Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 20, pp. 2347-2352, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00405-X

ENZYMATIC SYNTHESIS OF *(6R)*- AND *(6S)*-FLUOROSHIKIMIC ACIDS

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Abstract 6-Fluoroshikimates have interesting antibiotic properties. The enzymatic preparation of *(6R)*- and *(6S)*-fluoroshikimates from 3-fluorophosphoenolpyruvate is described. The multistep enzymatic conversion is followed by UV and ^{19}F NMR spectroscopy.

The shikimic acid pathway is an established target for herbicidal action.[1] Recently there has been interest in the pathway as a potential site of antimicrobial action following reports of the antibiotic properties of 6-fluoroshikimic acids.[2] It was reasoned that *(6R)*-6-fluoroshikimate might be converted *in vivo* into the corresponding *(6R)*-6-fluoro-5-enolpyruvylshikimate 3-phosphate [*(6R)*-fluoroEPSP, **8b**], which could inhibit the elimination reaction catalysed by chorismate synthase (Scheme 1). This hypothesis gained support when it was shown that *(6R)*- and *(6S)*-6-fluoroEPSPs (**8b** and **8c**) are competitive inhibitors of *Neurospora crassa* chorismate synthase.[3] Moreover, it has been shown that when **8b** is incubated with *Escherichia coli* chorismate synthase a stable flavin semiquinone radical is formed and the normal catalytic cycle is halted.[4] However these results do not account for the superior antibiotic action of the *(6S)*-6-fluoroshikimate (**6c**), nor why this activity is lost when the target bacterium is supplemented with *para*-aminobenzoic acid (PABA).[2] The recent demonstration that *(6S)*-6-fluoroEPSP (**8c**) is converted into the corresponding 6-fluorochorismate (**9b**) by chorismate synthase has prompted speculation that 2-fluorochorismate (**9b**) may inhibit PABA synthase.[5]

These studies highlight the utility of the 6-fluoroshikimates as precursors to mechanistic probes for chorismate synthase and PABA synthase, and have prompted several groups to attempt to synthesise them. Syntheses of both *(6R)*- and *(6S)*-fluoroshikimates from quinic acid have been published.[6] These syntheses have been improved,[7] but are still more than 10 steps. A more direct route to racemic methyl *(6S)*-fluoroshikimate has also been published.[8]

Our interest in the enzymology of the shikimate pathway prompted us to look for an enzymatic route to the 6-fluoroshikimates. Multistep enzymatic synthesis has recently been used to great effect to make isotopically labelled intermediates in studies on vitamin B_{12} biosynthesis,[9,10] and an extension of this approach seemed viable to make fluorinated substrate analogues. To achieve this goal we would use both isomers of 3-fluorophosphoenolpyruvate (3-fluoroPEP, **2b** and **2c**) as substrates for DAHP synthase, and then use the fluorinated products of each successive enzyme on the shikimate pathway as precursors for the next enzyme.

The conversion of 3-fluoroPEP to 3-fluoroDAHP (**3b** and **3c**) by DAHP synthase has previously been reported.[11] Furthermore *(3R)*-3-fluoroDAHP (**3c**) has been shown to be a substrate for dehydroquinate synthase in the presence of dehydroquinase.[12] 6-Fluorodehydroshikimate was assumed to be the product of this enzyme

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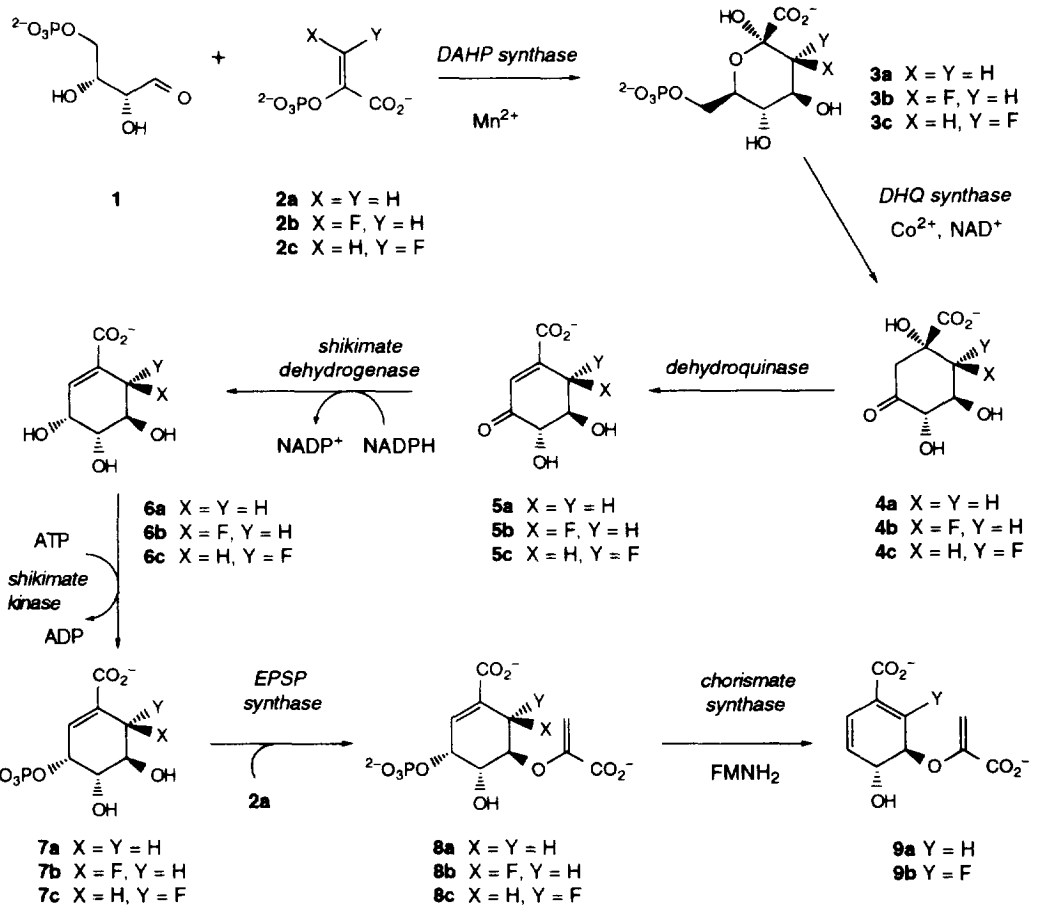
Scheme 1



reaction. We carried out a preliminary study to make 6-fluoroshikimate from 3-fluoroPEP using a mixture of recombinant DAHP synthase,[13] dehydroquinate synthase,[14] dehydroquinase[15] and shikimate dehydrogenase[16] together with their corresponding cofactors. The consumption of NADPH (followed spectrophotometrically at 340 nm) suggested a shikimate derivative was being formed.

This procedure was repeated on a small preparative scale to produce milligram amounts of fluoroshikimates.[17] The formation of fluoroDAHP from 3-fluoroPEP[18] (**2b**:**2c** = 88:12) and erythrose-4-phosphate (**1**) was catalysed by DAHP synthase. After consumption of fluoroPEP (decrease in absorbance at 234 nm), dehydroquinate synthase (with NAD^+) and dehydroquinase were added. The production of 6-fluorodehydroshikimate (**5b** and **5c**) was monitored at 234 nm. The final enzyme, shikimate dehydrogenase was then added (with NADPH). The reaction was considered complete when no further consumption of NADPH was observed after addition of extra shikimate dehydrogenase. The 6-fluoroshikimates (**6b** and **6c**) were purified using a combination of strong anion exchange and ion exclusion HPLC. This resolved the two epimers

and gave an isolated yield of each compound of approximately 17%. The 6-fluoroshikimates were identical to the corresponding synthetic samples[6] by ^1H and ^{19}F NMR spectroscopy.

The enzymatic transformation was studied in more detail by following each transformation by ^{19}F NMR spectroscopy. We wanted to investigate the reason for the modest overall yield (34%) and why approximately equimolar amounts of the two epimeric 6-fluoroshikimates (**6b** and **6c**) were isolated when a 88:12 mixture of **2b** and **2c** had been used.[19] Figure 1 shows representative spectra, the assignment of the ^{19}F NMR signals is given in Table 1, and the proportions of each fluorinated species in a transformation are recorded in Table 2.

Table 1: ^{19}F NMR chemical shifts and coupling constants of the fluorinated shikimate intermediates

	2b	**2c**	**3b**[a]	**3c**[a]	**4b**[a]	**4c**[a]	**4x**	**5b**[a]	**5c**[a]	**6b**[a]	**6c**[a]
shift:[b]	-141.4	-152.7	-206.0	-199.0[d]	-202.7	-202.3	-209.5	-194.5	-187.6	-189.4	-181.6
coupling	77.7	77.7	49.7	49.4	49.4	49.4	51.3	47.1	44.7	47.3	47.5
constants[e]			30.1	13.4	29.4	12.9	29.7	26.4	15.1	22.8	16.7

[a] Stereochemistry assigned on the basis of H_5-F_6 coupling constant. [b] ppm relative to CCl_3F = 0 ppm.

Table 2: The relative proportions[a] of the fluorinated intermediates determined by ^{19}F NMR spectroscopy

T (h)[b]	**2b**	**2c**	**3b**	**3c**	**4b**	**4c**	**4x**	**5b**	**5c**	**6b**	**6c**
0	88	12	-	-	-	-	-	-	-	-	-
1	27	-	63	10	-	-	-	-	-	-	-
4	-	-	63	-	14	10	14	-	-	-	-
6	-	-	57	-	6	6	16	11	5	-	-
7	-	-	54	-	-		17	-	-	17	12
23[c]	-	-	40	-	-		22	-	-	26	11

[a] Trifluoroacetate (-75.8 ppm) was used as an internal standard for determination of the concentrations of the fluorinated intermediates. ≥ 95% of the fluorine originally introduced as **2b** and **2c** was accounted for. [b] T = 0 h, spectrum then DAHP synthase added; T = 1 h, spectrum then dehydroquinate synthase added; T = 4 h, spectrum then dehydroquinase added; T = 5 h, additional dehydroquinate synthase and dehydroquinase added; T = 6 h, spectrum then shikimate dehydrogenase added. [c] After 23 hours, small concentrations of decomposition products began to appear.

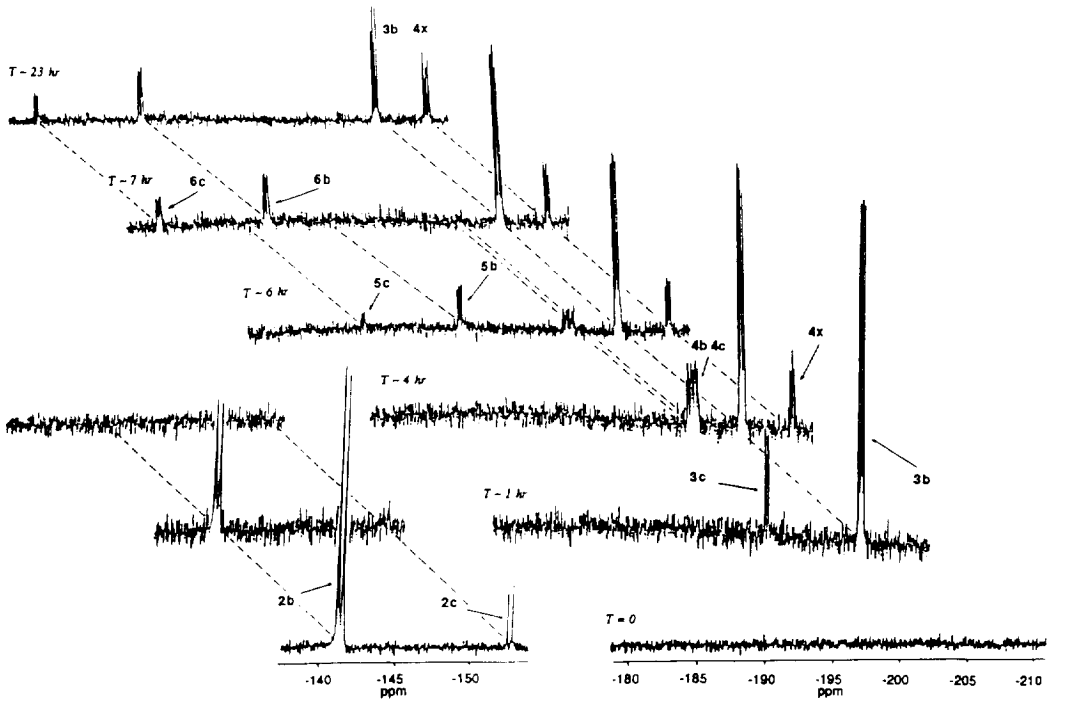


Figure1 Representative ^{19}F NMR spectra (235 MHz) of the enzymatic conversion of 3-fluoroPEPs (**2b** and **2c**, *Z:E* 88:12) to a mixture of 6-fluoroshikimates (**6b** and **6c**). The above spectra were recorded after 0, 1,,4, 6, 7, and 23 hours. The experimental details are described in reference 19.

The rates for each transformation were determined approximately from the average of the rate of disappearance of substrates and appearance of products by ^{19}F NMR spectroscopy (not all spectra shown). The following observations were made:

(i) Both *Z*- and *E*-fluoroPEP (**2b** and **2c**) are converted by DAHP synthase to the corresponding 3-fluoroDAHPs at approximately 2% the rate of PEP (**2a**). The slow conversion of *Z*-fluoroPEP has previously been reported.[11] The similarly slow conversion of *E*-fluoroPEP was unexpected, as a very large discrimation in favour of this isomer was seen in the analogous reaction catalysed by 3-deoxy-D-*manno*-2-octulosonate-8-phosphate (KDO8P) synthase.[20] Our NMR spectrocopic results confirm that *Z*-fluoroPEP (**2b**) is converted to *(3S)*-3-fluoroDAHP (**3b**) and *E*-fluoroPEP (**2c**) is converted to *(3R)*-3-fluoroDAHP (**3c**). This has previously been assumed.[11] The reaction with 3-fluoroPEP with DAHP synthase follows the same stereochemical course as the corresponding reaction using KDO8P synthase.[20]

(ii) The conversion of*(3S)*-3-fluoroDAHP (**3b**) to *(3S)*-fluorodehydroquinate (**4b**) is at least 10^3 times slower than the conversion of **3c** to **4c**. Furthermore conversion of **3b** yields an additional product **4x** which is not a substrate for dehydroquinase and slowly accumulates.

(iii) The reactions catalysed by dehydroquinase proceed more slowly with the fluorinated substrates **4b** and **4c**. This is in contrast to shikimate dehydrogenase reactions where the presence of fluorine in **5b** and **5c** appears to have little effect.

(iv) The respective yields of 6-fluoroshikimates do not reflect the initial distribution of fluorine in 3-fluoroPEP. The yields are a consequence of the preferential processing of **3c** and the partitioning of **3b** into **4b** and **4x**.

These experiments show that the enzymatic synthesis of 6-fluoroshikimates (**6b** and **6c**) from erythrose 4-phosphate (**1**) and 3-fluoroPEP (**2b** and **2c**) provides an attractive alternative to total synthesis. It has already been shown that the 6-fluoroshikimates can be enzymatically converted into the corresponding 6-fluoroEPSPs.[3] The results of the NMR spectroscopic study suggests that the relatively low overall yield of the fluorinated shikimates (**6b** and **6c**, 34%) results in part from the very slow reaction of **3b** and production of a side product **4x**. The structure of **4x** is under investigation.

Acknowledgement We thank the SERC for a postdoctoral grant for PJD, Dr Gareth Davies for authentic samples of 6-fluoroshikimates, and Mr David Fearing for recording the ^{19}F NMR spectra.

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(17) *Preparative experiment:* DAHP synthase (270 μL, 18 units) was added to a solution of cyclohexyl ammonium 3-fluoroPEP (16 mg, 61% pure, 0.030 mmol, 88% *Z* isomer), erythrose-4-phosphate

(sodium salt, 14 mg, 66% pure, 0.036 mmol), manganese sulphate (60 μM), cobalt sulphate (50 μM) in MOPS buffer (6 mL, 50 mM, pH 7) at 22 °C. The progress of the enzymatic reaction was followed by removing 10 μL aliquots, diluting them with 1 mL of water and measuring the absorbance at 234 nm. After 3 h, the conversion to 3-fluoroDAHP appeared to be complete. NAD^+ (100 μL, 1.5 mM soln, 0.15 μmol), dehydroquinase (7 μL, 4 units) and dehydroquinate synthase (600 μL, 18 units) were added and the reaction was monitored for the appearance of 6-fluorodehydroshikimate (234 nm). After 5 h NADPH (30 mg, 0.036 mmol) and shikimate dehydrogenase (1 μL, 8 units) were added. The consumption of NADPH was monitored at 340 nm and had stopped after 14 h. Additional shikimate dehydrogenase (1 μL, 8 units) was added and the incubation left for a further 4 hours. The reaction mixture was then concentrated *in vacuo* and injected onto a semiprep BioRad Organic Acids column (50 mM formic acid, 1.5 mL/min, 240 nm detection). The fractions containing 6-fluoroshikimates were concentrated and passed through a strong anion exchange column (potassium phosphate gradient 10-200 mM, pH 6.5, 4.5 mL/min, 240 nm detection) to remove contaminating $NADP^+$. This procedure yielded 17% of each epimer of 6-fluoroshikimate. The 6-fluoroshikimates were shown to be identical with synthetic samples[6] by ^1H and ^{19}F NMR spectroscopy and co-eluted on a BioRad Organic Acids HPLC column in 50 mM formic acid, 0.6 mL/min, retention times: *(6S)*-6-fluoroshikimate = 10.8 min, *(6R)*-6-fluoroshikimate = 12.1 min.

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(19) *^{19}F NMR experiment:* The activities of all enzymes were determined prior to each NMR experiment. The reaction mixture was maintained at pH 7.0 and 30 °C. Cyclohexyl ammonium 3-fluoroPEP (30 mg, 61%, 0.064 mmol, 88% *Z*), erythrose 4-phosphate (sodium salt, 26 mg, 66% pure, 0.077 mmol), trifluoroacetic acid (internal standard, 10 μl of a 10% soln in H_2O), MOPS (25 mg, 0.12 mmol), manganese sulphate (25 μL of a 2.4 mM soln) D_2O (160 μL), and MilliQ H_2O (490 μL) were combined in a 5 mm NMR tube. A ^{19}F NMR spectrum was recorded (T = 0) and DAHP synthase (500 μL, 27 units) was then added. After 1 h, cobalt sulphate (12 μL of a 7 mM soln), NAD^+ (10 μL of a 5 mM soln) and dehydroquinate synthase (400 μL, 18 units) were added. After 4 h, dehydroquinase (10 μL, 6 units) was added and after 5 h, more dehydroquinate synthase (60 μL, 3 units), dehydroquinase (10 μL, 6 units) and NAD^+ (10 μL of a 5 mM soln) were added. After 6 h, the potassium salt of NADPH (60 mg, 0.067 mmol) and shikimate dehydrogenase (10 μL, 10 units) were added.

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(*Received in Belgium* 26 *July* 1995; *accepted* 4 *September* 1995)