Specific Chemical Modification of Bacterial Type I Dehydroquinase – Opportunities for Drug Discovery

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Abstract

Type I Dehydroquinase (DHQ1) is a class I aldolase enzyme that catalyzes the reversible dehydration of 3-dehydroquinic acid to form 3-dehydroshikimic acid by multi-step mechanism that involves the formation of Schiff base species'. DHQ1 is present in plants and several bacterial sources but it does not have any counterpart in human cells. It has been suggested that DHQ1 may act as a virulence factor _in vivo_ and therefore a promising target in the search for new anti-virulence agents to combat widespread antibiotic resistance. This review covers recent progress in the structure-based design and chemical modifications caused by selective irreversible inhibitors. Computational studies aimed at understanding the experimentally obtained covalent modifications and inhibitory potencies of these inhibitors are also described.

The challenge of antibiotic resistance – an overview of the problem

The discovery and subsequent development of penicillin was one of the major achievements in medicine in the 20th century and this marked the beginning of the 'golden age' of antibiotic therapy. After the discovery of penicillin, a wide therapeutic arsenal was developed to combat various infectious diseases that had previously been potentially fatal. Unfortunately, the optimism associated with this success also led to the misconception that antibiotics were 'miraculous drugs' with 'power' that largely exceeded their real pharmacological properties. In most European countries, antibiotics are the second most widely used class of drugs after simple analgesics. The excessive use of these drugs, and in many cases their inappropriate use, in medicine, veterinary medicine and agriculture has led to a rapid and ever-increasing emergence of strains that are resistant to antibiotics. Today, infectious diseases caused by the development of drug resistance have become one of the most important public health issues of the early 21st century [1−5]. Severe hospital- and community-acquired bacterial infections are rising sharply, with antibiotic-resistant nosocomial infections being a major concern [6,7]. Antibacterial drug resistance also has a huge economic and social impact [8,9]. For example, the European Centre for Disease Prevention and Control (ECDC) estimates that each year antimicrobial resistance results in 25,000 deaths and related costs of over 1.5 billion € in healthcare expenses and productivity losses. Despite the recognized need for new antibiotics, the investment in antibiotic research and development by the major pharmaceutical companies has declined dramatically in the last 40 years [10]. Therefore, if we do not want to return to the pre-antibiotic era, it is necessary to develop alternative therapies, in particular drugs with new mechanisms of action, and to identify unexplored bacterial targets and gain a detailed knowledge of the catalytic mechanism and/or the binding determinants of those targets to combat the growth of antibiotic-resistant bacteria [11−13].

Anti-virulence therapies – an alternative

The most widely used strategy to combat bacterial infections is based on the disruption of their viability by preventing the synthesis and assembly of key components for bacterial survival [14]. Almost all clinically used antibiotics inhibit cell wall biosynthesis, DNA replication, RNA transcription, folate biosynthesis, or protein biosynthesis by blocking fewer than twenty-five targets [1]. Although this strategy is highly effective, it causes substantial stress to the bacterium and this favors the growing emergence of antibiotic-resistant strains [15]. Therefore, in addition to the discovery of novel compounds to target bacterial viability, in recent years a great deal of effort has been devoted to the development of alternative therapies that target bacterial virulence, i.e., the

ability of a pathogen to cause disease [14−22]. The attenuation of bacterial virulence makes the bacterium less able to establish successful infection and, in consequence, it is cleared by the host immune response [14, 15]. Therefore, anti-virulence drugs will disarm bacteria and will create an *in vivo* scenario similar to that achieved by vaccination with a live attenuated strain. Such a strategy might lessen the pressure for the development of resistance as most virulence traits are not essential for bacterial survival. In principle, these types of drugs will also preserve the gut microbiota.

Type I dehydroquinase – an anti-virulence target

Among the enzymes that may be promising targets in the search for new anti-virulence agents to combat resistant bacteria it is important to highlight type I Dehydroquinase (DHQ1). It has been suggested that this enzyme may act as a virulence factor *in vivo* as the deletion of the *aroD* gene, which encodes DHQ1 from *Salmonella typhi* and *Shigella flexneri*, has been proven to afford satisfactory live oral vaccines, with the latter providing monkeys with protection against oral challenge with live *S. flexneri* 2457T [23−28]. The important features of the DHQ1 enzyme have not gone unnoticed by the scientific community and a great deal of effort has been focused on the development of inhibitors of this enzyme. The structure-based design of inhibitors of this challenging enzyme has undoubtedly benefited significantly from extensive mechanistic and biochemical studies [29], the increasing availability of DHQ1 crystal structures from different bacterial sources, as well as from the recent advances in computational methods that have consolidated their value as important complementary tools that can assist in providing a detailed knowledge of the enzymatic mechanism, the enzyme binding requirements and the essential motions required for catalysis. This review will provide an overview of the recent achievements related to the irreversible inhibition (covalent modification) of the DHQ1 enzyme. A detailed view of the substrate binding requirements and the essential motion for catalysis is also described. Particular attention will be focused on explaining the chemical modifications caused by these inhibitors.

Dehydroquinase (3-dehydroquinate dehydratase, DHQ, EC 4.2.1.10) is the third enzyme in the shikimic acid pathway. This route involves seven enzymes that catalyze the sequential conversion of erythrose-4-phosphate and phosphoenolpyruvate to chorismic acid (Figure 1) [29]. The enzymes involved in the shikimic acid pathway are present in bacteria, fungi and higher plants, but they are absent in mammals. Some of these enzymes have also been detected in *Toxoplasma gondii* (which causes malaria) and *Plasmodium falciparum* extracts [30−32].

DHQ catalyzes the reversible dehydration of 3-dehydroquinic acid (**1**) to form 3-dehydroshikimic acid (**2**) [29,33]. This reaction is part of two metabolic pathways: the biosynthetic shikimic acid

pathway, which leads to chorismic acid, and the catabolic quinic acid pathway, in which quinic acid is converted into protocatechuate (Figure 1). Chorismic acid is the precursor in the synthesis of aromatic amino acids, L-Phe, L-Tyr and L-Thr, and other important aromatic compounds such as folate cofactors, ubiquinone and vitamins E and K. Whereas protocatechuate is converted via the β-adipate pathway to acetyl-CoA and succinyl Co-A. There are two distinct types of DHQ enzymes, known as type I (DHQ1, *aroD* gene) and type II (DHQ2, *aroD/aroQ* gene), which have different biochemical and biophysical properties and they do not show sequence similarity (Table 1) [34]. DHQ1, which is found in plants, fungi and many bacterial species such as *Salmonella typhi*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enterica subsp. enterica serovar typhimurium* and *Clostridium difficile*, is exclusively biosynthetic and is a heat-labile dimer with a subunit of around 27 KDa (Figure 2A) [35,36]. In contrast, DHQ2 has both biosynthetic and catabolic roles. It is a heat-stable dodecamer formed from a tetramer of trimers, where a trimer is the minimum catalytic unit of the enzyme [37] (Figure 2B), and it has a subunit of 16−18 KDa. DHQ2 is essential in pathogenic bacteria such as *Mycobacterium tuberculosis* (*aroD* gene), which is responsible for tuberculosis, and *Helicobacter pylori* (*aroD/aroQ* gene), which is the causative agent of gastric and duodenal ulcers and has also been classified as a type I carcinogen [38]. DHQ2 is a particularly attractive target for the development of new anti-tubercular drugs and alternative therapies for the treatment of *Helicobacter pylori* infections [39–42]. In this respect, compounds that target the DHQ2 enzyme would have an influence on bacterial viability. The fact that two DHQ enzymes have evolved to catalyze the same reaction by entirely different mechanisms and stereochemical courses has been used in the design of compounds that specifically inhibit either the type I or type II enzymes [34]. This review is focused on the DHQ1 enzyme.

DHQ1 mechanism – nucleophilic catalysis

DHQ1 is a class I aldolase enzyme that catalyzes the overall *syn* elimination of water from 3-dehydroquinic acid (**1**) by a multi-step mechanism that involves the formation of a Schiff base species. In the formation of such covalent intermediates Nature has found an excellent way to address mechanistically challenging transformations around the carbonyl group in aqueous solution [43−46]. Good examples are the PLP-dependent enzymes, which catalyze a wide range of reactions [47,48]. For enzyme-catalyzed reactions, covalent catalysis is a particularly effective strategy because: (a) it can place its substrate in the correct arrangement for further enantio- or diastereoselective transformations; (b) the formation of covalent intermediates in the reaction mechanism helps to stabilize subsequent transition states by lowering the activation energy; (c) it can generate a very high effective concentration of a desolvated nucleophile or base with the consequent

huge rate acceleration. Thus, considering that enzyme active sites are largely shielded from the solvent environment, usually by a substrate-covering loop that closes over the active site after substrate binding, the reactive side chain residues are likely to be desolvated and therefore a high effective concentration of a nucleophile or a base is achieved.

The reaction catalyzed by DHQ1 is initiated by the formation of a Schiff base between the C3 carbonyl group of the substrate and an essential lysine (Lys170 in *S. typhi*) (Figure 3). The role of the Schiff base is to act as an electron sink and it may also play a role in distorting the cyclohexyl ring to make the equatorial C2 hydrogen more reactive [49,50]. A basic residue of the active site then removes the C2 equatorial hydrogen (pro-*R*) to afford an enamine, which undergoes acid-catalyzed elimination of the C1 hydroxyl group − a reaction that is mediated by an essential histidine (His143 in *S. typhi*) acting as a proton donor [51]. The role in the enzymatic mechanism of this histidine has been the subject of much controversy. Site-directed mutagenesis and biochemical studies have shown that His143 is clearly involved in both the formation and subsequent hydrolysis of the Schiff base intermediates [52−56]. In addition, based on the proximity of His143 to the C2 carbon in the crystal of the substrate-Schiff base (PDB entry 3M7W, 1.95 Å), it was also suggested that this residue would also be the base that removes the C2 equatorial hydrogen [57] (Figure 4A). However, its role as a general base appears to be more complex because the replacement of His143 residue by and an alanine (variant H143A protein), which showed a 10^6-fold reduction in catalytic activity, is able to slowly transform substrate to product but stalls the hydrolytic release of product from the active site [55]. Computational studies suggest that the product-Schiff base hydrolysis is the rate-determining step [58−60]. The crystal structure of the reduced form of the product-Schiff base (PDB entry 1SFJ, 2.4 Å, [61]), which was obtained by reduction of the imine bond with sodium borohydride, has also been solved (Figure 4B) [62]. This covalent attachment of the product molecule to the DHQ1 from *E. coli* does not cause a significant change in the protein conformation but it causes a dramatic increase in the stability of the protein against proteolysis [63,64]. Thus, the melting temperature of the covalently modified protein is 40 ℃ higher than that of the unmodified one.

DHQ1 active site and substrate binding

Analysis of the amino acid sequence in various DHQ1 enzymes reveals that the active site is highly conserved (Figure 5). The available crystal structures of the Schiff base intermediates and Molecular Dynamics (MD) simulation studies carried out with the Michaelis complex of DHQ1 from *Salmonella typhi* (*St*-DHQ1) showed that the substrate binds in the active site by a series of strong electrostatic and hydrogen-bonding interactions [65]. This Michaelis complex was created from the

crystal structure of *St*-DHQ1 in complex with (2*R*)-2-methyl-3-dehydroquinic acid (PDB entry 4CNO, 1.5 Å, [65]), which is a substrate analog that does not form an imine linkage with the enzyme and leads to an open conformation of the substrate-covering loop. In particular, the substrate is anchored to the active site by a salt-bridge between its carboxylate group and the guanidinium group of Arg213, which has previously been identified by chemical modification as the key residue for carboxylate recognition [50,66], along with a bidentate hydrogen bond between the carboxylate group of the conserved Glu46 and the C4 and C5 hydroxyl groups of the substrate (Figure 4). The C4 hydroxyl group of the inhibitor also interacts by hydrogen bonding with the guanidinium group of Arg82 and a water molecule that is found in several crystal structures forming a bridge between Asp114 and Glu46, probably to avoid repulsion between the two residues. The C5 hydroxyl group establishes two additional hydrogen bonds, one with the guanidinium group of the conserved Arg48 and one with the loop that closes the active site. The latter interaction, along with the hydrogen bond between the carboxylate group of the substrate and the side chain of a conserved Gln236, contributes to close the active site tightly. Harris *et al.* [67] showed that the binding interactions involving the C4 hydroxyl group are very important for imine formation. Thus, whereas the removal of the C5 hydroxyl group in **1** cause a reduction in specificity by 10^3 ($k_{cat} = 3$ s^{-1}, K$_m$ = 700 µM; k_{cat}/ K$_m$ = 4.3 10^3 s^{-1} M^{-1}), removal of both C4 and C5 hydroxyl groups does not lead to formation of the Schiff base. Moreover, the C1 hydroxyl group of the ligand and the guanidinium group of Arg82 interact through a bridging water molecule. Finally, the appropriate disposition of the cyclohexane ring is controlled by key CH-π interactions between the phenyl ring of the functionally conserved Phe225 (in *S. typhi*) and the axial hydrogens of the cyclohexane ring of the inhibitor. This residue, which is embedded within an apolar pocket involving Ile201, Val139, Leu78 and Ala223, seems to act as a 'bottom lid' of the active site to isolate this part of the active site from the solvent environment and it also blocks one of the faces of the substrate for catalysis. Comparison of the amino acid sequence in several DHQ1 enzymes (Figure 5) revealed that in some cases this phenylalanine is replaced by a tyrosine, as for DHQ1 from *S. aureus* (*Sa*-DHQ1). In those cases, such interactions are only observed with the axial C2 hydrogen because the tyrosine phenol ring is shifted towards the essential lysine binding pocket. In both cases, the axial C2 hydrogen is protected by these aromatic residues.

MD simulation studies carried out on the Michaelis complex of *St*-DHQ1 suggest that the formation of this complex involves five key steps [65] (Figure 6): (1) folding of the substrate-covering loop triggered by the conserved Gln236; (2) reduction of the loop flexibility by a series of attractive hydrogen-bonding interactions involving Pro234, Ala233, Ser232 and Gln236 [66] residues that are located in the loop; (3) pushing the substrate into the bottom of the active site,

which involves Arg213 and Glu46 residues and the loop; (4) sealing of the active site with the entrance of the conserved Phe145 side chain into the active site after a small folding of the loop containing residues 144–151, a process that is triggered by a hydrogen-bonding interaction with the neighboring loop that contains residues 174–178; and (5) capture of the Phe145 side chain inside the active site by the conserved Met205 residue. These studies also showed that a closed conformation of the substrate-covering loop that seals the active site from the solvent environment is required for catalysis.

Covalent DHQ1 inhibitors

The first irreversible inhibitors of the DHQ1 enzyme, compounds **3−5**, were reported by Bugg *et al.* [68] (Figure 7). These substrate analogs with reactive functional groups, which were synthesized as racemic mixtures, proved to inhibit irreversibly DHQ1 from *E. coli* with K_i values between 0.4 and 1.1 mM, with epoxide **3** being the most potent of the three. However, details of the inhibition mechanism were not reported.

Later on, reasoning that the reaction catalyzed by DHQ1 involves the abstraction of the equatorial (pro-*R*) C2 hydrogen of 3-dehydroquinic acid (**1**), this hydrogen was replaced by a halogen, i.e., (2*R*)-2-fluoro- (**6a**) and (2*R*)-2-bromo-3-dehydroquinic acid (**6b**), as a strategy to inhibit the enzyme [69,70] (Figure 7). These compounds, which are substrates for the DHQ2 enzyme since they still contain the axial C2 hydrogen that is removed by the enzyme [71], proved to be time-dependent irreversible inhibitors of DHQ1 from *E. coli* with K_i values of 80 μM and 3.7 mM, respectively. The electrospray mass spectra of the partially inactivated enzyme with compounds **6** showed the presence of covalently modified proteins corresponding to an additional mass of 123, which were similar in both cases. It was suggested that this chemical modification is due to the formation of compound **10**, which would be obtained by a decarboxylation reaction (Figure 8). The distortion of the cyclohexyl ring induced by the formation of the Schiff base probably favors an antiperiplanar disposition of the carboxylate and the halide groups and this would allow the decarboxylation process to occur.

Recently, epoxides **7** and **8** were designed as irreversible inhibitors of the DHQ1 enzyme to study the binding requirements of the linkage to the enzyme [72]. Both epoxides proved to have a highly differentiated activity against *St*-DHQ1. The epoxide with the *R* configuration, compound **8**, proved to be a weak inhibitor with an IC$_{50}$ of 1 mM and this did not cause covalent modification of the enzyme. In contrast, the epoxide with the *S* configuration, compound **7**, was found to be a time-dependent irreversible inhibitor of *St*-DHQ1. A 274 μM concentration of epoxide **7** caused ~50% enzyme inactivation in ~30 minutes and ~75% enzyme inactivation in ~2 hours. These

studies also allowed the description of the first crystal structure of *St*-DHQ1 covalently modified by an inhibitor (PDB entry 4CLM) (Figures 9A and 9B). This structure, which was obtained by co-crystallization and was determined at 1.4 Å, revealed that surprisingly the modified ligand is covalently attached to the essential Lys170 through the formation of a stable Schiff base, which has two possible arrangements. The expected hydroxyl amino derivative that would be obtained after nucleophilic ring opening of the epoxide group in **7** by Lys170 was not obtained as electron density was not observed for the corresponding C3 hydroxyl group (Figure 9A). MD simulation studies carried out with the *St*-DHQ1/**7** binary complex suggest that the resulting *St*-DHQ1/**7** adduct would be obtained by activation of the epoxide oxygen by the essential histidine followed by nucleophilic ring opening of the epoxide by the lysine from the opposite face and subsequent dehydration and isomerization reactions. These studies provide strong evidence that the enzyme appears to be designed to form the substrate-Schiff base by activation and correct positioning of the oxygen atom of the ketone group in the natural substrate by His143 and subsequent nucleophilic attack by Lys170 from the *Si* face of the ketone group. This process requires an active site that is shielded from the solvent environment by the substrate-covering loop.

The proposed covalent modification mechanism is shown in Figure 10. Firstly, nucleophilic attack by the ε-amino group of the essential lysine to epoxide **7** by activation of the essential histidine would take place to afford adduct **10**. The resulting adduct could then be deprotonated by the neutral His143 to give the corresponding amino alcohol II. For the *St*-DHQ1 enzyme, a conserved water molecule (W173 in PDB entry 4CLM), which is also observed in all the available DHQ1 crystal structures, remains fixed between the side chain of the conserved Asp114 and Glu46 residues. In this arrangement, Asp114 would act as the base that deprotonates this water molecule to afford a hydroxide group, which triggers the elimination reaction. This hydroxide anion would be stabilized by the guanidinium group of Arg82. Dehydration of the C3 hydroxyl group would be mediated by His143 acting as a proton donor. As a result, enamine II, which is in equilibrium with its Schiff base **11**, would be obtained. The crystal structure of the latter adduct was obtained by co-crystallization of *St*-DHQ1 with epoxide **7** after several weeks (PDB entry 4CLM).

Non-covalent DHQ1 Inhibitors

Ratia *et al.* carried out a high-throughput screening study and identified three non-covalent inhibitors of DHQ1 from *Clostridium difficile*, compounds **12−14**, which had IC$_{50}$ values between 31 and 35 μM (Figure 11) [73,74]. NMR experiments showed that these compounds compete with the reaction product binding site. Considering that (a) these compounds proved to be selective inhibitors of the *C. difficile* enzyme but they were inactive against other DHQ1 enzymes such as the *S. enterica*

enzyme and (b) that the DHQ1 active site is highly conserved, the authors suggested that these compounds bind to the DHQ1 from *C. difficile* with the substrate-binding loop in the open conformation. The binding mode of these inhibitors is still unknown.

Future Perspective

After a gap in innovation of around 40 years (1960−2000) in the search for new antibiotic classes, and considering that very few new antibiotics have been brought to the market in the last decade, future research into the discovery of novel drugs and therapies promises to be exciting and challenging. Although the complexity of bacteria, which have had billions of years of evolution to cope with harsh environments, makes bacterial resistance an inevitable phenomenon, the development of drugs that deal with unexplored targets and/or with new mechanisms of action will help to reduce the rate and spread of this resistance. In this sense, anti-virulence drugs, which block the ability of bacteria to cause a disease or disrupt the interaction between the host and the pathogen, are attractive options. The significant effort devoted over the past few decades to understand how bacteria cause disease, along with the increasing availability of bacterial genome sequences, has created new opportunities for drug discovery that are increasingly being explored. More work needs to be done on the identification of unknown virulence factors involved in relevant pathogenic bacteria and a detailed knowledge of their mechanism of action needs to be gained. This knowledge will be an excellent starting point for the rational design of compounds and will provide a wider repertoire of agents to combat bacterial virulence.

Although only a few inhibitors of the type I dehydroquinase enzyme − a promising virulence factor *in vivo* − have been developed to date, preliminary *in vitro* anti-virulence studies show their ability to reduce bacterial virulence and this encourages further studies on this target. A wider variety of covalent and non-covalent inhibitors based on the scaffolds that have already been described is needed to improve on the anti-virulence activity achieved to date. Covalent inhibitors are particularly attractive due to their higher efficiency, their low sensitivity toward pharmacokinetic parameters and their longer duration of action [75,76]. However, care must be taken when selecting functional groups for specific covalent linkage to the enzyme to avoid the production of non-specific drugs. As for the other enzymes involved in the shikimic acid pathway, the binding requirements of this enzyme, in particular regarding the carboxylate recognition that is required for good binding affinity, and the high polarity of the residues involved in the substrate recognition represent the most challenging issues for drug design.

Executive summary

The challenge of antibiotic resistance – an overview of the problem

- Drugs with new mechanisms of action, the identification of unexplored bacterial targets as well as a detailed knowledge of the catalytic mechanism and/or the binding determinants of those targets to combat the growth of antibiotic-resistant bacteria is urgently needed.

Anti-virulence therapies – an alternative

- Anti-virulence drugs will disarm bacteria and will create an *in vivo* scenario similar to that achieved by vaccination with a live attenuated strain.

Type I dehydroquinase – an anti-virulence target

- Type I dehydroquinase is a promising target in the search of new anti-virulence agents to combat the widespread resistance to antibiotics.
- There are two distinct types of DHQ enzymes (DHQ1 and DHQ2) and these have different biochemical and biophysical properties.
- DHQ1 and DHQ2 enzymes catalyze the same reaction by very different mechanisms.

DHQ1 mechanism – covalent catalysis

- It is a class I aldolase involving the formation of Schiff-base species
- *Syn* elimination of water.

DHQ1 active site and substrate binding

- Details of the binding requeriments and essential motion for catalysis is described.

Covalent DHQ1 inhibitors

- First irreversible inhibitors, compounds **3−5**.
- (2*R*)-2-halo-3-dehydroquinic acids (**6**).
- Epoxides **7** and **8**.

Non-covalent DHQ1 inhibitors

- Non-covalent inhibitors discovered by high-throughput screening are disclosed.

Future perspective

Key Terms

Virulence: It is the magnitude of the morbidity and the increase in the likelihood of mortality resulting from the colonization and proliferation of bacteria in or on a host.

Nucleophilic catalysis: This type of enzymatic catalysis is a subtype of covalent catalysis and it involves nucleophilic attack of an active site residue to a reactive functional group of the substrate and subsequent formation of a covalent intermediate with the enzyme.

Molecular dynamics (MD) simulations: These computational studies provide information about the dynamic behavior of complex molecular systems such as receptors and enzymes that can be studied in the presence or absence of ligands in aqueous solution. In comparison with docking studies, in which the macromolecule is considered as a rigid system and the ligand is flexible, MD simulation studies consider both the receptor and the ligand to be flexible.

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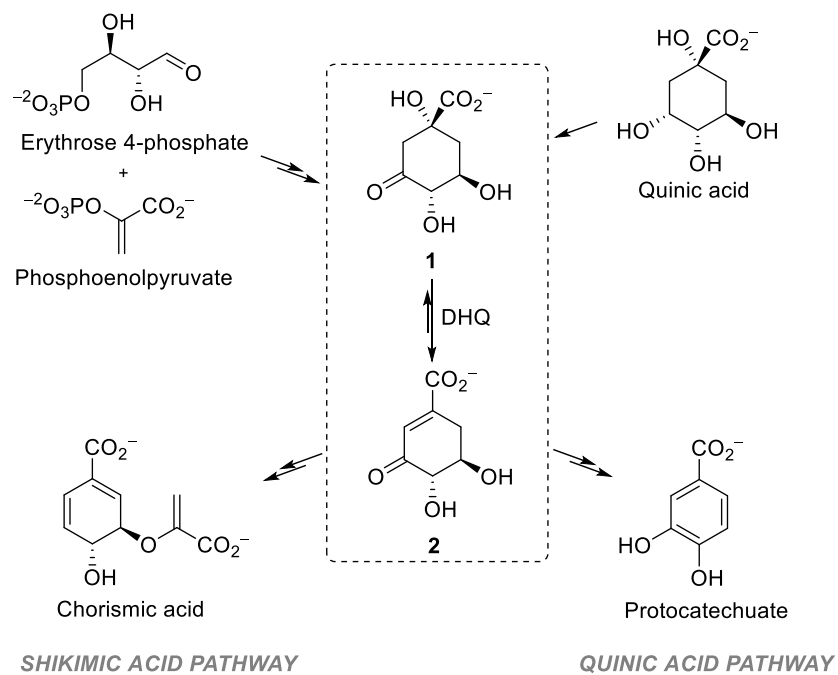
Figure 1. The shikimic acid pathway. The reaction catalyzed by the dehydroquinase enzyme is part of two metabolic pathways: the biosynthetic shikimic acid pathway leading to chorismic acid and the catabolic quinic acid pathway that converts quinic acid into protocatechuate.

Table 1. **Comparison of the different characteristics and mechanism of the DHQ enzymes**

	DHQ1	DHQ2
Structural characteristics	Heat-labile dimer Subunit 27 KDa	Heat-stable dodecamer (tetramer of trimers) Subunit 16−18 KDa
Bacterial sources	*Escherichia coli* *Salmonella typhi* *Staphylococcus aureus* *Salmonella enterica subsp. enterica serovar typhimurium* *Clostridium difficile*	*Mycobacterium tuberculosis* *Helicobacter pylori* *Streptomyces coelicolor* *Francisella novicida* *Acinetobacter baylyi* *Acinetobacter baumannii*
Mechanism of action	*syn* elimination of water equatorial (pro-*R*) C2 hydrogen covalent linkage to the enzyme (Lys) Schiff base species	*anti* elimination of water axial (pro-*S*) C2 hydrogen non-covalent linkage to the enzyme enolate intermediate
Essential residues for catalysis	Lys, His	Tyr, Arg, Asp, His
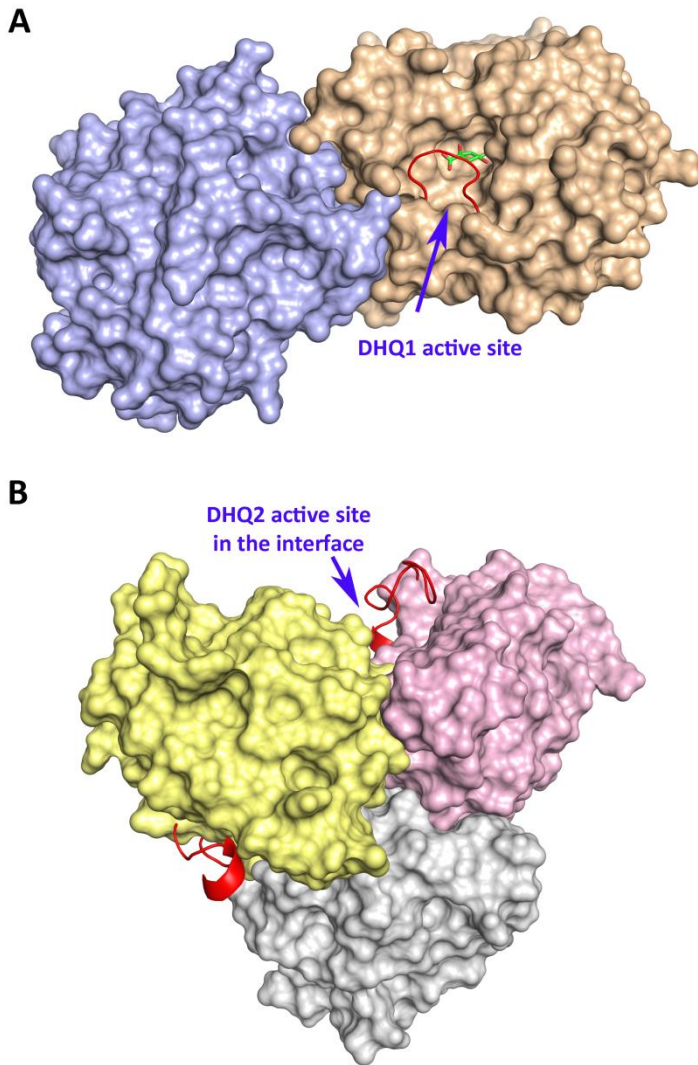
Figure 2. **Tridimensional structure of the DHQ enzymes**. A) DHQ1. B) DHQ2. Note how DHQ1 is a dimer whereas DHQ2 is a tetramer of trimers. Only the trimer is shown. Note how the DHQ2 active site is located in the interface between two chains. The substrate-covering loop for both enzymes is highlighted in red.
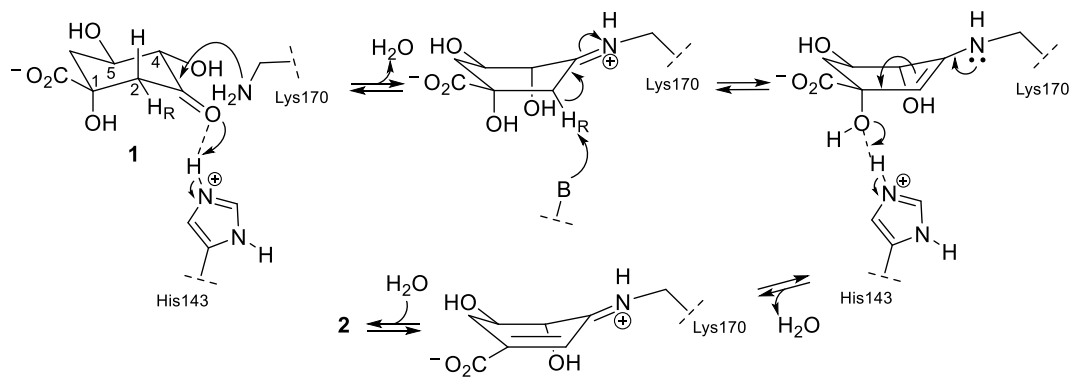
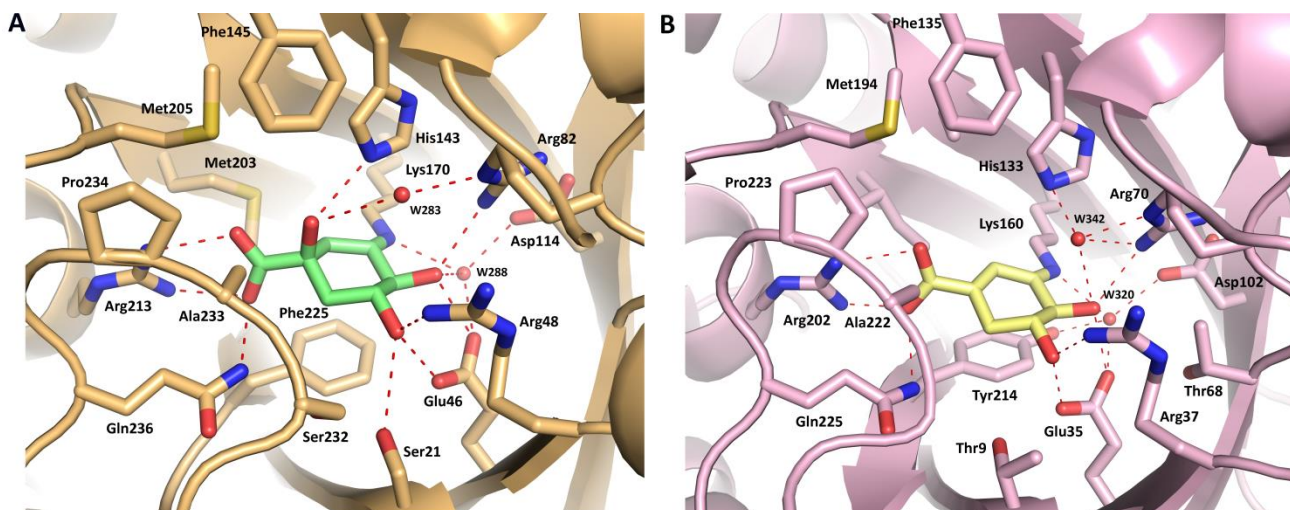**Figure 3. Proposed mechanism for the reaction catalyzed by DHQ1.**

Figure 4. **Crystal structures of DHQ1 substrate- and product-Schiff bases.** A) Substrate-Schiff base of DHQ1 from *S. typhimurium LT2* (PDB entry 3M7W, 1.95 Å, [51]). B) Product-Schiff base of DHQ1 from *S. aureus* (PDB entry 1SFJ, 2.4 Å, [48]). Hydrogen bonding and electrostatic interactions are shown as red dashed lines. Relevant residues are shown and labeled.

```
DHQ1_AA    MLIA--------------VPLDDTNFSENL---KKAKEKGADIVELRV
DHQ1_CD    MKRKVQVKNITIGEGRPKICVPIIGKNKKDIIKEAKELKDACLDIIEWRV
DHQ1_EC    MKT-VTVKDLVIGTGAPKIIVSLMAKDIASVKSEALAYREADFDILEWRV
DHQ1_SA    MTH-VEV----VATITPQL------YIEETLIQKINHRIDAIDVLELRI
DHQ1_SA2   MTH-VEV----VATIAPQL------SIEETLIQKINHRIDAIDVLELRI
DHQ1_ST    MKT-VTVKNLIIGEGMPKIIVSLMGRDINSVKAEALAYREATFDILEWRV
DHQ1_ST2   MKT-VTVRDLVVGEGAPKIIVSLMGKTITDVKSEALAYREADFDILEWRV
                                   :       *::* *:

DHQ1_AA    DQFSDTS-LNYVKEKLEEV--HSQGLKTILTIRSPEEGGREVKNREELFE
DHQ1_CD    DFFENVENIKEVKEVLYELRSYIHDIPLLFTFRSVVEGGEKLISRDYYTT
DHQ1_EC    DHYADLSNVESVMAAAKILRETMPEKPLLFTFRSAKEGGEQAISTEAYIA
DHQ1_SA    DQFENVT-VDQVAEMITKLKVMQDSFKLLVTYRTKLQGGYGQFTNDSYLN
DHQ1_SA2   DQIENVT-VDQVAEMITKLKVMQDSFKLLVTYRTKLQGGYGQFINDLYLN
DHQ1_ST    DHFMDIASTQSVLTAARVIRDAMPDIPLLFTFRSAKEGGEQTITTQHYLT
DHQ1_ST2   DHFANVTTAESVLEAAGAIREIITDKPLLFTFRSAKEGGEQALTTGQYID
           *    :    .  *     :         :.* *:  :**

DHQ1_AA    EL-----SPLSDYTDIELSSRGLLVK--L-YNITKEAGKKLIISYHNFEL
DHQ1_CD    LNKEISNTGLVDLIDVELFMGDEVID--EVVNFAHKKEVKVIISNHDFNK
DHQ1_EC    LNRAAIDSGLVDMIDLELFTGDDQVK--ETVAYAHAHDVKVVMSNHDFHK
DHQ1_SA    LISDLANINGIDMIDIEWQADIDIEKHQRIITHLQQYNKEVIISHHNFES
DHQ1_SA2   LISDLANINGIDMIDIEWQADIDIEKHQRIITHLQQYNKEVVISHHNFES
DHQ1_ST    LNRAAIDSGLVDMIDLELFTGDADVK--ATVDYAHAHNVYVVMSNHDFHQ
DHQ1_ST2   LNRAAVDSGLVDMIDLELFTGDDEVK--ATVGYAHQHNVAVIMSNHDFHK
                 *   *:*        .         :     :::* *:*.

DHQ1_AA    TPPNWIIREVLREGYRYG-GIPKIAVKANSYEDVARLLCISRQ-----VE
DHQ1_CD    TPKKEEIVSRLCRMQELGADLPKIAVMPQNEKDVLVLLEATNEMFKIYAD
DHQ1_EC    TPEAEEIIARLRKMQSFDADIPKIALMPQTSDVLTLLAATLEMQEQYAD
DHQ1_SA    TPPLDELQFIFFKMQKFNPEYVKIAVMPHNKNDVLNLLQAMSTFS-DTMD
DHQ1_SA2   TPPLDELQFIFFKMQKFNPEYVKIAVMPHNKNDVLNLLQAMSTFS-DTMD
DHQ1_ST    TPSAEEMVLRLRKMQALGADIPKIAVMPQSKHDVLTLLTATLEMQQHYAD
DHQ1_ST2   TPAAEEIVQRLRKMQELGADIPKIAVMPQTKADVLTLLTATVEMQERYAD
           **    :    :  .      *:*: .:.  **  **            :

DHQ1_AA    GEKILISMGDYGKISRLAGYVFGSVITYCSLEKAFAPGQIPLEEMVELRK
DHQ1_CD    RPIITMSMSGMGVISRLCGEIFGSALTFGAAKSVSAPGQISFKELNSVLN
DHQ1_EC    RPIITMSMAKTGVISRLAGEVFGSAATFGAVKKASAPGQISVNDLRTVLT
DHQ1_SA    CKVVGISMSKLGLISRTAQGVFGGALTYGCIGEPQAPGQIDVTDLKAQVT
DHQ1_SA2   CKVVGISMSKLGLISRTAQGVFGGALTYGCIGEPQAPGQIDVTDLKAQVT
DHQ1_ST    RPVITMSMAKEGVISRLAGEVFGSAATFGAVKQASAPGQIAVNDLRSVLM
DHQ1_ST2   RPIITMSMSKTGVISRLAGEVFGSAATFGAVKKASAPGQISVADLRTVLT
           : :**.   *  *** .  :**.. *:  .  .  ***** . ::

DHQ1_AA    KFYR--L
DHQ1_CD    LLHKSIN
DHQ1_EC    ILHQ--A
DHQ1_SA    L-----Y
DHQ1_SA2   L-----Y
DHQ1_ST    ILHN--A
DHQ1_ST2   ILHQ--A
```

Figure 5. Multiple sequence alignment. Amino acid sequence alignments for *Aquifex aeolicus (AA), Clostridium difficile (CD), Escherichia coli (EC), Staphylococcus aureus (SA), Staphylococcus aureus* (strain JH1, SA2), *Salmonella typhi (ST), Salmonella typhimurium LT2 (*ST2) DHQ1 enzymes. Protein sequences were aligned using the CLUSTAL W (1.83) multiple sequence alignment (https://www.ebi.ac.uk/Tools/msa/clustalw2/). Fully conserved (*, grey) and functionally conserved (:, black) residues are highlighted.
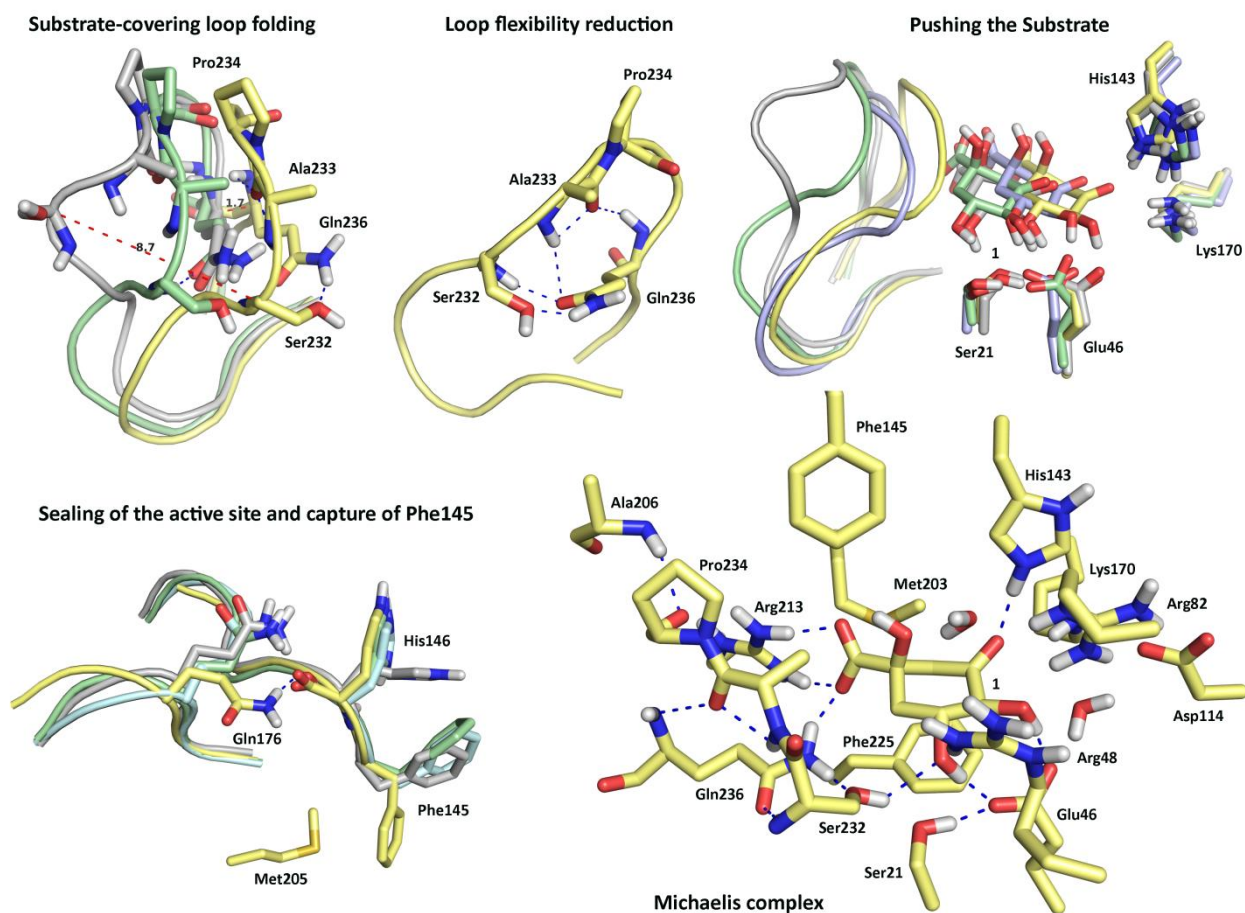
Figure 6. Michaelis complex formation process obtained by MD simulations studies. The process involves: (a) substrate-covering loop folding triggered by the conserved Gln236 residue; (b) Loop flexibility reduction by formation of several hydrogen-bonding interactions within the loop residues; (c) Pushing the substrate into the Lys/His binding pocket by a combined action of the loop and Glu46 side chain. (d) Sealing of the active site with the entrance of Phe145 into the active site and its capture by interaction with the Met205 side chain. As a result of this process the Michaelis complex is obtained. Relevant residues are shown and labeled.

3   **4**   **5**

6a X = F
6b X = Br

7   **8**
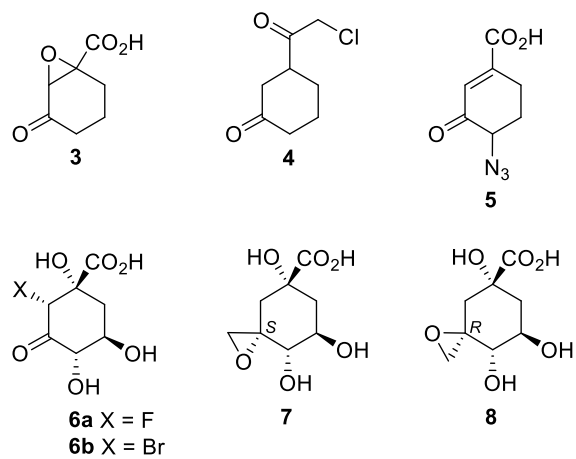
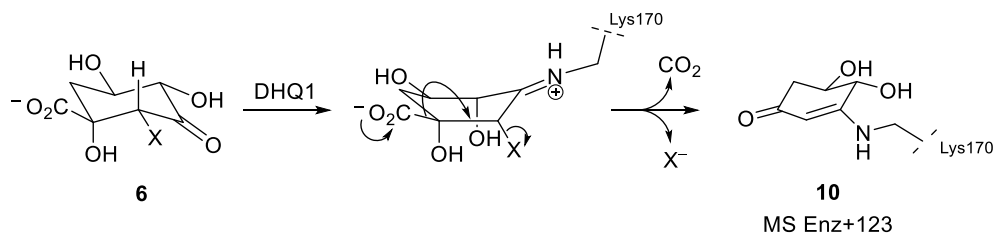**Figure 7**. **Irreversible inhibitors of DHQ1.**

Figure 8. **Proposed covalent modification of DHQ1 caused by irreversible inhibitors 6**.
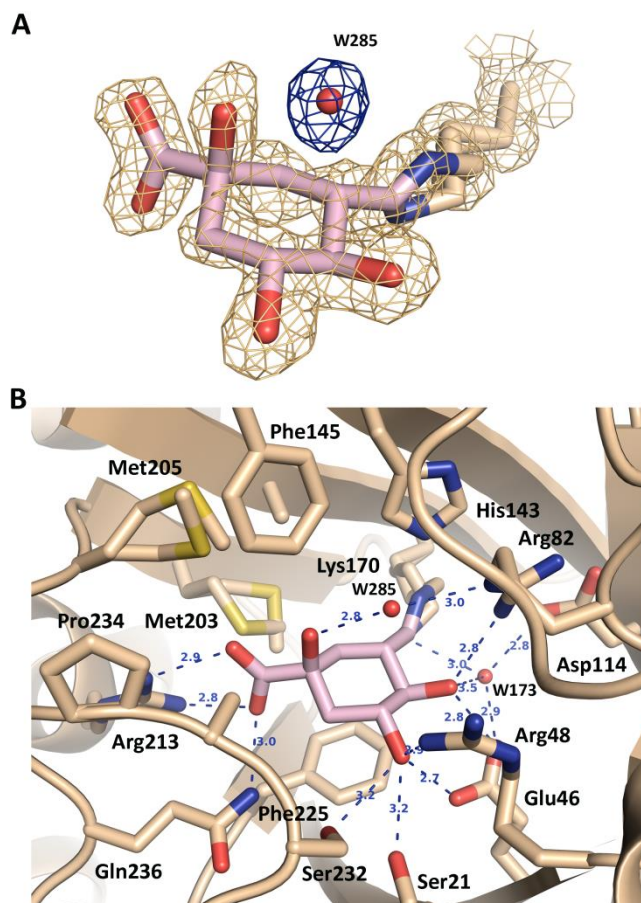
Figure 9. **Crystal structure of the *St*-DHQ1 covalently modified by epoxide 7**. A) Unbiased electron density for the modified inhibitor **7** (pink) and its covalent attachment to Lys170 of *St*-DHQ1 (light orange). A maximum-likelihood weighted $2F_o - F_c$ map contoured at 1σ is shown up to 1.6 Å around the inhibitor molecule (light orange) and water molecule W285 (blue). B) Interactions of the modified inhibitor **7** with *St*-DHQ1. Hydrogen bonding and electrostatic interactions (blue) between the ligand and the *St*-DHQ1 are shown. Relevant residues are shown and labeled.
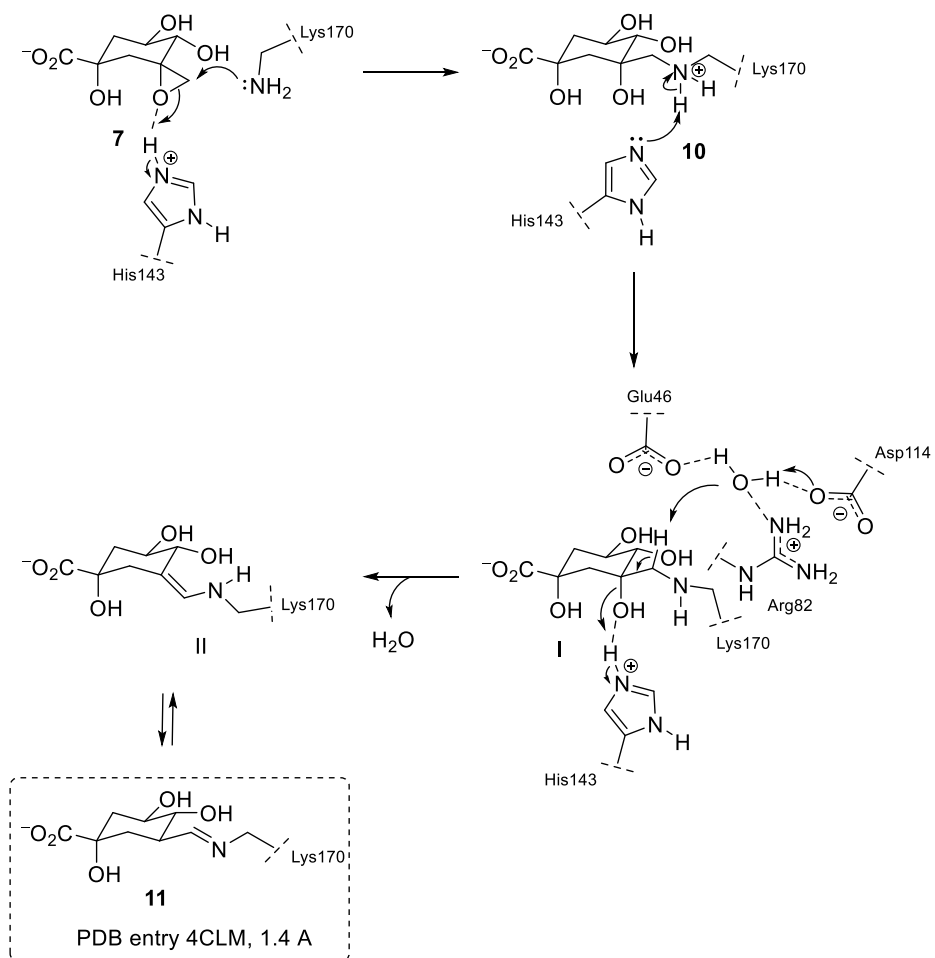
Figure 10. **Proposed covalent modification mechanism of** *St***-DHQ1 caused by irreversible inhibitor 7**.
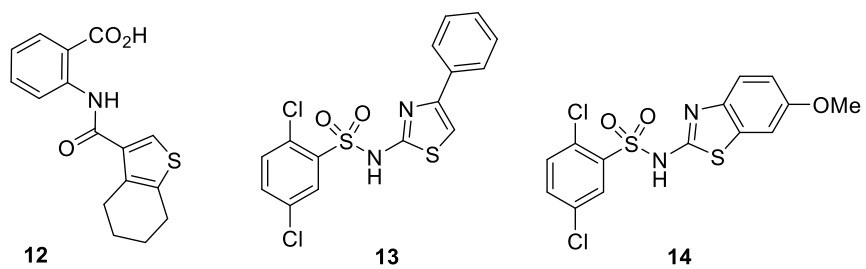
Figure 11. Non-covalent inhibitors of DHQ1 from *C. difficile* discovered by high-throughput screening.