MECHANISM-BASED INHIBITION OF 3-DEHYDROQUINATE SYNTHASE AND *MYO*-INOSITOL 1-PHOSPHATE SYNTHASE

By

Feng Tian

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ABSTRACT

MECHANISM-BASED INHIBITION OF 3-DEHYDROQUINATE SYNTHASE AND *MYO*-INOSITOL 1-PHOSPHATE SYNTHASE

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For the majority of enzymes that require nicotinamide adenine dinucleotide (NAD) for their activities, NAD serves as a cosubstrate which is consumed stoichometrically. However, DHQ synthase and MIP synthase employ NAD as a catalyst rather than a cosubstrate. For both enzymes, reduction of enzyme-bound NAD is followed by oxidation of the enzyme-bound NADH back to NAD during each turnover of substrate into product. To provide mechanistic insights of DHQ synthase and MIP synthase, synthetic inhibitors were developed and evaluated.

The role of inhibitors' ionization state in determining their binding to the DHQ synthase active site has received little attention. In the present study, dianionic and trianionic analogues of substrate DAHP were synthesized and their impact on active-site binding were compared. All but one of these inhibitors were synthesized via intermediacy of a butane 2,3-bisacetal-protected 3-dehydroquinate. Invariably dianionic analogues were weaker inhibitors than their trianionic counterparts. Carbocyclic tricarboxylates were the first examples of trianionic DHQ synthase inhibitors possessing neither a phosphate monoester nor a phosphonic acid. Carbocyclic inhibitors with malonyl and hydroxymalonyl groups bound to DHQ synthase as tightly as those possessing phosphorylmethyl and phophonoethyl moieties. These observations were consistent with the hypothesis that access of substrate and inhibitors to a trianionic ionization state is critical to binding at DHQ synthase's active site.

To take advantage of the presence of a metal ion at the active site of DHQ synthase, a mercapto group and an amino group were incorporated into DHQ synthase inhibitors

through regioselective oxirane opening of a common intermediate. The amino inhibitor was more effective than the mercapto inhibitor in disrupting enzymatic transformation. Enzyme-bound NADH formation was detected when each inhibitor was incubated with DHQ synthase. In vivo inhibition of DHQ synthase by the amino inhibitor was observed when *E. coli.* K-12 cells were grown up in M9 medium containing glucose and the amino inhibitor. The mercapto inhibitor failed to inhibit DHQ synthase in vivo. The extent of ionization state available to the metal-complexing moiety of each inhibitor seemed to correlate with the strength of inhibitor binding.

Two cyclic analogues of substrate glucose 6-phosphate were synthesized and tested for MIP synthase inhibition. None of them led to any detectable inhibition of MIP synthase. By contrast, acyclic substrate analogues were effective inhibitors and underwent enzyme-catalyzed oxidation. The results suggested that MIP synthase might efficiently and preferentially bind the acyclic form of D-glucose 6-phosphate for the enzymatic oxidation and an oxidized or ready-to-oxidize center was essential for a potent enzyme inhibitor.

A series of (*E*)- and (*Z*)-vinylphosphonates were synthesized and assayed for their inhibition potencies toward MIP synthase. (*E*)-Vinylphosphonate analogues were potent inhibitors, whereas (*Z*)-vinylphosphonate analogues exhibited no inhibitory activity. Furthermore, enzyme-bound NADH was detected when each (*E*)-vinylphosphonate analogue was incubated with the enzyme. These observations were in agreement with the hypothesis that MIP synthase preferentially stabilizes the (*E*)-conformation between C-5 carbon and phosphate groups of the substrate and employs the phosphate monoester for the ensuing enolization step. Thus, MIP synthase could be considered as a model of enzyme efficiency and enzyme opportunism.

To my parents and my wife

For their love and support

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Ac	acetyl
ADH	alcohol dehydrogenase
ADP	adenine diphosphate
AIBN	2,2'-azobisisobutyronitrile
Asp	aspartate
ATP	adenine triphosphate
Bn	benzyl
Boc	*tert*-butoxycarbonyl
Bu	butyl
Bz	benzoyl
CSA	(±)-10-camphorsulfonic acid
CI	chemical ionization
Cys	cysteine
DAHP	3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate
DBU	1,8-diazabicyclo[5,4,0]undec-7-ene
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DEAE	diethylaminoethyl
DHS	3-dehydroshikimate
DHQ	3-dehydroquinate
DIPEA	diisopropylethylamine
DMAP	4-(dimethylamino)pyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
DTT	dithiothreitol

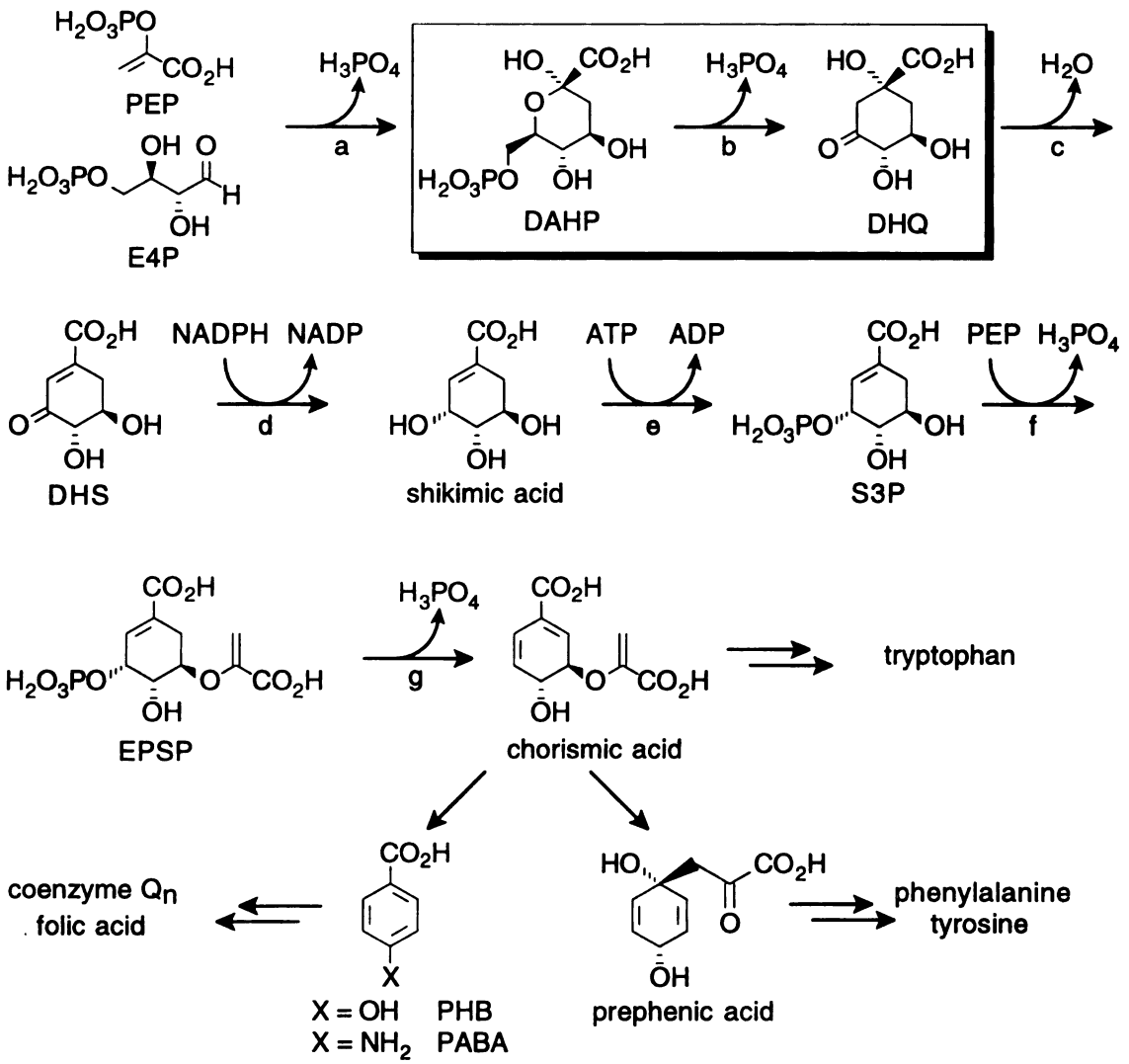
EI	electron impact
EPSP	5-enolpyruvylshikimate 3-phosphate
Et	ethyl
FAB	fast atom bombardment
Glu	glutamate
GTP	guanidine triphosphate
h	hour
His	histidine
HPLC	high pressure liquid chromatography
HRMS	high resolution mass spectrometry
Im	imidazole
IPTG	isopropyl-β-D-thiogalactopyranoside
k	rate constant
LB	luria broth
*m*CPBA	*m*-chloroperbenzoic acid
M	molar
Me	methyl
MIP	*myo*-inositol 1-phosphate
mL	milliliter
mM	millimolar
MOPS	4-morpholinepropanesulfonic acid
MS	mass spectrometry
min	minute
NAD	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
NADP	nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form

NBS	*N*-bromosuccinimide
NMO	*N*-methylmorpholine *N*-oxide
NMR	nuclear magnetic resonance
PABA	*p*-aminobenzoic acid
PHB	*p*-hydroxybenzoic acid
PEP	phosphoenolpyruvate
Ph	phenyl
PMB	*p*-methoxybenzyl
PMSF	*p*-toluenesulfonyl fluoroide
ppm	parts per million
*i*Pr	isopropyl
pyr	pyridine
rt	room temperature
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBAI	tetrabutyl ammonium iodide
TBDMS	*t*-butyldimethylsilyl
TEAB	triethylamine bicarbonate
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	trimethylsilyl
TPAP	tetrapropylammonium perruthenate
Tr	triphenylmethyl
Ts	*p*-toluenesulfonyl
TSP	sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$
Tf	trifluoromethanesulfonyl

CHAPTER 1

INTRODUCTION

Nicotinamide adenine dinucleotide (NAD) is involved in many enzymatic oxidoreductions. In most cases, the enzyme exploits NAD as a cosubstrate so that one equivalent of NADH is produced and then released from the active site along with one equivalent of product at the end of the catalytic cycle. There also exist a subset of enzymes[1] that utilize NAD catalytically even though the overall catalyzed transformation is redox neutral. The hallmark of these enzymes is that the initial reduction of enzyme-bound NAD to NADH is always coupled to an ensuing oxidation of the bound NADH back to NAD during each substrate turnover. In other words, this small group of enzymes employ NAD as a catalyst to build in transient functional groups and facilitate the overall transformation. In recognition that NAD is equivalent to an active site residue for this unique group of enzymes, we will refer to them as NADzymes, which include 3-dehydroquinate (DHQ) synthase,[2] *myo*-inositol 1-phosphate (MIP) synthase,[3] 2-deoxy-*scyllo*-inosose synthase,[4] *S*-adenosylhomocysteine hydrolase,[5] *S*-ribosylhomocysteine hydrolase,[6] and uridine diphosphate galactose 4-epimerase.[7] Besides hydride transfer reactions, NADzymes are able to catalyze eliminations, aldol condensations and Michael additions as well. These NADzymes play critical roles in such important biological processes as aromatic amino acid/aromatic vitamin biosynthesis, membrane biosynthesis and cellular signaling, antibiotic biosynthesis, methylation, and cell wall assembly.[1,4b] Understanding mechanisms of these NADzymes at the molecular level would help manipulate the corresponding biological processes when necessary. Along this line, two of the NADzymes, DHQ synthase and MIP synthase, were chosen as the subjects of this investigation.



(a) DAHP synthase; (b) DHQ synthase; (c) DHQ dehydratase; (d) shikimate dehydrogenase; (e) shikimate kinase; (f) EPSP synthase; (f) chorismate synthase.

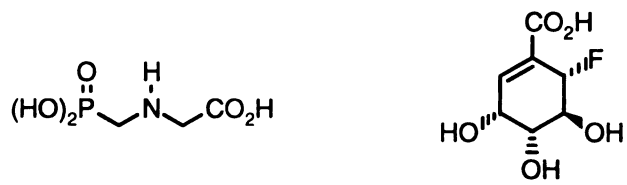
Figure 1. The shikimate pathway of aromatic amino acid biosynthesis.

The Shikimate Pathway: Target for Herbicides and Antimicrobial Agents

The shikimate pathway[8] in plants, fungi, and microorganisms is responsible for the biosynthesis of a spectrum of essential aromatic metabolites including three aromatic amino acids (tyrosine, tryptophan, and phenylalanine) as well as gallate, protocatechuate, *p*-aminobenzoate, folavonoids, and various quinones (Figure 1).

There are seven enzymes integrated in the shikimate pathway (Figure 1). The initial step of the aromatic biosynthetic pathway begins with the condensation of erythrose 4-phosphate (E4P) with phosphoenolpyruvate (PEP) to form 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) in the presence of DAHP synthase. DHQ synthase then expedites the conversion of DAHP into carbocyclic 3-dehydroquinate and inorganic phosphate. Elimination of water from DHQ is facilitated by DHQ dehydratase to produce 3-dehydroshikimate (DHS), which is, in turn, reduced by shikimate dehydrogenase to yield shikimate. Shikimate kinase-catalyzed phosphorylation with the aid of ATP generates shikimate 3-phosphate (S3P), followed by EPSP synthase-promoted condensation of S3P with PEP to afford 5-enolpyruvylshikimate 3-phosphate (EPSP). Finally, EPSP undergoes elimination of phosphate to give chorismate, the last common intermediate in the common pathway. Further processing of chorismate leads to aromatic amino acids and aromatic vitamins.

Through the course of evolution, nature obviated the shikimate pathway in mammals as the means to biosynthesize aromatic compounds. Humans rely on their diets for aromatic amino acids and aromatic vitamins. Thus agents able to block the common pathway critical in plants and microbes would have minimal adverse effect on humans. Consequently inhibition of enzymes within the shikimate pathway has become the subject of intense research with the aim to find potential herbicides and antimicrobial agents with low toxicity.



glyphosate (6*S*)-6-fluoroshikimic acid

Figure 2. Reagents disrupting aromatic biosynthesis.

For example, glyphosate (*N*-phosphonomethyl glycine, marketed as Roundup®, Figure 2) is a user-friendly, highly effective, broad spectrum herbicide.[9] Glyphosate's efficacy is attributed to its potent and competitive inhibition of EPSP synthase, the sixth enzyme of the shikimate pathway. Recently, evidence emerged indicating the presence of a functional shikimate pathway in apicomplexan parasites,[10] which cause substantial morbidity, mortality, and economic losses all over the world. Parasites rely on the shikimate pathway to supply chorismate as the starting material for biosynthesis of *p*-aminobenzoate and folic acid. Glyphosate was demonstrated to inhibit *in vitro* growth and survival of parasites (and associated disease states) such as *Toxoplasma gondii* (toxoplasmosis), *Plasmodium falciparum* (malaria), and *Cryptosporidium parvum* (diarrhea). Simultaneous administration of glyphosate and pyrimethamine, an antifolate drug which inhibits folic acid processing, rescued mice injected with lethal doses of *T. gondii*. This finding not only established glyphosate as a valuable lead compound for the discovery of novel antiparasitic drugs, but also highlighted the entire shikimate pathway as possible target for antimicrobial agents effective against bacterial and fungal pathogens and apicomplexan parasites.

(6*S*)-6-Fluoroshikimic acid (Figure 2) is another example of antibacterial agents disabling the aromatic biosynthetic pathway.[11] As an analogue of shikimic acid, (6*S*)-6-fluoroshikimic acid is processed by shikimate pathway enzymes to 6-fluorochorismate, which then inhibits biosynthesis of *p*-aminobenzoic acid. The MIC (minimal inhibition

concentration) values for (*6S*)-6-fluoroshikimic acid against bacteria are in the neighborhood of a few μg/mL. Its potency showcases the pivotal position of chemical disruption of aromatic amino acid and aromatic vitamin biosynthesis in the search for new antimicrobial agents.

Dysfunction of the shikimate pathway in a microbe would force the impaired microorganism to scavenge aromatic amino acids, coenzyme Q and folic acid or precursors to these aromatic vitamins from its growth environment. A large body of work has shown that inactivation of enzymes in the common pathway of microbes by genetic engineering can lead to severely attenuated growth when mutants are introduced into mammalian test subjects.[12] This establishes that microbes lacking a fully functional shikimate pathway are unable to scavenge the complete array of aromatic acids and vitamins efficiently. Moreover, mutations in various genes encoding common pathway enzymes have resulted in attenuation of virulence in a large number of different bacterial species. Live, attenuated strains of *Salmonella typhimurium* with mutation in the genes encoding DHQ synthase,[12e] DHQ dehydratase,[12b,c] EPSP synthase,[12a,f] and chorismate synthase[12b,d] have been extensively studied in mouse models as potential vaccines. The attenuated bacterial vaccine stimulates the host immune system into action against the disease state caused by the wild-type parent. These studies underscored the potential of chemical disruption of shikimate pathway as a new weapon against pernicious microbes.

In summary, an operative shikimate pathway is essential to the survival of plants, bacteria, and fungi as the main pipeline of aromatic amino acids and aromatic vitamins. Its absence from mammals makes enzymes within the shikimate pathway attractive targets for herbicides and antimicrobials. In face of the rising tide of antibiotic-resistance, inhibitors disrupting the common pathway might provide a surprising attack on pathogenic microbes. Hence, a study of the mechanism of particular enzymes constituting the shikimate pathway seems warranted.

Mechanistic Studies of DHQ Synthase

3-Dehydroquinate synthase is the second enzyme of the common pathway of aromatic amino acid biosynthesis. It requires the presence of NAD and a metal ion, such as cobalt(II) or zinc(II), for catalytic activity.[13a] DHQ synthase-catalyzed conversion of DAHP into DHQ is the first step to constitute the six-memebered carbocycle, which ultimately becomes the benzenoid portion of aromatic amino acids and a host of secondary metabolites.

The mechanism for DHQ synthase begins with oxidation of the C-5 alcohol of DAHP to the carbonyl of intermediate **A** by enzyme-bound NAD (Figure 3). Resulting acidification of the C-6 proton expedites the elimination of inorganic phosphate and forms the enol ether in intermediate **B**. Enzyme-bound NADH produced in the first step reduces the C-5 carbonyl back to the alcohol of intermediate **C** and regenerates NAD. Ring opening of intermediate **C** unmasks the C-2 carbonyl and an enol(ate) in intermediate **D**, which in turn forms DHQ via an intramolecular aldol condensation.

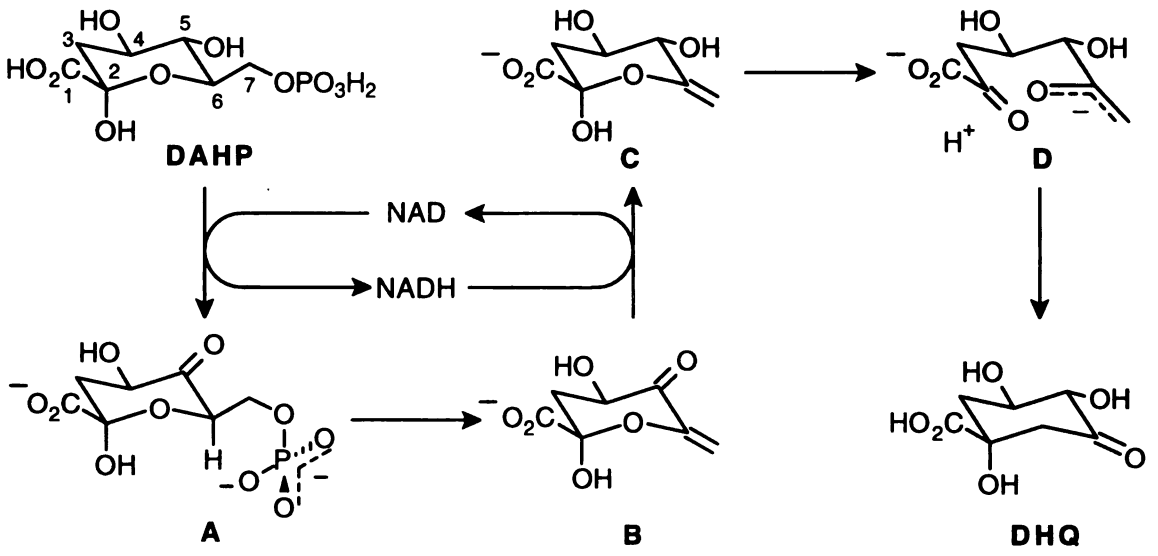


Figure 3. Proposed mechanism of DHQ synthase.

This proposed mechanistic pathway portrayed DHQ synthase as a catalytic marvel, catalyzing five reactions in a row: an oxidation, β-elimination, reduction, ring opening, and intramolecular aldol condensation. Initial support for this mechanism included several lines of evidence: (1) isotope exchange experiments[13b] established that proton at the C-6 position was lost to the medium during turnover; (2) kinetic isotope effects[13b,c] were observed at both the C-5 and C-6 positions, indicating that NAD oxidation of the C-5 alcohol and elimination of phosphate are both partially rate limiting steps; (3) NAD was indispensable to the stability and activity of DHQ synthase;[2,13b,d] and (4) a trapping experiment[13d] using tritiated sodium borohydride confirmed the transient existence of a keto group at C-5 position.

Availability of DHQ synthase[13e] and its substrate DAHP[13f] in reasonable quantities ushered in a new era of decoding the mechanism of DHQ synthase. Atomic absorption analysis demonstrated that the enzyme requires one divalent metal cation per monomer for activity.[13a] Though both Co(II) and Zn(II) can activate DHQ synthase, Zn(II) is believed to be the cofactor *in vivo* due to its bioavailability. DHQ synthase binds 1 equivalent of NAD with an apparent K_m of 80 nM.[13a] Enzyme-bound NADH was also detected during incubation of DHQ synthase with a number of carbocyclic substrate analogues.[13a]

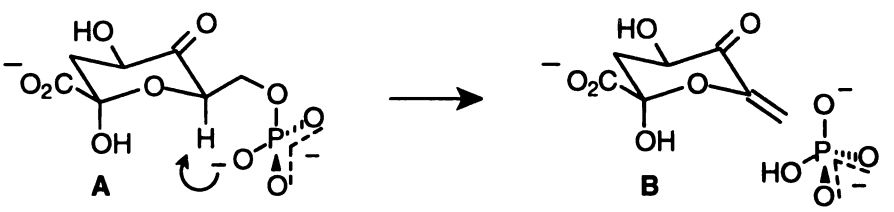
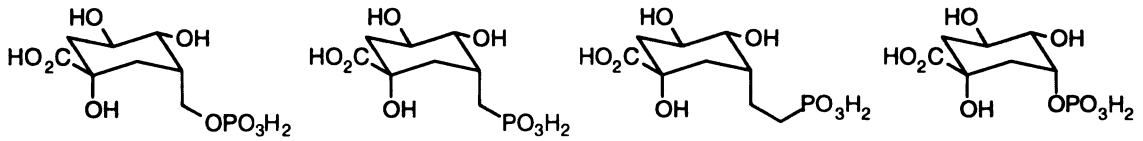


Figure 4. Self-mediated *syn*-elimination of inorganic phosphate.

Several reports also cast doubts on the role of DHQ synthase in each of the five steps during substrate turnover to product. For example, based on the finding that elimination of phosphate from intermediate **A** proceeds in *syn* fashion[13h] and that spatial

orientation of the phosphonate group of an inhibitor has a profound effect on both its inhibition potency and its tendency to undergo enzyme-catalyzed deuterium exchange[13i] at C-6 position, it was suggested that the phosphate monoester group of the substrate DAHP mediates its own elimination (Figure 4). In other words, the enzyme does not catalyze the second step, β-elimination. In a different study, *in situ* generated intermediate **C** was reported to undergo spontaneous ring opening and stereospecific ring closure to give DHQ in the absence of DHQ synthase.[13j] As a result, the enzyme's involvement in ring opening and aldo condensation steps was also questionable. The implication that intermediate **C** does not require the enzyme to control the aldol condensation stereochemistry along with the suggestion that phosphate catalyzes its own elimination led to a revised consideration of DHQ synthase. Instead of catalyzing five steps in one active site, DHQ synthase was suggested to only catalyze the initial oxidation and subsequent reduction. Hence, it might be more appropriate to regard DHQ synthase as a simple oxidoreductase.[13i]

However, reexamination of the spontaneous rearrangement of intermediate **C** showed that this nonenzymatic reaction does not proceed with complete stereospecificity at C-1 of DHQ.[13k] Therefore, it was assumed that DHQ synthase serves as a template to moderate, if not catalyze the last two steps. A recent study[13l] corroborated this suggestion. By challenging DHQ synthase with (*3R*)-3-fluoroDAHP and (*3S*)-3-fluoroDAHP, Scottish researchers demonstrated that fluorine substitution at C-3 could slow down the ring opening step and allow the fluorinated enol pyranose forms (analogues of intermediate **C**) to dissociate from the enzyme before completing the last two steps in the active site. Analysis of fluorinated products (analogues of DHQ) formed off the enzyme confirmed that epimerization at C-2 during aldol condensation readily occurred in the absence of enzyme. This finding articulated the subtle role that DHQ synthase plays in the cyclization step.



K_i = 30 x 10^{-9} M K_i = 55 x 10^{-9} M K_i = 0.30 x 10^{-6} M K_i = 0.53 x 10^{-6} M

Figure 5. 5-*epi*-Carbocyclic analogues.[14a]

An alternate approach to ascertaining whether an active site residue was involved in elimination of inorganic phosphate focused on challenging DHQ synthase with a series of carbocyclic analogues (Figure 5) whose functionalities at C-5 are epimeric relative to C-6 of DAHP.[14a] There are two possible scenarios: 1) If DHQ synthase employs an active site residue to mediate the elimination of inorganic phosphate, it is unlikely that the active site would tolerate the positioning of phosphoryl and phosphonyl groups at the same location where the active site basic residue supposedly resides. Consequently, inhibition of DHQ synthase by the 5-*epi*-carbocyclic analogues would not be possible. 2) If the phosphate monoester of DAHP catalyzes its own elimination, the active site may be quite tolerant of the type of stereochemical modifications in the *epi*-carbocycle series, and *epi*-Carbocyclic analogues might inhibit DHQ synthase. It turned out that all four *epi*-carbocyclic analogues were inhibitors of DHQ synthase. Such pronounced stereochemical and steric tolerance in active site binding supports the hypothesis that the phosphate monoester is functioning as the base to eliminate inorganic phosphate.

Further investigation of the involvement of DHQ synthase was focused on its possible role in stabilizing the conformational change concomitant with conversion of the C-6, sp^3-hybridized methine carbon of intermediate **A** to a C-6, sp^2-hybridized carbon of an E1cb intermediate or E1cb-like transition state. A series of cyclohexenyl and cyclohexylidene analogues (Figure 6) possessing strategically placed olefinic functionality were found to bind to DHQ synthase more tightly than similarly substituted cyclohexyl inhibitors.[14b] These results are in accordance with the hypothesis that DHQ synthase

actively stabilizes an E1cb intermediate or E1cb-like transition state. Thus DHQ synthase might not be a passive spectator during the self-elimination of inorganic phosphate from intermediate **A**.

The recently solved crystal structure of DHQ synthase[15] put all controversies to rest. Based on various interactions observed between the enzyme active site and carbaphosphonate (a substrate analogue), the enzyme is clearly not a bystander in catalysis. DHQ synthase stabilizes intermediates and impedes side reactions during substrate turnover. For instance, the enzyme provides a phosphate-binding pocket to accommodate the phosphate oxygens into a position where they can remove the proton from C-6 in the elimination step. Interactions with the active site residues practically freeze the conformation of the carboxylate and C-2 hydroxyl groups of intermediate **D** so that epimerization at C-1 of DHQ would be forestalled during the last two steps. Thus DHQ synthase is undoubtedly more than a simple oxidoreductase and its mechanism entails an orchestrated interplay between substrate, cofactors, and the enzyme active site.

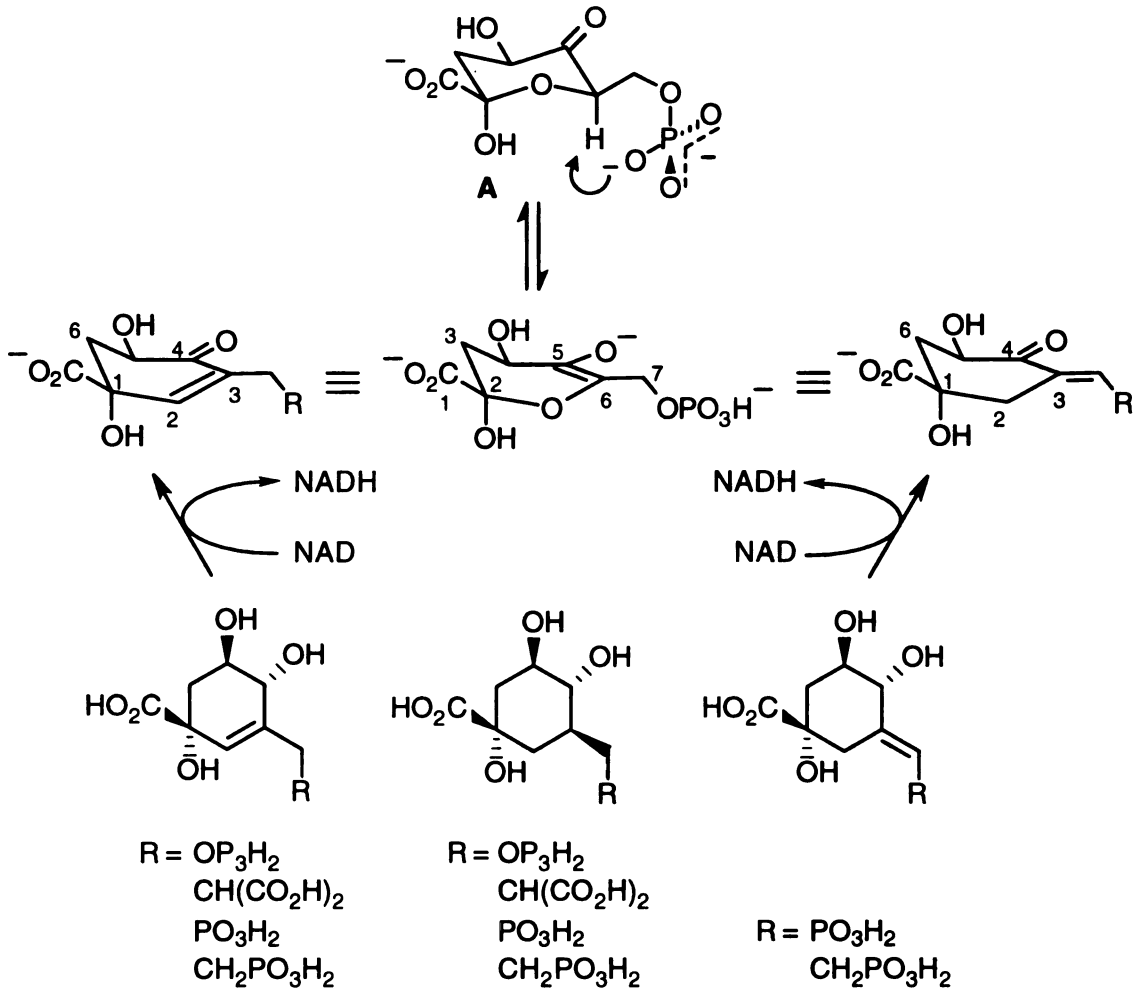


Figure 6. Mimics of an E1cb intermediate or E1cb-like transition state.[14b]

Phosphoinositide Signaling and Lithium Treatment

Advances in molecular and cellular biology have paved the way for studies on intracellular signal transduction pathways.[16] In general, intracellular pathways fall into two broad categories. One includes pathways that are controlled by receptor-coupled second messengers and regulated by neurotransmitters. The other embraces those that are controlled by receptors containing or attendant with protein tyrosine kinases and regulated by neurotrophic factors and cytokines. The phosphatidylinositol cascade[17] belongs to the first category (Figure 7). Research on the phosphatidylinositol pathway has emerged as an important area of research, owing to the pivotal role played by inositol lipids in cell physiology. In particular the phenomenon of transmembrane signaling, which has biomedical ramifications[16h] in a plethora of disease states, has been the focus of extensive studies.

After agonist (A) binds to its receptor protein (R) on the cell surface (Figure 7), GTP-binding protein (G-protein) activates phospholipase C (PLC), which in turn induces the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce two second messengers, *myo*-inositol 1,4,5-trisphosphate (1,4,5-IP_3) and 1,2-di-*O*-acylglycerol (DAG). While DAG activates protein kinase C (PKC), 1,4,5-IP_3 binds to receptors in the endoplasmic reticulum (ER) and enhances intracellular calcium concentration. Either way the external signal is transmitted into the cell. Upon accomplishing their missions, DAG is metabolized to phosphatidate (PA) and further to cytidine monophosphate-phosphatidate (CMP-PA), while 1,4,5-IP_3 is degraded via inositol monophosphates to inositol. Hydrolysis of inositol monophosphates including D-*myo*-inositol 1-phosphate (D-I-1-P), L-*myo*-inositol 1-phosphate (L-I-1-P) and *myo*-inositol 4-phosphate (I-4-P) is catalyzed by inositol monophosphatase. Reuptake of inositol and CMP-PA into the phosphatidylinositol cycle converts them back to phosphatidylinositol (PI) and phosphatidylinositol 4-phosphate (PIP).

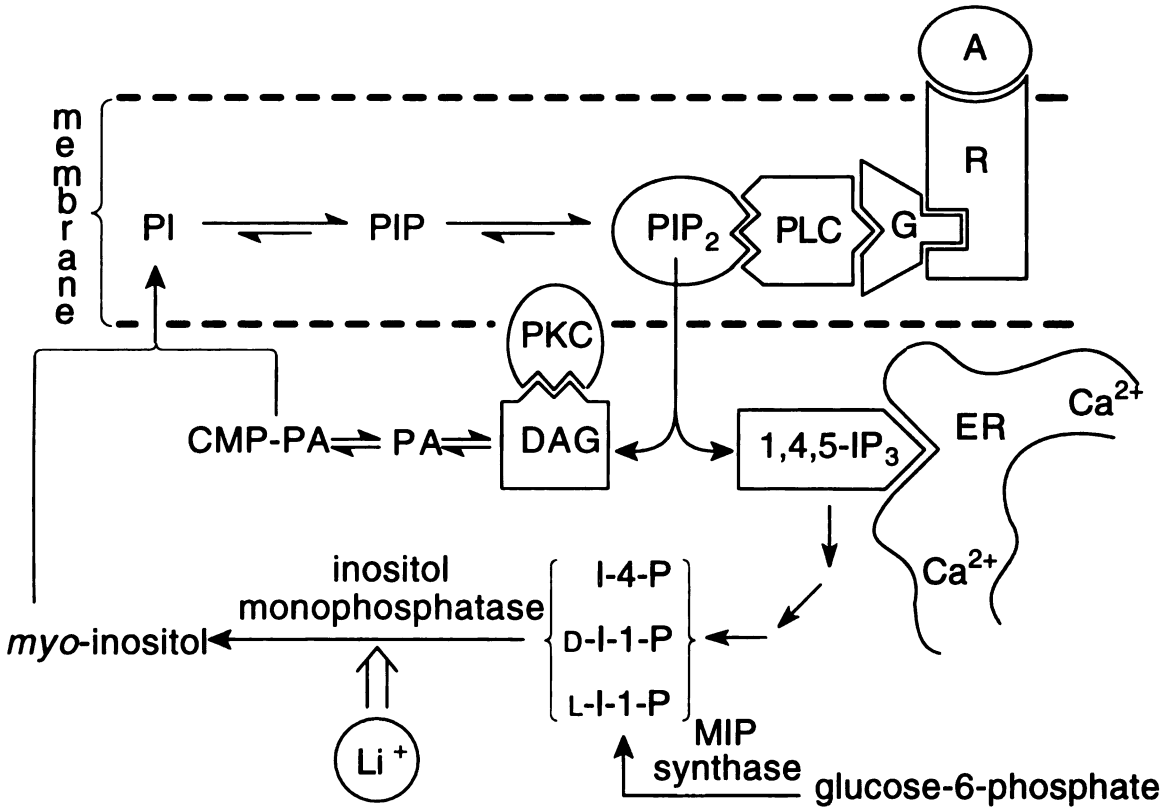


Figure 7. The phosphatidylinositol cascade.[17]

Lithium is an effective drug for the treatment and prevention of manic-depressive illness. A large amount of evidence has confirmed that the phosphoinositide cascade is a target for lithium in the brain.[18] Since the brain has limited access to inositol other than that derived from recycling of the inositol monophosphates, sufficient supply of *myo*-inositol in the brain can be crucial to the resynthesis of phosphoinositides and thus maintenance and efficacy of signal transduction. Lithium's effects were postulated to be the uncompetitive inhibition of inositol monophosphatase in the brain. Suppressing the hydrolysis of inositol monophosphates leads to the depletion of *myo*-inositol[19] and the scarcity of PIP_2, the precursor of second messenger 1,4,5-IP_3. Consequently the hyperactive neuron would be dampened since it loses a critical pool of PIP_2 for signal transduction.

Although lithium is the most common drug of choice for treating manic-depression, unwanted side effects, such as nausea, stomachache, loose stools, and hand tremor, somewhat detract from the value of the drug.[20] There is only a narrow margin between an effective therapeutic dose and the dose causing poisoning. In addition, there is a time lag between the administration of lithium salts and exhibition of antimanic effects. Consequently, other effective psychopharmacological treatments for manic depression would be highly desirable.

De novo Biosynthesis of *myo*-Inositol 1-Phosphate

Since the blood-brain-barrier blocks the penetration of dietary *myo*-inositol into the brain, access to *myo*-inositol is at the mercy of a de novo biosynthetic pathway,[21] which comprises two enzymes, MIP synthase and *myo*-inositol 1-phosphatase. Isomerization of D-glucose 6-phosphate to *myo*-inositol 1-phosphate (MIP) is catalyzed by MIP synthase. *myo*-Inositol 1-phosphate is then hydrolyzed to *myo*-inositol by *myo*-inositol 1-

phosphatase. De novo synthesis of *myo*-inositol 1-phosphate is the only biosynthetic route for the brain to get access to *myo*-inositol.

In theory, inhibition of MIP synthase in brain tissues could reduce *myo*-inositol levels as lithium treatment does, thus representing an alternate approach for treating manic depression. Whereas lithium therapy depletes the inositol pool and accumulates inositol monophosphates, DAG, and DAG-associated metabolites, selective inhibition of MIP synthase would reduce the amount of inositol and *myo*-inositol 1-phosphate but leave the levels of DAG and its related metabolites untouched. Accordingly inhibition of MIP synthase might have different neurological ramifications than that with lithium treatment. Inasmuch as DAG is a known endogenous activator of PKC, it is not inconceivable that lithium's indirect action in accumulating DAG and its metabolites might alter PKC-mediated modification of other proteins, which in turn modulate neuronal functions.[22] In this respect, inhibitors of MIP synthase might provide a more precise picture of lithium therapy and result in the identification of other targets and the design of mechanism-based mood stabilizers.

MIP synthase has been isolated from a variety of sources, such as yeast,[23] mammalian tissues,[24] fungi,[25] and plants.[26] Mechanistic studies indicated that MIP synthase from various sources share a common mechanism (Figure 8). Conversion of D-glucose 6-phosphate into *myo*-inositol 1-phosphate requires the oxidation of the C-5 alcohol of the acyclic form **A'** by enzyme-bound NAD to afford intermediate **B'**, 5-keto-glucose 6-phosphate. Deprotonation at C-6 generates the enol(ate) of intermediate **C'**. Subsequent intramolecular aldol condensation leads to intermediate **D'**, 2-inosose 1-phosphate. Reduction of the carbonyl of intermediate **D'** by NADH generated in the first step forms *myo*-inositol 1-phosphate and regenerates NAD.

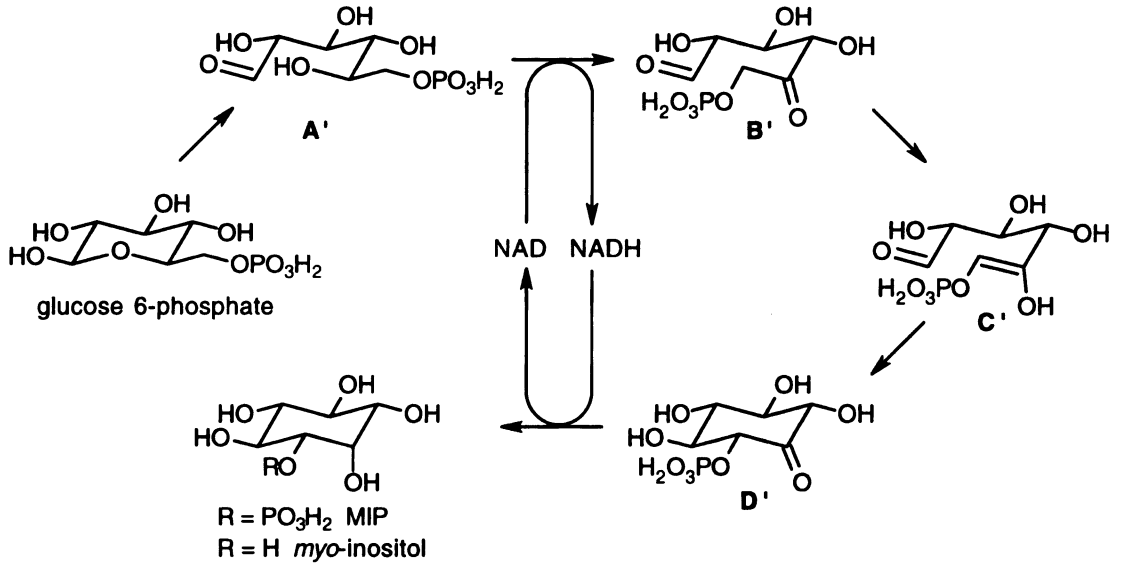


Figure 8. Proposed mechanism of MIP synthase.

Support for the mechanism of Figure 8 has come from various experiments: (1) MIP synthase requires NAD for its activity[24e,27] and enzyme-bound NADH is tightly associated with the enzyme. Hydride transfer apparently involves the pro-*S* hydrogen at the C-4 position of NADH.[28] In situ formation of NADH during substrate turnover has recently been observed by UV-vis spectroscopy.[29] (2) Trapping experiments[30] using tritiated sodium borohydride implied the transient existence of intermediate **D'**. (3) Isotope effects[31] were observed at both the C-5 and C-6 positions of D-glucose 6-phosphate, consistent with the presence of intermediates **B'** and indicating that the enolization step is partially rate limiting. (4) The *pro-R* hydrogen of the C-6 methylene group is preferentially removed during enolization.[32]

Recently intermediate **D'**, *myo*-2-inosose 1-phosphate, has been chemically synthesized.[33a] In the presence of *apo*MIP synthase reconstructed with NADH, intermediate **D'** was converted to *myo*-inositol 1-phosphate, concomitant with the regeneration of NAD at the active site. This finding confirmed the involvement of intermediate **D'** in MIP synthase-catalyzed reactions and demonstrated a half reaction that is essential to the overall turnover of substrate into product. Moreover, when incubated with

native MIP synthase possessing bound NAD, *myo*-2-inosose 1-phosphate inhibited the enzymatic transformation. In this case, intermediate **D'** behaves as a competitive inhibitor when it is introduced into the catalytic cycle at a premature stage. Inhibition might result from a kinetic barrier to release *myo*-2-inosose 1-phosphate caused by the absence of an active site reductant (NADH) when *myo*-2-inosose 1-phosphate is bound by MIP synthase.

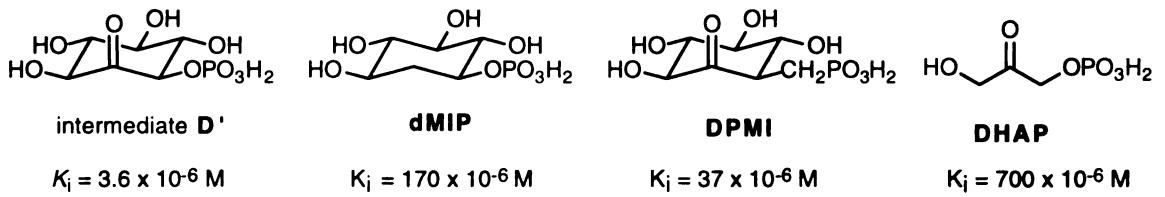


Figure 9. Intermediate **D'** and its analogues.[33]

One puzzle coming out of the above study was the presence of only minute amounts of the keto form of *myo*-2-inosose 1-phosphate in aqueous solution at biological pH. To determine the contribution of the keto form in *myo*-2-inosose 1-phosphate to active site binding and to gauge the utility of oxidized reaction center in designing MIP synthase inhibitiors, a seres of analogues (Figure 9) were examined for enzyme inhibition.[33b] A 47-fold reduction in inhibitor potency was observed when the oxidized reaction center of *myo*-2-inosose 1-phosphate was removed in **dMIP**. With a methylene group substituted for the phosphate monoester oxygen, **DPMI** existed in its keto form exclusively at biological pH and was as potent as *myo*-2-inosose 1-phosphate. DHAP is commercially available and exists as an approximately equal mixture of keto and hydrate forms at neutral pH. It was a much weaker inhibitor than **DPMI** or *myo*-2-inosose 1-phosphate. Nevertheless, it provides insights into the minimum set of structural requirements for inhibition of MIP synthase. Overall, these data suggest that incorporation of an oxidized reaction center into potential inhibitors is a general strategy for inhibiting MIP synthase.

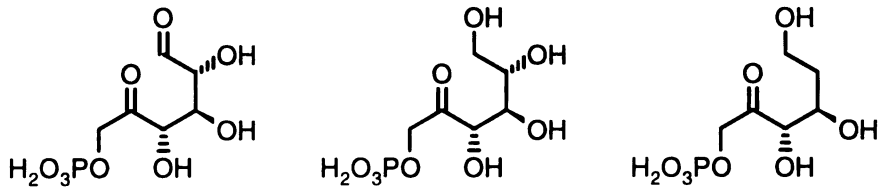


Figure 10. Intermediate **B'** and its analogues.[29]

Corroboration for the above hypothesis came from a parallel study of intermediate **B'**, 5-keto-D-glucose 6-phosphate, and its analogues (Figure 10).[29] Indeed, 5-keto-D-glucose 6-phosphate was another example that an intermediate along the reaction coordinate of MIP synthase inhibits the normal catalysis of the enzyme. To various degrees, analogues of intermediate **B'** disrupted the catalytic cycle of MIP synthase. Binding of intermediate **B'** or its analogues to the enzyme resulted in a nonnatural configuration involving a molecule having an oxidized reaction center being bound at an active site possessing NAD instead of NADH. The observed inhibitions by intermediate **B'** and its analogues reflected both the inability of the ternary enzyme-NAD-oxidized analogues complex to resume normal catalysis and the stabilization occurring between functional groups of intermediate **B'** and active site residues. It is noteworthy that intermediate **B'** and its analogues preferentially exist as cyclic hemiacetals in water as judged by their ^{1}H and ^{13}C NMR spectra. Presumably the agents responsible for the inhibition were the acyclic ketone forms in accord with the proposed mechanism. Therefore the extent of inhibition exhibited by intermediate **B'** and its analogues might in part reflect their propensity to form acyclic forms and unmask the oxidized reaction centers.

CHAPTER 2

INHIBITOR IONIZATION AS A DETERMINANT OF BINDING TO 3-DEHYDROQUINATE SYNTHASE

Design of Tricarboxylate Analogues

Stable analogues of reactive intermediates and transition states along enzyme reaction coordinates have typically been the focus of synthetic and enzymological attention.[34] For example, spirocyclic carbaphosphodiester **1** and spirocyclic keto carbaphosphodiester **2** (Figure 11) have been designed to be conformationally restrained mimics of the six-membered transition state wherein the methine proton in intermediate **A** is removed by the phosphate monoester.[35] If DHQ synthase accelerates the elimination of inorganic phosphate from reactive intermediate **A** by constraining the degrees of freedom available to the ionized phosphonomethyl group, spirocyclic **1** and **2** might be potent inhibitors of the enzyme.

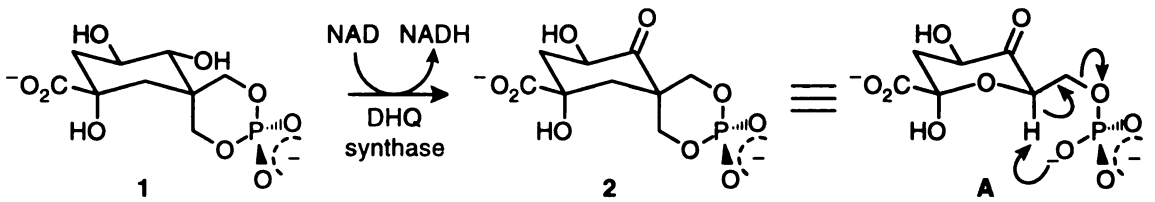


Figure 11. Spirocyclic carbaphosphodiester **1** and **2**.[35]

However, spirocyclic carbaphosphodiester **1** was only a modest inhibitor with K_i = 67 x 10^{-6} M even though it was oxidized at the active site of DHQ synthase. Spirocyclic carbaphosphodiester **2** failed to inhibit the enzyme completely. The modest inhibition of DHQ synthase by spirocyclic **1** might result from an inherent limitation to its ionization state instead of adverse steric interactions with the enzyme active site. Compared with previous studies, this result could be interpreted as suggesting a limited role for DHQ synthase during elimination of inorganic phosphate from intermediate **A** whereby the

enzyme merely ensures that either DAHP or intermediate **A** is in its trianionic ionization state. Another relevant study disclosed that for various phosphonate analogues of DAHP, the ratio of their inhibition constants versus K_m increased as the pH of the assay buffer was raised from pH 7.0 to pH 8.0.[13g] Since the phosphonates undergo their second ionization in the pH range studied, this trend suggested that it is the trianionic form of these phosphonate analogues that is bound to the enzyme. Except for the above two reports, comparatively little attention has been given to the impact of inhibitor ionization on binding to the active site of DHQ synthase.

Challenging the enzyme with a reactive intermediate or transition state analogue that can exist in a tribasic ionization state seems to be a prerequisite for tight binding to the active site. To gauge the contribution of different ionization states of the substrate to active site binding, analogues of the dianionic and trianionic forms of substrate DAHP would be ideal probes. Along this line, carbaphosphinate **3** and carbaacetate **4** were chosen as dianionic analogues of DAHP while carbasuccinate **5**, carbamalonate ether **6**, carbamalonate **7**, and carbahydroxymalonate **8** were designed as trianionic carbocyclic substrate analogues (Figure 12).

Besides spirocyclic carbaphosphodiester **1**, carbacarboxylate **9** (Figure 12) was the only literature example of a dianionic carbocyclic DHQ synthase inhibitor.[36] Known trianionic carbocyclic inhibitors of DHQ synthase included (Figure 12) carbocyclic phosphonates and phosphates having C-5 stereochemistry identical (**10-13**)[13g,l,m] and epimeric (**14-17**)[14a] to the stereochemistry at C-6 of DAHP, and C-1 epimeric carbaphosphonate **18**.[37] Tricarboxylates **5-8** would be the first examples of trianionic DHQ synthase inhibitors possessing neither a phosphate monoester nor a phosphonic acid. Since a greater variety of prodrug strategies are available for carboxylate conjugation relative to phosphonic acid and phosphate monoester conjugation, identifying the core structure of nonphosphorus functional groups in DHQ synthase inhibitors might pave the way for *in vivo* inhibition of DHQ synthase in microbes.

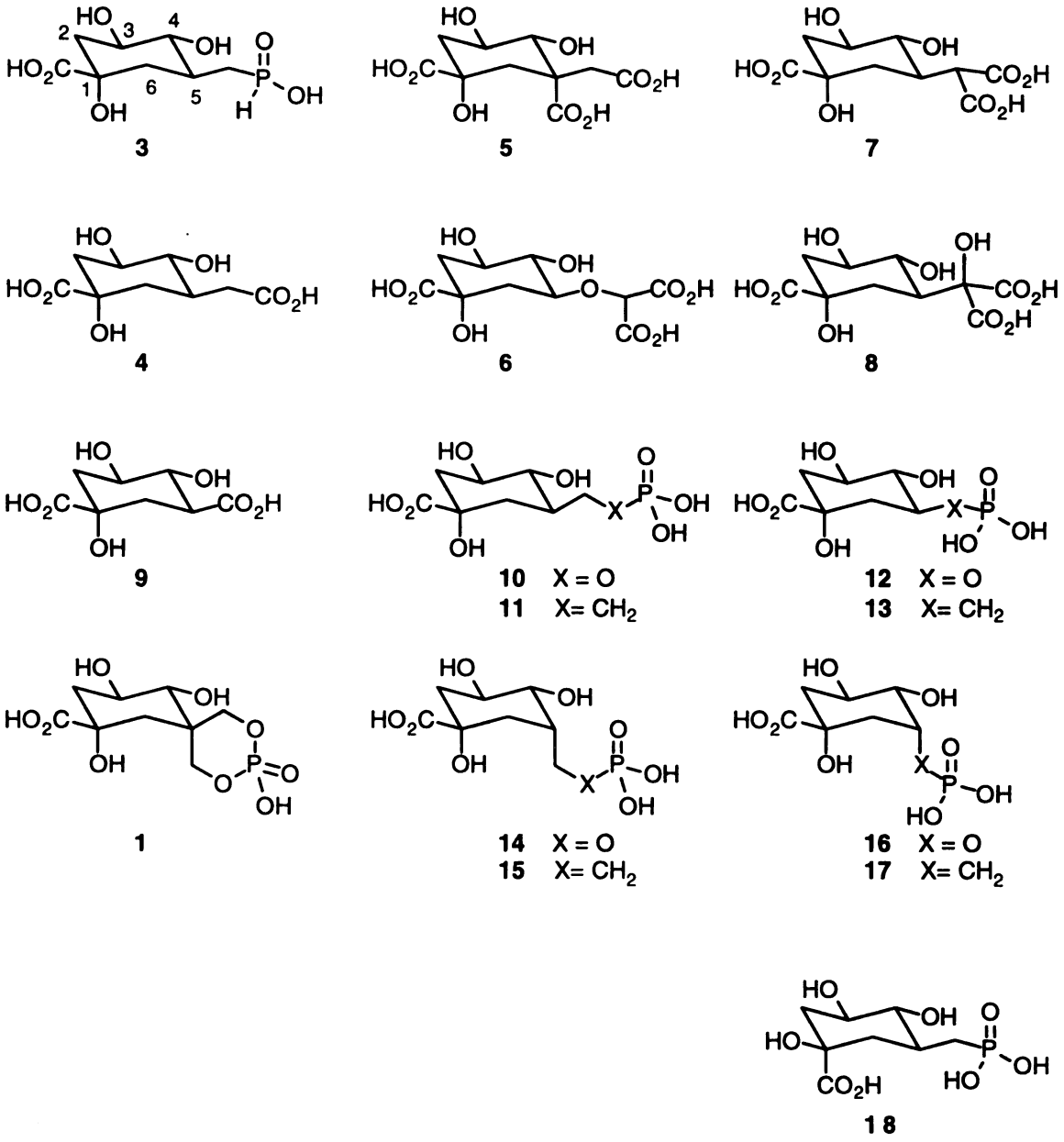
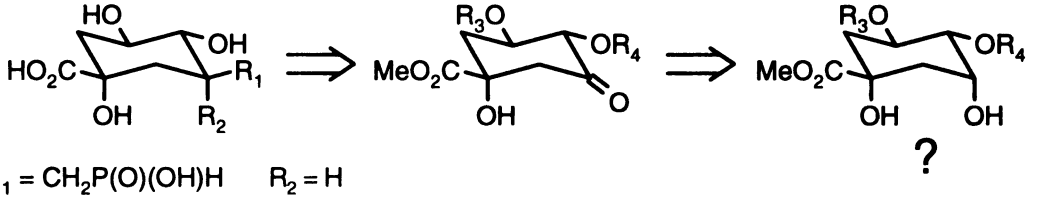


Figure 12. Carbocyclic inhibitors of DHQ synthase.

Synthetic Strategy: Utility of the Butane 2,3-Bisacetal Protecting Group

All of the targeted molecules (**3-8**) share a common six-membered ring with different substitution patterns at the C-5 position. One approach (Figure 13) to introduce functional groups at C-5 is to get access to a protected 3-dehydroquinate (DHQ) derivative, which in turn might come from a suitably protected (-)-quinic acid. This retrosynthetic analysis prompted the question of how to selectively protect the C-3,4 *trans* vicinal hydroxyls of quinic acid in the presence of C-4,5 *cis* vicinal diol.



3 $R_1 = CH_2P(O)(OH)H$ $R_2 = H$

4 $R_1 = CH_2COOH$ $R_2 = H$

5 $R_1 = CH_2COOH$ $R_2 = COOH$

6 $R_1 = OCH(COOH)_2$ $R_2 = H$

7 $R_1 = CH(COOH)_2$ $R_2 = H$

8 $R_1 = C(OH)(COOH)_2$ $R_2 = H$

Figure 13. Retrosynthetic analysis.

In recent years Ley and coworkers have developed the use of bisdihydropyrans (bis-DHP) and 1,1,2,2-tetramethoxycyclohexane (TMC) for the selective protection of 1,2 diequatorial diols (Figure 14) as their corresponding dispiroketals (Dispoke)[38] and cyclohexane 1,2-diacetals (CDA),[39] respectively. These protective groups proved to be effective in carbohydrate chemistry. High selectivity of both groups is presumably derived from the combination of two effects. One is the formation of the sterically less demanding *trans* ring fusion between the dioxane ring and the pyranoside. The other is the configurational control of the anomeric effect at the two acetal centers. However, bis-DHP requires multiple preparation steps[38b] while TMC demands an expensive starting material.

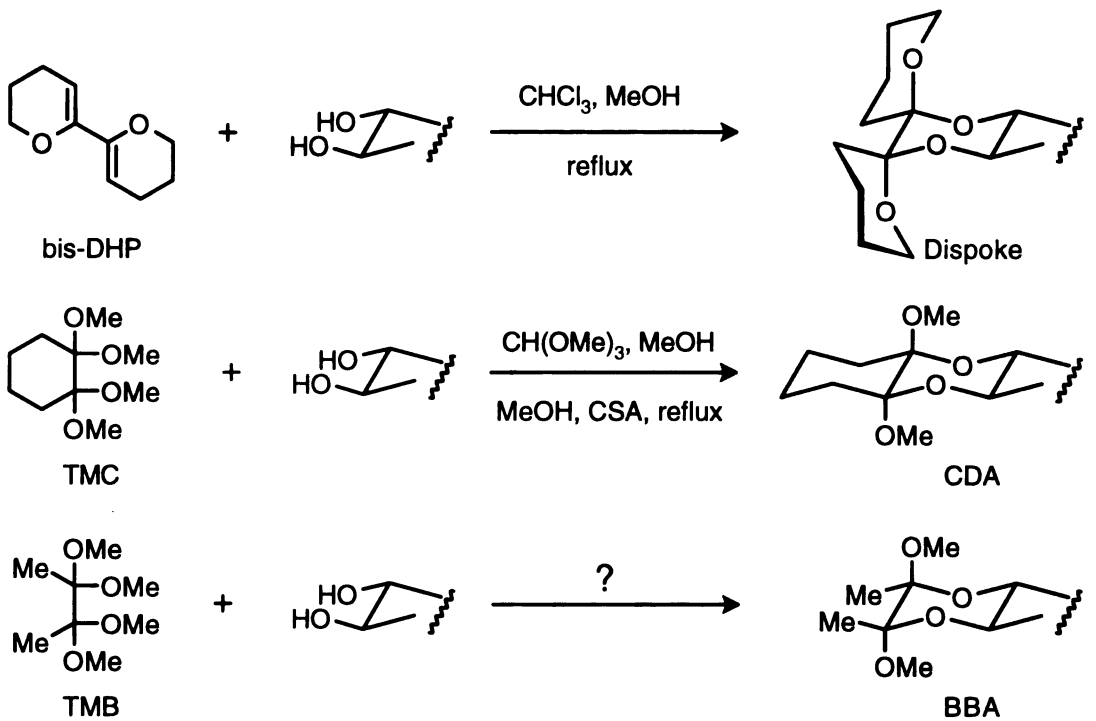
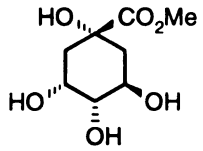
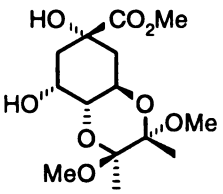
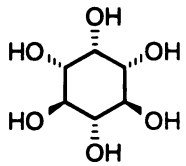
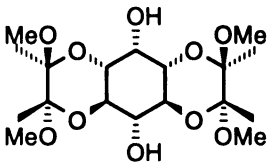
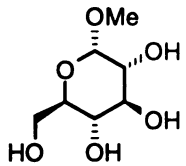
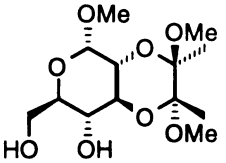
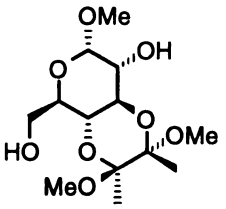
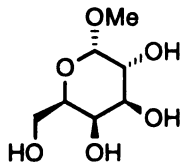
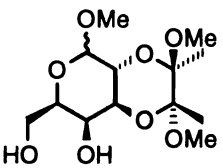
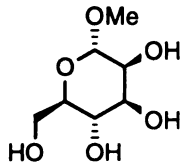
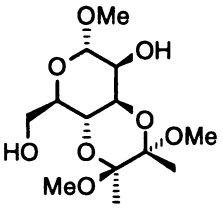


Figure 14. Selective protection of diequatorial diols.

Moreover, preparation of both bis-DHP and TMC involves chromatographic purification,[38b,39] which is undesirable for large scale synthesis. Apparently neither of these protecting groups would be the solution to our problem and a more practical approach akin to CDA and Dispoke protection seemed perspicacious.

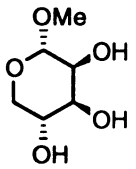
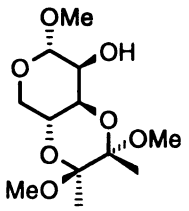
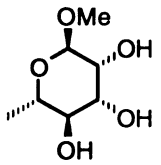
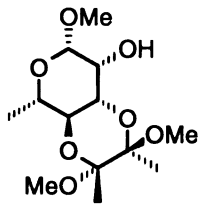
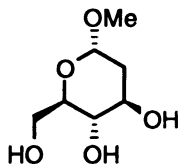
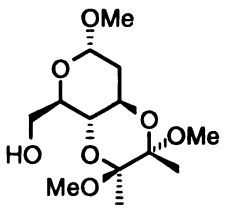
Inspection of 2,2,3,3-tetramethoxybutane (TMB), a truncated form of TMC, indicated that TMB could provide the same protection selectivity for vicinal diequatorial diols as TMC (Figure 14). Moreover, the reported protection of diethyl tartrate diol with monoacetal 3,3-dimethoxylbutan-2-one[40] suggested that TMB could form diacetals with vicinal diols. The TMB reagent was prepared on hundred-gram scale by acid-catalyzed acetalization of 2,3-butanedione in 50-60% yield after distillation. Selective protection of the C-3,4 vicinal diequatorial hydroxyls of methyl quinate as butane 2,3-bisacetals utilized reaction conditions similar to those employed for CDA protection. Quinic acid (50 g) was

Table 1. Butane 2,3-bisacetal protection of carbocycles and carbohydrates.[41]

Starting Material	Product	%Yield	Literature
 quinate	 **19**	87	
 *myo*-inositol	 **20**	79	66[a]
 methyl α-D-gluco-pyranoside	 **21**  **22**	82	80 / 68[b]
 methyl α-D-galaco-pyranoside	 **23** α/β = 10:1	54	46 / 76[b]
 methyl α-D-manno-pyranoside	 **24**	91	48 / -[b]

[a] Reference 42. [b] CDA protection/Dispoke protection, see reference 39.

Table 1. (cont'd)

Starting Material	Product	%Yield	Literature
 methyl α-D-lyxo-pyranoside	 **25**	83	45 / 62 [b]
 methyl α-L-rhamno-pyranoside	 **26**	81	74 / 47 [b]
 methyl 2-deoxy-α-D-gluco-pyranoside	 **27**	77	

[a] Reference 42. [b] CDA protection/Dispoke protection, see reference 39.

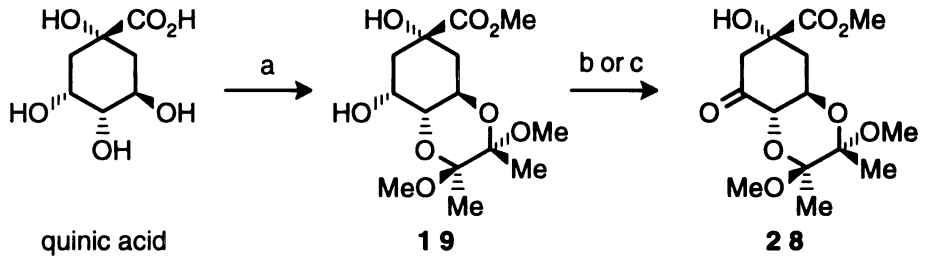
then routinely converted into the crystalline BBA-protected methyl quinate **19** as a one-pot procedure in over 80% yield (Table 1).[41]

The scope of BBA protection has been extended beyond quinic acid (Table 1). Reaction of *myo*-inositol with 2 equivalents of TMB yielded the highly symmetrical diprotected meso compound **20**. This protection pattern is reminiscent of protection of inositol with tetraisopropyldisiloxane groups[42] though BBA provides a cheaper and more efficient route. Reaction of TMB with carbohydrates attested to the versatility of BBA protection. For example, TMB reaction with methyl D-glucopyranoside gave an equimolar mixture of 2,3- and 3,4- regioisomers **21** and **22**, respectively. BBA protection of methyl D-galactopyranoside led to the desired products **23** with a certain degree(10:1 α/β) of epimerization at the anomeric center. Carbohydrate derivatives such as D-mannose, D-lyxose, L-rhamnose, and 2-deoxy-D-glucose also reacted with TMB and resulted in selective vicinal diol protection (Table 1).

Overall, the BBA protecting group appears to be a practical alternative to Dispoke or CDA for selective protection of vicinal diequatorial diols. A bonus of BBA protection is the simplicity of its ^{1}H NMR spectrum which displays four diagnostic singlets. The rigidity resulting from the formation of the 2,3-dimethoxyl-2,3-dimethyl-1,4-dioxane moiety also provides an element for conformational control that was exploited in subsequent reactions *vide infra*. The BBA protection is compatible with a wide variety of reagents and can be deprotected easily with aqueous trifluoroacetic acid with or without methylene chloride. Recently Ley and coworkers suggested a modified procedure for BBA protection.[43] It obviated the necessity to synthesize TMC, by simply generating TMC *in situ* from 2,3-butanedione, trimethyl orthoformate, and methanol under acidic conditions. The modification makes BBA protection a particularly appealing option for vicinal diequatorial diol protection.

C-1 Alcohol Directed Syntheses of Tricarboxylates **5-8**

Previous attempts to use DHQ as a starting material in the synthesis of carbocyclic DHQ synthase inhibitors were not successful due to DHQ's instability[44] under a variety of the conditions employed to selectively protect the molecule's hydroxyl groups. Development of the BBA protecting group[41] provided an expedient entry for the synthesis of the desired, protected DHQ intermediate (Figure 15). Quinic acid was quantitatively converted into its methyl ester by refluxing in methanol with Dowex 50 (H^+). BBA protection afforded diol **19**, whose C-5 hydroxyl group was oxidized with tetrapropylammonium perruthenate[45] (TPAP) and 4-methylmorpholine *N*-oxide (NMO) furnishing protected DHQ derivative **28** (Figure 15). Consistent with previous observations, large scale runs using TPAP gave incomplete reaction and resulted in lower yields even when acetonitrile was added.[45b] An alternative large-scale (30 g) oxidation of **19** employed KIO_4 and catalytic $RuCl_3$ in $CHCl_3/H_2O$ and afforded DHQ intermediate **28** in 77% yield. Although previous studies indicated that DHQ-type compounds were labi le to β-elimination and aromatization,[44] DHQ intermediate **28** proved to be reasonably stable under various reaction conditions. The carbonyl group in **28** provided a handle for carbon-carbon bond formation at C-5, which enabled syntheses of tricarboxylate analogues **5-8**.



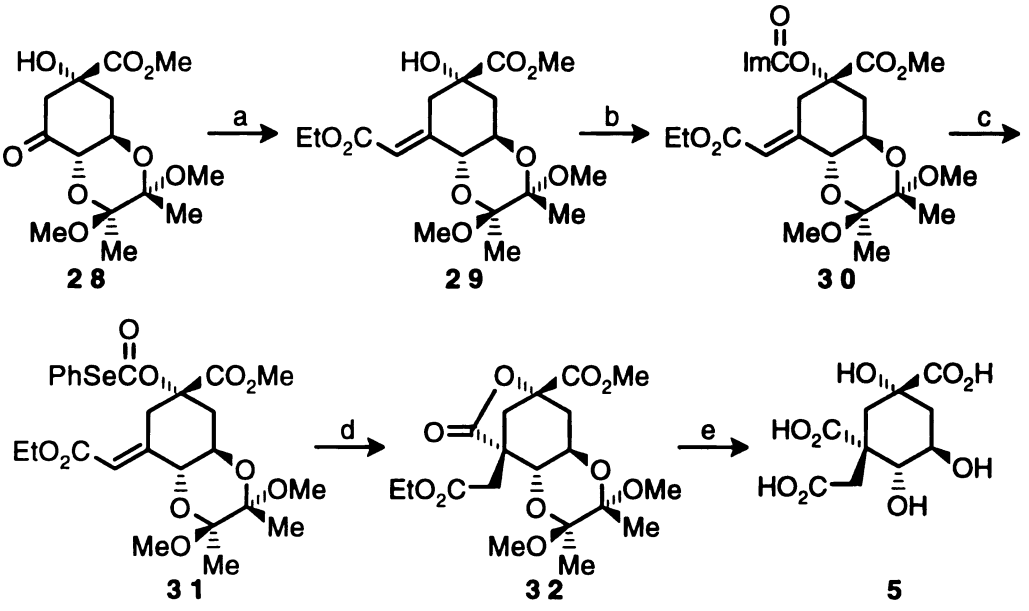
quinic acid **19** **28**

(a) (i) CH_3OH, Dowex 50 (H^+), reflux, (ii) 2,2,3,3-tetramethoxybutane, $CH(OCH_3)_3$, CH_3OH, CSA, reflux, 87%; (b) TPAP, NMO, CH_2Cl_2, CH_3CN, 92%; (c) KIO_4, K_2CO_3, $RuCl_3$, H_2O, $CHCl_3$, 77%.

Figure 15. Synthesis of DHQ derivative **28**.

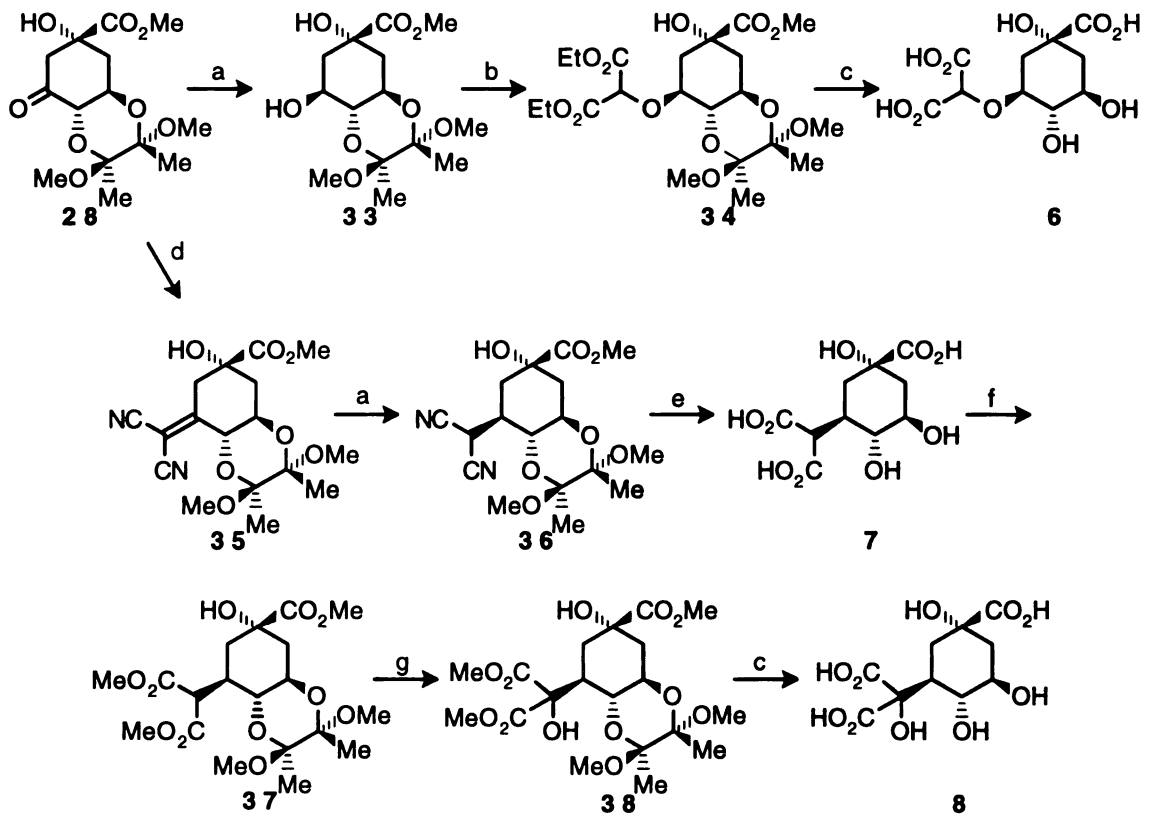
An essential feature of all tricarboxylate syntheses was the use of the C-1 alcohol to direct critical reactions. For example, the C-1 alcohol was left unprotected during reaction of DHQ intermediate **28** with (carbethoxymethylene)triphenylphosphorane (Figure 16). Protection of the C-1 alcohol of DHQ intermediate **28** as a trimethylsilyl (TMS) ether gave a slower condensation reaction that was accompanied by dehydration involving elimination of the protected C-1 alcohol. Phosphorane complexation with the C-1 alcohol was likely responsible for directing and accelerating the rate of the condensation reaction.[46]

The C-1 alcohol was also essential to establishing carbasuccinate's C-5 stereochemistry during radical cyclization of the C-1 phenylselenocarbonate **30**. Because of its steric environment, functionalization of the C-1 tertiary alcohol in dicarboxylate **29** required a two-step sequence instead of the conventional procedure exploited by Corey.[47a] Initial reaction of **29** with 1,1'-carbonyldiimidazole was followed by interception of the imidazoyl carbamate **30** with phenylselenol to afford phenylselenocarbonate **31**. Radical cyclization provided lactone **32** in 27% yield.[47] Formation of deoxygenation products epimeric at C-1 accounts for the modest yield of **32** and likely reflects C-1 carboxylate stabilization of a C-1 tertiary radical resulting from loss of CO_2. The BBA protecting group was easily removed in 95% aqueous trifluoroacetic acid (TFA) at room temperature. Subsequent hydrolysis of the esters and lactone under basic conditions followed by protonation using Dowex 50 (H^+) provided carbasuccinate **5**.



(a) $Ph_3P{=}CH_2CO_2Et$, CH_3CN, reflux, 90%; (b) Im_2CO, $ClCH_2CH_2Cl$, reflux, 82%; (c) PhSeH, $ClCH_2CH_2Cl$, reflux, 87%; (d) Bu_3SnH, AIBN, C_6H_6, reflux, 27%; (e) (i) TFA/H_2O (20:1, v/v), (ii) 0.2 N aqueous NaOH/THF (1:1, v/v), 100%.

Figure 16. Synthesis of carbasuccinate **5**.

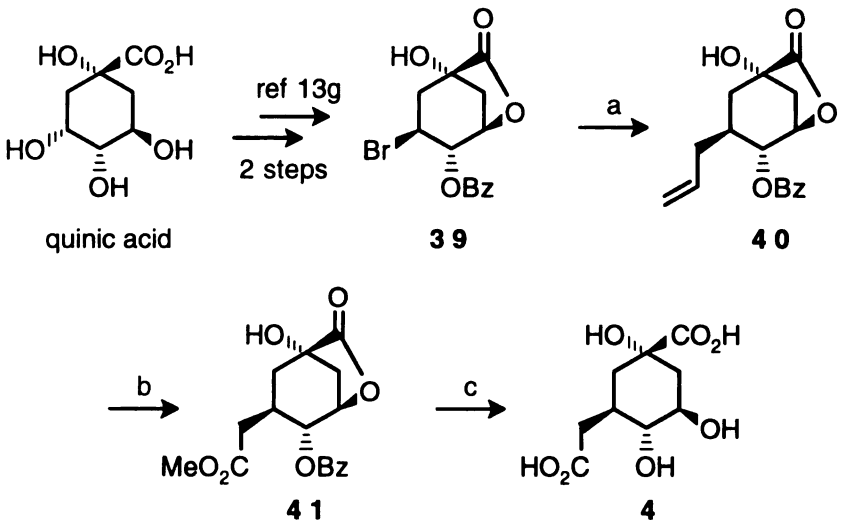


(a) $NaBH(OAc)_3$, CH_3CN/HOAc (1:1, v/v), **33**: 86%, **36**: 92%; (b) $N_2=C(CO_2Et)_2$, $Rh_2(OAc)_4$, C_6H_6, reflux, 75%; (c) (i) TFA/H_2O (20:1, v/v), (ii) 0.2 N aqueous NaOH/THF (1:1v/v), 100%; (d) $CH_2(CN)_2$, NH_4OAc, HOAc, C_6H_6, 90%; (e) (i) TFA/H_2O (20:1, v/v), (ii) 6 N aqueous HCl, reflux, (iii) 0.2 N aqueous NaOH/THF (1:1v/v), (iv) Dowex 50 (H^+), 95%; (f) (i) CH_2N_2, Et_2O, CH_3OH, (ii) 2,2,3,3-teratmethoxybutane, $CH(OCH_3)_3$, CH_3OH, CSA, reflux, 80% from **36**; (g) (i) $(CH_3)_3COK$, THF, 0 °C, (ii) 2-(benzenesulfonyl)-3-phenyloxaziridine, 75%.

Figure 17. Syntheses of tricarboxylates **6-8**.

The C-5 stereochemistry of carbamalonate ether **6**, carbamalonate **7**, and carbahydroxymalonate **8** all depended on C-1 alcohol direction of $NaBH(OAc)_3$ reductions (Figure 17). For example, reaction of DHQ intermediate **28** with $NaBH(OAc)_3$ exclusively gave *epi*-quinate **33**. The extremely high stereoselectivity of this reaction can best be explained by delivery of hydride to the face of the carbonyl as dictated by complexation of the C-1 alcohol with $NaBH(OAc)_3$.[13g,48] Coupling of *epi*-quinate **33** with diethyl diazomalonate to give protected malonate ether **34** was easily accomplished under $Rh_2(OAc)_4$ catalysis.[49] Subsequent ester and BBA removal using the aforementioned reaction conditions yielded carbamalonate ether **6**.

Reduction of the activated olefin of α,β-unsaturated carbamalonitrile **35** to give carbamalonitrile **36** (Figure 17) is also consistent with C-1 alcohol complexation of $NaBH(OAc)_3$.[48] α,β-Unsaturated carbamalonitrile **35** was obtained by reaction of DHQ intermediate **28** with malononitrile catalyzed by NH_4Ac and HOAc. Attempts to react DHQ intermediate **28** with diethyl malonate under basic conditions failed to yield a condensation product. Removal of the BBA protection group of **36** was followed by hydrolysis of the nitrile groups in refluxing 6 N hydrochloric acid, which also resulted in lactonization of the C-4 hydroxyl and malonyl carboxylate. This lactone was then hydrolyzed under basic conditions prior to Dower 50 (H^+) treatment to give the carbamalonate **7** triacid. Carbamalonate **7** was also converted into carbahydroxymalonate **8**. Esterification of **7** with diazomethane and reprotection of the C-3,4 *trans*-diol provided triester **37**, which underwent oxaziridine hydroxylation[50] yielding protected hydroxymalonate **38**. TFA-catalyzed removal of the BBA protecting group, base-catalyzed hydrolysis of the methyl esters, and treatment with Dower 50 (H^+) led to carbahydroxymalonate **8**.



(a) allyltributyltin, AIBN, C_6H_6, reflux; (b) (i) $NaIO_4$, $RuCl_3$, $CCl_4/CH_3CN/H_2O$ (2:2:3, v/v/v), (ii) CH_2N_2, Et_2O, CH_3OH, 65%; (c) (i) 0.2 N aqueous NaOH/THF (1:1, v/v), (ii) Dowex 50 (H^+), 100%.

Figure 18. Synthesis of Carbaacetate **4**.

Synthesis of Carbaacetate **4**

Access to carbaacetate **4** could be envisioned from stereospecific hydrogenation of the exocyclic alkene in dicarboxylate **29**. However, under various conditions[122] inseparable mixtures of hydrogenated products were obtained. Therefore, an alternative approach calling for the stereoselective alkylation at the C-5 position of quinate ring was attempted (Figure 18). Bromo lactone **39** and allyl lactone **40** were made from quinic acid following Knowles' procedure.[13g] Radical alkylation of **39** with allyltributyltin[51] gave the allyl derivative **40**. The selectivity was attributed to the stereoelectronic effect of the vicinal benzoate group, which stabilizes the axial antiperiplanar radical and enhances the electrophilicity of the radical by overlapping the orbital of the radical with the σ^* orbital of the benzoate group.[13g] Oxidation of the resulting C-5 allyl substituent of **40** with $NaIO_4$ and catalytic, *in situ* generated RuO_4[52] was followed by esterification to expedite purification of the protected carbaacetate. Subsequent basic hydrolysis and neutralization afforded carbaacetate **4**.

Enzymology of Carbocyclic Inhibitors **3-8**

DHQ synthase[13e] was purified from *E. coli* RB 791(*p*JB14). Enzyme assays were conducted in 50 mM MOPS buffer solution at pH 7.5 containing $CoCl_2$ (50 μM) and NAD (10 μM).[13a] DHQ dehydratase[52] was employed as the coupling enzyme to assay DHQ synthase activity. Dehydratase-catalyzed conversion of DHQ into 3-dehydroshikimate (DHS) and the increase in optical density at 234 nm resulting from formation of DHS provided a means for continuous quantitation of DHQ formation. The Michaelis constant (K_m) for DAHP binding to DHQ synthase has been taken as 4×10^{-6} M. Inhibition constants (K_i) were determined (Table 2) for carbocyclic inhibitors **3-8** (carbaphosphinate **3** was synthesized and evaluated by Dr. Jean-luc Monchamp). Interactions with DHQ synthase were further evaluated by whether observed inhibition was competitive,

noncompetitive, or linear mixed-type relative to substrate DAHP binding.[54a] Formation of NADH was measured by increases in absorbance at 340 nm when DHQ synthase was incubated with each inhibitor in the absence of substrate DAHP. This measure of whether the carbocyclic inhibitor's C-4 alcohol was in close enough proximity to the bound NAD for C-4 alcohol oxidation to occur provided a litmus test for the carbocyclic inhibitor's alignment in the active site.

Table 2. Inhibition of DHQ synthase by carbocyclic inhibitors **3-8**.

inhibitor	inhibition type	E-NADH formation	K_i (M)	K_i/K_m
3	competitive	+	20×10^{-6}	5
4	linear mixed-type[a]	–	3×10^{-6}	0.8
5	competitive	+	5×10^{-6}	1.3
6	competitive	+	7×10^{-6}	1.8
7	competitive	+	0.7×10^{-6}	0.2
8	competitive	+	0.3×10^{-6}	0.08

[a] $K_i' = 20 \times 10^{-6}$ M. Slopes and *y*-axis intercepts derived from double reciprocal plots were plotted as a function of inhibitor concentration. The base-line intercepts of the slope and *y*-axis intercepts, respectively, provided inhibition constants K_i and K_i'.[54]

Carbocyclic diacids carbaphosphinate **3** and carbaacetate **4** were modest inhibitors of DHQ synthase. Carbaacetate **4** was a linear mixed-type inhibitor (Table 2). While carbaphosphinate **3** was a significantly weaker inhibitor of DHQ synthase than carbaacetate **4**, its binding to the active site was entirely competitive relative to substrate DAHP. NADH formation was observed during carbaphosphinate **3** but not during carbaacetate **4** inhibition. Of the carbocyclic inhibitors **3-8** synthesized in this study, carbaacetate's

inhibition of DHQ synthase was the only instance where NADH formation was not observed.

The Importance of Trianionic Ionization States

Comparison of carbaphosphinate **3** and carbaphosphonate **13**[13g,i] (K_i = 0.8 x 10^{-9} M) highlights the importance of trianionic ionization states to active site binding. While the carbaphosphinate **3** is limited to a dianionic ionization state, carbaphosphonate **13** can exist in the active site as a trianion. The difference in available ionization states resulted in a 25,000-fold weaker inhibition of DHQ synthase by carbaphosphinate **3** relative to carbaphosphonate **13**. Another indication of the significance of a trianionic ionization state is the 560-fold weaker inhibition of DHQ synthase by spirocyclic carbaphosphodiester **1**[35] (K_i = 67 x 10^{-6} M) relative to carbaDAHP **10**[36,13i] (K_i = 0.12 x 10^{-6} M).

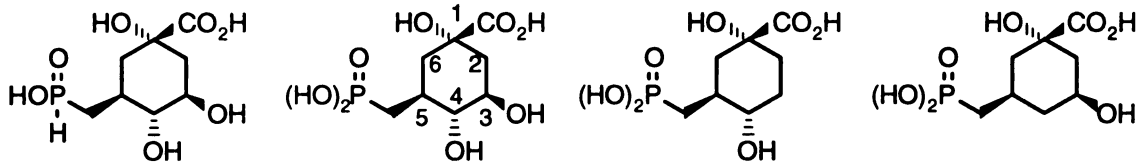


Figure 19. Carbocyclic inhibitors **3**, **13**, **42**, **43**

There appears an alternate explanation for the weaker inhibition of DHQ synthase by carbaphosphinate **3** relative to carbaphosphonate **13**. Even though a dibasic phosphate monoester (corresponding to an overall trianionic ionization state) at the active site of DHQ synthase would seem to ensure the optimum reaction environment for conversion of intermediate **A** to intermediate **B**, elimination of a monobasic phosphate monoester is also a mechanistic possibility. The structural difference between analogue **13** and **3** could be

perceived as a replacement of a monobasic phosphonate hydroxyl group in enzyme-bound, dianionic carbaphosphonate **13** with a hydrogen in the dominant tautomeric form of the phosphinate of enzyme-bound, dianionic carbaphosphinate **3**. Loss of active site interactions with a hydroxyl group might then explain the severely attenuated inhibitor potency of carbaphosphinate **3**. Active site interactions with hydroxyl groups in carbaphosphonate **13** can be approximated by the 275-fold and 675-fold reduction in inhibitor potency observed, respectively, for C-3 and C-4 monodeoxycarbaphosphonates[55] **42** and **43** relative to carbaphosphonate **13** (Figure 19). This reduction in inhibitor potency due to loss of active site interactions with a hydroxyl group is substantially less than the 25,000-fold loss of inhibition potency observed for carbaphosphinate **3** relative to carbaphosphonate **13**. The lack of access to a trianionic ionization state thus appears to be a better explanation for weaker inhibition observed for carbaphosphinate **3**.

Tricarboxylates as Analogues of Phosphate Monoester

The carbocyclic inhibitors **4-8** can be viewed as evolutionary extensions of the previously synthesized carbacarboxylate **9** (Figure 12), which was reported[36] to be a competitive inhibitor with K_i = 100 x 10^{-6} M. By inserting a methylene carbon between the carbocyclic ring and the carboxylate, inhibition of DHQ synthase improves by almost 30 fold based on the competitive inhibition constant K_i for carbaacetate **4** relative to that of carbacarboxylate **9**. The noncompetitive portion (K_i') of the linear mixed-type inhibition observed for carbaacetate **4** suggests that this inhibitor's interaction with DHQ synthase are not restricted to the enzyme's active site.[54] Providing access to a trianionic ionization state led to competitive inhibition for carbasuccinate **5**, carbamalonate ether **6**, carbamalonate **7**, and carbahydroxymalonate **8**. Employment of a malonate-type charged appendage results in improved active site interactions as indicated by the approximately 10-fold improvement in K_i values observed for tricarboxylates **7** and **8** relative to tricarboxylates **5** and **6**.

Titration of carbamalonate **7** indicated a single, large inflection point indicative of multiple proton dissociation at an apparent pK_a = 4.4. For comparison, two inflection points indicating apparent acid dissociation constants at pK_{a1} = 2.55 and pK_{a2} = 6.25 were observed during titration of DAHP.

Comparison of the newly synthesized tricarboxylate inhibitors with previously synthesized carbaphosphates and carbaphosphonates (see Figure 12, page 20) was also informative. Carbahydroxymalonate **8** is approximately as potent a competitive inhibitor of DHQ synthase as carbaDAHP[36,13i] **10** (K_i = 0.12 x 10^{-6} M). Both carbamalonate **7** and carbahydroxymalonate **8** are better competitive inhibitors than carbahomophosphonate[13g,14a] **11** (K_i = 1.7 x 10^{-6} M), carbaphosphate[13g,14a] **12** (K_i = 1.7 x 10^{-6} M), and C-5 epimers 5-(phosphonoethyl)-5-deoxyquinate[14a] **15** (K_i = 30 x 10^{-6} M) and 3-(phosphonooxy)-quinate[14a] **16** (K_i = 53 x 10^{-6} M). The only carbocyclic phosphate monoester synthesized prior to this study that is a substantially more potent competitive inhibitor than carbahydroxymalonate **8** is C-5 epimeric 5-[(phosphonooxymethyl]-5-deoxyquinate[14a] **14** (K_i = 7.0 x 10^{-9} M). Organophosponic acid analogues such as carbaphosphonate[13g,i] **13** (K_i = 0.8 x 10^{-9} M), C-1 epimeric carbaphosphonate[37] **18** (K_i = 1.8 x 10^{-9} M) and C-5 epimeric 5-(phosphonomethyl)-5-deoxyquinate[14a] **17** (K_i = 13 x 10^{-9} M) are more potent inhibitors of DHQ synthase. On the basis of these data, carbocyclic inhibitors with hydroxymalonyl and malonyl groups are apparently bound by DHQ synthase as tightly as carbocyclic inhibitors possessing phosphorylmethyl and phosphonoethyl moieties. The malonic acid and hydroxymalonic acid moieties are, not surprisingly, poor analogues of a nonisosteric phosphonic acid moiety of carbaphosphonate **13**.

Overall, the hydroxymalonic acid and malonic acid moieties of, respectively, carbahydroxymalonate **8** and carbamalonate **7** are excellent analogues of a phosphate monoester.[56] This follows from the competitive nature of these tricarboxylates' inhibition relative to substrate DAHP, the magnitude of their inhibition constants relative to those of carbaDAHP **10** and carbahomophosphonate **11**, and formation of NADH during DHQ

synthase inhibition. The development of tricarboxylate inhibitors of DHQ synthase is also important in terms of finding lead compounds for in vivo inhibition of DHQ synthase. Compared with phosphonate prodrugs, a wider range of prodrug strategies have been successfully employed for preparation of carboxylic acid prodrugs. For instance, α-C-(1,5-anhydro-7-amino-2,7-dideoxy-D-*manno*-heptopyranosyl)carboxylate is an inhibitor[57] of CMP-KDO synthetase. Though the drug's competitive inhibition potency was moderate (K_i = 4.0 x 10^{-6} M), a prodrug strategy relying on dipeptide transport was used to drastically increase the concentrations of this essentially nonpermeant inhibitor inside bacterial cytosols. Similar approaches could be applied to tricarboxylate inhibitors and the chemotherapeutic utility of *in vivo* DHQ synthase inhibition could ultimately be evaluated.

CHAPTER 3

BUIDING METAL-COMPLEXING MOIETIES INTO INHIBITORS OF 3-DEHYDROQUINATE SYNTHASE

Zinc: A Biological Metal

Among transition elements in biology, zinc is second only to iron in abundance. It is present in and indispensable to more than 300 proteins[58] representing all classes of enzymes categorized by the International Union of Biochemsitry (oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases). Zn(II)-metalloproteins participate in a wide variety of biological processes comprising gene replication and expression as well as protein biosynthesis and degradation.

Without speculating on the course of natural selection, but rather focusing on zinc's intrinsic chemical properties, zinc is well suited to play such an important role in enzyme catalysis.[59] (1) Zinc(II) ion has a completely filled *d* shell with 10 *d* electrons. As a result, Zn(II) has no ligand field stabilization energy when coordinated by ligands in any geometry. Its coordination number (nearly always 4 or 5 in proteins) and stereochemistry are entirely set by ligand charge and size. (2) Divalent zinc is inert to oxidoreductants present in the biological environment. It cannot generate radicals by itself. (3) Zn(II) is a borderline hard-soft acid. It can interact strongly with a variety of ligands including sulfur from cysteine (Cys), nitrogen from histidine (His), and oxygen from glutamate (Glu), aspartate (Asp), and water. (4) Its charge to radius ratio endows Zn(II) with the Lewis acidity necessary to polarize carbonyl groups and promote ionization of water at neutral pH. (5) Zn(II) readily undergoes ligand exchange and coordination geometry transformation. Consequently, biological Zn(II) is a redox-stable, coordinately flexible, substitutionally labile, stereochemically adaptable, Lewis-acidic metal center able to bind and activate substrate and then release product rapidly.

Structural analysis of zinc enzymes, in combination with enzymatic function studies, has improved our understanding of the interactions between the protein and zinc

that allow zinc to orchestrate a multiplicity of chemical reactions critical to life processes. Three types of zinc-binding motifs have been recognized so far: catalytic, cocatalytic, and structural.[58] In catalytic sites zinc normally forms complexes with any three nitrogen, oxygen, and/or sulfur donors of His, Glu, Asp, and Cys in the binding frequency His >> Glu > Asp > Cys. Water or substrate provide the fourth ligand to the catalytic zinc. They are activated for ionization, polarization, or displacement by ligands surrounding zinc. Cocatalytic zinc sites occur in multi-metal zinc enzymes where two or three zinc atoms are in close proximity with two of the zinc sites bridged by a single amino acid residue. Aspartate predominates in cocatalytic zinc sites where the frequency is Asp > His > Glu. Water and Asp have been found to be the bridging ligands and facilitate enzymatic transformations. Structural zinc often, but not exclusively, stabilizes the quaternary structure of proteins. Cysteine and histidine ligands are usually involved in structural zinc sites though other ligands such as aspartate have also been identified.

DHQ Synthase as a Metalloenzyme

DHQ synthase has been demonstrated to contain one tightly bound divalent metal cation per monomer for enzyme activity.[13a] On the basis of the greater bioavailability of Zn(II), it is likely that DHQ synthase is naturally a Zn(II)-metalloenzyme.[60] The catalytic role of the metal cation follows from a number of observations: (1) the enzyme rapidly loses activity upon incubation with EDTA, giving rise to a stable but catalytically inactive apoenzyme; (2) activity is fully restored by reconstitution with Co(II) and partially restored with other divalent metal cations including Zn(II); (3) the presence of the substrate DAHP protects the metal cation from sequestration by EDTA; (4) the dissociation rate of NAD depends on the metal cation bound at the active site. A recent study[61] reported that DHQ synthase could also be activated by the trivalent lanthanide cations Eu(III) and Sm(III), whose presence opens a new window for employing spectroscopic techniques[62] to further characterize the mechanism of DHQ synthase.

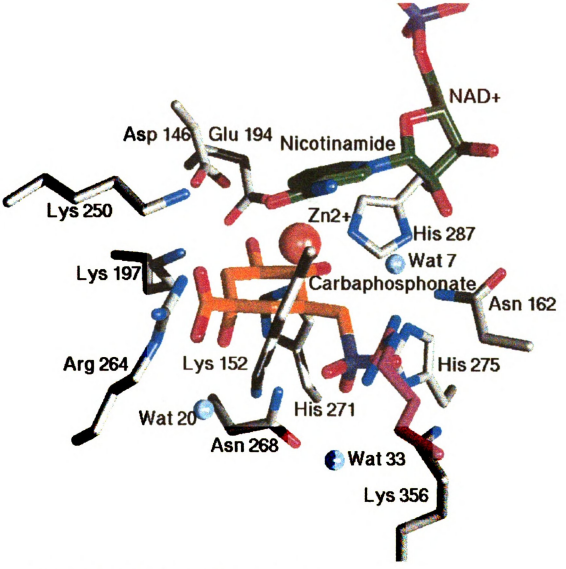


Figure 20. Active site of DHQ synthase.[15]

The catalytic role of metal ions was elucidated by the determination of the crystal structure of DHQ synthase (Figure 20) from *Aspergillus nidulans*.[15] Pentacoordinate Zn(II) was found complexing with Glu 194, His 271, His 287, and the C-3 and C-4 hydroxyl groups of carbaphosphonate **13**, a substrate analogue of DAHP. Through coordination, Zn(II) exhibits mechanistic prowess throughout the multistep conversion of substrate into product (Figure 21). For instance, one role of Zn(II) could be to facilitate proton shift and hydride transfer by polarizing the C-5 hydroxyl of DAHP and the C-5 carbonyl of intermediate **C** during oxidoreductions. This is reminiscent of the role of catalytic zinc in alcohol dehydrogenase (ADH) where zinc binds the substrate and facilitates hydride transfer. Indeed there is a striking similarity between the active sites of DHQ synthase and ADH in terms of the positioning of substrate, zinc and NAD.[15] Another role of zinc could be to clamp the C-4, C-5 edge of intermediate **D** through chelation of C-4 and C-5 hydroxyl groups during the intramolecular aldol condensation. Participation of zinc reinforces the rigidity of the C4, C-5 edge while permitting rotation of the C-5, C-6 carbon-carbon bond. As a result, the stereochemistry at C-1 of DHQ is controlled during carbon, carbon bond formation.

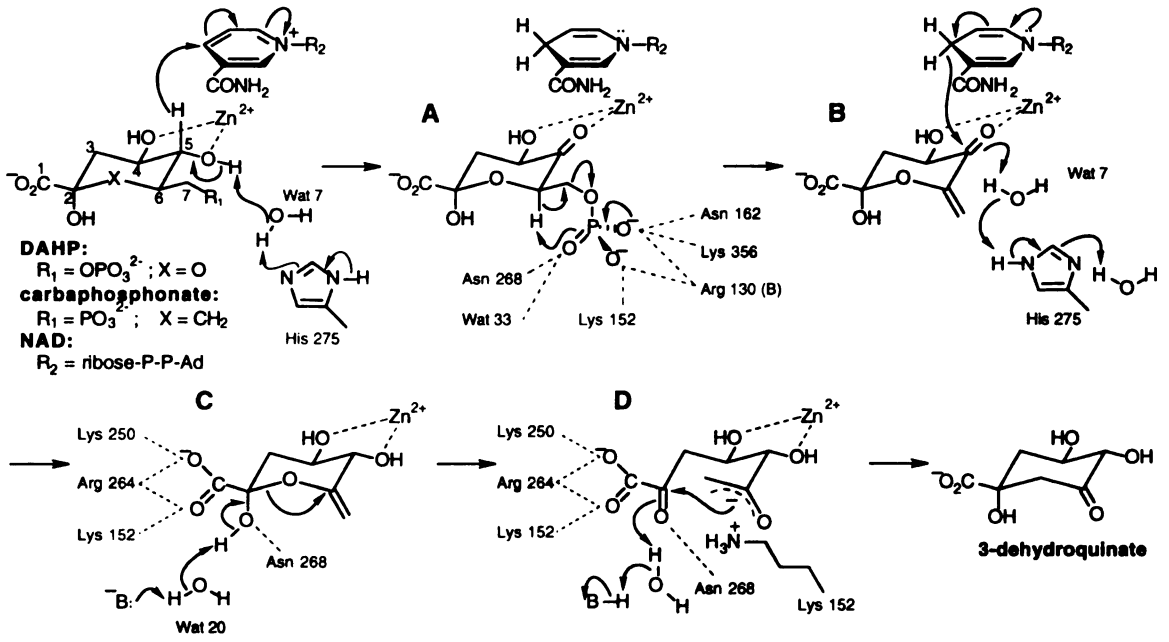


Figure 21. Postulated interactions occurred at the active site of DHQ synthase.[15]

Inhibitor Design

Zinc metalloenzymes have been the focus of medicinal chemistry as targets for inhibition leading to therapeutic effects. Inclusion of zinc-complexing moieties into inhibitor skeletons is one of the successful strategies to enhance inhibitory potency and selectivity. For example, captopril (Figure 22), an orally active agent against hypertension

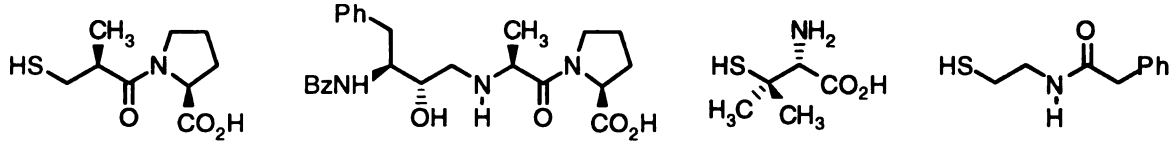


Figure 22. Inhibitors of zinc enzymes and a zinc-chelating agent.

and congestive heart failure, is a potent inhibitor[63] of angiotensin converting enzyme (ACE). It contains a thiol group designed to bind active site zinc in a monodentate fashion. The mercaptan is also the key functional group in the metabolism of captopril, with major metabolites being captopril disulfide and mixed disulfides with thiol-containing amino acids, peptides, and proteins.[64] Substrate-based alcohol inhibitors of ACE such as **44** (Figure 22) apparently employ a backbone secondary amine and an alcohol to complex active site Zn(II) in a bidentate fashion.[65] D-Penicillamine (Figure 22), a degradation product of penicillin, finds use as a therapeutic chelating agent to remove excess copper in treatment of Wilson's disease.[66a] Long-time administration of D-penicillamine has led to zinc deficiency, indicating that the drug also binds Zn(II) avidly.[66b] Class B β-lactamases are metalloproteins which require Zn(II) for their activity and help the organisms to resist the normally lethal action of β-lactam antibiotics. Recently a simple thiol **45** (Figure 22) was reported to be a competitive inhibitor of zinc-dependent β-lactamase of *Bacillus cereus*,[67] presumably due to thiol's coordination with zinc. Reversible inhibition of sheep liver sorbitol dehydrogenase (SDH) by various thiol compounds has been extensively

studied.[68] These inhibitors' potencies were ascribed to the Zn-complexing ability of the thiol group. A major incentive for studying SDH is to define the exact role of this enzyme in metabolism and in the etiology of diabetic complications.

Inspection of the active site of DHQ synthase (Figure 20) indicates that zinc coordinates with the C-3 and C-4 hydroxyl groups of carbaphosphonate **13** with a metal to oxygen distance of 2.3 Å and 2.2 Å, respectively. Consequently incorporation of Zn-complexing groups into substrate analogues seems to be an appealing strategy to enhance inhibitor's binding to the enzyme. Based on the observed trend that sulfur and nitrogen donors are common ligands to Zn(II) in nature, the C-3 hydroxyl group of cyclohexylidene phosphate **46**, a known inhibitor of DHQ synthase, was replaced with a mercapto group and an amino group to give mercaptan **47** and amine **48**, respectively (Figure 23).

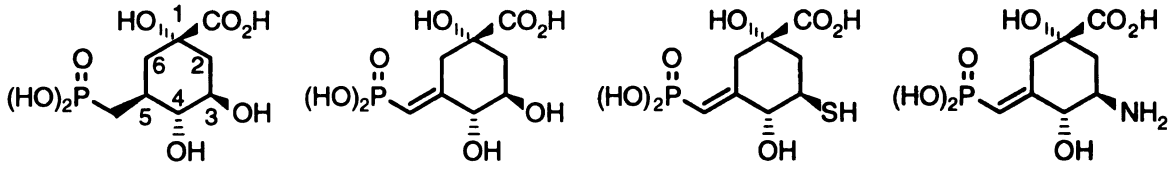


Figure 23. Target molecules **47** and **48**, and related inhibitors **13** and **46**.

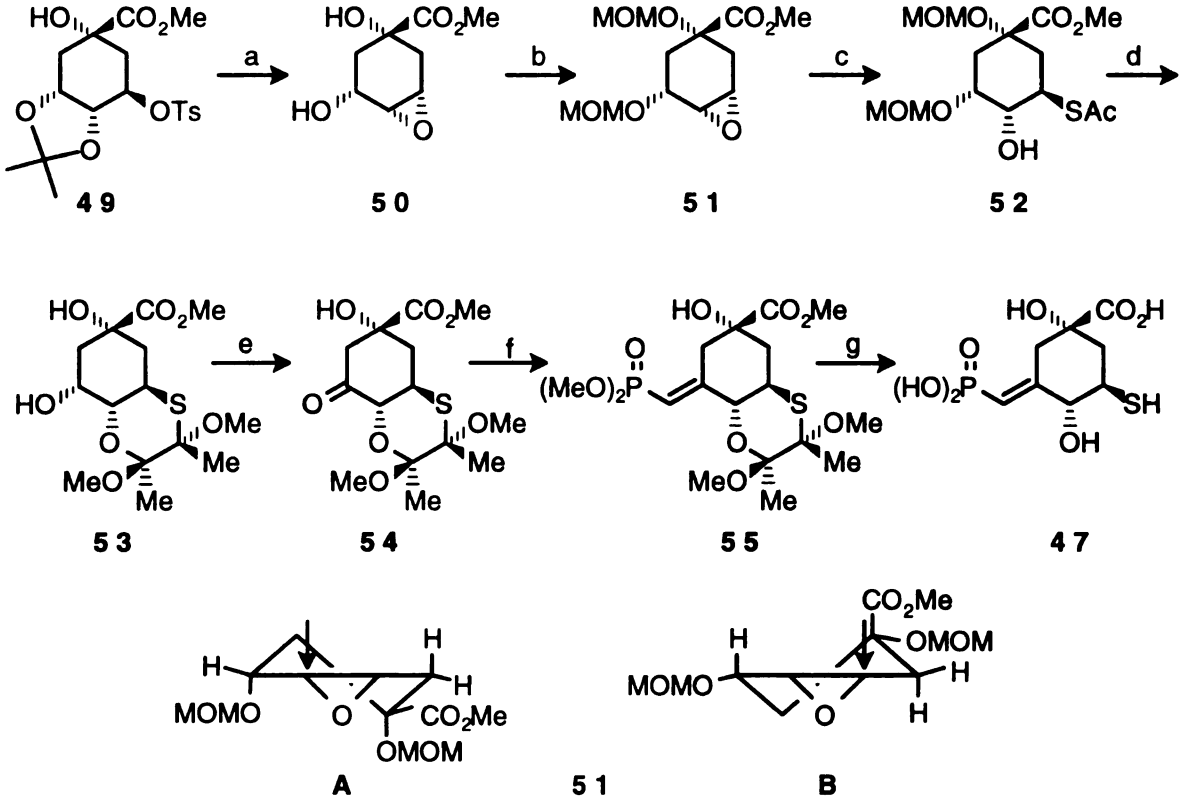
Choice of the cyclohexylidene backbone follows from the ease of synthesis[14b,69] of cyclohexylidene phosphonate **46** and the observation that this DHQ synthase inhibitor penetrates into the cytosol of *E. coli*.[70] Mercapto cyclohexylidene phosphonate **47** is intended to capitalize on the tight binding of Zn(II) with sulfhydryl groups as precedented by the potent inhibition of ACE by captopril and other thiol compounds.[71] Amino cyclohexylidene phosphonate **48** is designed to probe whether amine participation improves bidentate complexation of active site Zn(II) in DHQ synthase as what was observed for ACE inhibitor **44**,[65] which employs amine-based bidentate chelation of Zn(II). Another feature of mercaptan **47** and amine **48** is that substitution at C-3 would

not interfere with C-4 hydroxyl group oxidation at the active site. There are conflicting views in the literature as to the relationship of C-4 hydroxyl group oxidation to the strength of inhibitor binding.[13g,55] The intact C-4 hydroxyl group would allow the observation of enzyme-bound NADH formation if inhibitors **47** and **48** align in the active site of DHQ synthase similar to the substrate DAHP.

Synthesis of Mercaptan **47** and Amine **48**

Synthesis of mercaptan **47** and amine **48** exploited commercially available (-)-quinic acid as a starting material. Both inhibitors **47** and **48** were synthesized from an advanced intermediate MOM-protected epoxide **51** (Figures 24 and 25). The control of stereochemistry at C-3 hinged on regioselective and stereoselective nucleophilic attack on oxiranes. Choice of this strategy follows from previous syntheses of DHQ synthase inhibitors using nucleophilic heteromethylation[37a,72] of oxiranes derived from quinic acid and literature precedents of ring opening reactions occurred to oxiranes derived from shikimic acid.[73,74]

Methoxylmethyl (MOM)-protected epoxide **51** was derived (Figure 24) from tosylate **49**,[73a] readily available from (-)-quinic acid. Tosylate **49** underwent acidic cleavage of the acetonide group, followed by DBU treatment to give epoxide **50**. Free alcohols in **50** were subsequently protected as MOM ethers to produce epoxide **51**. Nucleophilic attack of thioacetic acid on epoxide **51** was then attempted to introduce a thioacetate group at C-3 position in a regioselective and stereoselective fashion. Analysis of the two conformations, conformer A and conformer B (Figure 24), available to epoxide **51** provides a prediction of the preferred regioselectivity during nucleophilic attack. Conformer A of **51** has two axial groups while conformer B has only one axial substituent.



(a) (i) AcOH/H_2O/THF (4:2:1, v/v/v), 70 °C, (ii) DBU, THF, 68%; (b) MOMCl, DIPEA, DMAP, CH_2Cl_2, 68%; (c) AcSH, pyr, 80%; (d) 2,3-butanedione, $HC(OCH_3)_3$, CSA, MeOH, reflux, 53%; (e) TPAP, NMO, 4 Å MS, CH_2Cl_2/CH_3CN, 71%; (f) tetramethyl methylenediphosphonate, *n*-BuLi, THF, -78 °C to rt, 54%; (g) (i) TMSBr, Et_3N, CH_2Cl_2, (ii) NaOH, THF/H_2O, (iii) 1,2-ethanedithiol, TFA/CH_2Cl_2, 0 °C, 91%.

Figure 24. Synthesis of mercaptan **47**.

Ab initio calculation (Spartan, Version 5.0) indicated that conformer B is more stable than conformer A. Electronegative inductive effect of the adjacent C-5 MOM ether favors attack at C-3,[72,73a] even though this requires a transdiaxial attack at the C-3 position in conformer B which will entail an adverse 1,3-diaxial interaction of the nucleophile with the C-1 carbomethoxy group. Indeed, C-3 thioacetate **52** was the major product isolated from the reaction of epoxide **51** with thioacetic acid in pyridine.

Refluxing thioacetate **52** in methanol with 2,3-butanedione, trimethyl orthoformate, and catalytic camphorsulfonic acid (CSA) resulted in (1) removal of two MOM protecting groups, (2) cleavage of the acetate group on the C-3 mercaptan, and (3) selective protection of 3,4-*trans*-mercapto alcohol as a butane 2,3-bisacetal (BBA) over the 4,5-*cis*-diol to afford BBA-protected mercapto alcohol **53**.[41,43] This reaction extended the synthetic application of the BBA group from protection of vicinal diequatorial diols to protection of vicinal diequatorial mercapto alcohols. Oxidation of the C-5 hydroxyl group in **53** with tetrapropylammonium perruthenate[45] (TPAP) and 4-methylmorpholine *N*-oxide (NMO) furnished ketol **54**. Reaction of ketol **54** with the lithium anion of tetramethyl methylenediphosphonate generated mercapto (*E*)-cyclohexylidene phosphonate **55**. Assignment of the stereochemistry for protected mercapto (*E*)-cyclohexylidene phosphonate **55** was based on ^{13}C NMR and the known relationship between $^{3}J(PC)$ and the dihedral angle.[75] Examination of the phosphorus couplings at C-4 (17 Hz) and C-6 (8 Hz) clearly supported the *E*-configuration of the double bond in compound **55**.

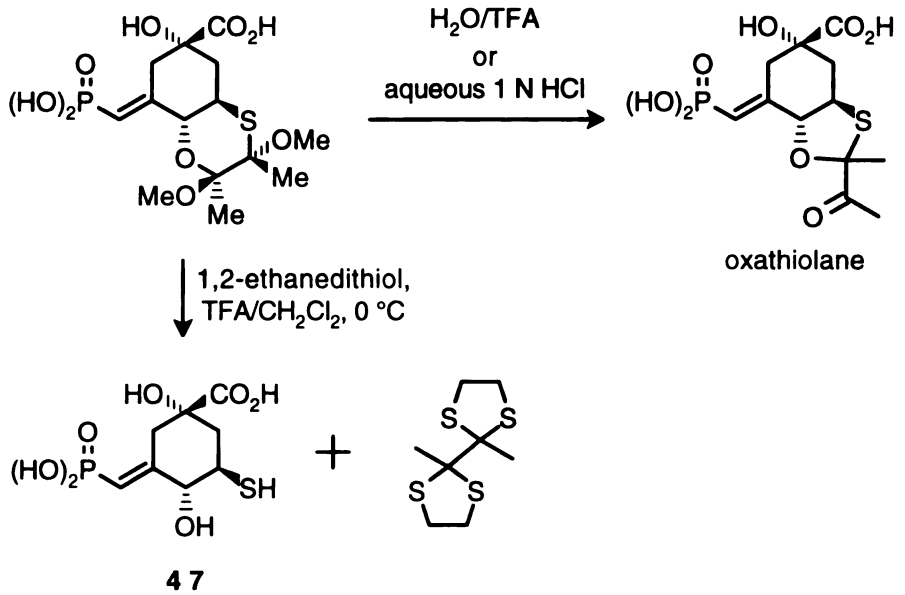
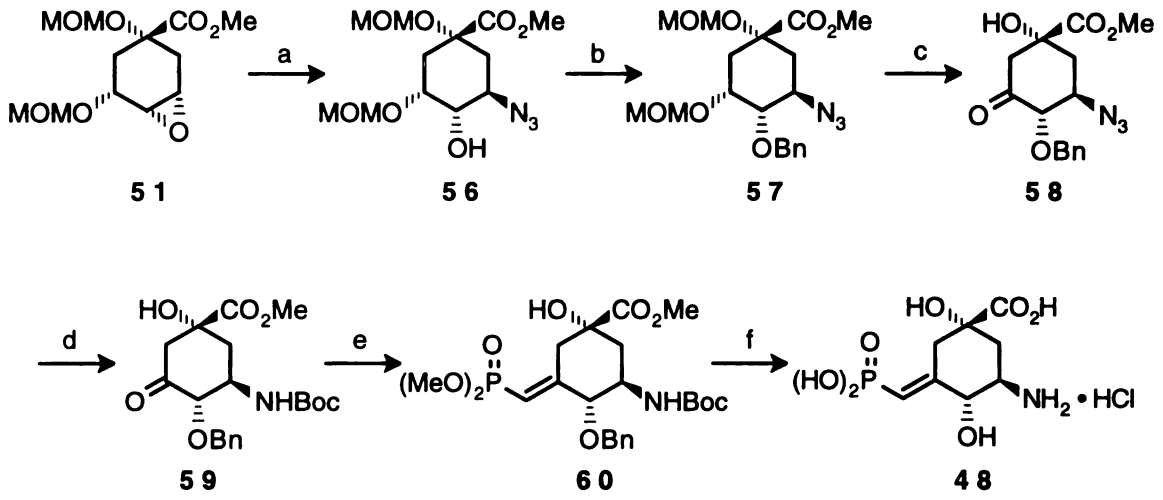


Figure 25. Removal of the BBA protecting group.

Deprotection of **55** began with transesterification of the phosphonate ester using trimethylsilyl bromide (TMSBr) followed by base hydrolysis of methyl ester (Figure 24). Removal of the BBA protective group with aqueous trifluoroacetic acid (H_2O/TFA = 1/20, v:v) or 1 N HCl led to formation of an oxathiolane between 3,4-mercapto alcohol and 2,3-butanedione as the major product (Figure 25). This oxathiolane resists further hydrolysis and decomposes over the time. In contrast, transacetalization with 1,2-ethanedithiol in a mixture of TFA and methylene chloride cleanly produced mercaptan **47** (Figure 25). The success of this deprotection procedure was accredited to the formation of stable 1,3-dithiane rings between carbonyl groups of 2,3-butanedione and 1,2-ethanedithiols, which shifts the equilibium towards the release of the 3,4-mercapto alcohol in mercaptan **47**.

The versatility of MOM-protected epoxide **51** was further demonstrated by the synthesis of amine **48** (Figure 26). Epoxide opening occurred at 75 °C with sodium azide[76] and ammonium chloride in aqueous methanol to give azido alcohol **56** selectively. The selectivity was again attributed to the electronegative inductive effect of the C-5 MOM

group.[72,73a] After protection of the C-4 alcohol as a benzyl ether and subsequent removal of the MOM ethers, the C-5 alcohol was oxidized with KIO_4 and catalytic $RuCl_3$ to give ketol **58**. Reduction of the C-3 azido group of **58** in the presence of di-*tert*-butyl dicarbonate (Boc_2O) and 10% Pd on carbon in EtOAc furnished *N*-Boc protected ketol **59** without deprotection of the benzyl group on the C-4 alcohol. Condensation of ketol **59** with tetramethyl methylenediphosphonate led to amino (*E*)-cyclohexylidene phosphonate **60**. A two-step deprotection procedure comprised of treatment with boron tribromide in methylene chloride and refluxing in 6 N HCl afforded amine **48** as its hydrogen chloride salt quantitatively.



(a) NaN_3, NH_4Cl, MeOH, H_2O, 75 °C, 90%; (b) NaH, BnBr, Bu_4NI, THF, 68%; (c) (i) HCl, MeOH, (ii) KIO_4, $RuCl_3$, K_2CO_3, $CHCl_3/H_2O$, 74%; (d) Boc_2O, EtOAc, 10% Pd/C, 78%; (e) tetramethyl methylenediphosphonate, *n*-BuLi, THF, -78 °C to rt, 38%; (f) (i) BBr_3, CH_2Cl_2, -78 °C to rt, (ii) 6 N HCl, reflux, 100%.

Figure 26. Synthesis of amine **48**.

Evaluation of Mercaptan **47** and Amine **48** as DHQ Synthase Inhibitors

DHQ synthase with Co(II) and NAD as cofactors was purified from an overproducing strain from *E. coli*.[13e] When mercaptan **47** and amine **48** was incubated with DHQ synthase and NAD, instantaneous formation of NADH was observed. This indicates that both inhibitors are positioned in the active site of DHQ synthase similarly to the substrate and other carbocyclic substrate analogues, which are likewise precedented to undergo oxidation with formation of enzyme-bound NADH.

Table 3. Inhibition of DHQ synthase by carbocyclic inhibitors **46-48**.

	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_i (M)	E-NADH formation
46[a]	8.0×10^{6}	2.3×10^{-3}	2.9×10^{-10}	+
47	3.0×10^{5}	1.1×10^{-3}	3.7×10^{-9}	+
48	5.5×10^{5}	4.5×10^{-4}	8.2×10^{-10}	+

[a]See ref 14b.

In vitro evaluation of inhibitors employed spectrophotometric detection at 234 nm of the DHS produced by DHQ-dehydratase-catalyzed dehydration of DHQ. Time-dependent inhibition was observed for both inhibitors. A series of progress curves were recorded and fitted to obtain the apparent first-order rate constant (k_{obsd}) for loss of enzyme activity.[14b,77] A linear plot of k_{obsd} versus inhibitor concentration was observed in both cases, suggesting a single-step, slowly-reversible competitive mechanism.[77,78] Dissociation rate constants (k_{off}) for the inhibitors were determined by incubating the enzyme with a near stoichiometric amount of inhibitor to form an inactive binary enzyme-inhibitor complex and by following the progress curve for recovery of enzyme activity upon 100-fold dilution of the binary complex into a high concentration of DAHP.[14b,77] The

association rate constants (k_{on}) were then calculated from equation 1 based on the known values of K_m, k_{off}, and k_{obsd} with corresponding substrate and inhibitor concentrations [S] and [I], respectively. Inhibition constant (K_i) of the inhibitors was then derived from equation 2. Dissociation rate constants, association rate constants, and inhibition constants for cyclohexylidene phosphonate **46**,[14b] mercaptan **47**, and amine **48** are provided in Table 3. Amine **48** (K_i = 8.2 x 10^{-10} M) is a 4.5-fold more potent inhibitor than mercaptan **47** (K_i = 3.7 x 10^{-9} M). However, neither is as potent as the parent cyclohexylidene phosphonate **46** (K_i = 2.9 x 10^{-10} M), the second most potent inhibitor of DHQ synthase thus far identified.[14b]

$$k_{on} = [(k_{obsd} - k_{off}) \times (1 + [S]/K_m)]/[I] \qquad (1)$$

$$K_i = k_{off}/k_{on} \qquad (2)$$

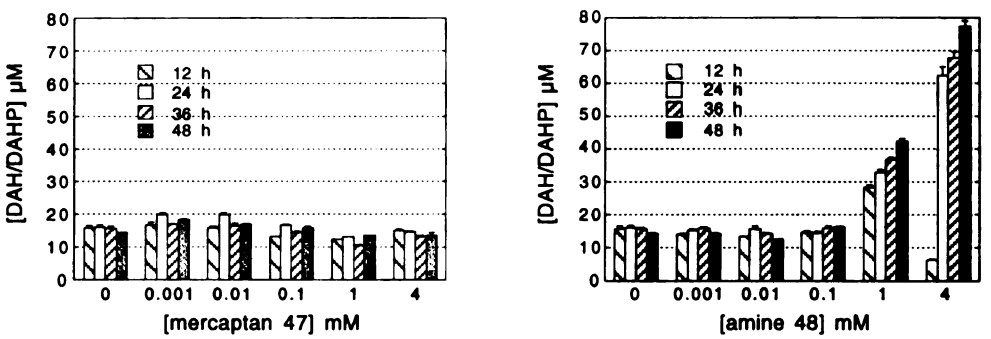


Figure 27. *In vivo* inhibition of DHQ synthase by mercaptan **47** and amine **48**.

In vivo inhibition of DHQ synthase was also evaluated. One approach began with the evaluation of whether mercaptan **46** or amine **47** is capable of penetrating into a microbe's cytosol (Figure 27). *E. coli* K-12 cells were grown in rich LB medium, harvested, and then resuspended in minimal M9 salts medium containing glucose and mercaptan **46** or amine **47**. At timed intervals, 3-deoxy-D-*arabino*-heptulosonic acid (DAH) and DAHP accumulation in the culture supernatant was assayed using the

thiobarbituric acid assay.[79] Previous studies in this group indicated that an indicator of *in vivo* inhibition of DHQ synthase is the accumulation of DAH in culture supernatant.[70] No DAH accumulation was observed for mercaptan **46**. However, increasing DAH accumulation was observed with increasing concentrations of amine **47**. A similar trend was also observed for cyclohexylidene phosphonate **45**.[70] These experiments established that amine **47** as well as its parent **45** can penetrate into *E. coli.* cytosol and inhibit DHQ synthase while mercaptan **46** fails to permeate bacterial membrane. It should be noted that the tendency of thiol groups to be oxidized to disulfides might have complicated the *in vivo* testing of mercaptan **46**.

Another approach to examining in vivo inhibition of DHQ synthase entailed impregnating discs with varying concentrations of mercaptan **46** or amine **47**. Then these discs were applied to the surface of two types of solid agar-containing medium which has had an aliquot of an *E. coli* K-12 culture freshly applied to its surface. One solid medium contained miminal M9 salts and glucose only while the other also contained supplements of L-phenylalanine, L-tyrosine, L-tryptophan, *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and 2,3-dihydroxybenzoic acid. In vivo inhibition of DHQ synthase would lead to no-growth zones around the discs on unsupplemented solid medium, and such effect would be reversed to certain extent on the supplemented medium. No growth inhibition zones were observed when mercaptan **46** (up to 1.6 mg/disc) or amine **47** (up to 4.0 mg/disc) was tested. These experiments did not bode well for medicinal applications of either compounds. Further modifications of both mercaptan **46** and amine **47** are required to enhance their capability of permeating into bacterial cyctosols.

Insights Gleaned from Inhibition of DHQ Synthase by Mercaptan **47** and Amine **48**

At first glance, the inferior inhibition exhibited by mercaptan **47** relative to amine **48** is a conundrum. The simplest answer would be the difference in the size of metal-complexing groups with the van der Waals radius. The radius of the thiol group is

estimated to be 1.8 Å and that of an amino group is about 1.5 Å relative to the 1.4 Å van der Waals radius of an alcohol. If the acitive site of DHQ synthase can not accommodate the bigger thiol group, mercaptan **47** stands no chance being a potent inhibitor. However, based on its k_{on} value and the instantaneous formation of enzyme-bound NADH when treating DHQ synthase with mercaptan **47**, mercaptan **47** seems to fit the active site of DHQ synthase.

Another possible explanation for the lack of improvement in inhibition by mercaptan **47** relative to amine **48** may arise from different intrinsic binding preference exhibited by Co(II) and Zn(II). Since DHQ synthase from *E. coli* is unstable with Zn(II) ligated at the active site, Co(II) was used as the cofactor in the current study. A mercapto group may not complex with Co(II) as strongly as an amino group. However, this does not reconcile with the Irving-Williams effect, which states that softer metal ions, such as Co(II) and Zn(II), favor ligands S>N>O.[80] Indeed, stability constants[80,81] of some oxygen, nitrogen, and sulfur chelates to Co(II) and Zn(II) are within the same range and follow the same stability trend. Since Zn(II) is spectroscopically silent, specific replacement of zinc ion by transitional metal ions is desired in studying solution chemistry. In practice, Co(II) was frequently employed as the substituent for Zn(II) due to their similarity in size and coordination properties.

The mercaptan ionization state is the remaining factor to consider as a potential explanation for the lack of improvement by mercaptan **47**. Cysteine and other thiol-containing agents bind Zn(II) through their thiolate ion. The deprotonation pH range for the thiol group in cysteine is between 8.5 and 9.5. For all the reported complexation between cysteine residues and zinc ions, ionization of the sulfhydryl groups is a necessary step. Bearing a phosphonic acid and a carboxylic acid, mercaptan **47** is expected to exist as a trianion at neutral pH. Further deprotonation of the C-3 thiol group to a tetraanion would be hampered as a consequence of the accumulation of negative charge on the molecule. Even after mercaptan **47** binds at the active site of DHQ synthase, there is no

basic residue in proximity to remove the acidic proton from the thiol group as judged from the crystal structure of DHQ synthase. Consequently the thiol group of mercaptan **47** might remain protonated at the enzyme active site. Compared with mercaptan **47**, protonated amine **48** is more likely to dissociate at pH 7. Although the pK_a of an ammonium group is around 10, dissociation of the C-3 ammonium group generates a trianion with negative charges on carboxylate and phosphonate. The aminophosphonic acid analogues of aspartic acid exist in a similar trianionic state and coordinate as tridentate ligands to transition metals.[82] The availability of the trianionic state to amine **48** might explain its enhanced inhibitory effect over mercaptan **47**. Likewise, the superiority of cyclohexylidene phosphonate **46** over mercaptan **47** and amine **48** is likely due to its ease to get access to the trianionic state.

Designing DHQ Synthase Inhibitors

An alcohol such as ethanol has an apparent pK_a of 16 and thus no tendency to dissociate a proton under normal physiological conditions. One aspect of alcohol oxidation catalyzed by alcohol dehydrogenase is that the enzyme facilitates the removal of the proton from the hydroxyl group. It is thought that zinc ion drastically lowers the pK_a of the alcohol so that proton abstraction readily occurs at the active site.[83] An important role proposed for the catalytic zinc atom in alcohol dehydrogenase entails stabilization of the alcoholate through complexation during the rate-limiting hydride transfer step.[83b]

The aforementioned hypothesis about the importance of available ionization state of an inhibitor may have ramifications relevant to designing DHQ synthase inhibitors. For example, mercaptan **61** (Figure 28) is expected to liberate the metal-complexing ability of the C-4 thiol group at the active site and thus to be a better inhibitor than mercaptan **47**. At neutral pH, mercaptan **61**, like mercaptan **47**, will presumably remain in its trianionic state without dissociation of the thiol group. However, once bound by the enzyme, thiol **61** might take advantage of the existing machinery at the active site of DHQ synthase and

dissociate to form the C-4 thiolate. The first step of the mechanism (Figure 21) of DHQ synthase involves hydride transfer from C-5 of DAHP to C-4 of the NAD nicotinamide moiety. In concert, a proton is lost from the C-5 hydroxyl group of DAHP. This proton could be relayed to a neighboring water molecule and further to a histine residue (His 275). Since NAD-coupled oxidation of a secondary thiol group to the corresponding thioketone has not been reported, it is unlikely that the C-4 thiol group of mercaptan **61** will be oxidized at the active site. However, the pK_a of C-4 thiol might be lowered thanks to the complexing zinc ion, and the same proton-shuffling system at the active site could be employed to deprotonate the C-4 thiol group of mercaptan **61**. The resulting thiolate would then ligate with the juxtaposed zinc ion. Potent inhibition of DHQ synthase might occur if this deprotonation step is allowed and irreversible. In addition, comparing inhibitory effects of mercaptans **47** and **61** might provide insights into another controversy: the importance of C-4 alcohol oxidation to inhibitor binding.[13g,55] Based on the previous argument, zinc complexation might enhance deprotonation of the ammonium salt of amine **62**. Amine **62** would then be expected to be a stronger inhibitor than amine **48**.

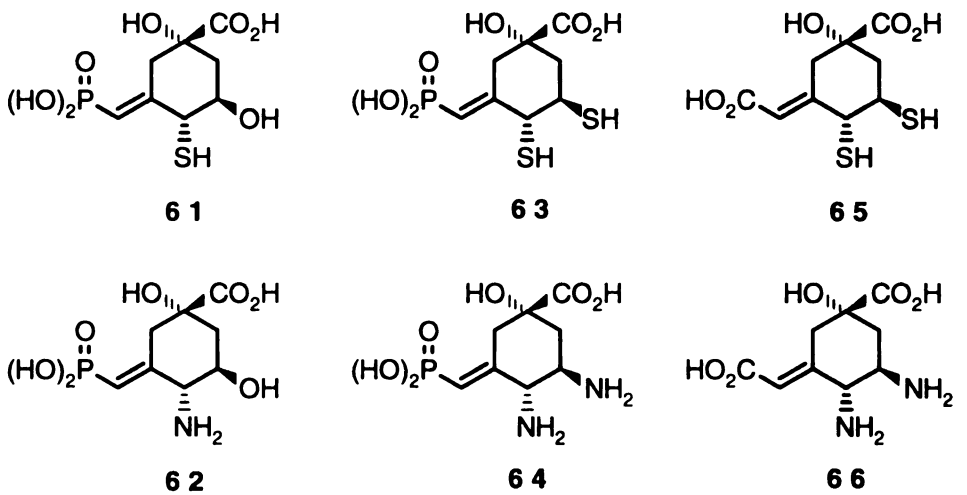


Figure 28. Potential inhibitors of DHQ synthase.

Wu and Walsh reported that simple dithiols such as dithiothreitol are time-dependent inactivators of a Zn(II)-dependent D,D-dipeptidase which is responsible for vancomycin resistance.[84] Since diamino and dimercapto ligands are earmarked as good chelating agents for metal ions, evaluation of compounds **63-66** (Figure 28) as possible inhibitors of DHQ synthase seems warranted. Extent of ionization is again a road block for dimercapto compounds. Even with the help of active site residues, it is questionable that both thiol groups of compound **63** will dissociate to afford the di-thiolate and add two more charges on the inhibitor. However, replacement of the dianionic phosphonate group in **63** with a monoanionic carboxylate group in **65** would render compound **65** more liable to thiol dissociation. If both thiols are readily ionized at the active site, tight binding of Zn(II) by dimercapto compound **66** will result in potent inhibition. A structurally-related compound, 2,3-dimercaptosuccinic acid (DMSA or succimer) is clinically used as an orally-active, chelating agent for removal of heavy metals such as lead and cadium.[84] In a clinically controlled trial in which half the subjects received DMSA and the other half received a placebo, DMSA increased the urinary excretion of lead, copper, mercury, and, to a lesser degree, zinc in the first 24 hours.[85]

By the same token, the extent that diamino compounds **64** and **66** exist in their respective trianionic forms at the active site of DHQ synthase will determine their inhibitory effects. Compared with dithiol compounds, diamine analogues might have the leverage to fully dissociate at the acitive site of DHQ synthase. Hence, they have a better chance to elevate their inhibition potencies with respect to their monoamine analogues. Moreover, compound **66** could be viewed as an unnatural dipeptide. It would be entertaining to study its permeability through cell membranes if it turns out to be a potent inhibitor of DHQ synthase.

In summary, mercaptan **47** and amine **48** were synthesized via regioselective and stereoselective epoxide opening reactions of a common intermediate. They are nanomolar-level inhibitors of DHQ synthase. Compared with the parent compound **46**, neither

mercaptan **47** nor amine **48** exhibited enhanced binding to the enzyme. This might reflect the reluctance of mercaptan **47** and amine **48** to unleash their metal-complexing groups at the active site. Results from this study could help refine the necessary ingredients of a recipe for DHQ synthase inhibition aiming at the metal ion cofactor. Furthermore, incorporation of amino and mercapto groups into the inhibitor skeleton might be used as an anchor to construct conjugates able to penetrate into the cytoplasm of cells more readily. In this respect, further modification of mercapto **47** and amine **48** seems justified.

CHAPTER 4

MIP SYNTHASE: A MODEL OF ENZYME EFFICIENCY AND ENZYME OPPORTUNISM?

Is MIP Synthase an Efficient Enzyme?

Conversion of D-glucose 6-phosphate into *myo*-inositol 1-phosphate (MIP) is catalyzed by MIP synthase.[21] It is the first step of de novo biosynthesis of *myo*-inositol, which is a cell membrane component and secondary messenger.[16] Employing β-nicotinamide adenine dinucleotide (NAD) as a cofactor, MIP synthase catalyzes an alcohol oxidation, enolization, intramolecular aldol condensation, and carbonyl reduction during each turnover (Figure 29). There is no net consumption of NAD during the whole catalytic cycle.

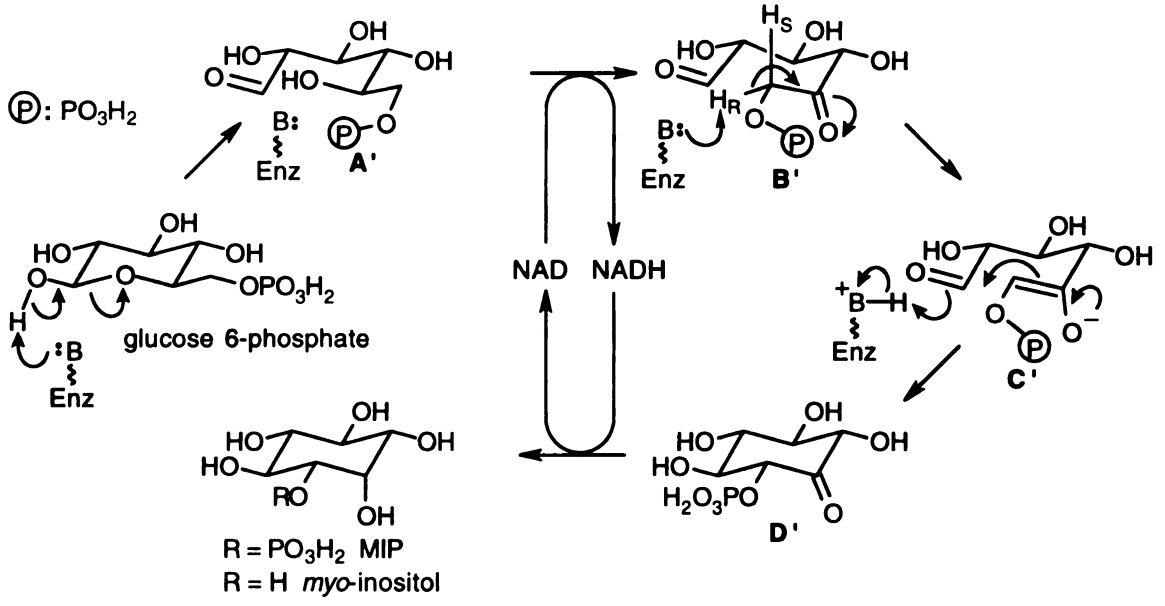


Figure 29. Mechanism of MIP synthase proposed by Floss.[32]

In principle, NAD-coupled oxidation only works on the acyclic form of D-glucose 6-phosphate which corresponds to less than 0.4% of the total population of D-glucose

6-phosphate in solution.[87] To pave the way for the oxidation, MIP synthase either abstracts the acyclic form of D-glucose 6-phosphate from the solution or first binds the cyclic form of D-glucose 6-phosphate then opens the pyranose ring prior to oxidation. To explore these opportunities, acyclic and cyclic analogues of D-glucose 6-phosphate would be ideal probes for the substrate specificity of MIP synthase.

In order to explain the stereospecificity of the deprotonation step, Floss[32] has proposed a mechanism for MIP synthase (Figure 29) wherein a single active site basic residue could catalyze the ring opening of the β-pyranoside of D-glucose 6-phosphate bound at the active site, the stereospecific loss of the 6-*pro-R* hydrogen of active site-generated 5-keto-D-glucose 6-phosphate (**B'**, Figure 29), and the subsequent intramolecular aldol condensation. The formulation by Floss of a single base and its conjugate acid to mediate all the nonredox steps separating substrate from product presents a model of a supremely efficient catalyst. However, one of the most intriguing discoveries[13h,i] made with DHQ synthase was the role that the phosphate monoester of substrate DAHP played during its own elimination. Could MIP synthase be as opportunistic as it is efficient and exploit the phosphate monoester of its own substrate as the base/conjugate acid to mediate the intramolecular aldol condensation? To explore this opportunity, a series of (*E*)- and (*Z*)-vinylphosphonates were examined for their respective inhibitory potencies.

Synthesis of Cyclic Substrate Analogues

To probe the preference of MIP synthase for the cyclic versus acyclic form of D-glucose 6-phosphate, a series of analogues (Figure 30) mimicking both forms were synthesized. Compounds **67-70** (Figure 30) were chosen as acyclic substrate analogues. Compared with D-glucose 6-phosphate, 1,5-anhydro-D-glucose 6-phosphate **67** is locked in an oxane ring and devoid of the anomeric hydroxyl group. 5-Thio-D-glucose

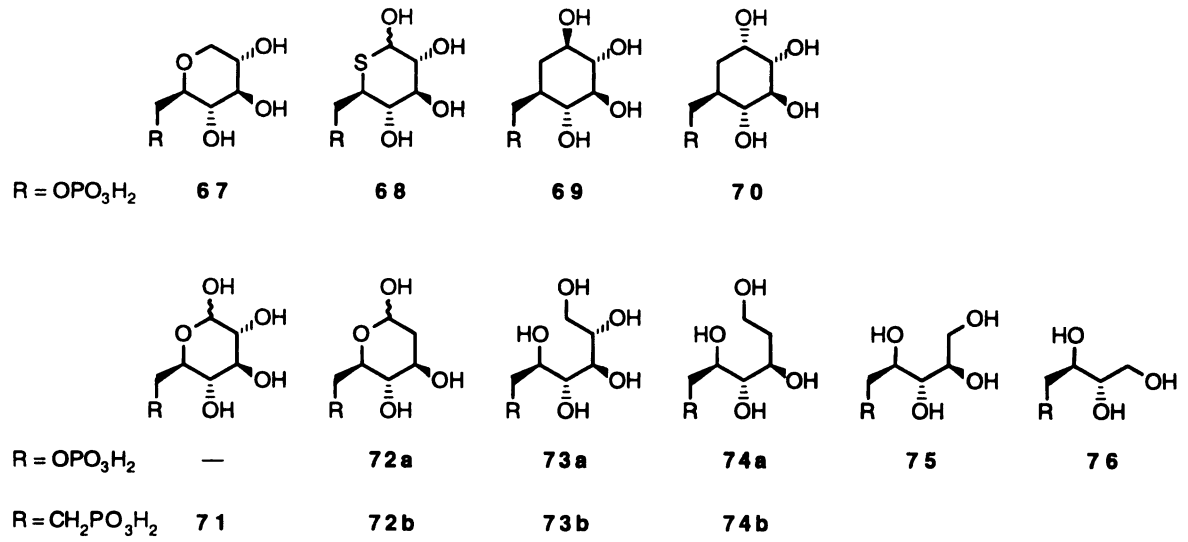
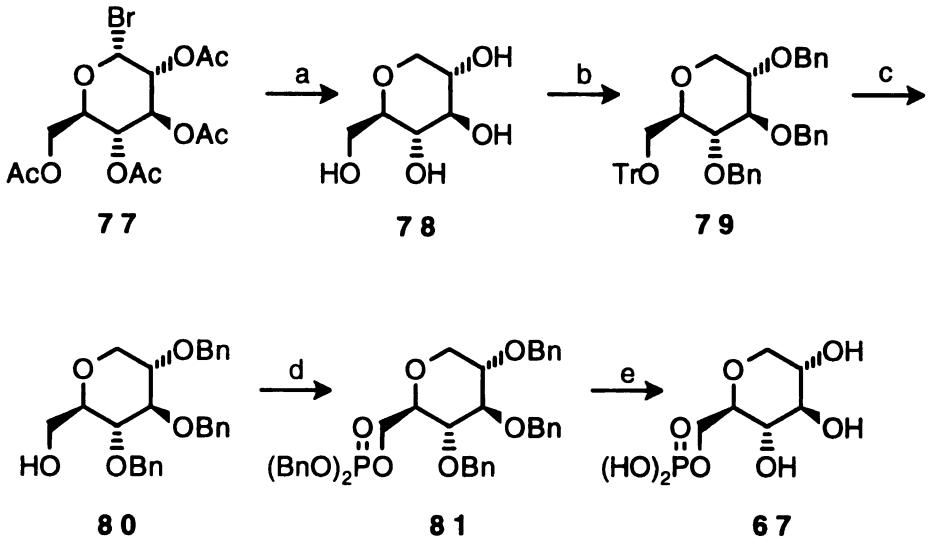


Figure 30. Cyclic and acyclic substrate analogues **67-76**.

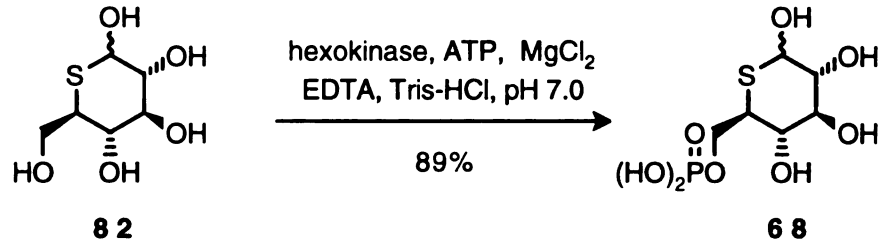
6-phosphate **68**[88] differs from D-glucose 6-phosphate in replacement of the pyranosyl oxygen with sulfur. Carbocyclic α-D-glucose 6-phosphate **69** and carbocyclic β-D-glucose 6-phosphate **70** substitute the pyranosyl oxygen of D-glucose 6-phosphate with a methylene group while mimicking the orientations of the anomeric hydroxyl group of the substrate respectively. If MIP synthase binds the cyclic form of substrate selectively, analogues **67-70** could be inhibitors of MIP synthase. Acyclic substrate analogues include compounds **73-76** (Figure 30). D-Glucitol 6-phosphate **73a** and its homophosphonate analogue **73b** as well as 2-deoxy-D-glucitol 6-phosphate **74a** and its homophosphonate analogue **74b** closely parallel the open chain form of D-glucose 6-phosphate. D-Arabinitol 5-phosphate **75** and D-erythritol 4-phsophate **76** are truncated acyclic analogues. If MIP synthase binds the acyclic form of D-glucose 6-phosphate selectively, inhibition of MIP synthase by compounds **73-76** would be possible.



(a) (i) $LiAlH_4$, Et_2O, C_6H_6, reflux, (ii) IR-120 (H^+), (iii) IR-120 (OH^-), 48%; (b) (i) TrCl, pyr, 75 °C, 85%; (ii) NaH, THF, BnBr, DMF, 88%; (c) HCO_2H, Et_2O, 60%; (d) *n*-BuLi, tetrabenzyl pyrophosphate, THF, -78 °C to 0 °C, 85%; (e) H_2, 10% Pd/C, THF/H_2O (2:1, v/v), 95%.

Figure 31. Synthesis of 1,5-anhydro-D-glucose 6-phosphate **67**.

Synthesis of 1,5-anhydro-D-glucose 6-phosphate **67** (Figure 31) began with $LiAlH_4$ reduction of glucopyranosyl bromide **77**.[89] Although radical debromination[90a] using one-pot addition of Bn_3SnH can also give the same 1-deoxy-glucose derivatives, the transient radical intermediate involved might rearrange to afford 2-deoxy glucose derivatives,[90b] which after phosphorylation would generate a known potent inhibitor *vide infra* and complicate kinetic analysis of MIP synthase inhibition. Selective protection of the C-6 primary alcohol as a trityl ether and subsequent perbenzylation furnished fully protected 1,5-anhydro-D-glucose **79**. Acidic hydrolysis unmasked the C-6 hydroxyl group which was phosphorylated using tetrabenzyl pyrophosphate. Palladium-catalyzed hydrogenolysis of **81** generated 1,5-anhydro-D-glucose 6-phosphate **67**.



82 **68**

Figure 32. Synthesis of 5-thio-D-glucose 6-phosphate **68**.

5-Thio-D-glucose 6-phosphate **68** was constructed by an enzymatic route[88a,91] (Figure 32). Selective phosphorylation of 5-thio-D-glucose **82** by ATP in aqueous solution was catalyzed by yeast hexokinase at 37 °C. TLC indicated that the reaciton was complete in 4 days. Compound **68** was purified by anion exchange chromatography. Analogues **69-75** were either purchased from commercial sources or synthesized by Drs. Jirong Peng or Marie Migauld.[29,92]

Enzymology of Cyclic and Acyclic Analogues: The Importance of an Oxidizable Center

MIP synthase was purified from an overproducing *E coli.* strain.[29,92] Assay of MIP synthase activity was carried out in a 50 mM Tris-HCl buffer (pH 7.7) containing 2 mM ammonium chloride, 0.2 mM dithiothreitol and 1 mM NAD at 37 °C. At timed intervals, aliquots were withdrawn, quenched with trichloroacetic acid, and assayed by a colorimetric method[93] for inorganic phosphate selectively released from MIP. This method is suitable for most analogues except for 5-thio-D-glucose 6-phosphate **68**, which decomposed and gave interfering inorganic phosphate under the assay conditions.[94] To circumvent this problem, an enzymatic assay was adopted which employs *myo*-inositol monophosphatase[92,95] to selectively dephosphorylate MIP in the presence of 5-thio-D-glucose 6-phosphate and D-glucose 6-phosphate. Compounds **69-76** were evaluated by Drs. Jirong Peng or Marie Migaud.[29,92] The following discussion includes the relevant data drawn from their studies.

None of the cyclic analogues **67-70** led to any detectable inhibition of MIP synthase. By contrast, acyclic substrate analogues **73-76** invariably were competitive inhibitors of MIP synthase. Similar to literature reports,[27] D-glucitol 6-phosphate **73a** was a moderate inhibitor with an inhibition constant (K_i) of 1.5 x 10^{-4} M. Its nonhydrolyzable mimic, acyclic D-glucitol 6-homophosphonate **73b** (K_i = 1.1 x 10^{-4} M) was an 83-fold more potent inhibitor relative to D-glucose 6-homophosphonate **71** (K_i = 4.9 x 10^{-3} M). Acyclic 2-deoxy-D-glucitol 6-phosphate **74a** (K_i = 2.3 x 10^{-6} M), the most potent inhibitor for MIP synthase, was also more potent than 2-deoxy-D-glucose 6-phosphate **72a** (K_i = 9.1 x 10^{-6} M). The same trend continued in 2-deoxy-homophosphonates series, where 2-deoxy-D-glucitol 6-homophosphonate **74b** (K_i = 5.8 x 10^{-6} M) was a more potent (12 fold) inhibitor than 2-deoxy-D-glucose 6-homophosphonate **72b** (K_i = 7.1 x 10^{-5} M). Truncated acyclic analogues of D-glucose 6-phosphate also demonstrated inhibitory effects on MIP synthase. D-Arabinitol 5-phosphate **75** (K_i = 1.7 x 10^{-5} M) and D-erythritol 4-phosphate **76** (K_i = 4.7 x 10^{-5} M) were competitive inhibitors. D-Erythritol 4-phosphate **76** marked the efficacy of four-carbon chain necessary for inhibitor binding since glycerol phosphate showed no inhibition of MIP synthase.

As expected, acyclic analogues **73-76** were susceptible to redox chemistry akin to the MIP synthase-catalyzed substrate oxidation.[29] When each of the analogues **73-76** was added to a solution of MIP synthase and NAD, a new absorbance at 348 nm was observed, reminiscent of the steady-state formation of NADH when the substrate D-glucose 6-phosphonate was used.[29] Addition of lactate dehydrogenase and pyruvate to the solution containing the binary complex of MIP synthase and the analogue failed to change the new absorbance, indicating that the NADH formed was tightly bound by the enzyme. These results suggested that acyclic analogues **73-76** were positioned in the active site of MIP synthase similarly to the substrate and oxidized with concomitant formation of enzyme-bound NADH. Oxidized acyclic analogues could be viewed as mimics of intermediate **B'** (**B'** Figure 29). The observed correlation between rates of enzymatic oxidation and the

potencies of inhibitors highlighted the impact of an inhibitor's readiness for oxidation on binding to the active site of MIP synthase.[29] Since MIP synthase is reported to strongly interact with oxidized intermediates and pre-oxidized substrate analogues,[29,33,92] it is conceivable that the success of acyclic analogues to inhibit MIP synthase might stem from tight binding of their oxidized forms to the active site of MIP synthase.

The failure of cyclic analogues **67-70** to inhibit MIP synthase corroborates the above hypothesis. 1,5-Anhydro-D-glucose 6-phosphate **67**, carbocyclic α-D-glucose 6-phosphate **69** and carbocyclic β-D-glucose 6-phosphate **70** are locked in six-membered rings and deprived of any possibility to undergo MIP synthase-catalyzed oxidation. 5-Thio D-glucose 6-phosphate **68** is expected to exist in a lower percentage of open chain form and have slower mutarotation rate than D-glucose 6-phosphate if the trend observed in 5-thio-D-glucose/D-glucose series[96] also applies here. Except for conceivable deformation caused by the sulfur atom in the ring, 5-thio-D-glucose 6-phosphate **68** could be regarded as the best mimic of D-glucose 6-phosphate among the analogues tested. If MIP synthase binds the acyclic form of the substrate, compound **68** has an additional advantage over the other cyclic analogue: it can undergo ring-opening slowly and thus be processed further at the active site. Nonetheless, it had no impact on the activity of MIP synthase. Since there has been no report about NAD-coupled oxidation of a thiol group into its corresponding thioketone, analogue **68** is considered inert to NAD oxidation. Hence, the lack of inhibition of MIP synthase observed for 5-thio-D-glucose 6-phosphate **68** is likely due to its inability to be oxidized by NAD. The absence of an oxidizable center in **68** led to substantially weak interactions with the acitive site. This is consistent with the premise that an oxidized or oxidizable center in a substrate analogue is indispensable to tight binding to MIP synthase.

The drastically different kinetic profiles of cyclic and acyclic analogues of D-glucose 6-phosphate contradicts the hypothesis that MIP synthase selectively binds the cyclic form of D-glucose 6-phosphate. Instead, MIP synthase seems to bind the acyclic form of the

substrate selectively. Considering the exceptional high mutarotation rate[97] of D-glucose 6-phosphate, it is unlikely that the ring-opening step of the pyranose ring would limit the supply of the acyclic form of D-glucose 6-phosphate in solution. Hence, MIP synthase can be viewed as an enzyme selectively binding the acyclic form of the substrate from a large excess of the cyclic form of the substrate.

Examining the Stereochemistry of the Deprotonation Step

Stereospecificity is a hallmark of enzymatic processes. Take, for instance, the enolization step during conversion of D-glucose 6-phosphate into *myo*-inositol 1-phosphate catalyzed by MIP synthase (Figure 29). Experiments[32] with stereospecifically tritiated substrate showed that MIP synthase catalyzes stereospecific loss of the 6-*pro-R* hydrogen of intermediate **B'** (Figure 29). The array of inhibitors identified for MIP synthase *vide supra* provided another window to follow the stereochemical course of this transformation. For example, previous studies showed that when 2-deoxy-D-glucitol 6-phosphate **74a**, the most potent inhibitor of MIP synthase, was incubated with the enzyme in the presence of NAD, enzyme-bound NADH was formed as evidenced by an increase in absorption around 340 nm.[29] Presumably, oxidized analogue 2-deoxy-5-keto-D-glucitol 6-phosphate **83** (an analogue of intermediate **B'**) was generated concurrently with NADH at the active site (Figure 33). Although oxidized analogue **83** could not complete the aldol condensation step analogous to the enzymatic pathway, it might undergo the stereospecific deprotonation step in the same fashion as intermediate **B'**. Provided the base responsible for proton removal can exchange its proton(s) freely with the solvent and the enolization and NAD-dependent oxidation steps are reversible, incubation of analogue **74a** with MIP synthase in D_2O could lead to the exchange of 6-*pro-R* hydrogen of **74a** with deuterium (Figure 33), a phenomenon particularly suited for ^1H NMR spectroscopy.

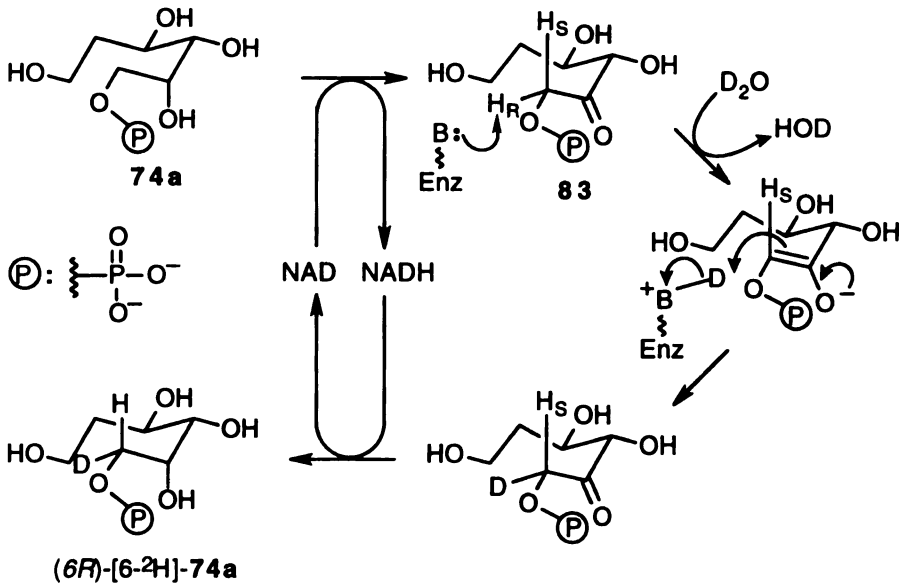


Figure 33. Enzyme-catalyzed stereospecific deuterium exchange.

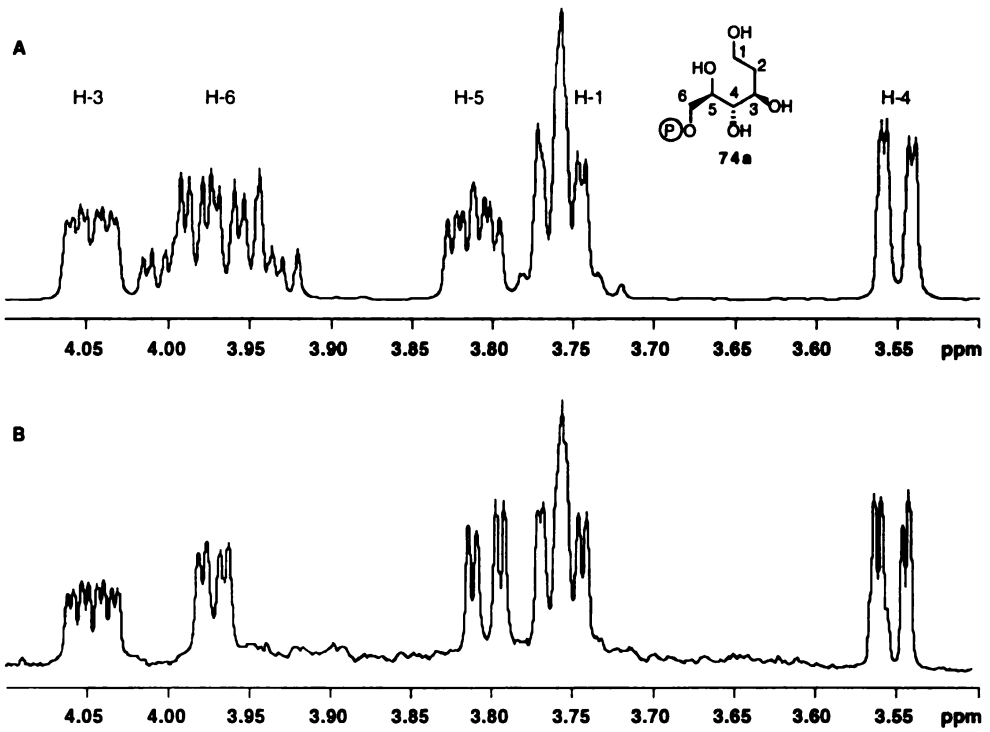


Figure 34. Partial 500 MHz ^{1}H NMR spectra of 2-deoxy-D-glucitol 6-phosphate **74a**.
Panel A: obtained at pD 7.7 in D_2O before treatment with MIP synthase.
Panel B: obtained at pD 7.7 in D_2O after incubation with MIP synthase.

Sodium borohydride reduction of commercially available 2-deoxy-D-glucose 6-phosphate and usual work-up yielded 2-deoxy-D-glucitol 6-phosphate **74a**. Assignment of the 500 MHz ^{1}H NMR spectrum of analogue **74a** (panel A, Figure 34) followed from a precedented NMR study[98] of 2-deoxy-D-glucitol (2-deoxy-D-arabino-hexitol). Compared with H-6*S* (δ 3.99, $J_{5,6S}$ = 2.6 Hz), the resonance for H-6*R* of analogue **74a** has an upfield chemical shift (δ 3.95) and a larger coupling constant with respect to H-5 ($J_{5,6R}$ = 5.2 Hz). This is in accordance with the trend observed in the ^{1}H NMR spectrum of 2-deoxy-D-glucitol (2-deoxy-D-arabino-hexitol). The resonance of H-5 (δ 3.81) exists as a ddd owing to couplings with H-6*R*, H-6*S*, and H-4 ($J_{5,6R}$ = 5.2 Hz, $J_{5,6S}$ = 2.6 Hz, and $J_{4,5}$ = 8.5 Hz).

2-Deoxy-D-glucitol 6-phosphate **74a** was incubated with MIP synthase at 37 °C in a D_2O-exchanged buffer containing Tris-CF_3CO_2D (50 mM, pD 7.7), NAD (10 mM), DTT (0.2 mM) and ND_4Cl (2 mM). After 24 hours, the changes in NMR revealed that one of the prochiral hydrogens on C-6 of analogue **74a** was stereospecifically deuterated. Based on coupling constants and chemical shifts, it is the H-6*R* proton whose signal disappeared with concomitant simplification of resonance for H-6*S* and H-5 to doublets of doublets (panel B, Figure 34). Pertinent resonance includes H-6*S* (δ 3.98, J_{POCH} = 7 Hz, $J_{5,6S}$ = 2.0 Hz) and H-5 (δ 3.80, $J_{5,6S}$ = 2.0 Hz, $J_{4,5}$ = 8.5 Hz). Hence, this NMR experiment provides evidence for the following: (1) the deprotonation step is stereospecific; (2) the first two enzymatic steps, namely oxidation and enolization (Figure 29), are reversible; and (3) the abstracted proton is exchangeable with the solvent.

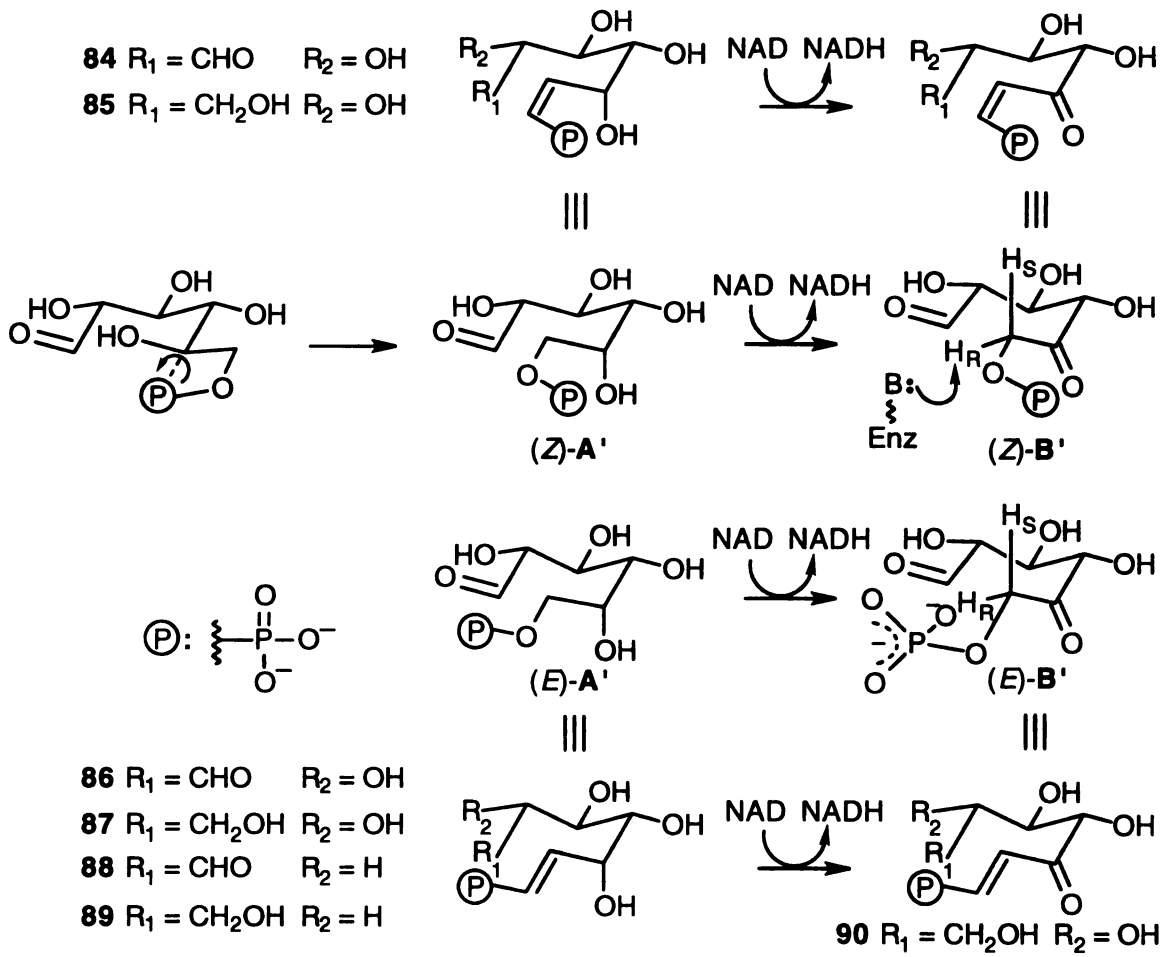


Figure 35. Design of vinylphosphonate inhibitors **84-90**.

(*E*)- And (*Z*)-Vinylphosphonates: Mimicking the Transition States of Intra- and Intermolecular Deprotonation

Floss[32] hypothesized that the β-pyranoside of D-glucose 6-phosphate was bound in a conformation where C-5, C-6, O-6, and phosphorus formed a plane with the phosphorus lying in the extension of the C-4/C-5 axis (Figure 35). After a basic, active-site residue catalyzes ring opening, rotation about the C-4/C-5 axis would lead to conformer (*Z*)-**A'**. Oxidation of the C-5 hydroxyl group would then lead to conformer (*Z*)-**B'** with the 6-*pro-R* hydrogen oriented for removal by the same basic, active-site residue, which later also catalyzes the intramolecular aldol condensation reaction. (*Z*)-Vinylphosphonates **84** and **85** (Figure 35) were designed to mimic the Floss-hypothesized ring-opened conformer (*Z*)-**A'**. In this conformation, adverse steric and electrostatic interactions would be minimized between the basic, active-site residue and the phosphate monoester. A binding pocket for the phosphate moiety in the active site positioning away from the basic residue might stabilize this conformation and facilitate the transformation. MIP synthase-catalyzed oxidation of **84** and **85** (Figure 35) would lead to mimics of (*Z*)-**B'** ready for intermolecular proton removal.

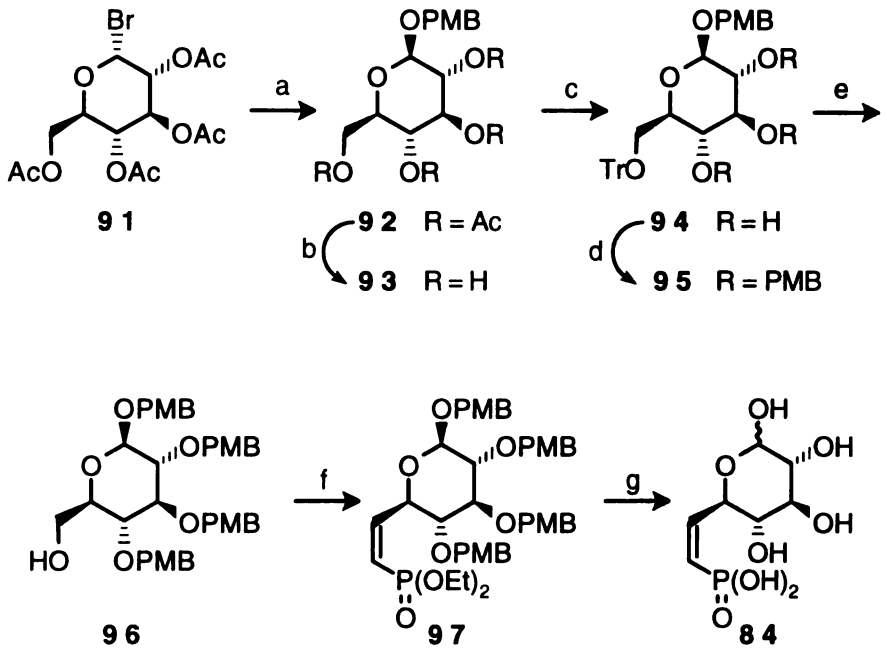
Recent studies suggested that MIP synthase might selectively bind the acyclic form of D-glucose 6-phosphate. Hence, the driving force to evolve such a versatile base residue at the active site diminishes. An alternative mechanism for the deprotonation step hinges on the opportunistic exploitation by the enzyme of the phosphate monoester group of the substrate as the catalytic base (Figure 35). This proposal enjoys the advantage of employing a strong base for the proton abstraction at physiological pH and avoiding repulsive electrostatic interactions between an enzymatic base and the charged phosphate monoester of the substrate at the active site. The (*E*)-vinylphosphonate series **86-89** (Figure 35) were designed to mirror an (*E*)-**A'** conformer which might be expected at the active site if the substrate's phosphate monoester were the base/conjugate acid mediating the enolization and aldol condensation steps. Their oxidized forms, such as

90 (Figure 35), could be regarded as analogues of intermediate (*E*)-**B'** poised for intramolecular proton removal.

Synthesis of Vinylphosphonates **84-89**

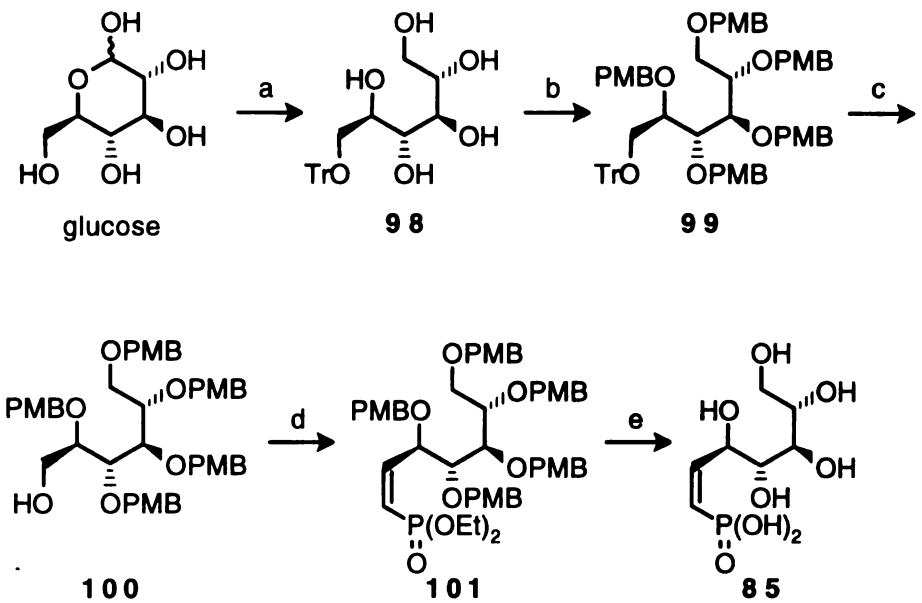
Synthesis of (*Z*)-vinylphosphonates **84** and **85** was contingent on Peterson-type olefination reactions (Figures 36 and 37).[13g] Koenigs-Knorr reaction[99] of glucopyranosyl bromide **91** with 4-methoxybenzyl alcohol gave 4-methoxybenzyl glucopyranoside **92** (Figure 36), which was hydrolyzed to polyol **93**. Selective protection of the C-6 primary alcohol as a triphenylmethyl ether and perbenzylation gave fully protected pyranoside **95**. After acidic hydrolysis unmasked the C-6 hydroxyl group, Swern oxidation[100] and subsequent Peterson olefination[101] afforded (*Z*)-vinylphosphonate **96**. Stepwise deprotection entailed reaction of the phosphonate diester with TMSBr in the presence of Et_3N and oxidation with DDQ[102] to remove three 4-methoxybenzyl groups. Pure (*Z*)-vinylphosphonate **84** was obtained after anion-exchange chromatography.

Attempted access to (*Z*)-vinylphosphonate **85** by $NaBH_4$ reduction of (*Z*)-vinylphosphonate **84** led to a mixture of inseparable isomerized products. Therefore, another route was designed (Figure 37). Tritylation of C-6 primary alcohol and reduction of the anomeric center of D-glucose afforded the trityl ether of D-glucitol **98**. Perbenzylation under phase-transfer conditions[103] and ensuing selective hydrolysis of the trityl ether gave alcohol **100**. Sequential Swern oxidation[100] and Peterson olefination[101] furnished (*Z*)-vinylphosphonate **101**. Deprotection of **101** involved transesterification of the phosphonate esters using TMSBr followed by DDQ-oxidation to afford (*Z*)-vinylphosphonate **85**.



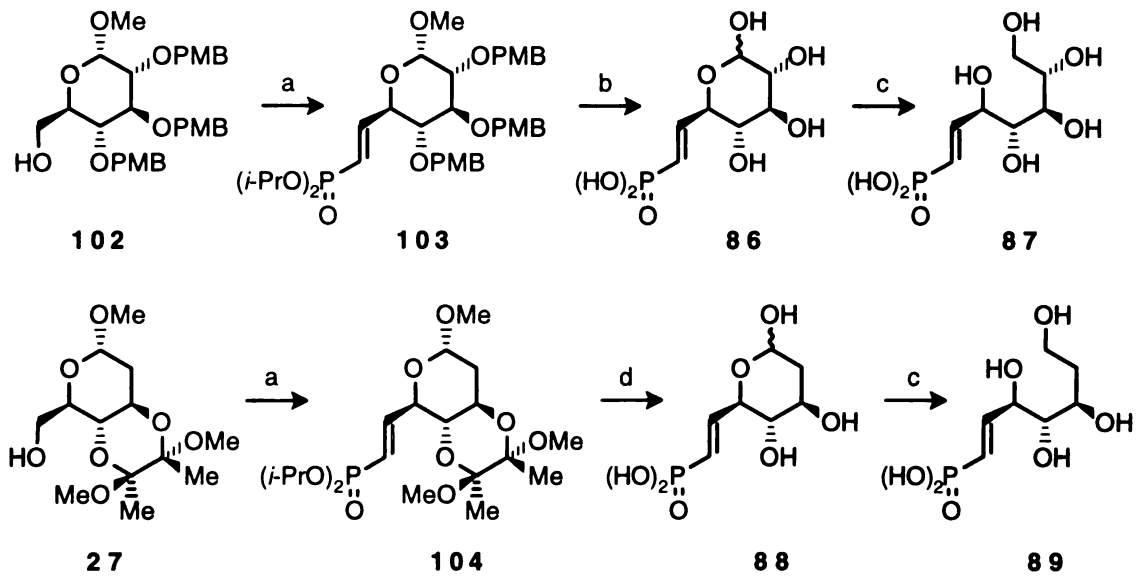
(a) 4-methoxybenzyl alcohol, $HgCl_2$, HgO, drierite, CH_2Cl_2, 85%; (b) $NaOCH_3$, MeOH, 87%; (c) TrCl, pyr, 75 °C, 76%; (d) NaH, PMBCl, DMF, 45 °C, 77%; (e) HCO_2H, Et_2O, 64%; (f) (i) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, -78 °C, (ii) $TMSCH_2P(O)(OEt)_2$, *n*-BuLi, THF, -78 °C, 27%; (g) (i) TMSBr, Et_3N, CH_2Cl_2, (ii) DDQ, CH_2Cl_2/H_2O (18:1, v/v), (iii) Ag 1 x 8 anion exchange, 61%.

Figure 36. Synthesis of (*Z*)-vinylphosphonate **84**.



(a) (i)TrCl, pyr, 75 °C, (ii) $NaBH_4$, EtOH/H_2O/CH_2Cl_2 (4:1:4, v/v/v), 41%; (b) PMBCl, *t*-amyl alcohol, TBACl, C_6H_6, aq. 50% NaOH, 78%; (c) H_2SO_4, H_2O, MeOH, 10 °C, 75%; (d) (i) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, -78 °C, (ii) $TMSCH_2P(O)(OEt)_2$, *n*-BuLi, THF, -78 °C, 22%; (e) (i) TMSBr, Et_3N, CH_2Cl_2, (ii) DDQ, CH_2Cl_2/H_2O (18:1, v/v), (iii) Ag 1 x 8 anion exchange, 54%.

Figure 37. Synthesis of (*Z*)-vinylphosphonate **85**.



(a) (i) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, -78 °C, (ii) tetraisopropyl methylenediphosphonate, *n*-BuLi, THF, -78 °C, **103**: 62%, **104**: 57%; (b) (i) DDQ, CH_2Cl_2/H_2O (18:1, v/v), (ii) aq. 6 N HCl, reflux, (iii) Ag 1 x 8 anion exchange, 60%; (c) $NaBH_4$, H_2O, 100%; (d) (i) TMSBr, Et_3N, CH_2Cl_2, (ii) aq. 6 N HCl, reflux, (iii) Ag 1 x 8 anion exchange, 100%.

Figure 38. Synthesis of (*E*)-vinylphosphonate **86-89**.

Synthesis of (*E*)-vinylphosphonate **86-89** (Figure 38) relied on Horner-Emmons reactions to control the configuration of the olefin group.[13g] Partially protected alcohol **102**[104] was oxidized to the corresponding aldehyde and subjected to olefination with the lithium salt of tetraisopropyl methylenediphosphonate to give (*E*)-vinylphosphonate **103**. After DDQ-oxidation removed three 4-methoxybenyl ether protective groups, refluxing in 6 N hydrochloric acid hydrolyzed both the pyranoside and the phosphonate diester. (*E*)-Vinylphosphonate **86** was purified by anion exchange chromatography. Sodium borohydride reduction of **86** afforded (*E*)-vinylphosphonate **87**.

A similar approach was applied for the synthesis of deoxy-(*E*)-vinylphosphonate **87** and **89** (Figure 38). BBA-protected 2-deoxy-D-glucose **27**[41] underwent Swern oxidation[100] and Horner-Emmons olefination to produce deoxy-(*E*)-vinylphosphonate **104**. A two-step deprotection consisted of reaction of the phosphonate diester with TMSBr in the presence of Et_3N and treatment with 6 N HCl at reflux gave deoxy-(*E*)-vinylphosphonate **88**. Subsequent reduction with $NaBH_4$ afforded deoxy-(*E*)-vinylphosphonate **89**. Analogue **90** was synthesized and evaluated by Dr. Jirong Peng.[29]

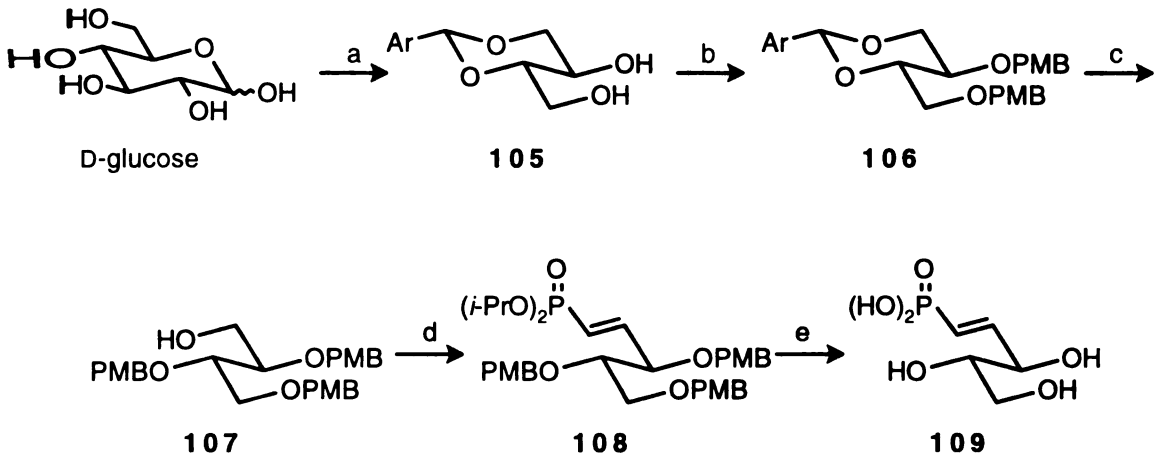
Enzymology of Vinylphosphonates **84-90**

None of the (*Z*)-vinylphosphonates **84** and **85** were inhibitors of DHQ synthase (Table 4). By contrast, all of the (*E*)-vinylphosphonates **84-90** were competitive inhibitors of the enzyme (Table 4). For instance, (*E*)-vinylphosphonate **86** was a competitive inhibitor (K_i = 1.1 x 10^{-3} M) and (*E*)-vinylphosphonate **87** was a competitive, slowly-reversible inhibitor (K_i = 30 x 10^{-6} M). In agreement with the well-established trend that removal of the C-2 hydroxyl group improves inhibition, deoxy-(*E*)-vinylphosphonates **88** was a competitive inhibitor (K_i = 0.10 x 10^{-3} M) while deoxy-(*E*)-vinylphosphonate **89** was a slowly-reversible inhibitor (K_i = 0.67 x 10^{-6} M) and the most potent inhibitor yet

Table 4. Inhibition of MIP synthase by vinylphosphonates **84-90**.

	inhibition type	k_{on} (M^{-1} s^{-1}) k_{off} (s^{-1})	K_i (M)	E-NADH formation
84	no inhibition			
85	no inhibition			
86	competitive		1.1×10^{-3}	+
87	slowly reversible	60 1.8×10^{-3}	30×10^{-6}	+
88	competitive		0.10×10^{-3}	+
89	slowly reversible	4.3×10^{3} 2.9×10^{-3}	0.67×10^{-6}	+
90[a]	competitive		81×10^{-6}	

[a] Reference 29.



Ar: 4-methoxyphenyl PMB: *p*-methoxybenzyl

(a) (i) 4-methoxybenzaldehyde dimethyl acetal, *p*-TsOH, DMF, 50 °C, (ii) $NaIO_4$, $NaHCO_3$, $MeOH/H_2O$, (iii) $NaBH_4$, $EtOH/H_2O$, 43%; (b) NaH, PMBCl, BuN_4I, DMF, 92%; (c) TMSCl, $NaBH_3CN$, CH_3CN, 0 °C to rt, 45%; (d) (i) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, -78 °C, (ii) tetraisopropyl methylenediphosphonate, *n*-BuLi, THF, -78 °C, 59%; (e) (i) TMSBr, Et_3N, CH_2Cl_2, (ii) DDQ, CH_2Cl_2/H_2O (18:1, v/v), (iii) Ag 1 x 8 anion exchange, 41%.

Figure 39. Synthesis of (*E*)-vinylphosphonate **109**.

identified for MIP synthase.

(*E*)-Vinylphosphonate **109** was then synthesized to examine if a shorter inhibitor with vinylphosphonate moiety can bind the enzyme as tightly as the substrate. D-Glucose was transformed to 2,4-benzylidene erythritol **105** in three steps (Figure 39). After protection of the 1,3-diol as their *p*-methoxybenzyl ethers, reductive ring opening[105] of the benzylidene protective group and purification gave primary alcohol **107**. Alcohol **107** was converted to fully protected (*E*)-vinylphosphonate **108** according to the usual two-step procedure. Deprotection and purification using aforementioned methods afforded (*E*)-vinylphosphonate **109**, which is a competitive inhibitor (K_i = 87 x 10^{-6} M) and induces E-NADH formation once it binds to MIP synthase. Compared with erythritol 4-phosphate **76**,[29] (*E*)-vinylphosphonate **109** was a twofold weaker inhibitor. Nevertheless, (*E*)-vinylphosphonate **109** presents another example of efficient active site binding brought about by the C-3 to C-6 portion of the glucose ring.

Intramolecular Proton Removal by the Phosphate Monoester of the Substrate

To gain additional insight into a possible intramolecular deprotonation mediated by the phosphate monoester of the substrate, *ab initio* calculations (Spartan, Version 5.0) were performed on intermediate **B'**. Initial minimization gave an acyclic, chair form conformer. The C(1) to C(4) backbone was then frozen and the dihedral angle of H_R-C(6)-C(5)-O(5) set to 90°, which is thought to be the ideal dihedral angle for enolization.[106a] Further minimization of this partially restricted conformer resulted in the *E*-conformation of intermediate **B'** (Figure 40). In this conformer, one peripheral oxygen O(7) of the phosphate monoester is held near H_R at a distance of 2.36 Å with an O(7)/H_R/C(6) bond angle of 107°. In addition, C(6) is the only carbon that bears negative charge (-0.16), indicating that C(6) is ready to give up one of the attached hydrogen and change into a carbanion for the aldol condensation step. Intramolecular deprotonation through a five-membered cyclic transition state during enolization is favored by a *5-exo-trig* mechanism

and documented by ample precedents.[106] The distance between C-1 carbonyl oxygen O(1) and the peripheral oxygen O(7) of phosphate monoester is 4.29 Å. After the enolization, the monobasic form of the phosphate monoester may relay its newly acquired hydrogen to the C-1 carbonyl during the aldol condensation step.

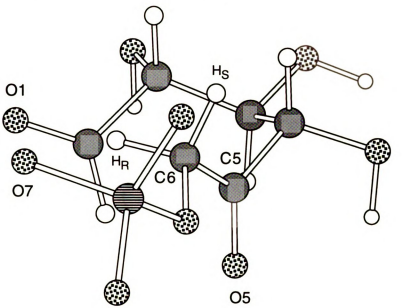


Figure 40. *Ab initio* calculation of intermediate **B'**.

As further evidence of the relevance of the (*E*)-**A'** conformation to MIP synthase catalysis, all of the (*E*)-vinylphosphonates **86**-**89** are oxidized upon binding to MIP synthase as judged by an increase in optical density at 340 nm (Table 4). The role of this oxidation in determining inhibitor potency can be assessed by 5-keto-D-glucitol 6-(*E*)-vinylhomophosphonate[29] **90** (Figure 35). This preoxidized analogue was a standard competitive inhibitor with K_i = 81 x 10^{-6} M (Table 4) which corresponds to a 2.6-fold reduction in inhibitory potency relative to (*E*)-vinylphosphonate **87**. The loss of slowly-reversible inhibitory behavior upon preoxidation of the C-5 hydroxyl group indicates that this oxidation of the C-5 hydroxyl is at the core of the slowly-reversible binding mode observed for (*E*)-vinylphosphonates **87** and **89**. It reconciles with the observed trend that

an inhibitor's propensity for enzymatic oxidation parallels its inhibitory caliber. Gross, Mehdi, and McCarthy[107] reported that (*E*)- and (*Z*)-fluorovinyl phosphonate analogues of glucose-6-phosphate had no inhibition effect on MIP synthase. In as much as the oxidation of C-5 alcohol would be impeded by the adjacent electronegative fluorine, it is likely that potential Michael acceptors, the enones, were unable to form in this case.

Is MIP synthase a passive observer during the deprotonation step? NMR studies of self-catalyzed deuterium exchange at C-6 of intermediate **B'** might provide some clues. Accessed by both ^{1}H and ^{13}C NMR spectroscopies, in solution intermediate **B'** exists mainly as a single pyranosyl hemiketal. In a D_2O-exchanged buffer containing imidazole-CF_3CO_2D (25 mM, pD 7.7) without MIP synthase, intermediate **B'** revealed no visible change in its ^{1}H spectrum even after two weeks. There are two possible explanations for the absence of deuterium exchange at C-6 in this experiment. On one hand, although the C-5 carbonyl group acidifies the C-6 methylene group, this effect is dampened in the stable cyclic hemiketal forms of intermediate **B'**. As a result, proton exchange with the solvent at the C-6 position is below the detection limit of ^{1}H NMR. On the other hand, when intermediate **B'** opens its pyranosyl ring and exists in its open chain form, it may not have access to the required optimal conformation for the intramolecular deprotonation before recycling back to its pyranosyl form. Either way, this finding suggests that one role of MIP synthase during enolization might be to stabilize the acyclic (*E*)-**B'** conformation for the recruitment of the phosphate monoester to accomplish intramolecular proton removal. This is consistent with the report on the base-catalyzed aldol condensation of intermediate **B'**.[123] In contrast to the stereospecific enzymatic reaction, base treatment (0.1 N NaOH) of intermediate **B'** yielded an isomeric mixture of inosose phosphates, which are epimeric with regard to both the hydroxyl group produced in the aldol condensation and the phosphate group. The formation of these products in chemically induced transformation of intermediate **B'** underscored the degree of control exerted by the MIP synthase on the putative intermediate **B'**.

During the normal catalytic cycle when intermediate **B'** formed at the active site of MIP synthase together with NADH, stereospecific deprotonation at C-6 of intermediate **B'** readily occurs.[32] Intermediate **B'** is a competitive inhibitor of native MIP synthase possessing bound NAD.[29] With such an unnatural configuration, intermediate **B'**-enzyme-NAD, whether or not MIP synthase can catalyze the stereospecific proton removal at C-6 position of intermediate **B'** remains an open question. Deuterium exchange at C-6 will not alter the splitting pattern of resonance for C-6 in ^{1}H NMR spectrum but will decrease the signal's integration value. However, it is rather difficult to determine the chirality of the remaining hydrogen directly from the ^{1}H NMR spectrum. Therefore, this experiment was not pursued in this study.

The complete lack of inhibition observed for the cyclic substrate analogues **67-70** and the inhibition observed for the acyclic substrate analogues **73-76** is consistent with MIP synthase selectively binding the acyclic form of substrate D-glucose 6-phosphate, which is less than 0.4% of the total population in solution.[87] Combined with the lack of inhibition observed for the (*Z*)-vinylphosphonates **84** and **85** and competitive inhibition and formation of NADH observed for (*E*)-vinylphosphonates **86-90**, MIP synthase's role in catalyzing the enolization step is reduced to stabilization of an (*E*)-**A'** conformer of intermediate **A'** whereby the phosphate monoester of the substrate mediates the intramolecular proton removal. Thus, MIP synthase can fairly be labeled as both an efficient and opportunistic enzyme.

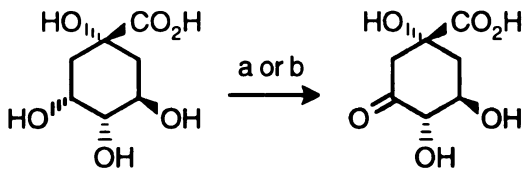
APPENDIX

APPENDIX

SYNTHESIS OF 3-DEHYDROQUINIC ACID AND (*6S*)-6-FLUOROSHIKIMIC ACID

Synthesis of 3-Dehydroquinic Acid

The first carbocyclic intermediate in the shikimate pathway is 3-dehydroquinic acid (DHQ). Several methods have been reported to prepare this important metabolite from the parent (-)-quinic acid.[108,109] For example, selective oxidation of the axial hydroxyl group at C-3 position of (-)-quinic acid was accomplished using either nitric acid[108] or platinum and oxygen[109] (Figure 41). However, the first method requires a chromatography purification to remove the by-product 5-dehydroquinic acid while the second one is of limited utility for large-scale synthetic work. To circumvent these problems, a synthetic route to DHQ based on the BBA protection methodology was examined.



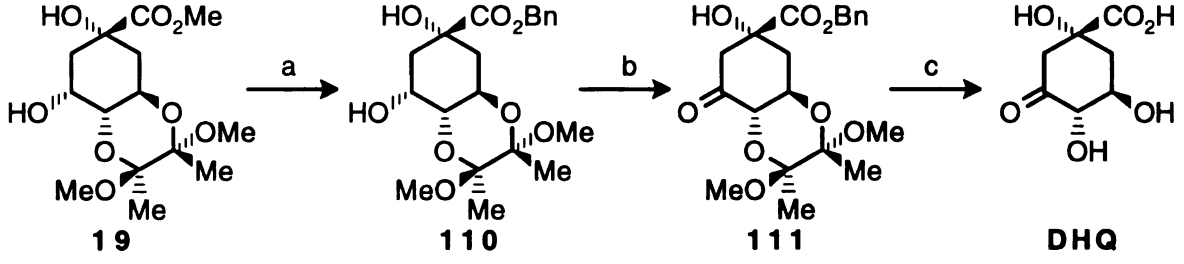
(-)-quinic acid DHQ

(a) HNO_3, H_2O, 60%; (b) PtO_2, O_2, H_2O, 100%.

Figure 41. Grewe's and Heyns's chemical synthesis of DHQ.

Quinic acid was esterified and then reacted with 2,2,3,3-tetramethoxybutane to selectively protect the vicinal diequatorial alcohols (Figure 15). BBA-protected quinate **19**[41] underwent transesterification (Figure 42) in the presence of Otera's catalyst[110] to give benzyl ester **110**, which was oxidized with KIO_4 and catalytic $RuCl_3$ to afford protected DHQ **111**. The BBA protective group was easily removed in 95% aqueous trifluoroacetic

acid at room temperature. Subsequent hydrogenolysis using 10% Pd on C furnished DHQ as the single product. Starting from quinic acid, 3-dehydroquinic acid was obtained after five steps with an overall yield of 41%. More than two grams of DHQ was made by this route, and each step of the route is amenable to scale-up.



(a) BnOH, 5 Å molecular sieves, 1-chloro-3-hydroxytetrabutyldi-stannoxane, toluene, reflux, 89%; (b) KIO_4, K_2CO_3 $RuCl_3$, H_2O, $CHCl_3$, 57%; (c) (i) TFA/H_2O (20:1, v/v), CH_2Cl_2, 92%, (ii) H_2, 10% Pd/C, THF/H_2O (1:1, v/v), 100%.

Figure 42. Synthesis of 3-dehydroquinic acid.

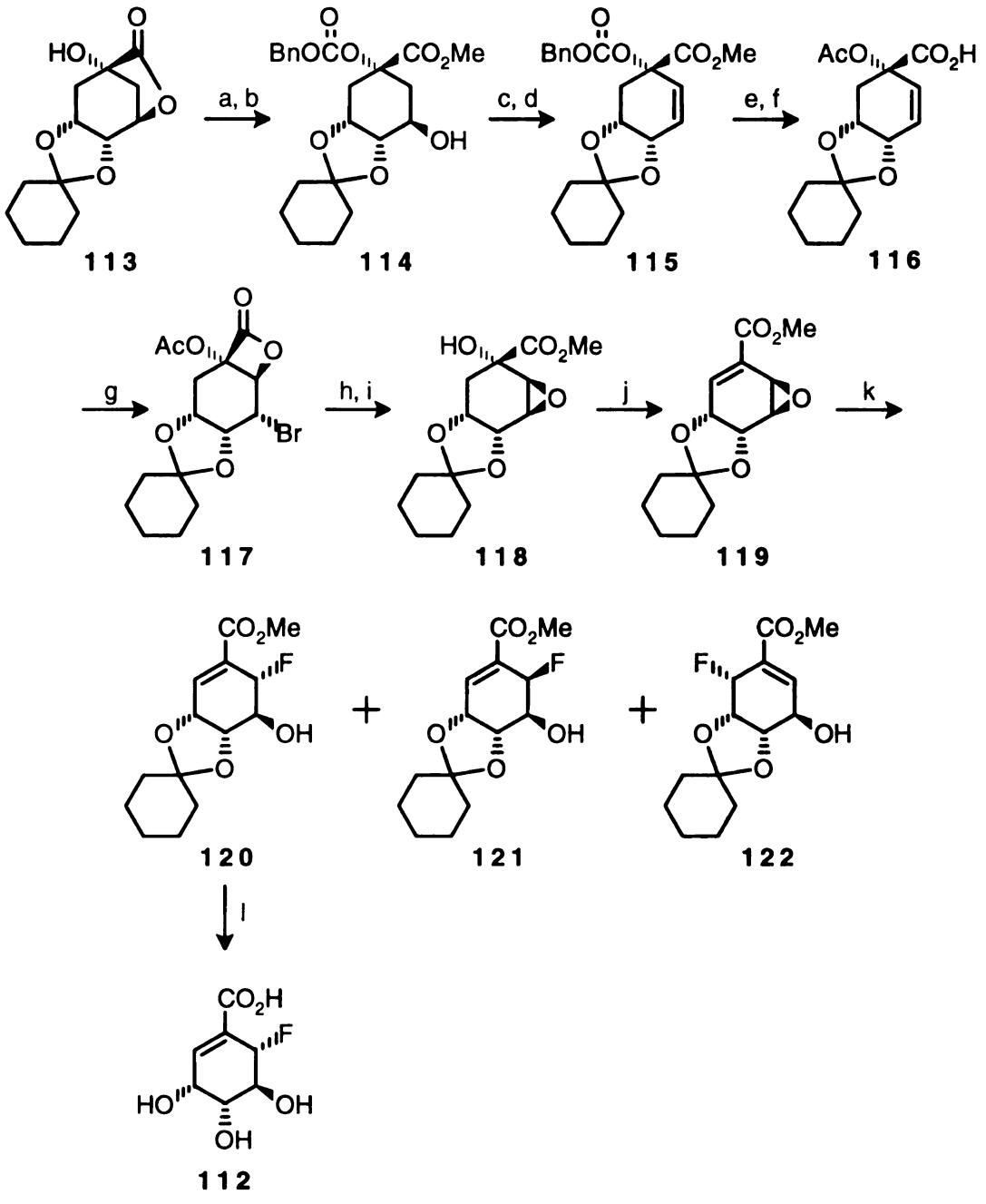
Synthesis of (*6S*)-6-Fluoroshikimic acid

Shikimic acid is a hydroaromatic intermediate in the common pathway of the aromatic amino acid biosynthesis (Figure 1). (*6S*)-6-Fluoroshikimic acid[11] is a fluorinated derivative of shikimic acid, which can be taken up by *E. coli* and transformed into 6-fluorochorismic acid via shikimate pathway enzymes.[11a] (*6S*)-6-Fluoroshikimic acid is a potent antibiotic with an MIC value of 0.1-0.5 μg/ML towards *E. coli*.[11] Its antibiotic activity was attributed to the disruption of *p*-aminobenzoic acid biosynthesis.[11a] However, resistance to (*6S*)-6-fluoroshikimic acid occurs at such high frequency that its use as an antibiotic is infeasible. In *E. coli*, this resistance was coupled with loss of the ability to transport shikimic acid.[11b]

An ongoing project in the Frost group is to use microbial biocatalyst to convert D-glucose into shikimic acid under fed-batch fermentor conditions. Mutagenic removal of shikimic acid uptake in *E. coli* would increase the yields and purity of shikimic acid during

fed-batch fermentor synthesis. Based on previous studies,[11b] (*6S*)-6-Fluoroshikimic acid might be used as an inducer to generate mutants which are deficient in shikimate uptake. A practical synthesis of gram quantities of (*6S*)-6-fluoroshikimic acid is highly desirable.

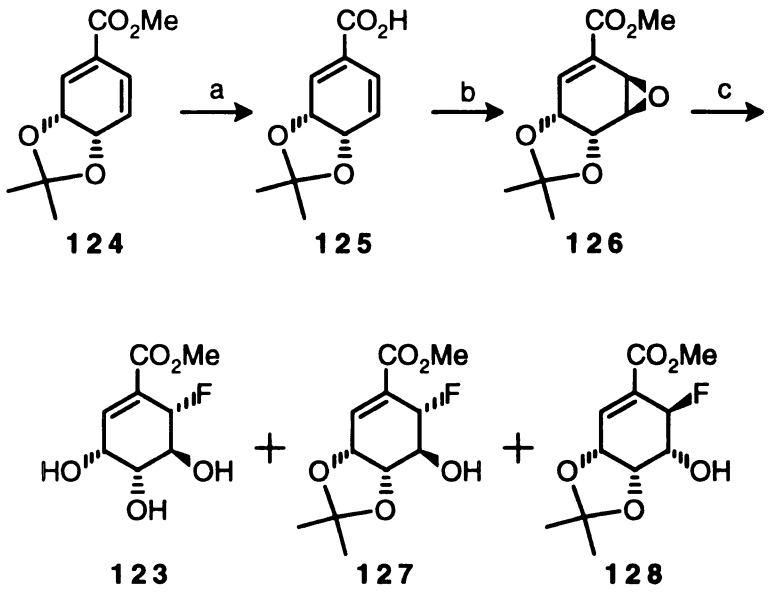
Sutherland and co-workers[111] reported the first chiral synthesis of (*6S*)-6-fluoroshikimic acid **112** from (-)-quinic acid (Figure 43). Lactone **113** was treated with benzyl chloroformate to protect the tertiary alcohol. Cleavage of the lactone ring with sodium methoxide in methanol generated alcohol **114**. Transformation of the hydroxyl group to a triflate followed by elimination of triflic acid with DBU yielded olefin **115**. Basic hydrolysis and subsequent acetylation produced acid **116**. Bromolactonization of acid **116** gave lactone **117**, which underwent ring opening of the lactone, hydrolysis of acetate group on the tertiary alcohol and epoxide formation to give **118**. Martin's reagent, the sulphurane $[PhC(CF_3)_2O]_2SPh_2$,[112] transformed **118** to allylic epoxide **119** which was treated with HF-pyridine[113] to introduce a fluorine atom. This epoxide ring opening was non-stereospecific, and three products **120**, **121**, and **122** were formed. After silica gel chromatography the desired compound **120** was obtained as the major product. Subsequent acidic hydrolysis and liquid chromatography purification afforded (*6S*)-6-fluoroshikimic acid **112** in 14 steps and with 4% overall yield.



(a) NaH, BnOCOCl, Bu_4NI, CH_2Cl_2, 92%; (b) NaOMe, MeOH, 81%; (c) $(CF_3SO_2)_2O$, pyridine, CH_2Cl_2, 96%; (d) DBU, $CHCl_3$; (e) KOH, H_2O, dioxane; (f) Ac_2O, pyridine; (g) $C_5H_6NBr_3$, $NaHCO_3$, H_2O, THF, 68% for steps d-g; (h) NaOMe, MeOH, 75%; (i) Bu_4NOAc, DMF, 82%; (j) $[PhC(CF_3)_2O]_2SPh_2$, 87%; (k) HF, pyridine, 54% total, 35% for **120**; (l) TFA, CH_2Cl_2, then 6 N HCl 59%.

Figure 43. Sutherland's synthesis of (*6S*)-6-fluoroshikimic acid **112**.

Campbell and Sainsbury[114] disclosed a synthesis of methyl (±)-(*6S*)-6-fluoroshikimate **123** (Figure 44). Hydrolysis of the racemic diene ester **124**[115] with pig-liver esterase[118] produced carboxylic acid **125**, which underwent regio- and stereoselective epoxidation with *m*CPBA followed by esterification to give epoxide **126**. When epoxide **126** was treated with hydrofluoric acid in pyridine, ring opening and removal of the acetonide protecting group occurred to afford methyl (±)-(*6S*)-6-fluoroshikimic acid **123** as the dominant product, together with trace amount of acetals **127** and **128**. Curiously further attempts to convert ester **123** to (±)-(*6S*)-6-fluoroshikimic acid **112** was not pursued.

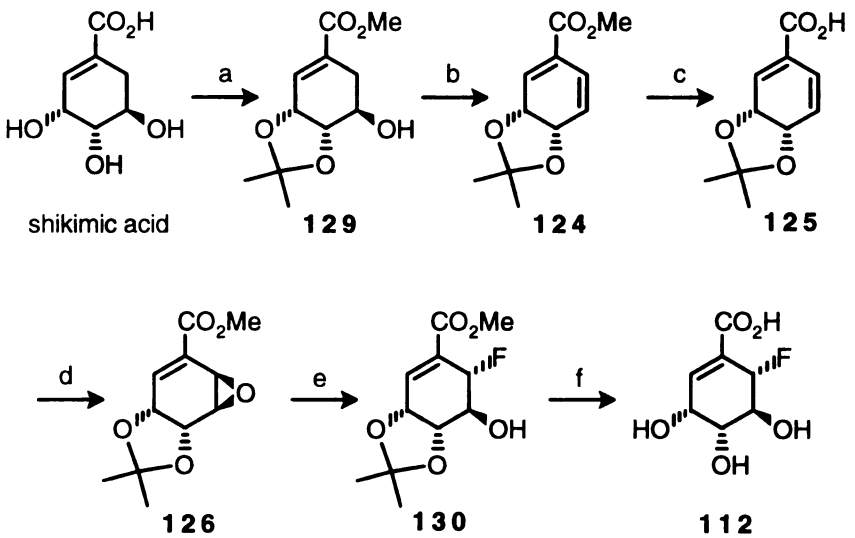


(a) pig liver esterase, aqueous phosphate buffer pH 7/acetone, 99%; (b) (i) *m*CPBA, $CHCl_3$, (ii) CH_2N_2, Et_2O, 56%; (c) HF-pyridine, CH_2Cl_2, 49% for **123**.

Figure 44. Synthesis of methyl (±)-(*6S*)-fluoroshikimate **123**.

Currently more than 50 g/L shikimic acid are synthesized under fed-batch fermentor conditions in the Frost group.[116] The availability of shikimic acid at low cost made possible the utilization of shikimic acid as a chiral synthon to synthesize

(*6S*)-6-fluoroshikimic acid **112** (Figure 45). Shikimic acid was esterified and reacted with 2,2-dimethoxypropane to selectively protect the *cis*-diol as its acetonide in **129**.[117] Treatment of alcohol **129** with trifluoromethanesulphonic anhydride gave the triflate which underwent elimination in the presence of DBU to afford diene ester **124**. Diene ester **124** was converted to epoxide **126** in analogy to Campbell and Sainsbury's synthesis.[114] Epoxide ring opening with HF-pyridine[113] and reprotection of *cis*-diol as its acetonide generated fluoroshikimate **130**. A two-step deprotection sequence consisted of pig liver esterase-catalyzed ester hydrolysis[118] and the removal of the acetonide protective group afforded (*6S*)-6-fluoroshikimic acid **112**. Starting from shikimic acid, (*6S*)-6-fluoroshikimic acid **112** was made in 11 steps with 10% overall yield.



(a) i) CH_3OH, Dowex 50 (H^+), reflux, ii) 2,2-dimethoxypropane, CH_3OH, camphorsulfonic acid, 88%; (b) i) $(CF_3SO_2)_2O$, pyridine, CH_2Cl_2, -30 °C, ii) DBU, toluene, 0 °C, 40%; (c) pig liver esterase, aqueous phosphate buffer pH 7/acetone, 85%; (d) i) *m*CPBA, $CHCl_3$, ii) CH_2N_2, ether, -20 °C, 56%; (e) i) HF-pyridine, CH_2Cl_2, -20 °C, ii) silica gel, -78 °C, iii) 2,2-dimethoxypropane, CH_3OH, camphorsulfonic acid, 61%; (f) (i) pig liver esterase, aqueous phosphate buffer pH 7/acetone, 4d, 64%; (ii) $AcOH/H_2O$, 70 °C, 98%.

Figure 45. Synthesis of (*6S*)-fluoroshikimic acid **112** from shikimic acid.

EXPERIMENTAL

General Information

General Chemistry

All air- and moisture-sensitive reactions were carried out in flame- or oven-dried glassware under a positive pressure of argon. Reagents or solutions sensitive to air or moisture were transferred to reaction flasks by syringes or cannula through rubber septa. Reactions were carried out at room temperature unless otherwise specified. Removal of solvents was accomplished using a Büchi rotary evaporator at water aspirator pressure or under high vacuum (lower than 0.5 mm Hg). Hydrogenation was performed with a Parr hydrogenation apparatus at 50 psi hydrogen pressure.

Reagents and Solvents

Water used in reactions or purifications was deionized and glass-distilled. Tetrahydrofuran (THF) was freshly distilled under nitrogen from sodium benzophenone ketyl. Dichloromethane and benzene were freshly distilled under nitrogen from calcium hydride. Pyridine, Et_3N and diisopropylamine were distilled from calcium hydride under nitrogen and stored over 4 Å molecular sieves under nitrogen. *N,N*-Dimethylformamide (DMF), dimethyl sulfoxide (DMSO) and acetonitrile were dried over 4 Å molecular sieves under nitrogen. 2,2'-Azobis(2-methylpropionitrile) (AIBN) was recrystallized from ethanol. *m*CPBA was purified by washing with phosphate buffer (1 N, pH 7.0), followed by extensive rinsing with water and drying under vacuum. Triphenylphosphine was recrystallized from hexane. 4-Dimethylaminopyridine (DMAP) was recrystallized from EtOAc. All other solvents and reagents were used as received from commercial sources or purified according to literature procedure.[119] After work up, organic solutions containing products were dried over anhydrous $MgSO_4$

Chromatography

Flash chromatography was carried out on 230-400 mesh silica gel 60 (Whatman). Radial chromatography was carried out with a Harrison Model 7924 Chromatotron, using 1, 2, or 4 mm thickness layers of silica gel 60 PF_{254} containing gypsum (E. Merck). Analytical thin-layer chromatography (TLC) was performed on precoated glass-backed plates of silica gel 60 (0.25 mm thickness, Whatman). TLC plates were visualized by ultraviolet light (254 nm), exposure to iodine vapor, immersion in 7% phosphomolybdic acid in ethanol followed by heating, or immersion in anisaldehyde stain (by volume: 93% ethanol, 3.5% sulfuric acid, 1% glacial acetic acid, and 2.5% anisaldehyde) followed by heating.

AG1-X8, Bio-gel A 0.5 was purchased from Bio-Rad, diethylaminoethyl (DEAE) cellulose (DE-52) from Whatman, Dyematrex Blue A from Amicon, and Dowex 50 (H^+) from Sigma. AG1-X8 anion exchange columns were eluted with $Et_3NH^+HCO_3^-$ (TEAB) buffer. TEAB buffer was prepared by bubbling CO_2 gas through an aqueous solution of E_3N until the pH reached 7.5 at 0 °C. Dowex 50 (H^+) was cleaned before use by the following procedure: a suspension of the resin was treated with bromine at pH 14, rinsed extensively with water, washed with 6 N aqueous hydrochloric acid followed by exhaustive rinsing with water until the filtrate was neutral. DEAE was routinely recycled and cleaned by eluting with 0.5 N ammonium chloride followed by exhaustive elution with water before use. All of the aqueous chromatographic purifications were carried out at 4 °C. High pressure liquid chromatography (HPLC) was carried out with a Rainin HPLC system using a 7.5 mm x 75 mm DEAE-5PW-TSK analytical HPLC column (purchased from Beckman).

Spectroscopic and Physical Measurements

Proton nuclear magnetic resonance (^{1}H NMR) spectra and carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Gemini 300, an Inova 300 or a VXR 500 spectrophotometer. Chemical shifts for ^{1}H NMR spectra are reported in parts per million (ppm) relative to internal tetramethylsilane (Me_4Si, δ = 0.00 ppm) when $CDCl_3$ and pyridine-*d5* were the solvent and to sodium 3-(trimethylsilyl)propionate-*2,2-3,3-d*$_4$ (TSP, δ = 0.00 ppm) when D_2O was the solvent. The following abbreviations are used to describe spin multiplicity: s (singlet), d (doublet), t (triplet), m (unresolved multiplet), br (broad peak), dd (doublet of doublets). Chemical shifts for ^{13}C NMR spectra are reported in parts per million (ppm) relative to $CDCl_3$ (δ = 77.0 ppm), pyridine-*d5* (135.5 ppm), or internal standard acetonitrile (CH_3CN, δ = 3.69 ppm) in D_2O.

Ultraviolet/visible spectra were recorded on a Hewlett Packard 8452A diode array spectrometer or a Perkin-Elmer Lambda 3B spectrometer. High resolution mass spectra (HRMS) were recorded on an MS50 or a VG 70S mass spectrometer. Fast atom bombardment (FAB) mass spectrometry was performed on a Kratos MS50 spectrometer employing glycerol as matrix. Combustion analysis were performed by Atlantic Microlab (Norcross, GA). Melting points were uncorrected and were determined using a Mel-Temp II melting point apparatus.

Assays

3-Deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) was synthesized according to the procedure of Frost and Knowles.[13f] DHQ synthase was purified from *E. coli* RB791 (*p*JB14) according to the procedure of Frost et al.[13e] High purity nicotinamide adenine dinucleotide (NAD, grade V-C) and D-glucose 6-phosphate was obtained from Sigma. MIP synthase was isolated from an overexpression strain of the *Saccharomyces cerevisiae INO1* locus in *E. coli* BL12 (DE3)/*p*T7-7/MIPSYN or from a yeast construct,[33b] *Saccharomyces cerevisiae* MW 5.55. Protein solutions were concentrated by ultrafiltration

(PM-10 Diaflo membranes or Centricon concentrators from Amicon). Protein concentrations were determined by Coomassie dye binding. Phosphorus was determined by the method of Ames and Avila.[120] DAHP was measured using the thiobarbiturate assay.[121] *myo*-Inositol 1-phosphate was quantified by the method of Barnett.[93]

Synthetic Procedures

Butane 2,3-Bisacetal (BBA) Protection.[41]

BBA-protected Methyl Quinate 19. A suspension of quinic acid (47.5 g, 0.247 mol) and Dowex 50 (H^+) (10 g) in MeOH (350 mL) was refluxed under Ar for 15 h. Heating was stopped, and the mixture was filtered to recover the acid catalyst. The cake was washed with MeOH (2x25 mL). To the combined filtrates were added trimethyl orthoformate (125 ml, 1.14 mol), 2,2,3,3-tetramethoxybutane (44.8 g, 0.251 mol), and (±)-10-camphorsulfonic acid (2.2 g, 0.010 mol). The resulting solution was refluxed for 22 h, cooled, and treated with powdered $NaHCO_3$ (8.9 g). Concentration afforded an orange suspension which was partitioned between EtOAc and saturated aqueous $NaHCO_3$. The aqueous layer was reextracted once with EtOAc and the combined organic layers were dried over $MgSO_4$. Filtration through silica and concentration afforded an orange oil which started to crystallize. Addition of EtOAc/hexane (1:5, v/v) caused complete crystallization into a single block. Recrystallization (EtOAc and hexane) afforded a white solid (63.9 g, 81%). The brown mother liquor was purified by flash chromatography (EtOAc) to afford an additional amount of product (4.9 g, 6%) as a white foamy solid: mp (crystals) 139.8-140.2 °C; ^{1}H NMR ($CDCl_3$) δ 4.31 (ddd, J= 12,10, 5 Hz, 1 H), 4.19 (ddd, J= 3, 3, 3 Hz, 1 H), 3.79 (s, 3 H), 3.60 (dd, 10, 3 Hz, 1 H), 3.26 (2 s, 2x3 H), 2.19 (ddd, J= 15, 3, 3 Hz, 1 H), 2.10 (ddd, J= 12, 5, 3 Hz, 1 H), 2.03 (dd, J= 15, 3 Hz, 1 H), 1.92 (dd, J= 12, 12 Hz, 1 H), 1.34 (s, 3 H), 1.30 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 174.1, 100.2, 99.6, 75.7,

72.6, 69.0, 62.3, 52.8, 47.8, 38.5, 37.3, 17.7, 17.5. Anal. Calcd for $C_{14}H_{24}O_8$: C, 52.49; H, 7.55. Found: C, 52.56; H, 7.52.

BBA-protected Inositol 20. A suspension of *myo*-inositol (0.591 g, 3.28 mmol) in methanol (10 mL), 2,2,3,3-tetramethoxybutane (1.19 g, 6.66 mmol), and trimethylorthoformate (2.9 mL, 26 mmol) was treated with CSA (0.042 g, 0.18 mmol). The resulting mixture was refluxed. A white precipitate appeared slowly overtime. After 135 h at methanol reflux, heating was stopped and the cool reaction mixture was filtered. The precipitate was washed with EtOAc/hexane (1:1, v/v). Drying under vacuum afforded the diprotected product (1.05 g, 79%) as a white solid: mp > 330 °C ; ^{1}H NMR (C_5D_5N) δ 4.80 (dd, *J*= 10, 9 Hz, 2, H), 4.71 (t, *J*= 2 Hz, 1 H), 4.28 (t, *J*= 9 Hz, 1 H), 4.16 (dd, *J*= 10, 2 Hz, 2 H), 3.34 (s, 6 H), 3.24 (s, 6 H), 1.45 (s, 6 H), 1.44 (s, 6 H); ^{13}C NMR (C_5D_5N) δ 100.1, 99.5, 71.0, 70.9, 70.0, 69.5, 47.7, 47.6, 18.1, 18.0; HRMS (FAB) calcd for $C_{18}H_{32}O_{10}$ (M - H^+) 407.1917, found 407.1912. Anal. Calcd for $C_{18}H_{32}O_{10}$: C, 52.93; H, 7.90. Found: C, 52.98; H, 7.90.

General Procedure for BBA Protection. A suspension of diol (2-5 mmol, 1 eq) in a solution of 2,2,3,3-tetramethoxybutane (1-1.2 eq), trimethylorthoformate (4 eq) in methanol (2-5 mL/mmol diol) was treated with camphorsulfonic acid (0.05 eq). After refluxing under Ar for 12-18 h, the reaction mixture was cooled, treated with powdered $NaHCO_3$ (ca. 500 mg), and concentrated under reduced pressure. Purification by flash chromatography or radial chromatography eluting with hexane/EtOAc (1:1, v/v) provided the protected carbohydrates.

BBA-protected Glucopyranoside (21 and 22). ^{1}H NMR ($CDCl_3$) δ 4.80 (d, *J*= 4 Hz, 1 H), 4.74 (d, *J*= 4 Hz, 1 H), 3.60-4.00 (m, 12 H), 3.43 (s, 3 H), 3.41 (s, 3 H), 3.30 (s, 3 H), 3.29 (s, 3 H), 3.26 (s, 6 H), 3.06 (br, 4 H), 1.34 (s, 3 H), 1.33 (s, 3 H), 1.31 (s, 3 H), 1.29 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 99.6, 99.4 (2), 99.3, 99.2, 97.8, 71.7, 69.8, 69.6, 69.5, 69.2, 68.0, 67.4, 65.6, 61.4, 60.7, 55.0, 54.8, 47.8, 47.7 (2), 47.6, 17.5 (2), 17.4 (2); HRMS (FAB) calcd for $C_{13}H_{24}O_8$ (M - H^+) 307.1393, found

307.1401. Anal. Calcd for $C_{13}H_{24}O_8 \cdot 1/2H_2O$: C, 49.20; H, 7.94. Found: C, 49.29; H, 7.82.

BBA-protected Galacopyranoside 23. mp 88-91 °C ; ^{1}H NMR ($CDCl_3$) δ 4.84 (d, *J*= 2 Hz, 1 H), 4.20 (dd, *J*= 10, 3 Hz, 1 H), 4.05-4.10 (m, 1 H), 3.80-4.00 (m, 5 H), 3.43 (s, 3 H), 3.26 (s, 3 H), 3.25 (s, 3 H), 2.77 (br, 2 H), 1.34 (s, 3 H), 1.31 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 100.1, 98.3 (2), 70.1, 69.0, 66.1, 65.0, 62.6, 55.1, 47.9 (2), 17.7, 17.6; HRMS (FAB) calcd for $C_{13}H_{24}O_8$ (M - H+) 307.1393, found 307.1380. Anal. Calcd for $C_{13}H_{24}O_8 \cdot 1/2H_2O$: C, 49.20; H, 7.94. Found: C, 49.37; H, 7.98. Spectral data for the β-anomer: ^{1}H NMR ($CDCl_3$) δ 4.42 (d, *J*= 8 Hz, 1 H), 3.80-4.05 (m, 6 H), 3.74 (dd, *J*= 10, 3 Hz, 1 H), 3.55 (s, 3 H), 3.28 (s, 3 H), 3.27 (s, 3 H), 2.73, (br, 2 H), 1.33 (s, 3 H), 1.32 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 101.9, 100.1, 99.7, 74.9, 70.2, 68.0, 66.8, 62.1, 56.7, 48.0, 47.9, 17.6, 17.5.

BBA-protected Mannopyranoside 24. ^{1}H NMR ($CDCl_3$) δ 4.75 (s, 1 H), 4.11 (dd, *J*= 10, 10 Hz, 1 H), 4.00 (dd, *J*= 10, 3 Hz, 1 H), 3.93 (s, 1 H), 3.70-3.85 (m, 3 H), 3.37 (s, 3 H), 3.28 (s, 3 H), 3.26 (s, 3 H), 3.07 (s, 1 H), 2.49 (br, 1 H), 1.32 (s, 3 H), 1.29 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 101.0, 100.2, 99.7, 70.5, 69.5, 68.0, 62.7, 61.1, 54.8, 48.0, 47.8, 17.7, 17.6; HRMS (FAB) calcd for $C_{13}H_{24}O_8$ (M - H^+) 307.1393, found 307.1404. Anal. Calcd for $C_{13}H_{24}O_8$: C, 50.64; H, 7.85. Found: C, 50.41; H, 7.90.

BBA-protected Lyxopyranoside 25. mp 63-64 °C ; ^{1}H NMR ($CDCl_3$) δ 4.68 (s, 1 H), 4.05-4.25 (m, 1 H), 3.90-3.95 (m, 2 H), 3.55-3.70 (m, 2 H), 3.37 (s, 3 H), 3.27 (s, 3 H), 3.26 (s, 3 H), 2.82 (br, 1 H), 1.33 (s, 3 H), 1.28 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 101.1, 100.4, 99.6, 69.5, 68.6, 62.7, 60.4, 54.8, 47.9, 47.8, 17.6 (2); HRMS (FAB) calcd for $C_{12}H_{22}O_7$ (M - H^+) 277.1287, found 277.1296. Anal. Calcd for $C_{12}H_{22}O_7$: C, 51.79; H, 7.97. Found: C, 51.50; H, 7.84.

BBA-protected Rhamnopyranoside 26. mp 144-147 °C; ^{1}H NMR ($CDCl_3$) δ 4.67 (s, 1 H), 3.90-4.00 (m, 2 H), 3.65-3.85 (m, 2 H), 3.36 (s, 3 H), 3.27 (s, 3 H), 3.24 (s, 3 H), 2.53 (br, 1 H), 1.32 (s, 3 H), 1.29 (s, 3 H), 1.28 (d, *J*= 6 Hz, 3 H); ^{13}C

NMR ($CDCl_3$) δ 100.8, 100.1, 99.7, 69.7, 68.3, 68.2, 66.3, 54.6, 48.0, 47.6, 17.7, 17.6, 16.5; HRMS (FAB) calcd for $C_{13}H_{24}O_7$ (M - H^+) 291.1444, found 291.1435. Anal. Calcd for $C_{13}H_{24}O_7$: C, 53.41; H, 8.27. Found: C, 53.33; H, 8.25.

BBA-protected 2-Deoxy glucopyranoside 27. ^1H NMR ($CDCl_3$) δ 4.84 (d, *J*= 3 Hz, 1 H), 4.12 (ddd, *J*= 13, 10, 5 Hz, 1 H), 3.70-3.85 (m, 3 H), 3.62 (dd, *J*= 10, 10 Hz, 1 H), 3.32 (s, 3 H), 3.28 (s, 3 H), 3.27 (s, 3 H), 2.37 (br, 1 H), 1.99 (dd, *J*= 13, 5 Hz, 1 H), 1.77 (ddd, *J*= 13, 13, 3 Hz, 1 H), 1.29 (s, 6 H); ^{13}C NMR ($CDCl_3$) δ 99.6, 99.5, 98.6, 69.9, 68.2, 64.6, 61.1, 53.4, 47.8, 47.6, 34.4, 17.6, 17.5. Anal. Calcd for $C_{13}H_{24}O_7$: C, 53.41; H, 8.27. Found: C, 53.23; H, 8.22.

Synthesis of Carbocyclic Inhibitors 4-8.[69]

Protected DHQ Intermediate 28. In the following order, H_2O (300 mL), KIO_4 (43.0 g, 187 mmol), K_2CO_3 (3.38 g, 24.5 mmol) and ruthenium trichloride (0.50 g, 1.9 mmol) were added to a solution of BBA-protected quinate **19**[41] (30.2 g, 94.2 mmol) in chloroform (300 mL). Vigorous stirring was continued at rt until the reaction was complete. The mixture was filtered through Celite, and the aqueous and organic phases separated. After saturation with NaCl, the aqueous layer was extracted with EtOAc (3x). The combined organic layers were dried and concentrated to give protected DHQ intermediate **19** as a white solid (23.0 g, 77%): ^1H NMR ($CDCl_3$) δ 4.43 (dd, *J* = 10, 1 Hz, 1 H), 4.26 (ddd, *J* = 13, 10, 4 Hz, 1 H), 3.85 (s, 3 H), 3.27 (s, 3 H), 3.24 (s, 3 H), 2.91 (dd, *J* = 14, 1 Hz, 1 H), 2.52 (dd, *J* = 14, 3 Hz, 1 H), 2.36 (dd, *J* = 13, 13 Hz, 1 H), 2.13 (ddd, *J* = 13, 4, 3 Hz, 1 H), 1.41 (s, 3 H), 1.31 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 199.4, 174.0, 100.4, 99.5, 77.1, 74.0, 66.9, 53.5, 48.9, 48.2, 47.9, 37.7, 17.6, 17.4. Anal. Calcd for $C_{14}H_{22}O_8$: C, 52.82; H, 6.97. Found: C, 52.65; H, 6.93.

Carbasuccinate Intermediate 29. A solution of intermediate **28** (0.661 g, 2.08 mmol) and (carbethoxymethylene)triphenylphosphorane (1.45 g, 4.16 mmol) in

anhydrous CH_3CN (10 mL) was refluxed for 15 min. After the solution was cooled to rt, the solvent was removed and the residue was purified by flash chromatography (hexane/EtOAc, 1:1, v/v) to give carbasuccinate intermediate **29** as a yellow foam (0.727 g, 90%): H^1 NMR ($CDCl_3$) δ 6.25 (d, J = 2 Hz, 1 H), 4.11-4.20 (m, 3 H), 4.05 (dd, J = 14, 3 Hz, 1 H), 3.97 (ddd, J = 11, 10, 5 Hz, 1 H), 3.80 (s, 3 H), 3.25 (s, 3 H), 3.22 (s, 3 H), 3.21 (s, 1 H), 2.34 (d, J = 14 Hz, 1 H), 2.12 (dd, J = 12, 12 Hz, 1 H), 2.00 (ddd, J = 13, 5, 3 Hz, 1 H), 1.36 (s, 3 H), 1.31 (s, 3 H), 1.28 (t, J = 7 Hz, 3 H); ^{13}C NMR ($CDCl_3$) δ 174.8, 166.8, 150.6, 115.1, 100.1, 99.4, 74.8, 72.9, 68.5, 60.0, 53.0, 47.9 (2), 37.3, 36.9, 17.6 (2), 14.1; HRMS (FAB) calcd for $C_{18}H_{28}O_9$ (M + H^+) 389.1812, found 389.1829. Anal. Calcd for $C_{18}H_{28}O_9$: C, 55.66; H, 7.27. Found: C, 55.59; H, 7.32.

Carbasuccinate Intermediate 31. 1,1'-Carbonyldiimidazole (0.415 g, 2.56 mmol) and carbasuccinate intermediate **29** (0.497 g, 1.28 mmol) were dissolved in anhydrous $ClCH_2CH_2Cl$ (5 mL) and heated at reflux. Heating was stopped 5 h later and the mixture cooled to rt. Ether was added and the organic layer was washed with water (1x) and brine (1x). Concentration yielded a yellow oil which was purified by flash chromatography (hexane/EtOAc, 1:1, v/v) to afford carbasuccinate intermediate **30** as a white foam (0.506 g, 82%): ^1H NMR ($CDCl_3$) δ 8.05 (d, J = 1 Hz, 1 H), 7.33 (dd, J = 2, 2 Hz, 1 H), 7.05 (dd, J = 2, 1 Hz, 1 H), 6.27 (m, 1 H), 4.59 (dd, J = 15, 3 Hz, 1 H), 4.21 (dd, J = 10, 2 Hz, 1 H), 4.08-4.16 (m, 2 H), 3.82 (s, 3 H), 3.75-3.84 (m, 1 H), 3.26 (s, 3 H), 3.21 (s, 3 H), 2.68 (ddd, J = 14, 5, 3 Hz, 1 H), 2.47 (dd, J = 15, 1 Hz, 1 H), 2.26 (dd, J = 14, 12 Hz, 1 H), 1.37 (s, 3 H), 1.32 (s, 3 H), 1.22 (t, J = 7 Hz, 3 H); ^{13}C NMR ($CDCl_3$) δ 169.4, 166.0, 147.4, 137.2, 130.7, 117.1, 116.8, 100.3, 99.7, 83.5, 72.5, 68.1, 60.3, 53.3, 48.2, 48.0, 35.3, 33.9, 17.6, 14.1; HRMS (FAB) calcd for $C_{22}H_{30}N_2O_{10}$ (M + H^+) 483.1979, found 483.1983.

Benzeneselenol (0.26 mL, 2.4 mmol) and carbasuccinate intermediate **30** (0.567 g, 1.17 mmol) were dissolved in anhydrous $ClCH_2CH_2Cl$ (5 mL) and the resulting

solution was heated at reflux overnight. Solvent removal and purification by flash chromatography (hexane/EtOAc, 5:1, v/v) yielded carbasuccinate intermediate **31** as a white solid (0.582 g, 87%): m.p. 174-176 °C; ^{1}H NMR ($CDCl_3$) δ 7.57-7.60 (m, 2 H), 7.30-7.38 (m, 3 H), 6.25 (s, 3 H), 4.64 (dd, J = 15, 3 Hz, 1 H), 4.10-4.22 (m, 3 H), 3.81 (ddd, J = 12, 10, 5 Hz, 1 H), 3.74 (s, 3 H), 3.23 (s, 6 H), 2.44 (ddd, J = 14, 4, 3 Hz, 1 H), 2.41 (dd, J = 14, 12 Hz, 1 H), 1.38 (s, 3 H), 1.31 (s, 3 H), 2.02 (dd, J = 14, 12 Hz, 1 H), 1.25 (t, J = 7 Hz, 3 H); ^{13}C NMR ($CDCl_3$) δ 169.8, 165.7, 165.2, 147.9, 135.4, 129.1, 129.0, 125.6, 116.1, 100.1, 99.5, 83.2, 72.3, 67.5, 59.9, 52.8, 47.9 (2), 35.8, 33.1, 17.5, 14.1; HRMS (EI) calcd for $C_{25}H_{32}O_{10}Se$ (M + H^+) 573.1238, found 573.1260. Anal. Calcd for $C_{25}H_{32}O_{10}Se$: C, 52.54; H, 5.65. Found: C, 52.45; H, 5.65.

Carbasuccinate Intermediate 32. A solution of AIBN (0.035 g, 0.21 mmol) and Bu_3SnH (0.86 mL, 3.2 mmol) in benzene (50 mL) was slowly added via a syringe pump (0.10 mmol/hr) to a refluxing solution of carbasuccinate intermediate **31** (1.21 g, 2.12 mmol) in benzene (25 mL). After completion of the addition, heating at reflux was continued for another 2 h. Solvent was removed under reduced pressure, and the residue was purified by radial chromatography (2 mm thickness, hexane/EtOAc, 5:1, v/v) to give carbasuccinate intermediate **32** as a white solid (0.23 g, 27%): m.p. 180-181 °C; ^{1}H NMR ($CDCl_3$) δ 4.07-4.19 (m, 2 H), 3.90-3.96 (m, 2 H), 3.83 (s, 3 H), 3.21 (s, 3 H), 3.18 (s, 3 H), 2.89 (d, J = 18 Hz, 1 H), 2.80 (d, J = 18 Hz, 1 H), 2.76 (dd, J = 12, 2 Hz, 1 H), 2.42-2.49 (m, 1 H), 2.34 (d, J = 12 Hz, 1 H), 1.98-2.06 (m, 1 H), 1.28 (s, 6 H), 1.26 (t, J = 7 Hz, 3 H); ^{13}C NMR ($CDCl_3$) δ 173.2, 170.0, 169.0, 101.1, 100.3, 81.6, 70.8, 66.8, 60.7, 52.9, 48.0, 47.8, 47.0, 42.9, 34.4, 31.7, 17.6, 17.5, 14.0; HRMS (FAB) calcd for $C_{19}H_{28}O_{10}$ (M - H^+) 415.1604, found 415.1600. Anal. Calcd for $C_{19}H_{28}O_{10}$: C, 54.80; H, 6.78. Found: C, 54.70; H, 6.73.

Carbasuccinate 5. Carbasuccinate intermediate **32** (0.152 g, 0.360 mmol) was stirred in CF_3CO_2H/H_2O (20:1, v/v, 3 mL) for 20 min. Water and CF_3CO_2H were removed in vacuo. The brown residue was stirred in a mixture of THF (10 mL) and

aqueous NaOH (0.2 N, 10 mL) for 24 h. The aqueous layer was washed with EtOAc (1x), passed down a Dowex 50 (H^+) column, and concentrated to afford carbasuccinate 3 as a colorless film (100% yield based on NMR analysis): ^{1}H NMR (D_2O, pH 7.5) δ 3.87 (m, 1 H), 3.47 (d, J = 10 Hz, 1 H), 2.67 (d, J = 14 Hz, 1 H), 2.56 (d, J = 14 Hz, 1 H), 2.36 (dd, J = 15, 2 Hz, 1 H), 1.82-2.03 (m, 3 H); ^{13}C NMR (D_2O, pH 7.5) δ 185.5, 184.9, 182.1, 80.8, 78.1, 71.7, 52.4, 49.3, 44.3, 42.8; HRMS (FAB) calcd for $C_{10}H_{11}O_9Na_3$ ($M + H^+$) 345.0174, found 345.0171.

Carbamalonate Ether Intermediate 33. Intermediate **28** (2.01 g, 6.31 mmol) and $NaBH(OAc)_3$ (5.35 g, 25.3 mmol) were dissolved in CH_3CN (10 mL) and HOAc (10 mL). The mixture was stirred at rt for 8 h. Solvents were removed in vacuo, and the residue was dissolved in ether and washed with aqueous $NaHSO_4$ (0.1 N). The aqueous layer was back extracted with ether (3x). The combined organic layers were then washed with aqueous phosphate buffer (2 N, pH 7), dried, and concentrated to a yellow solid. Purification by flash chromatography (hexane/EtOAc, 1:2, v/v) gave white, crystalline diol **33** (1.74 g, 86%): m.p. 164-166 °C; ^{1}H NMR ($CDCl_3$) δ 3.90-4.07 (m, 2 H), 3.80 (s, 3 H), 3.48 (dd, J = 10, 10 Hz, 1 H), 3.30 (s, 3 H), 3.26 (s, 3 H), 3.24 (s, 1 H), 2.58 (d, J = 2 Hz, 1 H), 1.99-2.06 (m, 1 H), 1.98 (dd, J = 12, 12 Hz, 1 H), 1.82-1.90 (m, 2 H), 1.35 (s, 3 H), 1.30 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 175.7, 99.6, 99.5, 76.3, 73.3, 66.9, 65.1, 53.2, 47.9, 47.8, 40.5, 37.9, 17.7 (2). Anal. Calcd for $C_{14}H_{24}O_8$: C, 52.49; H, 7.55. Found: C, 52.43; H, 7.51.

Carbamalonate Ether Intermediate 34. A solution of diethyl diazomalonate (0.239 g, 1.29 mmol) in benzene (2 mL) was slowly added via syringe pump (2.4 mmol/h) to a solution of carbamalonate ether intermediate **33** (0.206 g, 0.643 mmol) and $Rh_2(OAc)_4$ (0.004 g, 0.01 mmol) in refluxing benzene (10 mL). After completion of the addition, the mixture was refluxed for another 2 h. Removal of the solvent gave a green residue, which was purified by flash chromatography (hexane/EtOAc, 1:1, v/v) to yield carbamalonate ether intermediate **34** as a colorless oil (0.220 g, 72%): ^{1}H NMR ($CDCl_3$) δ 4.18-4.31 (m,

4 H), 3.98 (ddd, J = 12, 10, 5 Hz, 1 H), 3.89 (ddd, J = 11, 10, 5 Hz, 1 H), 3.79 (s, 3 H), 3.71 (dd, J = 10, 10 Hz, 1 H), 3.29 (s, 3 H), 3.28 (s, 1 H), 3.25 (s, 3 H), 2.23 (ddd, J = 13, 5, 2 Hz, 1 H), 1.95-2.05 (m, 1 H), 1.93 (dd, J = 12, 12 Hz, 1 H), 1.80 (ddd, J = 13, 5, 2 Hz, 1 H), 1.32 (s, 3 H), 1.29 (s, 3 H), 1.25-1.31 (m, 6 H); ^{13}C NMR ($CDCl_3$) δ 175.4, 167.6, 166.2, 99.5, 99.3, 80.0, 76.7, 75.6, 73.1, 65.3, 61.7, 61.6, 53.1, 47.9, 47.7, 39.9, 37.3, 17.6, 14.0, 13.9 Anal. Calcd for $C_{21}H_{34}O_{12}$: C, 52.60; H, 7.15. Found: C, 52.74; H, 7.20.

Carbamalonate Ether 6. Carbamalonate ether intermediate **34** (0.22 g, 0.48 mmol) was deprotected as described for carbasuccinate intermediate **32** to give carbamalonate ether **6** as a colorless film (100% yield based on NMR analysis): ^{1}H NMR (D_2O, pH 7.7) δ 4.98 (s, 1 H), 3.70-3.83 (m, 2 H), 3.54 (dd, J = 9, 9 Hz, 1 H), 2.25-2.31 (m, 1 H), 2.08-2.15 (m, 1 H), 1.97 (dd, J = 13, 12 Hz, 1 H), 1.90 (dd, J = 13, 12 Hz, 1 H); ^{13}C NMR (D_2O, pH 7.7) δ 180.2, 173.0, 172.9, 82.2, 81.4, 81.0, 76.0, 71.8, 42.2, 40.1; HRMS (FAB) calcd for $C_{10}H_{11}O_{10}Na_3$ ($M + H^+$) 361.0123, found 361.0122.

Carbamalonate Intermediate 35. To a solution of intermediate **28** (1.03 g, 3.23 mmol) in benzene (15 mL) was added malononitrile (0.235 g, 3.56 mmol), ammonium acetate (0.025 g, 0.32 mmol), and one drop of HOAc. The resulting mixture was stirred overnight at rt. Ether was added and the organic layer was washed with saturated aqueous $NaHCO_3$ (1x) and brine (1x). Drying and concentration of the organic layer afforded a yellow oil that was purified by flash chromatography (hexane/EtOAc, 1:1, v/v) to give carbamalonate intermediate **35** as a white crystalline solid (1.07 g, 90%): m.p. 189-190 °C; ^{1}H NMR ($CDCl_3$) δ 4.54 (d, J = 9 Hz, 1 H), 4.16 (ddd, J = 11, 9, 5 Hz, 1 H), 3.87 (s, 3 H), 3.62 (s, 1 H), 3.31 (s, 3 H), 3.24 (s, 3 H), 3.09 (dd, J = 14, 2 Hz, 1 H), 2.81 (d, J = 14 Hz, 1 H), 2.11 (dd, J = 14, 11 Hz, 1 H), 2.02 (ddd, J = 13, 5, 2 Hz, 1 H), 1.41 (s, 3 H), 1.29 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 173.5, 169.9, 112.3, 110.8, 101.1, 99.8, 85.3, 74.6, 74.1, 67.9, 53.8, 48.6, 48.1, 42.5, 37.5, 17.4, 16.7; HRMS

(EI) calcd for $C_{17}H_{22}N_2O_7$ (M + H^+) 367.1505, found 367.1502. Anal. Calcd for $C_{17}H_{22}N_2O_7$: C, 55.73; H, 6.05; N, 7.65. Found: C, 55.71; H, 6.04; N, 7.68.

Carbamalonate Intermediate 36. $NaBH(OAc)_3$ (2.31 g, 10.9 mmol) was dissolved in CH_3CN (10 mL) and HOAc (10 mL). After stirring at rt for 10 min, carbamalonate intermediate **35** (1.00 g, 2.73 mmol) was added as a solid in one portion. Stirring was continued overnight at rt. Solvents were removed in vacuo, and the residue was dissolved in ether and washed with aqueous $NaHSO_4$ (0.1 N, 1x). The aqueous layer was back extracted with ether (3x). Combined organic layers were then washed with aqueous phosphate buffer (2 N, pH 7), dried and concentrated to a yellow oil. Flash chromatography (hexane/EtOAc, 1:1, v/v) yielded carbamalonate intermediate **36** as a white crystalline solid (0.925 g, 92%): m.p. 158-159 °C; ^{1}H NMR ($CDCl_3$) δ 4.44 (d, J = 4 Hz, 1 H), 4.04 (ddd, J = 11, 10, 5 Hz, 1 H), 3.84 (s, 3 H), 3.66 (dd, J = 11, 10 Hz, 1 H), 3.52 (s, 1 H), 3.31 (s, 3 H), 3.26 (s, 3 H), 2.60-2.71 (m, 1 H), 2.11-1.89 (m, 4 H); ^{13}C NMR ($CDCl_3$) δ 174.9, 111.7, 110.7, 100.2, 99.7, 72.9, 70.4, 66.1, 53.4, 48.4, 48.0, 37.5, 37.3, 35.8, 23.2, 17.5 (2); HRMS (EI) calcd for $C_{17}H_{24}N_2O_7$ (M^+) 368.1583, found 368.1585. Anal. Calcd for $C_{17}H_{24}N_2O_7$: C, 55.42; H, 6.57; N, 7.61. Found: C, 55.28; H, 6.53; N, 7.50.

Carbamalonate 7. Carbamalonate intermediate **36** (2.0 g, 5.4 mmol) was stirred in CF_3CO_2H/H_2O (20:1, v/v, 5 mL) for 20 min. After concentration, the residue was dissolved in aqueous HCl (6 N, 20 mL) and heated at reflux for 2 h. After cooling to rt, the mixture was evaporated to dryness, dissolved in water, and the solution basicified by addition of aqueous NaOH to pH 12. The resulting solution was stirred overnight. The aqueous layer was washed with EtOAc (1x), passed down Dowex 50 (H^+), and concentrated to afford carbamalonate **7** as a colorless film (95% yield based on NMR analysis): ^{1}H NMR (D_2O, pH 7.9) δ 3.86 (d, J = 5 Hz, 1 H), 3.79 (ddd, J = 12, 10, 5 Hz, 1 H), 3.47 (dd, J = 11, 10 Hz, 1 H), 2.50-2.65 (m, 1 H), 2.09-2.19 (m, 1 H), 1.88-1.99 (m, 1 H), 1.86 (dd, J = 13, 12 Hz, 1 H); ^{13}C NMR (D_2O, pH 7.9) δ 180.8, 175.4, 174.8,

78.4, 76.6, 73.3, 55.0, 42.4, 40.8, 37.7; HRMS (FAB) calcd for $C_{10}H_{14}O_9$ (M + H^+) 279.0716, found 279.0711.

Carbahydroxymalonate Intermediate 37. Carbamalonate intermediate **36** (1.6 g, 4.3 mmol) was stirred in CF_3CO_2H/H_2O (20:1, v/v, 5 mL) for 20 min. CF_3CO_2H and water were removed in vacuo. The residue was dissolved and refluxed in aqueous HCl (6 N, 20 mL) for 2 h. After cooling to rt, the mixture was evaporated to dryness. The residue was dissolved in CH_3OH (100 mL), treated with an ethereal CH_2N_2 solution at -20 °C, and allowed to stand overnight. Evaporation gave a brown oil which was redissolved in CH_3OH (10 mL). To this solution was added 2,2,3,3-tetramethoxybutane (0.918 g, 5.15 mmol), $(CH_3O)_3CH$ (4.8 mL, 43 mmol), and (±)-10-camphorsulfonic acid (0.05 g, 0.2 mmol) and the solution was refluxed for 18 h. The resulting dark brown solution was treated with powdered $NaHCO_3$ (2 g) and concentrated to a brown residue, which was purified by flash chromatography (hexane/EtOAc, 1:1, v/v) to afford carbahydroxymalonate intermediate **37** as a colorless oil (1.49 g, 80%): ^1H NMR ($CDCl_3$) δ 3.99 (ddd, J = 12, 10, 5 Hz, 1 H), 3.95 (d, J = 4 Hz, 1 H), 3.77 (s, 3 H), 3.73 (s, 3 H), 3.72 (s, 3 H), 3.68 (dd, J = 10, 10 Hz, 1 H), 2.69-2.80 (m, 1 H), 1.82-2.01 (m, 4 H); ^{13}C NMR ($CDCl_3$) δ 175.7, 169.1, 168.6, 99.8, 99.4, 73.4, 71.3, 67.1, 53.0, 52.3, 52.0, 49.5, 47.9, 47.8, 37.5, 36.0, 35.4, 17.7, 17.6. Anal. Calcd for $C_{19}H_{30}O_{11}$: C, 52.53; H, 6.96. Found: C, 52.72; H, 7.01.

Carbahydroxymalonate Intermediate 38. To a solution of carbahydroxymalonate intermediate **37** (0.266 g, 0.613 mmol) in THF (10 mL) at 0 °C was added solid *t*-BuOK (0.151 g, 1.35 mmol) in one portion. After 20 min, the reaction mixture was cooled to -78 °C and stirring was continued at -78 °C for 15 min. A solution of 2-benzenesulfonyl-3-phenyloxaziridine[50] (0.320 g, 1.23 mmol) in THF (2 mL) was then cannulated into the reaction flask. The resulting mixture was stirred at -78 °C for 1 h and quenched with saturated aqueous NH_4Cl (10 mL). After addition of ether, the organic layer was separated and the aqueous layer was then extracted with ether (3x). The

combined organic layers were dried, filtered, and evaporated. The resulting yellow oil was purified by flash chromatography (hexane/EtOAc, 1:2, v/v) to give carbahydroxymalonate intermediate **38** as white crystals (0.208 g, 75 %):m.p. 206-207 °C; ^{1}H NMR ($CDCl_3$) δ 4.01 (ddd, J = 12, 10, 5 Hz, 1 H), 3.95 (s, 1 H), 3.81 (s, 3 H), 3.78 (s, 3 H), 3.77 (s, 3 H), 3.76 (dd, J = 11, 10 Hz, 1 H), 3.26 (s, 3 H), 3.21 (s, 3 H), 3.12 (ddd, J = 13, 11, 3 Hz, 1 H), 1.94 (dd, J = 12, 12 Hz, 1 H), 1.92 (dd, J = 13, 13 Hz, 1 H), 1.83 (ddd, J = 13, 5, 3 Hz, 1 H), 1.72 (br, 1 H), 1.55 (dt, J = 13, 3, 3 Hz, 1 H), 1.25 (s, 3 H), 1.18 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 176.1, 171.3, 170.2, 99.6, 99.4, 78.7, 73.6, 70.0, 66.7, 53.4, 53.3, 53.1, 48.5, 47.8, 40.5, 37.7, 35.0, 17.9, 17.5. Anal. Calcd for $C_{19}H_{30}O_{12}$: C, 50.66; H, 6.71. Found: C, 50.72; H, 6.73.

Carbahydroxymalonate 8. Carbahydroxymalonate intermediate **38** (0.21 g, 0.47 mmol) was deprotected as described for carbasuccinate intermediate **32** to afford carbahydroxymalonate **8** as a colorless film (100% yield based on NMR analysis): ^{1}H NMR (D_2O, pH 8.5) δ 3.84 (ddd, J = 12, 10, 5 Hz, 1 H), 3.53 (dd, J = 10, 10 Hz, 1 H), 2.93 (ddd, J = 13, 10, 3 Hz, 1 H), 2.14 (ddd, J = 13, 5, 3 Hz, 1 H), 1.89 (dd, J = 13, 13 Hz, 1 H), 1.82 (dd, J = 13, 12 Hz, 1 H), 1.72 (ddd, J = 14, 3, 3 Hz, 1 H); ^{12}C NMR (D_2O, pH 8.5) δ 180.7, 176.3, 175.4, 82.7, 76.7, 76.5, 73.2, 46.3, 42.5, 36.3; HRMS (FAB) calcd for $C_{10}H_{11}O_{10}Na_3$ (M + H$^+$) 361.0123, found 361.0129.

Carbaacetate Intermediate 41. To a vigorously stirred mixture of carbaacetate intermediate **40**[13m] (3.33 g, 11.0 mmol) in $CH_3CN/CCl_4/H_2O$ (16 mL/16 mL/24 mL) were added $NaIO_4$ (9.89 g, 46.2 mmol) and $RuCl_3 \cdot 7H_2O$ (0.063 g, 0.24 mmol). Stirring was continued overnight at rt. The biphase reaction mixture was filtered through Celite to remove suspended solids and allow the organic and aqueous layers to separate. After extraction of the aqueous layer with CH_2Cl_2 (3x), the combined organic layeres were dried, concentrated, redissolved in CH_3OH (20 mL), and treated with an ethereal CH_2N_2 solution at -20 °C. The solvent was evaporated and the residue was purified by flash chromatography (hexane/EtOAc, 1:1, v/v). Carbaacetate intermediate **41** was obtained as a

yellow oil (2.39 g, 65%): ^{1}H NMR ($CDCl_3$) δ 8.02-8.05 (m, 2 H), 7.58-7.64 (m, 1 H), 7.45-7.50 (m, 2 H), 5.14 -5.16 (m, 1 H), 4.91-4.93 (m, 1 H), 3.83 (br, 1 H), 3.66 (s, 3 H), 2.82-2.88 (m, 1 H), 2.39-2.75 (m, 5 H), 1.87 (d, J = 16 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 178.5, 171.9, 165.0, 133.6, 129.6, 129.5, 129.0, 128.6, 128.5, 75.3, 71.7, 70.4, 51.6, 38.4, 36.6, 36.5, 33.2; HRMS (FAB) calcd for $C_{17}H_{18}O_7$ (M + H^+) 335.1130, found 335.1133.

Carbaacetate 4. Carbaacetate intermediate **41** (0.23 g, 0.69 mmol) was stirred in a mixture of THF (10 mL) and aqueous NaOH (0.2 N, 10 mL) for 24 h. The aqueous layer was washed with EtOAc (1x), passed down a Dowex 50 (H^+) column, and concentrated in vacuo to yield carbaacetate **4** as a colorless film (100% yield based on NMR analysis): ^{1}H NMR (D_2O, pH 8.1) δ 3.77 (ddd, J = 12, 10, 5 Hz, 1 H), 3.23 (dd, J = 10, 10 Hz, 1 H), 2.08-2.34 (m, 4 H), 1.71-1.94 (m, 3 H); ^{13}C NMR (D_2O, pH 8.1) δ 181.1, 180.2, 80.1, 76.8, 73.1, 42.4, 40.5, 39.5, 37.7; HRMS (FAB) calcd for $C_9H_{12}O_7Na_2$ (M + H^+) 279.0457, found 279.0450.

Synthesis of Mercaptan 47 and Amine 48.

Epoxide 50. Tosylate **49**[73a] (28.1 g, 70.2 mmol) was dissolved in HOAc/H_2O/THF (4:2:1, v/v/v, 385 mL) and heated at 70 °C for 4 h. Solvents were removed, and the residue was azotroped with toluene (4x) and lyophilized overnight. The resulting solid was dissolved in THF (600 mL) and treated with DBU (10.6 mL, 70.9 mmol) at 0 °C. After stirred at rt for 12 h, the reaction mixture was concentrated under reduced pressure. The residue was purified by flash chromatography (hexane/EtOAc, 1:1, 1:3, 1:5, v/v) to give epoxide **50** (8.98 g, 68%) as a colorless liquid: ^{1}H NMR ($CDCl_3$) δ 4.66 (br, 1 H), 4.40, (d, J = 8 Hz, 1 H), 4.03-4.11 (m, 1 H), 3.77 (s, 3 H), 3.35-3.41 (m, 2 H), 2.43 (ddd, J = 15, 5, 1 Hz, 1 H), 2.00-2.06 (m, 1 H), 1.97 (d, J = 15 Hz, 1 H), 1.78 (dd, J = 13, 10 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 174.8, 73.3, 65.2, 54.8, 52.9,

52.6, 37.5, 32.7. Anal. Calcd for $C_8H_{12}O_5 \cdot 1/2H_2O$: C, 48.73; H, 6.64. Found: C, 48.84; H, 6.68.

MOM-protected Epoxide 51. To a solution of epoxide **50** (9.20 g, 48.9 mmol) in CH_2Cl_2 (100 mL) was added *N,N'*-diisopropylethyl amine (42.6 mL, 244 mmol), chloromethyl methyl ether (14.9 mL, 196 mmol) and DMAP (0.597 g, 4.89 mmol). After 24 h and 48 h, more *N,N'*-diisopropylethyl amine (8.0 mL, 46 mmol) and chloromethyl methyl ether (3.5 mL, 46 mmol) were added. When complete, the reaction mixture was concentrated to a brown oil which was partitioned between EtOAc and water. The aqueous layer was extracted with EtOAc (3x). Organic layers were combined and washed with saturated aqueous $CuSO_4$ (1x), saturated aqueous $NaHCO_3$ (1x) and brine (1x). Drying and concentration of the organic layer gave a brown oil which was purified by flash chromatography (hexane/EtOAc, 1:1, 1:2, v/v) to give MOM-protected epoxide **51** (9.20 g, 68%) as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 4.66 (br, 1 H), 4.77 (d, J = 8 Hz, 1 H), 4.75 (s, 2 H), 4.58 (d, J = 8 Hz, 1 H), 3.86 (ddd, J = 11, 5, 2 Hz, 1 H), 3.77 (s, 3 H), 3.41 (s, 3, H), 3.35 (s, 3 H), 3.31-3.34 (m, 2 H), 2.78 (ddd, J = 15, 5, 2 Hz, 1 H), 2.25 (ddd, J = 12, 6, 2 Hz, 1 H), 1.81-1.94 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 173.0, 95.5, 92.2, 77.6, 71.4, 56.0, 55.3, 53.6, 52.5, 51.5, 33.5, 30.9. Anal. Calcd for $C_{12}H_{20}O_7$: C, 52.16; H, 7.30. Found: C, 52.19; H, 7.24.

Thioacetate 52. To a solution of MOM-protected epoxide **51** (2.51 g, 9.08 mmol) in pyridine (5.88 mL) at 0 °C was added thioacetic acid (2.60 mL, 36.9 mmol). The reaction mixture was warmed to rt, stirred for 2 days. Concentration gave a yellow oil which was purified by flash chromatography (hexane/EtOAc = 5:1, 1:1, 1:2, 1:4, v/v) to give thioacetate **52** (2.52 g, 80%) as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 4.83 (d, J = 8 Hz, 1 H), 4.78 (d, J = 7 Hz, 1 H), 4.73 (d, J = 1 Hz, 1 H), 4.71 (d, J = 8 Hz, 1 H), 4.02-4.10 (m, 2 H), 3.73 (s, 3 H), 3.47 (s, 3 H), 3.46-3.54 (m, 1 H), 3.42 (s, 3 H), 3.24 (br, 1 H), 2.39-2.53 (m, 2 H), 2.36 (s, 3 H), 1.93-2.05 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 195.8,

173.6, 97.1, 93.0, 77.9, 76.5, 73.1, 56.7, 56.3, 52.7, 41.3, 36.7, 34.6, 31.0. Anal. Calcd for $C_{14}H_{24}O_8S$: C, 47.71; H, 6.86; S, 9.10. Found: C, 47.67; H, 6.94; S, 9.21.

BBA-protected mercapto alcohol 53. Thioacetate **52** (3.40 g, 9.65 mmol), butane-2,3-dione (1.02 mL, 11.6 mmol), trimethyl orthoformate (3.80 mL, 34.7 mmol), and (±)-10-camphorsulphonic acid (0.112 g, 0.05 mmol) were dissolved in degassed MeOH (40 mL) and heated under reflux for 20 h. After neutalized with Et_3N (1 mL), the reaction mixture was concentrated under reduce pressure to give a brown oil which was purified by flash chromatography (hexane/EtOAc, 1:1, 1:2, 1:4, v/v) to give BBA-protected mercapto alcohol **53** (1.71 g, 53%) as a white solid: ^{1}H NMR ($CDCl_3$) δ 4.60 (br, 1 H), 4.08-4.12 (m, 1 H), 3.78 (s, 3 H), 3.73 (dd, J = 11, 3 Hz, 1 H), 3.54 (ddd, J = 13, 11, 4 Hz, 1 H), 3.39 (s, 3 H), 3.32 (s, 3 H), 3.24 (br, 1 H), 2.25 (ddd, J = 15, 3, 3 Hz, 1 H), 2.07 (dd, J = 15, 3 Hz, 1 H), 1.92 (ddd, J = 13, 4, 3 Hz, 1 H), 1.81 (dd, J = 13, 13 Hz, 1 H), 1.40 (s, 3 H), 1.37 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 173.8, 101.1, 87.4, 74.4, 74.2, 68.7, 52.5, 49.1, 48.2, 37.6, 37.0, 30.0, 19.9, 17.3. Anal. Calcd for $C_{14}H_{24}O_7S$: C, 49.98; H, 7.19; S, 9.53. Found: C, 49.77; H, 7.14; S, 9.81.

BBA-protected Ketol 54. In the following order, BBA-protected mercapto alcohol **53** (1.80 g, 5.53 mmol), 4 Å molecular sieves (2.68 g), 4-methylmorpholine *N*-oxide (0.940 g, 8.03 mmol), and tetrapropylammonium peruthenate[45] (0.094 g, 0.268 mmol) were added to a vigorously stirred mixture of CH_2Cl_2 (11 mL) and CH_3CN (1.1 mL). After stirred at rt for 18 h, the reaction mixture was concentrated to a brown oil which was purified by flash chromatography (hexane/EtOAc, 1:1, 1:2, 1:4, v/v) to give BBA-proteted ketol **54** (1.27 g, 71%) as a white solid: ^{1}H NMR ($CDCl_3$) δ 4.45 (d, J = 11 Hz, 1 H), 3.84 (s, 3 H), 3.61 (ddd, J = 13, 11, 4 Hz, 1 H), 3.46 (br, 1 H), 3.37 (s, 3 H), 3.30 (s, 3 H), 2.95 (d, J = 14 Hz, 1 H), 2.58 (dd, J = 14, 3 Hz, 1 H), 2.29 (dd, J = 14, 13 Hz, 1 H), 1.94 (ddd, J = 14, 4, 3 Hz, 1 H), 1.49 (s, 3 H), 1.38 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 199.2, 173.6, 101.5, 87.6, 77.7, 75.6, 53.4, 49.4, 49.3, 48.7, 37.0, 36.3,

19.9, 17.3. Anal. Calcd for $C_{14}H_{22}O_7S$: C, 50.28; H, 6.63; S, 9.53. Found: C, 49.93; H, 6.54; S, 9.45.

BBA-protected Mercapto (*E*)-Cyclohexylidene Phosphonate 55. To a solution of tetramethyl methylenediphosphonate (0.563, 2.42 mmol) in THF (10 mL) at -78 °C was slowly added *n*-BuLi (2.5 M in hexane, 0.97 mL, 2.42 mmol). After 10 min at -78 °C, the resulting solution was added into a solution of BBA-protected ketol **54** (0.6755 g, 2.02 mmol) in THF (5 mL) at -78 °C. Ten min later the reaction mixture was warmed to rt and was stirred at rt overnight. Acetic acid (1 mL) was added and solvents were removed under reduced pressure. The resulting yellow oil was purified by radial chromatography (4 mm thickness, hexane/EtOAc, 1:2, 1:4, v/v) to afford BBA-protected mercapto (*E*)-cyclohexylidene phosphonate **55** as a white foam (0.479 g, 54%): ^{1}H NMR ($CDCl_3$) δ 6.05 (d, J = 18 Hz, 1 H), 4.27 (ddd, J = 10, 3, 2 Hz, 1 H), 3.78 (s, 3 H), 3.76 (d, J = 10 Hz, 1 H), 3.72 (d, J = 10 Hz, 1 H), 3.61 (dd, J = 14, 2 Hz, 1 H), 3.37 (s, 3 H), 3.28 (s, 3 H), 3.23-3.33 (m, 1 H), 2.55 (dd, J = 14, 3 Hz, 1 H), 2.01 (dd, J = 14, 13 Hz, 1 H), 1.86 (ddd, J = 14, 4, 3 Hz, 1 H), 1.44 (s, 3 H), 1.38 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 174.5, 156.6 (J_{PCC} = 8 Hz), 109.7 (J_{PC} = 190 Hz), 101.4, 87.5, 74.6, 74.3 (J_{PCCC} = 17 Hz), 52.8, 52.4 (J_{POC} = 6 Hz), 52.0 (J_{POC} = 6 Hz), 49.4, 48.4, 39.3 (J_{PCCC} = 8 Hz), 37.8, 36.9, 19.9, 17.5. Anal. Calcd for $C_{17}H_{29}O_9PS$: C, 46.36; H, 6.64; S, 7.28. Found: C, 46.46; H, 6.86; S, 7.04.

Mercapto (*E*)-Cyclohexylidene Phosphonate 47. BBA-protected mercapto (*E*)-cyclohexylidene phosphonate **55** (0.300 g, 0.681 mmol) was dissolved in CH_2Cl_2, and then Et_3N (0.50 mL, 3.6 mmol) and TMSBr (1.0 mL, 7.6 mmol) were added at rt. After 18 h, the brown reaction mixture was concentrated in vacuo. Aqueous NaOH (0.5 N, 8 mL) and THF (8 mL) were added, and the resulting solution was stirred vigorously at 4 °C. After 3 h, the solution was passed through a short column of Dowex 50 (H^+), which was rinsed with MeOH (3x). Eluates were combined, concentrated and azotroped with toluene (3x). The resulting foam was dissolved in CH_2Cl_2 (3 mL) and treated with

CF_3CO_2H (4 mL) and 1,2-ethanedithiol (0.4 mL) at 0 °C. After stirred at 0 °C for 2 h, the mixture was concentrated in vacuo, and the flask was rinsed with CH_2Cl_2 (4x3 mL) to remove the remaining 1,2-ethanedithiol. Further concentration gave mercapto (*E*)-cyclohexylidene phosphonate **47** as an off-white foam (0.176 g, 91%): ^{1}H NMR (D_2O) δ 6.68 (d, J = 18 Hz, 1 H), 4.03 (d, J = 10 Hz, 1 H), 3.44 (d, J = 15 Hz, 1 H), 3.05 (ddd, J = 10, 10, 6 Hz, 1 H), 2.66-2.72 (m, 1 H), 2.24-2.79 (m, 2 H); ^{13}C NMR (D_2O) δ 180.0, 159.7(J_{PCc} = 7 Hz), 115.9 (J_{PC} = 184 Hz), 79.6 (J_{PCCC} = 18 Hz), 77.7, 45.3, 44.1, 40.7 (J_{PCCC} = 8 Hz); ^{31}P NMR ($CDCl_3$) δ 16.7; HRMS (FAB) calcd for $C_8H_{12}O_7PS$ (M - H^+) 283.0041, found 283.0038.

Azido Alcohol 56. To a solution of MOM-protected epoxide **51** (9.20 g, 33.3 mmol) in MeOH/H_2O (270 mL, 8:1, v/v) was added NaN_3 (10.8 g, 166 mmol) and NH_4Cl (3.99 g, 74.6 mmol).[73,76] After the mixture was heated at 75 °C for 24 h, water was added to dissolve precipitated salts, and the solution was concentrated to remove methanol. The resulting aqueous phase was diluted to 100 mL and extracted with EtOAc (6x). Organic layers were combined and washed with brine (1x), dried and concentrated. The resulting yellow oil was purified by flash chromatography (hexane/EtOAc, 1:1, v/v) to afford azido alcohol **56** as a yellow oil (9.57 g, 90%): ^{1}H NMR ($CDCl_3$) δ 4.84 (d, J = 8 Hz, 1 H), 4.75 (d, J = 7 Hz, 1 H), 4.71 (d, J = 8 Hz, 1 H), 4.62 (d, J = 8 Hz, 1 H), 4.01-4.06 (m, 1 H), 3.92 (ddd, J = 9, 8, 4 Hz, 1 H), 3.75 (s, 3 H), 3.57-3.63 (m, 1 H), 3.44 (s, 3 H), 3.39 (s, 3 H), 3.15 (br, 1 H), 2.34-2.41 (m, 1 H), 2.25-2.32 (m, 1 H), 2.09 (dd, J = 15, 3 Hz, 1 H), 1.94 (dd, J = 14, 9 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 172.6, 96.4, 92.5, 77.5, 75.1, 72.3, 58.9, 56.2, 55.8, 52.5, 35.2, 33.0. Anal. Calcd for $C_{12}H_{21}O_7N_3$: C, 45.13; H, 6.63; N, 13.16. Found: C, 44.94; H, 6.68; N, 12.89.

Protected Azido Alcohol 57. To a suspension of NaH (0.936 g, 39.0 mmol) in THF (100 mL) at 0 °C was added a soluton of azido alcohol **56** (9.57 g, 30.0 mmol) in THF (40 mL). After stirring at rt for 20 min, BnBr (3.92 mL, 33 mmol) was added, followed by $Bu_4N^+I^-$ (0.554 g, 1.5 mmol). The mixture was stirred at rt overnight,

quenched by saturated aqueous NH_4Cl solution at 0 °C, and extracted with ether (6x). Organic layers were combined, washed with saturated aqueous $NaHCO_3$ (1x) and brine, dried and concentrated. The resulting residue was purified by flash chromatography (hexane/EtOAc, 5:1, 1:1, v/v) to afford protected azido alcohol **58** as a yellow oil (8.30 g, 68%): ^{1}H NMR ($CDCl_3$) δ 7.27-7.41 (m, 5 H), 4.84 (d, J = 8 Hz, 1 H), 4.64-4.79 (m, 4 H), 4.60 (d, J = 8 Hz, 1 H), 4.12 (ddd, J = 6, 3, 3 Hz, 1 H), 4.07 (ddd, J = 8, 8, 4 Hz, 1 H), 3.74 (s, 3 H), 3.43 (dd, J = 8, 3 Hz, 1 H), 3.38 (s, 6 H), 2.31-2.38 (m, 1 H), 2.24-2.29 (m, 1 H), 2.04-2.11 (m, 1 H), 1.97 (dd, J = 14, 8 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 172.7, 137.7, 128.3, 127.7 (2), 95.5, 92.4, 79.3, 77.3, 72.3, 70.4, 57.4, 56.2, 55.6, 52.4, 35.6, 32.7. Anal. Calcd for $C_{19}H_{27}O_7N_3$: C, 55.73; H, 6.65; N, 10.26. Found: C, 55.87; H, 6.62; N, 10.09.

Azido Ketol 58. To a solution of protected azido alcohol **58** (8.30 g, 20.3 mmol) in MeOH (100 mL) was added acetyl chloride (2.88 mL, 40.5 mmol). After stirring at rt overnight, the reaction mixture was concentrated under reduced pressure. The resulting yellow oil was dissolved in $CHCl_3$ (60 mL) in a flasked equipped with an overhead stirrer. In the following order, water (60 mL), KIO_4 (9.34 g, 40.6 mmol), K_2CO_3 (0.701 g, 5.08 mmol), and ruthenium trichloride (0.701g, 1.22 mmol) were added to the reaction flask. Vigorous stirring was continued at rt until the reaction was complete. The mixture was filtered through Celite and the aqueous and organic phases separated. After saturation with NaCl, the aqueous layer was extracted with EtOAc (3x). The combined organic layers were dried and concentrated to an oil, which was purified by flash chromatography (hexane/EtOAc, 1:1, 1:2, v/v) to afford azido ketol **58** as a white solid (4.80 g, 74%): ^{1}H NMR ($CDCl_3$) δ 7.30-7.45 (m, 5 H), 4.94 (d, J = 12 Hz, 1 H), 4.59 (d, J = 12 Hz, 1 H), 4.07 (ddd, J = 11, 10, 5 Hz, 1 H), 3.97 (dd, J = 10, 1 Hz, 1 H), 3.78 (s, 3 H), 3.70 (br, 1 H), 2.85 (d, J = 14 Hz, 1 H), 2.56 (dd, J = 14, 3 Hz, 1 H), 2.22 (ddd, J = 14, 5, 1 Hz, 1 H), 2.10 (dd, J = 14, 12 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 202.0, 173.4, 136.9, 128.3, 128.1, 127.9, 85.6, 73.7, 73.1, 60.9, 53.4, 48.8, 38.4.

Anal. Calcd for $C_{15}H_{17}O_5N_3$: C, 56.42; H, 5.31; N, 13.19. Found: C, 56.46; H, 5.31; N, 13.19.

Boc-protected Amino Ketol 59. Azido ketol **58** (1.50 g, 4.70 mmol) and di-*tert*-butyl dicarbonate (1.23 g, 5.64 mmol) were dissolved in EtOAc (10 mL) and hydrogenated over 10% Pd on C (0.3 g) at 50 psi H_2 pressure for 12 h. The mixture was filtered through Celite, and the filtrate was concentrated in vacuo. Purification by flash chromatrograpy (hexane/EtOAc, 1:2, v/v, EtOAc) gave Boc-protected amino ketol **59** as a white foam (1.45 g, 78%): ^{1}H NMR ($CDCl_3$) δ 7.27-7.40 (m, 5 H), 4.98 (d, J = 8 Hz, 1 H), 4.90 (d, J = 12 Hz, 1 H), 4.46 (d, J = 12 Hz, 1 H), 4.26 (br, 1 H), 3.92-4.20 (m, 1 H), 3.78 (s, 3 H), 3.69 (m, 1 H), 2.90 (d, J = 14Hz, 1 H), 2.57 (dd, J = 14, 3 Hz, 1 H), 2.50-2.55 (m, 1 H), 2.23-2.31 (m, 1 H), 1.43 (s, 9 H); ^{13}C NMR ($CDCl_3$) δ 203.2, 174.0, 155.4, 137.6, 128.3, 128.0, 127.8, 83.5, 79.8, 73.8, 72.6, 53.2, 51.4, 48.9, 28.3. Anal. Calcd for $C_{20}H_{27}O_7N$: C, 61.05; H, 6.92; N, 3.56. Found: C, 60.73; H, 6.73; N, 3.25.

Boc-protected Amino (*E*)-Cyclohexylidene Phosphonate 60. This was made from Boc-protected azido ketol **59** according to the procedure for BBA-protected mercapto (*E*)-cyclohexylidene phosphonate **55**. Boc-protected amino (*E*)-cyclohexylidene phosphonate **60** was obtained as a white foam (38%): ^{1}H NMR ($CDCl_3$) δ 7.32-7.40 (m, 5 H), 6.06 (d, J = 18 Hz, 1 H), 4.81 (d, J = 8 Hz, 1 H), 4.76 (d, J = 12 Hz, 1 H), 4.50 (d, J = 12 Hz, 1 H), 4.00-4.08 (m, 1 H), 3.89 (br, 1 H), 3.78 (s, 3 H), 3.76 (d, J = 3 Hz, 3 H), 3.74-3.81 (m, 1 H), 3.72 (d, J = 3 Hz, 3 H), 3.64 (d, J = 14 Hz, 1 H), 2.51 (dd, J = 14, 3 Hz, 1 H), 2.16-2.20 (m, 2 H), 1.48 (s, 9 H); ^{13}C NMR ($CDCl_3$) δ 174.6, 158.4 (J_{PCC} = 8 Hz), 155.3, 137.4, 128.4, 127.9 (2), 110.5 (J_{PC} = 189 Hz), 82.6 (J_{PCCC} = 18 Hz), 79.5, 74.0, 72.9, 53.2, 52.8, 55.6 (J_{POC} = 6 Hz), 52.1 (J_{POC} = 6 Hz), 39.3, 39.1 (J_{PCCC} = 8 Hz), 28.3. Anal. Calcd for $C_{23}H_{34}O_9NP$: C, 55.30; H, 6.86; N, 2.80. Found: C, 55.26; H, 6.79; N, 2.67.

Amino (*E*)-Cyclohexylidene Phosphonate 48. To a Boc-protected amino (*E*)-cyclohexylidene phosphonate **60** (0.462 g, 0.920 mmol) in CH_2Cl_2 (7 mL) at -78 °C was added boron tribromide (2.62 mL, 27.7 mmol). After 30 min, the cold bath was removed, and the mixture was stirred at rt overnight. Solvents were removed, and the residue was dissolved in aqueous 6 N HCl (5 mL) and refluxed for 3 h. Concentration under vacuo afforded amino (*E*)-cyclohexylidene phosphonate **48** as a yellow foam (0.246 g, 100%): ^{1}H NMR (D_2O, pH 7.9) δ 6.12 (d, J = 16 Hz, 1 H), 4.31 (d, J = 10 Hz, 1 H), 3.53 (d, J = 14 Hz, 1 H), 3.38 (ddd, J = 10, 10, 7 Hz, 1 H), 2.66 (d, J = 14 Hz, 1 H), 2.29-2.33 (m, 2 H); ^{13}C NMR (D_2O) δ 178.7, 157.3(J_{PCc} = 7 Hz), 117.0 (J_{PC} = 184 Hz), 76.0, 74.5 (J_{PCCC} = 19 Hz), 54.7, 39.8 (J_{PCCC} = 8 Hz), 38.2; ^{31}P NMR ($CDCl_3$) δ 16.3; HRMS (FAB) calcd for $C_8H_{13}O_7NP$ ($M - H^+$) 266.0430, found 266.0419.

Synthesis of 1,5-Anhydro-D-glucose 6-phosphate 67.

2,3,4-Tri-*O*-benzyl-6-*O*-triphenylmethyl-1,5-anhydro-D-glucose 79. 1,5-Anhydro-D-glucose (3.53 g, 21.5 mmol) and trityl chloride (5.99 g, 21.5 mmol) were dissolved in pyridine (25 mL) and heated at 75 °C for 24 h. The reaction mixture was poured into an ice-water mixture, acidified to pH 4 with aqueous 1 N HCl. Then the resulting mixture was extracted with CH_2Cl_2 (5x). Organic layers were combined, washed with saturated aqueous $NaHCO_3$ (1x) and brine (1x), dried, and concentrated to a yellow oil, which was purified by flash chromatography (hexane/EtOAc, 1:2, 1:4, v/v, EtOAc) to afford 6-*O*-triphenylmethyl-1,5-anhydro-D-glucose as an off-white solid (7.44 g, 85%). This was used directly in the following step.

To a suspension of NaH (1.93 g, 80.4 mmol) in DMF (100 mL) at 0 °C was added a soluton of 6-*O*-triphenylmethyl-1,5-anhydro-D-glucose (8.17 g, 20.1 mmol) in DMF (30 mL). After stirring at rt for 20 min, BnBr (7.41 mL, 62.3 mmol) was added. The mixture was stirred at rt overnight, quenched by MeOH (2 mL) at 0 °C, concentrated in vacuo to

give a yellow oil which was extracted with ether (6x). Organic layers were combined, washed with saturated aqueous $NaHCO_3$ (1x) and brine, dried and concentrated. The resulting residue was purified by flash chromatography (hexane/EtOAc, 5:1, 1:1, v/v) to afford 2,3,4-tri-*O*-benzyl-6-*O*-triphenylmethyl-1,5-anhydro-D-glucose **79** as a yellow oil (12.0 g, 88%): ^{1}H NMR ($CDCl_3$) δ 7.46-7.49 (m, 6 H), 7.14-7.37 (m, 22 H), 6.85-6.88 (m, 2 H), 4.95 (d, J = 11 Hz, 1 H), 4.84 (d, J = 11 Hz, 1 H), 4.66-4.75 (m, 3 H), 4.34 (d, J = 10 Hz, 1 H), 4.16 (dd, J = 14, 1 Hz, 1 H), 3.71-3.78 (m, 2 H), 3.55-3.64 (m, 2 H), 3.34-3.37 (m, 1 H), 3.16-3.78 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 143.8, 138.6, 138.3, 137.9, 128.7, 128.4 (2), 128.1, 128.0, 127.7, 127.6, 127.5, 126.9, 86.4, 86.2, 79.5, 78.7, 77.8, 75.7, 74.9, 73.2, 68.2, 62.6. Anal. Calcd for $C_{46}H_{44}O_5$: C, 81.63; H, 6.55. Found: C, 81.90; H, 6.62.

2,3,4-Tri-*O*-benzyl-1,5-anhydro-D-glucose 80. 2,3,4-Tri-*O*-benzyl-6-*O*-triphenylmethyl-1,5-anhydro-D-glucose **79** (12.0 g, 17.7 mmol) was dissolved in HCO_2H-ether (1:1, v/v, 100 mL). After the mixture was stirred at rt 1 h, ether and water were added, and phases were separated. The organic phase was washed with saturated aqueous $NaHCO_3$ and brine repeatedly until pH of the organic phase was neutral. Concentration of the organic layer gave an oil which was purified by flash chromatography (hexan/EtOAc, 1:1, 1:2, v/v) to afford fully protected 2,3,4-tri-*O*-benzyl-1,5-anhydro-D-glucose **80** as a colorless oil (4.6 g, 60%): ^{1}H NMR ($CDCl_3$) δ 7.28-7.35 (m, 15 H), 4.98 (d, J = 11 Hz, 1 H), 4.89 (d, J = 5 Hz, 1 H), 4.85 (d, J = 5 Hz, 1 H), 4.72 (d, J = 12 Hz, 1 H), 4.63 (d, J = 11 Hz, 1 H), 4.62 (d, J = 12 Hz, 1 H), 3.99 (dd, J = 11, 5 Hz, 1 H), 3.82 (dd, J = 12, 3 Hz, 1 H), 3.56-3.69 (m, 3 H), 3.48 (dd, J = 10, 9 Hz, 1 H), 3.27 (ddd, J = 10, 5, 3 Hz, 1 H), 3.22 (dd, J = 11, 10 Hz, 1 H), 1.82 (br, 1 H); ^{13}C NMR ($CDCl_3$) δ 138.6, 138.1, 138.0, 128.4 (2), 128.0, 127.9, 127.8, 127.6, 86.2, 79.7, 78.5, 77.6, 75.5, 75.1, 73.3, 67.9, 62.2. Anal. Calcd for $C_{27}H_{30}O_5$: C, 74.63; H, 6.96. Found: C, 74.49; H, 6.86.

2,3,4-Tri-*O*-benzyl-6-dibenzoxyphosphinoyl-1,5-anhydro-D-glucose 81. To a solution of 2,3,4-tri-*O*-benzyl-1,5-anhydro-D-glucose **80** (2.0 g, 4.60 mmol) in THF (20 mL) at -78 °C was added *n*-BuLi (1.6 M in hexane, 3.45 mL, 5.52 mmol). After the mixture was stirred at -78 °C for 10 min, a solution of tetrabenzyl pyrophosphate (3.22 g, 5.98 mmol) in THF (20 mL) was added into the reaction flask. After stirring 30 min at -78 °C, 2 h at 0 °C, and another 2h at rt, the reaction mixture was filtered, and the precipitates were washed with ether. The filtrate and washings were combined, washed with brine (1x), and concentrated to a brown oil which was purified by radial chromatography (4 mm thickness, hexane/EtOAc, 2:1, 1:1, v/v) to afford 2,3,4-tri-*O*-benzyl-6-dibenzoxyphosphinoyl-1,5-anhydro-D-glucose **81** as a yellow oil (2.71 g, 85%): ^{1}H NMR ($CDCl_3$) δ 7.24-7.35 (m, 25 H), 5.02 (d, J = 8 Hz, 4 H), 4.97 (d, J = 11 Hz, 1 H), 4.85 (s, 1 H), 4.81 (s, 1 H), 4.67 (d, J = 12 Hz, 1 H), 4.59 (d, J = 12 Hz, 1 H), 4.56 (d, J = 11 Hz, 1 H), 4.27 (ddd, J = 11, 6, 2 Hz, 1 H), 4.16 (ddd, J = 11, 7, 5 Hz, 1 H), 3.96 (dd, J = 11, 5 Hz, 1 H), 3.51-3.65 (m, 2 H), 3.44 (dd, J = 10, 8 Hz, 1 H), 3.31-3.36 (m, 1 H), 3.15 (dd, J = 11, 10 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 138.5, 138.0, 137.8, 135.8 (J_{POCC} = 7 Hz), 135.7 (J_{POCC} = 7 Hz), 128.4, 128.3 (2), 128.2, 127.8 (2x), 127.7 (2x), 127.5, 86.0, 78.2, 78.1 (J_{POC} = 7 Hz), 77.0, 75.4, 75.0, 73.1, 69.1 (J_{POC} = 6 Hz), 67.8, 66.5 (J_{POCC} = 6 Hz). Anal. Calcd for $C_{41}H_{43}O_8P$: C, 70.88; H, 6.24. Found: C, 70.72; H, 6.18.

1,5-Anhydro-D-glucose 6-phosphate 67. A solution of 2,3,4-tri-*O*-benzyl-6-dibenzoxyphosphinoyl-1,5-anhydro-D-glucose **81** (1.03 g, 1.48 mmol) was dissolved in THF/H_2O (1:1, v/v, 20 mL) and hydrogenated over 10% Pd on C (0.3 g) at 50 psi H_2 pressure for 3 h. The mixture was filtered through Celite, and the filtrate was concentrated in vacuo. The resulting residue was diluted to 100 mL, and the pH was adjusted to 7.5 with aqueous NaOH. This solution was applied to AG-1 X8 anion exchange resin (200 mL) which had been equilibrated with 100 mM $Et_3NH^+HCO_3^-$ (pH 7.5). After washing with water (100 mL), the column was eluted with a linear gradient (600 mL + 600 mL,

100-300 mM) of $Et_3NH^+HCO_3^-$ (pH 7.5). Fractions containing phosphorus were identified by the methods of Avila and Ames, concentrated, azotroped with 2-propanol (6x), dissolved in water, passed through a short column of Dowex 50 (H^+), and concentrated to dryness. 1,5-Anhydro-D-glucose 6-phosphate **67**was obtained as a colorless film (95%): ^1H NMR (D_2O) δ 4.04-4.22 (m, 2 H), 3.97 (dd, J = 11, 5 Hz, 1 H), 3.55-3.63 (m, 1 H), 3.41-3.46 (m, 3 H), 3.29 (dd, J = 11, 11 Hz, 1 H); ^{13}C NMR (D_2O) δ 81.5 (J_{POCC} = 8 Hz), 80.1, 72.0, 71.9, 71.7, 67.9 (J_{POC} = 5 Hz); ^{31}P NMR (D_2O) δ 0.1; HRMS (FAB) calcd for $C_6H_{12}O_8P$ ($M - H^+$) 243.0270, found 243.0258.

Synthesis of 5-Thio-D-glucose 6-phosphate 68. [88b, 91]

5-Thio-D-glucose 6-phosphate 68. Hexokinase (Sigma, type C-130) was dialyzed against Tris·HCl buffer (150 mM, pH 8.2) containing $MgCl_2$ (15 mM) and EDTA (0.25 mM). 5-Thio-D-glucose **82** (0.20 g, 1.02 mmol), ATP (0.80 g, 1.45 mmol), $MgCl_2$ (1 M, 0.76 mL, 0.76 mmol), EDTA (10 mM, 0.050 mL, 0.013 mmol) were added to a 50 mL Tris·HCl buffer (150 mM, pH 8.2) at 37 °C. After being kept at 37 °C for 10 min, the reaction mixture was charged with 2000 units of dialyzed hexokinase. The resulting solution was shaken at 37 °C. Another batch of hexokinase (400 units) was added after 12 and 24 h. After 36 h, TLC indicated that the reaction was complete. The solution was passed through a short Dowex 50 (H^+) column, and the column was washed with water (50 mL). Eluates were combined, diluted to 315 mL with water, neutalized to pH 7.5 with $NH_3 \cdot H_2O$, and applied to AG-1 X8 anion exchange resin (200 mL) which had been equilibrated with 50 mM $Et_3NH^+HCO_3^-$ (pH 7.5). After washed with water (100 mL), the column was eluted with a linear gradient (800 mL + 800 mL, 50-400 mM) of $Et_3NH^+HCO_3^-$ (pH 7.5). Fractions containing phosphorus were identified by the methods of Avila and Ames, concentrated, azotroped with 2-propanol (6x), dissolved in water, passed down a short column of Dowex 50 (H^+), and concentrated to dryness. 5-Thio-D-

glucose 6-phosphate **68** was obtained as a colorless film (89%): β-anomer: ^{1}H NMR (D_2O) δ 5.01 (d, J = 3 Hz, 1 H), 4.28-4.36 (m, 1 H), 4.15 (ddf, J = 11, 6, 3 Hz, 1 H), 3.73-3.81 (m, 1 H), 3.65-3.68 (m, 2 H), 3.30-3.35 (m, 1 H); ^{13}C NMR (D_2O) δ 77.9, 76.2, 75.9, 75.6, 67.1 (J_{PC} = 5 Hz), 44.0 (J_{POC} = 5 Hz); ^{31}P NMR (D_2O) δ 0.0; HRMS (FAB) calcd for $C_6H_{12}O_8PS$ ($M - H^+$) 274.9991, found 274.9998.

Synthesis of Vinylphosphonates 84-89.

4-Methoxybenzyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside 92. A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (20.5 g, 47.7 mmol) in CH_2Cl_2 (50 mL) was slowly added to a stirring suspension of 4-methoxybenzyl alcohol (11.9 mL, 95.4 mmol), HgBr (0.35 g, 0.95 mmol), yellow HgO (11.7 g, 52.5 mmol), and drierite (6 g) in CH_2Cl_2 (150 mL) at rt. The resulting suspension was stirred at rt overnight, filtered through Celite, and the residue washed with CH_2Cl_2. The filtrate and washings were combined, washed with water (1x), aqueous KBr (1 N, 1x), saturated aqueous $NaHCO_3$ (1x), and brine (1x). Drying and concentration of the organic layer gave a yellow oil which was purified by flash chromatography (hexane/EtOAc, 5:1, 1:1, v/v) to afford the tetraacetate **92** as a yellow oil (19.0 g, 85%): ^{1}H NMR ($CDCl_3$) δ 7.21 (d, J = 9 Hz, 2 H), 6.88 (d, J = 9 Hz, 2 H), 5.00-5.20 (m, 3 H), 4.82 (d, J = 12 Hz, 1 H), 4.56 (d, J = 12 Hz, 1 H), 4.52 (d, J = 8 Hz, 1 H), 4.28 (dd, J = 12, 5 Hz, 1 H), 4.19 (dd, J = 12, 2 Hz, 1 H), 3.81 (s, 3 H), 3.67 (ddd, J = 10, 5, 2 Hz, 1 H), 2.11 (s, 3 H), 2.02 (s, 3 H), 2.00 (s, 3 H), 1.99 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 170.5, 170.1, 169.3, 169.2, 159.4, 129.4, 128.4, 113.7, 98.7, 72.7, 71.7, 71.1, 70.3, 68.3, 61.9, 55.1, 20.6, 20.5 (3). Anal. Calcd for $C_{22}H_{28}O_{11}$: C, 56.40; H, 6.02. Found: C, 56.28; H, 5.73.

4-Methoxybenzyl β-D-glucopyranoside 93. Tetraacetate **92** (19.0 g, 40.6 mmol) and $NaOCH_3$ (0.110 g, 2.04 mmol) were dissolved in CH_3OH (150 mL) and stirred at rt overnight. Then the mixture was neutralized with Dowex 50 (H^+), filtered, and

concentrated to a yellow syrup. Recrystallization from EtOAc afforded the glucopyranoside **93** (10.6 g, 87%) as a white solid: m.p. = 131-133 °C; ^{1}H NMR (DMSO-d_6) δ 7.30 (d, J = 8 Hz, 2 H), 6.89 (d, J = 8 Hz, 2 H), 5.01-5.19 (br, 4 H), 4.74 (d, J = 11 Hz, 1 H), 4.49 (d, J = 11 Hz, 1 H), 4.20 (d, J = 8 Hz, 1 H), 3.72 (s, 3 H), 3.67-3.72 (m, 1 H), 3.48 (dd, J = 11, 5 Hz, 1 H), 3.01-3.16 (m, 4 H); ^{13}C NMR (DMSO-d_6) δ 158.7, 129.9, 129.4, 113.5, 101.8, 77.0, 76.8, 73.5, 70.2, 69.2, 61.2, 55.1. Anal. Calcd for $C_{14}H_{20}O_7 \cdot 1/2H_2O$: C, 54.36; H, 6.84. Found: C, 54.25; H, 6.53.

4-Methoxybenzyl 6-*O*-triphenylmethyl-β-D-glucopyranoside 94. Trityl chloride (10.1 g, 36.2 mmol) was added to a stirring suspension of glucopyranoside **93** (8.10 g, 28.3 mmol) in pyridine (100 mL) at rt. Overnight heating at 75 °C changed the suspension into a clear brown solution. Concentration gave a yellow gum which was partitioned between EtOAc and saturated aqueous NH_4Cl. After layers were separated, the organic layer was washed with saturated aqueous $CuSO_4$ (1x), saturated aqueous $NaHCO_3$ (1x), and brine (1x). Drying and concentration of the organic layer gave a yellow gum which was purified by flash chromatography (hexane/EtOAc, 1:1, 1:2, v/v) to afford the triol **94** as a yellow foam (11.7 g, 76%): ^{1}H NMR ($CDCl_3$) δ 7.42-7.48 (m, 6 H), 7.13-7.27 (m, 11 H), 6.78 (d, J = 9 Hz, 2 H), 4.77 (d, J = 11 Hz, 1 H), 4.54 (d, J = 11 Hz, 1 H), 4.22 (d, J = 5 Hz, 1 H), 3.68 (s, 3 H), 3.24-3.38 (m, 6 H); ^{13}C NMR ($CDCl_3$) δ 159.3, 143.8, 130.0, 128.9, 128.7, 127.8, 127.0, 113.8, 100.6, 86.6, 76.3, 74.6, 73.3, 71.1, 70.1, 63.9, 55.1. Anal. Calcd for $C_{33}H_{34}O_7$: C, 73.04; H, 6.32. Found: C, 72.76; H, 6.28.

4-Methoxybenzyl 2,3,4-tri-*O*-4-methoxybenzyl-6-*O*-triphenylmethyl-β-D-glucopyranoside 95. To a suspension of NaH (prewashed with hexane, 1.33g, 55.4 mmol) in DMF (50 mL) was added a solution of triol **94** (6.00 g, 11.1 mmol) in DMF (100 mL) at 0 °C. After the completion of the addition, the suspension was stirred at rt for 1 h and then charged with 4-methoxybenzyl chloride. The brown mixture was stirred at 45 °C overnight, quenched with CH_3OH (1 mL), concentrated to a black gum which was

partitioned between CH_2Cl_2 and saturated aqueous NH_4Cl. After layers were separated, the organic layer was washed with saturated aqueous $NaHCO_3$ (1x) and brine (1x). Drying and concentration of the organic layer gave a yellow oil which was purified by flash chromatography (hexane/EtOAc, 5:1, 1:1, v/v) to afford the trityl ether **95** as a yellow oil (7.65 g, 77%): ^{1}H NMR ($CDCl_3$) δ 7.52-7.62 (m, 6 H), 7.20-7.42 (m, 15 H), 6.68-6.94 (m, 10H), 5.00 (d, J = 11 Hz, 1 H), 4.92 (d, J = 11 Hz, 1 H), 4.82 (d, J = 11 Hz, 1 H), 4.67-4.76 (m, 3 H), 4.62 (d, J = 10 Hz, 1 H), 4.51 (d, J = 7 Hz, 1 H), 4.28 (d, J = 10 Hz, 1 H), 3.79 (2) (s, 3 H), 3.78 (s, 3 H), 3.75 (s, 3 H), 3.72-3.78 (m, 1 H), 3.53-3.63 (m, 3 H), 3.36-3.43 (m , 1 H), 3.25 (dd, J = 10, 4 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 159.3, 159.1 (2), 143.9, 130.8 (2), 129.8, 129.6, 129.4, 128.8, 127.7, 126.9, 113.8 (2), 113.7, 113.5, 101.9, 86.3, 84.4, 82.3, 77.6, 75.5, 74.6, 70.4, 62.4, 55.2. Anal. Calcd for $C_{57}H_{58}O_{10}$: C, 75.81; H, 6.47. Found: C, 75.84; H, 6.28.

4-Methoxybenzyl 2,3,4-tri-*O*-4-methoxybenzyl-β-D-glucopyranoside 96. Trityl ether **95** (4.20 g, 4.71 mmol) was dissolved in HCO_2H-Et_2O (1:1, v/v, 100 mL). After stirring at rt for 1h, the mixture was concentrated in vacuo to give a yellow foam. The foam was dissolved in CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$ (3x) and brine (1x). Drying and concentration of the organic layer gave a yellow foam which was purified by flash chromatography (hexane/EtOAc, 5:1, 1:1, v/v) to afford alcohol **96** as a white foam (2.0 g, 64%): ^{1}H NMR ($CDCl_3$) δ 7.16-7.3 (m, 8 H), 6.79-6.88 (m, 8 H), 4.80-4.86 (m, 3 H), 4.76 (d, J = 11 Hz, 1 H), 4.71 (d, J = 11 Hz, 1 H), 4.62 (d, J = 9 Hz, 1 H), 4.58 (d, J = 11 Hz, 1 H), 4.53 (d, J = 11 Hz, 1 H), 4.49 (d, J = 8 Hz, 1 H), 3.83 (dd, J = 12, 3 Hz, 1 H), 3.77 (s, 3 H), 3.76 (s, 6 H), 3.75 (s, 3 H), 3.66 (dd, J = 12, 5 Hz, 1 H), 3.59 (dd, J = 9, 9 Hz, 1 H), 3.49 (dd, J = 9, 9 Hz, 1 H), 3.40 (dd, J = 9, 8 Hz, 1 H), 3.30 (ddd, J = 9, 5, 3 Hz, 1 H), 1.95 (br, 1 H); ^{13}C NMR ($CDCl_3$) δ 159.3, 159.2, 159.1, 159.0, 130.7, 130.5, 130.1, 129.6 (2), 129.5, 129.4, 129.3, 113.7, 113.6 (2), 102.5, 84.2, 81.9, 77.2, 75.2, 75.0, 74.5, 74.4, 71.2, 61.9, 55.1. Anal. Calcd for $C_{38}H_{44}O_{10}$: C, 69.07; H, 6.71. Found: C, 68.99; H, 6.72.

4-Methoxybenzyl 6-deoxy-6-(*Z*)-diethoxyphosphonomethylene-2,3,4-tri-*O*-4-methoxybenzyl-β-D-glucopyranoside 97. To a stirred solution of oxalyl chloride (0.53 mL, 6.0 mmol) in CH_2Cl_2 (50 mL) at -78 °C was added DMSO (1.00 mL, 14.0 mmol) dropwise and the mixture was stirred for 10 min. Then a solution of alcohol **96** (1.60 g, 2.42 mmol) in CH_2Cl_2 (50 mL) was added into the reaction mixture. After 40 min at -78 °C, Et_3N (2.70 mL, 19.4 mmol) was added and the mixture was allowed to warm to rt and quenched with water. Ether was added and the resulting organic layer was separated and washed with aqueous $NaHSO_4$ (0.05 N, 1x), saturated aqueous $NaHCO_3$ (1x), and brine (1x). Drying and concentration of the organic layer gave the crude aldehyde which was used directly in the subsequent reaction.

Diethyl trimethylsilylmethylphosphonate (0.600 g, 2.66 mmol) was dissolved in THF (5 mL) and cooled to -78 °C. A solution of *n*-BuLi in hexane (1.6 M, 1.59 mL, 2.54 mmol) was added, and the mixture was stirred at -78 °C for 4 h. The aldehyde described above was dissolved in THF (10 mL) and added into the reaction flask. After stirring at -78 °C for 20 min, the mixture was warmed to rt. Acetic acid (3 mL) was added and the solvent was removed under reduced pressure. Purification by radial chromatography (4 mm thickness, hexane/EtOAc, 2:1, 1:1, v/v) afforded protected (Z)-vinylphosphonate **97** as a yellow oil (0.52 g, 27%): ^{1}H NMR ($CDCl_3$) δ 7.30 (d, J = 9 Hz, 2 H), 7.20 (d, J = 9 Hz, 6 H), 6.85 (d, J = 8 Hz, 2 H), 6.81 (d, J = 9 Hz, 6 H), 6.27 (ddd, J = 51, 13, 9 Hz, 1 H), 5.82 (dd, J = 16, 13 Hz, 1 H), 4.51-4.91 (m, 9H), 3.89-4.11 (m, 5 H), 3.75 (s, 6 H), 3.74 (2) (s, 3 H), 3.68 (dd, J = 9, 9 Hz, 1 H), 3.46 (dd, J = 9, 9 Hz, 1 H), 3.37 (dd, J = 9, 9 Hz, 1 H), 1.25 (dt, J = 22, 7 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 159.0, 158.9, 158.8, 147.2 (J_{PCC} = 2 Hz), 130.5, 130.4, 130.0, 129.4, 129.3, 129.2, 121.8 (J_{PC} = 182 Hz), 113.5, 113.4, 113.3, 113.2, 102.2, 83.6, 81.8, 80.4, 75.0, 74.0, 73.9, 70.9, 70.4 (J_{PCCC} = 8 Hz), 61.4 (J_{POC} = 2 Hz), 61.3 (J_{POC} = 2 Hz), 54.8, 16.0 (J_{POCC} = 6 Hz); ^{31}P NMR ($CDCl_3$) δ 11.0. Anal. Calcd for $C_{43}H_{53}O_{12}P$: C, 65.14; H, 6.74. Found: C, 65.29; H, 6.70.

6-Deoxy-6-(*Z*)-phosphonomethylene-D-glucose 84. Fully protected (*Z*)-vinylphosphonate **97** (1.17 g, 1.48 mmol) was dissolved in a solution of Et_3N (1.23 mL, 8.85 mmol) in CH_2Cl_2 (20 mL), and bromotrimethylsilane (TMSBr, 0.97 mL, 7.38 mmol) was added via syringe. After 12 h at rt, the brown solution was concentrated under reduced pressure. The residue was dissolved in THF/H_2O (1:1, v/v, 20 mL) and stirred at rt for 10 min. Methylene chloride was added and layers were separated. The aqueous layer was extracted with CH_2Cl_2 (6x). Organic layers were combined, dried, and concentrated to a yellow oil. This was dissolved in a mixture of CH_2Cl_2/H_2O (18:1, v/v, 19 mL), and 2,3-dichloro-5,6-dicyano-1,4-bezoquinone (DDQ, 1.67 g, 7.38 mmol) was added. The resulting purple solution was stirred for 4 h at rt, filtered through a short pad of celite, and residues were washed with water (10 mL). After layers were separated, the aqueous layer was washed with CH_2Cl_2 (2x) and EtOAc (1x), diluted to 200 mL, and the pH was adjusted to 7.5 with aqueous NaOH. This solution was applied to AG-1 X8 anion exchange resin (200 mL) which had been equilibrated with 100 mM $Et_3NH^+HCO_3^-$ (pH 7.5). After washing with water (100 mL), the column was eluted with a linear gradient (600 mL + 600 mL, 100-300 mM) of $Et_3NH^+HCO_3^-$ (pH 7.5). Fractions containing phosphorus were identified by the methods of Avila and Ames,[120] concentrated, azotroped with 2-propanol (6x), dissolved in water, passed down a short column of Dowex 50 (H^+), and concentrated to dryness. Glucose (*Z*)-vinylphosphonate **84** was obtained as a colorless film (61%): α-anomer: ^{1}H NMR (D_2O) δ 6.11 (ddd, J = 14, 13, 1 Hz, 1 H), 5.83 (ddd, J = 40, 13, 8 Hz, 1 H), 5.24-5.30 (m, 1 H), 5.24 (d, J = 4 Hz, 1 H), 3.77 (dd, J = 10, 9 Hz, 1 H), 3.54-3.57 (m, 1 H), 3.17 (dd, J = 10, 9 Hz, 1 H); ^{13}C NMR (D_2O) δ 137.8, 136.8 (J_{PC} = 143 Hz), 94.9, 76.2, 76.0, 74.4, 70.5 (J_{PCCC} = 8 Hz); ^{31}P NMR (D_2O) δ 13.2; β-anomer: ^{1}H NMR (D_2O) δ 6.11 (ddd, J = 14, 13, 1 Hz, 1 H), 5.83 (ddd, J = 40, 13, 8 Hz, 1 H), 4.88-4.96 (m, 1 H), 4.76 (d, J = 8 Hz, 1 H), 3.55 (dd, J = 9, 9 Hz, 1 H), 3.27 (dd, J = 10, 8 Hz, 1 H), 3.19 (dd, J = 10, 10 Hz, 1 H); ^{13}C NMR (D_2O) δ 137.6, 136.8 (J_{PC} = 179 Hz), 98.6, 79.2, 77.0, 75.8, 75.4 (J_{PCCC} = 8 Hz); ^{31}P

NMR (D_2O): δ 13.1; HRMS (FAB) calcd for $C_7H_{13}O_8P$ (M - H^+) 255.0270, found 255.0263.

6-*O*-Triphenylmethyl-D-glucitol 98. To a solution of glucose (10.0 g, 55.5 mmol) in pyridine (120 mL) was added triphenylmethyl chloride (17.0 g, 61.0 mmol). The suspension was stirred and heated at 75 °C for 8 h. Concentration in vacuo gave a yellow gum which was dissolved in CH_2Cl_2 and washed with saturated aqueous NH_4Cl (1x), saturated aqueous $NaHCO_3$ (1x), and brine (1x). Drying and concentration of the organic layer gave a yellow foam which was purified by flash chromatography ($CHCl_3$, $CHCl_3$/EtOH, 10:1, v/v) to afford trityl glucose as a yellow foam (21.0 g). This was dissolved in a mixture of EtOH/H_2O/CH_2Cl_2 (4:1:4, v/v/v, 360 mL) and transferred to a flask equipped with mechanical stirrer. Sodium borohydride (3.0 g, 79.3 mmol) was added in portions and stirring was continued for 4 h at rt. After neutralization with dry ice, the mixture was concentrated to a syrup. Purification by flash chromatography ($CHCl_3$, $CHCl_3$/EtOH, 10:1, 5:1, v/v) afforded trityl glucitol **98** as a white foam (9.66 g, 41 %): ^{1}H NMR (DMSO-d_6) δ 7.20-7.48 (m, 15 H), 4.89 (d, J = 6 Hz, 1 H), 4.57 (d, J = 5 Hz, 1 H), 4.45 (m, 1 H), 4.26 (d, J = 6 Hz, 1 H), 4.07 (d, J = 7 Hz, 1 H), 3.65-3.82 (m, 2 H), 3.48-3.56 (m, 1 H), 3.30-3.47 (m, 3 H), 3.12-3.19 (m, 1 H), 2.92-3.00 (m, 1 H); ^{13}C NMR (DMSO-d_6) δ 144.3, 128.5, 127.8, 126.9, 85.7, 73.8, 72.4, 70.2, 68.7, 66.3, 62.7. Anal. Calcd for $C_{25}H_{28}O_6$: C, 70.74; H, 6.65. Found: C, 70.59; H, 6.53.

1,2,3,4,5-Penta-*O*-4-methoxybenzyl-6-*O*-triphenylmethyl-D-glucitol 99. Into a flask fitted with an efficient mechanical stirrer were placed 50% aqueous NaOH (9.1 mL), benzene (9.1 mL), trityl glucitol **98** (1.70 g, 4.00 mmol), *tert*-amyl alcohol (0.2 mL), tetrabutylammonium chloride (1.12 g, 4.03 mmol), and 4-methoxybenzyl chloride (3.26 mL, 24.0 mmol). Stirring was continued overnight at rt. Benzene (150 mL) and water (50 mL) were added and layers were separated. The organic layer was washed with water (1x) and brine (1x). Drying and concentration of the organic layer gave a yellow oil which was purified by flash chromatography (hexane/EtOAc, 5:1, 2:1, v/v) to afford fully

protected glucitol **99** (3.18 g, 78%) as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.35-7.40 (m, 6 H), 7.07-7.16 (m, 15 H), 6.99 (d, J = 9 Hz, 2 H), 6.82 (d, J = 9 Hz, 2 H), 6.59-6.74 (m, 10 H), 4.53 (d, J = 12 Hz, 1 H), 4.52 (d, J = 11 Hz, 1 H), 4.42 (d, J = 3 Hz, 2 H), 4.41 (d, J = 11 Hz, 1 H), 4.35 (s, 2 H), 4.24 (s, 2 H), 4.20 (d, J = 11 Hz, 1 H), 3.87-3.92 (m, 1 H), 3.70-3.73 (m, 3 H), 3.65 (s, 3 H), 3.64 (s, 3 H), 3.63 (s, 3 H), 3.62 (2) (s, 3 H), 3.38-3.48 (m, 3 H), 3.25 (dd, J = 10, 5 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 158.9 (2), 158.8 (2), 144.0, 130.8, 130.4, 129.7, 129.6, 129.5, 129.1, 128.8 (2), 128.7, 127.6, 126.8, 113.6, 113.5, 113.4, 113.3, 86.6, 79.1, 78.2, 78.1 (2), 73.8, 73.3, 72.7, 72.4, 71.7, 70.4, 63.2, 55.1. Anal. Calcd for $C_{65}H_{68}O_{11}$: C, 76.15; H, 6.69. Found: C, 76.22; H, 6.74.

1,2,3,4,5-penta-*O*-4-methoxybenzyl-D-glucitol 100. To a solution of fully protected glucitol **99** (8.20 g, 8.00 mmol) in acetone (100 mL) was added H_2SO_4/MeOH (1:20, v/v, 100 mL) at 10 °C. The reaction mixture was warmed to rt, stirred for 30 min, quenched with water (100 mL), and diluted with ether (200 mL). After layers were separated, the aqueous layer was extracted with ether (5x). Organic layers were combined, washed with brine (1x), dried, filtered, treated with K_2CO_3 (0.5 g), and concentrated in vacuo. The residue obtained was purified by flash chromatography (hexane/EtOAc, 1:1, v/v) to afford alcohol **100** (4.70 g, 75%) as a yellow oil: ^{1}H NMR ($CDCl_3$)δ 7.14-7.24 (m, 10 H), 6.78-6.86 (m, 10 H), 4.67 (d, J = 11 Hz, 1 H), 4.64 (d, J = 12 Hz, 1 H), 4.54-4.58 (m, 3 H), 4.50 (d, J = 11 Hz, 1 H), 4.34-4.38 (m, 3 H), 4.27 (d, J = 11 Hz, 1 H), 3.70-3.89 (m, 5 H), 3.77 (2) (s, 3 H), 3.76 (3) (s, 3 H), 3.53-3.60 (m, 2 H), 3.43 (dd J = 10, 6 Hz, 1 H), 2.27 (br, 1 H); ^{13}C NMR ($CDCl_3$) δ 159.1, 130.6, 130.5 (2), 130.3, 129.8, 129.7, 129.6, 129.2, 129.1, 113.7, 113.6 (2), 79.5, 78.4 (2), 78.0, 74.0, 73.9, 72.8, 72.3, 70.9, 69.6, 60.8, 55.1. Anal. Calcd for $C_{46}H_{54}O_{11}$: C, 70.57; H, 6.95. Found: C, 70.59; H, 7.00.

6-Deoxy-6-(*Z*)-diethoxyphosphonomethylene-1,2,3,4,5-penta-*O*-4-methoxybenzyl-D-glucitol 101. This was made from alcohol **100** according to the

procedure for protected glucose (*Z*)-vinylphosphonate **97**. Protected glucitol (*Z*)-vinylphosphonate **101** was obtained as a yellow oil (22%): ^{1}H NMR ($CDCl_3$) δ 7.15-7.25 (m, 10 H), 6.77-6.83 (m, 10 H), 6.57 (ddd, J = 53, 17, 13 Hz, 1 H), 5.88 (dd, J = 17, 13 Hz, 1 H), 5.18 (dd, J = 10, 5 Hz, 1 H), 4.66 (d, J = 11 Hz, 1 H), 4.63 (d, J = 12 Hz, 1 H), 4.62 (d, J = 12 Hz, 1 H), 4.57 (d, J = 11 Hz, 1 H), 4.54 (d, J = 12 Hz, 1 H), 4.50 (d, J = 11 Hz, 1 H), 4.49 (d, J = 11 Hz, 1 H), 4.38 (d, J = 11 Hz, 1 H), 4.33 (s, 2 H), 3.90-4.11 (m, 6 H), 3.78 (s, 6 H), 3.77 (s, 3 H), 3.76 (s, 6 H), 3.70 (dd, J = 5, 5 Hz, 1 H), 3.56 (dd, J = 10, 5 Hz, 1 H), 3.49 (dd, J = 10, 6 Hz, 1 H), 1.22 (dt, J = 42, 7 Hz, 6 H); ^{13}C NMR ($CDCl_3$) δ 159.0, 158.9, 149.5 (J_{PCC} = 5 Hz), 131.1, 131.0 (2), 130.8, 130.5, 129.8, 129.6 (2), 129.2, 120.8 (J_{PC} = 184 Hz), 113.6, 113.5, 113.4, 80.3, 78.4, 78.2, 75.7 (J_{PCCC} = 7 Hz), 74.3, 73.7, 72.8, 72.7, 70.4, 70.3, 61.7 (J_{POC} = 5 Hz), 61.6 (J_{POC} = 6 Hz), 55.2, 16.3 (J_{POCC} = 6 Hz), 16.2 (J_{POCC} = 6 Hz); ^{31}P NMR ($CDCl_3$) δ 15.7. Anal. Calcd for $C_{51}H_{63}O_{13}P$: C, 66.94; H, 6.94. Found: C, 66.98; H, 6.84.

6-Deoxy-6-(*Z*)-phosphonomethylene-D-glucitol 85. This was made from protected glucitol (*Z*)-vinylphosphonate **101** according to the procedure for glucose (*Z*)-vinylphosphonate **84**. Glucitol (*Z*)-vinylphosphonate **85** was obtained as a colorless film (54%): ^{1}H NMR (D_2O) δ 6.22 (ddd, J = 46, 13, 9 Hz, 1 H), 5.98 (ddd, J = 16, 13, 1 Hz, 1 H), 4.93-4.99 (m, 1 H), 3.85 (ddd, J = 7, 5, 4 Hz, 1 H), 3.79 (dd, J = 5, 3 Hz, 1 H), 3.71 (dd, J = 12, 4 Hz, 1 H), 3.64 (dd, J = 7, 3 Hz, 1 H), 3.60 (dd, J = 12, 7 Hz, 1 H); ^{13}C NMR (D_2O) δ 146.4, 129.2 (J_{PC} = 172 Hz), 76.1, 75.4, 72.5, 70.8 (J_{PCCC} = 8 Hz), 65.2; ^{13}P NMR (D_2O) δ 10.9; HRMS (FAB) calcd for $C_7H_{15}O_8P$ (M - H^+) 257.0426, found 257.0437.

Methyl 6-deoxy-6-(*E*)-(di-1-methylethoxy)phosphonomethylene-2,3,4-tri-*O*-4-methoxybenzyl-α-D-glucopyranoside 103. To a stirred solution of oxalyl chloride (0.40 mL, 4.6 mmol) in CH_2Cl_2 (15 mL) at -78 °C was added DMSO (0.44 mL, 6.2 mmol) dropwise, and the mixture was stirred at -78 °C for 10 min. Then a solution of alcohol **102**[104] (1.72 g, 3.09 mmol) in CH_2Cl_2 (15 mL) was added into the

reaction mixture. After 40 min at -78 °C, Et_3N (1.30 mL, 9.28 mmol) was added, and the mixture was allowed to warm to rt and quenched with water. Ether was added, and the resulting organic layer was separated and washed with aqueous $NaHSO_4$ (0.05 N, 1x), saturated aqueous $NaHCO_3$ (1x), and brine (1x). Drying and concentration of the organic layer gave the crude aldehyde which was used as is in the subsequent reaction.

Tetraisopropyl methylenediphosphonate (1.48 g, 4.30 mmol) was dissolved in THF (10 mL) and cooled to -78 °C. A solution of *n*-BuLi in hexane (1.6 M, 2.70 mL, 4.32 mmol) was added dropwise, and the solution was stirred at -78 °C for 0.5 h. The aldehyde described above was dissolved in THF (10 mL) and added into the reaction flask. After stirred at -78 °C for 20 min, the mixture was warmed to rt. Acetic acid (3 mL) was added, and the solvents were removed under reduced pressure. Purification by radial chromatography (4 mm thickness, hexane/EtOAc, 2:1, 1:1, v/v) gave protected (*E*)-vinylphosphonate **103** as a yellow oil (1.37 g, 62%): ^{1}H NMR ($CDCl_3$) δ 7.22-7.31 (m, 6 H), 6.79-6.94 (m, 7 H), 6.04 (ddd, J = 21, 17, 2 Hz, 1 H), 4.88 (d, J = 11 Hz, 1 H), 4.72-4.78 (m, 3 H), 4.63-4.70 (m, 2 H), 4.60 (d, J = 12 Hz, 1 H), 4.54 (d, J = 4 Hz, 1 H), 4.50 (d, J = 10 Hz, 1 H), 4.15-4.25 (m, 1 H), 3.96 (dd, J = 9, 9 Hz, 1 H), 3.80 (s, 6 H), 3.79 (s, 3 H), 3.47 (dd, J = 10, 4 Hz, 1 H), 3.33 (s, 3 H), 3.18 (dd, J = 10, 9 Hz, 1 H), 1.32 (d, J = 6 Hz, 6 H), 1.25 (d, J = 6 Hz, 6 H); ^{13}C NMR ($CDCl_3$) δ 159.3, 159.2, 159.1, 146.8 (J_{PCC} = 6 Hz), 130.8, 130.1, 129.8, 129.6, 129.5, 119.4 (J_{PC} = 189 Hz), 113.8, 113.7, 98.0, 81.5, 81.3, 79.3, 75.4, 74.9, 73.0, 70.3 (J_{POC} = 5 Hz), 69.7 (J_{PCCC} = 22 Hz), 55.2, 24.0 (J_{POCC} = 3 Hz), 23.9 (J_{POCC} = 4 Hz); ^{31}P NMR ($CDCl_3$) δ 13.2. Anal. Calcd for $C_{38}H_{51}O_{11}P$: C, 63.85; H, 7.19. Found: C, 63.74; H, 7.04.

6-Deoxy-6-(*E*)-phosphonomethylene-D-glucose 86. Fully protected (*E*)-vinylphosphonate **103** (0.870 g, 1.22 mmol) was dissolved in CH_2Cl_2 (18 mL). Water (1 mL) and DDQ (1.67 g, 4.26 mmol) were added. The purple solution was stirred at rt for 4 h, filtered through a short pad of Celite, and residues were rinsed with water (2x). After layers were separated, the aqueous layer was washed with CH_2Cl_2 (2x) and

evaporated to dryness. Hydrochloric acid (6 N, 20 mL) was added, and the mixture was refluxed for 3 h, then concentrated to dryness. The residue was redissolved in water (200 mL), and the resulting solution was washed with EtOAc (2x), neutralized with aqueous NaOH, and applied to AG-1 X8 anion exchange resin (200 mL) which had been equilibrated with 100 mM $Et_3NH^+HCO_3^-$ (pH 7.5). After washing with water (100 mL), the column was eluted with a linear gradient (600 mL + 600 mL, 100-300 mM) of $Et_3NH^+HCO_3^-$ (pH 7.5). Fractions containing phosphorus were identified, concentrated, azotroped with 2-propanol (6x), dissolved in water, passed through a short column of Dowex 50 (H^+), and concentrated to dryness. Glucose (*E*)-vinylphosphonate **86** was obtained as a colorless film (0.189 g, 60%): α-anomer: ^{1}H NMR (D_2O) δ 6.50 (ddd, J = 21, 17, 6 Hz, 1 H), 6.07-6.20 (m, 1 H), 5.25 (d, J = 4 Hz, 1 H), 4.29-4.36 (m, 1 H), 3.73 (dd, J = 9, 9 Hz, 1 H), 3.57 (dd, J = 10, 4 Hz, 1 H), 3.28 (dd, J = 10, 9 Hz, 1 H); ^{13}C NMR (D_2O) δ 145.4 (J_{PC} = 6 Hz), 127.7 (J_{PCC} = 177 Hz), 95.1, 78.7, 78.4, 74.3 (J_{PCCC} = 22 Hz), 74.2; ^{31}P NMR (D_2O) δ 13.2; β-anomer: ^{1}H NMR (D_2O) δ 6.48 (ddd, J = 21, 17, 6 Hz, 1 H), 6.07-6.20 (m, 1 H), 4.70 (d, J = 8 Hz, 1 H), 3.94-4.01 (m, 1 H), 3.50 (dd, J = 9, 9 Hz, 1 H), 3.32 (dd, J = 10, 9 Hz, 1 H), 3.31 (dd, J = 12, 9 Hz, 1 H); ^{13}C NMR (D_2O) δ 144.7 (J_{PCC} = 6 Hz), 127.7 (J_{PC} = 177 Hz), 98.8, 78.2, 76.9, 75.6, 75.5 (J_{PCCC} = 40 Hz); ^{31}P NMR (D_2O) δ 13.1; HRMS (FAB) calcd for $C_7H_{13}O_8P$ (M - H^+) 255.0270, found 255.0265.

6-Deoxy-6-(*E*)-phosphonomethylene-D-glucitol 87. Glucose (*E*)-vinylphosphonate **86** (0.101 g, 0.391 mmol) was dissolved in water (5 mL) and $NaBH_4$ (0.088 g, 2.35 mmol) was added portionwise. After being stirred for 2 h, the reaction was quenched with Dowex 50 (H^+). The reaction mixture was passed through a short Dowex 50 (H^+) column and concentrated to dryness. After removing boric acid as an azeotrope with methanol (6x), the residue was dissolved in water, neutralized with aqueous NaOH, and concentrated to give glucitol (*E*)-vinylphosphonate **87** as a colorless film (100%): ^{1}H NMR (D_2O) δ 6.70 (ddd, J = 22, 17, 5 Hz, 1 H), 6.05-6.18 (m, 1 H), 4.32-

4.39 (m, 1 H), 3.57-3.84 (m, 5 H); ^{13}C NMR (D_2O) δ 150.1(J_{PCC} = 5 Hz), 124.5 (J_{PC} = 180 Hz), 76.0, 75.4, 74.3 (J_{PCCC} = 22 Hz), 65.2; ^{31}P NMR (D_2O) δ 15.0; HRMS (FAB) calcd for $C_7H_{15}O_8P$ (M - H^+) 257.0426, found 257.0417.

Protected 2-Deoxy-D-glucose-(*E*)-vinylphosphonate 104. According to the procedure for protected glucose (*E*)-vinylphosphonate **103**, BBA-protected 2-deoxy glucopyranoside **27** was transformed into protected 2-deoxy-D-glucose-(E)-vinylphosphonate **104**, which was obtained as a yellow oil (57%): ^1H NMR ($CDCl_3$) δ 6.88 (ddd, J = 23, 17, 4 Hz, 1 H), 6.07 (ddd, J = 21, 17, 2 Hz, 1 H), 4.88 (d, J = 4 Hz, 1 H), 4.59-4.71 (m, 2 H), 4.24-4.31 (m, 1 H), 4.15 (ddd, J = 12, 10, 4 Hz, 1 H), 3.33 (dd, J = 10, 10 Hz, 1 H), 3.31 (s, 3 H), 3.28 (s, 3 H), 3.20 (s, 3 H), 2.00 (dd, J = 12, 4 Hz, 1 H), 1.78 (ddd, J = 12, 12, 4 Hz, 1 H), 1.34 (s, 3 H), 1.32 (s, 3 H), 1.28-1.31 (m, 12 H); ^{13}C NMR ($CDCl_3$) δ 146.5 (J_{PCC} = 7 Hz), 119.5 (J_{PC} = 189 Hz), 100.0, 99.7, 98.6, 72.3, 70.2 (J_{POC} = 6 Hz), 68.8 (J_{PCCC} = 22 Hz), 64.8, 54.5, 47.8, 47.7, 34.3, 23.8 (J_{POCC} = 10 Hz, 2x), 17.6, 17.5; ^{31}P NMR ($CDCl_3$) δ 16.0. Anal. Calcd for $C_{20}H_{37}O_9P$: C, 53.09; H, 8.24. Found: C, 52.86; H, 8.19.

2,6-Dideoxy-6-(*E*)-phosphonomethylene-D-glucose 88. Protected 2-deoxy glucose (Z)-vinylphosphonate **104** (1.60 g, 3.54 mmol) was dissolved in a solution of Et_3N (3.0 mL, 21 mmol) in CH_2Cl_2 (30 mL), and TMSBr (2.33 mL, 17.7 mmol) was added via a syringe. After 12 h at rt, water was added and the brown solution was stirred at rt for another 30 min. After layers were separated, the aqueous layer was washed with ether (1x), and concentrated to a yellow oil. This was dissolved in aqueous HCl (6 N, 20 mL), stirred at rt for 6 h, and then concentrated under vacuo. The resulting residue was dissolved in water (200 mL), washed with EtOAc (2x), neutralized with aqueous NaOH, and applied to AG-1 X8 anion exchange resin (200 mL) which had been equilibrated with 100 mM $Et_3NH^+HCO_3^-$ (pH 7.5). After washed with water (100 mL), the column was eluted with a linear gradient (600 mL + 600 mL, 100-300 mM) of $Et_3NH^+HCO_3^-$ (pH 7.5). Fractions containing phosphorus were identified, concentrated,

azotroped with 2-propanol (6x), dissolved in water, passed down a short column of Dowex 50 (H^+), and concentrated to dryness. 2-Deoxy glucose (*E*)-vinylphosphonate **88** was obtained as a colorless film (0.81 g, 100%): α-anomer: ^{1}H NMR (D_2O) δ 6.64 (ddd, J = 22, 17, 6 Hz, 1 H), 6.08-6.22 (m, 1 H), 5.41 (d, J = 4 Hz, 1 H), 4.29-4.37 (m, 1 H), 3.96 (ddd, J = 12, 10, 5 Hz, 1 H), 3.17-3.30 (m, 1 H), 2.15 (dd, J = 13, 5 Hz, 1 H), 1.74 (ddd, J = 13, 10, 4 Hz, 1 H); ^{13}C NMR (D_2O) δ 148.0 (J_{PCC} = 5 Hz), 124.7 (J_{PC} = 181 Hz), 94.2, 78.1 (J_{PCCC} = 22 Hz), 77.1, 76.6, 39.9; ^{31}P NMR (D_2O) δ 15.0; β-anomer: ^{1}H NMR (D_2O) δ 6.63 (ddd, J = 22, 17, 5 Hz, 1 H), 6.08-6.22 (m, 1 H), 5.00 (dd, J = 10, 2 Hz, 1 H), 3.89-3.96 (m, 1 H), 3.74 (ddd, J = 12, 9, 5 Hz, 1 H), 3.17-3.30 (m, 1 H), 2.29 (ddd, J = 12, 5, 2 Hz, 1 H), 1.54 (dd, J = 12, 12, 10 Hz, 1 H); ^{13}C NMR (D_2O) δ 147.4 (J_{PCC} = 5 Hz), 124.6 (J_{PC} = 180 Hz), 96.3, 74.5 (J_{PCCC} = 22 Hz), 72.9, 70.5, 42.2; ^{31}P NMR (D_2O) δ 15.0; HRMS (FAB) calcd for $C_7H_{13}O_7P$ (M - H^+) 229.0321, found 239.0319.

2,6-Dideoxy-6-(*E*)-phosphonomethylene-D-glucitol 89. According to the procedure for glucitol (*E*)-vinylphosphonate **87**, 2-deoxy glucose (*E*)-vinylphosphonate **88** was transformed into 2-deoxy glucitol (*E*)-vinylphosphonate **89**, which was obtained as a colorless film (100%): ^{1}H NMR (D_2O) δ 6.80 (ddd, J = 23, 17, 5 Hz, 1 H), 6.06-6.20 (m, 1 H), 4.32-4.38 (m, 1 H), 3.98 (ddd, J = 9, 4, 4 Hz, 1 H), 3.73 (t, J = 6 Hz, 2 H), 3.43 (dd, J = 7, 3 Hz, 1 H), 1.68-1.87 (m, 2 H); ^{12}C NMR (D_2O) δ 152.1 (J_{PCC} = 5 Hz), 122.4 (J_{PC} = 181 Hz), 78.1, 74.1 (J_{PCCC} = 21 Hz), 61.2, 37.9; ^{31}P NMR (D_2O) δ 17.0; HRMS (FAB) calcd for $C_7H_{15}O_7P$ (M - H^+) 241.0477, found 241.0480.

Synthesis of (*E*)-Vinylphosphonate 109.

2,4-*O*-4-Methoxybenzylidene-D-erythritol 105. A mixture of D-glucose (40.0 g, 223 mmol), 4-methoxybenzaldehyde dimethyl acetal (20.3 g, 111 mmol), and

p-TsOH (1.0 g, 5.2 mmol) in anhydrous DMF (160 mL) was rotavaped at 50 °C for 8 h. Solid $NaHCO_3$ (1.0 g) was added, and solvents were removed under vacuo. The resulting foam was triturated with boiling EtOAc (3x). Combined EtOAc solutions were concentrated to about 200 mL, kept at 4 °C overnight, and filtered to afford 4,6-*O*-4-methoxybenzylidene-D-glucose as a white solid (21.2 g, 64%). This was used directly in the following reaction.

Sodium periodate (10.0 g, 46.9 mmol) and $NaHCO_3$ (0.99 g, 11.7 mmol) were added to a solution of 4,6-*O*-4-methoxybenzylidene-D-glucose (3.50 g, 11.7 mmol) in $MeOH/H_2O$ (2:1, v/v, 100 mL) at 0 °C. The resulting suspension was stirred at 0 °C for an hour and at rt for an additional hour. White precipitates were filtered off and washed with MeOH. The filtrate and washings were combined and evaporated to give a residue, which was dissolved in $CHCl_3$, washed with aqueous $Na_2S_2O_3$ (10%, 1x) and brine (1x). Drying and concentration of the organic layer afforded 2,4-*O*-4-methoxybenzylidene-D-erythrose as a white foam (2.10 g, 75%), which was used directly in the subsequent reaction.

A solution of $NaBH_4$ (0.630 g, 16.7 mmol) in H_2O (10 mL) was added dropwise to a solution of 2,4-*O*-4-Methoxybenzylidene-D-erythrose (2.00 g, 8.39 mmol) in EtOH (100 mL) at rt. The resulting mixture was stirred at rt for 5 h. After solvents were removed, the residue was dissolved in EtOAc, washed with Na_2SO_4 (10%, 1x) and brine. Drying and concentration of the organic layer left 2,4-*O*-4-methoxybenzylidene-D-erythritol **105** as a white solid (1.80 g, 90%): ^1H NMR ($CDCl_3$) δ 7.37-7.42 (m, 2 H), 6.86-6.91 (m, 2 H), 5.48 (s, 1 H), 4.33 (d, J = 5 Hz, 1 H), 4.15 (dd, J = 10, 5 Hz, 1 H), 3.78 (s, 3 H), 3.59-3.90 (m, 4 H), 3.54 (dd, J = 10, 10 Hz, 1 H), 2.91 (br, 1 H); ^{13}C NMR ($CDCl_3$) δ 160.8, 132.0, 128.4, 113.9, 101.4, 83.7, 71.8, 62.7, 62.6, 55.5. Anal. Calcd for $C_{12}H_{16}O_5$: C, 59.99; H, 6.71. Found: C, 60.09; H, 6.75.

1,3-Di-*O*-4-methoxybenzyl-2,4-*O*-4-methoxybenzylidene-D-erythritol 106. To a suspension of NaH (0.672 g, 28.0 mmol) in DMF (40 mL) at 0 °C was added a soluton of azido alcohol **56** (2.24 g, 9.32 mmol) in DMF (10 mL). After being stirred at rt

for 30 min, PMBCl (2.59 mL, 19.1 mmol) was added, followed by $Bu_4N^+I^-$ (0.172g, 0.466 mmol). The mixture was stirred at rt overnight and quenched by MeOH (1 mL) at 0 °C. After solvents were removed, the residue was partitioned between saturated aqueous NH_4Cl and ether. The aqueous layer was back extracted with ether (3x). Organic layers were combined, washed with saturated aqueous $NaHCO_3$ (1x) and brine, dried and concentrated. Purification by flash chromatography (hexane/EtOAc, 5:1, 1:1, v/v) afforded 1,3-di-*O*-4-methoxybenzyl-2,4-*O*-4-methoxybenzylidene-D-erythritol **106** as a white solid (4.14 g, 92%): ^{1}H NMR ($CDCl_3$) δ 7.40 (d, J = 9 Hz, 2 H), 7.27 (d, J = 9 Hz, 2 H), 7.14 (d, J = 9 Hz, 2 H), 6.81-6.88 (m, 6 H), 5.42 (s, 1 H), 4.58 (d, J = 12 Hz, 1 H), 4.49 (d, J = 12 Hz, 1 H), 4.46 (d, J = 11 Hz, 1 H), 4.40 (d, J = 11 Hz, 1 H), 4.26 (dd, J = 10, 4 Hz, 1 H), 3.76 (s, 6 H), 3.75 (s, 3 H), 3.72-3.83 (m, 3 H), 3.65 (ddd, J = 10, 9, 4 Hz, 1 H), 3.57 (dd, J = 10, 10 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 159.8, 159.3, 159.0, 130.2, 130.1, 129.8, 129.4 (2), 127.4, 113.7, 113.7, 113.5, 113.4, 100.9, 80.4, 72.9, 72.0, 69.3, 68.7, 68.2, 55.1. Anal. Calcd for $C_{28}H_{32}O_7$: C, 69.98; H, 6.71. Found: C, 70.12; H, 6.75.

1,2,3-Tri-*O*-4-methoxybenzyl-D-erythritol 107. A solution of TMSCl (4.31 mL, 34.0 mmol) in CH_3CN (34 mL) was added dropwise to a stirred mixture of 1,3-di-*O*-4-methoxybenzyl-2,4-*O*-4-methoxybenzylidene-D-erythritol **106** (2.72 g, 5.66 mmol), $NaBH_3CN$ (2.13 g, 34 mmol), and 3 Å molecular sieves (3.0 g) in CH_3CN (70 mL) at 0 °C.[105] After being stirred at rt for 5 h, the reaction mixture was filtered through Celite, and the filtrate was poured into an ice-cold $NaHCO_3$ solution. The resulting mixture was extracted with CH_2Cl_2 (5x), and the combined organic layers were washed with saturated aqueous $NaHCO_3$ (1x) and brine (1x). Drying and concentration of the organic layer gave a yellow oil which was purified by radial chromatography (hexane/EtOAc, 5:1, 1:1, v/v) to afford 1,2,3-tri-*O*-4-methoxybenzyl-D-erythritol **107** as a white solid (1.23 g, 45%): ^{1}H NMR ($CDCl_3$) δ 7.26 (d, J = 2 Hz, 2 H), 7.23 (d, J = 2 Hz, 2 H), 7.17 (d, J = 8 Hz, 2 H), 6.87 (d, J = 8 Hz, 2 H), 6.85 (d, J = 8 Hz, 2 H), 6.84 (d, J = 8 Hz, 2 H),

4.64 (d, J = 11 Hz, 1 H), 4.52 (d, J = 11 Hz, 1 H), 4.43-4.52 (m, 2 H), 3.79 (s, 3 H), 3.78 (s, 6 H), 3.54-3.74 (m, 6 H), 2.30 (br, 1 H); ^{13}C NMR ($CDCl_3$) δ 159.2, 159.1 (2), 130.2, 130.1, 129.5 (2), 129.3, 113.7, 78.0, 77.7, 72.9, 72.2, 71.9, 68.8, 61.4, 55.2. Anal. Calcd for $C_{28}H_{34}O_7$: C, 69.69; H, 7.10. Found: C, 69.71; H, 6.96.

Protected (*E*)-Vinylphosphonate 108. This was made from 1,2,3-tri-*O*-4-methoxybenzyl-D-erythritol **107** according to the procedure for protected glucose (*E*)-vinylphosphonate **103**. Protected (*E*)-vinylphosphonate **108** was obtained as a yellow oil (59%): ^{1}H NMR ($CDCl_3$) δ 7.16-7.27 (m, 6 H), 6.71-6.88 (m, 7 H), 5.99 (ddd, J = 21, 17, 1 Hz, 1 H), 4.49-4.60 (m, 3 H), 4.43 (s, 3 H), 4.32 (d, J = 12 Hz, 1 H), 4.10-4.80 (m, **3 H**), 3.79 (s, 6 H), 3.78 (s, 3 H), 3.55-3.65 (m, 3 H), 1.32 (d, J = 6 Hz, 6 H), 1.27 (d, J = 6 Hz, 6 H); ^{13}C NMR ($CDCl_3$) δ 159.0, 158.9, 158.8, 148.6 (J_{PCC} = 6 Hz), 130.1, 130.0, 129.6, 129.3, 129.2, 129.1, 120.5 (J_{PC} = 187 Hz), 113.5 (2), 79.5, 78.4 (J_{PCCC} = 22 Hz), 72.9, 72.2, 71.3, 70.3 (J_{POC} = 5 Hz), 68.7, 55.1, 24.1(J_{POCC} = 5 Hz), 24.0 (J_{POCC} = 6 Hz), 17.6, 17.5. Anal. Calcd for $C_{35}H_{47}O_9P$: C, 65.40; H, 7.37. Found: C, 65.48; H, 7.23.

(*E*)-Vinylphosphonate 109. This was made from protected (*E*)-vinylphosphonate **108** according to the procedure for glucose (*Z*)-vinylphosphonate **84**. (*E*)-Vinylphosphonate **109** was obtained as a yellow oil (41%): ^{1}H NMR (D_2O) δ 6.67 (ddd, J = 14, 10, 3 Hz, 1 H), 6.10 (ddd, J = 12, 10, 1 Hz, 1 H), 4.30-4.33 (m, 1 H), 3.70-3.76 (m, 2 H), 3.60 (dd, J = 7, 4 Hz, 1 H); ^{12}C NMR (D_2O) δ 150.2 (J_{PCC} = 5 Hz), 123.5 (J_{PC} = 181 Hz), 76.6, 74.8 (J_{PCCC} = 22 Hz), 65.0; ^{31}P NMR (D_2O) δ 15.6; HRMS (FAB) calcd for $C_5H_{10}O_6P$ (M - H^+) 197.0215, found 197.0217.

Synthesis of 3-Dehydroquinic Acid.

Benzyl ester 110. A mixture of BBA-protected quinate[41] **19** (10.0 g, 31.2 mmol), benzyl alcohol (6.46 mL, 62.4 mmol) and 1-chloro-3-hydroxytetrabutyldi-

stannoxane[110] (0.833 g, 1.56 mmol) in toluene (75 mL) were refluxed in a flask equipped with a Dean-Stark trap containing 5 Å molecular sieves (5 g). After 20 h solvents were removed and the resulting yellow oil was purified by flash chromatography (hexane/EtOAc, 1:1, 1:2, v/v) to give benzyl ester **110** as a yellow oil (11.0 g, 89%): ^{1}H NMR ($CDCl_3$) δ 7.32-7.34 (m, 5 H), 5.18 (m, 2 H), 4.40 (br, 1 H), 4.31 (ddd, J = 12, 10, 5 Hz, 1 H), 4.15-4.18 (m, 1 H), 3.65 (br, 1 H), 3.57 (dd, J = 10, 3 Hz, 1 H), 3.24 (s, 3 H), 3.23 (s, 3 H), 2.08-2.21 (m, 2 H), 2.01 (dd, J = 14, 3 Hz, 1 H), 1.93 (dd, J = 13, 12 Hz, 1 H), 1.33 (s, 3H), 1.28 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 173.5, 135.0, 128.5, 128.2, 128.0, 100.1, 99.6, 75.6, 72.7, 68.9, 67.4, 62.4, 47.8, 38.4, 37.3, 17.7, 17.5. Anal. Calcd for $C_{20}H_{28}O_8$: C, 60.59; H, 7.12. Found: C, 60.65; H, 7.15.

Protected DHQ 111. In the following order, H_2O (150 mL), KIO_4 (12.8 g. 55.5 mmol), K_2CO_3 (0.96 g, 6.9 mmol), and finally $RuCl_3$ (0.145 g, 0.555 mmol) were added to a solution of **110** (11.0 g, 27.7 mmol) in $CHCl_3$ (150 mL). Vigorous mechanical stirring was continued at rt until the reaction was complete. The mixture was filtered through Celite, and the aqueous and organic phases were separated. After saturation with NaCl, the aqueous layer was extracted with EtOAc (3x). Combined organic layers were dried and concentrated to give a yellow oil which was purified by flash chromatography (hexane:EtOAc, 1:1, 1:2, v/v) to give Protected DHQ **111** as a yellow oil (6.24 g, 57%): ^{1}H NMR ($CDCl_3$) δ 7.30-7.40 (m, 5 H), 5.23 (m, 2 H), 4.41 (dd, J = 10, 1 Hz, 1 H), 4.27 (ddd, J = 12, 10, 4 Hz, 1 H), 3.72 (br, 1 H), 3.25 (s, 3 H) 3.22 (s, 3 H), 2.90 (dd, J = 14, 1 Hz, 1 H), 2.51 (dd, J = 14, 3 Hz, 1 H), 2.38 (dd, J = 13, 13 Hz, 1 H), 2.13 (ddd, J = 13, 4, 3 Hz, 1 H), 1.39 (s, 3H), 1.30 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 199.3, 173.5, 134.5, 128.8, 128.7, 128.3, 100.5, 99.6, 77.2, 73.9, 68.4, 67.0, 48.9, 48.3, 47.9, 37.7, 17.6, 17.4. Anal. Calcd for $C_{20}H_{26}O_8$: C, 60.90; H, 6.65. Found: C, 60.73; H, 6.72.

3-Dehydroquinic Acid. Protected DHQ **111** (6.24 g, 15.8 mmol) was stirred in a mixture of CH_2Cl_2 (40 mL) and CF_3CO_2H/H_2O (20:1, v/v, 40 mL) for 2 h. Water and

CF_3CO_2H were removed in vacuo. The residue was azotroped with toluene (3x) and purified by flash chromatography (hexane:EtOAc, 1:1, 1:2, v/v, EtOAc) to give the benzyl ester of DHQ as a colorless oil (4.0 g, 92%): ^1H NMR ($CDCl_3$) δ 7.28-7.32 (m, 5 H), 5.15 (s, 2 H), 4.98 (br, 1 H), 4.79 (br, 1 H), 4.14-4.24 (m, 2 H), 3.80-4.06 (m, 1 H), 2.86 (d, J = 14 Hz, 1 H), 2.54 (d, J = 14 Hz, 1 H), 2.16-2.28 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 205.9, 173.2, 134.8, 128.7, 128.6, 128.2, 81.5, 74.1, 71.9, 68.0, 47.4, 39.6. This oil (4.0 g, 14.3 mmol) was dissolved in THF/H_2O (1:1, v/v, 3 mL) and hydrogenated over 10% Pd on C (0.82 g) at 50 psi H_2 pressure for 2 h. The mixture was filtered through Celite, and the residue was washed with H_2O (1x). Combined filtrates were concentrated in vacuo. 3-Dehydorquinic acid was obtained as a white foam (2.6 g, 100%), which was spectroscopically identical to previously synthesized samples.

Synthesis of (*6S*)-6-Fluoroshikimic Acid.

Diene Ester 124. To a solution of alcohol **129** (5.00 g, 21.9 mmol) in CH_2Cl_2 was added pyridine (5.31 mL, 65.7 mmol). After the solution was cooled to –30 °C, trifluoromethanesulfonic anhydride (4.05 mL, 24.1 mmol) was added dropwise. Stirring was continued at –30 °C for 30 min. Then the solution was warmed to rt, diluted with CH_2Cl_2, washed with $NaHSO_4$ (0.1 N, 2x), $NaHCO_3$ (1x) and brine. After drying and concentration, the residue was dissolved in and filtered through a short pad of silica gel which was further washed with hexane/EtOAc (1:1, v/v). The filtrate and washings were combined and concentrated to give the triflate as a yellow oil: ^1H NMR ($CDCl_3$) δ 6.99-7.01 (m, 1 H), 5.00 (ddd, J = 9, 8, 5 Hz, H), 4.84-4.87 (m, 1 H), 4.33 (dd, J = 8, 6, Hz, 1 H), 3.80 (s, 3 H), 3.06 (dd, J = 17, 5 Hz, H), 2.62 (dddd, J = 17, 9, 2, 2 Hz, 1 H), 1.47 (s, 3H), 1.42 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 165.2, 133.7, 128.9, 118.3 (J_{FC} = 319 Hz), 110.8, 85.7, 74.1, 72.2, 52.2, 28.0, 27.2, 25.4. This was used without further purification for the following reaction.

The triflate (4.0 g, 20.1 mmol) was dissolved in toluene, and DBU (4.26 mL, 28.5 mmol) was added at 0 °C. After stirred at 0 °C for 20 min, the reaction mixture was filtered through a short pad of silica gel, and the residue was washed with hexane/EtOAc (1:1, v/v). The filtrate and washings were combined and concentrated to give a yellow oil which was purified by flash chromatography (hexane:EtOAc, 2:1, 1:1, v/v) to give the benzyl ester of DHQ as a colorless oil (1.85 g, 40%): ^{1}H NMR ($CDCl_3$) δ 6.86-6.87(m, 1 H), 6.54 (dd, J = 10, 1 Hz, 1 H), 6.04 (dd, J = 10, 4 Hz, 1 H), 4.82 (dd, J = 9, 4 Hz, 1 H), 4.65 (dd, J = 9, 4 Hz, 1 H), 3.80 (s, 3 H), 1.41 (s, 3 H), 1.39 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 165.5, 133.4, 126.8, 125.3, 122.1, 105.3, 70.5, 69.4, 52.0, 26.5, 24.5. Anal. Calcd for $C_{11}H_{14}O_4$: C, 62.84; H, 6.71. Found: C, 62.76; H, 6.86.

(*6S*)-6-Fluoroshikimic acid was made from diene ester **124** according to published procedures.[111,114]

Enzymology

Purification of MIP Synthase from *E. coli*[29]

Assays.

Enzyme assay solutions (2 mL) were made up in deionized, glass-distilled water containing 50 mM Tris·HCl, pH 7.7, NH_4Cl (2 mM), DTT (0.2 mM), NAD (1 mM), and D-glucose 6-phosphate (5 mM). After incubation at 37 °C for 10 min, enzyme was added. At timed intervals (3 min) aliquots (0.2 mL) were withdrawn and quenched with 20% aqueous trichloroacetic acid (0.05 mL). Precipitated protein was removed by centrifugation. The quenched aliquots (0.1 mL) were incubated with aqueous $NaIO_4$ (0.2 M, 0.1 mL) at 37 °C for 1 h. After quenching excess $NaIO_4$ with aqueous Na_2SO_3 (1 M, 0.1 mL), the inorganic phosphate released was determined by the colorimetric method of Ames.[120] Meanwhile, phosphatase activities were assayed by the same procedure except that $NaIO_4$ was replaced by water. Progress curves were generated by plotting OD 820

versus time. Initial rates were then determined by linear regression analysis by using the computer program Kaleidagraph 2.0. The specific activity of MIP synthase preparations was calculated with the following equation:

$$\frac{\text{slope}}{\varepsilon} \times \frac{V_s}{V_c} \times \frac{V_a}{V_q} \times \frac{V_t}{\rho\ (\text{mg mL}^{-1}) \times V_\rho\ (\text{mL})}$$

where: slope is the slope obtained from linear regression fitting of progress curve,

ε is the extinction coefficient (26,000 OD L mole^{-1}),

V_s is the volume of final visualization solution,

V_c is the volume of aliquot taken for visualization,

V_a is the volume of aliquot withdrawn from the assay solution,

V_q is the volume of trichloroacetic acid used to quench the aliquot,

V_t is the total volume of the assay solution,

ρ is the enzyme concentration,

V_ρ is the volume of enzyme added.

Enzyme Purification.

Inocula (100 mL) were grown from a single colony of *E.coli* BL21 (DE3)/*p*T-7-7/MIPSYN in LB medium containing ampicillin (10 g tryptone, 5 g yeast extract, 10 g NaCl, and 75 mg ampicillin in 1 L water) at 37 ℃ to OD_{550} = 0.5 - 0.6. LB medium containing ampicillin (9 x 1 L) were inoculated with the inocula (10 mL each), and grown at 37 °C until OD_{550} = 0.5 - 0.7. IPTG (0.06 g for each flask) was then added and growth was continued for 5 h. Cells were harvested by centrifugation at 6000 g and resuspended

in buffer A (20 mM Tris·HCl pH 7.7, 10 mM NH_4Cl, 10 mM β-mercaptoethanol, and 0.5 mM PMSF). Cell lysate was prepared by French press disruption of the cells at 16,000 psi. After centrifugation (18,000 g, 4 °C, 25 min), the supernant was fractionated with ammonium sulfate. The 45% - 70% fraction was dissolved in buffer A (50 mL) and dialyzed against buffer A. Then the lysate was loaded onto a DEAE column (30 cm x 5 cm) equilibrated with buffer A and eluted with a linear gradient (1500 mL + 1500 mL, 0.01-0.25 M) of NH_4Cl in buffer A . Fractions showing MIP synthase activity were combined and concentrated (PM-10 Diaflo membranes, Amicon). The concentrate (about 5 mL) was then loaded onto a Bio Gel A 0.5 size exclusion column (80 cm x 2.5 cm) and eluted with buffer A (500 mL). Fractions containing MIP synthase were combined, concentrated, and stored at -80 °C. To remove the contaminating D-glucose 6-phosphate dehydrogenase, an enzyme solution was passed through a Blue A affinity column (2 mL) and eluted with buffer A. The eluted fractions (0.5 mL) with high concentration of protein were combined and concentrated with a Centricon concentrator (Amicon).

Enzyme Kinetics

Determination of Inhibition Constants (K_i) for Inhibitors of DHQ Synthase.[14b, 41]

To quantify the concentration of a given dicarboxylate or tricarboxylate inhibitor, the carboxylic acid solution was concentrated to dryness, exchanged twice with D_2O, and redissolved in a known volume of D_2O. From this stock solution, an aliquot was mixed with a known volume of sodium 3-(trimethylsilyl)propionate-2,2,3,3,-d_4 (TSP) solution (5.0 mM in D_2O). The concentration of the carboxylic acid was quantitated by comparison of the integration of selected proton resonances of the carboxylic acid with the integrated resonance of the internal TSP standard. Phosphur-containging inhibitiors were quantified by the method of Avila and Ames.[120]

DHQ synthase activity was assayed in a 1.0 mL solution of 3-(*N*-morpholino)propanesulfonate (MOPS) buffer (50 mM, pH 7.7) containing $CoCl_2$ (50 μM), NAD (10 μM), dehydroquinase (1 unit), and varying amounts of DAHP and of the enzyme inhibitor. After equilibration at rt, DHQ synthase (0.024 units) was added and the increase in absorbance at 234 nm was monitored over time. Initial rates were determined by linear squares fits of the progress curves and were used to determine inhibition constants.

Time-dependent inhibition was analyzed following literature procedures.[14b,77] Association rate constants (k_{on}) were determined after incubation of a range of inhibitor and substrate concentrations in the assay buffer at rt, followed by addition of enzyme (final concentration 0.04 μM) and monitoring the reaction over time. The progress curves were fitted to the equation: absorbance = $a + bt + ce^{-kt}$ (where a, b, and c are adjustable parameters, $k = k_{obsd}$ is the apparent first-order rate constant for loss of activity, and t is time), for portions of the progress curves where a control in the absence of inhibitor is linear. A plot of k_{obsd} versus [I] was linear and supported a one-step association mechanism of inhibition for which $k_{obsd} = k_{on}$ [I]/(1+ [S]/K_m) + k_{off}. The association rate (k_{on}) was obtained from the slope of this plot. Dissociation rate constants were determined by incubation of the enzyme (4 μM) and the inhibitor (8 μM) at rt. Aliquots were withdrawn at timed-intervals and diluted (100x) into assay buffer containing DAHP (0.5 mM) and dehydroquinase (1 unit). The progress curve was fitted to the equation: absorbance = $a + bt + ce^{-kt}$ (where $k = k_{off}$). The inhibition constant was then obtained from the equation $K_i = k_{off} / k_{on}$.

To measure enzyme-bound NADH formation during enzyme inhibition,[41] a 0.8 mL solution of MOPS buffer (50 mM, pH 7.7) containing $CoCl_2$ (50 μM), NAD (10 μM), and DHQ synthase (10 μM) was incubated at rt in a quartz cuvette. Spectra (190-820 nm) were measured to establish the base-line absorbance. A solution containing known concentration of an inhibitor was added to the cuvette and the change in absorbance at 340 nm was monitored.

Determination of Inhibition Constants (K_i) for Inhibitors of MIP Synthase.[29]

MIP synthase activity was assayed in a 2.0 mL solution containing 50 mM Tris·HCl, pH 7.7, NH_4Cl (2 mM), DTT (0.2 mM), NAD (1 mM), and varying amounts of D-glucose 6-phosphate and of the enzyme inhibitor. After incubation at 37 °C for 10 min, MIP synthase (0.01-0.02 units) was added and aliquots were withdrawn at timed intervals over a 30 min period. Initial rates were determined by linear squares fits of the progress curves and were used to determine inhibition constants.

Time-dependent inhibition was analyzed according to the procedure decribed for DHQ synthase. The association rate (k_{on}) was obtained from the slope of k_{obsd} (deduced from progress curves) versus [I]. Dissociation rate constants were determined by incubation of the enzyme (14 μM) and the inhibitor (1 mM) in a solution of Tris·HCl buffer (25 mM, pH 7.2) containing NAD (1 mM) at rt overnight. This was transferred to a Centricon-30 concentrator (Amicon) and exchanged five times with Tris·HCl buffer (25 mM, pH 7.2) containing NAD (1 mM). An aliquot was diluted (100x) and added to an assay buffer containing D-glucose-6-phosphate (10 mM). MIP synthase activity was assayed as described previously. The progress curve was fitted to the equation: absorbance $= a + bt + ce^{-kt}$ (where $k = k_{off}$) to determine the dissociation rate constant (k_{off}). The inhibition constant was then obtained from the equation $K_i = k_{off} / k_{on}$.

To measure enzyme-bound NADH formation during enzyme inhibition,[29] a solution of Tris·HCl buffer (25 mM, pH 7.2) containing NH_4Cl (2 mM), NAD (1 mM), DTT (0.2 mM), and MIP synthase (15 μM, 0.7 mL) was incubated at rt in a quartz cuvette for 10 min. Spectra (190-820 nm) were measured to establish the base-line absorbance. A solution containing known concentration of an inhibitor was added to the cuvette and the change in absorbance at 340 nm was monitored.

Deuterium Exchange at C-6 of 2-Deoxy-D-glucose 6-phosphate 74a Catalyzed by MIP Synthase.

A solution of buffer in D_2O was prepared by lyophilizing 25 mM Tris-trifluoroacetate buffer (pH 7.5) containing NH_4Cl (2 mM), NAD (1 mM), DTT (0.2 mM), and dissolving the residue in D_2O. A concentrated solution of MIP synthase in D_2O was prepared by repeatedly concentrating the enzyme solution by ultrafiltraion and rediluting with deuteriated buffer. To a solution of 2-deoxy-D-glucose 6-phosphate **74a** (1 mM) in deuteriated buffer (0.8 mL) at 37 °C was added MIP synthase (1 unit) in the same buffer. After 24 h at 37 °C, the solution was passed through a short column of Dowex 50 (H^+), and the eluates was concentrated under reduced pressure, redissolved in D_2O, and then lyophilized. The residue was then dissolved in D_2O and analyzed by ^1H NMR (500 MHz).

BIBLIOGRAPHY

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1 Frey, P. A. In *Pyridine Nucleotide Coenzymes*; Wiley: New York, 1987; part B, Chaper 13.

2 Srinivasan, P. R.; Rotchschild, J.; Sprinson, D. B. *J. Biol. Chem.* **1963**, *238*, 3176.

3 Loewus, F. A.; Kelly, S. *Biochem. Biophys. Res. Commun.* **1962**, *7*, 204.

4 (a) Kakinuma, K.; Ogawa, Y.; sasaki, T.; Seto, H.; Otake, N. *J. Am. Chem. Soc.* **1981**, *103*, 5614. (b) Kakinuma, K.; Ogawa, Y.; Sasaki, T.; Seto, H.; Otake, N. *J. Antibiot.* **1989**, *42*, 926.

5 Palmer, J. L.; Abeles, R. H. *J. Biol. Chem.* **1976**, *251*, 5817.

6 Duerre, J. A.; Walker, R. D. In *The Biochemistry of Adenosylmethionine*; Salvatore, F.; Borek, E.; Zappia, V.; Williams-Ashman, H.; Schlenk, F., Ed.; Columbia: New York, 1977, p. 43.

7 Maxwell, E. S. *J. Biol. Chem.* **1957**, *229*, 139.

8 (a) Haslam, E. *The Shikimate Pathway*; John Wiley and Sons: New York, 1974. (b) Weirs, U.; Edwards, J. M. *The Biosynthesis of Aromatic Compounds*; Wiley-Interscience: New York, 1980. (c) Ganem, B. *Tetrahedron***1978**, *34*, 3353.

9 (a) Grossbard, E.; Alkinson. D., Eds.; *The Herbicide Glyphosate*; Butterworths: Boston, 1985. (b) Devine, M. E.; Duke, S. O.; Fedlke, C. F. In *Physiology of Herbicide Action*; Prentice Hall: New Jersey, 1993; Chapter 13. (c) Franz, J. E., Mao, M. K.; Sikorski, J. A. In *Glyphosate: An Extraodinary Global Herbicide*; American Chemical Society Monograph, Washington, DC, 1996.

10 Roberts, F.; Roberts, C. W.; Johnson, J. J.; Kyle, D. E.; Krell, T.; Coggins, J. R.; Coombs, G.; Milhous, W.; Tzipori, D. V.M.; Ferguson, D.; Chakrabarti, D.; McLeod, R. *Nature* **1998**, *393*, 801.

11 (a) Davies, G. M.; Barrett-Bee, K. J.; Jude, D. A.; Lehan, M.; Nichols, W. W.; Pinder, P. E.; Thain, J. L.; Watkins, W. J.; Wilson, R. G. *Antimicrob. Agents Chemoth.* **1994**, *38*, 403. (b) Ewart, C. D. C.; Jude, D. A.; Thain, J. L.; Nichols, W. W. *Antimicrob. Agents Chemoth.* **1995**, *39*, 87.

12 (a) Hoiseth, S. K.; Stocker, B. A. *Nature* **1981**, *291*, 238. (b) Dougan, G.; Chatfield, S.; Pickard, D.; Bester, J.; O'Callaghan, D.; Maskell, D. *J. Infect. Dis.* **1988**, *158*, 1329. (c) Miller, I. A.; Chatfield, S.; Dougan, G.; Desilva, L.; Joysey, H. S.; Hormaeche, C. *Mol. Gen. Genet.* **1989**, *215*, 312. (d) O'Callaghan, D.; Maskell, D.; Liew, F. Y.; Easman, C. S.; Dougan, G. *Infect. Immun.* **1988**, *56*, 419. (e) Günel-Özcan, A.; Brown, K. A.; Allen, A. G.; Maskell, D. J. *Microbial Pathogen.* **1997**, *23*, 311. (f) Marskell, D. J.; Sweeney, K. J.; O'Callaghan, D.; Hormaeche, C. E.; Liew, F. Y.; Dougan, C. *Microb. Pathog.* **1987**, *2*, 211.

13 (a) Bender, S. L.; Mehdi, S.; Knowles, J. R. *Biochemistry* **1989**, *28*, 7555. (b) Rotenberg, S. L.; Sprinson, D. D. *J. Biol. Chem.* **1978**, *253*, 2210. (c) Le Marechal, P.; Azerad, R. *Biochimie* **1976**, *58*, 1123. (d) Maitra, U. S.; Sprinson, D.

B. *J. Biol. Chem.* **1978**, *253*, 5426. (e) Frost, J. W.; Bender, J. L.; Kadonaga, J. T.; Knowles, J. R. *Biochemistry* **1984**, *23*, 4470. (f) Frost, J. W.; Knowles, J. R. *Biochemistry* **1984**, *23*, 4465. (g) Bender, S. L.; Widlanski, T.; Knowles, J. R. *Biochemistry* **1989**, *28*, 7560. (h) Widlanski, T.; Bender, S. L.; Knowles, J. R. *J. Am. Chem. Soc.* **1987**, *109*, 1873. (i) Widlanski, T.; Bender, S. L.; Knowles, J. R. *J. Am. Chem. Soc.* **1989**, *111*, 2299. (j) Bartlett, P. A.; Satake, K. *J. Am. Chem. Soc.* **1988**, *110*, 1628. (k) Bartlett, P. A.; McLaren, K. L.; Marx, M. A. *J. Org. Chem.* **1994**, *59*, 2082. (l) Parker, E. J.; Coggins, J. R.; Abell, C. *J. Org. Chem.* **1997**, *62*, 8582. (m) Widlanski, T.; Bender, S. L.; Knowles, J. R. *Biochemistry* **1989**, *28*, 7572.

14 (a) Montchamp, J.-L.; Peng, J.; Frost, J. W. *J. Org. Chem.* **1994**, *59*, 6999. (b) Montchamp, J.-L.; Frost, J. W. *J. Am. Chem. Soc.* **1997**, *119*, 7645.

15 Carpenter, E. P.; Hawkins, A. R.; Frost, J. W.; Brown, K. A. *Nature* **1998**, *394*, 299.

16 (a) Morgan, N. G. *Cell Signalling*; Openg University Press: Mitlon Keynes, 1989. (b) Billington, D. C. *The Inositol Phosphates*; VCH: New York, 1993. (c) Potter, B. V. L.; Lampe, D. *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 1933. (d) Findley, J. *Chemistry in Britain* **1991**, 724. (e) Fisher S. K.; Heacock, A. M.; Agranoff, B. W. *J. Neurochem.* **1992**, *58*, 18. (f) Potter, B. V. L. *Nat. Prod. Rep.* **1990**, 1. (g) Downes, C. P.; MacPhee, C. H. *Eur. J. Biochem.* **1990**, *193*, 1. (h) Majerus, P. W.; Ross, T. S.; Cunningham, T. W.; Caldwell, K. K.; Jefferson, A. B.; Bansal, V. S. *Cell* **1990**, *63*, 459.

17 (a) Duman, R. S.; Heninger, G. R.; Nesteler, E. J. *Arch. Gen. Psychiatry* **1997**, *54*, 597. (b) Lenox, R. H.; Watson, D. G. *Clin. Chem.* **1994**, *40*, 309.

18 (a) Lenox, R. H. In *Molecular Mechanisms of Neuronal Responsiveness*; Ehrlich, Y.; Lenox, R. H.; Kornecki, E.; Berry, W. O., Ed.; Plenum Press: New York, 1987, p. 515. (b) Berridge, M. J.; Downes, P. F.; Hanley, M. R. *Biochem. J.* **1982**, *206*, 587. (c) Berridge, M. J.; Downes, P. F.; Hanley, M. R. *Cell* **1989**, *59*, 411. (d) Nahorski, S. R.; Ragan, C. I.; Challiss, R. A. J. *Trends Pharmacol. Sci.* **1991**, *12*, 297. (e) Sherman, W. R. In *Lithium and the Cell*; Birch. N. J., Ed.; Academic: London, 1991, p. 121.

19 (a) Kofman, O.; Belmaker, R. H. *Biol. Psychiatry* **1993**, *34*, 839. (b) Levine J.; Rapaport, A.; Lev, L.; Bersudsky, Y.; Kofman, O.; Belmaker, R. H.; Shapiro, J.; Agam, G. *Brain Research* **1993**, *627*, 168. (c) Benjamin, J.; Agam, G.; Levine, J.; Bersudsky, Y.; Kofman, O.; Belmaker, R. H. *Psychopharmacology Bulletin* **1995**, *31*, 167. (d) Belmaker, R. H.; Bersudsky, Y.; Benjamin, J.; Agam, G.; Levine, J.; Kofman, O. In *Depression and Mania: From Neurobiology to Treatment*; Gessa, G.; Fratta, W.; Pani, L.; Serra, G., Ed.; Raven: New York, 1995, p. 67.

20 Peet, M.; Pratt. J. P. *Drugs* **1993**, *46*, 7.

21 (a) Loewus, F. A.; Loewus, M. W. *Ann. Rev. Plant Physiol.* **1983**, *34*, 137. (b) Wong, Y.-H. H.; Sherman, W. R. *J. Biol. Chem.* **1985**, *260*, 11083.

22 Nishizuka, Y. *Science* **1992**, *258*, 607.

23 (a) Charlampous, F. C.; Chen, I.-W. *Methods Enzymol.* **1966**, *9*, 698. (b) Donahue, T. F.; Henry, S. A. *J. Biol. Chem.* **1981**, *256*, 7077.

24 (a) Maeda, T.; Eisenberg, F., Jr. *J. Biol. Chem.* **1980**, *255*, 8458. (b) Mauck, L. A.; Wong, Y.-H.; Sherman, W. R. *Biochemistry* **1980**, *19*, 3622. (c) Naccarata, W. F.; Ray, R. E.; Wells, W. W. *Arch. Biochem. Biophys.* **1974**, *164*, 194. (d) Pittner, F.; Hoffmann-Osternhof, O. *Monatsh. Chem.* **1976**, *107*, 793. (e) Barnett, J. E. G.; Corina, D. L. *Biochem. J.* **1968**, *108*, 125.

25 (a) Escamilla, J. E.; Contreras, M.; Martinez, A.; Zentella-Pina, M. *Arch. Biochem. Biophys.* **1982**, *218*, 275. (b) Pittner, F.; Torarora, I. I.; Kornitskaya, E. Y.; Khoklor, A. S.; Hoffmann-Ostenhof, O. *Mol. Cell Biochem.* **1979**, *25*, 43. (c) Zsindely, A.; Szabolcs, M.; Aradi, J.; Schblik, M.; Kiss, A.; Szabo, G. *Acta Biol. Acad. Sci. Hung.* **1977**, *28*, 281.

26 (a) Loewus, M. W.; Loewus, F. A. *Plant Physiol.* **1971**, *48*, 255. (b) Maiti, I. B.; Rosenfield, C.-L.; Loewus, F. A. *Phytochemistry* **1978**, *17*, 1185.

27 (a) Barnett, T. E. G.; Rasheed, A.; Cornia, D. L. *Biochem. J.* **1973**, *131*, 21. (b) Barnett, T. E. G.; Rasheed, A.; Cornia, D. L. *Biochem. Soc. Trans.* **1973**, *1*, 1267.

28 Byun, S. M.; Jenness, R. *Biochemistry* **1981**, *20*, 5174.

29 Peng, J. Ph.D. Dissertation, Michigan State University, 1996.

30 (a) Chen, C. H.-J.; Eisenberg, F., Jr. *J. Biol. Chem.* **1975**, *250*, 2963. (b) Eisenberg, F., Jr.; Maeda, T. In *Inositol and Phosphoinositides*; Bleasdale, J. E.; Eichberg, J.; Hauser, G., Ed.; Humana: New Jersey, 1985, p. 3.

31 (a) Bynn, S. M.; Jenness, R.; Ridley, W. P.; Kukwood, S. *Biochem. Biophys. Res. Commun.* **1973**, *54*, 961. (b) Chen, I.-W.; Charalampous, F. C. *Biochim. Biophys. Acta* **1967**, *136*, 568. (c) Loewus, M. W. *J. Biol. Chem.* **1977**, *252*, 7221.

32 (a) Otsuka, H.; Mascaretti, O. A.; Hurley, L. H.; Floss, H. G. *J. Am. Chem. Soc.* 1980, *102*, 6817. (b) Loewus, M. W.; Loewus, F. A.; Grillinger, G.-U.; Otsuka, H.; Floss, H. G. *J. Biol. Chem.* **1980**, *255*, 11710.

33 (a) Migaud, M. E.; Frost, J. W. *J. Am. Chem. Soc.* **1995**, *117*, 5154. (b) Migaud, M. E.; Frost, J. W. *J. Am. Chem. Soc.* **1996**, *118*, 495.

34 Schloss, J. V. *Acc. Chem. Res.* **1988**, *21*, 348. (b) Morrison, J. F.; Walsh, C. T. *Adv. Enzymol.* **1988**, *61*, 201.

35 Montchamp, J.-L.; Frost, J. W. *J. Org. Chem.* **1994**, *59*, 7596.

36 Nikolaides, N.; Ganem, B. *Tetrahedron Lett.* **1989**, *30*, 1461.

37 (a) Montchamp, J.-L.; Piehler, L. T.; Frost, J. W. *J. Am. Chem. Soc.* **1992**, *114*, 4453. (b) Piehler, L. T.; Montchamp, J.-L.; Frost, J. W.; Manly, C. J. *Tetrahedron* **1991**, *47*, 2423.

38 (a) Ley, S. V.; Woods, M.; Zanotti-Gerosa, A. *Synthesis* **1992**, 52. (b) Ley, S. V.; Leslie, R.; Tiffin, P. D.; Woods, M. *Tetrahedron Lett.* **1992**, *33*, 4767. (c) Ley, S. V.; Boons, G.-J.; Leslie, R.; Woods, M.; Hollinshead, D. M. *Synthesis* **1993**, 689.

39 Ley, S.V.; Priepke, H. W. M.; Warriner, S. L. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 2290.

40 Berens, U.; Leckel, D.; Oepen, S. C. *J. Org. Chem.* **1995**, *60*, 8204.

41 Montchamp, J.-L.; Tian, F.; Hart, M. E.; Frost, J. W. *J. Org. Chem.* **1996**, *61*, 3897.

42 Watanabe, Y.; Mitani, M.; Morita, T.; Ozaki, S. *J. Chem. Soc., Chem. Commun.* **1989**, 482.

43 Douglas, N. L.; Ley, S. V.; Osborn, H. M. I.; Owen, D. R.; Priepke, H. W. M.; Warriner, S. L. *Synlett* **1996**, 793.

44 (a) Delfourne, E.; Despeyroux, P.; Corrichon, L.; Véronique, J. *J. Chem. Res.* **1991**, 56. (b) Despeyroux, P.; Baltas, M.; Gorrichon, L. *Bull. Soc. Chim. Fr.* **1997**, *134*, 777.

45 (a) Griffith, W. P.; Ley, S. V. *Aldrichim. Acta* **1990**, *23*, 13. (b) Ley, S. V.; Norman, J.; Griffith, W. P.; Marsden, S. P. *Synthesis*, **1994**, 639.

46 Shing, T. K.; Tsui, H.-C.; Zhou, Z.-H. *J. Org. Chem.* **1995**, *60*, 3121.

47 (a) Singh, A. K.; Bakshi, R. K.; Corey, E. J. *J. Am. Chem. Soc.* **1987**, *109*, 6187. (b) Montchamp, J.-L. Ph.D. Dissertation, Purdue University, 1992.

48 (a) Saksena, A. K.; Mangiaracina, P. *Tetrahedron Lett.* **1983**, *24*, 273. (b) Turnbull, M. D.; Hatter, G.; Ledgerwood, D. E. *Tetrahedron Lett.* **1984**, *25*, 5449.

49 Paulissen, R.; Reimlinger, H.; Hayez, E.; Huber, A. J.; Teyssié, P. *Tetrahedron Lett.* **1973**, 2233.

50 (a) Davis, F. A.; Lamendola, J., Jr.; Nadir, U.; Kluger, E. W.; Sedergran, T. C.; Panunto, T. W.; Billmers, R.; Jenkins, R., Jr.; Turchi, I. J.; Watson, W. H.; Chen, J. S.; Kimura, M. *J. Am. Chem. Soc.* **1980**, *102*, 2000. (b) Davis, F. A.; Vishwakarma, L. C.; Billmers, J. M.; Finn, J. *J. Org. Chem.* **1984**, *49*, 3241.

51 Keck, G. E.; Yates, J. B. *J. Am. Chem. Soc.* **1982**, *104*, 5829.

52 Carlsen, P. H. J.; Katsuki, T.; Martin, V. S.; Sharpless, K. B. *J. Org. Chem.* **1981**, *46*, 3936.

53 Chaudhuri, S.; Duncan, K.; Coggins, J. R. *Methods Enzymol.* **1987**, *142*, 320.

54 (a) Segel, I. H. *Biochemical Calculations*, 2nd ed.; Wiley: New York, 1976. (b) Galli, A.; Mori, F. *J. Pharm. Pharmacol.* **1996**, *48*, 71.

55 (a) Montchamp, J.-L. Piehler, L. T.; Tolbert, T.; Frost, J. W. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1349. (b) Montchamp, J.-L.; Piehler, L. T.; Tolbert, T. J.; Frost, J. W. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 1403.

56 Sikorski, J. A.; Miller, M. J.; Braccolino, D. S.; Cleary, D. G.; Corey, S. D.; Font, J. L.; Gruys, K. J.; Han, C. Y.; Lin, K.-C.; Pansegrau, P. D.; Ream, J. E.; Schnur, D.; Shah, A.; Walker, M. C. *Phosphorus, Sulfur, and Silicon* **1993**, *76*, 115.

57 (a) Goldman, R.; Kohlbrenner, W.; Lartey, P.; Pernet, A. *Nature* **1987**, *329*, 162. (b) Hammond, S. M.; Claesson, A.; Jansson, A. M.; Larsson, L.-G.; Pring, B. G.; Town, C. M.; Ekström, B. *Nature* **1987**, *327*, 730.

58 (a) Vallee, B. L.; Auld, A. S. *Biochemistry* **1990**, *29*, 5647. (b) Vallee, B. L.; Auld, A. S. *Acc. Chem. Res.* **1993**, *26*, 543. (c) Auld, D. S. *Struct. Bond.* **1997**, *89*, 29.

59 (a) Lipscomb, W. N.; Sträter, N. *Chem. Rev.* **1996**, *96*, 2375. (b) Vallee, B. L. In *Zinc Enzymes*; Spiro, T. G. Ed.; John Wiley and Sons: New York, 1983, p 1. (c) Prasad, A. S. *Biochemistry of Zinc*; Plenum: New York, 1993.

60 Lambert, J. M.; Boocock, M. R.; Coggins, J. R. *Biochem. J.* **1985**, *226*, 817.

61 Moore, J. D.; Skinner, M. A.; Swatman, D. R.; Hawkins, A. R.; Brown, K. A. *J. Am. Chem. Soc.* **1998**, *120*, 7105.

62 Cotton, S. In *Lanthanides and Actinides*; Macmillan Education Ltd.: Houndmills, Basingstoke, Hampsshire, 1991; Chapter 2.

63 (a) Ondetti, M. A.; Rubin, B.; Cushman, D. W. *Science* **1977**, *196*, 441. (b) Ondetti, M. A.; Cushman, D. W. *CRC Crit. Rev. Biochem.* **1984**, *16*, 381.

64 (a) Lan, S.-J.; Weinstein, S. H.; Migdalof, B. H. *Drug Metab. Dispos.* **1982**, *10*, 306. (b) Drummer, O. H.; Worland, P. J.; Jarrott, B. *Biochem. Pharmacol.* **1983**, *32*, 1563. (c) Yeung, J. H. K.; Breckenridge, A. M.; Park, B. K. *Biochem. Pharmacol.* **1983**, *32*, 2467.

65 Gordon, E. M; Godfrey, J. D.; Pluscec, J.; Von Langen, D.; Natarajan, S. *Biochem, Biophys. Res, Commun.* **1985**, *126*, 419.

66 (a) *Copper in the Environment, Part II, Health Effects,* Nriagu, J. O., Eds; Wiley: New York, 1979, p 83. (b) *Trace Elements in Human Health and Disease*; Prasad, A. S., Eds.; Academic: New York, 1976, Vol 1.

67 Bounaga, S.; Laws, A. P.; Galleni, M.; Page, M. I. *Biochem. J.* **1998**, *331*, 703.

68 Lindstad, R. I.; Mckinley-Mckee, J. S. *Eur. J. Biochem.* **1996**, *241*, 142.

69 Tian, F.; Montchamp, J.-L.; Frost, J. W. *J. Org. Chem.* **1996**, *61*, 7373.

70 Chandran, S; Frost, J. W. Unpublished result.

71 Rich, D. H. In *Comprehensive Medicinal Chemistry*; Hansch, C.; Sammes, P. G.; Taylor, J. B., Ed.; Pergamon: New York, 1990, Vol 2, p 391.

72 Montchamp, J.-L.; Migaud, M. E.; Frost, J. W. *J. Org. Chem.* **1993**, *58*, 7679.

73 (a) Kim, C. U.; Lew W.; Williams, M. A.; Liu, H.; Zhang, L.; Swaminathan, S.; Bischofberger, N.; Chen M. S.; Mendel, D. B.; Tai, C. Y.; Laver, G.; Stevens, R. C. *J. Am. Chem. Soc.* **1997**, *119*, 681. (b) Rohloff, J. C.; Kent, K. M.; Postich, M. J.; Becker, M. W.; Chapman, H. H.; Kelly, D. E.; Lew, W.; Louie, M. S.; McGee, L. R.; Prisbe, E. J.; Schultze, L. M.; Yu, R. H.; Zhang, L. *J. Org. Chem.* **1998**, *63*, 4545.

74 (a) Pansegrau, P. D.; Anderson, K. S.; Widlanski, T.; Ream, J. E.; Sammons, R. D.; Sikorski, J. A.; Knowles, J. R. *Tetrahedron Lett.* **1991**, *32*, 2589. (b) Kim, C.-G.; Kirschning, A.; Bergon, P. Ahn, B. Y.; Wang, J. J.; Shibuya, M.; Floss, H. G. *J. Am. Chem. Soc.* **1992**, *114*, 4941.

75 (a) Thiem, J.; Meyer, B. *Org. Magn. Reson.* **1978**, *11*, 50. (b) Kozlov, V. A; Churusova, S. G.; Ivanchenko, V. I.; Negrebetskii, V. V.; Grapov, A. F.; Mel'nikov, N. N. *Zh. Obshch. Khim.* **1986**, *56*, 2013. (c) *Phosphorus-31 NMR Spectroscopy in Stereochemical Analysis*; Verkade, J. G., Quin, L. D., Eds.; VCH: Deerfield Beach, FL, 1987.

76 Caron, M.; Sharpless, K. B. *J. Org. Chem.* **1985**, *50*, 1557.

77 (a) Morrison, J. F.; Walsh, C. T. *Adv. Enzymol.* **1988**, *61*, 201. (b) Schloss, J. V. In *Target Sites of Herbicide Action*; Böger, P., Sandmann, G., Eds.; CRC: Boca Raton, FL., 1989. (c) Morrison, J. F.; Stone, S. R. *Comments Mol. Cell. Biophys.* **1985**, *2*, 347.

78 Duggleby, R. G.; Attwood, P. V.; Wallace, J. C.; Keech, D. B. *Biochemistry* **1982**, *21*, 3364.

79 Gollub, E.; Zalkin, H.; Sprinson, D. B. *Methods Enzymol.* **1971**, *17A*, 349.

80 (a) Sigel, H.; McCormick, D. B. *Acc. Chem. Res.* **1970**, *3*, 201. (b) Irving, H.; Williams, R. J. P. *Nature* **1948**, *162*, 746.

81 Laurie, S. H. In *Comprehensive Coordination Chemistry*; Wilkinson, G., Gillard, R., D, McCleverty, J. A., Eds; Pergamon: Oxford, 1987, Vol 2, p 739.

82 Kiss, T.; Farkas, E.; Kozlowski, H. *Inorg. Chim. Acta* **1989**, *155*, 281.

83 (a) Kvassmann, J.; Pettersson, G. *Eur. J. Biochem.* **1980**, *103*, 557. (b) Kvassmann, J.; Larsson, A.; Pettersson, G. *Eur. J. Biochem.* **1981**, *114*, 555. (c) Shore, J. D.; Gutfreund, H.; Brooks, R. L.; Santiago, D.; Santiago, P. *Biochemistry* **1974**, *13*, 4185.

84 Wu, Z.; Walsh, C. T. *J. Am. Chem. Soc.* **1996**, *118*, 1785.

85 (a) Mortensen, M. E. *J. Pediatr.* **1994**, *125*, 233. (b) Aposhian, H. V. *Annu. Rev. Pharmacol. Toxicol.* **1983**, *23*, 193. (c) Aposhian, H. V.; Aposhian, M. M. *Annu. Rev. Pharmacol. Toxicol.* **1990**, *30*, 279.

86 Grandjean, P. In *Abstracts from the Conference on the Role of Chelating Agents for the Prevention, Intervention, and Treatment of Exposures to Toxic Metals*, 22-23 September 1994, Reserch Triangle Park, NC; A19.

87 Schray, K. J.; Benkovic, S. J. *Acc. Chem. Res.* **1978**, *11*, 136.

88 (a) Chen, M.; Whistler, R. L. *Arch. Biochem. Biophys.* **1975**, *169*, 392. (b) Burton, L. E.; Wells, W. W. *Arch. Biochem. Biophys.* **1977**, *181*, 384.

89 (a) Redman, C. E.; Nieman, C. *Organic Syntheses*; Wiley: New York, 1955; Collect. Vol. 3, p 11. (b) Funabashi, M.; Hasegawa, T. *Bull. Chem. Soc. Jpn.* **1991**, *64*,

2528. (c) Maradufu, A.; Mackie, D. M.; Perlin, A. S. *Can J. Chem.* **1972**, *50*, 2617.

90 (a) Giese, B.; Dupuis, J. *Tetrahedron Lett.* **1984**, *25*, 1349. (b) Koch, A.; Lamberth, C.; Wetterich, F.; Giese, B. *J. Org. Chem.* **1993**, *58*, 1083.

91 Drueckhammer, D. G.; Wong, C.-H. *J. Org. Chem.* **1985**, *50*, 5912.

92 Migaud, M. E. Ph.D. Dissertation, Michigan State University, 1996.

93 Barnett, T. E. G.; Brice, R. E.; Corina, D. L. *Biochem. J.* **1970**, *119*, 183.

94 This contradicts the finding in ref. 88b. In our hands, the assay conditions used in ref. 88b resulted in substantial interference of inorganic phosphate released from 5-thio-D-glucose 6-phosphate.

95 Diehl, R. E.; Whiting, P.; Potter, J.; Gee, N.; Ragan, C. I.; Linemeyer, D.; Schoepfer, R.; Bennet, C.; Dixon, R. A. F. *J. Biol. Chem.* **1990**, *265*, 5946.

96 Grimshaw, C. E.; Whistler, R. L.; Cleland, W. W. *J. Am. Chem. Soc.* **1979**, *101*, 1521.

97 (a) Salas, M.; Viñuela, E.; Sols, A. *J. Biol. Chem.* **1965**, *240*, 561. (b) Bailey, J. M.; Fishman, P. H.; Pentchev, P. G. *J. Biol. Chem.* **1968**, *243*, 4827.

98 Lewis, D. *J. Chem. Soc. Perkin Trans. 2* **1990**, 1349.

99 Piel E. V.; Purves, C. B. *J. Am. Chem. Soc.* **1939**, *61*, 2978.

100 (a) Omura, K.; Swern, D. *Tetrahedron* **1978**, *34*, 1651. (b) Mancuso, A. J.; Swern, D. *Synthesis* **1981**, 165.

101 Savignac, P.; Mathey, F. *Synthesis* **1982**, 725.

102 Horita, K.; Yoshioka, T.; Tanaka, T.; Oikawa, Y.; Yonemitsu, O. *Tetrahedron* **1986**, *42*, 3021.

103 Szeja, W.; Fokt, I.; Grynkiewicz, G. *Recl. Trav. Chim. Pays-Bas* **1989**, *108*, 224.

104 Estevez, V. A.; Prestwich, G. D. *J. Am. Chem. Soc.* **1991**, *113*, 9885.

105 Johansson, R.; Samuelsson, B. *J. Chem. Soc. Perkin Trans. 1*, **1984**, 2371.

106 (a) Menger, F. M. *Tetrahedron* **1983**, *39*, 1013. (b) Menger, F. M.; Chow, J. F.; Kaiserman, H.; Vasquez, P. C. *J. Am. Chem. Soc.* **1983**, *105*, 4996. (c) Lansbury, P. T.; Mojica, C. A. *Tetrahedron Lett.* **1986**, *27*, 3967. (d) Blay, G.; Schrijvers, R.; Wijnberg, J. B. P. A.; Groot, A. D. *J. Org. Chem.* **1995**, *60*, 2188.

107 Gross, R. S.; Mehdi, S.; McCarthy, J. R. *Tetrahedron Lett.* **1993**, *34*, 7197.

108 (a) Grewe, R.; Jeschke, J. P. *Chem. Ber.* **1956**, *89*, 2080. (b) Grewe, R.; Haendler, H. *Biochem. Prep.* **1966**, *11*, 21.

109 (a) Heyns, K.; Gottschalk, H. *Chem. Ber.* **1961**, *94*, 343. (b) Knowles, P. F.; Haworth, R. D.; Haslam, E. *J. Chem. Soc.* **1961**, 1854. (c) Knowles, P. F.; Haworth, R. D.; Haslam, E. *Methods in Enzymology* **1963**, *6*, 498.

110 (a) Otera, J.; Yano, T.; Kawabata, A.; Nozaki, H. *Tetrahedron Lett.* **1986**, *27*, 2383. (b) Otera, J.; Dan-oh, N.; Nozaki, H. *J. Org. Chem.* **1991**, *56*, 5307.

111 Sutherland, J. K.; Watkins, W. J.; Bailey, J. P.; Chapman, A. K.; Davies, G. M. *J. Chem. Soc.* **1989**, 1386.

112 Arhart, R. J.; Martin, J. C. *J. Am. Chem. Soc.* **1972**, *94*, 5003.

113 Olah, G. A.; Welch, J. T.; Vankar, Y. D.; Nojima, M.; Kerekes, I.; Olah, J. A. *J. Org. Chem.* **1979**, *44*, 3872.

114 (a) Bowles, S.; Campbell, M. M.; Sainsbury, M. *Tetrahedron Lett.* **1989**, *30*, 3711. (b) Bowles, S.; Campbell, M. M.; Sainsbury, M.; Davies, G. M. *Tetrahedron* **1990**, *46*, 3981.

115 Campbell, M. M.; Kaye, A. D.; Sainsbury, M. *Tetrahedron Lett.* **1983**, *34*, 4745.

116 Karen, D.; Knop, D.; Frost, J. W. Unpublished result.

117 Chahoua, L.; Baltas, M.; Gorrichon, L.; Tisnès, P.; Zedde, C. *J. Org. Chem.* **1992**, *57*, 5798.

118 Sabbioni, G.; Jones, J. B. *J. Org. Chem.* **1987**, *52*, 4565.

119 Perrin, D. D.; Armarego, W. L. F.; Perrin, D. R. *Purification of Laboratory Chemicals*; Pergamon: New York, 1980.

120 (a) Ames, B. N. *Methods Enzymol.* **1966**, *8*, 115. (b) Avila, L. Z. Ph.D. Dissertation, Stanford University, 1990.

121 Gollub, E.; Zalkin, H.; Sprinson, D. B. *Methods Enzymol.* **1971**, *17A*, 349.

122 (a) Brown, J. M. *Angew. Chem. Int. Ed. Engl.* **1987**, *26*, 190. (b) Hoveyda, A. H.; Evans, D. A.; Fu, G. C. *Chem. Rev.* **1993**, *93*, 1307.

123 Kiely, D. E.; Sherman, W. R. *J. Am. Chem. Soc.* **1975**, *97*, 6810.