Plasmodium falciparum is the causative agent of the most serious and fatal malarial infections, and it has developed resistance to commonly employed chemotherapeutics. The *de novo* pyrimidine biosynthesis enzymes offer potential as targets for drug design, because, unlike the host, the parasite has not pyrimidine salvage pathway.In search for new *Plasmodium falciparum* dihydroorotate dehydrogenase (*Pf*DHODH) inhibitors as antimalarials, modifications of original 4-aminocoumarin scaffold were studied. 4-amino-3-benzylcoumarin derivatives are inactive against the recombinant enzyme, while results for the 4-amino-8-azacoumarin derivatives are still not available.

Investigation of novel malaria parasite enzyme (DHODH) inhibitors based on 4-amino-3-benzylcoumarin and 4-amino-8-azacoumarin scaffolds

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Malaria

Malaria is a parasitic disease caused by five species of the genus *Plasmodium* (*P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi*) that affect humans. The most deadly form of Malaria is due to *Plasmodium Falciparum* and it is mostly spread in African areas (Figure 1). In this region, *P. ovale* has replaced *P. vivax* since African people have lost the Duffy antigen which is the receptor *P. vivax* uses to infect human erythrocytes. *P. vivax* is less dangerous but more widespread, furthermore a dormant stage (hypnozoites), as well as in *P. ovale* infections, can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.[i]

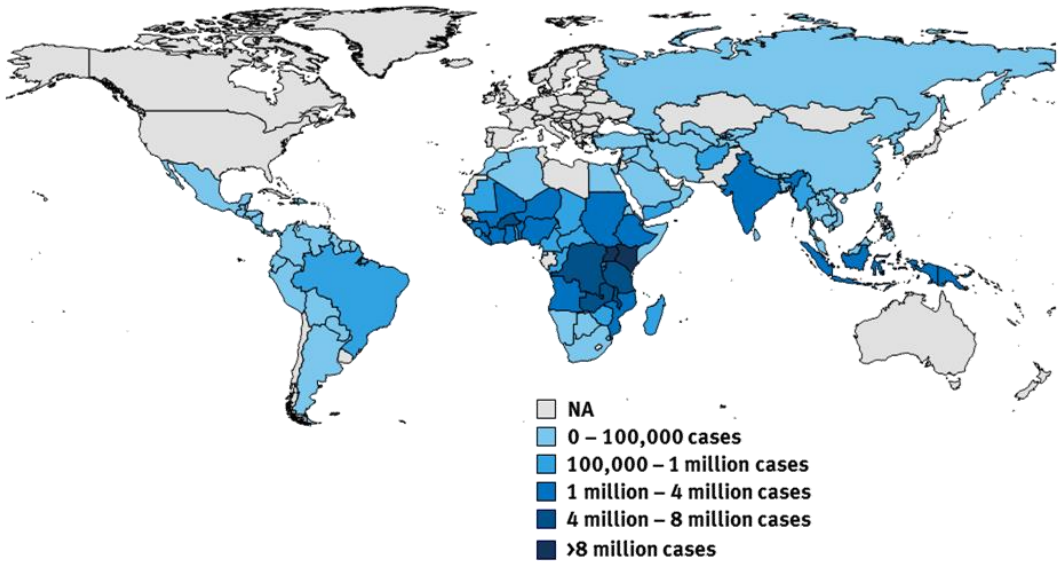


Figure 1. Reported malaria cases in 2011 (source: Kalser Family Foundation, www.GlobalHealthFacts.org, based on WHO, world malaria report 2012.

In 2011 there were an estimated 3.3 billion people at risk of Malaria and among these the more affected by the disease were children under five years of age and pregnant women. Today Malaria is a completely preventable and treatable disease if the currently recommended interventions are properly implemented. The parasite is transmitted by the bite of female mosquitoes of over 30 *Anopheles* species. *Anopheles Gambiae* is one of the most common vehicle of infection since it prefers to feed with human blood over animals and it is responsible for most infections in the sub-Sahara regions. The most effective efforts to defeat this plague and decrease the spreading of it are:

- vector (mosquito) control by using of insecticides treated nets (ITNs), indoor residual spraying and larval control (rainfalls can create collections of water where *Anopheles* eggs are deposited, and larvae and pupae develop into adulthood);

- chemoprevention;
- confirmation of malaria diagnosis;
- timely treatment with appropriate antimalarial medicines.

It is estimated that in 2010, 660,000 deaths were caused by Malaria and 219 million people were affected by the disease[ii]. However from 2000 to 2010 there has been a decrease in deaths rates of 26% and of 17% in the incidence rates but the eradication of the parasite is still far. More than 80% of deaths occur in 14 countries while 17 countries represent the 80% of worldwide cases.

Malaria treatments

The first medicine used to treat malaria was an active principle extracted from the bark of the cinchona tree called quinine. Quinine has a marked bitter taste which gives the well known taste to tonic water. In fact, originally tonic water contained just spring water and quinine and was consumed by British officers during colonial times, often served with gin, to avoid malaria contamination. Around mids of XXth century, chloroquine was introduced as a cheap malaria drug. It saved millions of lives and helped to reduce the malaria spread. However in few years, parasites developed resistant strains to chloroquine and the burden of malaria increased again. Chloroquine was replaced with sulfadoxine-pyrimethamine combination, but the drawback of important side-effects did not allow this treatment for prophylactic use[iii]. Again, the spread of resistance has ended the use of sulfadoxine-pyrimethamine alone.

Today the guidelines for treatment of chloroquine-resistant uncomplicated malaria from *P. falciparum* are atovaquone-proguanil (Malarone^{TM}); artemether-lumefantrine (Coartem^{TM}); quinine sulfate plus doxycycline, tetracycline or clindamycin; mefloquine (Lariam^{TM} and generics). Guidelines for treatment of chloroquine-sensitive uncomplicated malaria from *Pf* suggest the use of chloroquine phosphate (Aralen^{TM} and generics) or hydroxychloroquine (Plaquenil^{TM} and generics). Severe malaria should be treated with quinine gluconate plus doxycycline, tetracycline or clindamycin (intravenous); otherwise it should be treated with artesunate followed by one of the following: atovaquone-proguanil (Malarone^{TM}), doxycycline (clindamycin in pregnant women), or mefloquine.[iv]

Currently there are several drugs on market to treat malaria. The problem is the effectiveness of these drugs since the parasite develops resistance quite fast. In fact studies showed that the parasite uses a two-steps process which assists the plasmodium in finding productive solutions to new and unexpected evolutionary challenges[v]: it has been demonstrated that some haploid blood-stage parasites first survive antimalarial pressure through fortuitous DNA duplications that always included the DHODH gene. Parasites have different sized amplification units but they are always flanked by distant adenine/thymine tracks. Higher level amplification and resistance is attained using a

second, more efficient and more accurate, mechanism for head-to-tail expansion of the founder unit. Furthermore, recent studies confirmed the evidence of a decreased effectiveness of artemisinin combination therapies (ACT) in western Cambodia [vi] . Therefore, it is important to discover new targets and new chemotherapeutics to defeat this plague since the best vaccine candidate (RTS,S) provided just moderate protection in a phase 3 trial on African infants[vii].

Biological background

Dihydroorotate dehydrogenase (DHODH) is an enzyme that converts dihydroorotate to orotate by an oxidative reaction in the fourth, rate-limiting step of *de novo* synthesis of pyrimidines (Figure 2). Pyrimidines are essential in the synthesis of RNA and DNA which permit the transfer of genetic information from cells to daughter cells. If a cell is not able to synthetize own RNA or DNA, it will die. A human cell can acess pyrimidines by salvage of used pyrimidines or by *de novo* synthesis. If *de novo* synthesis is inhibited the cell will rely on the salvage pathway and the cell will not die, however since *Plasmodium* species lack the pyrimidine salvage pathway, inhibition of *de novo* synthesis in parasite cells will cause the death of those cells which makes the parasite vulnerable to inhibition of DHODH.

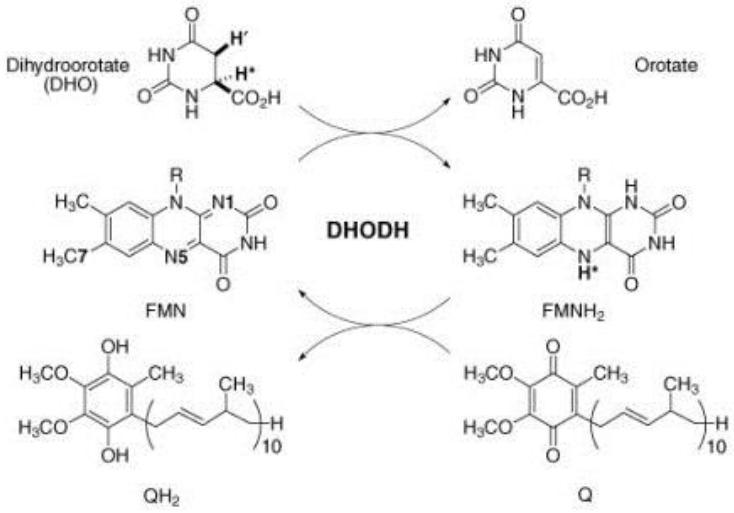


Figure 1. The fourth step in pyrimidine biosynthesis catalyzed by DHODH. Source: Liu *et al.*[viii]

Dihydroorotate dehydrogenase

DHODHs are divided into two classes depending on their primary structure, localization and final electron acceptor.[ix] Gram-positive bacteria and few unicellular eukaryotes are included in Class 1 and their DHODH is soluble and localized in the cytosol; Class 1 can be further divided in other subtypes, type 1A and 1B.[x] Unlike Class 1 DHODHs, in Class 2 the enzyme is linked to the inner membrane of mithocondria and it is found in Gram-negative bacteria, in humans, insects, plants and parasites. The overall structure of DHODHs is an α/β barrel which consists of eight parallel β strands surrounded by eight α

helices and two sets of antiparallel β strands at the top and the bottom of the barrel. The function of the DHODH can be simplified in two half-reactions: the first one, which is the oxidation of dihydroorotate into orotate with FMN as co-factor, is common to Class1 and 2; however, the two classes differ in the use of final electron acceptor during the second half-reaction of the enzyme: Class 2 enzymes use ubiquinone while Class 1-type A use fumarate as the latest electron acceptor[xi] and 1-B enzymes use first FAD and then NAD^+.[xii] This is not the only difference between Class1 and 2: a very important dissimilarity is the amino acid residue, which act as the catalytic base in the first step of the reaction. In class 2 DHODH the catalytic residue is a serine, which is very well conserved and it is the closest residue to C5 position of dihydroorotate. However, in Class 1 the catalytic base is not a serine, but a cysteine instead.[ixb] The role of the catalytic base is to remove the proton from C5 of dihydroorotate, but for doing it the residue must first be deprotonated: since there are not residues in its environment that can deprotonate it and the substrate contains a negative charge, it is assumable that the deprotonation is due to the substrate[xiii]. Another big difference between Class1 and 2 is the extended N terminus at the side of the main barrel, which is missing in Class1; in fact, the first part of the N-terminal domain is important for the binding to the mithocondrial membrane and allows the CoQ to reach the DHODH active site[viii]. N-terminal domain folds in two α-helices and a short 3_{10} helix, which are connected to the α/β barrel by a long loop. Liu *et al.* found that the N-terminal domain of the human enzyme in complex with brequinar, as well as with A77 1726 (the active form of leflunomide) hosts the quinone binding site and this function is kept also in *E. coli* [xii]. These α-helices and the side of the α/β barrel create an hydrophobic tunnel which ends up to FMN and was initially identified as the binding site for DHODH inhibitors and for CoQ. Further studies, however, demonstrated that ubiquinone binding site does not overlap with inhibitors site, but nevertheless the binding of an inhibitor prevent the binding of the ubiquinone.

Even if the fold and the active site of the hydrophobic tunnel is very similar among Class 2 enzymes, the regions near the binding site may differ from one organism to another. This is the reason why none of the inhibitors that bind to the human enzyme inhibit the *E. coli* enzyme (Liu S. *et al*)[viii].

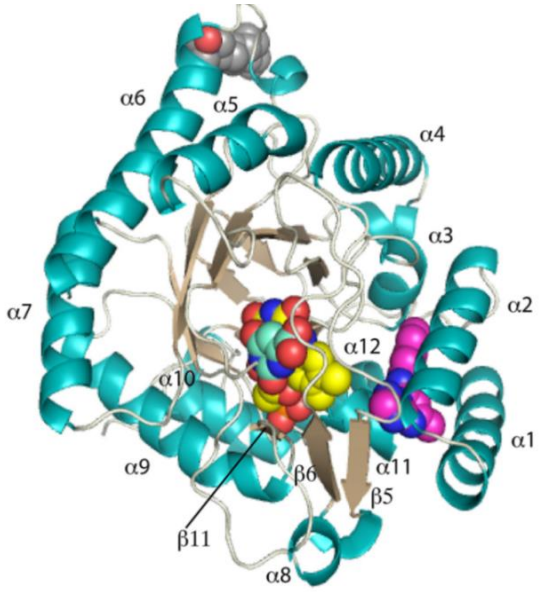


Figure 3. Ribbon diagram of a triazolopyrimidine inhibitor(**DSM1**) bound to *Pf*DHODH. Helices are displayed in *teal*, strands are displayed in *sand*, ligands are displayed as space filling balls with FMN in *yellow*, the inhibitor is in *pink*, orotate is in *turquoise*, and the bound detergent molecule is in *gray*. The position of the detergent corresponds to the position of the shortened surface loop truncated in the *Pf*DHODH_384–413 construct. *B*, A stereo rapresentation of the DSM1-binding site. Residues within 4 Å of the bound inhibitor are displayed. DSM1 is displayed in *pink*, and residues are displayed in *teal*. Nitrogen is displayed in *blue*, oxygen is in *red*, and sulfur is in *yellow*. Source: Deng *et al.*[xiv]

Species-selectivity

It has been found that good human DHODH inhibitors are not usually good *pf*DHODH inhibitors and vice versa[xv] and the reason can be found in the residues of the ubiquinone binding pocket. Deng *et al*.[xiv] found that inhibitors bind two sites in the *pf*DHODH. The site A is a highly conserved hydrophobic pocket in which some polar aminoacids (Hys185 and Arg265) assure the fundamental H-bridges with the inhibitor. Site B is a more hydrophobic pocket and it differs from species to species. The major feature of this pocket is its flexibility, which is illustrated by the binding mode of *pf*DHODH with A77 1726 not overlapping with specific *pf*DHODH inhibitors from a triazolopyrimidine series (DSM1; see Figure 3, DSM2, DSM74) except for in site A. The hydrophobic site B pocket can change its conformation by the rotation of the residue Phe188. In this way, two different pockets are defined and this explains why the plasmodium enzyme can host such different inhibitor scaffolds.

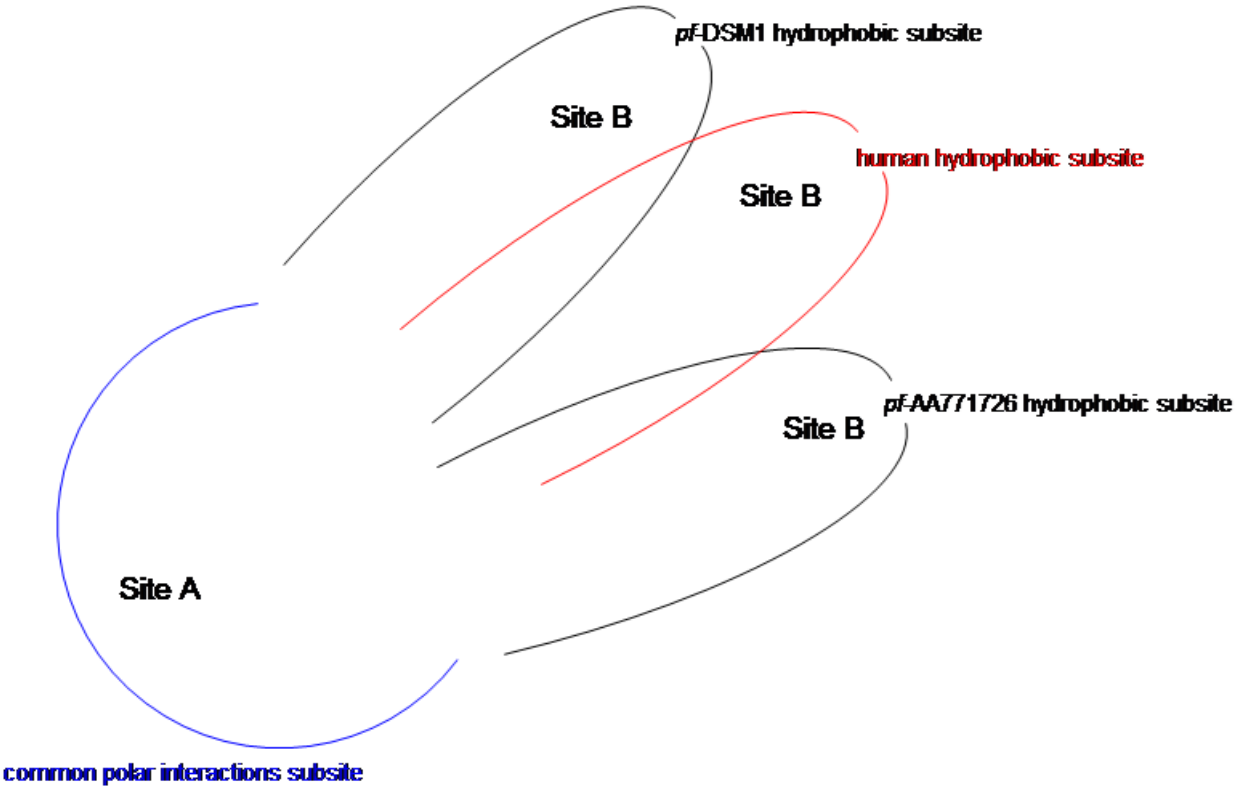


Figure 4. The picture shows the possible site B conformations of the parasite *pf*DHODH enzyme with two different inhibitors and the corresponding conformation in the humanDHODH binding site B.

The human enzyme has the same two sites for the inhibitor binding, with the site A very similar to the parasite one. However, site B occupies a completely different position in the plasmodium enzyme. In fact, site B in *pf*DHODH has two possible conformations that do not share the conformation of site B in *h*DHODH, which may explain why good *h*DHODH inhibitors are not good *pf*DHODH inhibitors (Figure 4).

Discussion

Probing 3-benzyl-4-aminocoumarins as novel *Pf*DHODH inhibitors

4-Aminocoumarins has already been proven as interesting *pf*DHODH inhibitors[xvi]. First, coumarins were designed to maintain the fundamental interactions between DSM1 compound and the *pf*DHODH pocket. These are two H-bonds occurring between the nitrogen in the naphtylamine position and histidine185 and between the pyridine nitrogen and arginine265 in **DSM1**[xvii,xiv]. The 4-aminocoumarin scaffold was *in silico* positioned in the binding site trying to mime the triazolopyrimidine in the *pf*DHODH-DSM1 complex (PDB 3I65). Calculations suggested that the ring oxygen may interact with the arginine residue, while the amino group in the 4-position may interact with the histidine. Some derivatives from this scaffold have been tested with the purified parasitic enzyme and with human enzyme. The most effective compound had an IC_{50} of 0.26µM ± 0.018 and more than 200 times less effective against the human enzyme, supporting the design hypothesis for these compounds (Figure 5).

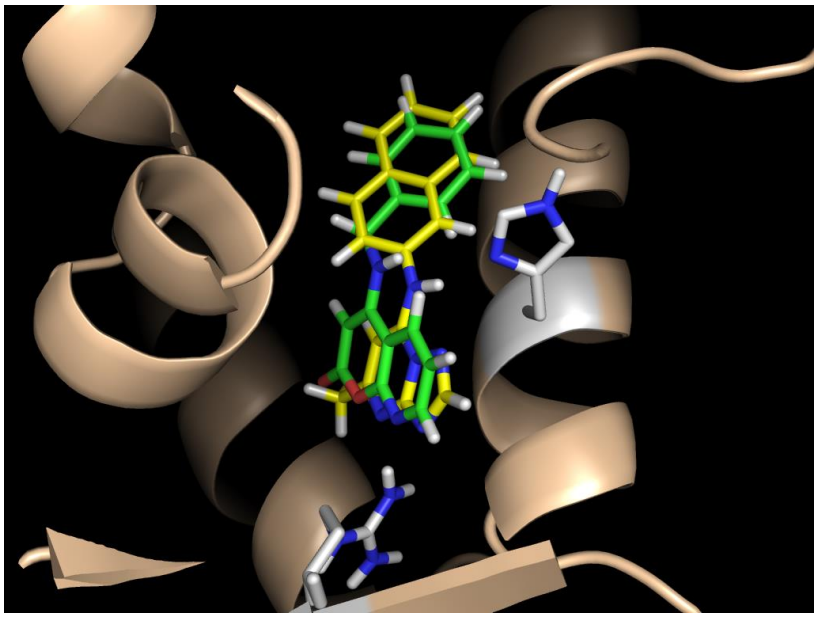


Figure 5. Predicted binding mode of (**15**) with the protein. The inhibitor is rappresented in green while **DSM1** in yellow; the arginine and the histidine are displayed in grey. Oxygen atoms are in red, nitrogen atoms in blue.

Even if 3-position lipophilic substitution in the coumarin scaffold has been proven beneficial for *h*DHODH 4-hydroxycoumarin inhibitors, it is not clear if the same substitution could increase the binding affinity between 4-aminocoumarin-inhibitors and *pf*DHODH. For this reason, a small series of 4-amino-3-benzylcoumarin derivatives (Figure 6) were synthetized and tested with the purified *pf*DHODH.

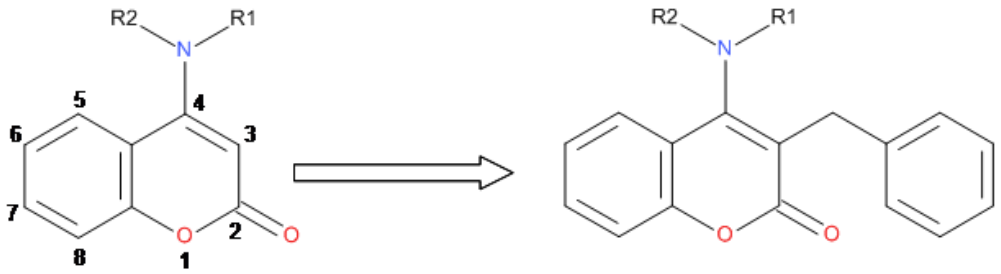
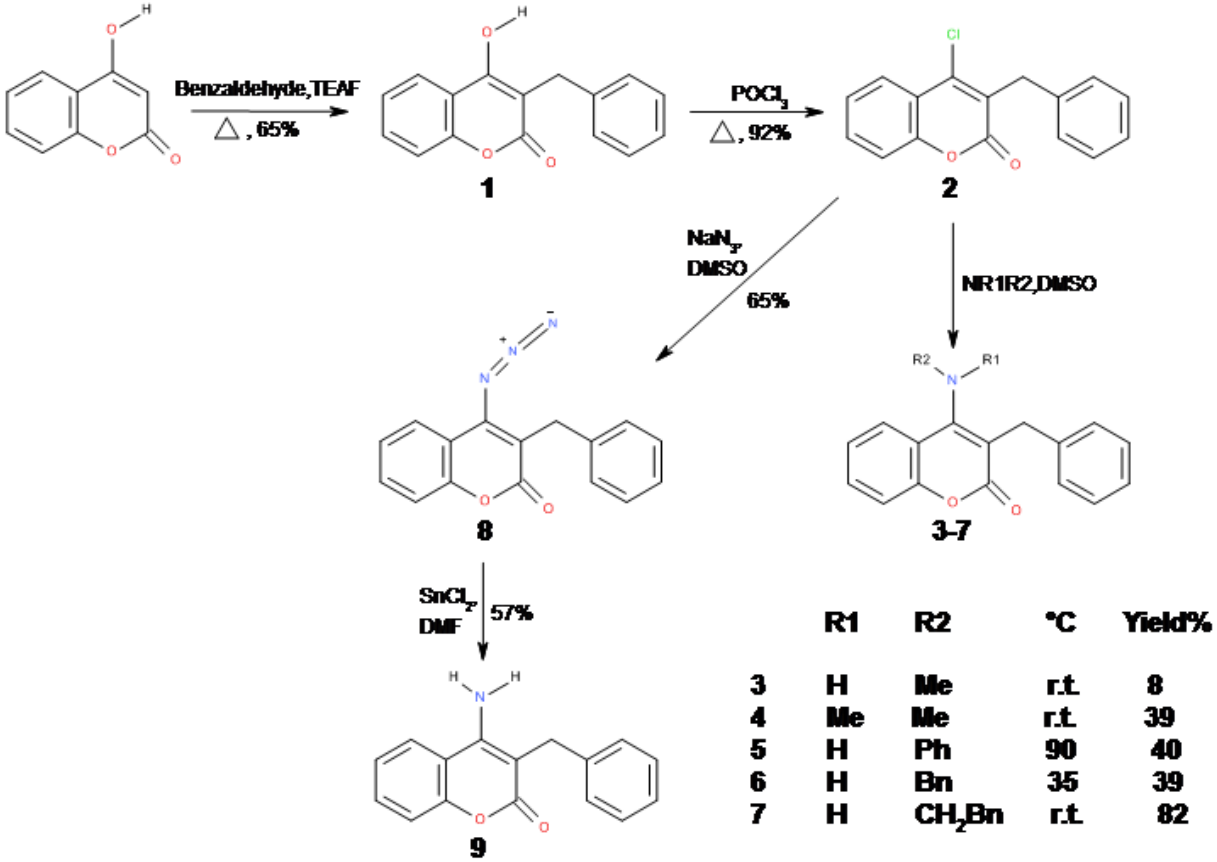


Figure 6. 4-Amino-3-benzylcoumarin derivatives.

The flexibility of the hydrophobic pocket in the binding site allows the expansion in the 4-position of the coumarin. For this reason we have synthetized six compounds (**4-8**,**10**) starting from the 4-amino derivative and increasing the length of the amino-alkyl/aromatic chain. Tested compounds have been prepared according to Scheme 1.



	R1	R2	°C	Yield%
3	H	Me	r.t.	8
4	Me	Me	r.t.	39
5	H	Ph	90	40
6	H	Bn	35	39
7	H	CH_2Bn	r.t.	82

Scheme 1. Synthesis of 4-amino-3-benzylcoumarin derivatives.

Probing 4-amino-8-azacoumarins as novel *pf*DHODH inhibitors

We also wanted to investigate if a change in the aromatic ring of the coumarin scaffold could be beneficial for inhibitors. The first idea was to introduce a nitrogen in the 8-position of the coumarin, as in that area the arginine residue of the binding site interact with the oxygen from the ring. Hence, the presence of another H-bond acceptor atom may increase the affinity between the scaffold and the active site. To investigate this hypothesis, molecular modeling was performed on 4-(benzylamino)pyrano[2,3-b]pyridin-2-one, comparing the position of the investigated 8-azacoumarin with DSM1.

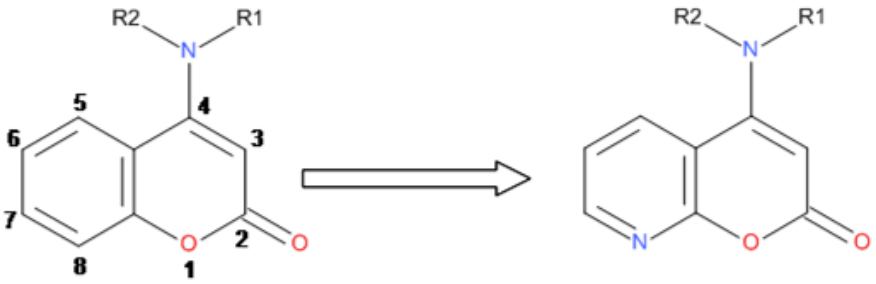


Figure 7. 4-Amino-8-azacoumarin derivatives.

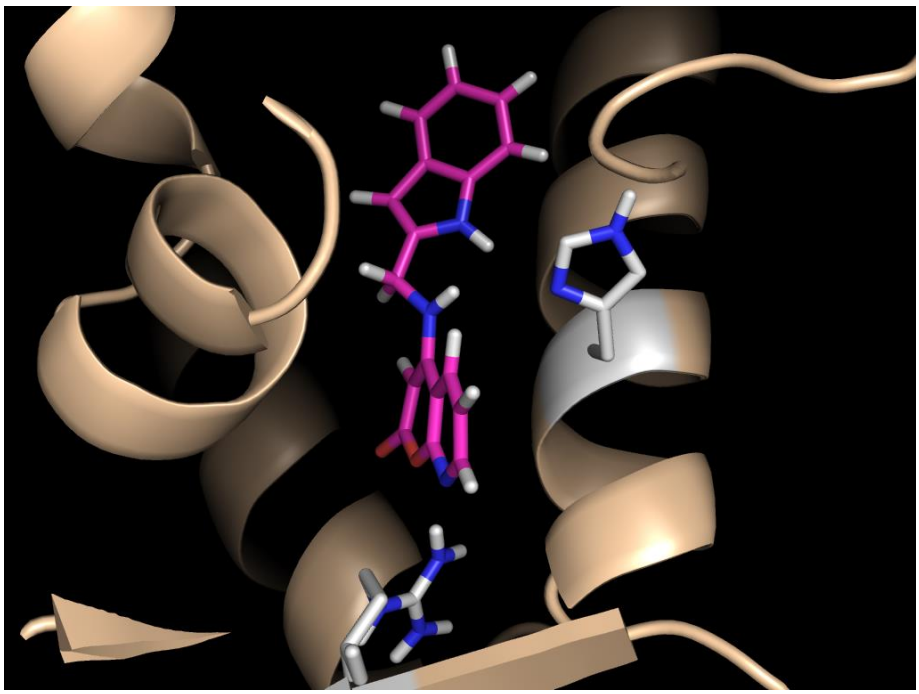


Figure 8. Predicted binding mode of compound (**16**) with the protein. In purple is depicted the inhibitor, in grey the arginine and the histidine residues; Oxygen atoms are displayed in red and nitrogen atoms in blue.

As shown from the modeling (Figure 8), the aza-compound fits well in the binding site and the new presumed inhibitor occupies the same position of DMS1, ensuring the correct position of the amino-nitrogen and the oxygen involved in the H-bond. From the modeling, it seems that the arginine residue may stand at the same distance from the pyridine-

nitrogen and the ring oxygen, so two different binding modes are possible: in case the H-bond involves the nitrogen instead of the oxygen, a shift of the molecule toward the mouth of the pocket may be considered (Figure 9). Previous studies showed that the benzyl-and the indol-derivatives of 4-aminocoumarin are micro molar inhibitors (IC_{50} around 12µM and 0.6µM, respectively) of *pf*DHODH (material unpublished). We wanted to investigate if the benzyl-, the indol- and the benzimidazol- derivatives of 4-amino-8-azacoumarin are more or less potent of the equivalent 4-aminocoumarin derivatives.

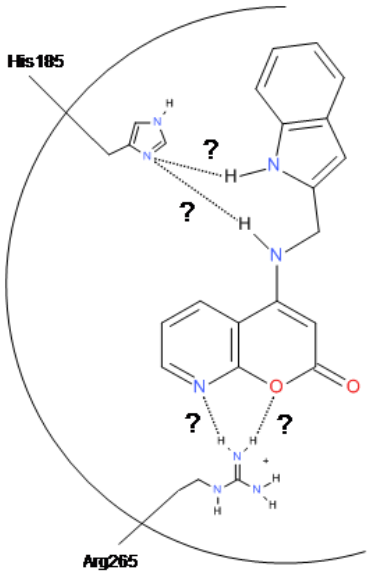
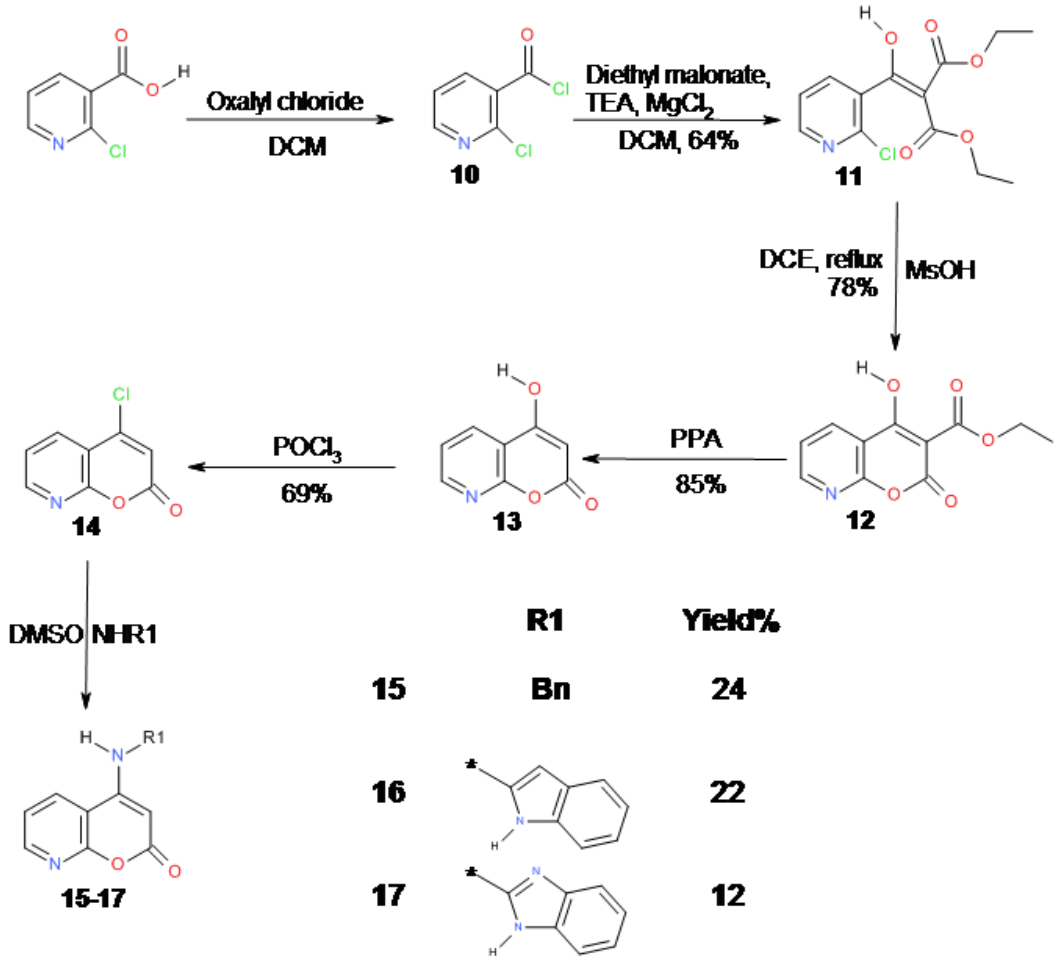


Figure 9. The picture shows the different interactions which may occur between the indol derivative and the binding site.

The azacoumarin-derivatives were synthetized according to Scheme 2.



Scheme 2. Synthesis of 4-amino-8-azacoumarin derivatives.

Conclusion

In summary, *in silico* design was performed in the search of potent *pf*DHODH inhibitors. The designed 3-benzyl-4-aminocoumarins were found to be inactive probably due to the steric hindrance of the aromatic moiety in 3-position. 4-Amino-8-azacoumarin derivatives will be tested soon. All the derivatives will be also tested with the human enzyme.

Chemistry

Unless otherwise noted, starting materials and reagents were obtained from commercial suppliers. NMR spectra were recorded on a Bruker Ultra Shield ^{TM} 400Plus spectrometer. Chemical shifts (δ), determined from residual solvent peaks, are reported in ppm relative to TMS while Js are in Hz. All the compounds were confirmed by mass spectrometry recorded on Waters Micromass Q-Tof (ESI+ ionization $[M+H]^+$). All the reactions were performed under nitrogen atmosphere and dry solvents were obtained from MBraun SPS-

800 solvents dispenser. All final compounds had a purity ≥95% (254nm) recorded on a Hewlett Packard series 1100 HPLC, column XTerra^{TM} MS C18 2.5µm, 2.1x50mm and when necessary, compounds were purified using a Gilson preparative HPLC, column Zorbax SB-C18 9.4mmx25cm.

Experimentals

3-Benzyl-4-hydroxy-chromen-2-one (**1**):[xviii]

Benzaldehyde (1.78 g, 0.017 mol) and 4-hydroxychromen-2-one (2.7 g, 0.017 mol) were added to triethylamine formate (18ml) prepared as follow: triethylamine (48.6 g, 0.48 mol) was added dropwise to formic acid (55.2 g, 1.2 mol) in an ice-bath. The reaction mixture were stirred and heated between 140-150 °C for 2 h. The reaction is allowed to cool around 50 °C and then it is poured into cold water (30 mL). The water is then acidified to pH 2 with 6M HCl. The suspension is stirred for 15' then the solid is filtered obtaining a light brown crude product that was crystallized from ethanol giving the desired product (2.745 g, 65% yield); ^{1}H-NMR(DMSO-d^{6}): 11.67(s, 1H); 7.98(d, 1H J=8.4Hz); 7.61(dd, 1H J=7.3-8.4Hz); 7.36(m, 2H); 7.25(m, 4H); 7.16(m, 1H); 3.89(s, 1H). ^{13}C-NMR(DMSO-d^{6}): 163.6; 162.2; 152.7; 140.4; 132.5; 128.8; 128.7; 126.4; 124.5; 123.9; 116.7(2C); 104.6; 29.1;

3-Benzyl-4-chloro-chromen-2-one (**2**):

3-Benzyl-4-hydroxy-chromen-2-one (2.485 g, 9.8 mmol) were heated to 100 °C in POCl3 (9 mL) for 6hours, then the excess of POCl3 was distilled off and the residues were dissolved in Ethyl acetate (40 mL). The organic phase was washed with diluted NaOH repeatedly, then washed with brine and dried over sodium sulfate. The solvent was then evaporated in vacuo giving a pale pink crude product that was purified by flash chromatography with toluene as eluent obtaining the pure product (2.442 g, 92% yield); ^{1}H-NMR($CDCl_3$): 7.88(dd, 1H J=8.54-1.54Hz); 7.55(ddd, 1H J=8.22-7.42-1.56Hz); 7.42(m, 2H); 7.31(m, 4H); 7.22(t, 1H J=7.3Hz); 4.16(s, 2H); ^{13}C-NMR($CDCl_3$): 160.8; 152.2; 146.3; 137.9; 132.6; 129.4; 129.0; 127.2; 127.0; 126.1; 125.1; 119.0; 117.0; 34.1; ESI-MS $[M+H]^+$: 271.

General procedure for amine derivatives (**3-7**)**:**[xix]

To DMSO (2.8 mL) was added 3-benzyl-4-hydroxy-chromen-2-one (250 mg, 0.92 mmol). Then 3eq of the corresponding amine were added to the suspension and the mixture was stirred at the required temperature (see below). The reaction was then cooled to room temperature and cold water (10 mL) was added obtaining a precipitate. The precipitate was filtered obtaining a crude solid that was purified by flash chromatography.

3-Benzyl-4-(methylamino)chromen-2-one (**3**):

The reaction was performed according to general procedure above. Reaction temperature and time: room temperature, 3h. Yield: 8%. ^{1}H-NMR($CDCl_3$): 7.69(dd, 1H J=8.14-1.29Hz); 7.49(ddd, 1H J=8.52-7.28-1.38Hz); 7.34(dd, 1H J=8.31-1.14Hz); 7.29-7.16(m, 6H); 4.10(s, 2H); 3.13(s, 3H); ^{13}C-NMR($CDCl_3$): 164.5; 153.7; 153.4; 140.2; 131.5; 129.1; 128.2; 126.7; 123.6; 123.3; 118.0; 116.2; 100.2; 34.3; 31.1; ESI-MS $[M+H]^+$: 266.

3-Benzyl-4-(dimethylamino)chromen-2-one (**4**):

The reaction was performed according to general procedure above. Reaction temperature and time: room temperature, 8h. Yield: 39%. ^{1}H-NMR($CDCl_3$): 7.68(dd, 1H J=8.07-1.54 Hz); 7.48(ddd, 1H J=8.68-7.21-1.57 Hz); 7.35(dd, 1H J=8.27-1.23 Hz); 7.29-7.16(m, 6H); 4.10(s, 2H); 2.95(s, 6H). ^{13}C-NMR($CDCl_3$): 164.9; 159.2; 153.5; 140.7; 131.1; 128.8; 128.3; 126.5; 126.1; 123.8; 119.8; 117.5; 115.9; 43.3; 32.4. ESI-MS $[M+H]^+$: 280.

4-Anilino-3-benzyl-chromen-2-one (**5**):

The reaction was performed according to general procedure above. Reaction temperature and time: 90°C, 6 days (1 eq. of sodium carbonate was added to the mixture to increase the attack from the aniline). Yield: 40%. ^{1}H-NMR($CDCl_3$): 7.44(ddd, 1H J=1.51-7.15-8.57 Hz); 7.36(dd, 1H J=1.02-8.34 Hz); 7.32-7.20(m, 8H); 7.02(m, 2H); 6.81(dd, 2H J=0.63-7.55 Hz); 4.04(s, 2H). ^{13}C NMR ($CDCl_3$): δ 163.9; 153.7; 149.0; 142.6; 138.8; 131.6; 129.9; 129.4; 128.5; 127.2; 125.9; 123.7; 123.6; 120.4; 117.6; 116.6; 112.6; 31.6. ESI-MS $[M+H]^+$: 328.

3-Benzyl-4-(benzylamino)chromen-2-one (**6**):

The reaction was performed according to general procedure above. Reaction temperature and time: 35°C, over night. Yield: 39%. ^{1}H-NMR($CDCl_3$): 7.59(dd, 1H J=8.17-1.19 Hz); 7.43(ddd, 1H J=7.24-7.04-1,46 Hz); 7.33-7.23(m, 3H); 7.20-7.06(m, 9H); 4.48(s, 2H); 3.90(s, 2H).^{13}C NMR ($CDCl_3$) δ 164.3; 153.6; 153.3; 139.8; 138.8; 131.6; 129.5; 129.2; 128.5; 128.2; 127.7; 126.8; 123.8; 123.6; 118.1; 116.2; 102.8; 51.4; 31.2. ESI-MS $[M+H]^+$: 342.

3-Benzyl-4-(2-phenylethylamino)chromen-2-one (**7**):

The reaction was performed according to general procedure above. Reaction temperature and time: room temperature, over night. Yield: 82%. ^{1}H-NMR($CDCl_3$): 7.54(dd, 1H J=1.38-

8.14 Hz); 7.48(ddd, 1H J=1.46-7.25-8.56 Hz); 7.36-7.16(m, 8H); 7.11(dd, 4H J=1.09-7.73 Hz); 3.92(s, 2H); 3.72(t, 2H J=6.88 Hz); 2.76(t, 2H J=6.86 Hz). ^{13}C NMR ($CDCl_3$): δ 164.3; 153.6; 153.5; 139.7; 137.9; 131.5; 129.4; 129.2; 128.3; 127.5; 126.8; 123.8; 123.6; 118.0; 116.4; 102.7; 48.2; 36.8; 31.1. ESI-MS $[M+H]^+$: 356.

4-Azido-3-benzyl-chromen-2-one (**8**):

3-Benzyl-4-chloro-chromen-2-one (300 mg, 1.1 mmol) were dissolved in dry DMF (5 mL), then sodium azide (107 mg, 1.6 mmol) was added to the solution. The mixture was stirred at room temperature over-night. Cold water (50 mL) was added to the mixture and the suspension was stirred for 20', then the precipitate was filtered and washed with water. A yellow solid was obtained (285 mg, Yield: 65%). ^1H-NMR($CDCl_3$): 7.66(dd, 1H J=1.33-8.05 Hz); 7.40(m, 1H); 7.24-7.03(m, 7H);3.99(s, 2H). ^{13}C NMR ($CDCl_3$): δ 162.5; 152.7; 146.2; 138.7; 132.6; 129.1; 128.6; 127.1; 124.8; 123.6; 118.2; 117.5; 116.8; 31.0. ESI-MS $[M+H]^+$: 278.

4-Amino-3-benzyl-chromen-2-one (**9**):[xx]

To a stirred solution of stannous chloride (205 mg, 1.08 mmol) in methanol (2.5 mL), 4-azido-3-benzyl-chromen-2-one (150 mg, 0.54 mmol) was added portion wise. The suspension was stirred at room temperature for 30' then cold water (15 mL) was added to the reaction and the mixture was stirred for 15' in an ice-bath. The precipitate was filtered and washed with water, obtaining a crude product that was purified by flash chromatography (eluent toluene/ethyl acetate 8:2) obtaining a pure product (69 mg, Yield: 57%). ^1H-NMR(DMSO): 8.06(d, 1H J=7.57 Hz); 7.57(t, 1H J=7.25 Hz); 7.40-7.05(m, 9H); 3.81(s, 2H). ^{13}C NMR (100 MHz, DMSO): δ 162.9; 152.9; 151.6; 140.8; 132.0; 128.6; 128.6; 126.2; 123.9; 123.6; 117.0; 115.2; 95.5; 29.4. ESI-MS $[M+H]^+$: 252.

2-Chloropyridine-3-carbonyl chloride (**10**):[xxi]

To a suspension of 2-chloropyridine-3-carboxylic acid (2 g, 13 mmol) in dry DCM (15 mL), was added dry DMF (0.1 mL) and oxalyl chloride (2.1 mL, 26 mmol) drop wise. the suspension was stirred at room temperature for 2h then the volatiles were removed under reduced pressure obtaining a pale-yellow crude product which was used for the next step without purification. The reaction was checked by TLC and ESI-MS, quenching a sample from the mixture in methanol. ESI-MS (sample + methanol) $[M+H]^+$: 172.

2-[(2-chloro-3-pyridyl)-hydroxy-methyl]malonic acid diethyl esther (**11**):[xxii]

Dry DCM (30 mL) was added to anhydrous magnesium chloride (870 mg, 9.1 mmol), then diethyl malonate (2.4 mL, 15.6 mmol) and triethylamine (4.3 mL, 31.2 mmol) were added. The mixture was stirred at room temperature for 1h, then a solution of 2-chloropyridine-3-carbonyl chloride (2.28 g, 13 mmol) in dry DCM (15 mL) was added drop wise and the mixture was stirred for 4h. the reaction was then poured in cold water (40 mL) and the water phase was extracted with DCM. The organic phases were collected, washed with brine and dried over sodium sulfate. The solvent was removed under reduced pressure obtaining a crude product which was purified by flash chromatography (eluent DCM/methanol 98:2). the pure product obtained is a red oil (2.49 g, Yield: 64%).^{1}H-NMR($CDCl_3$): 13.84(s, 1H); 8.45(dd, 1H J=4.86-1.94 Hz); 7.68(dd, 1H J=7.58-1.94 Hz); 7.30(dd, 1H J=7.58-4.86 Hz); 4.37(q, 2H J=7.13 Hz); 3.95(q, 2H J=7.13 Hz); 1.37(t, 3H J=7.14 Hz); 0.90(t, 3H J=7.14 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 174.9; 171.7; 164.7; 151.0; 149.0; 138.4; 131.4; 122.3; 102.9; 62.3; 61.0; 13.7; 13.3. ESI-MS $[M+H]^+$: 300.

Ethyl 4-hydroxy-2-oxo-pyrano[2,3-b]pyridine-3-carboxylate (**12**):[xxiii]

Diethyl 2-[(2-chloro-3-pyridyl)-hydroxy-methyl]propanedioate (4.17 g, 14 mmol) was dissolved in DCE (20 mL), then methansulfonic acid (0.1 mL) was added and the reaction was stirred at reflux for 90'. The volatiles were removed under reduced pressure obtaining a crude product which was purified by trituration with water obtaining the pure product (2.55 g, Yield: 78%). ^{1}H-NMR($CDCl_3$): 14.75(s, 1H); 8.68(dd, 1H J=4.76-1.98 Hz); 8.39(dd, 1H J=7.77-1.98 Hz); 7.38(dd, 1H J=7.77-4.76 Hz); 4.51(q, 2H J=7.13 Hz); 1.46(t, 3H J=7.13 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.3; 172.2; 159.6; 156.8; 155.2; 135.7; 121.4; 110.3; 94.1; 63.2; 13.8. ESI-MS $[M+H]^+$: 236.

4-Hydroxypyrano[2,3-b]pyridin-2-one (**13**):[xxiv]

Ethyl 4-hydroxy-2-oxo-pyrano[2,3-b]pyridine-3-carboxylate (2.54 g, 11 mmol) was added to polyphosphoric acid (20 g) and the mixture was heated to 127 °C (the reaction develops gas). When the mixture turned homogeneous and did not develop further gas, 50ml of water were added. The polyphosphoric acid dissolved slowly while a solid started to precipitate. The suspension was stirred in an ice-bath then the solid was filtered and washed with water and DCM, obtaining the product (1.5 g, Yield: 85%). ^{1}H-NMR(400 MHz, DMSO): 12.86(s, 1H); 8.57(dd, 1H J=4.74-1.86 Hz); 8.26(dd, 1H J=7.68-1.86 Hz); 7.45(dd, 1H J=7.68-4.78 Hz); 5.64(s, 1H). ^{13}C NMR (100 MHz, DMSO): δ 165.9; 162.1; 159.2; 152.0; 134.1; 121.5; 111.8; 92.0. ESI-MS $[M+H]^+$: 164.

4-Chloropyrano[2,3-b]pyridin-2-one(**14**):[xxv]

4-Hydroxypyrano[2,3-b]pyridin-2-one (400 mg, 2.4 mmol) was added to phosphorous oxychloride (5 mL) and the reaction was stirred at 100 °C for 15h. Part of the excess of the $POCl_3$ was removed by distillation and the residue was dispersed in DCM (50 mL). Then,

water (50 mL) was added and sodium hydroxide 5M was added until the pH was around 9. The water phase was extracted with DCM, the organic phases collected, washed with brine and dried over sodium sulfate. The solvent was removed under reduced pressure obtaining a crude product which was purified by flash chromatography on a silica plug (eluent DCM) obtaining a pure compound (308mg, Yield: 69%). ^{1}H-NMR(400 MHz, $CDCl_3$): 8.61(dd, 1H J=4.78-1.80 Hz); 8.26(dd, 1H J=7.79-1.83 Hz); 7.42(dd, 1H J=7.79-4.80 Hz); 6.69(s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 158.7; 158.2; 152.6; 148.9; 135.7; 121.8; 117.1; 114.0. ESI-MS $[M+H]^+$: 182.

4-(Benzylamino)pyrano[2,3-b]pyridin-2-one (**15**):[xix]

4-Chloropyrano[2,3-b]pyridin-2-one (100 mg, 0.55 mmol) were dissolved in dry DMSO (1.5 mL). Benzylamine (0.18 mL, 1.6 mmol) was then added and the reaction was stirred for 2h at room temperature. Cold water (4 mL) was added to the reaction and the precipitate was filtered, obtaining a crude product which was purified by flash chromatography (eluent DCM/methanol 96:4). A pure compound was obtained (33 mg, Yield: 24%). ^{1}H-NMR(400 MHz, DMSO): 8.61(dd, 1H J=7.90-1.70 Hz); 8.57-8.48(m, 2H); 7.47(dd, 1H J=7.86-4.74 Hz); 7.42-7.32(m, 4H); 7.27(m, 1H); 5.13(s, 1H); 4.53(d, 2H J=5.85 Hz). ^{13}C NMR (100 MHz, DMSO): δ 161.8; 158.8; 153.3; 151.4; 138.1; 133.3; 129.1; 127.8; 127.6; 120.8; 110.4; 83.2; 45.7. ESI-MS $[M+H]^+$: 253.

4-(1H-Indol-2-ylmethylamino)pyrano[2,3-b]pyridin-2-one (**16**):[xix]

4-Chloropyrano[2,3-b]pyridin-2-one (70 mg, 0.39 mmol) was dissolved In dry DMSO (1 mL), then 1H-indol-2ylmethanamine (113 mg, 0.8 mmol) was added and the reaction was stirred for 2.5h at room temperature. Cold water (4 mL) was added to the reaction and the precipitate was filtered obtaining a crude product which was purified by trituration in DCM obtaining a pure compound (25 mg, Yield: 22%). ^{1}H-NMR(400 MHz, DMSO): 11.08(s, 1H); 8.61(dd, 1H J=7.96-1.75 Hz); 8.54(dd, 1H J=4.74-1.73 Hz); 8.40(t, 1H J=5.49 Hz); 7.52-7.43(m, 2H); 7.34(dd, 1H J=0.81-8.04 Hz); 7.04(ddd, 1H J=8.16-7.09-1.21 Hz); 6.96(ddd, 1H J=7.98-7.14-1.04 Hz); 6.39(d, 1H J=1.07 Hz); 5.30(s, 1H); 4.66(d, 2H J=5.39 Hz). ^{13}C NMR (100 MHz, DMSO): δ 161.9; 158.8; 153.4; 151.4; 136.8; 135.6; 133.5; 128.5; 121.4; 120.8; 120.2; 119.5; 111.6; 110.4; 100.1; 83.2; 40.2. ESI-MS $[M+H]^+$: 292.

4-(1H-Benzimidazol-2-ylmethylamino)pyrano[2,3-b]pyridin-2-one(**17**):[xix]

4-Chloropyrano[2,3-b]pyridin-2-one (70 mg, 0.39 mmol) was dissolved in dry DMSO (1 mL), then 1H-benzimidazol-2-ylmethanamine (172 mg, 0.8 mmol) was added and the reaction was stirred at room temperature over-night. Cold water (4 mL) were then added to the reaction and the precipitate was filtered obtaining a crude product which was triturated twice in DCM obtaining a more pure product (14 mg, Yield: 12%).^{1}H-NMR(400 MHz, DMSO): 12.44(s, 1H); 8.67-8.51(m, 3H); 7.65-7.44(m, 3H); 7.16(m, 2H); 5.24(s, 1H);

4.76(d, 2H J=5.64 Hz). ^{13}C NMR(100 MHz, DMSO): δ161.9; 158.8; 153.8; 151.6; 151.5; 133.6; 122.3; 120.9; 110.5; 83.5; 41.0. ESI-MS $[M+H]^+$: 293.

*pf*DHODH inhibition assays

For determination of inhibition and IC_{50} values of *Plasmodium falciparum* DHODH the recombinant enzyme was used in an *in vitro* enzyme assay with N-terminally truncated recombinant DHODH[xxvi]. The assay is based on coupling of the ubiquinone reduction to the redox dye 2,6-dichloroindophenol (DCIP)[xxvii]. The reduction of DCIP was monitored photometrically by decreasing absorption at 600nm. The test solutions contained 60µM DCIP, 150 mM KCl, 50mM TRIS/HCl pH 7.8, 0.1% Triton X-100, 20µM decylubiquinone and 200µM DHO. Synthesized compounds were dissolved in DMSO and normally added to a final concentration of 1% DMSO. A higher concentration of DMSO, 5%, was used to prevent precipitation and measure inhibition of compounds with IC_{50} ‘s higher than 100µM. No inhibition of enzyme function was found in the control containing 5% DMSO.

Molecular modeling

The Schrodinger 2013 software suite was used for molecular modeling. Energy minimization was performed with Macromodel, utilizing the OPLS-2005 force field and the GB/SA model for water solvation. The Protein Preparation Wizard was utilized on the crystal structure, to add hydrogens, assign charges, and optimize hydrogen bond networks. The crystal waters with hydrogen bond interactions to the FMN prosthetic group were included in the model. Pictures were obtained from PyMOL.

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