3-Deoxy-D-*arabino*-heptulosonic Acid 7-Phosphate: Chemical Synthesis and Isolation from *Escherichia coli* Auxotrophs†

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ABSTRACT: A new chemical synthesis of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate is described and contrasted to isolation of the same molecule from the growth medium of several different auxotrophic strains of *Escherichia coli*. The higher yielding chemical synthesis begins with 2-deoxyglucose while the less time-intensive biological approach proceeds directly from glucose. Growth and accumulation aspects of whole cell biological synthesis are discussed along with various aspects of the biological purification protocol. Both approaches can be utilized to produce substantial quantities of methyl (methyl 3-deoxy-D-*arabino*-heptulopyranosid)onate, a key intermediate for semisynthetic 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate and a number of its derivatives.

The level of scrutiny to which an enzyme can be submitted is often determined by the ease, reproducibility, and flexibility of the protocol for the generation of the enzyme substrate and its analogues. Notably, advances in the enzymology of aromatic amino acid biosynthesis (the shikimate pathway) can be directly traced to improvements in the synthesis of various intermediates in this metabolic pathway.

3-Deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP)[1] (**9**) stands at the beginning of the enzyme-catalyzed cascade that starts with this seven-carbon carbohydrate and ends with the aromatic amino acids phenylalanine, tyrosine, and tryptophan. In prokaryotes and plants, DAHP is produced from phosphoenolpyruvate and erythrose 4-phosphate, the condensation of which is mediated by DAHP synthase. The DAHP is then converted into the six-membered cyclic intermediate dehydroquinic acid (Figure 1), a multistep transformation that is catalyzed by the enzyme dehydroquinate synthase (DHQ synthase). As a necessary preliminary to the investigation of the mechanism of the DHQ synthase catalyzed reaction, we required convenient access to both the substrate DAHP and the enzyme DHQ synthase. This paper reports the synthesis and biosynthesis of the substrate, and the following paper (Frost et al., 1984) presents the construction of a plasmid that allows the ready isolation of DHQ synthase in large amounts.

Two chemical routes have been reported for the synthesis of DAHP (Sprinson et al., 1963; Trigalo et al., 1975), and a single-step metal-catalyzed condensation of oxaloacetate and erythrose 4-phosphate to form a mixture of DAHP and its C-4 epimer (3-deoxy-D-*ribo*-heptulosonic acid 7-phosphate) has also been published (Herrmann & Poling, 1975). We have found, however, that these routes to DAHP are both too costly for the production of DAHP in quantity and too inflexible for the synthesis of the substrate analogues needed in our mechanistic studies on DHQ synthase. We have therefore elaborated a new chemical synthesis for DAHP and report the protocol for the isolation of DAHP from mutants of *Escherichia coli* lacking DHQ synthase. Both routes to DAHP are presented here, and their merits are compared.

Experimental Procedures

^{1}H and ^{13}C NMR spectra were recorded on a Jeol FX-270. Chemical ionization (CI) mass spectra were taken on a Kratos MS-50 L. Flash chromatography (Still et al., 1978) was carried out on silica gel 60 (40–63 μm, from E. Merck) and analytical thin-layer chromatography on precoated plates of silica gel 60 F-254 (0.25 mm, from E. Merck). Diethylaminoethylcellulose (DE-52) was from Whatman.

E. coli AB-2847 was a gift of B. Bachmann, Yale University. *E. coli* JB-5 resulted from a phage transduction from *E. coli* NK-6626 [see Frost et al. (1984)] generously provided by N. Kleckner of Harvard University. DAHP and its dephospho derivative were assayed by the method of Sprinson (Gollub et al., 1971).

2-Deoxy-D-arabino-hexose Propylene Dithioacetal (**2**). 2-Deoxyglucose (5.0 g, 0.030 mol) and 1,3-propanedithiol (3.84 g, 0.036 mol) were dissolved in a solution (80 mL) of concentrated hydrochloric acid (40 mL) and ethanol (40 mL). After this was allowed to stand overnight at room temperature, the reaction mixture solidified. This solid was isolated by filtration, washed with ethanol, and then dried in vacuo to yield a white solid (3.6 g). Ethanol (80 mL) was added to the filtrate, and the mixture left at 4 °C overnight to yield a second crop of crystals (2.7 g). The combined yield was 80% (based on 2-deoxyglucose): ^{1}H NMR (270 mHz, D_2O) [$(CH_3)_3Si(CH_2)_3SO_3H$] δ 1.70–1.95 (m, 2 H), 1.95–2.25 (m, 2 H), 2.80–3.10 (m, 4 H), 3.45 (dd, 1 H), 3.60–3.85 (m, 3 H), 4.17 (dq, 1 H), 4.25 (dd, 1 H); ^{13}C NMR (67.8 mHz, CD_3OD) (Me_4Si) δ 27.3, 30.7, 31.2, 40.7, 45.0, 65.1, 68.0, 73.1, 74.7.

2-Deoxy-3,4:5,6-di-O-isopropylidene-D-arabino-hexose Propylene Dithioacetal (**3**). 2-Deoxy-D-*arabino*-hexose propylene dithioacetal (**2**) (5.0 g, 0.020 mol) was dissolved in acetone (100 mL), and concentrated sulfuric acid (4 drops) was added. After this was allowed to stand overnight at room temperature, a few drops of concentrated ammonium hydroxide were added. The solid that precipitated was removed by filtration, and the filtrate was concentrated to dryness. Flash chromatography on silica (ethyl acetate–hexane, 1:1 v/v) afforded a white solid (5.5 g, 82%): ^{1}H NMR (270 mHz, $CDCl_3$) (Me_4Si) δ 1.34 (s, 3 H), 1.37 (s, 3 H), 1.39 (s, 3 H), 1.41 (s, 3 H), 1.83–2.05 (m, 2 H), 2.05–2.25 (m, 2 H), 2.80–2.97 (m, 4 H), 3.55 (t, 1 H), 3.90–3.97 (m, 1 H), 4.00–4.30 (m, 4 H); ^{13}C NMR (67.8 mHz, $CDCl_3$) (Me_4Si) δ 25.3, 26.0, 26.8, 27.0, 27.3, 29.7, 30.1, 39.8, 43.8, 67.7, 76.8, 76.9, 81.3, 109.3, 109.6.

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[1] Abbreviations: DAHP, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate; DAH, 3-deoxy-D-*arabino*-heptulosonic acid; DHQ, dehydroquinic acid.

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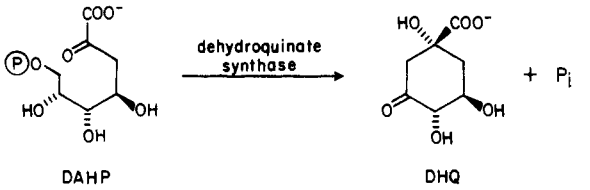


FIGURE 1: Reaction catalyzed by dehydroquinate synthase.

*Methyl 3-Deoxy-4,5:6,7-di-O-isopropylidene-*D*-arabino-heptulosonate* (**4**). 2-Deoxy-3,4:5,6-di-*O*-isopropylidene-D-*arabino*-hexose propylene dithioacetal (**3**) (1.0 g, 3 mmol) was dissolved in dry, degassed tetrahydrofuran (21 mL) under nitrogen. The temperature was lowered to −40 °C, and *n*-butyl lithium (4.0 mL, 6.6 mmol) was added by syringe. Metallation was allowed to proceed for 2 h at −40 °C. This solution was then added slowly to a solution of dry methyl chloroformate (9.3 mL, 0.12 mol) in dry, degassed tetrahydrofuran (9.3 mL) at −78 °C under nitrogen. After this was allowed to stand at −78 °C for 45 min, the solution was stirred at −40 °C for a further 45 min. The mixture was then warmed to room temperature and stirred for 1 h. Methylene chloride was then added to the solution, the precipitated lithium salts were removed by filtration, and the filtrate was concentrated under reduced pressure.

The resulting oil was dissolved in acetone (20 mL, containing 3% water v/v) and this solution slowly added to *N*-bromosuccinimide (4.8 g, 27 mmol) in acetone (80 mL, containing 3% water v/v) at 0 °C. When this was stirred for 15 min at 0 °C, a deep gold color developed. A solution of saturated aqueous sodium sulfite was added, and the solution was vigorously shaken until the gold color had been discharged. Hexane–methylene chloride (1:1 v/v) was added, and the organic layer was washed sequentially with water, 1 M sodium bicarbonate, water, and saturated sodium chloride. The solution was dried over magnesium sulfate, concentrated under reduced pressure, and subjected to flash chromatography (hexane–ethyl acetate, 2:1 v/v). Product **4** (0.54 g) was isolated in 60% yield: ^{1}H NMR (270 mHz, $CDCl_3$) (Me_4Si) δ 1.31 (s, 3 H), 1.37 (s, 6 H), 1.39 (s, 3 H), 3.23 (m, 2 H), 3.59 (t, 1 H), 3.80–4.07 (m, 2 H), 3.88 (s, 3 H), 4.14 (dd, 1 H), 4.43 (m, 1 H); ^{13}C NMR (67.8 mHz, $CDCl_3$) (Me_4Si) δ 25.2, 26.5, 26.9, 27.0, 42.8, 53.0, 67.8, 75.6, 76.8, 80.7, 109.7, 109.8, 161.1, 190.8.

*Methyl (Methyl 3-deoxy-*D*-arabino-heptulopyranosid)onate* (**5**). Methyl 3-deoxy-4,5:6,7-di-*O*-isopropylidene-D-*arabino*-heptulosonate (**4**) (2.1 g, 6.8 mmol) in methanol [70 mL, containing 4% HBr (w/v)] was refluxed for 24 h. After being cooled, the solution was neutralized by the addition of solid lead carbonate, with stirring. The excess lead carbonate was removed by filtration, and activated charcoal was added to the filtrate. The mixture was filtered through celite, and the filtrate was concentrated under reduced pressure. The resulting oil was triturated with ethyl acetate–tetrahydrofuran–hexane (7:3:1 v/v) to yield, after filtration, a white solid (0.75 g). The filtrate was subjected to flash chromatography (ethyl acetate–tetrahydrofuran–hexane, 7:3:1 v/v) and yielded an additional 0.12 g of white solid. The combined yield was 0.87 g (55%, based on **4**): ^{1}H NMR (270 mHz, D_2O) [$(CH_3)_3Si(CH_2)_3SO_3H$] δ 1.75 (t, 1 H), 2.36 (dd, 1 H), 3.25 (s, 3 H), 3.33–3.45 (m, 1 H), 3.53–3.64 (m, 1 H), 3.70–4.00 (m, 3 H), 3.87 (s, 3 H); ^{13}C NMR (67.8 mHz, D_2O) (CH_3CN) δ 39.3, 51.2, 53.9, 61.0, 68.6, 70.6, 74.6, 99.3, 170.6; mass spectrum (CI) of the tris(trimethylsilyl) derivative (relative intensity), 453 (43) ($M^+ + H$), 437 (100), 421 (48); mass spectrum (CI) of the underivatized material, 237 ($M^+ + H$).

*Methyl (Methyl 3-deoxy-*D*-arabino-heptulopyranosid)onate 7-(Diphenyl phosphate)* (**6**). Methyl (methyl 3-deoxy-D-*arabino*-heptulopyranosid)onate (**5**) (0.093 g, 0.4 mmol) was dissolved in dry pyridine (9 mL). After the addition of freshly distilled diphenyl phosphochloridate (0.040 mL, 0.2 mmol), the reaction was stirred under nitrogen at room temperature for 11 h. More diphenyl phosphochloridate (0.04 mL, 0.2 mmol) was then added, and the mixture was stirred for 3 h, followed by a final addition of diphenyl phosphochloridate (0.02 mL, 0.1 mmol) and stirring for 1 h. The reaction mixture was concentrated under reduced pressure and product water removed by the azeotropic distillation of two additions of toluene. Flash chromatography (ethyl acetate) of the mixture on silica yielded 0.093 g of product **6** (49%, based on **5**): ^{1}H NMR (270 mHz, $CDCl_3$) (Me_4Si) δ 1.63 (t, 1 H), 2.32 (dd, 1 H), 3.13 (s, 3 H), 3.28 (t, 1 H), 3.60–3.70 (m, 1 H), 3.75 (s, 3 H), 3.8–3.95 (m, 1 H), 4.45–4.60 (m, 2 H), 7.13–7.37 (m, 10 H); ^{13}C NMR (67.8 mHz, $CDCl_3$) (Me_4Si) δ 39.3, 50.8, 52.6, 68.1 (d), 68.8, 71.0, 72.7 (d), 99.2, 120.1, 120.2, 120.3, 120.4, 125.6, 125.6, 129.7, 129.9, 150.4, 168.4.

*Methyl (Methyl 3-deoxy-*D*-arabino-heptulopyranosid)onate 7-Phosphate* (**7**). The phosphate triester **6** (0.3 g, 0.64 mmol) was dissolved in methanol (20 mL) and vigorously stirred with platinum oxide (0.060 g) under hydrogen for 12 h. Additional catalyst (0.030 g) was then introduced and the hydrogenation continued for a further 12 h. The reaction mixture was filtered through celite, and the filtrate was concentrated to give 0.21 g of a clear, colorless oil (100%, based on **6**): ^{1}H NMR (270 mHz, D_2O) [$(CH_3)_3Si(CH_2)_3SO_3H$] δ 1.75 (t, 1 H), 2.35 (dd, 1 H), 3.24 (s, 3 H), 3.45 (t, 1 H), 3.68–3.76 (m, 1 H), 3.85 (s, 3 H), 3.80–3.97 (m, 1 H), 4.17–4.31 (m, 2 H); ^{13}C NMR (67.8 mHz, D_2O) (CH_3OH) δ 39.2, 51.2, 53.9, 65.3, 68.4, 70.2, 73.2 (d), 99.4, 170.3.

*Methyl 3-Deoxy-*D*-arabino-heptulopyranoside 7-Phosphate* (**8**). The methyl ester **7** (0.21 g, 0.68 mmol) was dissolved in 0.5 M potassium hydroxide (20 mL) and the mixture stirred for 5.5 h at room temperature. The reaction mixture was then passed down a column of Dowex 50 (H^+ form). Concentration of the eluate under reduced pressure gave 0.18 g of the acid product **8** (88%, based on **7**): ^{1}H NMR (270 mHz, D_2O) [$(CH_3)_3Si(CH_2)_3SO_3H$] δ 1.77 (t, 1 H), 2.36 (dd, 1 H), 3.27 (s, 3 H), 3.48 (t, 1 H), 3.63–3.76 (m, 1 H), 3.89–4.02 (m, 1 H), 4.20–4.33 (m, 2 H); ^{13}C NMR (67.8 mHz, D_2O) (CH_3CN) δ 39.3, 51.3, 65.6, 68.5, 70.3, 73.1 (d), 99.3, 171.8.

*3-Deoxy-*D*-arabino-heptulosonic Acid 7-Phosphate* (**9**). The methyl pyranoside **8** (0.18 g, 0.6 mmol) was dissolved in water (10 mL), and Dowex 50 (H^+ form) (10 mL) was added. The mixture was heated at 70 °C for 32 h. After this was cooled, the resin was removed by filtration, and the filtrate was neutralized to pH 7.1 with NaOH. This mixture was subjected to chromatography on a column (30 mL) of DE-52 equilibrated with 100 mM triethylammonium bicarbonate and eluted with 1 column volume of water followed by a linear gradient (200 mL + 200 mL, 100–300 mM) of triethylammonium bicarbonate, pH 7.1. Fractions containing DAHP (assayed by the thiobarbiturate method) were concentrated, and the buffer was removed by azeotropic distillation of three additions of 2-propanol. The resulting colorless oil was passed down Dowex 50 (H^+ form), and the eluate was brought to pH 7.6 by the addition of freshly prepared lithium hydroxide. The resulting white precipitate (110 mg) was isolated by filtration (63%, based on **8**): ^{1}H NMR (270 mHz, D_2O) [$(CH_3)_3Si(CH_2)_3SO_3H$] δ 1.81 (t, 1 H), 2.22 (dd, 1 H), 3.60 (t, 1 H), 3.82 (d, 1 H), 3.89–4.03 (m, 2 H), 4.05–4.15 (m, 1 H); ^{13}C NMR (67.8 mHz, D_2O) (CH_3CN) δ 39.7, 63.2, 68.8, 70.9,

73.6 (d), 96.9, 176.5; mass spectrum (CI) of the hexakis(trimethylsilyl) derivative (relative intensity), 721 (100) (M^+ + H), 705 (64), 693 (41), 649 (90), 631 (86). The material was shown to be a substrate for purified DHQ synthase (Frost et al., 1984) by using the thiobarbiturate assay (Gollub et al., 1971).

Microbiological Production of DAHP. (A) Protocol I. K_2HPO_4 (7.0 g), KH_2PO_4 (3.0 g), and $(NH_4)_2SO_4$ (1.0 g) were dissolved in water (1 L) in an Ehrlenmeyer flask (4 L) and autoclaved. $MgSO_4$ and glucose solutions were autoclaved separately, and these solutions containing glucose (5.0 g in 25 mL) and $MgSO_4$ (0.12 g in 1 mL) were added to the sterile salt solution. L-Tyrosine (0.008 g), L-phenylalanine (0.008 g), L-tryptophan (0.004 g), *p*-aminobenzoic acid (4×10^{-6} g), *p*-hydroxybenzoic acid (4×10^{-6} g), L-histidine (0.040 g), L-isoleucine (0.040 g), L-valine (0.040 g), and thiamin (0.001 g) were dissolved in H_2O (14.1 mL) and added by syringe to the solution of glucose and salts, through a sterilizing membrane. Strain JB-5 was grown up from a 3% inoculum (grown in the above medium) for 48 h at 37 °C. *E. coli* AB-2847 was treated identically, except that L-histidine, L-isoleucine, and L-valine were absent from the growth medium, and the DAHP was harvested after 32 h.

(B) Protocol II. Strain JB-5 was grown up from a 3% inoculum (grown in YT) in YT medium containing kanamycin (75 μg/mL) for 10 h at 37 °C. Cells were centrifuged at 8000*g* for 10 min and then resuspended in the same volume of the growth medium of protocol I. The cell suspension was immediately centrifuged, and the cells were resuspended in the growth medium of protocol I. Growth was continued for 47 h at 37 °C.

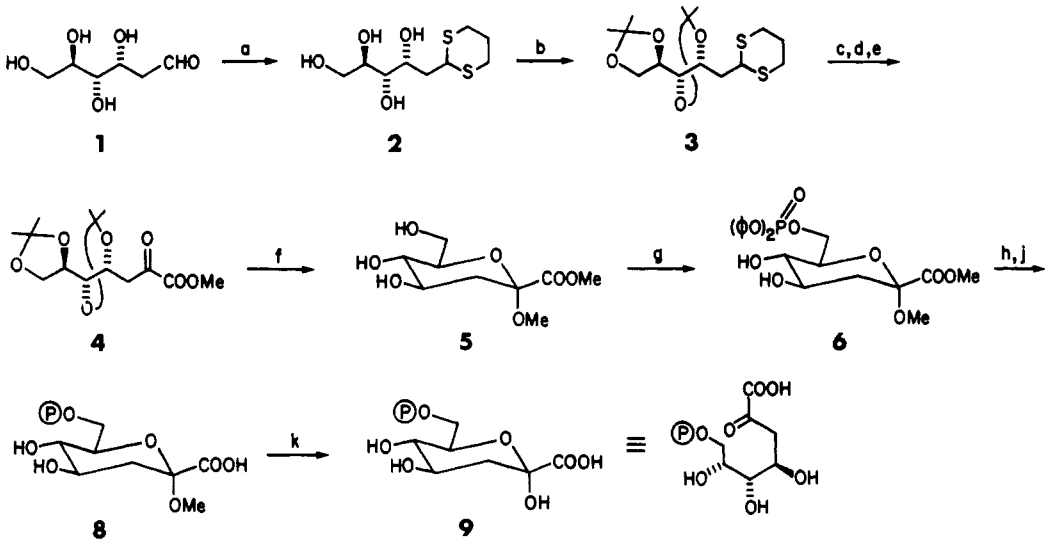
Isolation of DAHP. The cell suspension (1 L) from either of the above growth regimes was centrifuged at 8000*g* for 10 min, and the clear yellow supernatant was passed through a column (500 mL) of Dowex 50 (H^+ form) at 4 °C. The eluate was adjusted to pH 8.0 with freshly prepared 1 M lithium hydroxide. This solution was concentrated on a rotary evaporator, the temperature being kept below 30 °C. To the resulting oily residue, methanol (1 L) was added, and the mixture was vigorously stirred at 4 °C for 1 h. After removal of the precipitate by filtration, the filtrate was concentrated under reduced pressure. The residue was then dissolved in distilled, deionized water (1 L) and adjusted to pH 7.1 by the addition of 1 M lithium hydroxide. The solution was then loaded on a column (1 L) of DE-52 equilibrated with 100 mM triethylammonium bicarbonate, pH 7.1. The column was then washed with 1 L of water, and eluted with a linear gradient (4 L + 4 L, 100–300 mM) of triethylammonium bicarbonate, pH 7.1. Column fractions were assayed, and those containing DAHP and its dephospho derivative (DAH) were pooled separately and concentrated. After removal of buffer by three azeotropic distillations of added 2-propanol, the DAH and DAHP solutions were each passed down a column (100 mL) of Dowex 50 (H^+ form) at 4 °C. The DAH pool was concentrated directly. The DAHP pool was partially concentrated to remove residual carbon dioxide, the pH was adjusted to 7.6 with 1 M lithium hydroxide, and the solution then concentrated to dryness. Isolated yields based on the initial glucose present in the accumulation solution are shown in Table I. The ^1H NMR and ^{13}C NMR spectra of the material so isolated, and kinetic assay with purified DHQ synthetase, were identical with those of the synthetic DAHP (Figures 2 and 3). The DAH isolated as above was refluxed for 17 h in methanolic HBr (4% w/v). The methyl ester was isolated as described for the synthetic sample of **5** and afforded 0.16 g (2%, based on glucose). The ^1H and ^{13}C NMR spectra of this sample of **5** were identical with those of the synthetic material.

Results and Discussion

All chemical syntheses of DAHP basically require a one-carbon chain extension of 2-deoxy-D-glucose (**1**), specific phosphorylation at the primary hydroxyl group at C-7, and oxidation at C-2. Both of the routes to DAHP in the literature begin with **1**, and in the final step, the unprotected 3-deoxy-D-*arabino*-heptanoic acid 7-phosphate is oxidized to DAHP. The two methods differ in the intervening steps. The first reported synthesis (Sprinson et al., 1963) starts with a classic Kiliani chain extension to convert **1** to 3-deoxy-D-*arabino*-heptanoic acid. Subsequent steps involve tritylation of the primary hydroxyl followed by benzoylation of the secondary alcohols. Detritylation is followed by phosphorylation with diphenyl phosphochloridate. Deprotection of the benzoyl and phenyl protecting groups then leads to 3-deoxy-D-*arabino*-heptanoic acid 7-phosphate, which is then subjected to oxidation at C-2 by vanadium pentoxide. The more recent synthesis of DAHP (Trigalo et al., 1975) dispenses with a number of protection/deprotection steps by converting **1** to its methyl pyranoside and exploiting the possibility of preferential phosphorylation of the primary hydroxyl group. Subsequent deprotection of the phospho group and Kiliani chain extension then gives 3-deoxy-D-*arabino*-heptanoic acid 7-phosphate, which is oxidized at C-2 in the final step.

In our hands, the protection/deprotection sequence of the older approach was cumbersome, and the final oxidation of 3-deoxy-D-*arabino*-heptanoic acid 7-phosphate used in both the earlier routes gave a low yield of the desired product. Instead of using several steps for the chain extension of **1** and the oxidation at C-2 of 3-deoxy-D-*arabino*-heptanoic acid 7-phosphate, the synthesis described here allows the equivalent of these conversions to be accomplished in one operation (see Scheme I). Conversion of **1** to 2-deoxy-D-*arabino*-hexose propylene dithioacetal (**2**) is accomplished with acid catalysis. Subsequent reaction of **2** with acetone and a trace of sulfuric acid leads to 2-deoxy-3,4:5,6-di-*O*-isopropylidene-D-*arabino*-hexose propylene dithioacetal (**3**). Metallation of **3** with *n*-butyl lithium is followed by acylation with methyl chloroformate and removal of the propylene dithioacetal with *N*-bromosuccinimide in aqueous acetone. The metallation, acylation, and deprotection are carried out without isolation of intermediates. Methyl 3-deoxy-4,5:6,7-di-*O*-isopropylidene-D-*arabino*-heptulosonate (**4**) is converted to methyl (methyl 3-deoxy-D-*arabino*-heptulopyranosid)onate (**5**) in refluxing acidic methanol. Although the isopropylidene protecting groups are easily removed with gentle acid catalysis, simultaneous conversion to the methyl pyranoside required forcing conditions. The cyclic heptulosonate **5** is then phosphorylated with diphenyl phosphochloridate, giving methyl (methyl 3-deoxy-D-*arabino*-heptulopyranosid)onate 7-(diphenyl phosphate) (**6**). Removal of the phenyl ester groups by exhaustive hydrogenation is followed by basic hydrolysis of the methyl ester. Finally, the methyl pyranoside is hydrolyzed by heating with Dowex 50 (H^+ form) to yield DAHP (**9**), which is purified by ion-exchange chromatography. Intermediates **2**, **3**, and **5** are stable, crystalline compounds particularly well suited for accumulation for large-scale synthesis. All of the intermediates up to and including compound **6** are soluble in organic solvents, which facilitates more rapid manipulation than is possible for water-soluble intermediates. Overall, our route allows the production of DAHP in 6% yield (based on 2-deoxyglucose) in eight steps. The new synthetic scheme will allow the synthesis of a number of analogues of

Scheme I: Synthesis of 3-Deoxy-D-*arabino*-heptulosonic Acid 7-Phosphate (9)[a]



[a] a, HCl-HS(CH$_2$)$_3$SH-EtOH; b, (CH$_3$)$_2$CO-H$_2$SO$_4$; c, *n*-BuLi; d, CH$_3$OCOCl; e, *N*-bromosuccinimide-acetone; f, HBr-MeOH; g, (PhO)$_2$POCl-pyridine; h, PtO$_2$-H$_2$; j, KOH; k, Dowex 50 (H$^+$).

Table I: Microbiological Production of DAHP

E. coli	phenotype	protocol[a]	cell density (no./L)	concn DAH (mM)	concn DAHP (mM)	DAHP (μmol/10^8 cells)[b]
AB-2847	Aro B$^-$	I	8.3 × 10^8	0.54	0.22	92
JB-5	Aro B$^-$ Tyr R$^-$	I	5.5 × 10^8	1.3	0.26	284
JB-5	Aro B$^-$ Tyr R$^-$	II	10.5 × 10^8	3.1	0.49	342

[a] See Experimental Procedures. [b] Since DAH presumably derives from the dephosphorylation of DAHP, this column contains the values for DAH + DAHP.

DAHP (e.g., deoxy materials, phosphonates and homophosphonates, specifically labeled ^{3}H material, etc.) that are required for mechanistic investigation of the reaction catalyzed by dehydroquinate synthase. Finally, in a recent article, Aldersberg & Sprinson (1984) have reported an efficient synthesis of DAHP from **5**, which was synthesized earlier by these workers (Aldersberg & Sprinson, 1964).

Simultaneously with the chemical synthetic approach, we have evaluated the production of DAHP by mutants of *E. coli* that contain no dehydroquinate synthase. One of the difficulties of exploiting whole cells for the production of intermediary metabolites is the lack of control over contaminants that accumulate in the growth medium as a result of normal cellular processes. Purification of such metabolites necessarily involves the manipulation of large volumes of crude product and the isolation of the target molecule free from contaminants of closely similar structure and charge type. Our work first centered on *E. coli* AB-2847, a mutant that lacks dehydroquinate synthase (phenotype Aro B$^-$). These cells are grown in 0.5% glucose and minimal inorganic salts, supplemented with low levels of L-phenylalanine, L-tyrosine, L-tryptophan, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid. As the cells reach stationary phase, DAHP begins to accumulate in the growth medium. If this strain is first grown in rich medium, harvested, and then resuspended in the above minimal medium, no DAHP accumulates. The inability of *E. coli* AB-2847 to produce DAHP when first grown up in rich medium is probably due to the fact that, at a high level of aromatic amino acids, the synthesis of the enzymes of the shikimate pathway (most importantly for our purposes, DAHP synthase) is strongly repressed. We have therefore investigated DAHP production in a strain that lacks the repressor protein (phenotype Tyr R$^-$, which controls the transcription of the phenylalanine-sensitive and tyrosine-sensitive DAHP synthases).

During our construction of an *E. coli* strain that overproduces dehydroquinate synthase (Frost et al., 1984), strain JB-5, having the phenotype Aro B$^-$ Tyr R$^-$, was obtained. *E. coli* JB-5 was grown in the same minimal medium as *E. coli* AB-2847 (described above) but with the additional amino acid supplements of L-histidine, L-isoleucine, and L-valine. As the cells moved into stationary phase, DAHP was found in the growth medium. The amount of DAHP produced per cell was 3 times greater for JB-5 than for AB-2847 (see Table I). The final cell density was lower for *E. coli* JB-5, however, which meant that the *isolated* DAHP yield was only some 20% higher for strain JB-5. The lower cell density achieved by *E. coli* JB-5 could be increased by an initial growth in rich medium, followed by cell transfer to minimal medium. The initial growth in the rich medium led to a higher cell density without any repression of DAHP synthetase (since JB-5 is Tyr R$^-$). As a result, the yield of isolated DAHP from JB-5 first grown in rich medium was about twice that which could be obtained from AB-2847. A further advantage of *E. coli* JB-5 is its resistance to kanamycin, use of which in the medium prevents overgrowth by contaminating organisms.

An attempt was made to improve the production of DAHP by *E. coli* JB-5 still further, by transformation with pKB-45, a multicopy plasmid that codes for the tyrosine-sensitive isozyme of DAHP synthase. Since this isozyme of DAHP synthase is not under transcriptional control in *E. coli* JB-5, introduction of this plasmid should increase the intracellular concentration of tyrosine-sensitive DAHP synthase. When such transformants were tested for their ability to produce DAHP, no significant increase relative to the untransformed

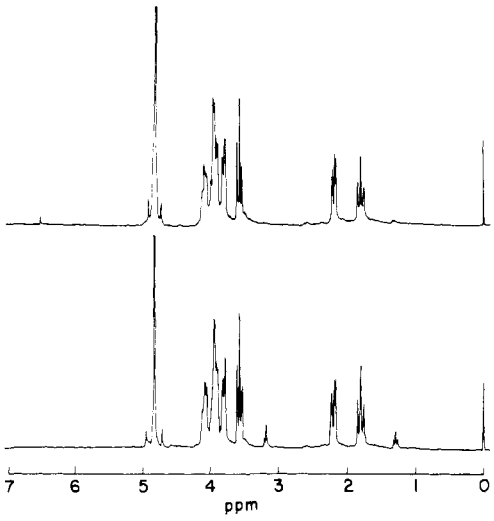


FIGURE 2: ^1H NMR spectra (D_2O) of DAHP from chemical synthesis (lower spectrum) and microbiological isolation (upper spectrum).

JB-5 was found. Evidently the levels of DAHP synthase are not limiting in JB-5 for the production of DAHP.

Both *E. coli* JB-5 and AB-2847 secrete considerable quantities of the dephospho material, 3-deoxy-D-*arabino*-heptulosonic acid (DAH) (see Table I). The amount of DAH relative to DAHP was found to be the same at the end of the growth period as it was at the end of the isolation procedure. The DAH is therefore not a product of the purification procedure. Furthermore, it is improbable that a biosynthetic pathway for DAH generation exists. Most likely, nonspecific phosphatases act on DAHP subsequent to its generation by DAHP synthase. Although *E. coli* AB-2847 could be grown and DAHP accumulated in media containing up to 0.1 M arsenate, molybdate, and fluoride, separately or together, the presence of these phosphatase inhibitors had no significant effect on the ratio of DAH to DAHP in the growth medium.

While anion-exchange chromatography is the obvious technique for the purification of DAHP and DAH, the large quantities of inorganic anions present in the growth medium must be reduced before columns of reasonable size can be used. Fortunately, the trilithium salt of DAHP is soluble in methanol whereas lithium phosphate and sulfate are not. This allows the selective removal of substantial amounts of the inorganic salts before the DAHP is subjected to anion-exchange chromatography on DEAE-cellulose.

The refinements reported here both in the strain of *E. coli* used and in the subsequent isolation procedure allow the ready preparation of 100-mg quantities of DAHP in a few days. As indicated by the ^1H NMR (Figure 2) and ^{13}C NMR (Figure 3) spectra, both the chemical synthesis and the microbiological isolation produce pure product.

Each of the routes to DAHP reported here, the microbiological and the chemical, has different advantages, and the approach of choice depends upon the aims of the investigation. If the target is simply to produce DAHP, it is probable that the microbiological route will be preferred, in that less skill and less time is required. On the other hand, the chemical route is essential for the concomitant production of substrate analogues. Moreover, methyl (methyl-3-deoxy-D-*arabino*-heptulopyranosid)onate (**5**) (Adlersberg et al., 1964) represents a useful convergence point between the microbiological and chemical routes to DAHP. As mentioned above, substantial

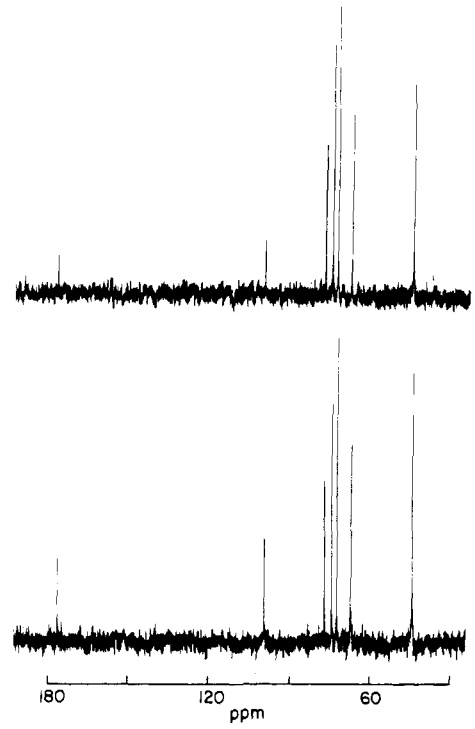


FIGURE 3: ^{13}C NMR spectra (D_2O) of DAHP from chemical synthesis (lower spectrum) and microbiological isolation (upper spectrum).

amounts of the dephospho analogue of DAHP are produced by the bacterium. Yet this material (DAH) need not be wasted: DAH produced microbiologically is readily converted by refluxing in acidic methanol into **5** from which DAHP can be made in four steps (Scheme I). The investigator thus has the option of producing the key intermediate **5** microbiologically in two steps from glucose. Together, microbiological and chemical synthesis can produce quantities of DAHP *and* a range of DAHP derivatives suitable for enzymological studies.

Acknowledgments

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Registry No. **1**, 154-17-6; **2**, 91294-63-2; **3**, 91294-64-3; **4**, 91294-65-4; **5**, 2880-95-7; **6**, 91382-79-5; **7**, 91382-80-8; **8**, 91382-81-9; 9, 2627-73-8; 1,3-propanedithiol, 109-80-8.

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