DESIGN, SYNTHESIS AND EVALUATION OF INHIBITORS FOR TRYTOPHAN INDOLE-LYASE, TYROSINE PHENOL-LYASE AND ENZYMATIC SYNTHESIS OF TRYPTOPHAN FROM GLYCEROL USING TRYPTOPHAN INDOLE-LYASE

by

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(Under the Direction of ROBERT S. PHILLIPS)

ABSTRACT

This dissertation is written in chapter format following the guidelines of the Graduate School of The University of Georgia. Chapter 1 will serve as an introduction to provide readers with relevant literature review. Chapters 2, 3 and 4 are copies of manuscripts prepared for submission to peer-reviewed journals. Chapter 5 provides a conclusion with emphasis on future research directions.

Part of my research interest focuses on the design, synthesis and evaluation of mechanism-based inhibitors for tryptophan indole-lyase (TIL) as an approach to a novel class of antibacterial treatment (Chapter 2). Our results indicated that homologation of the physiological substrate, L-tryptophan, at the Cα position effectively inhibited the activity of TIL while displaying high selectivity in preference to tryptophan synthase.

The accepted mechanism for TIL and tyrosine phenol-lyase (TPL) is remarkably similar. Therefore, we also extended our design and rationale in the search for potent mechanism-based

inhibitors of TPL. Synthesis, inhibition kinetics and pre-steady-state kinetic evaluation of inhibitors for TPL is discussed in Chapter 3.

TIL can also catalyze the reverse of its physiological reaction, *β*-substitution of indole, to yield L-tryptophan. As a proof-of-concept, we have demonstrated and validated the biosynthetic pathway for L-tryptophan from glycerol surplus by coupling natural glycerol metabolic enzymes of *Escherichia coli* with TIL expressed from *tnaA* plasmids (Chapter 4). This work presented an attractive and convenient alternative to the multi-step chemical synthesis for L-tryptophan, using a "one-pot" approach with whole-cell catalysis. The final chapter will provide a discussion on future research direction for the work reported in this dissertation.

INDEX WORDS: Tryptophan indole-lyase, tyrosine phenol-lyase, inhibitors, steady-state kinetics, pre-steady-state kinetics, glycerol surplus, *Escherichia coli* BL21 (DE3), plasmids, L-homotryptophan, L-bishomotryptophan, L-homotyrosine, L-bishomotyrosine.

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May 2013

DEDICATION

I would like to dedicate this work to my family as a way to thank each and every one for their unconditional love and support. To my parents, who taught me right from wrong and served as my role models in life. To my wife, Chilan, and my son, Jadon, who brought motivation, joy and laughter to my life. To my siblings, in-laws, nieces and nephews, who supported me unconditionally, taught me the important values of family and for doing the silly things to make me laugh. Without you, I will not be the person that I am today. Thank you and I love you all!

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LIST OF ABBREVIATIONS

DEAM	Diethyl acetamidomalonate ester
EC	Enzyme Commission code
E. coli	*Escherichia coli*
ESI-MS	Electrospray ionization mass spectrometry
HPLC	High performance liquid chromatography
NMR	Nuclear magnetic resonance
PLP	Pyridoxal-5'-phosphate
SOPC	*S*-(*o*-nitrophenyl)-L-cysteine
TIL	Tryptophan indole-lyase
TLC	Thin-layer chromatography
TPL	Tyrosine phenol-lyase
Trp	L-Tryptophan
Tyr	L-Tyrosine

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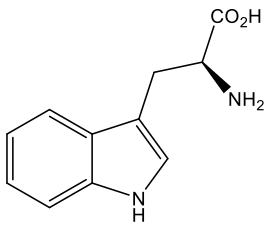
CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Synthesis of Tryptophan: Chemical and Biosynthesis

Tryptophan is one of 22 natural amino acids, and arguably, one that has the most interesting discovery (Figure 1.1). The name tryptophan came from the Greek combination for "illuminating by fragmentation".[1] The presence of indole, an aromatic substructure of tryptophan, was reported as early as 1831 in a reaction between dried pancreatic juice and aqueous chlorine solution described to give a red colored reaction.[2] However, it was until 1849 that indole was first isolated by Bopp from proteins.[3] Subsequent efforts to discover the amino acid continued until 1890 when it received the name tryptophan, for "illuminating by fragmentation", which reflects the discovery of its elusive existence.[4] In 1901, Hopkins and Cole reported the first isolation of tryptophan from protein extract.[5] Though, the proposed structure, as they believed tryptophan was a skatole derivative, was that of skatole-α-aminoacetic acid. By means of chemical synthesis, in 1907 Ellinger and Flamand provided evidence that the structure of tryptophan is indeed 2-amino-3-indolepropionic acid,[6] which is today's accepted structure for tryptophan.



L-Tryptophan (Trp, W)
2-Amino-3-indolepropionic Acid

Figure 1.1: Chemical structure of L-tryptophan

Modifications and alternative synthetic methodologies using indole or indole derivatives as starting materials for the synthesis of tryptophan were also reported.[7-10] Among those reported, a simplified synthetic route for D,L-tryptophan was reported by Snyder in 1955, using indole as a starting material (Figure 1.2).[11] Using Larock's palladium catalyzed heteroannulation of indole,[12-13] Cook *et. al.* previously reported[14-15] a convenient method for the synthesis of the optically active D- or L-isomer of tryptophan using the Schollkopf chiral auxiliary[16] (Figure 1.3). The selectivity of this method depends on the amino acid, valine, used in the synthesis of the chiral auxiliary. L-Valine (Val) was reported to give the D-isomer; whereas D-valine would give the L-isomer of the α-amino acid. More recently, Reisman *et. al.* also reported the asymmetric synthesis for Trp using (*R*)-BINOL-$SnCl_4$ complex[17] to give the optically active isomer in excellent enantiomeric excess.

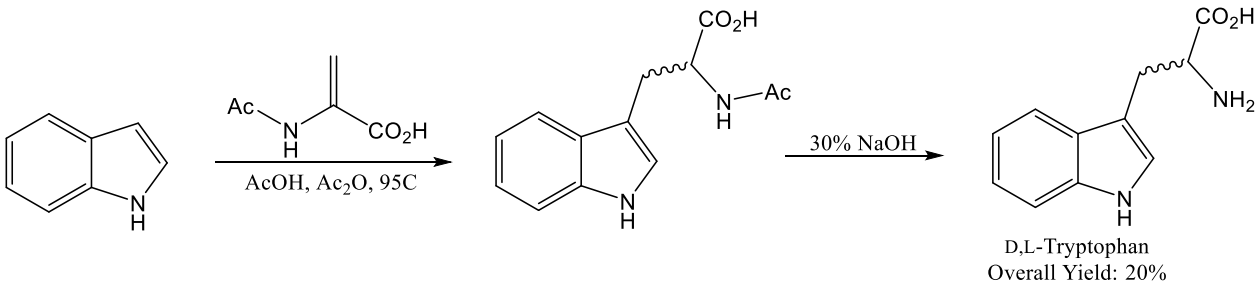


Figure 1.2: Synthesis of D,L-tryptophan reported by Snyder

As L-tryptophan (Trp) is an essential amino acid, it is only synthesized by plants, fungi and bacteria. The human body lacks the Trp operon required for expression of catalytic proteins necessary for Trp biosynthesis and therefore, we must uptake Trp from our dietary sources. The accepted biosynthetic pathway for Trp involved 13 steps, 12 enzymes, and divided into two

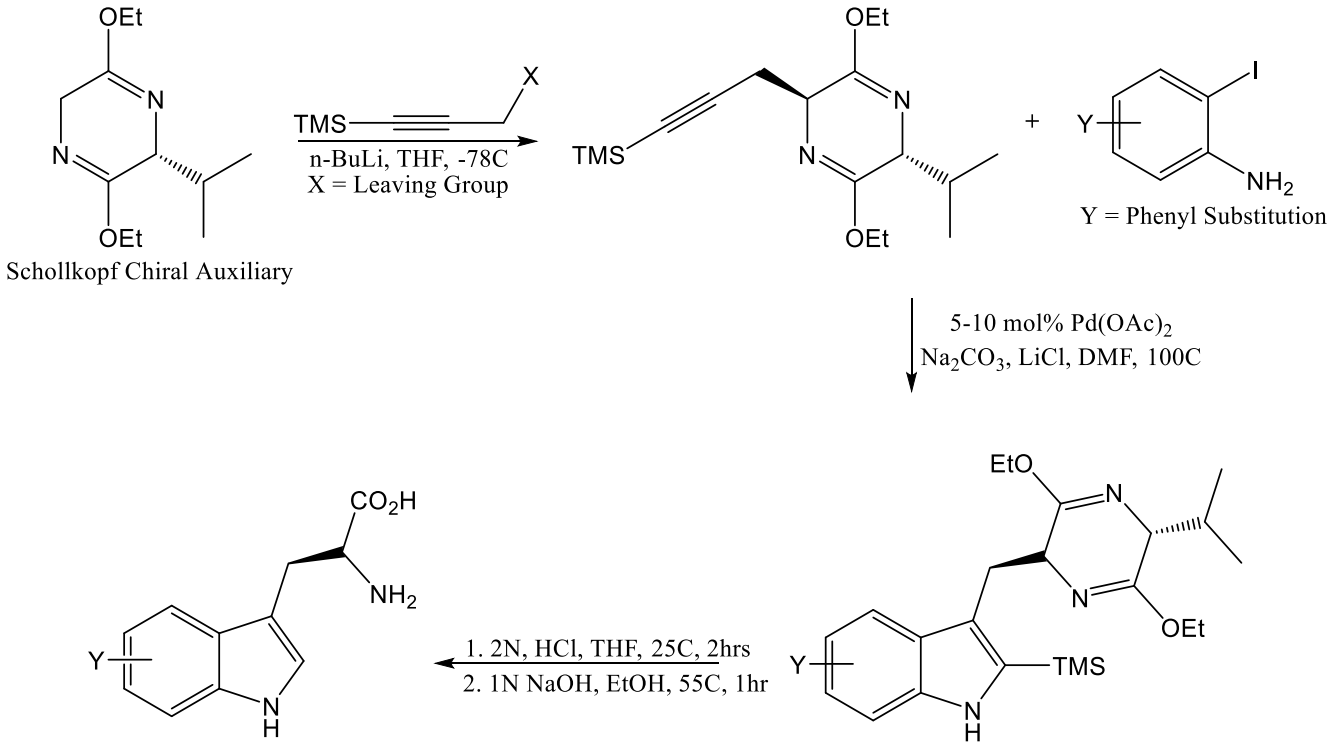


Figure 1.3: Asymmetric synthesis of L-tryptophan using the Schollkopf chiral auxiliary

separate pathways. The first pathway is commonly referred to as the shikimate pathway and the second is known as the biosynthesis of Trp. The shikimate pathway (Figure 1.4) is a metabolic pathway only found in plants and microorganisms that produces the precursor chlorismate required for the biosynthesis of aromatic amino acids Trp, L-phenylalanine (Phe), and L-tyrosine (Tyr).[18] Detail reviews of the shikimate pathway were previously discussed.[18-20] In addition, this pathway was also explored for the synthesis of a variety of aromatic compounds.[21] Condensation of phosphoenolpyruvate and erythrose-4-phosphate by 3-deoxy-D-arabino-hepulosonate-7-phosphate (DAHP) synthase yields DAHP and initiates the shikimate pathway. DAHP is further converted by 3-dehydroquinate (DHQ) synthase to form DHQ. Once formed, DHQ dehydratase converts it to 3-dehydroshikimate and subsequently to shikimate by shikimate dehydrogenase.

Shikimate is phosphorylated by shikimate kinase to give shikimate-3-phosphate which further undergoes condensation by 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase to give EPSP. The final step of this pathway is dephosphorylation of EPSP by chlorismate synthase giving chlorismate as a metabolite that can undergo many subsequent pathways, including the biosynthesis of aromatic amino acids.

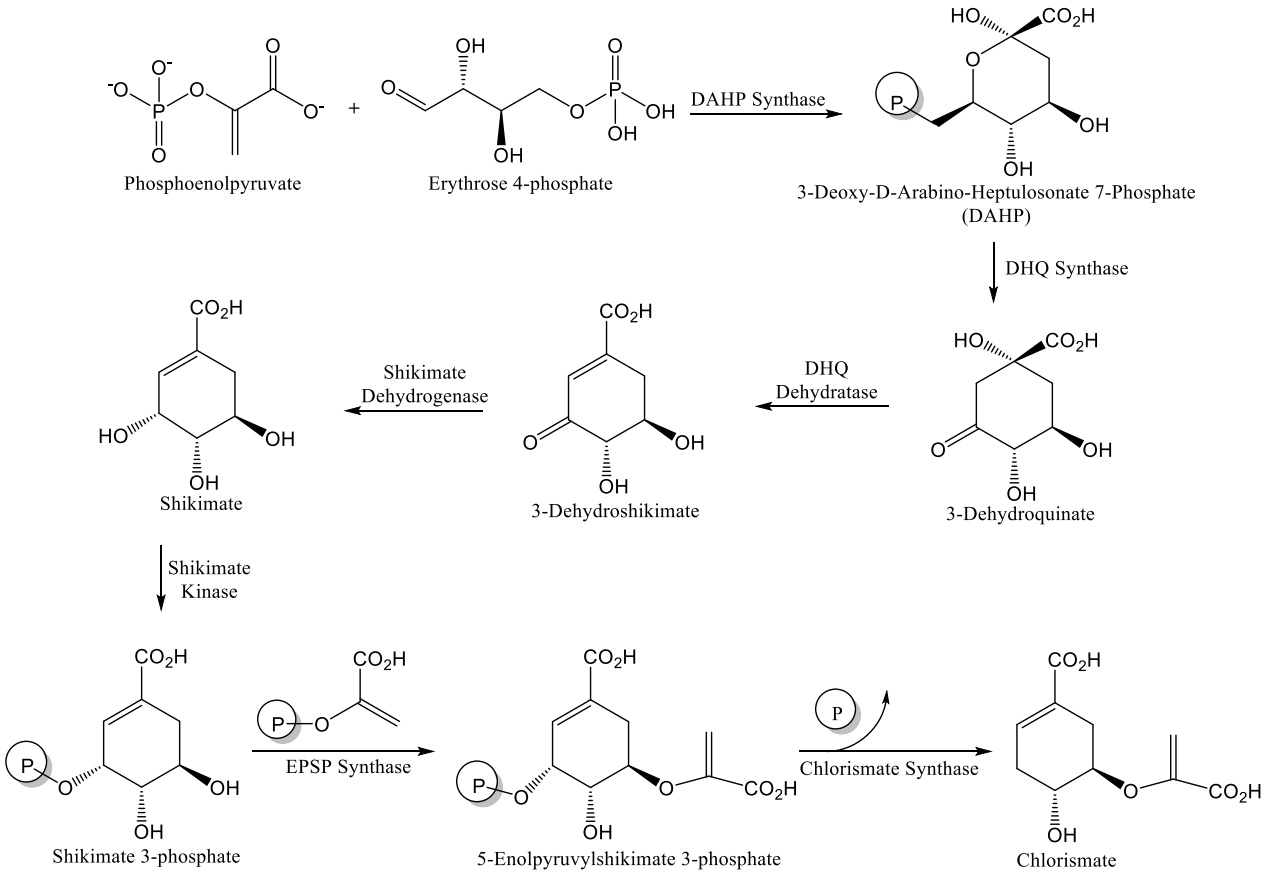


Figure 1.4: Biosynthesis of chlorismate: The precursor for tryptophan biosynthesis

Chlorismate subsequently undergoes six additional biotransformations catalyzed by five different enzymes, with Trp synthase (E.C. 4.2.1.20) catalyzing the last two steps to complete the

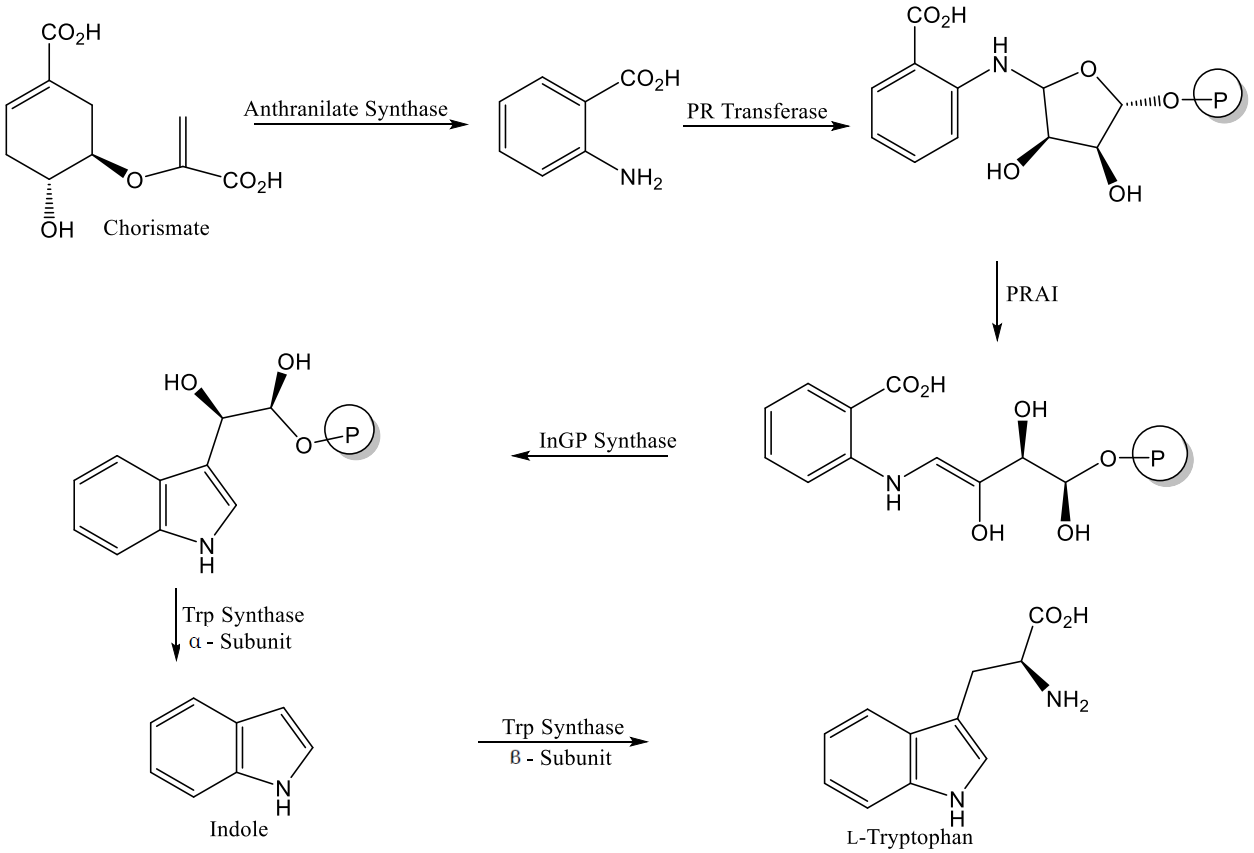


Figure 1.5: Biosynthesis of L-tryptophan

remaining pathway of Trp biosynthesis (Figure 1.5).[22] In the first step, chlorismate is converted to anthranilate by anthranilate synthase. Subsequent reaction with phosphoribosyl (PR) transferase converts it to phosphoribosylanthranilate (PRA). Opening of the ribosyl ring by PRA isomerase and recyclization by indole glycerol-3-phosphate (InGP) synthase yields InGP. This source of InGP is catalyzed by Trp synthase (Chapter 2) in two sequential steps to give Trp, the end product of the pathway. Trp synthase is an $\alpha_2\beta_2$ bienzyme complex with an intriguing "tunneling" mechanism and comprehensive reviews were published elsewhere.[23-25] InGP enters the α-subunit and gets converted to indole in the α-reaction. Interestingly, the two subunits are

interconnected by a channel approximately 25 Å in length, capable of accommodating four indoles.[26] Indole released from the α-subunit will travel, or "tunnel", its way to the active site of the β-subunit and initiates the β-reaction, which is also a pyridoxal-5'-phosphate (PLP)-dependent reaction.[27-28] The main function of the β-subunit is the catalytic condensation of indole with L-serine (Ser) to give Trp.

Even though chemical synthesis provides a virtually limitless number of possibilities to obtain Trp and its derivatives, they often suffer from difficulty of optical resolution or lengthy procedures of asymmetric synthesis to introduce chirality at the α-carbon. The biosynthesis of Trp from chlorismate has negligible industrial application in the production of Trp. In fact, it is common practice to synthesize Trp or derivatives from indole or substituted indole, respectively, catalyzed by Trp synthase in condensation with Ser.[29]

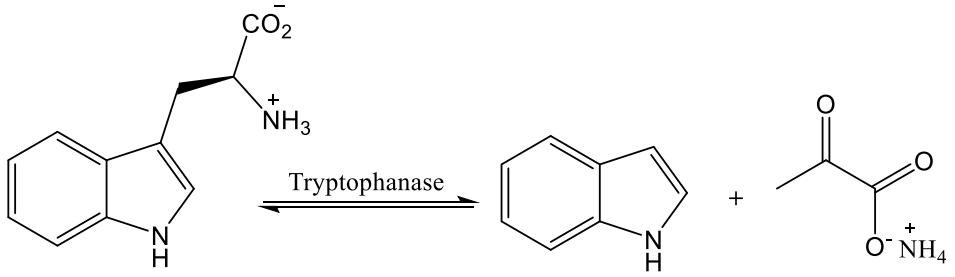


Figure 1.6: Cβ-Cγ Cleavage of L-tryptophan by tryptophan indole-lyase

Tryptophan indole-lyase (tryptophanase, TIL, E.C. 4.1.99.1) is a PLP-dependent bacterial enzyme that catalyzes the hydrolytic cleavage of the Cβ-Cγ bond of Trp (Chapter 2) to give indole and ammonium pyruvate (Figure 1.6).[30] As this reaction is reversible,[31] TIL can also

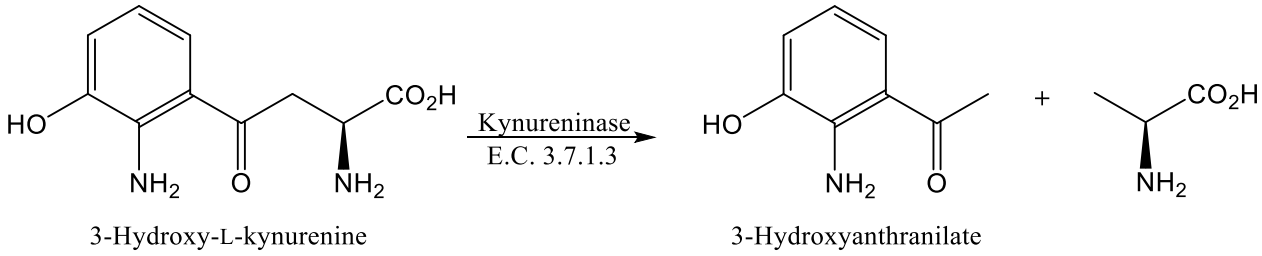
catalyze the reverse reaction, synthesis of Trp from indole and ammonium pyruvate (Chapter 4). The use of TIL in the synthesis of Trp, either by enzymatic synthesis[32] or whole-cell catalysis,[33] using indole and ammonium pyruvate were previously reported. The *in vivo* catalytic activity of TIL is highly specific for only the L-isomer of Trp. However, in a concentrated solution of diammonium hydrogen phosphate, the *in vitro* activity toward the D-isomer was also previously reported.[34-35] In addition, TIL was also previously reported to catalyze the *in vitro* enzymatic synthesis of Trp from condensation of Ser and indole, the physiological reaction of Trp synthase.[36]

1.2 Tryptophan Metabolism: Tryptophan Indole-Lyase and Kynureninase

Dietary Trp is subjected to two common metabolic fates. First, it can be delivered to tissues for protein synthesis. Second, the unused portion would undergo many important metabolic pathways which are critical and essential to functions of the human body. Among those is the serotonin pathway in which Trp serves as the precursor for the biosynthesis of melatonin[37] and serotonin,[38] two neurotransmitters regulating many of our neurological responses. Another important metabolic pathway is the kynurenine pathway. This pathway accounts for 99% of Trp metabolized by the liver, and is therefore, commonly referred to as the principle route of tryptophan catabolism.[39]

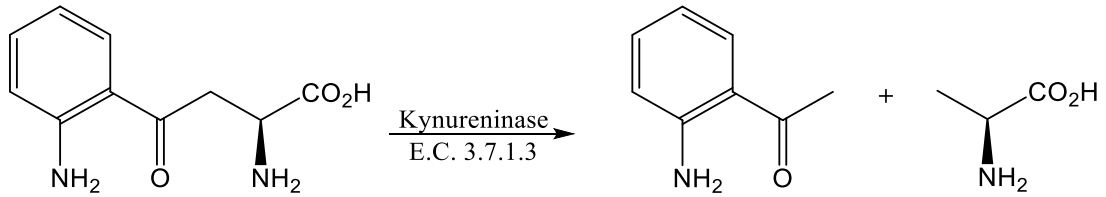
The kynurenine pathway commonly refers to the collective pathway of three separate segments.[40] Total metabolism of Trp following this pathway yields carbon dioxide (CO_2) and adenosine triphosphate (ATP) as end products. The first segment refers to the conversion of L-Trp to kynurenine. The next segment further metabolizes kynurenine to give

aminocarboxymuconic semialdehyde and quinolinate. The last segment results in total oxidation to give CO_2 and ATP. Tryptophan-2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO) are two enzymes that initiate and regulate the catabolism of tryptophan.[40-41] TDO is induced by tryptophan and metabolic steroids whereas IDO is induced by interferon-γ. The fact that tryptophan catabolism is triggered by different enzymes and induced by different agents suggests that there is a physiological switching mechanism between different states of the body.



3-Hydroxy-L-kynurenine 3-Hydroxyanthranilate

Figure 1.7: Activity of mammalian kynureninase



L-Kynurenine Anthranilate

Figure 1.8: Activity of kynureninase in microorganisms

Kynureninase (E.C. 3.7.1.3) is a PLP-dependent enzyme found in the kynurenine pathway. The physiological activity of kynureninase is different in humans and microorganisms.[42] In humans, kynureninase catalyzes the Cβ-Cγ bond cleavage of 3-hydroxy-L-kynurenine to give 3-hydroxyanthranilate and L-alanine (Figure 1.7).[43] Though, in bacteria and some fungi, kynureninase directly cleaves L-kynurenine to anthranilate and L-alanine (Figure 1.8).[44] With recent reports suggesting the activities of enzymes in the kynurenine pathway and their roles in neurodegenerative diseases, including Huntington's chorea[45] and Alzheimer's disease,[46] multiple sclerosis,[47] and HIV-related dementia,[48] much of the recent effort has focused on the design, synthesis, and evaluation of inhibitors for enzymes in the kynurenine pathway.

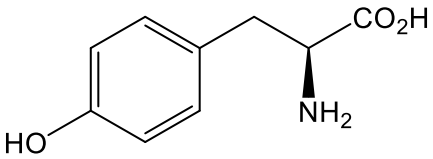
TIL discussed in the previous section is a bacterial enzyme also found in the metabolic pathway of Trp, catalyzing the hydrolytic cleavage of the Cβ-Cγ bond of Trp (Figure 1.6). Due to the difficulty of obtaining diffraction-quality crystals, the first structure of *Escherichia coli* TIL was not reported until 1991.[49] In its physiological reaction, indole, an unactivated carbon group with pKa of ~44, is the leaving group in this reaction. This fact is intriguing and suggests that an assisted-cleavage mechanism for indole is involved at the active site. Previous effort from our group and that of others (Chapter 2 and references therein) in elucidation of its mechanism has provided an explanation for this phenomenon, suggesting the formation of a quinonoid intermediate that undergoes proton transfer and carbon-carbon cleavage immediately prior to the departure of indole.

Interestingly, for many years indole produced from microorganisms was considered a metabolic waste product of Trp. However, recent reports suggested the role of metabolic indole as a bacterial signaling molecule and the activity of TIL as a promising target for antibacterial treatment. Evidence that metabolic indole has a critical role in bacterial biofilm formation was

previously reported by Di Martino *et. al.* through elegant experiments using Δ*tnaA* gene deletion and dose-dependent inhibition studies.[50-51] Recently, Lee *et. al.*[52] observed higher activity of TIL and indole production in high-resistance bacterial strains in the presence of antibiotics. Vega *et. al.*[53] also reported the overproduction of indole as a bacterial resistance mechanism in the presence of antibiotics. As TIL is a bacterial enzyme not found in eukaryotes, inhibition of its activity presents a novel and selective approach to treatment of biofilm formation and antibiotic resistance.

1.3 Tyrosine: Metabolism and Tyrosine Phenol-Lyase

L-Tyrosine (Tyr) is one of the 22 natural amino acids and is the second of three aromatic amino acids (Figure 1.9). Liebig first isolated Tyr,[54] along with leucine, in 1846 by reacting alkaline salt with casein and obtained a white needle-shaped solid that he did not elaborate with much description. In 1847, Liebig reported this white needle-shaped solid and gave it the name "tyrosine", a Greek word for cheese, reflecting the first source in which it was discovered.[55] However, it was until 1883 that the structure of tyrosine was first established through chemical synthesis by Erlenmeyer and Lipp.[56] Though, the synthesis reported was for tyrosine racemate but the optical activity of Tyr was also recognized during this time.[57]



L-Tyrosine (Tyr, Y)
S-2-Amino-3-(4-hydroxyphenyl)-propanoic acid

Figure 1.9: Structure of L-Tyrosine

Tyr is considered a conditional essential amino acid as the human body is capable of biosynthesizing Tyr. However, it requires Phe, an essential amino acid as a precursor. Tyr metabolism has a vital role in the human body as it is the precursor in the biosynthesis of neurotransmitters dopamine, norepinephrine and adrenaline, and also the precursor for melanins.[58] Many diseases are suggested to be related to Tyr metabolism, and among those, include Parkinson's disease, hypertension, hypothyroidism, as well as genetic-defective conditions, Hawksinuria and Alkaptonuria.[58]

Tyrosine phenol-lyase (TPL, E.C. 4.1.99.2) is a bacterial enzyme in one of Tyr metabolic pathway (Chapter 3 and references therein). Its physiological activity is the reversible hydrolytic cleavage of the Cβ-Cγ bond of Tyr to yield phenol and ammonium pyruvate. *In vitro*, TPL can also catalyze the β-elimination reactions for a diverse number of substrates. Interestingly, TPL and TIL from different bacteria share approximately 40% of their sequence identity leading to both enzymes adopting similar folds in their three-dimensional structures. Furthermore, the accepted mechanisms of both enzymes are remarkably similar. Substrate-bound complexes at the active site give rise to the external aldimines which undergo deprotonation at the α-carbon to give the quinonoid intermediates. The quinonoid intermediates, upon proton transfer and the Cβ-Cγ cleavage, give indole or phenol, respective to their substrates. Given the similarities in their mechanistic details, design for mechanism-based inhibitors of TIL (Chapter 2) is also speculated to be applicable to the design for mechanism-based inhibitors of TPL (Chapter 3).

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CHAPTER 2

DESIGN, SYNTHESIS AND EVALUATION OF INHIBITORS SELECTIVE FOR TRYPTOPHAN INDOLE-LYASE AND NOT TRYPTOPHAN SYNTHASE. TOWARD THE DEVELOPMENT OF ANTI-BIOFILM FORMATION[1]

[1]Do, Q. T., Nguyen, G. T., Celis, V., and Phillips, R. S. Design, Synthesis and Evaluation of Inhibitors Selective for Tryptophan Indole-Lyase and not Tryptophan Synthase. Toward the Development of Anti-biofilm Formation. (*To be submitted*)

2.1 Abstract

Recent reports suggested tryptophan indole-lyase (tryptophanase, TIL, E.C. 4.1.99.1) as a promising target for treatment of biofilm formation and antibiotic resistance. The report herein described the design, synthesis and evaluation of mechanism-based inhibitors for TIL. As previously suggested by our group that quinonoid is an intermediate in the physiological reaction of TIL, we anticipated that homologation of the physiological substrate, Trp, at the Cα position would provide analogues resembling the transition state and potentially inhibit the activity of TIL. Our results indicated that L-homotryptophan (**1a)** is a moderate inhibitor of TIL with K_i = 67 μM, whereas L-bishomotryptophan (**1b)** displayed potent inhibition with K_i = 4.7 μM. Pre-steady-state kinetics evaluation indicated the formation of an external aldimine and quinonoid with **1a** but only the formation of an external aldimine for **1b**, suggesting the differences in inhibition mechanism. In addition, Trp analogues evaluated as efficient inhibitors for TIL were also previously reported to display potency toward inhibition of Trp synthase, an enzyme widely distributed in plants, fungi and bacteria. Our results indicated that compound **1b** is at least 25-fold more selective toward TIL than Trp synthase. We desired to report that compound **1b** is comparable to the most potent inhibitor reported while displaying higher selectivity for TIL, suitable for the approach toward the development of an antibacterial treatment.

2.2 Introduction

Biofilm is a structured community of bacteria enclosed within a self-produced polysaccharide polymeric matrix. In a natural aquatic ecosystem, bacteria are found to exist in biofilm more than in their planktonic state. Formation of biofilms is critical for survival as

bacteria generally show higher resistance to biological and chemical attack as compared to their planktonic state.[1-6] Biofilm formation is a major concern for interference of machinery operability in industry,[7-8] failure of medical devices,[9] and infections of implants.[10] In addition, it is estimated that up to 80% of bacterial infection cases are caused by biofilm formation.[11] Each year, it costs the healthcare system billions of dollars in effort to combat infections and equipment failure resulting from biofilm formation.[6] Therefore, an understanding of the mechanistic details of biofilm formation and development of effective anti-biofilm treatments is of current interest in many laboratories.

Tryptophan indole-lyase (tryptophanase, TIL, E.C. 4.1.99.1) is a pyridoxal-5'-phosphate (PLP)-dependent bacterial enzyme that catalyzes the reversible hydrolytic cleavage of the Cβ-Cγ bond of Trp to yield indole and ammonium pyruvate (Figure 2.1).[12] For many years, this source of indole was considered a bacterial metabolic waste product of Trp. However, recent reports suggested that metabolic indole has a critical role in formation of biofilm in *Escherichia coli* (*E. coli*).[13-15] In addition, previous reports also suggested indole as a bacterial signaling molecule that regulates gene expression,[16] plasmid stability,[17] pathogenicity,[18] and antibiotic resistance.[19-20] Since TIL is a bacterial enzyme not found in eukaryotes, inhibition of its activity presents a selective and attractive approach for an anti-biofilm and antibacterial treatment.

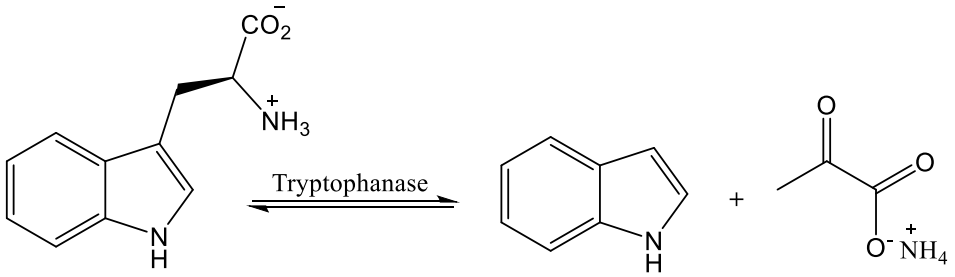


Figure 2.1: β-elimination of L-tryptophan to indole and ammonium pyruvate

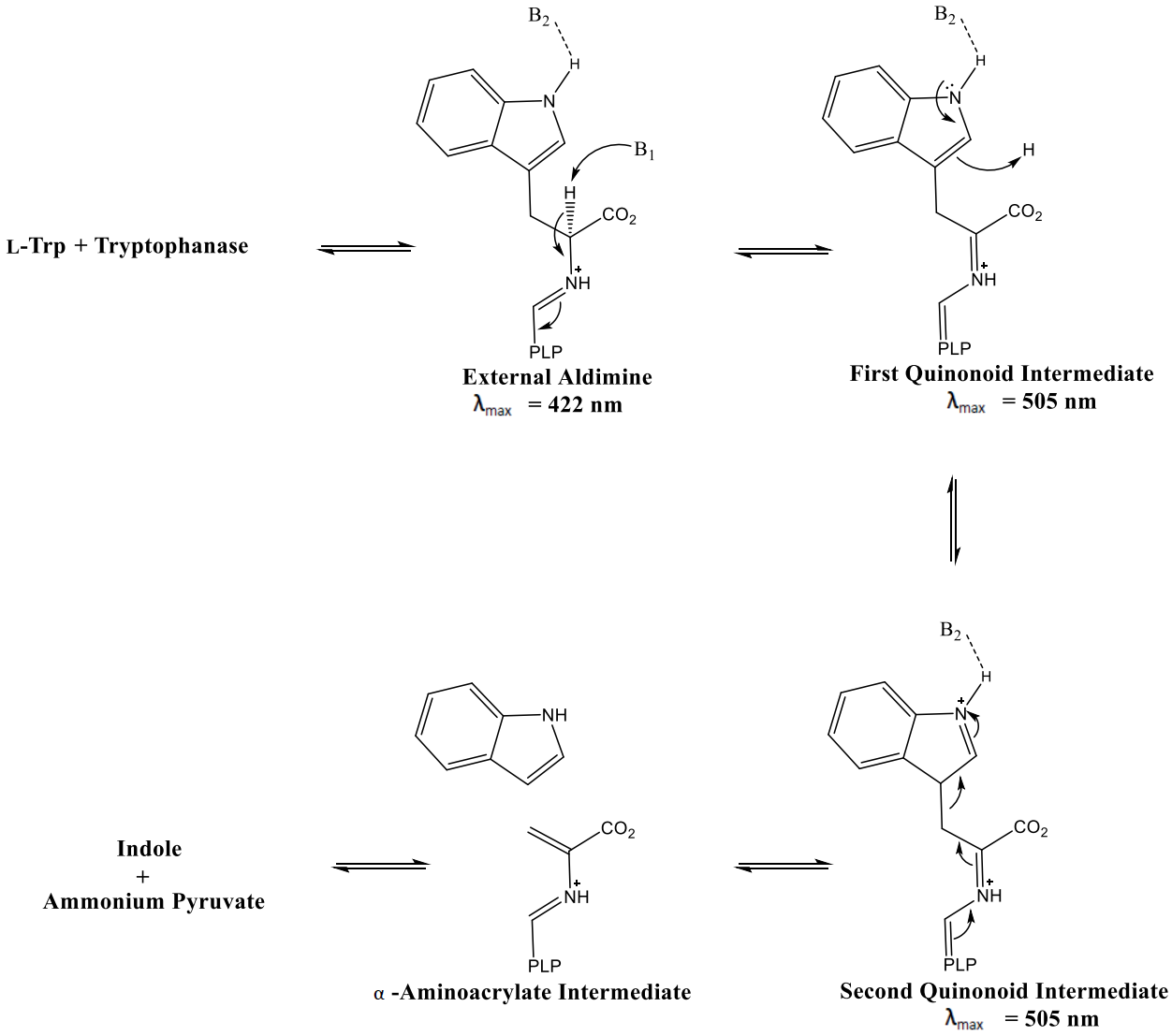
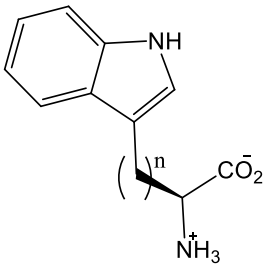


Figure 2.2: Mechanism of tryptophan indole-lyase in the cleavage of L-tryptophan

In addition to Trp, TIL can also catalyze the *in vitro* *β*-elimination for a series of substrates, including L-serine, *O*-alkyl-L-serines, *S*-alkyl-L-serines, *β*-chloroalanine, 2,3-diaminopropionate, *O*-acyl-L-serines, and *S*-(*o*-nitrophenyl)-L-cysteine (SOPC), as all of these substrates have a reasonable leaving group suitable for *β*-elimination.[21-23] However, the cleavage mechanism for its physiological substrate, Trp, is intriguing as indole is an unactivated aromatic

carbon group with pKa ~ 44 and departs as the leaving group in this reaction. Nevertheless, previous work from our group[24-29] and that of others[30-31] provided valuable insights for the catalytic mechanism of TIL (Figure 2.2).

The active form of *E. coli* TIL is a tetramer with PLP bound to Lys in the active site.[32] Once complexed, the substrate-bound PLP formed an external aldimine observed at λ_{max} = 422 nm.[25] Previously, it was suggested that indolenine was an intermediate in this reaction.[33] Further studies from our group suggested that following deprotonation of the Cα, departure of indole was accomplished by Cγ protonation followed by Cβ-Cγ bond cleavage of quinonoid intermediate, previously detected by absorbance at λ_{max} = 505 nm.[26] Based on the accepted mechanism of TIL, we were inclined to believe that L-homotryptophan (**1a**) and L-bishomotryptophan (**1b**), Trp homologues elongated at the Cα position resembling the transition state indolenine (Figure 2.3), can potentially act as potent competitive inhibitors for TIL. Discussed herein is the synthesis, inhibition kinetics and pre-steady-state kinetics evaluation of **1a** and **1b** as inhibitors for TIL. In addition, from our experience,[34] Trp analogues that are potent inhibitors of TIL are also potent inhibitors of Trp synthase, an enzyme widely distributed in plants, fungi and bacteria that catalyzes the last two steps of Trp biosynthesis.[35] Therefore, it is also of interest to determine the selectivity of **1a** and **1b** toward the activity of Trp synthase.



n = 1 = L-Tryptophan
n = 2 = L-Homotryptophan (**1a**)
n = 3 = L-Bishomotryptophan (**1b**)

Figure 2.3: Structures of L-tryptophan homologues resembling indolenine intermediate

2.3 Materials and Methods

Materials: Methylene chloride and ethanol were previously dried over CaH_2 and Mg, respectively, prior to use. Tryptophol (Acros), homotryptophol (Acros), triphenylphosphine (Aldrich), carbon tetrabromide (Janssen Chimica), diethyl acetamidomalonate ester (DEAM) (Aldrich), *Aspergillus acylase* I (0.43 unit/mg, Aldrich), indole (Aldrich), serine (USB Corp.), PLP (USB Corp.) and all other reagents (Fisher) were used without further purification. SOPC used in enzyme assays was prepared as previously described.[36] Enzyme assays were performed using distilled deionized water. All NMR data were collected on a 400 MHz Varian Mercury Plus NMR instrument and data were processed by MNova NMR processing software. ESI-MS experiments were performed on Perkin Elmer Sciex API I Plus.

Synthesis of L-Homotryptophan and L-Bishomotryptophan

3-(2-Bromoethyl)-1H-indole (**3a**). In a three-neck flask, tryptophol (2.00 g, 1 eq.) and triphenylphosphine (4.23 g, 1.3 eq.) was dissolved in dry CH_2Cl_2 (15 ml). In an addition funnel, carbon tetrabromide (5.35 g, 1.3 eq.) was dissolved in dry CH_2Cl_2 (5 ml) and added drop-wise, under inert atmosphere at 0ºC, until the addition was complete. The reaction was allowed to stir at room temperature for an additional 3 hours, or until complete disappearance of starting materials from TLC. Solvent was removed under reduced pressure and the residue was purified by column chromatography using hexane and ethyl acetate (EtOAc) to yield **3a** as an off-white solid. Yield: 2.64 g (95%). (Appendix) ^{1}H NMR ($CDCl_3$) δ (ppm): 3.32-3.36 (t, 2H), 3.62-3.66 (t, 2H), 7.10 (s, 1H), 7.12-7.16 (t, 1H), 7.19-7.23 (t, 1H), 7.37-7.39 (d, 1H), 7.59-7.61 (d, 1H), 8.04 (s, 1H).

Diethyl 2-(2-(1H-indol-3-yl)ethyl)-2-acetamidomalonate (**4a**). In a three-neck flask, DEAM (2.56 g, 1 eq.) was added to a solution of dry ethanol (20 ml) containing dissolved sodium metal (0.271 g, 1eq.) at 0ºC. The mixture was stirred for an additional 30 minutes at 0ºC. Then compound **3a** (2.64 g, 1 eq.) was added in and the solution was allowed to reflux under inert atmosphere for an additional 15 hours. Solvent was removed under reduced pressure and the residue was purified by column chromatography using hexane and EtOAc to yield **4a** as a white solid. Yield: 1.69 g (40%). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.18-1.22 (t, 3H), 2.00 (s, 3H), 2.63-2.67 (m, 2H), 2.79-2.83 (m, 2H), 4.07-4.21 (m, 4H), 6.85 (s, 1H), 6.97 (s, 1H), 7.09-7.12 (t, 1H), 7.16-7.20 (t, 1H), 7.33-7.35 (d, 1H), 7.54-7.56 (d, 1H), 8.00 (s, 1H).

2-Acetamido-4-(1H-indol-3-yl)butanoic acid (**5a**). In a round bottom flask, NaOH (0.225 g, 1.2 eq.) was dissolved in aqueous tetrahydrofuran (2:1, THF:H_2O, 45 ml) solution. Then compound **4a** (1.69 g, 1 eq.) was added in and the solution was allowed to reflux for 15 hours. The solvent was then removed under reduced pressure and the residue was taken up in water and EtOAc. The aqueous layer was acidified to pH 2 using 6M HCl and extracted with EtOAc. The organic layer was washed with water and dried over $MgSO_4$. Removal of solvent under reduced pressure give crude **5a** as a yellow solid which can be further purified by recrystallization in water to give pure **5a** as an off-white needle-shaped solid. Yield: 0.684 g (56%). ^{1}H NMR (DMSO) δ (ppm): 1.87 (s, 3H), 1.98-2.01 (m, 2H), 2.63-2.74 (m, 2H), 4.15-4.20 (m, 1H), 6.93-6.97 (t, 1H), 7.02-7.06 (t, 1H), 7.07 (s, 1H), 7.30-7.32 (d, 1H), 7.48-7.49 (d, 1H), 8.22-8.24 (1H), 12.51 (broad, 1H).

L-Homotryptophan (**1a**). In a 50 ml culture tube, compound **5a** (150 mg) was dissolved in potassium phosphate buffer (9 ml, 100 mM) and the final pH was adjusted to 8 using 6M NaOH. Then *Aspergillus acylase* I (45 mg) was added to the mixture and it was allowed to incubate

overnight at 37ºC, with stirring speed of 250 rpm (C25, New Brunswick Scientific). The resulting mixture was cooled, filtered and washed with cold water to give pure **1a** as a crystalline solid. No additional product was observed after readjusting pH of the filtrate to 6 using 6 M HCl. Attempts to readjust pH of the filtrate to 8, introducing additional enzyme and longer incubation time also did not result in additional product. Yield: 49.2 mg (78%). Unreacted **5a** can be recovered by acidifying the filtrate to pH 2 using 6 M HCl and extraction using EtOAc. ESI-MS M+1 (m/z): 219. ^{1}H NMR (D_2O/NaOD) δ (ppm): 1.72-1.88 (m, 2H), 2.62-2.66 (t, 2H), 3.12-3.16 (t, 1H), 6.97-7.01 (t, 1H), 7.06-7.09 (t, 2H), 7.32-7.34 (d, 1H), 7.53-7.55 (d, 1H).

L-Bishomotryptophan (**1b**)**.** Compound **1b** was obtained following the procedure described above for compound **1a**. Starting with homotryptophol **2b**, bromination using the Appel's salt yielded **3b** as a yellow oil (yield: 98%). Alkylation of **3b** with DEAM gave **4b** as an oil (yield: 58%). Ester hydrolysis and decarboxylation of **4b** in aqueous NaOH gave crude **5b** as a yellow solid which upon purification by recrystallization in water gave a white solid (yield: 55%). Enantioselective hydrolysis of *N*-acetyl using *Aspergillus acylase* I, as described above, gave **1b** as a white crystalline solid (yield: 71%).

Enzymes and Enzyme Assays

TIL was obtained and purified from *E. coli* JM101 containing plasmid *pMD6*, as previously described,[37] with *tnaA* gene being under natural regulation.[38] Enzyme assays were performed on a Cary 1 UV-visible spectrophotometer equipped with a Peltier-controlled six-cell changer. Enzyme activity was routinely determined as previously described,[39] by following the decrease in absorbance of SOPC at λ = 370 nm, in 50 mM phosphate buffer, pH 8.0, at 25°C. Enzyme concentration in solution was estimated spectrophotometrically from the absorbance of

holoenzyme at 278 nm.[37] Trp synthase was obtained and purified from *Salmonella typhimurium* as previously described.[34] Concentration and enzyme activity were determined, using the above spectrophotometer, by following the increase in absorbance of Trp at λ = 290 nm.[40]

Enzyme Inhibition Assays

TIL has relatively high *in vitro* activity for *β*-elimination of SOPC to give *o*-nitrothiophenol and ammonium pyruvate.[41] Enzyme inhibition assays for TIL were performed as previously described[37] by following the decrease in absorbance of SOPC at λ = 370 nm. Concentration of buffer, enzyme, cofactor, and total volume were constant whereas the concentration of substrate and inhibitors were varied for each assay. A typical enzyme inhibition assay contained potassium phosphate (50 mM), PLP (40 μM), with varying concentration of SOPC, **1a** or **1b** in a total volume of 600 μl. Inhibition assays for Trp synthase were performed as previously described,[34] by following the increase in absorbance of Trp at λ = 290 nm. Similarly, Trp synthase assays were performed in phosphate buffer (50 mM) containing PLP (40 μM), indole (0.1 mM), with varying concentration of serine, **1a** or **1b** in a total volume of 600 μl. Experimental velocities from inhibition assays were fitted and inhibition constant, K_i, was calculated using the FORTRAN program, COMPO, of Cleland.[42]

Rapid-Scanning Stopped-Flow Experiments

Prior to use, enzyme was incubated with 0.5 mM PLP for 30 minutes and subsequently purified by eluting through a Sepharose gel filtration column (PD-10, Pharmacia) to remove excess PLP. It was eluted with phosphate buffer (20 mM, pH 8, 0.16 M KCl) and rapid-scanning stopped-flow experiments were carried out in the same buffer. Rapid-scanning stopped-flow experiments were performed on an RSM-1000 spectrophotometer (OLIS, Inc.), equipped with a

stopped-flow cell mixer compartment of 1 cm path length capable of producing up to 1000 scans per second with a dead time of 2 ms.

2.4 Results and Discussion

Synthesis of L-Homotryptophan and L-Bishomotryptophan

Syntheses for racemates of **1a**[43] and **1b**[44] were previously reported but to our knowledge, no attempt for optical resolution of these compounds was reported in the literature. Elegant asymmetric syntheses of **1a** were also reported, using the Schollkopf chiral auxiliary[45] and Larock's heteroannulation.[46] However, both methods for asymmetric synthesis suffered from lengthy procedures to introduce the chiral center. Following a modified procedure,[35] we were able to obtain the *N*-acetyl derivatives **5** in three steps which can be enzymatically resolved to conveniently yield the optically active **1** (Figure 2.4). Tryptophol (**2a**) and homotryptophol (**2b**) were obtained from commercial sources and selected as starting materials. Bromination of compound **2** using phosphorus tribromide, in our hands, only gave modest yields consistent with previous report for this reaction.[47] Alternatively, the Appel's salt is a milder brominating agent for primary alcohols. When bromination was carried out using the Appel's salt, a clean conversion with high yield was achieved. Alkylation using one equivalent of DEAM afforded **4** in modest yield.[43] Hydrolysis using five equivalent of NaOH as reported by Snyder,[43] resulted in excess amount of salt recovered during the workup and did not provide the decarboxylated product **5**. However, when using 1.2 equivalent of NaOH, hydrolysis and decarboxylation was achieved in one step to yield the *N*-acetyl derivatives **5**. Enantioselective hydrolysis by *Aspergillus acylase* following the conditions reported for *N*-acetyl-α-amino acids[48] was difficult

due to low solubility of **5** in water. Alternatively, as this step was carried out in phosphate buffer at pH 8, we found that solubility was greatly enhanced. Hydrolysis of *N*-acetyl **5** following this condition yielded the optically active **1a** and **1b** which were conveniently recovered through simple filtration as both compounds have very low solubility in phosphate buffer.

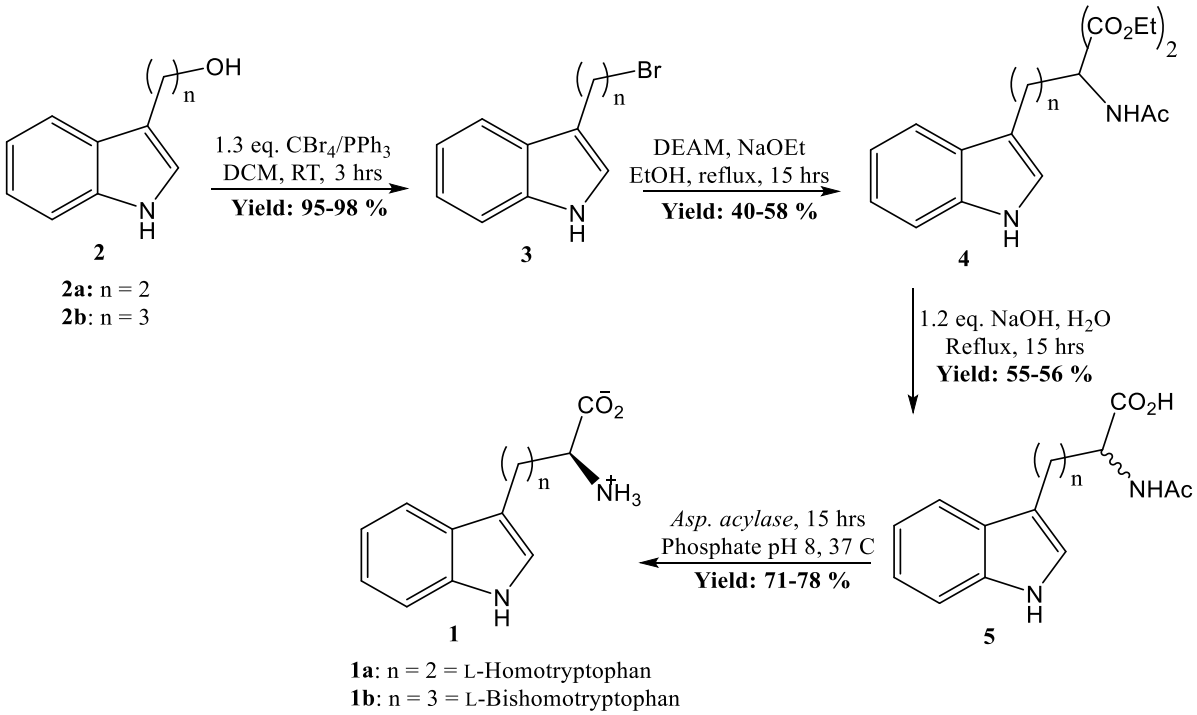


Figure 2.4: Synthesis of L-homotryptophan and L-bishomotryptophan

Enzyme Inhibition Kinetics

Experimental velocities obtained from inhibition assays were fitted to two equations (Equations 1 and 2) using the FORTRAN program, COMPO, of Cleland.[42] Our results indicated that **1a** exhibited moderate inhibition against TIL with K_i = 67 ± 12 μM. However, by further extending an additional methylene at the Cα position, compound **1b** displayed more than an

order of magnitude increase in potency as inhibitor for TIL, with K_i = 4.7 ± 0.5 μM. Previously, Phillips *et. al*. reported 2-oxindolyl-L-alanine and 2,3-dihydrotryptophan as two potent inhibitors for TIL (K_i = 2.5 and 4.5 μM, respectively).[34] To our knowledge, these compounds are the most potent inhibitors reported to date for TIL. Parola *et. al.* also reported the evaluation of Trp and anthraquinone derivatives as inhibitors for TIL, with very modest inhibition (K_i = 48 – 174 μM).[49] More recently, our group also reported the evaluation of several benzimidazole homologues of Trp as inhibitors (Figure 2.5) with the most efficient compound being 4-(benzimidazol-1-yl)-2-aminopropionic acid (**6b**), a benzimidazole derivative of homotryptophan, with K_i = 13 μM.[50] To our knowledge, **1b** is the only compound thus far to achieve a K_i in the same order as 2-oxindolyl-L-alanine and 2,3-dihydrotryptophan while displaying higher selectivity for TIL in comparison with Trp synthase.

$$v_{obs} = \frac{k_{cat}[S]}{K_m + [S]}$$

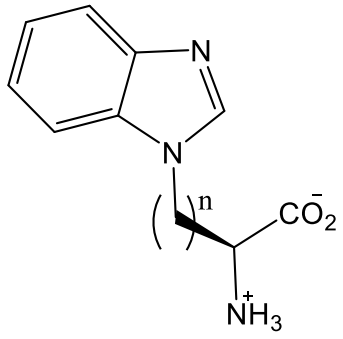
Equation 1

$$v_{obs} = \frac{k_{cat}[S]}{K_m\left(1 + \frac{[I]}{K_i}\right) + [S]}$$

Equation 2

It is interesting to note that from our previous work with benzimidazole derivatives,[50] by replacing the C-3 carbon of Trp with a nitrogen, compound **6a** was also a substrate for TIL. By extending the Cα position with one methylene, **6b** showed high efficiency as competitive inhibitor. However, further extension with a second methylene resulted in a great decrease in the potency of **6c**. Based on these results, our initial prediction was **1a** would show greater inhibition than **1b**. In contrast, **1b** was demonstrated to have a K_i of more than 10-fold higher in efficiency

than **1a**. As indole and imidazole are isoelectronic, this difference is probably attributed to their electrostatic potential influencing the binding of compounds **1** and **6** at the active site, rather than their three-dimensional structures in solution.



n = 1 = **6a**
n = 2 = **6b**
n = 3 = **6c**

Figure 2.5: Benzamidazole analogues of tryptophan as inhibitors of TIL[50]

Pre-Steady-State Kinetics

The reaction kinetics is relatively fast for monitoring through UV-Vis, and therefore, rapid-scanning stopped-flow experiments were conducted to determine the initial substrate-enzyme binding interaction. Pre-steady-state kinetics evaluation (Figure 2.6) suggested differences in inhibition mechanism of both compounds. For **1a**, an absorbance at λ_{max} = 420 nm followed by a second absorbance at λ_{max} = 505 nm were observed. We find this result consistent with the accepted mechanism for TIL in which the initial formation of aldimine gave rise to λ_{max} = 422 nm and subsequent formation of quinonoid intermediates gave rise to the absorbance at λ_{max} = 505 nm.[24] As a result, we assigned the absorbance at λ_{max} = 420 nm to the formation of an external aldimine, formed by complexing of **1a** with PLP at the active site. This was followed by

the absorbance at λ_{max} = 505 nm suggesting the formation of a quinonoid intermediate.[29] Therefore, we proposed that compound **1a** complexes with PLP at the active site, followed by deprotonation of Cα and transitioned to quinonoid intermediates but there was no indication for carbon-carbon bond cleavage leading to the departure of indole. With compound **1b**, only the absorbance at λ_{max} = 420 nm was detected and corresponded to the formation of an external aldimine. However, formation of quinonoid intermediates was not observed indicating that Cα deprotonation did not take place.

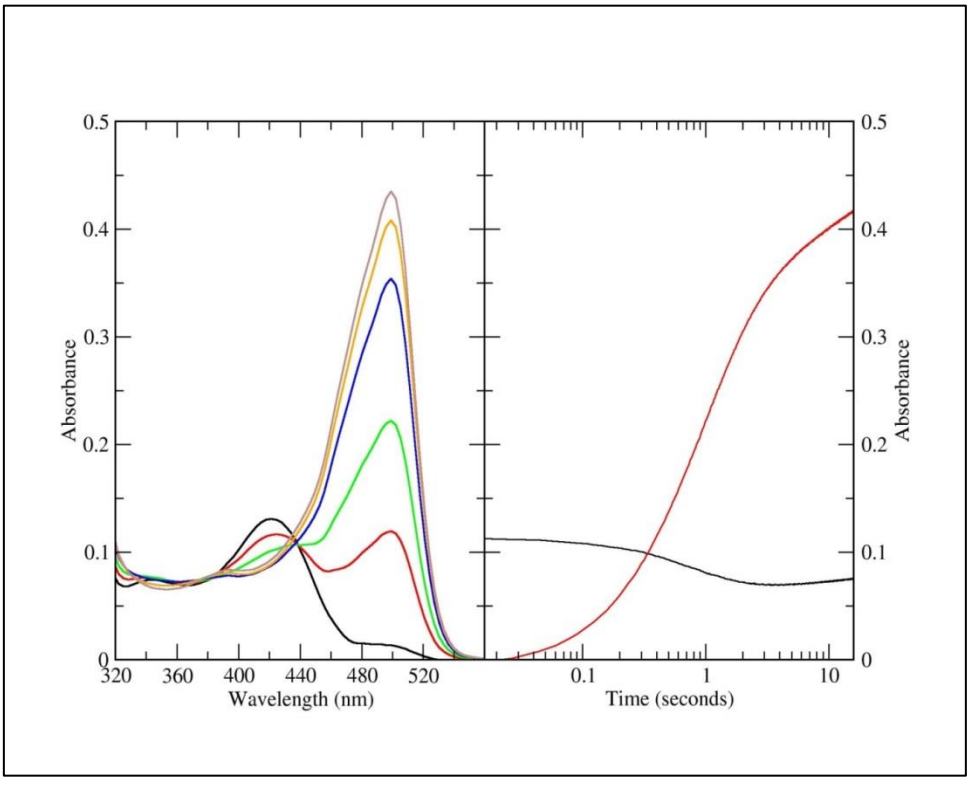


Figure 2.6a: Rapid-scanning stopped-flow for compound **1a**

Interestingly, by extending one additional methylene at the Cα position (**1a**), the reaction is mechanistically similar to that of the physiological substrate but carbon-carbon cleavage did not occur. This can be explained by the nonparticipation of C2-C3 double bond. In compound **1a**, the indole ring is positioned on the γ-carbon prohibiting the participation of C2-C3 π-electrons to participate in the β-elimination of indole. In the case of **1b**, with extension of two additional methylenes the compound adopted a different three-dimensional structure for the substituent at C3, with respect to the indole ring. Our explanation is that the Cα-proton is probably no longer within proximity for deprotonation to occur, which explains why the absorbance around λ_{max} = 505 nm for quinonoid intermediate was not observed.

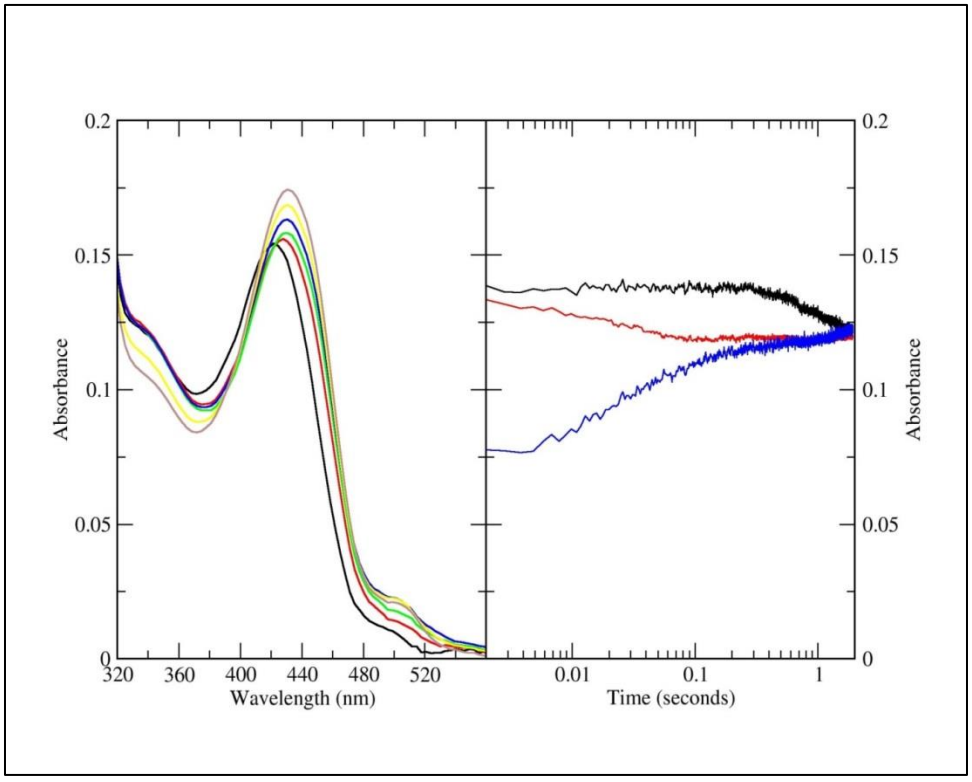


Figure 2.6b: Rapid-scanning stopped-flow of compound **1b**

Inhibition Assays with Tryptophan Synthase

Tryptophan synthase is also a PLP-dependent enzyme that catalyzes the last two steps of Trp biosynthesis.[35] From our experience,[34] Trp analogues that are potent inhibitors of TIL also showed nonselective potency toward Trp synthase. To our knowledge, the most efficient inhibitors of TIL are also the most efficient inhibitors of Trp synthase reported to date.[34] With regard to antibacterial development, an ideal inhibitor is one in which efficiently and effectively inhibits TIL but not Trp synthase, as Trp synthase is widely distributed in plants, fungi and bacteria. Assays for compounds **1a** and **1b** with Trp synthase displayed no measurable inhibition even at a concentration of 100 μM suggesting that both compounds were more selective for TIL than Trp synthase. Compound **1b**, in our estimate, is at least 25-fold more selective for TIL. To our knowledge, compound **1b** is the first to achieve the same order of inhibition efficiency of 2-oxindolyl-L-alanine and 2,3-dihydrotryptophan while displaying higher selectivity for TIL in comparison to Trp synthase.

2.5 Conclusion

With recent reports suggesting bacterial TIL as an attractive target for treatment development toward biofilm formation and antibiotic resistance, we expressed our interest in the design for mechanism-based inhibitors of TIL. Alternative to asymmetric syntheses, we reported a modified method of Snyder *et. al.* to conveniently obtain the optically active isomer of compounds **1a** and **1b**. Evaluation of **1a** in inhibition assays indicated that it is a moderate inhibitor for TIL with K_i = 67 μM and the presence of quinonoid intermediate was observed in pre-steady-state evaluation. In contrast, **1b** displayed potent inhibition for TIL with K_i = 4.7 μM

and formation of quinonoid intermediate was not observed indicating inhibition occurred prior to deprotonation of the Cα-proton of the external aldimine. In inhibition assays with Trp synthase, no inhibition was observed for both compounds up to 100 μM indicating higher selectivity for TIL in comparison with Trp synthase. To our knowledge, L-bishomotryptophan (**1b**) is the first inhibitor with efficiency in the same order as 2-oxindolyl-L-alanine and 2,3-dihydrotryptophan while displaying higher selectivity for TIL in preference to Trp synthase, suitable for the approach toward the development of an antibacterial treatment.

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CHAPTER 3

EVALUATION OF INHIBITORS FOR TYROSINE PHENOL-LYASE: AN EXTENSION FROM THE DESIGN OF INHIBITORS FOR TRYPTOPHAN INDOLE-LYASE[2]

[2]Do, Q. T., Nguyen, G. T., and Phillips, R. S. Evaluation of Inhibitors for Tyrosine Phenol-Lyase: An Extension from the Design of Inhibitors for Tryptophan Indole-Lyase. (*To be submitted*)

3.1 Abstract

The report herein described the design, synthesis, and evaluation of Tyr homologues and their *O*-methyl derivatives as inhibitors for tyrosine phenol lyase (TPL, E.C. 4.1.99.2). Recently, we reported that homologues of its physiological substrate, Trp, were efficient mechanism-based inhibitors of tryptophan indole-lyase (tryptophanase, TIL, E.C. 4.1.99.1). As the accepted mechanisms for TPL is remarkably similar, we desired to extend our design and rationale in the search for potent mechanism-based inhibitors of TPL. From our results, all Tyr homologues and derivatives exhibited an inhibition constant, K_i, in the range of 0.8 – 1.5 mM. Pre-steady-state kinetics data were very similar for all compounds tested and suggested the formation of quinonoid intermediates upon binding. In addition, we also observed a blue-shift for the absorbance of aldimine complexes of all Tyr homologues, suggesting a possible strain at the active site possibly due to accommodating elongated substrates.

3.2 Introduction

Tyrosine phenol-lyase (TPL, E.C. 4.1.99.2) is a pyridoxal-5'-phosphate (PLP)-dependent bacterial enzyme that catalyzes the reversible hydrolytic cleavage of the Cβ-Cγ bond of Tyr to phenol and ammonium pyruvate (Figure 3.1).[1-3] In addition to its physiological substrate, TPL also catalyzes the *in vitro* β-elimination for a series of substrates including L-serine, L-cysteine, *S*-methyl-L-cysteine, *O*-acetyl-L-serines, and *S*-(*o*-nitrophenyl)-L-cysteine (SOPC).[1-2,4] As the cleavage of Tyr is reversible, TPL was also utilized in the enzymatic production of Tyr[5] and β-substitution reactions in the synthesis of 3,4-dihydroxyphenyl-L-alanine (L-dopa),[6] and aza-L-tyrosine.[7]

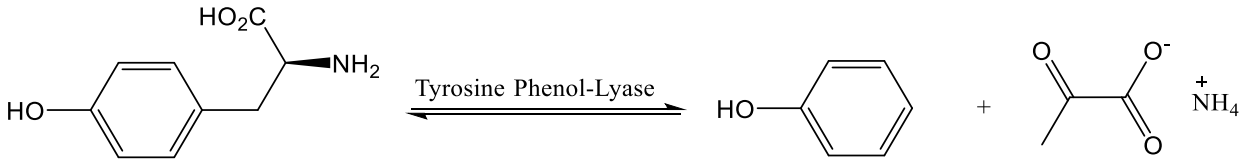


Figure 3.1: β-Elimination of L-tyrosine by tyrosine phenol-lyase

The postulated mechanism of TPL consists of several steps in which formation of quinonoid intermediate is the essential step of the enzyme's catalytic cycle (Figure 3.2).[8-12] The first step is transaldimination in which the internal aldimine is transformed to an external aldimine upon binding of the substrate. Subsequent deprotonation of the Cα proton, by the amino side chain of Lys257, gives rise to the quinonoid intermediate. Ketonization of phenol, followed by Cγ protonation, subsequently led to the cleavage of the Cβ-Cγ bond to form phenol and aminoacrylate. Previously, Phillips *et. al.*[8] and Milic *et. al.*[9-10] provided valuable insights in their reports, by isolating and characterizing stable quinonoid complexes and provided further evidence for quinonoid as the key intermediate in TPL mechanism. However, it remains unclear if Cγ proton transfer and Cβ-Cγ cleavage follow a concerted mechanism.

Tryptophan indole-lyase (tryptophanase, TIL, E.C. 4.1.99.1) is another PLP-dependent bacterial enzyme that catalyzes the reversible[13] hydrolytic cleavage of the Cβ-Cγ bond of Trp to indole and ammonium pyruvate.[14] Interestingly, TPL and TIL from various bacteria shared about 40% sequence identity[15] and both enzymes generally adopt similar three-dimensional folds.[16-17] In addition, the accepted mechanism for TIL[12,18-19] is remarkably similar to that suggested for TPL in the cleavage of their respective substrates (Figure 3.2). Recently, we reported the

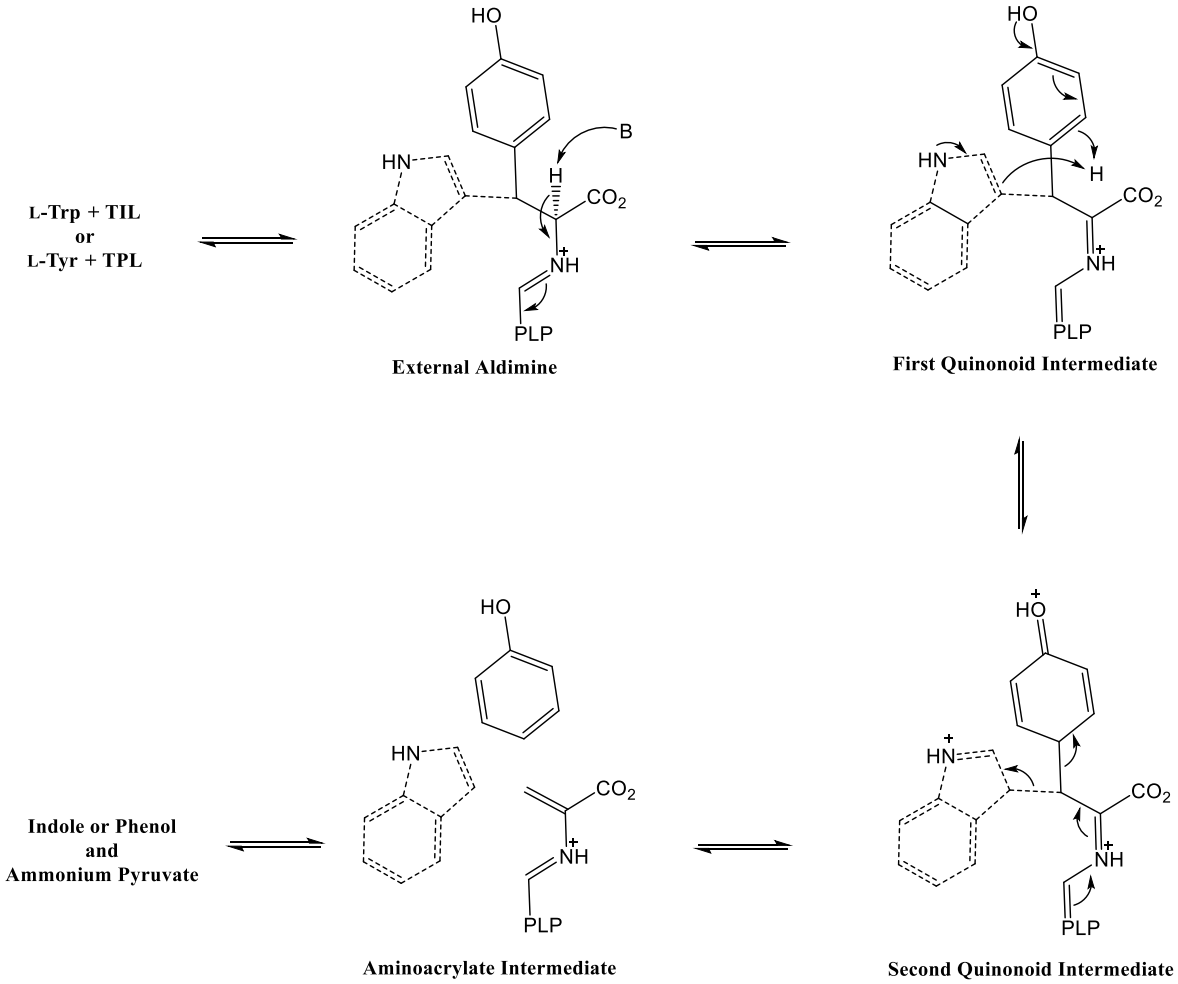
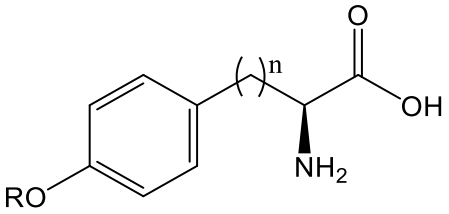


Figure 3.2: β-Elimination mechanism of TIL and TPL for Trp and Tyr, respectively

evaluation of mechanism-based inhibitors for TIL and discovered that homologation at the Cα position of the physiological substrate, Trp, effectively inhibited TIL activity.[20] Our rationale was to design a transition-state inhibitor resembling the elongated transition-state structure of indolenine intermediate. Since TIL and TPL have remarkably similar mechanisms and with encouraging results from our previous work, we are interested in extending our design and rationale in effort to search for new mechanism-based inhibitors of TPL.

In the present work, we speculated that inhibitors that structurally resemble the elongated transition-state structure of the keto-quinonoid intermediate can potentially inhibit the activity of TPL. Based on that rationale, we were inclined to believe that homologues of the physiological substrate, Tyr, can potentially inhibit TPL activity. This work described the synthesis, steady-state and pre-steady-state kinetic evaluations of L-homotyrosine (**1a**) and L-bishomotyrosine (**1b**) as mechanism-based inhibitors for TPL (Figure 3.3). The quinonoid intermediate was also suggested to undergo a closed transition state in which hydrogen bond from the hydroxyl group contributed to the Cα-Cβ-Cγ angle strain to assist in the cleavage of the Cβ-Cγ bond.[9] With this in mind, we also would like to report the evaluation of methylated derivatives of Tyr homologues, *O*-methyl-L-homotyrosine (**6a**) and *O*-methyl-L-bishomotyrosine (**6b**), as it would be of interest to explore whether methylation of the phenol OH would have an effect on the efficiency of the proposed compounds as inhibitors for TPL.



n = 1 = L-Tyr (R = H)
n = 2 = (R = H = **1a**; R = Me = **6a**)
n = 3 = (R = H = **1b**; R = Me = **6b**)

Figure 3.3: Structures of L-tyrosine and L-tyrosine homologues and derivatives.

3.3 Materials and Methods

Materials: Methylene chloride and ethanol were previously dried over CaH_2 and Mg, respectively, prior to use. 2-(4-Methoxyphenyl)ethanol (Acros), 3-(4-methoxyphenyl)propan-1-

ol (Aldrich), triphenylphosphine (Aldrich), carbon tetrabromide (Janssen Chimica), DEAM (Aldrich), *Aspergillus acylase* I (0.43 unit/mg, Aldrich), PLP (USB Corp.) and all other reagents (Fisher) were used without further purification. *S*-(*o*-nitrophenyl)-L-cysteine (SOPC) used in enzyme assays was prepared as previously described.[5] Enzyme assays were performed using distilled deionized water. All NMR data were collected on a 400 MHz Varian Mercury Plus NMR instrument and data were processed by MNova NMR processing software. ESI-MS experiments were performed on Perkin Elmer Sciex API I Plus.

Synthesis of L-Homotyrosine, L-Bishomotyrosine, and *O*-Methyl Derivatives

1-(2-bromoethyl)-4-methoxybenzene (**3a**). In a three-neck flask, 2-(4-methoxyphenyl)ethanol (3.00 g, 1 eq.) and triphenylphosphine (6.72 g, 1.3 eq.) were dissolved in dry CH_2Cl_2 (25 ml). In an addition funnel, carbon tetrabromide (8.50 g, 1.3 eq.) was dissolved in dry CH_2Cl_2 (15 ml) and added drop-wise under inert atmosphere, at 0ºC until the addition was complete. The reaction was allowed to stir at room temperature for an additional 4 hours, or until complete disappearance of starting materials from thin-layer chromatography (TLC). Solvent was removed under reduced pressure and the residue was purified by column chromatography using hexane and ethyl acetate (EtOAc) to yield **3a** as a clear oil. Yield: 4.11g (97%). (Appendix) ^{1}H NMR ($CDCl_3$) δ (ppm): 3.08-3.12 (t, 2H), 3.51-3.55 (t, 2H), 3.79 (s, 3H), 6.85-6.87 (d, 2H), 7.12-7.14 (d, 2H).

Diethyl 2-acetamido-2-(4-methoxyphenylethyl)malonate (**4a**). In a three-neck flask, DEAM (5.25 g, 1.3 eq.) was added to a solution of dry ethanol (40 ml) containing dissolved sodium metal (0.556 g, 1.3 eq.) at 0ºC. The mixture was stirred for an additional 30 minutes at 0ºC. Then compound **3a** (4.00 g, 1 eq.) was added in and the solution was allowed to reflux under inert atmosphere for an additional 15 hours. Solvent was removed under reduced pressure

and the residue was purified by column chromatography using hexane and EtOAc. Subsequent removal of eluting solvent under reduced pressure yielded compound **4a**. Yield: 3.30 g (51%). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.23-1.27 (t, 6H), 2.00 (s, 3H), 2.40-2.43 (m, 2H), 2.64-2.68 (m, 2H), 3.78 (s, 3H), 4.19-4.22 (m, 4H), 6.77 (s, 1H), 6.80-6.82 (d, 2H), 7.05-7.07 (d, 2H).

2-Acetamido-4-(4-methoxyphenyl)butanoic acid (**5a**). In a round bottom flask, NaOH (0.717 g, 2 eq.) was dissolved in aqueous tetrahydrofuran (1:1, THF:H_2O, 40 ml) solution. Then compound **4a** (3.15 g, 1 eq.) was added in and the solution was allowed to reflux for 15 hours. The solvent was then removed under reduced pressure and the resulting residue was taken up in water and EtOAc. The aqueous layer was acidified to pH 2 using 6M HCl and extracted with EtOAc. The organic layer was washed with water and dried over $MgSO_4$. Removal of organic solvent under reduced pressure gave **5a** as a white solid. Yield: 2.17 g (96%). ^{1}H NMR (DMSO) δ (ppm): 1.94 (s, 3H), 3.78 (s, 3H), 4.12-4.15 (m, 1H), 6.90-6.92 (d, 2H), 7.15-7.17 (d, 2H), 8.25-8.27 (d, 1H), 12.5 (b, 1H).

O-Methyl-L-homotyrosine (**6a**). In an Erlenmeyer flask, compound **5a** (2.00 g) was dissolved in potassium phosphate buffer (80 ml, 100 mM) and the final pH was adjusted to 8 using 6M NaOH. Then *Aspergillus acylase* I (200 mg) was added to the mixture and it was allowed to incubate overnight at 37ºC, with stirring speed of 250 rpm (C25, New Brunswick Scientific). The resulting mixture was cooled, filtered and washed with cold water to give pure **1a** as a crystalline solid. Yield: 573 mg (69%). ^{1}H NMR (D_2O/NaOD) δ (ppm): 1.61-1.70 (m, 2H), 2.38-2.42 (t, 2H), 3.03-3.06 (t, 1H), 3.62 (s, 3H), 6.76-6.78 (d, 2H), 7.05-7.07 (d, 2H).

L-Homotyrosine (**1a**). In a round-bottom flask, compound **6a** (0.1 g) was dissolved in concentrated hydriodic acid. The mixture was allowed to reflux overnight and hydriodic acid was

removed under reduced pressure. The residue was neutralized using phosphate buffer (pH 7). Solvent was again removed under reduced pressure and a minimal amount of cold water was added to the residue. The mixture was cooled, filtered and washed with water to give **1a** as a white solid. Yield: 68 mg (73%). ^{1}H NMR (D_2O/NaOD) δ (ppm): 1.54-1.62 (m, 2H), 2.24-2.28 (t, 2H), 3.04 (s, 1H), 6.34-6.36 (d, 2H), 6.79-6.81 (d, 2H).

O-Methyl-L-bishomotyrosine (**6b**) *and L-bishomotyrosine* (**1b**). Compounds **6b** and **1b** were obtained following the same procedure described above for **6a** and **1a**. Bromination of **2b** using the Appel's salt afforded **3b** as a clear liquid in excellent yield (92%). Subsequent alkylation using DEAM provided **4b** as a white solid (52%). Ester hydrolysis and decarboxylation of **4b** was achieved in one step by refluxing in aqueous NaOH to yield **5b** as a white solid in excellent yield (92%). Enantioselective hydrolysis of *N*-acetyl using *Aspergillus acylase*, as described above, provided **6b** in good yield (67%). Deprotection using hydriodic acid gave compound **1b** as a crystalline solid (69% yield).

Enzymes and Enzyme Assays

Enzyme was obtained from *Escherichia coli* SVS370/pTZTPL[11] and purified as previously described.[21] Purified enzyme was stored as aliquots at -78°C and thawed immediately prior to use. Enzyme assays were performed on a Cary I UV-visible spectrophotometer equipped with a Peltier-controlled six-cell changer and a thermoelectric block. Enzyme activity was routinely determined using SOPC as previously described,[6] by following the absorbance decrease at λ = 370 nm (Δε = 1.86 mM-1 cm-1).[22] Enzyme concentration was estimated from the absorbance at λ = 278 nm (A1% = 8.37)[23] assuming a subunit mass of 51 kDa.[16]

SOPC has very high binding affinity and was used as substrate in inhibition assays for TPL.[4] Inhibition assays were conducted on the same spectrophotometer under kinetic mode, using the same conditions previous reported for TIL,[20] by following the decrease in absorbance of SOPC at λ = 370 nm. All assays were conducted at 25°C and in a total volume of 600 μl. A typical assay would contain phosphate (50 mM), PLP (40 μM), and varying concentration of SOPC and inhibitors. Experimental velocities were analyzed and inhibition constant were calculated by the FORTRAN program, COMPO, of Cleland.[24]

Rapid-Scanning Stopped-Flow Experiment

Rapid-scanning stopped-flow experiments were performed on an RSM-1000 spectrophotometer (OLIS, Inc), equipped with a stopped-flow cell mixer compartment of 1 cm path length capable of producing up to 1000 scans per second with a dead time of 2 ms. Prior to performing kinetic experiments, stock enzyme was incubated with 0.5 mM PLP for 30 min at 30°C. Subsequently, enzyme was eluted through a short Sepharose gel filtration column (PD-10, Pharmacia) equilibrated with phosphate buffer (pH 8, 50 mM) to remove excess PLP and rapid-scanning experiments were carried out in the same buffer. Rapid-scanning stopped-flow data were processed and analyzed by GlobalWorks software supplied by OLIS.

3.4 Results and Discussion

Synthesis of homologues and derivatives

The synthesis and enzymatic resolution of **6b** and **1b**,[25-26] and the asymmetric synthesis of **6a**[27] and **1a**[28] were previously reported. In our hands, the most convenient method to obtain

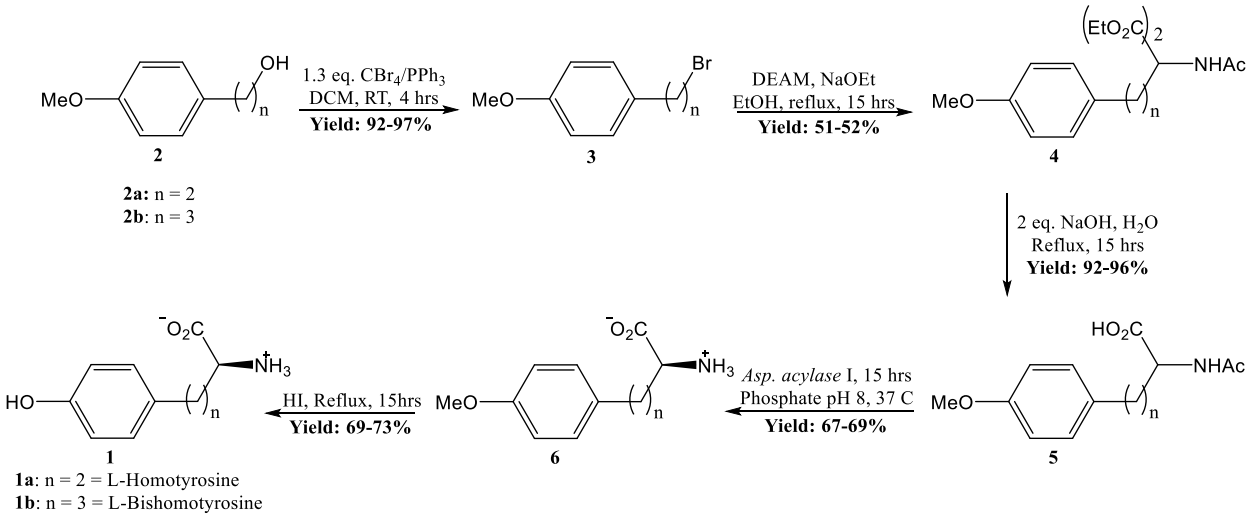


Figure 3.4: Synthesis of tyrosine homologues and *O*-methyl derivatives

the optically active Tyr homologues and their *O*-methyl derivatives was by following a modified method of Shimohigashi *et. al.*,[25] using DEAM as described previously (Figure 3.4).[20] 2-(4-methoxyphenyl)ethanol (**2a**) and 3-(4-methoxyphenyl)propanol (**2b**) are commercially available and were selected as starting materials. As Appel's salt is a milder reagent suitable for primary alcohols, when bromination was carried out using this reagent, we obtained excellent yield for **3**. Alkylation of **3** using DEAM in sodium ethoxide proceeded to give **4** in modest yield. Subsequent hydrolysis and decarboxylation of **4** was achieved in one step with excellent yield of **5** by refluxing with two equivalents of NaOH in aqueous THF. Enantioselective hydrolysis of **5** using *Aspergillus acylase*[29] was carried out in phosphate buffer at pH 8 instead of water due to low solubility of compounds **5** in water. Enzymatic resolution following this method proceeded to give the optically active **6** in good yield. Deprotection of **6** was previously reported to proceed in concentrated HCl, by refluxing for three days with optical activity remained unaffected.[25]

However, when this step was carried out in concentrated hydriodic acid, we recovered similar yields for compounds **1** within 15 hours of reflux.

Enzyme inhibition assays

Experimental velocities obtained from inhibition assays were fitted to two equations (Equations 1 and 2) and inhibition constants were calculated using the FORTRAN program, COMPO, of Cleland.[24] Our results indicated that compounds **1a, 1b**, **6a, and 6b** exhibited an inhibition constant in the range of 0.8-1.5 mM (Figure 3.5).

$$v_{obs} = \frac{k_{cat}[S]}{K_m + [S]} \qquad \text{Equation 1}$$

$$v_{obs} = \frac{k_{cat}[S]}{K_m\left(1 + \frac{[I]}{K_i}\right) + [S]} \qquad \text{Equation 2}$$

It is interesting to note that compounds **1a** and **6a**, homologues with extension of one methylene unit, displayed better inhibition than that of **1b** and **6b**, in which they were extended by two methylene units. It was previously suggested that the phenol OH is hydrogen bonded to Arg381 at the active site.[9] We were curious as to whether blocking off the hydrogen bond donor site with methylation would affect the inhibitory efficiency. Our results indicated a negligible difference between homologues **1a** and **1b** with their *O*-methyl derivatives **6a** and **6b**, suggesting that hydrogen bond between OH with that of Arg381 is not essential for the inhibitors evaluated in this work. Previously, Phillips *et. al.* reported the evaluation of 2- and 3-aza-L-tyrosines as inhibitors for TPL,[22] with K_i = 135 μM and 3.4 mM, respectively. In comparison, the rationale behind our design was to mimic the elongated structure of the keto-quinonoid transition state and deletion of the hydrogen bond donor site of phenol. However, the inhibition constants reported

herein has not surpassed that previously reported, indicating the binding affinity of these compounds for TPL is not as high as 2-aza-L-tyrosine.

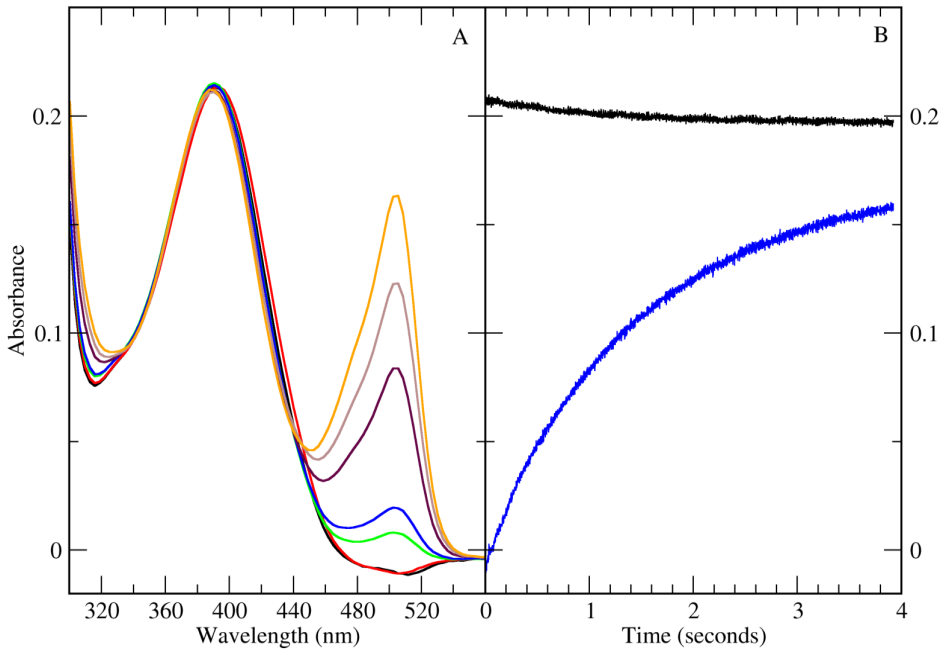
Inhibitors	**Inhibition Constant (K_i)**
L-Homotyrosine (**1a**)	0.80 mM ± 0.20 mM
L-Bishomotyrosine (**1b**)	1.50 mM ± 0.50 mM
O-Methoxy-L-homotyrosine (**6a**)	0.80 mM ± 0.17 mM
O-Methoxy-L-bishomotyrosine (**6b**)	1.04 mM ± 0.18 mM

Figure 3.5: Calculated inhibition constants

Pre-steady-state kinetics

The absorbance spectra from rapid-scanning stopped-flow experiments shared many similarities between all compounds tested (Figure 3.6). The cleavage of Tyr by TPL was previously reported to give rise to two characteristic absorbance at λ_{max} = 418 nm and 502 nm, corresponding to formation of external aldimine and quinonoid intermediate, respectively.[30] From our results, the presence of external aldimines was observed in all cases, indicated by characteristic absorbance around λ_{max} = 390 nm. In addition, the formation of quinonoid intermediates were also observed for all compounds, evident by absorbance around λ_{max} = 500 nm. From these data, we are confident that these inhibitors were bound to PLP at the active site leading to formation of external aldimines. Furthermore, deprotonation of the Cα proton has

taken place leading to formation of quinonoid intermediates as indicated by the increase in absorbance around λ_{max} = 500 nm.



Compound	$1/\tau_1$	$1/\tau_2$	E_{AL} λ_{max}, nm	E_Q λ_{max}, nm
1a	3.0 ± 0.4	(6.2 ± 0.5) x10^{-1}	387	505
1b	5.0 ± 0.3	2.39 ± 0.21	390	496
6a	5.3 ± 0.3	(1.40 ± 0.16) x10^{-1}	394	499
6b	0.66 ± 0.01	0.14 ± 0.01	387	496

Figure 3.6: Representative rapid-scanning stopped-flow data from compound **1a** (Appendix)

In evaluating these compounds, we also observed that the absorbance of aldimine intermediates for Tyr bishomologues blue-shifted slightly from that reported[30] in comparison

with Tyr being the substrate (Figure 3.6). The strain that exists in the family of aspartate aminotransferase (AAT) enzymes is well documented and a literature review is provided elsewhere.[31] From our results, we observed a blue-shift much more apparent with bishomologues of Tyr and we are attributing this shift in absorbance to the strain of aldimine at the active site.

3.5 Conclusion

With promising results from our previous work on mechanism-based inhibitors of TIL, we desired to extend our design and rationale to TPL since both enzymes shared remarkably similar mechanistic details in the cleavage of its physiological substrate. In addition, it was also suggested that the hydrogen bond from phenol of the substrate with Arg381 has an essential role in contributing to the Cα-Cβ-Cγ bond angle strain that assisted in cleavage of the Cβ-Cγ bond of the substrate. Based on the postulated mechanism of TPL, we desired to explore the effect of homologation of the Cα position and deletion of the hydrogen bond donor site of phenol from the physiological substrate, in the search for mechanism-based inhibitors of TPL. Our results indicated that the inhibition constant, K_i, of all inhibitors reported herein were in the range of 0.8-1.5 mM. Furthermore, pre-steady-state kinetic evaluations confirmed binding of these compounds at the active sites, suggesting the formation of aldimines and quinonoid intermediates.

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CHAPTER 4

ENZYMATIC SYNTHESIS OF TRYPTOPHAN FROM GLYCEROL USING TRYPTOPHAN INDOLE-LYASE[3]

[3]Do, Q. T. and Phillips, R. S. Enzymatic Synthesis of Tryptophan from Glycerol Using Tryptophan Indole-Lyase (*To be submitted*)

4.1 Abstract

The report herein described a new method for the enzymatic synthesis of L-tryptophan (Trp), a high-value essential amino acid, using glycerol as a starting material. High demand for renewable energy in recent years resulted in a surplus of glycerol, originated as a by-product of biodiesel processing. Glycerol surplus generated from biodiesel production resulted in the shutdown of glycerol plants and continued to be a disposal concern. By coupling of natural glycerol metabolic enzymes of *Escherichia coli* BL21 (DE3) with tryptophan indole-lyase expressed from pET15b:*tnaA* plasmids, Trp was produced from our method of whole-cell catalysis. The presence of Trp was detected by thin-layer chromatography with ninhydrin staining, high-pressure liquid chromatography at λ = 278 nm, and liquid chromatography coupled mass spectrometry. As a proof-of-concept, we have demonstrated and desired to report a convenient biosynthetic pathway for Trp from glycerol.

4.2 Introduction

In recent years, an increase in biodiesel production worldwide has resulted in a surplus of glycerol, originated as a by-product of biodiesel processing (Figure 4.1).[1] Biodiesel is commonly prepared by transesterification of animal fat, vegetable oil, or cooking oil employing low molecular weight alcohols in the presence of an alkaline salt.[2] It is estimated that for every 9 kg of biodiesel obtained, 1 kg of glycerol is produced as by-product.[3] In 2010, Europe produced an estimated $11.2e^6$ tons of biodiesel, whereas the United States on the other hand, produced an approximate $6.96e^6$ tons of biodiesel.[3] With the rate of biodiesel production on the rise, its unforeseen impact has resulted in the shut-down of several glycerol plants and continued to be a

disposal concern.[4-5] At present, the desired solution is to engineer methods for consumption of glycerol surplus, preferably in the conversion to high-value and marketable materials.

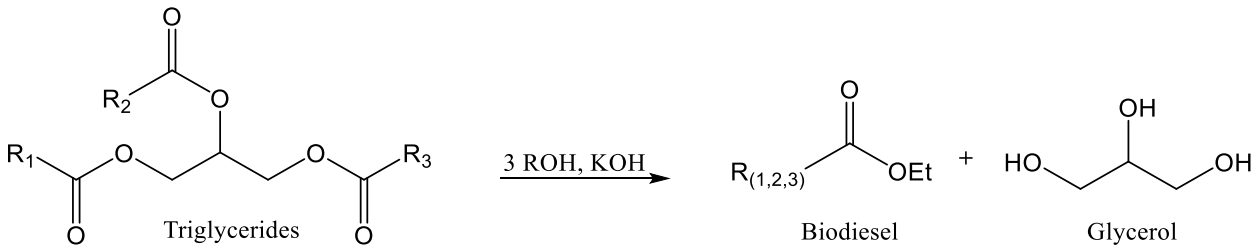

Figure 4.1: Biodiesel processing and generation of glycerol

Glycerol is taken up into the cells through facilitated diffusion, an energy-independent process catalyzed by glycerol facilitator (GlyF), a member of the broad family of aquaporins.[6] Once passed through the membrane, glycerol is phosphorylated by glycerol kinase to glycerol-3-phosphate to prevent back diffusion and initiate the glycerol dissimilation pathway through glycolysis.[7] Phosphorylation by glycerol kinase is the rate-limiting step in the dissimilation of glycerol and is regulated by feedback inhibition as the enzyme was reported to be inhibited by the glucose metabolite, fructose-1,6-diphosphate, which means this pathway is shut down in the presence of glucose.[8] To initiate glycerol dissimilation, glycerol-3-phosphate further undergoes oxidation to give dihydroxyacetone phosphate where it intercepts and funnels into the glycolysis pathway to serve as a source of carbon and energy, yielding pyruvate as the end product of this pathway (Figure 4.2).

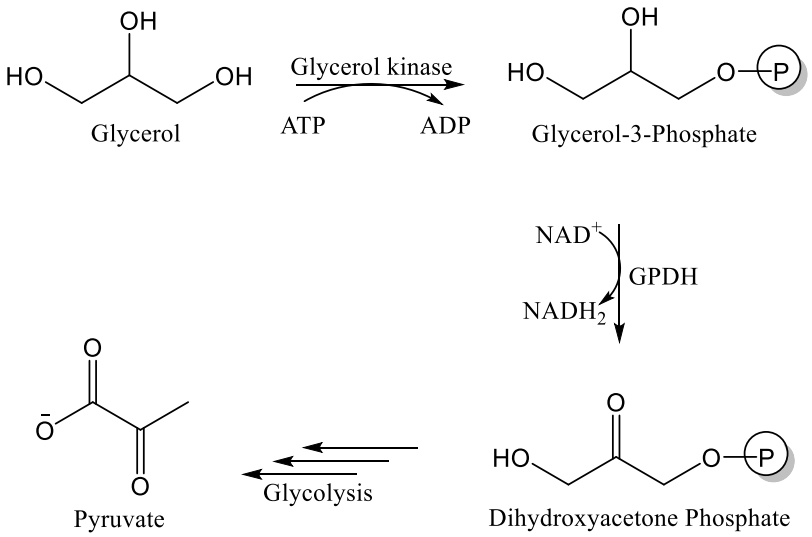

Figure 4.2: Glycerol metabolism and generation of pyruvate

Tryptophan indole-lyase (tryptophanase, TIL, E.C. 4.1.99.1), is a pyridoxal-5'-phosphate (PLP)-dependent enzyme, that catalyzes the hydrolytic cleavage of the Cβ-Cγ bond in Trp to give indole and ammonium pyruvate (Figure 4.3).[9] As this reaction is reversible[10] it was previously reported that TIL can also catalyze the reverse reaction, the enzymatic synthesis of Trp, using indole and ammonium pyruvate.[11-12] It is of our interest to utilize natural glycerol metabolic enzymes of *Escherichia coli* (*E. coli*), the workhorse of modern biotechnology, to convert glycerol to a source of metabolic pyruvate. As a proof-of-concept, the report herein described a method for utilization of metabolic pyruvate from glycerol together with pET15b:*tnaA* plasmids which overexpresses TIL, in the enzymatic production of Trp using *E. coli* BL21 (DE3) whole-cell catalysis.

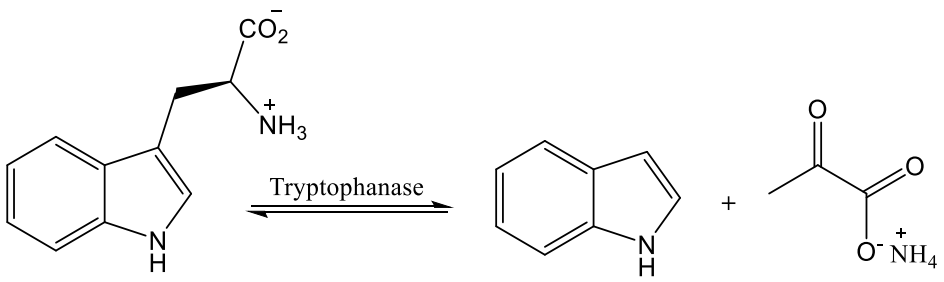

Figure 4.3: β-elimination of L-tryptophan to indole and ammonium pyruvate

4.3 Materials and Methods:

Materials: Indole (Aldrich) was purified by recrystallization from 1:10 EtOH/H_2O prior to use. Trp, ammonium chloride and ampicillin sodium salt (Fisher Scientific), anhydrous glycerol, 2-[4-(2-hydroxyethyl)-1-piperazin-2-yl]ethansulfonic acid (HEPES) and sodium chloride (J. T. Baker), PLP (USB Chemicals), isopropyl-β-D-1-thiogalactopyranoside (IPTG) (Roche chemicals), tryptone and yeast extract (Difco Laboratories Inc.) were used without further purification.

Strains and plasmids: *E. coli* strain BL21 (DE3) was selected as the host cells, containing pET15b:*tnaA* plasmids for overexpression of TIL. Enzyme activity was routinely determined by method previously described,[13] by following the absorbance of *S*-(*o*-nitrophenyl)-L-cysteine (SOPC) at λ = 370 nm.

Cell culture: Stock culture was incubated overnight at 37°C on agar plates. One colony was selected and used to inoculate 15 ml of Luria-Bertani (LB) broth[14] to reach the log phase. This culture was subsequently used to inoculate a larger medium and it was allowed to incubate for 15 hours. In glycerol-induced culture, M9 minimal medium[14] was supplemented with

glycerol as carbon source. Agar plates, LB broth and M9 medium all contained ampicillin at a concentration of 100 mg/L. All cultures were carried out at 37°C with stirring speed of 250 rpm (C25, New Brunswick Scientific). Cell pellets were collected by centrifugation, rinsed twice with saline solution (10g/L) before pellets were resuspended in the reaction medium. Dried-cell weight was determined by heating at 100°C for 12hrs.

Resting-cell reaction: HEPES buffer[14] used as reaction medium has a concentration of 0.1 M with pH adjusted to 7.5 using 6M NaOH. The medium also contained 1mM of IPTG to induce *tnaA* gene expression. In a typical experiment, the reaction medium is supplemented with indole (12.8 mM), ammonium chloride (16.6 mM), glycerol (0.4%), and PLP (10 mg/L). Once the cell pellet was resuspended into the reaction medium, the reaction was allowed to run at 37ºC with stirring speed of 250 rpm for up to 96 hours. Small aliquots of the reaction mixture were collected at different time intervals, centrifuged and analyzed for the presence of Trp.

Growing-cell reaction: M9 minimal medium was prepared as described above and used as the reaction medium in growing-cell experiments. In a typical experiment, a 2 ml culture at log phase, in minimal medium supplemented with glycerol, was used to inoculate 18 ml of the reaction medium containing glycerol, indole, ammonium chloride, ampicillin, and IPTG. The reaction was allowed to run at 37°C with stirring speed of 250 rpm for up to 30 hours. Small aliquots of the reaction mixture were collected at different time intervals, centrifuged and analyzed for presence of Trp.

Instrumentation and detection method: Trp detection by thin-layer chromatography (TLC) was carried out by elution of the reaction mixture on an aluminum-back silica plate, using a solution of ethanol and methylene chloride as eluent (3:5 EtOH:CH_2Cl_2). The developed TLC

plate was stained with 0.2% solution of ninhydrin. Upon heating at 60°C for several minutes, Trp-positive samples appeared as a purple stain. Trp has a characteristic absorption at $\lambda = 276$ nm and is readily detected. High-pressure liquid chromatography (HPLC) experiments were performed on a ThermoFisher P2000 pump equipped with a UV detector. A gradient mixture of MeOH and KH_2PO_4 (10 mM) was used as the eluting solution. Trp concentration was calculated from HPLC calibration standards. Liquid chromatography coupled mass-spec (LC-MS) experiments were performed on Perkin Elmer Sciex API I Plus.

4.4 Results and Discussion

The optimal activity of TIL in phosphate buffer pH 8 was previously reported.[10] In our hands, when early experiments were carried out in phosphate buffer, the presence of Trp was not detected until after approximately 24 hours and continued to increase over time and reached a maximum conversion after 4 days. Though, as we explored the reaction in HEPES buffer at pH 7.5, the presence of Trp was detected by TLC and HPLC within the first hour and continued to increase until the conversion reached a plateau after 24 hours (Figure 4.3). As the percent conversion remained unaffected under both conditions, the rate was dramatically enhanced in HEPES buffer. Phosphate buffer is commonly used in enzyme catalysis reaction for TIL but for whole-cell catalysis using *E. coli* BL21 (DE3) we observed a much faster rate with HEPES buffer as the reaction medium.

In our preliminary experiments, a large excess of unreacted indole remained in the mixture which affected the total percent conversion of the reaction. Following the conditions for resting-cells, we recovered Trp only up to 2% conversion from indole and the remainder of

indole stayed unreacted in the crude mixture. Our attempts to induce glycerol dissimilation in host cells by culturing in M9 minimal medium supplemented with glycerol was also proven unsuccessful as negligible difference was observed in the percent conversion.

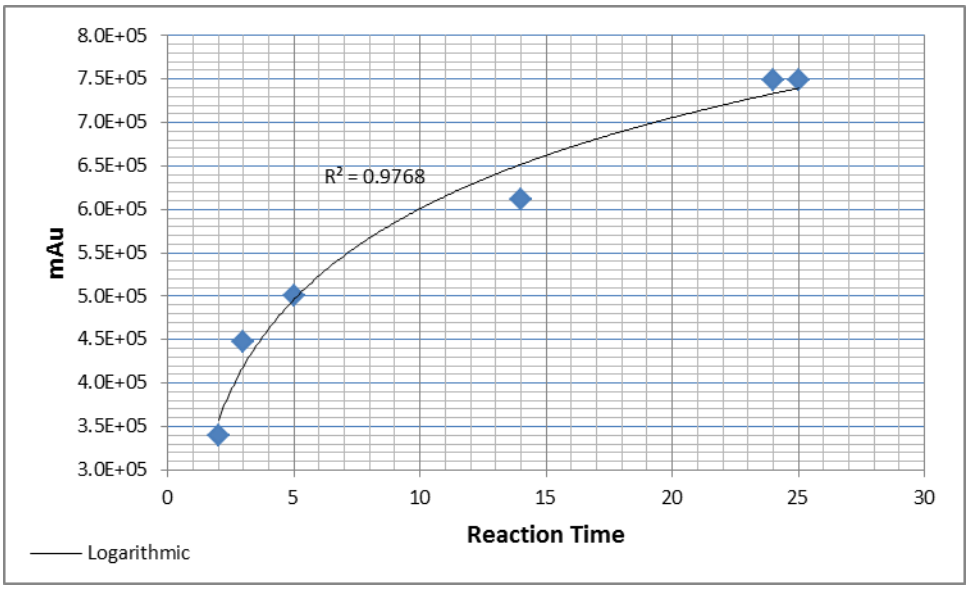

Figure 4.3: Tryptophan concentration in HEPES buffer as detected by HPLC at $\lambda = 278$ nm

In the absence of carbon source, bacterial cells utilize glycerol as their main source for carbon and energy. Therefore, a higher requirement for carbon and energy corresponds to a higher rate of glycerol dissimilation and in return would yield a higher production rate for metabolic pyruvate, which is critical for our approach. However, there is very minimal requirement for carbon and energy in resting-cells, as detailed in our approach, which could have affected the metabolism of glycerol and rate of pyruvate production in host cells. Based on this rationale, we were inclined to believe that carrying out catalysis using growing-cells (catalysis

during cell culture) would yield a higher conversion for Trp as carbon and energy requirement peaks during cell division.

Based on that rationale, we conducted experiments using the growing-cell approach. To eliminate all possible carbon sources except that from glycerol during cell growth and catalysis, both cell culture and reaction were carried out in M9 minimal medium supplemented with only glycerol, indole, and ammonium chloride. Our results indicated that the percent conversion was improved following this condition. At its peak, we detected a 10% conversion within 24 hours of reaction time. In addition, it is interesting to note that we did not detect the presence of Trp until after 5 hours. Using the growing-cell method, we observed that cell density was not high enough in the first few hours indicating that Trp was not produced until the bacterial culture reached the log phase. As the requirement for carbon and energy peak during the log phase, this suggested that the generation of metabolic pyruvate also peak during the log phase which explained the lag time in Trp production in comparison to resting-cell method. The reaction mixture was also analyzed by LC-MS with molecular ion (M+H m/z = 205.2 amu) indicating the presence of Trp (Figure 4.4).

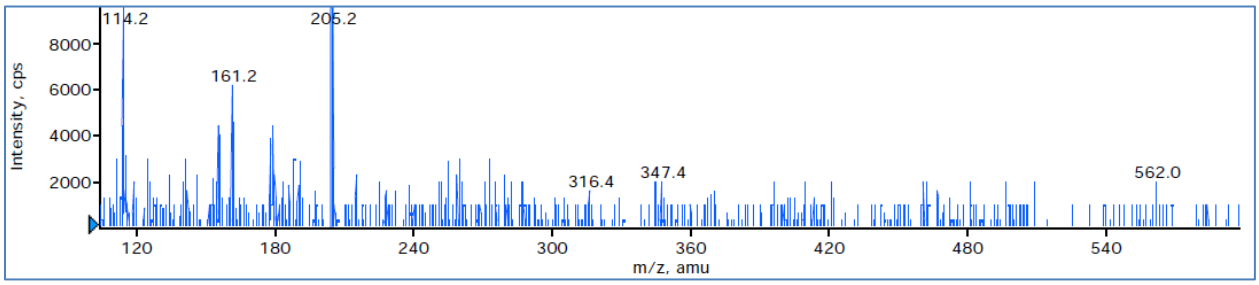

Figure 4.4: Detection of Trp by LC-MS

Metabolic pyruvate is the precursor for many pathways essential and critical for survival of bacterial cells, as it ultimately serves as their source for carbon and energy. Even though the report herein described an approach to utilize pyruvate generated by natural *E. coli* metabolism, we were inclined to believe that only a fraction of the total amount was efficiently used in the synthesis of Trp. Therefore, the remainder is likely to be consumed by the host cells to fulfill their carbon and energy requirement, which explained the low conversion and large excess of unreacted indole that remained in solution. From our results, we are also inclined to believe that utilizing an engineered strain capable of accumulating pyruvate from glycerol, similar to previous report,[15] would likely yield a more efficient conversion to Trp and this reflects the current effort in our laboratory.

4.5 Conclusion

A surge for demand of biodiesel in recent years has resulted in a surplus of glycerol, a by-product of biodiesel processing. As a proof-of-concept, we desired to report a method for utilizing glycerol in the synthesis of Trp, the rarest of 20 natural amino acids. We have demonstrated that Trp can be produced through either resting-cell or growing-cell method by utilizing the natural glycerol metabolic enzymes of *E. coli* BL21 (DE3) together with pET15b:*tnaA* plasmids. The presence of Trp was detected and confirmed by TLC, HPLC, and LC-MS.

4.6 References

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CHAPTER 5

CONCLUSION AND FUTURE WORK

With recent literatures suggesting the activity of tryptophan indole-lyase (TIL) as an attractive and selective target for the treatment of bio-film formation and antibiotic resistance, we set out to design, synthesize, and evaluate mechanism-based inhibitors for TIL. By exploring homologation of its physiological substrate at the α-carbon, we discovered that L-bishomotryptophan effectively inhibited the activity of TIL and is the first potent inhibitor reported to date that displayed excellent selectivity. Furthermore, we also provided insights for the enzyme-substrate binding interaction through pre-steady-state kinetic studies, suggesting the differences in inhibition mechanism of our inhibitors.

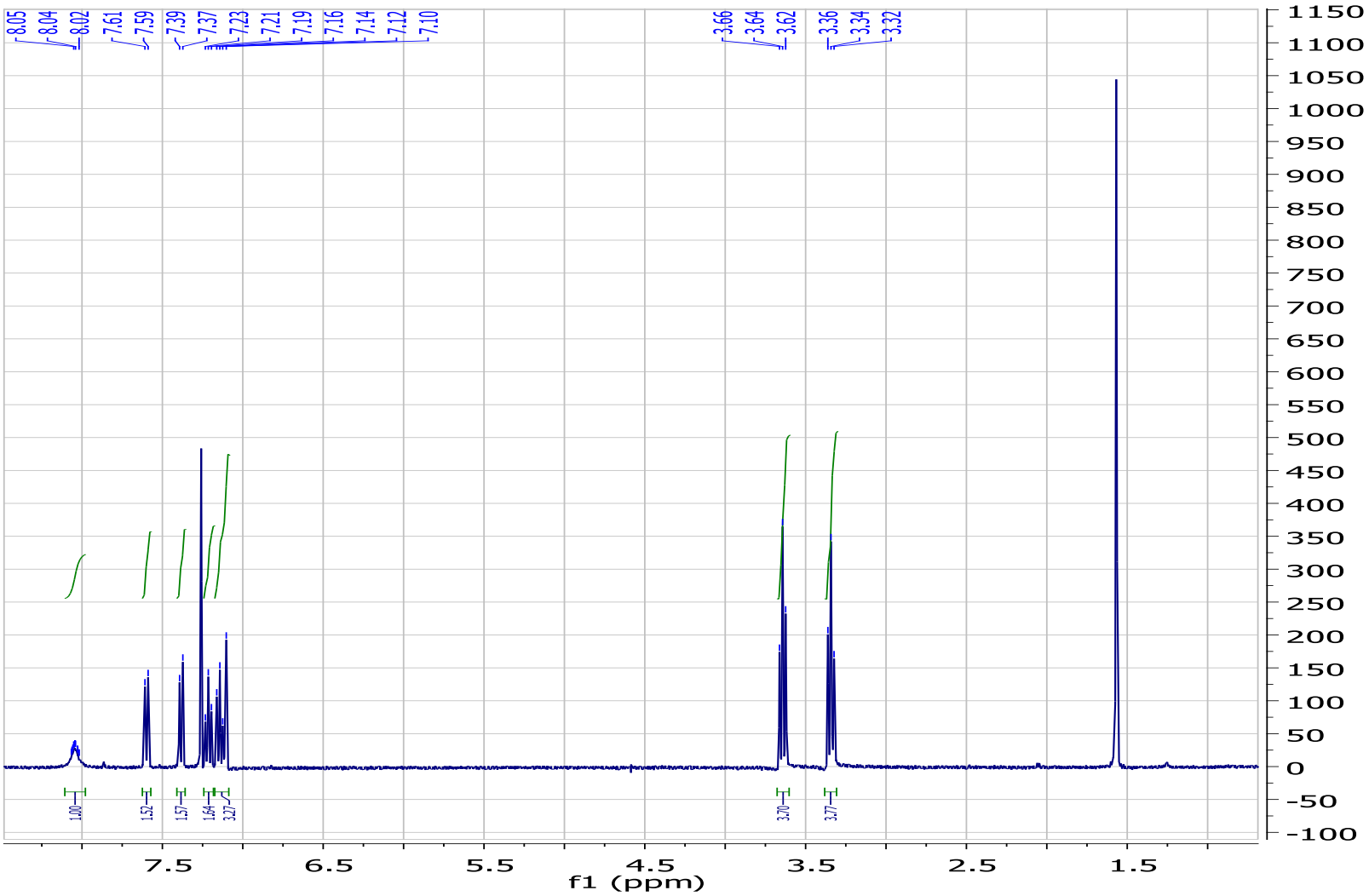
As tyrosine phenol-lyase (TPL) has a remarkably similar mechanism for cleavage of its physiological substrate, Tyr, we also extended our design and rationale in search of potent inhibitors for TPL. In addition to homologation, our design also included methylation of the phenol group of Tyr to study the effect on formation of ketoquinonoid intermediate. Our results indicated that homologation and methylation of the phenol group only yielded moderate inhibition for TPL. From our pre-steady-state kinetic experiments, we were able to gain valuable insights into the enzyme-substrate binding interactions with our inhibitors. Our data suggested that by homologating the α-carbon of the substrate, a substrate-induced strain was observed at the active site of TPL, indicated by a blue-shift in the absorbance of the external aldimines observed with both Tyr bishomologues.

The recent increase in global demand and production for biodiesel resulted in a surplus of glycerol, originated as a by-product in biodiesel processing. Glycerol surplus from biodiesel production resulted in the shutdown of many glycerol production plants and continued to be a disposal concern. We recently discovered a convenient method for the bioconversion of glycerol to a value-added amino acid, Trp, using *Escherichia coli* whole-cell catalysis. As a proof-of-concept, we have demonstrated and validated a convenient biosynthetic pathway for Trp from glycerol by coupling natural glycerol metabolic enzymes with *tnaA* plasmids in our one-pot biosynthetic approach.

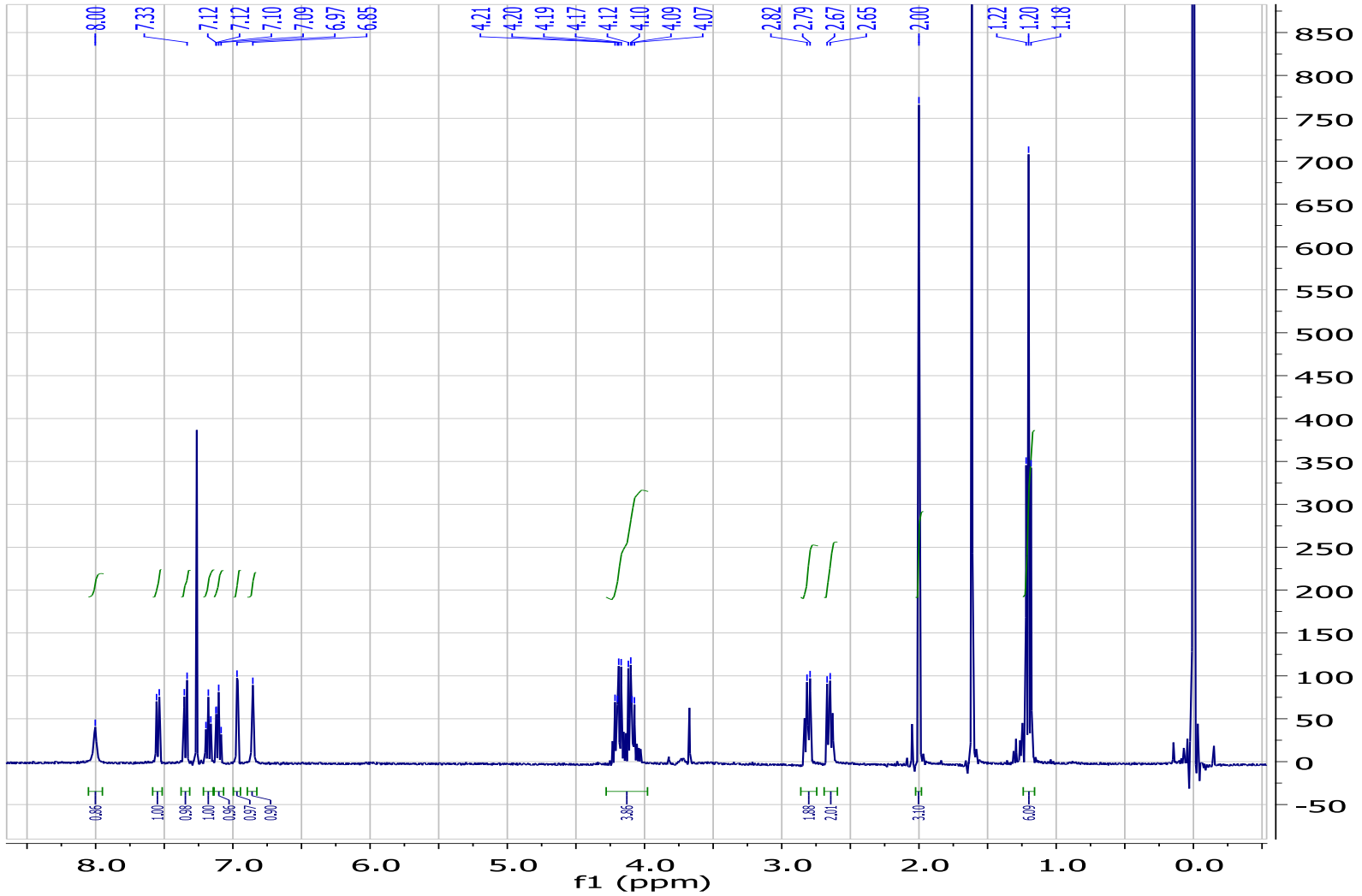
Since TIL can also catalyze the *in vitro* β-substitution reaction in the enzymatic synthesis of tryptophan derivatives, we speculated that the above method would also serve as an attractive and convenient route, alternative to chemical synthesis, to obtain Trp derivatives. In addition, TPL can also catalyze its reverse reaction, synthesis of Tyr from phenol and ammonium pyruvate. Therefore, we also speculated that the method presented is also applicable to the synthesis of Tyr and derivatives, by coupling glycerol metabolic enzymes with plasmids for expression of TPL. However, the validity of this approach for the biosynthesis of Trp derivatives and Tyr has not been explored by our group.

APPENDIX

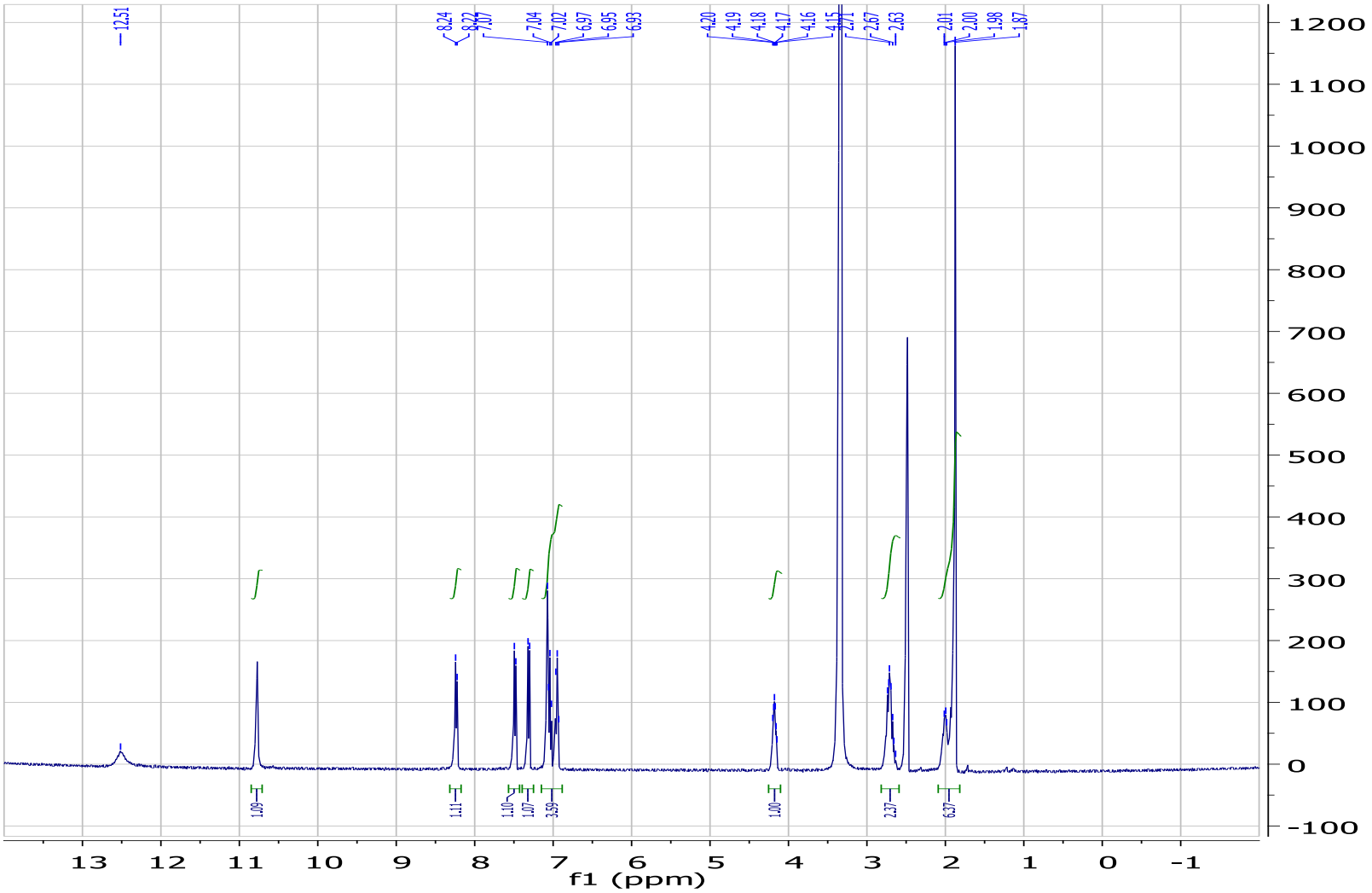
Appendices 2.1-2.6:	Synthesis of L-Homotryptophan
Appendices 2.7-2.12:	Synthesis of L-Bishomotryptophan
Appendices 3.1-3.6:	Synthesis of *O*-Methyl-L-Homotyrosine
Appendices 3.7-3.9:	Synthesis of L-Homotyrosine
Appendices 3.10-3.15:	Synthesis of *O*-Methyl-L-Bishomotyrosine
Appendices 3.16-3.18:	Synthesis of L-Bishomotyrosine
Appendices 3.19-3.22:	Pre-Steady-State Kinetics of Tyrosine Homologues



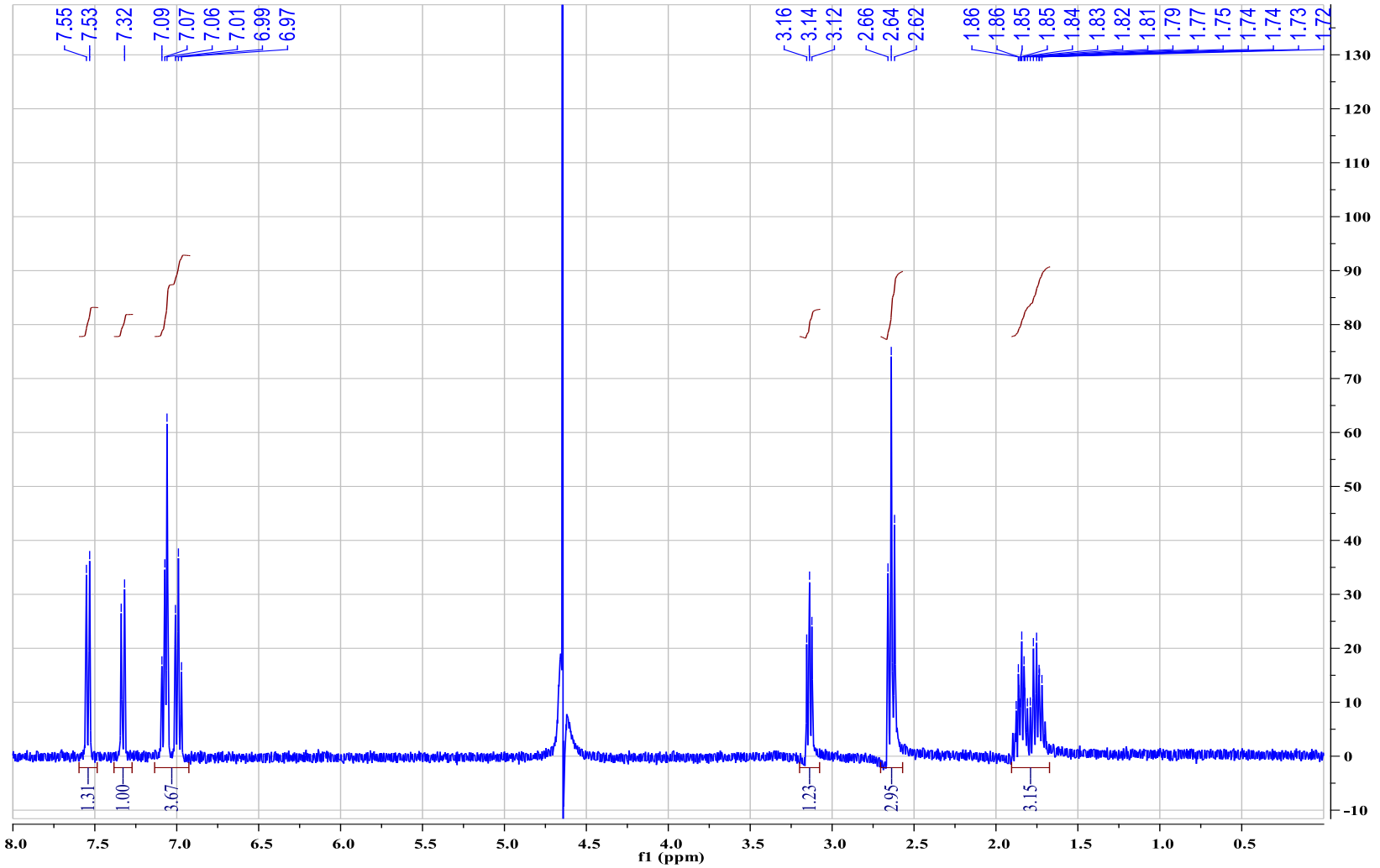
Appendix 2.1: 3-(2-Bromoethyl)-1*H*-indole (**3a**). ^{1}H NMR – Varian 400 MHz ($CDCl_3$) δ (ppm): 3.32-3.36 (t, 2H), 3.62-3.66 (t, 2H), 7.10 (s, 1H), 7.12-7.16 (t, 1H), 7.19-7.23 (t, 1H), 7.37-7.39 (d, 1H), 7.59-7.61 (d, 1H), 8.04 (s, 1H).



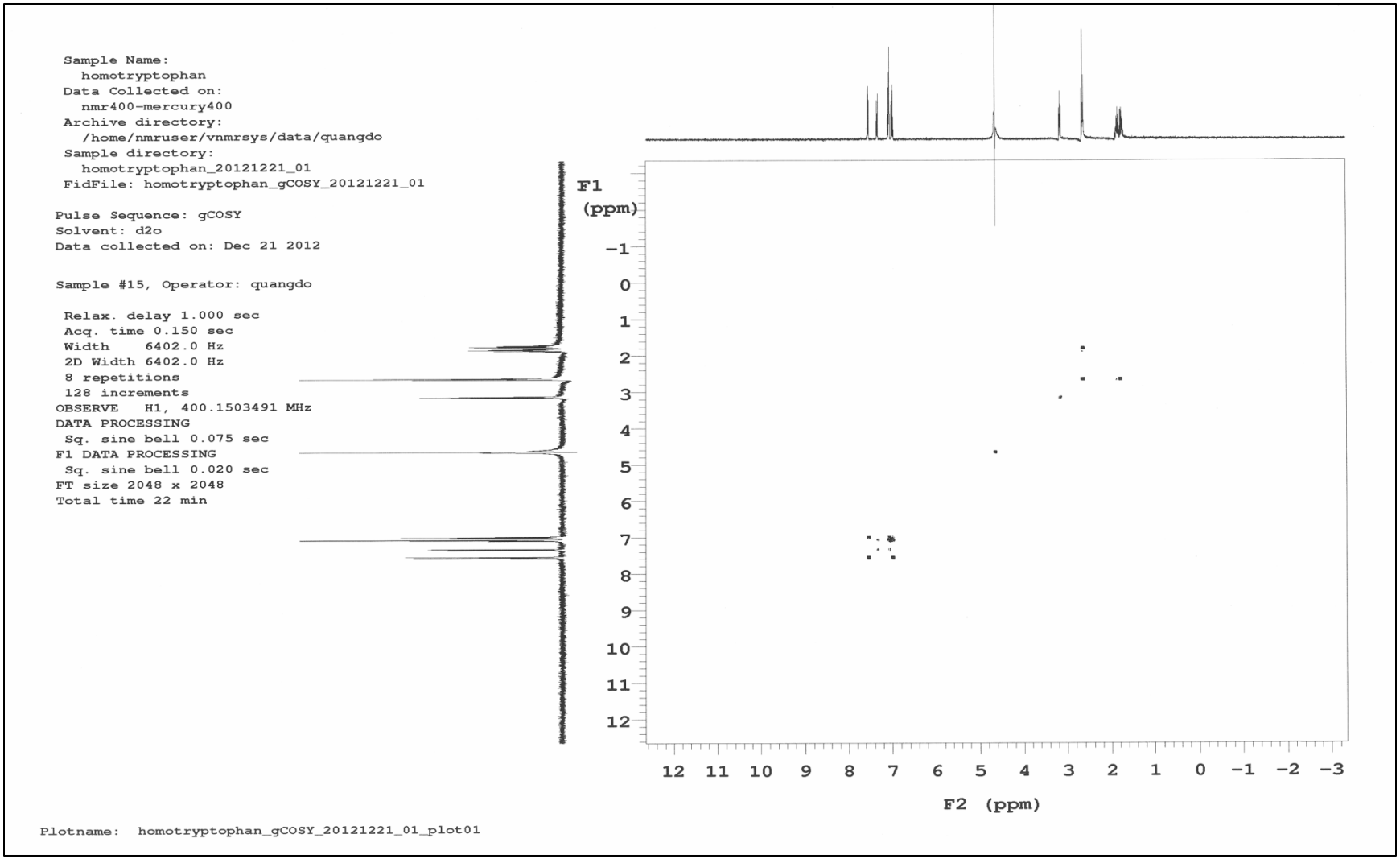
Appendix 2.2: Diethyl 2-(2-(1*H*-indol-3-yl)ethyl)-2-acetamidomalonate (**4a**). ^{1}H NMR – Varian 400 MHz ($CDCl_3$) δ (ppm): 1.18-1.22 (t, 3H), 2.00 (s, 3H), 2.63-2.67 (m, 2H), 2.79-2.83 (m, 2H), 4.07-4.21 (m, 4H), 6.85 (s, 1H), 6.97 (s, 1H), 7.09-7.12 (t, 1H), 7.16-7.20 (t, 1H), 7.33-7.35 (d, 1H), 7.54-7.56 (d, 1H), 8.00 (s, 1H).



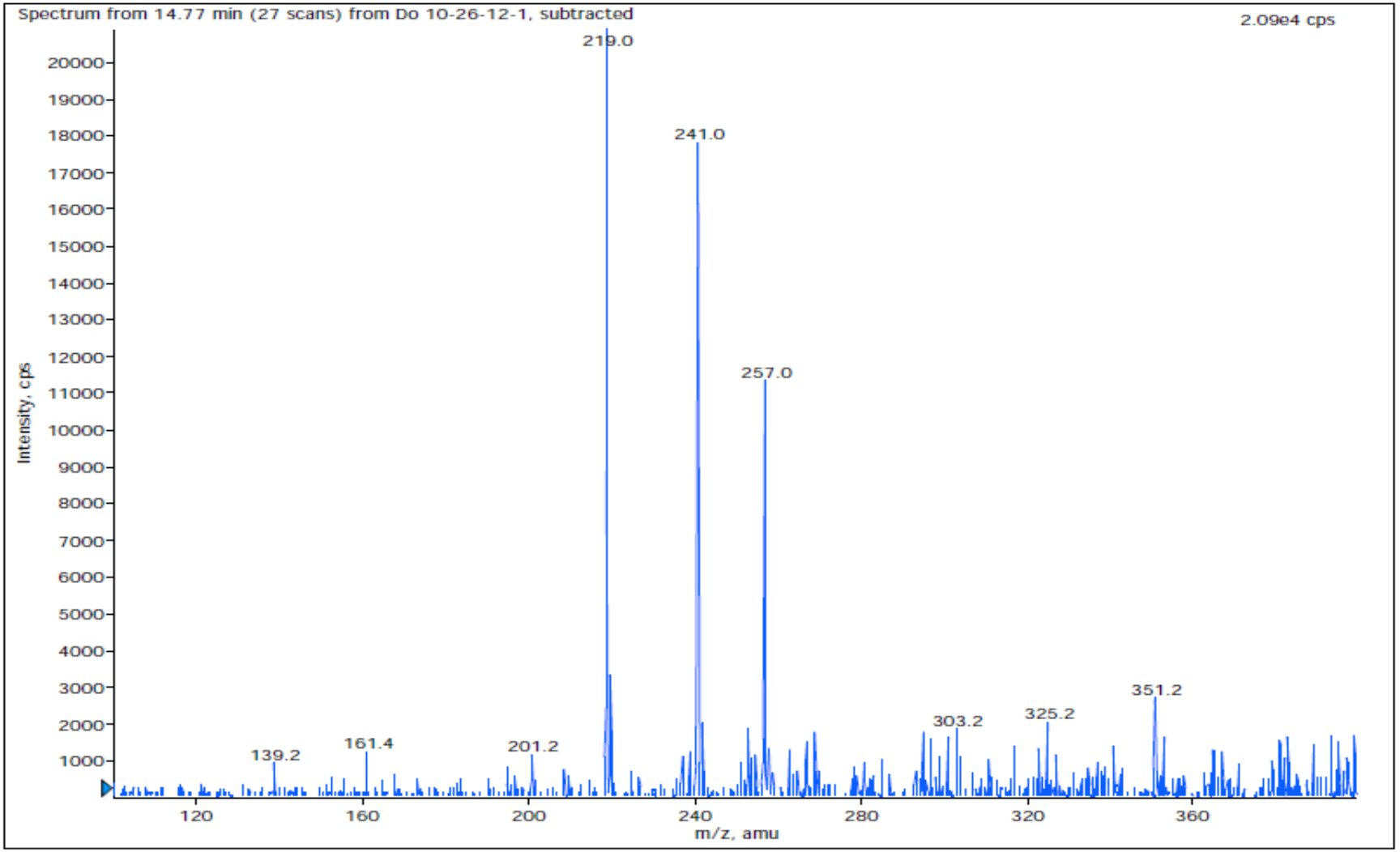
Appendix 2.3: 2-Acetamido-4-(1*H*-indol-3-yl)butanoic acid (**5a**). ^{1}H NMR – Varian 400 MHz (DMSO) δ (ppm): 1.87 (s, 3H), 1.98-2.01 (m, 2H), 2.63-2.74 (m, 2H), 4.15-4.20 (m, 1H), 6.93-6.97 (t, 1H), 7.02-7.06 (t, 1H), 7.07 (s, 1H), 7.30-7.32 (d, 1H), 7.48-7.49 (d, 1H), 8.22-8.24 (1H), 12.51 (broad, 1H).



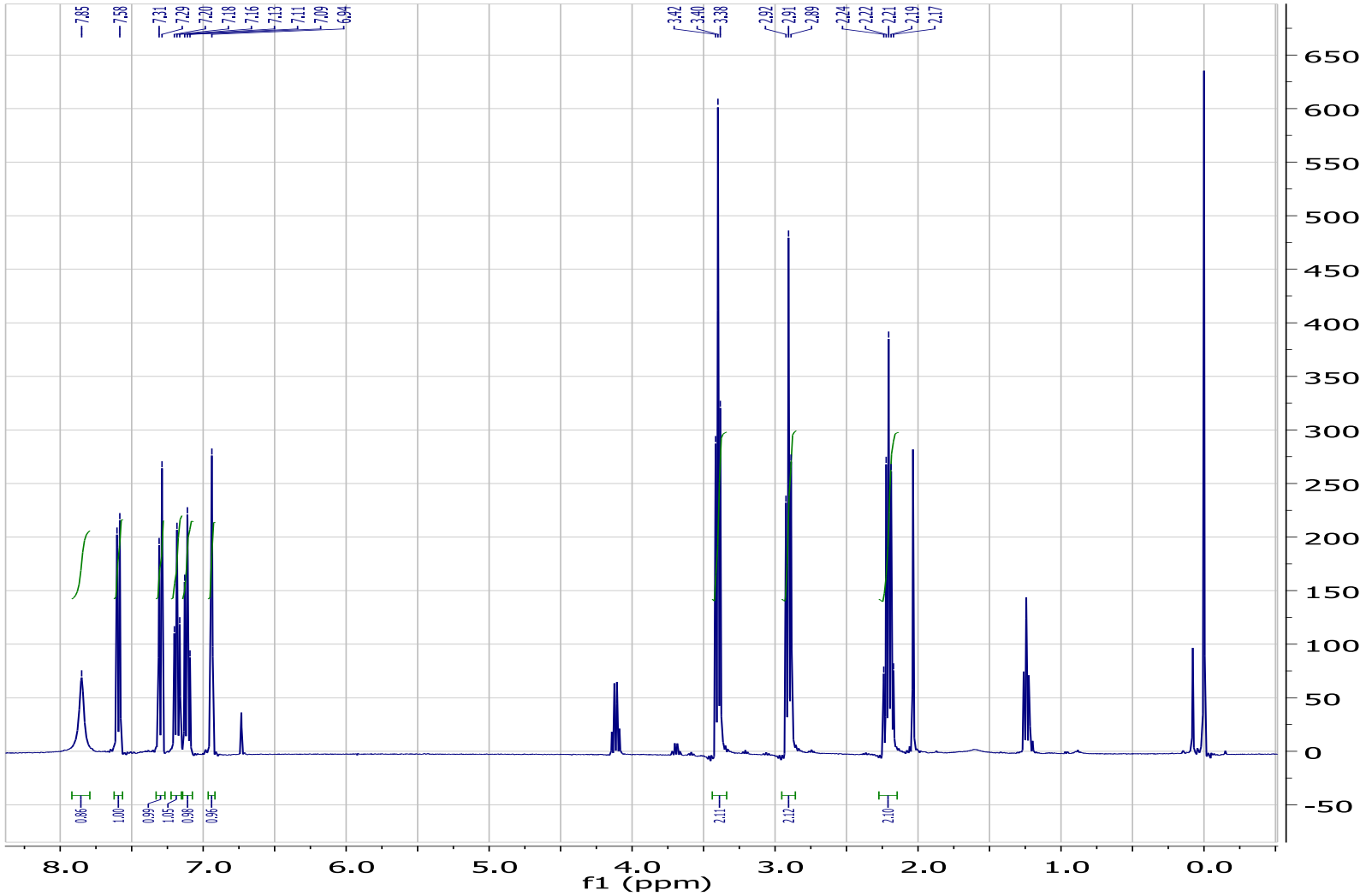
Appendix 2.4: L-Homotrytophan (**1a**). ^{1}H NMR – Varian 400 MHz (NaOD/ D_2O) δ (ppm): 1.72-1.88 (m, 2H), 2.62-2.66 (t, 2H), 3.12-3.16 (t, 1H), 6.97-7.01 (t, 1H), 7.06-7.09 (t, 2H), 7.32-7.34 (d, 1H), 7.53-7.55 (d, 1H).



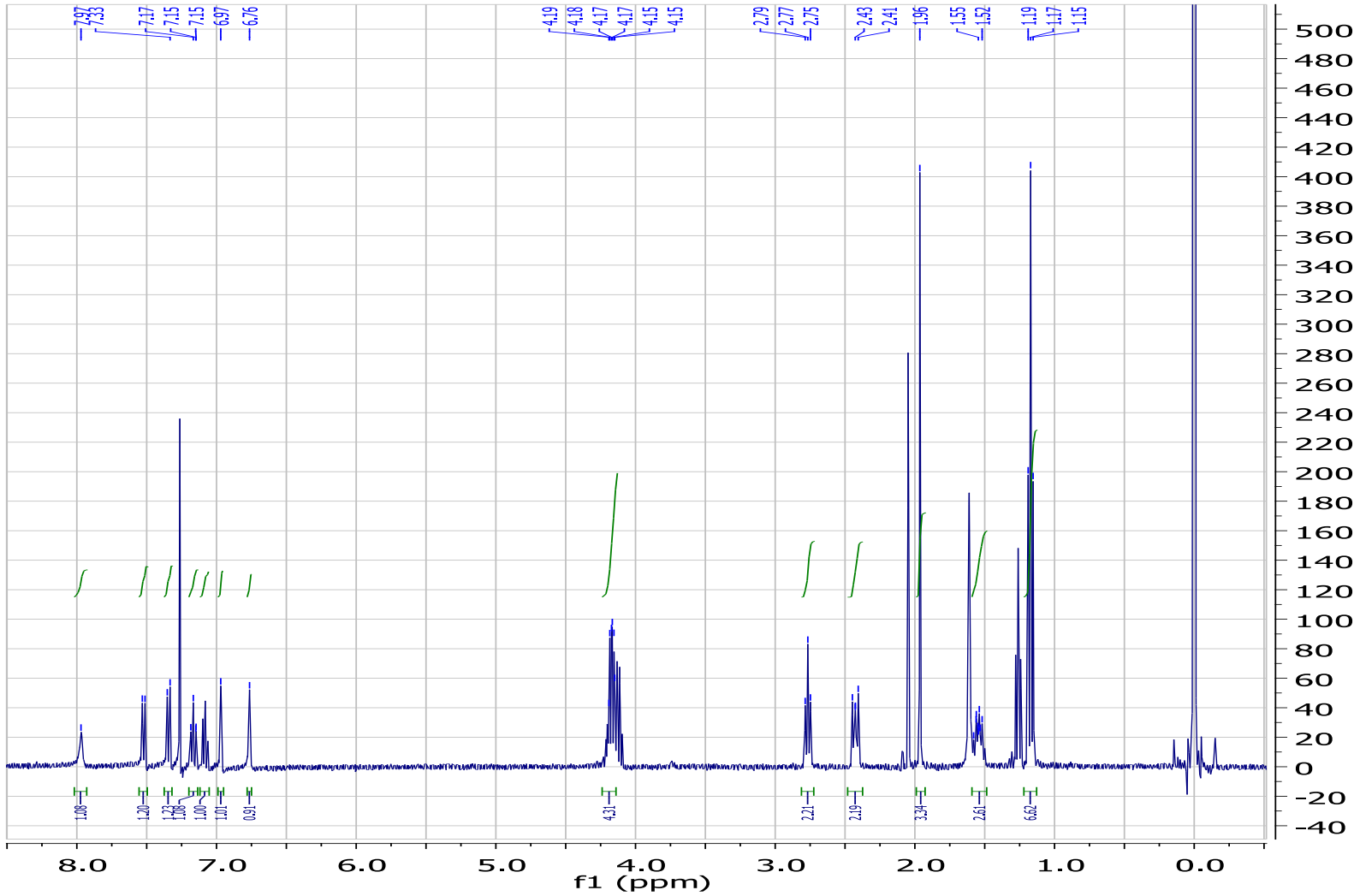
Appendix 2.5: L-Homotryptophan (**1a**). ^{1}H COSY-NMR – Varian 400 MHz (NaOD/D_2O).



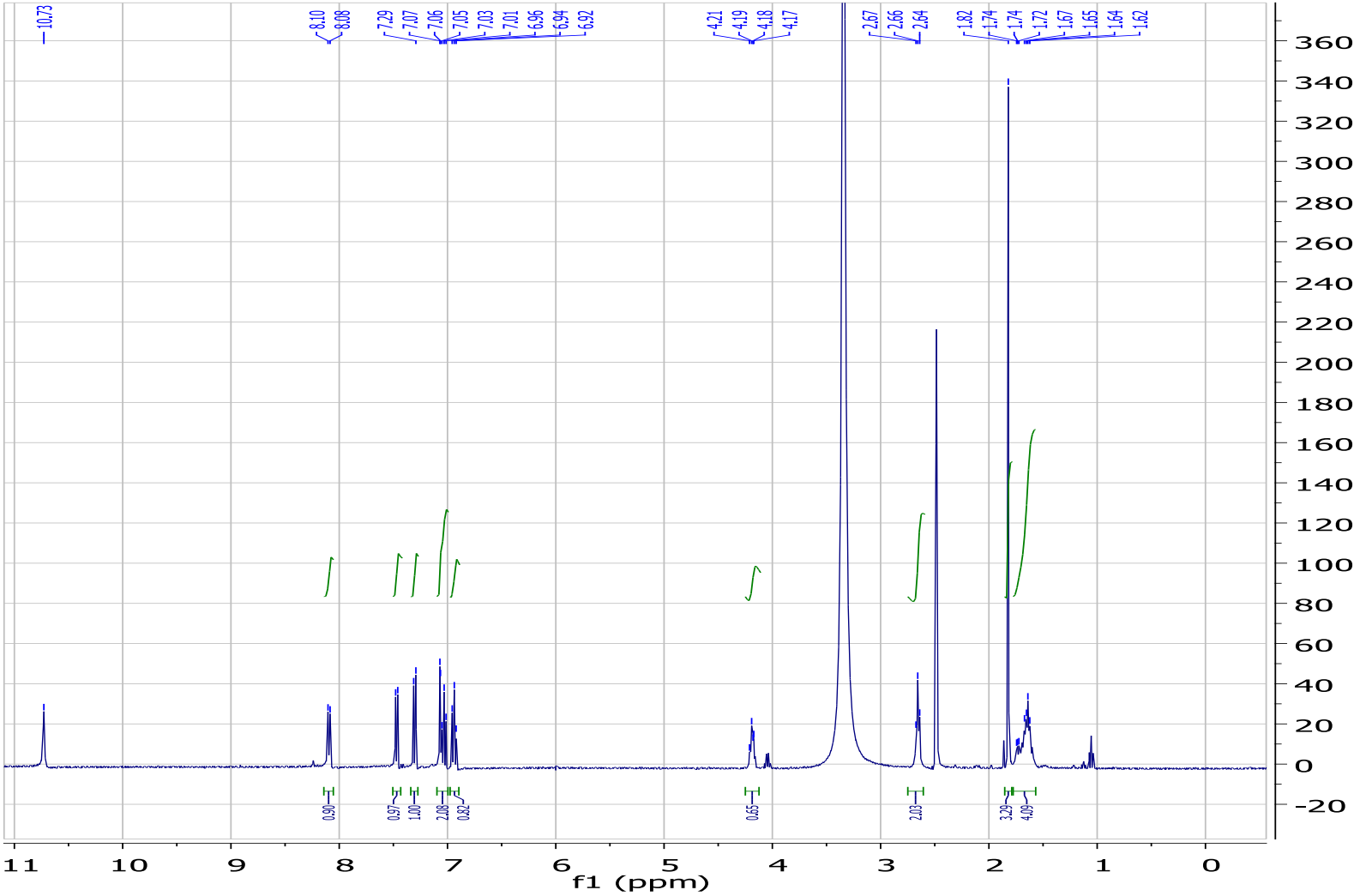
Appendix 2.6: L-Homotryptophan (**1a**). Electrospray Ionization Mass Spectrum - Perkin Elmer API I Plus ESI-MS (m/z): M+1 = 219; M+Na = 241; M+K = 257.



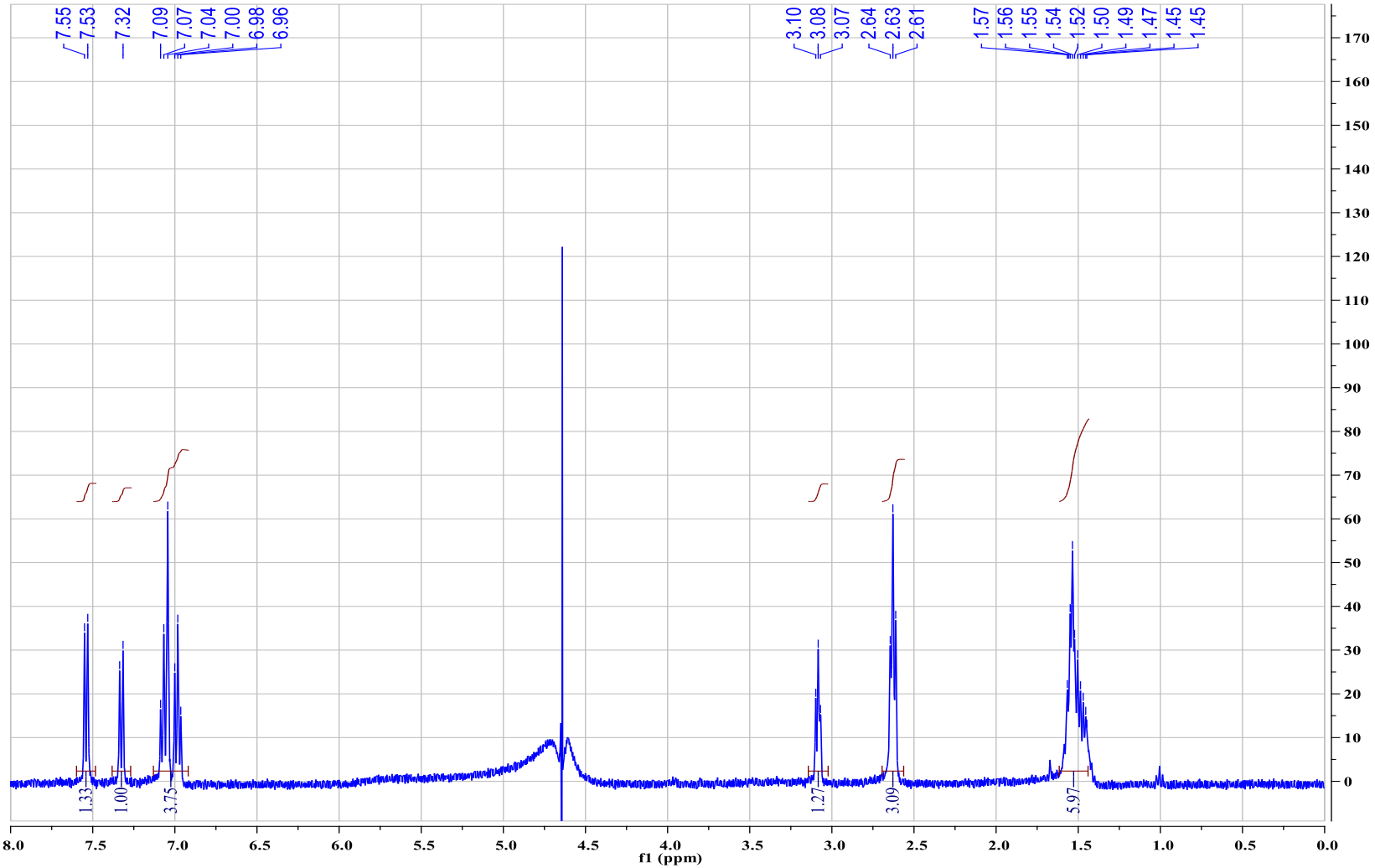
Appendix 2.7: 3-(3-Bromopropyl)-1*H*-indole (**3b**). ^{1}H NMR – Varian 400 MHz ($CDCl_3$) δ (ppm): 2.17-2.24 (m, 2H), 2.89-2.92 (t, 2H), 3.38-3.42 (t, 2H), 6.94 (s, 1H), 7.09-7.13 (t, 1H), 7.16-7.20 (t, 1H), 7.29-7.31 (d, 1H), 7.58-7.60 (d, 2H), 7.85 (broad, 1H).



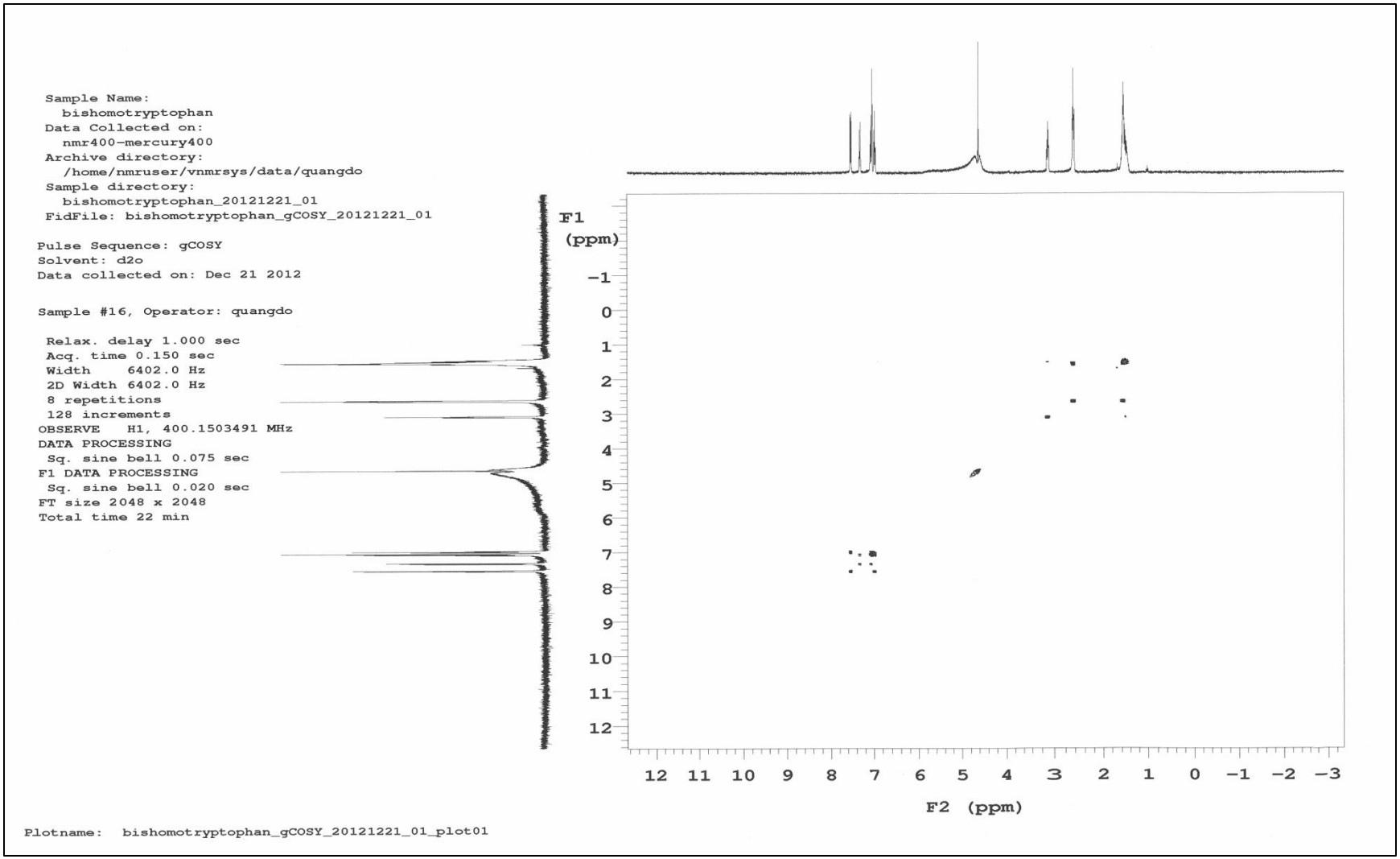
Appendix 2.8: Diethyl 2-(3-(1*H*-indol-3-yl)propyl)-2-acetamidomalonate (**4b**). ^{1}H NMR – 400 MHz ($CDCl_3$) δ (ppm): 1.15-1.19 (t, 3H), 1.50-1.58 (m, 2H), 1.96 (s, 3H), 2.41-2.45 (t, 2H), 2.75-2.79 (t, 2H), 4.15-4.19 (m, 4H), 6.76 (s, 1H), 6.97 (s, 1H), 7.06-7.10 (t, 1H), 7.15-7.18 (t, 1H), 7.33-7.35 (d, 1H), 7.51-7.53 (d, 1H), 7.97 (s, 1H).



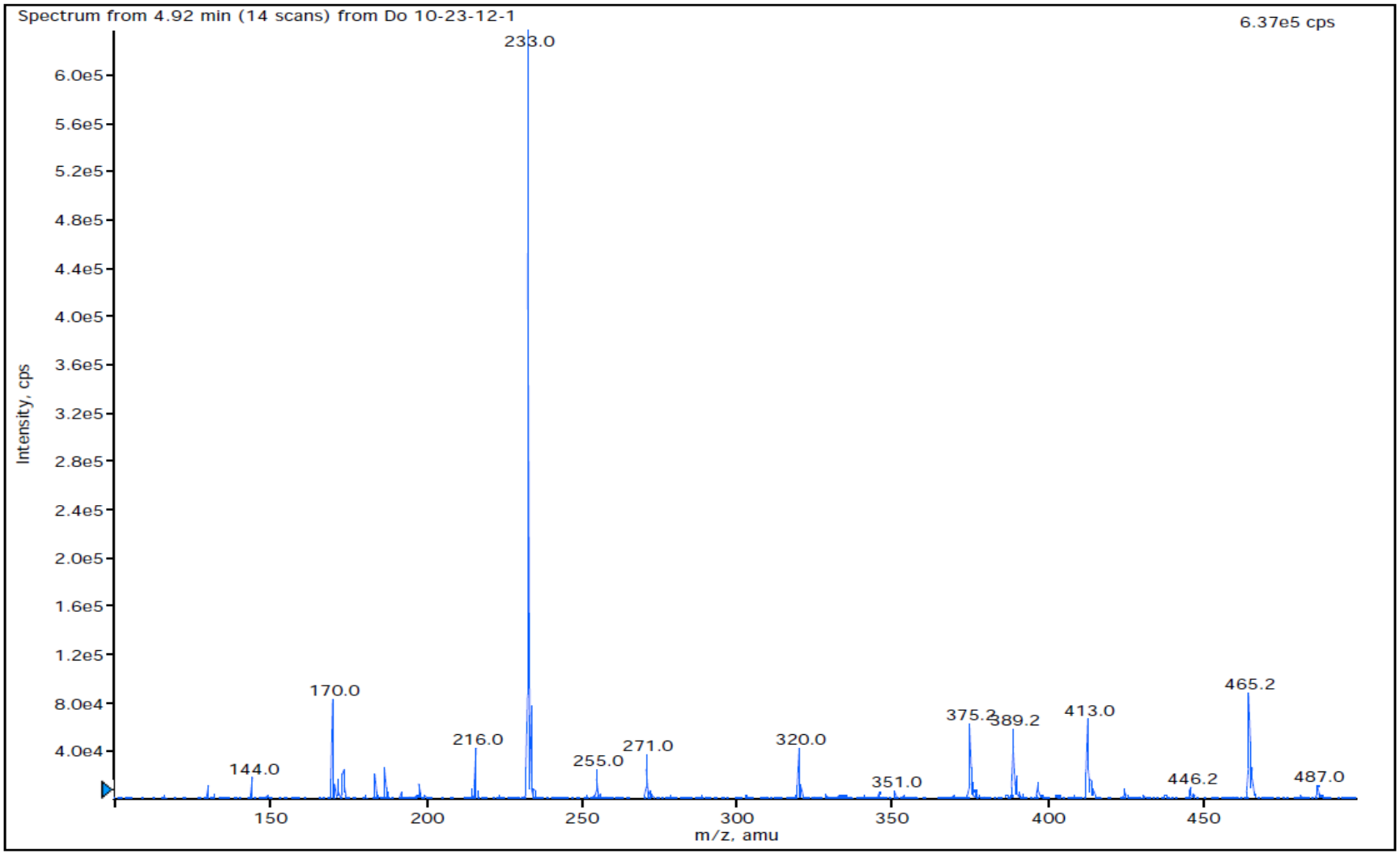
Appendix 2.9: 2-Acetamido-5-(1*H*-indol-3-yl)pentanoic acid (**5b**). ^{1}H NMR – Varian 400 MHz (DMSO) δ (ppm): 1.62-1.67 (m, 2H), 1.72-1.74 (m, 2H), 1.82 (s, 3H), 2.64-2.67 (t, 2H), 4.17-4.21 (m, 1H), 6.92-6.96 (t,1H), 7.01-7.05 (t, 1H), 7.06-7.07 (d, 1H), 7.29-7.31 (d, 1H), 7.46-7.48 (d, 1H), 8.08-8.10 (d, 1H), 10.73 (s, 1H).



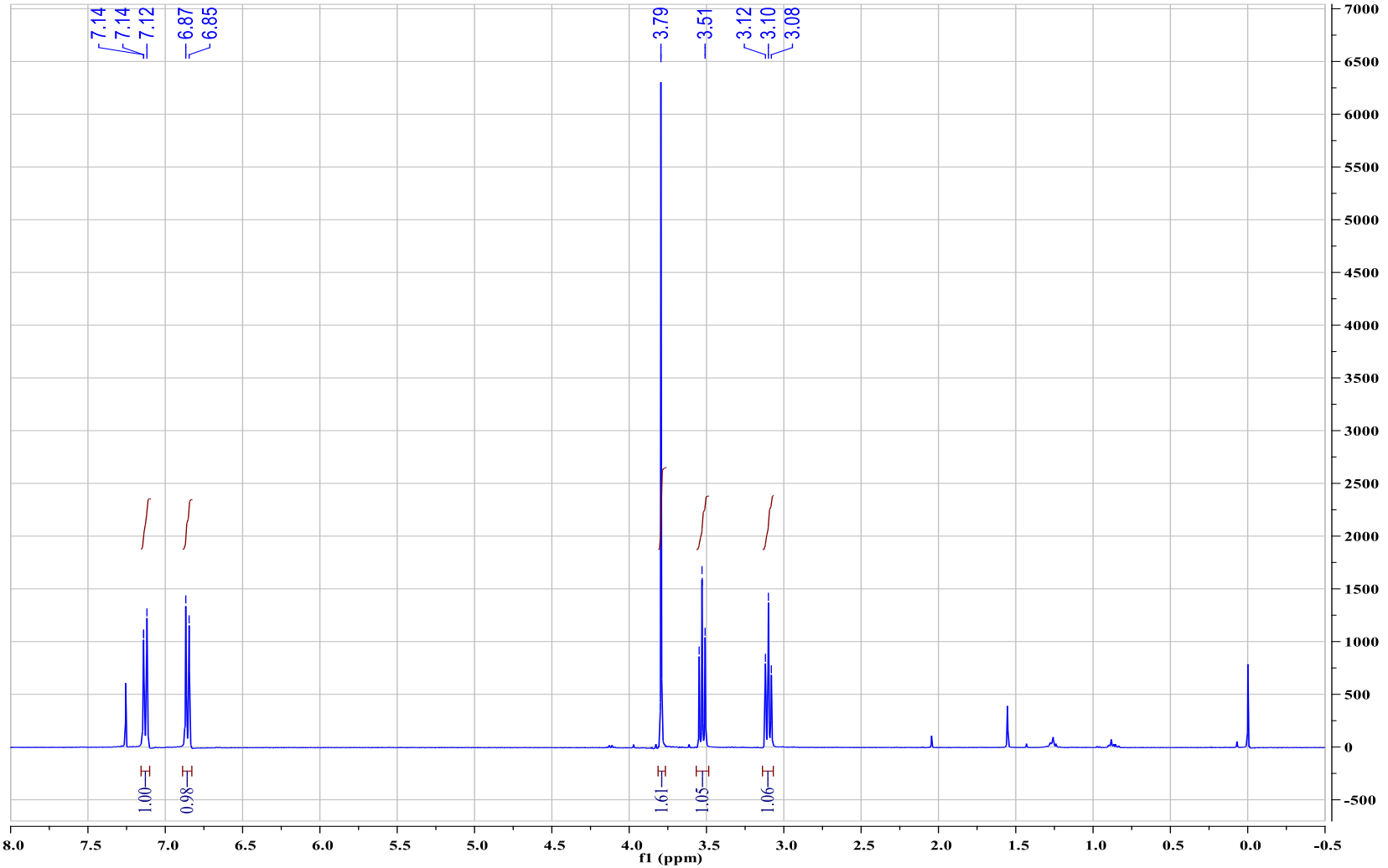
Appendix 2.10: L-Bishomotrytophan (**1b**). ^{1}H NMR – Varian 400 MHz (NaOD/ D_2O) δ (ppm): 1.45-1.57 (m, 4H), 2.61-2.64 (t, 2H), 3.07-3.10 (t, 1H), 6.96-7.00 (t, 1H), 7.04-7.09 (t, 2H), 7.32-7.34 (d, 1H), 7.53-7.55 (d, 1H).



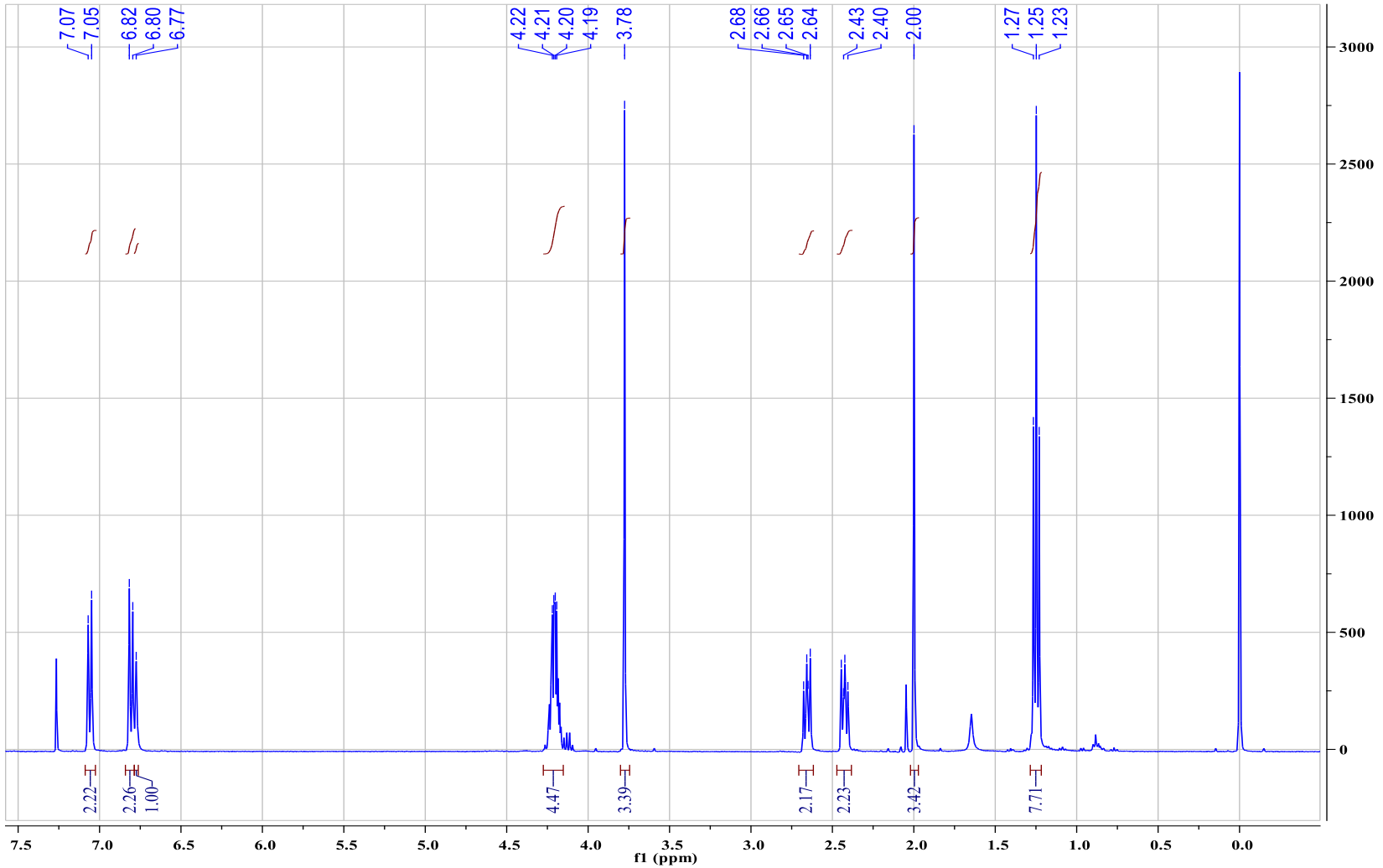
Appendix 2.11: L-Bishomotryptophan (**1b**). ^{1}H COSY NMR – Varian 400 MHz (NaOD /D_2O).



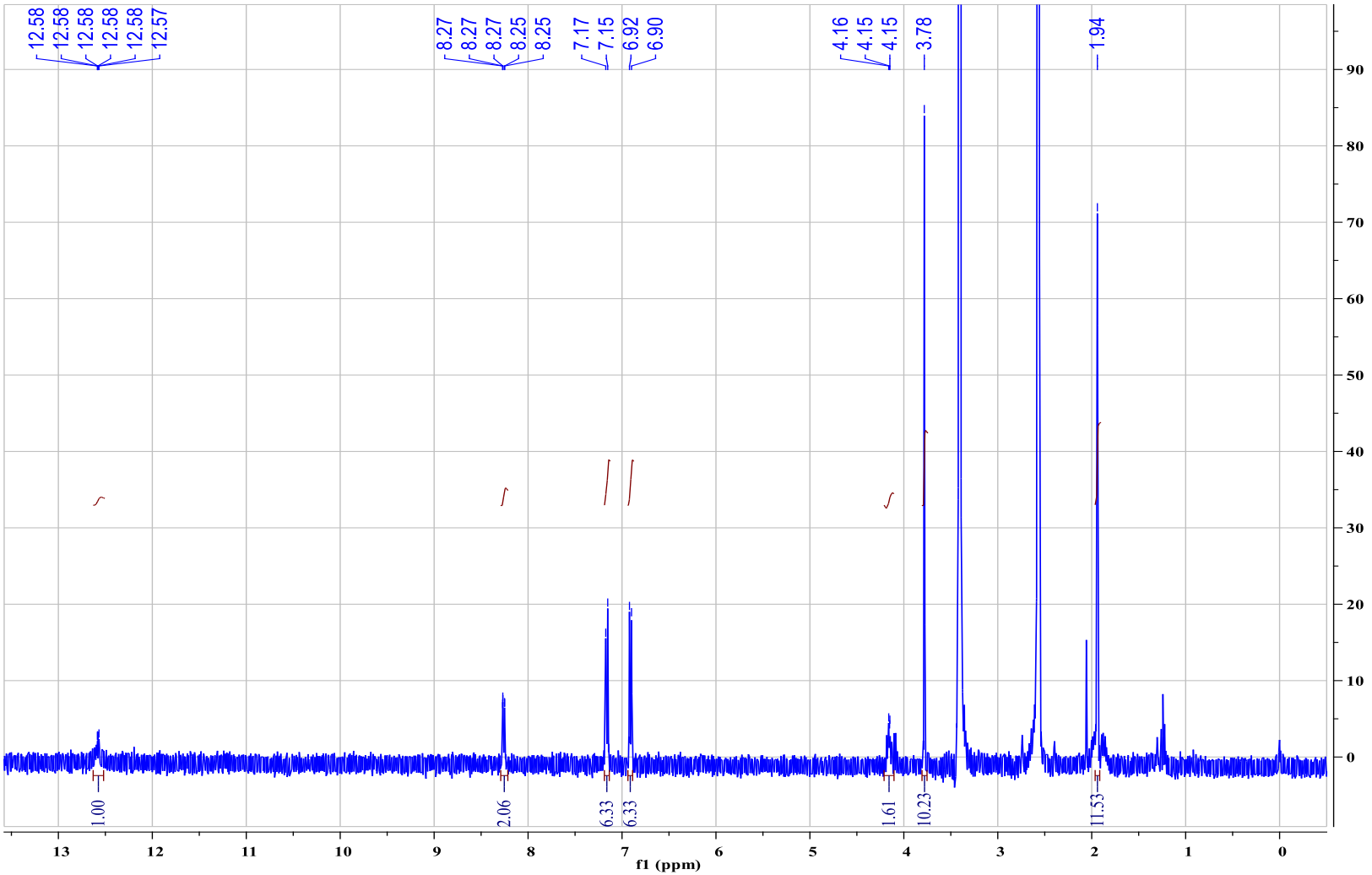
Appendix 2.12: L-Bishomotryptophan (**1b**). Electrospray Ionization Mass Spectrum - Perkin Elmer API I Plus ESI-MS (m/z): M+1 = 233.



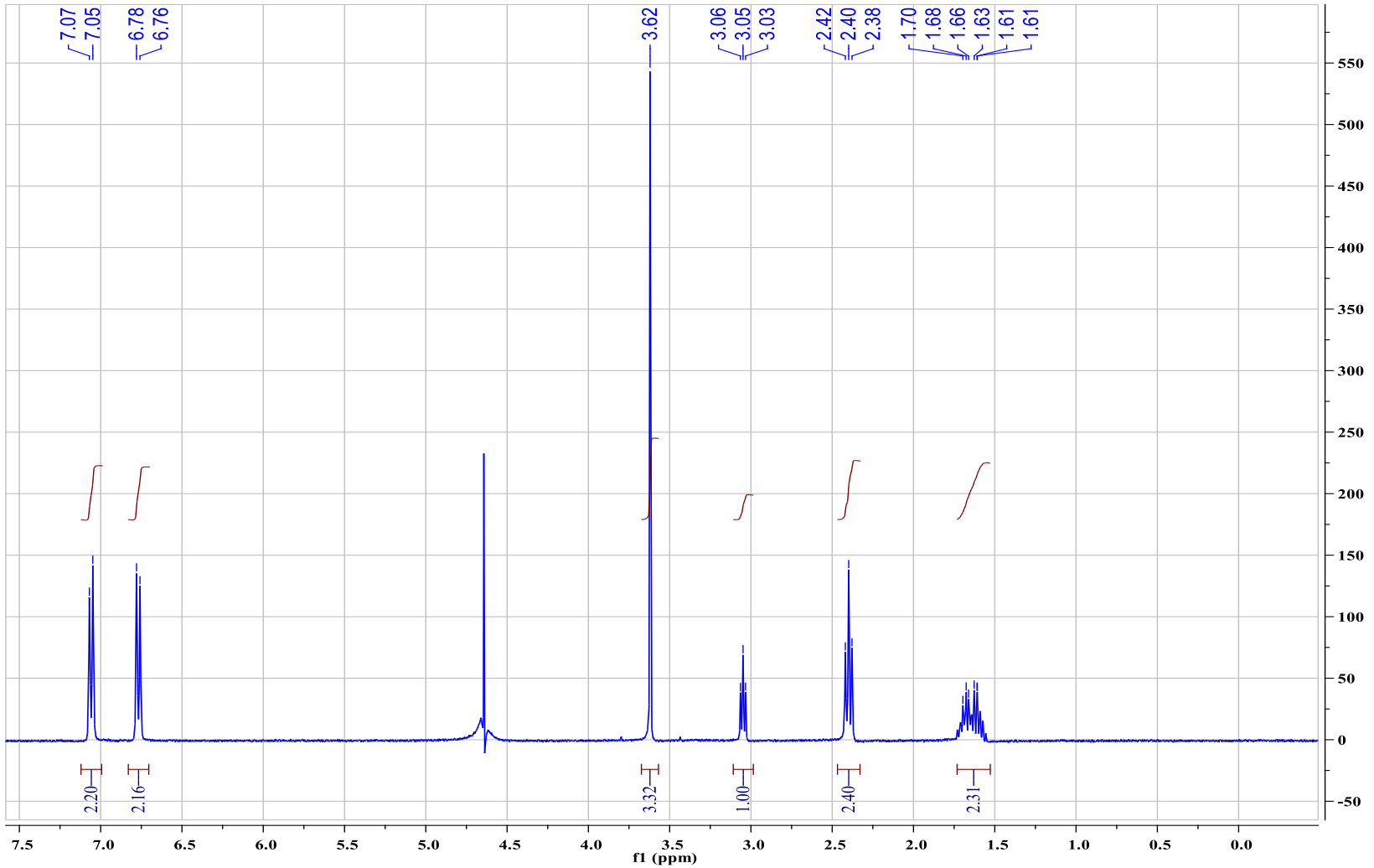
Appendix 3.1: 1-(2-Bromoethyl)-4-methoxybenzene (**3a**). ^{1}H NMR – Varian 400 MHz ($CDCl_3$) δ (ppm): 3.08-3.12 (t, 2H), 3.51-3.55 (t, 2H), 3.79 (s, 3H), 6.85-6.87 (d, 2H), 7.12-7.14 (d, 2H).



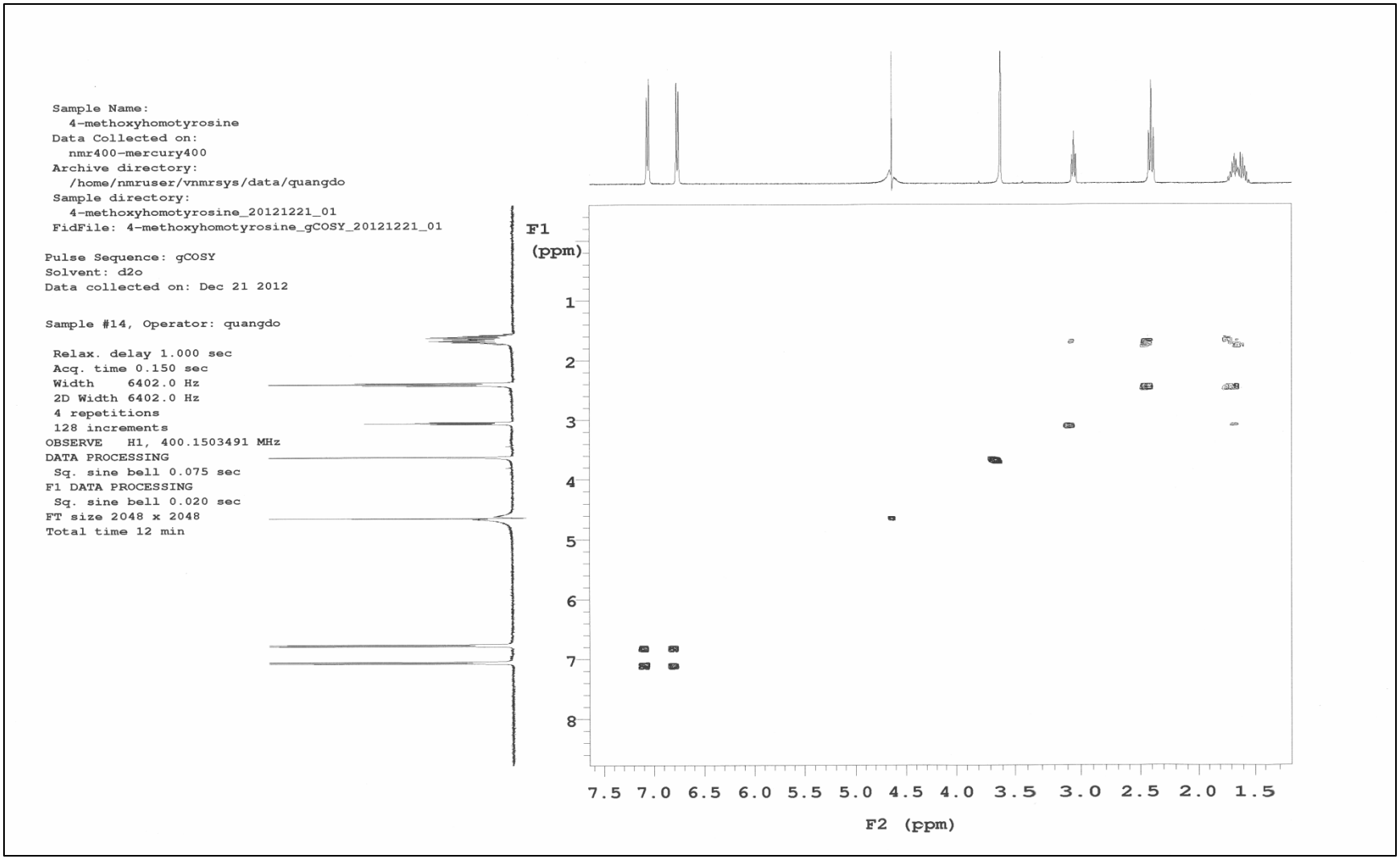
Appendix 3.2: Diethyl 2-acetamido-2-(4-methoxyphenethyl)malonate (**4a**). ^{1}H NMR – Varian 400 MHz ($CDCl_3$) δ (ppm): 1.23-1.27 (t, 6H), 2.00 (s, 3H), 2.40-2.43 (m, 2H), 2.64-2.68 (m, 2H), 3.78 (s, 3H), 4.19-4.22 (m, 4H), 6.77 (s, 1H), 6.80-6.82 (d, 2H), 7.05-7.07 (d, 2H).



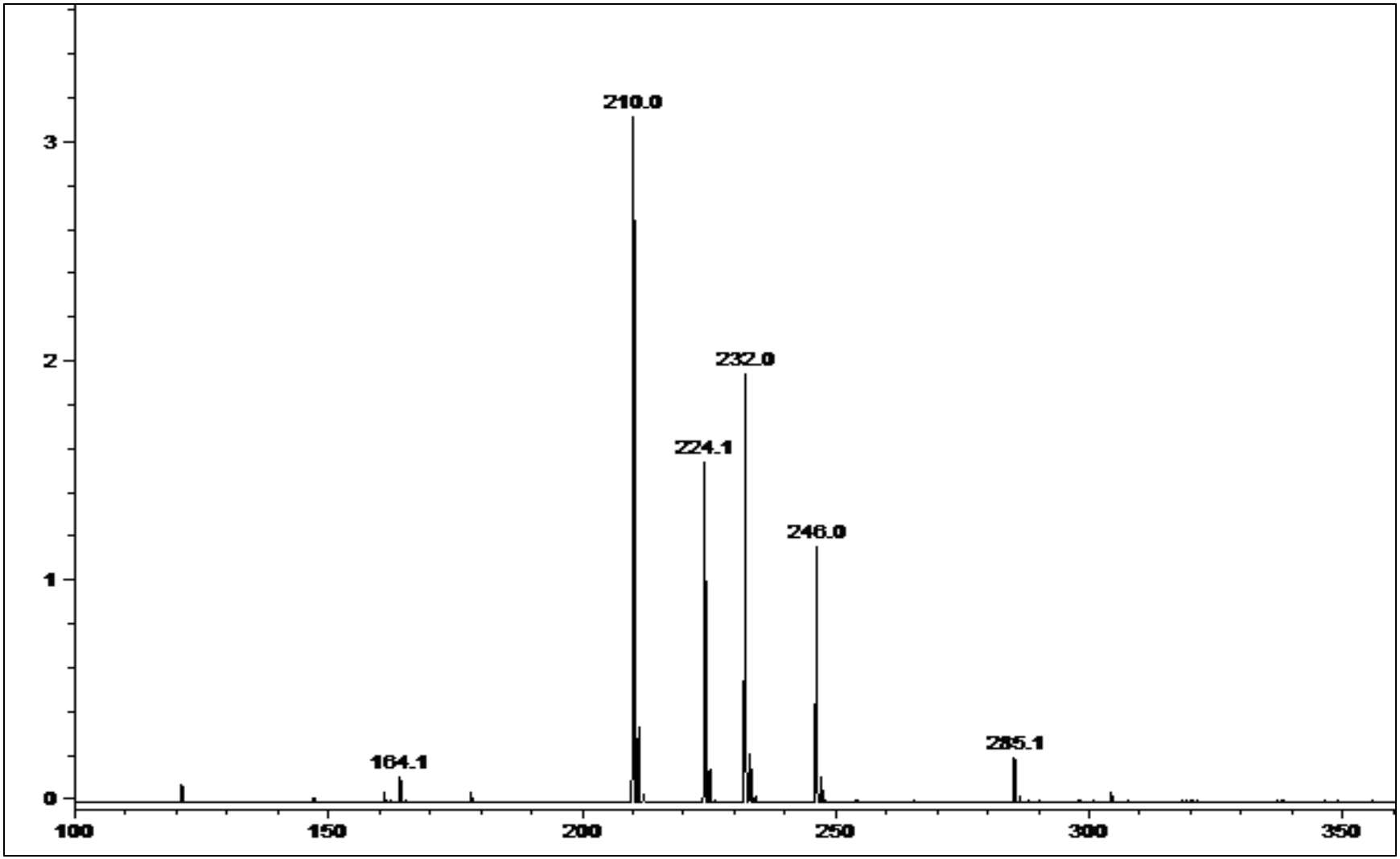
Appendix 3.3: 2-Acetamido-4-(4-methoxyphenyl)butanoic acid (**5a**). ^{1}H NMR – Varian 400 MHz (DMSO) δ (ppm): 1.94 (s, 3H), 3.78 (s, 3H), 4.12-4.15 (m, 1H), 6.90-6.92 (d, 2H), 7.15-7.17 (d, 2H), 8.25-8.27 (d, 1H), 12.5 (b, 1H).



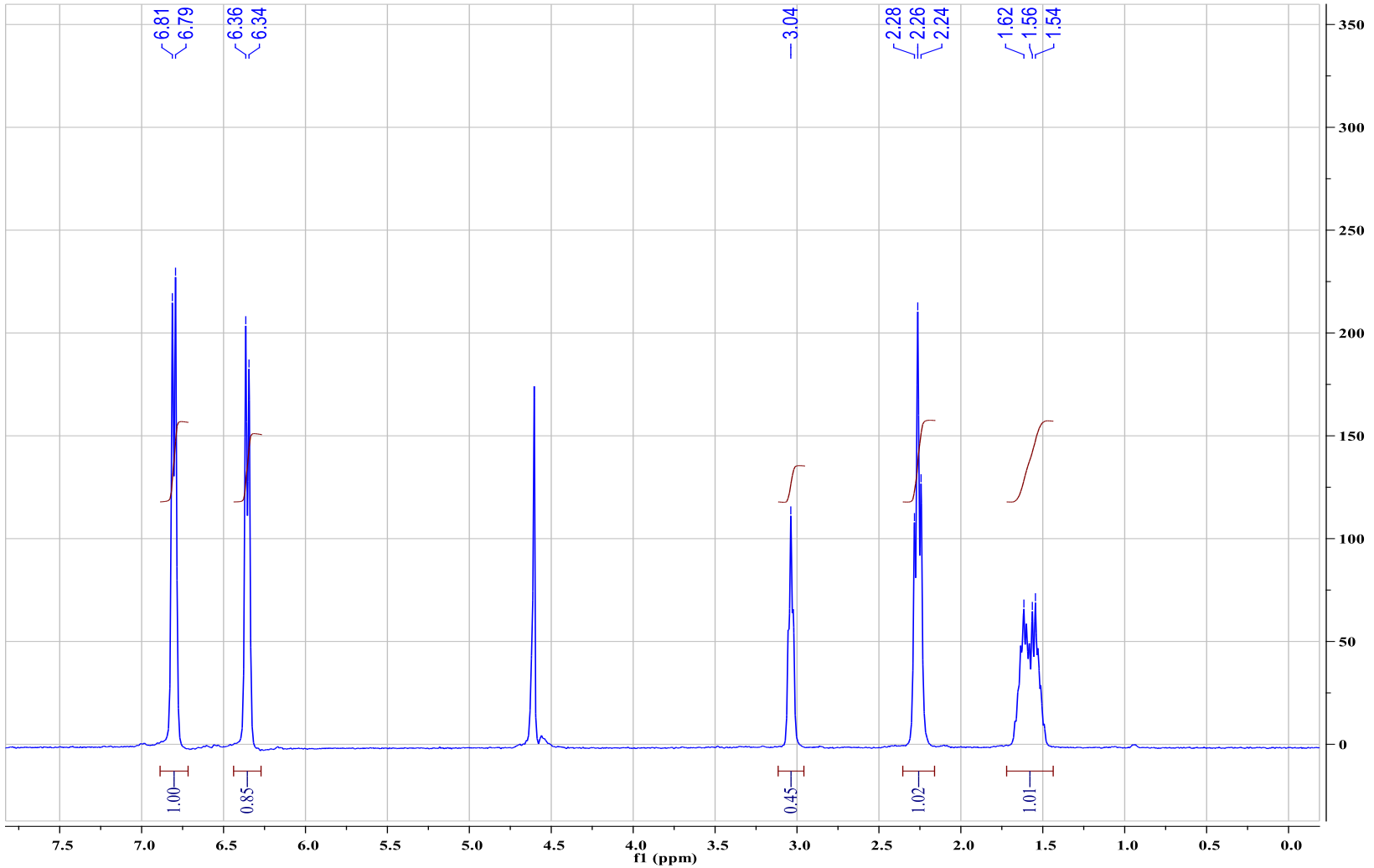
Appendix 3.4: *O*-methyl-L-homotyrosine (**6a**). ^{1}H NMR – Varian 400 MHz (NaOD/D_2O) δ (ppm): 1.61-1.70 (m, 2H), 2.38-2.42 (t, 2H), 3.03-3.06 (t, 1H), 3.62 (s, 3H), 6.76-6.78 (d, 2H), 7.05-7.07 (d, 2H).



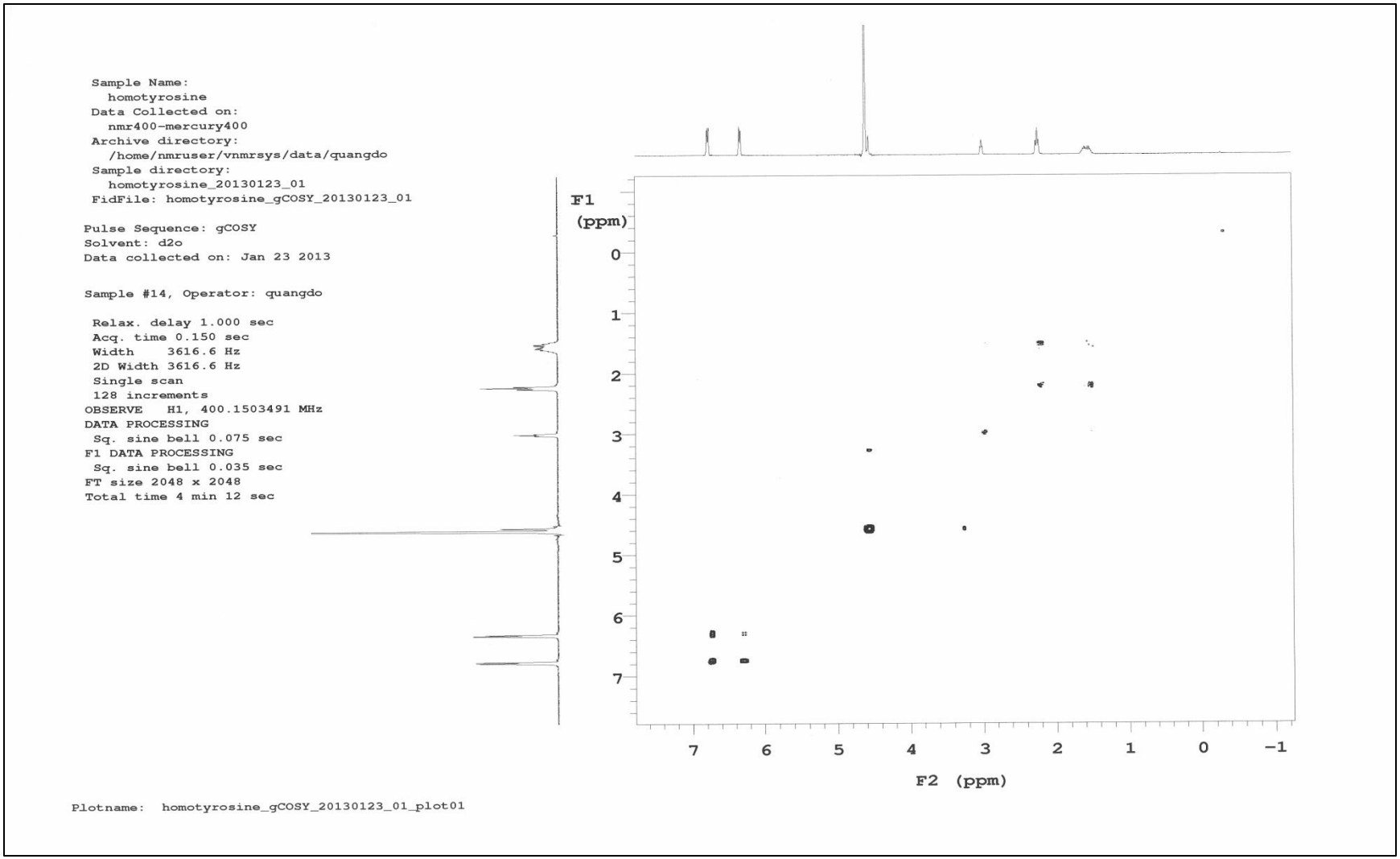
Appendix 3.5: *O*-methyl-L-homotyrosine (**6a**). ^{1}H COSY-NMR – Varian 400 MHz (NaOD/D_2O).



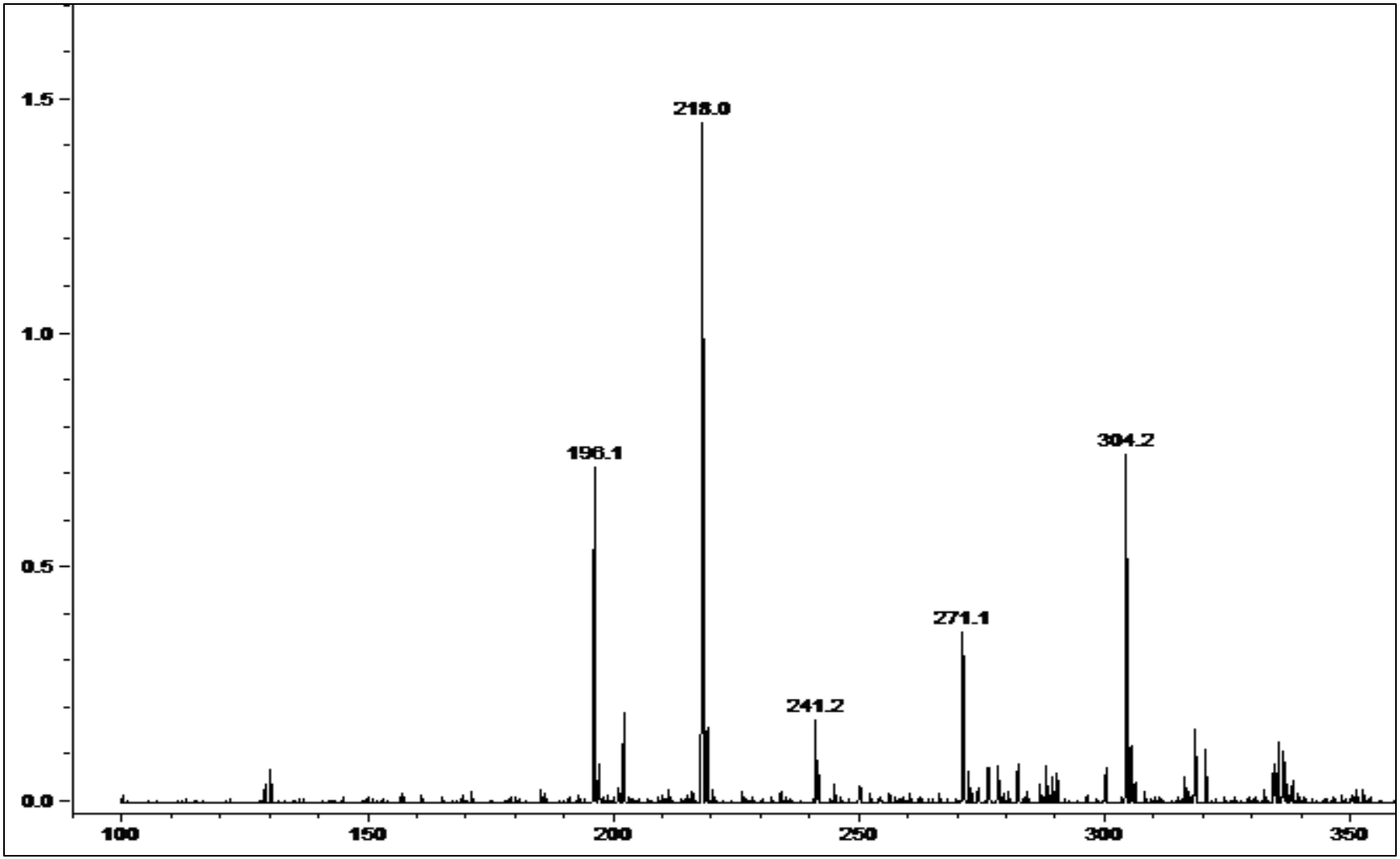
Appendix 3.6: *O*-methyl-L-homotyrosine (**6a**). Electrospray Ionization Mass Spectrum - Perkin Elmer API I Plus ESI-MS: M+1 = 210, M+Na = 232.



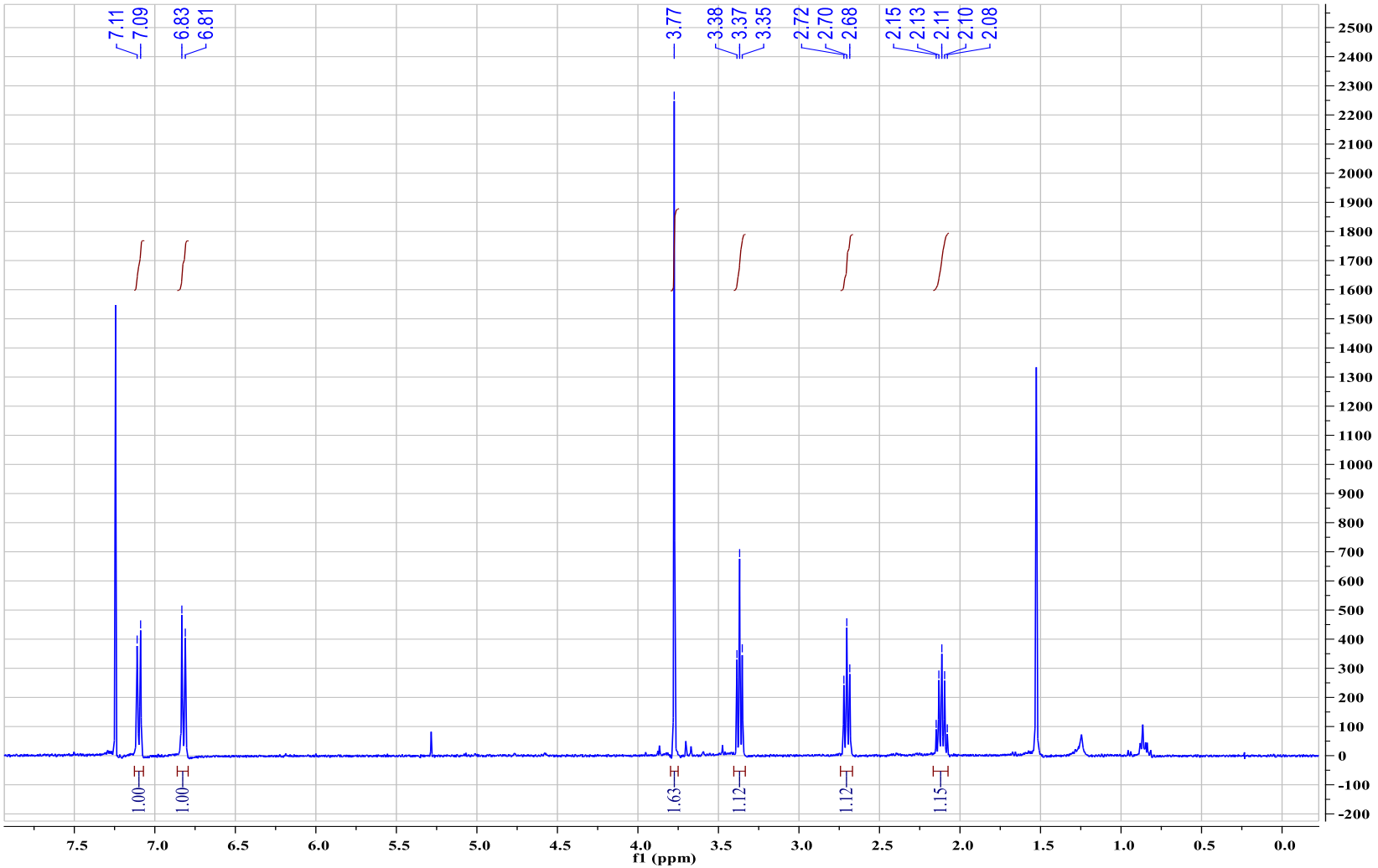
Appendix 3.7: L-Homotyrosine (**1a**). ^{1}H NMR – Varian 400 MHz (NaOD/D_2O) δ (ppm): 1.54-1.62 (m, 2H), 2.24-2.28 (t, 2H), 3.04 (s, 1H), 6.34-6.36 (d, 2H), 6.79-6.81 (d, 2H).



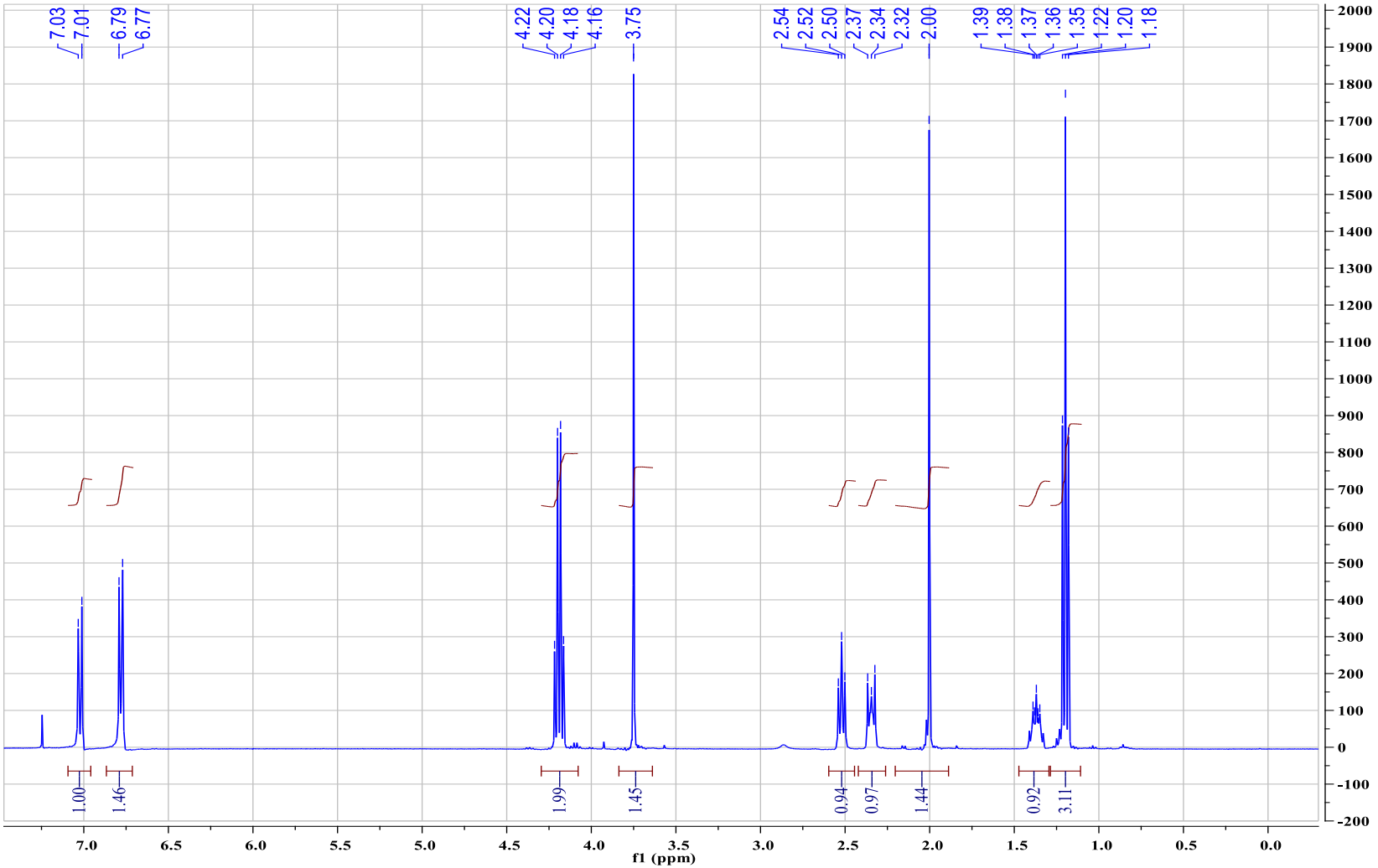
Appendix 3.8: L-Homotyrosine (**1a**). ^{1}H COSY-NMR – Varian 400 MHz ($NaOD/D_2O$).



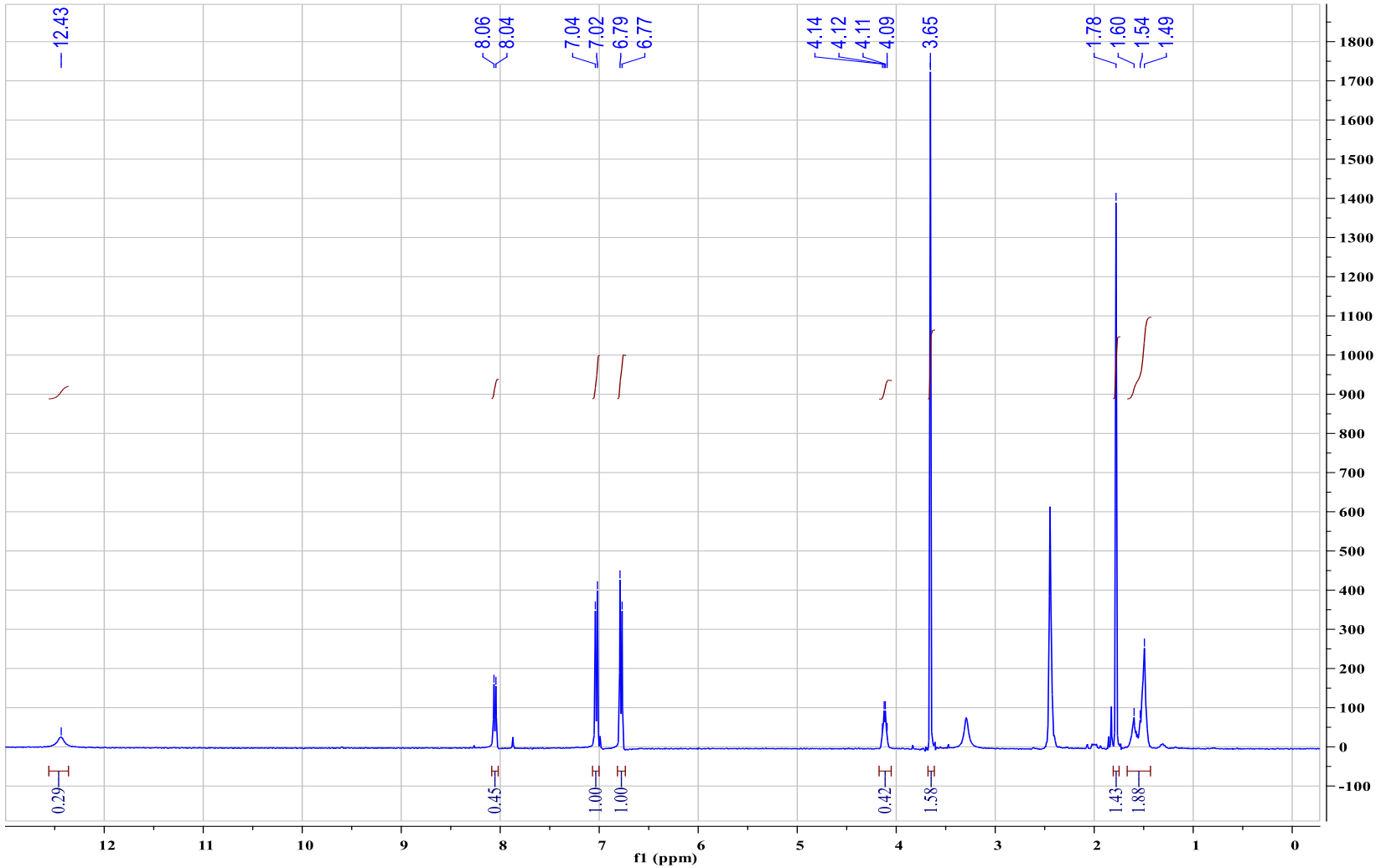
Appendix 3.9: L-Homotyrosine (**1a**). Electrospray Ionization Mass Spectrum - Perkin Elmer API I Plus ESI-MS: M+1 = 196, M+Na = 218.



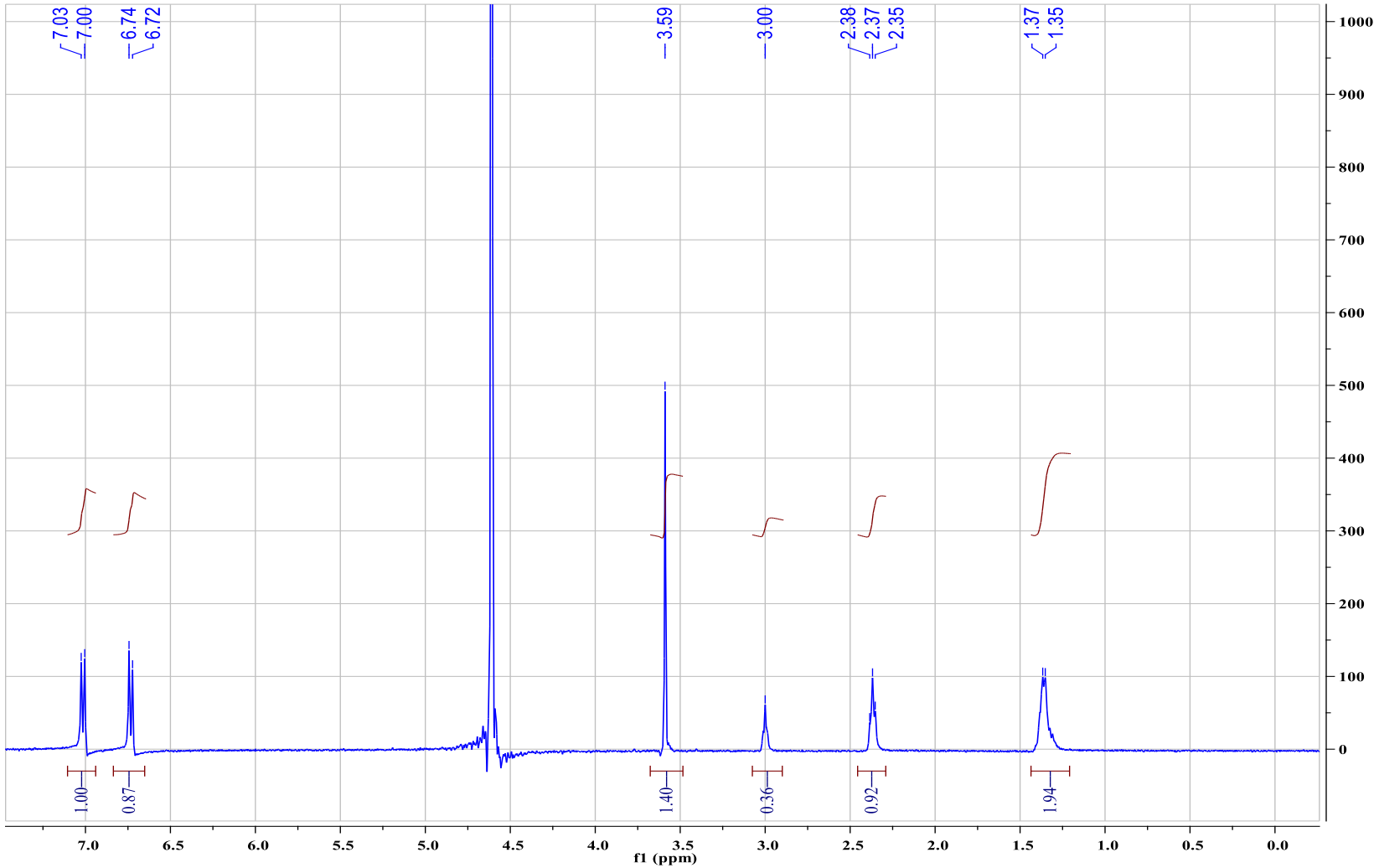
Appendix 3.10: 1-(3-Bromopropyl)-4-methoxybenzene (**3b**). ^{1}H NMR – Varian 400 MHz ($CDCl_3$) δ (ppm): 2.08-2.15 (m, 2H), 2.68-2.72 (t, 2H), 3.35-3.38 (t, 2H), 3.77 (s, 3H), 6.81-6.83 (d, 2H), 7.09-7.11 (d, 2H).



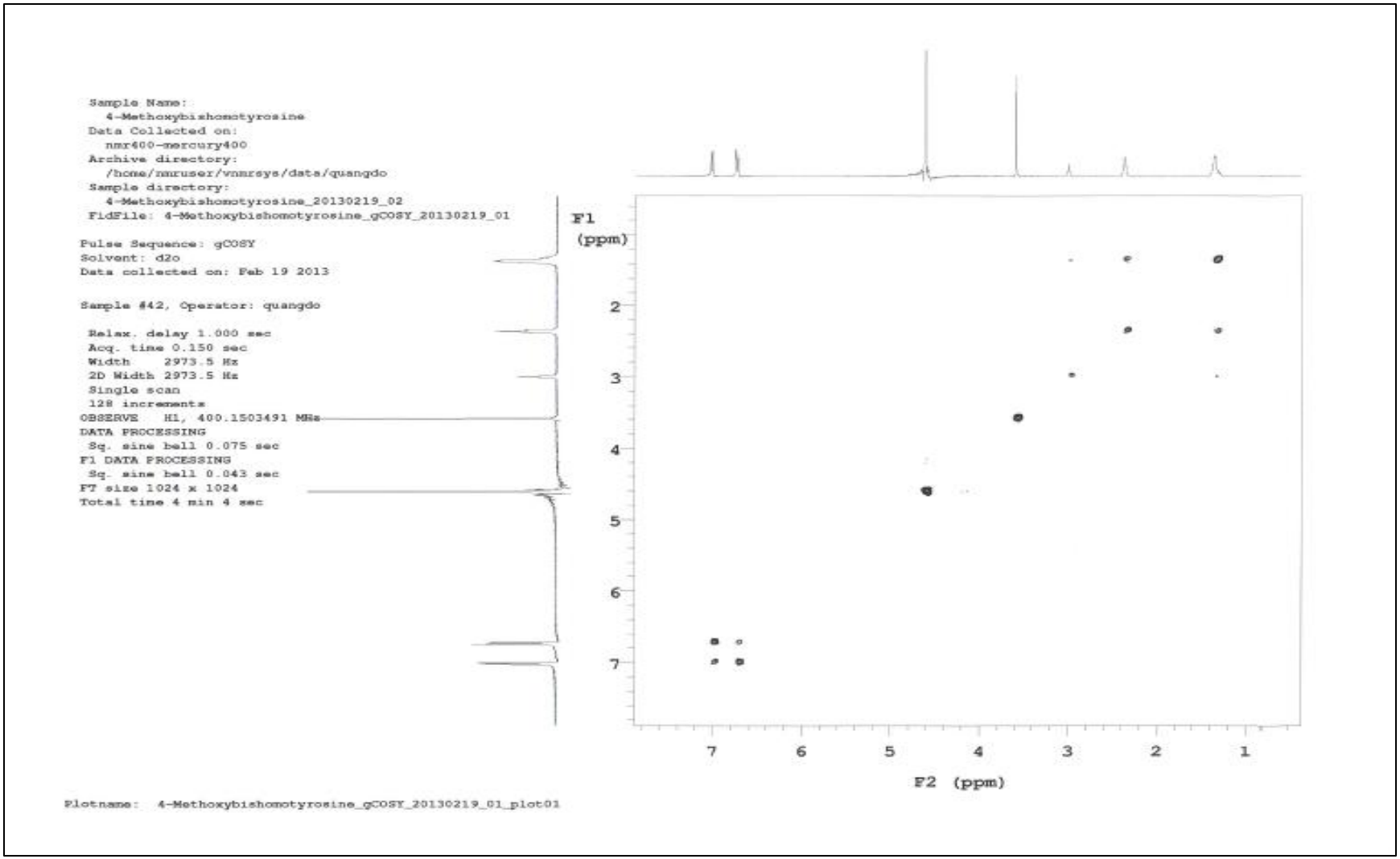
Appendix 3.11: Diethyl 2-acetamido-2-(3-(4-methoxyphenyl)propyl)malonate (**4b**). ^{1}H NMR – Varian 400 MHz ($CDCl_3$) δ (ppm): 1.18-1.22 (t, 6H), 1.35-1.39 (m, 2H), 2.00 (s, 3H), 2.32-2.37 (t, 2H), 2.50-2.54 (t, 2H), 3.75 (s, 3H), 4.16-4.22 (m, 4H), 6.77-6.79 (d, 2H), 7.01-7.03 (d, 2H).



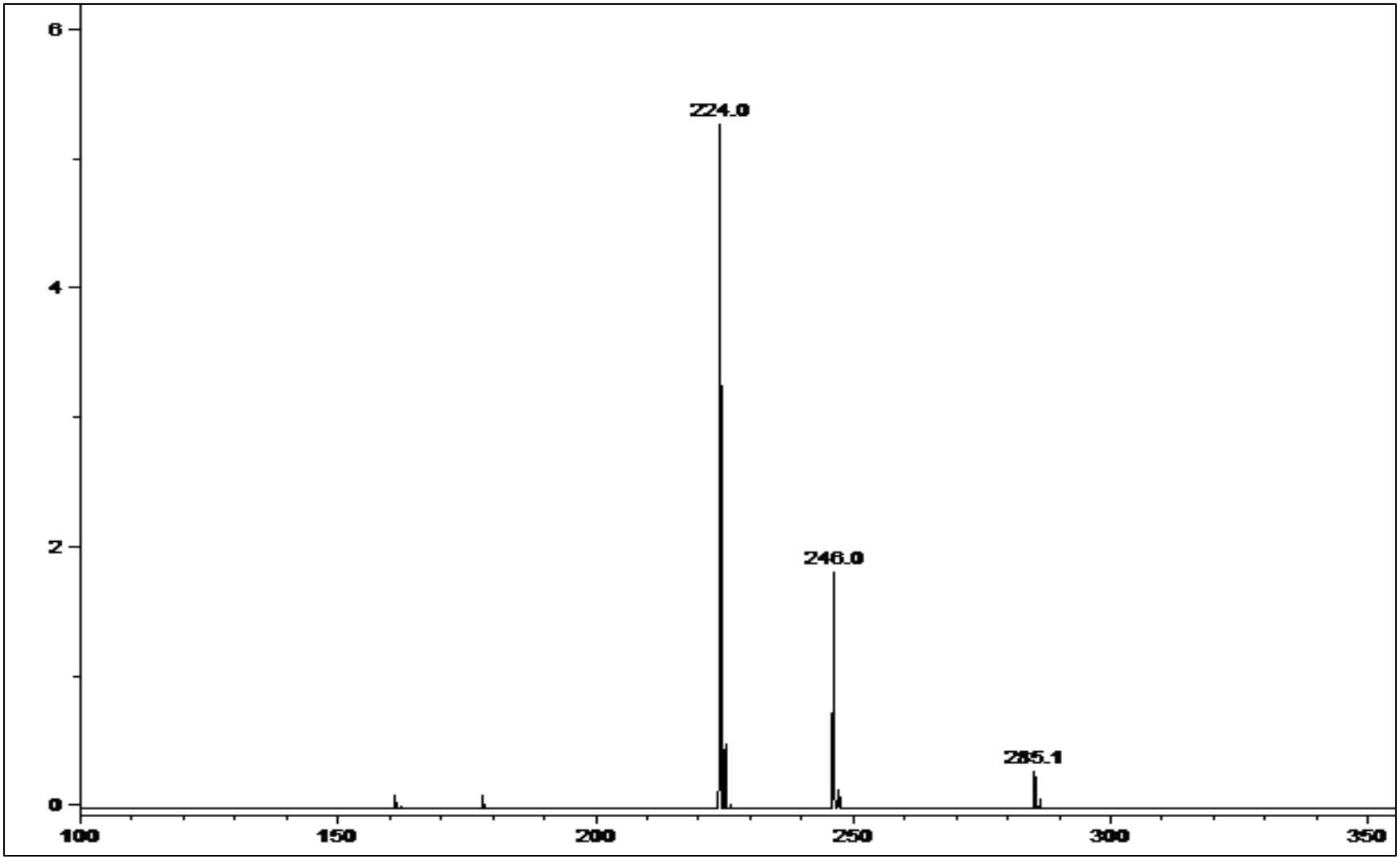
Appendix 3.12: 2-Acetamido-5-(4-methoxyphenyl)pentanoic acid (**5b**). ^{1}H NMR – Varian 400 MHz (DMSO) δ (ppm): 1.49-1.60 (m, 4H), 1.78 (s, 3H), 3.65 (s, 3H), 4.09-4.14 (m, 1), 6.77-6.79 (d, 2H), 7.02-7.04 (d, 2H), 8.04-8.06 (d, 2H), 12.43 (broad, 1H).



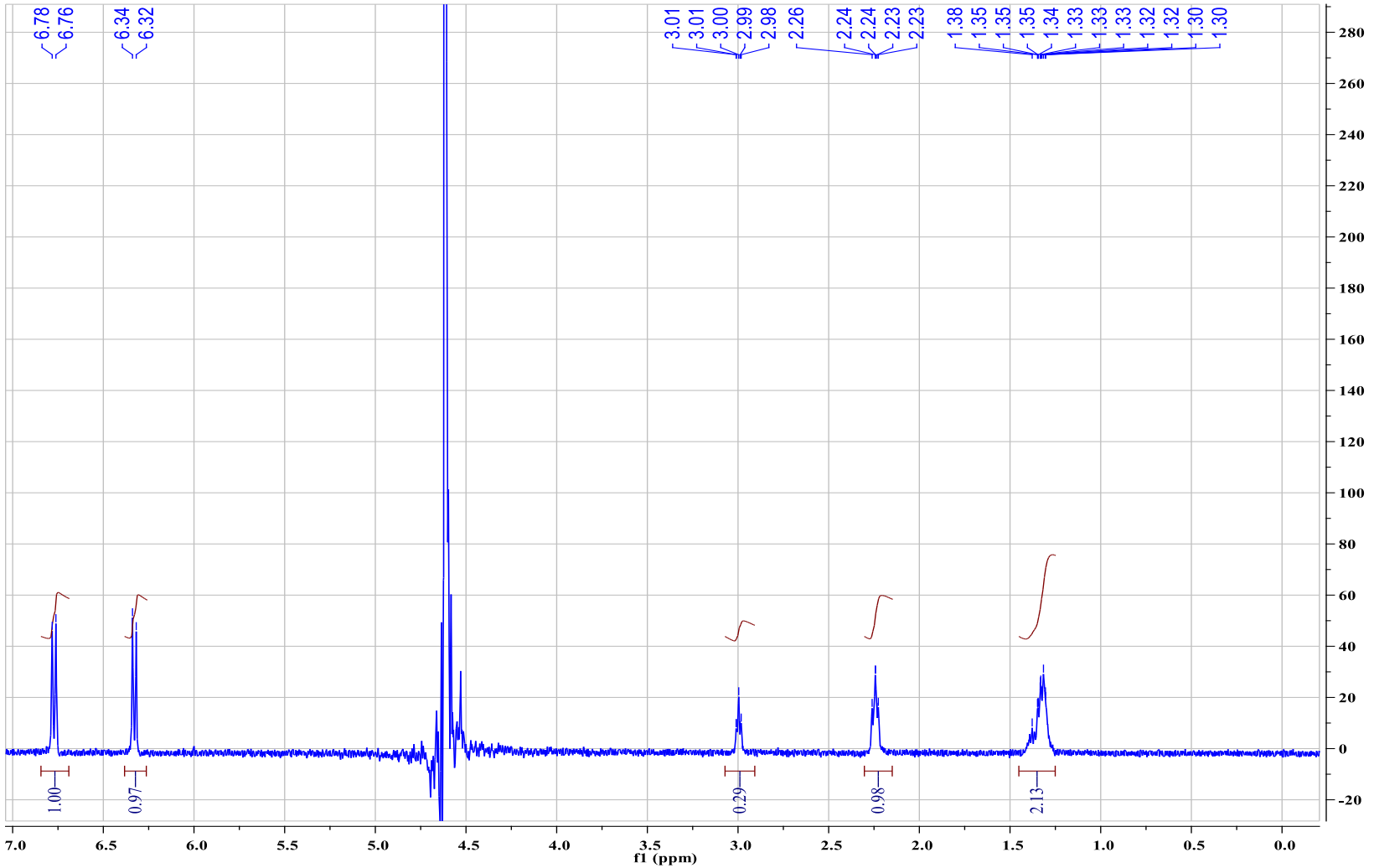
Appendix 3.13: *O*-methyl-L-bishomotyrosine (**6b**). ^{1}H NMR – Varian 400 MHz (NaOD/D_2O) δ (ppm): 1.35-1.37 (m, 4H), 1.35-1.38 (m, 2H), 3.00 (m, 1H), 3.59 (s, 3H), 6.72-6.74 (d, 2H), 7.00-7.03 (d, 2H).



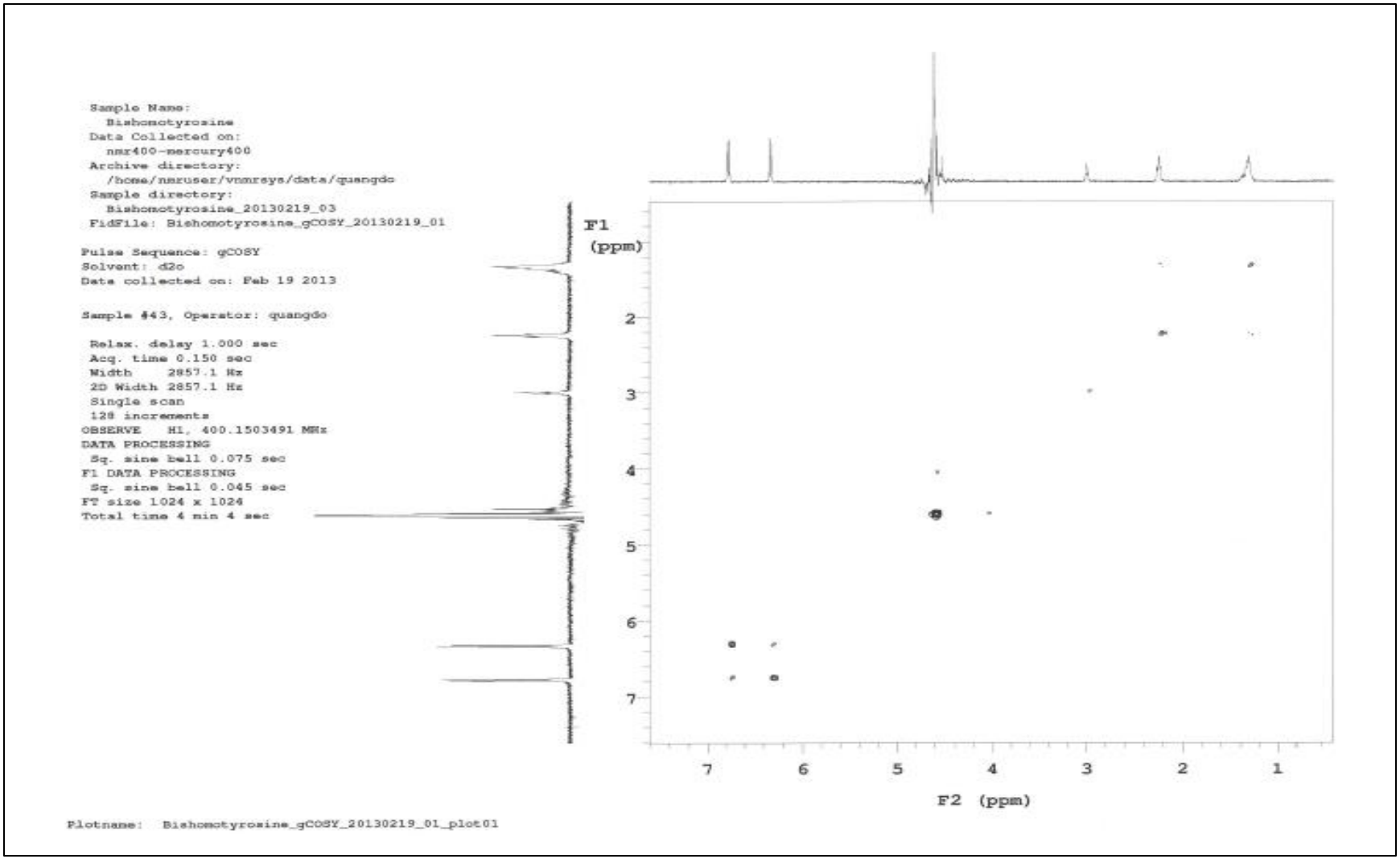
Appendix 3.14: *O*-methyl-L-bishomotyrosine (**6b**). ^{1}H COSY-NMR – Varian 400 MHz (NaOD/D_2O).



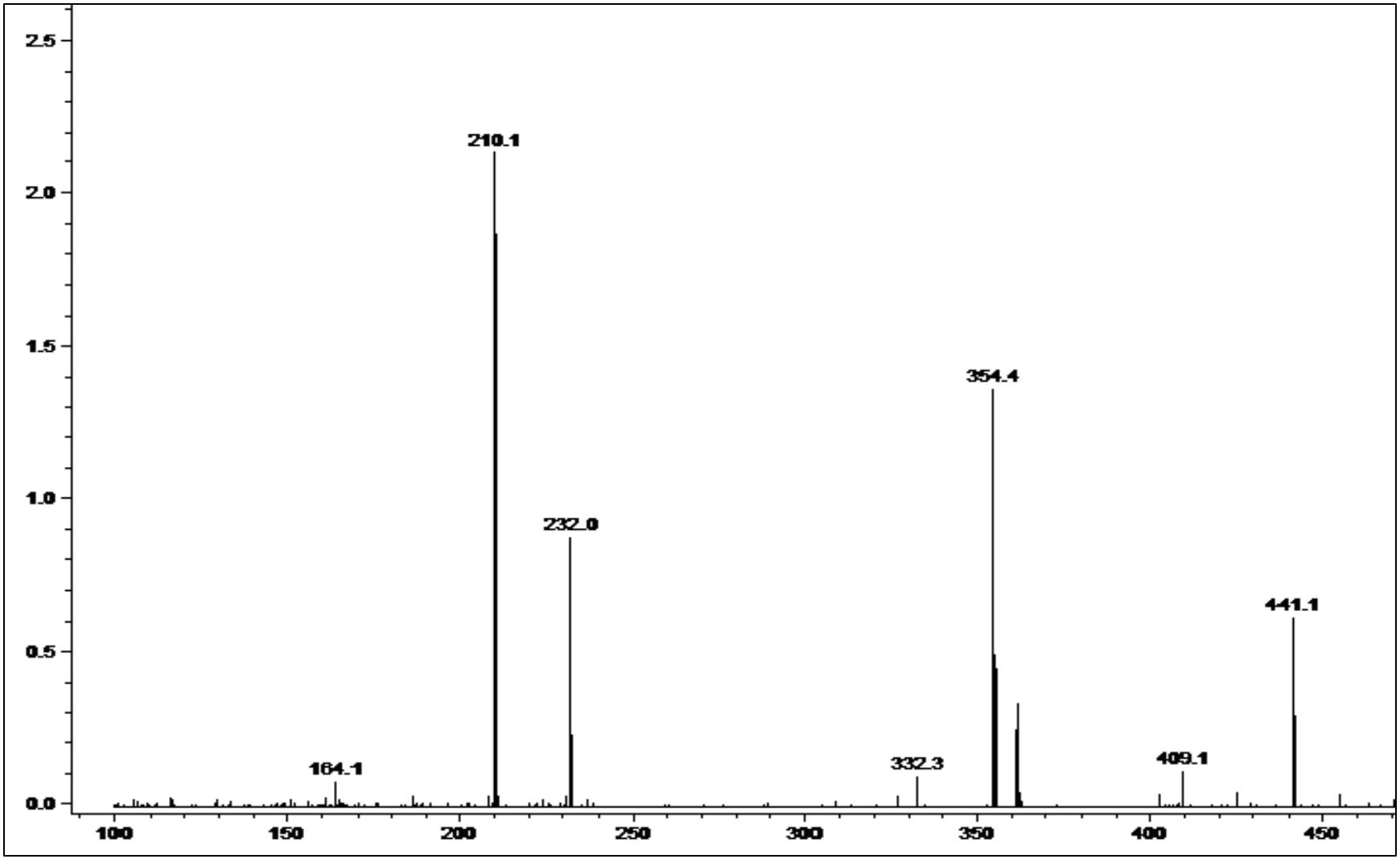
Appendix 3.15: *O*-methyl-L-bishomotyrosine (**6b**). Electrospray Ionization Mass Spectrum - Perkin Elmer API I Plus ESI-MS: M+1 = 224; M+Na = 246.



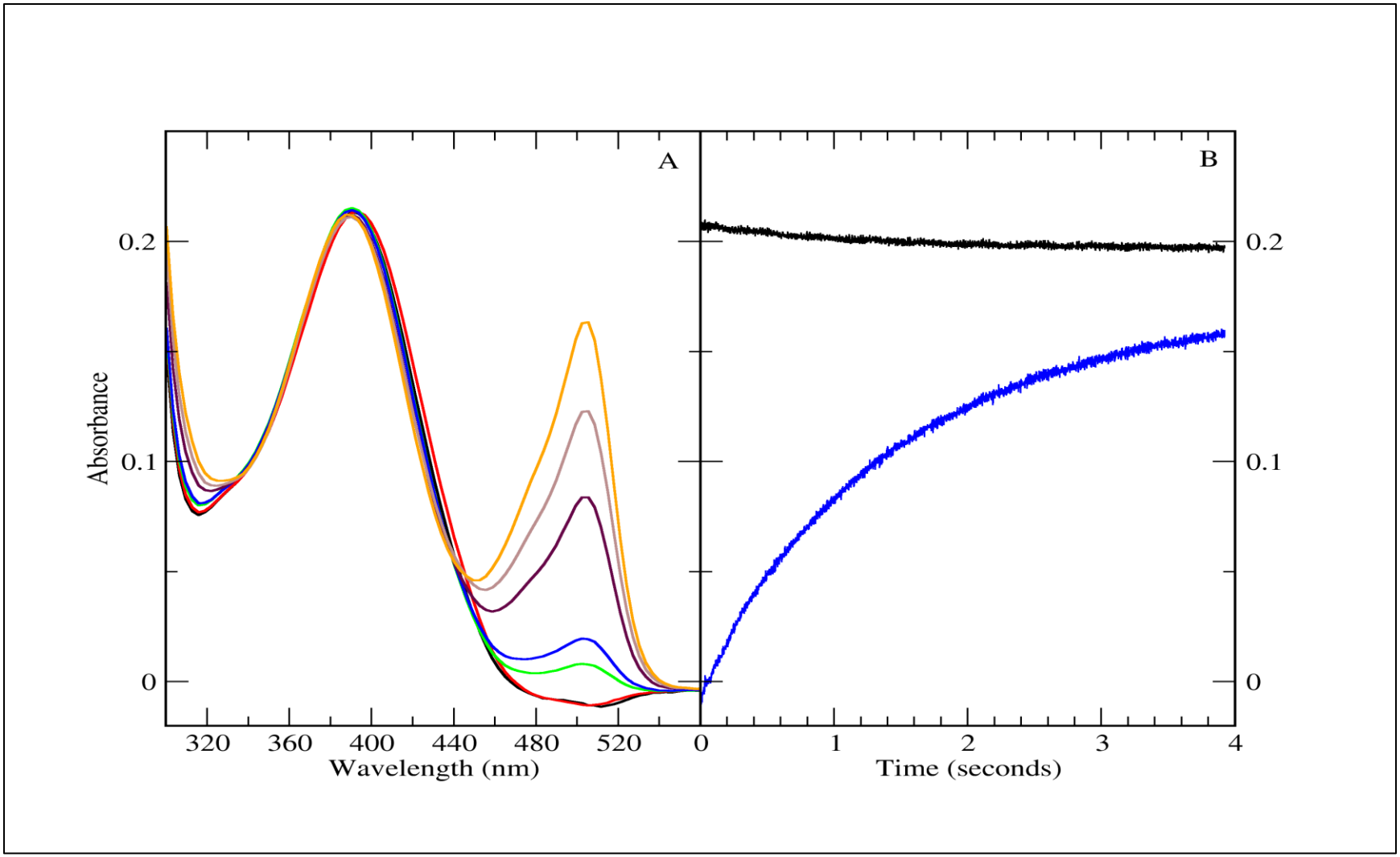
Appendix 3.16: L-Bishomotyrosine (**1b**). ^{1}H NMR – Varian 400 MHz (NaOD/D_2O) δ (ppm): 1.30-1.38 (m, 4H), 2.23-2.26 (m, 2H), 2.98-3.01 (m, 1H), 6.32-6.34 (d, 2H), 6.76-6.78 (d, 2H).



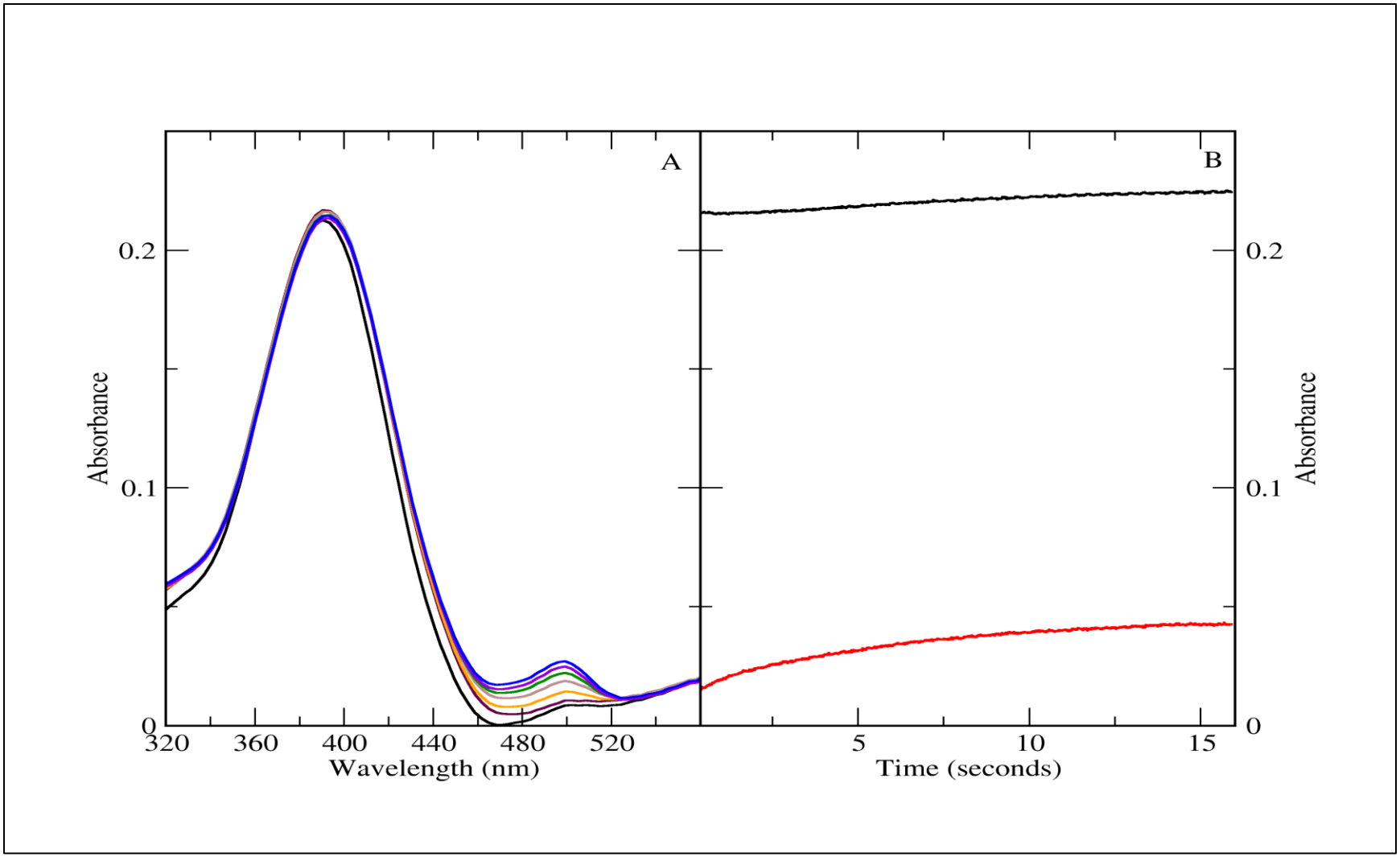
Appendix 3.17: L-Bishomotyrosine (**6b**). ^{1}H COSY-NMR – Varian 400 MHz (NaOD/D_2O).



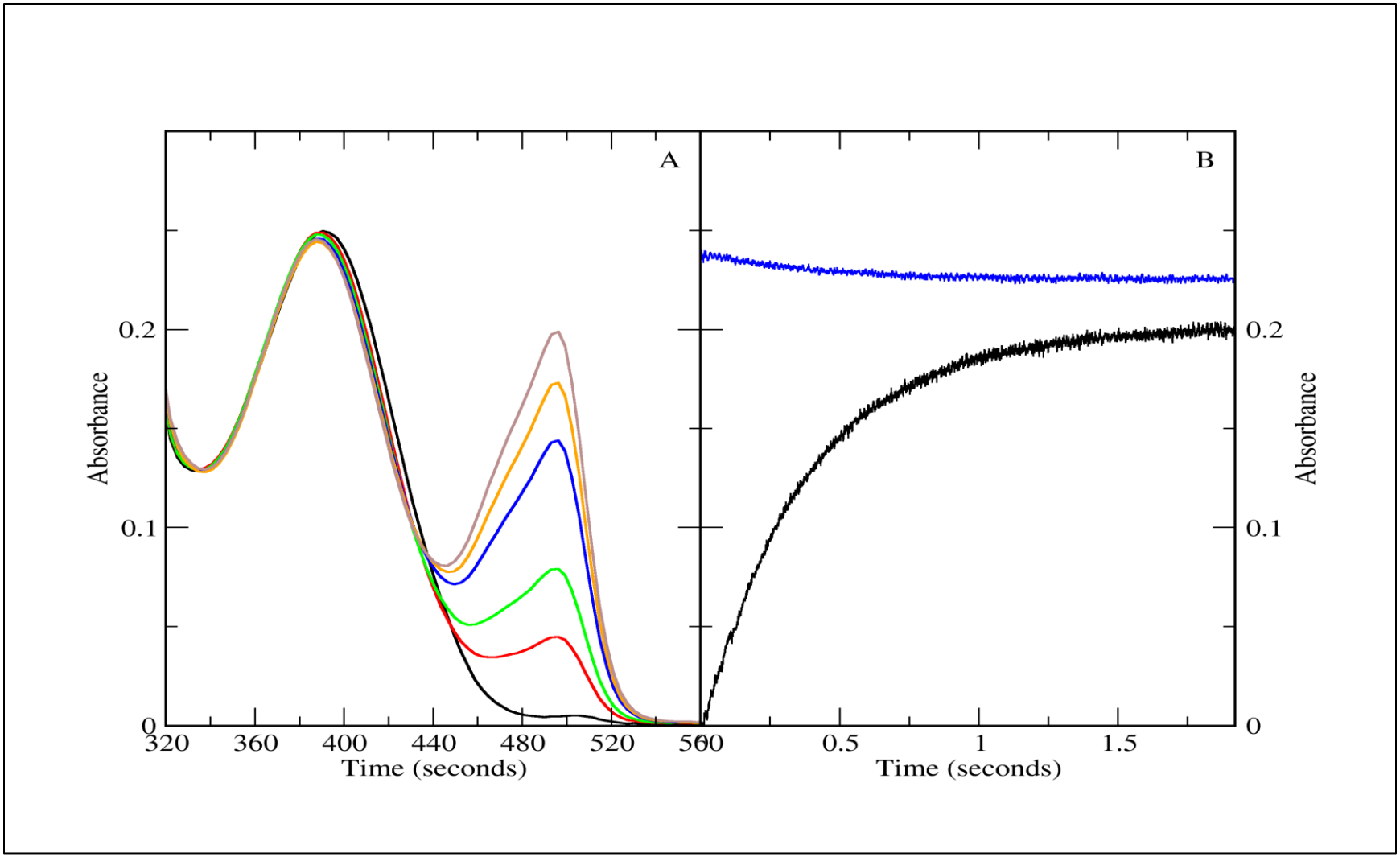
Appendix 3.18: L-Bishomotyrosine (**1b**). Electrospray Ionization Mass Spectrum - Perkin Elmer API I Plus ESI-MS: M+1 = 210, M+Na = 232.



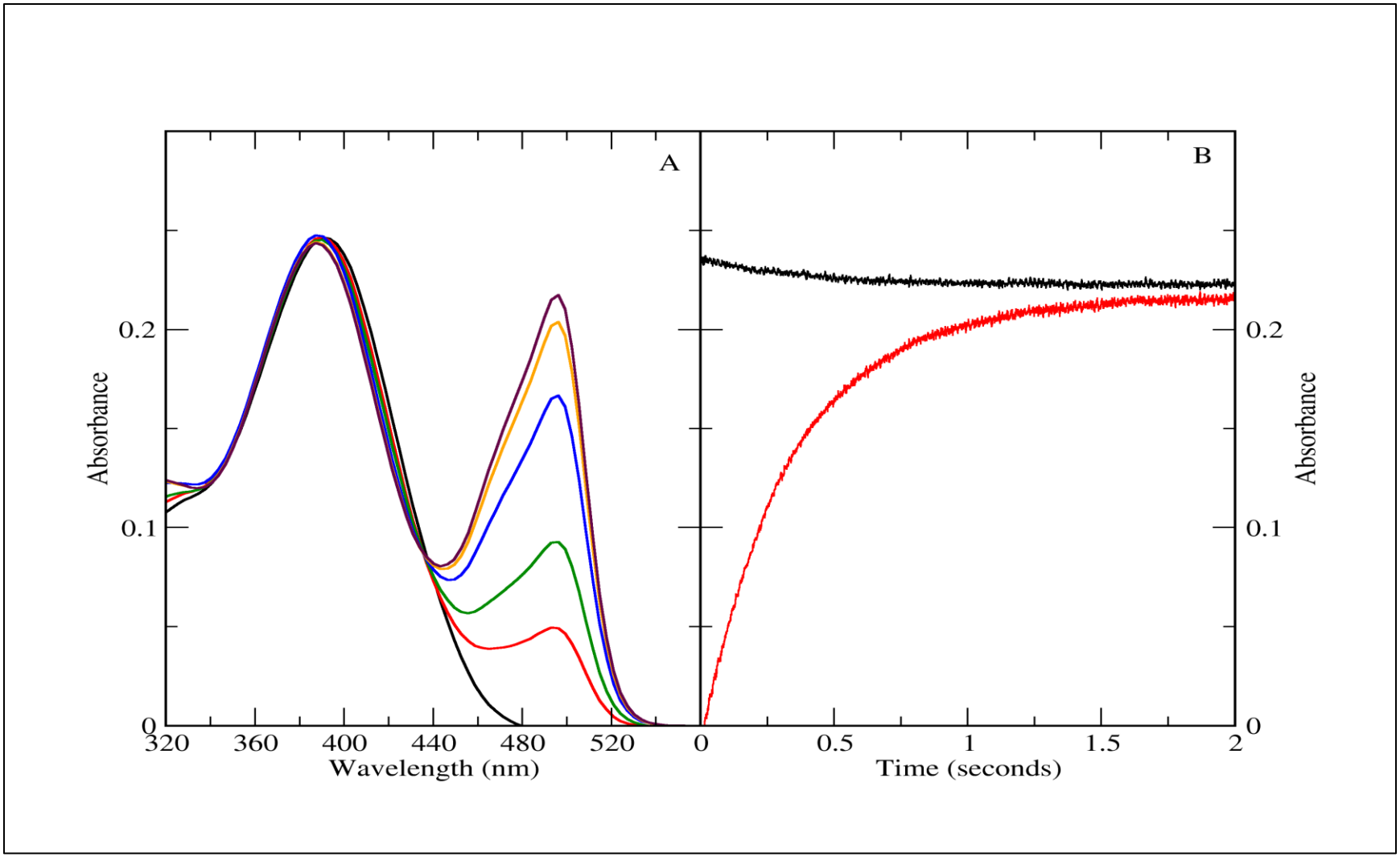
Appendix 3.19: Rapid-scanning stopped-flow of L-homotyrosine (**1a**) with tyrosine phenol-lyase.



Appendix 3.20: Rapid-scanning stopped-flow of *O*-methyl-L-homotyrosine (**6a**) with tyrosine phenol-lyase.



Appendix 3.21: Rapid-scanning stopped-flow of L-bishomotyrosine (**1b**) with tyrosine phenol-lyase.



Appendix 3.22: Rapid-scanning stopped-flow of *O*-methyl-L-bishomotyrosine (**6b**) with tyrosine phenol-lyase.