Communication

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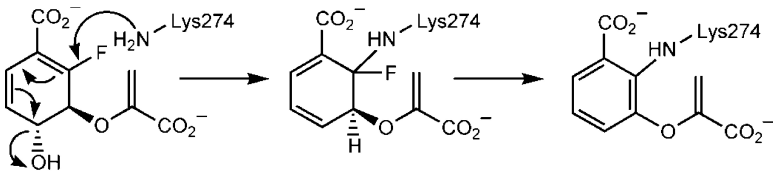

2-fluorochorismate

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Identification of 4-Amino-4-deoxychorismate Synthase as the Molecular Target for the Antimicrobial Action of (6*S*)-6-Fluoroshikimate

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The shikimate pathway leads to the aromatic amino acids and several key aromatic compounds. The pathway is an attractive target for antimicrobial agents as it is present in microorganisms but not in mammals. (6*S*)-6-Fluoroshikimate (**1a**) and (6*R*)-6-fluoroshikimate (**1b**) have antimicrobial activity against *Escherichia coli* with (6*S*)-6-fluoroshikimate being the more potent.[1] The 6-fluoroshikimates are taken up by *E. coli* and assumed to be transformed into fluorinated analogues of later-pathway intermediates which are the biologically active agents.[2] In this communication we provide evidence that the antimicrobial effect of (6*S*)-6-fluoroshikimate is ultimately the result of irreversible inhibition of 4-amino-4-deoxychorismate synthase (ADCS) by 2-fluorochorismate.

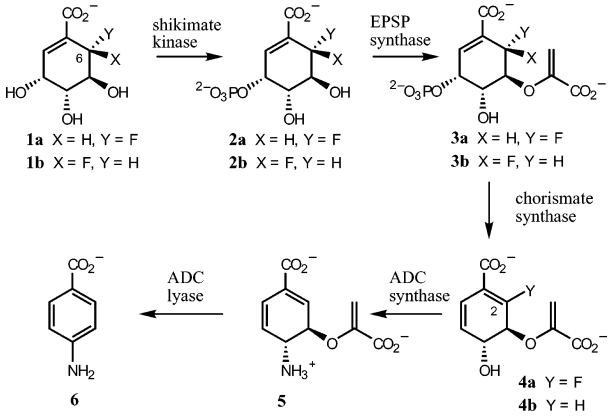
It has previously been shown that 6-fluoroshikimates can be phosphorylated in vitro by shikimate kinase to give the corresponding 6-fluoroshikimate 3-phosphates (**2a**, **2b**), which in turn are converted by 5-enol-pyruvylshikimate 3-phosphate (EPSP) synthase to 6-fluoroEPSPs (**3a**, **3b**) (Scheme 1).[3] On binding of (6*R*)-6-fluoroEPSP to *E. coli* chorismate synthase the enzyme's flavin cofactor is oxidized to a semiquinone radical[4] and (6*R*)-6-fluoroEPSP is converted to chorismate (**4b**).[5] Formation of this dead-end complex blocks the catalytic action of chorismate synthase.

Somewhat unexpectedly, (6*S*)-6-fluoroEPSP (**3a**) was found to inhibit *Neurospora crassa* chorismate synthase.[3] However, *E. coli* chorismate synthase was subsequently shown to transform (6*S*)-6-fluoroEPSP into 2-fluorochorismate (**4a**) albeit at a rate 270−370 times slower than the conversion of EPSP.[6] This observation, coupled with the ability of *p*-aminobenzoate to overcome the antimicrobial effect of (6*S*)-6-fluoroshikimate,[1] led to the hypothesis that the antimicrobial action of (6*S*)-6-fluoroshikimate is due to inhibition by 2-fluorochorismate of one of the enzymes involved in the conversion of chorismate to *p*-aminobenzoate.[6]

ADCS catalyzes the amination of chorismate (**4b**) at C4 to generate ADC (**5**), which is converted to the folate precursor *p*-aminobenzoate (**6**) by ADC lyase.[7,8] ADCS is a heterodimer consisting of a chorismate-aminating subunit, PabB, and a glutamine amidotransferase subunit, PabA. PabB is able to catalyze the ADCS reaction in vitro in the absence of PabA if a high concentration of ammonium ions is provided. We now report the inhibition of the PabB component of ADCS by 2-fluorochorismate and show how the mechanism of inhibition relates to a recently proposed mechanism for this enzyme. [9]

(6*S*)-6-FluoroEPSP was prepared from *E*-fluorophosphoenol−pyruvate and erythose-4-phosphate by the sequential reactions of DAHP synthase, dehydroquinate synthase, dehydroquinase, shikimate dehydrogenase, and EPSP synthase.[3,10] The (6*S*)-6-fluoroEPSP

Scheme 1. Conversions Catalyzed by Shikimate Kinase, EPSP Synthase, Chorismate Synthase, ADCS, and ADC Lyase on Fluorinated and Non-fluorinated Substrates



was then transformed to 2-fluorochorismate using chorismate synthase.[6] The production of 2-fluorochorismate was followed by ^{1}H and ^{19}F NMR spectroscopy. Chorismate synthase was removed from the preparation and the 2-fluorochorismate used for ADCS inhibition studies without further purification.

ADCS activity was monitored in the presence of ADC lyase, which converts ADC into *p*-aminobenzoate (**6**) (λ_{max} 265 nm). The activity assays were carried out in the absence of PabA with ammonium chloride (100 mM) present. Incubation of PabB (30 μM) with 2-fluorochorismate (100 μM) led to a rapid decrease in ADCS activity with complete inactivation of the enzyme after 5 min (inhibition data in Supporting Information). Addition of chorismate competitively reduced the rate of loss of activity, suggesting that 2-fluorochorismate was binding at the active site. Extensive washing of inactivated PabB with buffer did not restore enzyme activity, indicating that 2-fluorochorismate was irreversibly bound to the enzyme. Kinetic constants for the irreversible inhibition were determined by incubating PabB with various concentrations of 2-fluorochorismate and following the loss in ADCS activity. An approximate k_i of 1.0 min^{-1} and a K_I of 130 μM were determined.

Electrospray mass spectrometry was carried out on PabB that had been inactivated (approximately 90%) with 2-fluorochorismate. The sample was found to contain covalently modified PabB with a mass of 51167 ± 5 Da and a small amount of unmodified PabB with a mass of 50969 ± 5 Da. Hence 2-fluorochorismate was causing a covalent modification of 198 ± 10 Da to PabB.

To identify the residue undergoing covalent modification, both untreated and 2-fluorochorismate-inactivated PabB were digested with trypsin. The peptides produced were separated by HPLC on a C18 capillary column and analyzed by electrospray mass

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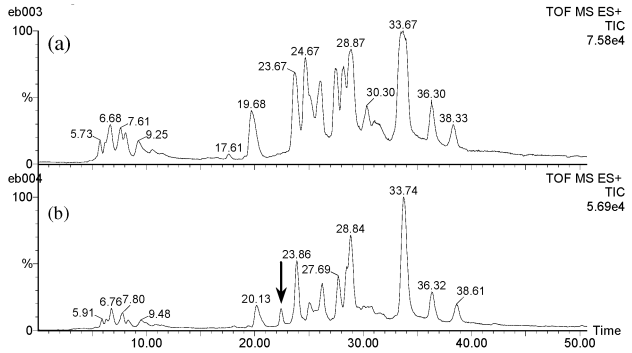


Figure 1. HPLC separation of peptides produced from the tryptic digest of PabB (a) and 2-fluorochorismate inactivated PabB (b). The arrow indicates a novel peptide with a monoisotopic mass of *m/z* 1087.6 $[M + H]^+$ in the digest of inactivated ADCS.

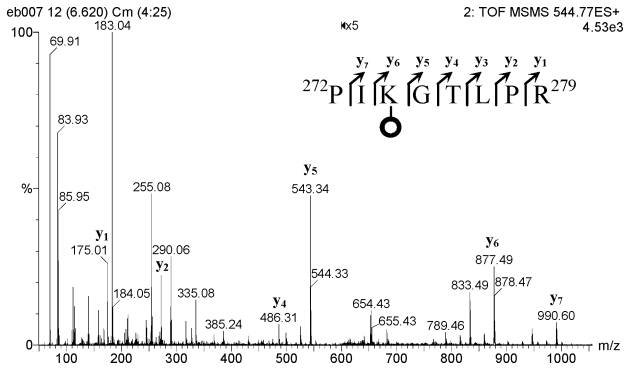


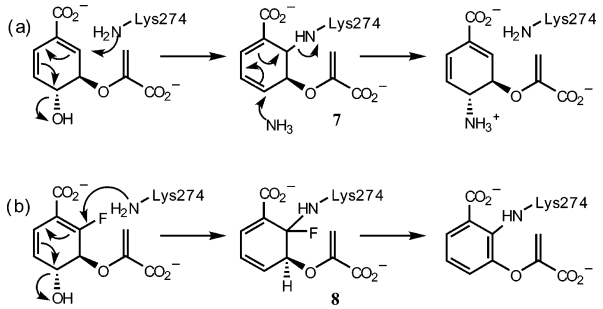
Figure 2. Sequencing of the *m/z* 1087.6 peptide by MS/MS revealed a modification of 206.1 Da on Lys-274. The y-ions are indicated.

spectrometry on a tandem quadrupole-time-of-flight instrument. A novel peptide with a monoisotopic mass of *m/z* 1087.6 $[M + H]^+$ was identified in the HPLC trace of the tryptic digest of 2-fluorochorismate-inactivated PabB by comparison with the trace of a digest of untreated PabB (Figure 1). Sequencing by tandem mass spectrometry (MS/MS) showed that the peptide corresponded to residues 272−279 of PabB with a modification of 206.1 Da on Lys-274 (Figure 2). Normally trypsin would have been expected to cleave on the carboxylic side of Lys-274, but it appears that the modification prevented this cleavage.

The discovery that 2-fluorochorismate covalently modifies Lys-274 of ADCS is especially interesting in relation to recent structural and mechanistic studies. The crystal structure of unliganded PabB has been reported.[11] Modeling chorismate into the enzyme active site shows that the ω-amino group of Lys-274 would be in close proximity to C2 of the bound substrate (Supporting Information). This observation and studies of the K274A mutant of PabB led to a novel proposal for the enzyme mechanism (Scheme 2a)[9] in which the ω-amino group of Lys-274 adds to chorismate at C2, leading to elimination of the C4 hydroxyl in an S_N2'' displacement reaction. If ammonia then attacks this enzyme-bound intermediate (**7**) at C4, another S_N2'' displacement would expel the Lys-274 side chain and form ADC.

This proposal provides a mechanistic framework for the inhibition of ADCS by 2-fluorochorismate (Scheme 2b).[9] If the side chain of Lys-274 similarly adds to C-2 of 2-fluorochorismate leading to elimination of the C4 hydroxyl in an S_N2'' displacement reaction,

Scheme 2. (a) Proposed Mechanism for ADCS. (b) Proposed Mechanism for the Irreversible Inhibition of ADCS by 2-Fluorochorismate



the fluorinated adduct (**8**) would result. Elimination of HF would then produce an aromatic species of molecular mass 206.1 Da irreversibly bound to Lys-274, in accord with the experimental observation.

This communication not only provides evidence that the antimicrobial effect of (6*S*)-6-fluoroshikimate is ultimately the result of irreversible inhibition of ADCS by 2-fluorochorismate, it also provides strong support for the recently proposed catalytic mechanism for ADCS.[9] 2-Fluorochorismate is the most potent inhibitor known for ADCS and may provide inspiration for a new generation of antimicrobial compounds.

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Supporting Information Available: Preparation of reagents and enzymes, kinetics of 2-fluorochorismate inactivation of PabB, and analysis of 2-fluorochorismate-inactivated PabB. This material is available free of charge via the Internet at http://pubs.acs.org.

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