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Review

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DXS as a target for structure-based drug design

In this review, we analyze the enzyme DXS, the first and rate-limiting protein in the methylerythritol 4-phosphate pathway. This pathway was discovered in 1996 and is one of two known metabolic pathways for the biosynthesis of the universal building blocks for isoprenoids. It promises to offer new targets for the development of anti-infectives against the human pathogens, malaria or tuberculosis. We mapped the sequence conservation of 1-deoxy-xylulose-5-phosphate synthase on the protein structure and analyzed it in comparison with previously identified druggable pockets. We provide a recent overview of known inhibitors of the enzyme. Taken together, this sets the stage for future structure-based drug design.

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Why is DXS a good drug target?

New anti-infective agents are urgently needed, as pathogens are developing that are increasingly resistant to front-line anti-infectives. Over the past decades, the number of newly introduced anti-infective drugs with a unique mode of action has strongly decreased. This can be ascribed to the high development cost for anti-infectives and to the fact that new anti-infectives are only used against multiresistant pathogens to delay the emergence of resistance. Because of this, the estimated revenue of new anti-infectives in the first year after introduction to the market is low, while a patent protection period of 20 years is starting on the day of the filing of the patent application. As a consequence, the number of major pharmaceutical companies that are developing new anti-infectives hast dropped from 18 in 1990 to only four in 2010 [1]. Since 2010, a number of actions have been taken to address this problem and increase the research output on novel antibiotics that start to show small effects [1]. Nevertheless, new anti-infectives and new targets for anti-infectives are needed, and an increasing number of academic groups have initiated research in this area; in particular, the treatment of so-called 'neglected diseases' is receiving increasingly more attention.

The recently discovered methylerythritol 4-phosphate (MEP) pathway seems to offer a variety of promising drug targets [2]. The pathway is named after its second intermediate, 2*C*-methyl-D-erythritol 4-phosphate and is one of the two distinct metabolic pathways leading to the universal building blocks for the biosynthesis of isoprenoids. This pathway is found in most bacteria, plants and protozoa, whereas higher eukaryotic organisms and some bacteria rely on the mevalonate pathway.

Thus, in pathogenic species that use the MEP pathway, it is possible to inhibit growth by selectively withdrawing a crucial source for the biosynthesis of several important secondary metabolites, while the eukaryotic/human mevalonate pathway is not affected [3]. This offers the potential for a new class of anti-infectives with minor side effects on the human body.

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The enzyme DXS catalyzes the first and rate-determining step of the MEP pathway, and is therefore of particular interest. In this review, we will investigate the DXS enzymes of the model organisms for bacteria and plants – *Escherichia coli* and *Arabidopsis thaliana*, respectively. We will also examine exemplary species for pathogenic bacteria (*Mycobacterium tuberculosis*) and for protozoan parasites (*Plasmodium falciparum)*. The bacterium *Deinococcus radiodurans* is also included, of which the only crystal structure in complex with thiamine diphosphate (ThDP) is known. In addition to reviewing the recent research done on this enzyme, we also focus on the structural properties of this enzyme and the consequences for a structure-based approach on inhibitor design against DXS enzymes of various species.

The MEP pathway is essential for some of the deadliest pathogens

Plasmodium falciparum

In recent years, substantial efforts have been made to eradicate malaria. The efforts resulted in a decreased death rate of 48% from 2000 to 2015 [4]. As a consequence of the widespread use of antimalarial drugs, the selection pressure for resistant parasites increased. The first resistant strains against artemisinin and its derivatives have emerged. Efforts to stop the spread of those resistant strains of *Plasmodium falciparum* failed and artemisinin-resistant *P. falciparum* is now widespread in East Asian countries [5,6]. In the light of the increasing spread of resistance, there is an urgent need for new antimalarial drugs with novel modes of action. In this context, the MEP pathway seems to be a rich source of promising drug targets [7]. The antibiotic fosmidomycin has been used since 2004 in the fight against malaria and was the first inhibitor targeting an enzyme of the MEP pathway, in particular, the enzyme DOXP reductoisomerase [8]. Although the drug fosmidomycin has poor pharmacokinetic properties and its use is therefore limited, it validated the MEP pathway as a good target for new anti-infectives [7].

Mycobacterium tuberculosis

The WHO lists tuberculosis as one of the ten most common causes of death worldwide [9]. Caused by *M. tuberculosis*, this infectious disease primarily affects the lungs of the patients. Around 30% of the world population is infected with tuberculosis, mainly in the latent state, which does not manifest any symptoms. The active state of tuberculosis is mostly (95%) found in developing countries.

Common treatment of tuberculosis consists primarily of a combination of the antibiotics rifampicin and isoniazid, and takes several months. Through the extended and inappropriate use of antituberculosis drugs, resistances have emerged against one or multiple antibiotics. In the case of multidrug-resistant tuberculosis, treatments with second-line antibiotics are available, which suffer from a number of side effects. In 2006, the first case of a tuberculosis strain was reported in Italy, which was resistant against all known antituberculosis drugs (totally drug-resistant tuberculosis [TDR-TB]) [10]. Since then, more reports on cases of TDR-TB have appeared (e.g., in Iran and India) [11]. The development of drugs against TDR-TB with new modes of action is therefore urgently needed. As a result, *M. tuberculosis* DXS of the MEP pathway is a promising target and is worth exploring in detail.

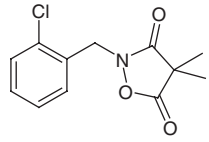
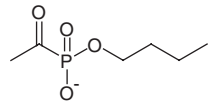
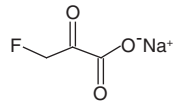
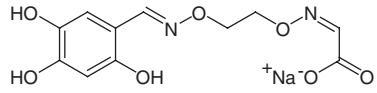
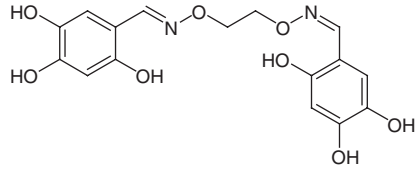
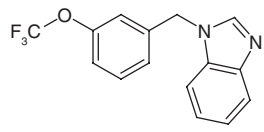
Herbicides

The first two enzymes of the MEP pathway are rate-limiting for the whole pathway [12]. The essential role of the MEP pathway in plants, for the synthesis of isoprenoids in the chloroplast, has attracted interest from two different fields.

First, the inhibition of the pathway results in plants that are unable to synthesize the terpene chlorophyll. This has been shown in several DXS knockout studies that show an albino phenotype owing to the inability to perform photosynthesis [13]. There is currently one known herbicide used, clomazone, which was introduced in 1986 and which targets the DXS enzyme of plants. Although it was long known that the herbicide affects the biosynthesis of diterpenes and tetraterpenes, the targeted protein could only be identified after the discovery of the MEP pathway in 1997 [14]. It took until 2013 to understand its mechanism of action. In living organisms, it is metabolized to ketoclomazone (Table 1) and undergoes ring opening before binding to DXS [15]. The molecule is, however, very small and has numerous unfavorable properties, such as high volatility, which limit its use as a herbicide.

Second, there is a strong interest in engineering the MEP pathway to increase the production of terpenoids in plants. Various natural substances are used in medicine, and their overexpression could significantly reduce the effort needed for the extraction from plant material, which often contains only a very small amount of the substance of interest [16]. However, genetically engineered enzymes are not directly aiding the discovery of new drugs, but the results obtained from enzyme function and pathway regulation should be transferable between the various homologs of DXS, owing to their high conservation of amino acids in the active site (see Figure 1 and their predicted 3D similarity [Figure 2]).

Table 1. Overview of the known inhibitors of DXS.

No.	Inhibitor	Structure	Enzyme	K_i (μM)	Mechanism of inhibition
1	Ketoclomazone		*Escherichia coli*	75	Uncompetitive to pyruvate
				220	Noncompetitive to D-GAP
2	Butylacetylphosphonate		*Mycobacterium tuberculosis*	4 ± 2	Competitive to pyruvate
			Yersinia pestis	7.5 ± 0.9	Noncompetitive to D-GAP
			Salmonella enterica	8.4 ± 0.4	
			E. coli	5.6 ± 0.8	
3	β-fluoropyruvate		*Plasmodium vivax*	35 ± 1.7	Competitive to pyruvate
				77 ± 7.2	Noncompetitive to D-GAP
			Plasmodium falciparum	43 ± 3.8	Competitive to pyruvate
				131 ± 10	Noncompetitive to D-GAP
4	Trihydroxybenzaldoximes Mixed oxime			18.4 ± 3.4	Noncompetitive to pyruvate Competitive to D-GAP
5	Symmetrical oxime			1.0 ± 0.2	Uncompetitive to pyruvate Competitive to D-GAP
6	1-(3-(trifluoromethoxy) benzyl)-1*H*-benzo[d] imidazole		*Deinococcus radiodurans*	151 ± 22	
7	Bacimethrin		*E. coli*		

D-GAP: D-Glyceraldehyde-3-phosphate.

The MEP pathway

Isoprenoids (or terpenoids) constitute a structurally diverse class of complex natural products, fulfilling a range of different functions such as protein degradation, apoptosis, influences on the cell wall, steroids, hormones, carotenoids, pigments and chlorophyll [17]. Isoprenoids are synthesized in all organisms using two common building blocks: isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP). Rohmer and Arigoni discovered that certain eubacteria, parasites and several plants use pyruvate and D-glyceraldehyde-3-phosphate (D-GAP) rather than acetyl-CoA, to produce the universal isoprenoid precursors [2,14,18]. This biosynthetic route, known as the MEP pathway, produces DMADP and IDP from a set of orthogonal enzymes nonexistent in higher organisms and mammals, which rely on the well-established mevalonate pathway [19].

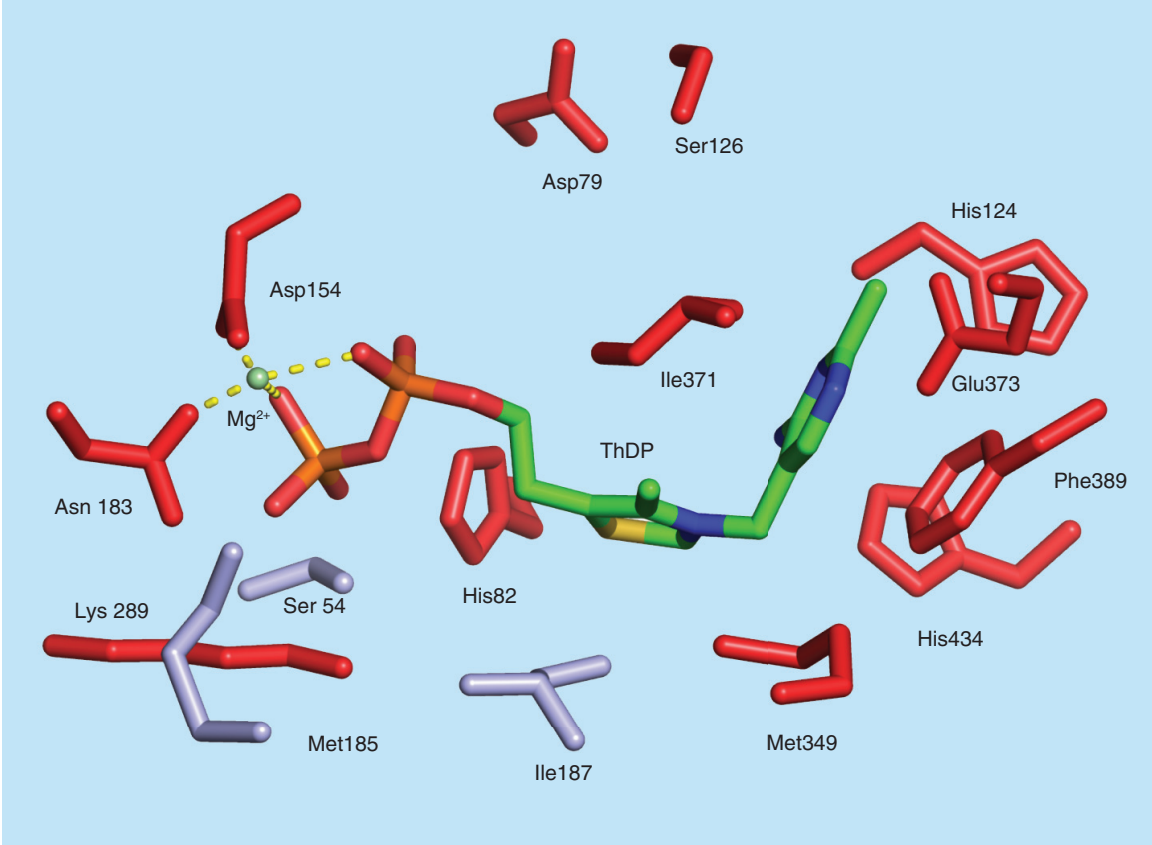


Figure 1. Mapping of the sequence conservation of the DXS enzymes from different species. Blue: Kow conservation; Red: High conservation; Green: ThDP.
ThDP: Thiamine diphosphate.

The MEP pathway starts with decarboxylation of pyruvate and D-GAP to produce 1-deoxy-D-xylulose 5-phosphate (DOXP) catalyzed by DXS and assisted by ThDP and Mg^{2+}. DOXP is converted to MEP by IspC. The enzyme IspD catalyzes the reaction between MEP and CTP to produce 4-diphosphocytidyl-2*C*-methyl-D-erythritol (CDP-ME) and diphosphate.

The ATP-dependent enzyme IspE catalyzes the formation of 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate from CDP-ME. In the subsequent step, IspF catalyzes the formation of 2*C*-methyl-D-erythritol-2,4-cyclodiphosphate from CDP-ME. In the final steps, IspG and IspH generate of IDP and DMADP (Figure 3).

The structural information for each step was elucidated in detail except for DXS, which is the first and rate-determining enzyme in the MEP pathway [14,20,21]. In bacteria, the product of the DXS enzyme, DOXP, is a branch point in the metabolic pathway. It is not only used as a substrate for isoprenoid biosynthesis in the MEP pathway, but also acts as a precursor in the biosynthesis of pyridoxine (vitamin B6) and thiamine (vitamin B1) [22]. Moreover, bacteria rely only on the MEP pathway as demonstrated by the fact that the DXS-knockout *E. coli* is not viable, but it can be rescued by media supplemented with vitamins B1, B6 and MEP. Vitamin B is needed because of the loss of the precursor DOXP for thiamine biosynthesis in the knockout strain. The supplement MEP is a downstream metabolite of the MEP pathway that restores the function of the rest of the pathway (Figure 3) [23].

Possible resistance development against drugs that target DXS

As the first enzyme of its pathway, DXS uses the substrates pyruvate and GAP. These metabolic intermediates are used for many different reactions in living organisms. Therefore, if the DXS enzyme is inhibited, its substrates do not accumulate. Pyruvate and GAP are instead processed in other metabolic pathways, or their production is downregulated [22]. An increase in substrate concentration is often observed for inhibition of enzymes in the middle of a pathway relying on specific substrates. The local concentrations of substrates reach high concentrations and may be able to reduce or completely revoke the effect of competitive inhibitors. Strictly, this would not be defined as a resistance, but as an error in target selection.

DXS is not only a promising drug target, it also seems to be robust against the most common mechanisms of resistance development. This should be relatively insensitive to intrinsic resistance arising by mutation. In most microorganisms, DXS is available as a single or double gene copy; therefore, there is a lower probability of a single-point mutation compared with targets that exist as several gene copies. In addition, the highly conserved active-site pocket gives hope to reduced endogenous resistance by mutation of the active site of the protein [24]. Often a resistance mutation bears some negative effect on the general fitness of the microorganism and is lost in the absence of selective pressure [24]. As a result, it is not a persistent mode of drug resistance. Another intrinsic resistance mechanism of microorganisms is the overexpression of the targeted gene [25]. In the case of the DXS enzyme, the first one in its pathway, it may well be possible that the depletion of some of the downstream metabolites upregulates gene expression of DXS. Overexpression of the DXS enzyme could influence the antiinfective effect of the drug in different ways, depending on the mechanism of inhibition of the drug.

In addition to intrinsic resistance, microorganisms can also acquire or develop resistance. This can be due to several mechanisms, which can be divided into three groups: reduction of the intracellular drug

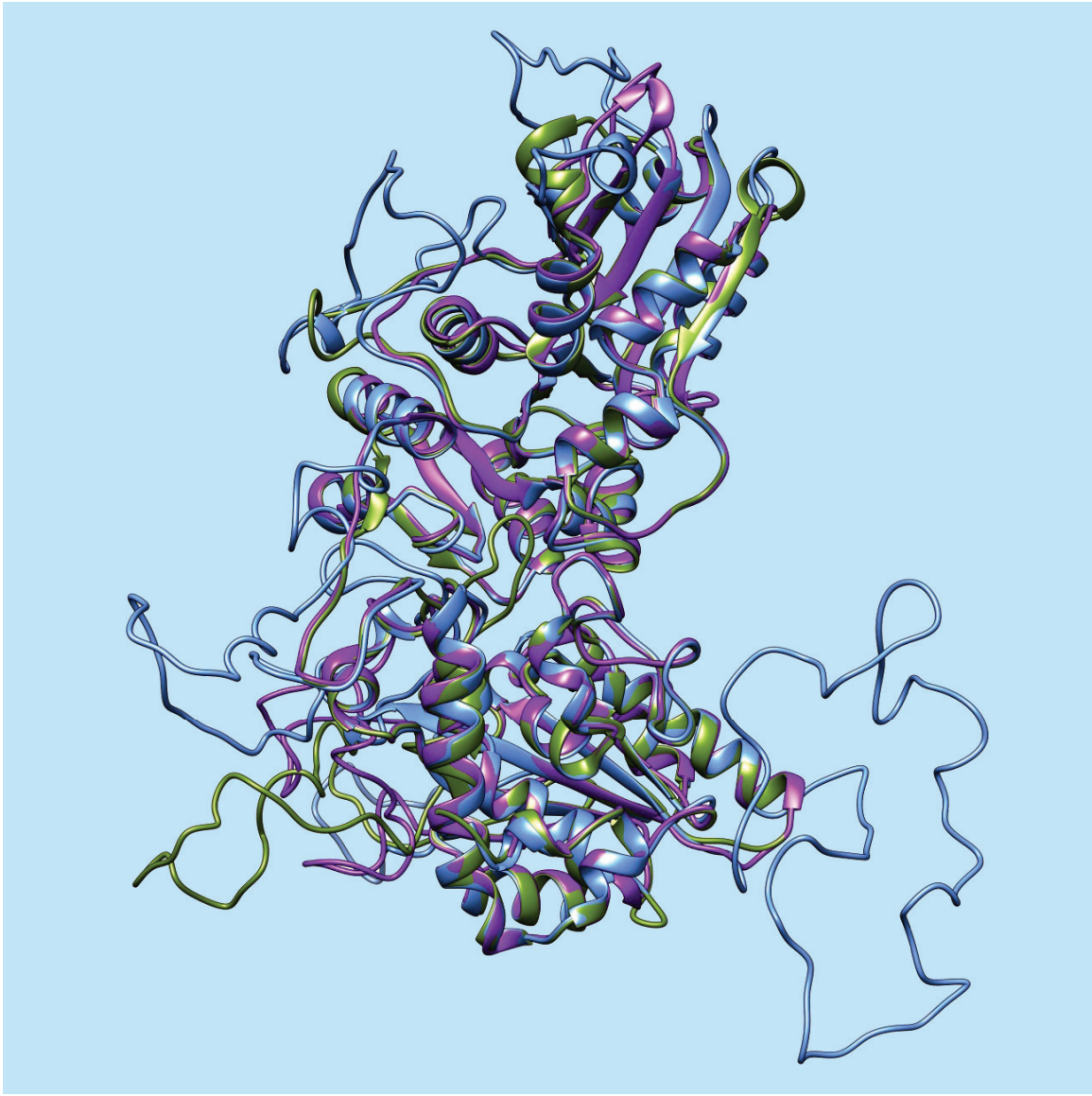


Figure 2. Overlay of the predicted structures of *Arabidopsis thaliana* (olive), *Mycobacterium tuberculosis* (purple) and *Plasmodium falciparum* (light blue). The domains of the protein are predicted similar to the *Deinococcus radiodurans* DXS template; the linker regions differ strongly in length and form random-coiled structures.
For color figires, please see online at: http://www.future-science.com/doi/full/10.4155/fmc-2017-0239

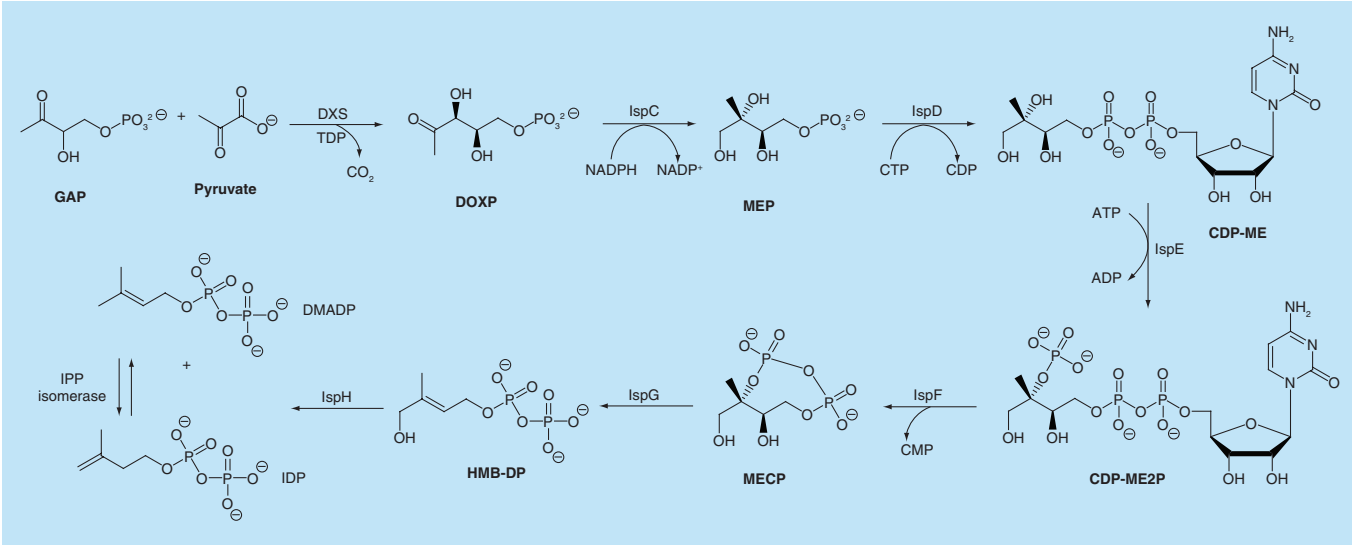


Figure 3. The methylerythritol 4-phosphate pathway for the biosynthesis of isopentenyl diphosphate and dimethylallyl diphosphate.
DMADP: Dimethylallyl diphosphate; IDP: Isopentenyl diphosphate; MEP: Methylerythritol 4-phosphate.

concentration, modification of the drug target and modification of the anti-infective drug [26]. Most of them are drug specific, but we can consider inhibitor classes such as ThDP or substrate analogs.

Reduced intracellular concentration is often achieved due to increased efflux by active transport of the drug. There are a range of thiamine transporters in nature, which could mutate to transport anti-infective thiamine analogs [27]. Most known transporters, however, act on the uptake of thiamine and might facilitate accumulation of the drug in the cell. In addition, if a microbacterial thiamine exporter would emerge as a resistance mechanism, it would be difficult to achieve a high selectivity for the drug in comparison with thiamine for the transporter, of which some are also known for their promiscuity to transport nicotinamide. The exporter would also actively transport the essential vitamin, thiamine, out of the cell, which is probably not beneficial for the viability of the microorganism [26,28]. For the second group of inhibitors, substrate analogues, active export would, in general, be possible, depending on the similarities of the inhibitor with the natural metabolites.

The structure of DXS

The structures of the DXS enzyme (EC 2.2.1.7) from *D. radiodurans* (PDB code: 2O1X) and *E. coli* (PDB code: 2O1S) have been solved using x-ray crystallography at resolutions of 2.9 and 2.4 Å, respectively [29]. In brief, the enzyme contains three subunits I, II and III. All three domains share an α/β-fold and consist of a central, parallel β-sheet, which is sandwiched by α-helices and closely resembles the transketolase (TK) and pyruvate dehydrogenase (PDH) E1 subunit (Figure 4) [29].

A key feature of both structures is that during crystallization, the enzyme probably undergoes partial digestion, and both known structures show no interpretable density for an internal loop (amino acids 199–243 and 183–240 in *D. radiodurans* and *E. coli*, respectively). There is no evidence yet whether the loop is missing due to intrinsic disorder or as a result of proteolytic cleavage during the crystallization process [29]. Importantly, all structural analyses in the literature and also in this review are based on the assumption that the x-ray crystal structures represent a catalytically competent form of DXS. This assumption is supported by the crystal structure of the enzyme TK, which shares the same overall fold but is missing in this loop [31].

Druggable pockets

We have recently reviewed the druggability of the enzyme DXS [30]. Using the program DoGSiteScorer, we identified the substrate-binding pocket and four other pockets as druggable pockets (Figure 4) [32]. DoGSiteScorer is an algorithm to predict possible binding pockets for drugs based on the 3D structure of a protein, paying special attention to the size, compactness and physicochemical properties of the pockets.

After pocket identification, the volume and surface of the pocket are calculated by the program using ellipsoids, which are fitted into the pocket. The functional groups within 4 Å of the pocket are identified and taken into account, along with the lipophilic surface. For the druggability prediction, a machine-learning technique is used, which was trained on published successful and failed inhibitor-design projects [33].

Sequence conservation

Sequence conservation was analyzed using the multiple sequence comparison by log-expectation (MUSCLE) algorithm [34]. The sequences of the DXS enzymes of the different species were retrieved from the UNIPROT database using accession numbers – *D. radiodurans*: Q9RUB5, *E. coli*: P77488 *P. falciparum*: O96694, *M. tuberculosis*: A5U634 and *A. thaliana:* Q38854 [35]. The sequences were selected to include common model organisms as well as interesting pathogens. The alignment is shown in Figure 5 and was mapped on to the crystal structure of *D. radiodurans* DXS (PDB code: 2O1X) using the program UCSF Chimera. A color gradient from blue to red represents an increasing conservation of amino acids between the homologues.

Mapping of the sequence conservation onto the 3D structure is shown in Figure 6. We chose a visualization of the surface of the protein to focus on solvent-accessible amino acids. The strongly conserved regions of the protein are colored in red and form well-defined regions. They can be divided into a group of amino acids contributing to conserved pockets and a group of single conserved amino acids. Single conserved amino acids occur at positions, which are likely important for the correct folding of the protein, for example, Pro363, which is found in all homologues at the end of helix 12 (residues 363–355). A proline at the end of a helix is well known to destabilize the helical structure [38]. These small conserved regions corresponding to single amino acids are unlikely to be suitable as targets for the development of binders and are not further investigated.

Conserved pockets, on the other hand, tend to play an important role in the function of the enzyme. Often the binding sites for substrates, cofactors or allosteric factors are evolutionarily conserved. Depending on the pocket, they are suitable to tightly bind a small molecule and therefore a good target for the structure-based development of inhibitors. This can be seen in Figures 4 & Figure 6, where the ThDP-binding site (pocket P0) shows an expected high degree of sequence conservation. The binding site of pyruvate is also proposed to

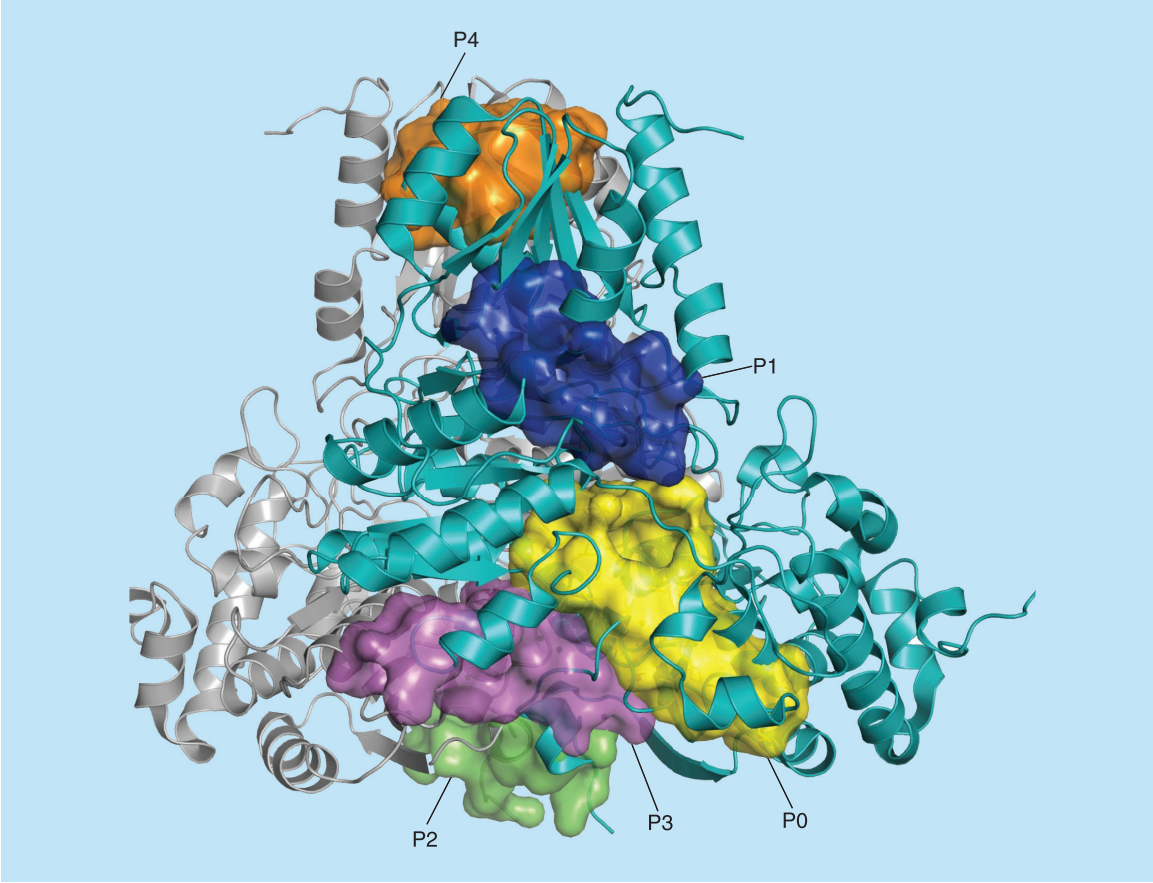


Figure 4. Co-crystal structure of the *Deinococcus radiodurans* DXS dimer with thiamine diphosphate (PDB code: 2O1X). The monomers of DXS are shown in gray and cyan. The druggable pockets are highlighted as colored surfaces (P0: yellow, P1: blue, P2: green, P3: magenta, P4: orange). For further details on the pocket identification, see [30].
PDB: Protein data bank; ThDP: Thiamine diphosphate.

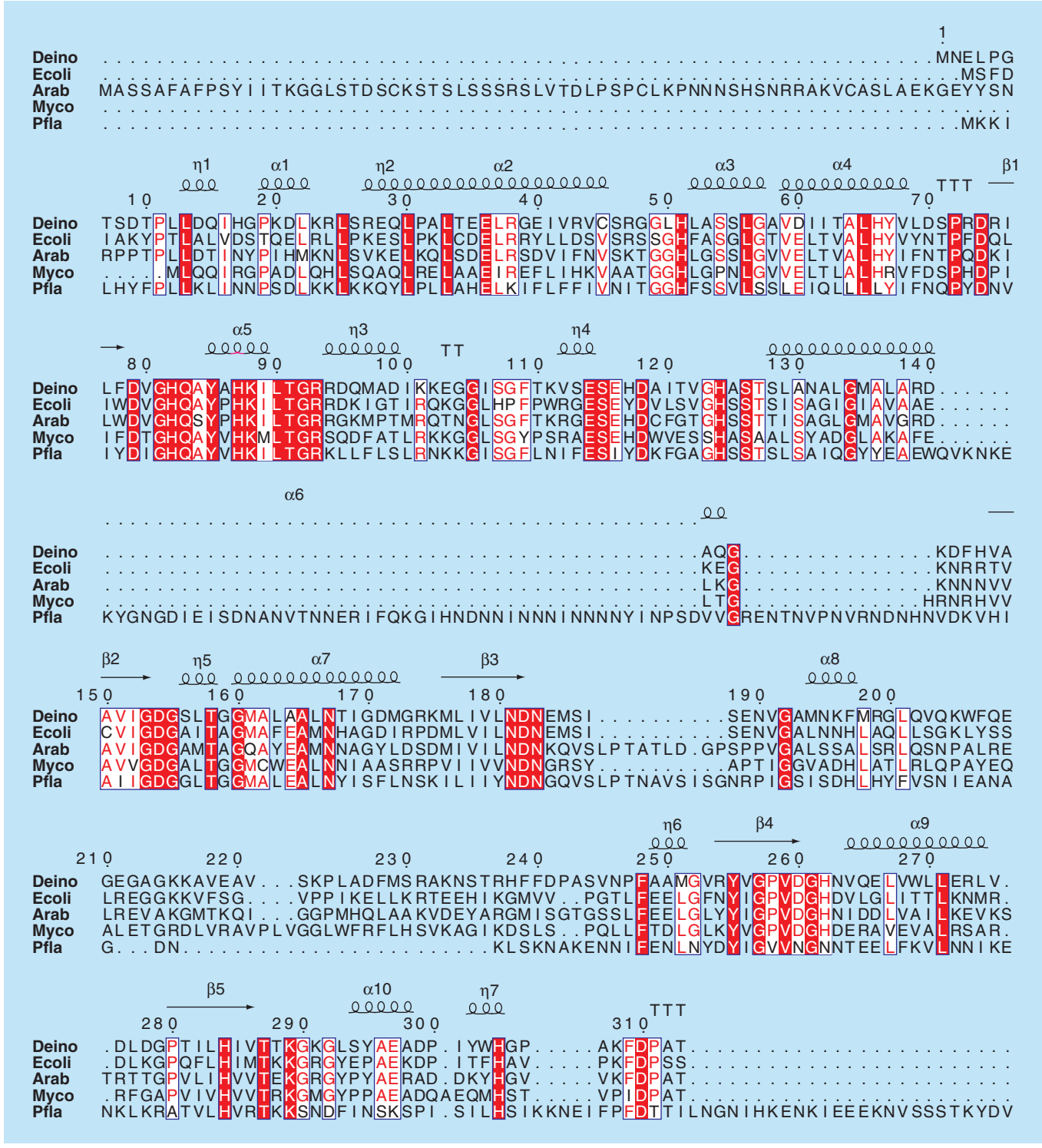


Figure 5. Structure-based sequence alignment of selected 1-deoxyxylulose-5-phosphate synthase proteins. The sequence of DXS from *Deinococcus radiodurans* (Deino; Q9RUB5) was aligned with *Escherichia coli* (*E. coli*; P77488), *Arabidopsis thaliana* (Arab; Q38854), *Mycobacterium tuberculosis* (Myco; A5U634) and *Plasmodium falciparum* (Pfla; O96694). Compared with Deino, the sequence identities of *E. coli*, Arab, Myco, Pfla are 45, 40, 40 and 33%, respectively. The sequence numbering and secondary-structure elements correspond to Deino. Conserved residues are shown in red color, while semiconserved residues are drawn in blue. The alignment was prepared with the program MUSCLE, and the figure was produced using the program ESpript [36,37].
MUSCLE: Multiple Sequence Comparison by Log-Expectation.

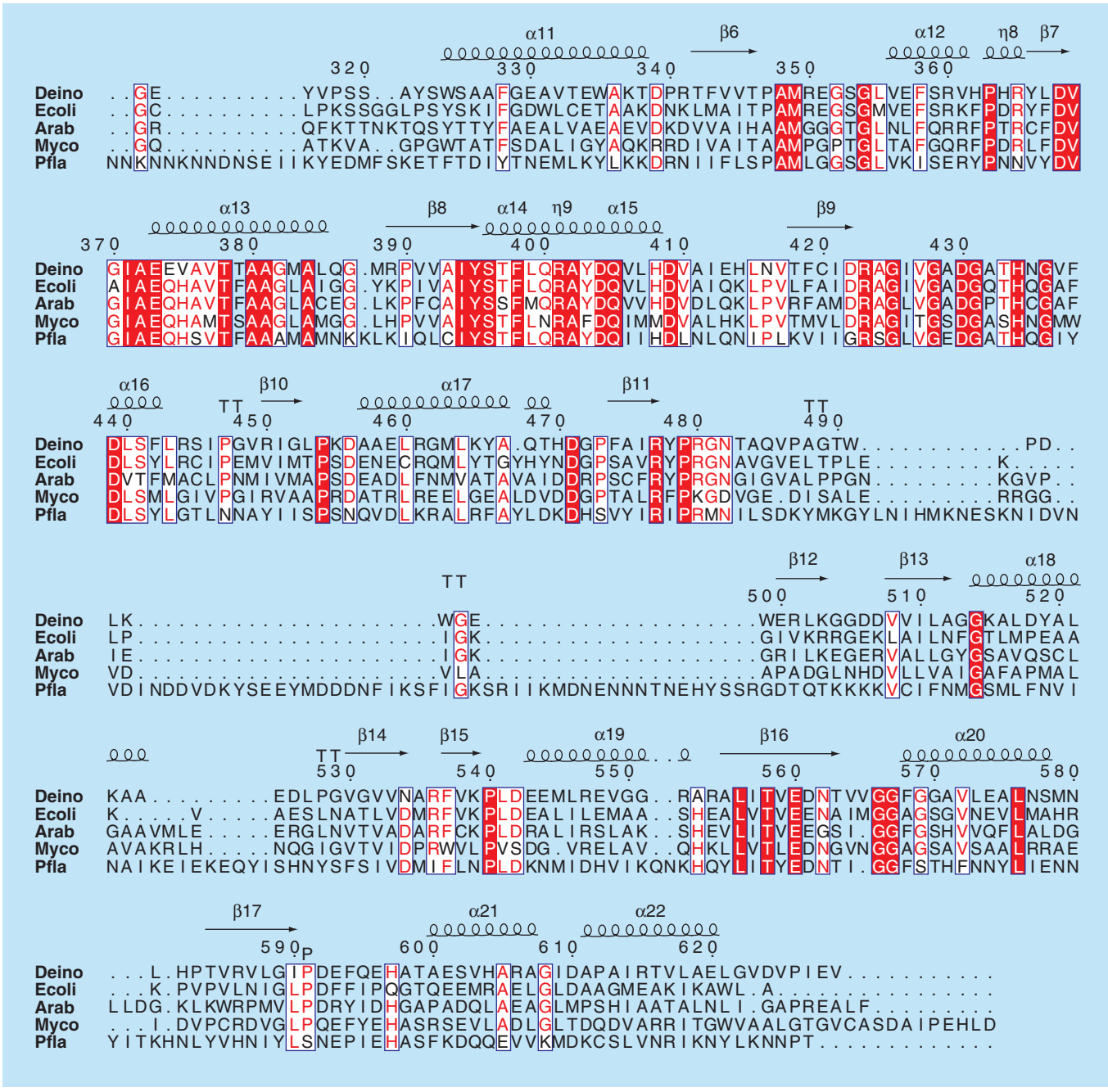


Figure 5. Structure-based sequence alignment of selected 1-deoxyxylulose-5-phosphate synthase proteins (cont. from previous page). The sequence of DXS from *Deinococcus radiodurans* (Deino; Q9RUB5) was aligned with *Escherichia coli* (*E. coli*; P77488), *Arabidopsis thaliana* (Arab; Q38854), *Mycobacterium tuberculosis* (Myco; A5U634) and *Plasmodium falciparum* (Pfla; O96694). Compared with Deino, the sequence identities of *E. coli*, Arab, Myco, Pfla are 45, 40, 40 and 33%, respectively. The sequence numbering and secondary-structure elements correspond to Deino. Conserved residues are shown in red color, while semiconserved residues are drawn in blue. The alignment was prepared with the program MUSCLE, and the figure was produced using the program ESpript [36,37].
MUSCLE: Multiple Sequence Comparison by Log-Expectation.

be in pocket P0, because it reacts with the C2 atom of the thiazolium ring of ThDP. The potential role of the other strongly conserved pockets is currently unknown. If the amino acids have no role in the stabilization of the protein structure, it is reasonable to propose that they fulfill a different important function in the protein, which makes them resistant to mutation. This is likely to be the role in the reaction or regulation of the enzyme. As such, these pockets are of interest for the development of allosteric inhibitors.

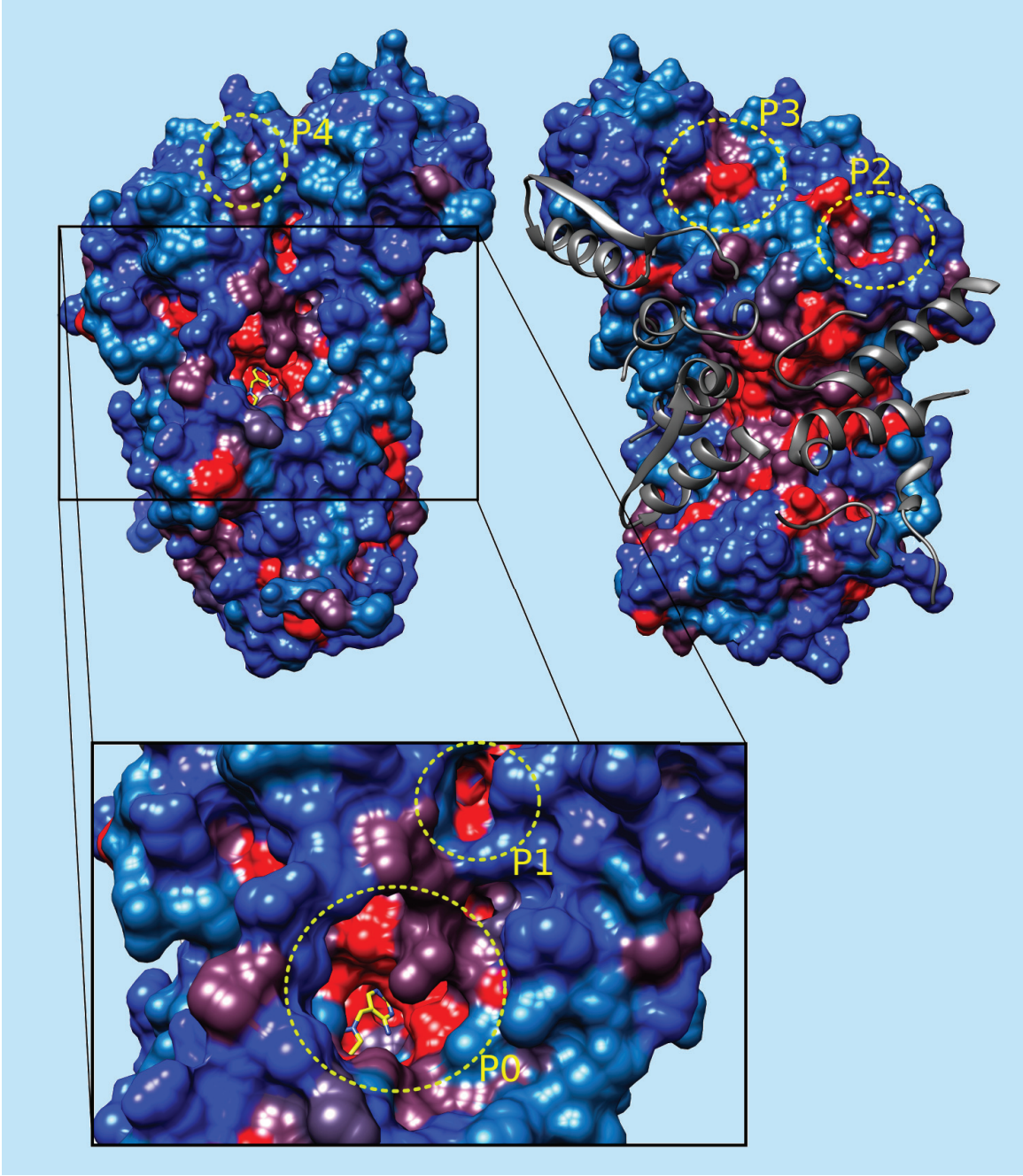


Figure 6. Mapping of the sequence conservation of the DXS enzymes from various species. Both sides of the enzyme and a closeup of the active site with bound cofactor (ThDP) are shown. Color code – blue: low conservation; red: high conservation; and gray: closely associated parts of the second protein from the DXS homodimer. Sequence alignment was done using MUSCLE on the sequences of *Escherichia coli, Arabidopsis thaliana, Plasmodium falciparum, Mycobacterium tuberculosis* and *Deinococcus radiodurans* DXS [34]. Mapping was done using UCSF Chimera [39].
DXS: 1-deoxyxylulose-5-phosphatesynthase; MUSCLE: Multiple Sequence Comparison by Log-Expectation; ThDP: Thiamine diphosphate.

The ThDP-binding pocket corresponding to P0 (Figure 6), which also represents the active site of the enzyme, is highly conserved through all species. In close proximity to the thiazolium ring of ThDP, there are some less conserved amino acids, such as Ile187, Glu184 and Met185. This pocket may be amenable to the binding of ThDP analogues as inhibitors against the whole DXS enzyme class. A minor drawback may

be the difficulty to achieve selectivity against human ThDP-binding enzymes, such as TK (Figure 1, comparison between DXS and TK). This has implications in off-target activities and may impose difficulties in the creation of a sufficiently specific DXS inhibitor for use as an anti-infective in humans.

In contrast, a small pocket in close proximity to the active site of the enzyme shows high conservation (Figure 6, the pocket on top of the ThDP-binding part). This region corresponds to the previously identified binding pocket P1 using the DoGSiteScorer algorithm. Therefore, it may be an interesting target for structure-based drug design (SBDD), as DoGSiteScorer indicates that it possesses suitable physicochemical properties to be addressed by small molecules, and our analysis here suggests that this region is strongly conserved in DXS enzymes. The human TK lacks this pocket, which may represent a possibility to address DXS selectively. The proximity to the catalytic domain of the enzyme suggests that it may have an influence on the mechanism. Furthermore, it shows a strong variability of amino acids at the entrance of the P1 pocket. If it is possible to address this pocket with a strongly binding fragment, it could be used as a scaffold for modifications, which interacts with a species-specific amino acid at the entrance of the pocket. In this way, it would be possible to develop a number of selective inhibitors against different species at the same time but targeting the same site. Considering the small size of the pocket, this should be possible without exceeding the recommended molecular weight of 500 g/mol of a drug-like molecule [40].

The predicted binding pockets P2 and P3 are close to the dimer interface. They show some sequence conservation but are more surface-exposed with a lower enclosure and a shallower depth of the pocket. In contrast to other ThDP-binding enzymes, DXS does not have its active site at the dimer interface. Therefore, these pockets at the interface are farther away from the active site and may not influence the function of the enzyme, but, instead, may play a role in the assembly of the homodimer. It is also possible that these pockets may play a role in subtle rearrangements of the monomer–monomer interaction during the catalytic cycle [41]; however, there are no data to support this. Additionally, this region of the crystal structures also possesses higher B factors than the other predicted pockets, indicative of a higher degree of mobility of the protein in this region. Therefore, calculations for this part of the enzyme may not be as accurate as for the rest because the use of a static structure for pocket identification may not be appropriate here. Thus, the pockets P2 and P3 may also be suitable for exploration, but are likely less desirable.

Pocket P4, which was identified by the DoGSiteScorer as a possible druggable pocket, shows low to no sequence conservation. This pocket may be used to selectively address only the *D. radiodurans* DXS enzyme. However, the low conservation suggests no participation of this region of the protein in the catalysis. A low conservation is also a hint to a high possibility of random mutation and therefore resistance development against drugs that target this part of the enzyme. Further investigation of the pocket P4 seems less promising.

At the 'back' of the protein is the protein–protein contact surface (Figure 6, right). Several helix–helix interactions can be seen, which stabilize the binding of the homodimer and are well conserved. For a better visualization of the interface, the parts of the binding partner, which are involved in interactions, are shown in a gray cartoon visualization. This part of the enzyme nicely shows the conservation of important amino acids at the interface and the variability at the amino acids, which are solvent-exposed.

In the future, these protein–protein interactions could also be explored as possible targets for drug design. For a long time, targeting protein–protein interactions with small-molecule inhibitors has been considered difficult. Recently, an increasing number of examples of successfully targeting protein–protein interactions in SBDD have been reported [42]. It could be possible that the protein follows a cooperative mechanism, in which the interactions between the two active sites could be influenced by a binder at the interface. If the enzyme is cooperative and it is possible to separate the homodimer by a small binder, this could be a different way to inhibit the DXS enzyme, achieving high selectivity, using the unique arrangement of its domains in comparison with ThDP-binding proteins of other enzyme classes.

Comparison of the DXS enzyme with TK

For the development of new anti-infectives, it is important to target the protein of interest with a high selectivity. ThDP is a cofactor, which is also used in the human body by several ThDP-binding proteins like TK, PDH and 2-oxoisovalerate dehydrogenase [27]. It is important to consider possible off-target effects on those human ThDP-binding enzymes during the development of new drugs. For a structural comparison, we performed a BLAST sequence analysis of the DXS enzyme and searched for human proteins with high similarity [43]. The non-DXS enzyme with the highest similarity in the results was the protein human TK and is used herein as an example for ThDP-binding proteins. The class of TK are enzymes, which transfer a ketol donor group to an accepting aldehyde

or ketone group [44]. For a structural comparison, the crystal structure of human TK (PDB code: 3OOY) was selected and compared with the structure of *D. radiodurans* DXS (PDB code: 2O1X).

The enzymes have a sequence identity of 23% and a similarity of 35% [45]. Although they have a rather different primary structure, it is remarkable that these enzymes show a very similar tertiary structure. Both enzymes consist of three domains and form a tightly associated homodimer. They show, however, significant differences in the arrangement of the three constituent domains. As a consequence, the two dimers also show a different orientation. A superimposition of the two enzymes is shown in Figure 1. The two parts of the TK and DXS dimers are colored in red and blue, and orange and light blue, respectively. They form the same overall structure however; it is possible to see four different parts of the protein due to the different arrangement of the domains.

The different arrangement has major consequences for the ThDP-binding pocket (Figures 1 & Figure 7, right). In contrast to the DXS enzyme, where the ThDP-binding domain is a deep pocket inside of each monomer, the ThDP-binding pocket of TK is located at the interface of the proteins and is formed by both chains (Figure 7, right). The linker between domains I and II of TK is with 95 amino acids longer than the 20 amino acid linker of the DXS enzyme and offers the possibility for the different arrangement of the enzyme [29]. Examining the ThDP-binding pocket at the level of the amino acids, a high similarity between the enzymes is apparent. The amino acids, which take part in binding ThDP, show a high conservation and bind ThDP in a similar strained conformation [46]. Some minor differences are observable, such as Met to Thr, Gln to His and His to Ser.

Reaction mechanism of the DXS-catalyzed condensation

The exact reaction mechanism of the DXS enzyme is still unclear; a proposed mechanism is shown in Figure 8. In general, the enzyme catalyzes the decarboxylation and head-to-tail condensation of pyruvate and D-GAP to DOXP (Figure 3). Earlier studies have shown that the enzyme follows a classical ping-pong mechanism, where the pyruvate likely binds first covalently to the C2-atom of the thiazolium ring of ThDP, and then undergoes decarboxylation and forms a carbon–carbon bond with the C1-atom of D-GAP [47].

In 2010, Sisquella *et al.* proposed an ordered reaction mechanism using a single-molecule force spectroscopy nanosensor [48]. In this study, they observed a twofold binding increase of D-GAP to the enzyme–ThDP complex in the presence of pyruvate. Later in 2012, Meyers and coworkers found that pyruvate and D-GAP can bind independently to the DXS enzyme and D-GAP was found to accelerate the decarboxylation of the lactyl-ThDP intermediate by a factor of 600. They proposed that it follows a rapid equilibrium, random, sequential mechanism [49]. It is now a general consensus that the DXS reaction mechanism differs from the typical ping-pong mechanism of ThDP-binding proteins. This is important for the evaluation of DXS as a target for drug design, as this diversity in mechanism from other ThDP-dependent proteins provides an opportunity to address the DXS enzyme selectively.

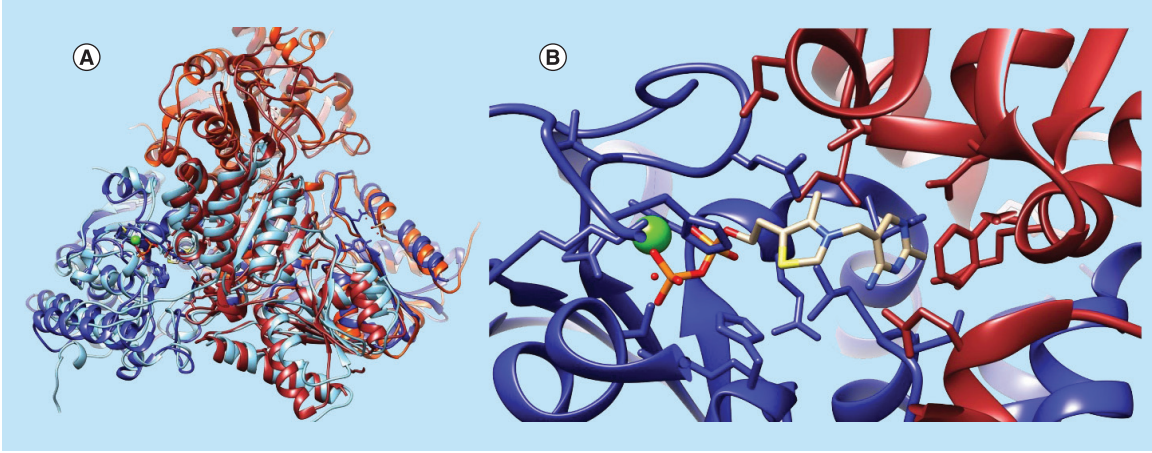


Figure 7. DXS in comparison with human TK. (A) Superposition of the *Deinococcus radiodurans* DXS enzyme with the human TK. The two chains of the human TK are colored in red and blue, and the chains of the DXS enzyme in the orange and light blue, respectively. The magnesium ion close to the ThDP is colored in green. **(B)** Active site of the human transketolase, which is formed at the interface of the two chains.
ThDP: Thiamine diphosphate; TK: Transketolase.

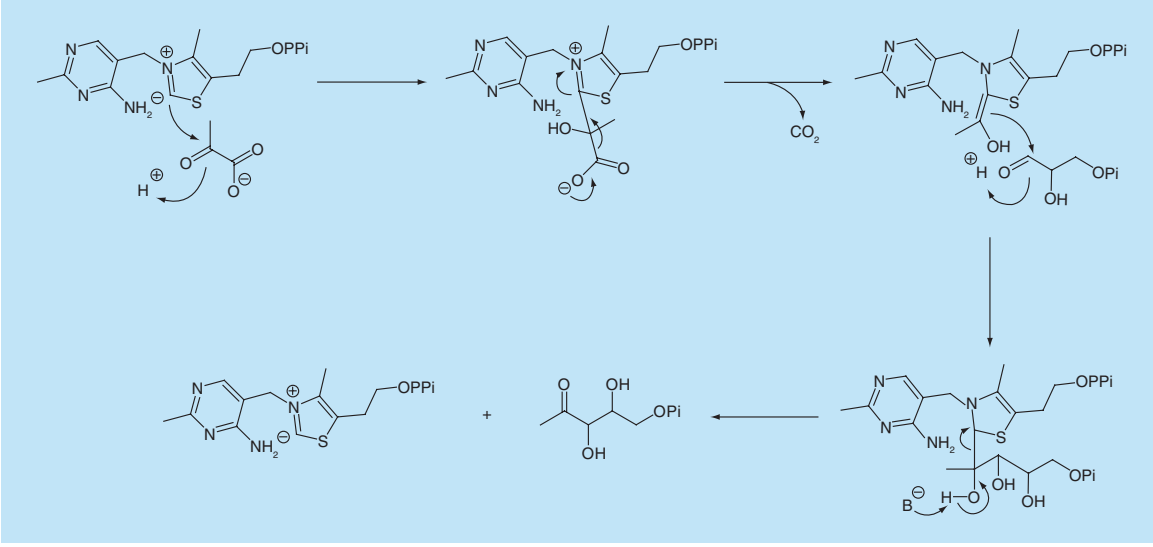


Figure 8. Proposed general reaction mechanism of the reaction catalyzed by the enzyme DXS. ThDP is shown in its ylide state, in which it is bound and activated in the binding pocket.
ThDP: Thiamine diphosphate.
Data taken from [47].

Mutational studies

To obtain more information about a reaction mechanism, site-directed mutagenesis is frequently used. By changing one specific amino acid and observing the consequence on the catalyzed reaction, the role of the mutated amino acid can often be identified.

The active site of the DXS enzyme is highly conserved, which can be seen in Figure 1, but not the entire conserved amino acids take an active part in the catalyzed reaction. Some of them may have other roles, such as binding the cofactor. An example of an essential amino acid that does not take part in the reaction is Phe398. It stabilizes the binding of the pyrimidine ring of the cofactor through π–π-stacking interactions.

In 2014, Querol-Audi *et al.* carried out mutations around the active-site pocket of *E. coli* DXS enzyme to determine their importance in catalysis. Mutational studies confirmed the importance of His49, Asp427, His431 and Glu370 (corresponding to His51, Asp430, His434 and Glu373 in the alignment in Figure 5 and structure of dr-DXS shown in Figure 1); for instance, mutation of Glu370 (Gln or Ala) resulted in a completely inactive enzyme, demonstrating its crucial role in cofactor deprotonation during catalysis [50]. The His431 mutation (Gln or Ala) resulted only in a 50% decrease in the reaction rate, suggesting a supportive role in catalysis. For the mutants of His49 and Asp427 (Gln, Asn or Ala), they observed a drop to only 2–10% of the wild-type activity, suggesting that these residues are critical in abstracting a proton from the donor substrate [50].

In 2016, Woodcock and coworkers also performed mutational studies on the *E. coli* and *D. radiodurans* DXS. They identified two important histidine amino acids, His82 and His304, in a close proximity of 3.7 Å, which form a hydrogen bond to the thiazolium ring of ThDP. Only one of them is needed, as they can replace each other in stabilizing the intermediate in a compensatory fashion [46]. They performed additional computational calculations and postulated that the activation of ThDP to an ylide in the active site proceeds via an activated water molecule instead of the previously assumed direct deprotonation by His434 [46].

Studies in plants

There are many protein-engineering studies that focus on optimization of the MEP pathway for an overexpression of plant secondary metabolites. One of their findings is a feedback inhibition of DXS by IDP and DMADP. They bind to the ThDP pocket, but not as tightly as ThDP because they are smaller in size and cannot form as many stabilizing interactions as ThDP [51,52]. Compounds IDP and DMADP act as natural competitive inhibitors, but using them as a scaffold for drug design does not seem to be advisable because of their ubiquity in living organisms [53].

Structural differences between the DXS homologues

In this section, we focus on the differences between the DXS homologues of the DXS enzymes of *E. coli*, *A. thaliana*, *M. tuberculosis*, *P. falciparum* and *D. radiodurans*.

The length of their primary amino acid sequence is between 600 and 700 amino acids. An alignment of the sequences using MUSCLE is shown in Figure 5. An exception is DXS of *P. falciparum*, which has a sequence length of 1200 amino acids, due to a signifi-

cant number of asparagine inserts. In general, proteins with multiple asparagine inserts have the tendency to form insoluble aggregates. However, these inserts are characteristics of *P. falciparum*, and they do not seem to affect its viability. The reasons for the insertions are still unknown. There are speculations that heat shock proteins play a major role in the metabolism of this organism, and the adaption may be due to the life cycle of the parasite with changing temperatures of the environment, depending on the host organism [54].

During SBDD against the DXS enzyme of *P. falciparum*, this major difference in the primary sequence in comparison with the other DXS enzymes should be considered. It may not be possible to apply the results of studies or computations from model enzymes to the DXS enzyme of *P. falciparum*. On the other hand, the high asparagine content is a rare property and may be an opportunity to achieve target selectivity.

Given the lack of structural information regarding crystallographic data, we decided to simulate the tertiary structure of the proteins of interest. The 3D structures were predicted using the SWISS-MODEL web service [55–57]. For all three proteins, the tool selected the already known structure of *D. radiodurans* as a template for prediction. It can be expected that the modeled structure shows similarities with the chosen template. The resulting models are shown in Figure 2. It can be seen that the secondary structure is mostly conserved between the homologues. The tertiary structure of the secondary motifs is also very similar. This may be because of the model used in the in the prediction algorithm, but it is also reasonable because all enzymes catalyze the same reaction and have conserved structural elements.

Most of the variability is found in the unordered regions at the surface and, in particular, in the loops that connect the three domains. Those loops are flexible regions, which are difficult to predict, and the modeled structures show only one of many possibilities. The longer amino acid sequence of *P. falciparum* manifests itself through a number of disordered loops. The differences in the random coiled regions between the homologues may be useful to target a specific species. This may be of particular interest when targeting the protein–protein interface. For further studies, experimentally determined crystal structures of the different homologues are needed.

Known Inhibitors of DXS

Ketoclomazone

Ketoclomazone (2-(2-chlorobenzyl)-4,4-dimethyl-isoxazolidine-3,5-dione), a derivative of the herbicide clomazone, is able to inhibit the DXS of *Chlamydomonas* with an IC_{50} value of 0.1 mM [58]. In 2010, Kuzuyama and coworkers demonstrated that it is also able to inhibit the DOXP synthase in *E. coli* and *Haemophilus influenzae*, with MIC values of 800 and 12.5 μg/ml, respectively. They proved its mechanism of action as an uncompetitive inhibitor with respect to pyruvate, which is able to bind only the enzyme–substrate complex (EA) and not the free enzyme (E). No information was provided about the inhibitor-binding site. A rescue screen using 1-deoxy-D-xylulose, which is phosphorylated in the bacteria to 1-deoxy-D-xylulose 5-phosphate (DOXP), the product of DXS, demonstrated that the DXS enzyme is the intracellular target of ketoclomazone [47].

Alkylacylphosphonate derivatives

Meyers and coworkers were able to achieve a selective inhibition against DXS by the design of alkylacylphosphonate derivatives [59]. By comparison of the dr-DXS crystal structure with other ThDP-dependent enzymes, they found that the DXS enzyme shows some peculiarities both in a distinct domain arrangement with a larger active site and in the formation of a ternary complex during the catalysis (Figures 1 & Figure 7). Therefore, selectivity for DXS over the other ThDP-dependent enzymes may be obtained by using un-natural bisubstrate analogues. On this basis, they designed and prepared a series of competitive inhibitors bearing a mimic donor substrate (pyruvate) and an apolar unnatural acceptor substrate that forms a dead-end complex with ThDP in the active pocket of the DXS enzyme. They synthesized a set of alkylacylphosphonate derivatives such as butylacetylphosphonate or methylacetylphosphonate [60].

Interestingly, phosphonates modified at the alkyl position rather than the acyl moiety have more influence on the inhibitory activity. These bisubstrate analogues are active against DXS enzymes with potencies in the low micromolar range, but, unfortunately, display very poor cell permeability. Continuing the selection of substrates able to mimic pyruvate, Battistini *et al.* demonstrated that β-fluoropyruvate, similarly to methylacetylphosphonate, displays competitive inhibition against pyruvate, while exhibiting noncompetitive inhibition versus D-GAP [61].

Hydroxybenzaldoximes

Next, Meyers and coworkers extended the idea of unnatural bisubstrate inhibition of DXS using an oxime moiety as a linker between the acceptor substrate and the moiety that should occupy the pyruvate-binding site. From the diversely decorated library, they identified two new hits (2,4,5-trihydroxybenzaldoxime and 3,4,5-trihydroxybenzaldoxime), exhibiting a K_i value in the low micromolar range.

They display a unique mode of inhibition compared with other inhibitors of ThDP-binding enzymes and are competitive with respect to D-GAP [62].

Thiamine derivatives

Recently, we performed *de novo* design of fragments and validated their binding mode in solution using the powerful STI NMR methodology [63], consisting of saturation-transfer difference NMR, transferred-NOE and the INPHARMA methodologies [64].

This study enabled us to discover a promising fragment (3-((1H-imidazol-1-yl)methyl)-5-methoxypyridine) featuring an IC_{50} value of 1.8 ± 0.5 mM. Fragment growing led to a small molecule (Figure 7 & Table 1) having a potency in the micromolar range, demonstrating the power of this NMR-based approach to guide fragment growing in the absence of protein x-ray crystallography. Therefore, the lack of structural information available for DXS could explain the very small number of inhibitors of this enzyme reported so far. We then carried out a structure-based study by constructing a homology model of *M. tuberculosis* DXS [65]. We docked a series of ThDP and thiamine derivatives both into the homology model constructed and into the crystal structure of *D. radiodurans* DXS as these ortholog enzymes have a high degree of similarity (68% sequence identity in the ThDP-binding pocket of *D. radiodurans* and *M. tuberculosis* DXS [66]). The obtained experimental results displayed remarkable differences in the binding mode of the inhibitors tested between the two enzymes and, in turn, these evidences could guide a rational design of new potential inhibitor for *M. tuberculosis* DXS.

Bacimethrin is a thiamine antagonist, which undergoes conversion to 2′-methoxythiamine diphosphate (MeOThDP) by enzymes producing ThDP. The metabolic product is a chemical analog of ThDP in which the C2′-methyl group of the 4′-aminopyridine ring is replaced by a 2′-methoxyl group. This natural antibacterial product was isolated from *Streptomyces albus* for the first time in 1987, and recently Jordan and coworkers studied the role of MeOThDP on five ThDP enzymes including DXS [67]. By using different mechanistic tools, they found that MeOThDP binds in the active site of ThDP-dependent enzymes. The difference between ThDP and MeOThDP is a methoxy substituent at position 2′ of ThDP. In their study, they investigated the influence of this modification on the reaction catalyzed by different ThDP-binding enzymes [68].

Using circular dichroism and fluorescence titration experiments they showed that the binding of ThDP to the enzyme is different for each enzyme class. They conclude that it should be possible to use the different ThDP-binding modes to target DXS selectively with a ThDP analog. For 2′-MeOThDP they found a different binding mode to the ThDP-binding pocket of DXS. Upon binding of 2′-MeOThDP to an inactive apoenzyme, it was possible to restore 9–14% of DXS enzyme activity. Investigation of other classes of ThDP-binding enzymes led from no activity restoration (2-oxoglutarate dehydrogenase) to up to 75% (PDH) activity restoration after binding 2′-MeOThDP to an apoenzyme. This points toward very different recognition of ThDP analogs in those enzymes (Figure 9).

Future perspective

The MEP pathway was discovered 20 years ago and is still not fully understood. The increasing amount of research activities on the MEP pathway will deliver interesting results over the next 5–10 years. Today's focus is still on determining the structures of the constituent enzymes and on elucidating their mechanism. With this information about the structure and mechanism of the MEP pathway, targeted manipulation may be possible. This could be used to develop new drugs and herbicides, modify plant metabolism or even explore yet unknown applications.

As more information becomes available about the various targets, the number of studies on the MEP pathway will increase. As a result, the number of inhibitors will also increase. By adopting state-of-the-art medicinal-chemistry strategies, undesired side- or off-target effects and unfavorable pharmacokinetic properties should be circumvented. Upon identification of a promising scaffold, SBDD and optimization become possible. A particular problem that has to be addressed is the high polarity of the active sites of enzymes in this pathway. As a consequence, strongly binding compounds will also probably be polar, which makes them less membrane-permeable, a problem, in particular, when targeting *P. falciparum*. The development of less polar prodrugs or screening in whole-cell assays may circumvent this problem. Comparable developments are expected in the field of herbicide development.

A different field of emerging research may be the genetic engineering of organisms to overexpress and modify isoprenoids. Nature offers many complex molecules that are used for different applications, but are hard to synthesize. They are still mainly extracted from

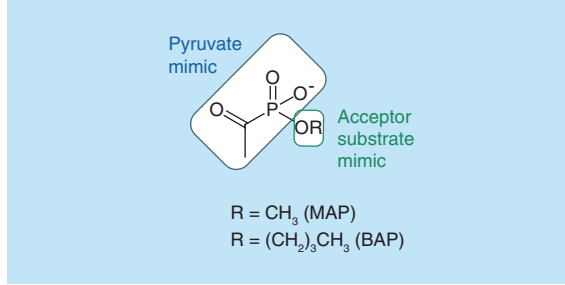


Figure 9. Designed alkylacylphosphonate derivatives as inhibitors of 1-deoxyxylulose-5-phosphate synthase.

natural sources, which are sometimes hard to cultivate and often contain only small amounts of the isoprenoid of interest. The amount of plant material needed for the extraction of secondary metabolites could be reduced by several orders of magnitude. This would free resources and may even enable the use of some isoprenoids in the first place.

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Executive summary

The methylerythritol 4-phosphate pathway

- The methylerythritol 4-phosphate (MEP) pathway was discovered in 1996 and is the second known pathway for the biosynthesis of the universal building blocks of isoprenoids. This complex class of secondary metabolites fulfils numerous essential roles in living organism like protein degradation and prenylation and is the basis for the biosynthesis of steroids, carotenoids, pigments and chlorophyll.
- The pathway is found in some bacteria, the chloroplasts of plants and protozoa, and the enzymes of the MEP pathway are therefore considered as possible new targets for the development of new anti-infectives against, for instance, malaria or tuberculosis.
- The MEP pathway is used in the chloroplasts of plants to deliver the universal building blocks for the biosynthesis of the isoprenoid chlorophyll, isopentenyl diphosphate and dimethylallyl diphosphate. The mode of action of the herbicide clomazone is based on the inhibition of this pathway and validates the MEP pathway as a target for further development of herbicides.

The structure of 1-deoxyxylulose-5-phosphate synthase

- The inhibitor class of thiamine diphosphate (ThDP) analogues is probably robust against microbial resistance development, in particular, against acquired resistance via increased efflux due to the important metabolic role and its structural similarity with the essential vitamin B1.
- The conservation of amino acids was mapped on the protein structure of *Deinococcus radiodurans* DXS. This is a useful visualization of amino acid sequence conservation and was used to discuss previously identified binding pockets; see Figure 6.
- Pocket P1 (Figures 4 & Figure 6) is in close proximity to the active site of the DXS enzyme, and has suitable physicochemical properties and a high sequence conservation. It seems to be a good target for structure-based drug design.

Structural differences

- A comparison of predicted structures of the DXS enzymes of *Arabidopsis thaliana, Mycobacterium tuberculosis* and *Plasmodium falciparum* based on the known crystal structures from *Escherichia coli* and *Deinococcus radiodurans* shows high structural conservation of the three subdomains of the DXS enzyme, but also variability of the linkers.
- A comparison of the DXS enzyme with other ThDP-binding enzymes gives a high overall structural similarity, but also showed some differences of the active site. Based on this, it may be possible to design a ThDP analogue that targets the DXS enzymes selectively in comparison with other ThDP-binding enzymes.

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