Effect of phosphorylation on the reaction rate of unnatural electrophiles with 2-keto-3-deoxy-6-phosphogluconate aldolase

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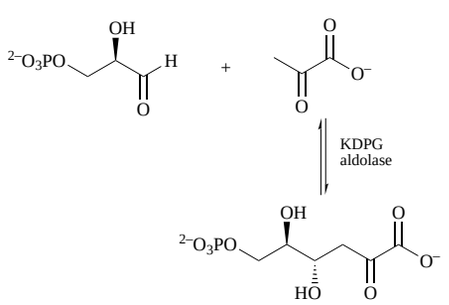
D-Glyceraldehyde is accepted as an electrophile by 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (EC 4.1.2.14) at 1% the rate of natural substrate, D-glyceraldehyde 3-phosphate. Accordingly, it was expected that addition of a phosphate moiety at C3 or C4 of unnatural aldehydes would enhance their activity as electrophilic substrates. Furthermore, phosphate would act as a useful protecting group during synthetic manipulations of the aldol adduct. A variety of phosphorylated and non-phosphorylated aldehydes were synthesized and evaluated as substrates for KDPG aldolase. Although small variations in reaction rate were observed, phosphorylation failed to provide a universal rate enhancement. Evaluation of substrate kinetic parameters revealed that the high rate of reaction of D-glyceraldehyde 3-phosphate compared to related electrophiles is entirely due to the efficiency of turnover with little change in binding exhibited among various substrates.

Introduction

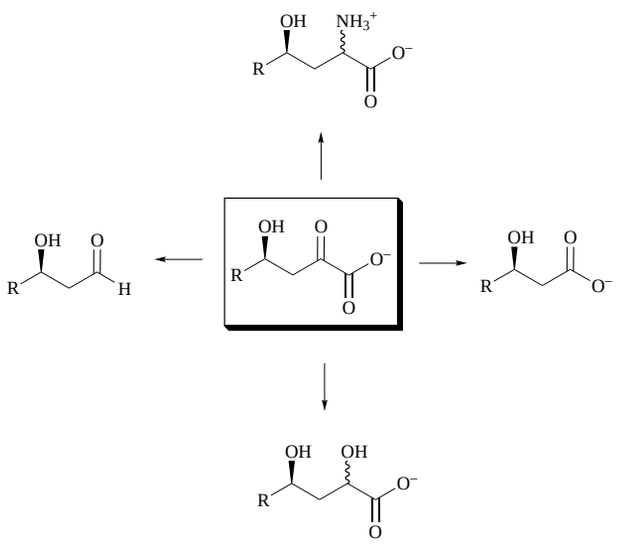
Recently, we have explored the utility of KDPG aldolases (EC 4.1.2.14) for stereocontrolled carbon–carbon bond formation.[1–3] Although aldolases are recognized as powerful catalysts, only a limited number of enzymes have been evaluated for synthetic utility.[4] This group includes the dihydroxyacetone phosphate aldolases, most notably fructose 1,6-diphosphate (FDP) aldolase and the pyruvate aldolases neuraminic acid aldolase[5–19] and 2-keto-3-deoxyoctanoate aldolase.[20] Use of the latter catalysts is limited by restricted substrate specificities, and the enzymes have been utilized primarily for the preparation of natural product analogs.[8,10,13,21–24] Furthermore, both neuraminic acid aldolase and 2-keto-3-deoxyoctanoate aldolase generate thermodynamic aldol addition products; diastereomeric product mixtures are common.[14,16,18,20]

Our research focuses on those aldolases that utilize pyruvate as the nucleophilic component in aldol addition. In particular, we have investigated 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase from *Escherichia coli*, *Pseudomonas putida* and *Zymomonas mobilis*. The enzymes accept a number of unnatural substrates at synthetically useful rates, show outstanding stability and resistance to organic cosolvent, and broad pH activity maxima.[1] Likewise, the enzymes appear to show complete kinetic diastereoselectivity in addition, providing products of high diastereomeric purity and predictable absolute configuration.

The products of pyruvate aldolase-catalyzed addition are 4-substituted 4-hydroxy-2-ketobutyrates (Scheme 1). This framework is ideally suited for further synthetic elaboration to a variety of motifs commonly found in biologically active



Scheme 1 The *in vivo* reaction catalyzed by KDPG aldolase



Scheme 2 Synthetic elaborations of 4-substituted-4-hydroxy-2-ketobutyrates

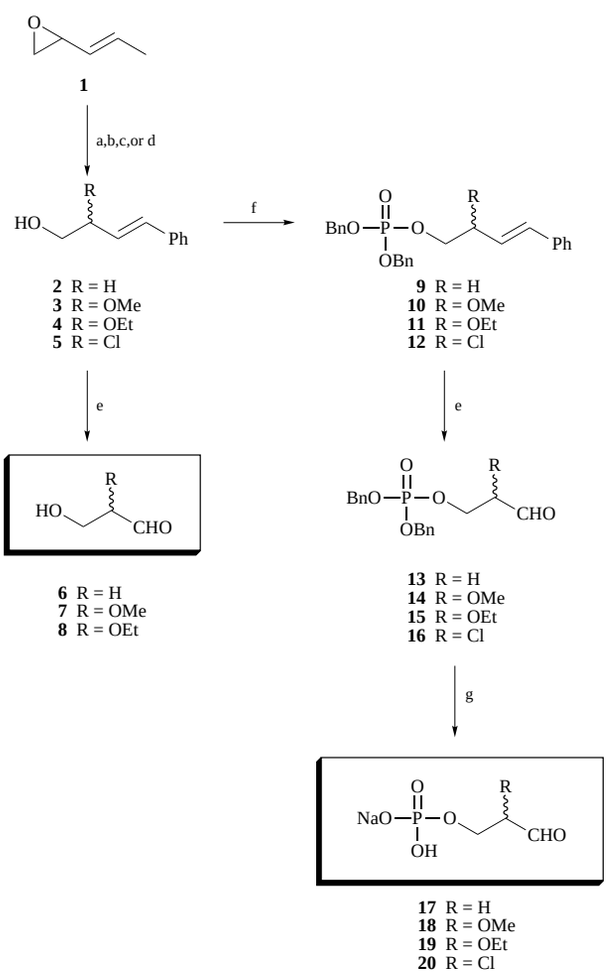
natural products (Scheme 2). For example, non-oxidative decarboxylation provides access to 2-deoxyaldose sugars. Similarly, the initial adduct can be readily transformed to the corresponding β-hydroxycarboxylic acid (oxidative decarboxylation), α,γ-dihydroxycarboxylic acid (reduction) or α-amino-γ-hydroxycarboxylic acid (reductive amination).[3] In each case, the required transformation can be carried out stereoselectively in aqueous solution without the need for protecting group chemistry: this varied and facile manifold is the source of the power of the pyruvate aldolases as catalysts for stereocontrolled carbon–carbon bond formation.

A drawback of the pyruvate aldolases is the relatively low rate of reactivity of unnatural electrophilic substrates as compared to the natural substrate. The greatest loss in reactivity appears to involve removal of the phosphate group of the natural electrophile: D-glyceraldehyde is accepted at roughly 1% the rate of D-glyceraldehyde 3-phosphate. Most unnatural electrophiles are accepted at or near the rate of D-glyceraldehyde. It should be noted that although the relative activity for D-glyceraldehyde as compared to D-glyceraldehyde 3-phosphate is low, the absolute specific activity remains high enough

for preparative scale syntheses. The specific activity of KDPG aldolase from *E. coli* using natural substrates is 400 U mg^{-1}; even if an unnatural aldehyde is accepted at only 1% the rate of the natural substrate, the absolute specific activity remains synthetically useful at 4 U mg^{-1}. We postulated that phosphorylation of electrophilic substrates might improve the rate of acceptance by KDPG aldolase. Furthermore, phosphorylation of the terminal hydroxy group of the electrophile prohibits cyclization of the product ketose through this hydroxy group, facilitating conversion of the aldol adduct to a variety of biologically relevant products. Here, we report the synthesis, enzymatic activity, and kinetic parameters of several phosphorylated and non-phosphorylated glyceraldehyde derivatives.

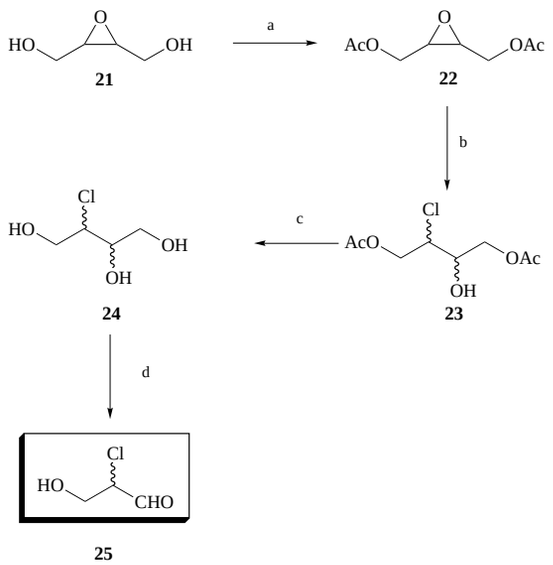
Results and discussion

Electrophilic aldehydes were prepared from either *trans*-cinnamaldehyde, according to the method of Whitesides[25] (Scheme 3), or from *cis*-butene-1,4-diol, according to the



Scheme 3 Synthesis of aldehydes from *trans*-cinnamaldehyde.[25] *Reagents*: a, DIBAL, CH_2Cl_2, −78 °C, quench MeOH, 76%; b, MeOH, H^+, 76%; c, EtOH, H^+, 71%; d, pyridinium hydrochloride, CH_2Cl_2, 77%; e, O_3, CH_2Cl_2–MeOH, −78 °C, then Me_2S, 65–69%; f, DDP, 1*H*-tetrazole, THF, then MCPBA, 67–78%; g, H_2, 10% Pd/C, THF, then aq. 0.1 M NaOH.

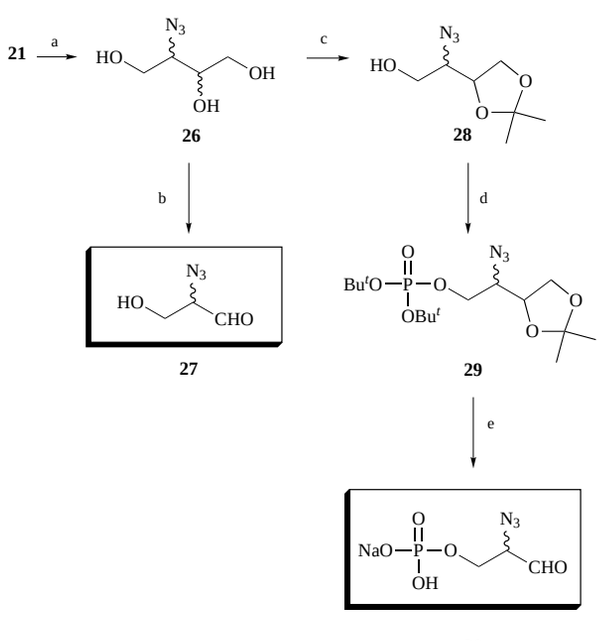
method of Wong[26] (Scheme 4). Addition of trimethylsulfonium ylide to *trans*-cinnamaldehyde readily furnishes (*E*)-1,2-epoxy-4-phenylbut-3-ene **1**. Selective opening of the epoxide at the allylic position with hydride, methanol, ethanol or chloride nucleophiles followed by ozonolysis provided electrophilic substrates **6**–**8**. Alternatively, the corresponding 2-substituted 4-phenylbut-3-en-1-ols **2**–**5** were phosphorylated with dibenzyl-*N*,*N*-diethylphosphoramidite (DDP). Oxidation to the dibenzyl



Scheme 4 Synthesis of aldehydes from *cis*-butene-1,4-diol.[26] *Reagents*: a, Ac_2O, pyridine, CH_2Cl_2, 95%; b, pyridinium hydrochloride, CH_2Cl_2, 72%; c, LAH, THF, 72%; d, $NaIO_4$, 95% EtOH.

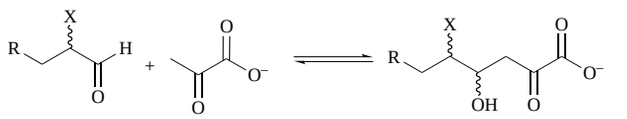
phosphate, ozonolysis and hydrogenolysis of the benzyl protecting groups provided the phosphorylated analogs **17**–**20**.

Attempts to purify 2-chloro-4-phenylbut-3-en-1-ol **5** afforded large amounts of epoxide **1**, proving this route to 2-chloro-3-hydroxypropanal **25** unsatisfactory. Alternatively, treatment of bis(acetoxymethyl)oxirane **22** with pyridinium hydrochloride followed by reductive removal of the acetate protecting groups and periodate cleavage of the vicinal diol yielded 2-deoxy-2-chloroglyceraldehyde **25**. Similarly, DDP was inappropriate for preparation of 2-deoxy-2-azido-glyceraldehyde 3-phosphate **30** since hydrogenolysis of the benzyl ether protecting groups was accompanied by reduction of the azide. Epoxide opening of bis(hydroxymethyl)oxirane **21** with sodium azide followed by protection of the vicinal diol **26** as the acetonide **28**, phosphorylation of the remaining hydroxy moiety, deprotection and oxidative cleavage of the vicinal diol provided the required phosphorylated derivative **30** (Scheme 5). While treatment of



Scheme 5 Synthesis of azido aldehydes. *Reagents*: a, NaN_3, NH_4Cl, MeOH, H_2O, 85%; b, $NaIO_4$, 95% EtOH, 64%; c, acetone, H^+, 0 °C, 86%; d, di-*tert*-butyl-*N*,*N*-diethylphosphoramidite, 1*H*-tetrazole, THF, then MCPBA, 79%; e, H^+, H_2O then $NaIO_4$.

Table 1 The effect of phosphorylation on the relative rates of KDPG aldolase conversion[a]



R	X	V_{rel}(EC[b])	V_{rel}(ZM[c])
OH	OH	100	100
H	OH	1	5
H	OP	6	18
OMe	OH	3	6
OMe	OP	0.5	4
OEt	OH	1	3
OEt	OP	5	9
Cl	OH	0.5	2
Cl	OP	1	1.5
N_3	OH	9	25
N_3	OP	3	2
OH	CH_2OH	11	30
OH	CH_2OP	120	140

[a] All assays were carried out with 50 mM electrophile and 50 mM pyruvate; substrates are at saturating, or k_{cat}, conditions. All electrophile substrates were tested as racemates, except for D-glyceraldehyde, D-erythrose and D-erythrose-4-phosphate. Reactions were conducted at pH 6.5 and 25 °C. [b] KDPG Aldolase from *E. coli*. [c] KDPG Aldolase from *Z. mobilis*.

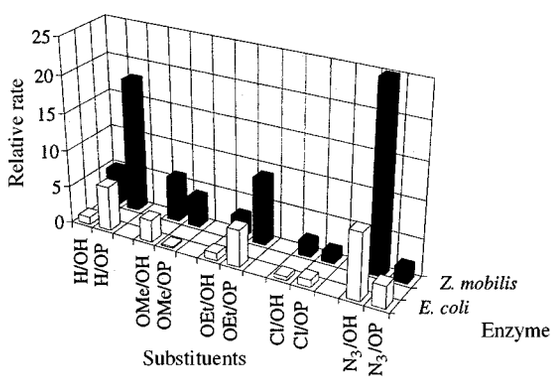


Fig. 1 Rate enhancement or depression with phosophorylated and non-phosophorylated aldehydes for KDPG aldolases from *E. coli* and *Z. mobilis*

triol **26** with acetone at 0 °C gave exclusively the five-membered 3,4-acetonide, treatment at higher temperatures gave increasing amounts of the undesired six-membered 1,3-acetonide.

Phosphorylated (**17**–**20**, **30**) and non-phosphorylated (**6**–**8**, **25**, **27**) electrophiles were investigated as substrates for both *E. coli* and *Z. mobilis* KDPG aldolases (Table 1). The purification and properties of these enzymes, as well as the assay procedure, have been previously described.[1,2,27–29] Briefly, discontinuous reactions were conducted with 50 mM electrophile, 50 mM nucleophile and the aldolase from *E. coli* or *Z. mobilis*; electrophile and nucleophile are at k_{cat} conditions. The extent of the reaction was monitored by measuring the amount of pyruvate remaining in the reaction *via* a coupled assay with L-lactic dehydrogenase.

The effect of phosphorylation on the rate of enzymatic reaction varies widely. Most importantly, *there is no systematic universal variation in reaction rate in response to phosphorylation*. With the unsubstituted and ethoxy-substituted derivatives the rates of conversion are increased three- to five-fold upon phosphorylation. Likewise, with erythrose the rate of reaction upon phosphorylation is increased twelve-fold. Alternatively, in the case of the methoxy- and azido-substituted derivatives, the rates of conversion of the phosphorylated analogs are reduced by a factor as high as ten. Finally, the rate of conversion of the chloro-substituted substrate is unaffected by phosphorylation.

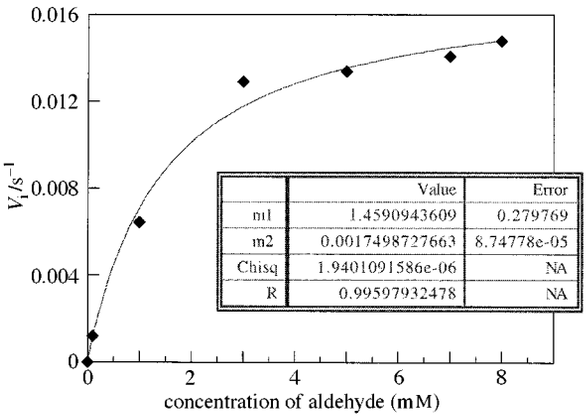


Fig. 2 Michaelis plot for varying D-glyceraldehyde with 50 mM pyruvate

Interestingly, the pattern of rate enhancement or depression is identical for the enzymes from *Escherichia* and from *Zymomonas* (Fig. 1). The *Zymomonas* enzyme accepts all unnatural substrates at significantly greater relative rates than does the *Escherichia* enzyme: this is consistent with our previous observation that the *Zymomonas* enzyme is the most promiscuous KDPG aldolase examined to data.[1]

Pseudo-first order kinetics were conducted with the *E. coli* KDPG aldolase for phosphorylated and non-phosphorylated aldehydes in order to further investigate the origin of the high activity of D-glyceraldehyde 3-phosphate. K_m and k_{cat} were determined for D-glyceraldehyde 3-phosphate, D-glyceraldehyde, D-erythrose 4-phosphate, D-erythrose, and 2-azido-2-deoxyglyceraldehyde with pyruvate under saturation conditions (Table 2). Likewise, kinetic constants were determined for pyruvate with each of the aldehydes, again under saturation conditions (Table 3). Although discontinuous assays typically reflect larger error than continuous assays, our graphs correlate well with the Michaelis equation (Fig. 2).

Evaluation of the parameters in Table 3 clearly reveals that variation in V_{rel} for electrophilic substrates is entirely due to variation in turnover rates. K_m for pyruvate as a function of electrophile identity does not vary within experimental error at saturating aldehyde concentrations. Michaelis constants for electrophilic substrates vary only modestly from 0.51–3.90 mM, at saturating pyruvate. In contrast, k_{cat} values spanned a broad range and in fact sufficiently explain the rate variation for electrophilic substrates. Thus, while unnatural electrophiles apparently bind at the KDPG aldolase active site, both the C2 hydroxy and the C3 phosphate are required for productive orientation in the active site. This same trend is mirrored in the requirement of the *E. coli* KDPG aldolase for the *R* configuration at C2 of the electrophile.

Even in the absence of a significant rate enhancement, phosphorylation of electrophile hydroxy groups provides unique differential protection of product ketoses that facilitates further synthetic manipulation to secondary targets. In this capacity, the phosphate group effectively blocks cyclization of the adduct through this hydroxy group, providing the product exclusively in the open-chain form (Scheme 6). The synthetic utility of KDPG aldolase is derived from the diverse range of products readily prepared from the initial adduct; modification often occurs at the C2 ketone. If the aldolase-catalyzed product exists exclusively in the cyclic form, as we have shown with KDG, manipulations at the ketone moiety are not accessible. Although phosphate has not been utilized as a protecting group in carbohydrate synthesis, it possesses characteristics that render it especially useful in aqueous enzymatic solution, where typical carbohydrate protecting groups have limited utility. Thus in addition to preventing cyclization, phosphorylation ensures aqueous solubility of both the electrophile

Table 2 Kinetic constants for various aldehydes with pyruvate at saturating kinetics

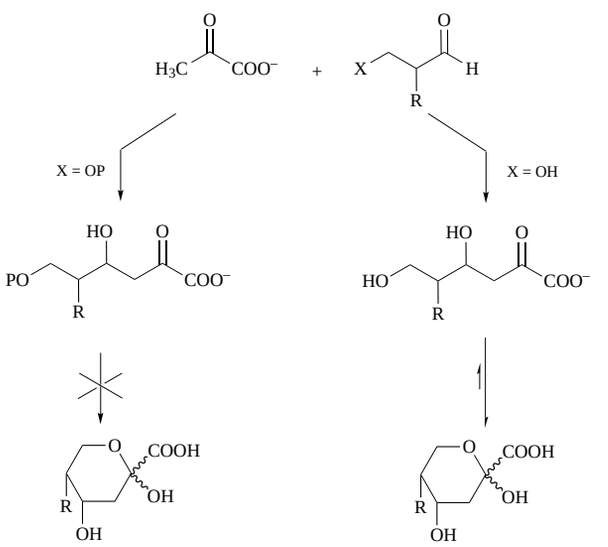
Aldehydes	K_m/mM	k_{cat}/s^{-1}	$(k_{cat}/K_m)/10^{-2}$ dm^3 s^{-1} mmol^{-1}
D-Glyceraldehyde 3-phosphate	3.90 ± 1.21	$2.5 \times 10^{-1} \pm 3.7 \times 10^{-2}$	6.4
D-Glyceraldehyde	1.46 ± 0.28	$1.7 \times 10^{-3} \pm 1 \times 10^{-4}$	1.2
D-Erythrose 4-phosphate	3.08 ± 1.68	$3.9 \times 10^{-3} \pm 8 \times 10^{-4}$	1.3
D-Erythrose	0.51 ± 0.08	$1.4 \times 10^{-4} \pm 6 \times 10^{-5}$	2.7
2-Azido-2-deoxyglyceraldehyde	1.62 ± 0.29	$2.9 \times 10^{-4} \pm 2 \times 10^{-5}$	1.8

Reactions were conducted at pH 6.5 and 25 °C.

Table 3 Kinetic constants for pyruvate with various aldehydes at saturating kinetics

Aldehydes	K_m/mM
D-Glyceraldehyde 3-phosphate	3.25 ± 0.51
D-Glyceraldehyde	2.81 ± 0.33
D-Erythrose-4-phosphate	2.92 ± 0.27
D-Erythrose	3.50 ± 0.22
2-Azido-2-deoxyglyceraldehyde	2.63 ± 0.36

Reactions were conducted at pH 6.5 and 25 °C.



Scheme 6 Phosphate as an effective protecting group

and the aldol adduct, an issue vital for enzymatic synthesis. Likewise, the phosphate protecting group can be readily and selectively removed *via* treatment with acid phosphatase in quantitative yields.

In summary, while phosphate functions as a useful protecting group in enzymatic aldol addition, phosphorylation produces a modest and unpredictable effect on reaction. Furthermore, variation in rate is solely a result of variation of substrate turnover.

Experimental

General procedures

Where necessary, solvents were dried and purified according to recommended procedures.[30] Light petroleum refers to the fraction boiling in the range 35–60 °C. Chromatography refers to the method of Still *et al.*[31] using silica gel 60 F_{254}, 230–400 mesh. Melting points were determined on a Thomas Hoover capillary melting point apparatus and are uncorrected. Where necessary, reactions were conducted under a dry argon atmosphere.

IR Spectra were recorded on a Bomem MB-100 spectrophotometer. ^{1}H NMR Spectra were recorded on a General Electric QE-300 spectrometer operating at 300.15 MHz. ^{13}C NMR Spectra were recorded on a General Electric QE-300 spectrometer operating at 74.48 MHz. Chemical shifts (δ in ppm) are given relative to those for Me_4Si. Accurate mass determinations were obtained from a JEOL JMS-SX 102 high resolution mass spectrometer. Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ. All chemicals and enzymes were purchased from Aldrich and Sigma Chemical Companies unless otherwise indicated. DDP was synthesized according to the method of Wong and co-workers.[32a] Protein concentration was determined by the Edelhoch method as previously described.[32b] Plasmids pTC 162[33] and pTC 190[34] were generous gifts from T. Conway, School of Biological Sciences, University of Nebraska. *E. coli* strain DF 214[35] was a gift from B. Bachmann, *E. coli* Genetic Stock Center, Yale University.

Growth of bacterial strains for KDPG aldolase. *E. coli* strains containing plasmids pTC 162 (expressing KDPG aldolase from *Z. mobilis*) and pTC 190 (expressing KDPG aldolase from *E. coli*) were grown according to previously published procedures.[27]

KDPG aldolase purification. *E. coli* KDPG aldolase was purified by differential dye–ligand chromatography by previously published procedures.[27] KDPG aldolase from *Z. mobilis* was purified by protamine sulfate precipitation, ammonium sulfate fractionation, ultrafiltration, and utilized for assays without further purification.

Determination of KDPG aldolase activity. KDPG aldolase activity was determined by a modification of a coupled assay with L-lactic dehydrogenase (L-LDH, EC 1.1.1.27, type II, rabbit muscle) previously described by Meloche and Wood.[36] 20 mM KH_2PO_4, pH 7.5 (3.0 ml), NADH (50 µl, 15 mg ml^{-1}), L-LDH (10 µl, 100 U) and KDPG aldolase (50 µl) were added to a 4.5 ml disposable cuvette. The reaction was initiated by the addition of KDPG (50 µl, 100 mg ml^{-1}, Li^+ salt).

Nucleophile assays. Nucleophile assays were performed on a 3 ml scale with varying nucleophile concentrations (0.1–50 mM) and electrophile concentration at 50 mM. Typically, 1–150 units of aldolase were used for each assay; lower concentrations of nucleophiles received less enzyme in order to monitor the aldolase-catalyzed reaction effectively. 100 µl Aliquots were taken at timely intervals and the aldolase in the aliquots destroyed with 7% perchloric acid (30 µl). The samples were neutralized with 1 M NaOH (20 µl) and diluted with 20 mM KH_2PO_4, pH 7.5 (1000 µl). The reactions were followed by monitoring the disappearance of pyruvate with L-LDH. In the case of lower nucleophile concentrations, assay reactions were scaled up in volume and a larger volume of sample was assayed to facilitate monitoring absorbance changes. Such scaling factors along with amount of enzyme were included in determination of kinetic parameters.

Electrophile assays. Electrophile assays were performed on a 3 ml scale with varying electrophile concentrations (0.1–50 mM) and nucleophile concentration at 50 mM. Typically, 1–150 units of aldolase were used for each assay; lower concentrations of electrophiles received less enzyme. 100 µl Aliquots were taken at timely intervals and the aldolase in the aliquots destroyed with 7% perchloric acid (30 µl). The samples were neutralized with 1 M NaOH (20 µl) and diluted with 0.1 M $Na_4P_2O_7$, pH 7.5 (1000 µl). The reactions were followed by monitoring the appearance of NADH with D-glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12, from rabbit muscle) or yeast aldehyde dehydrogenase.[37] In the case of lower electrophile concentrations, assay

reactions were scaled up in volume and a larger volume of sample was assayed to facilitate monitoring absorbance changes. Such scaling factors along with amount of enzyme were included in determination of kinetic parameters.

(*E*)-1,2-Epoxy-4-phenylbut-3-ene 1

Dry THF (35 ml) and DMSO (35 ml) were added to sodium hydride (2.9 g, 72.5 mmol) and the reaction was cooled in an ice–salt bath. A solution of trimethylsulfonium iodide (14.1 g, 70 mmol) in dry DMSO (50 ml) was added *via* cannula over a period of 5 min. *trans*-Cinnamaldehyde (3.08 g, 23.23 mmol) was added and the reaction stirred for 30 min at 0 °C and then at room temperature for one hour. Ice–water (150 ml) was slowly added. The product was extracted with CH_2Cl_2 (3 × 150 ml), dried ($MgSO_4$), filtered and concentrated *in vacuo* to provide a brown liquid. Vacuum distillation afforded epoxide **1** (2.71 g, 80%) as a pale yellow liquid. Spectral data are identical to those reported in the literature.[25]

(*E*)-4-Phenylbut-3-en-1-ol 2

DIBAL (1 M solution in CH_2Cl_2, 11 ml, 11 mmol) was added over a period of 5 min to epoxide **1** (1.3 g, 8.9 mmol) in dry CH_2Cl_2 (30 ml) at −78 °C. The reaction was stirred at room temperature for 3 h then cooled to −78 °C. Methanol (5 ml) was added dropwise and the reaction was stirred at −78 °C for 5 min then at room temperature for 10 min to produce a clear gel. The gel was washed with ethyl acetate (3 × 50 ml) and the solvent concentrated *in vacuo* to afford a colorless oil. Chromatography [ethyl acetate–light petroleum (1:1 v/v)] of crude product furnished alcohol **2** (1.0 g, 76%) as a colorless oil. Spectral data are identical to those reported in the literature.[38]

(*E*)-2-Methoxy-4-phenylbut-3-en-1-ol 3

Four drops of concentrated sulfuric acid were added to epoxide **1** (500 mg, 3.42 mmol) in anhydrous methanol (20 ml) and the reaction was refluxed for 3 h. $NaHCO_3$ was added until neutral pH was achieved. Filtration and concentration *in vacuo* furnished a pale yellow oil. Chromatography [ethyl acetate–light petroleum (1:1 v/v)] of the crude product afforded **3** (1.0 g, 76%) as a colorless oil. $\delta_H(CDCl_3)$ 7.30–7.08 (m, 5H), 6.50 (d, *J* 16, 1H), 5.94 (dd, *J* 7, 16, 1H), 3.80–3.72 (m, 1H), 3.55–3.51 (m, 2H), 3.25 (s, 3H), 3.04–2.98 (br s, 1H); $\delta_C(CDCl_3)$ 135.6, 133.4, 127.9, 127.3, 126.0, 125.8, 125.4, 82.6, 64.7, 55.9; ν_{max}/cm^{-1} 3406 (Calc. for $C_{11}H_{14}O_2$: C, 74.12; H, 7.92. Found: C, 73.89; H, 7.9%).

(*E*)-2-Ethoxy-4-phenylbut-3-en-1-ol 4

Four drops of concentrated sulfuric acid were added to epoxide **1** (602 mg, 4.12 mmol) in anhydrous ethanol (20 ml) and the reaction was refluxed for 3 h. $NaHCO_3$(s) was added until 'apparent' neutral pH was achieved. Filtration and concentration *in vacuo* produced a pale yellow oil. Chromatography [ethyl acetate–light petroleum (1:1 v/v)] of the crude product furnished **4** (562 mg, 71%) as a colorless oil. $\delta_H(CDCl_3)$ 7.30–7.09 (m, 5H), 6.51 (d, *J* 16, 1H), 5.97 (dd, *J* 7, 16, 1H), 3.92–3.26 (m, 1H), 3.62–3.48 (m, 3H), 2.90–2.75 (br s, 1H), 1.13 (t, *J* 7, 3H); $\delta_C(CDCl_3)$ 135.62, 132.87, 127.96, 127.94, 127.28, 127.26, 126.05, 125.91, 80.73, 64.75, 63.58, 14.68; ν_{max}/cm^{-1} 3345 (Calc. for $C_{12}H_{16}O_2$: C, 74.96; H, 8.39. Found: C, 74.89; H, 8.16%).

(*E*)-2-Chloro-4-phenylbut-3-en-1-ol 5

Pyridinium hydrochloride (1.23 g, 10.68 mmol) was added to epoxide **1** (1.3 g, 8.9 mmol) in dry CH_2Cl_2 (30 ml) and the reaction stirred at room temperature for 30 min. The organic layer was washed with water (3 × 30 ml), dried ($MgSO_4$), filtered and concentrated *in vacuo* to afford chlorohydrin **5** (1.25 g, 77%) as a pale yellow oil. $\delta_H(CDCl_3)$ 7.45–7.25 (m, 5H), 6.68 (d, *J* 16, 1H), 6.22 (dd, *J* 9, 16, 1H), 4.68–4.61 (m, 1H), 3.81 (t, *J* 5, 2H), 2.52 (br s, 1H); $\delta_C(CDCl_3)$ 135.02, 133.36, 127.94, 127.61, 126.07, 126.00, 125.78, 125.60, 65.73, 63.50; ν_{max}/cm^{-1} 3253.

(*E*)-4-Phenylbut-3-en-1-yl dibenzyl phosphate 9

DDP (1.47 g, 4.65 mmol) was added to a solution of alcohol **2** (530 mg, 3.58 mmol) and 1*H*-tetrazole (508 mg, 7.16 mmol) in dry THF (20 ml). The reaction was stirred at room temperature for 30 min and then cooled to −60 °C. 3-Chloroperoxybenzoic acid (85%; 2.18 g, 10.74 mmol) in CH_2Cl_2 was added. After stirring for 15 min at room temperature, 10% aq. $NaHSO_3$ (20 ml) was added and the reaction stirred for 10 min. Diethyl ether (100 ml) was added and the aqueous phase discarded. The ethereal phase was washed with 10% aq. $NaHSO_3$ (2 × 30 ml) and 5% aq. $NaHCO_3$ (2 × 30 ml), dried ($MgSO_4$), filtered and concentrated *in vacuo* to produce a colorless oil. Chromatography [CH_2Cl_2–acetone (15:1 v/v)] of the crude product yielded **9** (1.04 g, 71%) as a colorless oil. $\delta_H(CDCl_3)$ 7.58–7.26 (m, 15H), 6.34 (d, *J* 16, 1H), 6.02 (dt, *J* 7, 16, 1H), 4.95 (d, *J* 2, 2H), 4.92 (d, *J* 2, 2H), 4.00 (q, *J* 7, 2H), 2.46–2.37 (m, 2H); $\delta_C(CDCl_3)$ 136.5, 135.3, 135.2, 132.2, 127.9, 127.3, 126.7, 125.5, 124.2, 68.7, 68.6, 66.4, 33.2 (FAB HRMS: found: M^+, 409.1569; calc. for $C_{24}H_{26}O_4P$: *M*, 409.1569).

(*E*)-2-Methoxy-4-phenylbut-3-en-1-yl dibenzyl phosphate 10

The title compound was prepared in the same manner as **9**. Chromatography [CH_2Cl_2–acetone (20:1 v/v)] of the crude product provided **10** (1.11 g, 75%) as a colorless oil from alcohol **3** (601 mg, 3.38 mmol). $\delta_H(CDCl_3)$ 7.38–7.28 (m, 15H), 6.22 (d, *J* 16, 1H), 5.96 (dd, *J* 7, 16, 1H), 5.07–5.02 (m, 4H), 4.09–4.03 (m, 2H), 3.98–3.91 (m, 1H), 3.33 (s, 3H); $\delta_C(CDCl_3)$ 135.4, 135.2, 135.1, 134.0, 133.9, 128.0, 127.9, 127.5, 127.4, 127.3, 127.2, 126.0, 125.99, 124.4, 124.3, 80.0, 68.6, 66.6, 56.1 (FAB HRMS: found: M^+, 439.1839; calc. for $C_{25}H_{28}O_5P$: *M*, 439.1831).

(*E*)-2-Ethoxy-4-phenylbut-3-en-1-yl dibenzyl phosphate 11

The title compound was prepared in the same manner as **9**. Chromatography [CH_2Cl_2–acetone (20:1 v/v)] of the crude product afforded **11** (1.05 g, 78%) as a colorless oil from alcohol **4** (571 mg, 2.97 mmol). $\delta_H(CDCl_3)$ 7.52–7.24 (m, 15H), 6.49 (d, *J* 16, 1H), 5.91 (dd, *J* 7, 16, 1H), 4.98–4.84 (m, 4H), 4.02–3.90 (m, 3H), 3.54–3.42 (m, 1H), 3.39–3.26 (m, 1H), 1.07 (t, *J* 7, 3H); $\delta_C(CDCl_3)$ 135.47, 135.18, 133.31, 127.90, 127.33, 125.99, 125.90, 125.17, 78.28, 68.65, 68.58, 63.86, 14.71 (FAB HRMS: found: M^+, 453.1839; calc. for $C_{26}H_{30}O_5P$: *M*, 453.1831).

(*E*)-2-Chloro-4-phenylbut-3-en-1-yl dibenzyl phosphate 12

The title compound was prepared in the same manner as **9**. Chromatography [CH_2Cl_2–acetone (20:1 v/v)] of the crude product yielded **12** (862 mg, 67%) as a colorless oil from chlorohydrin **5** (532 mg, 2.91 mmol). $\delta_H(CDCl_3)$ 7.34–7.30 (m, 15H), 6.63 (d, *J* 16, 1H), 6.11 (dd, *J* 9, 16, 1H), 5.06–5.01 (m, 4H), 4.71–4.59 (m, 1H), 4.26–4.14 (m, 2H); $\delta_C(CDCl_3)$ 134.29, 127.97, 127.33, 127.27, 126.22, 126.16, 124.10, 68.92, 58.90 (FAB HRMS: found: M^+, 443.1179; calc. for $C_{24}H_{25}O_4{}^{35}ClP$: *M*, 443.1179).

3-Oxopropyl dibenzyl phosphate 13

Ozone was passed through a solution of alkene **9** (520 mg, 1.27 mmol) in CH_2Cl_2 (10 ml) and methanol (10 ml) at −78 °C until a blue color persisted. The reaction was then purged with nitrogen. Dimethyl sulfide (1.5 ml) was added and the reaction warmed to room temperature. The solvent was concentrated *in vacuo* to produce a colorless oil. Chromatography [ethyl acetate–light petroleum (4:1 v/v)] of the crude product furnished aldehyde **13** (293 mg, 69%) as a colorless oil. $\delta_H(CDCl_3)$ 9.64 (s, 1H), 7.36–7.30 (m, 10H), 5.04 (d, *J* 2, 2H), 4.28 (dt, *J* 6, 7, 2H), 2.70–2.64 (m, 2H); $\delta_C(CDCl_3)$ 198.3, 134.9, 127.9, 127.5, 127.4, 127.3, 68.8, 60.6, 42.0; ν_{max}/cm^{-1} 3361, 1724.

2-Methoxy-3-oxopropyl dibenzyl phosphate 14
The title compound was prepared in the same manner as **13**. Chromatography [ethyl acetate–light petroleum (3:1 v/v)] of the crude product yielded **14** (241 mg, 65%) as a colorless oil from alkene **10** (448 mg, 1.02 mmol). $\delta_H(CDCl_3)$ 9.58 (s, 1H), 7.37–7.26 (m, 10H), 5.08–4.96 (m, 4H), 4.34–4.10 (m, 3H), 3.49 (s, 3H); $\delta_C(CDCl_3)$ 199.7, 135.0, 134.9, 127.9, 127.3, 83.3, 68.8, 64.4, 57.9; ν_{max}/cm^{-1} 3353, 1738.

2-Ethoxy-3-oxopropyl dibenzyl phosphate 15
The title compound was prepared in the same manner as **13**. Chromatography [ethyl acetate–light petroleum (3:1 v/v)] of the crude product yielded **15** (267 mg, 68%) as a colorless oil from alkene **11** (472 mg, 1.04 mmol). $\delta_H(CDCl_3)$ 9.61 (s, 1H), 7.37–7.31 (m, 10H), 5.08–4.98 (m, 4H), 4.33–4.24 (m, 1H), 3.71–3.58 (m, 2H), 1.23 (t, *J* 7, 3H); $\delta_C(CDCl_3)$ 200.45, 134.97, 134.88, 127.92, 127.32, 81.74, 68.76, 66.03, 64.88, 14.56; ν_{max}/cm^{-1} 3348, 1739.

2-Chloro-3-oxopropyl dibenzyl phosphate 16
The title compound was prepared in the same manner as **13**. Chromatography [ethyl acetate–light petroleum (6:1 v/v)] of the crude product yielded **16** (562 mg, 45%) as a colorless oil from alkene **12** (1.5 g, 3.39 mmol). $\delta_H(CDCl_3)$ 7.38–7.30 (m, 10H), 5.45–5.20 (br s, 3H), 5.10–4.97 (m, 4H), 4.35–3.96 (m, 3H); ν_{max}/cm^{-1} 3368.

2-Deoxyglyceraldehyde 6, 2-methoxy-2-deoxyglyceraldehyde 7 and 2-ethoxy-2-deoxyglyceraldehyde 8
Aldehydes **6**–**8** were prepared by the ozonolysis of the corresponding olefins **2**–**4** as described for compound **13**. Chromatography [ethyl acetate–methanol (10:1 v/v)] of the crude products yielded aldehydes **6**–**8** as colorless oils in 65–69% yields. Concentration was determined by assay with aldehyde dehydrogenase.[39]

2-Deoxyglyceraldehyde 3-phosphate 17, 2-methoxy-2-deoxyglyceraldehyde 3-phosphate 18, 2-ethoxy-2-deoxyglyceraldehyde 3-phosphate 19 and 2-chloro-2-deoxyglyceraldehyde 3-phosphate 20
Aldehydes **17**–**20** were prepared *via* hydrogenolysis of the corresponding dibenzylphosphates **13**–**16**. A typical procedure is as follows: to compound **14** (160 mg, 0.44 mmol) in THF (10 ml) was added 10% Pd/C (16 mg). The solution was hydrogenated under atmospheric pressure for 30 min and filtered through Celite. The solution was concentrated *in vacuo* and water (10 ml) added. The pH was adjusted to 7.0 with 0.1 M NaOH and the solution lyophilized to produce **18** (86 mg) as a white solid. Aldehyde concentration was determined by assay with aldehyde dehydrogenase.[39]

***cis*-2,3-Epoxybutane-1,4-diol 21**
3-Chloroperoxybenzoic acid (85%, 60.0 g, 0.29 mmol) was added to *cis*-but-2-ene-1,4-diol (17.5 g, 0.20 mol) in CH_3CN (250 ml). The reaction remained at room temperature for 36 h without stirring. The crystalline side product was filtered off and water (600 ml) was added to produce a white precipitate. The solution was washed with $CHCl_3$ (2 × 300 ml) and the aqueous layer lyophilized to afford epoxide **21** (15.18 g, 73%) as a white solid. Spectral data are identical to those reported in the literature.[40]

2-Azido-2-deoxythreitol 26
NaN_3 (6.57 g, 101 mmol) and NH_4Cl (5.40 g, 101 mmol) were added to a solution of epoxide **21** (2.10 g, 20.20 mmol) in methanol (100 ml) and water (12 ml). The reaction was refluxed for 24 h and the solvent concentrated *in vacuo*. Ethanol (50 ml) was added and the precipitate filtered off. The procedure was repeated several times to remove excess NaN_3 and NH_4Cl to provide triol **26** (2.52 g, 85%) as a yellow liquid. Spectral data are identical to those reported in the literature.[26]

***cis*-1,4-Diacetoxy-2,3-epoxybutane 22**
Acetic anhydride (3.02 ml, 32.0 mmol) and DMAP (10 mg) were added to diol **21** (1.3 g, 12.5 mmol) in dry pyridine (15 ml). The reaction was stirred at room temperature for 4 h then washed with water (2 × 30 ml), sat. aq. $NaHCO_3$ (2 × 30 ml), 1 M HCl (2 × 30 ml) and sat. aq. NaCl (30 ml). The solution was dried ($MgSO_4$), filtered and concentrated *in vacuo* to produce a colorless oil. Chromatography [ethyl acetate–light petroleum (1:1 v/v)] of the crude product yielded diacetate **22** (2.23 g, 95%) as a white solid, mp 43–44 °C (EtOH); $\delta_H(CDCl_3)$ 4.30 (dd, *J* 4, 12, 2H), 4.11–4.02 (m, 2H), 3.28–3.22 (m, 2H), 2.07 (s, 3H); $\delta_C(CDCl_3)$ 169.85, 61.47, 52.56, 19.98; ν_{max}/cm^{-1} 1742. Lit.,[41] no spectral data given.

1,4-Diacetoxy-2-chloro-2-deoxythreitol 23
Pyridinium hydrochloride (2.22 g, 19.20 mmol) was added to epoxide **22** (2.0 g, 12.80 mmol) in dry CH_2Cl_2 (50 ml). The reaction was refluxed for 24 h. The solution was washed with water (3 × 30 ml), dried ($MgSO_4$), filtered and concentrated *in vacuo* to produce a colorless oil. Chromatography [CH_2Cl_2–acetone (20:1 v/v)] of the crude product yielded chlorohydrin **23** (1.77 g, 72%) as a colorless oil. $\delta_H(CDCl_3)$ 4.46–4.05 (m, 6H), 2.49 (d, *J* 7.5, 1H), 2.12 (s, 3H), 2.11 (s, 3H); $\delta_C(CDCl_3)$ 171.00, 170.73, 68.13, 65.20, 64.37, 59.29, 20.70, 20.63; ν_{max}/cm^{-1} 3456, 1735.

2-Chloro-2-deoxythreitol 24
$LiAlH_4$ (1 M solution in THF; 4 ml, 4 mmol) was added to diacetate **23** (2.40 g, 12.47 mmol) in dry THF (30 ml) at 0 °C. The reaction was stirred for 8 h at room temperature then cooled to 0 °C. Water (5 ml) was added dropwise to quench the reaction and the solvent was concentrated *in vacuo* to produce a white solid. Chromatography [CH_2Cl_2–acetone (5:1 v/v)] of the crude product yielded triol **24** (1.26 g, 72%) as a colorless oil. $\delta_H([^2H_6]acetone)$ 4.60 (br s, 1H), 4.35 (br s), 2H), 4.22–4.15 (m, 1H), 4.07–3.98 (m, 1H), 3.95–3.77 (m, 2H), 3.69–3.62 (m, 2H); $\delta_C([^2H_6]acetone)$ 70.21, 70.02, 63.29, 62.73; ν_{max}/cm^{-1} 3269 (Calc. for $C_4H_9ClO_3$: C, 34.28; H, 6.48. Found: C, 34.13; H, 6.33%).

3,4-*O*-Isopropylidene-2-azido-2-deoxythreitol 28
Three drops of concentrated sulfuric acid were added to triol **26** (900 mg, 6.12 mmol) in dry acetone (40 ml). The reaction was stirred 10 h at 0 °C. $NaHCO_3$(s) was added until 'apparent' neutral pH was reached, the solid filtered off and the solvent concentrated *in vacuo* to produce a colorless oil. Chromatography [ethyl acetate–light petroleum (1:1 v/v)] of the crude product furnished **28** (984 mg, 86%) as a colorless oil. $\delta_H(CDCl_3)$ 4.25 (q, *J* 6, 1H), 4.08 (dd, *J* 6, 8.5, 1H), 3.86 (dd, *J* 6, 8.5, 1H), 3.76–3.70 (m, 2H), 3.43 (q, *J* 6, 1H), 3.05 (br s, 1H), 1.48 (s, 3H), 1.37 (s, 3H); $\delta_C(CDCl_3)$ 109.07, 74.92, 65.28, 63.31, 61.34, 25.35, 24.26; ν_{max}/cm^{-1} 3416, 2114.

Di-*tert*-butyl 3,4-*O*-isopropylidene-2-azido-2-deoxythreitol 1-phosphate 29
DDP (1.25 ml, 4.48 mmol) was added to a solution of alcohol **28** (420 mg, 2.24 mmol) and 1*H*-tetrazole (856 mg, 4.48 mmol) in dry THF (30 ml). The reaction was stirred at room temperature for 30 min. The reaction was then cooled to −60 °C and a solution of 85% 3-chloroperoxybenzoic acid (1.36 g, 6.72 mmol) in CH_2Cl_2 (10 ml) added. After stirring 15 min at room temperature, 10% aq. $NaHSO_3$ (20 ml) was added and the reaction stirred 10 min. Diethyl ether (70 ml) was added and the aqueous phase discarded. The ethereal phase was washed with 10% aq. $NaHSO_3$ (2 × 30 ml) and 5% aq. $NaHCO_3$ (2 × 30 ml), dried ($MgSO_4$), filtered and concentrated *in vacuo* to produce a colorless oil. Chromatography [CH_2Cl_2–acetone (15:1 v/v)] of

the crude product yielded **29** (671 mg, 79%) as a colorless oil. $\delta_H(CDCl_3)$ 4.24 (q, *J* 6, 1H), 4.08 (dd, *J* 6, 8.5, 1H), 3.84 (dd, *J* 6, 8.5, 1H), 3.73 (d, *J* 6, 2H), 3.42 (q, *J* 6, 1H), 1.52 (s, 18H), 1.46 (s, 3H), 1.37 (s, 3H); $\delta_C(CDCl_3)$ 108.81, 82.31, 75.29, 65.56, 63.52, 61.25, 29.55, 25.43, 24.59; ν_{max}/cm^{-1} 2108 (FAB HRMS: found: M^+, 380.1947; calc. for $C_{15}H_{31}O_6N_3P$: *M*, 380.1950).

2-Chloro-2-deoxyglyceraldehyde 25

Sodium periodate (364 mg, 1.70 mmol) was added to triol **24** (202 mg, 1.44 mmol) in 95% ethanol (20 ml). The reaction was stirred for 5 h at room temperature. The solid was filtered off and the solution concentrated *in vacuo* to produce a white solid. Chromatography [ethyl acetate–methanol (10 : 1 v/v)] of the crude product yielded aldehyde **25** as a colorless oil. Aldehyde concentration was determined by assay with aldehyde dehydrogenase.[39]

2-Azido-2-deoxyglyceraldehyde 27

The title compound was prepared in the same manner as **25**. Chromatography [ethyl acetate–methanol (10 : 1 v/v)] of the crude product provided aldehyde **27** as a colorless oil. Aldehyde concentration was determined by assay with aldehyde dehydrogenase.[39]

2-Azido-2-deoxyglyceraldehyde 3-phosphate 30

One drop of concentrated HCl was added to compound **29** (620 mg, 1.63 mmol) in water (20 ml). The reaction was heated to 45 °C for 2 h and allowed to cool to room temperature. Sodium periodate (418 mg, 1.96 mmol) was added and the solution stirred for 2 h at room temperature. The pH of the reaction was adjusted to 7.0 with aq. 0.1 M NaOH and the solution lyophilized to produce aldehyde **30** (253 mg) as a white solid. Aldehyde concentration was determined by assay with aldehyde dehydrogenase.[39]

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