APPLICATION OF MUTANT VARIANTS OF SECONDARY ALCOHOL DEHYDROGENASE FROM *THERMOANAEROBACTER ETHANOLICUS* IN RACEMIZATION OF ENANTIOPURE SECONDARY ALCOHOLS IN ORGANIC SOLVENTS

BY

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A Dissertation Presented to the

DEANSHIP OF GRADUATE STUDIES

KING FAHD UNIVERSITY OF PETROLEUM & MINERALS

DHAHRAN, SAUDI ARABIA

In Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

In

CHEMISTRY

DECEMBER 2016

KING FAHD UNIVERSITY OF PETROLEUM & MINERALS

DHAHRAN- 31261, SAUDI ARABIA

DEANSHIP OF GRADUATE STUDIES

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This Thesis is dedicated to Dr. Rahim Karume

ACKNOWLEDGMENTS

I thank the Almighty ALLAH for enabling me to accomplish this piece of work.

I wish to express my sincere gratitude to many people who have helped in this work.

Particularly, I wish to thank Dr. Musa M. Musa for suggesting the research area and accepting to supervise me and for giving me the opportunity to work in his Laboratory. Thanks to my Advisor, Dr. Bassam M. El Ali and the Chairman of the Department of Chemistry, Dr. Abdulaziz A. Al-Saadi for the support and suggestions they offered.

Further, my gratitude goes to Dr. Samir M. Hamdan and Dr. Masateru Takahashi for the support offered. Part of the experimental work was conducted in Dr. Samir's Laboratory at KAUST. My sincere appreciation to Dr. Ashrof Ali and Dr. Alexis Mouanda for the guidance and accepting to be members of my doctoral committee. I am grateful to Prof. Muhammad Ntale from Makerere University for his endless support. My sincere appreciation to Prof. Claire Vieille, from Michigan State University, for providing the plasmids of TeSADH.

My special thanks to my entire family for the time they offered to live without me and for their support and encouragement during this doctoral pursuit.

Generous and financial support was received from the Ministry of Higher Education, Kingdom of Saudi Arabia through KFUPM. I acknowledge the support provided by King Abdulaziz City for Science and Technology through the Science and Technology Unit project No. 11-BIO1666-04, as part of the National Science, Technology and Innovation Plan.

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LIST OF ABBREVIATIONS

ADH	Alcohol Dehydrogenase
Ala	Alanine
[BMIM]PF_6	1-Butyl-3-methylimidazolium hexafluorophosphate
CALB	*Candida antarctica* Lipase B
CGATU	Cytosine, Guanine, Adenine, Thymine, Uracil, DNA bases
Conv.	Conversion
Csy	Cysteine
DKR	Dynamic Kinetic Resolution
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
E	Enantiomeric ratio
ee	enantiomeric excess
GC	Gas Chromatography
GTMS	(3-Glycidyloxypropyl)trimethoxysilane
Gly	Glycine
Ile	Isoleucine

IR	Infrared Spectroscopy
ITMS	Isobutyl(trimethoxy)silane
KR	Kinetic Resolution
mmol	millimole
MS	Mass Spectrometry
MTBE	Methyl *tert*-butyl ether
MTMS	Methyltrimethoxysilane
$NADP^+$	Nicotinamide Adenosine Dinucleotide Phosphate
NADPH	Nicotinamide Adenosine Dinucleotide Phosphate, Reduced
NMR	Nuclear Magnetic Resonance
rac	racemic
TeSADH	*Thermoanaerobacter ethanolicus* Secondary Alcohol Dehydrogenase
TMOS	Tetramethoxysilane
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
Trp	Tryptophan
VTMS	Vinyltrimethoxysilane

ABSTRACT

Full Name: Ibrahim Karume

Thesis Title: Application of mutant variants of secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* in racemization of enantiopure secondary alcohols in organic solvents

Major Field: Chemistry

Date of Degree: December 2016

The stereoselectivity of redox reactions of ketones and their corresponding alcohols was extensively studied. Various mutants of *Thermoanaerobacter ethanolicus* secondary alcohol dehydrogenase (TeSADH); W110A, W110G, W110V and I86A/W110A were used in this study. Medium engineering studies revealed a strong dependence of the enzyme's activity and stereoselectivity on the nature of solvent and cosolvent used. The tolerance of W110A TeSADH to elevated concentrations of organic media allowed coupling of racemization catalyzed by xerogel-immobilized W110A TeSADH with *Candida antarctica* lipase B-catalyzed kinetic resolution in a dual enzymatic dynamic kinetic resolution of racemic alcohols in hexane. For TeSADH-catalyzed reduction reactions in aqueous media, enzyme's enantioselectivity increased as the concentration of 2-propanol increases, while for oxidation reactions, the enantioselectivity decreased by reducing the concentration of acetone. Acetone concentration of 3% (v/v) as a cosubstrate allowed complete depletion of racemic alcohols to the corresponding ketones, which was followed by high stereoselective reduction using 2-propanol (30%, v/v) to predominantly produce *S* configured alcohols. Controlling enantioselectivity of TeSADH-catalyzed redox

reactions allowed for a single enzymatic deracemization approach of racemic alcohols in two steps and one pot. It also allowed for stereoinversion of the configuration of *R* alcohols by using a single mutant of TeSADH. *S* configured alcohols were produced in 70 to >99% *ee*.

ملخص الرسالة

الاسم الكامل: إبراهيم كرومي

عنوان الرسالة: أستخدام طفرات مختلفة من أنزيم كحول ثنائي نازع للهيدوجين من *Thermoanaerobacter ethanolicus* في تروسم الكحولات الثنائية الفراغية في المذيبات العضوية

التخصص: كيمياء

تاريخ الدرجة العلمية: رَبيع الأوّل ١٤٣٨

دُرست التفاعلات الإنتقائية الفراغية لعمليات اختزال الكيتونات وأكسدة الكحولات على نطاق واسع. في هذه الدراسة تم استخدام أنزيم كحول ثنائي نازع للهيدوجين من *Thermoanaerobacter ethanolicus* (TeSADH) بإحداث طفرات مختلفة تشمل W110A, W110G, W110V و I86A/W110A . وتم دراسة هذه التفاعلات في أوساط عضوية مختلفة وأشارت الدراسة الى اعتماد نشاط الأنزيم وانتقائيته على طبيعة الوسط المستخدم. سمحت قابلية TeSADH للعمل بأوساط عضوية بتراكيز عالية لمزج عمليات الروسمة المحفزة ب W110A TeSADH المثبت مع تفاعل الفصل الحركي المحفز بأنزيم *Candida antarctica* lipase B (CALB) ليؤدي إلى فصل حركي ديناميكي لكحولات راسمية باستخدام أنزيمين في الهكسان. بالنسبة لتفاعلات الإرجاع بواسطة المحفز TeSADH في الوسط المائي, تزايدت الإنتقائية الفراغية للأنزيم مع زيادة تركيز 2- بروبانول، بينما تناقصت الإنتقاءية الفراغية لتفاعلات الأكسدة مع تناقص تركيز الأسيتون. سمح تركيز الأسيتون 3% (حجم/حجم) كعامل مرافق باستنفاذ كامل لمزيج راسمي من الكحولات إلى الكيتونات المرافقة، والتي بدورها أتبعت بتفاعلات اختزال عالية الإنتقائية الفراغية باستخدام 2- بروبانول (30% ،حجم /حجم) لإنتاج كحولات (*S*) بانتقائية عالية. التحكم بالإنتقائية الفراغية لتفاعلات الإختزال والاكسدة بواسطة TeSADH مكنت ولأول مرة من تحويل مزيج راسيمي إلى كحول *S* في خطوتين بوعاء واحد وباستخدام أنزيم واحد, وكذلك سمحت لتحويل الكحول الثانوي *R* إلى التشكيل *S* بانتقائية عالية (من70 إلى 99% *ee*).

CHAPTER ONE

INTRODUCTION

1.1 Biocatalysis

Biocatalysis has manifested excellence in chemical transformations. The technique employs enzymes to perform a similar role to organic and inorganic catalysts. Biological catalysts reign traditional catalysts in several aspects. The cost effectiveness, the high stereospecificity and the need for less sophisticated reaction setups in biocatalyzed reactions make enzymes better alternatives to chemical catalysts in asymmetric synthesis. In the last few decades, the necessity for cheap and environmentally friendly methodologies has revolutionized synthetic organic chemistry. This campaign was triggered by an increasing demand for optically active compounds which serve as intermediates in food, agrochemicals, and pharmaceutical products. In drug efficacy, cases where an enantiomer is more active than the opposite enantiomer have been reported [1, 2]. New regulations such as the application of 'chiral switch'[3] for efficient drug delivery and prevention of adverse effects of drugs containing racemates have also been driving forces. For example, the (*S*)-isomer of Ibuprofen, a non-steroid inflammatory drug reaches a therapeutic concentration in blood faster than the racemic drug [1]. The initial use of racemic dopa in the treatment of Parkinson's disease had side effects such as nausea, vomiting, anorexia, involuntary movements, and granulocytopenia. These side effects reduce when a single enantiomer, L-dopa is used in addition to improved patient recovery [2]. (*S*)-4-(4-Hydroxyphenyl)-2-butanol known as (*S*)-rhododendrol has been used to prevent hepatotoxicity

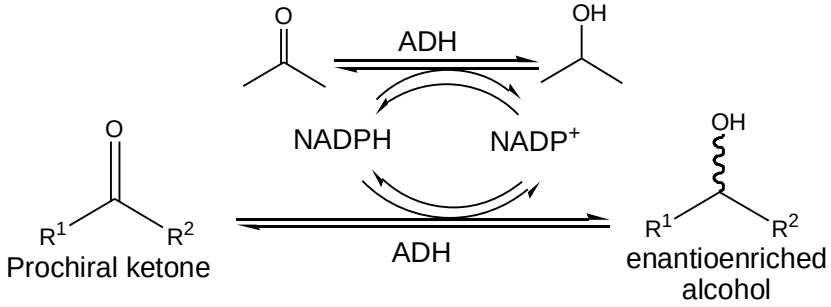
[4] and also as a bleaching agent in cosmetics [5], whereas 4-phenyl-2-butanol is a key building block of HIV protease inhibitors [6].

Asymmetric bioreduction of ketones to chiral alcohols has been employed in drug synthesis. For example, (3*S*,5*R*)-dihydroxy-6-(benzyloxy) hexanoic acid ethyl ester is produced via a bioreduction approach and is used as cholesterol lowering agents [7]. Both enantiomers of 6-hydroxybuspirone, an anxiety drug, have been synthesized by reduction of 6-keto buspirone catalyzed by (*S*)- and (*R*)-reductases from *Pseudomonas putida* and *Hansenula polymorpha*, respectively [8]. Enantiopure alcohols can also be obtained using alcohol dehydrogenases (ADHs), enzymes that catalyze the reversible asymmetric reduction of ketones and aldehydes to their corresponding alcohols. In this study, the candidates are mutants of secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* (TeSADH). Single and multi-site mutagenesis of wild-type TeSADH were performed to obtain mutants of interest; W110A, W110G, I86A/W110A, I86G/W110A, and I86G/W110G TeSADH. The effect of reaction medium and co-substrate concentration on the activity and stereospecificity of the mutants were investigated in oxidation, reduction, and racemization. Further, the reactions applied racemization catalysts in dynamic kinetic resolution (DKR) and in deracemization and stereoinversion of racemic alcohols in an attempt to obtain single enantiomers from racemates or to switch the configuration of enantiopure alcohols. DKR, a deracemization technique where a racemic mixture can be transformed to a single enantiomer in up to 100% yield with high enantioselectivity is an optimum alternative to kinetic resolution (KR) [9]. Controlled racemization of enantiopure compounds, which is extensively studied in the current work, is the key component to convert KR to DKR.

The success of an approach like enzymatic DKR requires a tandem biocatalytic setup with diverging reaction conditions. For instance, an aqueous environment required by ADH presents a challenge since most organic substrates are sparingly soluble in such media. This is also contrary to the requirements of *Candida antartica* lipase B (CALB)-catalyzed transesterification, the most popular lipase in KR [10] which requires non-aqueous environment for esterification. On the other hand, a rather less sophisticated approach discussed in this work is deracemization by stereoinversion, where less selective oxidation of an alcohol was followed by (*S*)-selective reduction of the intermediate prochiral ketone to produce the (*S*)-alcohol.

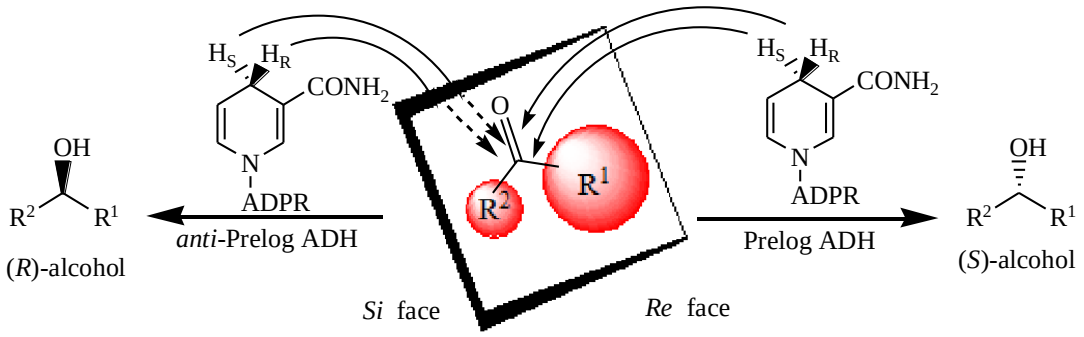
1.2 Alcohol dehydrogenases

Alcohol dehydrogenases (ADHs) catalyze the reversible asymmetric reduction of ketones and aldehydes to corresponding alcohols. In living organisms, these redox reactions require a coenzyme which is usually reduced nicotinamide adenine dinucleotide (NADH) or its phosphate (NADPH) acting as hydride sources for reduction and their oxidized forms NAD^+ or $NADP^+$ as hydride sinks for oxidation reactions [11]. The regeneration of the reduced and oxidized form of the coenzymes can be achieved cheaply by the use of 2-propanol and acetone, respectively, as cosubstrates (Scheme 1).



Scheme 1. Regeneration of coenzymes in ADH-catalyzed redox reaction.

Asymmetric reduction of ketones involves the addition of two hydrogen atoms to a carbonyl group forming an optically active alcohol. The stereochemical outcome was first explained by Prelog five decades ago [12]. In ADH-catalyzed reduction of ketones, the pro-(*R*) or pro-(*S*) hydride of the NAD(P)H can be delivered either from the *Re* face or *Si* face of a prochiral ketone forming an alcohol with *S* or *R* configuration, respectively, if the large group has higher Cahn-Ingold-Prelog priority than the small group of the ketone (Scheme 2) [12].



Scheme 2. Stereochemistry of hydride transfer by NAD(P)H in ADH-catalyzed reduction of ketones. ADPR= adenosine diphosphoribose, R^1 is more sterically hindered and has a higher Cahn-Ingold-Prelog priority than R^2.

According to Prelog's rule, the pro-(*R*) hydride of NAD(P)H is delivered from the *Re* face of a prochiral ketone producing *S*-configured alcohols in most of the cases. For example, Yeast ADH (YADH), Horse liver ADH (HLADH), *Thermoanerobium brockii* ADH (TbADH), *Rhodococcus ruber* ADH (RrADH) and *Thermoanaerobacter ethanolicus* secondary ADH (TeSADH) obey Prelog's rule, and thus termed Prelog ADHs [13, 14]. *Lactobacillus kefir* ADH (LkADH) [15] is an *anti*-Prelog ADH, a rare type of ADHs. In 1986, Keinan et al. hypothesized a model where they suggested that TbADH, an identical enzyme with TeSADH, has two hydrophobic pockets which differ in volume and affinity toward alkyl groups attached to the

carbonyl carbon. The small pocket has a higher affinity for alkyl groups, however, it only accommodates methyl, ethyl, isopropyl and cyclopropyl [14].

The hydrophobicity of the small pocket is exemplified in the asymmetric reduction by wild type TbADH in a study by Yang et al., in which 2-butanone was reduced to (*R*)-2-butanol contra to the normal *S*-configured alcohol as it is in the reduction of 2-pentanone which produced (*S*)-2-pentanol. This suggests the preference of the ethyl group to methyl in the small pocket yielding the (*R*)-enantiomer [16]. Keinan's hypothesis was later proven by X-ray characterization of TbADH which was found to contain a large and a small pocket (Figure 1) [17]. The crystal structure of TbADH has been resolved. It is a tetramer with each unit consisting of 352 amino acids with a zinc ion (Zn^{2+}) at the active site [18]. The structure has a crevice between the surface and the active site which serves as a pathway of the reactant and product in and out. It contains hydrophobic residues Ile-49, Leu-107, Trp-110, Tyr-267 and Cys-283 as well as Met-285 from another polypeptide which explains the high tolerance to organic medium exhibited by this enzyme as reported by Li et al. [19].

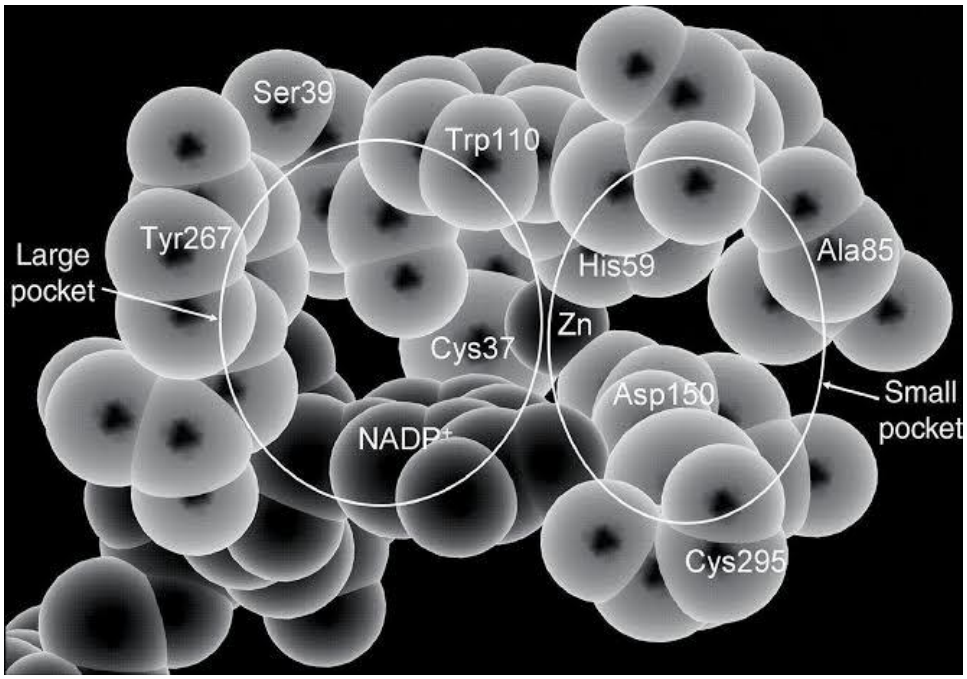
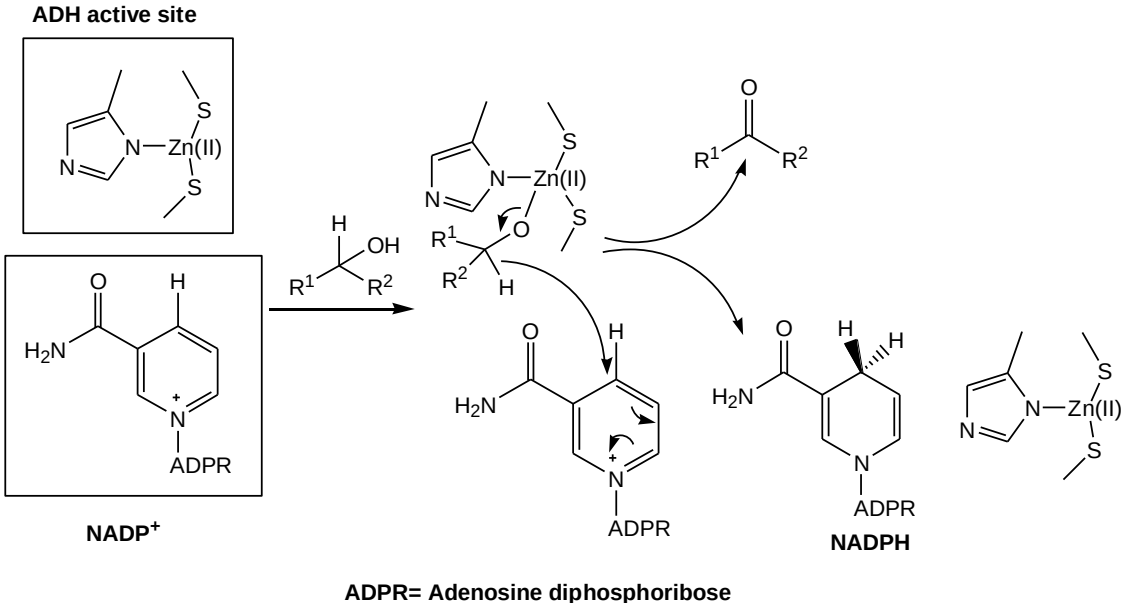


Figure 1. A model of a portion showing catalytic site in TbADH, an identical enzyme with wild TeSADH.

The positions of interest in the active site of TeSADH are Trp-110 at the large pocket and Ile-86 as well as Cys-295 in the small pocket. Interchanging the amino acids at these positions by less sterically demanding amino acids expands the active site, and thus allowing the accommodation of substrates that are not accepted by the wild-type enzyme [11]. Such modifications not only dictate the nature of substrates to be accepted but also improve or lower the enzymes stereospecificity.

YADH, HLADH, TbADH [14], and TeSADH [13] have been employed in the asymmetric reduction of prochiral ketones and stereospecific oxidation of alcohols [11]. However, only TbADH and TeSADH show tolerance to non-aqueous media with a noticeable degree of thermal stability. TeSADH tolerates temperatures up to 80 ^{o}C. TeSADH gains significant interests because of its high thermal stability as well as its high tolerance to organic solvents [20, 21].

In a TeSADH-catalyzed oxidation of an alcohol, the zinc coordinates through electrostatic interaction with the oxygen of the substrate. This makes the methine carbon more electrophilic and thus prone to nucleophilic attack, hence catalyzing asymmetric redox reaction of the intermediate carbonyl formed. The coenzyme is held and stabilized near the active site by hydrogen bonding between the hydroxyl groups of the ribose and the amino acids. The schematic of the simplified mechanism is summarized in Scheme 3.



Scheme 3. Suggested mechanism of ADH-catalyzed oxidation of a secondary alcohol.

1.2.1 Mutants of TeSADH

Several mutants of TeSADH have been designed and employed in reduction of prochiral ketones and racemization of enantiopure alcohols. Perhaps the most important one involves a single mutation at Trp-110 being replaced by alanine or glycine to expand the large pocket at the active site [17]. This allows accommodation of sterically hindered substrates like phenyl-ring-containing alcohols and their corresponding ketones that are not substrates for wild-type TeSADH. In this work, in addition to the single mutants, the double mutants; I86A/W110A, I86G/W110A and I86G/W110G TeSADH were constructed using multi-site-directed mutagenesis at both the small pocket (at Ile-86) and large pocket at the active site (Figure 2). Both the single and double mutants were employed in racemization and in asymmetric reduction to check substrate scope and stereospecificity.

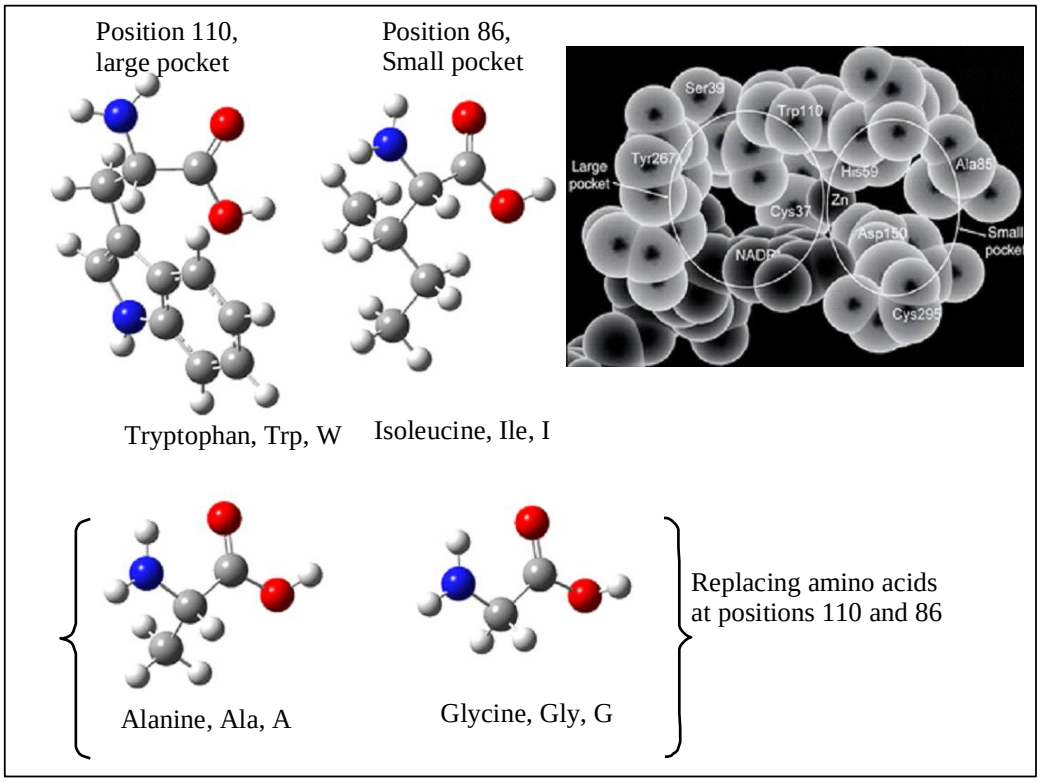


Figure 2. Amino acids to be interchanged. Red = oxygen, blue = nitrogen, grey = carbon, white = hydrogen.

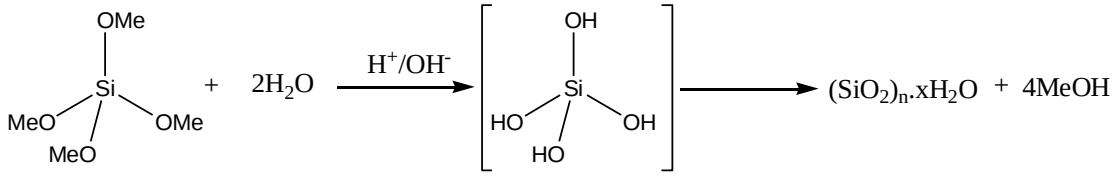
Unlike the wild-type TeSADH, a variety of ketones were successfully reduced with the genetically modified W110A TeSADH with the majority giving excellent conversions (>90%) and high enantioselectivities producing (*S*)-configured alcohols as the predominant product [11, 22]. An organic medium is crucial in dictating efficiency of the system since it allows sparingly soluble bulk substrates in satisfactory concentrations. However, this demands an immobilized system to prolong the activity of the enzyme in harsh media conditions contrary to their natural environment.

1.2.2 Immobilization of enzymes

Enzyme immobilization involves localizing the enzyme molecules on/in an inert molecule. Immobilization of enzymes has several advantages among which; easy handling, enhanced stability and recyclability where applicable Several immobilization techniques have been employed depending on the nature of the enzyme and the reaction. [23]. For instance, immobilization of lipase on acrylic resin as in the case of Novozyme-435, a commercially available lipase widely used in esterifications in organic media [24].

An immobilization method of interest is sol-gel encapsulation of alcohol dehydrogenase which involves trapping the enzyme in silica glasses synthesized by sol-gel method [23, 25]. In 1992, Ellerby et al. reported a novel technique for enzyme immobilization using the sol-gel method suitable for encapsulation of proteins without loss of biological activity [26]. The technique is less tedious, non-toxic and requires low-cost starting material and method of operation hence can be employed at both small and large scale. Synthesis of silica gels follows the same protocol which involves acid/base hydrolysis/alcoholysis of a silane of interest to form a uniform layer called sol which undergoes condensation to form $(SiO_2)_n$ framework that forms a

hydrogel after partial drying and a xerogel after prolonged drying conditions. The sol-gels are porous allowing diffusion of substrates in and out of the immobilized system. In addition, they retain sufficient water molecules to sustain the activity of an encapsulated enzyme [27]. The simplest and most common silane employed in sol-gel formation is tetramethyl orthosilicate (TMOS) (Scheme 4).

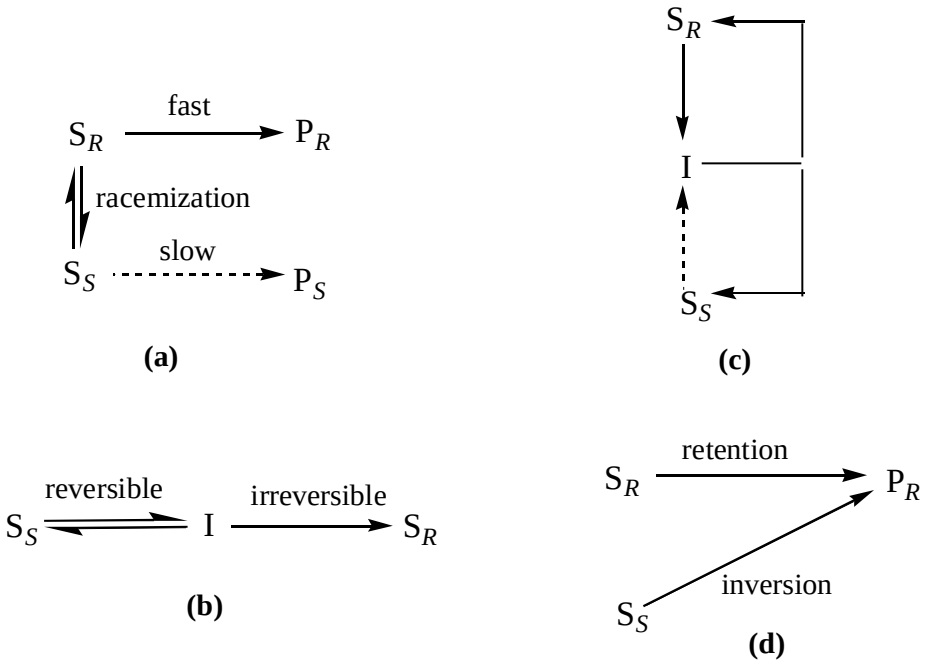


Scheme 4. Hydrolysis and condensation of TMOS during gel formation.

Xerogel-encapsulated W110A TeSADH has been successfully used in the asymmetric reduction of ketones in non-aqueous media [21]. The immobilized enzyme tolerated non-aqueous media like hexane and methyl *tert*-butyl ether (MTBE). In this work, TMOS based xerogel and hybrids synthesized by incorporating 30% of silanes with hydrophobic groups together with TMOS in the sol matrix were investigated. The incorporated silanes include; isobutyl(trimethoxy)silane (IBTMS), (3-glycidyloxypropyl)trimethoxysilane (GTMS), methyltrimethoxysilane (MTMS) and vinyltrimethoxysilane (VTMS) each having a non-hydrolyzable hydrophobic group. The increase of the hydrophobicity of the hybdrid-xerogel skeleton might enhance solvent and substrate diffusion in and out of the xerogel.

1.3 Deracemization of phenyl-ring-containing racemic secondary alcohols

Deracemization of alcohol racemates provides an attractive approach that could surpass the 50% yield limit by kinetic resolution. Deracemization strategies that have been developed include dynamic kinetic resolution, cyclic deracemization, stereoinversion and enantioconvergence (Scheme 5a-d) [9, 28-30].



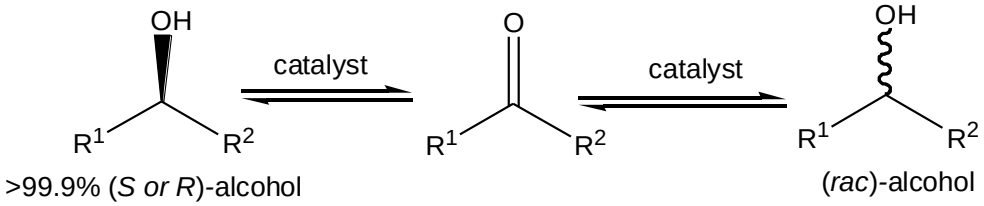
Scheme 5. Deracemization techniques: a) dynamic kinetic resolution; (b) cyclic deracemization; (c) stereoinversion; (d) enantioconvergence.

Dynamic kinetic resolution (DKR) (Scheme 5a) is the most advanced technique; it involves a fast reaction of one enantiomer in a kinetic resolution approach while the opposite enantiomer simultaneously racemizes to the KR preferred enantiomer. Stereoinversion (Scheme 5b) involves selective oxidation of one enantiomer which is then reduced with inversion of configuration to the opposite enantiomer. Cyclic deracemization (Scheme 5c) converts both

enantiomers to a common intermediate however one enantiomer reacts slowly resolving the racemate in the process. Enantioconvergence (Scheme 5d) converts both enantiomers to the same product with retention of configuration of one enantiomer and inversion of the opposite one giving a product with one configuration.

1.4 The concept and role of racemization

Racemization refers to partial stereoinversion of a pure enantiomer to yield a racemic mixture (Scheme 6).



Scheme 6. Racemization of a pure enantiomer to a racemate.

An efficient racemization is a necessity to convert a less active enantiomer to the opposite active one where applicable. Methods that employ transition metal complexes in racemization have been reported. Planar chiral ferrocene-ruthenium based complexes have been employed [31]. Shvo's chiral ruthenium complexes have been reported to catalyze racemization (Figure 3) [32].

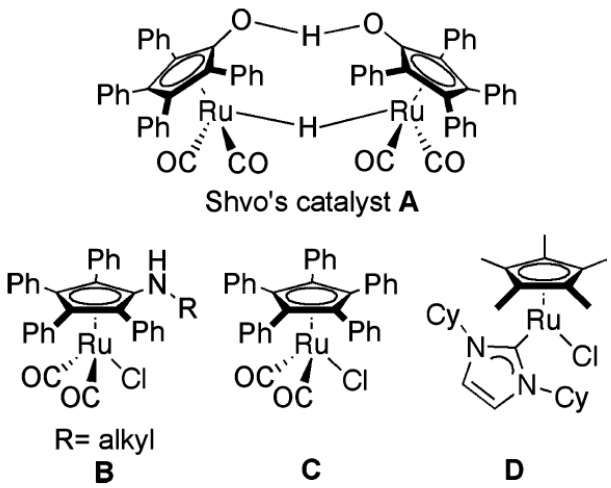
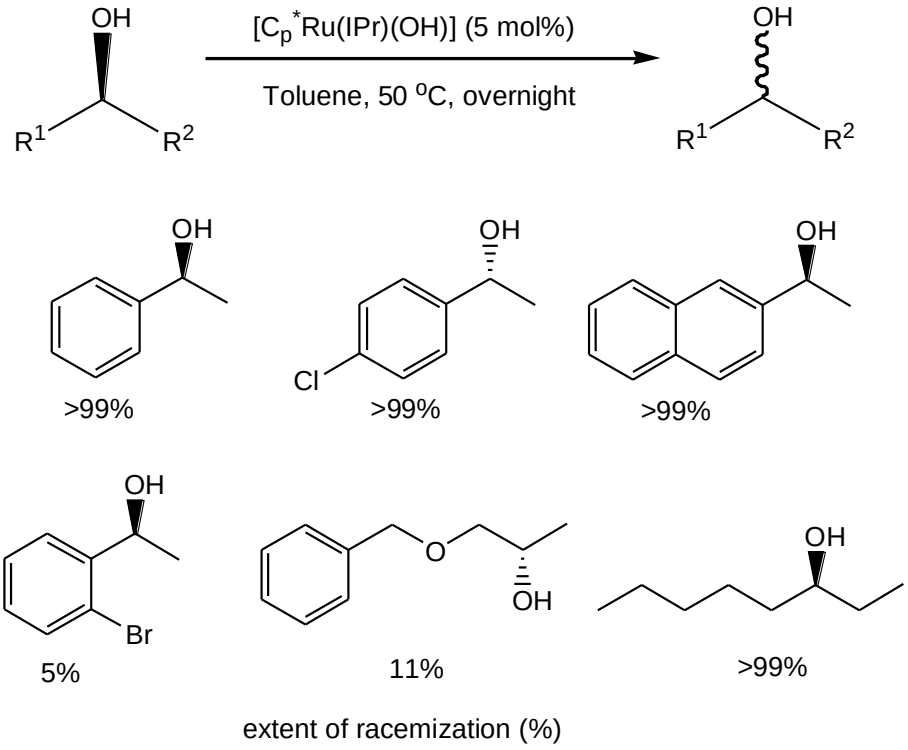


Figure 3. Shvo's ruthenium-based alcohol racemization catalysts.

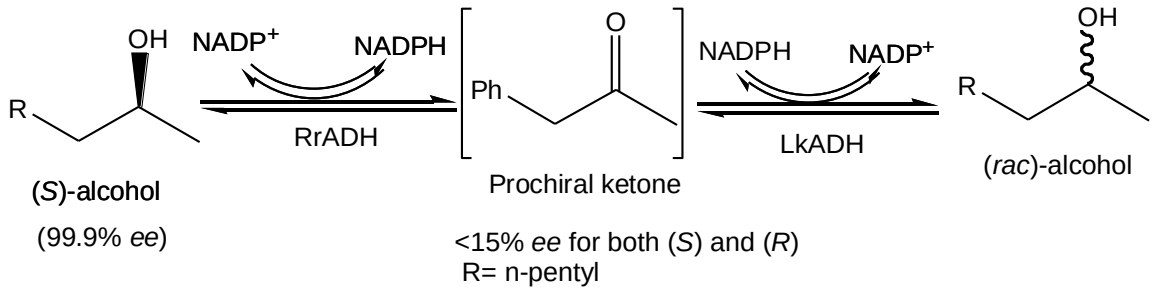
Nun et al. reported racemization of a wide range of enantiopure alcohols; including open chain, phenyl and biphenyl ring-containing pure alcohols using a ruthenium-based complex (Scheme 7) [33].



Scheme 7. Scope of the racemization reaction by [Cp*Ru(IPr)(OH)]. C_p= cyclopentyl, IPr= 2,6-diisopropylphenyl

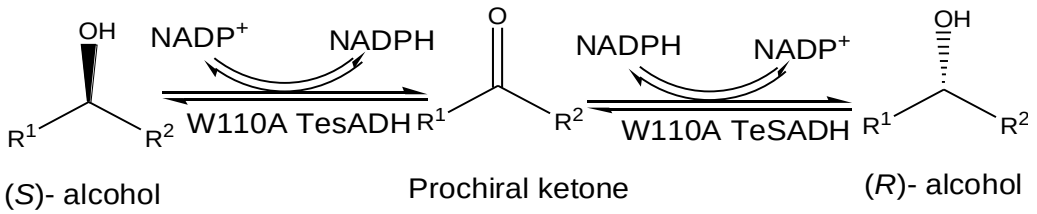
Organometallic catalyst systems have several drawbacks such as ligand decomposition under the experimental conditions employed, difficulty in product separation, production of undesirable by-products in addition to the expensive transition metals and sophisticated reaction setup which make the entire process costly and tedious. Further, as observed in Scheme 7, some catalyst systems have a limited substrate scope.

The use of enzymes presents a preferable alternative to transitional metal catalysis for being cheap and environmentally benign. Alcohol dehydrogenases are known to catalyze redox reactions interconverting alcohols and their corresponding ketones; however, high substrate stereospecificity of enzymes limits racemization. Biocatalytic racemization has several prerequisites among which; a single enzyme with incomplete stereoselectivity or two compatible highly selective enzymes with opposite stereopreference which demands a free exchange of cofactors between the enzymes. The use of two ADHs; RrADH and LkADH with opposite stereopreferences has been reported by Kroutil et al. in racemization of a range of enantiopure aliphatic and aromatic secondary alcohols [15]. The group also combined another commercially available Prelog ADH, from *Rhodococcus erythropolis* with the *anti*-Prelog LkADH in racemization (Scheme 8).



Scheme 8. Racemization using two enzymes with opposite stereopreferences.

Stereoselective reduction of prochiral ketones by W110A TeSADH and other ADHs may occur by delivering the hydride from the undesired *Si* face producing the *anti*-Prelog product, a phenomenon known as "selectivity mistake", leading to racemization (Scheme 9) [12]. Such selectivity mistakes may occur due to the reverse fit of substrates in the enzyme's active site.

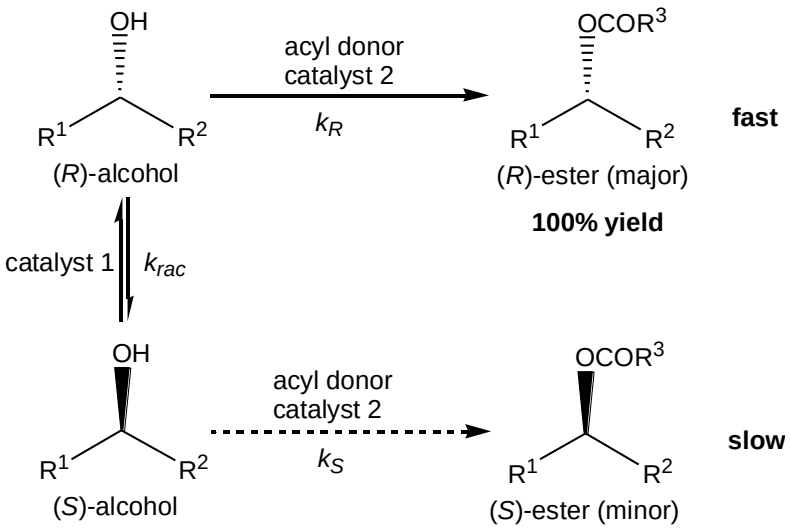


Scheme 9. Racemization by selectivity mistakes using a single enzyme.

Several mutants of TeSADH have been designed to expand substrate specificity and therefore recognize sterically hindered groups such as phenyl rings and long alkyl chains, which are not substrates for wild-type TeSADH. W110A TeSADH was used to racemize secondary alcohols with excellent outcomes, 4-15% ee was obtained starting with >99% ee (*S*) or (*R*)-1-phenyl-2-propanol. The reactions showed a high substrate and solvent dependency with barely any racemization with a few substrates [34].

1.5 Dynamic kinetic resolution of racemic phenyl-ring-containing alcohols

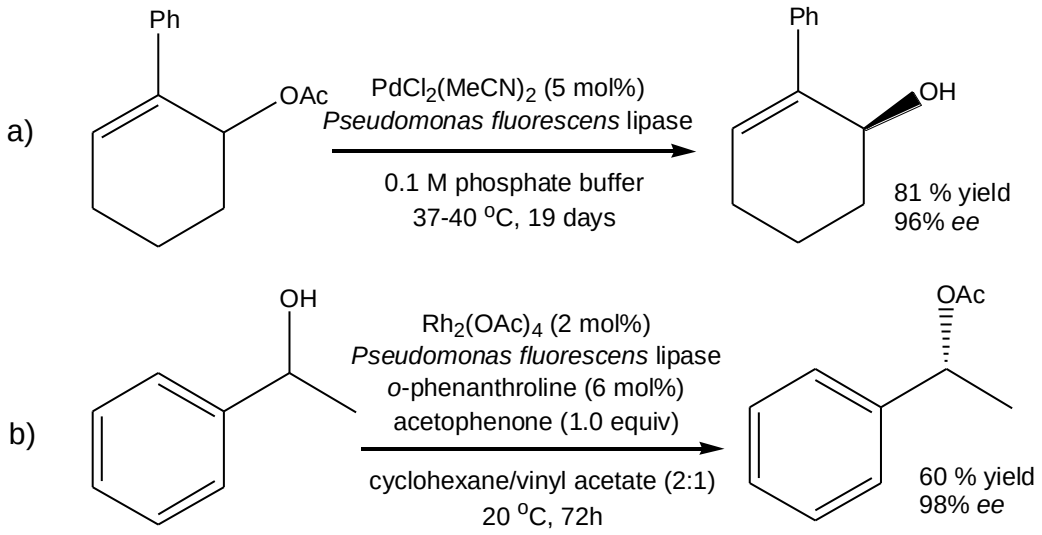
Dynamic kinetic resolution (DKR) provides an efficient synthetic route to enantiopure compounds such as alcohols, amines, amino acids and their derivatives overcoming the 50% yield by kinetic resolution (KR). The process involves simultaneous racemization with an appropriate KR, demanding a less stereoselective catalyst for racemization and a highly selective catalyst for the KR component. In order to ensure constant depletion of one enantiomer, the process requires relatively fast rate of racemization (k_{rac}), which should be faster or equal to the rate of KR of the more reactive enantiomer (k_R), and k_R and k_{rac} should be significantly faster than the rate of KR of the less reactive enantiomer (k_S) (Scheme 10) [9].



Scheme 10. Deracemization of a racemate into a single enantiomeric product by DKR.

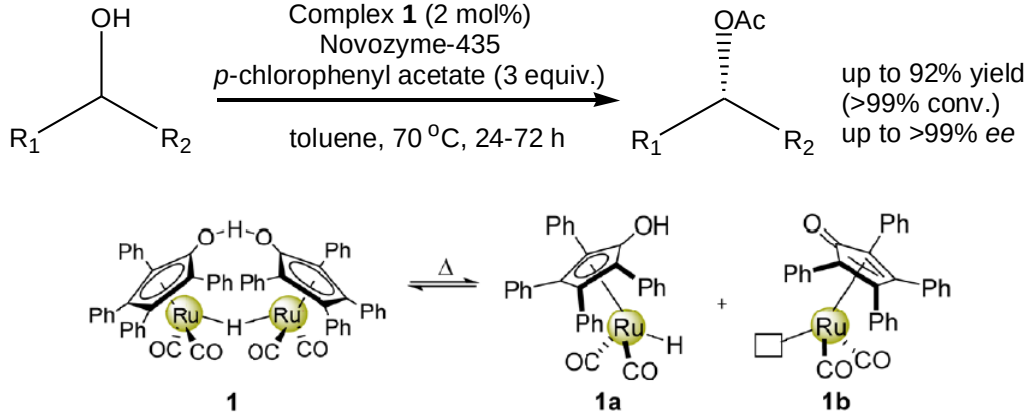
A positive entropy change thermodynamically favors racemization; however few racemases are available in nature since biocatalytic processes are highly stereospecific. Similarly, the same applies to KR where lipases and proteases are selective to (*R*)- and (*S*)-alcohols, respectively.

DKR that couples a KR with an in situ racemization is so far the most advanced deracemization approach surpassing 50% yield limit by KR to up to 100% theoretical yield. The method has mainly employed transition metal complexes [33] and chemo-enzymatic systems using palladium (Pd), rhodium (Rh) and ruthenium (Ru) complexes in combination with lipases. Non-enzymatic systems, for example, chiral ferrocene-Ru complexes [31, 35] have been employed in DKR of racemic alcohols. Early works reported by Williams' group employed $PdCl_2(MeCN)_2$ and $Rh_2(OAc)_4$ complexes to execute racemization in combination with *Pseudomonas fluorescens* lipase-catalyzed KR in deracemization of alcohols and their derivatives obtaining >95% *ee* from racemates by DKR (Scheme 11) [36].



Scheme 11. DKR employing Pd complexes and *Pseudomonas fluorescens* lipase.

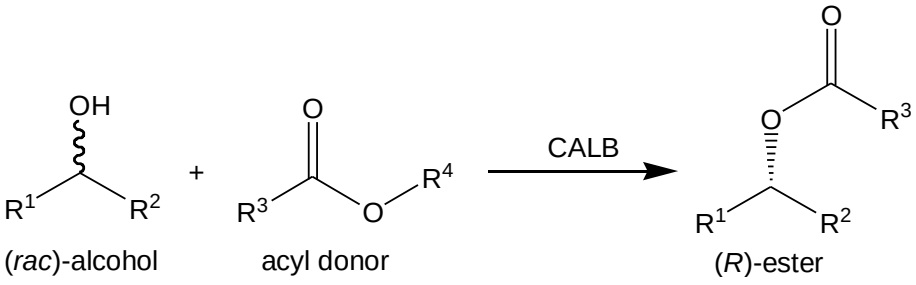
Despite the slow rate of the Williams' approach, it enlightened the possibility of combining enzymes with organometallic catalysts in DKR. Subsequent developments in attempt to develop a practical chemoenzymatic approach were conducted by Bäckvall *et al*. The Bäckvall group employed *Candida antarctica* lipase B (CALB) immobilized on acrylic resin to catalyze *R*-selective acylations with in situ racemization catalyzed by Shvo Ru-complexes (Scheme 12) [37, 38].



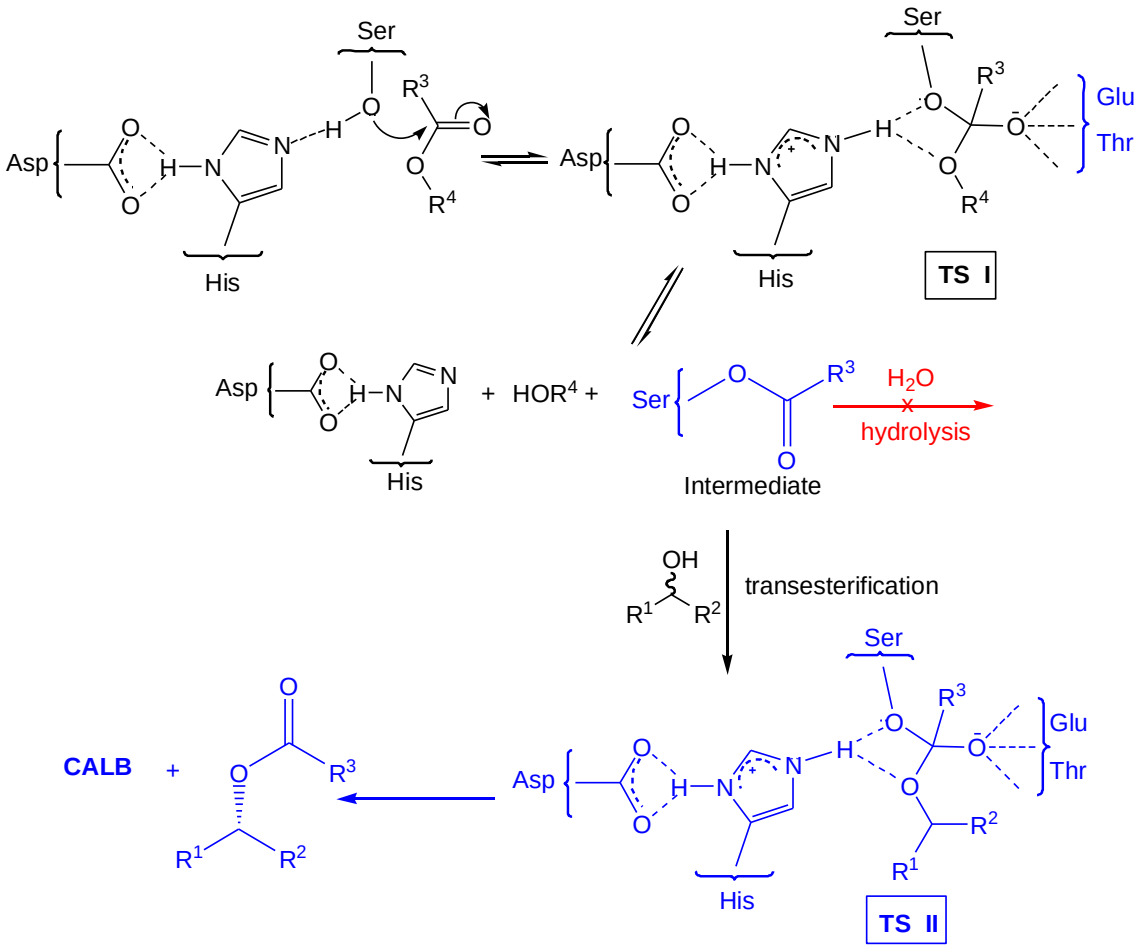
Scheme 12. DKR employing Shvo Ru-complexes and Novozyme-435

Racemization is mediated by monomeric species **1a** and **1b** (shown in Scheme 12) through an outer sphere redox mechanism. The heat requirement to generate the active species from complex **1** strictly limits this approach to thermostable lipases like Novozyme-435, the most popular lipase used in KR. Current works employing Ru-Ionic-Surfactant-Coated *Burkholderia cepacia* lipase [39] and core–shell beta–silicalite-1 micro composites with CALB [40] have been reported.

Lipases have natural tendency to select the *R*-enantiomer, nevertheless W104A CALB and proteases, for example, subtilisin have shown selectivity for the *S*-alcohols in DKR of secondary alcohols [41-43]. Lipases catalyze esterification processes (Scheme 13) and the reversible hydrolysis of the products. The enzyme has serine, histidine, and aspartate which coordinate at the active site through a series of hydrogen bonding to facilitate esterification as shown in the mechanism (Scheme 14). Transesterification strictly demands non-aqueous media. The presence of water triggers hydrolysis of the intermediate transition species and the product.



Scheme 13. CALB-catalyzed transesterification

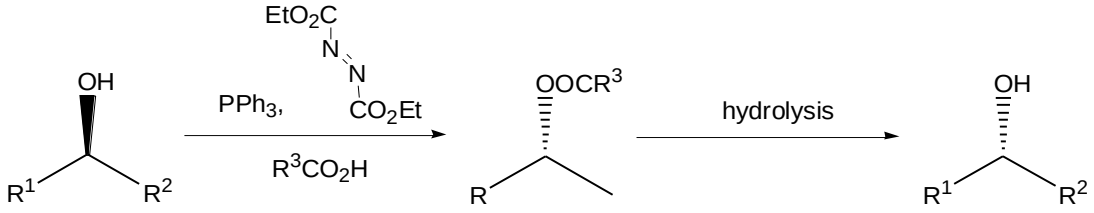


Scheme 14. Mechanism CALB-catalyzed transesterification [24].

Although both racemization and acylations proceed at very slow rates under mild conditions required by biocatalysts, this limitation could be surpassed by thermostable and organic media tolerant enzymes like W110A TeSADH in racemization in combination with Novozyme-435 (CALB). Novozyme-435 has manifested excellence in KR acylations whereas W110A TeSADH shows racemization activity in hexane [in this work] and therefore if compatible the two-enzyme combination enables a one-pot dual enzymatic DKR.

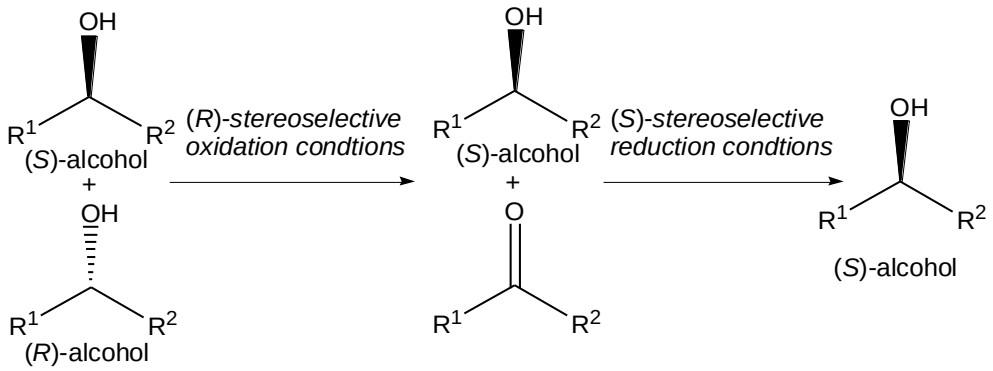
1.6 Stereoinversion of *R* configured alcohols using a single alcohol dehydrogenase

Several synthetic routes to chiral compounds have been devised considering the fact that one enantiomer is more effective in applications that demand optically active compounds, for instance, in drug delivery and activity [1, 2]. Enantiopure alcohols have been synthesized by asymmetric hydrogenation of the corresponding ketones [44] and from racemates by enantioconvergence processes that alter or retain configuration through opposite stereochemical pathways [9, 28-30]. A transformation of interest is one which inverts the configuration of one chiral alcohol to the opposite enantiomer. The outstanding methodology employed in inversion of stereochemistry is the Mitsunobu reaction [45] (Scheme 15), which since its invention has been applied in myriad syntheses [46].



Scheme 15. Mitsunobu stereoinversion of secondary alcohols.

Stereoinversion has been employed in deracemization methods using one [47, 48] or two catalysts [49]. Although several approaches employ enzymes to mediate deracemization via stereoinversion (Scheme 16), the use of multiple enzymes is inevitable and in some cases whole cells are used.



Scheme 16. Deracemization of alcohols via stereoinversion catalyzed by the multiple enzymes.

Single enantiomeric alcohols were achieved through concurrent oxidation and reduction by Kroutil and co-workers. They employed alcohol dehydrogenases (ADHs) with opposite stereopreferences [50, 51]. DSM 43066 ADH from *Rhodococcus erythropolis* (ReADH) for (*S*)-selective oxidation coupled with (*R*)-selective reduction of the intermediate ketone by LkADH to obtain the *R*-enantiomer, whilst the *S*-enantiomer was achieved by cells of *Alcaligenes faecalis* in (*R*)-oxidation and (*S*)-selective reduction by commercial *R. ruber* DSM 44541 ADH-A and ReADH [52]. Microorganisms have also been employed in deracemization. The bacterium *Sphingomonas paucimobilis* was successfully used to resolve 1-heteroaryl and 1-aryl-2-propanols where the (*S*)-enantiomer was oxidized to a ketone [53]. *S. paucimobilis* NCIMB 8195 successfully deracemized a wide range of a similar class of substrates to the (*R*)-enantiomer [54]. Benerjee and co-workers reported a one-pot stereoinversion of (*rac*)-1-(1-naphthyl)ethanol to its (*R*)-configured alcohol by stereoinversion using *Candida parapsilosis* [55]. Very recently, Rodrigues and co-workers used cells of *Candida albicans* CCT 0776 to deracemize 1-(4-substitutedphenyl)-1,2-ethanediol and its derivatives to the corresponding (*S*)-alcohols [56]. Multi-enzymatic approach suffers from problems associated with the incompatibility of the enzymes and difficulty in elucidating their reaction mechanism. In this work, focus was not only

on a method that employs a single enzyme in deracemization of racemates but also a single enzymatic protocol for stereoinversion of *R* configured enantiopure alcohols.

1.7 Statement of the problem

Optically active secondary alcohols have been achieved by asymmetric reduction of prochiral ketones or by KR of racemic alcohols. Dynamic kinetic resolution has shown to be an efficient alternative to KR. Racemization, the key step in a successful DKR, has been catalyzed by Ru, Ir or Fe-based complexes which are not only expensive but also employ sophisticated and less environmentally friendly reaction setups. The existence of racemases in nature is hindered by the high stereospecificity of biological systems. Nevertheless, ADHs have shown the potential to be used as racemases by taking advantage of the selectivity mistakes during interconversion between the alcohol and the ketone, which lead to a racemic mixture when reactions are given the sufficient time. W110A TeSADH was used in racemization of enantiopure phenyl-ring-containing alcohols; however, it was not effective for a wide spectrum of substrates. The limited substrate scope of TeSADH necessitates the design of new mutants with larger pockets which will definitely enhance the performance in racemization of enantiopure alcohols. Improving the activity of TeSADH in racemization will allow its use in situ with a CALB-catalyzed KR to achieve a dual enzymatic DKR. The compatibility of the two processes is however not achieved easily as the two enzymes work best in contrasting media. ADH-catalyzed racemization gives excellent results in aqueous media, which is required to maintain enzyme shape and activity, thus restricting its use in an organic solvent which also presents a problem of substrate solubility. To the contrary, lipase-catalyzed acylations require non-aqueous media for esterification. Regarding deracemization by purified enzymes, most of the work reported employ whole cells or more than

two enzymes with opposite stereopreferences. Challenges arise in ascertaining the mechanism when using whole cells and compatibility is paramount in cases where multiple enzymes operate in diverging reaction conditions which necessitate a single enzyme for the entire job. The success of ADH-catalyzed asymmetric redox reactions of phenyl-ring-containing alcohols is still a challenge not only because of the need for new mutants with extended pockets to expand substrate scope but also the need for further investigations on the reaction media and its effect on stereochemistry in oxidation and reduction as well as the compatibility of TeSADH-catalyzed racemization and CALB-catalyzed KR.

1.8 Objectives of the Study

The main objective of the study is to develop an efficient biocatalytic reaction system for the synthesis of single enantiomeric phenyl-ring-containing alcohols. The detailed objectives of the research are to:

i. study the effect of reaction media on stereoselectivity of TeSADH-catalyzed redox reactions of phenyl-ring-containing alcohols and their corresponding ketones,
ii. use site-directed mutagenesis to construct TeSADH mutants,
iii. study the effect of the new mutations of TeSADH in expanding substrate specificity of TeSADH,
iv. study the effect of new mutations of TeSADH on the stereoselective reduction of phenyl-ring-containing ketones,
v. conduct racemization of enantiopure (*R*)- and (*S*)-1-phenyl-2-propanol using W110A/I86G and W110G/I86G TeSADH mutants and compare their racemization activity with that obtained by W110A and W110G TeSADHs,

vi. employ the optimized reaction conditions using the best racemization mutant in (v) in racemization of other enantiopure phenyl-ring-containing alcohols,

vii. optimize the reaction conditions for racemization of enantiopure phenyl-ring-containing alcohols by using xerogel-encapsulated TeSADH mutant which has the best racemization activity,

viii. check the compatibility of mutant(s) TeSADH-catalyzed racemization of enantiopure alcohols with CALB-catalyzed KR in an effort to achieve a dual enzymatic DKR,

ix. optimize reaction condition for less selective oxidation of racemic alcohols and selective (*S*)-reduction of prochiral ketones to achieve single enantiomers by deracemization and stereoinversion,

x. isolate and characterize the products by polarimetry, gas chromatography (GC), mass spectrometry (MS), infrared (IR), and proton (^{1}H) and carbon-13 (^{13}C) nuclear magnetic resonance.

CHAPTER TWO

MEDIUM ENGINEERING OF TESADH CATALYZED REACTIONS

2.1 Medium effects on enzyme-catalyzed reactions

The percent conversion in a reaction is an indication of the activity of a particular enzyme. The reverse is not true since the reaction depends on several external factors in addition to the compatibility of a particular substrate with an active enzyme. The chemoselectivity of an enzyme observed from enantioselectivity of the products and stereopreference of chiral reactants not only depends on enzymes conformation and substrate's nature but is also greatly influenced by medium parameters such as temperature, pH and cosolvent all of which might directly or indirectly influence enzyme-substrate interaction by altering its active configuration.

The catalytic activity of an enzyme often varies depending on the reaction medium. An aqueous medium is a natural environment for ADHs which have also exhibited tolerance to organic medium [20]. The interaction of aqueous media with enzymes often changes their conformation which in turn influences the chemoselectivity. The multiple interactions make it difficult to come up with a general trend that correlates enzyme enantioselectivity and the reaction medium. For instance, water activity did not affect enantioselectivity of lipase-catalyzed esterification while an inverse relationship was observed in some reactions [57, 58]. More

important are organic solvents which enable enzymatic processes on a large scale in addition to coupling enzymatic processes with other catalytic systems.

Solvation of substrates and products by organic solvents slows enzyme catalyzed reactions and inhibitory effects can occur in cases where the solvent interacts with the enzyme. Solvents like DMSO, formamide, ethylene glycol and other polar solvents like methanol can dissolve more than 10 g/L of the enzyme which disrupts the enzymes tertiary structure resulting to inactivation [59, 60]. Interfacial tensions of the solvents have been found to inactivate enzymes with less rigid structures and the effect shows an inverse relationship with solvent hydrophobicity, log P [61]. More of interest is interactions affecting reaction equilibrium where the solvent also acts as a cosubstrate. The enantioselectivity of reduction of 2-butanone was found to increase upon increasing 2-propanol concentration [16]. A rationale to explain the outcome suggests an interaction between predominant *S*-product and TbADH-$NADP^+$ complex at a low cosubstrate concentration which lowers the *ee.* At higher cosubstrate concentration, the 2-propanol rather interacts with the enzyme-cofactor intermediate complex leaving much of the *S*-enantiomer thus increasing both the *ee* and conversion.

In this work, the effect of different water miscible and water immiscible organic solvents on enzyme activity and stereoselectivity were investigated. Diverse solvent/cosolvents systems were employed in racemization, oxidation, and reduction reactions. The enzyme was immobilized by encapsulation in a xerogel for reactions in the pure organic medium.

2.2 Experimental procedures

2.2.1 General

4-phenyl-2-butanone (**2a**), 4-(4'-methoxyphenyl)-2-butanone (**2b**), 4-(4'-hydroxyphenyl)-2-butanone (**2c**), 4-phenyl-3-butyn-2-one (**2d**), 4-phenyl-3-buten-2-one (**2e**), 1-(3ˈ-trifluoromethylphenyl)-2-propanone (**2f**), 1-(4'-chlorophenyl)-2-propanone (**2g**), 1-phenyl-2-butanone (**2i)**, (*rac*)-1-phenyl-2-propanol [(*rac*)-**1g**], and $NADP^+$ were used as purchased from Sigma-Aldrich; commercial grade solvents and other reagents were purchased from Sigma-Aldrich and used without further purification. (*rac*)-4-Phenyl-butanol [(*rac*)-**1a**], (*rac*)-4-(4ˈ-methoxyphenyl)-2-butanol [(*rac*)-**1b**], (*rac*)-4-(4ˈ-hydroxyphenyl)-2-butanol [(*rac*)-**1c**], (*rac*)-4-phenyl-3-butyn-2-ol [(*rac*)-**1d**], (*rac*)-4-phenyl-3-buten-2-ol [(*rac*)-**1e**] and (*rac*)-1-(3ˈ-trifluoromethylphenyl)-2-propanol [(*rac*)-**1f**] were synthesized by $NaBH_4$ reduction of their corresponding commercially available ketones as described [62].

2.2.2 General procedure for the oxidation of phenyl-ring-containing alcohols using mutants of TeSADH

The (*rac*)-alcohol (0.03 mmol) was added to a mixture containing enzyme (0.2 mg), $NADP^+$ (1.0 mg), acetone and Tris-HCl (50 mM, pH 8.0) in a 1.5-mL Eppendorf tube. The mixture was shaken at 180 rpm in a thermostat-controlled shaker at 50 °C for 24 h. The amount of acetone which is a cosubstrate in oxidation was varied (1-10% v/v) to obtain the optimal conditions. The effect of water-miscible and water-immiscible cosolvents on the activity and stereoselectivity of the enzymes in oxidation was studied by introducing a second cosolvent (25% v/v) into the reaction mixture.

2.2.3 General procedure for the reduction of phenyl-ring-containing ketones using mutants of TeSADH

The ketone (0.05 mmol)] was added to a mixture that contained enzyme (0.1 mg), $NADP^+$ (1.0 mg), 2-propanol and Tris-HCl buffer (50 mM, pH 8.0) in a 1.5-mL Eppendorf tube. The mixture was shaken at 180 rpm at 50 °C for 24 h. The amount of 2-propanol was varied (1-70%, v/v) to obtain the optimal conditions. The effect of water-miscible and water-immiscible cosolvents on the activity and stereoselectivity of the enzymes in reduction was studied by introducing a second cosolvent (25% v/v) into the reaction mixture.

2.2.4 Optimization of oxidation and reduction reactions

The optimal conditions for the reaction were determined by investigating the effect of the individual components of the reaction system. The enzyme load, coenzyme ($NADP^+$), cosubstrates (acetone and 2-propanol), temperature and time were varied accordingly to obtain the maximum activity and desired stereoselectivity of the enzymes.

2.2.5 Sample treatment prior analysis

The reaction mixture was extracted with diethyl ether (2 × 500 µL). The combined organic layers were dried over Na_2SO_4 and concentrated. For *ee* and percent conversion determination, the alcohols were derivatized by treatment with acetic anhydride (one drop) and pyridine (two drops) for 6 h prior to their analysis by chiral GC.

2.2.6 Characterization of reaction products

Capillary gas chromatography (GC) measurements were performed on Agilent 7890A gas chromatograph equipped with a flame ionization detector and an Agilent HP-Chiral-20B column (30 m, inner diameter 0.32 mm, film thickness 0.25 μm) using helium as the carrier gas. The following temperature program was used for the oven: 70 °C (initial, hold time 10 min) to 180 °C (final, hold time 20 min) at a rate of 5 °C/min. The injector temperature was 220 °C and the detector temperature was 230 °C. The gas flow rates for air, H_2, and He were 300, 30, and 15 mL/min, respectively. The split mode with a split ratio of 10:1 was used (Appendix A).

GC-MS analyses were performed using an instrument combined with an Agilent 5975C inert mass-selective detector with a triple- Agilent GC 7890A axis detector. The following temperature program was used for the GC oven: 70 °C (initial, hold time 10 min) to 180 °C (final, hold time 20 min) at a rate of 5 °C/min. The injector temperature was 220 °C and the detector temperature was 230 °C. The gas flow rates for air, H_2, and He were 300, 30, and 15 mL/min, respectively. The split mode with a split ratio of 10:1 was used (Appendix B).

NMR spectra were recorded on a JOEL JNM-LA500 FT NMR at 500 MHz (^{1}H) and at 125 MHz (^{13}C) at room temperature, using deuterated chloroform ($CDCl_3$) peak as an internal standard (Appendix C).

Infrared (IR) spectra were recorded using a NICOLET 6700 FT-IR spectrophotometer (Appendix D).

The *E* values were calculated according to the relationship [63]:

$$E = \frac{\ln[(1-c)(1-ee_s)]}{\ln[(1-c)(1+ee_s)]}$$ Equation 1

where *c* is percent conversion and ee_s is the *ee* of the unreacted substrate.

2.3 Results and Discussion

2.3.1 The effect of temperature and pH on oxidation and reduction

The effect of temperature on the percent conversion of the reaction was investigated by comparing oxidation and reduction reactions using 4-phenyl-2-butanol and 4-phenyl-2-butanone as model substrates. The reduction reaction proceeded to the same extent (99% conv.) at both room temperature (20 ^{o}C) and (50 ^{o}C) whereas the oxidation was slow at room temperature (21% conv.) but significantly improved (72% conv.) at 50 ^{o}C (Figure 4).

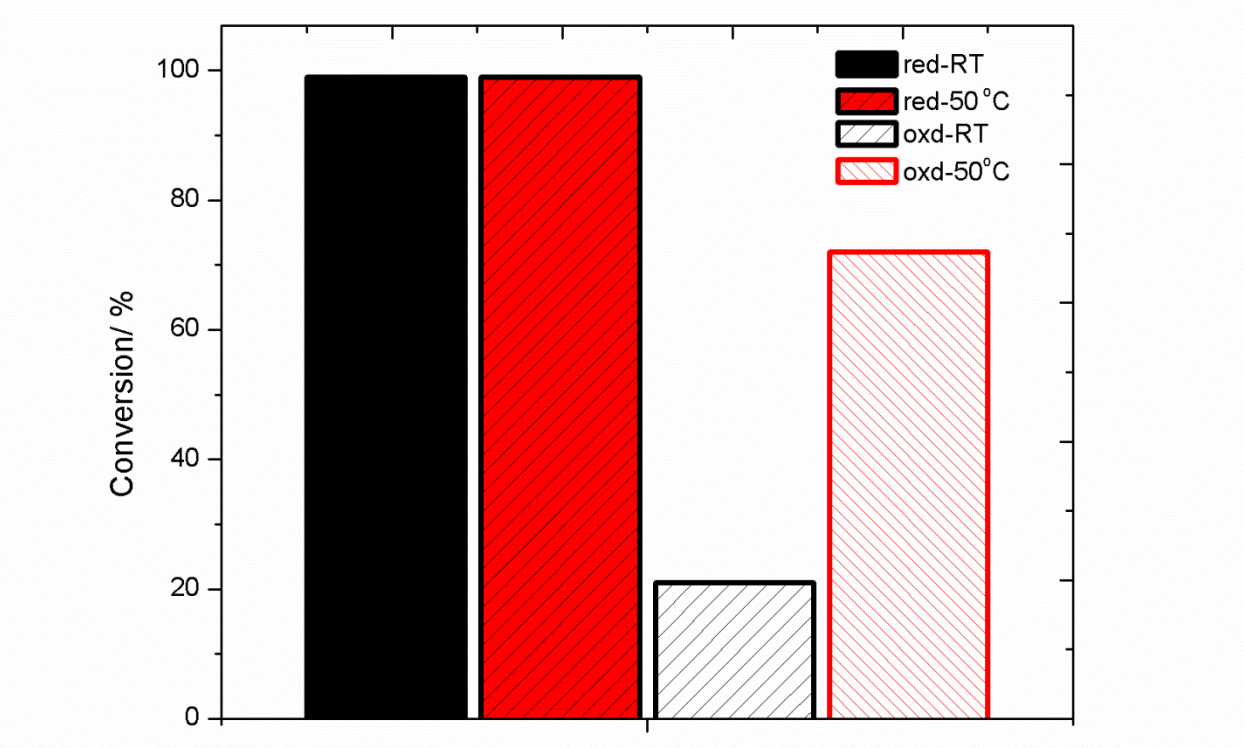
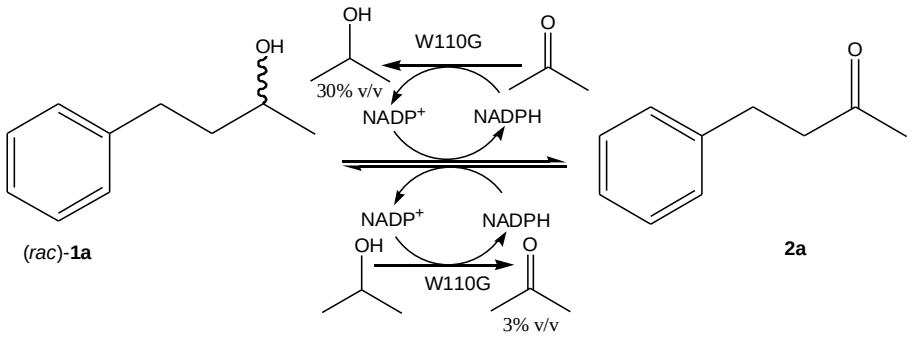


Figure 4. Comparison of oxidation and reduction reaction at room temperature and 50 ºC.

The effect of pH was investigated in the range 6.0 to 8.5 (Table 1). There was no detectable reaction at low pH (6.0) (Table 1, entry 1); this is attributed to the deactivation of the enzyme as anticipated for proteins in general since it alters the shape of the active site, which partly relies on hydrogen bonding. No significant difference in conversion was observed between pH 7.5 and 8.5, nevertheless, the W110A TeSADH stereoselectivity in oxidation reduced at pH 7.5 and 8.5 compared to 8.0(E = 1.0 vs 2.1) which concurred with the *ee* (94 vs 90%) at pH 8.0 vs 8.5 (Table 1, entries 2-4). In Table 1 are shown the conversions and stereospecificity of the oxidation of 4-phenyl-2-butanol as well as the conversion and stereoselectivity of the reduction of 4-phenyl-2-butanone using W110G TeSADH.

Table 1. The influence of pH on oxidation and reduction of 4-phenyl-2-butanol and 4-phenyl-2-butanone, respectively using W110G TeSADH.



		Oxidation[a]			Reduction[b]	
Entry	pH	Conv [%]	*E*(*S*/*R*)	*ee* [%]	Conv [%]	*ee* [%]
1	6.0	ND	NA	NA	ND	NA
2	7.5	82	1.0	4(*R*)	96	94(*S*)
3	8.0	72	2.1	46(*R*)	98	94(*S*)
4	8.5	82	1.0	2(*R*)	99	90(*S*)

Reaction conditions: oxidation performed using (*rac*)-**1a** (0.03 mmol)], W110G TeSADH (0.1 mg), $NADP^+$ (1.0 mg) and acetone (3%, v/v) in Tris-HCl buffer (pH 8.0, 50 mM, 97% v/v). [b]The reduction reaction was performed using **2a** (0.05 mmol)], W110G TeSADH (0.1 mg), $NADP^+$ (1.0 mg) and 2-propanol (30%, v/v) in Tris-HCl buffer (70% v/v). The reactions were carried independently.

2.3.2 Medium effect on the stereoselectivity and activity of TeSADH mutants

The effect of the cosubstrates and cosolvents on the activity and stereoselectivity of TeSADH mutants was investigated using various concentrations of the cosolvents (acetone and 2-propanol also function as cosubstrates) in the oxidation and reduction of 4-phenyl-2-butanol and 4-phenyl-2-butanone, respectively.

2.3.2.1 Effect of 2-propanol on the stereoselectivity and activity of W110G TeSADH-catalyzed reduction

The effect of 2-propanol on the stereoselectivity and activity of TeSADH was investigated in the reduction of 4-phenyl-2-butanone using W110G TeSADH (Figure 5). There was an exponential increase in conversion and activity between 1 and 10% (v/v) of 2-propanol (63-96% conv & 39-80% *ee S*). The *ee* steadily increased with the increase in the concentration of 2-propanol because the equilibrium is pushed in the forward reaction and hence reducing selectivity mistakes. However, the conversion sharply dropped at higher concentrations of 2-propanol (>30%, v/v) which is attributed to the precipitation of the enzyme by the cosubstrate.

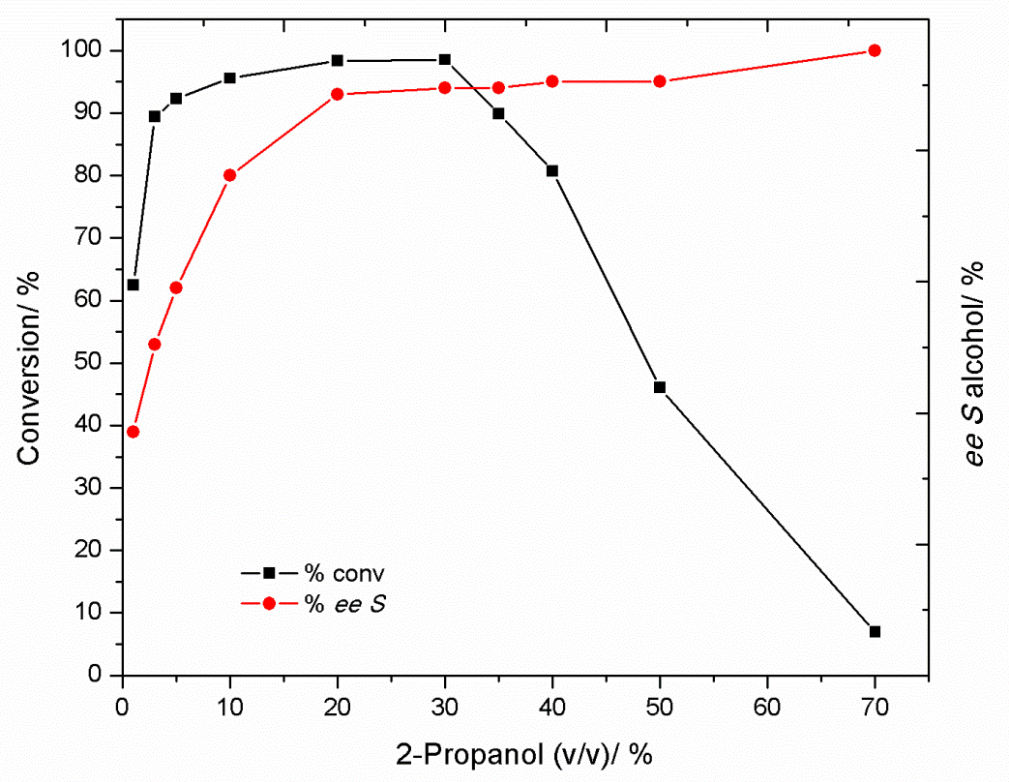


Figure 5. The effect of 2-propanol concentration on stereoselectivity and activity of the W110G TeSADH.

The influence of reaction dynamics on the stereoselectivity was further investigated by monitoring the reaction at a low concentration of 2-propanol (3%, v/v) to allow the reverse reaction (oxidation) and hence increase chances of selectivity mistakes. The conversion and *ee* at 30 ^{o}C to 60 ^{o}C were observed after time intervals (Figure 6). The reductions reached a similar extent (94-96% Conv. after 24 h) at all temperature conditions, however, the stereoselectivity varied greatly with temperature. The *ee* dropped sharply at 60 ^{o}C in the first 3 h (25% *ee*) compared to 49-73% *ee S*-alcohol in the same timeframe at a temperature between 30 and 50 ^{o}C. The sharp drop in *ee* observed at 60 ^{o}C after 3 h is attributed to fast kinetics at elevated temperatures which increased selectivity mistakes whereas the fairly maintained *ee* are likely due to a shortage of active enzyme to proceed the reaction suggesting some of the enzyme was denatured despite the heat tolerance by TeSADH. It was clearly manifested that the least stereoselective conditions can be achieved at low temperatures (2% *ee* at 40 ^{o}C vs. 18% *ee* at $\geq$ 50 ^{o}C) as shown in Figure 6. This could be due to the prolonged enzyme life at low temperatures under the less stereoselective medium conditions (3% of 2-propanol, v/v) used.

The effect of cosubstrates on the stereoselectivity was further investigated at 2-propanol (5 vs 30% v/v) in the reduction of selected ketones (Figure 7). Results are in agreement with the previously obtained with high *ee* at 30% (v/v) for **2a**, **2b** and 4-chlorophenylacetone (**2g**) while **2c** and **2d** were reduced with the same *ee* (>99%) regardless of the cosubstrate concentrations which is attributed to the possibility of a fixed alignment of the two substrates within the active site of W110G TeSADH as already discussed.

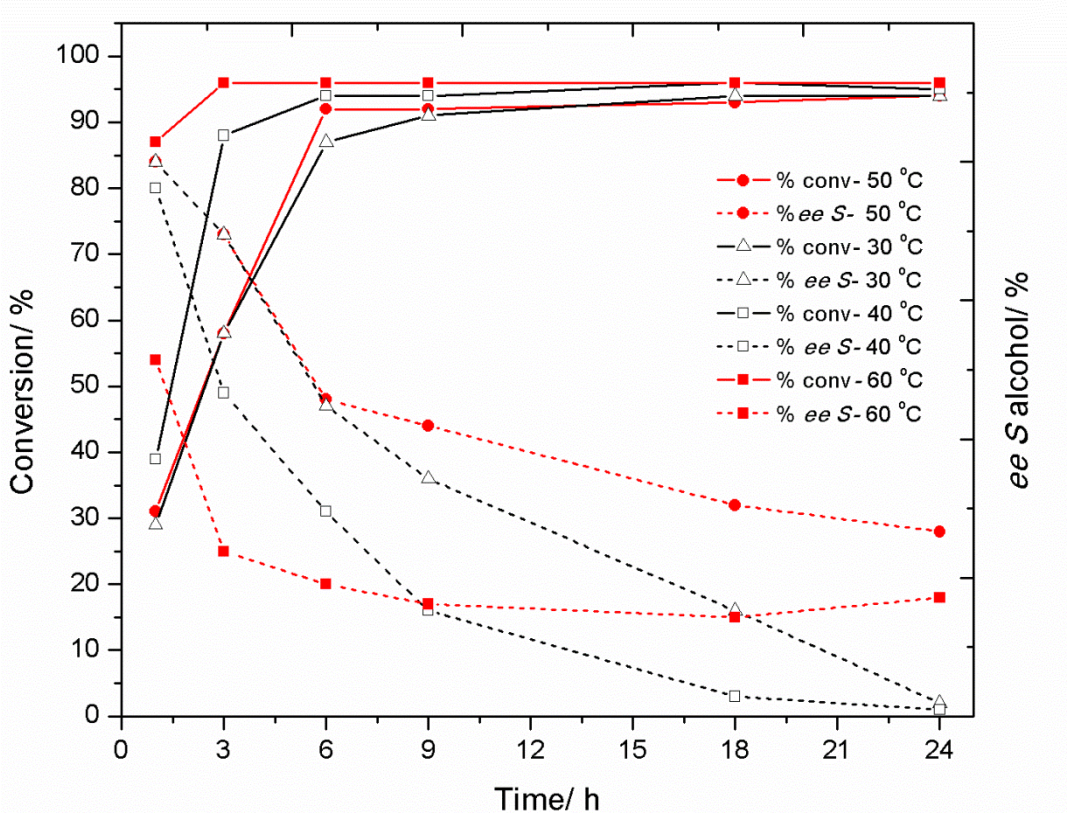


Figure 6. The effect of temperature on the activity and stereoselectivity of W110A TeSADH-catalyzed reduction of 4-phenyl-2-butanone in low stereoselective conditions (3% volume 2-propanol)

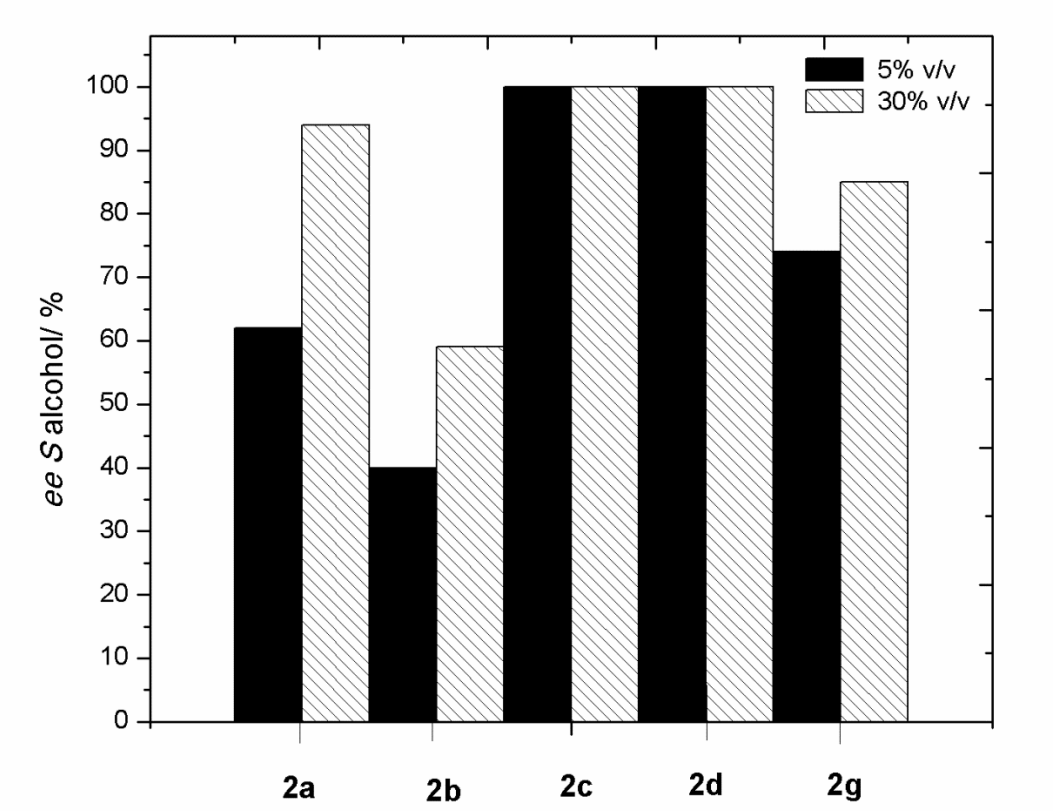
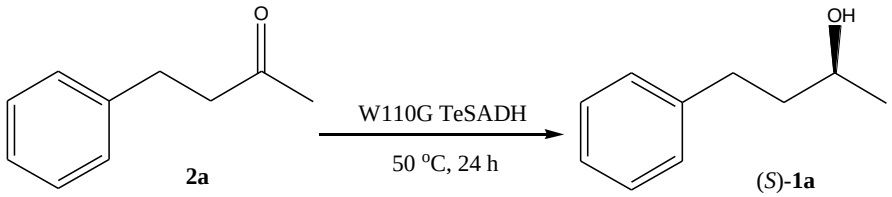


Figure 7. The stereoselectivity and reactivity of selected ketones using 2-propanol (5 and 30% v/v). Reactions were performed using **2a-g** (0.03 mmol), W110G TeSADH mutant (0.05 mg), $NADP^+$ (1.0 mg) in Tris-HCl/ 2-propanol at 50 ^oC for 24 h.

2.3.2.2 The effect of organic cosolvents on the stereoselectivity and activity of W110G TeSADH-catalyzed reduction

Table 2. The influence of water-miscible organic solvents on the activity and stereoselectivity of W110G TeSADH-catalyzed reduction of 4-phenyl-2-butanone.



2a (*S*)-**1a**

Entry	Cosolvent	LogP	pKa	Alcohol [%]	*ee* [%]
1	Tris-HCl	NA	NA	92	62
2	2-propanol	0.05	16.5	99	94
3	acetonitrile	-0.33	25.0	14	94
4	MeOH	-0.69	15.5	92	85
5	EtOH	-0.31	15.9	88	91
6	t-BuOH	0.58	16.5	65	93
7	DMSO	-1.35	35.0	79	81
8	ethylene glycol	-1.35	14.2	94	75
9	1,4-dioxane	-1.10	NA	69	93
10	DMF	-0.83	NA	82	89
11	THF	0.46	NA	0	NA

Reaction conditions: **2a** (0.03 mmol), W110G TeSADH mutant (0.05 mg), $NADP^+$ (1.0 mg) in Tris-HCl (pH 8.0, 50 mM)/ cosolvent/2-propanol (70:25:5, v/v/v) at 50 °C for 24 h.

The influence of water-miscible and -immiscible organic solvents on the activity and stereoselectivity of W110G TeSADH was investigated in oxidation and reduction reactions of 4-phenyl-2-butanol and 4-phenyl-2-butanone, respectively. In Table 2 are shown the conversion and *ee* obtained in the reduction of 4-phenyl-2-butanone in media constituting Tris-HCl/2-propanol/water-miscible organic solvent (70:5:25, v/v). Solvent properties like pKa and log P have no clear correlation with the activity and stereoselectivity of the enzyme. For example, DMF and THF are both water-miscible, however; the enzyme was inactive in the latter (Table 2, entries 10 and 11). In general, except 2-propanol which plays a dual role of cosolvent and cosubstrate, the rest of the solvents reduced the enzyme's activity observed from the decrease in conversions (<98%), however, the *ee* is significantly increased (>62%). Nevertheless, for some water-miscible solvents like methanol, ethanol, ethylene glycol and *tert*-butanol, the activity and stereoselectivity of W110G TeSADH correlated with pKa and log P as illustrated in Figure 8. The conversion bears an inverse relationship whereas the *ee* increased with pKa and log P.

The effect of water immiscible solvents on the activity and stereoselectivity of W110G TeSADH was also investigated. In Table 3 are shown the conversions and *ee* obtained in the reduction of 4-phenyl-2-butanone in biphasic systems. The enzyme showed similar activity in the majority of the solvents, however, there was a decrease in stereoselectivity at high log P (93-94% *ee* vs <84% for solvents with log P<3 and >3, respectively (Table 3, entries 1-6).

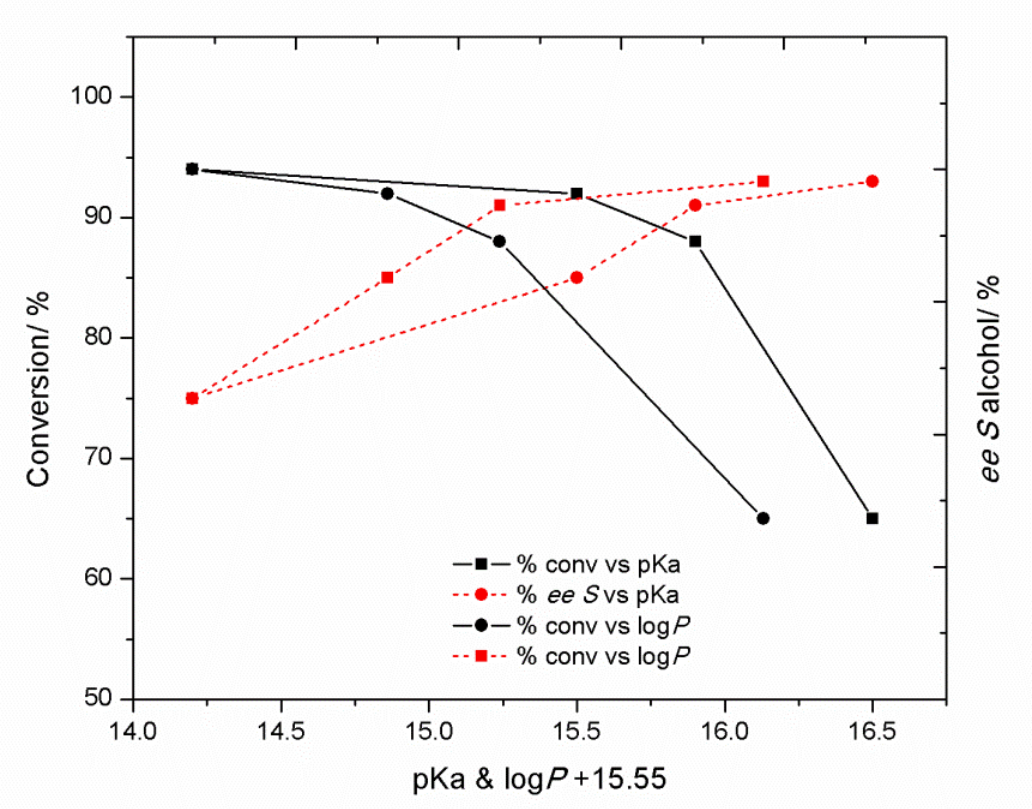
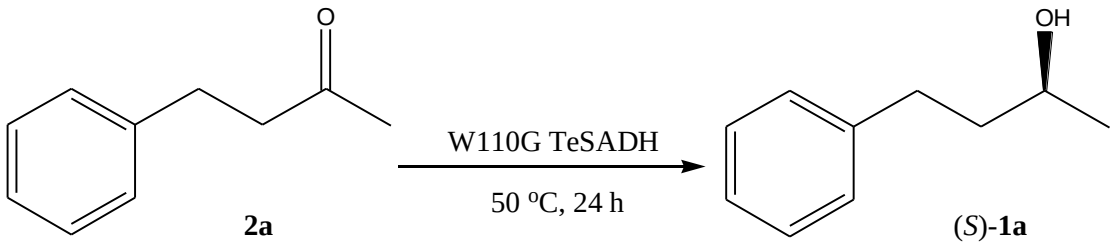


Figure 8. A correlation of log P and pKa with activity and enantioselectivity of W110G TeSADH-catalyzed reduction; pKa and log P are based on ethylene glycol, MeOH, EtOH, and *t*-BuOH.

Table 3. The influence of water-immiscible organic solvents on the stereoselectivity and activity of W110G TeSADH-catalyzed reduction of 4-phenyl-2-butanone.



2a (*S*)-**1a**

Entry	Cosolvent	LogP	Sol. [mg/L]	Alcohol [%]	*ee* [%]
1	MTBE	0.94	$2.6x10^{4}$	81	94
2	toluene	2.73	$5.2x10^{-4}$	95	93
3	hexane	3.76	9.5	77	82
4	octane	4.78	$7.0x10^{-3}$	95	79
5	2,2,4-TMP	4.37	insoluble	78	84
6	$[BMIM]PF_6$	NA	insoluble	86	84

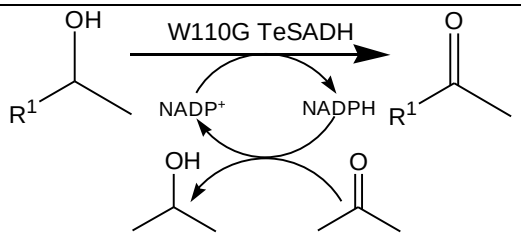
Reaction conditions: **2a** (0.03 mmol), W110G TeSADH mutant (0.05 mg), $NADP^+$ (1.0 mg) in Tris-HCl (pH 8.0, 50 mM)/ cosolvent/2-propanol (70:25:5, v/v/v) at 50 °C for 24 h. Sol. = solvent solubility in water

2.3.2.3 The effect of organic cosolvents on the stereospecificity and activity of TeSADH in oxidation reactions

The effect of organic solvents on the stereospecificity and activity of W110G TeSADH in oxidation was investigated in Tris-HCl media containing water-miscible or water-immiscible organic solvents. In Table 4 are shown the conversions obtained in the oxidation of 4-phenyl-2-butanol and 4-(4'-methoxyphenyl)-2-butanol using W110G TeSADH in Tris-HCl/ organic solvent (70:27 v/v) and acetone (3% v/v).

The stereospecificity and activity of W110G TeSADH varied greatly with the different solvents with no conclusive correlation. For instance, the oxidation of 4-(4'-methoxyphenyl)-2-butanol was highly stereospecific (*E*=2.3) in DMF as compared to less stereospecific oxidation (*E*= 1) observed in the majority of other water miscible solvents (Table 4, entries 2, 4 and 5) and hexane. It is worthy to mention that accumulation of the ketone product during oxidation largely relies on the stereoselectivity of the enzyme and the nature of the substrates.

Table 4. The influence of organic solvents on the stereoselectivity and activity of W110G TeSADH-catalyzed oxidation of 4-phenyl-2-butanol and 4-(4'-methoxyphenyl)-2-butanol.



			$R^1 = C_6H_5CH_2CH_2$			$R^1 = p$-MeO-$C_6H_4CH_2CH_2$		
Entry	Cosolvent	LogP	*E* (*S*/*R*)	Ketone [%]	*ee* [%]	*E* (*S*/*R*)	Ketone [%]	*ee* [%]
1	Tris-HCl	NA	2.1	72	46 (*R*)	1.1	92	10 (*R*)
2	EtOH	-0.31	NA	21	33 (*R*)	1.1	76	4 (*R*)
3	1,4-D	-1.10	NA	6	8 (*R*)	4.0	16	11 (*R*)
4	DMF	-0.83	1.1	86	8 (*R*)	2.3	70	50 (*R*)
5	MeOH	-0.69	1	87	>0.5	1.0	93	3 (*R*)
6	$(CH_2OH)_2$	-1.35	NA	22	35 (*R*)	1.0	92	1 (*R*)
7	hexane[b]	3.76	34.8	44	69 (*R*)	1	85	0.5>
8	toluene[b]	2.73	NA	22	48 (*R*)			
9	octane[b]	4.78	NA	42	74 (*R*)			

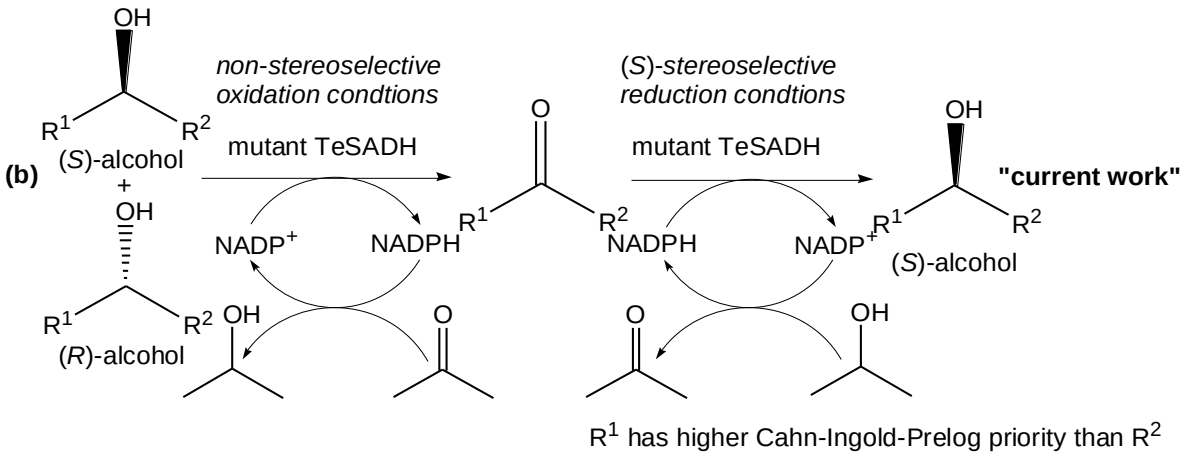
Reaction conditions: (*rac*)-alcohol (0.03 mmol), W110G TeSADH mutant (0.1 mg), $NADP^+$ (1.0 mg) in Tris-HCl (pH 8.0, 50 mM)/ water-miscible co-solvent/acetone (70:27:3, v/v/v) at 50 °C for 24 h. [b]Water immiscible co-solvents. 1,4-D=1,4-Dioxane

CHAPTER THREE

DERACEMIZATION OF PHENYL-RING-CONTAINING RACEMIC ALCOHOLS

3.1 Single enzymatic deracemization of phenyl-ring-containing racemic alcohols

In the previous investigations, it was observed that W110A and W110G TeSADH are not only capable of reducing phenyl-ring-containing ketones with high enantioselectivity [12] but also racemize the corresponding enantiopure alcohols by inducing "selectivity mistakes" [13]. In this deracemization approach, a single-enzyme-mediated two-step process was employed. The first step involved the non-stereoselective oxidation of racemic alcohols to ketones, followed by a stereoselective reduction to the corresponding *S*-configured alcohol in the same pot and without isolation of the ketone intermediate (Scheme 17). The stereoselectivity in the two reactions was controlled by varying the amounts of acetone and 2-propanol which act as cosubstrates in the oxidation and reduction reaction, respectively.



Scheme 17. Deracemization of alcohols via non-stereoselective oxidation followed by a stereoselective reduction catalyzed by the same enzyme.

3.2 Experimental procedures

3.2.1 General procedure for the deracemization and stereoinversion of phenyl-ring-containing alcohols

The (*rac*)-alcohol (0.03 mmol) was added to a mixture of TeSADH mutant (0.2 mg) and $NADP^+$ (1 mg) in Tris-HCl buffer solution (970 µL, 50 mM, pH 8.0) containing acetone (30 µL) in a 1.5 mL Eppendorf tube. The mixture was shaken at 50 °C for 24-36 h. The progress of the reaction was monitored by GC; if the oxidation was not complete, if applicable, fresh enzyme (0.1 mg) and acetone (30 µL) were added. The reduction was effected by adding a fresh enzyme (0.2 mg) and 2-propanol (30% v/v) to the same reaction vessel and the experiment was continued for 24 h.

3.2.2 Large-scale deracemization of phenyl-ring-containing racemic alcohols

A mixture of (*rac*)-alcohol (30 mg, final concentration 20 mM), W110G TeSADH (0.6 mg), and $NADP^+$ (2.0 mg) in Tris-HCl buffer solution (9.8 mL, 50 mM, and pH 8.0) containing acetone (0.2 mL) was placed in a round-bottomed flask equipped with a magnetic stirrer bar. The reaction mixture was stirred for 36 h at 50 °C at 200 rpm using a hot plate equipped with a thermocouple. After oxidation was complete (monitored by GC), 2-propanol (20 mL) and a fresh enzyme (0.4 mg) were added and the reaction mixture was stirred for 24 h. Fresh enzyme (0.2 mg) was added every 12 h during oxidation and reduction steps. The reaction mixture was extracted with diethyl ether (2 × 5 mL). The reaction mixture was derivatized prior to analysis as already described.

3.2.3 Design, synthesis and characterization of mutants of TeSADH

This section describes the design and development of single mutants, W110A, and W110G, as well as double mutants I86A/W110A, I86G/W110A and I86G/W110G. All mutants were developed by site-directed mutagenesis on the wild type TeSADH.

3.2.3.1 Construction of plasmids, gene expression, and purification of the enzymes

W110A TeSADH was constructed, expressed, and purified as previously described.[34] The mutations W110G and I86A/W110A were introduced into TeSADH using a QuikChange II site-directed mutagenesis kit (Agilent Technology) using the following primers: W110G: 5'-GGAATG CTGGCA GGCGGT AAATTT TCGAAT GTAAAA G-3' (forward) and 5'-CTTTTA CATTCG AAAATT TACCGC CTGCCA GCATTC C-3' (reverse); I86A/W110A: 5'-GTTGTG

CCAGCT GCGACC CCTGAT TGG-3', 5'-GAATGC TGGCAG GCGCGA AATTTT CGAATG-3' (forward) and 5'-CCAATC AGGGGT CGCAGC TGGCAC AAC-3', 5'-CATTCG AAAATT TCGCGC CTGCCA GCATTC-3' (reverse). The mutation sites (underlined nucleotides in the primer sequences) were confirmed by DNA sequence analysis. W110G and I86A/W110A were expressed in BL21(DE3) cells and purified as described previously, [17, 34] with some modifications.

Briefly, the TeSADH-overexpressing cells were collected by centrifugation (Sorvall RC-6 Plus centrifuge equipped with an F14-6X250Y rotor, Thermo Scientific) at 6,000 g and resuspended in lysis buffer [Tris-HCl (10 mM, pH 8.0), KCl (80 mM), 2-mercaptoethanol (5 mM), EDTA (1 mM)]. The cells were lysed using a cell disruptor (25,000 psi), and the debris was removed from the lysate by centrifugation (22,040 g for 30 min). The cleared supernatant was incubated at 85 °C for 15 min. The denatured protein was then removed by centrifugation (22,040 g for 30 min). The supernatant was loaded onto a 5 mL HisTrap FF column (GE Healthcare) and eluted using an imidazole gradient. Wild-type TeSADH, W110G TeSADH, and I86A/W110A TeSADH displayed similar behaviors throughout the purification procedure.

3.2.3.2 Molecular weight and DNA sequence of TeSADH

Inoculated cells mixed with loading dye were incubated for at 90^{o}C for 10 min. The mixture was loaded on Mini-Protean TGX Precast gels and subjected to electrophoresis using a Bio-Rad Mini Protean Tetra cell. Gel measure was done using visible light and compared to SM 0671 size marker. In Figure 9 is shown the gel diagram for W110G and I86A/W110A TeSADH showing the molecular weight of the two mutants (43 KD). Results from DNA sequencing of the purified mutants showed that the mutations were effected in W110G TeSADH (Figure 10).

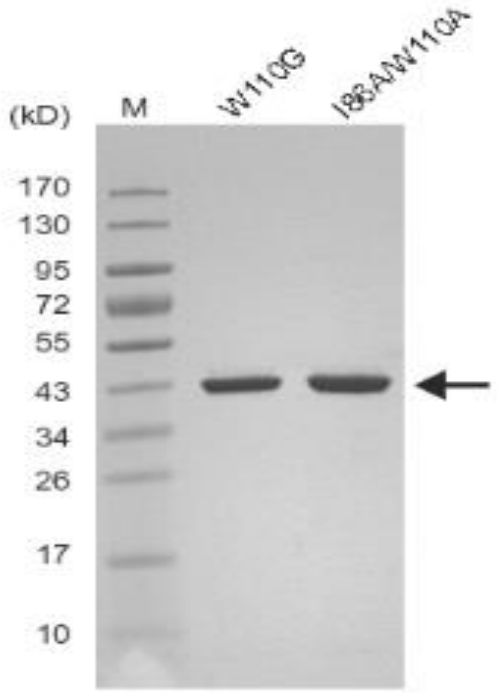


Figure 9. Gel diagram of purified W110G and I86A/W110A TeSADH.

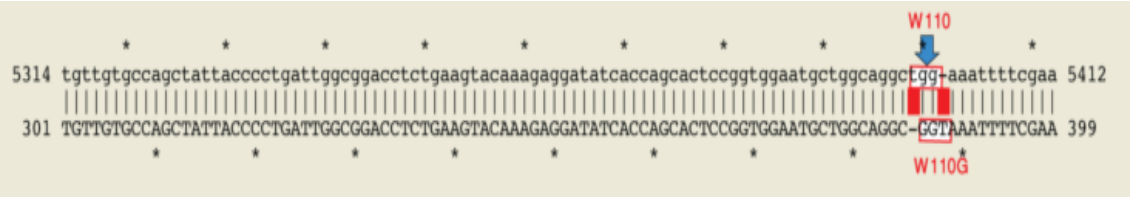


Figure 10. DNA sequence report confirming replacement of Trp-110 with Gly in W110G TeSADH mutant.

3.3 Results and Discussion

3.3.1 Comparison of TeSADH mutants in oxidation of phenyl-ring-containing racemic alcohols

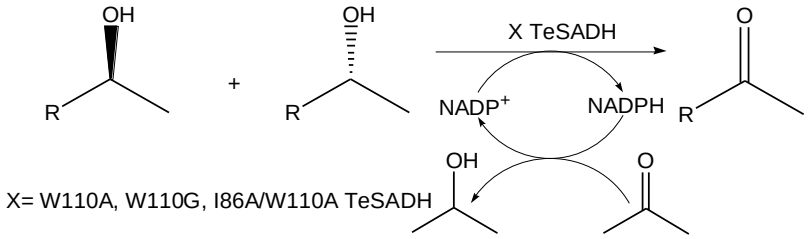
The activity and stereoselectivity of W110A, W110G, W110V and I86A/W110A were compared in the oxidation of racemic and *R* configured alcohols. In Table 5 are shown the conversion and stereoselectivity obtained in oxidation of different racemic alcohols using W110A, W110G, and I86A/W110A TeSADH mutants.

The activity of the mutants increased in the order W110G > W110A > I86A/W110A whereas a reverse trend was observed in stereoselectivity with W110G being least selective and the double mutants most selective. This was observed in the oxidation of all substrates investigated as indicated by the *E* values. The superior activity manifested by W110G TeSADH over other mutants is due to the poor enantioselectivity by this mutant. For instance, the $E \leq 2.1$ compared with >100 for W110A and I86A/W110A in the oxidation of (*rac*)-4-phenyl-2-butanol [(*rac*)-**1a**] (Table 5, entries 1, 3 and 5) which enables recognition of both enantiomers during oxidation. This is attributed to the extended large pocket at the active site of W110G TeSADH which enhances selectivity mistakes.

A contrast to this observation was recorded in I86A/W110A catalyzed oxidations which had highest selectivity (E>100) in the oxidation of (*rac*)-**1a** and (*rac*)-4-(4'-methoxyphenyl)-2-butanol [(*rac*)-**1b**] as compared to E=5.6 for W110A and negligible enantioselectivity using W110G TeSADH in the oxidation of the latter (Table 5, entries 6-8). The double mutant I86A/W110A has both the large and small pockets at the active site extended with the purpose of

lowering the stereoselectivity and enhancing the activity. The unanticipated poor performance (low conversion with high enantioselectivity) by the double mutant could be a result of the restructuring of the active site in favor of the outcome observed.

Table 5. Activity and stereoselectivity of W110A, W110G, and I86A/W110A TeSADH-catalyzed enantiospecific oxidation of phenyl-ring-containing racemic alcohols.



Entry	Mutant	Substrate	R	Ketone [%]	*ee* [%]	*E* (*S*/*R*)[c]
1	W110G	(*rac*)-**1a**	$C_6H_5CH_2CH_2$	72	46(*R*)	2.1
2	W110G	(*rac*)-**1a**	$C_6H_5CH_2CH_2$	>99[b]	-	-
3	W110A	(*rac*)-**1a**	$C_6H_5CH_2CH_2$	41	73(*R*)	>100
4	W110A	(*rac*)-**1a**	$C_6H_5CH_2CH_2$	60[b]	>99(*R*)	24
5	I86A/W110A	(*rac*)-**1a**	$C_6H_5CH_2CH_2$	11	26(*R*)	>100
6	W110G	(*rac*)-**1b**	*p*-MeO-$C_6H_4(CH_2)_2$	>99	-	-
7	W110A	(*rac*)-**1b**	*p*-MeO-$C_6H_4(CH_2)_2$	58	67(*R*)	5.6
8	I86A/W110A	(*rac*)-**1b**	*p*-MeO-$C_6H_4(CH_2)_2$	26	59(*R*)	>100
9	W110G	(*rac*)-**1c**	*p*-HO-$C_6H_4(CH_2)_2$	>99[b]	-	-
10	W110A	(*rac*)-**1c**	*p*-HO-$C_6H_4(CH_2)_2$	60[b]	99(*R*)	24
11	W110G	(*rac*)-**1f**	*m*-CF_3-$C_6H_4CH_2$	91[b]	1(*R*)	1.0
12	W110A	(*rac*)-**1f**	*m*-CF_3-$C_6H_4CH_2$	73[b]	32(*R*)	1.6
13	W110G	(*rac*)-**1h**	$C_6H_5CH_2$	80[b]	42(*R*)	1.7
14	W110A	(*rac*)-**1h**	$C_6H_5CH_2$	25	27(*R*)	12

Reaction conditions: (*rac*)-alcohol (*rac*)-alcohol (0.03 mmol), TeSADH mutant (0.1 mg), $NADP^+$ (1.0 mg), and acetone (3%, v/v). [b]TeSADH mutant (0.2 mg). [c]*E*: enantiomeric ratio, and can be calculated according to Equation 1.

3.3.2 Optimization of oxidation reaction at varying concentration of acetone

W110G TeSADH was the best choice due to its poor stereoselectivity compared to the other mutants. The oxidation reaction was optimized for the least enantioselective condition by conducting the oxidation of (*rac*)-**1a** at varying cosubstrate concentrations [1-10% (v/v) of acetone] (Figure 11). The enantioselectivity gradually increased with increase in the concentration of acetone whereas the conversion was low at 1% (v/v) acetone attributed to the slow equilibrium at low cosubstrate amounts. Increase to 3% (v/v) acetone significantly improved the reaction achieving the maximum conversion. The drop in conversion at a concentration of acetone higher than 3% (v/v) resulted from the decrease in the amount of active enzyme as protein precipitation was observed. Therefore, for the deracemization approach 3% (v/v) of acetone was optimum since low enantioselectivity and high conversion are vital. The accumulated ketone was monitored by chiral GC and GC-MS (Figure 12).

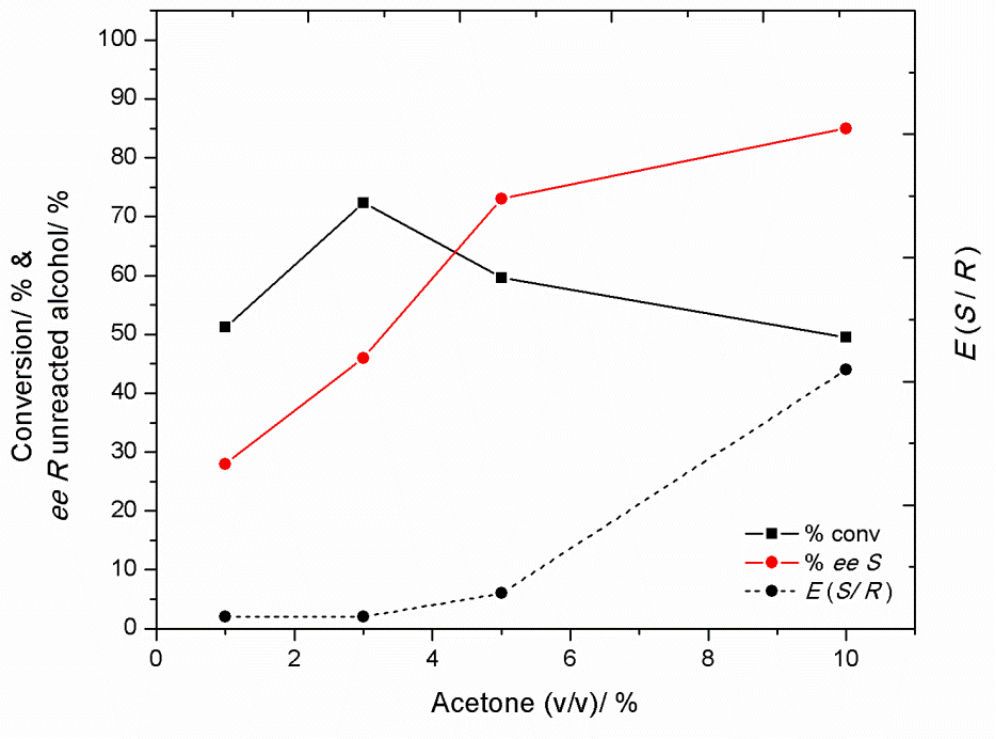


Figure 11. The effect of acetone concentration on the stereoselectivity and activity of the W110G TeSADH-catalyzed oxidation of (*rac*)-4-phenyl-2-butanol.

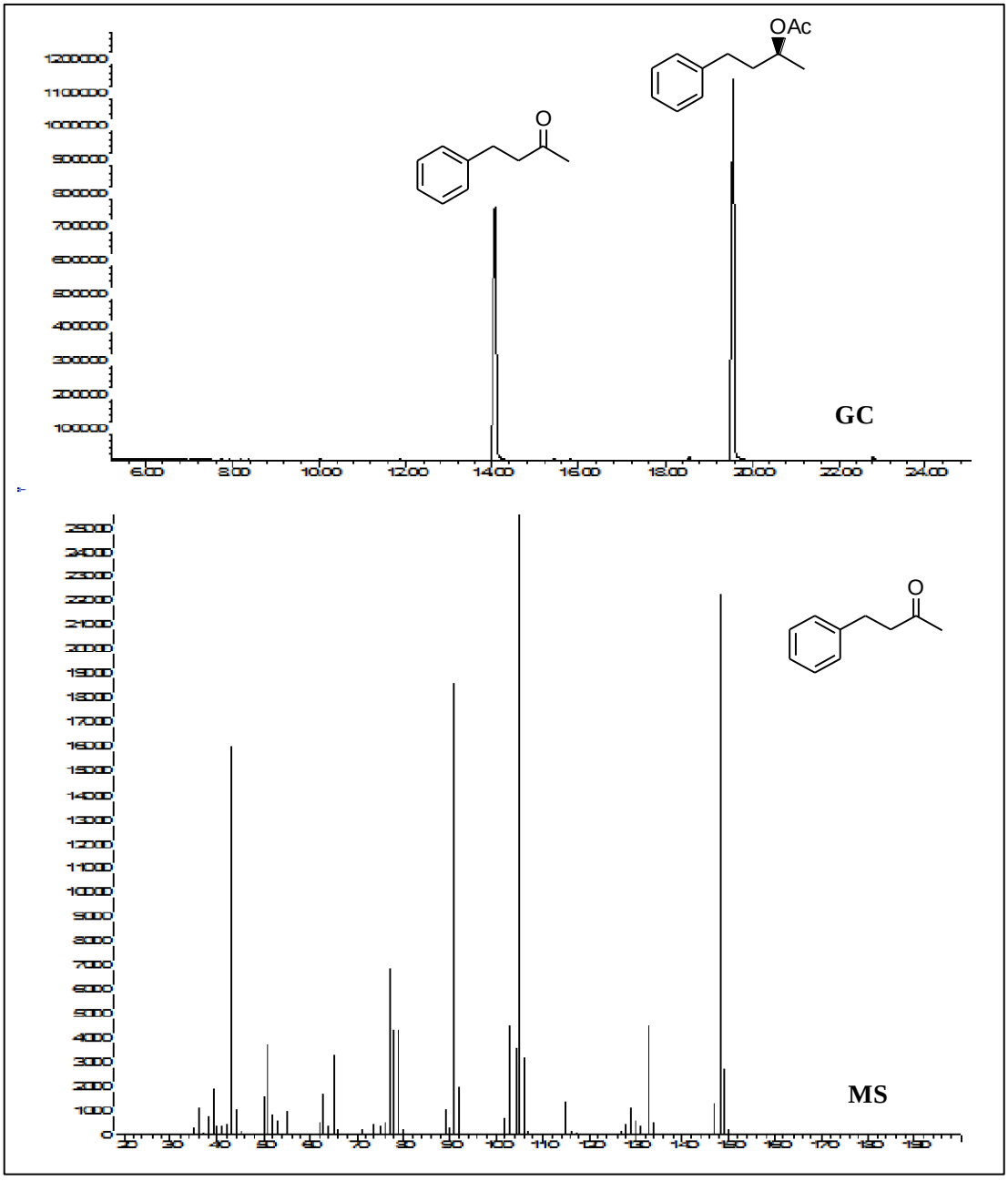


Figure 12. Gas chromatogram (top) and mass spectra of the ketone product of the oxidation of (*rac*)-4-phenyl-2-butanol with W110G TeSADH using 5% acetone.

3.3.3 Optimization of reduction reaction of phenyl-ring-containing ketones

3.3.3.1 Comparison of the stereoselectivity and activity of TeSADH mutants in reduction of 4-phenyl-2-butanone

The activity (measured as conversion) and stereoselectivity of W110A, W110G, I86A/W110A, I86G/W110A and I86G/W110G TeSADH were compared in the reduction of 4-phenyl-2-butanone in Tris-HCl/ 2-propanol (90/10, v/v). The activity was observed to increase in the order W110G > W110A > I86A/W110A while the stereoselectivity reduced in the order W110G < W110A < I86A/W110A (Figure 13). The I86G/W110A and I86G/W110G TeSADH showed no activity. The relatively low stereoselectivity of W110G as compared to W110A TeSADH is attributed to the further extension of the large pocket at the active site in the former which allows for more selectivity mistake producing the anti-Prelog alcohol and hence a reduction in *ee* (80 vs 94%) observed. I86A/W110A TeSADH showed an unanticipated higher activity than the W110A and W110G TeSADH mutants. The inactivity of I86G/W110A and I86G/W110G TeSADH is likely due to alteration of the active site which makes it inactive. There is also a possibility of losing the Zn^{2+} necessary to maintain the active site. Attempts to reactivate the double mutants by the addition of Zn^{2+} from an external source were futile.

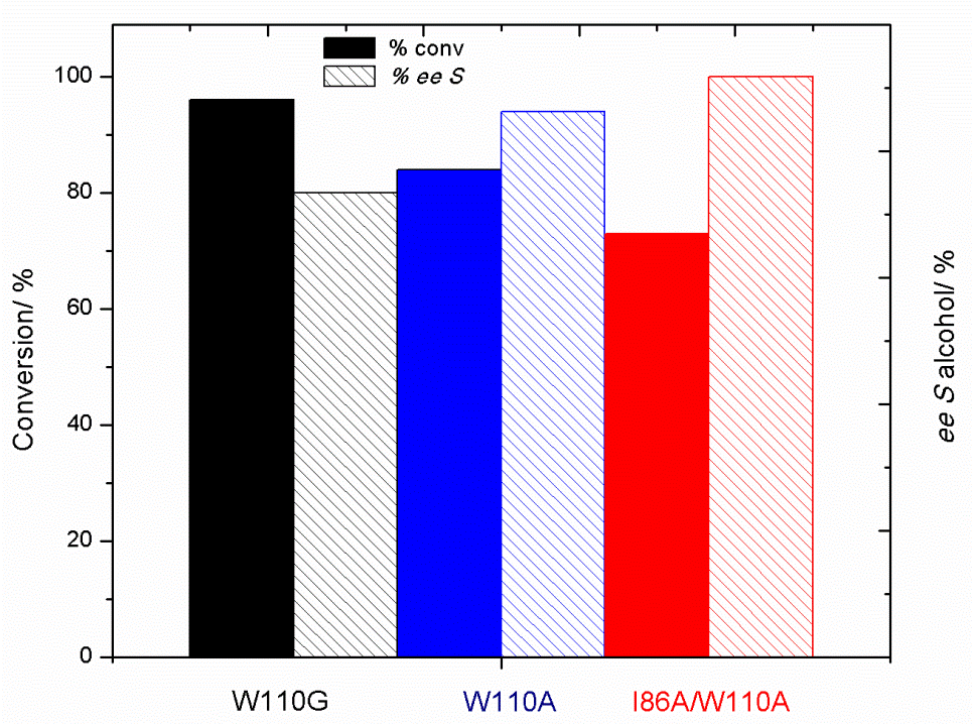


Figure 13. Comparison of the activity and stereoselectivity of mutants of TeSADH in reduction of 4-phenyl-2-butanone (I86G/W110A and I86G/W110G TeSADH showed no detectable activity).

Subsequently, the mutants were tested for activity by monitoring conversion of the cofactor $NADP^+$ to NADPH using UV-Vis spectroscopy (Figure 14). All the mutants except I86G/W110G exhibited activity observed from the conversion of $NADP^+$ to NADPH. The high selectivity of I86A/W110A and no detectable activity by I86G/W110A is perhaps due to undetermined effects arising from a change in the active configuration of the enzyme's active site.

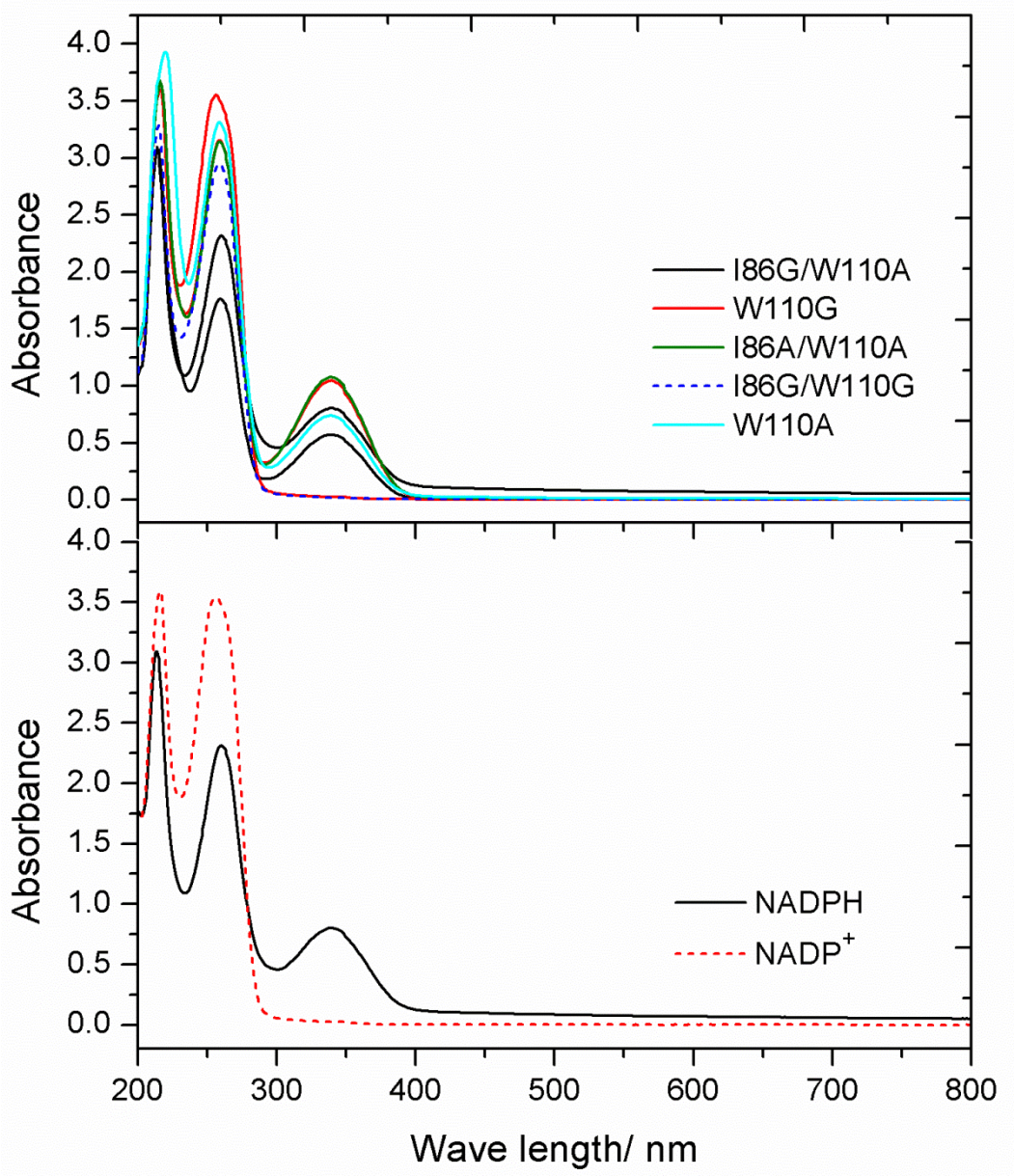
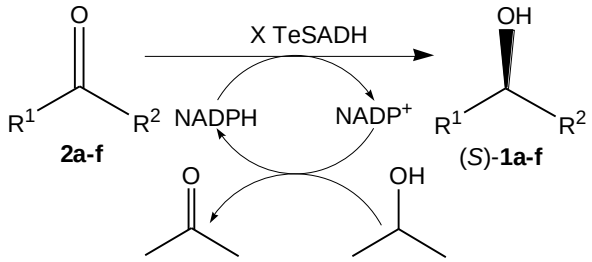


Figure 14. The activity of TeSADH mutants checked qualitatively by conversion of the cofactor $NADP^+$ to NADPH using 2-propanol.

The excellent activity and selectivity exhibited by W110A and W110G made them the mutants of choice for various studies in this work. The two mutants were further investigated in the reduction of selected ketones. In Table 6 are shown the conversions and *ee* obtained in the reduction of 4-phenyl-2-butanol (**2a**), 4-(4'-methoxyphenyl)-2-butanone (**2b**), 4-(4'-hydroxyphenyl)-2-butanone (**2c**), 4-phenyl-3-butyn-2-one (**2d**) and 1-(3'-trifluoromethylphenyl)-2-propanone (**2f**).

The W110G TeSADH mutant had a slight edge over the activity of W110A TeSADH while the later exhibited higher stereoselectivity in the reduction of **2a** and **2b** which concur with the earlier observation in Figure 13 and Table 6. It was also observed that some substrates dictate their own selectivity irrespective of the mutants. The same *ee* (>99%) suggesting no selectivity mistake was obtained in the reduction of 4-(4'-hydroxyphenyl)-2-butanone and 4-phenyl-3-butyn-2-one (Table 6, entries 5-8). This is attributed to hydrogen bonding between the *p*-hydroxyl group of **2c** and the amino acids at the active site which fixes the substrate in one position lowering the chances of selectivity mistakes. A similar fixed configuration might arise due to the rigid structure of **2d** and hence the single enantiomeric (*S*)-alcohols formed. Substrate influence on the stereoselective outcome was further exhibited in the reduction of **2f** where similar conversion and *ee* (>99 conv, 88-89% *ee*) were obtained (Table 6, entries 9 and 10).

Table 6. The stereoselectivity and activity of W110A, W110G, and I86A/W110A TeSADH-catalyzed reduction of phenyl-ring-containing ketones.



X= W110A, W110G TeSADH

Entry	Substrate	R^1	R^2	Mutant	Alcohol [%]	*ee* [%][c]
1	**2a**	$C_6H_5CH_2CH_2$	CH_3	W110G	>99	94
2	**2a**	$C_6H_5CH_2CH_2$	CH_3	W110A	>99	98
3	**2b**	*p*-MeO-$C_6H_4CH_2CH_2$	CH_3	W110G	88	72
4	**2b**	*p*-MeO-$C_6H_4CH_2CH_2$	CH_3	W110A	66	89
5	**2c**	*p*-HO-$C_6H_4CH_2CH_2$	CH_3	W110G	>99	>99
6	**2c**	*p*-HO-$C_6H_4CH_2CH_2$	CH_3	W110A	>99	>99
7	**2d**	$C_6H_5C_2$	CH_3	W110G	>99	>99
8	**2d**	$C_6H_5C_2$	CH_3	W110A	>99	>99
9	**2f**	*m*-CF_3-$C_6H_4CH_2$	CH_3	W110G	>99	88
10	**2f**	*m*-CF_3-$C_6H_4CH_2$	CH_3	W110A	>99	89

Reaction conditions: **2a-f** (0.03 mmol)], W110G TeSADH (0.1 mg), $NADP^+$ (1.0 mg) in Tris-HCl (pH 8.0, 50 mM)/ 2-propanol (70:30 v/v).

3.3.3.2 Optimization of reduction reaction at varying concentration of 2-propanol

For a successful deracemization approach, the high stereoselective reduction is paramount. A similar study to oxidation was conducted where the most stereoselective reduction conditions were determined by conducting reduction 4-phenyl-2-butanone at varying concentrations of 2-propanol [1-30% (v/v)] using W110A and W110G TeSADH (Figure 15). An exponential rise in conversion was observed between 1-10% (v/v) 2-propanol which steadily increased with increase in the cosubstrate concentration. The stereoselectivity of W110A TeSADH-catalyzed reduction steadily increased between 1-30% v/v 2-propanol with less variation whereas the stereoselectivity of W110G TeSADH significantly improved between the same concentration range an indication that the mutant can serve both low and high stereoselective demands. Maximum conversion and highest stereoselectivity were observed using both mutants at 30% (v/v) 2-propanol which was taken optimum for the deracemization approach. At concentrations >30% (v/v) despite the high stereoselectivity noticed, there was a drastic drop in conversion which is attributed to the observed enzyme precipitation reducing the amount of active enzyme.

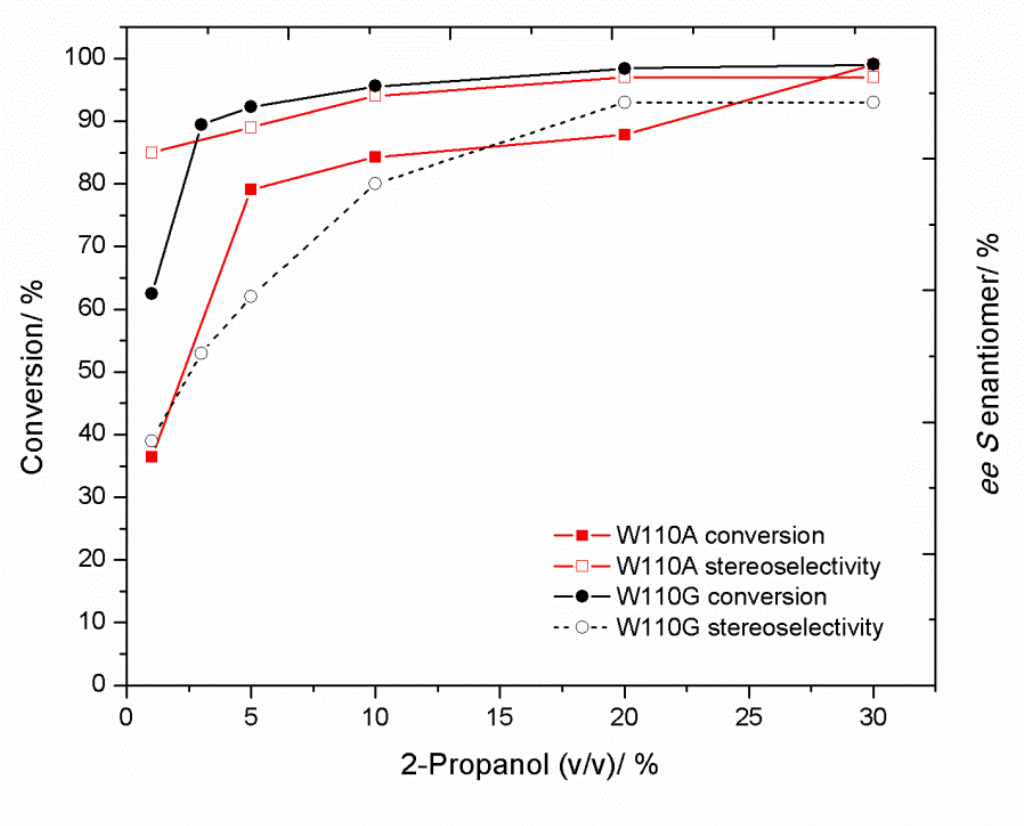


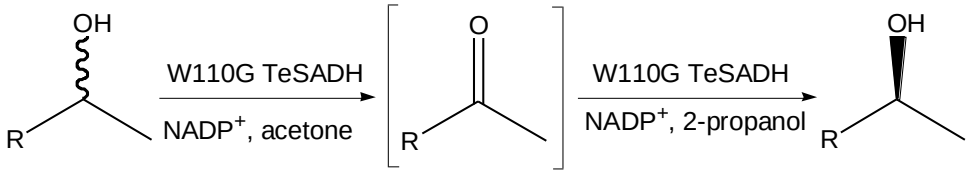
Figure 15. The effect of 2-propanol concentration on stereoselectivity and activity of the W110A and W110G TeSADH-catalyzed reduction reaction of 4-phenyl-2-butanone.

3.3.4 Deracemization of phenyl-ring-containing racemic alcohols

Initial attempts to deracemize racemic alcohols by oxidation under less stereoselective conditions (3% of acetone, v/v) for 24 h without complete depletion of the racemic alcohols followed by stereoselective reduction conditions (30% of 2-propanol, v/v) were not successful despite the ideal time allowance for both reactions when carried out independently as shown in Table 7.

In a modified approach, the racemic alcohols were oxidized under least selective oxidation condition (3% vol. acetone) to obtain the intermediate prochiral ketone. The reaction was monitored by GC until all the alcohol was fully oxidized where applicable. The intermediate without isolation was then subjected to the most selective condition (30% vol. 2-propanol) to yield the (*S*)-alcohol. In Table 8 are shown the *ee* of the recovered alcohol in deracemization of selected phenyl-ring-containing racemic alcohols.

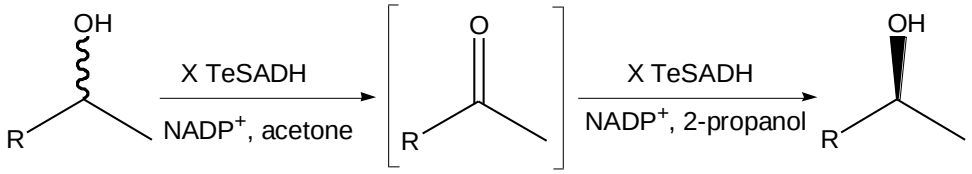
Table 7. Deracemization of phenyl-ring-containing racemic alcohols using W110G TeSADH without allowing maximum oxidation.



Entry	Substrate	R	Ketone [%]	*ee* [%]
1	(*rac*)-**1a**	$C_6H_5CH_2CH_2$	5	53(*S*)
2	(*rac*)-**1b**	*p*-MeO-$C_6H_4CH_2CH_2$	49	49(*S*)
3	(*rac*)-**1e**	$C_6H_5CH{=}CH$	32	76(*R*)
4	(*rac*)-**1h**	$C_6H_5CH_2$	19	21(*R*)

Reaction conditions: racemic alcohol (0.03 mmol), W110G TeSADH (0.2 mg), $NADP^+$ (1.0 mg), and acetone (3%, v/v); reduction reactions were performed using 2-propanol (30%, v/v).

Table 8. Deracemization of phenyl-ring-containing racemic alcohols catalyzed by X TeSADH (X=W110A or W110G).



Entry	Substrate	R	Mutant	Ketone [%]	*ee* (%)
1	(*rac*)-**1a**	$C_6H_5CH_2CH_2$	W110G	<0.5	94
2	(*rac*)-**1b**	*p*-MeO-$C_6H_4CH_2CH_2$	W110G	<0.5	72
3	(*rac*)-**1b**	*p*-MeO-$C_6H_4CH_2CH_2$	W110A	<0.5	87
4	(*rac*)-**1c**	*p*-HO-$C_6H_4CH_2CH_2$	W110G	<0.5	>99
5	(*rac*)-**1c**	*p*-HO-$C_6H_4CH_2CH_2$	W110A	<0.5	20
6	(*rac*)-**1f**	*m*-CF_3-$C_6H_4CH_2$	W110G	<0.5	76
7	(*rac*)-**1h**	$C_6H_5CH_2$	W110G	2	47
8	(*rac*)-**1h**	$C_6H_5CH_2$	W110A	<0.5	21(*R*)

Reaction conditions: racemic alcohol [(*rac*)-alcohol (0.03 mmol)] W110X TeSADH (0.2 mg), $NADP^+$ (1.0 mg), and acetone (3%, v/v); reduction reactions were performed using 2-propanol (30%, v/v).

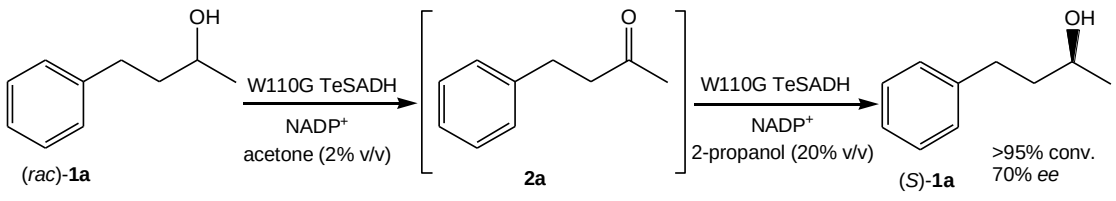
(*Rac*)-**1a** and **1b** were deracemized to the corresponding (*S*)-alcohol in 94% and 72% *ee*, respectively, using W110G TeSADH (Table 8, entries 1 and 2). A significant improvement in *ee* was achievable using a more selective W110A TeSADH mutant as observed in the deracemization of (*rac*)-**1b** where the alcohol was recovered in 87% *ee* compared to 72% *ee* (*S*)-**1b** using W110G TeSADH (Table 8,entry 3). The slight edge over W110G by W110A TeSADH in deracemization of (*rac*)-**1b** is attributed to the ability of both mutants to completely oxidize this substrate [(*rac*)-**1b]**, thus the determining step is the reduction reaction in which W110A TeSADH is more selective. On the contrary, the determinant step in deracemization of (*rac*)-**1c** is the oxidation step since intermediate ketone is selectively reduced to only the (*S*)-enantiomer regardless of the mutant and cosubstrate concentration (same *ee* obtained at 5% and 30% v/v 2-propanol, Figure 7). Hence the low enantioselectivity of W110G TeSADH allowed complete oxidation of (*rac*)-**1c** to the corresponding intermediate ketone which was reduced recovering >99% *ee* (*S*)-**1c** (Table 8, entry 4). The low *ee* of the recovered alcohol (20% *ee* (*S*)-**1c**) using W110A TeSADH is due to complete oxidation which leaves unreacted (*R*)-**1c** hence lowering the computed *ee* (Table 8, entry 5). Like in the deracemization of (*rac*)-**1c**, the poor oxidation of (*rac*)-**1h** during deracemization using W110A TeSADH left behind unreacted (*R*)-**1h** and hence the observed *R*-configured alcohol (21% *ee* (*R*) contra to the expected *S*-alcohol (Table 8, entry 8).

The formation of the (*R*)-enantiomer rather than the expected (*S*)-alcohol is a result of the low stereoselective reduction of the intermediate phenylacetone to low *ee* alcohol as previously reported [64]. Similarly, the low *ee* of the recovered (*S*)-**1h** (47% *ee*) after deracemization using W110G TeSADH despite the moderately high oxidation (80%) is due to poor stereoselective reduction of the intermediate phenylacetone (Table 8, entry 7). Nevertheless, the presence of the

trifluoromethyl substituent at the *meta* position significantly enhanced the oxidation of *rac*-(**1f**) to **2f** by both W110A and W110G TeSADH (Table 6, entries 11 and 12), which was reduced with relatively high stereoselectivity to give (*S*)-**1f** with 76% *ee* using the latter mutant (Table 8, entry 6).

3.3.5 Large scale deracemization

The single enzymatic deracemization was conducted on a large scale which would be of practical applications. The reactions were conducted using 0.2 mmol of the substrates (20 mM) which were deracemized to a similar extent like in the small scale. A slight modification in the procedure was made where the oxidation proceeded using 2% (v/v) acetone and the reduction reaction using 20% (v/v) 2-propanol (in 10 mL solvent) due to excessive precipitation of the enzyme above optimal concentrations (i.e 3% and 30%, respectively) for small scale reactions. Large scale deracemization of *rac*-**1a** and (*rac*)-**1c** [known as rhododendrol a naturally occurring alcohol which has been from *Rhododendron maximum*] [65] were achievable to a comparable extent to the small scale reactions (Schemes 18 and 19). Like the small scale, the reactions were progressively monitored by GC until complete oxidation of the racemates where applicable before switching to the reduction conditions as shown for the deracemization of *rac*-**1a** (Figure 16).



Scheme 18. Large scale deracemization of (*rac*)-4-phenyl-2-butanol using W110A TeSADH.

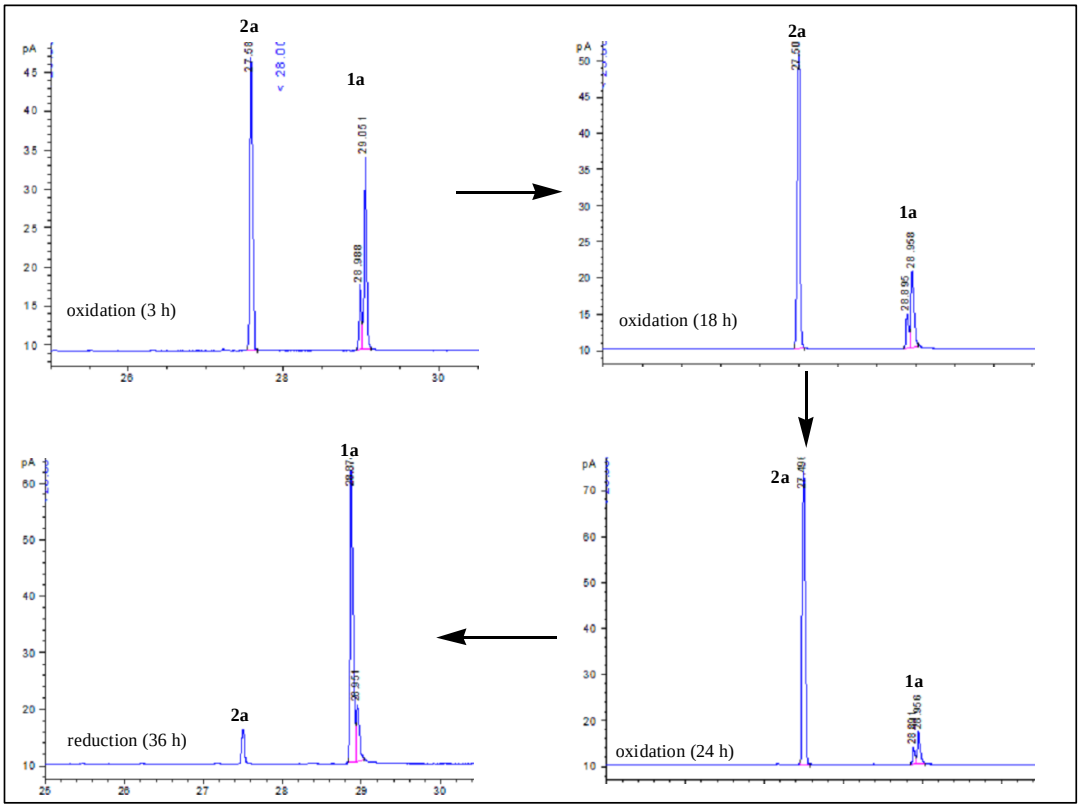
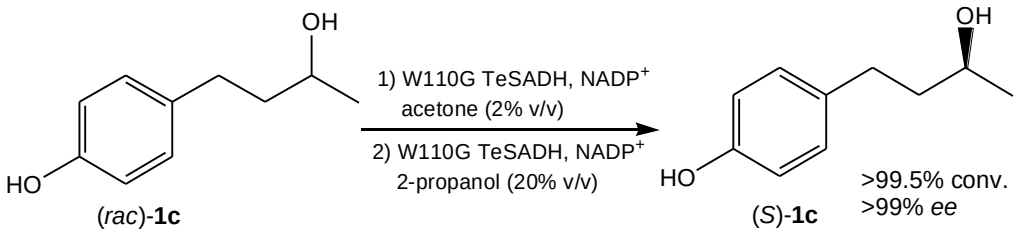


Figure 16. GC chromatograms for the oxidation and reduction of (*rac*)-4-phenyl-2-butanol during large scale deracemization.

Similarly, the deracemization of (*rac*)-**1c** (Scheme 19) was progressively monitored and, like the majority of the reactions, product characterization was done using mass spectrometry and NMR spectroscopy as seen in the spectra in Figures 17-19.



(*rac*)-**1c** (*S*)-**1c**

Scheme 19. Large scale deracemization of (*rac*)-4-(4ˈ-hydroxyphenyl)-2-butanol using W110G TeSADH.

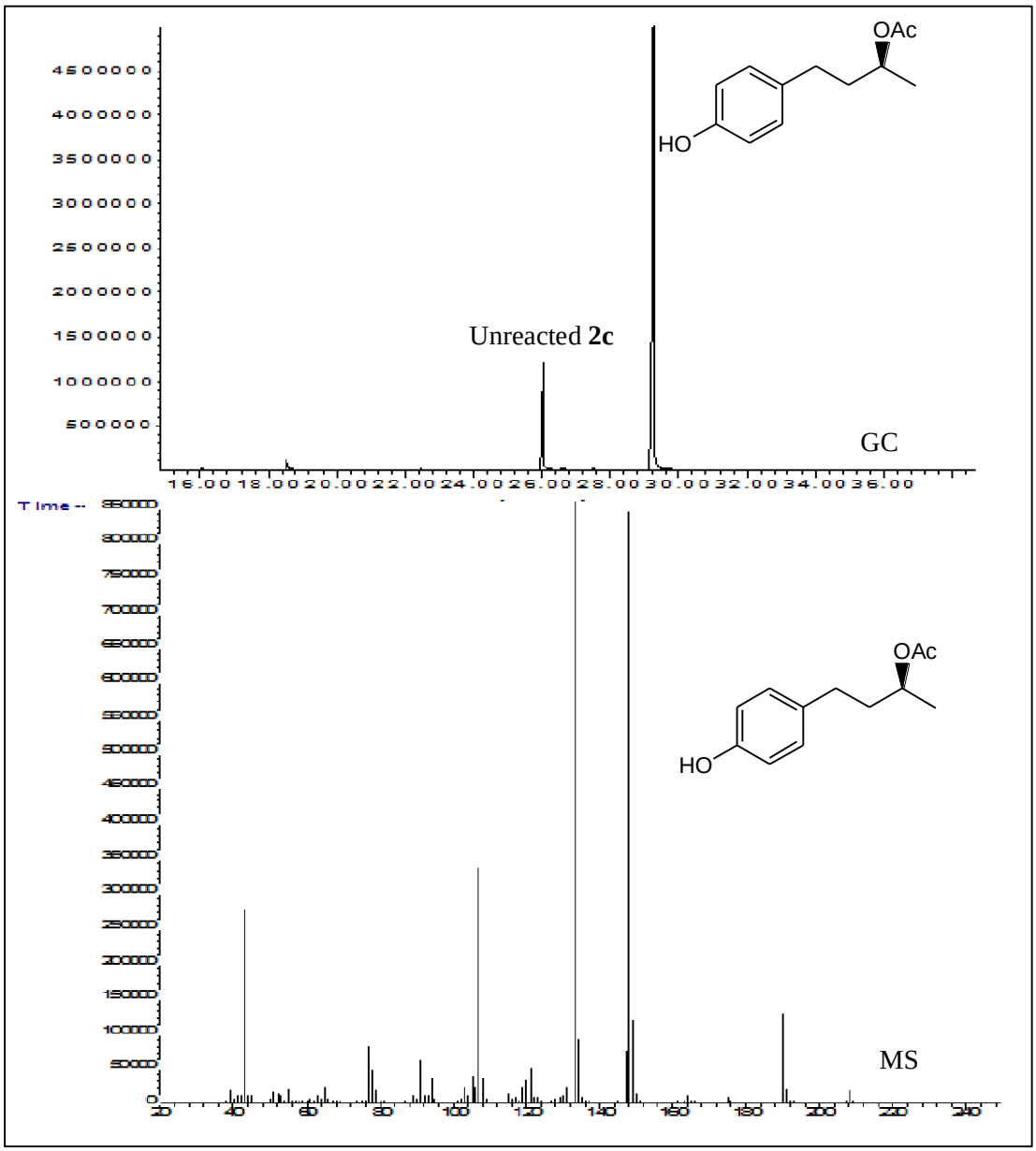


Figure 17. Monitoring of the deracemization of (rac)-4-(4ˈ-hydroxyphenyl)-2-butanol with W110G TeSADH prior to completion: gas chromatogram (top) and mass spectrum of the acetate derivative of the product

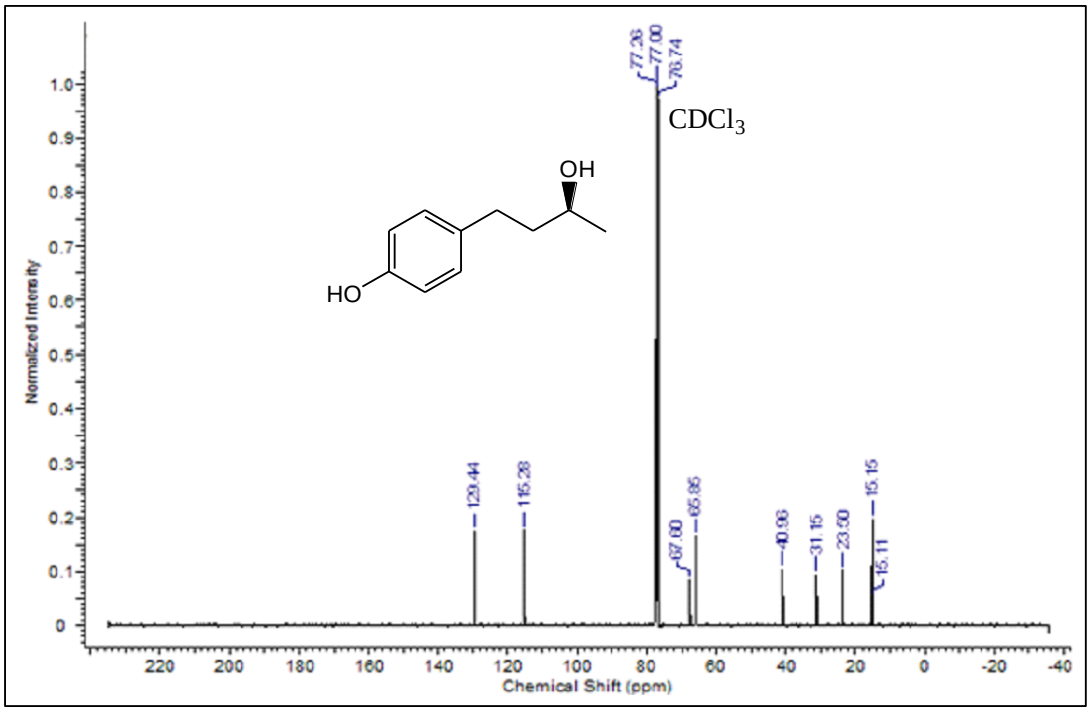


Figure 18. ^{13}C NMR spectrum of the deracemization product of (*rac*)-4-(4ˈ-hydroxyphenyl)-2-butanol.

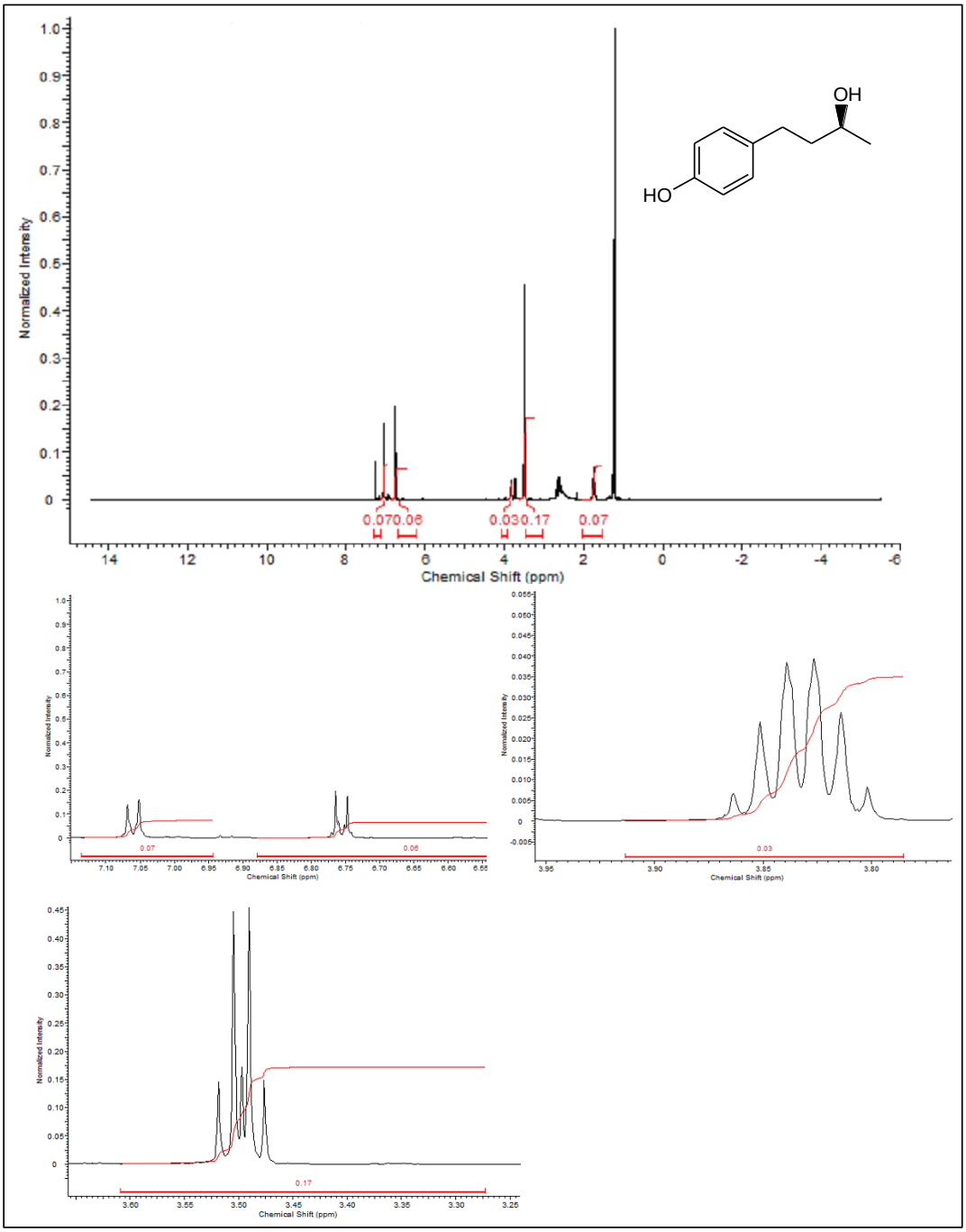
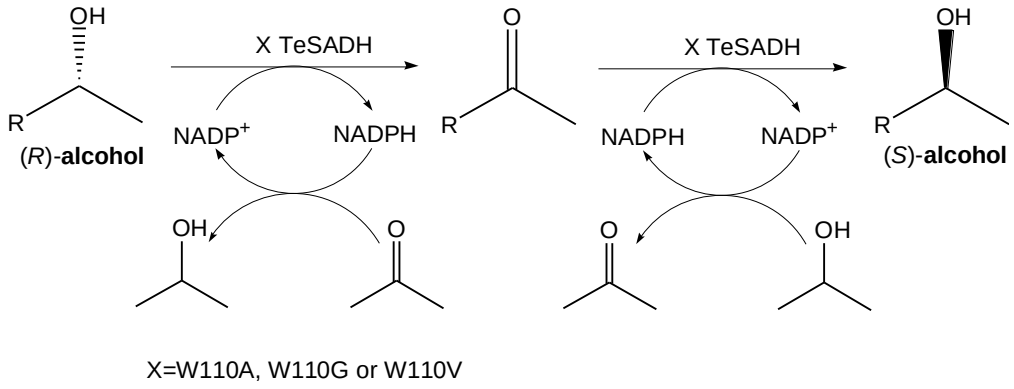


Figure 19. ^{1}H NMR spectrum of the deracemization product of (*rac*)-4-(4ˈ-hydroxyphenyl)-2-butanol.

CHAPTER FOUR

STEREOINVERSION OF *R*-CONFIGURED ALCOHOLS

Evidence from cited work on TeSADH show that the extended large pocket at the active site of W110A and W110G TeSADH allows the mutants to accommodate (*R*)-configured alcohols through selectivity mistakes. The inversion of stereochemistry reported herein relied on oxidation of the (*R*)-alcohol followed by (*S*)-selective reduction of the generated intermediate prochiral ketone (Scheme 20). Oxidations were performed at the least stereospecific conditions using acetone (3%, v/v) followed by high stereoselective reduction (using 30% of 2-propanol, v/v) of the intermediate prochiral ketone without isolation. W110A, W110G and W110V TeSADH mutants were compared in oxidation. The reaction rate for all the mutants was compared simultaneously in the oxidation of racemic, *S* and *R* enantiomers.



Scheme 20. Single enzymatic stereoinversion of *R* configured alcohols using TeSADH.

4.1 Experimental procedures

4.1.1 General procedure for the deracemization and stereoinversion of phenyl-ring-containing alcohols

The *R*-alcohol (0.03 mmol) was added to a mixture of TeSADH mutant (0.2 mg) and $NADP^+$ (1 mg) in Tris-HCl buffer (970 µL, 50 mM, pH 8.0) containing acetone (30 µL) in a 1.5-mL Eppendorf tube. The mixture was shaken at 180 rpm at 50 °C for 24-36 h. The progress of the reaction was monitored by GC; if the oxidation was not complete, if applicable, fresh enzyme (0.1 mg) and acetone (30 µL) were added. The reduction was effected by adding a fresh enzyme (0.2 mg) and 2-propanol (30%, v/v) to the same reaction vessel and the experiment was continued for 24 h.

4.1.2 Large-scale stereoinversion of phenyl-ring-containing *R*-alcohols

A mixture of (*R*)-alcohol (30 mg, final concentration 20 mM), W110G TeSADH (0.6 mg), and $NADP^+$ (2.0 mg) in Tris-HCl buffer solution (9.8 mL, 50 mM, and pH 8.0) containing acetone (2%, v/v) was placed in a round-bottomed flask equipped with a magnetic stirrer bar. The reaction mixture was stirred for 36 h at 50 °C at 200 rpm using a hot plate equipped with a thermocouple. After oxidation was complete (monitored by GC), 2-propanol (20%, v/v) and a fresh enzyme (0.4 mg) were added and the reaction mixture was stirred for 24 h. Fresh enzyme (0.2 mg) was added every 12 h during oxidation and reduction steps. The reaction mixture was extracted with diethyl ether (2 × 5 mL). The reaction mixture was derivatized prior to analysis as already described.

4.2 Results and Discussion

4.2.1 Comparison of TeSADH mutants in oxidation of opposing enantiomers

In an attempt to determine the ability of TeSADH mutants to invert the stereochemistry of (*R*)-alcohols, the extent of W110G, W110A, and W110V TeSADH-catalyzed oxidation of (*R*)-4-phenyl-2-butanol [(*R*)-**1a**], (*S*)-**1a** and (*rac*)-**1a** was monitored at specified intervals (Figure 20). Despite the relatively slow oxidation of the (*R*)-**3a**, a remarkable conversion comparable to (*S*)-**1a** (88% vs 90% conv.) was reached after 12 h.

It is worthy to mention that reduction was inevitable during oxidation generating the (*S*)-alcohol from the intermediate ketone with the *ee* of the unreacted alcohol 99%> which practically prevented complete oxidation (Figure 21). Poor oxidation of (*R*)-**1a** was observed with W110V TeSADH owing to its high stereoselectivity for the desired *S*-enantiomer. The relatively low conversion (<10%) which remained sluggish for hours could also be due to death of the enzyme after prolonged heating or poor tolerance to the water miscible acetone used as a cosubstrate. A sharp increase in oxidation of (*R*)-**1a** after 3 h (43% to 92% conv.) by W110A TeSADH is attributed to its higher stereospecificity as compared to W110G TeSADH which limits the parallel reduction of the accumulated ketone to predominant *S*-alcohol (W110G TeSADH reduction of the accumulated ketone is less stereoselective generating more of the starting material, i.e *R*-enantiomer during oxidation), the desired enantiomer of TeSADH hence the exponential rise.

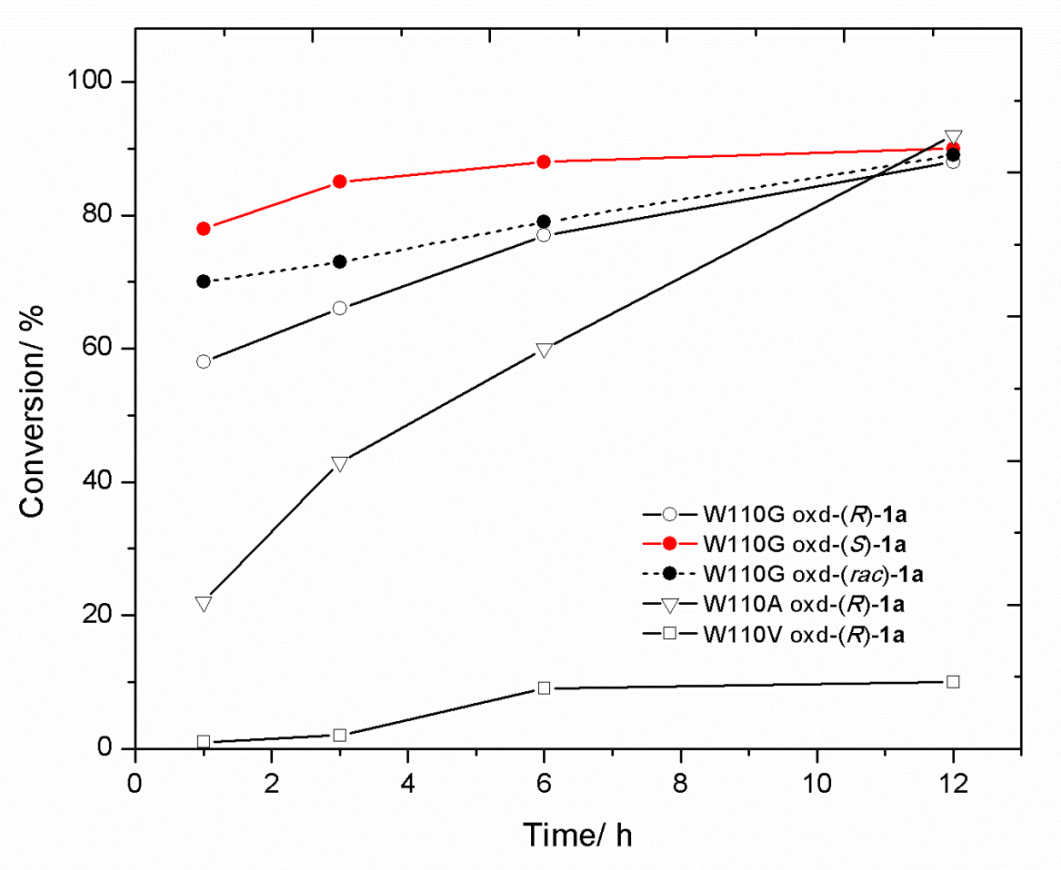


Figure 20. Oxidation of (*R*)-, (*S*)- and (*rac*)-**1a** at different time intervals using W110G TeSADH.

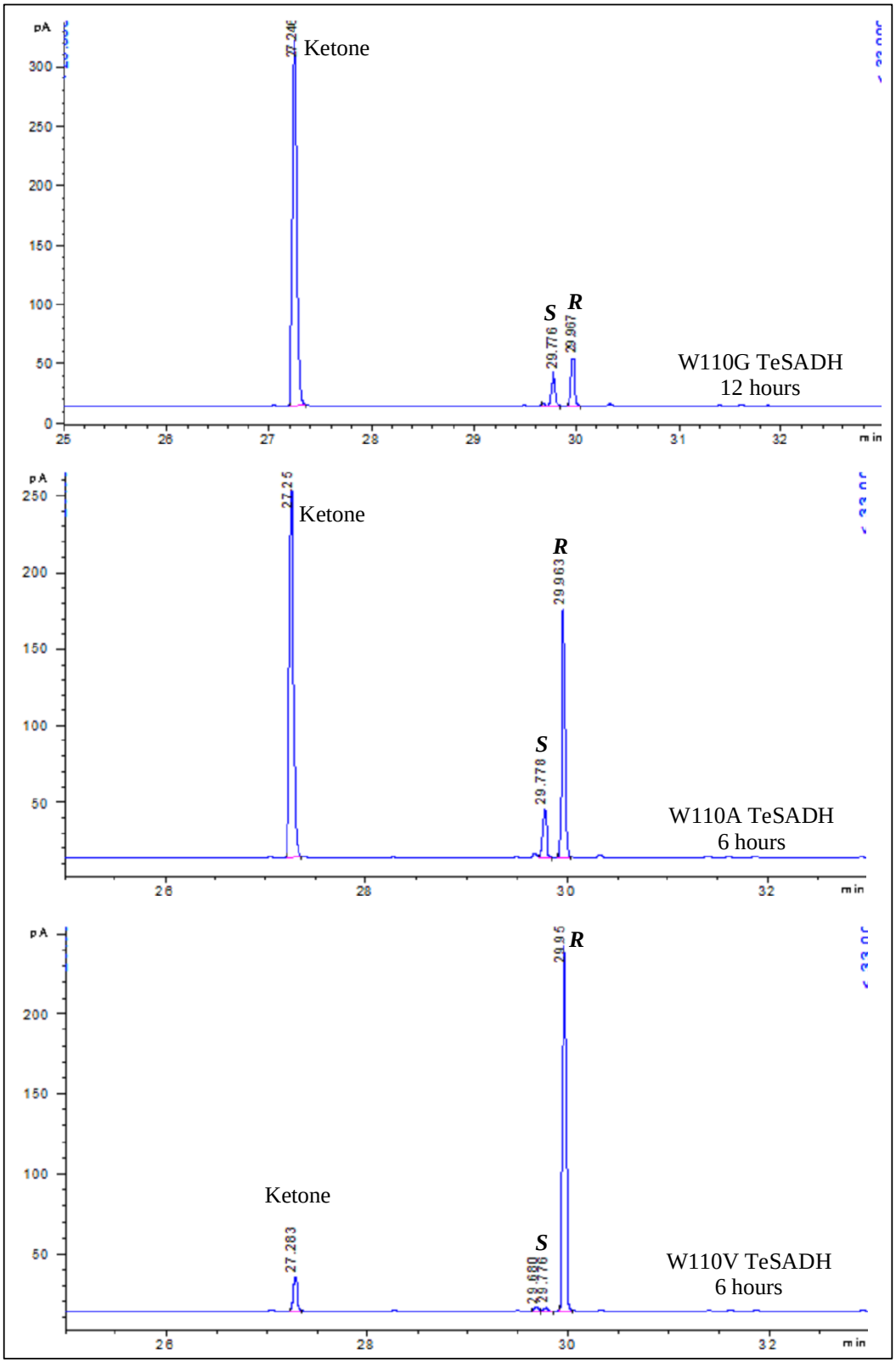
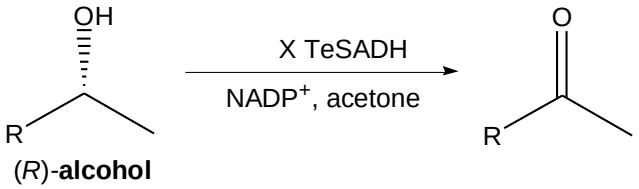


Figure 21. Generation of undesired *S*-enantiomer during oxidation of *R* alcohols

The oxidation study was extended to other selected substrates using W110A, W110G and W110V TeSADH using acetone (3%, v/v) as the optimum cosubstrate concentration as shown in Table 9. The poor performance by the W110V TeSADH in the oxidation (*R*)-**1a** (12% conv.) and (*R*)-**1h** (18% conv.) (Table 9, entries 3 and 14) as compared to W110A and W110G (>87% conv.) (Table 9, entries 1, 2, 12 and 13,) clearly manifests the essential role of the mutant's active site in achieving oxidation. The poor oxidation by W110V TeSADH is attributed to the reduced selectivity mistakes due to the relatively small size of the large pocket at the active site when valine replaces tryptophan. Excellent conversions (>99%) were obtained in the oxidation of (*R*)-**1b** using both W110A and W110G TeSADH (Table 9, entries 4 and 5). Similarly, the oxidation of (*R*)-**1c** was excellent (>99%) using W110G TeSADH while W110A TeSADH oxidized the substrate to 82% (Table 9, entries 6 and 7). There were no detectable oxidation of (*R*)-**1e** by all mutants due to the absence of selectivity mistakes. The double bond restricts rotation suggesting a fixed alignment of (*R*)-**1e** at the active site hence high preference for the desired *S*-enantiomer (Table 9, entries 8-10).

Table 9. Activity and stereospecificity of W110A, W110G, and W110V TeSADH-catalyzed oxidation of phenyl-ring-containing *R* configured alcohols.



(*R*)-**alcohol**

X=W110A, W110G or W110V

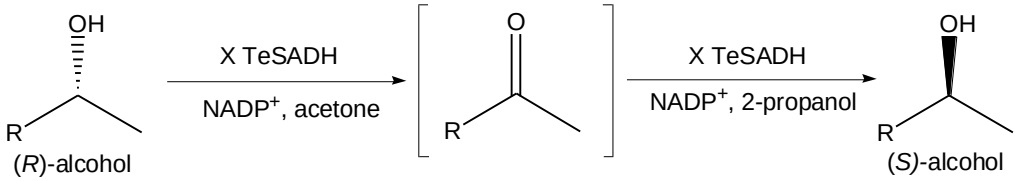
Entry	Mutant	Substrate	R	Ketone [%]	*ee* [%]
1	W110G	(*R*)-**1a**	$C_6H_5CH_2CH_2$	95	20(*R*)
2	W110A	(*R*)-**1a**	$C_6H_5CH_2CH_2$	96	(*rac*)
3	W110V	(*R*)-**1a**	$C_6H_5CH_2CH_2$	12[b]	91(*R*)
4	W110G	(*R*)-**1b**	*p*-MeO-$C_6H_4CH_2CH_2$	>99[a]	-
5	W110A	(*R*)-**1b**	*p*-MeO-$C_6H_4CH_2CH_2$	>99[a]	-
6	W110G	(*R*)-**1c**	*p*-HO-$C_6H_4CH_2CH_2$	>99	-
7	W110A	(*R*)-**1c**	*p*-HO-$C_6H_4CH_2CH_2$	82	>99(*R*)
8	W110G	(*R*)-**1e**	C_6H_5CH=CH	<0.5	87(*R*)
9	W110A	(*R*)-**1e**	C_6H_5CH=CH	<0.5	87(*R*)
10	W110V	(*R*)-**1e**	C_6H_5CH=CH	<0.5	87(*R*)
11	W110G	(*R*)-**1h**	$C_6H_5CH_2$	71[a]	3(*R*)
12	W110G	(*R*)-**1h**	$C_6H_5CH_2$	91	15(*R*)
13	W110A	(*R*)-**1h**	$C_6H_5CH_2$	87	4(*R*)
14	W110V	(*R*)-**1h**	$C_6H_5CH_2$	18	>99(*R*)

Reaction conditions: *R*-alcohol ((0.03 mmol), TeSADH mutant (0.4 mg), and $NADP^+$ (1.0 mg) in Tris-HCl buffer solution (1.0 mL, 50 mM, pH 8.0) containing acetone (3%, v/v), at 50 °C with shaking for 36 h. [a]TeSADH mutant (0.2 mg) and 24 h reaction time.

4.2.2 Stereoinversion of *R* configured alcohols using a single W110A and W110G TeSADH mutants

In an attempt to invert the stereochemistry, the oxidation of selected *R*-alcohols was monitored by GC until no further reaction. The equilibrium was then switched towards the reduction reaction by addition of 2-propanol (30% volume) which was the optimum concentration for (*S*)-selective reduction. W110V TeSADH mutant was not used in stereoinversion owing to the poor activity of the mutant in the oxidation reaction. In Table 10 are shown the percent recovery and *ee* of the alcohols after inversion of the stereocenter. *(R)*-**1a** (>99.9% *ee*) was inverted to 80% *ee*(*S*) and 86% *ee*(*S*) using W110G and W110A TeSADH, respectively (Table 10, entries 1 and 2). The presence of methoxy group at the *para* position of **1b** significantly reduced the stereoselectivity of both the oxidation and the reduction step in stereoinversion of (*R*)-**1b** which accounts for the moderate low *ee* of the recovered alcohol [82% *ee*(*S*)] using W110A TeSADH (Table 10, entry 3). On the contrary, the presence of the hydroxyl group at the *para* position of **1c** enhanced the stereoselectivity of reduction of the intermediate 4-(4ˈ-hydroxyphenyl)-2-butanone to >99% *ee* of (*S*)-**1c** using W110G TeSADH (Table 10, entry 4). Moderately low *ee* recovered using W110A TeSADH in the stereoinversion of (*R*)-**1c** is attributed to the unreacted *R* alcohol during the oxidation step (Table 10, entry 5). (*S*)-**1h** was recovered in the lowest *ee* (<35%) which is attributed to the low stereoselective reduction of the intermediate phenylacetone by both W110A and W110G TeSADH (Table 10, entries 6 and 7) as previously reported [64].

Table 10. Stereoinversion of phenyl-ring-containing enantiopure *R* alcohols catalyzed by X TeSADH (X=W110A or W110G).



Entry	Mutant	Substrate	R	Ketone (%)	Product *ee* (%)
1	W110G	(*R*)-**1a**	$C_6H_5CH_2CH_2$	4	80(*S*)
2	W110A	(*R*)-**1a**	$C_6H_5CH_2CH_2$	<0.5	86(*S*)
3	W110A	(*R*)-**1b**	*p*-MeO-$C_6H_4CH_2CH_2$	<0.5	82(*R*)
4	W110G	(*R*)-**1c**	*p*-HO-$C_6H_4CH_2CH_2$	<0.5	>99(*S*)
5	W110A	(*R*)-**1c**	*p*-HO-$C_6H_4CH_2CH_2$	<0.5	64(*S*)
6	W110G	(*R*)-**1h**	$C_6H_5CH_2$	<0.5	33(*S*)
7	W110A	(*R*)-**1h**	$C_6H_5CH_2$	<0.5	35(*S*)

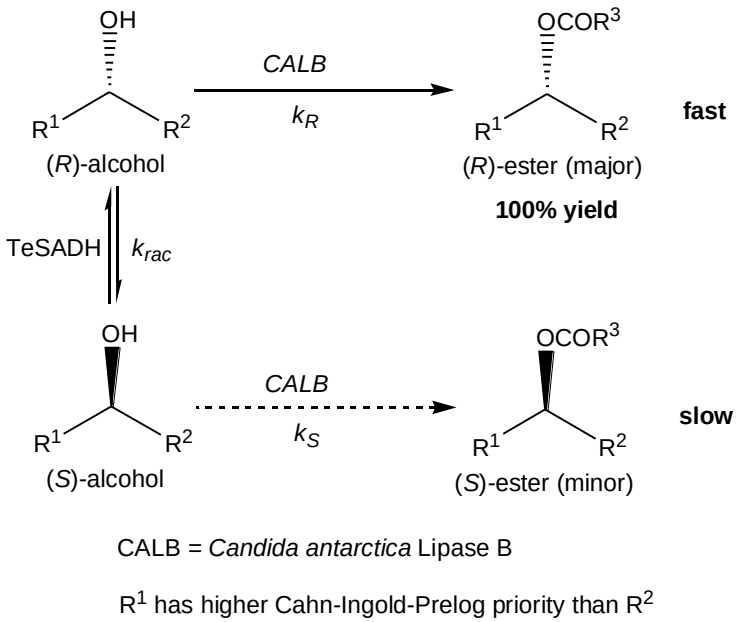
Reaction conditions: (*R*)-alcohol (0.03 mmol), W110X TeSADH (0.4 mg), and $NADP^+$ (1.0 mg) in Tris-HCl buffer solution (1.0 mL, 50 mM, pH 8.0) containing acetone (3%, v/v), at 50 °C with shaking at 180 rpm for 36 h; reduction reactions were performed using W110X TeSADH (0.2 mg) and $NADP^+$ (1.0 mg) in Tris-HCl buffer solution (1.0 mL, 50 mM, pH 8.0) containing 2-propanol (30%, v/v) at 50 °C with shaking for 24 h.

CHAPTER FIVE

DYNAMIC KINETIC RESOLUTION OF PHENYL-RING-CONTAINING RACEMIC ALCOHOLS

5.1 Dual enzymatic dynamic kinetic resolution

A racemization approach that utilizes a single enzyme in the organic medium was employed. An organic media is crucial in this work since it allows large scale reactions using the sparingly water-soluble substrates. It also enacts in situ reactions that strictly demand non-aqueous environment, for instance, lipase-catalyzed KR achieving dual enzymatic DKR. *Thermoanaerobacter ethanolicus* secondary alcohol dehydrogenase (TeSADH), an NADPH-dependent ADH, is well known for its high thermal stability and high tolerance to organic media [66]. The DKR approach utilizes W110A TeSADH in racemization and *Candida antarctica* lipase B, an *R*-selective enzyme in kinetic resolution by transesterification (Scheme 21).



Scheme 21. Dual enzymatic DKR by W110A TeSADH-catalyzed racemization and CALB-catalyzed kinetic resolution.

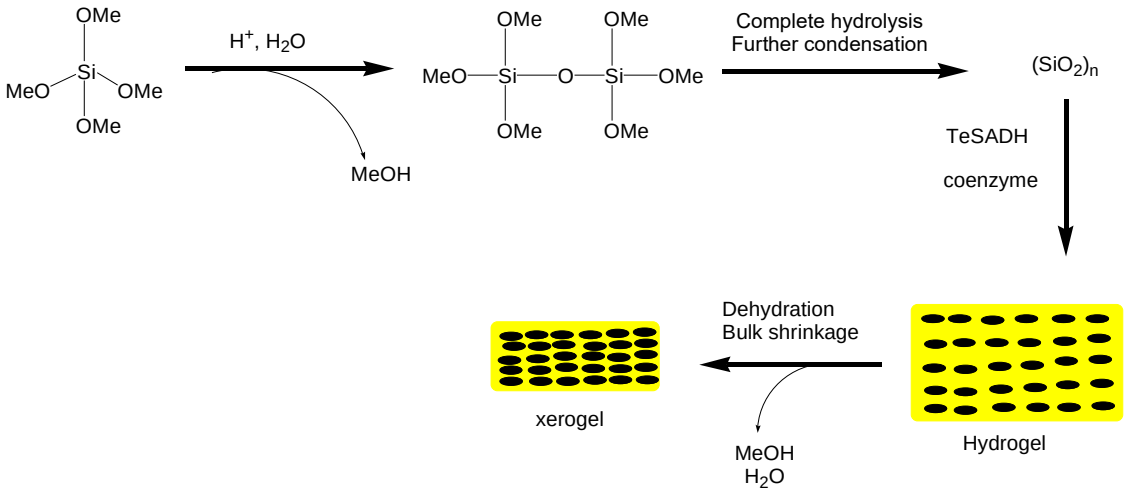
5.2 Experimental procedures

5.2.1 General

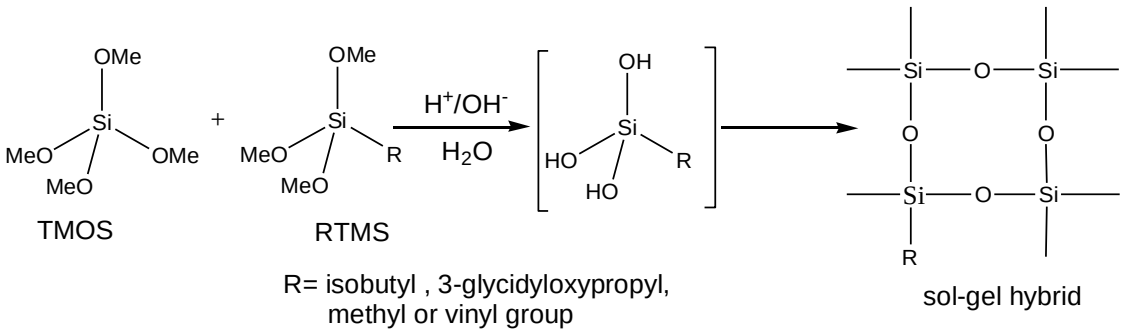
Compounds (*R*)-4-phenyl-2-butanol [(*R*)-**1a**], (*S*)-**1a**, (*R*)-1-phenyl-2-propanol [(*R*)-**1h**], (*S*)-**1h**, (*rac*)-**1h**, NADPH, $NADP^+$, CALB (Novozyme 435), and solvents (HPLC grade) were used as purchased from Sigma-Aldrich; (*R*)-**1i** was obtained by CALB-catalyzed kinetic resolution of (*rac*)-**1i** as reported;[67] (*rac*)-**1a**, **1b**, **1d** and **1i** were synthesized by $NaBH_4$ reduction of the corresponding commercially available ketones, as reported.[62]

5.2.2 Preparation of xerogel-encapsulated W110A TeSADH

Tetramethoxysilane (2035 µL, 1 mol eq) water (470 µL, 2 mol eq), and HCl (40 µL, 0.04 M) were vigorously shaken to form a sol. The enzyme stock containing $NADP^+$ (1.0 mg), NADPH (1.0 mg) and W110A TeSADH (0.2 - 0.6 mg) in Tris-HCl buffer solution (400 µL, pH 8.0) was gently mixed with an equal volume of the sol for 5 seconds and left to stand for 24 h in a closed Eppendorf tube to allow gel aging. The hydrogel containing the immobilized enzyme and coenzymes was left to dry in open air for 24 h forming the xerogel-encapsulated W110A TeSADH (Scheme 22). In a modified procedure silanes with hydrophobic groups (30% v/v) were incorporated in TMOS sol (Scheme 23).



Scheme 22. Xerogel synthesized from TMOS having $(SiO_2)_n$ framework.



Scheme 23. Hybrid sol-gel incorporated with hydrophobic in $(SiO_2)_n$ framework .

5.2.3 General procedure for racemization of enantiopure alcohol using xerogel-encapsulated W110 TeSADH

The enantiopure alcohol (0.05 mmol) and xerogel-encapsulated enzyme containing W110A TeSADH (0.2-0.6 mg), $NADP^+$ (1.0 mg) and NADPH (1.0 mg) in a solvent (1.0 mL) were placed in a 1.5-mL Eppendorf tube. The reaction mixture was shaken at 200 rpm in a thermostat-controlled shaker at 50 °C for 24 h. The reaction mixture was extracted with diethyl ether (2 × 500 µL). The reaction mixture was derivatized prior to analysis as already described.

5.2.4 Procedures for racemization using free enzyme (aqueous and biphasic)

(*S*)-1-Phenyl-2-propanol (0.05 mmol), W110A TeSADH (0.4mg), $NADP^+$ (1.0 mg) and NADPH (1.0 mg), were added to solvent (1.0 mL) in a round bottomed flask. The reaction in aqueous medium was conducted in Tris-HCl buffer (50 mM, pH 8.0)/ acetonitrile (95:5 v/v) whereas the biphasic reaction in Tris-HCl buffer (50 mM, pH 8.0)/hexane (50:50 v/v). The reaction mixture was shaken at 200 rpm in a thermostat-controlled shaker at 50 °C for 24 h. The reaction mixture was derivatized prior to analysis as already described.

The *E* values were calculated using the relationship shown in Equation 2 [63]:

$$E = \frac{\ln[1-c(1+ee_p)]}{\ln[1-c(1-ee_p)]}$$ Equation 2

where *c* is percent conversion and ee_p is the *ee* of the product.

5.2.5 General procedure for KR of racemic alcohols

The (*rac*)-alcohol (0.05 mmol)], acyl donor (0.1 mmol), CALB (1 mg), and hexane (1.0 mL) were placed in a 1.5 mL Eppendorf tube. The mixture was shaken at 50 rpm at 50 ^{o}C for 3-6 h (monitored by GC equipped with a chiral column). A portion (50 µL) from the reaction was dried over Na_2SO_4 prior to analysis. Retention times of the resultant peaks were compared with standard samples of enantiopure alcohols and esters.

5.2.6 General procedure for dual enzymatic DKR of racemic alcohols

Xerogel [containing W110A TeSADH (0.6 mg), NADPH (1.0 mg) and $NADP^+$ (1.0 mg)], CALB (1.0 mg), (*rac*)-alcohol (0.03 mmol) and isopropenyl acetate (0.06 mmol) in hexane (1.0 mL) were placed in the same Eppendorf tube ensuring no physical contact between the two enzyme systems as shown in Figure 22. The reaction mixture was shaken at 50 rpm at 50 ^{o}C for 24 h. The reaction content including the lipase and the xerogel were washed with ethyl acetate (2 × 500 µL). The combined organic layers were dried over Na_2SO_4 and concentrated. The *ee* of the ester derivatives was determined by chiral GC.

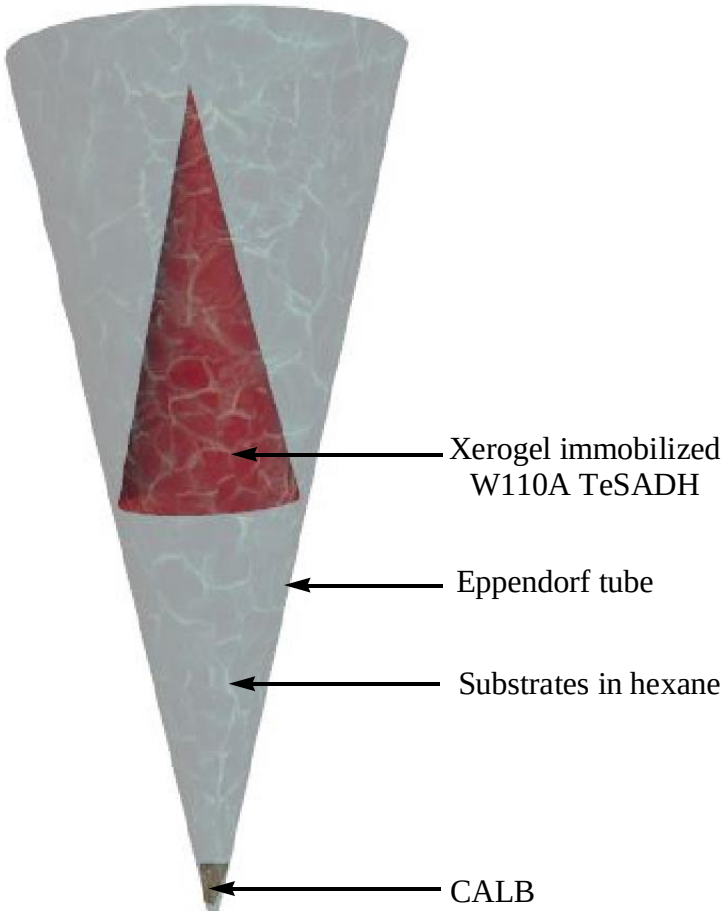


Figure 22. Reaction set-up for dual enzymatic DKR of phenyl-ring-containing secondary alcohols.

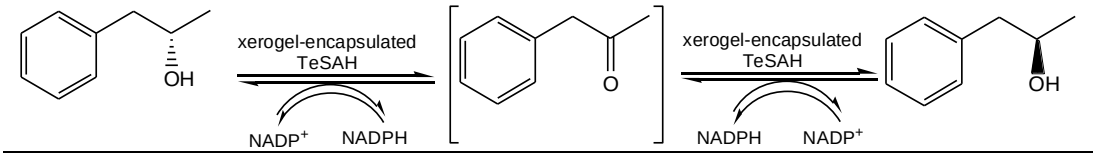
5.3 Results and Discussion

5.3.1 The effect of pH on racemization

The optimization of W110A TeSADH-catalyzed racemization reaction involved varying the pH for the reaction in aqueous media, nature of the organic solvent, substrate concentration and amount of enzyme loaded. In Table 11 are shown the percent *ee* of (*S*)-enantiomer obtained as a result of racemization of (*S*)-1-phenyl-2-propanol [(*S*)-**1h**] (>99.9% ee) at different pHs in Tris-HCl buffer (50 mM) containing acetonitrile as a cosolvent. (*S*)-**1h** was chosen for optimization because its corresponding ketone, phenylacetone, was reduced in low enantioselectivity by W110A TeSADH (37% *ee*), and thus represents an interesting substrate in racemization [22].

Enantiopure (*S*)-**1h** was racemized to 5% *ee* (*S*) at pH 8.0 whereas pH 8.8 (21% *ee*) allowed moderate racemization while and least racemization was observed at pH 6.5 (60% *ee*) (Table 11, entries 1, 2 and 4). The poor racemization at pH 6.5 and pH 8 is predictable since the low and high pH conditions favor oxidation and reduction, respectively. This makes racemization favorable at pH 8 since it falls between the redox pH range. W110G TeSADH had a slight edge in racemization (complete racemization was achieved) over W110A TeSADH due to the further extension of the large pocket at the active site in the former which increases chances of selectivity mistake and hence the enhanced racemization (Table 11, entry 3). W110G TeSADH mutant is more efficient in racemization of (*S*)-**1h** using the free enzyme in Tris-HCl (50 mM, pH 8) at all time intervals as observed in Figure 23. Time dependence reaction was performed using half the enzyme load (0.2 mg).

Table 11. The effect of pH on racemization of (*S*)-1-phenyl-2-propanol.



Entry	pH	*ee* (%)	
		before	after
1	6.5	>99.9 (*S*)	60 (*S*)
2	8.0	>99.9 (*S*)	5 (*S*)
3	8.0	>99.9 (*S*)	<0.5 (*S*)[b]
4	8.8	>99.9 (*S*)	21 (*S*)

Reaction conditions: (S)-**1h** (0.05 mmol), W110A TeSADH (0.4 mg), NADPH (1.0 mg), $NADP^+$ (1.0 mg), Tris-HCl buffer (950 µL, 50 mM) and CH_3CN (50 µL). Reactions were performed at 50°C for 24 h. [b]W110G TeSADH racemization

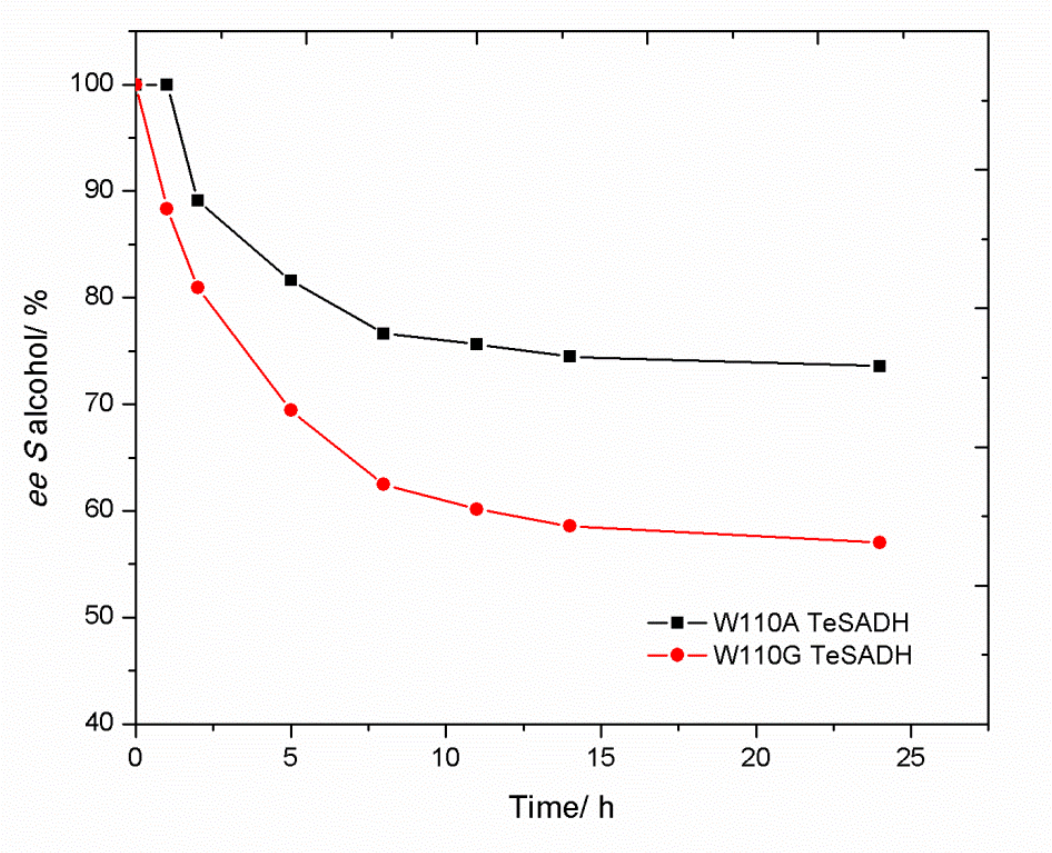


Figure 23. Comparison of W110A and W110G mutants of TeSADH in racemization of (*S*)-1-phenyl-2-propanol.

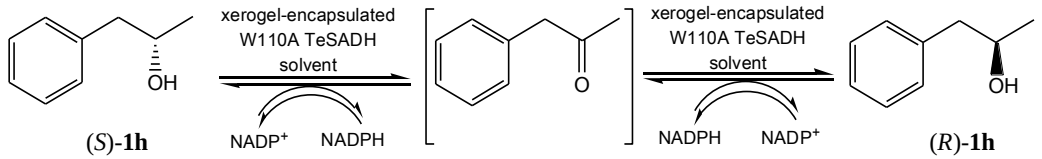
5.3.2 The efficiency of W110G TeSADH-catalyzed racemization in organic media

W110A TeSADH with $NADP^+$ and NADPH were immobilized in a xerogel using the sol-gel method as described previously [21]. The wet sol-gel (hydrogel) was dried to produce a xerogel. The encapsulated enzyme was employed in the racemization of (*S*)-1-phenyl-2-propanol in different organic solvents. (*S*)-**1h** was chosen as the model substrate since it has been reported as a substrate for W110A TeSADH [22] and its counterpart enantiomer as a substrate for CALB [68]. W110G exhibited less tolerance to organic solvents. In Table 12 are shown the *ee* obtained in racemization of (*S*)-**1h** in pure organic solvents.

Racemization was negligible in water-miscible organic solvents, for instance, methanol and DMSO (Table 12, entries 1 and 2), likely due to the removal of water molecules required to maintain the active configuration of the enzyme's active site. In general, the efficiency of W110A TeSADH-catalyzed racemization improved in solvents with high logP (Table 12, entries 5-12), with hexane and cyclohexane showing the highest racemization efficacy (Table 12, entries 6 and 7). It is worth to note the enzyme maintained full activity during the racemization of (*S*)-**1h** using xerogel-immobilized W110A TeSADH in Tris-HCl buffer solution, an indication that hydrophobic solvents like hexane restrict the diffusion of the substrate through the hydrophilic xerogel (SiO_2 framework) and hence the poor racemization in organic media. The xerogel contains water that is enough to maintain the enzyme's active shape [21] and perhaps too little to trigger CALB-hydrolysis of ester products.

The effect of water miscible solvents is further demonstrated at different concentrations of acetonitrile used as a cosolvent to facilitate substrate solubility in aqueous medium (Figure 24). Poor racemization was observed at higher concentrations of acetonitrile.

Table 12. Xerogel-encapsulated W110A TeSADH-catalyzed racemization of (*S*)-1-phenyl-2-propanol in organic media.



(*S*)-**1h** (*R*)-**1h**

Entry	Solvent	logP[a]	*ee* [%] *S* enantiomer	
			before	after
1	DMSO	-1.4	>99	>99
2	Methanol	-0.7	>99	>99
3	MTBE	0.9	>99	96
4	Dichloromethane	1.3	>99	>99
5	Toluene	2.7	>99	94
6	Cyclohexane	3.2	>99	84
7	Hexane	3.8	>99	81
8	Hexane	3.8	>99	63[b]
9	Heptane	4.3	>99	96
10	2,2,4-Trimethylpentane	4.4	>99	93
11	Octane	4.8	>99	92
12	Tris-HCl[c]	-	>99	59

Reaction conditions: (*S*)-**1h** (0.05 mmol), xerogel [W110A TeSADH (0.2 mg), NADPH (1.0 mg) and $NADP^+$ (1.0 mg)] in a solvent (1.0 mL) at 50 °C, 200 rpm, 24 h. [a]log *P* (partition coefficient) values based on octanol-water partition coefficient.[69] [b]0.6 mg of W110A TeSADH was used instead of 0.2 mg. [c]Aqueous medium [Tris-HCl (pH 8.0, 50 mM)/acetonitrile (95:5 v/v)]

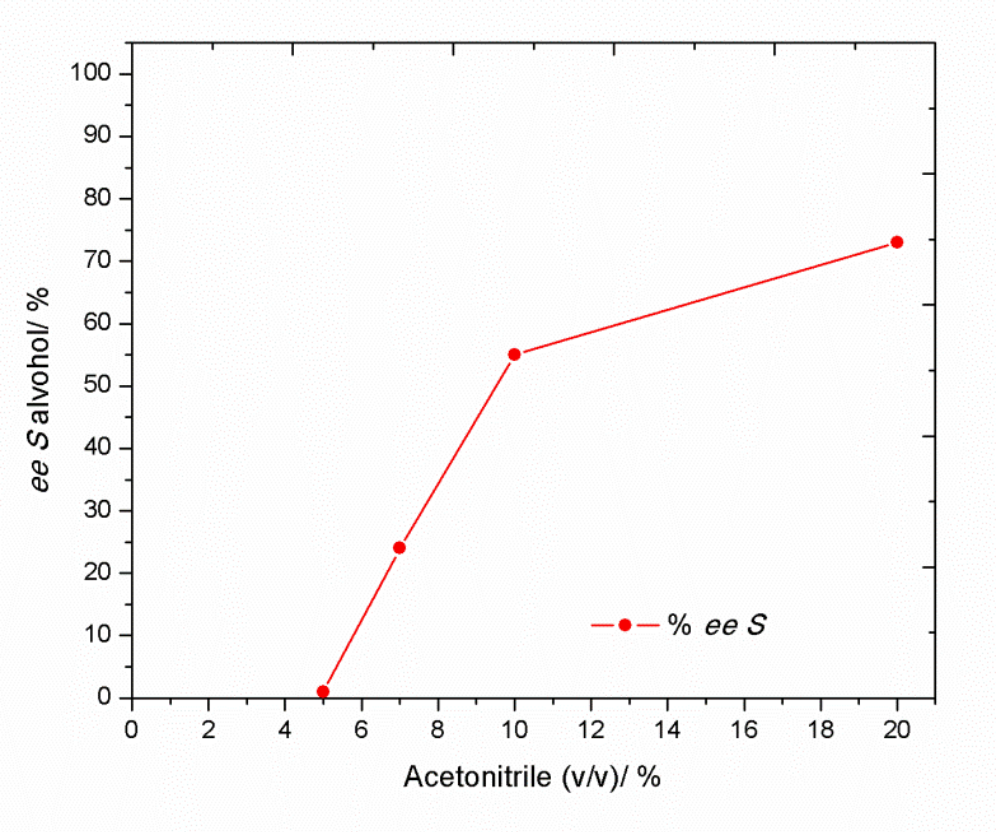


Figure 24. Effect of acetonitrile on enzyme activity and selectivity investigated in racemization of 1-phenyl-2-propanol racemization.

5.3.3 Comparison of racemization in aqueous, biphasic and organic media

The dependence of racemization on the reaction media was investigated in aqueous, biphasic and organic media. The racemization of (*S*)-**1h** at various time intervals using free W110A TeSADH in Tris-HCl buffer solution (aqueous medium) and in the biphasic system containing Tris-HCl and hexane (50% v/v) was compared with that catalyzed by xerogel-immobilized W110A TeSADH in hexane (Figure 25). Excellent racemization was observed after 20 h when in an aqueous medium (from >99% to 5% *ee*). Moderate racemization efficiency was noticed in a biphasic system (>99% to 33%). Racemization was rapid in the first 4 h in both aqueous (>99% to 54% *ee*) and in the biphasic system (>99% to 64% *ee*). The high racemization efficiency in both media is attributed not only to the abundance of water required to maintain the enzyme in its active conformation but also the free state of the enzyme which allows free movement of the substrate in and out of the active site. Xerogel-immobilized W110A TeSADH racemization of (*S*)-**1h** in improved hexane from 81% to 63% *ee*(*S*) when the enzyme load was increased from 0.2 mg to 0.6 mg, respectively. The poor racemization of the immobilized enzyme in hexane is attributed to the slow diffusion of the substrates in and out of the sol-gel. The use of immobilized ADHs in non-aqueous media to perform racemization of enantiopure alcohols has versatile applications.

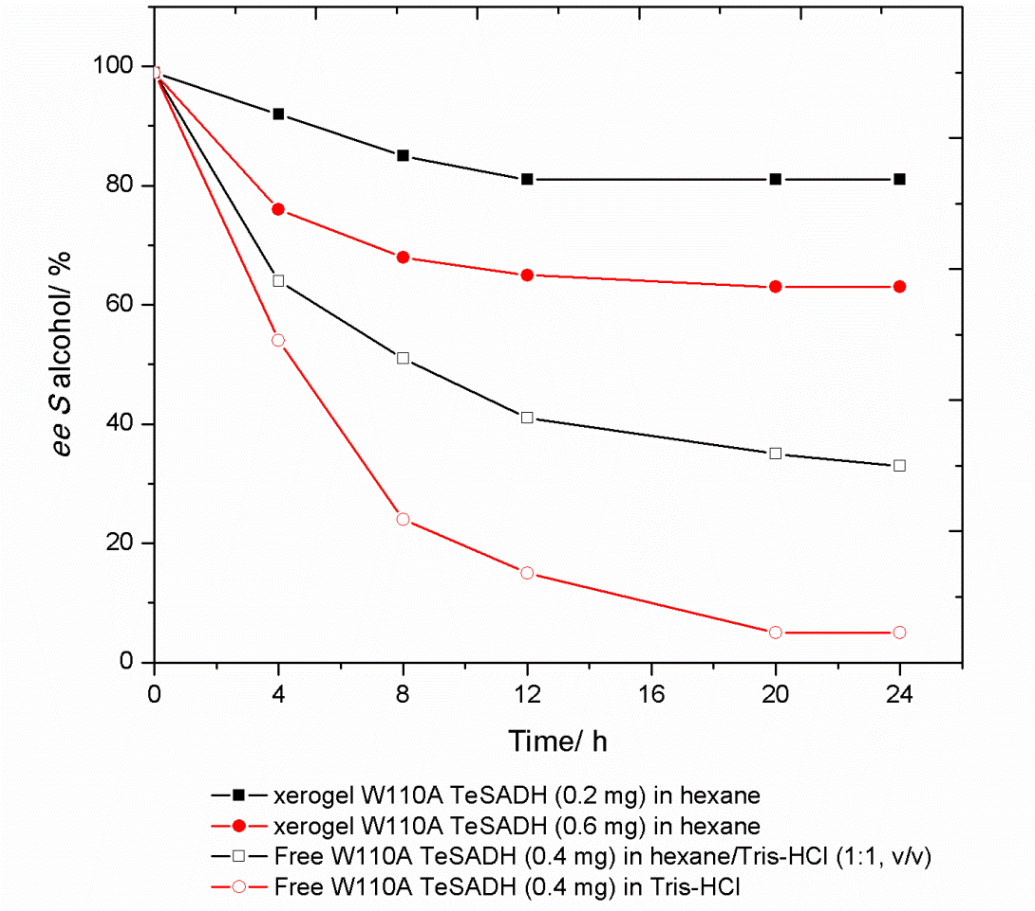
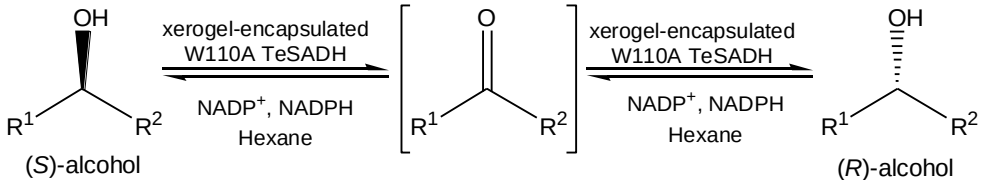


Figure 25. Comparison of racemization of (*S*)-1-phenyl-2-propanol using free and immobilized W110A TeSADH in aqueous, biphasic and organic media.

5.3.4 Racemization of selected enantiopure phenyl-ring-containing alcohols using xerogel-immobilized W110A TeSADH in hexane

Xerogel containing W110A TeSADH (0.6 mg) in hexane was the optimal condition for racemization. In Table 13 are shown the *ee* before and after racemization of enantiopure alcohols. A higher edge was observed in the racemization of (*R*)-**1h** as compared to that of (*S*)-**1h** (Table 13, entries 3 and 4). A similar trend was in the racemization of (*S*)-4-phenyl-2-butanol [(*S*)-**1a**] and (*R*)-**1a**, however, with lower efficiency (Table 13, entries 1 and 2). This is attributed to the structure of **1a**, which seems to properly fit in the active site of W110A TeSADH and thus increasing the substrates' stereospecificity of the enzyme and hence slowing racemization. TeSADH is reported to have two pockets within the active site, which are varying in size [17]. The relatively small size of **1h** might enable the entire molecule to fit in the large pocket with either direction allowing more selectivity mistakes hence the enhanced racemization. The improved racemization efficiency of (*R*)-alcohols compared to (*S*)-alcohols is attributed to the fact that W110A TeSADH obeys Prelog's rule, and thus expected to produce (*S*)-configured alcohols from the generated intermediate prochiral ketones whilst the (*R*)-configured alcohol is formed by selectivity mistakes. Least racemization efficiency was observed for (*R*)-1-phenyl-2-butanol [(*R*)-**1i**] (98% to 90% *ee*), likely due to the longer ethyl chain that might have restricted proper fitting of this enantiomer in the active site (i.e., high preference to (*S*)-enantiomer with less selectivity mistakes) (Table 13, entry 5).

Table 13. Xerogel-encapsulated W110A TeSADH-catalyzed racemization of selected enantiopure phenyl-ring-containing alcohols.



Entry	Substrate	R^1	R^2	*ee* [%] *S* enantiomer	
				before	*after*
1	(*S*)-**1a**	$C_6H_5CH_2CH_2$	CH_3	>99(*S*)	93(*S*)
2	(*R*)-**1a**	$C_6H_5CH_2CH_2$	CH_3	>99(*R*)	80(*R*)
3	(*S*)-**1h**	$C_6H_5CH_2$	CH_3	>99(*S*)	63(*S*)
4	(*R*)-**1h**	$C_6H_5CH_2$	CH_3	>99(*R*)	48(*R*)
5	(*R*)-**1i**	$C_6H_5CH_2$	CH_3CH_2	98(*R*)	90(*R*)

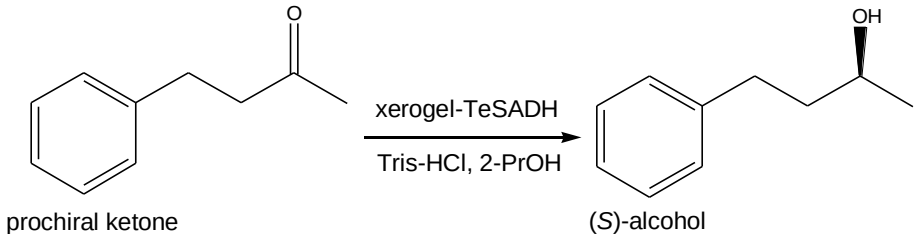
Reaction conditions: alcohol (0.04 mmol), xerogel [W110A TeSADH (0.6 mg), NADPH (1.0 mg) and $NADP^+$ (1.0 mg)] in hexane (1.0 mL) at 50 °C for 24 h.

5.3.5 Checking the efficiency of xerogel-immobilized enzyme

The poor racemization observed by the immobilized enzyme called for further investigation. In order the rule out whether the enzyme is killed during gel formation, the efficiency of the immobilized enzyme was investigated in the reduction of 4-phenyl-2-butanone using xerogel-encapsulated W110A and W110G TeSADH respectively which is crucial in racemization since the recovered *ee* of the alcohol relies on the selectivity mistakes during hydride delivery. The effect of incorporating a silane with the hydrophobic group in the TMOS xerogel was also studied. In Table 14 are shown the conversions and *ee* obtained in the reduction of 4-phenyl-2-butanol using xerogel-encapsulate TeSADH formed from TMOS and mixed silanes.

The immobilized-W110A TeSADH enzyme using TMOS retained full activity in the reduction of **2a** (>99% conv.) in Tris-HCl (aqueous medium) (Table 14, entry 1). However, low activity was observed in biphasic medium (Tris-HCl/hexane) attributed to poor diffusion of substrates in and out of the xerogel (Table 14, entry 2). Nevertheless, the moderately high conversion was observed using ethylene glycol (74% conv.) suggesting facilitated diffusion of substrates in and out of the immobilized enzyme. Attempts to incorporate silanes with hydrophobic moieties in the xerogel were futile (Table 14, entries 5-8). W110G TeSADH showed slightly lower activity than W110A TeSADH suggesting less tolerance to organic media (Table 14, entry 4).

Table 14. Reduction of 4-phenyl-2-butanone using xerogel-encapsulated TeSADH formed using TMOS and a mixture of silanes.



prochiral ketone (*S*)-alcohol

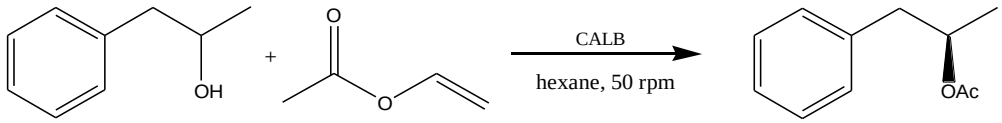
Entry	Xerogel	Alcohol [%]	*ee* [%]
1	TMOS	>99	97(*S*)[a]
2	TMOS	23	97(*S*)
3	TMOS	74	95(*S*)[b]
4	TMOS	20	95(*S*)[c]
5	GTMS/TMOS	15	>99(*S*)
6	ITMS/TMOS	4	>99(*S*)
7	MTMS/TMOS	1	>99(*S*)
8	VTMS/TMOS	1	>99(*S*)

Reaction conditions: 4-Phenyl-2-butanone (0.05 mmol), xerogel-immobilized enzyme [W110A (0.2 mg), $NADP^+$ (1 mg)], in hexane/2-propanol (7:3, v/v) at 50°C at 50 rpm for 24 h. [a]Tris-HCl buffer (pH 8, 50 mM) and [b]ethylene glycol used as a solvent. [c]W110G TeSADH used.

5.3.6 Optimization of lipase-catalyzed KR of alcohols

In an attempt to optimize KR, several variables were investigated in the reaction of (*rac*)-1-phenyl-2-propanol with vinyl acetate. Among studied were the effects of temperature, time, catalyst loading and concentration ratio of the alcohol to acyl donor used as shown in Table 15. An equimolar substrate ratio was sufficient, however, an excess of the acyl donor (2 mol eq) was chosen for the reactions to cater of loss of the acyl donor by evaporation during the reaction (Table 15, entry 7). Heating at 50 ^{o}C led to an exponential increase in the rate obtaining in as short as 8 h a similar conversion obtained at room conditions after 48 h (52% vs 60%, respectively) (Table 15, entries 3 and 5).

Table 15. KR dependency on temperature, time, substrate concentration and catalyst loading.



(*rac*)-**1h** (*R*)-**1h** ester

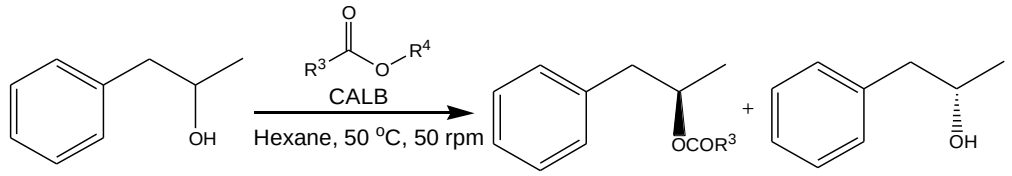
Entry	CALB/ mg	acyl/ mol eq.	Ester [%]	*ee* [%]
1	0.5	1	24	92(*R*)
2	1	0.5	28	93(*R*)
3	1	1	60	73(*R*)
4	1	1	39	74(*R*)[a]
5	1	1	52	81(*R*)[b]
6	2	1	81	28(*R*)
7	1	2	53	77(*R*)

Reaction conditions: RT (25°C) for 48 h using CALB, (*rac*)-**1h** (0.1 mmol), vinyl acetate in hexane (1.0 mL). [a]24 h, [b]50°C, 50 rpm, 8 h.

5.3.7 Comparison of selected acyl donors in CALB-catalyzed KR of racemic alcohols

The selectivity of different acyl donors was investigated in CALB-catalyzed KR of (*rac*)-1-phenyl-2-propanol. The reactions were conducted in hexane since it gave the best racemization results. The acyl donor plays a big role in *R*-selective acylation by CALB. In Table 16 are shown the conversion and stereoselectivity obtained in the acylation of (*rac*)-**1h** using different acyl donors. Racemization by xerogel-immobilized W110A TeSADH in hexane as the sole reaction medium allows its application in a one-pot dual enzymatic DKR when coupled with a CALB-catalyzed KR. A successful KR is crucial for DKR, which therefore demanded the optimization of KR to achieve the most active and highly stereoselective reaction conditions. Isopropenyl acetate and vinyl acetate showed similar reactivity (26% vs 31% conv.) but the former was the acyl donor of choice for DKR due to a slight edge in the stereoselectivity (E= 13-17 vs 10) (Table 16, entries 1-3). Methyl methoxy acetate showed excellent selectivity (E>100), however, the low conversion (5% conv.) is not suitable for a successful DKR (Table 16, entry 4). Moderate conversion and selectivity were observed for 2,2,2-trifluoroethyl butyrate (Table 16, entry 5).

Table 16. Comparison of the reactivity and selectivity of CALB-catalyzed kinetic resolution of (*rac*)-1-phenyl-2-propanol using selected acyl donors.



(*rac*)-**1h** (*R*)-**1h** ester (*S*)-**1h**

Entry	Acyl donor	Ester [%]	*ee* [%]	*E*(*R*/*S*)[b]
1	isopropenyl acetate	26	81(*R*)	13
2	isopropenyl acetate	48	78(*R*)[a]	17
3	Vinyl acetate	31	75(*R*)	10
4	methyl methoxy acetate	5	>99(*R*)	>100
5	2,2,2-Trifluoroethyl butyrate	15	87(*R*)	17

Reaction conditions: (*rac*)-**1h** (0.05 mmol), acyl donor **(**0.1 mmol) and CALB (1.0 mg) in hexane (1.0 mL) at 50 °C at 50 rpm for 3 h. [a]Reaction after 6 h. [b]*E* values were calculated according to Equation 1.

5.3.8 One-pot dual DKR of selected phenyl-ring-containing racemic alcohols

A stereoselective KR reaction compatible with an efficient racemization method is vital for a successful DKR. The compatibility of W110A TeSADH-catalyzed racemization and CALB-catalyzed KR of (*rac*)-1-phenyl-2-propanol in one-pot was investigated. Since the xerogel contains water, the two catalyst systems (i.e., xerogel-immobilized W110A TeSADH and CALB) were positioned in a way preventing direct contact of the two enzyme systems as explained in the experimental section [21]. The presence of water in the xerogel might slow down the CALB-catalyzed KR of alcohols due to the possibility of hydrolysis of the serine-acyl-donor intermediate in the active site of lipase. The excellent conversion with high *ee* (74% conv. & 68% *ee R*) obtained in deracemization of (*rac*)-**1h** via dual enzymatic DKR using CALB and W110A TeSADH with the calculated total composition of *R* enantiomers [(*R*)-**1h** and (*R*)-**1h** acetate ester) surpassing 50% (i.e. 62%) confirms the compatibility of CALB-catalyzed KR and W110A TeSADH-catalyzed racemization (Figure 26).

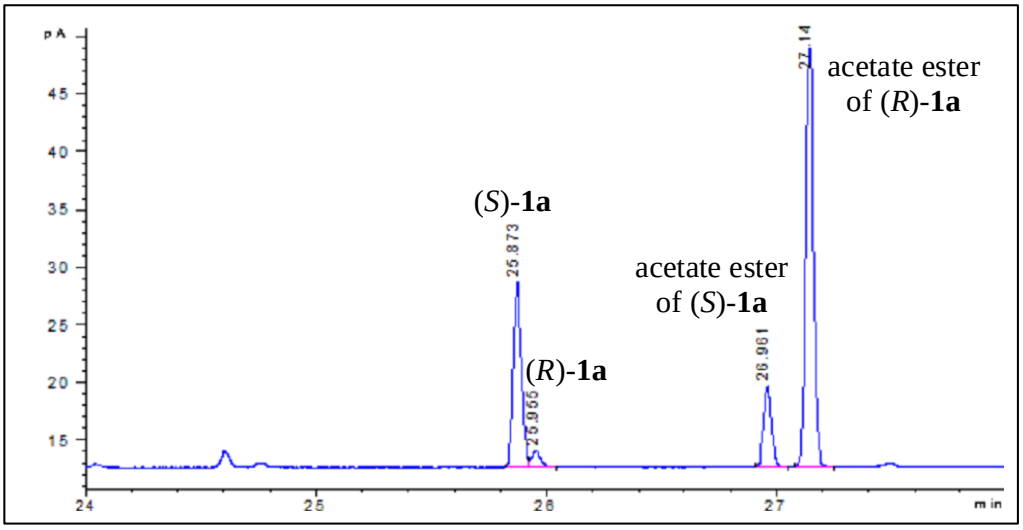
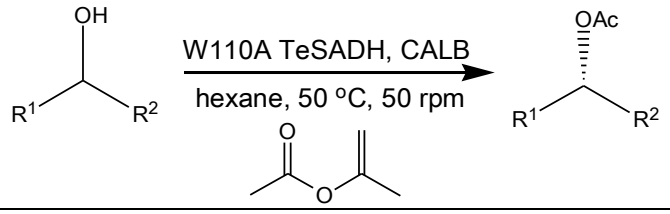


Figure 26. GC chromatogram of products of DKR of (*rac*)-**1a** using CALB-catalyzed KR and W110A TeSADH-catalyzed racemization in hexane.

Further, in Table 17 are shown the *ee* of *R*-ester obtained in DKR of selected racemic alcohols. Under the same reaction conditions, DKR of (*rac*)-**1a**, (*rac*)-**1b** and (*rac*)-4-phenyl-3-butyn-2-ol [(*rac*)-**1d**] gave similar results (69-71% conv. & 58-59% total *R*), (Table 17, entries 1-3). Lowest activity was observed with (*rac*)-**1i** (15% conv. & 52% *R*) attributed to both the poor racemization and slow KR of the substrate (Table 17, entry 5). Best DKR results obtained with (*rac*)-**1h** (74% conv. & 68% *ee*) is attributed to the good extent of racemization of its enantiopure forms by W110A TeSADH (Table 17, entry 4). Efficient DKR requires presence of a racemic alcohol during the course of reaction to minimize the selectivity mistakes that could be encountered in KR due to shortage of the desired enantiomer for the KR. Therefore efficient DKR is only possible when the rate of the racemization of the slow reacting *S*-enantiomer in KR is faster than the rate of the esterification of the fast reacting *R*-alcohol [70] which apparently is not the case in this research using due to the poor racemization by xerogel-immobilized W110A TeSADH hence the relative low outcome in DKR of majority of the substrates studied.

Table 17. W110A TeSADH-CALB one-pot DKR of selected racemic phenyl-ring-containing alcohols.



Entry	Substrate	R^1	R^2	Conv. (%)	*ee* (%)	Total *R* (%)[a]
1	(*rac*)-**1a**	$C_6H_5CH_2CH_2$	CH_3	70	66(*R*)	58
2	(*rac*)-**1b**	*p*-MeO-$C_6H_4CH_2CH_2$	CH_3	69	71(*R*)	59
3	(*rac*)-**1d**	C_6H_5CC	CH_3	71	63(*R*)	58
4	(*rac*)-**1h**	$C_6H_5CH_2$	CH_3	74	68(*R*)	62
5	(*rac*)-**1i**	$C_6H_5CH_2$	CH_3CH_2	15	99(*R*)	52

Reaction conditions: (*rac*)-alcohol (0.05 mmol), acyl donor **(**0.1 mmol), xerogel-immobilized enzyme [W110A (0.6 mg), $NADP^+$ (1.0 mg) and NADPH (1 mg)], CALB (1 mg) in hexane (1.0 mL) at 50 °C at 50 rpm for 24 h. [a]Total *R* (%)= *R*-alcohol + *R*-ester.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

Mutations in TeSADH were induced by site-directed mutagenesis at position 86 and 110 in the small and large pocket, respectively at the enzyme's active site. Parameters that control the stereoselectivity of the purified mutants were successfully studied to a reachable conclusion. W110G TeSADH with most extended large pocket showed the lowest stereoselectivity while W110A and the double mutant I86A/W110A TeSADH exhibited moderately higher stereoselectivity, however, the former had a higher edge in the oxidation of the racemic alcohols suggesting the W110A TeSADH allows selectivity mistakes and hence less selective than the double mutant. W110V TeSADH with relatively smaller large pocket was the most selective.

Medium engineering studies revealed a strong dependence of enzyme stereoselectivity on the nature of the solvent and cosolvent used. The stereoselectivity of oxidation decreased at low concentration of acetone while the reduction reaction was highly stereoselective at higher concentration of 2-propanol. 3% (v/v) acetone was optimal for the least enantioselective oxidation of alcohols to the corresponding ketones which was followed by highly stereoselective reduction using 30% (v/v) 2-propanol. The simple control of the oxidation and reduction pathways by varying the concentrations of acetone and 2-propanol cosubstrates was critical in successful deracemization of racemic alcohols using a single mutant of TeSADH. The concept was extendable to stereoinversion of *R* configured alcohols to *S* enantiomers. The use of a single enzyme advances the previous deracemization approaches that utilize at least two compatible enzymes or whole cells. In both studies excellent recovery of predominantly *S*-alcohol was

achievable. This concept should apply to ADH-catalyzed transformation reactions which can allow selectivity mistakes. Physical parameters like temperature were found have an effect on the stereoselectivity and activity of TeSADH. Selectivity of TeSADH catalyzed reduction was significantly reduced at room conditions. The activity of reduction of 4-phenyl-2-butanone was similar at room conditions and elevated temperature (50 ^{o}C) whereas the oxidation of 4-phenyl-2-butanol was significantly slow at room conditions.

The incorporation of water miscible and immiscible organic cosolvents still requires broad investigation. There was no clear correlation between the enzyme stereoselectivity and physical parameters like log P and pKa for the majority of the cosolvents in both oxidation and reduction reactions. Nevertheless, a deduction was reachable for W110G TeSADH-catalyzed reduction of 4-phenyl-2-butanone (study based on 25% volume of ethylene glycol, MeOH, EtOH and ^{t}BuOH as cosolvents) where the stereoselectivity increased with both log P and pKa whereas the conversion bared an inverse relationship.

The reactions in pure organic media required encapsulation of the enzymes. Tetramethoxysilane emerged the best silane for xerogel-immobilization of TeSADH and hexane was the best solvent. Only immobilized W110A TeSADH exhibited tolerance to pure organic media during racemization. Xerogel immobilized W110A TeSADH showed moderate activity in racemization of enantiopure phenyl-ring-containing alcohols in hexane contrary to aqueous media preferred by the enzyme. W110A TeSADH racemization in hexane was compatible with CALB-catalyzed KR which was applied in a dual enzymatic DKR of racemic alcohols in one pot. The relatively low efficiency of DKR is attributed to the poor racemization by the immobilized enzyme.

Biocatalysis is still a raw field that needs further exploration. Despite the successful application of W110G and W110A TeSADH mutants in stereoinversion and DKR, there is still a lot to be improved. Extensive mutations of TeSADH need to be conducted to achieve more efficient enzymes for racemization and stereoinversion. Developing new less stereoselective mutants of TeSADH is crucial for high racemization efficiency and less enantioselective oxidation of racemic alcohols which might improve the effectiveness of the reported deracemization techniques. Further investigation with regard to enzyme immobilization is crucial for reactions in organic media. There is a need to explore sol-gels synthesized from functionalized silanes with hydrophobic moieties that might improve substrate diffusion in and out of the xerogel enhancing the racemization efficiency for a more efficient DKR.

All in all the stereoselectivity of asymmetric redox reactions of alcohols and ketones was manipulated by protein engineering of TeSADH and by varying the reaction medium. Achieving less stereoselective conditions allowed racemization and poor enantioselective oxidation which were successfully utilized to deracemize alcohols by DKR and stereoinversion.

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APPENDIX A

STRUCTURES OF SUBSTRATES USED IN THIS STUDY

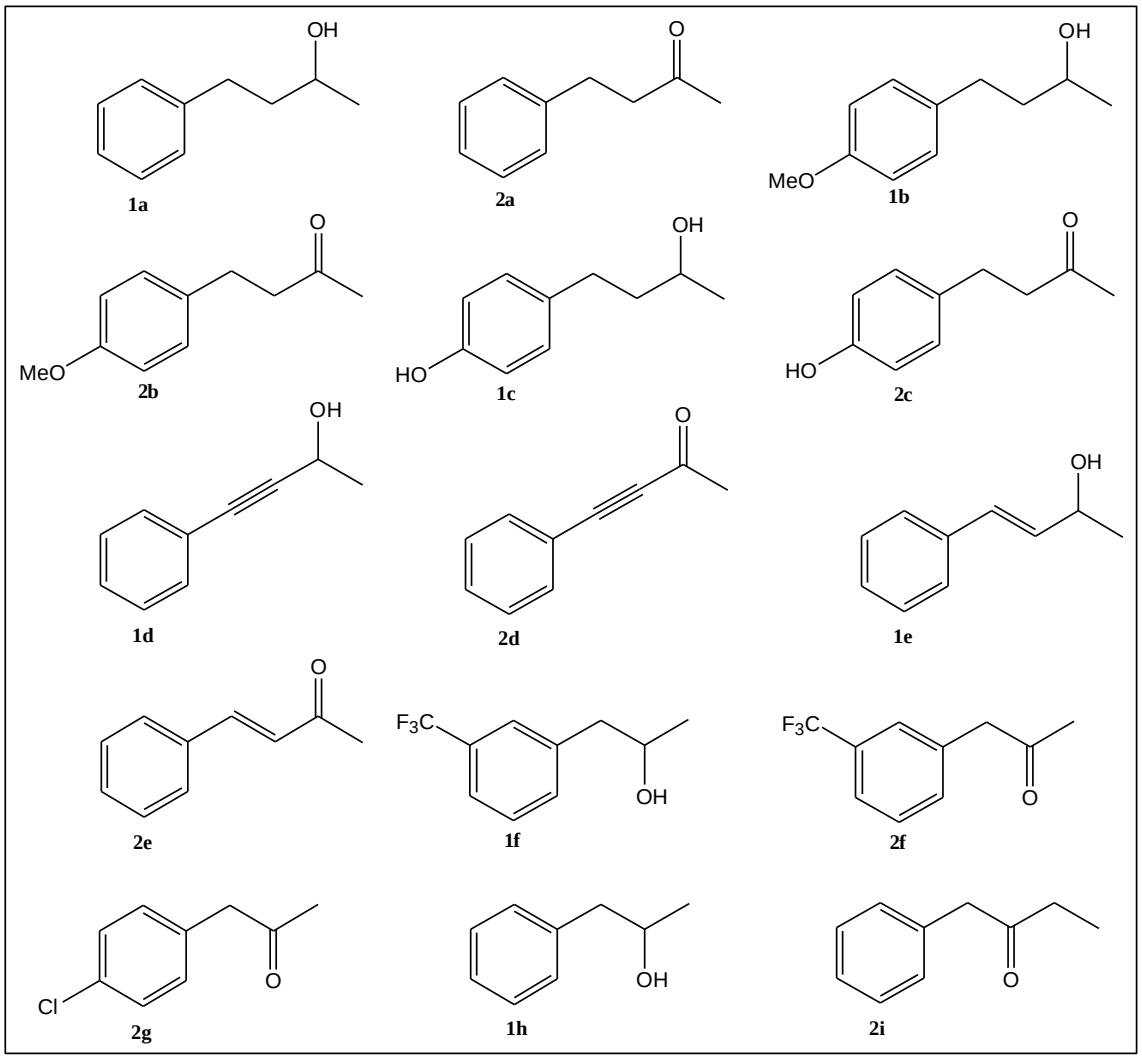

Figure S1. Structure of alcohol and ketones substrates.

APPENDIX B

GC CHROMATOGRAMS OF SELECTED REACTIONS

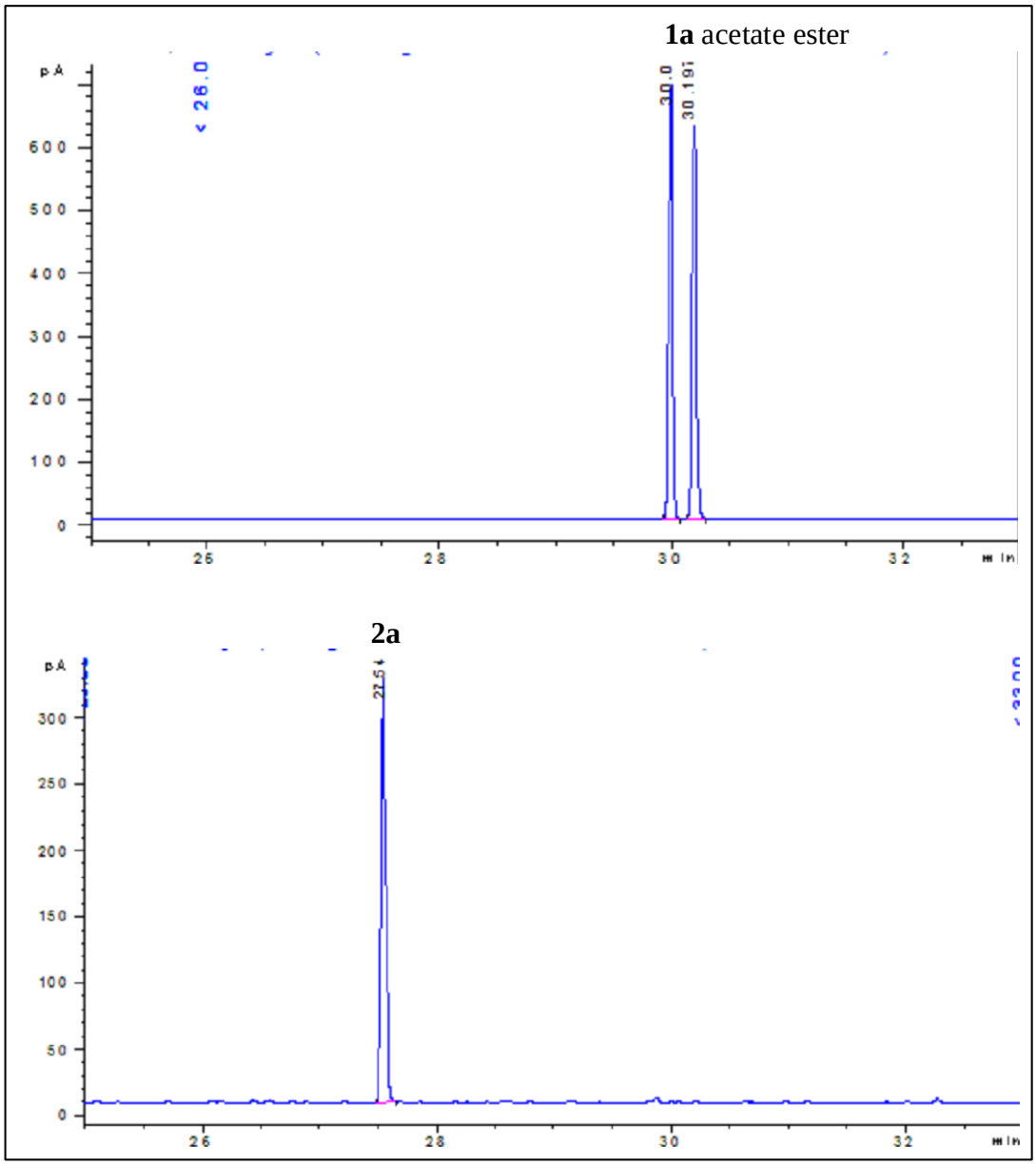

Figure S2. GC chromatograms of reference acetate ester of (*rac*)-**1a** and the correspond ketone **2a**.

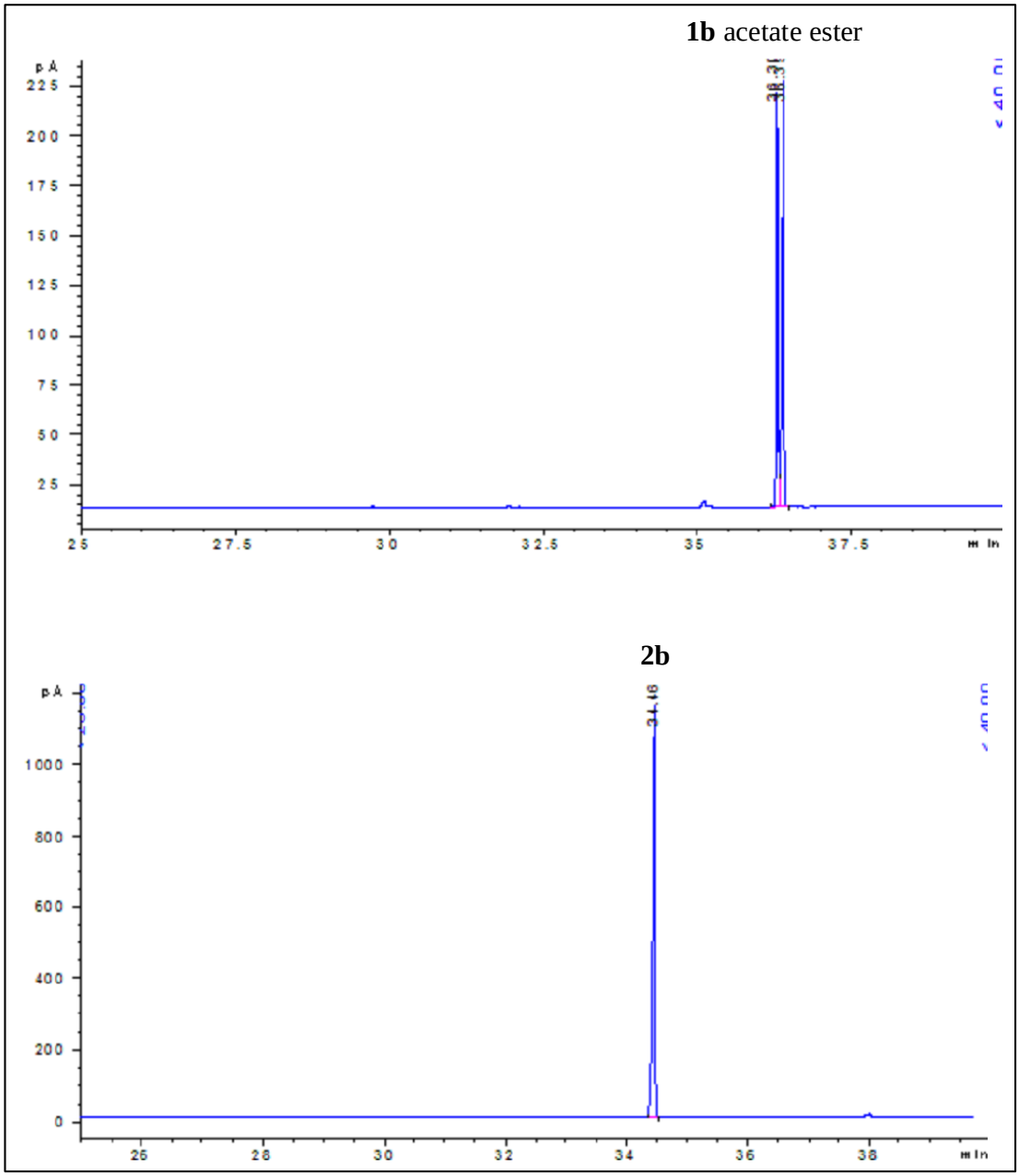

Figure S3. GC chromatograms of reference acetate ester of (*rac*)-**1b** and the correspond ketone **2b**.

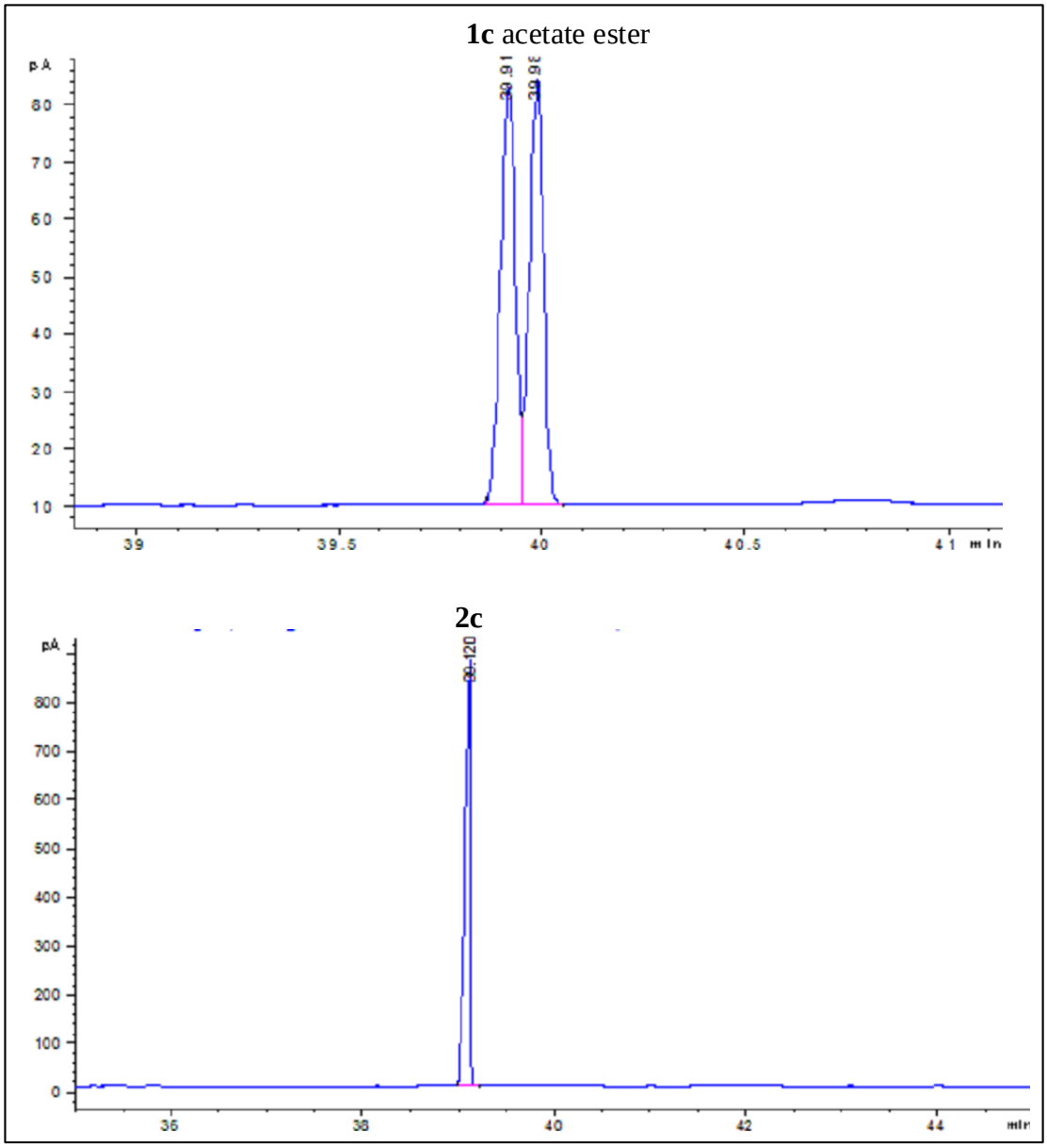

Figure S4. GC chromatograms of reference acetate ester of (*rac*)-**1c** and the correspond ketone **2c**.

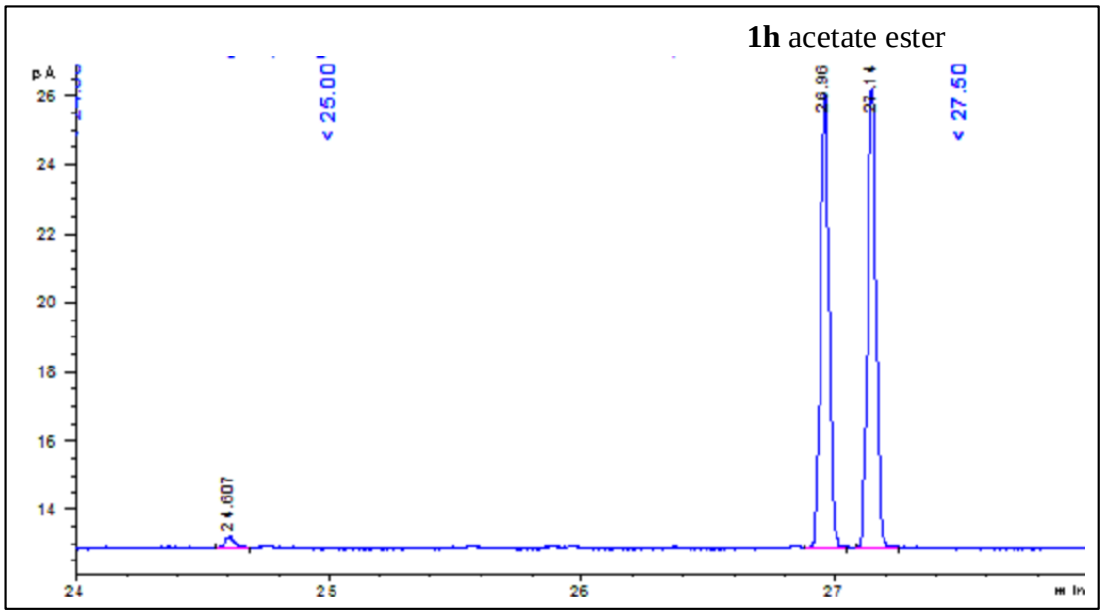

Figure S5. GC chromatogram of reference acetate ester of (*rac*)-**1h**

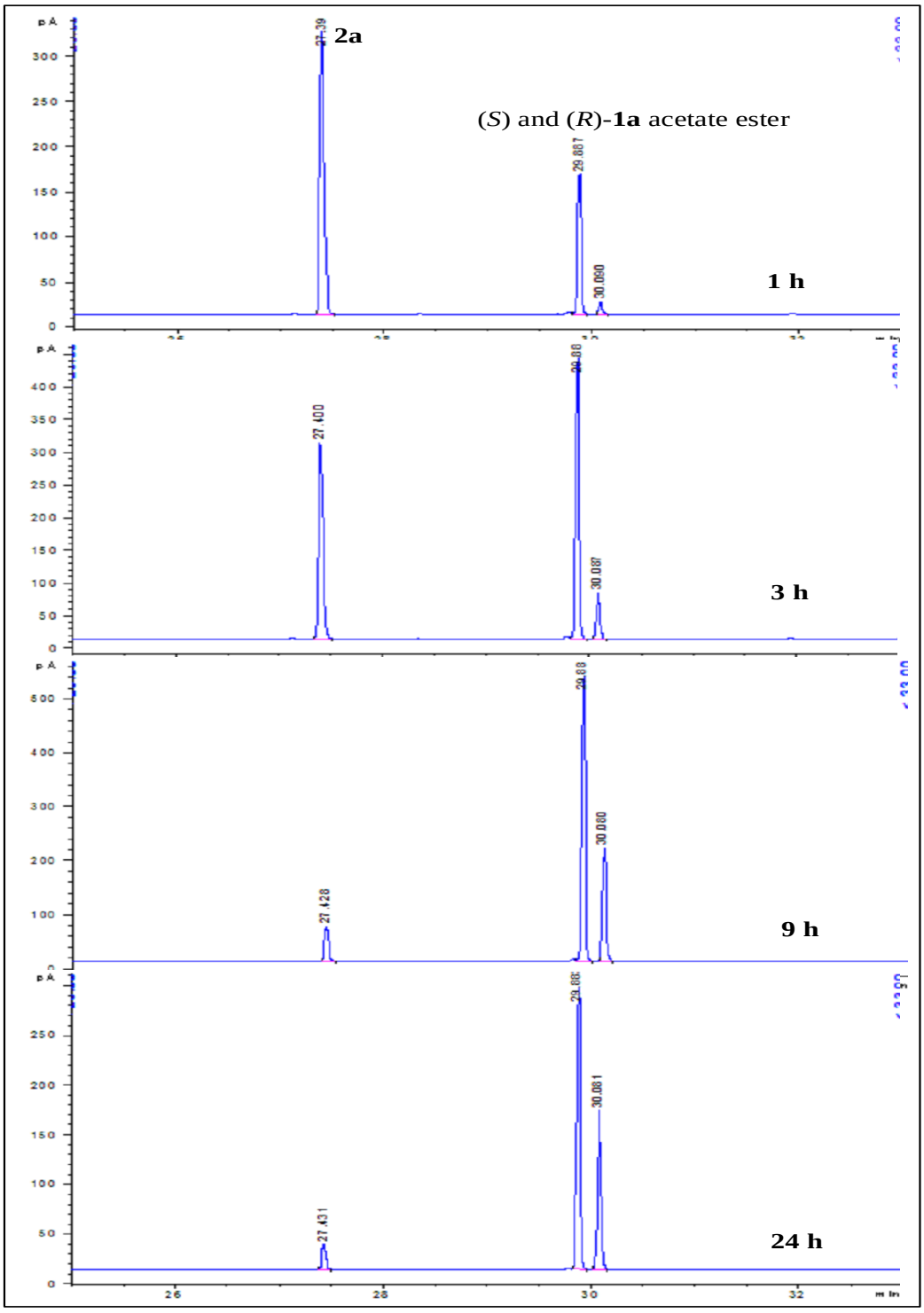

Figure S6. GC chromatograms obtained at selected time intervals in W110G TeSADH-catalyzed reduction of **2a** at 50 °C using 3% volume 2-propanol (Figure 6, main text).

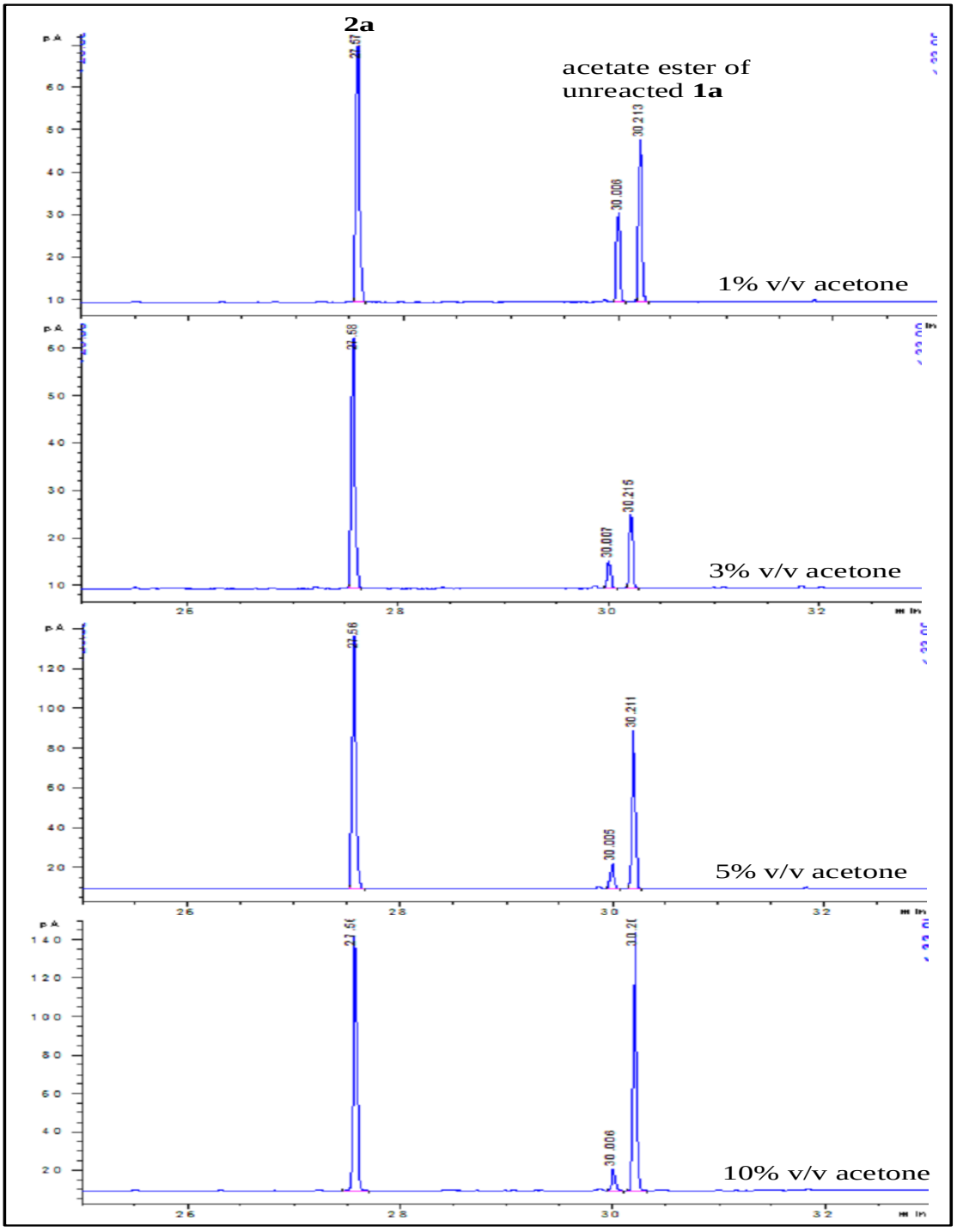

Figure S7. GC chromatograms obtained in W110G TeSADH-catalyzed oxidation of (*rac*)-**1a** at 50 ºC using 1-10% volume acetone (Figure 11, main text).

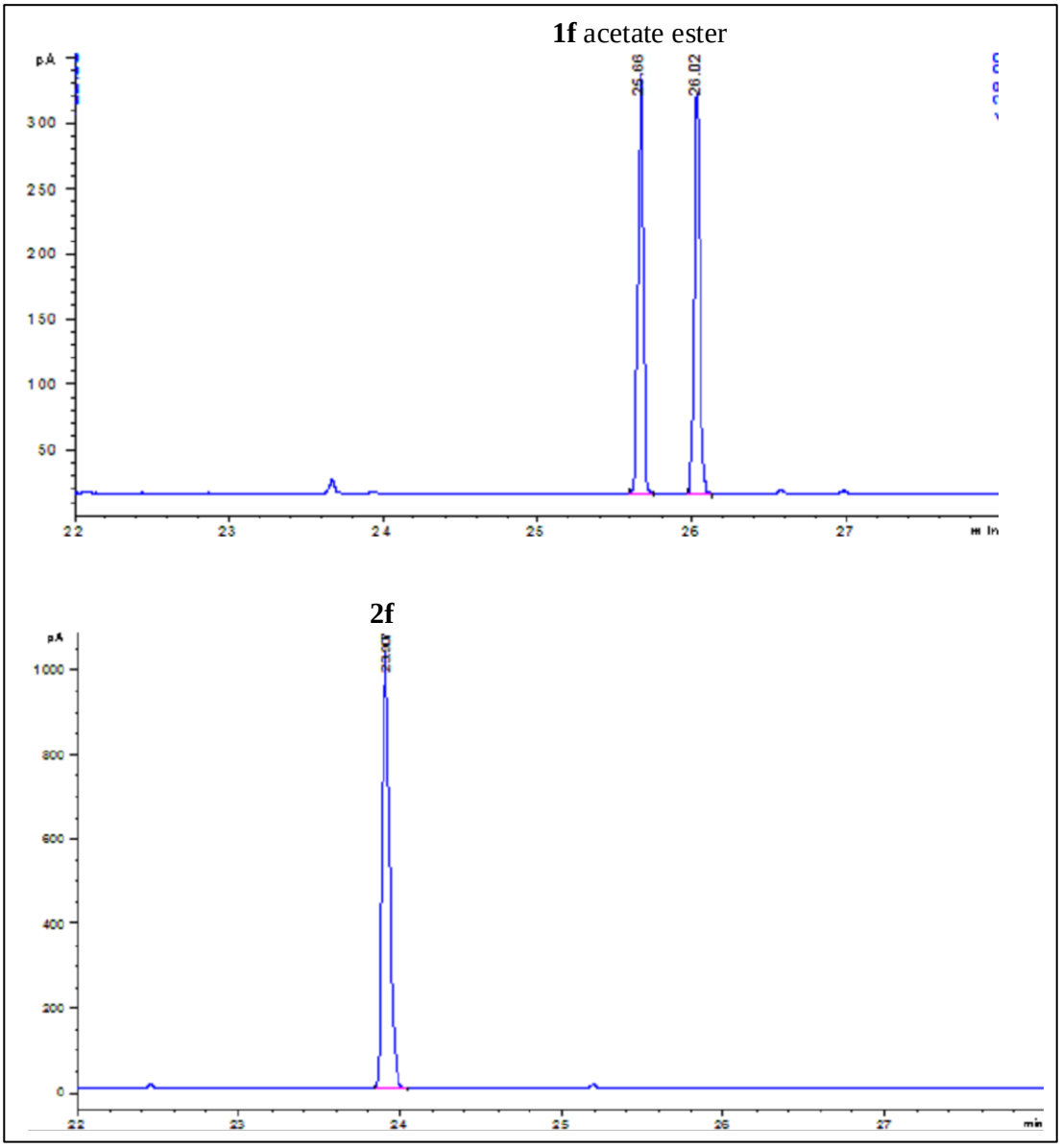

Figure S8. GC chromatograms of reference acetate ester of (*rac*)-**1f** and the correspond ketone **2f**.

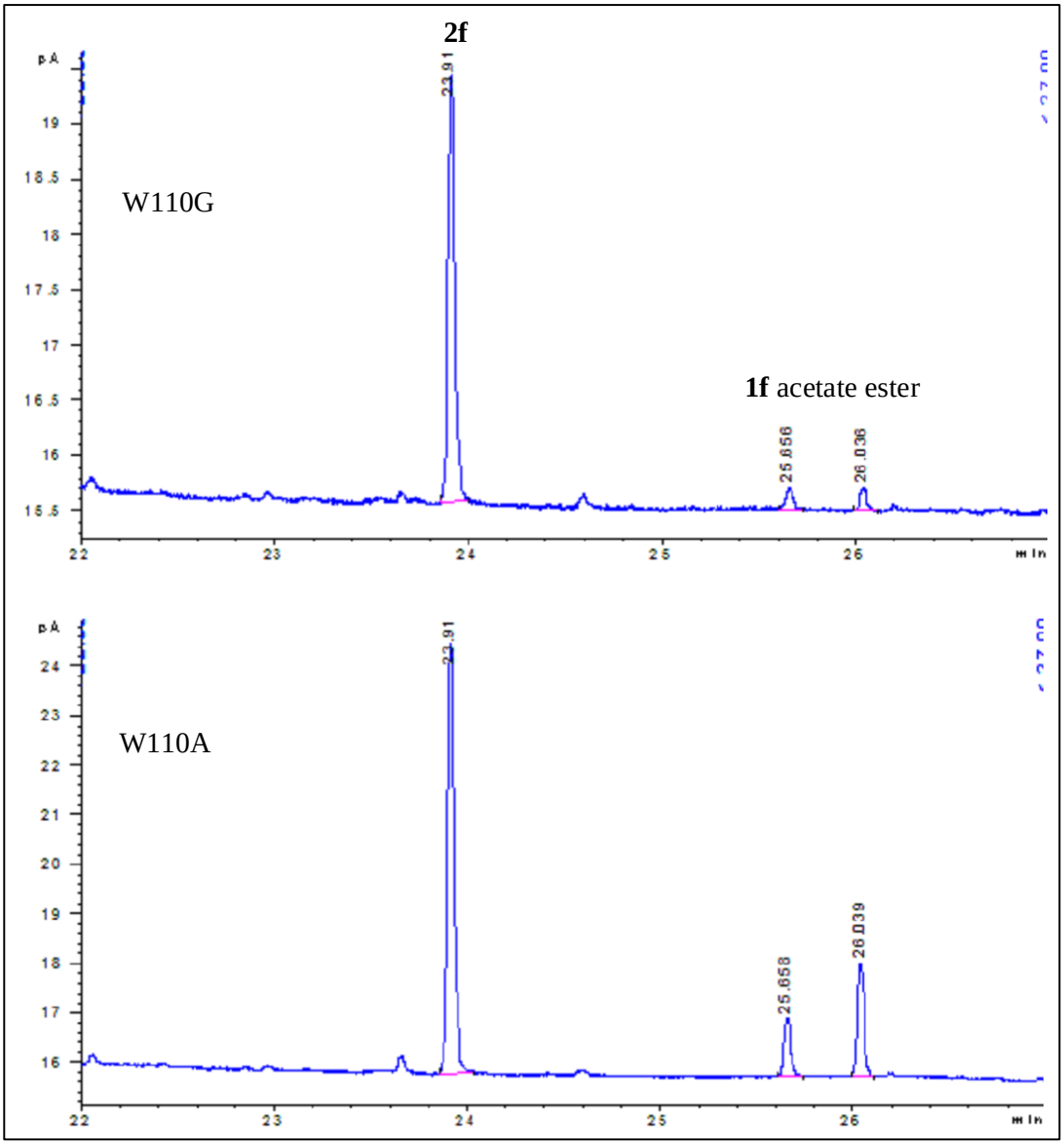

Figure S9. GC chromatograms of W110G and W110A TeSADH catalyzed-oxidation of (*rac*)-**1f** using 3% volume acetone (Table 5, entries 11 and 12).

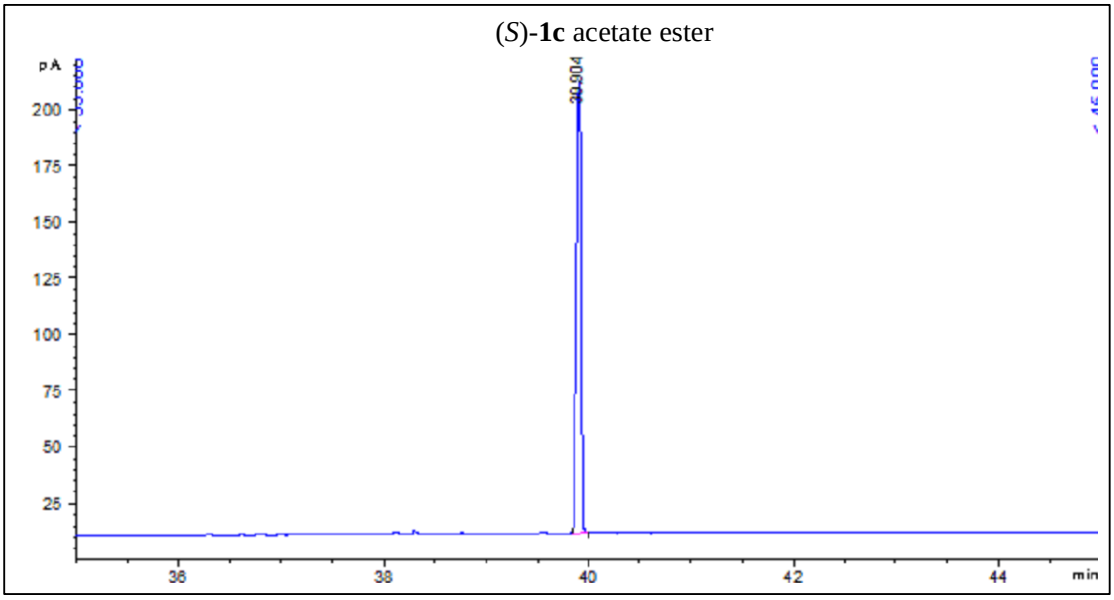

Figure S10. GC chromatogram of the acetate derivative of the product of the deracemization of **(*rac*)-1c** with W110G TeSADH (Table 8, entry 4).

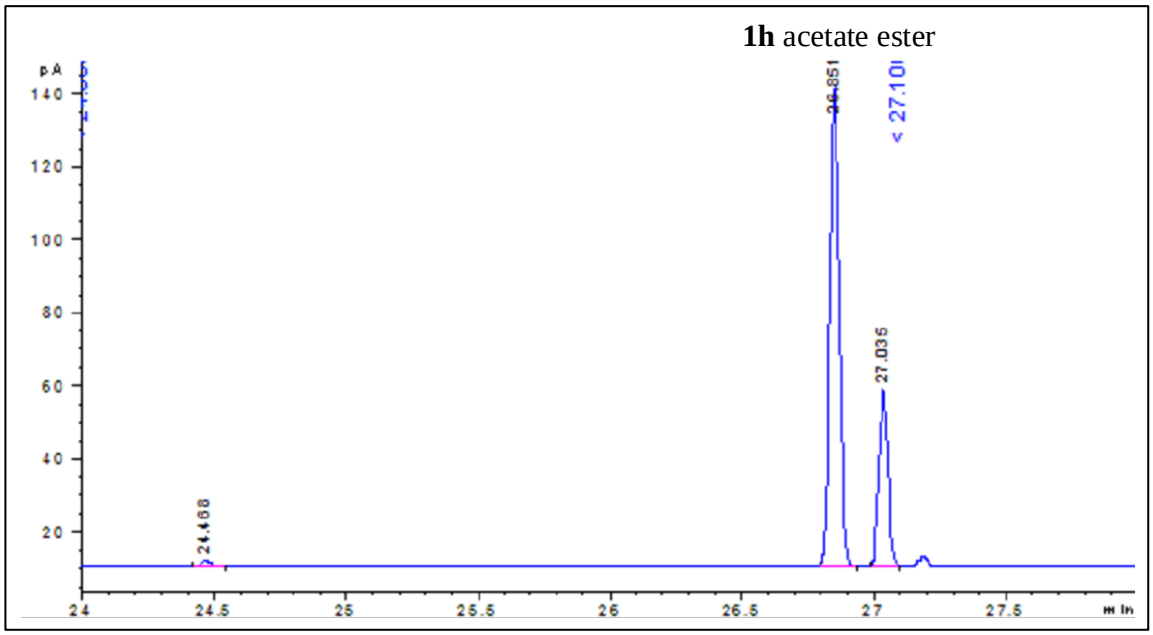

Figure S11. Gas chromatogram of the acetate derivative of the product of the deracemization of **(*rac*)-1h** with W110G TeSADH (Table 8, entry 7).

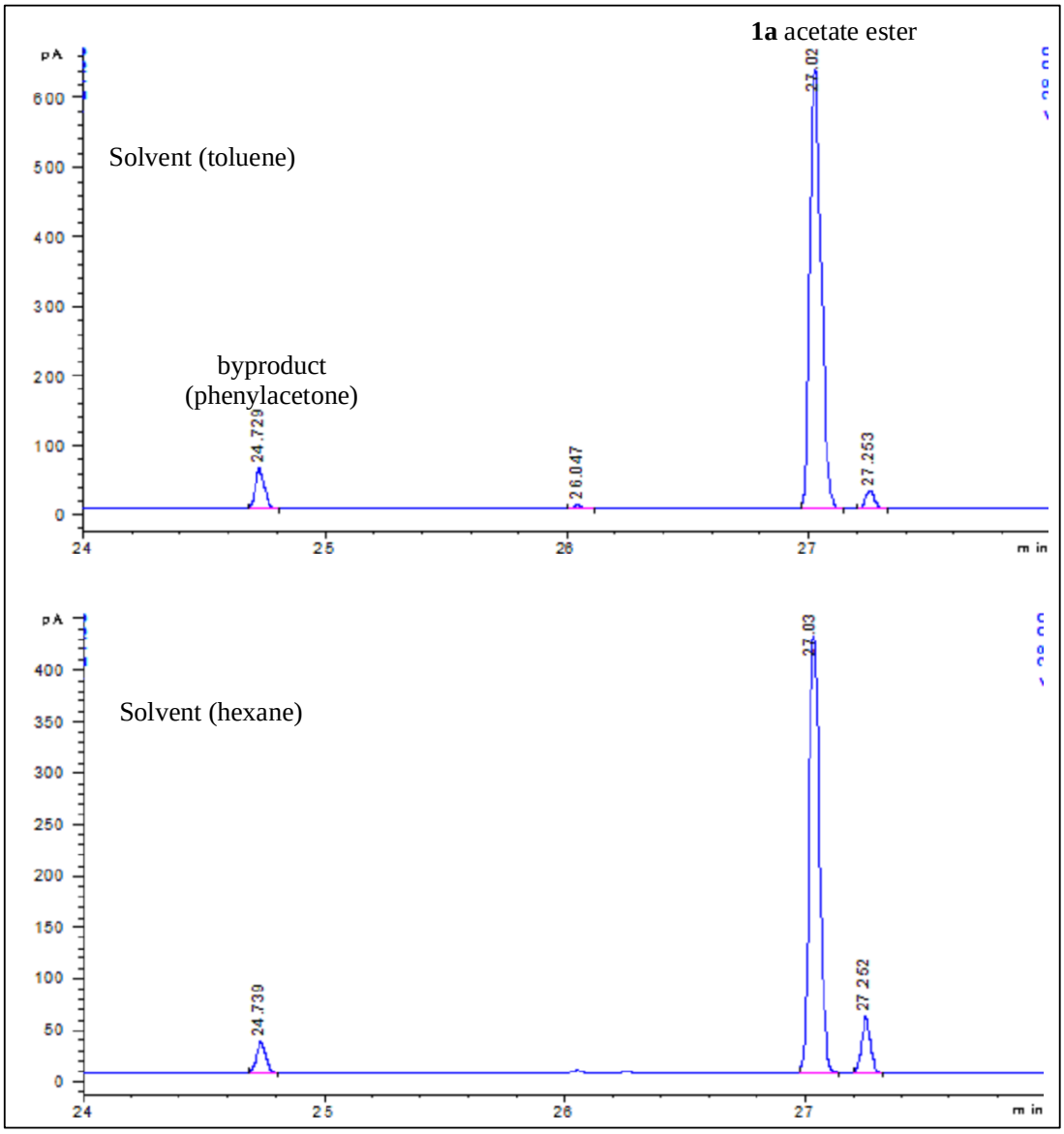

Figure S12. GC Chromatograms of xerogel-immobilized W110A TeSADH-catalyzed racemization of (*S*)-**1h** in toluene and hexane (Table 12, entries 5 and 8).

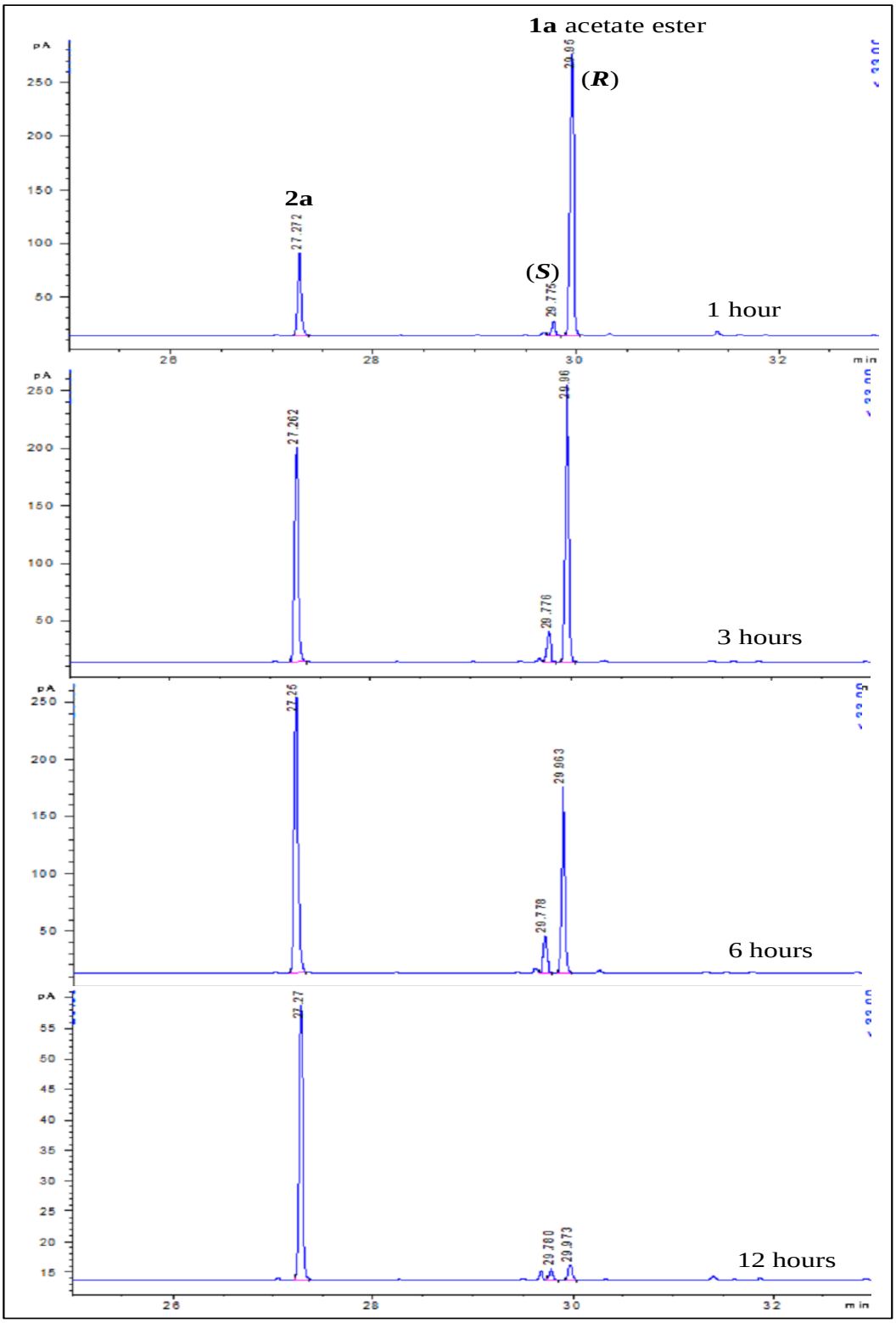

Figure S13. GC chromatograms of W110A TeSADH-catalyzed oxidation of (*R*)-**1a** at specified at time intervals (Figure 20, main text)

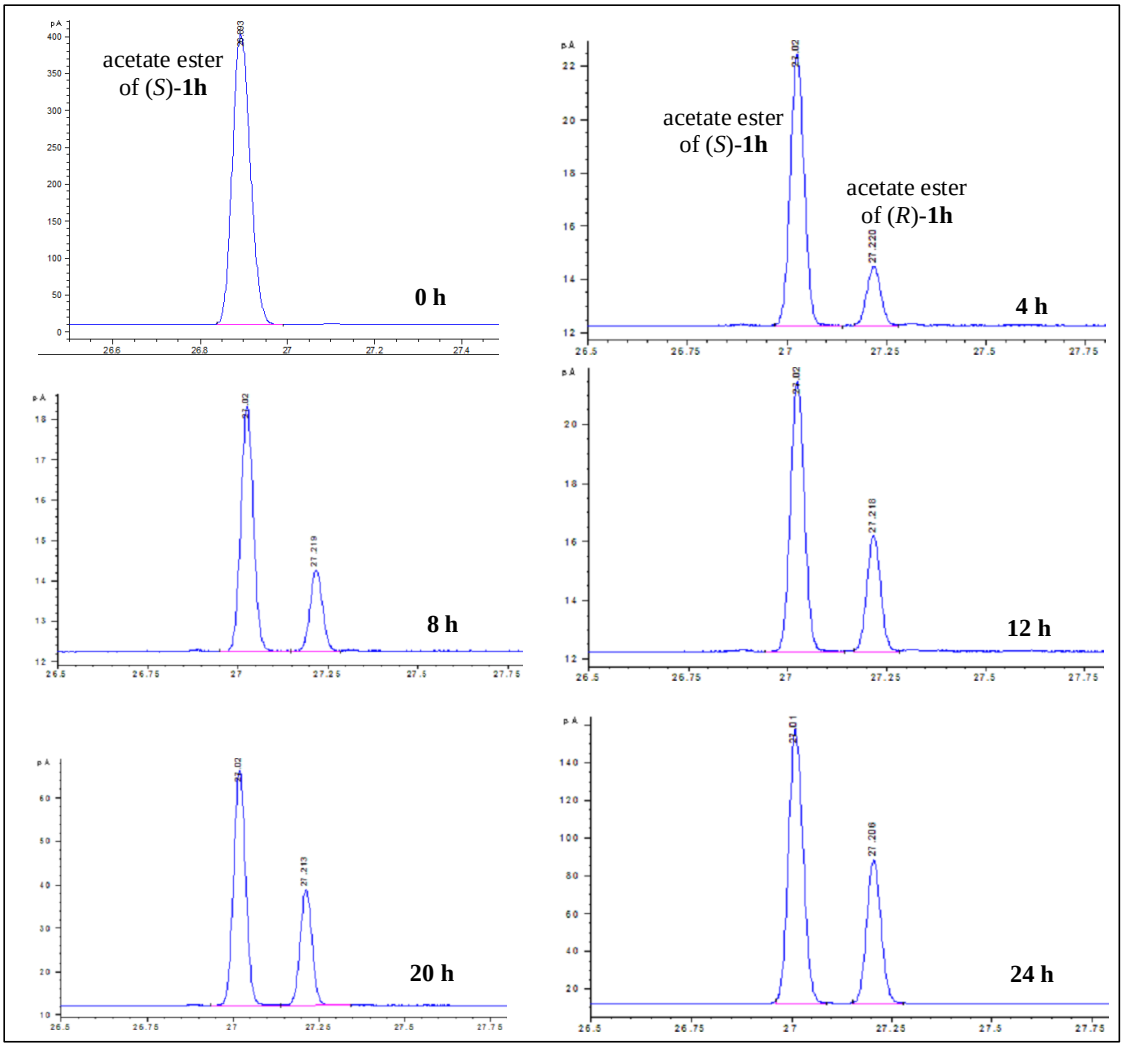

Figure S14. GC chromatograms of racemization of (*S*)-**1h** in biphasic medium-hexane/Tris-HCl at different time intervals (Figure 25, main text).

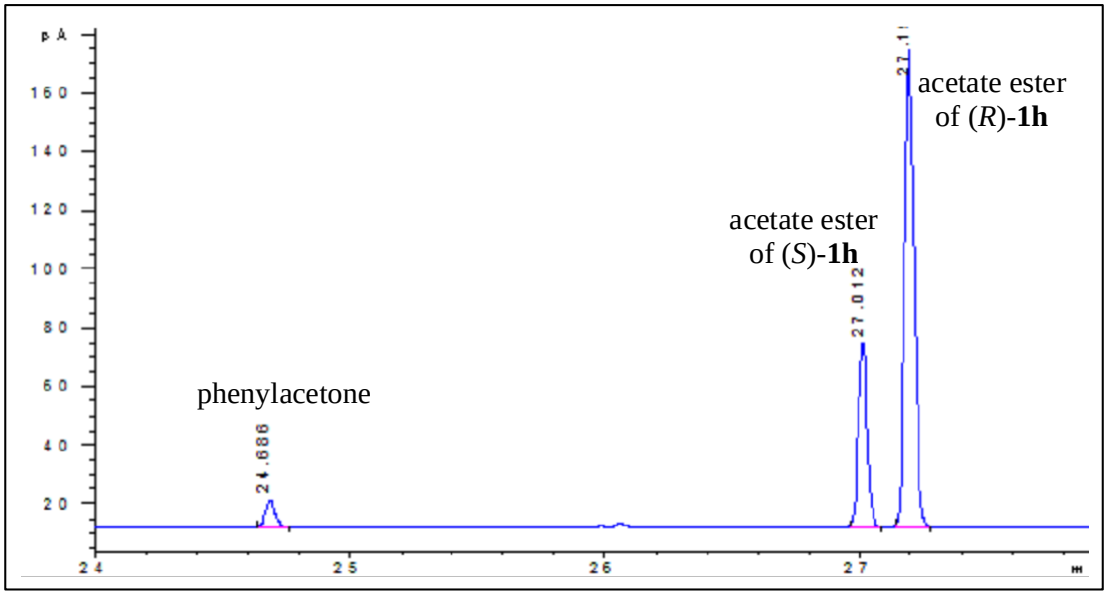

Figure S15. GC chromatogram of racemization of (*R*)-**1h** in hexane using xerogel-immobilized W110A TeSADH (Table 13, entry 4).

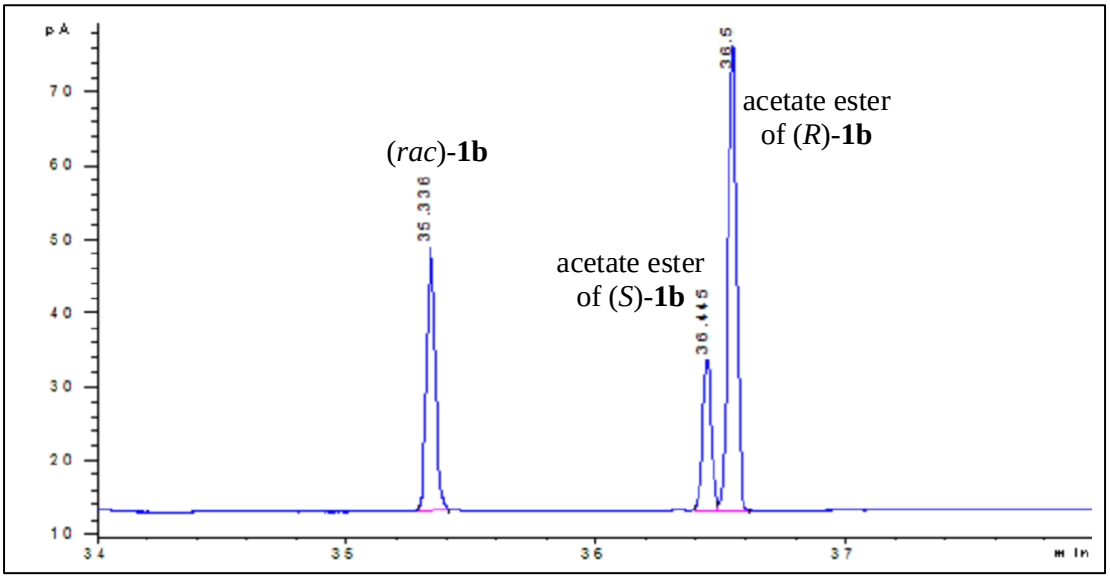

Figure S16. GC chromatogram of products of DKR of (*rac*)-**1b** using CALB-catalyzed KR and W110A TeSADH-catalyzed racemization (Table 17, entry 2).

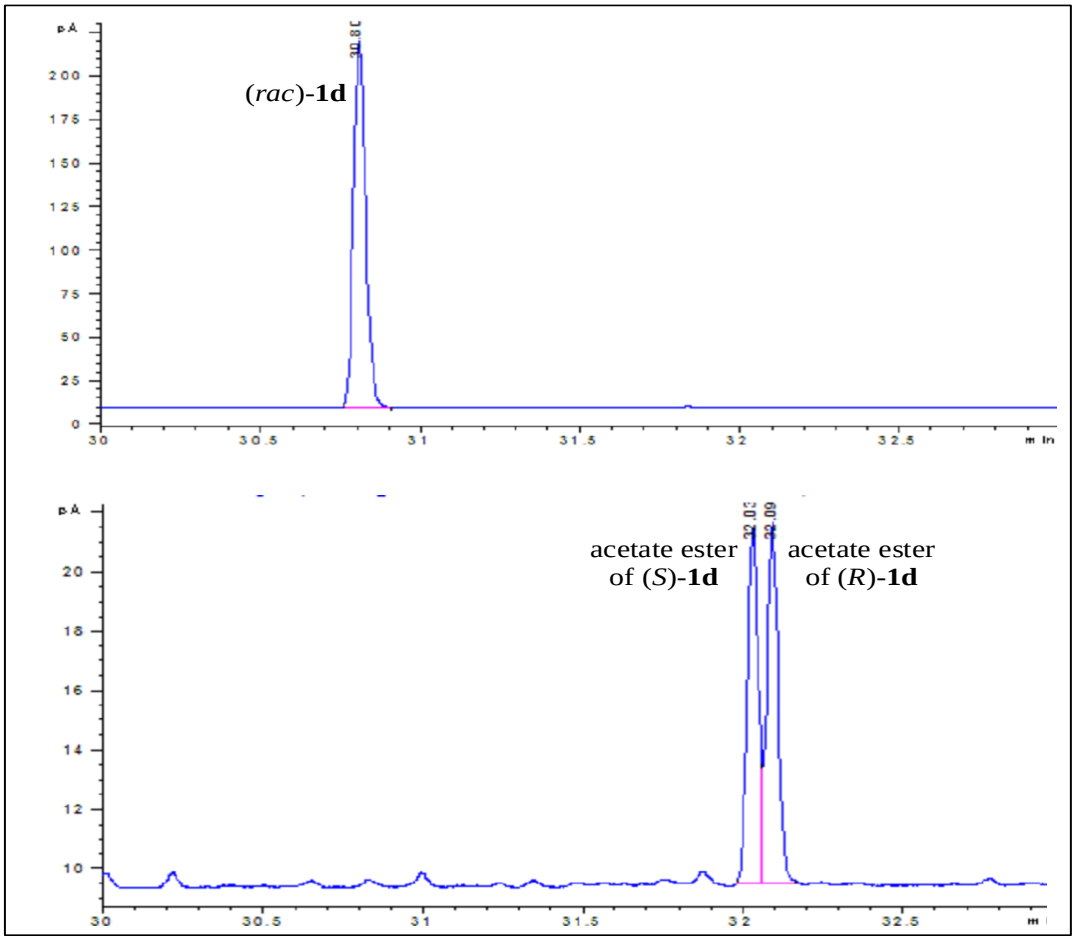

Figure S17. GC chromatograms of the reference (*rac*)-**1d** and the corresponding acetate ester derivative.

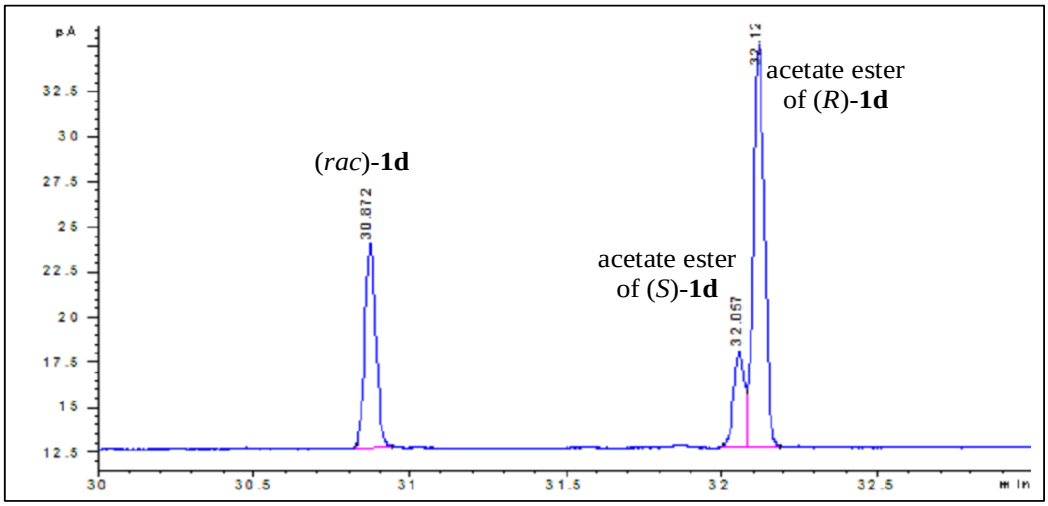

Figure S18. GC chromatogram of products of DKR of (*rac*)-**1d** using CALB-catalyzed KR and W110A TeSADH-catalyzed racemization (Table 17, entry 3).

APPENDIX C

GC-MS SPECTRA OF SELECTED REACTIONS

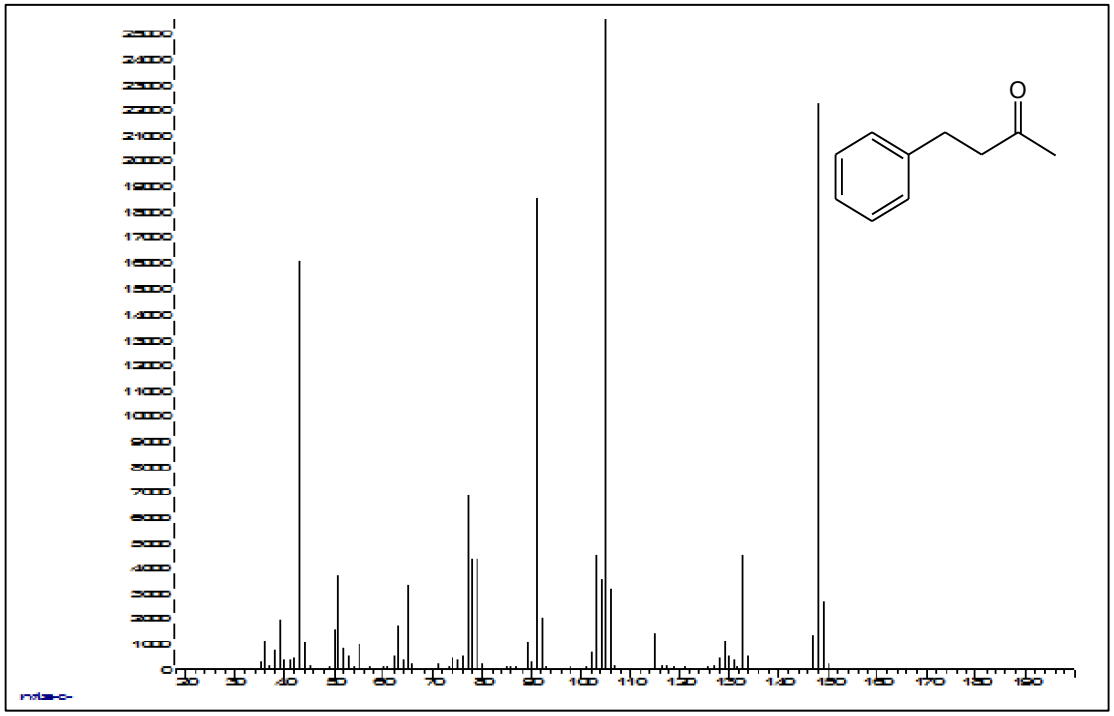

Figure S19. Mass spectrum of the product of the oxidation of (*rac*)-**1a** with W110G TeSADH using 5% acetone and of the acetate derivative of the unreacted alcohol.

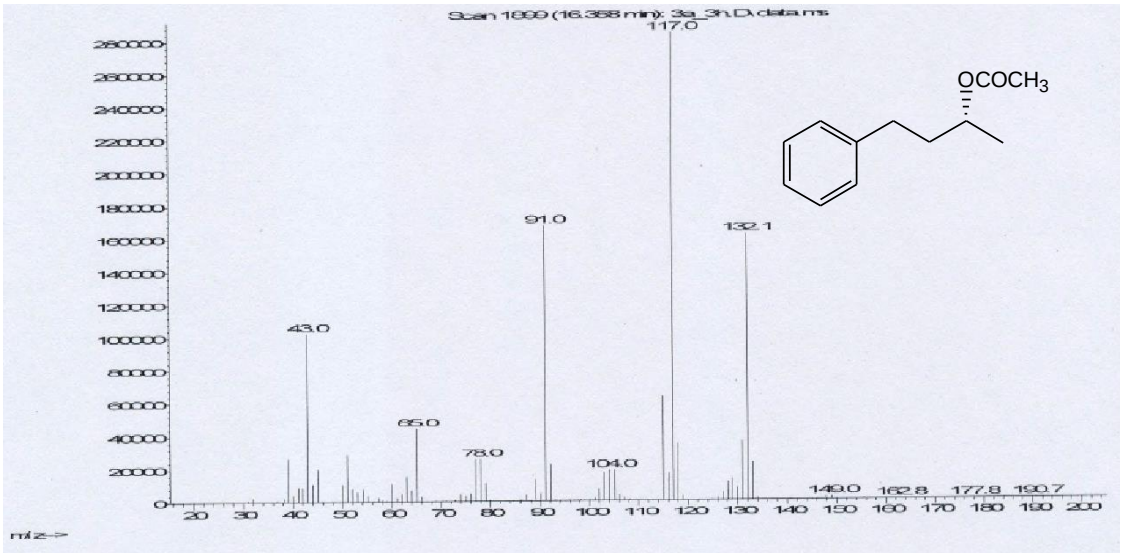

Figure S20. Mass spectrum of the product of the DKR of (*rac*)-**1a**.

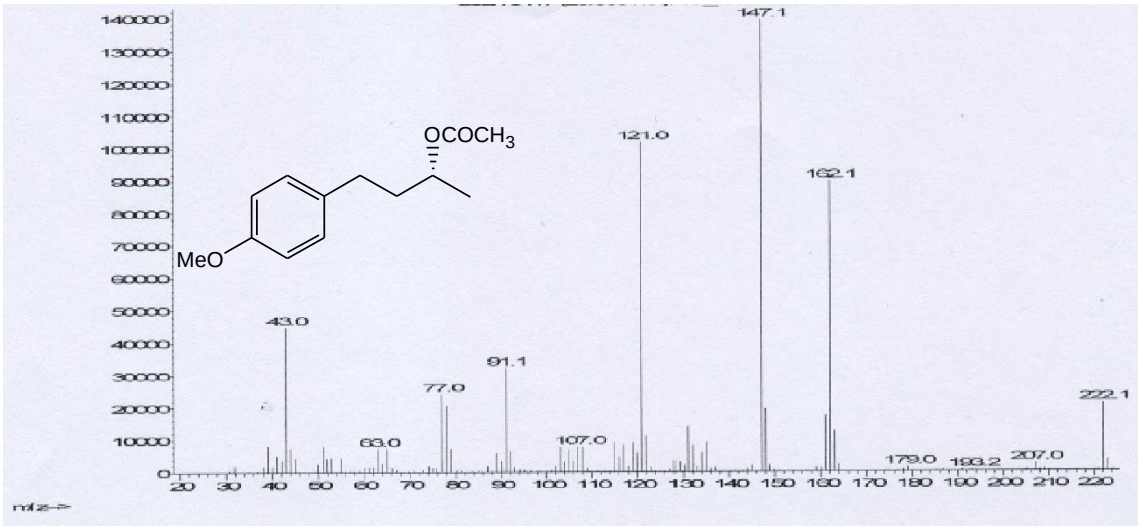

Figure S21. Mass spectrum of the product of the DKR of (*rac*)-**1b**.

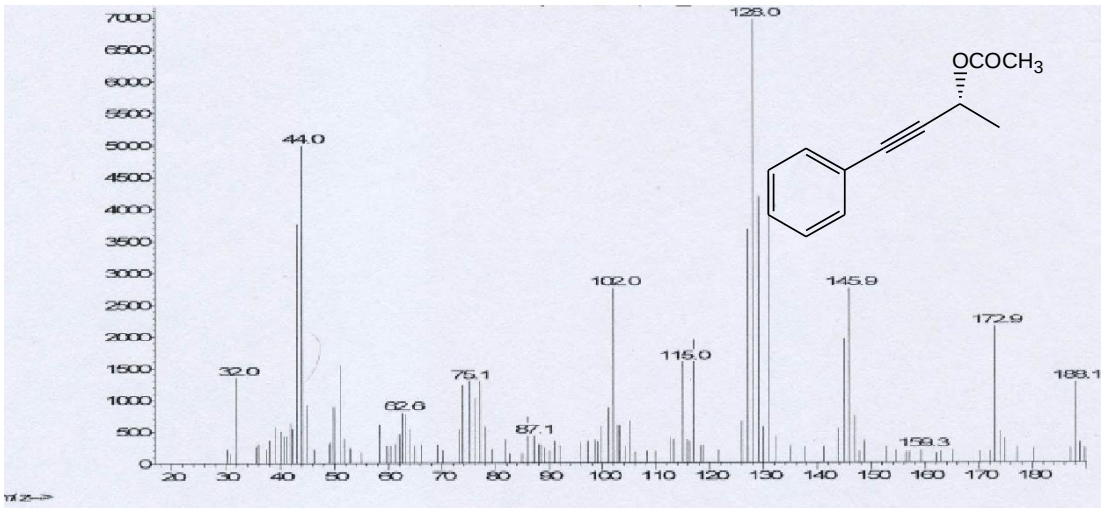

Figure S22. Mass spectrum of the product of the DKR of (*rac*)-**1d**.

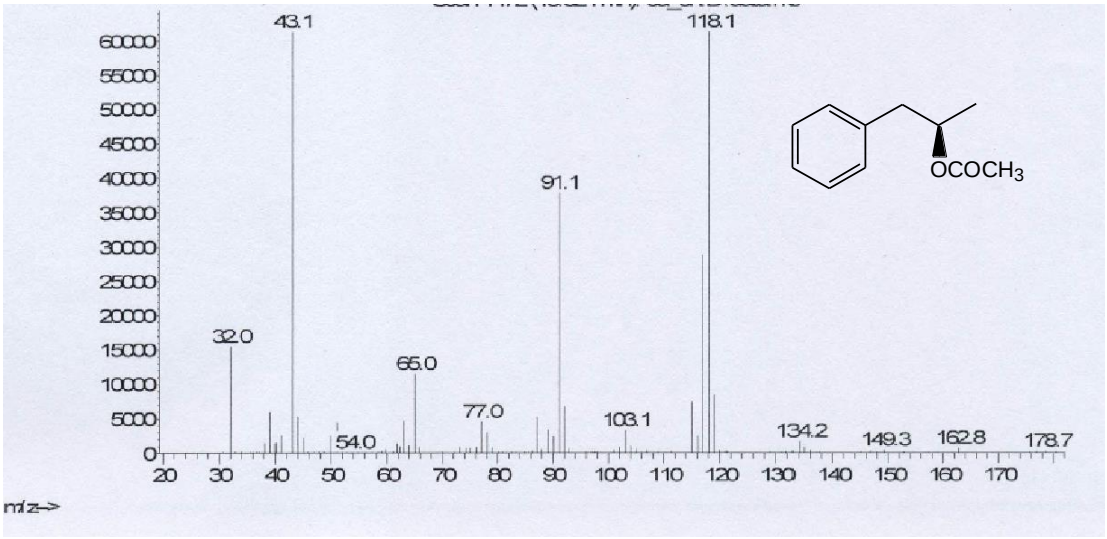

Figure 23. Mass spectrum of the product of the DKR of (*rac*)-**1h**.

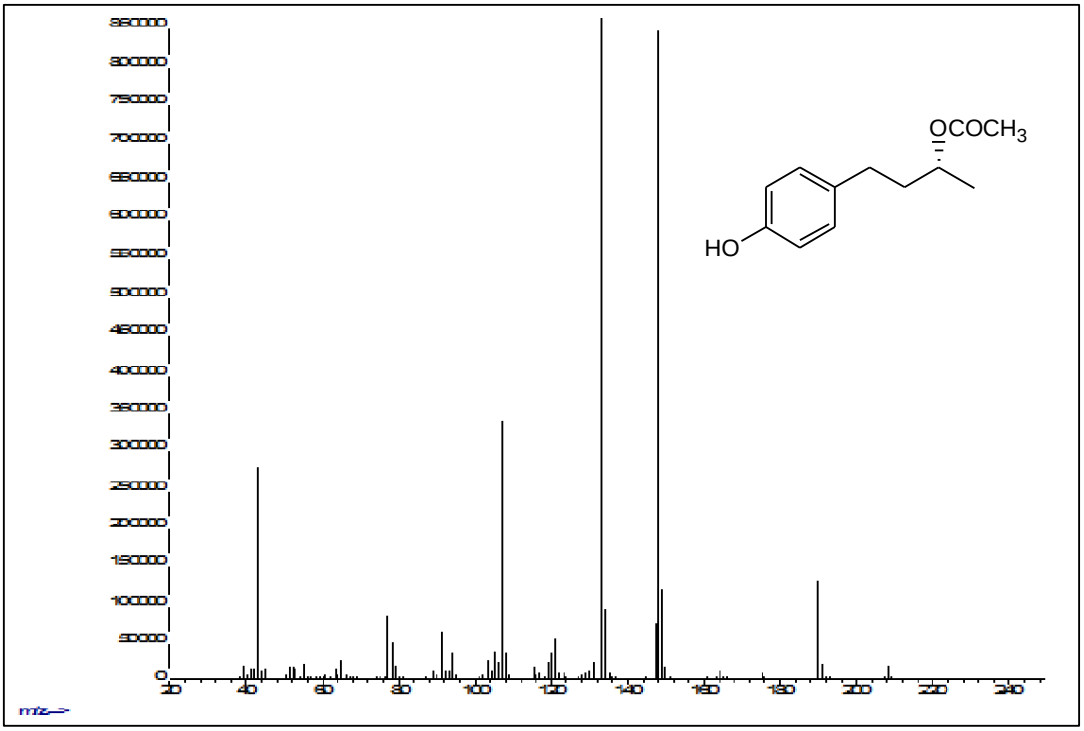

Figure S24. Mass spectrum acetate derivative of the product of deracemization of (*rac*)-**1c** with W110G TeSADH.

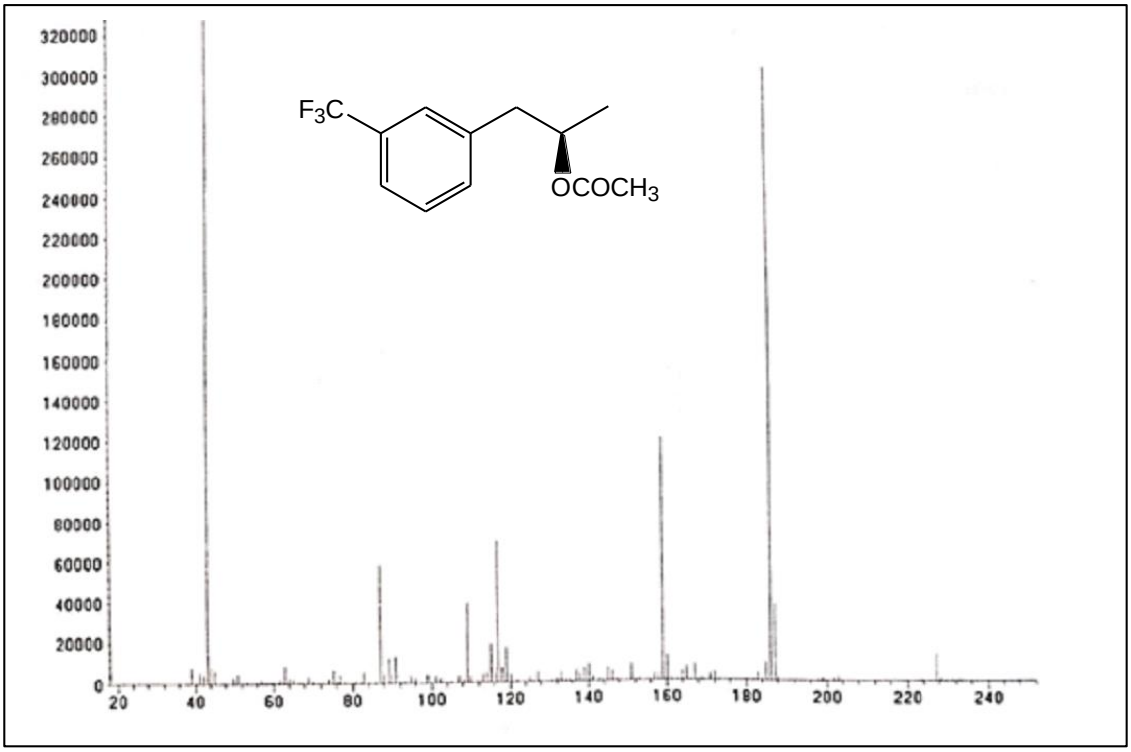

Figure S25. Mass spectrum acetate derivative of the product of deracemization of (*rac*)-**1f** with W110G TeSADH.

APPENDIX D

^{13}C and ^{1}H NMR-SPECTRA OF SELECTED REACTIONS

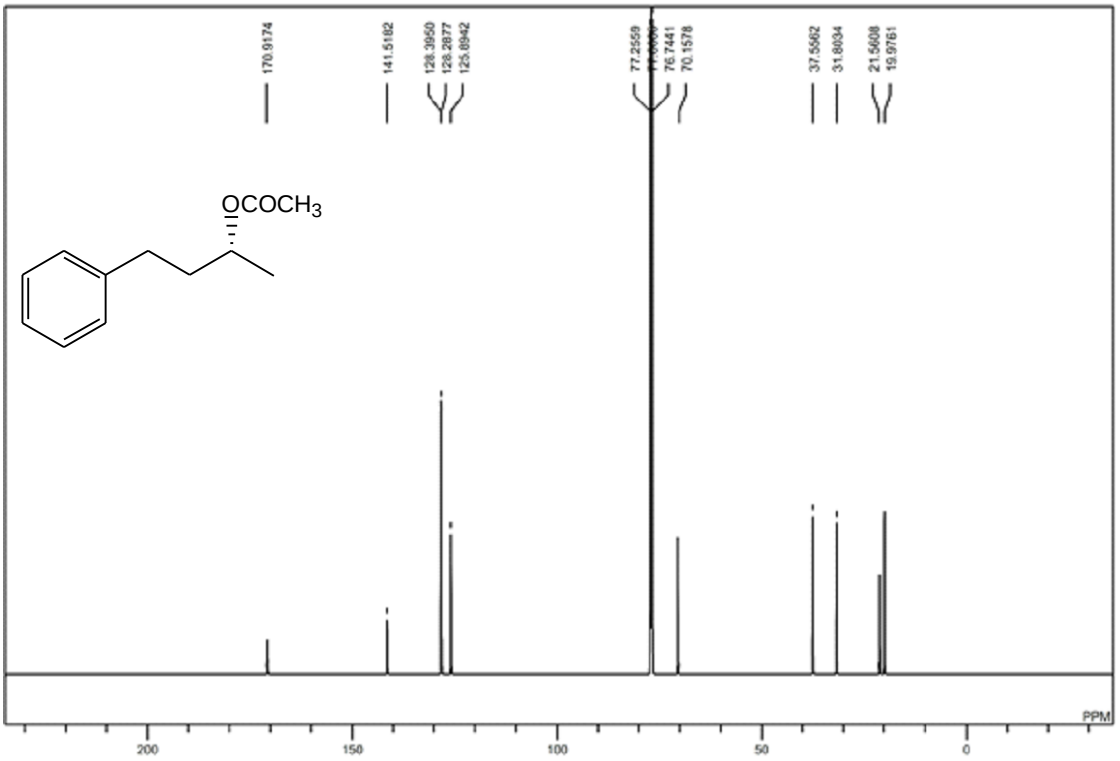

Figure S26. ^{13}C NMR spectrum of (*R*)-**acetate ester** produced by DKR of (*rac*)-**1a**.

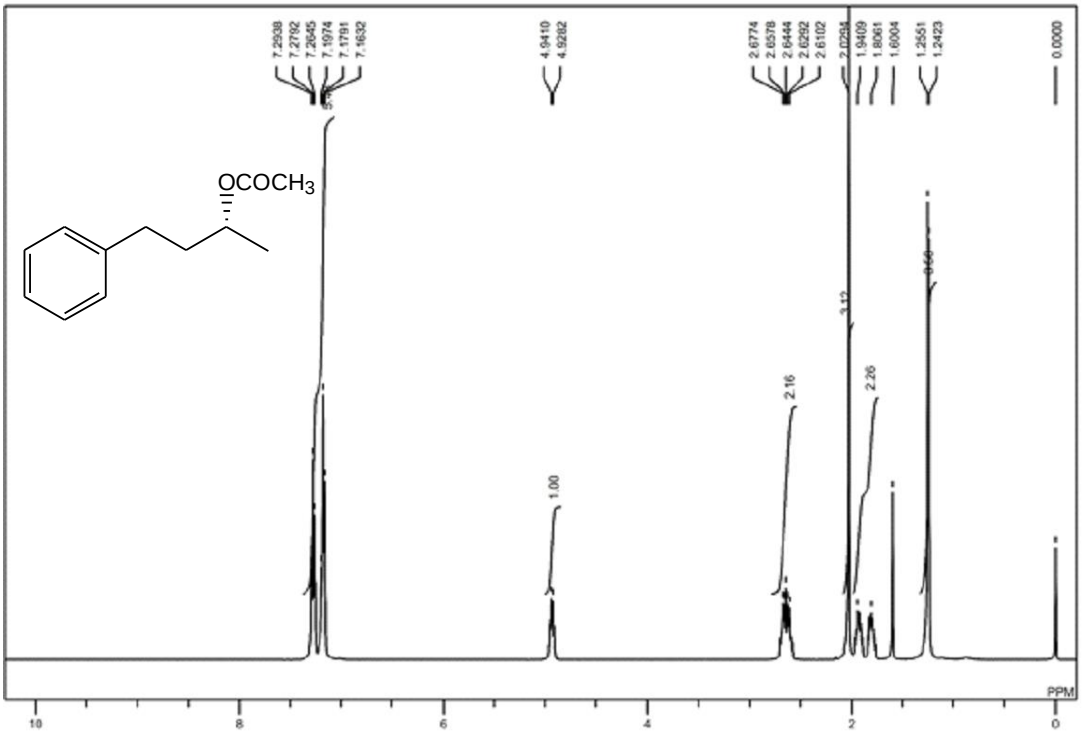

Figure S27. ^{1}H NMR spectrum of (*R*)-**acetate ester** produced by DKR of (*rac*)-**1a**.

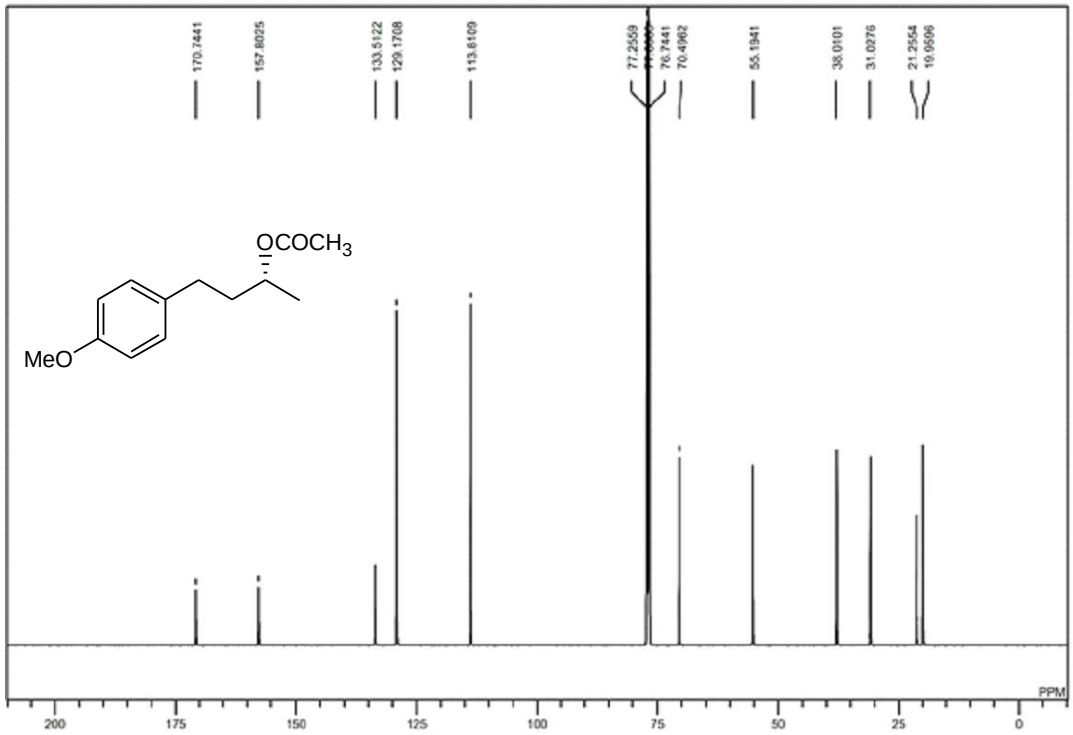

Figure S28. ^{13}C NMR spectrum of (*R*)-**acetate ester** produced by DKR of (*rac*)-**1b**.

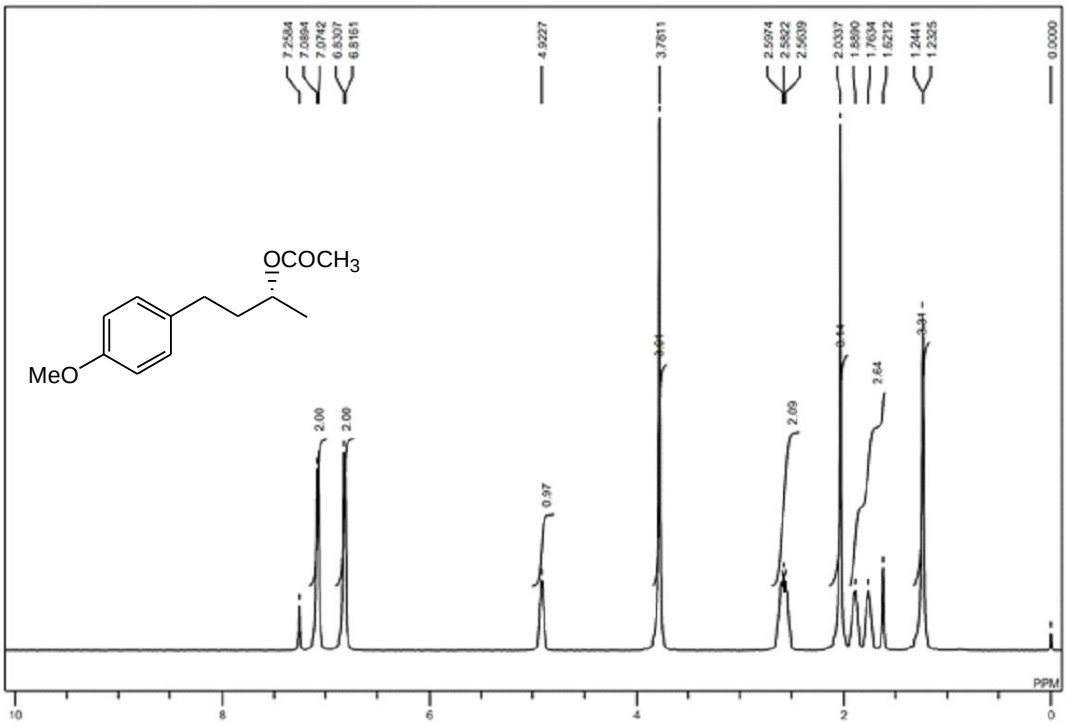

Figure S29. ^{1}H NMR spectrum of (*R*)-**acetate ester** produced by DKR of (*rac*)-**1b**.

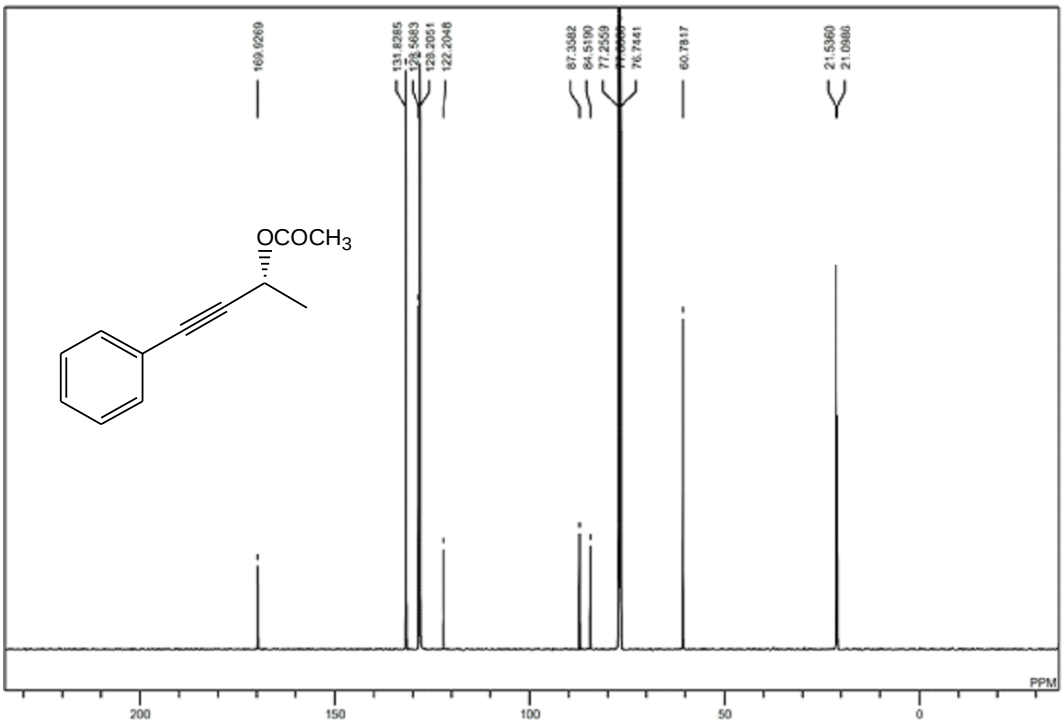

Figure S30. ^{13}C NMR spectrum of (*R*)-**acetate ester** produced by DKR of (*rac*)-**1d**.

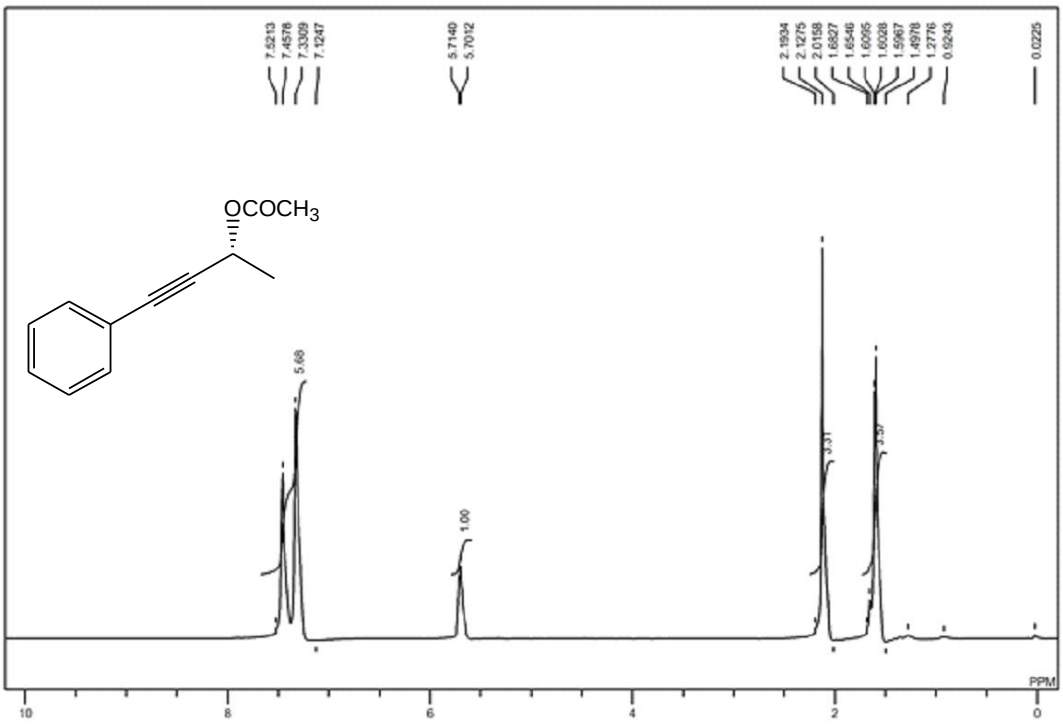

Figure S31. ^{1}H NMR spectrum of (*R*)-**acetate ester** produced by DKR of (*rac*)-**1d**.

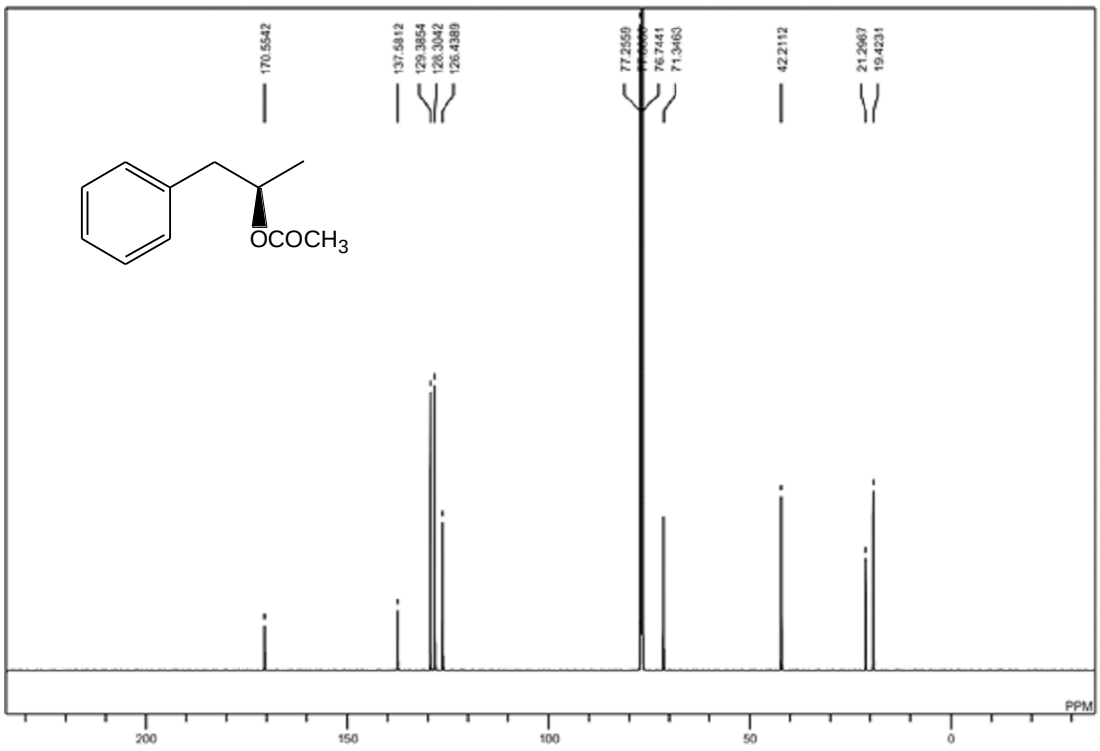

Figure S32. ^{13}C NMR spectrum of (*R*)-**acetate ester** produced by DKR of (*rac*)-**1d**.

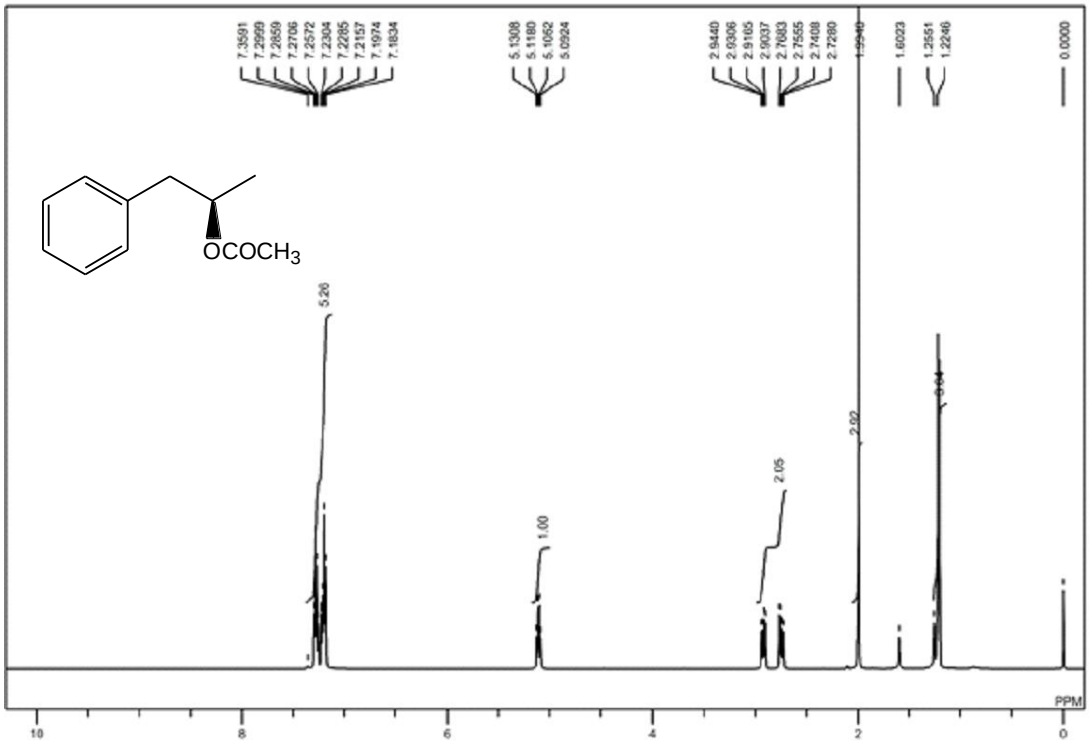

Figure S33. ^{1}H NMR spectrum of (*R*)-**acetate ester** produced by DKR of (*rac*)-**1h**.

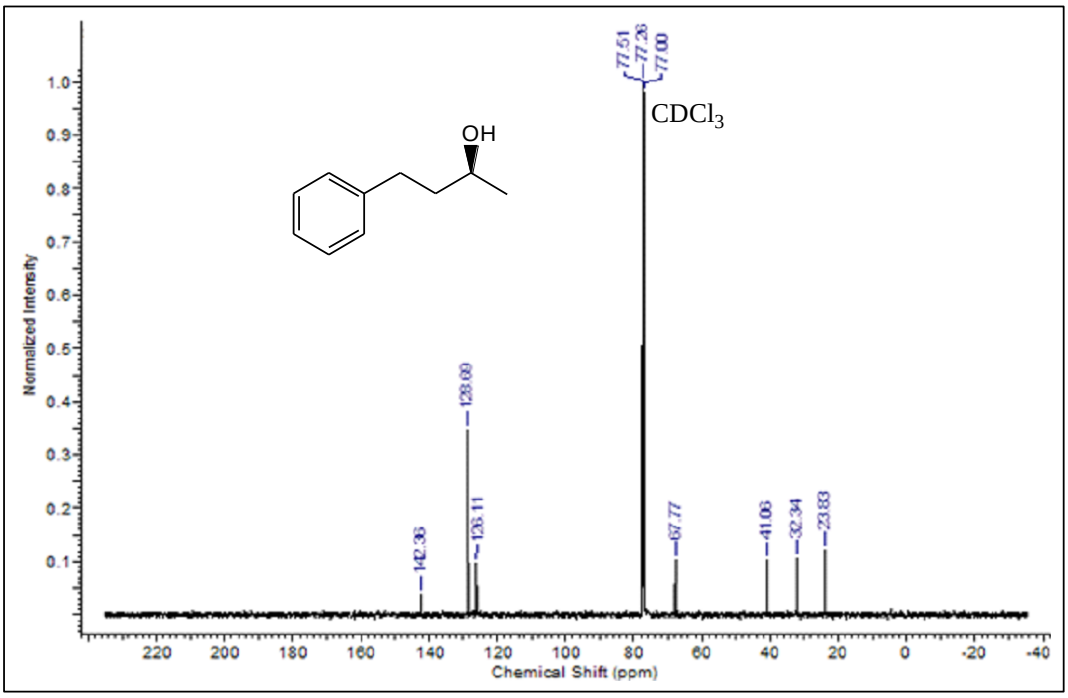

Figure S34. ^{13}C NMR spectrum of the deracemization product of (*rac*)-**1a**.

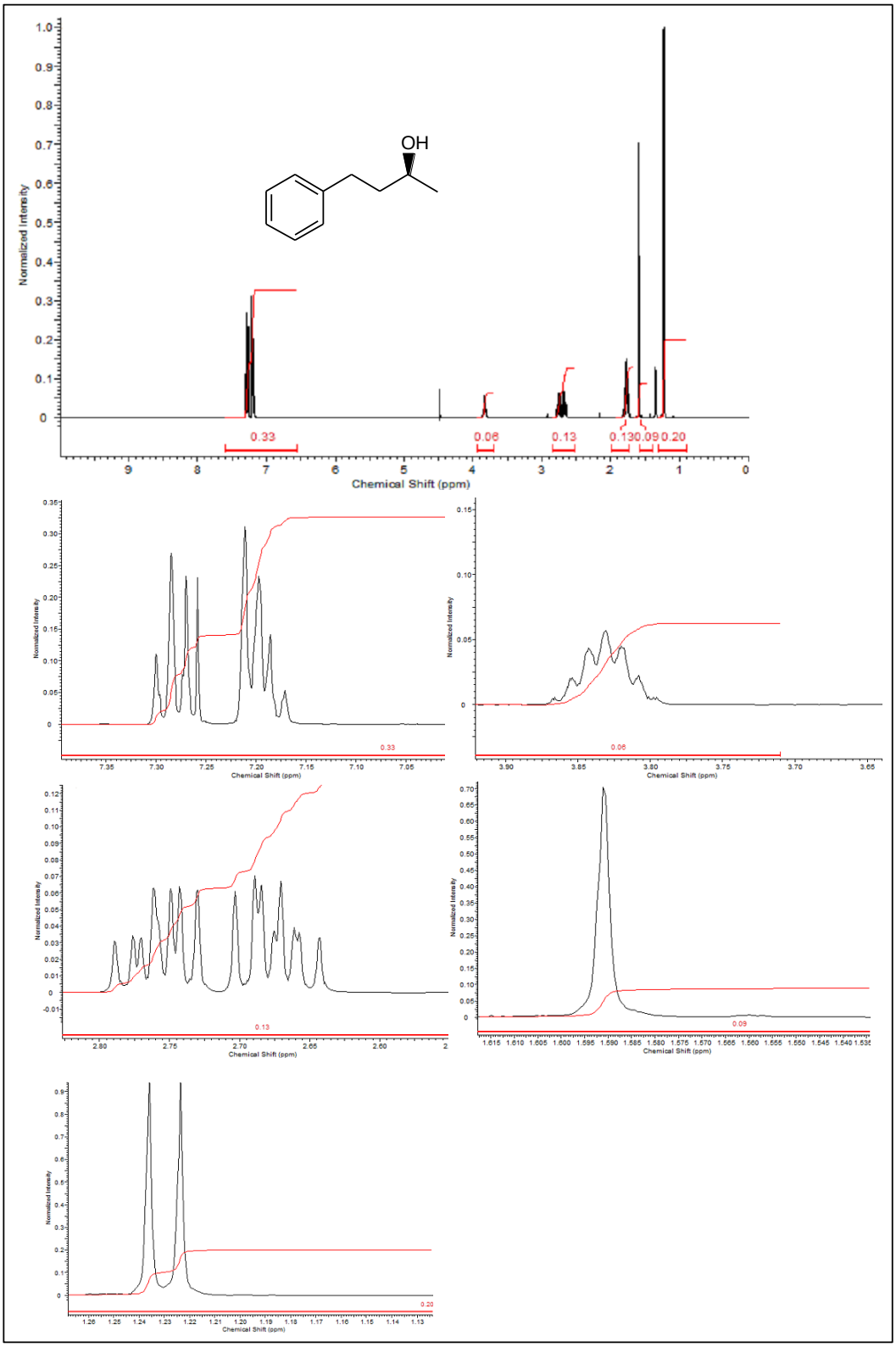

Figure S35. ^{1}H NMR spectrum of the deracemization product of (*rac*)-**1a**.

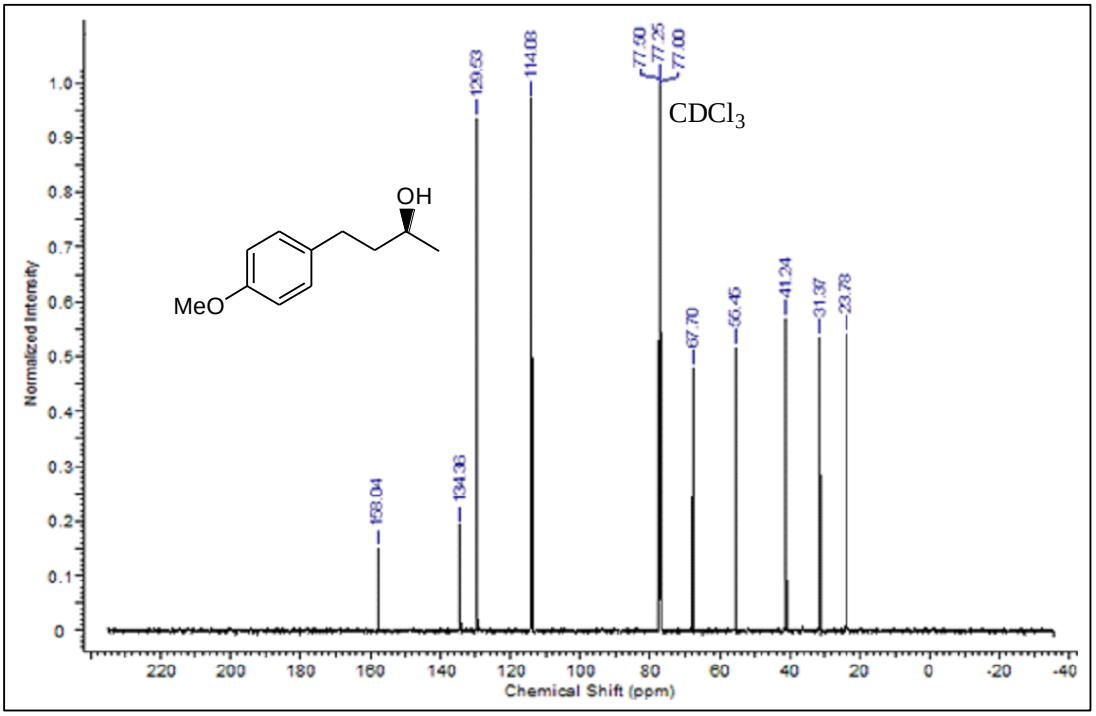

Figure S36. ^{13}C NMR spectrum of the deracemization product of (*rac*)-**1b**.

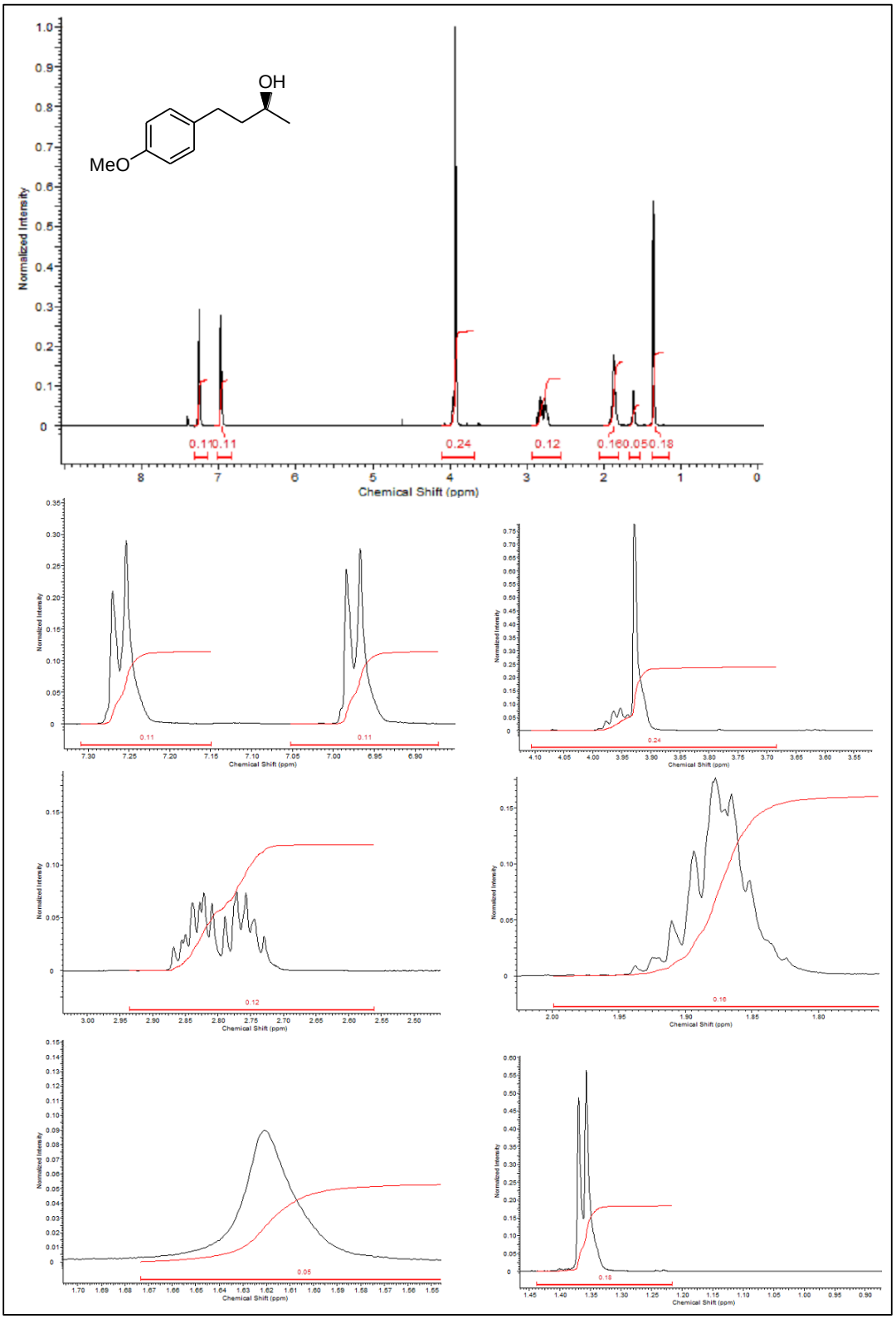

Figure S37. ^{1}H NMR spectrum of the deracemization product of (*rac*)-**1b**.

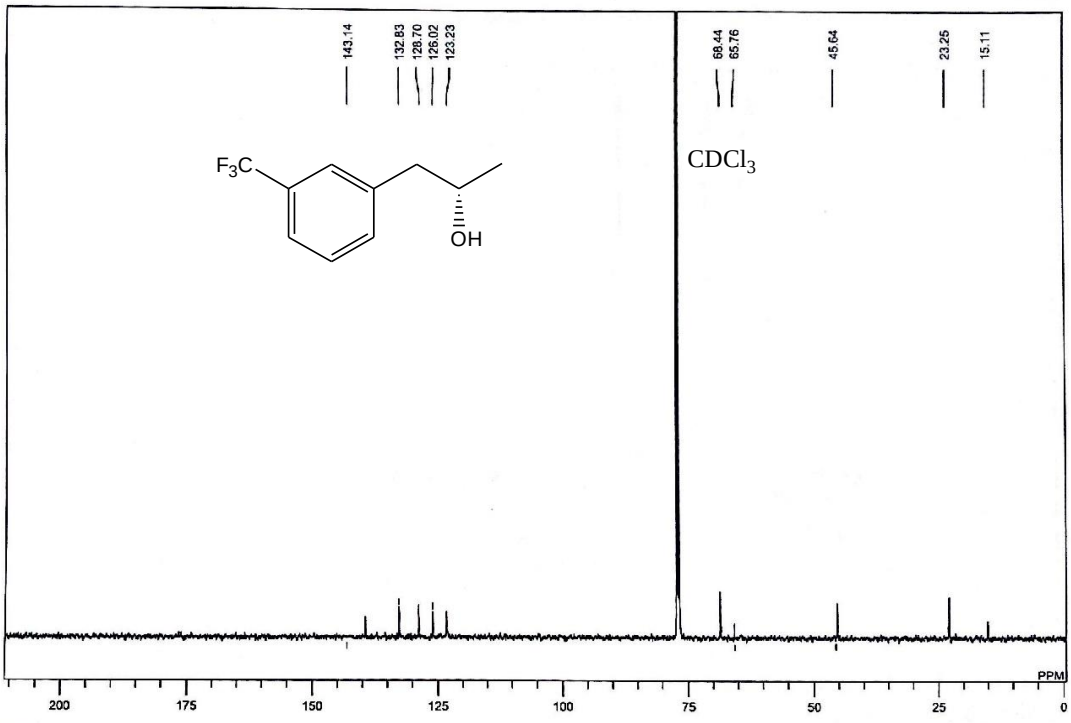

Figure S38. ^{13}C NMR spectrum of the deracemization product of (*rac*)-**1f**.

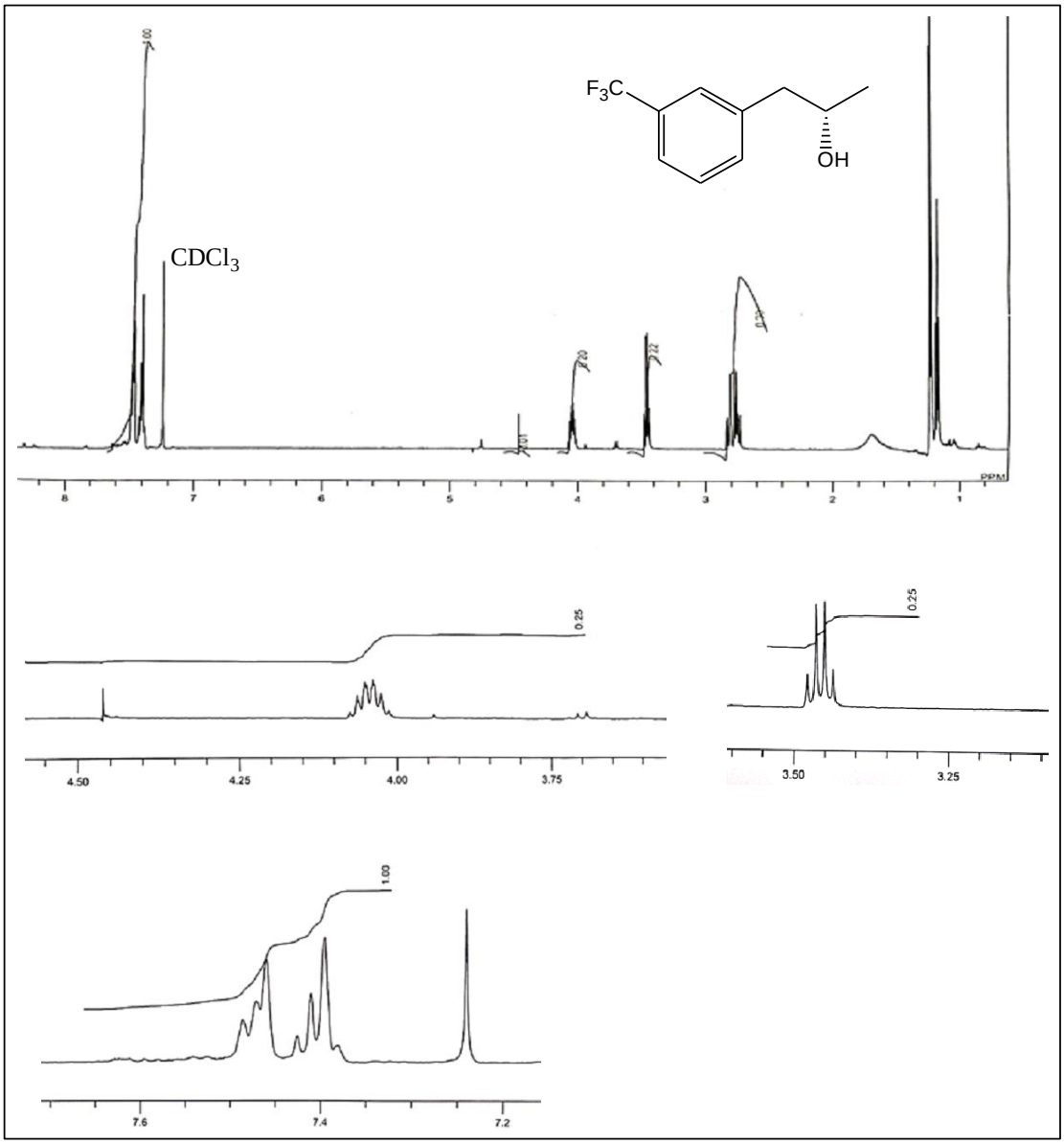

Figure S39. ^{1}H NMR spectrum of the deracemization product of (*rac*)-**1f**.

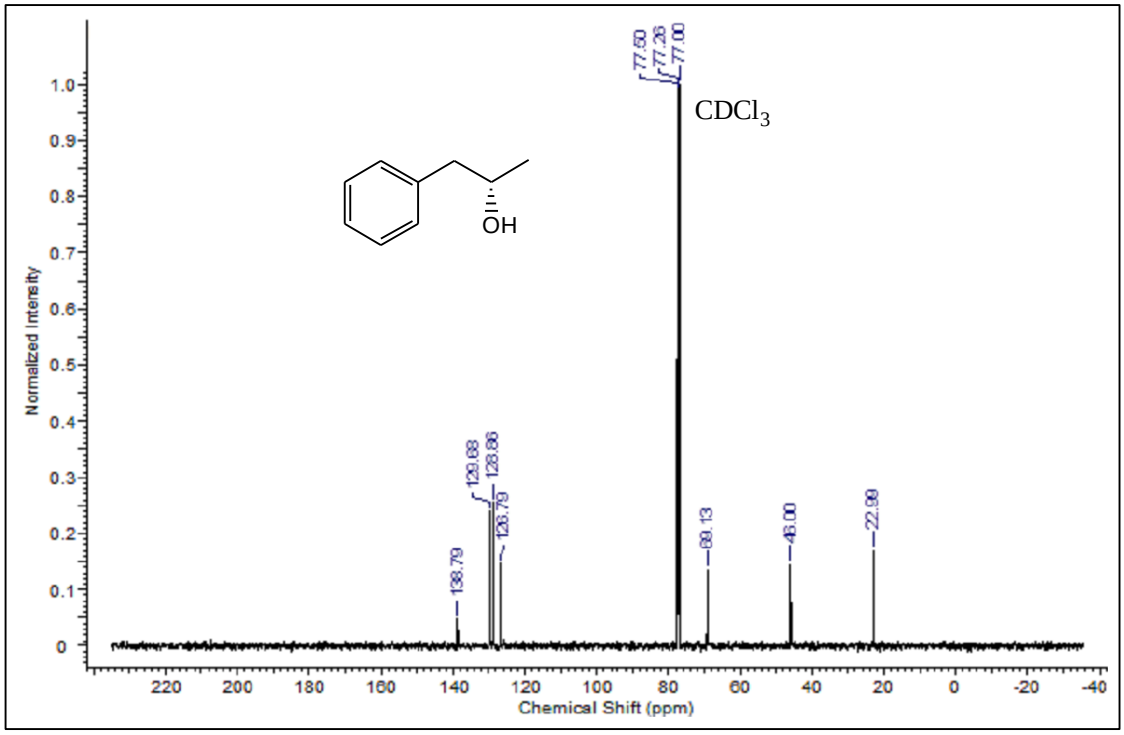

Figure S40: ^{13}C NMR spectrum of the deracemization product of (*rac*)-**1h**.

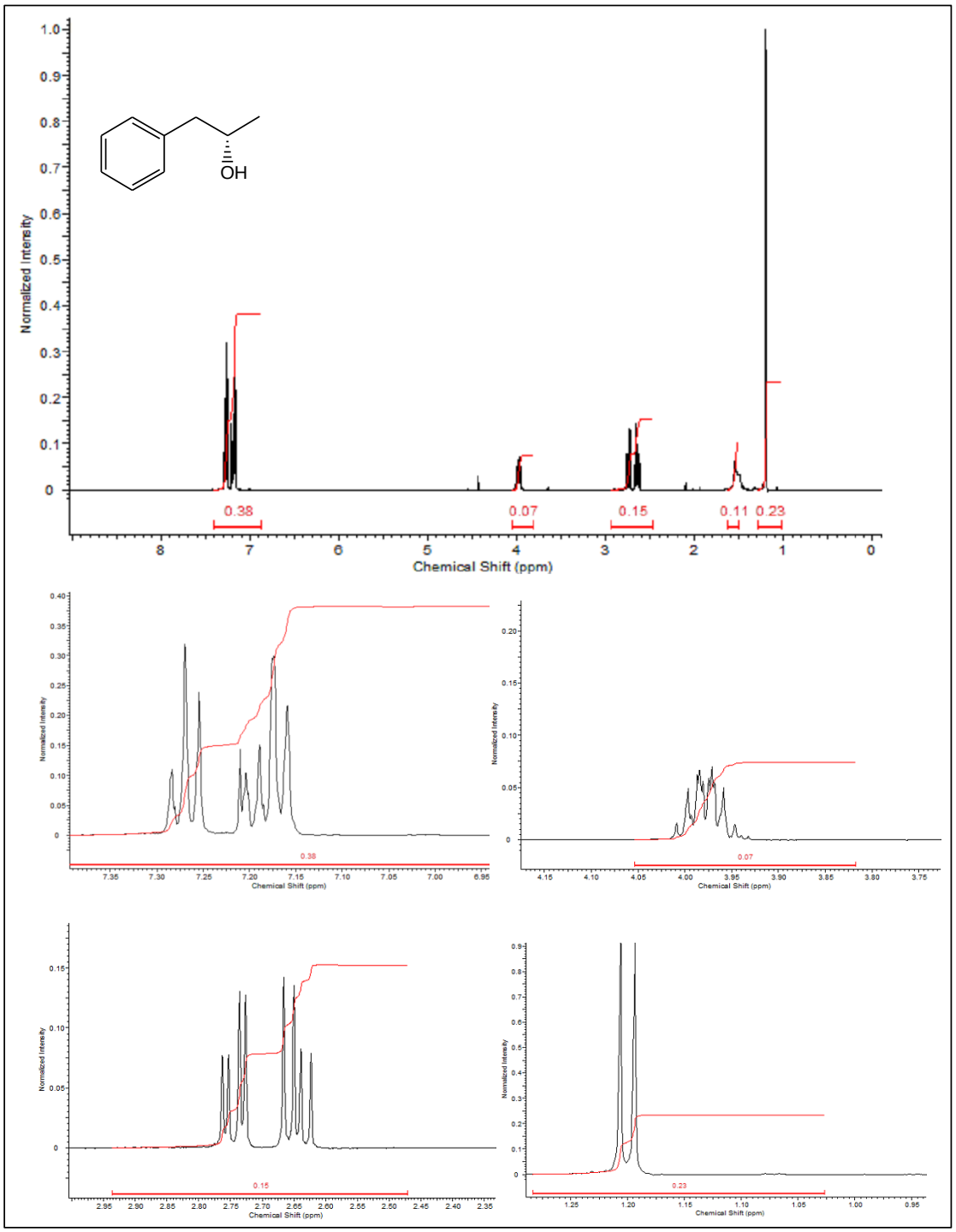

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Figure S41. ^{1}H NMR spectrum of the deracemization product of (*rac*)-**1h**.

APPENDIX E

IR SPECTRA OF SELECTED REACTIONS

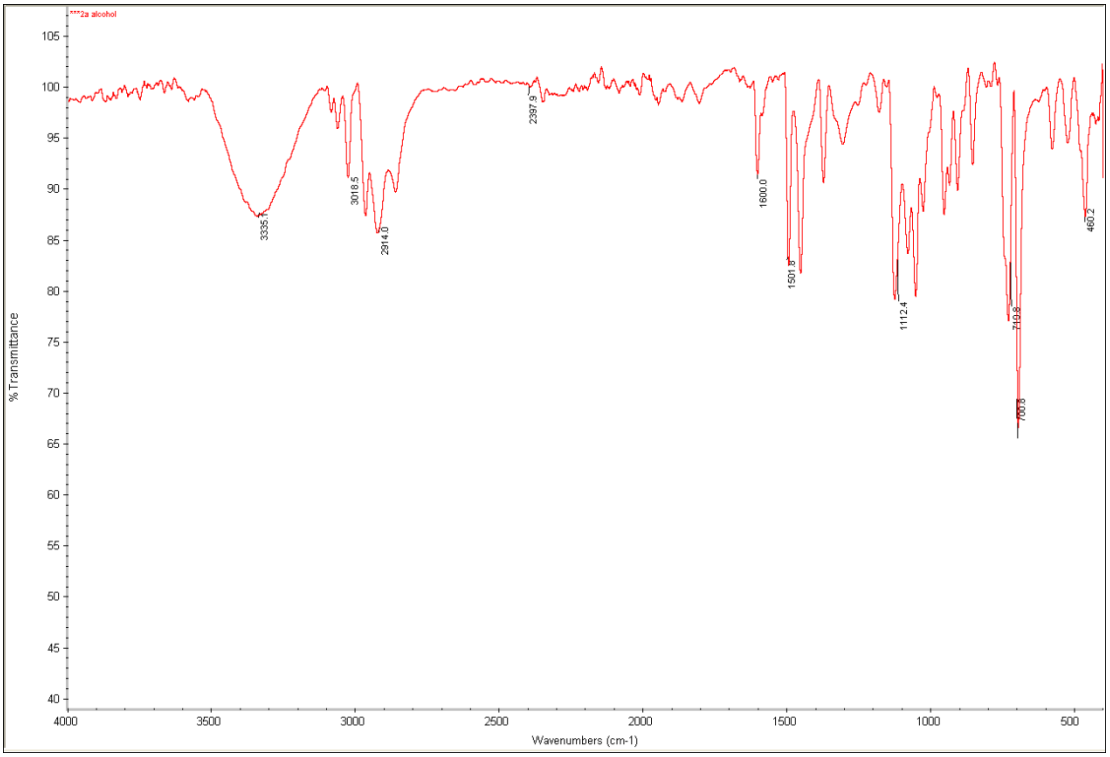

Figure S42. IR spectrum of the alcohol product of reduction of ketone **2a**.

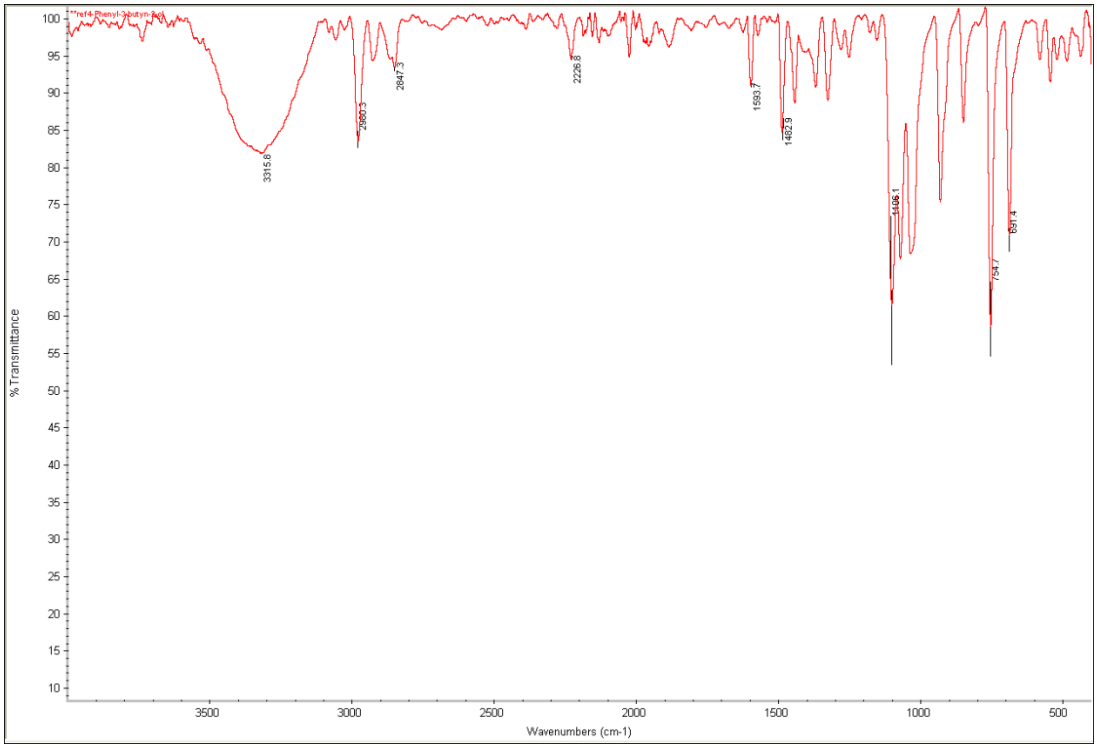

Figure S43. IR spectrum of the product of reduction of ketone **2d**.

CURRICULUM VITAE

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PERSONAL INFORMATION

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Marital Status: Married

Nationality: Ugandan

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EDUCATION

December 2016: **Ph.D.** - Chemistry, King Fahd University of Petroleum & Minerals (KFUPM), Dhahran, Kingdom of Saudi Arabia

March 2011: **M.Sc.** - Chemistry, Makerere University, Kampala, Uganda

January 2008: **B.Sc.** - Chemistry, Makerere University, Kampala, Uganda

RESEARCH EXPERIENCE

August 2012 to date: **Lecturer-B**, KFUPM

Devised a bio-catalyzed route to chiral alcohols using a single enzyme
Developed encapsulation supports for enzymes in harsh media environments
Engineered solvent systems that alter enzyme selectivity

August 2008 to July 2010: **Research Assistant,** International Science Program, bilateral arrangement between Makerere and Lund University, Sweden

Developed polyoxometalate-based catalyst systems for olefin epoxidation
Developed efficient heterogeneous supports for palladium nanoparticles for cross-coupling reactions

Instrumentation skills: Have hands-on experience in

Nuclear magnetic resonance spectroscopy
Bacteria culturing
DNA and Protein Purification
Gas chromatography
Mass spectrometry
IR spectroscopy
Ultraviolet-visible spectroscopy
Microscopy techniques
Familiar with most laboratory bench techniques

TEACHING EXPERIENCE

August 2012 to date: **Lecturer-B**, KFUPM, Dhahran, Saudi Arabia

January 2010 to July 2012: **Assistant Lecturer**, Makerere and Busitema University, Uganda

Duties

Conduct undergraduate lectures and practicals
Supervision of undergraduate research projects
Participate in curriculum development
Quality analyst for domestic products

RESEARCH INTERESTS

Chemistry of water environment
Biocatalysis
Microbiology
Corrosion science
Natural product chemistry
Medicinal chemistry
Organic synthesis
Environment hazard mitigation
Scientific green approaches
Community integrated projects and outreach

PUBLICATIONS

"Deracemization of secondary alcohols using a single alcohol dehydrogenase" Ibrahim Karume, Masateru Takahashi, Samir M. Hamdan and Musa M. Musa, *ChemCatChem.,* (**2016**), *8*, 1459-1463.

"Dual enzymatic dynamic Kinetic resolution by *Thermoanaerobacter ethanolicus* secondary alcohol dehydrogenase and *Candida antarctica* Lipase B" Ibrahim Karume, Musa M. Musa, Odey Bsharat, Masateru Takahashi, Samir M. Hamdan and Bassam El Ali, *RSC Adv.*, (**2016**), *6*, 96616–96622

"Thermoanaerobacter ethanolicus secondary alcohol dehydrogenase mutants with improved racemization activity" Musa M. Musa, Jay M. Patel, Christopher M. Nealon, Chang Sup Kim, Robert S. Phillips and Ibrahim Karume, *Journal of Molecular Catalysis B: Enzymatic*, (**2015**), *115*, 155-159.

Manuscripts under preparation

"Single enzymatic stereoinversion of phenyl-ring-containing *R* configured secondary alcohols" Ibrahim Karume, Masateru Takahashi, Samir M. Hamdan, Bassam El Ali and Musa M. Musa.

"Expanding substrate Specificity of phenyl-ring-containing ketones by using W110A/I86A *Thermoanaerobacter ethanolicus* Secondary Alcohol Dehydrogenase" Odey Bsharat, Ibrahim Karume, Claire Vieille, Sulayman Oladepo, Masateru Takahashi, Samir M. Hamdan, and Musa M. Musa.

CONFERENCE ABSTRACTS

"Compatibility of *Thermoanaerobacter ethanolicus* Secondary Alcohol Dehydrogenase and *Candida antarctica* Lipase B: An Approach for Dual Enzymatic Dynamic Kinetic Resolution" Ibrahim Karume, *CHEMINDIX* (**2016**), *Abstract Ref No. CI2016A-1050*, Manama, Bahrain.

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