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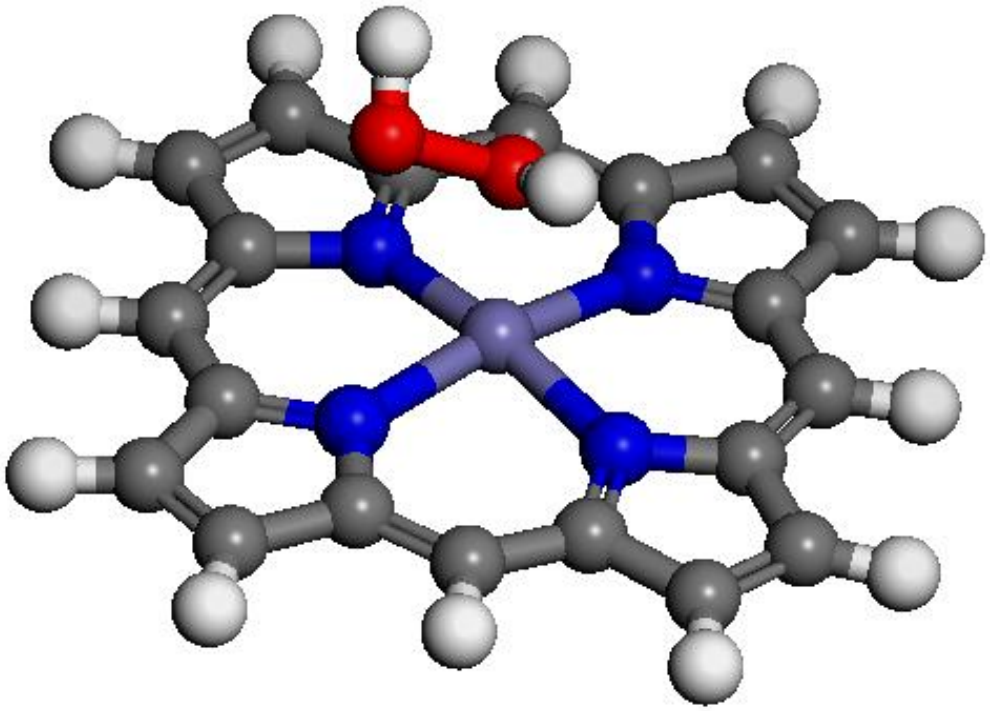

Inhibition of Enzymatic Degradation of Hydrogen Peroxide in Recycled Paper Pulp

A Quantum Chemical Calculations Study on Inhibition Strength

Bachelor's thesis in Chemical Engineering

Markus Enmark

Department of Chemistry and Biotechnology
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2014

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Cover:
[A model of a heme group inhibited by Hydrogen peroxide, the model was used in the quantum chemical calculations.]

Göteborg, Sweden 2014

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This thesis was performed as a part of the bachelor's degree in chemical engineering at Chalmers University of Technology starting March 17th and ending May 28th.

Markus Enmark
Göteborg, Sweden 2014

Abstract

The increasing environmental awareness all over the world is leading to increased recycling of paper. Recycling of paper pulp is a process carried out under mild conditions regarding temperature and pH. These mild conditions help to protect the recycled paper pulp from degrading during the process but it is also the underlying reason to the problem handled in this thesis. The conditions are very favorable for bacteria in the pulp, the bacteria present produce enzymes which are able to degrade the bleaching chemical hydrogen peroxide. Due to the degradation processes needs to add larger quantities of hydrogen peroxide to reach the desired level of whiteness. Some methods exist that are able to reduce this degradation but many are outdated and make use of hazardous chemicals.

This thesis investigates some of the methods used in bleaching of secondary fibers today such as usage of biocides, cross-linking, usage of formaldehyde releasing chemicals and active site inhibitors. Focus is put on active site inhibitors and quantum chemical calculations were made on four groups of inhibitors. The groups are: known inhibitors, inhibitors found from the literature study, inhibitors found from malaria research and potential new inhibitors. The primary goal was to see if quantum chemical calculations can be made to get a perception on which of the molecules that have the best inhibition properties. It is also of interest to see if a new inhibitor can be found to have inhibition properties better that those that have been presented and used before. Such molecules will be presented as possible leads for further studies.

It is known that the enzymes able to degrade hydrogen peroxide have similar structure. Many of them have a heme-group in the active site, thus this was the active site that calculations were based on. The enzymes have a small channel connecting the surface of the enzyme with the active site. This channel, called the substrate channel is 20 Å wide at the beginning and 3-5 Å wide at the end. This restricts the number of inhibitor available for usage significantly.

Results from calculations indicate that quantum chemical calculations might be a valuable tool to use when looking for potential inhibitors. The results showing inhibition strength, inhibition length and size of inhibitors also left several leads of molecules interesting for further studies. From the literature, urea and trimethylamine-N-oxide were found to have interesting properties. From the new potential inhibitors piperidine was found to have interesting properties, piperidine also turned out to be very toxic and thus might be improper for usage on industrial scale.

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1. Introduction

1.1 Background

The paper pulp industry, with an estimated annual production of about 380 million tons of paper pulp products worldwide, is a huge export factor for many countries [1]. The road from tree to paper is a relatively complex process carried out in a variety of processes optimized on account for the raw material used, the natural condition of the pulp, economy and the desired quality of output paper. The techniques used in the production processes have changed and improved ever since the beginning of the 19th century when man started using trees as raw material in the paper industry [2].

The technique used in the production process often depends on the purpose of the paper as well as economy and environmental rules and regulations. This thesis will not deal with all the different production techniques but will instead focus on recycling and the de-inking process of secondary fibers in particular. Secondary fibers contribute with a significant part of the produced paper pulp in the industry nowadays. In Europe e.g. 64.5% of all produced paper is said to be recycled, this contributes to less deforestation which of course is desirable [3]. The downside of the increasing recycling is that the de-inking phase is carried out with a lot of chemical additives which in many cases are harmful for human health and the environment. It is therefore of great interest to improve this process in every way possible. There are several ways to bleach the secondary fibers and one of the more environmentally benign processes involves bleaching with hydrogen peroxide.

One of the problems that occur when H_2O_2 is used is that it is decomposed to H_2O by certain enzymes that exist in the paper pulp. To reach the desired level of whiteness it is necessary to add more hydrogen peroxide than would have been necessary if the decomposition was not catalyzed by these enzymes [4]. It has been shown that the enzymes that naturally exist in the secondary fibers are catalase, peroxidase and haloperoxidase [5].

Several patents have existed that could obstruct the decomposition of hydrogen peroxide. BIM Kemi held 2 such patents, nowadays these patents have expired and are considered to contain hazardous chemicals.

1.2 Purpose

The starting point of this report is the licentiate thesis by Heino Kuusk *Enzymatic Degradation of Hydrogen Peroxide in a Recycled Paper Pulp Mill* [5]. By a literature study it will be examined if there is any further research in the field of enzymatic decomposition of hydrogen peroxide present at this point. Furthermore quantum chemical calculations will be carried out to examine if any environmentally benign chemicals can be found which can serve as inhibitors to the active site in the enzymes.

1.2.1 Research questions

Which methods are used today to decrease the decomposition of hydrogen peroxide in secondary fibers?

Is it possible to find a theoretically working inhibitor by using quantum chemical calculations?

How are the calculated inhibitors compared to those already in use, regarding bond energy, toxicity and availability?

1.2.2 Limitations

Results from the quantum chemical calculations will not be tested experimentally.

The active center will be limited to the heme group.

2. Theory

2.1 Bleaching methods

Largely speaking, the bleaching of paper pulp can be divided into two different processes. There are the mechanical pulp and the chemical pulp process. Mechanical pulp is the least environmentally harmful process and yields a higher paper pulp output ratio to the base wood input. The downside is the paper quality and brightness. This is because the fibers in the pulp are shortened in the process which leads to less consistent paper. Through the chemical pulp process it is easier to obtain better standards since it uses different chemicals to break down the lignin in the pulp while still maintaining the fiber length. Regardless of this, the mechanical pulp production has increased in production as of lately due to better production techniques [6].

Three different bleaching methods are used in the chemical pulp industry. They are classified after how much chlorine that is added in the bleaching. The most common method 1990 was bleaching with elementary chlorine. Obviously chlorine is very hazardous for both environment and human health, therefore it was slowly started to be replaced by Elementary Chlorine Free (ECF) bleaching which is the dominating process of today. The third method is called Totally Chlorine Free (TCF) bleaching; this method is as the name indicates free from both elementary chlorine and chlorine compounds [7][8]. One might think that TCF would be the most environmentally friendly process but this is not completely true. Studies shows that ECF leaves no bio accumulative footprints on the environment since the chemical compounds released in the wastewater can be degraded. Scientists still disagree about which one of TCF and ECF is the most preferable process. Some of the reasons why most paper pulp plants use the ECF are higher pulp yield, increased production, higher brightness, better pulp quality, simple production and customer demands [7].

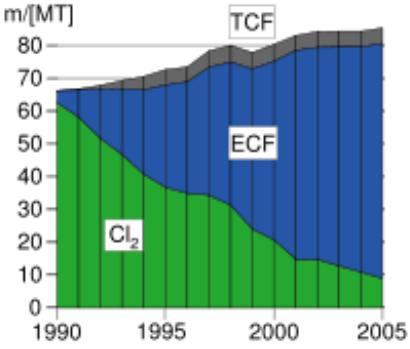


Figure 1 [8]

Shows how elementary chlorine free processes such as TCF and ECF have increased since 1990.

2.2 The deinking and bleaching process of secondary fibers

The market for secondary fibers is constantly growing with the increased consciousness for the environment. Just as with the bleaching of virgin fibers, secondary fibers have been transitioned into more environmentally benign methods lately. Recycled paper must go through a procedure roughly consisting of some mechanical methods such as filtration and

dispersion to get the pulp free from impurities. After that comes the chemical method consisting of flotation, washing and bleaching [9].

There are some problems with secondary fibers that restrict the usage of it. One of the most important downsides with recycled paper is that it cannot go through the process more than 4-6 times before the paper quality gets too poor. The reason is that every time the pulp gets treated the fibers are degraded and the paper quality gets poorer [9].

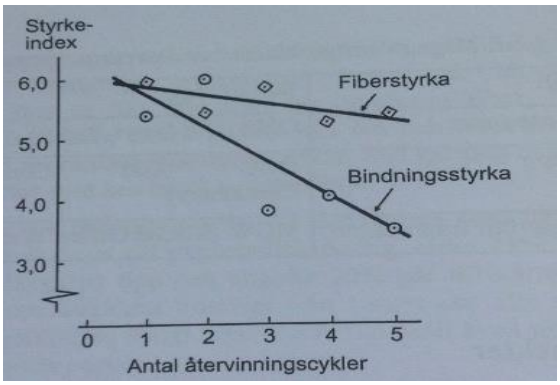


Figure 2 [9]

Shows how fiber strength and binding strength decreases with the number of recycling cycles.Tthe y-axis illustrates an index and the x-axis illustrates the number of cycles.

To counter this degradation the bleaching is carried out under mild conditions, for example the temperature is usually about 40 °C [10]. Unfortunately this temperature favors bacteria in the pulp and these bacteria contain the earlier mentioned hydrogen peroxide degrading enzymes [11]. The bacterium also makes it unsuitable to use this pulp in the production of food containers [9]. Another downside is that chemical and mechanical pulp is merged in the process and that makes the paper quality poorer and harder to control.

There are several different ways to bleach secondary fibers but the most common methods of today involve hydrogen peroxide in the bleaching.

2.3 Degradation of hydrogen peroxide

The problem that is investigated in this report is the degrading of hydrogen peroxide. Although this reaction is undesirable in bleaching processes it is actually a life essential mechanism taking place in all living cells to protect us from oxidative stress. The generic name of enzymes capable of degrading hydrogen peroxide is oxidoreductases. Furthermore these oxidoreductases can be divided into some different subgroups which all contain numerous of different degrading enzymes. There is the catalase group, the peroxidase group and the haloperoxidase group [5].

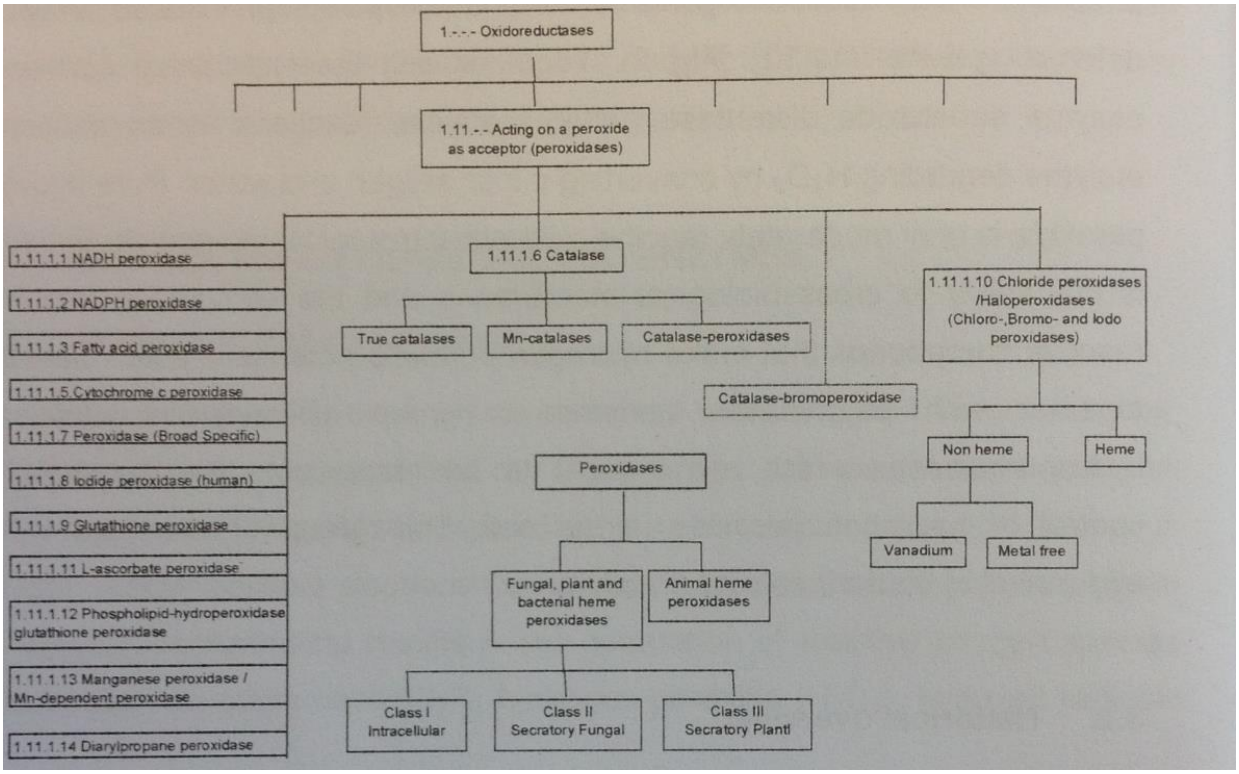


Figure 3 [5]

Shows the classification of the enzymes in the oxidoreductase family.

The majority of the degrading enzymes have a heme group in their active site; this heme group is the catalytic center which is the reason to the degradation. Although this report focus on the heme-group there are several other active sites as well, for example some sites contain a manganese or copper ion instead [5]. The most common active site is the one with the iron ion and that is why this report will focus on that active site and leave the others for further studies.

The reaction mechanism behind the decomposition is presented in H, Kuusk's report [5].

$$E\text{-}Fe^{III} + H_2O_2 \rightarrow E[\pi^{+\bullet}]\text{-}Fe^{IV}{=}O + H_2O \quad (1)$$

$$E[\pi^{+\bullet}]\text{-}Fe^{IV}{=}O + H_2O_2 \rightarrow E\text{-}Fe^{III} + O_2 + H_2O \quad (2)$$

$$E[\pi^{+\bullet}]\text{-}Fe^{IV}{=}O + HXOH \rightarrow E\text{-}Fe^{III} + XO + H_2O \quad (3)$$

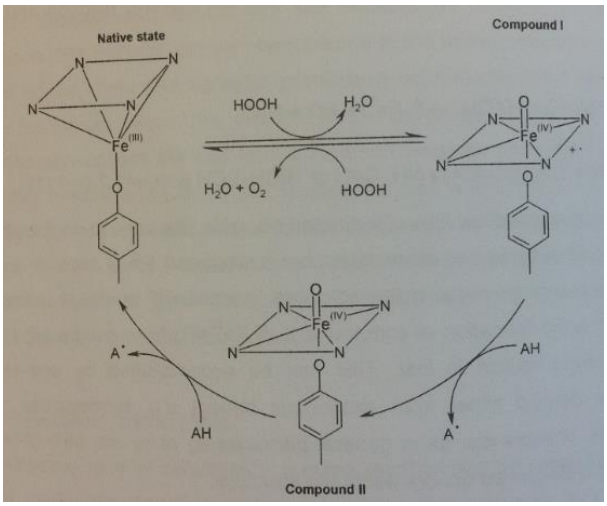


Figure 4 [5]

Illustrates how an inhibitor binds to the active site in the heme group.

This Reaction mechanism takes place at the active site of the enzyme. Notice that reaction (1), (2) and (3) illustrate the complete reaction mechanism. In this thesis focus is put on the first part of reaction (1) where hydrogen peroxide reacts with the heme^{3+} group. It has been found that all the different types of hydrogen peroxide degrading enzymes have similar structures. The similarities are for example that they have a tetrameric form consisting of 4 subunits and each subunit has some particular domains which are representative for all the enzymes [5]. The four domains are the N-terminal domain, the β-barrel domain, the wrapping domain and the α-helix domain. The exact purpose of the domains will not be brought up here since it is of less importance for this thesis work.

One thing that is of great importance though is the substrate channel. It is a narrow channel that connects the surface of the enzyme to the active site where the reaction occurs. The size of the funnel-shaped channel is 20-25 Å at the opening and only 3-5 Å at the end. This small passage is the reason why only small molecules with low polarity can pass through and reach the active site [5]. Hydrogen peroxide is a molecule with these properties, now the aim is to find a similar small molecule that binds to the active site with greater energy than hydrogen peroxide.

2.4 Existing inhibitors

There are several different methods used to prevent the decomposition of hydrogen peroxide at present. Some of the methods are out of date and some are still in use.

2.4.1 Biocides

One method that is used is addition of different biocides. These biocides intend to work on the bacteria itself instead of the enzymes they produce. By adding a biocide to the paper pulp the bacteria present gets killed and thus are unable to produce the hydrogen peroxide degrading enzymes. The biocides used are often environmentally hazardous and it is desirable to replace this method by something more environmentally benign.

2.4.2 Cross-linking

The definition of cross-linking is as the name suggests when a molecule creates a bond between two polymers and thereby binding them together. The cross-linking can occur either in polymers such as plastics, or in proteins e.g. peroxidase, catalase or haloperoxidase. These cross-links prevent the hydrogen peroxide molecule to reach the heme-group and thereby stopping it from being decomposed.

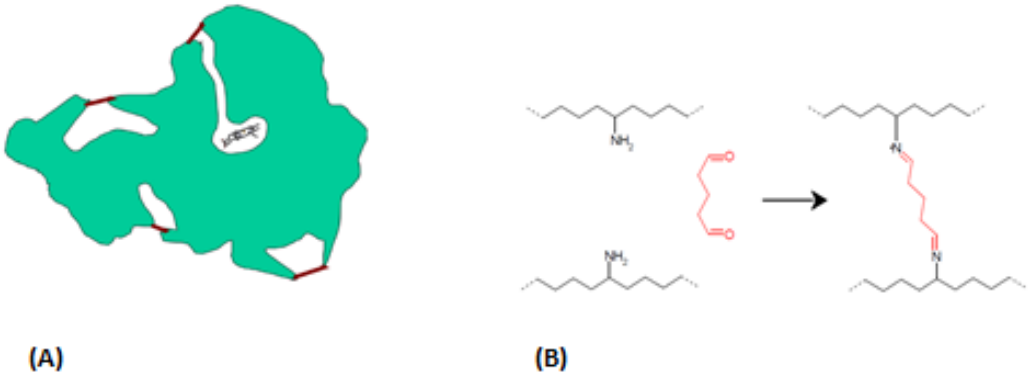


Figure 5 [10]

(A) *Illustrates how a cross-link prevents inhibitors from reaching the active site.*
(B) *Illustrates how a cross-linking molecule can form the cross-link between two polymer chains.*

BIM Kemi had a patent which listed a number of molecules that were able to cross-link the enzymes to reduce its decomposing ability. This patent was granted in 2001 and the molecules stated to cross-link the enzymes were either acetals or di-aldehydes [11].

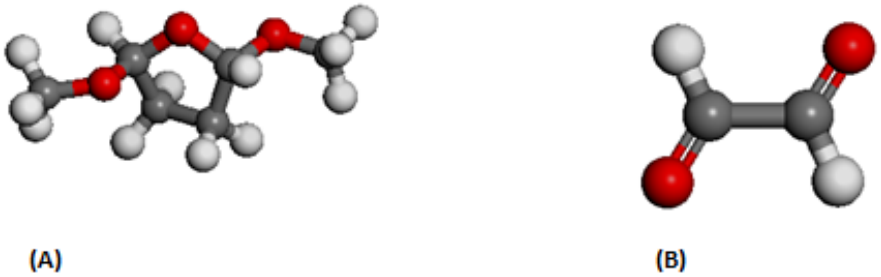


Figure 6 [11]

(A) *Shows an example of an acetal molecule able to cross-link.*
(B) *Shows an example of a di-aldehyde molecule able to cross-link.*

Since this patent was released it has been found by an American company named Buckman Laboratories International that the enzyme also can be inactivated by addition of an aldehyde functional polymer. The polymer proposed is a polyvinyl amide e.g. polyacryl amide or similar. This polymer must react with an aldehyde to produce the desired aldehyde functional polymer [12]. By studying the patent held by Buckman Laboratories it can be found that this patent also aims to inactivate the enzymes through the cross-linking technology [10].

2.4.3 Formaldehyde releasing chemicals

Since the licentiate thesis by Heino Kuusk was published back in 2001 some other innovations have been introduced. For instance BASF Ag holds a patent which aims to inhibit the enzymes by addition of either 1,3,5-Tris-(2-hy-droxyethyl)-1,3,5-hexahydrotriazine (HT) or Imidazo[4,5-d]imidazole-2,5-(1H, 3H)-dione-tetrahydro-1,3,4,6-tetrakis-(hydroxymethyl) (TD). These molecules are by themselves too big to inhibit the active site, so what happens is that they release formaldehyde which inhibits the enzyme [13].

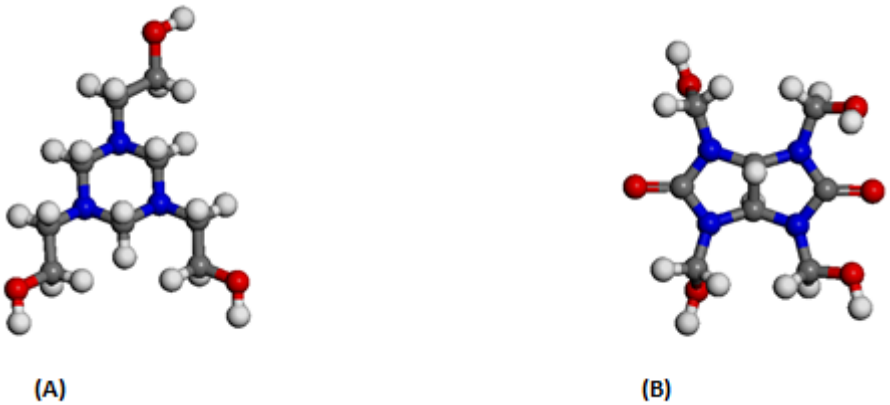


Figure 7 [13]

(A) *HT which is able to release formaldehyde to inhibit enzymes.*
(B) *TD which is able to release formaldehyde to inhibit enzymes.*

2.4.4 Active site inhibitors

In the licentiate thesis by Heino Kuusk a number of different inhibitors are listed which are all able to inhibit the active site of the heme group. These inhibitors do not meet the desired standards to be able for usage on industrial scale. The problem is often the toxicity of the molecule, economic aspects, environmental hazards and that the inhibitor only works in pure solutions i.e. in laboratory environment. Another criterion is that the inhibitor must bind irreversibly to the active site. The inhibitors listed by Heino Kuusk that do not meet the required standards are [5]:

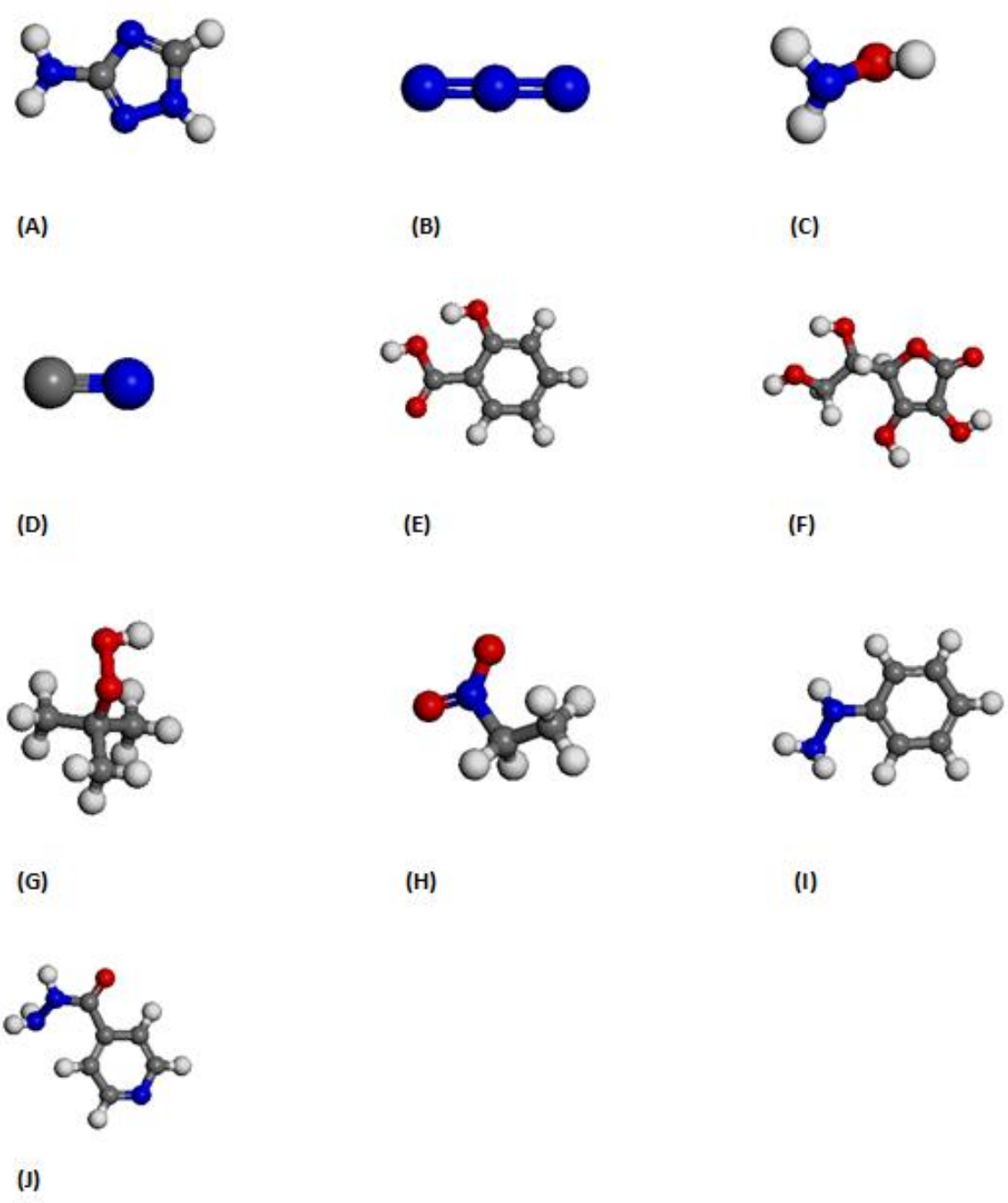


Figure 8

- ***(A)*** *3-amino-1,2,4-triazole*
- ***(B)*** *Azide*
- ***(C)*** *Hydroxylamine*
- ***(D)*** *Cyanide*
- ***(E)*** *Salicylic acid*
- ***(F)*** *Ascorbate*
- ***(G)*** *t-butyl hydroperoxide*
- ***(H)*** *Ethane nitronate*
- ***(I)*** *Phenylhydrazine*
- ***(J)*** *Isoniazid*

I will not state why each of these molecules do not fulfill the criteria for industrial usage. They will be used as reference molecules in the calculations and maybe they can give a lead to other possible inhibitors yet to be discovered. As earlier suggested it is hard to say whether a molecule would work appropriate before testing it on laboratory scale and then even on industrial scale but hopefully the calculations will give a hint on some candidate inhibitors to test later.

2.5 Malaria medication

By looking at the literature one particular article was found to link the problem presented in this thesis to another problem, the dreadful disease malaria.

Malaria is a lethal infection that prospers in the tropical parts of the world. The disease has for a long time been target for large scale medical research. Medication exists but the problem is that that the existing medicines are starting to face resistant bacteria [14]. When a human is infected with malaria, a blood feeding parasite is introduced to the circulatory system and travels to the liver where it stays and thrives. The blood feeding parasites are digesting the hemoglobin in the blood and free heme-groups are released. Free heme is toxic for living cells and also for the parasite, to counteract this toxicity the blood feeding parasites have developed a way to crystalize the free heme molecules through bio crystallization. The bio crystallization process combines the heme-groups according to figure 20 and the resulting crystal is called hemozoin. Hemozoin is essential for the survival of the malaria parasite.

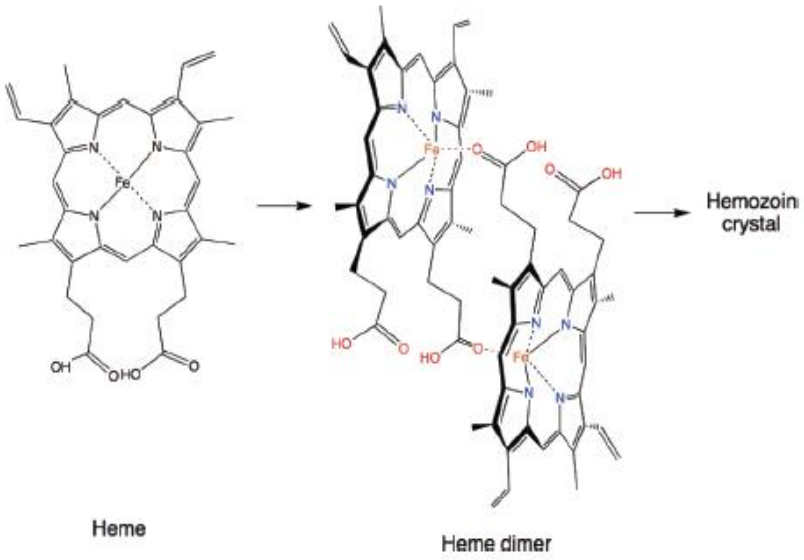


Figure 9

Shows how two heme groups can bind together to form a hemozoin crystal.

Most antimalarial drugs of today are working as inhibitors to free heme and thereby making it harder for the parasite to produce the hemozoin crystals, ultimately the parasite is suppressed by the absence of hemozoin. By looking at the medication present today it might be possible to find a lead to what may work as an inhibitor to the heme-group in the enzymes studied in this thesis. If comparing figure 9 with figure 10 C it can be seen that the bond formation between the two heme molecules is not so different from the bond between heme and hydrogen peroxide. Although it might be possible to find inhibitors this way, it is not certain that these inhibitors possess the properties needed to work on the enzymes present in paper pulp. Recall that the funnel that leads to the heme in catalase, peroxidase and haloperoxidase is about 20-25 Å at the opening and only 3-5 Å at the end.

In the article *Detection, Characterization, and Screening of Heme-Binding Molecules by Mass Spectrometry for Malaria Drug Discovery* K, Muñoz-Durango et.al commenced a mass spectrometry study to determine the bond strength of a wide range of possible inhibitors. The molecules tested were from the groups: Quinolines, Azoles and Artemisinin [14]. These molecules should be evaluated further in order to see if they might be appropriate for usage in bleaching of paper pulp as well. Nevertheless one shall have in mind that it is a big difference in properties between a drug, active in the human body, and an inhibitor that will be used on industrial scale.

2.6 Inhibition calculations

When calculating which inhibitor that would bind best to the heme group a program called Dmol3 can be used [15]. It is a quantum chemical calculation program that uses a theory called the density functional theory (DFT); this theory can be used to find out many different properties of a calculated molecule. It is a tool that is used as a replacement of the Schrödinger equation since it is not computationally practical to completely solve the Schrödinger equation for larger molecules [16]. It is an iterative calculation process that aims to find the lowest energy for an optimal geometry. The energy is a totality of several different components displayed below [17].

$$E = E_{NN} + E_T + E_V + E_{coul} + E_{exch} + E_{corr}$$

E_{NN} - Energy for nuclear repulsions
E_T - The electrons kinetic energy
E_V - Energy for nuclear electron attractions
E_{coul} - The energy for the repulsion of electrons, according to Coulombs law
E_{exch} - The electrons exchange energies
E_{corr} - Energy for interaction of electron pairs with different spin

The difference between DFT and the earlier very popular Hartree-Fock theory is that DFT accounts for the "electron correlation", E_{corr}, which is as the name suggests a factor that considers the interaction of electron pairs with opposite spin in a system. The electron pairs

do not want to come too close to each other because of the repulsive forces that they exert [16]. In this project it is of interest to find the bond strength of the inhibitor-heme group as well as the bond length of an inhibitor to the active site of the heme group. By optimizing the geometry of the heme group with the binding inhibitor the bond distance between the two can be measured. More about the settings and the usage of the program is found in section 3.3 Theory Method.

3. Methods and Materials

3.1 Procedure

The project started out by reading the material received from BIM Kemi e.g. the licentiate thesis by Heino Kuusk *Enzymatic Degradation of Hydrogen Peroxide in a Recycled Paper Pulp Mill* [5]. When the problem and earlier work was clear the planning report was written based on what was desired to get from this project. Then it was important to start reading more about the underlying theory of the problem. The first step was to find out more about what is used in the industry today and what the issues are with these additives. It was of great interest to see if it was possible to find any research regarding inhibition of hydrogen peroxide degrading enzymes that had been published after Heino Kuusk's licentiate study. By reading about the published research it was possible to create a perception on the characteristics of potential inhibitors and also find suggestions of inhibitors that might work. Inhibitors found from earlier patents, patents in use and those found in research papers were all listed and drawn in a program called Materials Studio [18]. Dmol3 is the program used in this project for the quantum chemical calculations [15]. The quantum chemistry theory is important to be familiar with in order to understand what the program calculates and to interpret all the results. The initiating calculations were made on the heme-group alone and after that the inhibitor alone. Then the heme-group and the inhibitor were placed in the same model at a bond distance of about 2.000 Å and optimized once again.

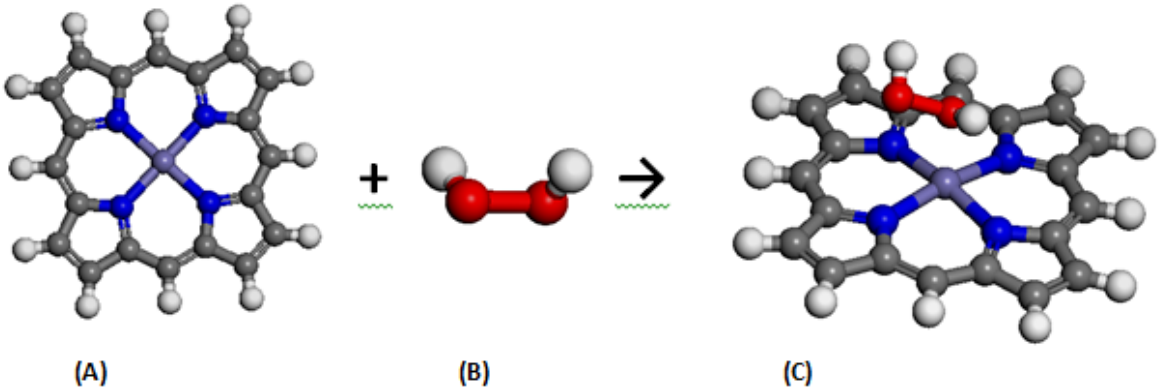


Figure 10

(A) *The Heme-group.*
(B) *Hydrogen peroxide, the inhibitor*
(C) *The heme group with the inhibitor bound to the iron ion.*

The bond strength could at that point be calculated according to the formula:

$$\Delta E(heme\ with\ an\ inhibitor) - \Delta E(heme) - \Delta E(inhibitor) = \Delta E$$

Before the calculations commenced, a study was completed on the inhibitors pKa values. This was done to evaluate to which extent the inhibitors would deprotonate in the pulp before the inhibition. Inhibitors with a pKa value below 7 were calculated as negatively charged.

Recall that the inhibitors cannot be larger than 3-5 Å to be able to reach the active site in the enzymes. Because of that it was necessary to measure the length of each inhibitor. This way it could be distinguished which inhibitors that suits for enzyme inhibition and which of them that only suits for free heme inhibition. The measurement was carried out in Materials Studio [18]; the inhibitors were measured where they were most narrow. The measurement tool measured the distance between the centers of the atoms. This fact means that the measurement is marginally shorter than its true value.

Another requirement for additives is that it needs to be environmentally benign. The inhibitors that showed interesting properties regarding inhibition energy and inhibition length were checked through Chalmers chemical handling system KLARA to see its toxic classification.

3.2 Materials

This thesis work was mainly theoretical and computational and the materials used were different computer software and search tools. Dmol3 was used for the calculations and is a program implemented in Materials studio 6.0; Materials studio 6.0 was helpful when drawing the molecules [15][18]. Excel was a great tool to get an overview of the results. Chalmers Library has been a great support for finding scientific articles as well as the search engines SciFinder, Web of Science and Summon.

3.3 Theory method

When using Dmol3 to calculate the energy and geometry of molecules there are several basis set settings that can be selected depending on the type of calculation executed. Another of the most significant settings is the "functional". In this thesis the plan was to use a functional called B3LYP which is a hybrid functional that mix the Hartree-Fock theory with DFT to obtain a very accurate output [19], it was later found that this functional was too time consuming. The functional was changed to PW91 which is a pure GGA functional and not a hybrid [20]. This functional turned out to be more time efficient and more appropriate for the time frame for this thesis. The offset by using PW91 is the quality in the calculations. B3LYP has been found to give exceptionally accurate result by using a mix of exchange correlation

from GGA and Hartree-Fock. Even though B3LYP is more accurate it will not affect the results so much since the same functional is used in every calculation which means that the comparison between molecules will be the same i.e. cancellation of errors.

The numerical basis set used was DNP with basis file 4,4 which is a numerical basis set with quality corresponding to valence double zeta, with polarization d-functions on heavy elements and p-functions on hydrogen [21].

The SCF tolerance value chosen was $1{,}0*10^{-5}$ eV. This value served as a threshold value for when the calculation should be considered converged.

The gradient convergence value chosen was $2{,}0*10^{-5}$ Hartree. This value served as a threshold value for when the geometry of the molecule should be considered optimized.

3.4 Data evaluation

When all the calculations were made according to the procedure above it was time to evaluate the results. As will be observed in the results-section, the inhibitors were divided into 4 groups depending on the background of the inhibitor. There is the “known inhibitors” group which consists of inhibitors found in Heino Kuusk’s licentiate report. This group will function as a reference group since it is known why these chemicals do not practically work. There is the “inhibitors found from the literature study” group which consists of different chemicals that have been mentioned as potential inhibitors in research reports since Heino Kuusk published his licentiate report in 2001. There is the “inhibitors found from malaria research” group which consist of inhibitors found in the article written by K, Muñoz-Durango et.al. [14]. Finally the last group is the ”potential new inhibitors” which consists of inhibitors that was found to be interesting calculation objects.

When evaluating the results several factors had to be taken into consideration. These factors were bond energy, how close the inhibitor binds to the active site, the size of the inhibitor and the toxicity of the inhibitors. These factors will be evaluated in the discussion and conclusion sections.

4. Results

Results will be displayed according to the categories: known inhibitors, inhibitors found from the literature study, inhibitors found from malaria research and potential new inhibitors. To begin with pictures of the calculation objects will be displayed. Then results in the form of inhibition energy, inhibition length and size of inhibitors will be presented in the same order as just mentioned. The most interesting leads will be discussed in the discussion section; the discussion is based on the results just mentioned as well as toxicity of the inhibitors found to be most interesting. In the graphs a neutral molecule will be displayed by the color blue and a deprotonated molecule with the color red.

The tables will display the data put in to the graphs. Extra information found in the tables is the pKa values. pKa values are shown for all the molecules where pKa value is significant for the result i.e. the inhibitor gets deprotonated to some extent. If the inhibitor cannot be deprotonated the table cell is filled out with N/A.

4.1 Known inhibitors

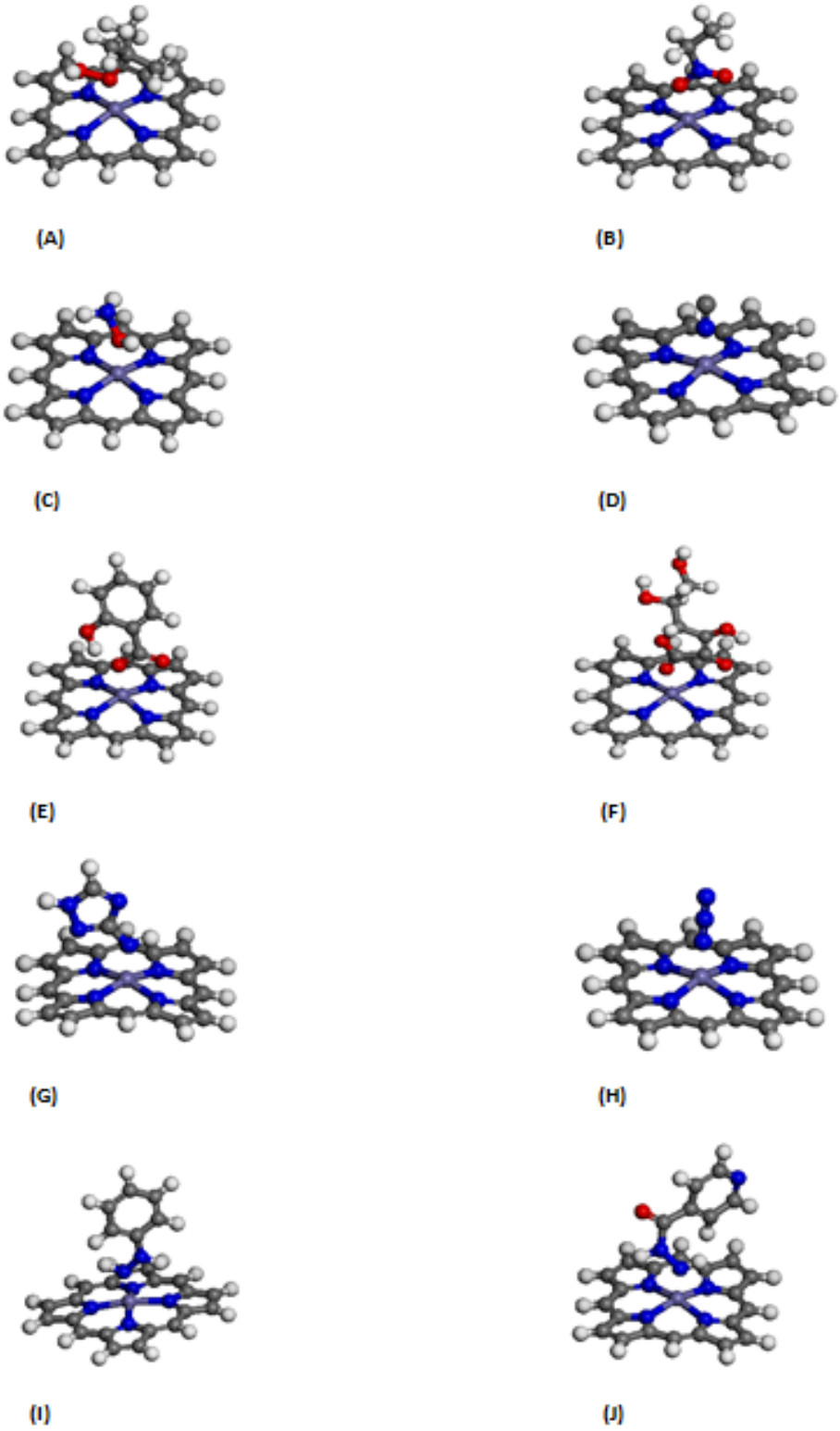


Figure 11

(A) *t-butyl hydroperoxide* **(B)** *Ethane nitronate* **(C)** *Hydroxylamine*

(D) *Cyanide* **(E)** *Salicylic acid* **(F)** *Ascorbate*

(G) *3-amino-1,2,4-triazole* **(H)** *Azide* **(I)** *Phenylhydrazine*

(J) *Isoniazid*

4.1.1 Inhibition energy

Table 1

Inhibition energy and pKa-values for the non-deprotonated inhibitors. Ethane nitronate says N/A since it is unable to get deprotonated. Hydrogen peroxide is displayed as a reference value.

Inhibitor	ΔE Total (kJ/mol)	pKa
t-butyl hydroperoxide	-205,5477695	12,69
Ethane nitronate	-206,8274382	N/A
Hydroxylamine	-140,9638826	13,7
Hydrogen Peroxide	-132,5313017	11,75

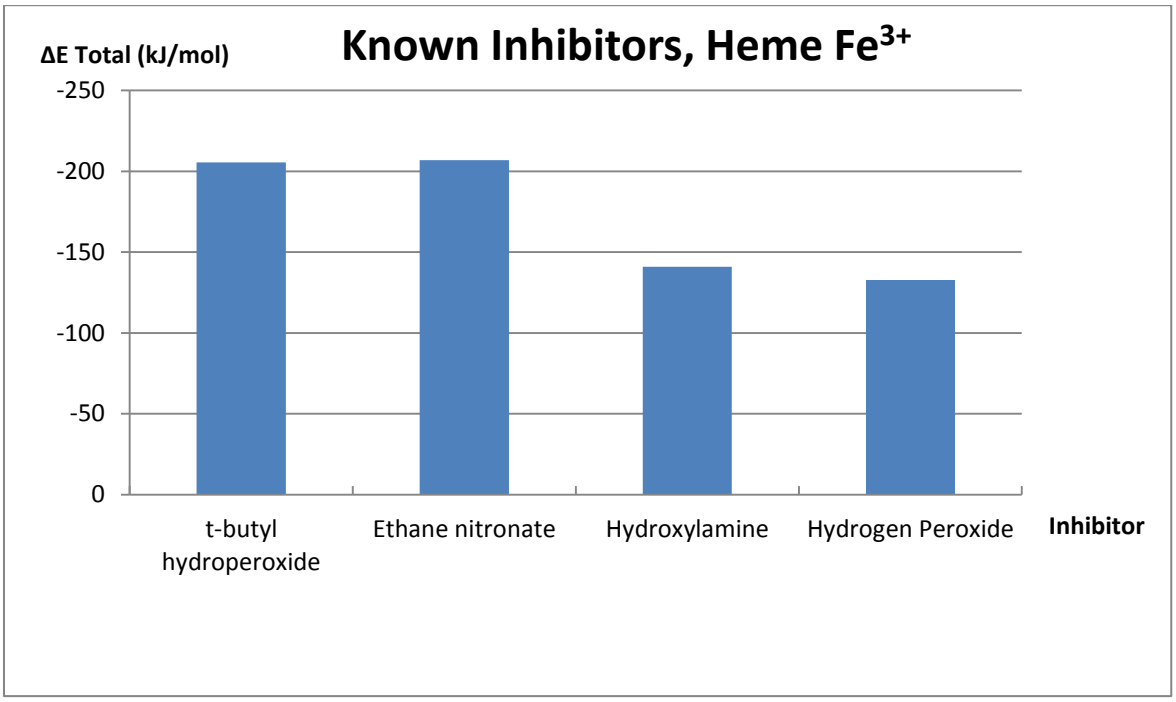


Figure 12

The bar- diagram displays the inhibition energy for the non-deprotonated inhibitors.

Table 2

Inhibition energy and pKa-values for the deprotonated inhibitors. Cyanide and azide says N/A since they are already deprotonated and unable to lose more protons.

Inhibitor	ΔE Total (kJ/mol)	pKa
Cyanide$^-$	-1287,635517	N/A
Salicylic acid$^-$	-1300,990123	2,97
Ascorbate$^-$	-1381,659136	4,47
3-amino-1,2,4-triazole$^-$	-1552,635371	4
Azide$^-$	-1294,497261	N/A
Phenylhydrazine$^-$	-1713,149252	5,21
Isoniazid$^-$	-1504,301492	1,82

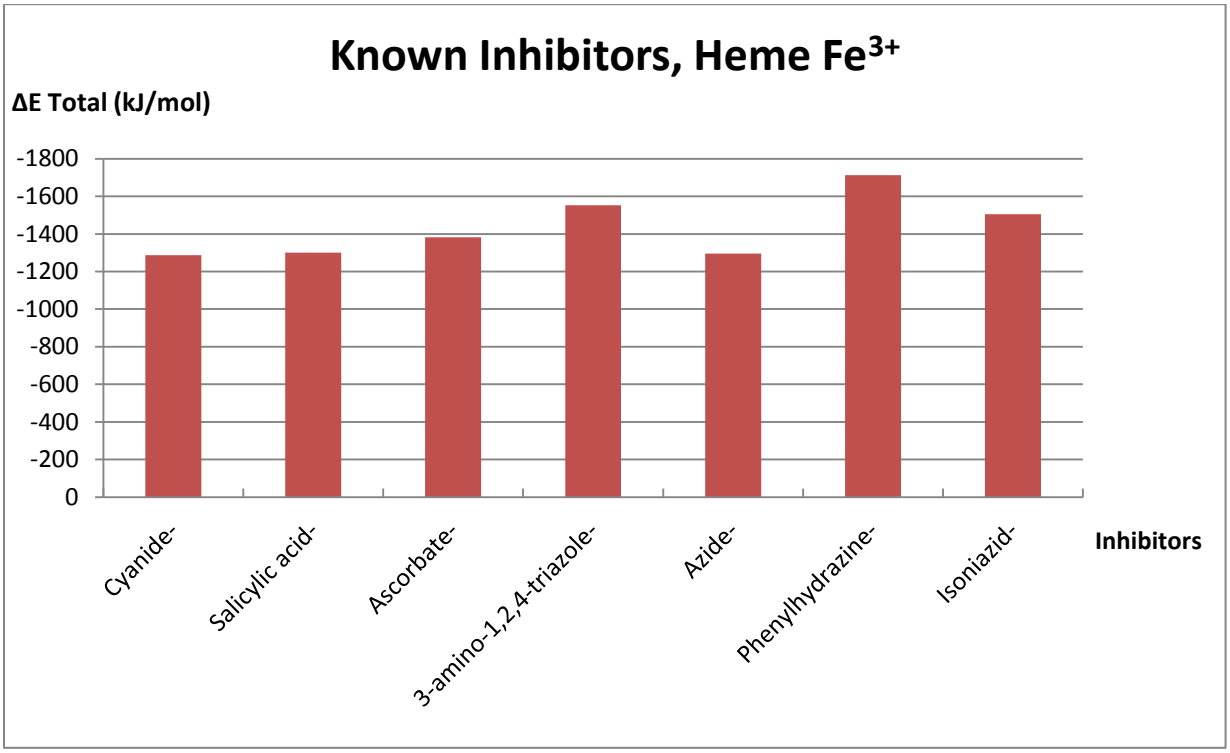


Figure 13

The bar- diagram displays the inhibition energy for the deprotonated inhibitors. Isoniazid$^-$ did not converge completely and its value is therefore not as exact as the others i.e. the iteration process was unable to reach the threshold value.

4.1.2 Inhibition length

Table 3

The inhibitors and how close they bind to the active site.

Inhibitor	Length (Å)
t-butyl hydroperoxide	2,132
Ethane nitronate	2,091
Hydroxylamine	2,156
Hydrogen Peroxide	2,182
Cyanide$^-$	1,844
Salicylic acid$^-$	2,026
Ascorbate$^-$	2,048
3-amino-1,2,4-triazole$^-$	1,779
Azide$^-$	1,782
Phenylhydrazine$^-$	1,791
Isoniazid$^-$	1,767

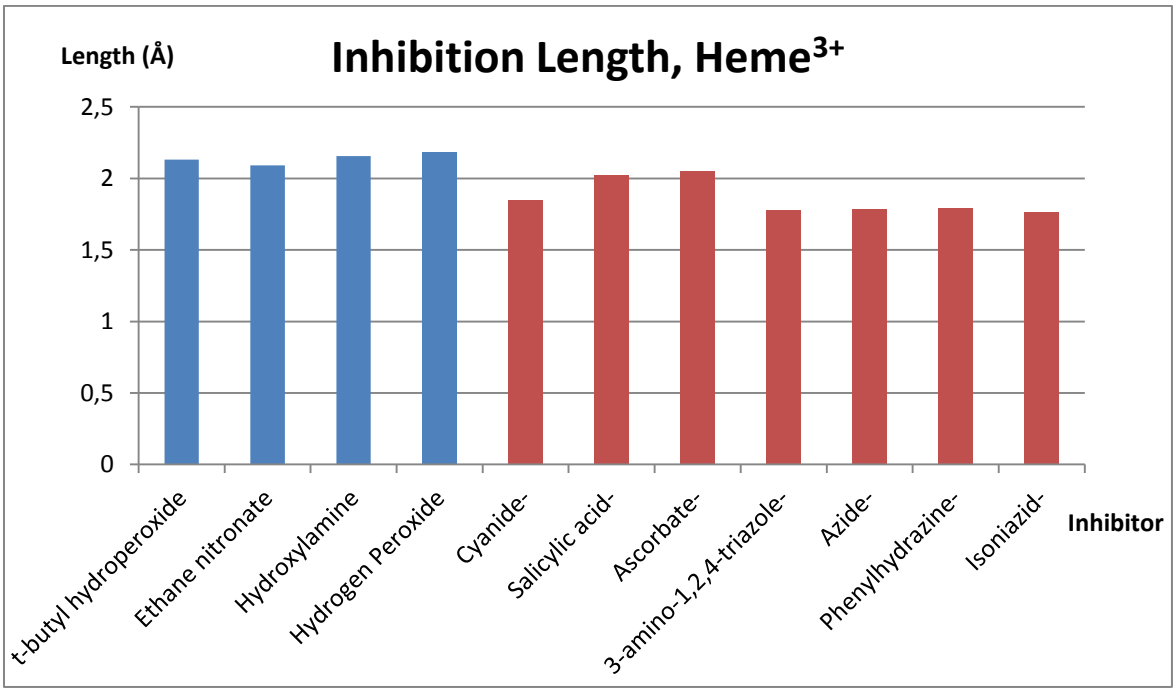


Figure 14

Displays the inhibition length.

4.1.3 Size of inhibitors

Table 4

The inhibitors and their size.

Inhibitor	Size (Å)
3-amino-1,2,4-triazole	2,595
t-butyl hydroperoxide	4,315
Ethane nitronate	2,798
Hydroxylamine	1,62
Hydrogen Peroxide	0,977
Cyanide$^-$	0,71
Salicylic acid$^-$	4,524
Ascorbate$^-$	4,049
3-amino-1,2,4-triazole$^-$	2,595
Azide$^-$	0,71
Phenylhydrazine$^-$	4,328
Isoniazid$^-$	4,289

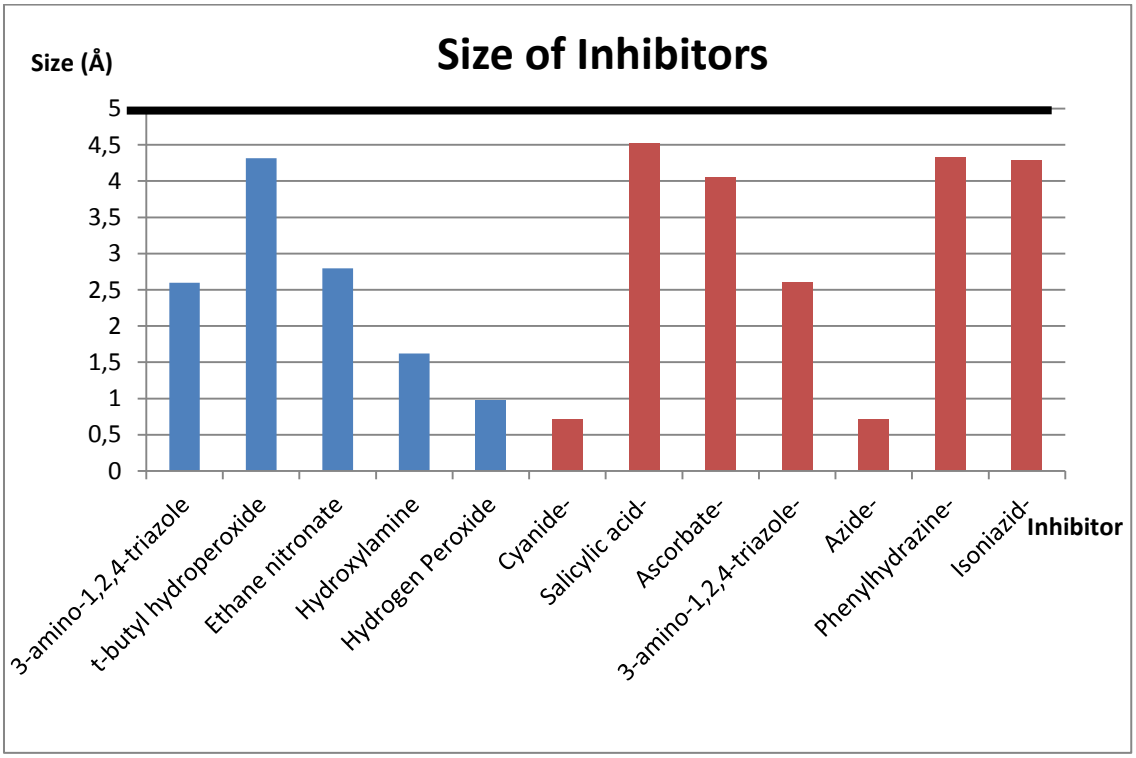


Figure 15

Displays the size of the inhibitors. Notice that a thick line is drawn at 5 Å since that is the upper limit for a molecules size to be able to pass through the funnel-shaped channel.

4.2 Inhibitors found from the literature Study

The following molecules have been found in several articles which all have claimed that they have the ability to inhibit catalases. The articles are: *Effects of Urea and Trimethylamine-N-Oxide on Enzyme Activity and Stability* by T, Mashino et.al [22], *Inhibition of Heme Peroxidases by Melamine* by P, Vanachayangkul et.al [23] and the patent *Method for the inactivation of enzymes* by S, Qureshi et.al [13].

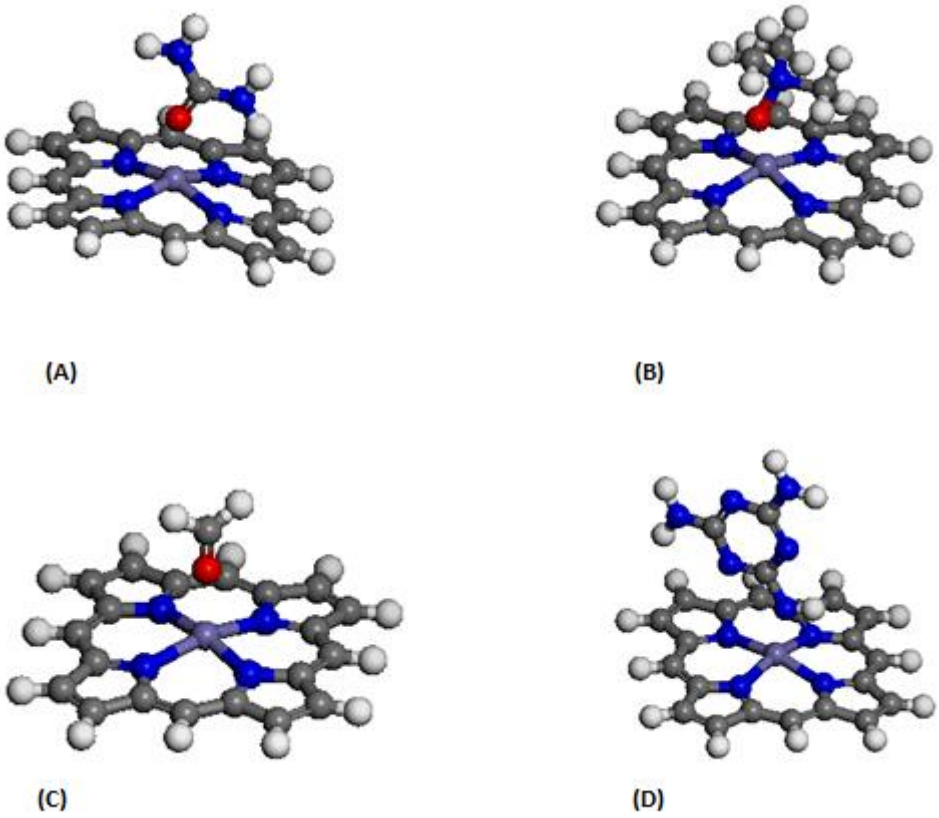


Figure 16

(A) *Urea*

(B) *Trimethylamine-N-Oxide*

(C) *Formaldehyde*

(D) *Melamine*

4.2.1 Inhibition energy

Table 5

Inhibition energy and pKa-values for the non-deprotonated inhibitors. Trimethylamine-N-Oxide says N/A since it is unable to get deprotonated. Hydrogen peroxide is displayed as a reference value.

Inhibitor	ΔE Total (kJ/mol)	pKa
Urea	-279,7567393	13,82
Trimethylamine-N-oxide	-371,4882962	N/A
Formaldehyde	-139,9701309	13,3
Hydrogen Peroxide	-132,5313017	11,75

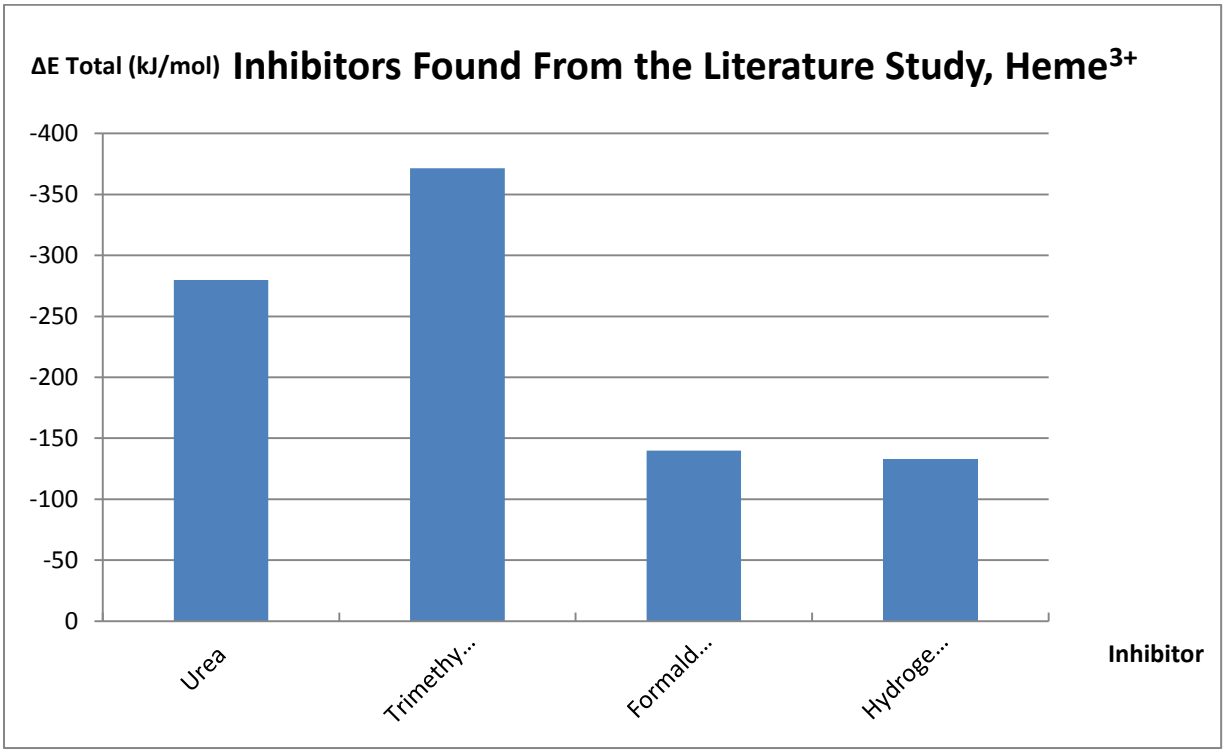


Figure 17

The diagram displays the inhibition energy for the non-deprotonated inhibitors.

Table 6

Inhibition energy and pKa-values for the non-deprotonated inhibitors. 3-amino-1,2,4-triazole⁻ is displayed as a reference value.

Inhibitor	ΔE Total (kJ/mol)	pKa
Melamine-	-1454,90796	5
3-amino-1,2,4-triazole-	-1552,635371	4

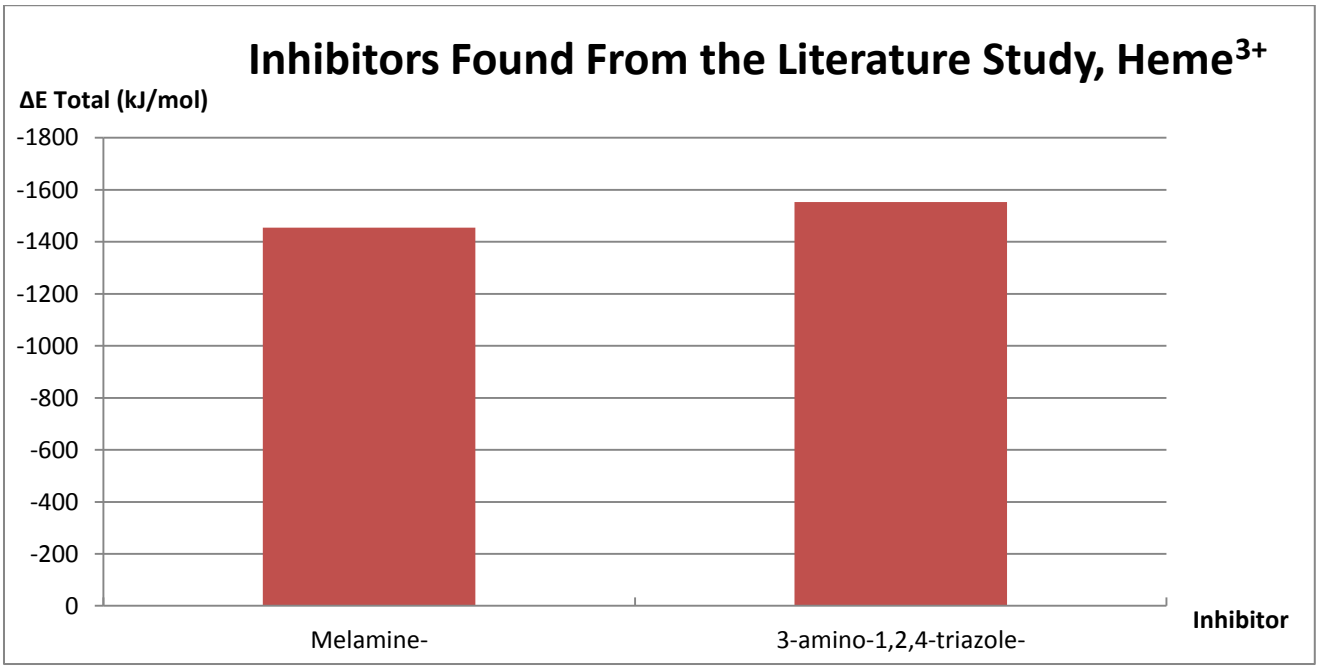


Figure 18

The diagram displays the inhibition energy for the deprotonated inhibitor melamine⁻ and 3-amino-1,2,4-triazole⁻ as a reference value .

4.2.2 Inhibition length

Table 7

The inhibitors and how close they bind to the active site.

Inhibitor	Length (Å)
Urea	1,944
Trimethylamine-N-oxide	1,911
Formaldehyde	2,075
Melamine-	1,84

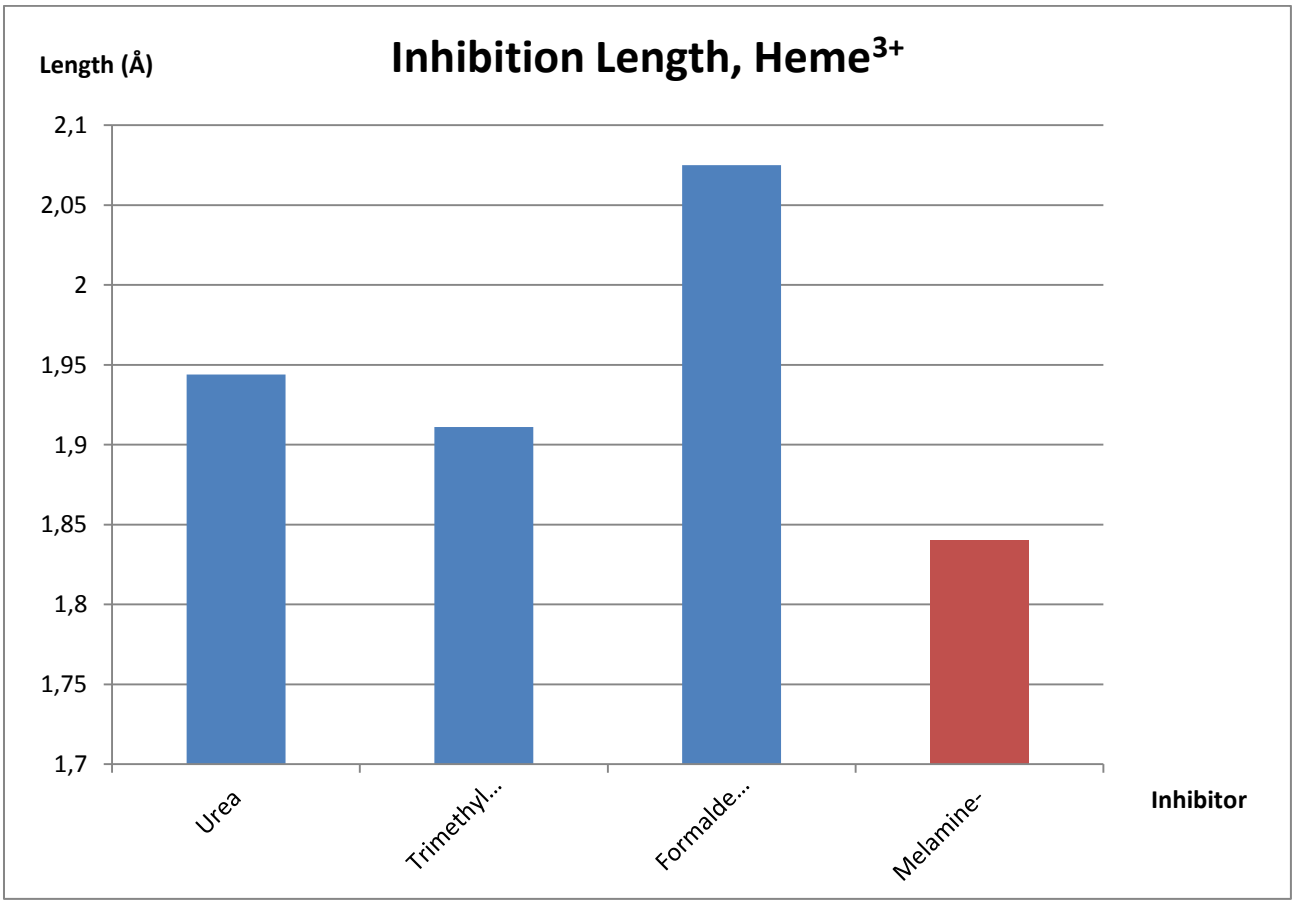


Figure 19

Displays the inhibition length.

4.2.3 Size of inhibitors

Table 8

The inhibitors and their size.

Inhibitor	Length (Å)
Urea	3,045
Trimethylamine-N-oxide	4,174
Formaldehyde	1,891
Melamine	5,46

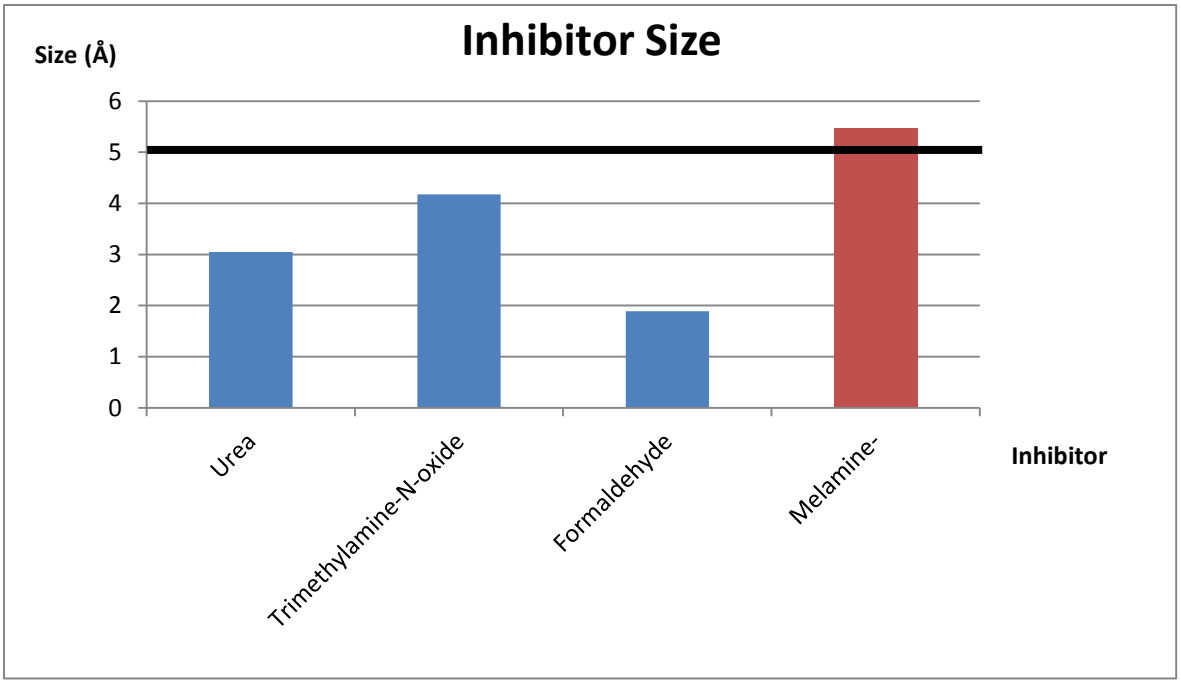


Figure 20

Displays the size of the inhibitors. Notice that melamine is slightly bigger than the tolerated 5 Å limit.

4.3 Inhibitors found from malaria research

The following molecules have been found in the article *Characterization, and Screening of Heme-Binding Molecules by Mass Spectrometry for Malaria Drug Discovery* K, Muñoz-Durango et.al [14].

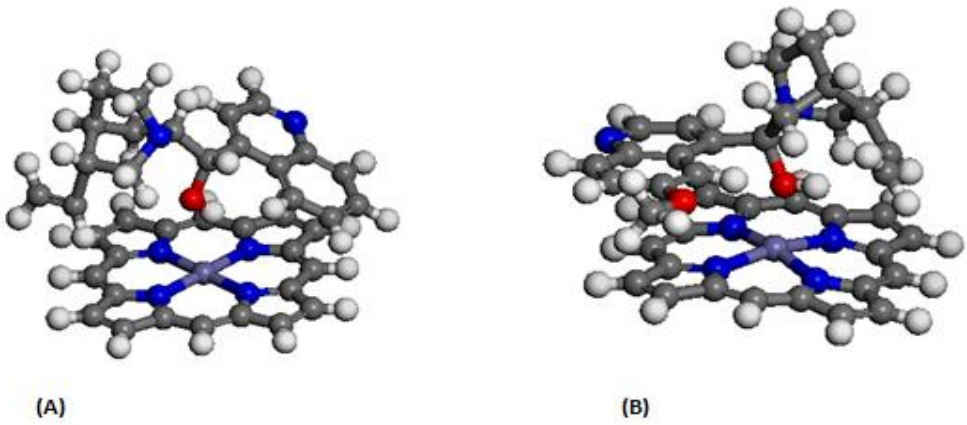


Figure 21

(A) *Cinchonidine*

(B) *Quinidine*

4.3.1 Inhibition energy

Table 9

Inhibition energy and pKa-values for the inhibitors. The molecules were not calculated in their deprotonated state despite their rather low pKa value. This was due to difficulties of getting the calculations to converge when performing calculations on deprotonated molecules.

Inhibitor	ΔE Total (kJ/mol)	pKa
Cinchonidine	-512,6693077	5,8
Quinidine	-531,2859405	4,2

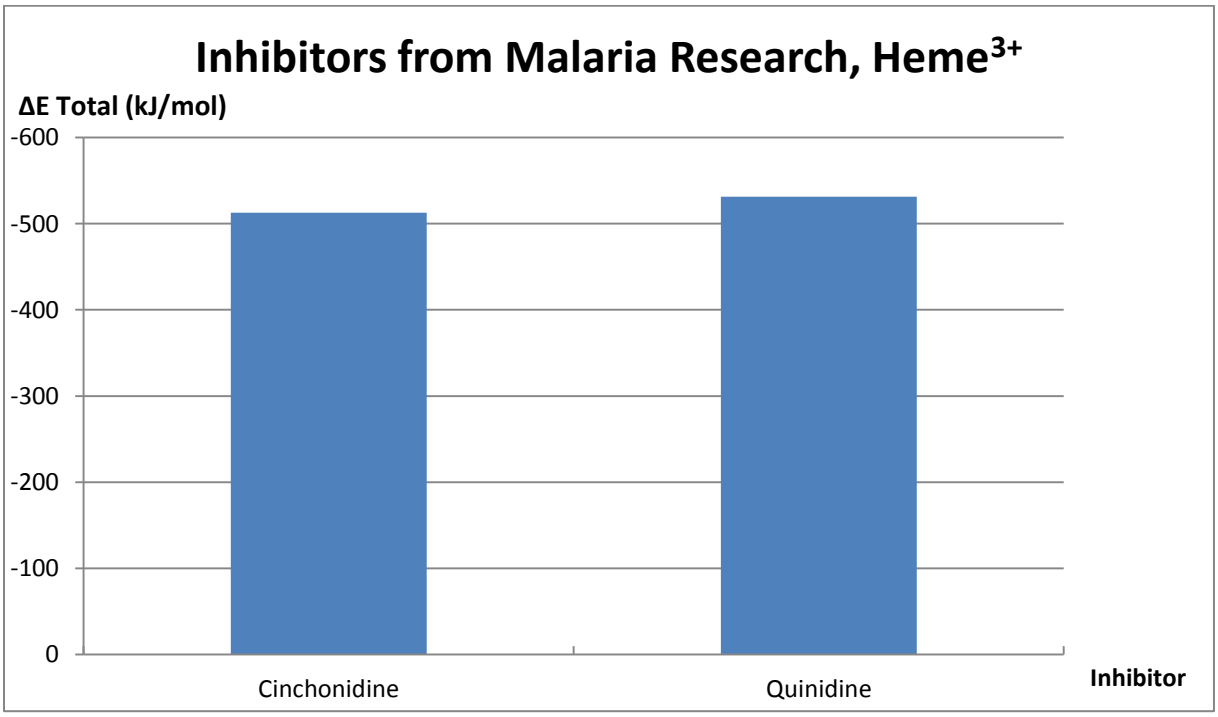


Figure 22

The diagram displays the inhibition energy for the two malaria medicines.

4.3.2 Inhibition length

Table 10

The inhibitors and how close they bind to the active site.

Inhibitor	Length (Å)
Cinchonidine	3,749
Quinidine	2,377

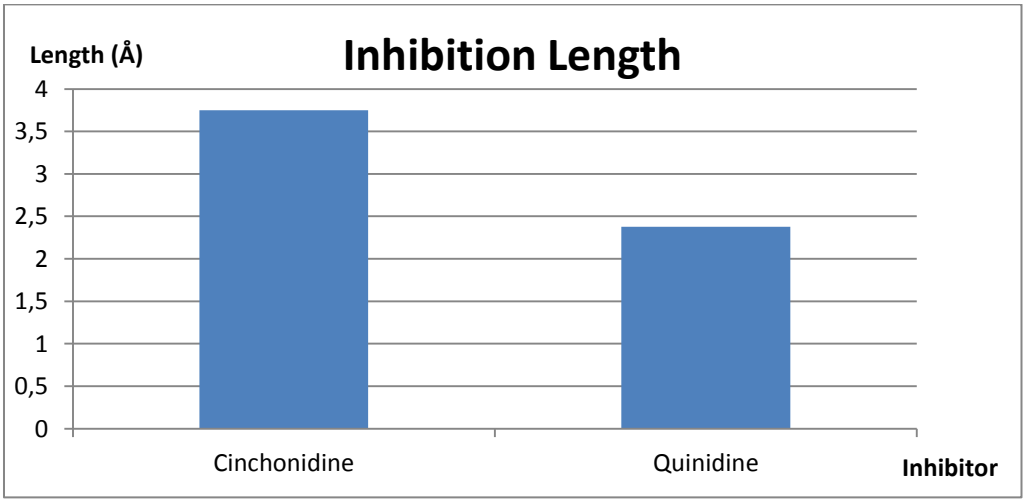


Figure 23

Displays the inhibition length.

4.3.3 Size of inhibitors

Table 11

The inhibitors and their size.

Inhibitor	Size
Cinchonidine	7,171
Quinidine	6,905

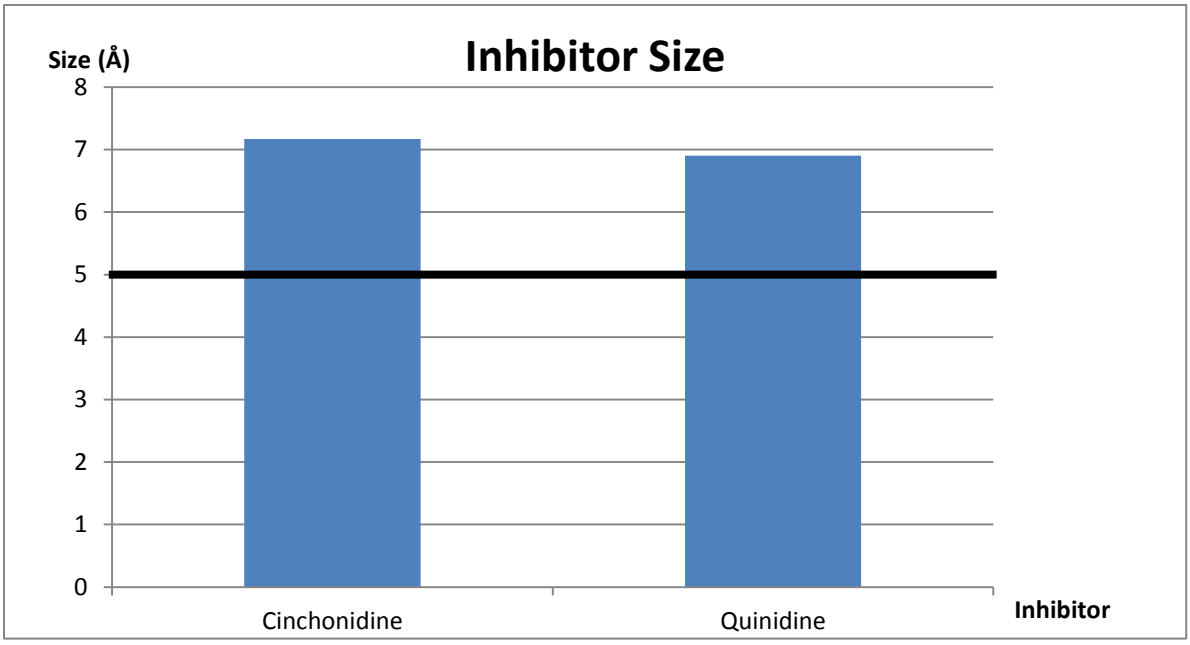
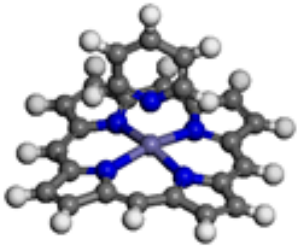


Figure 24

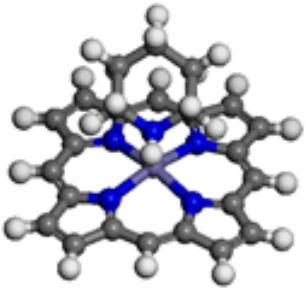
Displays the size of the inhibitors. Notice that both Cinchonidine and Quinidine is bigger than the tolerated 5 Å limit.

4.4 Potential new inhibitors

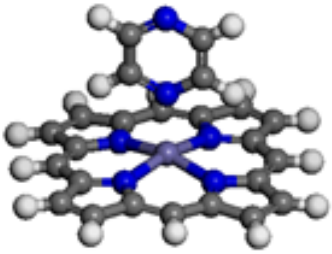
The inhibitors in this section have been calculated because of their interesting structure to see if they could successfully inhibit the active site. The choice of molecules is based on a perception, received from the literature study, of how a typical inhibitor may look like.



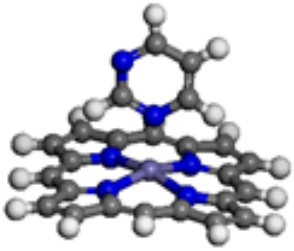
(A)



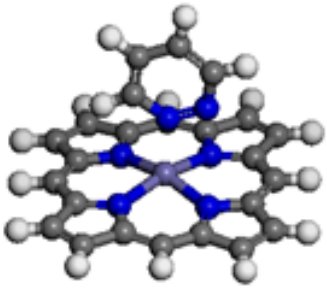
(B)



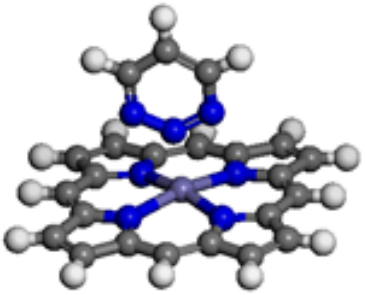
(C)



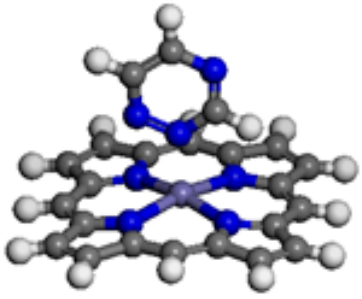
(D)



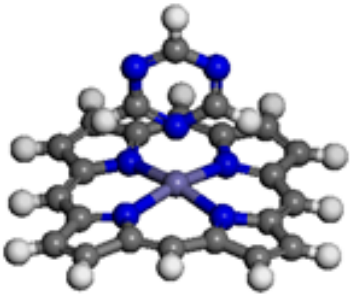
(E)



(F)



(G)



(H)

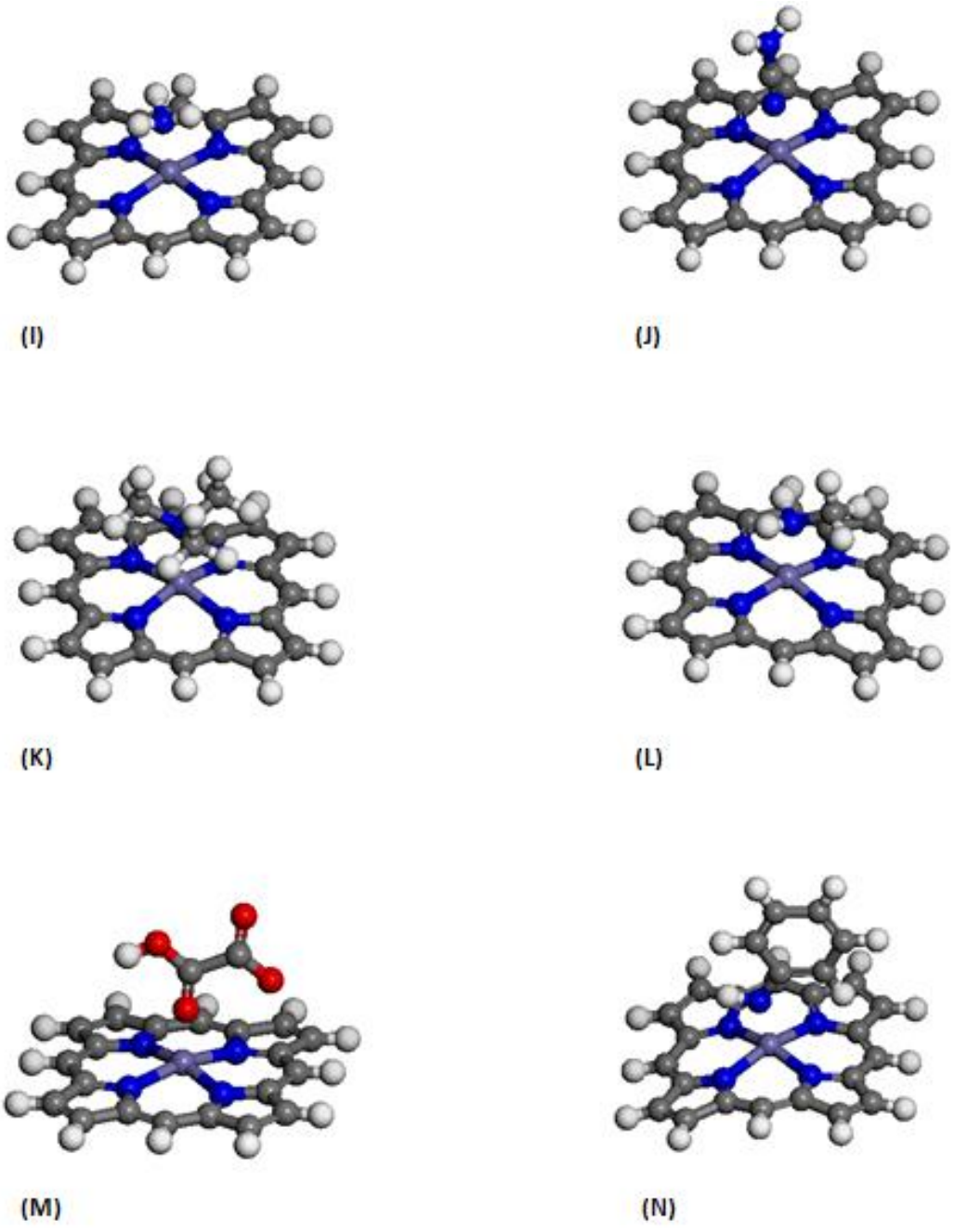


Figure 25

(A) *Pyridine*
(B) *Piperidine*
(C) *1,4-diazine*
(D) *1,3-diazine*
(E) *1,2-diazine*
(F) *1,2,3-triazine*
(G) *1,2,4-triazine*
(H) *1,3,5-triazine*
(I) *Ammonia*
(J) *Cyanamide*
(K) *Trimethylamine*
(L) *Methylamine*
(M) *Oxalic acid*
(N) *Aniline*

4.4.1 Inhibition energy

Table 12

Inhibition energy and pKa-values for the non-deprotonated inhibitors. The molecules that says N/A do so because they cannot get deprotonated further. Hydrogen peroxide is displayed as a reference value.

Inhibitor	ΔE Total (kJ/mol)	pKa
Pyridine	-146,5207534	N/A
Piperidine	-274,6945128	11,22
1,4-diazine	-91,41019565	N/A
1,3-diazine	-106,7675328	N/A
1,2-diazine	-163,2766944	N/A
1,2,3-triazine	-151,8723101	N/A
1,2,4-triazine	-123,0871157	N/A
1,3,5-triazine	-59,49934355	N/A
Ammonia	-64,03148165	9,25
Cyanamide	-97,9579301	N/A
Trimethylamine	-83,2887365	9,8
Methylamine	-83,1448591	10,66
Hydrogen Peroxide	-132,5313017	11,75

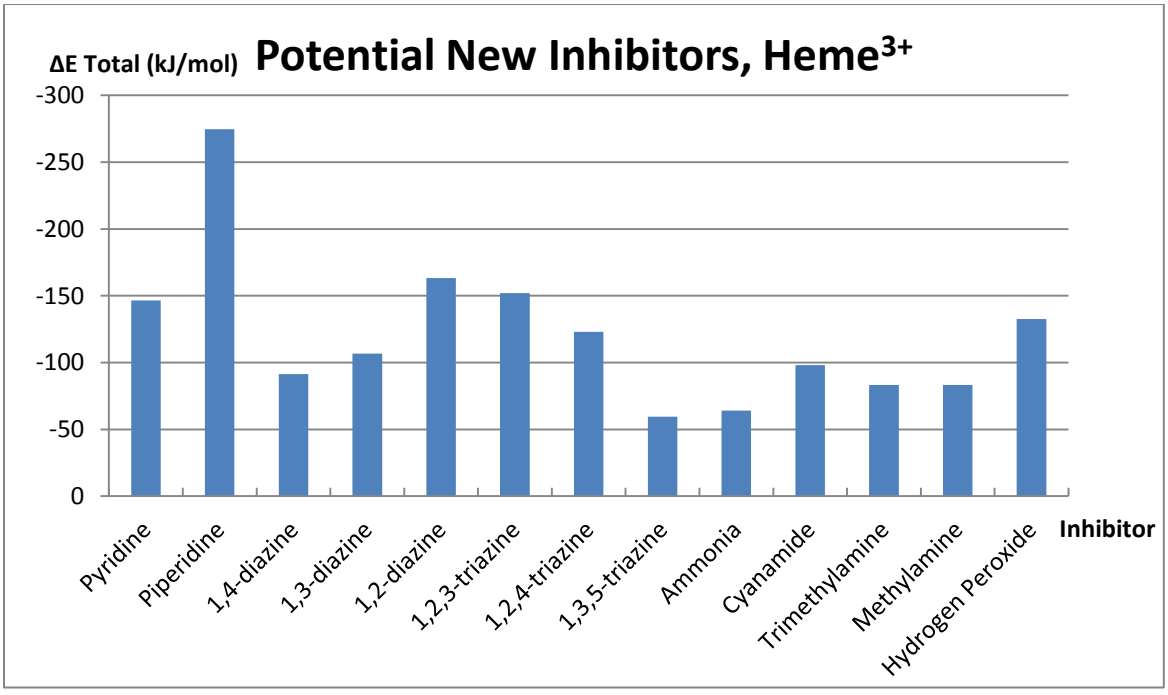


Figure 26

The diagram displays the inhibition energy for the non-deprotonated inhibitors.

Table 13

Inhibition energy and pKa-values for the non-deprotonated inhibitors. 3-amino-1,2,4-triazole is displayed as a reference value.

Inhibitor	ΔE Total (kJ/mol)	pKa
Oxalic acid$^-$	-1088,039231	1,25
Aniline$^-$	-1678,329608	4,87
3-amino-1,2,4-triazole$^-$	-1552,635371	4

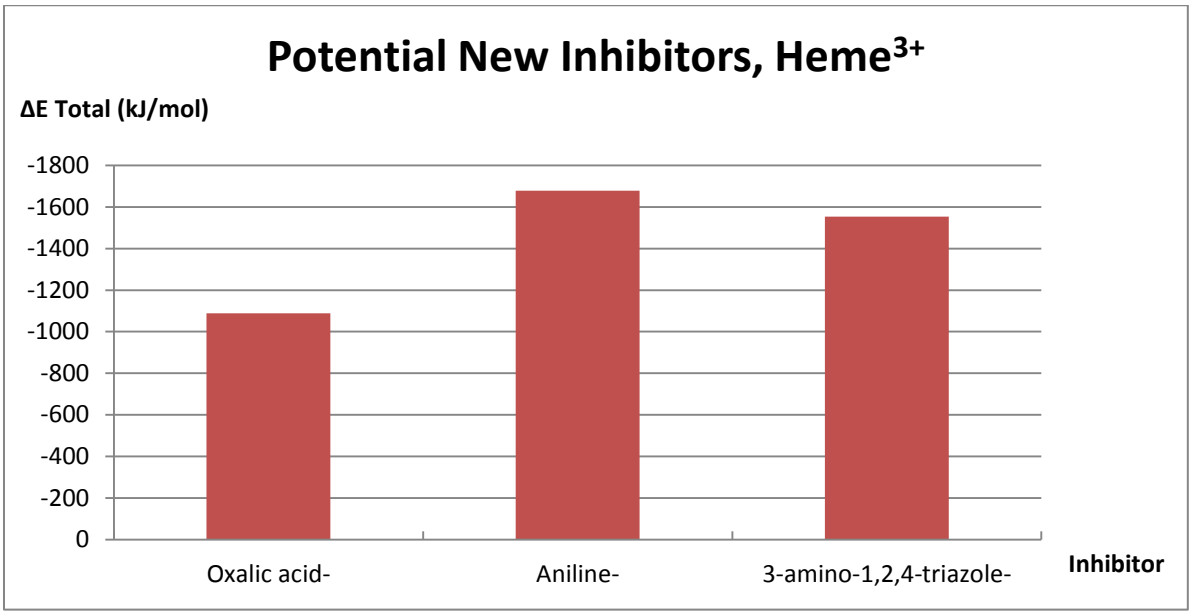


Figure 27

The diagram displays the inhibition energy for the deprotonated inhibitors oxalic acid$^-$ and Aniline$^-$ with 3-amino-1,2,4-triazole$^-$ as a reference value. Oxalic acid$^-$ did not converge completely and its value is therefore not as exact as the others i.e. the iteration process was unable to reach the threshold value.

4.4.2 Inhibition length

Table 14

The inhibitors and how close they bind to the active site.

Inhibitor	Length (Å)
Pyridine	1,95
Piperidine	2,034
1,4-diazine	2,142
1,3-diazine	2,086
1,2-diazine	2,089
1,2,3-triazine	1,954
1,2,4-triazine	1,964
1,3,5-triazine	1,97
Ammonia	1,987
Cyanamide	1,999
Trimethylamine	2,213
Methylamine	1,997
Hydrogen Peroxide	2,182
Oxalic acid$^-$	1,827
Aniline$^-$	1,788

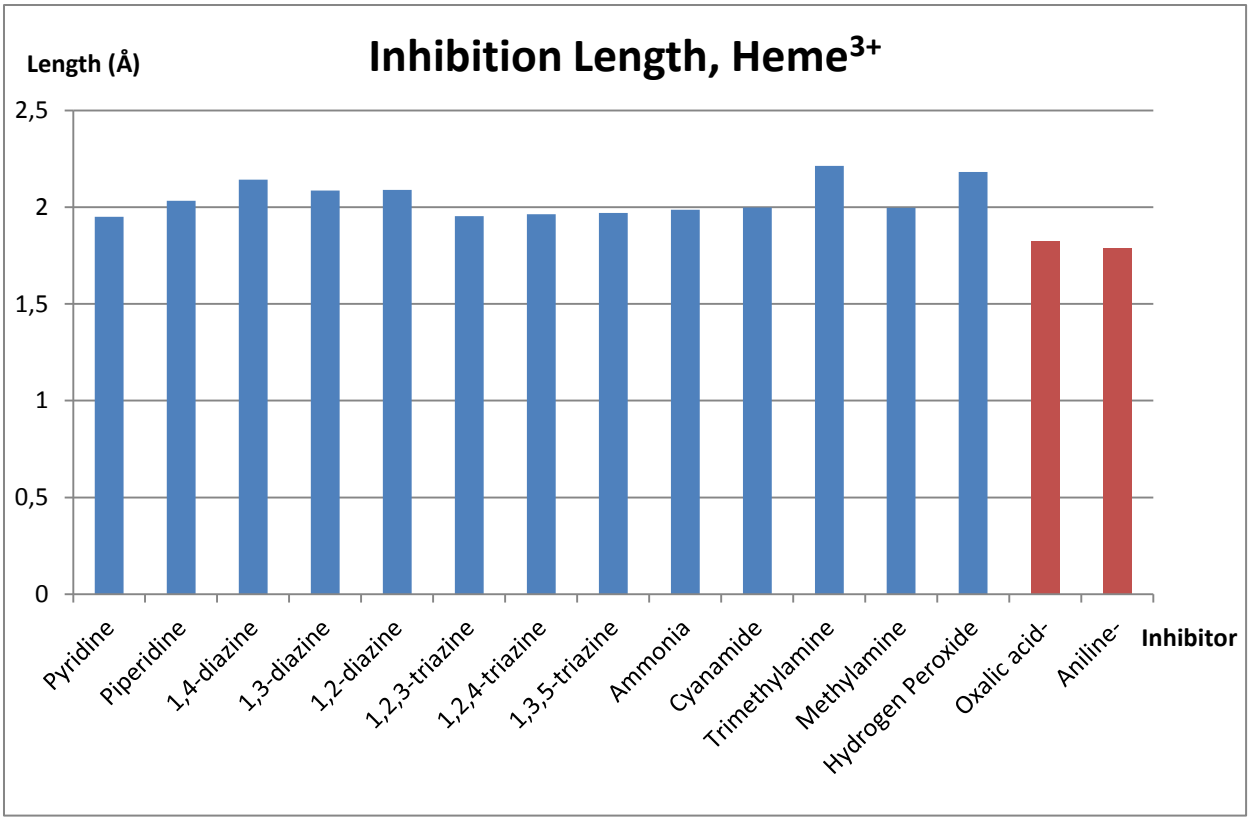


Figure 28

Displays the inhibition length.

4.4.3 Size of inhibitor

Table 15

The inhibitors and their size.

Inhibitor	Size (Å)
Pyridine	3,898
Piperidine	4,323
1,4-diazine	2,823
1,3-diazine	4,314
1,2-diazine	3,391
1,2,3-triazine	3,828
1,2,4-triazine	2,784
1,3,5-triazine	4,136
Ammonia	1,621
Cyanamide	1,686
Trimethylamine	4,179
Methylamine	1,78
Aniline	4,302
Oxalic acid$^-$	2,302
Aniline$^-$	4,318

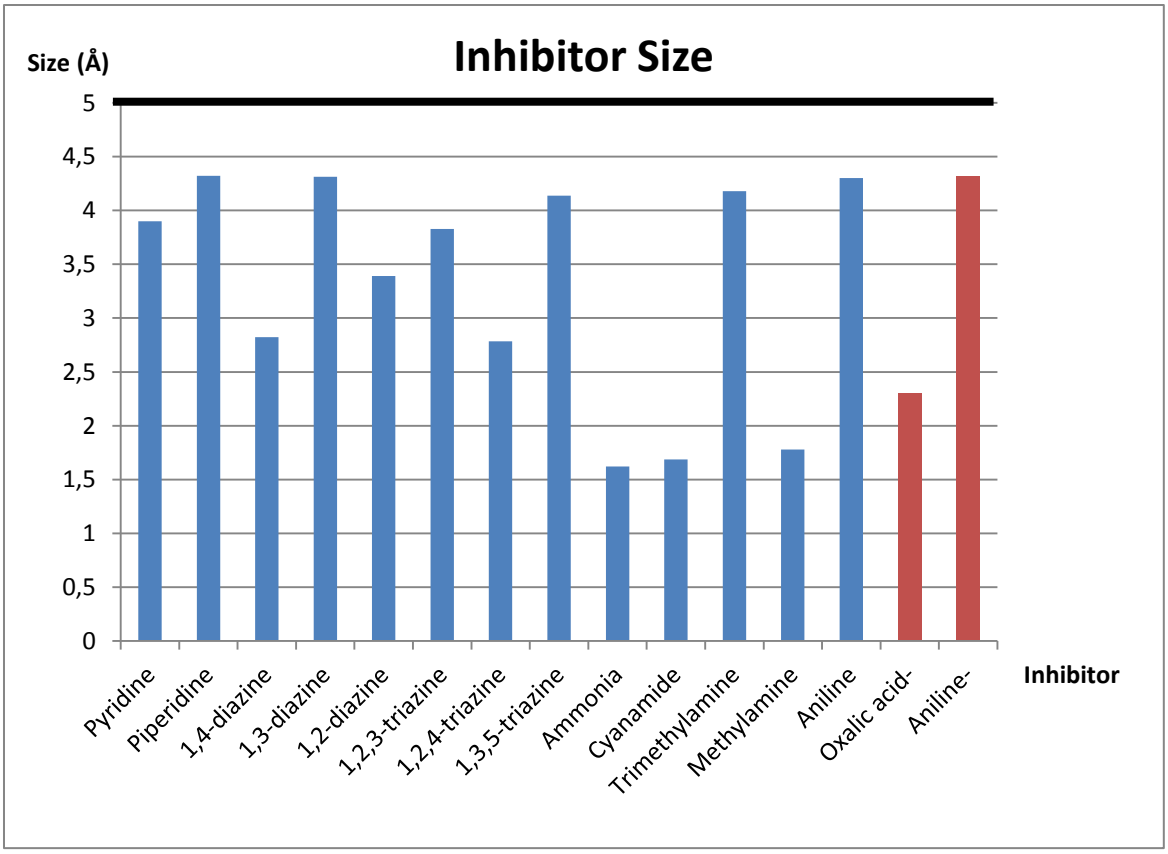


Figure 29

Displays the size of the inhibitors. Notice that all of the inhibitors are below the 5 Å limit.

4.5 Inhibition to heme^{4+}

Some of the molecules that were found to have interesting results were tested with heme^{4+} as well. These molecules were: 3-amino-1,2,4-triazole which was taken from the "known inhibitors"-group, trimethylamine-N-oxide which is one of the molecules found from the literature study and hydrogen peroxide which is the reference molecule.

4.5.1 Inhibition energy

Table 16

Inhibition energy and pKa-values for the all 3 molecules. pKa for Trimethylamine-N-Oxide says N/A since it is unable to get deprotonated. Hydrogen peroxide is displayed as a reference value.

Inhibitor	ΔE Total (kJ/mol)	pKa
3-amino-1,2,4-triazole-	-1996,619761	4
Trimethylamine-N-Oxide	-481,3452549	N/A
Hydrogen Peroxide	-168,4764972	11,75

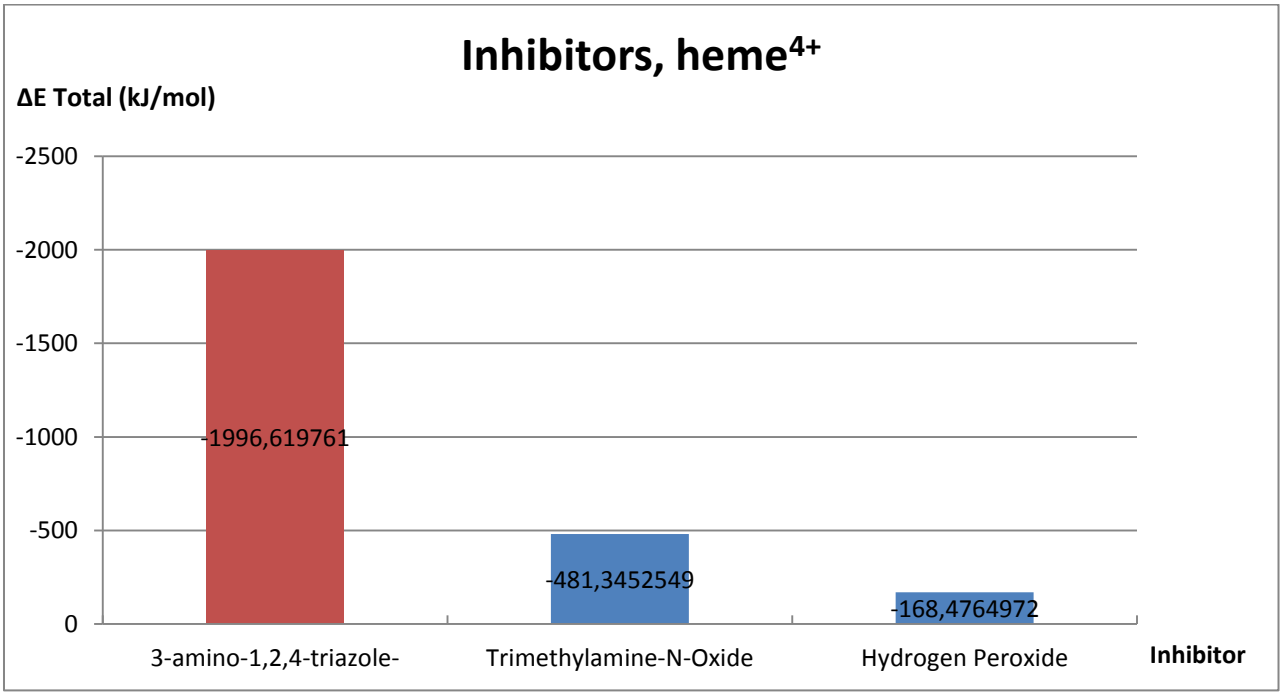


Figure 30

The diagram displays the inhibition energy for the inhibitors bound to heme^{4+}. Notice that the inhibition energy is much superior for deprotonated inhibitors.

4.5.2 Inhibition length

Table 17

The inhibitors and how close they bind to the active site.

Inhibitor	Length (Å)
3-amino-1,2,4-triazole$^-$	1,787
Trimethylamine-N-Oxide	1,878
Hydrogen Peroxide	2,189

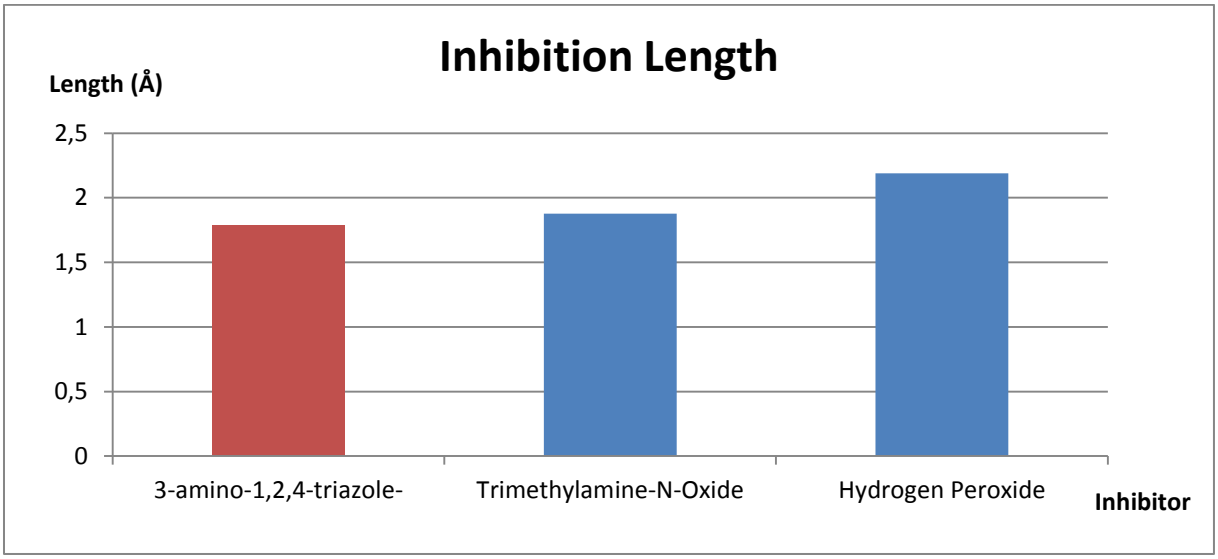


Figure 31

Displays the inhibition length.

5. Discussion

One of the reasons why the result section was divided into the categories: known inhibitors, inhibitors found from the literature study, inhibitors found from malaria research and potential new inhibitors was that it should be easy to compare the different categories and then be able to come to a conclusion. Therefore I will start this discussion by presenting my observations from each of the groups. Then I will present some overall observations of the results as well as thoughts about the work procedure and different choices made throughout the project.

5.1 Category 1: Known inhibitors

This category's main purpose was to act like a reference for the later calculations. Some of the inhibitors are known for good inhibition properties e.g. 3-amino-1,2,4-triazole which cannot be used industrially due to its toxicity.

From figure 34 it is clear that all of the three calculated molecules have better inhibition energy than hydrogen peroxide. This was clearly expected since they are all known to be able to inhibit the heme-group better than hydrogen peroxide. Nevertheless this can be seen as an indication on that the calculations might be accurate. One observation that is not as expected was that hydroxylamine had the lowest energy of the three inhibitors since hydroxylamine was earlier a patented inhibitor against hydrogen peroxide degradation.

When looking at figure 35 it can be seen that the inhibition energy is many times higher than for the non-deprotonated inhibitors. This is not just the case for the known inhibitors but for every inhibitor tested in the study. The reason behind this is that when a molecule loses a proton it becomes an ion which can form an ionic bond with the iron ion in the heme. This ionic bond is always much stronger than the dipole bond. This also leads to a shorter bond length which can be seen in the graphs presenting inhibition length. The result will be further discussed in section 5.6.

The next big question is whether inhibition strength and inhibition length is correlated? For figure 34 and 35 some correlation can be seen. Ethane nitronate which has the largest ΔE among the non-deprotonated molecules also has the shortest distance to the heme. With the deprotonated molecules it is harder to see clear correlation; phenylhydrazine which had the largest ΔE has among the shortest inhibition lengths, but not the shortest one.

Figure 37 shows that all the inhibitors would fit in the substrate-channel.

5.2 Category 2: Inhibitors found from the literature study

One of the more interesting inhibition energies was found from the literature. The ΔE result received from trimethylamine-N-oxide was very high compared to hydrogen peroxide and formaldehyde. This result indicates on good inhibition properties. Urea did not have just as good result but it was still interesting compared to the inhibition energies received from the calculations on the known inhibitors. Noteworthy is also that formaldehyde received a rather low inhibition energy. This result is a little surprising since one of the latest patents on the market mentions formaldehyde. The result from melamine, seen in figure 43, showed similar result to 3-amino-1,2,4-triazole thereby indicating that it could be a candidate for further studies.

Figure 44 further confirms the correlation between ΔE and inhibition length, a high ΔE-value gives a short bond distance.

When looking at the graph for the size of the inhibitors (Figure 45) it is shown that the melamine molecule is slightly larger than 5 Å. Even if melamine might be a good inhibitor for free heme it will most likely have problems to reach the active site in the enzymes to the same extent as the smaller molecules.

5.3 Category 3: Inhibitors found from malaria research

This category only contain the inhibitors cinchonidine and quinidine, the initial idea was to make calculations on all of the inhibitors mentioned in the article: *Detection, Characterization, and Screening of Heme-Binding Molecules by Mass Spectrometry for Malaria Drug Discovery* by K, Muñoz-Durango et. al. When I ran some of these molecules in Dmol3 they did not converge i.e. find the optimal energy. Hypothesizes on why they did not is that maybe the wrong base settings were used for the complex or maybe another atom from the inhibitor interacted with the heme-group and disturbed the calculation. However the inhibitors presented in the result section did converge when running them non-deprotonated. This is the reason why I calculated them in this state even though they both have a pKa value below 7.

Both cinchonidine and quinidine got large ΔE-values from the calculations. They even got higher inhibition energy than trimethylamine-N-oxide which indicates on good inhibition properties. The inhibition lengths on the other hand were rather long compared with all the other calculations. My theory why they have such long inhibition length is the size of the molecules. There are a lot more atoms in these molecules that are able to interact with the heme.

As it was expected the molecules were too big to be able to fit in the substrate-channel. Therefore they are not candidates for enzyme inhibition. However they are most likely able to inhibit free heme and free heme is also present in the process when bleaching secondary

fibers. Nevertheless one shall have in mind that these molecules are medicines and most likely very expensive to use in the industry.

5.4 Category 4: Potential new inhibitors

One particularly noteworthy result from this category is piperidine. It has significantly higher ΔE-value than the other calculated molecules. This was quite unexpected to me since I first assumed that the conjugated aromatics would bind best to the heme. The reason behind this result is that the nitrogen atom binding to the active site for piperidine has a localized lone pair. This lone pair cannot take place in a conjugation since the molecule does not have any. Therefore it does just exist in one place all the time, at the nitrogen atom, which explains the strong inhibition energy.

Figure 66 shows the deprotonated inhibitors oxalic acid and aniline. Oxalic acid did not show any good result regarding ΔE, aniline on the other hand had rather good inhibition energy. The problem with Aniline is its toxicity on human health which may make it improper for usage in the industry.

The inhibition lengths in this category also confirm that it seems to be a vague correlation between inhibition length and inhibition energy. As earlier mentioned other parameters play a part as well, for example molecule size and if the inhibitor exerts any other interaction with the heme-group.

When choosing the inhibitors in this category I considered the size of the substrate channel. That is why all of the molecules in figure 68 are smaller than 5 Å.

5.5 Inhibition to heme^{4+}

The reason why heme^{4+} was calculated as well was that I needed to see if my calculations on heme^{3+} can be applied to a different charge than the one calculated. Heme^{4+} is a rare form but do exist in enzymes. Looking at equations (1), (2) and (3) one can see that when Heme^{3+} reacts with hydrogen peroxide it forms heme^{4+}. The result from the three different inhibitors 3-amino-1,2,4-triazole, trimethylamine-N-oxide and hydrogen peroxide all shows that the inhibition energy raised almost 30% from the calculation with heme^{3+}. The interesting part is that all the inhibitors regardless of charge raised 30%. This result point toward the fact that the result received from calculations on heme^{3+} is representable for other heme charges as well.

When it comes to inhibition length it does not differ from Heme^{3+}.

5.6 pKa value and its significance

A substance pKa value shows to what extent it is deprotonated in an aqueous solution. I chose to calculate most molecules with a pKa value below 7 as deprotonated. One important thing to have in mind about pKa-value and deprotonating is that a low pKa value does not mean that most of the molecules deprotonate. When equilibrium state is reached in the pulp there will always be many non-deprotonated molecules left in the solution.

It is obvious that deprotonated molecules have better inhibition properties than those calculated as non-deprotonated. This is as earlier discussed expected from an ion-ion bond. A theory about what really happens is illustrated below. The hydrogen peroxide molecule was calculated it as non-deprotonated since it has a pKa-value of 11,75 but in this example I have calculated as deprotonated as well just to illustrate. The calculation model does not take into consideration that the complex is in an aqueous solution and neither does it consider pH-value of the solute. It is a model that calculates the properties of the molecules in vacuum. What happens in a real process is that equilibrium occurs according to the reaction formula below.

$$E\text{-}Fe^{III} + H_2O_2 + H_2O \rightarrow E\text{-}Fe^{III} + HO_2^- + H_3O^+$$

The large inhibition energy received from inhibitors calculated as deprotonated comes from the solvation energy of the hydroxonium ion. This means that if I could withdraw the energy from the reaction below from the energy of the heme-group with the deprotonated inhibitor it will give value closer to the real value. This is because the energy of the proton transfer is now being considered.

$$\Delta E = E\text{-}Fe^{III}\text{-}HO_2^- + H_3O^+ - E\text{-}Fe^{III}\text{-}H_2O_2 - H_2O$$

I show this with hydrogen peroxide as the inhibitor:

ΔE= -2402,7638 + (-76,7125) - (-2402,8744) - (-76,4357)= -0,1663 Ha

-0,1663 Ha= -436,5474 kJ/mol

This energy value is what it cost the inhibitor to deprotonate. Therefore the theory is that all the energies received from the calculations with a deprotonated inhibitor should be reduced by the ΔE corresponding to that reaction.

For hydrogen peroxide this gives us:

ΔE(total)= -1506,0314 - (-436,5474)= -1069,484 kJ/mol

This is still a high value; this indicates that the inhibitor gains more inhibition energy than it loses from deprotonating. It might also have to do with the fact that Dmol3 does not account for external conditions. If one wants to account for these external conditions the problem gets way more complicated at once.

Nevertheless the values received from the calculations can be seen as a first step in comparing different inhibitors and give some leads on which of them that might be appropriate to use.

From the reasoning above one can see that the more an inhibitor likes to get deprotonated the stronger the inhibition energy will get. It is already known that the inhibition process gets more efficient with increasing pH value, the reasoning above also confirms that this is the case. Favorable conditions when inhibiting enzymes in bleaching of secondary fibers are high temperature and high pH. The problem is that these conditions lead to yellowing and degradation of the pulp [4]. The objective for further research is to find an inhibitor that is efficient enough at lower pH-values.

6. Conclusion

Two of the newest patents found from the literature study could exert two different kinds of inhibition. One patent was found to use cross-linking by addition of an aldehyde functional polymer [12]. Another patent was found to use formaldehyde releasing chemicals. The chemicals added are either HT or TD from figure 8 and 9 [13]. Formaldehyde is the molecule to inhibit the enzyme from breaking down hydrogen peroxide. Newer patents than those just mentioned might exist but such patents were not found in this literature study.

The result from the calculations indicates that it is possible to get a good perception on which chemicals that are to prefer as inhibitors. Evaluation of the inhibition energies, inhibition length, size of inhibitor and possible toxicity of the inhibitors left me with several leads that would be interesting to further investigate.

From the literature study trimethyamine-N-oxide was found have the best inhibition energy as well as inhibition length. The size of the molecules shows that it is small enough. The safety data sheet specifies some degree of toxicity; it is irritating for the eyes, the lungs and the skin. Today the chemical is used for laboratory purposes mainly [24]. I recommend further research to investigate the chemical on a laboratory scale, to investigate the cost of the chemical and to see if it is available to buy in bulk.

The other chemical found from the literature study that showed interesting result was urea. It has lower inhibition energy than trimethylamine-N-oxide but much higher than hydrogen peroxide. The inhibition length is also a little longer than that for trimethylamine-N-oxide but it is still interesting. The size of the molecule suggests that it is small enough for inhibition. Urea is relatively safe for usage on industrial scale. I recommend further research to test this chemical on a laboratory scale.

The inhibitors used as malaria medication are too big to reach the active site in the enzymes. They could be able to inhibit free heme. I recommend further studies to investigate its properties on free heme. One shall also make sure that usage does not enhance the risk for resistant malaria parasites. Economic aspects were not considered in this thesis; therefore I suggest that the price of these medicines should be investigated before further studies are commenced with the purpose of finding an inhibitor for industrial purpose.

From the potential new inhibitors one particularly interesting inhibitor was found, piperidine. It has a high inhibition energy value as well as inhibition length. The size of the molecule is small enough to fit the substrate-channel. The safety data sheet specifies that the inhibitor is highly toxic, flammable and corrosive [25]. Even though piperidine might be a good inhibitor it might be too toxic to use on industrial scale. I suggest that further research continue to look at cyclic amines with localized lone pair electrons.

Lastly I suggest that further research should investigate pKa values significance on inhibition. It should also be tested if a deprotonated inhibitor is able to reach the active site in the enzymes.

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