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Investigation of Myo-Inositol 1-Phosphate Synthase
Catalytic Sequence
Utilizing Mechanism-Based Inhibitors

presented by

Marie E. Migaud

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of the requirements for

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Major professor

Date 7/18/96

INVESTIGATION OF *MYO*-INOSITOL 1-PHOSPHATE SYNTHASE CATALYTIC SEQUENCE UTILIZING MECHANISM-BASED INHIBITORS

By

Marie E. Migaud

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ABSTRACT

INVESTIGATION OF *MYO*-INOSITOL 1-PHOSPHATE SYNTHASE CATALYTIC SEQUENCE UTILIZING MECHANISM-BASED INHIBITORS

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The sequence of glucose 6-phosphate (G6P) conversion into *myo*-inositol 1-phosphate (MIP) catalyzed by MIP synthase, a NAD dependent enzyme, was investigated by examining active site interactions with analogues of substrates and reactive intermediates. Purification methods were developed to isolate homogeneous MIP synthase from *S. cerevisiae* and from genetically engineered *E. coli*.. New putative carbocyclic inhibitors of MIP synthase required the development of a strategy which could provide access to phosphonylated and phosphorylated inositol derivatives. This approach was based on the heteromethylation of highly substituted cyclohexene oxides in which boron played a key role either as a lewis acid during opening of the oxirane (using dialkyl lithiomethanephosphonate/boron trifluoride) or as a stabilizer of an adjacent carbanion on the attacking nucleophile [(lithiomethyl)dimesitylborane].

The question of whether the substrate in its α-pyranose, β-pyranose, or acyclic form binds to MIP synthase was examined by quantitating inhibition of MIP synthase by various substrate analogues. The importance of the oxygen pyranose and C-1 hydroxyl group was assessed by evaluating substrate analogues, such as 1,5-anhydro-glucitol 6-phosphate, and newly synthesized α- and β-carbocyclic glucose 6-phosphate, for inhibition. Active site interactions essential to binding the acyclic substrate after pyranosyl ring opening were investigated by studying newly synthesized deoxy-phosphosugars and other deoxy-glucitol 6-phosphate analogues. The nature of the rotation about the C4-C5

bond that must precede intramolecular aldol condensation was also investigated utilizing mono- and diphosphorylated carbocyclic species.

In addition to confirming that *myo*-2-inosose 1-phosphate, synthesized from *myo*-inositol, was an intermediate along the reaction coordinates, it was also shown to be a potent MIP synthase inhibitor. To probe the importance of the oxidized reaction center in *myo*-2-inosose 1-phosphate to active site interactions and to gauge the relative contribution of the keto form to these interactions, five analogues were examined as MIP synthase inhibitiors. 2-Deoxy-*myo*-inositol 1-phosphate was used to gauge the impact of removing the oxidized reaction center of *myo*-2-inosose 1-phosphate. 1-Deoxy-1-(phosphonomethyl)-*myo*-2-inosose, which exists in neutral aqueous solution exclusively in its keto form, was studied for its close structural similarity to *myo*-2-inosose 1-phosphate. MIP and *scyllo*-inositol phosphate were considered as monodeoxygenated analogues of the *myo*-2-inosose 1-phosphate hydrate form. Finally dihydroxyacetone 1-phosphate was examined to ascertain the structural requirements needed in addition to the oxidized reaction center for active site binding.

To my Parents.

ACKNOWLEDGMENTS

The present work would not have been possible if Prof. J. W. Frost had not shown great patience toward my lack of chemical skills, my poor English, and the many other imperfections that were my principle trademarks when I joined his group. Pr J. W. Frost taught me that knowledge in organic chemistry today can no longer be limited to an organic chemistry text book but requires an open-mind toward any aspects of chemistry and more generally of science. He also demonstrated that in order to succeed as a scientist, hard work, good thinking, and efficiency are required. Even if these requirements still need improving, the goals set by Prof. Frost, to be the best at what we do, will be the foundation of my future career as a scientist.

My deepest appreciation to Karen Frost for giving me support during my darkest hours and sharing some of the happy moments. At length, I thank her for her patience as a proof reader and all the important comments and advice she gave me when writing this thesis as she did with so many of my written works.

I thank Jean-Luc Montchamp who defined what an organic chemist should know and do. He had the strongest influence on my perception of chemistry and on my personal development. All the members of the Frost group from past to present that I had the chance to meet and work with have my most sincere gratitude for their patience. Finally, I want to thank Tara, Doug and Wanda, the friends I made along the way.

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LIST OF SCHEMES

LIST OF ABBREVIATIONS

Ac	acetyl
AIBN	2,2'-azobis(2-methylpropionitrile) or 2,2'-azobisisobutyronitrile
ATP	adenosine triphosphate
Bn	benzyl
Bu	butyl
Bz	benzoyl
CI	chemical ionization
DAHP	3-deoxy-D-arabino-heptulosonic acid 7-phosphate
DHQ	3-dehydroquinate
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis(β-aminoethyl ether)
EI	electron impact
Et	ethyl
FAB	fast atom bombardment
G6P	glucose 6-phosphate
h	hour
HPLC	high pressure/performance liquid chromatography
HRMS	high resolution mass spectrometry
1,4,5-IP_3	*myo*-inositol 1,4,5-triphosphate
MCPBA	*m*-chloroperbenzoic acid
Me	methyl
Mes	mesityl or 2,4,6-trimethylphenyl
MI	*myo*-inositol
MIP	*myo*-inositol 1-phosphate

MS	mass spectrometry
NAD	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
NBS	*N*-bromosuccinimide
NMR	nuclear magnetic resonance
PEP	phosphoenol pyruvic acid
Ph	phenyl
iPr	isopropyl
PI	phosphatidyl inositol
Pyr	pyridine
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBPP	tetrabenzyl pyrophosphate
TFAA	trifluoroacetic anhydride
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	trimethylsilyl
Ts	tosyl or *p*-toluenesulfonyl
UV	ultra-violet
Δ	heat at reflux

CHAPTER I

INTRODUCTION

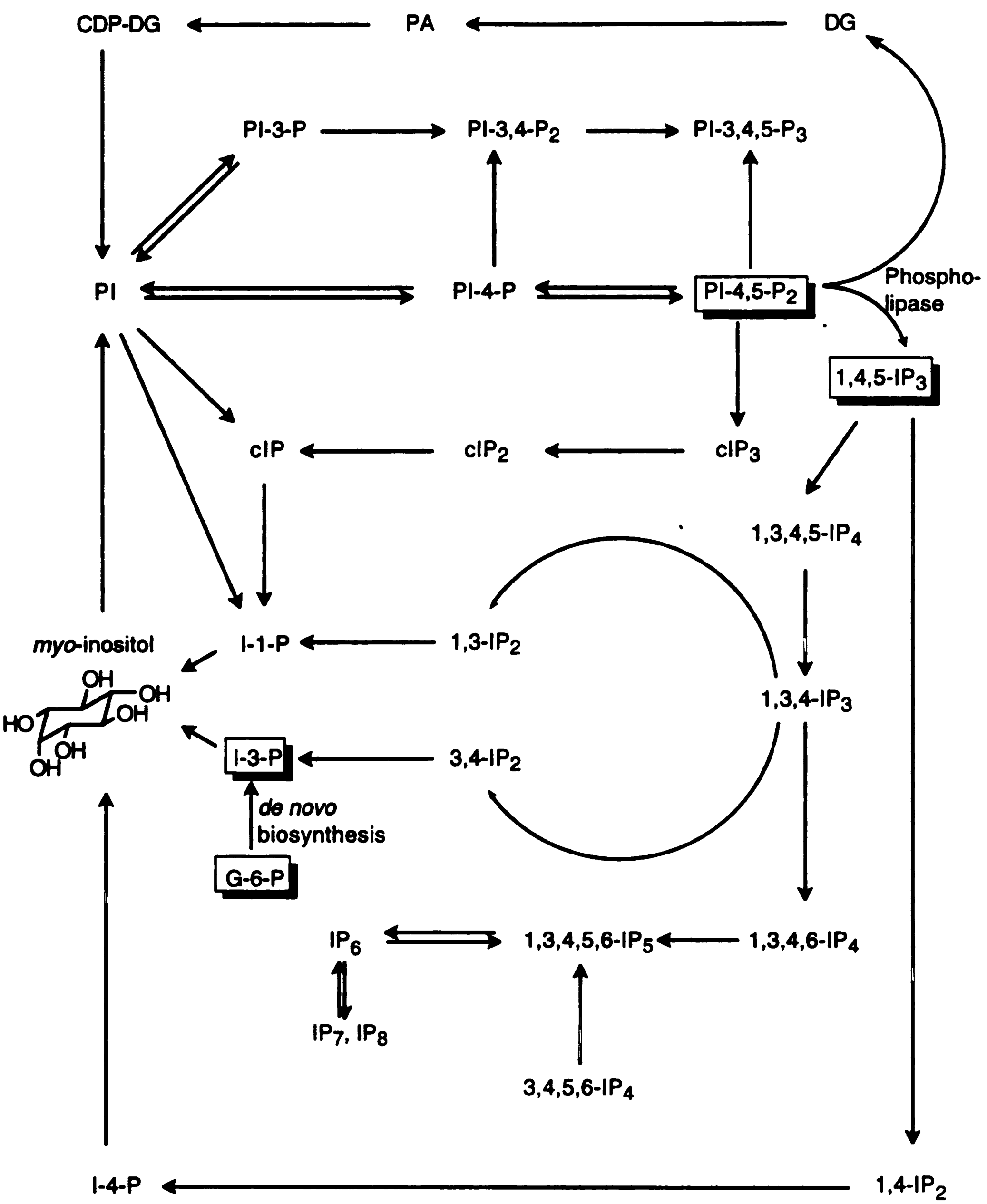
Cell Communication

Cells of multicellular organisms communicate with each others using substances such as ions, hormones and neurotransmitters. Lipophilic agents, like steroid hormones, pass through the lipid bilayer of the cell membrane and bind to specific intracellular receptors triggering the appropriate cellular response.[1a] Other hydrophilic species, which cannot cross the cellular lipid bilayer deliver their message by binding to specific receptors located on the cell surface. This type of signal transduction, also called transmembrane signaling,[1b] depends on surface receptors. If the agonist binds a receptor linked to an ion channel, the opening of this channel can modify the ionic flux in or out of the cell. Variation in the intracellular ion concentration can then activate intracellular enzymes evoking an ultimate response to the stimulatory cell surface ligand. Other agents transmit the cellular message by binding to specific receptors of the tyrosine kinases present on the cell surface. Tyrosine kinases are enzymes embedded in the cell membrane, that recognize agonists binding on the outer surface and possessing an active site on the inner surface membrane. Upon extracellular signaling, they activate intracellular enzymes by phosphorylating the tyrosine residues of these proteins.[1c]

For many water-soluble hormones, the signal transduction system requires a receptor which is coupled to the production of an internal signaling molecule via an intracellular effector. In this mode of signaling, the surface receptor is associated with a membrane bound GTP-binding protein (G-protein).[2] The family of G-proteins includes several members which regulate different intracellular pathways. Upon agonist binding to the receptor, the activated specific G-protein stimulates or inhibits other membrane bound enzymes that act as amplifiers. This, in turn, generates second messengers inside the cell. Only a few second messengers have been identified so far. They include adenosine 3',5' cyclic monophosphate, guanosine 3',5' cyclic monophosphate, diacylglycerol, Ca^{2+} and finally *myo*-inositol 1,4,5-triphosphate (1,4,5-IP_3).[3]

The second messenger 1,4,5-IP_3 is formed simultaneously with diacylglycerol by hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI-4,5-P_2, Scheme 1) catalyzed by a specific phospholipase activated in response to various stimuli. Secondary messenger 1,4,5-IP_3 is responsible for the mobilization of Ca^{2+} from cellular storage.[4] Continuous efforts to isolate new second messengers are now leading toward another inositol derivative, *myo*-inositol 1-2 cyclic,4,5-triphosphate[5] but the cellular properties of *myo*-inositol 1,4,5-triphosphate as well as its biological pathways (Scheme 1) are still the main focus of many research groups.[6]

To be an efficient intracellular activator, 1,4,5-IP_3 must be chemically altered as soon as the activation by the outer surface agonist is terminated. The depletion of the second messenger should then result in the interruption of the intracellular final response. The degradation of 1,4,5-IP_3 involves an intricate cascade of phosphorylation and dephosphorylation steps, creating a wide variety of intermediates as shown in Scheme 1.[7] The proposed routes of 1,4,5-IP_3 degradation, describing the phosphatidylinositide (PI) cascade, either lead to agonist-insensitive, highly phosphorylated inositols[8] (IP_6, IP_7, and IP_8) or to free *myo*-inositol (MI), the primary building block of most of the inositides, phosphatidylinositols and phosphorylated inositols.[9] Many intermediates involved in this latter route are sensitive to extracellular effectors.



DG= diacylglycerol
PA= phosphatidic acid
CDP-DG= CDP-diacylglycerol
CDP= cytosine diphosphate

PI= phosphatidylinositol
P= phosphate
IP= inositol phosphate
c= cyclic

I= inositol
Px= x-phosphate
IPx= inositol x-phosphate

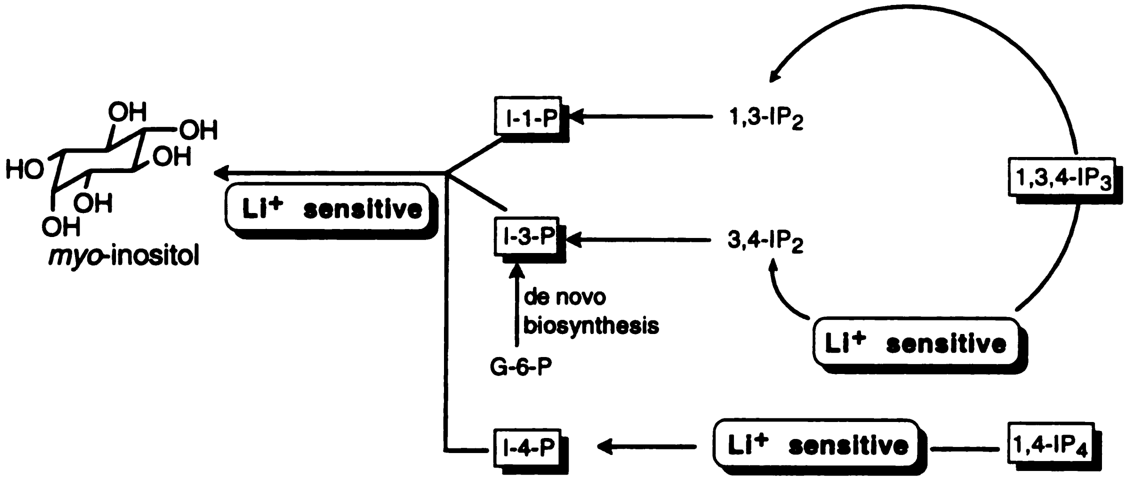
Scheme 1: PI cascade.[10]

Brain Cell Communication and Treatment of Mania by Lithium

In the brain, communication between neurons makes use of the signal transduction system involving 1,4,5-IP_3 and the PI cascade[11] and therefore requires suitable amounts of *myo*-inositol (MI). In most mammalian cells, MI is obtained from the diet. In brain cells, the major portion of MI is biosynthesized from D-glucose 6-phosphate since plasma MI does not effectively cross the blood-brain barrier.[12] The de novo synthesis of MI requires two independent enzymes, *myo*-inositol 1-phosphate (MIP) synthase which converts D-glucose 6-phosphate (G6P) into L-*myo*-inositol 1-phosphate (L-MIP or D-*myo*-inositol 3-phosphate) and MI monophosphatase which hydrolyzes L-MIP to MI and free inorganic phosphate.[13] The formation of L-*myo*-inositol 1-phosphate catalyzed by MIP synthase is the rate-limiting reaction in *myo*-inositol (MI) biosynthesis, since MIP monophosphatase is present in excess in all tissues.

Recently, the PI cascade has been associated with some manic disorders.[14] It is proposed that manic episodes are related to hyperactive neurons. Cellular signaling between hyperactive neurons and neighboring cells causes an increase in the turnover of the PI cascade. In the overstimulated neighboring cells, the constant resynthesis of the phosphatidylinositol 4,5-bisphosphate is required in order to maintain the intracellular level of 1,4,5-IP_3 as the primary response to the excess stimuli. The increased rate of hydrolysis of phosphatidylinositol 4,5-bisphosphate to 1,2-diacylglycerol and 1,4,5-IP_3 alters the electrical activity of the affected neurons. The abnormal electrical activity in the brain tissue surrounding the pathological cells is thought to result in manic episodes.

Lithium carbonate is used with variable success to treat manic disorder.[15] Its pharmaceutical value comes from its ability to inhibit various enzymes of the PI cascade, particularly MI monophosphatase. MI monophosphatase hydrolyzes de novo L-MIP as well as *myo*-inositol 4-phosphate and D-*myo*-inositol 1-phosphate (D-MIP).[9] MI monophosphatase is the sole enzyme responsible for the biosynthesis of free MI in brain cells. The inhibition of MI monophosphatase by Li^+ ions is unusual in that it is uncompetitive. Apparent inhibition constant K_i is 1 mM when DL-MIP is substrate and 0.26 mM when *myo*-inositol 4-phosphate is substrate.[16] Uncompetitive inhibition is characterized by the increase in inhibitor efficiency when the substrate concentration increases. This type of behavior allows selective inhibition of the MI monophosphatase in hyperactive neurones, since excessive 1,4,5-IP_3 turnover results in high *myo*-inositol monophosphates concentration. It has been reported that Li^+ traps a phosphoryl enzyme intermediate preventing subsequent nucleophilic attack by water.[17] *In vivo*, the inhibition of the PI cascade phosphatases by lithium results in the depletion of cellular MI.[18] The decrease in the MI availability to regenerate the phosphatidylinositol (4,5)-bisphosphate pool results in signal termination and provides control over manic episodes.[12b, 15] Effects of phosphatase inhibition are only observed in brain cells since no dietary extracellular MI can be pumped into the PI cascade.



Scheme 2: Li^+ inhibited enzymes.

Lithium Limitations and Alternative Approaches to Altering the PI Blockade

The established success of lithium as the therapeutic answer to mania is, however, not complete. A large quantity of lithium carbonate (> 2000 mg/day) is required to maintain an effective drug concentration at cellular level (0.5-1.2 mM), at the same time this therapeutic concentration is also relatively close to the toxicity level (3-5 mM) at which excessive dosage can be lethal.[19] In addition to MI monophosphatase, Li^+ inhibits other enzymes and causes accumulation of various metabolites with unknown and potentially harmful cellular functions. Along with the narrow therapeutic bracket of Li^+ dosages and its side effects, insensitivity to this drug has been observed in numerous patients.[19] In other cases, because of the required continual uptake, a decrease in the effectiveness of lithium to treat mania has been reported.[19]

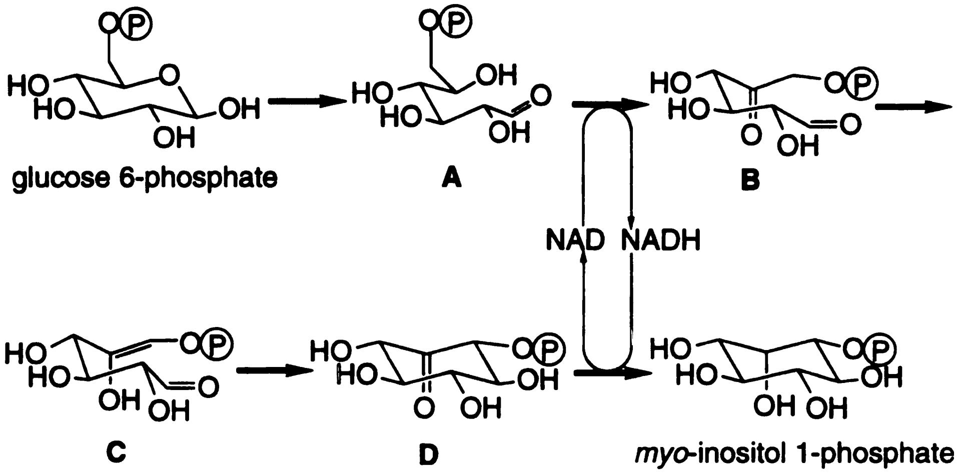
While most of the progress of the last ten years has been focused on the biological aspects of the PI signaling system,[6] current efforts are now directed towards selective interruption of this system.[20] MI monophosphatase has become a prime target in the search for new inhibitors of the PI cascade.[20, 21] A wide range of MI monophosphatase inhibitors have already been synthesized. An incomplete list includes deoxy-phosphoinositols, fluoro-phosphoinositols and polyphosphorylated inositols. However, the utility of these inhibitors in whole cell and *in vivo* studies is compromised by their lability towards ubiquitous non-specific phosphatases.

The three dimensional structure of MI monophosphatase, in conjunction with site-directed mutagenesis and kinetic studies, has recently led to identification of the active site and elucidation of the enzymatic mechanism.[22] These results should prove valuable in the development of novel inhibitors of MI monophosphatase. If the selective *in vivo* inhibition of the monophosphatase can be accomplished, the issues related to the accumulation of phosphorylated metabolites from the PI cascade and their side effects have yet to be considered. Problems associated with penetration of the blood-brain barrier by the inhibitor also remain to be addressed.

Disruption of the inositol cycle by inhibition of MIP synthase (the enzyme responsible for the de novo biosynthesis of MI) is an alternative approach. Inhibition of MIP synthase in the brain is also expected to reduce MI levels and may present pharmaceutical advantages. Alternatively, the ensuing reduced MIP levels may have very different neurological outcomes than that observed with lithium treatment and MI depletion. In this event, hypotheses for the role of MI concentrations controlling manic disorders would need adjustment. Upon inhibition of MIP synthase, accumulation of glucose 6-phosphate would be expected. However, under physiological conditions, D-glucose 6-phosphate is also the substrate of other intracellular enzymes[23] and therefore MIP synthase inhibition should not result in any substance accumulation. An initial basic and thorough understanding of MIP synthase and a methodical approach to inhibitor design are required to identify potent MIP synthase inhibitors. The scope of the present study is limited to the investigation of mechanistically challenging MIP synthase using strategically designed inhibitors. Neither the physiological effects of MIP synthase inhibition nor the *in vivo* behavior of the newly designed inhibitors have been considered during this investigation.

MIP Synthase: An NAD-Requiring Enzyme

MIP synthase (EC 5.5.1.4) catalyzes the isomerization of D-glucose 6-phosphate into L-MIP (Scheme 3).[13] In this transformation, the carbohydrate moiety is converted into a hexahydroxylated cyclohexane via an aldol condensation. MIP synthase requires *β*-nicotinamide adenine dinucleotide (NAD) to catalyze this reaction.[24] In most pyridinium nucleotide-dependent catalytic processes, either NAD or NADP is a cosubstrate. There is also a family of NAD-requiring enzymes where pyridine nucleotide plays the role of a catalytic prosthetic group.[24] These enzymes catalyze reactions such as epimerization, aldol reaction, cyclization, *α*, *β*-elimination, and decarboxylation, in which strong bonds with no obvious lability are cleaved. A transient oxidation of the appropriate substrate carbon along with the simultaneous reduction of the pyridinium nucleotide labilizes a scissile bond by introducing a carbonyl or an imine. The oxidized center is then reduced by the dihydropyridine nucleotide at a later step subsequent to the enzymatic transformation of the oxidized activated substrate. MIP synthase employs NAD as a prosthetic group which is neither consumed nor generated at the end of each catalytic cycle as shown in Scheme 3.



Scheme 3: MIP Synthase postulated mechanism.

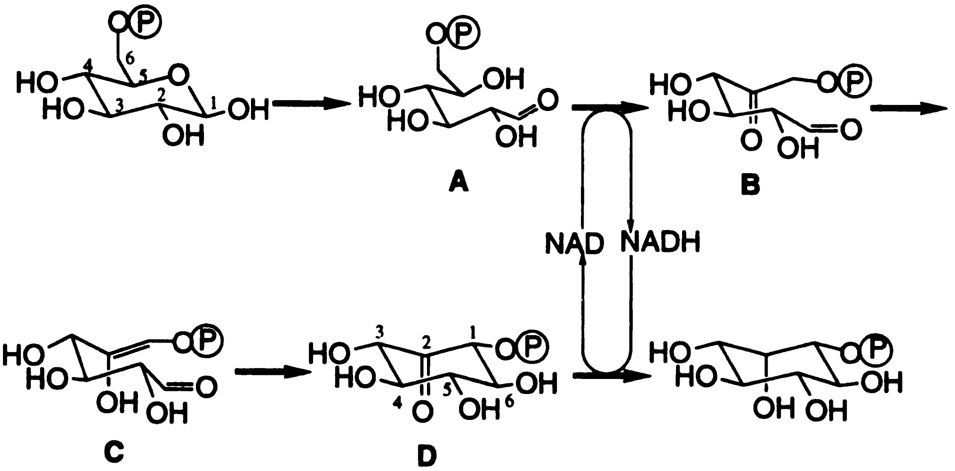
Loewus and Kelly were the first to propose that a cyclization mechanism involving the generation of a C-5 ketose prior to the formation of the C-C bond was the most likely pathway.[25] The mechanism of transformation seems to be the same regardless of the enzyme source. The enzyme-catalyzed reaction is proposed to begin with the binding of D-glucose 6-phosphate to the enzyme-NAD complex. The resulting open form **A** of glucose 6-phosphate is oxidized at the C-5 position with the simultaneous reduction of NAD to NADH and forms the xylo-hexos 5-ulose 6-phosphate **B**, in which the acidity of the *α* protons at C-6 is greatly increased. The subsequent enolization of **B**, also called 5-keto-glucose 6-phosphate, to intermediate **C** is followed by the aldol condensation reaction between C-6 and C-1. Intermediate **D**, *myo*-2-inosose 1-phosphate, is then reduced by the enzyme-bound NADH forming enzyme-bound NAD and MIP, which is subsequently released from the enzyme active site.

The reaction intermediates mentioned in this transformation have never been isolated and are presumed tightly bound to the enzyme active site. The difficulty in establishing the nature of the enzyme bound intermediate is not peculiar to this enzyme but is common to all enzymes of this NAD-requiring enzyme family. Evidence supporting the proposed mechanism and the involvement of the aforementioned intermediates is now presented.

MIP Synthase: A New Type of Aldolase

Some of the first experiments with MIP synthase were designed to categorize it as a type I or type II aldolase.[26] A type I aldolase is characterized by the formation of a Schiff base intermediate prior to the aldol condensation step. A type II aldolase requires the presence of a cation such as Co^{2+}, Zn^{2+} or Mg^{2+} to carry out the condensation. MIP synthase is competitively inhibited by classical aldolase type I inhibitors such as pyridoxal phosphate and trinitrobenzenesulfonic acid.[27] This inhibition ceases when the enzyme is preincubated with substrate and NAD. Hoffmann-Ostenhof isolated a ^{3}H-L-glucosyllysine residue when a MIP synthase reaction mixture was treated with [^{3}H]-$NaBH_4$, and thus presented MIP synthase as an aldolase type I.[28] Sherman,[29a] Eisenberg[29b] as well as Barnett,[30] however, found that the reduction of the reaction mixture with [^{3}H]-$NaBH_4$ did not furnish any lysyl derivative of glucose, regardless of the source of MIP synthase used. Another critical experiment using $H_2{}^{18}O$ showed that ^{18}O incorporation into the product was not the result of the hydrolysis of a Schiff base intermediate but came from the nonenzymatic exchange of the glucose aldehyde carbonyl with the medium.[31] These various observations established that MIP synthase could not be classified as a type I aldolase. However, MIP synthase was not inhibited by EDTA.[29b] Therefore, there is a possibility that MIP synthase does not utilize either the class I or the class II mechanism.

The understanding of the stereochemistry of conversion of D-glucose 6-phosphate into *myo*-inositol 1-phosphate has come about largely as a result of tracer experiments where ^{14}C-labeled D-glucose 6-phosphate in position C-1,[32] C-2,[33] or C-6[25] was converted to L-*myo*-inositol 1-phosphate with ^{14}C in position C-6, C-5 or C-1 respectively. It should be noted that the ring numbering in L-*myo*-inositol 1-phosphate is inversely related to the assignment in the carbon chain of the substrate. This important result proved that the phosphate of D-glucose 6-phosphate did not migrate during the transformation. Tritium labeling at C-2, C-3, C-4, C-5 or C-6 showed that the tritium at C-2, C-3 or C-4 had no effect on the rate of the reaction and therefore was not involved chemically in the conversion.[34] The tritiums at C-5[34] and C-6,[35,36] however, were responsible for kinetic isotopic effects. Oxidation of the C-5 hydroxyl and C5-C6 enolization are therefore partially rate determining.

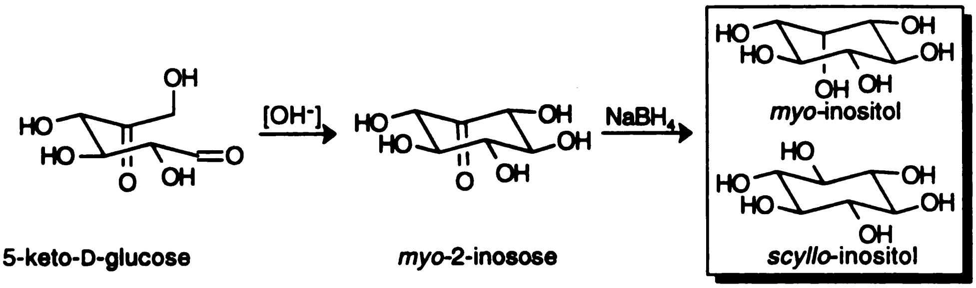


Scheme 4: MIP Synthase mechanism with labeled carbons.

When Barnett reconstituted *apo*-MIP synthase (enzyme lacking the cofactor) with [4-^{3}H]-NADH and incubated it with chemically prepared intermediate **B**,[30] radiolabeled glucose 6-phosphate along with traces of radiolabeled L-MIP were obtained. Jenness showed that the same *pro-S* proton of NADH was responsible for the reduction of intermediate **B** back to D-glucose 6-phosphate and for the reduction of intermediate **D** to L-MIP.[37] Therefore the reduction and oxidation of the cofactor occurs on the same side of its nicotinamide ring. Sherman corroborated the reversibility of the oxidation step by observing deuterium incorporation at the C-5 position of glucose 6-phosphate when substrate, enzyme and cofactor were incubated in D_2O medium.[38] Consequently, the reversibility of the oxidation step was established along with the evidence that the C-5 ketose **B** was a reaction intermediate. Sherman also demonstrated that the enolization step was reversible by establishing the incorporation of deuterium (11.5%) at the C-6 position of recovered D-glucose 6-phosphate.[38]

Sherman attempted to prove that all reaction intermediates were enzyme-bound. He simultaneously incubated D-glucose 6-phosphate and fully deuterated D-glucose 6-phosphate in the presence of MIP synthase and cofactor and found no detectable proton-deuterium exchange.[39] He deduced that the entire sequence of transformations from D-glucose 6-phosphate to L-MIP must occur in the enzyme active site. However, Jenness observed tritium incorporation into glucose 6-phosphate when a reaction mixture containing MIP synthase, substrate and NAD was incubated in the presence of [^{3}H]-NADH.[37] Jenness proposed that this incorporation was due to the exchange of NADH formed during the catalytic cycle in the enzyme active site with the [^{3}H]-NADH present in solution. This last experiment showed that reaction intermediates were not as tightly bound to MIP synthase as Sherman proposed. Mass spectroscopy of derivatized incubation products was the mode of detection used in Sherman's experiment, while direct detection of radioactive material on chromatography paper was utilized by Jenness. Such a difference in the sensitivity of the detection method might account for the difference in result and conclusion.

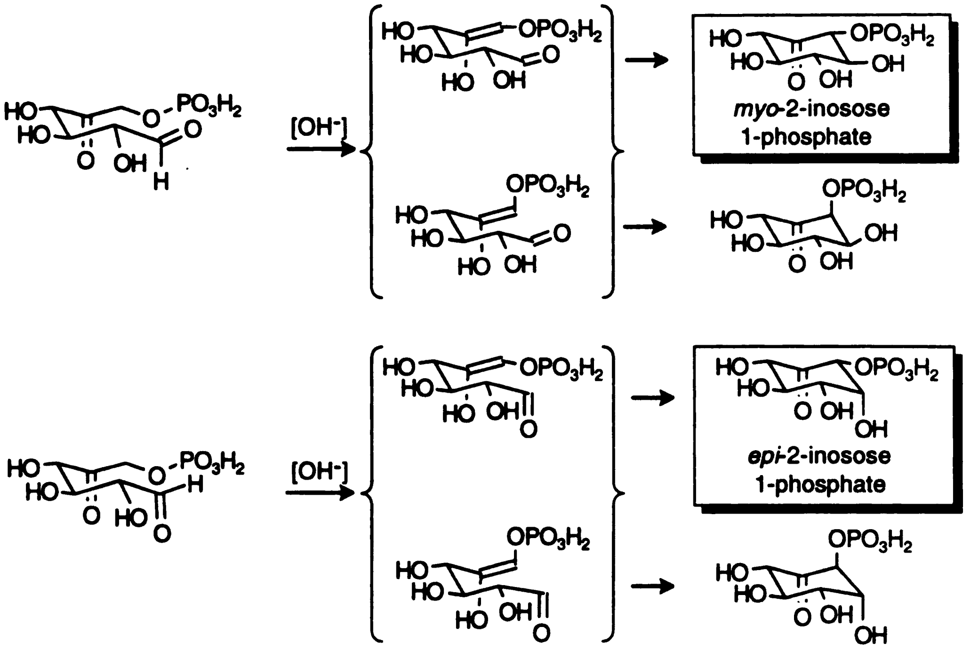
Kiely and Fletcher had shown that 5-keto-D-glucose was readily converted by dilute alkali to *myo*-2-inosose (Scheme 5).[40] The inosose was identified upon reduction to *myo*- and *scyllo*-inositols with $NaBH_4$ and GC/MS analysis of the fully silylated derivatives.



Scheme 5: Products of base catalysis of 5-keto-D-glucose cyclization.

Ketose **B**[41] (Scheme 6) was demonstrated by Sherman to undergo the same reaction giving, after reduction of the base-cyclized inosose phosphate, two major products identified as the *epi*-inositol 3-phosphate and D-*myo*-inositol 3-phosphate. The inososes were *epi*-2-inosose 1-phosphate and *myo*-2-inosose 1-phosphate respectively (Scheme 6).[42] Along with the two major products, other inositol phosphate species such as *scyllo*-inositol phosphate and *myo*-inositol 2-phophate were also detected.

MIP synthase might be responsible for the same type of base-catalyzed cyclization where the appropriate activation of the substrate is carried out by the enzyme oxido-reductase function. Loewus showed that only the C-6 *pro-R* hydrogen was lost during turnover and that the *cis*-enol **C** resulted directly from this selective removal.[43] The difference in stereoselectivity observed between base catalyzed cyclization and MIP synthase catalyzed glucose 6-phosphate cyclization clearly indicates the strong involvement of the enzyme active site during aldol condensation. Enzymatic-base-catalyzed enolization must be stereocontrolled and the orientation of the newly formed alcohol of *myo*-2-inosose 1-phosphate must be under strict enzymatic control.



Scheme 6: Products of base catalysis of intermediate **B** cyclization .

Eisenberg[44] attempted to establish the presence of *myo*-2-inosose 1-phosphate in MIP synthase active site during turnover by quenching the reaction mixture containing enzyme, NAD and glucose 6-phosphate with $[^3H]$-$NaBH_4$. ^3H-*scyllo*-Inositol 1-phosphate was then identified by paper chromatography. Sherman, Eisenberg, Barnett and others proposed that MIP synthase belongs to a new class of aldolase in which the conversion is catalyzed by a general base present in the enzyme active site.

Known Inhibitors of MIP Synthase: Substrate Analogues

Important advances have been made towards the understanding of the MIP synthase mechanism. However, little progress has been accomplished in designing inhibitors. The currently known inhibitors of MIP synthase are substrate analogues that are competitive inhibitors of variable potency. Table 1 constitutes a summary of the molecules that have been tested for MIP synthase inhibition. Intermediate **B**,[36,41] which was synthesized for mechanistic purposes, was never tested for inhibition despite being a reaction intermediate, and therefore likely to have high affinity for the active site. The most potent inhibitors were discovered by Barnett when he observed that glucitol 6-phosphate **8** inhibited MIP synthase at a micromolar level.[49] At the same time, the 2-deoxy substrate analogues, **3** and **9**, were observed to be also potent inhibitors.[49] The reduced form of the glucose 6-phosphate analogues were better inhibitors of MIP synthase than their oxidized precursors. These observations suggested that the open chain form of glucose 6-phosphate may bind to the enzyme before oxidation at C-5 occurs.[49] Mannose 6-phosphate, **5**, the epimer of glucose 6-phosphate at C-2, 2-deoxy-D-glucose 6-phosphate, **3** and 2-deoxy-2-fluoro-D-glucose 6-phosphate **4** are also substrates of MIP synthase, indicating that modifications at C-2 do not prevent binding.[46] The rate of conversion is much slower and impedes the sugar phosphates from being good substrates. Modifications at C-3 or C-4 result in complete loss of affinity.[46]

Table 1: Known inhibitors of MIP synthase.

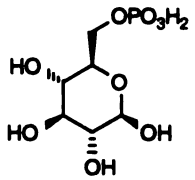
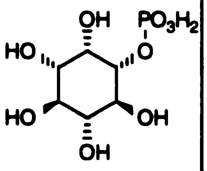
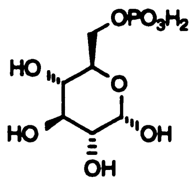
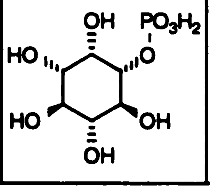
Structure	Source of MIP synt.	Inhi-bitor	K_i (mM)	Sub-strate	K_m (mM)	Structure of product	code	refe-rence
	-Bovine testis -Rat testis -Yeast - Pine pollen	-	-	yes	2.700 0.500 1.120 0.330		1	46 49 50 51
	-Bovine testis	yes	?	?	?		2	46

Table 1 (cont'd)

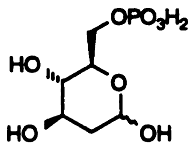
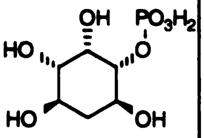
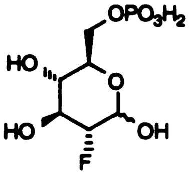
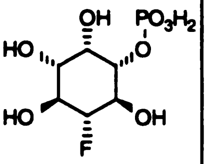
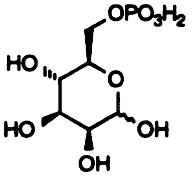
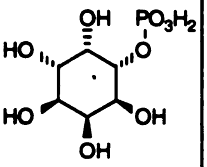
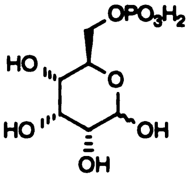
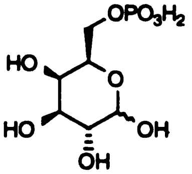
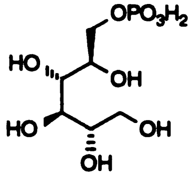
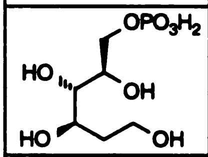
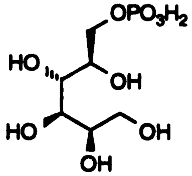
Structure	Source of MIP synt.	Inhibitor	K_i (mM)	Substrate	K_m (mM)	Structure of product	code	reference
	-Bovine testis -Rat testis -Pine pollen -yeast	yes	0.350 0.016 0.060 0.030	yes no - -	.230 - - -		3	49 46 50 51
	-Bovine testis	yes	0.330	yes	?		4	46
	-Bovine testis	yes	8.400	yes	5.800		5	3 46
	-Bovine testis	no	-	no	-		6	46
	-Bovine testis	no	-	no	-		7	46
	-Rat testis -Yeast	yes	0.340 0.250	-	-		8	49 50
	-Rat testis	yes	0.014	-	-		9	49
	-Rat testis	yes	0.120	-	-		10	49

Table 1 (cont'd)

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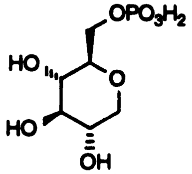
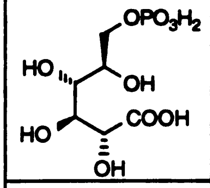
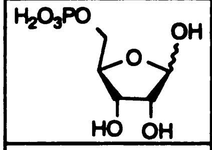
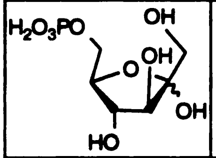
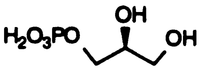
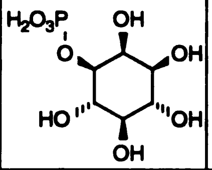
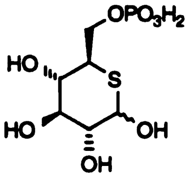
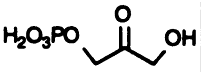
	-Rat testis	yes	>10.0	-	-		11	49
	-Rat testis	yes	3.300	-	-		12	49
	-Rat testis	no	-	-	-		13	49
	-Rat testis	yes	3.500	-	-		14	49
	-Rat testis	no	-	-	-		15	49
	-Bovine testis	no	-	-	-		16	46
		yes	0.330	-	-		17	52^a
							18	52^b

Remaining Challenges of MIP Synthase Mechanism

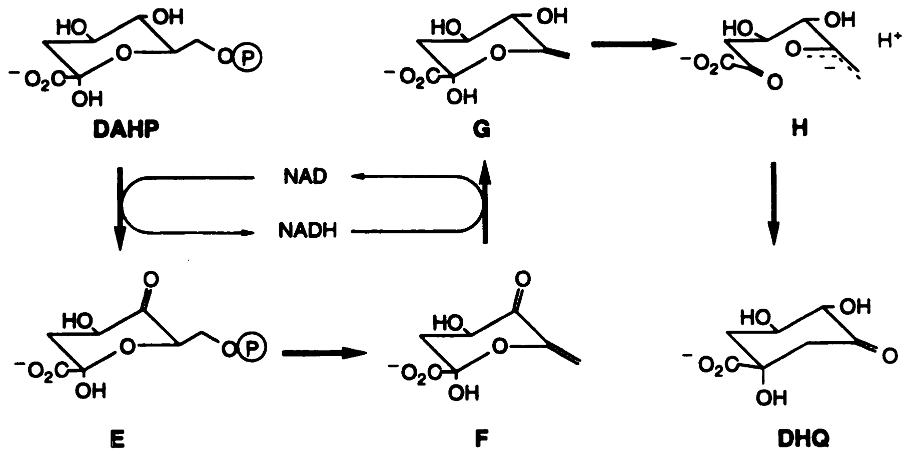
Although the mechanism shown in Scheme 3 accounts for the available data, a number of mechanistic questions remain. MIP Synthase obtained from various sources has been isolated with different amounts of bound-NAD, raising the question of which is the native form. MIP Synthase is usually isolated as a multimer containing 2, 3 or 4 identical subunits.[24] The number of NAD and substrate molecules bound to the enzyme during each catalytic conversion has not been determined. The conventional methods developed for other NAD-requiring enzymes, such as DHQ synthase,[45] cannot be applied to MIP synthase study since NAD falls off the active site by simple dialysis. NAD might be a tightly-bound prosthetic group which remains in the active site from one catalysis to the next, or the substrate and the cofactor might bind to the enzyme at the beginning of each turnover in either random or specific order.

It has been postulated that the β-anomer of glucose 6-phosphate is the substrate of MIP synthase and that the enzyme catalyzes the pyranose ring opening.[25] Sherman indicated that the β-anomer of glucose 6-phosphate maintained in pure anomeric form was converted to L-MIP while the presence of α -anomer decreased the catalytic rate.[46] The α -anomer was then described as an inhibitor of MIP synthase, however its aptitude as a substrate could not be disproved. If the α -anomer is a substrate, then the conversion calls for a rotation about the C1-C2 bond axis of the open form to afford the required stereochemistry at C-6 in the product. Based on similar considerations, MIP synthase might bind the appropriate acyclic form directly to avoid any ring opening step or potential rotation. This hypothesis is challenged by the low concentration of the acyclic form in solution (less than 0.4% of the total glucose 6-phosphate).[47]

Apart from the indirect indications obtained independently by Sherman[42] (chemical), Barnett[36] (enzymatic) and Eisenberg[44] (enzymatic) concerning the likelihood of intermediate **C** and *myo*-2-inosose 1-phosphate **D** as reaction intermediates, direct evidence of their participation in the enzymatic turnover is missing. Another controversy involves the enolization step of intermediate **B** to intermediate **C** which can either be catalyzed by the enzyme or by the phosphate moiety of the intermediate. At physiological pH, a dianionic phosphate ester is one of the strongest bases available. The phosphate group could, either solely or in conjunction with a base, catalyze the enolization-condensation step. This type of intramolecular catalysis has been extensively studied for DHQ (3-dehydroquinate) synthase, another NAD requiring enzyme.[48] DHQ synthase converts its substrate, in the presence of NAD, into product via successive oxidation, phosphate elimination, reduction and cyclization.[24] The removal of the phosphate moiety can be catalyzed by a base or by the phosphate itself. Various experimental results supported by strong steric and electrostatic considerations favored the self-catalyzed elimination hypothesis.[48b,c]

Strategy Developed for DHQ Synthase Applied to MIP Synthase

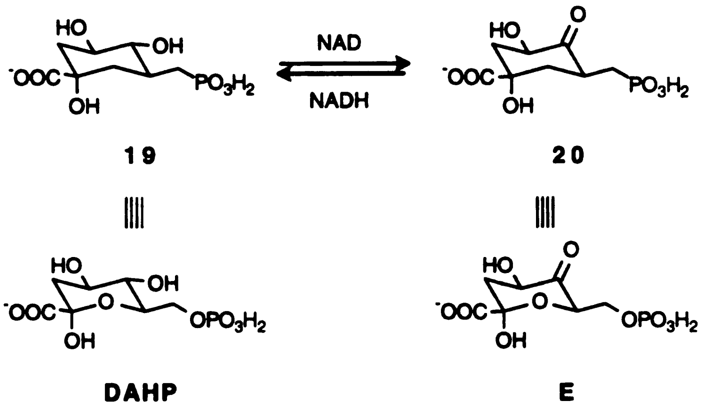
MIP synthase belongs to the same NAD-requiring enzyme family that uses NAD as a cofactor rather than as a substrate, as DHQ synthase.[24] Unlike MIP synthase, DHQ synthase has attracted considerable attention. Knowles and other groups have established the amount of NAD and metal cation such as Co^{2+} per enzyme,[45] along with important mechanistic features.[48a] This synthase catalyzes the conversion of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) into DHQ (3-dehydroquinate), precursor of phenylalanine, tyrosine, tryptophan and vitamins, via successive oxidation, elimination, reduction and condensation steps (Scheme 7). Because of its role in the aromatic amino acid biosynthesis,[53] this enzyme has become a tool in the discovery of new herbicides.[54] The unusually complex transformation that DHQ synthase catalyzes has made this synthase the paradigm of enzymes challenged by mechanism-based inhibitors.



Scheme 7: DHQ Synthase mechanism.

Various methods have been developed to inhibit DHQ synthase in vitro as well as in vivo.[54] These include substrate analogues,[48a,c,55] intermediate analogues[56] and transition state analogues[48b] based approaches. One strategy was to inhibit DHQ synthase with substrate analogues such as carbocyclic phosphonate analogues[48a,c] (for example: Scheme 8, **19**) which could be oxidized in the active site by NAD to an analogue of intermediate **E** but could not be processed further in the reaction.. The resulting tightly bound inhibitor-NADH-enzyme complex was stable. This type of inhibitor often leads to slowly reversible inhibition. It was proposed by Knowles that the type of inhibition depended on the inability of the enzyme to reduce the oxidized intermediate analogue back to the substrate analogue. However, the carbocyclic **DHAP** analogue missing the oxidizable C-5 hydroxyl group was also a slowly reversible inhibitor. This last result implied that slowly reversible inhibition was not solely due to the stability of the bound oxidized inhibitor-NADH-enzyme complex. An alternative strategy was to design inhibitors via a mechanism-based approach using the specific transient oxidation characteristic of the conversion. The reaction intermediate **E** is tightly bound to the NADH-bound active site of the enzyme. Challenging NAD-bound DHQ synthase with preoxidized intermediate

analogues of E, such as a carbocyclic ketophosphonate analogue[56] (Scheme 8, 20), resulted in very efficient apparent irreversible inhibition of the enzyme since DHQ synthase was unable to process the inhibitors.



Scheme 8: Mechanism-based inhibitors of DHQ synthase.

Synthesis of structural mimics of both putative reaction intermediates and transition states found along the enzyme reaction coordinate has proven to be a highly effective strategy for the discovery of potent enzyme inhibitors. The unique enzymology[56] associated with the inhibition of DHQ synthase by ketocarbaphosphonate **20** suggests a general strategy for irreversibly inhibiting enzyme that exploits nicotinamide adenine dinucleotide as a catalyst rather than a cosubstrate. This approach can be applied to the design of MIP synthase inhibitors. Three reaction intermediates **B**, **C** and **D** (Scheme 3) containing a carbonyl moiety and present in the MIP synthase active site at the same time as NADH are thought to be involved in the conversion of glucose 6-phosphate to L-MIP. These putative intermediates could be used as fundamental structures to develop a mechanism-based strategy for MIP synthase inhibition.

CHAPTER II

GENERAL METHODS DEVELOPED FOR ENZYME PRODUCTION AND SYNTHESIS OF INHIBITORS

PART I: ENZYME PRODUCTION

Purification of *myo*-Inositol 1-Phosphate Synthase from *S. cerevisiae*

MIP Synthase was first purified to homogeneity from bovine testis[38] and was soon after isolated from rat testis,[29a] pine pollen,[51] *Neurospora crassa*,[57] *Streptomyces griseus*[58] and *Saccharomyces cerevisiae*.[50] MIP monophosphatase can be isolated from the same organisms but rat and bovine brain are the sources of choice for homogeneous enzyme production.[59] All MIP synthases isolated to date are NAD dependent and have similar pH and temperature optima. They are all inhibited in a similar manner by known inhibitors such as substrate analogues (Table 1). This suggests that studies performed on MIP synthase isolated from one species are applicable to MIP synthases isolated from other sources.

Potential inhibitors synthesized during the course of this study were tested using MIP synthase isolated from *S. cerevisiae*. Enzyme purification would not be limited by availability of starting material since *S. cerevisiae* could readily be grown in the laboratory in multigram quantities. In contrast, this may have not been the case if MIP synthase had been purified from rat or bovine testis. Professor S. Henry kindly provided us with *S. cerevisiae* MW5.55,[60] a strain that produced amplified levels of MIP synthase due to the multiple copies of MIP synthase encoding gene, INO1 gene. Henry had purified MIP synthase from a wild type strain of *S. cerevisiae*.[50a] Modification of this procedure afforded homogeneous MIP synthase from MW5.55.[61]

Table 2: Purification of MIP synthase from *S. cerevisiae* MW5.55.

	total units[a]	specific activity[b]	x-fold purification	yield %
DEAE column	1.7[c]	0.025	1.0	100
Bio-Gel A column	1.7	0.035	1.4	102
HPLC DEAE column	1.5	0.220	8.8	92

[a]unit= 1 μmol of MIP formed per min at 37°C. [b]units/mg. [c]Prior to purification on DEAE column, the crude cell lysate was subjected to a streptomycin sulfate precipitation and an ammonium sulfate fractionation.

Fractions containing MIP synthase activity during purification of the enzyme were assayed following a procedure described by Henry.[50] MIP Synthase was incubated in the assay solution containing Tris-HCl, NH_4Cl and dithiothreitol at pH 7.2, with the appropriate concentrations of NAD and glucose 6-phosphate (G6P) at 37°C. In the case of samples prepared for column assay, a TCA solution (20%, w/v) was added to the reaction mixture after 1h of incubation. When kinetic assays were performed, aliquots were removed from the incubation solution at appropriate time points and added to a TCA solution (20%, w/v). The samples were then treated with aqueous $NaIO_4$ (0.2 N).[62] Sodium periodate oxidizes MIP formed during the reaction and releases inorganic phosphate while substrate glucose 6-phosphate remains almost inert to this treatment. The selectivity results from the rapid oxidation of *cis*-hydroxyl groups present in MIP as well as the stability of the glucose 6-phosphate oxidation product. The reaction mixtures were then quenched with aqueous Na_2SO_3. The amount of released inorganic phosphate was then determined by colorimetric assay. Assays of column fractions used a rapid and sensitive procedure employing malachite green and ammonium molybdate.[63] This method is very sensitive and thus useful for qualitative measurements. However, absorbance of assay solution varied with time and could not be used for experiments where accurate readings were required. Kinetic assays employed ascorbic acid[64] instead of malachite green to provide reliable spectrometric readings.

Even with overexpression of MIP synthase, enzyme activity could not be reliably assayed either in crude cell lysate or lysate after ammonium sulfate fractionation. Measurement of enzyme activity was based on quantitation of the inorganic phosphate selectively released from MIP during oxidation with sodium periodate. Phosphatase-mediated hydrolysis of substrate, D-glucose 6-phosphate, was likely responsible for the high background level of inorganic phosphate generation. This interfering enzyme activity was removed upon gradient elution from a DEAE cellulose column. Homogeneous enzyme was obtained after size exclusion chromatographic purification on a Bio-Gel A column followed by HPLC purification on a DEAE column. Enzyme homogeneity was confirmed by SDS-PAGE (Scheme 9).[137] The molecular weight of MIP synthase was determined to be 230± 20 kD by size exclusion HPLC and 240 kD by cross-linking using dimethyl suberimidate followed by SDS-PAGE under denaturating conditions.[138] Electrospray mass spectroscopy established the monomer molecular weight to be 61.919 ± 21 while size exclusion HPLC set the monomer molecular weight at 64.4 kD. Measured Michaelis constants (K_m) for D-glucose 6-phosphate and NAD were 1.2 mM and 17 μM, respectively.

Purification of *myo*-Inositol 1-Phosphate Synthase from *E. coli*

Although the purification of MIP synthase from *S. cerevisiae* resulted in homogeneous protein, 4 L of culture producing 60 g of wet cells yielded less than 2 units of activity corresponding to 10 mg of protein. In order to increase the availability of MIP synthase, Dr. D. Spear prepared a strain of *E. coli* that overexpressed the *S. cerevisiae* MIP synthase. Enzyme expression in *E. coli* was accomplished by using a T7 RNA polymerase expression system.

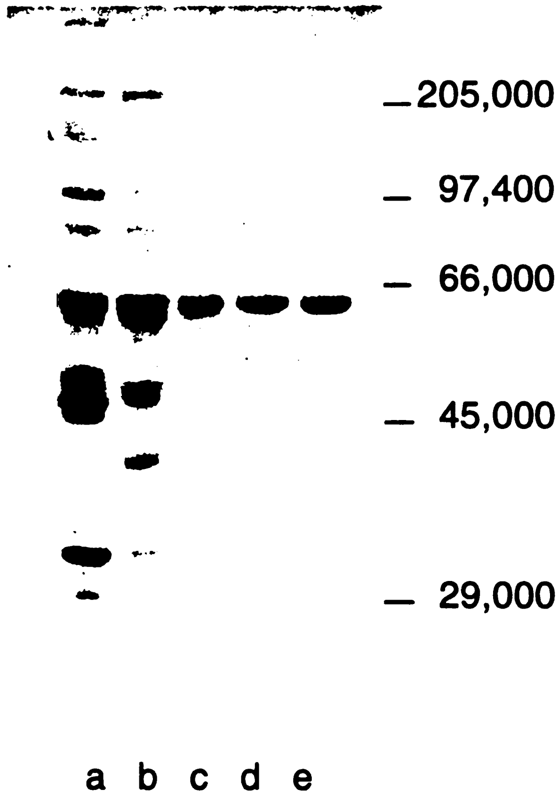
The *S. cerevisiae* INO1 gene, obtained from Prof. S. A. Henry, was removed from the original vector using restriction enzymes, reduced to the encoding open-reading frame by PCR and finally inserted in a plasmid containing T7 RNA polymerase promoter located a few bases upstream from the insertion site. The resulting vector pT7-7-MIPsynt was then used to transform *E. coli* BL21 (DE3), a strain that contains a chromosomal copy of IPTG inducible T7 RNA polymerase gene. IPTG binds to the repressor of T7 RNA polymerase gene freeing the promoter which initiates T7 RNA polymerase gene transcription. T7 RNA polymerase then binds to the T7 RNA polymerase promoter located on the plasmid and initiates the transcription of MIP synthase gene. In an ampicilin containing LB medium, the cells BL21 (DE3)/ pT7-7 MIPsynt were grown to reach a cell concentration corresponding to an absorbance of OD_{600nm}= 0.6-0.7, in the absence of IPTG. Then, the production of T7 RNA polymerase was induced by addition of IPTG (50 mg/L) which initiated the production of MIP synthase.

Table 3: Purification of MIP synthase isolated from *E. coli* BL21 (DE3)/ pT7-7 MIPsynt.

	total units[a]	specific activity[b]	x-fold purification	yield %
Crude lysate	5.6	0.073	1.00	100
Ammonium sulfate precipitation	3.9	0.092	1.26	70
DEAE column	4.0	0.241	3.30	71

[a]unit= 1 µmol of MIP formed per min at 37°C. [b]units/mg.

The initial steps of enzyme purification from *E. coli* were identical to those required for the purification of MIP synthase from *S. cerevisiae*, however the procedure was greatly simplified since the enzyme was homogeneous after the first DEAE column (Table 3). Enzyme homogeneity was confirmed by SDS-PAGE. MIP synthase isolated from *E. coli* and MIP synthase isolated from *S. cerevisiae* coeluted when coinjected on size exclusion HPLC column and displayed identical K_m. Size exclusion chromatography provides information on the molecular weight of large molecules under non-denaturating conditions. Although the enzyme activity remained low, the ease of this purification made *E. coli* MIP synthase suitable for kinetic purposes. A 1 L growth produced 5 g of wet cells and resulted in the production of 4.0 units of MIP synthase (20 mg of protein).



Scheme 9: MIP Synthase SDS-PAGE.

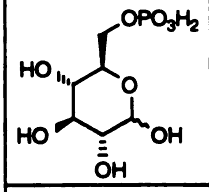
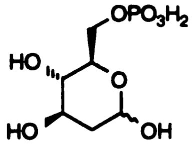
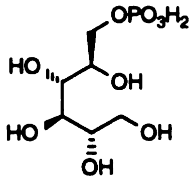
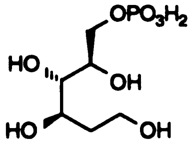
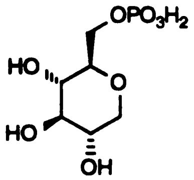
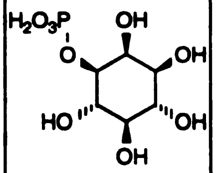
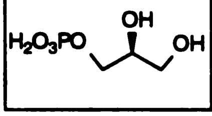
Limitation of MIP Synthase Production

The SDS-PAGE of BL21 (DE3)/ pT7-7 MIPsynt cell lysate showed a very wide band at 62 kD, corresponding to the MIP synthase monomer. The T7 expression system clearly overexpressed MIP synthase, although, the total activity of *E. coli* MIP synthase remained comparable to the enzyme activity obtained from the *S. cerevisiae*. Low enzyme activity could be related to an excessive expression of the MIP synthase gene. Formation of inactive protein aggregates may be responsible for the lower than expected enzyme activity. Inactive proteins could also originate from improper folding of the translation product. Chaperone enzymes, present in MW5.55 while absent from *E. coli*, could be involved in the folding of the tetrameric form of MIP synthase.

Known inhibitors of MIP synthase

Our initial investigations were performed using MIP synthase isolated from MW5.55. Therefore, consistency required that MIP synthase isolated from the same source be used for all the subsequent enzymatic studies. The inhibition experiments with 2-deoxy-glucose 6-phosphate **3** and 2-deoxy-glucitol 6-phosphate **9** carried out by Henry were repeated and inhibition constants (K_i) were calculated. Along with these two potent inhibitors, other substrate analogues were tested (Table 4). Competitive inhibition was observed for **3**, **8**, **9** and 1,5-anhydro-glucitol 6-phosphate **11**. The product, MIP **16**, only inhibited MIP synthase when present in millimolar concentration higher than 10 mM while glycerol 1-phosphate **15** did not have any effect on MIP synthase activity.

Table 4: MIP synthase inhibitors.

Structure	Substrate	Inhibitor	K_i (mM)	K_m (mM)	Abbr.	code
	yes	–		1.200	G6P	1,2
	–	yes	0.009			3
	–	yes	0.154			8
	–	yes	0.002			9
	–	yes	0.500			11
	–	no	I_{50}= 7.600		MIP	16
	–	no	–			15

Purification of MIP monophosphatase from *E. coli*

Continuous assay is the most efficient technique to obtain kinetic information about an enzymatic reaction. In this type of assay, the variation of concentration of a species present in the incubation mixture is followed spectroscopically over time. MIP Synthase does not produce spectroscopically detectable material, however a continuous coupled enzyme assay could be envisioned. In such an assay, a second enzyme present in the incubation mixture converts a product of the first enzymatic reaction into a spectroscopically detectable material. MIP monophosphatase could be used to develop an assay in which other enzymes such as *myo*-inositol dehydrogenase would be required to produce an UV active material. More simply, MI monophosphatase could be used in a coupled enzyme assay where MIP would be enzymatically hydrolyzed. This coupled enzyme assay would remove steps from the analysis since the periodate oxidation step would no longer be required for the inorganic phosphate release.

Table 5: Purification of MIP monophosphatase from *E. coli* BL21 (DE3)/ pBIMP.

	total units[a]	specific activity[b]	x-fold purification	yield %
Crude lysate	11.0	0.405	1.00	100
Heat treatment	13.3	0.709	1.75	121
Resource Q HPLC column	9.8	1.200	2.96	89

[a]unit= 1 µmol of MIP formed per min at 37°C. [b]units/mg.

MIP Monophosphatase was purified from *E. coli* BL21 (DE3)/ pBIMP which was provided by Dr. R. E. Diehls. *E. coli* BL21 (DE3)/ pBIMP expresses the bovine brain MIP monophosphatase from a T7- RNA polymerase promoter system.[66] Purification of

MIP monophosphatase from crude lysate utilized a heat-denaturating step followed by anion exchange chromatography on an HPLC chromatography and yielded homogenous MIP monophosphatase (Table 5).

As expected, the phosphatase has an absolute requirement for Mg^{2+}. The Michaelis constant K_m for DL-MIP has been determined to be 160 μM. DL-Glycerol 1-phosphate and dihydroxyacetone phosphate inhibit MIP monophosphatase but are also substrates.

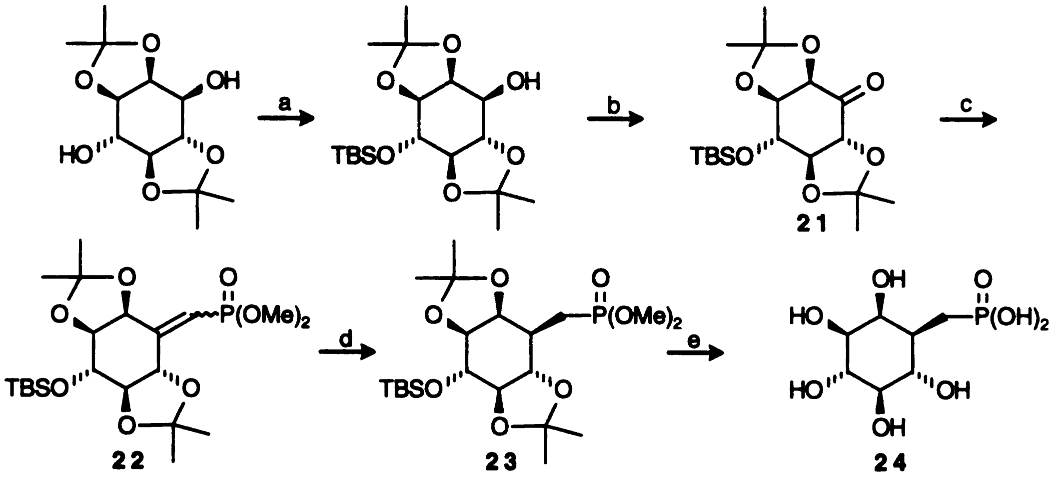
PART II: NUCLEOPHILIC EPOXIDE RING OPENING

Background

Phosphonates have found widespread use as isosteric analogues of biologically active phosphates.[67] Since phosphonates lack the scissile P-O bond, the main advantage gained over the corresponding phosphates, in enzymatic studies, is the stability towards the action of the phosphatases. As substrate or reaction intermediate analogues, they also have the advantage of being stable towards cyclization, migration and elimination. In many instances, their phosphate parents are so unstable that they cannot be isolated, much less synthesized. Therefore, since phosphonates are more manageable species, they have been under considerable scrutiny as potential enzyme inhibitors. Phosphonate characteristics have been advantageously used in the design of DHQ synthase inhibitors.[48,55,56] A trend observed in DHQ synthase inhibition has been that the phosphonate species bind the enzyme active site tighter than the phosphate counterparts. For example, the carbocyclic nonisosteric phosphonate analogue (**19**, Scheme 8) of DAHP ($K_m \approx$ 1-10 μM) is a potent (K_i= 5.4 nM, k_{on}= 1.4 x 10^5 $M^{-1}s^{-1}$, k_{off}= 7.5 x 10^{-4} s^{-1}), slowly-reversible inhibitor of DHQ synthase[48c] while the nonisosteric phosphate analogue equivalent is a weak competitive inhibitor (K_i= 2.75 μM).[48] The strategy developed in designing MIP synthase inhibitors is based on DHQ synthase inhibition studies. Therefore, phosphonate analogues of glucose 6-phosphate (**1** or **2**) derivatives and of intermediates **B**, **C** and **D** (Scheme 3) analogues must be considered as potential inhibitors of MIP synthase. Devising flexible routes to synthesize these species has become essential.

Many methods are available to introduce phosphonate moieties on linear carbon chains.[67] However, methods to obtain methylphosphonate derivatives of a cyclohexyl ring are not as commun. The literature is rich in examples where organometallics such as cuprates, Grignard reagents and organolithium compounds have been used to nucleophilically open epoxides. Only few of these reagents were successfully applied to the synthesis of phosphonate derivatives by nucleophilic opening of epoxides. One step synthesis of sterically hindered phosphonomethylated species by introduction of a phosphonomethyl moiety onto a sterically hindered cyclohexene oxide derivative has never been examined.[68]

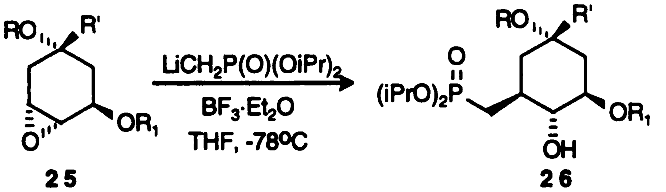
Kulagowski had synthesized (±) *myo*-inositol 1-phosphonate using Horner-Emmons methodology (Scheme 10).[69] The key intermediate was a derivative of *myo*-1-inosose **21** synthesized in five steps from *myo*-inositol. In our study, molecules designed as potential MIP synthase inhibitors required modification at the C-1 and C-2 position of *myo*-inositol. The intermediates presented in this synthesis do not offer simple access to the C-2 position. For this reason, this strategy could not be efficiently applied to the synthesis of the phosphonate series of putative MIP synthase inhibitors.



(a) (i) NaH, BnBr, DMF, 25°C, 45%, (ii) DBU, TBSCl, DMF, 60°C, 99%, (iii) $Pd(OH)_2$, 50 psi, H_2, EtOH, NEt_3, 25°C, 98%. (b) NCS, Me_2S, Toluene, -23°C, NEt_3, 83%. (c) n-BuLi, $(MeO)_2(O)PCH_2P(O)(OMe)_2$, THF, 73%. (d) PtO_2, 1 atm, H_2, EtOAc, 25°C, 99%. (e) (i) TMSBr, CH_2Cl_2, 25°C. (ii) 2:1 CF_3COOH/H_2O, 25°C, 78% from alkene.

Scheme 10: Kulagowski 's synthesis of (±) *myo*-inositol 1-phosphonate.

A strategy employing the direct phosphonomethylation of a cyclohexene oxide had been successfully developed in this laboratory by J-L. Montchamp for the synthesis of phosphonate inhibitors of DHQ synthase.[48,55c,56] Cyclohexene oxides **25** (Scheme 11) derived from quinic acid were treated at -78°C in THF with equimolar amounts of diisopropyl lithiomethylphosphonate and boron trifluoroetherate.[48a] Ring opening proceeded with high regioselectivity and overall yield. Use of nucleophile/Lewis acid combination for assisted nucleophilic addition onto oxiranes was first decribed by Ganem.[70] Shortly after this first successful phosphonomethylation of an hindered cyclohexene oxide was accomplished,[48a] a report appeared which described the utilization of this reagent in phosphonomethylation of oxetanes and more recently another review presented its use in phosphonomethylation of acyclic epoxides.[71]



25 **26**

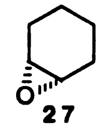
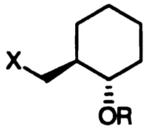
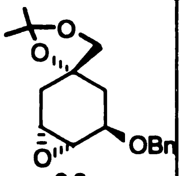
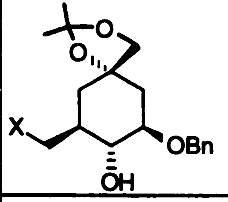
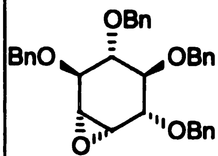
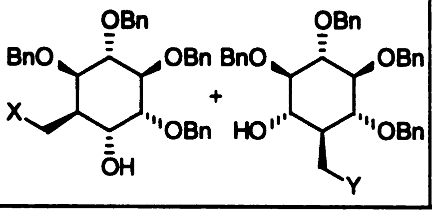
Scheme 11: Phosphonomethyl ring opening of cyclohexene oxide quinate derivatives.

When cyclohexene oxide derived from *myo*-inositol **29** was reacted with diisopropyl lithiomethylphosphonate under the same conditions, no phosphonomethylated material was formed. This lack of reactivity apparently reflected the known lack of reactivity of cyclohexene oxides[70] towards nuclephilic attack and the steric hindrance created by the benzyl ether groups surrounding this oxirane ring. A survey of the literature revealed a shortage of alternate methods for phosphonomethylation of hindered cyclohexene oxide systems. This prompted an examination of strategies relevant to introduction of a phosphonomethyl moiety into cyclohexene oxide **27** and sterically-hindered, substituted cyclohexene oxides, **28** and **29**.

Phosphonomethylation of Hindered Cyclohexene Oxide Systems

As a first step, parameters associated with reaction of the cyclohexene oxides **27**, **28** and **29** with diisopropyl lithiomethanephosphonate in the presence of boron trifluoride were varied. In the absence of boron trifluoride, reaction of **27**, **28** and **29** with diisopropyl lithiomethanephosphonate failed to yield detectable ring-opened product. Ring opening of **27** and **28** occurred in high yield and excellent regioselectivity in **28** at -78°C when a molar excess ranging from 1.1 to 2 equivalents of equimolar amounts of diisopropyl lithiomethanephosphonate and boron trifluoride was used (Table 6). However, ring opening of cyclohexene oxide **29** derived from *myo*-inositol only occurred when the temperature was raised to -40°C and 5 molar equivalents of diisopropyl lithiomethanephosphonate and 10 molar equivalents boron trifluoride were used. The ring opening yielded a mixture of regioisomers (Table 6) in an approximately 1:1 ratio.[72]

Table 6: Reaction of substituted and unsubstituted cyclohexene oxides with $(RO)_2P(O)CH_2Li/BF_3$ and with Mes_2CH_2Li.

Oxirane	Product	X	Y	R	co de	Yield
 **27**		$-P(O)(OiPr)_2$ -OH -I	_	Ac H H		71^a 82 92
 **28**		$-P(O)(OiPr)_2$ -OH -I	_	_		79 92 46
 **29**		$-P(O)(OiPr)_2$ $-P(O)(OMe)_2$	$-P(O)(OiPr)_2$ $-P(O)(OMe)_2$ -OH -I	_	 30 32	49 61 89 35

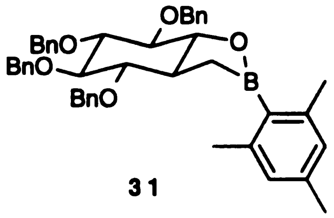
a: Combined yield for the oxirane ring opening and subsequent acetylation.

Formation of regioisomers was not unique to cyclohexene oxide **29** but was also observed for quinate-derived cyclohexene oxides **25** and **28** when reaction temperatures exceeded -78°C. The structure assignment of each regioisomer was achieved using a combination of two dimensional homonuclear (^1H, ^1H) COSY and heteronuclear (^1H, ^{13}C) HETCOR experiments.

Directness of Synthesis vs. Regioselectivity

The attenuated reactivity of diisopropyl lithiomethanephosphonate/boron trifluoride with cyclohexene oxide **29** and the loss of regioselectivity prompted examination of a less direct route to phosphonomethylation which relied on the reactivity of (lithiomethyl)dimesitylborane.[73] While the diisopropyl lithiomethanephosphonate/boron trifluoride combination utilizes boron as a Lewis acid, (lithiomethyl)dimesitylborane employs boron to stabilize an adjacent carbanion.[74] (Lithiomethyl)dimesitylborane has been principally used for hydroxymethylation of oxiranes in acyclic systems although reactivity on nonsubstituted cyclic systems has been reported.[75] Oxirane ring opening upon reaction with (lithiomethyl)dimesitylborane in THF was observed for the relevant cyclohexene oxides, **27**, **28** and **29**, and only one regioisomer was formed even though the reaction was run at room temperature. Direct oxidative work up[75] (NaOH, H_2O_2) afforded the desired hydroxymethylated ring systems (Table 6) while iodinolysis (I_2/MeONa[76a] or ICl/MeOH[76b]) of the purified organoborane intermediates afforded the iodomethane derivatives (Table 6) suitable for subsequent Arbuzov or Michaelis-Baker reactions.

The structure assignment and relative stereochemistry of the quinate derivatives isolated after ring opening and derivatization were established from NMR experiments. Such techniques were insufficient to establish the stereochemistry of the heteromethylated *myo*-inositol derivatives. Fortuitously, the purified intermediate organoborane product of the reaction of (lithiomethyl)dimesitylborane with **29** gave diffractable crystals, showing the intermediate as the oxiborinane **31** (Scheme 12). This bicyclic product was likely obtained from the intramolecular cyclization between the secondary alkoxide and the boronic moiety followed by the breaking of a C-B bond releasing a mesityl molecule upon protonolysis.[77] The structure of 1,3-diol **30**, derived from *myo*-inositol obtained by oxidative work up of **31** was then deduced along with the structure of **32** which could only be obtained when iodinolysis was performed with ICl[76b] in MeOH on purified oxiborinane **31**.



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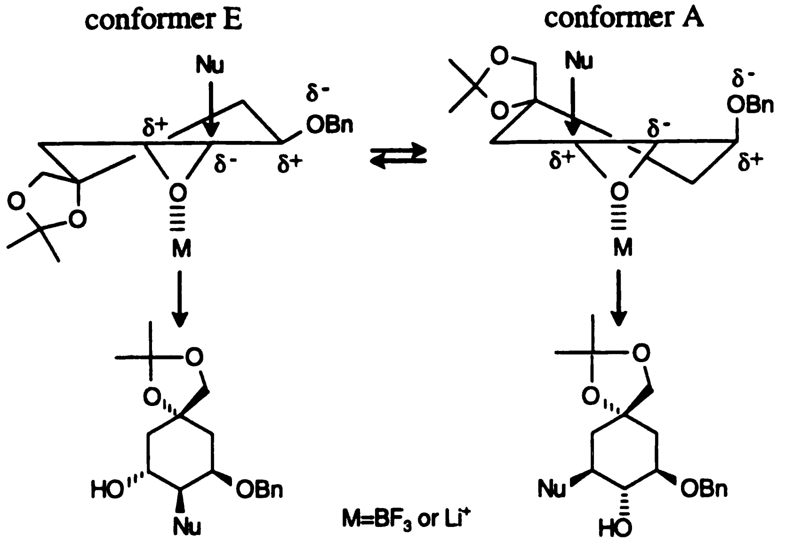
Scheme 12: Oxiborinane intermediate derived from *myo*-inositol.

Rationalization of the Regioselectivity

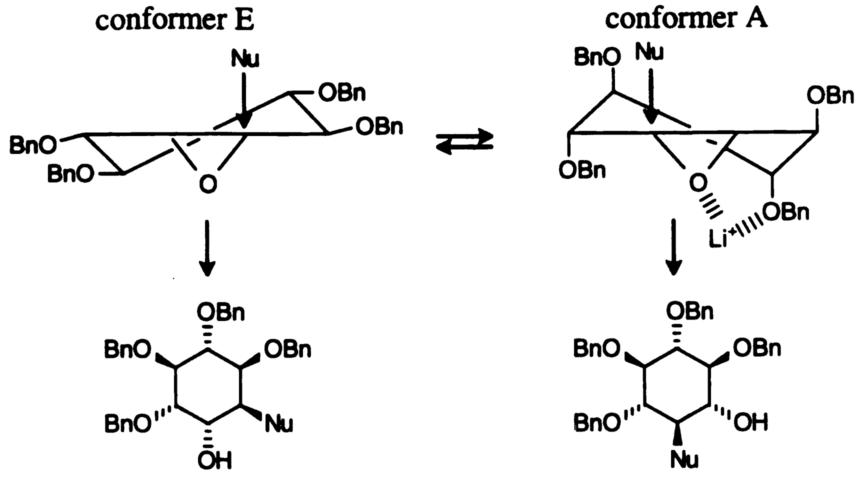
The regioselectivity observed during heteromethylation was identical for the reaction of the cyclohexene oxide **28** derived from quinic acid with diisopropyl lithiomethanephosphonate/boron trifluoride and with the (lithiomethyl)dimesitylborane. Product regioisomers were suggestive of a transition state (Scheme 13) involving transdiaxial attack[78] as predicted by the Fürst-Plattner's rule.[79] This regioselectivity is likely due to polarization of the bond between the C-5 carbon and oxirane oxygen caused by the inductive effect of the benzyloxy group attached at C-3. Transdiaxial attack at the partially electropositive C-5 carbon requires attack via the conformer A of Scheme 13.



Scheme 13: Regioselectivity of nucleophilic addition on quinate derived epoxide **28**.

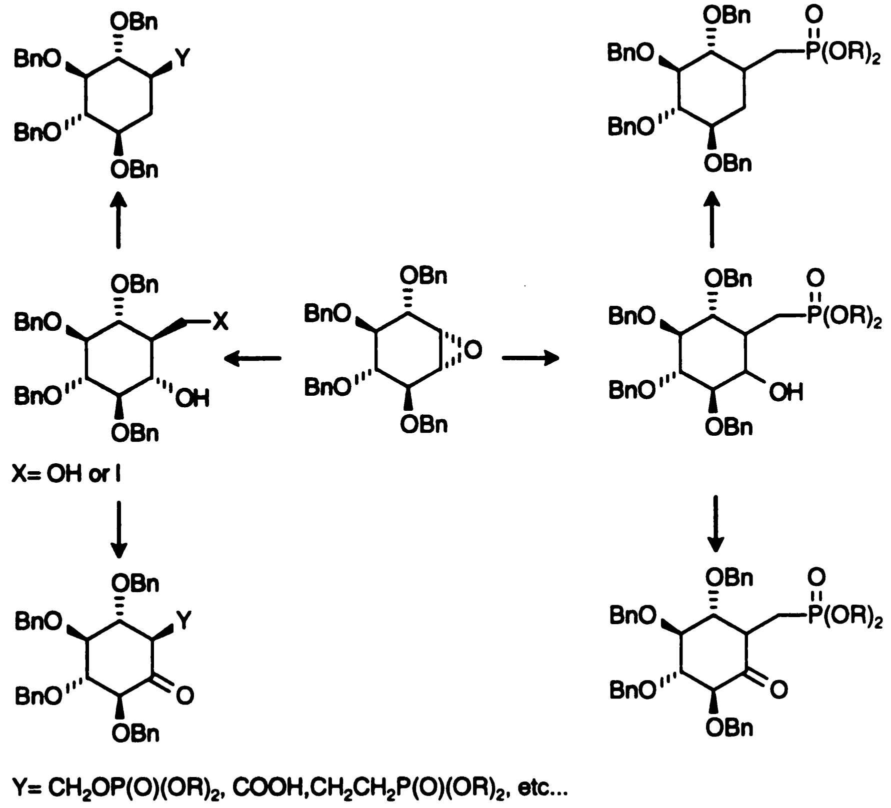
The lack of reactivity of an oxirane ring when cyclohexene oxides are reacted with diisopropyl lithiomethylphosphonate in the absence of boron trifluoride provides evidence of the BF_3 complexation with the oxirane oxygen in the ring opening transition state. Although complexes with the oxirane ring oxygen are implicated along with the polarization of one of the carbon-oxygen bonds of the oxirane, carbonium ion intermediacy during ring opening is doubtful. This follows from the lack of *cis*-substituted ring opened product during the reaction of quinate derived cyclohexene oxide **28** with either nucleophilic species. Lithium also appears to complex the oxygen of the oxirane ring based on the reactivity and the regioselectivity.

The regioselectivity observed for the reaction of the *myo*-inositol derived cyclohexene oxide **29** with (lithiomethyl)dimesitylborane suggested reaction via a transition state (Scheme 14) where transdiaxial nucleophilic attack[78] is occurring on axially substituted conformer A. The indicated dominance of axially substituted conformers in the ring opening transition states for both quinate **28** and *myo*-inositol **29** derived cyclohexene oxides is noteworthy. However, the factors responsible for the dominance of axially



Scheme 14: Regioselectivity of nucleophilic attack on *myo*-inositol derived epoxide **29**.

substituted conformers in the transition states are likely different for the two substituted cyclohexene oxides. The *myo*-inositol derived cyclohexene oxide **29** has two adjacent benzyloxy substituents. These substituents might inductively destabilize the formation of a complex between the boron trifluoride or lithium with oxirane oxygen. Diminished boron trifluoride complexation at -78°C probably accounts for the reduced reactivity of the diisopropyl lithiomethanephosphonate. An absence of preferential polarization of either of the oxirane carbon-oxygen bonds may account for the relaxed regioselectivity during the reaction of diisopropyl lithiomethanephosphonate. The regioselectivity observed with (lithiomethyl)dimesitylborane might reflect lithium simultaneous binding to the oxirane oxygen and the other oxygen of one of the benzyloxy groups.[80] The binding complex could stabilize the axially substituted conformer. In numerous reactions, lithium is precedented to be a divalent ligand[81] while boron trifluoride appears to be primarily a monovalent ligand.[82] Failure of the boron trifluoride to form a bridging complex to stabilize the axially substituted conformer may be an additional reason for the relaxed regioselectivity observed for the reactions of *myo*-inositol-derived cyclohexene oxide **29** with the dialkyl lithiomethanephosphonate.



Y= $CH_2OP(O)(OR)_2$, COOH,$CH_2CH_2P(O)(OR)_2$, etc...

Scheme 15: Possible generic targets as precursors of MIP synthase inhibitors.

The changes of regioselectivity observed for the reaction of cyclohexene oxides with dialkyl methylphosphonate in the presence of boron trifluoride etherate is a powerful tool to rapidly access phosphonate species. Lack of regioselectivity decreases yields but offers a wider choice of phosphonylated species. Alternatively, (lithiomethyl)-dimesitylborane's reactivity was uniformly high, irrespective of whether the cyclohexene oxide was unsubstituted or fully substituted (Table 6) and did not come at the price of sacrificed regioselectivities. This reagent stereoselectively introduces modifiable heteromethyl moieties onto cyclohexane ring and provides access to products such as phosphates, homophosphonates, carboxylic acids and others (Scheme 15). Phosphonates can be obtained from Arbuzov reaction on the suitable iodomethylated derivative.

Phosphates can be synthesized from the diols while chain elongation can yield homophosphonates and homophosphates.

Also, the newly formed secondary alcohol expands the scope of the possible targets. It can be oxidized to the ketone or reduced to the alkane. The methodology, presented here, offers facile access to new classes of MIP synthase inhibitors (Scheme 15). These inhibitors will principally be intermediate **D** (Scheme 3) and carbocyclic substrate analogues. Some of these putative inhibitors will be presented in the following chapters.

CHAPTER III

SUBSTRATE STRUCTURAL REQUIREMENTS FOR ACTIVE SITE BINDING AND CONVERSION

One of the difficulties in studying the mechanism of MIP synthase with the natural substrate is that the reaction appears to be essentially irreversible,[46] and the three presumed enzyme-bound intermediates cannot be examined under equilibrium conditions. However, the steps that occur prior to cyclization (that is substrate binding, substrate oxidation and enolization) have been shown to be reversible.[30,38] Enzyme interactions with substrate analogues unable to undergo cyclization might therefore provide insights relevant to several questions concerning the early steps of the enzyme catalyzed reaction. These queries concern the actual form of D-glucose 6-phosphate that binds to NAD-bound MIP synthase, the active site interactions essential to binding acyclic substrate after pyranosyl ring opening, and finally the nature of the rotation about the C4-C5 bond that must precede intramolecular aldol condensation.

Substrate Binding: Cyclic vs. Acyclic

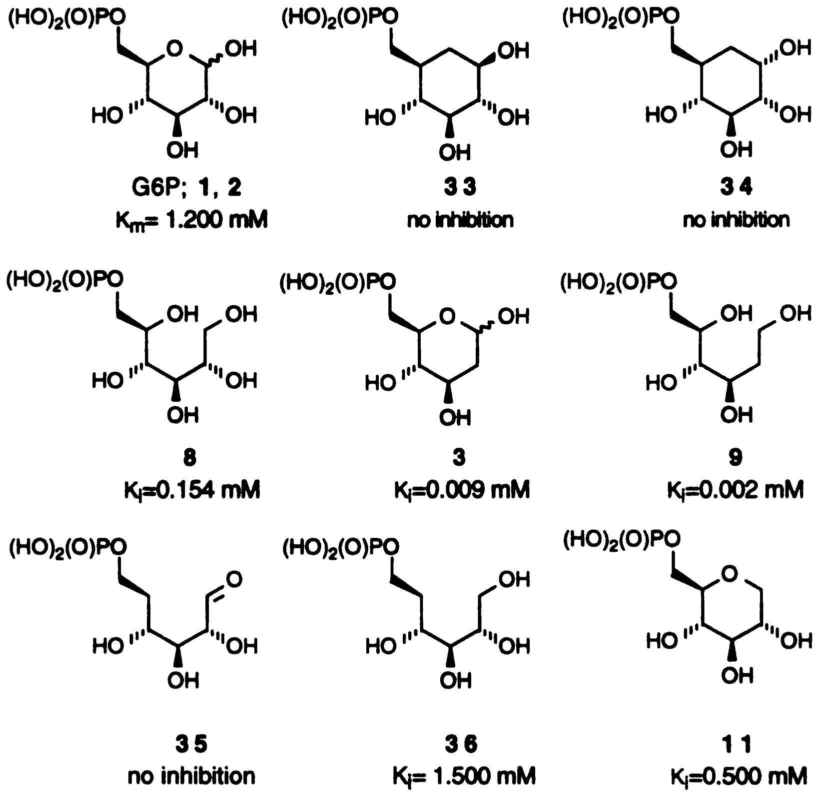
An essential feature of any enzymic reaction is the association of the enzyme and the substrate to form the enzyme-substrate complex. The formation of this complex is reversible and subject to a Michaelis constant K_m. Binding between enzyme and substrate is generally non-covalent, involving ionic and non-polar forces in addition to hydrogen bonds. In most cases, however, substrate specificity is due to more than exact complementarity between enzyme and substrate. Koshland proposed in his "induced fit" theory[83] that the binding of a substrate to enzyme might cause a conformational change in the enzyme and bring about a precisely controlled geometrical arrangement of the active site. In regard to MIP synthase, the question is which one of the three forms of glucose 6-phosphate binds to MIP synthase and causes the appropriate conformational changes for the catalysis to occur.

Benkovik[47] has determined the ratio of β-D-glucose 6-phosphate **1** and α-D-glucose 6-phosphate **2** at equilibrium in neutral pH solution, to be 1.63 to 1, respectively. In Sherman's experiments,[46] NAD-bound MIP synthase was incubated for a short period of time at low temperature (0-10°C) with anomerically pure α- or β-D-glucose 6-phosphate. Sherman demonstrated that the β-anomer of D-glucose 6-phosphate **1** was a substrate of MIP synthase and that α-D-glucose 6-phosphate **2** was an inhibitor. Because of the limitations of the experimental conditions, the possibility that α-D-glucose 6-phosphate **2** was also a substrate could not be rejected. However, for the reaction to proceed with the appropriate stereochemistry, the carbonyl at C-1 must be in the orientation obtained when the ring opening occurs from the β-anomer. Either β-D-glucose 6-phosphate **1** is the sole substrate of MIP synthase or the anomerization from the α-anomer to the β-anomer occurs in the enzyme active site, allowing MIP synthase to have access to each anomeric form of the substrate. Also, Sherman could not exclude the possibility that the acyclic glucose 6-phosphate[47] (<0.4 %) was a substrate and that the reaction rate differences observed between anomers were due to differential inhibition of MIP synthase, or differential ring opening rates.

To resolve which solution form of D-glucose 6-phosphate is bound by the active site, known inhibitors and new substrate analogues (Table 7) have been synthesized and evaluated for inhibition. Glucitol 6-phosphate **8**, 2-deoxy-glucose 6-phosphate **3** and its reduced form **9** were substrate analogues and known inhibitors of MIP synthase. The stereochemistry of the hydroxyl group at C-1 of glucose 6-phosphate has been fixed in β-

(**33**) and *α*- (**34**) carbaglucose 6-phosphate. If only one anomer of D-glucose 6-phosphate binds to the enzyme, then only one carbocycle should inhibit the synthase. The C-5 deoxy series (**35**, **36**) lacks the pyranosyl form in solution. 1,5-Anhydro-glucitol 6-phosphate **11** possesses the pyranosyl oxygen missing in **33** and **34** while lacking the equivalent of the anomeric hydroxyl of substrate.

Table 7: Substrate and substrate analogues: cyclic vs. acyclic.



G6P; **1**, **2**
K_m= 1.200 mM

33
no inhibition

34
no inhibition

8
K_i=0.154 mM

3
K_i=0.009 mM

9
K_i=0.002 mM

35
no inhibition

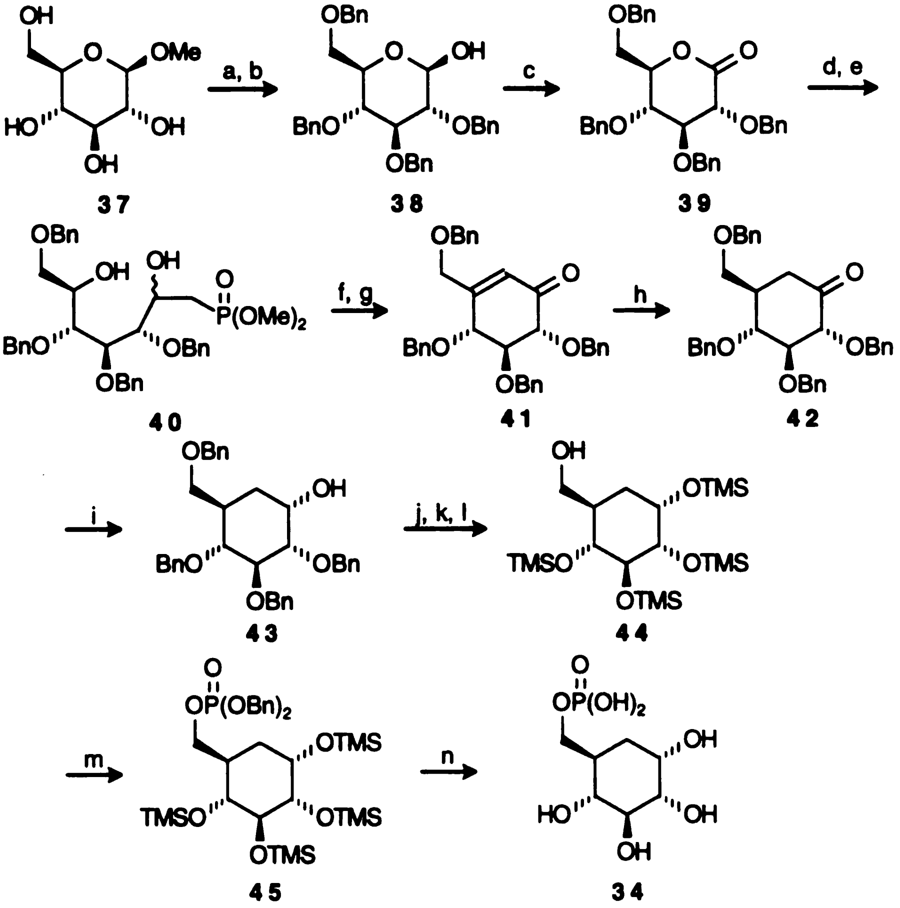
36
K_i= 1.500 mM

11
K_i=0.500 mM

Glucitol 6-phosphate **8** was obtained by reduction of D-glucose 6-phosphate with sodium borohydride. 2-Deoxy-glucitol 6-phosphate **9** was prepared in the same manner from **3**. 1,5-Anhydro-glucitol 6-phosphate **11** was prepared from the pentaacetylated *β*-D-glucose.[84] Tetraacetylated 1,5-anhydro-glucitol was produced upon reaction of the pentaacetylated glucose with 30% HBr in acetic acid and radical reduction of the halogenated product by tributyltin hydride.[85] Selective reduction at C-1 is favored because of the rate difference between radical hydride addition and C2-C1 acetate migration. Acetate migration to C-1 and reduction at C-2 position can be favored if tributyl hydride concentration is maintained low throughout the reaction.[85] 1,5-Anhydro-glucitol, obtained after methanolic deprotection, was silylated with HMDS and TMSCl in pyridine. The silyl group protecting the primary alcohol was then removed by treatment with K_2CO_3 in anhydrous methanol at 0°C.[86] Phosphitylation, using dibenzyl N,N-diisopropyl phosphoramidate, was followed by oxidation of the phosphite with *m*CPBA and hydrogenolysis, and yielded the 1,5-anhydro-glucitol 6-phosphate **11**. The C-5 deoxy glucose 6-phosphate **35** and its reduced analogue **36** were synthesized and assayed by Jirong Peng.

Numerous methods[87] had been developed to synthesize various carbocyclic sugar analogues. Most of these methods aimed at the simultaneous synthesis of diastereoisomers which were subsequently separated. Yet, synthesis of α-carbasugar glucose and β-carbasugar glucose phosphorylated species have never been reported in the literature.

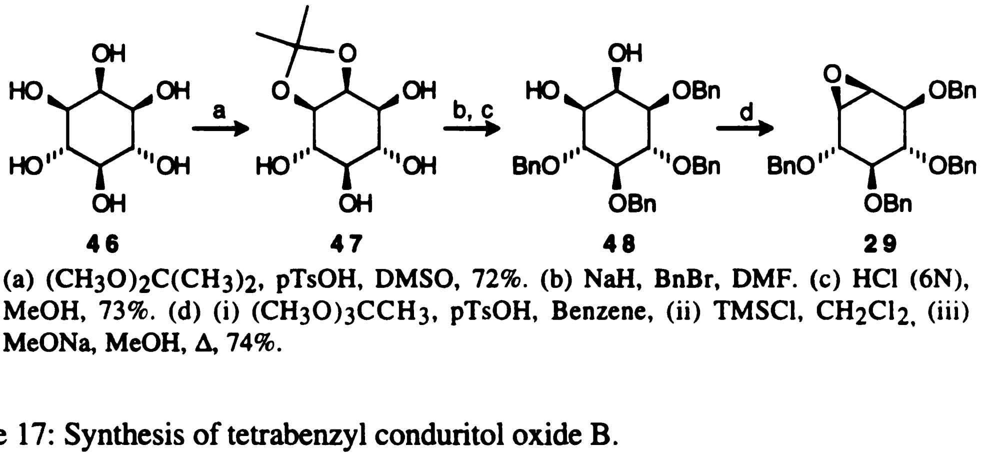
The synthesis of α-D-glucose 6-phosphate **34** followed an approach developed by Paulsen based on Horner-Emmons methodology.[87b] Attempts to reproduce Paulsen results in the synthesis of selectively protected carbaglucose were unsuccessful. A Japanese group had prepared the cyclic enone **41** by employing Horner-Emmons methodology.[88] Cyclic enone **41** was the precursor of α- and β-D-carbaglucose, in only seven steps from methyl β-D-glucose pyranoside **37** (Scheme 16). This route was then followed as an alternative to Paulsen's synthesis. After benzylation of methyl β-D-glucose pyranoside and removal of the methyl group under strong acidic conditions,[89] the benzylated hemiacetal was oxidized[90] to the lactone **39**. δ-Gluconolactone **39** was condensed with lithiomethane dimethylphosphonate and the lactol product of condensation then reduced with $NaBH_4$ to a diastereomeric mixture of diols **40**. Simultaneous oxidation of the two hydroxyl groups using Swern conditions[90] and base catalyzed cyclization with concomitant elimination of dimethyl phosphate, yielded the aforementioned enone **41**.



(a) NaH, BnBr, DMF, 0°C, 90%. (b) AcOH/H_2SO_4, 3/2, v/v. Δ, 48%. (c) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, -78°C, 96%. (d) $LiCH_2P(O)(OCH_3)_2$, THF, -78°C, 94%. (e) $NaBH_4$, THF, 97%. (f) DMSO, $(CF_3CO)_2O$, Et_3N, CH_2Cl_2. (g) K_2CO_3, Toluene, 51%. (h) $NaHCO_3$, $Na_2S_2O_3$, H_2O, C_6H_6, 80°C, Adogen 464, 98%. (i) L-Selectride®, THF, -78°C, 97%. (j) H_2, Pd/C, MeOH, 100%. (k) TMSCl, HMDS, Pyr, 97%. (l) K_2CO_3, MeOH, 0°C. (m) $(BnO)_2PNiPr_2$, CH_2Cl_2, *m*CPBA, 63%. (n) H_2, Pd/C, THF/H_2O, (4/1), 100%.

Scheme 16: Synthesis of *α*-carba-glucose 6-phosphate.

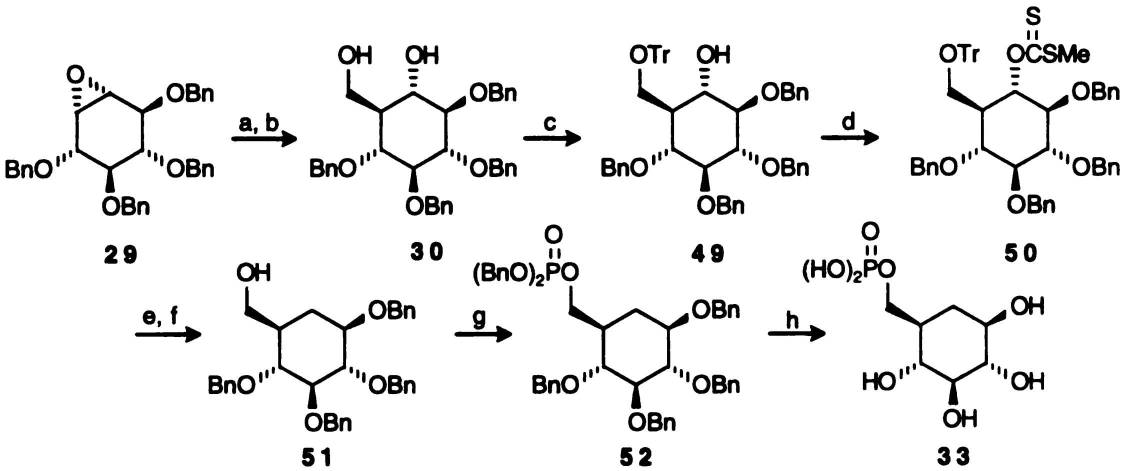
Thermodynamically controlled reduction[91] of the cyclohexenone afforded cyclohexanone **42** with all substituents in equatorial positions. Subsequent stereocontrolled reduction of the cyclohexanone[92] with L-Selectride® followed by hydrogenolysis yielded the fully deprotected α-carbocyclic glucose in 20% overall yield. Direct selective phosphorylation of this carbasugar with diphenylphosphorochloridate was unsuccessful. Enzymatic phosphorylation using an excess of hexokinase in the presence of ATP, PEP and pyruvate kinase[93] was also fruitless. The stepwise approach used for the phosphorylation of 1,5-anhydro-glucitol was successfully applied to the carbasugar and after hydrogenolysis, yielded α-carbaglucose 6-phosphate **34** as an enantiomerically pure product, in 63% yield from the unprotected carbasugar.



(a) $(CH_3O)_2C(CH_3)_2$, pTsOH, DMSO, 72%. (b) NaH, BnBr, DMF. (c) HCl (6N), MeOH, 73%. (d) (i) $(CH_3O)_3CCH_3$, pTsOH, Benzene, (ii) TMSCl, CH_2Cl_2, (iii) MeONa, MeOH, Δ, 74%.

Scheme 17: Synthesis of tetrabenzyl conduritol oxide B.

The carbocyclic analogue of the β-anomer of glucose 6-phosphate was synthesized by a shorter route as a racemic mixture using the heteromethylation methodology presented in the previous chapter. Fully benzylated conduritol oxide B, **29**, the key intermediate of this synthesis, was derived in 36% yield from *myo*-inositol **46** (Scheme 17). Selective protection of two *cis*-hydroxyl groups of *myo*-inositol **46**, followed by benzylation of the four remaining alcohols yielded, after the acid catalyzed removal of the acetonide, a racemic mixture of diol **48**. The resulting vicinal *cis*-diol was reacted with trimethyl orthoacetate followed with TMSCl to form the α-acetoxy chloride derivative that upon treatment with sodium methoxide in refluxing methanol afforded the conduritol oxide B **29**.



(a) $LiCH_2BMes_2$, THF, rt. (b) H_2O_2, NaOH, THF, 89%. (c) TrCl, DMAP, Pyr, 92%. (d) NaH, CS_2, MeI, THF, Δ, 69%. (e) Bu_3SnH, AIBN, C_6H_6. (f) MeOH, pTsOH, 93%. (g) $(BnO)_2PNiPr_2$, 1H-tetrazole, *m*CPBA, 92%. (h). H_2, Pd/C, THF/H_2O (4/1) 100%.

Scheme 18: Synthesis of β-carbaglucose 6-phosphate.

Hydroxymethylation of the cyclohexene oxide **29** yielded 1,3 diol **30** (Scheme 18), precursor of the carbocyclic β-anomer of glucose 6-phosphate. Initially, the synthesis was designed for the phosphate moiety to be introduced before the removal of the secondary hydroxyl group. However, when the phosphorylation step was carried out before the deoxygenation, low yields due to the formation of a purifiable cyclic phophodiester side product resulted. Such a material was isolated at the phosphorylation step whether NaH/TBPP[96] or $(BnO)_2PNiPr_2$/1H-tetrazole, followed by *m*CPBA oxidation [97] was used. It was also isolated when the monophosphorylated alcohol product of selective phosphorylation of the primary alcohol was treated with a base in the first step of the Barton-McCombie deoxygenation.[98] When the secondary alcohol was reacted with pentafluorophenyl chorothionocarbonyl in pyridine, only the cyclic phosphate product was obtained. A longer but more effective route was therefore chosen to synthesize

β-carbaglucose 6-phosphate **33**. After protection of the primary alcohol of **30** as a trityl ether,[99] the remaining secondary alcohol was reduced to the alkane via Barton-McCombie deoxygenation methodology.[98] The trityl group was removed under acidic conditions and the deprotected primary alcohol in **51** was phosphorylated. The racemicβ-carbaglucose 6-phosphate **33** was then obtained after hydrogenolysis.

Competitive inhibition of MIP synthase was obtained with 1,5-anhydro-glucitol 6-phosphate **11** while no inhibition was detected with either α- or β-carbaglucose 6-phosphate **33** and **34** (Table 7). Although the carbocyclic glucose 6-phosphates **33**, **34** and 1,5-anhydro-glucitol 6-phosphate **11** are similar structural analogues of the substrate, it is evident that a subtle structural alteration can have dramatic consequences in analogue binding affinity. Replacement of the pyranosyl ring oxygen in the substrate with a methylene group which characterizes carbocyclic analogue **33** and **34** resulted in a complete loss of active site binding. The removal of the hydroxyl group at the C-1 position did not modify the propensity for binding with the inhibition constant (K_i= 0.500 mM) for 1,5-anhydro-glucitol 6-phosphate being approximately equal to the Michaelis constant (K_m= 1.200 mM) for glucose 6-phosphate. 5-Thio-D-glucose 6-phosphate (K_i= 0.330 mM) had been observed to inhibit MIP synthase to similar extent as 1,5-anhydro-glucitol 6-phosphate.[52a] This fairly poor inhibition could be attributed either to the large sulfur atom of the heterocyclic ring or to the fact that 5-thio-D-glucose 6-phosphate undergoes only very slow ring opening. As the carbocyclic series, a complete loss of binding affinity was observed for 5-deoxy glucose 6-phosphate. The 5-deoxy-glucitol 6-phosphate poor inhibition could be attributed to the missing hydroxyl group involved in binding recognition or to the lack of pyranose oxygen. However, only a 10 fold decrease in enzyme affinity was observed between glucitol 6-phosphate and 5-deoxy glucitol 6-phosphate. The ring oxygen appears to be essential for active site recognition and binding of substrate pyranose, while the anomeric C-1 hydroxyl group seems to be involved in a latter stage of the transformation, such as in the enzyme-catalyzed ring opening.

In contrast to the previous sugar substrate analogues, both reduced sugar acyclic substrate analogues, glucitol 6-phosphate **8** and 2-deoxy-glucitol 6-phosphate **9**, were powerful competitive inhibitors of MIP synthase (Table 7). Barnett observed similar results[49] and concluded that, since the reduced linear sugars had such an enhanced affinity for the synthase active site, the acyclic form of G6P ought to be the enzyme's substrate. It is obvious that the dehydrogenation at C-5 and bond formation between C-1 and C-6 of G6P requires the presence of the open chain form of the substrate in the active site of the enzyme. However, in solution, the acyclic form of glucose 6-phosphate[47] is present in less than 0.4% and the flexibility of the open chain decreases the portion of substrate present in the appropriate configuration for binding. It would be advantageous for the enzyme to bind one or both of the cyclic forms of the substrate, open the hemiacetal ring, and then orient the molecule in a conformation suitable for ring closure. Studies on anomeric specificity of enzymes that act upon phosphorylated sugar substrates have shown that the substrate is rarely the acyclic sugar.[100] None of the enzymes involved in glycolysis or gluconeogenesis that use glucose 6-phosphate as a substrate bind only the acyclic form. The only argument that led Barnett to propose that the synthase binds the acyclic form of G6P was the fact that the linear substrate analogues were good inhibitors. Other factors besides substrate similarity could be considered to explain the greater inhibitory potency of the acyclic substrate analogues. The acyclic inhibitors can be considered as reaction intermediate analogues, and as reaction intermediate analogues, stronger interactions with the enzyme binding domain would be anticipated.

Barnett suggested that glucitol 6-phosphate **8** could be oxidized at the C-5 position and form an NADH-C-5-oxidized glucitol 6-phosphate-enzyme complex.[30] To establish whether such an oxidation occurred, the inhibitors containing an oxidable C-5 center (that is **3**, **8**, and **9**) were incubated, in the absence of substrate, with an equimolar amount of NAD and enzyme at 37°C. In another set of experiments carried out by Jirong Peng, the isosteric homophosphonate analogues of G6P, **8**, **3** and **9**, that are competitive inhibitors of MIP synthase, were submitted to the same enzymatic conditions.[102] None of these substrate analogues is turned-over by the enzyme, yet each of them is susceptible, in principle at least, to carry out redox chemistry at C-5. Incubation of each of these analogues with the synthase resulted in the establishment of an equilibrium mixture of NADH bound states which was measured by UV spectroscopy at 340 nm. The overlaid replots, giving NADH accumulation as a function of time for each species, indicated that the level and the rate of formation of NADH were related to the efficiency of the inhibitor. Acyclic reduced sugar analogues were oxidized faster than their cyclic sugar counterparts, yet a similar level of NADH was reached when the incubation time was long enough.

Sherman has indicated that MIP synthase could bind both of the cyclic forms of glucose 6-phosphate.[47] Binding of the substrate cyclic forms should be followed by ring opening and potential C1-C2 bond rotation which constitute a significant activation barrier. When under inhibition conditions, the formation of the first enzyme/linear reaction intermediate analogue/NAD-complex requires overcoming this activation barrier. Inhibitors which cannot be opened because of a lack of the anomeric hydroxyl group such as **11** or because of a high heterocyclic form stability such as **17**, do not pass the activation barrier and are not potent inhibitors. If an acyclic species binds to the enzyme, it is already in the reaction intermediate acyclic form. The activation barrier to reach the aforementioned complex does not apply to acyclic substrate analogue. NADH experiments showed that acyclic substrate analogues were oxidized faster by the NAD-enzyme complex than their sugar parents. The acyclic inhibitors were therefore in the appropriate position for oxidation faster than their sugar parents. These observations suggest that the complex formed between MIP synthase binding domain, NAD and acyclic substrate analogue were formed faster with an acyclic binding species than with a cyclic binding species . Inhibition constants are determined as a ratio ($K_i = K_{off}/K_{on}$) of the inhibitor/enzyme dissociation rate (K_{off}) to the inhibitor/enzyme binding rate (K_{on}). The K_{off} between cyclic and acyclic related species should be considered similar, since the C-1 hydroxyl group has been shown to be irrelevant to the initial binding efficiency. The K_{on} has been observed, however, to be larger for the acyclic species (NADH experiment). The faster the inhibitor gets in the acyclic reaction intermediate position, the better inhibitor it is.

Considering these results and observations, one can propose that MIP synthase binds each of the three forms of glucose 6-phosphate. Whether α-glucose 6-phosphate is a substrate has not been established, even though it binds to MIP synthase. Sherman's experiments presented β-glucose 6-phosphate as the preferred substrate, while the inhibition characteristics of acyclic species suggest that acyclic glucose 6-phosphate should be considered as a reaction intermediate that can be converted to MIP.

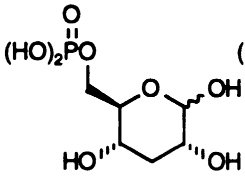
Active Site Interactions Essential to Binding Acyclic Intermediate A

Sherman had tested mannose 6-phosphate **5**, (epimer of G6P at C-2) and 2-deoxy-2-fluoro-glucose 6-phosphate **4** (Table 1) and showed that **5** was a weak inhibitor of MIP synthase isolated from bovine brain while **4** was a potent competitive inhibitor.[46] When **3** (2-deoxy-glucose 6-phosphate), **4** and **5** were incubated with MIP synthase in the presence of NAD, they were transformed into inositol derivatives.[46] Mannose 6-phosphate **5** was converted into *neo*-inositol 1-phosphate but was a poor substrate for the synthase with a K_m of 5.8 mM and a maximum velocity (V_{max}) 31 times slower than glucose 6-phosphate (K_m= 3.6 mM). 2-Deoxy-glucose 6-phosphate **3** was also substrate (K_m= 0.23 mM) but with a conversion rate 400 times slower than glucose 6-phosphate. 2-Deoxy-2-fluoro-glucose 6-phosphate **4** was also a substrate but was converted at an even slower rate. It is clear that although modification at C-2 prevents a sugar phosphate from being a good substrate, such a modification does not prevent binding. Sherman concluded that the specificity of MIP synthase was minimal for the C-2 position of G6P. The slow rate of conversion of these C-2 modified G6P analogues implied that the C-2 hydroxyl group is involved in specific interactions with residues of the active site during the latter steps of the conversion, such as cyclization. This suggests that either the hydroxyl group at C-2 induces conformational changes altering the enzyme into a catalytically active form or it is involved in restraining the oxidized intermediate into a suitable orientation for the intramolecular aldol condensation.

Sherman had also tested allose 6-phosphate **6** and galactose 6-phosphate **7** which are C-3 and C-4 epimers of glucose 6-phosphate (Table 1) and found that allose 6-phosphate **6** was a very weak competitive inhibitor while the C-4 epimer **7** was not bound by MIP synthase. He concluded that the enzyme affinity increased with each single-position epimer toward C-6 of the G6P structure.

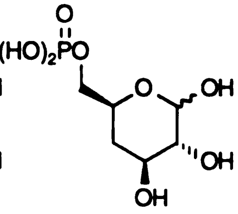
In order to investigate the nature of the interactions of these remaining hydroxyl groups with the enzyme active site, the kinetic parameters of the deoxy and reduced deoxy sugar substrate analogues (Table 8) were examined. This study provides a more detailed

Table 8: Deoxy glucose 6-phosphate analogue series[a]



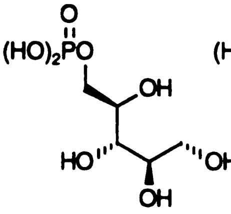
K_i= 0.59 mM

53



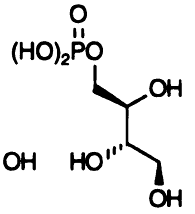
no inhibition

54



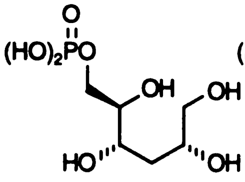
K_i= 0.017 mM

57



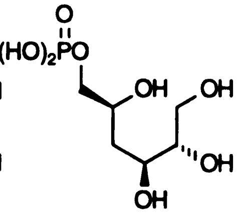
K_i= 0.047 mM

59



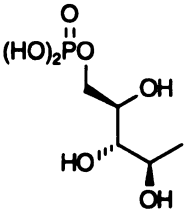
K_i= 0.26 mM

55



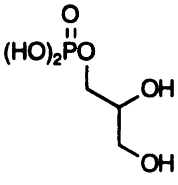
K_i= 0.93 mM

56



K_i= 0.008 mM

58



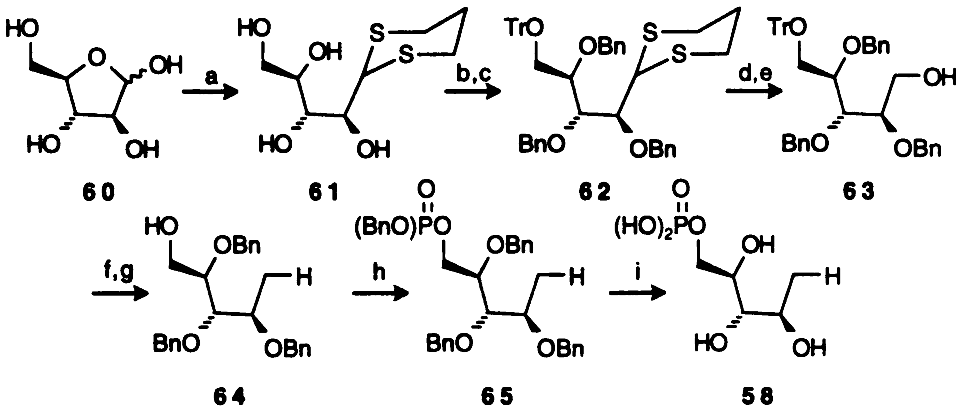
no inhibition

15

[a]Except for the arabinitol and glycerol series, the synthesis and the evaluation of these compounds were performed by Jirong Peng.

"map" of the key interactions between glucose 6-phosphate and the enzyme active site. The synthesis of the following deoxy series also allows us to identify the structurally simplest inhibitor of MIP synthase.

Arabinitol 5-phosphate **57** was obtained by reduction of the arabinose 5-phosphate with $NaBH_4$. Arabinose 5-phosphate was prepared following the method developed by Whitesides using D-arabinose **60** as starting material and a combination of PEP, ATP, pyruvate kinase and hexokinase as phosphorylating agents.[93]



(a) $(HSCH_2)_2CH_2$, HCl, EtOH, 70%. (b) TrCl, DMAP, Pyr. (c) NaH, BnBr, DMF, Bu_4NI, 64%. (d) NBS, CH_3CN, $CHCl_3$, 0°C. (e) $NaBH_4$, THF, 45%. (f) CBr_4, PPh_3, THF, Pyr, 98%. (g) (i) Bu_3SnH, AIBN, Benzene, (ii) *p*-TsOH, MeOH, 93%. (h) $(BnO)_2PNiPr_2$, 1H-tetrazole, CH_2Cl_2, *m*CPBA, 71%. (i) H_2, Pd/C, THF/H_2O, (4/1), 97%.

Scheme 19: Synthesis of 1-deoxy-arabinitol 5-phosphate

The 1-deoxy-arabinitol 5-phosphate **58** was also prepared from D-arabinose **60** (Scheme 19). After conventional acid catalyzed protection of the carbonyl of D-arabinose as a thioacetal **61**,[103] the primary alcohol was tritylated and the remaining secondary alcohols were benzylated. The thioacetal protecting group was removed with NBS[104] and the resulting carbonyl was reduced with $NaBH_4$. Exchange of the primary alcohol of intermediate **63** for a bromine was accomplished with carbon tetrabromide in the presence of triphenyl phosphine and pyridine in THF.[105] Pyridine prevented HBr formed in situ from catalyzing the trityl removal which resulted in overhalogenation of the material. Reduction of the halide derivative by tributyltin hydride was followed by trityl ether deprotection under acidic conditions which afforded **64**.[106] Phosphorylation of the alcohol yielded, after hydrogenolysis, the required 1-deoxy-arabinitol 5-phosphate **58**.

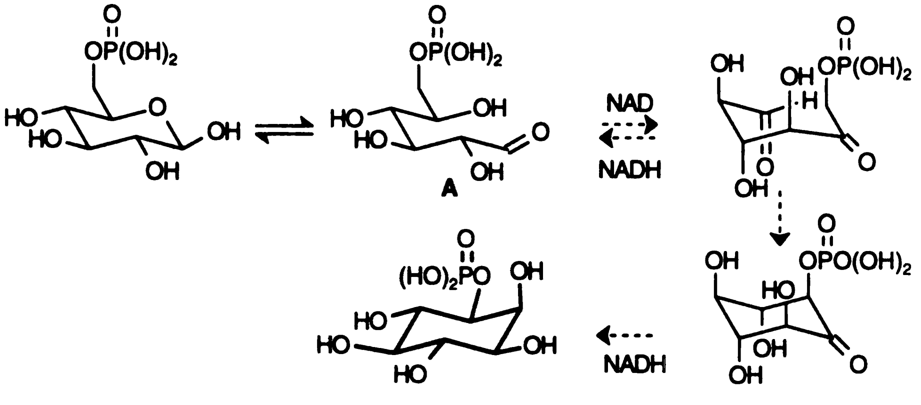
Once more, the reduced linear sugars are better inhibitors than their parent sugars. Unlike the C-2 deoxy series, the removal of the hydroxyl groups at C-3 and C-4 positions did not result in an increase for MIP synthase binding site affinity. On the contrary, removal of the hydroxyl groups at C-3 and C-4 resulted in a loss of enzyme affinity.

Arabinitol 5-phosphate **57** (K_i= 0.016 mM) and 1-deoxy arabinitol 5-phosphate **58** (K_i= 0.007 mM) were 10 and 20 times better inhibitors than glucitol 6-phosphate **8**, respectively. This result supported the hypothesis that neither C-1 or C-2 hydroxyl groups are involved in the recognition step. This increase in inhibition might be derived from a decrease in the chain flexibility and therefore an increase in the amount of material present in the appropriate configuration to bind MIP synthase. The removal of the C-2 hydroxymethyl moiety hardly decreased the affinity of the four carbon species **59** for the enzyme active site (K_i= 0.047 mM). However, no inhibition was observed when glycerol 1-phosphate **15** was tested against MIP synthase. Therefore, the C-3 and C-4 alcohols of glucose 6-phosphate are suspected to provide important binding interactions with the active site during the catalytic cycle. When tested for NADH accumulation, every single inhibitor containing an oxidizable C-5 hydroxyl group underwent oxidation. This implies that the molecules bind the active site in the same orientation as the substrate with respect to NAD and the catalytic residues.

Nature of the Rotation About the C4-C5 Bond

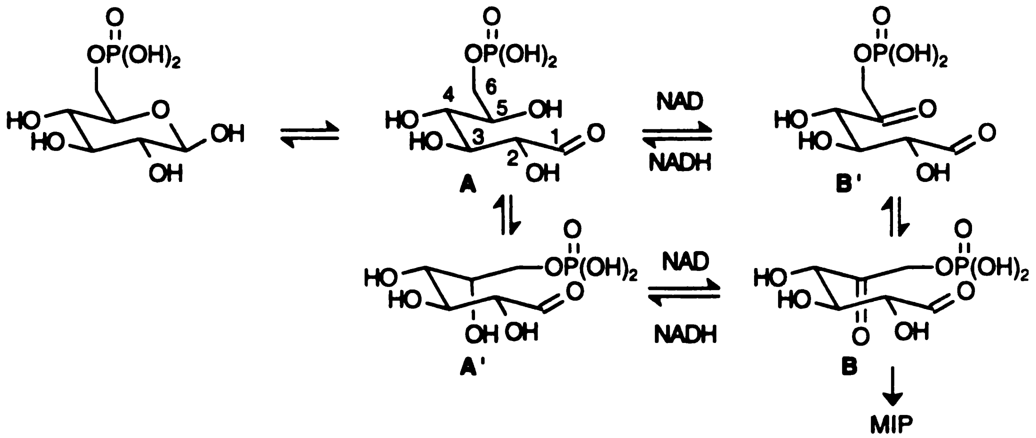
No non-charged species such as D-glucose or 2-deoxy-D-glucose has ever been found to inhibit MIP synthase.[51] Importance of the position of the phosphate moiety in the substrate has been studied by Jirong Peng. He observed that only isosteric homophosphonic acid substrate analogues where the phosphate monoester oxygen has been replaced with a methylene are good MIP synthase inhibitors.[102]

For the bond formation to occur between carbons C-1 and C-6 of the substrate, a rotation in the open form of the substrate must take place in order to bring carbons C-1 and C-6 in close proximity. Rotation around the C3-C4 bond of the C1-C4 edge of the carbohydrate without detachment of the C5-C6 edge would result in the formation of D-MIP (Scheme 20). Since D-*myo*-inositol 1-phosphate has never been isolated from MIP synthase reaction mixture, this last mode of rotation is not probable.



Scheme 20: Rotation around C3-C4 bond.

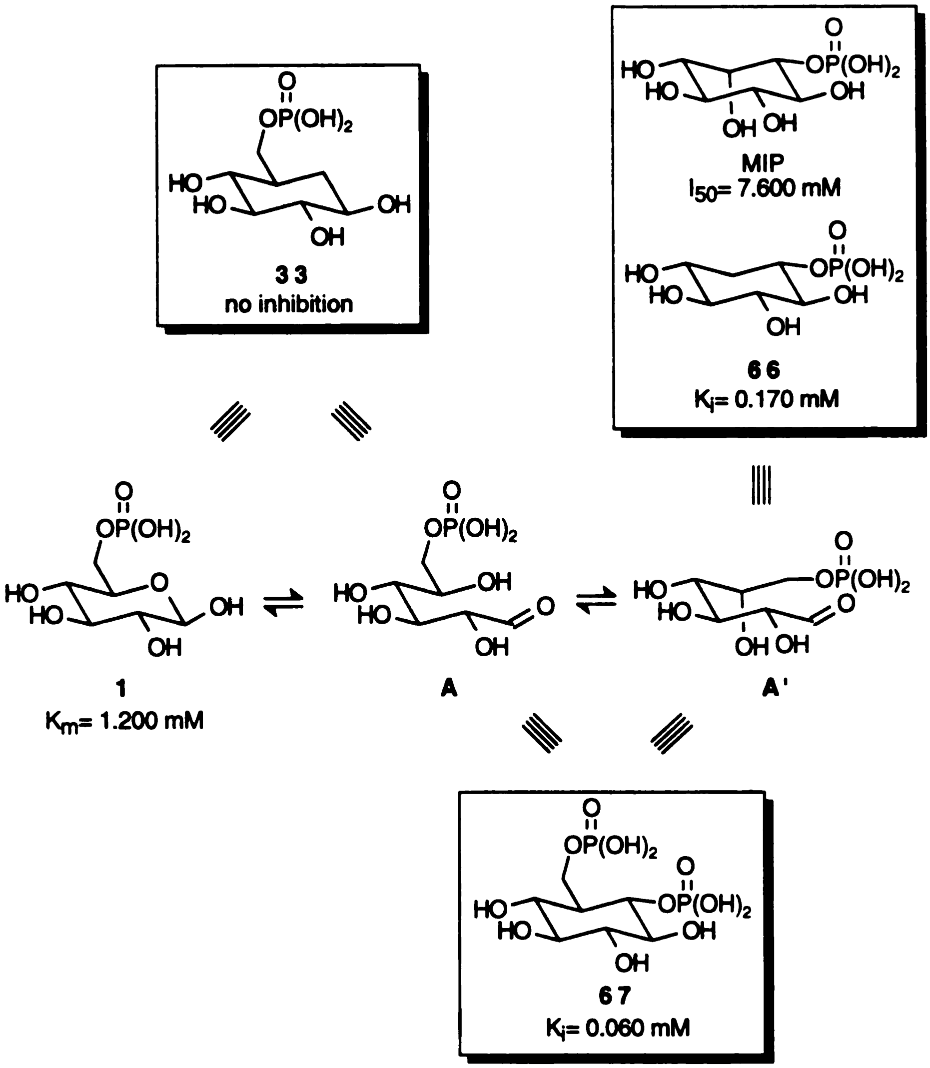
The rotation occurs around the C4-C5 bond of the linearized intermediate (**A** or **B'**, Scheme 21) without detachment from the active site of the C1-C4 edge of the carbohydrate. The 120° rotation occuring at C4-C5 might happen as soon as the ring opening step has taken place (**A**→**A'**) or after oxidation of the C-5 hydroxyl group (**B'**→**B**). The two possible sequences (**A**→**A'**→**B**) or (**A**→**B'**→**B**) are being investigated in order to understand what type of interactions the early transition states and reaction intermediates share with the active site. In this study, one should explore the position that the phosphate moiety occupies during the conversion.



Scheme 21: Possible sequences of oxidation/rotation.

In each proposed sequence, the phosphate moiety has to cover a large distance in the enzyme active site to place itself in the proper binding positions required by the conversion mechanism. One can wonder whether one or two phosphate binding pockets are required in the active site to convert G6P into MIP. In both cases, enzyme conformational changes have to be expected in order to carry out the conversion.[107]

In order to establish whether the phosphate moiety is interacting with one or two binding pockets during each turnover, the molecules presented in Scheme 22 were synthesized and tested as potential inhibitors of MIP synthase. Along with the identification of number of phosphate binding pockets, these species might provide some information about the conformation of the substrate that undergoes oxidation at C-5 (**A** or **A'**). *β*-Carba-glucose 6-phosphate **33** is considered an analogue of intermediate **A** while *myo*-inositol 1-phosphate and 2-deoxy-*myo*-inositol 1-phosphate **66** are considered as

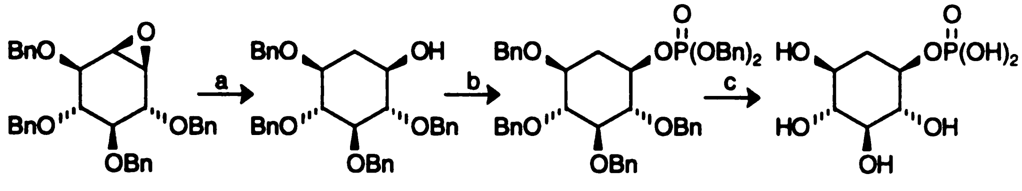


Scheme 22: Analogues of reaction intermediates of the early steps of the conversion.

analogues of putative intermediate **A'**. Finally, the diphosphate **67** is an analogue of both forms **A** and **A'**.

The synthesis and kinetic characteristic of *β*-carbaglucose 6-phosphate **33** have been described earlier in this chapter. *myo*-Inositol 1-phosphate (MIP) was synthesized[108] as a racemic mixture from *myo*-inositol **46**, which after selective protection of two vicinal *cis*-hydroxyl groups, acetylation of the remaining secondary alcohols, and removal of the acetonide protecting group under acidic conditions yielded the tetraacetylated diol. The diol was then phosphorylated in pyridine with diphenylphosphorochloridate.[109] Acetate and phenyl groups were removed by refluxing the protected *myo*-inositol phosphate in 1N aq NaOH and after passing the solution through a proton exchange resin Dowex 50, cyclohexylamine was added. The subsequent addition of acetone resulted in the precipitation of MIP which was then filtered.

The 2-deoxy-*myo*-inositol 1-phosphate **66** had been synthesized by Bruzik[110] but the route presented here (Scheme 23) was shorter. Reduction of tetrabenzyl conduritol oxide B **29** with Super-Hydride® led to a regioisomeric mixture consisting of a 3:1 mol ratio of 2-deoxy-*myo*-inositol **68** and 1-deoxy-*myo*-inositol.[111] Chromatographic purification of the regioisomers followed by phosphorylation of the alcohol **68** using NaH, TBPP as phosphorylating agent and hydrogenolysis of the fully benzylated phosphate ester yielded 2-deoxy-*myo*-inositol 1-phosphate **66**.

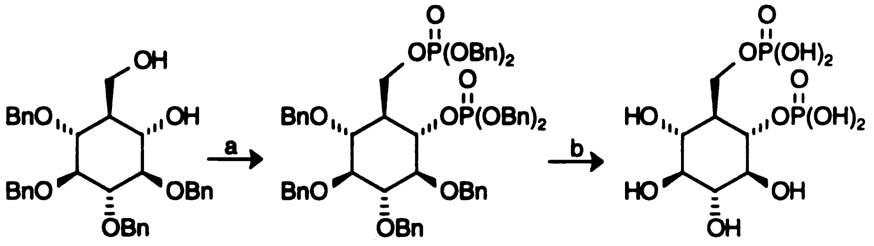


29 **68** **69** **66**

(a) $LiB(C_2H_5)_3H$, THF, -78°C to rt, 59%. (b) (i) NaH, THF, Δ, (ii) TBPP, Δ, 62%. (c) H_2, Pd/C, THF/H_2O (4:1), 100%.

Scheme 23: Synthesis of 2-deoxy-*myo*-inositol 1-phosphate.

The diphosphate **67** (Scheme 24) was synthesized from 1,3-diol **30** presented in the previous chapter. Both alcohols were simultaneously phosphitylated with dibenzyl N,N-diisopropyl phosphoramidate in the presence of 1H-tetrazole in CH_2Cl_2 then converted to phosphates by oxidation with *m*CPBA. Hydrogenolysis afforded the diphosphorylated material.



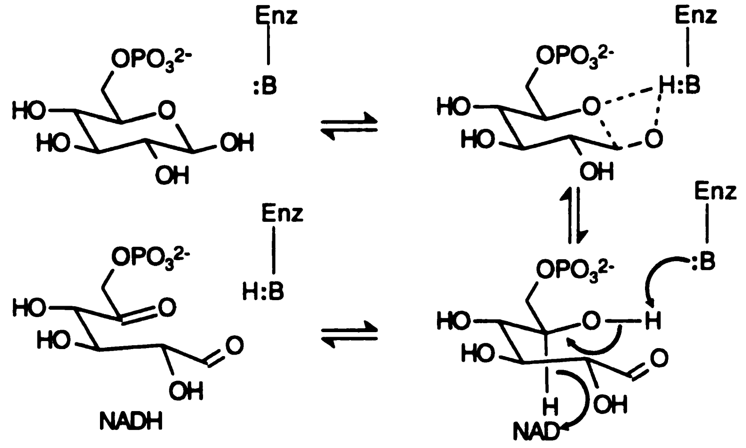
30 **70** **67**

(a) $(BnO)_2PNiPr_2$, 1H-tetrazole, *m*CPBA, CH_2Cl_2, 62%. (b) H_2, Pd/C, THF/H_2O, (4:1), 100%.

Scheme 24: Synthesis of 2-deoxy-2-(phosphoroxymethyl) *scyllo*-inositol 1-phosphate.

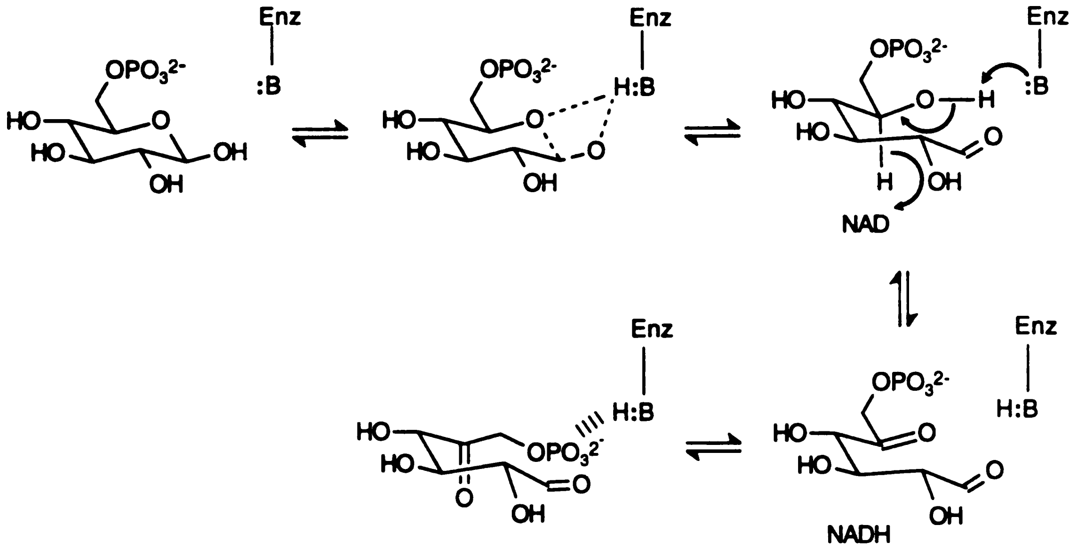
β-Carbaglucose 6-phosphate **33**, considered as an intermediate **A** analogue in Scheme 22, did not bind to MIP synthase. Diphosphate **67**, the other analogue of intermediate **A** and **A'**, was a good inhibitor of MIP synthase. While 2-deoxy-*myo*-inositol 1-phosphate **66** was a fairly good inhibitor, the product of the conversion, MIP, did not inhibit the synthase unless its concentration exceeded millimolar levels. This loss of binding affinity resulting from the introduction of a hydroxyl group vicinal to the secondary phosphate monoester indicates that the position of the C-5 glucose 6-phosphate hydroxyl group is under tight steric control from the enzyme active site residues.

The diphosphate **67** was found to bind to MIP synthase-NAD complex. This result was somewhat surprising since this molecule had twice the charge as the intermediates it was supposed to mimic. Increase in charge did not impede the analogue from sharing interactions with the enzyme active site residues. Therefore, each phosphate moiety must share interactions with distinct active site residues. This suggests that the phosphate moiety occupies two different binding pockets during the conversion of G6P into MIP. The diphosphate should be considered an analogue of the transition state of the rotation step rather than an analogue of reaction intermediates **A** and **A'** if the rotation occurs before oxidation or of intermediates **B'** and **B** if the rotation succeeds oxidation. If the oxidation step happens before the rotation step, the sequence would then be **A→B'→B**, where a single basic residue can participate in the ring opening and the oxidation step (Scheme 25).



Scheme 25: General base catalyzed ring opening and oxidation rotation.

The present observations are suggestive of the primary phosphate monoester interaction with two different binding pockets of the active site. One pocket would be involved in recognizing the substrate phosphate monoester and linear intermediates prior to rotation while the second would be responsible for binding intermediate primary phosphate monoester after C4-C5 bond rotation. One of the residues of the second phosphate binding pockets proposed to be involved in the enzyme catalyzed reaction could be the same base employed in the rotation and oxidation steps (Scheme 26). The protonated base, obtained from the oxidation of C-5 hydroxyl group could be involved in the rotation step by stabilizing the phosphate charges.



Scheme 26: One base as catalytic residue in ring opening, oxidation and rotation.

The nicotinamide ring of NAD must be located adjacent to the sugar within glucose 6-phosphate binding site since there is direct hydrogen transfer between C-5 of G6P and C-4 of nicotinamide ring. Jenness had shown[37] using radiolabeling technique that the hydride transferred from the C-5 position to NAD during the oxidation step was returned to *myo*-2-inosose 1-phosphate by NADH at the reduction step. The hydrogen *pro*-S of NADH is exclusively involved in the transformations. Since the same face of the nicotinamide ring participates in the oxidation and reduction steps, two scenarios concerning the place of NADH in the active site can be conceived. The first scenario involves the migration of the reduced cofactor during turn-over. Glucose 6-phosphate present in the active site in the **A** conformation is first oxidized by NAD to **B'**. This oxidation requires the nicotinamide ring to be located below the plane of the pyranose ring. The phosphate group then migrates to form the reaction intermediate **B** and the conversion carries on. If NADH reduces *myo*-2-inosose 1-phosphate by equatorial hydride delivery, then the reduced nicotinamide ring has to migrate from below to above the plane of the intermediate in order to stereoselectively reduce the inosose. The sequence **A→B'→B**

(Scheme 21) implies the migration of the reduced cofactor in the active site during the conversion of substrate to product. The second scenario precludes any migration of NADH. Glucose 6-phosphate present in the active site in the **A** conformation, first changes conformation to **A'** and is then oxidized by NAD to intermediate **B** (Scheme 21). In this scenario, the reduction of NAD to NADH and the reverse oxidation require the nicotinamide ring to be constantly present in the plane above the pyranose ring. Therefore this mechanism would not require any migration of the reduced cofactor .

For most NAD requiring enzymes,[24] the cofactor is tightly bound to the enzyme active site. However, MIP synthase loses NAD (K_m= 16 μM) fairly easily when substrate is absent from the solution. Dr. M. Hager tested ADPR, an NAD analogue lacking the nicotinamide ring, as potential inhibitor of MIP synthase and found that ADPR was a very weak inhibitor ($K_{iADPR} \approx$ 350 μM).[112] ADPR displays 20 times less affinity for MIP synthase active site than NAD. Since the sugar part of the cofactor does not share strong interactions with the enzyme active site, the reduced nicotinamide ring moiety might be the part of the cofactor involved in the most important interactions with the active site.

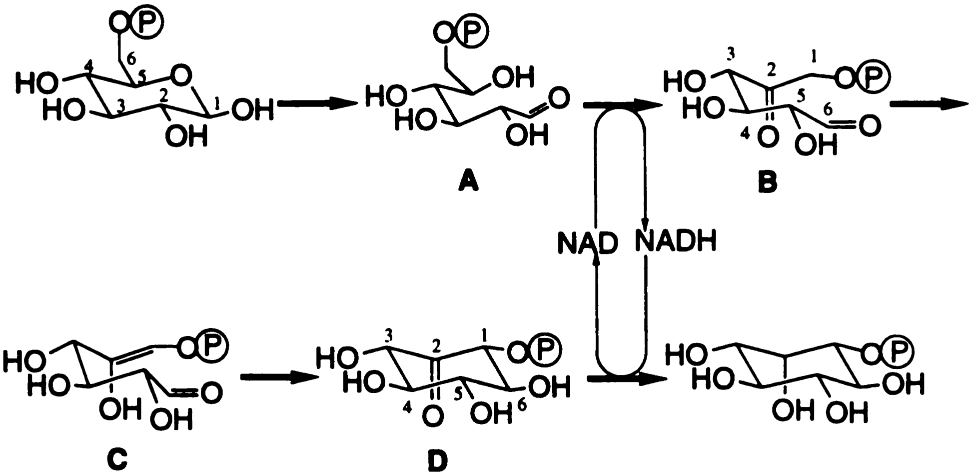
If the intermediate **A** is initially oxidized to intermediate **B'** then converted to intermediate **B** by rotation of the phosphate moiety around the C4-C5 bond of the substrate, then the reduced nicotinamide adenine dinucleotide must rotate to occupy the appropriate position for the reduction of *myo*-2-inosose 1-phosphate **D** (Scheme 3) to MIP. The dihydronicotinamide should undergo the conformational reorientation required for the transport of the hydride at any steps during the conversion. Even though this type of migration can be possible, movement of a molecule such as NADH in the enzyme active site is somewhat arguable, considering the least motion principle so common to enzymes.[107] The sequence **A→B'→B** cannot be discarded as a possible succession of events, but, the sequence **A→A'→B** is more likely to occur since no migration of the cofactor is required during turnover of the substrate to product.

In conclusion, studies of substrate analogues suggest that all forms of G6P bind MIP synthase. Cyclic glucose 6-phosphates could be considered as substrates while the acyclic glucose 6-phosphate would bind as an actual reaction intermediate. Acyclic inhibitors can be viewed as reaction intermediate analogues, which would explain their efficiency in MIP synthase inhibition. Results also indicated that the C-3 and C-4 hydroxyl groups are important for enzyme recognition and binding while the C-1 is required in the ring opening step and the C-2 is involved in the enolization/condensation steps. It was shown that the phosphate moiety probably binds to two different locations of the enzyme active site. The first binding pocket would be used for recognition and initial steps (binding and ring opening) while the second would be involved in the latter steps of the conversion (probably oxidation and condensation).

CHAPTER IV

USE OF REACTION INTERMEDIATE AND ANALOGUES AS INHIBITORS OF MIP SYNTHASE

DHQ synthase is a premier example of the steady evolution of mechanism-based strategies directed toward inhibition of an enzyme. Effective competitive inhibition of DHQ synthase was first achieved with organophosphonate analogues of substrate DAHP ($K_i \approx 10^{-6}$ M).[55a,b] Efficiency in DHQ synthase inhibition was then improved by a 1000 fold with slow-binding, carbacyclic analogues[48,55c,d] of DAHP (for example: **17**, Scheme 6). Finally, apparent irreversible inhibition of DHQ synthase was achieved in the presence of ketocarbaphosphonate **18**,[56] a preoxidized reaction intermediate analogue (Scheme 6). This last approach using preoxidized reaction center containing species could be successfully applied to design inhibitors of MIP synthase. Carbonyl-containing analogues of reaction intermediates **B**, **C** and **D** (Scheme 27) might be effective inhibitors of MIP synthase and result in inhibition other than competitive. Identification of a new type of MIP synthase inactivator would be valuable for enzyme inhibition as well as for mechanistic studies.



Scheme 27: Postulated MIP synthase mechanism, MIP numbering of carbons.

Background

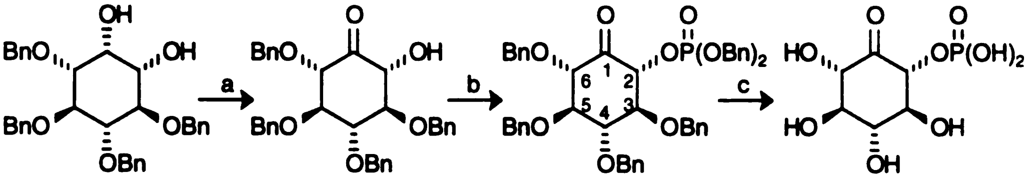
Formation of a tightly bound enzyme-NADH-oxidized substrate analogue inhibitor complex has been reported to be accountable for slow-binding inhibition of DHQ synthase.[48a] In the case of MIP synthase, it has been shown that the enzyme tightly binds species which can effectively challenge the substrate for active site occupancy and which can be linearized as intermediate **A** or **B** analogues. NADH formation and concomitant oxidation of inhibitors have also been observed, yet they resulted in competitive inhibition (Chapter III). The MIP synthase-generated, carbonyl-containing form of inhibitors have to be reduced by NADH before their release from the enzyme active site. The reduction required for the release of the inhibitor would not be possible if the species binding to MIP synthase containing NAD was already oxidized. Challenging DHQ synthase with species containing an oxidized reaction center led to time dependent apparent irreversible inhibition.[56] Similar inhibition of MIP synthase could be conceived if the enzyme is unable to clear bound species from its active site for a lack of NADH. A substrate analogue with its reaction center already oxidized, such as intermediate **B**, **C** or **D** analogues could bind to the enzyme-NAD complex. Lacking NADH, the enzyme active site would not be able to reductively process the oxidized center. Release of the intermediate analogue may then be slow or possibly irreversible due to binding interactions between the oxidized center and the enzyme active site.

Indirect evidence supporting the catalytic role of NADH and the involvement of putative intermediates **B**, **C** and **D** (Scheme 27) in the conversion of D-glucose 6-phosphate to *myo*-inositol 1-phosphate catalyzed by MIP synthase follows from a number of experiments presented in earlier chapters. Multiple experiments strongly supported the fact that **B** is a reaction intermediate. In contrast, experiments designed to establish binding of *myo*-2-inosose 1-phosphate **D** to the catalytic active site of MIP synthase during turnover are less conclusive. Intermediate **D** has never been synthesized, characterized, or used to demonstrate its half reaction with the enzyme active site possessing bound NADH.

As potential enzyme inhibitors, stable analogues of reactive intermediates have typically been the focus of synthetic and enzymological attention given the reactivity and relative instability of most reaction coordinate intermediates. In contrast, intermediate **B** was easily synthesized by various routes and has already been shown to interact with NADH-bound MIP synthase active site.[30] Challenging NAD-bound MIP synthase with intermediate **B** and intermediate **B** analogues is now carried out by Jirong Peng. Sherman[49] isolated *scyllo*-inositol-phosphate after reduction of what he proposed to be intermediate **D** obtained from base catalyzed cyclization of **B**. He showed that putative reaction intermediate **D** should be stable in solution since its reduced forms could be isolated. *myo*-2-Inosose 1-phosphate **D** (Scheme 27) appears to be a molecule possessing sufficient stability to allow its synthesis and isolation as well as its characterization as actual reaction intermediate.

Intermediate D: *myo*-2-Inosose 1-Phosphate

Previous attempts to synthesize *myo*-2-inosose 1-phosphate have been reported to be unsuccessful, but no indication of the strategy employed for this endeavor was given.[37] In the present approach, intermediate **D** was synthesized as a racemic mixture in 15% overall yield from *myo*-inositol.[113] The dibutyl stannylene acetal of diol **48** (Scheme 28) was prepared by refluxing **48** in methanol in the presence of dibutyl tin oxide.[114] After methanol removal and exchange for dichloromethane, the tin derivative was oxidized with bromine in the presence of tributyl tin methoxide used as a proton trap.[115] The products of oxidation constituted a 3:1 regioisomeric mixture from which ketone **71** was purified by crystallization. The structure of ketone **71** was confirmed by comparison of the product of hydrogenolysis to commercially available *myo*-2-inosose. Subsequent reaction of ketone **71** with sodium hydride and tetrabenzylpyrophosphate afforded benzylated phosphoroketone **72** and an α,β-unsaturated phosphoroketone resulting from the elimination of C-3 benzyl alcohol of **72**. Although the elimination side product could be minimized by use of NaH that was free of NaOH contamination, chromatography followed by crystallization was required to obtain pure phosphoroketone **72**. All the benzyl protecting groups were then removed in a single step by catalytic hydrogenolysis to afford *myo*-2-inosose 1-phosphate **D**.

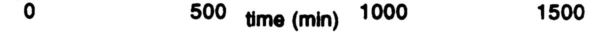


48 **71** **72** **73, D**

(a) (i) Bu_2SnO, MeOH, (ii) Br_2, Bu_3SnOMe, 50%. (b) (i) NaH, THF, 0°C, (ii) TBPP, NaH, 0°C to rt, 61%. H_2, Pd/C, THF/H_2O, (6:1), 99%.

Scheme 28: Synthesis of *myo*-2-inosose 1-phosphate, **D**.

In order to support the hypothesis that *myo*-2-inosose 1-phosphate **D** was a reaction intermediate in the conversion of G6P into MIP, a similar experiment to the one carried out by Barnett[30] on intermediate **B** was elaborated. MIP synthase was treated with activated charcoal at 4°C for 20 min. NAD-complexed charcoal was then removed by centrifugation and filtration. Addition of NAD and substrate to a solution containing *apo*MIP synthase showed that only a few percent of the enzyme activity were lost during the charcoal treatment. NAD-reconstituted enzyme had recovered more than 90% of its original specific activity. When *apo*MIP synthase was treated with NADH, addition of putative intermediate **D** resulted in a loss of NADH that was followed by UV spectroscopy at 340 nm. The time-dependent decrease in NADH concentration is shown Scheme 29. The decrease corresponds to the oxidation of NADH to NAD. Formation of *myo*-inositol 1-phosphate from *myo*-2-inosose 1-phosphate was established by FAB (fast atom bombardment) mass spectrometry. These results confirm the enzyme-catalyzed half reaction whereby *myo*-2-inosose 1-phosphate **D** is reduced by NADH resulting in product *myo*-inositol 1-phosphate formation. Accumulation of MIP without formation of G6P also indicates that the cyclization step is not reversible.

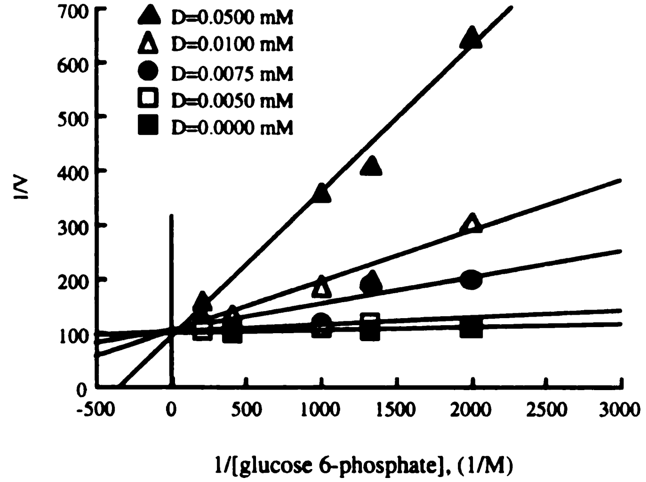


(▲) NADH oxidation rates for control solutions containing either MIP synthase reconstituted with NADH or intermediate **D** and NADH. (■) NADH oxidation rates for a solution containing MIP synthase reconstituted with NADH and intermediate **D**.

Scheme 29: NADH depletion rates.

Inhibition of MIP Synthase by Its Own Reaction Intermediate

The nature of the interaction between reaction intermediate **D** and MIP synthase depended on the redox state of the enzyme-bound cofactor. When *myo*-2-inosose 1-phosphate challenged the substrate G6P for the active site of MIP synthase with bound NAD, competitive inhibition was observed (Scheme 30). The observed inhibition constant (K_i) of 3.6×10^{-6} M contrasts with the Michaelis constant (K_m) of 1.2×10^{-3} M for substrate G6P. *myo*-2-Inosose 1-phosphate is among the most potent inhibitors of MIP synthase. It is worth noting that this inhibition occurs in the absence of the ternary complexes consisting of MIP synthase with bound NAD and linear intermediate **A** analogue or NADH and oxidized inhibitor which were proposed as the causes of efficient inhibition of MIP synthase by substrate analogues (Chapter III).



Scheme 30: Intermediate **D** Lineweaver-Burk replot.

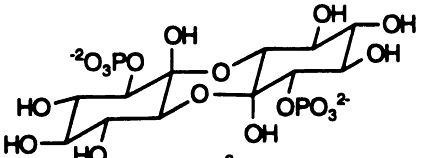
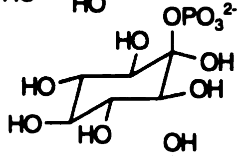
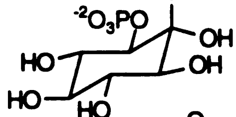
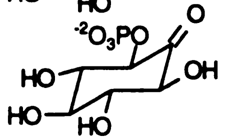
When compared to the results obtained for DHQ synthase inhibition by intermediate analogues possessing preoxidized reaction center, the present results are somewhat disappointing. Native DHQ synthase challenged with ketocarbaphosphonate **18** resulted in apparent irreversible inhibition while native MIP synthase challenged with preoxidized species resulted in conventional competitive inhibition yielding an inhibition constant which is slightly more potent than substrate analogue inhibitors (Table 6). Explaining the discrepancy in the effectiveness of the strategy requires a close examination of the interactions between intermediate **D** and MIP synthase active site.

Aqueous Solution Chemistry of Intermediate D

Catalytic hydrogenation of the fully benzylated precursor in THF/H_2O is the final step in the synthesis of *myo*-2-inosose 1-phosphate. After removal of the THF/H_2O, exchangeable protons in the residue were replaced with deuterium atoms to facilitate ^1H NMR analysis by redissolving the residue in D_2O followed by concentration. This exchange protocol was repeated three times prior to redissolving in DMSO-d_6 or D_2O for NMR characterization. The keto form of *myo*-2-inosose 1-phosphate **73** (Table 9) was the only species in DMSO-d_6 to be detected by ^1H, ^{13}C and ^{31}P NMR. Confirmation of the structure assignment followed from two-dimensional homonuclear (^1H, ^1H) COSY and heteronuclear (^1H, ^{31}P) HMQC.

Samples of *myo*-2-inosose 1-phosphate treated identically to those analyzed in DMSO-d_6 yielded multiple species when dissolved in D_2O. Structures consistent with the assigned ^1H and ^{13}C NMR resonances are displayed in Table 9. For aqueous solutions of *myo*-2-inosose 1-phosphate at pH 2, a ^{13}C NMR resonance at δ 206 consistent with the keto form **73** of the carbonyl was evident, although another resonance consistent with the chemical shift of a hydrated inosose **74** carbonyl was observed at δ 96. Analysis of this same solution by negative ion FAB MS indicated a molecular ion at (M-H^+) 257 for the carbonyl form of *myo*-2-inosose 1-phosphate **73** and a molecular ion at (M-H^+) 275 for the hydrate form **74** of *myo*-2-inosose 1-phosphate. Hydration of *myo*-2-inosose in aqueous medium is precedented with ^{13}C NMR resonances reported at δ 206.6 and 95 for the carbonyl carbons of the keto and hydrated forms.[116]

Table 9: *myo*-2-Inosose 1-phosphate forms in solution.

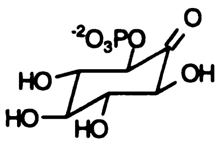
		pH 2 DMSO	pH 2 D_2O	pH 7 D_2O
	**76**	no	no	yes
	**75**	no	yes?	yes?
	**74**	no	yes	yes
	**73**	yes	yes	no

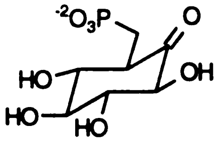
After adjustment of *myo*-2-inosose 1-phosphate solutions from pH 2 to pH 7, resonances consistent with keto form **73** of intermediate **D** could no longer be detected by either ^1H or ^{13}C NMR. Hydrated form **74** was still in evidence along with resonances indicating the presence of newly formed species. ^1H NMR analysis suggested that one of these newly formed species was dimeric *myo*-2-inosose 1-phosphate **76**. The presence of dimeric *myo*-2-inosose 1-phosphate **76** was confirmed by negative ion FAB MS with the observation of a molecular ion at (M-H^+) 515.

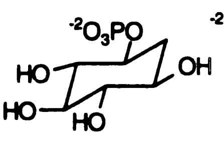
Based on NMR analysis, the keto form of intermediate **D** in neutral aqueous solution could not be detected. Reduction of intermediate **D** in neutral aqueous solution by MIP synthase with bound NADH, occurs either if the substrate is *myo*-2-inosose 1-phosphate ketone form **73** in equilibrium with the other solution forms of intermediate **D**, or if other solution species are also substrate. In the same manner, inhibition at pH neutral of NAD-bound MIP synthase by intermediate **D** can be due to keto form **73** present in steady-state concentration or to other solution species.

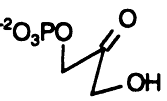
To determine the ketone's contribution to active site interactions for *myo*-2-inosose 1-phosphate and to gauge the utility of reaction center oxidation in designing MIP synthase inhibitors, a series of *myo*-2-inosose 1-phosphate analogues (Table 10) were examined.[61] These analogues include 1-deoxy-1-(phosphonoxymethyl)-*myo*-2-inosose **77**, dihydroxyacetone 1-phosphate **78**, 2-deoxy-*myo*-inositol 1-phosphate **67**, *myo*-inositol 1-phosphate MIP and *scyllo*-inositol phosphate **79**. By substituting the phosphorylated oxygen of *myo*-2-inosose 1-phosphate with a methylene group in 1-deoxy-1-(phosphonoxymethyl)-*myo*-2-inosose **77**, the reduced substituent electronegativity adjacent to the carbonyl was anticipated to ensure the presence of a sizable concentration of keto form at pH 2. The keto form of dihydroxyacetone 1-phosphate **78** is known to be one of the dominant forms of this molecule present in neutral aqueous solutions. 2-deoxy-*myo*-inositol 1-phosphate **67** was used to evaluate the impact of completely removing the oxidized reaction center of *myo*-2-inosose 1-phosphate. MIP and *scyllo*-inositol 1-phosphate **79** are considered analogues of intermediate **D** hydrated form, **74**.

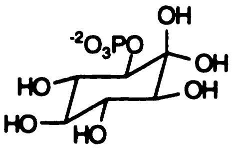
Table 10: Intermediate **D** and intermediate **D** analogues.

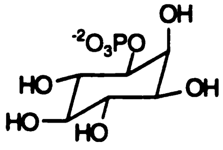

73

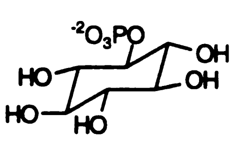

77


67


78


74

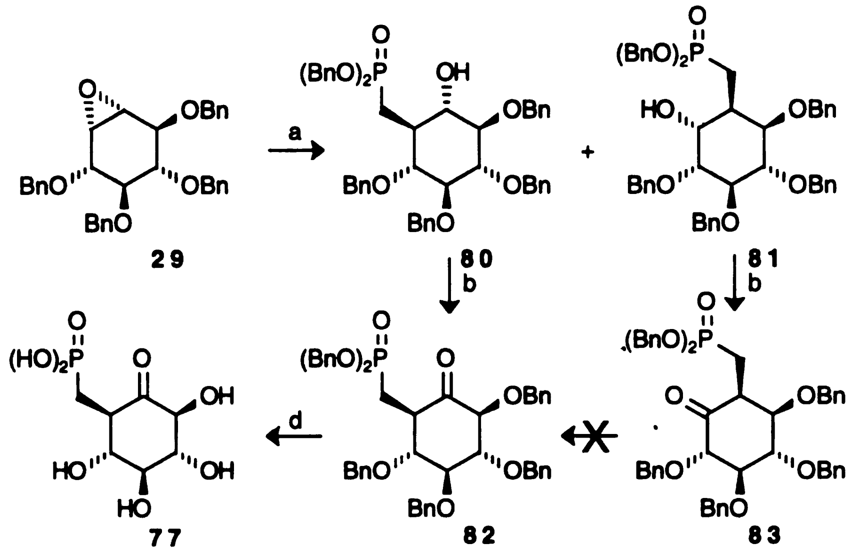

MIP


79

Reaction of Preoxidized Reaction Centers with NADH Catalyzed by MIP Synthase

NADH reconstituted enzyme experiment is the only method available to indicate that MIP synthase has access to the keto form of *myo*-2-inosose 1-phosphate in neutral aqueous solution. Confirmation that *apo*MIP synthase reconstituted with NADH is a valid method for detecting small, steady-state concentrations of *myo*-2-inosose 1-phosphate keto form **73** became possible with the synthesis 1-deoxy-1-(phosphonoxymethyl)-*myo*-2-inosose[61] **77** and the preparation of dihydroxyacetone phosphate.

Reaction of dibenzyl (lithiomethyl) phosphonate with tetrabenzyl conduritol oxide B **29** (Scheme 17) in the presence of boron trifluoride etherate as a Lewis acid provided racemic phosphonomethylated regioisomers **80** and **81** (Scheme 31). Separation of **80** from **81** led to diffractable crystals of **81** that were used to confirm the structure assignments by X-ray crystallography. This reaction follows the methodology presented in Chapter II. Swern oxidation[90] of regioisomers **80** and **81** in separate reactions afforded the respective phosphonomethylated inosose **82** and **83** in approximately equal yields. Chemical epimerization of the axial phosphonomethyl substituent of inosose **83** was explored as a potential route for obtaining additional amounts of the desired phosphonomethylated inosose **82** possessing all equatorial ring substituents. However, acidic conditions such as THF/aq HCl (1 N) or THF/aq CH_3COOH afforded only unreacted material, even when treated at reflux. Basic conditions such as THF/aq. NaOH, or THF/LDA yielded the α,β-unsaturated phosphonomethylated inosose resulting from elimination of the C-5 benzyl ether or unreacted starting material if the reactions were carried out at low temperature. Consequently, only inosose **82** was used to complete the synthesis of **77**. Catalytic hydrogenolysis removed all the benzyl protecting groups in a single step. Modification of the solution pH from neutral by either addition of base or acid yielded an α,β-unsaturated phosphonomethylated inosose. Facile elimination was also observed when tetrabenzylated 1-(dimethyl phosphonomethyl) *myo*-2-inosose, obtained from phosphonomethylation of conduritol oxide derivative **29** with dimethyl (lithiomethyl) phosphonate, was treated with TMSBr[117] to remove the methyl groups of the phosphonate moiety.



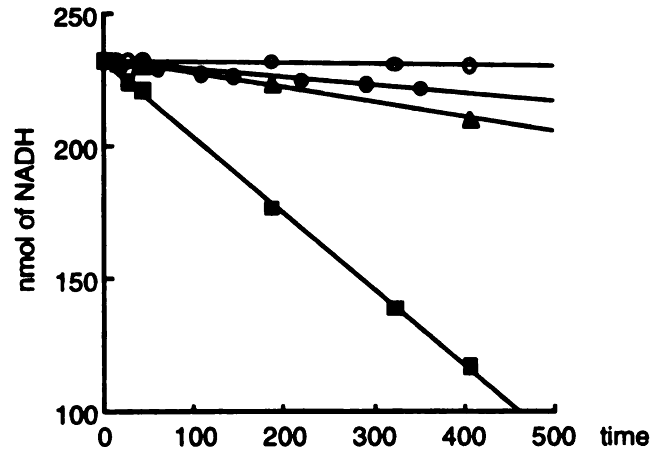
(a) $LiCH_2PO(OBn)_2$, BF_3-OEt_2, THF, -78°C to -20°C, 74%; (b) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, -78°C, (**82**) 89%, (**83**) 94%; (d) H_2, Pd/C THF/H_2O, 4:1, 100%

Scheme 31: Synthesis of 1-deoxy-1-(phosphonoxymethyl)-*myo*-2-inosose.

Attempts were made to obtain a phosphonate analogue of **80** starting from **32**, the iodomethyl-derivative product of (lithiomethyl) dimesityl borane methodology (Chapter II). After acetylation of the secondary alcohol, various reaction conditions were examined in an attempt to introduce a phosphonate moiety via Arbuzov methodology.[118] Trimethyl phosphine, triethyl phosphine and triisopropyl phosphine were examined as reagents for Arbuzov reaction. Parameters such as temperature, pressure (sealed tube), and concentration were also modified. Either starting material was recovered or complete decomposition occured. This type of behavior from the *myo*-inositol series has already been reported by Kulagowski.[69] Attempts to carry out Michaelis-Baker methodology on modified diol **30** were also unsuccessful. Diol **30** was derivatized as a tosylate and the second alcohol was oxidized under various conditions. The product of oxidation was never isolated in good yields or as a clean material. Direct phosphonomethylation methodology was the only method affording access to the appropriate precursors of 1-deoxy-1-phosphonomethyl-*myo*-2-inosose. The key features of 1-deoxy-1-(phosphonoxymethyl)-*myo*-2-inosose **77** are its close structural similarity to *myo*-2-inosose 1-phosphate **73** and the presence of only the keto form of the oxidized reaction center in aqueous solution at pH neutral, confirmed by ^{1}H NMR, ^{13}C NMR and (^{1}H, ^{1}H) COSY.

Dihydroxyacetone phosphate was prepared by hydrolysis of the commercially available dihydroxyacetone phosphate diethyl ketal in the presence of Dowex 50 ($^+$H form).[119] In neutral aqueous solution, dihydroxyacetone phosphate exists as an approximately 1:1 mixture of ketone **78** and hydrate. Other than its defined solution chemistry, dihydroxyacetone phosphate provides insights into the minimum subset of *myo*-2-inosose 1-phosphate's structural features required for active site binding.

*Apo*MIP synthase treated with NADH was oxidized by 1-deoxy-1-(phosphonoxymethyl)-*myo*-2-inosose **77**, although at a slower rate than that observed for intermediate **D** (Scheme 32). Dihydroxyacetone 1-phosphate **78** also oxidized (Scheme 32) the complex of *apo*MIP synthase and NADH. Weaker interaction between dihydroxyacetone phosphate **78** and MIP synthase with bound NADH are indicated by the slower rate that this enzyme complex is oxidized by dihydroxyacetone phosphate **78** relative to *myo*-2-inosose 1-phosphate **D** and 1-deoxy-(phosphonomethyl)-*myo*-2-inosose **77**. Establishing that the enzyme-bound NADH oxidation was due to the reduction of 1-deoxy-1-(phosphonoxymethyl)-*myo*-2-inosose **77** and dihydroxyacetone 1-phosphate **78** followed, respectively, from detection of 1-deoxy-1-(phosphonoxymethyl)-*myo*-inositol and glycerol 3-phosphate formation by FAB mass spectroscopy. Therefore, NADH-bound MIP synthase experiment is valuable to detect the keto form **73** of intermediate **D** in aqueous solution at pH 7 since NADH-bound MIP synthase reduces species present in solution in keto form.



NADH oxidation rates for a control solution (o) containing only the complex of NADH and apoMIP synthase relative to this same solution upon addition of **79** (●), **77** (▲) and myo-2-inosose 1-phosphate **D** (■).

Scheme 32: NADH depletion rates.

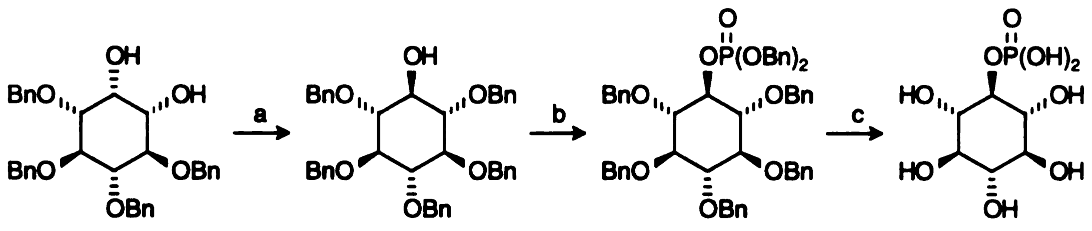
When incubation of *apo*MIP synthase in the presence of NADH and intermediate **D** was long enough, FAB MS did not detect any *myo*-2-inosose 1-phosphate. It is thought that *myo*-2-inosose 1-phosphate was totally reduced to *myo*-inositol 1-phosphate, even though intermediate **D** had been synthesized as a racemic mixture. Such a result would indicate either that MIP synthase is not enantioselective at this stage of the transformation or that *myo*-2-inosose 1-phosphate enantiomers can exchange in solution and maintain a racemic equilibrium. Appropriate amount of NADH-treated enzyme and extended reaction time resulted in total reduction (based on FAB MS results) of dihydroxyacetone phosphate **78** into glycerol 3-phosphate. Under similar conditions, only partial reduction of racemic 1-deoxy-1-(phosphonoxymethyl)-*myo*-2-inosose **77** was recorded. Mass spectroscopy showed that 1-deoxy-1-(phosphonoxymethyl)-*myo*-inositol and 1-deoxy-1-(phosphonoxymethyl)-*myo*-2-inosose **77** were present in comparable quantities. When more NADH-bound MIP synthase was added to the solution, no further consumption of NADH was recorded by UV spectroscopy. This suggests that MIP synthase only reduces one of the two 1-deoxy-1-(phosphonoxymethyl)-*myo*-2-inosose **77** enantiomers and therefore is enantioselective. This result could imply that in aqueous solution at neutral pH, the various forms of intermediate **D** are in equilibrium and that ultimately these forms are converted to the keto form **73** which is then reduced by NADH-bound MIP synthase. This keto form is at least present in the active site when the reduction by NADH occurs. Racemic intermediate **D** can only be converted to enantiomerically pure L-MIP if DL-intermediate **D** can equilibrate to L-intermediate **D** via a mesomeric intermediate. Such an intermediate could be species **75** (Table 9). The hydrated form of *myo*-2-inosose has been reported as a strong singlet by ^{1}H NMR.[119] A similar singlet is observed in ^{1}H NMR spectrum of *myo*-2-inosose 1-phosphate in D_2O at pH 2 and pH 7. To this strong singlet could be assigned structure **75**. However, no further NMR data, FAB MS or other spectrometric methods were available to confirm the presence of such a species in solution.

Preoxidized Reaction Center Inhibition of MIP Synthase

In the presence of *apo*MIP synthase initially reconstituted with NADH, *myo*-2-inosose 1-phosphate **D**, 1-deoxy-1-(phosphonoxymethyl)-*myo*-2-inosose **77** and dihydroxyacetone phosphate **78** were converted to MIP, 1-deoxy-1-(phosphonoxymethyl)-*myo*-inositol and glycerol 3-phosphate, respectively, along with the oxidation of enzyme-bound NADH. These same species competitively inhibited the native form of MIP synthase having enzyme-bound NAD. The respective inhibition constants are given Table 11.

During turnover, oxidized reaction intermediates (**B**, **C** and **D**) are present in MIP synthase active site simultaneously with NADH. Potent inhibition of NAD-bound MIP synthase by preoxidized center containing species might result from only modest active site interactions and an absence of a kinetically accessible escape route for the release of the bound inhibitor. Inhibition of MIP synthase by reaction intermediate and reaction intermediate analogues can also be interpreted as arising from strong interactions between functional groups possessed by **D** and its analogues and the active site residues normally responsible for binding the reactive intermediate. Inhibition of native MIP synthase by intermediate **D** might thus be due to the stabilizing interactions between enzyme and one of its reaction intermediates. The oxidized reaction center could be the functional group responsible for initiating tight binding and efficient inhibition. This section is aimed at probing the importance of the oxidized reaction center in **D** to the active site interactions and gauge the relative contribution of the keto form to these interactions.[61]

The impact of completely removing the oxidized reaction center of *myo*-2-inosose 1-phosphate **73** was assessed by 2-deoxy-*myo*-inositol 1-phosphate **67**. To estimate whether the hydrated form **74** is able to bind NAD-bound MIP synthase active site, MIP and *scyllo*-inositol 1-phosphate **79** are considered as monodeoxy analogues of the inosose hydrate **74**. *scyllo*-Inositol 1-phosphate **79** was synthesized from diol **48** (Scheme 33).[120] A refluxing benzene solution containing diol **48**, benzyl bromide, sodium hydride and a catalytic amount of tetrabutylammonium iodide was used to selectively benzylate the equatorial hydroxyl of diol **48**. Successive Swern oxidation of the remaining hydroxyl



a. i. NaH, BnBr, Bu_4NI, C_6H_6, rt, 62%, ii. $(COCl)_2$, DMSO, CH_2Cl_2, Et_3N, iii. $NaBH_4$, EtOH, 25%; b. $(iPr)_2NP(OBn)_2$, ^1H-tetrazole, CH_2Cl_2, *m*CPBA, 82%; c. H_2, Pd/C, THF/H_2O, 4:1, 100%.

Scheme 33: Synthesis of *scyllo*-inositol phosphate.

group and sodium borohydride reduction yielded a separable 3:1 mixture of *myo*- and *scyllo*-inositol derivatives. The pentabenzylated *scyllo*-inositol was hydrogenolized after phosphorylation to yield *scyllo*-inositol 1-phosphate **79**.

Table 11: Inhibition of MIP synthase.

	Type of Inhibition	K_i	K_m/K_i	pH
Intermediate D	competitive	3.6×10^{-6}	330	7.2
67	competitive	170×10^{-6}	7.1	7.2
77	competitive	160×10^{-6}	7.5	6.4
	competitive	37×10^{-6}	32	7.2
	competitive	6.4×10^{-6}	190	8.0
78	competitive	7.0×10^{-4}	1.7	7.2
MIP	competitive	$I_{50} = 7.6 \times 10^{-3}$	-	7.2
79	no inhibition	-	-	7.2

Table 11 summarizes the results of measured MIP synthase inhibition. The K_i for 2-deoxy-*myo*-inositol 1-phosphate **67** provides a measure of the oxidized center's contribution to the active site interactions between MIP synthase and *myo*-2-inosose 1-phosphate. Complete removal of the oxygen of the oxidized center *myo*-2-inosose 1-phosphate to give the methylene group in **67** resulted in a 47-fold reduction in inhibitory potency. 1-Deoxy-1-(phosphonoxymethyl)-*myo*-2-inosose **77** was a better inhibitor of MIP synthase at pH 7.2 than **67** but a significantly weaker inhibitor relative to *myo*-2-inosose 1-phosphate. The inhibitory potency of 1-deoxy-1-(phosphonoxymethyl)-*myo*-2-inosose was observed to vary significantly with solution pH (Table 11). Inhibition constants of 160, 37 and 6.4 μM were measured at, respectively, pH 6.4, 7.2 and 8.0. This variation in the active site interactions as a function of solution pH has been observed for other phosphonate monoesters and may reflect the reduced acidity of the second dissociable proton of a phosphonic acid relative to the acidity of the second dissociable proton of a phosphate monoester.

Tritrimetric analysis of *myo*-2-inosose 1-phosphate indicated two inflection points. Although *myo*-2-inosose 1-phosphate exists as a mixture of solution forms, these two inflection points can be broadly interpreted to correspond to dissociable protons with pK_{a1}= 2.5 and pK_{a2}= 5.6. Tritrimetric inflection points of glucose 6-phosphate were determined to correspond to dissociable protons with pK_{a1}= 2.2 and pK_{a2}= 5.9. The acidic protons of 1-deoxy-1-(phosphonoxymethyl)-*myo*-2-inosose were characterized by noticeably reduced acidity with dissociation constants K_{d2}= 2.5 x 10^{-4} and K_{d1}= 5.0 x 10^{-8} (pK_{a1}= 3.6 and pK_{a2}= 7.3). Under more basic conditions (pH 8.0), the higher concentration of the dianionic form of DPMI may account for the phosphonic acid's improved inhibitory potency.

Although 1-deoxy-1-(phosphonoxymethyl)-*myo*-2-inosose was nearly as potent an inhibitor as *myo*-2-inosose 1-phosphate at the solution pH where it was in its fully dissociated ionization state, this inhibition was much weaker than expected. After all, 1-deoxy-1-(phosphonoxymethyl)-*myo*-2-inosose **77** is an analogue that existed exclusively in its keto form and is structurally similar to the *myo*-2-inosose 1-phosphate keto form **73**. Reduction in binding affinity between enzyme active site and inhibitor may have resulted from replacement of the phosphate monoester oxygen with a methylene group in 1-deoxy-1-(phosphonoxymethyl)-*myo*-2-inosose **77**. Nonetheless, inhibition of MIP synthase by 1-deoxy-1-(phosphonoxymethyl)-*myo*-2-inosose **77** establishes that the keto form of *myo*-2-inosose 1-phosphate **73** is probably an inhibitor. However, what if the hydrate form of *myo*-2-inosose 1-phosphate contributes along with the keto form to MIP synthase inhibition? This could explain why *myo*-2-inosose 1-phosphate, where only small, steady state levels of a keto form are available, is almost as good an inhibitor of MIP synthase as its phosphonate analogue.

To examine possible inhibition of MIP synthase by the hydrate form of myo-2-inosose 1-phosphate, MIP and *scyllo*-inositol 1-phosphate were tested as inhibitors. As shown in Table 11, these species, whose structures were displayed in Table 10, do not bind to MIP synthase. The presence of one hydroxyl group situated adjacent to the phosphate monoester does not lead to enzyme inhibition. This contrasts with 2-deoxy-*myo*-inositol 1-phosphate where a methylene group adjacent to the phosphate monoester does lead to enzyme inhibition. If the hydrate species of *myo*-2-inosose 1-phosphate was responsible to some extent for MIP synthase inhibition, one of these "monodeoxy hydrate" species should bind to some extent to the enzyme active site. The lack of significant enzyme inhibition observed for MIP and **79** is not consistent with the hydrate form of *myo*-2-inosose 1-phosphate contributing significantly to enzyme inhibition.

While 1-deoxy-(phosphonomethyl)-*myo*-2-inosose was investigated for its structural similarities with the keto form **73** of intermediate **D**, dihydroxyacetone phosphate **78** was examined to ascertain the structural requirements needed in addition to the oxidized reaction center for active site binding. Although dihydroxyacetone phosphate **78** is a much weaker inhibitor relative to *myo*-2-inosose 1-phosphate and 1-deoxy-(phosphonomethyl)-*myo*-2-inosose **77** (Table 11), inhibition of MIP synthase is still significant in the light of the K_m for substrate G6P. This level of inhibition is even more noteworthy given the absence in dihydroxyacetone phosphate **78** of the cyclic, six-membered ring along with three of the hydroxyl groups found on *myo*-2-inosose 1-phosphate. The importance of the peroxidized center is emphasized when dihydroxyacetone phosphate **78** is compared to DL-glycerol 1-phosphate (Table 8) for MIP synthase inhibition. Dihydroxyacetone phosphate **78** can also be viewed as an analogue of acyclic intermediate **B** as well as an analogue of cyclic intermediate **D**.

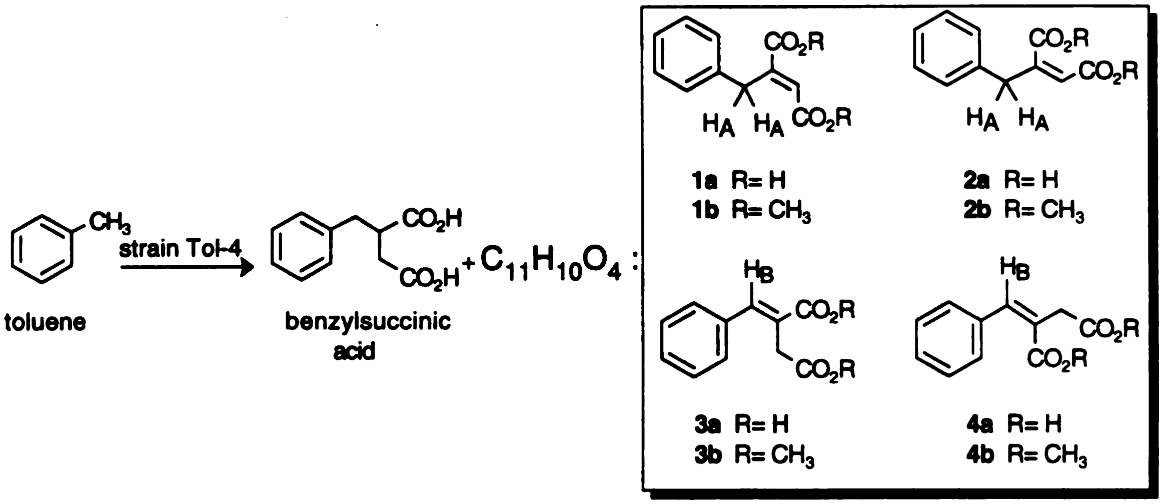
The complex of MIP synthase, NAD and intermediate **D** is not a naturally occurring configuration and yet is a fairly tight complex since micromolar level inhibition is obtained. Reduction of intermediate **D** and oxidation of NADH may be critical to driving an enzyme conformational change that ultimately facilitates the release of MIP. In this respect, inhibition of MIP synthase may not be due to strong stabilizing interactions between active site and *myo*-2-inosose 1-phosphate. Inhibition may be the result of kinetic barriers to the release of *myo*-2-inosose 1-phosphate caused by the absence of reducing equivalents when this intermediate is bound by the native NAD-MIP synthase complex. Simple competitive inhibition of MIP synthase might result from the impossibility for the enzyme to create a tight NAD bound MIP synthase intermediate **D** complex. NAD and NADH are more easily removed from or introduced into MIP synthase active site than into DHQ synthase. The latter binds its cofactors more tightly. It has been shown that some substrate analogues, inhibitors of MIP synthase, were oxidized by NAD-bound MIP synthase. This observation has also been made for DHQ synthase. However, the kinetic outcomes of such a reaction were different. Oxidizable substrate analogues were all competitive inhibitors of MIP synthase while certain inhibitor DAHP analogues[48,55] were slow binding inhibitors. The improvement in inhibition when NAD-bound MIP synthase was to be challenged by putative intermediate and intermediate analogues was not as intense as it was expected. All of these observations clearly distinguish MIP synthase from DHQ synthase in its manner of handling inhibitors. However, it is clear that MIP synthase inhibition by preoxidized center containing intermediate analogues is a valid strategy but it is limited by the versatility of MIP synthase catalytic cycle.

CHAPTER V

ADDENDUM

SYNTHESIS AND CHARACTERIZATION OF FOUR DICARBOXYLIC ACIDS WITH THE CHEMICAL FORMULA: $C_{11}H_{10}O_4$

Benzylsuccinic acid and benzylfumaric acid (**1a**, Scheme 34) have been reported to accumulate during anaerobic degradation of toluene under denitrifying conditions by strains T1,[121] *Pseudomonas* sp. strain T[122] and *Thauera aromatica* K172[123] as well as under sulfate-reducing conditions by strain PRTOL.[124] *Azoarcus tolulyticus* Tol-4,[125] a microbe newly characterized by the Tiedje laboratory at Michigan State University, has likewise been discovered to accumulate two metabolites during anaerobic degradation of toluene under denitrifying conditions. One of these metabolites was identified as benzylsuccinic acid.[125] However, identification of the second metabolite proved to be more elusive. Electron impact mass spectroscopy (EIMS) of the *bis*-silylated end-product established the metabolite molecular weight as being *m/z* 196, corresponding to the molecular formula $C_{11}H_{10}O_4$. The unknown metabolite was either a phenylmethylbutenedioic acid (**1a** and **2a**, Scheme 34) or a phenylmethylene-butanedioic acid (**3a** and **4a**, Scheme 34). Beyond the carbon backbone pattern of the second metabolite, the location and the substitution pattern of the double-bond were not identifiable. Benzylfumaric acid [*E*-(phenyl-methyl)butene-dioic acid] (**1a**), benzylmaleic acid [*Z*-(phenylmethyl)butenedioic acid] (**2a**), *E*-phenyl-itaconic acid [*E*-(phenylmethylene)butanedioic] (**3a**) and Z-phenylitaconic acid [Z-(phenylmethylene)butanedioic] (**4a**) had to be considered as candidate structures for the second metabolite (Scheme 34).[126]

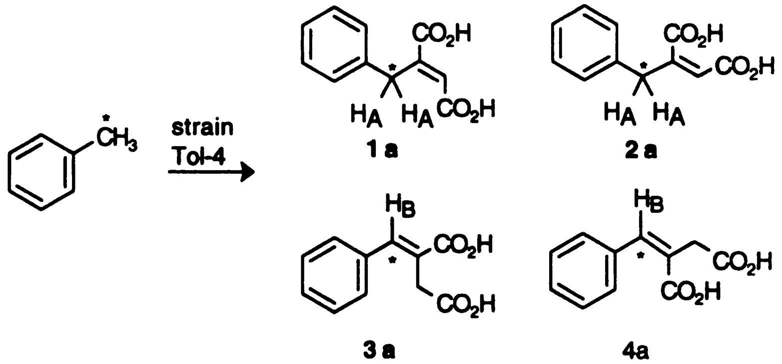


Scheme 34: Possible structures for the second metabolite.

Benzylfumaric acid **1a** had been identified by previous studies as the second end-product formed during the anaerobic microbial degradation of toluene.[121,122] Assignment of a structure to the second metabolite was primarily based on EIMS spectra. Benzylmaleic acid[121] **2a** was thought to be another possible metabolite but because of the similarity of its EIM spectrum, it could not be differentiated from benzylfumaric acid **1a**. Use of this spectroscopic technique to distinguish between the structure of the individual phenylmethylbutenedioic acids (**1a** and **2a**) and the phenylmethylenebutanedioic (**3a** and **4a**) is potentially problematic. Double-bond migration and interconversion of *E*- and *Z*-isomers are phenomena that are documented to occur during MS analysis of olefins.[127] Also, chemical manipulations of the unknown material were required prior to spectroscopic analysis. Metabolites were derivatized as methyl esters in order to allow their injection on a gas chromatography (GC) column in line with mass spectrometer chambers. Three acid derivatization methods had been devised.[121] They included treatment with MeOH, H_2SO_4, and H_2O (2:1:1, v/v), with MeOH and H_2SO_4 (1:1, v/v), or with MeOH and BCl_3. These derivatization techniques also call for concern. Acid-catalyzed migration and isomerization of the double bonds during derivatization of the dioic acids to the corresponding diesters would greatly complicate structure assignments.

Double-bond migration and isomerization would not be a problem with nuclear magnetic resonance (NMR) analysis. ^1H and ^{13}C NMR was then chosen to characterize the second metabolite of toluene anaerobic degradation isolated by Tiedje. However, comparison of the sample spectral data to literature ^1H and ^{13}C NMR spectral data was not feasible. NMR information was only available for **3a**.[128]

Anaerobic degradation of [methyl-^{13}C]toluene by strain Tol-4 was carried out in the Tiedje laboratory by J. Chee-Sanford. The ^{13}C labeled material was isolated by HPLC with a C_{18} reverse phase column and then analyzed by ^1H NMR. The ^{13}C label should result in a large split of the signal corresponding to the proton attached to the labeled carbon (Scheme 35) relative to the same resonance in a metabolite derived from unlabeled toluene. If the metabolite was, as expected from previously published work, benzylfumaric acid **1a**, a methylene carbon should be ^{13}C labeled.



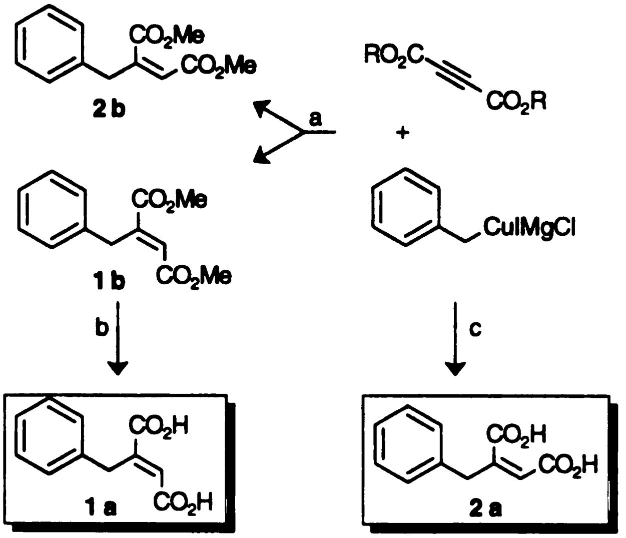
Scheme 35: ^{13}C Labeled toluene and possible ^{13}C labeled metabolites.

Rather surprisingly, the ^1H NMR resonance displaying the expected splitting caused by the ^{13}C labeling occurred at a frequency well downfield of what could be assigned to a methylene proton (H_A, Scheme 35). This was not consistent with the degradation product being either benzylfumaric acid **1a** or maleic acid **2a**. However, such a chemical shift was consistent with ^{13}C-labeling of a vinyl carbon (H_B, Scheme 35) of *E*-phenylitaconic acid **3a** or *Z*-phenylitaconic acid **4a**. Additional evidence was therefore needed to confirm that the unknown metabolite was a phenylmethylenebutanedioic acid (**3a** or **4a**) and to differentiate between an *E* or *Z* olefin substitution pattern. This necessitated the synthesis and detailed spectroscopic characterization of each phenylmethylbutenedioic acid (**1a**, **2a**) and phenylmethylenebutanedioic acid (**3a**, **4a**). For proper comparison and study of GC-EIMS technique reliability to analyze such metabolites, dimethyl ester derivatives of each acid were also synthesized and characterized. Published routes to individual dioic acids and diesters span approximately 30 years of chemical literature. However, some protocols required substantial optimization to afford the desired products.

Synthesis of Dioic Acids of Molecular Formula $C_{11}H_{10}O_4$ and Their Corresponding Dimethyl Esters

Dioic acids **1a**, **3a** and **4a** were synthesized as the corresponding dimethyl diesters **1b**, **3b** and **4b**. The free dioic acids were then obtained by hydrolysis under basic conditions of the corresponding dimethyl diesters. Dimethyl benzylmaleate **2b** was synthesized but it was not the precursor for benzylmaleic acid **2a** . Benzylmaleic acid **2a** was synthesized by deprotection of the corresponding trimethylsilyl diester.

Dimethyl benzylfumarate **1b** and dimethyl benzylmaleate **2b** were prepared by nucleophilic attack of the complex [BnMgCuClI] on dimethyl acetylenedicarboxylate (Scheme 36).[129] Dimethyl benzylmaleate **2b** was obtained in 40% yield while dimethyl benzylfumarate **1b** was formed in 30% yield. This result was only in partial agreement with the published procedure since only dimethyl benzylmaleate **2b** had been observed formerly. The published procedure called for the reaction to be carried out at -78°C but, higher yields and more rapid reaction was observed when the temperature was maintained at -40°C. Quenching of the reaction was particularly important. When the reaction was quenched by addition of water at either temperature, all four dimethyl diester isomers were observed in various proportions. When quenched with saturated NH_4Cl solution, the reaction only afforded dimethyl benzylmaleate **2b** and dimethyl benzylfumarate **1b**.

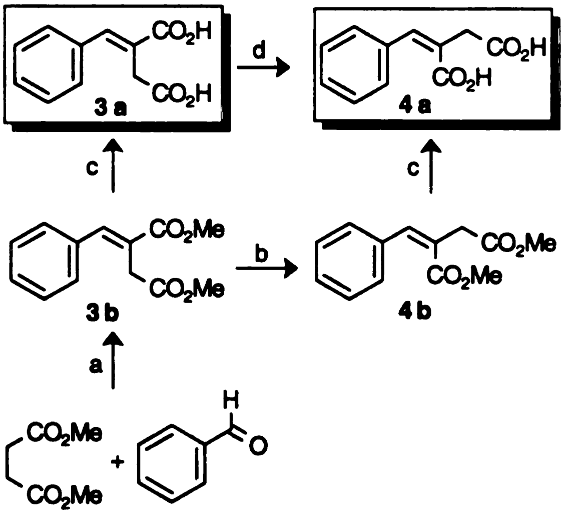


(a) THF, -40°C, R= Me. (b) 0.1N aq NaOH/THF (1:4).
(c) THF, -40°C, R= $SiMe_3$.

Scheme 36: Synthesis of benzylfumaric and benzylmaleic acid and ester derivatives.

In concentrated basic conditions (1N NaOH/THF, 4:1, v/v), **1b** as well as **2b** are deprotected with simultaneous modification of the carbon backbone. Dimethyl benzylfumarate **1b** was the most stable to strong basic conditions of the two, yet benzylfumaric acid **1a** was isolated after hydrolysis in a 9:1 ratio with *E*-phenylitaconic acid **3a**. Under these conditions, dimethyl benzylmaleate **2b** was only converted to *E*-phenylitaconic acid **3a** and benzylfumaric acid **1a.** Dilute basic (10 mM aq NaOH; THF, 1:4, v/v) aqueous treatment of **1b** afforded benzylfumaric acid **1a**, while dimethyl benzylmaleate **2b** was still isomerized to benzylfumaric acid **1a** (Scheme 36). Benzylmaleic acid **2a** was then synthesized by reacting bis(trimethylsilyl)-2-butynedioate with benzyl magnesium bromide in the presence of cuprous iodide, followed by acid hydrolysis, as previously described.

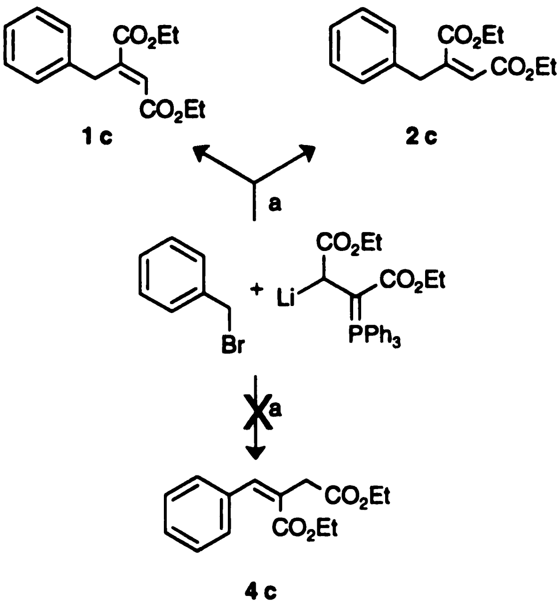
Dimethyl *E*-phenylitaconate **3b** was obtained following a modified procedure which involved the condensation of dimethyl succinate on benzaldehyde catalyzed by MeONa (Scheme 37).[130] Acid quench was necessary to prevent the rapid hydrolysis of one of the two methyl esters. Dimethyl *E*-phenylitaconate **3b** was the major product of the reaction, while dimethyl *Z*-phenylitaconate **4b** and dimethyl benzylfumarate **1b** were isolated in trace amounts. Production, in reasonable yield, of dimethyl *Z*-phenylitaconate **4b** was accomplished by photochemical isomerization of dimethyl *E*-phenylitaconicate **3b**.[131] When taken in a mixture of acetone and $CHCl_3$ and irradiated at 300 nm in a Rayonnet apparatus, the dimethyl *E*-phenylitaconicate **3b** isomerized into dimethyl *Z*-phenylitaconate **4b** in a maximal 60% conversion. Modification of the solvent system for benzene did not improve yields nor reaction rates. Separation of **4b** from **3b** was easily performed by silica gel chromatography.



(a) Na, MeOH. (b) CH_3Cl, Acetone, 300 nm (c) 0.1N aq NaOH/THF (1:4). (d) Acetone/H_2O 1.1 eq $NaHCO_3$, (1/1), 300 nm

Scheme 37: Synthesis of *E*- and *Z*-phenylitaconic acids and ester derivatives.

E-Phenylitaconic acid **3a** is easily obtained as the product of base catalyzed deprotection of dimethyl *E*-phenylitaconate **3b**. When treated with 1N aqueous NaOH in THF, dimethyl *Z*-phenylitaconate **4b** partially isomerized to a 3:1 mixture of **4a** and **3a**. However, *Z*-phenylitaconic acid **4a** could be obtained either by deprotection of **4b** under dilute basic conditions or by irradiation of *E*-phenylitaconic acid[132] **3a** at 300 nm in a mixture of acetone and H_2O containing 1.1 mole equivalents of sodium bicarbonate. C_{18} Reverse phase HPLC purification of each acid was performed prior to NMR analysis.



(b) THF, -78°C.

Scheme 38: Actual products of condensation.

A more recent publication claimed that diethyl *Z*-phenylitaconate and diethyl benzylfumarate could be synthesized from diethyl fumarate (Scheme 38).[133] In this synthesis, an adduct formed from reaction of triphenylphosphine and diethyl fumarate was treated with LDA followed by addition of benzyl bromide.[134] The nucleophilic displacement was said to yield diethyl *Z*-phenylitaconate **4c** and diethyl benzylfumarate **1c** after work-up. When these reaction conditions were used, the isolated products were identified as diethyl benzylfumarate **1c** and diethyl benzylmaleate **2c**. To confirm these results, the same experiment was carried out with dimethyl fumarate. Reaction products were compared to previously synthesized dimethyl diesters. Dimethyl *Z*-phenylitaconate was not formed in the reaction.

Chromatographic Characteristics of Dioic Acids of Molecular Formula $C_{10}H_{10}O_4$ and Their Dimethyl Esters

The original strategy for identifying the unknown metabolite formed during anaerobic degradation of toluene by strain Tol-4 was based on the previously reported derivatization of the metabolites to form dimethyl diesters. These esterified components were then to be analyzed by GC according to their retention times and coinjection with synthesized samples. Correlations of fragmentation patterns obtained from EIMS was to provide further means of analysis. Synthetic dimethyl diesters **1b**, **2b**, **3b** and **4b** were separated by GC with base line resolution. However, isomerizations observed during base-catalyzed deprotection of the esters might occur during derivatization of the dioic acids under strong acidic conditions. This possibility prompted the examination of the previously reported derivatization methods. For each acid derivatization method, each dioic acid was treated following published procedure. The product of each reaction mixture was then coinjected on a GC column with the synthesized dimethyl diester which should result of the derivatization.

Treatment of benzylfumaric **1a**, benzylmaleic acid **2a**, and *E*-phenylitaconic acid **3a** at 50°C for 20 min with MeOH-H_2SO_4-H_2O (2:1:1, v/v) led to the exclusive formation of dimethyl diesters **1b**, **2b**, and **3b** with no apparent double-bond migration or isomerization. However, treatment of *Z*-phenylitaconic acid **4a** under the same conditions resulted in only partial derivatization, with no dimethyl *Z*-phenylitaconate **4b** observable by GC. Similar results were observed when dioic acids **1a**, **2a**, **3a** and **4a** were treated at 50°C for 20 min with MeOH-H_2SO_4 (1:1, v/v). Overall, reaction of the dioic acids with

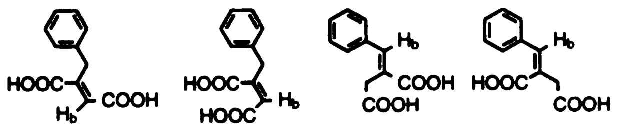
BCl_3 at 50°C in methanol for 20 min proved to be the most useful derivatization protocol. Each of the four dioic acids was dimethylated without any detectable double-bond migration or isomerization.

The next concern was the EIMS analysis of derivatized metabolites. A key argument in previous work was the presence of a tropylium ion ($C_7H_7^+$) at *m/z* 91, which was interpreted as being indicative of the benzyl substituent in dimethyl benzylfumarate **1b** and dimethyl benzylmaleate **2b**. The tropilium ion's presence seemed inconsistent with the absence of a benzyl substituent in dimethyl *E*- or *Z*- phenylitaconate **3b** and **4b**. The synthesis of each dimethyl esters provided an opportunity to study MS fragmentation patterns in detail. A similarly intense fragment at *m/z* 91 corresponding to tropylium ion was observed in the spectrum of each dimethyl diester. In fact, the EIMS fragmentation patterns of compounds **1b, 2b, 3b** and **4b** were essentially identical. Ionization was evidently introducing enough energy into these systems for double-bond migration and isomerization to occur.

Identification of the Second Metabolite by NMR Spectroscopy

Synthetic dioic acids **1a, 2a, 3a** and **4a** were separable with nearly baseline resolution by HPLC with a C_{18} reverse-phase column. The HPLC retention times and coinjection with synthesized dioic acids provide the first line of evidence needed for the direct identification of toluene degradation products. High field NMR analysis provides the second line of evidence for the proper structural determination (Table 12).

Table 12: Chemical shifts of diagnostic protons.



	1a	**2a**	**3a**	**4a**
H_b (vinyl)	6.85	5.84	7.92	6.89
H_a (methylene)	4.17	3.71	3.56	3.44

The ^1H NMR chemical shifts (Table 12) of the vinyl and methylene proton resonances were usually the most useful for identification of dioic acid. Chemical shifts recorded for diester species are very similar to those of their acid parents and are therefore usable for structure determination. The phenylmethylbutenedioic acids (**1a** and **2a**, Table 12) are characterized by downfield chemical shift of the methylene protons and upfield chemical shift of the vinylic proton when compared to the chemical shifts of the methylene and vinylic protons of the phenylmethylenebutanedioic acids (**3a** and **4a**, Table 12). Anisotropic deshielding of the vinylic proton H_b of benzylfumaric acid **1a** attributable to a carboxylate carbonyl explains its downfield chemical shift relative to **2a**. The same effect is also responsible for the downfield chemical shift of the H_a methylene protons of **1a** and **2a**. The diagnostic downfield chemical shift of the vinylic proton H_b of *E*- and *Z*-phenylitaconic acids **3a** and **4a** is attributed to the electron withdrawing effect of *gem*-phenyl ring. Anisotropic deshielding of the vinylic proton H_b of *E*-phenylitaconic acid **3a** attributable to the carboxylate carbonyl located in *cis*-position to the vinylic proton H_b explains the 1ppm value increase in chemical shift relative to **4a**. While the vinylic proton H_b chemical shift of *Z*-phenylitaconic acid **4a** and of benzylfumaric acid **1a** are almost identical, the difference in chemical shift of their methylene protons H_a permit to discriminate between **1a** and **4a**.

Critical supporting evidence for the ^{1}H NMR and ^{13}C NMR assignment followed from use of rotating frame Overhauser effect spectroscopy. The observed NOEs are summarized in Table 13. Measured NOEs attendant radiation of vinyl protons (H_b) were the most diagnostic. Effects were recorded as strong, medium or weak. For example when a protons is irradiated, the transfer of energy to neighboring protons is observed by a change in peak intensities. The difference between spectra taken before radiation and after radiation shows a variation in peak intensity of the protons which are in close proximity.

Table 13:

		H_a (methylene)	H_b (vinyl)	H_c (aromatic)
1a	H_a	-	w	m
	H_b	w	-	a
	H_c	a	a	-
2a	H_a	-	s	m
	H_b	s	-	a
	H_c	a	a	-
3a	H_a	-	a	m
	H_b	a	-	m
	H_c	m	m	-
4a	H_a	-	m	a
	H_b	s	-	m
	H_c	a	m	-

[a] s: strong; m: medium; w: weak; a: absent

Strong NOE effects were observed for benzylmaleic acid **2a** and *Z*-phenylitaconic acid **4a**. Upon irradiation of the methylene protons H_a of benzylmaleic acid **2a**, the intensity of the vinylic proton H_b was largely modified, indicating the protons proximity. The same change in intensity was observed for the methylene protons H_a when the vinylic proton H_b was irradiated. As it could be expected, irradiation of the aromatic protons of benzylmaleic acid **2a** had no effect on its methylene protons H_a or the vinylic proton H_b. In a similar fashion, the closeness between the methylene protons H_a and the vinylic proton H_b of *Z*-phenylitaconic acid **4a** was characterized by a strong NOE effect upon their individual irradiation. Irradiation of the aromatic protons of *Z*-phenylitaconic acid **4a** resulted in a change of intensity of the vinylic proton H_b which indicates the vinylic proton H_b vicinity to the aromatic ring. Weaker NOE effects were registered for benzylfumaric acid **1a** and *E*-phenylitaconic acid **3a** and are indicative of an increased distance between protons. After NOE measurements have been obtained, the structure assignment of each acids becomes even more straightforward than the assignment based on chemical shift.

With synthesized samples of the dioics acids on hand, multiple lines of spectroscopic evidence for the assigned dioic acid structures, and baseline HPLC resolution of the dioic acids, attention turned to the unknown metabolite formed by *A. tolulyticus* Tol-4. The isolated quantities of metabolite were adequate for ^{13}C NMR, ^{1}H NMR, and assignment of NOE correlations. Measured retention times in addition to coinjection with synthetic sample of **1a**, **2a**, **3a** and **4a** by using an HPLC fitted with a C_{18} reverse-phase column provided the necessary confirming data. On the basis of this information, the second metabolite formed along with benzyl succinic acid during anaerobic degradation of toluene by *A. tolulyticus* Tol-4 is *E*-phenylitaconic acid **3a**.

Previous reports of formation of benzylfumaric acid **1a** need to be reconciled with the present observations and characterizations. The sample derivatization techniques employed in those earlier studies did not result in any isomerization or migration of the double-bond in the newly synthesized dioic acids. Only incomplete dimethyl diester formation of *Z*-phenylitaconic acid was observed for two of the three derivatization methods. However, reliance on EIMS analysis for structure identification is not recommended. The identical MS spectra obtained for synthesized samples of compounds **1b**, **2b**, **3b** and **4b** clearly indicate the limited utility in identifying structures of dioic acids of molecular formula $C_{11}H_{10}O_4$ based on fragmentation patterns. Mass spectroscopy using fast atom bombardment technique (FAB) could be used for direct molecular weight determination of the metabolite without esterification. The similarity observed between fragmentation patterns of the various dimethyl diester was also observed for the dioic acids' fragmentation patterns. Thus, mass spectrometry is not the appropriate tool to identify the structure of species of molecular formula $C_{11}H_{10}O_4$. However, resolution of the microbial products of anaerobic toluene metabolism in other organisms should now be straightforward, since all products can be resolved by HPLC analysis. ^1H NMR chemical shift and NOE correlations can also be used for comparison and assignment.

EXPERIMENTAL

General

Spectroscopic and Physical Measurements

Proton nuclear magnetic resonance spectra (^1H NMR), carbon nuclear magnetic resonance spectra (^{13}C NMR) and two-dimensional magnetic resonance spectra (HMQC, COSY, ROESY) were recorded on a Gemini 200, Gemini 300, VXR 300S, or VXR 500S spectrometer. ^1H NMR spectra were recorded at 200, 300 and 500 MHz. Chemical shifts are reported in parts per million (ppm) relative to internal tetramethylsilane (Me_4Si, δ= 0.00 ppm) with $CDCl_3$ as solvent, relative to CHD_2COCD_3 (δ= 2.04 ppm) when samples were dissolved in acetone-d_6, relative to CHD_2SOCD_3 (δ= 2.49 ppm) when samples were dissolved in DMSO-d_6, or relative to sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (TSP, δ= 0.00 ppm) when D_2O was the solvent. Multiplicity is indicated using the following notation: s (singlet), d (doublet), t (triplet), q (quartet), m (unresolved multiplet), br (broad), dd (doublet of doublet), etc. ^{13}C NMR spectra were recorded at 50, 75, 125 MHz. Chemical shifts for ^{13}C NMR spectra are reported (in parts per million) relative to $CDCl_3$ (δ= 77.0 ppm), to $(CD_3)SOCD_3$ (δ= 39.5 ppm) in DMSO, to CD_3COCD_3 (δ= 29.8 ppm) in acetone or internal acetonitrile (CH_3CN, δ= 3.69 ppm) in D_2O. ^{31}P NMR spectra were recorded at 121 MHz and 202 MHz. Chemical shifts obtained for ^{31}P NMR spectra are reported (in parts per million) relative to external 85% phosphoric acid (δ= 0.00 ppm). COSY (Correlated Spectroscopy) spectra were recorded using a 500 MHz Varian VXR at controlled temperature (± 0.1°C). A spectral window of about 4000 Hz was used in both f_1 and f_2 dimensions and increments of 16 scans were collected. HMQC (^1H, ^{31}P) (Hetero-Multinuclear Quadrupolar Correlation) spectra were recorded using a 500 MHz Varian VXR at controlled temperature (± 0.1°C). A spectral window of about 3000 Hz was used in f_1 dimension while a spectral window of about 8000 Hz was used in f_2 dimension. Increments of 8 scans were collected. ROESY (Rotating frame OverHauser Effect Spectroscopy) spectra were recorded in phase-sensitive mode using a 500 MHz Varian VXR at controlled temperature (± 0.1°C). A mixing time of 0.08 s was used while the pulse delay was maintained at 2 s. A spectral window of about 4000 Hz was used in both f_1 and f_2 dimensions and increment of 16 scans were collected.

A Perkin-Elmer Model 1800 FTIR spectrometer and a Nicolet IR/42 spectrometer were used for infrared spectra which were recorded in wavenumbers (cm^{-1}). Peak intensity is indicated using the following notation: s (strong), m (medium), w (weak). Low resolution electron impact (EI) and chemical ionization (CI) mass spectra (MS) were recorded on a Finnigan 4000 mass spectrometer and high resolution mass spectra (HMRS) were recorded on Kratos MS50 or MS25 mass spectrometers. FAB mass spectra were obtained using a JEOL HX-110 double focusing mass spectrometer or a Kratos MS50 mass spectrometer employing glycerol as a matrix. Ultraviolet/visible spectra were recorded on a Perkin-Elmer Lambda 3B spectrophotometer. The gas chromatography (GC) data were collected on a Hewlett Packard 5890 equipped with a DB-1 column (0.25 mm ID, 30m L). The reactions involving ultra-violet irradiation were performed with a Rayonnet apparatus at 300 nm. Single X-ray structure analysis was conducted by Dr. P. Fanwick of the Purdue X-ray crystallography facility. Elemental analyses were performed by Atlantic Microlab Inc. (Norcross, GA).

General Synthetic Chemistry

All reactions requiring anhydrous or inert conditions were carried out in flame- or oven dried glassware under a positive atmosphere of argon or nitrogen. Solutions or liquids were introduced using oven-dried syringes or cannula through rubber septa. All reactions were stirred magnetically using Teflon-coated stir bars unless otherwise noted. In the cases requiring heating, the reactions were warmed using an electrically-heated silicon oil bath, and the stated reaction temperature is the temperature of the bath, or were conducted at reflux using a heating mantle. In the cases requiring -78°C cooling, the reactions were chilled with a dry ice/acetone bath. Organic solutions obtained after an aqueous work-up were dried over $MgSO_4$. Removal of solvents was accomplished using a Büchi rotary evaporator at water aspirator pressure or under high vacuum (0.5 mm Hg). Hydrogenolyses were carried out with a Parr hydrogenation apparatus (Parr Instrument Co.) at the hydrogen pressure indicated.

Reagents and Solvents

Tetrahydrofuran (THF) was distilled under nitrogen from sodium benzophenone ketyl. Benzene (C_6H_6) was distilled from sodium under nitrogen (N_2). Methylene chloride (CH_2Cl_2) was distilled from calcium hydride under nitrogen. THF, C_6H_6, and CH_2Cl_2 were distilled immediately prior to use. Pyridine, triethylamine (TEA), diisopropylamine and toluene were distilled from calcium hydride and stored under nitrogen or argon (Ar) with activated Linde 4Å molecular sieves. Methanol (MeOH), acetonitrile (CH_3CN) and N, N-dimethylformamide (DMF) were dried over activated 3Å molecular sieves. Water used in reaction solutions was deionized and glass distilled. *N*-Bromosuccinimide was recrystallized from water and dried under vacuum. 2,2'-Azobis(2-methylpropionitrile) (AIBN) was recrystallized from ethanol. *m*-Chloro-benzoic acid was washed from *m*-chloro-perbenzoic acid (80% w/w) with phosphate buffer, *m*-chloro-perbenzoic acid was then dried under vacuum. All other solvents and reagents were used as received from commercial sources or purified according to : Perrin, D. D.; Armarego, W. L. F.; Perrin, D. R. *Purification of Laboratory Chemicals*; Pargamon Press; New York, 1980.

Chromatography

Chromatographic solvents, ethyl acetate (EtOAc) and hexane were distilled from calcium hydride prior to use. Flash chromatography was carried out on 230-400 mesh silica gel. Radial chromatography (preparative centrifugal thin-layer chromatography, PCTLC) was performed on a Harrison Model 7924 chromatotron, using 1, 2 or 4 mm layers of silica gel 60 PF254 containing gypsum (E. Merck). Thin layer chromatography (TLC) was performed using Merck silica gel 60A PF254 glass-baked plates (0.25 mm thickness) or Whatman silica gel 60A PF254 glass baked plates (0.25 mm thickness). TLC plates were visualized by ultraviolet light (254 nm), immersion in anisaldehyde stain (by volume: 93% ethanol, 3.5 % sulfuric acid, 1% acetic acid and 2.5 % anisaldehyde) followed by heating or immersion in 7% phosphomolybdic acid in ethanol followed by heating.

Ion exchange resins were obtained from Bio-Rad, except Dowex-50 which was purchased from Sigma. Dowex-50 was cleaned before use by treatment of a water suspension of the gel with bromine at pH 14, extensive rinsing with water, followed by conversion to the acid form with 6N aqueous hydrochloric acid. Anion exchange purifications were performed at 4°C and employed AG1-X8 resin.

High pressure liquid chromatography (HPLC) was carried out with a Rainin system or a biocompatible Rainin system. HPLC Purifications of synthetically prepared materials were performed on a Rainin instrument using a Microsorb reverse phase C_{18} semi-preparative (5 µm, 21.4 mm ID, 25 cm L) column. Retention times on reverse phase C_{18} were obtained using a Microsorb reverse phase C_{18} analytical (5 µm, 4.6 mm ID, 25 cm L) column. Thin layer chromatography (TLC) was performed using Whatman silica gel C_{18} reverse phase 60Å PF254 glass plates (0.25 mm thickness). Analytical Beckman DEAE TSK-PW5 column was used for weak anion exchange high pressure liquid chromatography, and Resource Q (1 mL, purchased from Bio-Rad) was used for anion exchange high pressure liquid chromatography. All solvents and solutions were filtered through 0.5 µm pore size filter and degassed prior to use. Samples were filtered through 0.5 µm pore size microfilter prior to injection.

Enzymology Generalities

Glucose 6-phosphate and nicotinamide adenine dinucleotide (98%) were purchased from Sigma. High purity NAD grade V-C was required for K_m determination since lower grades of NAD contained contaminants that potentially inhibit MIP synthase and interferes with the assay. Protein concentration was assayed by the Bradford dye-binding procedure with protein assay solution purchased from Bio-Rad.[135] A standard curve was obtained using bovine serum albumin.

In the following experimental descriptions, "reaction" refers to mixing the enzyme *myo*-inositol 1-phosphate synthase with substrate glucose 6-phosphate, regardless of what other component (inhibitor, cofactor, etc....) is present. The expression "progress curve" refers to the plot of inorganic phosphate concentration versus time. One unit of enzyme activity represents 1µmol of substrate converted to product per min at 37°C.

All protein manipulation was carried out at 4°C. Protein solutions were concentrated by ultra filtration (PM-10 Diaflo membranes from Amicon). Dialysis tubing (12000-14000 molecular cutoff from Spectrator) was cleaned prior to use. Centrifugations, unless specified otherwise, were performed at 18000g for 20 min at 4°C.

MIP Synthase from *S. cerevisiae* MW5.55

General

Unless otherwise noted, all experiments were conducted in a phosphate free buffer solution consisting of deionized, glass distilled water containing Tris-HCl (20 mM), NH_4Cl (14 mM) and dithiothreitol (0.2 mM) pH 7.2. The substrate, cofactor and inhibitor concentration were calculated by substraction of the inorganic phosphate concentration from the total phosphorus concentration, each determined by Ames procedure.[64]

Whatman (diethylaminoethyl) cellulose (DE52), Bio-Gel A 0.5 obtained from Bio-Rad, a 7.5 mm x 7.5 cm DEAE-5PW TSK analytical (purchased from Beckman) HPLC column were used during the purification. Buffers included buffer A: Tris-HCl (20 mM), NH_4Cl (20 mM), 2-mercaptoethanol (10 mM), PMSF (0.5 mM), pH 7.2, and buffer B: Tris-HCl (20 mM), NH_4Cl (250 mM), 2-mercaptoethanol (10 mM), PMSF (0.5 mM), pH 7.2.

Aliquots of fractions obtained during purification of the enzyme were incubated in the assay solution containing NAD (1 mM) and D-glucose 6-phosphate (5 mM) at 37°C for 1 h prior. The reaction was stopped by addition of TCA (20%, w/v). Subsequent incubation of 0.1 mL of the reaction mixture with aqueous $NaIO_4$ (0.2 M, 0.10 mL) for 1 h at 37°C was followed by addition of aqueous Na_2SO_3 (1.5 M, 0.1 mL). Released inorganic phosphate was measured using the method of Eisenberg[13a] that employs malachite green and reads at 660 nm, and subsequently indicated fractions containing MIP synthase.

Isolation of MIP Synthase

Saccharomyces cerevisiae MW5.55 was provided by Professor S. A. Henry. The growth medium (YEPD) was prepared in distilled, deionized water and contained Bacto peptone (20 g), Bacto yeast extract (10 g) and D-glucose (10 g). The culture growth was carried out at 30°C. *S. cerevisiae* MW5.55 cells (50 g) were harvested by centrifugation at 6000 g, washed twice with buffer A and resuspended in 75 mL of the same buffer. Cell lysate was prepared by disruption of the cells by French press (16000 psi). The lysate was then centrifuged at 6000 g for 10 min to remove intact cells which were resuspended in buffer A (50 mL) and lysed by French press. The cell lysate fractions were combined after centrifugation, and streptomycin sulfate in buffer A (25%, w/v) was added to the supernatant to a final concentration of 2% (w/v). After 20 min of gentle stirring, the solution was centrifuged. Powdered ammonium sulfate was added slowly to the supernatant to give a final concentration of 229 g/L (40% saturation). After being stirred for 20 min the precipitated protein was removed by centrifugation. Ammonium sulfate was added to the supernatant to a final concentration of 483 g/L (75% saturation) and stirred for 20 min. The precipitated protein was collected as pellets by centrifugation, resuspended in buffer A, dialyzed against buffer A and layered onto DE52 (250 mL) equilibrated with buffer A. Elution with buffer A (50 mL) was followed by elution with a linear gradient (500 mL + 500 mL, buffer A-buffer B). Fractions containing MIP synthase were combined. Ammonium sulfate was added to give a final concentration of 523 g/L (80% saturation) and stirred for 20 min. The precipitated protein was collected by centrifugation (18000 g) for 10 min after which the pellets were resuspended in buffer B and applied to the Bio-Gel column (500 mL) equilibrated with buffer B. After elution of the protein with buffer B (500 mL), the fractions containing MIP synthase were combined and concentrated. The concentrate was then dialyzed against buffer A, and applied onto the HPLC DEAE column equilibrated with buffer A. MIP synthase was eluted with a linear gradient (100:0 buffer A/buffer B-20:80 buffer A/buffer B). Fractions containing MIP synthase were concentrated and stored at -80°C.

Polyacrylamide gel electrophoresis of purified MIP synthase in the presence of sodium dodecyl sulfate was performed according to Laemmli.[136] The stacking gel was buffered with Tris-HCl at pH 6.8 and the separating gel (10% acrylamide) was buffered with Tris-HCl at pH 8.8. Typically, 20 µg of protein was loaded per lane. Gels were fixed with acetic acid solution and visualized with Coomasie Brilliant Blue. Standards used for molecular weight determination included carbonic anhydrase (MW 29,000), ovalbumin (MW 45,000), bovine serum albumin (MW 66,000), phosphorylase b (MW 97,400), β-galactosidase (MW 116,000) and myosin (MW 205,000). Cross-linking of purified MIP synthase followed the protocol of Stach[137] and entailed reaction (in a total volume of 0.20 mL) of MIP synthase (0.15 mg/mL) with dimethyl suberamidate (0-10 mg/mL) in 0.20 M borate, pH 9.5, for 2 h at 25°C. Addition of sodium dodecyl sulfate (10% , w/v, 0.03 mL) and β-mercaptoethanol (0.0005 mL) was followed by incubation for 1 h at 37°C. Subsequent separation by polyacrylamide gel electrophoresis followed the procedure of Davies.[138] The separating gel (5% acrylamide) was buffered with 0.1 M sodium borate and 0.1 M sodium acetate, pH 8.5 and contained sodium dodecyl sulfate (0.1%). Electrospray data were obtained using a VG plateform (VG Organic, Manchester, England).

Enzyme Kinetics

All kinetic information are based on initial velocity kinetics data. Specific activity, Michaelis constant (K_m) and inhibition constants (K_i) were determined in an assay solution (1.5 mL) containing Tris-HCl (20 mM), NH_4Cl (14 mM) and dithiothreitol (0.2 mM), pH 7.2. MIP Synthase was incubated in the assay solution with the selected concentration of NAD, D-glucose 6-phosphate, and inhibitors at 37°C. Aliquots (0.15 mL) were removed every 2 min, added to a TCA solution (20%, w/v, 0.05 mL) and, subsequently incubated with aqueous $NaIO_4$ (0.2 M, 0.10 mL) for 1 h at 37°C. The reaction mixture was quenched by addition of aqueous Na_2SO_3 (1.5 M, 0.1 mL). Released inorganic phosphate was measured using the method of Ames. Specific activities were calculated when D-glucose 6-phosphate and NAD were 5 mM and 1 mM, respectively. Michaelis constants (K_m) of D-glucose 6-phosphate and NAD were calculated from linear regression analysis of the progress curves. These curves were obtained for various concentrations of substrate ranging from 0.5 mM to 5 mM (NAD, 5 mM) and for various concentrations of cofactor ranging from 5 µM to 100 µM (substrate, 5 mM). Linear regression of the resulting progress curves was applied to calculate velocities for each reaction using the following equation:

$$v= [\text{ slope x } 10^6 \text{ x } V_s \text{ x } (V_q+ V_a)]/ [\varepsilon \text{ x } V_c \text{ x } V_a]$$

where:

- v is the velocity (mM min^{-1})
- slope is the slope obtained from linear regression (OD_{820nm} min^{-1})
- 10^6 is a conversion factor (µM M^{-1})
- V_s is the volume of chemical visualization (mL)
- V_q is the volume of solution used to quenched the time point (mL)
- V_a is the volume of aliquot removed from the time point (mL)
- V_c is the volume of time point used for visualization (mL)
- ε is the extinction coefficient (26,000 L M^{-1})

The resulting data was plotted as 1/v versus 1/[S] (Lineweaver-Burk plot), where v is the velocity, [S] is the substrate concentration. When inhibitor concentration is varied, [I] is the inhibitor concentration. The value for the inhibition constant (K_i) was extracted from the above plots using the following equation:

$$v/V_{max}= [S] \text{ x } \{K_m (1+ [I]/K_m)+ [S]\}$$

In cases where enzyme specific activity could not be determined by calculating the amount of released inorganic phosphate from MIP oxidation, the amount of residual glucose 6-phosphate was used to estimate the specific activity. This assay employs glucose 6-phosphate dehydrogenase. It was used to determine MIP and dihydroxyacetone phosphate inhibition constants and to estimate the I_{50} values of *myo*-2-inosose 1-phosphate and of its phosphonate analogue **77**. MIP Synthase, glucose 6-phosphate, inhibitor, NAD and buffer were incubated for the appropriate time period. Aliquots (0.1 mL) were taken from the incubation mixture at the required time point and added to a 100°C pre-heated tube where the enzymatic reaction was stopped. The tube containing the aliquot was then cooled to rt, and a buffered solution (2.5 mL) containing triethanolamine (0.1 M, pH 7.6), $MgCl_2 \cdot 6H_2O$ (5 mM) and NAD (0.5 mM) and glucose 6-phosphate dehydrogenase was added. Five units of glucose 6-phosphate dehydrogenase per 250 mL of buffered assay solution were used. Amount of glucose 6-phosphate remaining from MIP synthase incubation is equal to the amount NADH produced and determined by UV spectrometry (OD_{340nm}). Velocity was calculated from the replot of NADH concentration as a function of time.

Preparation of *apo*MIP synthase began with incubation at 4°C of native enzyme (0.5×10^{-3} g) in 2 mL of the aforementioned incubation solution lacking NAD and containing activated charcoal (0.2 g). This solution was stirred for 15 min and then centrifuged for 10 min in a microfuge at 4°C. A soluiton of the resulting *apo*MIP synthase (1.0×10^{-6} M) and NADH (0.23×10^{-3} M) were incubated together for 5 min at 37°C and then *myo*-2-inosose 1-phosphate added to give a concentration of this reaction coordinate intermediate of 0.23 mM. Oxidation of the NADH was monitored with a Perkin Elmer 3λ Spectrophotometer by scanning between 400 nm and 250 nm or by following the decerase in absorbance at 340 nm.

Enzyme Purifications from *E. coli*

General

E. coli BL21(DE3) are host cells used for the production of MIP synthase and *myo*-inositol monophosphatase. The growth LB medium is constituted of Bacto tryptone (10 g/L), Bacto yeast extract (5 g/L) and NaCl (10 g/L) in 1 L of glass-distilled, deionized water. LB-plate contained (per L) Bacto tryptone (10 g/L), Bacto yeast extract (5 g/L), NaCl (10 g/L) and Difco agar (15 g/L). Appropriate antibiotic concentrations are obtained by addition of sterile filtered antibiotic solution to pre-autoclaved liquid media.

Competent *E. coli* BL21(DE3) cells were prepared using a procedure modified from Maniatis.[139] One milliliter of an overnight culture (12 h) was used to inoculate 100 mL of LB in a 500 mL Erlenmeyer flask which contained the appropriate antibiotics. Cells were grown at 37°C with gentle shaking (180 rpm) until they reached the mid-log phase of growth (OD_{600nm}= 0.4). All manipulations were then carried out on ice during the remaining portion of the procedure. Cells were harvested (4000 g, 5 min, 4°C), washed in 100 mL of cold 0.9% NaCl, and resuspended in 50 mL of cold 100 mM $CaCl_2$. The cells were kept on ice for at least 30 min and then pelleted by centrifugation (4000 g, 5 min, 4°C). Cells were resuspended in 4 mL of cold 15 % glycerol in 100 mM $CaCl_2$, and 0.25 mL aliquots were dispensed into sterile 1.5 mL microfuge tubes and immediately frozen in liquid nitrogen. Competent cells were maintained at -70°C for a few months.

Competent cells were thawed on ice for 15 min before transformation. Manipulation of DNA followed methods described by Maniatis.[139] Plasmid DNA (stored in TE buffer at 4°C) was added to a 0.25 mL aliquot of competent cells and the solution was gently mixed. TE buffer contains Tris (10 mM), pH 8 and EDTA (1 mM). After standing for 30 min on ice, the cells were heat-shocked at 42°C for 2 min and then placed on ice for 1 min. One milliliter of LB (no antibiotic) was added to the solution, which was then incubated at 37°C for 1 h without shaking. The cells were harvested by microcentrifugation (15 s), resuspended in a small volume of LB (0.1 mL) and plated onto the appropriate selective medium. Small scale isolation of plasmids to confirm the plasmid identity relied on the alkaline lysis method described by Maniatis.[139]

Isolation of MIP Synthase from *E. coli* BL21(DE3)/pT7-7MIPsynt.

Competent *E. coli* BL21(DE3) cells were transformed with plasmid pT7-7MIPsynt. prepared by Dr. D. Spear. LB medium (5 mL) containing 20 mg/mL ampicilin was inoculated by a single colony of *E. coli* strain BL21(DE3) bacteria containing MIP synthase expression construct. Growth conditions of 37°C and rapid shaking were maintained until an A_{600nm} of 0.9 was reached. Cells were then spun down and resuspended in LB/Amp (30 mL) fresh medium. One liter of LB/Amp was inoculated with the 30 mL browth and growth was maintained until an A_{600nm} of 0.9. The inducer, isothiopropyl-β-galactoside (IPTG), was then added to a final concentration of 0.4 mM, and shaking continued for 3 h. The cells (2.3 g of wet cells) were harvested by centrifugation at 6000 g, washed twice with buffer A and resuspended in 10 mL of the same buffer. Cell lysate was prepared by two successive disruptions of the cells by French press (16000 psi). The cell lysate fractions were combined after centrifugation and powdered ammonium sulfate was added slowly to the supernatant (31 mL) to give a final concentration of 229 g/L (40% saturation). After being stirred for 20 min the precipitated protein was removed by centrifugation. Ammonium sulfate was added to the supernatant (33 mL) to a final concentration of 483 g/L (75% saturation) and stirred for 20 min. The precipitated protein was collected as pellets by centrifugation, resuspended in buffer A (10 mL), dialyzed against buffer A and layered onto DE52 (70 mL) equilibrated with buffer A. Elution with buffer A (30 mL) was followed by elution with a linear gradient (200 mL + 200 mL, buffer A-buffer B).

Fractions containing MIP synthase were combined, concentrated and stored at -80 °C. Kinetic experiments were performed as previously described.

Isolation of *myo*-Inositol Monophosphatase from *E. coli* BL21(DE3)/pBIMP

Competent *E. coli* BL21(DE3) cells were transformed with plasmid pBIMP obtained from Dr. R. E. Diehl. LB medium (5 mL) containing 20 mg/mL ampicilin was inoculated by a single colony of *E. coli* strain BL21(DE3) bacteria, containing MI monophosphatase expression construct. Growth conditions of 37°C and rapid shaking were maintained until an A_{600nm} of 0.9 was reached. Cells were then spun down and resuspended in LB/Amp (30 mL) fresh medium. One liter of LB/Amp was inoculated with the 30 mL broth and growth was pursued until an A_{600nm} of 0.9. The inducer, isothiopropyl-*β*-galactoside (IPTG), was then added to a final concentration of 0.4 mM, and shaking continued for 4 h. The cells (1.8 g of wet cells) were pelleted, resuspended in 4 mL of a buffer C constituted of EGTA (0.1 mM), EDTA (0.5 mM) and Tris-HCl (20 mM) at pH 7.8. Cell lysate was prepared by disruption of the cells by sonication (at 0°C, 3 x 1 min). The lysate was then centrifuged at 6000 g for 20 min to remove intact cells which were resuspended in buffer C (10 mL) and lysed by sonication. The cell lysate fractions were combined after centrifugation then heated for 2 min at 68°C and centrifuged at 4°C. The supernatant (11 mL) was then frozen at -80°C in 1 mL aliquot. After thawing and filtering, each sample was applied onto the HPLC Resource Q column equilibrated with buffer C. MIP synthase was eluted with a linear gradient of NaCl (from 100% buffer C to 100% buffer C + 0.3 M NaCl). Fractions containing MI monophosphatase were concentrated and stored at -80 °C.

Enzyme Kinetics

All kinetic information are based on initial velocity kinetics data. Specific activity, Michaelis constant (K_m) and inhibition constants (K_i) were determined in an assay solution (1.5 mL) containing Tris-HCl (50 mM), KCl (250 mM) and $MgCl_2$ (3 mM), pH 8.0. MI Monophosphatase was incubated in the assay solution with the selected concentration of MIP and inhibitors at 37°C. Aliquots (0.15 mL) were removed every 2 min, added to a TCA solution (20%, w/v, 0.05 mL) and, subsequently, released inorganic phosphate was measured using the method of Ames.[64]

Synthetic Procedure

Regioisomer I: [1R*(1α,2β,3α,4β,5α,6β)]-2,3,4,5-Tetrakis(benzyloxy)-6-[(diisopropoxyphosphinyl)methyl]cyclohexan-1-ol.

Regioisomer II: [1S*(1α,2α,3β,4α,5β,6β)]-2,3,4,5-Tetrakis(benzyloxy)-6-[(diisopropoxyphosphinyl)methyl]cyclohexan-1-ol.

Table 6, X= $-P(O)(OiPr)_2$, Y= $-P(O)(OiPr)_2$

A solution of *n*-butyl lithium in hexane (1.6 M, 0.97 mL, 1.56 mmol) was added to THF (5 mL) at -78°C under N_2. To this solution was slowly added a solution of diisopropyl methanephosphonate (0.28 g, 1.56 mmol) in THF (5 mL). This solution was stirred for 30 min at -78°C. Boron trifluoride etherate (0.19 mL, 1.56 mmol) was added immediately followed by a solution of cyclohexene oxide **29** (0.16 g, 0.31 mmol) in THF (5 mL). The solution was stirred at -78°C for 3 h. More boron trifluoride etherate (0.19 mL, 1.56 mmol) was added. The solution was warmed to -40°C and stirred for 12 h. Saturated aqueous $NaHCO_3$ was then added. The aqueous layer was extracted with ether, and the combined organic layers were washed with brine, followed by water, and dried. The solvents were evaporated under reduced pressure and the crude was first purified by flash chromatography (EtOAc/ hexane, 1:1, v/v) and then by radial chromatography (2-mm thickness of silica gel, EtOAc/hexane, 1:1, v/v). The faster eluting regioisomer I was isolated as an oil which slowly solidified at rt (0.09 mmol, 28%). The slower eluting regioisomer II was isolated as an oil (0.07 mmol, 21%). Regioisomer I: ^{1}H NMR ($CDCl_3$) δ 7.20-7.40 (m, 20 H), 4.55-5.05 (m, 11 H), 3.30-3.90 (m, 4 H), 1.80-2.35 (m, 3 H), 1.50 (br, 1 H), 1.25-1.50 (m, 19 H); ^{13}C NMR ($CDCl_3$) δ 139.1, 138.8, 138.6, 128.5, 128.4, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 86.0, 85.2, 82.8, 79.7 (J_{PCC}= 7 Hz), 75.8, 75.6, 75.1, 73.8 (J_{PCCC}= 3 Hz), 72.0, 71.8, 70.9 (J_{POC}= 7 Hz), 70.6 (J_{POC}= 7 Hz), 41.7 (J_{PCC}= 4 Hz), 25.3 (J_{PC}= 141 Hz), 24.3, 24.2, 24.1, 24.0; IR (neat NaCl) 3380 (w), 1460 (m), 1450 (s), 1210 (s); MS *m/z* (rel inten) EI 91 (100). CI 107 (100), 703 (M+H$^+$, 25); HRMS (CI) calcd for $C_{41}H_{52}O_8P$ (M+H$^+$) 703.3400, found 703.3400; Anal. Calcd for $C_{41}H_{51}O_8P$: C, 70.07; H, 7.31. Found: C, 70.31; H, 7.05. Regioisomer II: ^{1}H NMR ($CDCl_3$) δ 7.00-7.45 (m, 20 H), 4.40-4.80 (m, 10 H), 3.90-4.05 (m, 1 H), 3.55-3.80 (m, 2 H), 3.25-3.45 (m, 2 H), 2.60-2.80 (m, 1 H), 2.45 (br, 1 H), 2.00-2.30 (m, 1 H), 1.00-1.60 (m, 13 H); ^{13}C NMR ($CDCl_3$) δ 138.7, 138.6, 138.4, 137.9, 128.4, 128.3, 128.2, 127.9, 127.8, 127.7, 127.6, 127.5, 81.7, 81.5, 79.7, 78.1 (J_{PCCC}= 13 Hz), 76.0, 75.9, 75.5, 72.4 (J_{POC}= 7 Hz), 70.6, 70.2 (J_{POC}= 7 Hz), 67.8, 35.5 (J_{PCC}= 4 Hz), 25.1 (J_{PC}= 134 Hz), 24.1, 24.0, 23.9; IR (neat NaCl) 3550 (s), 1460 (s), 1402 (s), 1255 (m); MS *m/z* (rel inten) EI 91 (100). CI 703 (M+H$^+$, 100), 704 (29); HRMS (CI) calcd for $C_{41}H_{52}O_8P$ (M+H$^+$) 703.3400, found 703.3386; Anal. Calcd for $C_{41}H_{51}O_8P{\cdot}H_2O$: C, 68.30; H, 7.41. Found: C, 68.03; H, 7.23.

Regioisomer I: [1R*(1α,2β,3α,4β,5α,6β)]-2,3,4,5-Tetrakis(benzyloxy)-6-[(dimethoxyphosphinyl)methyl]cyclohexan-1-ol.

Regioisomer II: [1S*(1α,2α,3α,4β,5β,6β)]-2,3,4,5-Tetrakis(benzyloxy)-6-[(dimethoxyphosphinyl)methyl]cyclohexan-1-ol.

Table 6. X= -P(O)(OMe)$_2$, Y= -P(O)(OMe)$_2$

A solution of *n*-butyl lithium in hexane (1.6 M, 8.45 mL, 13.5 mmol) was added to THF (20 mL) at -78°C under N_2. To this solution was slowly added a solution of dimethyl methanephosphonate (1.67 g, 13.5 mmol) in THF (25 mL). The resulting solution was stirred for 30 min at -78°C. Boron trifluoride etherate (1.66 mL, 13.5 mmol) was added immediately followed by a solution of cyclohexene oxide **29** (2.35 g, 4.50 mmol) in THF (25 mL). The solution was stirred at -78°C for 3 h. More boron trifluoride etherate (1.66 mL, 13.5 mmol) was added. The solution was warmed to -40°C and stirred for 12 h. Saturated aqueous $NaHCO_3$ was then added. The aqueous layer was extracted with ether and the combined organic layers were washed with brine, followed by water, and dried. The solvents were evaporated under reduced pressure and the crude was first purified by flash chromatography (EtOAc/hexane, 1:1, v/v) and then by radial chromatography (2-mm thickness of silica gel, EtOAc/hexane, 1:1, v/v). The faster eluting regioisomer I was isolated as an oil (1.54 mmol, 34%) which slowly solidified at rt. The slower eluting regioisomer II was isolated as an oil (1.19 mmol, 27%) which slowly solidified at rt. Regioisomer I: ^{1}H NMR ($CDCl_3$) δ 7.20-7.40 (m, 20 H), 4.80-5.05 (m, 7 H), 4.67 (d, *J*= 11 Hz, 1 H), 3.75 (d, *J*= 2 Hz, 3 H), 3.70, (d, *J*= 2 Hz, 3 H), 3.40-3.65 (m, 5 H), 1.80-2.40 (m, 3 H); ^{13}C NMR ($CDCl_3$) δ 138.7, 138.4, 138.3, 138.2, 128.3, 128.0, 127.8, 127.7, 127.6, 127.5, 86.0, 85.1, 82.7, 79.4 (J_{PCCC}= 7 Hz), 75.8, 75.6, 75.5, 75.1, 73.1 (J_{PCCC}= 3 Hz), 52.7(J_{POC}= 7 Hz), 52.3 (J_{POC}= 7 Hz), 41.4 (J_{PCC}= 4 Hz), 22.6 (J_{PC}= 139 Hz); IR (neat NaCl) 3550 (w), 3360 (s), 1452 (s), 1260 (s); MS *m/z* (rel inten) EI 91 (100). CI 615 (100) 647 (M+H$^+$, 5); HRMS (FAB) calcd for $C_{37}H_{44}O_8P$ (M+H$^+$) 647.2774, found 647.2768. Anal. Calcd for $C_{37}H_{43}O_8P \cdot 1/3H_2O$: C, 68.08; H, 6.74. Found: C, 68.09; H, 7.17. Regioisomer II: ^{1}H NMR ($CDCl_3$) δ 7.15-7.40 (m, 20 H), 4.60-4.90 (m, 8 H), 4.40-4.50 (m, 1 H), 4.12 (ddd, J= 12, 5, 3 Hz, 1 H), 3.87 (dd, J= 9, 9 Hz, 1 H), 3.69 (d, J= 5 Hz, 3 H), 3.64 (d, J= 5 Hz, 3 H), 3.63-3.70 (m, 1 H), 3.51 (dd, J= 9, 9 Hz, 1 H), 2.97 (br, 1 H), 2.70-2.95 (m, 1 H), 2.35 (ddd, J= 20, 16, 3 Hz, 1 H), 1.45 (ddd, J= 17, 16, 12 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 138.5, 138.2, 137.8, 128.2, 128.1, 127.7, 127.6, 127.5, 127.4, 127.3, 81.5, 81.3, 79.4, 77.8 (J_{PCCC}= 13 Hz), 75.6, 75.3, 72.3, 67.7, 52.3 (J_{POC}= 7 Hz), 52.1 (J_{POC}= 7 Hz), 35.5 (J_{PCC} = 4 Hz), 19.9 (J_{PC}= 143 Hz); IR (neat NaCl) 3545 (m), 3400 (s), 1455 (s), 1370 (m), 1255 (s); MS *m/z* (rel inten) EI 91 (100). CI 647 (M+H$^+$, 100), 648 (37). FAB 647 (36), 648 (15); HRMS (FAB) calcd for $C_{37}H_{44}O_8P$ (M+H$^+$) 647.2774, found 647.2767; Anal. Calcd for $C_{37}H_{43}O_8P \cdot 1/2H_2O$: C, 67.77; H, 6.76. Found: C, 67.77; H, 6.76.

Preparation of solution of (lithiomethyl)dimesitylborane.

Solutions of (lithiomethyl)dimesitylborane in THF were prepared freshly prior to addition to cyclohexene oxides as described by Pelter *et al.*[73] The following is a representative procedure:

To a solution of mesityl bromide (1.50 g, 7.47 mmol) in THF (15 mL) cooled at -78°C was added *tert*-butyl lithium in pentane (1.7 M, 8.8 mL, 15.0 mmol) under N_2. The solution became yellow and salts rapidly formed. After 15 min, the cold bath was replaced by a water bath at rt. After an additional 15 min, the homogeneous orange solution was cannulated into a solution of dimesitylmethylborane (1.80 g, 6.79 mmol) in THF (10 mL) at rt. The pale orange solution slowly turned darker and was stirred for 1 h at rt before use.

[1R*(1α,2β,3α,4β,5α,6β)]-2,3,4,5-Tetrakis(benzyloxy)-6-(hydroxymethyl)cyclohexan-1-ol, 30.

Table 6. X= OH

To a solution of cyclohexene oxide **29** (1.44 g, 2.00 mmol) in THF (25 mL) was added a (lithiomethyl)dimesitylborane solution in THF (1.28 M, 15 mL, 20 mmol) at rt under N_2. After 12 h, saturated aqueous $NaHCO_3$ and ether (20 mL) were added. The organic layer was washed with brine, dried and concentrated under reduced pressure. The intermediate **31** was purified by radial chromatography (2-mm thickness of silica gel, EtOAc/hexane, 1:5, v/v) to yield a white solid which crystallized from CCl_4 (1.41 g). To a solution of this intermediate (0.21 g, 0.32 mmol) in THF (5 mL) under N_2 were added MeOH (2 mL) followed by aqueous NaOH (5N, 0.16 mL, 0.81 mmol). The solution was then chilled to 0^oC and aqueous H_2O_2 (30% w/v, 0.50 mL, 0.81 mmol) was slowly added. The mixture was then stirred at rt for 6 h. Saturated aqueous $NaHCO_3$ (20 mL) was added, followed by ether. The organic phase was washed with brine, followed by water, and then dried. After removal of the solvents under reduced pressure, the diol was purified by radial chromatography (2-mm thickness of silica gel, EtOAc/hexane, 1:1 v/v). The diol was obtained as an oil (0.27 mmol, 89%) which slowly solidified at rt. ^1H NMR ($CDCl_3$) δ 7.20-7.30 (m, 20 H), 4.80-5.00 (m, 6 H), 4.60-4.75 (m, 2 H), 3.91 (dd, *J*= 11, 3 Hz, 1 H), 3.73 (dd, *J*= 11, 4 Hz, 1 H), 3.60 (dd, *J*= 9, 9 Hz, 1 H), 3.50-3.55 (m, 2 H), 3.35-3.45 (m, 2 H), 2.30 (br, 2 H), 1.60-1.75 (m, 1 H); ^{13}C NMR ($CDCl_3$) δ 138.6, 138.5, 138.2, 128.8, 128.7, 128.6, 128.5, 128.3, 128.2, 128.1, 128.0, 127.8, 86.3, 85.1, 83.2, 77.5, 75.7 (2C), 75.5, 75.3, 70.5, 60.4, 46.0; IR (neat NaCl), 3535 (b), 3340 (b), 1450 (w), 1258 (s); MS *m/z* (rel inten) EI 91 (100). CI 107 (100), 555 ($M+H^+$, 4); HRMS (CI) calcd for $C_{35}H_{39}O_6$ ($M+H^+$) 555.2747 found 555.2736; Anal Calcd for $C_{35}H_{38}O_6$: C, 75.09; H, 6.90. Found: C, 75.52; H, 6.95.

[1R*(1α,2β,3α,4β,5α,6β)]-2,3,4,5-Tetrakis(benzyloxy)-6-(hydroxymethyl)cyclohexan-1-ol, 32.

Table 6. X= I

To a solution of cyclohexene oxide **29** (3.00 g, 5.73 mmol) in THF (25 mL) was added a (lithiomethyl)dimesitylborane solution in THF (0.72 M, 25 mL, 28.6 mmol) at rt under N_2. After 12 h, a solution of sodium acetate in MeOH (1 M, 57.3 mL, 57.3 mmol) was added, followed by a solution of ICl in MeOH (1 M, 28.6, 28.6 mmol). After 30 min at rt, saturated aqueous $NaHCO_3$ was added, followed by ether. The organic layer was washed with brine, dried, and concentrated under reduced pressure. Purification by flash chromatography (EtOAc/hexane, 1:1, v/v) afforded an intermediate which was then dissolved in anhydrous THF. A solution of sodium acetate in MeOH (1 M, 57.3 mL, 57.3 mmol) was added under N_2, followed by a solution of ICl in MeOH (1 M, 28.6 mL, 28.6 mmol). After 8 h at rt, saturated aqueous $NaHCO_3$ was added, followed by ether. Aqueous fraction was extracted with ether and the combined organic layers were washed with brine, dried, and concentrated under reduced pressure. The residue was purified by radial chromatography (4-mm thickness of silica gel, EtOAc/hexane, 1:5, v/v) to afford the iodide as a white solid (2.00 mmol, 35%). ^1H NMR ($CDCl_3$) δ 7.20-7.45 (m, 20 H), 4.60-5.05 (m, 8 H), 3.65-3.80 (m, 3 H), 3.40-3.65 (m, 4 H), 2.38 (br, 1 H), 1.00-1.15 (m, 1 H); ^{13}C NMR ($CDCl_3$) δ 138.2, 138.1, 128.7, 128.4, 128.2, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 85.7, 84.5, 83.1, 79.5, 75.8, 75.7, 75.5, 70.9, 43.9, 10.4; IR (neat NaCl), 3535 (m), 3450 (w), 1452 (s); MS, *m/z* (rel inten) EI 91 (100). CI 181 (100), 573 (46), 574 (11); HRMS (CI) calcd for $C_{35}H_{36}O_5I$ (M-H$^+$) 663.1608, found 663.1601; Anal. Calcd for $C_{35}H_{37}O_5I$: C, 63.26; H, 5.61. Found: C, 63.47; H, 5.63.

α-D-Glucofuranosyl bromide.

Pentaacetylated *β*-D-glucopyrannose (1.00 g, 2.56 mmol) was dissolved in CH_2Cl_2 (5 mL) and 8.3 mL of a HBr solution in acetic acid (30%, w/v) was added. After 3 h of stirring, the solution was poured into an iced saturated aqueous $NaHCO_3$ solution (50 mL). Organic material was extracted with CH_2Cl_2. Combined organic layers were washed with water and brine, dried, and finally concentrated to an oil. The crude was 1-bromo-tetraacetyl-glucose (2.48 mmol, 97%).[140] ^1H NMR ($CDCl_3$) δ 6.54 (d, *J*= 2 Hz 1 H), 5.57 (dd, *J*= 10, 10 Hz, 1 H), 5.18 (dd, *J*= 9, 10 Hz, 1 H), 4.86 (dd, *J*= 2, 10 Hz, 1 H), 4.27-4.40 (m, 2 H), 4.12 (m, 1 H), 2.11 (s, 3 H), 2.10 (s, 3 H), 2.06 (s, 3 H), 2.04 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 170.1, 169.5, 169.4, 169.1, 86.5, 71.9, 70.3, 69.9, 66.9, 60.7, 20.3, 20.2.

This oil (0.54 g, 1.31 mmol) was dissolved in C_6H_6 under Ar and heated at reflux after addition of AIBN (0.02 g, cat.) and tributyltin hydride (0.71 mL, 2.63 mmol). After 12 h, C_6H_6 was removed under reduced pressure and the residue was filtered through a pad of silica gel. The crude tetraacetyl-1,5-anhydro-glucitol[85] (0.90 mmol, 69%) was recovered as an oil. ^1H NMR ($CDCl_3$) δ 5.14 (dd, *J*= 8,9 Hz, 1 H), 4.91 (dd, *J*= 9, 9 Hz, 1 H), 4.14 (dd, *J*= 2, 9 Hz, 1 H), 3.97-4.10 (m, 2 H), 3.51-3.61 (m, 1 H), 3.26 (dd, *J*= 10, 10 Hz, 1 H), 2.00 (s, 3 H), 1.95 (m, 9 H); ^{13}C NMR ($CDCl_3$) δ 169.8, 169.5, 169.0, 168.8, 75.8, 73.1, 68.5, 68.0, 66.2, 61.7, 20.0, 19.9.

1,5-Anhydro-glucitol.

Crude tetraacetyl-1,5-anhydro-glucitol (0.22 g, 0.66 mmol) in 21 mL of aq. MeOH ($MeOH/H_2O$, 20:1, v/v) was stirred at rt with an excess of K_2CO_3 for 5 h. MeOH was removed under reduced pressure and H_2O (5 mL) was added. The aqueous layer was thoroughly extracted with EtOAc and concentrated to yield 1,5-anhydro-glucitol[141] (0.65 mmol, 98%) as a white solid. ^1H NMR (D_2O) δ 3.92 (dd, *J*= 5, 11 Hz, 1 H), 3.82 (dd, *J*= 2, 11 Hz, 1 H), 3.62 (ddd, *J*= 2, 5, 11 Hz, 1 H), 3.54 (ddd, *J*= 6, 10, 11 Hz, 1 H), 3.38 (dd, *J*= 10, 10 Hz, 1 H), 3.26- 3.43 (m, 2 H), 3.20 (dd, *J*= 11, 11 Hz, 1 H); ^{13}C NMR (D_2O) δ 76.8, 74.0, 66.3, 65.9, 65.4, 57.5.

1,5-Anhydro-glucitol 6-phosphate, 11.

HMDS (1 mL) and TMSCl (2 mL) were successively added under Ar to 1,5-anhydro-glucitol (0.54 g, 3.33 mmol) in pyridine (10 mL). After 2 h, the solution was filtered through Celite. H_2O (50 mL) and hexane (50 mL) were added to the filtrate. The aqueous layer was extracted with hexane and the combined organic fractions were washed with saturated aqueous $CuSO_4$, water, brine, and dried. After removal of hexane under reduced pressure, fully silylated 1,5-anhydro-glucitol (3.26 mmol, 98%) was isolated as an oil. ^1H NMR ($CDCl_3$) δ 3.88 (dd, J= 5, 11 Hz, 1 H), 3.80 (dd, J= 2, 11 Hz, 1 H), 3.62 (dd, J= 6,11 Hz, 1 H), 3.45-3.55 (m, 1 H), 3.35-3.45 (m, 2 H), 3.13 (dd, J= 11, 11 Hz, 1 H), 3.10-3.18 (m, 1 H); ^{13}C NMR ($CDCl_3$) δ 81.5, 80.2, 71.9, 71.8, 70.3, 62.8, 1.3, 0.9, 0.5, -0.3.

Pentasilyl-1,5-anhydro-glucitol (0.58 g, 1.44 mmol) was dissolved in MeOH and stirred at 0°C for 30 min with solid K_2CO_3 (0.005 g, 0.05 mmol). MeOH was then removed under high vacuum and freshly distilled CH_2Cl_2 was added. Dibenzyl N,N-diisopropyl phosphoramidate (0.54 g, 1.60 mmol) was added, followed by 1H-tetrazole (0.02 g, 0.30 mmol), and the solution was stirred for 2 h, then cooled at -78°C. After addition of MCPBA (0.50 g, 2.90 mmol), the solution was stirred for 30 min. The excess of MCPBA was reduced by a saturated solution of $Na_2S_2O_3$ and simultaneously, benzoic acid was neutralized by a saturated solution of Na_2CO_3. Aqueous layer was extracted with CH_2Cl_2 and the combined organic fractions were washed with brine, dried and finally concentrated. The crude was then purified by radial chromatography (2-mm thickness of silica gel, hexane/EtOAc, 9:1, v/v) to yield the fully protected 1,5-anhydro-glucitol 6-phosphate (0.50 mmol, 35%). ^1H NMR ($CDCl_3$) δ 7.20- 7.40 (m, 10 H), 5.05 (s, 2 H), 5.03 (s, 2 H), 4.30 9 (ddd, J= 2, 8, 11 Hz, 1 H), 4.03 (ddd, J= 6, 10, 11 Hz, 1 H), 3.81 (dd, J= 5, 11 Hz, 1 H), 3.40-3.50 (m, 1 H), 3.30-3.40 (m, 1 H), 3.20-3.30 (m, 1 H), 3.08 (dd, J= 10, 11 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 135.8, 135.7, 129.9, 129.4, 128.4, 128.3, 127.9, 127.8, 127.7, 127.6, 127.3, 79.3, 79.2 (J_{POCC}= 6 Hz), 71.5, 71.5, 70.1, 69.0, 69.0, 67.2 (J_{POC}= 5 Hz), 1.2, 0.8, 0.4.

Protected anhydro-glucitol phosphate (0.32 g, 0.50 mmol) in THF (2 mL) was added to a heterogeneous solution of 10% Pd on C in 5 mL of THF/H_2O (4:1, v/v). The suspension was hydrogenated at 50 psi H_2 for 12 h. Catalyst removal by filtration through Celite and removal of the solvents yielded 1,5-anhydro-glucitol 6-phosphate **11** (0.48 mmol, 96%).[84] ^{1}H NMR (D_2O) δ 4.17 (dd, *J*= 6, 11 Hz, 1 H), 4.06 (ddd, *J*= 5, 6, 11 Hz, 1 H), 3.94 (dd, *J*= 5, 11 Hz, 1 H), 3.60-3.61 (m, 1 H), 3.37-3.50 (m, 2 H), 3.40 (dd, *J*= 5, 5 Hz, 1 H), 3.26 (dd, *J*= 10, 11 Hz, 1 H); ^{13}C NMR (D_2O) δ 79.2 (J_{POCC}= 8 Hz), 77.7, 69.7, 69.6, 69.3, 65.4 (J_{POC}= 6 Hz); MS *m/z* (rel inten) FAB 93 (100), 133 (13), 195 (32), 225 (11), 245 (M+H$^+$, 6); HRMS (FAB) calcd for $C_6H_{13}O_8P$ (M+H$^+$) 245.0426 found 245.0434.

2,3,4,6-Tetra-O-benzyl-α-D-glucose, 38.

A mixture of commercial methyl α-D-glucopyranoside **37** (4.84 g, 25.0 mmol), benzyl bromide (19.2 g, 112 mmol), tetrabutylammonium iodide (1.00 g, 3.00 mmol) and DMF (50 mL) was efficiently stirred at 0°C under Ar when NaH (4.91 g, 112 mmol) was added as a solid. The solution was maintained at 0°C for 1 h, then warmed to rt and stirred for 2 h. After addition of ether (200 mL), the organic fraction was washed with NH_4Cl saturated solution and brine, dried, and finally concentrated. The fully protected methyl α-D-glucopyranoside (22.3 mmol, 90%) was obtained as an oil. ^{1}H NMR ($CDCl_3$) δ 7.20-7.40 (m, 18 H), 7.10-7.15 (m, 2 H), 4.97 (d, *J*= 11 Hz, 1 H), 4.78-4.87 (m, 2 H), 4.77 (d, *J*= 14 Hz, 1 H), 4.60-4.68 (m, 2 H), 4.58 (d, *J*= 14 Hz, 1 H), 4.41-4.53 (m, 2 H), 3.98 (dd, *J*= 9, 9 Hz, 1 H), 3.53-3.80 (m, 5 H), 3.46 (s, 3 H); ^{13}C NMR ($CDCl_3$)δ 138.6, 138.0, 137.9, 137.7, 128.2, 128.1, 127.9, 127.7, 127.6 (2C), 127.5, 127.4 (2C), 127.3, 97.9, 81.9, 79.6, 75.5, 74.7, 73.2, 73.1, 69.8, 54.9.

The residue (12.2 g, 22.3 mmol) was mixed with a solution (167 mL) of glacial acetic acid and 4N H_2SO_4 (AcOH/4N H_2SO_4, 16:9, v/v), and heated at reflux of water for 12 h. H_2O (50 mL) was added, the mixture was cooled in an ice bath and the crystallized product was removed by filtration. The crystals were washed with aqueous acetic acid (200 mL, AcOH/H_2O, 1:1, v/v), and finally dried under high vacuum in the presence of P_2O_5, yielding tetrabenzyl-D-glucose **38** (10.8 mmol, 48%).[89] ^1H NMR ($CDCl_3$) δ 7.20-7.40 (m, 18 H), 7.10-7.15 (m, 2 H), 5.20 (d, *J*= 3 Hz, 1 H), 5.00-4.42 (m, 8 H), 3.95-4.10 (m, 2 H), 3.50-3.70 (m, 4 H); ^{13}C NMR ($CDCl_3$) δ 138.8, 138.7, 138.6, 138.5, 138.4, 138.0, 137.9, 137.8, 137.7, 128.2, 128.1, 127.9, 127.7, 127.6 (2C), 127.5, 127.4 (2C), 127.3, 97.5, 91.2, 84.5, 81.7, 79.9, 77.6, 75.7, 75.0, 74.7, 74.6, 73.4, 73.2, 70.2, 68.5, 66.3.

2,3,4,6-Tetra-O-benzyl-α-D-glucono-1,5-lactone, 39.

DMSO (3.06 mL, 43.1 mmol) in CH_2Cl_2 (50 mL) was slowly added to a -78°C solution of oxalyl chloride (1.90 mL, 21.5 mmol) in CH_2Cl_2 (25 mL) and stirring continued for 30 min at -78°C. Hemiacetal **38** (5.67 g, 10.8 mmol) in CH_2Cl_2 (25 mL) was then added under Ar and the reaction mixture stirred for an additional 2 h. After subsequent addition of anhydrous Et_3N (11.7 mL, 86.2 mmol), the reaction was warmed to rt and quenched with water (50 mL). The aqueous layer then extracted with CH_2Cl_2. The combined organic layers were washed with 0.1N HCl solution followed by brine, dried and concentrated. The crude product was 2,3,4,6-tetra-O-benzyl-α-D-glucono-1,5-lactone **39** (10.3 mmol, 96 %).[88] ^1H NMR ($CDCl_3$) δ 7.10-7.40 (m, 20 H), 4.95 (d, *J*= 9 Hz, 1 H), 4.35-4.70 (m, 7 H), 4.10 (d, *J*= 4 Hz, 1 H), 3.90 (dd, *J*= 9, 9 Hz, 1 H), 3.87 (dd, *J*= 9, 9 Hz, 1 H), 3.53-3.70 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 168.8, 137.2, 137.1, 136.6, 128.0, 127.9, 127.8, 127.6, 127.5, 127.4, 127.3, 80.3, 73.3, 73.2, 73.1, 72.9, 67.8.

3,4,5,7-Tetra-O-benzyl-1-deoxy-1-(dimethoxyphosphoryl)-*α*-D-*gluco*-2-heptulopyranose.

A solution of *n*-butyl lithium in *n*-hexane (1.6 N, 12.9 mL, 20.6 mmol) was added to a solution of dimethyl methanephosphonate (2.23 mL, 20.6 mmol) in THF (50 mL) under Ar at -78°C with stirring. After 30 min, a solution of 2,3,4,6-tetra-O-benzyl-*α*-D-gluconolactone **39** (5.40 g, 10.3 mmol) in THF (20 mL) was added to the previous solution. Stirring continued for an additional 1 h. The mixture was removed from the cooling bath and allowed to warm to rt. After 1 h, ice-cold 10% aqueous NH_4Cl (w/v) solution (50 mL) was added to the reaction mixture, and the resulting oily substance was extracted with EtOAc. The combined organic layers were washed with 2N HCl, saturated $NaHCO_3$, water and finally brine, dried, and concentrated. Crude product was chromatographed on silica gel and yielded lactol 3,4,5,7-tetra-O-benzyl-1-deoxy-1-(dimethoxyphosphoryl)-*α*-D-*gluco*-2-heptulopyranose[88] (9.72 mmol, 94 %) as a white solid. ^{1}H NMR ($CDCl_3$) δ 7.10-7.40 (m, 20 H), 5.74 (br, 1 H), 4.94 (d, J= 13 Hz, 1 H), 4.88 (s, 2 H), 4.85 (d, J= 13 Hz, 1 H), 4.64 (d, J= 12 Hz, 1 H), 4.55-4.61 (m, 1 H), 4.43 (s, 2 H), 4.11 (dd, J= 9, 9 Hz, 1 H), 4.07 (ddd, J= 2, 4, 9 Hz, 1 H). 3.50-3.75 (m, 11 H), 3.30 (d, J= 10 Hz, 1 H), 2.31 (dd, J= 14, 16 Hz, 1 H), 1.66 (m, 1 H); ^{13}C NMR ($CDCl_3$) δ 139.0, 138.9, 138.8, 138.7, 128.2, 128.0, 127.9, 127.8, 127.6, 127.5, 127.4, 127.3, 96.3, 96.2, 82.7, 82.6, 82.2, 82.0, 77.9, 75.0, 74.6, 74.3, 72.7, 70.6, 68.0, 52.7 (J_{POC}= 6 Hz), 51.2 (J_{POC}= 6 Hz), 33.2 (J_{PC}= 135 Hz).

A mixture of 3,4,5,7-tetra-O-benzyl-1-deoxy-1-(dimethoxyphosphoryl)-D-*glycero*-D-*gulo*-heptitol and 1,3,4,5-tetra-O-benzyl-7-deoxy-7-(dimethoxyphosphoryl)-D-*glycero*-D-*gulo*-heptitol, 40.

To a stirred solution of 3,4,5,7-tetra-O-benzyl-1-deoxy-1-(dimethoxyphosphoryl)-*α*-D-*gluco*-2-heptulopyranose (6.30 g, 9.72 mmol) in THF (20 mL) was added $NaBH_4$ (0.78 g, 20.6 mmol), and stirring was continued for 12 h at rt. Insoluble material was removed by filtration and washed with THF. The filtrate and the washings were combined and concentrated. The residue was partitioned between EtOAc (50 mL) and water (50 mL). The combined organic layers were washed with H_2O and brine, then dried and concentrated. After flash chromatography (EtOAc/Hexane, 1:1, v/v) a mixture of the two epimers[88] (9.42 mmol, 97 %) **40** was obtained. The NMR data of this mixture were not interpretable.

[4S-(4α,5β,6α)]-4,5,6-Tris-benzyloxy-3-[(benzyloxy)methyl]cyclohex-2-en-1-one, 41.

A solution of TFAA (0.67 mL, 4.74 mmol) in CH_2Cl_2 (5 mL) was added dropwise to a stirred solution of DMSO (0.67 mL, 9.48 mmol) in CH_2Cl_2 (20 mL) at -78°C under Ar, and stirring was continued for 30 min. To this solution was added dropwise a solution of the (dimethoxyphosphoryl)heptitol derivative (0.79 g, 1.18 mmol) in CH_2Cl_2 (5 mL). Stirring was maintained for 1 h, period after which Et_3N (2.60 mL, 18.95) was added. The mixture was removed from the cooling bath after 30 min and allowed to warm to 0°C. A 2N HCl solution (20 mL) was added to the mixture. The organic soluble material was then extracted with CH_2Cl_2 and the combined organic layers were washed with brine, dried and concentrated. The residue was then taken up in toluene (10 mL). To this solution was added 18-crown-6 (0.10 g, 0.23 mmol) and potassium carbonate (0.50 g, 3.70 mmol). The mixture was stirred for 8 h at rt. Insoluble material was then removed by filtration through Celite and washed with toluene. The filtrate and the washings were combined, washed with 2N HCl and brine, dried, and finally concentrated. The residue was chromatographed on silica gel (EtOAc/Hexane, 1:9, v/v) and afforded the branched unsaturated inosose derivative[88] (0.60 mmol, 51 %) **41** as a colorless syrup. ^1H NMR ($CDCl_3$) δ 7.20-7.40 (m, 20 H), 5.90 (dd, J= 1, 4 Hz, 1 H), 4.98 (d, J= 14 Hz, 1 H), 4.60-4.80 (m, 4 H), 4.37-4.50 (m, 2 H), 4.12-4.35 (m, 3 H), 4.07 (dd, J= 7, 8 Hz, 1 H), 3.92 (d, J= 13 Hz, 1 H), 3.58 (dd, J= 9, 4 Hz, 2 H); ^{13}C NMR ($CDCl_3$) δ 139.5, 138.4, 138.0, 137.9, 128.5, 128.4, 128.3, 127.9, 127.8, 127.6, 127.5, 127.4, 127.3, 124.5, 79.0, 78.7, 78.6, 74.5, 73.9, 72.6, 72.4, 70.0, 64.8.

[2R-(2α,3β,4α,5β)]-2,3,4-Tris-benzyloxy-5-[(benzyloxy)methyl]-cyclohexanone, 42.

Enone **41** (1.50 g, 2.88 mmol) and 3 drops of phase transfer catalyst Adogen 464 in C_6H_6 (50 mL) were vigorously stirred under Ar at reflux of C_6H_6 with a solution of sodium dithionite (1.25 g, 7.21 mmol) and $NaHCO_3$ (1.15 g, 14.4 mmol) in water (30 mL). The reaction was monitored by NMR. After cooling, the aqueous layer was separated and extracted twice with ether. The combined organic layers were washed with brine, dried, and concentrated. The residue was purified by radial chromatography (4-mm thickness of silica gel, EtOAc/Hexane, 1:5, v/v) and afforded cyclohexanone **42** (2.83 mmol, 98 %) as an oil which solidified at rt. ^1H NMR ($CDCl_3$) δ 7.15-7.40 (m, 20 H), 4.96 (d, *J*= 9 Hz, 1 H), 4.95 (s, 1 H), 4.90 (d, *J*= 5 Hz, 1 H), 4.77 (d, *J*= 11 Hz, 1 H), 4.56 (d, *J*= 6 Hz, 1 H), 4.52 (d, *J*= 7 Hz, 1 H), 4.40 (s, 2 H), 4.11 (d, *J*= 10 Hz, 1 H), 3.90 (dd, *J*= 9, 9 Hz, 1 H), 3.75 (dd, *J*= 4, 9 Hz, 1 H), 3.69 (dd, *J*= 10, 9 Hz, 1 H), 3.35 (dd, *J*= 3, 9 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 205.5, 138.3, 138.2, 137.8, 137.5, 128.2, 128.1, 128.0, 127.9, 127.8, 127.6, 127.5, 127.4, 85.9, 85.6, 79.2, 75.6, 75.2, 73.2, 72.9, 68.5, 39.9, 39.0; Anal. Calcd for $C_{35}H_{36}O_5$: C, 78.34; H, 6.76. Found: C, 78.11; H, 6.79.

[1S-(1α,2α,3β,4α,5β)]-2,3,4-Tris-benzyloxy-5-[(benzyloxy)methyl]-cyclohexan-1-ol, 43.

To a solution of cyclohexanone (1.07 g, 2.00 mmol) **42** in THF (15 mL) stirred at -78°C under Ar was added dropwise a solution of L-Selectride® (1N, 2.20 mL, 2.20 mmol) in THF. The solution was stirred for 1 h, then warmed to 0°C. After 10 min, 2 mL of H_2O were added followed by 1.60 mL of 5N aq NaOH (8.00 mmol) and 0.70 mL of

H_2O_2 (6.00 mmol, 30%, w/v). The oxidation reaction was quenched after 30 min by addition of Na_2SO_3 (10 mL). The organic soluble material was extracted with ether and the combined organic layers were washed with brine, dried, and concentrated. Radial chromatography (2-mm thickness of silica gel, EtOAc/hexane, 1:3, v/v) of the crude afforded cyclohexanol (1.94 mmol, 97 %) **43** as a white solid. ^{1}H NMR ($CDCl_3$) δ 7.15-7.40 (m, 20 H), 4.86 (m, 3 H), 4.66 (s, 2 H), 4.55 (d, *J*= 4 Hz, 1 H), 4.53 (d, *J*= 7 Hz, 1 H), 4.40 (s, 2 H), 4.09 (m, 1 H), 3.86 (dd, *J*= 9, 9 Hz, 1 H), 3.74 (dd, *J*= 4, 9 Hz, 1 H), 3.50 (dd, *J*= 9, 9 Hz, 1 H), 3.40 (ddd, *J*= 2, 4, 9 Hz, 1 H), 2.60 (br, 1 H), 2.19 (ddd, *J*= 2, 11, 14 Hz, 1 H), 1.94 (ddd, *J*= 4, 4, 15 Hz, 1 H), 1.57 (ddd, *J*= 2, 14, 15 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 138.9, 138.7, 138.5, 138.0, 128.4, 128.2, 128.0, 127.7, 127.6 (2C), 127.5, 127.4, 83.5, 83.2, 80.7, 75.6, 75.5, 72.9, 72.4, 69.7, 66.4, 36.6, 30.1; MS *m/z* (rel inten) (FAB) 91 (100), 136 (18), 181 (22), 539 ($M+H^+$, 1); HRMS (FAB) calcd for $C_{35}H_{39}O_5$ ($M+H^+$) 539.2797, found 539.2789; Anal. Calcd for $C_{35}H_{38}O_5 \cdot 1/2H_2O$: C, 76.76; H, 7.17. Found: C, 76.80; H, 7.09.

[1S-(1α,2α,3β,4α,5β)]-2,3,4-Tris-hydroxyl-5-[(hydroxy)methyl]-cyclohexan-1-ol, 45.

Cyclohexan-1-ol **43** (0.31 g, 0.61 mmol) in MeOH (0.5 mL) was added to 10% Pd on C (0.10 g) in 5 mL of MeOH. Hydrogenation at 50 psi H_2 for 12 h was followed by filtration of the suspension through Celite and concentration which afforded α-carba-glucose[87b] (0.61 mmol, 100 %) as a white solid. ^{1}H NMR (D_2O) δ 4.06 (ddd, *J*= 2, 4, 8 Hz, 1 H), 3.63-3.65 (m, 2 H), 3.56 (dd, *J*= 9, 9 Hz, 1 H), 3.40 (dd, *J*= 3, 9 Hz, 1 H), 3.25 (dd, *J*= 9, 10 Hz, 1 H), 1.70-1.90 (m, 1 H), 1.47 (dddd, *J*= 3, 4, 9, 9 Hz, 1 H); ^{13}C NMR (D_2O) δ 80.4, 79.9, 79.0, 74.8, 68.2, 44.0, 36.1.

[1S-(1α,2α,3β,4α,5β)]-1,2,3,4-Tetrakis-silyloxy-5-[(silyloxy)methyl]-cyclohexane.

To a solution of α-carbaglucose (0.18 g, 1.00 mmol) in pyridine (5 mL) stirred under Ar at rt was added 0.5 mL of HMDS and 1.0 mL of TMSCl. The solution turned cloudy white within 5 min. After 1 h of stirring, the mixture was filtered through Celite. The residue was rinsed with hexane. H_2O was added to the filtrate and the aqueous layer was extracted with hexane. The combined organic layers were thoroughly washed with aqueous $CuSO_4$ (5%, w/v), water and brine, then dried. The fully silylated α-carbaglucose (0.97 mmol, 97 %) was obtained after removal of hexane under reduced pressure. ^{1}H NMR ($CDCl_3$) δ 3.90 (ddd, J= 2, 4, 8 Hz, 1 H), 3.74 (dd, J= 9, 9 Hz, 1 H), 3.68 (dd, J= 5, 9 Hz, 1 H), 3.50 (dd, J= 3, 9 Hz, 1 H), 3.29 (dd, J= 8, 9 Hz, 1 H), 3.17 (dd, J= 2, 9 Hz, 1 H), 1.87-2.02 (m, 1 H), 1.60 (ddd, J= 3, 4, 14 Hz, 1 H), 1.34 (ddd, J= 2, 10, 14 Hz, 1 H), 0.1-0.2 (m, 45 H); ^{13}C NMR ($CDCl_3$) δ 76.0, 74.8, 74.6, 71.6, 62.8, 39.3, 32.6, 1.3, 1.0, 0.6, 0.3, -0.5.

[1S-(1α,2α,3β,4α,5β)]-1,2,3,4-Tetrakis-silyloxy-5-[(phosphonoxy)-methyl]-cyclohexane, 45.

A solution of the fully silylated α-carbaglucose (0.60 g, 1.12 mmol) and K_2CO_3 (0.05 g, cat.) in MeOH (20 mL) was stirred at 0°C for 15 min. MeOH was then rapidly removed under high vacuum and CH_2Cl_2 (20 mL) was added. Dibenzyl N,N-phosphoramidate (0.46 g, 1.33 mmol) and 1H-tetrazole (0.02 g, 0.32 mmol) were added and, upon stirring under Ar, the solution became cloudy. The solution was cooled to -78°C and MCPBA (0.47 g, 2.66 mmol) was added. A saturated $Na_2S_2O_3$ aqueous solution (20 mL) was added along with saturated aqueous Na_2CO_3 (20 mL). After extraction of the organic soluble material with CH_2Cl_2, the combined organic layers were washed with brine, dried, and concentrated. Radial chromatography (2-mm thickness of silica gel, hexane/EtOAc, 9:1, v/v) of the residue yielded the protected phosphorylated α-carbaglucose **45** (0.71 mmol, 63 %). ^{1}H NMR (CDCl$_3$) δ 7.25-7.32 (m, 10 H), 4.90-5.03 (m, 4 H), 3.95-4.02 (m, 3 H), 3.74 (m, 1 H), 3.62 (dd, *J*= 9, 9 Hz, 1 H), 3.13 (dd, *J*= 9, 10 Hz, 1 H), 3.01 (dd, *J*= 3, 10 Hz, 1 H), 1.95-2.01 (m, 1 H), 1.50 (ddd, *J*= 3, 3, 15 Hz, 1 H), 1.10-1.20 (m, 1 H); ^{13}C NMR (CDCl$_3$) δ 138.0, 137.9, 128.5, 128.4, 127.8, 127.7, 75.6, 74.4, 74.2, 71.1, 69.1 (2C, J_{POC}= 5 Hz), 68.6 (J_{POC}= 5 Hz), 38.3 (J_{POCC}= 7 Hz), 32.1, 1.27, 1.0, 0.5, 0.2; ^{31}P NMR δ +0.4; MS *m/z* (rel inten) FAB 91 (100), 147 (45), 269 (10), 727 (M+H$^+$, 0.5), 749 (M+Na$^+$, 1); HRMS (FAB) calcd for $C_{33}H_{59}O_8PNaSi_4$ (M+Na$^+$) 749.2922 found 749.2900.

[1S-(1α,2α,3β,4α,5β)]-1,2,3,4-Tetrakis-hydroxyl-5-[(phosphonoxy)-methyl]-cyclohexane, 34.

Phosphorylated tetra-silylated *α*-carbaglucose **45** (0.53 g, 0.77 mmol) in THF (2 mL) was added to 10% Pd on C (0.10 g) in 5 mL of an aqueous THF solution (4:1, THF/H$_2$O, v/v). Hydrogenation at 50 psi H$_2$ for 12 h was followed by filtration of the suspension through Celite and concentration to afford *α*-carbaglucose 6-phosphate (0.77, 100 %). ^{1}H NMR (D$_2$O) δ 4.02-4.13 (m, 2 H), 3.94-4.03 (m, 1 H), 3.55 (dd, *J*= 11, 10 Hz, 1 H), 3.41 (dd, *J*= 4, 10 Hz, 1 H), 3.32 (dd, *J*= 10, 10 Hz, 1 H), 1.80-2.03 (m, 2 H), 1.54 (m, 1 H); ^{13}C NMR (D$_2$O)δ 80.3, 79.8, 78.1, 74.8, 72.6, 43.1 (J_{POCC}= 6 Hz), 36.0; ^{31}P NMR (D$_2$O) δ +0.2; MS *m/z* (rel inten) FAB 242 (23), 269 (17), 259 (M+H$^+$, 4), 281 (M+Na$^+$, 100); HRMS (FAB) calcd for $C_7H_{15}O_8PNa$ (M+Na$^+$) 281.0402 found 281.0407.

[1R*-(1α,2α,3α,4β,5α,6β)]-3,4,5,6-Tetrakis-benzyloxy-cyclohexa-1,2-diol, 48.

To a heterogeneous mixture of *myo*-inositol (54.0 g, 300 mmol) and 2,2-dimethoxypropane (120 mL, 1.10 mol) in DMSO (180 mL) was added *p*-toluenesulfonic acid (0.35 g, 0.002 mmol). Upon heating at 110°C under N$_2$, the solution became homogeneous. The heat was maintained for 6 h, after which period DMSO was distilled off under high vacuum. The residue was triturated in EtOAc and the crystals were filtered. Tetrol **47** (216 mmol, 72%) was isolated as a white solid. To a mixture of tetrol **47** (6.00 g, 25.0 mmol), benzyl bromide (18.1 g, 102 mmol) and tetrabutyl ammonium iodide (0.50 g, 1.25 mmol) in DMF (100 mL) stirred under Ar at 0°C was added NaH (4.8 g, 110 mmol) as a powder. The solution was stirred for 2 h at 0°C, then warmed to rt where stirring was maintained for 1 h. Ice-cold saturated NH_4Cl solution (100 mL) was added to the reaction mixture, followed by CH_2Cl_2 (200 mL). The aqueous layer was extracted with CH_2Cl_2 and the combined organic layers were washed with brine, dried, and concentrated. The residue was dissolved in a MeOH/ 6N aq HCl (3:1, v/v) solution (160 mL) which was then refluxed for 12 h. After cooling, CH_2Cl_2 was added and the aqueous layer was extracted three times with CH_2Cl_2. The combined organic fractions were washed with brine, dried, and concentrated to afford a solid which was recrystallized in CH_2Cl_2 /Hexane (50:1, v/v). Diol **48**[142] (18.3 mmol, 73 %) was obtained as a white powder after filtration of the solvents. ^1H NMR ($CDCl_3$) δ 7.20-7.45 (m, 20 H), 4.75-5.00 (m, 5 H), 4.72 (s, 2 H), 4.56 (s, 2 H), 4.20 (dd, *J*= 3, 3 Hz, 1 H), 3.97 (dd, *J*= 9, 10 Hz, 1 H), 3.82 (dd, *J*= 9, 10 Hz, 1 H), 3.40-3.52 (m, 2 H), 2.20 (br, 1 H); ^{13}C NMR ($CDCl_3$) δ 138.6, 129.9, 128.8, 128.5, 128.1, 128.0, 127.9, 83.2, 81.6, 81.3, 80.0, 75.9, 75.6, 75.5, 72.7, 71.7, 69.1.

[1R*-(1α,2α,3α,4β,5α,6β)]-3,4,5,6-Tetrakis-benzyloxy-cyclohexene oxide, 29.

To a heterogeneous solution of 1,4,5,6 - tetrabenzyl - *myo*-inositol **48** (4.41 g, 8.17 mmol) in C_6H_6 (20 mL) was added trimethylorthoacetate (1.33 g, 9.81 mmol) under N_2, followed by *p*-toluenesulfonic acid (0.15 g, 0.82 mmol). The solution was stirred vigorously at rt for 12 h. A few drops of pyridine were added and C_6H_6 was removed under reduced pressure. CH_2Cl_2 (25 mL) was added, followed by chlorotrimethylsilane

(3.0 mL, 24.5 mmol). The solution was heated at reflux for 3 h and, after cooling, H_2O (20 mL) was added. Organic soluble material was extracted with CH_2Cl_2 and the combined organic layers were then washed with aqueous $CuSO_4$ (3%, w/v), water, and brine, dried and finally concentrated under reduced pressure. MeOH (25 mL) was added, followed by powdered MeONa (0.45 g, 8.17 mmol), and the solution was refluxed under N_2. After 4 h, the solution was cooled and powdered $NaHCO_3$ was added followed by ether. The aqueous fraction was extracted with ether and the combined organic layers were washed with brine and dried. After removal of the solvents, the resulting solid was recrystallized from a solution of EtOAc and hexane (EtOAc/hexane, 1:9, v/v) to afford epoxide **29** (6.05 mmol, 74%) as a white solid. ^1H NMR ($CDCl_3$) δ 7.20-7.40 (m, 20 H), 4.70-4.88 (m, 8 H), 3.90 (dd, *J*= 2, 8 Hz, 2 H), 3.63 (dd, *J*= 8, 10 Hz, 1 H), 3.47 (dd, *J*= 8, 10 Hz, 1 H), 3.30 (dd, *J*= 1, 4 Hz, 1 H), 3.20 (d, *J*= 4 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 128.5, 128.4, 128.3, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 83.4, 79.3, 79.2, 79.0, 75.9, 75.5, 73.3, 73.1, 55.2, 53.9; IR (neat NaCl) 3440 (s), 3090 (m), 3060 (m), 3025 (s), 2915 (m), 2900 (m), 2870 (m), 1640 (w), 1605 (w), 1495 (m), 1395 (m), 1365 (s), 1255 (m); MS *m/z* (rel inten) EI 91 (100), CI 91 (60), 107 (13), 179 (16), 181 (100), 182 (14), 271 (24), 431 (15), 521 ($M-H^+$, 3); HRMS (FAB) calcd for $C_{34}H_{33}O_5$ ($M-H^+$) 521.2328 found 521.2326. Anal. Calcd for $C_{34}H_{34}O_5 \cdot 1/2H_2O$: C, 76.81; H, 6.63. Found: C, 76.81; H, 6.60.

[1S*(1α,2β,3α,4β,5α,6β)]-2,3,4,5-Tetrakis-benzyloxy-2-[(triphenylmethoxy)-methyl]-cyclohexan-1-ol, 49.

To a solution of diol **32** (0.42 g, 0.76 mmol) in CH_2Cl_2 (10 mL) was added triphenylmethyl chloride (0.23 g, 0.83 mmol) and Et_3N (0.12 g, 1.20 mmol) under Ar at rt. After 12 h, CH_2Cl_2 (20 mL) and saturated NH_4Cl solution (20 mL) were added. The combined organic layers were washed with water and brine, dried, and finally concentrated. The crude was purified by radial chromatography (EtOAc/hexane, 1:3, v/v) and afforded the tritylated alcohol **49** (0.70 mmol, 92%). ^{1}H NMR (CDCl$_3$) δ 7.10-7.50 (m, 33 H), 6.90-6.97 (m, 2 H), 4.75-5.00 (m, 9 H), 4.34 (d, *J*=10 Hz, 1 H), 3.85 (dd, *J*= 9, 10 Hz, 1 H), 3.55-3.80 (m, 4 H), 3.38-3.51 (m, 2 H), 2,42 (br, 1 H), 1.72 (dddd, *J*= 2,2,9,10 Hz, 1 H); ^{13}C NMR (CDCl$_3$) δ 143.7, 138.6, 138.5, 138.4, 138.2, 128.8, 128.7, 128.5, 128.4, 128.2, 128.1, 127.9, 127.8, 127.7, 86.7, 86.3, 85.5, 83.0, 77.3, 75.8, 75.7, 75.6, 75.0, 70.0, 58.5, 45.9. MS *m/z* (rel inten) FAB 153 (100), 259 (18), 553 (3), 705 (M-$C_7H_7^+$, 1), 795 (M-H^+, 2.5).

[1S*(1α,2β,3α,4β,5α,6β)]-2,3,4,5-Tetrakis-benzyloxy-2-[(triphenyl-methoxy)-methyl]-1-O-methylthionocarbonate-cyclohexane, 50.

To a solution of alcohol **49** (0.27 g, 0.34 mmol) in THF (10 mL) stirred under Ar was added a suspension of NaH (0.03 g, 0.68 mmol) in THF. The solution was stirred at rt for 1 h, then carbon disulfide was added (0.25 mL, 3.30 mmol) and stirring was maintained for 12 h. Addition of methyl iodide (0.5 mL, 3.00 mmol) and heating at reflux for 2 h turned the solution dark red. After cooling, saturated aqueous NH_4Cl (20 mL) was added, along with ether (20 mL). The aqueous layer was extracted with ether and the combined organic fractions were washed with brine, dried, and concentrated. Radial purification (2-mm thickness of silica gel, EtOAc/Hexane, 1:5, v/v) afforded the xanthate **50** (0.23 mmol, 69%) as a solid. ^{1}H NMR (CDCl$_3$) δ 7.05-7.50 (m, 33 H), 6.88-6.98 (m, 2 H), 6.30-6.40 (m, 1 H), 4.80-4.95 (m, 4 H), 4.65-4.75 (m, 4 H), 3.97 (d, *J*= 9 Hz, 1 H), 3.60-3.80 (m, 5 H), 3.50 (s, 2 H), 2.50 (s, 3 H), 2.20 (m, 1 H); ^{13}C NMR (CDCl$_3$) δ 214.7, 143.4, 138.5, 138.4, 138.2, 137.8, 129.1, 128.8, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7., 127.6, 127.2, 127.0, 126.9, 126.8, 86.7, 85.9, 83.8, 82.4, 79.6, 75.9, 75.6, 75.5, 73.7, 58.2, 44.9, 19.2. MS *m/z* (rel inten) FAB 153 (100), 305 (30), 795 (M-$C_7H_7^+$, 1), 871 (M-H+, 0.25).

[1R-(1a,2b,3a,4b,5a)]-1,2,3,4-Tetrakis-benzyloxy-5-[(hydroxy)methyl]-cyclohexane.

Xanthate **50** (0.12 g, 0.14 mmol) was dissolved in C_6H_6 (20 mL) under Ar and heated at reflux for 12 h in presence of AIBN (0.005 g, 0.03 mmol) and tributyltin hydride (0.07 mL, 0.28 mmol). C_6H_6 was then removed and MeOH (10 mL) along with *p*-toluenesulfonic acid (0.01 g, 0.06 mmol) were added. The solution was stirred for a 3 h period after which the solvent was removed under reduced pressure and the residue was chromatographed (EtOAc/Hexane, 1:3, v/v) to afford alcohol **51**[87b] (0.13 mmol, 93 %) as an oil. ^1H NMR ($CDCl_3$) δ 7.22-7.40 (m, 20 H), 4.97 (dd, *J*= 1, 11 Hz, 3 H), 4.84 (dd, *J*= 1, 11 Hz, 2 H), 4.60-4.70 (m, 3 H), 3.60-3.63 (m, 2 H), 3.50-3,60 (m, 3 H), 3.43 (dd, *J*= 9, 9 Hz, 1 H), 2.04 (ddd, *J*= 2, 3, 9 Hz, 1 H), 1:20-1.40 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 138.8, 138.1, 128.6, 128.4, 128.3, 128.2, 128.1, 128.0 (2C), 127.9, 127.8, 127.7, 127.6, 127.5, 86.3, 85.9, 82.1, 80.1, 75.8, 75.1, 72.3, 64.6, 40.2, 29.9.

[1R-(1a,2b,3a,4b,5a)]-1,2,3,4-Tetrakisbenzyloxy-5-[(dibenzyloxy-phosphonyloxy)methyl]-cyclohexane, 52.

A mixture of alcohol **51** (0.08 g, 0.13 mmol), dibenzyl-N,N-diisopropyl phosphoramidate (0.09 g, 0.26 mmol) and 1H-tetrazole (0.002 g, 0.03 mmol) in CH_2Cl_2 was stirred at rt for 4 h under Ar. The solution was then cooled to -78°C, where MCPBA (0.09 g, 0.52 mmol) was added as a solid. After stirring for 15 min, saturated aqueous $Na_2S_2O_3$ (20 mL) was added along with CH_2Cl_2 (20 mL). The aqueous layer was extracted with CH_2Cl_2 and the combined organic layers were washed with brine, dried, and concentrated. Radial chromatography (2-mm thickness of silica gel, hexane/EtOAc, 5:1, v/v) of the residue afforded the fully benzylated β-carbaglucose 6-phosphate **52** (0.12 mmol, 92%) as an oil. ^1H NMR ($CDCl_3$) δ 7.00-7.40 (m, 30 H), 4.94 (s, 2 H), 4.92 (s, 2 H), 4.65-4.84 (m, 6 H), 4.51 (m, 2 H), 4.41 (dd, *J*= 9, 9 Hz, 1 H), 4.04 (m, 1 H), 3.92 (m, 1 H), 3.36 (m, 2 H), 3.18 (m, 1 H), 1.92 (ddd, *J*= 2, 2, 12 Hz, 1 H), 1.42-1.60

(m, 1 H), 1.10-1.26 (m, 1 H); ^{13}C NMR ($CDCl_3$) δ 138.6, 138.5, 138.3, 128.6, 128.4, 128.3, 128.0, 127.9 (2C), 127.7 (2C), 127.6, 127.5, 86.0, 85.6, 79.8, 79.6, 75.8, 75.7, 75.2, 72.3, 69.4 (2C, J_{POC}= 7 Hz), 67.5 (J_{POC}= 4 Hz), 38.7 (J_{POCC}= 7 Hz), 29.5; MS *m/z* (rel inten) 183 (100), 437 (14), 617 (22), 707 ($M-C_7H_7^+$, 42), 797 ($M-H^+$, 2.5).

[1R-(1a,2b,3a,4b,5a)]-1,2,3,4-Tetrakis-hydroxyl-5-[(phosphonoxy)methyl]-cyclohexane, 33.

Fully benzylated β-carbaglucose 6-phosphate **52** (0.10 g, 0.12 mmol) in THF (2 mL) was added to 10% Pd on C (0.05 g) in 10 mL of an aqueous THF solution (4:1, THF/H_2O, v/v). Hydrogenation at 50 psi H_2 for 12 h was followed by filtration of the suspension through Celite and concentration to afford β-carbaglucose 6-phosphate (0.04 g, 100 %). ^1H NMR (D_2O) δ 3.92 (m, 2 H), 3.50-3.60 (m, 1 H), 3.39 (ddd, *J*= 8, 9, 9 Hz, 1 H), 3.30 (dd, *J*= 9, 10 Hz, 1 H), 3.15 (dd, *J*= 10, 10 Hz, 1 H), 1.90 (ddd, *J*= 3, 4, 13 Hz, 1 H), 1.50-1.63 (m, 1 H), 1.26 (ddd, *J*= 12, 12, 13 Hz, 1 H); ^{13}C NMR; MS *m/z* (rel inten) FAB 127 (50), 171 (100), 183 (85), 257 ($M-H^+$, 23); HRMS calcd for $C_7H_{15}O_8P$ ($M-H^+$) 257.0426 found 257.0421.

Arabinitol 5-phosphate, 57.

To a solution of PEP^-K^+ (1.10 g, 7.30 mmol) and water (10 mL) in a 100-mL Erlenmeyer flask were added arabinose **60** (1.09 g, 7.27 mmol), ATP (0.13 g, 0.20 mmol) and $MgCl_2.6H_2O$ (0.22 g, 1.00 mmol). The pH was adjusted to pH 7.6 with 1N NaOH, the solution was degassed with Ar for 30 min, and 0.1 mL of mercaptoethanol was added. Hexokinase (1000 U) and pyruvate kinase (50 U) were added. The flask was then stoppered and agitated at 150 rpm on an orbital shaker at rt. Analysis by ^{31}P NMR monitored the formation of arabinose 6-phosphate. After 2 days 500 U of hexokinase along with ATP (0.15 g, 0.20 mmol) were added to the reaction mixture. The volume of the solution was reduced to 2 mL under high vacuum, and the crude was purified by aqueous chromatography on AG-1 X8 resin by eluting with a TEAB gradient (50 mM-500 mM). After assay and concentration of appropriate fractions to a 5 mL solution, the residue was passed through a cation exchange column (H^+-Dowex 50). The elute was neutralized and concentrated to dryness to yield arabinose 5-phosphate containing some residual PEP. Arabinose 5-phosphate (0.50 g, 2.00 mmol) was dissolved in H_2O and $NaBH_4$ was added as a powder. After stirring for 12 h, the solution was loaded on an AG-1 X8 resin column and the product was eluated with a TEAB gradient (50 mM-500 mM). The fractions containing arabinitol 5-phosphate were concentrated and passed through a cation exchange column (H^+-Dowex). The elute was neutralized and concentrated to dryness, which yielded arabinitol 5-phosphate **57**[143] (0.15 mmol, 75%). ^1H NMR (D_2O) δ 4.05 (ddd, *J*= 11, 5, 2 Hz, 1 H), 3.92-4.10 (m, 1 H), 3.82 (ddd, *J*= 1, 7, 7 Hz, 1 H), 3.72-3.79 (m, 1 H), 3.54 (d, *J*= 6 Hz, 2 H), 3.52 (dd, *J*= 1, 6 Hz, 1 H); ^{13}C NMR (D_2O) δ 70.5, 70.3, 69.9, 67.8, 63.5.

2,3,4-Tris-benzyl-5-[(triphenyl)methyl]-D-*arabino*-1,3-propyldithiane, 62.

Arabinose **60** (25 g, 166 mmol) was added to 200 mL of a pre-mixed solution of EtOH and 12N HCl (EtOH/HCl, 1:1, v/v), immediately followed by 1,3-dithiopropane. The solution was stirred at rt for 6 h, then poured into 300 mL of EtOH and placed at -20°C. After 12 h, the crystals formed upon standing were filtered and rinsed with ice-cold EtOH (200 mL). The filtrate was placed at -20°C. A second crop was collected after 24 h. Thioacetal arabinose (116 mmol, 70 %) was isolated as a white solid. ^{13}C NMR ($CDCl_3$) δ 143.1, 136.1, 128.4, 127.6, 126.9, 86.6, 71.6, 69.7, 69.1, 65.1, 46.7, 26.6, 26.0, 25.1. Thioacetal arabinose **61** (1.00 g, 4.2 mmol) was dissolved in pyridine (5 mL) and stirred for 12 h under Ar in presence of tritylchloride (1.74 g, 6.25 mmol) and DMAP (0.02 g, 0.18 mmol). Pyridine was then removed under reduced pressure and EtOAc (50 mL) was added along with 50 mL of aq. $CuSO_4$ (3%, w/v). The aqueous layer was extracted with EtOAc and the combined organic fractions were washed with water and brine, dried, and concentrated. The residue was dissolved in DMF (100 mL) under Ar. To this solution was added benzyl bromide (2.51 g, 14.7 mmol) and tetrabutyl ammonium iodide (0.08 g, 0.2 mmol). After cooling the mixture to 0°C, NaH was added as a solid and the solution was warmed to rt after 2 h of stirring at 0°C. The reaction was quenched after 2 h with an ice-cold NH_4Cl saturated solution. CH_2Cl_2 was added to the solution and the organic soluble materials were extracted. Combined organic fractions were washed with water and brine, dried, and concentrated. The residue was then chromatographed on silica and yielded the fully protected thioacetal arabinose[144] **62** (2.70 mmol, 64%). ^1H NMR ($CDCl_3$) δ 7.00-7.53 (m, 30 H), 4.40-4.90 (m, 6 H), 4.27-4.40 (m, 2 H), 4.15-4.20 (m, 1 H), 4.00-4.10 (m, 1 H), 3.77-3.90 (m, 1 H), 3.62-3.75 (m, 1 H), 2.65-2.80 (m, 2 H), 2.50-2.65 (m, 2 H), 1.75-1.92 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 143.6, 138.4, 138.3, 138.1, 129.2, 128.4, 128.3, 128.2, 128.1, 128.0, 127.8, 127.7, 127.6, 127.5, 127.4, 127.3, 127.2, 127.1, 127.0, 126.9, 126.8, 126.6, 86.4, 81.0, 78.9, 78.5, 78.4, 74.4, 74.2, 72.9, 71.3, 68.6, 49.7, 29.8, 29.4, 25.7.

2,3,4-Tris-benzyl-5-triphenylmethyl-arabinitol, 63.

N-Bromosuccinimide (0.26 g, 1.46 mmol) was added to a 0°C chilled mixture of CH_3CN/H_2O (35:8, v/v). The thioacetal (0.55 g, 0.73 mmol) **62** dissolved in $CHCl_3$ (2 mL) was rapidly added to this solution which turned gold-yellow. After 5 min of stirring, the reaction was quenched with saturated $Na_2S_2O_3$ solution (20 mL). CH_2Cl_2 (20 mL) was added and the aqueous layer was extracted with CH_2Cl_2. Combined organic fractions were washed with brine, dried, and concentrated. The crude was dissolved in EtOH (10 mL) and $NaBH_4$ (0.04 g, 1.15 mmol) was added. After stirring at rt for 30 min, EtOH was removed under reduced pressure and CH_2Cl_2 (20 mL) was added along with saturated aqueous NH_4Cl (20 mL). After extraction of the aqueous layer, the combined organic fractions were washed with brine and dried, and concentrated. Radial chromatography afforded the alcohol **63** (0.33 mmol, 45%) as an oil. ^1H NMR ($CDCl_3$) δ 7.40-7.50 (m, 6

H), 7.10-7.42 (m, 22 H), 7.05-7.10 (m, 2 H), 4.80 (d, *J*= 12 Hz, 1 H), 4.52 (d, *J*= 4 Hz, 4 H), 4.48 (d, *J*= 12 Hz, 1 H), 3.80-3.94 (m, 2 H), 3.60-3.90 (m, 3 H), 3.55 (dd, *J*= 3, 11 Hz, 1 H), 3.39 (dd, *J*= 5, 10 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 143.9, 138.4, 138.3, 138.1, 128.7, 128.5, 128.3, 128.1, 127.8, 127.7, 127.6, 127.5, 127.1, 126.9, 86.8, 79.2, 79.0, 78.8, 73.9, 72.6, 63.0, 61.8; Anal Calcd for $C_{42}H_{44}O_5 \cdot 1/2H_2O$: C, 80.20; H, 6.58. Found C, 79.86; H, 6.78.

1-Deoxy-1-bromo-2,3,4-tribenzyl-5-[(triphenyl)methyl]-arabinitol.

To a solution of the alcohol (0.60 g, 0.90 mmol) in THF (20 mL) were added pyridine (0.05 mL, 0.90 mmol), triphenylphosphine (0.47 g, 1.80 mmol) and tetrabromomethane (0.75 g, 2.25 mmol). The solution was stirred for 2 h under Ar at rt. THF was then removed under reduced pressure and the residue was chromatographed (EtOAc/Hexane, 1:3, v/v). After removal of the solvents, 1-deoxy-1-bromo-2,3,4-tribenzyl-5-[(triphenyl)methyl]-arabinitol (0.89 mmol, 98 %) was obtained as a white solid. ^{1}H NMR ($CDCl_3$) δ 7.40-7.55 (m, 4 H), 7.15-7.40 (m, 24 H), 7.00-7.10 (m, 2 H), 4.76 (d, *J*= 12 Hz, 1 H), 4.46-4.59 (m, 3 H), 4.41 (dd, *J*= 11, 11 Hz, 1 H), 4.14 (dd, *J*= 3, 6 Hz, 1 H), 3.94-4.04 (m, 1 H), 3.78-3.86 (m, 1 H), 3.62 (dd, *J*= 3, 9 Hz, 1 H), 3.41 (m, 2 H), 3.30 (dd, *J*= 6, 12 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 143.9, 138.4, 138.1, 128.7, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.6, 127.5, 127.4, 127.2, 126.9, 86.8, 79.3, 78.5, 78.0, 74.5, 73.1, 72.1, 62.6, 31.5; Anal. Calcd for $C_{45}H_{43}O_4Br$; C, 74.27; H, 5.96. Found C, 79.21; H, 6.08.

1-Deoxy-2,3,4-tris-benzyl-arabinitol, 64.

The arabinitol bromo derivative (0.65 g, 0.89 mmol) was dissolved in C_6H_6 (20 mL) under Ar and heated at reflux for 12 h in the presence of AIBN (0.005 g, 0.03 mmol) and tributyltin hydride. Benzene was then removed and MeOH (10 mL) along with *p*-toluenesulfonic acid (0.05 g, 0.30 mmol) were added. The solution was stirred for 3h, period after which the solvent was removed under reduced pressure and the residue was chromatographed (EtOAc/Hexane, 1:3, v/v) to afford alcohol **64** (0.83 mmol, 93%) as an oil. ^{1}H NMR (CDCl$_3$) δ 7.20-7.40 (m, 15 H), 4.42-4.88 (m, 6 H), 3.82-3.94 (m, 4 H), 3.67 (dd, *J*= 4, 6 Hz, 1 H), 1.26 (d, *J*= 6 Hz, 3 H); ^{13}C NMR (CDCl$_3$) δ 138.6, 138.2, 138.1, 128.7, 128.4, 128.3, 128.2, 127.9, 127.8, 127.7, 127.5, 81.9, 79.3, 74.8, 74.6, 71.7, 71.1, 60.9, 16.2; Anal. Calcd for $C_{26}H_{30}O_4$: C, 76.82; H, 7.44. Found: C, 76.30; H, 7.09.

1-Deoxy-2,3,4-tribenzyl-5-[(dibenzyloxy)phosphonyl]-arabinitol, 65.

A mixture of alcohol **64** (0.53 g, 0.83 mmol), dibenzylphosphoramidate (0.54 g, 1.56 mmol) and 1H-tetrazole (0.01 g, 0.18 mmol) in CH_2Cl_2 (20 mL) was stirred at rt for 4 h under Ar. The solution was then cooled to -78°C, where MCPBA (0.54 g, 3.12 mmol) was added as a solid. After stirring for 15 min, saturated $Na_2S_2O_3$ solution (20 mL) was added along with CH_2Cl_2 (20 mL). The aqueous layer was extracted with CH_2Cl_2 and the combined organic layers were washed with brine, dried, and concentrated. Radial chromatography of the residue afforded the fully benzylated 1-deoxy-arabinitol 5-phosphate **65** (0.59 mmol, 71%) as an oil. ^{1}H NMR (CDCl$_3$) δ 7.20-7.40 (m, 25 H), 4.95-5.14 (m, 4 H),4.50-4.72 (m, 4 H), 4.50-4.72 (m, 5 H), 4.30-4.50 (m, 3 H), 4.28 (ddd, *J*= 3, 9, 3 Hz, 1 H), 3.72-3.80 (m, 2 H), 3.54 (ddd, *J*= 2, 2, 6 Hz, 1 H), 1.21 (d, *J*= 6 Hz, 1 H); ^{13}C NMR (CDCl$_3$) δ 138.6, 138.2, 138.1, 128.7, 128.6, 128.4, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.6, 127.5, 127.4, 127.3, 81.3, 78.1 (J_{POCC}= 7 Hz), 74.6, 74.3, 72.1 71.1, 69.2 (J_{POC}= 6 Hz), 69.1 (J_{POC}= 6 Hz), 67.1 (J_{POC}= 5 Hz), 16.1; MS *m/z* (rel inten) 91 (100), 181 (20), 667 (M+H$^+$, 1<); HRMS (FAB) calcd for $C_{40}H_{44}O_7P$ (M+H$^+$) 667.2825, found 667. 2829.

1-Deoxy-arabinitol 5-phosphate, 58.

Fully benzylated 1-deoxy-arabinitol 5-phosphate **65** (0.30 g, 0.45 mmol) in THF (2 mL) was added to 10% Pd on C (0.05 g) in 10 mL of an aqueous THF solution (4:1,

THF/H_2O, v/v). Hydrogenation at 50 psi H_2 for 12 h was followed by filtration of the suspension through Celite and concentration to afford 1-deoxy-arabinitol 5-phosphate (0.44 mmol, 97%). ^1H NMR (D_2O) δ 4.01 (dddd, *J*= 3, 5, 5, 6 Hz, 1 H), 3.79 (dd, *J*= 3, 6 HZ, 1 H), 3.49-3.73 (m, 2 H), 3.36 (dd, *J*= 3, 5 Hz, 1 H), 1.20 (d, *J*= 6 Hz); ^{13}C NMR (D_2O) δ 74.8, 71.8, 66.7, 63.3, 18.6; MS m/z (rel inten) FAB 107 (36), 136 (82), 154 (100), 219 (7), 239 ($M+Na^+$, 3), 251 ($M+2Na^+$, 4); HRMS (FAB) calcd for $C_5H_{13}O_7PNa$ ($M+Na^+$) 239.0297, found 239.0303.

[1R* (1α, 2β, 3α, 4β, 5α)] - 2, 3, 4, 5 - Tetrakis(benzyloxy) cyclohexan -1- ol, 68.

To a solution of the fully benzylated conduritol oxide **29** (0.21 g, 0.04 mmol) in THF (5 mL), stirred at -78 °C under Ar was added a solution of Super-Hydride® in THF (1 M, 1.61 mL, 1.61 mmol). After 10 min, the solution was slowly warmed to rt and stirred for 2 h. A saturated solution of NH_4Cl (10 mL) was added to the reaction mixture cooled to 0°C. The aqueous layer was extracted with ether and the combined organic fractions were washed with brine, dried, and concentrated. Radial chromatography (2 mm thickness, 1:1 EtOAc/ hexane, v/v) afforded the alcohol **68** as an oil (0.13 g, 59%). ^1H NMR ($CDCl_3$) δ 7.15- 7.40 (m, 20 H), 4.82-4.98 (m, 3 H), 4.70- 4.80 (m, 2 H), 4.55-4.70 (m, 3 H), 3.34- 3.55 (m, 4 H), 3.23 (dd, *J*= 10, 11 Hz, 1 H), 2.20- 2.40 (br, 1 H) , 2.28 (ddd, *J*= 10.5, 2.0, 2.0 Hz, 1 H), 1.37 (ddd, *J*= 10.5, 10.5, 10.5 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 138.6, 138.4 (2C), 138.2, 128.6, 128.4, 128.3, 127.9, 127.8, 127.7, 127.6, 127.5, 85.7, 83.2, 77.4, 75.7 (2C), 75.4, 72.3, 68.3, 33.9; MS *m/z* (rel inten). EI 91 (100), 181 (12), 433 (11); FAB 181 (100), 271 (10), 433 (5), 523 ($M-H^+$, 12); HRMS (FAB) calcd for $C_{34}H_{35}O_5$ ($M-H^+$) 523.2485, found 523.2487.

[1R* (1α, 2β, 3α, 4β, 5α)] - 2, 3, 4, 5 - Tetrakis(benzyloxy) -1- [(dibenzyloxyphosphonyl)oxy] cyclohexane, 69.

Sodium hydride (0.03 g, 0.69 mmol) as a suspension in THF (2 mL) was added under Ar to a solution of alcohol **68** (0.12 g, 0.23 mmol) in THF (8 mL) at rt. The solution was heated at reflux for 2 h and kept at the same temperature for 3 h after the addition of tetrabenzylpyrophosphate (0.25 g, 0.46 mmol). The reaction was quenched at rt by addition of a saturated solution of NH_4Cl (10 mL). Extraction of the aqueous layer with ether, followed by concentration of the dried combined organic fractions yielded the crude product which was purified by radial chromatography (2-mm thickness of silica gel, 1:3 EtOAc/ hexane, v/v). The phosphate triester **69** was isolated as an oil (0.12 g, 62%) along with some unreacted alcohol **68** (0.04 g, 30%). ^1H NMR ($CDCl_3$) δ 7.10- 7.40 (m, 30 H), 4.75- 5.00 (m, 6 H), 4.79 (d, *J*= 6 Hz, 4 H), 4.54 (dd, *J*= 1, 8 Hz, 2 H), 4.20- 4.32 (m, 1 H), 3.51 (dd, *J*= 9, 9 Hz, 1 H), 3.48 (dd, *J*= 8, 9 Hz, 1 H), 3.44 (dd, *J*= 8, 9 Hz, 1 H), 3.36-3.45 (m, 1 H), 2.55 (ddd, *J*= 5, 5, 12 Hz, 1 H), 1.55 (ddd, *J*= 12, 12, 12 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 138.5, 138.2, 138.0, 135.6, 135.7, 128.5, 128.4, 128.3, 128.2, 127.9, 127.8, 127.7 (2C), 127.5, 127.4, 85.0, 83.9 (J_{POCC}= 7.4 Hz) 82.5, 76.6, 76.1, 75.9 (J_{POC}= 6 Hz), 75.8, 75.5, 72.5, 69.2 (J_{POC}= 5 Hz), 69.1 (J_{POC}= 5 Hz), 33.7; MS *m/z* (rel inten) FAB 109 (100), 123 (57), 181 (24), 785 ($M+H^+$, 3); HRMS (FAB) calcd for $C_{48}H_{50}O_8P$ ($M+H^+$) 785.3243, found 785.3249.

[1R* (1α, 2β, 3α, 4β, 5α)] - 2,3,4,5 - Tetrakis(hydroxy) -1- phosphonoxy cyclohexane, 66.

Benzylated phosphate triester **69** (0.12 g, 0.14 mmol) in THF (2 mL) was added to 10% Pd on C (0.05 g) in 5 mL of THF/H_2O (4:1, v/v). The suspension was hydrogenated at 50 psi H_2 for 12 h. Catalyst removal by filtration through Celite and removal of the solvents yielded the phosphate monoester **66** as an oil (0.04 g, 100%). ^1H NMR (D_2O) δ 4.66 (m, 1 H), 3.83 (m, 1 H), 3.39 (m, 1 H), 3.13 (m, 2 H), 2.21 (m, 1 H), 1.40 (ddd, *J*= 12, 12, 12 Hz, 1 H); ^{13}C NMR (D_2O) δ 79.7, 78.7 (J_{POCC}= 6 Hz), 76.8 (J_{POC}= 7.5 Hz), 76.7, 71.3, 38.8; MS *m/z* (rel inten) FAB 62 (72), 91(78), 154 (70), 183 (100), 243 (M-H$^+$, 80); HRMS (FAB) calcd for $C_6H_{12}O_8P$ (M-H$^+$) 243.0270, found 243.0274.

[1S*(1α,2β,3α,4β,5α,6β)]-3,4,5,6-tetrakisbenzyl-2-deoxy-2-[(dibenzyloxy)phosphonoroxymethyl]-1-[(dibenzyloxy)phosphonyl]-*scyllo*-inositol, 70.

A mixture of alcohol **30** (0.40 g, 0.74 mmol), dibenzylphosphoramidate (1.01 g, 2.94 mmol) and 1H-tetrazole (0.02 g, 0.36 mmol) in CH_2Cl_2 (10 mL) was stirred at rt for 4 h under Ar. The solution was then cooled to -78°C, where MCPBA (1.01 g, 5.88 mmol) was added as a solid. After stirring for 15 min, saturated aqueous $Na_2S_2O_3$ (30 mL) was added along with CH_2Cl_2 (30 mL). The aqueous layer was extracted with CH_2Cl_2 and combined organic fractions were washed with brine, dried, and concentrated. Radial chromatography of the residue afforded the fully benzylated diphosphotriester **70** (0.46 mmol, 62%) as an oil. ^{1}H NMR ($CDCl_3$) δ 7.10- 7.40 (m, 38 H), 7.03 (dd, *J*= 2, 7 Hz, 2 H), 4.65-5.10 (m, 17 H), 4.54 (dd, *J*= 7, 8 Hz, 2 H), 4.39 (ddd, *J*= 1, 1, 9 Hz, 1 H), 3.57 (dd, *J*= 7, 7 Hz, 2 H), 3.37-3.50 (m, 2 H), 1.86 (m, 1 H); ^{13}C NMR ($CDCl_3$) δ 138.5, 138.4, 138.0, 135.9 (J_{POCC}= 4 Hz), 135.8 (J_{POCC}= 4 Hz), 128.5, 128.4, 128.3, 128.3 (2C), 128.3, 128.1, (2C), 128.0, 127.9, 1278, 127.7, 127.6, 127.5, 127.3, 127.2, 127.1 127.0, 85.3, 82.9, 82.2 ,75.8, 75.6 (J_{POC}= 6 Hz), 75.4, 74.7, 69.3 (J_{POC}= 4 Hz), 69.2 (J_{POC}= 4 Hz), 62.1 (J_{POC}= 5 Hz), 45.5 (J_{POCC}= 4 Hz, J_{POCC}= 9 Hz); Anal Calcd for $C_{63}H_{64}O_{12}P_2{\cdot}H_2O$; C, 69.22; H, 6.08. Found; C, 68.89; H, 6.04.

[1S*(1α,2β,3α,4β,5α,6β)]-2-deoxy-2-[phosphonoroxymethyl]-*scyllo*-inositol 1-phosphate, 67.

Benzylated diphosphate triester **70** (0.48 g, 0.46 mmol) in THF (2 mL) was added to 10% Pd on C (0.05 g) in 5 mL of THF/H_2O (4:1, v/v). The suspension was hydrogenated at 50 psi H_2 for 12 h. Catalyst removal by filtration through Celite and removal of the solvents yielded the phosphate monoester **67** as an oil (0.46 mmol, 100%). ^{1}H NMR (D_2O) δ 4.22 (m, 2 H), 4.15 (dd, *J*= 10, 10 Hz, 1 H), 3.50-3.65 (m, 2 H), 3.41 (dd, *J*= 9, 10 Hz, 1 H), 3.34 (dd, *J*= 9, 10 Hz, 1 H), 1.74-1.83 (m, 1 H); ^{13}C NMR (D_2O) δ 76.2, 75.9, 75.7, 75.2 (J_{POC}= 5 Hz), 73.5, 68.1, 60.9 (J_{POC}= 5 Hz), 45.7 (J_{POCC}= 5 Hz, J_{POCC}= 8 Hz); MS *m/z* (rel inten) FAB 93)60), 115 (100), 137 (14), 185 (30), 207 (45), 251 (88), 377 (M+Na$^+$, 53), 399 (M+2Na$^+$, 40); HRMS (FAB) calcd for $C_7H_{15}O_{12}P_2Na$, 377.0015 found 377.0014.

***myo*-Inositol 1-phosphate.**

To an heterogeneous mixture of *myo*-inositol (54 g, 300 mmol) and 2,2-dimethoxypropane (120 mL, 1.10 mol) in DMSO (180 mL) was added *p*-toluenesulfonic acid (0.35 g, 0.002 mmol). Upon heating at 110°C under N_2, the solution became homogeneous. The heat was maintained for 6 h, after which period DMSO was distilled off under high vacuum. The residue was triturated in EtOAc and the crystals were filtered. Tetrol **47**[145] (200 mmol, 67%) was isolated as a white solid. Tetrol **47** (4.40 g, 20 mmol) dissolved in pyridine (50 mL) was stirred at rt for 12 h in the presence of acetic anhydride (2.0 mL). The solution was filtered through Celite, concentrated, and the residue was purified on silica gel (hexane/EtOAc, 1:1, v/v) to yield the tetraacetylated *myo*-inositol derivative[145] (12.1 mmol, 61%). ^{1}H NMR ($CDCl_3$) δ 5.50 (dd, *J*= 10, 10 Hz, 1 H), 5.24-5.40 (m, 2 H), 5.07 (dd, *J*= 9, 10 Hz, 1 H), 4.53 (dd, *J*= 9, 10 Hz, 1 H), 4.28 (dd, *J*= 9, 9 Hz, 1 H), 2.13 (s, 3 H), 2.08 (s, 3 H), 2.03 (s, 3 H), 2.02 (s, 3 H), 1.61 (s, 3 H), 1.35 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 169.6, 169.3, 169.0, 110.6, 75.6, 73.0, 72.0, 70.7, 69.2, 68.5, 26.9, 25.2, 20.4, 20.2, 20.1.

The tetraacetylated *myo*-inositol derivative was stirred for 12 h in an aqueous acetic acid solution (H_2O/AcOH, 2:8, v/v) at rt. After concentrating of the solution, acetic acid and H_2O were azeotroped with toluene. The residue (3.05 g, 8.76 mmol) was then dissolved in pyridine (35 mL) and stirred at rt under N_2 for 24 h in the presence of diphenylphosphorochloridate (4.71 g, 17.53 mmol). The solution was filtered through Celite and concentrated. Attempts to properly purify the residue on silica gel were unsuccessful. ^{1}H NMR ($CDCl_3$) δ 7.08-7.30 (m, 10 H), 5.58 (dd, *J*= 9, 10 Hz, 1 H), 5.25-5.50 (m, 1 H), 5.13 (dd, *J*= 9, 9 Hz, 1 H), 4.95 (dd, *J*= 3, 9 Hz, 1 H), 4.26 (m, 1 H), 3.78 (d, *J*= 10 Hz, 1 H), 2.09 (s, 6 H), 2.07 (s, 6 H); ^{13}C NMR ($CDCl_3$) δ 170.9, 170.1, 169.8, 169.5, 137.5, 128.7, 127.9, 125.0, 72.2, 71.1, 70.7, 69.7, 69.5, 69.3, 20.5, 20.4, 20.2, 20.1.

The fully protected *myo*-inositol phosphoester was dissolved in a solution of THF and 5N NaOH (1:1, v/v) and stirred for 12 h at rt. THF was removed under reduced pressure and the pH of the solution was modified to pH 7 by addition of 5N HCl. The neutral solution was then loaded on an pre-equilibrated AG1-X8 resin column. A salt gradient was then applied to elute *myo*-inositol 1-phosphate off the resin. The gradient was initiated at 50 mM of TEAB buffer and terminated at 500 mM. TEAB buffer is prepared by mixing the appropriate concentration of Et_3N in water and bubbling CO_2 gas into the solution until neutral pH is obtained. Fractions containing *myo*-inositol 1-phosphate, assayed by the method of Ames[64] were concentrated. Triethylamine was azeotroped with isopropanol. The residue was then passed through a Dowex-50 column. The elute was neutralized and concentrated to dryness. *myo*-inositol 1-phosphate sodium salt[94] (2.53 mmol, 29% from diol) was obtained as a hydroscopic gum. ^{1}H NMR (D_2O) δ 4.25 (dd, *J*= 3, 3 Hz, 1 H), 4.04 (ddd, *J*= 3, 9, 10 Hz, 1 H), 3.75 (dd, *J*= 10, 10 Hz, 1 H), 3.50-3.70 (m, 2 H), 3.31 (dd, *J*= 10, 10 Hz, 1 H); ^{13}C NMR (D_2O) δ 77.2, 75.8, 75.0 (J_{POCC}= 8 Hz), 74.3, 74.2, 73.8.

[2S*(2α, 3β, 4α, 5β, 6α)] -2,3,4,5 Tetrakis(benzyloxy)-6-hydroxy cyclohexan-1- one, 71.

Dibutyltin oxide (1.15 g, 5.09 mmol) was added to a CH_3OH (10 mL) solution of 1,4,5,6-tetrabenzyl-*myo*-inositol **48** (2.50 g, 4.63 mmol). After heating this heterogeneous solution at reflux under N_2 until disappearance of dibutyltin oxide, MeOH was removed under reduced pressure. The resulting oil was dissolved in CH_2Cl_2 (10 mL) under N_2 and tributyltin methoxide (0.74 g, 2.32 mmol) added. A CH_2Cl_2 solution of Br_2 (0.5 M) was then added dropwise at rt until an orange color persisted for 5 min. The reaction was then quenched with addition of a 10% Na_2SO_3 solution (10 mL). Extraction of the aqueous layer with CH_2Cl_2 was followed by washing the combined organic layers with brine. After drying and concentrating, the crude product was purified on silica gel (1:3 EtOAc/hexane, v/v). Isolated product was redissolved in ether (10 mL), a few drops of hexane were added and the flask was cooled to 4°C after crystal formation began. After a second crop of crystals was collected from the filtrate, ketone **71** was obtained as a white powder (1.24 g, 50%). ^1H NMR ($CDCl_3$) δ 7.15-7.45 (m, 20 H), 4.70-5.00 (m, 8 H), 4,52 (d, *J*= 11 Hz, 1 H), 4.25 (d, *J*= 9 Hz), 3.87 (dd, *J*= 9, 9 Hz, 1 H), 3.68 (dd, *J*= 9, 9 Hz, 1 H), 3.45 (dd, *J*= 9, 9 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 204, 138.1, 138, 137.9, 137, 128.4, 128.3, 128, 127.9, 127.8, 127.7, 127.6, 83.6, 83.1, 81.5, 81.3, 77.2, 75.9 (2C), 75.3, 73.4; IR (neat NaCl) 3494 (w), 1734 (m); MS *m/z* (rel inten) EI 91 (100), 181 (11); CI 91 (84), 181 (100), 537 ($M-H^+$, 6), 538 (M^+, 2), 539 ($M+H^+$, 2); FAB 181 (100), 537 ($M-H^+$, 1); HRMS (FAB) calcd. for $C_{34}H_{33}O_6$ ($M-H^+$) 537.2277, found 537.2278; Anal. Calcd for $C_{34}H_{34}O_6 \cdot 1/2\ H_2O$: C, 74.57; H, 6.44. Found: C, 74.86; H, 6.40.

Inosose 2 : *meso* [2S*(2α, 3β, 4α, 5β, 6α)]-2,3,4,5,6- pentahydroxy cyclohexan-1- one

Ketone **71** (0.30 g, 0.56 mmol) in THF (5 mL) was added to 10% Pd on C (0.05 g) in 10 mL of an aqueous THF solution (4:1, THF/H_2O, v/v). Hydrogenation at 50 psi

H_2 for 2 h was followed by filtration of the suspension through Celite and concentration to a white solid (0.096 g, 96%). For ^{13}C and ^{1}H NMR characterization, product was dissolved in H_2O and the solution was adjusted to pH 7 by addition of NaOH. Concentration and redissolving product in D_2O was repeated 3x. Synthesized *myo*-2-inosose was found to be identical by ^{1}H NMR to *myo*-2-inosose purchase from Aldrich. ^{1}H NMR (D_2O) δ 4.41 (d, *J*= 11 Hz, 2 H), 3.83 (dd, *J*= 11 Hz, 2 H), 3.43 (dd, *J*= 11 Hz, 1 H); ^{13}C NMR (D_2O) δ 208.8, 97, 78.9, 77.2, 76.7, 76, 75.9.

[2R*(2α, 3β, 4α, 5β, 6α)] -2,3,4,5 Tetrakis(benzyloxy)-6-[(dibenzyloxyphosphonyl)oxy] cyclohexan-1- one, 72.

Sodium hydride (0.41 g,1.00 mmol) as a suspension in THF (5 mL) was slowly added under N_2 to a solution of cyclohexanone **71** (0.54 g,1.01 mmol) in THF (5 mL) at 0°C. After reaction for 1 h, tetrabenzylpyrophosphate (0.82 g, 1.55 mmol) in THF (5 mL) was added under N_2. The reaction solution became turbid with the formation of a white precipitate during subsequent stirring under N_2 for 1 h at 0°C. After quenching with saturated NH_4Cl (20 mL) and extraction with ether the combined organic layers were washed with brine, dried, and concentrated. Purification by radial chromatography (4 mm, 1:3 EtOAc/ hexane, v/v) and crystallization from CH_3CN at 4°C afforded benzylated phosphorocyclohexanone **72** (0.49 g, 61%) as a white solid. ^{1}H NMR ($CDCl_3$) *δ* 7.15-7.45 (m, 30 H), 5.36-5.21 (m, 2 H), 4.74-5.07 (m, 10 H), 4.49 (d, *J*= 11 Hz, 1 H), 4.23 (dd, *J*= 1, 10 Hz, 1 H), 3.91 (dd, *J*= 9 Hz, 1 H), 3.64 (dd, *J*= 9 Hz, 1 H), 3.63 (dd, *J*= 9 Hz, 1 H); ^{13}C NMR ($CDCl_3$) *δ* 128.3, 128.1, 128, 127.9, 127.8, 127.7 (2), 127.6, 127.5, 127.4, 127.3, 83.7, 81.6, 81.3, 81.2, 80.3 (J_{POC}= 7 Hz), 76.1, 76, 75.7, 73.5, 69.9 (J_{POC}= 5 Hz), 69.3 (J_{POC}= 5 Hz); IR (neat NaCl) 3476 (b), 1754 (m), 1455 (s), 1275 (s); MS *m/z* (rel inten) EI 91 (100), 277 (14); CI 91 (70), 279 (11), 369 (100); FAB 181 (100), 799 ($M+H^+$, 13); HRMS (FAB) calcd for $C_{48}H_{48}O_9P$ ($M+H^+$) 779.3036, found 799.3017; Anal. Calcd for $C_{48}H_{47}O_9P \cdot 1/3H_2O$: C,71.63; H, 5.97. Found: C, 71.4; H, 5.88.

[2S*(2α, 3β, 4α, 5β, 6α)]-2,3,4,5 tetrakis(hydroxy)-6-phosphonoxy cyclohexan-1- one, 73, D.

Benzylated phosphoroketone **72** (0.51 g, 0.63 mmol) in THF (5 mL) was added to 10% Pd on C in 10 mL of THF/water (4:1, v/v) (10 mL). The suspension was hydrogenated at 50 psi H_2 for 1 h. Catalyst removal by filtration through Celite and removal of the solvent yielded intermediate **D**. Addition of H_2O (5 mL) and adjustment of the solution to pH 2 with addition of HCl was followed by removal of solvent under high vacuum to afford intermediate **D** as a hygroscopic foam (0.16 g, 99%). ^1H NMR (DMSO-*d6*) δ 4.69 (dd, *J*= 9, 10 Hz, 1 H), 4.10 (dd, *J*= 1, 10 Hz, 1 H), 3.50 (dd, *J*= 10, 10 Hz, 1 H), 3.50 (dd, *J*= 9, 9 Hz, 1 H), 3.16 (dd, *J*= 10, 10 Hz, 1 H), 3.00 (dd, *J*= 9, 10 Hz, 1 H); ^{13}C NMR, (DMSO) δ 202,80.4, 76.6, 74.7, 73.7, 73.6; ^{31}P NMR (DMSO) δ 0.4; MS *m/z* (rel. inten.) FAB 257 (60), 239 (28), 97 (100), 79 (56); HRMS (FAB) Calcd. for $C_6H_{10}O_9P$ (M-H$^+$) 257.00625, found 257.00602.

^1H NMR (D_2O, pH 2) *myo*-2-inosose 1-phosphate δ 4.74 (dd, *J*= 9, 10 Hz, 1 H), 4.33 (dd, *J*= 1, 10 Hz, 1 H), 3.75 (dd, *J*= 9.5, 9.5 Hz, 1H), 3.45 (dd, *J*=9, 9 Hz, 1 H), 3.28-3.36 (m, 1H); ^{13}C NMR (D_2O), pH 2, δ 205.2, 96.5, 82.9, 82.7, 82.6, 78.8, 76.9, 76.7, 76.6, 76.5, 76.1, 75.6, 75.5, 75.4, 75.2, 75.1, 74.5.

^1H NMR (D_2O, pH 7) *myo*-2-inosose 1-phosphate hemiacetal dimer δ 4.66-4.73 (m, 2 H), 4.33 (d, *J*= 10 Hz, 2 H), 3,76 (dd, *J*= 9.5, 9.5 Hz, 2 H), 3.43 (dd, *J*= 10, 10 Hz, 2 H), 3.29-3.35 (m, 2 H); ^{13}C NMR (D_2O), pH 7, δ 94.5, 78.8, 77.9, 76.7, 76.4, 74.9, 74.3, 73.9, 73.4.

[1R* (1α, 2β, 3α, 4β, 5α, 6β)] - 2, 3, 4, 5 - Tetrakis(benzyloxy) -6- [(dibenzyloxyphosphinyl)methyl]cyclohexan -1- ol, 80 and **[1S* (1α, 2α, 3β, 4β, 5α, 6β)] - 2, 3, 4, 5 - Tetrakis(benzyloxy) -6- [(dibenzyloxyphosphinyl)methyl]cyclohexan-1-ol, 81.**

A solution of *n*-butyl lithium in hexane (1.6 N, 12.0 mL, 19.2 mmol) was added to THF (20 mL) at -78 °C under N_2. To this solution was slowly added dibenzyl methanephosphonate (5.29 g, 19.2 mmol) in THF (20 mL). After stirring for 30 min at -78°C, boron trifluoride etherate (4.71 mL, 19.2 mmol) was added immediately followed by tetrabenzyl conduritol oxide B **29** (2.00 g, 3.83 mmol) in THF (5 mL). The solution was stirred at -78 °C for 3 h and more boron trifluoride etherate (4.71 mL, 19.2 mmol) was added. The solution was then allowed to warm to -20 °C and stir for 12 h at this temperature. Saturated aqueous $NaHCO_3$ was then added. The aqueous layer was extracted with ether and the combined organic layers were washed with brine followed by water, dried, and finally concentrated. The crude product was first purified by flash chromatography (1:1 EtOAc/hexane, v/v), then by radial chromatography (4-mm thickness of silica gel, 1:1 EtOAc/ hexane, v/v). The faster eluting regioisomer **80** was isolated as an oil (0.91 g, 30%) which slowly solidified at rt. ^{1}H NMR ($CDCl_3$) δ 6.85-7.40 (m, 30 H), 4.70-5.10 (m, 11 H), 4.57 (d, *J*= 16 Hz, 1 H), 3.98 (d, *J*= 4 Hz, 1 H), 3.60-3.75 (m, 1 H), 3.35-3.55 (m, 3 H), 1.80-2.40 (m, 3 H); ^{13}C NMR ($CDCl_3$) δ 138.8, 138.4, 138.3, 136, 129.5, 129.4, 128.7, 128.5, 128.4, 128.3, 128.1, 127.9, 127.8, 127.6, 127.5, 127.2, 85.8, 85, 82.7, 79.4 (J_{PCCC}= 7 Hz), 75.7, 75.5, 75.4, 74.9, 73.1, 67.5 (J_{POC}= 7 Hz), 67.3 (J_{POC}= 7 Hz), 41.5 (J_{PCC}= 3 Hz), 24.0 (J_{PC}=139 Hz); IR (neat NaCl) 3400(b), 1210(s); MS *m/z* (rel inten) EI 91 (100). CI 91 (100). FAB 799 (25), 181 (100); HRMS (FAB) calcd for $C_{49}H_{52}O_8P$ (M+H$^+$) 799.3399, found 799.3383; Anal. Calcd for $C_{49}H_{51}O_8P \cdot 1/2\, H_2O$: C, 72.85; H, 6.36. Found: C, 72.81; H, 6.52. The slower eluting regioisomer **81** was isolated as an oil (1.37 g, 44%) which slowly crystallized at rt: ^{1}H NMR ($CDCl_3$) δ 7.15-7.40 (m, 30 H), 4.65-5.10 (m, 8 H), 4.55 (s, 2 H), 4.50 (s, 2 H), 4.40-4.55 (m, 1 H), 4.04 (ddd, *J*= 14, 4, 2 Hz, 1 H), 3.81 (dd, *J*= 9, 9 Hz, 1 H), 3.64 (dd, *J*= 9, 3 Hz, 1 H), 3.45 (dd, *J*= 9, 9 Hz, 1 H), 2.75-2.95 (m, 1 H), 2.50-2.60 (br, 1 H), 2.41 (ddd, *J*= 21, 16, 3 Hz, 1 H), 1.48 (ddd, *J*= 18, 16, 12 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 139.3, 138.9, 138.4, 129.5 129.1, 129.0, 128.9, 128.7, 128.5, 128.4, 128.3, 128.1, 82.1, 82.0, 80.3, 78.6, 78.4, 76.4, 76.1, 72.9 (2C), 68.2 (J_{POC}= 14 Hz), 67.9 (J_{POC}= 8 Hz), 36.1 (J_{PCC}= 4 Hz), 22.1 (J_{PC}= 143 Hz); IR (neat NaCl) 3380(s), 1210(s); MS *m/z* (rel inten) EI 91 (100). CI 91 (29), 353 (100). FAB 181 (100), 799 (45), 800 (30); HRMS (FAB) calcd for $C_{49}H_{52}O_8P$ ($M+H^+$) 799.3399, found 799.3391; Anal. Calcd for $C_{49}H_{51}O_8P \cdot H_2O$: C, 72.04; H, 6.54. Found: C, 72.34; H, 6.64.

[2S* (2α, 3β, 4α, 5β, 6α)] - 2, 3, 4, 5 - Tetrakis(benzyloxy) -6-[(dibenzyloxyphosphinyl)methyl]cyclohexan-1-one, 82.

DMSO (0.17 g, 2.15 mmol) in CH_2Cl_2 (5 mL) was slowly added to a -78°C solution of oxalyl chloride (0.49 mL, 0.98 mmol) in CH_2Cl_2 (2 mL) and stirring continued for 35 min. at -78°C. Alcohol **80** (0.26 g, 0.35 mmol) in CH_2Cl_2 (3 mL) was then added under N_2 and the reaction mixture stirred for an additional 2 h. After subsequent addition of anhydrous Et_3N, the reaction was warmed to rt and quenched with water (2 mL). The aqueous layer then extracted with CH_2Cl_2. The combined organic layers were washed with 0.1N HCl solution followed by brine, dried and concentrated. After radial chromatography (2-mm thickness of silica gel, 1:1 EtOAc/ hexane, v/v) the product **82** (0.23 g, 89%) was obtained. ^1H NMR ($CDCl_3$) δ 7-7.5 (m, 30 H), 4.65-5.05 (m, 10 H), 4.53 (d, *J*= 11 Hz, 1 H), 4.27 (d, *J*= 11 Hz, 1 H), 4.22 (d, *J*= 9 Hz, 1 H), 3.92 (dd, *J*= 9, 9 Hz, 1 H), 3.59 (dd, *J*= 9, 9 Hz, 1 H), 3.27 (dd, *J* = 8, 11 Hz, 1 H), 2.82-3.06 (m, 1 H), 2.49 (ddd, *J*= 9, 15, 18 Hz, 1 H), 2.05 (ddd, *J*= 2, 8, 15 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 202.2 (J_{PCCC}= 3 Hz), 138, 137.9, 137.5, 137.4, 128.5, 128.3, 128.2, 127.9, 127.8 (2C), 127.7, 127.4, 127.3, 85.6, 84.4, 81.8, 79.3 (J_{PCCC}= 12 Hz), 75.6, 75.4, 74.8, 72.9, 67.2, 67.1, 47.5 (J_{PCC}= 4 Hz), 20.6 (J_{PC}= 143 Hz); IR (neat NaCl), 3450(b), 1735(s), 1210(s); MS *m/z* (rel inten) FAB 105 (100), 797 (89); HRMS (FAB) calcd for $C_{49}H_{50}O_8P$ ($M+H^+$) 797.3243, found 797.3268; Anal. Calcd for $C_{49}H_{49}O_8P.2H_2O$: C, 70.66; H, 6.41. Found: C, 70.67; H, 6.01.

[2S* (2α, 3β, 4α, 5β, 6α)]- 2, 3, 4, 5 -Tetrakis(benzyloxy) -6-[(dibenzyloxyphosphinyl)methyl]cyclohexan-1-one, 83.

Alcohol **81** (0.15 g, 0.19 mmol) was oxidized as described for alcohol **80** and afforded the ketone **83** as a yellow oil (0.14 g, 94% yield). ^1H NMR ($CDCl_3$) δ 7.10-7.50 (m, 30 H), 4.42-5.08 (m, 11 H), 4.35 (d, *J*= 11 Hz, 1 H), 4.34 (d, *J*= 10 Hz, 1 H), 4.12 (dd, *J*= 3, 3 Hz, 1 H), 3.89 (dd, *J*= 3, 5 Hz, 1 H), 3.79 (dd, *J*= 5, 10 Hz, 1 H), 3.00 (dddd, *J*= 3, 5, 11, 11 Hz, 1 H), 2.45 (ddd, *J*= 5, 16, 19 Hz, 1 H), 1.99 (ddd, *J*= 9, 16, 17 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 206.1 (J_{PCCC}= 11 Hz), 138.1, 138, 137.8, 137.6, 137.3, 137.2, 136, 29.3, 129.2, 129.1, 128.5, 128.3, 128.2, 127.9, 127.7, 127.6, 127.3, 127.2, 81.9, 81.4, 79.4, 76.7, 74.3, 74, 72.2, 72, 67.3 (J_{POC}= 3 Hz), 67.2 (J_{POC}= 4 Hz), 44.3, 21.8 (J_{PC}= 145 Hz); IR (neat NaCl) 3450(b), 1735(s), 1210(s); MS *m/z* (rel inten); FAB 797 (97); HRMS (FAB) calcd for $C_{49}H_{50}O_8P$ ($M+H^+$) 797.3243, found 797.3271; Anal Calcd for $C_{49}H_{50}O_8P \cdot 3H_2O$: C, 69.18; H, 6.51. Found: C, 69.16; H, 6.25.

[2R* (2α, 3β, 4α, 5β, 6α)]- 2, 3, 4, 5 -Tetrakis(hydroxy) -6-[phosphinylmethyl]cyclohexan-1-one, 77.

The benzylated phosphonoketone **82** (0.18 g, 0.22 mmol) in THF (5 mL) containing $NaHCO_3$ (0.02 g, 0.22 mmol) was added to 10% Pd on C (0.06 g) in 10 mL of THF/water (4/1, v/v). The suspension was hydrogenated at 50 psi H_2 for 1 h. The ketone **77** (0.04 g, 100% yield) was obtained as a white solid after filtration through Celite, removal of the organic solvent under reduced pressure, adjustment to pH 7, and final concentration to dryness: ^1H NMR (D_2O) δ 4.23 (d, *J*= 10 Hz, 1 H), 3.67 (dd, *J*= 9 Hz, 1 H), 3.20 (dd, *J*= 10 Hz, 1 H), 3.13 (dd, *J*= 9, 10 Hz, 1 H), 2.83 (m, 1 H), 2.22 (ddd, *J*= 9, 16, 18 Hz, 1 H), 1.91 (ddd, *J*=2, 16, 17 Hz, 1 H); ^{13}C NMR (D_2O) δ 206.8, 79.6, 77.4, 75.7, 74.4, 74.3, 49.7 (J_{PCC}= 4 Hz), 24.5 (J_{PC}= 138 Hz); MS *m/z* (rel. inten) FAB 255 (22); HRMS (FAB) calcd for $C_7H_{12}O_8P$ (M-H$^+$) 255.0283, found 255.0283.

1,3,4,5,6-Pentakis-benzyl-*myo*-inositol.

Diol **48** (0.40 g, 0.74 mmol) was dissolved in C_6H_6 (40 mL) along with benzyl bromide (0.13 g, 0.75 mmol), tetrabutyl ammonium iodide (0.05 g, cat) and NaH (0.03 g, 0.75 mmol). The solution was refluxed for 12 h under Ar. After addition of NH_4Cl saturated solution (20 mL) and CH_2Cl_2 (20 mL), the aqueous layer was extracted with CH_2Cl_2 and the combined organic fractions were washed with brine, dried and concentrated. Radial chromatography (2-mm thickness of silica gel, hexane/EtOAc, 5:1, v/v) afforded the pentabenzylated *myo*-inositol[146] (0.46 mmol, 62%). ^1H NMR ($CDCl_3$) δ 7.20-7.40 (m, 25 H), 4.80-4.92 (m, 6 H), 4.80 (s, 4 H), 4.22 (dd, J= 2, 2 Hz), 4.00 (dd, *J*= 9, 9 Hz, 2 H), 3.45 (dd, *J*= 9, 9 Hz, 1 H), 3.38 (dd, *J*= 2, 10 Hz, 2 H). ^{13}C NMR (D_2O) δ 138.7, 137.9, 128.5, 128.4, 128.3, 127.9, 127.8, 127.5, 126.9, 83.1, 81.1, 79.7, 75.0, 72.7, 67.4.

Pentakis-benzyl-*scyllo*-inositol.

DMSO (1.78 mL, 7.12 mmol) in CH_2Cl_2 (10 mL) was slowly added to a -78°C solution of oxalyl chloride (1.78 mL, 3.6 mmol) in CH_2Cl_2 (10 mL) and stirring continued for 35 min. at -78°C. Pentabenzylated *myo*-inositol (0.56 g, 0.89 mmol) in CH_2Cl_2 (10 mL) was then added under Ar and the reaction mixture stirred for an additional 2 h. After subsequent addition of anhydrous Et_3N (2.00 mL, 14.2 mmol), the reaction was warmed to rt and quenched with water (20 mL). The aqueous layer then extracted with CH_2Cl_2. The combined organic layers were washed with 0.1N HCl solution followed by brine, dried, and concentrated. After radial chromatography (2-mm thickness of silica gel, 1:1

EtOAc/ hexane, v/v) pentabenzylated *scyllo*-inosose[145] (0.67 mmol, 75%) was obtained as a white solid. *scyllo*-Inosose (0.42 g, 0.67 mmol) was dissolved in EtOH (15 mL) and $NaBH_4$ (0.03 g, 0.8 mmol) was added. The solution was stirred under Ar at rt for 3 h. EtOH was removed under reduced pressure and CH_2Cl_2 (20 mL) was added along with saturated aqueous NH_4Cl. After extraction of the aqueous layer, washing of the combined organic fractions with brine and concentration, the organic residue was chromatographed on silica gel. Pentabenzylated *myo*-inositol (0.50 mmol, 75%) was the first eluated species. Pentabenzyl *scyllo*-inositol[145] (0.17 mmol, 25%) was then isolated as a solid. ^1H NMR ($CDCl_3$) δ 7.20-7.40 (m, 25 H), 4.86-4.95 (m, 10 H), 3.50-3.68 (m, 4 H), 3.38-3.50 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 138.5, 138.4, 128.5, 128.4, 127.9 (2C), 127.8, 127.7, 83.1, 82.7, 82.4, 75.9, 75.8, 75.5, 74.4 .

scyllo-Inositol phosphate.

A mixture of pentabenzyl *scyllo*-inositol (0.11 g, 0.17 mmol), dibenzyl-N, N-diisopropyl phosphoramidate (0.12 g, 0.34 mmol) and 1H-tetrazole (0.005 g, 0.08 mmol) in CH_2Cl_2 was stirred at rt for 4 h under Ar. The solution was then cooled to -78°C, where MCPBA (0.12 g, 0.68 mmol) was added as a solid. After stirring for 15 min, saturated aqueous $Na_2S_2O_3$ solution (10 mL) was added along with CH_2Cl_2 (10 mL). The aqueous layer was extracted with CH_2Cl_2 and the combined organic layers were washed with brine, dried, and concentrated. Radial chromatography of the residue afforded the fully benzylated diphosphotriester (0.14 mmol, 82%) as an oil. ^1H NMR ($CDCl_3$) δ 7.18-7.42 (m, 38 H), 7.02-7.10 (m, 3 H), 4.95-5.10 (m, 4 H), 4.70-4.95 (m, 8 H), 3.63 (s, 3 H). ^{13}C NMR ($CDCl_3$) δ 137.9, 137.7, 136.4, 135.7, 128.0, 127.9, 127.9, 127.8,127.7,127.5, 127.,3,127.3, 127.2, 127.0, 126.9, 126.5, 82.2, 81.9, 80.4 (2C), 79.8 (J_{POC}= 7 Hz), 75.5, 74.7, 68.8 (J_{POC}= 7 Hz).

Fully protected *scyllo*-inositol phosphate (0.12 g, 0.14 mmol) in THF (5 mL) was added to 10% Pd on C (0.05 g) in 10 mL of an aqueous THF solution (4:1, THF/H_2O, v/v). Hydrogenation at 50 psi H_2 for 2 h was followed by filtration of the suspension through Celite and concentration to a white solid yielded *scyllo*-inositol phosphate[146] (0.04 g, 100 %). ^1H NMR (D_2O) δ 3.85-4.00 (m, 1 H), 3.42-3.54 (m, 2 H), 3.27-3.40 (m, 3 H); ^{13}C NMR (D_2O) δ 84.6 (J_{POC}= 4.5 Hz), 78.7, 78.4 (2C), 77.9 (2C).

EXPERIMENTALS (ADDENDUM)

Dimethyl benzylfumarate, 1b. and **Dimethyl benzylmaleate, 2b.**

A 2 N solution of benzyl magnesium chloride (12.0 mmol) in dry THF was added dropwise over 10 min to a suspension of CuI (2.28 g, 12.0 mmol) in THF (25 mL) kept at -40°C under Ar . The heterogeneous solution was stirred for 1 h at -40°C, then the dimethyl acetylenedicarboxylate (1.42 g, 10.0 mmol) in THF (5 mL) was added to the reaction mixture which immediately turned dark red. After stirring for 2 h at -40°C, the reaction was quenched by addition of a saturated NH_4Cl solution (40 mL), and slowly warmed to rt where the organic soluble products were extracted with ether (40 mL, 3x). The combined organic layers were washed with brine, dried, and concentrated to an oil which was purified first by radial chromatography (hexane) then by reverse phase HPLC. Dimethyl benzylfumarate **1b** (0.73 g, 31%) and dimethyl benzylmaleate **2b** (0.94 g, 40%) were obtained as oils . **Dimethyl benzylfumarate 1b** ^1H NMR ($CDCl_3$) δ 7.30-7.15 (m, 5 H), 6.85 (s, 1 H), 4.20 (s, 2 H), 3.80 (s, 3 H), 3.74 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 166.9, 166.0, 145.9, 138.0, 128.8, 128.6, 128.5, 128.4, 128.3, 129.2, 126.7, 126.3, 52.5, 51.8, 32.9; MS *m/z* (rel inten) EI 91 (12), 115 (92), 174 (35), 202 (100), 234 (14); HRMS (EI) calcd for $C_{13}H_{14}O_4$, 234.0891, found 234.0890; Anal. Calcd for $C_{13}H_{14}O_4$: C, 66.66; H, 6.02. Found: C, 66.62; H, 6.03. **Dimethyl benzylmaleate 2b** ^1H NMR ($CDCl_3$) δ 7.15-7.35 (m, 5 H), 5.66 (s, 1 H), 3.77 (s, 3 H), 3.70 (s, 3 H), 3.66 (s, 2 H); ^{13}C NMR ($CDCl_3$) δ 168.7, 165.4, 149.0, 135.5, 129.3, 128.7, 128.4, 127.2, 121.1, 52.3, 51.8, 40.0; MS *m/z* (rel inten) EI 91 (20), 115 (100), 174 (35), 202 (98), 234 (5); HRMS (EI) calcd for $C_{13}H_{14}O_4$, 234.0891, found 234.0892; Anal. Calcd for $C_{13}H_{14}O_4$: C, 66.66; H, 6.02. Found: C, 66.39; H, 6.03.

Dimethyl *E*-phenylitaconate, 3b.

Na metal (0.51 g, 22.0 mmol) was slowly added to CH_3OH (50 mL) maintained at 0°C under Ar. After complete disappearance of Na, dimethyl succinate (1.61 g, 11.0 mmol) was added immediately followed by benzaldehyde (1.06 g, 10 mmol). The solution was warmed to rt, stirred for 1 h, and then heated at reflux for 2 h. After cooling the reaction mixture to 0°C, saturated NH_4Cl solution was added (25 mL); this was immediately followed by acidification to pH 2 with the dropwise addition of HCl (1 N). The aqueous layer was then extracted three times with ether (50 mL) and the combined organic fractions were washed with brine, dried, and concentrated to an oil which was purified by radial chromatography (hexane). Pure dimethyl *E*-phenylitaconate **3b** was obtained as a yellow oil (1.52 g, 65%). ^1H NMR ($CDCl_3$) δ 7.85 (s, 1 H), 7.40-7.45 (m, 5 H), 3.77 (s, 3 H), 3.66 (s, 3 H), 3.52 (s, 2 H); ^{13}C NMR ($CDCl_3$) δ 171.4, 167.6, 141.9, 134.8, 128.9, 128.8, 128.5, 125.8. 52.1, 52.0, 33.3; MS *m/z* (rel inten) EI 91 (20), 115 (100), 174 (43), 202 (41), 234 (48); HRMS (EI) calcd for $C_{13}H_{14}O_4$, 234.0891, found 234.0890; Anal. Calcd for $C_{13}H_{14}O_4$: C, 66.66; H, 6.02. Found: C, 66.94; H, 6.09.

Dimethyl *Z*-phenylitaconate, 4b.

Dimethyl *E*-phenylitaconate (0.34 g, 1.45 mmol) was added to a mixture of $CHCl_3$ (10 mL) and acetone (5 mL). After dissolved O_2 was removed by bubbling Ar through the solution for 20 min, the solution was irradiated at 300 nm for 12 h at rt. Dimethyl *Z*-phenylitaconate was isolated as the single product of the reaction along with some unreacted material. Purification by radial chromatography (hexane) led to the isolation of dimethyl *Z*-phenylitaconate **4b** (0.20 g, 60%). ^1H NMR ($CDCl_3$) δ 7.45-7.35 (m, 5 H), 6.88 (s, 1 H), 3.71 (s, 3 H), 3.63 (s, 3 H), 3.47 (s, 2 H); ^{13}C NMR ($CDCl_3$) δ 171.1, 168.1, 139.5, 135.4, 128.5, 128.2, 128.1, 128.0, 126.3, 52.0, 51.6, 40.6; MS *m/z* (rel inten) EI 91 (46), 115 (100), 174 (88), 202 (100), 234 (14); HRMS (EI) calcd for

$C_{13}H_{14}O_4$ 234.0891, found 234.0890; Anal. Calcd for $C_{13}H_{14}O_4$: C, 66.66; H, 6.02. Found: C, 66.49; H, 6.08.

Benzylfumaric acid, 1a.

Dimethyl benzylfumarate **1b** (0.23 g, 1.00 mmol) was dissolved in 10 mL of a THF/aq NaOH (10 mM) solution (4:1, v/v). The deprotection was quenched after 12h of stirring at rt by acidification of aqueous HCl (1N, 5 mL). After extraction of the organic soluble diacid with ether (10 mL, 3x), the combined ethered fractions were washed with brine and dried. Pure benzylfumaric acid **1a** was obtained as an oil (0.20 g, 0.97 mmol) after removal of the solvents. ^{1}H NMR (CD_3COCD_3) δ 7.31 (d, *J*= 10 Hz, 2 H), 7.24 (dd, *J*= 10, 10 Hz, 2 H), 7.14 (dd, *J*= 10, 10 Hz, 1 H), 6.85 (s, 1 H), 4.17 (s, 2 H); ^{13}C NMR (CD_3COCD_3) δ 170.9, 170.0, 146.7, 140.1, 129.8, 128.9, 128.3, 126.4, 33.7; MS *m/z* (rel inten) FAB 205 (100), 161 (40), 117 (4), 91 (0.5); HRMS (FAB) calcd for $C_{11}H_9O_4$, 205.05008 (M-H$^+$), found 205.04959.

Bis(trimethylsilyl)-2-acetylenedicarboxylate

Acetylene dicarboxylic acid (5.70 g, 50.0 mmol) was dissolved under Ar in 33 mL of a THF/hexane solution (1:2, v/v) and slowly added to a solution of hexamethyl disilazane (5.40 g, 33.3 mmol) dissolved in hexane (25 mL). The mixture was stirred for 15 min and then vacuum filtered. Pure bistrimethylsilyl acetylenedicarboxylate was obtained as a yellow oil after removal of the solvents (8.97 g, 97%). ^{1}H NMR ($CDCl_3$) δ 0.42 (s); ^{13}C NMR ($CDCl_3$) δ 150.6, 74.8, -0.8.

Benzylmaleic acid, 2a.

A solution of 2N benzyl magnesium chloride (11.5 mmol) in dry THF was added dropwise, over 10 min, to a suspension of CuI (2.20 g, 11.5 mmol) in THF (40 mL) maintained at -40°C under Ar . The heterogeneous solution was stirred for 1 h at -40°C.

Bistrimethylsilyl acetylenedicarboxylate (2.48 g, 9.61 mmol) in THF (10 mL) was subsequently added to the reaction mixture, which immediately turned dark red. After stirring for 2 h at -40°C, the reaction was quenched by addition of a saturated NH_4Cl solution (40 mL) immediately followed by a dropwise addition of HCl (1N) up to pH 2. The heterogeneous solution was then slowly warmed to rt and the organic soluble products were extracted with ether (40 mL, 3x). Benzylmaleic acid **2a** was the only diacid observable in the crude obtained after the combined organic layers were washed with brine, dried, and concentrated. Pure Benzylmaleic acid **2a** was obtained as an oil by reverse phase HPLC purification (0.79 g, 40%). ^1H NMR (CD_3COCD_3) δ 7.22-7.35 (m, 5 H), 5.84 (s, 1 H), 3.71 (s, 2 H); ^{13}C NMR (CD_3COCD_3) δ 169.5, 166.9, 149.6, 137.5, 130.1, 129.4, 127.6, 122.7, 40.8; MS *m/z* (rel inten) FAB 205 (100), 161 (25), 117 (2.5), 91 (2), HRMS (FAB) calcd for $C_{11}H_9O_4$, 205.05008 ($M-H^+$), found 205.05080.

***E*-Phenylitaconic acid, 3a.**

Dimethyl *E*-phenylitaconate **3b** (0.23 g, 1.00 mmol) was dissolved in 10 mL of a THF/aq NaOH (10 mM) solution (4:1, v/v). After 15 min of stirring at rt, the deprotection was quenched by acidification to pH 2 with 1N HCl aq. The organic soluble diacid was immediately extracted with ether (3x) and the combined organic fractions were then washed with brine and dried. After removal of the solvents, *E*-phenylitaconic acid **3a** was crystallized from $CHCl_3$ as a white solid (0.19 g, 93%). ^1H NMR (CD_3COCD_3) δ 7.92 (s, 1 H), 7.35-7.55 (m, 5 H), 3.56 (s, 2 H); ^{13}C NMR (CD_3COCD_3) δ 172.4, 168.8, 141.8, 136.1, 129.9, 129.7, 129.5, 127.8, 33.8; MS *m/z* (rel inten) FAB 205 (100), 161 (43), 117 (6), 91 (5); HRMS (FAB) calcd for $C_{11}H_9O_4$, 205.05008 ($M-H^+$), found 205.05037.

Z-Phenylitaconic acid, 4a.

Dimethyl Z-phenylitaconate **4b** (0.23 g, 1.00 mmol) was dissolved in 10 mL of a 4:1 THF/aq NaOH (10 mM), v/v, solution. The deprotection was quenched after 12h of stirring at rt by acidification of aqueous HCl (1N, 5 mL). After extraction of the organic soluble diacid with ether (10 mL, 3x), the combined ethered fractions were washed with brine and dried. Pure Z-phenylitaconic acid **4a** was obtained as an oil (0.18 g, 88%) after removal of the solvents.

Another method to synthesize *Z*-phenylitaconic acid **4a** from *E*-phenylitaconic acid **3a** was used to confirm the structural assignment of compound **4a**. *E*-phenylitaconic acid **3a** (0.21 g, 1 mmol) was dissolved in 10 mL of a Acetone/H_2O solution (1:1, v/v) containing 1.1 eq of $NaHCO_3$. Dissolved O_2 was removed by bubbling Ar through the solution for 20 min, after which the solution was irradiated for 12 h. *Z*-Phenylitaconic acid **4a** (0.12 g, 60%) was extracted with ether (15 mL, 3x), and the combined organic fractions were washed with brine, dried, and concentrated. *Z*-phenylitaconic acid **4a** was isolated from *E*-phenylitaconic acid **3a** (0.08 g, 40%) by reversed-phase HPLC.

^{1}H NMR (CD_3COCD_3) δ 7.4 (d, *J*= 10 Hz, 2 H), 7.20-7.34 (m, 3 H), 6.89 (s, 1 H), 3.44 (s, 2 H); ^{13}C NMR (CD_3COCD_3) δ 173.0, 169.6, 138.5, 136.7, 129.6, 128.6, 41.5; MS *m/z* (rel inten) FAB 205 (35), 161 (17), 117 (9), 91 (1); HRMS (FAB) calcd for $C_{11}H_9O_4$, 205.05008 (M-H$^+$), found 205.05091.

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