An Improved Preparation of D-Glyceraldehyde 3-Phosphate and Its Use in the Synthesis of 1-Deoxy-D-xylulose 5-Phosphate

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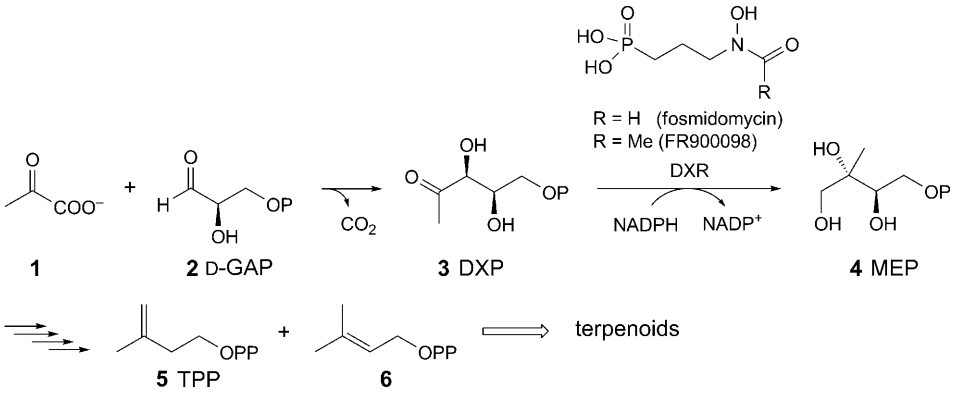
D-Glyceraldehyde 3-phosphate (=D-GAP; **2**) was prepared by an improved chemical method (*Scheme 2*), and it was then employed to synthesize 1-deoxy-D-xylulose 5-phosphate (= DXP; **3**) which is enzymatically one of the key intermediates in the MEP (**4**) terpenoid biosynthetic pathway (*Scheme 1*). The recombinant DXP synthase of *Rhodobacter capsulatus* was used to catalyze the condensation of D-glyceraldehyde 3-phosphate (**2**) and pyruvate (=2-oxopropanoate; **1**) to produce the sugar phosphate **3** (*Scheme 2*). The simple two-step chemoenzymatic route described affords DXP (**3**) with more than 70% overall yield and higher than 95% purity. The procedure may also be used for the synthesis of isotope-labeled DXP (**3**) by using isotope-labeled pyruvate.

Introduction. – Terpenoid natural products are found essentially in all forms of life. The more than tens of thousands of naturally occurring terpenes comprise physiologically important compounds such as vitamines A and D, cholesterol, steroid hormones, chlorophylls, carotenoids, and pharmaceutically active compounds like taxol, artemisinin, and ginkgolides, just to mention a few. The pioneering studies of *Bloch* and coworkers by using yeast and animal cells have led to the elucidation of the mevalonate (MVA) pathway of terpenoid biosynthesis [1]. Recently, *Arigoni*, *Rohmer*, and coworkers independently made the discovery by studies on certain bacteria that there is a second biochemical pathway of terpenoid biosynthesis, namely the 2-methyl-D-erythritol 4-phosphate (= MEP; **4**) pathway (*Scheme 1*) [2][3]. In this nonclassic biosynthetic pathway, the key intermediate is 1-deoxy-D-xylulose 5-phosphate (= DXP; **3**) which is converted into MEP (**4**). The first committed intermediate **3** of this route to **4** by a two-step process catalyzed by the DXP reductoisomerase (DXR) in the present of NADPH, is biosynthesized from pyruvate (=2-oxopropanoate; **1**) and D-glyceraldehyde 3-phosphate (=D-GAP; **2**) by the catalytic action of DXP synthase (= DXS), a thiamine diphosphate dependent enzyme. Subsequently, **4** is transformed by four consecutive enzyme reactions into 3-methylbut-3-en-1-yl diphosphate (= isopentenyl diphosphate = IPP; **5**) and 3-methylbut-2-en-1-yl diphosphate (= dimethylallyl diphosphate; **6**), the two universal building blocks for the terpenoids [4]. Recent research has established that all enzymes of this new pathway are unexplored targets for the design of novel types of antibacterial and antiparasitic drugs [5][6]. The concept has been validated by the discovery that fosmidomycin, a long-

Scheme 1. *1-Deoxy*-D-*xylulose 5-Phosphate* (**3**) *as Precursor for Terpenoids* via *the MEP* (**4**) *Pathway*



known antibiotic and its acetyl congener FR900098 are indeed inhibitors of DXR, the second enzyme of the MEP pathway [3], and are active against bacteria as well as the malaria parasite [7–10]. To find new DXR inhibitors and determine their bioactivity, there is now a demand for larger quantities of DXP (**3**), the natural substrate of DXR.

Over the last two decades, some chemical and enzymatic methods including labeling with stable or radioactive isotopes, have been established by several groups due to the biological importance of DXP [11]. Generally, two strategies were exploited in the chemical synthesis of this sugar phosphate. Efficient approaches to the target compound employed commercially available starting materials which already possess the required configuration at certain C-atoms corresponding to the two asymmetric centers of DXP [12–16]. These 6–8 steps reaction sequences afforded optically pure final products with low to medium overall yields. In addition, the iterative protection and deprotection steps were often critical in these schemes, decreasing the overall yield and requiring purification of the final product. A second method involved the preparation of the required DXP C-atom chain from achiral precursors. In this case, the two chiral centers of the target molecule were produced by asymmetric dihydroxylation of an achiral α,β-unsaturated pentan-2-one derivatives with a chiral osmium tetraoxide complex (5–7 steps, 84–87% ee values, and 6.5–23% overall yields) [17–19].

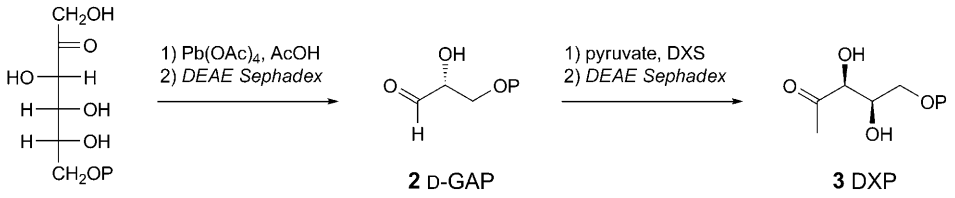
In comparison to chemical syntheses, the enzymatic procedures always have the combined advantages of *i*) short reaction time, *ii*) easy access to isotope-labeled products from commercially available precursors, *iii*) virtually perfect stereoselectivity, *iv*) the simplicity of the one-pot reaction conditions in aqueous solution. Therefore, enzymatic synthesis of DXP (**3**), especially multiple-isotope-labeled DXP with the recombinant DXS represents an attractive alternative to chemical synthesis. However, the enzyme-mediated DXP synthetic methodology has not been fully explored up to now. *Taylor* and co-workers [12] reported an enzymatic route in which **3** was produced from D-fructose 1,6-diphosphate and pyruvate in 47% yield by the co-action of aldolase, triosephosphate isomerase (TIM), and partially purified DXS of *Escherichia coli*. The medium yield may result from the $BaCl_2$ precipitation/redissolution steps for the purification of the final product from the reaction buffer. Another way was set up by *Kis*, *Eisenreich*, and co-workers [20] who used recombinant DXS of *Bacillus subtilis*

to prepare isotope-labeled DXP from isotope-labeled glucose and/or isotope-labeled pyruvate as starting materials with an overall yield of *ca.* 50%. Totally 7 enzymes and an ATP-recycling system were involved in this cascade reaction, and the ion-exchange process for purification of the final product from the reaction buffer was really time-consuming.

There should be a most straightforward way to prepare DXP (**3**) enzymatically from pyruvate (**1**) and D-GAP (**2**; *Scheme 1*). But this strategy is always hampered by the lack of good-quality D-GAP since commercially available **2** is either racemic or enantiomerically impure and expensive. Although D-GAP plays a central role in fermentation and the glycolysis of carbohydrates, the methods for the preparation of this triose phosphate are rare. *Ballou* and *Fischer* published the first chemical synthesis of this bioactive compound in which D-mannitol was used as starting material [21]: Starting from 1 kg of D-mannitol only few grams of the target compound **2** were obtained after a 9-step conversion. A practical preparation of D-GAP (**2**) was reported by *Barker* and co-workers [22]. In this method, D-fructose 6-phosphate was oxidized by $Pb(OAc)_4$ to produce 2-*O*-glycoloyl-D-glyceraldehyde 3-phosphate and after subsequent mild acid hydrolysis of this intermediate and anion exchange with *DEAE Sephadex*, D-GAP was obtained in 81% yield and at least 95% purity. The main problem of this process is that the purification step took a too long time, which can lead to partial decomposition of the unstable phosphate **2** even when operating at 4°.

In this article, an improved procedure for the purification of D-GAP (**2**) is disclosed. Meanwhile, an enzymatic preparation of DXP (**3**) by using **2**, recombinant DXS of *R. capsulatus*, and commercially available pyruvate (**1**) was established (*Scheme 2*). DXP could thus be obtained in more than 70% overall yield and high purity (> 95%). This route can also be used for the preparation of isotope-labeled DXP if isotope-labeled pyruvate is used.

Scheme 2. *Chemical Synthesis of* D-*Glyceraldehyde 3-Phosphate* (**2**) *and Enzymatic Preparation of 1-Deoxy*-D-*xylulose 5-Phosphate* (**3**)



Results and Discussion. – Basically, the synthesis of D-GAP (**2**) followed the published procedure with minor improvements [22], and the crude product obtained as yellowish oil could be used for the preparation of DXP (**3**) without further workup. First, the *DEAE Sephadex* purification of the product **2** was done in accordance with the described method, but either the yield or the purity was lower than the published ones. To solve the problem, we tried elution buffers with different pH values and different total volumes. The data (see *Table*) showed that the best results were obtained when an elution buffer of pH 6.5 and 500 ml of total volume were selected. The yield of D-GAP (**2**) at pH 7.0 and 500 ml total volume was a little better, but the product was

Table. *Optimization of* D-*GAP* (**2**) *Purification on* DEAE-Sephadex A-25[a])

Buffer and volume	pH Value	Yield [%]	Purity [%][b])
0.05M AcONa + 0.6M AcONa, 250 ml each	5.5	63	90
	6.0	72	93
	6.5	83	96
	7.0	85	86
0.05M AcONa + 0.6M AcONa, 500 ml each	5.5	55	86
	6.0	62	91
	6.5	73	91
	7.0	77	82

[a]) Column was operated at 4° with a flow rate of 2.5 ml/min. [b]) Determined by ^{1}H-NMR.

slightly contaminated by glycolic acid (=2-hydroxyacetic acid). After the anion-exchange process, the fractions containing **2** were pooled and treated with *Dowex 50w* × *8* resin to remove Na^+, and the acidic eluate was concentrated *in vacuo* to give pure **2**.

DXP (**3**) was then prepared conveniently from pyruvat (**1**), DXS of *R. capsulatus*, and crude or purified D-GAP (**2**). To get a maximum production of **3**, the ratio **1/2** was determined as 2 : 1, the concentration of the DXS was determined as 0.83 μg/μl, the pH of the reaction buffer was adjusted to 7.5, and the incubation was done at 37° overnight. The final product was also purified by a combination of anion-exchange on *DEAE Sephadex* and then cation-exchange on *Dowex 50w* × *8*.

In conclusion, we have improved the preparation of the biologically important triose phosphate D-GAP (**2**) and set up a straightforward two-step chemoenzymatic synthesis of DXP (**3**), the key intermediate of the MEP (**4**) biosynthetic pathway for terpenoids, by achieving a higher yield and purity of **2**. Moreover, isotopically labeled DXP should be readily accessible by this route from isotope-labeled pyruvate.

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Experimental Part

General. Plasmid pet11a-pFMH30 for the synthesis of recombinant DXS of *R. capsulatus* was a kind gift from Prof. *C. Dale Poulter*, Chemistry Department of the University of Utah. The bacterial strain *E. coli* BL21(DE3) was from the stock of this institute, Ni-NTA agarose resin was from *Qiagen*, sodium pyruvate was from *Fluka*, and *Dowex® 50w* × *8–200* resin (H^+ form, 100–200 mesh) and *DEAE Sephadex A-25* (Cl^- form) were from *Sigma*. All other chemicals used were of anal. reagent grade. ^{1}H- and ^{31}P-NMR: *Varian-Inova-400* NMR spectrometer in D_2O; δ in ppm, J in Hz. MS: *Thermo Fisher LTQ XL* mass spectrometer; m/z (rel. %).

Preparation of DXS. Recombinant *R. capsulatus* DXS was prepared according to the published procedures with plasmid pet11a-pFMH30 transformed *E. coli* BL21-DE3 competent cells [23][24]. The yield of the protein was 21 mg per liter medium.

D-*Glyceraldehyde 3-Phosphate* (= *(2*R*)-2,3-Dihydroxypropanal 3-Phosphate* = *(2*R*)-2-Hydroxy-3-oxopropyl Dihydrogen Phosphate* = D-GAP; **2**). At r.t., disodium D-fructose 6-phosphate (1.0 g, 3.3 mmol) was moistened with H_2O (1.6 ml) and then dissolved in glacial AcOH (132 ml) with efficient

stirring. Upon dissolution of the salt, conc. H_2SO_4 (0.36 ml) was added, and $Pb(OAc)_4$ (5.9 g, 13.3 mmol) was added during 15 min in 10 portions. After 2 h, oxalic acid dihydrate (1.6 g, 12.7 mmol) was added, and stirring was continued for an additional 30 min. The suspension was centrifuged[1]) at 4000 rpm at 4° for 10 min, and the supernatant was concentrated at 30° *in vacuo* to *ca.* 5 ml by means of a rotary evaporator. The residual solid was washed with H_2O (3×20 ml), the concentrate and washings were combined, and $Ba(OAc)_2$ (3.3 g, 12.9 mmol) was added with efficient stirring at 4° for 15 min. The white suspension was centrifuged at 4000 rpm at 4° for 10 min, the residual solid was washed with H_2O (2×5 ml), and the supernatant and washings were combined and treated with *ca.* 60 ml of *Dowex 50w* × *8* resin (H^+ form). The suspension was filtrated, and the acidic soln. was concentrated to *ca.* 3 ml and stored at 25° for 18 h. Then the soln. was evaporated *in vacuo* to give crude **2** as yellowish oil. For purification, the crude **2** was dissolved in H_2O (2 ml) and the pH of the soln. adjusted to pH 6.5 with 2M NaOH. Then the soln. was applied to a 1.0×18 cm column of *DEAE Sephadex A-25* (acetate form) at 4°, and the product was eluted with a linear gradient of AcONa (0.05M, pH 6.5, and 0.6M, pH 6.5, 250 ml each, flow rate 2.5 ml/min). The combined fractions containing **2** were treated with excess *Dowex 50w* × *8* resin (H^+ form) at r.t., and concentrated twice *in vacuo* at 30° to remove AcOH: oily **2** (0.47 g, 84.8%). ^{1}H-NMR: 4.95 (*d*, $J = 5.6$, H–C(1)); 4.00 (*m*, CH_2(3)); 3.67 (*m*, H–C(2)). ^{31}P-NMR (121.5 MHz): 2.32 (*s*). ESI-MS (neg.): 169 (100, $[M-H]^-$).

1-Deoxy-D-*xyl-2-ulose 5-Phosphate* = *1-Deoxy*-D-threo-*pent-2-ulose 5-Phosphate* = *(2*R,*3*S*)-2,3-Dihydroxy-4-oxopentyl Dihydrogen Phosphate* = DXP; **3**). To a soln. containing 120 mM *Tris* · HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM thiamine pyrophosphate, and 2 mM dithiothreitol, purified **2** (100 mg, 0.59 mmol) or crude **2** (145 mg, containing 45 mg of glycolic acid) and sodium pyruvate (**1** · Na^+; 130.3 mg, 1.18 mmol) were added. After the pH of the mixture was adjusted to 7.5 with 2M NaOH, the reaction was initiated by addition of recombinant DXS of *R. capsulatus* (1 mg, total volume 1.2 ml) and incubated at 37° overnight. Then the mixture was directly applied to a 1.5×18 cm column of *DEAE Sephadex A-25* (formate form, 25 ml) at 4° which had been equilibrated with 80 ml of 0.06M $HCOONH_4$ (pH 8.0) beforehand. The product was then eluted with a linear gradient of 0.06–0.68M $HCOONH_4$ (0.06M and 0.62M, pH 8.0, 250 ml each, flow-rate 2.5 ml/min, fractions of 15 ml). The combined fractions containing **3** were treated with excess *Dowex 50w* × *8* resin (H^+ form) to remove NH_4^+. The acidic eluate was collected on ice and concentrated repeatedly *in vacuo* to remove HCOOH: oily yellowish **3** (105 mg, 83.2%). ^{1}H-NMR: 2.29 (*s*, Me(1)); 4.00 (*m*, CH_2(5)); 4.35 (*ddd*, $J = 2.0, 2.0, 1.0$, H–C(4)); 4.48 (*d*, $J = 2$, H–C(3)). ^{31}P-NMR (121.5 MHz): 1.14 (*s*). ESI-MS (neg.): 213 (100, $[M-H]^-$).

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[1]) In [22], the suspension was filtered through *Celite*. The process took a too long time, especially when it was done at 4°.

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