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PAPER

Fluorinated substrates result in variable leakage of a reaction intermediate during catalysis by dehydroquinate synthase†

Leonardo Negron[a] and Emily J Parker*[b]

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Incubation of (3*S*)-3-fluoro-3-deoxy-D-*arabino*-heptulosonate 7-phosphate with dehydroquinate (DHQ) synthase from three phylogenetically distinct sources resulted in the production of (6*S*)-6-fluoroDHQ and its epimer 1-*epi*-(6*S*)-6-fluoroDHQ. The differences in the product ratios of the reactions catalysed by each enzyme imply that 1-*epi*-(6*S*)-6-fluoroDHQ formation occurs by an unusual partial leakage of a reaction intermediate from the enzyme.

Introduction

The shikimate pathway is the biosynthetic pathway responsible for producing the precursors of the aromatic amino acids, folic acid and ubiquinones, which are vital for plants, fungi and bacteria.[1,2] The absence of the pathway in humans makes the enzymes of the shikimate pathway prime targets for the development of new antibacterial drugs.[3]

Dehydroquinate synthase (DHQS, EC 4.6.1.3) is the second enzyme of the shikimate pathway. DHQS catalyses the five step transformation of the seven carbon sugar 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAH7P, **1**) to the carbocycle dehydroquinate (DHQ, **5**). Multiple studies have described in detail the mechanism of most of the steps carried out by DHQS.[2–5] The complexity of the reaction sequence has led to considerable debate over the role of the enzyme in each of the reaction steps.[6,7] Consideration has particularly centred about the role of the enzyme in the final cyclisation step, which involves an intramolecular aldol reaction resulting in the formation of carbocycle, DHQ **5**. This step is preceded by ring opening of the enolpyranose intermediate **4**, rotation about the bond between C5 and C6, and attack on the *re* face of the C2 carbonyl by the C7 enolate-carbon to form DHQ **5** (Fig. 1).[8]

The extent to which the enzyme controls the cyclisation step and whether this last step occurs on or off the enzyme has been questioned. Bartlett and Satake showed that the non-enzymatic cyclisation which spontaneously and rapidly follows the deprotection of *O*-nitrobenzyl-enolpyranose analogue **6**, gives both DHQ **5** and a small amount of 1-*epi*-DHQ **7**.[7] Epimer formation has not been reported for the DHQS catalysed reaction with

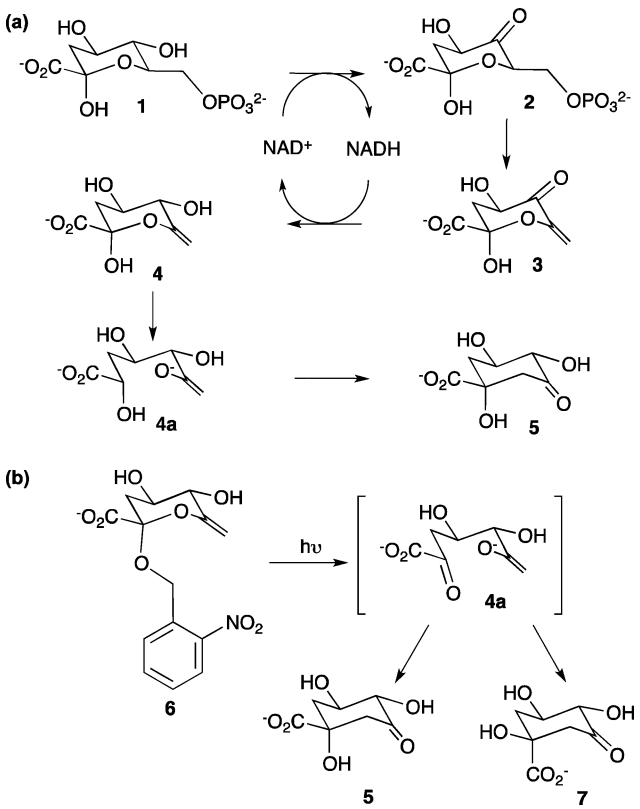

Fig. 1 Products formed in the presence and absence of DHQS. (a) The mechanism for conversion of DAH7P **1** to DHQ **5** in the presence of DHQS,[9] (b) the formation of DHQ **5** and 1-*epi*-DHQ **7** in the absence of DHQS when the ring-opened enolpyranose intermediate **4a** is allowed to undergo cyclisation free of the stereochemical constraints of the enzyme. The enolpyranose intermediate **4** is generated in solution *via* the photo-deprotection of *O*-nitrobenzyl enolpyranose **6**.[10]

[a]*Institute of Fundamental Sciences, Massey University, Private Bag, 11222, Palmerston North, New Zealand*

[b]*Biomolecular Interaction Centre and Department of Chemistry, PO Box 4800, University of Canterbury, Christchurch, New Zealand. E-mail: emily.parker@canterbury.ac.nz; Fax: + 64 3 364 2100; Tel: + 64 3 364 2871*

† Electronic supplementary information (ESI) available: Sequence alignment of DHQS enzymes; NMR spectra for DAH7P and 3-fluoroDAH7Ps; ^{19}F NMR time-course experiments for the reactions of 3-fluoroDAH7P with *E.coli*, *P. furiosus* and *A. chinensis* DHQS; ^{19}F NMR experiments for the conversion of 3-fluoroDAH7P into 3-fluoroDHS; Determination of the stability of 3-fluoroDHQ. See DOI: 10.1039/c0ob01141j

DAH7P. This formation of 1-*epi*-DHQ **7** in the non-enzymatic reaction suggested that the enzyme is responsible for ensuring the overall stereochemical control of the reaction, and that the enol-pyranose intermediate **4** is unlikely to be released from the enzyme active site prior to the formation of DHQ **5**. The precise stereochemical control observed for the enzymatic reaction is not possible if the intermediate is released prior to cyclisation.

Carpenter and co-workers determined the structure of *Aspergillus nidulans* DHQS and showed the interactions between the active site amino acids of DHQS and carbaphosphonate (a carbocyclic analogue of DAH7P), NAD^+, and zinc.[11] Interactions between the carboxylate moiety attached to C2 and the side chains of Lys152, Lys250 and Arg264 restrict rotation of the bond between C2 and C3 of the ring-opened enolpyranose intermediate **4a**, thereby ensuring that the enolate attacks the *re* face of the ketone to generate the correct stereochemistry of DHQ **5** (Fig. 2). Given the conformation constraints imposed on the enolpyranose intermediate and its ring opened form, by interactions with the enzyme, it would be difficult for the enzyme to support the formation of an epimeric product.

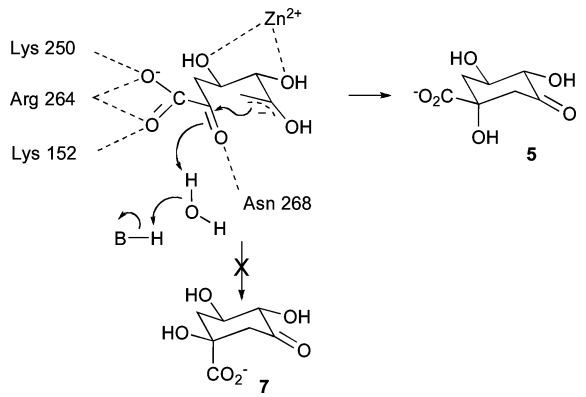

Fig. 2 Predicted interaction of active site residues of *A. nidulans* DHQS with the ring opened enolpyranose intermediate **4a**. These interactions prevent the rotation of the bond between C2 and C3 ensuring the production of a single isomer.[11] All these residues are conserved in the DHQSs from *E. coli*, *A. chinensis* and *P. furiosus* used in this study (see ESI†).

Previously it has been shown that the incubation of a modified substrate containing fluorine at C3 on DAH7P in the axial position, (3*S*)-3-fluoroDAH7P **8**, with DHQS from *Escherichia coli* DHQS resulted in the formation of both the expected reaction product, (6*S*)-6-fluoroDHQ **9**, and its C1 epimer, 1-*epi*-(6*S*)-6-fluoroDHQ **10** at a ratio of 2 : 1 (Fig. 3).[12] This result closely mirrored that of the non-enzymatic conversion of *O*-nitrobenzyl-enolpyranose analogue **6** to DHQ **5** and 1-*epi*-DHQ **7**, and was interpreted as early disassociation of the (3*S*)-3-fluoroenol-pyranose intermediate from the enzyme leading to the ring forming step in solution with the formation of the two fluorinated products. In contrast, incubation of (3*R*)-3-fluoroDAH7P **11**, containing fluorine in the equatorial position, with *E. coli* DHQS resulted in the formation of (6*R*)-6-fluoroDHQ **12**, and none of its corresponding C1 epimer.

In this study, (3*S*)-3-fluoroDAH7P **8** and (3*R*)-3-fluoroDAH7P **11** were synthesised and assayed as substrates for three phylogenetically distinct sources of DHQS. These sources were *E. coli* (Bacteria), *Actinidia chinensis* var. or New Zealand Kiwifruit (Eukarya), and *Pyrococcus furiosus* (Archaea). Our objective was to assess the extent of stereochemical control on the reaction chemistry provided by the different enzymes in the presence of fluorinated substrates, and to determine whether fluorine substitution gives rise to complete or partial leakage of the reaction intermediate from each of the enzymes. Our results reveal a delicate partitioning between on-enzyme reaction and premature release of the reaction intermediate. Moreover, we demonstrate that employing DHQS enzymes from different sources alters this balance.

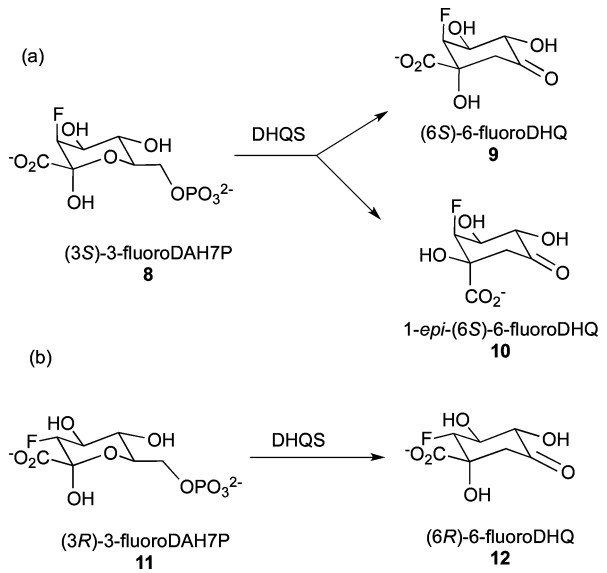

Fig. 3 Formation of DHQ products with fluorinated starting materials in the presence of *E. coli* DHQS. (a) formation of both (6*S*)-6-fluoroDHQ **9** and 1-*epi*-(6*S*)-6-fluoroDHQ **10** from (3*S*)-3-fluoroDAH7P **8**; (b) formation of (6*R*)-6-fluoroDHQ **12** from (3*R*)-3-fluoroDAH7P **11**.

Results

Fluorinated substrates (3*R*)-3-fluoroDAH7P **11** and (3*S*)-3-fluoroDAH7P **8** were prepared enzymatically from the reaction of erythrose 4-phosphate (E4P) and 3-fluorophosphoenolpyruvate (3-fluoroPEP) catalysed by the first enzyme of the shikimate pathway, DAH7P synthase (DAH7PS, EC 2.5.1.54).[13] The reactions of (3*R*)-3-fluoroDAH7P **11** and (3*S*)-3-fluoroDAH7P **8** catalysed by DHQS enzymes from different sources were monitored directly by ^{19}F NMR spectroscopy.

Incubation of (3*R*)-3-fluoroDAH7P **11** with DHQS from each of the three DHQS enzymes resulted in the rapid formation of (6*R*)-6-fluoroDHQ **12** as the only detectable product (Fig. 4). The chemical shift of the fluorine signal of (3*R*)-3-fluoroDAH7P **11** using ^{19}F NMR is –198.9 ppm and that of (6*R*)-6-fluoroDHQ **12** is further upfield at –202.2 ppm. The equatorial positioning of the fluorine in both starting material and product was clear from the magnitudes of the coupling constants between the fluorine and the adjacent proton at C4 for (3*R*)-3-fluoroDAH7P **11** and C5 for (6*R*)-6-fluoroDHQ **12**. The fluorine signal is split by coupling both to the geminal proton (~50 Hz) and to the axial proton on the adjacent carbon (~12 Hz for (6*R*)-6-fluoroDHQ). There is additional splitting observed for (6*R*)-6-fluoroDHQ **12** due to ω-coupling (^4J) between the C6 fluorine and the C2 equatorial

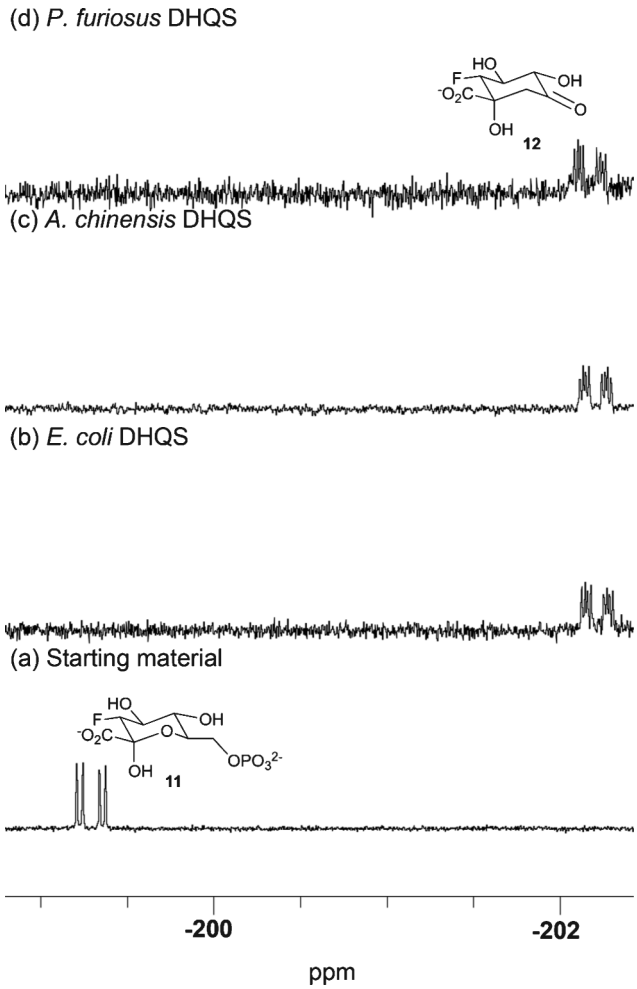

Fig. 4 ^{19}F NMR (376 MHz) spectra showing formation of (6*R*)-6-fluoroDHQ **12** by *P. furiosus*, *A. chinensis* and *E. coli* DHQS.

proton. No other products with a similar splitting pattern to that of the starting material were observed. UV spectrophotometric analysis of the products formed from (3*R*)-3-fluoroDAH7P in the presence of DHQS, by using DHQase to form the corresponding (6*S*)-6-fluorodehydroshikimate (chromophore at 230 nm) indicated complete conversion of (3*R*)-3-fluoroDAH7P **11** into (6*R*)-6-fluoroDHQ **12**, by all DHQS enzymes, in line with the observations made in the ^{19}F NMR spectra.

In marked contrast to the single product observed from the reaction of (3*R*)-3-fluoroDAH7P **11** with *E. coli* DHQS, reaction of (3*S*)-3-fluoroDAH7P **8** resulted in the formation of both (6*S*)-6-fluoroDHQ **9** and 1-*epi*-(6*S*)-6-fluoroDHQ **10** at 68 and 31 percent respectively (Fig. 5b, based on peak integration). This ratio of products for *E. coli* DHQS is consistent with that observed in earlier studies.[12] These two diastereomers have distinct ^{19}F NMR chemical shifts, at –209.5 ppm for 1-*epi*-(6*S*)-6-fluoroDHQ **10** and –202.6 ppm for (6*S*)-6-fluoroDHQ **9**. The axial position of the fluorine in both products was confirmed by analysing the splitting pattern of the fluorine resonance. The coupling constant between the fluorine at C6 and the axial proton at C5 for both (6*S*)-6-fluoroDHQ **9** and 1-*epi*-(6*S*)-6-fluoroDHQ **10** is ~30 Hz, which is consistent with its axial positioning.

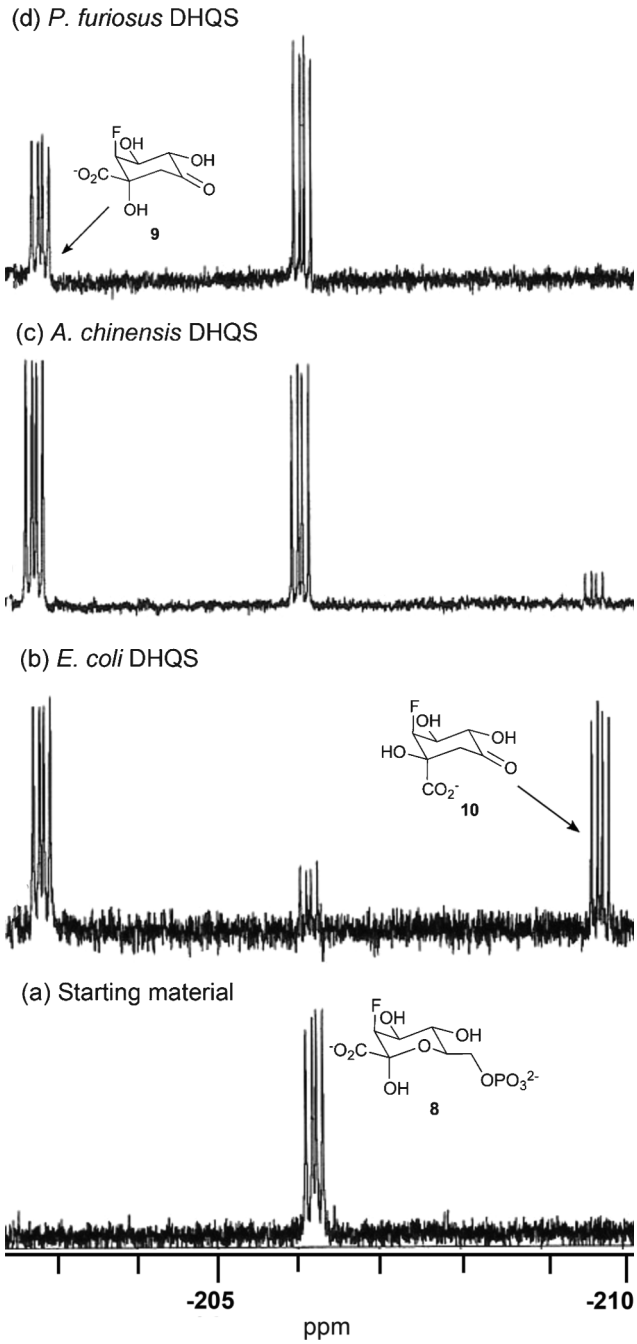

Fig. 5 ^{19}F NMR spectra (376 MHz) showing the partitioning results for all three enzymes incubated with (3*S*)-3-fluoroDAH7P **8**. Ratios of the two products (6*S*)-6-fluoroDHQ **9** and 1-*epi*-(6*S*)-6-fluoroDHQ **11** were determined by peak integration (69 : 31 for *E. coli* DHQS, 93 : 7, for *A. chinensis* DHQS and 100 : 0 for *P. furiosus* DHQS).

When the DHQS from *A. chinensis* was used in place of the *E. coli* DHQS, a significantly different ratio of the two isomeric products was obtained. (6*S*)-6-FluoroDHQ **9** was formed in considerably greater proportion (93%) with this enzyme than 1-*epi*-(6*S*)-6-fluoroDHQ **10** (7%) (Fig. 5c).

In contrast to what was observed with *E. coli* DHQS and *A. chinensis* DHQS, incubation of *P. furiosus* DHQS with (3*S*)-3-fluoroDAH7P **8** resulted in the formation of (6*S*)-6-fluoroDHQ

Table 1 Michaelis constants for *E. coli* DHQS, *A. chinensis* DHQS, and *P. furiosus* DHQS for DAH7P **1**, (3*S*)-3-fluoroDAH7P **8** and (3*R*)-3-fluoroDAH7P **11**

Enzyme	Substrate	K_M (μM)	$k_{cat.}$ (s^{-1})
E. coli DHQS	DAH7P	5.7 ±0.2	16.0 ±0.2
	(3*S*)-3-fluoroDAH7P	21 ±4	0.30 ±0.02
	(3*R*)-3-fluoroDAH7P	1.6 ±0.1	2.50 ±0.01
A. chinensis DHQS	DAH7P	3.2 ±0.2	0.50 ±0.01
	(3*S*)-3-fluoroDAH7P	64 ±2	0.90 ±0.01
	(3*R*)-3-fluoroDAH7P	8.7 ±0.5	0.30 ±0.01
P. furiosus DHQS	DAH7P	3.7 ±0.2	3.0 ±0.1
	(3*S*)-3-fluoroDAH7P	29 ±1	1.3 ±0.1
	(3*R*)-3-fluoroDAH7P	2.0 ±0.2	3.3 ±0.1

9 exclusively; no 1-*epi*-(6*S*)-6-fluoroDHQ **10** was observed (see Fig. 5d).

Unlike the reactions of (3*R*)-3-fluoroDAH7P **11**, complete conversion of (3*S*)-3-fluoroDAH7P **8** was not observed. Kinetic analysis shows that (3*S*)-3-fluoroDAH7P is a considerably poorer substrate for the enzyme than both (3*R*)-3-fluoroDAH7P **11** and DAH7P **1** (Table 1). It is likely that loss of enzyme activity over the extended time period of the assay was responsible for the incomplete reaction. In all cases, the ratio of the two products, (6*S*)-6-fluoroDHQ **9** and 1-*epi*-(6*S*)-6-fluoroDHQ **10**, was constant for the reaction period.

Discussion

The findings of these experiments clearly indicate that substitution of fluorine into DAH7P disrupts the enzymic reaction, and this disruption is apparent when the fluorine occupies the axial position. The ratio of (6*S*)-6-fluoroDHQ **9** and 1-*epi*-(6*S*)-6-fluoroDHQ **10** formed depends on the particular DHQS that was employed; *E. coli* DHQS gave the greatest proportion of epimer. On the other hand when *P. furiosus* enzyme was the catalyst, only (6*S*)-6-fluoroDHQ **9** was formed.

Formation of 1-*epi*-(6*S*)-6-fluoroDHQ **10** results from attack of the *si* face of the C2 carbonyl by the C7 enolate, rather than by attack on its *re* face. The previous observation of partitioning observed in the non-enzymatic reaction from the deprotection of *O*-nitrobenzyl enol-pyranose **6** (Fig. 1b), and the constraints on rotation to expose the *si* face to attack imposed by the active site (Fig. 2), imply clearly that C1 epimer formation occurs off the enzyme. The two most likely explanations for the partitioning observed for the enzymatic reactions with 3-fluoroDAH7Ps is that either both epimer and expected reaction product ((6*S*)-6-fluoroDHQ **9** and 1-*epi*-(6*S*)-6-fluoroDHQ **10**) are formed off the enzyme, or that there is partial leakage of the enolpyranose intermediate to allow some product to form in solution.

In these experiments different ratios of epimeric products were observed arising from (3*S*)-3-fluoroDAH7P **8** for each of the different enzymes used. If the 3-fluoroenolpyranose intermediate was released by the enzyme entirely, so that further reaction of this intermediate was free from enzymic stereochemical constraints in the aldol reaction cyclisation step, the product ratio would be expected to be independent of the enzyme source. It therefore seems likely that the product distribution reflects partial leakage of the intermediate from the enzyme.

Fluorine adjacent to the anomeric centre is known to stabilise the pyranose form of sugar.[14] This stabilisation may increase the lifetime of the 3-fluoroenolpyranose intermediate so that the dissociation pathway competes more effectively with the on-enzyme ring opening, allowing for partial leakage of the intermediate from the enzyme. The different ratios of products observed demonstrate that partitioning between on-enzyme reaction and intermediate release depends on the enzyme used.

Analysis of active site residues of the three enzymes employed in these experiments shows that all key amino acids identified as forming direct interactions with the substrates and involved in catalysis are conserved (Fig. S1, ESI†). However, there is significant sequence variation more remote from the active site. The sequence identity between the *A. chinensis* and *E. coli* enzyme is the highest at 52%, whereas the *P. furiosus* DHQS shares only 37% and 30% identity with the *E. coli* and *A. chinensis* enzymes. Intermediate release and onward on-enzyme reaction are clearly energetically delicately balanced, and subtle changes in dynamic properties are likely responsible for the differences observed in these experiments. This is supported by the results observed for the *P. furiosus* enzyme. The likely operating temperature for this enzyme is far closer to 100 °C, as the natural environment for this archaeal organism is geothermally heated marine sediments. In these studies the *P. furiosus* DHQS was used well below its expected temperature maximum (50 °C), due to substrate stability constraints. It is expected that the normal conformational flexibility and dynamic range of this enzyme is considerably lower at this temperature, thereby slowing down intermediate release.

When the intramolecular aldol reaction takes place away from the enzymic template, this step is no longer under the stereochemical constraints that the enzyme imposes on the reaction of the natural substrates. The position of fluorine is expected to influence the relative energies of the transition states for carbocycle formation in the intramolecular aldol reaction. Transition states for the solution reaction would be expected to favour configurations in which the fluorine occupies a position perpendicular to the carbonyl bond and away from the direction of attack of the incoming nucleophile in accordance with Felkin Anh principles modified for hyperconjugative effects (Fig. 6).[15] The expected chair-like transition state predicted for the enzymatic reaction, is therefore not favourable for the enolate intermediate derived from (3*S*)-3-fluoroDAH7P **8**, and conformational change for the solution reaction would be expected. The equatorial position of the fluorine in (3*R*)-3-fluoroDAH7P **11** means that the same enzymic chair-like transition state would be favoured, explaining the observation that (6*R*)-6-fluoroDHQ **12** is formed exclusively.

Conclusions

These studies highlight subtle differences in enzymic properties that are associated with natural sequence variations. These findings reinforce the important role of the enzyme dehydroquinate in guiding the correct stereochemical outcome for the product. They also illuminate how finely balanced energetic reaction pathways that control enzymic reaction outcomes are, where the simple displacement of a proton by fluorine in DAH7P is sufficient to disrupt catalytic function.

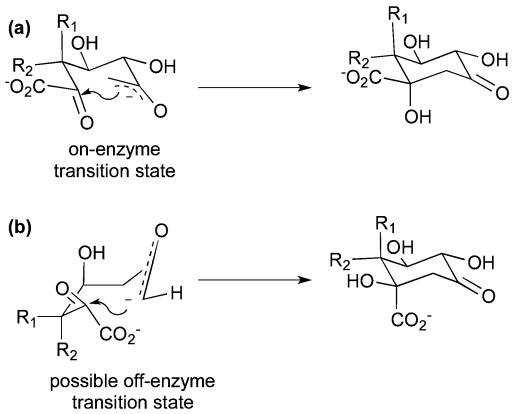

Fig. 6 Possible transition states for (a) DHQ, and (b) 1-*epi*-DHQ production. When R_1 = F and R_2 = H, (b) places the fluorine substituent perpendicular to the carbonyl bond and away from the direction of attack on the enolate nucleophile. Transition state in (a) is favoured by the enzyme-catalysed reaction, and when R_2 = F and R_1 = H, places the fluorine in the favoured position for the intramolecular aldol reaction.

Experimental section

Purification of enzymes

E. coli DAH7PS was expressed and purified as previously described.[16]

For the purification of *P. furiosus* DHQS, *E. coli* BL21(DE3)-Rosetta cells containing the expression plasmid pT7-*Pfu* DHQS were grown overnight at 37 °C in Luria-Bertani (LB) medium supplemented with ampicillin (100 μg mL^{-1}) and chloramphenicol (34 μg mL^{-1}). This culture was used to inoculate 500 mL of fresh medium in a 1000 mL flask, and growth continued with shaking at 37 °C. Isopropyl-β-D-thiogalactopyranoside (IPTG, Applichem) was added at 1 mM to mid-logarithmic phase cultures (OD600 ~0.6) to induce expression. Cells were harvested by centrifugation (4 °C, 4000*g*, 20 min) 16 h after induction. Cell pellets were resuspended in 50 mM 1,3-bis(tris(hydroxymethyl)methylamino) propane (BTP, Sigma) buffer containing 2 mM dithiothreitol (DTT, BDH), 0.5 mM NAD^+ (Sigma), and 200 mM KCl (Ajax Chemicals), pH 6.8 and lysed by sonication at 30 °C. The cell lysate was heat treated at 70 °C for 20 min, cooled to 5 °C and centrifuged (4 °C, 10000*g*, 20 min).

P. furiosus DHQS was further purified by anion-exchange chromatography using a Source Q 15 column (Amersham Biosciences). After filtering through a 0.45 micron filter, the supernatant from the heat treatment step was diluted with buffer A (50 mM BTP with 10 μM EDTA, pH 6.8) and loaded onto the anion-exchange column. *P. furiosus* DHQS was eluted at 90 mM NaCl by applying a linear gradient of NaCl at 2 mL min^{-1} using buffer B (50 mM BTP, 10 μM EDTA with 1 M NaCl, pH 6.8). Fractions with DHQS activity were pooled and concentrated using a 10 kDa cut-off concentrator (Vivascience). The concentrate was aliquoted (50 μL), frozen in liquid nitrogen and stored at –80 °C.

E. coli DHQS was expressed and purified according to previously described procedures.[5] Purified *A. chinensis* (Kiwifruit) DHQS was provided by Plant and Food Research (Auckland, New Zealand). Sequences of all three enzymes and their alignment are shown as Fig. S1 in the ESI†. The relative molecular masses of the purified recombinant enzymes, as determined by electrospray ionisation mass spectrometry, were 37,397 Da for the *P. furiosus* DHQS monomer, 38,888 Da for the *E. coli* DHQS monomer, and 45,189 Da for the His-tagged *A. chinensis* DHQS monomer, in close agreement with the calculated masses of 37, 394, 38,881 and 45,176 Da respectively.

For the kinetic measurements the third enzyme in the shikimate pathway, dehydroquinase (DHQase, EC4.2.1.10) was required. *E. coli* DHQase was prepared from *E. coli* AB2848/pKD201 cells[17] containing the *E. coli* DHQase gene, grown overnight at 37 °C in LB medium supplemented with ampicillin (100 μg mL^{-1}). Over-expressed *E. coli* DHQase was purified by anion exchange chromatography using SourceQ resin using a linear gradient of NaCl (Buffer A: 50 mM BTP with 10 μM EDTA, pH 7.5; Buffer B: 50 mM BTP, 10 μM EDTA (Sigma) with 1 M NaCl, pH 7.5). Fractions containing purified DHQase were pooled and concentrated.

For the purification of *P. furiosus* DHQase *E. coli* BL21(DE3)-Rosetta cells, transformed with the expression plasmid pT7-*P. furiosus* DHQase, were grown at 37 °C in LB medium supplemented with ampicillin (100 μg mL^{-1}) and chloramphenicol (34 μg mL^{-1}). Expression of *P. furiosus* DHQase was induced with IPTG as described for *P. furiosus* DHQS and purified based on the method of Schofield and co-workers.[18] Soluble protein from crude lysate was subjected to heat treatment at 70 °C in lysis buffer (50 mM BTP, 2 mM DTT, 200 mM KCl, 1 mM EDTA, pH 7.5). The resulting protein suspension was centrifuged to obtain supernatant which was then subjected to size-exclusion chromatography on a Superdex S200 HR 10/300 column (Amersham Biosciences) and eluted under isocratic conditions at 0.4 mL min^{-1} (Buffer: 10 mM BTP, 10 μM EDTA, 50 mM KCl, pH 6.8). Fractions containing purified *P. furiosus* DHQase were pooled and concentrated.

Preparation of 3-fluorophosphoenolpyruvate (3-fluoroPEP)

(*Z*)-3-FluoroPEP and (*E*)-3-fluoroPEP were prepared by the procedures reported by Bergmann and Shahak, and later modified by Stubbe and Kenyon.[19] This reaction resulted in the formation of a 9 : 1 ratio of the *Z* and *E* isomers of 3-fluoroPEP. To generate a mixture of 3-fluoroPEP with a higher percentage of (*E*)-3-fluoroPEP, the 9 : 1 mixture of (*Z*/*E*)-3-fluoroPEP was photoisomerised by exposure to ultraviolet light at neutral pH. This reaction resulted in the formation of a 58 : 42 mixture of (*Z*/*E*)-3-fluoroPEP (see ESI†).

Preparation of DAH7P 1

DAH7P **1** was prepared enzymatically from E4P[20] and phosphoenolpyruvate (PEP, Research Chemicals) using DAH7PS[21] PEP, (0.88 mM), E4P (0.88 mM), and $ZnCl_2$ (100 μM) were incubated in the presence of *E. coli* DAH7PS 100 μL, 10 mg mL^{-1}) in 20 mL of 50 mM BTP buffer pH 6.8 at 25 °C. The reaction was monitored by tracking the disappearance of PEP ($\varepsilon = 2.8 \times 10^3$ M^{-1} cm^{-1} at 232 nm, pH 6.8 at 25 °C). Upon completion, the reaction mixture was filtered through a 10 kDa molecular weight cut-off concentrator (MWC) in order to remove DAH7PS. The filtrate was loaded onto an anion exchange column and a linear gradient from 0 to 500 mM of ammonium bicarbonate was applied over a span of two hours. DAH7P eluted at 150 mM of ammonium bicarbonate. The purified fractions were pooled and concentrated

via lyophilisation to obtain DAH7P 1 as a white solid (5 mg, 99.7%). Purified DAH7P was characterised by enzymatic analysis involving the conversion of DAH7P to dehydroshikimate (DHS) utilising both DHQS and DHQase. The formation of DHS (ε = 1.2×10^4 M^{-1} cm^{-1} at 25 °C) was monitored spectroscopically at 234 nm.

^{1}H NMR (500 MHz, D_2O): δ 4.06 (2H, m), 3.87 (1H, ddd, *J* 5.1, 8.9 and 11.7 Hz), 3.79 (1H, d, *J* = 8.9 Hz, H6), 3.45 (1H, dd, *J* = 9.6 and 9.6 Hz), 2.2 (1H, dd, *J* = 5.1 and 13 Hz), 1.75 (1H, dd, *J* = 12 and 12 Hz); ^{13}C NMR (100 MHz, D_2O): δ 178.4, 98.7, 75.4 (d, *J* = 6.8 Hz), 73.0, 71.3, 66.9 (d, *J* = 4.5 Hz) 41.6; *m/z* (–ve ESMS) 287($M - H^-$, 80%, 143($M - 2H^{2-}$, 100%).

Preparation of (3*S*)-3-fluoroDAH7P 8

To prepare (3*S*)-3-fluoroDAH7P **8** $ZnCl_2$ (100 μM), E4P (23 mg, 0.12 mmol), and a 9 : 1 mixture of (*Z*/*E*)-3-fluoroPEP monocyclohexylammonium salt (23 mg, 0.08 mmol) were dissolved in 10 mL of 50 mM BTP buffer pH 6.8 at 25 °C and allowed to incubate in the presence of 10 μL of *E. coli* DAH7PS (10 mg mL^{-1}) for one hour. Additional aliquots of *E. coli* DAH7PS were added until all of the (*Z*/*E*)-3-fluoroPEP was consumed. The consumption of (*Z*/*E*)-3-fluoroPEP was determined by monitoring the disappearance of the signal generated by this compound at 232 nm by UV spectroscopy. Upon completion, the reaction was filtered through a 10 kDa MWC concentrator in order to remove DAH7PS. The filtrate containing a 9 : 1 mixture of (3*S*)-3-fluoroDAH7P and (3*R*)-3-fluoroDAH7P was loaded onto an anion exchange column and a linear gradient from 0 to 500 mM of ammonium bicarbonate was applied over a span of two hours. (3*S*)-3-fluoroDAH7P eluted at 130 mM of ammonium bicarbonate. The purified fractions of (3*S*)-3-fluoroDAH7P were pooled and concentrated *via* lyophilisation to obtain (3*S*)-3-fluoroDAH7P **8** as a white solid (2 mg, 9%).

^{1}H NMR (500 MHz, D_2O): δ 4.79, (1H, dd, *J* = 49.2 and 2.6 Hz), 4.1 (2H, m), 3.93 (1H, ddd, *J* = 30.2, 9.8 and 2.6 Hz), 3.85 (1H, d, *J* 9.9 Hz), 3.37 (1H, dd, *J* 9.9 and 9.9 Hz); ^{13}C NMR (100 MHz, D_2O): δ 178.7, 98.0 (d, *J* = 28 Hz), 94.0 (d, *J* = 173 Hz, C3), 75.8, 72.4 (d, *J* 18 = Hz), 68.7, 65.5;^{19}F NMR (235 MHz, D_2O): δ 205.3 (dd, *J* = 49.2 30.2 Hz. 96%), –215.4 (dd, *J* = 51.8 and 29.0 Hz, 4.2%); *m/z* (–ve ESMS) 305.0088 (M – H, 100%). Enzymatic analysis *via* UV spectrophotometry at 230 nm following treatment with DHQS and DHQase showed 66% conversion of (3*S*)-3-fluoroDAH7P to (6*S*)-6-fluoroDHS.[12]

Preparation of (3*R*)-3-fluoroDAH7P 11

To obtain (3*R*)-3-fluoroDAH7P **11**, the same reaction mixture was prepared as above except 23 mg (0.08 mmol) of a 58 : 42 mixture of (*Z*/*E*)-fluoroPEP monocyclohexylammonium salt was used. Upon completion of the enzymatic reaction, the mixture was filtered to remove enzyme and the filtrate was loaded onto an anion exchange column and a linear gradient from 0 to 500 mM of ammonium bicarbonate was applied over a span of two hours. (3*R*)-3-fluoroDAH7P eluted at 170 mM of ammonium bicarbonate. The purified fractions of (3*R*)-3-fluoroDAH7P were pooled and concentrated *via* lyophilisation to obtain (3*R*)-3-fluoroDAH7P **11** as a white solid (1 mg, 10%).

^{1}H NMR (400 MHz, D_2O): δ 4.53, (1H, dd, *J* = 49.6 and 9.4 Hz), 4.0 (2H, m), 3.87 (1H, ddd, *J* = 13.3, 9.4 and 9.4 Hz), 3.83 (1H, d, *J* = 9.7 Hz), 3.57 (1H, dd, *J* = 9.7 and 9.7 Hz); ^{13}C NMR (100 MHz, D_2O): δ 178.3, 95.4 (d, *J* = 28 Hz), 92.1 (d, *J* = 186 Hz), 73.1 (d, *J* = 6.6 Hz), 72.7 (d, *J* = 17.5 Hz C4), 69.5 (d, = *J* 7.9 Hz), 64.5; ^{19}F NMR (235 MHz, D_2O): δ 197.7 (dd, *J* = 49.6 and 13.3 Hz); *m/z* (–ve ESMS) 305.0091(M – H, 100%). Enzymatic analysis *via* UV spectrophotometry following treatment with DHQS and DHQase at 230 nm showed 100% conversion of (3*R*)-3-fluoroDAH7P to (6*R*)-6-fluoroDHS.[12]

Accurate concentrations of DAH7P, (3*S*)-3-fluoroDAH7P, and (3*R*)-3-fluoroDAH7P were determined by the Lanzetta assay[22] using potassium dihydrogen phosphate standards ranging from zero to 51 μg mL^{-1}. DAH7P samples were incubated for 1 h with alkaline phosphatase (1U). Standards and samples were then reacted with Lanzetta reagent for 20 min and the absorbance for each was read at 630 nm. The Lanzetta reagent was composed of a 3 : 1 : 0.1 mixture of malachite green (0.045%), ammonium molybdate (4.2%), and Triton X 100 (1.5%).

^{19}F NMR experiments

^{19}F NMR spectra (376 MHz) showing the partitioning results for all three enzymes incubated with a mixture of (3*S*)-3-fluoroDAH7P **8** and (3*R*)-3-fluoroDAH7P **11** generated *in situ* utilising *E. coli* DAH7PS, E4P and a 9 : 1 mixture of (*Z*/*E*)-3-fluoroPEP. Conditions for the NMR experiments shown were: 45 mM (*Z*/*E*)-fluoroPEP, 47 mM E4P, 50 μM $ZnCl_2$, 30 μM NAD^+, and 20% D_2O in 50 mM BTP buffer, pH 7 at 25 °C (final volume 550 μL). *E. coli* DAH7PS[12] (0.4 mg mL^{-1}) was added to the NMR tube and the reaction was monitored overnight by ^{19}F NMR spectroscopy. Spectra showing the formation of 3-fluoroDAH7P are provided in the ESI.† DHQS (0.4 mg mL^{-1}) from each of the three sources was added to three identical NMR tubes and the reaction was monitored for a further 12 h by ^{19}F NMR spectroscopy. For *P. furiosus* DHQS the pH was adjusted to 7 at 50 °C prior to adding DHQS. A multi-experiment protocol was setup whereby 120 experiments were performed at 1 experiment every 5 min. The time-courses for each experiment are shown in the ESI.†

Kinetic measurements

In vitro kinetic experiments were performed spectroscopically using a coupled enzyme continuous assay to determine the activity of DHQS. In the presence of excess DHQase, the rate of formation of DHS served as a quantitative measure for the activity of DHQS. The appearance of DHS was monitored spectrophotometrically at 234 nm ($\varepsilon = 1.2 \times 10^4$ M^{-1} cm^{-1} at 25 °C, pH 6.8). In the case of the fluorinated substrates, the appearance of (6*S*)-6-fluoroDHS and (6*R*)-6-fluoroDHS was monitored at 230 nm ($\varepsilon = 9.6 \times 10^3$ M^{-1} cm^{-1} and 3.4×10^3 M^{-1} cm^{-1} respectively) at 25 °C pH 6.8.[12] Measurements were made in a 1 cm path length quartz cuvette maintained at the required temperature in a thermostated cuvette holder.

Assays were performed at 25 °C for *E. coli* DHQS and *A. chinensis* DHQS, and at 60 °C for *P. furiosus* DHQS. All assay mixtures included $ZnCl_2$ (100 μM), and NAD^+ (29 μM) in 50 mM BTP buffer with 10 μM EDTA. Initial rates of reaction were

determined by a least-squares fit of the initial rate data. One unit (1 U) of enzyme activity is defined as the production of 1 μmol of shikimate product per minute at 25 °C for *E. coli* DHQS and *A. chinensis* DHQS, and at 60 °C for *P. furiosus* DHQS. Specific activity is defined as the production of 1 μmol of dehydroshikimate product per minute at 25 °C per mg of protein (U mg^{-1}). K_m and k_{cat} values were determined by fitting the data to the Michaelis–Menten equation using GraFit 5 (Erithacus Software Limited, 2006). The total volume for all assays was 1000 μL. Errors between measurements were no more than 10%.

Steady state kinetics for *E. coli* DHQS with DAH7P. The standard assay reaction mixtures contained DAH7P (0.5 to 42 μM) at pH 6.8. The mixture was preincubated at 25 °C for 1 min followed by addition of 20 μL of *E. coli* DHQase (10 mg mL^{-1}). After a further minute of incubation, the reaction was initiated by the addition of 20 μL of *E. coli* DHQS (7 nM).

Steady state kinetics for *A. chinensis* DHQS with DAH7P. The standard assay reaction mixture contained DAH7P (5 to 500 μM) at pH 6.8. The mixture was preincubated at 25 °C for 1 min followed by addition of 20 μL of *E. coli* DHQase (10 mg mL^{-1}). After a further minute of incubation, the reaction was initiated by the addition of 5 μL of *A. chinensis* DHQS (86 nM).

Steady state kinetics for *P. furiosus* DHQS with DAH7P. The standard assay reaction mixture contained DAH7P (1 to 70 μM) at pH 6.7 at 60 °C. The mixture was preincubated at 60 °C for 5 min followed by addition of 20 μL of *P. furiosus* DHQase (4.1 mg mL^{-1}). After a further minute of incubation, the reaction was initiated by the addition of 10 μL of *P. furiosus* DHQS (56 nM).

Steady state kinetics for *E. coli* DHQS with (3*S*) - 3-fluoroDAH7P. The standard assay reaction mixture contained (3*S*)-3-fluoroDAH7P (5 to 240 μM) at pH 6.8 at 25 °C. The mixture was preincubated at 25 °C for 1 min followed by addition of 20 μL of *E. coli* DHQase (10 mg mL^{-1}). After a further minute of incubation, the reaction was initiated by the addition of 20 μL of *E. coli* DHQS (7 nM).

Steady state kinetics for *A. chinensis* DHQS with (3*S*)-3-fluoroDAH7P. The standard assay reaction mixture contained (3*S*)-3-fluoroDAH7P (8 to 93 μM) at pH 6.8 at 25 °C. The mixture was preincubated at 25 °C for 1 min followed by addition of 20 μL of *E. coli* DHQase (10 mg mL^{-1}). After a further minute of incubation, the reaction was initiated by the addition of 10 μL of *A. chinensis* DHQS (86 nM).

Steady state kinetics for *P. furiosus* DHQS with (3*S*)-3-fluoroDAH7P. The standard assay reaction mixture contained (3*S*)-3-fluoroDAH7P (10 to 680 μM) at pH 6.7 at 60 °C. The mixture was preincubated at 60 °C for 5 min followed by addition of 20 μL of *P. furiosus* DHQase (4.1 mg mL^{-1}). After a further minute of incubation, the reaction was initiated by the addition of 10 μL of *P. furiosus* DHQS (56 nM).

Steady state kinetics for *E. coli* DHQS with (3*R*)-3-fluoroDAH7P. The standard assay reaction mixture contained (3*R*)-3-fluoroDAH7P (2 to 160 μM), at pH 6.8 at 25 °C. The mixture was preincubated at 25 °C for 1 min followed by addition of 20 μL of *E. coli* DHQase (10 mg mL^{-1}). After a further minute of incubation, the reaction was initiated by the addition of 5 μL of *E. coli* DHQS (35 nM).

Steady state kinetics for *A. chinensis* DHQS with (3*R*)-3-fluoroDAH7P. The standard assay reaction mixture contained (3*R*)-3-fluoroDAH7P (4 to 160 μM) at pH 6.8 at 25 °C. The mixture was preincubated at 25 °C for 1 min followed by addition of 20 μL of *E. coli* DHQase (10 mg mL^{-1}). After a further minute of incubation, the reaction was initiated by the addition of 4 μL of *A. chinensis* DHQS (0.13 μM).

Steady state kinetics for *P. furiosus* DHQS with (3*R*)-3-fluoroDAH7P. The standard assay reaction mixture contained (3*R*)-3-fluoroDAH7P (4 to 241 μM) at pH 6.7 at 60 °C. The mixture was preincubated at 60 °C for 5 min followed by addition of 20 μL of *P. furiosus* DHQase (4.1 mg mL^{-1}). After a further minute of incubation, the reaction was initiated by the addition of 10 μL of *P. furiosus* DHQS (56 nM).

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