Tetrahedron Letters,Vol.29,No.51,pp 6779-6782,1988 0040-4039/88 $3.00 + .00
Printed in Great Britain Pergamon Press plc

SPECIFICITY OF *E. COLI* SHIKIMATE DEHYDROGENASE TOWARDS ANALOGUES OF 3-DEHYDROSHIKIMIC ACID

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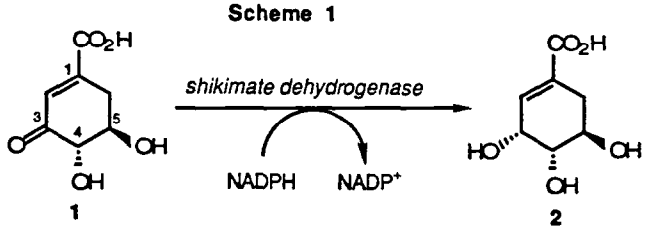
Summary: Analogues of 3-dehydroshikimic acid which lack the C-4 and C-5 hydroxyl groups have been synthesised and assayed as substrates for shikimate dehydrogenase. The presence of the C-4 hydroxyl group is found to be very important for specificity, whereas the C-5 hydroxyl group is not. The enzyme exhibits enantioselectivity at C-1 and C-4 of the racemic substrate analogues.

Scheme 1



1 2

The reduction of 3-dehydroshikimic acid (**1**) to shikimic acid (**2**) catalysed by shikimate dehydrogenase (EC 1.1.1.25; Scheme 1) is the fourth step of the shikimate pathway.[1] Early studies on the *Pisum sativum* (pea seedling) enzyme indicated that it is strongly selective for NADPH over NADH,[2] and the reverse reaction of this enzyme has been shown to be inhibited by a number of simple aromatic compounds.[3] *E. coli* shikimate dehydrogenase has recently been purified to homogeneity and sequenced,[4] which has made possible active site studies on this enzyme. In order to investigate enzyme-substrate interactions, analogues of 3-dehydroshikimate have been synthesised which lack the C-4 and C-5 hydroxyl groups, and the carbon-carbon double bond (Scheme 2).

The dideoxy analogue (**4**) was synthesised by chromic acid oxidation of methyl cyclohex-1-enecarboxylate, followed by hydrolysis.[5] The 5-deoxy analogue (**6**) was synthesised by α-hydroxylation of (**3**) via MCPBA oxidation of its silyl enol ether (**5**), followed by careful hydrolysis. The dideoxy-dihydro analogue (**7**) was synthesised by catalytic hydrogenation of (**3**), followed by hydrolysis. The natural substrate 3-dehydroshikimic acid (**1**) was prepared from shikimic acid by the method of Ganem.[6]

3-Dehydroshikimic acid and the three analogues were assayed as substrates for shikimate dehydrogenase, by monitoring the decrease in absorbance of NADPH at 340 nm. All were found to be substrates, and all showed Michaelis-Menten kinetics. Values of K_m and k_{cat} were determined from Lineweaver/Burk plots[7] and are given in Table 1.

Table 1[a]

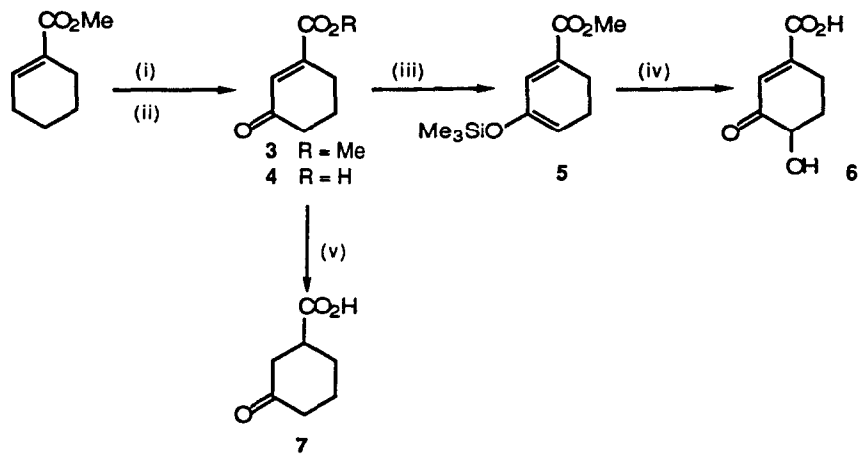
Substrate	K_m (mM)	k_{cat} (s^{-1})[b]	k_{cat}/K_m ($M^{-1}s^{-1}$)
3-Dehydroshikimate (**1**)	0.090	100	1.1×10^6
5-deoxy analogue (**6**)	0.140[c]	75	5.3×10^5
dideoxy analogue (**4**)	17.0	0.060	3.5
dideoxy-dihydro analogue (**7**)	0.75[c]	0.095	1.3×10^2

a Assays were carried out in 100 mM potassium phosphate buffer (pH 7.0) at 20 °C.

b Values of k_{cat} are based on protein concentrations obtained from Bradford protein assays.

c Values corrected for enantiomeric specificity.

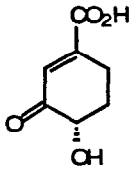
Scheme 2



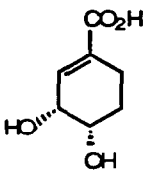
(i) CrO_3, AcOH, 34%. (ii) K_2CO_3, MeOH/H_2O, 60%.
(iii) LDA, THF, -78°C; TMSCl, 100%.
(iv) MCPBA, CH_2Cl_2; 10% HCl/THF, 50%; $NaHCO_3$, MeOH/H_2O, 20%.
(v) H_2/Pd/C, MeOH, 60%; K_2CO_3, MeOH/H_2O, 60%.

Measurement of k_{cat}/K_m with natural and deoxy- substrates allows the estimation of apparent binding energies between each hydroxyl group and the enzyme.[8] The results in Table 1 indicate that for the C-5 hydroxyl group the apparent binding energy (ΔG_{app}) is 1.8 kJ mol^{-1}, whereas for the C-4 hydroxyl group ΔG_{app}= 29 kJ mol^{-1}. This demonstrates that removal of the C-5 hydroxyl group has little effect on specificity, but removal of the C-4 hydroxyl group is very significant. The observed loss of specificity on removal of the C-4 hydroxyl group of ΔG_{app}= 29 kJ mol^{-1} suggests removal of a hydrogen bonding interaction between substrate and a charged group at the enzyme active site.[8] This is in agreement with the observation by Balinsky and Davies that the most effective aromatic inhibitors of the reverse reaction of *P. sativum* shikimate dehydrogenase possessed a *para*-hydroxyl group.[4] From examination of the pH/rate profile they suggested that this hydroxyl group forms a hydrogen bond to either a cysteine or an α-NH_2 group on the enzyme.[9]

As the 5-deoxy analogue (**6**) was a racemic mixture, it was of interest to study whether the enzyme had selectively reduced one enantiomer of (**6**). Chemical reduction of (**6**) with sodium borohydride gave a 1:2 mixture of *cis*-(**8**) and *trans*-(**9**) diols, which were separated by HPLC.[10] Their respective methyl esters were characterised by proton NMR spectroscopy: the *cis*-diol showed a 2% NOE between the C-3 and C-4 protons, whereas the *trans*-diol showed no such NOE.[11] Treatment of (**6**) with shikimate dehydrogenase and NADPH rapidly gave the *cis*-diol (**8**) only, as observed by HPLC, indicating that the enzyme had selectively reduced the S enantiomer (**6a**). This is the enantiomer corresponding to the configuration of the natural substrate (**1**). However, incubation with a large amount of enzyme eventually led to the appearance of the *trans*-diol (**9**), indicating that the enzyme would also reduce the R enantiomer very slowly. If left to reach equilibrium, equal amounts of the *cis*- and *trans*- diols were produced, and 4.4% of the substrate remained, corresponding to an equilibrium constant of 22. This is comparable with the reported value for 3-dehydroshikimic acid of 28.[2]



6a



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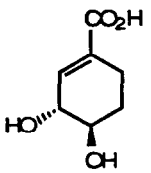

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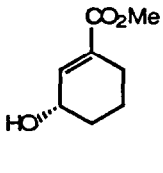

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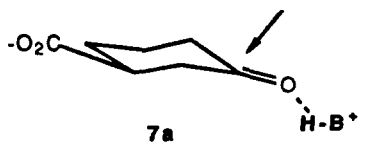
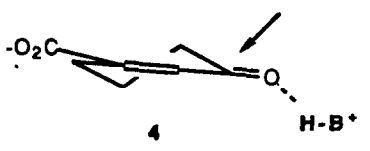
Treatment of the dideoxy analogue (**4**) with shikimate dehydrogenase and NADPH,[12] followed by methylation with diazomethane, gave the allylic alcohol (**10**). The optical purity of (**10**) was confirmed by addition of the chiral shift reagent $Eu(tfc)_3$ to a solution of (**10**) in CD_2Cl_2. No splitting of the vinylic proton signal (δ 6.81 ppm) was observed in the proton NMR spectrum, whereas treatment of a racemic sample of (**10**) under the same conditions gave rise to two well-resolved peaks. Studies to confirm the absolute configuration of (**10**) are in progress.

Treatment of the dideoxy-dihydro analogue (**7**) with shikimate dehydrogenase and NADPH,[12] followed by methylation with diazomethane, gave uniquely the *trans*- product (**11**). This was identified by ^1H and ^{13}C NMR spectroscopy,[13] and showed that the enzyme had selectively reduced the S enantiomer (**7a**). This enantiomer can adopt a conformation with the carbonyl group pointing down below the plane of the ring. It is probable that such a conformation would be able to form a hydrogen-bond with the group that protonates the carbonyl in the reduction of the natural substrate (Scheme 3).

Scheme 3



7a



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In conclusion: shikimate dehydrogenase has been shown to reduce a series of analogues of 3-dehydroshikimic acid stereospecifically. The C-4 hydroxyl group appears to provide an important binding interaction with the enzyme, and the enzyme in turn selects the dehydroshikimate-like enantiomer at C-4, although it will convert the other enantiomer very slowly.

Acknowledgements. The authors would like to thank J. Greene, University of Glasgow, for purification of *E. coli* shikimate dehydrogenase, A. Leech, University of Cambridge, for NMR spectroscopy, and the S.E.R.C. and The Research Corporation Trust for financial support.

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10. All HPLC separations were carried out on a Bio-Rad Aminex HPX-87H Organic Acids column, using 50 mM formic acid as eluant.
11. All new compounds were fully characterised by NMR, IR and HRMS.
12. Enzymic reductions of (**4**) and (**7**) were carried out on a 5-10 mg scale by stirring overnight at room temperature in 100 mM potassium phosphate buffer (pH 7.0) in the presence of 50-100 units *E. coli* shikimate dehydrogenase and 1 molar equivalent of NADPH. Isolated yields of (**10**) and (**11**) were 85% and 90% respectively.
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(Received in UK 31 October 1988)