

**BIOCATALYTIC ASYMMETRIC REDUCTION OF α -
HALOACETOPHENONE ANALOGS USING
THERMOANAEROBACTER PSEUDETHANOLICUS
SECONDARY ALCOHOL DEHYDROGENASE**

BY

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I dedicate this work to my parents whose support and undying love have been a constant propellant in this journey. |

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LIST OF ABBREVIATIONS

ADH	:	Alcohol dehydrogenase
<i>ee</i>	:	Enantiomeric excess
GC	:	Gas Chromatography
CIP	:	Cahn-Ingold Priority
HLADH	:	Horse liver alcohol dehydrogenase
KR	:	Kinetic Resoluion
DKR	:	Dynamic Kinetic Resoluion
LkADH	:	<i>Lactobacillus kefir</i> alcohol dehydrogenase
LsADH	:	<i>Leifsonia</i> sp. alcohol dehydrogenase
LbADH	:	<i>Lactobacillus brevis</i> alcohol dehydrogenase
NMR	:	Nuclear Magnetic Resonance
NADH	:	Nicotinamide-adenine dinucleotide
NADPH	:	Nicotinamide-adenine dinucleotide phosphate
PpYSDR	:	<i>Pseudomonas putida</i> short-chain dehydrogenase
PpKR3	:	<i>Paraburkholderia phymatum</i> short-chain dehydrogenase
TeSADH	:	<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 : Muhammad Abdulrasheed
Thesis Title : Biocatalytic Asymmetric Reduction of α -Haloacetophenone Analogs
Using *Thermoanaerobacter pseudethanolicus* Secondary Alcohol
Dehydrogenase
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Enantiopure 2-halo-1-phenylethanol analogs are important building blocks that could be used for the synthesis of pharmaceuticals, agrochemicals and fine chemicals. This study explores the asymmetric reduction of 2-haloacetophenone analogs to obtain their corresponding optically active 2-halo-1-phenylethanol using secondary alcohol dehydrogenase from *Thermoanaerobacter pseudethanolicus* (*TeSADH*) mutants. Specifically, Δ P84/A85G and P84S/A85G *TeSADH* mutants were examined for the asymmetric reduction of 2-haloacetophenones to generate both enantiomers of optically active halohydrins in high enantioselectivities. The asymmetric reduction of substituted 2-haloacetophenones using the Δ P84/A85G *TeSADH* mutant yielded their corresponding (S)-2-halo-1-phenylethanols with high enantiopurity. Conversely, the P84S/A85G *TeSADH* mutant produced (R)-alcohols when reducing 2-chloro-4'-bromoacetophenone and 2-chloro-4'-chloroacetophenone, while generating the (S)-configured halohydrin from 2-chloro-4'-fluoroacetophenone. These findings contribute to the understanding and application of *TeSADH* in synthesizing optically active compounds.

ملخص الرسالة

الاسم الكامل	:	محمد عبدالرشيد
عنوان الرسالة	:	الإختزال غير المتماثل لمركبات ألفا هالوجين أسيتوفينون باستخدام طفرات في الأنزيمات الحرارية (<i>TeSADH</i>) نازعة هيدروجين الكحول الثانوي
التخصص الرئيسي	:	الكيمياء
التاريخ الدرجة العلمية	:	ديسمبر 2023

يعتبر المتخايل الضوئي النقي لمركبات 2-هالوجين-1-فينيل إيثانول لبنات أساسية تستخدم في اصطناع المواد الصيدلانية والكيمويات الزراعية والمواد الكيميائية الدقيقة. استخدمت هذه الدراسة أن الاختزال غير المتماثل لمركبات ألفا هالوجين أسيتوفينون للحصول على 2-هالوجين-1-فينيل إيثانول الفعال ضوئياً باستخدام نازعات هيدروجين الكحول من طفرات في الأنزيمات الحرارية (*TeSADH*)، وعلى وجه التحديد الطفرات $\Delta P84/A85G$ و $P84S/A85G$. حيث لوحظ أن نواتج التفاعل ذات انتقاء تماثلي عالي.

أظهر الاختزال غير المتماثل لمستبدلات ألفا هالوجين أسيتوفينون باستخدام المتحور $\Delta P84/A85G$ من *TeSADH* مردود عالي من المتخايل الضوئي اس. وبالمقابل، أنتج استخدام المتحور $P84S/A85G$ من *TeSADH* متخايل ضوئي عشكل كحولات ار وذلك من الركازة 2-كلورو-4-برومو أسيتوفينون و2-كلورو-4-كلورو أسيتوفينون؛ بينما أنتج كحولات اس وذلك مع الركازة 2-فلورو-4-برومو أسيتوفينون. ساهمت هذه النتائج في فهم وآلية عمل *TeSADH* في تصنيع المركبات المتخايلة ضوئياً.

CHAPTER 1

INTRODUCTION AND LITERATURE RIVIEW

1.1 Background

Optically active alcohols are essential building blocks in industries such as pharmaceutical, fragrance and agrochemical industries. These alcohols are typically obtained in the industry through kinetic resolution (KR), where a stereoselective catalyst selectively converts only one of the enantiomers and leaves the other one untouched. Hence, KR is in theory limited to a 50% yield of enantiopure alcohol, which makes it an unattractive approach.¹ Another promising approach is dynamic kinetic resolution (DKR), which is capable of achieving a theoretical yield up to 100%.² This approach features an in-situ racemization of the alcohol, which is accompanied by a KR reaction. However, a major challenge encountered in DKR lies in the complexity of orchestrating more than one catalyst to function simultaneously within a single reaction vessel. Consequently, asymmetric reduction of prochiral ketones presents a straight forward approach towards getting optically active alcohols, which can be achieved through the use of either a chemocatalyst or a biocatalyst. Biocatalysts have recently attracted a lot of attention owing to their superior characteristics. They tend to be more stereo-, regio- and chemo-selective, less expensive, work in mild condition and importantly, they are potentially more environmentally friendly than organometallic and organocatalysts.³

Alcohol dehydrogenases (ADHs) are essential enzymes with significant potentials in biocatalytic applications. These versatile enzymes catalyze the reversible reduction of aldehydes or ketones to their corresponding alcohols by relying on nicotinamide-adenine dinucleotide (NADH) or its phosphate (NADPH) as hydride sources, making them valuable tools for the synthesis of important compounds (**Figure 1**). ADHs exhibit high substrate specificities (i.e., limited substrate scope) and excellent regio- and stereo-selectivities, enabling the production of enantiopure products. It is of great interest to expand their specificities to non-natural substrates. Moreover, the ability to modulate ADHs activities through protein engineering strategies enhances their applicability in tailored biotransformation processes such as the switch of natural enantioselectivity. This is of great interest because only a few ADHs show the *anti*-Prelog enantioselectivity. As a result, the exploitation of ADHs in biocatalysis holds great promise for the sustainable production of fine chemicals and pharmaceuticals, offering greener and more efficient synthetic routes. ADHs have been extensively used in interconversion between aldehydes and ketones and their corresponding alcohols.⁴ There are many ADHs that are commercially available such as yeast ADH (YADH), horse liver ADH (HLADH), and *Thermoanaerobium brockii* ADH (TbADH).⁵⁶

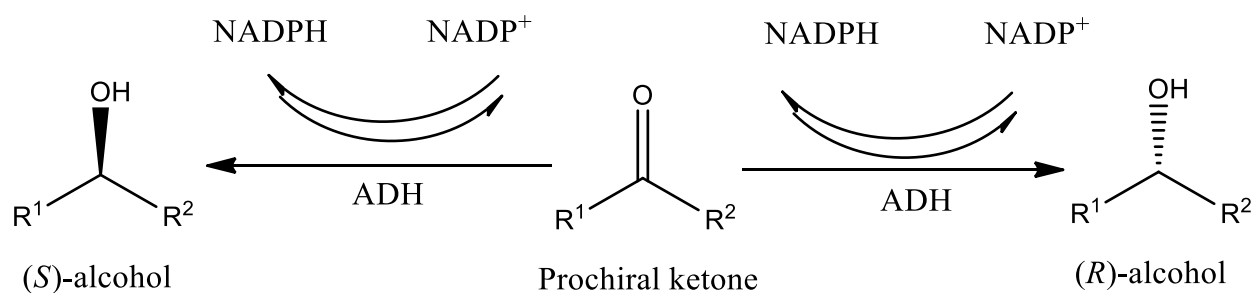


Figure 1. Asymmetric reduction of prochiral ketones using ADHs. R¹ exhibits higher Cahn-Ingold-Prelog priority than R².

Optically active alcohols that are generated by asymmetric reduction of α -haloacetophenones are important building blocks for pharmaceutical chemicals.⁷ For example, 2-chloro-1-(3,4-dichlorophenyl)ethanol is crucial in the synthesis of sertraline, an antidepressant drug.⁸ In this work, we proffer the use of *Te*SADH mutants in the asymmetric reduction of α -haloacetophenone analogs (**Figure 2**).

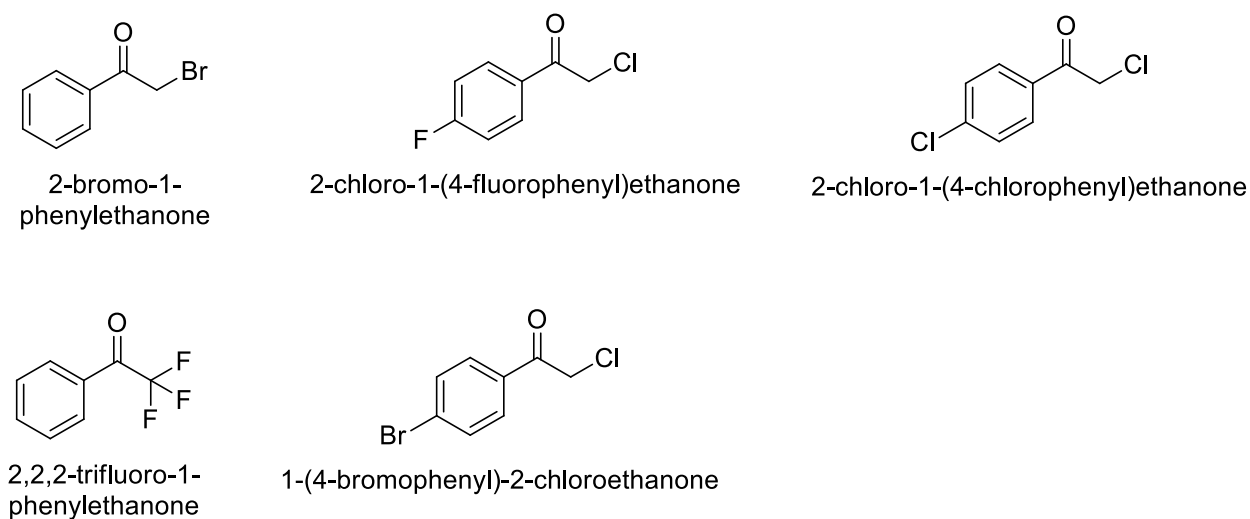
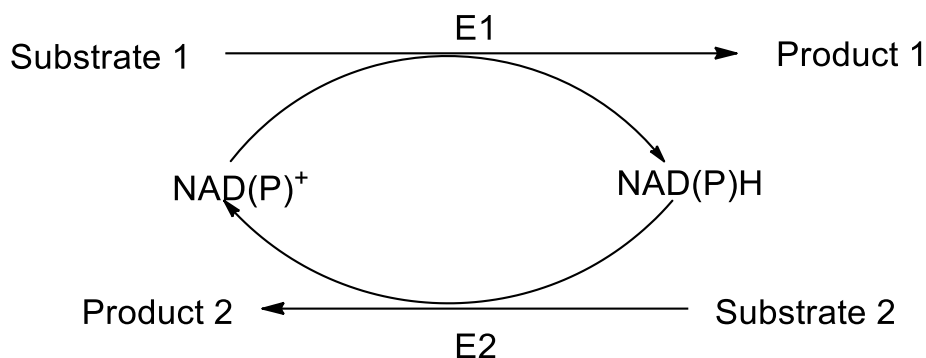


Figure 2. List of substrates reduced by various *Te*SADH mutants in the current study.

1.2 Alcohol Dehydrogenases in Asymmetric Redox Reactions.

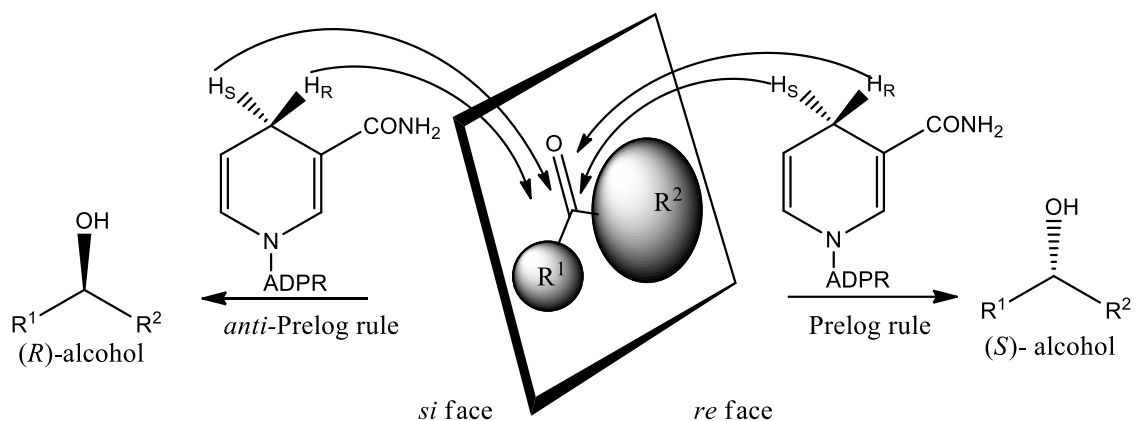
Alcohol dehydrogenases (ADHs, EC 1.1.1.X, X = 1 or 2) catalyze the reversible asymmetric reduction of ketones and aldehydes to their corresponding alcohols. This transformation requires hydride from nicotinamide adenine dinucleotide (NADH) or its phosphate (NADPH) for reduction reactions, while their oxidized forms (NAD^+ or NADP^+) serve as co-enzymes for oxidation reactions. These co-enzymes can be regenerated through either an enzyme-coupled approach, for example; using glucose dehydrogenase, glucose and gluconolactone, or through substrate-coupled approach, for example using acetone and 2-propanol co-substrates (**Scheme 1**).



Scheme 1. Enzymatic regeneration of cofactors (for substrate-coupled approach, the two enzymes are the same, $\text{E1} = \text{E2}$; for enzyme-coupled regeneration, E1 and E2 represent two different enzymes).

There are four possible ways of delivering the hydride from the co-enzyme to a prochiral ketones in ADH-catalyzed asymmetric reduction. Either the pro-(*R*) or pro-(*S*) of the co-enzyme can be delivered to the *si* face or the *re* face of the ketone (**Scheme 2**). ADHs that bind prochiral ketones in such a way that the hydride from co-enzyme is being delivered

through the *re* face of the ketones are said to follow Prelog's rule, thereby producing (*S*)-alcohols when the higher Cahn–Ingold–Prelog (CIP) priority is exhibited by the large substituent of the prochiral ketone. However, in cases where the smaller group on the ketone exhibits higher CIP priority, (*R*)-alcohol is produced. Examples of Prelog ADHs are YADH, HLADH, *Tb*ADH and *Te*SADH. There are only few examples of ADHs that do not follow the Prelog's rule (termed *anti*-Prelog), which include *Lactobacillus brevis* ADH (*Lb*ADH)⁹ and *Leifsonia* sp. ADH (*Ls*ADH)¹⁰.



Scheme 2. Stereochemical outcome of ADH-catalyzed asymmetric reduction. R^2 exhibits higher Cahn-Ingold-Prelog priority than R^1 . ADPR = adenosine diphosphoribose.

1.2.1 Secondary ADH from *Thermoanaerobacter pseudoethanolicus*

Secondary ADH from *Thermoanaerobacter pseudoethanolicus* ATCC 33223 (*Te*SADH, EC 1.1.1.2) was isolated by Wiegel and Ljungdahl.¹¹ *Te*SADH, as described by Ljungdahl and coworkers¹² is identical to the commercially available *Thermoanaerobacter brockii* secondary ADH.^{13,14} *Te*SADH is a robust enzyme that is thermally stable¹⁵ and tolerates high concentrations of organic solvents, up to more than 30 % (v/v) 2-propanol.¹⁶ Just like

most ADHs, *Te*SADH also displays Prelog's stereopreference.¹⁷ The active site model of *Tb*SADH (**Figure 3**) was proposed by Keinan and coworkers, which suggests that *Tb*SADH features two hydrophobic pockets that differ in sizes and affinities towards alkyl groups of ketones and secondary alcohol substrates.^{5,18}

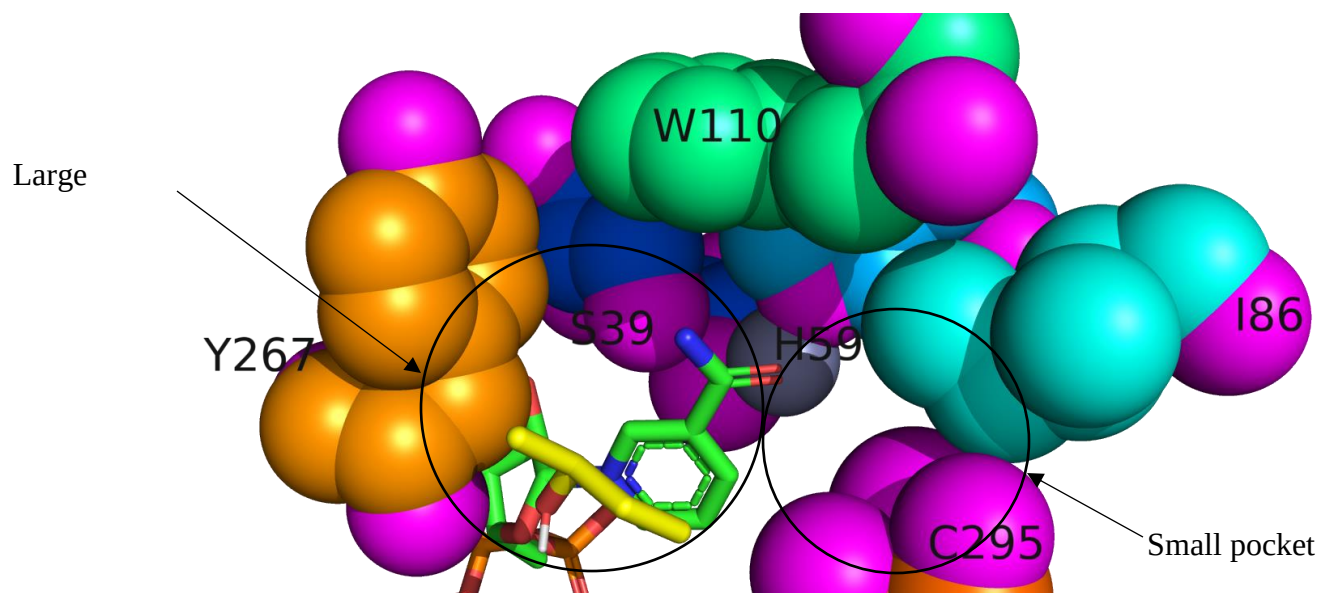
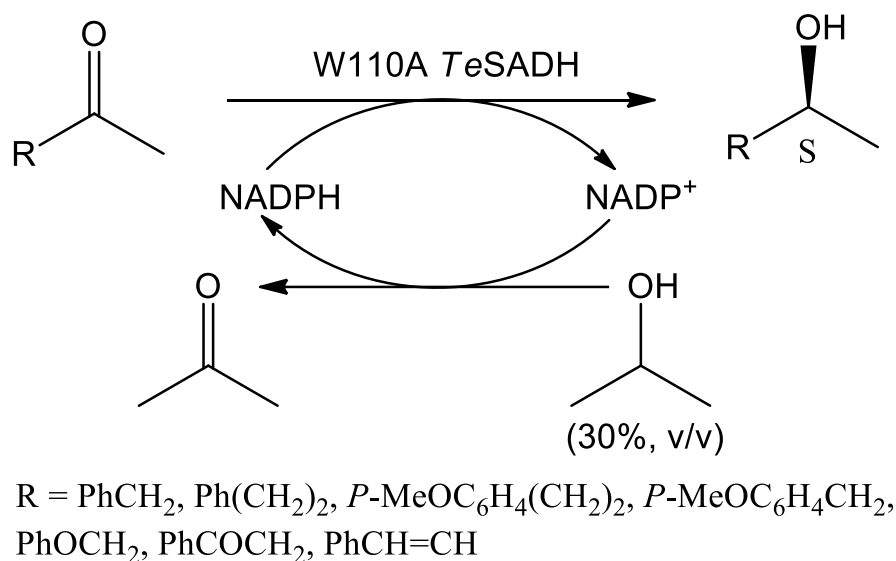


Figure 3. The structure of the active site of *Tb*SADH.

Due to the interesting features of *Te*SADH, several research groups have contributed to the substrate scope of *Te*SADH or *Tb*SADH.^{19–24} Although the smaller pocket shows higher binding affinity towards alkyl groups of the prochiral ketone, it cannot accommodate substrates that are larger than isopropyl such as aryl rings. *Te*SADH and *Tb*SADH are restricted by a limited substrate scope, confining their activity primarily to smaller substrates like aliphatic ketones and their respective alcohols. Extensive efforts have been made in order to make its substrate scope wider through protein engineering. According to the crystal structure of *Tb*SADH-2-butanol complex reported by Shoham and co-workers (PDB 1BXZ)²⁵, W110, S39, Y267, and M285 are amino acids that line the large pocket

while A85, I86, L294 and C295 are lining the smaller pocket. Phillips and coworkers described a *TeSADH* mutant that accepted aryl-ring-containing ketones and their corresponding alcohols. This was achieved by expanding the larger pocket through a single point mutation of W110A. Several aryl-ring-containing prochiral ketones have been reduced to their (*S*)-alcohols by W110A *TeSADH* (**Scheme 3**).^{26,27}

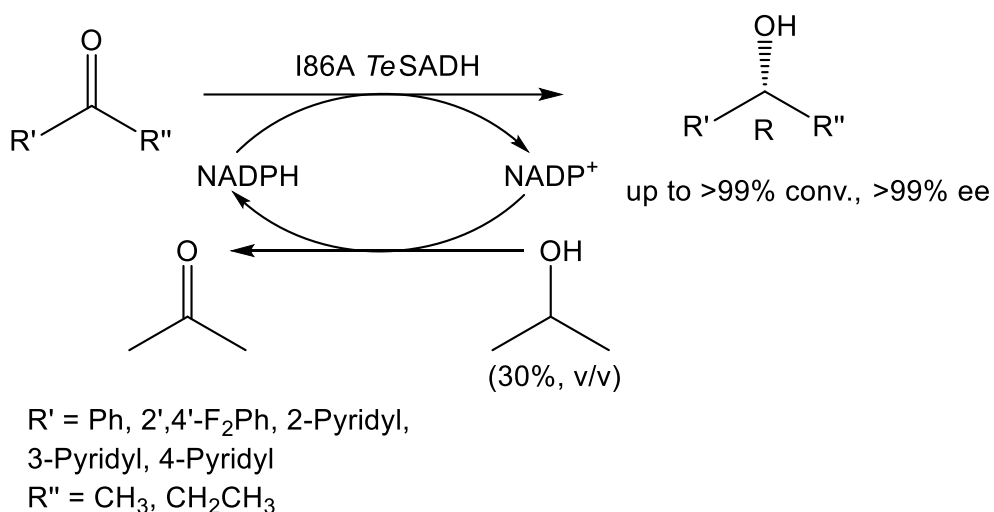


Scheme 3. Asymmetric reduction of aryl-ring-containing ketones by using

W110A *TeSADH*.

Another important mutant of *TeSADH* is the I86A *TeSADH*. This mutant accepts acetophenone and its analogs to produce the corresponding (*R*)-alcohols (**Scheme 4**).¹⁷ This interesting mutant switches the stereopreference of the wild-type enzyme to the highly desirable *anti*-Prelog stereopreference by a single point mutation. Nevertheless, I86A *TeSADH* could not accommodate substituted acetophenones as substrates. Thus, further mutations were carried out to expand the smaller pocket; these mutants are I86A/C295A,

A85G/I86A/C295A, I86A/V115A/C295A and I86A/T153A/C295A, which accommodate substituted acetophenones.^{28,29} Other mutations on *Te*SADH have been reported to carry out asymmetric reduction of difficult to reduce bulky-bulky ketones^{30,31} and 2-tetralones.³² Due to the heightened interest in the use of biocatalysts, there is still need to expand the substrate scope for *Te*SADH further and to tune its stereopreference.



Scheme 4. Asymmetric reduction of phenyl and heteroaryl ketones using I86A *Te*SADH.

1.3 Asymmetric Reduction of α -Haloacetophenone Analogs.

Enantiopure halohydrins are essential synthons of a large variety of compounds such as flavorants, agricultural chemicals, natural products and pharmaceutical products (**Figure 4**). For example, (*S*)-2-chloro-1-(2',4'-dichlorophenyl)ethanol can be used in the synthesis of ticonazole, which is used to treat vaginal candidiasis and superficial fungal infections of the skin,^{33,34} (*R*)-2-chloro-1-phenylethanol can be used for the synthesis of mirabegron (β -

3 adrenergic receptor agonist),³⁵ (S)-2-chloro-1-(3,4-difluorophenyl)ethanol can be used to synthesize ticagrelor (a receptor antagonist).³⁶

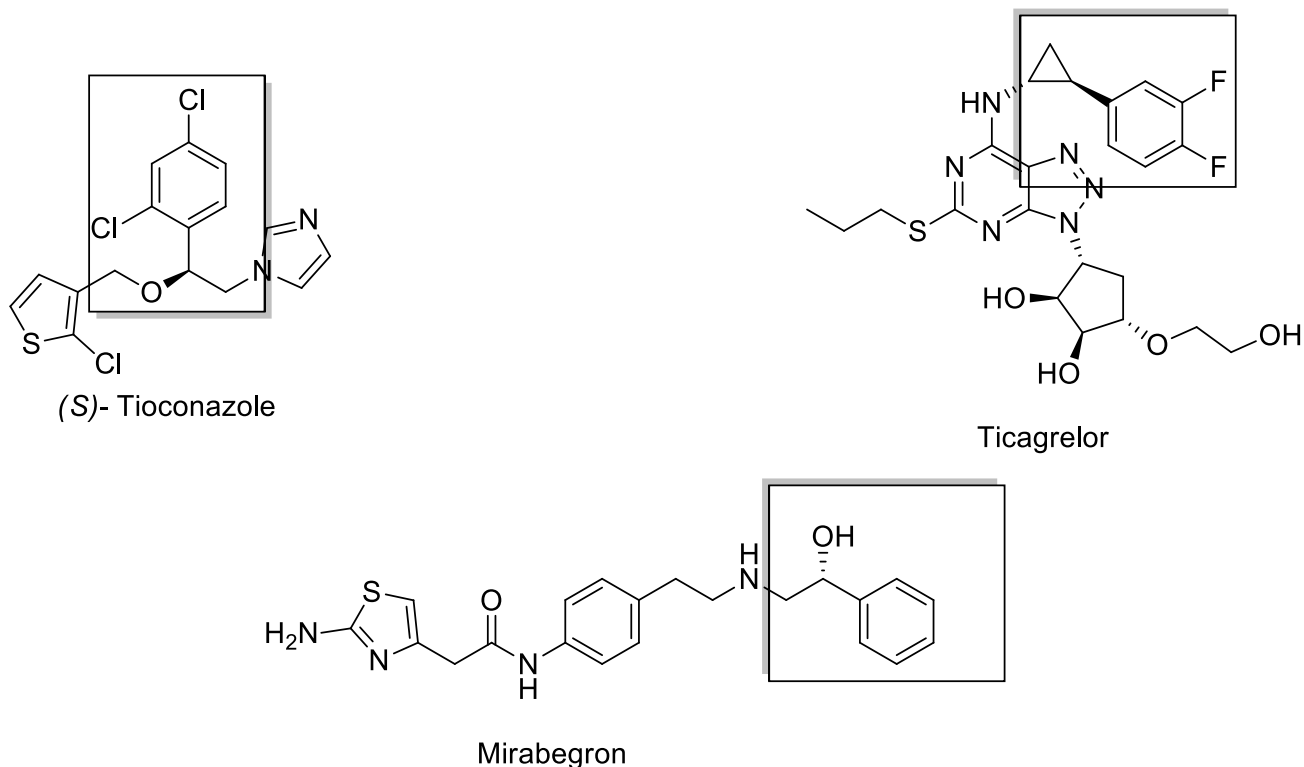
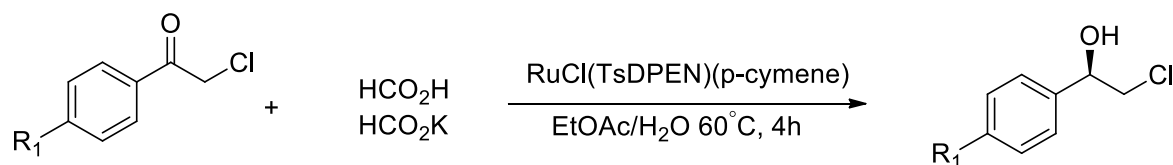


Figure 4. Examples of pharmaceutical products with chiral halohydrins as building blocks.

There is great interest in the synthesis of enantiopure halohydrins because they can be easily converted to various synthetic intermediates such as epoxides, azido alcohols, diols, hydroxyl nitriles and amino alcohols. Halohydrins have been synthesized using chemo- and biocatalytic methods through either kinetic resolution of their racemates or asymmetric reduction of their corresponding α -haloacetophenones.

Yuki and coworkers reported the reduction of α -substituted acetophenones using bifunctional oxo-tethered ruthenium(ii) catalysts producing the corresponding (*R*)-2-

bromo and (*R*)-2-chlorohydrins with an ee of between 82-96% (**Scheme 5**).³⁷ However, this method suffers from formation of by-products such as diols, carbonates and epoxides. Biocatalysts have the advantage of working under mild conditions in aqueous media, and perform asymmetric reduction of prochiral ketones with higher enantioselectivities. These eliminate the formation of by-products and thus make it preferable for the delicate asymmetric reduction of α -substituted acetophenones.



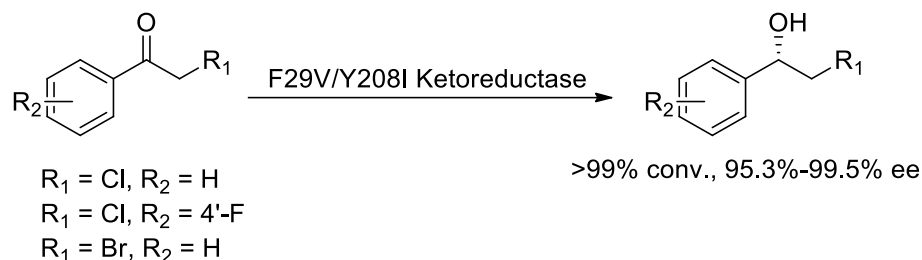
TsDPEN = N-(p-toluenesulfonyl)-1,2-diphenylethylenediamine)

R ₁	%ee
H	95
Cl	93
OMe	94
Ph	95
F	92
I	79
NMe ₂	87
	96

Scheme 5. Selective asymmetric transfer hydrogenation of α -substituted acetophenones with bifunctional oxo-tethered ruthenium(ii)catalysts.

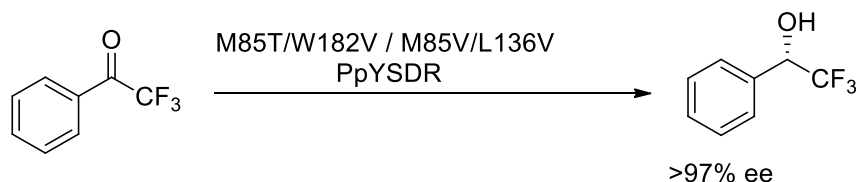
You and his coworkers reported the synthesis of enantiopure *anti*-Prelog halohydrins using a ketoreductase from *Candida glabrata*.³⁸ They formed six mutants by replacing F92 with I, S, T, A, V, and L. All these mutants displayed a switched enantioselectivity giving the corresponding (*S*)-alcohol (i.e., *anti*-Prelog mode) as opposed to the wild type enzyme.

They performed a double mutation by changing Y208 to six other smaller groups, while F208V served as the parent mutant. F29V/Y208I afforded the *anti*-Prelog product with the best conversion and enantiomeric excess (**Scheme 6**).



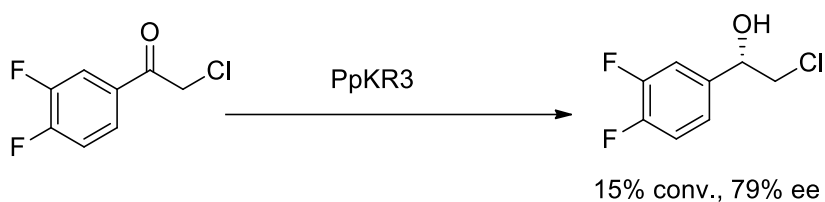
Scheme 6. Asymmetric reduction of α -haloacetophenone analogs using F29V/Y208I mutant of a ketoreductase.

More so, Yu and coworkers carried out a site-directed mutagenesis on M85, L136, W182 and M187 active sites of an NADH-dependent short-chain dehydrogenase/reductase from *Pseudomonas putida* (PpYSDR).⁷ The resultant mutants were shown mostly to have *anti*-Prelog stereopreference different from the wild-type enzyme in the asymmetric reduction of halogenated acetophenones. For example, M85T/W182V and M85V/L136V PpYSDR afforded (*S*)-2,2,2-trifluoro-1-phenylethanol from its corresponding prochiral ketone in high optical purity (**Scheme 7**).



Scheme 7. Production of (*S*)-2,2,2-trifluoro-1-phenylethanol using M85V/L136V PpYSDR.

As aforementioned, only few wild-type enzymes have been reported to follow *anti*-Prelog rule in asymmetric reduction of α -haloacetophenones. Among the few examples is a report by Xu and his coworkers.³⁹ They reported four wild-type enzymes that were able to asymmetrically reduce α -chloroacetophenone to (S)-2-chloro-1-phenylethanol with excellent conversion and optical yield. More so, Wang and coworkers identified an NADH-dependent short-chain dehydrogenase (PpKR3) from *Paraburkholderia phymatum*, which converted 3',4'-difluoro-2-chloroacetophenone to its corresponding (S)-halohydrin (**Scheme 8**).⁴⁰



Scheme 8. Asymmetric reduction of 3',4'-difluoro-2-chloroacetophenone using PpKR3.

CHAPTER 2

OBJECTIVES OF THE STUDY

Although optically active α -halohydrins are important in the synthesis of essential chemicals, reports of ADHs that act as catalysts in such transformations are few. In this work, several mutants of *TeSADH* are tested for the symmetric reduction of α -haloacetophenones. These mutants include A85G/186A/C295A, Δ P84/A85G, P85S/I86A, and A85G/186A/Q101A, which were identified based on previous reports that indicate the possibility for these mutants to accommodate the proposed α -haloacetophenones, and reduce them in *anti*-Prelog mode.

The main objectives of the research were to:

- i- Use A85G/186A/C295A, Δ P84/A85G, P85S/I86A, and A85G/186A/Q101A mutants of *TeSADH* in the asymmetrical reduction of various substituted α -haloacetophenones.
- ii- Confirm the chemical identity of the produced alcohols using NMR, IR, and GC-MS.
- iii- Determine the stereochemical outcome of the produced alcohols by chiral GC and by optical rotation.

CHAPTER 3

EXPERIMENTAL PROCEDURES

3.1 General

Capillary gas chromatographic measurements were performed on a GC equipped with a flame ionization detector and an HP chiral-20B column (30 m, 0.32 mm [i.d.], 0.25 μ m film thickness) using Helium as the carrier gas. Nuclear magnetic resonance (NMR) spectra were recorded on a 600 or 400 MHz spectrometer at 600 or 400 MHz (^1H) and at 150 or 100 MHz (^{13}C) at room temperature using the solvent peak as an internal standard. Commercial grade solvents were used without further purification. NADP^+ and all substrates were used as purchased from commercial sources.

3.2 Gene Expression and Purification of *Te*SADH Mutants

I86A, A85G/186A/C295A, P84S/186A, and Δ P84/A85G mutants of *Te*SADH were constructed, expressed and purified as previously reported.⁴¹

3.3 Reduction of Ketones Using NaBH₄

Racemic alcohols were produced from their corresponding ketones using NaBH₄ as described in the literature previously with minor modifications.⁴² To a solution of ketone (1.0 mmol) in methanol (5.0 mL) incubated in an ice bath, sodium borohydride (2.0 mmol) was added. When the reduction was complete (confirmed by TLC) a few drops of 1.0 M HCl were added. The solvent was evaporated then distilled water (10 mL) was added, and the solution was extracted with diethyl ether (3 × 10 mL). The organic layers were combined and dried over Na₂SO₄. The solvent was evaporated and the residue was subjected to column chromatography (silica gel) using mixtures of hexane/ethyl acetate as eluents when the conversion was not complete.

3.4 Asymmetric Reduction of Ketones Using *TeSADH* Mutants

The enzymatic reduction of ketones were carried out by using an NADPH recycle system as described previously.² Reactions were conducted in 1.5-mL reaction tubes consisting of α -haloacetophenones (10mM), NADP⁺ (1.0 mg), Tris-HCl buffer solution (700 μ L, pH 7.0), 2-propanol (300 μ L), and *TeSADH* mutant (1.6 μ M, final concentration in reaction mixture). The mixture was shaken at 180 rpm and 50 °C for 14 h. The reaction progress was monitored using thin layer chromatography. The mixture was then extracted with diethyl ether (500 μ L × 2). The organic layer was dried with sodium sulfate, and then concentrated to dryness.

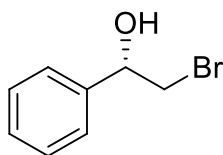
3.5 GC Analysis (chiral)

The percent *ee* values of the alcohol products were determined using an Agilent 7890A capillary gas chromatography (GC), equipped with a flame ionization detector and an HP chiral-20B column (30 m, 0.32 mm [i.d.], 0.25 μ m film thickness) using Helium as the carrier gas. The temperature program was set as: oven 100 °C (initial, hold time 10 min) to 200 °C (final, hold time 30 min) at 5 °C/min rate; injector 220 °C, detector 230 °C; and 15 mL/min Helium flow. The injection volume was 5.0 μ L splitless.

3.6 GC-MS Analysis (non-chiral):

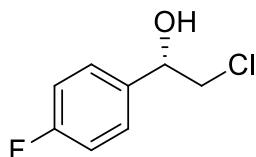
GC-MS analysis was conducted using Agilent GC model 7890A combined with Agilent 5975C Inert MSD with Triple-Axis Detector using capillary column [(5% phenyl)-methylpolysiloxane, 30 m $\times$ 320 μ m $\times$ 0.25 μ m]. The following temperature program was used for GC: oven 70 $^{\circ}$ C (initial, hold time 5 min) to 290 $^{\circ}$ C (final, hold time 5 min) at 5 $^{\circ}$ C/min rate; injector 250 $^{\circ}$ C; detector 300 $^{\circ}$ C, and 15 mL/min Helium flow. The injection volume was 1.0 μ L with split ratio 10:1.

3.7 Characterization of Products



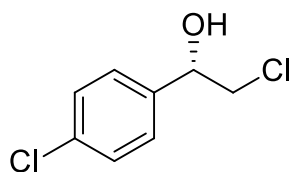
(*rac*)-2-Bromo-1-phenylethanol [(*rac*)-**1b**]: $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ = 7.39-7.33 (m, 5H), 4.94 (dd, J = 9.0, 3.3 Hz, 1H), 3.65 (dd, J = 10.5, 3.3 Hz, 1H), 3.55 (dd, J = 6.48, 9.04 Hz, 1H) ppm. $^{13}\text{C-NMR}$ (151 MHz, CDCl_3) δ 140.3, 128.7, 128.5, 126.0, 73.8, 40.3.

(*S*)-2-Bromo-1-phenylethanol [(*S*)-**1b**]: $[\alpha]_D^{20}$ = +31.2 (c 0.5, CHCl_3) >99 % ee. [Lit +39.8 (c 1.2, CHCl_3), 91% ee *S*].⁴³



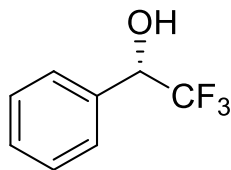
(*rac*)-2-Chloro-1-(4-fluorophenyl)ethanol [(*rac*)-**2b**]: $^1\text{H-NMR}$ (600 MHz, CDCl_3): δ = 7.38-7.35 (m, 2H), 7.09-7.05 (m, 2H), 4.89 (dd, J = 8.8, 3.4 Hz, 1H), 3.71 (dd, J = 11.2, 3.4 Hz, 1H), 3.62 (dd, J = 11.3, 8.8 Hz, 1H) ppm. $^{13}\text{C-NMR}$ (151 MHz, CDCl_3) δ 161.9, 135.7, 127.9, 115.9, 73.6, 51.0.

(*S*)-2-Chloro-1-(4-fluorophenyl)ethanol [(*S*)-**2b**]: $[\alpha]_{\text{D}}^{25}$ = +34.77 (c 0.29, CHCl_3) 99 % ee. [Lit. +52.8 (c 1.0, CHCl_3), 98.4 ee *S*].⁴⁴



(*rac*)-2-Chloro-1-(4-chlorophenyl)ethanol [(*rac*)-**3b**]: $^1\text{H-NMR}$ (600 MHz, CDCl_3) δ 7.35-7.25 (m, 4H), 4.87 (dd, J = 8.7, 3.4 Hz, 1H), 3.71 (dd, J = 11.2, 3.4 Hz, 1H), 3.59 (dd, J = 11.3, 8.8 Hz, 1H), $^{13}\text{C-NMR}$ (151 MHz, CDCl_3) δ 138.4, 134.3, 128.9, 127.5, 73.4, 50.8.

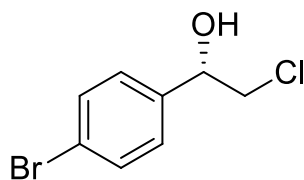
(*R*)-2-chloro-1-(4-chlorophenyl)ethanol [(*R*)-**3b**]: $[\alpha]_{\text{D}}^{25}$ = -4.15 (c 0.2, CHCl_3) 94 % ee. [Lit. +27.2 (c = 3.45 CHCl_3), 86 % ee *S*].⁴⁵



(*rac*)-2,2,2-Trifluoro-1-phenylethanol [(*rac*)-**4b**]: $^1\text{H-NMR}$ (600 MHz, CDCl_3) δ 7.48-7.40 (m, 5H), 5.02 (q, J = 6.8 Hz, 1H), 2.24 (s, 1H). $^{13}\text{C-NMR}$ (151 MHz, CDCl_3) δ 134.2 (Cq),

129.6 (CH), 128.7 (CH), 127.6 (CH), 124.4 (q, $J_{\text{CF}} = 282$ Hz, Cq), 72.8 (q, 2 $J_{\text{CF}} = 32$ Hz, CH-OH) ppm.

(S)-2,2,2-Trifluoro-1-phenylethanol [(S)-**4b**]: $[\alpha]_{\text{D}}^{25} = +11.3$ (c 0.22, CHCl_3). [Lit. -5.60 (c 1.16, CHCl_3), 22% ee *R*].⁴⁶



(R)-2-Chloro-1-(4-chlorophenyl)ethanol [(R)-**5b**]: $[\alpha]_{\text{D}}^{25} = -5.8$ (c 0.26, CHCl_3). [Lit. -39.4 (c 1.0, CHCl_3), 99.6% ee *R*].⁴⁵

CHAPTER 4

RESULTS AND DISCUSSION

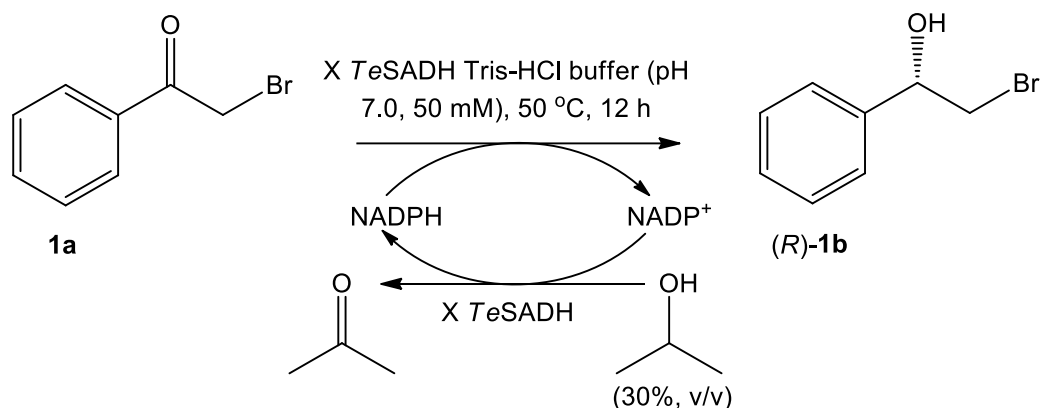
In this study, we constructed four mutants of *TeSADH* namely, I86A, A85G/I86A/C295A, P84S/I86A, and Δ P84/A85G to carry out the asymmetric reduction of α -haloacetophenone analogs which have never been reported to be reduced using *TeSADH*. These mutants were designed to expand the smaller binding pocket of this enzyme as well as to break the rigidity (conferred by proline) of the loop lining the active site.³¹ A85, I86, and C295 line the small pocket of *TeSADH*, and thus mutations at these sites with sterically less demanding amino acids is expected to improve the substrate scope of *TeSADH*. These point mutations were proven before to reduce acetophenone analogs in *anti*-Prelog mode.^{47–49}

To evaluate the performance of these *TeSADH* mutants, we conducted reduction reactions using 2-bromoacetophenone (**1a**) as the substrate. The reactions were carried out in Tris-HCl buffer solutions (pH 7.0, 50 mM) containing 2-propanol (30% v/v), which served as both a co-substrate for NADPH regeneration and a co-solvent to enhance the solubility of phenyl-ring-containing hydrophobic substrates, which are sparingly soluble in aqueous media. Reactions under slightly acidic or basic pH conditions resulted in formation of by-products including the 1-phenyl-1,2-ethanediol and 1-phenylethanol.

All tested mutants successfully accepted **1a** as a substrate, affording (*S*)-2-bromo-1-phenylethanol [(*S*)-**1b**] with excellent conversion and enantioselectivity (>99%), except

when using I86A, which resulted in a lower conversion (71%), **Table 1**. Interestingly, the production of (*R*)-alcohol, note the inverted Cahn-Ingold-Prelog priority for all substrates reported in the current study, contradicted our expectations based on the design of these *TeSADH* mutants. These mutants were intended to expand the smaller pocket of *TeSADH* and should have resulted in inversion of the enzyme's stereopreference. Our findings suggest that the presence of 2-bromo group in **1a** is crucial for its proper orientation in the active site, allowing a *re*-face attack (i.e., Prelog mode). This is supported by a previous report of I86A *TeSADH*-catalyzed reduction of acetophenone, which produced (*R*)-1-phenylethanol (i.e., *anti*-Prelog mode).⁴⁷

Table 1. Asymmetric reduction of 2-bromoacetophenone using *TeSADH* mutants.



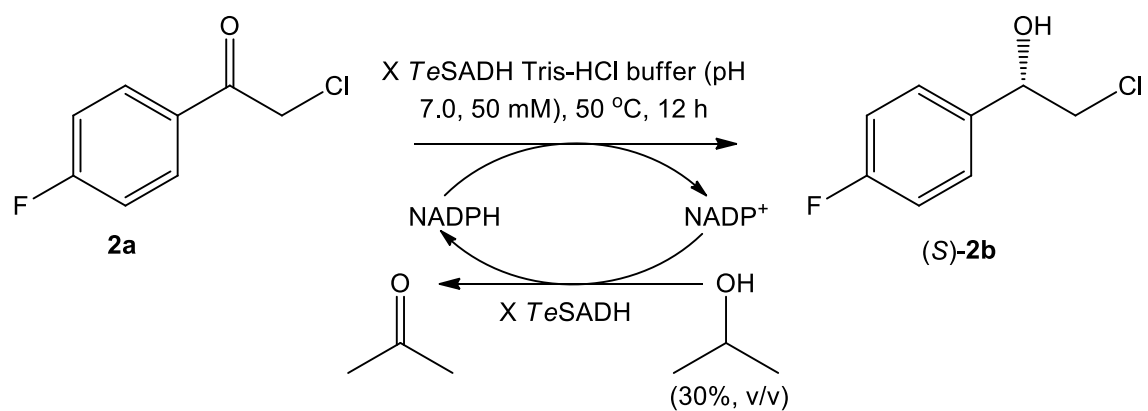
X	Conv. (%)	ee (%)
A85G/186A/C295A	>99	>99 S
P84S/I86A	>99	>99 S
ΔP84/A85G	>99	>99 S
I86A	71	>99 S

Reduction of 2-chloro-4'-fluoroacetophenone (**2a**) using all mutants resulted in formation of (S)-2-chloro-1-(4'-fluorophenyl)-1-ethanol [(S)-**2b**] with very high enantioselectivity. Notably, P84S/I86A or ΔP84/A85G mutants showed improved conversion yields for this substrate, **Table 2**. Reduction of 2-chloro-4'-chloroacetophenone (**3a**) using I86A, A85G/186A/C295A and P84S/I86A *TeSADH* mutants resulted in production of (R)-2-chloro-1-(4'-chlorophenyl)-1-ethanol [(R)-**3b**] with high enantioselectivity (>99% ee), but in low to moderate conversion. On the other hand,

Δ P84/A85G mutant produced (S)-**3b** with low conversion but high enantioselectivity,

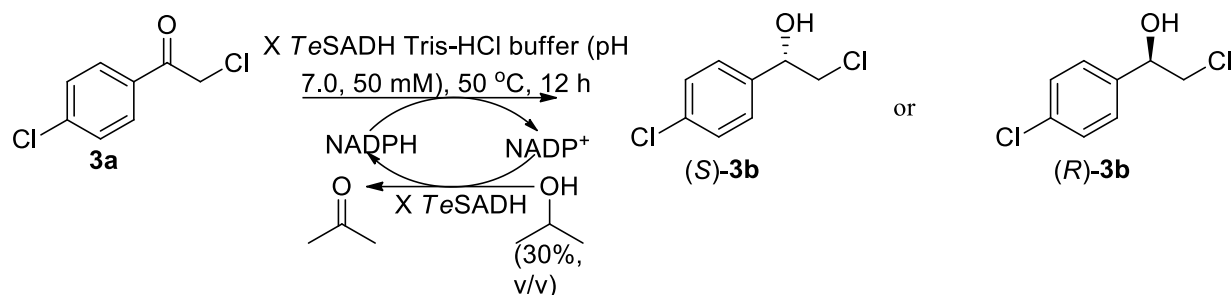
Table 3.

Table 2. Asymmetric reduction of 2-chloro-4'-fluoroacetophenone using *TeSADH* mutants.



X	Conv. (%)	ee (%)
A85G/186A/C295A	78	>99 S
P84S/I86A	>99	>99 S
Δ P84/A85G	98	>99 S
I86A	48	96 S

Table 3. Asymmetric reduction of 2,4'-dichloroacetophenone using *TeSADH* mutants.



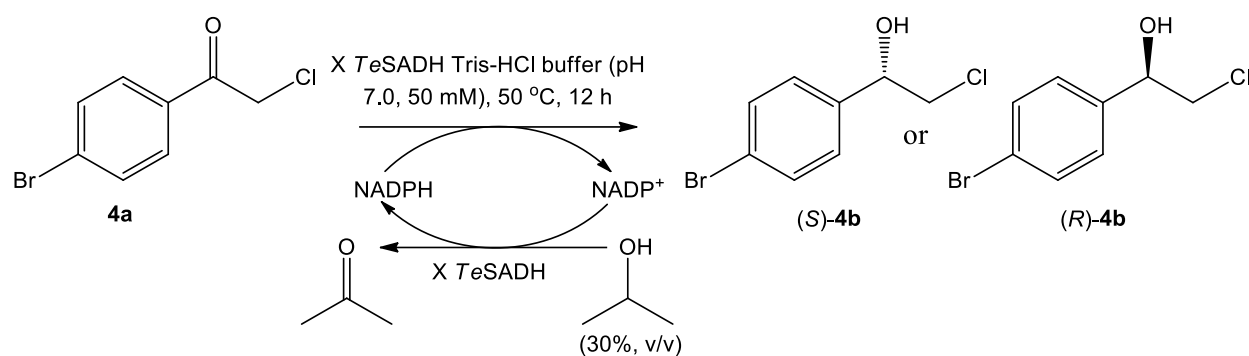
X	Conv. (%)	ee (%)
A85G/I86A/C295A	10	>99 <i>R</i>
P84S/I86A	66	>99 <i>R</i>
ΔP84/A85G	37	88 <i>S</i>
I86A	12	>99 <i>R</i>

Asymmetric reduction of 2-chloro-4'-bromoacetophenone (**4a**) using P84S/I86A and ΔP84/A85G mutants quantitatively yielded (*R*)-2-chloro-1-(4'-bromophenyl)-1-ethanol [(*R*)-**4b**], and (*S*)-**4b**, respectively, with high enantioselectivity (>99% ee), **Table 4**. These results obtained from the asymmetric reduction of **2a-4a** highlight the influence of the halogen identity on the phenyl ring in controlling the stereopreference of *TeSADH*-catalyzed asymmetric reductions of substituted α-haloacetophenones. Finally, reduction of 2,2,2-trifluoroacetophenone (**5a**) using P84S/I86A *TeSADH* resulted in quantitative formation of (*S*)-2,2,2-trifluoro-1-phenylethanol [(*S*)-**5b**] with high enantioselectivity. The

same enantiomer, yet with a lower yield, was produced when using P84S/I86A *TeSADH*,

Table 5.

Table 4. Asymmetric reduction of 2-chloro-4'-bromoacetophenone using *TeSADH* mutants.



X	Conv. (%)	ee (%)
A85G/186A/C295A	16	Rac
P84S/I86A	>99	>99 <i>R</i>
ΔP84/A85G	>99	>99 <i>S</i>
I86A	10	Rac

Table 5. Asymmetric reduction of 2,2,2-trifluoroacetophenone using *TeSADH* mutants.

Reaction scheme showing the asymmetric reduction of 2,2,2-trifluoroacetophenone (**5a**) to (S)-2,2,2-trifluoro-1-phenylethanol ((S)-**5b**) using *TeSADH* mutants. The reaction conditions are: X *TeSADH*, Tris-HCl buffer (pH 7.0, 50 mM), 50 °C, 12 h. A cofactor cycle is shown where NADPH is oxidized to NADP⁺ and then regenerated to NADPH by the reduction of 2-butanone to 2-butanol (30% v/v) using X *TeSADH*.

X	Conv. (%)	ee (%)
A85G/I86A/C295A	>99	>99 S
P84S/I86A	43	>99 S
ΔP84/A85G	1	ND ^a
I86A	1	ND ^a

^anot determined

The use of A85G/I86A/C295A, P84S/I86A and ΔP84/A85G *TeSADH* mutants showed better performance than that of I86A *TeSADH* in asymmetric reduction of the tested para-substituted α-haloacetophenones. Interestingly, P84S/I86A *TeSADH* displayed enantiocomplementary stereopreference to ΔP84/A85G *TeSADH* in the asymmetric reduction of **3a** and **4a**, which is consistent with a previous report for asymmetric reduction of bulky-bulky ketones that exhibit phenyl-ring-containing groups on both sides.⁵⁰ Moreover, ΔP84/A85G *TeSADH* consistently displayed *anti*-Prelog stereopreference,

producing the (*S*)-halohydrins, in reduction of para-substituted **2a-4a** ketones. With substrates **1a**, **2a** and **5a**, both P84S/I86A and Δ P84/A85G mirrored the same trend observed in I86A and A85G/186A/C295A mutants but with better conversions and ee. Notably, the substrate **2a**, despite having a substituent at the para-position, interacts with the enzymes similarly to the unsubstituted acetophenone, which could be attributed to the smaller size of fluorine when compared with chlorine and bromine.

CHAPTER 5

CONCLUSION

In conclusion, this study investigated the asymmetric reduction of α -haloacetophenone analogs using four mutants of *TeSADH* (I86A, A85G/186A/C295A, P84S/186A, and Δ P84/A85G). Notably, P84S/I86A and Δ P84/A85G mutants exhibited improved performance compared to I86A and A85G/186A/C295A mutants, with better conversions and enantiomeric excess. Interestingly, P84S/I86A and Δ P84/A85G mutants displayed enantiocomplementary stereopreference in the reduction of 2-chloro-4'-chloroacetophenone and 2-chloro-4'-bromoacetophenone. These findings demonstrate the potential of guided mutagenesis in expanding the substrate scope of *TeSADH* and provide valuable insights into the influence of halogen identity on stereopreference in enzyme-catalyzed asymmetric reductions. The results pave the way for further studies on other α -haloacetophenone analogs using these mutants and suggest that P84S/I86A and Δ P84/A85G mutants are promising candidates for future studies. Overall, this study contributes to the development of more efficient and selective biocatalysts for organic synthesis.

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SUPPLEMENTARY MATERIALS

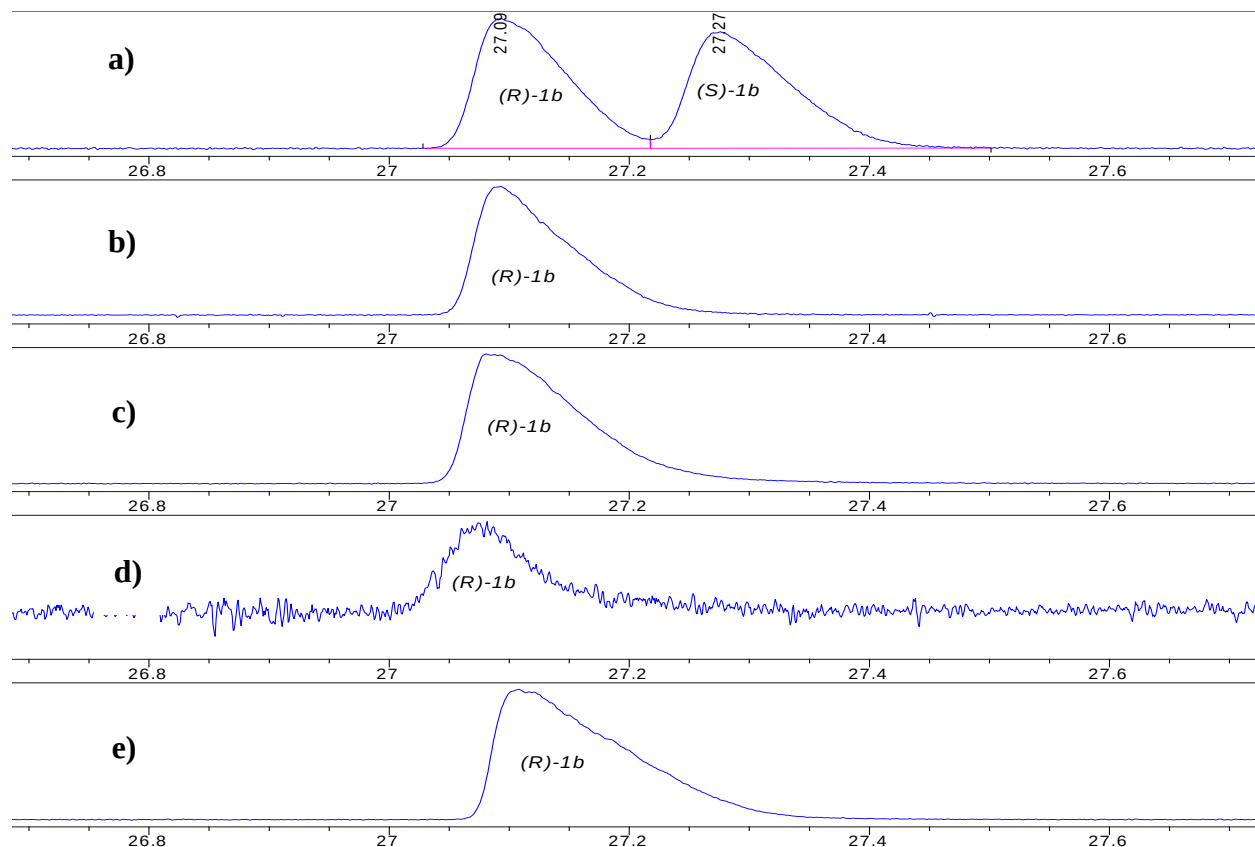


Figure S1. Gas chromatograms of: a) (*rac*)-**1b** produced from reduction of **1a** using NaBH₄. (*R*)-**1b** from reduction of **1a** using; b) A85G/I86A/C295A *TeSADH*, c) P84S/I86A *TeSADH*. d) Δ P84/A85G *TeSADH*, e) I86A *TeSADH*.

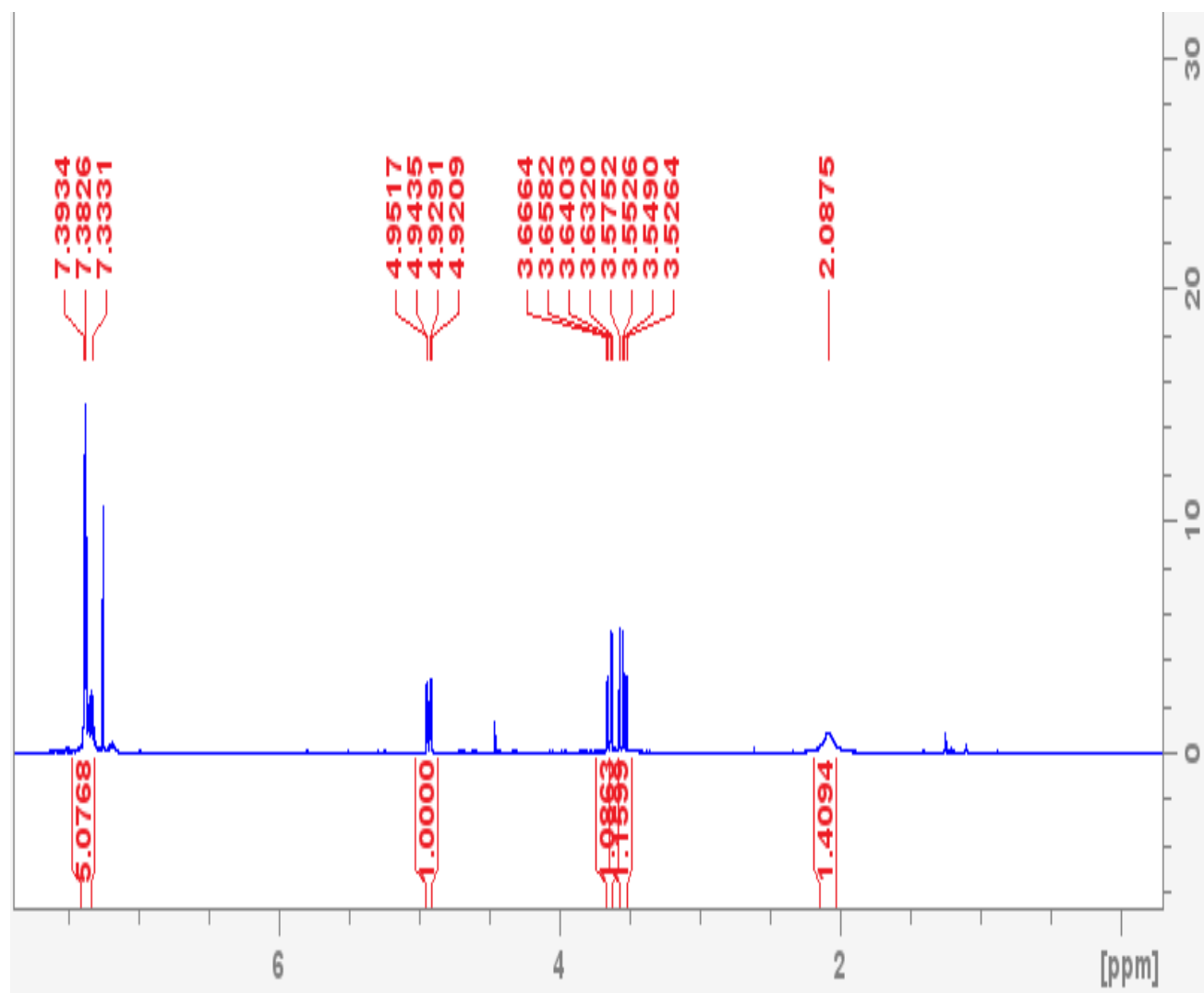


Figure S2. ^1H -NMR spectrum of *(rac)*-**1b** produced from reduction of **1a** with NaBH_4 .

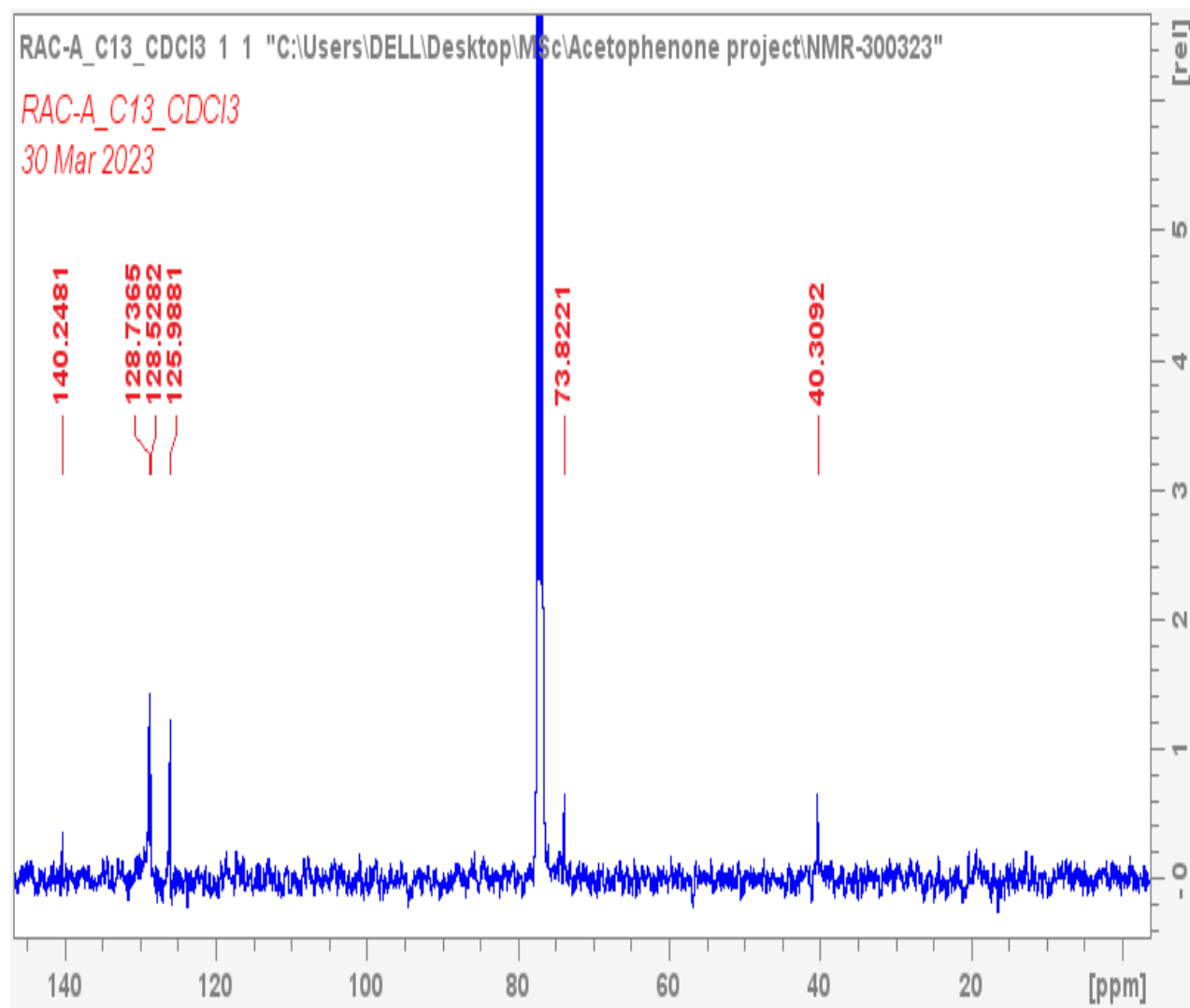


Figure S3. ^{13}C -NMR spectrum of (*rac*)-**1b** produced from reduction of **1a** with NaBH_4 .

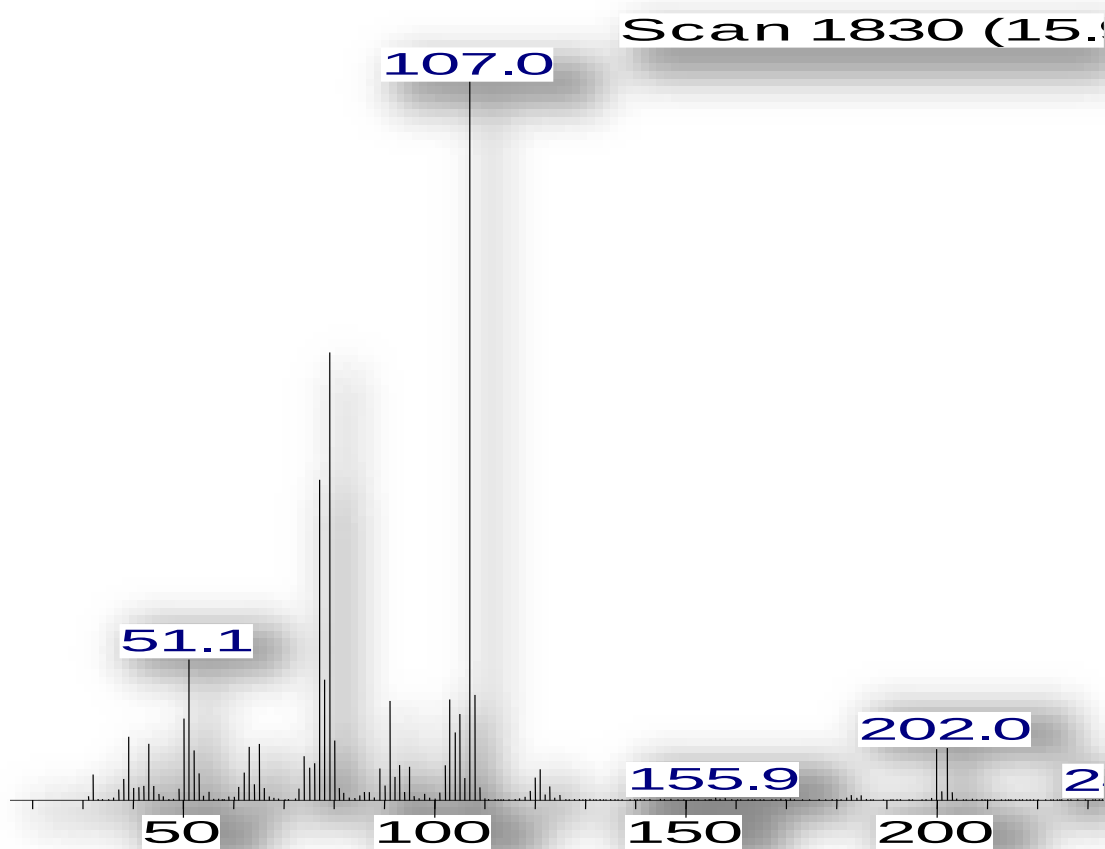


Figure S4. Mass spectrum of (R)-1b from reduction of 1a using P84S/I86A.

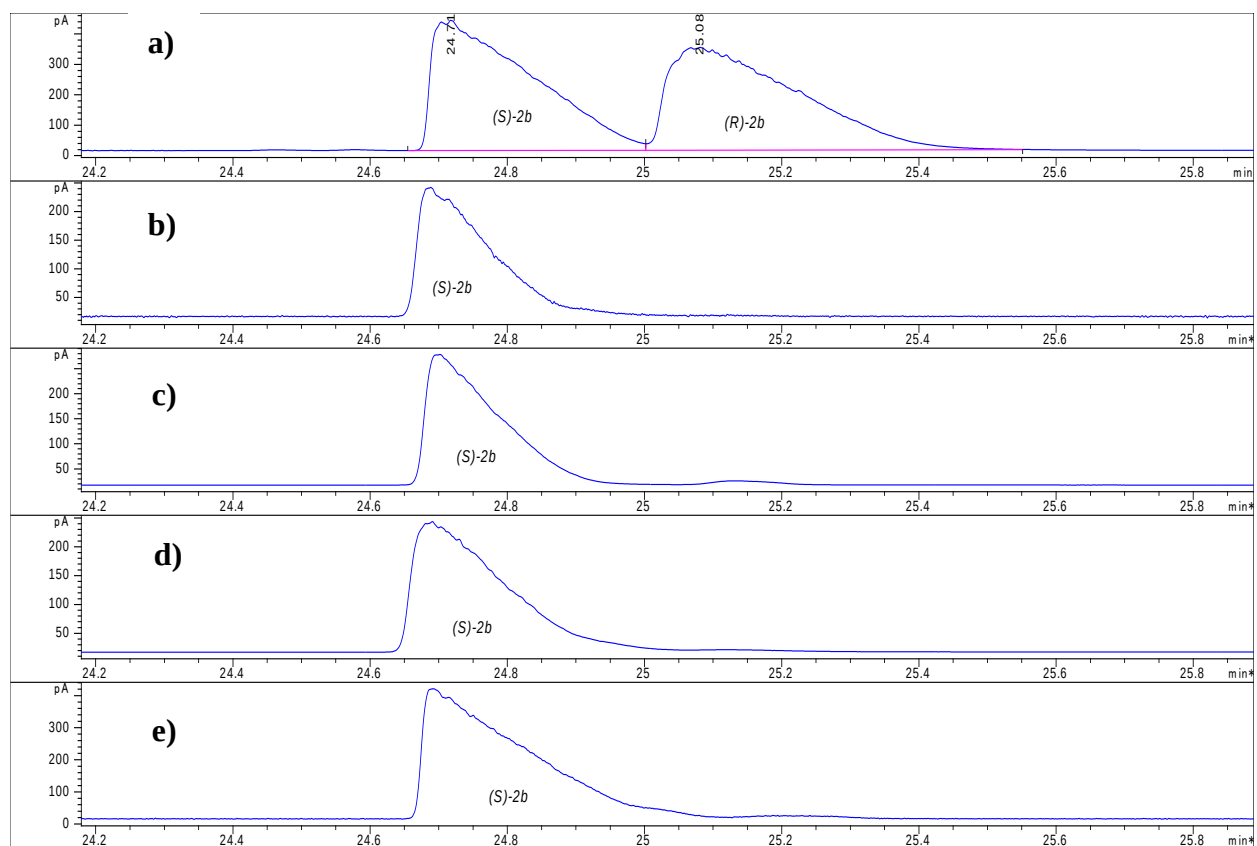


Figure S5. Gas chromatogram of: **a)** (*rac*)-**2b** produced from reduction of **2a** using NaBH₄. (*S*)-**2b** from reduction of **2a** using; **b)** A85G/I86A/C295A *TeSADH*, **c)** P84S/I86A *TeSADH*. **d)** ΔP84/A85G *TeSADH*, **e)** I86A *TeSADH*.

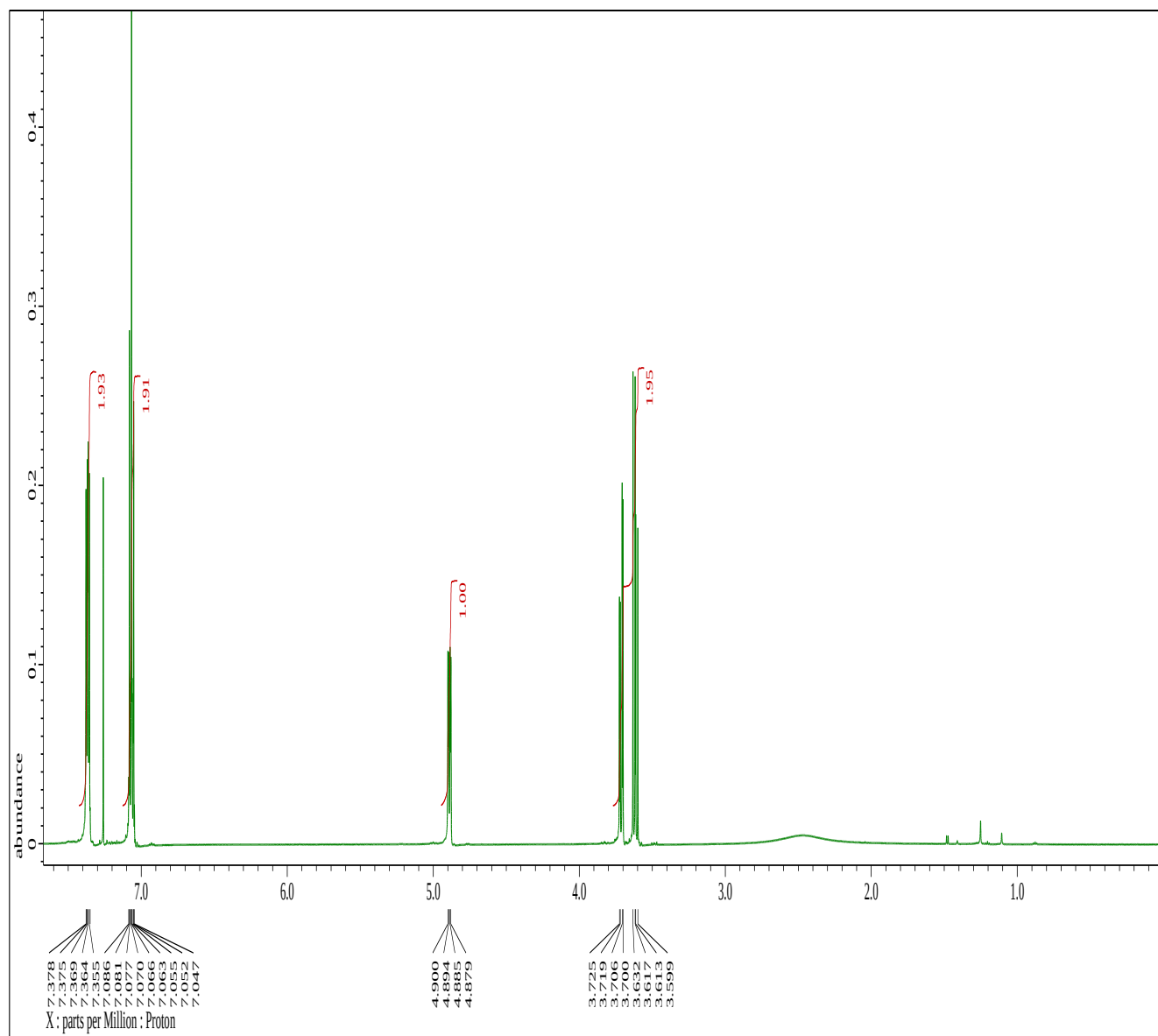


Figure S6. ^1H -NMR spectrum of *(rac)*-**2b** produced from reduction of **2a** with NaBH_4 .

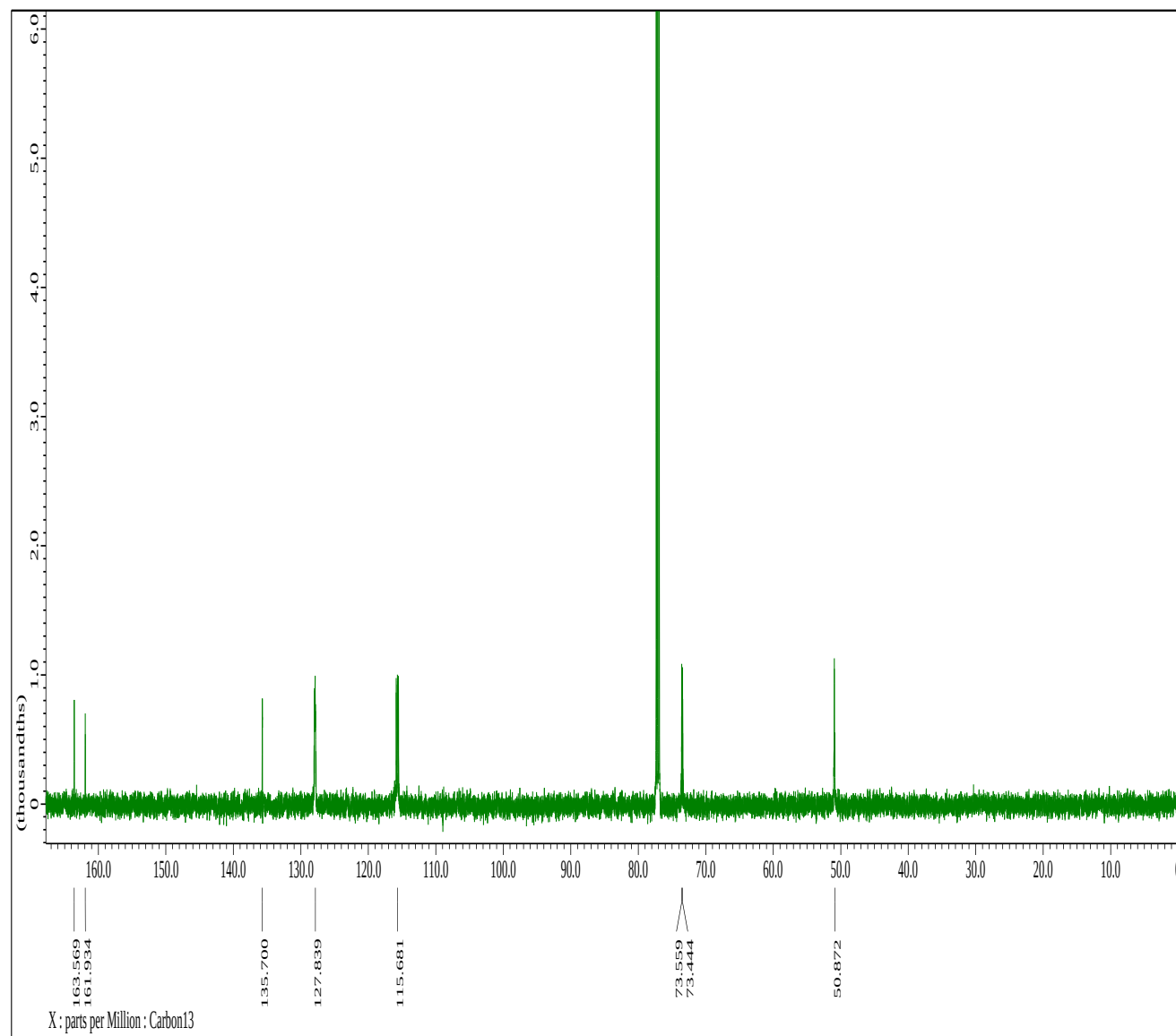


Figure S7. ^{13}C -NMR spectrum of *(rac)*-2b produced from reduction of 2a with NaBH₄.

Abundance

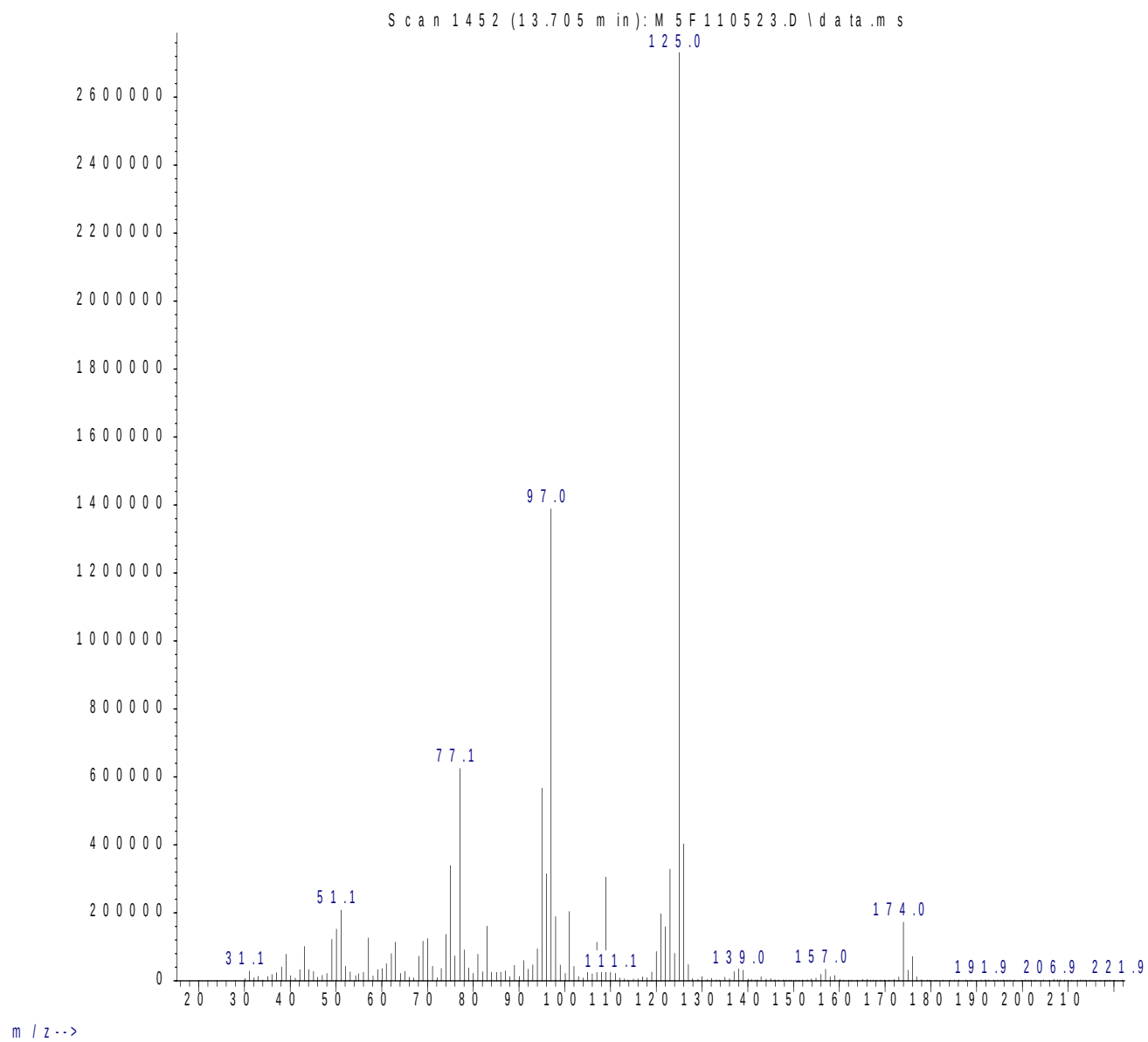


Figure S8. Mass spectrum (S)-**2b** produced from reduction of **2a** using Δ P84/A85G TeSADH.

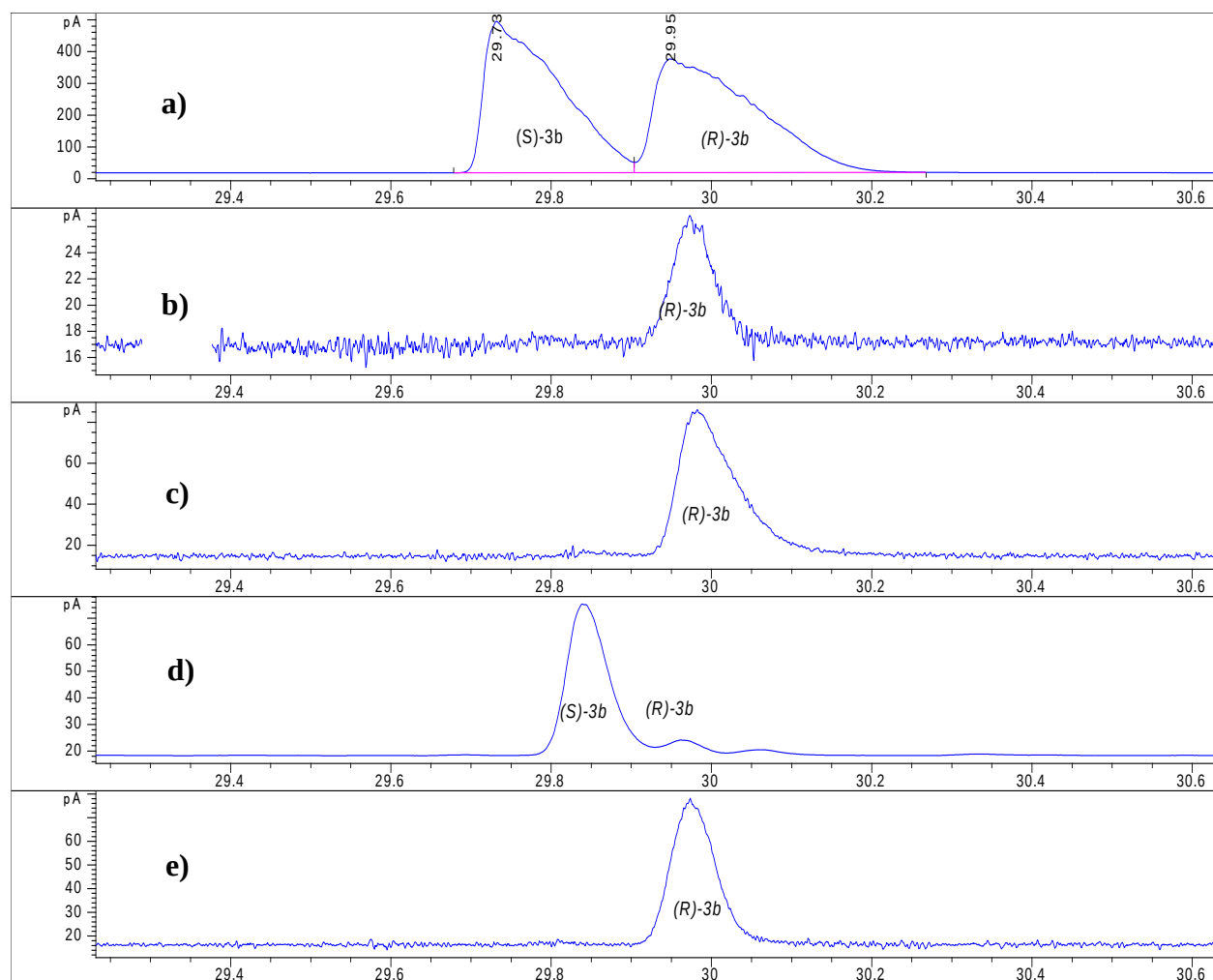


Figure S9. Gas chromatogram of: **a)** *(rac)*-**3b** produced from reduction of **3a** using NaBH_4 . *(R)*-**3b** from reduction of **3a** using; **b)** A85G/I86A/C295A *TeSADH*, **c)** P84S/I86A *TeSADH*, **e)** I86A *TeSADH*. **d)** *(S)*-**3b** from reduction of **3a** using Δ P84/A85G *TeSADH*

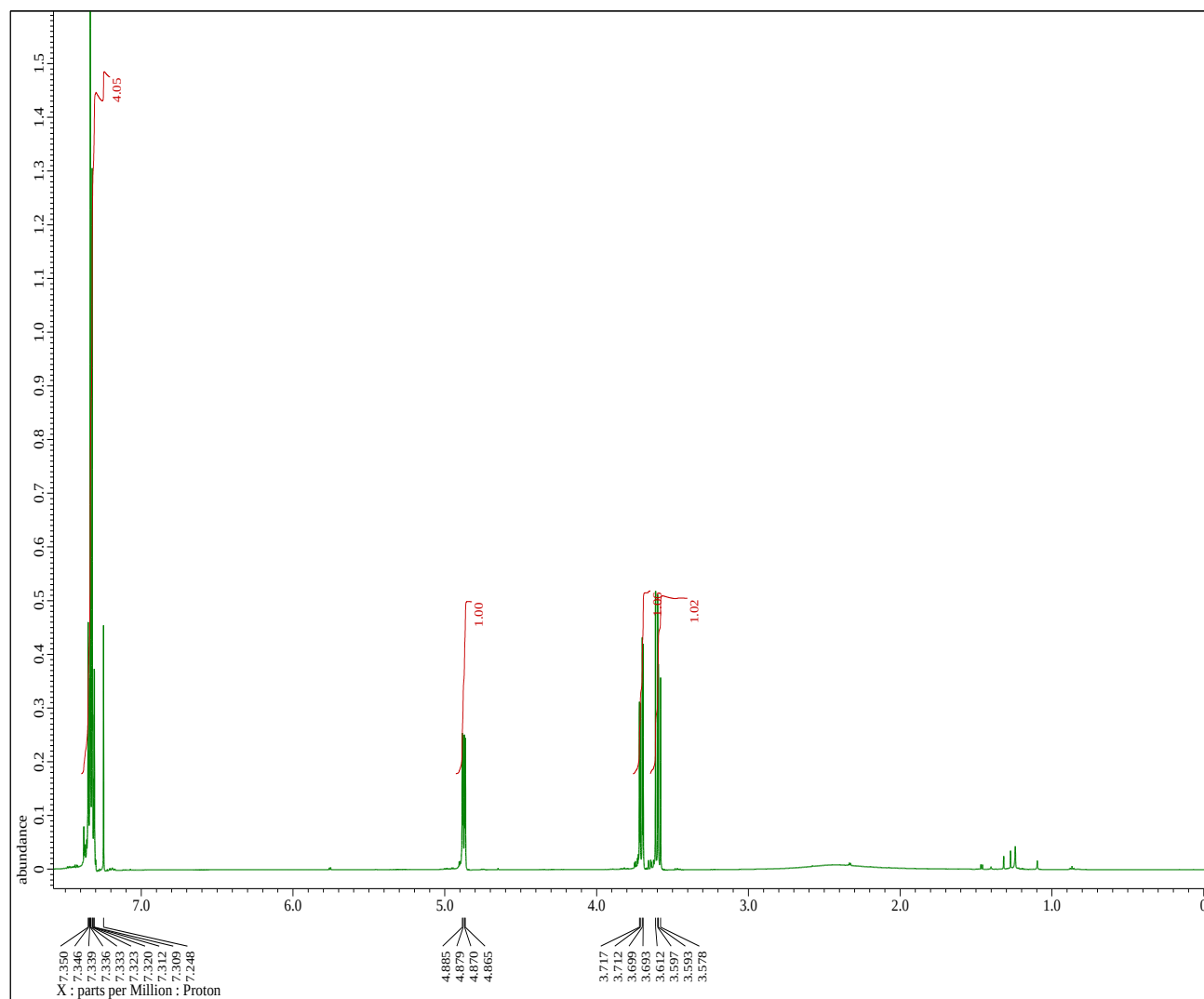


Figure S10. ¹H-NMR spectrum of (rac)-3b produced from reduction of 3a with NaBH₄.

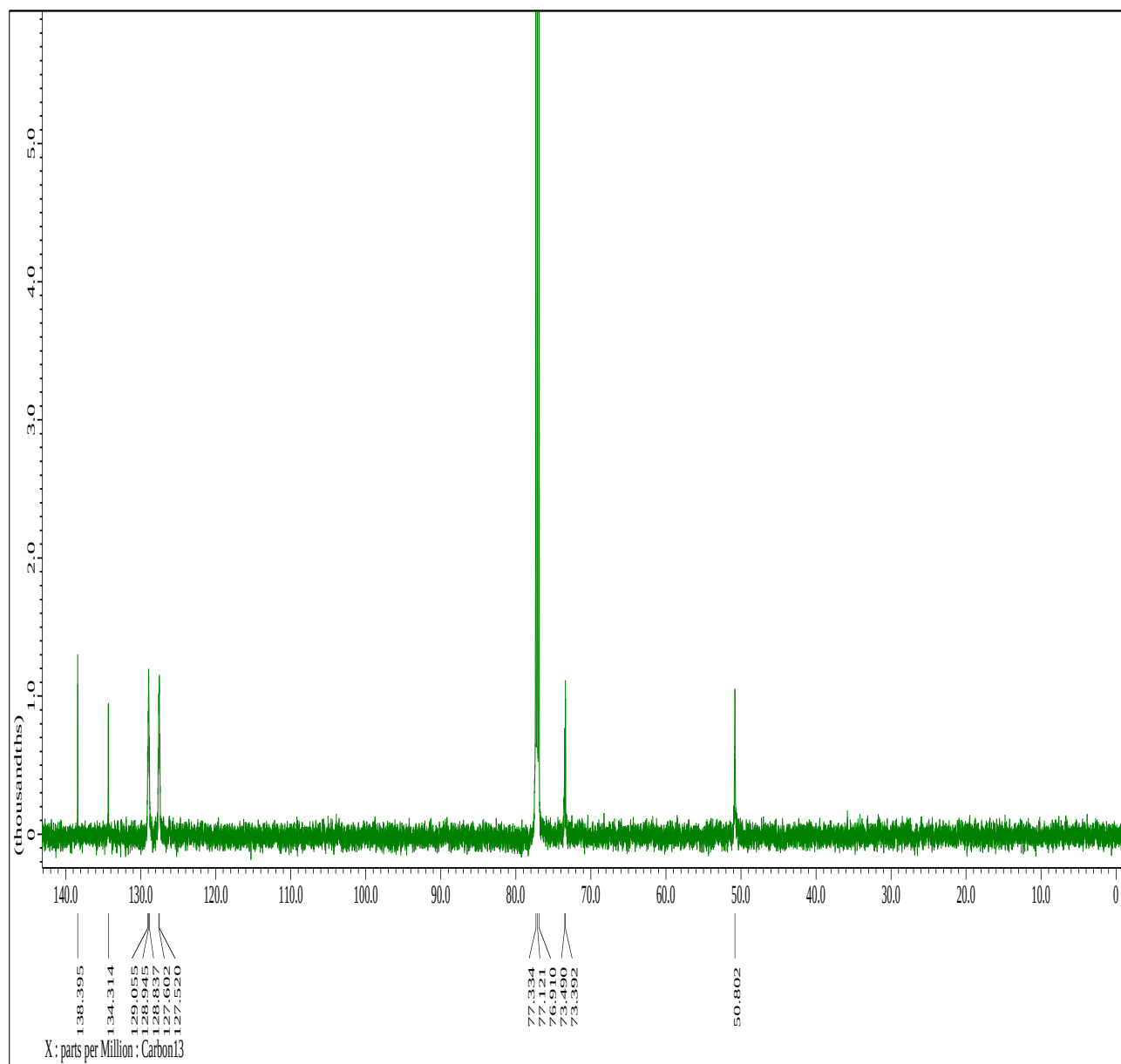


Figure S11. ^{13}C -NMR spectrum of *(rac)*-**3b** produced from reduction of **3a** with NaBH₄.

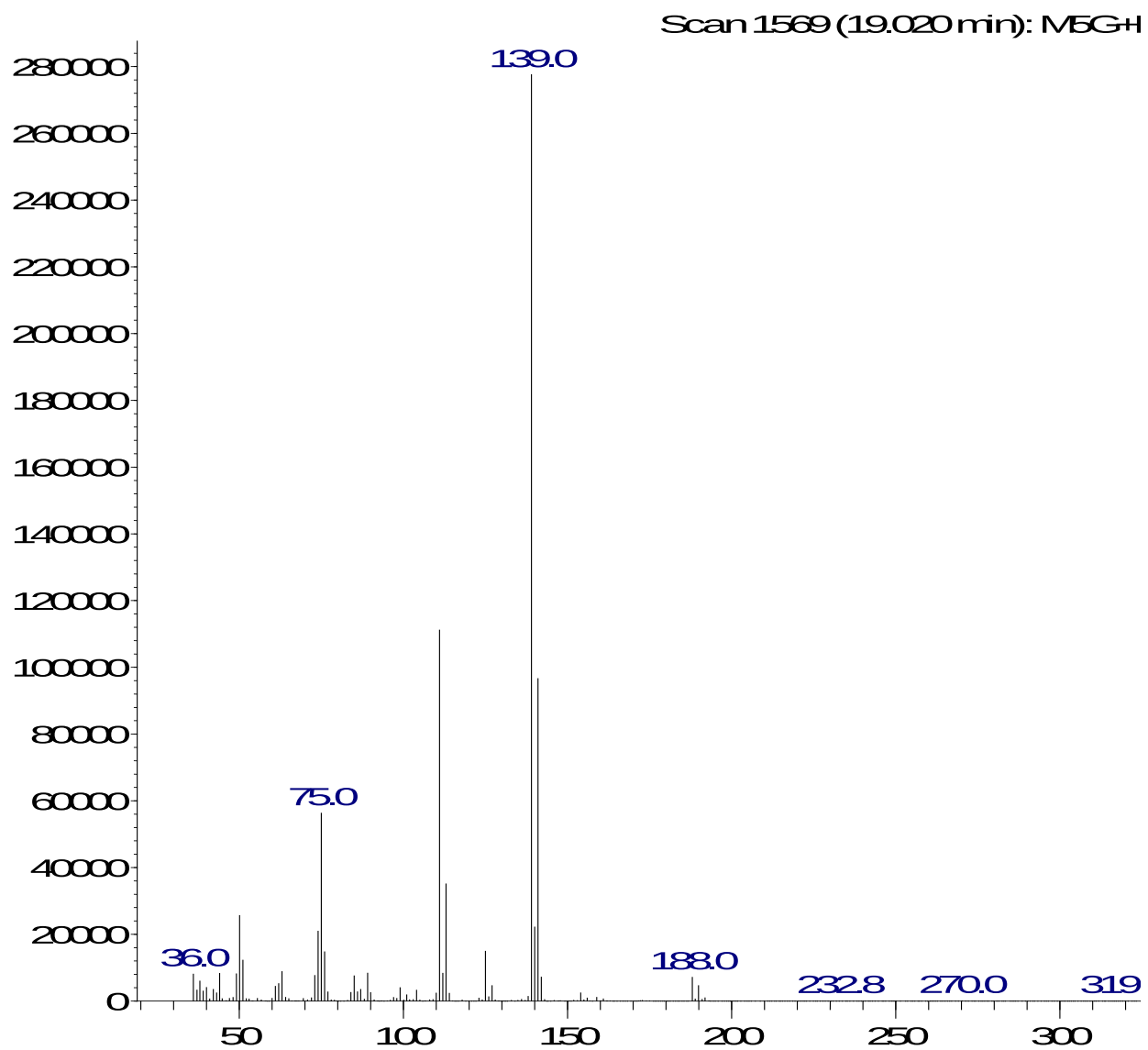


Figure S12. Mass spectrum (S)-**3b** produced from reduction of **3a** using Δ P84/A85G *TeSADH*.

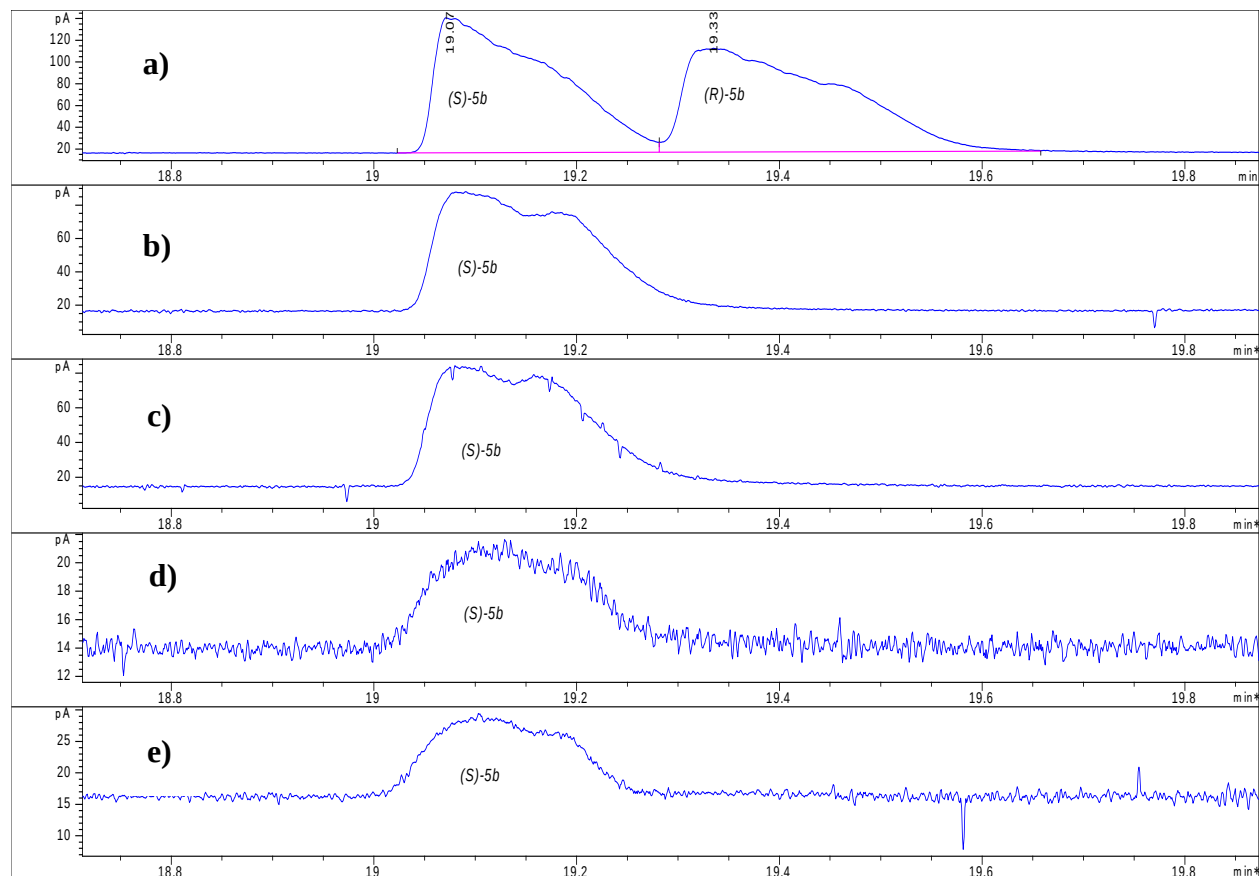


Figure S13. Gas chromatogram of: **a)** (*rac*)-5b produced from reduction of 5a using NaBH_4 . (*S*)-5b from reduction of 5a using; **b)** A85G/I86A/C295A TeSADH, **c)** P84S/I86A TeSADH. **d)** $\Delta\text{P84/A85G TeSADH}$, **e)** I86A TeSADH.

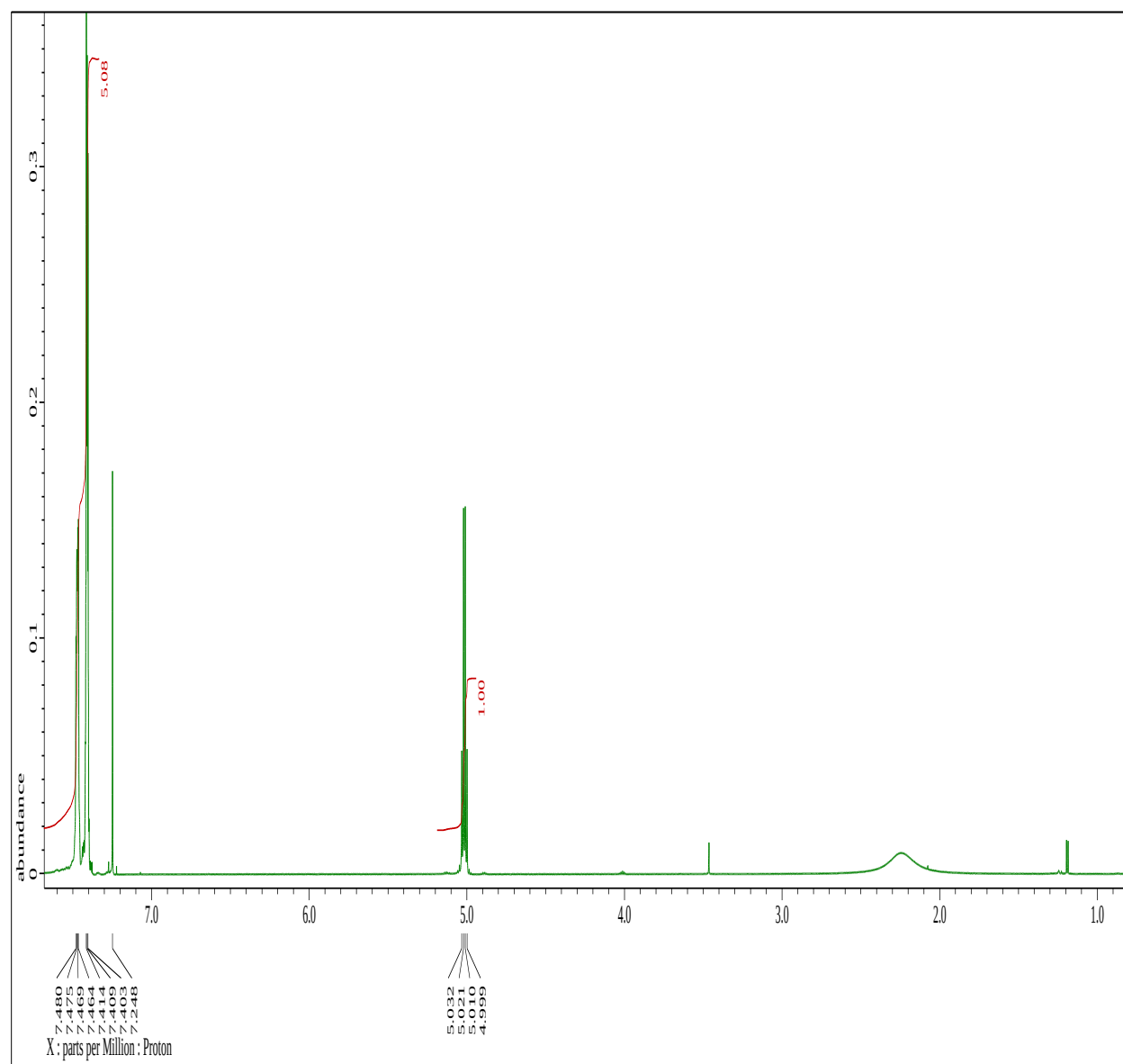


Figure S14. ^1H -NMR spectrum of (*rac*)-**5b** produced from reduction of **5a** with NaBH_4 .

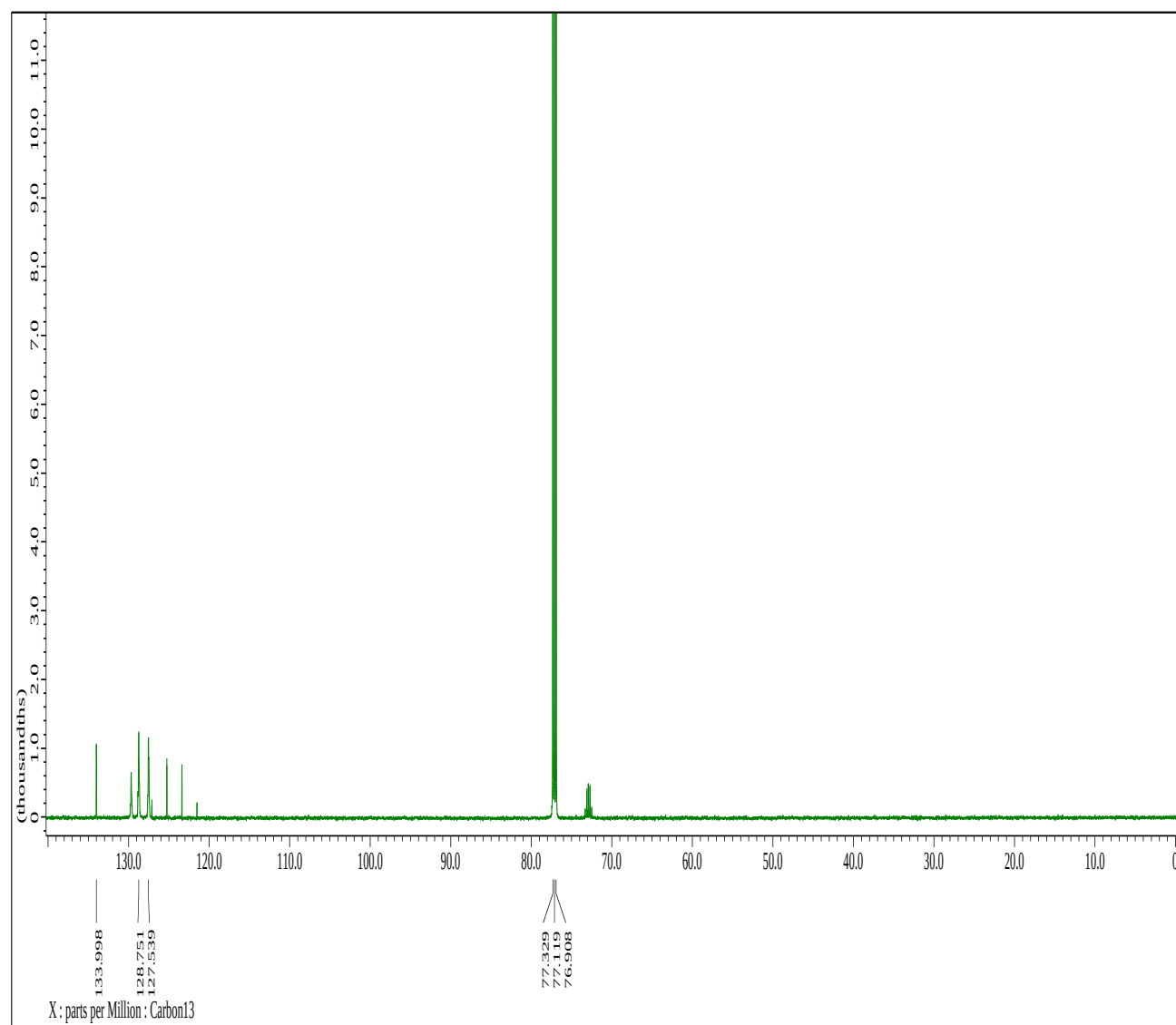


Figure S15. ^{13}C -NMR spectrum of *(rac)*-5b produced from reduction of 5a with NaBH_4 .

Abundance

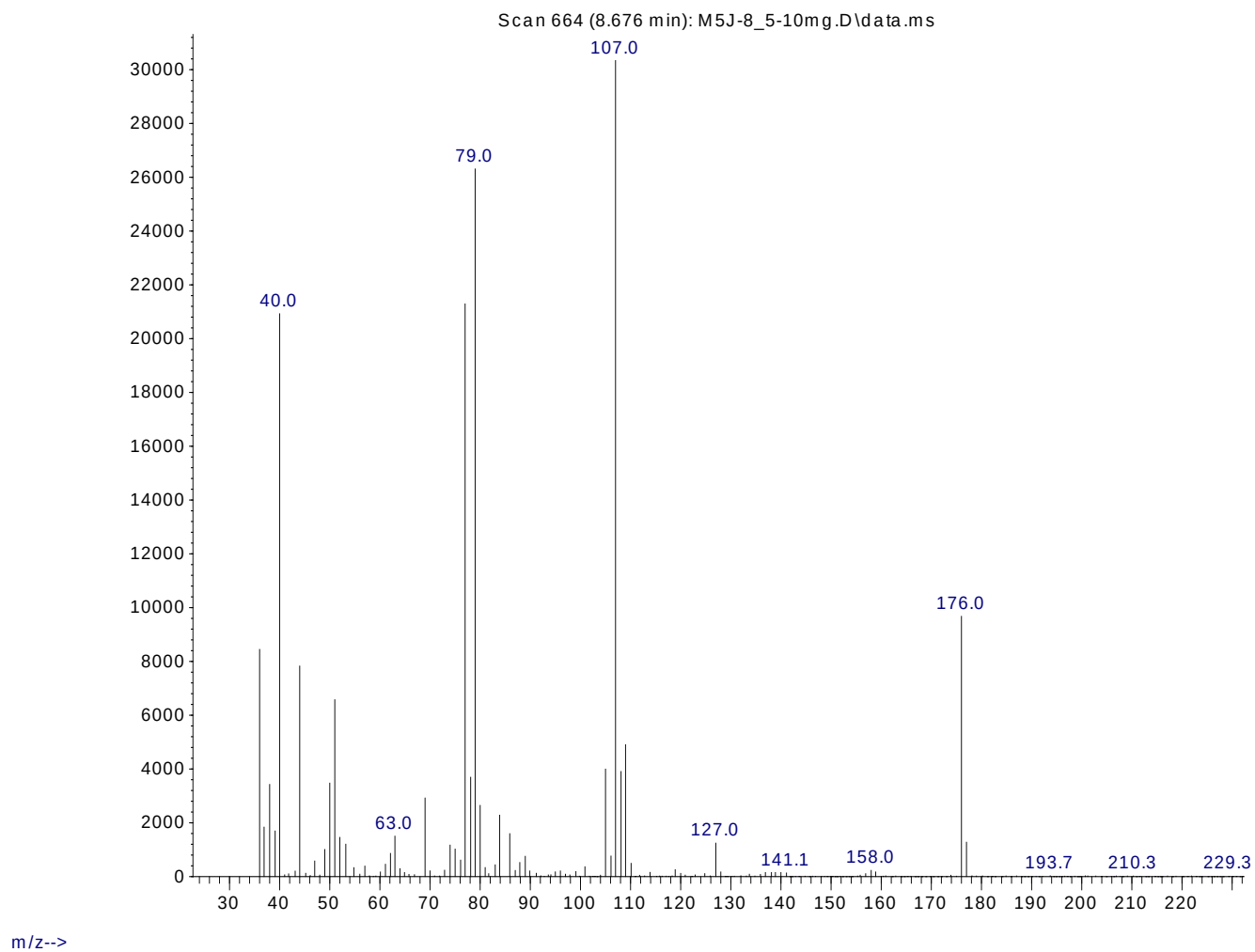


Figure S16. Mass spectrum (S)-**5b** produced from reduction of **5a** using P84S/I86A *TeSADH*.

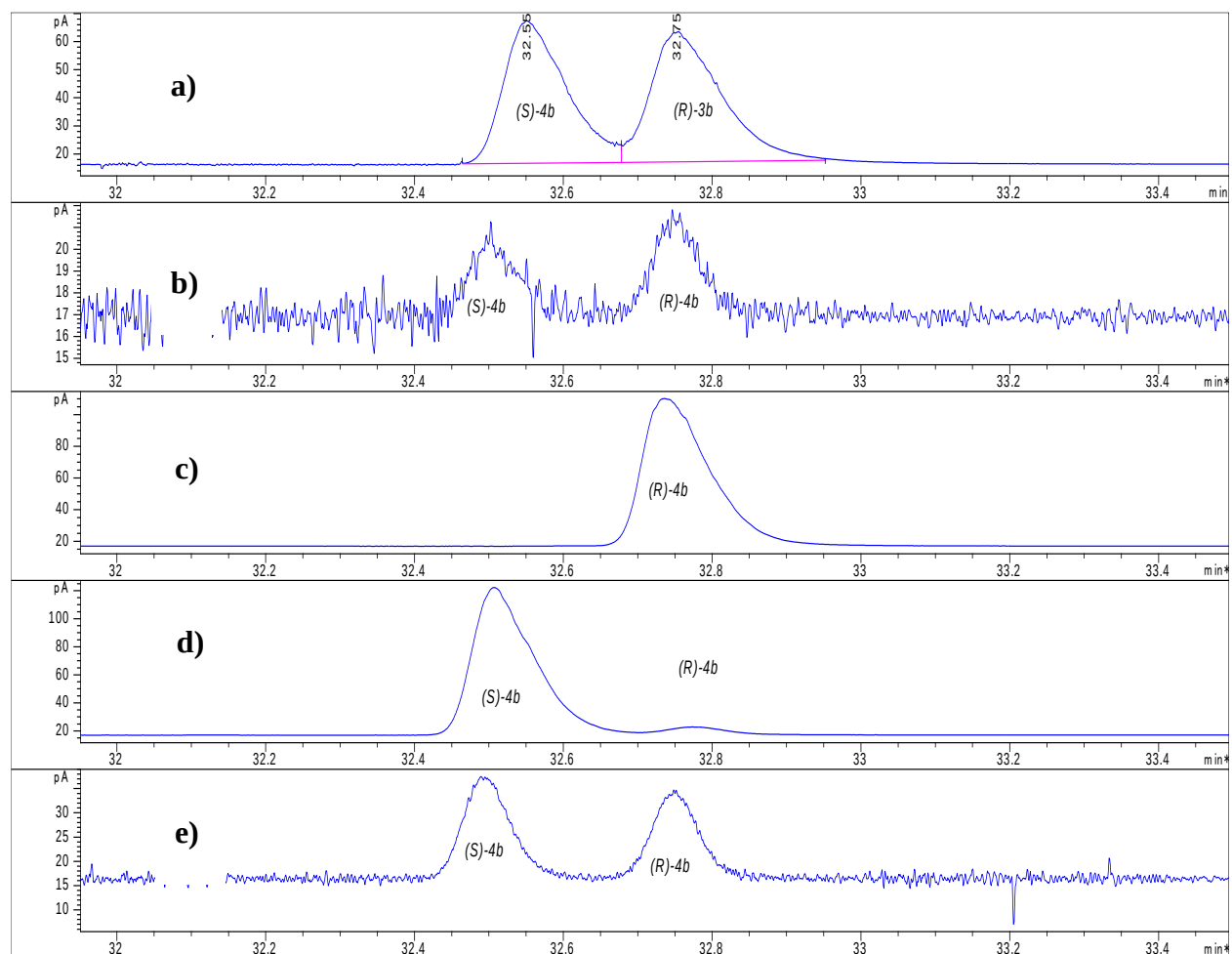


Figure S17. Gas chromatogram of: (*rac*)-**4b** produced from reduction of **4a** using **a)** NaBH₄, **b)** A85G/I86A/C295A, **e)** I86A. **c)** (*R*)-**4b** from reduction of **4a** using P84S/I86A *Te*SADH. **d)** (*S*)-**4b** from reduction of **4a** using ΔP84/A85G *Te*SADH.

Abundance

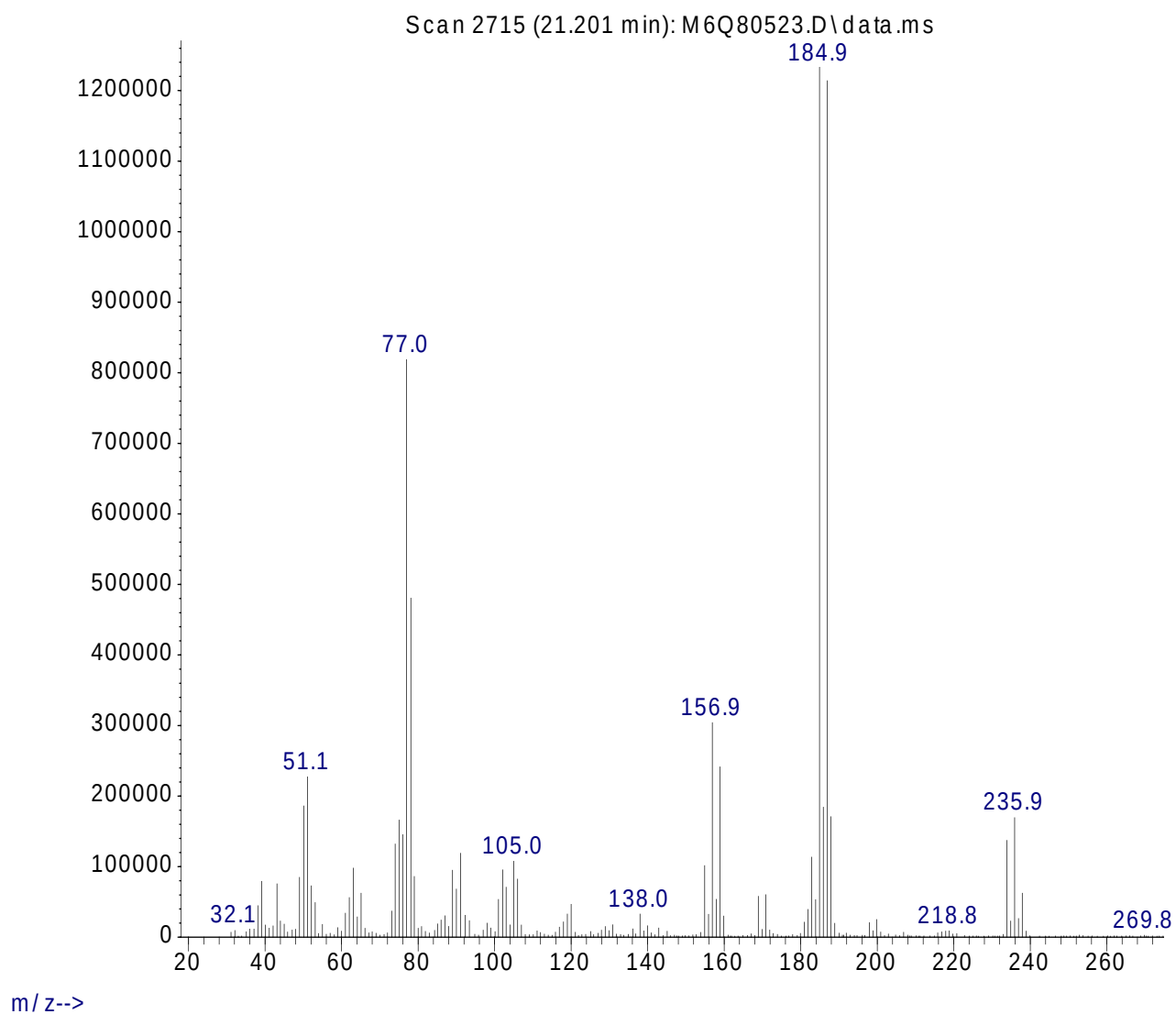


Figure S18. Mass spectrum (S)-**4b** produced from reduction of **4a** using Δ P84/A85G *TeSADH*.

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EDUCATION

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BSc. Chemistry, Ahmadu Bello University Zaria, Nigeria, January, 2020.

PUBLICATIONS

- “Biocatalytic asymmetric reduction of prochiral bulky-bulky ketones” A. E. Sardauna, M. Abdulrasheed, A. Nzila, M. M. Musa. Molecular Catalysis 2023, 541, 113099.
<https://doi.org/10.1016/j.mcat.2023.113099>.

- “Asymmetric Reduction of α -Haloacetophenone Analogs Using *Thermoanaerobacter pseudethanolicus* Secondary Alcohol Dehydrogenase” Muhammad Abdulrasheed, Auwal Eshi Sardauna, Mouheddin T. Alhaffar, Masateru Takahashi, Samir M. Hamdan, Musa M. Musa.

CONFERENCES

- Presented “Asymmetric reduction of α -haloacetophenones” at NextGenBiocat conference, TU Graz, Austria, 2023.

AWARDS

- Awarded King Fahd University of Petroleum and Minerals graduate scholarship.
- Best student in organic/polymer synthesis (KFUPM).
- Deanship of Research Oversight and Coordination conference grant.
- NNPC/TOTAL merit annual scholarship.
- Nigeria Federal Government merit-based scholarship.