2019-01-01

Ibuprofen as a Probe of Lipid Metabolism

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IBUPROFEN AS A PROBE OF LIPID METABOLISM

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Dedicación

Esta tesis la dedico a mi Madre María Mata,

a mi Padre Antonio Rosales,

y a mis hermanos Guadalupe, Elizabeth, y Jesús

que son mi gran inspiración en la vida.

IBUPROFEN AS A PROBE OF LIPID METABOLISM

by

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THESIS

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

Department of Chemistry and Biochemistry

THE UNIVERSITY OF TEXAS AT EL PASO

August 2019

Acknowledgements

I would like to thank my graduate advisor, Dr. James Salvador, for his encouragement, serenity and support in this project. I would also like to thank all my committee members for consenting to contribute in revising my thesis and reviewing my defense: Dr. Juan C. Noveron, Dr. Wen-Yee Lee, Dr. Siddarthra Das and Dr. Salvador. I would especially like to thank the following people who have helped me during my graduate studies and facilitated my laboratory work: Brian Z. Alawneh, Erin J. Quintana, Laura Borunda, Thomas Guinto, Jeffrey Richards, and Dr. Saideh Mortazavi. Lastly, I would like to thank my family, specifically my parents Maria Mata and Antonio Rosales, and my siblings: Guadalupe, Elizabeth, and Jesus; and my friends: Veronica, Marisol, Jose, Joshua, Freda, and Tariqul for their encouragement. I also acknowledge the financial support of the UTEP Department of Chemistry and Biochemistry and the DMR PREM Program (NSF-1205302) during these master's studies.

Abstract

The hypothesis that deracemization of racemic ibuprofen (IBU) occurs via the enolization of a *Candida rugosa* lipase (CRL)-ibuprofen ester (Scheme 1.6.2) was disproven by reacting racemic IBU under the conditions that were used to deracemize ibuprofen methyl ester (IME), namely pH 7.6. At pH 7.6, IME through IBU butyl esters were not formed *in-situ* and racemic IBU was not deracemized by reacting the corresponding primary alcohol with racemic IBU in the presence of CRL.

At pH 7.6, hexyl through dodecyl IBU esters were formed *in-situ* by reacting the corresponding primary alcohol with racemic IBU in the presence of CRL. The longer chain alcohols reacted faster following the trend: hexanol, octanol, decanol, and dodecanol with values 9.0×10^{-4}, 2.3×10^{-3}, 3.9×10^{-3}, and 6.4×10^{-3} hr^{-1}, respectively.

Initially it was believed that the formation of ester corresponded with an increase of (*R*)-IBU but after isolating (*R*)-IBU and IBU dodecyl ester, in a corresponding 48 and 45 % respective yield, it was determined that the monitoring of the reactions by chiral HPLC was affected by the difficulties in recovering the ester. The esters formed are therefore probably only of (*S*)-IBU.

Molecular docking studies indicated that IBU through IBU propyl esters enter the hydrophobic pocket of CRL, isobutyl group first. However, longer chain IBU esters bind with the primary alkyl group in the hydrophobic pocket first.

In addition, lower concentrations of the substrates, not using 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, using the CRL supernatant versus the whole commercial enzyme, and reacting at pH 6.0 versus 7.6 all led to faster reactions of racemic IBU and dodecanol.

For the reaction of racemic IBU and *n*-butanol catalyzed by CRL, formation of ester was favored at pH 6.0 and hydrolysis of the same ester was favored at pH 7.6. The hydrolysis of larger chain IBU esters was much slower. The observed slower hydrolysis is due to the hydrophobic anchoring of the primary alkyl group of long chain IBU esters in the hydrophobic pocket of CRL.

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List of Abbreviations

BA - Binding Affinity

COX - Cyclooxygenase

CRL - *Candida Rugosa* Lipase

DKR - Dynamic Kinetic Resolution

DMF - N, N-Dimethylformamide

DMSO - Dimethylsulfoxide

E - Entantiomeric Ratio

GIT - Gastrointestinal Tract

IBU - Ibuprofen

IME - Ibuprofen Methyl Ester

KR - Kinetic Resolution

NSAID - Non-Steroidal Anti-Inflammatory Drugs

VR - Visual Representation

Chapter 1: Introduction

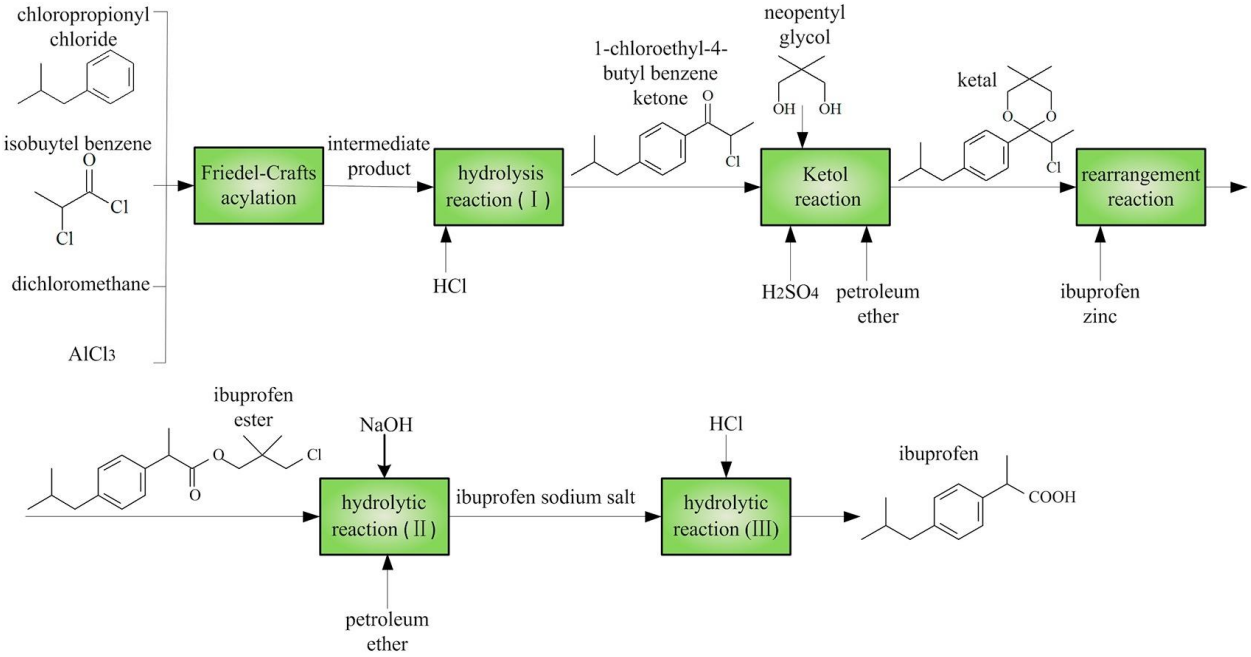
1.1 Enantiomers

Enantiomers are asymmetric (chiral) mirror-image compounds. Many pharmaceuticals are chiral but are sold as equal mixtures of enantiomers called a racemic mixture or racemate. Enantiomers have identical physical and chemical properties in symmetric environments, therefore, racemic mixtures are difficult to separate.

In asymmetric environments such as biological systems, enantiomers have different activities.[1,2] There are many examples where one enantiomer of a racemic pair exhibits the medicinal activity of a drug, while the other enantiomer lacks the activity or has harmful side effects.[3] An example is the sedative thalidomide, which was sold in many countries around the world from 1957-1961. Thalidomide was removed from retail when it was found to cause birth defects. (*R*)-thalidomide caused the sedative effects, while (*S*)-thalidomide caused birth defects.[4]

1.2 Ibuprofen

Ibuprofen (IBU) is one of the most common non-steroidal anti-inflammatory drugs (NSAID) consumed today for treatment of headaches, fever, muscle, and joint pain. It is part of a family of 2-arylpropionic acids or profens such as naproxen and ketoprofen. In the United States, it has long been sold as a racemic mixture. For example, the synthesis of racemic IBU using an aryl-1,2-translocation rearrangement was developed by Shandong Xinhua Pharmaceutical Company.[5] The six-step process is shown in Scheme 1.1.

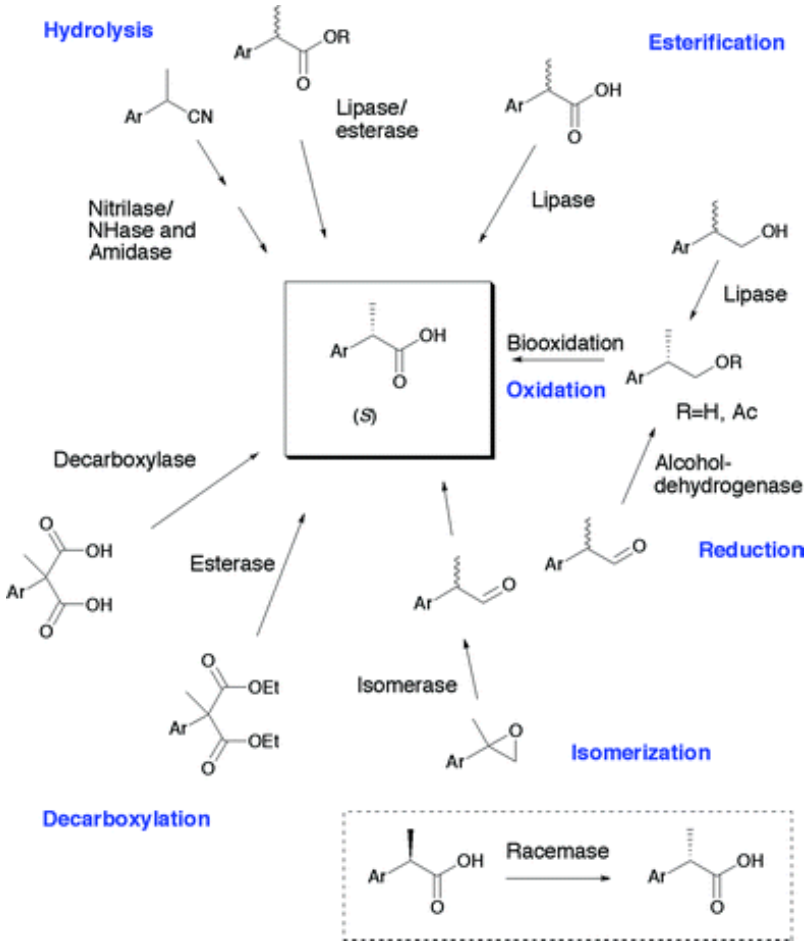


Scheme 1.1 The Flow Chart of Racemic IBU Synthesis using Aryl-1,2-Translocation Rearrangement[5]

Although this method of producing racemic IBU is effective at an industrial scale, the production of enantiopure IBU has been inadequate due to the difficulties of separating the enantiomers of a racemic mixture.[6] Therefore, the development of new separation methods for racemic IBU remains a large area of pharmaceutical research.[7] Efforts have been made to stereo-specifically and enzymatically-catalyze the resolution of IBU enantiomers.[8] In addition, enzyme catalysis offers a more environmentally benign synthesis with enantio-, chemo- or regioselectivities.[9] Subsequently, a number of enzymatic processes have been developed on an industrial scale.[10] In profen synthesis, substantial work has been demonstrated in the biocatalysis industry with different types of enzymes and reactions as shown in Scheme 1.2.

The function of NSAID's is hindering the cyclooxygenase (COX) pathway by the inhibition of prostaglandin synthesis.[11] The (*S*)-enantiomer of IBU is more effective at inhibiting COX than the (*R*)-enantiomer.[12] The (*R*)-enantiomer also produces undesired effects in the

gastrointestinal tract (GIT), lipid metabolism, and membrane function.[13] For these reasons, (*S*)-IBU has greater value than racemic IBU.



Scheme 1.2 Diverse Biocatalytic Reactions for the Separation of Profen Enantiomers[14]

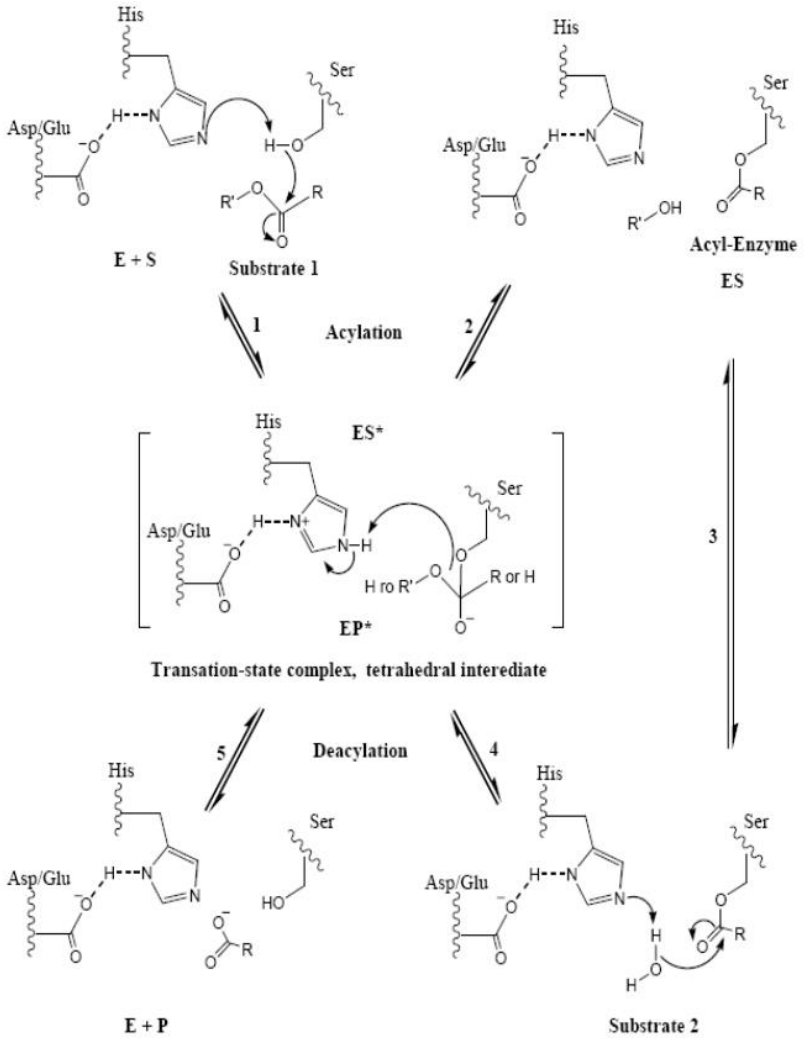
1.3 Lipase

Lipase are biocatalysts in organic synthesis that catalyze Kinetic Resolution (KR) reactions.[15] They are found in bacteria, fungi, plants and animals that function by hydrophobic/hydrophilic interfacial activation preceding to an increase in hydrolytic activity.[16] They hydrolyze triglycerides into fatty acids and glycerol. Their application ranges from detergents, foods, pulp/paper, and catalysts in industry.[17] They are widely used due to their commercial availability, low cost, not requiring a cofactor, catalysis in both aqueous and non-aqueous solvents, and recoverability.[18]

They also have broad substrate spectra, high activities and perform adequately in organic synthesis, including the resolution of racemic mixtures of compounds.[19] Among their advantages is their stereoselectivity in synthesis involving the resolution of racemates.[20] Numerous lipase reactions involve the asymmetrization of prochiral and meso-diols or the Dynamic Kinetic Resolution (DKR) of racemic primary and secondary alcohols via enzyme-catalyzed acyl transfer.[21] It has been observed that the enantio-preference of an enzyme is not reversed when an organic solvent substitutes water because, in terms of equilibrium, hydrolysis and esterification are reactions in opposite directions.[22]

Lipase have a common catalytic triad active site consisting of a serine, histidine, and aspartic/glutamic acid which assists serine to attack an acyl donor, hence, they can also be referred to as serine hydrolase.[23] The tandem deprotonation of the serine hydroxyl group by the imidazole of histidine in coordination with the aspartate/glutamate carboxyl groups is a charge relay network that makes the hydroxyl oxygen more nucleophilic.[24]

In addition, up to four hydrogen bonds with other enzyme residues increase the catalytic activity of the lipase. These hydrogen bonds are essential for diastereomeric discrimination of racemic substrates.[25] Thus, the enantiomeric preference of a lipase relies in the substrate's esterification/hydrolysis mechanism as shown in Scheme 1.3 as well as the entire shape of the enzyme pocket.

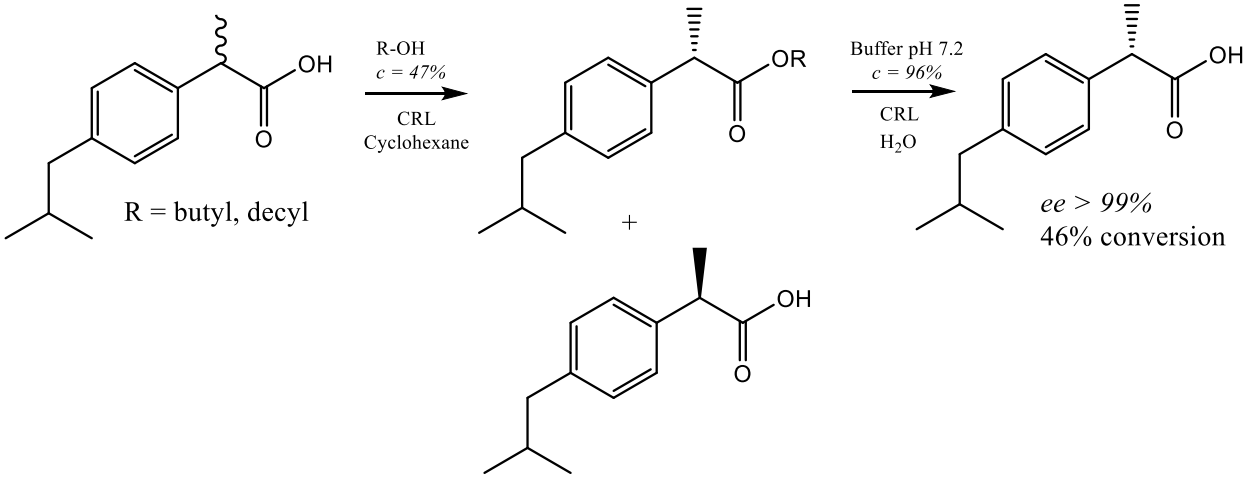


Scheme 1.3 Literature Mechanism of Lipase Catalyzed Esterification and Hydrolysis[26]

The mechanism shown in Scheme 1.3 is a two-step reaction which requires two incoming substrates. In the first step, serine is acylated with the carboxyl group of the acyl donor (**Substrate 1**) via enzyme substrate addition (**E + S**) and through a tetrahedral intermediate (**EP***) releases an alcohol or water molecule forming an acyl-enzyme (**ES**) complex. In the second step, deacylation of the **ES** complex is caused by an attack of a hydroxyl group in the nucleophile (**Substrate 2**), which can be water or an alcohol. The formation of a second **EP***, gives the free enzyme (**E**) and the acyl product (**P**). In KR reactions of alcohols, the second **EP*** is imperative because the alcohol replaces **Substrate 2** in the formation of the **EP*** complex.[26] Thus, the formation and collapse of the intermediate complex **ES** and **EP*** determines the stereoselectivity of lipase-catalysed hydrolysis.[27]

1.4 Kinetic Resolution (KR) of IBU Enantiomers

Since the separation of racemic mixtures is difficult, the discovery of enzyme catalyzed KR of enantiomers is a method of separating them.[28] For instance, *Candida rugosa* lipase (CRL) has been used to enantioselectively esterify racemic IBU[29] and hydrolyze racemic IBU esters.[30] From the work of Chavez et. al.[27], an enantioselective esterification of racemic IBU, followed by the separation of unreacted (*R*)-IBU from the (*S*)-IBU alkyl ester and subsequent hydrolysis of the (*S*)-IBU ester, successfully separated pure (*S*)-IBU in a 46 out of a 50 % possible yield from racemic IBU (Scheme 1.4).

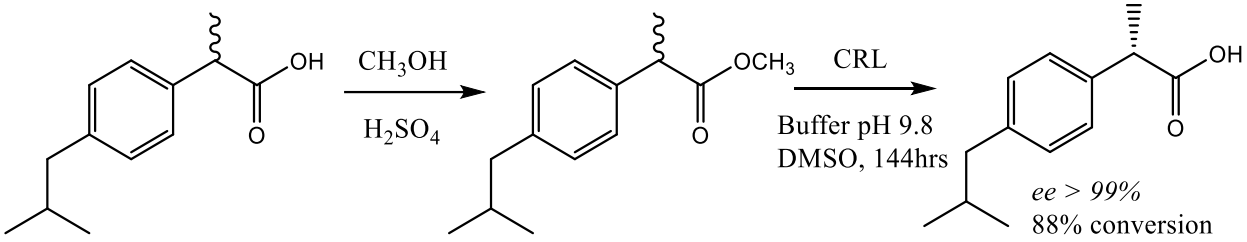


Scheme 1.4 KR of (*S*)-IBU via Enantioselective Esterification and Hydrolysis with CRL[27]

Following Le Chatelier's principle for these KR reactions, it was observed that esterification was preferred in a non-polar solvent (cyclohexane) whereas hydrolysis was preferred in a polar solvent (water). The Enantiomeric Ratio (*E*) was almost 3 times higher for the formation of the decyl versus butyl esters, i.e., 130 vs 46, respectively, which means that longer chains have higher selectivity for the formation of (*S*)-IBU esters. Overall, the resolution of the decyl ester was better as it allowed for a more facile separation of the (*S*)-IBU decyl ester from unreacted (*R*)-IBU. The only drawback of the decyl system was that (*S*)-IBU decyl ester took 96 hours to hydrolyze versus 48 hours in the butyl system.

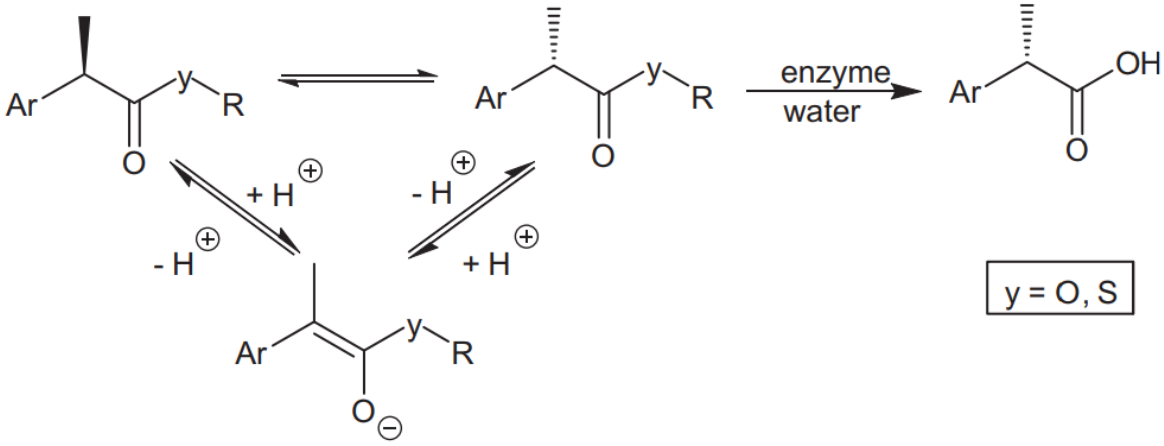
1.5 Dynamic Kinetic Resolution (DKR) of Racemic IBU

In the process of learning how to enzymatically resolve IBU enantiomers, Chavez et. al.[31] discovered that (*R*)-IBU can be converted to (*S*)-IBU in a two-step process (Scheme 1.5) and therefore eliminate the waste usually associated with only separating enantiomers. The first step was to form racemic IBU methyl ester (IME) by Fischer esterification followed by hydrolysis of this ester with CRL in a high pH buffer and co-solvent dimethylsulfoxide (DMSO) to help solubilize IME. The overall result was the isolation of 88 % pure (*S*)-IBU from racemic IBU.



Scheme 1.5 Conversion of Racemic IME to (*S*)-IBU[31]

The proposed mechanism for this DKR was the *in-situ* base catalyzed equilibration of IME enantiomers followed by the known preference for hydrolysis of (*S*)-IME catalyzed by CRL as outlined in Scheme 1.5.1.

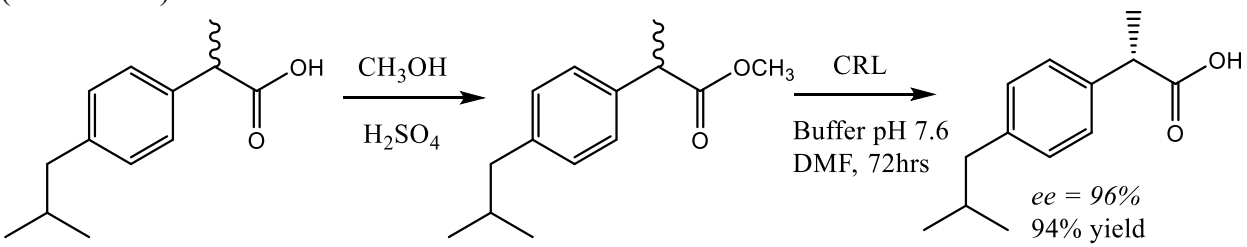


Scheme 1.5.1 First Proposed Mechanism of DKR of Racemic IME[31]

1.6 Isomerase Activity of CRL

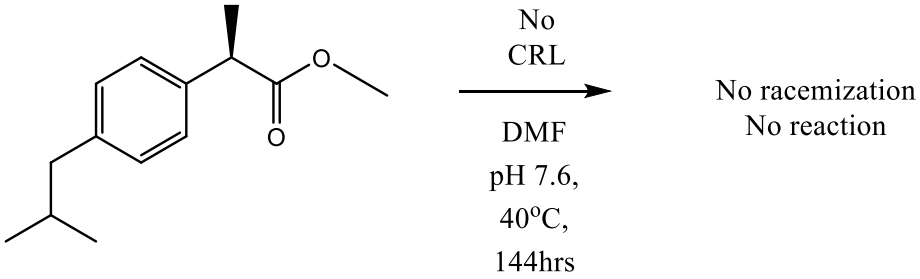
However, Mortazavi et. al.[8] observed that commercial CRL powder in lactose is acidic and therefore the optimal pH for the DKR of racemic IME is 7.6. In addition, N, N-dimethylformamide

(DMF) was found to be a better co-solvent than the DMSO co-solvent used in previous work (Scheme 1.6).



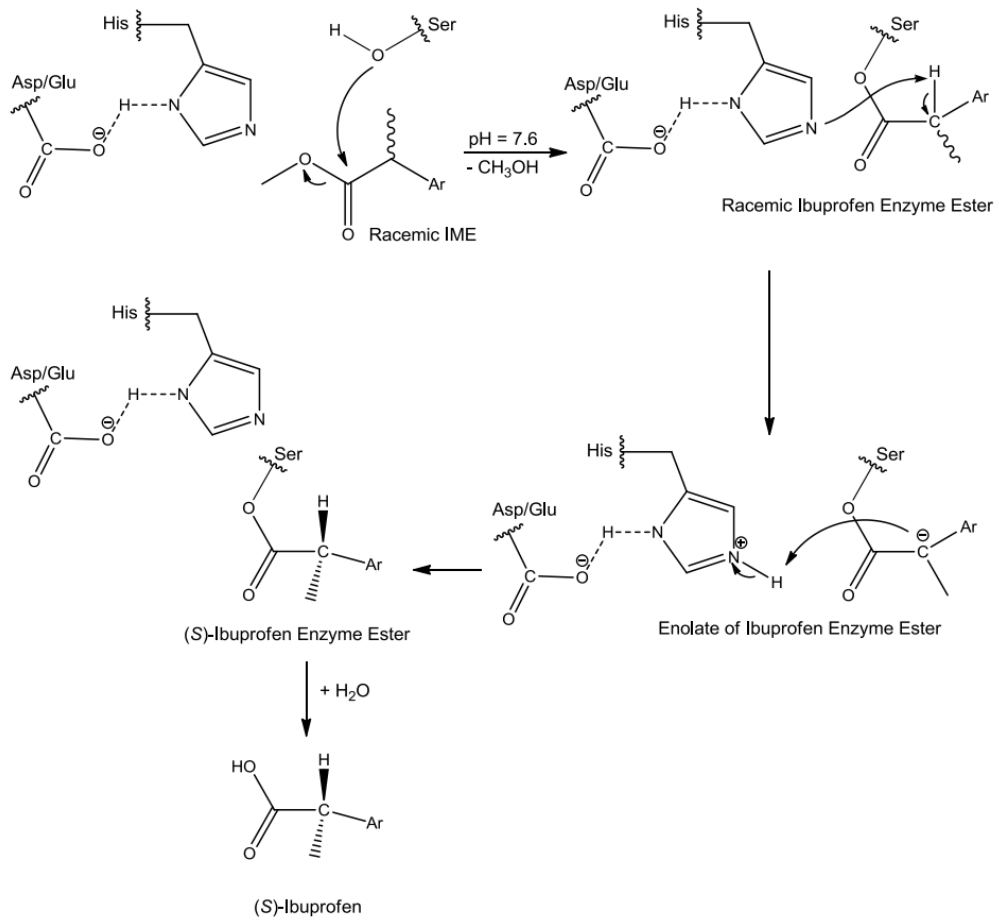
Scheme 1.6 Optimized Conversion of Racemic IME to (*S*)-IBU[8]

Because racemization of (*R*)-IME without CRL was not observed at pH 7.6 (Scheme 1.6.1), the previous enolization hypothesis was ruled out, and a new CRL isomerase mechanism was proposed.



Scheme 1.6.1 Attempted Racemization of (*R*)-IME without CRL[8]

Since the mechanism of the observed DKR of racemic IME requires CRL and an acyl-enzyme intermediate that was hypothesized to form at the active site of serine hydrolase for both enzyme catalyzed esterifications and hydrolysis reactions, a second hypothesis for how this reaction occurred was proposed (Scheme 1.6.2).



Scheme 1.6.2 Second Proposed Mechanism of DKR of Racemic IME[8]

The proposed mechanism begins with the formation of racemic-IBU enzyme ester, followed by deprotonation of the chiral center at the alpha position of the (*R*)-IBU enzyme ester by the nearby histidine (pKa 6.0) of the catalytic triad. Re-protonation of the enolate intermediate to form (*S*)-IBU enzyme ester and subsequent hydrolysis was used to explain the deracemization of racemic IBU to mostly (*S*)-IBU.

Because Scheme 1.3 indicates that the (*R*)-IBU enzyme ester could be formed without starting from racemic IME, the initial goal of this research was to determine if (*S*)-IBU could be synthesized by reacting racemic IBU with CRL at pH 7.6. If this occurred, the conversion of racemic IBU could be accomplished without having to synthesize its methyl ester, making the whole deracemization process more efficient.

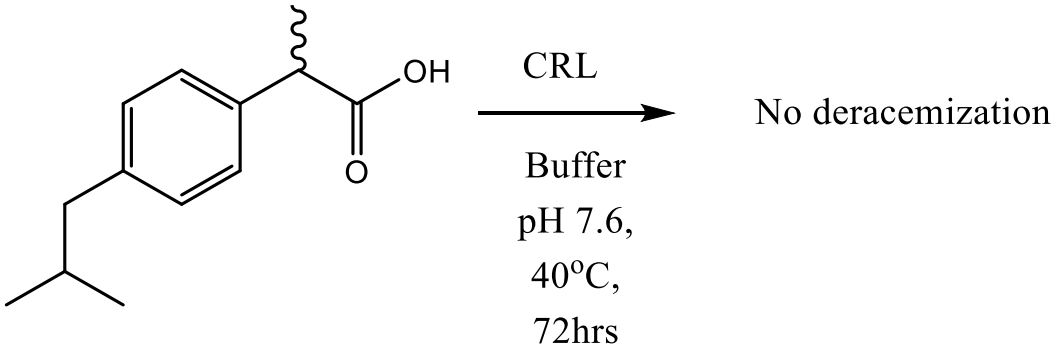
Chapter 2: Results and Discussion

2.1 Isolation of Racemic IBU from Commercial Tablets

Generic over the counter 200 mg IBU tablets purchased from Sam's Club were immersed in acetone to separate racemic IBU from the coating and fillers of the tablets. Once the tablets were swollen with acetone, IBU was released by piercing the tablets with a spatula. Vacuum filtration with a Buchner funnel was performed to separate the IBU acetone solution from the insoluble material. The IBU solution was then transferred to a round bottom flask and the acetone was evaporated under reduced pressure using a rotary evaporator to give a quantitative yield of racemic IBU (i.e., 4 g from 20 pills).

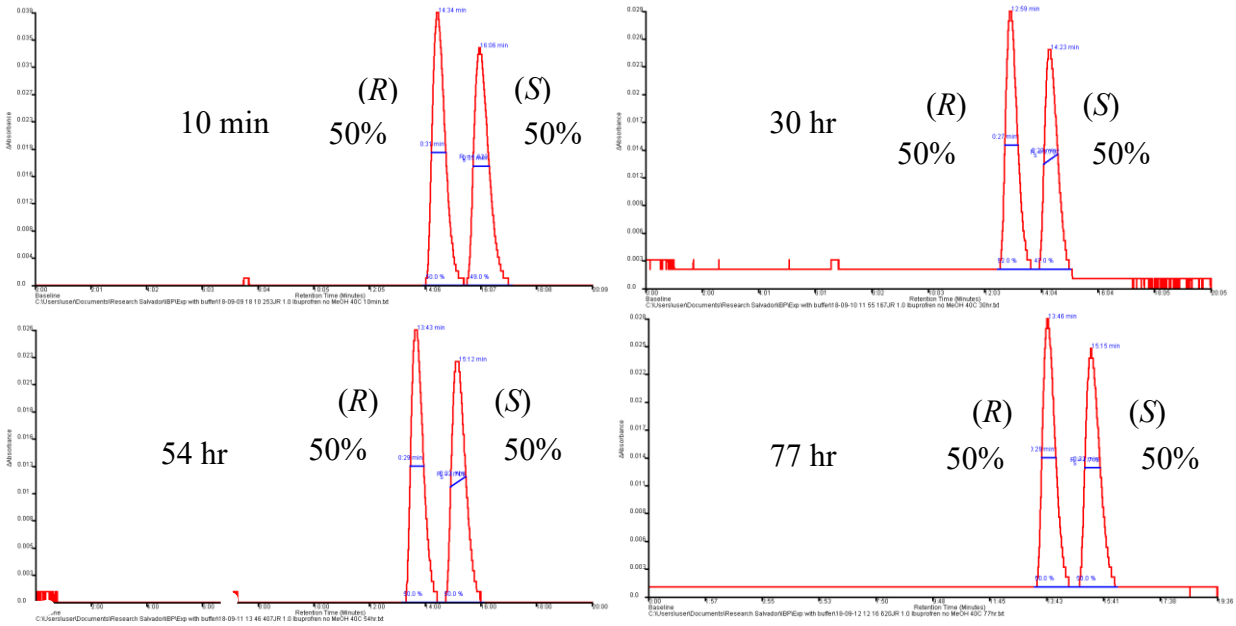
2.2 Reaction of Racemic IBU and CRL

To test if the mechanism postulated by Mortazavi et. al.[8] (Scheme 1.6.2) was valid, racemic IBU was reacted under the same conditions that were used to deracemize racemic IME (Scheme 2.1). DMF was omitted since racemic IME was not introduced in the reaction.



Scheme 2.1 Reaction of Racemic IBU with CRL and HEPES Buffer at pH 7.6

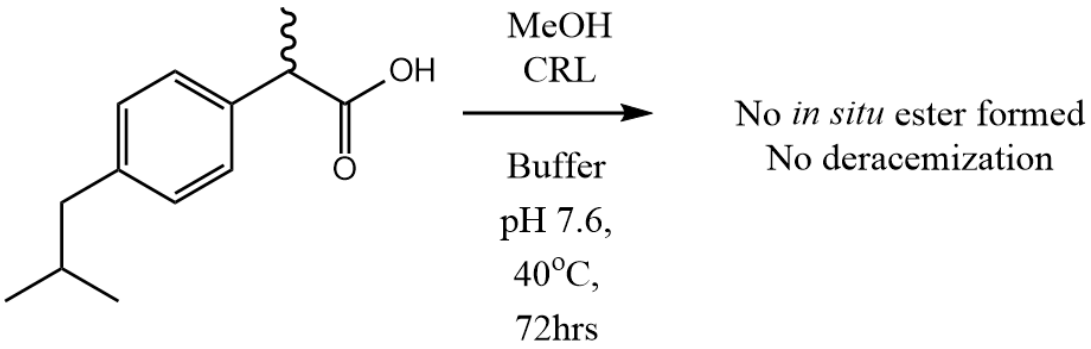
If deracemization occurred by enolizing an IBU-enzyme ester, then IME is not required as the enzyme ester is an intermediate in CRL mediated reactions (Scheme 1.3). However, no deracemization was detected by chiral phase HPLC (Chromatograms 2.1) when racemic IBU was reacted with CRL, thus implying that an ester *is* required to achieve deracemization.



Chromatograms 2.1 Reaction of Racemic IBU with CRL and HEPES Buffer at pH 7.6

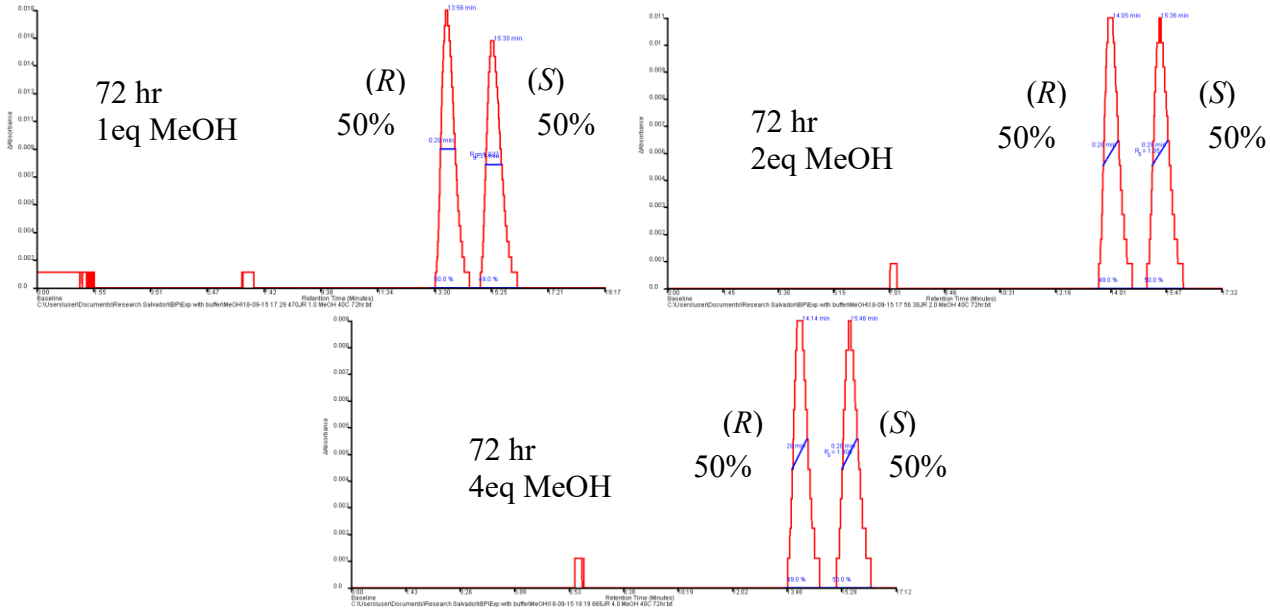
2.3 Reaction of Racemic IBU, Methanol, and CRL

Since CRL catalyzes the formation of esters as well as their hydrolysis, depending upon the solvent used,[32] and racemic IME can be converted to (*S*)-IBU with CRL under controlled conditions (Scheme 1.6), *in-situ* formation of IME was attempted to see if deracemization was possible without having to first perform a Fischer esterification. Racemic IBU and methanol in different ratios were reacted with CRL under the same conditions as racemic IME (Scheme 2.2).



Scheme 2.2 Reactions of Racemic IBU, Methanol, and CRL with HEPES Buffer at pH 7.6

Nevertheless, no esterification with methanol as a co-reactant or deracemization of racemic IBU was observed because Chromatograms 2.2 shows no ester peaks and a 50/50 mixture of IBU.



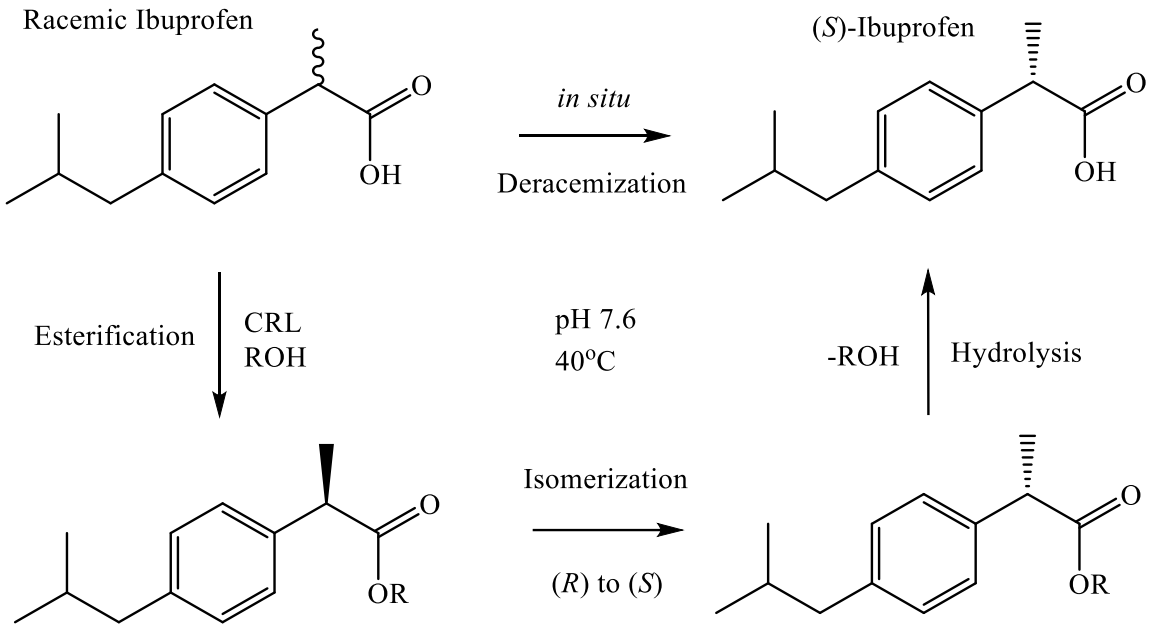
Chromatograms 2.2 Reactions of Racemic IBU, Methanol, and CRL

2.4 Reactions of CRL with Racemic IBU and Different Chain Length Primary Alcohols with HEPES Buffer at pH 7.6

2.4.1 Hypothesis of the Reactions of CRL with Racemic IBU and Different Chain Length Primary Alcohols with HEPES Buffer at pH 7.6

Since the native solvent of CRL is water and its initial use was to hydrolyze esters, non-polar solvents such as cyclohexane[33] have been used to drive the equilibrium towards forming esters of (*S*)-IBU with high enantioselectivity with water as a byproduct.

Although esterification of racemic IBU with methanol in water was not observed, it was hypothesized that because CRL has a hydrophobic pocket to bind long chain fatty acids, perhaps IBU esters could be formed *in-situ* with long chain primary alcohols in water and that an inversion of configuration from (*R*) to (*S*)-IBU ester followed by hydrolysis of (*S*)-IBU ester would produce (*S*)-IBU just as racemic IME was converted to (*S*)-IBU (Scheme 2.3).



R = ethyl, propyl, butyl, hexyl, octyl, decyl, dodecyl

Scheme 2.3 Hypothesized Deracemization of Racemic IBU with Different Chain Length Alcohols using CRL and HEPES Buffer at pH 7.6

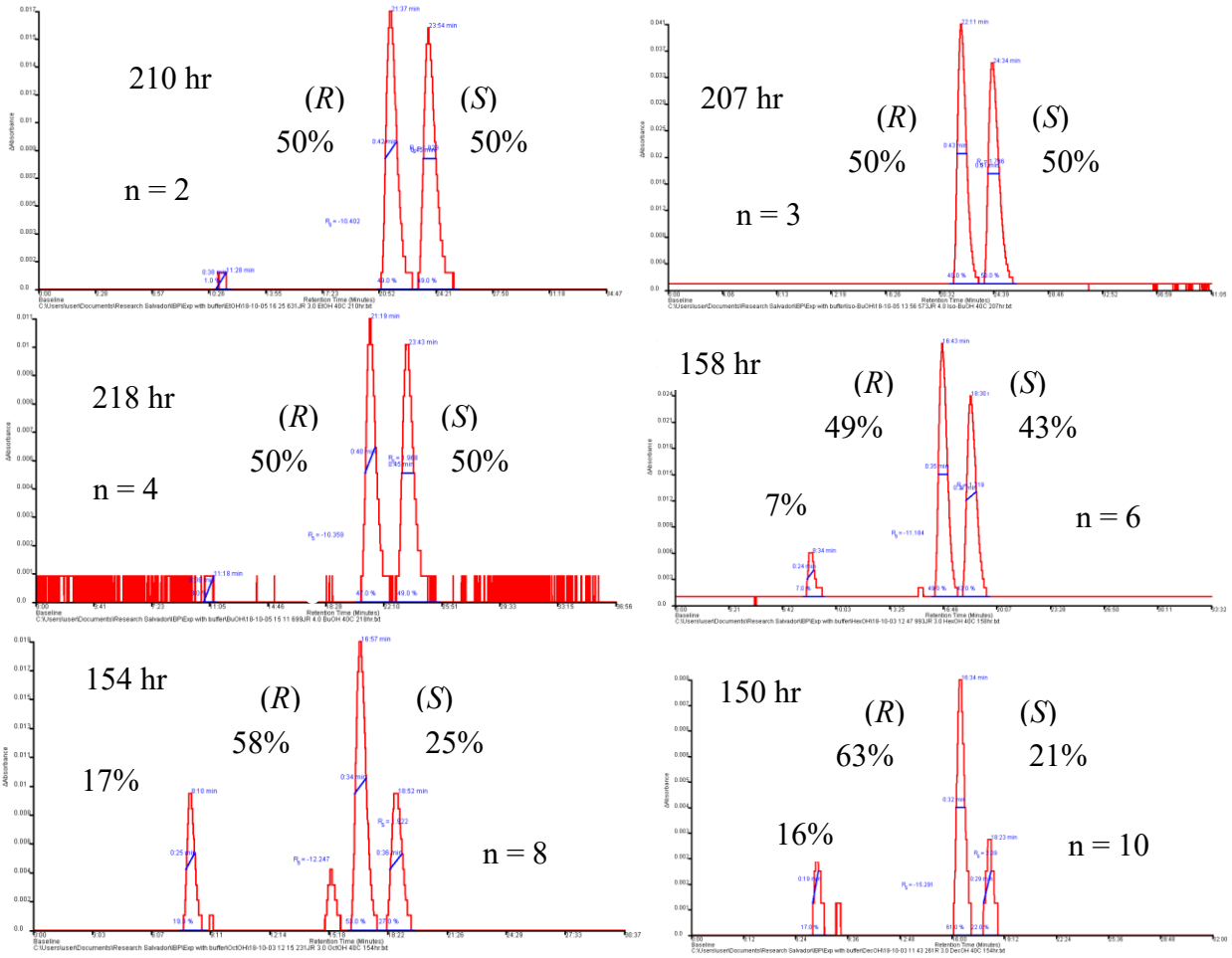
2.4.2 Analysis of Racemic IBU, Different Chain Length Alcohols, and CRL with HEPES Buffer at pH 7.6

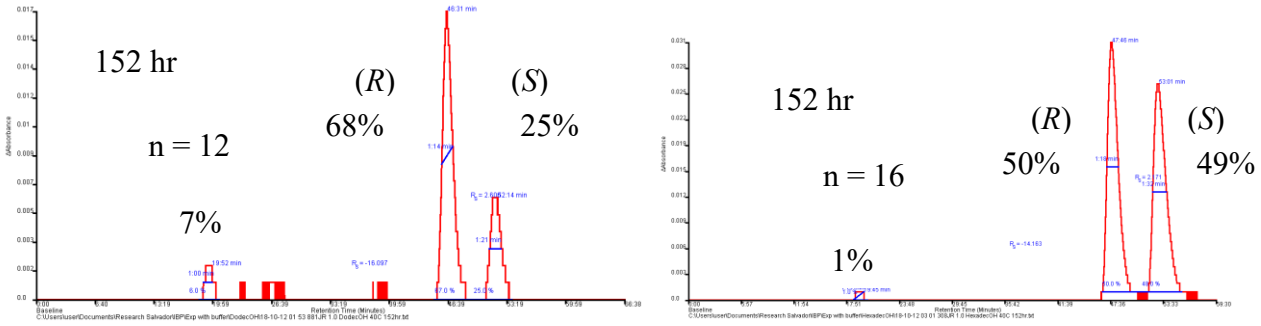
From the work of Mortazavi et. al.[8], these reactions were maintained at pH 7.6 using a HEPES buffer by adding drops of 1 M NaOH or HCl and were kept at a constant temperature of 40°C in a sand bath with a transformer while stirring. For the analysis of the IBU esters, aliquots of the reaction were acidified to a pH of ~ 5 using 1M HCl and then extracted with hexanes to be injected into a chiral column HPLC. The solvent used to carry the sample through the HPLC Chiracel OJ-column was 3 % isopropanol in hexanes. Injections were performed to separate (*R*) and (*S*)-enantiomers of IBU to gradually see a decrease of the (*S*)-IBU peak as an IBU ester was formed.

2.4.3 Results of Reactions of Racemic IBU, Different Chain Length Alcohols, and CRL with HEPES Buffer at pH 7.6

These reactions were performed to test whether increasing the chain length of the alcohol would alter the preference of ester formed *in-situ* and in the same reaction, hydrolysis of the preferred ester would lead to the formation of more than 50 % (*S*)-IBU.

However, ester formation was only observed for chain lengths 6, 8, 10, and 12 as peaks eluding before the IBU enantiomers. In these cases, the percentage of (*S*)-IBU always decreased with an apparent increase of (*R*)-IBU as the chain length increased, shown in Chromatograms 2.3.





Chromatograms 2.3 Deracemization of Racemic IBU to (*R*)-IBU with Different Chain Length Alcohols ($H(CH_2)nOH$) using CRL and HEPES Buffer at pH 7.6

From these chromatograms, the rate of deracemization was measured as a function of alcohol chain length with a particular interest in the difference in the rate of even numbered carbons. Also, by comparing the rate of esterification of each ester formed in terms of their chain length, their enantiomeric preference of ester formed in solution was determined. Although the equilibrium to form ester is unfavorable in water, chiral IBU esters were formed *in-situ* to allow deracemization to occur as was observed for racemic IME. These percentages can be plotted as (*R*)-IBU as a function of time (Chart 2.1) to determine the rate of esterification of (*S*)-IBU with increasing chain length (Chart 2.2).

Chart 2.1 Long Chain Length IBU Esters formed *in-situ* with CRL and HEPES Buffer at pH 7.6

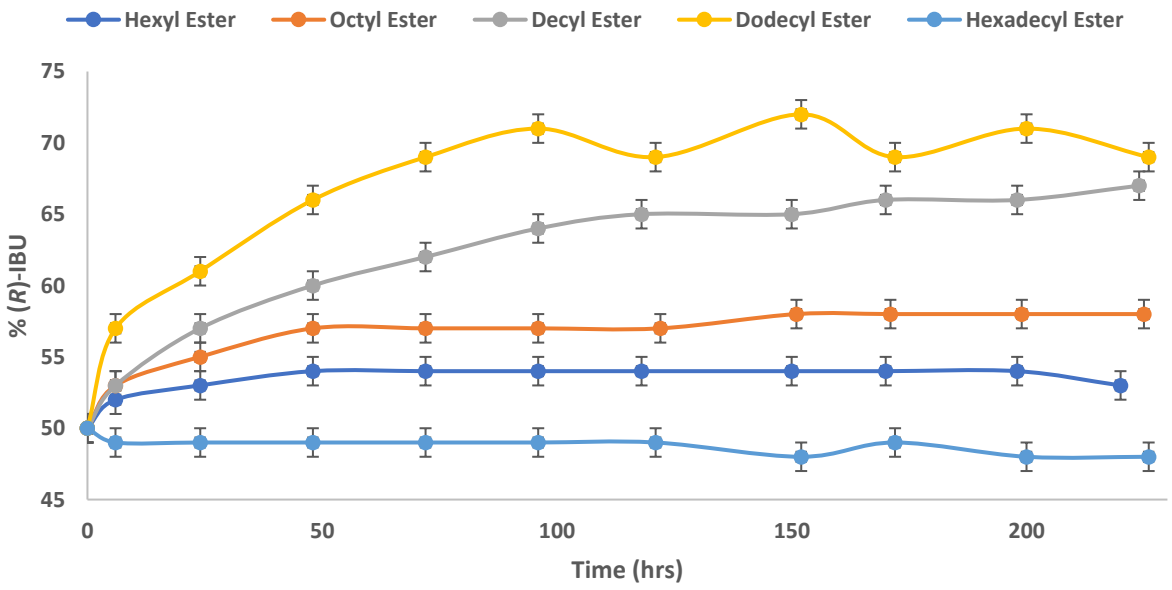
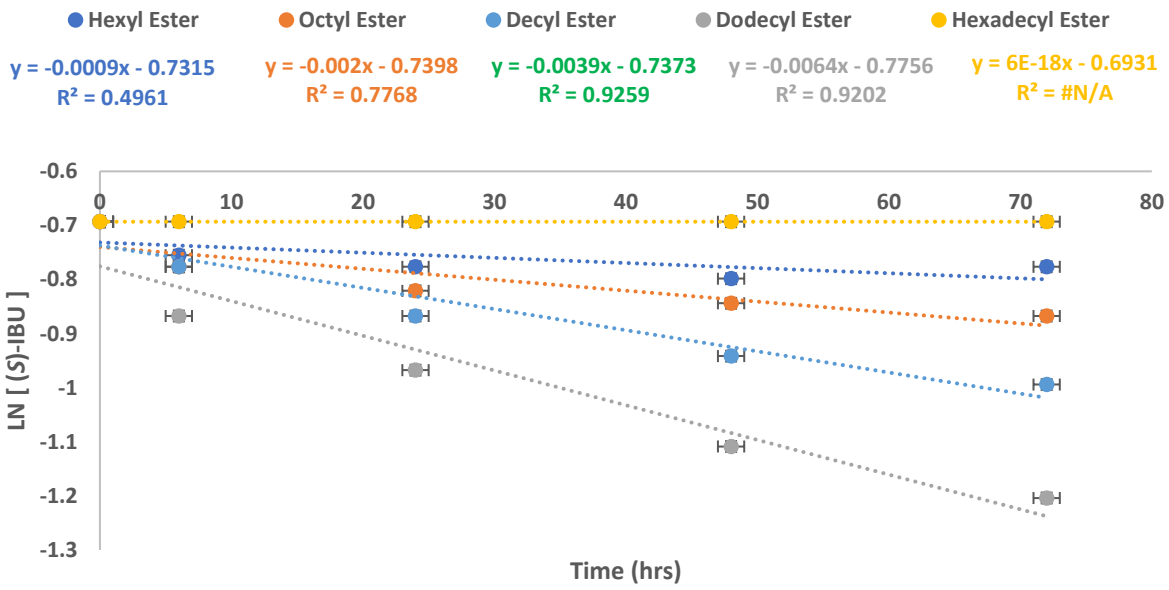


Chart 2.1 illustrates the percentages of (*R*)-IBU formed as a function of time obtained from HPLC chromatograms. Firstly, this chart indicates that shorter chain length alcohols (methanol to butanol) *do not* form observable amounts of IBU esters in water. Secondly, longer chain length even numbered alcohols (hexanol to dodecanol) *do* form observable amounts of IBU esters that elude before the IBU enantiomers in all HPLC chromatographs. Though tetradecanol (myristyl alcohol) was not available to test, hexadecanol (cetyl alcohol) did not seem to react at all because it was insoluble in water.

Other aspects to note were the apparent increase in % (*R*)-IBU with longer chain length alcohols, up to a point, and the observation that with longer chains, deracemization appeared to be favored in the opposite direction than deracemization of IME, (*S*)-IBU appeared to be converted to (*R*)-IBU.

Chart 2.2 Rates of Esterification of Long Chain Length IBU Esters formed *in-situ* with CRL and HEPES Buffer at pH 7.6



This is further evident in Chart 2.2 which shows the rate of esterification of (*S*)-IBU with different alcohols by plotting the natural logarithm of [(*S*)-IBU] versus time, assuming pseudo first-order kinetics, to obtain a first-order rate constant of each alcohol. These apparent rate constants depict that as the chain length of the alcohol increases, the rate of esterification increases

as well, following the trend: hexanol, octanol, decanol, and dodecanol with values 9.0 x 10^{-4}, 2.0 x 10^{-3}, 3.9 x 10^{-3}, and 6.4 x 10^{-3} hr^{-1}, respectively. Interestingly, dodecanol had the highest % (*R*)-IBU (~72%) and higher rate of esterification (6.4 x 10^{-3} hr^{-1}).

To understand the apparent reversal in enantioselectivity between IME and longer chain length IBU esters reacting with CRL, molecular docking studies were performed. Since a hydrophobic pocket is present in CRL's X-Ray crystal structure[34], a Visual Representation (VR) of each IBU ester ligand-binding site is shown in the following section.

2.5 Molecular Docking Calculations

Molecular docking is a method which predicts the preferred orientation of one molecule to another when bound to each other.[35] The preferred orientation is used to predict the Binding Affinity (BA) between the two molecules. In this case, AutoDock Vina[36] was used predict the conformation of IBU esters to the appropriate binding site of CRL.

AutoDock Vina uses an empirical scoring function that approximates the ligand binding free energy (ΔG). This docking function is based on electrostatic, Van der Waals, column energy, internal ligand strain, and other energetic parameters. The scoring function of ΔG (Equation 2.1) is calculated by six pair-wise evaluations (V) and an estimate of the conformational entropy lost upon binding. The energetic terms include evaluations for Van Der Waals dispersion/repulsion, hydrogen bonding, electrostatics, and solvation.[37]

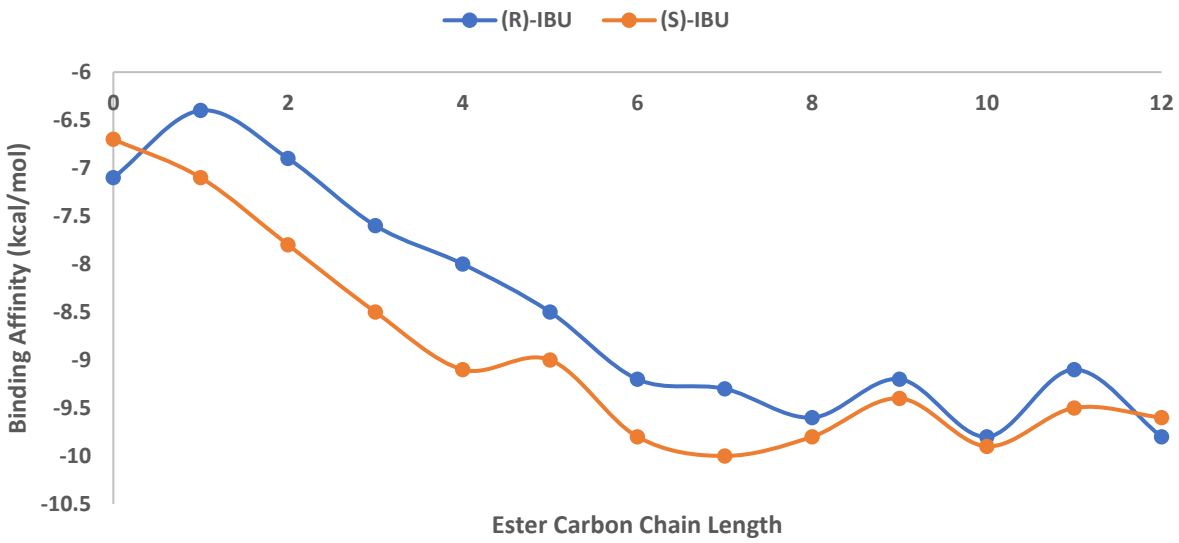
$$\Delta G = (V_{bound}^{L-L} - V_{unbound}^{L-L}) + (V_{bound}^{P-P} - V_{unbound}^{P-P}) + (V_{bound}^{P-L} - V_{unbound}^{P-L} + \Delta S_{conf})$$

$$V = W_{vdw}\sum_{i,j}\left(\frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^{6}}\right) + W_{hbond}\sum_{i,j}E(t)\left(\frac{C_{ij}}{r_{ij}^{12}} - \frac{D_{ij}}{r_{ij}^{10}}\right) + W_{elec}\sum_{i,j}\frac{q_i q_j}{e(r_{ij})r_{ij}} + W_{sol}\sum_{i,j}\left(S_i V_j + S_j V_i\right)e^{(-r_{ij}^2/2\sigma^2)}$$

Equation 2.1 Ligand Binding ΔG Calculated by 6 Pair-Wise Evaluations (V).[37]

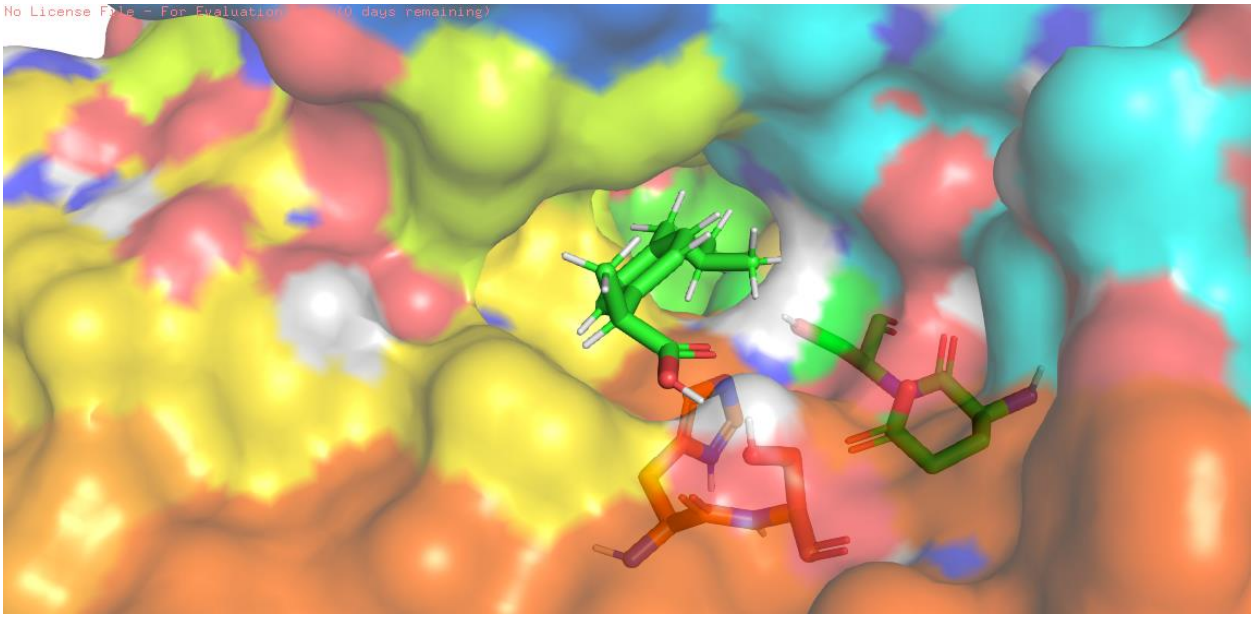
Molecular docking calculations were done for each IBU enantiomer and ester carbon chain length.

2.5.1 Binding Affinity (BA) Values of (*S*) and (*R*) Enantiomers of Different IBU Esters



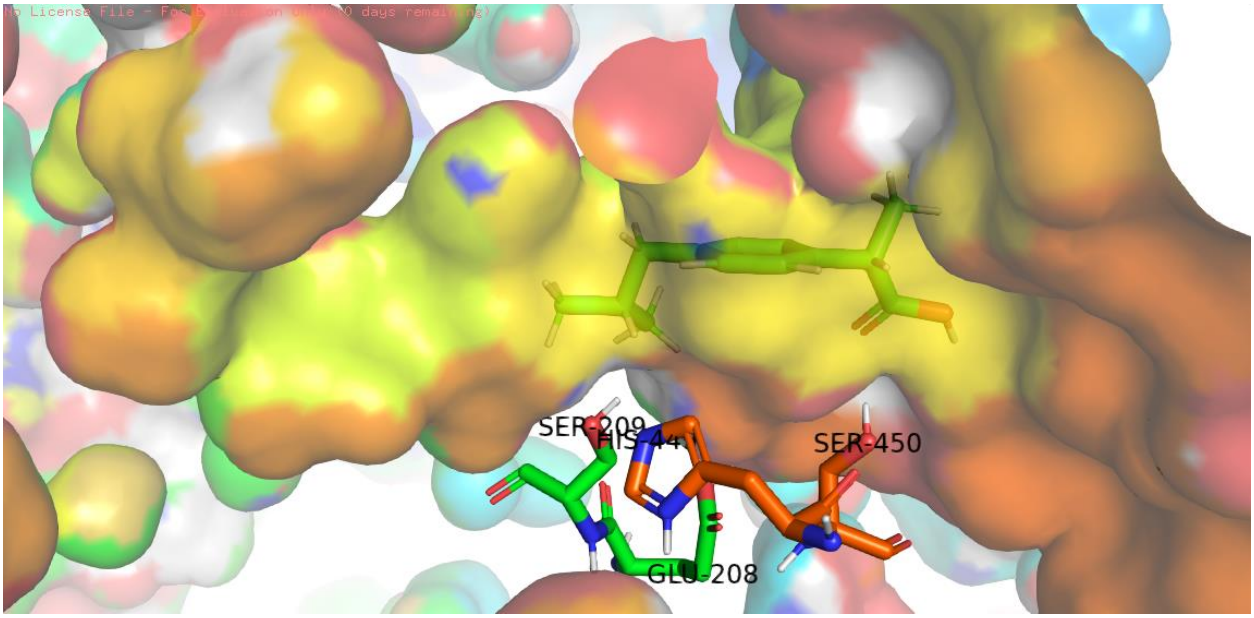
From the BA values of each IBU enantiomer and ester carbon chain length shown, as the ester carbon chain length increases, so does the BA. Also, the enantiomeric preference seems to favor (*S*)-IBU binding throughout 1-11 ester carbon chain lengths, however, the preference is for (*R*)-IBU binding over (*S*)-IBU and for (*R*)-IBU dodecyl ester binding over (*S*)-IBU dodecyl ester.

2.5.2 VR of (*S*)-IBU Ligand Binding to Catalytic Site of CRL (Front View)



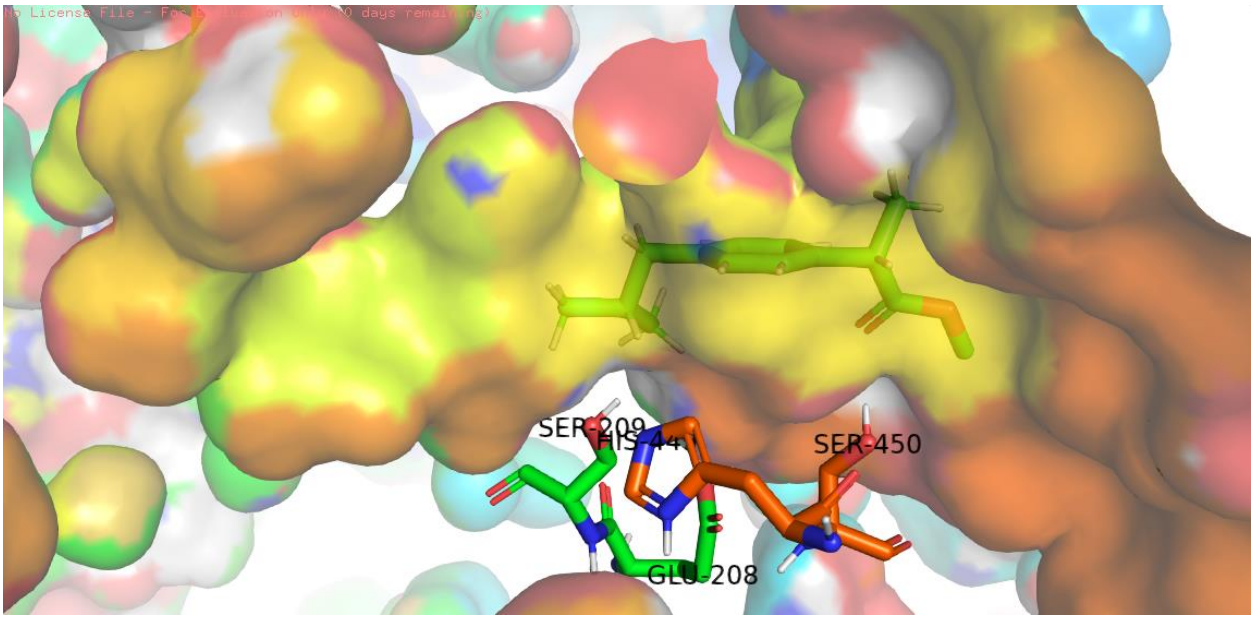
A front view of (*S*)-IBU bound to CRL is shown to demonstrate that the less-polar isobutyl group reaches inside the hydrophobic pocket instead of the more-polar *trans* propanoic acid group.

2.5.3 VR of (*S*)-IBU Ligand Binding to Catalytic Site of CRL (Side View)



A side view shows (*S*)-IBU oriented the same way in CRL with the hydrophobic pocket to the left and the active site on the right.

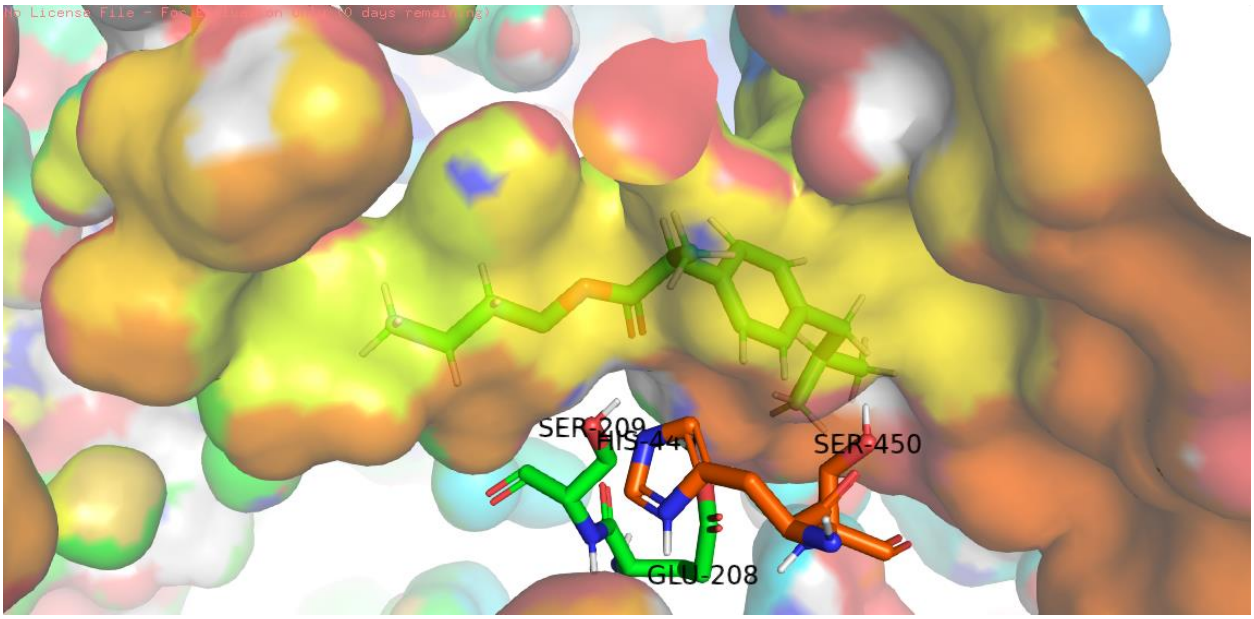
2.5.4 VR of (*S*)-IME Ligand Binding to Catalytic Site of CRL (Side View)



(*S*)-IME bound to CRL also prefers to bind the isobutyl group into the hydrophobic pocket (left side) even though the ester is less polar than the parent propanoic acid group of IBU. The

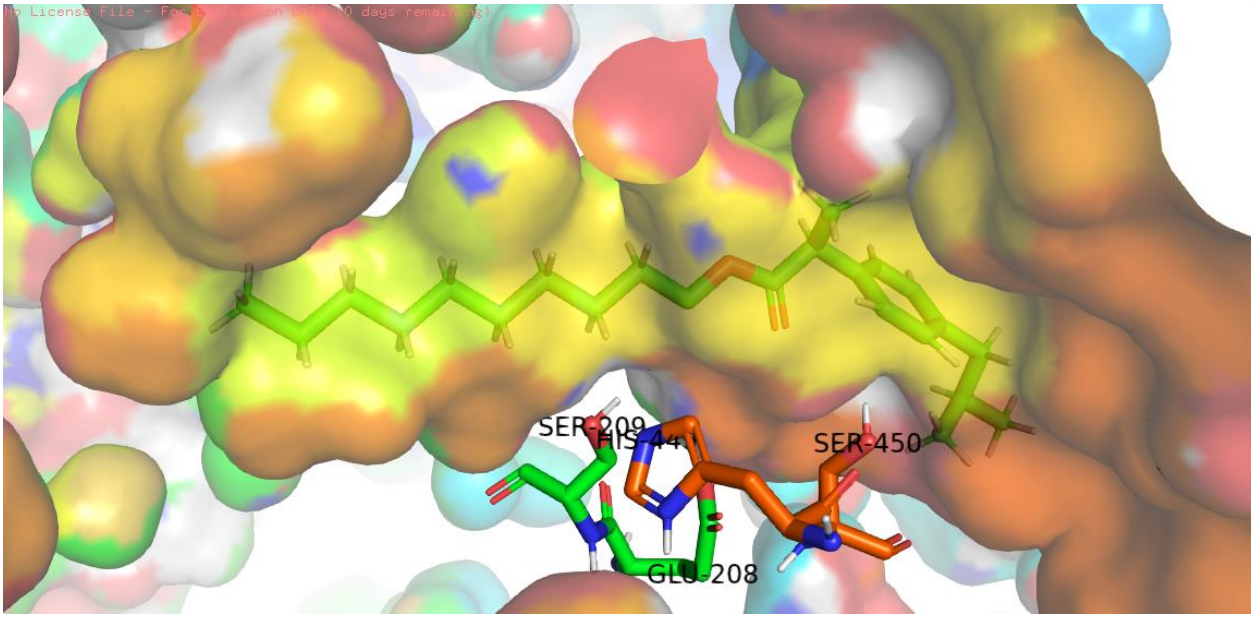
calculated BAs are in line with the reactivity that Chavez and Mortazavi et. al.[8,31] observed for the hydrolysis of (*S*)-IME over (*R*)-IME.

2.5.5 VR of (*S*)-IBU Butyl Ester Ligand Binding to Catalytic Site of CRL (Side View)



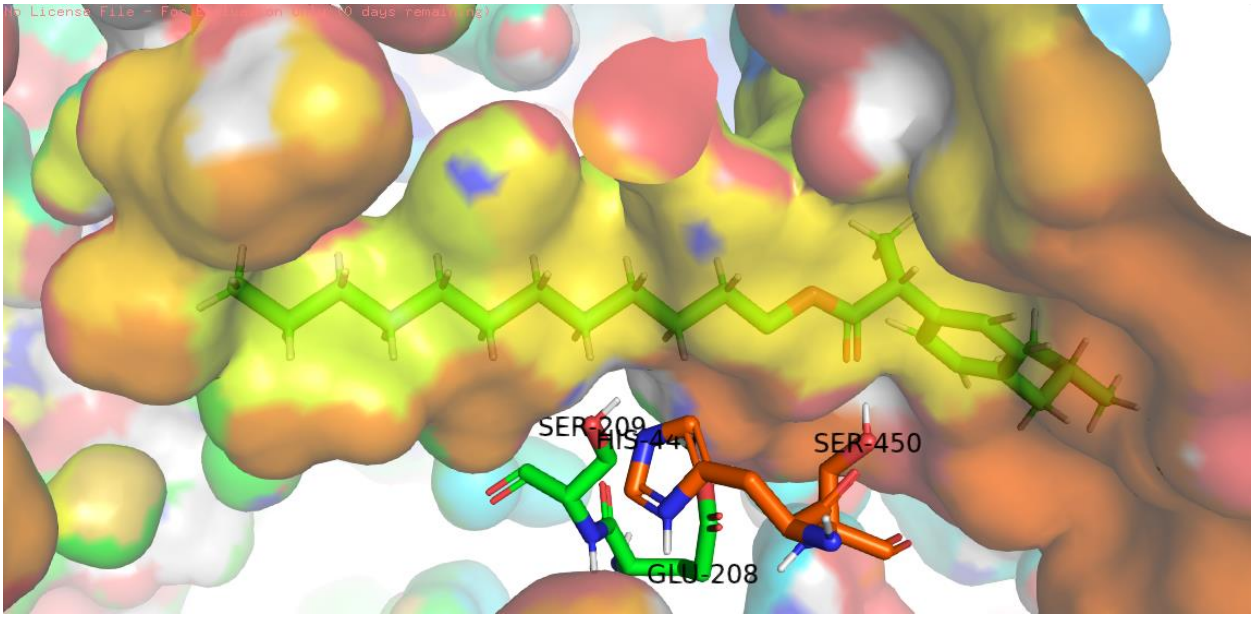
Once the propanoic acid is esterified with a longer chain length alcohol (e.g. butanol), the ester functional group prefers to bind with the hydrophobic pocket of CRL (towards the left side of the illustration). This is evident because the hydrophobic residues (yellow) is distributed throughout the channel and the ester is more tightly bound.

2.5.6 VR of (*S*)-IBU Decyl Ester Ligand Binding to Catalytic Site of CRL (Side View)



As the chain length gets longer (e.g. decyl ester), the ester becomes more tightly bound to the hydrophobic pocket of CRL as given by increasing BA values. Since the hydrophobic pocket is meant to bind fatty acids, the longer decyl group would slow down the hydrolysis of the IBU decyl ester since the orientation is reversed from the way fatty acids should enter the hydrophobic channel. Perhaps, the observed faster formation of longer chain IBU esters is really an indication of a slower rate of hydrolysis as evident by the work of Chavez et. al.[27] in observing that (*S*)-IBU decyl ester hydrolysis is slower than (*S*)-IBU butyl ester.

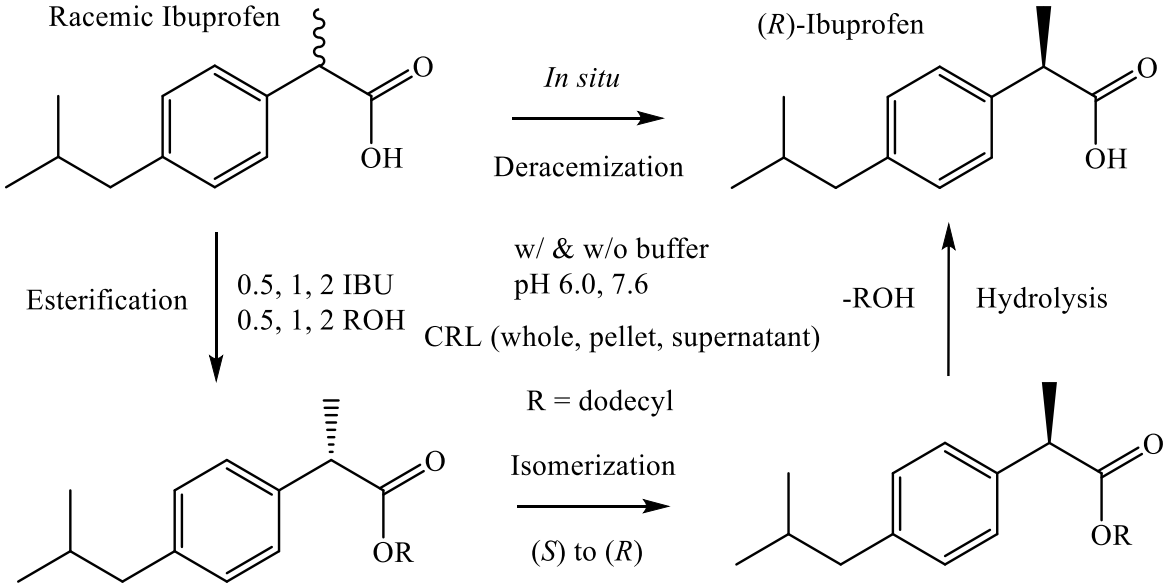
2.5.7 VR of (*R*)-IBU Dodecyl Ester Ligand Binding to Catalytic Site of CRL (Side View)



As expected, the dodecyl ester has the highest BA value due to its tight binding inside the hydrophobic pocket which would make hydrolysis even more difficult.

2.6 Esterification Reactions of Racemic IBU, Dodecanol, and CRL

Another attempt to probe the esterification mechanism was to react racemic IBU, dodecanol, and CRL using different equivalents of reactants, at a lower pH, and using different CRL components as shown in Scheme 2.4.



Scheme 2.4 *In-situ* Esterification of Racemic IBU with Dodecanol using Different Amounts of Reactants, Alternating pH Values, and CRL Components

The purpose of this study was to see how altering the amount of the reactants as well as the pH affected the conversion of (*S*)-IBU to (*R*)-IBU. These reactions were limited to dodecanol because this alcohol seemed to produce the most ester and (*R*)-IBU. Because commercial CRL is a mixture of enzymes[38] and lactose, separating its components by solubility was also studied. Once this was done, the aqueous soluble fraction reactions appeared to be more pH stable, therefore, reactions with and without HEPES buffer were also studied.

Because of the concern that the apparent increase in (*R*)-IBU may be due to separation of the hydrophobic IBU dodecyl ester, which is not accounted for in the HPLC analysis, from this point on of the research, the reactions were sampled while they were still stirring before the aliquots were acidified.

2.6.1 Reactions of Dodecyl IBU Ester formed *in-situ* with CRL by Varying the Amounts of IBU and Dodecanol using HEPES Buffer at pH 7.6

From Chart 2.3, when the amount of racemic IBU is increased but the amount of dodecanol is kept constant, the % of (*R*)-IBU produced is less. Similarly, in Chart 2.3.1, when the amount of dodecanol is increased but the amount of racemic IBU is kept constant, the % of (*R*)-IBU produced is less. These trends may indicate that as the racemic IBU or dodecanol concentrations increase, (*R*)-IBU, dodecanol, or IBU dodecyl ester may be inhibiting the overall reaction. From Charts 2.4 and 2.4.1, a decrease in the rate of esterification of (*S*)-IBU is more pronounced by varying the amount of racemic IBU.

Chart 2.3 IBU Dodecyl Ester formed *in-situ* with CRL by Varying the Amounts of IBU

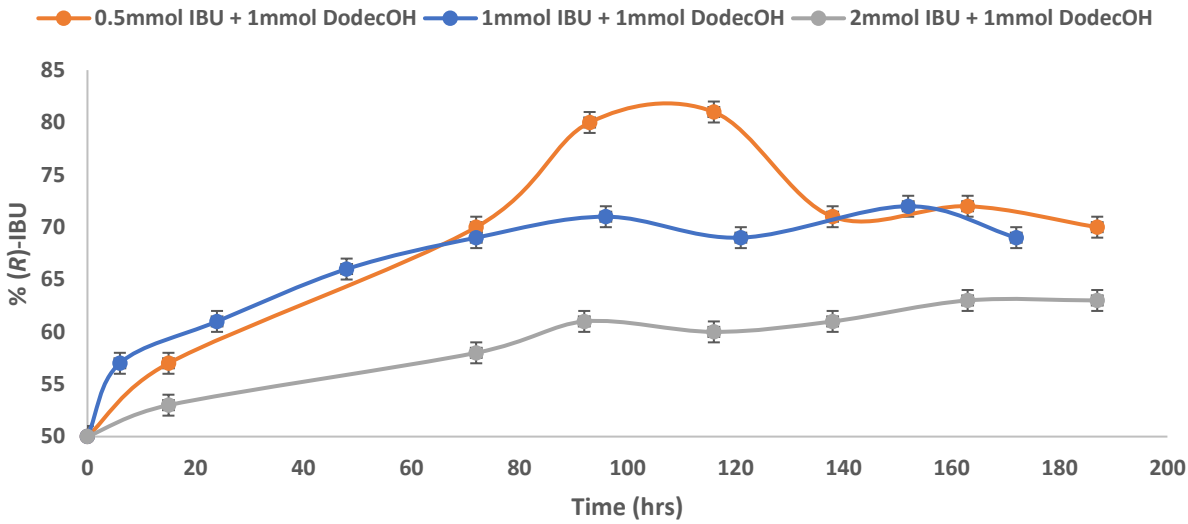


Chart 2.3.1 IBU Dodecyl Ester formed *in-situ* with CRL by Varying the Amounts of Dodecanol

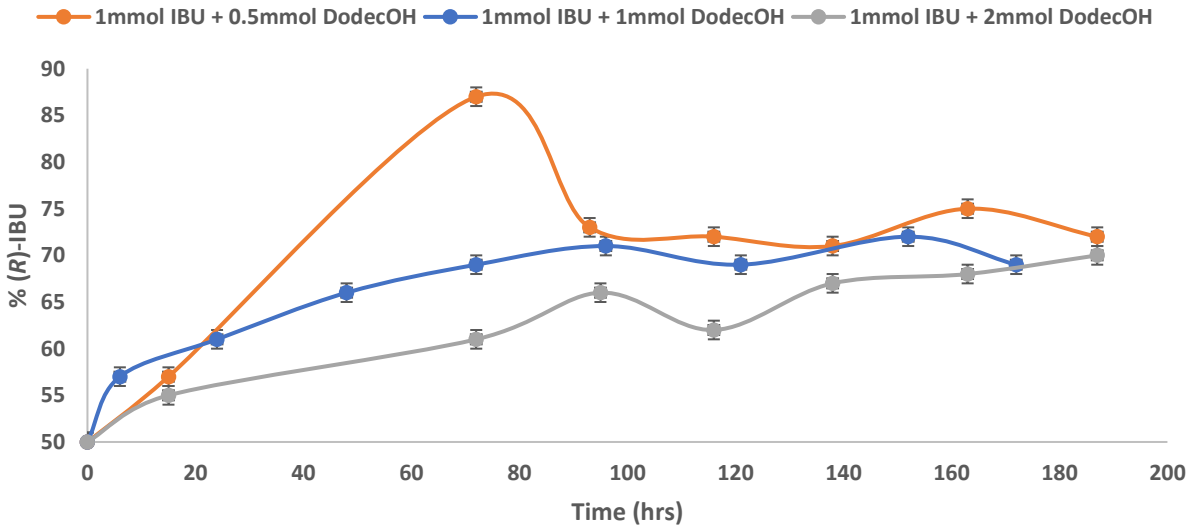


Chart 2.4 Rates of Esterification of IBU Dodecyl Ester formed *in-situ* with CRL by Varying the Amounts of IBU

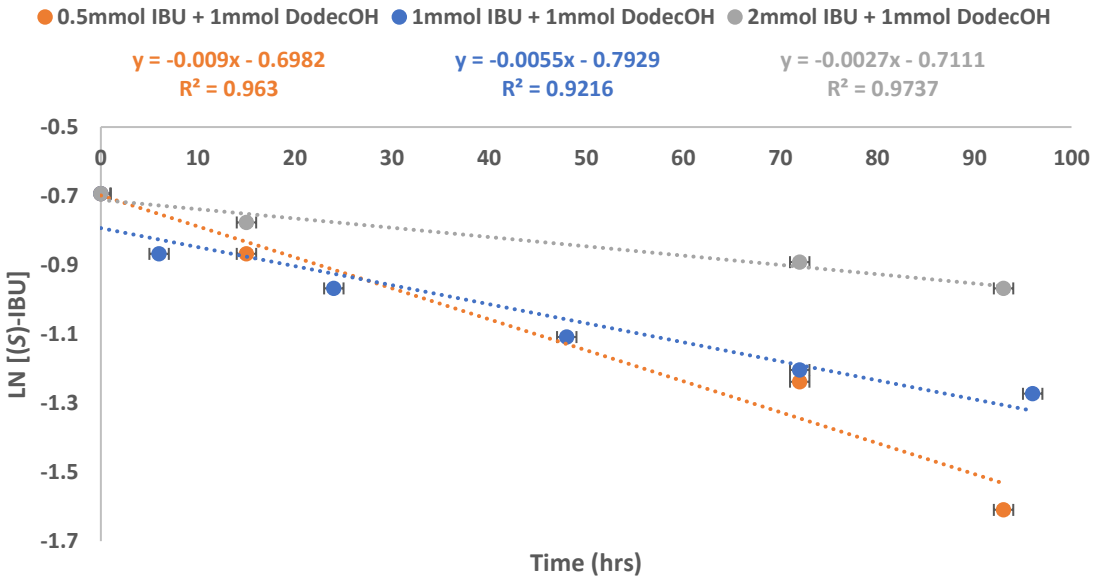
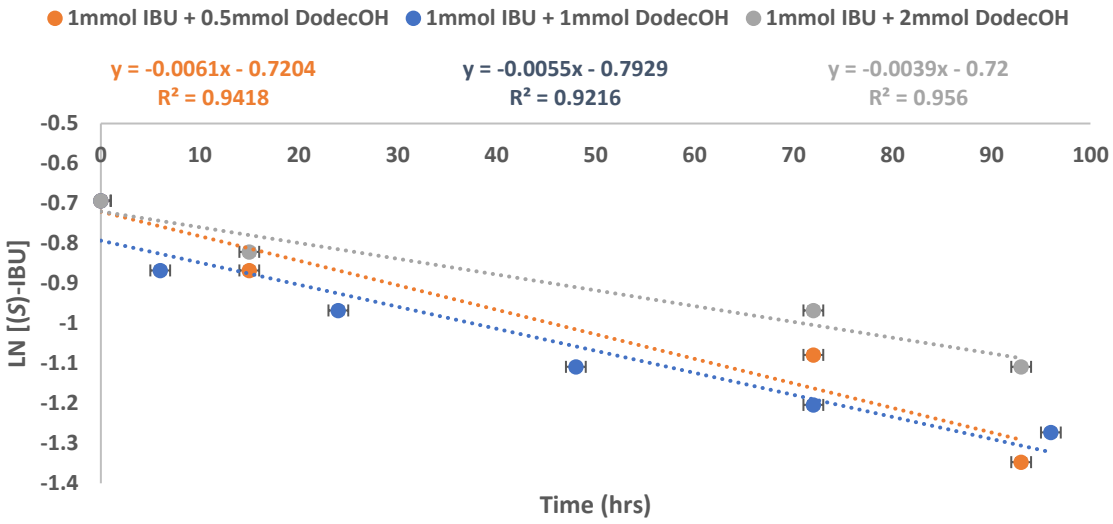


Chart 2.4.1 Rates of Esterification of IBU Dodecyl Ester formed *in-situ* with CRL by Varying the Amounts of Dodecanol



2.6.2 Reactions of IBU Dodecyl Ester formed *in-situ* with Different Components of CRL and HEPES Buffer at pH 7.6

Observing that commercial CRL does not completely dissolve in water, the majority of the insolubles were separated as a pellet by centrifugation. The supernatant was further filtered

through GeneJET Spin Columns & Collection Tubes in a centrifuge. The activity of the whole commercial CRL, the pellets and the supernatant were measured as shown (Charts 2.5 and 2.5.1.)

Chart 2.5 IBU Dodecyl Ester formed *in-situ* with Different Components of CRL

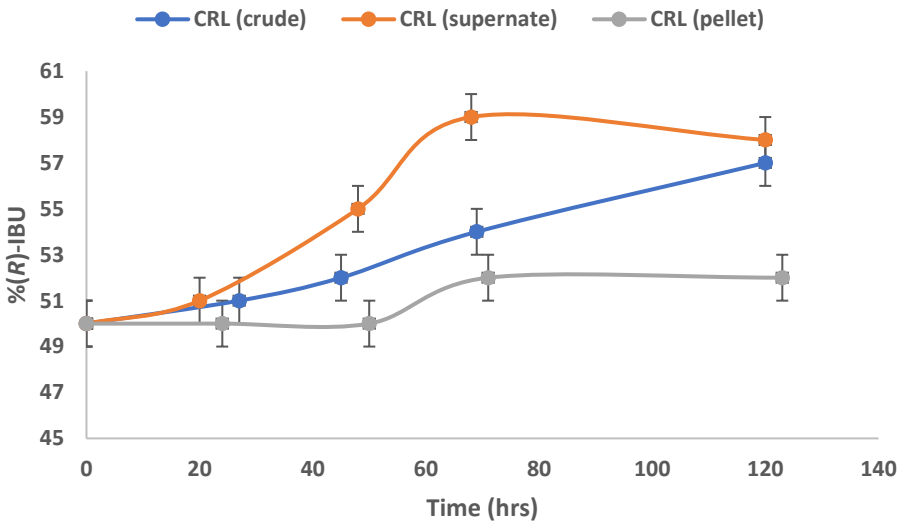
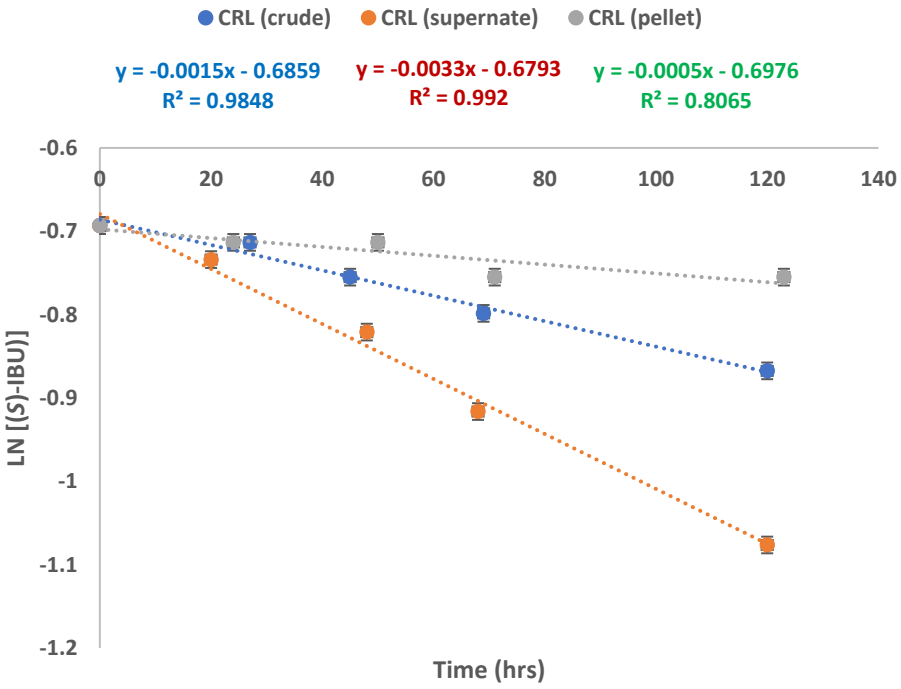


Chart 2.5.1 Rates of Esterification of IBU Dodecyl Ester formed *in-situ* with Different Components of CRL



Interestingly, the rate of esterification was greatest for the supernatant and the least for the material in the solid pellet, although for longer times the amount of excess (*R*)-IBU went down. With this observation, all further reactions were performed only with the supernatant extract. In addition, it was observed that not as much 1 M NaOH or HCl was needed to maintain the pH of the reactions at 7.6. In fact, in most cases the pH of the original CRL reaction had to be treated with HCl between measurements.

2.6.3 Reactions of IBU Dodecyl Ester formed *in-situ* with CRL at pH 6 without HEPES Buffer.

To test whether the esterification of (*S*)-IBU involves the isomerization to (*R*)-IBU dodecyl ester and subsequent hydrolysis to (*R*)-IBU, the reaction of racemic IBU and dodecanol with CRL was performed at pH 6 rather than 7.6. To do this, the HEPES buffer was omitted, which was now possible because the pH did not appear to change as much using only the CRL supernatant. A lower pH was not tested because IBU separates from water at pH 5 and below, which the basis of the sampling of the reactions is outlined in section 2.4. A higher pH was not tested because in aqueous organic medium, at neutral or basic pH, CRL probably assumes a less flexible conformation[39].

The first observation (Chart 2.6) was that the reaction at pH 7.6 without HEPES buffer was 9.6 times faster than with the buffer present. Second, the reaction at pH 6 was 4.3 times faster than the reaction at pH 7.6 leading to a much more apparent formation of (*R*)-IBU at 6 hours as shown in Chart 2.6.1.

Chart 2.6 IBU Dodecyl Ester formed *in-situ* with CRL by Varying the pH Values

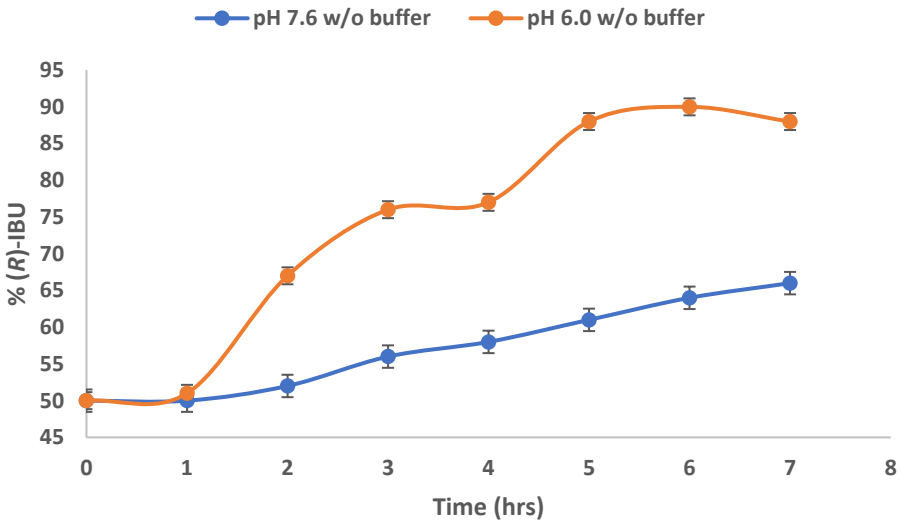
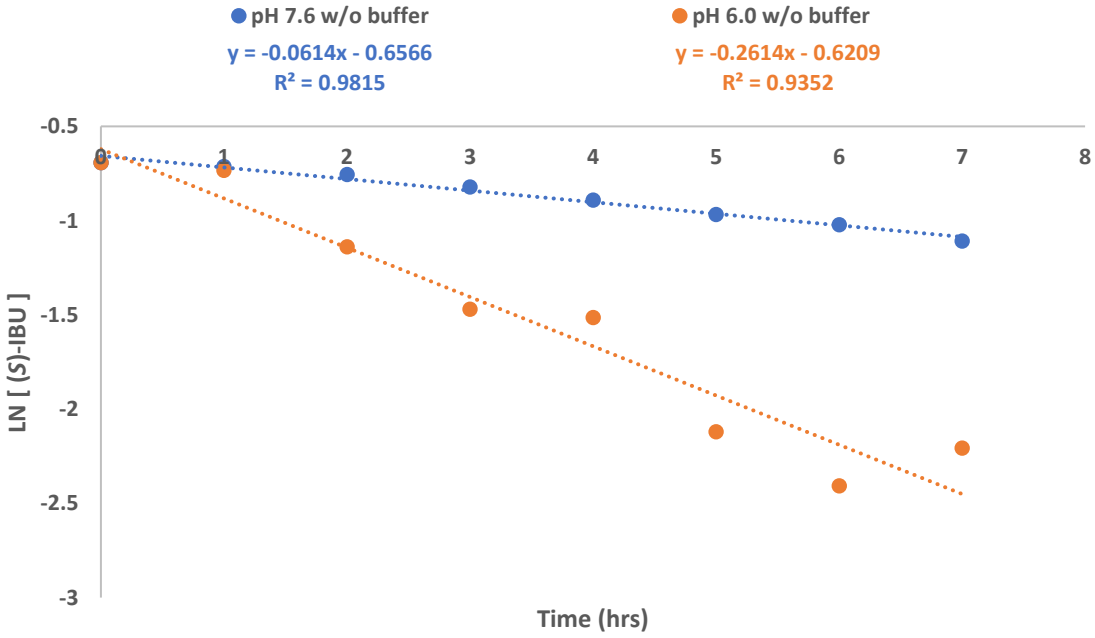


Chart 2.6.1 Rates of Esterification of IBU Dodecyl Ester formed *in-situ* with CRL by Varying the pH Values



To test whether the apparent increase in (*R*)-IBU, as monitored by chiral HPLC, from the reaction of racemic IBU with dodecanol and CRL supernatant at pH 6.0 was occurring, it was decided that the products, ester and IBU, of these reactions needed to be isolated.

2.7 Results of the Isolation from the Reactions of Racemic IBU, Dodecanol, and CRL without HEPES Buffer at pH 6.0

After the reactions were carried out at a mmol scale (0.206g), the whole mixture was acidified to a pH of 5.0 and extracted with hexanes. There was a concern that not all the ester was extracted because there was an intractable film of greasy material (dodecanol) on all the glassware. Nevertheless, the hexanes extract was evaporated, and the remaining oil was subjected to radial chromatography, initially eluding the ester with hexanes and then using 15% ethyl acetate/hexanes to isolate IBU. The identity of the components was confirmed by chiral HPLC. The chromatography fractions were evaporated to give a 45% yield (0.168g, 0.45mmol) of ester and a 48% yield (0.098g, 0.48mmol) of (*R*)-IBU.

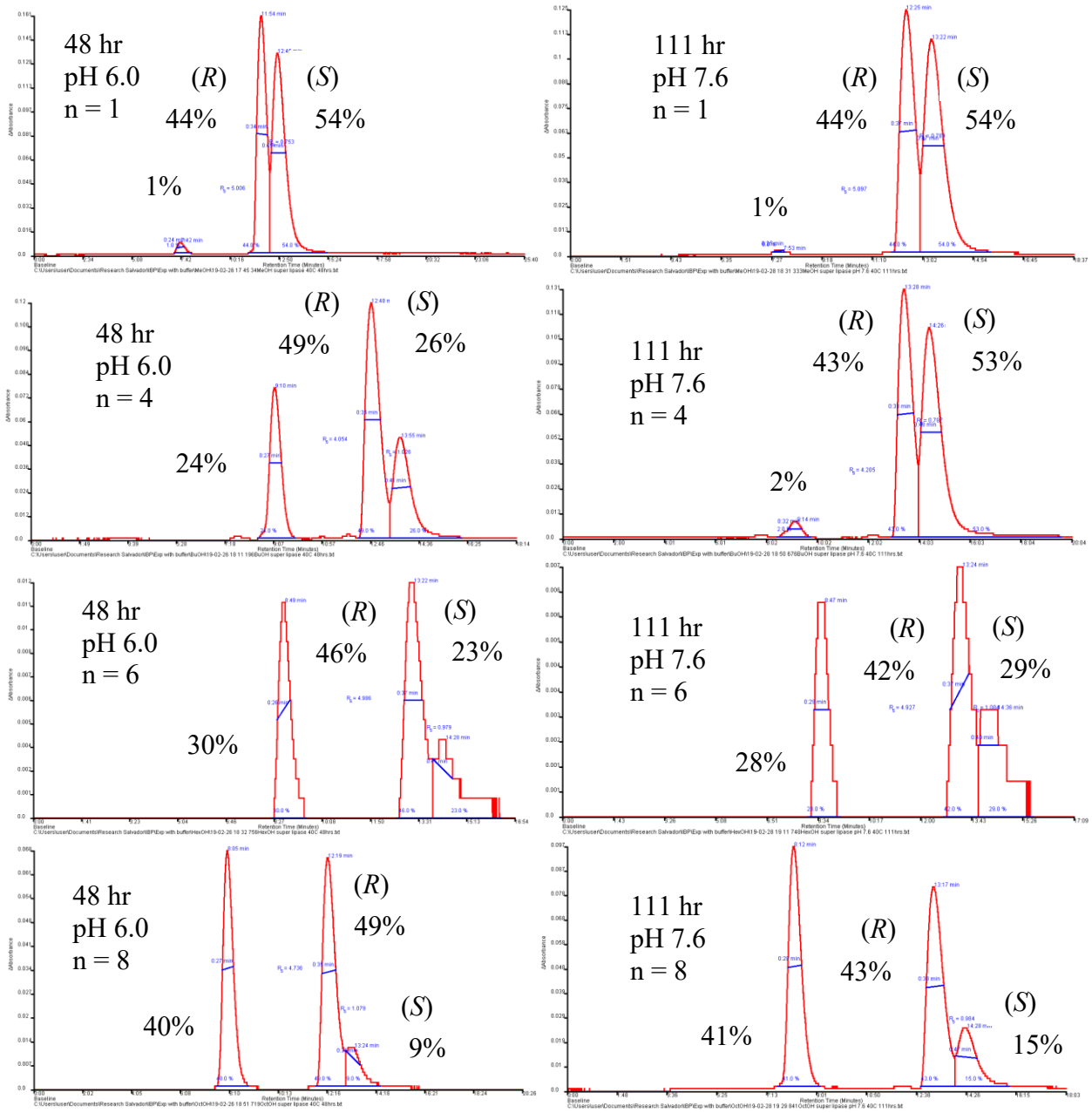
From the % yields of the isolated (*R*)-IBU, the sampling of the previous reactions was in-accurate, probably because the IBU dodecyl ester separated from the reactions had adhered to the glassware. Also, by gathering the aliquots from the surface or the stirring reactions, it did not increase its recovery. In addition, isolating close to 100 % of the theoretical (*R*)-IBU yield indicates that no deracemization was occurring and that the ester formed was that of (*S*)-IBU.

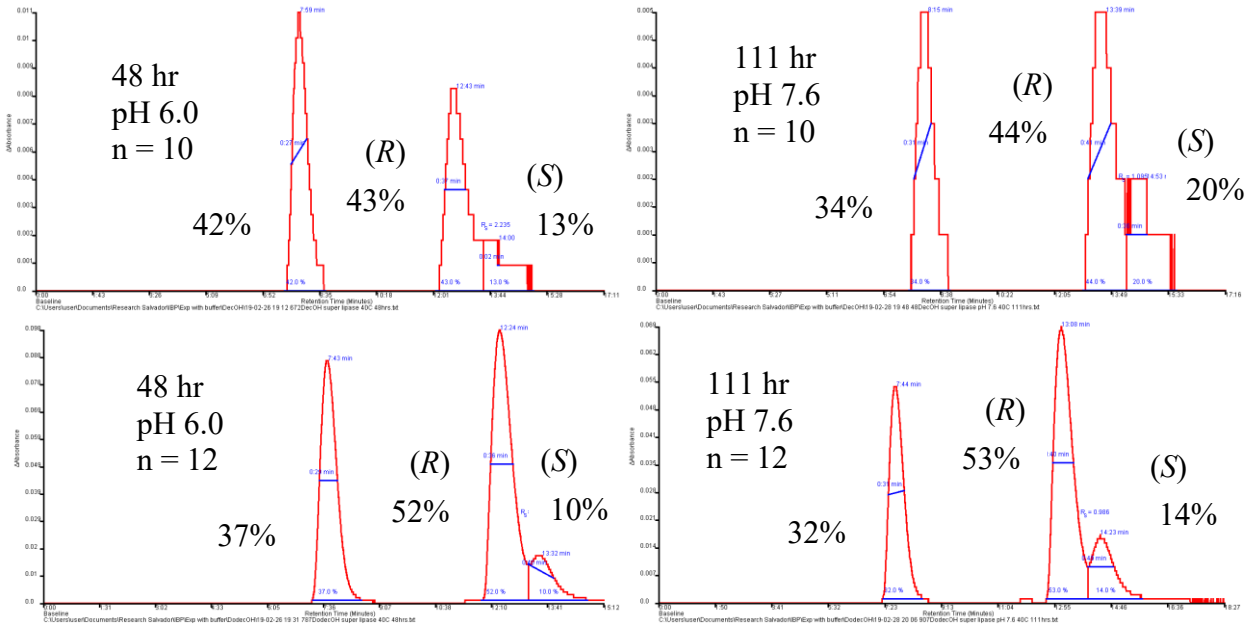
Noting that the esterification of dodecanol was better at pH 6.0 than 7.6, it was decided that the esterification of racemic IBU with different chain length alcohols would be tested at pH 6.0.

2.8 Results of the Reactions with Racemic IBU, Different Chain Length Alcohols ($H(CH_2)nOH$), and CRL without HEPES Buffer at pH 6.0

As shown on the left side of Chromatograms 2.4, IBU butyl ester was formed after 2 days, which was not observed at pH 7.6. All larger esters were formed in greater amounts in 2 days at pH 6.0 when compared to 6 days at pH 7.6 as previously demonstrated in Chromatograms 2.3.

Since the IBU butyl ester was not observed at pH 7.6, the reversibility of ester formation was tested by increasing the pH of all the primary alcohol reactions to 7.6. Surprisingly, only the IBU butyl ester was almost completely hydrolyzed back to IBU. The other esters, chain lengths 6 to 12, did not appear to hydrolyze significantly in less than 5 days.





Chromatograms 2.4 Reactions of Racemic IBU, Different Chain Length Primary Alcohols (H(CH₂)nOH), and CRL without HEPES Buffer at pH 6.0

Chapter 3: Conclusions

3.1 Reaction of Racemic IBU and CRL

From the previously proposed hypothesis by Mortazavi et al.[8], that deracemization of IME occurs by the formation of an enzyme-IBU ester followed by enolization/hydrolysis, was disproven by reacting racemic IBU under the same conditions but without DMF.

3.2 Reactions of CRL with Racemic IBU and Different Chain Length Primary Alcohols with HEPES Buffer at pH 7.6

Under the condition previously observed for deracemization of IBU, namely pH 7.6, the formation of IBU esters with primary alcohols was observed for chain lengths 6-12 but not 1-4 or 16.

3.3 Molecular Docking Calculations

Docking studies on IBU and IBU esters indicate that their BA for CRL increases with chain length for (*S*) over (*R*) esters except for (*R*)-IBU over (*S*)-IBU and (*R*)-IBU dodecyl ester over (*S*)-IBU dodecyl ester. Also, IBU through IBU propyl esters dock with the isobutyl group in first as opposed to the primary alkyl group for longer chain esters.

3.4 Esterification Reactions of Racemic IBU, Dodecanol, and CRL

The reactions of CRL proceeded faster with lower concentrations of racemic IBU and dodecanol. Also, the reactions of racemic IBU and dodecanol were more pH stable and proceeded faster using the CRL supernatant extract compared to using the whole commercial enzyme or the insoluble pellet from centrifugation. This indicates that the CRL enzyme is water soluble.

Esterification of racemic IBU and dodecanol proceeded two orders of magnitude faster at pH 6 than at 7.6 and the reactions of racemic IBU and dodecanol at pH 7.6 were faster without HEPES buffer.

3.5 Isolation from the Reactions of Racemic IBU, Dodecanol, and CRL without HEPES Buffer at pH 6.0

(*R*)-IBU was isolated in a 48 % yield from the reactions of racemic IBU and dodecanol at pH 6.0. This along with the observation that reactions were faster at pH 6 versus 7.6, indicates that the observed esterifications do not involve deracemization of IBU but instead were the preference for formation of longer chain length esters that separated from solution.

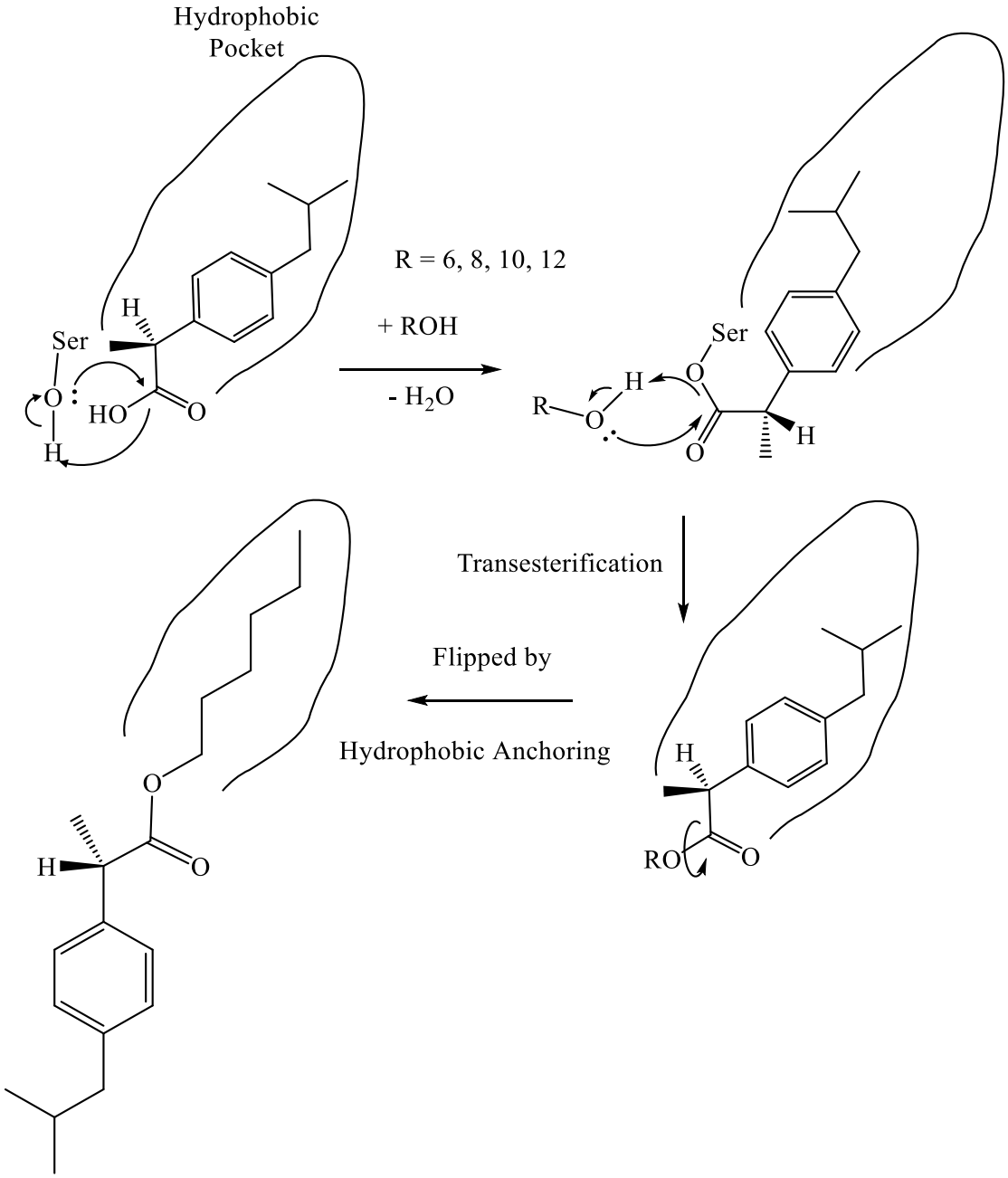
3.6 Reactions with Racemic IBU, Different Chain Length Primary Alcohols, and CRL without HEPES Buffer at pH 6.0

(*S*)-IBU butyl ester could be formed at pH 6.0 in 2 days and hydrolyzed almost completely at pH 7.6 over 5 days whereas larger esters did not hydrolyze reversibly over the same time. This indicates that the faster rate of formation of longer chain esters is probably an indication of a higher rate of hydrolysis of shorter chain esters at pH 7.6.

3.7 Potential Health Problems with the Use of Racemic IBU

Racemic IBU esters have been reported to cause gastrointestinal problems such as ulcers, bleeding, irritation, and inflammation even more so than IBU.[40,41,42] This work shows that long chain primary alcohols can be used to esterify (*S*)-IBU with CRL although they are difficult to hydrolyze, which could be a health concern not only because of the previously reported problems with IBU esters, but also because these type of intractable esters may be formed and accumulated in the human body.

A working hypothesis of why longer chain (*S*)-IBU esters are difficult to hydrolyze is shown in Scheme 3.1. If, like a fatty acid, (*S*)-IBU enters the CRL hydrophobic pocket with the isobutyl chain first, forms an enzyme ester with the serine of the active site, is trans-esterified to a long chain (*S*)-IBU ester, and the long chain alkyl group binds strongly to the hydrophobic pocket as indicated in the docking studies of Section 2.5, then the hydrolysis of long chain (*S*)-IBU esters would be disfavored because of hydrophobic anchoring.



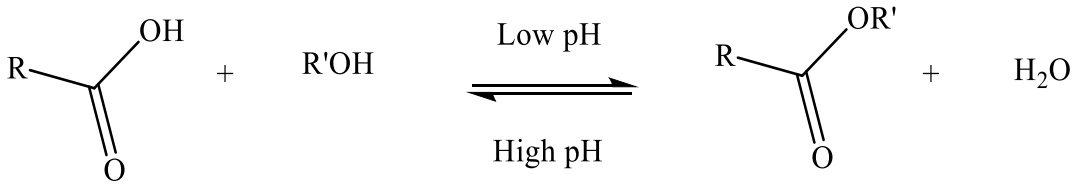
Scheme 3.1: Esterification Reaction Scheme of (*S*)-IBU with Long Chain Length Alcohols and the Hydrophobic Anchoring of Long Chain (*S*)-IBU Esters

3.8 Resolution of Racemic IBU by Reversible Esterification of (*S*)-IBU with Butanol and CRL in Water, a Potential Green Chemistry Application

Since lipase catalyzes several biotransformations,[43,44,45] the reactions presented in this thesis are the basis of metabolism of all living organisms involving a carboxyl group that reacts via esterification, transesterification, and hydrolysis.[46,47,48] Most of these reactions consist of an alcohol and a carboxylic acid in the presence of organic non-polar solvents.[49,50,51,52] However, the use of polar solvents, e.g. water, was shown in this work to be technically feasible and offers significant cost savings. Furthermore, the absence of non-polar solvents makes the process environmentally friendly, since most non-polar solvents are toxic and polluting agents.[53]

The present work evaluates the performance of CRL for the esterification of butanol with IBU as well as longer chain length primary alcohols (e.g. hexyl, octyl, decyl, and dodecyl) in a polar solvent system. Experimental runs were carried out to analyze the influence of alcohol chain length (C4 to C12) and IBU concentration on the esterification yield and productivity. The results allowed a better understanding of the pH influence in this environmentally friendly process of producing ester at a lower pH (6.0) and hydrolyzing ester at a higher pH (7.6). This microscopic reversibility also has the advantage of a higher productivity when compared with a non-polar solvent system.[54]

It is also interesting to speculate that as observed with CRL, a pH of 6 favors the formation of (*S*)-IBU butyl ester raising the pH, and a pH of 7.6 favors the hydrolysis of this ester lowering the pH, as would be expected by Le Chatelier's Principle as outlined in Scheme 3.2. Since the introduction of carbon dioxide and other acid producing pollutants are acidifying the environment, perhaps obesity or the accumulation of fat catalyzed by lipase is a way for nature to counter this effect.



Scheme 3.2 The pH Dependency of CRL to Esterify at Low pH and Hydrolyze at High pH.

Chapter 4: Experimental

4.1 Materials

Candida rugosa lipase (CRL, 1176 units/mg of solid), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), hexanes (98.5% CHROMASOLV), ethyl acetate, methanol, ethanol, butanol, 2-propanol, propanol, hexanol, octanol, decanol, dodecanol, and hexadecanol were purchased from the Sigma-Aldrich Chemical Company. Racemic IBU (200mg/tablet) was isolated from inexpensive commercial tablets purchased from Sam's Club. All other chemicals and analytical grade reagents were from commercial sources and were used without further purification.

4.2 Equipment

4.2.1 Solvent Evaporation

An IKA™ RV 10 rotary evaporator was utilized to remove volatile organic solvents from different reaction mixtures (Figure 4.2.1).

Figure 4.2.1 Rotary Evaporator for Removing Organic Solvents

4.2.2 Chiral Column High Performance Chromatography

A Chiralcel OJ chiral column (Diacel Chemical Industries) High Performance Liquid Chromatography (HPLC) with a Spectra Physics: Spectra System P1500 gradient pump and a Milton Roy UV detector set at 256 nm (Figure 4.2.2) programmed with Netbeans® and Java® software were employed to quantify, identify, and monitor all analyses at room temperature. The chiral column is efficient of resolving IBU and IBU esters with a mobile phase of hexanes/2-propanol (97/3, v/v), a flow rate of 1 mL/min, and helium solvent degassing. Analytes of 5.0-10.0 µL were introduced into the pump injector at room temperature (22ºC).

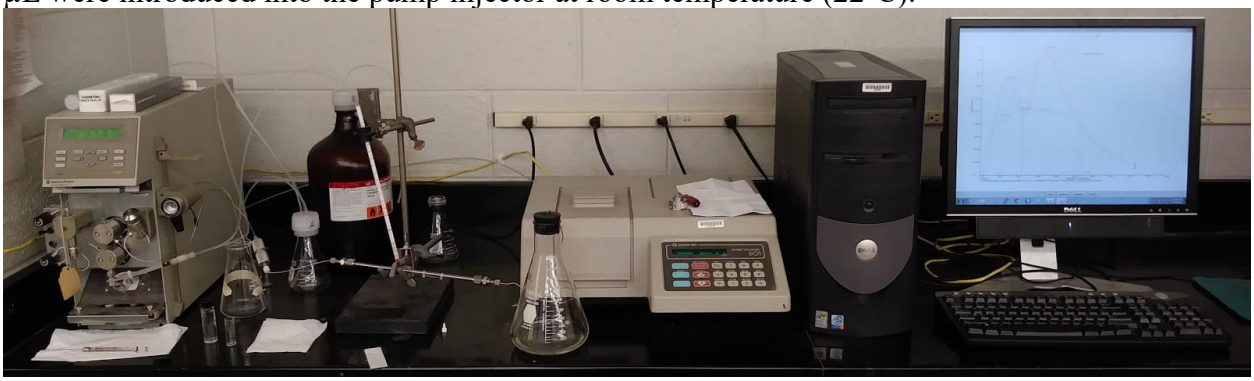


Figure 4.2.2 HPLC Equipment and Software Used for the Analysis of IBU Esters.

4.2.3 pH Measurements

An Oakton bench pH/Ion Meter (pH 1100 series) was used to measure the pH of buffered solutions and of the reaction mixtures before and after adding the lipase (Figure 4.2.3).

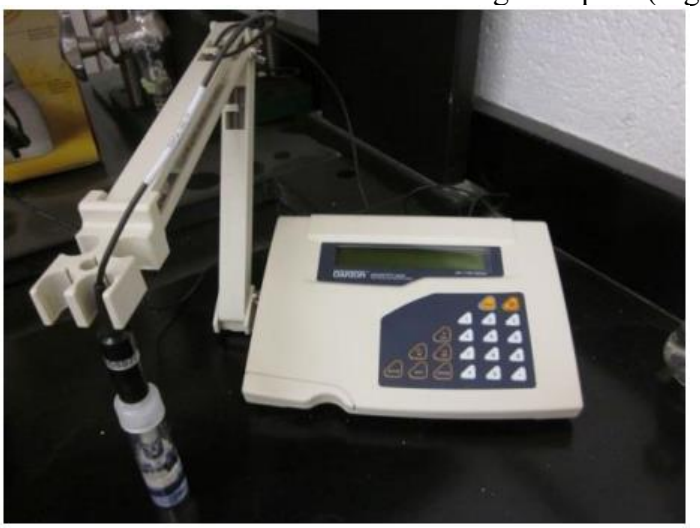


Figure 4.2.3 pH/Ion Meter for Measuring the pH of Buffer Solutions.

4.2.4 Centrifugation

Separations of CRL extracts were done with a Fisher Scientific: Safety Centrifuge (Figure 4.2.4) using GeneJET Spin Columns & Collection Tubes (50 units).

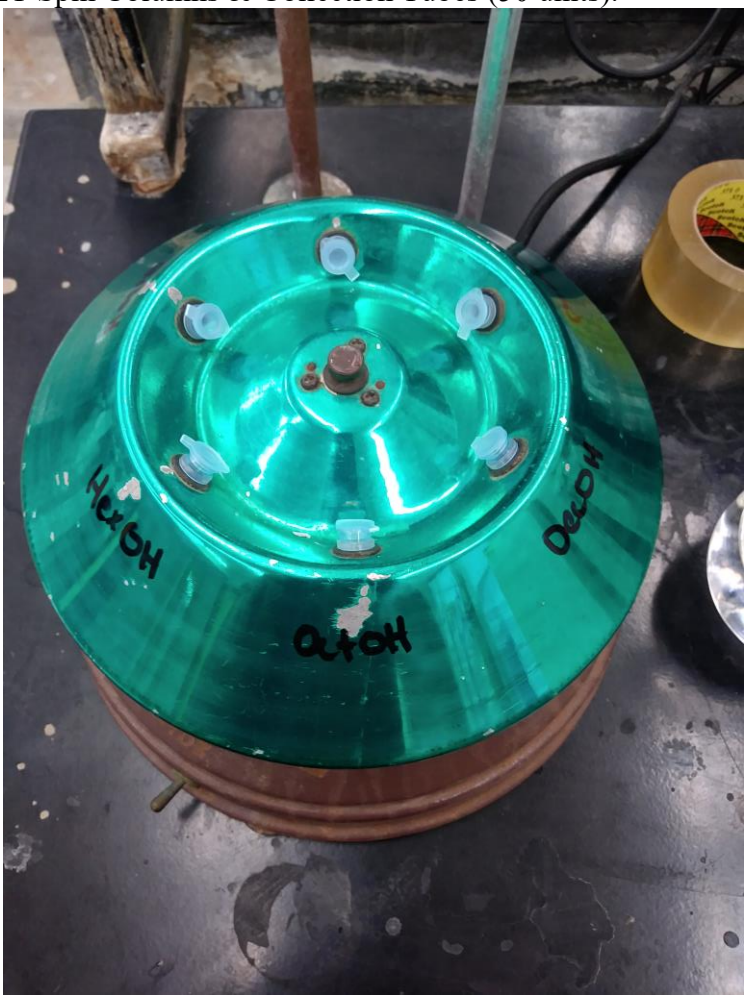


Figure 4.2.4 Safety Centrifuge with GeneJET Spin Columns & Collection Tubes.

4.2.5 Nuclear Magnetic Resonance (NMR) Spectrometry

Proton Nuclear Magnetic Resonance (1H NMR) and carbon-13 (13C) NMR spectra of crude reagents and isolated products were recorded on a Bruker-Spectrospin 300 MHz spectrometer (Figure 4.2.5) with deuterated chloroform ($CDCl_3$) as the solvent and tetramethylsilane (0.03% v/v) as an internal standard.

Figure 4.2.5 Bruker-Spectrospin 300 MHz Nuclear Magnetic Resonance Spectrometer.

4.2.6 Horizontal Bulb to Bulb Distillation

A Kuelgrohr apparatus (Sigma Aldrich Chemical Company, Inc.) was used to purify compounds after rotary evaporation by distillation at higher vacuum and by removal of high boiling point solvents. An ice water bath was used to trap distillates in the bulb and liquid nitrogen was used to trap volatiles before the vacuum pump (Figure 4.2.6).

Figure 4.2.6 Kuelgrohr Horizontal Distillation for Purifying Compounds

4.2.7 Radial Chromatography

A Chromatotron 7924 T (Figure 4.2.7) with silica gel plate (4 mm thickness) and a mobile phase of 100% hexanes was used to moisten the plate and to separate the IBU esters from racemic IBU. An aliquot of the sample (~1 g) was introduced into the sorbent rotor silica plate as it rotated. A UV lamp was utilized to observe the bands as they eluded from the center to the outside of the plate. Afterward a final wash with methanol of the plate was performed to remove impurities.

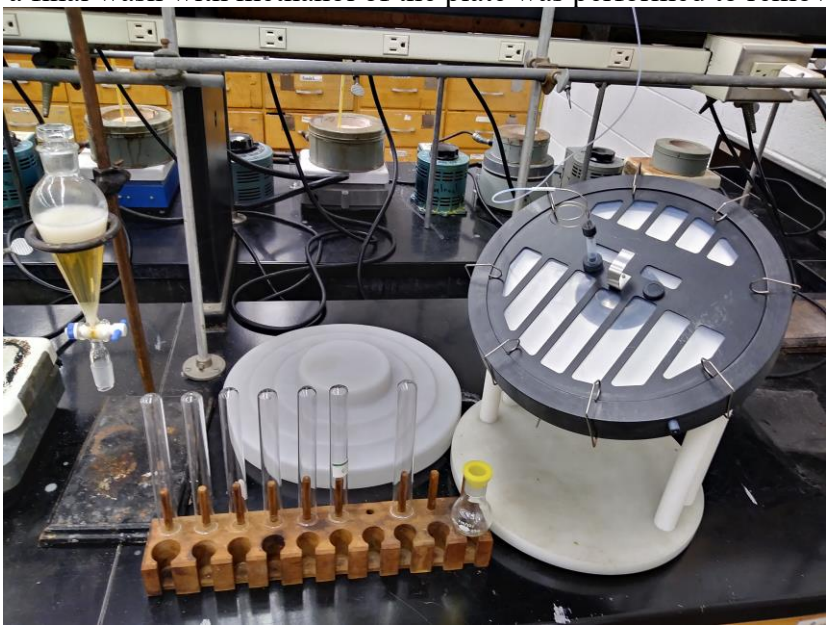


Figure 4.2.7 Chromatotron 7924 T for Separating Compounds

4.2.8 Heating and Stirring

Heating wells of various sizes filled with sand along with electrical variable autotransformers (0-140 V, 10 A, 50/60 Hz) were utilized as heating source to warm up all the reactions to 40 °C. Various types of magnetic stirrers were used to mix all the reactions while heating. The reaction setups with sand bath heating sources are illustrated in Figure 4.2.8.

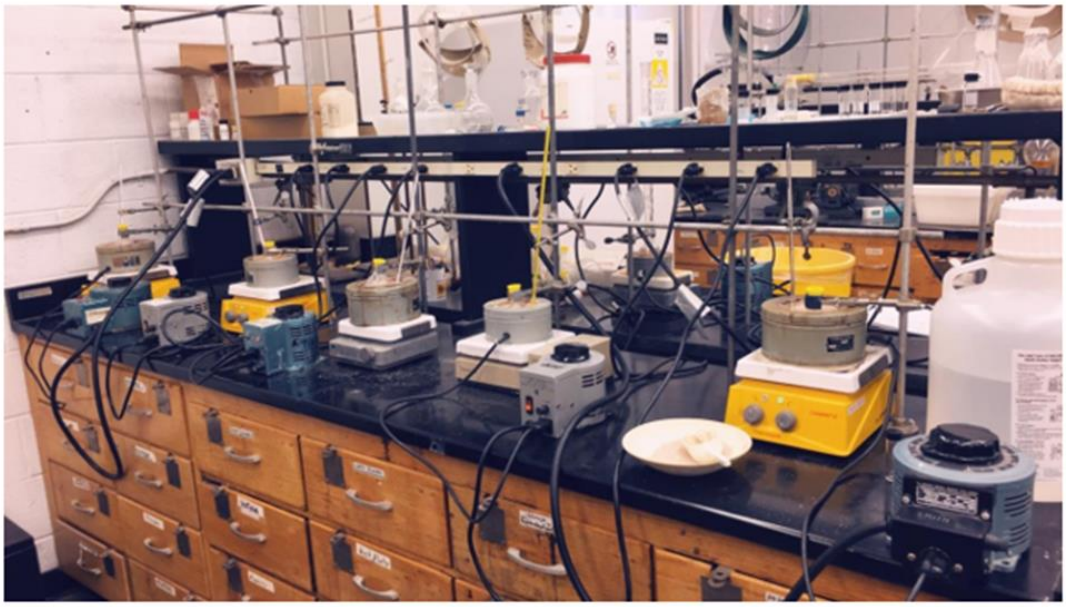


Figure 4.2.8 Reaction Setups of the Experiment with Variations of Alcohol and IBU.

4.2.9 Mass Measurements

A Sartorius Basic balance (Figure 4.2.9) with up to 4-digit accuracy was utilized for all mass measurements.

Figure 4.2.9 Sartorius Balance

4.3 Experimental

4.3.1 Racemic IBU Extraction from Tablets

Isolation of racemic IBU from commercial tablets (200 mg/tablet) was done (Figure 4.3.1) by suspending twenty tablets in 100 mL acetone for 30 minutes (5 mL/tablet) inside a clean and dry 125 mL beaker. Vacuum filtration was done to separate the coating and the filler from the filtrate containing the dissolved racemic IBU in acetone. The solvent was removed by vacuum distillation on the rotary evaporator, and the remaining white powder was used for the subsequent reactions listed below.

Figure 4.3.1 Extraction and Filtration from IBU Tablets.

4.3.2 Reaction of Racemic IBU and CRL

To a clean and dry 25mL round bottom flask was added 206.3mg of racemic IBU, 0.9g of CRL, 1.19g of HEPES buffer, and 10mL of H_2O. The initial pH of the solution was ~6.0 and was adjusted by adding 1M NaOH (~10mL) into the solution. Once the solution reached the optimal pH of 7.6, the reaction flask was covered with a plastic stopper and placed into a heating well filled with sand. A variable transformer and an alcohol thermometer were used to maintain the temperature at 40^{o}C for 72 hours. The reaction was monitored via chiral column HPLC analysis by taking aliquots of the reaction and acidifying it to a pH ~5.0 with 1M HCl followed by extraction with hexanes. 10µL of the organic layer was injected into the chiral column HPLC for analysis of IBU.

4.3.3 Reaction of Racemic IBU, Methanol, and CRL

To a clean and dry 25mL round bottom flask was added 206.3mg of racemic IBU, 0.9g of CRL, 1.19g of HEPES buffer, 40.5µL of methanol and 10mL of H_2O. The initial pH of the solution was ~5.0 and was adjusted by adding 1M NaOH dropwise (~10mL) into the solution. Once the solution reached the optimal pH of 7.6, the reaction flask was covered with a plastic stopper and placed into a heating well filled with sand. A variable transformer and an alcohol thermometer were used to maintain the temperature at 40ºC for 72 hours. The reaction was monitored via chiral column HPLC analysis by taking aliquots of the reaction and acidifying it to a pH ~5.0 with 1M HCl followed by extraction with hexanes. 10µL of the organic layer were injected into the chiral column HPLC for analysis of IBU.

4.3.4 Reactions of Racemic IBU, Different Chain Length Alcohols, and CRL with HEPES buffer at pH 7.6

To clean and dry 25mL round bottom flasks were added 206.3mg of racemic IBU, 0.9g of CRL, 1.19g of HEPES buffer, 1 mmol of each alcohol (methanol, ethanol, propanol, etc.) and 10mL of H_2O. The initial pH of the solutions was ~5.0 and were adjusted by adding 1M NaOH dropwise (~10mL) into the solutions. Once the solutions reached the optimal pH of 7.6, the reaction flasks were covered with plastic stoppers and placed into heating wells filled with sand. Variable transformers and alcohol thermometers were used to maintain the temperature at 40ºC for ~200 hours. The reactions were monitored via chiral column HPLC analysis by taking aliquots of the reactions and acidifying them to a pH ~5.0 with 1M HCl followed by extraction with hexanes. 10µL of the organic layers were injected into the chiral column HPLC for analysis of IBU esters.

4.3.5 Reactions of IBU Dodecyl Ester formed *in-situ* with CRL by Varying the Amounts of IBU and Dodecanol with HEPES buffer at pH 7.6

To clean and dry 25mL round bottom flasks were added alternating 0.5/ 1.0/ 2.0 mmol amounts of racemic IBU, 0.9g of CRL, 1.19g of HEPES buffer, alternating 0.5/ 1.0/ 2.0/ mmol amounts of dodecanol and 10mL of H_2O. The initial pH of the solutions was ~5.0 and were

adjusted by adding 1M NaOH dropwise (~10mL) into the solutions. Once the solutions reached the optimal pH of 7.6, the reaction flasks were covered with plastic stoppers and placed into heating wells filled with sand. Variable transformers and alcohol thermometers were used to maintain the temperature at 40ºC for ~200 hours. The reactions were monitored via chiral column HPLC analysis by taking aliquots of the reactions and acidifying them to a pH ~5.0 with 1M HCl followed by extraction with hexanes. 10µL of the organic layers were injected into the chiral column HPLC for analysis of IBU esters.

4.3.6 Reactions of IBU Dodecyl Ester formed *in-situ* with Different Components of CRL and HEPES buffer at pH 7.6

To clean and dry 25mL round bottom flasks were added 0.5 mmol of racemic IBU, 0.9g of CRL (crude)/ 10mL of CRL (supernate)/ 0.3g of CRL (pellet), 1.19g of HEPES buffer, 0.5 mmol of dodecanol and 10mL of H_2O. The initial pH of the solutions was ~5.0/ ~6.0/ ~5.0, respective of each component, and were adjusted by adding 1M NaOH dropwise (~10mL) into the solutions. Once the solutions reached the optimal pH of 7.6, the reaction flasks were covered with plastic stoppers and placed into heating wells filled with sand. Variable transformers and alcohol thermometers were used to maintain the temperature at 40ºC for ~200 hours. The reactions were monitored via chiral column HPLC analysis by taking aliquots of the reactions and acidifying them to a pH ~5.0 with 1M HCl followed by extraction with hexanes. 10µL of the organic layers were injected into the chiral column HPLC for analysis of IBU esters.

4.3.7 Reactions of IBU Dodecyl Ester and CRL formed *in-situ* without HEPES Buffer by Varying the pH Values

To clean and dry 25mL round bottom flasks were added 1.0 mmol of racemic IBU, 20mL of CRL supernatant and 1.0 mmol of dodecanol. The initial pH of the solutions was ~6.0 and one of them was adjusted by adding 1M NaOH dropwise (~5mL) into the solution. Once the solution reached the optimal pH of 7.6, the reaction flasks were covered with plastic stoppers and placed into heating wells filled with sand. Variable transformers and alcohol thermometers were used to maintain the temperature at 40ºC for ~12 hours. The reactions were monitored via chiral column

HPLC analysis by taking aliquots of the reactions and acidifying them to a pH ~5.0 with 1M HCl followed by extraction with hexanes. 10µL of the organic layers were injected into the chiral column HPLC for analysis of IBU esters

4.3.8 Reactions of Racemic IBU, Different Chain Length Alcohols, and CRL without HEPES buffer at pH 6.0

To clean and dry 25mL round bottom flasks were added 206.3mg of racemic IBU, 20mL of CRL supernatant and 1 mmol of each alcohol (methanol, ethanol, propanol, etc.). The initial pH of the solutions was ~6.0 and the reaction flasks were covered with plastic stoppers and placed into heating wells filled with sand. Variable transformers and alcohol thermometers were used to maintain the temperature at 40ºC for ~72 hours. Afterwards, they were adjusted by adding 1M NaOH dropwise (~5mL) into the solutions until they reached the optimal pH of 7.6. The reactions were monitored via chiral column HPLC analysis by taking aliquots of the reactions and acidifying them to a pH ~5.0 with 1M HCl followed by extraction with hexanes. 10µL of the organic layers were injected into the chiral column HPLC for analysis of IBU esters.

4.3.8 Molecular Docking Calculation Parameters

Ligand coordinate pdb files were generated with an Online SMILES Translator and Structure File Generator (https://cactus.nci.nih.gov/translate/) of (*S*/*R*)-IBU and each (*S*/*R*)-IBU ester (methyl to dodecyl) created in ChemDraw Prime 16.0 sdf files. A protein coordinate pdb file was obtained from the Protein Data Bank (www.pdb.org) website with the pdb ID: 3RAR. In the Autodock Vina: Tools program, appropriate hydrogen atoms were added to the ligand and protein structures with the subtraction of water molecules and current ligands (CA, IAN, NAG) found in 3RAR's structure. The rotatable bonds were fixed to non-rotatable on each ligand's aliphatic ester carbons and were all saved as pdbqt files for further calculations. The grid box coordinate dimension/size were: center X = 70, center Y = 54, center Z = -21, size X = 40, size Y = 40, and size Z = 40 with a 1Å spacing. The calculations generated output files of each (*S*/*R*)-IBU ester ligand binding site which were then analyzed with the PyMOL 2.3.0 program.

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Appendix

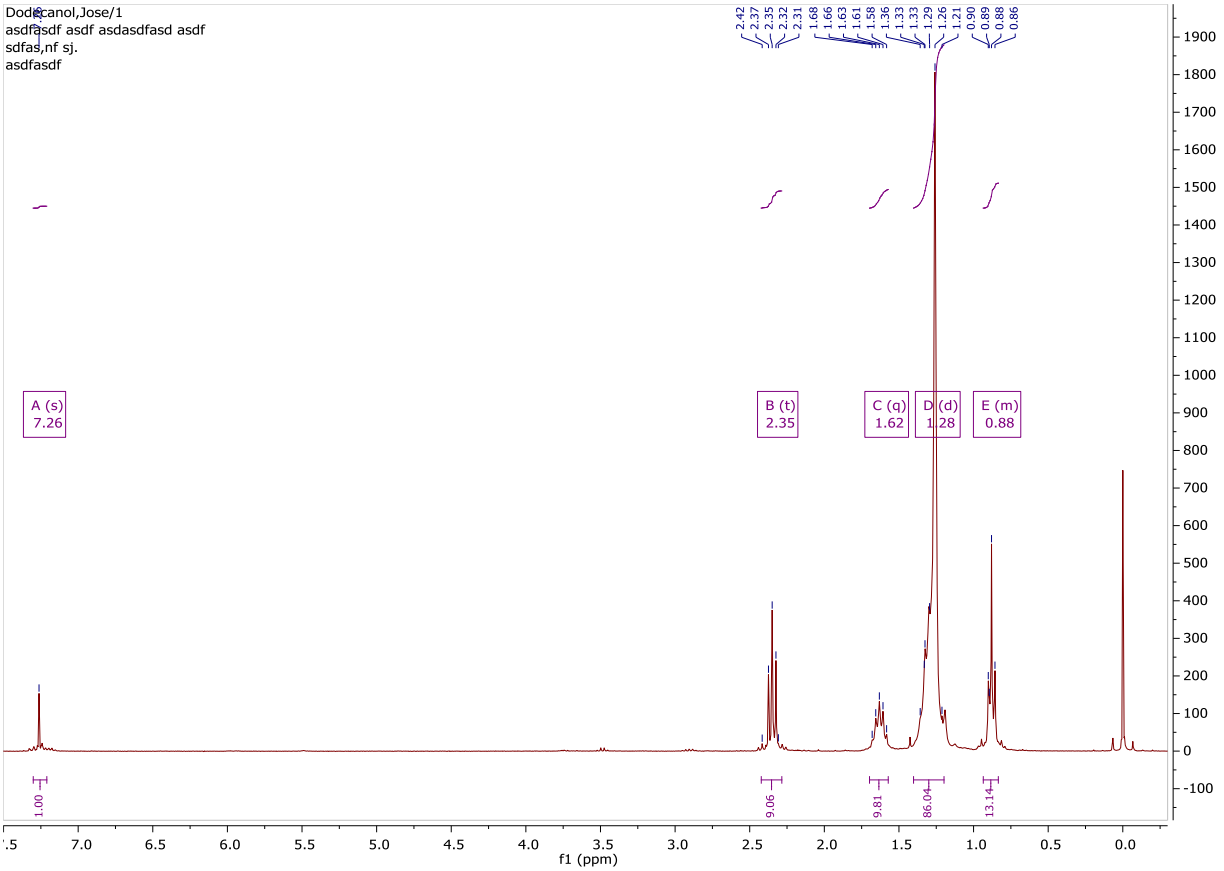

Figure 1. Proton ^{1}HNMR Spectrum of (S)-IBU Dodecyl Ester

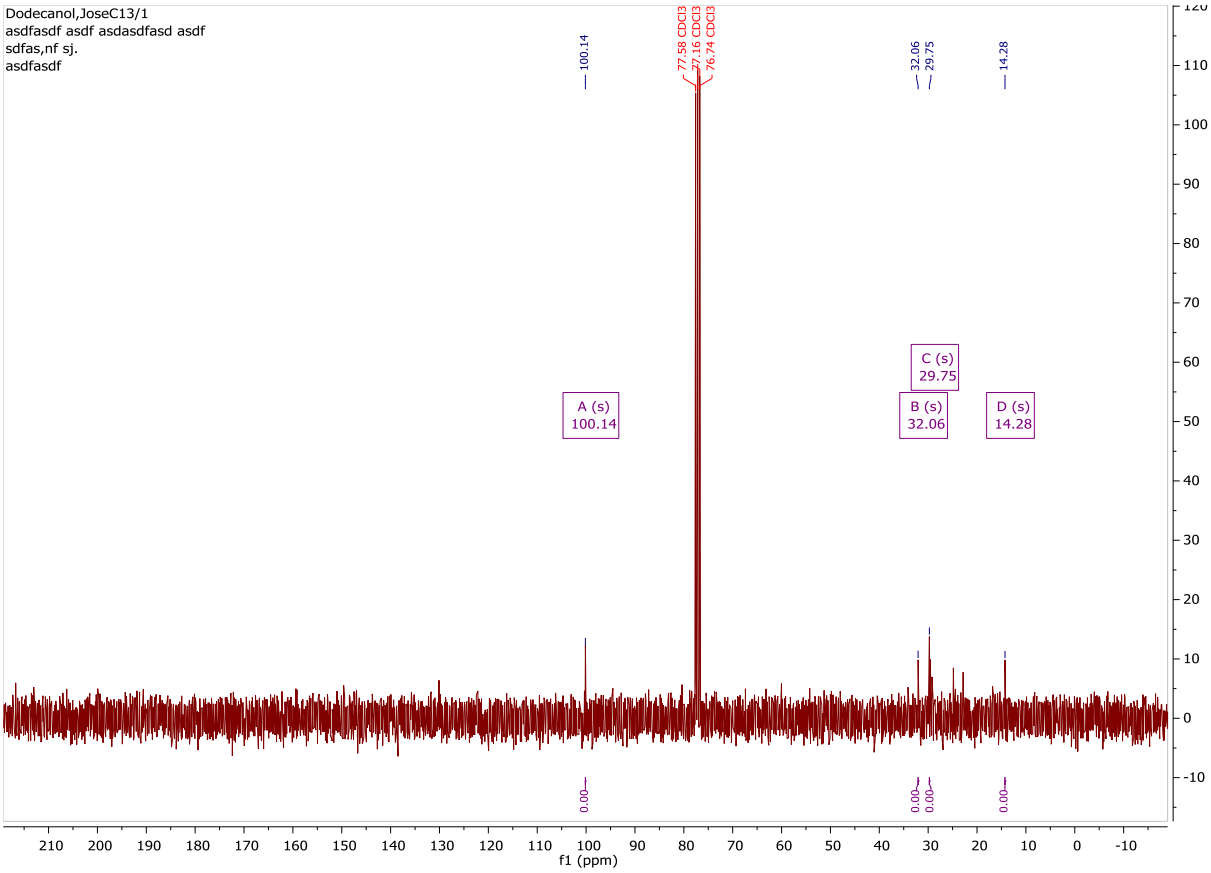

Figure 2. Carbon-13 NMR Spectrum of (S)-IBU Dodecyl Ester

Vita

Jose Antonio Rosales was born in Los Angeles, California to Antonio Rosales and Maria Mata. He graduated with a Bachelor of Science degree in chemistry from the University of Texas at El Paso (UTEP) in 2018. From 2015 to 2019, he held an Academic Tutor and Laboratory Assistant position for the department of chemistry at El Paso Community College (EPCC). He started his Master of Science degree in chemistry at UTEP in June of 2018 and joined Dr. James Salvador's research group. While pursuing his Master's, he worked as a teaching assistant for the department of chemistry and biochemistry. In the fall of 2019, he will continue his doctoral studies at UTEP and will become a chemistry instructor at EPCC.

Jose has been the recipient of scholarships and an internship which include: EPCC-UTEP Bridges to the Baccalaureate Research Program, UTEP System Louis Stokes Alliance for Minority Participation (LSAMP) Summer Research Academy, intern at the Centre National de la Recherche Scientifique (CNRS): Laboratoire de Coordination de Chimie (LCC), the UTEP National Science Foundation (NSF) Partnership in Research and Education in Materials (PREM) research program, and recently, the UTEP Doctoral Excellence Fellowship Award. He has presented his research at international, national, and local conferences such as the Border Biomedical Research Center (BBRC) conference (2015), Campus Office of Undergraduate Research Initiatives (COURI) symposium at UTEP (2015-2018), LSAMP research conference (2016), Louisiana State University (LSU) US/France/Belgium international Research Experience for Undergraduates (iREU) Site in Translational Chemistry (2017), Emerging Researchers National (ERN) conference (2017), Annual Biomedical Research Conference for Minority Students (ABRCMS) (2017), and the 255th American Chemical Society (ACS) National Meeting (2017). His current goal is to publish his master's and doctoral research work, pursue a postdoctoral position, and teach.

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