

**TUNING THE STEREOPREFERENCE OF
THERMOANAEROBACTER PSEUDOETHANOLICUS
SECONDARY ALCOHOL DEHYDROGENASE FOR
ASYMMETRIC REDUCTION OF PHENYL-RING-
CONTAINING KETONES**

BY

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I dedicate this work to my parents, Alh. Ahmad Sardauna and Haj. Jamila Sardauna, whose unwavering support, encouragement, and love have been my pillars of strength throughout this academic journey.

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LIST OF ABBREVIATIONS

ADH	:	Alcohol dehydrogenase
<i>ee</i>	:	Enantiomeric excess
GcAPRD	:	<i>Geotrichum candidum</i> NBRC 4597
GC	:	Gas Chromatography
HLADH	:	Horse liver alcohol dehydrogenase
LkADH	:	<i>Lactobacillus kefir</i> alcohol dehydrogenase
NMR	:	Nuclear Magnetic Resonance
NADH	:	Nicotinamide-adenine dinucleotide
NADPH	:	Nicotinamide-adenine dinucleotide phosphate
SSCR	:	<i>Sporobolomyces salmonicolor</i> AKU4429 (SSCR)
<i>TeSADH</i>	:	<i>Thermoanaerobacter pseudethanolicus</i> secondary ADH
TbADH	:	<i>Thermoanaerobium brockii</i> secondary alcohol dehydrogenase
Tris-HCl	:	Tris(hydroxymethyl)aminomethane hydrochloride
WT	:	Wild-Type
YADH	:	Yeast alcohol dehydrogenase

ABSTRACT

Full Name : Auwal Eshi Sardauna
Thesis Title : Tuning the Stereopreference of *Thermoanaerobacter pseudoethanolicus* Secondary Alcohol Dehydrogenase for Asymmetric Reduction of Phenyl-Ring-Containing Ketones
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The biocatalytic asymmetric reduction of prochiral ketones is an appealing method for generating optically active secondary alcohols. Alcohol dehydrogenases (ADHs) are enzymes that facilitate the reversible conversion of ketones into their corresponding optically active secondary alcohols. For ADH-catalyzed asymmetric reduction of prochiral ketones, most ADHs adhere to Prelog's rule in determining the stereopreference. In this study, we present mutations of *Thermoanaerobacter pseudoethanolicus* secondary ADH (*TeSADH*) that are capable of reducing phenyl-ring-containing ketones to their corresponding alcohols in *anti*-Prelog mode. We showed that P84 plays a crucial role in adjusting the stereopreference of *TeSADH* in the asymmetric reduction of phenyl-ring-containing ketones. More specially, deletion of P84 accompanied by A85G *TeSADH* is proven to exhibit *anti*-Prelog stereopreference, which is opposite to that of *TeSADH*. This mutant was shown to accommodate a wide range of substrates. The ability to create mutants of the same enzyme that exhibit opposite stereopreferences is highly intriguing as it allows for fascinating transformations such as bienzymatic racemization, which can be utilized in dynamic kinetic resolution, and stereoinversion to be conducted using two mutants of the same enzyme.

ملخص الرسالة

الاسم الكامل: أول إشي ساردونا

عنوان الرسالة: ضبط الانتقاء الفراغي لنازعات الهيدروجين الكحولية لعملية للاختزال الغير المتماثل لمجموعة من مركبات الكيتون الحاوية على حلقة فينيل

التخصص: الكيمياء

تاريخ الدرجة العلمية: ديسمبر 2023

يُعد التحفيز الحيوي لامتناظر في تفاعلات إختزال الكيتونات إلى كحولات فعالة ضوئياً من الطرق المثيرة للإهتمام. نازعات الهيدروجين الكحولية (ADHs) هي عبارة عن أنزيمات تسهل التحويل العكسي للكيتونات إلى كحولات ثانوية فعالة ضوئياً. نازعات الهيدروجين الكحولية (ADHs) هي عبارة عن أنزيمات تسهل التحويل العكسي للكيتونات إلى كحولات ثانوية فعالة ضوئياً. معظم هذه النازعات (ADHs) تلتزم بقاعدة بريلوغ في تحديد الانتقاء الفراغي. في هذه الدراسة، استخدمنا طفرات في بكتيريا *Thermoanaerobacter pseudothanolicus* الثانوية ADH (*TeSADH*) القادرة على تقليل الكيتونات المحتوية على حلقة الفينيل إلى الكحولات المقابلة لها في وضع مضاد - بريلوغ. تبين أن P84 يلعب دوراً حاسماً في ضبط الانتقاء الفراغي لـ *TeSADH* في تفاعل الاختزال غير المتماثل لمجموعة من مركبات الكيتون الحاوية على حلقة فينيل. وبشكل أكثر تحديداً، أثبت أن حذف P84 بواسطة A85G *TeSADH* يُظهر التفضيل الفراغي مضاد - بريلوغ. وبشكل أكثر تحديداً، أثبت أن حذف P84 بواسطة A85G *TeSADH* يُظهر التفضيل الفراغي مضاد - بريلوغ، وهو ما يتعارض مع نتائج لـ *TeSADH*. وتبين أيضاً أن هذا التحور قادر على استيعاب مجموعة واسعة من الركائز.

إمكانية أحداث تحورات في الأنزيم للحصول على افضلية فراغية معاكسة للأنزيم نفسه، هي أمر يثير الاهتمام حيث يتيح إمكانية اجراء تحولات رائعة باستخدام أنزيمين في تفاعلات تحويل مزيج رازمي إلى أحد المتخاليات الضوئية، والذي يمكن استخدامه في تحديد الحركية الديناميكية، وكذلك الانتقاء الفراغي باستخدام متحورين لنفس الأنزيم.

CHAPTER 1

INTRODUCTION

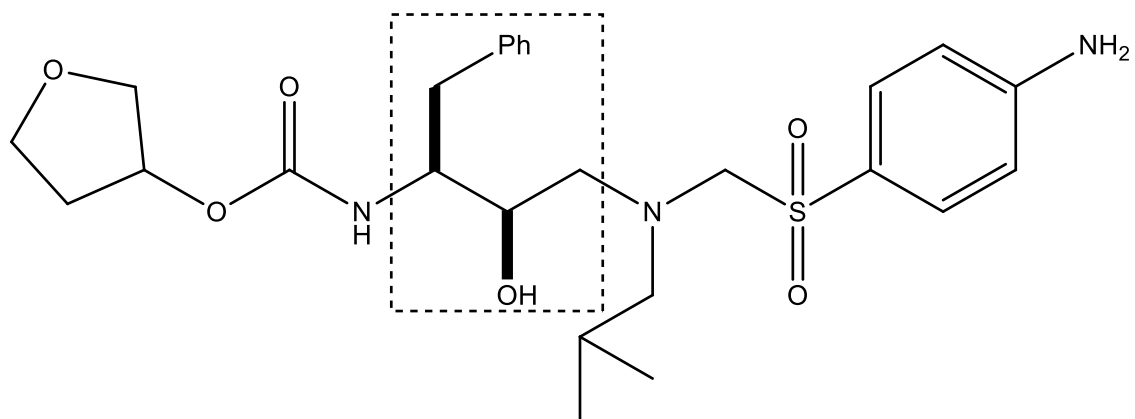
1.1 Background

Enantiomerically pure alcohols serve as synthons in drug production and other important chemicals; this makes them very vital and highly sourced in the pharmaceutical, petroleum, agrochemical, and food industries. optically pure alcohols are usually produced by kinetic resolution in the industry using their racemates where two enantiomers react at different rates which results in alcohols enriched with one enantiomer.^{1,2} However, one major setback of this approach is that it has an utmost 50% yield of product with great enantiopurity.^{3,4} Deracemization of racemic alcohols is another viable approach to produce optically pure alcohols,^{5,6} however, this approach requires two systems of compatible reactions to work in a one pot system. This brings about the challenge faced in searching or looking for a new technique for deracemization. Optically active alcohols can be obtained from the asymmetric reduction of their corresponding ketones, which is usually by the use of chemical catalysts or using biocatalysts, with the latter being more advantageous because of certain factors ranging from being highly chemo-, regio- and stereoselective to the fact that they are sustainable and economical.^{7,8} Biocatalysis aligns with the majority of the principles of green chemistry, offering sustainability through cost-effectiveness and reusability.⁹ This creates an opportunity for the widespread adoption of

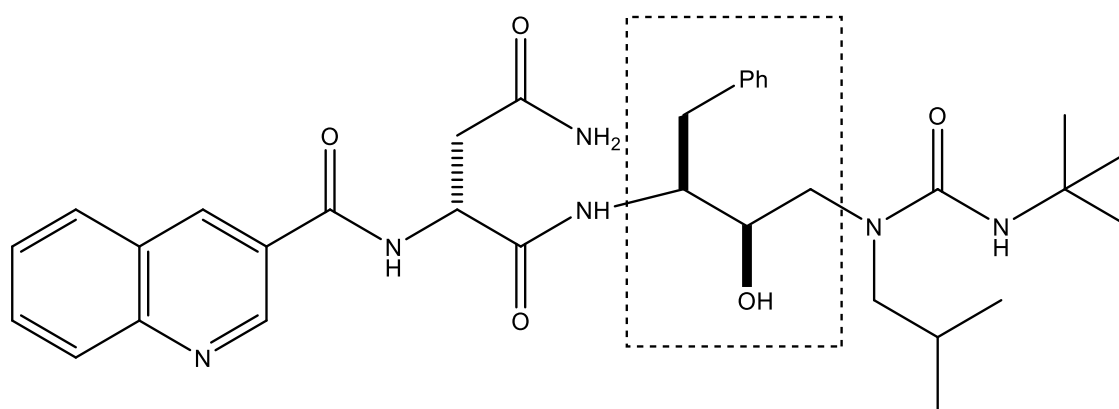
biocatalytic methods, which have proven to be both economical and environmentally beneficial.^{10,11}

Asymmetric reduction of phenyl-ring-containing ketones has proven to be very vital to several sectors such as pharmaceutical industries because it paves way for production of optically active alcohols which serve as synthons in the production of active pharmaceutical ingredients (API) such as optically active 4-phenyl-2-butanol an important synthon in the synthesis of inhibitors used in the treatment of AIDS such as SC-52151 and VX-478.¹² In some cases, optically active alcohols serve as API, for instance enantiopure 4-(4'-hydroxyphenyl)-2-butanol, which is commercially known as rhododendrol and plays an important role in pharmaceutical industries as it serves as antihepatotoxicity and its glucoside is used as an inhibitor for melanin.¹³

Among the commonly used methods to synthesize alcohols that are optically active is the usage of alcohol dehydrogenase (ADHs) as a catalyst in the asymmetric reduction of prochiral ketones. ADHs make use of nicotinamide-adenine dinucleotide (NADH) or its phosphate (NADPH) as cofactor that serves as a hydride supplier. A number of ADHs such as *Thermoanaerobium brockii* secondary ADH (TbSADH),¹⁴ *Sporobolomyces salmonicolor* AKU4429 carbonyl reductase (SsCR),¹⁵ *Lactobacillus kefir* DSM 20587 ADH (LkADH),¹⁶ *Thermoanaerobacter ethanolicus* secondary ADH (TeSADH),^{17,18} horse liver ADH (HLADH) have been reported to have achieved this asymmetric reduction of prochiral ketones.^{19,20}



VX-478 (Vertex - Glaxo Wellcome)



SC-52151 (Searle - Monsanto)

Figure 1. Important pharmaceutical compounds containing enantiopure 4-phenyl-2-butanol entities.

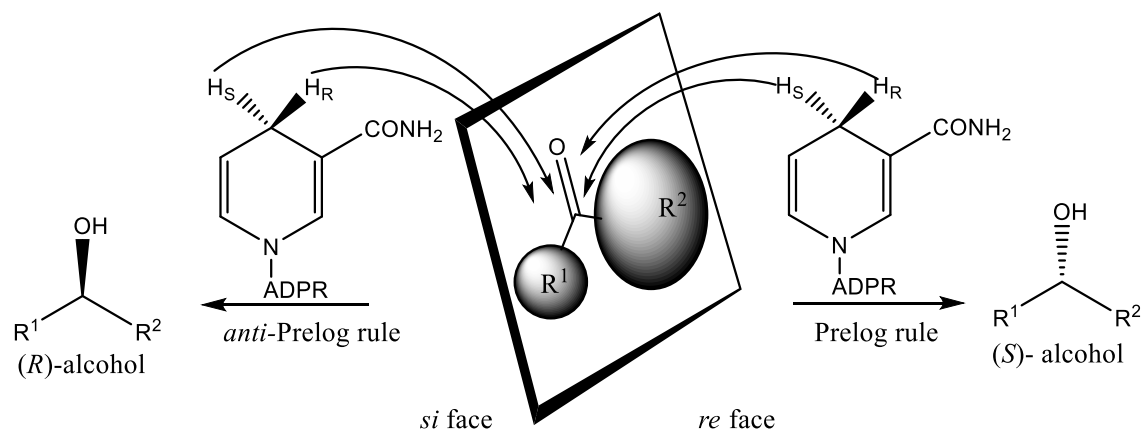
The successful application of biocatalysis relies on fine-tuning the substrate specificity and stereopreferences of enzymes as well as their chemo-, regio-, and stereoselectivities.²¹ Most of the known ADHs exhibit Prelog's stereopreference, for which the hydride of the cofactor is delivered from the *re* face of a prochiral ketone producing the corresponding (S)-configured alcohol when the large alkyl group of the ketones exhibits higher Cahn-Ingold-Prelog priority, which calls for more ADHs with *anti*-Prelog's stereopreference.²²

Herein, we introduce *TeSADH* mutants, capable of reducing phenyl-ring-containing ketones in *anti*-Prelog mode, which is the opposite stereopreference of *TeSADH* and the previously reported W110X *TeSADH* mutants (X = A, G, V).²³ The findings presented here will facilitate the development of additional *anti*-Prelog *TeSADH* mutants, expanding the possibilities for their application.

1.2 Asymmetric Redox Reactions catalyzed by ADHs

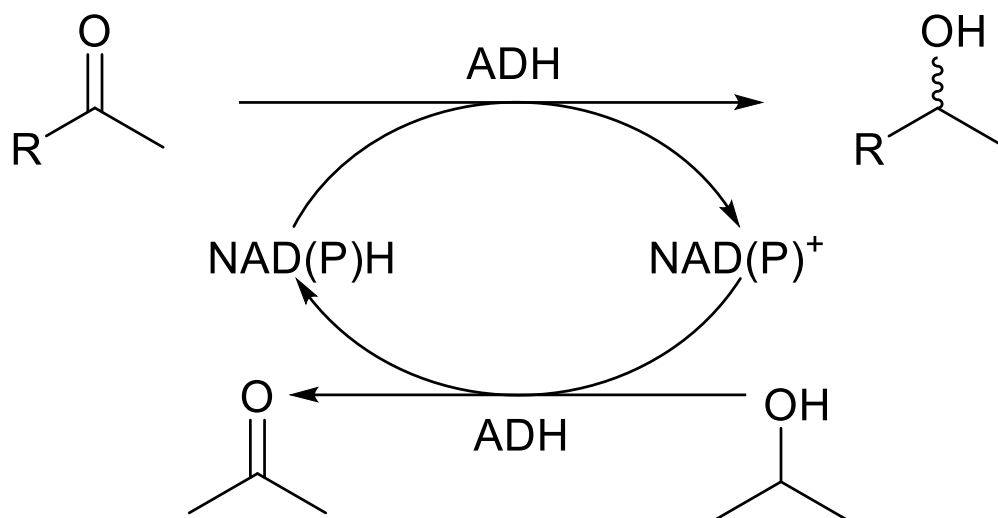
Aldehydes and ketones can be reduced reversibly to their respective alcohols using alcohol dehydrogenases (ADHs, EC 1.1.1.X, X = 1 or 2) as catalysts. This biological process requires a cofactor, typically NADH or NADPH, that acts as the source of hydride to enable the reduction, while its oxidized entities facilitate the oxidation of primary or secondary alcohols using the oxidized forms of the cofactor.^{19,24–27}

In the reduction of prochiral ketones catalyzed by ADH, hydride can be delivered from either the *si* or *re* face of the ketones. This delivery leads to the production of alcohols with *R* or *S* configuration, respectively, assuming that the large group of the ketone has a higher Cahn-Ingold-Prelog priority than that of the smaller group.²⁸ Most of the known ADHs follow Prelog's rule, in which the hydride of the coenzyme is delivered from the *re* face of the ketone, resulting in the production of *S*-configured alcohols. Notable examples of these ADHs that obey Prelog's rule include horse liver ADH, yeast ADH, *TbSADH*, and *TeSADH*. However, there are exceptions like *Lactobacillus kefir* ADH, which has proven to be an *anti*-Prelog ADH, representing a unique kind of ADHs. The process of addition of hydride in reduction of ketones catalyzed by ADH is summarized in Scheme 1.



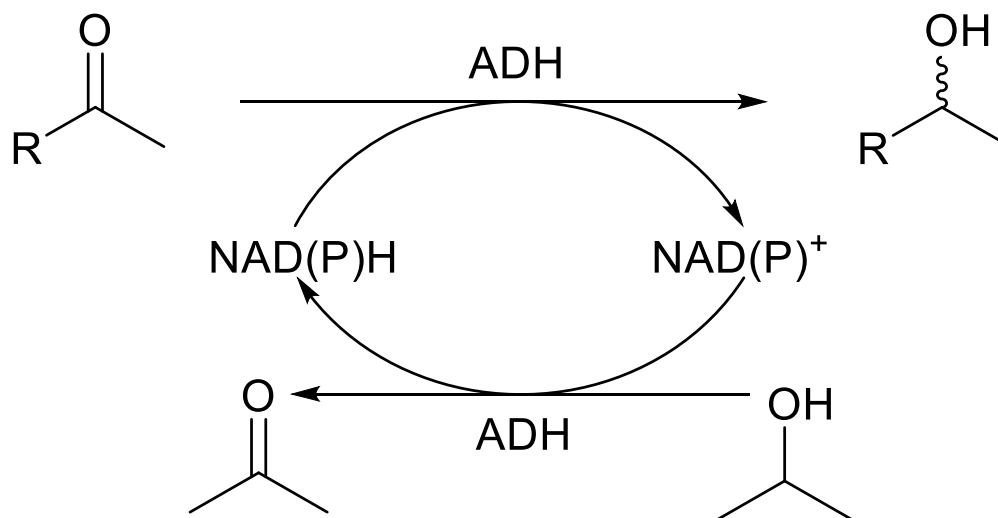
Scheme 1. The process of hydride transfer in reduction of prochiral ketones catalyzed by ADHs. R² is more sterically hindered and exhibits a higher Cahn-Ingold-Prelog priority than that of R¹.

The cofactor NAD(P)H is usually recycled through enzyme coupled or substrate coupled approaches. The latter being the simplest of the two requires the use of a single ADH which at the same time transforms the substrate and a secondary substrate (called co-substrate) usually a small aliphatic moiety such as 2-propanol in its active site (Scheme 2).^{29–36} However, the co-substrate must be in excess because the reaction procedure is hindered by thermodynamic equilibrium, therefore addition of more co-substrate will push the equilibrium more to the product side at the same time increasing the solubility of the primary substrate thereby increasing the rate of conversion. This approach is faced with one major setback due to the fact that the ADH is usually rendered inactive with increasing concentration of the co-substrate, so finding an ADH that is stable in the presence of high concentrations of the co-substrate is crucial for the successful use of the substrate coupled approach.^{37,38}



Scheme 2. NAD(P)H cofactor recycling system via the substrate coupled approach.

This set back by the substrate coupled approach can be avoided by using an irreversible enzyme coupled system which requires using two enzymatic reactions as shown in Scheme 3 thereby making the process more complex than the substrate coupled approach and will require different recycling method for oxidation and reduction reactions.³⁷

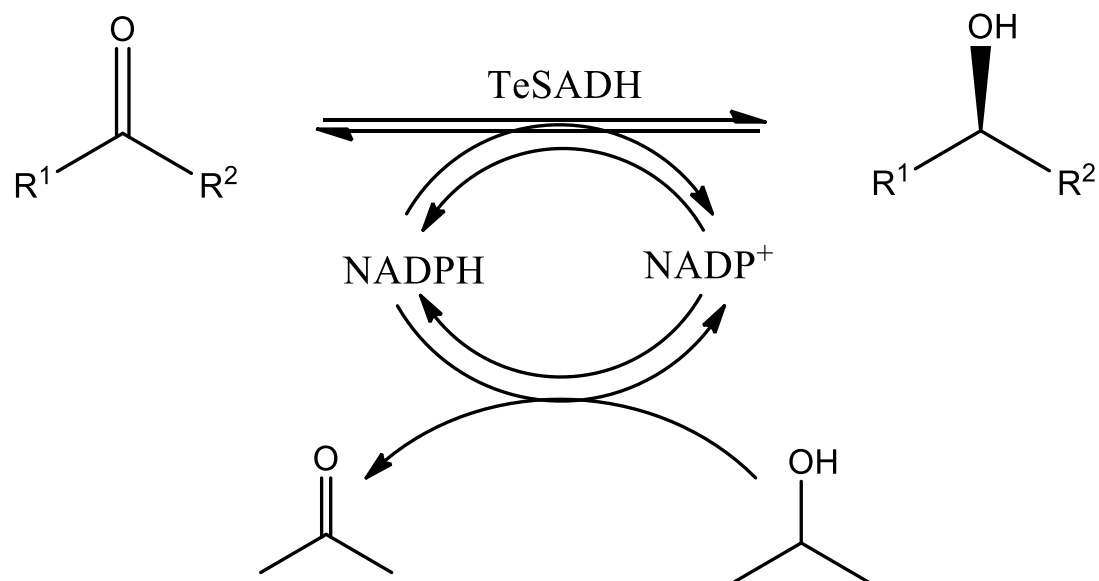


Scheme 3. NAD(P)H cofactor recycling system via the enzyme coupled approach.

One intriguing ADH is *TeSADH*, which has proven successful in performing reversible asymmetric reduction of ketones. This enzyme is robust due to its ability to withstand harsh conditions such as high temperatures (up to 80 °C) and high concentrations of organic solvents.³⁹

1.3 Secondary ADH from *Thermoanaerobacter psuedoethanolicus*

Secondary ADH from *Thermoanaerobacter pseudethanolicus* (*TeSADH*, EC 1.1.1.2), an NADPH-dependent ADH, proves to be a highly effective biocatalyst in various synthetic applications.⁴⁰ Its efficiency can be attributed to several factors. Firstly, *TeSADH* demonstrates high activity and stereoselectivity when accepting alcohols and their corresponding ketones as substrates. This characteristic enhances its utility in synthetic processes. Secondly, *TeSADH* exhibits remarkable resistance to denaturation even in the presence of high concentrations of organic solvents.⁴¹ This advantageous feature allows for the utilization of co-substrates such as 2-propanol and acetone in high concentrations in the reduction and oxidation routes, respectively. Consequently, the coenzyme can be efficiently regenerated using substrate coupled approach, enabling the process to function catalytically as depicted in Scheme 4. Furthermore, *TeSADH* displays notable thermal stability, adding to its overall versatility and reliability.



Scheme 4. Coenzyme regeneration system in *TeSADH*-catalyzed reactions using co-substrate-coupled approach.

Keinan and coworkers proposed that *TbSADH* active site, an enzyme that is identical to *TeSADH*,⁴⁰ comprises two distinct pockets: a large one and a small one.¹⁴ The smaller pocket exhibits greater affinity for alkyl chains, albeit it cannot accommodate groups that are more sterically hindered than isopropyl.¹⁴ In 1998, *TbSADH* crystalline structure was elucidated, providing valuable insights into the architecture of the functional site, enabling the construction of beneficial *TeSADH* mutants.⁴² Notably, W110 was identified as an interesting site for mutations, being in the larger pocket, as depicted in Figure 2.⁴³ Phillips and coworkers demonstrated that *TeSADH* specificity of substrate was modified by W110A mutation, allowing the accommodation of aryl ketones and corresponding alcohols, in accordance to Prelog's rule.⁴⁴

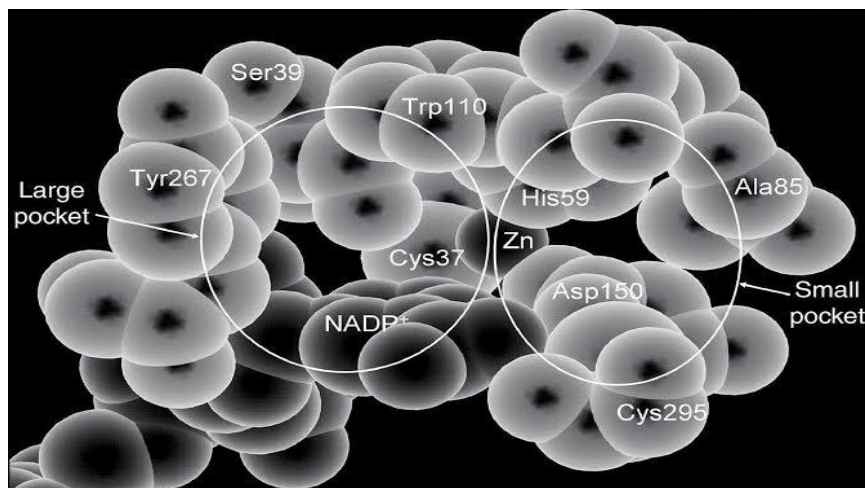


Figure 2. The structure of the active site of *TeSADH* and *TbADH* (Protein Eng. Des. 2007, 20, 47-55).

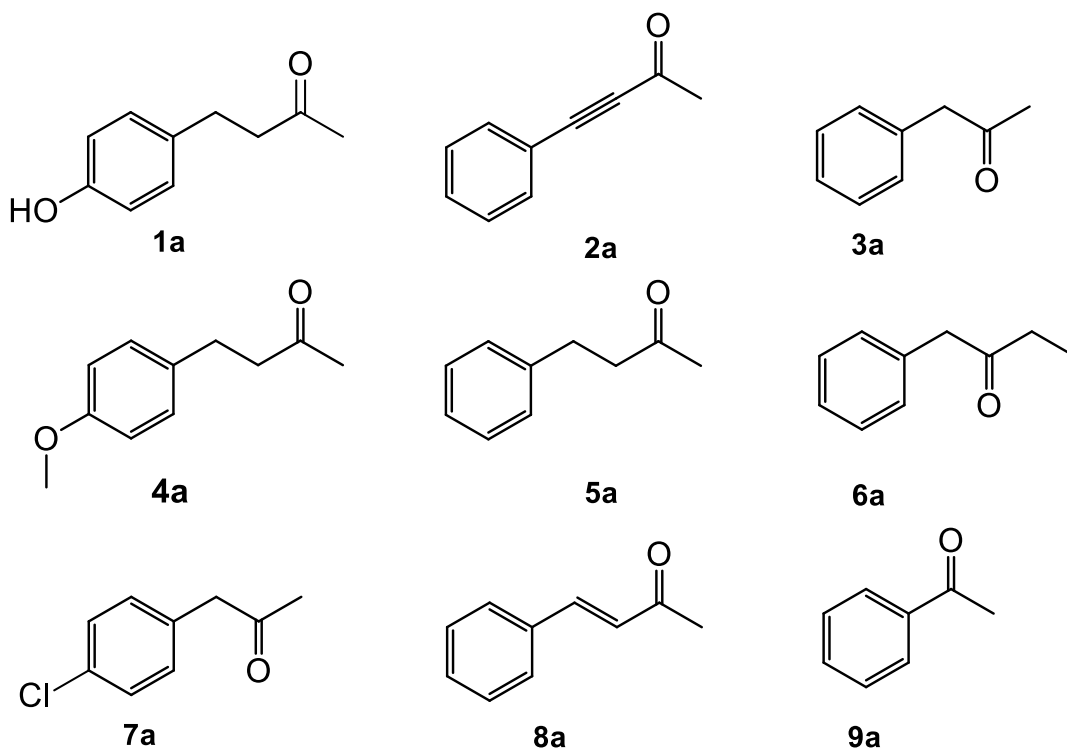
1.4 Significance of Study

The key objective of this work is to synthesize optically active alcohols that serve as synthons in the production of pharmaceutical compounds and other fine chemicals. Δ P84, A85G and Δ P84/A85G mutants were designed and tested in the asymmetric reduction of ketones containing aryl rings previously reduced using W110 mutants following the Prelog orientation with the aim of switching the stereopreference of *TeSADH* to *anti*-Prelog.

More specifically, the objectives of this research are listed below.

1. Design Δ P84, A85G and Δ P84/A85G mutants of *TeSADH* believed to be capable of accepting bulky-small ketone substrates.

2. Test the activity of these mutants in the asymmetric reduction of aryl-ring-containing ketones listed in Scheme 5.
3. Characterize the chemical identity of the reaction products using ^1H and ^{13}C NMR and MS.
4. Distinguish the stereochemical identity of the produced alcohols by chiral GC.
5. Compare the results of the asymmetric reduction of aryl-ring containing ketone substrates using $\Delta\text{P84/A85G}$ mutant which was capable of switching the stereopreference of *TeSADH* with those reported using *W110V* mutant.



Scheme 5. List of substrates used for this work.

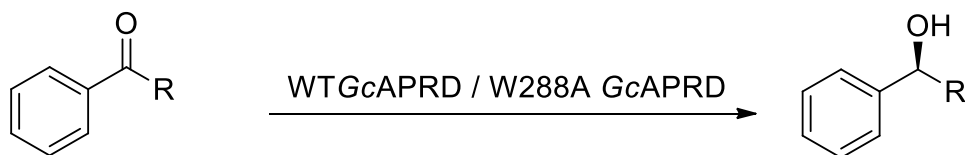
CHAPTER 2

LITERATURE REVIEW

2.1 Biocatalytic Asymmetric Reduction Using ADHs

The asymmetric reduction of phenyl-ring-containing ketones using ADHs ranging from whole cells to purified enzymes have been reported over the years with majority reported to follow Prelog's rule.²⁸ Prochiral ketones containing phenyl rings were reduced using WT *Geotrichum candidum* NBRC 4597 (*GcAPRD*) and W288A mutant as seen in Table 1.⁴⁵ These reactions produced the *S*-enantiomer of the alcohol products with that of the WT having enantiomeric excess of >99% although it accepted only small alkyl chains such as methyl, ethyl, and propyl.

Table 1. asymmetric reduction of ketones using *Geotrichum candidum* NBRC 4597 mutants.

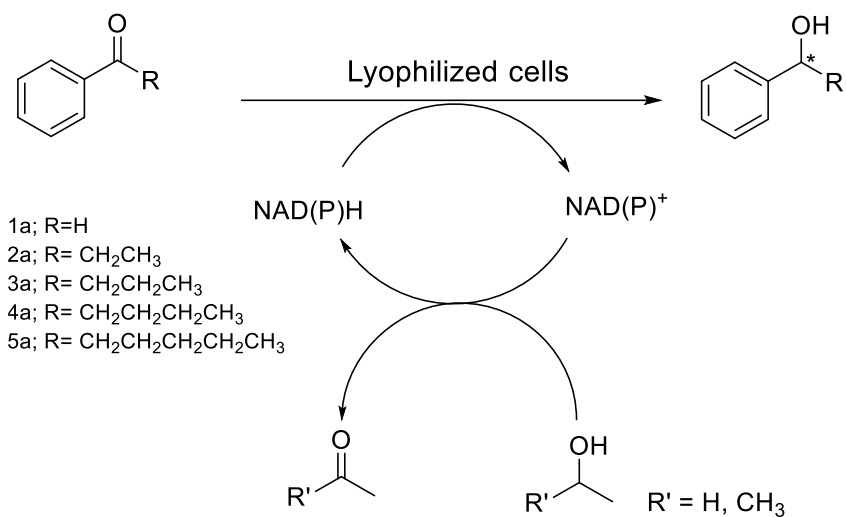


1a; R=Me, 2a; R= Et
 3a; R= *n*-Pr, 4a; R= *n*-Bu
 5a; R= *n*-Pen, 6a; R= *n*-Hex

Substrate	<i>ee</i> (%)	
	WT	W288A
1a	>99	46
2a	>99	64
3a	>99	86
4a	-	99
5a	-	99
6a	-	74

Kroutil and colleagues reported the asymmetric reduction of phenyl-ring-containing ketones using lyophilized cells of microorganisms coupled with either 2-propanol or ethanol as co-substrate.⁴⁶ This resulted in optically active alcohols having medium to very high enantioselectivity as seen in Table 2 with that of *Rhodococcus ruber* DSM 44541 showing a switch in stereopreference .

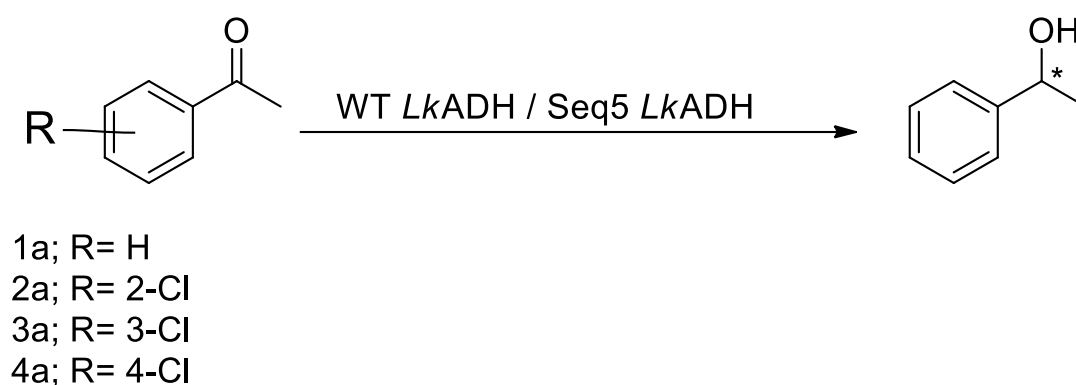
Table 2. Asymmetric reduction of phenyl-ring containing ketones using lyophilized cells.



microorganism	Substrate	ee (%)	
		(2-PrOH)	(EtOH)
<i>Ralstonia</i> sp. DSM 6428	1a	40 (S)	-
<i>Ralstonia</i> sp. DSM 6428	2a	50 (S)	60 (S)
<i>Ralstonia</i> sp. DSM 6428	3a	84 (S)	87 (S)
<i>Ralstonia</i> sp. DSM 6428	4a	77 (S)	90 (S)
<i>Ralstonia</i> sp. DSM 6428	5a	75 (S)	95 (S)
<i>S. yanoikuyae</i> DSM 6900	1a	-	49 (S)
<i>S. yanoikuyae</i> DSM 6900	2a	66 (S)	59 (S)
<i>S. yanoikuyae</i> DSM 6900	3a	84 (S)	87 (S)
<i>S. yanoikuyae</i> DSM 6900	4a	86 (S)	79 (S)
<i>S. yanoikuyae</i> DSM 6900	5a	97 (S)	89 (S)
<i>Rhodococcus ruber</i> DSM 44541	1a	99 (S)	99 (S)
<i>Rhodococcus ruber</i> DSM 44541	2a	99 (S)	40 (S)
<i>Rhodococcus ruber</i> DSM 44541	3a	-	40 (R)
<i>Rhodococcus ruber</i> DSM 44541	4a	-	53 (R)
<i>Rhodococcus ruber</i> DSM 44541	5a	-	49 (R)

Shao and coworkers reported the asymmetric reduction of substituted and unsubstituted acetophenones using WT and Y190P/I144V/L199V/E145C/M206F (seq 5) mutant of *Lactobacillus kefir* DSM 20587 (*LkADH*) with both showing opposite stereopreferences in the reduction reaction of all the substrate except that of 4-chloro-1-phenylethanone which resulted in the *anti*-Prelog product for both WT and seq 5 mutant (Table 3).¹⁶

Table 3. Asymmetric reduction of phenyl-ring-containing ketones using *LkADH* mutants.

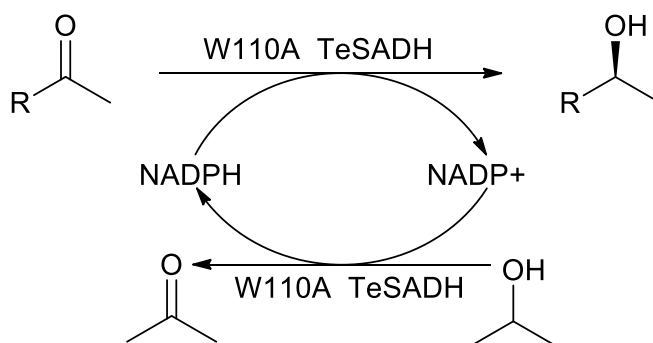


Substrate	<i>ee</i> (%)	
	WT	Seq 5
1a	>99 (<i>R</i>)	36 (<i>S</i>)
2a	65 (<i>R</i>)	>99 (<i>S</i>)
3a	>99 (<i>R</i>)	70 (<i>S</i>)
4a	>99 (<i>R</i>)	82 (<i>R</i>)

Phillips and coworkers asymmetrically reduced phenyl-ring-containing ketones employing W110A mutant of *TeSADH*, these ketones were successfully reduced to a single enantiomer following the Prelog's rule with high conversion and *ee* as seen in Table 4.

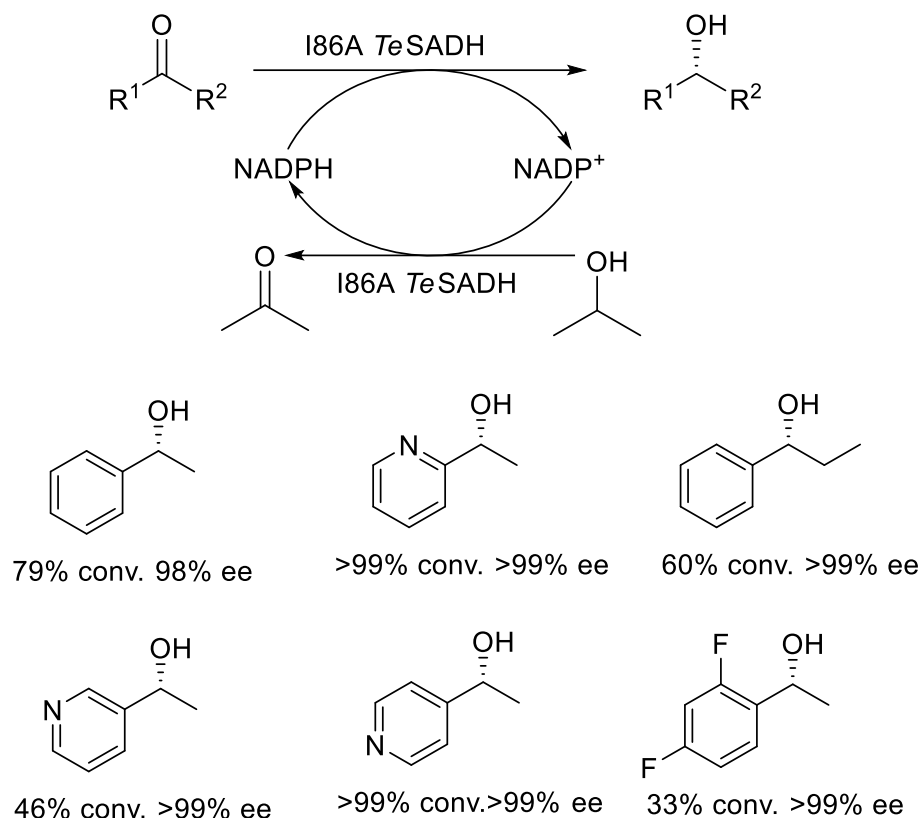
They then produced the opposite enantiomer of the produced alcohols by kinetic resolution with high enantiomeric purity.⁴⁴

Table 4. Asymmetric reduction of prochiral ketones by W110A *TeSADH* mutant.



R	Conv. (%)	<i>ee</i> (%)
PhCH ₂ CH ₂	99	> 99
Ph(C=O)CH ₂	98	> 99
(<i>E</i>)-Ph-HC=CH	64	> 99
<i>p</i> -MeOC ₆ H ₄ (CH ₂) ₂	87	91
PhOCH ₂	> 99	> 99
PhCH ₂	95	37
<i>p</i> -MeOC ₆ H ₄ CH ₂	97	> 99

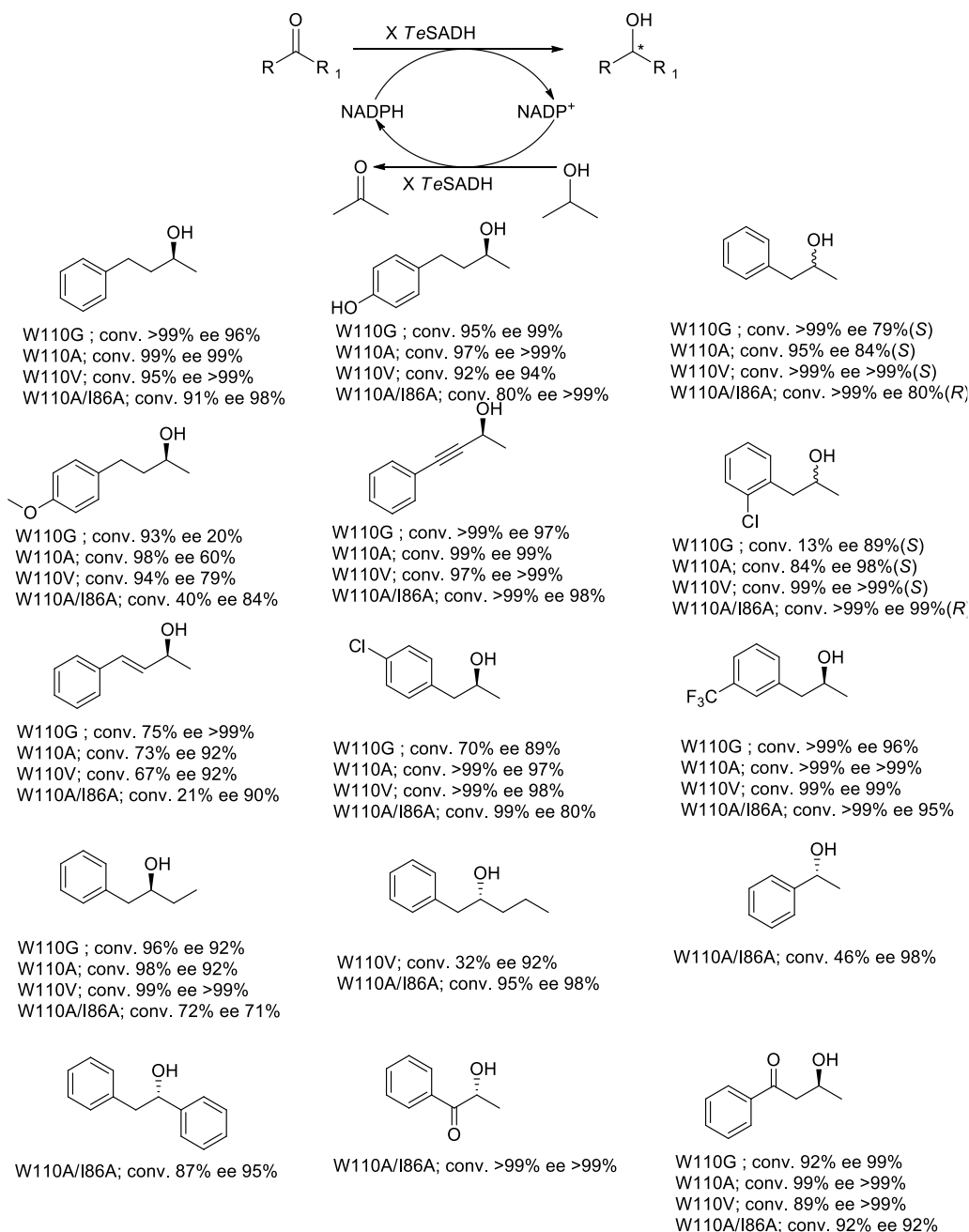
Subsequently, it was reported that a single mutation on the active site of *Te*SADH (I86A), which lines the smaller pocket of the enzyme, was capable of changing the enantioselectivity of the enzyme to *anti*-Prelog's mode in the asymmetric reduction of ketones containing phenyl and heterocyclic rings with appreciable conversions and high enantiomeric purity (Scheme 6).⁴⁷ However, I86A *Te*SADH had a very narrow substrate scope.



Scheme 6. Asymmetric reduction of ketones by I86A *Te*SADH mutant.

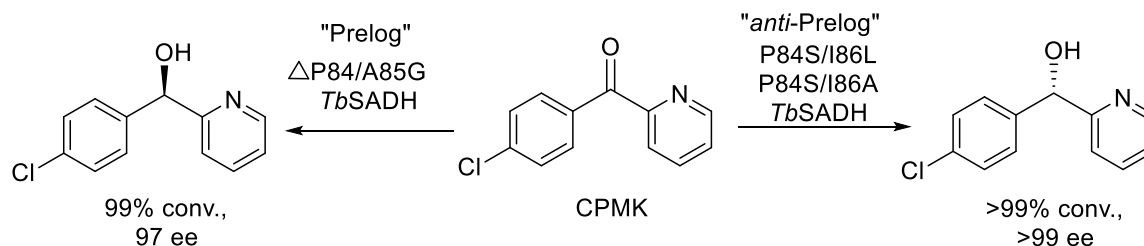
Musa and coworkers screened the asymmetric reduction of various aryl-ring-containing ketones using single (W110A, W110V and W110G) and double (W110A/I86A) mutations of *Te*SADH (Scheme 7). This resulted in optically active alcohols with *S*-configuration that

are having enantiomeric excesses and conversions greater than 99% with the double mutant having the ability to tune the stereopreference of the enzyme from Prelog to *anti*-Prelog in asymmetric reduction of phenylacetone.²³



Scheme 7. Asymmetric reduction of phenyl-ring containing ketones using several mutants of *TeSADH*.

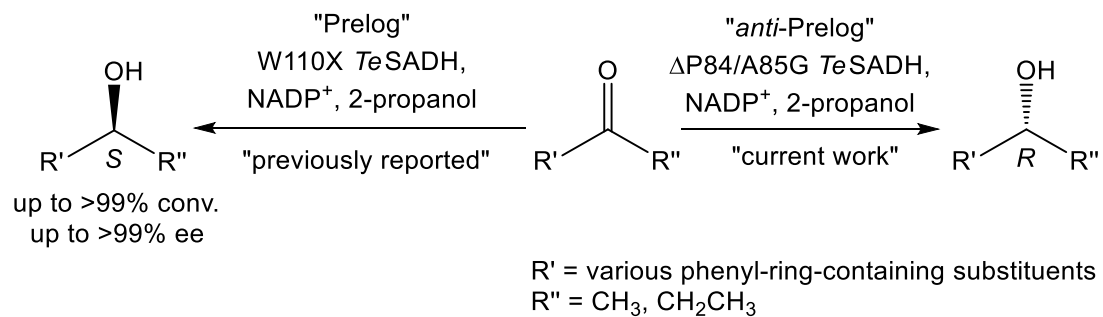
In a study by Sun and coworkers,⁴⁸ the significance of P84, which possesses a unique characteristic of having only one rotatable angle compared to other amino acids, was reported in controlling the stereopreference of *TbSADH* during the asymmetric reduction of bulky-bulky ketones containing an aryl ring on both sides of the carbonyl group (Scheme 8).



Scheme 8. Switch in enantioselectivity of *TbSADH* for the asymmetric reduction of CPMK.

Motivated by this research, we envisaged employing a similar strategy to modulate the stereopreference of *TeSADH* in the asymmetric reduction of ketones that possess bulky-small substituents, which exhibit significant differences in their sizes.

This research work presents the $\Delta\text{P84/A85G}$ *TeSADH* mutant, which is capable of reducing phenyl-ring-containing prochiral ketones in *anti*-Prelog mode, in contrast to the stereopreference of W110 *TeSADH* mutants (Scheme 9) reported previously.²³ This study aims at the investigate of the stereopreference and the substrate scope of $\Delta\text{P84/A85G}$ *TeSADH*.



Scheme 9 . Asymmetric reduction of phenyl-ring-containing prochiral ketones using the enantiocomplementary W110X and P84/A85G mutants of *TeSADH*. X = V, A, G.

CHAPTER 3

EXPERIMENTAL PROCEDURES

3.1 General

Ketone substrates (**1a-8a**), NADP⁺, racemic alcohols (**3b**, **5b** and **9b**), solvents, and sodium sulfate were purchased from commercial sources and used without further treatment. All gas chromatography (GC) analyses were conducted using a capillary Agilent GC model 7890A equipped with an HP chiral-20B column (30 m, 0.32 mm [i.d.], 0.25 μ m film thickness) using helium as the carrier gas with a flame ionization detector. GC-MS analyses were performed using an Agilent 5975C with a triple-Axis detector combined with an Agilent GC 7890A. Nuclear magnetic resonance (NMR) spectra were recorded in CDCl₃ on an FT-NMR at 600 MHz (¹H) and 150 MHz (¹³C) at room temperature using CDCl₃ peak as an internal standard. All buffer solutions were adjusted to pH 8 at room temperature.

3.2 GC-MS Analysis

The following temperature program was used for the GC oven: 80 °C (initial, hold time of 5 min) to 300 °C (final, hold time of 10 min) at a rate of 3 °C/min. The injector temperature was 250 °C and the detector temperature was 310 °C. The gas flow rates for air, H₂, and Helium were 300, 30, and 20 mL/min, respectively. The splitting mode with a split ratio of 10:1 was used.

3.3 Gene Expression and Purification of *TeSADH* Mutants

Δ P84, A85G and Δ P84/A85G mutants of *TeSADH* were constructed, expressed and purified as previously reported.⁴⁹

3.4 Asymmetric Reduction of Ketones Using *TeSADH* Mutants

The asymmetric reduction of ketones catalyzed by *TeSADH* mutants was done by following previously reported procedures with minor modifications as needed.^{43,44} Briefly, a mixture of the ketone substrate (6.8 mM), *TeSADH* mutant (final concentration of 2.5 μ M), NADP⁺ (1.3 mM), was placed in a plastic tube containing Tris-HCl buffer solution (pH 8.0, 50 mM, 750 μ L) and 2-propanol (20%, v/v). The reaction mixture was then shaken at 50 °C and 180 rpm for 12 hours (reaction progress was monitored by a Gas chromatogram). This was followed by extraction using diethyl ether (2 $\times$ 500 μ L). The combined organic layer was dried with sodium sulfate and concentrated to dryness.

3.5 Determination of Enantiomeric Excess

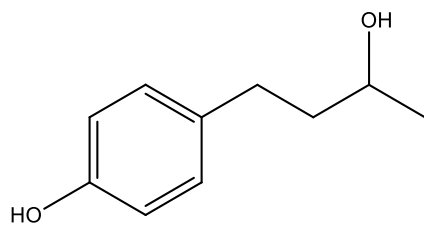
The produced alcohols were transformed to their corresponding acetate esters by treatment with one drop of acetic anhydride and two drops of pyridine three hours prior to their analysis by chiral GC⁵⁰. The following method was used in the GC analysis: (a) for the acetate esters of **3b**, **5b**, **6b**, **7b** and **8b**: initial oven temperature was 100 °C for 5 min to 180 °C for 10 min at 5 °C/min; injector 220 °C, detector 230 °C; and the helium at 14.2 mL/min. The volume injected was 1.0 μ L with split ratio of 10:1. (b) for acetate esters of **1b**, **2b** and **4b**: initial oven temperature was 80 °C for 5 min to 180 °C for 10 min at 5 °C/min; injector 220 °C, detector 230 °C; and the helium at 14.2 mL/min. The volume injected was 1.0 μ L with split ratio of 10:1.

3.6 Determination of Absolute Configuration

To identify the stereochemical identity of these alcohols, the products with available standards were co-injected along with their standards while others were co-injected with products of the asymmetric reduction using W110V mutant of *TeSADH* which from previous reports is known to produce the *S*-enantiomer of the alcoholic products.

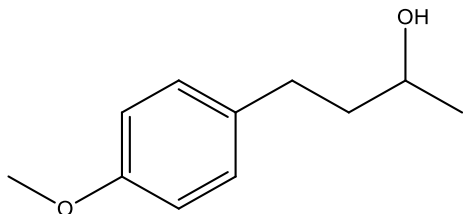
3.7 Reduction of Ketones Using NaBH₄

Racemic alcohols (**1b**, **4b**, **6b** and **8b**) were obtained via reduction of their corresponding ketones by NaBH₄ using a previously published procedure with minor modifications.⁵¹ The ketone (1.0 mmol) was dissolved in 5.0 mL of methanol at 0 °C, sodium borohydride (2.0 mmol) was then added. The solution was stirred for 15 minutes, and the reaction was monitored by TLC. After the reaction was complete, a few drops of 1.0 M HCl was added then the solvent was evaporated under vacuum. Water was added and the solution was extracted with anhydrous ethyl acetate (2 × 5.0 mL). The combined organic layer was dried with sodium sulphate. The solvent was then evaporated under vacuum to obtain pure racemic alcohols.

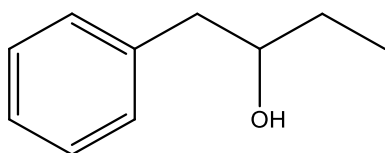


4-(4'-Hydroxyphenyl)-2-butanol (1b); ¹H-NMR (600 MHz, CDCl₃) δ 1.22 (d, *J* = 6.2 Hz, 3H), 1.69-1.75 (m, 2H), 2.57-2.69 (m, 2H), 3.81-3.85 (m, 1H), 6.74 (d, *J* = 8.6 Hz, 2H), 7.04 (d, *J* = 8.4 Hz, 2H) ppm. ¹³C-NMR (CDCl₃) δ 23.7, 31.3, 41.1, 67.8, 115.4, 129.5,

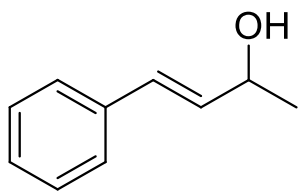
134.0, 153.9 ppm. The ^1H and ^{13}C spectroscopic data were in accordance with those reported previously.¹³



4-(4'-Methoxyphenyl)-2-butanol (4b); ^1H -NMR (600 MHz, CDCl_3) δ 1.21 (d, $J = 6.2$ Hz, 3H), 1.69-1.75 (m, 2H), 2.58-2.63 (m, 1H), 2.66-2.71 (m, 1H), 3.78 (s, 3H), 3.79-3.82 (m, 1H), 6.82 (d, $J = 8.6$ Hz, 2H), 7.11 (d, $J = 8.8$ Hz, 2H) ppm. ^{13}C -NMR (CDCl_3) δ 23.7, 31.3, 41.2, 55.4, 67.6, 113.9, 129.4, 134.1, 157.8 ppm. The ^1H and ^{13}C spectroscopic data were in accordance with those reported previously.⁴⁴



1-Phenyl-2-butanol (6b); ^1H -NMR (600 MHz, CDCl_3) δ 0.99 (t, $J = 7.5$ Hz, 3H), 1.52-1.59 (m, 3H), 2.64 (dd, $J = 13.6, 8.4$ Hz, 1H), 2.83 (dd, $J = 13.6, 4.3$ Hz, 1H), 3.72-3.77 (m, 1H), 7.21-7.32 (m, 5H) ppm. The ^1H spectroscopic data were in accordance with those reported previously.²³



4-Phenyl-2-buten-2-ol (8b); ^1H -NMR (600 MHz, CDCl_3) δ 1.37 (d, $J = 6.4$ Hz, 3H), 1.63 (s, 1H), 4.46-4.51 (m, 1H), 6.26 (dd, $J = 15.9, 6.4$ Hz, 1H), 6.56 (d, $J = 15.8$ Hz, 1H), 7.22-7.38 (m, 5H) ppm. ^{13}C -NMR (CDCl_3) δ 23.5, 69.1, 126.5, 127.7, 128.6, 129.5, 133.6, 136.7 ppm. The ^1H and ^{13}C spectroscopic data were in accordance with those reported previously.⁵²

CHAPTER 4

RESULTS AND DISCUSSION

In this study, we aimed to explore the role of Δ P84 mutant of *Te*SADH for the asymmetric reduction of bulky-small ketones such as 4-phenyl-2-butanone and 1-phenyl-2-propanone, which were previously reported to be reduced by W110 mutants in Prelog mode,²³ with the aim of switching the stereopreference of *Te*SADH to the *anti*-Prelog mode. Previous research had highlighted the importance of proline mutation in switching the stereopreference of *Te*SADH in asymmetric reduction of bulky-bulky ketones.⁴⁸

To assess the effect of the proline deletion on the stereopreference and selectivity in the asymmetric reduction of bulky-small ketones, we generated Δ P84, A85G, Δ P84/A85G mutants of *Te*SADH using site-directed mutagenesis. It should be noted that deletion of proline-84 results in a shift of each of the amino acids that follow this site (i.e., A85 become A84). A85G mutation, along with other site mutations, has been shown before to extend the size of the small binding pocket of *Te*SADH,^{53,54} thus expanding the substrate scope of *Te*SADH. We chose to check the asymmetric reduction using the individual omission of proline and mutation of A85 to G as well as using Δ P84/A85G to determine whether or not proline omission is responsible for the switch in stereopreference. We then tested these mutants in the asymmetric reduction of selected phenyl-ring-containing ketones that had previously been shown to be substrates for W110 *Te*SADH mutants.^{55,56}

The asymmetric reduction reactions were carried out in Tris-HCl buffer solution (50 mM, pH 8.0) containing 2-propanol (20%, v/v), which acts as both a cosolvent and co-

substrate to regenerate the cofactor NADPH. The reduction of 4-(4'-hydroxyphenyl)-2-butanone (**1a**) using A85G *TeSADH* resulted in the production of (S)-4-(4'-hydroxyphenyl)-2-butanol [*S*-(**1b**)] in low conversion and high enantioselectivity (Table 6). The reduction reaction of **1a** using Δ P84/A85G *TeSADH* mutant quantitatively produced *R*-(**1b**) with high enantioselectivity (Table 7). These results suggest that Δ P84/A85G *TeSADH* is capable of tuning the stereopreference of *TeSADH* to *anti*-Prelog mode, and the inversion of stereopreference in this double mutant may be attributed to proline deletion rather than A85G mutation.

After confirming the catalytic activity of Δ P84/A85G *TeSADH* mutant in the asymmetric reduction of **1a**, we proceeded to test its efficiency in asymmetric reduction of selected phenyl-ring containing ketone substrates. These selected substrates were previously been reported to follow Prelog's rule in their asymmetric reduction using W110 mutants of *TeSADH*.^{55,56} The reduction of 4-phenyl-3-butyne-2-one (**2a**) using Δ P84/A85G and Δ P84 *TeSADH* mutants resulted in formation of (*R*)-4-phenyl-3-butyne-2-ol [*R*-(**2b**)] with higher conversion and enantioselectivity observed for the double mutant (Table 7). In contrast, the reduction using A85G mutant yielded the opposing (*S*)-**2b** (Table 6), consistent with previous findings using W110 mutants (Table 8). These results further support the notation that the reversed stereopreference in Δ P84/A85G *TeSADH* is a consequence of proline-84 deletion.

Similarly, the asymmetric reduction of 1-phenyl-2-propanone (**3a**) using Δ P84/A85G *TeSADH* under the same reaction conditions resulted in production of (*R*)-1-phenyl-2-propanol [*R*-(**3b**)] with both high conversion and enantioselectivity (Table 7).

However, the reduction of **3a** using the single mutants Δ P84 and A85G was not possible as shown in Table 5 and Table 6, respectively.

The reduction of 4-(4'-methoxyphenyl)-2-butanone (**4a**) using Δ P84/A85G *TeSADH* produced (*R*)-4-(4'-methoxyphenyl)-2-butanol [*R*-(**4b**)] with high conversion and enantioselectivity. In contrast, reduction of this substrate using A85G produced *S*-(**4b**) in high enantioselectivity but low conversion (Table 6), while Δ P84 *TeSADH* showed almost no conversion. Δ P84/A85G *TeSADH*-catalyzed reduction of 4-phenyl-2-butanone (**5a**) resulted in formation of (*R*)-4-phenyl-2-butanol [(*R*)-**5b**] with high conversion and good enantioselectivity (Table 7), whereas the single mutants showed no conversion. It is worth mentioning that the optically active **5b** is an important synthon in the synthesis of inhibitors used in the treatment of AIDS such as SC-52151 and VX-478 (Figure 1).¹²

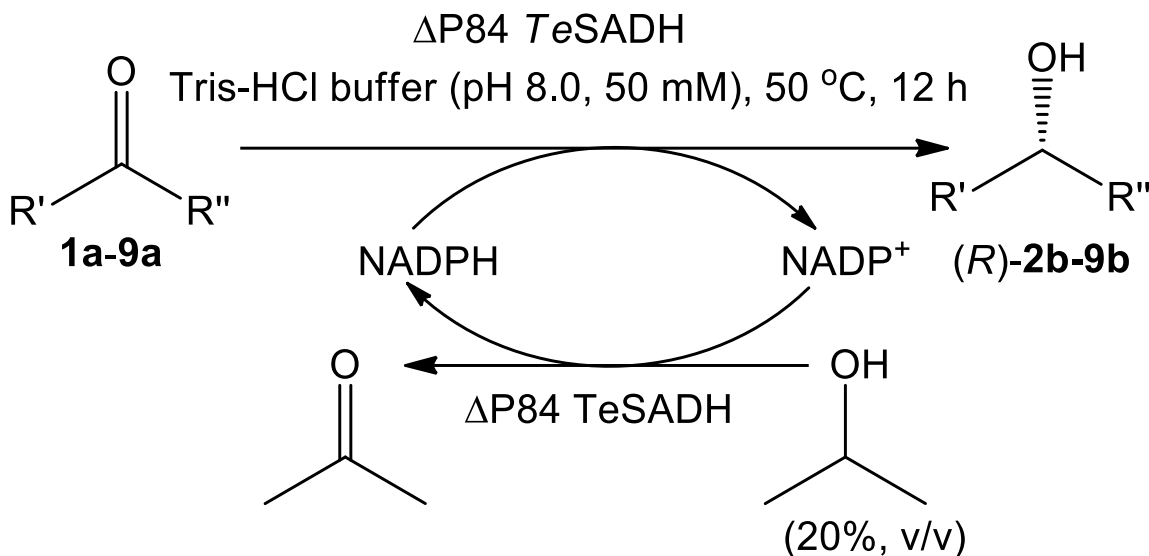
The reduction of 1-phenyl-2-butanone (**6a**) using Δ P84/A85G resulted in the formation of the corresponding (*R*)-configured 1-phenyl-2-butanol [(*R*)-**6b**] with high enantioselectivity and conversion. No conversion was observed using Δ P84 and A85G mutants of *TeSADH*. 4-Chlorophenylacetone (**7a**) was reduced to (*R*)-**7b** using Δ P84/A85G *TeSADH* mutant with high conversion and enantioselectivity contrary to that using A85G which resulted in the opposite enantiomer (*S*)-**7b** with a very low conversion (Table 6). The asymmetric reduction of *E*-4-phenyl-3-buten-2-one (**8a**) using Δ P84/A85G *TeSADH* resulted in (2*R*,3*E*)-4-phenyl-3-buten-2-ol [(*R*)-**8b**] with high conversion and enantioselectivity (Table 7). In contrast, very low conversions were observed in the asymmetric reduction of **8a** using Δ P84 and A85G mutants of *TeSADH*.

The substrates discussed above were previously reduced using W110 *TeSADH* in Prelog mode, resulting in the production of their corresponding (*S*)-configured alcohols

with high conversions and enantioselectivities (Table 8).^{55,57} In contrast, Δ P84/A85G *Te*SADH accommodates these substrates in a manner that allows a *Si*-face attack, leading to the production of their corresponding (*R*)-configured alcohols. Therefore, Δ P84/A85G *Te*SADH is a stereocomplementary mutant to W110 *Te*SADH mutants (Scheme 9).

The asymmetric reduction of acetophenone (**9a**) using the studied mutants of *Te*SADH (Δ P84, A85G and Δ P84/A85G) resulted in production of (*R*)-1-phenylethanol [(*R*)-**9b**], contrary to the previously reported inability of W110 *Te*SADH mutants to reduce **9a** (Table 8). Among the mutants, the double mutant Δ P84/A85G exhibited the highest conversion and enantioselectivity (Table 7). It is worth noting that I86A *Te*SADH, which has a very limited substrate scope, was previously reported to reduce **9a** in *anti*-Prelog mode. The ability of Δ P84/A85G *Te*SADH to reduce the substrates **1a-8a**, which are also accommodated by W110 *Te*SADH, as well as **9a**, which is accommodated by I86A *Te*SADH, highlights the potential of Δ P84/A85G *Te*SADH as a mutant with wide substrate scope.

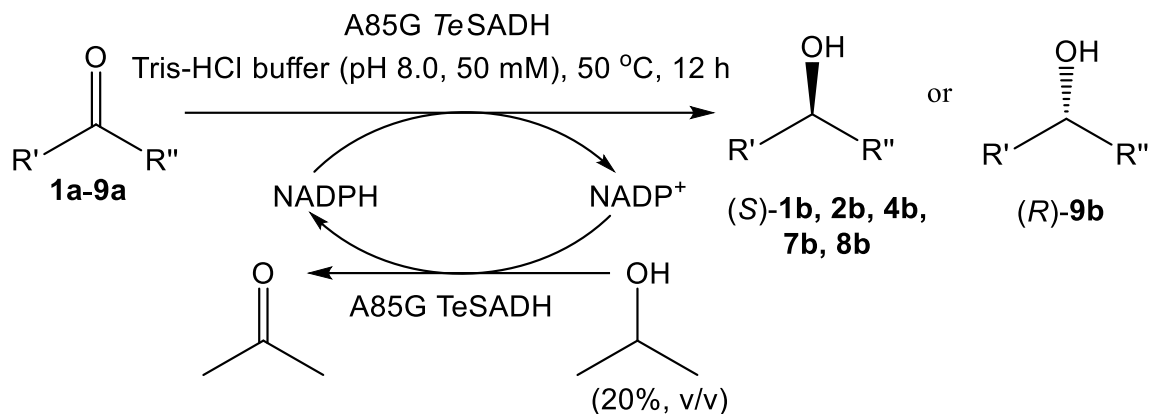
Table 5. Asymmetric reduction of phenyl-ring-containing ketones using Δ P84 mutant of TeSADH.^a



R'	R''	Substrate	Conv. (%) ^b	ee (%) ^c
<i>p</i> -HO-C ₆ H ₄ -CH ₂ CH ₂	CH ₃	1a	<5	>99 <i>R</i>
Ph-C $\equiv$ C	CH ₃	2a	51	76 <i>R</i>
PhCH ₂	CH ₃	3a	1	nd
<i>p</i> -MeO-C ₆ H ₄ -CH ₂ CH ₂	CH ₃	4a	1	nd
PhCH ₂ CH ₂	CH ₃	5a	nr	-
PhCH ₂	CH ₃ CH ₂	6a	nr	-
<i>p</i> -Cl-C ₆ H ₄ -CH ₂	CH ₃	7a	nr	-
(<i>E</i>)-Ph-HC=CH	CH ₃	8a	6	>99 <i>R</i>
Ph	CH ₃	9a	16	>99 <i>R</i>

^aUnless otherwise stated, reactions were performed in Tris-HCl buffer solution (pH 8.0, 50 mM) containing 2-propanol (20%, v/v) with total volume of 1.0 mL using the following expressed in final concentration in the reaction mixture; substrate (6.8 mM), TeSADH mutant (2.5 μ M), NADP⁺ (1.3 mM). The reaction mixture was then shaken at 50 °C and 180 rpm for 12 hrs. ^bPercent conversion was determined by GC. ^cThe % ee of the acetate ester derivatives of the alcohols produced was determined by GC using a chiral stationary phase. nd: not determined.

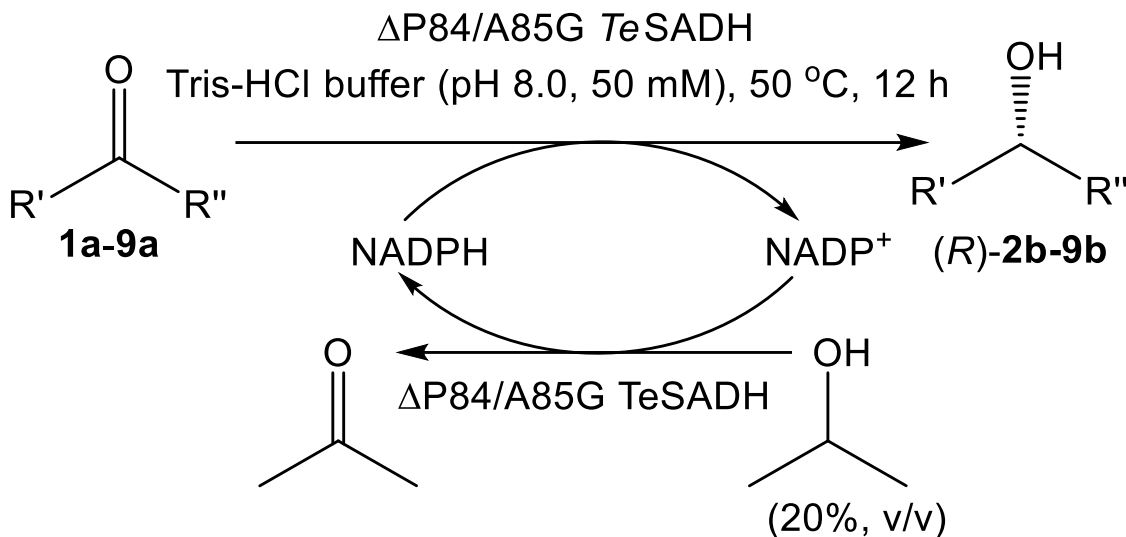
Table 6. Asymmetric reduction of phenyl-ring-containing ketones using A85G mutant of TeSADH.^a



R'	R''	Substrate	Conv. (%) ^b	ee (%) ^c
<i>p</i> -HO-C ₆ H ₄ -CH ₂ CH ₂	CH ₃	1a	11	>99 <i>S</i>
Ph-C≡C	CH ₃	2a	86	>99 <i>S</i>
PhCH ₂	CH ₃	3a	nr	-
<i>p</i> -MeO-C ₆ H ₄ -CH ₂ CH ₂	CH ₃	4a	29	>99 <i>S</i>
PhCH ₂ CH ₂	CH ₃	5a	nr	-
PhCH ₂	CH ₃ CH ₂	6a	nr	-
<i>p</i> -Cl-C ₆ H ₄ -CH ₂	CH ₃	7a	5	98 <i>S</i>
(<i>E</i>)-Ph-HC=CH	CH ₃	8a	9	84 <i>S</i>
Ph	CH ₃	9a	94	98 <i>R</i>

^aUnless otherwise stated, reactions were performed in Tris-HCl buffer solution (pH 8.0, 50 mM) containing 2-propanol (20%, v/v) with total volume of 1.0 mL using the following expressed in final concentration in the reaction mixture; substrate (10 mM), TeSADH mutant (2.5 μM), NADP⁺ (1.3 mM). The reaction mixture was then shaken at 50 °C and 180 rpm for 12 hrs. ^bPercent conversion was determined by GC. ^cThe % ee of the acetate ester derivatives of the alcohols produced was determined by GC using a chiral stationary phase. nd: not determined.

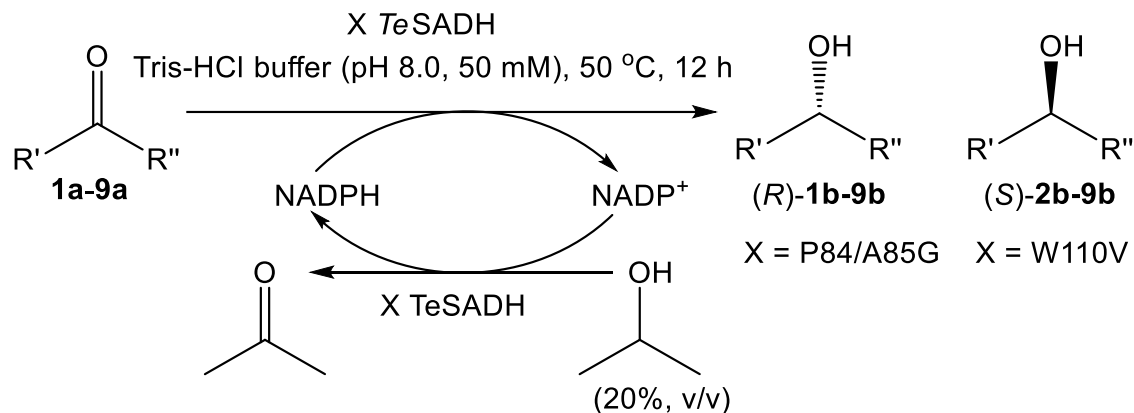
Table 7. Asymmetric reduction of phenyl-ring-containing ketones using Δ P84/A85G mutant of *TeSADH*.^a



R'	R''	Substrate	Conv. (%) ^b	ee (%) ^c
<i>p</i> -HO-C ₆ H ₄ -CH ₂ CH ₂	CH ₃	1a	99	>99 <i>R</i>
Ph-C $\equiv$ C	CH ₃	2a	88	95 <i>R</i>
PhCH ₂	CH ₃	3a	96	95 <i>R</i>
<i>p</i> -MeO-C ₆ H ₄ -CH ₂ CH ₂	CH ₃	4a	98	96 <i>R</i>
PhCH ₂ CH ₂	CH ₃	5a	98	80 <i>R</i>
PhCH ₂	CH ₃ CH ₂	6a	91	99 <i>R</i>
<i>p</i> -Cl-C ₆ H ₄ -CH ₂	CH ₃	7a	90	96 <i>R</i>
(<i>E</i>)-Ph-HC=CH	CH ₃	8a	94	99 <i>R</i>
Ph	CH ₃	9a	99	99 <i>R</i>

^aUnless otherwise stated, reactions were performed in Tris-HCl buffer solution (pH 8.0, 50 mM) containing 2-propanol (20%, v/v) with total volume of 1.0 mL using the following expressed in final concentration in the reaction mixture; substrate (6.8 mM), *TeSADH* mutant (2.5 μ M), NADP⁺ (1.3 mM). The reaction mixture was then shaken at 50 °C and 180 rpm for 12 hrs. ^bPercent conversion was determined by GC. ^cThe % ee of the acetate ester derivatives of the alcohols produced was determined by GC using a chiral stationary phase. nd: not determined.

Table 8. Asymmetric reduction of phenyl-ring-containing ketones using Δ P84/A85G and W110V mutants of *TeSADH*.^a



R'	R''	Substrate	X			
			Δ P84/A85G		W110V ^d	
			Conv. (%) ^b	ee (%) ^c	Conv. (%) ^b	ee (%)
<i>p</i> -HO-C ₆ H ₄ -CH ₂ CH ₂	CH ₃	1a	99	>99 <i>R</i>	92	94 <i>S</i>
Ph-C $\equiv$ C	CH ₃	2a	88	95 <i>R</i>	97	>99 <i>S</i>
PhCH ₂	CH ₃	3a	96	95 <i>R</i>	>99	>99 <i>S</i>
<i>p</i> -MeO-C ₆ H ₄ -CH ₂ CH ₂	CH ₃	4a	98	96 <i>R</i>	94	79 <i>S</i>
PhCH ₂ CH ₂	CH ₃	5a	98	80 <i>R</i>	95	>99 <i>S</i>
PhCH ₂	CH ₃ CH ₂	6a	91	99 <i>R</i>	99	>99 <i>S</i>
<i>p</i> -Cl-C ₆ H ₄ -CH ₂	CH ₃	7a	90	96 <i>R</i>	>99	98 <i>S</i>
(<i>E</i>)-Ph-HC=CH	CH ₃	8a	94	99 <i>R</i>	67	92 <i>S</i>
Ph	CH ₃	9a	99	99 <i>R</i>	nr	-

^aUnless otherwise stated, reactions were performed in Tris-HCl buffer solution (pH 8.0, 50 mM) containing 2-propanol (20%, v/v) with total volume of 1.0 mL using the following expressed in final concentration in the reaction mixture; substrate (6.8 mM), *TeSADH* mutant (2.5 μ M), NADP⁺ (1.3 mM). The reaction mixture was then shaken at 50 $^\circ$ C and 180 rpm for 12 hrs. ^bPercent conversion was determined by GC. ^cThe % ee of the acetate ester derivatives of the alcohols produced was determined by GC using a chiral stationary phase. nd: not determined. ^dReaction was previously reported by Musa and his coworkers.²³

CHAPTER 5

CONCLUSION

In conclusion, our study has demonstrated that deletion of proline in *TeSADH* can effectively switch its stereopreference in the asymmetric reduction of bulky-small ketones, such as those with an aryl-ring-containing moiety on one side and a methyl or ethyl on the other side. The coupling of Δ P84 and A85G mutants opened up the small pocket in the active site of *TeSADH*, allowing for the reduction of these substrate in an *anti*-Prelog mode leading to the opposing stereopreference to that of the previously reported W110 *TeSADH* mutants. Δ P84/A85G *TeSADH* also accepts acetophenone, which is not a substrate for the previously reported W110 mutants, which reflects the extended substrate scope of this double mutant. The Development of such *TeSADH* mutants that exhibit *anti*-Prelog stereopreference, and are enantiocomplementary to existing mutants of *TeSADH*, will enable the incorporation of these enantiocomplementary mutants in interesting reactions such as deracemization via stereoinversion. It will also allow for the independent production of both enantiomers of secondary alcohols in high enantiopurities via asymmetric reduction of their prochiral ketones. These findings highlight the potential of *TeSADH* mutants in the synthesis of enantiomerically pure alcohols with therapeutic applications.

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SUPPORTING INFORMATION

GC Chromatograph

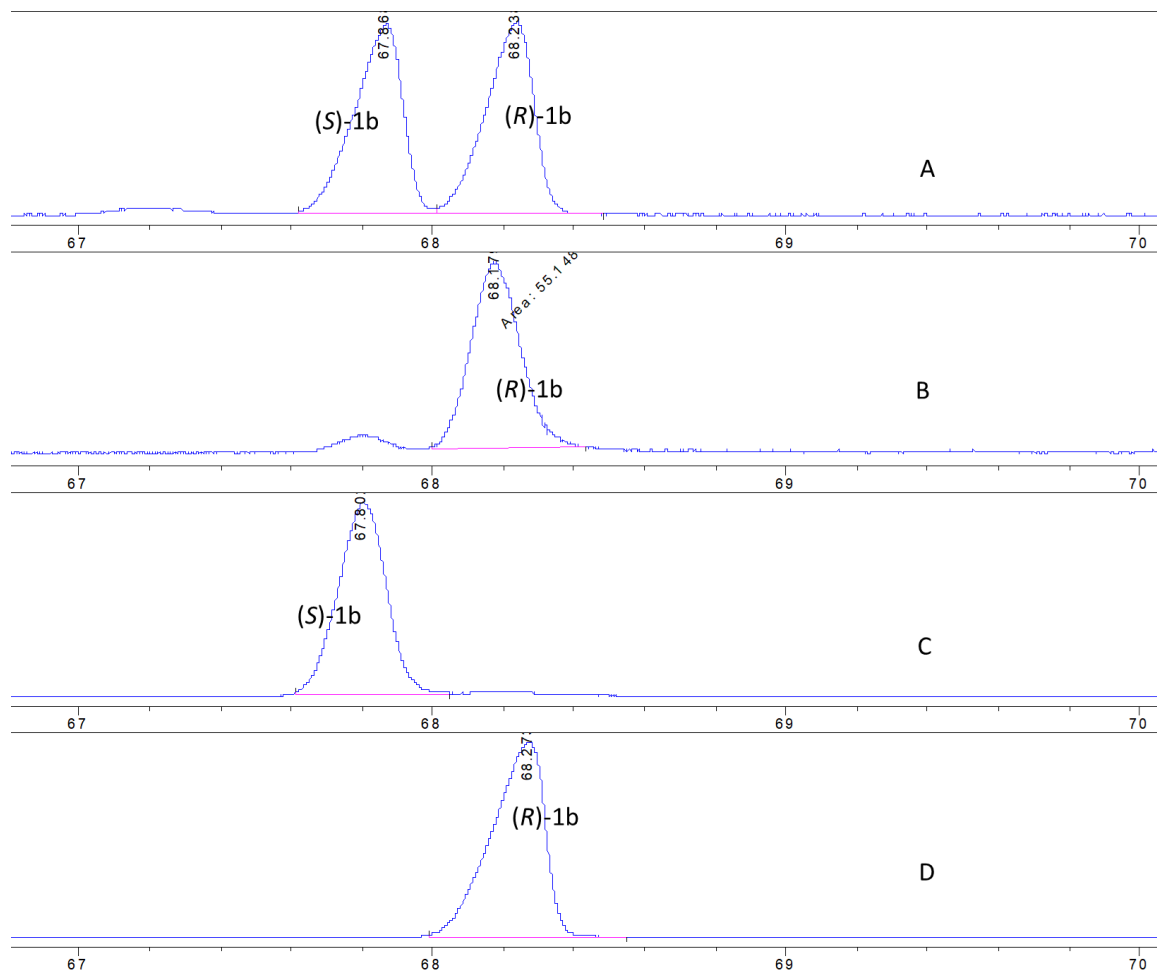


Figure 3. Gas chromatogram of the acetate derivatives of products of the reduction of 4(4'-hydroxyphenyl)-2-butanone (**1a**) using: (A) NaBH₄, (B) Δ P84 *TeSADH*, (C) A85G *TeSADH* and (D) Δ P84/A85G *TeSADH*.

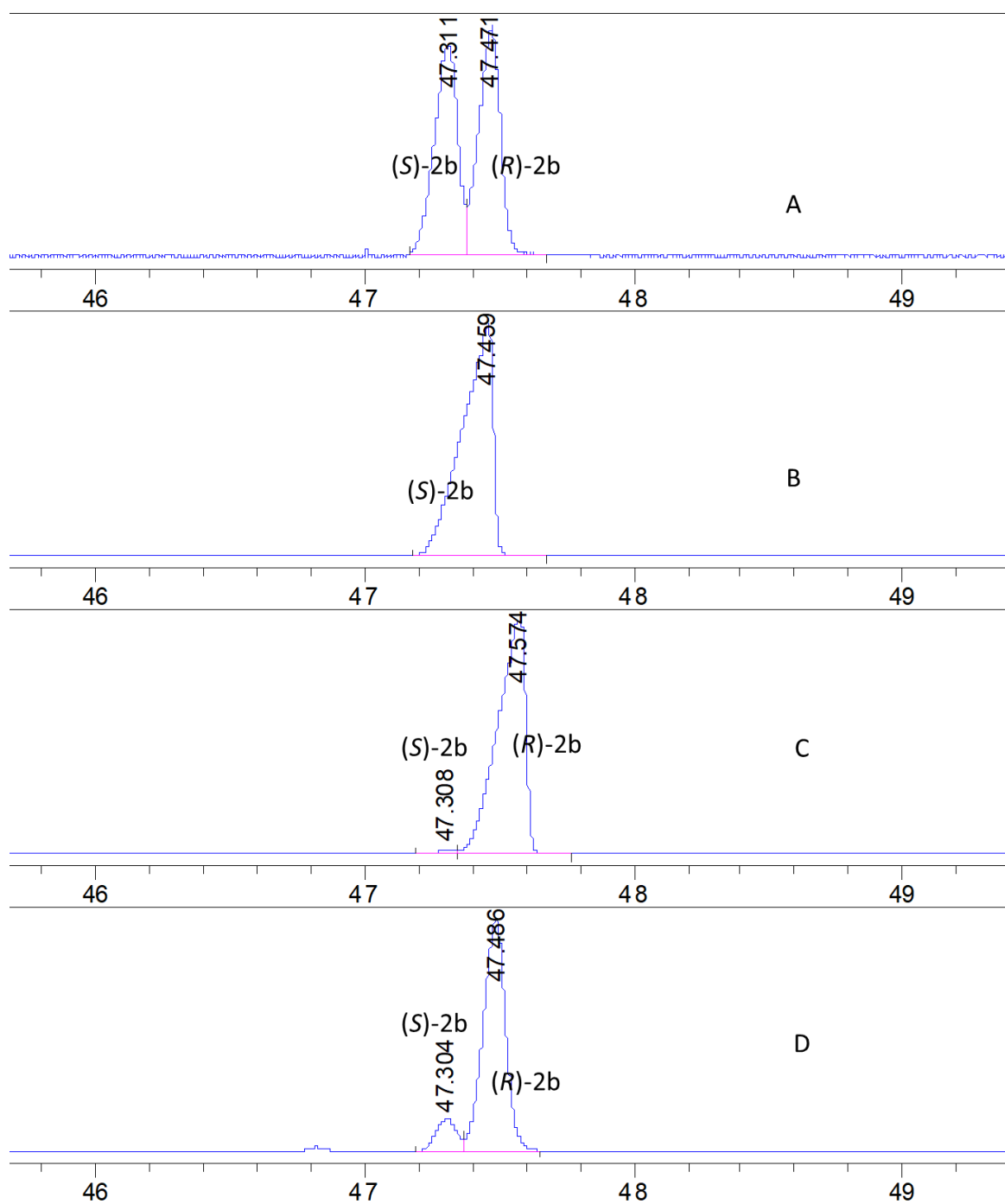


Figure 4. Gas chromatograms of the acetate derivatives of products of the reduction of 4-phenyl-2-butyne-3-one (**2a**) using: (A) NaBH_4 , (B) A85G *TeSADH*, (C) $\Delta\text{P84/A85G}$ *TeSADH* and (D) ΔP84 *TeSADH*.

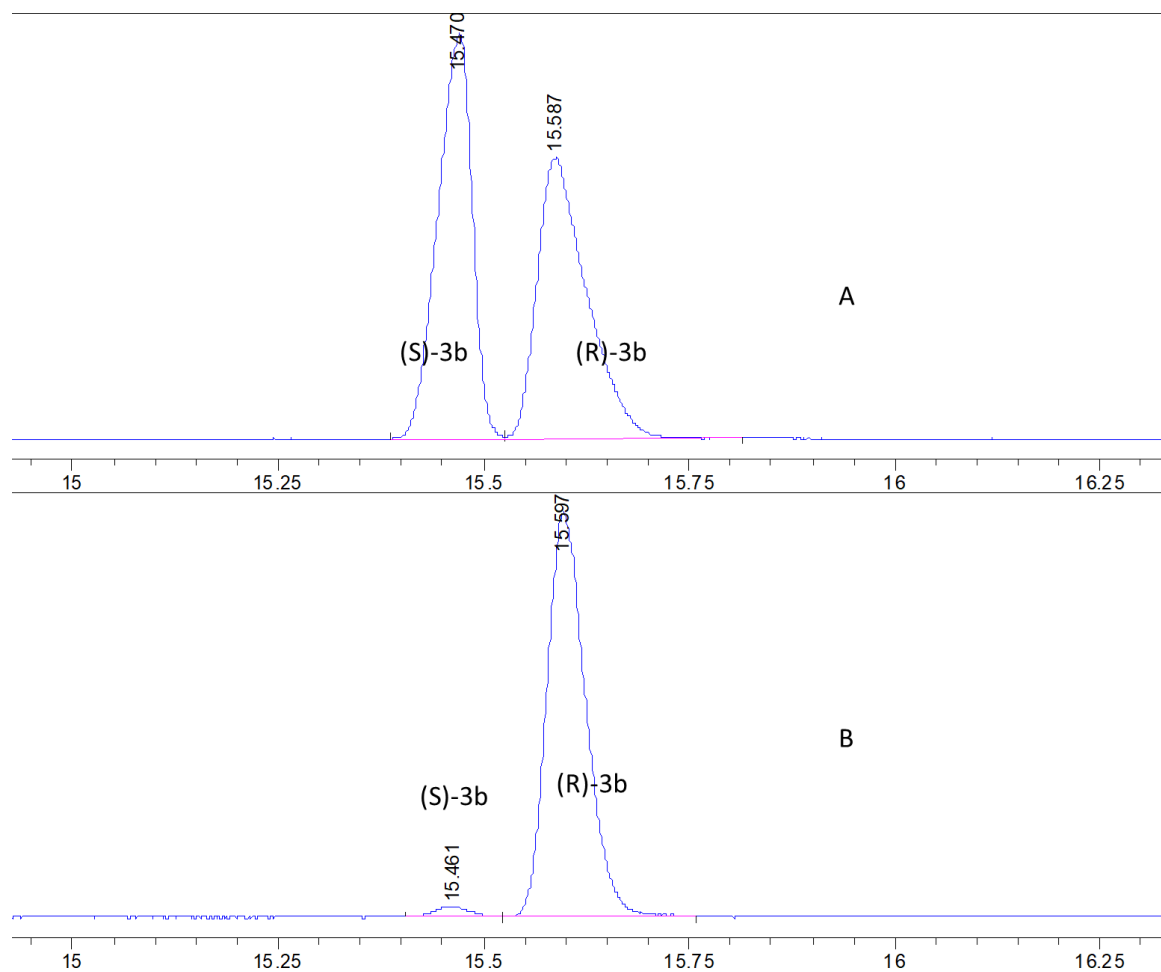


Figure 5. Gas chromatograms of the acetate derivatives of products of the reduction of 1-phenyl-2-propanone (**3a**) using: (A) NaBH_4 and (B) $\Delta\text{P84/A85G TeSADH}$.

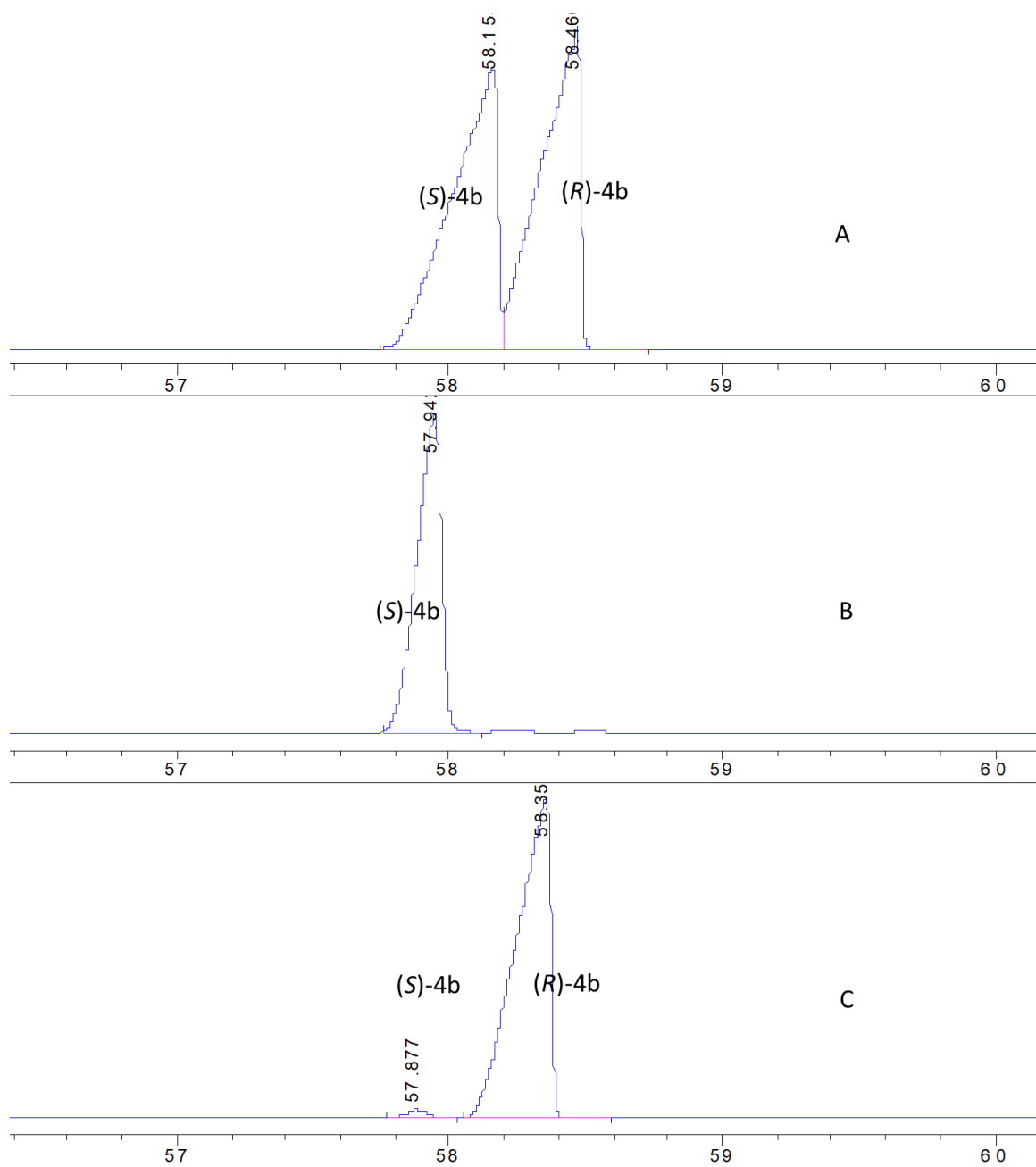


Figure 6. Gas chromatograms of the acetate derivatives of products of the reduction of 4(4'-methoxyphenyl)-2-butanone (**4a**) using: (A) NaBH₄, (B) A85G *Te*SADH, (C) Δ P84/A85G *Te*SADH.

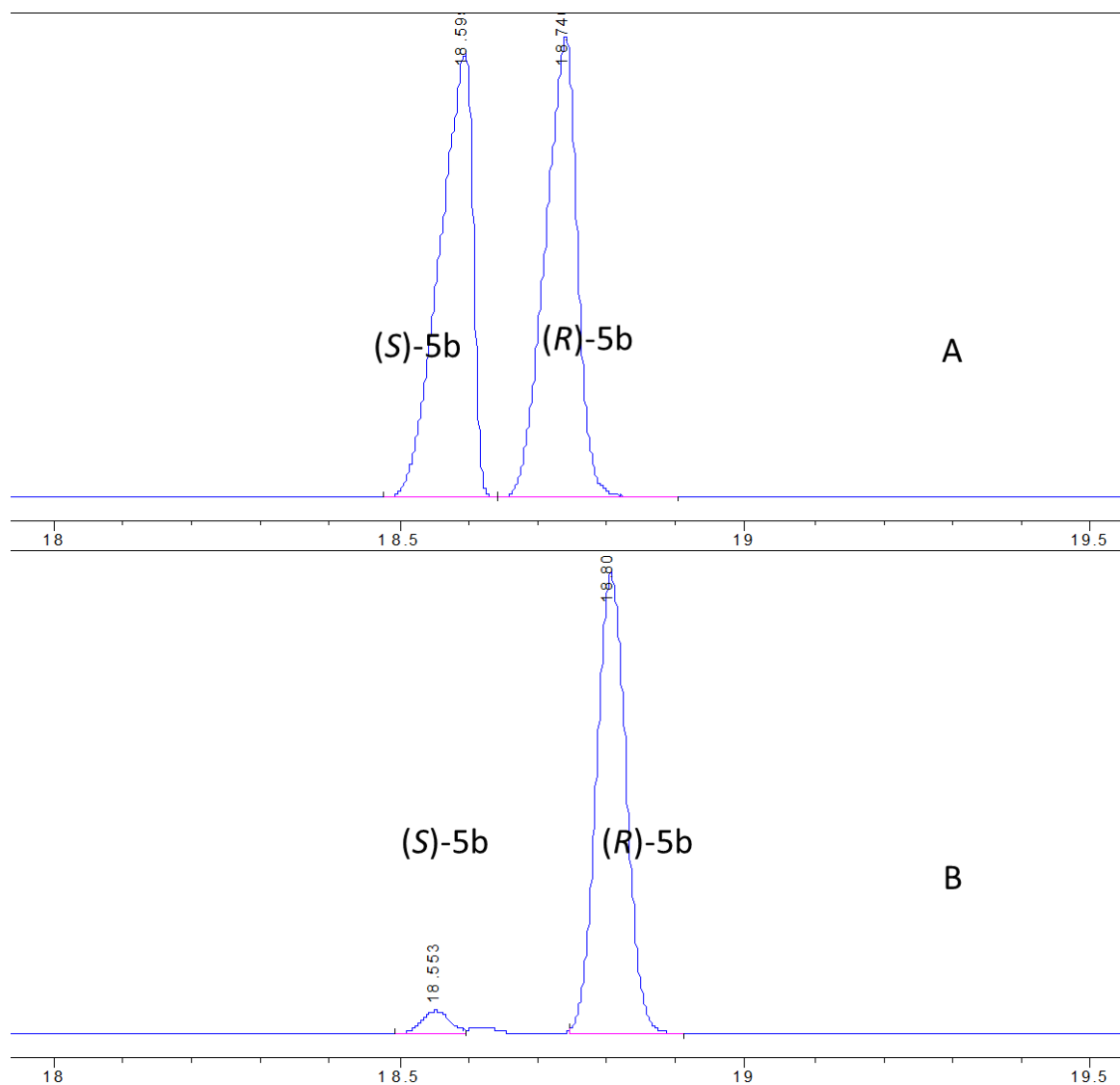


Figure 7. Gas chromatograms of the acetate derivatives of products of the reduction of 4-phenyl-2-butanone (5a) using: (A) NaBH₄ and (B) ΔP84/A85G *TeSADH*.

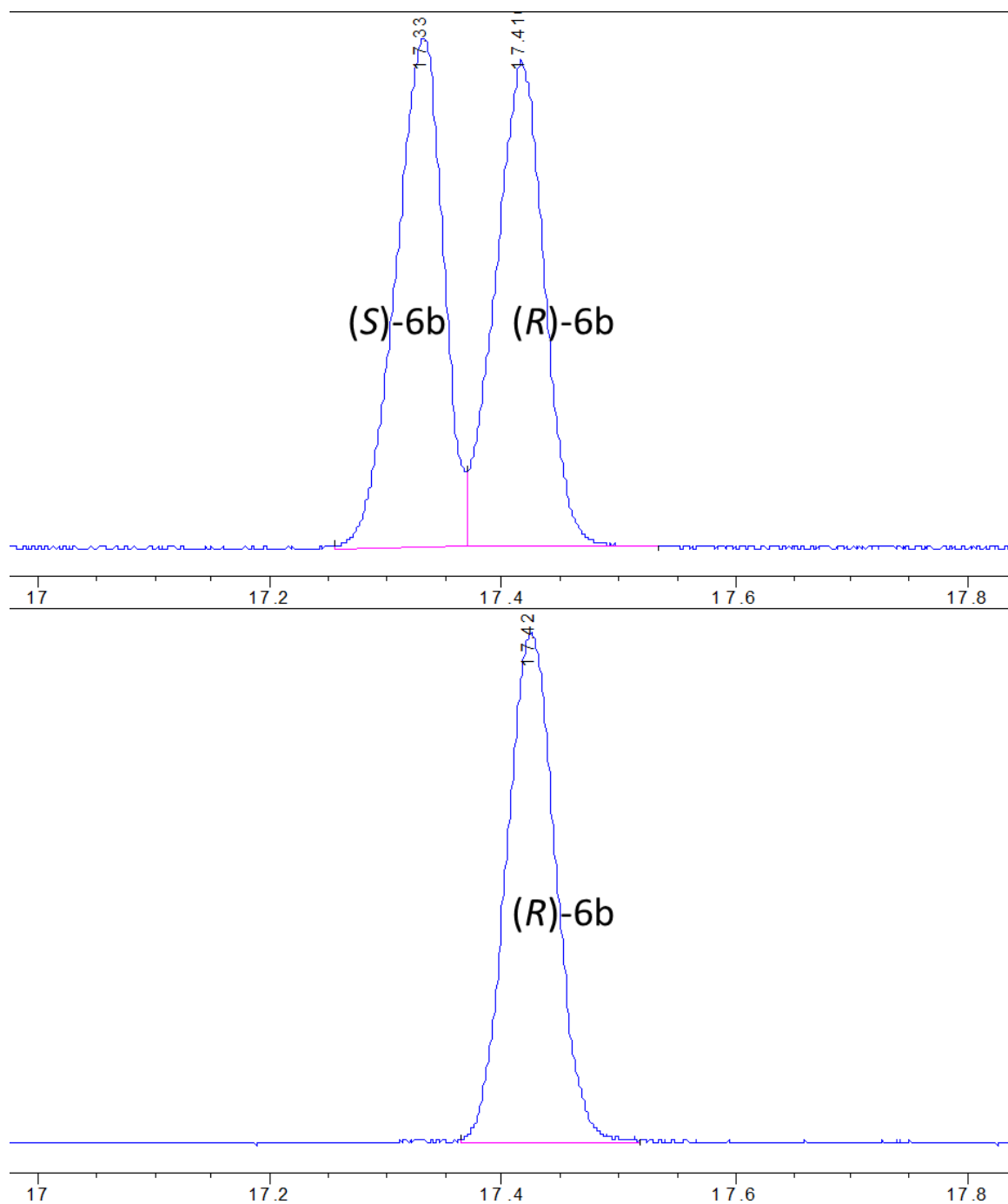


Figure 8. Gas chromatograms of the acetate derivatives of products of the reduction of 1-phenyl-2-butanone (**6a**) using: (A) NaBH₄ and (B) Δ P84/A85G TeSADH.

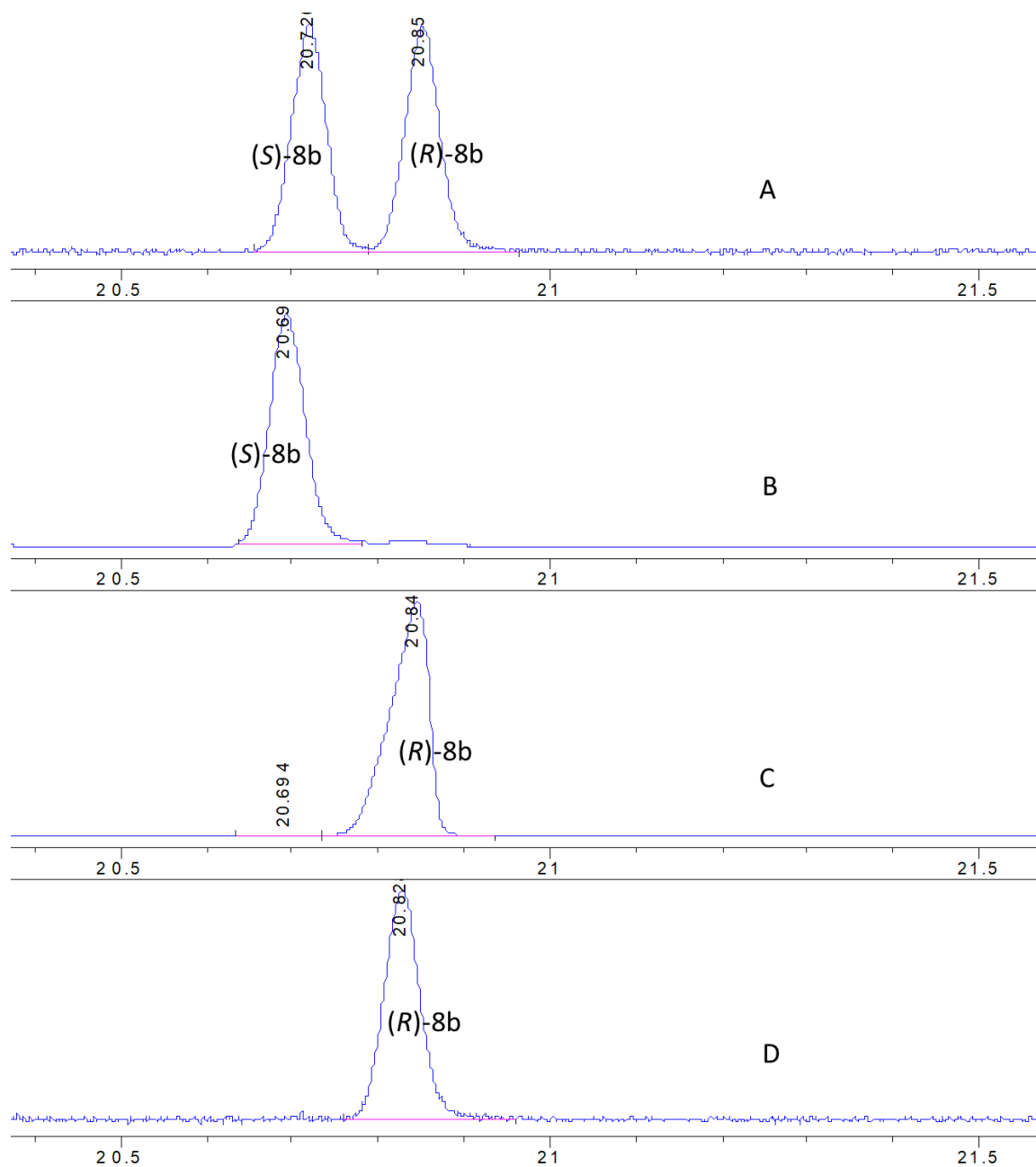


Figure 9. Gas chromatogram of the acetate derivative of products of the reduction of 4-phenyl-2-buten-3-one (**8a**) using: (A) NaBH_4 , (B) A85G *TeSADH*, (C) $\Delta\text{P84/A85G}$ *TeSADH* and (D) ΔP84 *TeSADH*.

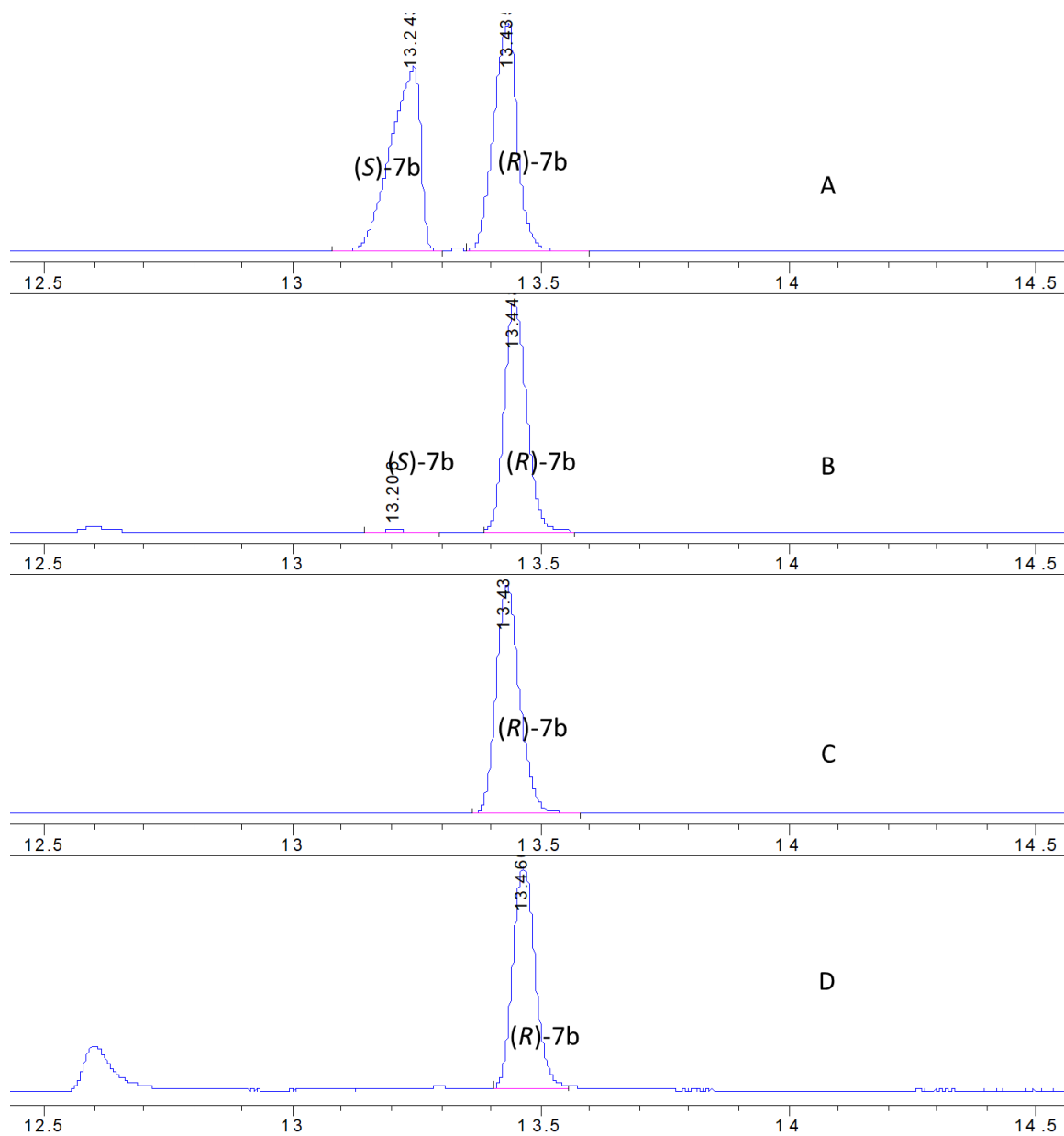


Figure 10. Gas chromatograms of the acetate derivatives of products of the reduction of Acetophenone (**9a**) using: (A) NaBH₄, (B) A85G *TeSADH*, (C) ΔP84/A85G *TeSADH* and (D) ΔP84 *TeSADH*.

Mass spectra

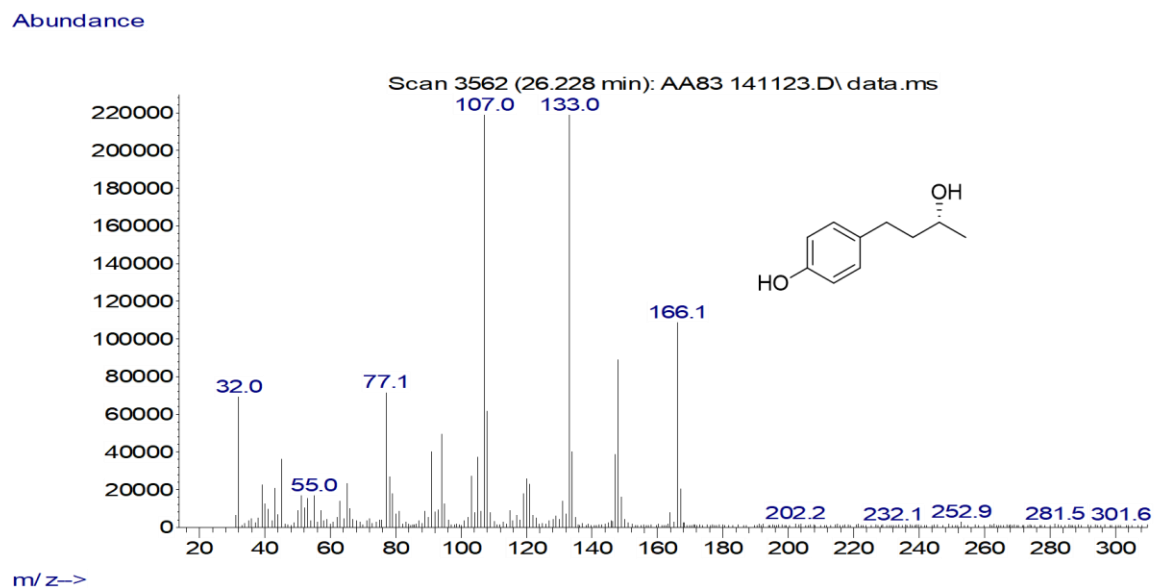


Figure 11. Mass spectrum of the asymmetric reduction product (*R*)-4-(4-hydroxyphenyl)-2-butanol (**1b**) using Δ P84/A85G *Te*SADH.

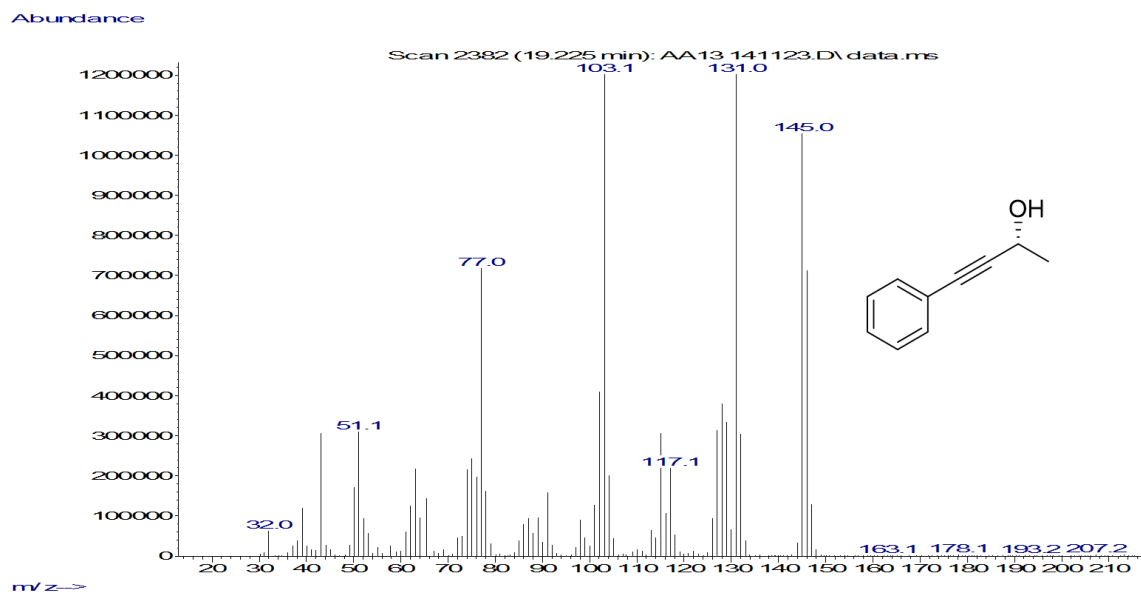


Figure 12. Mass spectrum of the asymmetric reduction product (*R*)-4-phenyl-2-butyn-3-ol (**2b**) produced by asymmetric reduction of the corresponding ketones using Δ P84/A85G TeSADH.

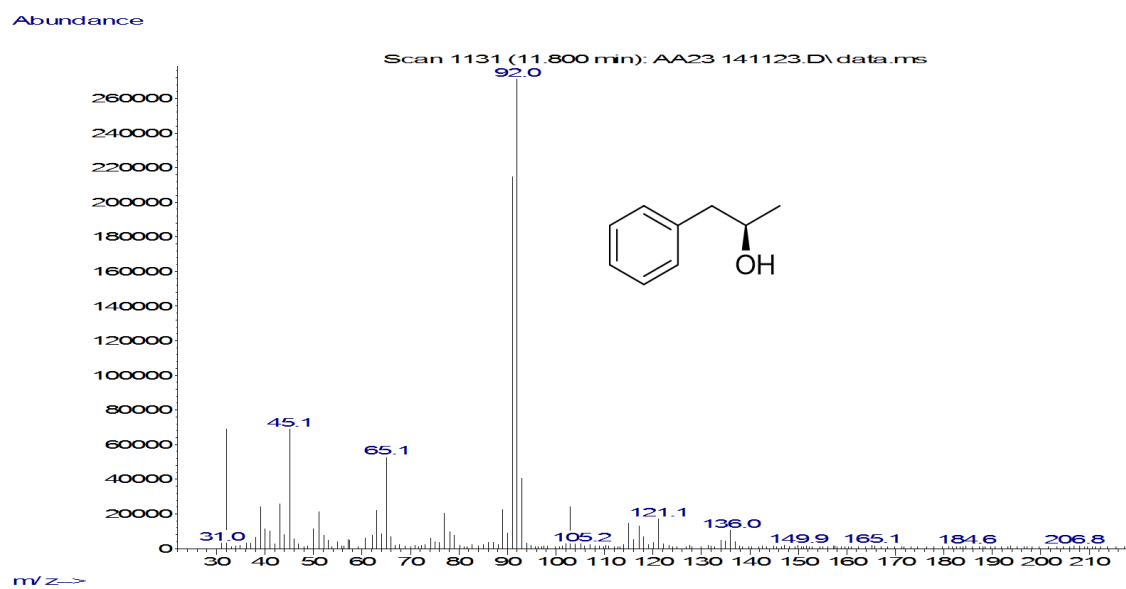


Figure 13. Mass spectrum of the asymmetric reduction product (*R*)-1-phenyl-2-propanol (**3b**) using Δ P84/A85G *Te*SADH.

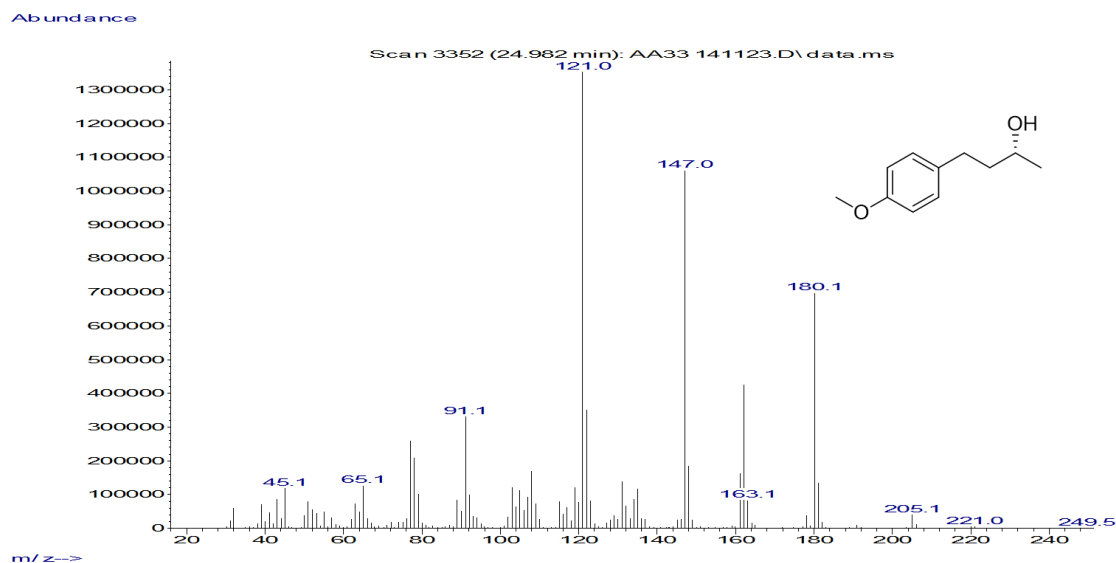


Figure 14. Mass spectrum of the asymmetric reduction product (*R*)-4-(4'-methoxyphenyl)-2-butanol (**4b**) using Δ P84/A85G *TeSADH*.

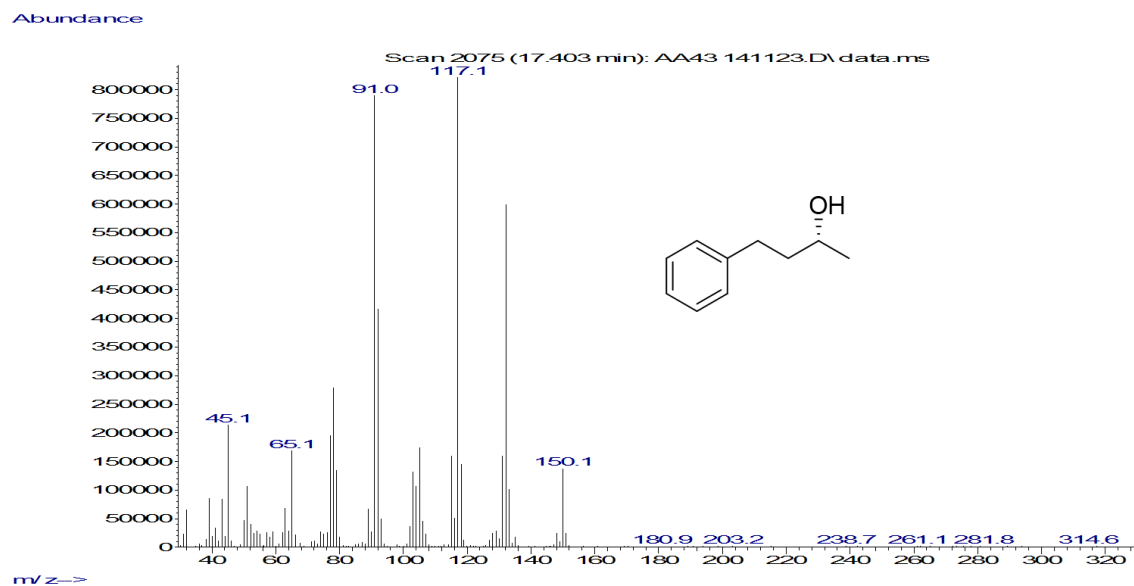


Figure 15. Mass spectrum of the asymmetric reduction product (*R*)-4-phenyl-2-butanol (**5b**) using Δ P84/A85G *Te*SADH.

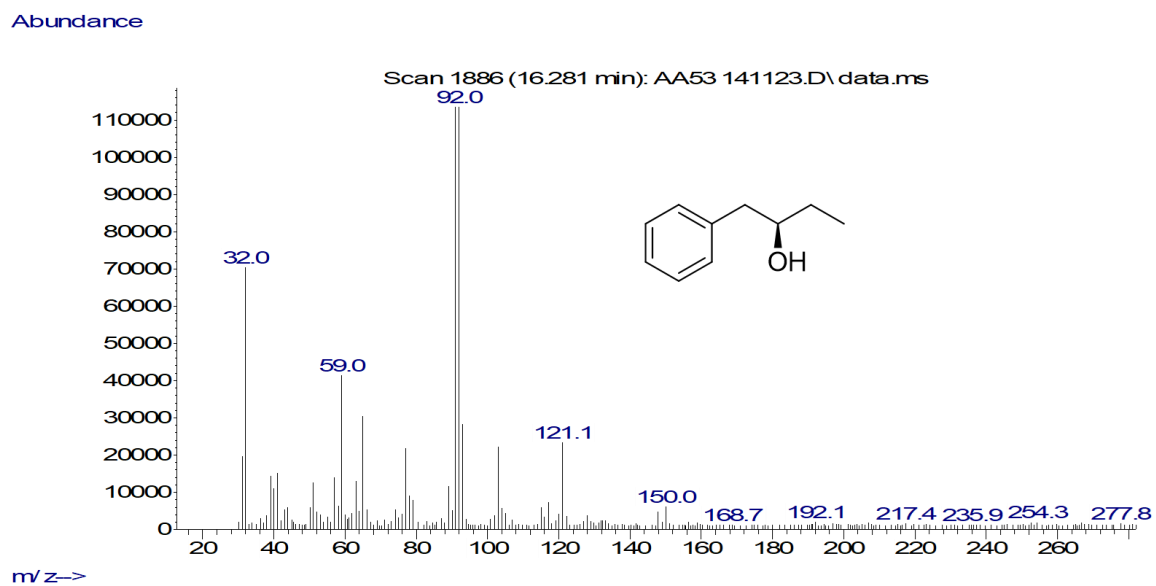


Figure 16. Mass spectrum of the asymmetric reduction product (*R*)-1-phenyl-2-butanol (**6b**) using Δ P84/A85G *Te*SADH.

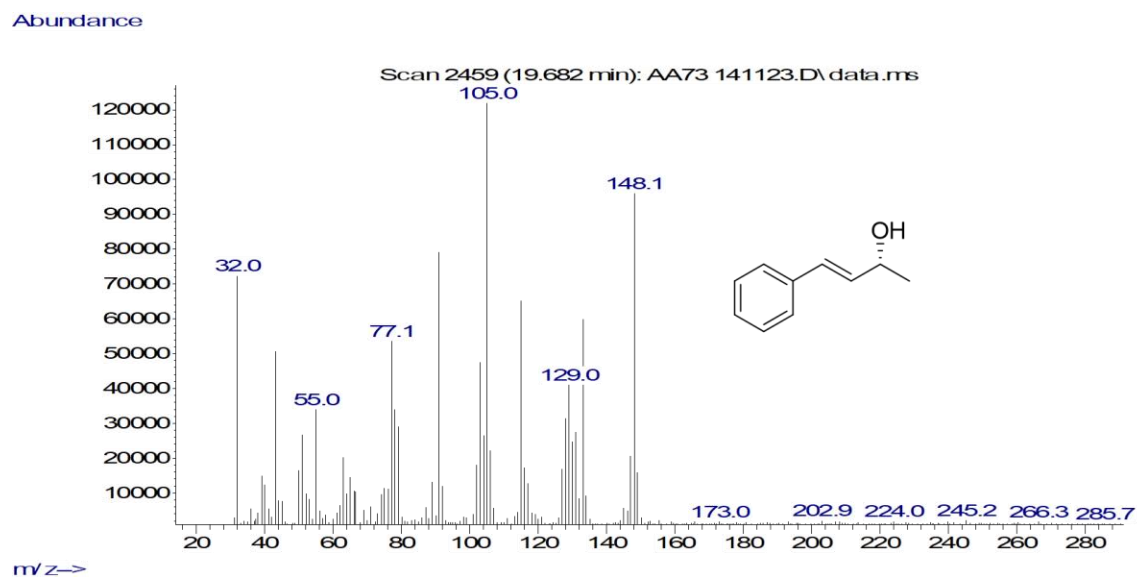


Figure 17. Mass spectrum of the asymmetric reduction product (*R*)-4-phenyl-2-buten-3-ol (**8b**) using Δ P84/A85G TeSADH.

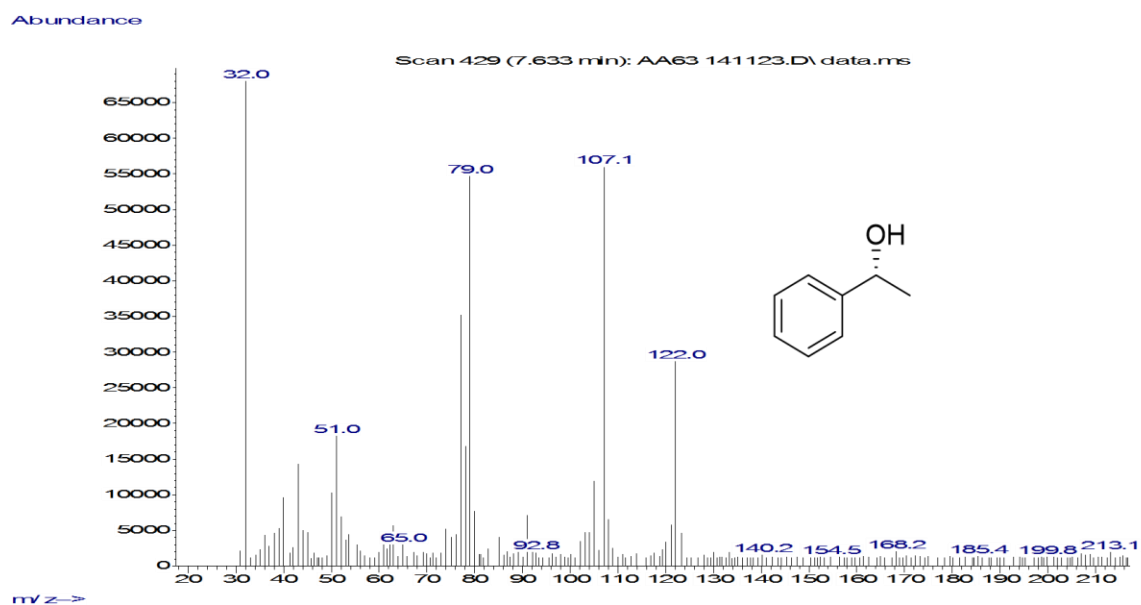


Figure 18. Mass spectrum of the asymmetric reduction product (*R*)-1-phenylethanol (**9b**) using Δ P84/A85G *Te*SADH.

NMR spectra

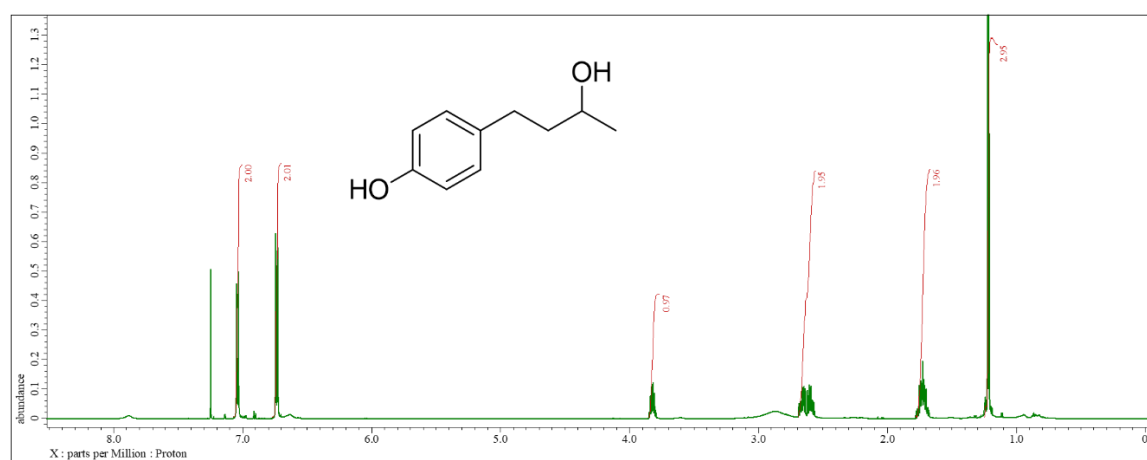


Figure 19. ^1H NMR spectrum of 4-(4'-hydroxyphenyl)-2-butanol (**1b**) produced by reduction of its ketone using NaBH_4 .

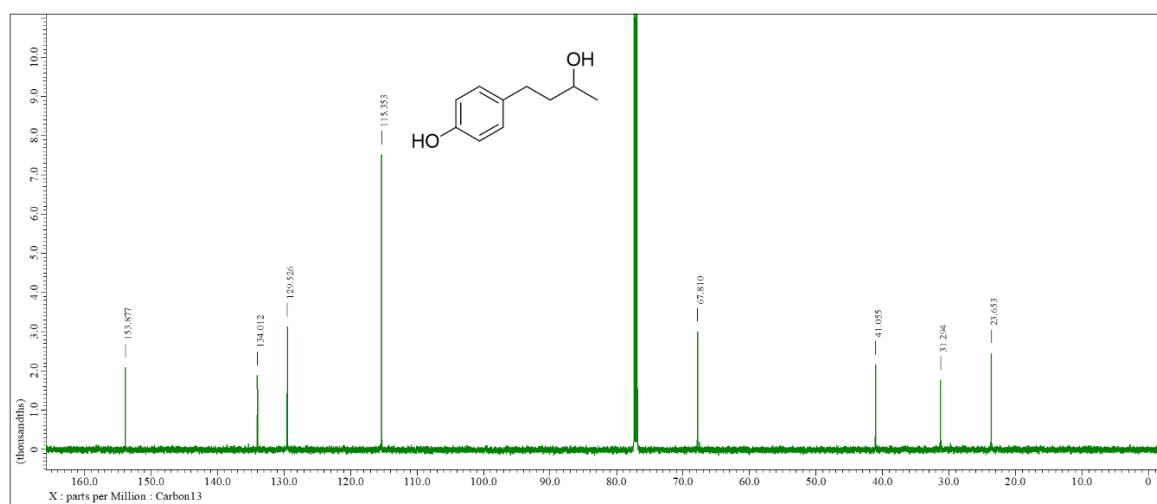


Figure 20. ^{13}C NMR spectrum of 4-(4'-hydroxyphenyl)-2-butanol (**1b**) produced by reduction of its ketone using NaBH_4 .

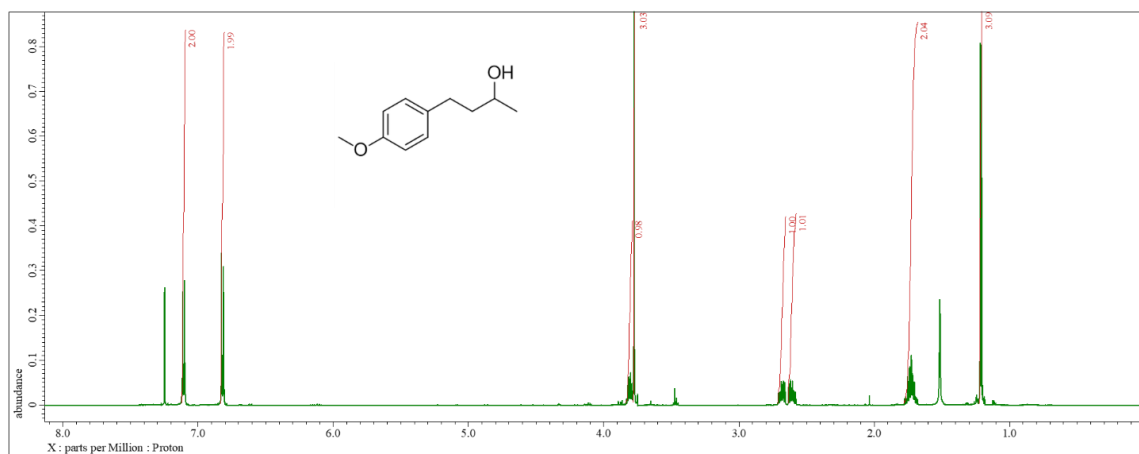


Figure 21. ^1H NMR spectrum of 4-(4'-methoxyphenyl)-2-butanol (**4b**) produced by reduction of its ketone using NaBH_4 .

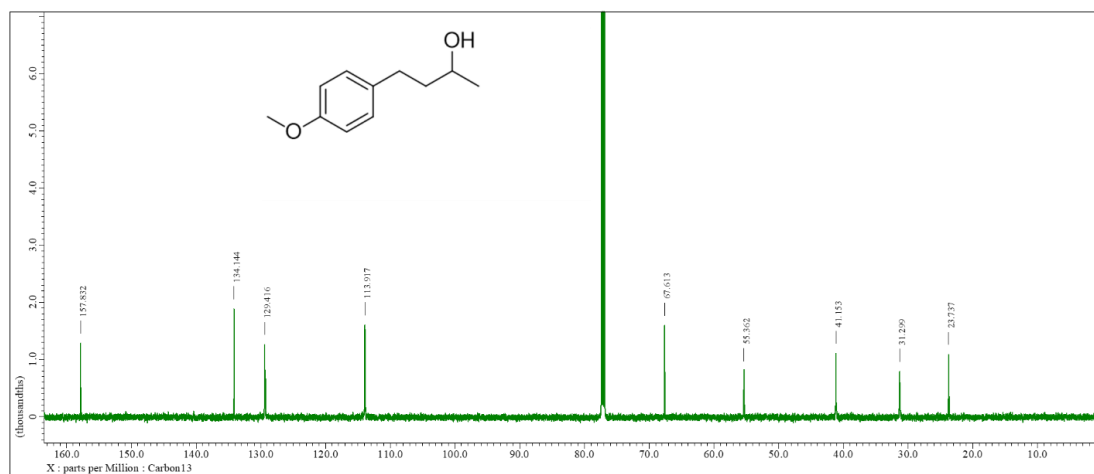


Figure 22. ^{13}C NMR spectrum of 4-(4'-methoxyphenyl)-2-butanol (**4b**) produced by reduction of its ketone using NaBH_4 .

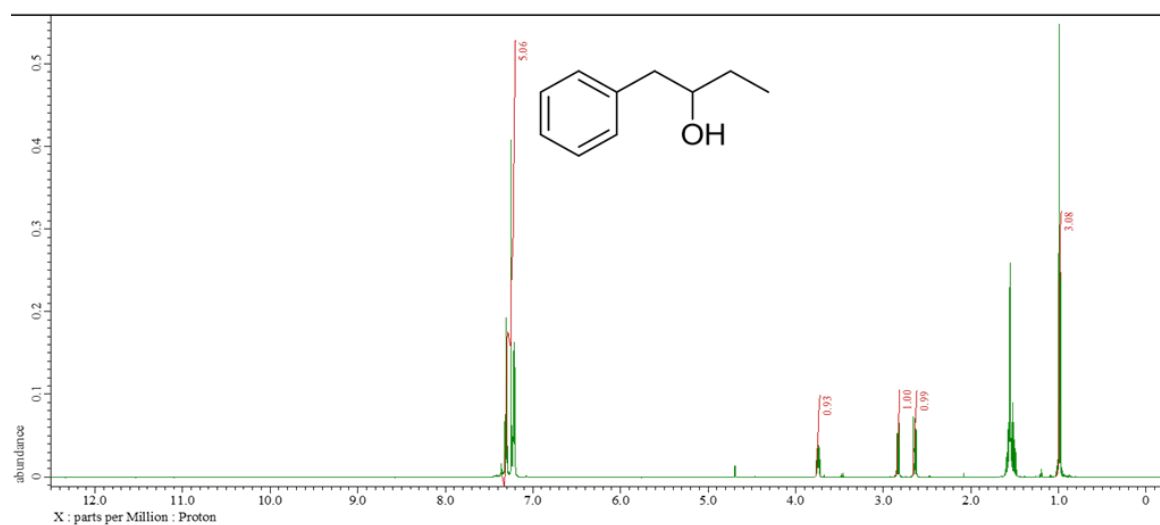


Figure 23. ^1H NMR spectrum of 1-phenyl-2-butanol (**6b**) produced by reduction of its ketone using NaBH_4 .

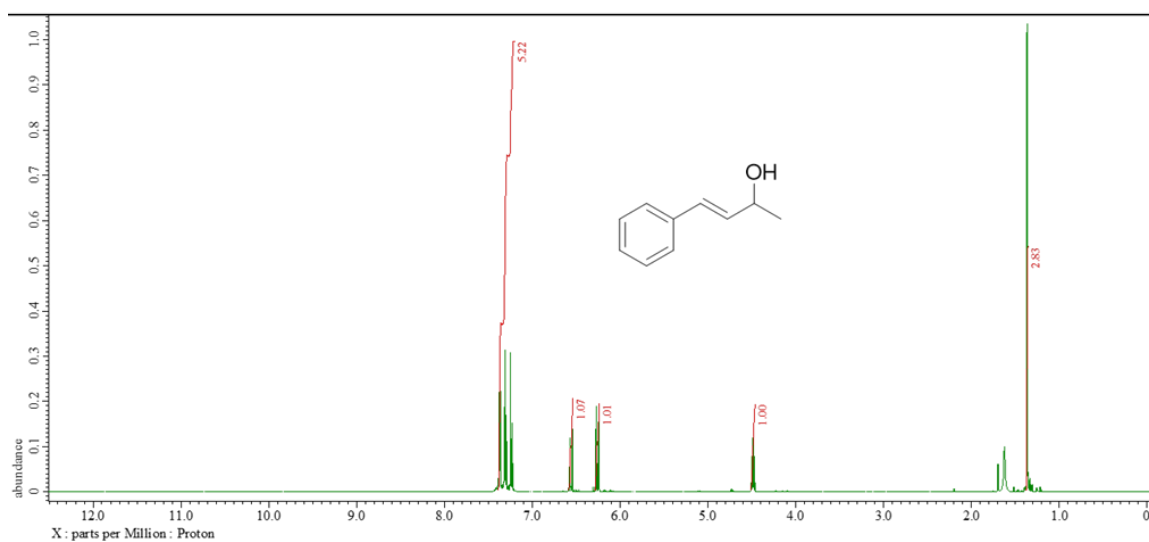


Figure 24. ^1H NMR spectrum of 4-phenyl-2-buten-3-ol (**8b**) produced by reduction of its ketone using NaBH_4 .

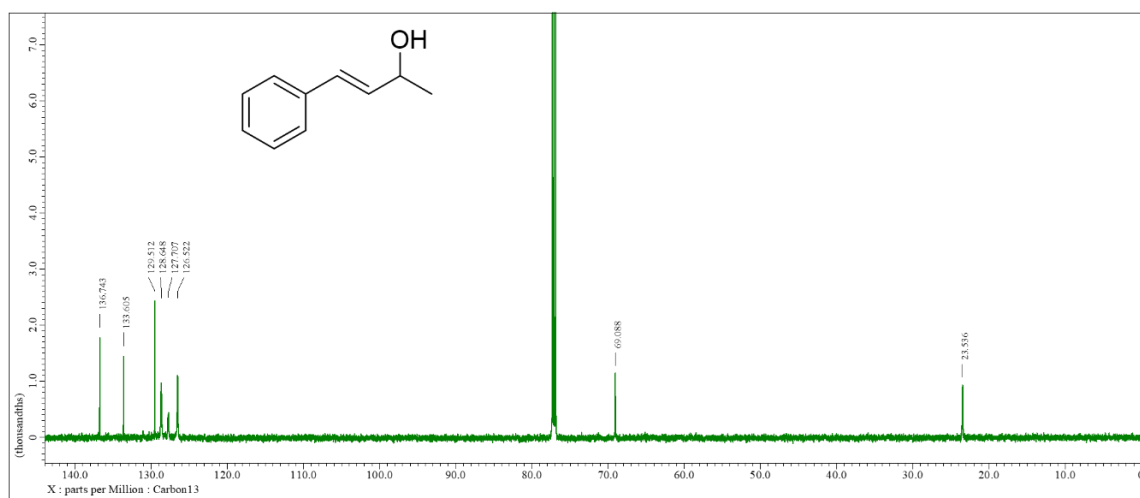


Figure 25. ^{13}C NMR spectrum of 4-phenyl-2-buten-3-ol (**8b**) produced by reduction of its ketone using NaBH_4 .

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M. Biocatalytic asymmetric reduction of prochiral bulky-
bulky ketones. Molecular Catalysis 541, 113099 2023