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Synthesis and evaluation of a mechanism-based inhibitor of KDO8P synthase

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Abstract—The enzyme 3-deoxy-D-*manno*-2-octulosonate-8-phosphate (KDO8P) synthase catalyzes the condensation reaction between phosphoenolpyruvate (PEP) and D-arabinose 5-phosphate (A5P) to produce KDO8P and inorganic phosphate. In attempts to investigate the lack of antibacterial activity of the most potent inhibitor of KDO8P synthase, the amino phosphonophosphate **3**, we have synthesized its hydrolytically stable isosteric phosphonate analogue **4** and tested it as an inhibitor of the enzyme. The synthesis of **4** was accomplished in a one step procedure by employing the direct reductive amination in aqueous media between unprotected sugar phosphonate and glyphosate. The analogue **4** proved to be a competitive inhibitor of KDO8P synthase with respect to both substrates A5P and PEP binding. In vitro antibacterial tests against a series of different Gram-negative organisms establish that both inhibitors (**3** and **4**) lack antibacterial activity probably due to their reduced ability to penetrate the bacterial cell membrane.

Keywords: KDO8P synthase; Lipopolysaccharide; Enzyme inhibitors; Isosteric phosphonate; Antibacterial drug

1. Introduction

The rapid spread of antibiotic resistance in Gram-negative bacteria has prompted a continuing search for new agents against this important class of bacterial pathogens.[1] Because the biosynthesis of lipopolysaccharide is unique to Gram-negative bacteria[2] and required by them for growth and virulence,[3] the site-specific sugar found almost exclusively in all bacterial lipopolysaccharides, 3-deoxy-D-*manno*-2-octulosonic acid (KDO), has been identified as a target and most efforts to design synthetic inhibitors have been aimed towards inhibiting this sugar's biosynthesis and processing.[4] Indeed, some of the potent inhibitors of CMP-KDO synthetase have shown in vivo antibacterial activity.[5] 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, which is a key enzyme that controls the carbon flow in the biosynthetic formation of KDO.[4] The enzyme catalyzes the unusual aldol-type condensation of phosphoenolpyruvate (PEP) with D-arabinose 5-phosphate (A5P) to produce KDO8P and inorganic phosphate (P_i, Scheme 1).[6]

The mechanism of this reaction is still uncertain. Most recent biochemical[7] and structural[8] studies suggest a reaction pathway involving the formation of an *acyclic* bisphosphate intermediate **2**, however to date there is no direct evidence for the existence of **2** as a true enzymatic intermediate. Recently, the first indirect support to the concept of this mechanism was provided. The stable analogue of the putative oxocarbenium **1**, the amino

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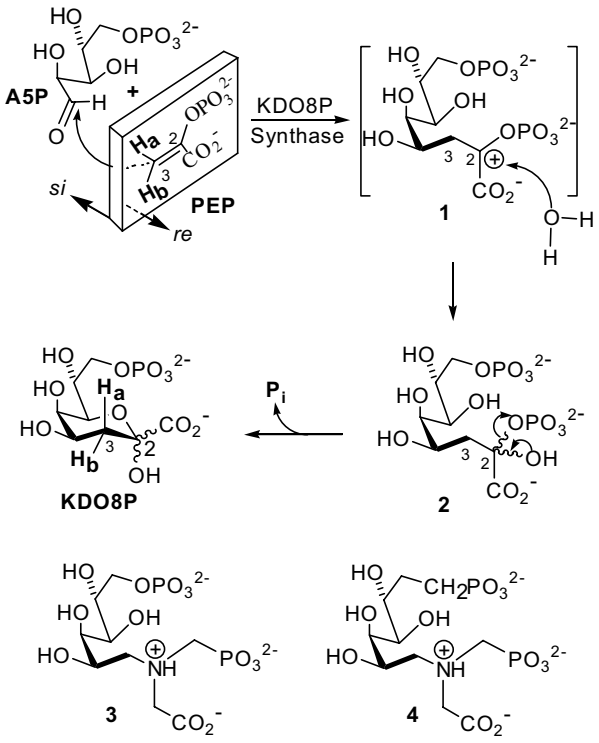
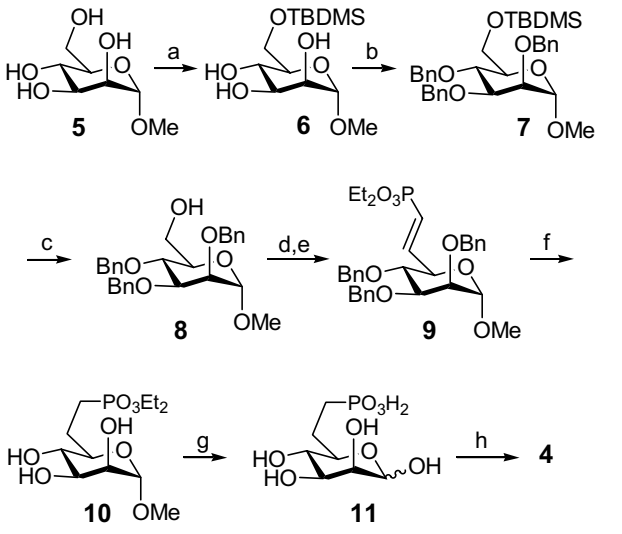


Scheme 1. Proposed mechanism for KDO8P-synthase-catalyzed reaction.



Scheme 2. Reagents and conditions: (a) TBDMSCl, pyridine, DMAP, rt; (b) BnBr, NaH, DMF, rt; (c) 1 M H_2SO_4 (cat.), MeOH, rt; (d) PCC, CH_2Cl_2, rt; (e) $CH_2(PO_3Et_2)_2$, *n*-BuLi, THF, −78 °C; (f) H_2, Pd/C, EtOH, rt; (g) i: $(Me)_3SiBr$, CH_2Cl_2, rt, ii: Dowex H^+, H_2O, reflux; (h) glyphosate, $NaBH_3CN$, MeOH/H_2O, pH 6.2, 80 °C.

phosphonophosphate **3**, was synthesized and tested as inhibitor of the synthase.[9a] Compound **3** was found to be a potent mechanism-based inhibitor of KDO8P synthase,[9b] but failed to inhibit bacterial growth.[10] This lack of antibacterial activity was suspected to be the result of either the inability of the inhibitor **3** to cross the cytoplasmic membrane,[5] or the hydrolysis of its crucial C-6 phosphate monoester by various phosphatases.[11] While both of these reasons could explain why **3** does not have antibacterial activity, we decided first to focus on the hydrolysis issue, which is indeed a major drawback with small molecule drugs containing phosphate monoesters.[12] For this purpose, we designed the amino bisphosphonate **4**, which is an isosteric phosphonate analogue of **3** and should be stable towards the action of bacterial phosphatases. In this paper we describe the synthesis of the analogue **4** and its evaluation as inhibitor of KDO8P synthase. The examination of **4** against a series of different Gram-negative bacteria is also reported.

2. Results and discussion

2.1. Preparation of 4

Our approach in this search for new inhibitors of KDO8P synthase was to develop synthetic strategy that avoids lengthy protection/deprotection steps. Both analogues **3** and **4** are similar and their structures can be divided in two parts: sugar part and *N*-phosphono methyl glycine (glyphosate) part. Therefore, it was assumed that the direct reductive amination in aqueous media between unprotected sugar part (with a free reducing end) and the commercial glyphosate would yield the desired structures. Indeed, using this strategy, we previously reported on a very efficient, one-step synthesis of **3**[9b] and its various ^{13}C and ^{15}N-labeled analogues, which were successfully used for structure–function investigation of the KDO8P synthase.[8b,13] A same approach led us to synthesize the target amino bisphosphonate **4** in a one-step procedure by directly coupling the isosteric phosphonate analogue of mannose-6-phosphate (compound **11**) with glyphosate. The overall synthetic pathway to **4**, as illustrated in Scheme 2, starts from the commercial methyl α-D-mannopyranoside **5** and includes eight chemical steps with an overall yield of 16%.

Treatment of **5** with *tert*-butyldimethylsilyl chloride in pyridine, in the presence of 4-DMAP as a catalyst, selectively protected primary hydroxyl to afford the 6-silyloxy mannoside **6** in 85% isolated yield. Benzylation of **6** with benzyl bromide (BnBr) yielded fully protected derivative **7** (76%), which was desilylated under mild acidic condition (catalytic amount of 1 M H_2SO_4 in MeOH) to give the primary alcohol **8** (97%).

The introduction of the phosphonomethylene moiety at the side chain of the mannose derivative **8** was accomplished in three chemical steps. The primary alcohol **8** was oxidized with pyridinium chlorochromate (PCC) to afford the corresponding aldehyde (65% isolated

yield), which was found to be stable and could be isolated in pure form by column chromatography on silica gel. Treatment of this aldehyde with tetraethyl methylenediphosphonate ylide by Wittig–Horner reaction afforded the vinyl phosphonate **9** as an *E* isomer in 83% yield.[14] Hydrogenolysis (H_2, Pd/C in MeOH) of **9** allowed the reduction of the double bond and the simultaneous removal of the benzyl groups to afford the phosphonate **10** in almost quantitative yield.

Treatment of **10** with trimethylsilyl bromide was followed by treatment with Dowex (H^+) cation exchange resin to afford the completely deprotected phosphonate **11** as a mixture of anomers in 88% yield.[15] The direct reductive amination between **11** and glyphosate [$NaBH_3CN$ in MeOH/H_2O (1:1), pH 6.2, 80 °C][9b] was monitored by ^{31}P NMR. The product was purified on AG 1x8 ion-exchange column (triethylammonium bicarbonate buffer, pH 7.5, 0–0.5 M linear gradient), followed by treatment with Dowex (K^+) cation exchange column to afford the target amino bisphosphonate **4** as a potassium salt in 53% yield. The spectral characterization of purified **4** using 2D-COSY, ^1H, ^{13}C, ^{31}P NMR, and mass spectra were all consistent with this structure.

2.2. Inhibition of KDO8P synthase with 4

The analogue **4** was evaluated as an inhibitor of homogeneous KDO8P synthase by using a well established procedure reported earlier.[9] 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 the enzyme. The kinetic measurements by this procedure assume 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 $50 \pm 5\,\mu M$ (Fig. 1a). Since the structure **4** combines the key features of both substrates, A5P and PEP, into a single molecule, it was expected that **4** should be competitive inhibitor with respect to both PEP and A5P. Examination of **4** as inhibitor against A5P binding (Fig. 1b) reveals that it serves as a competitive inhibitor with apparent inhibition constant of $50 \pm 8\,\mu M$.

Comparison of the K_i value of **4** against PEP binding (50 μM) with that of the amino phosphonophosphate **3** (3.3 μM) determined under the same experimental conditions,[9a] reveals that **4** is more than 15-fold weaker inhibitor than **3**. A similar preference in binding of phosphate esters against their phosphonate mimics is precedent.[16] In general, enzymes bind isosteric phosphonate analogues several times less tightly than the naturally occurring phosphates.[17] This has been attributed to several contributing factors: small differences in bonding geometries; steric interactions with the methylene hydrogens; deletion of a hydrogen bond to the phosphate oxygen; and incomplete ionization of phosphonate diacids. Each of these factors could plausibly explain the observed reduced affinity of **4** to that of **3**. Nevertheless, both of these inhibitors were further subjected to in vitro antibacterial tests against a series of different Gram-negative bacteria.

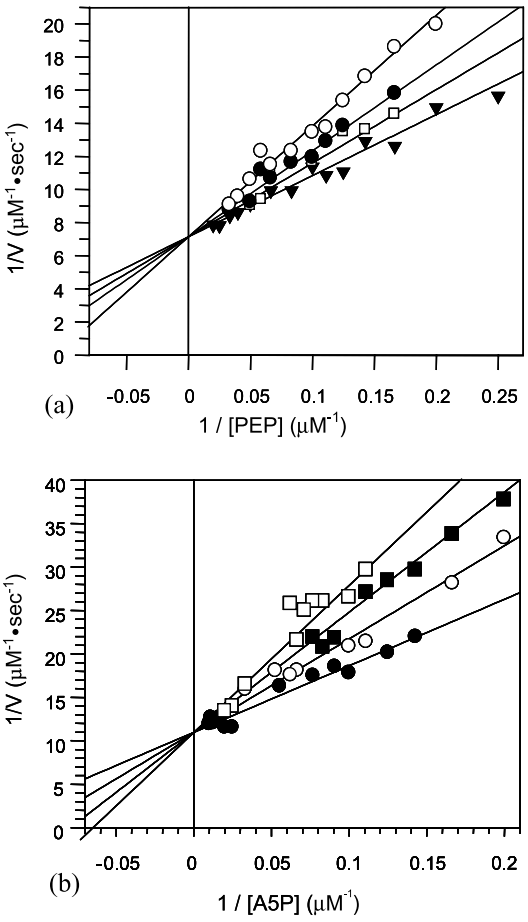


Figure 1. Inhibition of KDO8P synthase by the analogue **4**. Double-reciprocal plots of initial velocities are given as a function of (a) PEP, when the A5P concentration was 500 μM, and the inhibitor concentrations were none (▼), 10 μM (□), 20 μM (●) and 40 μM (○); (b) A5P, when the PEP concentration was 200 μM, and the inhibitor concentrations were none (●), 20 μM (○), 40 μM (■), and 60 μM (□). The assays were carried out at 37 °C, pH 7.3 in a reaction buffer consisting 0.1 M Tris/HCl, BSA (0.1 mg/mL), PEP, A5P, inhibitor **4** and ~30 mU of homogeneous KDO8P synthase in a final volume of 1 mL. All samples were assayed in triplicate and analogous results were obtained in two to four different experiments.

2.3. Antibacterial activity test

In vitro antibacterial activities of **3** and **4** were evaluated by using disc diffusion tests[18] on wild-type *Escherichia coli* strain K12, and on various *E. coli* strains mutated in

the *lpxA* and *lpxC* genes including: SM101,[19a] R477-10,[19b] and D22.[19c] These mutant strains are defective in lipid A biosynthesis and therefore show increased sensitivity to a range of antibiotics. Clinically important antibiotics, ampicillin, and kanamycin, were used for the comparison and for the control. Unfortunately, no detectable inhibition was measured with both **3** and **4** even at concentrations >1 mg per disc, while a considerable large zone of inhibitions was observed with both ampicillin and kanamycin even at concentrations <10 μg per disc. Therefore, we conclude that the problem associated with the lack of antibacterial activity of these inhibitors (**3** and **4**) lies in their low permeability through the bacterial cell membrane, and not due to the hydrolysis of the crucial C-6 phosphate monoester of **3** as it would be anticipated before this work. This observation indicates that in order to redesign these inhibitors (as well as other future analogues of the putative intermediate **2**) as potential antibacterials, their impermeability through the bacterial cell membrane should be solved. Indeed, in an attempt to circumvent this barrier, several transport devices in which the inhibitor to be transported is attached to L-amino acid,[20a] to a small peptide,[5] or is not peptide-linked,[11,12,20b] have been reported. At this stage, each of these devices might serve as a possible candidate to overcome the impermeability problem of KDO8P synthase inhibitors. Solution of this problem should be one of the most important issues for the design of new antibacterial drugs that target the KDO biosynthesis.

In summary, we have synthesized an isosteric phosphonate analogue of the most potent inhibitor of KDO8P synthase and tested it as an inhibitor of the enzyme. The new analogue binds to the enzyme with an affinity lower than the phosphate inhibitor, and in vitro antibacterial tests establish that both the inhibitors lack antibacterial activity probably due to their reduced ability to penetrate the bacterial cell membrane. The synthesis of isosteric phosphonate analogues conjugated to various transport systems is now being considered.

3. Experimental

3.1. General

The homogeneous KDO8P synthase (specific catalytic activity of 16 U/mg) was isolated from the overproducing strain *E. coli* BL21 (DE3), following the procedure of Ray[6] with some modifications.[16b] A5P was prepared enzymatically according to the procedure of Whitesides and co-workers.[21] All other chemicals were received from Aldrich or from Sigma and used without further purification, unless noted. In all the synthetic work, reactions were performed under argon atmosphere unless otherwise noted. All solvents used in the synthetic work were anhydrous and received from Aldrich or Sigma, unless noted. THF was dried over sodium and freshly distilled in the presence of benzophenone prior to use.[22] Flash column chromatography[23] was performed on Silica Gel 60 (70–230 mesh). Reactions were monitored by TLC on Silica Gel 60 F254 (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 solution[24] was used for the detection of the compounds containing phosphate monoesters.

3.2. 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-400 and Bruker Avance-500 spectrometers, and chemical shifts reported (in ppm) are 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-400 (100.6 MHz) and Bruker Avance-500 (125.8 MHz) spectrometers, and the chemical shifts reported (in ppm) are relative to the external sodium 2,2-dimethyl-2-silapentane sulfonate ($\delta = 0.0$) in D_2O. ^{31}P NMR spectra were recorded on a Bruker AC-200 spectrometer at 81.0 MHz and the chemical shifts reported (in ppm) are relative to external orthophosphoric acid ($\delta = 0.0$) in D_2O. All the coupling constants (J) are in Hz. Mass spectra were obtained on a TSQ-70B mass spectrometer (Finnigan Mat) by negative chemical ionization (NCI) and by fast-atom bombardment (FAB) in glycerol matrices or on a Bruker Daltomics Apex-III using the method of electrospray ionization (ESI).

3.3. Inhibition study

3.3.1. Enzyme assays. Unless otherwise stated, the enzyme activity was assayed in a 1.0 mL reaction buffer consisting of 0.1 M Tris/HCl (pH 7.3), A5P (0.5 mM, >50K_m), PEP (0.5 mM, >50K_m), and BSA (0.1 mg/mL). All solutions except enzyme were filtered through Millipore type-HA filters (0.45 μm) before use. Following equilibration at 37 °C for 2 min, KDO8P synthase (20 μL, at a final concentration of approximately 10 nM) was added. 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[25] is based on the absorbance difference at 232 nm between PEP ($\varepsilon = 2840\ M^{-1}{\cdot}cm^{-1}$) and the other substrates and products ($\varepsilon < 60\ M^{-1}{\cdot}cm^{-1}$) under the assay conditions. The concentrations of PEP and A5P were determined precisely by quantitative assaying of the P_i released by alkaline phosphatase.[26] 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. The protein concentration of the enzyme fractions was determined using the Bio-Rad protein assay with bovine serum albumin as a standard. During the enzyme purification, the enzyme activity was assayed by thiobarbituric acid assay method.[6]

3.3.2. Enzyme inhibition study. In order to determine the K_i value of inhibitor **4** against PEP binding, the reaction solutions were prepared in 1.0 mL of a reaction buffer consisting of 0.1 M Tris/HCl (pH 7.3), BSA (0.1 mg/mL), the constant saturating concentration of A5P (0.5 mM $>50K_m$) and variable concentrations of PEP (4–50 μM). Four inhibitor concentrations were examined (0, 10, 20, 40 μM) and for each inhibitor concentration variable concentrations of PEP (4–50 μM) were used. Following equilibration at 37 °C for 2 min, KDO8P synthase (20 μL) was added. The rate measurements were made as described above, while a 5 s delay was allowed following initiation of the reaction. The initial rate was then determined by a least squares fitting of the first 10% of the progress curve (between 20 and 200 s, depending on the initial concentration of PEP) to a straight line. All samples were assayed in triplicate and analogous results were obtained in two 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 GraFit5 program. The K_i value was calculated either from the above treatment, or from the secondary replots of the slopes from initial double-reciprocal plots ($1/V$ vs $1/[S]$) versus inhibitor concentration[27] and found to be 50 ± 5 μM against PEP (Fig. 1a).

The determination of K_i value of inhibitor **4** against A5P binding was carried out in a similar manner, while the constant saturating concentration of PEP (0.5 mM, $>50K_m$) and variable concentrations of A5P (5–100 μM) were used. Four inhibitor **4** concentrations were examined (0, 20, 40, 60 μM) and for each inhibitor concentration, variable concentrations of A5P (5–100 μM) were used. The rate measurements were made as described above. From received data, the K_i value was calculated as described above and found to be 50 ± 8 μM against A5P (Fig. 1b).

3.3.3. In vitro antibacterial tests. The Gram-negative bacterial strains used in this study were wild-type *E. coli* K12 and its mutants: SM101,[19a] R477-10,[19b] and D22,[19c] which are known as holding increased sensitivity to a range of antibiotics. The test strains were grown to A_{600} 0.2–0.4 at 37 °C in LB broth. A volume of 100 μL culture of each strain was plated on LB agar plate using top agarose. The 6 mm discs (Schleicher & Schuell, Germany) were saturated with 500 and 1000 μg of either inhibitor **3** or **4** dissolved in water and after 10 min drying at room temperature the discs were placed on the freshly plated cells and incubated overnight at 37 °C. The control experiments with ampicillin and kanamycin were prepared in a same manner, but only 10–30 μg of each antibiotic were used. The diameter of growth inhibition was detected as a zone clearing around each disc after overnight incubation.

3.4. Synthetic procedures

3.4.1. Methyl 6-*O*-*tert*-butyldimethylsilyl-α-D-mannopyranoside (6). To a solution of compound **5** (10 g, 51.5 mmol) in 20 mL pyridine were added TBDMSCl (8.53 g, 56.65 mmol), and catalytic amount of 4-di methylaminopyridine at 0 °C. The reaction mixture was stirred at room temperature and the reaction progress was monitored by TLC (MeOH–$CHCl_3$, 9:1). After completion, reaction mixture was diluted with EtOAc, and then washed with 3% H_2SO_4, saturated $NaHCO_3$, water, and saturated NaCl. The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The crude material was purified by flash chromatography to give **6** as a white solid (13.42 g, 85%). ^1H NMR ($CDCl_3$, 500 MHz): δ 0.07 (s, 6H, OSi(CH_3)$_2$*t*-Bu), 0.87 (s, 9H, OSiC(CH_3)$_3$Me$_2$), 3.32 (s, 3H, OCH_3), 3.52 (ddd, 1H, $J_{5,6}$ 5.5 Hz, $J_{5,6'}$ 4.5 Hz, $J_{4,5}$ 9.5 Hz, H-5), 3.66 (t, 1H, $J_{3,4} = J_{4,5} = 9.5$ Hz, H-4), 3.75 (dd, 1H, $J_{3,4}$ 9.5 Hz, $J_{2,3}$ 3.5 Hz, H-3), 3.80 (dd, 1H, $J_{6,6'}$ 11 Hz, $J_{5,6}$ 5.5 Hz, H-6), 3.87 (dd, 1H, $J_{1,2}$ 1.5 Hz, $J_{2,3}$ 3.5 Hz, H-2), 3.89 (d, 1H, $J_{1,2}$ 1.5 Hz, H-1). ^{13}C NMR ($CDCl_3$, 125.8 MHz): δ 4.7 (OSi(CH_3)$_2$*t*-Bu), 19.0 (OSiC(CH$_3$)$_3$Me$_2$), 25.8 (OSiC(CH_3)$_3$Me$_2$), 54.7 (OMe), 64.3 (C-6), 69.5 (C-4), 70.4 (C-2), 71.7 (C-5), 72.0 (C-3), 100.7 (C-1); CIMS m/z 309.1 (MH^+, $C_{13}H_{28}O_6Si$ requires 308.4).

3.4.2. Methyl 2,3,4-tri-*O*-benzyl-6-*O*-*tert*-butyldimethylsilyl-α-D-mannopyranoside (7). To a solution of compound **6** (10.12 g, 32.8 mmol) in DMF (20 mL) was added NaH (5.67 g, 49.2 mmol). The reaction mixture was stirred at room temperature for 1 h. BnBr (14.04 mL, 39.36 mmol) was added to this mixture at 0 °C and the reaction progress was monitored by TLC with two solvent systems: 100% EtOAc and EtOAc–hexane (1:9). After completion, the reaction mixture was diluted with EtOAc and then washed with water and saturated NaCl solution. The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The crude material was purified by flash chromatography to give **7** (14.37 g, 76%). ^1H NMR ($CDCl_3$, 500 MHz): δ 0.04, 0.05 (2s, 2×3H, OSi(CH_3)$_2$*t*-Bu), 0.86 (s, 9H, OSiC(CH_3)$_3$Me$_2$), 3.28 (s, 3H, OCH_3), 3.55 (m, 1H, H-5), 3.74 (t, 1H, $J_{1,2} = J_{2,3} = 2$ Hz, H-2), 3.79–3.84 (m, 2H, H-6, and H-3), 3.86–3.90 (m, 2H, H-6′, and

H-4), 4.60 (d, 1H, *J* 11 Hz, C′*H*HPh), 4.61 (s, CH_2Ph), 4.65 (d, 1H, *J* 12.5 Hz, C″*H*HPh), 4.70 (d, 1H, $J_{1,2}$ 2 Hz, H-1), 4.72 (d, 1H, *J* 12.5 Hz, C″*H*HPh), 4.90 (d, 1H, *J* 11 Hz, C′*H*HPh), 7.24–7.34 (m, 15H, $3C_6H_5$). ^{13}C NMR ($CDCl_3$, 125.8 MHz): δ −5.3, −5.2 (2×s, OSi(CH_3)$_2$*t*-Bu), 25.9 (OSi*C*(CH_3)$_3$Me$_2$), 54.4 (OMe), 62.8 (C-6), 72.1, 72.5, 75.0 (3×s, 3×*C*H_2Ph), 73.1 (C-2), 74.9 (C-3), 77.2 (C-4), 80.2 (C-5), 98.6 (C-1), 127.4–128.3 (m, 15×$C_{arom.}$), 138.5, 138.7, 138.8 (3×s, 3×$C_{arom.}$). CIMS *m*/*z* 579.4 (MH^+, $C_{34}H_{46}O_6Si$ requires 578.8).

3.4.3. Methyl 2,3,4-tri-*O*-benzyl-α-D-mannopyranoside (8). To a stirred solution of compound **7** (5.51 g, 9.52 mmol) in 15 mL of MeOH was added catalytic amount of 1 M solution of H_2SO_4 in MeOH at 0 °C. The reaction mixture was stirred at room temperature and the reaction progress was monitored by TLC using two solvent systems: EtOAc–hexane (1:9) and EtOAc–hexane (1:1). After completion, the reaction mixture was diluted with EtOAc, and then washed with saturated $NaHCO_3$, water, and saturated NaCl. The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The crude material was purified by flash chromatography to give **8** (4.29 g, 97%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.31 (s, 3H, OCH_3), 3.64 (ddd, 1H, $J_{5,6}$ 4 Hz, $J_{5,6'}$ 3 Hz, $J_{4,5}$ 9.5 Hz, H-5), 3.79 (dd, 1H, $J_{6,6'}$ 12 Hz, $J_{5,6}$ 4 Hz, H-6), 3.82 (m, 1H, $J_{2,3}$ 3 Hz, H-2), 3.87 (dd, 1H, $J_{6,6'}$ 12 Hz, $J_{5,6'}$ 3 Hz, H-6′), 3.93 (dd, 1H, $J_{3,4}$ 9.5 Hz, $J_{2,3}$ 3 Hz, H-3) 3.40 (t, 1H, $J_{3,4} = J_{4,5} = 9.5$ Hz, H-4), 4.65 (s, CH_2Ph), 4.68 (d, 1H, *J* 11 Hz, C′*H*HPh), 4.71 (d, 1H, *J* = 12.5 Hz, C″*H*HPh), 4.74 (s, H-1), 4.79 (d, 1H, *J* 12.5 Hz, C″H*H*Ph), 4.96 (d, 1H, *J* 11 Hz, C′*H*HPh), 7.18–7.38 (m, 15H, $3C_6H_5$). ^{13}C NMR ($CDCl_3$, 125.8 MHz): δ 54.8 (OMe), 62.3 (C-6), 69.5 (C-5), 72.2 (*C*H_2Ph), 73.0 (*C*″H_2Ph), 74.2 (C-2), 74.9 (C-4), 75.6 (C′H_2Ph), 80.2 (C-3), 99.3 (C-1), 127.6–128.4 (m, 15×$CH_{arom.}$), 138.5, 138.8, 138.9 (3×s, 3×$C_{arom.}$). CIMS *m*/*z* 465.0 (MH^+, $C_{28}H_{32}O_6$ requires 464.5).

3.4.4. Methyl 2,3,4-tri-*O*-benzyl-6-deoxy-6-diethoxyphosphinylmethylene-α-D-mannopyranoside (9). Solution of compound **8** (1.06 g, 2.28 mmol) in dichloromethane (5 mL) was added to the mixture of PCC (2.0 g, 4.67 mmol), and activated molecular sieves (5 g) in dichloromethane (20 mL) at 0 °C. The reaction mixture was stirred at room temperature and the reaction progress was monitored by TLC EtOAc–CH_2Cl_2 (1:9). After completion, the reaction crude was passed through Celite, and the solvent was removed under reduced pressure. The crude material was purified by flash chromatography to give the corresponding aldehyde (0.68 g, 65%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.36 (s, 3H, OCH_3), 3.74 (t, 1H, $J_{1,2} = J_{2,3} = 2.8$ Hz, H-2), 3.92 (dd, 1H, $J_{2,3}$ 2.8 Hz, $J_{3,4}$ 7.7 Hz, H-3), 4.03 (dd, 1H, $J_{4,5}$ 8.8 Hz, $J_{3,4}$ 7.7 Hz, H-4), 4.07 (dd, 1H, $J_{4,5}$ 8.8 Hz, $J_{5,CHO}$ 0.47 Hz, H-5), 4.56–4.69 (m, 3CH_2Ph), 4.83 (d, 1H, $J_{1,2}$ 2.8 Hz, H-1), 7.20–7.41 (m, 15H, $3C_6H_5$), 9.71 (d, 1H, $J_{5,CHO}$ 0.47 Hz, CHO). ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 55.5 (OMe), 72.4 (m, 2×$CH_3C$$H_2$OP), 73.0 (s, $CH_3C$$H_2$OP), 74.1 (C-2), 74.6 (C-4), 76.2 (C-5), 79.2 (C-3), 99.6 (C-1), 127.6–128.5 (m, 15×$CH_{arom.}$), 137.0, 138.7, 139.0 (3×s, 3× $C_{arom.}$), 197.8 (CHO). FABMS *m*/*z* 463.1 (MH^+, $C_{28}H_{30}O_6$ requires 462.5). To a solution of tetramethyl methylenediphosphonate (0.64 g, 2.21 mmol) in THF (5 mL) at −78 °C was added *n*-butyllithium (0.28 mL of 1.6 M solution in hexane). To this mixture was added a solution of the above aldehyde (0.68 g, 1.47 mmol) in THF (5 mL) and stirring was continued for about 30 min at −78 °C. The mixture was allowed to warm to 0 °C and the reaction progress was monitored by TLC EtOAc–CH_2Cl_2 (1:9). The reaction was quenched by the addition of a few drops of AcOH solution in THF. The reaction crude was diluted with EtOAc, and then washed with saturated $NaHCO_3$, water, and saturated NaCl. The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The crude material was purified by flash chromatography to give **9** (0.73 g, 83%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.29 (t, 6H, *J* 7.2 Hz, 2×C$H_3$$CH_2$OP), 3.27 (s, 3H, OCH_3), 3.70 (t, 1H, $J_{3,4} = J_{4,5} = 9.3$ Hz, H-4), 3.77 (dd, 1 H, $J_{1,2}$ 1.7 Hz, $J_{2,3}$ 3.0 Hz, H-2), 3.88 (dd, 1H, $J_{2,3}$ 3.0 Hz, $J_{3,4}$ 9.3 Hz, H-3), 4.01–4.17 (m, 5H, 2×CH_3CH_2OP, and H-5), 4.56 (d, 1H, *J* 10.6 Hz, C*H*HPh), 4.59 (d, 1H, *J* 10.6 Hz, C′*H*HPh), 4.63 (d, 1H, *J* 10.6 Hz, C′H*H*Ph), 4.68 (d, 1H, *J* 12.4 Hz, C″*H*HPh), 4.71 (d, 1H, $J_{1,2}$ 1.7 Hz, H-1), 4.75 (d, 1H, *J* 12.4 Hz, C″H*H*Ph), 4.87 (d, 1H, *J* 10.6 Hz, CH*H*Ph), 6.11 (ddd, 1H, $J_{5,7}$ 1.8 Hz, $J_{6,7}$ 17.2 Hz, $J_{7,P}$ 21.3 Hz, H-7), 6.94 (ddd, 1H, $J_{5,6}$ 4.3 Hz, $J_{6,7}$ 17.2 Hz, $J_{6,P}$ 22.4 Hz, H-6), 7.23–7.35 (m, 15H, $3C_6H_5$). ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 16.3 (s, *C*$H_3$$CH_2$OP), 16.7 (s, *C*′$H_3$$CH_2$OP), 55.0 (s, OMe), 61.8 (m, 2×$CH_3C$$H_2$OP), 71.2 (d, $J_{5,P}$ 21.4, C-5), 72.3 (s, *C*′H_2Ph), 72.9 (s, *C*″H_2Ph), 74.8 (C-2), 75.3 (*C*H_2Ph), 78.3 (C-4), 80.1 (C-3), 99.3 (C-1), 118.1 (d, $J_{7,P}$ 188.8 Hz, C-7), 127.6–128.4 (m, 15×$CH_{arom.}$), 148.0, 148.4, 148.7 (3×s, 3×$C_{arom.}$), 148.9 (d, $J_{6,P}$ 5.94, C-6). ^{31}P NMR ($CDCl_3$, 81.0 MHz): δ 16.4. CIMS *m*/*z* 597.3 (MH^+, $C_{33}H_{41}O_8P$ requires 596.6).

3.4.5. Methyl 6-deoxy-6-diethoxyphosphinylmethyl-α-D-mannopyranoside (10). A mixture of compound **9** (0.62 g, 1.05 mmol), and Pd/C (178 mg) in EtOH (10 mL) was stirred under H_2 (1 atm) and the reaction progress was monitored by TLC using two solvent systems: EtOAc–hexane (7:3) and MeOH–$CHCl_3$ (1:4). After completion, the catalyst was removed by filtration through Celite, and filtrate was concentrated under reduced pressure. The crude material was purified by flash chromatography to give **10** in (0.34 g, 100%): ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.30 (t, 6H, *J* 7.1 Hz, 2 × C$H_3$$CH_2$OP), 1.71–1.90 (m, 2H, H-6, and H-7), 2.0–2.2 (m, 2H, H-6′, and H-7′), 2.25 (s, 1H, OH), 3.30 (s, 3H, OCH_3), 3.47

(td, 1H, $J_{5,6} = J_{4,5} = 9.4$ Hz, $J_{5,6'} = 2.4$ Hz, H-5), 3.56 (t, 1H, $J_{3,4} = J_{4,5} = 9.4$ Hz, H-4), 3.71 (dd, 1H, $J_{2,3}$ 2.9 Hz, $J_{3,4}$ 9.4 Hz, H-3), 3.89 (dd, 1H, $J_{2,3}$ 2.9 Hz, $J_{1,2}$ 1.1 Hz, H-2), 4.01–4.11 (m, 4H, 2×CH_3CH$_2$OP), 4.41 (s, 1H, OH), 4.55 (s, 1H, OH), 4.64 (d, 1H, $J_{1,2}$ 1.1 Hz, H-1). ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 16.1 (*C*H$_3$-CH$_2$OP), 16.6 (*C*′H$_3$CH$_2$OP), 20.7 (d, $J_{7,P}$ 140.8 Hz, C-7), 24.1 (C-6), 54.9 (OMe), 61.3 (d, $J_{C,P}$ 5.9 Hz, 2 × CH$_3$*C*H$_2$OP), 62.0 (d, $J_{C,P}$ 6.02 Hz, 2×CH$_3$*C*′H$_2$OP), 70.2 (C-2), 70.7 (C-4), 71.2 (d, $J_{5,P} = 15.05$, C-5), 71.8 (C-3), 100.9 (C-1). ^{31}P NMR ($CDCl_3$, 81.0 MHz): δ 31.8. CIMS m/z 329.0 (MH^+, $C_{12}H_{25}O_8P$ requires 328.2).

3.4.6. 6-Deoxy-6-dihydroxyphosphinylmethyl-α-D-mannopyranoside potassium salt (11). To a solution of compound **10** (0.32 g, 0.97 mmol) in 12 mL of dichloromethane was added trimethylsilyl bromide (3.30 mL, 25.21 mmol). The reaction mixture was stirred at room temperature and the reaction progress was monitored by TLC MeOH–$CHCl_3$ (1:4). After completion, the solvent was then removed under reduced pressure. The residue was diluted with 15 mL of H_2O and stirred for 30 min. Then Dowex H^+ was added and the solution was heated at reflux for 12 h. The Dowex H^+ was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was diluted with H_2O, applied to AG 1×8 ion-exchange column (16×2.8 cm) and eluted with H_2O to elute inorganic salt and then with a linear gradient of triethylammonium bicarbonate (1.2 L, 0–0.5 M, pH 7.5). Fractions containing the product was combined and lyophilized. The residue was passed through a Dowex (K^+) column to give anomeric mixture of **11** (0.85 g, 88%): ^{1}H NMR (D_2O, 400 MHz): δ 1.21–1.35, 1.45–1.65, 1.85–2.02 (m, 8H, H-6a, H-6′a, H-6b, H-6′b, H-7a, H-7′a, H-7b, H-7′b), 3.19 (td, 1H, $J_{4b,5b} = J_{5b,6'b} = 9.6$ Hz, $J_{5b,6b}$ 2.5 Hz, H-5b), 3.38 (t, 1H, $J_{4b,5b} = J_{3b,4b} = 9.6$ Hz, H-4b), 3.46 (t, 1H, $J_{4a,5a} = J_{3a,4a} = 9.7$ Hz, H-4a), 3.52 (dd, 1H, $J_{3b,4b}$ 9.6 Hz, $J_{2b,3b}$ 3.3 Hz, H-3b), 3.64 (td, 1H, $J_{5a,4a} = J_{5a,6'a} = 9.7$ Hz, $J_{5a,6a}$ 2.6 Hz, H-5a), 3.71 (dd, 1H, $J_{4a,3a}$ 9.7 Hz, $J_{2a,3a}$ 3.4 Hz, H-3a), 3.83 (dd, 1H, $J_{2a,3a}$ 3.4 Hz, $J_{1a,2a}$ 1.4 Hz, H-2a), 3.84 (d, 1H, $J_{2b,3b}$ 3.3 Hz, H-2b), 4.77 (s, 1H, H-1b), 5.04 (d, 1H, $J_{1a,2a}$ 1.4 Hz, H-1a). ^{13}C NMR (D_2O, 100.6 MHz): δ 70.4 (C-4b), 71.0 (C-4a), 71.6 (C-3a), 72.4 (C-2a), 72.9 (C-2b), 73.6 (d, $J_{5a,P}$ 15.9 Hz, C-5a), 74.4 (C-3b), 77.5 (d, $J_{5b,P}$ 15.9 Hz, C-5b), 94.1 (C-1b), 94.9 (C-1a). ^{31}P NMR (D_2O, 81.0 MHz): δ 22.8. ESIMS m/z 335.2 (MH^+, $C_7H_{13}O_8PK_2$ requires 334.4).

3.4.7. Synthesis of the target aminobisphosphonate (4). To a solution of the potassium salt of compound **11** (100 mg, 0.3 mmol) and glyphosate (101 mg, 0.6 mmol) in 6 mL of MeOH–H_2O (1:1) was added triethylamine to adjust the pH of the solution to about 6.2. Then the reaction mixture was treated with sodium cyanoborohydride (103.5 mg, 1.6 mmol) and stirred at 80 °C. The pH of the mixture was kept constant (pH 6.2) by addition of 5% solution of acetic acid whenever the pH was higher than that. The reaction progress was monitored by ^{31}P NMR and after the completion, the solvent was removed under reduced pressure. The residue was diluted to 50 mL H_2O, applied to AG 1×8 ion-exchange column (16×2.8 cm), eluted with H_2O to elute inorganic salt and by-products and then with a linear gradient of triethylammonium bicarbonate (1.2 L, 0–0.5 M, pH 7.5). Fractions containing the product were combined and then lyophilized. The residue was passed through a Dowex (K^+) column to give pure **4** as a potassium salt (89.5 mg, 53%). ^{1}H NMR (D_2O, 500 MHz): δ 1.45–1.53 (m, 2H, H-6′, and H-7′), 1.65–1.74 (m, 1H, H-7), 1.89–1.97 (m, 1H, H-6), 3.09–3.19 (m, 2H, NCH_2PO$_3^{2-}$), 3.39 (dd, 1H, $J_{1,1'}$ 13 Hz, $J_{1',2}$ 10.5 Hz, H-1′), 3.52–3.58 (m, 2H, H-4, and H-5), 3.61 (dd, 1H, $J_{1,1'}$ 13 Hz, $J_{1,2}$ 3 Hz, H-1), 3.74 (d, 1H, $J_{2,3}$ 8 Hz, H-3), 3.81 (d, 1H, $J_{H,H'}$ 16 Hz, NCH*H*′CO$_2^-$), 4.03 (m, 2H, $J_{H,H'}$ 16 Hz, NCH*H*′CO$_2^-$, and H-2). ^{13}C NMR (D_2O, 125.8 MHz): δ 23.9 (d, $J_{7,P}$ 134.6 Hz, C-7), 27.3 (d, $J_{6,P}$ 3.77 Hz, C-6), 52.7 (d, $J_{C,P}$ 125.7 Hz, N*C*H$_2$PO$_3^{2-}$), 58.4 (d, $J_{C,P}$ 4 Hz, N*C*H$_2$CO$_2^-$), 59.2 (d, $J_{C,P}$ 5 Hz, C-1), 64.9 (C-2), 70.7 (d, $J_{C,P}$ 16.35 Hz, C-5), 71.3 (C-3), 71.9 (C-4), 173.2 (CO$_2^-$). ^{31}P NMR (D_2O, 81.0 MHz): δ 7.7 (P from glyphosate moiety), 26.8 (P at side chain of molecule). ESIMS m/z 563.1 (MH^+, $C_{10}H_{18}O_{12}P_2K_3$ requires 562.6).

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