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Microbial synthesis of 3-deoxy-D-*erythro*-hex-2-ulosonic acid 6-phosphate

Bernd R. Knappmann [a], Mohamed A. El-Nawawy [b], Hans G. Schlegel [b] and Maria-Regina Kula [a,*]

[a] *Institut für Enzymtechnologie der Heinrich-Heine Universität Düsseldorf, Postfach 2050, W-5170 Jülich (FRG)*

[b] *Institut für Mikrobiologie der Georg-August-Universität Göttingen, Griesebachstr. 8, W-3400 Göttingen (FRG)*

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ABSTRACT

A microbial route was explored for the synthesis of 3-deoxy-D-*erythro*-hex-2-ulosonic acid 6-phosphate (2-keto-3-deoxy-6-phosphogluconate, KDPG). Two strains of bacteria, *Alcaligenes eutrophus* H16 F34 (DSM 529) and *Escherichia coli* DF 71 (CGSC 4880), lacking in KDPG-aldolase activity were tested for excretion of KDPG. Using pyruvate and gluconate as carbon sources, *Alcaligenes eutrophus* H16 F34 accumulated and excreted 3-deoxy-D-*erythro*-hexulosonic acid 6-phosphate into the culture broth, while the *E. coli* strain, using pyruvate and glucuronate, failed. KDPG was isolated from the culture supernatant of *Alcaligenes eutrophus* H16 F34 in 78% yield and 5 g scale with respect to the consumed gluconate.

INTRODUCTION

"There is no convenient chemical means available for synthesizing KDPG." This disappointing statement was made in 1966 by Meloche and Wood[1]. 3-Deoxy-D-*erythro*-hex-2-ulosonic acid 6-phosphate (2-keto-3-deoxy-6-phospho-gluconate, KDPG), was first proposed in 1952 by Entner and Doudoroff[2] as an intermediate in glucose metabolism and later shown to be part of an alternate glucose degradative pathway in some bacteria[3]. These microorganisms belong to the group of pseudomonads and *Zymomonas*, and are lacking the key enzyme of glycolysis, fructose-1,6-bisphosphate aldolase (EC 4.1.2.13). For further investigations of this unusual way of glucose degradation, the so-called Entner–Doudoroff or KDPG-pathway, KDPG and other sugar-derivatives had to be prepared. This was first accomplished by isolation from natural sources[4]. KDPG was synthesized enzymi-

* Corresponding author.

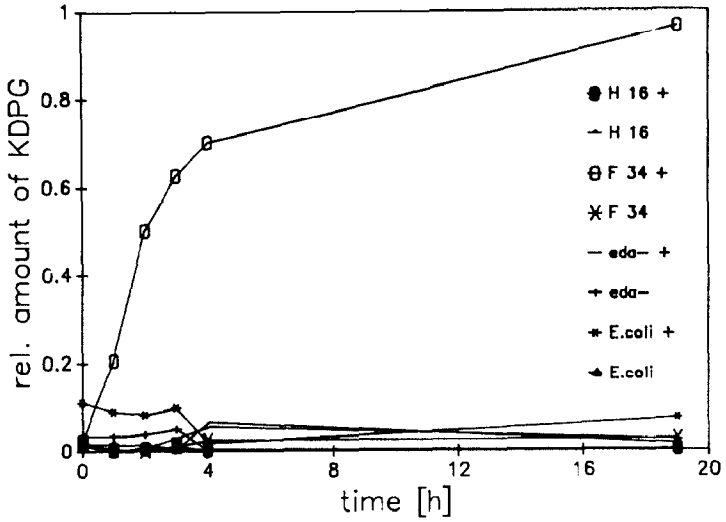


Fig. 1. KDPG-excretion of several bacterial strains. Two strains of *A. eutrophus* (H16, DSM 428, and H16 F34, DSM 529) and two *E. coli*-strains ("wildtype", DSM 498, and the KDPG-aldolase-negative mutant eda$^-$, CGSC 4880) were grown on pyruvate as carbon source. + Indicates addition of D-gluconate in the case of *Alcaligenes*-strains or D-glucuronate in the case of *E. coli* strains, respectively. KDPG-content of the culture broth was determined by the TBA-assay.

cally with whole cells[5], or with more or less crude preparations of KDPG-aldolase[3] (EC 4.1.2.14)[3], or 6-phosphogluconic acid dehydratase[1,6–8] (EC 4.1.2.12). The chemical synthesis of KDPG was first achieved in 1975 by Trigalo et al.[9] by oxidation of glucometasaccharinic acid 6-phosphate. To our knowledge, no other attempt to synthesize KDPG has been published so far. This report explores a microbial route to KDPG.

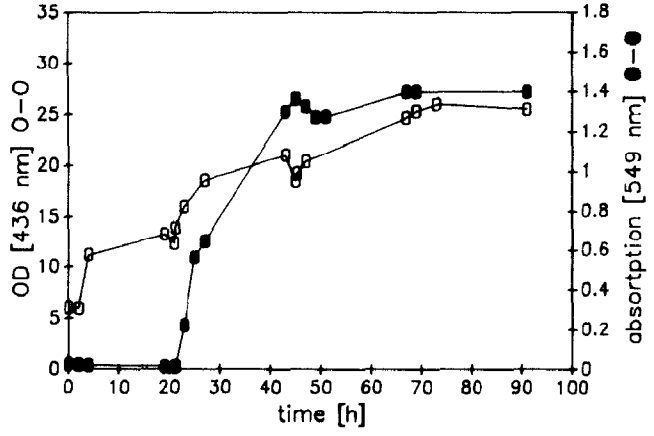


Fig. 2. Cultivation of *Alcaligenes eutrophus* F34 H16. The growth of the culture was followed by measuring the absorbance at 436 nm (○———○). Excretion of KDPG was determined with the TBA-assay and recorded as absorbance at 549 nm (●———●).

DISCUSSION

Two strains of microorganisms lacking KDPG-aldolase activity were tested for excretion of KDPG, *Alcaligenes eutrophus* H16 F34 (DSM 529) [10] and *Escherichia coli* DF 71 (CGSC 4880)[11]. *A. eutrophus* degrades hexoses via the Entner–Doudoroff pathway, while *E. coli* has to be induced to use this pathway to produce KDPG by feeding with uronic acids. These strains were compared to the "wild-type" strains *Alcaligenes eutrophus* H16 (DSM 428) and *Escherichia coli* (DSM 498) with respect to their excretion of KDPG upon addition of D-gluconate (for *A. eutrophus*) or D-glucuronate (for *E. coli*). With pyruvate as the sole carbon source, no excretion of KDPG was detectable. Upon addition of D-gluconate or D-glucuronate, respectively, *A. eutrophus* H16 F34 was the only strain that excreted KDPG into the culture broth, while the mutant *E. coli* strain, known to accumulate KDPG when fed with D-glucuronate, excreted no detectable amounts of KDPG (Fig. 1). The strain *Alcaligenes eutrophus* H16 F34 was employed for the microbial production of KDPG, because larger amounts and simpler purifications could be expected compared to *E. coli*. As shown in Fig. 2, KDPG was detectable in the culture broth of *A. eutrophus* H16 F34 a short time after addition of D-gluconate. KDPG was isolated in 78% yield (relative to consumed D-gluconate) from the spent medium of *A. eutrophus* H16 F34 by a multi-step procedure[12]. The cell-free broth was lyophilized, and material soluble in absolute ethanol was extracted from the solids. KDPG was solubilized in 40% ethanol, further purified by anion exchange chromatography and finally precipitated as the lithium salt. This

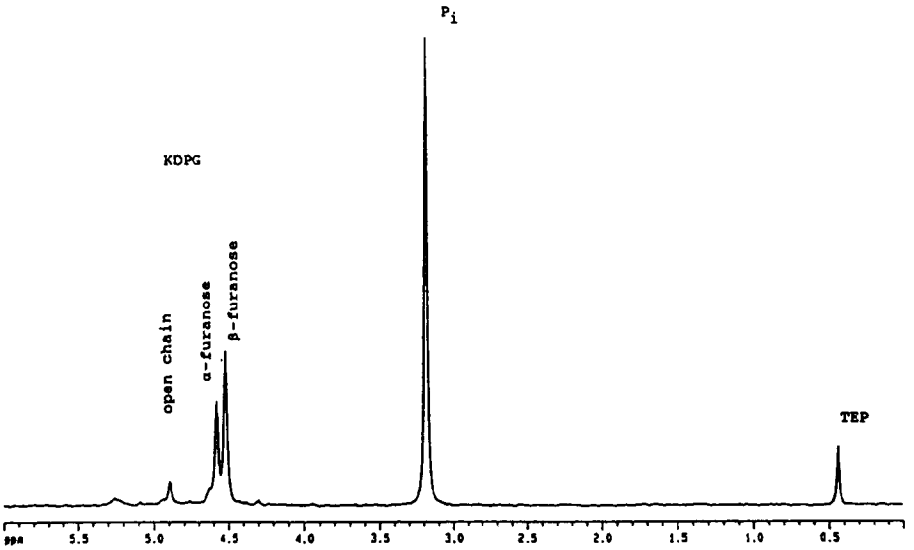
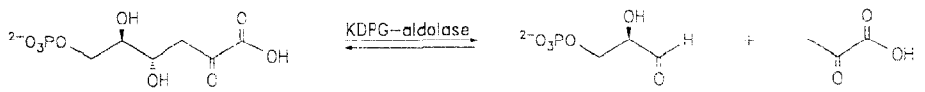


Fig. 3. ^{31}P NMR of KDPG in aqueous solution, pH 8.2. TEP (triethylphosphate) was used as standard. Inorganic phosphate (P_i) was used for correlation. Three forms of KDPG (open chain, α- and β-furanose) are distinguishable.



3-Deoxy-D-*erythro*-hex-2-ulosonic acid 6-phosphate

Scheme 1. In vivo reaction of KDPG-aldolase.

purification scheme resulted in 5-g quantities of KDPG, 82% pure as shown by a colorimetric[13] and a coupled enzymic test[14]. ^{31}P NMR data are presented in Fig. 3. The three signals arise from the α-furanose (37%), β-furanose (52%) and open-chain form (11%) of KDPG. The relative proportions are in agreement with the ^{13}C NMR data reported by Midelfort et al.[15]. Residual components in the preparation were inorganic salts, mainly phosphates[16], and small amounts of organic material which can be separated by gel permeation chromatography, if desired. Other sugar phosphates are present, if at all, only in very small amounts.

In order to produce KDPG by an alternative chemo-enzymic way, various unphosphorylated intermediates were synthesized by chemical methods[17–21] and tested for enzymic phosphorylation using hexokinase[22,23] or gluconate kinase, respectively. Under the reaction conditions employed (pH 6.9, ambient temperature, 1.6 molar excess of ATP), and with hexokinase alone, only a slow phosphorylation of 3-deoxy-D-*erythro*-hexos-2-ulose, glucometasaccharinic acid and 3-*deoxy*-D-ribo- and -D-*arabino*-4-lactone was observed after ~ 20 days.

The microbial production described here is therefore best suited when large amounts of KDPG are required.

EXPERIMENTAL

General methods. — Thin-layer chromatography (TLC) was performed according to Stahl and Kaltenbach[24] with 10:10:3 MeOH–$CHCl_3$–H_2O as mobile phase. *p*-Anisaldehyde or H_2SO_4 in MeOH (20% v/v) were used as spray reagents. Phosphorylated compounds were chromatographed and detected following the method of Hanes and Isherwood[25]. Carbohydrates and phosphorylated compounds were separated by precipitation according to Umbreit et al.[4]. HPLC was carried out using a carbohydrate resin column (Chromatographie Service, Langerwehe, FRG) and H_2SO_4 (4 mM) as eluent (0.5 mL/min). Carbohydrates and phosphorylated compounds were monitored spectrophotometrically at 200 nm. Aldehydes and ketones were detected using the semicarbazide method of MacGee and Doudoroff[3]. 3-Deoxy sugars and α-keto acids were detected by the thiobarbituric acid (TBA) method as described by Weissbach and Hurwitz[13]. Gluconate was determined by a method described by Möllering and Bergmeyer[12]. Phosphate was detected by the method of Taussky and Shorr[16]. KDPG[14] and pyruvate[26] were determined enzymically in lactate dehydrogenase-linked spectrophotometric assays

at 340 nm. Glyceraldehyde 3-phosphate was determined enzymically according to the product information "enzymatic assay of D-glyceraldehyde-3-phosphate" of Sigma Chemical Co. Optical rotations were measured in a Perkin–Elmer 241 polarimeter (Überlingen, FRG). NMR data were obtained from ^{13}C and ^{1}H NMR spectra measured in D_2O solutions with a Varian VXR 300 spectrometer at 75 and 300 MHz. Chemical shifts were measured from the resonance signals of Me_4Si or sodium 4,4-dimethyl-4-silapentanoate. ^{31}P NMR spectra were measured for solutions in H_2O, with a Bruker AMX 400 spectrometer. Chemical shifts were measured from the resonance signal of triethyl phosphate.

Microorganisms. — Two KDPG-aldolase negative strains of microorganisms were used, *Alcaligenes eutrophus* H16 F34 (DSM 529) and *Escherichia coli* DF 71 (eda^-, CGSC 4880). Both strains were compared to the "wild type" strains *Alcaligenes eutrophus* H16 (DSM 428) and *Escherichia coli* (DSM 498). The strain *Escherichia coli* DF 71 (cda^-, CGSC 4880) developed by Fradkin and Fraenkel[11] was a gift of Dr. B. J. Bachmann from the *E. coli* Genetic Stock Center, Yale University, New Haven, CT, USA.

Nutrient agar. — The medium consisted of peptone (5.0 g), meat extract (3.0 g), agar (15 g), and H_2O (to 1 L). The final medium had a pH of ~ 7.0.

Mineral media. — Solution A1: $Na_2HPO_4 \cdot 2H_2O$ (4.5 g), KH_2PO_4 (1.5 g), NH_4Cl (1.0 g), and H_2O (to 900 mL); solution A2: $MgSO_4 \cdot 7\ H_2O$ (0.25 g), and H_2O (to 100 mL); solution B: $FeNH_4$ citrate (125 mg), $CaCl_2 \cdot 2\ H_2O$ (250 mg), and H_2O (to 250 mL); solution C: Na pyruvate (12.5 g) and H_2O (to 50 mL). All solutions were sterilized separately by autoclave.

To solution A1 (900 mL) were added: solution A2 (100 mL), trace element solution SL 6 (0.1 mL; ref. 27), and of solution B (10 mL). The complete mineral medium had a pH between 7.0 and 7.2.

Small-scale cultivation. — Colonies of *Alcaligenes eutrophus* H16 F34 or *Escherichia coli* (CGSC 4880), respectively, were transferred from a nutrient agar plate and suspended in 100-mL Erlenmeyer-flasks, containing complete mineral medium (10 mL) with addition of solution C (0.1 mL). Cultures were grown overnight on a rotary shaker (150 rpm, 30°C) and used as inoculum for 30 mL-cultures in 300-mL Erlenmeyer-flasks, containing solution C (0.4 mL). After 12–24 h, these cultures were transferred to 1-L cultures in 5-L dented flasks containing solution C (12 mL). Cultures were grown overnight and used for studies of KDPG-excretion. Cells were harvested by centrifugation and resuspended in freshly prepared complete mineral medium (A 436 nm = 8.0). Sodium pyruvate (150 mg per 50 mL of cell-suspension) was used as the carbon source, while D-gluconate (for *A. eutrophus*, 50 mg per 50 mL cell-suspension) and D-glucuronate (for *E. coli*, 50 mg per 50 mL cell-suspension) were used as starting material for KDPG-synthesis. *Alcaligenes eutrophus* H16 F34 excreted KDPG shortly after addition of gluconate. In suspensions of *E. coli*, KDPG was not detectable (Fig. 1).

Large-scale cultivation. — Cultivation was performed in a Biostat E-bioreactor (Braun, Melsungen, FRG) with a working volume of 10 L. Nutrient solution (6 L),

containing the minerals for 8 L, were inoculated with two 1-L cultures of *Alcaligenes eutrophus* H16 F34. Solution C was added (128 mL) and cultivation started (30°C, 400 rpm, 2 L air/min). Solution C (128 mL) and a solution of sodium D-gluconate (8 g/40 mL H_2O) were added 24 and 48 h later. Cultivation was stopped 48 h after the last addition.

Isolation of KDPG from the culture broth. — Cells were removed by centrifugation and the culture broth (8 L) was lyophilized. The dry solids were suspended in abs EtOH (10 vol with respect to the solid) and centrifuged. The pellet was washed with 5 portions of abs EtOH (5 × 50–100 mL each), and the supernatant was discarded. Then the pellet was resuspended in aq EtOH (40% EtOH, v/v, 1 L), centrifuged, and washed with aq EtOH (40% EtOH, v/v, 5 × 50–100 mL). The supernatants were pooled (~ 1400 mL), concentrated to 140 mL and acidified (pH 2) with Dowex 50W × 8, H^+ form. The resin was removed and washed with water. Liquids were collected, neutralized with NaOH, and concentrated (10–20 mL). The solution was adjusted to pH 7 and applied to a column (5.2 × 40 cm) of Dowex 1 × 8, Cl^- form, 200–400 mesh. The column was washed with a small portion of water and eluted with a linear gradient of 0 → 0.2 M HCl (2 L), followed by stepwise elution with 0.5 M HCl (0.5 L). The eluate was collected in fractions (30 mL). Fractions were tested for KDPG with the TBA-assay. Two TBA-positive peaks were eluted, separately pooled and neutralized (pH 6.9) with LiOH (1 M), concentrated (5–10 mL), brought to pH 7.6 with LiOH and precipitated for 12–24 h with abs EtOH (20 volumes) at 4°C. The two precipitates were collected by centrifugation, washed twice with abs EtOH (4°C) and dried. KDPG-aldolase assay, TLC, and NMR-data showed the second pool to be the Li salt of KDPG. The salt (5.1 g) was obtained in 82% purity as shown by treatment with KDPG-aldolase and determination of the cleavage products, pyruvate and glyceraldehyde 3-phosphate, with coupled enzymic tests. For that, a solution of the salt (0.1%, 250 μL) was pipetted into a cuvette containing triethanolamine buffer (0.1 M, 1.4 mL, pH 7.6), NADH (30 mM, 40 μL), H_2O (1.29 mL), and lactate dehydrogenase (5 mg/mL, 10 μL). Absorption was measured at 340 nm before addition of the KDPG-aldolase (10 μL) to start the reaction, and after the reaction had gone to completion. From the difference in absorption, the molar amount of NADH consumed was calculated ($\in = 6.3$ cm^2/μmol), which is equal to the amount of KDPG present in the sample. Glyceraldehyde 3-phosphate was determined in a similar way using glyceraldehyde 3-phosphate dehydrogenase as the auxiliary enzyme. Pyruvate and glyceraldehyde 3-phosphate were detected in equimolar amounts (0.71 μmol) corresponding to 82% of the initial weight. For determination of KDPG with the TBA-assay[13], a standard curve with a KDPG-solution of known concentration was used; $[\alpha]_D^{22} + 1.6°$ (*c* 3.5, H_2O).

The yield of KDPG represents 78% of the consumed gluconate. KDPG was separated from organic impurities and inorganic phosphate by gel permeation chromatography (2.6 × 80 cm, P2 Bio-Gel extra fine, BioRad, München) with water as eluent (6 ml/h) using ~ 200 mg material per run. By TLC and HPLC of

the product from the main peak, no contamination was detectable. After lyophilization, pure KDPG could be obtained in 70% yield (140 mg, 55% with respect to the consumed gluconate); $[\alpha]_D^{22} + 1.8°$ (*c* 1.4, H_2O), lit. [9] $[\alpha]_D^{22} + 6.5°$ (*c* 1, H_2O); ^{13}C NMR (D_2O): α-furanose δ 44.50 (C-3), 65.55 (d, $J_{6,P}$ 5.1 Hz, C-6), 72.63 (C-4), 86.30 (d, $J_{5,P}$ 7.5, C-5), 104.93 (C-2), and 177.46 (C-1); β-furanose δ 44.56 (C-3), 66.21 (d, $J_{6,P}$ 5.0, C-6), 72.58 (C-4), 86.68 (d, $J_{5,P}$ 8.5, C-5), 104.73 (C-2), and 178.04 (C-1); ^1H NMR (D_2O): α-furanose δ 2.03 (dd, 1 H, $J_{3,3'}$ 14.1, $J_{3,4}$ 3.4 Hz, H-3), 2.57 (dd, 1 H, $J_{3',4}$ 7.4 Hz, H-3′), 3.94 (m, 2 H, H-6,6′), 4.27 (bq, 1 H, $J_{5,6} \approx J_{4,5} \approx 4.7$ Hz, H-5), 4.41 (bdt, 1 H, $J_{3,4} \approx J_{4,5} \approx 3.7$ Hz, H-4); β-furanose δ 2.31 (dd, 1 H, $J_{3,3'}$ 13.7, $J_{3,4} = 6.4$ Hz, H-3), 2.42 (dd, 1 H, $J_{3',4}$ 6.8 Hz, H-3′), 3.94 (m, 2 H, H-6,6′), 4.15 (bq, $J_{4,5} \approx J_{5,6} \approx 4.9$ Hz, H-5), and 4.47 (dt, $J_{4,5}$ 5.0 Hz, H-4). The α- and β-assignments were based on published data[15].

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