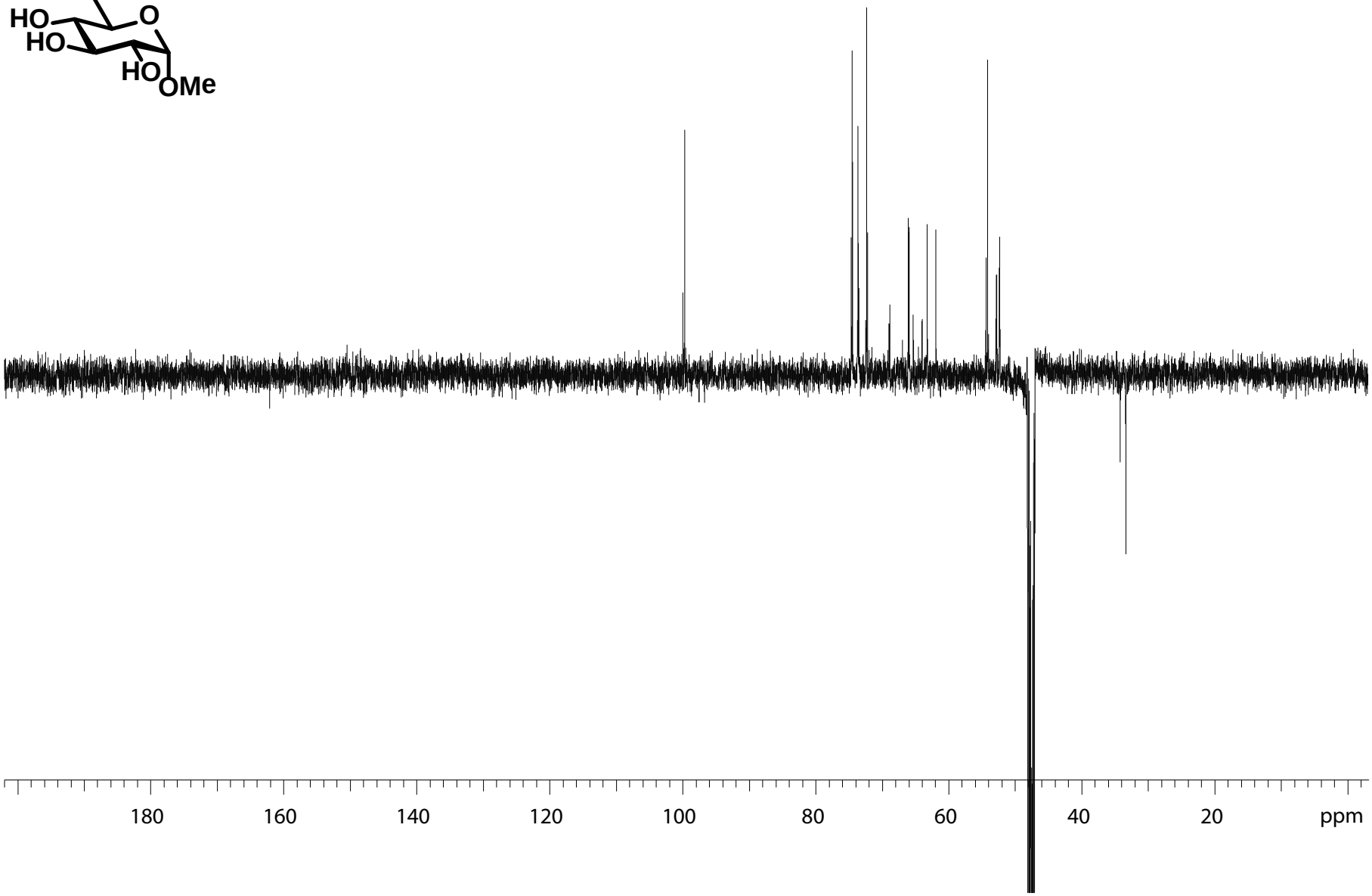
498.124 MHz H1 1D in cd3od



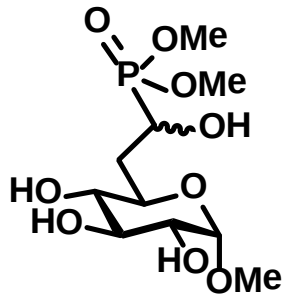


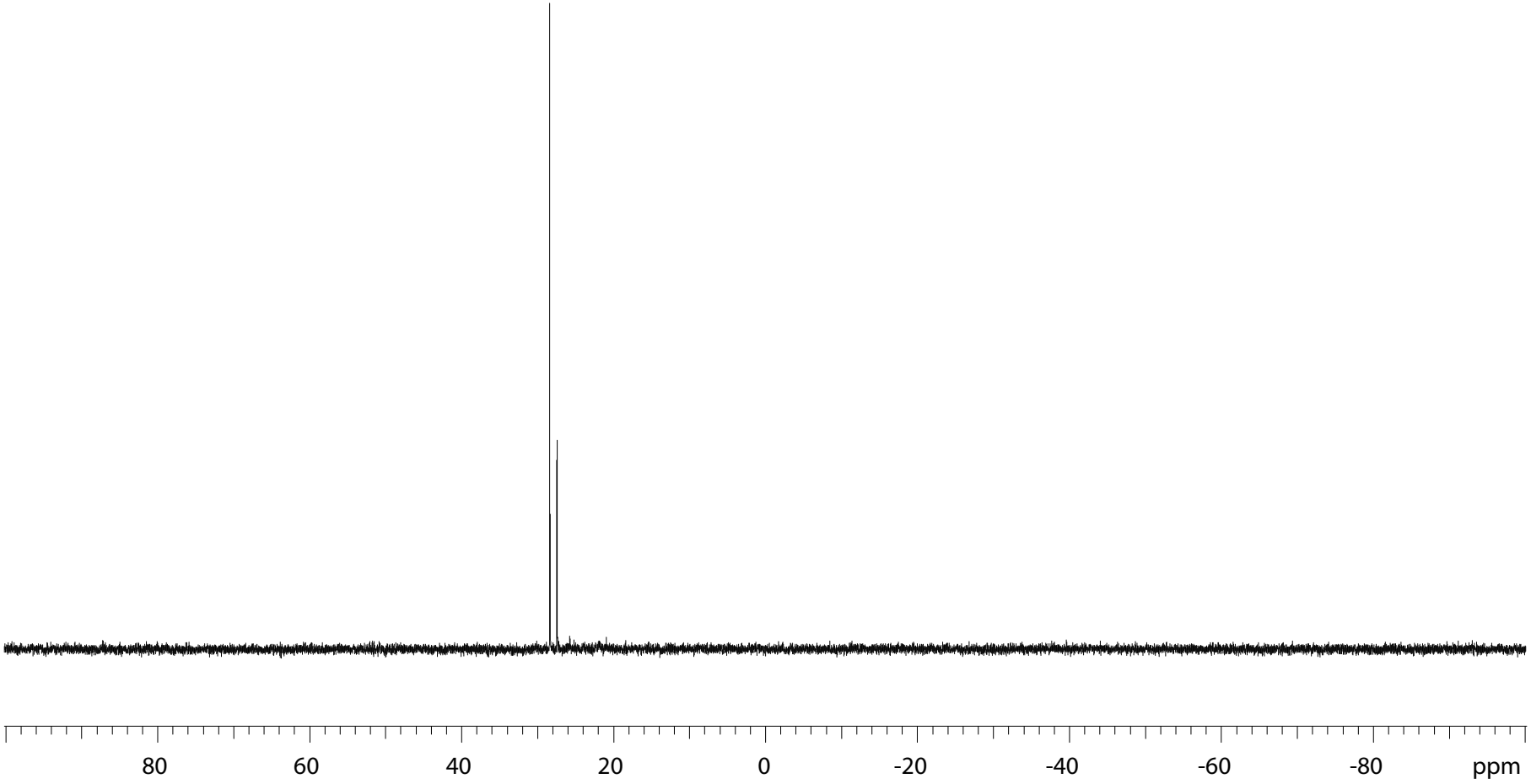
125.693 MHz C13[H1] APT_ad in cd3od



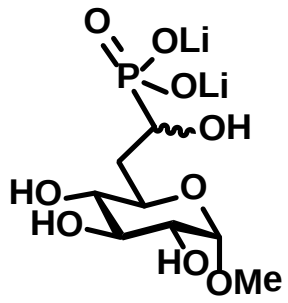


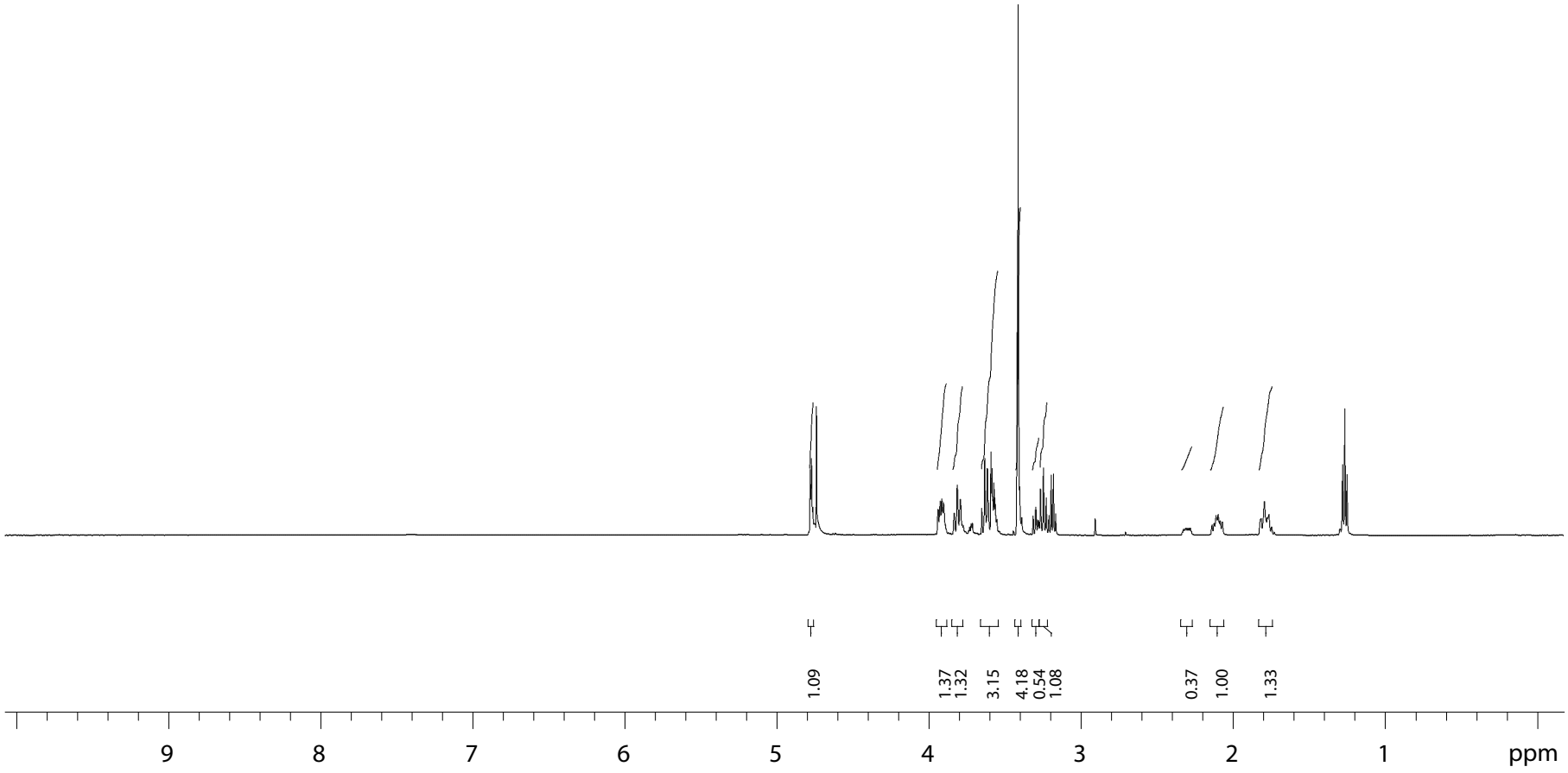
201.643 MHz P31[H1] 1D in cd3od



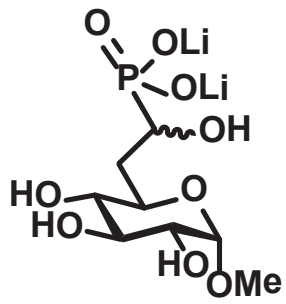


499.816 MHz H1 PRESAT in d2o

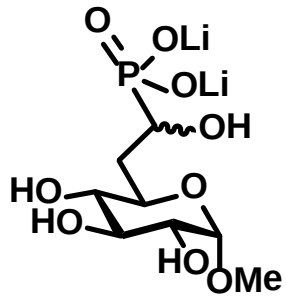


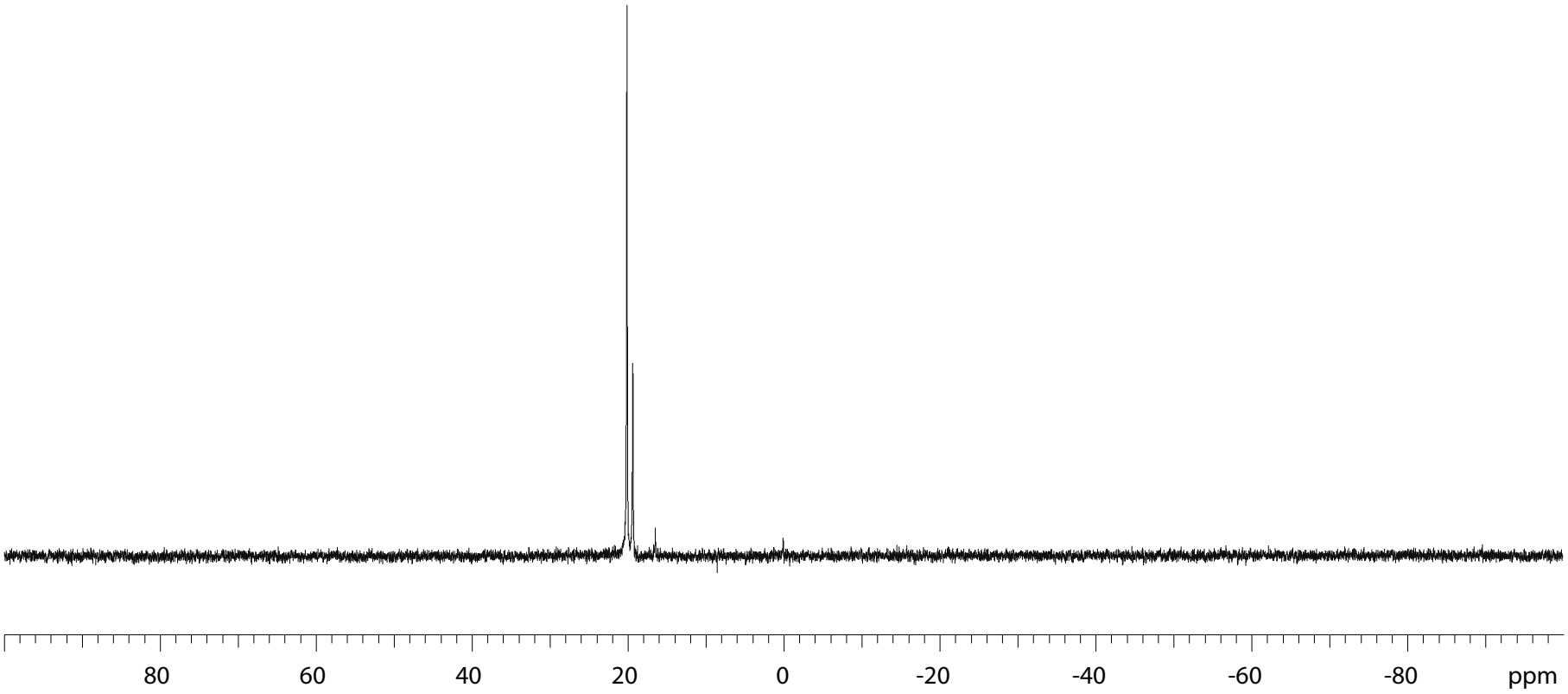


125.693 MHz C13[H1] APT_ad in d2o

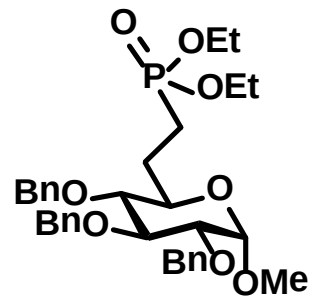


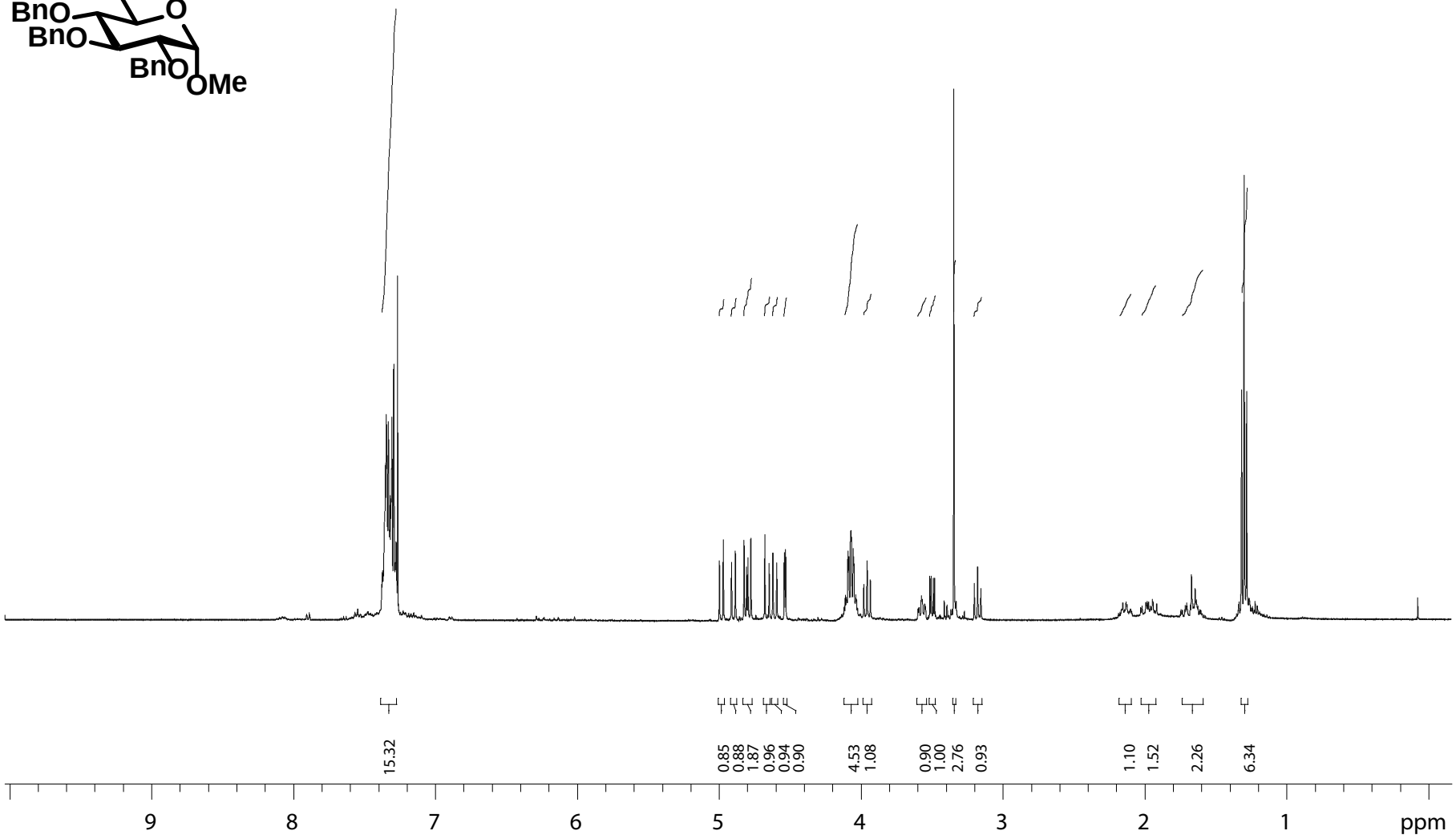
161.839 MHz P31[H1] 1D in d2o



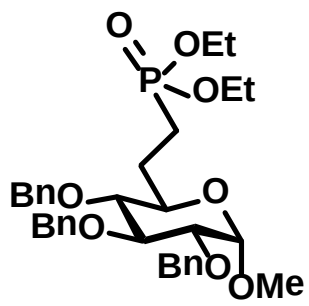


399.794 MHz H1 1D in cdcl3

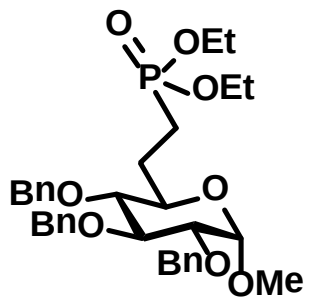


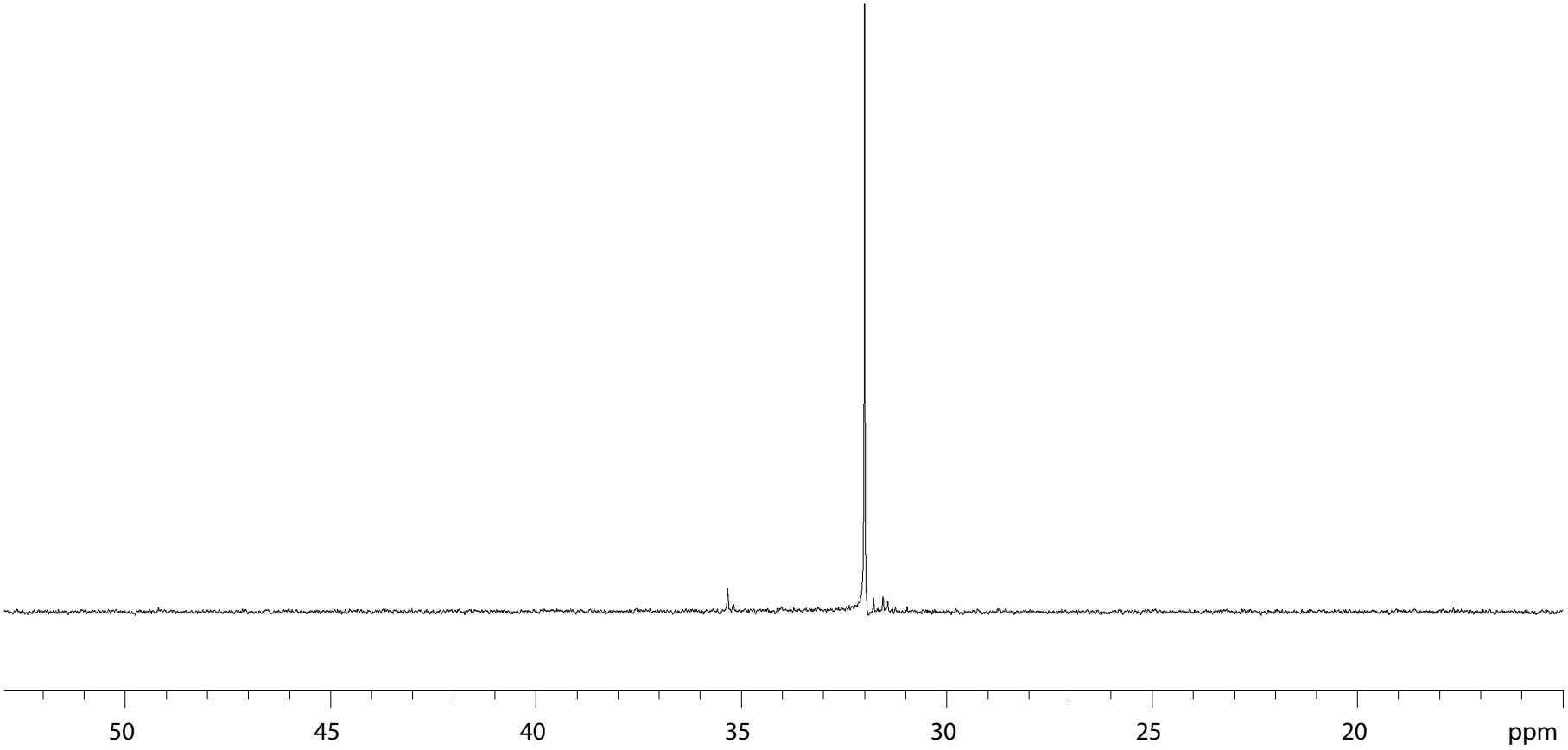


125.692 MHz C13[H1] APT_ad in cdcl3

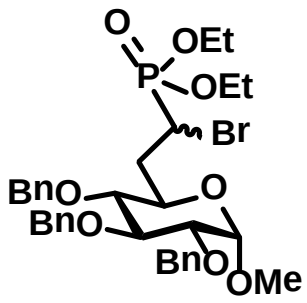


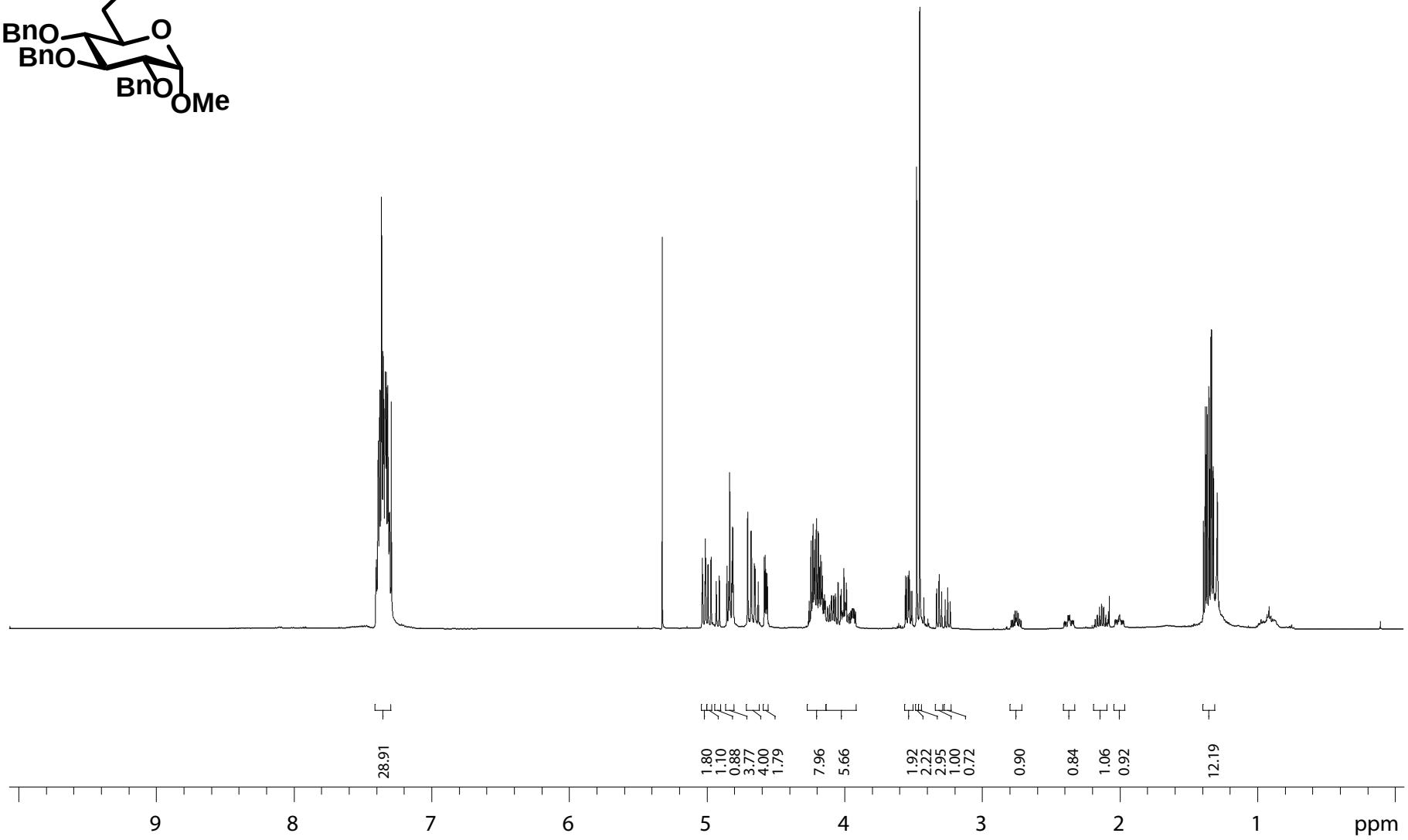
161.839 MHz P31[H1] 1D in cdcl3



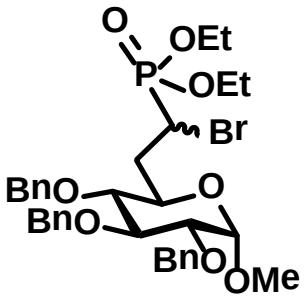


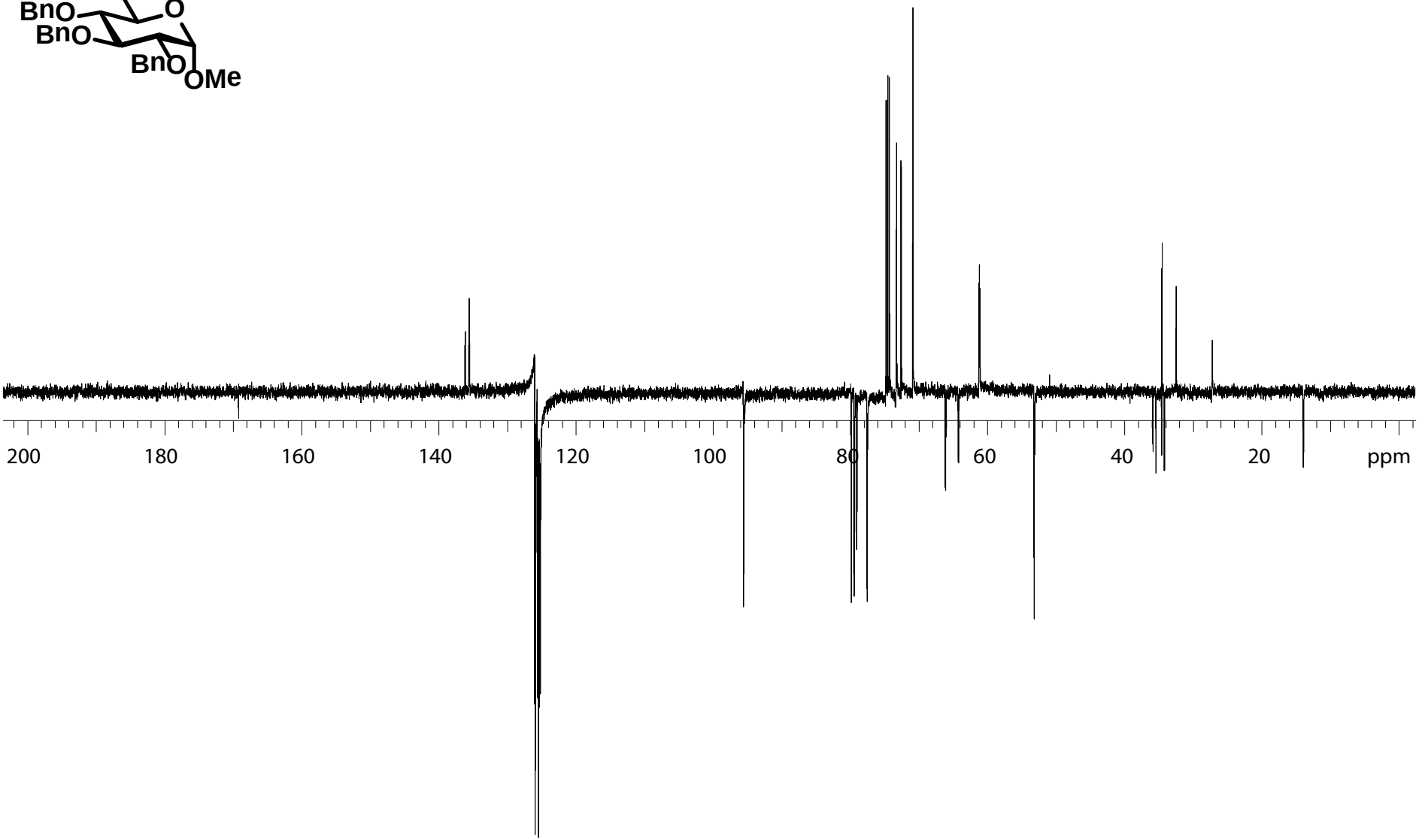
499.815 MHz H1 1D in cdcl3



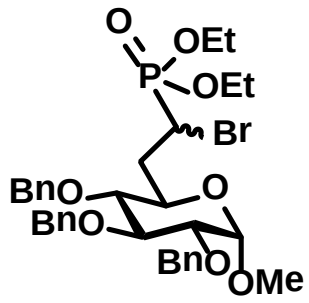


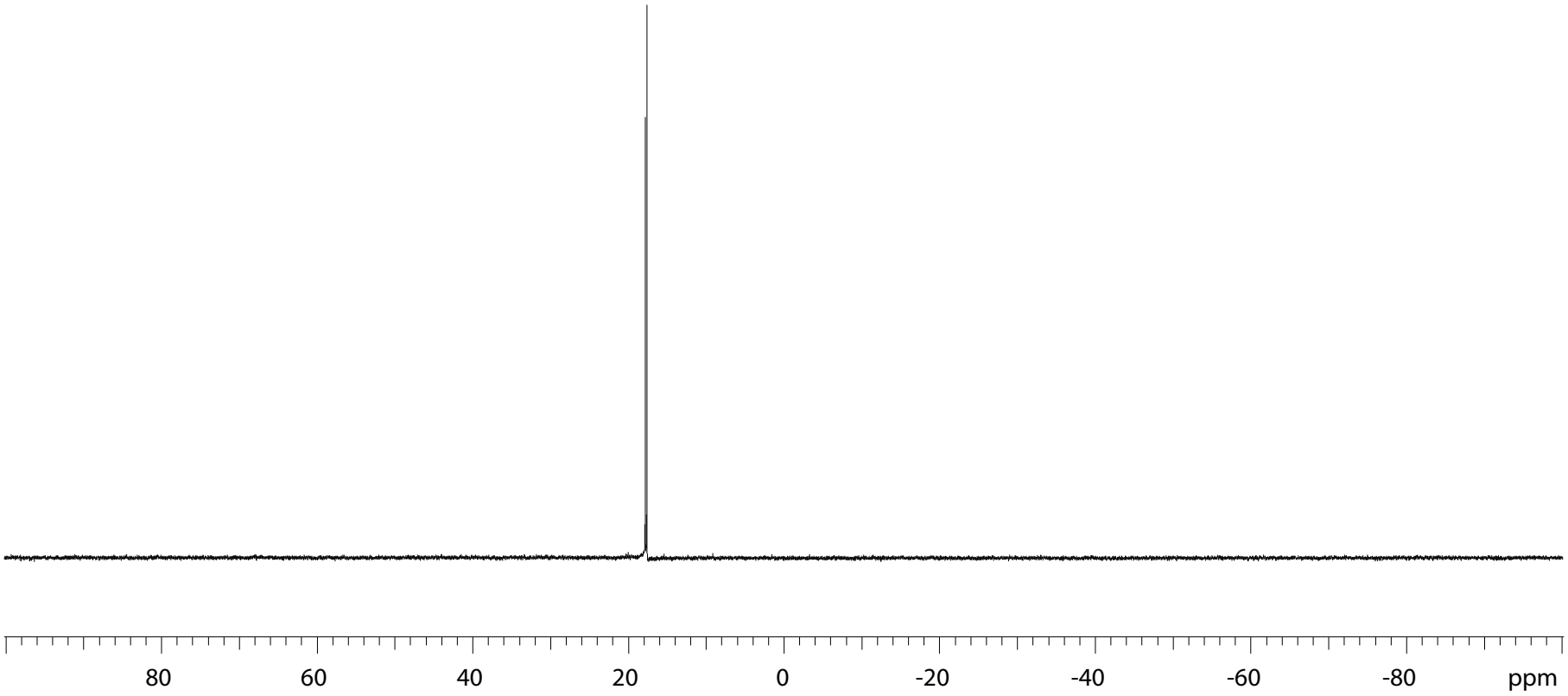
125.693 MHz C13[H1] DEPT_chempack in cdcl3



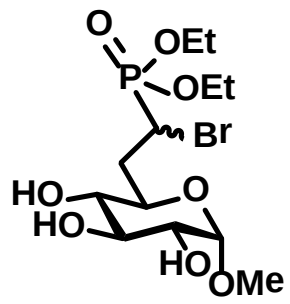


201.643 MHz P31[H1] 1D in cdcl3

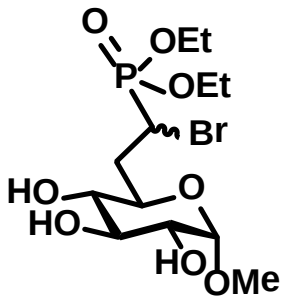




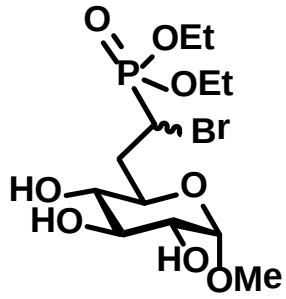
498.124 MHz H1 1D in cd3od

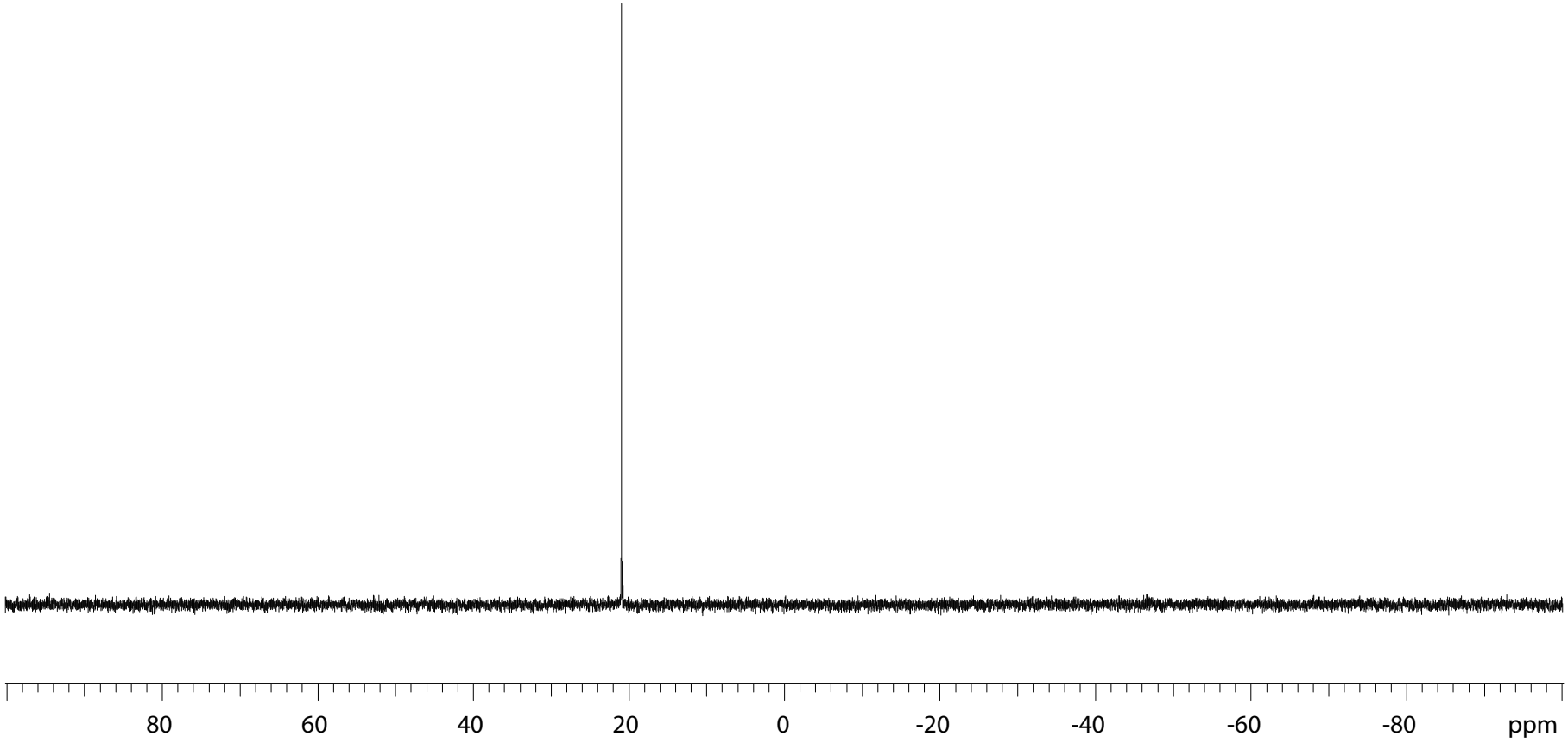


125.693 MHz C13[H1] DEPT_chempack in cd3od

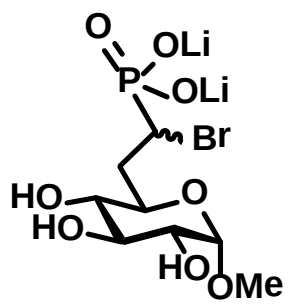


201.643 MHz P31[H1] 1D in cd3od

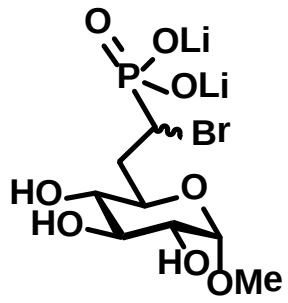




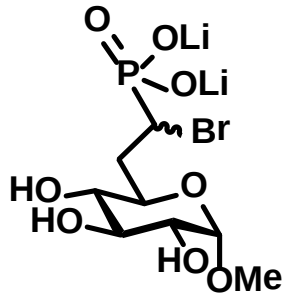
498.123 MHz H1 1D in d2o

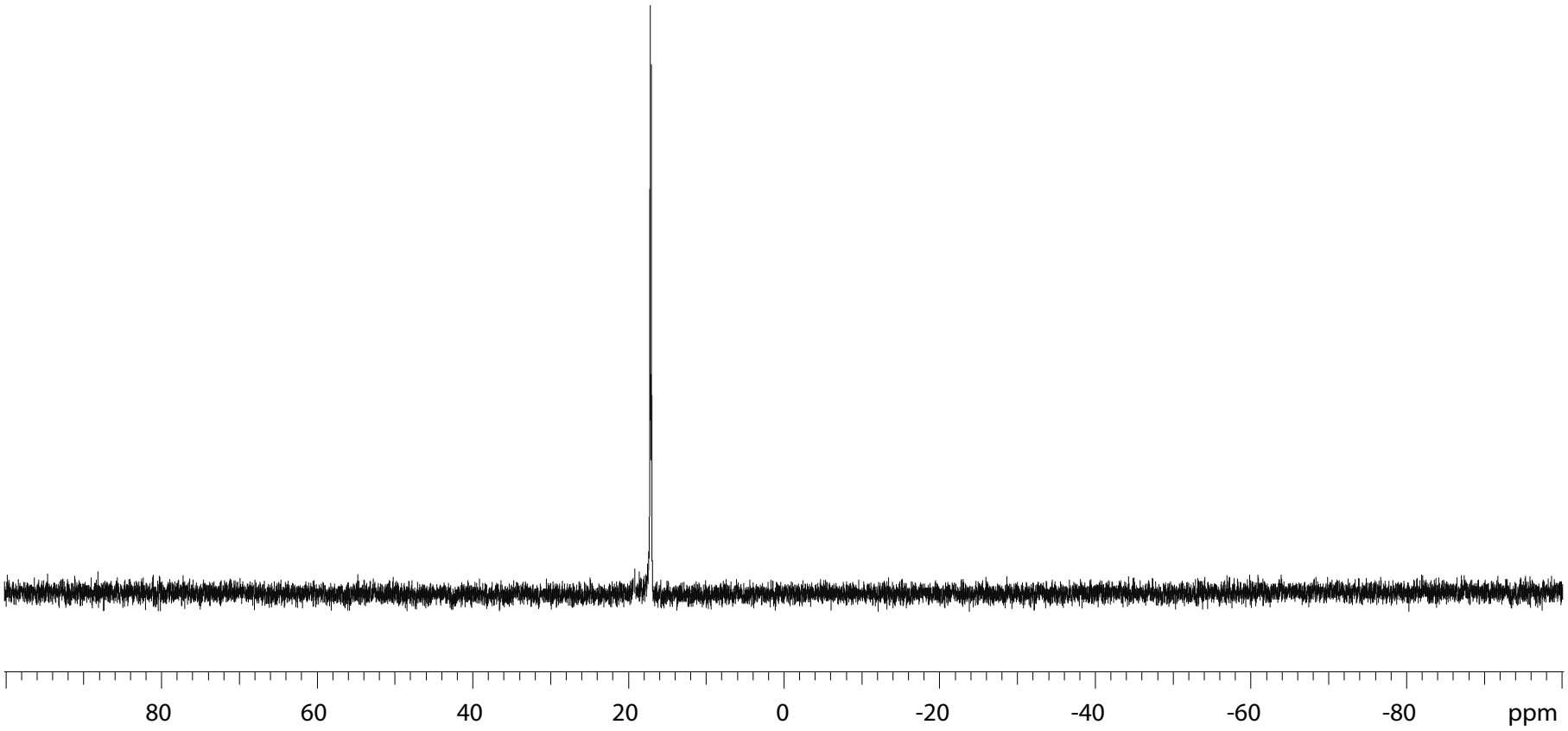


125.693 MHz C13[H1] APT_ad in d2o

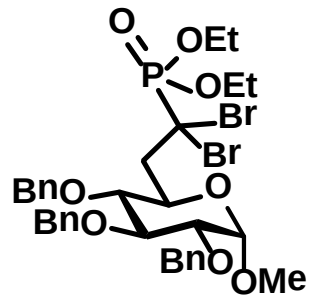


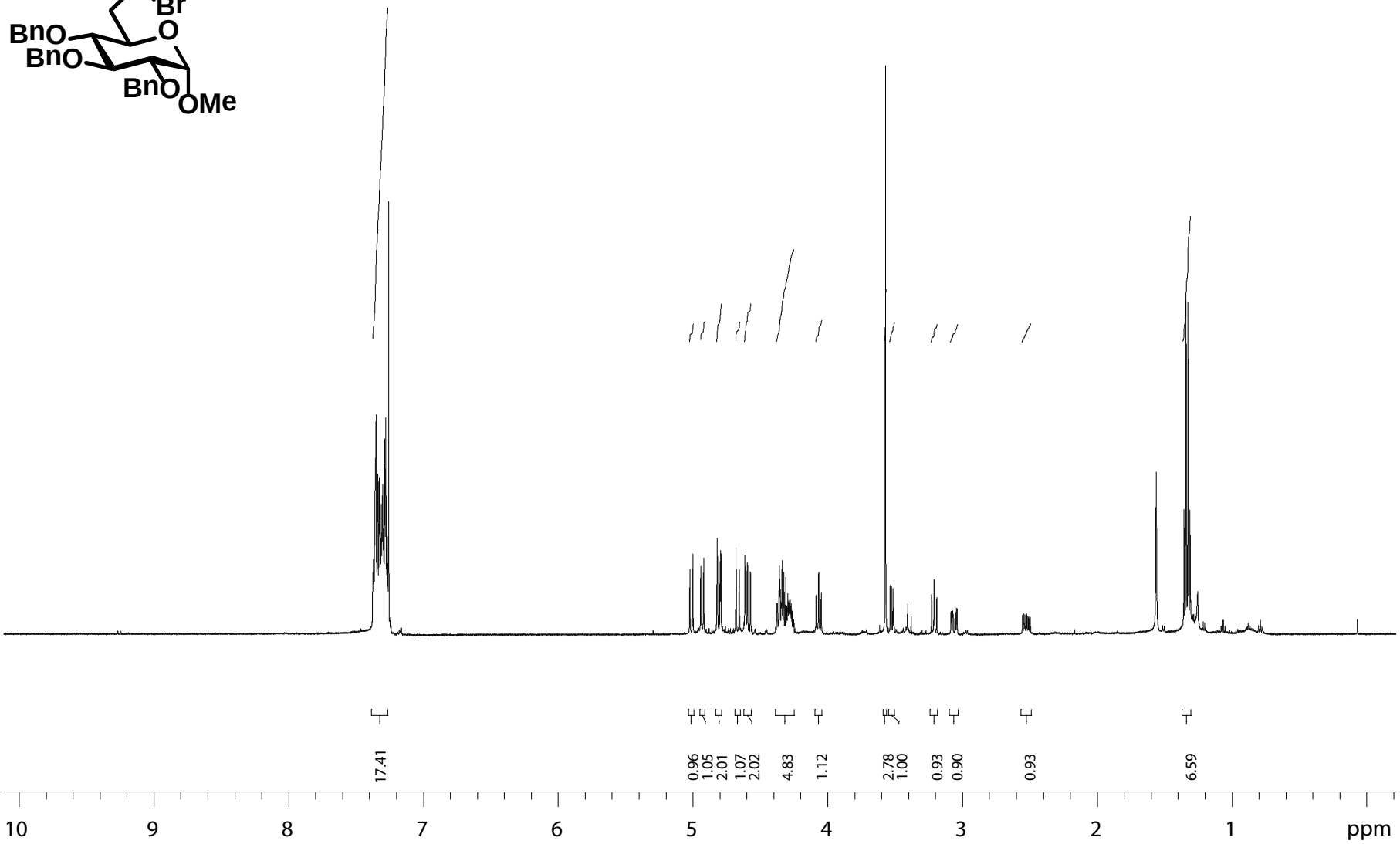
201.642 MHz P31[H1] 1D in d2o



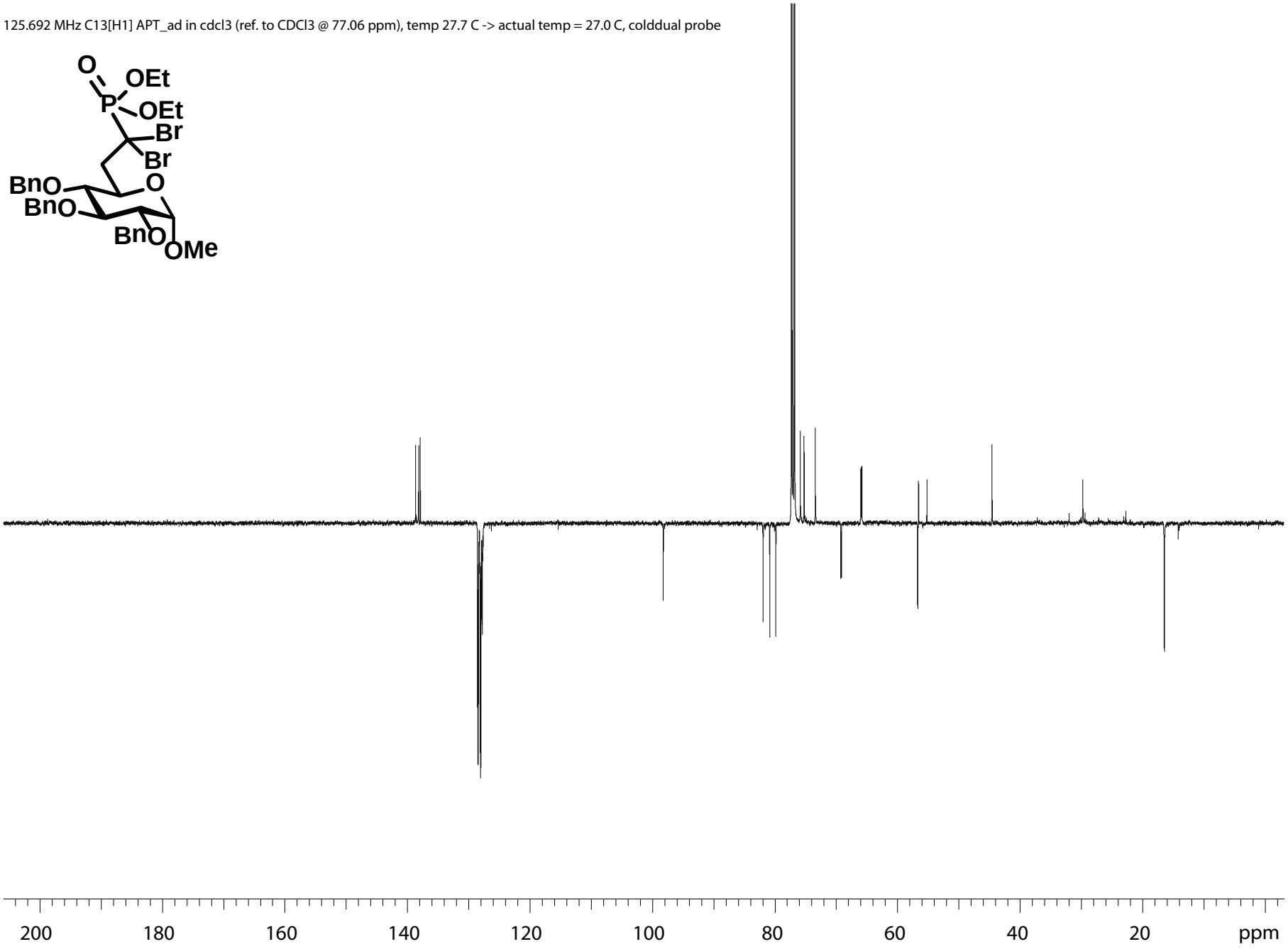


498.122 MHz H1 1D in cdcl3

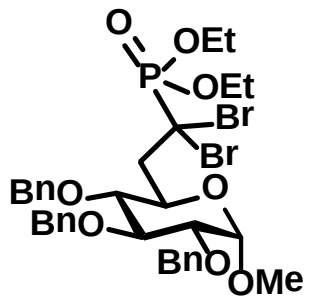


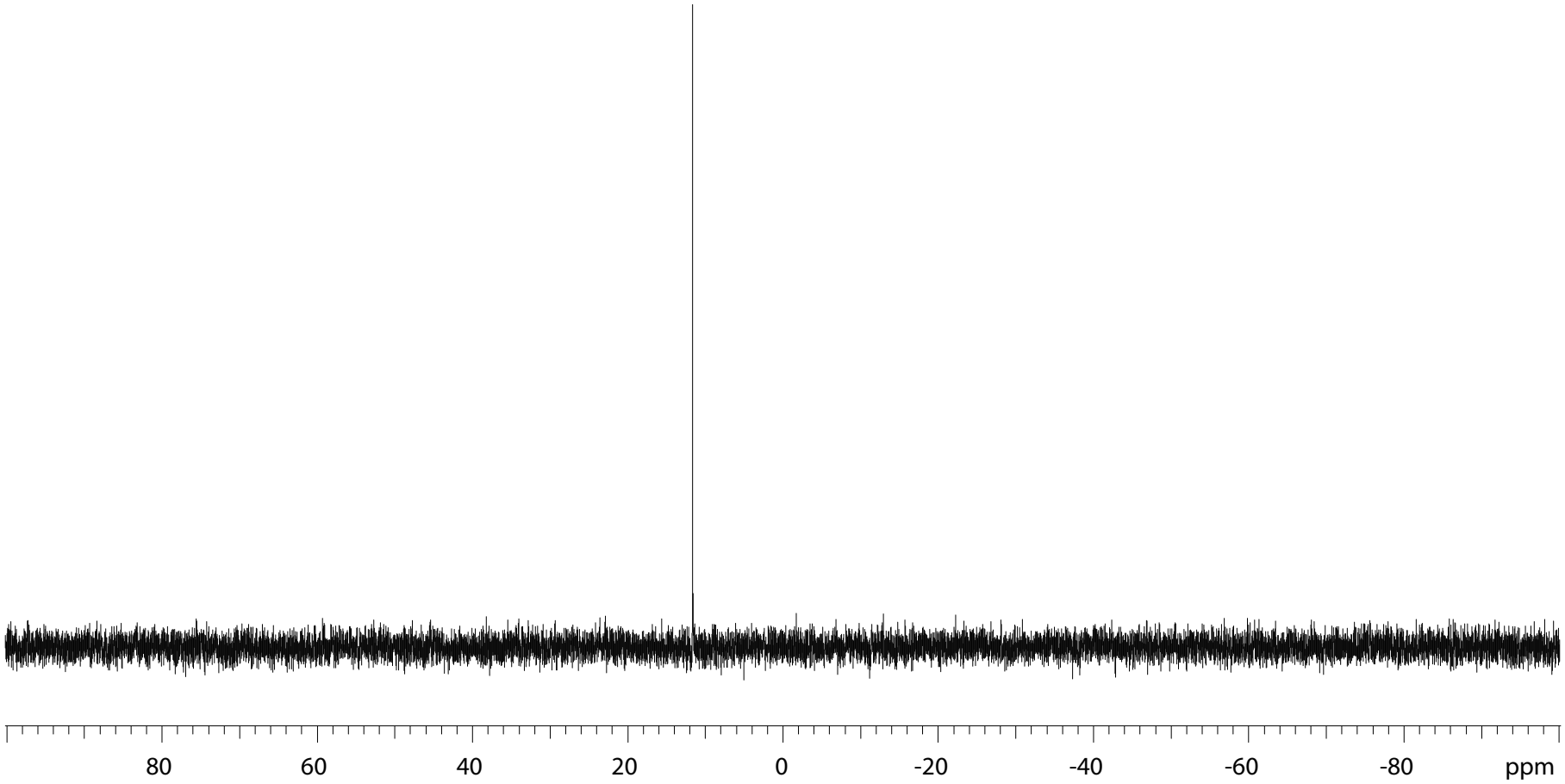


125.692 MHz C13[H1] APT_ad in cdcl3 (ref. to CDCl3 @ 77.06 ppm), temp 27.7 C -> actual temp = 27.0 C, colddual probe

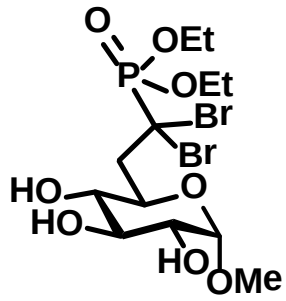


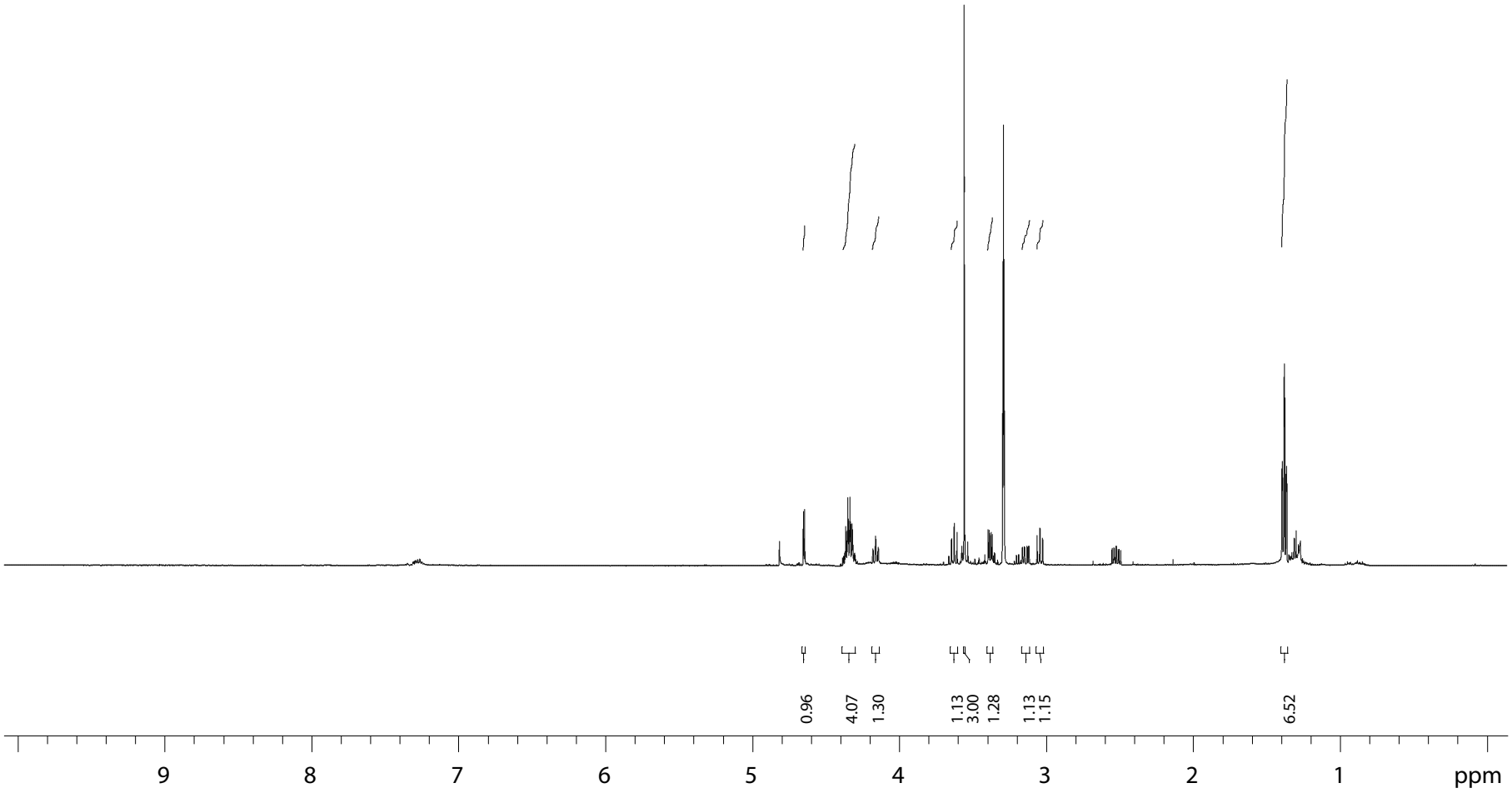
201.642 MHz P31[H1] 1D in cdcl3



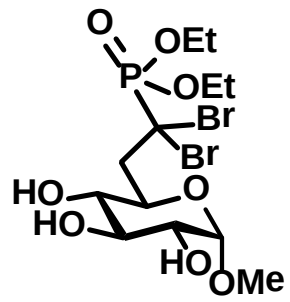


498.124 MHz H1 1D in cd3od

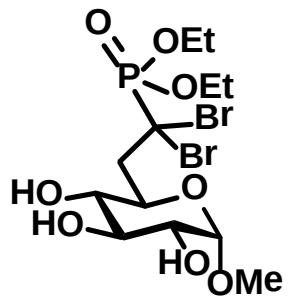


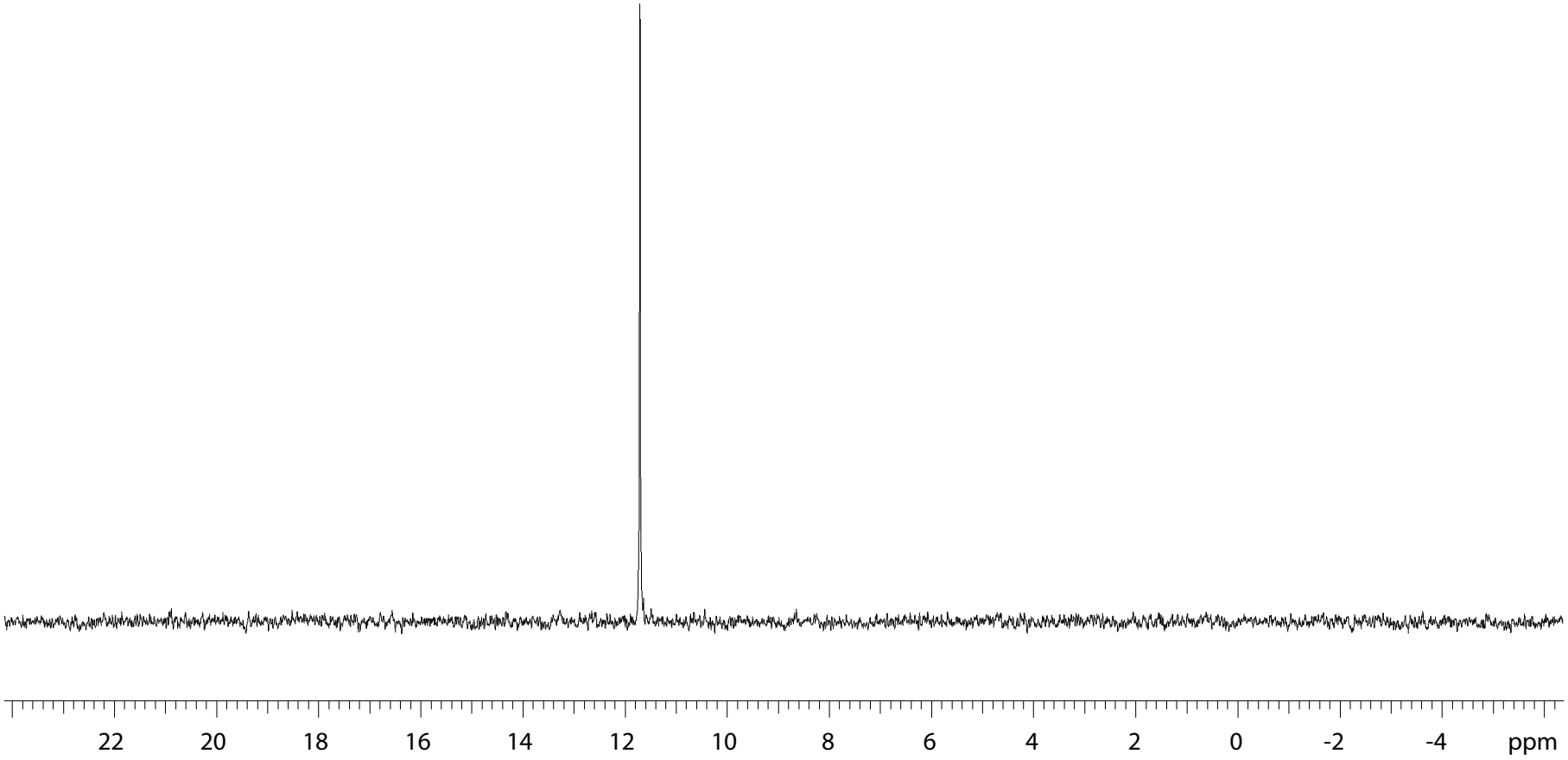


125.693 MHz C13[H1] APT_ad in cd3od

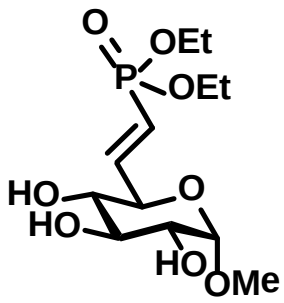


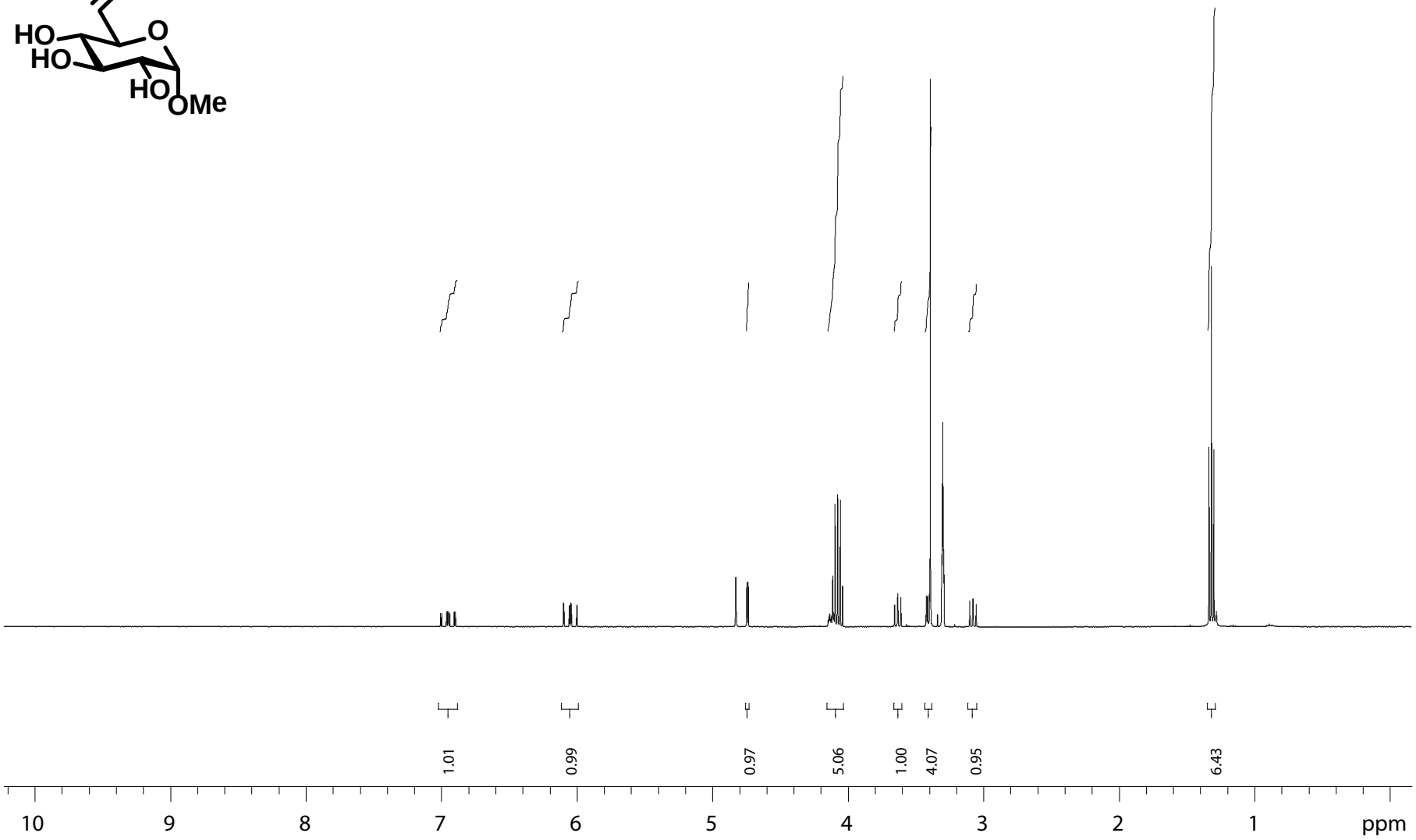
201.643 MHz P31[H1] 1D in cd3od



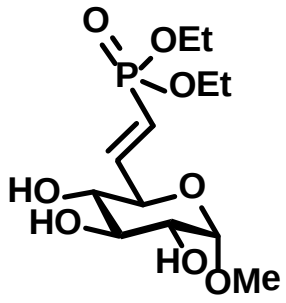


399.796 MHz H1 1D in cd3od





125.693 MHz C13[H1] APT_ad in cd3od



161.840 MHz P31[H1] 1D in cd3od