[Frontiers in Bioscience 18, 944-969, June 1, 2013]

The shikimate pathway in apicomplexan parasites: Implications for drug development

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1. ABSTRACT

The shikimate pathway provides basic building blocks for a variety of aromatic compounds including aromatic amino acids, ubiquinone, folate and compounds of the secondary metabolism. The seven enzymatic reactions of the pathway lead to the generation of chorismate from simple products of the carbohydrate metabolism, namely erythrose 4-phosphate and phosphoenolpyruvate. The shikimate pathway is present in plants, bacteria, fungi and chromalveolata to which the apicomplexan parasites belong. As it is absent from humans, the enzymes of the shikimate pathway are attractive targets for antimicrobial drug development. Inhibition of the pathway is effective in controlling growth of certain apicomplexan parasites including the malaria parasite *Plasmodium falciparum.* Yet, despite being an attractive drug target, our knowledge of the shikimate pathway in this parasite group is lacking. The current review summarizes the available information and discusses aspects of the genetic organization of the shikimate pathway in apicomplexan parasites. Compounds acting on shikimate pathway enzymes will be presented and discussed in light of their impact for antiapicomplexan/antiplasmodial drug development.

2. INTRODUCTION

The syntheses of many aromatic compounds rely on chorismate as a precursor, which is produced by the shikimate pathway. This pathway is present in bacteria, plants, fungi and certain protozoans including apicomplexan parasites (1, 2), but is absent in the animal kingdom. Thus the enzymes catalyzing the transformation of the shikimate pathway present suitable targets for herbicides and antimicrobials (3, 4).

The pathway comprises seven enzymatic reactions performed by seven different enzymes (Figure 1). The first step is the condensation of phosphoenolpyruvate (PEP) with erythrose 4-phosphate (E4P) to 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP), catalyzed by DAHP synthase (DAHPS). DAHP is accepted by the dehydroquinate synthase (DHQS) and converted to 3-dehydroquinate (5). This reaction encompasses a transient NAD^+-dependent redox step that facilitates hydrogen and phosphate elimination followed by reorganization of the ring to establish a cyclohexanone structure (6). In the following steps 3-dehydroquinate becomes dehydrated by 3-dehydroquinate dehydratase (DHQase) and reduced to

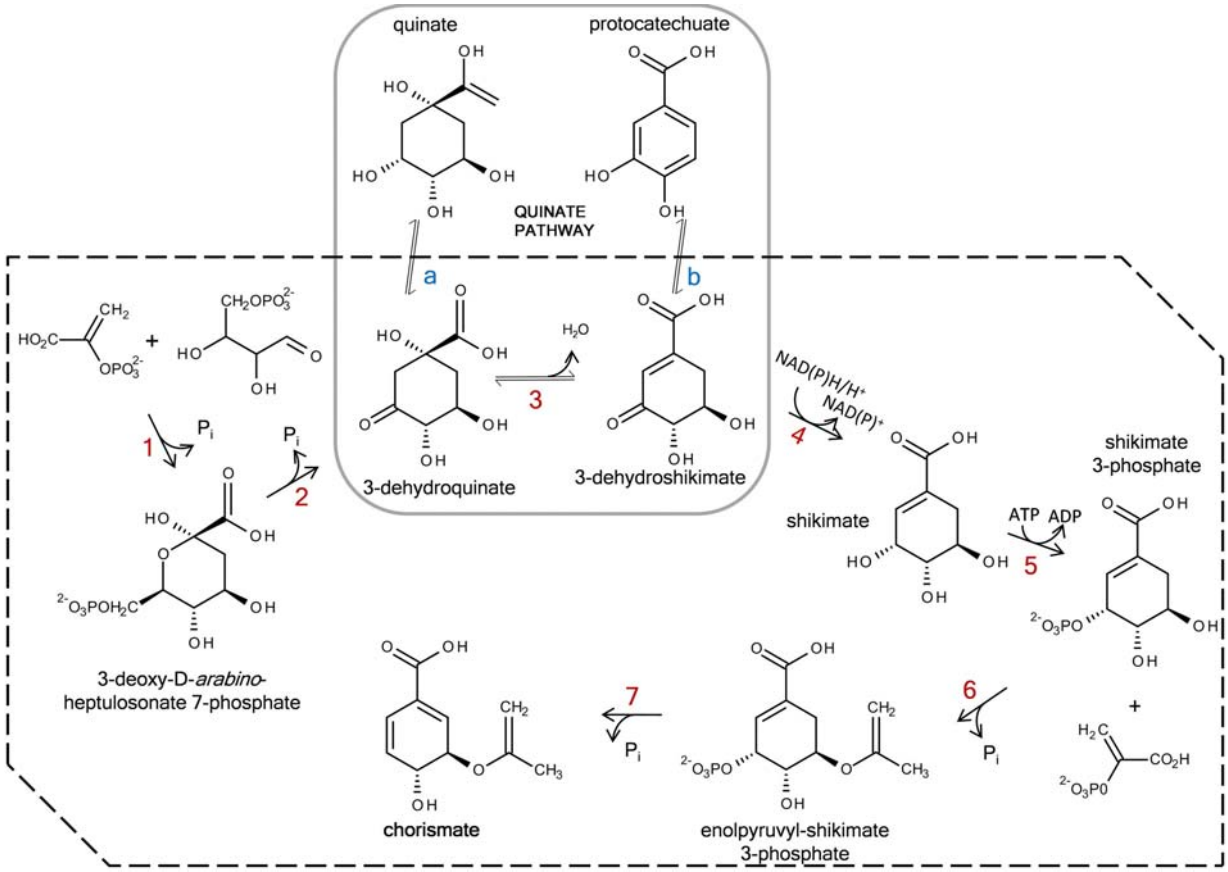

Figure 1. Overview of the shikimate and quinate pathway. The individual steps are catalyzed by 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (1), deydroquinate synthase (2), 3-dehydroquinase (3), shikimate dehydrogenase (4), shikimate kinase (5), 5-enolpyruvylshikimate-3-phosphate synthase (6) and chorismate synthase (7). 3-dehydroquinase (3) and shikimate dehydrogenase (4) catalyze steps within the shikimate and the quinate pathway. Quinate dehydrogenase (qut B) (a) and dehydroshikimate dehydratase (qut C) (b) are restricted to the quinate pathway. In the majority of cases, shikimate dehydrogenase use $NADP^+$ as cofactor, however, *e.g.* YidB, utilises NAD^+ and $NADP^+$ as acceptor (4).

shikimate by shikimate dehydrogenase (SDH). The latter step is NADPH-dependent. Phosphorylation of shikimate by the shikimate kinase (SK) yields shikimate-3-phosphate, which is converted to 5-enolpyruvylshikimate-3-phosphate (EPSP) by 5-enolpyruvylshikimat-3-phosphate synthase (EPSPS) at the expense of yet another molecule of PEP. EPSPS is specifically inhibited by glyphosate, a potent herbicide (3). Finally, chorismate synthase (CS) eliminates a hydrogen and phosphate from EPSP to yield chorismate, the product of the common shikimate pathway.

Chorismate itself is used as precursor for (i) the synthesis of the three aromatic amino acids tyrosine, phenylalanine and tryptophan, (ii) the production of ubiquinone, (iii) the generation of *para*-aminobenzoic acid and (iv) biosynthesis of vitamin K. In plants, chorismate is a major building block for the synthesis of a variety of secondary metabolites such as betalains, flavonoids and phenylpropanoids like lignin (7, 8). Bacteria also use chorismate for the biosynthesis of siderophores (9).

3. BIOCHEMICAL CHARACTERISTICS OF SHIKIMATE PATHWAY ENZYMES

3.1. 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (DAHPS; EC 2.5.1.54)

3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase catalyzes the first committed step in the shikimate pathway, an aldol reaction between phosphoenolpyruvate and erythrose 4-phosphate to produce 3-deoxy-D-*arabino*-heptulosonate 7-phosphate. The aldol-like condensation of PEP and E4P is stereospecific for both substrates with the *si* face of PEP attacking the *re* face of E4P (10, 11). It was a matter of debate whether all DAHPSs have a strict requirement for a divalent metal ion in the active site for activity. Indeed, the fact that an absolute conserved metal binding motif is found in all DAHPSs suggests that all DAHPS enzymes are metalloenzymes (12).

Two major families of DAHPS are found in nature: the AroAI family containing the smaller type I enzymes (30-40 kDa), which are mainly of bacterial origin (13) and the AroAII family defined as the "plant-like" DAHPSs consisting of the larger type II proteins (>50

Table 1. Abbreviations used for shikimate pathway enzymes

Enzmye	Organism	Properties
1. 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (DAHPS; EC 2.5.1.54)		
AroI	mainly prokaryotic and fungal species	DAHPS isozymes of about 30-40 kDa in size
$AroI_{\alpha}$	e.g. *E. coli* AroF	Extensions to the N-terminus and α2-β3 loop forming a domain that binds Tyr or Phe
$AroI_{\beta}$	e.g. *Thermotoga maritima*, *Pyrococcus furiosus*, *M. tuberculosis;B. subtilis*	catalytic barrel only or catalytic barrel with N-terminal or C-terminal extensions
aroF/aroFp	*E. coli*, *A. nidulans*	DAHPS isozyme - feedback inhibition via Tyr
aroG/aroGp	*E. coli*, *A. nidulans*	DAHPS isozyme - feedback inhibition via Phe
aroH	*E. coli*	DAHPS isozyme - feedback inhibition via Trp
ARO3	*S. cerivisiae*	DAHPS isozyme - feedback inhibition via Phe and to a lesser extent via Trp
ARO4	*S. cerivisiae*	DAHPS isozyme - feedback inhibition via Tyr and to a lesser extent via Trp
AroII	Plants, prokaryotic (*e.g. Streptomyces*) and fungal species (*e.g. N. crassa*), *T. gondii*	DAHPS isozyme of >50 kDa in size
2. AroB - Dehydroquinate synthase (DHQS; EC 4.2.3.4)		
3. AroD – Dehydroquinase (DHQase; EC 4.2.1.10)		
4. Shikimate dehydrogenase (SDH; EC 1.1.1.25)		
AroE	*all prokaryotes*	prototypical SDH, accepts shikimate as substrate
YdiB	*e.g. E.coli*, *Pseudomonas putida*	accepts quinate and shikimate as substrate and uses NAD^+ and $NADP^+$ as cofactor
YdiB2	e.g. *P. putida*	accepts quinate and shikimate as substrate but phylogenetically distinct from YdiB
RifII	*e.g. Amycolatopsis mediterranei*, *P. putida*	part of the aminoshikimate biosynthetic pathway
SdhL	*H. influenza*, *P. putida*	accepts quinate and has a 1,000 fold lower activty for shikimate than *E. coli* AroE
Ael1	*P. putida*	AroE-like with enzymatic properties distinct from any other SDH familiy
5. Shikimate kinase (SK; EC 2.7.1.71)		
SK I/AroL	*E. coli*	*aroL* gene is controlled TrpR and TyrR; K_m for shikimate - 20 mM
SKII/AroK	*E. coli, M. tuberculosis; H. influenza*	*aroK* gene is constitutively expressed; K_m for shikimate is 200 µM
6. AroA - 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS; EC 2.5.1.19)		
7. AroC - Chorismate synthase (CS; EC 4.2.3.5)		
AROM complex - pentafunctional complex encoded by the locus *aromA* that comprises the DHQS, EPSPS, SK, SDH and DHQase		

kDa), which are primarily found in plants (see Table 1). The former family is further divided into the subfamilies $AroAI_{\alpha}$ and $AroI_{\beta}$ (13, 14). One striking feature of the DAHPSs is that despite their large sequence variability all enzymes share a conserved tertiary core structure, a $(\beta/\alpha)_8$ TIM barrel fold. This core catalytic barrel is decorated with diverse small domains that are implicated in allosteric regulation of enzyme activity (14).

DAHPS is the enzyme with the largest number of allosteric regulatory patterns thus far observed (15). Several organisms express isozymes that show different sensitivities to pathway end products. For example, *Escherichia coli* and *Neurospora crassa* possess three isozymes each, which are differentially regulated by Phe, Tyr and Trp (15). In contrast, *Saccharomyces cerevisiae* has two isozymes inhibited by either Phe or Tyr (16). DAHPS of *Bacillus subtilis* has an active chorismate mutase domain fused to its N-terminus that confers feedback inhibition by chorismate and prephenate (14). The DAHPS of *Thermotoga maritima* also possesses an additional domain fused to its N-terminus, which is similar to a domain implicated in allosteric regulation of amino acid biosynthesis (ACT) (17-19). The DAHPS of *Mycobacterium tuberculosis* has both an extension and an additional internal loop for the binding of regulatory amino acids. Its enzyme activity is fine-tuned by combinations of phenylalanine and tryptophan or tyrosine and tryptophan (20). Bentley has explained the occurrence of different regulation modes of DAHPS isozymes by the "endo-exo" orientation of a given organism. "Exo-oriented" organisms such as *E. coli* and some yeast strains must adapt efficiently to the exogenous availability of each individual aromatic amino acid and thus possess isozymes that are repressed by a single aromatic amino acid. Whereas "endo-oriented" organisms such as cyanobacteria regulate the flow through the shikimate pathway by pathway intermediates and usually have DAHPS of the single effector type (15).

Genes encoding DAHPS function are present in *Toxoplasma gondii*, *Neospora caninum* and *Eimeria*

tenella. The respective gene IDs are TGGT1_065100, NCLIV_004821and ETH_00003830, respectively. However, it has to be mentioned that the only complete gene sequence available is that of *T. gondii* (21). Blast searches using the predicted protein sequences of the aforementioned genes did not reveal additional apicomplexan DAHPS genes. *T. gondii*, *N. caninum and E. tenella* DAHPSs belong to the AroAII type family. However, with a predicted molecular mass of 67.4 kDa *T. gondii* DAHPS is significantly larger than the previously reported type II enzymes. This is caused by numerous insertions into the protein sequence (21). To the best of our knowledge no biochemical information of an apicomplexan DAHPS is presently available except that DAHPS activity was observed in crude extracts of the malarial parasite *Plasmodium falciparum* (22).

3.2. Dehydroquinate synthase (DHQS; EC 4.2.3.4)

Dehydroquinate synthase belongs to the superfamily of sugar phosphate cyclases and catalyzes the cyclisation of its sugar phosphate substrate 3-deoxy-D-*arabino*-heptulosonate 7-phosphate to 3-dehydroquinate (23). The complex multistep reaction catalyzed by DHQS is initiated by the oxidation of the primary alcohol group at C-4 to facilitate proton abstraction at C-5 and the elimination of the phosphate group. After reduction of the keto-group at C-4, proton abstraction at the C-1 hydroxyl group results in ring opening to an anionic intermediate and finally attack of the carbanion at the C-1 keto function to close the ring to yield 3-dehydroquinate (5). The transient oxidation-reduction reaction is performed by NAD^+. In addition DHQS requires either Co^{2+} or Zn^{2+} to assist catalysis (24, 25). The N-terminal domain of DHQS contains a Rossmann fold involved in NAD^+ binding. Strikingly, DHQS binds NAD^+ in an inverted orientation to that found in other common Rossmann fold proteins (5).

There has been some controversy whether Co^{2+} or Zn^{2+} is the actual metal cofactor in nature. Although the Co^{2+}-form of the enzyme is more stable and exhibits a higher specific activity, it is quite likely that Zn^{2+} is the actual cofactor due to its higher bioavailability when compared to Co^{2+} (25).

The enzyme assembles in a functional homodimer with each protomer being composed of an N-terminal α/β domain and a C-terminal α-helical domain. The C-terminal domain contains the residues required for catalysis and for substrate and Me^{2+} binding. Binding of Me^{2+} is ensured by a pentafunctional coordination of the ion. The active site is formed between the two domains and contains 13 active site residues mainly provided by the C-terminal domain, which are strictly conserved among DHQSs (5, 23). Three of these are involved in the coordination of the Me^{2+} ion, the others in substrate binding and catalysis (5, 23). This overall organization is typical for all sugar phosphate cyclases. However, each subclass is characterized by a unique signature of binding pocket residues (23).

Genes coding for a DHQS activity have been identified in *T. gondii*, *N. caninum, E. tenella* and all *Plasmodium* species as part of the AROM complex (see below) (21). The respective sequences of *T. gondii*, *N. caninum* and *E. tenella* encode the two conserved histidines being absolutely required for catalysis (5). These are missing in all *Plasmodium* species. At this stage it cannot be ruled out that the *Plasmodium* enzyme employs an alternative catalytic mechanism. Thus, the definitive answer whether *Plasmodium* possesses a functional DHQS activity awaits further experimental clarification. So far, no DHQS activity has been reported in crude parasite extracts for any apicomplexan parasite or for a recombinant protein.

3.3. Dehydroquinase (DHQase; EC 4.2.1.10)

Dehydroquinate dehydratase (dehydroquinase, DHQase) catalyzes the third step in the shikimate pathway, which is the dehydration of 3-dehydroquinate to 3-dehydroshikimate. Dehydroquinases are classified into two distinct types (I and II), which are structurally and mechanistically different (see Table 1) (26, 27). Type I DHQases catalyze the *syn* dehydration of 3-dehydroquinate through the formation of a Schiff base, the reaction catalyzed by type II dehydroquinases involves an *anti*-elimination of water via an enolate intermediate (28). Type I dehydroquinases form homodimers with a subunit size of 26-28 kDa, which are heat-sensitive. In contrast, type II dehydroquinases build up dodecamers with a subunit size of 16-18 kDa that are heat-resistant (29). Type I enzymes are found in plants, fungi and many bacterial species and are exclusively involved in the biosynthesis of chorismate. Biosynthetic type II dehydroquinases are present in bacterial pathogens such as *M. tuberculosis* and *Helicobacter pylori*. On the other hand, catabolic type II dehydroquinases, enabling the use of quinic acid as carbon source for the formation of protocatechuate, are found in many fungal species (see Figure 1) (30-32). Quinate, which comprises about 10% by weight of decaying leaf litter, is used as an abundant carbon source in many fungi (33). Whereas the fungal DHQase of the quinate pathway is encoded in a cluster of eight genes that is transcriptionally regulated in response to the presence of quinate, the respective enzyme of the shikimate pathway is part of the so-called AROM complex (for details see below) (21, 34, 35). Remarkably, the 3-dehydroquinases of the catabolic quinate pathway and the AROM complex do not possess any significant sequence similarity and have most likely developed through convergent evolution (36, 37). In the fungal system, both type I and type II dehydroquinases can complement loss of function mutants in either the quinate or the shikimate pathway (38, 39).

Again, *dhqase* genes have been identified as part of the AROM complex in *T. gondii, N. caninum* and all *Plasmodium* species (see later) (21). *T. gondii*, and *N. caninum* DHQases are grouped together and possess the typical signatures of type I DHQase (21). Albeit, it is quite likely that the plasmodial DHQase species also belong to the type I enzymes, their signatures are not as distinct, which is why a biochemical characterization should be used for final assignment. The partial *arom* complex sequence of *E. tenella* is lacking a significant portion of the *arom* gene including the *dhqase* sequence and thus the putative *E. tenella* DHQase cannot be assigned. A *T. gondii* DHQase

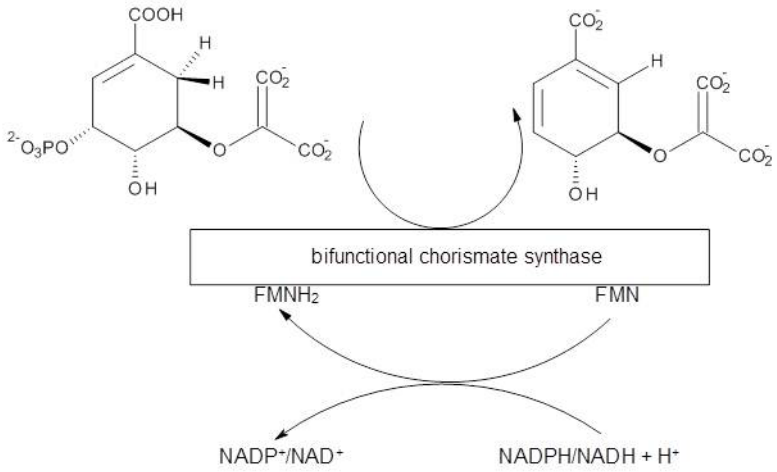

Figure 2. The catalytic activity of bifunctional CS. This class of enzymes reduces oxidized FMN to the fully reduced cofactor $FMNH_2$ at the expense of NADPH or NADH (*M. tuberculosis*). With the reduced cofactor bound to the active site multiple turnover of the substrate EPSP can occur.

activity has been partially purified from *T. gondii* extracts and was shown to be heat-sensitive classifying it as a typical type I DHQase (1).

3.4. Shikimate dehydrogenase (SDH; EC 1.1.1.25)

Shikimate dehydrogenases (SDH) form a large superfamily that is divided into six distinct enzyme groups differing in enzymatic characteristics and function (see Table 1) (40). The prototypical SDH, AroE, catalyzes the reversible NADPH-dependent reduction of 3-dehydroshikimate to shikimate, the fourth reaction of the shikimate pathway. Further members of the SDH superfamily are (i) YdiB that accepts either quinate or shikimate as substrate and is supposed to function as a quinate/shikimate dehydrogenase (41); (ii) YdiB2 a second shikimate/quinate dehydrogenase but phylogenetically distinct from YdiBs (40); (iii) RiflI first identified in *Amycolatopsis mediterranei,* which is part of the aminoshikimate biosynthetic pathway resulting in the synthesis of 3-amino-5-hydroxybenzoate the starter unit for the synthesis of ansamycins (rifamycin B) and mitomycins (42); (iv) SdhL, a SDH like protein from *Haemophilus influenza*, that does not recognize quinate as substrate and has a 1,000 fold lower activity than *E. coli* AroE when shikimate is used as substrate (43); and (v) Ael1 (AroE-like 1) which compared to AroE possesses a unique binding/catalytic motif and enzymatic properties distinct from any other SDH subfamily (40). All bacteria analysed by Singh and coworkers contained an AroE SDH homolog indicating a vertical descent during speciation and further reflecting the ancestral nature of *aroE* and the shikimate pathway, whereas the other SDH homologs seem to have evolved through independent evolution and acquisition via horizontal gene transfer (40). In *E. coli*, *aroE*-loss of function mutants are auxotrophic for the aromatic amino acids an indication for the essentiality of its gene product and the shikimate pathway (44, 45).

AroE/SDH orthologs have a bipartite structure composed of a $NADP^+$-binding domain and a catalytic domain. The $NADP^+$ binding domain consists of a typical Rossmann fold and a unique glycine-rich P-loop with a conserved sequence motif, GAGGXX, that results in a nonstandard binding mode with the nicotinamide and ribose moieties disordered in the binary complex (46). The catalytic domain has an open twisted α/β motif (46). There is a deep pocket between the $NADP^+$-binding domain and the catalytic domain with a narrow entrance. It has been suggested that the flexibility of the nicotinamide mononucleotide portion of NADPH is required to enable the substrate 3-deydroshikimate to enter the pocket and to allow for the release of the product shikimate (46). SDHs have been mostly found as monomers or dimers (41, 47).

SDH activity has already been described in *P. falciparum* lysates in the late 1980s (22). Genes encoding a SDH activity have been identified in *T. gondii*, *N. caninum* and all *Plasmodium* species as part of the AROM complex (see below) (21). Again, it has to be noted that the SDH signatures are less pronounced in the *Plasmodium* species than in the other apicomplexan parasites harbouring an AROM complex. So far, all apicomplexan parasites with a complete AROM complex possess a prototype AroE SDH. No point can be made on *E. tenella* SDH, since its gene sequences are not contained in the partial sequence of the *E. tenella arom* gene. Whether there exist other SDH variants of the non AroE type in apicomlexan parasites is not known (1, 21).

3.5. Shikimate kinase (SK; EC 2.7.1.71)

Shikimate is converted into shikimate-3-phopshate (S3P) by shikimate kinase, which catalyzes the phosphorylation of the 3-hydroxy group using ATP as a co-substrate. SK belongs to the same structural family as the nucleoside monophosphate (NMP) kinases to which adenylate kinase, guanylate kinase, uridylate-cytidylate kinase and thymidylate kinase belong (48). NMP kinases as well as shikimate kinase display a typical α/β/α fold consisting of a central five-strand parallel β-pleated sheet

surrounded by eight α-helixes (49). NMP kinases consist of three domains: the NMP-binding domain, the LID domain and the core domain (50). In SK, the shikimate-binding (SB) domain is equivalent to the NMP-binding domain of NMP kinases. As exemplified for adenylate kinase, NMP kinases undergo large conformational changes during catalysis involving the NMP-binding and LID domain (51, 52). Subsequently, a detailed analysis of the global movements of *M. tuberculosis* SK upon substrate binding and catalysis identified four domains in SK: the extended SB (substrate binding) domain, which includes the NMP-binding domain of NMP kinases, the nucleotide binding domain, the LID and the reduced core domain (51).

Three functional motifs involved in nucleotide binding have been identified in shikimate kinase, a Walker A-motif, a Walker B-motif and an adenine binding loop (49). The Walker A-motif (GXXXXGKT/S), which is located between ß1 and α1, forms the phosphate-binding loop (P-loop), a giant anion hole that accommodates the ß-phosphate of the ADP by providing several backbone amides (49, 53). The second conserved motif ZZDXXG, known as the Walker B-motif (where Z represents hydrophobic and X any residue), is modified in SKs to ZZZTGGG and is located in the C-terminal segment of ß3 of the central ß sheet (49, 53, 54). It is involved in hydrogen bonding of the γ-phosphate (49). The adenine binding motif (I/VDXXX(X)XP) links ß5 and α8 and enwraps the adenine moiety of ATP (49, 55).

Shikimate kinase activity has been reported in *T. gondii* lysates and crude extracts of *P. falciparum* (1, 22). Shikimate kinase encoding sequences have been identified in all apicomplexan parasites having an AROM complex except for *E. tenella*, where the sequence of the *arom* gene is incomplete (21, 56). The SK moiety of the bipartite enzyme of *P. falciparum* contains one complete and one partial match to the PROSITE motif characteristic for known SK sequences (56). Although sequence similarities and conservation argue for a gene encoding a SK activity, clarification of its function still awaits experimental proof.

3.6. 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; 2.5.1.19)

The 5-enolpyruvylshikimate-3-phosphate synthase is a carboxyvinyl transferase producing enolpyruvyl shikimate 3-phosphate and phosphate from shikimate 3-phosphate and PEP. This reaction is quite unusual in the sense that PEP is used as the donor of the enolpyruvyl group. The only other enzymes known to catalyze a carboyxyvinyl transfer using PEP as substrate are UDP-*N*-acetylglucosamine enolypyruvyl transferase (MurA) and UMP-enolpyruvyl transferase (NikO). MurA catalyzes the first committed step in the biosynthesis of peptidoglycans and NikO the transfer of enolpyruvyl to the 3'-hydroxyl group of UMP in nikkomycin biosynthesis (57, 58).

The sequence of events involved in substrate binding and product release for EPSPS has been resolved by the group of Johnson (59). S3P binds to the catalytic site of EPSPS first before PEP is attached and P_i is released prior to EPSP. The EPSPS and MurA reactions proceed through an addition step forming a tetrahedral intermediate followed by an elimination step to form the enolpyruvyl product (60, 61). In the case of EPSPS, the tetrahedral complex is formed by S3P and PEP. EPSPS speeds up the breakdown of the tetrahedral intermediate by more than 10^5-fold when compared to a non-enzymatic breakdown (62). The elimination step involves a cationic intermediate/transition state that is stabilized by positively charged side chains of active side residues in form of an electrostatic sandwich (63).

EPSPS is a monomeric enzyme with a molecular mass of roughly 46-48 kDa. The enzyme consists of two domains each comprising three copies of a βαβαββ-folding unit (64). MurA and NikO apparently have the same 3D-structure (58, 65).

Based on the inhibitory potential of the herbicide glyphosate, EPSPSs are grouped into two major classes, class I enzymes that are sensitive to glyphosate and class II enzymes being tolerant to glyphosate (66). It is assumed that both types have evolved from a common ancestor and diverged into these two families (67). Glyphosate is a potent inhibitor of plant and *E. coli* EPSPS (3, 68), whereas EPSPSs from *Staphylococcus aureus*, *Streptococcus pneumonia*, *Pseudomonas* spec. strain PG2982 and *Agrobacterium* spec. strain CP4 are glyphosate tolerant (69-72). The molecular basis for glyphosate resistance has been resolved for the prototypical class II enzyme of *Agrobacterium* spec. strain CP4. Glyphosate binds to the CP4 EPSP synthase in a condensed conformation that does not inhibit the enzyme. A single point mutation in the active site (Ala-100-Gly) allows glyphosate to bind in its extended conformation thereby restoring its inhibitory potential (73).

EPSPS encoding sequences are present in all apicomplexan parasites harbouring an AROM complex (see below) (21, 56). All seventeen amino acids interacting with either the substrate or glyphosate are conserved in the EPSPS moiety of the *P. falciparum* enzyme and are invariant residues in all plasmodial orthologs, suggesting that the respective plasmodial genes are indeed encoding EPSPS (56). Further support comes from inhibition studies that demonstrate that glyphosate inhibits the growth of *P. falciparum* albeit at very high concentrations (1). In addition, the growth of *T. gondii* and *Cryptosporidium parvum* was inhibited by glyphosate. Despite the fact that glyphosate is a very specific inhibitor of EPSPS and does not affect the bacterial enolpyruvyl transferases MurA and NikO, it is quite likely that its target in *Cryptosporidium* is not EPSPS, since up to now no *epsps* gene could be identified in this parasite species (58, 68).

3.7. Chorismate synthase (CS; EC 4.2.3.5)

The reaction catalyzed by CS involves an 1,4-*anti*-elimination of the 3-phosphate group and the C-(*proR*) hydrogen from EPSP (74-76). Extensive mechanistic studies by Thorneley, Bornemann and Macheroux have led to a detailed mechanistic proposal that explains the obligate role of reduced FMN in the catalytic mechanism and

rationalizes the C-O and C-H bond breaking steps (77-80). CSs differ in the way reduced FMN is provided. In general, two classes of CSs are distinguished: (i) monofunctional CSs depend on an exogenous source for free reduced FMN and (ii) bifunctional CSs possess an intrinsic flavin reductase activity requiring NAD(P)H for the reduction of FMN (81). Whereas monofunctional CSs are found in plants and bacteria, bifunctional CSs seem to be restricted to fungi. However exceptions to this general rule are the CSs of *M. tuberculosis* and *B. subtilis*. Whereas *B. subtilis* CS forms a heterotrimeric complex consisting of CS, a NADH:FMN reductase and deydroquinate synthase (76, 82), *Mt*CS seems to be a truly bifunctional enzyme. However as it is found for *B. subtilis* CS, *M. tuberculosis* CS utilises NADH instead of NAD(P)H for the reduction of FMN (83). Furthermore, CS from *Euglena gracilis*, a ciliated protozoan, shares bifunctionality with the fungal enzymes raising the question of the distribution of this class of enzymes among eukaryotes (84).

In the crystal, CS occurs as a tetramer composed of a dimer of dimers, but in solution it can appear as dimers, tetramers, or a mixture of these two forms (85, 86).

Chorismate synthase genes are present in all apicomplexan species with an AROM complex. The gene IDs are ETH_00032645, TGGT1_020040, NCLIV_023120 and PF3D7_0623000 for *E. tenella*, *T. gondii*, *N. caninum* and *P. falciparum*, respectively. In contrast to the AROM complex that does not show significant amino acid identities between the cyst-forming coccidians, *E. tenella*, *T. gondii* and *N. caninum*, on the one and the *Plasmodium* species on the other hand, apicomplexan CSs are fairly conserved ranging from 37.7% to 77.1% amino acid identity. The CSs of *T. gondii* and *N. caninum* are most closely related as reflected by their amino acid identity of 77.1%. Their amino acid identity with *E. tenella* and *P. falciparum* ranges from 38.9 to 44.8%. *T. gondii* CS mRNA is found in all parasite stages: the unsporulated and sporulated oocyst, the fast dividing tachyzoite and the slowly dividing bradyzoite. Highest expression is found after sporulation as well as in tachyzoites and bradyzoites (ToxoDB). *Tg*CS has also been detectecd in *T. gondii* tachyzoites by mass spectrometry (87). In red blood cells, *Pf*CS mRNA peaks roughly 20-35 hours after infection (88). Using mass spectrometry, peptides of *Pf*CS have been identified in schizont stages and in stage V gametocytes (89-91).

The only apicomplexan CS, which has been characterised in more detail, is *Pf*CS (92). It appears as 60 kDa protein on Western blots and is found in the cytosolic fraction of all parasite blood stage forms. In addition, two bands with a slightly different electrophoretic mobility were detected in the membrane and organelle fraction of the uninuclear ring and trophozoite stages, which may present modified forms of the enzyme in these stages (92). Immunofluorescence analyses revealed a scattered distribution of the enzyme in the parasite cytosol, where it appears to concentrate - at least in part - to distinct foci in contrast to many other cytosolic proteins that are evenly disseminated.

*Pf*CS activity could not be detected in crude extracts of *P. falciparum* lysates. Therefore a recombinant protein has been used to determine enzymatic characteristics of the *P. falciparum* enzyme. CS activity of the recombinant protein was very low compared to the ones of the *E. coli* and *N. crassa* enzymes (92). Despite this fact, it was shown that *Pf*CS does not possess an intrinsic flavin reductase activity and therefore the plasmodial protein was classified as monofunctional similar to plant and bacterial CS. However, this classification awaits further experimental verification using protein with a higher specific activity (see below).

Ehammer *et al.* established a cellular screen to discriminate between mono- and bifunctional CSs. This *in vivo* screen is based on a chorismate synthase-deficient *S. cerivisiae* strain that is unable to grow on medium lacking aromatic amino acids. While bifunctional chorismate synthases are able to complement the CS-deficient strain, monofunctional are only able to rescue growth if the CS-deficient strain is co-complemented with a bacterial NADPH:FMN oxidoreductase (81). Complementation with *Tg*CS restored the growth of the CS-deficient yeast strain in the absence of the NADPH:FMN oxidoreductase albeit clearly less vigorous as other bifunctioncal CSs. *Pf*CS, however, did not confer prototrophy to the CS-deficient yeast strain either in the absence or presence of the NADPH:FMN oxireductase (81). Recombinantly expressed *Tg*CS was shown to utilize NADPH for the reduction of FMN and hence clearly has intrinsic NADPH:FMN oxidoreductase activity as suggested by the complementation assay, which unequivocally classified *Tg*CS as a bifunctional enzyme. The lack of complementation by *Pf*CS was not due to a lack in expression but most likely caused by instability or inactivity of the protein in yeast (81). Interestingly the distribution of bifunctional CSs exhibiting NADPH:FMN oxidoreductase activity concurs in the presence of the pentafunctional AROM complex, since an AROM complex is present in *T. gondii* and *Tetrahymena thermophila* (21, 93). Richards and coworkers suggested that the *arom* gene was inherited vertically from the last common eukaryotic ancestor to fungi and chromoalveolates during evolution (93). This might also be the case for the gene encoding the bifunctional CS. Since *P. falciparum* also belongs to the chromoalveolates it is anticipated that it also possesses a bifunctional CS. It is, however, currently unclear whether *P. falciparum* is an exception to this rule.

4. GENETIC ORGANIZATION AND REGULATION OF THE SHIKIMATE ENZYMES

The genetic organization of the shikimate pathway enzymes varies between organisms. Several modifications occur; however, in this article only the ones affecting larger taxonomic groups are discussed. In bacteria, the seven enzymes of the shikimate pathway are encoded as single monofunctional enzymes, whose genes are spread over the genome (Figure 3) (45). Plants have a similar organization with the exception of 3-dehydroquinate dehydrogenase and shikimate dehydrogenase, which are expressed as a bifunctional

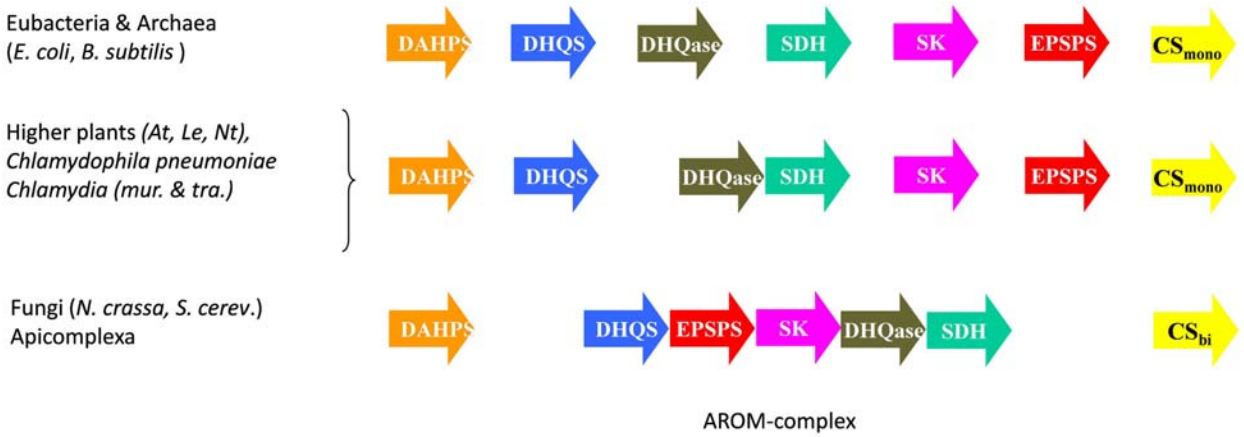

Figure 3. Genetic organization of the shikimate pathway in bacteria (first row), higher plants (second row) and fungi and Apicomplexa (last row). In bacteria, each enzyme is encoded by a single gene. Plants have a similar organization like bacteria except for DHQase and SDH which are fused to a bifunctional enzyme complex. In contrast, the genes of DHQS, EPSPS, SK, SDH and DHQase are fused to a pentafunctionals complex in fungi and certain apicomplexan species.

fusion protein (Figure 3) (94). However, fungi like *N. crassa* and *S. cerevisiae* and certain apicomplexan parasites display a pentafunctional polypeptide, called the AROM complex (21, 95, 96). The AROM complex is a heterodimer that catalyzes the enzymatic steps 2 to 6 of the shikimate pathway thereby converting 3-deoxy-D-*arabino*-heptulosonate-7-phosphate into 5-enolpyruvylshikimate-3-phosphate. The respective enzymatic entities DHQS, DHQase, SDH, SK and EPSPS are present in an altered order: the N-terminus is formed by DHQS, followed by EPSPS, SK, DHQase and finally SDH that is located at the very C-terminus of the polypeptide (Figure 3). In organisms containing an AROM complex, the last enzyme in the shikimate pathway, chorismate synthase, usually displays another interesting feature: it possesses an additional flavin reductase activity, classifying it as bifunctional enzyme, while the CSs of plants and bacteria is monofunctional (see above, (81)).

Since the AROM complex is the organizational structure that was found in several apicomplexan parasites it will be discussed in more detail (21). The domain structure and interaction within the AROM complex was addressed by several studies expressing distinct domains of the *aromA* gene in either prokaryotic or fungal expression systems (31). The DHQS domain can be stably expressed in *E. coli* (97). Only a modest expression of the DHQase domain in *E. coli* has been achieved (98). Attempts to express a monofunctional SDH either in *E. coli* or in *Aspergillus nidulans* failed (31), however, a bifunctional enzyme specifying DHQase and SDH activities was successfully produced in *A. nidulans* indicating that an interaction between these two domains stabilises both activities (31). Attempts to express a monofunctional EPSPS domain in *E. coli* failed, whereas a bifunctional protein consisting of the complete DHQS and the EPSPS domains was produced and enzymatically active, suggesting that EPSPS depends on the DHQS domain in a cis context for its enzymatic activity (31). The emerging picture from these observations suggests that the AROM complex can be divided into two parts functioning independently of each other. One of these parts is composed of DHQS and EPSPS whereas the other one consists of SK, SDH and DHQase (31). There is a substantial interaction between domains within each of the two parts resulting in a stabilization and thus potentially maximisation of the enzymatic activities (31).

The AROM protein has been attributed properties such as metabolic channelling, catalytic facilitation and coordinate regulation (see below) (31). These properties have been further examined by investigating the leakage of metabolites from the AROM complex and by analysing whether there is evidence for a preferential flux of DHQ and DHS through the AROM protein under *in vivo* conditions. To address the leakage of metabolites, an *A. nidulans* strain was constructed lacking the *qutE* encoded DHQase but constitutively expressing the quinate dehydrogenase and the dehydroshikimate dehydratase (for reactions of the pathway see Figure 1). When this strain was complemented with *aromA*, transformants were obtained albeit at a low frequency on quinate-minimal medium containing quinate as the only carbon source, suggesting that the AROM complex converts DHQ from the quinate pathway to DHS that - upon release - is further used in the quinate pathway (99). Thus the AROM complex seems to be leaky *in vivo*. In order to address the extent of leakage from the AROM complex, a mutant strain of *A. nidulans* was constructed that constitutively expressed the quinate dehydroshikimate dehydratase at varying concentration from 0.2 to 30 fold when compared to wild type levels. When this mutant was grown on minimal medium using glycerol as carbon source, augmented activity resulted in enhanced growth impairment, which could be counterbalanced by the addition of aromatic amino acids, indicating that the quinate dehydroshikimate dehydratase feeds a considerable portion of the shikimate pathway DHS into the quinate pathway (99). An n-fold increase in enzyme concentration resulted in an n-fold increase of the quinate pathway end product protocatechuic

acid, indicating that the AROM complex possesses very poor channelling properties. Since high expression levels of quinate dehydroshikimate dehydratase in the range of 12-30 fold over wild-type level did not completely inhibit growth of the above mutant, it has been suggested that the AROM complex has a low channelling function most likely because of the close proximity of the five active sites (31, 99).

While the genetic organisation of the shikimate pathway in *Plasmodium* has been an enigma for many years, it now became obvious that all apicomplexan parasites being equipped with a shikimate pathway metabolic function exhibit the same overall structural organization of the pathway, namely as a pentafunctional AROM complex (21, 56). Genes encoding the AROM complex have been identified in the following apicomplexan parasites: *T. gondii* (gene ID: TGGT1_055170), *N. caninum* (gene ID: NCLIV_053120), *E. tenella* (gene ID: ETH_00015150) and the various plasmodial species (*P. falciparum:* gene ID PF3D7_0206400, *P. vivax*: gene ID: PVX_003750, *P. knowlesi*: gene ID PKH_041350, *P. berghei*: gene ID PBANKA_030400 and *P. chabaudi*: gene ID PCAS_030620). In the case of *E. tenella* only a partial sequence of the *arom* is available encoding *dhqs* and parts of *epsps*, whilst the sequences coding for *sk*, *dhqase* and the *sdh* are missing. Fungal AROM complexes vary in size from about 110 (*A. nidulans*) to roughly 175 kDa (yeast), whilst apicomplexan AROM complexes range in size from 231 kD (*P. chabaudi*) to 362 kD (*T. gondii*) and are thus significantly larger than their fungal orthologs (100-102). This increase in size is mainly due to numerous insertional sequences not found in the fungal counterparts. The AROM complexes of *T. gondii* and *N. caninum* share 64 % amino acid identity and 42.3 % and 38.5 % with the partial *E. tenella* AROM complex. However, no significant homologies exist between the AROM complexes of the cyst-forming coccidians and the plasmodial species. The immense size of the apicomplexan AROM complexes poses a major obstacle for further characterization. Their sheer size turns expression of these proteins into a tremendous challenge and thereby has so far impeded their biochemical characterization.

Regulation of a pathway can occur in numerous ways, *e.g*. by i) transcriptional control through activation/suppression of gene expression (for instance in tissue-specific expression) or by ii) posttranslational controls leading *e.g.* to feedback inhibition through the alteration of the enzymatic activities. The carbon flow through the shikimate pathway itself is either controlled by feedback inhibition or via synthesis of by-products like quinic acid. DAHPS, SK and CS catalyze irreversible steps of the shikimate pathway, whereas EPSPS catalyzes a reversible reaction that favours the formation of products (103). Of these four enzymes, DAHPS and SK are often controlled on transcriptional level and/or by the down-regulation of their enzymatic activities (104).

In gram-negative bacteria, such as *E. coli*, transcription of shikimate pathway genes is controlled by the TrpR and TyrR repressor (105, 106). The first enzyme of the pathway, DAHPS, exists in three distinct versions (*aro*F, *aro*G, *aro*H) (see Table 1), which are repressed by the amino acids phenylalanine, tyrosine and tryptophan (107). Total cellular activity of the three DAHPS is unequally distributed with highest contribution of the Phe-regulated DAHPS aroG, followed by the Tyr-inhibited aroF and only 1 % of enzymatic activity added by the Trp-controlled aroH (108). For the following enzymes DHQS, DHQase and SDH, no significant change in expression could be detected in *E. coli* upon amino acid starvation of auxotrophic mutants (108). In *E. coli*, there exist two isoforms of SK, SK I/AroK and SK II/AroL (see Table 1), which are located on different parts of the bacterial chromosome and also in distinct operons (109-112). Unlike the *aroL* gene, which is controlled by the TrpR and TyrR repressor, the *aroK* gene seems to be constitutively expressed (109, 113). The affinities of the two SKs for shikimate differ significantly and have been determined to 200 μM for SK II and > 20 mM for SK I (57, 114). AroK can substitute for loss of AroL function, however double knockouts are auxotrophic for aromatic amino acids (115). It was suggested that AroL is the major enzyme of the shikimate pathway with AroK having a minor role; however, it should be noted that other bacteria like *H. influenza* and *M. tuberculosis* encode only AroK and no AroL orthologs (116, 117).

Amino acid biosynthesis in yeast is a highly concerted system and is generally controlled by the transcription factor Gcn4 (118, 119). The genes of the shikimate pathway and aromatic amino acid biosynthesis are activated by Gcn4p during amino acid starvation (120, 121). It has been suggested that the organization in a pentafunctional AROM complex allows for a coordinated regulation of the individual enzymatic activities through channelling of reaction intermediates. However the AROM complex displays only a low channelling function *in vivo* suggesting that such a control is not in place (31, 99). Nevertheless, this type of assembly results in a stoichiometric amount of enzymes and therefore alleviates enzymatic control. Similar to *E. coli*, fungal species such as *S. cerivisiae*, *A. nidulans* and *N. crassa* possess DAHPS isogenes that are differentially regulated by Phe, Tyr or Trp (see Table 1) (122-127). *S. cerivisiae* ARO3 is controlled by feedback inhibition of Phe whereas ARO4 is regulated by Tyr (122-125). In addition, both isoenzymes are controlled by tryptophan as an additional effector, however, to a lesser extent than by the actual regulating amino acid (118). Similar to *S. cerivisiae*, *A. nidulans* aroGp is differentially regulated by Phe and aroFp by Tyr (126). In *N. crassa*, three allosterically inhibitable DAHPS have been described responding to Trp, Phe and Tyr (127). However, only the Trp-sensitive DAHPS has been further characterized (128, 129).

In plants, the enzymes of the shikimate and the aromatic amino acids biosynthesis pathways are located in the chloroplast, but there is also evidence for cytosolic reactions (93, 130). Similar to the situation in *E. coli*, the individual plant enzymes of the shikimate pathway are encoded by single genes except for DHQase and SDH which form a bifunctional enzyme complex (94). The shikimate pathway of plants seems to be mostly regulated at the level of gene expression rather than at the

posttranscriptional level in contrast to most microbial species (131). Plant DAHPSs are only weakly affected by aromatic amino acids or their precursor metabolites (131). The weak effects of aromatic amino acids on the plant chloroplast DAHPS, however, suggest that although the enzyme has lost its sensitivity to aromatic amino acids their binding sites are still preserved (131, 132). In general the regulation of the plant shikimate pathway enzymes seems to be more complex than that of the bacterial ones. Although it was thought that regulation primarily occurs at the chorismate branch point, substantial evidence has been obtained that additional control levels exist. For example, EPSPS mRNA was upregulated upon inhibition of histidine biosynthesis, sulphate starvation and in response to infection by a fungal pathogen (133-135). Furthermore, glyphosate treatment caused a several fold increase of DAHPS activity (135, 136). Depending on the species, plants contain two to three DAHPS isozymes (137, 138). In *Arabidopsis*, the *dahps1* gene is induced upon physical wounding, methyljasmonate, infiltration with *Pseudomonas syringae*, the redox state and abscisic acid (132, 138-142). Moreover, redox regulation of *Arabidopsis dahps1* by thioredoxin connects the shikimate pathway carbon flow with the electron flow in photosystem I (132, 138). The bifunctional DHQase-SDH is found at the branch point to quinate biosynthesis for which dehydroquinate and dihydroshikimate serve as substrates (15). In plants, quinate is found as a precursor for chlorogenic acids and as quinate-*p*-coumaryl ester as intermediate in lignin biosynthesis (143, 144). Fusion of DHQase and SDH deprives dihydroshikimate from quinate metabolism and preserves it for the shikimate pathway by direct channelling of the intermediate to the active site of SDH (145). The final product of the complex, shikimate, is then processed by SK. In tomato, only one chloroplast located SK has been isolated, by contrast *Arabidopsis* possesses two isoforms, SKI and SKII (146). Whereas At*sk*2 is mainly expressed in early embryogenesis and in vegetative tissue throughout development, At*sk*1 is near or below background levels in vegetative tissues and is only expressed at higher levels in mature embryos and senescing leaves (146). It has been suggested that SKs act as a regulatory node in plants enabling the flux towards distinct secondary metabolite pools (147). Up-regulation of *sk* transcripts is induced in response to heat stress and recovery (*sk1*), upon inoculation with spores of the oomycete *Phytophthora infestans* (*sk2*) and in response to biotic stresses (147, 148). Moreover, a chloroplast SK from spinach is inhibited by ADP suggesting a dependency of SK activity on the cellular ATP level (149). Whereas only one *EPSPS* has been identified in *Brassica* and *Petunia, Arabidopsis* and tomato contain two alleles (150). In *Petunia*, *EPSPS* expression is developmentally regulated and differentially expressed in different tissues, while in tomato the levels of *EPSPS* transcripts vary only slightly between organs (150).

5. TARGETING THE SHIKIMATE PATHWAY - ENZYMES OF THE SHIKIMATE PATHWAY AS ANTIMICROBIAL AND ANTIPARASITIC DRUG TARGETS

The shikimate pathway has attracted considerable attention as potential drug target for combating diseases, including malaria, tuberculosis and pneumonia (4, 151, 152). The best-known and already commercially used target is EPSPS, which is inhibited by glyphosate (3), a herbicide marketed by Monsanto as RoundUp®. Research on inhibitors of shikimate pathway enzymes is a very active field in particular with regard to novel antimycobacterial agents (4). In the following chapter, compounds acting on shikimate pathway enzymes and their potential mechanism of action will be described in more detail. To prevent possible confusion, please note that the numbering of compounds refers to the numbering in the original publications to facilitate locating of compounds therein.

5.1. 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase

The first committed step in the shikimate pathway is the stereospecific aldol reaction between PEP and E4P resulting in the production of DAHP and inorganic phosphate. With the exception of one study by Grison and coworkers in 2005 (153), to the best of our knowledge all other reports on DAHPS inhibitors are from the laboratory of Emily Parker, University of Canterbury, Christchurch, New Zealand (154-157). Grison and coworkers designed compounds where the C-O phosphate bond of the aldose phosphate was replaced by a stable C-C link (see *e.g.* compound 8e/8'e). These compounds were tested for their effect on bacterial growth. *S. aureus* was used as a member of gram-positive bacteria and *E. coli*, *Pseudomonas aeruginosa* and *Yersinia enterocolitica* as representatives of gram-negative bacteria. Growth of all bacterial strains was affected by compound 8e/8′e, which was synthesised from D-arabinose as a precursor and it was suggested that the observed inhibition occurs through the inhibition of DAHPS (Figure 4) (153). While there is little information on DAHPS inhibitors, research on inhibitors of the 3-deoxy-D-*manno*-2-octulosonate 8-phosphate synthase (KDOPS), an enzyme involved in bacterial cell wall biosynthesis that catalyzes a similar aldol-like reaction as DAHPS, is far more active. Despite the fact that the mechanisms used to activate their aldose substrates are different between DAHPS (substrate: E4P) and KDOPS (substrate: arabinose 5-phosphate), both reactions involve a stereospecific attack of the *si* face of PEP on the *re* face of the aldehyde of the aldose substrate resulting in the cleavage of the C-O bond of the PEP phosphate group (158-160). This prompted Walker and coworkers to synthesize compound **8** as an analogue of inhibitors **5** and **6** of KDOPS (see Figure 4; DAHPS inhibitor **(8)** not to be confused with compound 8e/8'e). Compound **8** is a very slow binding inhibitor and has an IC_{50} on *E. coli* DAHPS of 6.6 μM (156). The slow formation rate of the inhibitor substrate complex relative to substrate consumption did not allow for the determination of the inhibition constant (156). In a second approach, Walker and coworkers synthesized substrate analogues of PEP that mimic the PEP-portion of the proposed oxocarbenium intermediate (compounds **4** and **5**) and enantiomeric phospholactates (compounds (*R*)-**6** and (*S*)-**6**) mimicking the proposed phosphohemiketal reaction intermediate (157). All inhibitors were found to be competitive inhibitors of the DAHPS with respect to PEP. The vinyl phosphonates **4** and **5** exhibited the highest inhibition constants with 4.7 and 8.8 μM, respectively. With regard to the phospholactones, their stereochemistry

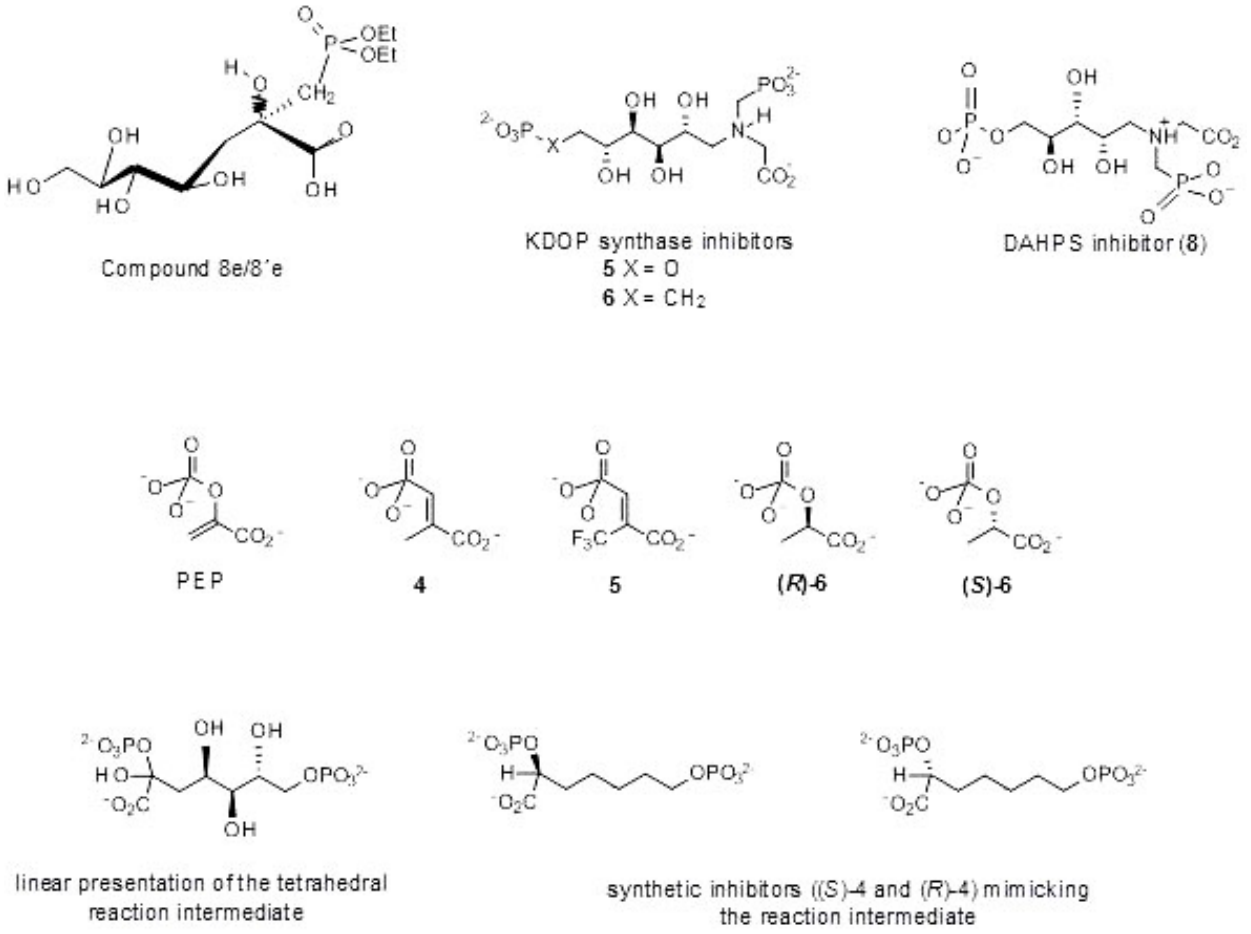

Figure 4. DAHPS inhibitors. Numbering of the compounds refers to the numbering in the original publications. Consult text for further information

had a strong effect on their inhibitory potential with the (*R*)-form being a 10-fold better inhibitor (*K*i of 49 μM) than the (*S*)-form (*K*i of 670 μM). In a follow up study, inhibitors targeting the PEP binding site were extended in order to assess the phosphate-binding site of E4P. However, only a small increase in the inhibitory potential was observed for these dual site inhibitors (155). The attack of an active site water of DAHPS on the central carbon of PEP is expected to generate a tetrahedral reaction intermediate although both the substrate PEP and the product DAHP possess planar geometry at C2 (154). By combining mechanism based design with molecular modelling of a tetrahedral reaction intermediate into the active site of *M. tuberculosis* DAHPS, Reichau and coworkers designed and synthesised inhibitors mimicking this intermediate (Figure 4, (*R*)-4 and (*S*)-4)). (*R*)-4 and (*S*)-4 are the first potent inhibitors of *Mt*DAHPS with K_i values of 360 and 620 nM, respectively (154).

As far as we know, DAHPS inhibitors have not been tested for growth inhibition of apicomplexan parasites.

5.2. Dehydroquinate synthase

Dehydroquinate synthase catalyzes the conversion of DAHP to 3-dehydroquinate (see Figure 1). The early steps of this reaction have been probed with a series of substrate analogues that were supposed to structurally prevent the β-elimination of inorganic phosphate, the committed step in the conversion of DAHP to DHQ (161). One of these compounds, carbaphosphonate (CBP), turned out to be a very potent inhibitor of DHQS with a K_i value of 0.8 nM (Figure 5) (161). CBP is a competitive, but slow binding inhibitor (161, 162). In a follow up study, Montchamp and Frost incorporated strategically placed double bonds in cyclohexyl inhibitors of DHQS to improve their efficacy (161, 162). All cyclohexenyl inhibitors investigated were slowly-reversible inhibitors with K_i values in the nano- and subnanomolar range and had a higher inhibitory activity than their cyclohexyl analogues (162). The cyclohexenyl analogue of CBP, cyclohexenyl phosphate, turned out to be the most potent inhibitor of DHQS with a K_i of 0.12 nM. In addition, one compound of the cyclohexenyl series, cyclohexenyl tricarboxylate, is the first example for an inhibitor of

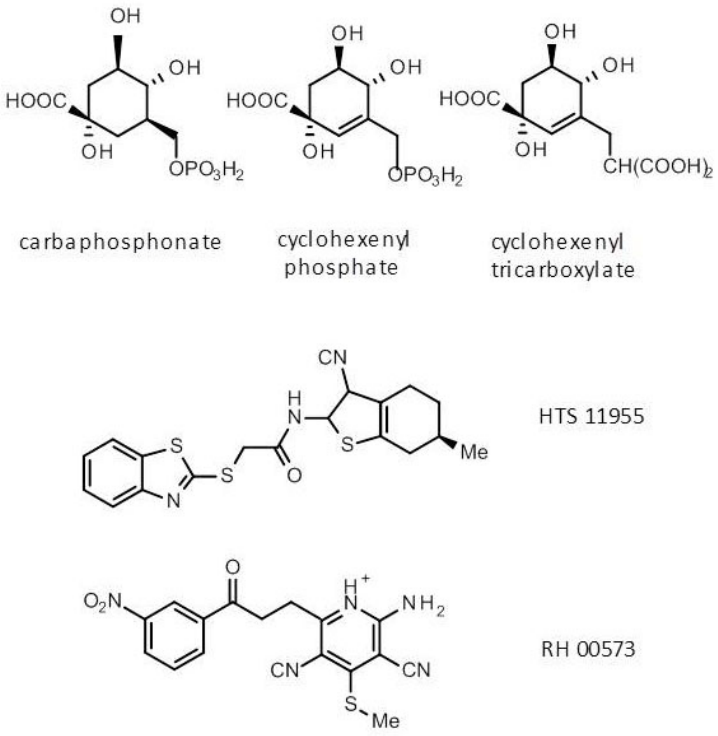

Figure 5. DHQS inhibitors. Consult text for further information.

DHQS in the low nM range that lacks a phosphonic ester or phosphate monoester group (162). Virtual screening of the Maybridge database has been applied in a structure-based approach to identify inhibitors of *H. pylori* DHQS. IC_{50} values of the two best compounds, HTS 11955 and RH 00573 were in the lower µM range (Figure 5) (163).

As before, none of these DHQS inhibitors have been tested concerning their potential to inhibit the growth of apicomplexan parasites.

5.3. Dehydroquinase

Type I and type II dehydroquinases catalyze the dehydration of 3-dehydroquinate to 3-dehydroshikimate by different mechanisms. Accordingly, inhibitors for each type were identified either through enzyme mechanistic studies or by inhibitor screening (28). As apicomplexan parasites contain a classical pentafunctional AROM complex with a type I DHQase, only type I DHQase inhibitors will be discussed here (21). In type I DHQases, dehydration of DHQ proceeds via imine formation with a highly conserved active-site lysine, Lys 170 (26, 164). Crystal structures of *Salmonella enterica* and *Clostridium difficile* type I DHQases provided crucial insight into the reaction indicating that an active site histidine assumes different catalytic roles in the formation as well as in the hydrolysis of the covalent Schiff base intermediates and in catalytic dehydration (165).

Bugg and coworkers identified substrate analogues of DHQ that irreversibly inhibit *E. coli* DHQase, which were based on a cylcohexane ring bearing an electrophile, a carboxylate and a ketone functionality (166). Active compounds of these series were an epoxide, a chloromethylketone and an azide (Figure 6 (24), (25) and (27), respectively). All three compounds irreversibly inhibited *E. coli* DHQase with K_i values of 400 µM, 680 µM and 1.1 mM at a maximal rate of inhibition (k_i) of 2.5 x 10^{-3} s^{-1}, 5.6 x 10^{-4} s^{-1} and 3.9 x 10^{-4} s^{-1}, respectively (166). Inhibition by these compounds was significantly impaired or completely abolished by addition of the substrate, marking these compounds as competitive inhibitors of the enzyme (166).

Another possible strategy for the inhibition of type I DHQases was identified through the functional analysis of a surface loop of *S. enterica* type I dehydroquinase, which closes over the active site upon substrate binding (167). Closure of this loop is functionally important for catalysis. It is assumed to assist substrate binding through direct interaction and to provoke a conformational change of Arg213 that thereby adopts its substrate binding position (167). Compounds stabilising the open configuration or destabilising the closed state, thus slowing down enzymatic activity may work as effective allosteric inhibitors of type I dehydroquinase (167).

All of these DHQase inhibitors are still waiting for being tested for their effect on apicomplexan parasites.

5.4. Shikimate dehydrogenase

AroE has been determined as a promising target for the discovery of novel antimicrobial agents. However,

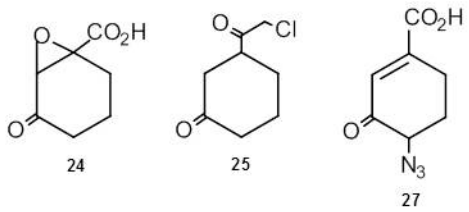

Figure 6. DHQase inhibitors. Consult text for further information.

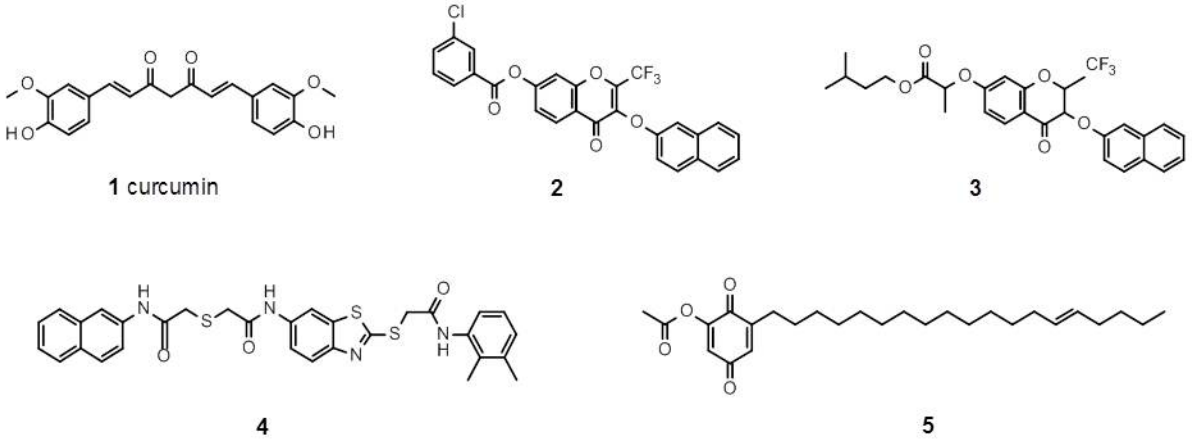

Figure 7. SDH inhibitors. Consult text for further information.

given the diversity of SDHs, the precise extent to which other SDHs may compensate for AroE loss of function has to be clarified. The potential ability of additional SDHs in the genome of a given pathogen to complement for loss of AroE function might render a chemotherapeutic approach that targets this enzyme largely ineffective and impractical (40). Thus the set of SDHs present in a given pathogen as well as their enzymatic characteristic with respect to substrate specificities and affinities need to be established first in order to allow a conclusive statement on whether such an approach is promising.

As far as we know there is only one report on SDH inhibitors, where the *aroE* gene encoded SDH from *H. pylori* was used in a high throughput screening. This approach resulted in the identification of five novel *Hp*SDH inhibitors: curcumin (**1**), 3-(2-naphthyloxy)-4-oxo-2-(trifluoromethyl)-4*H*-chromen-7-yl 3-chlorobenzoate (**2**), butyl 2-{[3-(2-naphthyloxy)-4-oxo-2-(trifluoromethyl)-4*H*-chromen-7-yl]oxy}propanoate (**3**), 2-({2-[(2-{[2-(2,3-dimethylanilino)-2-oxoethyl]sulfanyl}-1,3-benzothiazol-6-yl)amino]-2 oxoethyl}sulfanyl)-N-(2-naphthyl)acetamide (**4**), and maesaquinone diacetate (**5**) exhibiting IC_{50} values on *Hp*SDH of the 15.4, 3.9, 13.4 and 3.5 µM, respectively (168). For the chemical structure of the compounds consult Figure 7.

Compound **4** is a competitive inhibitor of the substrate shikimate and compounds **2** and **3** competitive inhibitors with respect to NADP. Compounds **1** and **5** did not show competitive inhibition with shikimate or NADP, suggesting that they are not competitive inhibitors of the enzyme (168). Of the five compounds only substances **1**, **2** and **5** had a moderate effect of *H. pylori* growth with MIC values of 16, 16 and 32 µg/mL, respectively, whereas compounds **3** and **4** did not show any significant inhibition (168). Curcumin is considered as the most active constituent of the perennial herb *Curcuma longa*, which is a common spice in curries of South Asian and Middle Eastern cuisine. In a second study, its effect on 65 clinical *H. pylori* isolates from Kolkata was investigated resulting in MIC variations ranging from 5 to 50 µg/mL (169). Furthermore, its efficacy in reducing gastric damage was histologically investigated in a mouse system. Curcumin was highly effective in restoring the *H. pylori* induced gastric damage and in the eradication of *H. pylori* from infected mice (169). Whether the observed effects are indeed caused by an inhibition of *Hp*SDH remains to be established. It has to be mentioned that curcumin has pleiotropic effects ranging from antitumor, to anti-inflammatory and antiinfectious activities, which in part are caused by the inhibition of the transcription factor NF-κB (170–172).

Again to the best of our knowledge, SDH inhibitors have not been tested for their effect on apicomplexan parasites.

5.5. Shikimate kinase

SK catalyzes the transfer of a phosphate group from ATP to shikimate resulting in the production of shikimate 3-phosphate and ADP. SK is recognized as a promising antibacterial target and was subject of extensive investigations. Despite the fact that functional and structural studies of SKs from several bacteria have provided deep insight into ligand binding and catalysis,

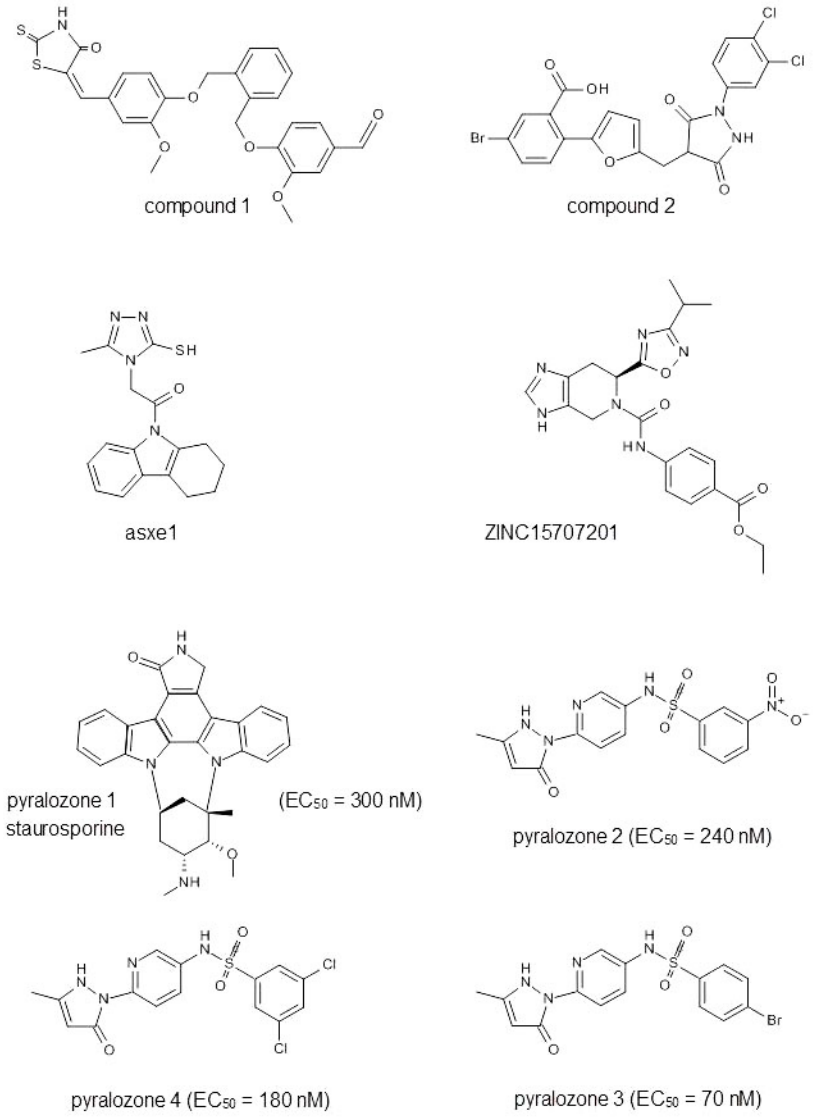

Figure 8. SK inhibitors. Consult text for further information.

only a very limited number of SK inhibitors are available to date of which most were identified by molecular docking simulations. Furthermore, only a few compounds were tested on the enzyme and for their potential to inhibit bacterial growth. It is quite obvious that the paucity of information on SK inhibitors does not reflect the real situation. A possible explanation for this might be that compounds active against SK are retained by pharmaceutical companies for reasons of intellectual property rights. Evidence for this assumption is provided by a publication from the Novartis background addressing the elimination of nonstoichiometric enzyme inhibitors from high throughput screening (HTS) hit lists where *Mt*SK was assessed as one of the enzyme targets (173).

Using HTS, Han and collaborators identified two inhibitors against *H. pylori* SK: compound **1** (3-methoxy-4-{[2-({2-methoxy-4-[(4-oxo-2-thioxo-1,3-thiazolidin-5-ylidene)methyl]phenoxy}methyl)benzyl]oxy}benzaldehyde) and compound **2** (5-bromo-2-(5-{[1-(3,4-dichlorophenyl)-3,5-dioxo-4-pyrazolidinylidene]methyl}-2-furyl)benzoic acid) (Figure 8) (174). Both compounds inhibited bacterial growth with an IC_{50} of 5.5 and 6.4 μM, respectively. Whereas compound **1** is an uncompetitive inhibitor for both substrates shikimate and MgATP exhibiting a K_i of 9.48 μM, compound **2** is a competitive inhibitor for shikimate and a noncompetitive inhibitor for its second substrate with a K_i of 2.19 μM (174). The binding site of *Hp*SK is composed of three sub-pockets: the short arm, the long arm and the corner of the L-shaped surface channel. Docking of compound **1** and **2** into the binding pocket of *Hp*SK revealed that compound **1** is supposed to bind to the corner sub-pocket and compound **2** to the short arm of the binding channel explaining their mode of actions. Binding to the corner sub-pocket has no effect on shikimate or MgATP binding but is supposed to abolish the transfer of the

phosphate from MgATP to shikimate. Binding to the short arm, the binding site of shikimate, results in a competition with shikimate (174).

Although SK of *M. tuberculosis* is a validated drug target, since it is essential for its survival (116), to our knowledge no inhibitors have been reported that are effective on *M. tuberculosis* growth. Nevertheless, there have been a few reports on compounds being inhibitory or potentially inhibitory for *Mt*SK. With the help of ultrafiltration-liquid chromatography/mass spectrometry ligand assays, Mulabagal and Calderón were able to identify four pyrazolones including staurosporine (known as a non-selective "protein kinase" inhibitor) effective against *Mt*SK with half maximum effective concentrations in the nanomolar range of 70, 180, 240 and 300 nM (Figure 8) (175). Further, crystallographic information was used to assist molecular docking simulations for the drug-discovery process (176-178). Structure based virtual screening by Segura-Cabrera and Rodríguez-Pérez resulted in 644 putative hits, which were mainly triazole/tetrazole heteroaromatic systems (178). The most potent compound asxe1 had an eHTS score of -7.252 (see Figure 8) (178). Molecular docking simulations performed by Vianna and Azevedo resulted in 20 selected molecules of which nine including staurosporine matched the Lipinski's role of five (176). One of the top-scoring compounds, ZINC 15707201, is presented in Figure 8. Furthermore, a dipeptide inhibitor (RD) was identified in an *in silico* structure-based approach with a predicted binding affinity of 5.5 nM, being 8000 times better than the substrate shikimate (177).

SDH inhibitors were not tested for their effect on apicomplexan parasites. However, it has to be stated that staurosporine inhibits *P. falciparum* growth with IC_{50} values in the low nanomolar range (179). Whether this observed effect is indeed caused by the inhibition of *Pf*SDH or by parasite protein kinases remains to be established.

5.6. 5-Enolpyruvylshikimate-3-phosphate synthase

EPSPS catalyzes the condensation of shikimate 3-phosphate and PEP to 5-enolpyruvylshikimate-3-phosphate. EPSPS is considered as an attractive target for the development of novel antibiotics. For instance, virulence of *S. aureus*, *S. pneumonia* and *Bordetella bronchiseptica* is impaired in knockouts where the *epsps* gene has been deleted (180-182). In addition, many bacteria such as *M. tuberculosis*, *P. aeruginosa*, *Vibrio cholera* and *Y. pestis* rely on the shikimate pathway for the production of salicylate, a precursor in siderophore biosynthesis, for pathogenicity (183, 184). Moreover, glyphosate, a herbicide and the active constituent of Roundup®, which specifically targets EPSPS, inhibits the growth of the apicomplexan parasites *T. gondii*, *P. falciparum* and *C. parvum,* albeit its antiparasitic properties are very poor and IC_{90} values were in the low millimolar range (1). It is notable that genome sequencing of *C. hominis, muris* or *parvum* revealed no genetic evidence for the presence of an *epsps* gene in these parasite species suggesting that the shikimate pathway may not be present. Inhibition by glyphosate may thus be due to the effect on another so far unknown target.

Glyphosate, the prototype inhibitor of EPSPS, is a potent and specific inhibitor of EPSPS and a valuable lead compound in the search for novel antimicrobial drugs and herbicides (3, 68, 185). Glyphosate binds to the PEP binding site of EPSPS and mimicks an intermediate state of the ternary enzyme-substrate complex (68, 185). Despite the fact that the mode of action of glyphosate on EPSPS is well understood, intense efforts to identify compounds with a better efficacy than glyphosate on type I EPSPSs have largely failed (186, 187). Analogues of the tetrahedral reaction intermediate (TI) have been synthesized substituting the labile ketal phosphate moiety by phosphonate or stabilizing the ketal phosphate by introducing fluorine substituents (188). Despite these efforts, only a few analogues with a higher potency than glyphosate were identified so far (Figure 9) (188-190). The most potent inhibitors are the (*R*) enantiomeric form of the difluoromethyl derivative exhibiting a K_i of 4 nM on the *Petunia hybrida* EPSPS, followed by the (*R*)-phosphonate TI analogue and the (*S*) and (*R*)-trifluoromethyl TI analogues with K_i values of 15, 26 and 32 nM, respectively (188). The phosphonate TI analogues exhibited a pronounced stereoselective effect with the (*S*)-enantiomeric form having a roughly 1000-fold higher K_i than the (*R*)-form (188). This finding was even more puzzling, since the configuration of the (*S*)-phosphonate corresponds to that of the virtual TI and molecular docking experiments failed to explain the stereoselective effect. Structure resolution revealed that the (*R*)-form causes a conformational change of the strictly conserved Arg124 and Glu314 residues in the active site thereby inducing substantial changes in the amino-terminal globular domain of the protein (190). The authors suggested that the conformational flexibility of EPSPS might promote the tight binding of structurally diverse ligands and that, in case of the (*R*)-phosphonate, structural changes occurring during the open-closed transition of EPSPS are modified as a result of the inhibitor action (190). It is worthy to note that most inhibitor studies concentrated on type I EPSPSs. To our knowledge the only report on inhibitor studies with type II enzymes were by Funke and collaborators (66). Using class II EPSPSs from *S. aureus* and *Agrobacterium* spec. strain CP4, these authors could prove that class II EPSPSs are in general 400 times less susceptible to inhibition by TI analogues. The conformational change of active site residues determined upon inhibitor binding to the type I EPSPS of *E. coli*, was not observed in the class II enzyme of *Agrobacterium* (66). It seems that the active site of class II EPSPSs do not possess the same flexibility to accommodate TI analogues as do class I enzymes and that the analogues are therefore forced to undergo conformational changes leading to less favourable inhibitory activities (66).

5.7 Chorismate synthase

CS catalyzes the elimination of a hydrogen and phosphate group from 5-enolpyruvylshikimate-3-phosphate to yield chorismate (see Figure 1). Based on the knowledge that the *C*-6 pro-*R* hydrogen is lost during formation of chorismate from EPSP, Balasubramanian and coworkers

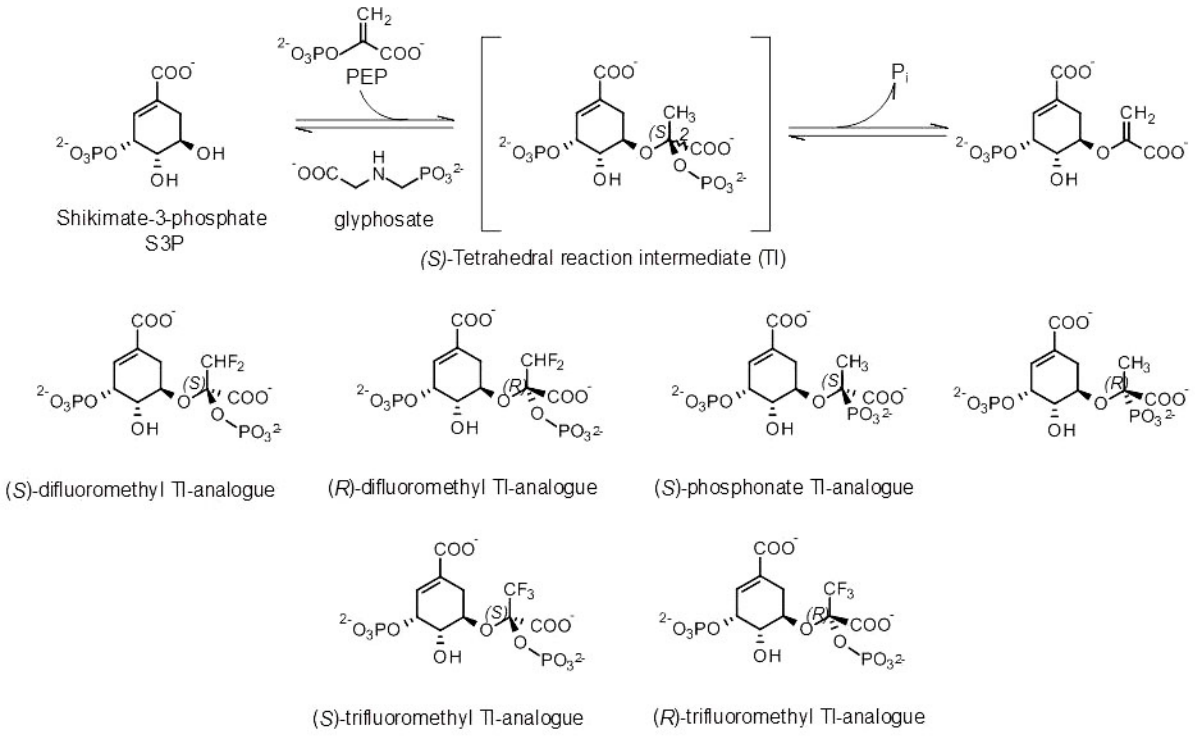

Figure 9.: EPSPS inhibitors. The upper part of the Figure displays the reaction mechanisms with the tetrahedral reaction intermediate (TI). The lower part shows analogues of the tetrahedral reaction intermediate that are described in the text. Consult text for further information.

designed shikimic acid analogs in which each of the hydrogens on C6 was replaced by fluorine (191) (Figure 10).

As anticipated both substances were substrates of *E. coli* shikimate kinase and EPSP synthase and were converted into the diastereoisomeric 6-fluoro-EPSP derivatives (192). The compound in which the *C*-6 pro-*R* hydrogen was replaced by fluorine was found to bind to the monofunctional *E. coli* chorismate synthase *in vitro*, where it caused oxidation of the enzyme-bound reduced flavin. It was not further converted, since there was neither a release of phosphate nor chorismate or other products (192). As expected from the reaction mechanism, the compound in which the *C*-6 pro-*S* is substituted by fluorine is transformed into 2-fluoro-chorismate, however, at a rate being 2 orders of magnitude slower than that of the normal substrate (192, 193). Interestingly, (6*S*)-6-fluoro-EPSP and (6*R*)-6-fluoro-EPSP did not exhibit the same mode of inhibition on the bifunctional *N. crassa* CS. Here, both fluoro-substituted EPSPs where found to exhibit a clear competitive inhibition with the CS substrate EPSP and where shown to lack any irreversible modification of *N. crassa* CS, since no loss of enzyme activity was observed in the presence of inhibitor over a time period of 1 h (191). While the *E. coli* enzyme was considerably more affected by (6*R*)-6-fluoro-EPSP, the *N. crassa* CS was more severely inhibited by (6*S*)-6-fluoro-EPSP (K_i of about 0.2 µM for (6*S*)-6-fluoro-EPSP and 3.0 µM (6*R*)-6-fluoro-EPSP) (191). These findings suggest that mono- and bifunctional CSs can also be distinguished on the basis of their inhibition mode by the two fluoro-EPSP analogs. In light of this, it would be interesting to see which kind of inhibition these inhibitors exhibit on *Pf*CS.

When tested for antibacterial growth inhibition, the two stereoisomers of 6-fluoroshikimic acid showed a pronounced enantiomeric effect with the (6*S*) being at least 125 fold more effective than (6*R*) indicating that the antimicrobial activity of (6*S*)-6-fluoroshikimic acid is not mediated by the inhibition of chorismate synthase itself but rather by the inhibition of a downstream enzyme utilizing 2-fluorochorismate (192). Bulloch *et al.* were able to show that 2-fluorochorismate is a competitive inhibitor for chorismate of the 4-amino-4-deoxychorismate synthase (ADC) inhibiting the enzyme with a k_i of 1.0 min^{-1} and K_I of 130 µM. 2-fluorochorismate irreversibly modifies the chorismate aminating subunit PabA of ADC at lysine 274 (194).

When tested on *P. falciparum*, (6*R*)-6-fluoro-shikimic acid was a more potent inhibitor of parasite growth than (6*S*)-6-fluoro-shikimic acid with IC_{50} of 15 µM and 270 µM, respectively (152). Provided that (as outlined above) the two enantiomeric forms of 6-fluoro-shikimic acid can indeed be utilized to distinguish between mono- and bifunctional CSs, the higher sensitivity of *P. falciparum* towards (6*R*)-6-fluoro-shikimate may suggest that *Pf*CS is indeed a bifunctional enzyme. McRobert *et al.* further tested an expanded set of fluorinated shikimate

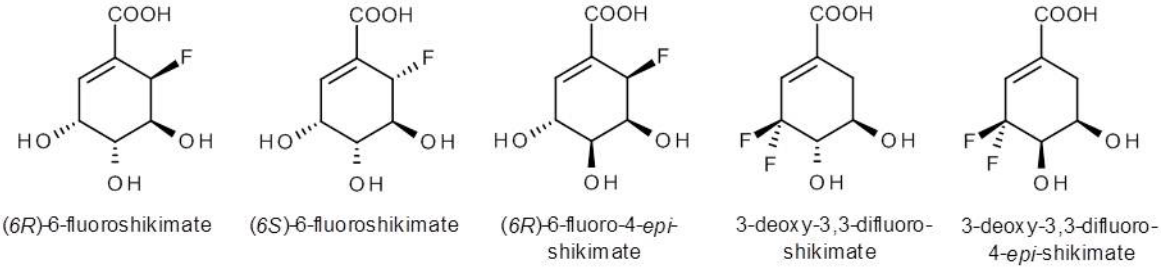

Figure 10. CS inhibitors. Consult text for further information.

analogs, which consisted of a stereoisomer of (6*R*)-6-fluoro-shikimate, namely (6*R*)-6-fluoro-4-epi-shikimic acid with the C-4 OH group occupying the opposite plane, 3-desoxy-3,3-difluoro-shikimate and its stereoisomer 3-desoxy-3,3-difluoro-4-epi-shikimate and 2-fluoroshikimate (Figure 10) (195). Based on their potency on parasite growth inhibition, the following ranking in effectiveness was made by the authors: 6*R* > 6*S* > 6*R*-4-epi > 3,3 difluoro-4-epi > 3,3 difluoro > 2-fluoro-shikimate with IC_{50} values of 15, 270, 360, 810 and 750 μM, respectively (an IC_{50} value for 2-fluoro-shikimate was not provided by the authors). Further, the IC_{50} value for (6*R*)-6-fluoro-4-epi-shikimic acid given in the publication (0.36 μM) was corrected according to the statement in the text that its IC_{50} value is 12-fold higher than that of (6*R*)-6-fluoro-shikimate) (152, 195). When tested in combination with atovaquone, a competitive inhibitor of the quinol oxidation site of the mitochondrial cytochrome bc1 complex resulting in a collapse of the mitochondrial membrane potential, the four shikimate analogs 6*R*, 6*R*-4-epi, 3,3 difluoro and 3,3 difluoro-4-epi-shikimate exhibited an synergistic effect (196-198). However, when tested in combination with pyrimethamine, an antimalarial drug acting on the parasitic dihydrofolate reductase (199, 200), only (6*R*)-6-fluoro-shikimic acid and (6*R*)-6-fluoro-4-epi-shikimic acid had an additive effect on the effectiveness of pyrimethamine, whereas (6*S*)-6-fluoro-shikimic acid did not enhance pyrimethamine inhibition (again it has to be noted that the data of Table 1, which is cited in this context, contradicts the statement of the authors within the text of the publication according to which only 6*R*)-6-fluoro-shikimic acid had an additive effect (195)).

6. CONCLUSION

The appearance of the shikimate pathway in apicomplexan species concurs with their phylogenetic grouping (201, 202). It is found in the cyst-forming coccidians represented by *T. gondii*, *E. tenella* and *N. caninum*, which fall into one group, and the haemosporidians represented by the *Plasmodium* lineage. Surprisingly, no signs for the shikimate pathway have been found in the sister group of the haemosporidians, the piroplasmids, represented by *Babesia* and *Theileria* species (201). Further, it seems to be missing in the *Cryptosporidium* lineage that is basal to apicomplexan and separate from coccidia (201). The organization of the apicomplexan shikimate pathway is similar to that found in fungal species and is characterized by the presence of monofunctional DAHPS, bifunctional CS and the fusion of DQHS, EPSPS, SK, DHQase and SDH to a pentafunctional AROM complex. There are significant differences between the shikimate pathway enzymes of the cyst-forming coccidians and the plasmodial lineage: (i) Whereas the former contain gene sequences coding for DAHPS, the *dahps* genes of the latter escaped identification either because they are too diverged not allowing their detection by common biocomputational applications or because they are simply not present, although DAHPS activity has been observed in crude extracts of *P. falciparum* (22). (ii) The AROM complexes of the cyst-forming coccidians and the haemosporidians differ significantly, which is already demonstrated by the lack of any meaningful sequence similarity. Whilst the AROM complexes of the cyst-forming coccidians contain typical signatures characteristic for all five AROM enzymatic entities, DHQase and SDH escaped reliable identification in the *Plasmodium* lineage. (iii) Further, the DHQSs of the cyst-forming coccidians contain the catalytic histidines being essential for catalytic activity, which are absent from the plasmodial counterparts (5) and (iv) *Tg*CS truly is a bifunctional enzyme as demonstrated in the above described complementation assay. *Pf*CS was not able to complement loss of CS function and thus its true nature awaits further clarification. In summary, it can be stated that the organization of the shikimate pathway in the cyst-forming coccidians is akin to that in fungal species. The shikimate pathway of haemosporidians, however, seems to be far more divergent. Up to now it is still unsettled whether the plasmodial lineage really contains a fully functional shikimate pathway. However, we have clear indications that the AROM complex is essential for *Plasmodium* survival. Given that this also holds true for the cyst-forming coccidians *T. gondii*, *E. tenella* and *N. caninum*, the shikimate pathway may present an attractive target for drugs directed against these two coccidian groups. Whether there will be a "block-buster" effective against both phylogenetic lineages may be doubted, since the differences are quite pronounced. However, as far as there is no detailed characterization of coccidian shikimate pathway enzymes, it can also no be excluded. The most promising candidate for such an efficient drug might be the highly conserved CS.

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Abbreviations: 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (DAHPS), deydroquinate synthase (DHQS), dehydroquinase (DHQase), shikimate dehydrogenase (SDH), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), chorismate synthase (CS), 3-deoxy-D-manno-2-octulosonate 8-phosphate synthase (KDOPS), 4-amino-4-deoxychorismate synthase (ADC), phosphoenolpyruvate (PEP), erythrose 4-phosphate (E4P), 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP), dehydroquinate (DHQ), dehydroshikimate (DHS), shikimate-3-phopshate (S3P), 5-enolpyruvylshikimate-3-phosphate (EPSP), carbaphosphonate (CBP)

Key Words: Shikimate pathway, AROM complex, *Plasmodium*, Apicomplexan, Drug target, Review

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