Chemical and Enzymatic Synthesis of 1-Deoxy-D-xylulose-5-phosphate

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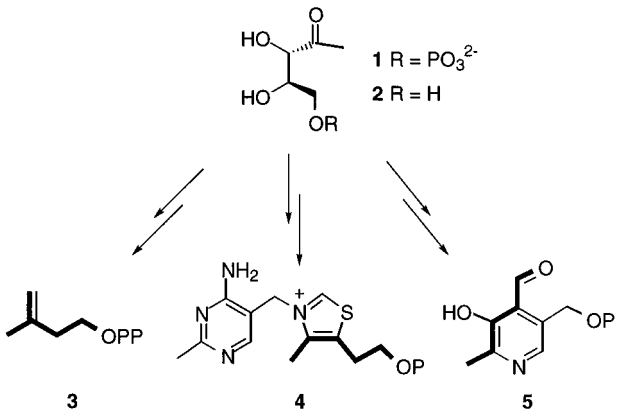
1-Deoxy-D-xylulose-5-phosphate **1** or the corresponding nonphosphorylated sugar, **2**, has been identified as a precursor in three major biosynthetic pathways (Scheme 1). In higher plants, algae, and bacteria, a nonmevalonate pathway to isopentenyl diphosphate **3** which utilizes **1** or **2** has been demonstrated.[1] In bacteria, **1** or **2** is involved in the biosynthesis of thiamin pyrophosphate **4**.[2] In *Escherichia coli*, it has been demonstrated that one of the enzymes required for the biosynthesis of the thiazole moiety of thiamin (ThiSG) catalyzes the epimerization of **1** but not **2.** This suggests that **1** is the precursor to the thiazole.[3] Last, labeling studies have shown that **1** or **2** is the precursor to pyridoxal **5**.[4] While numerous routes to **2** have been published,[5] a synthesis of **1** has not yet been described. In this paper we report a chemical and an enzymatic syntheses of **1**. The availability of **1** should facilitate studies on the mechanistic enzymology of all three biosynthetic pathways.

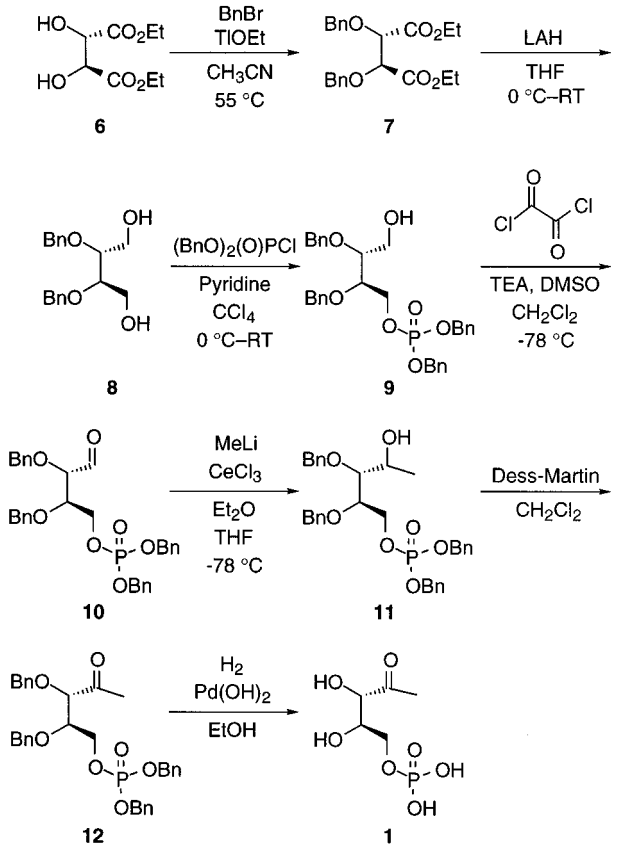
The chemical synthesis of **1** is outlined in Scheme 2. Benzylation of diethyl-D-tartrate **6** followed by LAH reduction gave **8**.[6] Phosphorylation of **8** using dibenzyl phosphorochloridate resulted in a mixture of **9**, the corresponding diphosphate, and starting material **8**, which was recycled. Swern oxidation of **9** gave **10**. Methyl addition using the in situ generated methyl cerium reagent gave **11,** which was oxidized to **12** using the Dess−Martin reagent. Ketone **12** is stable when stored at −20 °C and was quantitatively deprotected using catalytic hydrogenation to **1** in an overall yield of 5%.

E. coli 1-deoxy-D-xylulose-5-phosphate synthase catalyzes the condensation of pyruvate **16** and glyceraldehyde 3-phosphate **14** to give **1** (Scheme 3). This thiamin-dependent enzyme has been overexpressed at a high level (∼10% of total soluble protein) and purified[7] and can be used for the enzymatic synthesis of **1**.

Scheme 1



Scheme 2



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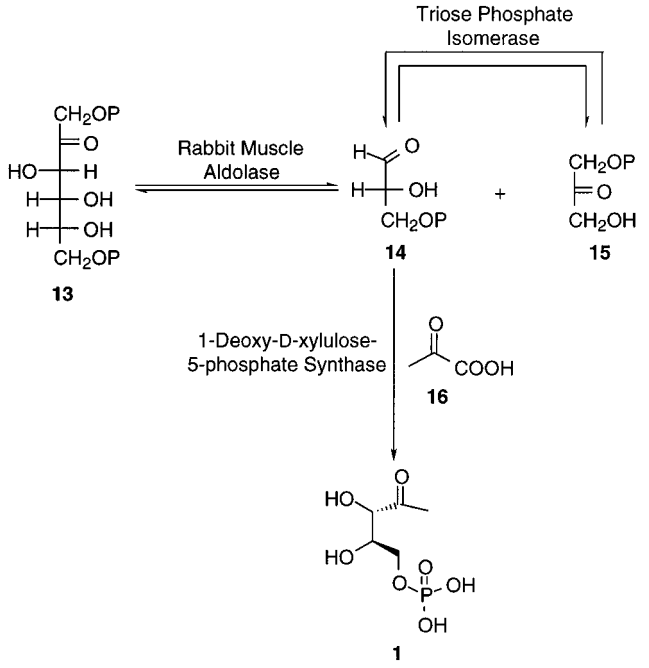
Scheme 3



Treatment of **13** and **16** with rabbit muscle aldolase, triosephosphate isomerase, and partially purified 1-deoxy-D-xylulose-5-phosphate synthase gave **1** in 47% yield, which was easily purified by precipitation as its barium salt using a variation of the method of Cardini and Leloir.[8] The in situ generation of **14** from fructose-1,6-diphosphate **13** is preferable to directly adding it to the reaction mixture, since commercially available preparations of **14** are enantiomerically impure and expensive.

The synthetic and biological methods reported in this study are simple and should be easily reproduced by organic chemists and biochemists for use in studies on the biosynthesis of isoprenoids, thiamin, and pyridoxol. In addition, isotopically labeled **1** should be readily attainable using these routes and commercially available labeled precursors.

Experimental Section

General. Mass spectra were obtained in the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois. ^{1}H and ^{13}C NMR were collected on a Varian XL-400 spectrometer. Chemical shifts are reported relative to $\delta(CHCl_3) = 7.24$ ppm and $\delta(H_20) = 4.65$ ppm. ^{31}P spectra were calibrated against an external H_3PO_4 standard set at $\delta = 0.00$ ppm and were not decoupled. The ^{13}C spectrum of compound **1** was calibrated against an internal standard of ethanol at 56.80 and 16.15 ppm. Unless stated otherwise, all reagents and chemicals were purchased from commercial sources and used as supplied. All reactions were carried out under an inert (nitrogen or argon) atmosphere with dry, freshly distilled solvents unless stated otherwise. Dibenzyl phosphorochloridate was prepared according to the procedure of Smith.[9] Anhydrous cerium chloride was prepared according to the method of Imamoto et al.[10] The Dess–Martin reagent was prepared according to the original procedure of Dess and Martin,[11] and thoroughly dried in vacuo before use. EM Science Silica gel 60 F-254 was purchased from Krackeler Scientific (Albany, New York). For thin-layer chromatography analysis, Merck precoated glass backed TLC plates (silica gel 60, F-254, 250 μm) were used. The Q-Sepharose HP anion-exchange column was from Pharmacia Biotech, (Uppsala, Sweden). The Aminex 87 H HPLC column was from Bio-Rad Laboratories GmbH (Munich, Germany). The Centriprep-30 ultrafiltration membrane was from Amicon (Witten, Germany). Rabbit muscle aldolase, triosephosphate isomerase, and alkaline phosphatase were from Boehringer Mannheim (Mannheim, Germany). The fructose-1,6-diphosphate was purchased from Biomol (Hamburg, Germany).

(2*S*,3*S*)-2,3-Bis(benzyloxy)succinic Acid Diethyl Ester 7. Thallous ethoxide (3.87 g, 0.0155 mol, 1.1 mL) and benzyl bromide (3.99 g, 0.0233 mol, 2.77 mL) were added to a stirred solution of diethyl-D-tartrate (1.6 g, 0.00776 mol) in dry acetonitrile (20 mL) at 45 °C. Stirring was continued for 48 h at 55 °C and then for 12 h at room temperature. The reaction mixture was filtered through Florisil to remove thallium bromide, and the acetonitrile was removed in vacuo. Flash chromatography (15:85 ethyl acetate:hexane) of the resulting oil gave 2.00 g of a white solid in 88% yield ($R_f = 0.32$, 15:85 ethyl acetate:hexane). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.29 (m, 10H), 4.87 (d, $J = 12.1$ Hz, 2H), 4.45 (d, $J = 12.1$ Hz, 2H), 4.39 (s, 2H), 4.12 (m, 4H), 1.18 (t, $J = 7.1$ Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 169.22, 137.10, 128.47, 128.39, 128.05, 78.47, 73.3, 61.43, 14.20. MS (CI + CH_4): 295 ($M^+ - 91$).

(2*R*,3*R*)-2,3-Bis(benzyloxy)butane-1,4-diol 8. Lithium aluminum hydride (452 mg, 0.0119 mol) was added over 30 min to a stirred solution of diester **7** (2.00 g, 0.00518 mol) in dry THF (35 mL) at 0 °C. The mixture was warmed to room temperature and stirring was continued for 3 h. The reaction mixture was cooled to 0 °C, quenched by slow addition of THF (35 mL), water (0.5 mL), 2M sodium hydroxide (0.5 mL), and water (0.5 mL), and filtered. The filtrate was dried over anhydrous magnesium sulfate, and THF was removed in vacuo to give 1.30 g of a white solid in 83.3% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.34 (m, 10H), 4.66 (s, 4H), 3.75 (m, 6H), 2.57 (br s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 138.10, 128.73, 128.17, 128.14, 79.07, 72.75, 60.94. MS (CI + CH_4): 303.

(2*R*,3*R*)-Phosphoric Acid Dibenzyl Ester 2,3-Bis(benzyloxy)-4-hydroxybutyl Ester 9. Dibenzyl phosphorochloridate (0.0028 mol) dissolved in CCl_4 (6 mL) was added to a stirred solution of diol **8** (650 mg, 0.00215 mol) in pyridine (25 mL) at 0 °C. The reaction was stirred at room temperature for 12 h and quenched by addition of water (2 mL), and the solvents were removed in vacuo. Flash chromatography (20:80 ethyl acetate: methylene chloride) gave 479 mg of a yellow oil in 40% yield ($R_f = 0.22$, 25:75 ethyl acetate:methylene chloride). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.32 (m, 20H), 5.04 (m, 4H), 4.61 (m, 4H), 4.29 (m, 1H), 4.16 (m, 1H), 3.77 (m, 2H), 3.61 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 138.08, 137.94, 135.89 (d), 128.73, 128.7, 128.62, 128.57, 128.24, 128.08, 128.03, 78.38, 77.97, 77.89, 73.27, 73.00, 69.49 (d), 67.00 (d), 61.37. ^{31}P NMR (160 MHz, $CDCl_3$) δ: −0.151.

(2*R*,3*S*)-Phosphoric Acid Dibenzyl Ester 2,3-Bis(benzyloxy)-4-oxobutyl Ester 10. Dimethyl sulfoxide (330 mg, 0.00423 mol, 327 μL) was added with stirring to a solution of oxalyl chloride (0.00184 mol) in methylene chloride (4.9 mL) at −78 °C. The mixture was stirred for 2.5 min and warmed to −10 °C, and alcohol **9** (450 mg, 0.000799 mol), dissolved in methylene chloride (5 mL), was added. After 10 min, triethylamine (471 mg, 0.00465 mol, 648 μL) was added, the reaction mixture stirred for 5 min and then warmed to room temperature. Water (5 mL) was added, and the resulting mixture was extracted with chloroform (4 × 5 mL). The organic extracts were combined, washed with water (2 × 5 mL) and brine (2 × 5 mL), and dried with anhydrous magnesium sulfate, and the solvent was removed in vacuo. Flash chromatography (15:85 ethyl acetate: methylene chloride) gave 323 mg of a white solid in 73% yield ($R_f = 0.40$, 15:85 ethyl acetate:methylene chloride). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.60 (d, $J = 1$ Hz, 1H), 7.31 (m, 20H), 5.01 (m, 4H), 4.55 (m, 4H), 4.15 (m, 2H), 3.92 (m, 1H), 3.84 (dd, $J =$ 1 Hz, $J = 3.8$ Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 202.44, 137.25, 136.94, 135.82 (d), 128.79, 128.74, 128.61, 128.47, 128.44, 128.29, 128.24, 128.18, 128.16, 82.11, 77.35, 77.27, 73.79, 73.32, 69.64 (d), 65.01 (d). ^{31}P NMR (160 MHz, $CDCl_3$) δ: −1.094. MS (CI + CH_4): 561.

(2*R*,3*R*)-Phosphoric Acid Dibenzyl Ester 2,3-bis(benzyloxy)-4-hydroxypentyl Ester 11. Ceric chloride was dried at 140 °C in vacuo for 2 h, cooled to room temperature, dissolved

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in THF (5 mL), and stirred at room temperature for 2 h. Methyl iodide (1 g, 7 mmol, 439 μL) was added with stirring to fresh lithium shavings in diethyl ether (3.5 mL). This mixture was stirred for 30 min at room temperature under a reflux condenser. Then, 500 μL of the resulting methyllithium solution was added to the cerium chloride−THF mixture at −78 °C and stirred for 1 h. Aldehyde **10**, dissolved in THF (5 mL), was added dropwise to the methyllithium−cerium chloride mixture, and stirring was continued at −78 °C for 3 h. The reaction mixture was quenched by addition of saturated ammonium chloride (0.5 mL) and warmed to room temperature. Water was added (10 mL) and the aqueous phase was extracted with chloroform (3 × 5 mL). The organics were combined, extracted with brine (3 × 5 mL), and dried over anhydrous magnesium sulfate, and the solvents were removed in vacuo. Flash chromatography (20:80 ethyl acetate:methylene chloride) gave 105 mg of the two possible diastereomers as a yellow oil in 33% yield (R_f = 0.35, 20:80 ethyl acetate:methylene chloride). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.27 (m, 20H), 5.05 (m, 4H), 4.58 (m, 4H), 4.21 (m, 2H), 3.90 (m, 2H), 3.33 (m, 1H), 1.19 and 1.12 (d, J = 6.3 and 6.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 138.05, 137.97, 137.92, 137.58, 135.87 (d), 128.72, 128.70, 128.67, 128.65, 128.60, 128.57, 128.53, 128.50, 128.36, 128.27, 128.13, 128.07, 128.06, 128.03, 127.96, 127.91, 81.84, 80.83, 78.31 (d), 77.91 (d), 74.82, 73.66, 73.31, 73.17, 69.50 (d), 69.41, 67.35, 67.27 (d), 66.99 (d), 66.88, 20.35, 19.91. ^{31}P NMR (160 MHz, $CDCl_3$) δ: −0.717, −0.845.

(2*R*,3*S*)-Phosphoric Acid Dibenzyl Ester 2,3-Bis(benzyloxy)-4-oxopentyl Ester 12. Dess−Martin reagent (258 mg, 0.000608 mol) and **11** (100 mg, 0.00017 mol) were stirred in methylene chloride (15 mL) for 12 h at room temperature. Diethyl ether (10 mL) was added, and the reaction was quenched by the addition of saturated sodium thiosulfate (5 mL) and saturated sodium bicarbonate (5 mL). The mixture was stirred until clear and the aqueous phase was extracted with diethyl ether (2 × 10 mL). The organics were combined and washed with saturated sodium bicarbonate (2 × 10 mL), water (10 mL), and brine (10 mL). The organics were dried over anhydrous magnesium sulfate, and the solvent was removed in vacuo to give 73 mg of a yellow oil in 74% yield (R_f = 0.4, 20:80 ethyl acetate:methylene chloride). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.31 (m, 20H), 4.99 (m, 4H), 4.48 (m, 4H), 4.09 (m, 2H), 3.92 (m, 1H), 3.85 (d, J = 3.4 Hz, 1H), 2.09 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 210.30, 137.43, 137.02, 135.85 (d), 128.84, 128.73, 128.59, 128.49, 128.39, 128.20, 83.74, 78.40 (d), 74.09, 73.73, 69.63, 65.50 (d), 27.96. ^{31}P NMR (160 MHz, $CDCl_3$) δ: −0.385. MS (CI + CH_4): 575.

1-Deoxy-D-xylulose-5-phosphate 1. Compound **12** (10 mg, 0.0000174 mol) was dissolved in absolute ethanol (8 mL). Pearlman's catalyst (20% $Pd(OH)_2$ on carbon) was added (12 mg) and the reaction was stirred for 6 h at room temperature under a positive pressure of hydrogen. The catalyst was removed by filtering through Celite, and the solvent was removed in vacuo to give 4 mg of a clear film in 99% yield. ^{1}H NMR (400 MHz, D_20) δ: 4.27 (s, 1H), 4.19 (t, J = 6.1 Hz, 1H), 3.81 (m, 2H), 2.11 (s, 3H). ^{13}C NMR (100 MHz, D_20) δ: 212.08, 76.12, 69.33 (d), 65.17 (d), 25.15. ^{31}P NMR (160 MHz, D_2O) δ: 0.837.

Partial Purification of 1-Deoxy-D-xylulose-5-phosphate Synthase. *E. coli* JM109 (pUCBM20*dxs*)[7] was grown in LB medium supplemented with ampicillin (100 mg/L) at 37 °C, induced at an OD_{600} of 0.8 with IPTG (0.4 mm), and harvested by centrifugation after an additional 4 h of growth. The cell pellet was resuspended in buffer A (50 mm Tris-HCl, 1 mm DTT, 0.5 mm thiamin pyrophosphate, 5 mm $MgCl_2$, pH 7.5), sonicated in a Branson sonicator (10 30-s pulses at 40 W output, duty cycle 50%), and centrifuged (1 h at 38000*g*). Ammonium sulfate (225 g/L, 40% saturation) was added to the resulting cell-free extract at 4 °C. The mixture was centrifuged (1 h at 38000*g*), and the precipitate was dissolved in buffer A, desalted by repeated ultrafiltration through a YM30 membrane, and applied to a 100 mL bed volume Q-Sepharose HP anion-exchange column. The enzyme eluted in a linear NaCl gradient at 0.1−0.2 m NaCl. The purification was monitored by SDS−PAGE, which indicated that the partially purified enzyme was 70% pure.

Enzymatic Synthesis of 1-Deoxy-D-xylulose-5-phosphate, 1. Fructose-1,6-diphosphate **13** (trisodium salt, 406 mg) and pyruvate **16** (sodium salt, 220 mg) were dissolved in 36 mL of buffer A to give final concentrations of 25 mM **13** and 50 mM **16**. Triosephosphate isomerase (200 units), rabbit muscle aldolase (40 units), and partially purified 1-deoxy-D-xylulose-5-phosphate synthase (4 mg) in a volume of 4 mL of buffer A were added. To monitor the reaction progress, samples were removed, dephosphorylated with alkaline phosphatase, and analyzed for **2** by HPLC (Aminex 87 H column, eluted with 6 mM H_2SO_4, and monitored at 195 nm, retention time of **2** was 12 min). After 12 h the reaction was judged to be 80% complete. Another 203 mg of **13** and 110 mg of **16** were added to the reaction mix. After 24 h the reaction was over 90% complete.

Purification of the Enzymatically Synthesized 1-Deoxy-D-xylulose-5-phosphate, 1. The reaction mixture was deproteinized by ultrafiltration using a Centriprep-30 membrane. A 4 mL portion of 1 M $BaCl_2$ solution was added and the cloudy mixture was centrifuged for 10 min at 3000 rpm. The pellet was washed with water (2 × 10 mL) and **1** was precipitated from the washings by addition of ethanol (60 mL) followed by standing at −20 °C for 30 min. After centrifugation (10 min, 3000 rpm) the pellet was washed twice with ice cold 70% ethanol (2 × 5 mL) and dried. The pellet was redissolved in water (8 mL), insoluble material was removed by centrifugation, and the solvent removed using a Speed Vac to give **1** as a light orange powder (707 mg, 47% yield). ^{1}H NMR (400 MHz, D_20) δ: 4.35 (s, 1H), 4.17 (t, J = 5.9 Hz, 1H), 3.71 (m, 2H), 2.12 (s, 3H).[12]

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(12) The C3 epimer of **1** can be clearly differentiated by NMR from **1** and the stereochemistry at C4 is determined by the stereochemistry of **14**. Therefore, the configuration of the enzymatic product is 3*S*,4*R*. In addition, the enzymatic product is a substrate for ThiSG. The enantiomer of **1** would not be a substrate.