Computational studies of the mechanisms of chemical reactions involved in mechanism-based inactivation of cytochrome P450 enzymes

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**Computational Studies of the Mechanisms of Chemical
Reactions Involved in Mechanism-Based Inactivation of
Cytochrome P450 Enzymes**

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SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

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List of Abbreviations

P450	Cytochrome P450
SRSs	Substrate Recognition Sites
Cpd I	Compound I
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NADH	Nicotinamide Adenine Dinucleotide
FAD	Flavin Adenine Dinucleotide
FMN	Flavin Mononucleotide
DDIs	Drug-Drug Interactions
MBI	Mechanism-Based Inactivation
HTS	High Throughput Screening
MM	Molecular Mechanics
QM	Quantum Mechanics
QM/MM	Quantum Mechanics and Molecular Mechanics
PDB	Protein Data Bank
MD	Molecular Dynamics
HF	Hartree-Fock
DFT	Density Functional Theory
MP2	The Second-Order Møller-Plesset Perturbation Theory
LSDA	Local Spin Density Approximation
GGA	Generalized Gradient Approximation

List of Abbreviations (cont.)

ONIOM	Our own N-layered Integrated molecular Orbital and molecular Mechanics
ME	Mechanical Embedding
EE	Electronic Embedding
LSCF	Local Self-Consistent Field
GHO	Generalized Hybrid Orbitals
UDMH	1,1-dimethylhydrazine
UB3LYP	unrestricted-B3LYP functional
ZPE	Zero Point Energy
SCRF	Self-Consistent Field
RC	Reactant Complex
TS	Transition State
Pro	Product
HAT	Hydrogen Atom Transfer
PCET	Proton-Coupled Electron Transfer
SOMO	Singly Occupied Molecular orbital
HOMO	Highest Occupied Molecular Orbital
LUMO	Lowest Unoccupied Molecular Orbital
IRC	Intrinsic Reaction Coordinate
MIC	Metabolic-Intermediate Complex
ADF	Amsterdam Density Functional
EDA	Energy Decomposition Analysis
NMH	<i>N</i> -methylhydroxylamine

List of Abbreviations (cont.)

SNOs	Spin Natural Orbitals
BDE	Bond Dissociation Energy
VB	Valence Bond
ET	Electron Transfer
PT	Proton Transfer
ABT	1-Aminobenzotriazole
BBT	<i>N</i> -benzyl-1-aminobenzotriazole
TDDFT	Time-Dependent Density Functional Theory
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectroscopy

Abstract

The cytochrome P450 enzymes (P450s) play crucial roles in xenobiotic metabolism, especially drug metabolism. Even though metabolism is fundamentally an essential process, metabolic products could also lead to adverse effects. For example, in mechanism-based inactivation (MBI), a reactive metabolite acts as an inhibitor of a P450 and thereby reduces or eliminates the enzymatic activity. Such inhibition can have great influence on the metabolic profiles of drugs. An in-depth understanding of MBI of P450s will provide clues as to how to prevent this unwanted consequence during a therapeutic period. The complex mechanisms of the reactions of P450s are hard to probe experimentally. Computational studies, based on small models or realistic models, are indispensable for gaining valuable insight into the enzymatic reactions. Through this thesis, computational methods, such as density functional theory (DFT), quantum mechanics (QM), and hybrid quantum mechanics/molecular mechanics (QM/MM) calculations are applied to some reactions of P450s pertaining to MBI. Through computational studies, valuable insights into MBI processes have been derived, which I believe is an important first step for the design of drugs free from MBI.

Chapter 1 Introduction

1.1 Cytochrome P450s

Cytochromes P450 (P450s) are a superfamily of hemoprotein enzymes and are available in all kinds of organisms (bacteria, fungi, plants, and animals). P450s are responsible for the catalysis of a variety of chemical reactions in biological systems.[1-7] Such P450-catalyzed reactions pertain to the detoxification of xenobiotic compounds, biosynthesis, bioremediation, degradation of biochemical compounds.[5, 8-12] The first clue to the nature of this enzyme came from the spectroscopic studies of the microsomal heme proteins in 1958 by Klingenberg, which provided a unique spectrum that differed from the known Cytochromes.[13] At that time, the enzyme was known as an unknown carbon monoxide binding pigment in rat liver microsomes. The term cytochrome P450 was coined in 1962 and came from the behavior of the P450s, which bound to membranes within cells, and their carbon monoxide bound form had an absorption maximum at 450 nm. Most of other hemoproteins have an absorption maximum at 420 nm.[14-17] To date, more than 18,000 P450 isoforms have been identified.[18-20] Of these, 57 P450 isoforms are available in humans. They are expressed mainly in the liver and small intestine. The main functions of P450s in human body are the metabolism of xenobiotic compounds through a variety of reactions (hydroxylation, epoxidation, C-, N-, S-oxidation, etc.) and the biosynthesis of biologically active molecules (e.g. hormones and lipid signaling molecules). In xenobiotic metabolism, P450s generally increase the polarity of substrates to facilitate their excretion as water soluble compounds.[1, 2, 5, 21, 22] Moreover, P450s are responsible for the phase I metabolism of drugs (which are considered as xenobiotic compounds).[21, 23-28] In plants, P450s provide a wide range of functions in herbicide resistance, pigment and hormone

syntheses.[29-31] The P450s in microorganisms such as fungi serve in cell protection and also facilitate pathogenesis processes.[32-35]

Table 1 Classification of P450 families.

P450 families	Organisms
CYP1-49	Animals
CYP51-69	Yeast, Fungi
CYP71-99	Plants
CYP101-299	Bacteria
CYP301-499	Animals
CYP501-699	Lower eukaryotes
CYP701-999	Plants
CYP3001-4999	Animals
CYP5001-6999	Lower eukaryotes

Because there are a large number of isoforms, a systematic rule for nomenclature has been established to organize them as categorized systems. This system provides the information on the families and subfamilies to individual names. It is based on the level of amino-acid sequence identity. The names of P450 isoforms always start with CYP that refers to cytochrome P450. Individual families are designated by the number and the letter that indicates the subfamily name. The individual isoform in a given subfamily is specified by the last number.[9, 21, 36, 37] In the same family, individual isoforms share more than 40% amino acid identity. Those isoforms with sequences that are more than 55% identical are classified as the same subfamily. Finally, those isoforms, which share at least 97% amino acid sequence identity, are considered as analogous isoforms.

Insignificant differences could occur owing to the genetics of individual persons.[1, 26, 27, 36] As mention above, P450s are found in all kinds of organisms, and thus new P450 isoforms have been and would be identified. Some block numbers are reserved for newcomers. Examples of the block number classification are shown in Table 1.[26] The substrates of P450 enzymes are usually hydrophobic compounds because the active-site of P450s is highly hydrophobic.[3, 5, 8] The substrate recognition of CYP450s is governed by the substrate recognition sites (SRSs). The SRSs consist of the B'-helix region, some parts of the F and G helices, a part of the I helix, the K helix of the β_2 connecting region, and the β_4 hairpin (Figure 1). If the amino acid sequences of SRSs are modified, the substrate specificity of P450s will be altered. Even within the same subfamily of P450s that have at least 55 % identity, if those differences appear in the SRSs, the specificity and activity of P450s are different. Moreover, when the SRSs are mutated in the individual isoform, the substrate specificity is altered. For example, hydroxylation of plant fatty acids is catalyzed by members of the CYP94A subfamily. CYP94A1-3 are responsible for this reaction in *Vicia sativa*. In the case of CYP94A1, the fatty acid substrate is hydroxylated at the ω -position, whereas the product of CYP94A2 depends on the substrate chain-length.[30, 38]

Figure 1 Structure of a P450 isoform CYP101A1.

It is well known that the catalytic center of P450 enzymes is a cysteinate-heme species. The fifth ligand of the heme is a cysteinate group and the sixth coordination site is open for O₂ and substrates. When the oxygen molecule binds to this site, the catalytic cycle will be triggered (Figure 2). The active species, which is formed during the catalytic cycle is an oxoiron(IV) (or ferryl) protoporphyrin π -cation radical species, called Compound I (Cpd I). The formation of Cpd I is a two-electron reduction process (Figure 2). Two electrons are supplied from the protein framework that consists of nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide (NADH) and redox partner proteins (i.e. flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN)), which are known as P450 oxidoreductases.[17, 22, 36, 39-42] The electron transfer process is initiated when the FAD is reduced by

NADPH. The reduced FAD (known as FADH₂) transfers electrons to the nearby FMN, to form FMNH₂ is formed. Finally, electrons from FMNH₂ are passed to the P450 system (Figure 3). The heme iron receives the electrons and uses them to activate the molecular oxygen in the catalytic cycle.[3, 27, 36, 37, 42-52] It should be noted that the spin state of the species varies depending on the coordination state of the central iron atom. The hexacoordinated resting state is in equilibrium between the low-spin or high-spin states, whereas the pentacoordinate, which is formed after the removal of the distal ligand, prefers the high-spin state.

Figure 2 Catalytic cycle of P450s.

A variety of P450 enzymes have been identified so far. They can be categorized into three broad groups based on their localization in cells: microsomal-type, mitochondrial-type, and cytosolic-type. As for the first two types, they are embedded in

the membranes of respective organisms.[17, 37, 53, 54] In eukaryotic cells, the most abundant are these types. In contrast, the cytosolic-type is the most abundant form in prokaryotic cells. Even though individual P450 isoforms share a small amino-acid sequence identity between P450 families, the overall folding topology appears to be remarkably similar.[45] Thus, the members of P450 superfamily share a common structural framework (Figure 1). There are relatively high α -helix content in the C-terminal half and high β -sheet content in the N-terminal half. The α -helices are identified by letters A-L. The particular site of the I helix near the protein center is situated in the reaction pocket. The cysteinato proximal ligand of heme active species is located in the K helix. The heme moiety is embedded inside the protein close to the I and L helices.[45, 55]

Figure 3 Mechanism of electron transfer from NADPH to cytochrome P450.

Elementary studies on the reaction catalyzed by P450s elucidated several key evidences for the properties of Cpd I. Ullrich and coworkers[56, 57] found that the reactions of P450 are similar to that of the trifluoroperoxy acid reaction, which has been known as electrophilic hydroxylation. Thus, the active species of P450 might be considered as the electrophilic species. Hydroxylation is the most common reaction of P450s. Regioselectivity of reactions was studied by Grove and coworkers.[58, 59] They suggested that the iron-oxo species might activate the C-H bond and produce a substrate

radical. A hydroxylated product was formed presumably *via* a subsequent radical rebound, which is very rapid. This C-H activation requires a radical active species, which can trigger the catalytic activity. This result suggested that the iron-oxo species might carry unpaired electrons as a radical intermediate.

Over the past few decades, reactions of Cpd I have attracted attention of many theoretical chemists.[47, 49, 60-70] Preliminary theoretical results suggested that the Cpd I should be triradicaloid species. In most of the previous studies, the small model of Cpd I used for theoretical studies consisted of the porphyrin ring, which represents the protoporphyrin IX ring. To represent the cysteinato ligand, there are several choices i.e. thiolate (SH^-), methyl mercaptide (SCH_3^-), and also cysteinato (full cysteine) ligands. Ogliaro and co-workers[71] characterized the electronic structure of representative small models of Cpd I theoretically. As shown in Figure 4, they argued that the thiolate ligand is more suitable to describe both the geometry and electronic structure of Cpd I than mercaptide ligand. As we have seen in the EPR, Mössbauer, XANES spectroscopic studies, two unpaired electrons reside on the ferryl moiety and they are coupled with another electron in the a_{2u} orbital (Figure 5). On the ferryl moiety, two unpaired electrons reside in the π^* orbital and thus form a triplet spin state and the one on a_{2u} can be spin up or spin down. Hence, this coupling results in two low-lying electronic ground states, $S=1/2$ and $3/2$ (doublet and quartet, respectively). [9, 11, 48, 60-62, 65, 72] Theoretical studies showed that the energy gap between these two ground states is small and regardless of basis set used. In addition, the cysteinato ligand is regarded as an electron-withdrawing group. It was shown that the thiolate ligand has greater electron-withdrawing ability than mercaptide ligand. Therefore, the thiolate ligand is usually used as an axial ligand in a Cpd I small model. The author also uses this model when necessary.

Figure 4 Comparison of the electronic properties of the axial ligand.[69, 73, 74]

As mentioned above, P450s are responsible for drug metabolism. Their metabolites are usually excreted as water-soluble compounds. Therefore, the concentration of drugs in the body should be maintained in the appropriate range for the best efficacy of the drug. The appropriate range of a drug concentration is called “therapeutic window”.[75] If the concentration of a drug goes below the lower bound, the treatment will be unsuccessful. On the other hand, the high concentration will cause toxicity (Figure 6). This concentration depends on the clearance rate, which is affected by many factors such as the age, gender, and body weight. Thus, drug prescription is different from person to person. Even though we carefully take these factors into account, the rate of drug metabolism may be affected by other unexpected factors such as disturbance of P450s by other drugs (drug-drug interactions) or chemicals in foods (drug-food interaction). Hence, minimization of these interactions is one of the most important parts in drug discovery processes.

Figure 5 Electronic structure of Cpd I in the doublet (quartet) state.

Two major interfering phenomena result from induction and inhibition. In the case of induction, the drug clearance rate is accelerated, resulting in a lower concentration of the drug than expected. The acceleration usually occurs in the presence of an inducer, which stimulates the metabolic system but inducer itself cannot be or difficult to be metabolized. The induction period may range from an hour to days. Decreasing the drug level leads to a drug failure. By contrast, the inhibition will impede or terminate the drug metabolism. The drug level in the body is increased and results in toxicity when it exceeds the upper bound of the therapeutic window. The inhibition effect is more harmful than induction and sometimes leads to death. Several serious cases have been reported.[76-79]

Figure 6 Schematic of therapeutic window.[75]

In general, the relation between an enzyme and a substrate is considered as “lock and key” in their mutual binding. The lock can be activated only by the suitable key.[80] This statement reflects the fact that the enzymatic activities have the specificity to substrates. However, substrate-like compounds can reduce the enzymatic activity by altering the shape of the active site or obstructing the active site. There are four types of inhibitors: competitive, non-competitive, uncompetitive, and mechanism-based inhibitors. Schematic illustrations of these types are given in Figure 7.

Figure 7 Schematic illustrations of different types of enzyme inhibition.

Figure 8 Schematic of MBI cycle.

In this research, the metabolism of mechanism-based inhibitors and also their inhibition approaches have been investigated theoretically. This kind of inhibitor binds to the same active site and triggers the catalytic pathway in the same way as what substrates do, but the subsequent metabolite is not released or the release from the enzyme is delayed. This process is called mechanism-based inactivation (MBI).

Inhibitors usually consist of functional groups like alkyne, alkene, furan, thiol, amine, etc. The inhibition can be classified into two types: quasi-irreversible and irreversible processes (Figure 8). Metabolites may bind to the apoprotein or heme prosthetic group covalently in an irreversible fashion, resulting in permanent inactivation of the enzymatic activity. By contrast, the metabolites bind to heme-iron moiety quasi-irreversibly. In the latter case, it will not cause any damage to the enzyme after metabolites are released. Understanding the mechanism of MBI will assist in minimizing the side effect caused by drugs and other xenobiotics. Furthermore, many mechanism-based inhibitors are used as reaction phenotyping to evaluate the possible reaction pathway of similar substrates both experimentally and theoretically. [81, 82]

1.2 Computational methods in drug discovery

In pharmaceutical industry, it takes an average of 14 years and highly costly to investigate and eventually launch a new drug.[81] The major part of the cost is accounted for by the clinical trial and the development phase, while most of the time is spent on initial optimization of drug candidates. To date computer technology has had a significant impact on the advances in many areas of chemistry and biology. In drug discovery, computational methods have been developed and increasingly used to improve the quality of hit identification and lead optimization. One example of useful methods is so-called high throughput virtual screening (HTVS).[83, 84] In addition to these computational studies on binding affinity of drugs, studies of drug metabolism are essential for the safety of drugs. Metabolites must be isolated and shown to be nontoxic. Before clinical trials, new drugs should be tested *in vitro* and *in vivo*. However, compared to the studies of binding affinities, which are essentially described well by nonbonded classical interactions, computational studies of MBI are rather scarce. The

main reason is that the study requires the use of quantum mechanical approaches such as density functional theory and hybrid methods (quantum mechanics and molecular mechanics, QM/MM).[85, 86] Conventional biomolecular docking should also be used in combination. In my thesis, I tackle this less explored area of research using computational approaches.

Biomolecular docking

Biomolecular docking has become a key method that allows selection of drug candidates. This method is used to predict plausible conformations and orientations of ligands within an active site or target area. Various docking algorithms have been proposed to predict the pose of ligands reliably on the basis of scoring functions. The scoring functions are designed to evaluate the interaction between the guest and the enzyme active site. These scoring methods are based on the simple forms of interactions such as electrostatic and van der Waals interactions. Although this technique is easy to use and useful, the docking techniques are based on nonbonded interactions. QM methods are necessary to investigate biochemical reactions that have implications in pharmaceutical and toxicological properties.[87-89]

In general, molecular docking is used to obtain the most plausible ligand conformations within the target protein (host). The model of a complex is based on the known 3D structures of proteins and ligands. Protein structures can be taken from the PDB website. Currently, 109,457 structures are deposited on the PDB website, and the number is increasing (Figure 9).[90] With a large number of potential lead compounds, a major technical issue is how to run high throughput screening (HTS) efficiently and at low costs. In this context, docking can be a useful approach. In practice, most of docking software programs treat the host and guest molecules as rigid bodies. However,

protein structures are essentially flexible, and the flexibility influences the binding modes of a ligand. Therefore, the protein flexibility is one of the major issues that have to be included in the docking simulation and also in the development of docking software.

Figure 9 Yearly growth of the published PDB structures.[90]

Molecular dynamics simulation of proteins and biomolecules

Molecular dynamics (MD) simulation is a technique for studying the time dependent properties of a molecular system. The macroscopic properties of a system are predicted statistically from the motion at the microscopic level. To run MD simulations, one must obtain force acting on each atom. There are two main approaches for doing

this: classical and ab initio (quantum).[91, 92] In the first approach, molecules are treated as balls and springs (classical objects) and classical mechanics is employed. In contrast, ab initio MD simulation describes the quantum nature of bonds and valence electrons based on the quantum mechanical equation. At the same time, the nuclei (and perhaps also inner core electrons) are described by the classical mechanics. The latter method is capable of describing chemical reactions in which bonds are formed or broken. However, it requires extremely high computational resources. Hence, the classical MD simulation is a more practical and widely used approach for biomolecular simulations.

The algorithm for the classical MD simulation includes the time integration of Newton's equation of motion. For simplicity, let r_i represent the Cartesian coordinates (x_i, y_i, z_i). Then the equation can be written as

$$F_i(t) = \frac{m_i d^2 r_i(t)}{dt^2} \quad (\text{eq. 1})$$

where F_i is the force acting on the i th particle at the time (t), r_i is the position the changes with time, and m_i is the mass of the particle. The second derivative of the position with respect to time is the acceleration of a particle. To solve the above equation, the initial position and velocity of particle must be specified. However, the integration of this equation is executed with a finite period of time, to derive a set of particle positions, $r_i(t)$ and velocity, $v_i(t)$ at each time. The kinetic energy and temperature of the system can also be evaluated. The position of a particle after Δt can be defined as

$$r_i(t + \Delta t) \cong 2r_i(t) - r_i(t - \Delta t) + \frac{F_i(t)}{m_i} \Delta t^2 \quad (\text{eq. 2})$$

It should be noted that there are some limitations in the classical MD simulation. First, MD is based on MM force fields, which cannot describe the behaviour of electrons

in a molecule. Thus, the classical MD simulation cannot simulate the dynamics of bond breaking or bond formation processes in enzymatic reactions. Nevertheless, the MD simulation can assist the theoretical chemist in identifying key molecules or residues in certain behaviour of large molecules. Currently, many available force fields are available such as AMBER,[93] GROMACS,[94] OPLS,[95] and CHARMM,[96] which are different in mathematical forms of energy functions, and have proven useful in describing the properties of biomacromolecules. However, MD simulation has the limitation of time and size. The time scale of the biological reaction can range from picoseconds to microseconds to minutes. The larger the time scale and the size of system are, the larger the required computational resources will be.

Force fields

In MD simulation and other MM calculations, they employ force fields, which parameterize energy as a function of nuclear coordinates of molecules. These parameters are defined from either experimental or QM computational results. Force fields can well demonstrate the macroscopic properties of a given system. The dynamics of atoms are defined by classical mechanics (Newton's equation of motion), whereby the properties of a system evolve with time. The main propose of the force fields and MD simulation is to define the potential energy of a system. A known problem of MD is that the system is trapped in a local minimum of its potential energy surface (Figure 10).

Figure 10 Potential energy surface.

Force fields are assigned according to the standard building blocks of a molecule, which define the atom types and chemical environment of individual atoms. There are two types of force fields; all-atom and united-atom force fields. An all-atom force field provides the parameters to every atom type in a molecule, whereas a united-atom force field regards a few atoms comprising a subunit one atom type. The precise force fields will produce more realistic and reliable descriptions of molecules. However, the existing force fields are not capable of representing all atom types or molecule types. Therefore, occasionally, force fields for specific molecules must be newly parameterized.

Currently, there are many force fields available. However, the basic mathematical structures of different force fields are more or less the same. The total energy is the sum of bonded and non-bonded terms at a given molecular geometry and interactions. The general equation of energy can be written as

$$E_{FF} = E_{str} + E_{bend} + E_{tor} + E_{vdW} + E_{el} \quad (\text{eq. 3})$$

First three terms describe the bonded interactions (geometry) of a molecule i.e. stretching, bending and torsional energies, respectively. E_{vdW} and E_{el} terms represent non-bonded interactions i.e., van der Waals and electrostatic interactions, respectively.

Density functional theory

Density functional theory (DFT) has been developed since 1960s and has become a mainstay in many scientific fields such as chemistry, materials science, engineering, biology, and pharmaceutical sciences. DFT is superior to the conventional quantum chemistry methods in terms of the balance between accuracy and computational cost.[97-102] Currently, a number of software packages that can execute DFT calculations are available.

DFT assumes that energy is a functional of electron density (ρ). Here, a functional means a function of a function. Electron density can be viewed as a function of nuclear coordinates (r), while the energy depends on the electron density and therefore is a functional. Thus, if we know the electron density of a system, we should be able to determine its energy. The validity of this assumption was proved by Hohenberg and Kohn.[103] They proved that there is a one to one correspondence between the ground state electron density and the external potential. The electron density is obtained by integrating the square of a wave function over $3(N-1)$ spatial variables and N spin variables. Thus, the density depends only on three coordinates, regardless of the system's size. This is in contrast to a wave function, which contains $3N$ spatial together with N spin coordinates.

The energy functional in DFT can be divided into three parts, kinetic energy ($T[\rho]$), attraction between nuclei and electrons ($E_{ne}[\rho]$), and electron-electron repulsion ($E_{ee}[\rho]$) (nuclear-nuclear repulsion becomes a constant after applying the Born-

Oppenheimer approximation). However, explicit forms of $T[\rho]$ and $E_{ee}[\rho]$ are not known. To circumvent this problem, a practical method of determining energy within DFT was proposed by Kohn and Sham.[104, 105] They introduced a method for determining the kinetic energy of non-interacting electrons ($T_s[\rho]$), which has a large contribution to the exact kinetic energy. The non-interacting electrons are described by a Slater determinant of Kohn-Sham orbitals. It is assumed that the density of the non-interacting electrons (eq. 4) can represent the real density.

$$\rho(r) = \sum_{i=1}^N |\phi_i(r)|^2 \quad (\text{eq. 4})$$

Then the equation reduces to

$$\left(-\frac{\hbar^2}{2m} \nabla^2 + v_{\text{eff}}(r) \right) \phi_i(r) = \epsilon_i \phi_i(r) \quad (\text{eq. 5})$$

where $v_{\text{eff}}(r)$ is the Kohn-Sham potential. The total energy of the non-interacting electrons is written as

$$E_{DFT}[\rho] = T_s[\rho] + E_{ne}[\rho] + J[\rho] + E_{xc}[\rho] \quad (\text{eq. 6})$$

In which

$$E_{ne}[\rho] = \sum_a \int \frac{Z_a \rho(r)}{|R_a - r|} dr \quad (\text{eq.7})$$

$$J[\rho] = \frac{1}{2} \int \int \frac{\rho(r)\rho(r')}{|r - r'|} dr dr' \quad (\text{eq.8})$$

and $E_{xc}[\rho]$ is the exchange-correlation energy.

Unfortunately, the exact form of the exchange-correlation energy is not known. There are many exchange-correlation functionals, which have been developed so that they satisfy different fundamental quantum mechanical requirements or the parameters reproduce experimental data.[106] Some electron correlation effects are included in the Kohn-Sham treatment. The Kohn-Sham method is generally faster than many correlated

electronic structure methods and more accurate than the Hartree-Fock method. Sometimes, the accuracy of DFT calculation is comparable to second-order Møller-Plesset perturbation theory (MP2) calculation (a post Hartree-Fock method).

Kohn-Sham DFT is classified into several types. The five well-known classes are local spin density approximation (LSDA), the generalized gradient approximation (GGA), meta-GGA, hybrid-GGA, and meta-hybrid-GGA. Among them, the simplest method is LSDA in which energy depends only on electron density. Energy in GGA methods is dependent not only on the electron density, but also on the reduced gradient. Meta-GGA is similar to GGA, but depends on the kinetic energy density. The other two hybrid methods combine a GGA or meta-GGA and the Hartree-Fock (HF) exchange energy.[99]

Our own *N*-layered Integrated molecular orbital and molecular mechanics (ONIOM)

Multiscale approaches have been developed to deal with large molecular systems. Even though the fast and powerful computers have been developed, it is still difficult to determine quantum mechanically and accurately the properties of large complex chemical systems such as supramolecules and biomacromolecules. In multiscale approaches, the entire system is partitioned into subsystems. Each subsystem will be treated using different levels of theory. In 1970, Honing and Karplus introduced a method to combine QM and MM calculations. Since then, this hybrid method has been developed extensively.[107] In 2013, The Nobel prize in chemistry was awarded for the development of the multiscale method to deal with large molecular systems. [108]

A multiscale, multi-level calculation method, which combines an accurate QM method and a classical force field (MM), is called QM/MM method. In the QM/MM

method, a QM approach is used for the model system at which, for example, a chemical reaction occurs. The remaining system is treated using a MM method. The total energy of the entire system can be evaluated by two different schemes: additive and subtractive schemes (eqs. 9-10).[109-111]

$$E^{add} = E_{qm}(QM) + E_{mm}(MM) + E_{qm-mm} \quad (\text{eq.9})$$

$$E^{sub} = E_{mm}(\text{system}) + E_{qm}(QM) - E_{mm}(QM) \quad (\text{eq.10})$$

As we can see in eqs. 9 and 10, the additive scheme evaluates the total energy as the sum of the energies of the QM system ($E_{qm}(QM)$) and the MM system ($E_{mm}(MM)$) and the interaction energy between the QM and MM systems (E_{qm-mm}). On the other hand, the subtractive scheme uses multiple layers to describe a system. In the case of a two-layered system, the energy of the entire system is expressed as the sum of the MM energy of the entire system ($E_{mm}(\text{system})$) and the QM energy of the QM system ($E_{qm}(QM)$), minus the MM energy of the QM system ($E_{mm}(QM)$). This scheme is also known as an “extrapolative” approach.[112-114] The latter scheme can be extended to multiple (more than 2) layer models, and such a layered method is generally called Our own *N*-layered Integrated molecular Orbital and molecular Mechanics (ONIOM).

The ONIOM method has several advantages over the additive QM/MM scheme:

1) The lack of an energy coupling (E_{qm-mm}) term avoids the over- and undercounting of interactions between QM and MM atoms; 2) besides the QM:MM combination, it can be applied with different levels of QM methods (QM1:QM2); 3) the *n*-layered system can be implemented to computational programs with no special amendments needed. Nevertheless, high quality of the MM force field parameters is required for accurate calculations.

Similar to DFT calculations, properties of the model system are determined in QM/MM by solving the Schrödinger equation approximately. The effect of point

charges in the surrounding amino acid residues in the model system can also be included. In the simplest scheme, the charges in both of the QM and MM systems are constants and thus the QM wave function is not polarized by the surrounding MM charges. This scheme is called “Mechanical Embedding (ME)”. The accuracy of this scheme depends on the accuracy of the charges for the model system that are specified by the user. For a better description of the model system, the polarization of the wave function induced by the surroundings should be included, *via* so-called “Electronic Embedding (EE)”. In general, the electrostatic interaction between the QM and MM regions could be better described by the EE scheme than by the ME scheme. However, the EE scheme requires a higher computational cost than ME.

In QM/MM, the entire system is divided into the QM and MM regions. The ideal partitioning involves no scission of a covalent bond between these regions. In practical cases, it is inevitable to cut a few covalent bonds. When a covalent bond is cut, a special treatment is necessary to modify the chemical state of the boundary. There are three well-known boundary schemes for the treatment, which are detailed below.

i) Link atoms

An additional atom is introduced to saturate the dangling bond in the QM region: the added atom is called link-atom. In most cases, a hydrogen atom is used. This link atom is not a part of the real system. The QM calculation will be performed with the saturated QM region. The presence of an independent link atom might increase the degrees of freedom as well as the computational complication. These unnecessary degrees of freedom can be eliminated when the position of an introduced link atom depends on the position of frontier atoms. This position is placed along the cut bond. As shown in Figure 11, the position of link-atom can be determined explicitly with a scale factor (g). [115]

Figure 11 Link atom and equation for determining its position.

ii) Boundary atoms

In this scheme, an MM atom, which is connected to a QM atom, is replaced by a special MM atom, namely, a so-called boundary atom. This atom participates in the normal MM calculation and the QM calculation as well. This scheme prevents an unnecessary degrees of freedom caused by additional atoms. In this case, the MM parameters of the boundary atom should reproduce the electronic properties of the MM atom that is included in the QM calculations.[116, 117]

iii) Frozen localized orbitals

Instead of using an additional atom or a special atom, a dangling bond may be saturated by hybrid orbitals.[110] These orbitals are kept frozen along the link bond between the QM and MM regions and do not participate in SCF iterations. There are two widely used methods: Local Self-Consistent Field (LSCF)[118, 119] and Generalized Hybrid Orbitals (GHO).[120] The differences of these methods lie in where and how the frozen orbital is placed (Figure 12). In LSCF, the frozen orbital is placed on the QM frontier atom. One hybrid orbital that is linked to the MM region is kept frozen. This orbital lies along the link bond and is not optimized as a part of SCF cycles.

Similar to the LSCF method, frozen hybridorbitals are placed on the MM frontier atom. In the case of sp^3 hybridization, three of them are kept frozen. The improvement of this method over the previous one is due to the fact that the population of the frozen orbital that is connected to the QM region is optimized during the SCF cycles without altering the fundamental constant shape.

Figure 12 Comparison of frozen orbital schemes. The frozen orbitals are in red.

To minimize the systematic error, an appropriate choice of cut bonds should be made. Because the H atom is usually used as a link atom, using a non-polar bond that does not participate in the hyperconjugation effect (e.g. benzene ring) can minimize the polarization problem nearby the frontier atoms. Therefore, a C-C single bond that is connected using sp^3 - sp^3 hybridization is an ideal choice. However, if this choice cannot be made, a partial double bond can be chosen.[110, 111, 121]

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Chapter 2 Mechanism-Based Inactivation of Cytochrome P450 by 1,1-Dimethylhydrazine

2.1 Introduction

Cytochrome P450 enzymes are powerful catalysts existing in nature for oxidative biotransformation of drugs and other xenobiotic compounds.[1-7] The essential functions of P450s should not therefore be disturbed. However, some drugs or their metabolic products are known to cause a major problem that is known as drug-drug interactions (DDIs). In the worst case, DDIs can lead to withdrawal of a drug from the market. In order not to waste the budget allocated for research and development of drugs, it is necessary to detect this problem in the early stage of drug discovery. Many reliable methods have been proposed to predict DDIs through both theoretical and experimental approaches. Mechanism-based inactivation (MBI) of P450s is one of the major types of DDIs. The metabolic products could bind to the heme-iron center, the heme prosthetic group, or surrounding amino acids, thereby leading to diminished enzymatic activity. MBIs can be classified into two major classes: irreversible and quasi-irreversible MBI.[8-14] Detailed studies of molecular interactions are required to understand the metabolic reactions and the reactivity of metabolic products in MBI processes. Besides the laboratory experiments, theoretical studies using quantum mechanical methods can play useful roles in delineating the complete pictures of MBIs.[15-18]

Amine containing drugs have been used in pharmaceutical treatments for pain relief, antidepressant medication, anti-malaria, etc.[13, 19-22] However, their long-term use might cause unwanted problems such as hepatitis, convulsions, or cancer.[23, 24] Hydrazine-type compounds have been used as the representative of amine-containing

drugs in many studies.[25-27] Their metabolite can act as P450 inhibitors to impede the catalytic activity. The metabolic product of 1,1-dialkylhydrazine forms a metabolic intermediate (MI) with the heme-iron center.[28, 29] The catalytic mechanism was proposed by Correia and Ortiz de Montellano; in their proposed mechanism, the reaction is initiated by H-abstraction, which is followed by either another H-abstraction or N-hydroxylation (Figure 1).[9] However, the detailed mechanism is still unclear. Therefore, we performed a computational study on the reaction between 1,1-dimethylhydrazine (UDMH) and P450 compound I (iron(IV)-oxo species, Cpd I). Before our study, several studies have been conducted on the Cpd I catalysed nitrogen-containing compounds, although as far as we are aware, no theoretical study has been performed for the MBI of 1,1-dialkylhydrazines.[30-38] For example, Rydberg and Olsen found that the plausibility of the N-oxidation and H-abstraction mechanisms is comparable in the first step of the reaction of a primary amine (propane-2-amine).[39] However, the later studies on other nitrogen containing compounds found that preferential mechanism is varied. For example, the N-oxidation is preferred in nitroso formation from methylamine, while the H-abstraction is more favourable in aniline, trimethylamine (TMA), and dimethylamine (DMA) metabolisms.[16, 24, 35, 40] In our study, both mechanisms are examined. In addition, H-abstraction from methyl group can occur, and it could initiate a dealkylation mechanism.

Figure 1. Proposed mechanism of the MBI of P450 by UDMH.

2.2 Methodology

Our Cpd I model consists of simple porphine (POR) ring and thiolate ligands (SH⁻) that represent protoporphyrin IX and cysteinato axial ligands, respectively. Geometries of key species were optimized using the unrestricted B3LYP method (UB3LYP) in combination with the SDD (for Fe) and 6-31G(d) (for others) basis set (B1).[41-45] Frequency calculations were also performed at the same level to characterize the local minima, transition states, and to obtain zero point energy (ZPE) values. The optimized geometries were subjected to single-point calculations with the 6-311+G(d,p) basis set (B2). However, the gas phase calculation may not be able to describe the effect of protein environment. To describe the protein effect, the self-

consistent reaction field (SCRF) method was used.[46] Assuming that the chlorobenzene solvent ($\epsilon = 5.7$) can describe the protein effect reasonably well, we used this solvent. OPBE(SCRF)/B2/B3LYP/B1 and B3LYP/Def2-TZVP//B3LYP/B1 calculations were also tested for some species.[47, 48] All calculations were performed using Gaussian 09 software.[49] DFT-D3 dispersion energy was also determined using the DFTD3 program to correct the interaction energy.[50, 51]

2.3 Results and Discussion

Four possible reaction pathways were examined (Figure 2), which differ in the initial step. Path A begins with H-abstraction from the terminal N-H bond of the hydrazine. Path B involves N-oxidation on the N2 atom. H-abstraction from the methyl group and N-oxidation on N1 initiates path C and path D, respectively. The doublet, quartet, and sextet spin states were considered.

Figure 2. Model for computational analysis in our study and the four paths (A–D) considered.

The first path we investigated is H-abstraction from the N-H bond (Path A). The reactant complex (RC) is stabilized by a H-bonding interaction between UDMH and the oxo moiety of Cpd I. A comparison of different spin states suggests that the lowest-energy state is the doublet state, with the quartet state lying slightly higher in energy. However, this small difference suggested that the ground state could be in thermal equilibrium between the doublet and quartet states. By contrast, the sextet state lies around 10 kcal/mol higher. To validate the relative stability of spin states of RC, the OPBE functional was also tested (Table S2, Appendix A); this functional provided the same trend. This result is consistent with previous computational studies on the iron(IV)-oxo species.[52] The first H-abstraction step via TS1A (Figure 4) shows a relatively low energy barrier in the gas phase (Figure 3). Interestingly, quartet shows a lower barrier than doublet state, although this trend is inverted, and the barrier is relatively low or barrierless in the doublet state in the polar environment. The H-abstraction barriers were also evaluated using a higher level of theory. In the cases of primary amines, H-abstraction barrier from the N-H bond has been calculated as 12-17 kcal/mol.[17, 39] Thus, the barrier for the hydrazine reaction is lower than those for primary amines. One of the reasons will be the weaker N-H bond strength in hydrazines. In fact, additional calculations showed that bond dissociation energy (BDE) of the UDMH N-H bond is much smaller than that of methylamine ($\Delta E = 18.3$ kcal/mol) at the B3LYP(SCRF)/B2//B1+ZPE(B1) level.

Figure 3. Energy diagram (in kcal mol⁻¹) for Path A, as obtained at the B3LYP/B2//B1+ZPE(B1) and B3LYP(SCRf)/B2//B1+ZPE(B1) levels.

Figure 4. Transition states on Path A optimized at the B3LYP/B1 level. Bond distances are shown in Å, and $r(\text{Fe-N})$ refers to the mean value of the four Fe-N distances.

The H-abstraction step result in the formation of a stable substrate radical intermediate and ferryl-hydroxyl complex (Compound II or Cpd II). Substrate radical undergoes a second H-abstraction by Cpd II. This step requires a small energy. The 1,1-

dimethylaminonitrene is formed together with a ferric complex with a water molecule bound as the sixth axial ligand (resting state). Spin density analyses on the complex suggested that the 1,1-dimethylaminonitrene molecule is a closed-shell singlet species. We also performed calculations on an isolated 1,1-dimethylaminonitrene, and the calculations suggested that it has a singlet state. The singlet state is more stable than triplet state by 19.5 and 21.4 kcal/mol at B3LYP/B2//B1+ZPE(B1) level in the gas phase and in the chlorobenzene solvent, respectively.

The alternative pathway for the radical intermediate is a rebound to form a *N*-hydroxylated product. Nevertheless, our attempts to obtain the rebound pathway were not successful. *N*-hydroxyl intermediate was found only on path B.

The second pathway (path B) is initiated by N-O bond formation (*N*-oxidation mechanism). The transition state for the N-O bond formation was found to be much more stable in the doublet state rather than other two spin states (Figure 5). The N-O bond is formed as a result of two-electron transfer from the lone pair of nitrogen to Cpd I. The spin densities analysis revealed that two electrons are completely transferred in a single step. The negative values of spin density on UDMH of TS1B indicated that two-electron transfer occurs in a quasi-stepwise manner; the α -electron (spin-up) is shifted earlier. In the doublet state, this mode of electron transfer stabilizes TS1B, because the remaining β -electron (spin-down) is able to transfer into the Fe=O π^* orbital. On the other hand, the remaining electron on UDMH in TS1B is an α -electron (spin-up) in the case of the quartet state. Instead of Fe=O π^* , the second electron must transfer to the high-lying σ^*_{z2} orbital (Figure 6). This explains the higher barrier in the quartet state. The proton transfer can take place via a proton-shuffle mechanism to generate the *N*-hydroxyl intermediate (Int2B). The proton-shuffle event is assisted by an equatorial pyrole nitrogen. This mechanism is crucial in acid-base catalysts[53, 54] and was

recently suggested to operate in other reactions of P450s.[17] The nitrene is formed via a proton transfer (TS3B). This final step seems to be facile especially in the doublet and sextet spin states.

Figure 5. Energy diagram (in kcal mol⁻¹) for Path B, as obtained at the B3LYP/B2//B1+ZPE(B1) and B3LYP(SCRF)/B2//B1+ZPE(B1) levels.

Figure 6. Electron-shift patterns for the three spin states.

Reactant complex (RC) is stabilized by H-bonding interaction (Figure 8). However, H-abstraction can also take place on the methyl group (Path C). The energy profile is shown in Figure 8. In this case, the first H-abstraction occurs via TS1C. The

doublet TS1C has the lowest energy among the three states. In the doublet and quartet states, hydroxylated species (Int2C) is formed by a rebound mechanism and no barrier was found. Interestingly, hydroxylated species was not observed in the sextet state. The second H was abstracted from N-H bond without barrier. Unlike other two spin states, azomethine imine was formed (Int1C).

Figure 7. Transition states on Path B optimized at the B3LYP/B1 level. Bond distances are shown in Å, and $r(\text{Fe-N})$ refers to a mean value of the four Fe-N distances.

Figure 8. Energy diagram for Path C (in kcal mol⁻¹), as obtained at the B3LYP/B2//B1+ZPE(B1) and B3LYP(SCRFF)/B2//B1+ZPE(B1) levels. The optimized geometry of TS1C is also shown, with bond distances shown in Å.

Figure 9. Energy diagram for Path D (in kcal mol⁻¹), as obtained at the B3LYP/B2//B1+ZPE(B1) and B3LYP(SCRF)/B2//B1+ZPE(B1) levels. The optimized geometry of TS1D is also shown, with bond distances shown in Å.

The final path (path D) proceeds through the N1-oxidation, which results in the N-oxide complex (Figure 9). As mentioned above, the unpaired electron on N1 attacks the ferryl-oxo moiety. Compared to path B (N2-oxidation), the N1-oxidation is more favorable by a few kcal/mol. The lowering of the N-oxidation barrier originates from the attachment of two electron-donating groups (methyl) on N1, which stabilize the intermediate formation. A one-electron transferred intermediate (Int1D) was found only in the sextet state. The intrinsic reaction coordinate (IRC) calculations were performed for TS1D. On the RC side, this transition state is connected to Int1A (radical intermediate) instead, unlike our expectation (Figure 10). The backward IRC calculation led to a less stable conformation of the substrate (Figure 11). Its H-abstraction seems to be feasible, thus the backward IRC calculation provided Int1A instead. The IRC

calculations of the first transition state for Path A-C were also performed. They led to RC, which has the stable UDMH conformation. The IRC results gave a complete picture of path D, which suggests that the N1-oxidation might occur after the first H-abstraction step on path A, and its occurrence is unlikely in view of the high energy barrier (> 20 kcal/mol in both gas phase and polar environment).

Figure 10. IRC calculation from TS1D toward the reactant in doublet state.

Figure 11. Two conformers of UDMH. The values in parentheses are relative energies calculated at the B3LYP/B2//B1+ZPE(B1) and B3LYP(SCRF)/B2//B1+ZPE(B1) levels.

Table 1 Proton affinity (PA)^a evaluated at the B3LYP/B2(SCRf)//B1 level

	E(B1)	E(B2,gas)	E(B2,solv)
	[kcal/mol]	[kcal/mol]	[kcal/mol]
UDMH (Conf-1 N1 protonated)	-221.3	-218.4	-263.9
UDMH (Conf-1 N2 protonated)	-215.9	-212.0	-259.6

^a PA = E(protonated UDMH) – E(neutral UDMH) + ΔZPE(B1)

In real proteins, there are some tritratable amino acid residues such as arginine and glutamic acid around the active site.[38] A comparison of the basicity between UDMH and the arginine side chain suggests that the protonated form of our substrate is unlikely to occur during the reactions. Nevertheless, it is worthwhile to consider the protonated UDMH for better understanding of its reactivity toward Cpd I. The proton affinity of UDMH was calculated to be 263.9 and 259.6 kcal/mol for N1 and N2, respectively, at the B3LYP/B2(SCRf)//B1+ZPE level (Table 1). Therefore, the N1-protonated form is more reasonable. Here path A-C were investigated, but path D was not considered because the N-oxidation cannot occur when the N1 is protonated.

Figure 12. Energy diagram for the reactions of protonated UDMH with doublet Cpd I (in kcal mol⁻¹), as obtained at the B3LYP/B2//B1+ZPE(B1) and B3LYP(SCRF)/B2//B1+ZPE(B1) levels.

The reaction energy diagrams were obtained in the doublet state (Figure 12). Regarding neutral UDMH, the most favorable path is H-abstraction from N2-H bond (path A). However, in the protonated form, the relaxed energy scan calculations using the distance between the H atom on N2 and the oxo moiety of Cpd I as the fixed parameter resulted in a continuous increase in energy, and transition state of path A was not acquired. Nevertheless, the transition states of path B and C were obtained (TS1Bp and TS1Cp). They were much less stable than their respective transition states of the neutral UDMH and led to different intermediates. Thus, neutral UDMH is preferred in the reaction of MBI.

The nitrene product of paths A and B can form a complex with ferric-heme and yield an MI complex (MIC) by replacing the water coordinating ligand. The optimized geometries of MICs are shown in Figure 13. The computational result is consistent with the previous spectroscopic study by Hines and Prough, in which the ferrous complex was found as the ground state. Although the spin state of the nitrene metabolite was not confirmed.[28] Compared to the other two states, its Fe-N distance is relatively short because of the vacant σ^*_{22} orbital in ferric-heme complex. The calculated interaction energy (ΔE) between nitrene product and ferric-heme complex suggested the stronger interaction than ferric-resting state (Table 2). The dispersion correction was made using the DFT-D3 method together with B2 basis set and the SCRF method. The calculated interaction energy of MIC was predicted as 13-14 kcal/mol stronger than that for the resting state. These results showed that the binding of MIC is stronger than that of the resting state, which is the precursor of reactive Cpd I.

Figure 13. B3LYP/B1-optimized geometry of the MIC in various spin states (doublet/quartet /sextet). Bond distances are given in Å, and bond angles are in degrees.

Table 2. Interaction energy (ΔE in kcal/mol)^[a] for the coordination of aminonitrene and water to ${}^6[\text{Fe(III)(Por)(SH)}]$ to form a complex in the doublet state. In parentheses are shown ΔE values with DFT-D3 corrections.

ϵ	1,1-dimethylaminonitrene	water
1.0	−5.8 (−16.2)	1.3 (−3.3)
5.7	−4.3 (−14.7)	4.1 (−0.5)

^[a] Defined as $\Delta E = E(\text{MIC or resting state}) - E(1,1\text{-dimethylaminonitrene or water}) - E({}^6[\text{Fe(III)(Por)(SH)}])$, and calculated at the B3LYP(gas, $\epsilon=1.0$)/B2//B1+ZPE and B3LYP(SCRF, $\epsilon=5.7$)/B2//B1+ZPE levels. A larger negative value means stronger binding.

2.4 Conclusion

The catalytic reactions between UDMH and P450 Cpd I were investigated by performing a series of DFT calculations. A comparison of energy diagrams suggests that the sequential H-abstractions from the N2-H bonds is more plausible than N-oxidation or H-abstraction from the H-C methyl group. This mechanism differs from previous studies on the similar class of compound (tertiary amine), which TMA preferred the H-abstraction from H-C bond rather than N-oxidation, while H abstraction from N-H was more favored in aniline metabolism.[39, 55] The reaction of protonated UDMH was also investigated. However, it is unlikely that the reaction of the protonated form occurs during the reactions because the energy barrier is much higher than the case of neutral UDMH. The nitrene MI binds strongly to the ferric-heme center and thereby interferes with the catalytic cycle that forms a reactive intermediate Cpd I.

2.5 References

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Chapter 3 H-Abstraction in the Final Step of Nitrosoalkane Formation in the Mechanism-Based Inactivation of Cytochrome P450 by Amine-Containing Drugs

3.1 Introduction

Human cytochrome P450 enzymes (P450s) have been known as essential biological catalysts with wide variety of substrate specification for several decades.[1-8] A few P450 isoforms such as CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 are involving in drug metabolisms, especially in phase I metabolism.[9-11] However, enzymatic functions of these P450s could be altered with multiple-drugs administration, which is known as drug-drug interactions (DDIs).[10, 12, 13] The undesirable interactions can cause a serious problem or lead to death.[14-17] The severe cases usually involve particular type of DDIs, mechanism based inactivation (MBI).[18] The inactivation process proceeds through the metabolic intermediate (MI) formation *via* a P450 catalyzed reaction. To prevent those problems, *in silico* methods have been promising as a rational and worthwhile tool to disclose the plausible reaction mechanisms. Studies of the catalytic functions of P450s using density functional theory (DFT) have become increasingly dominant in this field with acceptable cost and accuracy.[19-23]

Amine groups such as alkylamines (Figure 1) are often found in calcium channel blockers, macrolide antibiotics, monoamine oxidase inhibitors, etc.[24-27] Their MIs have been reported to form a quasi-irreversible type MBI.[28-30] A proposed metabolic pathway is shown in Figure 2. Initially, tertiary alkylamine (1) is dealkylated to a secondary alkylamine (2). The metabolism of secondary alkylamine proceeds either Path I or II to form a nitrosoalkane metabolite. In either case, it is widely believed that a *N*-hydroxyalkylamine intermediate (4 and 5).[31-33] The resultant MI can coordinate

to the heme-iron center to form a metabolic intermediate complex (MIC). The spectroscopic signature of the MIC is the Soret absorbance peak at 455 nm.[34]

Figure 1. Examples of amine-containing calcium channel blockers that act as mechanism-based inactivators.

Figure 2. Possible pathways of metabolic-intermediate complex (MIC) formation starting from a tertiary amine.[35] Some of the alkyl groups are replaced by CH₃ for simplicity.

In this study, we focus on the final step of nitrosoalkane formation (5-7) and the coordination of nitrosoalkane(7) to the heme-iron center. Our model system consists of an iron(IV)-oxo porphyrin π -cation radical intermediate (compound I, Cpd I of P450) and *N*-methylhydroxylamine as a substrate. Our substrate might undergo autooxidation without Cpd I.[33, 36] This step is not necessarily clear, and our assumption here is that Cpd I is the reactive species of the reaction. We performed density functional theory

(DFT) calculations to elucidate the plausible reaction mechanism and the coordination behavior of MI to both ferric and ferrous heme complexes.

3.2 Methodology

The B3LYP functional has been shown to give reliable results in many studies of P450s.[20, 37-39] In this study, a few transition states could not be determined with this functional. Therefore, The M06 functional[40] was employed for the geometry optimization calculations, in conjugation with the SDD effective core potential basis set for Fe and the 6-31G(d) basis set for the other atoms, henceforth called B1.[41, 42] The optimized geometries were subjected to single point calculations using the B3LYP functional and the 6-311+G(d,p) basis set (B2) for all important species. The zero-point energy (ZPE) corrections were estimated using frequency calculations at the M06/B1 level. To mimic the polar environment of the enzyme, the implicit polar solvent was taken into account with the IEFPCM self-consistent reaction field (SCRF) method. As the solvent chlorobenzene has a dielectric constant (ϵ) value of 5.6968 was employed.[43] Many of the previous studies of metabolic reactions of P450s indicated that the doublet spin state.[20, 22, 37, 39, 44-46] Therefore, the doublet ground state was considered in this study.

The interaction between the nitrosoalkane MI and the heme in MIC was also investigated. The M06 functional tended to overestimate the stability of the high spin state of pentacoordinate P450 intermediate (Table 1). Therefore, we mainly used the B3LYP(SCRF)/B2//M06/B1 data for the discussion of MICs. The dispersion effect was evaluated using the B3LYP-D3 method with Becke-Johnson (BJ) damping.[47-51] Energy decomposition analysis (EDA) was performed for MIC at the B3LYP/TZP level using the M06/B1 optimized geometries.[52-55] The mechanism of MI formation was

explored using Gaussian 09 software,[56] whereas the EDA calculations of MICs were performed using the Amsterdam Density Functional (ADF) program.[57]

Table 1. Energy data for the MIC formation at M06(SCRF)/B2//M06/B1

	E [au]	ZPE [au]
¹ [Fe(II)(Por)SH]	−2650.499887	0.282631
⁵ [Fe(II)(Por)SH]	−2650.535686	0.278476
² [Fe(III)(Por)SH]	−2650.378398	0.284859
⁶ [Fe(III)(Por)SH]	−2650.412653	0.282962
nitrosomethane	−169.743770	0.043344
¹ MIC(II), N-bound	−2820.293417	0.329955
² MIC(III), N-bound	−2820.150151	0.330849
¹ MIC(II), O-bound	−2820.266030	0.328153
² MIC(III), O-bound	−2820.136354	0.329466

3.3 Results and Discussion

The Cpd I model that we used is shown in Figure 3a. The thiolate ligand and porphine ring were used instead of the real cystainato ligand and protoporphyrin IX, respectively. There are four possible paths (A-D) for the reaction between Cpd I and *N*-methylhydroxylamine (NMH, Figure 3b): H-abstraction from the O-H bond, H-abstraction from the N-H bond, N-oxidation, and H-abstraction from the methyl group, respectively.

The energy profile and key geometries for path A are shown in Figures 4a and 5a, respectively. The reactant complex (RCa) is stabilized by the hydrogen bonding interaction between the hydroxyl group of NMH and oxo-moiety of Cpd I. The first H-abstraction has no barrier, and TS1a is lower in energy by 0.3 kcal/mol than RCa. The

first H-abstraction step leads to a radical intermediate (Int1a). The subsequent H-abstraction occurs from the adjacent N-H bond to form a nitrosoalkane product (PROa). This path A seem to be feasible with relatively low or no barrier.

Figure 3. (a) Cpd I model; (b) Hydroxylamine and three possible pathways considered (paths A–D); (c) MIC model.

The reactant complex of pathway B (RCb) is somewhat different from the previous one. RCb is slightly less stable than RCa by a few kcal/mol because of the weaker H-bonding interaction between the N-H bond and the oxo moiety. The first H-abstraction occurs from the N-H bond. This step had a small energy barrier of around 2 kcal/mol. Similar to path A, the first H-abstraction results in formation of a stable radical intermediate (Int1b). The second H-abstraction from the O-H bond has no barrier and then forms a nitrosomethane product (PROb). As in pathway A, the sequential H-abstractions from O-H and N-H bonds are accessible. These two pathways produce a nitrosomethane product.

Figure 4. Energy diagrams (in kcal/mol) for (a) path A, (b) path B, (c) path C, and (d) path D, obtained at the B3LYP(SCRF)/B2//M06/B1 level with zero-point energy corrections.

Figure 5. Optimized geometries of species on (a) path A, (b) path B, (c) path C, and (d) path D. Key bond distances are shown in Å.

In contrast to the above sequential H-abstractions from the O-H and N-H bonds, the barrier for H-abstraction from the C-H bond of the methyl group is much higher than that for path A and B (TS1D, 11.2 kcal/mol), and the reactant complex (RCd) on this path is less stable than RCa by a few kcal/mol. The following H-abstraction from the O-H bond proceeds without any barrier to form formaldonitrone (PROd). A comparison of

paths A, B, and C suggested that the H-abstraction barriers are related to the bond dissociation energy of each X-H bond (X=O, N and C), respectively, as shown in Table 2.

Table 2. Bond dissociation energy

	M06/B1	M06(SCRf)/B2	B3LYP(SCRf)/B2
	[kcal/mol]	//M06/B1	//M06/B1
		[kcal/mol]	[kcal/mol]
O–H bond	68.3	68.8	66.1
N–H bond	75.0	76.7	75.2
C–H bond	94.4	92.1	90.2

Finally, we considered the N-oxidation pathway (path C). The energy barrier is 13.3 kcal/mol, which is higher than those for the three other paths. However, this barrier is still considered relative low. The recent study by Taxak and co-workers[22] suggested that the N-oxide intermediate (int1c) can be converted to a *N,N*-dihydroxy type diol intermediate. However, the further dehydrogenation of the diol intermediate is difficult and requires a high energy barrier (around 35 kcal/mol).

The resultant nitrosomethane from path A and B can form a MIC with the heme-iron center. Two different oxidation states of the iron center (+2 and +3) were examined. We call them ¹MIC(II) and ²MIC(III), respectively, and the superscript indicates the electronic spin state of MICs. For each of these MICs, there are two binding modes of nitrosomethane: O-bound and N-bound modes. The geometries of MICs were optimized at the M06/B1 level. The optimized geometries are shown in Figure 6 along with their relative energies. It was found that the N-bound mode is more favorable than O-bound mode in ¹MIC(II) and ²MIC(III) by 11.8 and 4.2 kcal/mol, respectively, at the

B3LYP(SCRf)/B2 level. These results are in a good agreement with the X-ray crystallographic structures.[58, 59] For the N-bound geometry, the Fe-N distance of $^1\text{MIC(II)}$ is relatively shorter than that of $^2\text{MIC(III)}$. This trend indicated that the interaction between nitrosomethane MI and ferrous heme might be stronger than that in the ferric MIC.

The binding strengths of these MICs can be evaluated systematically by the interaction energy (ΔE), or the energy change for these processes:

where Por and SH refers to porphine and thiolate ligands, respectively. Above relationships are derived from the reaction between the high-spin pentacoordinate heme complex and the nitrosomethane to form an MIC.[8] These interaction energies were calculated using three functionals (i.e. M06, B3LYP, and B3LYP-D3). All methods predicted that the ferrous heme is preferred as a MIC over the ferric heme (Table 3). This is consistent with the spectroscopic observation by Lindeke and co-workers.[60]

Figure 6. M06/B1-optimized geometries of $^1\text{MIC(II)}$ and $^2\text{MIC(III)}$: (a) the N-bound form and (b) the O-bound form. Key distances are given in Å. The values below the geometries are relative energies (kcal/mol) obtained at the M06(SCRf)/B2 level

(¹MIC(II)/²MIC(III)), while the values in parentheses are relative energies obtained at the B3LYP(SCRF)/B2 level.

Table 3. Interaction energies (kcal/mol) calculated for the MIC(II) and MIC(III) ^a.

	$\Delta E(\text{M06})$	$\Delta E(\text{B3LYP})$	$\Delta E(\text{B3LYP-D3})^b$
MIC(II)	-3.7	-2.1	-13.6
MIC(III)	6.8	-0.4	-10.5

^a Obtained from B2(SCRF) single-point calculations on the M06/B1-optimized geometries, with the M06/B1 zero-point energy effect included. ΔE was calculated as $E(\text{MIC}) - E([\text{Fe}(\text{Por})(\text{SH})]) - E(\text{nitrosomethane})$.

^b With B3LYP-D3(BJ) corrections.

The bonding interactions between pentacoordinate heme complexes and nitrosomethane intermediate were further investigated using EDA. This method is useful for interpreting chemical bonds or nonbonded interactions, and the interaction energy (ΔE_{int}) between those fragments is decomposed into three terms (eq.1); (i) the quasiclassical electrostatic interaction energy between charge densities of the fragments (ΔE_{elstat}), (ii) the exchange repulsion between the fragments due to Pauli's principle (ΔE_{Pauli}), and (iii) the energy gain due to orbital mixing of the fragment (ΔE_{oi}). Hence, the sum of the interaction energy term is different from the interaction energy evaluated by the previous approach, because the geometries and spin states of fragments differ in the two approaches.

$$\Delta E_{\text{int}} = \Delta E_{\text{elstat}} + \Delta E_{\text{Pauli}} + \Delta E_{\text{oi}} \tag{eq.1}$$

The calculated decomposed energies are shown in Table 4. Even though there are several differences depending on how the interaction energy is evaluated, the total

interaction energy of $^1\text{MIC(II)}$ was again found to be slightly larger than that of $^2\text{MIC(III)}$. The major contribution in $^1\text{MIC(II)}$ came from the electrostatic and orbital interactions. As mention above, the Fe-N distance in $^1\text{MIC(II)}$ is relatively shorter than in $^2\text{MIC(III)}$. To make a fair comparison, the Fe-N distance in $^1\text{MIC(II)}$ was elongated to 2.01279 Å, which is equal to that in $^2\text{MIC(III)}$. This geometry is called ' $^1\text{MIC(II)}$ '. The elongation did not affect to the total interaction energy significantly. These results implied that the formal charge of Fe center might be an important factor for their interaction. Closer inspection of individual decomposed energy terms showed that the magnitude of electrostatic stabilization in ' $^1\text{MIC(II)}$ ' was ~ 8 kcal/mol smaller than $^2\text{MIC(III)}$. The orbital interaction energies are almost the same, whereas the Pauli repulsion energy for ' $^1\text{MIC(II)}$ ' was smaller by 13 kcal/mol. Our analyses suggested that $^1\text{MIC(II)}$ is preferred because of its smaller Pauli's repulsion. As we saw in the previous section, the Fe-N distance was allowed to be shorter in $^1\text{MIC(II)}$. When both fragments come closer, the Pauli repulsion is increased by 58.0 kcal/mol, however, the other two terms are increased in magnitude as well. Thus, the overall interaction is slightly changed. However, the sum of electrostatic and Pauli repulsion terms are smaller than the orbital stabilization energy. It is clear that the latter effect is important in stabilizing the complexes.

Table 4. Summary of B3LYP/TZP-EDA-derived energy terms for several MICs (in kcal/mol).

Energy term	¹ MIC(II)	¹ MIC(II) ^a	² MIC(III)
Electrostatic (ΔE_{elstat})	-104.1	-67.9	-75.8
Repulsion (ΔE_{Pauli})	148.0	90.0	103.1
Orbital interaction (ΔE_{oi})	-67.4	-45.1	-45.8
Total interaction (ΔE_{int})	-23.6	-22.9	-18.4

^a The Fe–N distance of the geometry of ¹MIC(II) was elongated to 2.01279 Å (which is the same as the distance in ²MIC(III)), while keeping all the other internal coordinates unchanged.

3.4 Conclusions

According to the calculated reaction energy profiles, the pathways involving two sequential H-abstractions from either O–H or N–H bonds are more favorable than the N-oxidation and C–H activation pathways. The O–H bond activation is more facile than N–H bond activation by a few kcal/mol, but the difference in the stability of these pathways is small. Hence, the initial step of N-methylhydroxylamine metabolism could occur *via* H-abstraction from either the O–H or N–H bond, depending on the initial configuration of the substrate in the active site. The nitroso metabolite preferred to bind with Heme-iron center with N atom rather than the O atom. The further analysis on the MIC predicted that the binding energy of MIC(II) is greater than that of MIC(III). The EDA revealed the stronger interaction comes from a smaller Pauli repulsion in ¹MIC(II)['], meanwhile, electrostatic and orbital interactions are almost similar for both ¹MIC(II)['] and ²MIC(III).

3.5 References

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Chapter 4 An Attempt to Evaluate the Effect of Proton-Coupled Electron Transfer on the H-Abstraction Step of the Reaction between 1,1-Dimethylhydrazine and Cytochrome P450 Compound I

4.1 Introduction

Over the past several decades, the mechanisms of reactions between the active species of P450, compound I (Cpd I), and many organic compounds have been elucidated by experimental and theoretical approaches. The molecular-level knowledge thus obtained is conducive to the research of toxicology and drug discovery.[1-9] The most common reaction that P450 Cpd I can effect is hydroxylation of a C-H bond, which can be even an inert C-H bond of an alkane.[10-13] The hydroxylation reaction is initiated by the H-abstraction (C-H bond activation) from the substrate by the iron(IV)-oxo moiety of P450s, followed by a radical rebound step. P450s can also activate X-H bonds, where X can be N or O, but in these cases, rebound may not occur. The mechanism of X-H activation by P450s has inspired efforts to prepare many synthetic catalysts.[14-18] H-abstraction is an important step for many critical processes in living organisms,[19-23] and it can be classified roughly into two classes: hydrogen atom transfer (HAT) and proton-coupled electron transfer (PCET).[24-27] We simply consider HAT as involving three electrons in the three relevant atomic orbitals (AOs), whereas PCET requires three electrons and five AOs. Previous computational studies revealed that the latter class is important in many chemical and biochemical reactions, because PCET can lower the energy barrier of reactions. It has also been found in P450 reactions, e.g. Cpd I formation from Cpd 0,[4] the C-C bond formation in P450 StaP.[28] Recently, we have studied the reaction between 1,1-dimethylhydrazine (unsymmetrical dimethylhydrazine, UDMH) and P450 Cpd I using DFT calculations (see Chapter

2).[29] We found that the nitrene formation from UDMH required a low or no barrier. Our additional calculations also showed that homolytic bond dissociation energy (BDE) was 73.8 kcal/mol for UDMH. Recently, H-abstraction reactions of heme and non-heme catalysts have been studied computationally by Usharani *et al.*[30] They observed a unique feature in the spin natural orbitals (SNOs) for the transition states of PCET reactions, thereby demonstrating that SNOs can be used to distinguish between HAT and PCET reactions. When the PCET character is dominant, the SNO is a π - or π -lone-pair orbital rather than a orbital localized onto the X-H breaking bond ($X = C, N, O, \dots$). Therefore, this characteristic is in line with the PCET definition that tells that the electron is transferred *via* an alternative route. On the other hand, in the case of HAT, the SNO is localized on the X-H breaking bond, which indicates that the electron is transferred directly from the X-H σ bond.

In this study, we attempted to use this concept to understand the low-energy barrier observed in Chapter 2. Thus, we investigated the H-abstraction step of UDMH by Cpd I by using the SNO technique. Furthermore, the H-abstraction reaction of UDMH was compared to the H-abstraction from cyclohexane (CHA), cyclohexene (CHE), and 1,4-cyclohexadiene (CHD) (Figure 1). Qualitative valence bond (VB) modeling was also performed to rationalize the effect of PCET on the potential energy surface.[31-34]

4.2 Methodology

We investigated the H-abstraction reaction shown in Figure 1. In the case of UDMH, we adopted the optimized structure and some data from Chapter 2. The CHA, CHE, and CHD compounds were chosen, in which H-abstraction is regarded essentially as HAT. DFT calculations were performed using Gaussian 09 software.[35] The B3LYP

functional was used together with the SDD (for Fe) and 6-31G(d) (for others) basis sets (B1), for geometry optimization and frequency calculations. Single-point calculations were performed at the B3LYP/6-311+G(d,p) level (B2). The polar environment of the protein was simulated using the SCRF method with the chlorobenzene solvent (dielectric constant, $\epsilon = 5.6968$).[36-41] The SNO analysis was carried out using the 'pop=SpinNatural' keyword of Gaussian 09. The SNO which has an occupation number close to -1 was visualized using MOLEKEL software.[42] The homolytic bond dissociation energy (BDE) of substrates was calculated using the G4 method at 298.15 K and 1 atm.[43]

Figure 1. (a) The reaction and (b) substrates considered in this study.

4.3 Results and Discussion

Energy barriers for the H-abstraction reactions of different substrates are compared in Table 1. The G4 calculated BDE for UDMH is 80.2 kcal/mol, which is somewhat larger than the previous DFT calculated value in Chapter 2. This result indicates that DFT tends to underestimate the homolytic BDE values of this compound. In the cases of C-H substrates, the G4 calculated BDEs decreased in the order CHA >

CHE > CHD, the H-abstraction barriers also followed the same trend. The PCET character is supposedly small in these compounds, although the number of π -electrons in these substrates varies in CHA, CHE, and CHD, and it may affect PCET to some extent. The plot for UDMH did not fall in the relationship between the BDE values and the H-abstraction barriers for the C-H substrates. As we can see in Table 1, the BDE of UDMH is larger than that of CHD by 5 kcal/mol. However, the energy barrier is significantly lower by 6.1 kcal/mol. Similar BDE values were obtained for UDMH and CHE (82.9 kcal/mol), but the H-abstraction barrier of CHE is higher than that of UDMH by 10 kcal/mol. Thus, it is clear that there is some stabilization effect existing in the transition state of UDMH, which lowers the barrier.

Table 1. Calculated barrier heights ($\Delta E(\text{TS})$), reaction energies ($\Delta E(\text{Int})$), and BDEs. All values are given in kcal/mol unit.

substrate	$\Delta E(\text{TS})^a$	$\Delta E(\text{Int})^a$	BDE ^b
UDMH	-0.1 ^c	-17.7 ^c	80.2
CHA	11.9	2.1	97.6
CHE	9.9	-12.4	82.9
CHD	6.0	-20.8	75.2

^a Calculated at the B3LYP(SCRf)/B2//B3LYP/B1+ZPE level. ^b Calculated at the G4 level. ^c Taken from Chapter 2.[29]

Following the work by Usharani *et al.*, [30] the SNOs of transition states for H-abstraction were analyzed. As shown in Figure 2, clear distinctions between HAT and PCET were observed for these substrates. In the reaction of UDMH, the SNO is not distributed on the breaking N-H bond, but a large distribution is found on the nitrogen

atom instead. The shape of SNO looks like the lone-pair orbital of the nitrogen atom, the distribution of which is perpendicular to the N-H bond. A similar spin distribution pattern has been found recently in the reactions of aromatic amines.[44] On the Cpd I moiety, a small distribution is found on the oxo moiety, while a large SNO distribution is observed on the porphine and thiolate ligands (a_{2u} orbital). This distribution pattern indicates that electron transfer occurs from the lone-pair orbital of the substrate to the a_{2u} orbital, and in concert with this, a bare proton is transferred from the nitrogen atom to the oxo moiety. In the C-H bond activation processes, the SNO distribution is similar to that for other HAT reactions,[25, 26] particularly the HAT of iron-oxo species.[30] In the HAT cases, the major distribution was observed on the breaking C-H bond and the oxo moiety. This indicates that there is electron transfer from the C-H σ bond to Cpd I. The SNO analysis suggests H-abstraction is characterized as PCET in the reaction of UDMH, whereas it is essentially HAT in the reactions of the C-H substrates.

Figure 2. SNOs for (a) UDMH, (b) CHA, (c) CHE, and (d) CHD.

Attempts were then made to quantify the extent to which the barrier for the UMDH reaction is lowered by the PCET effect. Specifically, we plotted the calculated barrier heights against the BDEs (Figure 3). We have seen in Table 1 that the C-H substrates have somewhat higher barriers than UMDH. This distinction is now more clearly seen in Figure 3, and from the gap between the UMDH barrier and the barriers

for the C–H substrates, the additional transition-state stabilization in the case of UMDH is estimated to be 9 kcal/mol at the theoretical levels employed here.

Figure 3. Plot of $\Delta E(\text{TS})$ against BDE for the four substrates.

The effect of PCET on the barrier height was also approximated in the quantitative approach. The calculated energy barriers were plotted against the BDEs (Figure 3). As mentioned in Table 1, the energy barrier increases with increasing BDE in the case of C–H substrates. The relationship between the energy barrier and BDE of UDMH is significantly off the line for C–H substrates. We estimated the stabilization energy of PCET in UDMH using the vertical gap between point and trend line, which is calculated as around 9 kcal/mol at the theoretical level used here.

Figure 4. (a) Notation for VB analysis, and (b) electronic occupation of the d-block and a_{2u} of doublet Cpd I.

We attempted to understand the origin of the different reactivity of substrates in terms of a qualitative VB diagram. Because our system consists of Cpd I, which has many possible electronic structures, the VB structures were carefully constructed. Illustration of each VB structure is given in Figure 4a. The system was split into three moieties: the substrate, the iron-oxo, and the unit containing the porphine and thiolate ligands. The valence electrons of each moiety are represented by dots. The iron(IV) center has four electrons ($8-4 = 4$) in the d-block and two of them are housed in the low-lying d_{xy} orbital, which are less reactive than the unpaired electrons in the two π* orbitals (Figure 4b). The other one unpaired electron lies in the a_{2u} type orbital (formed by a mixing of porphine and thiolate orbitals), where bears the cation radical. Finally, the oxo moiety has a -2 charge, which has eight electrons around this moiety. The sum of charges here is +3, but our system is neutral. The implicit -3 charges came from the deprotonated porphine (-2) and thiolate (-1) ligands, which are less important in our VB model.

The VB modeling technique has been applied to many reaction systems to gain chemical insight. This approach was previously applied to the rebound step of P450 reactions.[32, 45, 46] As mentioned above, P450 Cpd I has many valence electrons,

therefore, the number of possible VB states is large. In this study, we chose key structures based on fragment orbitals to understand the contribution of PCET to the H-abstraction step. Thus, the model system was partitioned into three fragments as described above. VB structures I-VIII were chosen that are assumed to make significant contributions to the ground state potential energy surface of H-abstraction step. In this case, the ionic states are Structure I-VI and the other two structures corresponding to covalent states of HAT. All ionic structures are associated with electron transfer (ET) and/or proton transfer (PT) between fragments, while the HAT structures involve pure covalent interactions. In Figure 5, electrons are represented by dots and covalent interactions represented by lines.

Figure 5. Detailed explanations for the VB structures considered.

We began by considering the stability of ionic species along the reaction coordinate, which is, in this case, the O-H distance.

Structure I: It is stable on the RC side because of the strong ionic character of the N-H bond of UDMH. The reactive Cpd I has a stabilized tri-radicaloid character. The Cpd I is an electrophilic species and not very basic. Hence, the stability of this structure decreases along the reaction coordinate.

Structure II: This species can be generated from ET from the nitrogen lone-pair electron to the ligand moiety (a_{2u} orbital) of Cpd I, thus yielding a neutral ligand moiety. In this case, the N-H bond is covalent interaction. However, the structure is destabilized when the N-H bond is elongated. Compared to structure I, this state is not very stable because the single electron transfer from the substrate to a_{2u} is unlikely. Nevertheless, the single electron transfer can sometimes occur.[28, 47, 48]

Structure III: This state could be less stable than the previous ones at the initial state. Initially, the N-H bond is an ionic bond. An electron is transferred from the nitrogen lone pair to the ligand moiety to yield this structure. The energy of this structure can decrease as a result of the proton migration along the reaction coordinate. The product side of this structure is the iron(IV)-hydroxo species (Compound II), which is relatively stable. Because this process includes the ET to in initial state and is followed by proton transfer, we can assume this structure to represent PCET. DFT calculations do not yield a stable intermediate for a stepwise ET+PT mechanism. Therefore, we can assume that the ET and PT are rather concerted.

Structure IV: This structure is similar to Structure III, but an electron of the substrate is shifted to the iron-oxo moiety instead. The oxidation state of the iron center turns to +3. As mentioned above, the route of proton is different from that of an electron that involves different orbitals in the case of PCET. This applies to this structure. Similar to structure III, structure IV is stabilized as the reaction progresses because of the electron rich iron-oxo species.

Structure V: It does not involve any ET from the substrate to the Cpd I moiety; however, there is an ET from the iron-oxo moiety to the a_{2u} orbital. There is also a covalent bonding interaction between the iron-oxo and the hydrogen of UDMH. On the initial geometry, it is less stable than Structures I-IV because of the long O-H distance. However, along reaction coordinate, the O-H distance is shortened, and thus the structure is stabilized. The spin density analysis in DFT calculations clearly shows that the electronic state of radical intermediate and Cpd II is similar to this VB state, with the numbers of unpaired electrons on the iron-oxo and substrate units being two and one, respectively. Even though this state is similar to Structure III at the intermediate geometry, the O-H interaction in this state is covalent interaction rather than ionic. Hence, this VB state is relatively stable compared with Structure III.

Structure VI: This ionic state is formed by ET from UDMH to the a_{2u} moiety. In addition, there is a covalent interaction between the oxo and the UDMH hydrogen. This state is highly unstable and will be destabilized along the reaction coordinate as a result of the formation of a cationic substrate.

The total contribution of ionic structures I-VI is represented by the red curve in Figure 6a. The ground-state ionic potential energy surface is contributed mainly from structures I, II and V. In particular, structure III is important which corresponds to the PCET process. This structure is stable in the reaction of the N-H substrate, but it is not for C-H substrates because there is no high-lying lone-pair orbital and the ionic structure is not very stable in C-H substrates.

Figure 6. VB correlation diagrams for the H-abstraction from UDMH. (a) The ionic curve and its components, and (b) the total energy curve created from the resonance of the HAT and ionic states.

The last two structures (VII and VIII) represent pure covalent structures. The difference between these structures is the covalent interaction between UDMH hydrogen and nitrogen atom or oxo moiety. As we seen in the Figure 6b, the structure VIII is less stable than structure VII in the RC geometry, whereas the relative stability is inverted as the reaction progresses to the intermediate geometry. The crossing point in the diagram indicates the transition state of this reaction. As a result of a mixing of the covalent curves in Figure 6b and the ionic curve, the transition state for the N-H substrate is stabilized significantly. Nevertheless, the ionic curve of the C-H substrate does not stabilize the transition state of this reaction as effectively as the reaction of the N-H substrate. Therefore, our qualitative VB modeling suggests that it is mainly due to

the PCET that the transition state of H-abstraction reaction is stabilized in the reaction of UDMH.

4.4 Conclusion

According to our interpretation, the H-abstraction step of the reaction between UDMH and P450 Cpd I is stabilized by the PCET effect. The DFT and G4 calculations revealed this stabilization amounts to about 9 kcal/mol. The SNO analysis also suggested clear distinctions between the HAT and PCET processes in the reaction of P450. The qualitative VB modeling substantiated the notion that the PCET stabilizes the transition state for the reaction of the N-H substrate compared to the cases of C-H substrates.

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Chapter 5 Benzyne Formation in the Mechanism-Based Inactivation of Cytochrome P450 by 1-Aminobenzotriazole and *N*-Benzyl-1-Aminobenzotriazole: Computational Insights

5.1 Introduction

As we have seen in the previous chapters, P450s are responsible for the oxidative biotransformation of both endogeneous and exogeneous compounds.[1-21] Different isoforms of P450 share significant structural similarity.[6, 22, 23] It has been established that the Cpd I species containing the heme and cysteine ligands is the reactive intermediate of P450s, which is generated in the catalytic cycle. However, many chemicals can inhibit the metabolic function of P450s in a variety of ways.[5, 21, 24-29] Among them, the mechanism-based inactivation (MBI) of P450s can cause adverse effects or even lead to death. Despite such remarkable downsides, MBI also has several benefits.[18, 30] For example, MBI is employed to investigate the reaction progress of drugs and drug-like compounds during in early stage of drug discovery by both *in vivo* and *in vitro* experiments.[31-33] As such, the molecular level understanding of MBI mechanism is important not only for predict and minimize the issues during the pharmaceutical treatment, but also for making drug design precise.[34-37] However, the detailed mechanisms of many MBI processes are still far from complete. We believe that quantum mechanical approaches play crucial roles in elucidating the mechanisms of MBI.[38]

As mentioned above, MBI has been used to understand the reaction mechanism, and this technique known as reaction phenotyping.[31] For these applications, 1-aminobenzotriazole (ABT) has been known as a useful, non-specific P450 inhibitor.[33] Ortiz de Montello and co-workers proposed that the inhibition occurs as a result of the formation of benzyne from ABT and its covalent modification of the heme moiety.[39-

41] Later, Mathews and Bend reported that *N*-benzyl-1-aminobenzotriazole (BBT), an ABT analogue provides the same consequence.[42] In addition, different reactive intermediates were observed in the guinea pig hepatic and pulmonary microsomes instead.[43-46] Therefore, in the case of BBT, benzyne does not appear to be the only product. However, it seems to us that the process of benzyne formation seems to be particularly important in the context of MBI. Therefore, we decided to study the benzyne formation mechanism from both ABT and BBT compounds.

Three possible reaction mechanisms were examined (Figure 2). Path A involves two sequential H-abstractions from exocyclic N-H bonds. After the first H-abstraction, the rebound of radical intermediate could occur to form a *N*-hydroxylated intermediate (Path B). Both paths result in the nitrene formation. Then, the resultant nitrene species is decomposed into two nitrogen molecules and a benzyne molecule. In addition to these pathways, the lone pair of nitrogen atom may be able to attack the oxo moiety of Cpd I.[47] Therefore, this *N*-oxidation mechanism (Path C) was also examined. In this case, after the *N*-oxidation step, the *N*-hydroxylation takes place. Subsequently, the pathway is merged to Path B. The reaction of an ABT analogue, BBT, was also explored, and it turns out that this reaction involves somewhat different pathways and products.

Figure 1. ABT and BBT.

Figure 2. Possible mechanisms of benzyne formation from ABT. L stands for the porphyrin ligand of a P450.

5.2 Methodology

All DFT calculations were performed using Gaussian 09 software.[48] The unrestricted B3LYP (UB3LYP) method and the SDD (for Fe) and 6-31G(d) (for the remaining atoms) basis sets (B3LYP/B1) were utilized for geometry optimization and frequency calculations. The optimized geometries were subjected to energy refinement at the UB3LYP/6-311+G(d,p) level (B3LYP/B2). In the latter calculations, the polarizability of protein environment was imitated by self-consistent reaction field (SCRF) with the chlorobenzene solvent ($\epsilon = 5.6968$). Zero-point energies (ZPEs) were obtained from frequency calculations at the UB3LYP/B1 level.[49-53] Time-dependent DFT (TDDFT) calculations were performed at the B3LYP(SCRF)/B2 level for selected species to investigate the feasibility of electron transfer. The homolytic N-H bond

dissociation energy (BDE) of exocyclic N-H of ABT was evaluated by G4 enthalpy calculations at 298.15 K and 1 atm.[54]

5.3 Results and Discussion

First, the reaction of ABT was investigated. The first H-abstraction from the exocyclic amine is shared by Paths A and B. The G4 calculated N-H BDE for ABT was 83.6 kcal/mol, which is somewhat higher than the homolytic BDE of N-H bond of UDMH in Chapter 4 (80.2 kcal/mol).[55] From these values, we can predict that the ABT is slightly less reactive than UDMH. Indeed, the energy barrier of the first H-abstraction (TS1a) is calculated as 6.4 kcal/mol (Figure 4), which is higher than that of UDMH by 6.5 kcal/mol. In general, H-abstraction from an X-H bond by Cpd I provides a substrate radical and the ferryl-hydroxo species (Cpd II). The electronic reorganization during this step is shown in Figure 3b. One electron is transferred from the substrate to the a_{2u} orbital of Cpd I; the latter is formed by hybridization of the porphine and thiolate orbitals (Figure 3a). This H-abstraction yields a doubly occupied a_{2u} orbital. For the second H-abstraction to occur, electronic reorganization of Int1a2 was necessary by an electron that is shifted from the a_{2u} to the π^* orbital to form Int1a2'. The electronic reorganization requires only 3.7 kcal/mol and thus should not be difficult. The second H-abstraction has only a small energy barrier (TS2a). The resultant nitrene state (Int2a) had an odd geometry, in which the N1-N2 bond is almost cleaved ($r = 1.92 \text{ \AA}$). This is not consistent with the conventional picture (Figure 2). Finally, the decomposition of Int2a results in formation of the product complex (Pro) containing benzyne, two nitrogen molecules, and the ferric resting state of heme moiety. The final step has a barrier of only 6.4 kcal/mol.

Figure 3. a) Schematic electron occupation diagram for Cpd I in the doublet state b) electronic reorganization.

Figure 4. Energy profiles (kcal/mol) for the reaction of ABT (paths A and B), as determined at the B3LYP(SCRf)/B2//B3LYP/B1+ZPE level.

Figure 5. Optimized TS geometries for paths A and B of the reaction of ABT. Key bond distances are shown in Å.

The rebound mechanism to form the *N*-hydroxylated intermediate (Path B) was investigated after the first H-abstraction. The new N-O bond formation passes through TS1b with a barrier of 9.1 kcal/mol. This barrier is slightly higher than that of the second H-abstraction in Path A by several kcal/mol. Hence, Path B is less favorable than Path A. Further investigations showed that the nitrene intermediate from Int1b proceeds *via* the proton transfer with the assistance of the pyrrole nitrogen atom of the porphine ring (TS2b). This step required high activation energy (27.4 kcal/mol), which is too high for this process to occur. So, the benzyne formation from ABT is likely to occur *via* Path A rather than Path B.

As shown in Figure 4, the second H-abstraction requires a smaller amount of energy than radical rebound. It was shown in the previous Chapter that H-abstraction from H-X bonds (X = N or O) can be classified as PCET. The additional SNOs analyses

were performed for the transition state of H-abstraction steps. Interestingly, the SNOs for both TS1a and TS2a showed the PCET character, where the SNO was not localized on the breaking N-H bonds. As a result of PCET, the transition state is stabilized and more stable than the radical rebound transition state.

Figure 6. Spin natural orbitals (B3LYP/B1) for (a) TS1a and (b) TS2a.

Another interesting point is the geometry of the resultant nitrene compound, in which the N1-N2 distance is longer than expected. The spin density analysis provided a close-shell singlet character on the nitrene. Therefore, the isolated nitrene compound was further investigated. The nitrene compound might have singlet or triplet ground states. Both spin states were examined and their geometries are shown in Figure 7. The calculations showed a larger N1-N2 distance in the triplet geometry ($\Delta r_{\text{N1-N2}} = 0.8 \text{ \AA}$) because the spin density is localized on the N atoms. The singlet is more stable by 1.1 kcal/mol. As a matter of fact, Int2a is decomposed into singlet state products. Hence, the nitrene species is likely to have the singlet state in Int2a.

Figure 7. B3LYP/B1-optimized geometries of the nitrene species in Int2a in the (a) singlet and (b) triplet states. The N1–N2 distances (in Å) are shown in red, and the relative energies evaluated at the B3LYP(SCRf)/B2//B3LYP/B1+ZPE level is shown in italic font. For the triplet species, the B1 spin population values are shown in parentheses.

The N-oxidation pathway (Path C) was also determined. The energy profile is shown in Figure 8. The N-oxidation is a two-electron transfer process, which differs from the H-abstraction. Two electrons are shifted from the exocyclic nitrogen atom of ABT to Cpd I. The diagram shows that the energy barrier is the highest of all barriers in the three paths (22.3 kcal/mol). If Int1c happens to be formed, a N-hydroxylated product (Int3c) can be formed with relatively low barriers. Int3c is identical to Int1b. Hence, this path later will be merged into the Path B and again encounters the very high barrier of 28.4 kcal/mol to form a benzyne product.

Figure 8. Energy profile (kcal/mol) for the reaction of ABT (path C), as determined at the B3LYP(SCRf)/B2/B3LYP/B1+ZPE level.

The reaction mechanism of BBT was then explored. In the calculations on ABT, the *N*-oxidation pathway (Path C) had a high barrier than the other two. Therefore, this *N*-oxidation was not considered for BBT. The first H-abstraction from the N-H bond has an energy barrier of 6.4 kcal/mol (TS1a-II), which is the same as the barrier for the ABT reaction. Besides H-abstraction from this exocyclic N-H bond, there is another possibility of H-abstraction from the benzylic C-H bond (Path D). Therefore, this possibility was also assessed. However, this reaction was less probable because it has a significantly higher barrier than that for the N-H bond. Therefore, the first step of benzyne formation from BBT should occur from H-abstraction from the N-H bond

rather than the C-H bond. Furthermore, the TS1a-II geometry look similar to that of TS1a (Figure 10).

Because there is only one N-H bond in BBT, the second step could undergo either H-abstraction from the C-H bond or radical rebound from Int1a-II. Similar to Int1a, an electronic reorganization is needed to yield Int1a-II', which can undergo the second H-abstraction. The second H-abstraction from the benzylic C-H bond (TS2a-II) was found to be facile with a barrier of 5.5 kcal/mol. On the other hand, the radical rebound can proceed from Int1a-II *via* TS1b-II without electronic reorganization. However, it needs a higher energy barrier ($\Delta E = 5.3$ kcal/mol), so that the rebound is less favorable. The two-sequential H-abstraction steps yield Int2a-II (containing a hydrazone compound). Int2a-II can also be formed from H-abstraction from the C-H bond (Figure 9). Int2a-II did not seem to decompose easily to form benzyne. Hence, we propose that another catalytic cycle is necessary to form benzyne.

Figure 9. Energy profiles (kcal/mol) for the first step of the reaction of BBT, as determined at the B3LYP(SCRf)/B2/B3LYP/B1+ZPE level.

Figure 10. Optimized TS geometries for the first step of the reaction of BBT. Key bond distances are shown in Å.

The second catalytic cycle began with the C-oxidation to form a new C-O bond through TS3a. The energy profile is shown in Figure 11. The C-O bond formation has an energy barrier of 16.3 kcal/mol and leads to Int3a-II. The nitrene compound formation could proceed via C-N bond cleavage. It was found that this bond cleavage is feasible with a small energy barrier. Together with the nitrene compound, the benzaldehyde is also formed. The nitrene species later undergoes the same non-catalytic decomposition as the Int2a of ABT (Figure 4). Thus, after two catalytic cycle, benzyne can be formed from BBT.

We also found another pathway in which an oxaziridine ring closure is possible from Int3a-II to Int4a'-II via the TS4a'-II. The different energy between TS4a-II and TS4a'-II is small (< 1 kcal/mol), and this species is less stable than the nitrene intermediate. The oxaziridine might also be involved in the apoprotein modification or other processes. It was suspected that a similar species is formed in the 3-substituted indazole metabolism by CYP3A1 and CYP3A2 (Figure 13) in a covalent modification of DNA.[56]

Figure 11. Energy profiles (kcal/mol) for the second step of the reaction of BBT, as obtained at the B3LYP(SCRF)/B2/B3LYP/B1+ZPE level.

Figure 12. Optimized TS geometries for the second step of the reaction of BBT. Key bond distances are shown in Å.

Figure13. Previously proposed oxaziridine formation during the P450-mediated reaction of an indazole-type compound.[56]

We also performed TDDFT calculations at the B3LYP(SCRF)/B2 level for the RC and RC-II states of ABT and BBT, respectively. The key molecular orbitals that are relevant to electron transfer or excitation from the substrate to Cpd I for ABT are shown in Figure 14. The excitation energy was calculated as 28.6 kcal/mol for RC, and 28.5 kcal/mol for RC-II, which are larger than the barriers for the H-abstraction (6.4 kcal/mol). Therefore, the H-abstraction seems to constitute the most plausible pathway.

Figure 14. Orbitals relevant to electron transfer: the π -type molecular orbitals (HOMO-2 and HOMO-3) and the a_{2u} -type orbital (LUMO) for the RC state of ABT reaction.

5.4 Conclusion

A series of DFT calculations provided valuable insights into the mechanisms of benzyne formation from ABT and BBT in the reactions with P450 Cpd I. In particular,

we showed that the pathway involving two sequential H-abstraction steps is much more favorable in the reaction of ABT substrate than *N*-hydroxylation and *N*-oxidation, which have high energy barriers. Our additional analysis found that the H-abstraction transition states are stabilized by the PCET, similar to the reaction of UDMH. The nitrene intermediate decomposes easily into benzyne and two nitrogen molecules. In the reaction of BBT, again H-abstraction from the N-H bond triggers the MBI process. However, it is followed by H-abstraction from the nearby benzylic C-H bond instead, because of the lack of a second hydrogen atom on the exocyclic amine. In contrast to ABT, the two sequential H-abstraction steps result in formation of a hydrazone molecule, and thus benzyne formation from the hydrazone should require another catalytic turnover. Another electron transfer mechanism was also examined, but the results confirmed that the H-abstraction is the most plausible pathway in these reactions.

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Chapter 6 Density Functional Theory and QM/MM Studies of the Mechanism-Based Inactivation of Cytochrome P450 by Bergamottin

6.1 Introduction

The metabolic profiles of drugs are a major concern in drug discovery processes. Human P450s are responsible for the metabolism of approximately 75% of clinical drugs, and many of them are found in the small intestine and liver cells.[1-6] In general, P450-mediated metabolism increases the solubility of drugs and other xenobiotic molecules, thereby aiding their excretion in the water soluble urine or other forms.[7-9] It is also known that P450-mediated metabolism sometimes causes adverse effects that result in increased or decreased activity of a P450. Human P450s generally have broad substrate specificity; many different drugs can be metabolized by a common isoform. Therefore, if the activity of a P450 is altered, its negative influence on metabolism could be large, giving rise to the issue of drug-drug interaction. Drugs and reactive substrates typically contain several functional groups that undergo reactions with reactive Cpd I. Some P450 isoforms, e.g. CYP3A4, have a large active site that allows multiple orientations of a substrate, thus providing various possible oxidized products.[10-12] For example, aflatoxin B1 could be hydroxylated at the 3 α or epoxidized at the 8,9 double bond positions. Reactions at these particular sites yield different forms of products. The hydroxylated product is nontoxic, whereas the epoxidized product is highly toxic.[13, 14](Figure1)

Figure 1. Metabolic sites of aflatoxin B1.

In plants, there are numerous chemical substrates that have a therapeutic effect. However, some of them could also obstruct the catalytic activity of P450s through MBI. A well-known mechanism-based inhibitor is grapefruit, which is a fruit in *Citrus* genus and is found in subtropical areas. There are more than 30 clinical drugs,[15-20] which are inhibited by grapefruit juice. The inhibition effect of grapefruit juice was discovered by Bailey et al., who did *in vivo* experiments to study the interaction between ethanol and felodipine, a medicine for the treatment of chest pain and hypertension.[19] Their experiment showed that the concentration of the drug in a patient's blood was three times as high as that in the normal condition. Moreover, the interaction between ethanol and felodipine was not detected.[21] The *in vitro* experiment demonstrated that the active component of grapefruit juice is flavonoid or furanocoumarin (Figure 2), which does not exist as much in other *Citrus* fruits.[22-24] Recently, the furanocoumarin compounds in grapefruit juice have been identified as a CYP3A4 inhibitor.[18, 22, 25, 26] Among them, Goosen et al. found that bergamottin is the most effective inhibitor,[21] which was shown to inhibit many CYP450s. In humans, CYP3A4 is the main target of this grapefruit juice effect. The metabolites of bergamottin covalently modify the apoprotein of CYP3A4. Lin and co-workers[27] used the liquid chromatography-tandem mass spectroscopy (LC-MS/MS) analysis to detect the increment of mass of an enzyme, which is digested by trypsin. They discovered that the increase in mass corresponded to the mass of 6'-7'-dihydroxylbergamottin plus one oxygen atom (388 Da). These compounds are the oxidative products of a bergamottin compound. The experiment also revealed that the furan moiety was oxidized to form either furanoepoxide or γ -ketoenal (Figure 3).[28-30] In 2012, the target residue was identified by Lin et al. In their study, Glutamine 273 (Q273) formed a covalent bond

with the reactive MI and inactivated the enzymatic activity. They also found that the mass of a target residue increases by the mass of the proposed metabolites plus one oxygen atom. Moreover, there is no evidence of hemodestruction and binding to other residues. The reactivity of a potent mechanism-based inhibitor, furanoepoxide, was studied computationally by Taxak et al. They proposed that there are three possible inhibition pathways: (i) transformation of furanoepoxide to *cis*-2-butene-1,4-dial (BDA), (ii) hydrolysis by water molecule(s) in the active-site cavity, and (iii) the epoxide ring opening by a nucleophilic residue.[31] Of these, the last pathway is the most favored. In addition, BDA was found to form a pyrrolin-2-one with the hepatic protein and can cause cancer.[29, 31, 32]

Figure 2. Three major components of grapefruit juice.

Previous computational studies of the reactions between organic substrates and Cpd I in MBI were conducted mostly by using truncated QM models.[31, 33-41] Although the QM calculations provide valuable insights into reaction mechanisms, they neglect the effect of the surrounding protein environment. In QM models, this effect was modeled typically by using implicit solvent models, but explicit effects such as hydrogen bonding were largely neglected.[42-46] The hybrid QM/MM method has emerged as a promising method for dealing with large and complex molecular systems such as enzymes. In the QM/MM approximation, a QM method is employed to describe

the region where a chemical reaction takes place. A less important part is dealt with by an MM method.[47-53] The QM/MM method has become an important tool for the studies of P450s.[14, 54-63] In the above-mentioned example, aflatoxin B1 can be oxidized at two sites. QM calculations using a QM model allow the substrate to move freely above the reactive Cpd I, because there is no steric restriction. Thus, the selectivity and role of surrounding residues cannot be investigated. On the other hand, the QM/MM method has the advantage that the steric, electrostatic, and polarization effects can be taken into account, and thus the model becomes more realistic. Understanding substrate-enzyme reactions is the first important step in the development of enzyme inhibitors. QM/MM helps us do so in a reliable manner. Moreover, QM/MM will also provide valuable insights into MBI processes.

Figure 3. Proposed bergamottin metabolism pathways.

A previous study by Lin et al. showed that the inactivation of P450s by bergamottin results in modification of the enzyme and is time- and concentration-dependent.[26, 27] Furthermore, they concluded that bergamottin could be metabolized *via* various pathways to provide electrophilic MIs.[15, 26, 27, 64] The mechanism of inactivation of P450s caused by bergamottin is likely to involve epoxidation of the furan

moiety.[65, 66] However, the metabolic reactions are so complicated that the entire mechanism is not yet clear.

In this study, the mechanisms of the reactions involved in this type of MBI are studied by DFT and QM/MM calculations. We used a small model consisting of reactive Cpd I and furanocoumarin. As in the previous studies, a thiolate (-SH) ligand is used to represent the cysteinato ligand, and porphine is used in place of photoporphyrin IX.[40, 41, 67, 68] A geranyl-oxy chain of bergamottin is replaced by -H (Figure 4) to reduce the number of atoms in the substrate molecule. The reaction begins with a C-O bond formation between Cpd I and the furan ring. The furan-containing compounds are assumed to follow a mechanism whereby a metabolic intermediate binds covalently to a protein *via* a nucleophilic amino acid residue of P450s.

6.2 Methodology

6.2.1 Small model calculations using DFT

All DFT calculations on the small truncated model were performed using Gaussian 09 software.[69] Reactions between Cpd I and furanocoumarin (psoralen) in the doublet, quartet, and sextet spin states were considered (Figure 4). Two possible pathways were examined (Figure 5). Path A involves the formation of furanoepoxide, which is followed by ring-opening to form γ -ketoenal. Path B is a direct γ -ketoenal formation pathway via the radical rearrangement of the radical intermediate. Geometries of key species were fully optimized using the unrestricted B3LYP method in conjunction with the SDD (for Fe) and 6-31G(d) (for others) basis sets (B1) in the gas phase.[70-73] Frequency calculations were also performed at the same level of theory. The local minima had no imaginary frequencies and the transition states had only one imaginary frequency. The intrinsic reaction coordinate (IRC) calculations were used to

confirm that the transition states are connected to the local minima as expected. Optimized structures were subjected to single point calculations using the 6-311+G(d,p) basis set (B2) in both the gas phase and the chlorobenzene intrinsic solvent included with the IEFPCM method.[74] The chlorobenzene has a dielectric constant (ϵ) of 5.7, which was used to model the polar environment of the enzyme. Energies presented in this study include the zero-point energy (ZPE) obtained from B3LYP/(B1) frequency calculations.

P450 Cpd I model

Figure 4. Model for the computational study.

Figure 5. Proposed mechanism of furanocoumarin oxidation by P450s. Path A is initiated by epoxide formation, and Path B leads to direct γ -ketoenal formation.

6.2.2 Setup of a QM/MM model

The crystal structure of CYP3A4 was obtained from the Protein Data Bank (PDB) website.[75] The 3NXU structure that contains CYP3A4 in complex with the ritonavir ligand was selected on the basis of the relatively high resolution (2.0 Å) and the geometry of the ligand in the active site (Figure 6).[76] This X-ray crystal structure contains two monomers, and chain A was used in this study. Non-terminal missing residues were appended using Modeller software.[77] The ritonavir ligand was removed to secure space for our substrate. A bergamottin model was built and optimized with the B3LYP functional and the 6-31G(d) basis set. The optimized structure was used for docking simulation within the CYP3A4 active site using AutoDock Vina.[78] The grid box dimension was specified as 15×15×15 Å (Figure 7). The center of the box is placed around 8 Å above the Fe atom. Substrate molecule consists of a rigid fragment (furanocoumarin) and a flexible fragment (geranyl side chain).

Figure 6. Comparison of the structures of a) ritonavir and b) bergamottin.

Figure 7. Grid box for docking simulation.

6.2.3 Determination of MM parameters of bergamottin

Because MM parameters of bergamottin are not available, AMBER parameters for bergamottin were generated for the QM optimized geometry, using Antechamber software.[79] Restrained electrostatic potential (RESP) charges were calculated at the same level of theory. The optimized geometry of bergamottin is shown in Figure 8. Electrostatic potential values at grid points were generated using the “pop=MK” keyword. The MM parameters for bergamottin are shown in Appendix E.

Figure 8. a) Optimized geometry of bergamottin and b) electrostatic potential surface.

6.2.4 Selection of structures for QM/MM calculations

The docking simulation produced different orientations of the substrate in the active-site cavity. The substrates in different orientations were added to the enzyme structure in PDB format. Structures of the substrate for QM/MM calculations were selected when the site of metabolism (furan ring) was close to Cpd I. Afterwards, the starting structures from docking simulation were relaxed by a series of partial MM minimizations (Figure 9). During the QM/MM calculations, the residues that are located 15 Å away from the Fe center were fixed, while only the inner residues were allowed to move freely.

Figure 9. Flowchart of setup.

6.2.5 QM/MM calculations

The QM region consists of the oxo-iron-porphyrin (without side chains of the heme), the cysteinato ligand side chain, and the furanocoumarin moiety of bergamottin (Figure 10). MM calculations were performed with the AMBER force field. QM calculations were performed with the B3LYP functional and the SDD (for Fe) and 6-

31G(d) (for the rest atoms) basis sets. Geometry optimization was carried out with the mechanical embedding (ME) scheme of ONIOM. The energies of the optimized structures were refined by performing single point calculations with the electronic embedding (EE) scheme at the same DFT level. The doublet spin state was considered. All ONIOM QM/MM calculations were performed with Gaussian 09 software.[80-82]

Figure 10. QM region definition. QM atoms are shown in ball-and-stick style and MM atoms are shown in other styles.

6.3 Results and Discussions

6.3.1 Small model calculations

The two possible oxidation pathways share the first step of the reaction, namely C=C bond activation, which provides a radical intermediate (Int1). The optimized geometries of key species are shown in Appendix E. In the reactant complex (RC), there is a weak hydrogen bonding interaction between Cpd I and substrate, and the H atom points toward the oxo-moiety. The O-H-C moiety is almost linear (173-178°). The important geometric parameters in this stage are similar for all spin states (C-O distance

~3.2 Å, Fe-O distance ~2.0 Å, and Fe-S distance ~2.5 Å). At this point, the doublet and quartet states are almost equally stable with a small difference in energy (0.3 kcal/mol), whereas the sextet state lies 10.5 kcal/mol above the doublet and quartet states (Figures 11 and 12). There is the first transition state (TS1), for the new C-O bond formation. The C-O distance at TS1 is around 2 Å in all spin states. The Fe-O bonds are slightly elongated. Interestingly, the Fe-S distance in the sextet state increases significantly, while it decreases in the other two states. The different trend can be explained by the different electronic configurations during the reaction process. Initially, an electron is transferred from the substrate to d_{z^2} -type molecular orbital in the sextet state, which results in an increase in the Fe-S distance. By contrast, in the other two states, an electron shift occurs to the a_{2u} orbital (Figure 13). The energies of the transition states are 12.2 (doublet), 13.9 (quartet) and 18.2 (sextet) kcal/mol. In particular, the energy barrier in the sextet state is the lowest (around 7 kcal/mol relative to the RC in the sextet state), and is 5 and 4 kcal/mol higher than those of TSs of doublet and quartet states, respectively, in the gas phase. The stabilization of the sextet transition state can be explained by the enhanced exchange stabilization. Here, the electron transfer from the substrate to the d_{z^2} -type molecular orbital of the iron-oxo unit results in an increase in the number of unpaired electrons on iron.[39] In the doublet state, the Int1 species could not be obtained, but the substrate was transformed directly to the furanoepoxide intermediate on Pathway A. Int1 was available only in the quartet and sextet states. At Int1, the Fe-O bond was elongated to 1.92 (quartet) and 1.88 (sextet) Å, and the corresponding energies are -12.1 and -7.3 kcal/mol, respectively. At Int1, the Fe-S bond was also shortened (Figure S1, Appendix E). Hence, in these spin states, the epoxide ring closure reaction is considered to follow the radical rebound mechanism. There is a small rebound energy barrier in the quartet, where an electron from the substrate radical

could fill the σ^* orbital of the iron center. However, in the doublet state reaction, radical electron is transferred to the singly occupied π^* orbital (Figure 13).[39, 40, 67, 68] Whereas Int1 in the quartet state can enter both of the two pathways. As shown in Figure 13, the unpaired electron of the radical will be transferred to d-block orbitals. In the case of the sextet state, there is a large repulsive interaction with the incoming electron. Therefore, rebound of the radical is more difficult in the sextet state than in the quartet and doublet states.

Figure 11. Relative energy profiles for the C=C activation step driven by P450 Cpd I, as obtained at the B3LYP/B2+ZPE(B1) and B3LYP(SCRF)/B2+ZPE(B1) levels. The red, black and blue lines indicate the doublet (D), quartet (Q), and sextet (S) spin states, respectively. Relative energies are given in kcal/mol.

Starting from the radical intermediate (Int1), there are two proposed mechanistic pathways (Figure 12). In the case of pathway A, the substrate radical will rebound to the oxo moiety. There is a small barrier in the B2 gas phase calculations but it is barrierless in the chlorobenzene solvent (TS2_A has a lower energy than Int1) in the quartet state. The furanoepoxide intermediate (Int2_A) is more stable in the quartet state species than in the doublet state by 3 kcal/mol. According to Taxak's report in 2013,[31] a furan metabolite (furanoepoxide compound) can be transformed to *cis*-2-butene-1,4-dial. Thus, the Int2_A is proposed to be transformed to a γ -ketoenal compound. Our calculated energy barriers are around 11 and 16 kcal/mol. The additional calculations of furanoepoxide conversion were performed in the absence of the heme moiety. The calculated barrier is 11 kcal/mol in the gas phase and 16 kcal/mol in chlorobenzene solvent. Therefore, the furanoepoxide transformation can occur without the assistance of the heme. The γ -ketoenal complex (Int3_A) also favors the quartet state. A comparison between these two intermediates shows that the γ -ketoenal complex is slightly more stable.

In the case of Pathway B, radical rearrangement of Int1 takes place. To explore the potential energy surface of this reaction, the C₁-O₁ (Figure 4) distance was elongated. The energy barriers are smaller than those for TS1_A; hence, Pathway B is kinetically favored. The spin density value at the radical center changes significantly in both the quartet and sextet states during the radical rearrangement. In the quartet state, spin density at the radical center decreases from 0.58 to 0.28. The spin density was distributed on other atoms in the substrate and also on the a_{2u} orbital, particularly on the -SH part of Cpd I. In the sextet state, the spin density at the carbon changes dramatically from -0.65 to -0.03. Spin density was also distributed to other atoms of the substrate and was transferred mainly to the oxygen and the a_{2u} orbital. The spin density values changed

from 0.25 to 0.02 at the oxygen and from 0.78 to 0.42 for the -SH moiety. The reason the radical rearrangement is more feasible than the radical rebound reaction is because Cpd I is highly electron deficient, which receives an electron from the substrate radical in Int1. In comparison, the γ -ketoenal has lower energies than their respective furanoepoxide product (by 2-3 kcal/mol) in the polar environment, whereas in the gas phase, γ -ketoenal is a bit higher in energy. At TS2_B, the C-O bond distance is around 1.30 Å, which is shorter than a single bond but longer than a double bond. Hence, the C-O bond has some double bond character. Subsequently, a γ -ketoenal intermediate (Int2_B) is formed. The Fe-O distance increased significantly from Int1. Therefore, it may also be possible for the γ -ketoenal product to be isolated from the heme. The aldehyde C-O distance in MIs in both quartet and sextet states is 1.22 Å. As a result, the distances between the heme iron and reactive metabolites are large, which suggests that they could not have interacted with each other. Therefore, these reactive metabolites do not coordinate with the heme group and can be released from the enzyme pocket easily, which assists the covalent modification of a target residue.[27]

Figure 12. The second step of oxidation of furanocoumarin compound by P450 from Int1. On the left-hand side is the radical rearrangement (Pathway B) and on the right-

hand side is radical rebound (Pathway A). The red, black and blue lines indicate the doublet (D), quartet (Q), and sextet (S) states, respectively. Relative energies obtained at the B3LYP/B2+ZPE(B1) and B3LYP(SCRFF)/B2+ZPE(B1) and are given in kcal/mol.

Figure 13. Orbital diagram showing the electronic change during the reaction process.

Figure 14. Location of Gln273 in CYP3A4 (PDB code 3NXU).

6.3.2 Enzyme system

The original enzyme structure was obtained from the PDB website. The bergamottin molecule was docked to the active site using AutoDock Vina. The docking simulation yielded 20 different substrate conformations (Figure 15). Four suitable substrate conformations at which the furan moiety lied close to the reactive oxo group were selected as the starting structure for QM/MM studies (Figure 16). Because the X-ray crystal structure did not contain the hydrogen atoms, their positions were added using LEAP software of AMBER. AMBER topology and coordination files are used to generate the starting PDB file for ONIOM input file generation by GaussView. The four docking geometries can be classified into two groups (Group A and B, Figure 16), depending on the orientation of the plane of furanocoumarin.

Figure 15. Docking results. Selected results are highlighted with frames.

6.3.3 QM/MM calculations

The oxidation of furan moiety of bergamottin could yield a furanoepoxide *via* epoxidation or γ -ketoenal *via* the radical rearrangement. It is known that the metabolic intermediate of this reaction inhibits an inactivation of CYP3A4.[20, 28-30, 83] DFT calculations on a small model was performed first. It was found that the epoxidation is feasible and no radical intermediate was found on the doublet-state energy surface. The conversion of furanoepoxide to γ -ketoenal is possible. The higher spin state had an alternative path in which γ -ketoenal could be formed directly from the radical intermediate. Because the enzyme structure is included in the QM/MM model, sequential MM minimizations were performed in order to relax the crystal structure and generate a decent starting geometry. Initially, the positions of hydrogen atoms and side chains were minimized without allowing the backbone movement. In the subsequent geometry optimization, those residues which were located at least 15 Å away from Cpd I Fe center were fixed. The full MM minimization was performed for the inner free constraint region. The resulting structures were used as the initial geometries for ONIOM calculations.

Figure 16. Selected docking results.

The first step of furan oxidation was investigated using the ONIOM method in order to include the effect of the protein environment. The optimized RC geometries are shown in Figure 17. The distances between the oxo moiety and its target C atom are 2.81, 2.82, 2.94, and 3.60 Å, for Geom1, 2, 3, and 4, respectively. The interaction between the bergamottin substrate and the surrounding amino residues were investigated. Residue S119 forms a H-bonding interaction with the coumarin oxygen atoms in Geom1 and 2, while its H-bonding interaction is formed with the geranyl oxygen in Geom3 and 4. In Geom1 and 2, T309 can form a H-bonding interaction with the furan's oxygen atom, but it cannot in Geom3 and 4 (Figure 18). The group of

arginine residues close to the propionate sidechain of the heme also stabilizes the electronegative part of substrate. The orientations of substrate are determined by the active-site residues i.e. F108, S119, F211, F213, F241, I301, F304, I369, A370 and L482 (Figure 19). Compared to the small DFT model, it is clear that the direction of the furanocoumarin moiety is restricted. The flexible geranyl tail also plays a crucial role in the substrate accommodation. Thus, through its interaction with the amino acid residues could restrict the orientation of the furanocoumarin moiety. Nevertheless, 6 of 20 docking conformations are suitable for the tail oxidation which could lead to 6'-7' or 2'-3' oxidative products (Figure 15).

The reaction energy profile is shown in Figure 20. The extrapolated barriers for C-O bond formation are varied from 12 – 20 kcal/mol in ME, and 14 – 22 kcal/mol in EE. The highest barrier is found in Geom3. However, the key parameters of the QM region of TS1 are similar (Figure 21). The origin of different barrier will be explained later. The radical intermediate was found in this protein environment, whereas the epoxide intermediate was directly formed in the doublet state in the small model. The energy of radical intermediate are -10.5, -10.7, -6.8, and -11.4 kcal/mol, respectively.

Geom1

$$\angle \text{Fe-O-C} = 134.9^\circ$$

Geom2

$$\angle \text{Fe-O-C} = 132.1^\circ$$

Geom3

$$\angle \text{Fe-O-C} = 134.4^\circ$$

Geom4

$$\angle \text{Fe-O-C} = 167.6^\circ$$

Figure 17. Optimized geometries of RC.

Figure 18. Optimized RC geometries along with a few surrounding charge-stabilizing residues.

Figure 19. Optimized RC geometries along with key residues.

Next, the origin of the high barrier obtained for Geom3 was investigated. In our ONIOM calculations, the total energy is calculated based on the subtractive scheme (eq. 10, Chapter 1). Therefore, the relative energy between different stationary point geometries can be rewritten as:

$$\Delta E = \Delta E_{QM,model} + \Delta E_{MM,real} - \Delta E_{MM,model} \quad (\text{eq. 1})$$

Combining the MM parts into a single term yields

$$\Delta E = \Delta E_{QM} + \Delta E_{MM}; \Delta E_{MM} = \Delta E_{MM,real} - \Delta E_{MM,model} \quad (\text{eq. 2})$$

where ΔE is the total relative energy, ΔE_{QM} and ΔE_{MM} are relative QM energy and MM energy between a given state (e.g., a transition state) and the reference state (i.e., RC), respectively. The latter term is the combination of the MM energy of the real system and the QM region (eq. 2); this term is usually close to zero in an ideal situation where MM is as accurate as QM. Because the explicit protein structure is included in the calculation, the orientation of a substrate is influenced by both electrostatic and steric effects. These energy terms provide insight into the stabilizing or destabilizing effects on reaction pathways (Appendix E).

Figure 20. Extrapolated and QM energy profile for the C-O bond formation.

Firstly, the QM energy of QM region was plotted against the reaction process (Figure 20). The energy barriers of Geom1, 2 and 4 are 13.3, 13.8, 16.2 kcal/mol, respectively for ME, and 15.7, 11.2, and 13.1 kcal/mol for EE, whereas it is 17.5 and 25.4 kcal/mol for Geom3 in ME and EE, respectively. The relative energies were

calculated from TS1-RC for each model are shown in Appendix E. The examples are also shown below.

<p>Geom1 (ME)</p> $\Delta E = \Delta E_{\text{QM,model}} + (\Delta E_{\text{MM,real}} - \Delta E_{\text{MM,model}})$ $12.8 = 13.3 + (152.3 - 152.8)$		<p>Geom3 (ME)</p> $\Delta E = \Delta E_{\text{QM,model}} + (\Delta E_{\text{MM,real}} - \Delta E_{\text{MM,model}})$ $19.7 = 17.5 + (232.2 - 230.0)$
<p>Geom3 - Geom1 (ME)</p> $\Delta \Delta E = \Delta \Delta E_{\text{QM,model}} + (\Delta \Delta E_{\text{MM,real}} - \Delta \Delta E_{\text{MM,model}})$ $19.7 - 12.8 = (17.5 - 13.3) + [(2.2) - (-0.5)]$ $6.9 = \underline{4.2} + (2.7)$		

According to the above calculations, the major cause of the different energy came from the QM energy term. Thus, the QM region was inspected in detail. The group spin densities are shown in Table S6 in Appendix E. An analysis of the electron distribution shows a clear indication of α -electron (spin-up) transfer, where the negative value remained on the substrate radical. The spin distributions in RC and TS1 of all model are almost the same, whereas the Int1 showed some distinction. Previous studies show that the α -electron of the substrate is usually transferred to the a_{2u} orbital in the doublet state, as is indeed the case with Geom1, 2, and 4. However, in the case of Geom3, the α -electron was shifted to the σ^* orbital instead, which provides the unusually high barrier. The electronic configurations are shown in Figure 22. The occupation of electron in σ^* orbital also elongates the Fe-S distance (2.58 Å in RC and 2.83 Å in Int1). This situation usually occurs when the C-C double bond approaches the active species in the upright position. The unusual electron transfer also gives a less stable Int1 in Geom3. These computational results suggest that if the substrate happens

to take such a configuration at which the furan carbon comes just above the oxoiron(IV) unit, the subsequent C–O bond formation is not necessarily favorable.

Figure 21. TS1 geometries.

Figure 22. Electronic configuration from RC to Int1 for each models.

6.4 Conclusion

The CYP450-catalyzed oxidation of furanocoumarin has been investigated using DFT and QM/MM calculations. The calculations suggest that there are two major reactive intermediates that are formed after the reaction. The formation of both intermediates involve the C-O bond formation in the first step, and the resultant intermediate then possibly undergoes two types of reactions. In the first possible pathway, there is a radical rebound to form an epoxide moiety in the furanoepoxide intermediate, whereas in the second pathway, there is a radical rearrangement to form a γ -ketoenal intermediate. A previous study suggested that these two MIs can be transformed to each other with a relative low barrier. Interestingly, the calculation of doublet state simultaneously gave furanoepoxide as a reactive intermediate because the radical electron is transferred to the porphine cation radical easily. In contrast, in the other two high spin states, a new C-O bond formation and the radical intermediate were

seen. According to this study, the stability of both MIs are comparable. Our calculations clearly show that the oxidation of a furanocoumarin compound would present two competitive reactive intermediates, which are electrophilic species and have the ability to react with nucleophilic residues on a protein. QM/MM calculations also provided insights into the effect of protein environment. In the future, QM/MM calculations will be performed more extensively for full comprehension of the reaction pathway.

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Appendix A Chapter 2

Table S1. Raw energy data for the reaction between P450 Cpd I and hydrazine
(a) B3LYP/B1

	ΔE [kcal/mol]	$\Delta(E+ZPE)$ [kcal/mol]	E [Eh]	ZPE [Eh]	E+ZPE [Eh]
2RC	0.0	0.0	-1776.773735	0.397767	-1776.375968
4RC	0.2	0.3	-1776.773393	0.397893	-1776.375500
6RC	9.8	8.4	-1776.758095	0.395487	-1776.362608
2TS1A	4.3	3.5	-1776.766900	0.396478	-1776.370421
4TS1A	1.0	0.6	-1776.772175	0.397132	-1776.375043
6TS1A	10.8	8.8	-1776.756601	0.394616	-1776.361984
2Int1A	-14.2	-14.9	-1776.796413	0.396760	-1776.399653
4Int1A	-14.2	-14.9	-1776.796440	0.396703	-1776.399736
6Int1A	-10.8	-14.0	-1776.790911	0.392632	-1776.398279
2TS2A	-10.6	-12.0	-1776.790631	0.395572	-1776.395059
4TS2A	-10.6	-11.8	-1776.790561	0.395772	-1776.394789
6TS2A	-7.4	-11.4	-1776.785592	0.391501	-1776.394091
2TS1B	10.9	10.6	-1776.756387	0.397337	-1776.359050
4TS1B	19.4	19.6	-1776.742759	0.398082	-1776.344677
6TS1B	19.2	17.5	-1776.743170	0.395047	-1776.348123
2Int1B	-24.4	-21.0	-1776.812665	0.403236	-1776.409430
4Int1B	-15.0	-13.0	-1776.797561	0.400843	-1776.396718
6Int1B	-19.1	-17.9	-1776.804145	0.399626	-1776.404519
2TS2B	-4.2	-2.9	-1776.780381	0.399830	-1776.380551
4TS2B	-2.1	-1.5	-1776.777017	0.398633	-1776.378383
6TS2B	-1.4	-1.9	-1776.775985	0.397047	-1776.378938
2Int2B	-30.8	-29.1	-1776.822754	0.400390	-1776.422364
4Int2B	-26.4	-25.7	-1776.815842	0.398871	-1776.416972
6Int2B	-30.4	-30.3	-1776.822111	0.397857	-1776.424254
2TS3B	-22.4	-22.5	-1776.809399	0.397572	-1776.411827
4TS3B	-11.1	-13.3	-1776.791421	0.394212	-1776.397209
6TS3B	-15.5	-18.3	-1776.798368	0.393192	-1776.405176
2TSC	10.4	8.3	-1776.757145	0.394340	-1776.362805
4TSC	12.3	9.3	-1776.754192	0.393043	-1776.361149
6TSC	15.5	10.8	-1776.748956	0.390163	-1776.358794
2IntC	-38.4	-38.1	-1776.834943	0.398241	-1776.436702
4IntC	-32.2	-33.3	-1776.825119	0.396041	-1776.429079
6IntC	-35.5	-37.4	-1776.830309	0.394738	-1776.435571
2Int2C	-53.8	-51.2	-1776.859480	0.401999	-1776.457481
4Int2C	-51.2	-49.5	-1776.855361	0.400559	-1776.454802
6Int2C	-55.7	-54.7	-1776.862459	0.399295	-1776.463163
2TS1D	7.2	7.4	-1776.762258	0.398137	-1776.364121
4TS1D	14.1	14.6	-1776.751228	0.398490	-1776.352738
6TS1D	16.8	15.1	-1776.746961	0.395028	-1776.351934
6Int1D	11.6	10.0	-1776.755269	0.395291	-1776.359977
6TS2D	13.1	11.3	-1776.752894	0.394962	-1776.357932
2Int2D	-24.1	-21.0	-1776.812103	0.402674	-1776.409429
4Int2D	-17.8	-16.1	-1776.802111	0.400491	-1776.401620
6Int2D	-20.9	-19.9	-1776.806992	0.399304	-1776.407688
2Pro	-38.0	-38.4	-1776.834323	0.397230	-1776.437093
4Pro	-32.1	-33.7	-1776.824821	0.395104	-1776.429717
6Pro	-35.5	-38.0	-1776.830278	0.393761	-1776.436518

(b) B3LYP/B2//B3LYP/B1

	ΔE [kcal/mol]	$\Delta(E+ZPE)$ [kcal/mol]	E [Eh]	ZPE [Eh]	E+ZPE [Eh]
2RC	0.0	0.0	-2916.943574	0.397767	-2916.545806
4RC	0.5	0.5	-2916.942854	0.397893	-2916.544961
6RC	12.1	10.7	-2916.924288	0.395487	-2916.528801
2TS1A	3.9	3.1	-2916.937298	0.396478	-2916.540820
4TS1A	0.9	0.6	-2916.942062	0.397132	-2916.544930
6TS1A	12.8	11.0	-2916.923226	0.394907	-2916.528318
2Int1A	-17.2	-17.8	-2916.970960	0.396760	-2916.574200
4Int1A	-17.7	-18.4	-2916.971838	0.396703	-2916.575135
6Int1A	-9.1	-12.3	-2916.958011	0.392632	-2916.565379
2TS2A	-14.8	-16.2	-2916.967150	0.395572	-2916.571578
4TS2A	-14.8	-16.0	-2916.967091	0.395772	-2916.571319
6TS2A	-7.0	-11.0	-2916.954780	0.391501	-2916.563278
2TS1B	9.9	9.6	-2916.927804	0.397337	-2916.530467
4TS1B	19.3	19.5	-2916.912885	0.398082	-2916.514803
6TS1B	23.4	21.7	-2916.906322	0.395047	-2916.511275
2Int1B	-22.3	-18.9	-2916.979163	0.403236	-2916.575928
4Int1B	-12.1	-10.2	-2916.962827	0.400843	-2916.561984
6Int1B	-13.5	-12.3	-2916.965088	0.399626	-2916.565462
2TS2B	-1.5	-0.2	-2916.945973	0.399830	-2916.546143
4TS2B	2.3	2.9	-2916.939884	0.398633	-2916.541251
6TS2B	4.6	4.1	-2916.936250	0.397047	-2916.539203
2Int2B	-30.0	-28.3	-2916.991359	0.400390	-2916.590970
4Int2B	-25.9	-25.2	-2916.984891	0.398871	-2916.586020
6Int2B	-27.0	-27.2	-2916.986648	0.397563	-2916.589086
2TS3B	-25.1	-25.2	-2916.983516	0.397572	-2916.585944
4TS3B	-13.0	-15.2	-2916.964254	0.394212	-2916.570042
6TS3B	-15.5	-18.3	-2916.968203	0.393192	-2916.575012
2TSC	8.6	6.5	-2916.929830	0.394340	-2916.535489
4TSC	9.6	6.6	-2916.928296	0.393043	-2916.535253
6TSC	16.9	12.1	-2916.916691	0.390163	-2916.526528
2IntC	-45.3	-45.0	-2917.015835	0.398241	-2916.617594
4IntC	-39.1	-40.2	-2917.005911	0.396041	-2916.609870
6IntC	-39.8	-41.7	-2917.006940	0.394738	-2916.612202
2Int2C	-56.9	-54.3	-2917.034294	0.401999	-2916.632295
4Int2C	-54.1	-52.3	-2917.029760	0.400559	-2916.629201
6Int2C	-56.2	-55.2	-2917.033115	0.399295	-2916.633820
2TS1D	6.2	6.4	-2916.933703	0.398137	-2916.535565
4TS1D	13.4	13.9	-2916.922216	0.398490	-2916.523726
6TS1D	18.9	17.2	-2916.913426	0.395028	-2916.518398
6Int1D	14.9	13.4	-2916.919756	0.395291	-2916.524465
6TS2D	17.0	15.2	-2916.916522	0.394962	-2916.521560
2Int2D	-22.2	-19.2	-2916.979024	0.402674	-2916.576350
4Int2D	-22.2	-20.5	-2916.979024	0.400491	-2916.578533
6Int2D	-15.9	-14.9	-2916.968835	0.399304	-2916.569532
2Pro	-42.0	-42.4	-2917.010534	0.397230	-2916.613304
4Pro	-36.2	-37.9	-2917.001331	0.395104	-2916.606227
6Pro	-36.9	-39.4	-2917.002326	0.393761	-2916.608566

(c) B3LYP(SCRf)/B2//B3LYP/B1

	ΔE [kcal/mol]	$\Delta(E+ZPE)$ [kcal/mol]	E [Eh]	ZPE [Eh]	E+ZPE [Eh]
2RC	0.0	0.0	-2916.959485	0.397767	-2916.561718
4RC	0.2	0.3	-2916.959171	0.397893	-2916.561278
6RC	10.9	9.5	-2916.942053	0.395487	-2916.546566
2TS1A	0.7	-0.1	-2916.958305	0.396478	-2916.561827
4TS1A	1.4	1.0	-2916.957266	0.397132	-2916.560134
6TS1A	13.4	11.4	-2916.938102	0.394616	-2916.543485
2Int1A	-17.1	-17.7	-2916.986757	0.396760	-2916.589997
4Int1A	-17.8	-18.4	-2916.987798	0.396703	-2916.591095
6Int1A	-9.5	-12.7	-2916.974649	0.392632	-2916.582017
2TS2A	-14.2	-15.6	-2916.982187	0.395572	-2916.586615
4TS2A	-14.3	-15.5	-2916.982262	0.395772	-2916.586490
6TS2A	-7.4	-11.4	-2916.971354	0.391501	-2916.579853
2TS1B	5.4	5.1	-2916.950909	0.397337	-2916.553573
4TS1B	16.9	17.1	-2916.932517	0.398082	-2916.534435
6TS1B	22.4	20.7	-2916.923752	0.395047	-2916.528705
2Int1B	-22.8	-19.3	-2916.995758	0.403236	-2916.592522
4Int1B	-14.6	-12.6	-2916.982700	0.400843	-2916.581857
6Int1B	-14.0	-12.8	-2916.981749	0.399626	-2916.582123
2TS2B	-1.2	0.0	-2916.961470	0.399830	-2916.561640
4TS2B	3.2	3.7	-2916.954450	0.398633	-2916.555816
6TS2B	4.3	3.8	-2916.952699	0.397047	-2916.555652
2Int2B	-27.8	-26.1	-2917.003739	0.400390	-2916.603349
4Int2B	-25.6	-24.9	-2917.000244	0.398871	-2916.601373
6Int2B	-27.2	-27.1	-2917.002790	0.397857	-2916.604934
2TS3B	-29.6	-29.7	-2917.006664	0.397572	-2916.609091
4TS3B	-18.2	-20.4	-2916.988517	0.394212	-2916.594306
6TS3B	-25.5	-28.3	-2917.000078	0.393192	-2916.606887
2TSC	8.4	6.3	-2916.946089	0.394340	-2916.551748
4TSC	10.3	7.4	-2916.943009	0.393043	-2916.549967
6TSC	17.9	13.1	-2916.930955	0.390163	-2916.540793
2IntC	-45.0	-44.7	-2917.031270	0.398241	-2916.633028
4IntC	-41.4	-42.5	-2917.025475	0.396041	-2916.629434
6IntC	-40.0	-41.9	-2917.023166	0.394738	-2916.628428
2Int2C	-55.6	-52.9	-2917.048080	0.401999	-2916.646080
4Int2C	-55.3	-53.5	-2917.047594	0.400559	-2916.647035
6Int2C	-56.1	-55.2	-2917.048939	0.399295	-2916.649643
2TS1D	2.4	2.7	-2916.955597	0.398137	-2916.557460
4TS1D	11.6	12.1	-2916.940994	0.398490	-2916.542504
6TS1D	19.2	17.5	-2916.928883	0.395028	-2916.533855
6Int1D	15.2	13.6	-2916.935292	0.395291	-2916.540001
6TS2D	17.3	15.5	-2916.931920	0.394962	-2916.536958
2Int2D	-22.4	-19.3	-2916.995190	0.402674	-2916.592516
4Int2D	-22.4	-20.7	-2916.995190	0.400491	-2916.594699
6Int2D	-16.4	-15.4	-2916.985609	0.399304	-2916.586305
2Pro	-42.4	-42.8	-2917.027132	0.397230	-2916.629902
4Pro	-38.7	-40.4	-2917.021137	0.395104	-2916.626034
6Pro	-37.3	-39.8	-2917.018952	0.393761	-2916.625192

Table S2. Relative energies calculated by different methods

(a) RC at the OPBE(SCRF)/B2//B3LYP/B1 level

	ΔE [kcal/mol]	$\Delta(E+ZPE)$ [kcal/mol]	E [Eh]	ZPE [Eh]	E+ZPE [Eh]
2RC	0.0	0.0	-2916.609709	0.397767	-2916.211942
4RC	3.2	3.2	-2916.604664	0.397893	-2916.206771
6RC	14.6	13.2	-2916.586403	0.395487	-2916.190916

(b) Barrier height for Path A at the OPBE(SCRF)/B2//B3LYP/B1 level

	ΔE [kcal/mol]	$\Delta(E+ZPE)$ [kcal/mol]	E [Eh]	ZPE [Eh]	E+ZPE [Eh]
2RC	0.0	0.0	-2916.609709	0.397767	-2916.211942
2TS1A	1.2	0.4	-2916.607856	0.396478	-2916.211378

(c) Barrier height for Path A at the B3LYP(SCRF)/Def2-TZVP//B3LYP/B1 level

	ΔE [kcal/mol]	$\Delta(E+ZPE)$ [kcal/mol]	E [Eh]	ZPE [Eh]	E+ZPE [Eh]
2RC	0.0	0.0	-2917.140484	0.397767	-2916.742717
2TS1A	0.7	-0.1	-2917.139302	0.396478	-2916.742824

Table S3. Raw energy data for the reaction between P450 Cpd I and N1-protonated hydrazine

(a) B3LYP/B1

	ΔE [kcal/mol]	$\Delta(E+ZPE)$ [kcal/mol]	E [Eh]	ZPE [Eh]	E+ZPE [Eh]
RCp	0.0	0.0	-1777.184504	0.414250	-1776.770254
TS1Bp	44.5	44.4	-1777.113622	0.414103	-1776.699519
Int1Bp	-12.1	-16.4	-1777.203808	0.407459	-1776.796349
TS1Cp	31.3	26.6	-1777.134577	0.406673	-1776.727904
Int1Cp	19.9	17.4	-1777.152758	0.410304	-1776.742453

(b) B3LYP/B2//B3LYP/B1

	ΔE [kcal/mol]	$\Delta(E+ZPE)$ [kcal/mol]	E [Eh]	ZPE [Eh]	E+ZPE [Eh]
RCp	0.0	0.0	-2917.349688	0.414250	-2916.935438
TS1Bp	42.7	42.6	-2917.281597	0.414103	-2916.867494
Int1Bp	-10.0	-14.2	-2917.365585	0.407459	-2916.958126
TS1Cp	27.5	22.8	-2917.305788	0.406673	-2916.899115
Int1Cp	14.2	11.7	-2917.327059	0.410304	-2916.916754

(c) B3LYP(SCRF)/B2//B3LYP/B1

	ΔE [kcal/mol]	$\Delta(E+ZPE)$ [kcal/mol]	E [Eh]	ZPE [Eh]	E+ZPE [Eh]
RCp	0.0	0.0	-2917.407613	0.414250	-2916.993363
TS1Bp	32.4	32.3	-2917.356024	0.414103	-2916.941921
Int1Bp	-4.5	-8.8	-2917.414823	0.407459	-2917.007364
TS1Cp	28.4	23.6	-2917.362423	0.406673	-2916.955750
Int1Cp	14.7	12.2	-2917.384213	0.410304	-2916.973908

Table S4. B3LYP/B1 Mulliken group spin populations and group charges of the stationary points for the reaction between P450 Cpd I and hydrazine

(a) Spin population

	Fe	O	Por	SH	UDMH
2RC	1.47	0.81	-0.49	-0.78	0.00
4RC	1.12	0.84	0.47	0.56	0.01
6RC	3.05	0.70	1.16	0.09	0.00
2TS1A	1.54	0.69	-0.09	-0.36	-0.78
4TS1A	1.36	0.79	0.23	0.09	0.53
6TS1A	3.46	0.38	0.62	0.65	-0.11
2Int1A	1.98	0.24	0.01	-0.28	-0.95
4Int1A	1.94	0.28	0.02	-0.24	1.01
6Int1A	4.05	0.34	0.74	0.82	-0.96
2TS2A	1.19	-0.04	-0.04	-0.67	0.56
4TS2A	2.12	0.19	0.02	-0.32	0.99
6TS2A	4.04	0.30	0.72	0.64	-0.70
2TS1B	1.43	0.58	-0.20	-0.26	-0.54
4TS1B	2.09	0.41	0.29	-0.11	0.32
6TS1B	3.93	0.12	0.44	0.80	-0.29
2Int1B	1.02	0.02	0.04	-0.08	0.00
4Int1B	2.65	0.06	0.36	-0.07	0.01
6Int1B	4.14	0.07	0.38	0.40	0.00
2TS2B	0.91	0.09	0.07	-0.07	0.01
4TS2B	2.88	0.09	-0.02	0.04	0.02
6TS2B	4.08	0.24	0.32	0.31	0.05
2Int2B	1.10	0.00	0.03	-0.13	0.00
4Int2B	2.72	0.02	0.39	-0.14	0.01
6Int2B	4.08	0.00	0.51	0.40	0.00
2TS3B	1.09	0.03	0.04	-0.16	0.00
4TS3B	2.80	0.10	0.21	-0.13	0.03
6TS3B	4.12	0.18	0.42	0.28	0.01
2TSC	1.70	0.14	-0.22	-0.08	-0.54
4TSC	1.56	0.65	-0.06	0.20	0.65
6TSC	3.98	0.16	0.50	0.85	-0.49
2IntC	1.04	-0.01	-0.08	0.04	0.00
4IntC	2.63	0.03	-0.06	0.40	0.00
6IntC	4.13	0.03	0.42	0.41	0.00
2Int2C	1.06	0.00	-0.09	0.03	0.00
4Int2C	2.58	0.00	-0.01	0.43	0.00
6Int2C	4.08	0.00	0.51	0.41	0.00
2TS1D	1.46	0.55	-0.22	-0.17	-0.63
4TS1D	2.05	0.39	-0.10	0.29	0.37
6TS1D	3.46	0.37	0.55	0.73	-0.11
6Int1D	3.95	0.18	0.47	0.86	-0.47
6TS2D	3.90	0.13	0.42	0.85	-0.30
2Int2D	1.05	0.01	-0.09	0.03	0.00
4Int2D	2.63	0.04	-0.08	0.40	0.01
6Int2D	4.13	0.05	0.39	0.42	0.01
2Pro	1.12	0.00	0.03	-0.15	0.00
4Pro	2.73	0.03	0.37	-0.13	0.00
6Pro	4.13	0.04	0.41	0.42	0.00

(b) Charge

	Fe	O	Por	SH	UDMH
2RC	0.54	-0.42	-0.06	-0.09	0.04
4RC	0.54	-0.42	-0.06	-0.10	0.04
6RC	0.62	-0.46	-0.06	-0.14	0.03
2TS1A	0.41	-0.55	-0.12	-0.45	0.71
4TS1A	0.47	-0.48	-0.06	-0.28	0.36
6TS1A	3.25	0.62	0.60	0.11	0.42
2Int1A	0.36	-0.65	0.02	-0.32	0.58
4Int1A	0.36	-0.65	0.02	-0.32	0.59
6Int1A	0.82	-0.76	-0.11	-0.50	0.56
2TS2A	0.35	-0.64	0.08	-0.25	0.46
4TS2A	0.35	-0.64	0.08	-0.25	0.45
6TS2A	0.83	-0.76	-0.17	-0.48	0.57
2TS1B	0.42	-0.42	-0.15	-0.38	0.53
4TS1B	0.42	-0.41	-0.08	-0.44	0.51
6TS1B	0.76	-0.51	-0.66	-0.14	0.54
2Int1B	0.25	-0.44	0.00	-0.56	0.74
4Int1B	0.47	-0.46	-0.18	-0.51	0.68
6Int1B	0.54	-0.45	-0.69	-0.09	0.70
2TS2B	0.25	-0.49	-0.06	-0.36	0.65
4TS2B	0.34	-0.52	-0.08	-0.43	0.69
6TS2B	0.53	-0.50	-0.51	-0.18	0.66
2Int2B	0.27	-0.56	0.05	-0.50	0.74
4Int2B	0.44	-0.53	-0.13	-0.43	0.66
6Int2B	0.46	-0.58	-0.50	-0.03	0.65
2TS3B	0.19	-0.70	-0.04	-0.56	1.10
4TS3B	0.41	-0.75	-0.15	-0.58	1.08
6TS3B	0.55	-0.76	-0.14	-0.74	1.09
2TSC	0.44	-0.52	-0.34	-0.05	0.47
4TSC	0.42	-0.54	-0.36	-0.01	0.49
6TSC	0.86	-0.66	-0.49	-0.10	0.40
2IntC	0.24	-0.77	-0.55	0.05	1.04
4IntC	0.42	-0.78	-0.46	-0.15	0.98
6IntC	0.49	-0.78	-0.65	-0.05	0.99
2Int2C	0.26	-0.62	-0.50	0.08	0.78
4Int2C	0.41	-0.63	-0.37	-0.10	0.69
6Int2C	0.47	-0.63	-0.50	-0.02	0.68
2TS1D	0.43	-0.48	-0.39	-0.14	0.58
4TS1D	0.45	-0.46	-0.46	-0.12	0.59
6TS1D	0.76	-0.45	-0.38	-0.08	0.15
6Int1D	0.79	-0.56	-0.62	-0.10	0.50
6TS2D	0.76	-0.54	-0.68	-0.11	0.57
2Int2D	0.25	-0.45	-0.54	0.02	0.72
4Int2D	0.45	-0.46	-0.50	-0.17	0.67
6Int2D	0.53	-0.46	-0.68	-0.08	0.69
2Pro	0.26	-0.78	0.05	-0.54	1.01
4Pro	0.44	-0.77	-0.15	-0.47	0.96
6Pro	0.51	-0.77	-0.05	-0.65	0.97

Table S5. B3LYP/B1Mulliken group spin populations and group charges for the stationary points for the reaction between P450 Cpd I and N1-protonated hydrazine

(a) Spin population

	Fe	O	Por	SH	Hydrazine
RCp	1.54	0.60	-0.58	-0.58	0.01
TS1Bp	1.26	-0.14	-0.10	0.08	-0.10
Int1Bp	0.98	0.46	-1.04	0.07	0.52
TS1Cp	2.14	0.02	-0.62	-0.17	-0.37
Int1Cp	2.11	0.09	-0.18	-0.03	-0.99

(b) Charge

	Fe	O	Por	SH	Hydrazine
RCp	0.52	-0.58	0.07	0.08	0.92
TS1Bp	0.29	-0.38	-0.25	0.11	1.23
Int1Bp	0.30	-0.30	0.33	0.13	0.55
TS1Cp	0.38	-0.70	0.16	0.15	1.02
Int1Cp	0.32	-0.76	-0.11	0.23	1.31

Table S6. Proton affinity calculations

(a) B3LYP/B1

	E(B1) [au]	ZPE [au]	E(B1)+ZPE [au]
UDMH (Conf-1 N1 protonated)	-190.846639	0.125272	-190.721368
UDMH (Conf-1 N2 protonated)	-190.837188	0.124421	-190.712767
UDMH (Conf-1)	-190.478615	0.109853	-190.368762
UDMH (Conf-2)	-190.473883	0.109995	-190.363888

(b) B3LYP/B2//B1 and B3LYP(SCRf)/B2//B1

	B2(gas) [au]	B2(solv) [au]	E(B2,gas)+ZPE [au]	E(B2,solv)+ZPE [au]
UDMH (Conf-1 N1 protonated)	-190.911654	-190.988586	-190.786383	-190.863315
UDMH (Conf-1 N2 protonated)	-190.900557	-190.980883	-190.776136	-190.856462
UDMH (Conf-1)	-190.548223	-190.552688	-190.438370	-190.442835
UDMH (Conf-2)	-190.543912	-190.548701	-190.433917	-190.438706

(c) Proton affinity (PA) ^a

	E(B1) [kcal/mol]	E(B2,gas) [kcal/mol]	E(B2,solv) [kcal/mol]
UDMH (Conf-1 N1 protonated)–UDMH (Conf-1)	-221.3	-218.4	-263.9
UDMH (Conf-1 N2 protonated)–UDMH (Conf-1)	-215.9	-212.0	-259.6

^a PA = E(protonated UDMH) – E(neutral UDMH) + ΔZPE(B1)

(d) Relative energy of two UDMH conformations

	E(B1) [kcal/mol]	E(B2,gas) [kcal/mol]	E(B2,solv) [kcal/mol]
UDMH (Conf-1)	0.0	0.0	0.0
UDMH (Conf-2)	3.1	2.8	2.6

Table S7. N–H bond dissociation energy of UDMH

(a) B3LYP/B1

	E(B1) [au]	ZPE [au]	E(B1)+ZPE [kcal/mol]
UDM-Heterolytic	-189.808439	0.092363	-119048.639804
UDM-Homolytic	-189.846878	0.096664	-119070.061975
UDMH	-190.478615	0.109853	-119458.206470
H	-0.500273	0.000000	-313.925935

(b) B3LYP/B2//B1 and B3LYP(SCRF)/B2//B1

	B2(gas) [au]	B2(solv) [au]	E(B2,gas)+ZPE [kcal/mol]	E(B2,solv)+ZPE [kcal/mol]
UDM-Heterolytic	-189.907440	-189.992735	-119110.764312	-119164.287358
UDM-Homolytic	-189.913723	-189.919682	-119112.007722	-119115.747239
UDMH	-190.548223	-190.552688	-119501.886528	-119504.687856
H	-0.502156	-0.502174	-315.107598	-315.118830

(c) N-H dissociation

	E(B1) [kcal/mol]	E(B2,gas) [kcal/mol]	E(B2,solv) [kcal/mol]
Heterolytic dissociation	409.6	391.1	340.4
Homolytic dissociation	74.2	74.8	73.8

Table S8. N–H bond dissociation energy of methylamine

(a) B3LYP/B1

	E(B1) [au]	ZPE [au]	E(B1)+ZPE [kcal/mol]
Methylamine	-95.851370	0.064441	-60107.208300
Methylamine radical	-95.188580	0.049087	-59700.935118
Methylamine ion	-95.164978	0.046152	-59687.966756
H	-0.500273	0.000000	-313.925935

(b) B3LYP/B2//B1 and B3LYP(SCRf)/B2//B1

	B2(gas) [au]	B2(solv) [au]	E(B2,gas)+ZPE [kcal/mol]	E(B2,solv)+ZPE [kcal/mol]
Methylamine	-95.893656	-95.897285	-60133.743104	-60136.020273
Methylamine radical	-95.225601	-95.229859	-59724.166524	-59726.838647
Methylamine ion	-95.236434	-95.327481	-59732.806074	-59789.939057
H	-0.502156	-0.502174	-315.107598	-315.118830

(c) N-H dissociation

	E(B1) [kcal/mol]	E(B2,gas) [kcal/mol]	E(B2,solv) [kcal/mol]
Heterolytic dissociation	419.2	400.9	346.1
Homolytic dissociation	92.3	94.5	94.1

Table S9. Energy data for the MI complex and the resting state

(a) B3LYP/B1

	E(B1) [au]	ZPE [au]	$\Delta E(B1)$ [kcal/mol]	$\Delta(E(B1)+ZPE)$ [kcal/mol]
1nitrene	-189.232558	0.084575	0.0	0.0
3nitrene	-189.204272	0.084193	17.7	17.5
2[Fe(III)(Por)(SH)]	-1511.151994	0.285186	6.5	7.9
4[Fe(III)(Por)(SH)]	-1511.155260	0.284281	4.5	5.3
6[Fe(III)(Por)(SH)]	-1511.162390	0.283011	0.0	0.0
2MIC	-1700.408659	0.372549	0.0	0.0
4MIC	-1700.394957	0.369838	8.6	6.9
6MIC	-1700.399584	0.368549	5.7	3.2
water	-76.407023	0.021132		
2[Fe(III)(H2O)(Por)(SH)]	-1587.579586	0.310140		

(b) B3LYP/B2//B1 and B3LYP(SCRf)/B2//B1

	B2(gas) [au]	B2(solv) [au]	$\Delta(E(B2, gas)+ZPE)$ [kcal/mol]	$(\Delta E(B2, solv)+ZPE)$ [kcal/mol]
1nitrene	-189.296780	-189.304798	0.0	0.0
3nitrene	-189.265352	-189.270308	19.5	21.4
2[Fe(III)(Por)(SH)]	-2651.228595	-2651.239546	3.7	3.5
4[Fe(III)(Por)(SH)]	-2651.231403	-2651.244604	1.9	0.3
6[Fe(III)(Por)(SH)]	-2651.234463	-2651.245074	0.0	0.0
2MIC	-2840.545391	-2840.561614	0.0	0.0
4MIC	-2840.529635	-2840.548074	9.9	8.5
6MIC	-2840.530089	-2840.545431	9.6	10.2
water	-76.458362	-76.464461		
2[Fe(III)(H2O)(Por)(SH)]	-2727.696675	-2727.709022		

(3) B3LYP-D3 (BJ-damping) dispersion energy

	Edisp (au)
1nitrene	-0.01012514
6[Fe(III)(Por)(SH)]	-0.11516325
2MIC	-0.14197972
water	-0.00057391
2[Fe(III)(H2O)(Por)(SH)]	-0.12311549

Figure S1. Optimized geometries

(d) Path-C

(e) Path-D

Figure S2. Optimized geometries for the reaction between P450 Cpd I and N1-protonated hydrazine

(a)

RCp

(b) Path-B

TS1Bp

Int1Bp

(c) Path-C

TS1Cp

Int1Cp

Figure S3. IRC calculations from $^2\text{TS1A}$

(a) Toward the reactant side

(b) Toward the product side

Figure S4. IRC calculations from $^2\text{TS1B}$

(a) Toward the reactant side

(b) Toward the product side

Figure S5. IRC calculations from $^2\text{TS1C}$

(a) Toward the reactant side

(b) Toward the product side

Figure S6. IRC calculations from $^2\text{TS1D}$

(a) Toward the reactant side

(b) Toward the product side

Appendix B Chapter 3

Table S1. Raw energy data obtained at the M06/B1 level.

	ΔE [kcal/mol]	$\Delta(E+ZPE)$ [kcal/mol]	E [au]	ZPE [au]
RCa	0.0	0.0	-1756.414741	0.357489
TS1a	0.9	-2.0	-1756.413279	0.352849
INT1a	-25.8	-26.5	-1756.455886	0.356353
TS2a	-24.4	-25.2	-1756.453580	0.356094
INT2a	-28.4	-29.2	-1756.459967	0.356123
INT2a'	-28.0	-29.4	-1756.459384	0.355321
TS3a	-20.6	-25.3	-1756.447616	0.350030
PROa	-43.8	-44.8	-1756.484531	0.355869
RCb	2.3	2.2	-1756.411027	0.357343
TS1b	5.7	2.9	-1756.405688	0.353073
INT1b	-17.3	-18.8	-1756.442248	0.355056
INT1b'	-24.6	-25.8	-1756.454008	0.355615
TS2b	-19.8	-24.2	-1756.446316	0.350478
PROb	-44.0	-45.3	-1756.484898	0.355515
TS1c	9.8	8.7	-1756.399062	0.355628
INT1c	-54.9	-53.4	-1756.502264	0.359927
RCd	0.8	0.9	-1756.413535	0.357759
TS1d	8.4	5.4	-1756.401394	0.352718
PROd	-48.5	-48.3	-1756.492052	0.357877

Table S2. Raw energy data obtained at the M06(SCRF)/B2//M06/B1 level.

	ΔE [kcal/mol]	$\Delta(E+ZPE)$ [kcal/mol]	E [au]	ZPE [au]
RCa	0.0	0.0	-2896.499043	0.357489
TS1a	0.2	-2.7	-2896.498710	0.352849
INT1a	-25.0	-25.7	-2896.538924	0.356353
TS2a	-24.2	-25.0	-2896.537532	0.356094
INT2a	-26.7	-27.5	-2896.541513	0.356123
INT2a'	-27.2	-28.6	-2896.542387	0.355321
TS3a	-25.4	-30.1	-2896.539588	0.350030
PROa	-47.1	-48.1	-2896.574149	0.355869
RCb	2.2	2.1	-2896.495519	0.357343
TS1b	7.3	4.5	-2896.487381	0.353073
INT1b	-17.0	-18.5	-2896.526127	0.355056
INT1b'	-22.1	-23.2	-2896.534198	0.355615
TS2b	-17.1	-21.5	-2896.526280	0.350478
PROb	-47.1	-48.4	-2896.574158	0.355515
TS1c	12.2	11.0	-2896.479656	0.355628
INT1c	-31.5	-29.9	-2896.549192	0.359927
RCd	-0.5	-0.3	-2896.499799	0.357759
TS1d	10.2	7.2	-2896.482823	0.352718
PROd	-57.1	-56.9	-2896.590067	0.357877

Table S3. Raw energy data obtained at the B3LYP(SCRf)/B2//M06/B1 level.

	ΔE [kcal/mol]	$\Delta(E+ZPE)$ [kcal/mol]	E [au]	ZPE [au]
RCa	0.0	0.0	-2897.499259	0.357489
TS1a	2.7	-0.3	-2897.495033	0.352849
INT1a	-18.8	-19.5	-2897.529230	0.356353
TS2a	-18.6	-19.5	-2897.528965	0.356094
INT2a	-20.7	-21.5	-2897.532185	0.356123
INT2a'	-20.9	-22.2	-2897.532493	0.355321
TS3a	-22.0	-26.7	-2897.534303	0.350030
PROa	-41.3	-42.3	-2897.565024	0.355869
RCb	2.1	2.0	-2897.495937	0.357343
TS1b	6.6	3.9	-2897.488664	0.353073
INT1b	-13.9	-15.5	-2897.521461	0.355056
INT1b'	-15.6	-16.7	-2897.524047	0.355615
TS2b	-15.6	-20.0	-2897.524142	0.350478
PROb	-40.2	-41.4	-2897.563270	0.355515
TS1c	14.5	13.3	-2897.476131	0.355628
INT1c	-25.5	-24.0	-2897.539970	0.359927
RCd	2.9	3.0	-2897.494691	0.357759
TS1d	14.2	11.2	-2897.476705	0.352718
PROd	-51.2	-51.0	-2897.580884	0.357877

Table S4. Mulliken group spin populations.

(a) M06/B1

	Fe	O	Por	SH	Substrate
RCa	1.59	0.60	−0.70	−0.50	0.01
TS1a	1.82	0.46	−0.63	−0.20	−0.45
INT1a	2.28	0.18	−0.24	−0.25	−0.98
TS2a	2.29	0.17	−0.23	−0.25	−0.98
INT2a	2.34	0.16	−0.25	−0.27	−0.98
INT2a'	2.39	0.15	−0.27	−0.31	−0.96
TS3a	1.85	0.03	−0.19	−0.20	−0.49
PROa	1.20	0.00	−0.13	−0.07	0.00
RCb	1.55	0.63	−0.65	−0.51	−0.01
TS1b	1.87	0.29	−0.41	−0.29	−0.47
INT1b	2.23	0.21	−0.23	−0.21	−1.00
INT1b'	2.44	0.14	−0.27	−0.32	−0.99
TS2b	2.32	0.06	−0.31	−0.36	−0.71
PROb	1.23	0.00	−0.14	−0.10	0.00
TS1c	2.07	−0.24	−0.25	−0.15	−0.43
INT1c	1.21	−0.01	−0.14	−0.07	0.01
RCd	1.63	0.56	−0.64	−0.49	−0.07
TS1d	2.12	−0.06	−0.27	−0.27	−0.52
PROd	1.22	0.00	−0.14	−0.09	0.00

(b) M06(SCRf)/B2//M06/B1

	Fe	O	Por	SH	Substrate
RCa	1.50	0.70	-0.80	-0.39	-0.01
TS1a	1.62	0.54	-0.55	-0.11	-0.51
INT1a	2.18	0.20	-0.24	-0.17	-0.97
TS2a	2.19	0.19	-0.23	-0.17	-0.98
INT2a	2.28	0.17	-0.29	-0.19	-0.97
INT2a'	2.38	0.14	-0.34	-0.22	-0.96
TS3a	1.14	0.01	-0.11	-0.01	-0.03
PROa	1.12	0.00	-0.12	0.00	0.00
RCb	1.48	0.73	-0.78	-0.42	-0.01
TS1b	1.64	0.44	-0.44	-0.19	-0.45
INT1b	2.15	0.20	-0.25	-0.13	-0.98
INT1b'	2.40	0.15	-0.30	-0.25	-1.01
TS2b	2.16	0.07	-0.25	-0.27	-0.71
PROb	1.12	0.00	-0.11	-0.01	0.00
TS1c	1.79	-0.01	-0.18	-0.07	-0.53
INT1c	1.09	0.00	-0.11	0.00	0.01
RCd	1.52	0.68	-0.76	-0.41	-0.02
TS1d	1.91	0.03	-0.23	-0.18	-0.54
PROd	1.12	0.00	-0.11	-0.01	0.00

(c) B3LYP(SCRf)/B2//M06/B1

	Fe	O	Por	SH	Substrate
RCa	1.50	0.77	-0.87	-0.40	-0.01
TS1a	1.62	0.59	-0.56	-0.08	-0.57
INT1a	2.07	0.23	-0.27	-0.05	-0.98
TS2a	2.12	0.21	-0.28	-0.05	-1.00
INT2a	2.17	0.18	-0.31	-0.06	-0.98
INT2a'	2.23	0.15	-0.32	-0.08	-0.97
TS3a	1.08	0.00	-0.10	0.05	-0.02
PROa	1.09	0.00	-0.14	0.05	0.00
RCb	1.45	0.80	-0.82	-0.42	-0.01
TS1b	1.64	0.50	-0.46	-0.15	-0.52
INT1b	2.01	0.24	-0.24	-0.01	-1.01
INT1b'	2.29	0.14	-0.32	-0.11	-1.01
TS2b	1.16	0.01	0.09	-0.01	-0.25
PROb	1.07	0.00	-0.13	0.06	0.00
TS1c	1.77	0.06	-0.26	-0.01	-0.56
INT1c	1.07	0.01	-0.14	0.05	0.00
RCd	1.52	0.75	-0.82	-0.42	-0.03
TS1d	1.91	0.11	-0.34	-0.11	-0.58
PROd	1.09	0.00	-0.13	0.04	0.00

Table S5. Mulliken group charges.

(a) M06/B1

	Fe	O	Por	SH	Substrate
RCa	0.49	-0.48	0.00	-0.04	0.03
TS1a	0.44	-0.63	-0.18	-0.09	0.46
INT1a	0.40	-0.69	-0.28	0.07	0.49
TS2a	0.37	-0.68	-0.27	0.07	0.52
INT2a	0.40	-0.71	-0.25	0.08	0.47
INT2a'	0.38	-0.72	-0.21	0.10	0.45
TS3a	0.31	-0.78	-0.34	0.08	0.73
PROa	0.21	-0.77	-0.51	0.08	1.00
RCb	0.49	-0.45	-0.04	-0.03	0.04
TS1b	0.42	-0.54	-0.22	-0.05	0.40
INT1b	0.36	-0.68	-0.27	0.05	0.54
INT1b'	0.36	-0.74	-0.20	0.11	0.47
TS2b	0.34	-0.81	-0.26	0.13	0.60
PROb	0.21	-0.77	-0.52	0.08	1.00
TS1c	0.37	-0.49	-0.33	-0.01	0.46
INT1c	-1.50	0.08	0.37	0.52	0.53
RCd	0.48	-0.49	-0.03	-0.03	0.08
TS1d	0.43	-0.59	-0.26	0.00	0.41
PROd	0.22	-0.79	-0.51	0.08	0.99

(b) M06(SCRF)/B2//M06/B1

	Fe	O	Por	SH	substrate
RCa	-1.04	-0.36	1.94	-0.63	0.09
TS1a	-0.89	-0.47	1.64	-0.80	0.52
INT1a	-1.75	-0.40	2.50	-0.65	0.30
TS2a	-1.25	-0.35	2.05	-0.69	0.24
INT2a	-0.73	-0.39	1.51	-0.64	0.25
INT2a'	-0.75	-0.50	1.58	-0.65	0.31
TS3a	0.14	-0.65	0.55	-0.90	0.86
PROa	-0.58	-0.34	1.04	-0.76	0.64
RCb	-0.83	-0.36	1.70	-0.64	0.13
TS1b	-1.09	-0.36	1.70	-0.75	0.50
INT1b	-1.84	-0.37	2.46	-0.58	0.33
INT1b'	-1.35	-0.54	2.17	-0.60	0.32
TS2b	-0.47	-0.56	1.35	-0.70	0.39
PROb	-1.05	-0.26	1.36	-0.71	0.65
TS1c	-1.78	-0.39	2.37	-0.68	0.48
INT1c	-0.57	-0.01	1.01	-0.63	0.20
RCd	-1.04	-0.35	1.90	-0.60	0.08
TS1d	-0.93	-0.42	1.62	-0.73	0.46
PROd	-1.05	-0.42	1.51	-0.75	0.71

(c) B3LYP(SCRf)/B2//M06/B1

	Fe	O	Por	SH	substrate
RCa	-1.43	-0.28	2.31	-0.70	0.09
TS1a	-1.38	-0.40	2.07	-0.86	0.57
INT1a	-2.22	-0.32	2.93	-0.69	0.30
TS2a	-1.73	-0.30	2.47	-0.72	0.28
INT2a	-1.30	-0.30	2.03	-0.67	0.24
INT2a'	-1.13	-0.39	1.97	-0.69	0.23
TS3a	-0.18	-0.60	0.99	-0.95	0.74
PROa	-0.79	-0.30	1.33	-0.81	0.57
RCb	-1.31	-0.26	2.13	-0.70	0.14
TS1b	-1.64	-0.25	2.13	-0.81	0.57
INT1b	-2.33	-0.39	2.92	-0.63	0.43
INT1b'	-1.70	-0.43	2.51	-0.63	0.25
TS2b	-0.62	-0.58	1.44	-0.94	0.70
PROb	-1.22	-0.22	1.63	-0.75	0.55
TS1c	-2.18	-0.22	2.70	-0.75	0.46
INT1c	-0.80	0.07	1.25	-0.67	0.15
RCd	-1.51	-0.24	2.31	-0.66	0.10
TS1d	-1.53	-0.30	2.13	-0.79	0.49
PROd	-1.35	-0.38	1.88	-0.79	0.63

Table S6. Homolytic dissociation energy.

(a) M06/B1

	E [au]	ZPE [au]
Substrate	-170.909330	0.069055
Hydrogen atom	-0.497919	0.000000
C-radical	-170.246410	0.054535
N-radical	-170.278153	0.055325
O-radical	-170.289185	0.055698

(b) M06(SCRF)/B2//M06/B1

	E [au]	ZPE [au]
Substrate	-170.977699	0.069055
Hydrogen atom	-0.499894	0.000000
C-radical	-170.316580	0.054535
N-radical	-170.341834	0.055325
O-radical	-170.354757	0.055698

(c) B3LYP(SCRF)/B2//M06/B1

	E [au]	ZPE [au]
Substrate	-171.091355	0.069055
Hydrogen atom	-0.502174	0.000000
C-radical	-170.430894	0.054535
N-radical	-170.455610	0.055325
O-radical	-170.470556	0.055698

(d) Bond dissociation energy

	M06/B1 [kcal/mol]	M06(SCRF)/B2 //M06/B1 [kcal/mol]	B3LYP(SCRF)/B2 //M06/B1 [kcal/mol]
O-H bond	68.3	68.8	66.1
N-H bond	75.0	76.7	75.2
C-H bond	94.4	92.1	90.2

Table S7. Energy data for the MIC formation.

(a) M06/B1

	E [au]	ZPE [au]
¹ [Fe(II)(Por)SH]	-1510.431413	0.282631
⁵ [Fe(II)(Por)SH]	-1510.493827	0.278476
² [Fe(III)(Por)SH]	-1510.380705	0.284859
⁶ [Fe(III)(Por)SH]	-1510.429771	0.282962
nitrosomethane	-169.686295	0.043344
¹ MIC(II), N-bound	-1680.170716	0.329955
² MIC(III), N-bound	-1680.099546	0.330849
¹ MIC(II), O-bound	-1680.144894	0.328153
² MIC(III), O-bound	-1680.085446	0.329466

(b) M06(SCRF)/B2//M06/B1

	E [au]	ZPE [au]
¹ [Fe(II)(Por)SH]	-2650.499887	0.282631
⁵ [Fe(II)(Por)SH]	-2650.535686	0.278476
² [Fe(III)(Por)SH]	-2650.378398	0.284859
⁶ [Fe(III)(Por)SH]	-2650.412653	0.282962
nitrosomethane	-169.743770	0.043344
¹ MIC(II), N-bound	-2820.293417	0.329955
² MIC(III), N-bound	-2820.150151	0.330849
¹ MIC(II), O-bound	-2820.266030	0.328153
² MIC(III), O-bound	-2820.136354	0.329466

(c) B3LYP(SCRF)/B2//M06/B1

	E [au]	ZPE [au]
¹ [Fe(II)(Por)SH]	-2651.357412	0.282631
⁵ [Fe(II)(Por)SH]	-2651.378144	0.278476
² [Fe(III)(Por)SH]	-2651.236956	0.284859
⁶ [Fe(III)(Por)SH]	-2651.243553	0.282962
nitrosomethane	-169.854083	0.043344
¹ MIC(II), N-bound	-2821.243632	0.329955
² MIC(III), N-bound	-2821.102857	0.330849
¹ MIC(II), O-bound	-2821.223045	0.328153
² MIC(III), O-bound	-2821.094733	0.329466

Scheme S1. Possibility of outer-sphere electron transfer.

The Cpd I moiety within the RC state has a triradicaloid character, while the substrate has a closed-shell character (see RCa above). We attempted to obtain a state that has a closed-shell porphine ligand and a substrate radical. However, attempts to obtain such an electron-transferred state failed.

Appendix C Chapter 4

Table S1. B3LYP/B1 Mulliken group spin populations and group charges for the transition states

(a) Spin population

	Fe	O	SH	Por	Subs.
CHA	1.82	-0.21	-0.03	-0.20	-0.39
CHE	1.78	-0.17	-0.05	-0.25	-0.31
CHD	1.67	0.23	-0.21	-0.36	-0.33
UMDH	1.45	0.67	-0.26	-0.40	-0.47

(b) Charge distribution

	Fe	O	SH	Por	Subs.
CHA	0.45	-0.51	0.01	-0.27	0.32
CHE	0.46	-0.46	-0.01	-0.25	0.26
CHD	0.50	-0.50	-0.07	-0.23	0.29
UMDH	0.47	-0.51	-0.13	-0.25	0.42

Appendix D Chapter 5

Table S1. Raw energy data.

(a) ABT

	E(B1) [hartree]	ZPE [hartree]	E(B2) [hartree]	$\Delta(E(B2)+ZPE)$ [kcal/mol]
RC	-2037.480392	0.411264	-3177.731427	0.0
TS1a	-2037.461827	0.405229	-3177.715245	6.4
Int1a	-2037.488063	0.409734	-3177.741908	-7.5
Int1a2	-2037.490850	0.409549	-3177.743938	-8.9
Int1a2'	-2037.485545	0.408436	-3177.736811	-5.2
TS2a	-2037.475253	0.403732	-3177.729021	-3.2
Int2a	-2037.502858	0.408023	-3177.760526	-20.3
TS3a	-2037.485369	0.402830	-3177.745222	-13.9
Pro	-2037.538505	0.398620	-3177.805967	-54.7
TS1b	-2037.474474	0.409304	-3177.726930	1.6
Int1b	-2037.507994	0.413517	-3177.756394	-14.3
TS2b	-2037.452262	0.407759	-3177.705448	14.1
TS1c	-2037.442547	0.411417	-3177.696049	22.3
Int1c	-2037.485497	0.414651	-3177.734113	0.4
TS2c	-2037.473870	0.409805	-3177.721688	5.2
Int2c	-2037.478397	0.413720	-3177.728305	3.5
TS3c	-2037.474952	0.413105	-3177.725640	4.8

(b) BBT

	E(B1) [hartree]	ZPE [hartree]	E(B2) [hartree]	$\Delta(E(B2)+ZPE)$ [kcal/mol]
RC-II	-2307.836375	0.520692	-3448.156353	0.0
TS1a-II	-2307.816903	0.514592	-3448.140084	6.4
Int1a-II	-2307.846299	0.519400	-3448.168409	-8.4
Int1a'-II	-2307.836689	0.516291	-3448.158116	-3.9
TS2a-II	-2307.831189	0.513115	-3448.153425	-2.9
Int2a-II	-2307.919464	0.520061	-3448.245275	-56.2
TS1b-II	-2307.827292	0.518462	-3448.150331	2.4
Int1b-II	-2307.862075	0.522028	-3448.181665	-15.0
TS1d-II	-2307.802021	0.514900	-3448.125109	16.0
RC2	-2306.628813	0.496529	-3446.940357	0.0
TS3a-II	-2306.598004	0.494899	-3446.912680	16.3
Int3a-II	-2306.634620	0.497564	-3446.947639	-3.9
TS4a-II	-2306.618366	0.494569	-3446.934041	2.7
Int4a-II	-2306.645288	0.493576	-3446.963506	-16.4
TS4a'-II	-2306.623870	0.496527	-3446.934841	3.5
Int4a'-II	-2306.628165	0.497835	-3446.936139	3.5

Appendix E Chapter 6

Table S1. Raw energy data obtained at the B3LYP/SDD,6-31G(d) level

CpdI + Furanocoumarin

	ΔE (kcal/mol)	$\Delta(E+ZPE)$ (kcal/mol)	E (a.u.)	ZPE (a.u.)	E+ZPE (a.u.)
2RC	0.0	0.0	-2234.729782	0.431854	-2234.297927
4RC	0.1	0.2	-2234.729629	0.432021	-2234.297609
6RC	9.8	8.5	-2234.714153	0.429744	-2234.284409
2TS	13.7	13.3	-2234.708028	0.431216	-2234.276812
4TS	13.7	13.5	-2234.707951	0.431499	-2234.276452
6TS	17.4	14.8	-2234.702021	0.427678	-2234.274343
4Int1	-13.4	-13.5	-2234.751076	0.431646	-2234.319429
6Int1	-11.3	-13.3	-2234.747822	0.428701	-2234.319121
4TS2	-12.6	-13.3	-2234.749795	0.430676	-2234.319119
6TS2	-11.0	-13.2	-2234.747256	0.428295	-2234.318961
2Int2	-32.1	-30.0	-2234.780928	0.435143	-2234.345785
4Int2	-31.3	-30.3	-2234.779638	0.433443	-2234.346195
6Int2	-35.5	-35.3	-2234.786396	0.432197	-2234.354199
2TS3	-17.4	-17.1	-2234.757513	0.432358	-2234.325155
4TS3	-12.6	-13.7	-2234.749924	0.430212	-2234.319712
6TS3	-14.8	-16.5	-2234.753431	0.429231	-2234.324201
2Pd	-28.5	-28.3	-2234.775149	0.432175	-2234.342974
4Pd	-26.2	-27.1	-2234.771512	0.430343	-2234.341169
6Pd	-32.9	-34.8	-2234.782283	0.428892	-2234.353391
4PB.TS	-12.6	-13.7	-2234.749874	0.430153	-2234.319721
6PB.TS	-11.0	-13.2	-2234.747256	0.428291	-2234.318965
4PB.Prod	-26.1	-27.2	-2234.771399	0.430141	-2234.341258
6PB.Prod	-29.8	-31.8	-2234.777215	0.428658	-2234.348557

Table S2. Raw energy data obtained at the B3LYP/6-311+G(d,p) level

CpdI + Furanocoumarin

	ΔE (kcal/mol)	$\Delta(E+ZPE)$ (kcal/mol)	E (a.u.)	ZPE (a.u.)	E+ZPE (a.u.)
2RC	0.0	0.0	-3375.014549	0.431854	-3374.582694
4RC	0.2	0.3	-3375.014195	0.432021	-3374.582174
6RC	12.1	10.8	-3374.995262	0.429744	-3374.565519
2TS	13.1	12.7	-3374.993734	0.431216	-3374.562518
4TS	13.5	13.2	-3374.993088	0.431499	-3374.561589
6TS	20.1	17.5	-3374.982531	0.427678	-3374.554853
4Int1	-12.9	-13.1	-3375.035180	0.431646	-3374.603534
6Int1	-6.1	-8.0	-3375.024219	0.428748	-3374.595471
4TS2	-10.8	-11.6	-3375.031809	0.430676	-3374.601133
6TS2	-5.1	-7.3	-3375.022651	0.428295	-3374.594356
2Int2	-29.0	-27.0	-3375.060794	0.435143	-3374.625651
4Int2	-28.3	-27.3	-3375.059582	0.433443	-3374.626140
6Int2	-30.5	-30.3	-3375.063216	0.432197	-3374.631019
2TS3	-15.5	-15.2	-3375.039264	0.432358	-3374.606906
4TS3	-10.0	-11.1	-3375.030541	0.430212	-3374.600329
6TS3	-12.6	-14.2	-3375.034581	0.429231	-3374.605350
2Pd	-29.0	-28.8	-3375.060719	0.432175	-3374.628544
4Pd	-26.7	-27.7	-3375.057153	0.430343	-3374.626810
6Pd	-32.3	-34.1	-3375.066003	0.428892	-3374.637111
4PB.TS	-11.4	-12.4	-3375.032679	0.430153	-3374.602526
6PB.TS	-10.4	-12.6	-3375.031086	0.428291	-3374.602795
4PB.Prod	-26.7	-27.8	-3375.057087	0.430141	-3374.626946
6PB.Prod	-28.2	-30.2	-3375.059553	0.428658	-3374.630895

Table S3. Raw energy data obtained at the B3LYP(SCRf)/6-311+G(d,p) level

CpdI + Furanocoumarin

	ΔE (kcal/mol)	$\Delta(E+ZPE)$ (kcal/mol)	E (a.u.)	ZPE (a.u.)	E+ZPE (a.u.)
2RC	0.0	0.0	-3375.034751	0.431854	-3374.602896
4RC	0.2	0.3	-3375.034422	0.432021	-3374.602401
6RC	11.8	10.5	-3375.015976	0.429744	-3374.586232
2TS	12.6	12.2	-3375.014593	0.431216	-3374.583377
4TS	14.1	13.9	-3375.012227	0.431499	-3374.580728
6TS	20.8	18.2	-3375.001568	0.427678	-3374.573890
4Int1	-12.0	-12.1	-3375.053852	0.431646	-3374.622206
6Int1	-5.4	-7.3	-3375.043348	0.428748	-3374.614600
4TS2	-13.0	-13.7	-3375.05545	0.430676	-3374.624774
6TS2	-5.8	-8.1	-3375.044068	0.428295	-3374.615772
2Int2	-27.7	-25.7	-3375.078921	0.435143	-3374.643778
4Int2	-29.0	-28.0	-3375.080979	0.433443	-3374.647536
6Int2	-30.5	-30.3	-3375.083359	0.432197	-3374.651162
2TS3	-17.7	-17.4	-3375.062955	0.432358	-3374.630597
4TS3	-13.8	-14.8	-3375.056666	0.430212	-3374.626454
6TS3	-14.6	-16.3	-3375.058067	0.429231	-3374.628836
2Pd	-30.4	-30.2	-3375.083128	0.432175	-3374.650953
4Pd	-29.8	-30.7	-3375.082177	0.430343	-3374.651834
6Pd	-31.9	-33.7	-3375.085568	0.428892	-3374.656676
4PB.TS	-13.8	-14.8	-3375.056697	0.430153	-3374.626544
6PB.TS	-12.5	-14.7	-3375.05468	0.428291	-3374.626390
4PB.Prod	-29.8	-30.9	-3375.082301	0.430141	-3374.652159
6PB.Prod	-30.3	-32.3	-3375.083021	0.428658	-3374.654363

Table S4. Mulliken group spin densities and group charges for optimized geometries

(a) Spin density

	Fe	O	Por	SH	substrate
2RC	1.32	0.80	-0.56	-0.56	0.00
4RC	1.19	0.85	0.45	0.51	0.00
6RC	3.18	0.61	0.87	0.33	0.00
2TS	1.63	0.26	-0.34	-0.24	-0.31
4TS	1.30	0.79	0.14	0.36	0.41
6TS	3.84	0.15	0.45	0.85	-0.29
4Int1	2.28	0.06	-0.16	-0.01	0.83
6Int	4.16	0.25	0.69	0.79	-0.88
4TS2	2.55	-0.09	-0.10	0.26	0.37
6TS2	4.16	0.20	0.71	0.63	-0.70
2Int	1.09	0.00	-0.09	0.00	0.00
4Int2	2.60	0.00	-0.03	0.43	0.00
6Int2	4.08	0.00	0.51	0.41	0.00
2TS3	1.08	-0.03	-0.14	0.00	0.09
4TS3	2.57	-0.02	-0.12	0.15	0.41
6TS3	4.08	0.00	0.51	0.41	0.00
2Pd	1.08	0.00	-0.09	0.02	-0.01
4Pd	2.61	0.01	-0.03	0.42	0.00
6Pd	4.01	0.00	0.52	0.41	0.00
4PB.TS	2.57	-0.02	-0.12	0.15	0.41
6PB.TS	4.16	0.20	0.71	0.63	-0.70
4PB.Prod	2.61	0.01	-0.04	0.42	0.00
6PB.Prod	4.09	0.00	0.50	0.40	0.00

(b) Charge distribution

	Fe	O	Por	SH	substrate
2RC	0.57	-0.43	-0.08	-0.05	-0.01
4RC	0.56	-0.43	-0.07	-0.05	-0.01
6RC	0.64	-0.46	-0.13	-0.04	-0.01
2TS	0.50	-0.45	-0.24	-0.06	0.26
4TS	0.48	-0.44	-0.27	0.00	0.23
6TS	0.87	-0.56	-0.45	-0.10	0.23
4Int1	0.43	-0.49	-0.25	0.05	0.26
6Int	0.86	-0.57	-0.39	-0.12	0.22
4TS2	0.46	-0.45	-0.39	-0.09	0.48
6TS2	0.83	-0.55	-0.43	-0.15	0.31
2Int	0.31	-0.42	-0.48	0.07	0.52
4Int2	0.46	-0.41	-0.40	-0.10	0.46
6Int2	0.48	-0.43	-0.49	-0.01	0.45
2TS3	0.30	-0.36	-0.45	0.08	0.42
4TS3	0.47	-0.41	-0.43	-0.14	0.51
6TS3	0.49	-0.43	-0.51	-0.01	0.46
2Pd	0.27	-0.33	-0.49	0.05	0.49
4Pd	0.45	-0.33	-0.41	-0.13	0.41
6Pd	0.48	-0.38	-0.47	0.00	0.37
4PB.TS	0.46	-0.45	-0.37	-0.07	0.43
6PB.TS	0.79	-0.54	-0.40	-0.13	0.28
4PB.Prod	0.45	-0.32	-0.42	-0.13	0.42
6PB.Prod	0.51	-0.35	-0.53	-0.03	0.40

Figure S1. Geometries of key species Doublet/Quartet/Sextet

Figure S2. Spin density distribution of key parts and carbon atoms

Doublet/Quartet/Sextet

Bergamottin AMBER Parameters

=== Prep File ===
0 0 2

This is a remark line
molecule.res

LIG XYZ 0

CHANGE OMIT DU BEG

0.0000							
1	DUMM	DU	M	999.000	999.000	-999.000	0.000
2	DUMM	DU	M	999.000	-999.000	999.000	0.000
3	DUMM	DU	M	-999.000	999.000	999.000	0.000
4	C1	CA	M	-1.217000	0.692000	-0.641000	-0.401352
5	C2	CA	B	-1.891000	1.678000	0.093000	0.545359
6	O1	OS	S	-1.350000	2.900000	0.393000	-0.434540
7	C3	C	B	-0.008000	3.176000	-0.023000	0.815764
8	C4	CA	B	0.690000	2.122000	-0.824000	-0.528898
9	C5	CA	S	0.132000	0.947000	-1.124000	0.170777
10	H2	HA	E	0.659000	0.216000	-1.700000	0.083429
11	H1	HA	E	1.682000	2.320000	-1.174000	0.193402
12	O3	O	E	0.537000	4.216000	0.261000	-0.503931
13	C9	CA	B	-3.190000	1.471000	0.570000	-0.550062
14	C8	CB	S	-3.771000	0.241000	0.279000	0.337928
15	C7	CB	S	-3.142000	-0.768000	-0.442000	0.032298
16	C10	C*	B	-4.075000	-1.834000	-0.506000	-0.324301
17	C11	CA	B	-5.197000	-1.412000	0.173000	-0.003916

18	O2	OS	E	-5.020000	-0.155000	0.652000	-0.199363
19	H5	H4	E	-6.084000	-1.995000	0.305000	0.162865
20	H4	HA	E	-3.936000	-2.780000	-0.986000	0.176670
21	H3	HA	E	-3.710000	2.220000	1.129000	0.243234
22	C6	CA	M	-1.836000	-0.542000	-0.915000	0.200983
23	O4	OS	M	-1.187000	-1.497000	-1.624000	-0.245762
24	C12	CT	M	-0.112000	-2.188000	-1.047000	-0.376749
25	H6	H1	E	0.538000	-2.471000	-1.848000	0.237169
26	H7	H1	E	-0.534000	-2.938000	-0.410000	0.237169
27	C13	CM	M	0.665000	-1.260000	-0.167000	-0.468245
28	H8	HA	E	0.890000	-0.268000	-0.501000	0.481752
29	C14	CM	M	1.078000	-1.669000	1.036000	0.370170
30	C15	CT	3	0.764000	-3.056000	1.505000	-0.537418
31	H9	HC	E	0.207000	-3.570000	0.750000	0.152992
32	H10	HC	E	1.675000	-3.583000	1.696000	0.152992
33	H11	HC	E	0.185000	-3.007000	2.404000	0.152992
34	C16	CT	M	1.855000	-0.740000	1.917000	-0.624096
35	H12	HC	E	2.463000	-1.351000	2.551000	0.203721
36	H13	HC	E	1.150000	-0.060000	2.347000	0.203721
37	C17	CT	M	2.805000	0.092000	1.066000	0.345282
38	H14	HC	E	2.204000	0.601000	0.342000	-0.052104
39	H15	HC	E	3.416000	0.651000	1.743000	-0.052104
40	C18	CM	M	3.720000	-0.816000	0.306000	-0.652399
41	H16	HA	E	3.481000	-1.854000	0.203000	0.202825
42	C19	CM	M	4.838000	-0.330000	-0.244000	0.465924

43	C21	CT	3	5.173000	1.122000	-0.102000	-0.419988
44	H20	HC	E	6.096000	1.325000	-0.603000	0.104300
45	H21	HC	E	4.393000	1.712000	-0.537000	0.104300
46	H22	HC	E	5.268000	1.366000	0.935000	0.104300
47	C20	CT	M	5.754000	-1.239000	-1.003000	-0.419988
48	H17	HC	E	6.590000	-0.679000	-1.368000	0.104300
49	H18	HC	E	5.226000	-1.670000	-1.828000	0.104300
50	H19	HC	E	6.101000	-2.018000	-0.357000	0.104300

LOOP

C5 C1
O2 C8
C6 C7

IMPROPER

C6 C2 C1 C5
C9 C1 C2 O1
C4 O3 C3 O1
C3 C5 C4 H1
C1 C4 C5 H2
C2 C8 C9 H3
C9 C7 C8 O2
C10 C6 C7 C8
C11 C7 C10 H4
C10 H5 C11 O2
C1 C7 C6 O4

C14	C12	C13	H8
C13	C15	C14	C16
C19	C17	C18	H16
C18	C21	C19	C20

DONE

STOP

== Force Field parameter file ==

MASS

CA	12.010	0.360
OS	16.000	0.465
C	12.010	0.616
HA	1.008	0.135
O	16.000	0.434
CB	12.010	0.360
C*	12.010	0.360
H4	1.008	0.135
CT	12.010	0.878
H1	1.008	0.135
CM	12.010	0.360
HC	1.008	0.135

BOND

CA-CA	478.400	1.387
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CA-OS	392.600	1.357
OS-C	411.300	1.343
C -CA	449.900	1.406
C -O	648.000	1.214
CA-HA	344.300	1.087
CA-CB	411.700	1.434
CB-CB	418.300	1.429
CB-OS	392.600	1.357
CB-C*	418.300	1.429
C*-CA	411.700	1.434
C*-HA	344.300	1.087
CA-H4	344.300	1.087
OS-CT	301.500	1.439
CT-HI	337.300	1.092
CT-CM	328.300	1.508
CM-HA	344.300	1.087
CM-CM	418.300	1.429
CT-HC	337.300	1.092
CT-CT	303.100	1.535

ANGLE

CA-CA-OS	71.200	121.430
CA-CA-CA	67.200	119.970
CA-CA-HA	50.300	119.700
CA-CA-CB	66.000	120.100

CA-OS-C	68.200	106.900
OS-C -CA	71.680	112.135
OS-C -O	76.200	122.430
C -CA-CA	67.900	120.700
C -CA-HA	48.000	119.700
CA-C -O	72.800	119.120
CA-CB-CB	67.700	111.040
CA-CB-OS	71.200	121.430
CB-CA-HA	50.300	119.700
CB-CB-C*	67.900	110.700
CB-OS-CA	66.000	113.140
CB-CB-OS	71.200	121.430
CB-C*-CA	67.700	111.040
CB-C*-HA	50.300	119.700
CB-CA-OS	71.200	121.430
C*-CB-CA	67.700	111.040
C*-CA-OS	71.200	121.430
C*-CA-H4	50.000	120.940
CA-C*-HA	50.300	119.700
OS-CA-H4	52.400	112.690
CA-OS-CT	64.200	112.090
OS-CT-H1	50.900	108.700
OS-CT-CM	68.300	108.880
CT-CM-HA	45.100	120.000
CT-CM-CM	64.300	123.420

H1-CT-H1	39.400	108.350
H1-CT-CM	47.000	110.490
HA-CM-CM	50.300	119.700
CM-CT-HC	47.000	110.490
CM-CT-CT	63.700	110.960
CT-CM-CT	62.700	116.520
HC-CT-HC	39.400	108.350
CT-CT-HC	46.400	110.050

DIHEDRAL

CA-CA-OS-C	1.000	1.050	180.000	2.000
CA-CA-CA-CB	1.000	3.625	180.000	2.000
CA-CA-CA-HA	1.000	6.650	180.000	2.000
CA-CA-CA-C	1.000	6.650	180.000	2.000
CA-CA-CB-CB	1.000	4.000	180.000	2.000
CA-CA-CB-C*	1.000	4.000	180.000	2.000
CA-CA-OS-CT	1.000	1.050	180.000	2.000
CA-CA-CA-CA	1.000	3.625	180.000	2.000
CA-CA-CA-OS	1.000	6.650	180.000	2.000
CA-OS-C -CA	1.000	2.700	180.000	2.000
CA-OS-C -O	1.000	2.700	180.000	2.000
CA-CA-CB-OS	1.000	6.650	180.000	2.000
OS-CA-CA-CB	1.000	6.650	180.000	2.000
OS-CA-CA-HA	1.000	6.650	180.000	2.000
OS-C -CA-CA	1.000	2.175	180.000	2.000

OS-C -CA-HA	1.000	2.175	180.000	2.000
C -CA-CA-HA	1.000	6.650	180.000	2.000
CA-CA-C -O	1.000	2.175	180.000	-2.000
CA-CA-C -O	1.000	0.300	0.000	3.000
HA-CA-CA-HA	1.000	6.650	180.000	2.000
HA-CA-C -O	1.000	2.175	180.000	2.000
CA-CB-CB-C*	1.000	4.000	180.000	2.000
CA-CB-CB-CA	1.000	4.000	180.000	2.000
CA-CB-OS-CA	1.000	1.050	180.000	2.000
CB-CB-C*-CA	1.000	4.000	180.000	2.000
CB-CB-C*-HA	1.000	6.650	180.000	2.000
CB-CB-CA-OS	1.000	6.650	180.000	2.000
CB-OS-CA-C*	1.000	1.050	180.000	2.000
CB-OS-CA-H4	1.000	1.050	180.000	2.000
CB-CB-CA-HA	1.000	6.650	180.000	2.000
CB-CB-OS-CA	1.000	1.050	180.000	2.000
CB-C*-CA-OS	1.000	6.650	180.000	2.000
CB-C*-CA-H4	1.000	4.000	180.000	2.000
CB-CA-OS-CT	1.000	1.050	180.000	2.000
C*-CB-CB-OS	1.000	6.650	180.000	2.000
C*-CB-CA-OS	1.000	6.650	180.000	2.000
CA-C*-CB-CA	1.000	4.000	180.000	2.000
OS-CB-CA-HA	1.000	6.650	180.000	2.000
OS-CB-CB-CA	1.000	6.650	180.000	2.000
OS-CA-C*-HA	1.000	6.650	180.000	2.000

H4-CA-C*-HA	1.000	6.650	180.000	2.000
HA-C*-CB-CA	1.000	6.650	180.000	2.000
CA-OS-CT-HI	1.000	0.383	0.000	3.000
CA-OS-CT-CM	1.000	0.383	0.000	3.000
OS-CT-CM-HA	1.000	0.000	0.000	2.000
OS-CT-CM-CM	1.000	0.000	0.000	2.000
CT-CM-CM-CT	1.000	6.650	180.000	-2.000
CT-CM-CM-CT	1.000	1.900	180.000	1.000
H1-CT-CM-HA	1.000	0.000	0.000	2.000
H1-CT-CM-CM	1.000	0.380	180.000	-3.000
H1-CT-CM-CM	1.000	1.150	0.000	1.000
CM-CM-CT-HC	1.000	0.380	180.000	-3.000
CM-CM-CT-HC	1.000	1.150	0.000	1.000
CM-CM-CT-CT	1.000	0.000	0.000	2.000
HA-CM-CM-CT	1.000	6.650	180.000	2.000
CM-CT-CT-HC	1.000	0.156	0.000	3.000
CM-CT-CT-CM	1.000	0.156	0.000	3.000
CT-CM-CT-HC	1.000	0.000	0.000	2.000
CT-CM-CT-CT	1.000	0.000	0.000	2.000
CT-CT-CM-HA	1.000	0.000	0.000	2.000
HC-CT-CT-HC	1.000	0.150	0.000	3.000
HC-CT-CM-HA	1.000	0.000	0.000	2.000

IMPROPER

CA-CA-CA-CA	1.1	180.0	2.0
CA-CA-CA-OS	1.1	180.0	2.0
CA-O -C -OS	1.1	180.0	2.0
C -CA-CA-HA	1.1	180.0	2.0
CA-CA-CA-HA	1.1	180.0	2.0
CA-CB-CA-HA	1.1	180.0	2.0
CA-CB-CB-OS	1.1	180.0	2.0
C*-CA-CB-CB	1.1	180.0	2.0
CA-CB-C*-HA	1.1	180.0	2.0
C*-HA-CA-OS	1.1	180.0	2.0
CA-CB-CA-OS	1.1	180.0	2.0
CM-CT-CM-HA	1.1	180.0	2.0
CM-CT-CM-CT	1.1	180.0	2.0

NONBON

CA	1.9080	0.0860
OS	1.6837	0.1700
C	1.9080	0.0860
HA	1.4870	0.0157
O	1.6612	0.2100
CB	1.9080	0.0860
C*	1.9080	0.0860
H4	1.4870	0.0157
CT	1.9080	0.1094
H1	1.4870	0.0157

CM	1.9080	0.0860
HC	1.4870	0.0157

Table S5. ONIOM raw energy data

a) Extrapolated energy - ONIOM(UB3LYP/SDD(Fe),6-31G(d)(others):AMBER)
Mechanical Embedding

	Geom1		Geom2	
	E(a.u.)	$\Delta E(\text{kcal/mol})$	E(a.u.)	$\Delta E(\text{kcal/mol})$
RC	-2411.713248	0.0	-2411.884244	0.0
TS1	-2411.692805	12.8	-2411.861800	14.1
Int1	-2411.729968	-10.5	-2411.901347	-10.7

	Geom3		Geom4	
	E(a.u.)	$\Delta E(\text{kcal/mol})$	E(a.u.)	$\Delta E(\text{kcal/mol})$
RC	-2411.834377	0.0	-2411.910779	0.0
TS1	-2411.802985	19.7	-2411.889510	13.3
Int1	-2411.845206	-6.8	-2411.928952	-11.4

b) Extrapolated energy - ONIOM(UB3LYP/SDD(Fe),6-31G(d)(others):AMBER)
Electronic Embedding

	Geom1		Geom2	
	E(a.u.)	$\Delta E(\text{kcal/mol})$	E(a.u.)	$\Delta E(\text{kcal/mol})$
RC	-2411.615378	0.0	-2411.782227	0.0
TS1	-2411.591812	14.8	-2411.759935	14.0
Int1	-2411.626735	-7.1	-2411.800266	-11.3

	Geom3		Geom4	
	E(a.u.)	$\Delta E(\text{kcal/mol})$	E(a.u.)	$\Delta E(\text{kcal/mol})$
RC	-2411.717615	0.0	-2411.790994	0.0
TS1	-2411.681994	22.4	-2411.767418	14.8
Int1	-2411.726305	-5.5	-2411.808555	-11.0

c) QM energy - ONIOM(UB3LYP/SDD(Fe),6-31G(d)(others):AMBER) Mechanical
Embedding

	Geom1		Geom2	
	E(a.u.)	$\Delta E(\text{kcal/mol})$	E(a.u.)	$\Delta E(\text{kcal/mol})$
RC	-2388.509499	0.0	-2388.508602	0.0
TS1	-2388.488366	13.3	-2388.486603	13.8
Int1	-2388.52619	-10.5	-2388.523409	-9.3

	Geom3		Geom4	
	E(a.u.)	$\Delta E(\text{kcal/mol})$	E(a.u.)	$\Delta E(\text{kcal/mol})$
RC	-2388.514056	0.0	-2388.512393	0.0
TS1	-2388.486166	17.5	-2388.486549	16.2
Int1	-2388.526749	-8.0	-2388.525974	-8.5

d) QM energy - ONIOM(UB3LYP/SDD(Fe),6-31G(d)(others):AMBER) Electronic Embedding

	Geom1		Geom2	
	E(a.u.)	$\Delta E(\text{kcal/mol})$	E(a.u.)	$\Delta E(\text{kcal/mol})$
RC	-2388.570899	0.0	-2388.579881	0.0
TS1	-2388.545825	15.7	-2388.562032	11.2
Int1	-2388.579789	-5.6	-2388.597781	-11.2

	Geom3		Geom4	
	E(a.u.)	$\Delta E(\text{kcal/mol})$	E(a.u.)	$\Delta E(\text{kcal/mol})$
RC	-2388.594283	0.0	-2388.567126	0.0
TS1	-2388.553854	25.4	-2388.546286	13.1
Int1	-2388.59843	-2.6	-2388.587007	-12.5

e) MM,real energy - ONIOM(UB3LYP/SDD(Fe),6-31G(d)(others):AMBER) Mechanical Embedding

	Geom1		Geom2	
	E(a.u.)	$\Delta E(\text{kcal/mol})$	E(a.u.)	$\Delta E(\text{kcal/mol})$
RC	-23.017276	0.0	-23.185862	0.0
TS1	-22.774509	152.3	-22.947283	149.7
Int1	-3.188739	12442.6	-2.025014	13278.6

	Geom3		Geom4	
	E(a.u.)	$\Delta E(\text{kcal/mol})$	E(a.u.)	$\Delta E(\text{kcal/mol})$
RC	-23.124886	0.0	-23.21427461	0.0
TS1	-22.754815	232.2	-22.92299233	182.8
Int1	1.725646	15593.9	-2.208135196	13181.6

f) MM,real energy - ONIOM(UB3LYP/SDD(Fe),6-31G(d)(others):AMBER) Electronic Embedding

	Geom1		Geom2	
	E(a.u.)	$\Delta E(\text{kcal/mol})$	E(a.u.)	$\Delta E(\text{kcal/mol})$
RC	-23.01727601	0.0	-23.18586259	0.0
TS1	-22.77450924	152.3	-22.94728197	149.7
Int1	-3.188758508	12442.6	-2.024976899	13278.7

	Geom3		Geom4	
	E(a.u.)	$\Delta E(\text{kcal/mol})$	E(a.u.)	$\Delta E(\text{kcal/mol})$
RC	-23.12488608	0.0	-23.21427451	0.0
TS1	-22.75481397	232.2	-22.92299081	182.8
Int1	-21.48116613	1031.4	-1.921476621	13361.4

g) MM,model energy - ONIOM(UB3LYP/SDD(Fe),6-31G(d)(others):AMBER)
Mechanical Embedding

	Geom1		Geom2	
	E(a.u.)	ΔE(kcal/mol)	E(a.u.)	ΔE(kcal/mol)
RC	0.186473	0.0	0.016484	0.0
TS1	0.429930	152.8	0.250620	146.9
Int1	20.015038	12442.6	21.177508	13278.7

	Geom3		Geom4	
	E(a.u.)	ΔE(kcal/mol)	E(a.u.)	ΔE(kcal/mol)
RC	0.195436	0.0	0.184111	0.0
TS1	0.562004	230.0	0.479969	185.7
Int1	25.044103	15592.8	21.194843	13184.4

h) MM,model energy - ONIOM(UB3LYP/SDD(Fe),6-31G(d)(others):AMBER)
Electronic Embedding

	Geom1		Geom2	
	E(a.u.)	ΔE(kcal/mol)	E(a.u.)	ΔE(kcal/mol)
RC	0.027203266	0.0	0.016484234	0.0
TS1	0.271477812	153.3	0.250620341	146.9
Int1	19.85818703	12444.1	21.35292375	13388.8

	Geom3		Geom4	
	E(a.u.)	ΔE(kcal/mol)	E(a.u.)	ΔE(kcal/mol)
RC	-0.001554369	0.0	0.009592953	0.0
TS1	0.373326759	235.2	0.298141419	181.1
Int1	1.647448698	1034.8	21.30007135	13360.0

Table S6. ONIOM spin densities

a) ONIOM(UB3LYP/SDD(Fe),6-31G(d)(others):AMBER) Mechanical Embedding

	Geom1				
	Fe	O	Por.	Cys.	Subs.
RC	1.27	0.84	-0.29	-0.82	0.01
TS1	1.71	0.31	-0.29	-0.39	-0.34
Int1	1.97	0.19	-0.18	-0.14	-0.84

	Geom2				
	Fe	O	Por.	Cys.	Subs.
RC	1.26	0.84	-0.29	-0.81	0.01
TS1	1.68	0.33	-0.31	-0.36	-0.34
Int1	2.08	0.19	-0.18	-0.19	-0.89
Geom3					

	Fe	O	Por.	Cys.	Subs.
RC	1.31	0.81	-0.32	-0.80	0.01
TS1	1.96	0.29	-0.28	-0.62	-0.34
Int1	2.73	0.22	-0.20	-0.82	-0.93

	Geom4				
	Fe	O	Por.	Cys.	Subs.
RC	1.25	0.86	-0.30	-0.80	0.00
TS1	1.83	0.27	-0.28	-0.48	-0.34
Int1	2.12	0.15	-0.18	-0.17	-0.92

b) ONIOM(UB3LYP/SDD(Fe),6-31G(d)(others):AMBER) Electronic Embedding

	Geom1				
	Fe	O	Por.	Cys.	Subs.
RC	1.34	0.79	-0.58	-0.55	0.01
TS1	1.37	0.64	-0.69	-0.38	0.06
Int1	2.14	0.15	-0.20	-0.18	-0.91

	Geom2				
	Fe	O	Por.	Cys.	Subs.
RC	1.35	0.77	-0.67	-0.46	0.01
TS1	1.40	0.61	-0.70	-0.31	0.01
Int1	2.16	0.15	-0.19	-0.20	-0.91

	Geom3				
	Fe	O	Por.	Cys.	Subs.
RC	1.41	0.73	-0.50	-0.64	0.01
TS1	1.83	0.38	-0.56	-0.57	-0.08
Int1	2.81	0.16	-0.29	-0.75	-0.93

	Geom4				
	Fe	O	Por.	Cys.	Subs.
RC	1.32	0.80	-0.53	-0.59	0.00
TS1	1.73	0.37	-0.54	-0.43	-0.12
Int1	2.22	0.11	-0.19	-0.21	-0.93

Decomposed energy analysis of C-O formation barrier

$$\Delta E = \Delta E_{QM,model} + (\Delta E_{MM,real} - \Delta E_{MM,model})$$

Mechanical Embedding

Geom1	12.8 = 13.3 + (152.3 – 152.8)
Geom2	14.1 = 13.8 + (149.7 – 146.9)
Geom3	19.7 = 17.5 + (232.2 – 230.0)
Geom4	13.3 = 16.2 + (182.8 – 185.7)

Electronic Embedding

Geom1	14.8 = 15.7 + (152.3 – 153.3)
Geom2	14.0 = 11.2 + (149.7 – 146.9)
Geom3	22.4 = 25.4 + (232.2 – 235.2)
Geom4	14.8 = 13.1 + (182.8 – 181.1)

List of Publications

1. Hajime Hirao, Pratanphorn Chuanprasit, Ying Yi Cheoung, Xiaoqing Wang, *How Is a Metabolic Intermediate Formed in the Mechanism-Based Inactivation of Cytochrome P450 by Using 1,1-Dimethylhydrazine: Hydrogen Abstraction or Nitrogen Oxidation?* Chem. Eur. J. 2013; 7361-7369. (Chapter 2, **Our cover art was adopted for the front page of the journal**)
2. Hajime Hirao, Nandun M Thellamurege, Pratanphorn Chuanprasit, Kai Xu, *Importance of H-abstraction in the Final Step of Nitrosoalkane Formation in the Mechanism-Based Inactivation of Cytochrome P450 by Amine-Containing Drugs.* Int. J. Mol. Sci. 2013; 24692-24705. (Chapter 3, **One of the journal's most highly accessed**)
3. Hajime Hirao, Pratanphorn Chuanprasit, *An Attempt to Evaluate the Effect of Proton-Coupled Electron Transfer on the H-abstraction Step of the Reaction between 1,1-Dimethylhydrazine and Cytochrome P450 Compound I,* Chem. Phys. Lett. 2015; 188-192. (Chapter 4)
4. Pratanphorn Chuanprasit[⌘], Shu Hui Goh[⌘], Hajime Hirao, *Benzyne Formation in the Mechanism-Based Inactivation of Cytochrome P450 by 1-Aminobenzotriazole and N-Benzyl-1-Aminobenzotriazole: Computational Insights,* ACS Catal. 2015; 2952-2960 ([⌘] Both authors contribute equally to the production of this paper) (Chapter 5, **Featured in the Virtual Special Issue of ACS Catalysis entitled 'Catalysis in Singapore'**)
5. Pratanphorn Chuanprasit, Hajime Hirao, *Density Functional Theory and QM/MM Studies of the Mechanism-Based Inactivation of Cytochrome P450 by Bergamottin,* to be submitted. (Chapter 6)

Other Publications

1. Haiyan Xu, Peng Yang, Pratanphorn Chuanprasit, Hajime Hirao, Jianrong (Steve) Zhou, *Nickel-Catalyzed Asymmetric Transfer Hydrogenation of Hydrazones and Other Ketimines*. *Angew. Chem. Int. Ed.* 2015; 5112-5116.

List of Presentations

1. Pratanphorn Chuanprasit and Hajime Hirao. *Computational Studies on Grapefruit Juice Effect on Cytochrome P450*, Pure and Applied Chemistry International Conference 2014 (PACCON2014), 8th-10th January, 2014 (poster).
2. Pratanphorn Chuanprasit, Shu Hui Goh, and Hajime Hirao*. *Benzyne Formation in the Mechanism-Based Inactivation of Cytochrome P450 by 1-Aminobenzotriazole and N-Benzyl-1-Aminobenzotriazole: Computational Insights*, The Chemical Bonds at the 21st Century, 14th-18th June, 2015 (poster).
3. Pratanphorn Chuanprasit, Jianrong (Steve) Zhou*, and Hajime Hirao*. *ONIOM-DFT Study of Nickel-Catalyzed Asymmetric Hydrogenation of Hydrazones*, The Chemical Bonds at the 21st Century, 14th-18th June, 2015 (poster).
4. Pratanphorn Chuanprasit, and Hajime Hirao*. *An Attempt to Evaluate the Effect of Proton-coupled Electron Transfer on the H-abstraction Step of the Reaction Between 1,1-dimethylhydrazine and Cytochrome P450 Compound I*, The Chemical Bonds at the 21st Century, 14th-18th June, 2015 (poster, **Selected as one of the 5 awardees of the Best Poster Award**)