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Elaboration of a General Strategy for Inhibition of myo-Inositol 1-Phosphate Synthase: Active Site Interactions of Analogues Possessing Oxidized Reaction Centers

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Abstract: In addition to being an intermediate along the reaction coordinate catalyzed by *myo*-inositol 1-phosphate (MIP) synthase, *myo*-2-inosose 1-phosphate is a 3.6 μM competititve inhibitor of the enzyme at pH 7.2. To probe the importance of the oxidized reaction center in *myo*-2-inosose 1-phosphate to active site interactions and gauge the relative contribution of the keto form to these interactions, three analogues have been examined for MIP synthase inhibition. 2-Deoxy-*myo*-inositol 1-phosphate is used to gauge the impact of removing the oxidized reaction center of *myo*-2-inosose 1-phosphate. 1-Deoxy-1-(phosphonomethyl)-*myo*-2-inosose, with a methylene group substituted for the phosphate monoester oxygen of *myo*-2-inosose 1-phosphate, exists in neutral aqueous solution exclusively in its keto form. Dihydroxyacetone phosphate provides insights into the minimum set of structural requirements for inhibition of MIP synthase. 2-Deoxy-*myo*-inositol 1-phosphate was a 170 μM competitive inhibitor at pH 7.2, which is a 47-fold reduction in inhibitor potency relative to *myo*-2-inosose phosphate. Competitive inhibition of MIP synthase by 1-deoxy-1-(phosphonomethyl)-*myo*-2-inosose was dependent on solution pH with inhibition constants of 6.4, 37, and 160 μM measured, respectively, at pH 8.0, 7.2, and 6.4. Dihydroxyacetone phosphate was a 700 μM competitive inhibitor of MIP synthase at pH 7.2. The measured inhibition constants do not allow MIP synthase inhibition to be unambiguously assigned to the keto form of *myo*-2-inosose 1-phosphate. However, the examined analogues of *myo*-2-inosose 1-phosphate do establish the importance of active site interactions with an oxidized reaction center and suggest that this approach may be a general strategy for inhibiting MIP synthase.

As a membrane component and secondary messenger, inositol (Scheme 1) and its various phosphate esters play critical roles in both cellular structure and regulation of cellular processes in eucaryotes.[1] The first step in the de novo biosynthesis of inositol is the formation of *myo*-inositol 1-phosphate from D-glucose 6-phosphate in a reaction catalyzed by *myo*-inositol 1-phosphate (MIP) synthase (Scheme 1).[2] In vivo inhibition of MIP synthase might lower cellular inositol levels, thereby altering inositol-based signal transduction. Reduced inositol concentrations in brain tissue have been proposed as the mode of action for Li^+ in the treatment of manic depression.[1b,c]

For MIP synthase, each turnover of substrate into product formally requires an oxidation, enolization, intramolecular aldol condensation, and reduction. β-Nicotinamide adenine dinucleotide (NAD) is used as a catalyst rather than a cosubstrate.

[⊗] Abstract published in *Advance ACS Abstracts,* January 1, 1996.

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(2) For an overview of MIP synthase enzymology, see: Frey, P. A. In *Pyridine Nucleotide Coenzymes: Chemical, Biochemical and Medical Aspects*; Dolphin, D., Poulson, R., Avromovic, O., Eds.; Wiley: New York, 1987; Part B, p 461.

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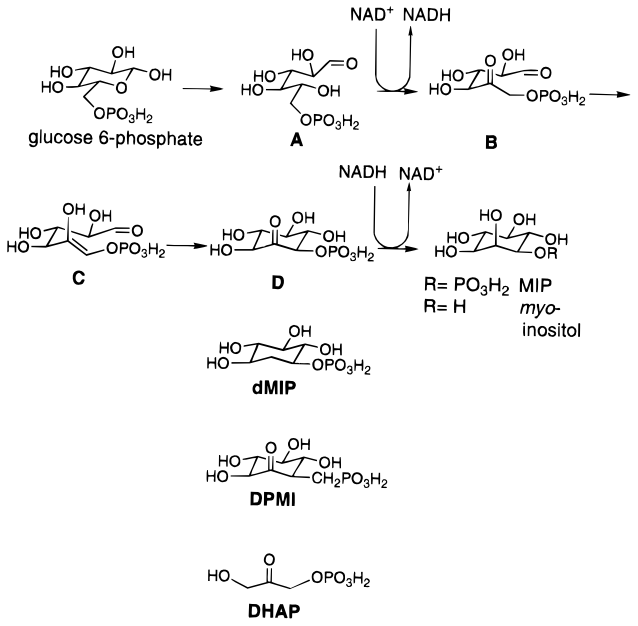
Scheme 1



Evidence supporting the catalytic role of NADH and the involvement of intermediates **B** and **D** (Scheme 1) follows from a number of experiments. Incubation of independently synthesized intermediate **B** with *apo*MIP synthase reconstituted with [4-^{3}H]NADH resulted in formation of D-[5-^{3}H]glucose 6-phosphate and [^{3}H]-*myo*-inositol 1-phosphate.[3] Addition of tritiated sodium borohydride to a solution containing MIP synthase, alkaline phosphatase, and D-glucose 6-phosphate led to the isolation of *myo*- and *scyllo*-[^{3}H]inositol.[4] Isotope effects observed with D-[5-^{3}H]glucose 6-phosphate are also consistent with intermediacy of **B** and **D**.[5]

Most enzymes that use NAD as a catalyst begin their catalytic cycle with the oxidation of an alcohol reaction center in the substrate. NAD is converted to NADH along with an intermediate where the reaction center is in a ketone oxidation state. Both NADH and the ketone intermediate remain bound at the enzyme active site. A substrate analogue with its reaction center already oxidized challenges the enzyme−NAD complex with a ketone reaction center. Lacking NADH, the enzyme active site cannot reductively process the oxidized center. Release of the substrate analogue may be slow due to binding interactions between the oxidized center and the enzyme active site. Oxidation of a reaction center leads to time-dependent, irreversible inhibition of DHQ synthase,[6] another enzyme which employs NAD as a catalyst. MIP synthase has recently been demonstrated to be inhibited by one of its own intermediates, *myo*-2-inosose 1-phosphate (**D**, Scheme 1).[7] Understanding the role of the oxidized reaction center of *myo*-2-inosose 1-phosphate in active site interactions is complicated by the molecule's solution chemistry. Only small concentrations of the keto form of *myo*-2-inosose 1-phosphate are present in aqueous solution at neutral pH.

To determine the ketone's contribution to active site interactions for *myo*-2-inosose 1-phosphate and to gauge the utility of reaction center oxidation in designing MIP synthase inhibitors, a series of *myo*-2-inosose 1-phosphate analogues were examined for enzyme inhibition. These analogues included 2-deoxy-*myo*-inositol 1-phosphate (dMIP, Scheme 1), 1-deoxy-1-(phosphonomethyl)-*myo*-2-inosose (DPMI, Scheme 1), and dihydroxyacetone 1-phosphate (DHAP, Scheme 1). dMIP was used to evaluate the impact of completely removing the oxidized reaction center of *myo*-2-inosose 1-phosphate. By substituting the phosphorylated oxygen of *myo*-2-inosose 1-phosphate with a methylene group in DPMI, the reduced substituent electronegativity adjacent to the carbonyl was anticipated to ensure the presence of sizable concentrations of keto-DPMI.[8] The keto form of DHAP is known to be one of the dominant forms of this molecule present in neutral aqueous solutions.[9] DHAP also promised insights into the minimum subset of *myo*-2-inosose 1-phosphate's structural features required for active site binding.

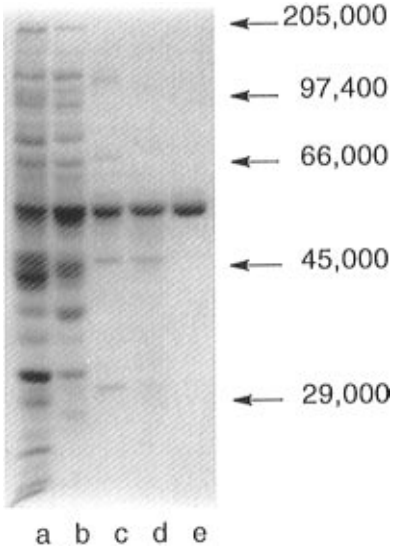


Figure 1. SDS-PAGE gel electrophoresis of enzyme solutions after the following purification steps: (lane a) crude cell lysate; (lane b) ammonium sulfate fractionation; (lane c) DEAE column; (lane d) Bio-Gel A; (lane e) HPLC DEAE column.

Table 1. Purification of MIP Synthase from *S. cerevisiae* MW5.55

	total units[a]	specific activity[b]	x-fold purification	yield %
DEAE column	1.7[c]	0.025	1.0	100
Bio-Gel A column	1.7	0.035	1.4	100
HPLC DEAE column	1.5	0.220	8.8	88

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

Results

Enzyme Purification and Characterization. MIP synthase was purified to homogeneity (Figure 1) by a modification (Table 1) of a published procedure.[10] A yeast construct, *Saccharomyces cerevisiae* MW5.55, was used as the source of MIP synthase.[11] MW5.55 expresses amplified levels of the enzyme due to multiple genomic insertions of the *INO1* locus which encodes MIP synthase.[11] Even with overexpression of MIP

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(4) (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., Eds.; Humana: New Jersey, 1985; p 3.
(5) Loewus, M. W. *J. Biol. Chem.* **1977**, *252*, 7221.
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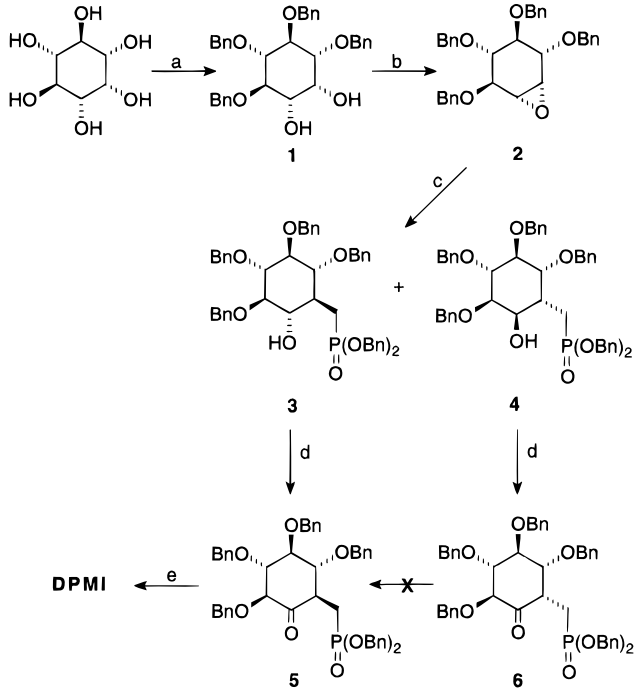
Scheme 2[a]

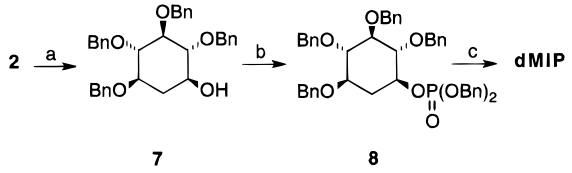


[a] (i) $(CH_3O)_2C(CH_3)_2$, *p*-TsOH, DMSO, 110 °C, (ii) NaOH, BnBr, 120 °C, (iii) aq HCl, 6 N, CH_3OH, reflux, 48%; (b) (i) $CH_3C(OCH_3)_3$, *p*-TsOH, C_6H_6, (ii) TMSCl, CH_2Cl_2, reflux (iii) $NaOCH_3$, CH_3OH, reflux, 74%; (c) $LiCH_2PO(OBn)_2$, BF_3-OEt_2, THF, −78 °C to −20 °C, 74%; (d) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, −78 °C, 5 89%, 6 94%; (e) H_2, Pd/C, THF/H_2O (4:1), 100%.

synthase, enzyme activity could not be reliably assayed either in crude cell lysate or in lysate after ammonium sulfate fractionation. Measurement of enzyme activity was based on quantitation of the inorganic phosphate selectively released from MIP during oxidation by sodium periodate.[12] Phosphatase-mediated hydrolysis of substrate D-glucose 6-phosphate was likely responsible for the high background levels of inorganic phosphate generation. This interfering enzyme activity was removed (Table 1) upon gradient elution from a DEAE cellulose column. Homogeneous enzyme (Figure 1) was obtained after size exclusion chromatographic purification on a Bio-Gel A column followed by HPLC purification using a DEAE column. The molecular weight of MIP synthase was determined to be 230 kD by size exclusion HPLC and 240 kD by chemical cross-linking using dimethyl suberimidate followed by SDS-PAGE gel electrophoresis under denaturing conditions. Electrospray mass spectrometry established the monomer molecular weight to be 61 919 ± 21 while size exclusion HPLC set the monomer molecular weight at 64.4 kD. Measured Michaelis constants (K_m) for substrate D-glucose 6-phosphate and β-nicotinamide adenine dinucleotide were, respectively, 1.2 mM and 17 μM.

Synthesis of *myo*-2-Inosose 1-Phosphate Analogues. Hydrolysis of the commercially available DHAP dimethyl ketal affords easy access to DHAP. In neutral aqueous solution DHAP exists as an approximately 1:1 mixture of ketone and hydrate. Access to structurally more complex DPMI followed from phosphonomethylation[13] of fully benzylated conduritol oxide B (**2**, Scheme 2).[14] This key intermediate in the synthesis was obtained in 36% overall yield from *myo*-inositol. Selective

Scheme 3[a]



[a] (a) $LiB(C_2H_5)_3H$, THF, −78 °C to room temperature, 59%; (b) (i) NaH, THF, reflux, (ii) TBPP, reflux, 62%; (c) H_2, Pd/C, THF/H_2O (4:1), 100%.

protection and deprotection of *myo*-inositol formed a racemic α-acetoxy chloride that upon treatment with $NaOCH_3$ in refluxing CH_3OH afforded the fully benzylated conduritol oxide B (**2**, Scheme 2). Reaction of dibenzyl (lithiomethyl)phosphonate with benzylated conduritol oxide B in the presence of boron trifluoride etherate as a Lewis acid catalyst then provided racemic, phosphonomethylated regioisomers **3** and **4**. Transdiaxial opening of the equatorially substituted oxirane conformer leads to phosphonomethylated regioisomer **4**, while regioisomer **3** most likely results from transdiaxial opening of the axially substituted conformer of benzylated conduritol oxide B. Mechanistic interpretation of the regioselectivity observed during the phosphonomethylation reaction has been presented elsewhere.[13] The structure assigned to regioisomer **4** was confirmed by X-ray crystallography.

Swern oxidation of regioisomers **3** and **4** in separate reactions afforded the respective phosphonomethylated inososes **5** and **6** in approximately equal yields. Chemical epimerization of the axial phosphonomethyl substituent of inosose **6** would afford the desired phosphonomethylated inosose **5** possessing all equatorial ring substituents. However, acidic conditions afforded only unreacted **6**. An α,β-unsaturated, phosphonomethylated inosose resulting from elimination of the C-5 benzyl ether was the only product observed under basic reaction conditions. Consequently, only inosose **5** was used to complete the synthesis of DPMI. Catalytic hydrogenation using Pd on C removed all of the benzyl protecting groups in a single step. Based on ^1H NMR, ^{13}C NMR, and two-dimensional, homonuclear (^1H,^1H) COSY, the keto form of DPMI was the only species present in aqueous solution at neutral pH.

Benzylated conduritol oxide **2** was also the key intermediate in the synthesis (Scheme 3) of dMIP. Reduction of epoxide **2** with lithium triethylborohydride led to a regioisomeric mixture consisting of a 3:1 mol ratio of 2-deoxy-*myo*-inositol (**7**) and 1-deoxy-*myo*-inositol. Chromatographic separation of the regioisomers followed by phosphorylation of 2-deoxy-*myo*-inositol (**7**) afforded fully protected dMIP. Product dMIP was obtained after hydrogenolysis removed all of the benzyl protecting groups in a single step. Only one species was present in aqueous solutions of dMIP at neutral pH based on NMR analysis.

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

In D_2O solution, multiple forms of *myo*-2-inosose 1-phosphate

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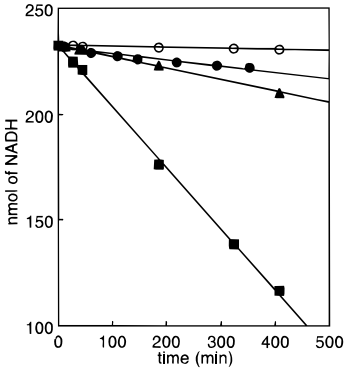

Figure 2. NADH oxidation rates for a control solution (○) containing only the complex of NADH and *apo*MIP synthase relative to this same solution upon addition of DHAP (●), DPMI (▲), and *myo*-2-inosose 1-phosphate (■).

were evident. Samples of *myo*-2-inosose 1-phosphate treated identically to those analyzed in DMSO-d_6 yielded multiple species when dissolved in D_2O. For aqueous solutions of *myo*-2-inosose 1-phosphate at pH 2.0, a ^{13}C NMR resonance at δ 206 consistent with the keto form of the carbonyl was evident, although another resonance consistent with the chemical shift of a hydrated inosose carbonyl was observed at δ 96. Analysis of this same solution by negative ion FAB indicated a molecular ion at $(M - H^+)$ 257 for the carbonyl form of *myo*-2-inosose 1-phosphate and a molecular ion at $(M - H^+)$ 275 for the hydrated form of *myo*-2-inosose 1-phosphate. Hydration of *myo*-2-inosose in aqueous medium is precedented with ^{13}C NMR resonances reported at δ 206.6 and 95.0 for the carbonyl carbons of the keto and hydrated forms.[15]

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

The inability to detect the keto form of *myo*-2-inosose 1-phosphate in aqueous solutions at pH 7 by ^1H or ^{13}C NMR led to the development of an approach that relied on enzymatic detection.[7] MIP synthase was treated with activated charcoal to remove the bound NAD cofactor. Reconstitution of the *apo*enzyme with NADH created a complex that was oxidized in the presence of *myo*-2-inosose 1-phosphate.[7] Oxidation was followed by loss of absorbance at 340 nm, and product *myo*-inositol 1-phosphate formation was corroborated by FAB mass spectrometry. These data indicated that MIP synthase has access to the keto form of *myo*-2-inosose at the neutral pH values at which enzyme activity was measured.

Confirmation that *apo*MIP synthase reconstituted with NADH is a valid method for detecting small, steady-state concentrations of the keto form of *myo*-2-inosose 1-phosphate became possible with the synthesis of DPMI. The key features of DPMI relevant to this verification issue were its close structural similarity to *myo*-2-inosose 1-phosphate and the presence of only the keto form of the oxidized center in aqueous solution at neutral pH. *Apo*MIP synthase reconstituted with NADH was oxidized by DPMI, although at a slower rate than that observed for *myo*-2-inosose 1-phosphate (Figure 2). DHAP also oxidized (Figure 2) the complex of *apo*MIP synthase and NADH. Establishing that enzyme-bound NADH oxidation was due to reduction of DPMI and DHAP followed, respectively, from detection of 1-deoxy-1-(phosphonomethyl)-*myo*-inositol and glycerol 3-phosphate formation by FAB mass spectrometry.

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Table 2. Inhibition of MIP Synthase

	type of inhibition	K_i	K_m/K_i	pH
intermediate **D**	competitive	3.6×10^{-6}	330	7.2
dMIP	competitive	170×10^{-6}	7.1	7.2
DPMI	competitive	160×10^{-6}	7.5	6.4
	competitive	37×10^{-6}	32	7.2
	competitive	6.4×10^{-6}	190	8.0
DHAP	competitive	7.0×10^{-4}	1.7	7.2

MIP Synthase Inhibition. At pH 7.2, *myo*-2-inosose 1-phosphate was a competitive inhibitor of MIP synthase with an inhibition constant (K_i) of 3.6 μM.[7] The Michaelis constant (K_m) for D-glucose 6-phosphate, the substrate of MIP synthase, was 1.2 mM. For MIP synthase in its native state with complexed NAD, dMIP and DPMI were both competitive inhibitors (Table 2) at pH 7.2 with respective K_i values of 170 and 37 μM.

The K_i for dMIP inhibition provides a measure of the oxidized center's contribution to active site interactions between MIP synthase and *myo*-2-inosose 1-phosphate. Complete removal of the oxygen of the oxidized center in *myo*-2-inosose 1-phosphate to give the methylene group in dMIP resulted in a 47-fold reduction in inhibitory potency. DPMI was a better inhibitor of MIP synthase at pH 7.2 than dMIP but a significantly weaker inhibitor relative to *myo*-2-inosose 1-phosphate. However, DPMI inhibitory potency was observed to vary significantly with solution pH (Table 2). Inhibition constants of 160, 37, and 6.4 μM were measured for DPMI at, respectively, pH 6.4, 7.2, and 8.0. This variation in active site interactions as a function of solution pH has been observed for other phosphonate analogues of phosphate monoesters and may reflect the importance of the phosphonic acid ionization state to active site interactions.[16]

Titrimetric analysis of *myo*-2-inosose 1-phosphate indicated two inflection points. Although *myo*-2-inosose 1-phosphate exists as a mixture of solution forms, these two inflection points can be broadly interpreted to correspond to dissociable protons with $pK_{a1} = 2.5$ and $pK_{a2} = 5.6$. No ambiguity is associated with the determined titrimetric inflection points of substrate D-glucose 6-phosphate which correspond to dissociable protons with $pK_{a1} = 2.2$ and $pK_{a2} = 5.9$. Relative to *myo*-2-inosose 1-phosphate and D-glucose 6-phosphate, the dissociable protons of DPMI were characterized by a noticeably reduced acidity with $pK_{a1} = 3.6$ and $pK_{a2} = 7.3$. Under more basic conditions (pH 8.0), the higher concentration of the dianionic form of DPMI may therefore account for the phosphonic acid's inhibitory potency ($K_i = 6.4$ μM) more closely approximating the inhibition constant ($K_i = 3.6$ μM) for *myo*-2-inosose 1-phosphate under less basic (pH = 7.2) solution conditions.

DHAP was also a competitive inhibitor (Table 2) of MIP synthase with $K_i = 0.7$ mM. Although DHAP is a much weaker inhibitor relative to *myo*-2-inosose 1-phosphate and DPMI, inhibition of MIP synthase by DHAP is still significant in light of the K_m for substrate D-glucose 6-phosphate. This level of inhibition is even more noteworthy given the absence in DHAP of the cyclic, six-membered ring along with three of the

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hydroxyl groups found in *myo*-2-inosose 1-phosphate and DPMI. Beyond the weaker interaction between DHAP and native MIP synthase with bound NAD, weaker interactions with *apo*MIP synthase reconstituted with NADH are indicated by the slower rate that this enzyme complex is oxidized by DHAP relative to *myo*-2-inosose 1-phosphate and DPMI.

Discussion

The conceptually simple replacement of a phosphoryl group with a phosphonomethyl group transformed a molecule (*myo*-2-inosose 1-phosphate) lacking spectroscopically detectable concentrations of its keto form into a molecule (DPMI) where the keto form is the only spectroscopically detectable species present in aqueous solution. Alternatively, the equivalent of removing most of the functional groups found in *myo*-2-inosose 1-phosphate resulted in DHAP, where the keto form of the molecule was one of the two major species in aqueous solution at neutral pH. Both approaches yielded competitive inhibitors of MIP synthase.

Although DPMI was nearly as potent an inhibitor as *myo*-2-inosose 1-phosphate at the solution pH where it was in its fully dissociated ionization state, this inhibition was much weaker than might have been expected. Afterall, DPMI is an analogue that existed exclusively in its keto form and is structurally very similar to *myo*-2-inosose 1-phosphate. Reduction in binding interactions between enzyme and inhibitor may have resulted from replacement of the phosphate monoester oxygen of *myo*-2-inosose 1-phosphate with the methylene group in DPMI. But what if the hydrate form of *myo*-2-inosose 1-phosphate contributes along with the keto form to MIP synthase inhibition? This might explain why *myo*-2-inosose 1-phosphate, where only small, steady state levels of a keto form are available, is almost as good an inhibitor of MIP synthase as DPMI. Inhibition of MIP synthase by DPMI establishes that the keto form of *myo*-2-inosose 1-phosphate is probably an inhibitor. However, attributing inhibition of MIP synthase exclusively to the keto form of *myo*-2-inosose 1-phosphate is premature.

MIP synthase inhibition by DHAP is particularly intriguing. DHAP can be viewed as an analogue of acyclic intermediate **B** as well as an analogue of cyclic intermediate **D**. Addition of methine and methylene groups with attached hydroxyl groups to DHAP, depending on the stereochemical configuration of chiral centers, may improve inhibitor potency. Intermediate **B** may even be an MIP synthase inhibitor. Although intermediate **B** has previously been synthesized, its potential use as an inhibitor of MIP synthase was not examined.[3] A large number of acyclic analogues possessing oxidized reaction centers may well warrant synthesis and evaluation for inhibition of MIP synthase given the diversity of DHAP derivatives and reaction coordinate **B** analogues that can be envisioned.

DPMI, dMIP, DHAP, and *myo*-2-inosose 1-phosphate attest to the importance of MIP synthase active site interactions with oxidized reaction centers. DPMI and *myo*-2-inosose 1-phosphate are structurally similar molecules possessing oxidized reaction centers. Both are potent inhibitors of MIP synthase. Structurally related dMIP, which lacks an oxidized reaction center, is a severely attenuated inhibitor of MIP synthase relative to DPMI and *myo*-2-inosose 1-phosphate. DHAP, which shares a minimum of structural similarities beyond its oxidized center with *myo*-2-inosose 1-phosphate and DPMI, is a competitive inhibitor. While the issue remains as to whether MIP synthase binds to the keto, hydrate, or both of these forms of the oxidized centers, the sum total of the data suggests that incorporation of an oxidized reaction center into potential inhibitors is a general strategy for inhibiting MIP synthase.

Experimental Section

General Enzymology. Protein concentration was assayed by the Bradford dye-binding procedure[17] with protein assay solution purchased from Bio-Rad. A standard curve was obtained using bovine serum albumin. Fractions containing MIP synthase activity during purification of the enzyme were assayed by following the procedure described by Henry.[10] Specific activities, Michaelis constants (K_m), and inhibition constants (K_i) were determined in an assay solution (1.5 mL) containing Tris-HCl (20 mM), NH_4Cl (14 mM), and dithiothreitol (0.2 mM), pH 7.2. MIP synthase was incubated in the assay solution with the selected concentrations of NAD^+, D-glucose 6-phosphate and inhibitors at 37 °C. Aliquots (0.15 mL) were removed every 2 min, added to a TCA solution (20%, w/v, 0.05 mL) and subsequently incubated with aqueous $NaIO_4$ (0.2 M, 0.10 mL) for 1 h at 37 °C. The reaction mixture was quenched by addition of aqueous Na_2SO_3 (1.5 M, 0.1 mL). Released inorganic phosphate was determined by the colorimetric method of Ames.[18] One unit of activity is defined as the formation of 1 μmol of MIP/min. Specific activities were calculated with D-glucose 6-phosphate and NAD^+ concentrations of 5 and 1 mM, respectively. Michaelis constants (K_m) of D-glucose 6-phosphate and NAD^+ were calculated from linear regression analysis of progress curves. The curves were obtained for varying concentrations (0.5, 1.0, 1.5, 2.0, and 5.0 mM) of D-glucose 6-phosphate at 5 mM NAD^+ and for varying concentrations (5, 10, 20, 50, and 100 μM) of NAD^+ at 5 mM D-glucose 6-phosphate. Determination of inhibition constants (K_i) employed Lineweaver−Burk analysis of enzyme velocity in the aforementioned pH 7.2 assay buffer with 5 mM NAD^+ and varying concentrations of D-glucose 6-phosphate (0.5, 1.0, 1.5, 2.0, and 5.0 mM), dMIP (0, 10, 50, 100, 250, and 500 μM), DMPI (0, 10, 50, 100, and 150 μM), and DHAP (0, 100, 250, 500, and 1000 μM). The preparation of the *apo*MIP synthase and the reconstitution experiments were performed by following the procedure previously described.[7]

Saccharomyces cerevisiae MW5.55 was provided by Professor Susan A. Henry. The growth medium (YEPD) was prepared in distilled, deionized water and contained Bacto peptone (20 g), Bacto yeast extract (10 g), and D-glucose (10 g). The culture growth was carried out at 30 °C. Whatman ((diethylamino)-ethyl)cellulose (DE52), Bio-Gel A 0.5 obtained from Bio-Rad, and a 7.5 mm × 7.5 cm DEAE-5PW TSK analytical (purchased from Beckman) HPLC column were used during the purification. Buffers included buffer A [Tris-HCl (20 mM), NH_4Cl (20 mM), 2-mercaptoethanol (10 mM), and PMSF (0.5 mM), pH 7.2] and buffer B [Tris-HCl (20 mM), NH_4Cl (250 mM), 2-mercaptoethanol (10 mM), and PMSF (0.5 mM), pH 7.2]. All protein manipulation was carried out at 4 °C. Protein solutions were concentrated by ultrafiltration (PM-10 Diaflo membranes from Amicon). Centrifugations, unless specified otherwise, were performed at 18000*g* for 20 min at 4 °C.

Purification of MIP Synthase. *S. cerevisiae* MW5.55 cells (50 g) were harvested by centrifugation at 6000*g*, washed twice with buffer A, and resuspended in 75 mL of the same buffer. Cell lysate was prepared by French press disruption of the cells at 16 000 psi. The lysate was then centrifuged at 6000*g* for 10 min to remove intact cells which were then resuspended in buffer A (50 mL) and lysed by French press. The cell lysate fractions were combined after centrifugation, and streptomycin

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(18) Ames, B. N. *Methods Enzymol.* **1966**, *8*, 115.

sulfate in buffer A (25%, w/v) was added to the supernatant to a final concentration of 2% (w/v). After 20 min of gentle stirring, the solution was centrifuged. Powdered ammonium sulfate was added slowly to the supernatant to give a final concentration of 229 g/L (40% saturation). After being stirred for 20 min, the precipitated protein was removed by centrifugation. Ammonium sulfate was added to the supernatant to a final concentration of 483 g/L (75% saturation) and stirred for 20 min. The precipitated protein was collected as a pellet by centrifugation, resuspended in buffer A, dialyzed against buffer A, and loaded onto DE52 (250 mL) equilibrated with buffer A. Elution with buffer A (50 mL) was followed by elution with a linear gradient (500 mL + 500 mL, buffer A/buffer B). Fractions containing MIP synthase were combined, and ammonium sulfate was added to give a final concentration of 523 g/L (80% saturation). After stirring for 20 min, precipitated protein was collected by centrifugation. The pellet was resuspended in buffer B and applied to the Bio-Gel column (500 mL) equilibrated with buffer B. Fractions containing MIP synthase obtained after elution with buffer B (500 mL) were combined and concentrated. The concentrate was then dialyzed against buffer A and injected on the HPLC DEAE column equilibrated with buffer A. MIP synthase was eluted with a linear gradient (100:0 buffer A/buffer B to 20:80 buffer A/buffer B). Fractions containing MIP synthase were concentrated and stored at −80 °C.

Polyacrylamide gel electrophoresis of purified MIP synthase in the presence of sodium dodecyl sulfate was performed according to Laemmli.[19] The stacking gel was buffered with Tris-HCl at pH 6.8, and the separating gel (10% acrylamide) was buffered with Tris-HCl at pH 8.8. Typically, 20 μg of protein was loaded per lane. Gels were fixed with acetic acid solution and visualized with Coomasie Brilliant Blue. Standards used for molecular weight determination included carbonic anhydrase (MW 29 000), ovalbumin (MW 45 000), bovine serum albumin (MW 66 000), phosphorylase b (MW 97 400), β-galactosidase (MW 116 000), and myosin (MW 205 000). Cross-linking of purified MIP synthase followed the protocol of Stach[20] and entailed reaction (in a total volume of 0.20 mL) of MIP synthase (0.15 mg/mL) with dimethyl suberimidate (0−10 mg/mL) in 0.20 M borate, pH 9.5, for 2 h at 25 °C. Addition of sodium dodecyl sulfate (10%, w/v, 0.03 mL) and β-mercaptoethanol (0.0005 mL) was followed by incubation for 1 h at 37 °C. Subsequent separation by polyacrylamide gel electrophoresis followed the procedure of Davies.[21] The separating gel (5% acrylamide) was buffered with 0.1 M sodium borate and 0.1 M sodium acetate, pH 8.5, and contained sodium dodecyl sulfate (0.1%). Electrospray data were obtained using a VG plateform (VG Organic, Manchester, England).

General Chemistry. See ref 22 for general experimental information dealing with synthetic manipulations. ^{1}H NMR spectra were recorded at 200, 300, or 500 MHz. Chemical shifts are reported (in part per million) relative to C*H*D$_2$SOCD$_3$ (δ 2.49 ppm) when samples were dissolved in DMSO-d_6. ^{13}C NMR spectra were recorded at 50, 75, or 125 MHz. Chemical shifts are reported (in part per million) relative to (*C*D$_3$)$_2$SO (δ 39.5 ppm) in DMSO-d_6. Low-resolution electron impact (EI) and chemical ionization (CI) mass spectra (MS) were recorded on a Finnigan 4000 mass spectrometer, and high-resolution mass spectra (HRMS) were recorded on a Kratos MS50 or MS25 mass spectrometer. FAB mass spectra were obtained using a JEOL HX-110 double-focusing mass spectrometer or a Kratos MS50 mass spectrometer. A Nicolet IR/42 spectrometer was used for infrared spectra which were recorded in wavenumbers (cm^{-1}).

[1R*-(1α,2*β*,3α,4*β*,5α,6*β*)]-2,3,4,5-Tetrakis(benzyloxy)-6-[[bis(benzyloxy)phosphinyl]methyl]cyclohexan-1-ol (3) and [1S*-(1α,2α,3*β*,4*β*,5α,6*β*)]-2,3,4,5-Tetrakis(benzyloxy)-6-[[bis-(benzyloxy)phosphinyl]methyl]cyclohexan-1-ol (4). A solution of *n*-butyllithium in hexane (1.6 N, 12.0 mL, 19.2 mmol) was added to THF (20 mL) at −78 °C under N_2. To this solution was slowly added dibenzyl methylphosphonate[13] (5.29 g, 19.2 mmol) in THF (20 mL). After stirring for 30 min at −78 °C, boron trifluoride etherate (4.71 mL, 19.2 mmol) was added, immediately followed by tetrabenzylconduritol oxide B[14] (2.00 g, 3.83 mmol) in THF (5 mL). The solution was stirred at −78 °C for 3 h, and more boron trifluoride etherate (4.71 mL, 19.2 mmol) was added. The solution was then allowed to warm to −20 °C and was stirred for 12 h at this temperature. After addition of saturated aqueous $NaHCO_3$, the aqueous layer was extracted with ether and the combined organic layers were washed with brine followed by water, dried, and concentrated. This crude product was purified by flash chromatography (1:1 EtOAc/hexane, v/v) followed by radial chromatography (4 mm thickness, 1:1 EtOAc/ hexane, v/v). The faster eluting regioisomer **3** was isolated as an oil (0.91 g, 30%) which slowly solidified at room temperature (rt): ^{1}H NMR ($CDCl_3$) δ 6.85−7.40 (m, 30 H), 4.70−5.10 (m, 11 H), 4.57 (d, J = 16 Hz, 1 H), 3.98 (d, J = 4 Hz, 1 H), 3.60−3.75 (m, 1 H), 3.35−3.55 (m, 3 H), 1.80−2.40 (m, 3 H); ^{13}C NMR ($CDCl_3$) δ 138.8, 138.4, 138.3, 136.0 (J_{POCC} = 6 Hz), 135.9 (J_{POCC} = 7 Hz), 129.5, 129.4, 128.7, 128.5 (2C), 128.4, 128.3, 128.1, 127.9, 127.8, 127.6, 127.5, 85.8, 85, 82.7, 79.4 (J_{PCCC} = 7 Hz), 75.7, 75.5, 75.4, 74.9, 73.1, 67.5 (J_{POC} = 7 Hz), 67.3 (J_{POC} = 7 Hz), 41.5 (J_{PCC} = 3 Hz), 24.0 (J_{PC} = 139 Hz); IR (neat) 3400 (b), 1210 (s); MS *m/z* (relative intensity) EI 91 (100), CI 91 (100), FAB 799 (M + H$^+$, 25), 181 (100); HRMS (FAB) calcd for $C_{49}H_{52}O_8P$ (M + H$^+$) 799.3399, found 799.3383. Anal. Calcd for $C_{49}H_{51}O_8P\cdot{}^1/_2H_2O$: C, 72.85; H, 6.36. Found: C, 72.81; H, 6.52. The slower eluting regioisomer **4** was isolated as an oil (1.37 g, 44%) which slowly crystallized at rt: ^{1}H NMR ($CDCl_3$) δ 7.15−7.40 (m, 30 H), 4.65−5.10 (m, 8 H), 4.55 (s, 2 H), 4.50 (s, 2 H), 4.40−4.55 (m, 1 H), 4.04 (ddd, J = 14, 4, 2 Hz, 1 H), 3.81 (dd, J = 9, 9 Hz, 1 H), 3.64 (dd, J = 9, 3 Hz, 1 H), 3.45 (dd, J = 9, 9 Hz, 1 H), 2.75−2.95 (m, 1 H), 2.50−2.60 (br, 1 H), 2.41 (ddd, J = 21, 16, 3 Hz, 1 H), 1.48 (ddd, J =18, 16, 12 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 139.3, 138.9, 138.4, 136.8 (J_{POCC} = 3 Hz), 136.7 (J_{POCC} = 3 Hz), 129.2, 129.1, 129.0, 128.9, 128.7, 128.5, 128.4, 128.3, 128.1, 82.1, 82.0, 80.3, 78.6, 78.4, 76.4, 76.1, 72.9 (2C), 68.2 (J_{POC} = 14 Hz), 67.9 (J_{POC} = 8 Hz), 36.1 (J_{PCC} = 4 Hz), 22.1 (J_{PC} = 143 Hz); IR (neat) 3380(s), 1210(s); MS *m/z* (relative intensity) EI 91 (100), CI 367 (83), 353 (100), 91 (29), FAB 800 (30), 799 (M + H$^+$, 45), 181 (100); HRMS (FAB) calcd for $C_{49}H_{52}O_8P$ (M + H$^+$) 799.3399, found 799.3391. Anal. Calcd for $C_{49}H_{51}O_8P\cdot H_2O$: C, 72.04; H, 6.54. Found: C, 72.34; H, 6.64.

[2S*-(2α,3*β*,4α,5*β*,6α)]-2,3,4,5-Tetrakis(benzyloxy)-6-[[bis-(benzyloxy)phosphinyl]methyl]cyclohexan-1-one (5). DMSO (0.17 g, 2.15 mmol) in CH_2Cl_2 (5 mL) was slowly added to a −78 °C solution of oxalyl chloride (0.49 mL, 0.98 mmol) in CH_2Cl_2 (2 mL), and stirring continued for 35 min at −78 °C. Alcohol **3** (0.26 g, 0.35 mmol) in CH_2Cl_2 (3 mL) was then added under N_2, and the reaction mixture stirred for an additional 2 h. After addition of anhydrous triethylamine, the reaction was warmed to rt and quenched with water (2 mL). The aqueous layer was then extracted with CH_2Cl_2 (3×). The combined

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(21) Davies, G. E.; Stark, G. R. *Proc. Natl. Acad. Sci. U.S.A.* **1970**, *66*, 651.
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organic layers were washed with 0.1 N aqueous HCl solution followed by brine, dried, and concentrated. Radial chromatography (2 mm thickness, 1:1 EtOAc/ hexane, v/v) afforded phosphonomethylated inosose product **5** as an oil (0.23 g, 89%): ^{1}H NMR ($CDCl_3$) δ 7−7.5 (m, 30 H), 4.65−5.05 (m, 10 H), 4.53 (d, J = 11 Hz, 1 H), 4.27 (d, J = 11 Hz, 1 H), 4.22 (d, J = 9 Hz, 1 H), 3.92 (dd, J = 9, 9 Hz, 1 H), 3.59 (dd, J = 9, 9 Hz, 1 H), 3.27 (dd, J = 8, 11 Hz, 1 H), 2.82−3.06 (m, 1 H), 2.49 (ddd, J = 9, 15, 18 Hz, 1 H), 2.05 (ddd, J = 2, 8, 15 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 202.2 (J_{PCCC} = 3 Hz), 138, 137.9, 137.5, 137.4, 136.1 (J_{POCC} = 6 Hz), 136 (J_{POCC} = 6 Hz), 128.5, 128.3 (2C), 128.2, 127.9, 127.8 (2C), 127.7, 127.4, 127.3, 85.6, 84.4, 81.8, 79.3 (J_{PCCC} = 12 Hz), 75.6, 75.4, 74.8, 72.9, 67.2, 67.1, 47.5 (J_{PCC} = 4 Hz), 20.6 (J_{PC} = 143 Hz); IR (neat), 3450(b), 1735(s), 1210(s); MS *m/z* (relative intensity) FAB 797 (M + H^+, 89), 105 (100); HRMS (FAB) calcd for $C_{49}H_{50}O_8P$ (M + H^+) 797.3243, found 797.3268. Anal. Calcd for $C_{49}H_{49}O_8P\cdot 2H_2O$: C, 70.66; H, 6.41. Found: C, 70.67; H, 6.06.

[2*S-(2α,3*β*,4α,5*β*,6α)]-2,3,4,5-Tetrakis(benzyloxy)-6-[[bis(benzyloxy)phosphinyl]methyl]cyclohexan-1-one (6).** Alcohol **4** (0.15 g, 0.19 mmol) was oxidized as described for alcohol **3**. Phosphonomethylated inosose **6** was obtained as a yellow oil (0.14 g, 94% yield): ^{1}H NMR ($CDCl_3$) δ 7.10−7.50 (m, 30 H), 4.42−5.08 (m, 11 H), 4.35 (d, J = 11 Hz, 1 H), 4.34 (d, J = 10 Hz, 1 H), 4.12 (dd, J = 3, 3 Hz, 1 H), 3.89 (dd, J = 3, 5 Hz, 1 H), 3.79 (dd, J = 5, 10 Hz, 1 H), 3.00 (dddd, J = 3, 5, 11, 11 Hz, 1 H), 2.45 (ddd, J = 5, 16, 19 Hz, 1 H), 1.99 (ddd, J = 9, 16, 17 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 206.1 (J_{PCCC} = 11 Hz), 138.1, 138, 137.8, 137.7, 137.6, 137.3, 137.2, 136, 129.3, 129.2, 129.1, 128.5, 128.3, 128.2, 127.9, 127.7, 127.6, 127.3, 127.2, 81.9, 81.4, 79.4, 76.7, 74.3, 74, 72.2, 72, 67.3 (J_{POC} = 3 Hz), 67.2 (J_{POC} = 4 Hz), 44.3, 21.8 (J_{PC} = 145 Hz); IR (neat) 3450 (b), 1735 (s), 1210(s); MS *m/z* (relative intensity) FAB 797 (M + H^+, 97); HRMS (FAB) calcd for $C_{49}H_{50}O_8P$ (M + H^+) 797.3243, found 797.3271. Anal. Calcd for $C_{49}H_{50}O_8P\cdot 3H_2O$: C, 69.18; H, 6.51. Found: C, 69.16; H, 6.25.

[2*R-(2α,3*β*,4α,5*β*,6α)]-2,3,4,5-Tetrahydroxy-6-[phosphonomethyl]cyclohexan-1-one (DPMI).** The benzylated phosphono ketone **5** (0.18 g, 0.22 mmol) in THF (5 mL) containing $NaHCO_3$ (0.02 g, 0.22 mmol) was added to 10% Pd on C (0.06 g) in 10 mL of THF/water (4/1, v/v). The suspension was hydrogenated at 50 psi H_2 for 1 h. Filtration through Celite, removal of THF under reduced pressure, adjustment of the remaining aqueous solution to pH 7, and final concentration to dryness afforded DMPI as a white solid (0.04 g, 100% yield): ^{1}H NMR (D_2O) δ 4.23 (d, J = 10 Hz, 1 H), 3.67 (dd, J = 9 Hz, 1 H), 3.20 (dd, J = 10 Hz, 1 H), 3.13 (dd, J = 9, 10 Hz, 1 H), 2.83 (m, 1 H), 2.22 (ddd, J = 9, 16, 18 Hz, 1 H), 1.91 (ddd, J = 2, 16, 17 Hz, 1 H); ^{13}C NMR (D_2O) δ 206.8, 79.6, 77.4, 75.7, 74.4, 74.3, 49.7 (J_{PCC} = 4 Hz), 24.5 (J_{PC} = 138 Hz); MS *m/z* (relative intensity) FAB 255 (22); HRMS (FAB) calcd for $C_7H_{12}O_8P$ (M − H^+) 255.0283, found 255.0283.

[1*R-(1α,2*β*,3α,4*β*,5α)]-2,3,4,5-Tetrakis(benzyloxy)cyclohexan-1-ol (7).** To a solution of the fully benzylated conduritol oxide **2** (0.21 g, 0.04 mmol) in THF (5 mL), stirred at −78 °C under argon, was added a solution of Super-Hydride in THF (1 M, 1.61 mL, 1.61 mmol). After 10 min, the solution was slowly warmed to rt and stirred for 2 h. A saturated solution of NH_4Cl (10 mL) was added to the reaction mixture cooled to 0 °C. The aqueous layer was extracted with ether, and the combined organic fractions were washed with brine, dried, and concentrated. Radial chromatography (2 mm thickness, 1:1 EtOAc/ hexane, v/v) afforded the alcohol **7** as an oil (0.13 g, 59%): ^{1}H NMR ($CDCl_3$) δ 7.15−7.40 (m, 20 H), 4.82−4.98 (m, 3 H), 4.70−4.80 (m, 2 H), 4.55−4.70 (m, 3 H), 3.34−3.55 (m, 4 H), 3.23 (dd, J = 10, 11 Hz, 1 H), 2.20−2.40 (br, 1 H), 2.28 (ddd, J = 10.5, 2.0, 2.0 Hz, 1 H), 1.37 (ddd, J = 10.5, 10.5, 10.5 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 138.6, 138.4 (2C), 138.2, 128.6, 128.4, 128.3, 127.9, 127.8, 127.7, 127.6, 127.5, 85.7, 83.2, 77.4, 75.7 (2C), 75.4, 72.3, 68.3, 33.9; MS *m/z* (relative intensity), EI 91 (100), 181 (12), 433 (11), FAB 181 (100), 271 (10), 433 (5), 523 (M − H^+, 12); HRMS (FAB) calcd for $C_{34}H_{35}O_5$ (M − H^+) 523.2485, found 523.2487.

[1*R-(1α,2*β*,3α,4*β*,5α)]-2,3,4,5-Tetrakis(benzyloxy)-1-[[bis(benzyloxy)phosphonyl]oxy]cyclohexane (8).** Sodium hydride (0.03 g, 0.69 mmol) as a suspension in THF (2 mL) was added under argon to a solution of alcohol **7** (0.12 g, 0.23 mmol) in THF (8 mL) at rt. The solution was heated at reflux for 2 h and kept at the same temperature for 3 h after the addition of tetrabenzylpyrophosphate (0.25 g, 0.46 mmol). The reaction was quenched at rt by addition of a saturated solution of NH_4Cl (10 mL). Extraction of the aqueous layer with ether, followed by concentration of the dried combined organic fractions yielded the crude product which was purified by radial chromatography (2 mm thickness, 1:3 EtOAc/hexane, v/v). The phosphate triester **8** was isolated as an oil (0.12 g, 62%) along with some unreacted alcohol **7** (0.04 g, 30%): ^{1}H NMR ($CDCl_3$) δ 7.10−7.40 (m, 30 H), 4.75−5.00 (m, 6 H), 4.79 (d, J = 6 Hz, 4 H), 4.54 (dd, J = 1, 7.5 Hz, 2 H), 4.20−4.32 (m, 1 H), 3.51 (dd, J = 8.7, 8.7 Hz, 1 H), 3.48 (dd, J = 7.5, 8.5 Hz, 1 H), 3.44 (dd, J = 8.5, 8.7 Hz, 1 H), 3.36−3.45 (m, 1 H), 2.55 (ddd, J = 4.5, 4.5, 12 Hz, 1 H), 1.55 (ddd, J = 12, 12, 12 Hz, 1 H); ^{13}C NMR ($CDCl_3$) δ 138.5, 138.2, 138.0, 135.6, 135.7, 128.5, 128.4, 128.3, 128.2, 127.9, 127.8, 127.7 (2C), 127.5, 127.4, 85.0, 83.9 (J_{POCC} = 7.4 Hz) 82.5, 76.6, 76.1, 75.9 (J_{POC} = 6 Hz), 75.8, 75.5, 72.5, 69.2 (J_{POC} = 5 Hz), 69.1 (J_{POC} = 5 Hz), 33.7; MS *m/z* (relative intensity) FAB 109 (100), 123 (57), 181 (24), 785 (M + H^+, 3); HRMS (FAB) calcd for $C_{48}H_{50}O_8P$ (M + H^+) 785.3243, found 785.3249.

[1*R-(1α,2*β*,3α,4*β*,5α)]-2,3,4,5-Tetrahydroxy-1-(phosphonooxy)cyclohexane (dMIP).** Benzylated phosphate triester **8** (0.12 g, 0.14 mmol) in THF (2 mL) was added to 10% Pd on C (0.05 g) in 5 mL of THF/H_2O (4:1, v/v). The suspension was hydrogenated at 50 psi H_2 for 12 h. Catalyst removal by filtration through Celite and removal of the solvents yielded the phosphate monoester dMIP as an oil (0.04 g, 100%): ^{1}H NMR (D_2O) δ 4.66 (m, 1 H), 3.83 (m, 1 H), 3.39 (m, 1 H), 3.13 (m, 2 H), 2.21 (m, 1 H), 1.40 (ddd, J = 12, 12, 12 Hz, 1 H); ^{13}C NMR (D_2O) δ 79.7, 78.7 (J_{POCC} = 6 Hz), 76.8 (J_{POC} = 7.5 Hz), 76.7, 71.3, 38.8; MS *m/z* (relative intensity) FAB 62 (72), 91(78), 154 (70), 183 (100), 243 (M − H^+, 80); HRMS (FAB) calcd for $C_6H_{12}O_8P$ (M − H^+) 243.0270, found 243.0274.

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Supporting Information Available: Text describing experimental procedures, the ORTEP plot, tables of crystallographic details, final positional and thermal parameters, bond lengths, and bond angles for phosphonomethylated inositol **4** (27 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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