Pergamon

Bioorganic & Medicinal Chemistry 7 (1999) 2671–2682

BIOORGANIC & MEDICINAL CHEMISTRY

Towards the Development of Novel Antibiotics: Synthesis and Evaluation of a Mechanism-Based Inhibitor of Kdo8P Synthase

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Received 25 February 1999; accepted 25 May 1999

Abstract—The design and two synthetic pathways to aminophosphonate **4** which mimics the ionic and steric properties of putative oxocarbenium intermediate **3** in the Kdo8P synthase-catalyzed reaction are reported. It was found that **4** is a slow-binding, most potent inhibitor of the enzyme yet tested, with a K_i value of 0.4 μM.

Introduction

The rapid spread of antibiotic resistance in Gram-negative bacteria has prompted a continuing search for new agents with antibacterial activity against this important class of bacterial pathogen.[1] Because the biosynthesis of lipopolysaccharide is unique to Gram-negative bacteria[2–4] and required for their growth and virulence,[5–7] attempts have been made to discover antibacterial agents acting at this site. However, since the biosynthesis of lipopolysaccharide requires a rather large number of enzymatic steps,[8] a site-specific constituent of this macromolecule - the unusual 8-carbon sugar 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) has been identified and most efforts to rationally design synthetic inhibitors have been aimed towards the inhibition of this sugar's biosynthesis.[9,10] Indeed, some of the potent inhibitors of CMP-Kdo synthetase have shown in vivo antibacterial activity.[11,12] These results have prompted us to a further design of synthetic molecules exhibiting selective activity against Gram-negative bacterial cells.

For this purpose we have selected the 3-deoxy-D-*manno*-2-octulosonate-8-phosphate (Kdo8P) synthase [EC 4.1.2.16] which catalyzes the unusual condensation of phosphoenolpyruvate (PEP) with D-arabinose 5-phosphate (A5P) to produce Kdo8P and inorganic phosphate (P_i).[13] The catalytic mechanism of this enzyme has been the subject of considerable controversy. Earlier studies by Hedstrom and Abeles[14] demonstrated that the Kdo8P-synthase-catalyzed reaction proceeds through the C–O bond cleavage of PEP and postulated a mechanism involving the formation of acyclic intermediate **1** (Scheme 1, path a). Recently,[15–17] an alternative pathway in which the formation of cyclic bisphosphate **2** (Scheme 1, path b) as a reaction intermediate has been proposed. This proposal was mainly based on the results accumulated through the synthesis and evaluation of various analogues of the substrates[18–20] and product[15–21] as mechanistic probes of the synthase.

Although the above proposals were based on reasonable chemical grounds, the distinction between these two mechanistic pathways and the detailed insight into the nature of the reaction intermediates awaited the application of pre-steady-state kinetic measurements, using rapid-quench techniques,[22,23] in combination with the chemical studies.[24] These two parallel studies have clearly demonstrated that the reaction does not operate through the formation of **2** as a reaction intermediate. This observation left us with an acyclic intermediate mechanism (Scheme 1, path a), although, to date, there is no evidence available for the existence of **1** as a true intermediate. In addition, the timing of the condensation step is an unresolved issue and, therefore, both a synchronous generation of new C−C and C−O bonds, or a stepwise process (through the formation of the transient oxocarbenium intermediate **3**), can be suggested as possible mechanisms that may lead to the formation of **1**.

Since many mechanistic questions for the action of the synthase are still unresolved, the rational design of

Key words: Kdo8P synthase; lipopolysaccharide; mechanism-based inhibitors.

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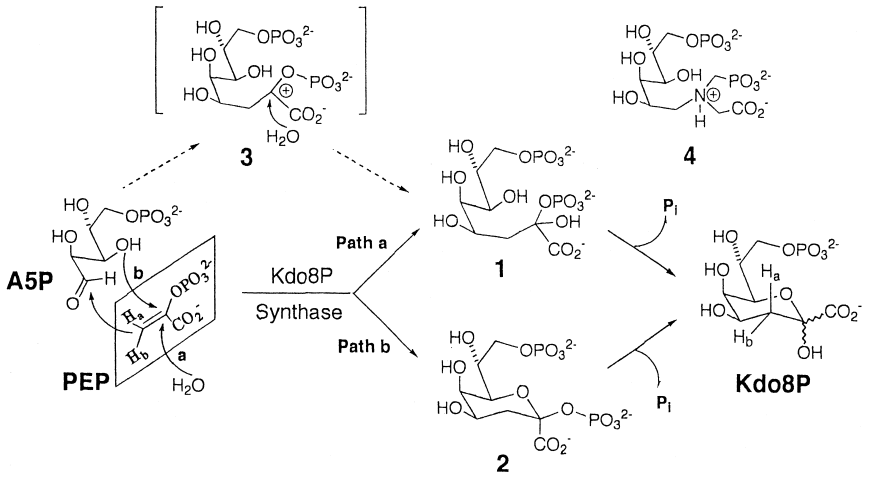
0968-0896/99/$ - see front matter
PII: S0968-0896(99)00233-3



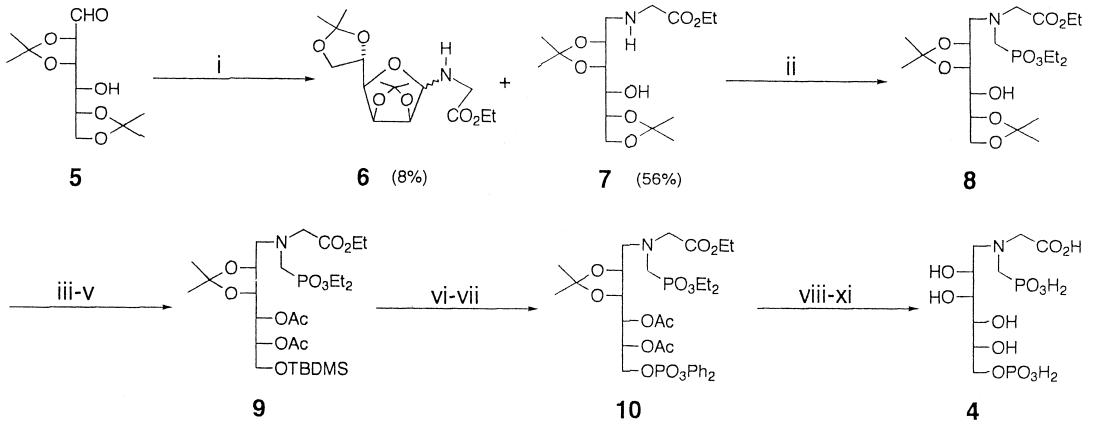
Scheme 1. Proposed mechanistic pathways for the reaction catalyzed by Kdo8P synthase.

potent inhibitors of this enzyme is a difficult target. On the other hand, it is noteworthy that all the above mechanistic proposals are largely based on the results accumulated through the evaluation of various synthetic compounds as mechanistic probes of the enzyme. Therefore, in keeping with the possibility that the condensation process in Scheme 1 might be a stepwise process, we should expect the stable analogues of cation **3** to serve as potent inhibitors of the enzyme. Close structural analogues of **3** not only incorporate the binding determinants of both substrates, but may also take advantage of any stabilization that **3** could enjoy in the active site. In this account, we describe the synthesis and inhibition studies of the amino phosphonate analogue **4**.[25] Here we provide evidence that compound **4**, which mimics the structural and electrostatic properties of the putative intermediate **3**, is a slow binding inhibitor of the enzyme. Also outlined is the first very short, one-step synthesis of **4** from the commercially available compounds. The applications of these results to Kdo8P-synthase-catalyzed reaction are discussed.

Results

Synthesis of the amino phosphonate analogue 4

Synthesis of **4** was accomplished in 12 steps starting from D-mannose via the known acetonide **5**[26] as illustrated in Scheme 2. Direct reductive amination of **5** ($NaBH_3CN$, MeOH) with glycine ethyl ester resulted in acyclic secondary amine **7** in 56% isolated yield, along with 12% of the starting acetonide **5** and ~8% of cyclic unreduced amine **6**. Attempts to improve the yield of **7** by adding excess reagent or using different reducing reagents [$NaBH_4$ or $NaBH(OAc)_3$][27,28] proved to be less satisfactory. For example, sodium triacetoxyborohydride [$NaBH(OAc)_3$] gave the cyclic amine **6** as a major product. Although this product could be reduced in the following step by $NaBH_3CN$ to afford the required acyclic amine **7**, we preferred the direct reductive amination procedure since the reaction products and the unreacted starting material could easily be separated by simple chromatography.



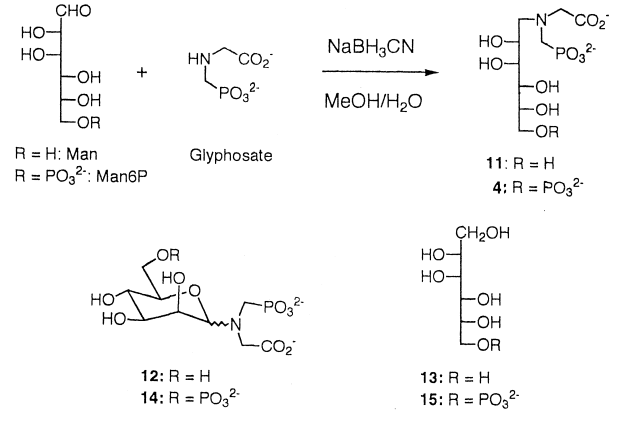
Scheme 2. Reagents and conditions: (i) $H_2NCH_2CO_2Et$, $NaBH_3CN$, MeOH; (ii) $TfOCH_2PO_3Et_2$, sat. $NaHCO_3$, CH_2Cl_2, 65%; (iii) TFA, MeOH/H_2O; (iv) TBDMSCl, Py, DMAP; (v) Ac_2O, Py, 30% over three steps; (vi) H_2SO_4, MeOH; (vii) $(PhO)_2P(O)Cl$, Py, DMAP, 48%, two steps; (viii) TMSBr, CH_3CN, Et_3N; (ix) H_2, PtO_2, MeOH, HOAc; (x) Dowex (H^+), H_2O; (xi) KOH, H_2O, 35% from **10**.

The phosphonomethyl function was successfully introduced at secondary amine by treatment of **7** with diethyl phosphonomethyltriflate[29] to afford the phosphonate **8** in 65% isolated yield. A variety of methods were examined to effect the deprotection and selective phosphorylation of **8** at C-6. Most were plagued by multiple side products and/or product decomposition. These problems were circumvented by using the following five step procedure. Treatment of **8** with mild acidic condition (0.16 M TFA in MeOH:H_2O (5:3)) caused selective deprotection of 5:6-*O*-isopropylidene moiety to afford the corresponding triol as a major product. Treatment of this triol with *tert*-butyldimethylsilyl chloride (TBDMSCl) resulted in the primary silylated product, which was isolated as its diacetate **9** (30% yield for three steps). Desilylation in mild acidic condition and then phosphorylation with diphenylphosphochloridate afforded compound **10** in 48% yield. Deprotection of the phosphonate with TMSBr was followed by hydrogenolysis and then by sequential treatment with acid (Dowex H^+) and aqueous base (1 M KOH) to provide the target compound **4** (35% yield from **9**). The spectral characterization of purified **4** using 2D-COSY, ^1H, ^{13}C, ^{31}P NMR, and mass spectra were all consistent with this structure.

One-step synthesis of 4

The above described synthesis to **4** was accomplished in 12 steps from D-mannose with an overall yield of about 3%. Thus, although successful, this procedure was too long and gave a limited amounts of the final product (**4**), which in turn has impeded further studies on the enzyme-inhibitor interaction, especially the detailed investigation of the kinetics of the inhibition. To overcome this problem, we have developed very efficient one-step synthesis to **4** from the commercially available starting materials. This synthetic strategy includes the direct reductive amination in aqueous media between unprotected D-mannose 6-phosphate (Man6P) and *N*-phosphonomethyl glycine (glyphosate) (Scheme 3).

In order to elaborate the suitable condition for such a reaction, initially we tested the procedure with the simple sugar D-mannose. Treatment of D-mannose and glyphosate with $NaBH_3CN$ in MeOH:H_2O (1:1) at pH 6.2, 80°C, gave the product **11** in 60% isolated yield, along which the unreduced anomeric amine **12** (5%) and D-mannitol **13** (7%) were also isolated. We found that the amine **12** could be completely converted to the desired product **11** by addition of excess $NaBH_3CN$ and continuation of the reaction for longer times. During this study we also found that the reaction pH and the temperature are crucial parameters for the reaction success. For example, we have shown that the optimal pH range is between 6 and 7, and the optimal temperature is 80°C. At pH > 7, the reaction becomes very slow, and at pH < 7 the sugar is reduced faster to the respective alditol.[27,28]



Scheme 3. One step synthesis of **4** from D-mannose 6-phosphate (Man6P) and glyphosate.

Using the above procedure, we have synthesized the target inhibitor **4** from glyphosate and D-mannose 6-phosphate in 60% isolated yield. The reaction progress was monitored by proton decoupled ^{31}P NMR as illustrated in Fig. 1. The resonance of the phosphonate of glyphosate (8.6 ppm), and the resonance of the phosphate of mannose 6-phosphate (1.8 ppm), are gradually diminished by the time and replaced by the resonances of the product **4** (2.3 ppm, phosphate fraction, and 7.3 ppm, phosphonate fraction). All the mannose 6-phosphate was converted to the product in about 20 hours. The formation of byproduct **14** could be completely suppressed by adding excess $NaBH_3CN$.

Inhibition of the synthase with 4

Initially, the analogue **4** was evaluated as an inhibitor of homogeneous Kdo8P synthase by using a well established procedure reported earlier.[15,16,21] In this procedure, the inhibition constant is measured from the initial velocity studies, while the substrates (A5P and PEP) and the inhibitor (**4**) are preincubated at constant temperature and the reaction is initiated by addition of

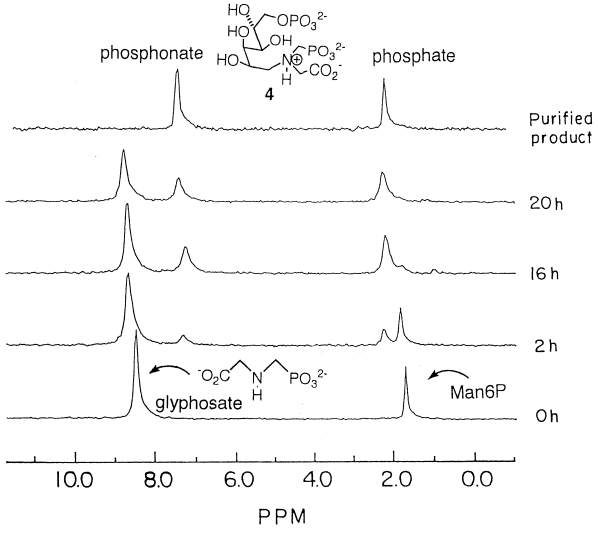


Figure 1. A timecourse of ^{31}P NMR spectra showing the one-step conversion of D-mannose 6-phosphate (Man6P) and glyphosate to compound **4**. The mixture of Man6P (1 equiv) and glyphosate (2 equiv) in MeOH:D_2O (1:1) solution was treated with $NaBH_3CN$ (8 equiv) at pH 6.2, 80°C.

the enzyme. The kinetic measurements by this procedure assumes that substrates and inhibitor are in a rapid equilibrium with the enzyme and that the steady-state conditions are established instantaneously within the initiation of the reaction. Using this experimental procedure, we found that **4** is a competitive inhibitor against PEP binding, and the apparent inhibition constant was estimated to be 3.3 ± 0.3 μM.[25] However, upon close investigation we found that **4** is a slow binding inhibitor to the enzyme. This phenomena is clearly seen from the progress curves illustrated in Fig. 2. Thus, while the enzyme reaction without the presence of inhibitor (curve A) is a straight line, the presence of inhibitor into the reaction mixture resulted convex and concave lines (curves B and C, respectively). Curve B indicates the slow development of the enzyme inhibition when the enzyme is added last to the reaction mixture containing both substrates (A5P and PEP) and inhibitor (**4**). Complementarily, the curve C indicates the slow recovery of enzyme activity when the enzyme was preincubated with inhibitor **4** and the reaction was initiated by addition of the substrates.

To obtain the dissociation constant, the rate constants for association to and dissociation from the enzyme were measured according to the method of Williams and Morrison.[30] Firstly, the values of k_{obs} were measured by fitting the progress curves of the slow development of inhibition at various concentrations of the inhibitor to eq. (1) (see Experimental). The observed values of k_{obs} were then plotted against inhibitor concentration, according to eq (2), as illustrated in Figure 3. The dissociation rate (k_{off}) was determined to be 0.0022 s^{-1} from the vertical intercept of the plot. The association rate constant (k_{on}) was calculated from the slope, using [PEP] = 240 μM and K_m (PEP) = 7.89 μM,

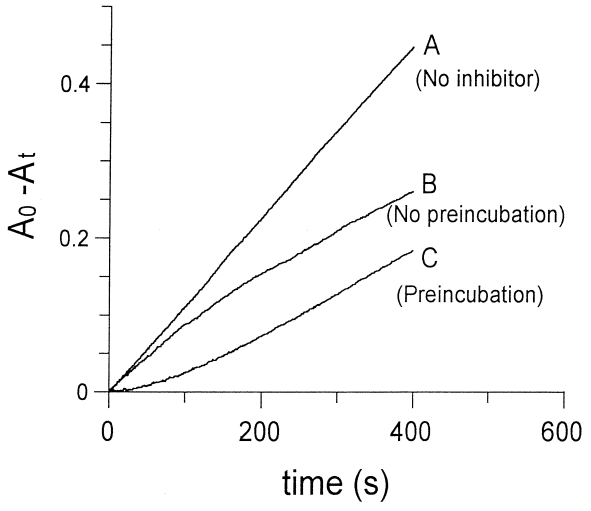


Figure 2. Progress curves for Kdo8P-synthase-catalyzed reaction in the presence of the inhibitor **4**. Curve A, the control experiment without the presence of inhibitor. Curve B, the reaction was initiated by addition of enzyme (30 nM) to the assay mixture (0.1 M Tris/HCl, pH 7.3) containing PEP (240 μM), A5P (1 mM), BSA (0.1 mg/mL), and inhibitor **4** (26.6 μM). Curve C, the enzyme (1.5 μM) was first preincubated with **4** (1.33 mM) at 0°C for 30 min, and then diluted 50-fold into the assay buffer pH 7.3 containing PEP (240 μM), A5P (1 mM), and BSA (0.1 mg/mL). The absorbance was monitored at 232 nm.

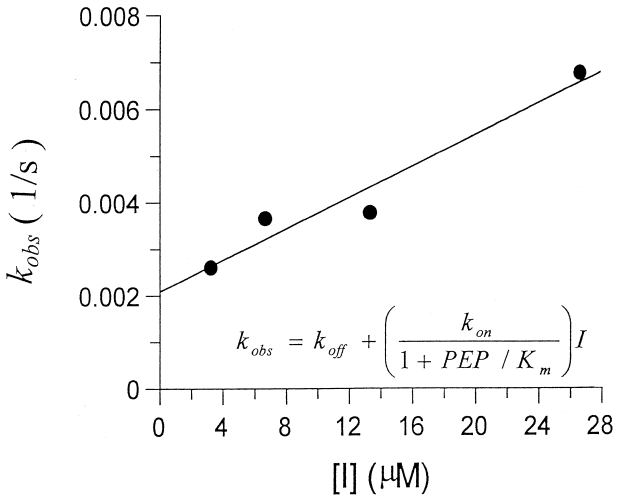


Figure 3. The plot of k_{obs} versus inhibitor (**4**) concentration [*I*]. The values of k_{obs} were obtained by fitting the progress curves of inhibition (curve B, Fig. 2) with different inhibitor concentrations (1.6–26.6 μM) to the expression: $A_0 - A_t = at + b\ e^{-kt} + c$. The values of k_{off} and k_{on} were calculated from the plot and eq (2) (see insert).

and found to be 528 M^{-1} s^{-1}. From these rate constants, K_i was calculated to be 0.42 μM, which is nearly one order of magnitude smaller than that obtained by regular initial velocity study (3.3 μM).

Although from the above data it is clear that the inhibitor **4** exhibits the characteristics of a slow binding inhibitor, as seen from Figure 2, the same steady-state velocities are reached within a short period of time (a few minutes) with and without preincubation of the enzyme with the inhibitor **4**. These results indicate that there is no significant depletion of substrate (PEP) before the equilibria are established. Under these circumstances, according to Williams and Morrison,[30] if we set up our assay conditions so that the enzyme will be preincubated with **4** and PEP, and the reaction will be initiated by addition of the second substrate (A5P), then we might be able to determine the true K_i value through a classical approach of initial velocity measurements. For this purpose it is also advisable[30] that the ratio of the inhibitor concentration to the concentration of the enzyme will be high enough so that the changes in the concentration of free inhibitor on formation of the enzyme-inhibitor complex will be sufficiently small. All these precautions would minimize changes in substrate concentration over the period required for attainment of equilibria, and the data may be analyzed on the basis of Michaelis–Menten kinetics.

Using the above modified assay procedure, the K_i of **4** was measured from the initial velocity data and found to be 0.37 μM (Fig. 4). This value is consistent with that determined by slow-binding studies (0.42 μM), and establishes that the initial velocity study is still practical and valid for the slow binding inhibitor such as compound **4**.

Examination of the binding of 4 as a function of pH

From the above data, one can now assume that the observed inhibition with **4** is the result of its considerable

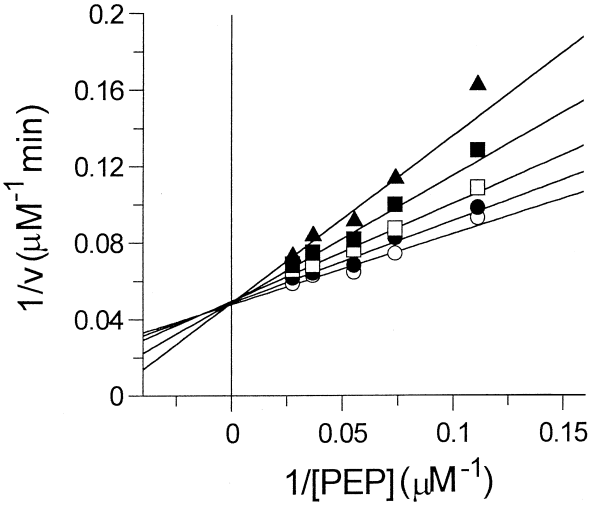


Figure 4. Inhibition of Kdo8P synthase by **4** under slow-binding conditions. Double-reciprocal plots of initial velocities are given as a function of PEP concentration when the A5P concentration was 500 μM, and the inhibitor concentrations were none (○), 0.0532 μM (●), 0.1064 μM (□), 0.26 μM (■), and 0.532 μM (▲). For the assays, the enzyme (1 μM) was first preincubated with various concentrations of PEP (266-1333.3 μM) and of **4** (0–17.73 μM) at 0°C for 30 min. The reactions were initiated by addition of aliquots (30 μL) of the above preincubated enzyme mixture to the reaction buffer containing 0.1 M Tris/HCl, pH 7.3, BSA (0.1 mg/mL), and A5P (0.5 mM) at 37°C.

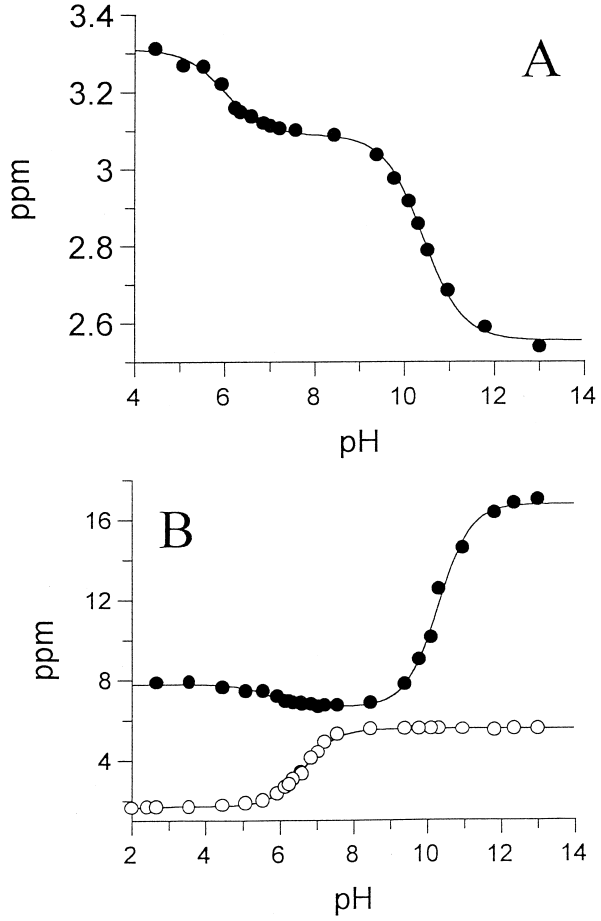


Figure 5. Titration curves for compound **4** ^{1}H NMR (A) and ^{31}P NMR (B) chemical shifts (δ, ppm) against pH are plotted. A sample of (20 mM) in D_2O was titrated between pH 2.0 and 13.0. (A), ^{1}H NMR chemical shifts of the methylene protons ($CH_2PO_3^{2-}$) are plotted versus pH. (B), ^{31}P NMR chemical shifts of the phosphonate (●) and the phosphate (○) are plotted versus pH.

fit to putative oxocarbenium intermediate **3**. Alternatively, the tetrahedral geometry at the ammonium center in **4** and its acyclic structure, also suggests that the enzyme•**4** binary complex may actually mimic the enzyme-bound acyclic intermediate (enzyme•**1**). In order to provide the distinction between these two possibilities, it was necessary to test whether the ammonium ion or the free amine form of **4** is responsible for the strong interaction with the enzyme. For this purpose we have examined the binding affinity of **4** to the enzyme as a function of pH. Firstly, we determined the second pK_a values for the phosphate and phosphonate and the pK_a of the amine in **4** by titration using ^{1}H and ^{31}P NMR (Fig. 5).

The ^{1}H δ titration curve (Fig. 5A) was obtained by following the chemical shift changes of the methylene protons, located alfa to the phosphonate moiety, as a function of pH. Because the close vicinity of these protons to the phosphonate and amine groups, their chemical shift is very sensitive to the ionization states of both phosphonate and amine. The ^{31}P δ titration curve (Fig. 5B) of the phosphonate group of **4** is unusual in that the shielding trend observed for anion formation is reversed to deshielding upon deprotonation of the ammonium anion. The calculated pK_as from the titration curves in Figure 5A and B are summarized in Table 1. It should be noted that the chemical shift changes and the shape of ^{31}P δ versus pH curve for the phosphonate group of **4**, as well as the calculated pK_as for the phosphonate and the amine functions, are very similar to that of glyphosate.[31]

The inhibition constants of **4** at various pH are listed in Table 2 as the ratio of the K_m value for the substrate PEP to the K_i value. The inhibition constants were measured from initial velocity studies by using the above mentioned modified procedure, and in all cases the inhibition was found to be competitive. Since the second pK_as for the phosphate and phosphonate groups in **4** were determined to be 6.66 and 5.68 respectively (Table 1), both groups are close to be fully ionized when pH is above 7.3 (for example, from Fig. 5B it was calculated that at pH 7.3 and 8.0, the phosphate group is 81% and 95.7% ionized). In addition, the pK_a of the amine group is 10.3. Therefore, if we assume that the above pK_a values are not significantly changed upon the binding of the inhibitor **4** to the enzyme, than the observed changes in the inhibition ability of **4** at pH

Table 1. Summary of calculated pK_a values from Figure 5[a]

Nucleus	RPO_3H^-	$ROPO_3H^-$	R_3NH^+
^{31}P	5.68 ± 0.095	6.66 ± 0.016	10.3 ± 0.037
^{1}H	6.07 ± 0.084	ND[b]	10.4 ± 0.027

[a] All pK_as were calculated by nonlinear regression fit using the commercial software GraFit PC-IBM program.[38]
[b] ND, this titration was not determined.

Table 2. Binding affinity (K_m/K_i) of **4** as a function of pH[a]

Buffer	pH	K_m of PEP (μM)	K_i of **4** (μM)	K_m/K_i
Tris/HCl	7.3	7.89	0.37	21.3
	8.0	15.58	0.39	40.0
	8.7	43.48	0.31	140.3
Glycine/KOH	8.6	16.64	3.5	4.75
	9.3	87.56	0.95	92.2

[a] The K_m values for PEP and the K_i values for **4** were measured by using the modified initial velocity studies (see text) in which the reactions were initiated by addition of preincubated enzyme with both PEP and the inhibitor **4**. In all experiments the final concentration of the enzyme was 30 nM.

> 7.3 (Table 2) should predominantly be the result of the change in the protonation state of the amine group. Furthermore, it should be also noted that in Table 2 while we are expressing the relative binding affinities of PEP and **4** as a ratio of K_m/K_i, we assume that the K_m of PEP represents its dissociation constant. Unfortunately, at this stage of investigation, we could only presuppose this approximation. This because our previous study[23] demonstrated that the Kdo8P synthase is isolated with **1** equiv of noncovalently bound PEP which may serve to enhance enzyme stability. Therefore, the determination of K_d value for PEP is complicated by the fact that the enzyme tends to lose activity when PEP is removed.

From the data in Table 2, it is clear that the relative binding affinity of **4** is enhanced as the pH increases. Although such an elevated binding of **4** is most significant at higher pH values (the increase of about 20-fold from pH 8.6 to pH 9.3), it should be noted that in the pH range examined (7.3–9.3), only very small fraction (0.1–9%) of **4** exists in its free amine form (Fig. 5). Therefore, the observed data suggests about the tendency that the free amine form of **4** might be much stronger inhibitor than its ammonium form.

Discussion

Synthesis of inhibitor 4

The analogue **4** described in this paper was designed to resolve several questions concerning the proposed mechanism of Kdo8P synthase shown in Scheme 1. Initially **4** was synthesized in 12 chemical steps starting from D-mannose with an overall yield of about 3%. This procedure, although successful, has impeded the detailed studies of the enzyme-inhibitor interaction because low availability of the final product. Therefore, we have developed a very efficient synthesis that employs the direct reductive amination in aqueous media between unprotected D-mannose 6-phosphate and glyphosate to provide in one step over 60% isolated yield of the target aminophosphonate **4**. In addition to its general application, this method provides the easiest and simplest way for the preparation of isotopically labeled analogues of **4** which might be used for the structure–function investigation of the enzyme. Studies in this direction using several ^{13}C and ^{15}N-labeled analogues of **4** are underway.

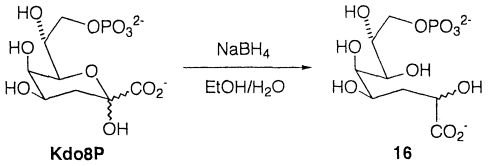
Interaction of 4 with the enzyme

A synthetic sample of **4** was evaluated as an inhibitor of Kdo8P synthase. Firstly, it appears that **4** is a slow binding inhibitor to the enzyme, therefore, its true K_i value cannot be measured from initial velocity data by using conventional assay procedures. To obtain the dissociation constant, the rate constants for association to and dissociation from the enzyme were measured, and found to be 528 M^{-1} s^{-1} and 0.0022 s^{-1} respectively. From these rate constants, K_i was calculated to be 0.42 μM at pH 7.3. With the dissociation constant of 0.42 μM, compound **4** represents the most potent inhibitor to the enzyme tested to date. Comparison of this value with the K_i of the product Kdo8P (600 μM),[20] and with the K_m of PEP (8 μM), reveals that **4** binds to the enzyme 1500-fold more tightly than Kdo8P and 20-fold more tightly than PEP. In addition, compound **4** is the first bisubstrate inhibitor of the enzyme that combines key features of A5P and PEP into a single molecule and has an acyclic structure.

The observed time-dependent interaction between the enzyme and inhibitor **4** could involve the rapid formation of enzyme•**4** complex, which then undergoes a slow isomerization to an (enzyme•**4**)* complex. This might fit to the scenario in which the enzyme binds the ammonium form of **4** and then undergoes a slow deprotonation to form (enzyme•**4**)* complex that consists **4** in a free amine form. The observed same K_i values of **4** in pH range 7.3–8.7 (Table 2) supports this suggestion. Alternatively, the slow establishment of the equilibrium between the inhibitor **4**, enzyme, and enzyme•**4** complex could be due to the low value of the pseudo-first-order rate constant with respect to enzyme for the formation of enzyme•**4** complex, or of the first-order rate constant for the dissociation of enzyme•**4** complex. At this stage of investigation, all the suggestions to account for which of the above factors are really responsible will be only speculation. A more detailed characterization of the interaction of **4** with the active site residues of the enzyme, by using solution and solid-state NMR techniques with the isotopically labeled analogues of **4**, is underway.

Secondly, the examination of the binding affinity of **4** as a function of pH (Table 2) suggests that the free amine form of the inhibitor might binds to the enzyme much tighter than the charged ammonium form. For example, the K_m/K_i values at pH 7.3 and 8.7 were determined to be 21.3 and 140.3, respectively. This result is consistent with the scenario that the enzyme•**4** binary complex may actually mimics the enzyme bound acyclic intermediate (enzyme•**1**), and not the enzyme•**3** complex, as it would be anticipated because the presence of positive charge in **4**. This result provides some support to the possibility that the formation of acyclic bisphosphate intermediate **1** (Scheme 1, path a) may occurs through a concerted mechanism.

In addition, Ray and co-workers[10] have reported that borohydride reduction of Kdo8P gives a mixture of the two epimeric 2-dihydro analogues of Kdo8P (structures **16**, Scheme 4) and that this material does not function as an inhibitor of the synthase. If the reaction proceeds upon the formation of intermediate **1** (Scheme 1, path a), then one should expect the acyclic analogues **16**, which mimic the proposed intermediate **1**, to be inhibitors of the enzyme. However, the observed inert nature of **16** cannot rationally be explained. We, therefore, reinvestigated this issue by synthesizing the mixture of analogues **16** and evaluated it as inhibitor of the synthase. Our results clearly show that **16** functions as competitive inhibitor with a K_i value of 0.5 mM. This observation, coupled with the results with inhibitor **4**, provides additional support for the concept of a mechanism[23] that proceeds through the involvement of an acyclic intermediate **1** (Scheme 1, path a).



Scheme 4. Synthesis of C2-epimeric mixture of compound **16**.

In summary, a mechanism-based inhibitor, the aminophosphonate **4**, and an acyclic analogue of the product Kdo8P (structures **16**), have been synthesized and evaluated as inhibitors of Kdo8P synthase. It was found that **4** functions as a slow-binding inhibitor with a dissociation constant of 0.42 μM, and thus represents the most potent inhibitor to the enzyme tested to date. In addition, a very simple and efficient one-step synthesis of the inhibitor **4** from the commercial compounds has been successfully accomplished. This synthetic method, in addition to its general application, also paves the way for the production of in vivo inhibitors of the LPS biosynthesis which might have commercial future as novel antibacterial drugs. The in depth studies of this enzyme mechanism, including a detailed description of the interaction of **4** with the active site residues of the enzyme, and the synthetic effort to prepare more advanced analogues of the putative intermediate **1** are underway and will be reported in due course.

Experimental

General methods

The homogeneous Kdo8P synthase (specific catalytic activity 9 U/mg) was isolated from the overproducing strain *Escherichia coli* DH5α (pJU1), as previously described.[16,21] A5P and Kdo8P were prepared enzymatically according to the procedure of Whitesides.[32] All other chemicals were received from Aldrich or from Sigma and used without further purification, unless noted. In all the synthetic work described, reactions were performed under dry argon atmosphere unless otherwise noted. All solvents were dried over standard drying agents[33] and freshly distilled prior to use. Flash column chromatography[34] was performed on Silica Gel 60 (70-230 mesh). Reactions were monitored by TLC on Silica Gel 60 F_{254} (0.25 mm, Merck) and detected by charring with a yellow solution containing $Na_2MoO_4{\cdot}4H_2O$ (5 g) and cerric ammonium nitrate (5 g) in 10% H_2SO_4 (300 mL). Soviet spray[35] solution was used for the detection or qualitative analysis of the compounds containing phosphate mono-esters.

Spectral methods

Spectrophotometric measurements were made on a Hewlett–Packard 8452A diode array spectrophotometer using 1 cm path-length cells with a thermostatic cell holder, and a circulating water bath at the desired temperature. ^{1}H NMR spectra were recorded on a Bruker AM-200 or AM-400 spectrometer, and chemical shifts reported (in ppm) relative to internal tetramethylsilane ($\delta = 0.0$) with $CDCl_3$ as the solvent and relative to HOD ($\delta = 4.63$) with D_2O as the solvent. ^{13}C NMR spectra were recorded on a Bruker AM-200 (50.3 MHz) or AM-400 (100.6 MHz) spectrometer, and the chemical shifts reported (in ppm) relative to external sodium 2,2-dimethyl-2-silapentane sulfonate ($\delta = 0.0$) in D_2O. ^{31}P-NMR spectra were recorded on a Bruker AM-200 spectrometer at 81.0 MHz and the chemical shifts reported (in ppm) relative to external orthophosphoric acid ($\delta = 0.0$) in D_2O. All the coupling constants (J) are in Hz. Mass spectra were obtained by the use of a TSQ-70B mass spectrometer (Finnigan Mat) using fast-atom bombardment (FAB) in glycerol matrices.

Inhibition study

Enzyme assays. Unless otherwise stated, the enzyme activity was assayed in 1.0 mL of a reaction buffer consisting of 0.1 M Tris/HCl (pH 7.3), PEP (0.2 mM, $27K_m$), BSA (0.1 mg/mL), and A5P (0.5 mM, $20K_m$). Following equilibration at 37°C for 2 min, Kdo8P synthase (10 μL, at a final concentration of approximately 30 nM) was added, and the decrease in the absorbance difference between 232 and 350 nm (as internal reference) was monitored as a function of time (MS-DOS UV/VIS software). This method[36] is based on the absorbance difference at 232 nm between PEP ($\varepsilon = 2840$ M^{-1} cm^{-1}) and the other substrates and products ($\varepsilon <$ 60 M^{-1} cm^{-1}) under the assay conditions. The concentrations of PEP, A5P and of **4** were determined precisely by quantitative assaying of the P_i released by alkaline phosphatase.[37] In each case, to ensure complete hydrolysis of the phosphate monoester, the aliquots of the incubation mixture with alkaline phosphatase were tested by ^{31}P-NMR. One unit of the enzyme activity is defined as the amount that catalyzes the consumption of 1 μmol of PEP per minute at 37°C.

Regular initial velocity study. In order to determine the steady-state kinetic parameters, K_m for PEP and K_i of **4**, the reaction solutions were prepared as described above but with a constant (500 μM, $20K_m$) A5P concentration and variable (8–40 μM) PEP concentrations. The rate measurements were taken as described above, while a 5

s delay was allowed following the initiation of the reaction. The initial rate was then determined by a least-squares fitting of the first 10% of the progress curve (between 10 and 60 s, depending on the initial concentration of PEP) to a straight line. The K_m for PEP was 8 μM and the K_m for A5P was 25 μM. Four inhibitor concentrations were examined, and for each inhibitor concentration, four concentrations of PEP (8–40 μM) were used. All samples were assayed in triplicate, and analogous results were obtained in two to four different experiments. The data were fitted to the competitive model using the equation: $Y = V\ [S]/[K\ (1 + [I]/K_i) + [S]]$, employing the commercial software GraFit program.[38] The K_i value was calculated either from the above treatment, or from the secondary replots of the slopes from initial double-reciprocal plots ($1/v$ versus $1/[S]$) versus inhibitor concentration.[39]

Initial velocity study under slow-binding conditions. The enzyme (1 μM) was preincubated with the variable concentrations of **4** (0−17.73 μM) and the variable concentrations of PEP (266–133.3 μM) at 0°C for 30 min. An aliquot of the enzyme mixture (30 μL) was then added to a 1.0 mL reaction buffer preequilibrated at 37°C for 2 min, and consisting of 0.1 M Tris/HCl, pH 7.3, A5P (0.5 mM, = 20 K_m) and BSA (0.1 mg/mL). The rate measurements were performed immediately with the above addition, and the initial rate was then determined as described above. Using this procedure, the K_i value was calculated to be 0.37 μM (Fig. 4).

Using the similar experiments, the K_i values of **4** were also measured as a function of pH. Two different buffer systems were used: Tris/HCl (pH = 7.3, 8.0, 8.7) and Glycine/KOH (pH = 8.6, 9.3). The final concentrations of the enzyme and of BSA in all these experiments were 30 nM and 0.1 mg/mL, respectively. The final concentrations of A5P, PEP, and **4** varied at different pH values.

Progress curves. Progress curves (Fig. 2) were obtained by two different experimental procedures. First, the slow development of the inhibition was determined by continuous monitoring the disappearance of PEP after initiation of the reaction by addition of the enzyme (30 nM). Reaction mixtures contained Tris/HCl buffer (pH = 7.3), PEP (240 μM), A5P (1 mM), BSA (0.1 mg/mL) and various concentrations of inhibitor **4** (1.625–26.6 μM). Second, for determination the slow recovery of enzyme activity after preincubation with inhibitor, the experiments were performed as follow. The mixtures of enzyme (1.5 μM) and inhibitor **4** (0–1.33 mM) were preincubated in Tris/HCl buffer (pH = 7.3) for 30 min at 0°C. An aliquot (20 μL) of the incubation mixture was then diluted 50-fold into a 1.0 mL reaction mixture containing Tris/HCl buffer (pH = 7.3), PEP (240 μM), A5P (1 mM) and BSA (0.1 mg/mL) to give a final enzyme concentration of 30 nM. The reaction progress was followed by continuous monitoring the disappearance of PEP at 232 nm.

Data analysis. The regression curves (disappearance of PEP, 232 nm) were converted to the progress curves of product formation by calculating $A_0 - A_t$, where A_0 is the absorbence of PEP at 0 time, and A_t is the absorbence of PEP at time t. The first 400 s of the observed progress curves were fitted to the eq (1), from which the values of k_{obs} were calculated. The parameters a, b, and c in eq (1) are constants.

$$A_0 - A_t = at + be^{-kt} + c \quad (1)$$

The values of k_{on} and k_{off} were then determined from the plot of k_{obs} as a function of [I] according the eq (2):[30,40]

$$k_{obs} = k_{off} + \left(\frac{k_{on}}{1 + [PEP]/K_m}\right)[I] \quad (2)$$

NMR titration. The potassium salt of **4** (8 mg) was dissolved in water (2 mL) and treated with Dowex 50W (H^+ form) to adjust the pH to about 2. The solution was filtrated and lyophilized. The residue was dissolved in D_2O (0.5 mL), and titrated by careful addition of KOD (0.5N in D_2O). At each pH, the solution was transferred into NMR tube, and ^1H and ^{31}P NMR were acquired at 25°C. The titration curves were obtained by plotting the corresponding ^{31}P or ^1H NMR chemical shifts against pH. All pK_as were calculated by non-linear regression fit using the commercial software GraFit PC-IBM program.[38]

Synthetic procedures

1-Deoxy-1-[(2-ethoxy-2-oxoethyl)amino]-2,3:5,6-di-*O*-isopropylidene-D-mannitol (6). To a solution of isopropylidene derivative **5**[26] (4 g, 15.4 mmol) and glycine ethyl ester hydrochloride (2.16 g, 15.4 mmol) in methanol (140 mL) was added sodium cyanoborohydride (0.49 g, 7.6 mmol). The reaction mixture was stirred at room temperature until the reactants were consumed (68 h), as determined by TLC. The reaction mixture was diluted with EtOAc (100 mL), washed with water and saturated NaCl, dried ($MgSO_4$) and evaporated to dryness. Purification by chromatography on silica gel (EtOAc: hexane, 1:3) afforded the secondary amine **7** (3.02 g, 56.4%), along with unreacted **5** (0.5g, 12.5%) and the glycosyl amine **6** (0.41 g, 7.7%) as a byproduct. Data for **7**: ^1H NMR ($CDCl_3$, 400 MHz) δ 1.24 (t, 3H, J = 7.1 Hz, $CO_2CH_2CH_3$), 1.33 (s, 3H, Me), 1.35 (s, 3H, Me), 1.37 (s, 3H, Me), 1.51 (s, 3H, Me), 2.73 (dd, 1H, J = 12.7, 2.0 Hz, H-1a), 3.10 (dd, 1H, J = 12.7, 4.0 Hz, H-1b), 3.32 (d, 1H, J = 17.3 Hz, N-CHHCO$_2$), 3.45 (d, 1H, J = 17.3 Hz, N-CHHCO$_2$), 3.49 (d, 1H, J = 7.9 Hz, H-4), 4.01 (ddd, 1H, J = 7.9, 7.6, 3.1 Hz, H-5), 4.08–4.14 (m, 2H, 2H-6), 4.16 (q, 2H, J = 7.1 Hz, $CO_2CH_2CH_3$), 4.34 (ddd, 1H, J = 7.9, 4.0, 2.0 Hz, H-2), 4.38 (d, 1H, J = 7.9 Hz, H-3); ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 14.13 ($CO_2CH_2CH_3$), [24.54, 25.33, 26.31, and 26.89 (4CH_3)], 47.66 (NCH_2CO_2), 50.29 ($CO_2CH_2CH_3$), 60.98 (C1), [67.66, 70.58, 75.44, 76.00, 76.68 (C2, C3, C4, C5, C6)], 108.03 and 109.10 [$C(CH_3)_2$], 171.07 (CO_2); CIMS m/z 348.1 (MH^+, $C_{16}H_{30}O_7N$ requires 348.4). Data for **6**

(mixture of two anomers): ^{1}H NMR ($CDCl_3$,400 MHz) δ 1.23 (t, 3H, $J=7.1$ Hz, $CO_2CH_2CH_3$), 1.24 (t, 3H, $J=7.1$ Hz, $CO_2CH_2CH_3$), [1.29, 1.30, 1.31, 1.33, 1.39, 1.41, 1.43, 1.46 (8s, 24H, Me of isopropylidene groups), 3.52 (d, 1H, $J=17.2$ Hz, $NCHHCO_2$), 3.63 (d, 1H, $J=17.2$ Hz, $NCHHCO_2$), 3.90 (dd, 1H, $J=7.9$, 3.7 Hz, H-3b), 3.92 (d, 1H, $J=4.48$, H-2a), 4.00–4.05 (m, 3H, H-6b, H-5b, H-3a), 4.13 (q, 2H, $J=7.1$ Hz, $CO_2CH_2CH_3$), 4.15 (q, 2H, $J=7.1$ Hz, $CO_2CH_2CH_3$), 4.306–4.326 (m, 3H, H-4a, H-6b, H-4b), 4.46 (d, 1H, $J=5.9$ Hz, H-1b), 4.57 (dd, 1H, $J=6.08$, 3.54 Hz, H-5a), 4.65 (dd, 1H, $J=6.07$, 3.44 Hz, H-6a), 4.67 (s, 1H, H-1a), 4.74 (dd, 1H, $J=5.96$, 3.62 Hz, H-2b); ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 14.15 ($CO_2CH_2CH_3$), [24.64, 25.24, 25.29, 25.75, 25.98, 26.85, $C(CH_3)_2$)], 46.84 (NCH_2CO_2), 48.05 ($CO_2CH_2CH_3$), [60.72, 60.94, 66.84, 66.95, 73.21, 79.45, 79.68, 79.73, 80.31, 85.62, 91.13, 94.69 (C1, C2, C3, C4, C5, C6)], {109.01, 109.18, 112.57, 112.68 [$C(CH_3)2$}, [171.86, 172.39 (CO_2)]; CIMS m/z 346.1 (MH^+, $C_{16}H_28O_7N$ requires 346.4).

1-Deoxy-1-[(diethylphosphonomethyl)(2-ethoxy-2-oxoethyl)amino]-2,3:5,6-di-*O*-isopropylidene-D-mannitol (8). To a stirred mixture of compound **7** (0.9 g, 2.6 mmol) and Na_2CO_3 (0.98 g) in 8 mL CH_2Cl_2:H_2O (1:1) was added diethylphosphonomethyltriflate (0.83 g, 3.11 mmol). The resulting mixture was stirred for 2 h at 45°C. Additional portions of the triflate (0.83g, 3.11 mmol), Na_2CO_3 (0.9 g), CH_2Cl_2 (4 mL) and H_2O (4 mL) were added, and the reaction mixture was heated at reflux for 2 h. After cooling to room temperature the reaction mixture was extracted with CH_2Cl_2 (3×20 mL), dried ($MgSO_4$) and concentrated under a vacuum. Chromatography of the crude residue (EtOAc:hexane, 3:2) furnished **8** (0.84 g, 65%), along with unreacted **7** (20.6 mg, 2.3%). Data for **8**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.22 (t, 3H, $J=7.1$ Hz, $CO_2CH_2CH_3$), 1.28 (t, 3H, $J=7.1$ Hz, Me of PO_3Et_2), 1.28 (t, 3H, $J=7.1$ Hz, Me of PO_3Et_2), 1.30 (s, 3H, Me), 1.31 (s, 3H, Me), 1.35 (s, 3H, Me), 1.43 (s, 3H, Me), 3.14-3.16 (m, 2H, 2H-1), 3.19 (dd, 1H, $J=15.9$, 9.6 Hz, $NCHHPO_3$), 3.24 (dd, 1H, $J=15.9$, 10.6 Hz, $NCHHPO_3$), 3.48 (dd, 1H, $J=7.0$, 0.7 Hz, H-4), 3.64 (d, 1H, $J=18.0$ Hz, $NCHHCO_2$), 3.69 (d, 1H, $J=18.0$ Hz, $NCHHCO_2$), 3.95 (dd, 1H, $J=6.2$, 3.2 Hz, H-6a), 3.98–4.04 (m, 1H, H-5), 4.04–4.156 (m, 7H, H-6b and OCH_2CH_3), 4.28(dd, $J=7.2$, 0.7 Hz, H-3), 4.35 (ddd, 1H, $J=7.2$, 5.5, 5.0 Hz, H-2); ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 14.19 ($CO_2CH_2CH_3$), {16.37, 16.48 [$PO_3(CH_2CH_3)2$]}, {24.72, 25.24, 26.65, 26.81 [$C(CH_3)2$]}, 50.27 (d, $J=159.8$ Hz, NCH_2P), 55.21 (d, $J=9.2$ Hz, NCH_2CO_2), 56.20 (d, $J=4.9$ Hz, $CO_2CH_2CH_3$), 60.37 (C1), 61.95 (d, $J=5.8$ Hz, $PO_3CH_2CH_3$), 62.10 (d, $J=6.5$ Hz, $PO_3CH_2CH_3$), [67.28, 70.47, 76.06 (bs), 76.20 (C2, C3, C4, C5, C6)], {108.13, 109.17 [$C(CH_3)2$]}, 170.89 (CO_2); ^{31}P NMR (proton decoupled, $CDCl_3$, 81.0 MHz) δ 22.86; CIMS m/z 497.9 (MH^+, $C_{21}H_{41}O_{10}NP$ requires 498.5).

1-Deoxy-1-[(diethylphosphonomethyl)(2-ethoxy-2-oxoethyl)amino]-2,3-*O*-isopropylidene-4,5-di-*O*-acetate-6-[(*tert*-butyl)dimethylsilyl]-D-mannitol (9). To a stirred solution of **8** (4.6 g, 9.24 mmol) in methanol (130 mL) was added 0.33 M solution of trifluoroacetic acid in MeOH:H_2O (1:3, 130 mL). The reaction progress was monitored by TLC (silica gel, 10% MeOH:$CHCl_3$). After 1.5 h, the reaction was quenched by addition of Amberlite IR-45 (OH^- form) resin until the pH was neutral (pH paper). The mixture was filtrated and solvent was removed in vacuum. Column chromatography (2.5–7.0% MeOH:$CHCl_3$) afforded the target triol (2.011 g, 47.5%) as an oil: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.25 (t, 3H, $J=7.1$ Hz, $CO_2CH_2CH_3$), 1.295 (t, 3H, $J=7.1$ Hz, Me of PO_3Et_2), 1.300 (t, 3H, $J=7.1$ Hz, Me of PO_3Et_2), 1.33 (s, 3H, Me), 1.44 (s, 3H, Me), 3.07 (dd, 1H, $J=14.3$, 4.8 Hz, H-1a), 3.17 (dd, 1H, $J=14.3$, 6.2 Hz, H-1b), 3.20 (d, 2H, $J=10.4$ Hz, NCH_2PO_3), 3.59 (d, 1H, $J=18.0$ Hz, $NCHHCO_2$), 3.68 (d, 1H, $J=18.0$ Hz, $NCHHCO_2$), 3.75 (dd, 1H, $J=11.8$, 2.8 Hz, H-6a), 3.85 (dd, 1H, $J=11.8$, 2.8 Hz, H-6b), 3.95 (m, 1H, H-4), 4.05–4.14 (m, 5H, H-5, $2CH_2$ of PO_3Et_2), 4.14 (q, 2H, $J=7.1$ Hz, CH_2 of CO_2Et_2), 4.33 (ddd, 1H, $J=6.6$, 6.2, 4.8 Hz, H-2), 4.38 (dd, 1H, $J=6.6$ and 3.4 Hz, H-3); ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 14.24 ($CO_2CH_2CH_3$), [16.37, 16.48 ($PO_3CH_2CH_3$)], [25.12, 27.20 ($C(CH_3)_2$)], 50.81 (dd, $J=162.2$ Hz, NCH_2P), 55.83 (d, $J=6.8$ Hz, $CO_2CH_2CH_3$), 55.95 (d, $J=8.5$ Hz, NCH_2CO_2), 60.51 (C1), 62.21 (d, $J=6.9$ Hz, $POCH_2CH_3$), 62.55 (d, $J=7.0$ Hz, $POCH_2CH_3$), [63.66, 69.02, 73.03, 76.06, 77.0 (C2, C3, C4, C5, C6)], 107.99 [$C(CH_3)2$], 170.75 (CO_2); ^{31}P NMR (proton decoupled, $CDCl_3$, 81.0 MHz) δ 22.87 ppm; CIMS m/z 458.0 (MH^+, $C_{18}H_{37}O_{10}NP$ requires 458.4).

The above triol (50 mg, 0.11 mmol) was dissolved in dry pyridine (5 mL), to which dimethyl-*tert*-butyl-chlorosilane (50 mg, 0.33 mmol) and catalytic amount 4-dimethylaminopyridine were added at 0°C. The reaction mixture was stirred at room temperature for 2 h, then diluted with EtOAc and washed with saturated $NaHCO_3$. The organic fraction was dried ($MgSO_4$) and concentrated to give the corresponding silylated product. This crude product, without further purification, was dissolved in dry pyridine (2 mL) and acetic anhydride (0.5 mL) was added. The reaction mixture was stirred at room temperature for 1.5 h, then diluted with EtOAc, washed with saturated $NaHCO_3$, dried ($MgSO_4$), and evaporated to dryness. Purification by chromatography on silica gel (EtOAc:hexane, 3:2) gave pure product **9** (67 mg, 93%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.02 (s, 6H, $SiMe_2$), 0.85 (s, 9H, SiC*t-Bu*), 1.24 (t, 3H, $J=7.1$ Hz, $CO_2CH_2CH_3$), 1.297 (t, 3H, $J=7.1$ Hz, Me of PO_3Et_2) 1.301 (t, 3H, $J=7.1$ Hz, Me of PO_3Et_2), 1.30 (s, 3H, Me), 1.43 (s, 3H, Me), 2.02 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.78 (ddd, 1H, $J=13.7$, 3.6, 2.8 Hz, H-1a), 2.99 (dd, 1H, H-1b, $J=13.7$, 8.1 Hz), 3.19 (dd, 1H, $J=16.0$, 8.0 Hz, $NCHHP$), 3.26 (dd, 1H, $J=16.0$, 11.8 Hz, $NCHHP$), 3.61 (dd, 1H, $J=11.3$, 6.8 Hz, H-6a), 3.62 (d, 1H, $J=18.0$ Hz, $NCHHCO_2$), 3.73 (d, 1H, $J=18.0$ Hz, $NCHHCO_2$), 3.93 (dd, 1H, $J=11.3$, 4.0 Hz, H-6b), 4.067–4.158 (m, 6H, $3CH_2CH_3$), 4.29 (dd, 1H, $J=6.4$, 2.9 Hz, H-3), 4.35 (ddd, 1H, $J=8.0$, 6.4, 3.6 Hz, H-2), 4.97 (ddd, 1H, $J=6.8$, 4.7, 4.0 Hz, H-5), 5.09 (dd, 1H, $J=4.7$, 2.9 Hz, H-4); ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 5.46 ($SiMe_3$), 14.17 ($SiCMe_3$), 14.23 ($CO_2CH_2CH_3$), [16.42, 16.54 ($PO_3CH_2CH_3$)], [20.92, 21.04 (CH_3CO)], 25.66 [$C(CH_3)_2$], 25.76 ($SiCMe_3$), 26.74

[C(CH_3)2], 49.91 (d, J=162.2 Hz, NCH_2P], 55.18 (d, J=10.6 Hz, NCH_2CO_2), 55.95 (d, J=3.8 Hz, $CO_2CH_2CH_3$), 60.23 (C1), 61.26 (C6), 62.08 (d, J=6.9 Hz, POCH_2CH_3), [69.68, 74.10, 75.61, 76.87 (C2, C3, C4, C5, C6)], 108.90 [C(CH_3)2], [169.71, 170.10, 17.06 (3CO_2)]; ^{31}P NMR (proton decoupled, $CDCl_3$, 81.0 MHz) δ 23.05; CIMS m/z 656.0 (MH^+, $C_{28}H_{55}O_{12}NPSi$ requires 656.79).

1-Deoxy-1-[(diethylphosphonomethyl)(2-ethoxy-2-oxoethyl)amino]-2,3-*O*-isopropylidene-4,5-di-*O*-acetate-6-(diphenylphosphoryl)-D-mannitol (10). Compound **9** (20 mg, 0.03 mmol) was dissolved in methanol (3 mL) and sulfuric acid (1M in MeOH, 1.5 mL) was added at 0°C. The reaction progress was monitored by TLC (EtOAc: hexane, 4:1). After being stirred for 2.5 h at 0°C, then reaction mixture was diluted with EtOAc, washed with saturated $NaHCO_3$ and saturated NaCl solutions, dried over $MgSO_4$ and concentrated under a vacuum. Chromatography of the oily residue (EtOAc:hexane, 3:1 to 9:1) afforded 20 mg (78%) of pure desilylated product: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.22 (t, 3H, J=7.14 Hz, $CO_2CH_2CH_3$), 1.27 (t, 3H, J=7.10 Hz, CH_3 of PO_3Et_2), 1.27 (t, 3H, J=7.10 Hz, CH_3 of PO_3Et_2),1.30 (s, 3H, Me of isopropylidene), 1.42 (s, 3H, Me of isopropylidene), 2.01 (s, 3H, OAc), 2.09 (s, 3H, OAc), 2.81 (dd, 1H, J=13.6, 3.0 Hz, H-1a), 3.05 (dd, 1H, J=13.6, 6.0 Hz, H-1b), 3.14 (dd, 1H, J=16.0, 12.7 Hz, NCH$H$$PO_3$), 3.24 (dd, 1H , J=16.0 and 7.0 Hz, NC$H$$HPO_3$), 3.56 (ddd, 1H, J=12.0, 4.5 Hz, H-6a), 3.59 (d, 1H, J=16.0 Hz, NCH$H$$CO_2$), 3.70 (d, 1H, J=16.0 Hz, NC$H$$HCO_2$), 3.85 (dd, 1H, J=12.0, 5.2 Hz, H-6b), 4.04–4.15 (m, 9H), 4.37–4.38 (m, 2H, H-2 and H-3), 5.06 (ddd, 1H, J=5.2, 4.5, and 4.5 Hz, H-5), 5.20 (dd, 1H, J=4.5, 3.2 Hz, H-4); ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 14.23 (CO_2CH_2 CH_3), [16.38, 16.50 ($PO_3(CH_2CH_3)_2$], [20.90, 21.05 (2CH_3CO)], {25.59, 26.86 [C(CH_3)2]}, 55.56 (d, J=9.9 Hz, NCH_2CO_2), 56.26 (dd, J=4.4 Hz, $CO_2CH_2CH_3$), [60.22, 60.33 (C1 and C6)], 62.13 [d, J=6.9 Hz, $PO_3(CH_2CH_3)_2$], 62.27 [d, J=6.9 Hz, $PO_3(CH_2CH_3)_2$], [69.59, 73.49, 75.64, and 76.25 (C2, C3, C4, C5)], 108.90 [C(CH_3)], [170.03, 170.58, and 170.99 (3CO_2)]; ^{31}P NMR (proton decoupled, $CDCl_3$, 81.0 MHz) δ 22.77 ppm; CIMS m/z 542.0 (MH^+, $C_{22}H_{41}O_{12}NP$ requires 542.5).

The above-mentioned desilylated product (1.9 g, 3.5 mmol) was dissolved in dry pyridine (20 mL) to which diphenylphosphochloridate (1.88 g, 7 mmol) and 4-dimethylaminopyridine (0.2 g) were added until the pH of the resulted solution was basic (pH∼8 by pH paper). The reaction mixture was stirred at room temperature for about 1 h, then diluted with EtOAc, washed with saturated $NaHCO_3$, and dried over $MgSO_4$. The solvent was evaporated under reduced pressure, and the residue was purified by chromatography on a silica gel column (EtOAc:hexane, 4:1) to give 1.64 g of pure **10** (60.6% yield) along with unreacted desilylated material (0.41 g, 21.6%). Data for **10**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.23 (t, 3H, J=7.1 Hz, Me of CO_2Et), 1.29 (t, 3H, J=7.1 Hz, Me of PO_3Et_2), 1.29 (t, 3H, J=7.1 Hz, Me of PO_3Et_2), 1.30 (s, 3H, Me of isopropylidene), 1.43 (s, 3H, Me of isopropylidene), 1.89 (s, 3H, OAc), 2.05 (s, 3H, OAc), 2.6 (dd, 1H, J=13.6, 2.8 Hz, H-1a), 2.99 (dd, 1H, J=13.6, 7.6 Hz, H-1b), 3.178 (d, 1H, J=9.0 Hz, NC$H$$HPO_3$), 3.18 (d, 1H, J=10.8 Hz, NCH$H$$PO_3$), 3.57 (d, 1H, J=18.0 Hz, NCH$H$$CO_2$), 3.70 (d, 1H, J=18.0 Hz, NCH$H$$CO_2$), 4.06–4.14 (m, 6H), 4.25–4.33 (m, 2H, H-3, H-6a), 4.38 (ddd, 1H, J=8.1, 7.6, and 2.8 Hz, H-2), 4.73 (ddd, 1H, J=11.6. 7.0, and 2.2 Hz, H-6b), 5.12–5.14 (m, 2H, H-4, and H-5), 7.11–7.20 (m, 5H, Ph), 7.29–7.37 (m, 5H, Ph); ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 14.20 ($CO_2CH_2CH_3$), {16.40, 16.51 [$PO_3(CH_2CH_3)_2$]}, [20.64, 20.99 (2CH_3 CO)], {25.42, 26.36 [C(CH_3)2]}, 49.85 (d, J=162.1 Hz, NCH_2P), 54.8 (d, J=10.0 Hz, NCH_2CO_2), 56.07 (d, J=4.1 Hz, $CO_2CH_2CH_3$), 60.27 (C1), 61.99 (d, J=5.5 Hz, POCH_2CH_3), 62.10 (d, J=5.1 Hz, POCH_2CH_3), 66.53 (d, J=5.5 Hz, C6), 72.85 (d, J=7.2 Hz, C5), [69.25, 74.87, 76.58 (C2, C3, C4)], 109.29 [C(CH_3)2], [119.96, 119.98, 120.06, 125.34, 129.74, 150.44, 150.59 (aromatic carbons)], [169.70, 170.03, 170.97 (3CO_2)]; ^{31}P NMR (proton undecoupled, $CDCl_3$, 81.0 MHz) δ -13.9 (dd, J=7.0 and 6.5 Hz, CH_2OP), 22.91 (dd, J=9.0 and 10.8 Hz, CH_2P); CIMS m/z 773.8 (MH^+, $C_{34}H_{50}O_{15}NP_2$ requires 774.7).

1-Deoxy-1-[[*N*-(phosphonomethyl)-2-oxoethyl]amino]-D-mannitol-6-phosphate (4). To a stirred solution of **10** (860 mg,1.11 mmol) and triethylamine (0.46 mL, 3.33 mmol) in CH_3CN (15 mL) was added bromotrimethylsilane (0.67 mL, 4.44 mmol) at 0°C. After 1 h, another portion of triethylamine and TMSBr were added, and the reaction mixture was stirred for additional 2 h. The reaction was quenched by addition of methanol (15 mL) and water (15 mL), concentrated on a rotary evaporator to a small volume (∼5 mL), and to a resulted solution added 30 mL water. This solution was washed with ether (3×20 mL), and the resulted water fraction was lyophilized. The residue was dissolved in methanol (10 mL) and the pH was adjusted to ∼3 by addition of acetic acid. To this solution PtO_2 (0.2 g) was added, and the mixture was stirred under H_2 at 1 atm for 40 h. The reaction progress was monitored by ^{31}P NMR. The catalyst was removed by filtration through Celite, and the filtrate was washed with methanol. The washes were combined and solvent was evaporated under vacuum. The residue was dissolved in water (10 mL), the pH was adjusted to 1 with Dowex 50W (H^+ form), and the resulted mixture was stirred at room temperature for 40 h. After removal of the resin by filtration, the solvent was lyophilized. The resulted material was dissolved in water (10 mL), pH was adjusted to 13 with 2 M KOH, and stirred at room temperature for 5 h. The mixture was neutralized with Dowex (H^+), filtrated, and lyophilized to give the crude product **10**. This product was purified by using anion-exchange chromatography, by passing through the column of AG 1X8 (HCO_3^- form), eluting with a linear gradient of triethylammonium bicarbonate buffer (0–0.7M, pH 7.5). Fractions were analyzed for inorganic phosphate after digestion with $HClO_4$. The active fractions containing the product were then combined and lyophilized to give 0.22 g (34.6%) of pure **10**: ^{1}H NMR (D_2O, pD=13.0, 400 MHz) δ 2.53 (d, 1H, J=10.2 Hz, C$H$$HPO_3$), 2.53 (d, 1H, J=11.8 Hz, CH$H$$PO_3$), 2.62 (dd, 1H, J=13.5, 7.6 Hz, H-1a), 2.85 (ddd, 1H, J=13.5, 4.0 Hz, H-1b),

3.18 (dd, 1H, $J=16.4$ Hz, NC*H*HCO$_2$), 3.29 (d, 1H, $J=16.4$, NCH*H*CO$_2$), 3.61 (dd, 1H, $J=8.3$, 0.9 Hz, H-3), 3.63 (ddd, 1H, $J=8.0$, 4.8, 2.2 Hz, H-5), 3.68 (dd, 1H, $J=8.0$, 0.9 Hz, H-4), 3.69 (ddd, 1H, $J=8.3$, 7.6, 4.0 Hz, H-2), 3.76 (ddd, 1H, $J=11.5$, 7.4, 4.8, H-6a), 3.85 (ddd, 1H, $J=11.5$, 6.5, 2.2 Hz, H-6b); ^{13}C NMR (D$_2$O, 100 MHz) δ 56.80 (d, $J=141.8$ Hz, N*C*H$_2$P), 61.77 (d, $J=7.45$, C1), 63.14 (d, $J=5.57$ Hz, N*C*H$_2$CO$_2$), 68.17 (d, $J=5.59$ Hz, C6), 70.42 (C2), 71.70 (C4), 72.93 (d, $J=5.5$ Hz, C5), 74.33 (C3), 182.76 (CO$_2$); ^{31}P NMR (proton undecoupled, D$_2$O, 81.0 MHz) δ 5.42 (dd, $J=6.5$, and 7.4 Hz, CH$_2$OP), 16.94 (dd, $J=10.2$ and 11.8 Hz, CH$_2$P); FAB mass spectrum m/z 414.1 (MH$^+$, $C_9H_{22}O_{13}NP_2$ requires 414.2).

1-Deoxy-1-[[*N*-(phosphonomethyl)-2-oxoethyl]amino]-D-mannitol (11). Glyphosate (52 mg, 0.308 mmol) and D-mannose (75 mg, 0.417 mmol) were mixed in MeOH:H$_2$O (1:1, mL), and the pH of the resulted mixture was adjusted to 6.2 with triethylamine. To this mixture was added sodium cyanoboronhydride (30 mg, 0.5 mmol), and stirred at 80°C until all the glyphosate was consumed (72 h) as determined by monitoring the reaction progress by ^{31}P NMR. During the reaction, the additional portions of D-mannose (75 mg, 0.417 mmol, every 24 h) and sodium cyanoboronhydride (30 mg, 0.5 mmol, every 6 h) were added. The pH of the mixture was kept constant (6.2) by addition of 5% solution of acetic acid whenever it was required. The reaction mixture was evaporated to remove MeOH. The residue was diluted to 50 mL with H$_2$O and purified by passing through AG 1×8 ion-exchange column (1 x 2.8 cm), eluting with 300 mL water to give D-mannitol **13**, followed by the elution with a linear gradient of triethylammonium bicarbonate (1.2 L, 0–0.5M, pH = 7.5). Fractions containing the product were combined and lyophilized. The residue was dissolved in water and passed through a Dowex 50W (K$^+$ form) column to give 112.5 mg (81%) of pure **11** as a potassium salt: ^{1}H NMR (D$_2$O, pD = 11.0, 400 MHz) δ 2.53 (d, 2H, $J=10.8$ Hz, HNCH$_2$P), 2.62 (dd, 1H, $J=13.5$, 7.4 Hz, H-1), 2.84 (dd, 1H, $J=13.5$, 4.2 Hz, H-1′), 3.19 (d, 1H, $J=16.8$ Hz, NC*H*HCO$_2$), 3.25 (d, 1H, $J=16.8$ Hz, NCH*H*CO$_2$), 3.47 (dd, 1H, $J=11.7$, 5.2 Hz, H-6), 3.54–3.58 (m, 2H, H-3, H-5), 3.62 (d, 1H, $J=7.8$ Hz, H-4), 3.64–3.66 (m, 1H, H-2), 3.67 (dd, 1H, $J=11.7$ and 2.4 Hz, H-6′); ^{13}C NMR (D$_2$O, pD = 11.0, 50.3 MHz) δ 56.78 (d, $J=142.0$ Hz, *C*H$_2$P), 61.28 (d, $J=6.3$ Hz, C1), 63.31 (d, $J=5.7$ Hz, *C*H$_2$CO$_2$), 65.86 (C6), 70.92 (C2), 73.69 (C5); ^{31}P NMR (proton decoupled, D$_2$O, pD = 11.0, 81.0 MHz) δ 16.90.

One-step preparation of 4. Barium salt of D-mannose 6-phosphate (50 mg, 0.126 mmol) was dissolved in aqueous solution of AcOH (5%, v/v, 2 mL), and passed through a short column of Dowex 50W (H$^+$ form). The resulted acid solution was lyophilized, and the observed residue was dissolved in a mixture of MeOH:H$_2$O (1:1, mL), to which glyphosate (5 mg, 0.33 mmol) was added and the pH of the solution was adjusted to 6.2 with triethylamine. The mixture was treated with sodium cyanoboronhydride (30 mg, 0.5 mmol), and stirred at 80°C. During the reaction, the pH of the mixture was kept constant (about 6.2) by addition of 5% AcOH whenever it was desired, and the additional portions of cyanoboronhydride (30 mg, 0.5 mmol) were added after every 6 h. The reaction progress was followed by ^{31}P NMR (Fig. 1). After 24 h, the reaction mixture was evaporated to remove MeOH, then diluted to 50 mL with H$_2$O, and applied on a column of AG 1×8 (16×2.8 cm). The product was eluted with a linear gradient of triethylammonium bicarbonate buffer (1.2 L, 0–0.6M, pH = 7.5). Fractions containing the product were combined and lyophilized. The residue was passed through a column of Dowex 50W (K$^+$ form) to give 40.5 mg (60%) of pure **4** as a potassium salt, along with 1.2 mg (3%) of D-mannitol-6-phosphate **15** and 1.5 mg (5.2%) of unreacted D-mannose 6-phosphate. All the spectral data of the compound **4**, isolated with this procedure was identical to that obtained by the procedure in Scheme 2. Data for **15**: ^{1}H NMR (potassium salt in D$_2$O, pD = 10.4, 400 MHz) δ 3.51 (dd, 1H, $J=11.1$, 5.6 Hz, H-1), 3.60–3.66 (m, 3H, H-2, H-3, H-5), 3.71 (dd, 1H, $J=11.1$, 2.6 Hz, H-1′), 3.74 (d, 1H, $J=8.5$ Hz, H-4), 3.83 (dd, 2H, $J=7.5$, 3.4 Hz, 2×H-6); ^{13}C NMR (D$_2$O, 100 MHz) δ 65.79, 67.81, 71.20 (d, $J=11.9$ Hz), 71.91 (d, $J=8.0$ Hz), 72.88, 73.59; ^{31}P NMR (proton decoupled, D$_2$O, 81.0 MHz) δ 3.39.

Synthesis of compound 16. To a solution of Kdo8P (triethylammonium salt, 0.758 g, 1.76 mmol) in water (15 mL) was added the solution of sodium borohydride (0.2 g, 5.29 mmol) in ethanol (3 mL) at 0°C. The reaction was stirred at 0°C and the progress was monitored by thiobarbituric acid assay[13] in which the disappearance of the ketosidic linkage of the starting material (Kdo8P) could be clearly monitored. After 2 h the second portion of the sodium borohydride (0.2 g, 5.29 mmol) was added and the reaction was stirred to the completion (2 h). The cold solution was first acidified to pH 2 with 1 N HCl, then after 20 min at 0°C the pH was adjusted to 7.3 with 1N KOH. The resulted mixture was purified by ion-exchange chromatography, using AG 1×8 (HCO$_3^-$ form), eluting with a linear gradient of triethylammonium bicarbonate buffer (0–0.6 M, pH 7.5). Fractions were analyzed for inorganic phosphate after digestion with HClO$_4$. The active fractions were then combined and concentrated until dry. The residue was dissolved in water, passed through a column of Dowex 50W (K$^+$ form), and concentrated by lyophilization to give the highly purified product **16** (223 mg, 32%) as a mixture of two diastereomers: ^{1}H NMR (potassium salt in D$_2$O, 400 MHz) δ 1.64 (ddd, 1H, $J=14.6$, 7.2, and 1.7 Hz, H-3b′), 1.68 (ddd, 1H, $J=14.6$, 10.6 and 3.0 Hz, H-3b), 1.85 (ddd, 1H, $J=14.6$, 10.3 and 2.2 Hz, H-3a), 2.08 (ddd, 1H, $J=14.6$, 5.3 and 3.0 Hz, H-3a′), 3.55 (dt, 1H, $J=6.8$ and 1.3 Hz, H-5), 3.65–3.74 (m, 1H, H-7), 3.78 (dt, 1H, $J=8.7$ and 1.3 Hz, H-4), 3.79–3.88 (m, 2H, 2H-8), 3.92 (ddd, 1H, $J=6.0$, 2.53 and 1.0 Hz, H-6), 4.07 (dd, 1H, $J=10.3$ and 3.0 Hz, H-2). ^{13}C NMR (D$_2$O, 100 MHz) δ 40.53 (C3), 40.72 (C3′), 68.76, 70.57, 71.30, 71.43, 71.95, 72.76, 73.42, 74.55, 74.81, 183.73 (C1), 184.34 (C1′). ^{31}P NMR (proton decoupled, D$_2$O, 81.0 MHz) δ 3.13; FAB mass spectrum m/z 396.9 (MH$^+$, $C_8H_{15}O_{11}PK_2$ requires 396.2).

Acknowledgements

This work was supported by the United States–Israel Binational Science Foundation (BSF), Jerusalem, Israel (Grant No. 97-00356). Technical support was provided by the Technion Institute of Catalysis Science and Technology. V.B. acknowledges the financial support by the Center of Absorption in Science, Ministry of Immigration Absorption and the Ministry of Science and Arts, State of Israel (Gilady Program). We thank Professor D. Khananashvili from Tel-Aviv University for helpful discussions about slow-binding inhibition.

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