The versatility of phosphoenolpyruvate and its vinyl ether products in biosynthesis

The diverse enzymes that use phosphoenolpyruvate as a substrate lie at the heart of cellular energy metabolism, as well as a number of critical biosynthetic pathways. The versatility of the enol ether linkage is reflected not only in the rich chemistry and enzymology of PEP, but also in the variety of metabolites in which the high-energy enol ether linkage is preserved.

Chemistry & Biology February 1996, **3**:83–91

Phosphoenolpyruvate (PEP) is a highly functionalized, chemically versatile molecule used at several intersections of cellular energy metabolism and biosynthesis. Here, we first examine the distinct types of reactions that the three-carbon skeleton of PEP can undergo, which fall into two main classes depending on whether PEP undergoes enzymatic cleavage of the P–O bond or the C–O bond (Fig. 1). We focus in particular on the mechanism of enol ether transfer catalyzed by either of two enzymes, UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) or 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase. In the second half of this review, we explore the remarkably diverse biosynthetic fates that await the enol ether products generated from PEP by these enzymes.

Enzymatic cleavage routes of PEP

P–O cleavage

As a trapped enol, PEP is both a thermodynamically activated phosphorylating agent ($\Delta G = -14.8$ kcal mol^{-1}) and a kinetically stable precursor of a carbanion equivalent at carbon 3 (C_3), which can be generated in the active site of an enzyme by P–O cleavage (Fig. 1, path a). This carbanion equivalent can be directed to react with electrophilic cosubstrates (Fig. 2), undergoing protonation, carboxylation (with concomitant C–C bond formation), or even addition of electrophilic phosphorus in the PEP mutase-mediated generation of the C–P bond of phosphonopyruvate [1,2]. PEP thus serves the dual roles of phosphoryl transfer agent and versatile biosynthon, acting as a regiospecific C_3 carbanion precursor in C–C bond formation during fixation of CO_2, in carbohydrate metabolism, and in C–P bond construction in naturally occurring phosphinates and phosphonates.

C–O cleavage and the net aldol condensation

A second enzymatic route to cleavage of PEP (illustrated in Fig. 1, paths b_1 and b_2) is catalyzed by one of four different enzymes, either from the shikimate pathway of microbes or plants [3], or from the pathways involved in the biosynthesis of bacterial peptidoglycan or lipopolysaccharide. The net aldol condensations of PEP with erythrose-4-P or arabinose-5-P both follow path b_1; these reactions are catalyzed by 3-deoxy-D-*arabino*-2-heptulosonate-7-phosphate (DAHP) synthase (Fig. 3a) and 3-deoxy-D-*manno*-2-octulosonate-8-phosphate (KDO8P) synthase (Fig. 3b), which function in aromatic amino acid and bacterial *O*-antigen biosynthetic pathways, respectively. Although these reactions might be expected to proceed with P–O cleavage and generation of the C_3 carbanion equivalent described above, experiments using [^{18}O]PEP (labeled as shown in Fig. 1) have established that ^{18}O is not retained in the sugar product. Thus, these reactions must proceed with C–O cleavage [4–7]. In this reaction, the C_2 atom of PEP reacts as a latent electrophile, while C_3 of PEP serves as a nucleophile.

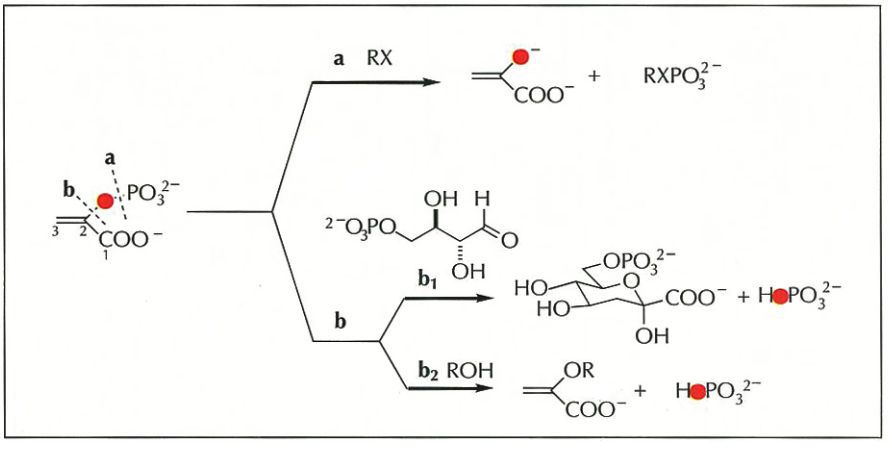

Fig. 1. Enzymatic cleavage routes of PEP.

 ISSN 1074-5521

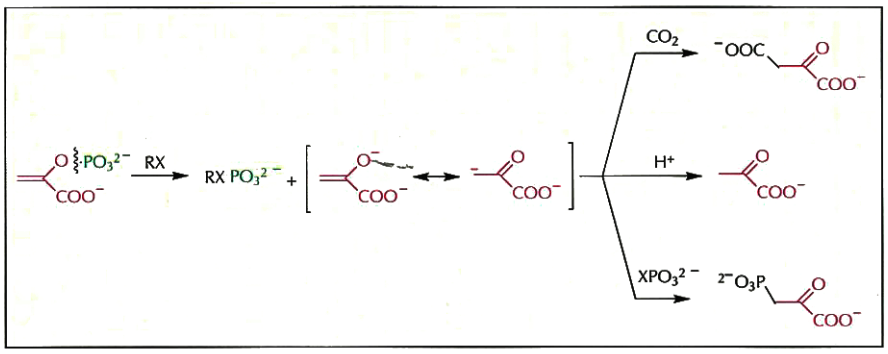

Fig. 2. Cleavage of the P–O bond of PEP and generation of a C_3 carbanion equivalent.

Stereochemical studies using 3-^{2}H-PEP isomers as substrates for KDO8P synthase have shown that (*Z*)-3-^{2}H-PEP yields (3*S*)-^{2}H-KDO8P, whereas (*E*)-3-^{2}H-PEP yields (3*R*)-^{2}H-KDO8P, consistent with 2-*si* face addition to PEP [8,9]. Interestingly, these stereochemical studies also reveal that H_Z and H_E are distinguishable throughout the reaction, arguing against protonation at C_3 of PEP and formation of a torsiosymmetric C_3 methyl group at any stage in catalysis. The timing of C–C bond formation (which occurs by attack of the π electrons from the *si* face of the PEP double bond on the *re* face of the aldehyde) relative to the addition of the 3-OH of arabinose-5-P onto the C_2 of PEP (the electrophile) has not yet been determined. Both synchronous addition (Fig. 3c, path a) and a stepwise mechanism (C–C bond formation leading to a C_2 oxocarbenium capture by intramolecular addition of the 3-OH of the sugar moiety; Fig. 3c, path b) have been suggested as mechanisms that could yield the proposed pyranose bisphosphate intermediate

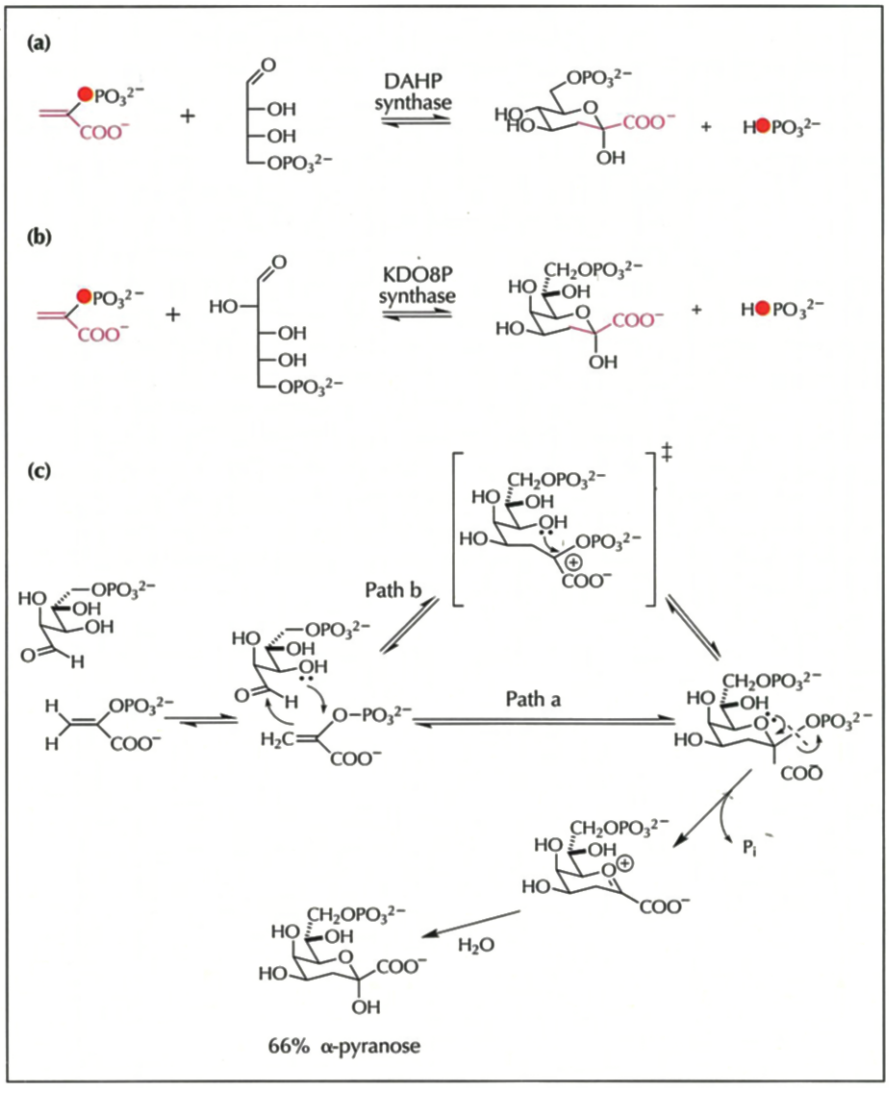

Fig. 3. Net aldol condensation reactions involving PEP. **(a)** The reaction of PEP with erythrose-4-P, catalyzed by DAHP synthase. **(b)** The reaction of PEP with arabinose-5-P, catalyzed by KDO8P synthase. **(c)** Proposed mechanism of catalysis by KDO8P synthase.

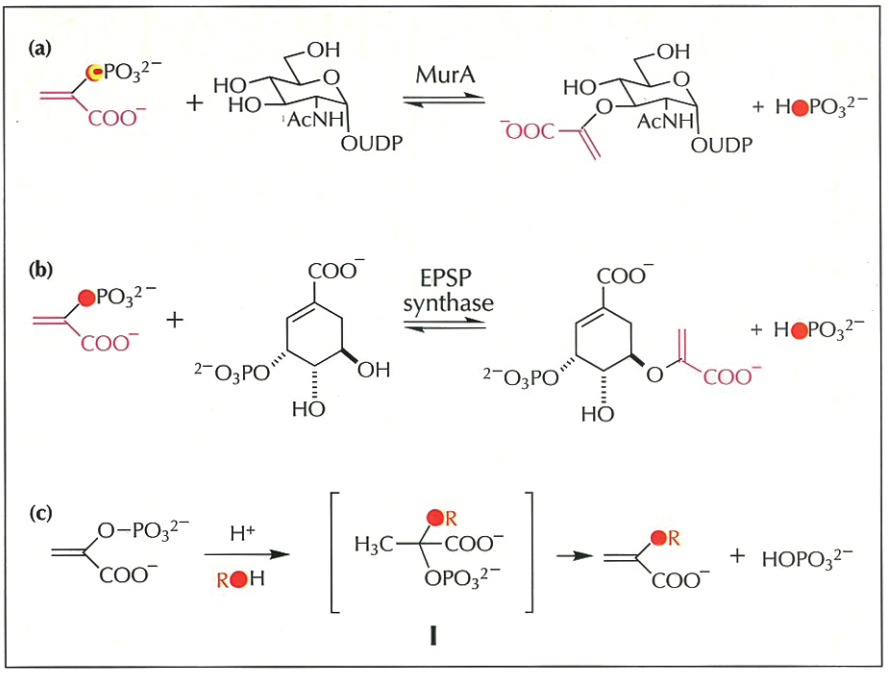

Fig. 4. Enzymatic enol ether transfer reactions catalyzed by **(a)** MurA and **(b)** EPSP synthase. **(c)** Enol ether transfer proceeds through a tetrahedral intermediate [14–17, 19].

[10,11]. Loss of P_i would accomplish the observed C-OPO_3^{2-} cleavage [6,7] via an oxocarbenium ion, which is then hydrated to yield the 2:1 mix of α- and β-pyranose products.

C–O cleavage and enol ether transfer

A second distinct class of enzymatic reaction involving C–O cleavage of PEP and the reactivity of C_2 of PEP as an electrophile (Fig. 1, path b_2) is represented by the reactions catalyzed by MurA, an enzyme involved in bacterial peptidoglycan biosynthesis (Fig. 4a), and EPSP synthase, which is part of the shikimate pathway (Fig. 4b). These enzymes catalyze the enol ether transfer from PEP to the respective cosubstrate alcohols and have been of particular interest not only because of the unusual chemistry involved, but also because they are both the targets of commercially important inhibitors. EPSP synthase is targeted by the herbicide glyphosate [12], and MurA is the site of action of the antibiotic fosfomycin [13].

Rapid-quench studies of EPSP synthase [14,15] led to the isolation and NMR characterization of a tetrahedral adduct, normally formed and decomposed in the enzyme's active site, that is kinetically and chemically competent. An analogous ketal has now been characterized as an intermediate in MurA catalysis [16,17], so the reactions shown in Figure 4a,b follow the pathway shown in Figure 4c, where the C_3 of PEP goes from sp^2 hybridization to the sp^3 C_3 methyl group in the tetrahedral adduct **I** and back to sp^2 in the vinyl ether product.

As in the case of the KDO8P synthase reaction, the timing of the enzymatic addition of ROH across the vinyl double bond of PEP was difficult to resolve. The reaction could be stepwise, for example, being initiated by protonation at the C_3 terminus of the enol to yield a C_2 oxocarbenium ion transition state. Alternatively, it could involve synchronous addition of H^+ and RO^- to give the ketal directly. The timing of the elimination of phosphate and deprotonation at C_3 from the ketal intermediate **I** (to complete a net enolpyruvyl transfer and yield a vinyl ether product; Fig. 4c) has also been uncertain. In particular, the mechanism by which the non-acidic hydrogen of the methyl group of intermediate **I** is readily made labile has been unclear.

Probing enzyme reaction mechanisms using 3-fluoro analogs of phosphoenolpyruvate

The question of whether the addition and elimination steps in enolpyruvyl transfer are synchronous or stepwise has been addressed by recent studies with (*E*)- and (*Z*)- isomers of 3-fluorophosphoenolpyruvate (FPEP) on both MurA [18,19] and EPSP synthase ([20] and D.H.K, unpublished results). The 3-fluoro analog has been shown to be a pseudosubstrate, generating the fluoromethyl analogs **II** or **III** of the normal tetrahedral intermediates (Fig. 5) in the active site of MurA or EPSP synthase, respectively. However, breakdown of the intermediate analogs in the forward direction (and the

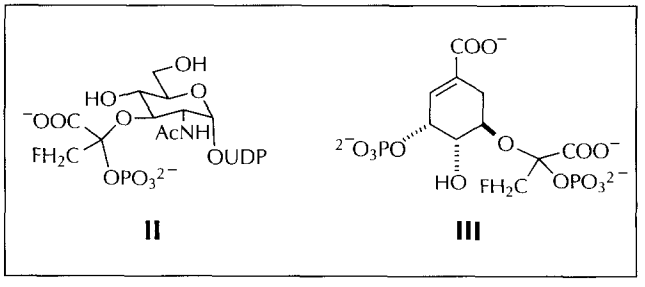

Fig. 5. Fluoromethyl analogs of the normal tetrahedral intermediates formed at the active site of MurA (**II**) or EPSP synthase (**III**). The electronic effect of F substitution for H slows enzymatic catalysis and produces stoichiometric titration of the two enzymes.

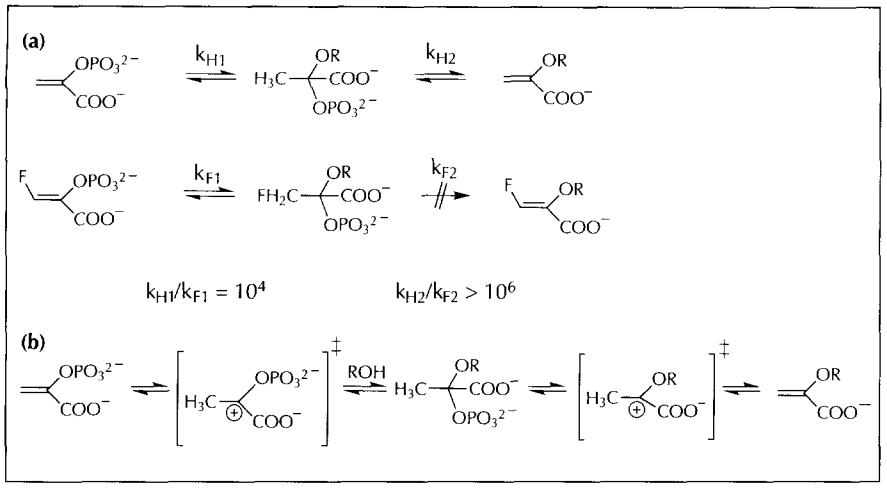

Fig. 6. Kinetic analysis of MurA-catalyzed enol ether transfer using (*Z*)-FPEP (ROH = UDP-GlcNAc). **(a)** Both k_{F1} and k_{F2} are greatly depressed by fluorine substitution. **(b)** Proposed mechanisms of addition and elimination involving oxocarbenium ion transition states.

reverse direction) has been so slowed by the electronic perturbation of the F-for-H substitution that each enzyme comes to a halt in mid-catalytic cycle, resulting in time-dependent inhibition. For MurA, kinetic analysis has revealed a 10^4-fold rate reduction in forming the fluoromethyl adduct **II** and a greater than 10^6-fold reduction in the rate of its breakdown compared to the native substrate (Fig. 6a). These studies provide strong evidence for oxocarbenium ion transition states in both formation and decomposition of the normal reaction intermediate (Fig. 6b) [19]. A similar argument has been put forth for EPSP synthase [21], based on the behavior of analog **III** [20] and analogous chemically-synthesized difluoro analogs [22]. Comparable rate depressions, effected electronically by fluorine substituents, have also been observed in prenyl transferase reactions [23] and have been interpreted as indicating the presence of carbocationic intermediates.

An ancillary benefit of the robust kinetic stability of the fluoromethyl analogs **II** and **III** ($t_{1/2}$ > 500 h at pH 8.0 for analog **II**, after enzyme denaturation and release of the adduct from the active site) has been the opportunity to generate chiral -CHDF fluoromethyl diastereomeric adducts, using (*E*)- or (*Z*)-FPEP in D_2O. These adducts were isolated from the self-inactivated MurA and EPSP synthase active sites and subjected to stereochemical analysis, establishing that the stereochemistry of addition at C_3 of PEP is to the *si* face in both enzymatic enolpyruvyl transfer reactions ([24] and D.H.K, unpublished results). The relative stereochemistry of the pair of addition and elimination steps is also constrained (*syn/anti* or *anti/syn*) in the reactions catalyzed by MurA (as shown by studies on (*Z*)- and (*E*)-phosphoenolbutyrate) [25] and by EPSP synthase (as shown by studies on (*Z*)- and (*E*)-^{3}H-PEP) [26,27]. This leaves only the absolute stereochemistry of addition of the cosubstrate alcohol at C_2 of PEP to be determined to complete the stereochemical description of enzymatic enolpyruvyl transfer.

Fates of vinyl ether metabolites

Four enzymatic fates for vinyl-ether-containing molecules are illustrated in Figure 7. The first three substrates (Fig. 7 a–c) are clearly in the greater shikimate pathway, while the fourth (Fig. 7d) is the second step in bacterial peptidoglycan biosynthesis.

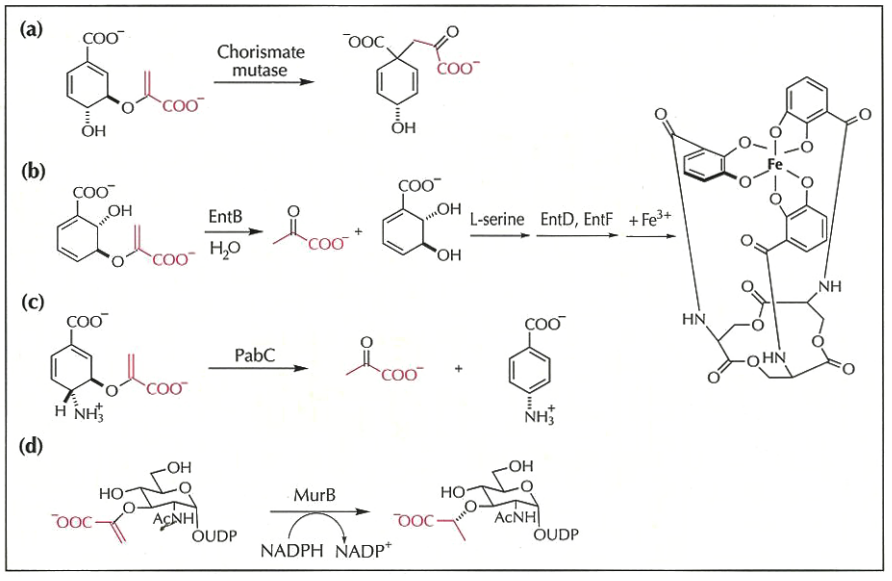

Fig. 7. Four enzymatic fates for vinyl-ether-containing molecules. EPSP can be converted to chorismate, isochorismate or 4-amino-4-deoxychorismate, which are substrates for the reactions shown in **(a)**, **(b)** and **(c)**, respectively. **(d)** In the second step of peptidoglycan assembly, the vinyl ether, enolpyruvyl-UDP-GlcNAc is reduced by MurB to yield the lactyl ether UDP-*N*-acetylmuramic acid.

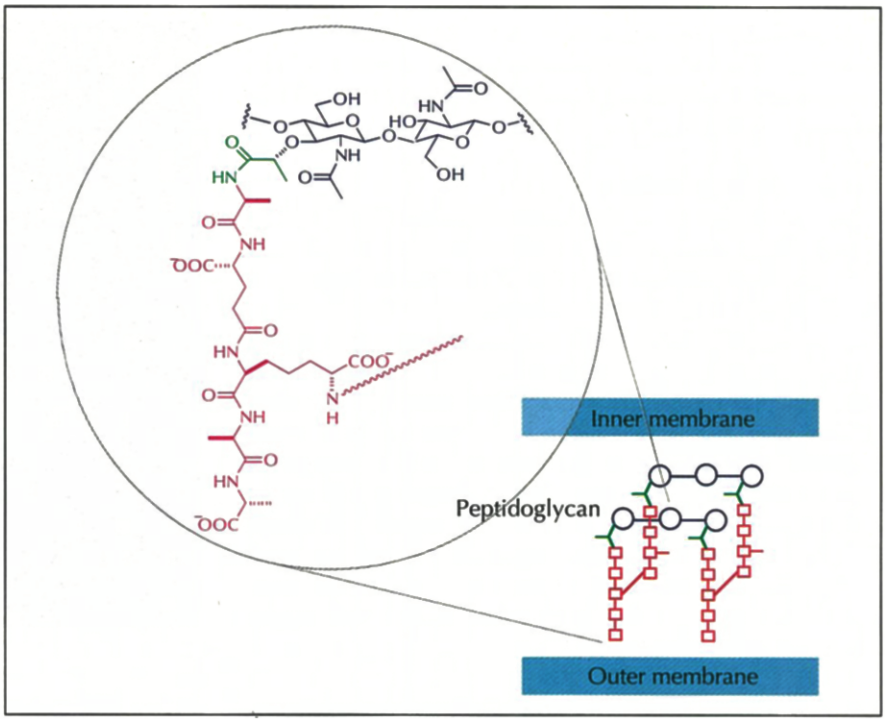

Fig. 8. Molecular view of peptidoglycan structure. The D-lactyl ether stem of muramic acid (green) serves as the bridge between the disaccharide portion (blue) and pentapeptide portion (magenta) of the bacterial cell wall.

The shikimate pathway

EPSP, generated as shown in Figure 4b, can be converted enzymatically to any one of the substrates in the reactions shown in Figure 7a–c. Chorismate (the substrate in Fig. 7a) is produced by a 1,4-elimination of P_i from EPSP whereas the curious 1,5-isomerization catalyzed by the enzyme isochorismate synthase (EntC) [28] yields isochorismate (the substrate in Fig. 7b). Isochorismate, in turn, is processed by PabA and PabB [29] to the 4-amino-4-deoxychorismate substrate of the reaction catalyzed by PabC (Fig. 7c).

The four transformations shown in Figure 7 encompass some interesting biological chemistry. The chorismate mutase reaction (Fig. 7a) is the celebrated 3,3-sigmatropic rearrangement with migration of the enolpyruvyl moiety and C–C bond formation in prephenate. This reaction has been described in detail [30,31] and therefore is not further analyzed here. The product prephenate has the pyruvyl side chain in place to yield, on enzymatic aromatization, either phenylpyruvate or *p*-hydroxyphenylpyruvate, which are on the routes to phenylalanine and tyrosine synthesis, respectively. Figure 7b depicts a vinyl ether hydrolysis, most likely by water addition at C_2 of the vinyl ether, catalyzed by the enzyme EntB [32]. The dihydrodihydroxybenzoate product is subsequently dehydrogenated by EntA [33] to the catechol, which, after conversion to the L-seryl amide, is trimerized to yield the iron-hexacoordinating ligand of enterobactin, an extracellular siderophore responsible for iron uptake in some bacteria. A third distinctive biosynthetic fate (Fig. 7c) for the vinyl ether functionality in the chorismate family is the eliminative aromatization of 4-amino-4-deoxychorismate, catalyzed by PabC, to yield the vitamin PABA, a key precursor for the folate

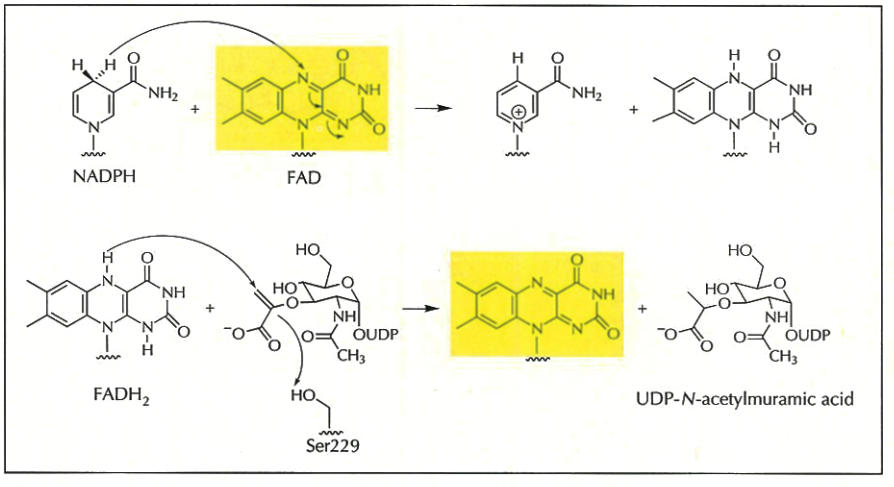

Fig. 9. Mechanism of the reaction catalyzed by MurB. The bound FAD acts as the mediator of hydride transfer between NADPH and enolpyruvyl-UDP-GlcNAc.

cofactors. An analogous enzymatic elimination of the isomeric 2-amino-2-deoxychorismate occurs in the formation of anthranilate in the biosynthetic pathway to tryptophan [34,35].

The peptidoglycan pathway

The fourth fate for a vinyl ether is exemplified by the reduction of enolpyruvyl-UDP-GlcNAc in the second step of peptidoglycan assembly (Fig. 7d), resulting in a lactyl ether, UDP-*N*-acetylmuramic acid. The carboxylate of the lactyl ether is the site for subsequent stepwise addition of amino acids to build up the peptidyl strands of the peptidoglycan. In a mature cross-linked peptidoglycan layer, the lactyl bridge is the key covalent linker between the repeating disaccharyl unit and each peptide strand (Fig. 8). The naturally occurring epoxypropane phosphonate, fosfomycin, is a covalent inhibitor of MurA [13] and thus an antibacterial drug. MurB (see below) is an equally valid novel target for antibacterial agents, since failure to construct the lactyl ether bridge will totally block cell-wall assembly.

On purification, the vinyl ether reductase MurB contained one equivalent of tightly bound FAD, the riboflavin-based redox coenzyme. The bound FAD was observed to undergo reduction to $FADH_2$ by NADPH and then reoxidation by the enol ether substrate [36]. In deuterium-labeling studies, hydrogen from the (4*S*)-locus of NADPH was found at the C_3 methyl group of the lactyl moiety of UDP-*N*-acetylmuramic acid and a solvent-derived hydrogen atom ended up at C_2 to yield the (2*R*)- or D-lactyl group. The redox process is then a net hydride addition to the vinylic terminus of the enol ether and proceeds via hydride transfer to and from N_5 of FAD to yield a $FADH_2$ intermediate in the enzyme active site — a two-step process, as shown in Figure 9.

The X-ray structure of MurB complexed with enolpyruvyl-UDP-GlcNAc has recently been solved

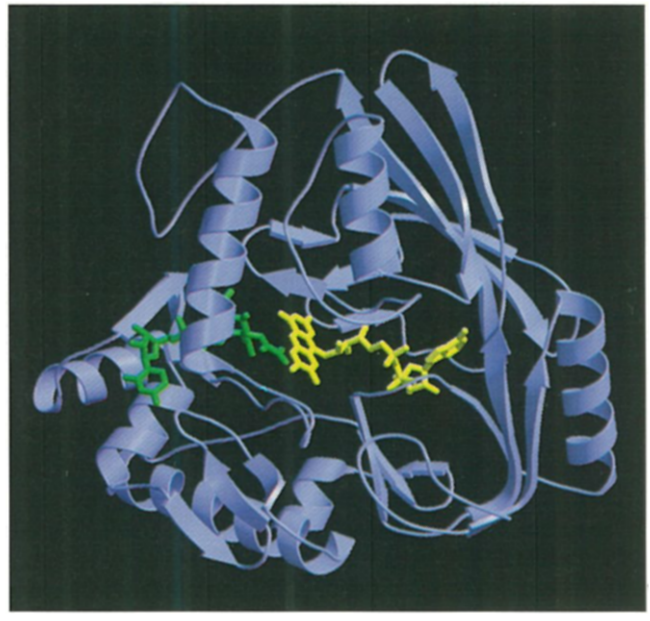

Fig. 10. The structure of the enolpyruvyl-UDP-*N*-acetylglucosamine-MurB complex [37]. The FAD is shown in yellow and the enolpyruvyl substrate in green.

(Fig. 10) and shows the apposition (~3 Å separation) of N_5 of the isoalloxazine ring of FAD and the vinylic double bond of the substrate [37]. Using the X-ray structures of both enolpyruvyl-UDP-GlcNAc and (*E*)-enolbutyryl-UDP-GlcNAc in complex with the enzyme, it was possible to predict the stereochemical outcome at C_3 of the product (Fig. 11). This prediction was confirmed using (*E*)-enolbutyryl-UDP-GlcNAc as an alternative substrate in D_2O (Fig. 12a), which, as expected, gave a product with a (2*R*, 3*R*)-$^2H_2$ hydroxybutyryl moiety [38].

Use of this four-carbon enolbutyryl substrate with MurB also revealed isomerization to the (*Z*)-geometric

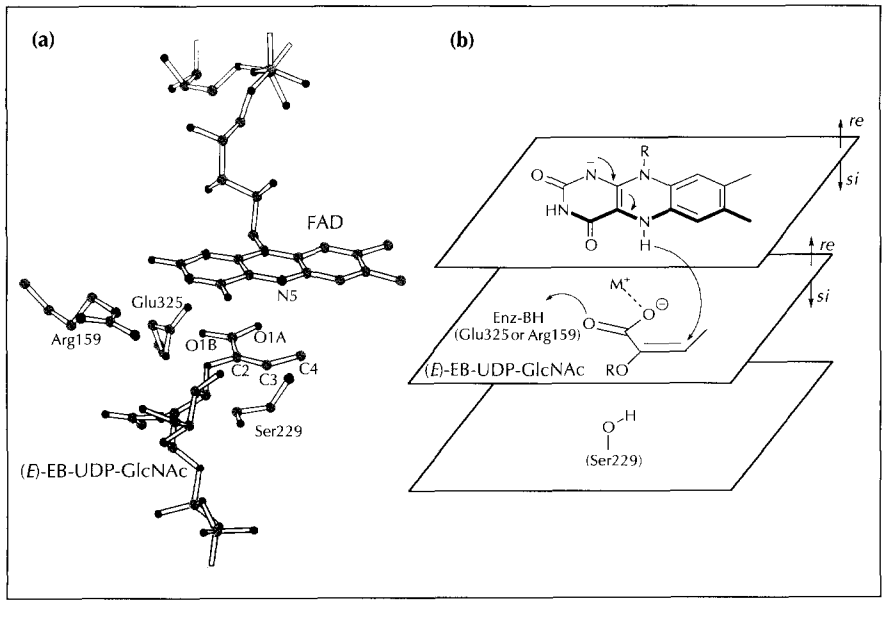

Fig. 11. Stereochemistry of addition to the vinyl ether. **(a)** A close-up view of the active site of the (*E*)-enolbutyryl-UDP-GlcNAc–MurB complex (adapted from [37]) reveals the predicted stereochemistry of addition to the vinyl bond, shown schematically in **(b)** (adapted from [38]). This prediction has been confirmed biochemically (see text). EB, enolbutyryl.

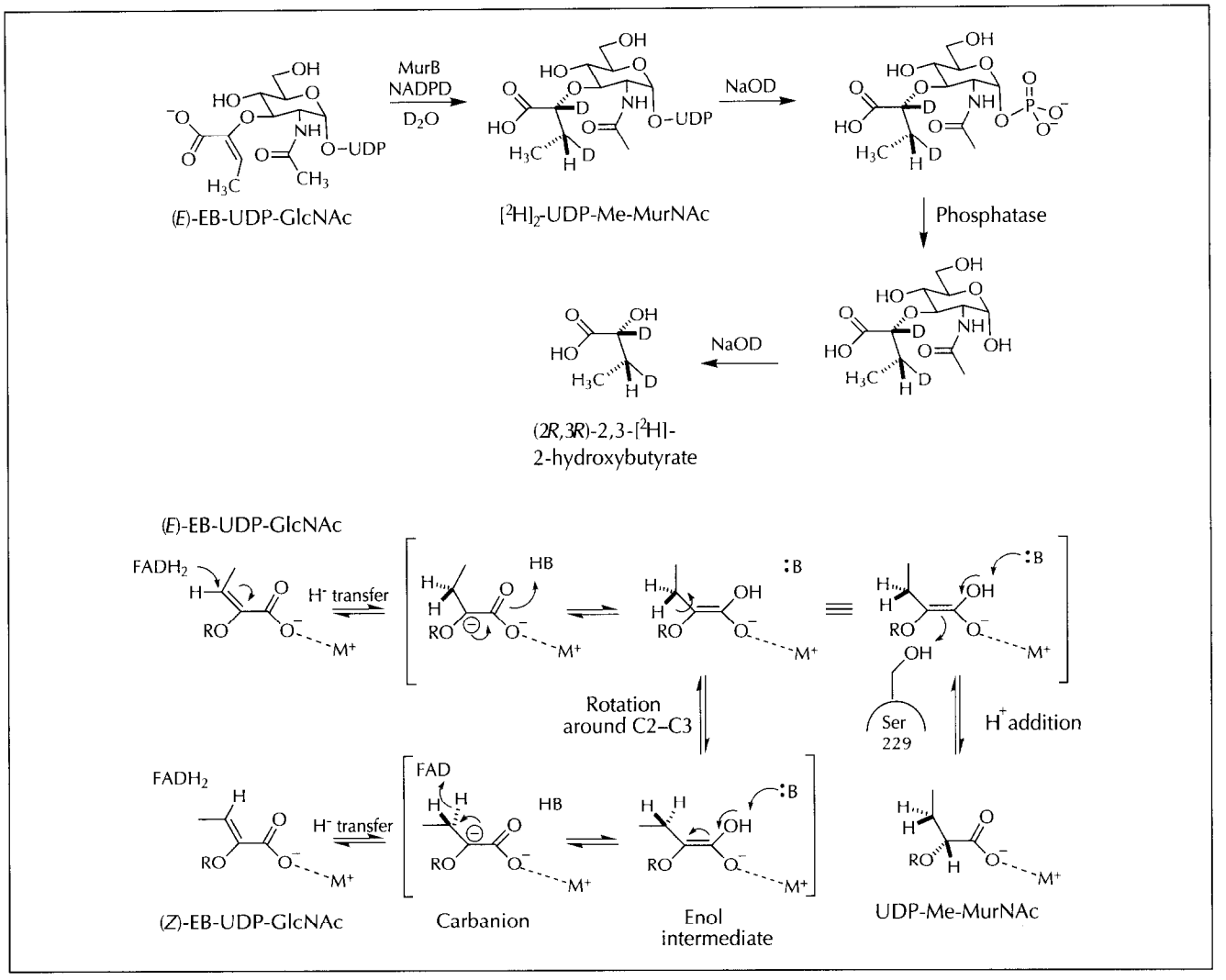

Fig. 12. Biochemical confirmation of the stereochemistry of the product of reduction by MurB, using the (*E*)-enolbutyryl substrate. **(a)** Reaction in D_2O followed by removal of UMP, dephosphorylation and removal of the muramyl moiety yields the (2*R*,3*R*)-$^2H_2$ species. **(b)** Isomerization to the substrate with the (*Z*)-geometry is in competition with reduction. This configuration-equilibrating side reaction indicates a reaction pathway in which H^- addition at C_3 precedes H^+ addition at C_2. EB, enolbutyryl; B, base. Reprinted with permission from [38].

substrate isomer in competition with reduction (Fig. 12b). D_2O kinetic and product isotope effects and product isotope composition indicated that this is due to partitioning of a long-lived C_2 carbanion intermediate, formed from initial hydride addition at C_3 [38]. The carbanion intermediate undergoes either protonation at C_2, to yield the reduction product UDP-*N*-acetylmuraminic acid, or rotation around the C_2–C_3 axis and H-transfer back to FAD, to yield the *Z*-isomer of the enol ether substrate. This configuration-equilibrating side reaction at C_3 is highly diagnostic of a reaction sequence in which H^- addition at C_3 precedes H^+ addition at C_2.

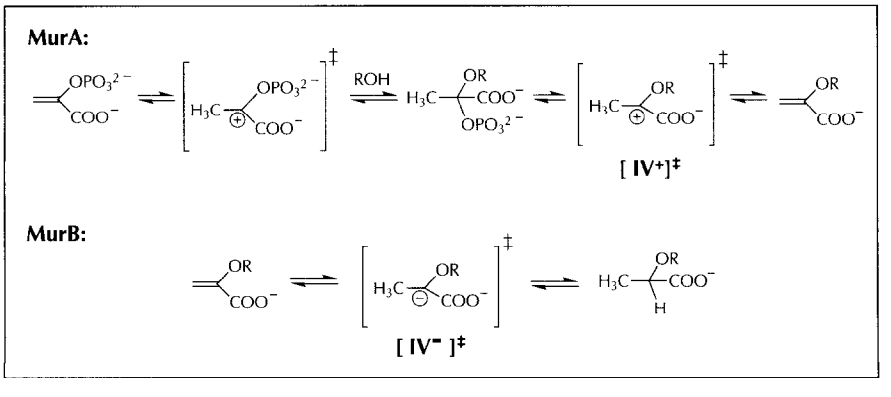

Fig. 13. The reactions catalyzed by MurA and MurB generate transition states (**IV$^+$** and **IV$^-$**, respectively) that contain the same carbon skeleton but have opposite charges on the C_2 atom.

Reverse polarity of consecutive intermediates in an enzymatic pathway

The sequence of reactions catalyzed by MurA and MurB, which creates the simple lactyl ether bridge between glycan and peptide strands in bacterial cell wall biosynthesis, reveals the chemical versatility of the enol ester in PEP and of the enol ether in enolpyruvyl-UDP-GlcNAc, respectively. There is evidence for both the positively charged C_2 oxocarbenium ion transition state in the breakdown of the tetrahedral adduct in MurA catalysis [19] and the corresponding negatively charged enolate/C_2 oxocarbanion transition state in MurB catalysis [38]. Thus, the same carbon skeleton forms transition states of opposite polarities in consecutive reactions (Fig. 13, $[\mathbf{IV}^+]^\ddagger$ and $[\mathbf{IV}^-]^\ddagger$), dramatically emphasizing the biosynthetic utility and versatility of the vinyl ether linkage.

Summary

PEP has a great deal of functionality packed into a small skeleton. Its thermodynamic activation in phosphoryl transfer is balanced by adequate kinetic stability in aqueous media at physiological pH. PEP can act either as a nucleophile at C_3 or as an electrophile at C_2, depending on the timing and regiospecificity of O–P and C–O cleavage. The vinyl ether products from MurA and EPSP synthase then reveal their rich chemistry in the biological rearrangements, hydrolysis, eliminations and reductions that are seen in the several pathways described here. Selective inhibition of these biochemical transformations has clear utility in agricultural and human health sectors.

Acknowledgements: This work was supported in part by NIH Grant 49338-02 to C.T.W.; D.H.K. was supported by Medical Scientist Training Program and Pharmacological Sciences Training Grants from the NIH, and W.J.L. was supported by a postdoctoral fellowship from the National Sciences and Engineering Research Council of Canada.

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