Fall 12-2009

Tuning Pig Liver Esterase Enantioselectivity for the Synthesis of Unnatural Serine and Tyrosine Analogues

Dale Anthony Rosado Jr.
University of Southern Mississippi

The University of Southern Mississippi

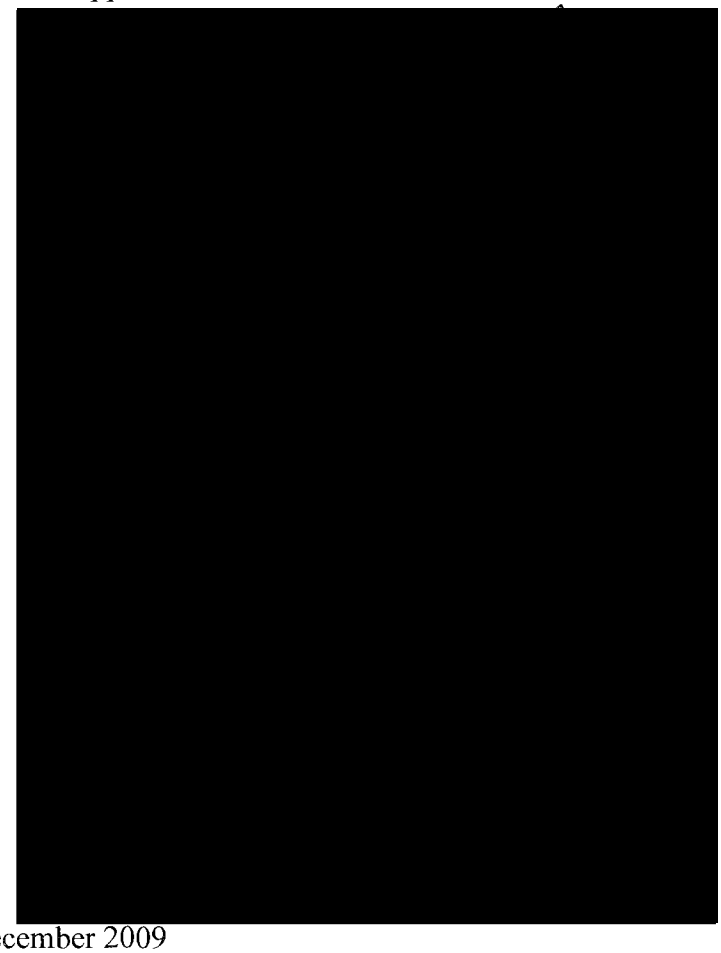
TUNING PIG LIVER ESTERASE ENANTIOSELECTIVITY FOR THE SYNTHESIS OF UNNATURAL SERINE AND TYROSINE ANALOGUES

by

Dale Anthony Rosado, Jr.

A Dissertation
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

Approved:



December 2009

The University of Southern Mississippi

TUNING PIG LIVER ESTERASE ENANTIOSELECTIVITY FOR THE SYNTHESIS OF UNNATURAL SERINE AND TYROSINE ANALOGUES

by

Dale Anthony Rosado, Jr.

Abstract of a Dissertation
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

December 2009

ABSTRACT

TUNING ESTERASE ENANTIOSELECTIVITY FOR THE SYNTHESIS OF UNNATURAL SERINE AND TYROSINE ANALOGUES

by

Dale Anthony Rosado, Jr.

December 2009

The synthesis of disubstituted and homologated unnatural amino acids and the development of a mass spectrometry based assay for enantioselectivity are presented here. Disubstituted and homologated unnatural amino acids have proven to be effective for the treatment of diseases and for use as imaging agents. Peptides that contain unnatural amino acids have also proven to be effective treatments for various disease states including cancer. The potential to evaluate such unnatural amino acids for pharmaceutical properties is limited by the available syntheses. No method currently exists that will allow for the synthesis of homochirally similar unnatural amino acids from a common intermediate. Herein is described a method that will allow for the synthesis of both enantiomers of disubstituted and homologated serine and tyrosine analogues from a common malonate half ester intermediates *via* several well-known functional group transformations and protecting group chemistry. The described synthetic methodology has resulted in the synthesis of both enantiomers of α-methyl-, $\beta^{2,2}$-, and $\beta^{3,3}$-serine and both enantiomers of α-methyl-, $\beta^{2,2}$-, $\beta^{3,3}$-, and $\gamma^{4,4}$- tyrosine analogues and one enantiomer of a $\gamma^{2,2}$- tyrosine analogue.

Synthesis of the aforementioned unnatural amino acids requires a chiral malonate half ester. To obtain this half ester intermediate, a malonate diester is enantioselectively

hydrolyzed by Pig Liver Esterase (PLE) to give an excess of one enantiomer of a malonate half ester over the other. The enantiomeric composition of the unnatural amino acids is dependent on the enantioselectivity of the enzyme. An increase in the enantioselectivity of the enzyme is needed if less than 97% enantiomeric excess (*ee*) is obtained from the enzymatic hydrolysis. This process can consume a tremendous amount of time and materials. This dissertation also describes the development of a high throughput electrospray-mass spectrometry based assay for determining the enantioselectivity of PLE for malonate diesters. The mass spectrometry assay has been utilized to evaluate the hydrolysis of three malonate diesters under a variety of cosolvent, buffer, and pH conditions. The PLE hydrolysis of a diester that was used to synthesize unnatural serine analogues was improved from 70% to greater than 97% *ee*. The hydrolysis of another diester that was used to synthesize unnatural tyrosine analogues was improved from 63% to 85% *ee*. This assay has proven to be a powerful tool for the enhancement of the enantiomeric composition of malonate half esters that can be used for the synthesis of disubstituted and homologated unnatural amino acids.

ACKNOWLEDGEMENTS

I would like to thank my wife, Martha Rosado, for her patience, love, and support during the duration of my doctoral work. A special thanks to my mother, Eva Rosado, for her numerous sacrifices for me and my siblings without which I might not be the person that I am today.

I would like to express my gratitude to my doctoral advisor, Dr. Douglas Masterson, whose guidance and friendship have helped me to succeed in my doctoral studies. I would like to express my appreciation to Dr. Jeffrey Evans, Dr. Wujian Miao, Dr Hans Schanz, and Dr. Karl Wallace for their advice, support, and guidance throughout my studies. I would also like to thank The Department of Chemistry and Biochemistry for the many opportunities that have satisfied my curiosity and advanced my scientific skill set.

I would like to acknowledge The American Chemical Society, Wiley and Sons, and BMJ Publishing Group Ltd. for permission to reproduce figures contained within this dissertation.

TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF ILLUSTRATIONS

Figure

LIST OF SCHEMES

Scheme

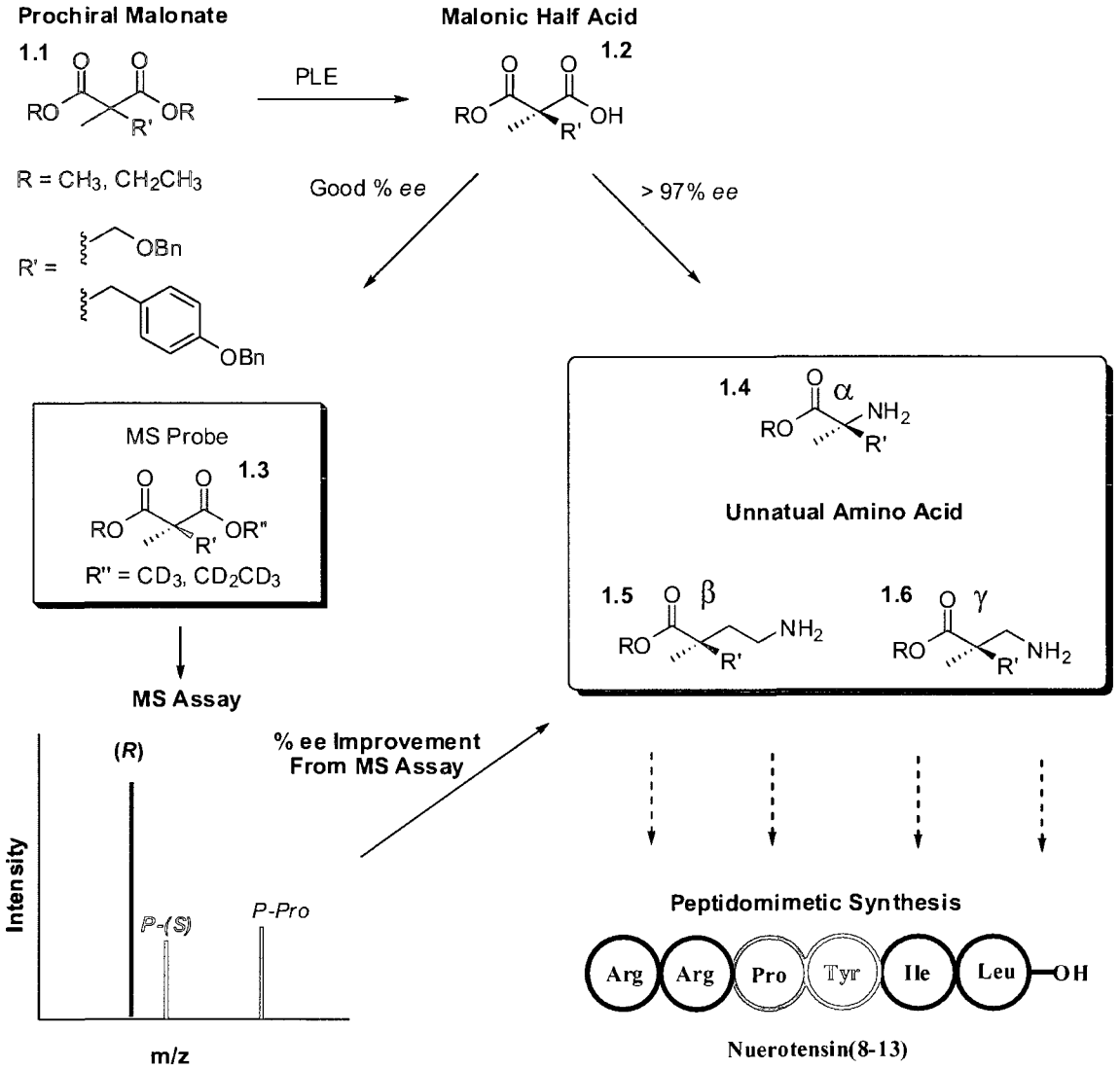
CHAPTER I

PEPTIDOMIMETICS, UNNATURAL AMINO ACIDS, AND MASS SPECTROMETRY: TYING IT ALL TOGETHER

Introduction

The National Research Council published "Beyond the Molecular Frontier" to state the challenges facing chemical scientists in the 21st century. One of the stated goals for synthetic chemists is to develop new syntheses using enzymes as catalysts to make compounds and materials of value.[1] The publication also states that compounds of pharmaceutical interest, either synthons or complete compounds, are perhaps the most important of the current synthetic endeavors. The overall goal of research in the Masterson research group is to synthesize small peptidomimetic compounds and test their potential for use as pharmaceutically active compounds, both *in vitro* and *in vivo*, as compared to the natural peptide. This dissertation will detail the efforts to create a series of homochirally similar unnatural serine and tyrosine analogues from a common intermediate (Scheme 1.1). The common intermediate is synthesized by use of pig liver esterase as a desymmetrization enzyme. The primary disadvantage of our proposed synthesis is that PLE may not yield compounds of sufficient enantiomeric composition (greater than 97% enantiomeric excess (*ee*)) for further synthesis. In an attempt to solve this problem, a high-throughput MS based assay that allows for the monitoring of the enantioselectivity of enzymes under a variety of conditions was devised. Once the enantioselectivity problem has been solved, synthesis of our unnatural amino acids can continue, which may ultimately result in a peptidomimetic compound with increased

biological stability and activity that may be used as a receptor specific imaging or a anti-disease compound.

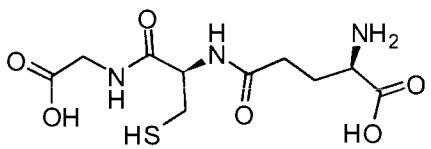


Scheme 1.1 The development of novel unnatural tyrosine analogues for incorporation into neurotensin peptidomimetic compounds

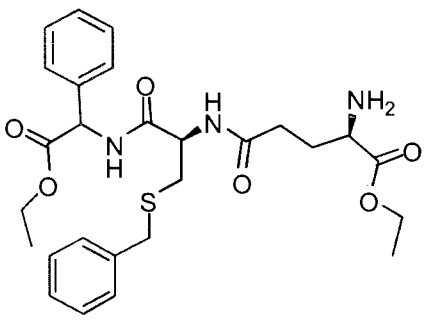
Literature Review

Peptidomimetics

A peptide is defined as two or more amino acids that are covalently joined by an amide bond. Peptides can be composed of two to fifty amino acids.[2] Peptides are involved in many functions throughout the body including peptide hormones, signaling, clearing of xenobiotics as peptide conjugates, and as antibiotics. Peptides are made and / or released as needed and are degraded or deactivated as soon as their function has been fulfilled. The efficiency of this process is increased by the high level of specificity exhibited by receptors for specific peptides.



1.7 Glutathione



1.8 TER199

Figure 1.1. The primary structure of glutathione and the TER199 glutathione derivative

Metabolic diseases are the result of a cell that has lost control of this regulation which can be caused by environmental or biological events. Peptidomimetic compounds are structurally similar and can be derived from natural peptides that are involved in some form of a disease state (up-regulation or down-regulation of genes, peptide aggregates, etc.). These compounds are designed to mimic the biological activity of a natural peptide or inhibit a receptor or enzyme. These compounds also display increased *in vivo* properties such as stability to the cellular machinery that is normally responsible for degradation of natural peptides. Synthesis of peptidomimetic compounds can be accomplished by covalent modification of the natural peptide, replacement of specific

residues with unnatural amino acids, or a combination of both.[3] An example of a small peptidomimetic compound is the TER199 (γ-glutamyl-*S*-(benzyl)cysteinyl-*R*-(2)-phenyl glycine diethyl ester, **1.8**) glutathione (**1.7**) derivative of which the cysteine residue had been S-benzylated and glycine has been replaced by phenyl glycine (Figure 1.1). This compound has shown the ability to inhibit a specific isozyme of glutathione S-transferase (GSTP1-1), which is responsible for metabolizing multiple xenobiotics, including anti-cancer medication, through glutathione conjugation and can be responsible for drug resistance. Inhibition of this isozyme with TER 199 led to prolonged uptake and activity of several drugs that had previously shown resistance.[4]

It has been shown that cancerous cells in various tumor tissues express large quantities of peptide hormones and, more importantly, peptide hormone receptors.[5,6] Many of these peptide hormones and receptors are expressed in specific tissues and should not be expressed in normal tissues elsewhere in the body. Another important characteristic of peptide hormones is that after binding to their receptor, they can be internalized into cells.[7,8] This can result in an increase in concentration of these peptides in tumor cells. These properties make peptide hormones promising candidates for evaluation as potential peptidomimetic drugs. Somatostatin (SST, **1.9**) is one such peptide hormone that has received much attention. Naturally, SST functions to regulate the secretion of growth hormone, insulin, glucagon, secretin, and vasoactive intestinal polypeptide.[9] Given the broad regulatory ability of SST, this peptide hormone was chosen for evaluation as one of the first peptidomimetic drugs. The initial research was focused on creation of an imaging agent. However, somatostatin has proven to be a very efficient hormone peptidomimetic at treating a variety of cancers by providing regulation of

mitosis.[10] This has led to development and FDA approval of several synthetic peptide drugs which have comparable activity to somatostatin, but have increased half-lives *in vivo* (somatostatin half-life = 2 to 3 min, octreotide half-life = 90 min).[9,11,12] They are available under the names octreotide (**1.10**), lanreotide (**1.11**), and vapreotide (**1.12**, Figure 1.2). The major advantage of these peptidomimetic drugs is that minimum chemical modification of natural peptide hormones can lead to compounds that are sufficiently stable to proteolysis and have high affinity and selectivity for the receptor of the native peptide. This is in contrast to the tremendous amount of trial and error that is involved in developing a new drug candidate.

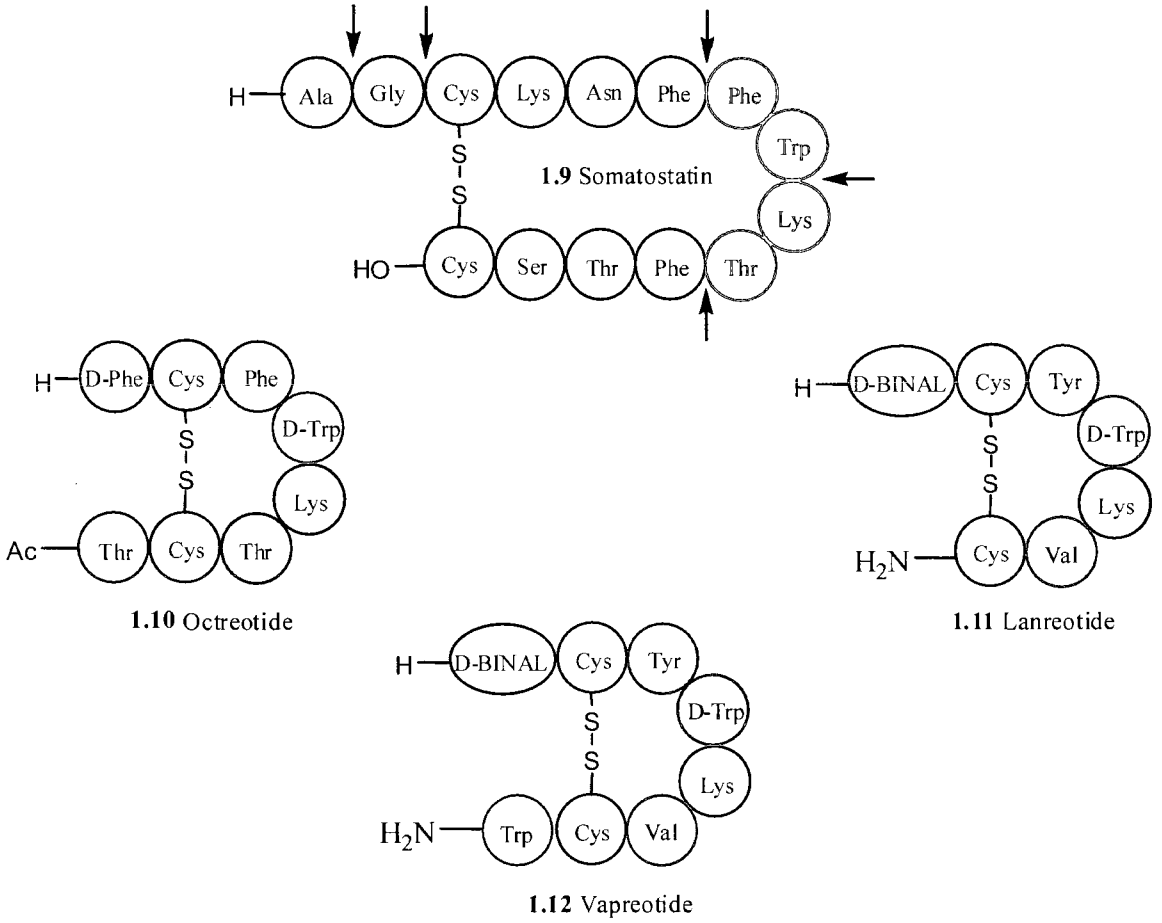


Figure 1.2. The native primary structure of Somatostatin (**1.9**) with protease cut sites indicated by arrows. Octreotide (**1.10**) Landtreotide (**1.11**) and Vaprotide (**1.12**) both with indicated modification of the active residues (highlighted in blue)

Picking a Target: Neurotensin

It is now clear that peptidomimetic compounds have a multitude of uses in the pharmaceutical world. With an unknown number of peptides performing various functions in the body, it is necessary to determine a target peptidomimetic compound. An ideal target will 1) be a small peptide composed of a minimum number of active amino acid residues and 2) contain minimum protease cut sites. The small nature of the peptides should make them amenable to efficient solid phase peptide synthesis. Neurotensin (NT,

1.13, Figure 1.3) is a peptide hormone which meets the desired properties. Literature has also shown that this peptide exhibits potential for imaging and cancer treatment.[5,6] However, the short half-life of NT in the blood (1.5 minutes) limits the potential use of natural NT.[13] As discussed earlier, this problem may be solved by the incorporation of unnatural amino acids into protease specific cut-sites in the peptide. It has been shown that replacement of several specific amino acid residues with unnatural analogues (unnatural side chains) has lent dramatically to the stability of these NT analogues in human serum. Furthermore, some of the existing NT analogues exhibit increased binding affinity for the NT receptor.[13]

1.13

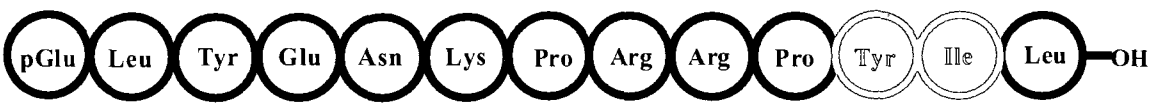


Figure 1.3 Condensed structure of Neurotensin. The protease cleavage site of interest between the tyr-ile bond is highlighted in red

Neurotensin is a 13 residue peptide hormone. Neurotensin was discovered in 1973 by Carraway *et al.*[14] The amino acid sequence was later determined and the peptide was synthesized by solid-phase peptide synthesis. Neurotensin is found primarily in the central nervous system and the intestinal tract. In the central nervous system, NT functions as a neurotransmitter or neuromodulator of dopamine transmission and in anterior pituitary hormone secretion.[15] In the digestive tract, NT functions as a local hormone that exerts paracrine and endocrine modulations of the digestive tract and the cardiovascular system in mammals.[15,16] NT has also been shown to be involved in immune response in the gut, as well as a growth response in a variety of other tissues.[15]

Neurotensin and the NT receptor have been found to be over expressed in Ewing's sarcoma (65%), mengiomas (52%), astrocytoma (43%), medulloblastoma (38%), medullary thyroid carcinoma (29%), and small lung cell cancer (25%).[17] Additionally NT receptors have also been found in prostate cancer cell lines[18], breast cancer cell lines[19], pancreatic cancer cell lines[5], and colon cancer cell lines[15]. In 1998, Reubi, et al. confirmed the presence of NT receptors in human exocrine pancreatic tumors (Figure 1.4). This was accomplished by testing 24 patients with ductal pancreatic adenocarcinomas, 20 with endocrine pancreatic cancers, 18 patients with chronic pancreatitis, and 10 healthy patients. Acetyl-NT(1-13) was labeled at 3-Tyr with ^{125}I, apposed to 3H hyperfilms (Where a tissue slice containing a radioactive isotope is exposed to film by placing the tissue directly on the film.), and subjected to x-ray cassettes for seven days. Reubi was able to conclude that NT receptors were present in 75% of the human ductal adenocarcinomas examined and up to 83% of the highly differentiated tumors expressed NT receptors. Equally important was the fact that NT receptors were not found in the pancreatic tissue of healthy patients or in the pancreatic tissue of patients with chronic pancreatitis (a disorder that can lead to pancreatic adenocarcinomas).[5] The high level of specificity exhibited by NT for exocrine tumors

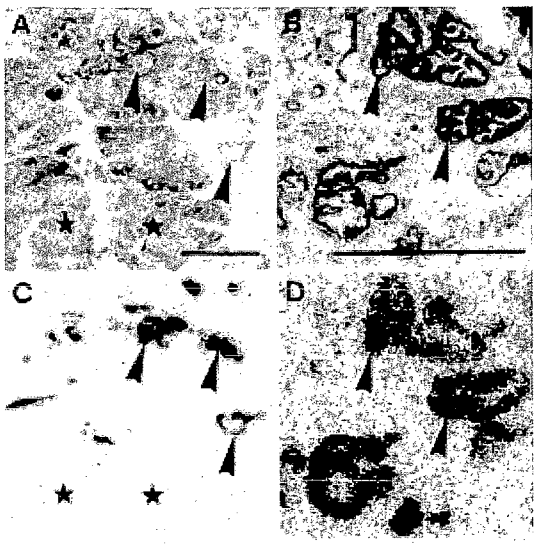


Figure 1.4. A & B. Autoradiograms of a Haematoxylin-Eosin stained pancreatic tumor. C & D Autoradiodiagram of the same tumor with ^{125}I-NT [5] Reproduced with permission from BMJ publishing Group Ltd

combined with previously developed Octreoscan (a labeled somatostatin analogue specific for types endocrine pancreatic cancer (since NT receptors were not expressed in the endocrine pancreatic cancer)) could make NT a powerful therapeutic tool able to selectively identify the presence and type of tumors (Figure 1.5).[5]

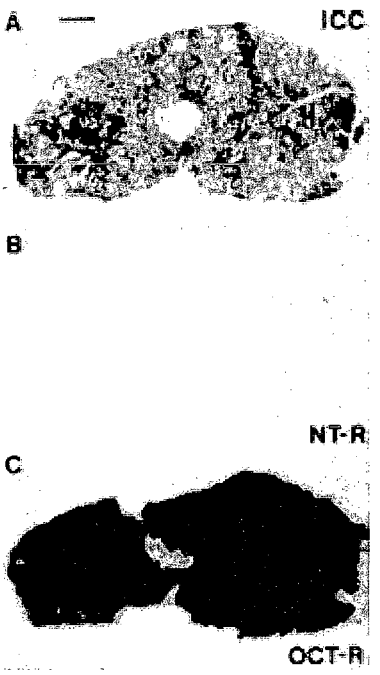


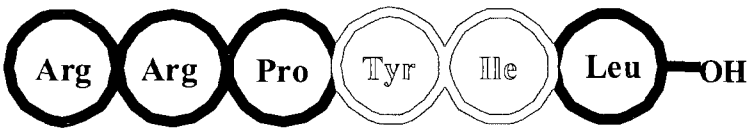
Figure 1.5. Autoradiodiagram of an endocrine pancreatic tumor A. Haematoxylin-Eosin B. ^{125}I-NT C. ^{125}I-Octreotide [5] Reproduced with permission from BMJ Publishing Group Ltd

Neurotensin: Evaluation of Primary and Secondary Peptide Structure for Design of a Peptidomimetic Compound

It is important to know the binding site of peptides when designing peptidomimetic drugs. Some peptide hormones may bind to several different subtypes of receptors; each with varying affinity. If peptidomimetic NT analogues exhibit internalization it may allow for efficient use of the analogues for imaging. [20-22] To date, three subtypes of the NT receptor have been discovered.[15,16] The most important characteristic of the 3 NT receptor subtypes in regard to this dissertation is that they all recognize the same C-terminal 8-13 sequence and have similar structure-function with regard to NT.[16] To determine if NT exhibits internalization upon binding to the NTS, Chabry *et al.*[7] used a radio-labeled NT analogue. It was found that after 1 hour of incubation with NTS-1 cells, at 37 °C, 56% of ^{125}I-NT that bound to NTS-1, was internalized into cells that had

been transfected with NTS-1. This was further confirmed by using NT labeled with a fluorescent tag. It has also been observed that all three subtypes of NTS participate in internalization.[8] The internalization of the NT/NTS means that NT has potential for use as an imaging agent or a receptor-specific drug. The genes for NTS-1 have been shown to be fairly conserved in rats, mice, and humans.[16] The conserved nature of these genes makes rats and mice ideal candidates for *in vivo* testing of potential NT analogues.

When designing peptidomimetic compounds it is necessary to determine two properties of the peptide of interest: 1) the smallest peptide that retains biological activity and 2) the active residues of that segment. Once the active residues are determined, these residues can then be replaced with homologous unnatural amino acids or with other unnatural amino acids that retain the properties of the residue of interest. Determination of the shortest active fragment of NT was performed by Carraway et al. Using native NT 1-13 as a control, NT fragments synthesized that were truncated at both the N and C termini. The biological activity of each fragment was then tested using rats and guinea pigs.[23] It was determined that these NT analogues maintained their biological activity (60 to 70% of native NT activity) as long as the last six residues remained intact (NT(8-13), **1.14**, Figure 1.6).[24] St-Pierre et al. determined which residues were responsible for binding to the NTS by systematically replacing specific amino acids with either similar amino acids, *D*-amino acids, or alkylated amino acids. It was determined that 8-Arg, 9-Arg, and 10-Pro were required for binding. Removal of 8-Arg or substitution of 9-Arg with *D*-Arg



1.14

Figure 1.6. NT(8-13) Smallest NT analogue that maintains biological activity

resulted in significant loss of binding to the NTS-1. The same effect was seen when 10-Pro was replaced with *D*-Pro. This was proof that L-Pro is required for the correct conformation for binding to the NT receptor. The most important finding was that replacement of 11-Tyr with any amino acid (*D*-Tyr, Phe, *D*-Phe, Ala, Leu, *D*-Trp, and Tyr(OMe)) but Trp led to a dramatic loss in activity. This led St-Pierre, et al. to conclude that the 11^{th} residue must be an aromatic *L*-amino acid capable of H-bonding.[24]

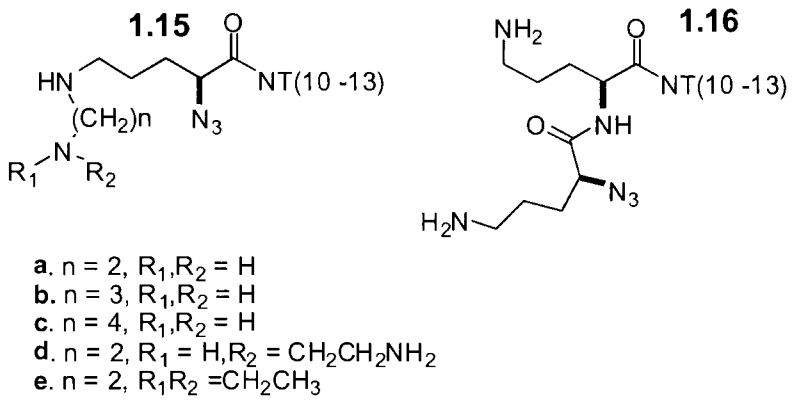
The 3-dimensional structures of peptides are also important in designing peptidomimetic compounds. The spatial arrangement of the functional groups of a peptide can greatly influence binding affinity. Therefore, it is important that a peptidomimetic compound display a similar 3-D structure as compared to the native peptide. This means that it is important to know the solution and binding conformations of the peptide of interest. Nieto et al. were able to confirm that NT(1-13) has no stable secondary structure in solution. This was accomplished by a ^{1}H-NMR study of NT(1-13), NT(1-8), and NT(8-13) in the presence of a temperature gradient (-5 to 38°C), denaturing agents (6M urea), and a pH gradient. No significant difference in conformation was seen for NT(1-13), NT(1-8), or NT(8-13) in the presence of any of the above conditions. Furthermore, no presence of peptide aggregates were found in concentrated solutions of NT(1-13). It was concluded that NT(1-13) assumes a random coil in solution.[25] It is also necessary to determine if NT adopts a particular conformation upon binding to the NTS. This was accomplished by using ^{13}C, ^{15}N-labeled NT(8-13) peptides that were bound to the lipid reconstituted NTS-1.[26] Two dimensional ^{13}C-^{13}C (2Q,1Q) NMR correlation experiments were used due to the method's ability to select for pairs of nearby ^{13}C-^{13}C nuclei (directly bonded). This study determined that the 10-Pro residue with a ψPro

dihedral angle of 146 ± 15° allows NT and NT analogues to assume a β-strand conformation when bound to NTS-1. This means that any structural modification that allows the peptidomimetic compound to retain or more readily assume the binding conformation may increase the binding affinity of the peptidomimetic compound for the NTS.

In light of the discovery that NT and NTS are over expressed in cancer tissue, several different approaches to adapting NT and truncated NT analogues for use as cytotoxic drugs and/or imaging agents have been developed. As mentioned earlier, there are two primary problems that must be overcome when designing NT analogues: 1) the NT analogue must be stable to endogenous proteases and 2) the binding affinity to NT receptors must be retained or improved upon. Knowledge of prior attempts to make a NT peptidomimetic compound is necessary to pick an amino acid residue on which to focus our synthetic efforts. The primary focus for the development of NT analogues is the NT(8-13) fragment. The labile nature of NT(8-13) has been attributed to the 8-Arg- 9-Arg, 10-Pro- 11-Tyr, and the 11-Tyr- 12-Ile peptide bonds.[13,24,27-32] Therefore, modification at these residues has been the primary focus of recent NT research.

Several groups have recently shown that replacement of either the 8-Arg with a single basic amino acid, or both the 8-Arg -9-Arg with either two new basic amino acids or a single amino acid capable of mimicking both 8-Arg -9-Arg, has increased binding affinity to NTS-1 compared to NT(8-13) or NT(8-19).[13,27-32] Some of these groups have also addressed the liability of the 11-Tyr -12-Ile peptide bond by replacing the Ile residue with bulkier unnatural amino acids. Linquist et al. [33] working with a proposed binding model of NT(8-13), developed NT analogues in which the 9-Arg residue was replaced

with azido acids **1.15a-e** and **1.16** in Figure 1.7 that could mimic the 8-Arg- 9-Arg, but reduce the molecular weight of the analogue.[33] These NT analogues were compared to NT(9-13) (hNTR-1 affinity = 19.0 ± 2.0 nM) and it was found that every analogue showed a higher binding (hNTR-1 affinity = 3.9 – 14.7 nM) affinity to the hNTS-1 than NT(9-13), with the exception of the NT(9-13) analogue containing compound **5,** which had a comparable binding affinity (hNTR-1 affinity = 28 ± 5.0 nM) to NT(9-13).

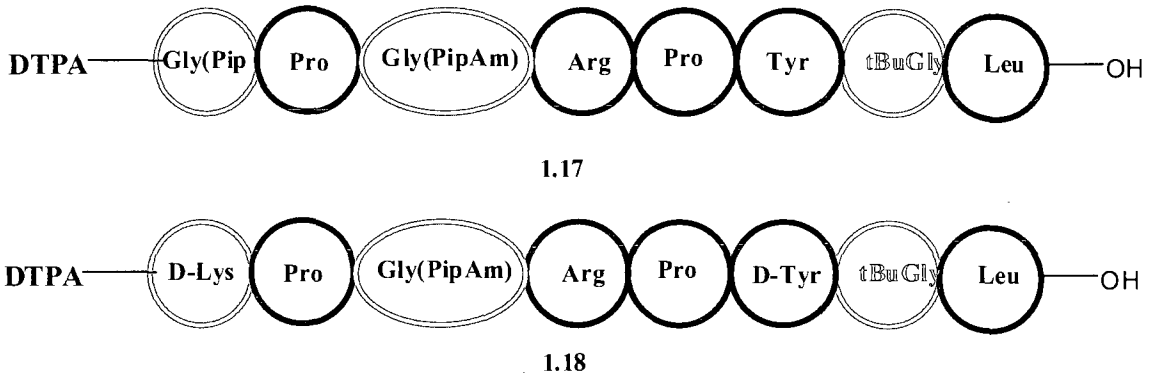


a. n = 2, R_1,R_2 = H
b. n = 3, R_1,R_2 = H
c. n = 4, R_1,R_2 = H
d. n = 2, R_1 = H,R_2 = $CH_2CH_2NH_2$
e. n = 2, R_1R_2 =CH_2CH_3

Figure 1.7. 8-Arg- 9-Arg mimics synthesized by Linquist et al[33]

Achilefu et al. synthesized NT(8-13) analogues that contained unnatural amino acids to replace several specific residues (Figure 1.8).[13] These NT(8-13) analogues contained a diethylene tetramine pentaacetic acid (DTPA) moiety capable of chelating ^{111}In (**1.17**, Figure 1.8), which was used for nuclear imaging. It was observed that **1.17** had a IC_{50} value of 0.3 nM (compared to NT(1-13) IC_{50} = 0.2 nM and NT(8-13) IC_{50} = 0.4 nM). To test stability, **1.17** was incubated in rat serum and urine for 4 hours at which time fractions were taken and injected into reverse-phase HPLC (RP-HPLC). It was observed that **1.17** was 96% intact in serum and 94 % intact in urine. It was found that replacement of 12-Ile with t-butyl glycine dramatically improved the stability of **1.17** over other analogues containing 12-Ile. It was also stated that replacement of 11-Tyr

with *D*-Tyr in the same position (**1.18**) gave the greatest stability of the NT analogues in this study. However, the IC_{50} value for **1.18** was greater than 1000 nM confirming St Pierre's earlier statement that there was a decrease in binding of NT(8-13) analogues that contained D-Tyr.[24]



1.17

1.18

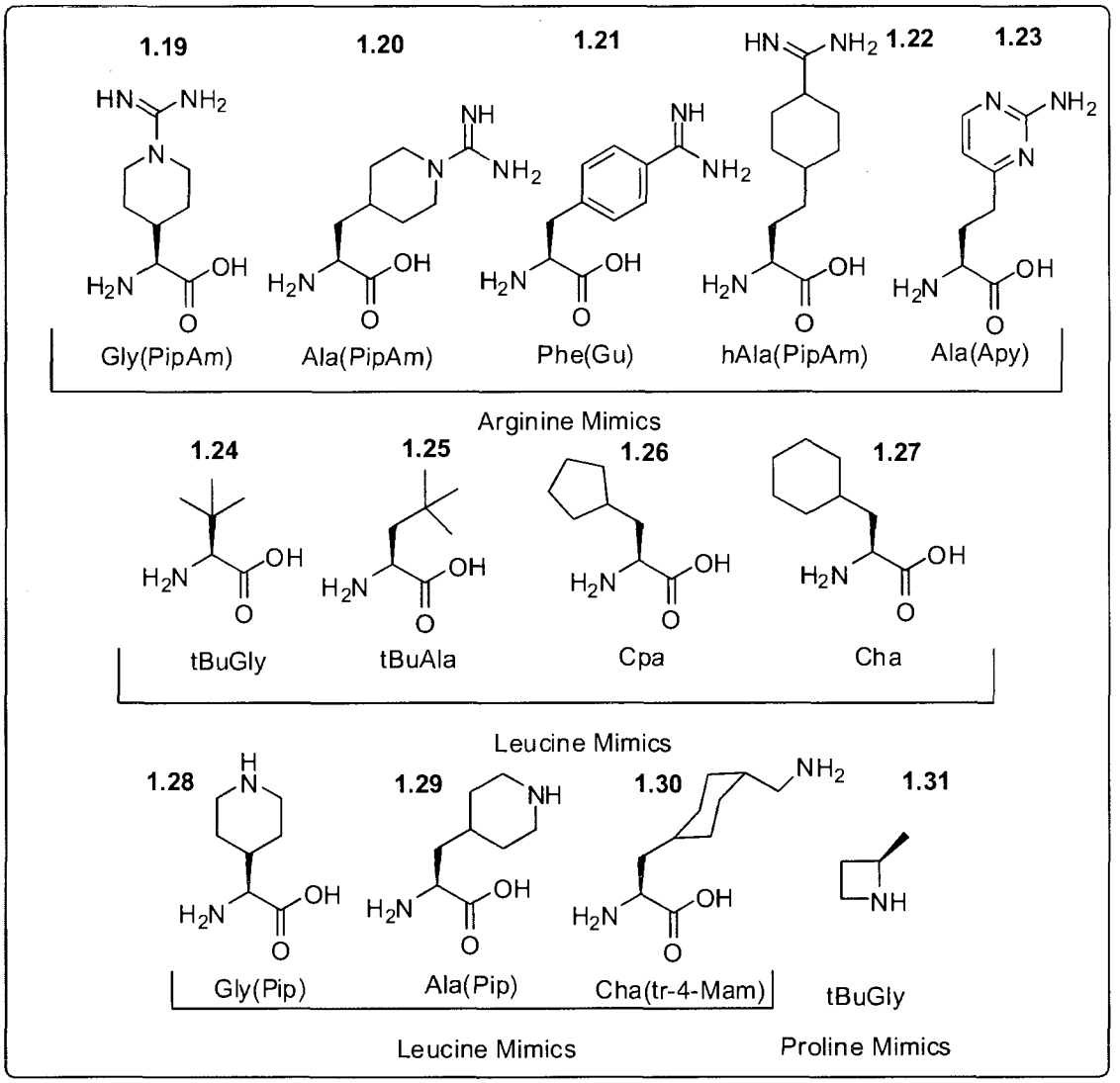
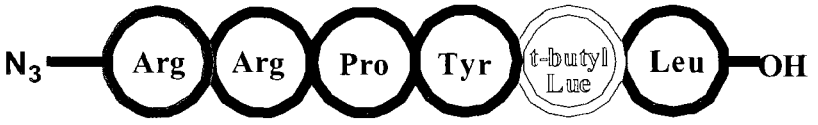


Figure 1.8. DTPA labeled NT(6-13) analogue containing a 9-Arg mimic (**1.17**) and DTPA labeled NT(6-13) analogue containing D-Tyr (**1.18**). The unnatural amino acids (**1.19-1.31**) incorperated into NT(6-13) analogues synthesized by Achilefu, et al.[13]

Kokko et al. reported that truncated analogues of NT with N-terminus azide moieties have the ability to cross the blood-brain barrier (BBB).[29] These NT analogues were of interest as possible anti-psychotics. Incorporation of the azido group, as well as replacement of the 12-Ile residue with a L-*tert*-Lue residue, was shown to dramatically increase the stability of the NT analogues from a half-life of 5.78 minutes (NT(8-13) Figure 1.14) to a half-life of 24 hours. The NTS showed no tolerance for **1.32** (Figure 1.9) after 5 days of increased loading (5 mg / kg body weight). Furthermore, this compound did not induce catalepsy (a common side effect of antipsychotic drugs where the body becomes rigid while a person is unconscious). Compound **1.32** also exhibited a long half-life ($t_{1/2}$ = 24hours) and the azido group was shown to have little or no effect on the binding affinity of **1.32** to the NT receptors as compared to NT(8-13).[29] This example of the ability of an NT analogue to cross the BBB further demonstrates the invaluable utility of peptides like NT as tools and templates for pharmaceuticals.



1.32

Figure 1.9. NT(8-13) analogue (**1.32**) capable of crossing thr blood-brain barrier

Only one group has studied the effects of replacing the 10-Pro residue on binding affinity. Based on the dihedral angles of the NTS-1 bound NT(8-13) deduced by Luca et al.[26], Bitterman et al. proceeded to incorporate functionalities that would allow NT analogues to maintain a stable binding conformation. Replacement of the 10-Pro residue has been approached by substitution with 4,4 spirolactams. Bitterman et al. found that compound **1** (Figure 1.10), which had a ψPro dihedral angle of + 128.7°, showed a 1000

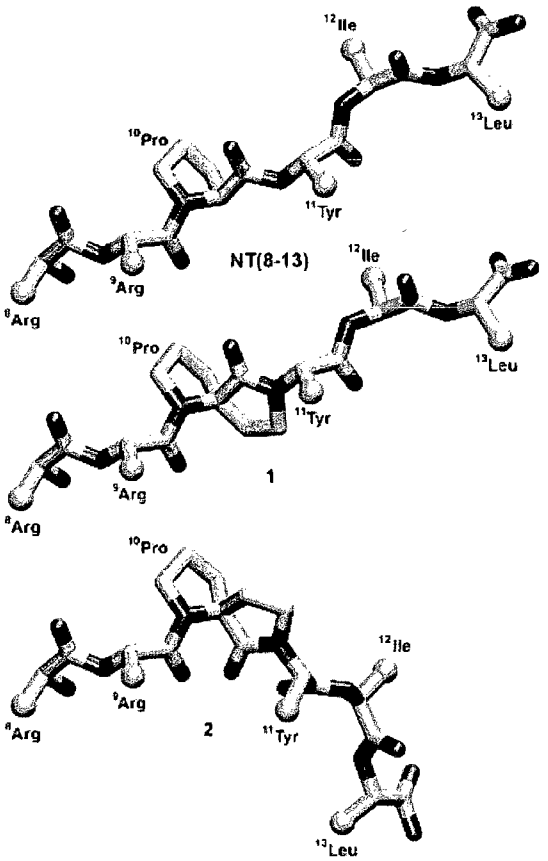


Figure 1.10. A representation of the peptide backbone of NT(8-13) and spirolactam containing analogues **1** and **2** [34] Reproduced with permission from the American Chemical Society

fold increase (K_i = 12 ± 0.73 nM) in binding affinity to porcine NTS-1 when compared to the more flexible [11-NMeTyr]NT(8-13) (K_i = 12 ± 150 nM, The [11-NMeTyr]NT(8-13) was used to mimic the loss of hydrogen bonding due to the spirolactam structure of compound **1**.). Compound **2**, with a ψPro dihedral angle of -128.7° exhibited a dramatic loss of binding affinity (17000 ± 1200 nM). Compound **1** does exhibit lower binding affinity for the NTS-1 than NT(1-13) (K_i = 1.3 nM). It was concluded that this was due to the loss of hydrogen bonding ability of compound **1**.[34] The results of this experiment specifically demonstrate the necessity of the correct conformation in NTS-1 binding.

One human trial has been carried out to date using a NT analogue. Buchegger et al.[35] conducted the first clinical trial of a ^{99m}Tc-NT(8-13) analogue with the following structure: [^{99m}Tc(CO)$_3$](NαHis)Ac-Lys-(CH$_2$-NH)-Arg-Pro-Tyr-Tle-Leu. The NT analogue was stabilized by replacing the 8-Arg- 9-Arg bond with a pseudopeptide bond and replacing the 12-Ile residue with a *t*-butyl Leu. This analogue was chosen due to its

binding ability to the NTS-1 and its long half-life. Four volunteers were used in the study, each having been previously diagnosed with ductal pancreatic adenocarcinomas. All patients were injected an average of 20 hours before a surgery was conducted to remove the tumors. Whole-body scintographies were conducted pre-surgery on two of the patients and abdominal scintographies were conducted on all four patients. Patient 4 showed moderate tumor uptake and the tumor was visible in the abdominal scintogram. Presence of the radiotracer uptake by the tumor in patient 4 was seen even after 20 hours. The half-life of the ^{99m}Tc-NT(8-13) analogue was determined by drawing blood samples at various times between 17 and 383 minutes. The *in vivo* half-lives in humans were determined to be 1.7-6.3 hours. The test results were a confirmation of earlier tests performed in rats.[35]

In summary, it is clear that modification of the 8, 9 Arg-Arg bond has led to NT analogues with sufficient $t_{1/2}$ and binding ability. Replacement of the 12-Ile with an unnatural amino has also lent to the stability of NT analogues, possibly due to the creation of steric bulk at the Tyr-Ile cut site. It has also been shown that NT analogues that maintain the binding conformation of NT have a higher binding affinity than analogues that contain residues that result in modified 3-D structure.

Description of Dissertation Research

Synthesis of Unnatural Tyrosine Analogues

Interestingly, with all the research done on NT analogues and all of the amino acids replaced to increase half-life or affinity, the question of modification of the Tyr^{11} residue to increase the half-life still remains somewhat ambiguous. It was concluded from the work of St-Pierre et al. that the residue in this position must be the *L*-isomer of an aromatic amino acid capable of hydrogen bonding.[24] Achilefu et al. later confirmed that binding was decreased when Tyr^{11} was replaced with a *D*-Tyr, but stability was greatly increased.[13] However, to our knowledge, no group has explored the replacement of the Tyr^{11} residue with an *D*-α-methyl Tyr, $\beta^{2,2}$ Tyr, or $\beta^{3,3}$ Tyr. It is for this reason that our synthetic efforts will focus on the creation of both enantiomers of a series of unnatural tyrosine analogues for eventual incorporation into NT analogues for use as potential peptidomimetic compounds.

Several α-methyl amino acids exhibit medicinal properties[36] and potential for use as imaging agents.[37] Several medicinally active compounds are currently available that contain β and γ amino acids.[38-41] Literature has also shown that incorporation of α-methyl and homolongated amino acids (*i.e.* β and γ-amino acids) can induce secondary structure and

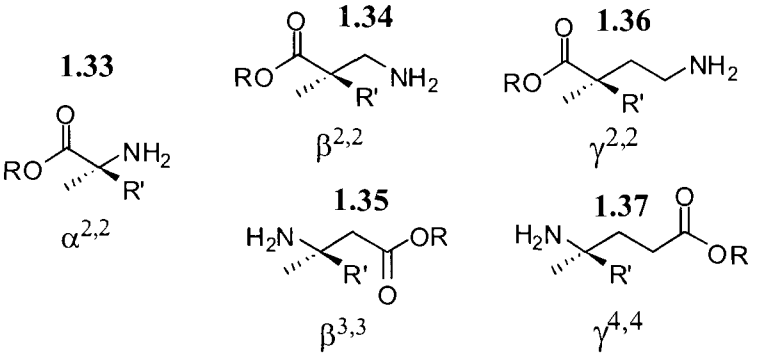


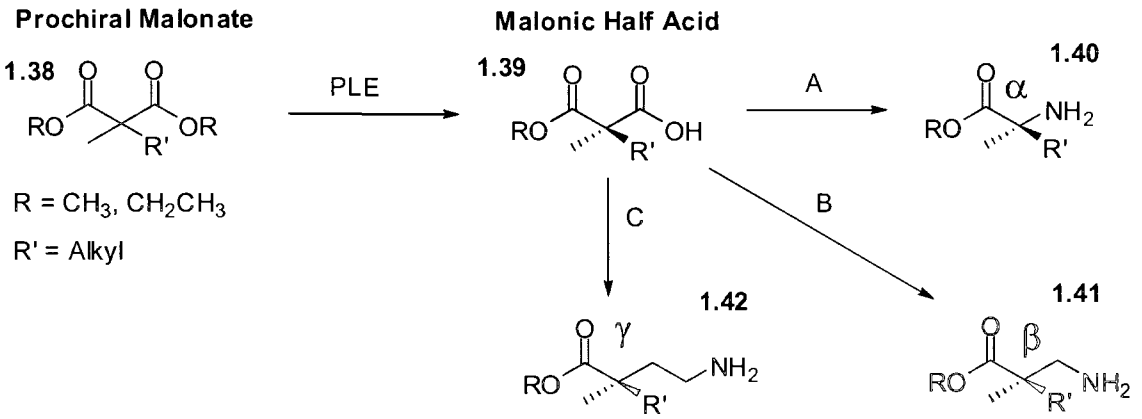
Figure 1.11. Structure of the classes of unnatural amino acids synthesized in this dissertation

increase the proteolytic stability of peptides.[42-46] Incorporation of unnatural tyrosine residues into NT(8-13) may result in a pharmaceutically active peptidomimetic compound. Tyrosine analogues are needed in order to synthesize these peptidomimetic compounds. For this reason, the primary focus of this dissertation will be the synthesis of the α-methyl, β-, and γ- classes of the homolongated, disubstituted unnatural amino acids (Figure 1.11). Each of theses classes of unnatural amino acids exhibits unique properties when incorporated into peptides. These properties create a need for an efficient synthesis of both enantiomers of each class of unnatural amino acids. Currently, synthesis of a variety of homolongated amino acids requires many different synthetic paths. This makes synthesis of a series of homologous amino acids a daunting task. This section will outline our approach to the synthesis of α-methyl (**1.33**), $\beta^{2,2}$ (**1.34**), $\beta^{3,3}$ (**1.35**), $\gamma^{2,2}$ (**1.36**), and $\gamma^{4,4}$ (**1.37**) tyrosine residues from a common intermediate.

One popular method is to generate an unnatural amino acid from a natural amino acid.[42,43,47] The disadvantage of this method is that the types of unnatural amino acids that can be synthesized are limited. This method requires careful control of reaction conditions without which may lead to racemization through epimerization at the α-carbon. Another popular approach is to use chiral catalyst to generate the unnatural amino acids.[48] This requires the use of expensive metal catalysts and may require an extensive amount of trial and error to select ligands that will give the desired stereochemistry and enantiomeric composition. Transition metal catalysts may also have significant problems with functional group tolerance which can limit their application. The shared disadvantage of both of these methods is that they draw from a pool of chiral molecules which can cause additional detriment to the cost effectiveness of the synthesis.

The ideal synthetic scheme should make use of simple chemistry, use cost-effective starting materials, have common synthetic intermediates, use common chemistry, and induce or conserve the configurations of the starting materials. One candidate that fits these criteria is malonic diesters. The acidity of the α-protons of malonates makes the generation of enolates efficient. The resulting enolate can then nucleophilically attack a variety of electrophiles to generate a tetrahedral center. This creates a pro-chiral compound that can be converted to a chiral compound by hydrolysis of one of the esters. The result is a half-ester that can be transformed to a variety of functional groups. If the starting material is a 2-methyl malonate diester (**1.38**), the derivatives will contain a neopentyl-like framework that does not racemize through epimerization. Saponification of the malonate diester with one equivalent of base results in a racemic half-ester that can be used in synthesis, but would have to be resolved into enantiomers at some point. Resolution of the racemic half-ester results in a maximum yield of 50 % for each enantiomer. A better approach is to use a stereoselective synthesis. One possible solution to the aforementioned problem of obtaining good chemical yields with high enantioselectivity is the use of an enzyme for desymmetrization of a malonate. Esterases are enzymes that are involved in the metabolism of the various dietary esters.[49] In organic chemistry, esterases have found use in hydrolysis of the alkoxy groups of esters to form an acid and an alcohol. Pig Liver Esterase (PLE) is an esterase that has proven to be fairly indiscriminate toward substrates. Depending on the substrate and the conditions, the hydrolysis of a malonate has the potential to yield a chiral half ester of significant enantiomeric enrichment. [49] The chiral half-ester can then be modified *via* several well known reactions to yield both enantiomers of α-methyl, $\beta^{2,2}$-, $\beta^{4,4}$-, $\gamma^{2,2}$-, and

$\gamma^{4,4}$- amino acids (Scheme 1.2). The acid can be converted to an amine by use of the Curtius rearrangement (Scheme 1.2, path A).[50] Manipulation of protecting groups, followed by sequential utilization of the Ardnt-Eistert synthesis, Wolff rearrangement and Curtius rearrangement yields $\beta^{2,2}$, $\beta^{3,3}$ amino acids (Scheme 1.2, Paths B).[47] Synthesis of the $\gamma^{2,2}$- and $\gamma^{4,4}$- amino acids can be accomplished through use of protecting group manipulation, reduction of the acid to an aldehyde, a Wittig reaction, a reduction, and a Curtius rearrangement (Scheme 1.2, Paths D).[42]



Scheme 1.2. Proposed synthesis of the unnatural amino acid analogues

High Throughput Assay for Enantioselectivity

The major problem encountered with enantioselective synthesis using enzymes is that the enzyme does not always yield a high enantiomeric excess. In this case, a mixture of enantiomers is obtained which is not useful for further synthesis when the target compound is of pharmaceutical interest. Several solutions are currently available. An enzyme can be modified by site-directed-mutagenesis to yield hydrolysis products in higher % *ee*.[51-53] This approach requires mutations through modification in the DNA that code for specific amino acids. This method requires knowledge of the DNA that codes

for the enzyme. To further complicate this method, knowledge of the active site and mechanism must be known. This can be very time consuming and requires a great deal of biochemical experience. A simpler method to tune the enantioselectivity of enzymes is solvent engineering.[54] This process uses various organic cosolvents to change some characteristic of the enzyme such as the conformation of the active site. Although the exact cause of the change in enantioselectivity may not be known, the effect is well documented.[55-57] The following will outline the efforts that have been made in the area of termed "combinatorial asymmetric catalysis" and our proposal of a method that will greatly contribute to the evaluation of malonate substrates for current and new enzymes, under a variety of conditions.

The rate limiting steps in determining if an enzyme is stereoselective with regard to a particular substrate is the hydrolysis time and purification of the product. Typical rates of enzymatic hydrolyses can in buffer alone vary greatly from a few hours to a few weeks. Since chiral analytical GC and HPLC columns are sensitive to contaminants, the product of an enzymatic hydrolysis must be at least partially purified to remove buffer, salts, and protein. It is now clear that this method is not efficient; neither in materials nor time. To compound this problem, use of organic cosolvents has been shown to slow the rate.[54,55,57] Problems like these must be solved in order for further development of syntheses that incorporate the use of enzymes. For us, the challenge lies in determining a method that will allow for the efficient analysis of the enantiomeric composition of the products from enzymatic reactions. The method should include 1) the ability to analyze of a large range of cosolvent conditions with various enzymes, 2) be applicable to multiple substrates, and 3) ideally be automated.

Mass Spectrometry (MS) offers an ideal solution. Each sample can be acquired in a short period of time. Very small quantities of material are needed for MS analysis, so small quantities of probes are needed for each assay. Since the structures of the molecules of interest are very similar, this method would also be applicable to multiple substrates. Most mass spectrometers are equipped with auto samplers that would allow for automated analysis. Several mass spectrometry based assays have been developed to monitor the enantiomeric composition of the products from enzymes or chiral organometallic catalysts.[58] However, none have been used to monitor the desymmetrization of malonic diesters. The primary strength displayed in our assay is that the probes need only be enantiomerically enriched, not enantiomerically pure. This allows for recycling of material from enzymatic hydrolysis that yield good % *ee* but that is not synthetically useful. After the assay is complete, the % *ee* obtained from the hydrolysis can be corrected to give the actual % *ee*. We have used this assay to significantly improve the enantioselectivity of PLE for two malonate diesters. Once conditions have been determined that give high enantioselectivity for the malonate acid/esters, they can be used for further synthesis. The unnatural amino acids obtained from these syntheses can then be incorporated into NT(8-13) analogues. These peptidomimetic NT analogues can then be evaluated for biological activity and stability.

Conclusions

The success seen in SST and NT analogues has proven the potential for the therapeutic use of peptidomimetic drugs. In order to advance this area of research, new developments in synthetic methodology for unnatural amino acids must be developed and optimized. This will require an adequate supply of cost effective, chiral synthons. It

seems quite likely that enzymes will fulfill these two requirements. However, discovery of new and more efficient enzymes will require a significant amount of work. Methods will have to be developed that will allow chemist to efficiently determine the types of substrates that each enzyme may accept. If chiral products are obtained from these enzymes, additional work will be needed to efficiently assay the resulting enantioselectivity. With this all in mind, this dissertation will focus primarily on A) the development of methodology for synthesis of a series of homochirally similiar α-methyl, β-, and γ-unnatural amino acid analogues B) Application of that methodology for synthesis of an series on unnatural tyrosine analogues and C) the development of a mass spectrometry based enantioselectivity assay for substrate specific, cosolvent engineering for the PLE hydrolysis of malonate diesters.

Hypotheses

Hypothesis 1 of this dissertation states that a series of homochirally similar serine and tyrosine analogues may be synthesized from a common malonate half ester intermediate by the utilization of several well known organic transformations and protecting group chemistry. The syntheses of the unnatural serine and tyrosine analogues will be divided into three parts and described in Chapters II-IV. Chapter II will detail part A of hypothesis 1: the efforts to synthesize α-methyl serine and α-methyl tyrosine. Chapter III will detail part B of hypothesis 1: the efforts to synthesize $\beta^{2,2}$-serine, $\beta^{3,3}$-serine, $\beta^{2,2}$-tyrosine, and $\beta^{3,3}$tyrosine. Chapter IV will details part C of hypothesis 1: the efforts to synthesize $\gamma^{2,2}$-tyrosine and $\gamma^{4,4}$-tyrosine.

Hypothesis 2 of this dissertation states that a mass spectrometry assay may be devised to determine the enantiomeric composition of malonate half esters that are

yielded as the result of an enantioselective enzymatic hydrolysis. Chapter V will detail the efforts to create and validate such an assay. Chapter VI will conclude this dissertation with future work that may be used to solve the encountered synthetic problems and outline future directions for the mass spectrometry assay for enantioselectivity.

References

(1) National Research Council Committee on Challenges for the Chemical Sciences in the 21st Century. *Beyond the Molecular Frontier: Challenges for Chemistry and Chemical Engineering*; National Academies Press, 2003.

(2) Nelson, D. L.; Cox, M. M. *Lehninger Principles of Biochemistry*; Third ed.; Worth Publishers: New York, NY, 2000.

(3) Silverman, R. D.*The Organic Chemistry of Drug Design and Drug Action* ; Second ed.; Hayhurst, J., Ed.; Elsevier: 2004, p 47-50.

(4) O'Brien, M. L.; Vulevic, B.; Freer, S.; Boyd, J.; Shen, H.; Tew, K. D. *Journal of Pharmacology and Experimantal Therapeutics* **1999**, *291*, 1348-1355.

(5) Reubi, J. C.; Waser, B.; Friess, H.; Buchler, M.; Laissue, J. *Gut* **1998**, *42*, 546-550.

(6) Reubi, J. C.; Waser, B.; Schaer, J.; Laissue, J. A. *International Journal of Cancer* **1999**, *82*, 213-218.

(7) Chabry, J.; Botto, J.-M.; Nouel, D.; Beaudet, A.; Vincent, J.-P.; Mazella, J. *Journal of Biological Chemistry* **1995**, *270*, 2439-2442.

(8) Chabry, J.; Gaudriault, G.; Vincent, J.; Mazella, J. *Journal of Biological Chemistry* **1993**, *268*, 17138-17144.

(9) Lamberts, S.; van der Lely, A.; Hofland, L. *European Journal of Endocrinology* **2002**, *146*, 701-705.

(10) Tomassetti, P.; Migliori, M.; Caletti, G. C.; Fusaroli, P.; Corinaldesi, R.; Gullo, L. *New England Journal of Medicine* **2000**, *343*, 551-554.

(11) Nicholls, J.; Wynick, D.; Domin, J.; Sandler, L. M.; Bloom, S. R. *Clinical Endocrinology* **1990**, *32*, 545-550.

(12) Janecka, A.; Zubrzycka, M.; Janecki, T. *The Journal of Peptide Research* **2001**, *58*, 91-107.

(13) Achilefu, S.; Srinivasan, A.; Schmidt, M. A.; Jimenez, H. N.; Bugaj, J. E.; Erion, J. L. *Journal of Medicinal Chemistry* **2003**, *46*, 3403-3411.

(14) Carraway, R.; Leeman, S. E. *Journal of Biological Chemistry* **1973**, *248*, 6854-6861.

(15) Reubi, J. C. *Endocrine Reviews* **2003**, *24*, 389-427.

(16) Vincent, J.-P.; Mazella, J.; Kitabgi, P. *Trends in Pharmacological Sciences* **1999**, *20*, 302-309.

(17) Reubi, J.; Waser, B.; Schaer, J.; Laissue, J. *International Journal of Cancer* **1999**, *82*, 213-218.

(18) Seethalakshmi, L.; Mitra, S. P.; Dobner, P. R.; Menon, M.; Carraway, R. *The Prostate* **1997**, *31*, 183-192.

(19) Souaze, F.; Dupouy, S.; Viardot-Foucault, V.; Bruyneel, E.; Attoub, S.; Gespach, C.; Gompel, A.; Forgez, P. *Cancer Research* **2006**, *66*, 6243-6249.

(20) Nussenzveig, D.; Heinflink, M.; Gershengorn, M. *Journal of Biological Chemistry* **1993**, *268*, 2389-2392.

(21) Benya, R.; Fathi, Z.; Battey, J.; Jensen, R. *Journal of Biological Chemistry* **1993**, *268*, 20285-20290.

(22) Lameh, J.; Philip, M.; Sharma, Y.; Moro, O.; Ramachandran, J.; Sadee, W. *Journal of Biological Chemistry* **1992**, *267*, 13406-13412.

(23) Carraway, R.; Leeman, S. *Peptides: Chemistry, Structure, and Biology: Proceedings of the Fourth American Peptide Symposium*; 4th ed. New York, N.Y. , 1975.

(24) St-Pierre, S.; Lalonde, J. M.; Gendreau, M.; Quirion, R.; Regoli, D.; Rioux, F. *Journal of Medicinal Chemistry* **1981**, *24*, 370-376.

(25) Nieto, J. L.; Rico, M.; Santoro, J.; J., H.; Bermejo, F. J. *International Journal of Peptide and Protein Reserach* **1986**, *28*, 315-323.

(26) Luca, S.; White, J. F.; Sohal, A. K.; Filippov, D. V.; van Boom, J. H.; Grisshammer, R.; Baldus, M. *Proceedings of the National Academy of Science* **2003**, *100*, 10706-10711.

(27) Nock, B. A.; Nikolopoulou, A.; Reubi, J. C.; Maes, V.; Conrath, P.; Tourwe, D.; Maina, T. *Journal of Medicinal Chemistry* **2006**, *49*, 4767-4776.

(28) Kokko, K. P.; Hadden, M. K.; Orwig, K. S.; Mazella, J.; Dix, T. A. *Journal of Medicinal Chemistry* **2003**, *46*, 4141-4148.

(29) Kokko, K. P.; Hadden, M. K.; Price, K. L.; Orwig, K. S.; See, R. E.; Dix, T. A. *Neuropharmacology* **2005**, *48*, 417-425.

(30) Garcia-Garayoa, E.; Blauenstein, P.; Bruehlmeier, M.; Blanc, A.; Iterbeke, K.; Conrath, P.; Tourwe, D.; Schubiger, P. A. *Journal of Nuclear Medicine* **2002**, *43*, 374-383.

(31) Garcia-Garayoa, E.; Maes, V.; Blauenstein, P.; Blanc, A.; Hohn, A.; Tourwe, D.; Schubiger, P. A. *Nuclear Medicine and Biology* **2006**, *33*, 495-503.

(32) Bergmann, R.; Scheunemann, M.; Heichert, C.; Mading, P.; Wittrisch, H.; Kretzschmar, M.; Rodig, H.; Tourwe, D.; Iterbeke, K.; Chavatte, K. *Nuclear Medicine and Biology* **2002**, *29*, 61-72.

(33) LundquistIv, J. T.; Bullesbach, E. E.; Dix, T. A. *Bioorganic & Medicinal Chemistry Letters* **2000**, *10*, 453-455.

(34) Bittermann, H.; Einsiedel, J.; Hubner, H.; Gmeiner, P. *Journal of Medicinal Chemistry* **2004**, *47*, 5587-5590.

(35) Buchegger, F.; Bonvin, F.; Kosinski, M.; Schaffland, A. O.; Prior, J.; Reubi, J. C.; Blauenstein, P.; Tourwe, D.; Garcia Garayoa, E.; Bischof Delaloye, A. *Journal of Nuclear Medicine* **2003**, *44*, 1649-1654.

(36) Cativiela, C.; Diaz-de-Villegas, M. D. *Tetrahedron: Asymmetry* **1998**, *9*, 3517-3599.

(37) Flamen, P.; Bernheim, N.; Deron, P.; Caveliers, V.; Chavatte, K.; Franken, P. R.; Bossuyt, A. *European Journal of Nuclear Medicine* **1998**, *25*, 177-181.

(38) Steer, D. L.; Lew, R. A.; Perlmutter, P.; Smith, I. A.; Aguilar, M. *Current Medicinal Chemistry* **2002**, *9*, 811.

(39) Hom, R. K.; Fang, L. Y.; Mamo, S.; Tung, J. S.; Guinn, A. C.; Walker, D. E.; Davis, D. L.; Gailunas, A. F.; Thorsett, E. D.; Sinha, S.; Knops, J. E.; Jewett, N. E.; Anderson, J. P.; John, V. *Journal of Medicinal Chemistry* **2003**, *46*, 1799-1802.

(40) Sun, I. C.; Chen, C. H.; Kashiwada, Y.; Wu, J. H.; Wang, H. K.; Lee, K. H. *Journal of Medicinal Chemistry* **2002**, *45*, 4271-4275.

(41) Trabocchi, A.; Guarna, F.; Guarna, A. *Current Organic Chemistry* **2005**, *9*, 1127-1153.

(42) Hintermann, T.; Gademann, K.; Jaun, B.; Seebach, D. *Helvetica Chimica Acta* **1998**, *81*, 983-1002.

(43) Obrecht, D.; Bohdal, U.; Daly, J.; Lehmann, C.; Schonholzer, P.; Muller, K. *Tetrahedron* **1995**, *51*, 10883-10900.

(44) David L. Steer, R. A. L., Patrick Perlmutter, A. Ian Smith, Marie-Isabel Aguilar *Current Medicinal Chemistry* **2002**, *9*, 811-822.

(45) Frackenpohl, J.; Arvidsson, P. I.; Schreiber, J. V.; Seebach, D. *ChemBioChem* **2001**, *2*, 445-455.

(46) Hanessian, S.; Luo, X.; Schaum, R.; Michnick, S. *Journal of the American Chemical Society* **1998**, *120*, 8569-8570.

(47) Linder, M.; Steurer, S.; Podlech, J. *Organic Syntheses* **2002**, *79*, 154.

(48) Liu, M.; Sibi, M. P. *Tetrahedron* **2002**, *58*, 7991-8035.

(49) Bornscheuer, U. T.; Kazlauskas, R. J. *Hydrolases in Organic Synthesis: Regioselective and Stereoselective Biotransformations*; Second ed.; Wiley-VCH, 2006.

(50) Kedrowski, B. L. *Journal of Organic Chemistry* **2003**, *68*, 5403-5406.

(51) Becker, S.; Hobenreich, H.; Vogel, A.; Knorr, J.; Wilhelm, S.; Rosenau, F.; Jaeger, K.; Reetz, M.; Kolmar, H. *Angewandte Chemie International Edition* **2008**, *47*, 5085-5088.

(52) Reetz, M.; Wilensek, S.; Zha, D.; Jaeger, K. *Angewandte Chemie International Edition* **2001**, *40*, 3589-3591.

(53) Reetz, M. T.; Carballeira, J. D. *Nature Protocols* **2007**, *2*, 891-903.

(54) Guanti, G.; Banfi, L.; Narisano, E.; Riva, R.; Thea, S. *Tetrahedron Letters* **1986**, *27*, 4639-4642.

(55) Guanti, G.; Banfi, L.; Narisano, E. *Tetrahedron Letters* **1989**, *30*, 2697-2698.

(56) Lam, L.; Hui, R.; Jones, B. *The Journal of Organic Chemistry* **1986**, *51*, 2047-2050.

(57) Wallert, S.; Drauz, K.; Grayson, I.; Groger, H.; Dominguez de Maria, P.; Bolm, C. *Green Chemistry* **2005**, *7*, 602-605.

(58) Reetz, M. T.; Becker, M. H.; Klein, H.-W.; Stockigt, D. *Angewandte Chemie-International Edition* **1999**, *38*, 1758-1761.

CHAPTER II

α- METHYL AMINO ACIDS

Literature Review

Introduction

The previous chapter contains a discussion of the properties that unnatural amino acids can produce in peptidomimetic compounds. The need for an efficient synthesis of unnatural tyrosine analogues for incorporation in neurotensin peptidomimetic compounds was also outlined. This chapter will focus on part A of hypothesis 1, which is the development of synthetic methodology for both enantiomers of α-methyl tyrosine from a common intermediate. Discussed within Chapter 2 is the synthesis of both enantiomers of α-methyl serine with subsequent application of this methodology for the synthesis of both enantiomers of α-methyl tyrosine. To accomplish this, a malonate diester that contains a substituent that is analogous to a protected a serine side-chain was synthesized. The enantioselectivity of Pig Liver Esterase (PLE) was evaluated for this substrates, and the resulting malonate half ester that was obtained from the hydrolysis was used to synthesize both enantiomers of α-methyl serine. The same methodology was utilized to synthesize both enantiomers of α-methyl tyrosine with the long term goal of incorporation into neurotensin peptidomimetic compounds. This chapter will illustrate the current syntheses of α-methyl amino acids, our proposed methodology, and the results and conclusions of our work.

Properties of α-Methyl Amino Acid

Recent research into α-methyl amino acids has shown promise for use in many different areas. Several α-methyl amino acids (Figure 2.1) such as α-methyl aspartic acid (**2.1**) are potent enzyme inhibitors (aspartate amino transferase is inhibited by **2.1**).[1] Literature has shown application of ^{123}I- α-methyl-*L*-tyrosine (**2.2**) as a potent tumor imaging agent. Flamen et al. observed ^{123}I- α-methyl-*L*-tyrosine scintography was able to identify previously confirmed tumors in the head and neck of 10 out of 11 patients (91%).[2] Other α-methyl amino acids (Figure 2.1) such as α-methylalanine (**2.3**), α-methylvaline (**2.4**), α-methylphenylalanine (**2.5**), α-methylleucine (**2.6**), and α-methylserine (**2.7**) have shown the ability to induce specific secondary structures (i.e. helices and β-turns) in peptides that are not observed in peptides containing natural amino acids.[1,3,4] The presence of unnatural α-amino acids that induce

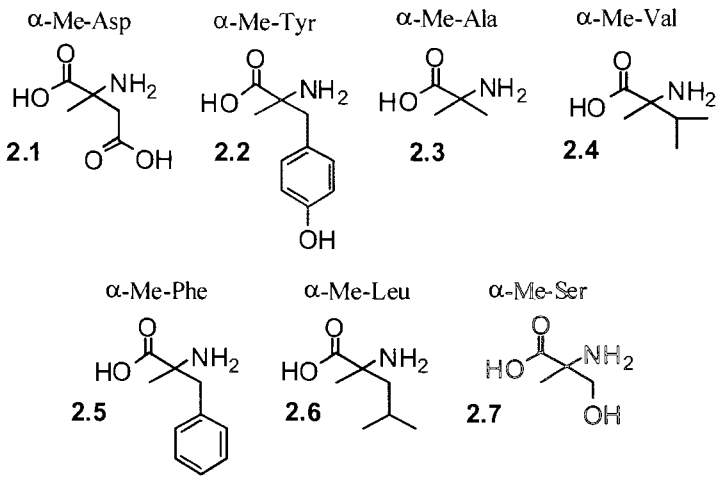
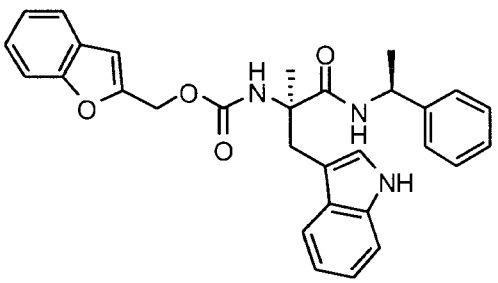


Figure 2.1. α-methyl amino acids that have seen use as imaging agents,enzyme inhibitors, or as conformation inducers in peptidomimetics. The blue (α-methyl tyrosine) and red (α-methyl serine) are the residues of interest for this chapter.



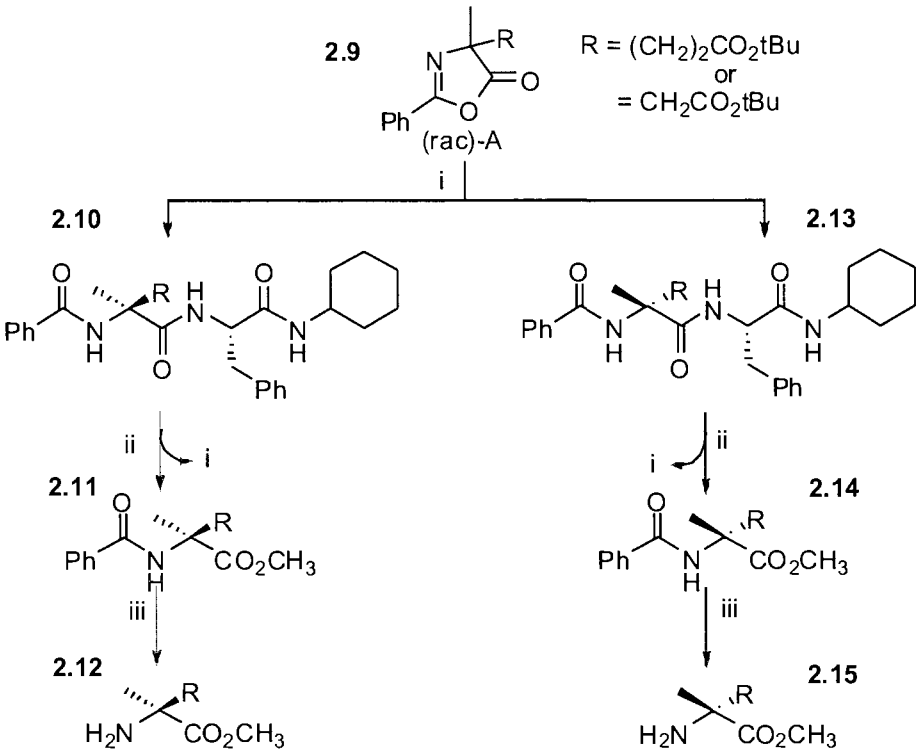
2.8 Tachykinin Receptor Antagonist PD 154075

Figure 2.2. Structures of a receptor antagonist containing α-methyl tryptophan

binding conformation results in an increase in the affinity of the receptor for the peptide.[5] In this manner, these α-methyl amino acids may be used to improve the binding affinities of peptidomimetic compounds for specific receptor targets. Several pharmaceutically active compounds, including some antibiotic peptides, contain α-methyl amino acids that increase the stability of the compound (Figure 2.2).[6] Our interest in α-methyl amino acids arises from the ability of α-methyl amino acids to increase the stability of peptidomimetic compounds and to induce conformation changes that facilitates binding of peptidomimetic compounds.

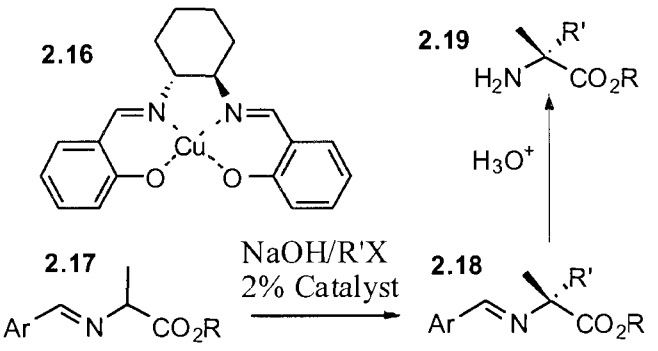
Synthesis of α-Methyl Amino Acids

Asymmetric synthesis of α-methyl amino acids is not a trivial task. Much work has been done to develop synthetic methodology that is efficient and cost effective enough to use on an industrial scale. Based on the numerous uses of this class of unnatural amino acids already established, commercially available quantities of α-methyl amino acids could prove to be invaluable synthetic and as pharmacological tools. Cativiela stated in his review of the synthesis of quaternary α-amino acids that the extensive use of quaternary α-amino acids was limited only by the availability of enantiomerically pure compounds in large quantities.[1] There are several methods currently used to synthesize α-methyl amino acids. Direct methylation of an amino acid



Scheme 2.1. Azalactones Synthesis of α-methyl-Asp and Glu amino acids
i: L-Phe-cyclohexylamide
ii: CF_3SO_3H, MeOH, reflux
iii: 25% HCl (aq), dioxane

Schiff's base would lead to racemic α-methyl amino acids, and is therefore not of suitable utility in methyl amino acid synthesis. Previous synthetic methodologies have made use of auxiliaries to confer chirality to the α-methyl amino acids or the α-methyl amino acid was synthesized racemically and later resolved as a pair of diastereomers using a chiral auxiliary.[1] Obrecht et al. synthesized enantiomerically pure α-methyl aspartic acid and α-methyl glutamic acid *via* racemic azlactones (Scheme 2.1), which were later resolved into enantiomers by reaction with *L*-Phe-cyclohexylamide.[3] The diastereomers formed were separable by flash chromatography. The amide bond of the diastereomers was cleaved by CF_3SO_3H, yielding the free α-methyl aspartic acid and α-methyl glutamic acid. The major disadvantage of this method is that 50% chemical yield is the maximum chemical yield

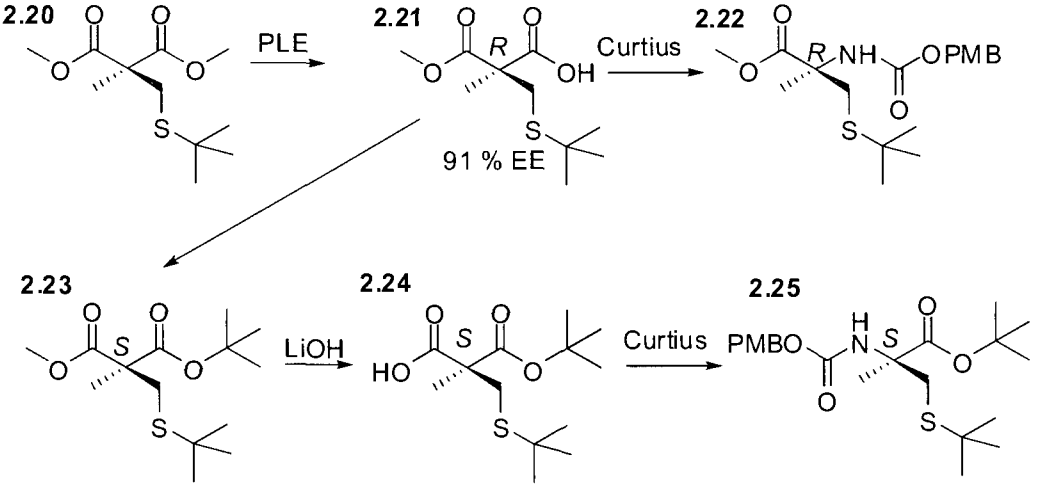


Scheme 2.2 . Use of copper(salen) catalyst for synthesis of α-methyl amino acids

possible for each enantiomer. This synthesis is problematic in that low yields are obtained and a chiral compound had to be used for resolution of the racemates. One possible solution to the problems present with resolutions is the use of stereoselective transition metal catalysis. Belokon et al. have made extensive use of copper(salen) complexes (**2.16**) to synthesize a variety of α-dialkyl amino acids from alanine enolates (Scheme 2.2).[7-10] The chemistry is mild and results in up to 92% *ee*. Once the alkylation has been achieved, the protecting groups can be cleaved by treatment with aqueous acid. Both enantiomers could be obtained in these syntheses by changing the chiral ligand. The disadvantages of this methodology are 1) the amount of time that is required to tune the enantioselectivity of the catalyst, 2) chiral ligands are required for asymmetric induction which can be expensive, and 3) transition metal catalyst can be sensitive to atmospheric conditions.

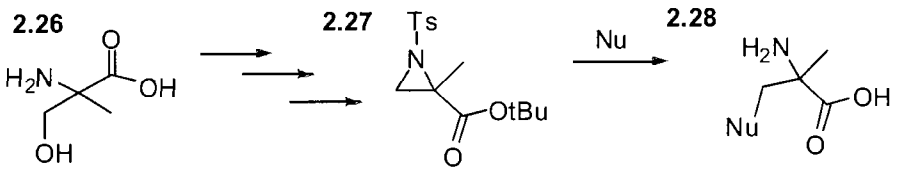
Enzymes offer a solution to the majority of the aforementioned problems with the asymmetric synthesis of α-methyl amino acids. They are catalytic, generally tolerant to variety of experimental conditions, tolerate a variety of functional groups, and can result in highly enantioselective products (> 97% *ee*).[11] Kedrowski reported the synthesis of both enantiomers of α-methyl cysteine (Scheme 2.3) by a procedure that involved enzymatic hydrolysis, using PLE, of **2.20** to **2.21** in 97 % chemical yield and 91% *ee*. [12] The half-ester was subjected to a Curtius rearrangement to give the protected (*R*)-α-

methylcysteine (**2.22**) in 87% yield. Furthermore, **2.21** was *tert*-butylated to give **2.23**. The methyl ester of **2.23** was saponified with LiOH and subjected to a Curtius rearrangement to give the protected (*S*)-α-methylcysteine (**2.25**) in 92% yield. This synthesis demonstrates a method that allows the synthesis of both enantiomers of α-methyl cysteine in good enantiomeric and chemical yields from a common intermediate. This synthesis demonstrates the careful manipulation of orthogonal protecting groups that is necessary in this type of synthesis. The use of a common intermediate, orthogonal protecting group strategies, and enzymes utilized in this synthesis are appealing. The malonate half ester intermediate has the potential to be used in the synthesis other classes of unnatural amino acids. The synthetic work discussed in this dissertation is our efforts to extend this methodology to synthesize a class of α-methyl and homolongated unnatural amino acids from a similar intermediate.



Scheme 2.3. Synhesis of (*R*)-α-methyl Cysteine (**2.22**) and (*S*)-α-methyl Cysteine (**2.25**)[12]

It was decided to begin our synthesis with a prototype amino acid. It was found that α-methyl serine would be an interesting target. Literature has shown that α-methyl serine has found significant use as a synthetic intermediate for the production of other α-methyl amino acids.[13,14] As an example, α-methyl serine can be converted to an aziridine 2-methyl carboxylate derivative.[14] With these substrates, nucleophilic attack occurs regioselectively at the C3 position when organocuprates and other mild nucleophiles are used. The result is an α-methyl amino acid with a side chain that corresponds to the nucleophile that was used (Scheme 2.4). This means that an efficient synthesis of α-methyl serine may lead to the synthesis of multiple classes of α-methyl amino acids with moderate to good yields (48-84 %). The primary interest of this chapter is the synthesis of the α-methyl tyrosine series. However, we evaluated our synthetic methodology on synthesis of the serine series due the significant synthetic potential of the analogues. To accomplish this, diesters which contain protected amino acid side chains were synthesized. These diesters were subjected to a hydrolysis with PLE which yielded a half ester. This half ester was converted to both enantiomers of our desired α-methyl amino acids by careful manipulation of protecting groups and by use of the Curtius rearrangement. The starting materials for a **2.31a** with a protected serine side chain (**2.30a**) are commercially available and there is literature precedence for synthesis and

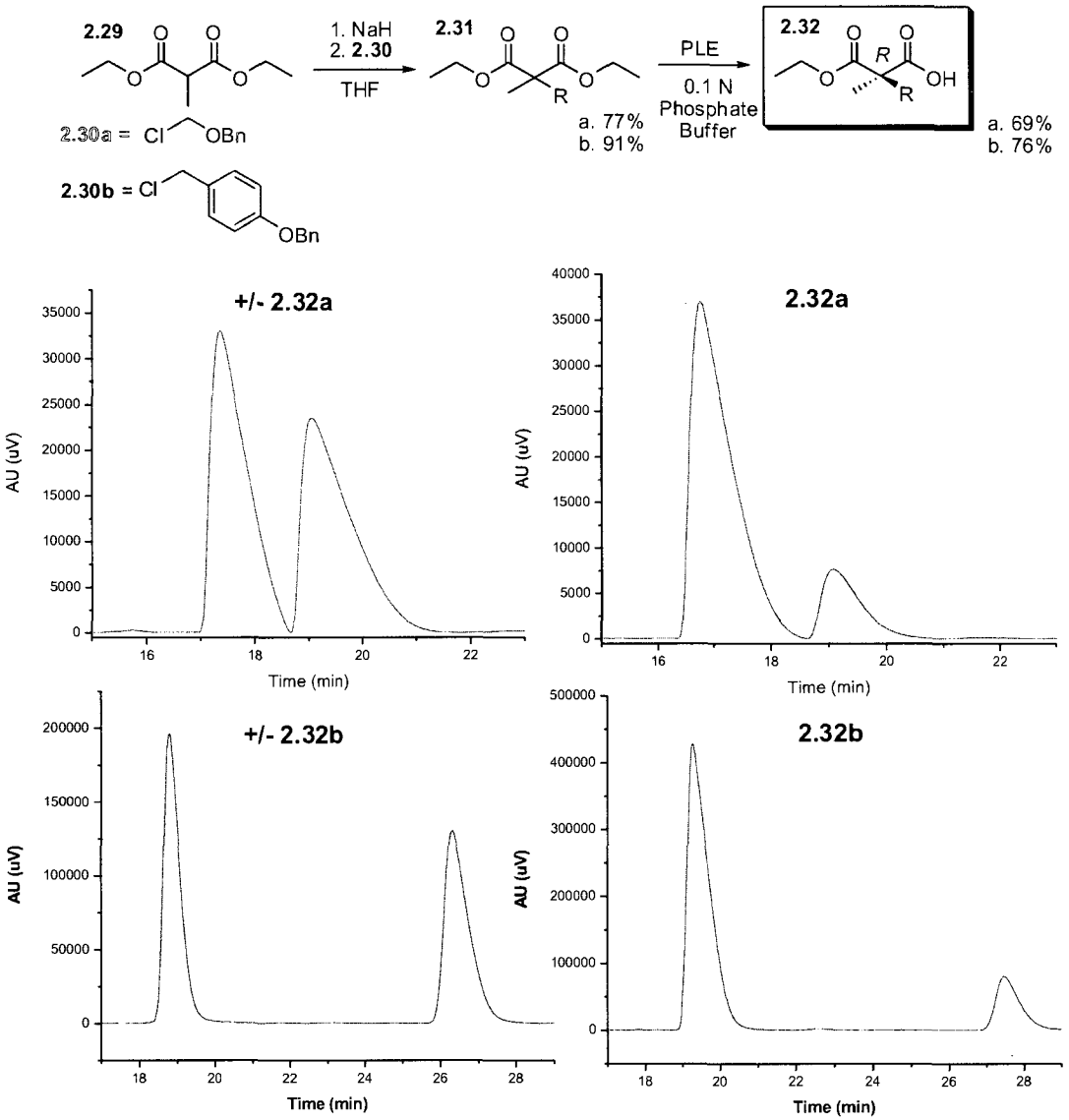


Scheme 2.4 . Conversion of α–methyl seine (**2.26**) into an aziridine (**2.27**) followed by nucleophilic attack of the β-carbon to yield a new α-methyl amino acid (**2.28**)

PLE hydrolysis.[15] Although synthesis of the tyrosine diester (**2.31b**) has been previously accomplished, the side chain is not commerically available and was synthesized.[16] However, the PLE hydrolysis of **2.31a** has not been previously accomplished. Considering these factors, it was clear that the synthesis of the tyrosine analogues would be more tedious. It is for this reason that we decided to evaluate our methodology by synthesis of both enantiomers of α-methyl serine.

Results and Discussion

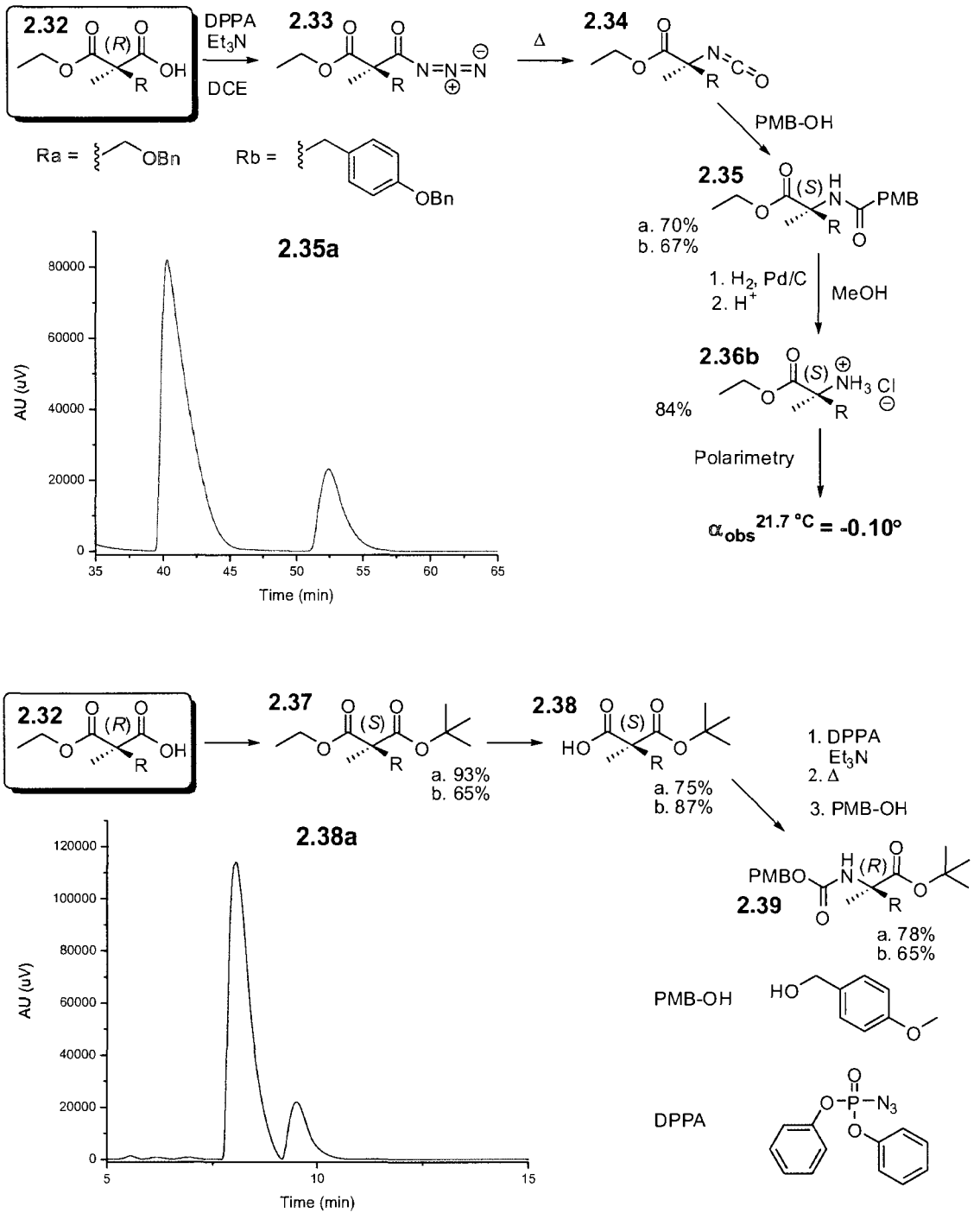
The first step in synthesis of α-methyl serine is the synthesis of diethyl 2-(benzyloxymethyl)-2-methylmalonate (Scheme 2.5, **2.31a**). To accomplish this, the enolate of **2.29** was generated upon addition to a suspension of NaH in anhydrous THF. Alkylation with Benzylchloromethylether (**2.30a**) gave compound **2.31a** in 77% yield. Generation of the half ester intermediate (**2.32a**) took place through a PLE hydrolysis in 0.1 N pH 7.4 phosphate buffer. The hydrolysis was based on previous literature and was monitored by titration of the acid with a Titrino® in pH stat mode. [11,12,17,18] The reaction was complete after one equivalent of NaOH was consumed (approximately 24 hours). Compound **2.32a** is the common intermediate for synthesis of our unnatural serine analogues. Chiral HPLC showed that compound **2.32a** exhibited 70% *ee* as compared to the racemic material that was generated by saponification with one equivalent of NaOH. Polarimetry was used to verify that the enzyme showed selectivity of the *R* enantiomer of **2.32a**. Both the enantiomeric composition and the absolute configuration was consistent with literature values.[15]



Scheme 2.5. Synthesis of the **2.31a** and **2.31b** with subsequent PLE hydrolysis to **2.32a** and **2.32b**

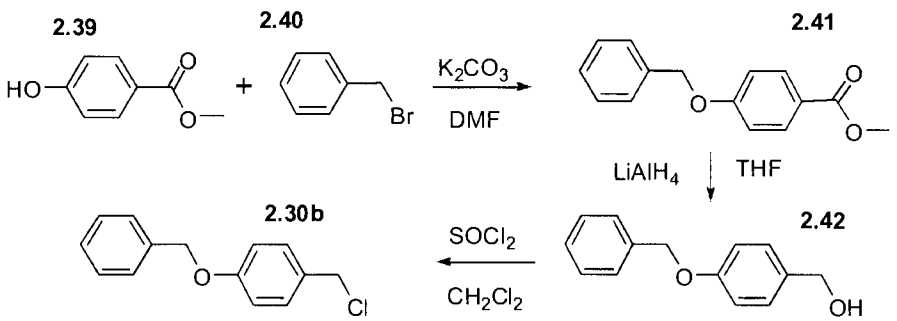
To synthesize the protected (*S*)-α-methyl serine (Scheme 2.6, **2.36a**), **2.32a** was treated with a slight excess of diphenylphosophoroazide (DPPA) at room temperature to generate an acyl azide (**2.33a**). Heating of this acyl azide resulted in a Curtius rearrangement to an isocyanate (**2.34a**) which was quenched with an excess of *p*-methoxybenzyl alcohol (PMB-OH) to give the **2.36a** in 70% yield. The synthesis of the protected (*R*)-α-methyl serine (**2.39a**) required manipulation of the protecting groups.

Compound **2.32a** was treated with isobutylene (IBE) in the presence of catalytic amounts sulfuric acid to give a 93% yield of **2.37a**. Saponification of the ethyl ester with NaOH resulted in a *tert*-butyl half ester (**2.38a,** 75% yield) that was subjected to a Curtius rearrangement under the aforementioned conditions to give **2.39a** in 78% isolated yield. Chiral HPLC was conducted on both the protected **2.35a** and the **2.38a** proved that the enantiomeric composition of **2.32a** was retained throughout the synthesis.[19]



Scheme 2.6. Curtius rearrangement of the malonic half esters to protected amino acids. The chiral HPLC chromatograms of **2.34a** (red) and **2.38a** (blue) prove that the composition of enantiomers is retained during synthesis

The positive results seen with the synthesis of α-methyl serines were encouraging, so it was decided to proceed with the synthesis of both enantiomers of α-methyl tyrosine. The first step was to synthesize the desired side chain. The phenol of the tyrosine analogue was protected as a benzyl ether due to stability and ease of removal by hydrogenolysis.[20] A suitable method for preparation of the benzylchlorobenzylether (**2.30b**) was found in the literature.[21] The preparation of **2.30b** followed the literature preparation with the exceptions that we used a methyl ester (**2.41**) instead of an ethyl ester and generated an alkylchloride instead of an alkylbromide (Scheme 2.7). This alkyl chloride was added dropwise as a THF solution to a stirring solution of the enolate of diethyl methyl malonate that was generated as described above. This gave **2.31b** in 91 % yield after precipitation from pentane. To facilitate the PLE hydrolysis, **2.31b** was crushed into a fine powder and dispersed in buffer. Although hydrolysis of **2.31b** has not been previously accomplished, there is literature precedence for the PLE hydrolysis of similar diesters yielding good enantioselectivity.[22] The crystal structure for PLE has yet to be



Scheme 2.7. Synthesis of **2.30b**

determined, so we made use of a model of the active site of PLE proposed by Jones (Figure 2.3 with compound **2.31b** imposed) and the PLE hydrolysis of similar substrates to predict that the *R* configuration would be obtained.[23] It was found that a large ratio of buffer to starting material (800 mL for 4 grams of diester) is necessary for the hydrolysis of this substrate to proceed. The hydrolysis was complete after 5 days and resulted in 76% yield of **2.32b**. This is the common intermediate needed for synthesis of both enantiomers of α-methyl tyrosine. The enantiomeric composition of **2.32b** was determined by chiral HPLC to be 63% *ee*. Compound **2.32b** was converted to the protected (*S*)-α-methyl tyrosine (**2.35b**, 67% yield) by means of the Curtius arrangement discussed above. The amino ester was then deprotected by hydrogenolysis with Pd on carbon black. A biphasic extraction was performed using methanol and pentane to remove the *p*-methoxy anisole byproduct to give 84% yield of the deprotected amino ester **2.36b**. The specific rotation of this amino ester was compared to that of an authentic sample to determine that the absolute configuration of **2.32b** was *R*. The synthesis of (*R*)-α-methyl tyrosine was similar to the synthesis of (*R*)- α-methyl serine. It was initially thought that the activated aromatic ring of **2.32b** might lead to *tert*-butylation at the ortho position from the benzyl ether. However, we were able to obtain 65% isolated yield of **2.37b** without any detectable presence of over alkylated byproducts. Saponification with NaOH led to 87% yield of half ester **2.38b**. Curtius rearrangement yielded the protected

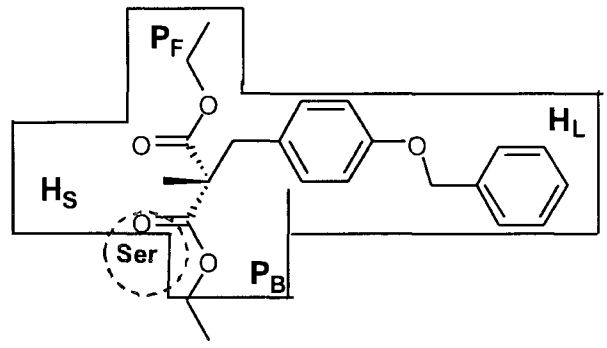


Figure 2.3 . Active site model of PLE proposed by Jones with compound **2.31b** imposed

(*R*)-α-methyl tyrosine (**2.39b**) in 65% isolated yield. Chiral HPLC of both the *R* and *S* α-methyl tyrosine proved that the enantiomeric composition was retained throughout the syntheses.

Conclusions

The above chapter outlined the synthesis for both enantiomers of α-methyl serine and α-methyl tyrosine. The PLE hydrolysis of the starting diesters yielded good enantioselectivity for both to yield common intermediates that were used to synthesize both enantiomers of the α-methyl amino acids. Workup of the tyrosine half ester (**2.32b**) has proven to be to be quite tedious due to the tremendous amount of buffer necessary for PLE hydrolysis and the emulsions that forms during extraction. Kedrowski's methodology was extended to include the synthesis of both enantiomers of α-methyl serine and α-methyl tyrosine from a common malonate half ester. It was proven that the protecting groups used can be removed and replaced selectively without racemization. It was also shown that the Curtius rearrangement proceeds without any change in enantioselectivity. The α-methyl serine analogues have potential to be a synthetic intermediate of both enantiomers of other α-methyl amino acids. The α-methyl tyrosine analogues have potential use in peptidomimetic drug synthesis. The primary problem with our current synthesis is that the PLE hydrolysis of both **2.31a** and **2.31b** does not result in great enantioselectivity. Although the half ester discussed in this chapter will be used to prove the remainder of our synthetic methodology (Chapters III and IV), we have developed an assay that we hope to use to solve the enantioselectivity problem. This assay is will be discussed thoroughly in Chapter V.

Experimental

General

Tetrahydrofuran was distilled over sodium, under a nitrogen atmosphere, prior to use. Methylene chloride and 1,2-dichloroethane were distilled from calcium hydride under a nitrogen atmosphere prior to use. Triethylamine was distilled from sodium hydroxide pellets under a nitrogen atmosphere prior to use. Diphenylphosphorylazide was prepared using a literature procedure.[24] All other chemicals and enzymes were obtained from Aldrich Chemical and used as received unless otherwise noted. Benzylchloromethyl ether (**2.30a**) was purchased from Sigma and distilled over calcium chloride before use. Diethyl 2-(benzyloxymethyl)-2-methylmalonate (**1**) was synthesized according to a literature procedure.[19] All NMR spectra were acquired with a Varian Mercury 300 MHz spectrometer and referenced to either residual solvent protons or to TMS. IR spectra were acquired with a Thermo-Nicolet Nexus 470-FT-IR using a diamond anvil ATR accessory. UV/Vis spectra were acquired with an HP 8452 spectrophotometer. Optical rotation measurements were acquired with a Rudolph Research Autopol III autopolarimeter using a 1dm cell at ambient temperature. TLC analysis was performed on EMD science silica coated aluminum plates and visualized using UV or phosphomolybic acid stain. Flash chromatography was performed using Silicycle silica gel (Silia-P). Radial chromatography was performed using a Harrison Research Chromatotron with Analtech precoated plates. Chiral HPLC was performed using a LabAlliance Series III isocratic pump coupled to a LabAlliance Model 500 UV/Vis detector. HPLC chromatograms were recorded using the Peaksimple® data acquisition system and software. Chiral HPLC was performed using a Chiralcel OJ-H analytical column or a Chiralcel AD-H column from

Chiral Technologies, Inc. All HPLC retention times are listed in minutes. HRMS analysis was performed at Old Dominion University on an Apex FT-MS using a 1:1 THF:MeOH solvent system with added NaCl to observe sodium adducts of the compounds of interest. Melting points were determined in an open capillary tube using a Hoover melting point apparatus and are uncorrected.

Diethyl 2-(benzyloxymethyl)-2-methylmalonate (2.31a): A 1.60 g (46 mmol) portion of NaH (60% dispersion in mineral oil) was dispersed in 50 mL of dry THF and a solution of diethyl methylmalonate (6.67 g, 38.3 mmol in 5 mL anhydrous THF) was added dropwise to the NaH suspension at 0 °C. The solution was allowed to stir at 0 °C until no further gas evolution was observed. The reaction mixture was removed from the ice bath and allowed to stir at ambient temperature for 90 minutes. A solution of **2.30a** (6.00 g, 38.3 mmol, in 5 mL anhydrous THF) was added dropwise and the solution was heated to solvent reflux for 12 hours. The resulting suspension was diluted with 50 mL of ether and placed in a separatory funnel. The suspension was then washed three times with brine and three times with 10% HCl. The organic layer was dried over $MgSO_4$, filtered, and concentrated by rotary evaporation. The crude material was purified by column chromatography (30% EtOAc / 70% hexanes) to give 8.63 g (29.3 mmol, 77% yield) of **2.31a** as a clear and colorless viscous liquid.

(*R*)-2-(4-(benzyloxymethyl)-3-ethoxy-2-methyl-3-oxopropanoic acid (2.32a): An amount of 7.09 g (24.1 mmol) of **2.31a** was dispersed in 450 mL of rapidly stirring phosphate buffer (0.1 N pH 7.4). 90.4 mg of PLE (24 units / mg, 90 units / mmol of

substrate) was dissolved in 1.0 mL of 3M $(NH_4)_2SO_4$ and added to the buffer solution and the pH of the reaction mixture was maintained using a 798 MPT Titrino® in the pH stat mode. The Titrino® was set to titrate to a volume of 19.9 mL (24.1 mmol) of 1.21 M NaOH. The hydrolysis proceeded for 20 hours, after which NaOH was added to make the solution sufficiently basic. The aqueous solution was then extracted with three 300 mL volumes of ether. The aqueous layer was acidified using concentrated HCl to a pH of 2. The aqueous layer was then extracted with three volumes of ether. The organic layer was dried over $MgSO_4$, filtered, concentrated *via* rotary evaporation, and placed under high vacuum to yield 4.42 g (16.6 mmol, 69% yield, 70% *ee*) of a viscous, clear liquid. The absolute stereochemistry was determined by optical rotation and comparison to literature values. $[\alpha]_D^{22} = +7.69°$ (c =0.208, MeOH). The % *ee* was determined by analytical chiral HPLC (Chiracel OJ-H, 257 nm, flow rate = 1 mL / minute, 4% *i*PrOH / 96% hexane) $Rt_{(R)} = 16.90$, $Rt_{(S)}$ 19.20.

(*S*)-Ethyl3-(benzyloxy)-2-((4-methoxybenzyloxy)carbonylamino)-2-methylpropanoate (2.35a): An amount of 0.67 g (2.51 mmol) of **2.32a** was dissolved in 10.00 mL of 1,2-dichlrorethane followed by addition of 596 µL (2.77 mmol) of DPPA and 739 µL (5.30 mmol) of Et_3N was added and the solution was brought to reflux solvent for 1.5 hours, at which time 432 µL (3.48 mmol) of PMB-OH was added and the solution was heated to reflux solvent for 12 hours. The solution was concentrated by rotary evaporation. The protected amino ester was then purified by silica chromatography using 5% MeOH / 95% $CHCl_3$ (R_f= 0.60). This gave 0.70 g (1.74 mmol, 70% yield, 70% *ee*) of a transparent, viscous oil. λ_{max} = 286 nm, $[\alpha]_D^{23} = -1.8°$ (c = 0.164 ,CH_2Cl_2),

IR (cm^{-1}) 3420 (br), 1717 , ^{1}H-NMR (300 MHz, $CDCl_3$) 1.22 (3H, t, J = 7 Hz), 1.54 (3H, s), 3.70 (1H, d, J = 9 Hz), 3.78 (4H, m), 4.18 (2H, m), 4.48 (2H, q, J = 12 Hz), 5.01 (2H, s), 5.77 (1H, bs), 6.87 (2H, d, J = 9 Hz), 7.30 (7H, m) ^{13}C-NMR (75 MHz, $CDCl_3$) 14.2, 20.3, 55.3, 60.4, 61.9, 66.3, 72.7, 73.3, 113.9, 127.6, 127.8, 128.4, 128.7, 130.0, 137.8, 155.2, 159.6, 172.7. HRMS: $[C_{22}H_{27}NO_6Na]^+$ Calcd = 424.1736 Obsd = 424.1739. The % *ee* was determined by analytical chiral HPLC (Chiracel, OJ-H, 286 nm, 1 mL / minute, 4% *i*PrOH / 96% hexane) $Rt_{(S)}$ = 46.42 $Rt_{(R)}$ = 61.45.

(*S*)-1-*tert*-Butyl 3-ethyl 2-(benzyloxymethyl)-2-methylmalonate (2.37a): An amount of 1.26 g (4.73 mmol) of **2.32a** was dissolved in 5 mL of dry CH_2Cl_2 and placed in a 20 mL sealable pressure tube at -10 °C. 300 μL of conc. H_2SO_4 and 3 mL of condensed isobutylene were added, the vial was capped and the reaction was allowed to stir overnight a room temperature. The flask was then placed in an ice bath for 15 minutes. The tube was opened and allowed to stir at room temp for 2 hours to allow evaporation of any remaining IBE. The solution was diluted with 25 mL of CH_2Cl_2 and washed three times with 1.0 M NaOH, dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give a clear, viscous oil. 1.42 g (4.4 mmol 93 % yield, TLC (10% *i*PrOH / 90% hexane) R_f = 0.536 $[\alpha]_D^{21}$ = + 0.60° (c = 0.100 , CH_2Cl_2). IR (cm^{-1}) 1725, ^{1}H-NMR (300 MHz, $CDCl_3$) 1.24 (3H, t, J = 7 Hz), 1.42 (9H, s), 1.49 (3H, s), 3.77 (2H, m), 4.17 (2H, q, J = 7 Hz), 7.30 (5H, m). ^{13}C-NMR (75 MHz, $CDCl_3$) 14.3, 18.6, 28.0, 55.6, 61.3, 73.0, 73.6, 81.8, 127.7, 127.8, 128.5, 138.3 169.9, 171.2. HRMS $[C_{18}H_{26}O_5Na]^+$ calcd = 345.1672, obsd = 345.1683.

(*S*)-2-(benzyloxymethyl)-3-*tert*-butoxy-2-methyl-3-oxopropanoic acid (2.38a): An amount of 2.03g (6.30 mmol) of **2.37a** was dissolved in 50 mL of EtOH. 0.50 g (3.30 mmol) of LiOH was dissolved in three mL of water and added to the reaction flask. The reaction mixture was allowed to stir at room temperature for 48 hours. Then, 150 mL of 1.0 M NaOH was added to the reaction mixture and the basic aqueous layer was then washed three times with 150 mL portions of Et_2O. The aqueous layer was acidified to pH 1.0 using cold 10% HCl and extracted with three portions of Et_2O. The organic layer was then combined, dried over $MgSO_4$, filtered, and concentrated by rotary evaporator to give 1.38g (4.70 mmol, 75% yield) of a clear viscous liquid which crystallized upon standing to a white solid. Mp = 70-76°C, TLC (10 % *i*PrOH / 90% hexane), R_f = 0.24, $[\alpha]_D^{24}$= -3.8° (c = 0.03 ,$CHCl_3$) IR (cm^{-1}) 3250, 1708 ^{1}H-NMR (300MHz, $CDCl_3$) 1.45 (9H, s), 1.48 (3H, s), 3.49 (2H, s), 3.77 (1H, d, *J* = 11 Hz), 3.80(1H, d, *J* = 11 Hz), 4.56 (2H, s), 7.30 (5H, m) ^{13}C-NMR (75 MHz, $CDCl_3$) 18.7, 27.9, 55.4, 73.1, 73.7, 82.8, 127.7, 127.8, 128.5, 137.8, 170.3, 176.2. HRMS $[C_{16}H_{22}O_5Na]^+$ calcd = 317.1365, obsd = 317.1355.

(*R*)-*tert*-butyl-3–(benzyloxy)-2-((4-methoxybenzyloxy)carbonylamino)-2-methylpropanoate (2.39a): An amount of 0.150 g (0.51 mmol) of **2.38a** was dissolved in 10 mL of 1,2-Dichloroethane followed by addition of 155 µL (0.56 mmol) of DPPA and 300 µL (2.13 mmol) of Et_3N was added and the solution was heated to reflux solvent for three hours, at which time 95 µL (0.77 mmol) of PMB-OH was added and the solution was allowed to reflux overnight. The mixture was then cooled and diluted with 50 mL of $CHCl_3$ and washed three times with 1.0 M NaOH. The organic layer was passed through a small plug of silica gel, dried over $MgSO_4$, filtered, and concentrated by

rotary evaporation to give an orange, transparent, viscous oil. This was purified by radial chromatography (Silica, 50 % Et_2O / 50% hexane) to give 0.170 g (0.40 mmol, 78 % yield, 70% *ee*) of a clear viscous oil. TLC (50% Et_2O / 50% hexane) $R_f = 0.36$, $[\alpha]_D^{24.0} = +0.80°$ (c = 0.05 ,CH_2Cl_2), IR 3419, 1717. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.43 (9H, s), 1.50 (3H, s), 3.67 (1H, d, *J* = 9 Hz), 3.79 (1H, d, *J* = 9 Hz), 3.79 (3H, s), 3.85 (1H, d, *J* = 9 Hz), 4.44 (1H, d, *J* = 12 Hz), 4.54 (1H, d, *J* = 12 Hz), 5.01 (2H, s), 5.80 (1H, bs), 6.86 (2H, d, J = 9 Hz), 7.30 (7H, m) ^{13}C-NMR (75 MHz, $CDCl_3$) 20.3, 28.0, 55.4, 60.7, 66.2, 72.9, 73.5, 82.2, 140.0, 127.7, 127.8, 128.5, 128.9, 130.0, 138.0, 155.2, 159.6, 171.9. HRMS: $[C_{24}H_{31}NO_6Na]^+$ calcd= 452.2049 obsd = 452.2033. The % *ee* was determined by analytical chiral HPLC (Chiralcel AD-H, 257 nm, 4% *i*PrOH / 96% hexane, 1 mL / minute) $Rt_{(S)} = 11.72$, $Rt_{(R)} = 13.73$.

Benzylchlorobenzyl ether (2.30b): Methyl 4-(benzyloxy)benzoate was prepared by an established synthesis, with the exception that the ethyl ester is used in the literature.[21] An amount of 6.40 g (168.5 mmol) of $LiAlH_4$ was suspended in 200 mL of dry THF in a 1000 mL RBF and placed in an icebath. 27.22g (112.4 mmol) of methyl 4-(benzyloxy)benzoate (**2.41**) was dissolved in 200 mL of dry THF and added dropwise to the stirring $LiAlH_4$ solution over 40 minutes. The reaction was allowed to stir overnight at ambient temperature, and heated to reflux solvent for 2 hours. The reaction was allowed to cool and 20 mL of ddH_2O was added to the reaction mixture to remove any unreacted $LiAlH_4$. Then, 20 mL of 1M NaOH was added to quench the $LiAlH_4$ and precipitate the Al as Al_2O_3. The solution was decanted, diluted with 200 mL Et_2O washed three times with brine, dried over $MgSO_4$, concentrated with a rotary evaporator, and

placed under reduced pressure to give 23.95 g (111.8 mmol, 99% yield) of **2.**42 as a solid, white powder. TLC (10% MeOH / 90% $CDCl_3$) of benzyloxybenzyl alcohol R_f= 0.56. Then, 8.23 g (38.4 mmol) of **2.42** was dissolved in 150 mL of dry CH_2Cl_2 at 0 °C. Then, 26.70 mL (384 mmol) of $SOCl_2$ was added slowly. The solution was heated to reflux solvent for 17 hours. The excess CH_2Cl_2 and $SOCl_2$ was removed by a rotary evaporation, and placed under reduced pressure for 4 hours to give (**2.30b**) as a white powder, which was used in the next step without further purification.

Diethyl 2-(4-(benzyloxy)benzyl)-2-methylmalonate (2.31b): A 250 mL 3-neck round-bottom flask was charged with 100 mL of dry THF and 1.70 g (41.9 mmol) of NaH (60% dispersion in mineral oil) under a N_2 atmosphere. A solution of diethyl methylmalonate (6.07 g, 34.9 mmol in 5 mL dry THF) was added dropwise to the NaH suspension at 0 °C. The solution was allowed to stir at 0 °C until no further gas evolution was observed. The reaction mixture allowed to stir at ambient temperature for 90 minutes. A solution of **2.30b** (6.00 g, 38.3 mmol, in 30 mL dry THF) was added dropwise and the resulting solution was heated to reflux solvent for 17 hours. The resulting suspension was diluted with 100 mL of ether and placed in a separatory funnel. The suspension was then washed three times with brine and three times with 10% HCl, dried over $MgSO_4$, filtered, and concentrated in by rotary evaporation. The crude material was dissolved in warm pentane and cooled to -25 °C for 5 hours. The resulting white solid was isolated by vacuum filtration and washed with three 20 mL portions of cold pentane to give 11.73 g (31.7 mmol, 91% yield) of **2.31b** as a white amorphous solid. TLC (20% EtOAc / 80% hexane) R_f = 0.30. Mp = 49° C, IR (cm^{-1}) 1727 , ^{1}H-NMR (300 MHz, $CDCl_3$) 1.25 (6H, t, J = 7

Hz), 1.33 (3H, s), 3.17 (2H, s), 4.19 (4H, q, J = 7 Hz), 5.02 (s, 2H), 6.92 (2H, d, J = 9 Hz), 7.04 (2H, d, J = 9 Hz) 7.38 (5H, m). ^{13}C-NMR (75 MHz, $CDCl_3$) 14.3, 19.9, 40.5, 55.1, 61.5, 70.2, 114.7, 127.7, 128.1, 128.7, 128.8, 131.4, 137.2, 158.0, 172.2. HRMS: $[C_{22}H_{26}O_5Na]^+$ Calcd = 393.1678, Obsd = 393.1672.

(±)-2-(4-(benzyloxy)benzyl)-3-ethoxy-2-methyl-3-oxopropanoic acid (±2.32b): An amount of 1.00 g (2.70 mmol) of **2.31b** was dissolved in 5 mL of 100% EtOH. To this 0.151 g (2.70 mmol) of KOH was added and allowed to stir for 24 hours at room temperature. The reaction was then diluted 50 mL of Et_2O and extracted three times with 60 mL portions of NaOH, which were then combined and acidified using 12.1 M HCl. This acidic solution was then extracted three times with Et_2O. This organic solution was dried over $MgSO_4$, filtered, concentrated by rotary evaporator, and placed under high vacuum to give 0.51 g (55% yield, 1.50 mmol) of a white solid.

(*R*)-2-(4-(benzyloxy)benzyl)-3-ethoxy-2-methyl-3-oxopropanoic acid (2.32b): An amount of 4.00 g (10.8 mmol) of **2.31b** was dispersed in 600 mL of rapidly stirring phosphate buffer (0.1 N, pH 7.4). 100 mg of PLE (2100 units) was dissolved in 1.0 mL of 3M $(NH_4)_2SO_4$ and added to the buffer solution. The pH of the reaction mixture was maintained using a 798 MPT Titrino® in the pH stat mode. The Titrino® was set to titrate to a volume of 10.2 mL (1 eq of 1.06 M NaOH). The hydrolysis proceeded for 20 hours, after which time 40 mL of 1.06 M NaOH was added to make the solution sufficiently basic. The aqueous solution was then extracted three times with 300 mL portions of ether. The aqueous layer was acidified using concentrated HCl to pH = 2. The

aqueous layer was then extracted three times with CH_2Cl_2. The organic layer was dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give 2.81 g (8.21 mmol, 76% yield, 63% *ee*) of a white, amorphous solid. TLC (20% EtOAc / 80% hexane) R_f = 0.25. $[\alpha]_D^{22}$ = -1.00° (c =0.07, CH_2Cl_2). Mp = 99.0 °C ^{1}H-NMR (300 MHz, $CDCl_3$) 1.22 (3.0 H, t, *J* = 7 Hz), 1.40 (3H, s), 3.15 (1H, d, *J* = 14 Hz), 3.25 (1H, d, *J* = 14 Hz), 4.21 (2H, q, *J* = 7 Hz), 5.02 (2H, s), 6.88 (2H, d, *J* = 8 Hz), 7.08 (2H, d, *J* = 8Hz), 7.36 (5H, m), 11.50 (1H, bs). ^{13}C - NMR (75 MHz, $CDCl_3$) 14.2, 20.1, 40.1, 55.2, 62.0, 70.1, 114.8, 127.7, 128.1, 128.2, 128.8, 131.4, 137.1, 158.1, 172.8, 178.0. HRMS: $[C_{20}H_{22}O_5Na]^+$ Calcd = 365.1365 Obsd = 365.1359. Chiral HPLC (Chiracel AD-H, 282 nm, flow rate = 1 mL / minute, 4% *i*PrOH / 96% hexane) $Rt_{(R)}$ = 20.87, $Rt_{(S)}$ 30.18.

(*S*)-ethyl-3–(benzyloxy)-2-((4-methoxybenzyloxy)carbonylamino)-2-methylpropanoate (2.35b): An amount of 0.310 g (0.90 mmol) of **2.32b** was dissolved in 10 mL of Dichloroethane followed by addition of 215 μL (0.996 mmol) of DPPA and 409 μL (2.72 mmol) of Et_3N was added and the solution was heated to reflux solvent for 1.5 hours, at which time 169 μL (1.36 mmol) of PMB-OH was added and the solution was allowed to reflux for 15 hours. The mixture was then cooled and diluted with 20 mL of chloroform and washed with 10% HCl. The organic layer was dried, concentrated by rotary evaporation and purified by flash chromatography (SiO_2, 50% Et_2O / 50% Hexane) to give 0.29 g (0.61 mmol, 67% yield, 63% *ee*) of a clear viscous oil. TLC (50% Et_2O / 50% hexane) R_f = 0.35. $[\alpha]_D^{17.8}$ = +24.2° (c = 0.07, CH_2Cl_2). IR (cm^{-1}) 3426, 3354, 1716. ^{1}H-NMR (300MHz, $CDCl_3$) 1.27 (3H, t, *J* = 7Hz), 1.61 (3H, s), 3.09 (1H, d, *J* = 13 Hz), 3.36 (1H, d, *J* = 13 Hz), 3.80 (3H, s), 4.18 (1H, m), 5.00 (2H, s), 5.00 (1H, d, *J* = 12 Hz),

5.10 (1H, d, J = 12 Hz), 5.44 (1H, bs),6.78 (2H, d, J = 9 Hz), 6.89 (4H, m), 7.37 (7H, m). ^{13}C-NMR (75MHz, $CDCl_3$) 14.3, 23.8, 41.0, 55.5, 60.9, 61.9, 66.4, 70.1, 114.0, 114.7, 127.7, 128.1, 128.6, 128.8, 128.9, 130.3, 131.1, 137.2, 154.9, 157.9, 159.7, 173.8. HRMS: $[C_{28}H_{31}NO_6Na]^+$ calcd= 500.2049 obsd = 500.2044. The % *ee* was determined by analytical chiral HPLC (Chiralcel AD-H, 282 nm, 1 mL / minute, 4% *i*PrOH / 96% hexane) $Rt_{(S)}$ = 34.38, $Rt_{(R)}$ = 39.35.

(*S*)-α-Methyl Tyrosine Ethyl Ester (2.36b): An amount of 0.500 g of Pd/C was placed in a 50 mL RBF and carefully wetted with 3 mL of THF. Then, 0.270 g of **2.35b** was dissolved in 8 mL of dry THF and slowly added to the flask. The solution was degassed with hydrogen gas for 15 minutes and then placed under a Hydrogen blanket for 20 hours. The disappearance of **2.35b** was monitored by TLC (50% Et_2O / 50% hexane). The reaction was filtered through a celite pad to remove the Pd/C and concentrated by rotary evaporation. The material was then dissolved in 10 mL of 3N methanolic HCl and washed twice with pentane and concentrated by rotary evaporator to give 0.124 g (0.477 mmol, 84% yield) a white hygroscopic amorphous solid. Polarimetry was performed in 1.21 M HCl. Due to the hygroscopic nature of **2.36b**, an accurate mass of the sample was difficult to obtain. The levorotary direction of rotation ($\alpha_{obs}^{21.7}$ = -0.10°, 1.2 M HCl) of **2.36b** matched that of an authentic sample. ^{1}H-NMR (300 MHz, CD_3OD) 1.26 (3H, t, J = 7 Hz), 1.54 (3H, s), 2.95 (1H, d, J = 14 Hz), 3.15 (1H, d, J = 14 Hz), 4.23 (2H, m), 6.73 (2H, d, J = 9Hz), 6.97 (2H, d, J = 9Hz).

1-*tert*-Butyl 3-ethyl 2-(4-(benzyloxy)benzyl-2-methylmalonate (2.37b): An amount of 0.568 g (mmol) of **2.32b** was dissolved in 5 mL of dry CH_2Cl_2 and placed in a 20 mL pressure vial at -10 °C (NaCl/icebath) . Then, 150 µL (2.70 mmol) of concentrated H_2SO_4 and 3 mL of condensed isobutylene was added, the vial was capped and the reaction was allowed to stir overnight a room temperature. The flask was then placed in an icebath at 0 °C for 15 minutes and opened to allow evaporation of any remaining IBE. The solution was diluted with 25 mL of CH_2Cl_2 and washed three times with 1.0 M NaOH, dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give a clear, viscous oil. The material was purified by centrifugal chromatography (Silica, 30% Et_2O / 70% hexane) to give 0.419 g (1.10 mmol, 65% yield) of a clear, viscous oil. TLC $R_f = 0.41$, IR (cm^{-1}) 1724 cm^{-1}, ^{1}H-NMR (300 MHz, $CDCl_3$) 1.26 (3H, t, $J = 7.2$ Hz), 1.28 (3H, s), 1.44 (9H, s), 3.09 (3H, d, $J = 14$ Hz), 3.16 (1H, d, $J = 14$ Hz), 4.18 (2H, q, $J = 7$ Hz), 5.02 (2H, s), 6.86 (2H, q, $J = 9$ Hz), 7.06 (2H, d, $J = 9$ Hz), 7.37 (5H, m).^{13}C-NMR (75 MHz, $CDCl_3$) 14.3, 19.8, 40.2, 55.5, 61.2, 70.1, 81.7, 114.6, 127.6, 128.15, 128.76, 128.9, 131.4, 137.2, 157.8, 171.2, 172.4. HRMS: $[C_{24}H_{30}O_5Na]^+$ calcd= 421.1985 obsd = 421.1972.

2-(4-(Benzyloxy)benzyl)-3-tert-butoxy-2-methyl-3-oxopropanoic acid (2.38b): An amount of 2.62g (6.58 mmol) of **2.37b** was dissolved in 20 mL of 95% EtOH. 1.58 mL (19.72 mmol) of 12.50 M NaOH solution was added to the reaction flask. The reaction mixture was allowed to stir at room temperature for 48 hours. The reaction was diluted with 30 mL of 1.0 M NaOH, washed three times with Et_2O, acidified to pH 1.0, extracted into Et_2O and concentrated by rotary evaporator to give 2.12 g (5.72 mmol, 87% yield) of a clear, viscous, liquid which later crystallized to a white solid. Mp = , TLC (20% *i*PrOH

/ 80% hexane) R_f = 0.48, IR (cm^{-1}) 3230, 1750, 1698. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.35 (3H, s), 1.45 (9H, s), 3.13 (1H, d, *J* = 14 Hz), 3.19 (1H, d, *J* = 14 Hz), 5.01 (2H, s), 6.88 (2H, d, *J* = 9 Hz), 7.10 (2H, d, *J* = 9 Hz), 7.36 (5H, m), 10.4 (1H, bs), ^{13}C-NMR ($CDCl_3$) 20.1, 27.9, 40.5, 55.6, 70.0, 82.6, 114.6, 127.6, 128.1, 128.4, 128.7, 131.4, 137.1, 157.9, 171.4, 178.2. HRMS: $[C_{22}H_{26}O_5Na]^+$ calcd = 393.1678, obsd = 393.1668

(*R*)-*tert*-Butyl-3-(4-(benzyloxy)phenyl)-2-((4-methoxybenzyloxy)carbonylamino)-2-methylpropanoate (2.39b): An amount of 0.300 g (0.81 mmol) of **2.38b** was dissolved in 10 mL of Dichlrorethane in a 50 mL RBF and 192 µL (0.89 mmol) of DPPA and 739 µL (4.90 mmol) of Et_3N was added and the solution was heated to reflux solvent for 3 hours, at which time 151 µL (1.20 mmol) of PMB-OH was added. The solution was allowed to reflux overnight, diluted with 50 mL of $CHCl_3$, washed three times with 1.0 M NaOH, filtered through silica gel, and concentrated by rotary evaporation to give a orange, transparent, viscous oil that was purified by centrifugal chromatography (Silica, 50% Et_2O / 50% hexane) to give 0.266 g (0.53 mmol, 65% yield, 60% *ee*) of a clear, viscous oil. TLC (50% Et_2O / 50% hexane) R_f = 0.31. IR (cm^{-1}) 3350, 1708. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.43 (9H, s), 1.59 (3H, s), 3.05 (1H, d, *J* = 14 Hz), 3.37 (1H, d, *J* = 14 Hz), 3.79 (3H, s), 4.97 (1H, d, *J* = 12 Hz), 4.99 (2H, s), 5.01 (1H, d, *J* = 12 Hz), 5.51 (1H, bs), 6.78 (2H, d, *J* = 9Hz), 6.89 (2H, d, *J* = 9 Hz), 6.93 (2H, d, *J* = 9Hz), 7.32 (2H, *J* = 9 Hz), 7.40 (5H, m), ^{13}C-NMR (75 MHz, $CDCl_3$) 24.1, 28.0, 40.5, 55.4, 61.0, 66.1, 70.0, 82.4, 113.9, 114.5, 127.6, 128.0, 128.7, 128.9, 129.0, 130.2, 131.2, 137.2, 154.8, 157.8, 159.6, 172.8. . HRMS: $[C_{30}H_{35}N\,O_6Na]^+$ calcd = 528.2357, obsd = 528.2342. The

% *ee* was determined by analytical chiral HPLC (Chiralcel AD-H, 282 nm, 4% *i*PrOH / 96% hexane, 1mL / minute) $Rt_{(S)}$ = 20.63, $Rt_{(R)}$ = 23.97.

References

(1) Cativiela, C.; Diaz-de-Villegas, M. D. *Tetrahedron: Asymmetry* **1998**, *9*, 3517-3599.

(2) Flamen, P.; Bernheim, N.; Deron, P.; Caveliers, V.; Chavatte, K.; Franken, P. R.; Bossuyt, A. *European Journal of Nuclear Medicine* **1998**, *25*, 177-181.

(3) Obrecht, D.; Bohdal, U.; Daly, J.; Lehmann, C.; Schonholzer, P.; Muller, K. *Tetrahedron* **1995**, *51*, 10883-10900.

(4) Tanaka, M. *Chemical and Pharmaceutical Bulletin* **2007**, *55*, 349-358.

(5) Wang, D.; Cole, P. A. *Journal of the American Chemical Society* **2001**, *123*, 8883-8886.

(6) Humphrey, J. M.; Chamberlin, A. R. *Chemical Reviews* **1997**, *97*, 2243-2266.

(7) Belokon, Y. N.; North, M.; Kublitski, V. S.; Ikonnikov, N. S.; Krasik, P. E.; Maleev, V. I. *Tetrahedron Letters* **1999**, *40*, 6105-6108.

(8) Belokon, Y. N.; Davies, R. G.; North, M. *Tetrahedron Letters* **2000**, *41*, 7245-7248.

(9) Belokon, Y. N.; North, M.; Churkina, T. D.; Ikonnikov, N. S.; Maleev, V. I. *Tetrahedron* **2001**, *57*, 2491-2498.

(10) Belokon, Y. N.; Davies, R. G.; Fuentes, J. A.; North, M.; Parsons, T. *Tetrahedron Letters* **2001**, *42*, 8093-8096.

(11) Bornscheuer, U. T.; Kazlauskas, R. J. *Hydrolases in Organic Synthesis: Regioselective and Stereoselective Biotransformations*; Second ed.; Wiley-VCH, 2006.

(12) Kedrowski, B. L. *Journal of Organic Chemistry* **2003**, *68*, 5403-5406.

(13) Boulton, L.; Stock, H.; Raphy, J.; Horwell, D. *Journal of the Chemical Society, Perkin Transactions 1* **1999**, *1999*, 1421-1430.

(14) Burgaud, B. G. M.; Horwell, D. C.; Padova, A.; Pritchard, M. C. *Tetrahedron* **1996**, *52*, 13035-13050.

(15) Heidel, H.; Huttner, G.; Zsolnia, L. *Zeitschriftz fur Naturforschung. B, A Journal of Chemical Sciences* **1995**, *50*, 729-734.

(16) Peet, N. P.; Lentz, N. L.; Office, E. P., Ed. C07D 473/06, A61K 31/52

1992, p 42.

(17) Luyten, M.; Muller, S.; Herzog, B.; Keese, R. *Helvetica Chimica Acta* **1987**, *70*, 1250-1254.

(18) Guanti, G.; Banfi, L.; Narisano, E.; Riva, R.; Thea, S. *Tetrahedron Letters* **1986**, *27*, 4639-4642.

(19) Masterson, D. S.; Roy, K.; Rosado, D. A.; Fouche, M. *Journal of Peptide Science* **2008**, *14*, 1151-1162.

(20) Greene, T. W.; Wuts, P. G. *Protecting Groups in Organic Chemistry*; Third ed.; John Wiley and Sons, 1999.

(21) Blanco, L.; Bloch, R.; Bugnet, E.; Deloisy, S. *Tetrahedron Letters* **2000**, *41*, 7875-7878.

(22) Bjorkling, F.; Boutelie, J.; Gatenbeck, S.; Hult, K.; Norin, T. *Tetrahedron Letters* **1985**, *26*, 4957-4958.

(23) Toone, E. J.; Werth, M. J.; Jones, B. *Journal of the American Chemical Society* **1990**, *112*, 4946-4952.

(24) Wolff, O.; Waldvogel, S. R. *Synthesis* **2004**, 1303-1305.

CHAPTER III

$\beta^{2,2}$- AND $\beta^{3,3}$-AMINO ACIDS

Literature Review

Introduction

In the previous chapter, the synthesis of α-methyl serine and α-methyl tyrosine analogues from a common intermediate was discussed. Part B of hypothesis 1 states that both enantiomers of two classes of β-amino acids may be synthesized from a malonate half-ester intermediate. This chapter will detail the synthesis of both enantiomers of $\beta^{2,2}$ and $\beta^{3,3}$ serine and tyrosine analogues from the same malonate half ester common intermediate that was used to synthesize the α-methyl serine and α-methyl tyrosine amino acids. Beta amino acids differ from α-amino acids by an additional methylene group in the carbon backbone between the amine and carboxylic acid groups. There are several different types of β-amino acids (Figure 3.1). The two of interest in the context of this dissertation are the $\beta^{3,3}$- amino acids (**3.2**) and the $\beta^{2,2}$-amino acids (**3.3**). The $\beta^{2,2}$- amino acids have a (HOOC-CR,R'–CH_2-NH_2) connectivity, while the $\beta^{3,3}$ amino acids have a (HOOC-CH_2-CR,R'-NH_2) connectivity. There are a limited number of available syntheses for both classes of β-amino acids that will be discussed here in.

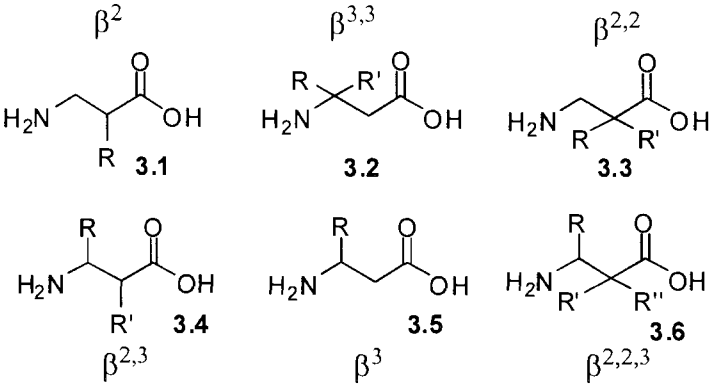
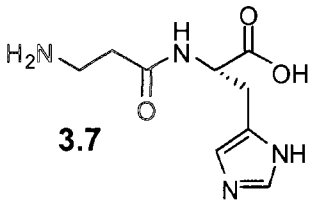


Figure 3.1. Various β-amino acids

Properties of β-Amino Acids and β-Peptides

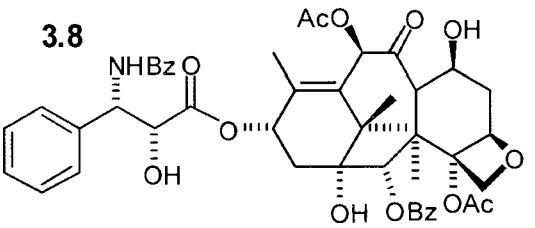
Interest in β-amino acids can be found in literature references dating back to at least 1948.[1] Generally, β-amino acids are considered to be unnatural amino acids, but many examples exist where β-amino acids are present in peptides found in various natural sources including prokaryotes and eukaryotes. One specific example of a β-amino acid present in humans is the dipeptide carnosine (β-Gly-His, Figure 3.2, **3.7**), which is present in muscle tissue and has potent anti-oxidant properties.[1,2] Currently marketed drugs have also made use of β-amino acids. The anti-tumor drug taxol® (Figure 3.3, **3.8**) contains a 2-hydroxy β-phenylglycine substituent.[1]



3.7

Figure 3.2 . L-Carnosine with highlighted β-glycine

β-Amino acids, like their α-methyl amino acid counterparts, have shown very interesting properties as peptidomimetics. It has been established that common proteolytic enzymes can degrade β-amino acids, but at a much slower rate than α-amino acids.[3,4] These properties have made β-amino acids a lucrative research avenue for peptidomimetic research.[1] Seebach et al. have synthesized a series of β-peptides composed entirely of β-amino acids of different types (β^2-, β^3-, $\beta^{2,2}$-, $\beta^{2,3}$-, $\beta^{2,2,3}$- and alternating β^2- and β^3-amino acids, examples are in Figure 3.1, **3.9-3.13**).[5] They tested these β-peptides in mixtures of enzymes, of high specific activity that would degrade the analogous α-peptides quickly. The enzymes were chosen to represent the member of the



3.8

Figure 3.3. Taxol® with highlighted α-hydroxy-β-phenylglycine

major classes of peptidases such as serine, aspartic, and metallo- proteases. To accomplish this, a commercially available mixture of enzymes called Pronase (EC 3.4.24.4) that is derived from fungus was used. Pronase has been well documented in the literature to cleave most types of peptide bonds. Other select enzymes also used were proteinase K, amidase, penicillin amidase, and β-lactamase. Once the enzymatic degradation tests were started, aliquots were taken at predetermined times and analyzed by HPLC and MS to determine the degradation times and the products of the enzymatic degradation. It was observed that several of the β-peptides were intact after 48 hours whereas the analogous α-peptides had degraded within one hour (results shown in Table 3.1). This stability has been confirmed in mammals, insects, plants, and microorganisms.[4]

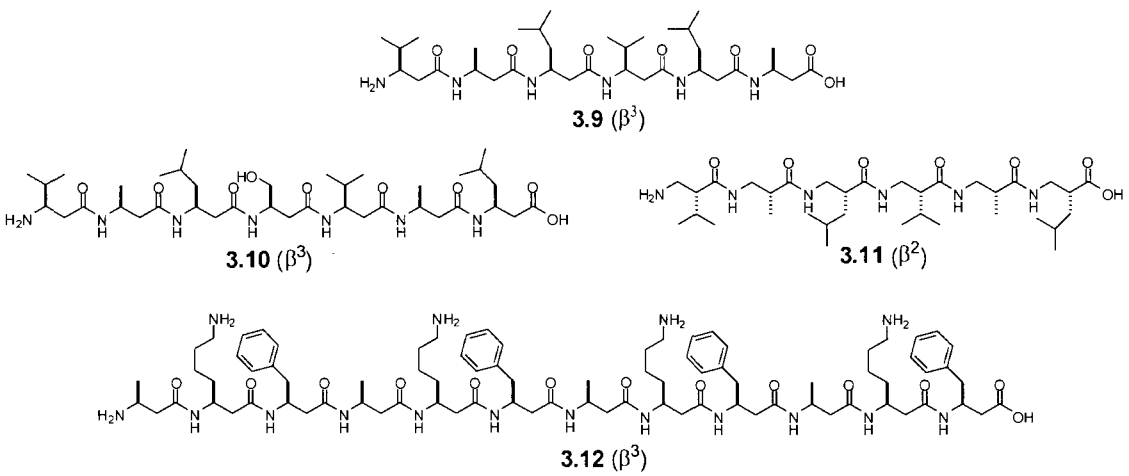


Figure 3.4. β–Peptides tested for stability to proteases (see Table 3.1)

Table 3.1. β-peptide stability to enzymatic degradation

Entry	Enzyme	Origin of the enzyme	α	3.9	3.10	3.11	3.12
1	Pronase	Streptomyces griseus	+		-	-	-
2	proteinase K	Tritirachium album	+		-	-	-
3	pepsin	hog stomach	+	-	-	-	-
4	chymotrypsin	bovine pancreas	+	-	-	-	-
5	elastase	hog pancreas	+	-	-	-	-
6	trypsin	hog pancreas	+	-	-	-	-
7	carboxypeptidase A	bovine pancreas	+	-	-	-	-
8	leucyl aminopeptidase	porcine kidney	+	-	-	-	-
9	proteinase	Bacillus substilis var. biotecus A	+			-	-
10	peptidase	porcine intestinal mucosa	+			-	-
11	pronase E	Streptomyces griseus	+			-	-
12	penicillin amidase	Eschericha coli			-		-
13	β-lactamase	Enterobacter cloacae			-		-
14	amidase	Pseudomonas aeruginosa	-		-		-
15	20 S proteasome	human erythrocytes					-

Results of the peptidase degradation studies conducted on α- and β- peptides from Figure 3.4. Each substrate was incubated with the enzyme for 48 hours. A (+) indicates degradation had taken place. A (–) sign indicates that no detectable degradation had taken place.

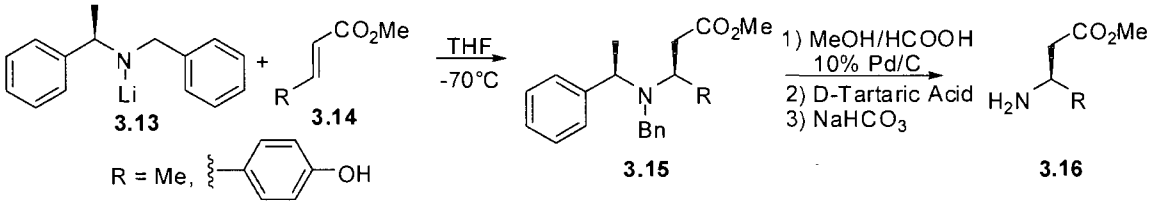
It has been shown that β-amino acids induce secondary structure. Recently, Seebach et al. have shown that a peptide composed of as few as four β-amino acid residues can form stable secondary structures in solution.[5,6] Literature has also shown that simple acyclic β-peptides are capable of forming stable secondary structures found in α-peptides such as helices, parallel and anti-parallel β-pleated sheets, and hairpins. It has also been observed that β-peptides form unique secondary structures such as tubular stacking, ribbons, and alternative helices.[5] The unique secondary structure and stability has yielded unique pharmacological properties in peptidomimetic compounds that

contain β-amino acid residues.[4] Inhibition of cholesterol binding has been observed in cyclic β-peptides that was not observed in peptides composed of the L-enantiomer of natural amino acids.[7] Cyclic somatostatin mimics that are composed entirely of β^3-amino acids have been shown to bind to the somatostatin receptor by mimicking the β-turn structure of the natural peptide.[4,8] Cyclic β-peptides have shown anti-proliferate effects on leukemia, ovarian cancer, breast cancer, renal cancer, lung cancer, and cancer of the central nervous system.[9] Peptides composed of β-amino acids have been designed that can interact with DNA in the same fashion as the natural DNA replication enzymes.[10] It has even been shown that small cationic β-peptide oligomers such as octa-arginine can carry unnatural cargo such as magnetic beads into the nucleus of a cell and interact with the DNA directly.[4] The properties outlined above clearly illustrate the need for efficient syntheses of β-amino acids. The following section will outline the methods that are currently used to synthesize this class of unnatural amino acids.

Synthesis of β-Amino Acids

The properties of β-amino acids and β-peptides outlined above clearly demonstrate the need for synthetic methodology that will allow for efficient synthesis of a variety of classes of β-amino acids. There are many different strategies that have been developed to synthesize β-amino acids. Many of these methods draw from the "chiral pool" to introduce chirality into the β-amino acids.[11-13] Davies et al. reported the synthesis of β^3-amino acids by Michael-type addition of chiral lithium amides to an α,β-unsaturated ester.[14] To accomplish this, the chiral lithium amide (**3.13**) was added to an α,β-unsaturated ester (**3.14**), which yielded the tertiary amine (**3.15**). This tertiary amine (**3.15**) was then treated with Pd/C in MeOH/formic acid to deprotect the amine. The

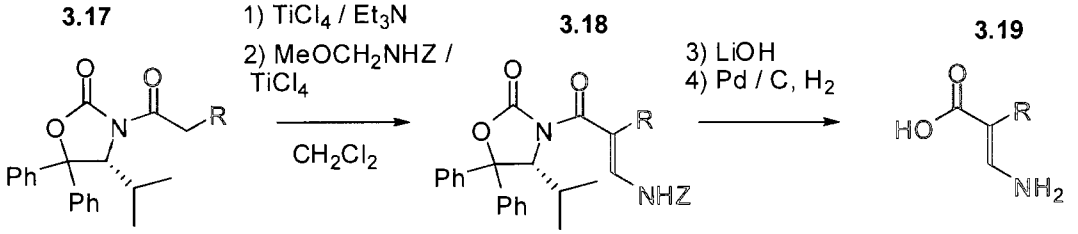
crude β-amino ester (**3.16**) was then purified by crystallization with D-tartaric acid. Using this methodology, this group synthesized β^3-Ala and β^3-Tyr in moderate chemical yields with 99% – 100% *ee*. This methodology was adopted by several other groups to synthesize several different β^3-amino acids.[15] The main disadvantage of this methodology is the use of chiral reactants and a chiral resolving agent, which can be expensive and hamper the possibility of industrial application. Furthermore, this methodology is limited to the synthesis of a single class (β^3) of β-amino acids.



Scheme 3.1. β-Amino acid synthesized by Davies et al.[15]

The synthesis of β^2- amino acids requires a different synthetic methodology. The addition of the $-CH_2-$ must take place between the amine functional and the α-carbon. Several approaches to the synthesis of β^2-amino acid using the enolate of an N-acyl-oxazolidin-2-ones to perform a Mannich reaction to a $XHC{=}NHZ^+$ equivalent (Z = benzyloxycarbonyl, X = Cl, OH, OMe).[16-19] As an example, gram quantities of β^2-amino acids with proteinogenic and unnatural side chains have been conducted using the 4-isopropyl-5,5-diphenyloxazolidin-2-one (DIOZ) chiral auxiliary (Scheme 3.2).[17] Since both enantiomers of the DIOZ auxiliary are commercially available, both enantiomers of the β^2-amino acids can be synthesized using this method. To accomplish the synthesis, the DIOZ auxiliary was N-acylated with an acyl chloride that contains the desired side chain. The Ti, B, or Li enolate was generated followed by addition of the appropriate

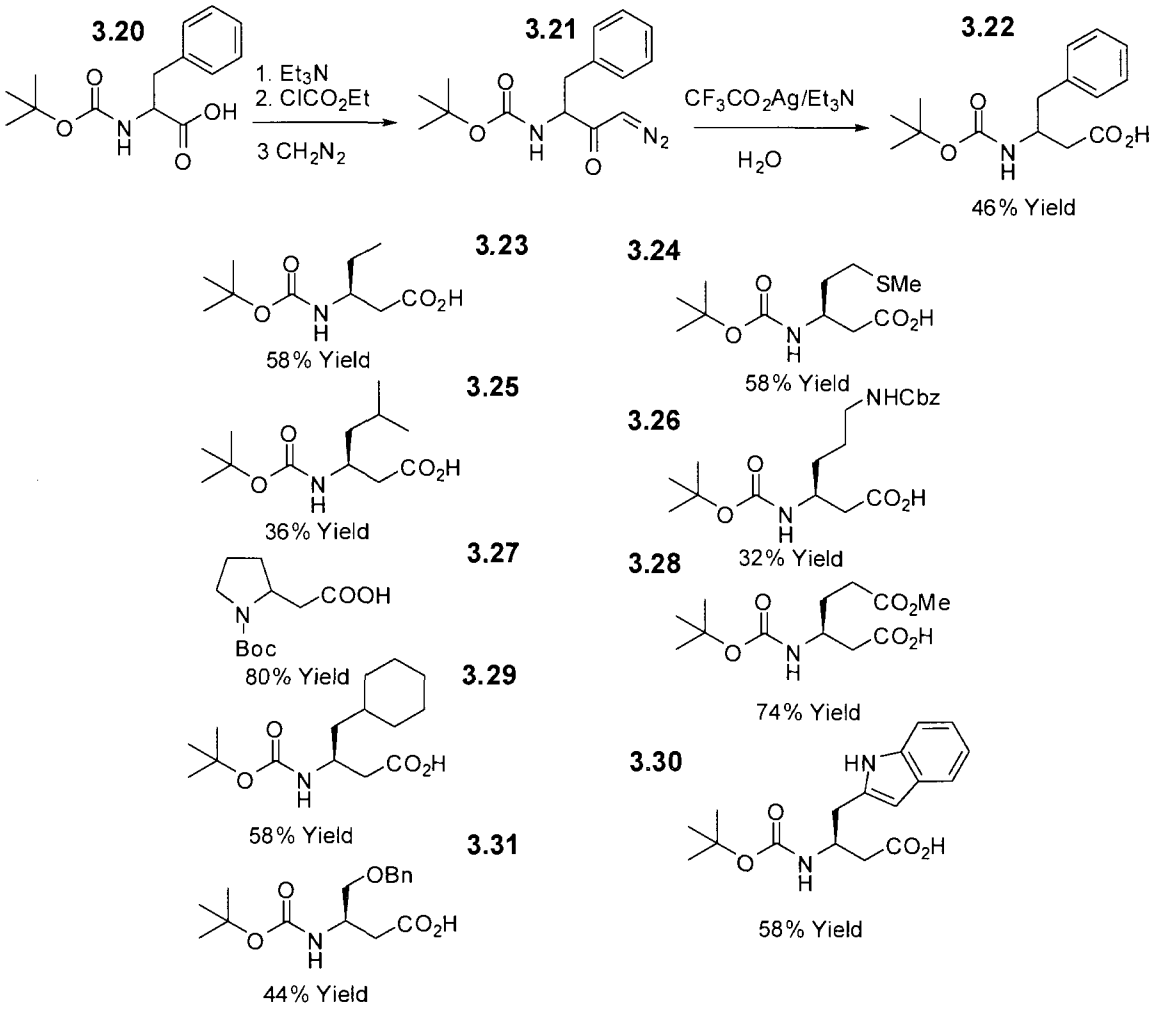
electrophile (CH_3OCH_2NHZ). The DIOZ auxiliary can then be cleaved using base and hydrogenolysis to give the free β^2-amino acid. Yields of 55-90 % and diastereoselectivities of 80 to > 97 % were achieved with 16 β-amino acids using this method (Scheme 3.2). The ability to synthesize both enantiomers of the β^2-amino acids in good to excellent yields and selectivity make this method a powerful technique. The disadvantage of this method, like the Davies method, is that expensive chiral materials (the N-acyl DIOZ auxiliary cost $ 685,000 / mol!) are required to induce the desired chiral center.



Scheme 3.2 . Synthesis of β^2-amino acids via the nucleophilic addition of a Ti, B, or Li enolate to an imine (Mannich Reaction).

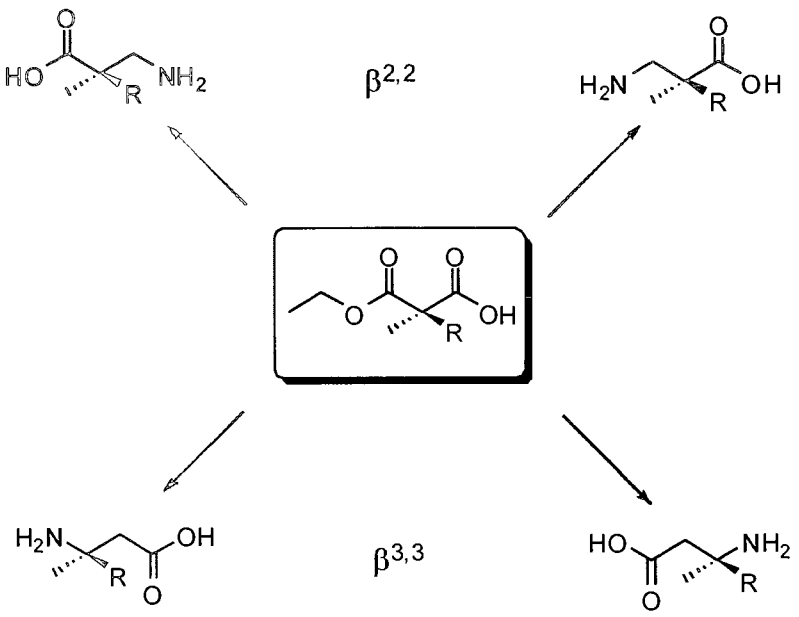
A popular method to synthesize β^3-amino acids that has been widely applied and is inspiration for the work contained in this dissertation is the *homo*-elongation of N-Boc protected α-amino acids.[20,21] Several different classes of β^3-amino acids have been synthesized using this methodology (Scheme 3.3). This method uses Ardnt-Eistert methodology, which is considered by most to be the best method for elongation of a carboxylic acid by one carbon.[11,22] This method involves activation of the acid (**3.20**) to an acid-anhydride, which is then treated with CH_2N_2 to give a diazoketone (**3.21**). The diazoketone is heated in the presence of a silver catalyst to give the free acid (**3.22**).

There are two main problems with this synthetic route that make it of limited utility: 1) the products are generally in low yields due to formation of a cyclic byproduct[11], and 2) this method yields only the β^3-amino acids.



Scheme 3.3 . β-Amino acids synthesized by Arndt-Eistert/ Wolff rearrangement of natural and unnatural α-amino acids

All of the methods described above require the presence of chiral center to facilitate asymmetric synthesis, either in the reactant or as an auxiliary. In Chapter II, the desymmetrization of a prochiral malonate diester with PLE was introduced. The chiral half-ester product served as a common synthetic intermediate that was converted to both enantiomers of α-methyl serine and α-methyl tyrosine by means of protecting group manipulation and a Curtius rearrangement of the acid functionality. Our group has contributed to the synthesis of β-amino acids by utilizing the same malonate half-ester to synthesize both enantiomers of $\beta^{2,2}$- and $\beta^{3,3}$- amino acids.[23] To accomplish this, the acid functionality is used to homologate the carbon backbone via an Ardnt-Eistert synthesis followed by a Wolff rearrangement.[20,21] If water is a component of the Wolff rearrangement, the result is a carboxylic acid that has a new methylene spacer between the acid and the α-carbon. This carboxylic acid can be converted to an amine by use of the Curtius rearrangement to yield a protected $\beta^{2,2}$-amino acid. Manipulation of the protecting groups with subsequent application of the Curtius and Wolff rearrangements results in the synthesis of the enantiomer $\beta^{2,2}$-amino acid and both enantiomers of the



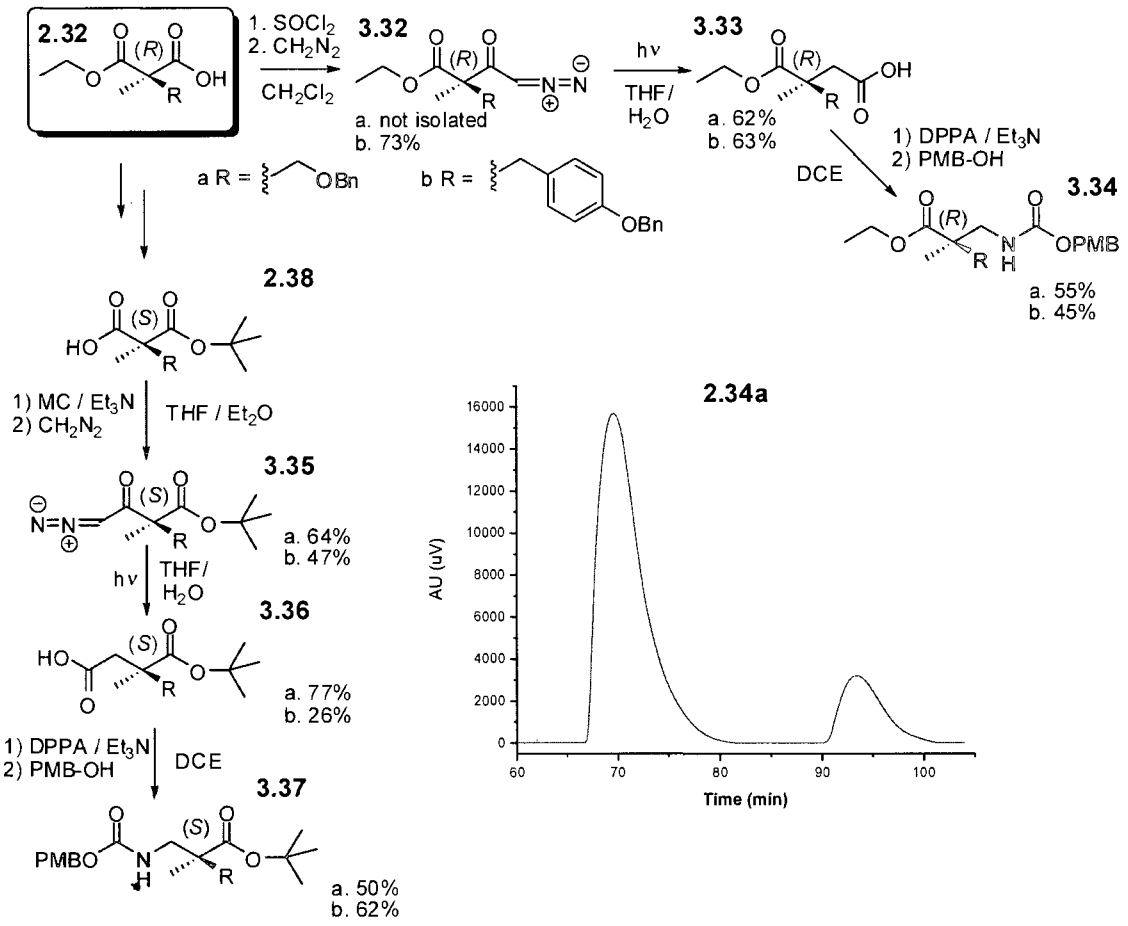
Scheme 3.4 . Generation of both enantiomers of $\beta^{2,2}$- and $\beta^{3,3}$- amino acids from a common malonate half ester intermediate

$\beta^{3,3}$-amino acids. The following will describe the development of the synthesis of both enantiomers of $\beta^{2,2}$- and $\beta^{3,3}$- serine and tyrosine analogues from the same common malonate half-ester intermediates (**2.32a** and **2.32b**) that were discussed in Chapter II. As discussed in Chapter II, the serine analogues functioned as a prototype for the development of the needed synthetic methodology. After synthesis of the serine analogues had been achieved, the same methodology was applied to the synthesis of tyrosine analogues.

Discussion and Results

To synthesize the (*S*) enantiomer of $\beta^{2,2}$-serine the half-ester intermediate **2.32a** was treated with thionyl chloride to generate an acid chloride. Addition of diazomethane yielded a diazoketone **3.32a** (Ardnt-Eistert synthesis). Our initial attempts of a Ag^{+} catalyzed Wolff rearrangement failed. One possible explanation of this failure is that the diazoketone functionality is in close proximity to a neopentyl backbone and the catalysis can not proceed due to steric congestion. Literature findings support this hypothesis and note that the Wolff rearrangement of sterically hindered diazoketones can be accomplished by photolysis.[21] Synthesis of the desired compound was accomplished when the diazoketone was subjected to intense UV light in the presence of water. This resulted in a new half acid (**3.33b**, 63% yield) that has been homologated by one methylene group. Analysis by chiral HPLC proved that there was no racemization of the chiral center during the Ardnt-Eistert synthesis and the Wolff rearrangement (Scheme 3.5). This half-ester was subjected to a Curtius rearrangement and quenched with PMB-OH to yield the protected (*S*)-$\beta^{2,2}$-serine (**3.34a**, 55% yield). The (*R*)-$\beta^{2,2}$-serine enantiomer was generated using a similar synthetic sequence with the exceptions that

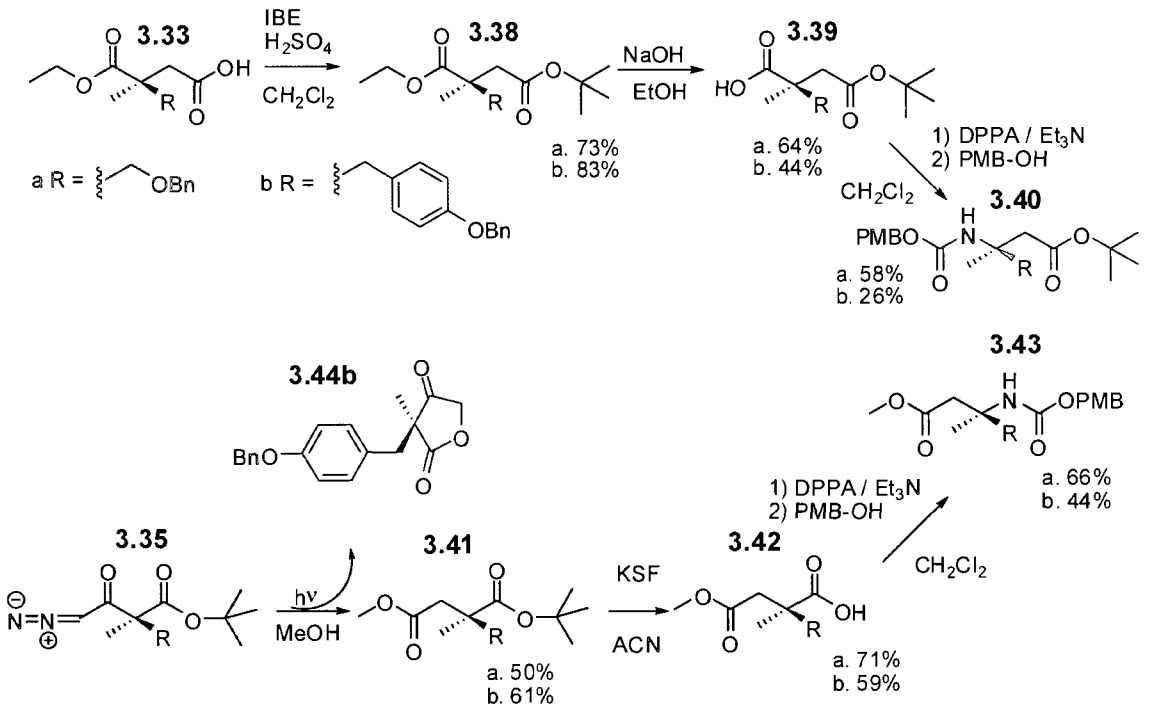
half-ester **2.38a** (Synthesis of **2.38a** from common intermediate **2.31a** is detailed in Chapter II) was used as the starting acid and a different method to activate the acid was required. The acid sensitive nature of the *tert*-butyl ester of **2.38a** did not allow for activation of the acid with thionyl chloride due to the HCl that is formed as a byproduct of the reaction. An alternate path was activation of the acid as a mixed anhydride in the presence of a proton scavenger. Compound **2.38a** was treated with methyl chloroformate in the presence of triethyl amine to give mixed anhydride that was quenched with diazomethane to give the desired diazoketone **3.35a** in 65 yield.[24] A Wolff rearrangement yielded **3.36a** (77%) which was subjected to a Curtius rearrangement to give the (*R*)-$\beta^{2,2}$ serine (**3.37a)** in 50% yield (70% *ee*). Analysis by chiral HPLC showed that the enantiomeric composition was retained throughout each step of the synthesis.



Scheme 3.5. Generation of both enantiomers of $\beta^{2,2}$- and $\beta^{3,3}$- amino acids from a common malonate half-ester intermediate. The chiral HPLC of **3.34a** shows that the enantiomeric composition of **2.32a** was retained throughout both the Wolff rearrangement and Curtius rearrangement

Both enantiomers of the $\beta^{3,3}$-serine analogues can be synthesized using intermediates from the $\beta^{2,2}$-serine synthetic path. To synthesize the (*S*)-enantiomer, Compound **3.33a** was converted to **3.38a** (73% yield) by treatment with isobutylene (IBE) and catalytic amounts of sulfuric acid. Saponification of the ethyl ester of **3.38a** led to a half-ester **3.39a** (64% yield) that was subjected to a Curtius rearrangement to yield **3.40a** in 58 % yield. Analysis by chiral HPLC showed that the enantiomeric ratio of **2.32a** was retained throughout the entire synthesis. To generate the (*R*)-enantiomer, **3.35a** was subjected to a Wolff rearrangement in the presence of methanol which yielded **3.41a**

(50% yield). The *tert*-butyl ester of **3.35a** must be cleaved to give the free acid functionality. Hydrolysis of the *tert*-butyl ester can be accomplished by using catalytic amounts of H_2SO_4, but a *tert*-butyl cation scavenger (i.e. anisole) would be necessary to prevent *tert*-butylation of the aromatic functionality of the protecting group.[25] A more efficient solution is the use of KSF montmorillonite clay to cleave the *tert*-butyl ester.[26] This method is chemoselective for the *tert*-butyl ester. The clay also acts as a *tert*-butyl cation scavenger and can be removed by vacuum filtration. Treatment of **3.41a** with KSF clay in refluxing acetonitrile gave 71% yield of **3.42a**. The free acid was then subjected to a Curtius rearrangement to give the (*R*)-$\beta^{3,3}$-serine (**3.42a)** in 66% yield. HPLC analysis of **3.43a** proved that the enantiomeric composition of **2.32a** (70% *ee*) was preserved throughout the synthesis.



Scheme 3.6. Synthesis of both enantiomers of $\beta^{3,3}$-serine and tyrosine from intermediates generated in the $\beta^{2,2}$ synthetic path.

The synthesis of the $\beta^{2,2}$- and $\beta^{3,3}$-tyrosine series was carried out using a similar synthetic methodology. It was decided at this point, to proceed with racemic synthesis of the tyrosine series. This decision was due to the large amount of time each PLE hydrolysis requires and the tedious workup to obtain **2.32b** that is required. Once the synthesis of the tyrosine analogues has been optimized, each amino acid can be synthesized as needed using **2.32b** from the PLE hydrolysis. To synthesize the (*R*)-$\beta^{2,2}$-tyrosine , Compound **2.32b** was subjected to an Ardnt-Eistert synthesis to give diazoketone **3.32b** (73% yield). This was followed by a Wolff rearrangement in the presence of water to give the desired half ester (**3.33b**, 63 % yield) that had been homologated by one methylene group. Compound **3.34b** (45% yield) was obtained by a Curtius rearrangement of **3.33b**. The (*S*)- $\beta^{2,2}$-tyrosine was synthesized by homologation of **2.38b** to **3.36b** (26% yield) using a Wolff rearrangement that was followed by a Curtius rearrangement which was quenched with PMB-OH yielded the (*S*)-$\beta^{2,2}$-Tyrosine (**3.37b**, 62% yield).

To synthesize the (*R*)-$\beta^{3,3}$-tyrosine, **3.33b** was *tert*-butylated using isobutylene with catalytic amounts of sulfuric acid to give **3.38b** in 82% yield. The ethyl ester was then saponified to give **3.39b** (% yield). This half-ester (**3.39b**) was subjected to a Curtius rearrangement and quenched with PMB-OH to give the (*R*)-$\beta^{3,3}$-tyrosine (**3.40b**, 26% yield). The (*S*)-$\beta^{3,3}$-tyrosine was synthesized by Wolff rearrangement of **3.35b** in methanol to give **3.41b** in 61% yield. The *tert*-butyl ester was then cleaved by the KSF clay method to give **3.42b** (59% yield).[26] The acid moiety of **3.42b** was converted to the (*S*)- $\beta^{3,3}$-tyrosine (**3.43b**, 44% yield) by means of a Curtius rearrangement with subsequent quenching of the isocyanate with PMB-OH. One problem encountered with

the synthesis of the $\beta^{2,2}$- and $\beta^{3,3}$-tyrosine (**3.37b**) was the lower yield of the Wolff rearrangement. This is due to the formation of a keto-alcohol intermediate that cyclizes to give **3.44b**. Optimization of the reactions condition for the Wolff rearrangement of the diazoketones may lead to an increased yield of each. It is possible that these intermediates were also formed in earlier Wolff rearrangements using the **2.32b** and **2.35b**.

Conclusions

It has been shown in this chapter that both enantiomers of $\beta^{2,2}$- and $\beta^{3,3}$- serine and tyrosine analogues can be synthesized from a common malonate half-ester intermediate. As outlined in Chapter 2, this same common intermediate can be used to synthesize both enantiomers of the α-methyl serine and α-methyl tyrosine series. Also seen in Chapter 2 is that the common malonate half-ester intermediate is generated using simple, cost-effective starting materials and an enzyme to catalytically provide the needed chirality. The synthetic methodology outlined above had proven to be efficient. The chemistry used in the synthesis of the $\beta^{2,2}$- and $\beta^{3,3}$-amino acids is similar (Ardnt-Eistert synthesis, Wolff rearrangement, and the Curtius rearrangement). Clearly put, both enantiomers of 3 different classes of unnatural amino acids have been successfully synthesized from the same common intermediate. This synthetic methodology has the potential to generate a large number of unnatural amino acids for use in peptidomimetic compounds. The ability to synthesize these classes from a common intermediate and with common chemistry may allow this methodology to be utilized by biologists and biochemists that do not have access to a full scale synthetic laboratory. Additionally, the use of common synthetic methodology may allow a library of $\beta^{2,2}$- and $\beta^{3,3}$-amino acids

to be efficiently synthesized and incorporated into the same peptide in a combinatorial fashion. This will allow for the possible evaluation of an entire library of peptidomimetic compounds.

Experimental

See Chapter II (pgs 43-44) for general experimental. Diazomethane was generated using the Sigma-Aldrich mini-diazomethane apparatus and the procedure. Diazald was prepared using an Organic Syntheses preparation.[27] Separation of the enantiomers of the $\beta^{2,2}$- and $\beta^{3,3}$- tyrosine analogues by chiral HPLC was not accomplished with either the AD-H or OJ-H chiral columns.

(*R*)-Ethyl 2-(benzyloxymethyl)-4-diazo-2-methyl-3-oxobutanoate (3.32a): An amount of 1.99 g (7.48 mmol) of **2.32a** was dissolved in 10 mL of dry CH_2Cl_2 and 8.10 mL (113 mmol) $SOCl_2$ was added slowly to the solution. The solution was heated to reflux solvent and allowed to reflux overnight. The excess CH_2Cl_2 and $SOCl_2$ were removed by rotary evaporation and the acid chloride was placed under vacuum for 3 hours to remove any residual $SOCl_2$. The acid chloride was dissolved in dry CH_2Cl_2 and added dropwise to an ethereal solution of anhydrous diazomethane (13.6 mmol) containing 1.13 mL (7.51 mmol) of Et_3N *via* syringe and allowed to react without stirring overnight at 0 °C. The excess diazomethane was then removed by bubbling dry N_2 into the solution for 15 minutes and the triethyl ammonium salt is removed by gravity filtration. The resulting solution was then concentrated using a rotary evaporator and placed under vacuum in the dark for 3 hours to give 2.59 g of crude diazoketone **3.32a** as an orange, transparent, viscous oil. This material was used in the next step without further purification. IR (cm^{-1})

2106, 1727. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.24 (3H , t, J = 7 Hz), 1.47 (3H, s), 3.78 (2H, q, J = 9 Hz), 4.18 (2H, q, J = 7 Hz), 4.54 (2H, s), 5.51 (1H, s), 7.29 (5H, m), ^{13}C-NMR (75 MHz, $CDCl_3$) 14.3, 18.5, 54.6, 58.8, 61.8, 73.1, 73.7, 127.8, 127.9, 128.6, 138.1, 171.6, 192.0.

(*S*)-3-(4-(benzyloxy)methyl)-4-ethoxy-3-methyl-4-oxobutanoic acid (3.33a): An amount of 2.59 g of **3.32a** (9.24 mmol) was placed in a 25 mL roundbottomed flask and dissolved in 10 mL of 30% H_2O / 70% THF was added. Nitrogen was bubbled through the resulting solution for 15 minutes to deoxygenate the solution. The solution was then irradiated with a 500W Hanovia lamp at a distance of approximately 10 cm. The photo-induced Wolff was monitored by the disappearance of the diazo stretch by IR (2106 cm^{-1}). The reaction was complete after 48 hours, diluted with 50 mL of Et_2O and extracted three times with 1.0 M NaOH. The basic aqueous layers were combined, acidified to pH = 1.0 with concentrated HCl, and extracted three times with Et_2O. These Et_2O washes were combined, dried over $MgSO_4$, filtered, concentrated by rotary evaporation, and placed under high vacuum for 3 hours to give 1.31 g (4.70 mmol, 62% yield, 66% *ee*) of a clear viscous oil. TLC (25% *i*PrOH / 75% Hexane) R_f = 0.41, $[\alpha]_D^{22.0}$ = -3.2° (c = 0.061, CH_2Cl_2), IR 3350, 1707. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.22 (3H, t, J = 7 Hz), 1.32 (3H, s), 2.62 (1H, d, J = 16 Hz), 2.90 (1H, d, J = 16 Hz), 3.52 (1H, d, J = 9 Hz), 3.65 (1H, d, J = 9 Hz) 4.16 (2H, q, J = 7 Hz), 4.51 (2H, s), 7.30 (m, 5H) ^{13}C-NMR (75 MHz, $CDCl_3$) 14.2, 20.9, 45.4, 61.2, 73.4, 74.4, 127.6, 127.8, 128.5, 138.2, 175.0, 177.4. HRMS: [$C_{15}H_{20}O_5Na^+$] calcd = 303.1203, Obsd = 303.1209. The % *ee* was determined

by analytical chiral HPLC (Chiracel OJ-H, 257 nm, flow rate = 1 mL / minutes, 4% *i*PrOH / 96% Hexane) $Rt_{(S)}$ = 15.35, $Rt_{(R)}$ = 16.82 .

(*S*)-ethyl 3-(benzyloxy)-2-(((4-methoxybenzyoxy)carbonylamino)methyl)-2-methylpropanoate (3.34a): An amount of 0.50 g (1.80 mmol) of **3.33a** was dissolved in 10mL of 1,2-Dichlroethane in a 50 mL roundbottomed flask with a magnetic stirbar. 415 µL (1.98 mmol) of DPPA and 750 µL of Et_3N (5.38 mmol) was added and the solution was brought to reflux solvent for 3 hours, at which time 335 µL (2.70 mmol) of PMB-OH was added and the solution was again heated to reflux solvent. The solution was diluted with 50 mL of $CHCl_3$ and washed three times with 1.0 M NaOH and three times with 10% HCl. The organic layer was dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give an orange, transparent, viscous oil that was purified by flash chromatography (35 mL of silica gel, 5% *i*PrOH / 95% hexane) to give 0.414 g (1.00 mmol, 55% yield, 68% *ee*) of a clear, viscous oil. TLC R_f = 0.24, $[\alpha]_D^{22.0}$ = -0.9° (c = 0.0378, CH_2Cl_2), IR 1726. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.15 (3H, s), 1.41 (3H, t, *J* = 7 Hz), 3.41 (1H, d, *J* = 9 Hz), 3.49 (2H, dd, *J* = 6 Hz), 3.64 (1H, d, *J* = 9 Hz), 3.80 (3H, s), 4.13 (2H, q, *J* = 2 Hz), 4.48 (2H, s), 5.01 (2H, s), 5.25 (1H, bt, *J* = 6 Hz), 6.88 (2H, d, *J* = 9 Hz), 7.29 (m, 7H) ^{13}C-NMR (75 MHz, $CDCl_3$) 14.3, 18.6, 45.7, 48.2, 55.5, 61.1, 66.7, 73.5, 74.5, 114.0, 127.6, 127.8, 128.6, 128.9, 130.1, 138.1, 157.0, 159.7, 175.1. HRMS: $[C_{23}H_{29}NO_6Na]^+$ calcd = 438.1887 obsd = 438.1879. The % *ee* was determined by analytical chiral HPLC (Chiracel OJ-H, 282 nm, 5% *i*PrOH / 95% hexane) $Rt_{(S)}$ = 69.73, $Rt_{(R)}$ = 93.73.

(*S*)-*tert*-butyl-2-(benzyloxymethyl)-4-diazo-2-methyl-3-oxobutanoate (3.35a) An amount of 1.31 g (4.50 mmol) of **2.38a** and 1.42 mL of Et_3N (9.50 mmol) was dissolved in 25 mL of dry THF under a blanket of N_2 at -50 °C. Then, 360 μL (4.70 mmol) of methyl chloroformate was added slowly to the solution *via* syringe. The solution was allowed to stir at – 50 °C for 3 hours. An ethereal solution of anhydrous diazomethane (21.6 mmol) was added drop-wise to the mixed anhydride solution *via* a dry syringe and allowed to react without stirring overnight at 0 °C. The excess diazomethane was then removed by bubbling dry N_2 into the solution for 15 minutes and the solution was filtered to remove the triethylammonium salt. The crude **3.35a** was purified using 40% Et_2O / 60% hexane and a flash column with 200 mL of silica gel. The pure fractions were then concentrated using a rotary evaporator and placed under vacuum in a flask wrapped in foil for 3 hours to give 0.91 g (2.89 mmol, 64% yield) of an orange, transparent, viscous oil. TLC (40% Et_2O / 60% hexane) $R_f = 0.38$; IR (cm^{-1}) 2106, 1725. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.44 (3H, s), 1.45 (9H, s), 3.73 (1H, d, $J = 9$ Hz), 3.80 (1H, d, $J = 9$ Hz), 4.55 (2H, s), 5.50 (1H, s), 7.33 (5H, m), ^{13}C-NMR (75 MHz, $CDCl_3$) 18.5, 28.0, 54.1, 59.4, 73.2, 73.7, 82.1, 127.7, 127.8, 128.5, 138.1, 170.6, 192.3

(*R*)-3-(Benzyloxymethyl)-4-*tert*-butyl-3-methyl-4-oxobutanoic acid (3.36a) An amount of 0.20 g (0.63 mmol) of **3.35a** was dissolved in 10 mL of 30% water / 70% THF in a 25 mL roundbottomed flask. Dry N_2 was passed through the solution for 15 minutes to deoxygenate the solution. The solution was irradiated with a 500W Hanovia lamp at a distance of approximately 10 cm. The photo-induced Wolff was monitored by the disappearance of the diazo stretch at 2106 cm^{-1} by IR spectroscopy. The reaction was

complete after 48 hours. The solution was then diluted with 50 mL of Et_2O and extracted three times with 1.0 M NaOH. The basic aqueous layers were combined, acidified to pH ≈ 2.0 with 10% HCl, and extracted three times with CH_2Cl_2. These CH_2Cl_2 washes were combined, dried over $MgSO_4$, filtered, concentrated by rotary evaporation, and placed under vacuum for 3 hours to give 0.15 g (0.49 mmol, 77% yield) of a clear, viscous oil. $[\alpha]_D^{22.5} = +3.6°$ (c = 0.05, CH_2Cl_2) IR 1705. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.29 (3H, s), 1.42 (9H, s,), 2.56 (1H, d, *J* = 16 Hz), 2.87 (1H, d, *J* = 16 Hz), 3.48 (1H, d, *J* = 9 Hz), 3.65 (1H, d, *J* = 9 Hz) 4.52 (2H, s), 7.31 (7H, m) ^{13}C-NMR (75 MHz, $CDCl_3$) 21.1, 28.0, 39.5, 45.9, 73.5, 74.7, 81.3, 127.6, 127.8, 128.5, 138.3, 174.2, 177.6 HRMS: $[C_{17}H_{24}O_5Na]^+$ calcd = 466.2200, obsd = 466.2193.

(*R*)-*tert*-Butyl-3-(benzyloxy)-2-(((4-methoxybenzyloxy)carbonylamino)methyl)-2-methylpropanoate (3.37a): An amount of 0.15g (0.49 mmol) of **3.36a** was dissolved in 10 mL of 1,2-dichlroethane in a 25 mL round-bottomed flask with a magnetic stirbar. Then, 113 µL (0.54 mmol) of DPPA and 739 µL of Et_3N (5.30 mmol) was added and the solution was brought to reflux solvent for 3 hours, at which time 91 µL (0.73 mmol) of PMB-OH was added and the solution was again brought to reflux solvent. The solution was cooled to room temperature and diluted with 50 mL of $CHCl_3$ and washed 3 times with 1.0 M NaOH and two times with water. TLC showed a baseline impurity that was removed by filtration through silica gel. The solution was then dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give a orange, transparent, viscous oil. This was purified by flash chromatography (200 mL of silica gel, R_f = 0.65, 30% EtOAc / 70% hexane) to give 0.11 g (50% yield, 0.24 mmol, 69% *ee*) of a clear, viscous oil.

$[\alpha]_D^{22.0}$ = +1.5° (c = 0.03 ,CH_2Cl_2) IR 3420, 1717. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.11 (3H, s), 1.41 (9H, s), 3.45 (3H, m), 3.60 (1H, d, *J* = 9 Hz), 3.78 (3H, s), 4.48 (2H, m), 5.00 (2H, s), 5.31 (1H, bt, *J* = 6 Hz), 6.87 (2H, d, *J* = 9 Hz), 7.28 (7H, m) ^{13}C-NMR (75 MHz, $CDCl_3$) 18.6, 28.1, 45.7, 48.5, 55.4, 66.5, 73.5, 74.8, 81.1, 114.0, 127.5, 127.7, 128.5, 128.9, 130.1, 138.2, 156.9, 159.6, 174.2. HRMS: $[C_{25}H_{33}NO_6Na]^+$ calcd = 466.2200 obsd = 466.2193. The % *ee* was determined by analytical chiral HPLC (Chiracel OJ-H, 282 nm, 6% *i*PrOH / 94% hexane) $Rt_{(S)}$ = 29.24, $Rt_{(R)}$ = 41.78.

(*S*)-4-*tert*-butyl 1-ethyl 2-(benzyloxymethyl)-2-methylsuccinate (3.38a): An amount of 0.540 g (1.93 mmol) of **3.33a** was dissolved in 5 mL of dry CH_2Cl_2 and placed in a 20 mL pressure vessel at -10 °C. Then, 250 µL of H_2SO_4 and 15 mL of condensed isobutylene were added, the vial was sealed and the reaction was allowed to stir overnight a room temperature. The flask was then placed in an icebath at 0 °C for 15 minutes then opened and allowed to stir at room temp for 2 hours to allow evaporation of any remaining isobutylene. The solution was diluted with 25 mL of Et_2O and washed three times with 1.0 M NaOH, dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give a clear, viscous oil. 0.51 g (4.4 mmol, 73% yield). TLC (50% Et_2O / 50% hexane) R_f = 0.34, $[\alpha]_D^{22.7}$ = -3.8° (c = 0.02, CH_2Cl_2) IR (cm^{-1}) 1726 ^{1}H-NMR (300 MHz, $CDCl_3$) 1.24 (3H, t, *J* = 7 Hz), 1.30 (3H, s), 1.41 (9H, s), 2.46 (1H, d, *J* = 16 Hz), 2.74 (1H, d, *J* = 16 Hz), 3.54 (1H, d, *J* = 9, 1 H), 3.59 (1H, d, *J* = 9Hz), 4.15 (2H, q, *J* = 7 Hz), 4.51 (2H, s), 7.31 (5H, m) ^{13}C-NMR (75 MHz, $CDCl_3$) 14.3, 20.7, 28.2, 40.9, 45.6, 60.8, 73.4, 74.8, 80.8, 127.6, 127.7, 128.5, 138.5, 170.6, 175.1. HRMS: $[C_{19}H_{28}O_5Na]^+$ calcd = 359.1829, obsd = 359.1824.

(*S*)-2-(benzyloxymethyl)-4-*tert*-butoxy-2-methyl-4-oxobutanoic acid (3.39a): An amount of 0.658 g (2.00 mmol) of **3.39a** was dissolved in 7 mL of THF. 0.140 g (5.9 mmol) of LiOH was dissolved in 3 mL of water and added to the reaction flask. The reaction mixture was allowed to stir at room temperature for 72 hours. The THF was removed by rotary evaporation and 30 mL of 1.0 M NaOH was added to the reaction mixture. The basic aqueous layer was then washed 3 times with 50 mL portions of Et_2O, acidified to pH 1.0 using cold 10% HCl, then extracted with three portions of Et_2O. The Et_2O extracts were combined and concentrated by rotary evaporation then placed under vacuum to give 0.395 g (1.30 mmol, 64% yield) of a clear, viscous, liquid. TLC (20% *i*PrOH / 80% hexane) $R_f = 0.48$, $[\alpha]_D^{21.3} = -2.9°$ (c = 0.02 ,$CHCl_3$) IR (cm^{-1}) 1726. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.36 (3H, s,), 1.42 (9H, s), 2.51 (1H, d, J = 16 Hz), 2.75 (1H, d, J = 16 Hz), 3.59 (2H, s), 4.55 (2H, s), 4.99 (2H, s), 7.33 (5H, m) ^{13}C-NMR (75 MHz, $CDCl_3$) 20.6, 28.1, 40.9, 45.6, 73.6, 74.4, 81.3, 127.7, 127.8, 128.6, 138.2, 170.5, 181.3 HRMS: $[C_{17}H_{24}O_5Na]^+$ calcd = 331.1516, obsd = 331.1509.

(*S*)-*tert*-Butyl 4-(benzyloxy)-3-((4-methoxybenzyloxy)carbonylamino)-3-methylbutanoate (3.40a): An amount of 0.395 g (1.30 mmol) of **3.39a** was dissolved in 10 mL of 1,2-dichlroethane in a 25 mL roundbottomed flask with a magnetic stirbar. Then, 304 µL (1.40 mmol) of DPPA and 739 µL of Et_3N (5.30 mmol) was added and the solution was brought to reflux solvent for 3 hours, at which time 587 µL (2.00 mmol) of PMB-OH was added and the solution was brought to reflux solvent. The solution was diluted with 50 mL of $CHCl_3$ and washed three times with 1.0 M NaOH, three times with 10 % HCl, and one time with Brine. The crude material was then filtered through a plug

of silica gel to remove a baseline impurity. The solution was dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give an orange, transparent, viscous oil that was purified by flash chromatography (200 mL of silica gel, 50% Et_2O / 50% hexane, R_f = 0.18) to give 0.366 g (0.76 mmol, 58% yield, 69% *ee*) of a clear, viscous oil. $[\alpha]_D^{21.8}$ = -3.20° (c = 0.09, CH_2Cl_2) IR 3361, 1726. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.41 (12H, s), 2.64 (1H, d, *J* = 14 Hz), 2.72 (1H, d, *J* = 14 Hz), 3.59 (2H, m), 3.79 (3H, s), 4.51 (2H, s), 4.99 (2H, s), 5.57 (1H, bs), 6.87 (2H, d, *J* = 8.55 Hz), 7.30 (7H, m) ^{13}C-NMR (75 MHz, $CDCl_3$) 22.2, 28.2, 42.2, 54.6, 55.4, 66.1, 73.4, 74.5, 81.0, 114.0, 127.7, 127.8, 128.5, 128.9, 130.0, 138.2, 155.2, 159.6, 170.7 HRMS: $[C_{25}H_{33}NO_6Na]^+$ calcd = 466.2200 obsd = 466.2190. The % *ee* was determined by analytical chiral HPLC (Chiracel OJ-H, 6% *i*PrOH / 94% hexane, 286 nm, flow rate = 1 mL / minute) $Rt_{(S)}$ = 29.24, $Rt_{(R)}$ = 41.78.

(*R*)-1-*tert*-butyl-4-methyl-2-(benzyloxymethyl)-2-methylsuccinate (3.41a): An amount of 0.70 g (2.20 mmol) of **3.35a** was dissolved in 10 mL of dry MeOH in a 25 mL roundbottomed flask. Dry N_2 was passed through the solution for 15 minutes to deoxygenate the solution. The solution was irradiated with a 500W Hanovia lamp. The photo-induced Wolff was monitored by the disappearance of the diazo stretch (2106 cm^{-1}). The reaction was complete after 48 hours and the crude reaction mixture was concentrated by rotary evaporation, and placed under high vacuum for 3 hours to give a clear viscous oil that was purified by flash chromatography (40% Et_2O / 60% hexane, R_f = 0.38) to give 0.35 g (1.10 mmol, 50% yield) of a clear, viscous oil. $[\alpha]_D^{21.0}$ = + 5.4° (c = 0.08 ,CH_2Cl_2) IR 1724, . ^{1}H-NMR (300 MHz, $CDCl_3$) 1.27 (3H, s), 1.43 (9H, s), 2.53 (1H, d, *J* = 16 Hz), 2.79 (1H, d, *J* = 16 Hz), 3.50 (1H, d, *J* = 9 Hz), 3.61 (1H, d, *J* = 9 Hz),

3.63 (2H, s), 4.51 (2H, s), 7.31 (5H, m). ^{13}C-NMR (75 MHz, $CDCl_3$) 21.0, 28.0, 39.3, 46.0, 51.5, 73.4, 74.7, 80.8, 127.55, 127.64, 128.4, 128.5, 138.5, 172.1, 174.0. HRMS: $[C_{18}H_{26}O_5Na]^+$ calcd = 345.1672 , obsd = 345.1669.

(*R*)-2-((benzyloxy)methyl)-4-methoxy-2-methyl-4-oxobutanoic acid (3.42a): In a 50 mL roundbottomed flask 0.352 g (1.10 mmol) of **3.41a** was dissolved in 20 mL of dry CH_3CN and 1 mL of water was added. Then, 1.0 g of KSF Montmorillonite clay was added and the solution was heated to reflux solvent. The reaction was monitored by TLC (40% Et_2O / 60% hexane) and was determined to be complete after 7 hours. The KSF clay was removed by vacuum filtration, and the filtrate was washed with CH_3CN. The CH_3CN was removed by rotary evaporation and placed under vacuum for 3 hours to give a clear viscous oil that was purified by flash chromatography (10% MeOH / 90% $CHCl_3$, R_f = 0.50) to give 0.207 g (0.77 mmol, 71% yield) of a clear, viscous oil. $[\alpha]_D^{21.2}$ = +4.8° (c = 0.041, CH_2Cl_2). IR (cm^{-1}) 1736, 1703. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.34 (3H, s), 2.62 (1H, d, *J* = 16 Hz), 2.82 (1H, d, *J* = 16 Hz), 3.56 (1H, d, *J* = 9 Hz), 3.62 (3H, s) 3.65 (1H, d, *J* = 9 Hz), 4.52 (2H, s), 7.31 (5H, m), 10.00 (1H, bs). ^{13}C-NMR (75 MHz, $CDCl_3$) 20.8, 28.9, 38.9, 45.5, 51.8, 73.5, 74.0, 127.7, 127.8, 128.5, 138.1, 171.9, 181.2. HRMS: $[C_{14}H_{18}O_5Na]^+$ calcd = 289.1046, obsd = 289.1039.

(*R*)-methyl-4-(benzyloxy)-3-(((4-methoxybenzyoxy)carbonylamino)methyl)-3-methylbutanoate (3.43a): In a 25 mL roundbottomed flask 0.182 g (0.68 mmol) of **3.42a** was dissolved in 10 mL of 1,2-dichlroethane. The solution was stirred and 159 µL (0.75 mmol) of DPPA and 739 µL of Et_3N (5.30 mmol) was added and the solution was

brought to reflux solvent for 3 hours, at which time 127 μL (1.00 mmol) of PMB-OH was added and the solution was again heated to reflux solvent. The solution was cooled and diluted with 50 mL of $CHCl_3$ and washed three times with 1.0 M NaOH and two times with water. The organic layer was then filtered through a plug of silica gel to remove a baseline impurity. The solution was then dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give an orange, transparent, viscous oil that was purified by flash chromatography (200 mL of silica gel, 30% EtOAc / 70% hexane) to give 0.195 g (0.49 mmol, 72% yield, 66% *ee*) of a clear viscous oil. TLC $R_f = 0.39$, $[\alpha]_D^{14.3} = +1.7°$ (c = 0.04 ,CH_2Cl_2) IR 3369, 1717,. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.43 (3H, s), 2.70 (1H, d, J = 14 Hz), 2.81 (1H, d, J = 14 Hz), 3.58 (5H, s), 3.79 (3H, s), 4.49 (2H, s), 4.99 (2H, s), 5.50 (1H, bs), 6.87 (2H, d, J = 7 Hz), 7.30 (7H, m) (75 MHz, $CDCl_3$) 22.4, 40.7, 51.7, 54.6, 55.4, 66.1, 73.4, 74.6, 114.0, 127.75, 127.8, 128.1, 128.5, 128.7, 128.9, 130.0, 138.2, 138.1, 155.3, 159.6, 171.8. HRMS: $[C_{22}H_{27}NO_6Na]^+$ calcd = 424.1701, obsd = 424.1720. The % *ee* was determined by analytical chiral HPLC (chiracel OJ-H, 280nm, 4% *i*PrOH / 96% hexane) $Rt_{(S)} = 99.703$, $Rt_{(R)} = 122.183$.

(±)-Ethyl 2-(4-(benzyloxy)benzyl)-4-diazo-2-methyl-3-oxobutanoate (3.32b): An amount of 2.00 g (5.84 mmol) of **2.32b** was and 6.31 mL (87.6 mmol) $SOCl_2$ was dissolved in 30 mL of dry CH_2Cl_2 and the solution was heated to reflux solvent. After refluxing overnight, the excess CH_2Cl_2 and $SOCl_2$ were removed by rotary evaporation and the acid chloride was placed under reduced pressure with an N_2 cold finger for 3 hours. An ethereal solution of CH_2N_2 was generated *via* Aldrich's procedure for an alcohol free solution using the mini-Diazald apparatus and allowed to dry over KOH for

3 hours. The concentration of CH_2N_2 was determined by UV/Vis at 410 nm ($\varepsilon = 7.2$) to be 20 mmol.[28] The acid chloride was then dissolved in anhydrous CH_2Cl_2 and added dropwise to the ethereal CH_2N_2 solution and allowed to react without stirring overnight at 0 °C. The excess CH_2N_2 was then removed by bubbling dry N_2 into the solution for 15 minutes. The solution was washes three times with 10% HCl, dried over $MgSO_4$, filtered, and concentrated using a rotary evaporator and placed under high vacuum in a flask wrapped in foil for 3 hours to give 2.92 g of a orange, transparent, viscous oil. Purification by flash chromatography (300 mL of silica gel, 50% Et_2O/ 50% hexane) gave 1.56 g (73% pure yield, 4.26 mmol) TLC (50% Et_2O / 50% hexane) $R_f = 0.35$, IR (cm^{-1}) 1716, 2102. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.26 (3H, t, $J = 7$ Hz), 1.28 (3H, s), 3.05 (1H, d, $J = 14$ Hz), 3.25 (1H, d, $J = 14$ Hz), 4.18 (2H, q, $J = 7$ Hz), 5.03 (2H, s), 5.42 (1H, s), 6.87 (2H, d, $J = 9$ Hz), 7.04 (2H, d, $J = 9$ Hz), 7.38 (5H, m), ^{13}C-NMR (75 MHz, $CDCl_3$) 14.2, 19.5, 40.7, 54.2, 59.0, 61.7, 70.1, 114.6, 127.6, 128.1, 128.6, 128.7, 131.4, 131.7, 157.9, 172.7, 193.1

(±)-3-(4-(benzyloxy)benzyl)-4-ethoxy-3-methyl-4-oxobutanoic acid (3.33b: An amount of 1.56 g (4.26 mmol) of **3.32b** was placed in a 25 mL roundbottomed flask and dissolved in 13 mL of 23% H_2O / 77% THF. Nitrogen was bubbled through the solution for 15 minutes to deoxygenate the solution. The solution was then irradiated with a 500W Hanovia lamp at a distance of approximately 10 cm. The photo-induced Wolff was monitored by the disappearance of the diazo stretch at 2102 cm^{-1} by IR. The reaction was complete after 48 hours and the solution was then diluted with 30 mL of Et_2O and extracted 3 times with 1.0 M NaOH, acidified to pH ≈ 1.0 with 1.21 M HCl, and

extracted three times with Et_2O. These Et_2O washes were combined, dried over $MgSO_4$, filtered, concentrated by rotary evaporation, and placed under reduced pressure to give 0.95 g (2.67 mmol, 63 % yield) of a clear viscous oil. TLC (50% Et_2O / 50% hexane) R_f = 0.22. IR (cm^{-1}) 1716.0 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.20 (3H, t, *J* = 7 Hz), 1.25 (3H, s), 2.42 (1H, d, *J* = 17), 2.79 (1H, d, J = 17 Hz), 2.88 (2H, s), 4.12 (2H, q, *J* = 7 Hz), 5.03 (2H, s), 6.88 (2H, d, J = 9 Hz), 7.02 (2H, d, J = 9 Hz) 7.38 (5H, m). ^{13}C-NMR (75 MHz, $CDCl_3$) 14.2, 22.1, 41.6, 43.9, 45.2, 61.0, 70.1, 127.7, 128.1, 128.7, 128.9, 131.5, 137.1, 157.9, 176.0, 177.8. HRMS: $[C_{21}H_{24}O_5Na]^+$ calcd = 379.1516 , obsd = 379.1507.

(±)-ethyl 2-(4-(benzyoxy)benzyl)-3-((4-methoxybenzyoxyl)carbonylamino)-2-methylpropanoate (3.34b): An amount of 0.153 g (0.31 mmol) of **3.33b** was dissolved in 10 mL of 1,2-dichlroethane. Then, 100 µL (0.47 mmol) of DPPA and 253 µL of Et_3N (1.70 mmol) was added and the solution was heated to reflux solvent for 3 hours, at which time 80 µL (0.64 mmol) of PMB-OH was added and the solution was brought to reflux solvent. The solution was diluted with 30 mL of $CHCl_3$ and washed three times with 10 % HCl. TLC showed a baseline impurity that was removed by filtration through silica gel. The material was then dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give a orange, transparent, viscous oil that was purified by flash chromatography (200 mL of silica gel, 10% *i*PrOH / 90% hexane) to give 0.094 g (0.19 mmol, 45% yield) of a clear, viscous oil. TLC R_f = 0.31, IR (cm^{-1}) 1706. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.16 (3H, s), 1.22 (3H, t, J = 7 Hz), 2.82 (2H, s), 3.33 (2H, m), 3.80 (3H,s), 4.11 (2H, q, J =7 Hz), 5.02 (4H, s), 5.12 (1H, bt, J = 7 Hz), 6.87 (4H, m), (2H, d, J = 9

Hz) 7.34 (7H, m). ^{13}C-NMR (75 Hz, $CDCl_3$) 14.4, 20.6, 42.5, 47.4, 55.5, 61.0, 66.8, 70.2, 114.1, 114.7, 127.7, 128.7, 130.2, 137.2, 156.9, 157.9, 159.8, 176.2. HRMS: $[C_{29}H_{33}NO_5Na]^+$ calcd = 514.2200, obsd = 514.2188.

(±)-*tert*-butyl 2-(4-(benzyloxy)benzyl)-4-diazo-2-methyl-3-oxobutanoate (3.35b) An amount of 2.12 g (3.34 mmol) of **2.38b** and 1.81 mL of Et_3N (12.0 mmol) was dissolved in 30 mL of anhydrous THF under a blanket of N_2 at 0 °C. Then, 606 μL (8.58 mmol,) of MC was added slowly to the solution via syringe. The solution was allowed to stir at 0 °C for 3 hours. An ethereal solution of anhydrous diazomethane (21 mmol) was added drop-wise to the mixed anhydride solution *via* a syringe and allowed to react without stirring overnight at 0 °C. The excess diazomethane was then removed by bubbling dry N_2 into the solution for 15 minutes and washed 3 times with 10% HCl, dried over $MgSO_4$, filtered, concentrated by rotary evaporation, and purified by flash chromatography using 50% Et_2O / hexane and 200 mL of silica gel to give 1.07 g (2.71 mmol, 47% yield) of an orange, transparent, viscous oil. TLC (50 % Et_2O / 50% hexane) R_f = 0.39. IR (cm^{-1}) 2104, 1703. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.24 (3H, s), 1.45 (9H, s), 3.02 (1H, d, J = 14 Hz), 3.19 (1H, d, J = 14 Hz), 5.03 (2H, s), 5.41 (1H, s), 6.87 (2H, d, J = 9 Hz), 7.08 (2H, d, J = 9 Hz) 7.33 (5H, m), ^{13}C-NMR (75 MHz, $CDCl_3$) 19.7, 28.0, 40.5, 53.9, 59.6, 70.1, 82.2, 127.7, 128.1, 128.8, 129.0, 131.6, 137.2, 157.9, 171.8, 193.6

(±)-3-(4-(Benzyloxy)benzyl)-4-*tert*-butoxy-3-methyl-4-oxobutanoic acid (3.36b) An amount of 0.60 g (1.54 mmol) of **3.36b** was dissolved in 13 mL of 23% H_2O / 77% THF. Anhydrous N_2 was passed through the solution for 15 minutes to deoxygenate the

solution. The solution was then irradiated with a 500 W Hanovia lamp at a distance of approximately 10 cm. The photo-induced Wolff was monitored by the disappearance of the diazo stretch at 2104 cm^{-1} by IR spectroscopy. The reaction was complete after 48 hours. The solution was diluted with 20 mL of Et_2O and extracted three times with 1.0 M NaOH. The basic aqueous layers were combined, acidified to pH ≈ 2.0 with 10 % HCl, and extracted three times with CH_2Cl_2. The CH_2Cl_2 washes were combined, dried over $MgSO_4$, filtered, concentrated by rotary evaporation, and placed under reduced pressure to give 0.154 g (0.40 mmol, 26% yield) of a clear, viscous oil. IR (cm^{-1}) 1707. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.20 (3H, s), 1.41 (9H, s), 2.36 (1H, d, J = 17 Hz), 2.71 (1H, d, J=17 Hz), 2.86 (2H, s), 5.03 (2H, s), 6.88 (2H, d, J = 9 Hz), 7.05 (2H, d, J = 9 Hz) 7.38 (5H, m) ^{13}C-NMR (75 MHz, $CDCl_3$) 22.4, 28.0, 41.8, 43.7, 45.6, 70.1, 81.2, 114.6, 127.7, 128.1, 128.8, 129.2, 131.7, 137.2, 157.8, 175.3, 178.1. HRMS: $[C_{23}H_{28}O_5Na]^+$ calcd = 407.1829, obsd = 407.1823.

(±)-*tert*-Butyl 2-(4-(benzyloxybenzyl)-3-((4-methoxybenzyloxy)carbonylamino)-2-methylpropanoate (3.37b): An amount of 0.15g (0.40 mmol) of **3.36b** was dissolved in 10 mL of 1,2-dichlroethane. Then, 94 μL (0.43 mmol) of DPPA and 796 μL of Et_3N (1.20 mmol) was added and the solution was heated to reflux solvent for 3 hours, at which time 49 μL (0.40 mmol) of PMB-OH was added and the solution was heated to reflux solvent. The solution was cooled to room temperature and diluted with 15 mL of CH_2Cl_2, washed three times with 10% HCl, and filtered through silica gel. The solution was concentrated by rotary evaporation to give a orange, transparent, viscous oil. This was purified by flash chromatography using 50 % Et_2O / 50% hexane and 70 mL of silica

gel to give 0.128 g (0.25 mmol, 62 % yield) of a clear, viscous oil. TLC (50% Et_2O / 50% hexane) R_f = 0.24, IR 3356, 1712. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.12 (3H, s), 1.43 (9H, s), 2.81 (2H, s), 3.31 (2H, m), 3.80 (3H, s), 5.40 (4H, m), 5.16 (1H, bt, J = 7 Hz), 6.89 (4H, m), 7.07 (2H, d, *J* = 9 Hz), 7.28 (5H, m) ^{13}C-NMR (75 MHz, $CDCl_3$) 20.8, 28.2, 42.2, 47.5, 48.6, 55.4, 66.7, 70.1, 81.4, 114.1, 114.6, 127.7, 128.1, 128.7, 129.2, 130.2, 131.4, 137.2, 156.9, 157.8, 159.7, 175.4. HRMS: $[C_{31}H_{37}NO_6Na]^+$ calcd = 542.2513, obsd = 542.2506.

(±)-4-*tert*-butyl 1-ethyl 2-(4-(benzyloxy)benzyl)-2-methylsuccinate (3.38b): An amount of 0.95 g (2.67 mmol) of **3.33b** was dissolved in 5 mL of anhydrous CH_2Cl_2 and placed in a 20 mL pressure vessel at 0 °C. Then, 100 µL of H_2SO_4 and 2.00 mL of condensed isobutylene (IBE) were added, the vial was sealed and the reaction was allowed to stir overnight a room temperature. The flask was placed in an icebath at 0 °C for 15 minutes and then opened and allowed to stir at room temp for 15 minutes. The solution was diluted with 40 mL of Et_2O and washed three times with 1.0 M NaOH, dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give a clear, orange, viscous oil. 0.91 g (2.21 mmol, 83% yield). TLC (50 % Et_2O/Hexane) R_f = 0.53, IR (cm^{-1}) 1724 ^{1}H-NMR (300MHz, $CDCl_3$) 1.20 (3H, s), 1.22 (3H, t, *J* = 7 Hz), 1.43 (9H, s), 2.31 (1H, d, *J* = 16 Hz), 2.67 (1H, d, *J* = 16 Hz), 2.80 (1H, d, *J* = 13 Hz), 2.90 (1H, d, *J* = 13 Hz), 4.12 (2H, m), 5.03 (2H, s), 6.88 (1H, d, *J* = 9 Hz), 7.02 (1H, d, *J* = 9 Hz), 7.39 (5H, m) ^{13}C-NMR (75MHz, $CDCl_3$) 14.3, 21.9, 28.2, 43.4, 43.9, 45.4, 60.7, 70.1, 80.8, 114.6, 127.7, 128.1, 128.8, 129.3, 131.6, 137.2, 157.8, 170.8, 176.1. HRMS: $[C_{25}H_{32}O_5Na]^+$ calcd = 435.2141, Obsd = 435.2139.

(±)-2-(4-(benzyloxy)benzyl)-4-*tert*-butoxy-2-methyl-4-oxobutanoic acid (3.39b): An amount of 0.35 g (0.85 mmol) of **3.38b** was dissolved in 5 mL of EtOH and 582 μL (7.27 mmol) of a 12.5 M NaOH solution was added to the reaction flask. The reaction mixture was allowed to stir at room temperature for 72 hours. The solution was diluted with 10 mL 1.0 M NaOH, washed three times with Et_2O, acidified with cold 10% HCl and, extracted into Et_2O. The resulting solution was dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give 0.14 g (0.38 mmol, 44% yield) of a clear, viscous, liquid. TLC (50% Et_2O / 50% hexane) R_f = 0.07, IR (cm^{-1}) 1724, 1698. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.22 (3H, s,), 1.44 (9H, s), 2.33 (1H, d, J = 16 Hz), 2.66 (1H, d, J = 16 Hz), 2.90 (2H, m), 5.02 (2H, s), 6.90 (2H, d, J = 9 Hz), 7.07 (2H, d, J = 9 Hz), 7.33 (5H, m) ^{13}C-NMR (75 MHz, $CDCl_3$) 21.7, 28.2, 43.3, 43.5, 45.4, 70.2, 81.3, 114.7, 127.7, 128.1, 128.8, 137.2, 157.9, 170.6, 183.0. HRMS: $[C_{23}H_{28}O_5Na]^+$ calcd = 407.1829, obsd = 407.1824.

(±)-*tert*-Butyl 4-(4-(benzyloxy)phenyl)-3-((4-methoxybenzyloxy)carbonylamino)-3-methylbutanoate (3.40b): In a 25 mL roundbottomed flask 0.143 g (0.37 mmol) of **3.39b** was dissolved in 10 mL of 1,2-dichlroethane. Then, 88 μL (0.41 mmol) of DPPA and 168 μL of Et_3N (1.16 mmol) was added and the solution was allowed to stir for 30 minutes and brought to reflux solvent for 2 hours, at which time 69 μL (0.56 mmol) of PMB-OH was added and the solution was again heated to reflux solvent for 72 hours. The solution was cooled and diluted with 20 mL of $CHCl_3$ and washed two times with 10% HCl, filtered throught silica gel, and concentrated by rotary evaporation to give a

transparent, viscous oil. This was purified by preperative TLC using 50% Et_2O / 50% hexane) to give 0.05 g (0.10 mmol, 26% yield) of a clear viscous oil. TLC R_f = 0.38, IR 3389, 1717. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.33 (3H, s), 1.44 (9H, s), 2.38 (1H, d, J = 14 Hz), 2.64 (1H, d, J = 14 Hz), 3.02 (2H, s), 3.81 (3H, s), 5.02 (2H, s), 5.04 (2H, s), 5.17 (1H, bs), 6.83 (2H, d, J = 9 Hz), 6.90 (2H, d, J = 9 Hz), 7.37 (7H, m) ^{13}C-NMR (75 MHz, $CDCl_3$) 24.6, 28.3, 43.3, 44.2, 54.8, 55.5, 66.1, 70.1, 81.2, 114.0, 114.5, 127.7, 128.8, 130.2, 137.3, 157.8, 159.7, 170.9. HRMS: $[C_{31}H_{37}NO_6Na]^+$ calcd = 542.2513 Obsd = 542.2511.

(±)-1-*tert*-butyl-4-methyl-2-(4-(benzyloxy)benzyl)-2-methylsuccinate (3.41b): An amount of 1.07 g (2.71 mmol) of **3.35b** was dissolved in 10 mL of HPLC MeOH in a 25 mL round-bottomed flask. The solution was degassed for 15 minutes with dry N_2. The solution was irradiated with a 500W Hanovia lamp. The photo-induced Wolff was monitored by the disappearance of the diazo stretch (2104 cm^{-1}). The reaction was complete after 48 hours and the crude reaction mixture was concentrated by rotary evaporation, and placed under high vacuum for 3 hours to give a clear viscous oil that was purified by flash chromatography using 50% Et_2O / 50% hexane and 200 mL of silica gel. TLC (50% Et_2O / 50% hexane, R_f = 0.44) to give 0.66 g (1.63 mmol, 61% yield) of a clear, viscous oil. IR (cm^{-1}) 1720. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.18 (3H, s), 1.42 (9H, s), 2.34 (1H, d, J = 16 Hz), 2.67 (1H, d, J = 16 Hz), 3.65 (2H, s), 5.03 (2H, s), 6.88 (2H, d, J = 9 Hz), 7.05 (2H, d, J = 9 Hz) 7.38 (5H, m). ^{13}C-NMR (75 MHz, $CDCl_3$) 20.1, 33.3, 41.8, 43.8, 45.7, 51.6, 70.1, 80.8, 114.5, 127.7, 128.1, 128.8, 129.4, 131.7, 137.2, 157.8, 172.2, 175.1. HRMS: $[C_{24}H_{30}O_5Na]^+$ calcd = 421.1985 , obsd = 421.1984

(±)-2-(4-(benzyloxy)benzyl)-4-methoxy-2-methyl-4-oxobutanoic acid (3.42b): In a 25 mL roundbottomed flask 0.66 g (1.66 mmol) of **3.41b** was dissolved in 10 mL of dry CH_3CN and 1 mL of water was added followed by addition of 1.0 g of KSF Montmorillonite Clay. The resulting solution was heated to reflux solvent. The reaction was monitored by TLC (50% Et_2O / 50% hexane) and was determined to be complete after 4 days. The KSF clay was removed by vacuum filtration, and the filtrate was washed with CH_3CN. The CH_3CN was concentrated by rotary evaporation to give a clear viscous oil that was purified by flash chromatography (5% MeOH / 95% $CHCl_3$, R_f = 0.55) to give 0.34 g (0.98 mmol, 59 % yield) a white amorphous solid. Mp = 99 – 103 °C IR (cm^{-1}) 2935, 1735, 1697. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.26 (3H, s), 2.43 (1H, d, J = 16 Hz), 2.74 (1H, d, J = 16 Hz), 3.68 (3H, s), 5.03 (2H, s), 6.90 (2H, d, J = 9 Hz), 7.07 (2H, d, J = 9 Hz), 7.38 (5H, m), ^{13}C-NMR (75 MHz, $CDCl_3$) 21.9, 41.4, 43.6, 45.3, 51.9, 70.2, 114.7, 127.7, 128.2, 128.7, 128.8, 131.6, 137.2, 138.0, 172.0, 181.5, HRMS: $[C_{20}H_{22}O_5Na]^+$ calcd = 365.1359, obsd = 365.1360.

(±)-methyl-4-(4-(benzyloxy)phenyl)-3-((4-methoxybenzyoxy)carbonylamino)-3-methylbutanoate (3.43b): In a 50 mL roundbottomed flask 0.34 g (0.99 mmol) of **3.42** was dissolved in 20 mL of 1,2-dichlroethane. Then, 245 µL (1.14 mmol) of DPPA and 467 µL of Et_3N (3.11 mmol) was added and the solution was allowed to stir for 30 minutes, then brought to reflux solvent for 2 hours, at which time 193 µL (1.55 mmol) of PMB-OH was added and the solution was again heated to reflux solvent for 72 hours. The solution was cooled and diluted with 20 mL of $CHCl_3$ and washed two times with

10% HCl, filtered through silica gel, and concentrated by rotary evaporation to give a transparent, viscous oil. This was purified by flash chromatography using 30% EtOAc / 70% hexane) to give 0.21 g (0.44 mmol, 44% yield) of a clear viscous oil. TLC $R_f = 0.29$, IR (cm^{-1}) 3459, 3378, 1723, 1711. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.33 (3H, s), 2.56 (1H, d, $J = 14$ Hz), 2.86 (1H, d, $J = 14$ Hz), 2.98 (1H, d, $J = 14$ Hz), 3.10 (1H, d, $J = 14$ Hz), 3.65 (3H, s), 3.81 (3H, s), 5.03 (2H, s), 5.06 (2H, s), 6.85 (2H, d, $J = 9$ Hz), 6.92 (2H, d, $J = 9$ Hz),7.00 (2H, d, $J = 9$ Hz), 7.30 (7H, m) (75 MHz, $CDCl_3$) 24.7, 42.6, 43.2, 51.7, 54.6, 55.4, 66.1, 70.0, 114.0, 114.5, 127.6, 128.1, 128.7, 129.0, 129.1, 130.2, 131.8, 137.2, 155.1, 157.7, 159.6, 172.8. HRMS: $[C_{28}H_{31}NO_6Na]^+$ calcd = 500.2044, obsd = 500.2041.

References

(1) David L. Steer, R. A. L., Patrick Perlmutter, A. Ian Smith, Marie-Isabel Aguilar *Current Medicinal Chemistry* **2002**, *9*, 811-822.

(2) Nelson, D. L.; Cox, M. M. *Lehninger Principles of Biochemistry*; Third ed.; Worth Publishers: New York, NY, 2000.

(3) Hanson, H. T.; Smith, E. L. *Journal of Biological Chemistry* **1948**, *175*, 833-848.

(4) Seebach, D.; Gardiner, J. *Accounts of Chemical Research* **2008**, *41*, 1366-1375.

(5) Frackenpohl, J.; Arvidsson, P.; Schreiber, J.; Seebach, D. *ChemBioChem* **2001**, *2*, 445-455.

(6) Daura, X.; Gademann, K.; Schafer, H.; Jaun, B.; Seebach, D.; van Gunsteren, W. F. *Journal of the American Chemical Society* **2001**, *123*, 2393-2404.

(7) Moritz, W.; Helmut, H.; Stefan, A.; Dieter, S. *Helvetica Chimica Acta* **1999**, *82*, 1774-1783.

(8) Gademann, K.; Ernst, M.; Seebach, D.; Hoyer, D. *Helvetica Chimica Acta* **2000**, *83*, 16-33.

(9) Gademann, K.; Seebach, D. *Helvetica Chimica Acta* **2001**, *84*, 2924-2937.

(10) Kenji, N.; James, G.; Thierry, K.; Dieter, S. *Helvetica Chimica Acta* **2006**, *89*, 3087-3103.

(11) Liu, M.; Sibi, M. P. *Tetrahedron* **2002**, *58*, 7991-8035.

(12) Evans, D. A.; Wu, L. D.; Wiener, J. J. M.; Johnson, J. S.; Ripin, D. H. B.; Tedrow, J. S. *Journal of Organic Chemistry* **1999**, *64*, 6411-6417.

(13) Soloshonok, V. A.; Fokina, N. A.; Rybakova, A. V.; Shishkina, I. P.; Galushko, S. V.; Sorochinsky, A. E.; Kukhar, V. P.; Savchenko, M. V.; Svedas, V. K. *Tetrahedron: Asymmetry* **1995**, *6*, 1601-1610.

(14) Davies, S. G.; Ichihara, O. *Tetrahedron: Asymmetry* **1991**, *2*, 183-186.

(15) Abdel-Magid, A. F.; Cohen, J. H.; Maryanoff, C. A. *Current Medicinal Chemistry* **1999**, *6*, 955-970.

(16) Hintermann, T.; Gademann, K.; Jaun, B.; Seebach, D. *Helvetica Chimica Acta* **1998**, *81*, 983-1002.

(17) Dieter, S.; Laurent, S.; Fran; ccedil; ois, G.; Pascal, B.; auml; dler; Corinna, J.; ger; Delphine, J.; Sascha, K.; eacute; rald, L.; Yogesh; Mahajan, R.; Peter, M.; Radovan, S.; Bernd; Schweizer, W. *Helvetica Chimica Acta* **2003**, *86*, 1852-1861.

(18) Arvanitis, E.; Ernst, H.; Ludwig, A. A.; Robinson, A. J.; Wyatt, P. B. *Journal of the Chemical Society, Perkin Transactions 1* **1998**, 521-528.

(19) Evans, D. A.; Rieger, D. L.; Bilodeau, M. T.; Urpi, F. *Journal of the American Chemical Society* **2002**, *113*, 1047-1049.

(20) Linder, M.; Steurer, S.; Podlech, J. *Organic Syntheses* **2002**, *79*, 154-160.

(21) Kirmse, W. *European Journal of Organic Chemistry* **2002**, 2193-2256.

(22) Silverman, R. D.; Second ed.; Hayhurst, J., Ed.; Elsevier: 2004, p 47-50.

(23) Masterson, D.; Roy, K.; Rosado, D.; Fouche, M. *Journal of Peptide Science* **2008**, *14*, 1151-1162.

(24) Gérald, L.; Peter, M.; Delphine, J.-L.; Francesco, R.; Dieter, S. *Helvetica Chimica Acta* **2004**, *87*, 3131-3159.

(25) Greene, T. W.; Wuts, P. G. *Protecting Groups in Organic Chemistry*; Third ed.; John Wiley and Sons: Hoboken, NJ, 1999.

(26) Yadav, J. S.; Subba Reddy, B. V.; Sanjeeva Rao, K.; Harikishan *Synlett* **2002**, *5*, 826-828.

(27) de Beor, T. J.; Backer, H. J. *Organic Syntheses* **1954**, *34*, 96-99.

(28) Gassman, P. G.; Greenlee, W. J. *Organic Syntheses* **1973**, *53*, 38-42.

CHAPTER IV

$\gamma^{2,2}$- and $\gamma^{4,4}$- AMINO ACIDS

Literature Review

Introduction

The γ-amino acid class of unnatural amino acids has received a large amount of interest from scientists in the academic and industrial communities. This class of unnatural amino acids is similar to β-amino acids in that they have increased conformation ability and have been shown to add secondary structure to peptides with as few as four residues.[1] This is due to the presence of two additional methylene carbons between the amine and carboxylic acid substituents of the γ-amino acids. Part C of Hypothesis 1 states that a series of homochirally similar γ-amino acids may be synthesized from a common intermediate. There are too many different classes of γ-amino acids to be covered in this dissertation (Figure 4.1). For this reason, this chapter will focus on the synthesis of the $\gamma^{2,2}$- (**4.4**) and $\gamma^{4,4}$- (**4.6**)class of unnatural amino acids, in a straight-forward manner.

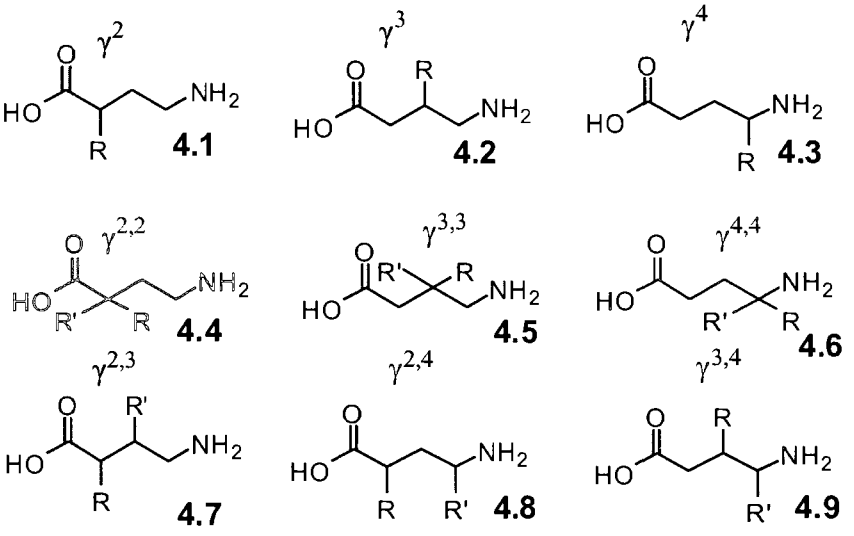


Figure 4.1. Different classes of mono and di-substituted γ-amino acids

Like the α-methyl (Chapter 2), $\beta^{2,2}$-, and $\beta^{3,3}$- (Chapter 3), It is proposed here in that both enantiomers of $\gamma^{2,2}$- and $\gamma^{4,4}$- amino acids can be synthesized from a common malonate half ester intermediate by means of straight-forward chemical transformations.[2] As outlined in the previous chapters, we intend to incorporate these γ-amino acids into peptidomimetic neurotensin analogues for the purpose of testing the biological properties of such peptidomimetic compounds.

Properties of γ-Amino Acids and γ-Peptides

Several pharmaceutical compounds that are currently marketed contain γ-amino acids. To date, the α-hydroxy and β-hydroxy γ-amino acids are the most synthesized class of γ-amino acids due to their pharmacological activity.[3-6] One example is a statine-based peptidomimetic inhibitor of Human β-secretase (**4.10**), which has an IC_{50} = 0.3 μM.

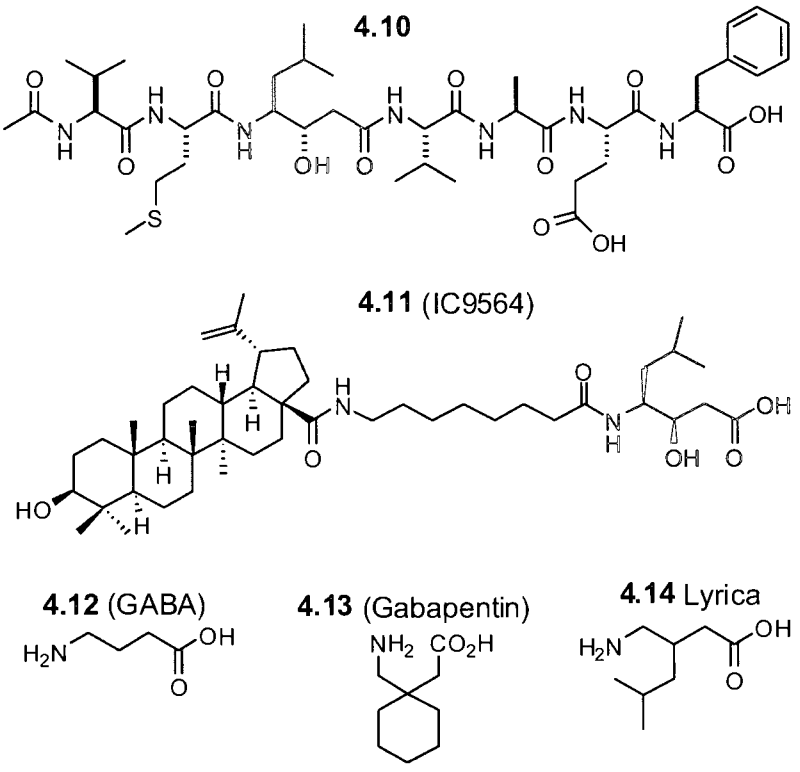


Figure 4.2. γ-Amino acids pharmaceuticals

This compound contains a 3-hydroxy γ^4–Leu at its core.[5] Another example is a compound believed to function as an HIV fusion inhibitor known as IC9564 (**4.11**), which also contains a 3-hydroxy γ^4–Leu, but at its C-terminus.[4] Interest in γ-amino acids

is not limited to compounds that contain a γ-amino acid residue. There has been a great deal of interest in the synthesis of analogues of γ-amino butyric acid (GABA, **4.12**) analogues. GABA is an achiral γ-amino acid that functions in the central nervous system.[3] One GABA analog, Gabapentin (a $\gamma^{3,3}$-amino acid, **4.13**), is currently used as an anticonvulsant. Another GABA analogue is marketed under the Lyrica® trade name. Lyrica is a γ^3-amino acid (**4.14**) that has found use as a treatment for neuropathic pain.[7] This is an encouraging example of the development of a pharmaceutical compound that was developed in an academic laboratory and then marketed by a major pharmaceutical company.

Peptides containing or composed of γ-amino acids have been investigated for their ability to form stable secondary structures. Hanessian et al. performed solution NMR studies of γ-tetra-, hexa-, and octapeptides as well as variants of theses peptides containing α-methylated γ-amino acids.[1] They found that γ-peptides with as few as four residues can form stable helical structures. They also found that substitution of (*S*)-α-methyl γ-amino acids into the γ-peptides had no detrimental effect on the helical structures, but (*R*)-α-methyl γ-amino acid containing peptides did not exhibit helical structures. These characteristics may allow for the control of the secondary structure of peptides that contain α-methyl γ-amino acids by manipulation of the stereochemistry of the γ-amino acids. To further explore the ability of the γ-amino acids to form secondary structure, Hinterman et al. performed solution NMR studies of a γ-hexapeptide, and compared their findings to data for similar α- and β-

4.15

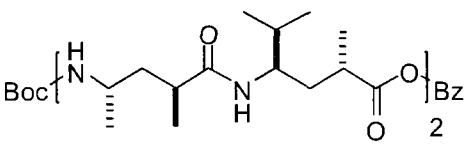


Figure 4.3. γ-Amino acid tetramer capable of forming an α-helix

peptides containing analogous residues (Figure 4.4).[8] They found that the γ-hexapeptide formed a right-handed helical structure with a 5-Å pitch and 14-membered hydrogen bonded ring (Figure 4.5). The β-peptide gave a left-handed helical secondary structure with 5-Å pitch and 14-membered hydrogen bonded ring (Figure 4.5). The α-peptide has the typical right-handed helix (α-helix) with a 5-Å pitch and 14-membered hydrogen bonded ring (Figure 4.5). Interestingly, it was found that the stability of the helix increased with the homolongation of the amino acids.

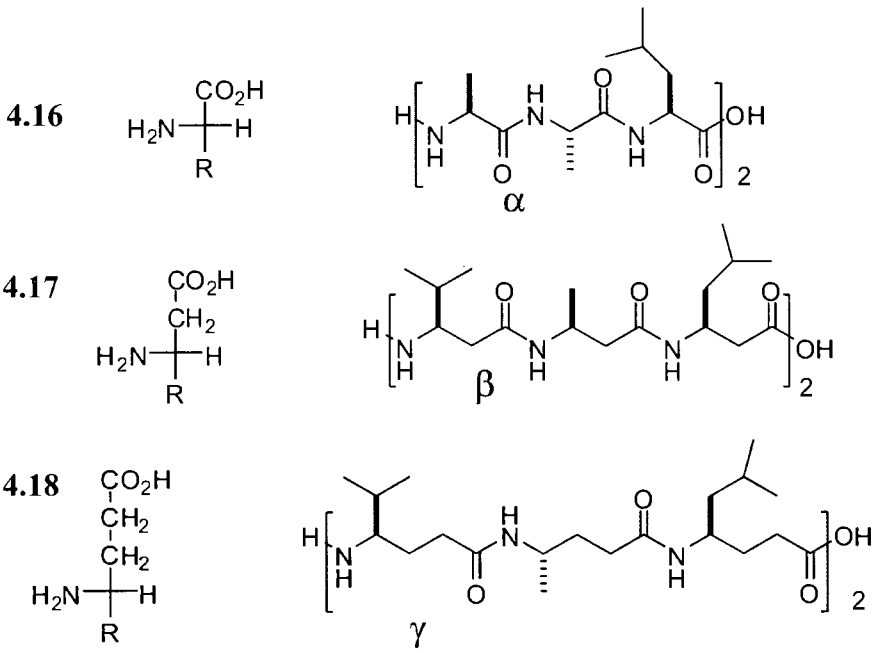


Figure 4.4 Analogous α-,β-, and γ-Peptides studied by Hinterman, et al.

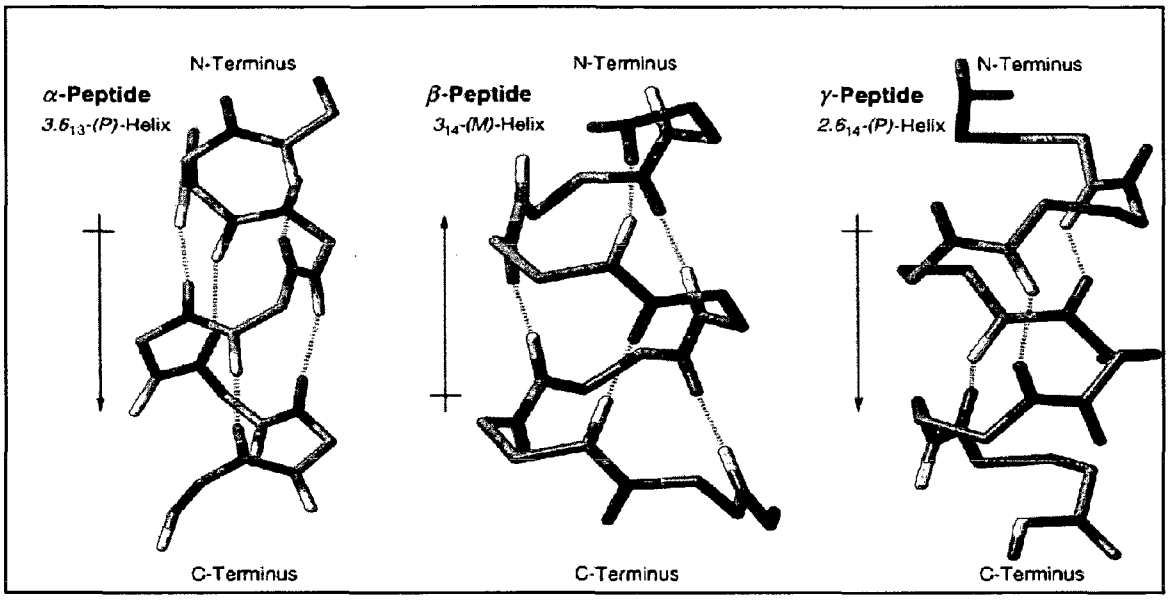
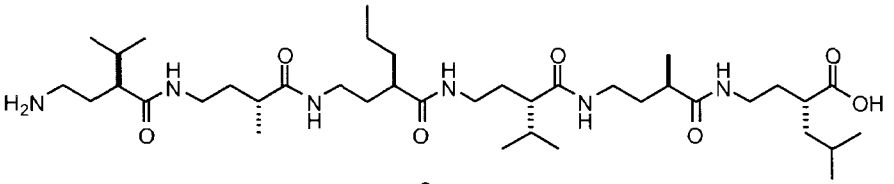


Figure 4.5. Secondary structure representations for analogous α-, β-, and γ- peptides. Reproduced with permission John Wiley and Sons Inc.

Seebach et al. also synthesized and evaluated the biological stability of γ-peptides (Figure 4.6, **4.19**) by the subjecting the γ-peptides to mixtures of peptidases that quickly degrade natural peptides.[9] The γ-peptides were shown to be intact for 48 hours under enzymatic conditions that would degrade the natural (α-peptides) completely within one hour. This stability led to the synthesis and evaluation of cyclic somatostatin analogues that were composed of 2 γ-amino acids.[10] These analogues were capable of binding to the somatostatin receptor by mimicking the β-turn structure of somatostatin. These



4.19 γ^2-peptide

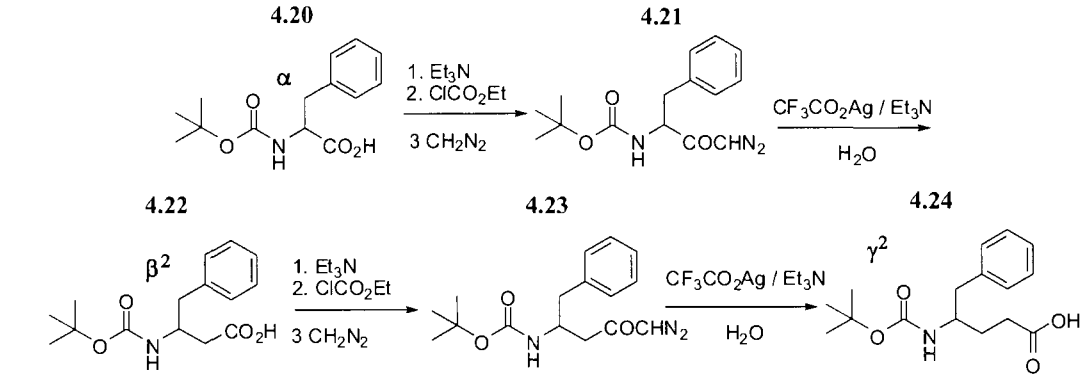
Figure 4.6. γ^2-Peptide synthesized and investigated for stability by Seebach et al.[9]

experiments demonstrated that peptidomimetic compounds containing γ-amino acids were capable of mimicking the conformations of natural peptides which allow the peptidomimetics to bind to natural receptors. This is a significant find due to the pharmacological properties of somatostatin analogues (see Chapter I).[11-14] The stability of γ-peptides combined with the ability to bind to natural receptors, make theses compounds promising peptidomimetic compounds.

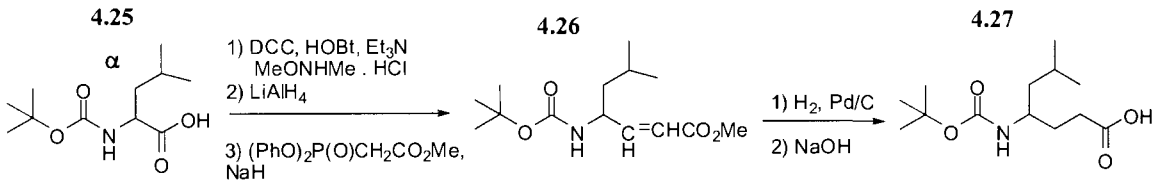
Synthesis of γ-Amino Acids

There are fewer synthetic methodologies in the literature for γ-amino acids than for α-methyl and β-amino acids. These methods include but are not limited to chemoenzymatic routes,[6,15,16] transition metal catalytic routes,[17,18] and multiple synthetic routes that make use of compounds from the chiral pool to generate γ-amino acids.[3,19] One method that has influenced the work of our group involves homolongation of N-Boc protected α-amino acids using double Ardnt-Eistert syntheses and Wolff rearrangements to produce a γ-amino acid (Scheme 4.1A).[20] While this homolongation method worked well for the synthesis of β-amino acids (Chapter III),[21] use of a second Ardnt-Eistert / Wolff rearrangement resulted in low yields of the γ-amino acids (25%). This was primarily due to the formation of a cyclic imino anhydride which is less reactive toward nucleophiles than the mixed anhydride. As a more productive alternative, the same group was able to get reasonable yield through the conversion of Boc-protected α-amino acids into Weinreb amides. The Weinreb amides were reduced to α-amino aldehydes and converted into α,β-unsaturated γ-amino methyl esters *via* a Witting type reaction, which results in an α,β-unsaturated diester (**4.26**). The olefin functionality was reduced with

Pd/C and the methyl ester was saponified, which yielded the N-Boc γ-amino acid in good yields (Scheme 4.1, 55-72% yield).[20]



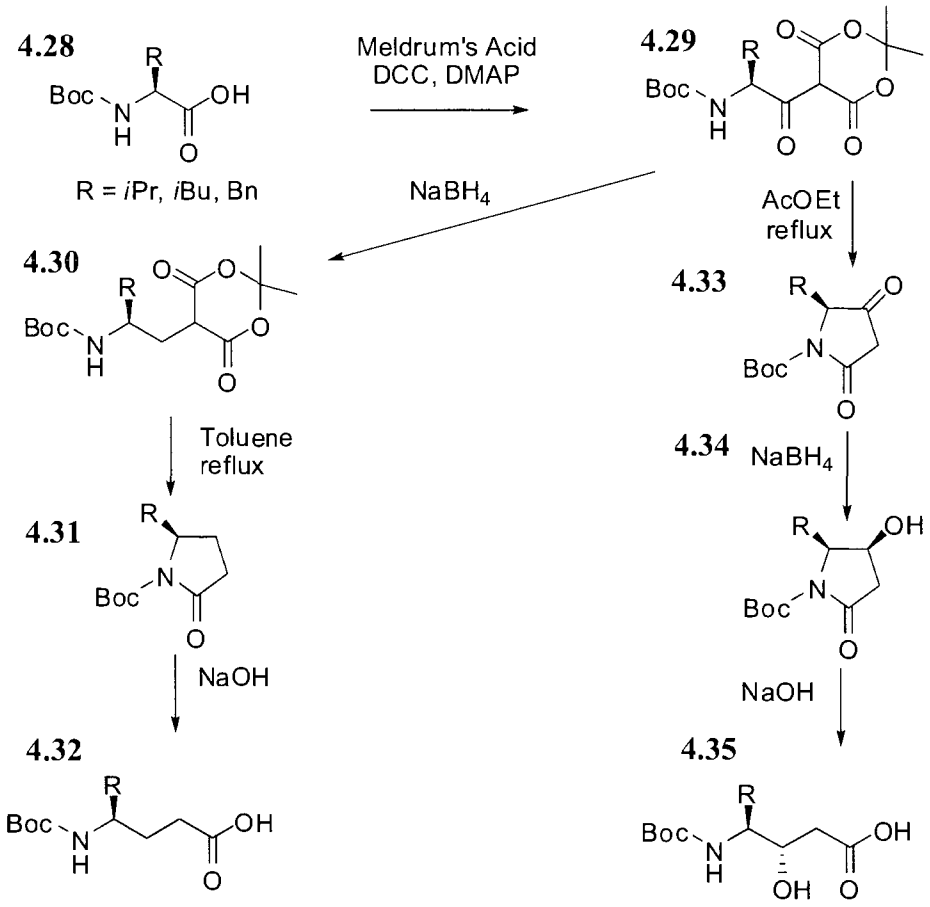
A) Double Arndt-Eistert / Wolff Synthesis of γ-Phenylalanine



B). Witting reaction to synthesize N-Boc γ-leucine

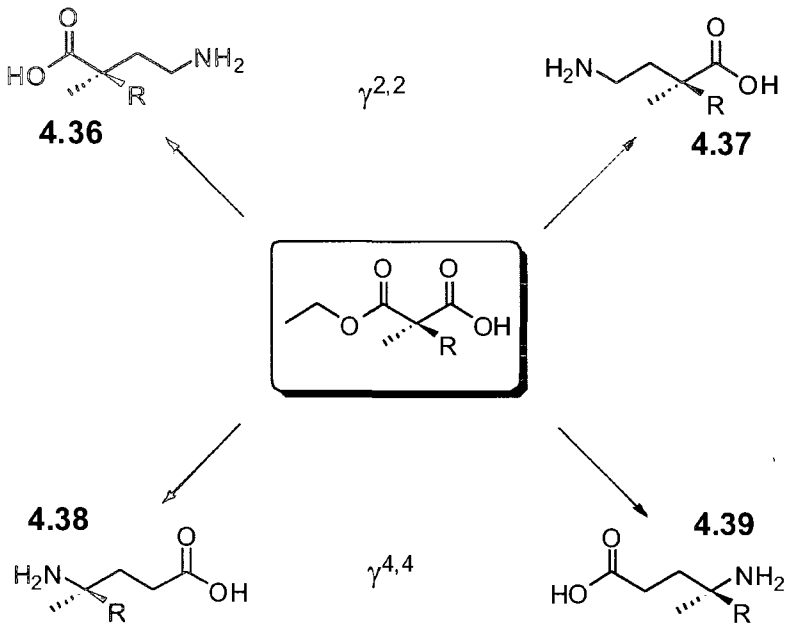
Scheme 4.1. Two syntheses of γ-amino acids from α-amino acids

Another popular route to the synthesis of γ-amino acids by elongation of a N-Boc α-amino acids was developed by Smrcina et al. (Scheme 4.2).[22] This method uses an α-amino acyl Meldrum's acid derivative (**4.29**), which can be heated to accomplish the decarboxylative ring closure to the N-Boc lactam (**4.31**). The lactam can then be hydrolyzed to give the N-Boc γ-amino acid (**4.32**). By altering the sequence of reduction and decarboxylative ring closure, a 3-hydroxy N-Boc γ-amino acid can be synthesized (**4.35**). The biological activity, stability to proteolytic degradation, and unique structural characteristics stability of γ-peptides denote the need for efficient syntheses of γ-amino acids.



Scheme 4.2 . Homolongation of an α-amino acid to a γ-amino acid using Meldrum's acid

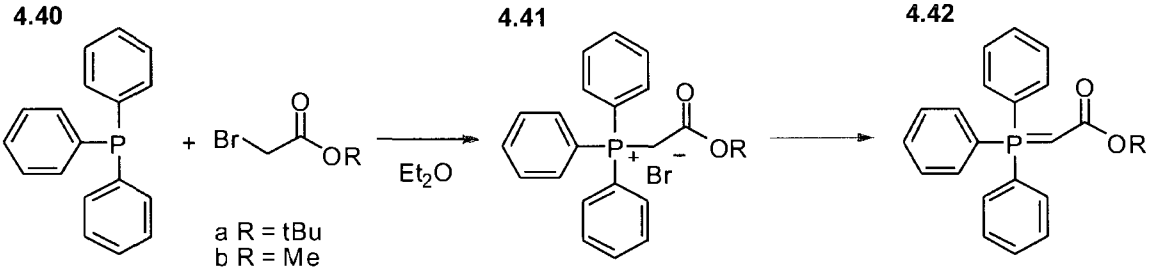
It is clear from the literature review above that there are many different methods currently used to synthesize γ-amino acids. However there are no methods currently available to synthesize the $\gamma^{2,2}$- and $\gamma^{4,4}$- class of unnatural amino acids. It is the intention of this work to contribute to the current synthetic methodology by providing an efficient method to synthesis both enantiomers of $\gamma^{2,2}$- and $\gamma^{4,4}$-tyrosine analogues. The same common malonate half-ester intermediate that was used in the Chapters 2 and 3 was used to synthesize the desired γ-amino acids. It is demonstrated that the use of the Wittig reaction to homologate the malonate half esters (**2.32b** and **2.38b**) yields intermediates that can be converted into both enantiomers of the desired amino acids.[23] The synthetic methodology developed in this chapter will eventually be used to synthesize amino acid analogues that will be incorporated into neurotensin analogues.



Scheme 4.3 . Generation of both enantiomers of $\gamma^{2,2}$-and $\gamma^{4,4}$-amino acids from a common malonate half ester intermediate

Results and Discussion

Like the previous chapters, our synthetic plan was to synthesize both enantiomers of the $\gamma^{2,2}$- and $\gamma^{4,4}$-amino acids using the same common intermediate (**2.32b**) that was used to synthesize both enantiomers of the α-methyl, $\beta^{2,2}$-, and $\beta^{3,3}$-amino acids (Chapters II and III). The γ-amino acids discussed in this section will be synthesized using familiar chemistry that was also used for the synthesis of the α-methyl, $\beta^{2,2}$-, and $\beta^{3,3}$-amino acids (protecting group chemistry and the Curtius rearrangement).[2] It is important to note that the compounds in this chapter are synthesized using racemic half-esters. As explained in Chapter III, the use of **+/-2.32b** is due to the difficulty in obtaining large enough quantities of **2.32b** for parallel synthesis. For the purpose of clearly illustrating my synthetic strategy, the major enantiomer of **2.32b** obtained from the PLE hydrolysis has been used to demonstrate the products that should be obtained in the following synthesis. The synthesis of the γ-tyrosine series was accomplished by conversion of **2.32b** was to a mixed anhydride by treatment with methyl chloroformate

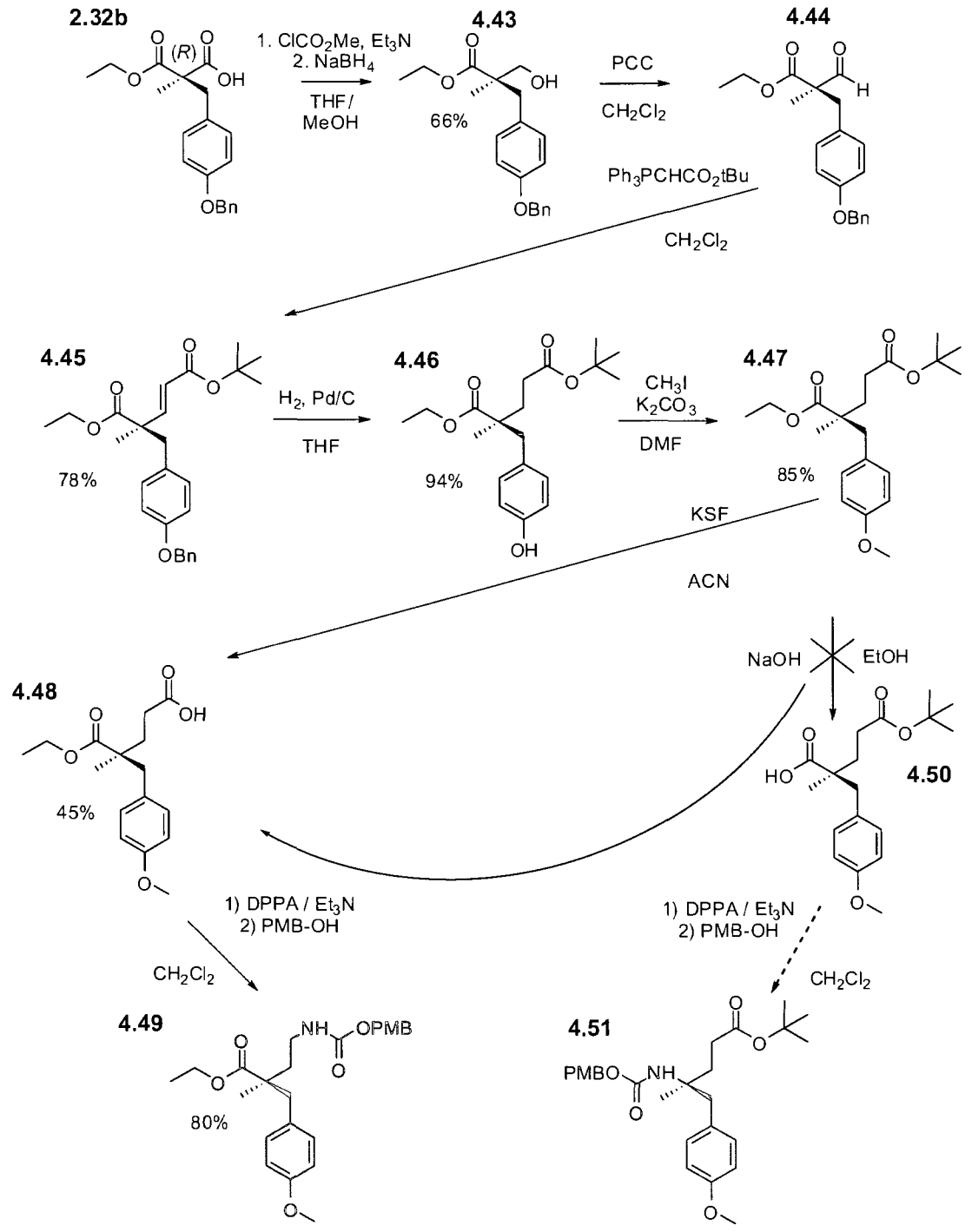


Scheme 4.4 . Synthesis of the needed Wittig reagents

(MC). This

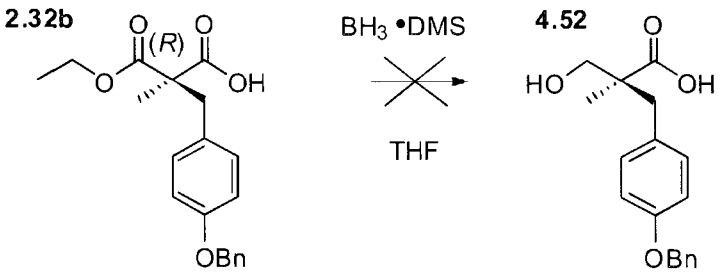
mixed anhydride was reduced to **4.43** using $NaBH_4$ with slow addition of methanol.[24]

The alcohol ester (**4.43**) was oxidized to **4.44** (66% yield) using a standard PCC oxidation. The pyridinium chlorochromate (PCC) was removed by filtration through silica and **4.44b** was used without further purification due to the instability of the aldehyde.[8,24] To facilitate the Wittig reaction, **4.40A** (synthesized in house, Scheme 4.4) was added to **4.44** and allowed to reflux for 5 days to give 78% yield.[23,25,26] The reaction was fairly slow and sterics are thought to be the largest factor in the slow rate of the reaction. The resulting α,β-unsaturated diester (**4.45**) was reduced using palladium that was absorbed onto amorphous carbon, under a hydrogen atmosphere to give **4.46** in 94% yield. The free phenol functional group of **4.46** was converted to a methyl-phenyl ether by treatment with methyliodide to give **4.47** (85% yield). To synthesize the (*R*)-$\gamma^{2,2}$-tyrosine, **4.47** was treated with KSF montmorillonite clay in refluxing acetonitrile to give **4.48** in 45% yield.[27] A subsequent Curtius rearrangement generated an isocyanate that was quenched with PMB-OH to give **4.49** (80% yield). In an attempt to synthesize the (*S*)-$\gamma^{4,4}$-tyrosine, **4.32** was treated with NaOH to saponify the ethyl ester. Unfortunately, the product of the saponification was **4.48** instead of **4.50**. The *tert*-butyl ester seems to be of sufficient distance from the neopentyl backbone of **4.47** to allow the *tert*-butyl ester to be saponified at a faster rate than the ethyl ester. This was confirmed by repetition of the experiment which resulted in the same outcome. Another route will have to be developed to obtain the (*S*)-$\gamma^{4,4}$-tyrosine (**4.51**). This alternate route is discussed in Chapter VI of this dissertation.

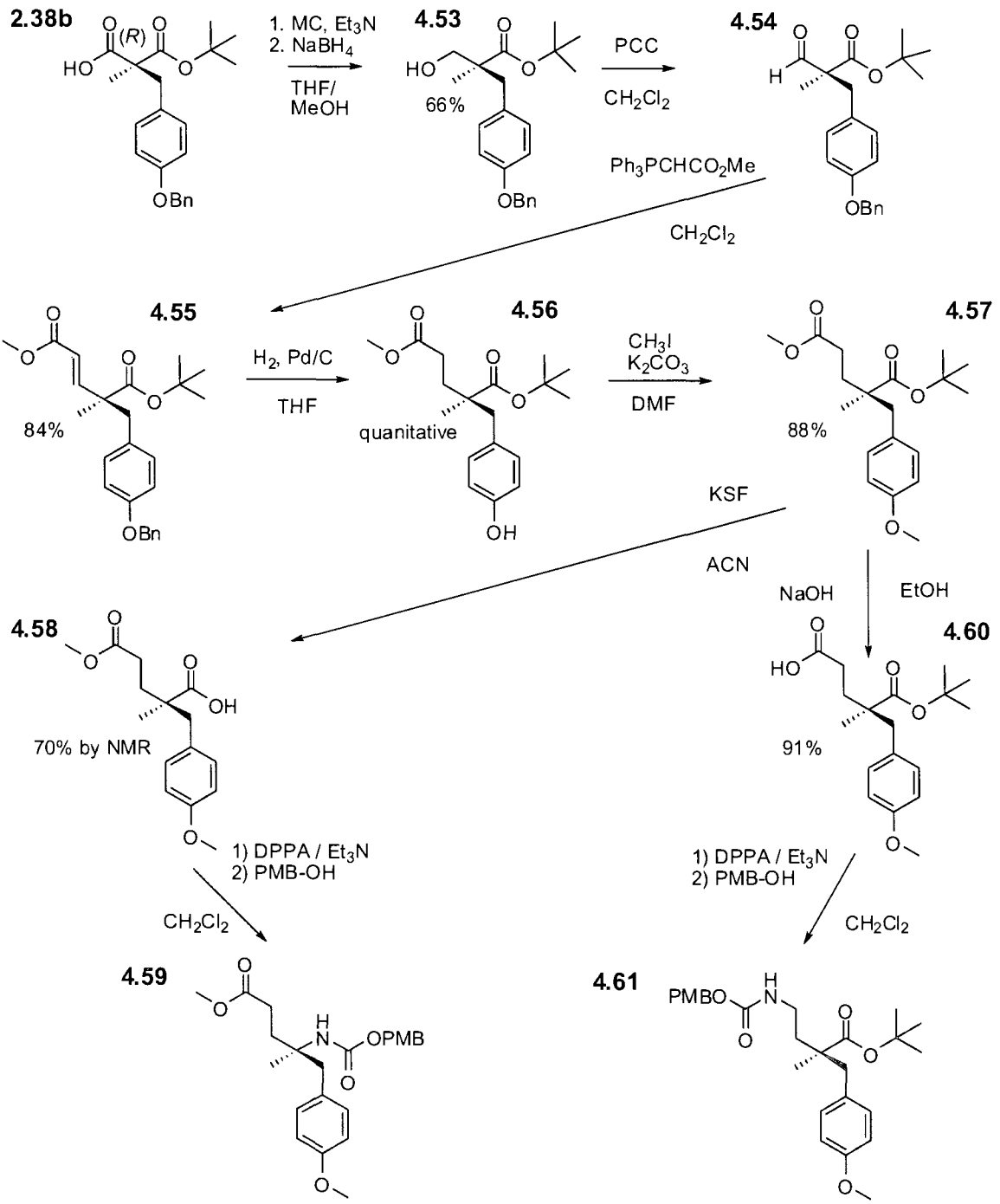


Scheme 4.5 . Generation of one enantiomer of $\gamma^{2,2}$ -tyrosine (**4.49**) and the attempted synthesis of one enantiomer of $\gamma^{4,4}$-tyrosine (**4.51**) from a common malonate half ester intermediate.

The (*S*)-$\gamma^{2,2}$-tyrosine and (*R*)-$\gamma^{4,4}$-tyrosine were synthesized in a similar methodology to the synthesis in the previous paragraph. To synthesize the necessary ester alcohol we first attempted a borane reduction of the ester functionality of **2.32b** (Scheme 4.6).[24] The free ester can then be esterified with methyl iodide to give the needed ester-alcohol. Although our group has successfully reduced half-ester *via* this method before, a mixture of five products was obtained from this reaction with **2.32b**. Rather than pursue a method that required a tedious purification, another route to the needed ester alcohol was taken. Compound **2.38b** was reduced to **4.53** in 66% yield by conversion to a mixed anhydride with subsequent reduction using $NaBH_4$. Compound **4.53** was oxidized to the ester-aldehyde (**4.54**) by mean of a PCC oxidation. Treatment with Wittig reagent (**4.42b**) yielded **4.55** (84% yield). Compound **4.55** was reduced to **4.56.** Saponification of **4.60** with NaOH produces **4.60** (91% yield). When **4.60** is subjected to a Curtius rearrangement, the result is **4.61** (61% yield). When the *tert*-butyl ester is hydrolyzed with KSF clay, the result is **4.58**. This compound is converted to **4.62** in by means of a Curtius rearrangement that was quenched with PMB-OH.



Scheme 4.6. Reduction of half ester **2.32b** to an acid alchohol **4.52** was successful, but as a mixture of 5 products



Scheme 4.7 . Generation of the other enantiomer of $\gamma^{2,2}$ -tyrosine (**4.61**) and one enantiomer of $\gamma^{4,4}$-tyrosine (**4.59**) from a common malonate half ester intermediate.

Conclusions

The biological activity and proteolytic stability of γ-amino acids and of compounds composed of or containing γ-amino acids makes this class of amino acids interesting pharmacores. The desire to study the properties of γ-amino acids has led to the development of several unique syntheses. However, each class of γ-amino acid requires a different synthetic strategy. The synthesis detailed in this chapter clearly demonstrates that a combination of the Wittig reaction, the Curtius rearrangement, and protecting group chemistry can be used to synthesize the $\gamma^{2,2}$- and $\gamma^{4,4}$-tyrosine analogues from a common synthetic intermediate in good yields. Although the amino acids synthesized in this chapter were synthesized racemically, one can clearly see that the methodology can be applied to the synthesis of enantiomerically enriched γ-amino acids. Furthermore, the Wittig reaction is known to proceed without scrambling of the stereochemistry of α-amino aldehydes and we hypothesize that the same result will be seen when applied to our substrates.[20] The conclusion for this chapter illustrates the synthetic potential of the malonate half-esters. In previous chapters (Chapters 2 and 3), it has been shown that the malonate half-ester intermediate (**2.32b**) can be transformed to both enantiomers of α-methyl, $\beta^{2,2}$-,$\beta^{3,3}$-tyrosine analogues using a common intermediate. In this chapter, It has been demonstrated that the same intermediate (**2.32b**) and both enantiomers of the $\gamma^{2,2}$- and one enantiomer of the $\gamma^{4,4}$-tyrosine analogues using known protecting group chemistry and functional group transformations (Ardnt-Estert, Wolff Reaction, and Wittig Reaction). The future directions section of this dissertation (Chapter 6) will discuss a potential method that may the used to synthesize the (*S*)-$\gamma^{4,4}$-tyrosine analogue.

It is our hope that peptidomimetic compounds containing these unnatural amino acids can be synthesized and will display unique biological properties.

Experimental

See Chapter II (pgs 43 to 44) for general experimental. The Wittig salts were prepared in house *via* a literature procedure using triphenyl phosphine and the appropriate α-bromoester.[26] Treatment of the Wittig salt with aqueous NaOH yielded the desired Wittig reagents. Chiral HPLC separation of the γ-amino acids was not successful with the available OJ-H or AD-H chiral columns.

(±)-Ethyl 2-(4-(benzyloxy)benzyl)-3-hydroxy-2-methylpropanoate (4.43): An amount of 5.53 g (16.2 mmol) of **2.32b** was dissolved in 100 mL of anhydrous THF and 1.30 mL (17.01 mmol) of methyl chloroformate was added at 0 °C. The reaction stirred for 60 minutes followed by addition of 1.84 mL (24.30 mmol) of Et_3N and stirring for an additional 40 minutes. Then, 1.84 g of $NaBH_4$ was added and the solution was allowed to stir for an additional 20 minutes at 0 °C. Addition of 10 mL of MeOH took place over 30 minutes and the reaction was allowed to slowly warm to room temperature overnight. Cold 10% HCl was added at 0 °C to quench the excess $NaBH_4$. The reaction was diluted with 100 mL of Et_2O, washed three times with 10% HCl, dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give a clear, viscous liquid that was purified by flash chromatography with 50% Et_2O / 50% hexane and 400 mL of silica gel to give 3.50 g (10.7 mmol, 66% yield) of a clear, viscous liquid. TLC (50% Et_2O / 50% hexane) R_f = 0.18, IR (cm^{-1}) 3885, 1716. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.10 (3H, s), 1.24 (3H, t, J = 7 Hz), 2.50 (1H, bs), 2.82 (1H, d, J = 14 Hz), 2.91 (1H, d, J = 14 Hz), 3.52 (1H, d, J = 11

Hz), 3.57 (1H, d, J = 11 Hz), 4.15 (2H, q, J = 7 Hz), 5.03 (2H, s), 6.88 (2H, d, J = 9 Hz), 7.08 (2H, d, J = 9 Hz), 7.38 (5H, m). (75 MHz, $CDCl_3$) 14.3, 19.5, 41.3, 48.8, 61.0, 70.13, 114.58, 127.7, 128.1, 128.8, 129.1, 131.5, 157.7, 177.1. HRMS: $[C_{20}H_{24}O_4Na]^+$ calcd = 351.1567, obsd = 351.1562.

(±)-1-*tert*-Butyl 5-ethyl 4-(4-benzyloxy)benzyl)-4-methylpent-2-enedioate (4.45): An amount of 3.37 g (10.2 mmol) of **4.33** and 2.65 g (12.31 mmol) of PCC were dissolved in 50 mL of dry CH_2Cl_2 at room temperature. Conversion of the alcohol functionality to an aldehyde was complete after 48 hours by TLC. The reaction was filtered through a silica plug. Then, 4.63 g (12.31 mmol) of *tert*-butyl triphenyl phosphonate was added to the resulting solution. The solution was heated to reflux solvent for 72 hours, then filtered through a silica plug and purified by flash chromatography to give 3.39 g (7.99 mmol, 78% yield) of a clear, viscous oil. TLC (15% Et_2O / 85% hexane) R_f = 0.15, IR (cm^{-1}) 1710. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.23 (3H, t, J = 7 Hz), 1.26 (3H, s,), 1.49 (9H, s), 2.83 (1H, d, J = 13 Hz), 3.05 (1H, d, J = 13 Hz), 4.14 (2H, m), 5.02 (2H, s), 5.72 (1H, d, J = 16 Hz), 6.86 (2H, d, J = 9 Hz), 7.01 (2H, d, J = 9 Hz), 7.08 (1H, d, J = 16 Hz), 7.38 (5H, m) ^{13}C-NMR (75 MHz, $CDCl_3$) 14.4, 20.4, 28.3, 44.6, 49.8, 61.4, 70.1, 80.7, 114.6, 122.4, 127.7, 128.1, 128.7, 128.8, 131.5, 137.2, 149.7, 157.9, 165.9, 174.1.$[C_{26}H_{32}O_5Na]^+$ calcd = 447.2142, obsd = 447.2138.

(±)-5-*tert*-Butyl-1-ethyl 2-(4-hydroxybenzyl)-2-methylpentanedioate (4.46): An amount of 3.39 g (7.99 mmol) of 4.46 was dissolved in 40 mL of distilled THF and 0.50 g of Pd/C was added as a THF slurry. The solution was degassed with H_2 and allowed to

stir over an H_2 atmosphere. The reaction was monitored by TLC and was complete after 48 hours. The Pd/C was removed by filtration through a Celite plug and the solvent was removed by rotary evaporation to give 2.52 g (7.49 mmol, 94% yield) a clear viscous liquid. R_f = 0.09 (50% Et_2O / 50% hexane), IR (cm^{-1}) 3406, 1724, 1698. ^{1}H-NMR (300MHz, $CDCl_3$) 1.08 (3H, s,), 1.25 (3H, t, *J* = 7 Hz), (9H, s),1.71 (1H, m), 2.06 (1H, m), 2.24 (2H, m), 2.67 (1H, d, *J* = 13 Hz), 2.91 (1H, d, *J* = 13 Hz), 4.12 (2H, m), 6.08 (1H, bd, *J* = 62 Hz), 6.66 (2H, d, *J* = 9 Hz), 6.94 (2H, d, *J* = 9 Hz). ^{13}C-NMR (75MHz, $CDCl_3$) 14.3, 20.8, 28.3, 31.4, 33.8, 45.0, 47.0, 60.8, 80.9, 115.1, 128.9, 131.4, 155.0, 173.5, 176.8. HRMS: $[C_{19}H_{28}O_5Na]^+$ calcd = 359.1829, obsd = 359.1827.

(±)-5-*tert*-Butyl 1-ethyl 2-(4-methoxybenzyl)-2-methylpentanedioate (4.47): An amount of 2.52 g (7.49 mmol) of **4.46** was dissolved in 20 mL of anhydrous DMF followed by addition of 1.55 g (11.23 mmol) of anhydrous K_2CO_3 and dropwise addition of 701 µL (11.2 mmol) of CH_3I. The reaction was allowed to stir for 48 hours, was diluted with Et_2O, washed with ddH_2O, dried over $MgSO_4$, and concentrated by rotary evaporation to give 2.24 g (6.93 mmol, 85 % yield) of a clear viscous oil. R_f = 0.37 (1:1 Et_2O : hexane), IR (cm^{-1}) 1723. ^{1}H-NMR (300MHz, $CDCl_3$) 1.07(3H, s,), 1.23 (3H, t, *J* = 7 Hz), 1.42 (9H, s), 1.71 (1H, m), 2.03 (1H, m), 2.31 (2H, m), 2.65 (1H, d, *J*= 13 Hz), 2.75 (1H, d, *J* = 13 Hz), 3.76 (3H, s), 6.78 (2H, d, *J* = 9 Hz), 7.01 (2H, d, *J* = 9 Hz). ^{13}C-NMR (75MHz, $CDCl_3$) 14.4, 20.8, 28.2, 31.3, 34.0, 44.8, 46.9, 55.3, 60.6, 80.4, 113.6, 129.5, 131.3, 158.4, 172.9, 176.3. HRMS: $[C_{20}H_{30}O_5Na]^+$ calcd = 373.1985, obsd = 373.1984.

(±)-5-ethoxy-4-(4-methoxybenzyl)-4-methyl-5-oxopentanoic acid (4.48): In a 25 mL roundbottomed flask 0.50 g (1.43 mmol) of **4.47** was dissolved in 15 mL of dry CH_3CN and 1 mL of water was added followed by addition of 1.0 g of KSF Montmorillonite clay. The resulting solution was heated to reflux solvent and the reaction was determined to be complete after 4 days by TLC. The KSF clay was removed by vacuum filtration and the solution was concentrated by rotary evaporation to give a clear viscous oil that was purified by flash chromatography to give 0.24 g (0.65 mmol, 45% yield) a clear viscous liquid. TLC (5% MeOH / 95% $CHCl_3$, Rf = 0.58), IR (cm^{-1}) 1706. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.09 (3H, s), 1.22 (3H, t, J = 7 Hz), 1.73 (1H, m), 2.09 (1H, m), 2.36 (2H, m), 2.67 (1H, d, J = 14 Hz), 2.94 (1H, d, J = 14 Hz), 3.67 (3H, s) 4.11 (2H, q, J = 7 Hz), 6.79 (2H, d, J = 9 Hz), 7.00 (2H, d, J = 9 Hz), 9.14 (1H, bs). ^{13}C-NMR (75MHz, $CDCl_3$) 14.3, 20.8, 30.0, 33.5, 44.7, 46.8, 55.3, 60.8, 113.6, 129.2, 131.2, 158.4, 176.2, 179.5. HRMS: $[C_{16}H_{22}O_5Na]^+$ calcd = 317.1359, obsd = 317.1355.

(±)-Ethyl 2-(4-methoxybenzyl)-4-((4-methoxybenzyloxy)carbonylamino)-2-methylbutanoate (4.49): An amount of 0.24 g (0.82 mmol) of **4.48** was dissolved in 20.00 mL of CH_2Cl_2 in a 50 mL RBF with a magnetic stirbar. 193 µL (0.90 mmol) of DPPA and 368 µL of Et_3N (2.45 mmol) was added and the reaction was allowed to stir for 30 minutes. The solution was brought to reflux solvent for 3hours, at which time 152 µL (1.22 mmol) of PMB-OH was added and the solution was heated to reflux solvent. The solution was concentrated by rotary evaporation and the resulting mixture was purified by flash chromatography (200 mL of silica gel) to give 0.28 g (0.65 mmol, 80% yield) of a clear, viscous oil. TLC (50% EtOAc / 50% hexane) R_f = 0.40. IR (cm^{-1}) 3366,

1712. ^{1}H-NMR ($CDCl_3$) 1.13 (3H, s), 1.21 (3H, t, J = 7.2 Hz), 1.58 (2H,s), 1.97 (2H, s), 2.68 (1H, d, J = 13 Hz), 2.90 (1H, d, J = 13 Hz), 3.21 (2H, m), 3.77 (3H,s), 3.80 (3H, s), 4.08 (2H, q, J =7.1 Hz), 4.75 (1H, bs), 5.01 (2H, s), 6.79 (2H, d, J = 9 Hz), 6.88 (2H, d, J = 9 Hz), 7.00 (2H, d, J = 9 Hz), 7.28 (2H, d, J = 9 Hz). ^{13}C-NMR (75MHz, $CDCl_3$) 14.4, 21.0, 37.6, 38.7, 45.2, 46.1, 55.4, 60.8, 113.6, 114.0, 128.8, 129.2, 130.2, 131.3, 156.45, 158.5, 159.7, 176.7. HRMS: $[C_{24}H_{31}NO_5Na]^+$ calcd = 452.2044, obsd = 452.2042.

(±)-*tert*-Butyl 2-(4-(benzyloxy)benzyl)-3-hydroxy-2-methylpropanoate (4.53): An amount of 2.16 g (5.83 mmol) of **2.38b** was dissolved in 20 mL of anhydrous THF and 471 µL (6.12 mmol) of methyl chloroformate and 1.32 mL (8.75 mmol) of Et_3N was added at 0 °C. The reaction stirred for 2 hours followed by addition of 0.61 g of $NaBH_4$. The reaction was then allowed to stir for 41 hours at room temperature. However, the reaction had not proceeded. Then, 5 mL of EtOH was added over 1 hour. The reaction was complete after 24 hours by TLC. Cold 10% HCl was added at 0 °C to quench the excess $NaBH_4$. The reaction was diluted with 30 mL of Et_2O, washed three times with 10% HCl, dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give a clear, viscous liquid that was purified by flash chromatography with 50% Et_2O / 50% hexane and 400 mL of silica gel to give 3.50 g (10.7 mmol, 66% yield) of a white amorphous solid. Mp = 71 – 74 °C. TLC (30% EtOAc / 70% hexane) R_f = 0.36, IR (cm^{-1}) 3459, 3378, 1723, 1711. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.05 (3H, s), 1.45 (3H, s), 2.60 (1H, t, J = 7 Hz), 2.77 (1H, d, J= 14 Hz), 2.91 (1H, d, J = 14 Hz), 3.49 (2H, d, J = 7 Hz), 5.03 (2H, s), 6.88 (2H, d, J = 9 Hz), 7.11 (2H, d, J = 9 Hz), 7.39 (5H, m). (75 MHz,

$CDCl_3$) 19.7, 28.2, 40.2, 49.0, 67.2, 70.1, 81.4, 114.5, 127.7, 128.1, 131.7, 137.2, 157.7, 176.7. HRMS: $[C_{22}H_{28}O_4Na]^+$ calcd = 379.1880, obsd = 379.1878.

(±)-5-*tert*-Butyl 1-methyl 4-(4-(benzyloxy)benzyl)-4-methylpent-2-enedioate (4.55): An amount of 0.95 g (2.67 mmol) of **4.53** and 0.69 g (3.20 mmol) of PCC were dissolved in 25 mL of dry CH_2Cl_2 at room temperature. Conversion of the alcohol functionality to an aldehyde was complete after 48 hours by TLC. The reaction was filtered through a silica plug and 0.89 g (3.20 mmol) of methyl triphenyl phosphonate was added to the resulting solution. The solution was heated to reflux solvent for 72 hours, then an additional 0.89 g of methyl triphenyl phosphonate and 840 µL of diisopropyl ethylamine were added and the reaction was allowed to reflux for an additional 48 hours. The reaction solution was filtered through a silica plug and purified by flash chromatography to give 0.92 g (2.24 mmol, 84% yield) a clear, viscous oil. TLC (10% Et_2O / 90% hexane) R_f = 0.17, IR (cm^{-1}) 1720. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.23 (3H, s,), 1.43 (9H, s), 2.87 (1H, d, J = 13 Hz), 3.00 (1H, d, J = 13 Hz), 3.75 (3H, s), 5.03 (2H, s), 5.78 (1H, d, J = 16 Hz), 6.87 (1H, d, J = 9 Hz), 7.04 (1H, d, J = 9 Hz), 7.18 (1H, d, J = 16 Hz), 7.39 (5H, m) ^{13}C-NMR (75 MHz, $CDCl_3$) 20.5, 28.1, 44.4, 50.4, 51.8, 70.1, 81.8, 114.5, 120.0, 127.8, 128.1, 128.7, 128.9, 131.6, 137.2, 151.7, 157.8, 167.1, 173.2. $[C_{25}H_{30}O_5Na]^+$ calcd = 433.1985, obsd = 433.1982.

(±)-5-*tert*-Butyl 1-methyl 2-(4-hydroxybenzyl)-2-methylpentanedioate (4.56): An amount of 0.92 g (2.24 mmol) of **4.55** was dissolved in 30 mL of distilled THF and 0.50 g of Pd/C was added as a THF slurry. The solution was degassed with H_2 and allowed to

stir over an H_2 atmosphere. The reaction was monitored by TLC and was complete after 48 hours. The Pd/C was removed by filtration through a Celite plug and the solvent was removed by rotary evaporation to give 0.75 g (quantitative) of a clear viscous liquid. R_f = 0.18 (50% Et_2O / 50% hexane), IR (cm^{-1}) 3411, 1712. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.04 (3H, s), 1.44 (9H, s), 1.71 (1H, m), 2.06 (1H, m), 2.31 (2H, m), 2.64 (1H, d, J = 14 Hz), 2.89 (1H, d, J = 14 Hz), 3.68 (3H, s), 6.69 (1H, d, J = 9 Hz), 6.99 (1H, d, J = 9 Hz) ^{13}C-NMR (75MHz, $CDCl_3$) 20.9, 28.2, 30.3, 34.0, 44.8, 47.3, 52.0, 81.1, 115.0, 129.1, 131.6, 154.9, 174.6, 175.9. $[C_{18}H_{26}O_5Na]^+$ calcd = 345.1672, obsd = 345.1671.

(±)-1-*tert*-Butyl 5-methyl 2-(4-methoxybenzyl)-2-methylpentanedioate (4.57): An amount of 0.72 g (2.24 mmol) of **4.56** was dissolved in 15 mL of acetone followed by addition of 0.31 g (2.24 mmol) of anhydrous K_2CO_3 and dropwise addition of 210 µL of CH_3I. The reaction was allowed to stir at reflux solvent for 48 hours, filtered to remove the K_2CO_3, and concentrated by rotary evaporation to give 0.66 g (1.96 mmol, 88% yield) of a clear viscous oil. TLC (50% Et_2O / 50% hexane) R_f = 0.32, IR (cm^{-1}) 1717, 1737. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.03 (3H, s,), 1.44 (9H, s), 1.70 (1H, m), 2.04 (1H, m), 2.64 (2H, m), 2.64 (1H, d, J = 14 Hz), 2.93 (1H, d, J = 14 Hz), 3.67 (3H, s), 3.78 (3H, s), 6.80 (2H, d, J = 9 Hz), 7.06 (2H, d, J = 9 Hz). ^{13}C-NMR (75 MHz, $CDCl_3$) 20.9, 28.2, 30.0, 44.6, 47.4, 51.9, 55.3, 80.8, 113.5, 129.6, 131.5, 158.4, 174.1, 175.5. HRMS: $[C_{19}H_{28}O_5Na]^+$ calcd = 359.1829, obsd = 359.1826.

(±)-5-Methoxy-2-(4-(methoxybenzyl)-2-methyl-5-oxopentanoic acid (4.58): In a 25 mL round-bottomed flask 0.66 g (1.66 mmol) of **4.57** was dissolved in 10 mL of dry

CH_3CN and 1 mL of water was added followed by addition of 1.0 g of KSF Montmorillonite Clay. The resulting solution was heated to reflux solvent. The reaction was monitored by TLC (50% Et_2O / 50% hexane) and was determined to be complete after 4 days. The KSF clay was removed by vacuum filtration, and the filtrate was washed with CH_3CN. The CH_3CN was concentrated by rotary evaporation to give a clear viscous oil that was purified by flash chromatography (5% MeOH / 95% $CHCl_3$, Rf = 0.55) to give 0.34 g (0.98 mmol, 59% yield) a white amorphous solid. IR (cm^{-1}) 1732, 1697. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.11 (3H, s), 1.77 (1H, m), 2.09 (1H, m), 2.39 (2H, m), 2.72 (1H, d, J = 14 Hz), 2.98 (1H, d, J = 14 Hz), 3.67 (3H, s), 3.78 (3H, s), 6.81 (2H, d, J = 9 Hz), 7.08 (2H, d, J = 9 Hz). ^{13}C-NMR (75MHz, $CDCl_3$) 20.7, 28.9, 33.7, 44.6, 46.9, 51.9, 55.4, 113.7, 129.0, 131.4, 158.6, 174.0, 182.4 HRMS: $[C_{15}H_{20}O_5Na]^+$ calcd = 303.1203, obsd = 303.1200.

(±)-*tert*-Butyl 4-((4-methoxybenzyloxy)carbonylamino)-5-(4-methoxyphenyl)-4-methylpentanoate (4.59): An amount of 0.11 g (0.39 mmol) of **4.58** was dissolved in 10 mL of methylene chloride. Then, 93 µL (0.47 mmol) of DPPA and 177 µL of Et_3N (1.18 mmol) was added and the solution was brought to reflux solvent for 1.5 hours, at which time 79.9 µL (1.5 eq, 0.64 mmol) of PMB-OH was added and the solution was heated to reflux solvent for 17 hours. The excess solvent was removed by rotorary evaporation followed by purification *via* flash chromatography (200 mL of silica gel, 50% Et_2O / 50% hexane) to give 0.088 g of a clear, viscous oil. TLC R_f = 0.42, Presence of **4.59** was detected by ^{1}H-NMR and ESI-MS ($[M + Na]^+$ m/z = 438). However, the oil was found to be composed primarily of the isocyanate formed from the Curtius rearrangement of **4.58**

($[M + Na]^+$ m/z = 300). This is evidence that the reaction was preceding, but longer times or a higher boiling point solvent may be required.

(±)-5-*tert*-Butoxy-4-(4-methoxybenzyl)-4-methyl-5-oxopentanoic acid (4.60): An amount of 0.37 g (1.09 mmol) of **4.57** was dissolved in 10 mL of EtOH and 0.13g (3.26 mmol) of NaOH was dissolved in 1 mL of water and added to the reaction solution. The reaction mixture was allowed to stir at room temperature for 48 hours. The solution was diluted with 15 mL water, washed three times with Et_2O, acidified with cold 10% HCl and, extracted into Et_2O. The resulting solution was dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give 0.32 g (0.99 mmol, 91% yield) of a clear, viscous, liquid. TLC (50% Et_2O / 50% hexane) R_f = 0.25. IR (cm^{-1}) 1707. ^{1}H-NMR (300MHz, $CDCl_3$) 1.05 (3H, s,), 1.44 (9H, s), 1.70 (1H, m), 2.05 (1H, m), 2.35 (2H, m) 2.65 (1H, d, J = 14 Hz), 2.92 (1H, d, J = 14 Hz), 3.78 (3H, s), 6.80 (2H, d, J = 9 Hz), 7.06 (2H, d, J = 9 Hz). ^{13}C-NMR (75MHz, $CDCl_3$) 21.0, 28.2, 30.1, 33.8, 44.6, 47.2, 55.4, 81.0, 113.5, 129.5, 131.5, 158.4, 175.5, 180.0. HRMS: $[C_{18}H_{26}O_5Na]^+$ calcd = 345.1672, obsd = 345.1672.

(±)-tert-Butyl 2-(4-methoxybenzyl)-4-((4-methoxybenzyloxy)carbonylamino)-2-methylbutanoate (4.61): An amount of 0.32 g (0.96 mmol) of **4.60** was dissolved in 10 mL of CH_2Cl_2 in a 50 mL RBF with a magnetic stirbar. Then, 228 µL (1.06 mmol) of DPPA and 435 µL of Et_3N (2.89 mmol) was added and the solution was stirred for 30 minutes at room temperature. The solution was heated to reflux solvent for 3 hours, at which time 179 µL (1.44 mmol) of PMB-OH was added and the solution was brought to

reflux solvent. The solution was diluted with 20 mL of CH_2Cl_2, filtered through a plug of silica gel, and concentrated by rotary evaporation to give a orange, transparent, viscous oil that was purified by flash chromatography (200 mL of silica gel) to give 0.27 g (0.59 mmol, 61% yield) of a clear, viscous oil. TLC (30% EtOAc / 70% hexane) $R_f = 0.27$. IR (cm^{-1}) 3358, 1709. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.08 (3H, s), 1.42 (9H, s), 1.54 (1H, m), 1.89 (1H, m), 2.65 (1H, d, J = 13 Hz), 2.87 (1H, d, J = 13 Hz), 3.20 (2H, m), 3.78 (3H, s), 3.80 (3H, s), 4.75 (1H, bs), 5.01 (2H, s), 6.79 (2H, d, J = 9 Hz), 6.87 (2H, d, J = 9 Hz), 7.04 (2H, d, J = 9 Hz), 7.28 (2H, d, J = 9 Hz). ^{13}C-NMR (75MHz, $CDCl_3$) 21.3, 28.2, 37.7, 38.9, 45.0, 46.5, 55.4, 55.5, 66.6, 81.0, 113.5, 114.1, 128.9, 129.4, 130.2, 131.5, 156.5, 158.5, 159.7, 175.9. HRMS: $[C_{26}H_{35}NO_6Na]^+$ calcd = 480.2357, obsd = 480.2355.

References

(1) Hanessian, S.; Luo, X.; Schaum, R.; Michnick, S. *Journal of the American Chemical Society* **1998**, *120*, 8569-8570.

(2) Masterson, D.; Roy, K.; Rosado, D.; Fouche, M. *Journal of Peptide Science* **2008**, *14*, 1151-1162.

(3) Trabocchi, A.; Guarna, F.; Guarna, A. *Current Organic Chemistry* **2005**, *9*, 1127-1153.

(4) Sun, I. C.; Chen, C. H.; Kashiwada, Y.; Wu, J. H.; Wang, H. K.; Lee, K. H. *Journal of Medicinal Chemistry* **2002**, *45*, 4271-4275.

(5) Hom, R. K.; Fang, L. Y.; Mamo, S.; Tung, J. S.; Guinn, A. C.; Walker, D. E.; Davis, D. L.; Gailunas, A. F.; Thorsett, E. D.; Sinha, S.; Knops, J. E.; Jewett, N. E.; Anderson, J. P.; John, V. *Journal of Medicinal Chemistry* **2003**, *46*, 1799-1802.

(6) Gou, D. M.; Liu, Y. C.; Chen, C. S. *The Journal of Organic Chemistry* **2002**, *58*, 1287-1289.

(7) Dworkin, R. H.; Kirkpatrick, P. *Nat Rev Drug Discov* **2005**, *4*, 455-456.

(8) Hintermann, T., Gademann, K.; Juan, B.; Seebach, D. *Helvetica Chimica Acta* **1998**, *81*, 983-1002.

(9) Frackenpohl, J.; Arvidsson, P.; Schreiber, J; Seebach, D. *ChemBioChem* **2001**, *2*, 445-455.

(10) Dieter, S.; Laurent, S.; Meinrad, B.; Daniel, H. *Angewandte Chemie International Edition* **2003**, *42*, 776-778.

(11) Chabry, J.; Botto, J.-M.; Nouel, D.; Beaudet, A.; Vincent, J.-P.; Mazella, J. *Journal of Biological Chemistry* **1995**, *270*, 2439-2442.

(12) Chabry, J.; Gaudriault, G.; Vincent, J.; Mazella, J. *Journal of Biological Chemistry* **1993**, *268*, 17138-17144.

(13) Lamberts, S.; van der Lely, A.; Hofland, L. *European Journal of Endocrinology* **2002**, *146*, 701-705.

(14) Tomassetti, P.; Migliori, M.; Caletti, G. C.; Fusaroli, P.; Corinaldesi, R.; Gullo, L. *New England Journal of Medicine* **2000**, *343*, 551-554.

(15) Forro, E.; Fulop, F. *European Journal of Organic Chemistry* **2008**, 5263-5268.

(16) Felluga, F.; Pitacco, G.; Valentin, E.; Venneri, C. D. *Tetrahedron Assymmetry* **2008**, *19*, 945-955.

(17) Enders, D.; Wang, C.; Bats, J. W. *Angewandte Chemie International Edition* **2008**, *47*, 7539-7542.

(18) Deng, J.; Duan, Z.-C.; Huang, J.-D.; Hu, X.-P.; Wang, D.-Y.; Yu, S.-B.; Xu, X.-F.; Zheng, Z. *Organic Letters* **2007**, *9*, 4825-4828.

(19) Aquilera, J.; Gutierrez-Abad, R.; Mor, A.; Moglioni, A. G.; Y., M. G.; Ortunu, R. M. *Tetrahedron Asymmetry* **2008**, *19*, 2864-2869.

(20) Hintermann, T.; Gademann, K.; Jaun, B.; Seebach, D. *Helvetica Chimica Acta* **1998**, *81*, 983-1002.

(21) Kirmse, W. *European Journal of Organic Chemistry* **2002**, 2193-2256.

(22) Smrcina, M.; Majer, P.; Majerova, E.; Guerassina, T. A.; Eissenstat, M. A. *Tetrahedron* **1997**, *53*, 12867-12874.

(23) Smith, M. B.; March, J. *March's Organic Chemistry: Reactions, Mechanisms, and Structure*; 5th ed.; John Wiley & Sons: New York, NY, 2001.

(24) Fadel, A.; Garcia-Argote, S. *Tetrahedron Assymmetry* **1996**, *7*, 1159-1166.

(25) Corey, H. S.; McCormick, J. R. D.; Swensen, W. E. *Journal of the American Chemical Society* **1964**, *86*, 1884-1885.

(26) Tran, Y. S.; Kwon, O. *Organic Letters* **2005**, *7*, 4289-4291.

(27) Yadav, J. S.; Subba Reddy, B. V.; Sanjeeva Rao, K.; Harikishan *Synlett* **2002**, *5*, 826-828.

CHAPTER V

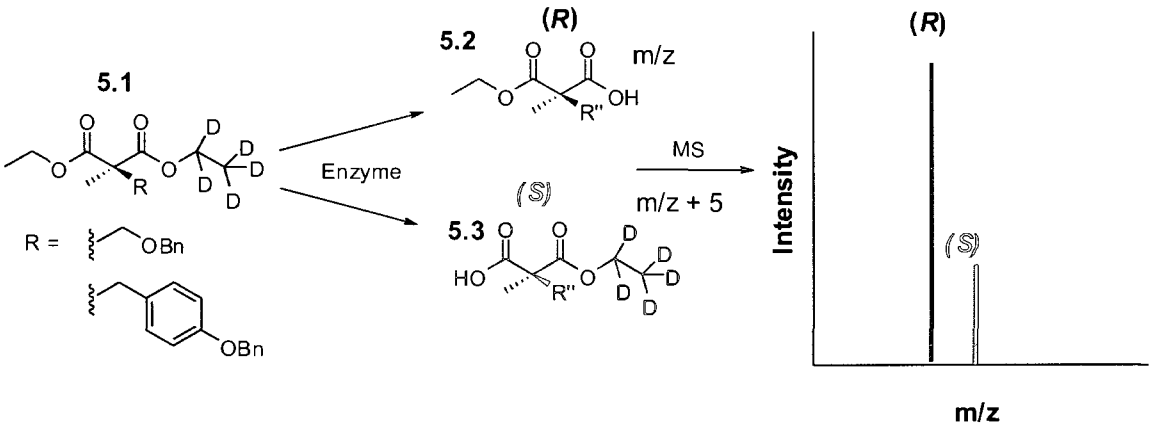
MASS SPECTROMETRY BASED ENANTIOSELECTIVITY ASSAY FOR MALONATE DIESTERS

Literature Review

Introduction

Enzymes display great utility for use as synthetic tools. This was exemplified by the cover story of a 2006 issue of Chemical and Engineering News titled: "Enzymes at Work".[1] This article highlights the advantages of the few enzymes that are currently used in industry. Enzymes catalyze a variety of stereo-selective reactions such as C-C, C-O, and C-N bond cleavage. This article points out that, with the evolution of rapid screening, optimization, commercial availability, and ease of use, enzymes will become a necessity in future drug discovery. It was demonstrated in the Chapter II that enzymes can be used in the synthesis of chiral malonate half esters. Chapters II, III, and IV describe how these chiral half esters can be transformed into a series of homochirally similar disubstituted unnatural amino acids.[2] A crucial part of our synthetic methodology is to generate the malonate half esters in as close to optically pure form as possible. The enantiomeric composition of the amino acids is dependent on the enantioselectivity of PLE for the malonate diester substrate. In Chapter I, it was noted that PLE hydrolysis of **2.31a** gave **2.32a** in 70% *ee*, where as the PLE hydrolysis of **2.31b** to **2.32b** showed 50% *ee*. The selectivity of PLE for these two substrates was sufficient to prove our synthetic methodology, but greater enantioselectivity is needed in order to achieve our goal of providing an efficient synthesis for these unnatural amino acid classes in optically pure form. There are several possible solutions to this problem which include using different

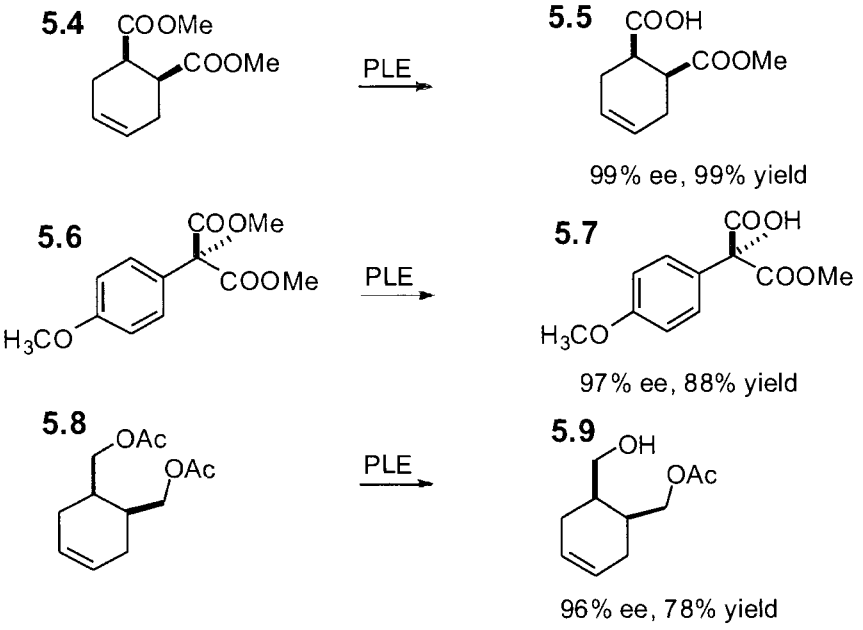
desymmetrization enzymes, altering the protecting groups of our diesters, or modification of the medium in which the enzymatic hydrolysis is performed. While each of these is a possible solution, determining the outcome of each variable, or a combination of variables, is a very time consuming process. This process is made even more difficult by the amount of time that is required for each hydrolysis (1 day to 2 weeks). For these reasons, an efficient high-throughput screening process is needed. As is often stated, necessity is the mother of invention and this was the case with the evolution of hypothesis 2 of this dissertation. Hypothesis 2 states that a high-throughput mass spectrometry (MS) based assay for malonate diesters may be performed by monitoring the ratio of the mixture of labeled and non-labeled half esters that results from the enzymatic hydrolysis of a deuterium labeled, enantiomerically enriched D_5 / H_5-diester (Scheme 5.1). This chapter will focus on 1) the development of a high throughput assay, 2) determination of conditions that improve the enantioselectivity of PLE for **2.32a** and **2.32b** using this MS assay, and 3) validation of the methodology by scale-up of each successful for comparison by chiral HPLC.



Scheme 5.1. General scheme for a mass spectromety based assay to monitor the enantioselectiviy of enzymes for malonate diesters

Modifying the Enantioselectivity of Pig Liver Esterase

Multitudes of papers and several books have been published on the use of enzymes in organic synthesis. One example of such enzymes is PLE, which is a serine protease that consists of three subunits. This esterase is commercially available as a mixture of isozymes that is isolated by homogenation of pig livers. One kg of the esterase mixture can be isolated from 4 kg of pig liver. There were 64 million pigs slaughtered in the U.S. from January to July, 2009.[3] This exemplifies the availability of this esterase mixture. PLE has proven to be promiscuous for many different chiral resolutions and prochiral desymmetrizations, giving both excellent enantioselectivity and excellent chemical yields (Scheme 5.2).[4] These hydrolyses are typically performed in aqueous buffer. This makes the chemistry environmentally friendly, whereas most chiral ligand asymmetrization catalysts use organic solvents that must be disposed. The crystal



Scheme 5.2. Different esters hydrolyzed by PLE

structure of PLE has not been determined which means that the mechanism for PLE selectivity is unknown. However, Jones et al. were able to create a model of the active site of PLE (Figure 5.1).[5] This model was based on over 100 symmetrical diester substrates that had been previously hydrolyzed by PLE. The model contains two polar pockets (P_F and P_B) and two hydrophobic pockets (H_S and H_L) with the active serine residue within close vicinity of the P_B pocket. The model predicts that the ester to be hydrolyzed must be within the 1 Å serine sphere, with the remaining groups best fit into the remaining H and P pockets. The substrate will be hydrolyzed based on the fit of the remaining groups in these pockets. The unhydrolyzed ester will fit into the P_F pocket and the largest non-polar side chain should fit into the H_L pocket. Since the model was proposed, rabbit and human carboxypeptidase, which are closely related to PLE (75% and 76% conserved, respectively), has verified the model.[6]

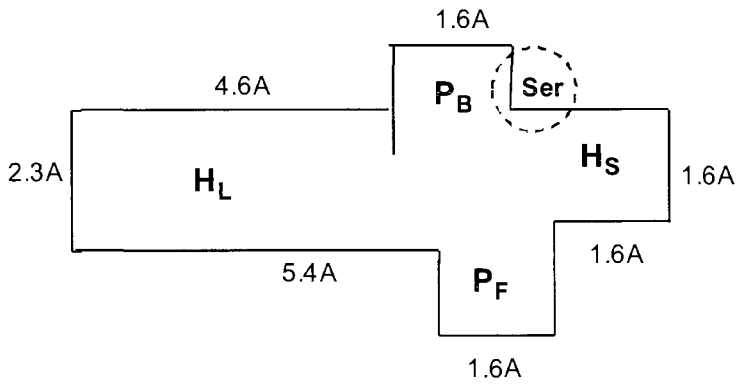
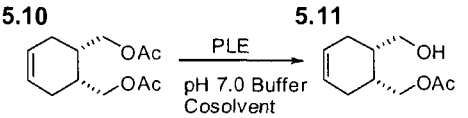


Figure 5.1. PLE active site model proposed by Jones, et al.

The synthetic utility of enzymes for asymmetric synthesis is undisputable.[1,4] However, the case often arises when PLE does not provide a high enantioselectivity for a particular hydrolysis. In such cases several strategies have been developed to increase the enantioselectivity of the enzyme. Given the model in Figure 5.1, the enantiomeric composition of the products is highly dependant on fit of the substrate into the active site of PLE. Modification of protecting groups or addition of a cleavable auxiliary can result in increased enantioselectivity. For example, it has been shown that PLE exhibits higher selectivity for malonic methyl esters over ethyl esters.[2,7-9] While covalent modifications of a substrate molecule have proven successful in increasing enantioselectivity, they require a large amount of synthesis (racemic standards are required) and workup before analysis can be performed. Another method involves the direct modification of the biocatalyst by modification of the proteins primary structure through biochemical exchange of single or multiple amino acid residues. Many groups have made extensive use of directed evolution of enzymes.[10-18] This approach utilizes random mutations to generate mutants that are then screened for the desired properties. The best candidates are then subjected to directed evolution to create a more efficient enzyme for the desired task.[10,11] While directed evolution has been successful, it requires thorough knowledge of biochemical techniques and requires a great deal of time to generate successful mutants. For the synthetic chemist, one of the more promising methods of increasing enantioselectivity has been termed reaction media engineering



Organic Cosolvent	Relative Rate [%]	Optical Purity [%ee]
None	100	55
20% DMSO	70	59
40% DMSO	28	72
20% DMF	35	84
5% t-BuOH	70	94
10% t-BuOH	44	96

Scheme 5.3. Effect of cosolvent on PLE enantioselectivity

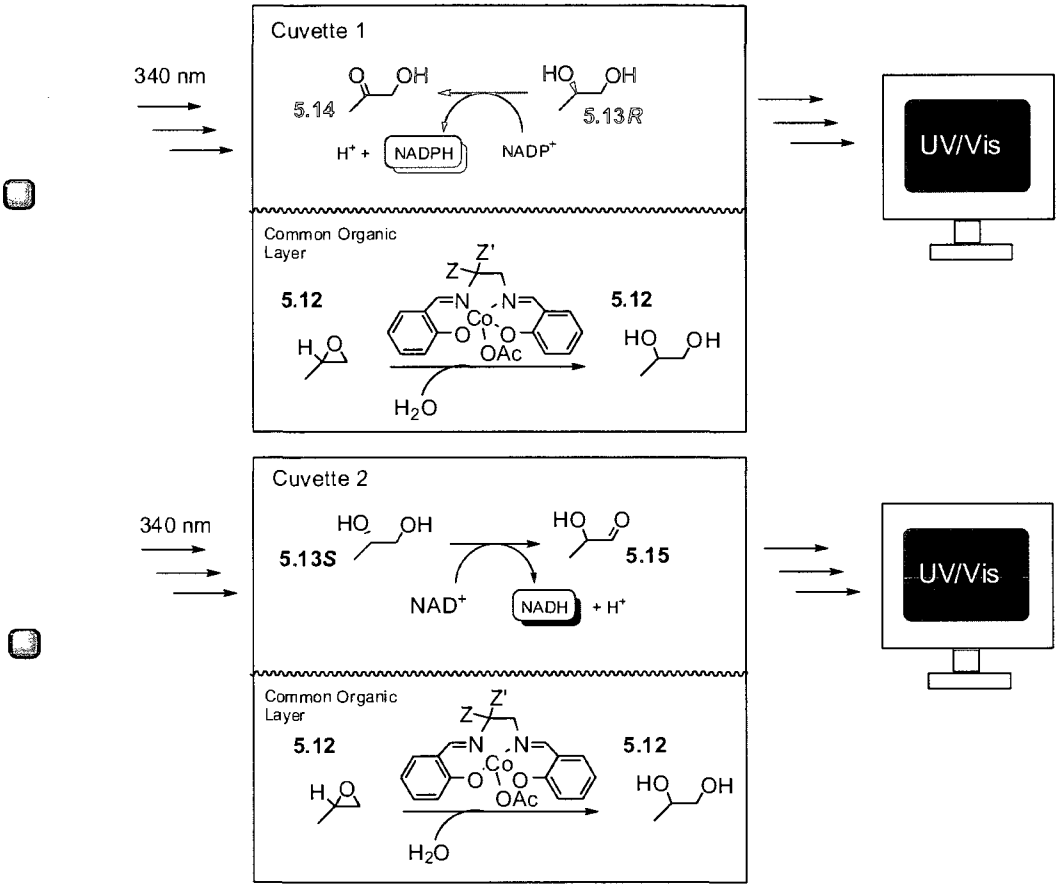
which utilizes the addition of organic solvents that can modify the rate of hydrolysis, the chemical yield and/or the enantioselectivity of the enzyme (Scheme 5.3).[19] Although the effect seen with cosolvents is well documented, the cause remains unknown. Alcohols have by far been the most successful cosolvents.[19-22] The largest improvements in enantioselectivity is typically seen when a sterically hindered alcohol, such as isopropanol, is used. However, polar aprotic cosolvents have also seen extensive use. The disadvantage of using cosolvents is that the rate of the hydrolysis can be dramatically decreased. [19-21] One possible solution to the decrease in rate of hydrolysis is the use of small amount of ionic liquids as additives. Wallert et al. have shown that addition of 0.1% to 5% of an ionic liquid to alcohol cosolvent systems results in increased the enantioselectivity and rate of hydrolysis for PLE using malonate diester substrates.[22] It is clear from the literature review above that a method to evaluate the enantioselectivity of enzymatic hydrolyses under a variety of conditions is necessary.

High Throughput Assays for Enantioselectivity

Many approaches have been developed that screen catalysts for activity. The development of a combinatorial approach by Menger et al. has lead to a tremendous surge in screening of catalyst.[23] The analysis of chiral mixtures using high throughput screening should be simple, quick, efficient, and use very little consumable material (i.e. solvents). This approach has been termed combinatorial asymmetric catalysis. This section will outline the methods that show the most potential for high throughput screening of enantioselectivity.

A popular method for the high throughput quantitation and kinetics is UV/Vis because of the fast scan rates and the technology (i.e. plate readers) that have been

developed to increase the throughput of this type of assay.[24,25] However, obtaining information about the enantiomeric composition produced is difficult.[26] Berkowitz et al. have develop an In Situ Enzyme Screening (ISES) assay that uses a biphasic, dual cuvette UV/Vis assay to screen Co(III) transition metal catalysts for rate and ability to kinetically resolve a chiral diol. In this assay, the chiral catalyst hydrolyzes (±)-propylene oxide (**5.12**) to a chiral diol in the organic phase. The diol can then diffuse into the aqueous phase which contains a non-selective alcohol dehydrogenase enzyme that uses $NADP^+$ as a coenzyme to convert **5.12** into **5.14**. The conversion of $NADP^+$ to NADPH during oxidation of the alcohol is monitored by UV/Vis (at 340 nm) to determine the rate of the reaction. The second cuvette contains a stereoselective alcohol dehydrogenase that catalyzes the conversion of **5.13*S*** to **5.15** and monitors conversion of NAD^+ to NADH. An estimation of resolving ability of the chiral catalyst can be made by comparing the rates of hydrolyses from the two cuvettes. The assay has been successful in ligand tuning for several Co (III) catalyst.[27,28] The main drawbacks of the ISES method are: 1) the catalyst must be water stable and 2) finding an enzyme that is selective for the product diols can be difficult and time consuming.[27]



Scheme 5.4. A schematic representation of the ISES assay developed by Berkowitz, et al.[27-28]

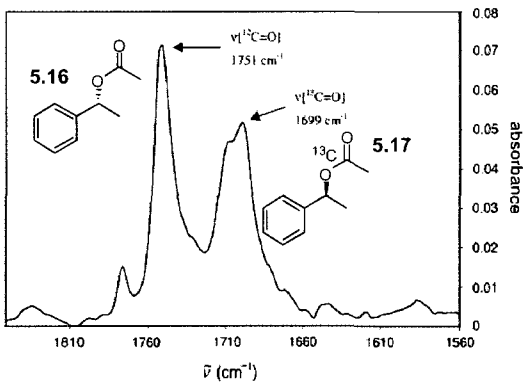


Figure 5.2. FTIR spectra of (*R*)-1-Phenylethylacetate at 1751 cm^{-1} and (*S*)-1-Phenylethyl-1-^{13}C-acetate at 16 cm^{-1}

Infrared spectrometry has also seen use in the screening of chiral catalyst for the kinetic resolution of phenylethylacetate (**5.16**) and phenylethylacetamide.[29] This method makes use of isotopic labeling of the carbonyl with ^{13}C. These carbonyl compounds have a unique and strong

absorbance at 1600 to 1800 cm^{-1}. The large shift in cm^{-1} exhibited by the ^{13}C-labeled carbonyl (40 to 50 cm^{-1}) as compared to the unlabeled carbonyl, makes this method useful for the analysis of samples with multiple components. Once the spectrum has been taken, application of the Beer-Lambert law allows for quantitation of each enantiomer produced from the catalysis. This method is capable of determining the enantiomeric composition of 10,000 samples per day with < 7% error as compared to chiral GC. The assays can be performed directly from biological supernant. The major disadvantage of this method is that the molar absorptivity coefficient (ε) must be determined prior to application of the assay. However, this method has shown utility as a cost-effective assay for enantioselectivity. Another derivation of this method is IR thermography which has allowed for the visualization of kinetic and enantioselectivity of catalysts through use of an IR camera to monitor the heat of reaction.[30-32]

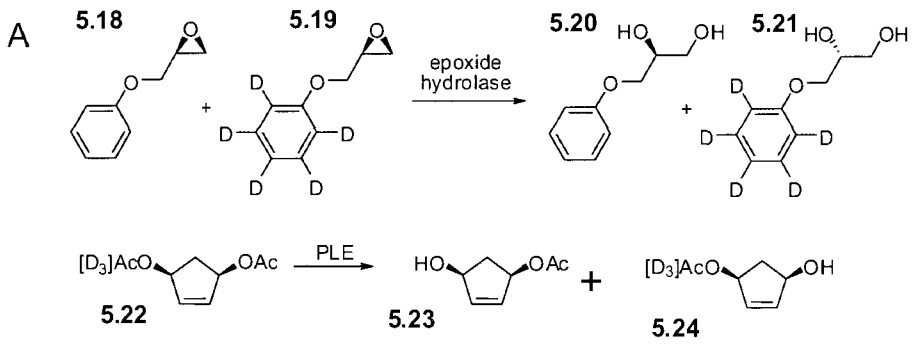
Microarrays have also found use in the determination of % *ee*.[33] This method makes use of probes that fluoresce at different wavelengths depending on the enantiomeric composition. To use this methodology, amine functionalized glass slides are derivatized with D or L N-Boc-protected amino acids (the samples to be analyzed). This is followed by acetylation of any remaining free amines and deprotection of the amino acids. The amino acids on the slide are then subjected to a coupling reaction with a mixture of D and L proline which had been attached to a fluorescent reporter at the N-terminus. Each enantiomer of the reporter reacts with a specific enantiomer of the amino acids. When these samples are scanned with a laser, the samples fluoresce at wavelengths that vary between red and green which is related to the enantiomeric composition of the amino acids. The fluorescent wavelength of each sample can be used to directly

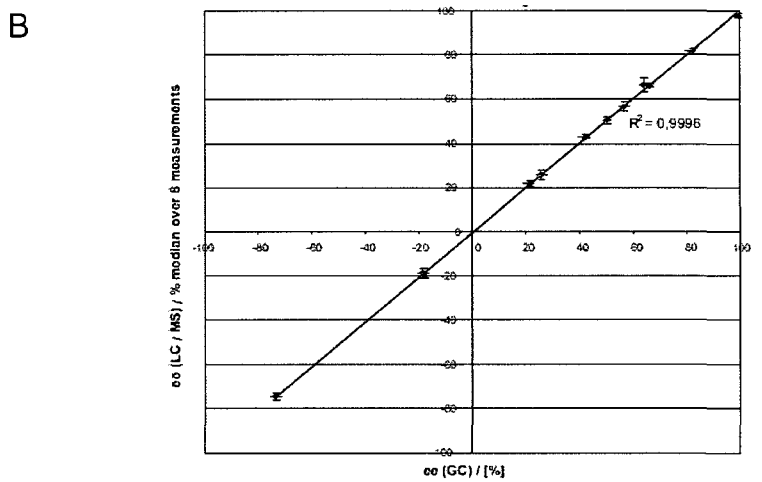
determine the enantiomeric composition. This method allowed for the determination of enantiomeric composition of 15,500 samples on a single glass slide with an average error of < 8% *ee*.

An NMR spectroscopy based assay has been developed to observe the resolution of ±- 1-phenylethyl acetate by a lipase. This technology is based on NMR flow cell technology and has been able to obtain 1400 % *ee* determinations per day.[34] Later application of chemical shift imaging allowed for an increase of up to 5600 *ee*-determinations per day.[35] One form of this assay is based on the observance of the ^{1}H-NMR of observance of acetic acid that is generated from the enzymatic resolution of pseudo-racemic mixtures of S-^{13}C-1-phenylethyl acetate and the unlabeled R-1-phenylethyl acetate. The protons from the methyl group of the labeled acetic acid are observed as a doublet and can be distinguished from the singlet observed from the non-labeled acetic acid. Integration of these peaks allows for determination of the enantiomeric composition of the chiral alcohols with approximately five percent error.

Mass Spectrometry has the potential to be the highest throughput method of all of the analytical methods discussed in this dissertation. Multiple approaches have been evaluated for the determination of enantiomeric composition by mass spectrometry. The sensitivity of modern mass spectrometry instrumentation allows for use of very small quantities of analyte. Methods have been developed that make use of the formation of complexes with chiral selectors in the gas phase[36,37], supramolecular complexes[38,39], and transition metal complexes of pseudo-enantiomers.[40-42] Although these methods have been successful, some require complex mathematical corrections and require extensive work to expand to new analytes. An attractive method for high-throughput screening of

enzyme selectivity was developed by Reetz et al. that makes use of pseudo-enantiomer mixtures or pseudo-meso esters in which strategic parts of the molecule of interest were deuterium labeled to give mass spectrometry probes (**5.18**, **5.19**, and **5.21**, Scheme 5.5).[13,16] The probes were subjected to enzymatic reactions and the resulting products (**5.20** and **5.21**) from the reaction were analyzed by mass spectrometry. Direct comparison of the sodium adducts of **5.20** and **5.21** analyte allow for direct determination of the enantiomeric composition. The % *ee* values obtained from the MS analysis of various compositions of **5.20** and **5.21** were compared to values found by chiral GC to determine the validity of the method (Scheme 5.5). It was found that the two methods correlated extremely well for an enantiomeric excess of either enantiomer. The most impressive aspect of this methodology was that it allows for the analysis of 10,000 samples per day with less than three precent error.

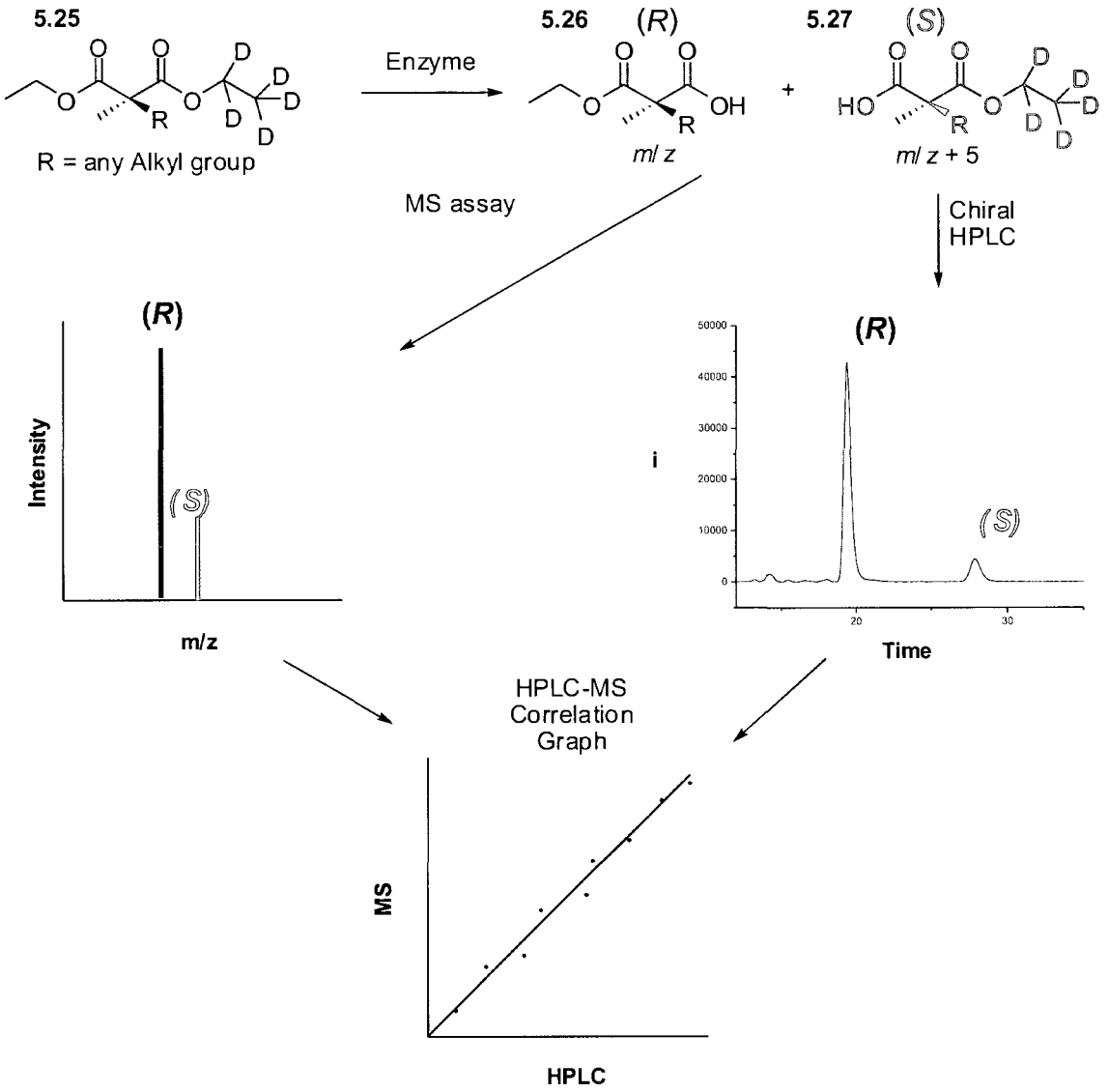




Scheme 5.5. A) Substrates and reaction from the high-throughput MS assay for enzyme enantioselectivity composition developed Reetz B) The correlation plot of data obtained from the MS assay and chiral GC analysis from the epoxide hydrolase hydrolysis of **5.18** and **5.19**

To our knowledge, no assay has been developed to screen for the enantioselectivity in the enzymatic hydrolysis of malonate diesters. In the previous Chapters, the synthetic utility of malonate half-esters for the synthesis of multiple classes of disubstituted unnatural amino acids was demonstrated. The incorporation of both enantiomers of these unnatural amino acids into neurotensin peptidomimetics requires that the selectivity of the PLE hydrolysis of the malonate diesters be improved. In this Chapter, several possible methods were outlined that can be used to monitor the increase

in enantioselectivity of enzymes. Reaction media engineering seems to be the simplest approach to modifying the enantioselectivity of enzymes. However, the cause of the change in enantioselectivity seen with cosolvents is not well understood. This means that there is no defined approach to determine which solvent will give the best result. In order to effectively evaluate the numerous possible combinations of solvents, enzymes, and buffers, a high throughput assay for the enzymatic enantioselectivity of malonate diesters must be developed. The analytical capacity of the mass spectrometry assay similar to the one developed by Reetz should allow us to obtain the highest throughput without custom equipment.[13,16] The following section will address the development of a mass spectrometry based assay for the enzymatic hydrolysis of malonate diesters. This work will use deuterium labeling to create *pseudo*-prochiral malonate diesters (**5.25**) that can be subjected to enzymatic hydrolysis and monitored by mass spectrometry (Scheme 5.6). The results of the assay will also be monitored by chiral HPLC to validate our assay.



Scheme 5.6. Scheme for the proposed mass spectometry based assay for *pseudo*-prochiral malonate diesters

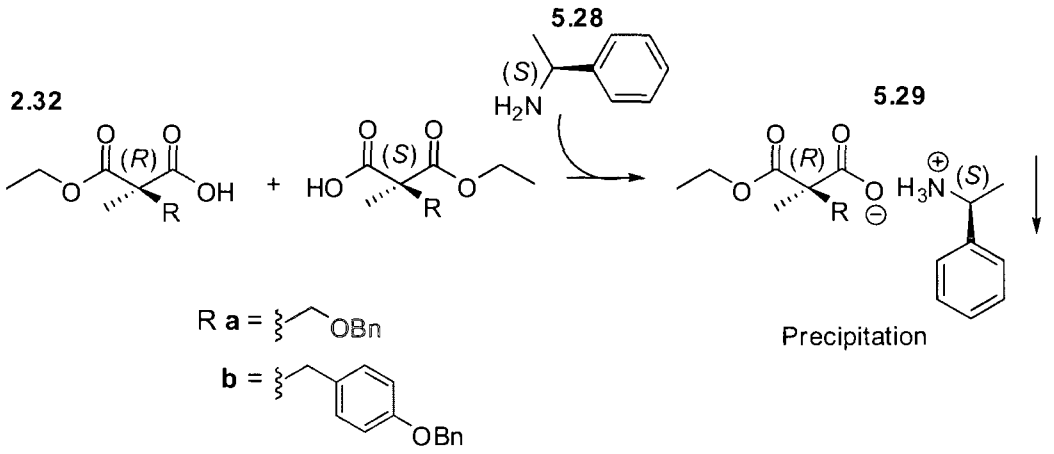
Discussion and Results

The first step in the development of an MS-based assay is the synthesis of deuterium labeled probe molecules (**5.31a** and **5.31b**). Initially, we sought to create enantiomerically pure half esters (**2.32a** and **2.32b**) in order to design an MS based assay similar to the one developed by Reetz.[13] To accomplish this we attempted to resolve **2.32a** as a diastereomeric α-methylbenzyl ammonium salt in diisopropyl ether by a

literature procedure (Scheme 5.7).[43] We were able to obtain the desired ammonium salt, but when the resulting half ester was analyzed by chiral HPLC we observed no change in enantiomeric excess. In an attempt to resolve **2.38b**, we followed the same literature procedure and modified our solvent composition to include a variety of solvents and combination of solvents. Unfortunately, we were unable to obtain the α-methylbenzyl ammonium salt of **2.32b**. We also attempted to purify the (*R*)-enantiomers of **2.38a** and **2.38b** by semi-preparative chiral HPLC. Unfortunately, we were only able to purify milligram quantities after several days of continual injections. The breakthrough in the probe synthesis came when we realized that we should be able to use a probe that was not enantiomerically pure. Cawley et al. were able to prove this concept mathematically and provide a simple formula to correct for enantiomerically impure probes (Figure 5.3).[44] Application of this methodology would lend two primary strengths to our assay by 1) making the probe synthesis more efficient by removing the resolution step and 2) allowing us to recycle our enantiomerically enriched half-esters obtained from PLE hydrolyses.

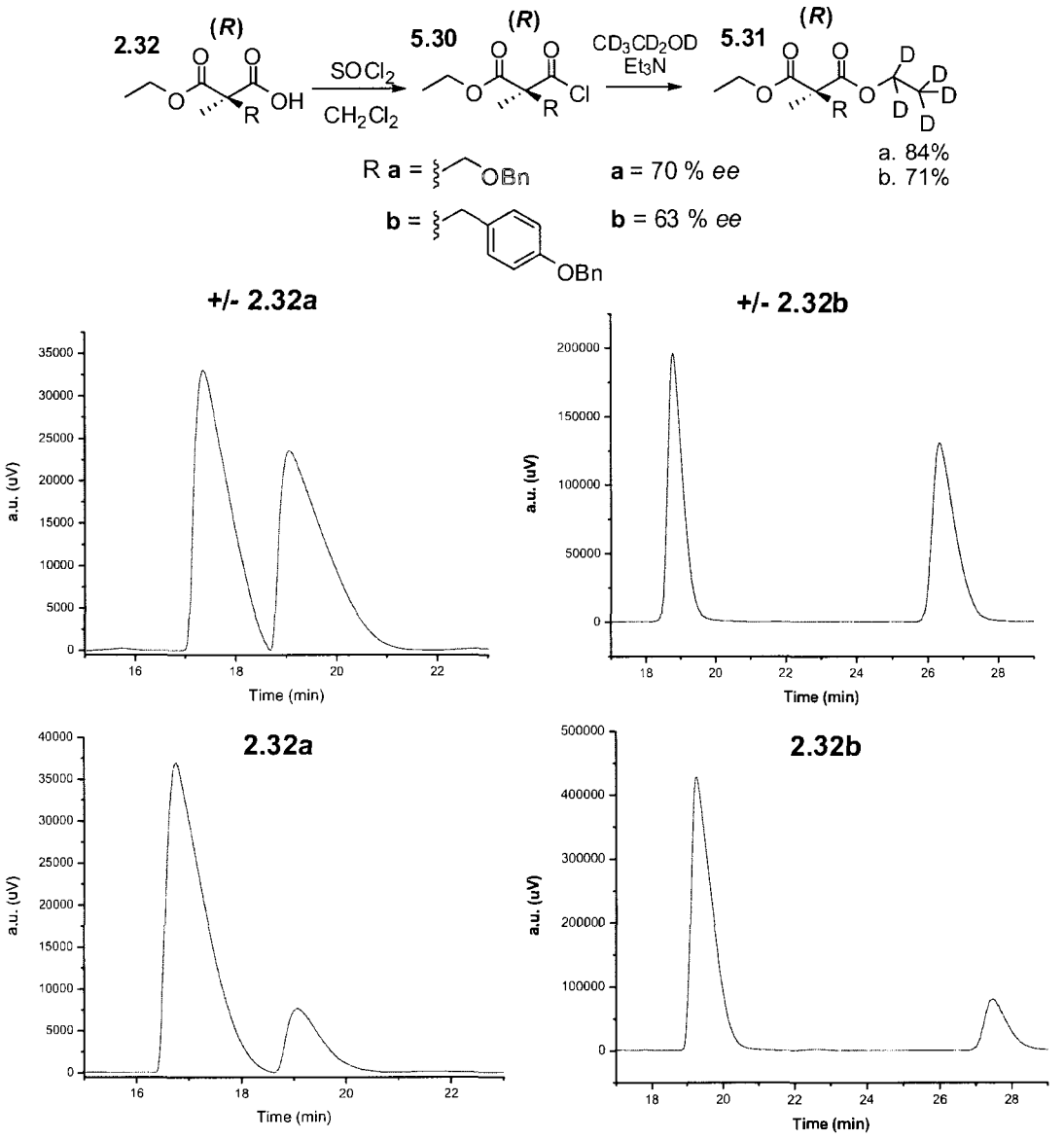
$$\frac{\text{Observed Enantiomeric Excess}}{\text{Starting \% ee of Probe}} = \text{Corrected Enantiomeric Excess}$$

Figure 5.3. The equation used to correct for the purity of the MS probes



Scheme 5.7. Resolution of **2.32a** and **2.32b** by formation of a diastereomeric ammonium salt with α-methylbenzyl amine

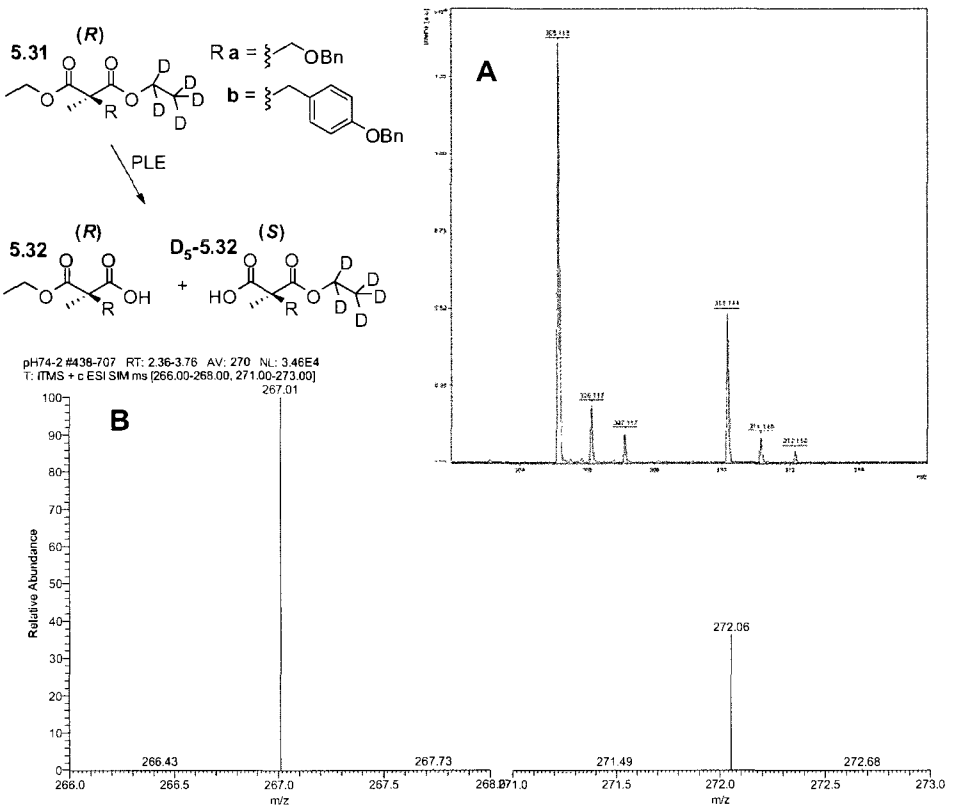
Synthesis of the probe **5.31a** was initiated by performing a PLE hydrolysis of **2.31a** to **2.32a**. Compound **2.32a** was analyzed by chiral HPLC which confirmed that the half-ester was produced in 68% *ee*. Conversion to **5.31a** was accomplished by activation of **2.32a** as an acid chloride followed by quenching of the acid chloride with D_6-ethanol. This gave **5.31a** in 84% isolated yield. Compound **5.31b** was synthesized using the same methodology (63% yield). Chiral HPLC analysis of **2.32b** confirmed that the half-ester was produced in 63% *ee*. Racemic half-esters (±-**2.32a** and ±-**2.32b**) were also synthesized by saponification of **2.32a** and **2.32b** with NaOH. These racemic half-esters were esterified using the D_5 / H_5 diesters using the described acid chloride method.



Scheme 5.8. Synthesis of probes **5.31a** and **5.31b** for the MS assay for enzyme selectivitywith the correspnding chiral HPLC chromatograms

Once the probes had been synthesized, we proceeded with a preliminary assay to determine if the proposed methodology would provide the expected result and whether ESI or MALDI-ToF mass spectrometry would be most convenient. To determine the validity of our proposed assay, PLE hydrolyses of probes **5.31a** and **5.31b** were performed using 10 mg of each probe in 1.5 mL of buffer. The resulting mixture of D_5

and H_5 half-esters were then analyzed by chiral HPLC, ESI-ion trap mass spectrometry, and LDI-ToF mass spectrometry. Laser Desorption Ionization Time-of-Flight (LDI-ToF) was used to analyze the assay due to the interference caused by matrix in the low molecular weight region seen in MALDI-ToF. This was possible because the aromatic ring on our substrates is able to absorb the laser radiation and facilitate ionization.[45] Analysis by ESI and LDI-ToF MS showed that the PLE hydrolysis of **5.31a** proceeded with an observed 74% *ee* and 69% *ee*, respectively after correction (Scheme 5.8). Analysis by chiral HPLC revealed that the PLE hydrolysis of **5.31a** proceeded with 67% *ee*. Hydrolysis of probe **5.31b** in pH 7.4 phosphate buffer gave 66% *ee* and 58% *ee* after correction, respectively. Chiral HPLC analysis confirmed that the hydrolysis of **5.31b** proceeded with 62% *ee*. Statistical analysis of the results from the hydrolysis of probes **5.31a** and **5.31b** revealed less than four percent standard deviation for ESI-MS, LDI-ToF-MS, and chiral HPLC. This means that both LDI-ToF and ESI MS are viable methods that give reliable results. These observations confirm that our assay provides valid data that can be used to evaluate the PLE hydrolysis of our MS probes (**5.31a** and **5.31b**).



Scheme 5.9. The mass spectra from the PLE hydrolysis of probe **5.31a** in pH 7.4 phosphate buffer A) The LDI-ToF spectra B) The ESI spectra in SIM mode

The next task was to determine which method of analysis (ESI or LDI-ToF) gave the most reproducible results as compared to chiral HPLC. It has been observed that pH has an effect on the selectivity of some enantioselective enzymes.[4,46] For this reason, it was decided to begin the assay by evaluating the mid buffering range of the phosphate buffer (pH 6.2 to 8.2) that is used as a solvent for the hydrolysis. The assay was performed in triplicate for pH 6.5, 7.0, 7.4, and 8.0. The results obtained from the three analytical methods (ESI-MS, LDI-ToF-MS, and chiral HPLC) were then compared graphically to determine how well the methods correlate (Figure 5.4). The trends observed in the graphs indicate that there was little variance in enantioselectivity over the pH range of the phosphate buffer. The same minor changes in enantiomeric composition

were observed with all three techniques which should allow either mass spectrometry technique to be utilized for analysis of our assays. It was decided that ESI-MS was the best technique to use to analyze our assays due to the ease of sample preparation. The LDI-ToF technique required more tedious sample preparation to remove salts that interfere with the laser desorption ionization process. The LC-MS technique allows us to use a divert valve to remove buffer and salts from our sample before it is injected into the mass spectrometer (Figure 5.5). With sound methodology for our assay now available, we proceeded to evaluate various cosolvents.

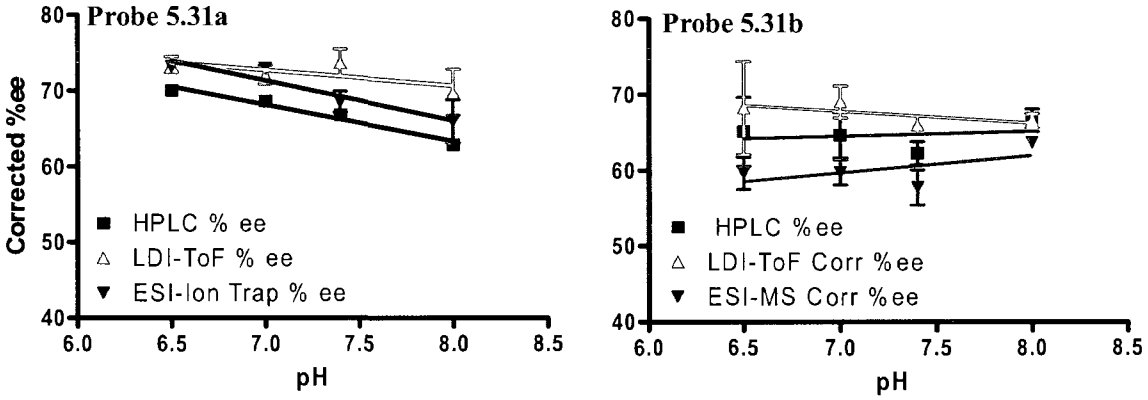


Figure 5.4. A comparison of ESI-MS and LDI-ToF MS corrected % *ee* to the results obtained from chiral HPLC to determine how the methods correlate

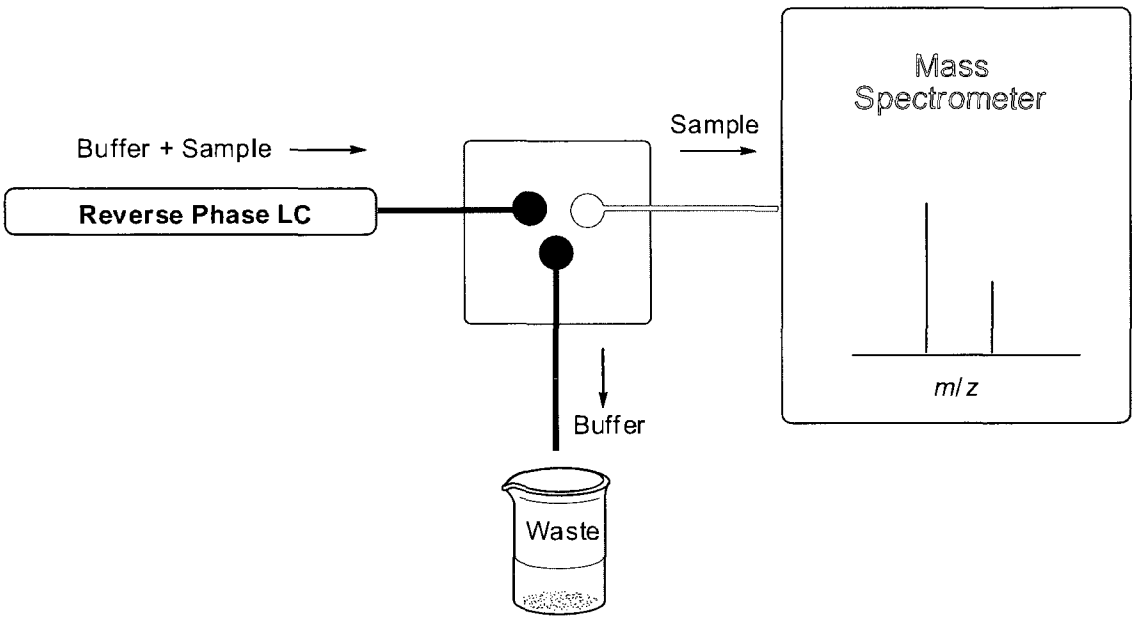


Figure 5.5. Schematic of the divert valve used in the ESI-MS assay for enantioselectivity

It is well known that inorganic buffers like phosphate and sulfate buffers suppress ion formation in mass spectrometry and can clog the inlet to an LC-MS system due to the non-volatile nature of the buffer salts.[45] For this reason, we wanted to determine if we could use a buffer that was compatible with mass spectrometry such as the TRIS buffer. We also wanted to determine if the buffer that was used had an affect on the enantioselectivity of PLE. We chose to evaluate TRIS, bis-tris-propane, imidazole, glycine-glycine, and tricine as the medium for

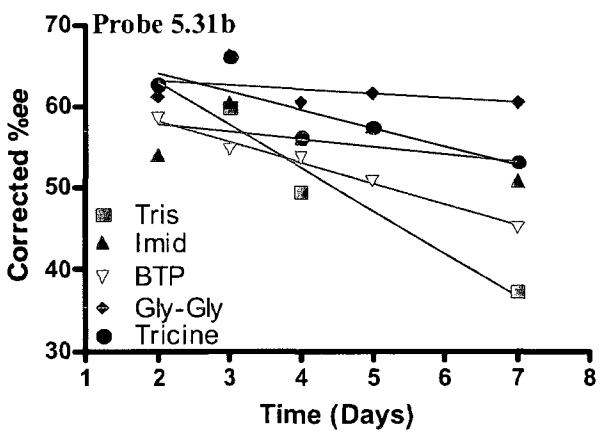


Figure 5.6. Buffer effects on the PLE hydrolysis of Probe **5.31b**

the hydrolysis of probe **5.32b**. A phosphate buffer control was also used. The general approach to the assay involved placing 10 mg of each probe in a microcentrifuge with the appropriate buffer. A PLE solution (50 mg / mL) was made and 4 units of enzyme were added to each tube. The assays were allowed to proceed for three days for probe **5.32a** and five days for probe **5.32b**. To analyze the samples, 200 μL of each sample was placed in an autosampler vial and diluted with 200 μL of one percent acetic acid in MeOH. An injection of 1 μL of the mixture was made into the LC-MS system. The eluant from the LC system was diverted for two minutes to elute the buffer before the remainder of the eluant was injected into the MS detector. We found that the first two to three days of hydrolyses proceeded with a similar selectivity to that seen in the phosphate buffer control (Figure 5.5). However, it was observed that a loss of selectivity was seen as the reaction proceeded beyond three days. This may have been due to buffer catalyzed racemic hydrolysis of the probes or reaction of the buffer with the probe. These experiments allowed for the conclusion that phosphate buffer was the better choice for buffering of our assays.

Several literature sources have cited that polar aprotic cosolvents can induced an increase in enantioselectivity in various enzymatic hydroyses.[4,20,47] We proceeded to apply our assay to evaluate several of the more commonly used cosolvents like DMSO, DMF, MeCN, THF, Dioxane, Diglyme, and Triglyme with probes **5.32a** and **5.32b**. Cosolvent compositions of 5% to 20% in pH 7.4 phosphate buffer were used. The results are described as an increase or decrease in enantioselectivity relative to controls that were performed in phosphate buffer (**5.32a** = 68% *ee*, **5.32b** = 63% *ee*). The experimental results are shown in Figure 5.6. A slight increase in selectivity of PLE was seen with both

5.32a and **5.32b** with most of the cosolvents. The exception was MeCN where we observed decreased enantioselectivity initially with probe **5.32a** followed by an increase in selectivity with concentrations above 10%. The same result was obtained with the hydrolysis of **5.32b** with MeCN resulted in a decrease in enantioselectivity with concentrations up to 15%, followed by a slight increase in enantioselectivity at 20% MeCN. The hydrolysis of probe **5.32b** in MeCN cosolvents resulted in a decrease in enantioselectivity. The observed increase in enantioselectivity observed (6% for **5.32a** and 14% for **5.32b**) with the polar aprotic cosolvents is synthetically insignificant which lead to evaluation of other cosolvents systems.

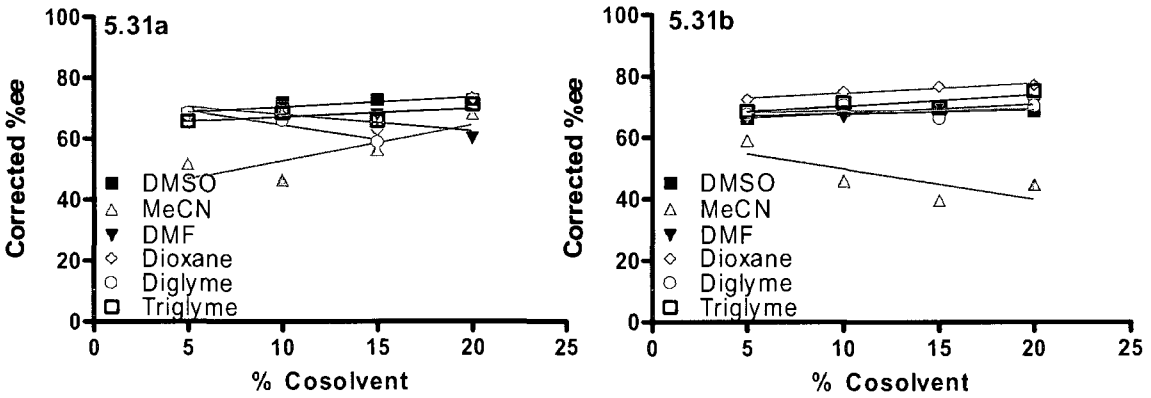


Figure 5.7. Results from the PLE hydrolysis of probes **5.31a** and **5.31b** in polar aprotic cosolvents

Literature reports have reported that alcohol cosolvents are most effective as additives to improve the enantioselectivity of the PLE hydrolysis of malonate diesters.[22] We began our alcohol cosolvent assay by examining mixtures of methanol, ethanol, *isopropanol*, and *tert*-butanol with pH 7.4 phosphate buffer. The solvent composition ranged from 5% to 30% of each of the alcohols. When we began analyzing the results of the assay from probe **5.32a** using the Select Ion Monitoring (SIM) mode for the MeOH

cosolvent reactions, we noticed that there were two resolved peaks in the LC-MS chromatogram that were isobaric (m/z = 267). The same sample was analyzed again using the full scan mode of the ion trap. We observed five identifiable peaks in the chromatogram when only two should have been present. When these peaks were subjected to MS^n analysis by collision induced dissociation (CID), we found that the additional compounds were the result of transesterification with the methanol cosolvent (Figure 5.8). We saw the same result when ethanol was used as a cosolvent. We determined the transesterification was taking place by looking for the $[M+Na]^+ = 317$ that corresponds to compound **2.31a**. When this diester is hydrolyzed by PLE, we observe a ratio of m/z = 267 / m/z = 272 that was not representative of the enantiomeric composition of the hydrolysis. This was confirmed by chiral HPLC analysis of the same samples. Transesterification with primary alcohols clearly illustrates a limit of this assay. For this reason, we ceased the use of methanol and ethanol as cosolvents in these assays.

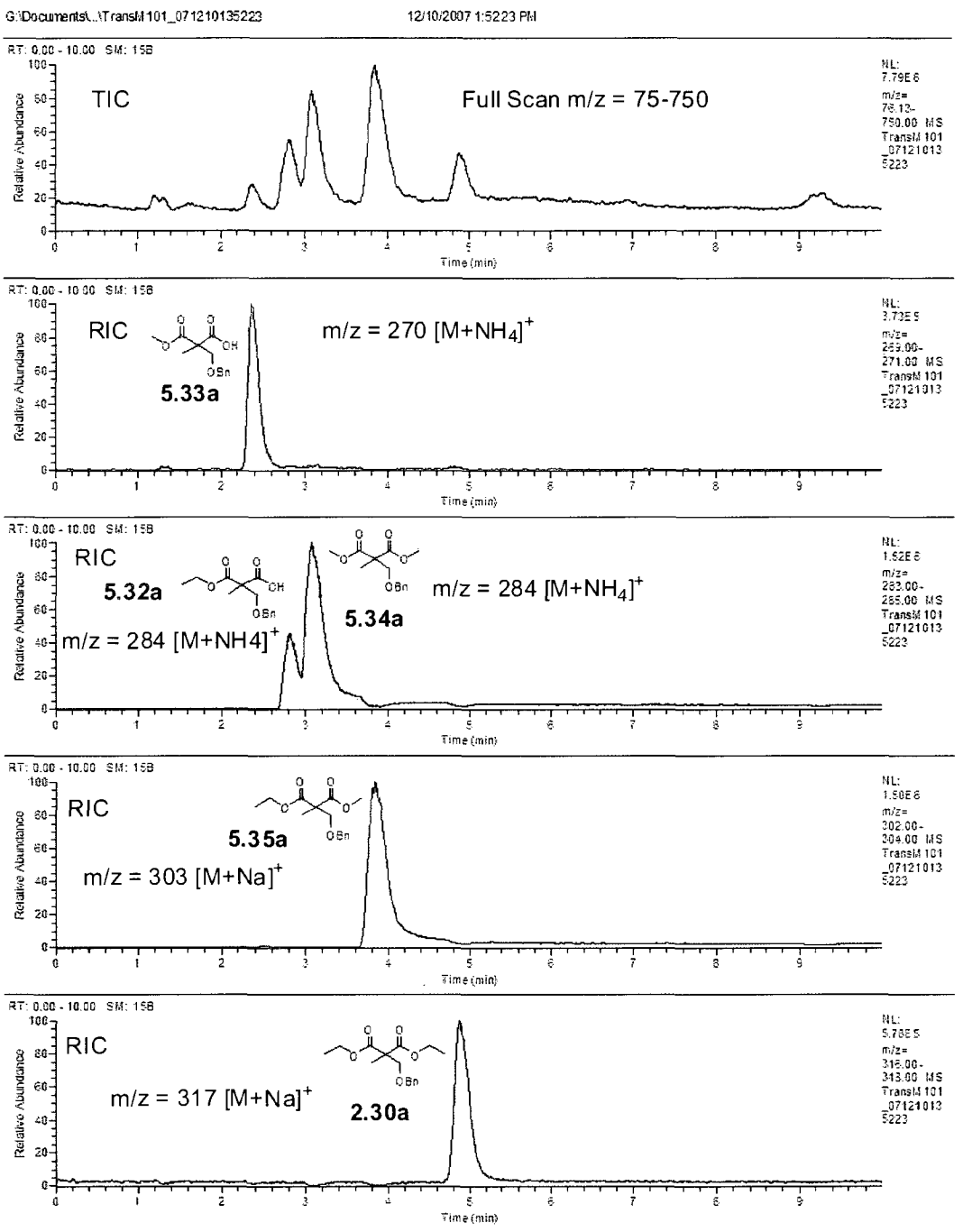
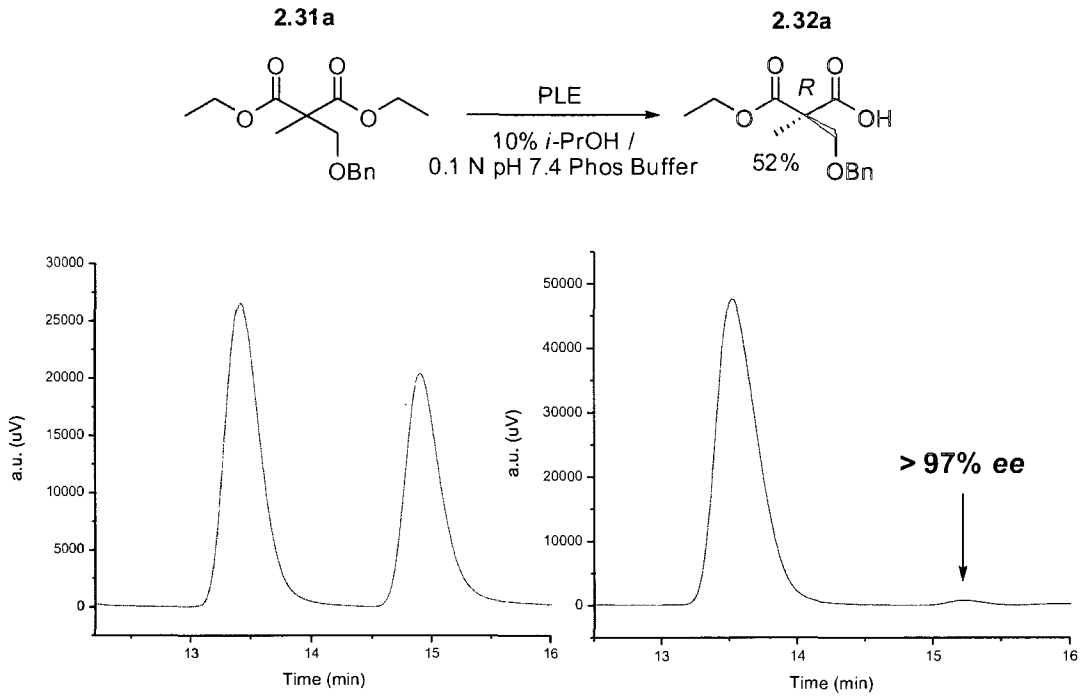


Figure 5.8. LC-MS chromatograms of the results of the PLE hydrolysis of probe **5.31a** in 10% MeOH / pH 7.4 phosphate buffer cosolvent

We obtained the most interesting results with the isopropanol and *tert*-butanol cosolvent assays. Probe **5.32a** showed an increase from 70% *ee* to 85% *ee* with the use of 10% tert-butanol as a cosolvent. A similar result was seen with probe **5.32b**, where an increase from 63% *ee* to 85% *ee* was seen with 30% tert-butanol. Interestingly isopropanol gave the best results of all of the evaluated cosolvents. We observed a substantial increase in the enantiomeric composition of probe **5.32a** from 70% *ee* to 103% *ee* (overestimation due to error from the MS measurement) when 10% isopropanol in pH 7.4 phosphate buffer was used as the solvent. When probe **5.32b** was subjected to the assay with 30% isopropanol cosolvent, an increase in enantiomeric composition from 63% *ee* to 90% *ee* was observed. To verify the results of these assays 100 mg of **2.31a** and **2.32b** were hydrolyzed in the appropriate cosolvent isopropanol cosolvent. The hydrolysis of **2.31a** to **2.32a** using 10% isopropanol in pH 7.4 phosphate buffer as the reaction medium resulted in > 97% *ee*, which confirmed the increase in enantiomeric composition seen in the MS assay (Scheme 5.10). As seen in previous reports, we observed a significant decrease in rate when > 30% cosolvent is used. In concentrations of the cosolvent over 30% the signals from the mass spectrometers was too weak to calculate an accurate enantiomeric composition. The overestimation of the enantiomeric composition seen in the MS assay is thought to be the result of the error associated the initial chiral HPLC determination of the probe composition and the error associated with the mass spectrometry assay.



Scheme 5.10. PLE hydrolysis of **2.31a** to **2.32a** in 10% *i*PrOH in pH 7.4 phosphate buffer with the corresponding chiral chromatograms

The reasoning behind the modification of selectivity seen with cosolvent use is not understood. Matson, et al. has observed the occurrence of a buffer acting as an alternative nucleophile to water.[48] This was considered as a possible explanation to the increase in selectivity seen with the PLE hydrolysis of probes **5.32a** and **5.32b** when *isopropanol* and *tert*-butanol were used as cosolvents. There was a small quantity of the isopropyl ethyl diester of probe **5.36a** observed in the full scan LC-MS chromatogram (Figure 5.8). This lends evidence to isopropanol acting as an alternative nucleophile to water which would lead to transesterification of a single enantiomer of the half ester. However, there was no transesterification observed with the use of *tert*-butanol as a cosolvent which also resulted in increased enantioselectivity with PLE. Experiments to determine if transesterification is the cause of the increase in selectivity will be discussed in Chapter VI.

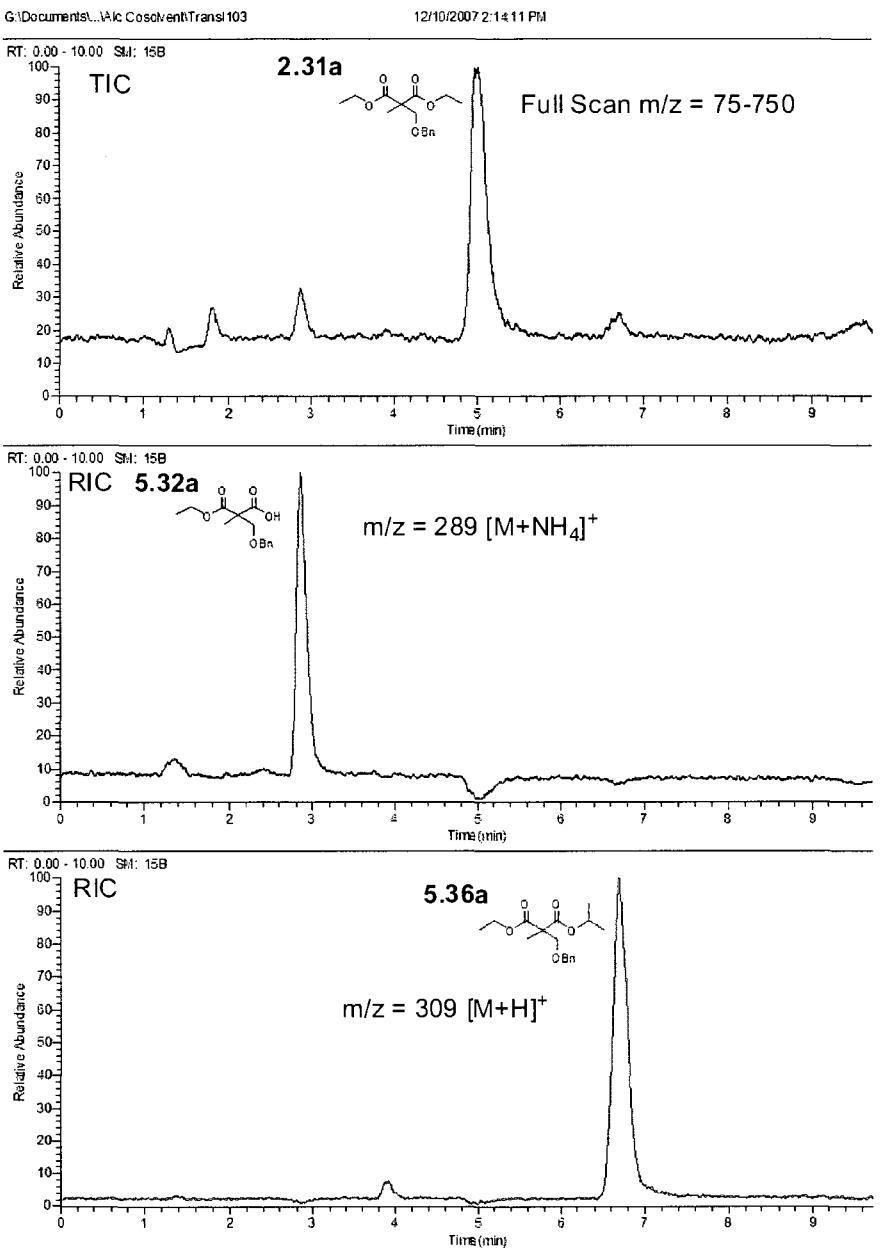
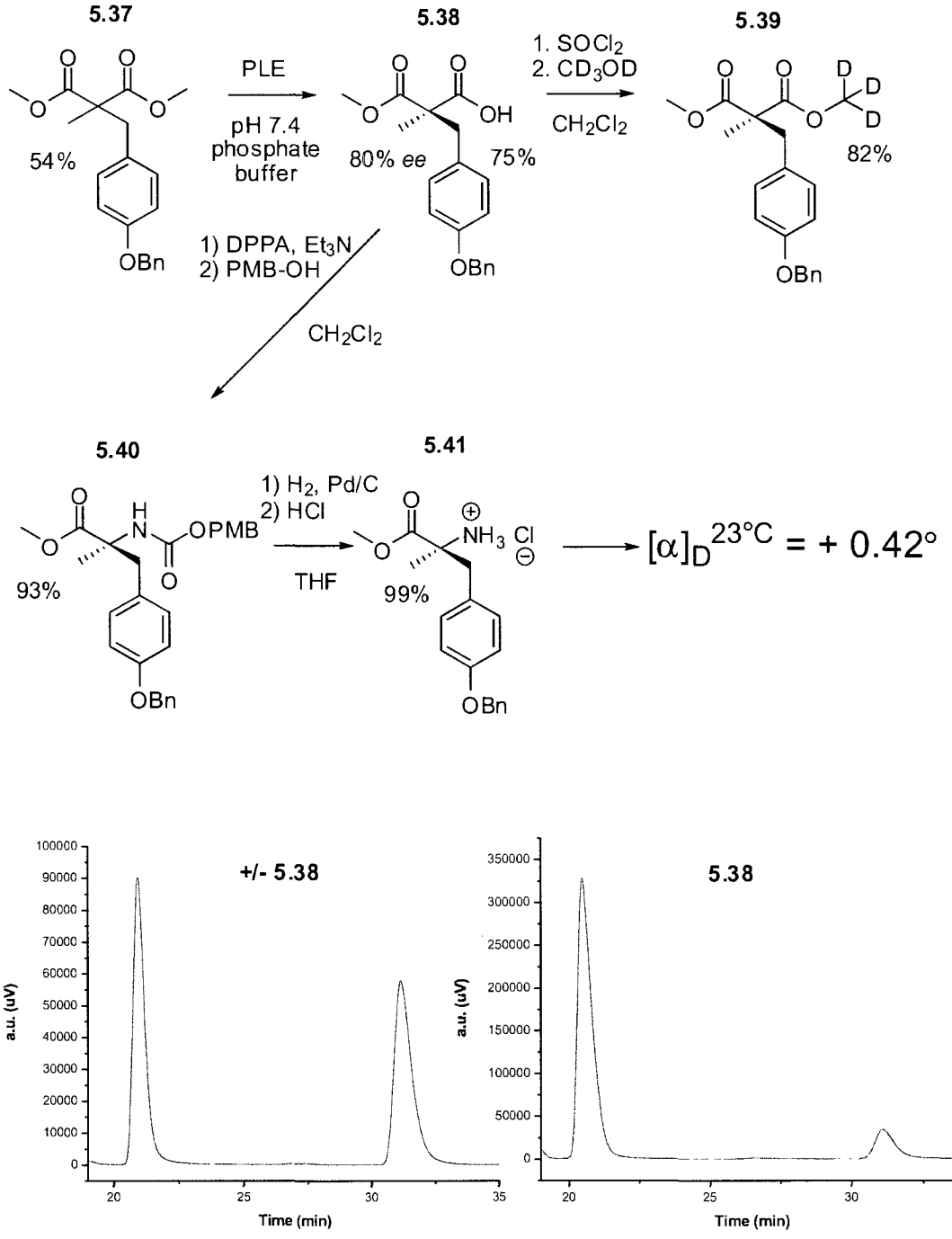


Figure 5.9. LC-MS chromatograms of the results of the PLE hydrolysis of probe **5.31a** in 10% iPrOH / pH 7.4 phosphate buffer cosolvent

We were very pleased with the increase from 70% *ee* to > 97% *ee* seen with the PLE hydrolysis of **2.31a** to **2.32a**. We had hoped that a similar result would be seen with the PLE hydrolysis of **2.31b** to **2.32b**. However, we were only able to increase selectivity from 63% *ee* to 85% *ee* with < 10% yield of **2.32b**. While this is a significant increase, it is not of sufficient enantiomeric purity for to complete synthesis of our desired unnatural tyrosine analogues. Literature has shown that the PLE hydrolysis of methyl diesters yield higher selectivity than ethyl diesters.[2,7-9] It is for this reason that the PLE hydrolysis of **5.36** to **5.38** was evaluated in hopes that the selectivity would be > 97% *ee*. Synthesis of **5.36** was initiated by generation of the enolate of dimethyl methyl malonate followed by quenching of the enolate with benzylchlorobenzyl ether (54% yield). Compound **5.37** was subjected to a PLE hydrolysis in pH 7.4 phosphate buffer to give **5.38** in 75% yield. Chiral HPLC analysis of **5.38** showed that the hydrolysis proceeded in 80% *ee*. This is also a significant improvement in selectivity, but is still not of adequate optical purity. An MS probe was synthesized by converting **5.38** to an acid chloride, then quenching the acid chloride with CD_3OD to give **5.39** in 82% yield. The absolute configuration of **5.38** was determined by synthesis of α-methyl tyrosine methyl ester (**5.41**) from **5.38** (Scheme 5.11) followed by comparison of the optical rotation to an authentic sample of α-methyl tyrosine methyl ester. The observed dextrorotary optical rotation matched that of the authentic sample of (*S*)-α-methyl tyrosine which allows for the conclusion that the PLE hydrolysis of **5.37** yields the *R*-enantiomer of **5.38**.



Scheme 5.11. Synthesis of MS probe **5.39** with the corresponding chiral HPLC chromatogams and synthesis of α-methyl tyrosine to determine the configuration of **5.38**

Probe **5.39** was subjected to hydrolysis in the same cosolvent systems used with probes **5.31a** and **5.31b**. This included isopropanol, *tert*-butanol, and the polar aprotic

cosolvents used in the previous assays. We hoped to see the same increase in enantioselectivity with the methyl diester (probe **5.39**) that we observed with the ethyl diester (**5.31b**). Surprisingly, it was observed that hydrolysis of probe **5.39** in low concentrations of cosolvents resulted in a decrease in enantioselectivity. The result was that the enantioselectivity of probe **5.39** seen with 10% isopropanol in pH 7.4 phosphate buffer cosolvent was decreased to the value seen in the hydrolysis of probe **5.31b** in phosphate buffer (Table 5.1). It was observed that the hydrolysis of **5.39** in 30% isopropanol / 70% phosphate buffer yielded > 100% *ee* and the PLE hydrolysis of **5.39** in 30% *tert*-butanol / 70% phosphate buffer yielded 85% *ee*, by mass spectrometry. However, a scale-up of both reactions resulted in < 20% *ee* by chiral HPLC (Scheme 5.11). The over estimation in enantioselectivity was due to low conversion of **5.39**. The exact cause of this change in enantioselectivity is unknown and confirms that the results of hydrolyses with PLE in organic cosolvents are not well understood.

Table 5.1. A comparison of the corrected MS reults from the PLE hydrolysis of probes **5.31b** and probe **5.39**.

Cosolvent	Probe 5.31b Corrected % ee			Probe 5.39 Corrected % ee		
	10%	20%	30%	10%	20%	30%
i-PrOH	71	79	90	64	62	103
t-BuOH	71	77	85	62	61	85
DMSO	72	73	n/a	76	68	67
MeCN	46	45	n/a	65	71	n/a
DMF	67	72	n/a	67	65	78
Dioxane	75	77	n/a	67	69	n/a
Diglyme	71	71	n/a	70	69	69
Triglyme	72	75	n/a	71	70	70
Control	63			80		

The result of the PLE hydrolysis of probe **5.39** in 10% *i*-PrOH (highlighted in blue) is the same as that seen in the PLE hydrolysis of **5.31b** in buffer alone

To further demonstrate the validity of our assay, it was necessary to create a correlation plot to compare the values obtained from our mass spectrometry assay and the values found by chiral HPLC. In addition to the points obtained from our assays, we synthesized **+/-5.32a** by saponification with NaOH in isopropanol and mixed this with the products from the PLE hydrolysis of probe **5.32a** (68% *ee*) to generate enantiomeric compositions from 20-45% *ee*. The mixtures were analyzed by our mass spectrometry assay and by chiral HPLC to provided enough points in a broad enough range of enantiomeric composition for sufficient line fitting. The results of this line fitting are displayed in Figure 5.10. The excellent fit of the data to the line, the slope of the line, and the near zero Y-intercept illustrates the validity of our assay. The slight deviations seen are likely the result of the error associated with the Y-axis of the graph. There are two sources of error associated with the values plotted on the Y-axis; the error from the initial determination of enantiomeric composition of the half-ester used to make a probe and the error associated with the mass spectrometry measurements. This error results in an underestimation of enantiomeric composition by up to 5% to 8% *ee*, but the assay is still capable of determining conditions to optimize the selectivity of PLE for malonate diesters.

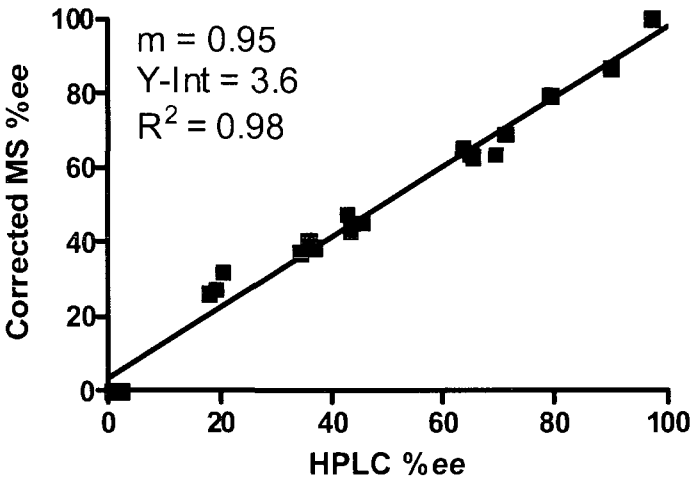


Figure 5.10. Correlation of HPLC% *ee* and corrected MS % *ee*

Conclusions

The field of combinatorial asymmetric catalysis has seen much attention from scientists in academia and industry. The utility of this methodology is undisputable for optimization of chiral catalysts. In this chapter, a high-throughput mass spectrometry based assay has been developed that has allows for the evaluation of the enantioselectivity of PLE for three substrates under a variety of cosolvent conditions. This assay was used to find conditions that gave increased enantioselectivity for two of the diesters of interest.[49] Furthermore, the data obtained from the MS assays was plotted against the data obtained from chiral HPLC to create a linear correlation graph. The regression analysis of this graph further validates that our assay is capable of determining the enantioselectivity of enzymes for malonate diesters over a broad range of enantioselectivities. This approach should allow us to synthesize mass spectrometry probes from any half ester of moderate selectivity from an enzymatic hydrolysis. Probes of lower enantiomeric composition will result in intensities that are closer to equal by mass spectrometry. The 5% to 8% *ee* error observed in the assay of probes 5.31a and 5.31b should result in higher error with probes of lower enantiomeric composition, as compared to chiral HPLC. For this reason, better results should be observed with probes that are greater than 50% *ee*. However, empirical evidence is needed to determine the lower limit of probe enantiomeric composition that can be used for this assy. It is the intention of our research group to use this assay to further improve the enantiomeric composition of compound **2.32a** for synthesis of the unnatural tyrosine analogues seen in Chapters II through IV. This assay will also be used to increase the selectivity of various enzymes for other malonate diesters of interest.

Experimental

General

All other chemicals and enzymes were obtained from Aldrich Chemical and used as received unless otherwise noted. Mass Spectrometry grade methanol was purchased from Aldrich. HPLC grade water was purchased from Fisher. Acetic Acid was purchased from Aldrich. Auto sampler vials and caps were purchased from VWR. Samples were analyzed using either a ThermoFisher LXQ ESI-Ion trap mass spectrometer coupled to a ThermoFisher Accela HPLC system or a Bruker Microflex MALDI mass spectrometer in the LDI mode. Chiral HPLC was performed using a LabAlliance Series III isocratic pump coupled to a LabAlliance Model 500 UV/Vis detector. HPLC chromatograms were recorded using the Peaksimple® data acquisition system and software. Chiral HPLC was performed using a Chiralcel OJ-H analytical column or a Chiralcel AD-H column from Chiral Technologies, Inc.

Enzyme Assays (General procedure)

Approximately 10 mg of the probe under study (**5.31a** or **5.31b**) was placed in a 2.0 mL microcentrifuge tube along with 1.5 mL of reaction media (buffer or buffer / cosolvent mixtures). A 3 μL aliquot of a 50 mg/mL PLE solution (in 0.1 M phosphate buffer) was added (4.1 units total). The samples were loaded into an Eppendorf™ Thermomixer at 25 °C and 1400 RPM mixing rate. The samples were incubated and mixed continuously for three to five days for **5.31a** and **5.31b** respectively. Aliquots were taken at desired time intervals and analyzed by either LC-MS or LDI-ToF MS as described below. The observed % *ee* values were corrected for the optical purity of the

probe by dividing the observed % *ee* by the optical purity of the probe as determined by chiral HPLC analysis.[50]

LDI-ToF MS

A 200 μL aliquot of reaction media was placed in a glass vial and acidified with 30 μL of 10% HCl solution. The acidified media was then extracted with 200 μL of CH_2Cl_2. A 2 μL aliquot of the organic phase was spotted directly onto a polished steel Microscout® chip (Bruker Daltonics). The sample was allowed to dry at ambient temperature by gently passing a stream of air over the Microscout® chip. An average of 800 laser pulses were collected for each sample and the intensities of the peaks were used for calculation of enantiomeric excess.

ESI-ION TRAP MS

A 200 μL aliquot of reaction media was placed in a glass autosampler vial and diluted with 200 μL of methanol. The Accela autosampler was programmed to inject 1 μL onto a Hypersil Gold RP HPLC column (50 x 2.1 mm, ThermoFisher). The mobile phase was 70:30 methanol:water at a flow rate of 100 μL / minute. The LC-MS system was programmed to divert the initial two minutes of flow to waste to desalt the sample. The data was collected for the chromatographic peak in SIM mode and the intensities were used to calculate enantiomeric excess.

(*R*)-2-(4-(benzyloxymethyl)-3-ethoxy-2-methyl-3-oxopropanoic acid (2.31a):

Compound **2.31a** was synthesized using the procedure outlined in Chapter 2. The characterization data match that of authentic material. The % *ee* was determined by analytical chiral HPLC on either a Chriacel OJ-H (257 nm, flow rate = 1 mL / minute,

4% *i*PrOH / 96% hexane) $Rt_{(R)}$ = $Rt_{(R)}$ = 16.90, $Rt_{(S)}$ 19.20 or Chiracel AD-H (45°C, 257 nm, flow rate = 1 mL/minute, 4% *i*PrOH / 96% hexane) $Rt_{(R)}$ = 13.65, $Rt_{(S)}$ 15.20.

D^5-Diethyl 2-(benzyloxymethyl)-2-methylmalonate (5.31a): An amount of 0.45 g (1.7 mmol) of **2.32a** was dissolved in 25 mL of CH_2Cl_2 in a 50 mL round-bottom flask with a magnetic stirbar. Thionyl Chloride (1.8 mL, 25.2 mmol) was added and the solution was heated to reflux solvent for 19 hours. The solution was concentrated in vacuo to remove excess thionyl chloride and solvent. The acyl chloride was dissolved in 10 mL of dry CH_2Cl_2. A solution of 220 μL of Et_3N in 1 mL of d^6-ethanol was added dropwise to the stirring acyl chloride under a dry nitrogen atmosphere. The reaction was stirred overnight at ambient temperature. The reaction was diluted with 20 mL of ether and washed three times each with 10% HCl and 1.0 M NaOH. The organic layer was dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give **5.31a** as a clear, viscous oil 0.43 g (1.41 mmol 84% yield) TLC (30% ether / 70% hexane) R_f = 0.28. IR (cm^{-1}) 1727, ^{1}H-NMR (300MHz, $CDCl_3$) 1.21 (3H, t, *J*=7Hz), 1.52 (3H, s), 3.82 (2H, s), 4.16 (2H, q, *J*=7Hz), 4.53 (3H, s), 7.28 (5H, m). ^{13}C-NMR (75MHz, $CDCl_3$) 14.2, 18.6, 55.0, 61.5, 72.8, 73.6, 81.8, 127.6, 127.8, 128.5, 138.2, 170.8. HRMS: $[C_{16}H_{17}D_5O_5Na]^+$ Calcd = 322.1679, Obsd = 322.1670.

(±)-D^5/H^5 -2-(4-(benzyloxymethyl)-3-ethoxy-2-methyl-3-oxopropanoic acid (±-5.32a): An amount of 4.00g (13.6 mmol) of **2.31a** was dissolved in 11 mL of Ethanol and 0.790 g of KOH was added in a 50 mL round-bottom flask with a magnetic stirbar. After 48 hours, the reaction was diluted with 20 mL of water and extracted three times with 20 mL

of ether. The aqueous layer was then acidified to pH 2 and extracted three times with 20 mL of CH_2Cl_2, the combined organic layers were dried over $MgSO_4$, filtered and concentrated in vacuo to give the racemic half ester **2.32a** (2.21 g , 8.30 mmol, 61% yield). Then, ±-**2.32** (0.446 g, 1.68 mmol) was esterified by the same procedure reported to prepare **5.31a** to give 0.422 g (1.41 mmol, 84% yield) ±-**5.31a** diester. The racemic D^5/H^5 diester (0.357 g) was dissolved in a 80% *i*PrOH / 20% H_2O solution containing 95.5 μL of a 50% w/v aqueous NaOH solution. The reaction was monitored by TLC and was complete after 48 hours. The reaction mixture was diluted with 20 mL of 1.0 M NaOH and extracted three times with Et_2O. The aqueous layer was acidified to pH 2 and extracted three times with CH_2Cl_2, dried over $MgSO_4$, filtered and concentrated by rotary evaporation to give racemic D^5/H^5 half ester (**±5.32a**) (0.209 g, 0.775 mmol (based on average molecular weight), 65% yield). TLC (5% MeOH / 95% CH_2Cl_2) R_f = 0.317. ^{1}H-NMR (300MHz, $CDCl_3$) 1.23 (1.5H, t, J = 7 Hz), 1.54 (3H, s), 3.81 (2H, s), 4.20 (1H, q, J = 7 Hz), 4.55 (2H, s), 7.28 (5H, m). The % *ee* was determined by analytical chiral HPLC (Chiracel AD-H, 257 nm, flow rate = 1 mL / minute, 4% *i*PrOH / 96% hexane) $Rt_{(R)}$ = 14.97, $Rt_{(S)}$ 17.62.

(*R*)-2-(4-(benzyloxy)benzyl)-3-ethoxy-2-methyl-3-oxopropanoic acid (2.32b): Compound **2.32b** was synthesized according to the procedure outlined in Chapter 2. The % *ee* was determined by analytical chiral HPLC (Chiracel AD-H, 282 nm, flow rate = 1 ml / minute, 4% *i*PrOH / 96% hexane) $Rt_{(R)}$ = 20.87, $Rt_{(S)}$ 30.18.

(±) D^5/H^5-2-(4-(benzyloxy)benzyl)-3-ethoxy-2-methyl-3-oxopropanoic acid (+/-5.32b): An amount of 3.24 g (8.75 mmol) of **2.31b** was dissolved in 20 mL of ethanol

and 701 μL of a 50% w/v aqueous NaOH solution was added in a 50 mL round-bottom flask with a magnetic stirbar. After 24 hours, the reaction was diluted with 40 mL of water and washed three times with Et_2O. The aqueous layer was then acidified to pH 2 and extracted three times with CH_2Cl_2, dried over $MgSO_4$, filtered and concentrated in vacuo to give the racemic (2.27 g, 6.62 mmol, 76% yield) half-ester (±**2.32b**). The characterization data was identical to that reported in the literature.[51] Compound +/-**2.32b** was then esterified by the same procedure reported for **5.31b** to give 0.391g (1.043 mmol, 71% yield) of the racemic D^5/H^5 diester. The racemic D^5/H^5 diester (0.618g, 1.646 mmol) was then dissolved in 13 mL of a 80% *i*PrOH / 80% water solution and 132 μL of 50% w/v aqueous NaOH solution was added. The reaction was monitored by TLC (5% MeOH / 95% CH_2Cl_2) and was completed after 48 hours. The reaction was diluted with 20 mL of 1.0 M NaOH and washed three times with Et_2O. The aqueous layer was then acidified to pH 2 and extracted three times with CH_2Cl_2, dried over $MgSO_4$, filtered and concentrated in vacuo to give 0.49 g (0.078 Mol, 87% yield) of the racemic D^5/H^5 half ester (**+/-5.32b**). TLC (5% MeOH / 95% CH_2Cl_2) $R_f = 0.51$. ^{1}H-NMR (300MHz, $CDCl_3$) 1.26 (1.5H, t, $J = 7$ Hz), 1.40 (3H, s), 3.13 (1H, d, $J = 14$ Hz), 3.25 (1H, d, $J = 14$ Hz), 4.20 (1H, q, $J = 7$ Hz), 5.01 (2H, s), 6.88 (2H, d, $J = 8$ Hz), 7.08 (2H, d, $J = 8$ Hz)7.38 (5H, m).

D^5- Diethyl 2-(4-(benzyloxy)benzyl)-2-methylmalonate (5.31b): An amount of 0.50 g (1.46 mmol) of **2.32b** (from PLE hydrolysis) was dissolved in 15 mL of Methylene Chloride in a 25 mL round-bottom flask with a magnetic stirbar. Thionyl Chloride (2 mL, 27.8 mmol) was added and the solution was brought to reflux solvent for 15 hours.

The solution was concentrated in vacuo and the crude acyl chloride was dissolved in 10 mL of dry CH_2Cl_2.Then, 220 μL of Et_3N was dissolved in 1 mL of d^6-ethanol and added dropwise to the stirring acyl chloride. The reaction stirred for 15 hours at ambient temperature under an N_2 blanket. The reaction was diluted with 20 mL of Et_2O and washed three times each with 10% HCl and three times with1.0M NaOH. The organic layer was dried over $MgSO_4$, filtered, and concentrated in vacuo to give **5.31b** as a white amorphous solid. TLC: (10% EtOAc / 90% hexane) R_f= 0.22. This gave 0.39 g (1.04 mmol, 71% yield) of a white amorphous solid. Mp = 49° C, IR (cm^{-1}) 1726 , ^{1}H-NMR (300 MHz, $CDCl_3$) 1.24 (3H, t, *J* = 7 Hz), 1.33 (3H, s), 3.17 (2H, s), 4.18 (2H, q, *J* = 7 Hz), 5.01 (2H, s) 6.86 (2H, d, *J* = 9 Hz), 7.04 (2H, d, *J* = 9 Hz) 7.37 (5H, m). ^{13}C-NMR (75 MHz, $CDCl_3$) 13.1 (sept, *J* = 20 Hz), 14.2, 19.8, 40.4, 55.0, 60.6 (quint, *J* = 22 Hz), 61.5, 70.1, 114.6, 127.6, 128.0, 128.5, 128.7, 131.3, 137.1, 157.9, 172.1. HRMS: $[C_{22}H_{21}D_5O_5Na]^+$ calcd = 398.1992 obsd = 398.1983.

Dimethyl 2-(4-(benzyloxy)benzyl)-2-methylmalonate (5.37): Compound **5.37** was synthesized by a variation of a previous of literature procedure.[52] A 250 mL 3-neck round-bottom flask was charged with 100 mL of dry THF, a stirbar, and 1.30 g (32.4 mmol) of NaH (60% dispersion in mineral oil) under a nitrogen blanket. The flask was placed in an ice bath and allowed to stir for 15 minutes. A solution of dimethyl methylmalonate (3.95 g, 27 mmol in 5 mL of anhydrous THF) was added dropwise to the NaH suspension at 0 °C. Once H_2 evolution had ceased, the solution was allowed to stir at ambient temperature for 30 minutes. A solution of 1-(benzyloxy)benzyl)-4-(chloromethyl)benzene (5.78 g, 27.0 mmol, in 30 mL of anhydrous THF) was added

dropwise. The resulting solution was heated to reflux solvent for 15 hours. The solution was then allowed to cool to ambient temperature and 1 mL of water was added to quench any remaining NaH. The resulting suspension was diluted with 100 mL of ether, washed three times with brine, three times with 10% HCl, dried over $MgSO_4$, filtered, and concentrated by rotary evaporation. The crude material was dissolved in 20 mL of MeOH and 20 mL of ddH_2O was slowly added to precipitate the pure **5.37** which was isolated by vacuum filtration and dried over H_2SO_4 to give 5.01g (14.4 mmol, 54% yield) of a white amorphous solid. TLC (50% Et_2O / 50% hexane) $R_f = 0.40$.

(*R*)-2-(4-(Benzyloxy)benzyl)-3-methoxy-2-methyl-3-oxopropanoic acid (5.38): An amount of 2.67 g (7.80 mmol) of **5.37** was dispersed in 600 mL of rapidly stirring phosphate buffer (0.1 N, pH 7.4). Then, 40 mg of PLE (702 units) was dissolved in 1.0 mL of 3M $(NH_4)_2SO_4$ and added to the buffer solution. The pH of the reaction mixture was maintained using a 798 MPT Titrino® in the pH stat mode. The Titrino® was set to titrate to a volume of 7.50 mL (1 eq of 1.04 M NaOH). The hydrolysis proceeded for 96 hours, after which time the solution was made basic by addition of 1.04 M NaOH. The aqueous solution was then extracted three times with 300 mL portions of ether and the aqueous layer was acidified using concentrated H_2SO_4 to a pH of 2. The aqueous layer was then extracted three times with CH_2Cl_2 and four times with Et_2O. The organic layers were combined, dried over $MgSO_4$, filtered, concentrated by rotary evaporation, and purified by flash chromatography to give 1.93 g (5.88 mmol, 75% yield, 80% *ee*) of a white, amorphous solid. TLC (5% MeOH / 95% CH_2Cl_2) $R_f = 0.33$. $[\alpha]_D^{24} = -5.10°$ (c =8.00, $CHCl_3$). Mp = 142 - 146 °C. IR (cm^{-1}) 1734, 1707. ^{1}H-NMR (300MHz, $CDCl_3$)

1.42 (3H, s), 3.13 (1H, d, J = 14 Hz), 3.25 (1H, d, J = 14 Hz), 3.76 (2H, s), 5.03 (2H, s), 6.89 (2H, d, J = 9 Hz), 7.06 (2H, d, J = 9 Hz), 7.38 (5H, m). ^{13}C-NMR (75MHz, $CDCl_3$) 20.4, 41.2, 53.0, 55.1, 70.2, 114.8, 127.7, 128.1, 128.2, 128.8, 131.3, 137.1, 158.2, 173.2, 176.7. HRMS: $[C_{19}H_{20}O_5Na]^+$ Calcd = 351.1203 Obsd = 351.1199. The % *ee* was determined by analytical chiral HPLC (Chiracel AD-H, 282 nm, flow rate = 1 ml / minute, 4% *i*PrOH / 96% hexane, 42 °C) $Rt_{(R)}$ = 20.52, $Rt_{(S)}$ 31.15.

(+/-)-2-(4-(Benzyloxy)benzyl)-3-methoxy-2-methyl-3-oxopropanoic acid (5.38): An amount of 5.01 g (14.6 mmol) of **5.37** was dissolved in 70 mL of MeOH and 1.17 mL (14.6 mmol) of a 12.50 M NaOH was added and the solution was allowed to stir at ambient temperature for 48 hours. The reaction was then diluted with 50 ml of 1.0 M NaOH, washed three times with Et_2O, acidified with concentrated H_2SO_4 at 0 °C, and extracted into Et_2O. The Et_2O was dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to give 3.68 g (11.2 mmol, 77% yield) of a white, amorphous solid.

D_3-Dimethyl 2-(4-(benzyloxy)benzyl)-2-methylmalonate (5.39): An amount of 0.23 g (0.70 mmol) of **(*R*)-5.38** (from PLE hydrolysis) was dissolved in 10 mL of anhydrous CH_2Cl_2 and 757 µL of $SOCl_2$ (10.5 mmol) was added and the solution was brought to reflux solvent for 19 hours. The solution was concentrated in vacuo and the crude acyl chloride was dissolved in 1 mL of D_3-methanol. Then, 1 mL $SOCl_2$ and 10.5 µL of Et_3N was added and the reaction was allowed to stir at room temperature for 17 hours. The solution was diluted with 10 mL of CH_2Cl_2 and washed three times each with 10% HCl. The organic layer was dried over $MgSO_4$, filtered, and concentrated by rotary evaporation

to give 1.99g (0.58 mmol, 82% yield) of **5.39** as a white amorphous solid. TLC (30% EtOAc / 70% hexane) R_f= 0.41, Mp = 75 - 78° C, IR (cm^{-1}) 1720, ^{1}H-NMR (300 MHz, $CDCl_3$) 1.36 (3H, s), 3.18 (2H, s), 3.73 (3H, s), 5.03 (2H, s), 6.88 (2H, d, *J* = 9 Hz), 7.03 (2H, d, *J* = 9 Hz) 7.39 (5H, m). ^{13}C-NMR (75 MHz, $CDCl_3$) 19.9, 40.7, 52.7, 55.2, 70.1, 114.7, 127.7, 128.1, 128.4, 128.7, 131.3, 137.2, 158.0, 172.6. HRMS: $[C_{20}H_{19}D_3O_5Na]^+$ Calcd = 368.1548 Obsd = 368.1542.

(*S*)-methyl-3-(4-(benzyloxy)phenyl)-2-(benzyloxycarbonylamino)-2-methylpropanoate (5.40): An amount of 1.00 g (3.05 mmol) of **5.38** was dissolved in 10 mL of Dichloroethane and 722 µL (3.35 mmol) of DPPA and 1.37 µL (9.12 mmol) of Et_3N was added and the solution was allowed to stir at room temp for 30 minutes. The solution was heated to reflux solvent for 1.5 hours, at which time 473 µL (4.58 mmol) of benzyl alcohol was added and the solution was allowed to reflux for 17 hours. The mixture was then cooled and diluted with 20 mL of CH_2Cl_2, washed two times with 10% HCl, filtered through a plug of silica gel, and concentrated by rotary evaporation. The residue was purified by flash chromatography (40% EtOAc / 60% hexane) with 200 mL of silica gel to give 1.23 g (2.84 mmol, 93% yield, 80% *ee*) of a clear, viscous oil. TLC (40% EtOAc / 60% hexane) Rf = 0.44. $[\alpha]_D^{23.5}$ = -27.1° (c = 10.3, MeOH). IR (cm^{-1}) 3352, 1719. ^{1}H-NMR (300 MHz, $CDCl_3$) 1.66 (3H, s), 3.16 (1H, d, *J* = 14 Hz), 3.39 (1H, d, *J* = 14 Hz), 3.77 (3H, s), 5.03 (2H, s), 5.11 (1H, d, *J* = 12 Hz), 5.22 (1H, d, *J* = 12 Hz), 5.55 (1H, bs), 6.84 (2H, d, *J* = 9 Hz), 6.92 (2H, d, *J* = 9 Hz), 7.41 (10H, m). ^{13}C-NMR (75 MHz, $CDCl_3$) 23.6, 41.0, 52.7, 61.0, 66.5, 70.0, 114.7, 127.6, 128.1, 128.2, 128.3,

128.4, 128.6, 128.7, 131.0, 136.7, 137.1, 154.7, 157.9, 174.2. HRMS: $[C_{26}H_{27}NO_5Na]^+$ calcd = 456.1781 obsd = 456.1773.

(*S*)-α-Methyl Tyrosine Methyl Ester (5.41): An amount of 0.50 g of Pd / C was carefully suspended in 5 mL of MeOH. Then, 1.26 g (2.85 mmol) of **5.40** was dissolved in 30 mL of MeOH and slowly added to the flask. The solution was degassed with hydrogen gas for 15 minutes placed under a Hydrogen blanket for 19 hours. The disappearance of **5.40** was monitored by TLC (40% EtOAc / 60% hexane). The reaction was filtered through a celite pad to remove the Pd/C and concentrated by rotary evaporation. The material was dissolved in 10 ml of 3N methanolic HCl to form the chloride salt of α-methyl tyrosine and the excess MeOH was removed by rotary evaporation to give a clear, viscous liquid. The liquid was washed three times with Et_2O which resulted in the formation 0.69g (2.81 mmol, 99% yield) of a white amorphous solid. The dextrorotary direction of rotation $[\alpha]_{obs}^{23\ °C} = +0.42°$ (c = 7.1, MeOH) of **5.41** matched that of an authentic sample $[\alpha]_{obs}^{23\ °C} = +0.33$ (c = 5.0, MeOH). ^{1}H-NMR (300 MHz, CD_3OD) 1.47 (3H, s), 2.88 (1H, d, J = 14 Hz), 3.08 (1H, d, J = 14 Hz), 6.66 (2H, d, J = 8 Hz), 6.88 (2H, d, J = 9 Hz).

References

(1) Thayer, A. M. *Chemical and Engineering News* **2006**, *84*, 15-25.

(2) Masterson, D.; Roy, K.; Rosado, D.; Fouche, M. *Journal of Peptide Science* **2008**, *14*, 1151-1162.

(3) Agricultural Statistics Board. "2009 Liverstock Slaughter," National Agricultural Statistics Service, 2009.

(4) Bornscheuer, U. T.; Kazlauskas, R. J. *Hydrolases in Organic Synthesis: Regioselective and Stereoselective Biotransformations*; Second ed.; Wiley-VCH, 2006.

(5) Toone, E. J.; Werth, M. J.; Jones, B. *Journal of the American Chemical Society* **1990**, *112*, 4946-4952.

(6) Bornscheuer, U. T.; Kazlauskas, R. J. *Hydrolases in Organic Synthesis: Regioselective and Stereoselective Biotransformations*; Second ed.; Wiley-VCH, 2006.

(7) Fadel, A.; Garcia-Argote, S. *Tetrahedron Assymmetry* **1996**, *7*, 1159-1166.

(8) de María, P. D.; Kossmann, B.; Potgrave, N.; Buchholz, S.; Trauthwein, H.; May, O.; Gröger, H. *Synlett* **2005**, 1746-1748.

(9) Kedrowski, B. L. *Journal of Organic Chemistry* **2003**, *68*, 5403-5406.

(10) Crameri, A.; Raillard, S.-A.; Bermudez, E.; Stemmer, W. P. C. *Nature* **1998**, *391*, 288.

(11) Reetz, M.; Wilensek, S.; Zha, D.; Jaeger, K. *Angewandte Chemie International Edition* **2001**, *40*, 3589-3591.

(12) Stemmer, W. P. C. *Nature* **1994**, *370*, 389.

(13) Reetz, M. T.; Schrader, W.; Eipper, A.; Pugh, J. *Canadian Journal of Chemistry* **2002**, 626-632.

(14) Detlev, B.; Martin, L.; Li-Wen, W.; Manfred, T. R. *Angewandte Chemie International Edition* **2006**, 45, 2463-2466.

(15) Reetz, M.; Becker, H.; Liebl, M.; Alois, F. *Angewandte Chemie International Edition* **2000**, *39*, 1236-1239.

(16) Reetz, M. T.; Becker, M. H.; Klein, H.-W.; Stockigt, D. *Angewandte Chemie-International Edition* **1999**, *38*, 1758-1761.

(17) Reetz, M. T.; Carballeira, J. D. *Nature Protocols* **2007**, *2*, 891-903.

(18) Becker, S.; Hobenreich, H.; Vogel, A.; Knorr, J.; Wilhelm, S.; Rosenau, F.; Jaeger, K.; Reetz, M.; Lolmar, H. *Angewandte Chemie International Edition* **2008**, *47*, 5085-5088.

(19) Guanti, G.; Banfi, L.; Narisano, E.; Riva, R.; Thea, S. *Tetrahedron Letters* **1986**, *27*, 4639-4642.

(20) Guanti, G.; Banfi, L.; Narisano, E. *Tetrahedron Letters* **1989**, *30*, 2697-2698.

(21) Lam, L. K. P.; Hui, R. A. H. F.; Jones, J. B. *The Journal of Organic Chemistry* **1986**, *51*, 2047-2050.

(22) Wallert, S.; Drauz, K.; Grayson, I.; Groger, H.; Dominguez de Maria, P.; Bolm, C. *Green Chemistry* **2005**, *7*, 602-605.

(23) Menger, F. M.; Eliseev, A. V.; Migulin, V. A. *The Journal of Organic Chemistry* **1995**, *60*, 6666-6667.

(24) David, B. B.; Mohua, B.; Sungjo, C. *Angewandte Chemie International Edition* **2002**, *41*, 1603-1607.

(25) Berkowitz, D. B.; Maiti, G. *Organic Letters* **2004**, *6*, 2661-2664.

(26) Sareen, R.; Bornscheuer, U.; Mishra, P. *Analytical Biochemistry* **2004**, *333*, 193-195.

(27) Dey, S.; Karukurichi, K. R.; Shen, W.; Berkowitz, D. B. *Journal of the American Chemical Society* **2005**, *127*, 8610-8611.

(28) Sangeeta, D.; Douglas; Powell, R.; Chunhua, H.; David; Berkowitz, B. *Angewandte Chemie International Edition* **2007**, *46*, 7010-7014.

(29) Patrick Tielmann, M. B., Martin Luft, Manfred T. Reetz, *Chemistry - A European Journal* **2003**, *9*, 3882-3887.

(30) Taylor, S. J.; Morken, J. P. *Science* **1998**, *280*, 267.

(31) Arnold, H.; Hans-Werner, S.; Wilhelm, F. M. *Angewandte Chemie International Edition* **1998**, *37*, 2644-2647.

(32) Reetz, M.; Becker, M.; Klaus, M. K.; Holzwarth, H. *Angewandte Chemie International Edition* **1998**, *37*, 2647-2650.

(33) Korbel, G. A.; Lalic, G.; Shair, M. D. *Journal of the American Chemical Society* **2001**, *123*, 361-362.

(34) Reetz, M.; Eipper, A.; Tielmann, P.; Mynott, R. *Advanced Synthesis & Catalysis* **2002**, *344*, 1008-1016.

(35) Reetz, M. T.; Tielmann, P.; Eipper, A.; Ross, A.; Schlotterbeck, G. *Chemical Communications* **2004**, 1366-1367.

(36) Zu, C.; Brewer, B. N.; Wang, B.; Koscho, M. E. *Analytical Chemistry* **2005**, *77*, 5019-5027.

(37) Yao, Z.-P.; Wan, T. S. M.; Kwong, K.-P.; Che, C.-T. *Analytical Chemistry* **2000**, *72*, 5394-5401.

(38) Shizuma, M.; Imamura, H.; Takai, Y.; Yamada, H.; Takeda, T.; Takahashi, S.; Sawada, M. *International Journal of Mass Spectrometry* **2001**, *210-211*, 585-590.

(39) Masami, S.; Yamaoka, H.; Takai, Y.; Kawai, Y.; Yamada, H.; Azuma, T.; Fujioka, T.; Tanaka, T. *International Journal of Mass Spectrometry* **1999**, 123-130.

(40) Markert, C.; Rosel, P.; Pfaltz, A. *Journal of the American Chemical Society* **2008**, *130*, 3234-3235.

(41) Markert, C.; Pfaltz, A. *Angewandte Chemie International Edition* **2004**, *43*, 2498-2500.

(42) Szewczyk, J. W.; Zuckerman, R. L.; Bergman, R. G.; Ellman, J. A. *Angewandte Chemie International Edition* **2001**, *40*, 216-219.

(43) Heidel, H.; Huttner, G.; Zsolnia, L. *Zeitschriftz fur Naturforschung. B, A Journal of Chemical Sciences* **1995**, *50*, 729-734.

(44) Cawley, A.; Duxbury, J. P.; Kee, T. P. *Tetrahedron: Asymmetry* **1998**, *9*, 1947-1949.

(45) de Hoffmann, E.; Stroobant, V. *Mass Spectrometry: Principles and Applications*; Third ed.; John Wiley and Sons Ltd., 2007.

(46) Schmitke, J. L.; Wescott, C. R.; Klibanov, A. M. *Journal of the American Chemical Society* **1996**, *118*, 3360-3365.

(47) Carrea, G.; Ottolina, G.; Riva, S. *Trends in Biotechnology* **1995**, *13*, 63-70.

(48) Mattson, A.; Boutelje, J.; Csoregh, I.; Hjalmarsson, M.; Jacobsson, U.; Lindback, M.; Norin, T.; Szmulik, P.; Hult, K. *Bioorganic and Medicinal Chemistry* **1994**, *2*, 501-508.

(49) Masterson, D.; Rosado, D.; Nabors, C. *Tetrahedron Assymmetry* **2009**, *20*, 1476-1486.

(50) Cawley, A.; Duxbury, J. P.; Kee, T. P. *Tetrahedron: Asymmetry* **1998**, *9*, 1947-1949.

(51) Heidel, H.; Huttner, G.; Zsolnai, L. *Zeitschrift für Naturforschung* **1995**, *50*, 729-734.

(52) Annunziata, R.; Benaglia, M.; Cinquini, M.; Cozzi, F.; Pitillo, M. *The Journal of Organic Chemistry* **2001**, *66*, 3160-3166.

CHAPTER VI

FUTURE DIRECTIONS

Introduction

It seems that each experiment preformed in the laboratory brings another question that would be interesting to answer. The work described in this dissertation is no exception to this. There have been many interesting discussions on the possible uses of the compounds that have been synthesized during the duration of this project. However, research is a story that is always in the process of being completed. This chapter will focus on what is needed to complete the work described in Chapters II though V with some possible expansions of the project. Methods that may be used to obtain compound **2.32b** in greater than 97% *ee*, including possible conversion of **2.32a** into **2.32b** will be discussed. This will include application of the mass spectrometry based assay that was outlined in Chapter 6.[1] Also discussed is a potential method to increase the throughput of our mass spectrometry assay. Once compound **2.32b** is obtained in sufficient optical purity, synthesis and testing of neurotensin (NT) analogues will proceed. Hopefully, future work on this project will allow for completion of this project and application of the described unnatural amino acids for use in NT peptidomimetic compounds.

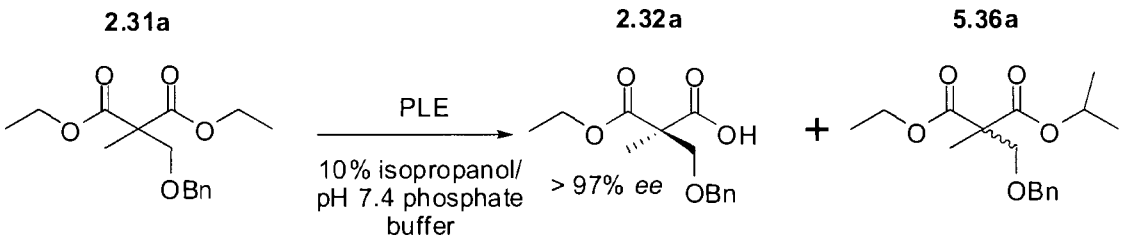
Future Directions

Does Transesterification Influence the Enantioselectivity of Pig Liver Esterase?

As demonstrated in Chapter V, use of various concentrations of isopropanol and *tert*-butanol as cosolvents gave a significant increase in the enantioselectivity of the PLE hydrolysis of probes **5.31a**, **5.31b**, and **5.39**.[1] Attempts have been made to establish predictive models for the enantioselectivity of enzymes in cosolvents.[2] It was concluded

that any correlations seen with the dielectric constant or hydrophobicity (logP) of the cosolvents were enzyme and substrate specific and were not sufficient for establishing a model for other substrates. In our studies of PLE enantioselectivity it was also observed that the effects of each cosolvent were dependant on the substrate that was being studied (see Table 5.1, page 151).

It is possible that some solvents, or even buffers, act as nucleophiles and assist in the observed increase in enantioselectivity of PLE. Some groups have observed the buffer acting as an alternate nucleophile to water. The buffer reacts with a single enantiomer of the product when formed in the enzyme active sight; leading to an the increase in enantioselectivity.[3] It may be possible that the increase in enantioselectivity seen in the PLE hydrolysis of our diesters with isopropanol cosolvent is due, in part, to isopropanol acting as an alternative nucleophile to water (Scheme 6.1). Formation of the **5.36a** has been observed when isopropanol was used as a cosolvent for the PLE hydrolysis of **5.31a**. Isolation of the isopropyl ethyl diester (**5.36a**), followed by analysis by chiral HPLC would allow us to determine if transesterification contributed to the observed increase in enantioselectivity. However, it is also worth noting that the increased selectivity seen when *tert*-butanol was used as a cosolvent was not accompanied by any detectable transesterification. This means that although transesterification may play a role in increasing enantioselectivity, it is not the only contributing factor. It may be that the effect of cosolvents on enzymatic hydrolyses is far too complex to be narrowed to a single mechanism and will require extensive study, but this is beyond the scope of this dissertation.



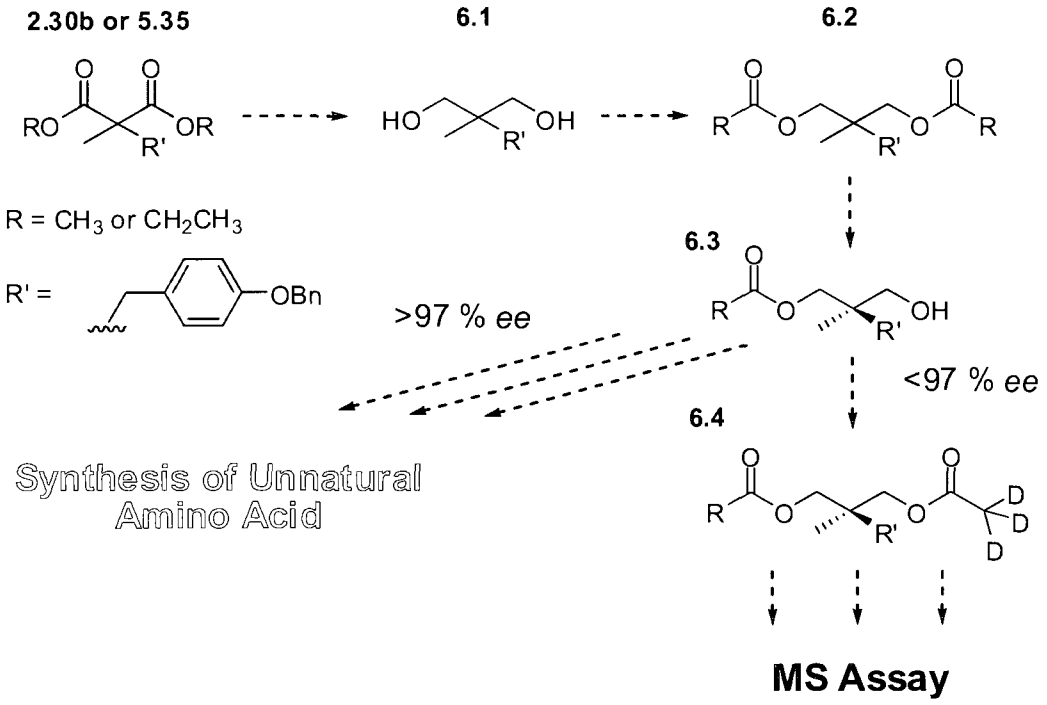
Scheme 6.1. Transesterification observed in the PLE hydrolysis of **2.31a** in 10% *i*PrOH / pH 7.4 phosphate buffer

*Increasing Enantioselectivity of PLE for **2.32b** above 85% ee*

The effect of cosolvents on the enantioselectivity of PLE is well documented even though the mechanism remains unknown. In Chapter 5, it was demonstrated that the PLE hydrolysis of **2.31b** to **2.32b** in 30% isopropanol / 70% phosphate buffer could be obtained in 85% *ee*. It was also noted that concentrations of the cosolvents above 30% led to a low rate of conversion and low yield (less than 10%). Further work is needed to determine conditions that yield greater than 97% *ee* for the PLE hydrolysis of **2.31b**. It may be possible to add ionic liquids to increase the rate of hydrolysis with higher concentrations of cosolvents.[4] This may result in an increase in enantioselectivity above 85% *ee* for **2.32b**. If addition of ionic liquids and / or higher concentrations of cosolvents to the PLE hydrolysis of **2.31b** fails to yield greater than 97% *ee*, it will be necessary to find other enzymes that are capable of producing high enantioselectivity for ester hydrolysis. Horse liver esterase, rabbit liver esterase, choline esterase, and Bacillus *substilis* esterase have proven to be enantioselective enzymes, and could be evaluated for hydrolysis of our diesters.[5-8]

In addition to the numerous available esterases, there are also a number of lipases that have been shown to resolve chiral alcohols by cleavage of acetates or resolve chiral acids by cleavage of esters.[9,10] Some lipases, such as *pseudomonas* lipase, have shown the

ability to resolve prochiral diesters.[11,12] Porcine pancrease lipase has been used for resolution of chiral diesters.[13] These lipases can be evaluated for selectivity with prochiral malonate diester substrates using our mass spectrometry based assay for enantioselectivity. If the diester probes fail to yield notable enantioselectivity with lipases, the diesters can be converted to diacetates that may be enantioselectively hydrolyzed with lipases.[14] The conversion of **2.31b** or **5.57** to diacetates can be accomplished by reduction of the diester of interest to a diol by treatment with lithium aluminium hydride, followed by acetylation with acetic anhydride.[15] Similar diacetates have been hydrolyzed selectively by Lipases. The resulting diacetate can be evaluated with various lipases and esterases and if conditions are found that result in greater than 97% *ee*, the half acetates can be can be converted to a series of unnatural amino acids. However, if the enantioselectivity is less than 97% *ee*, the half-acetate can be converted to a probe (**6.4**) that can be used in our mass spectrometry assay for enantioselectivity. This can be accomplished by treating **6.3** with D_3-acetic anhydride. Cosolvents have the same effect on lipases that is seen with PLE.[2,14] This should allow **6.4** to be used as a probe for media engineering of lipases under a variety of conditions.

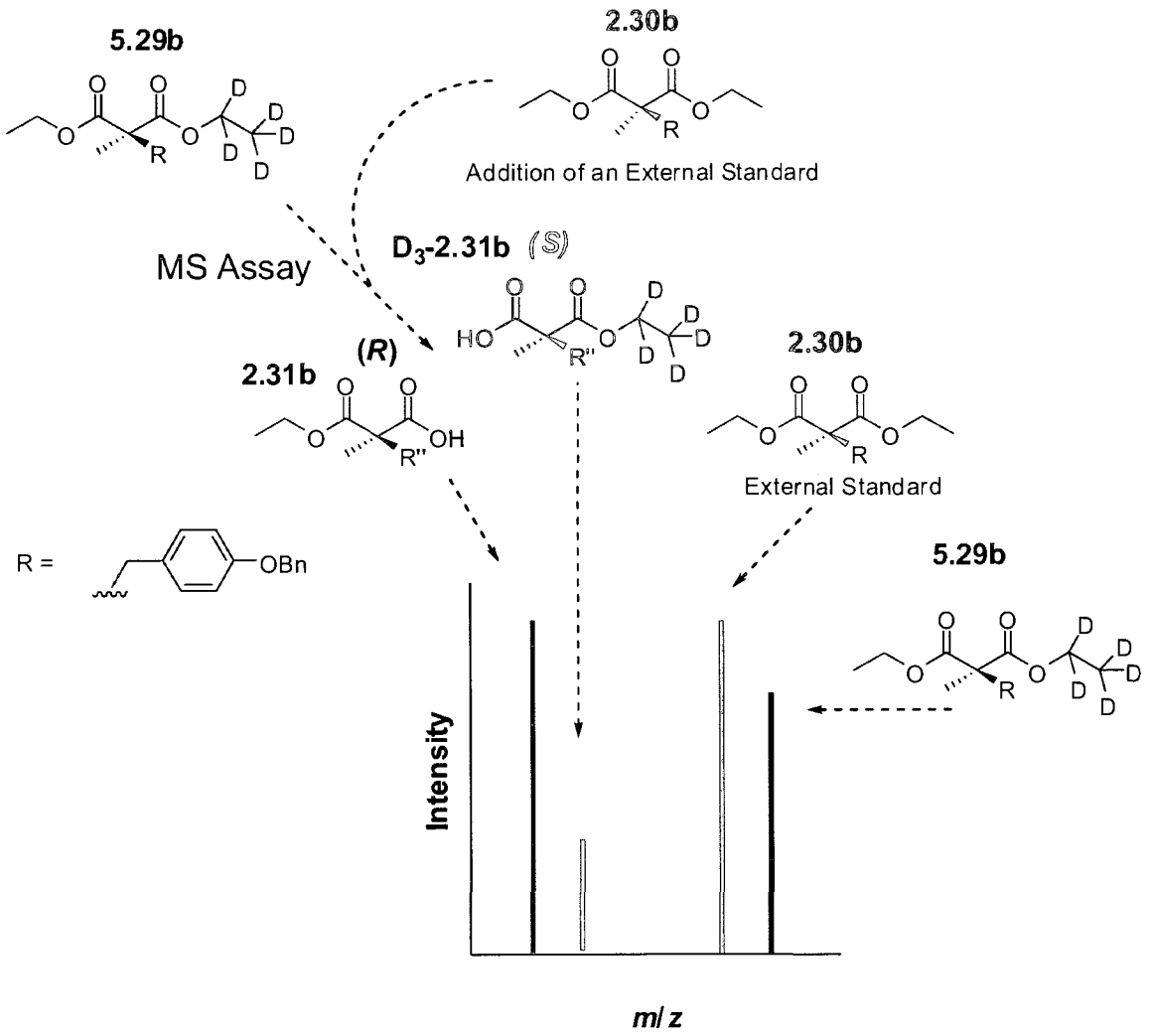


Scheme 6.2. Conversion of a **2.31b** or **5.37** into a diacetate subsequent synthesis of a D_3-diacatate mass spectrometry probe (**6.4**)

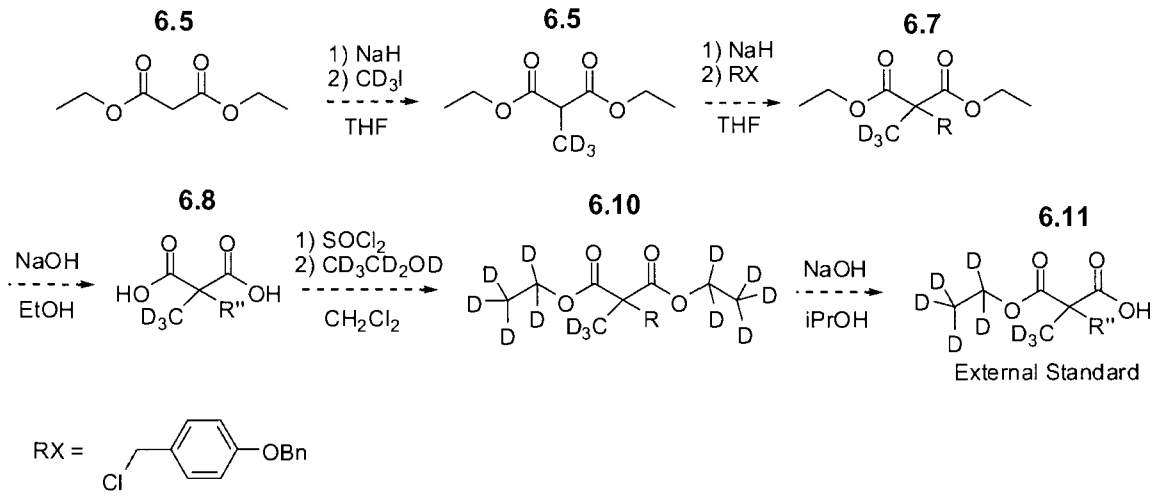
Obtaining Quantitative Information for the Mass Spectrometry Assay

We would like to extend the mass spectrometry assay for enantioselectivity to give information on the rate and yield of the enzymatic reactions. One possibility that would allow us to monitor both rate and yield of the desymmetrization of a malonate diester is the isotopic dilution method for quantitation.[16] This method requires the addition of an isotopomer as an external standard directly before analysis of the sample by LC-MS. The isotopomer should be ionized to the same extent as the molecule of interest, so if the concentration of the standard is known, the concentration of the analyte can be calculated. To use this method for our MS assay would require addition of an isotopomer of the diester probes or half ester product to the aliquots to be analyzed. This is convenient because the H_{10} or H_6- diesters (**2.31a**, **2.31b**, or **5.37**) are intermediates in

synthesis of the D_5/H_5-diester probes and can be used as the needed isotopomer. As an example, use of the H_{10} diester (**2.31b**) would allow us to monitor the rate of the disappearance of the **5.31b** (Scheme 6.3). The rate of the disappearance of the **5.31b** should be equal to the rate of the reaction. This relationship should also exist for the yield of the reaction. However, if there is any decarboxylation of the **2.32b**, the yield could appear to be higher than the actual yield of the reaction because the amount of decarboxylated half-ester would not be factored into the calculations for yield. To resolve this issue, an isotopomer of the half-ester could be used to directly monitor the rate and yield of the half ester probe. This would require the synthesis of a D_8-half ester that could be used as an external standard (Scheme 6.4). However this synthesis will require the use and waste of multiple deuterated materials.



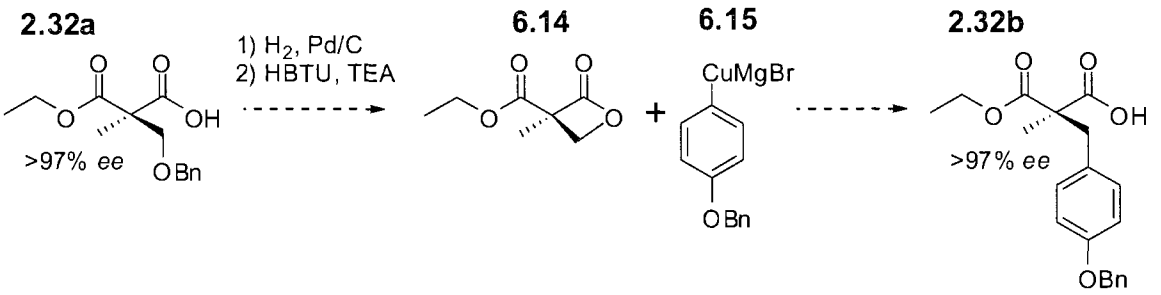
Scheme 6.3. Example of the addition of an H_{10}-diester as an external probe and the representative mass spectra



Scheme 6.4. Synthesis of a D_8-half ester as an external probe

If all of the methods described above fail to allow for the production of **2.31b** in greater than 97% *ee*, it may be possible to convert **2.31a** to **2.31b**. Literature has shown that ring opening of β-lactones synthesized from α-methyl serine with nucleophiles has led to the synthesis of various α-methyl amino acids.[17,18] The nucleophile reacts almost exclusively at the β-carbon which give an α-methyl amino acid. Smith et al. were able to successfully ring open **6.12** (Scheme 6.5) with a variety of organocuprates to yield 11 α-methyl amino acids with proteinogenic and non-proteinogenic sidechains.[19] It may be possible to convert **2.31a** to a β-lactone (**6.14**) by hydrogenolysis of the benzyl protecting group followed by treatment with O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexaflourophosphate (HBTU).[19] Treatment of **6.14** with **6.15** should result in the desired **2.31b** in greater than 97% *ee*. The procedure outlined by Smith et al. made use of catalytic amount of copper. However, it is possible that the organocuprate may react with ester moiety of the **6.14**. For this reason, it may be necessary to use stoichiometric amounts of copper to transform **6.14** to **2.32b**. If **2.32b** can be obtained in greater than

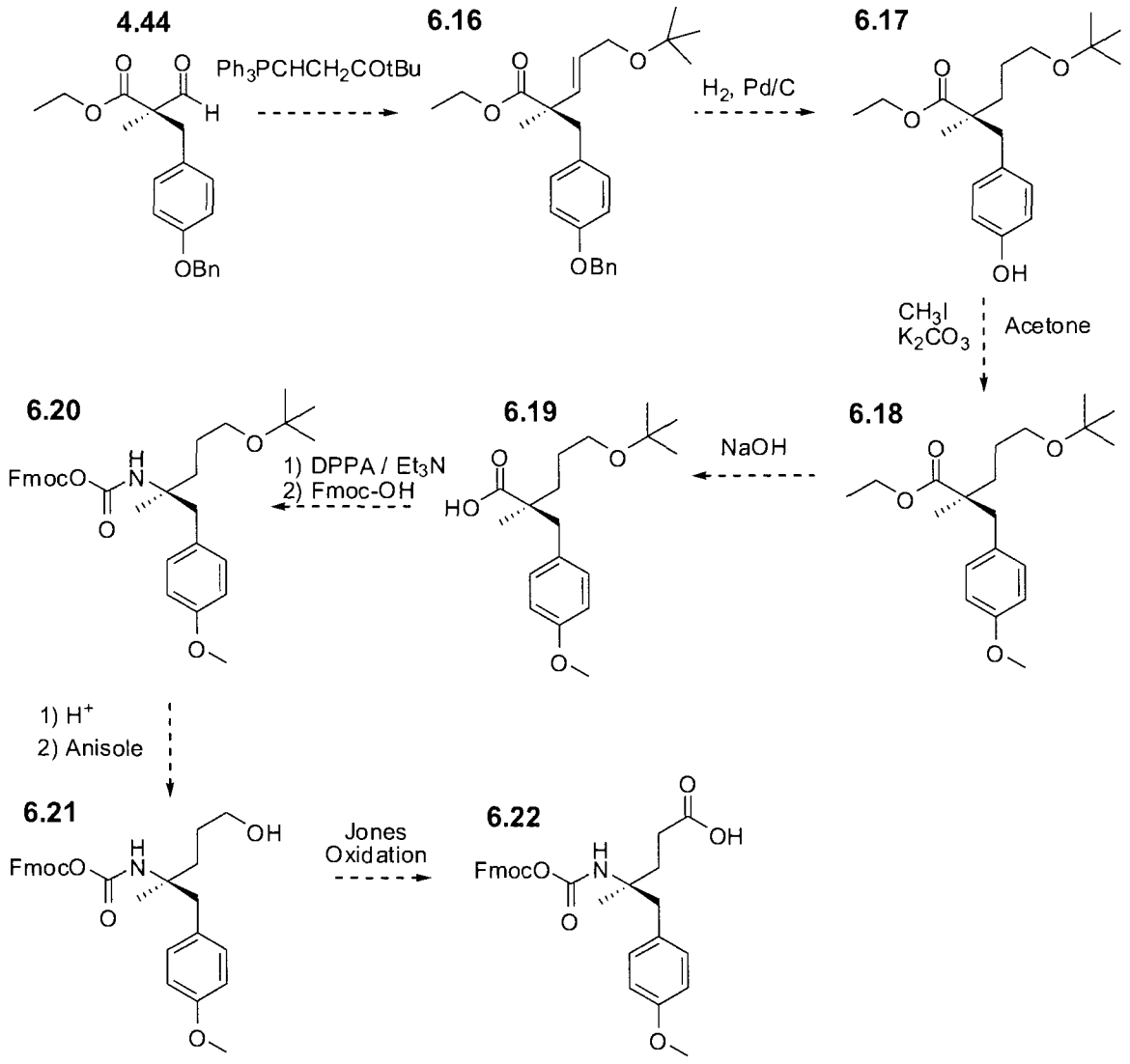
97% *ee* through one of the methods outlined above, synthesis of the desired unnatural amino tyrosine analogues can proceed.



Scheme 6.5. Synthesis of a **2.32b** from **2.32a** through a β-lactone intermediate

Synthesis of (S)-$\gamma^{4,4}$-Tyrosine

In Chapter IV, it was noted that the attempted saponification of **4.47** did not yield **4.50**, but instead hydrolysis of the *tert*-butyl ester was observed to give **4.48** (see Scheme 4.5 on page 105). A solution to this problem is to change the Wittig reagent to a *tert*-butyl ether instead of a *tert*-butyl ester (total synthesis illustrated in Scheme 6.6). This should allow for the ester of **6.19** to be saponified. Compound **6.19** must be subjected to a Curtius rearrangement that is quenched with Fmoc-OH to generate **6.20** before the *tert*-butyl ether is cleaved by treatment with H^+ in the presence of a *tert*-butyl ion scavenger such as anisole.[20] Otherwise, a competing nucleophile may be generated that can react with the isocyanate that is formed in the Curtius rearrangement to give a cyclic carbamate. Compound **6.21** can be oxidized to the an acid by treatment with Jones reagent to yield the desired (*S*)-$\gamma^{4,4}$-tyrosine (**6.22**).[21]



Scheme 6.6 . Alternative synthesis of (*S*)- $\gamma^{4,4}$-tyrosine (**6.22**)

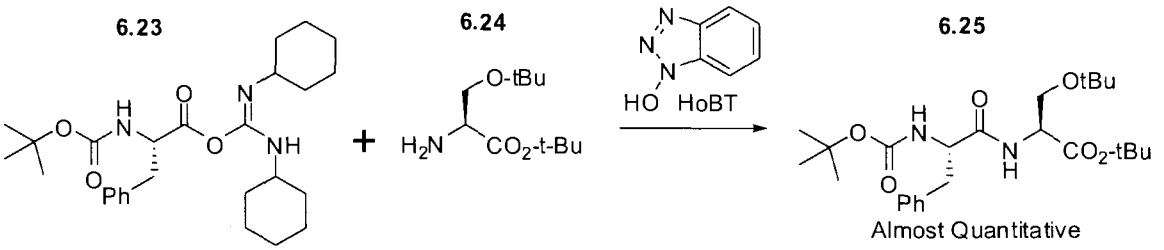
Synthesis and Evaluation of the Stability of Neurotensin Peptidomimetics

Synthesis of neurotensin (NT) analogues can be conducted once conditions have been determined that yield acceptable optical purity for **2.32b** and the desired unnatural tyrosine analogues have been synthesized. There are two options to obtain the desired neurotensin peptidomimetics: 1) the peptidomimetics can be synthesized commercially using our unnatural tyrosine analogues or 2) the peptidomimetics can be synthesized in

our laboratory using solid-phase peptide synthesis. The peptidomimetic can then be tested *via* a previously developed assay for neurotensin degradation. Since NT(8-13) is a small peptide and should be relatively easy to synthesize, I will discuss method that will allow for the completion of this research project in house.

There are several published procedures for the solid phase synthesis of neurotensin.[22-28] Chang et al. first used a 4-(hydroxymethyl)phenylacetamidomethyl-resin (PAM) for the solid phase synthesis NT analogues.[29] It was found that the PAM resin gave double the yield of the more commonly used oxymethyl-copoly-(styrene-divinylbenzene) resin. The reason for the increased yield was that the resin-amino acid linkage for PAM was 100 fold more stable during peptide synthesis than the oxymethyl-copoly-(styrene-divinylbenzene) resin. For this reason, a PAM resin should be used for the solid phase peptide synthesis of our NT(8-13) analogues with the unnatural Tyr residues. A convergent synthetic strategy can be used to limit the number of steps, and increase the yield of the peptide synthesis. A convergent synthesis will increase the overall yield of the peptide synthesis by decreasing the total number of steps required as compared to a linear (one amino acid at a time) synthesis. For example, if each step in a linear peptide synthesis results in a 90 % yield, the six-residue NT(8-13) would be coupled with a 50 % overall yield. Therefore, each step that is removed from the synthesis by use of a convergent strategy should result in a 10% increase in overall yield of the peptide. This is particularly important because multiple NT analogues will be synthesized. Each cycle also requires 8 to 16 hours for coupling of each amino acid residue.[30] Any step which limits the amount of time that the synthesis requires will make the entire peptide synthesis more efficient. Amino acid dimers, such as the 8-Arg -9-Arg,

can be synthesized in solution *via* Dicyclohexylcarbodiimide (DCC) amino acid coupling in the presence of N-hydroxybenzotriazole (HOBt) using Boc (*tert*-Butoxycarbonyl) protected amino acids (Scheme 6.7).[31] This will allow us to make bulk quantities of the required amino acid dimers that can be used in the synthesis of each NT(8-13) analogue.



Scheme 6.7. Example of a DCC peptide coupling of amino acids in the presence of HoBT

Synthesis of our NT(8-13) analogues can be accomplished using methods similar to the those used by Bitterman et al.[30] The Lue[13] preloaded PAM resin is commercially available. The preloaded Leu residue can be deprotected by treatment with 50% TFA / CH_2Cl_2 / Indole, followed by neutralization with N,N-Diisopropylethylamine (DIPEA). The Ile[12] can be coupled to Lue[13] by treatment of the Boc-Ile with 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU,3-5 eq) to activate the acid of Ile[12], followed by addition of DIPEA and 8-16 hours of agitation. Complete acylation can be monitored by the Kaiser test (Ninhydrin test). The remainder of the amino acid residues can be coupled using the same procedure with exception of the Pro residue. The Pro residue must be coupled using Fmoc-Pro that is activated by bis-(trichloromethyl)carbonate (BTC) in the presence of 2,6-lutidine. The F-moc group can be removed with piperidine / diazobicycloundecane (DBU) to facilitate the next coupling step. Once peptide synthesis is complete, the Boc-group can be removed by treatment with trifluoroacetic acid.[30] Removal of the arginine and tyrosine protecting groups and

cleavage of the peptide from the resin can be facilitated by treatment with fluoric acid in the presence of a *tert*-butyl cation scavenger.[30] The peptide can then be purified by reverse phase preparative HPLC and is ready to be subjected to degradation studies.

Degradation studies can be conducted using established literature procedures.[27,32-34] For controls, native neurotensin and NT(8-13) can be used. Both are commercially available. This will allow us to compare the stability of our NT(8-13) analogues to the natural NT and NT(8-13) fragments. Our NT(8-13) will be incubated in a mixture of enzymes which exhibit high specific activity for the Pro-Tyr and Tyr-Ile peptides bonds. Pepsin and Chymotrypsin, as examples, hydrolyze peptide bonds on the amino-terminal side of aromatic amino acid residues.[35] To mimic *in vivo* conditions in the human body, our neurotensin analogues can be incubated in human serum (50% serum / 50% phosphate buffered Saline (PBS)) at 37 °C. At preset integrals an aliquot can be taken and reverse phase HPLC can be used to determine the amount of degradation that has taken place.[27,33,34] The degradation products can be determined by LC-MS.

Kokko et al. developed an *in vitro* degradation assay that utilizes MALDI-ToF mass spectrometry to monitor the degradation of neurotensin in rat serum.[36] The neurotensin analog of interest will be incubated in rat serum, at 37 °C. At preset intervals a 20 μL aliquot will be removed and added to 80 μL of an alcohol solution (3:1 MeOH/EtOH). This solution will then be vortexed for 30 seconds, followed by centrifugation for 10 minutes. Then, one μL of the solution will be mixed with one μL of an external standard (Kemptide, 1.30 nmol in saline) and added to six μL of matrix. Next, 0.5 μL of the mixture will be spotted on a target plate and analyzed by MALDI-ToF MS. The masses observed in the mass spectrum should allow us to determine the degradation

products from our neurotensin analogues. We should be able to determine the rate of the hydrolysis by direct comparison of the intensities of the fragments to the intensities of the external standard.

Conclusions

Hopefully, with the future work outlined in this chapter, the enantiomeric composition of **2.32b** can be improved to greater than 97% ee. This should allow for complete synthesis of both enantiomers of the α-methyl, $\beta^{2,2}$-, $\beta^{3,3}$-, $\gamma^{2,2}$- and $\gamma^{4,4}$-tyrosine analogues. Once the desired unnatural tyrosine analogues have been obtained, synthesis and testing of neurotensin peptidomimetics can proceed. Hopefully, the end result of this research project will be the development of neurotensin peptidomimetics that display increased *in vivo* stability and that retain the ability to interact with the neurotensin receptors with high affinity. This may allow for the exploration of new cancer drugs or new imaging agents that may specifically target cancerous cells.

References

(1) Masterson, D.; Rosado, D.; Nabors, C. *Tetrahedron Assymmetry* **2009**, *20*, 1476-1486.

(2) Carrea, G.; Ottolina, G.; Riva, S. *Trends in Biotechnology* **1995**, *13*, 63-70.

(3) Mattson, A.; Boutelje, J.; Csoregh, I.; Hjalmarsson, M.; Jacobsson, U.; Lindback, M.; Norin, T.; Szmulik, P.; Hult, K. *Bioorganic and Medicinal Chemistry* **1994**, *2*, 501-508.

(4) Wallert, S.; Drauz, K.; Grayson, I.; Groger, H.; Dominguez de Maria, P.; Bolm, C. *Green Chemistry* **2005**, *7*, 602-605.

(5) Tanyeli, C.; Turkut, E. *Tetrahedron Assymmetry* **2004**, *15*, 2057-2060.

(6) Sano, S.; Hayashi, K.; Miwa, T.; Ishii, T.; Fujii, M.; Mima, H.; Nagao, Y. *Tetrahedron Letters* **1998**, *39*, 5571-5574.

(7) Nakazawa, K.; Hayashi, M.; Tanaka, M.; Aso, M.; Suemune, H. *Tetrahedron Assymmetry* **2001**, *12*, 897-901.

(8) Fotakopoulou, I.; Barbayianni, E.; Constantinou-Kokotou, V.; Bornscheuer, U. T.; Kokotos, G. *Journal of Organic Chemistry* **2007**, *72*, 782-786.

(9) Haase, B.; Schneider, M. P. *Tetrahedron Asymmetry* **1993**, *4*, 1017-1026.

(10) Tanaka, M.; Demizu, Y.; Nagano, M.; Hama, M.; Yoshida, Y.; Kurihara, M.; Suemune, H. *The Journal of Organic Chemistry* **2007**, *72*, 7750-7756.

(11) Smith, G. B.; Bhupathy, M.; Denzeny, G. C.; Douglas, A. W.; Lander, R. J. *Journal of Organic Chemistry* **1992**, *57*, 4544-4546.

(12) Hughes, D. L.; Song, Z.; Smith, G. B.; Bergan, J. J.; Dezeny, G. C.; Reider, P. J. *Tetrahedron Assymmetry* **1993**, *4*, 865-874.

(13) Jampel-Guibe, E.; Rousseau, G.; Salaun, J. *Journal of the Chemical Society, Chemical Communications* **1987**, 1080-1081.

(14) Guanti, G.; Banfi, L.; Narisano, E. *Tetrahedron Letters* **1989**, *30*, 2697-2698.

(15) Hultin, P. G.; Mueseler, F. J.; Jones, J. B. *The Journal of Organic Chemistry* **2002**, *56*, 5375-5380.

(16) de Hoffmann, E.; Stroobant, V. *Mass Spectrometry: Principles and Applications*; Third ed.; John Wiley and Sons Ltd., 2007.

(17) Kudaj, A.; Olma, A. *Tetrahedron Letters* **2007**, *48*, 6794-6797.

(18) Smith, N. D.; Goodman, M. *Organic Letters* **2003**, *5*, 1035-1037.

(19) Smith, N. D.; Wohlrab, A. M.; Goodman, M. *Organic Letters* **2005**, *7*, 255-258.

(20) Greene, T. W.; Wuts, P. G. *Protecting Groups in Organic Chemistry*; Third ed.; John Wiley and Sons: Hoboken, NJ, 1999.

(21) Berkowitz, D. B.; Maiti, G. *Organic Letters* **2004**, *6*, 2661-2664.

(22) Kokko, K. P.; Hadden, M. K.; Price, K. L.; Orwig, K. S.; See, R. E.; Dix, T. A. *Neuropharmacology* **2005**, *48*, 417-425.

(23) Hultsch, C.; Pawelke, B.; Bergmann, R.; Wuest, F. *Bioorganic & Medicinal Chemistry* **2006**, *14*, 5913-5920.

(24) Bergmann, R.; Scheunemann, M.; Heichert, C.; Mading, P.; Wittrisch, H.; Kretzschmar, M.; Rodig, H.; Tourwe, D.; Iterbeke, K.; Chavatte, K. *Nuclear Medicine and Biology* **2002**, *29*, 61-72.

(25) Gargia-Garayoa, E.; Maes, V.; Blauenstein, P.; Blanc, A.; Hohn, A.; Tourwe, D.; Schubiger, P. A. *Nuclear Medicine and Biology* **2006**, *33*, 495-503.

(26) Achilefu, S.; Srinivasan, A.; Schmidt, M. A.; Jimenez, H. N.; Bugaj, J. E.; Erion, J. L. *Journal of Medicinal Chemistry* **2003**, *46*, 3403-3411.

(27) Nock, B. A.; Nikolopoulou, A.; Reubi, J. C.; Maes, V.; Conrath, P.; Tourwe, D.; Maina, T. *Journal of Medicinal Chemistry* **2006**, *49*, 4767-4776.

(28) Carraway, R.; Leeman, S. E. *Journal of Biological Chemistry* **1975**, *250*, 1912-1918.

(29) Chang, D.; Rackur, G.; Folkers K. *Bioorganic Chemistry* **1979**, *8*, 41-44.

(30) Bittermann, H.; Einsiedel, J.; Hubner, H.; Gmeiner, P. *Journal of Medicinal Chemistry* **2004**, *47*, 5587-5590.

(31) Bruckner, R. *Advanced Organic Chemistry: Reaction Mechanisms*; Harcourt Academic Press: Burliington, Mass, 2002.

(32) Frackenpohl, J.; Arvidsson, P. I.; Schreiber, J. V.; Seebach, D. *ChemBioChem* **2001**, *2*, 445-455.

(33) de Visser, M.; Janssen, P. J. J. M.; Srinivasan, A.; Reubi, J. C.; Waser, B.; Erion, J. L.; Schmidt, M. A.; Krenning, E. P.; de Jong, M. *European Journal of Nuclear Medicine & Molecular Imaging* **2003**, *30*, 1134-1139.

(34) Gargia-Garayoa, E.; Blauenstein, P.; Bruehlmeier, M.; Blanc, A.; Iterbeke, K.; Conrath, P.; Tourwe, D.; Schubiger, A. *The Journal of Nuclear Medicine* **2002**, *43*, 374-383.

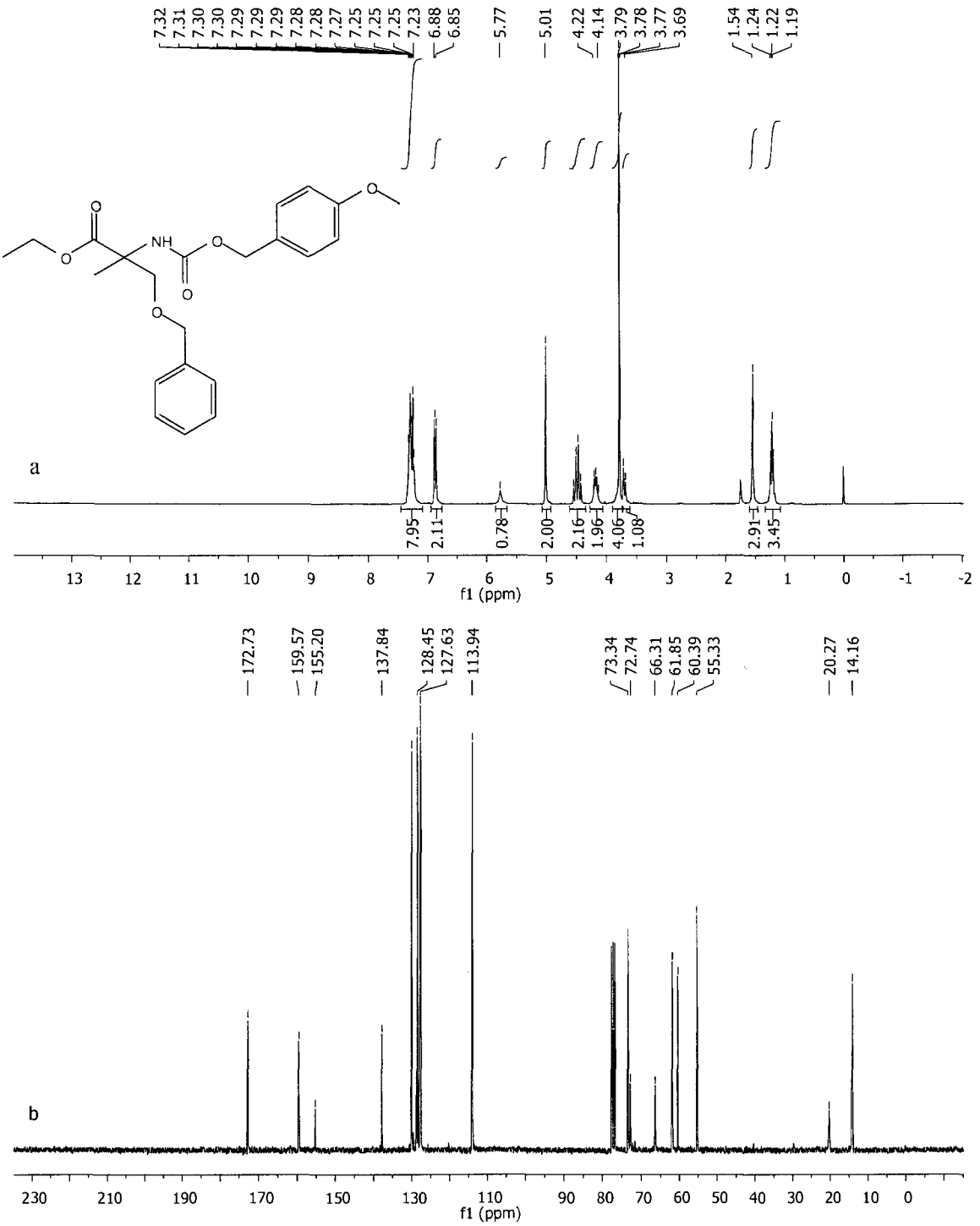
(35) Nelson, D. L.; Cox, M. M. *Lehninger Principles of Biochemistry*; Third ed.; Worth Publishers: New York, NY, 2000.

(36) Kokko, K. P.; Hadden, M. K.; Orwig, K. S.; Mazella, J.; Dix, T. A. *Journal of Medicinal Chemistry* **2003**, *46*, 4141-4148.

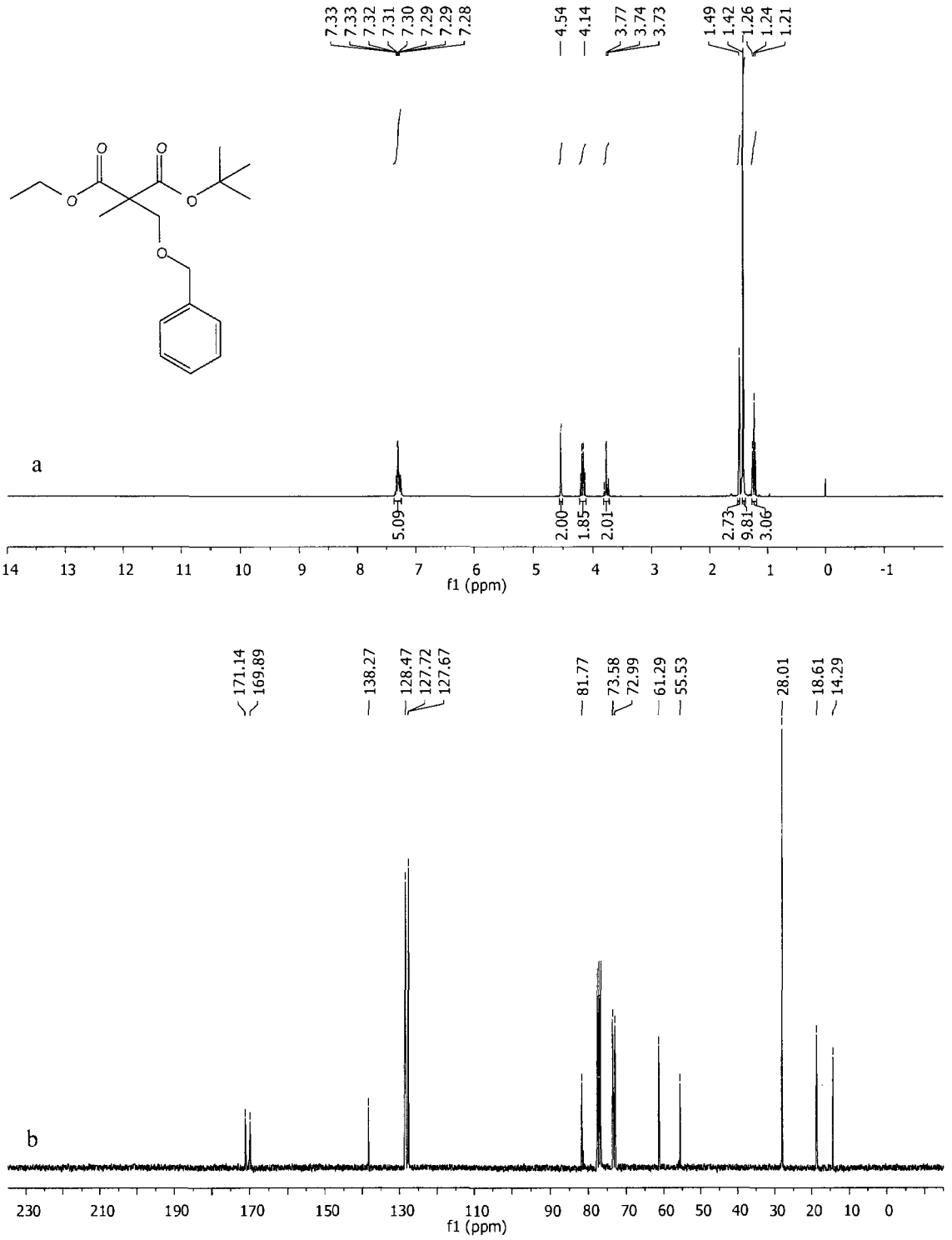
APPENDIX

NMR SPECTRA

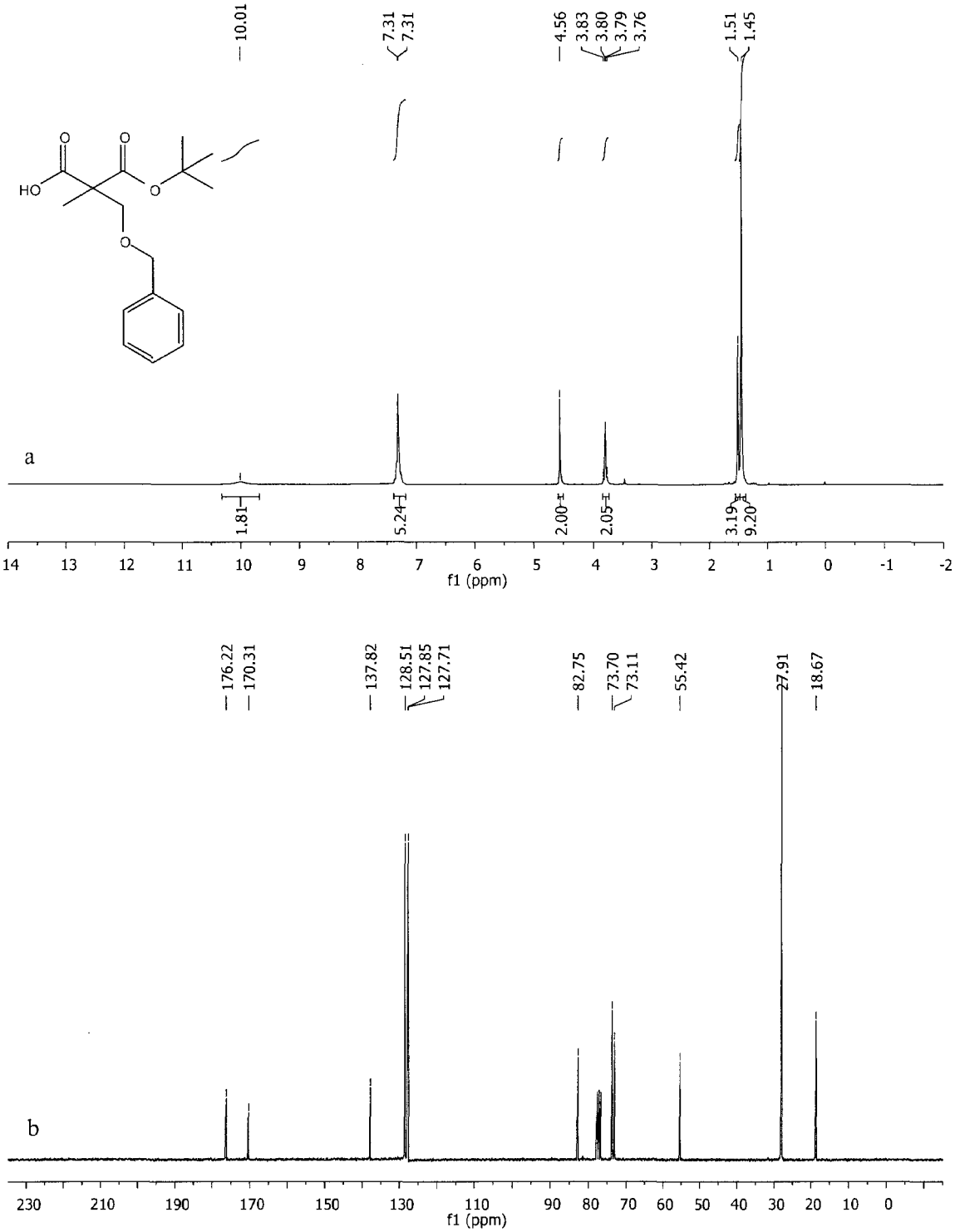
Chapter II



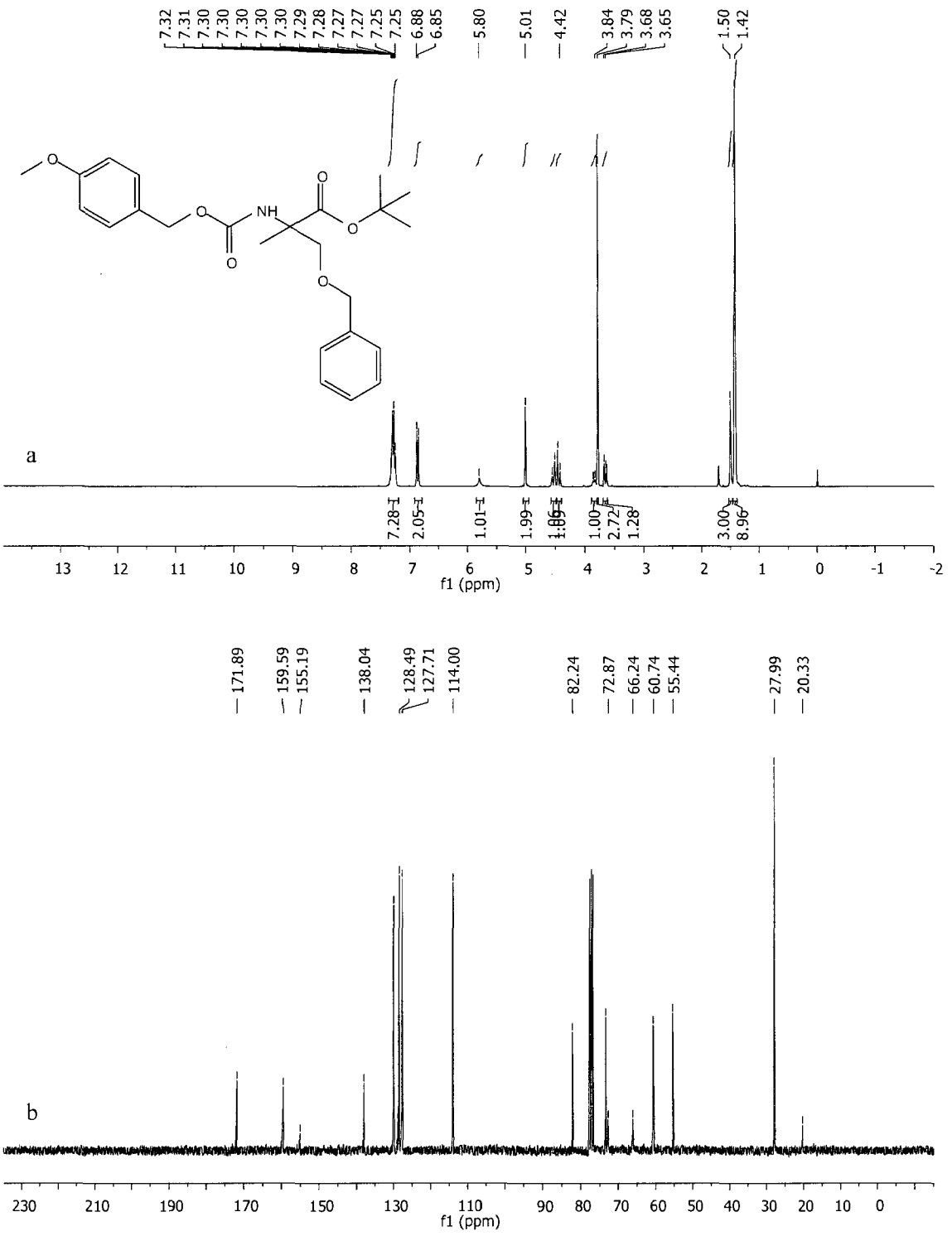
A.1. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **2.35a**



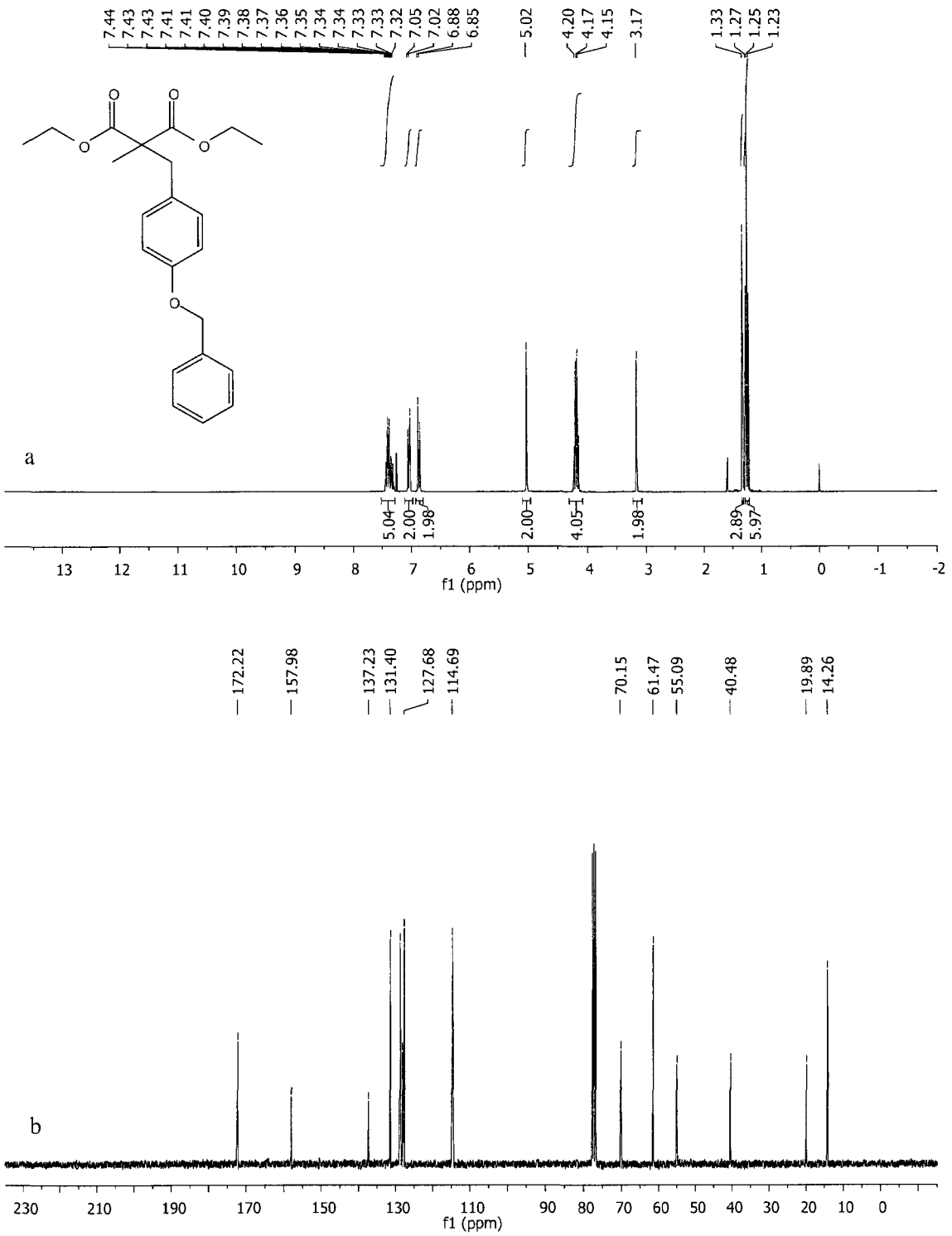
A.2. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **2.37a**



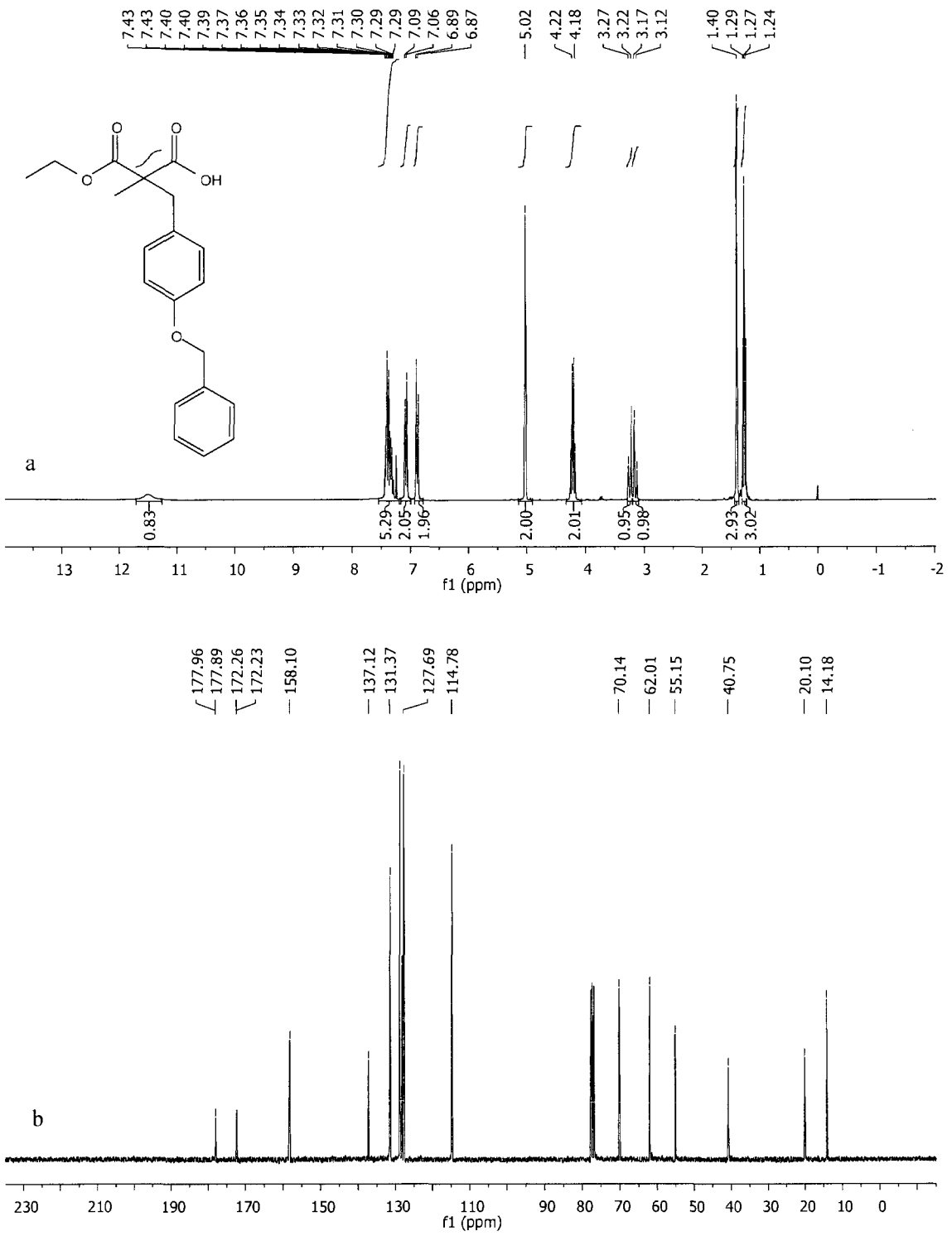
A.3. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **2.38a**



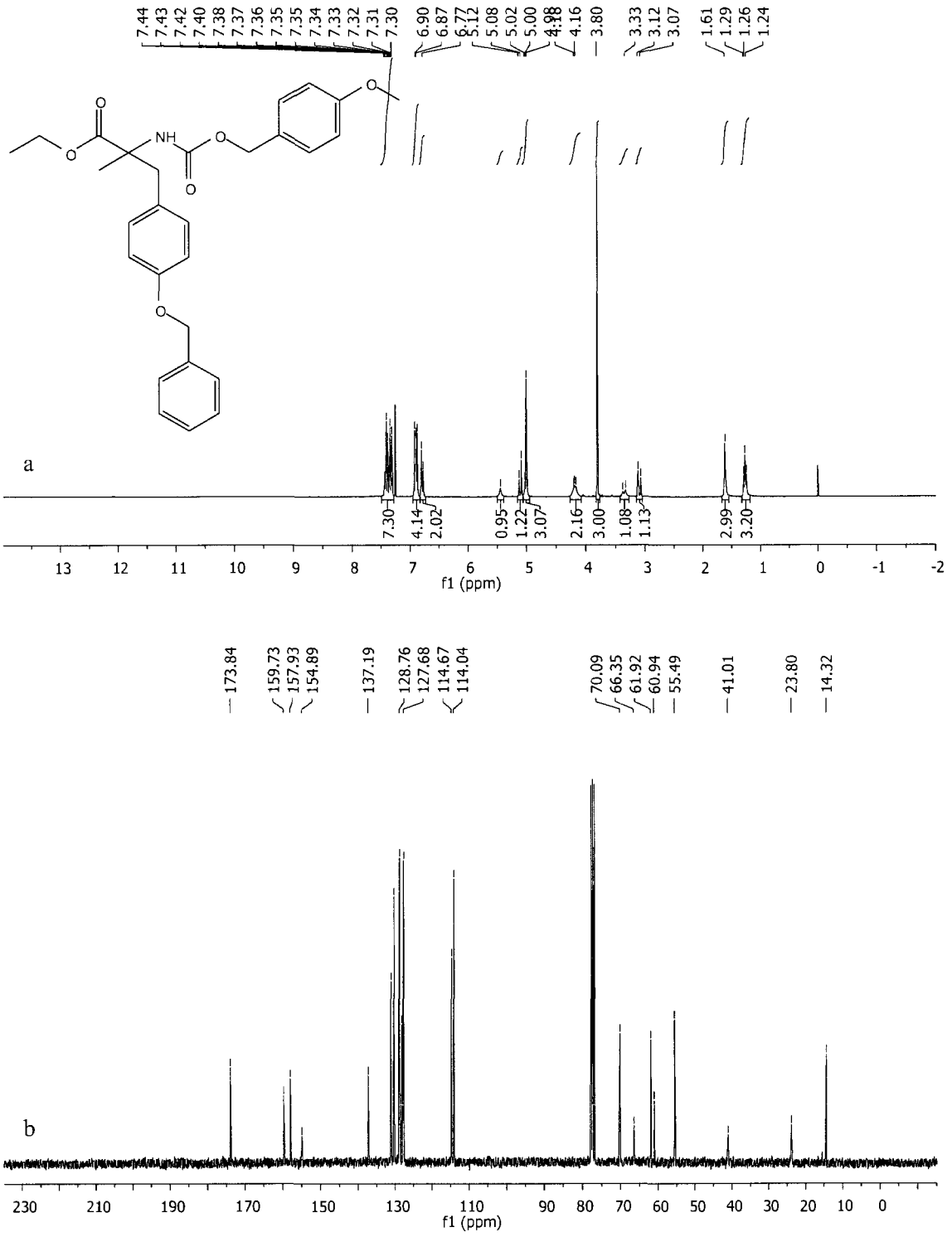
A.4. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **2.39a**



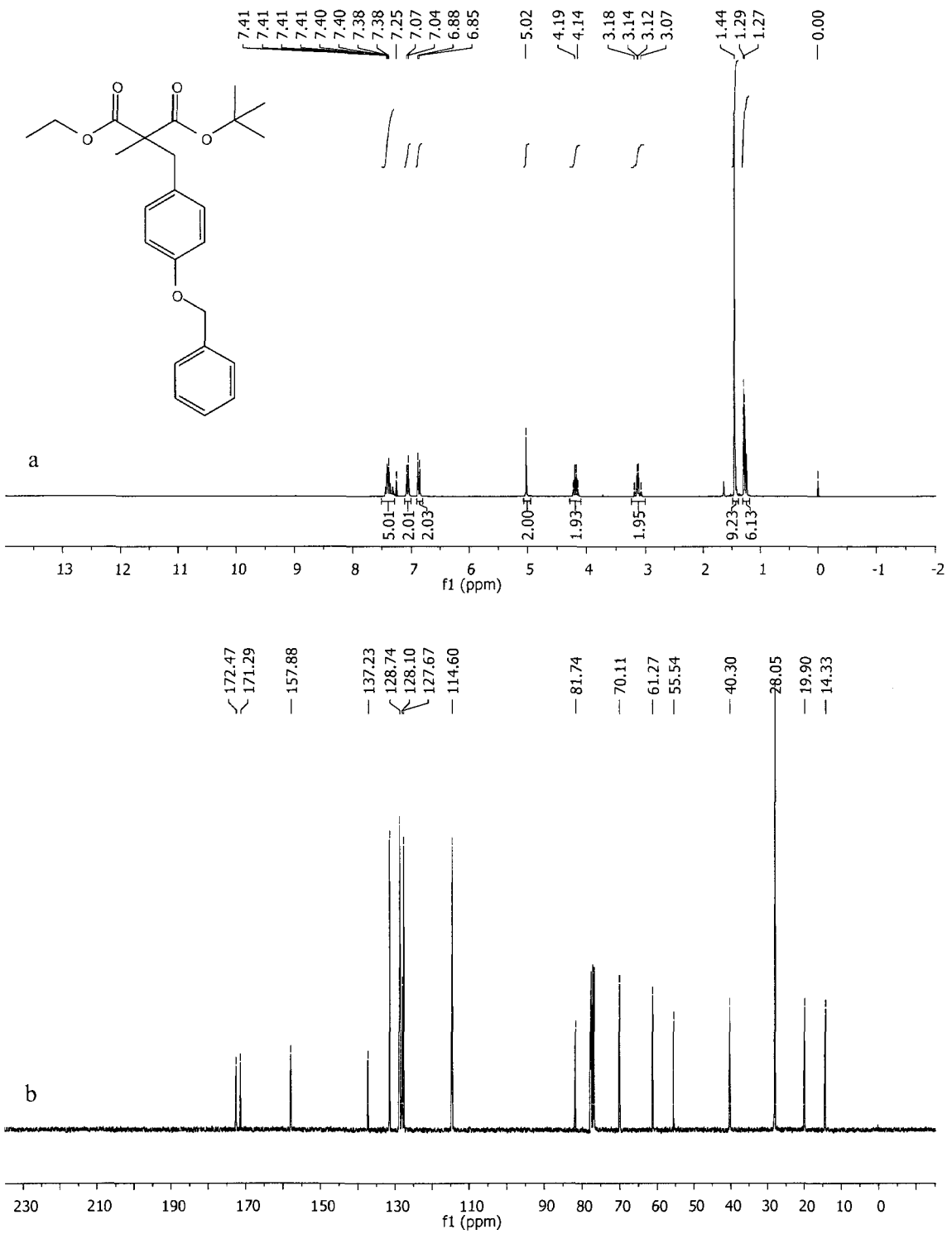
A.5. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **2.31b**



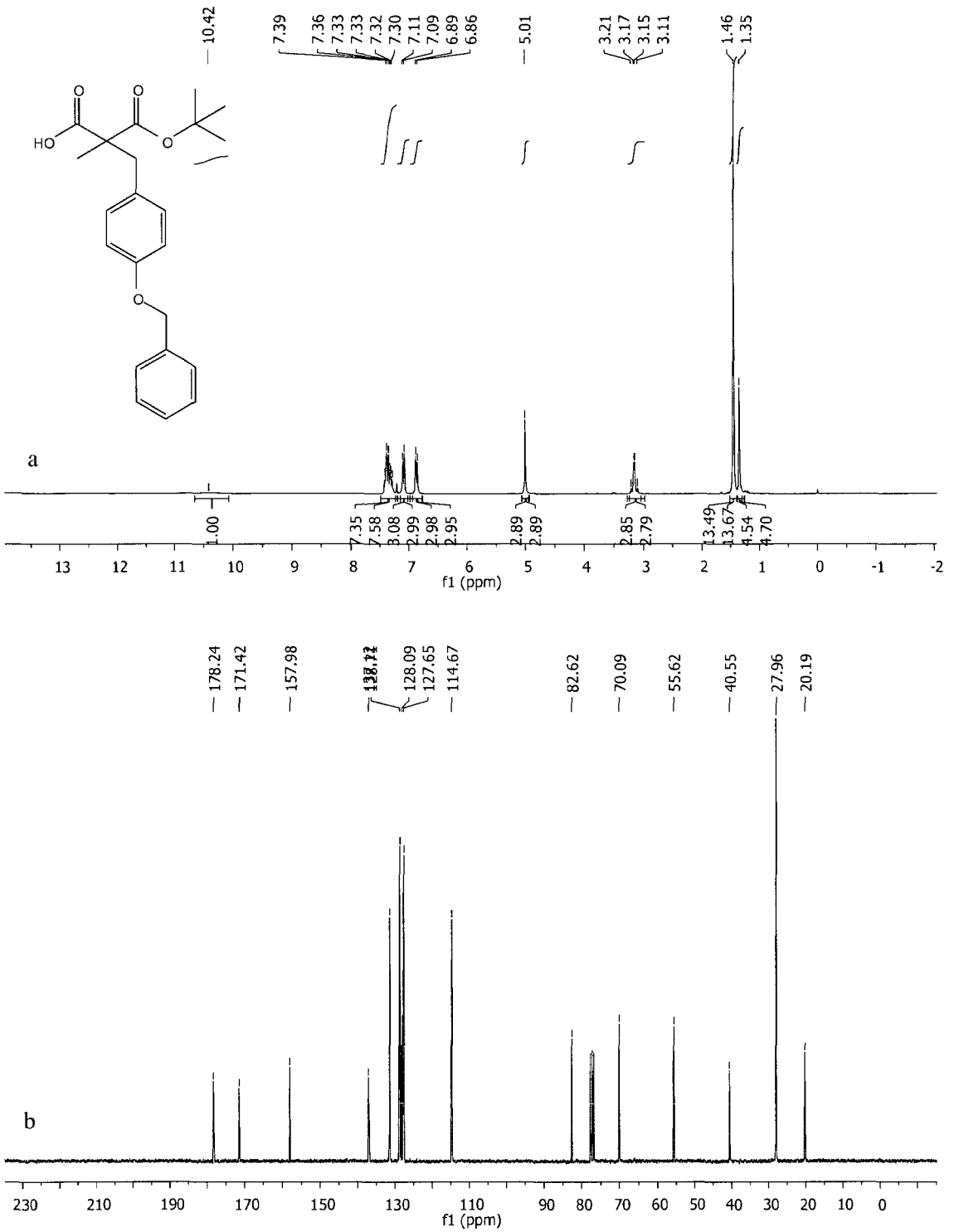
A.6. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **2.32b**



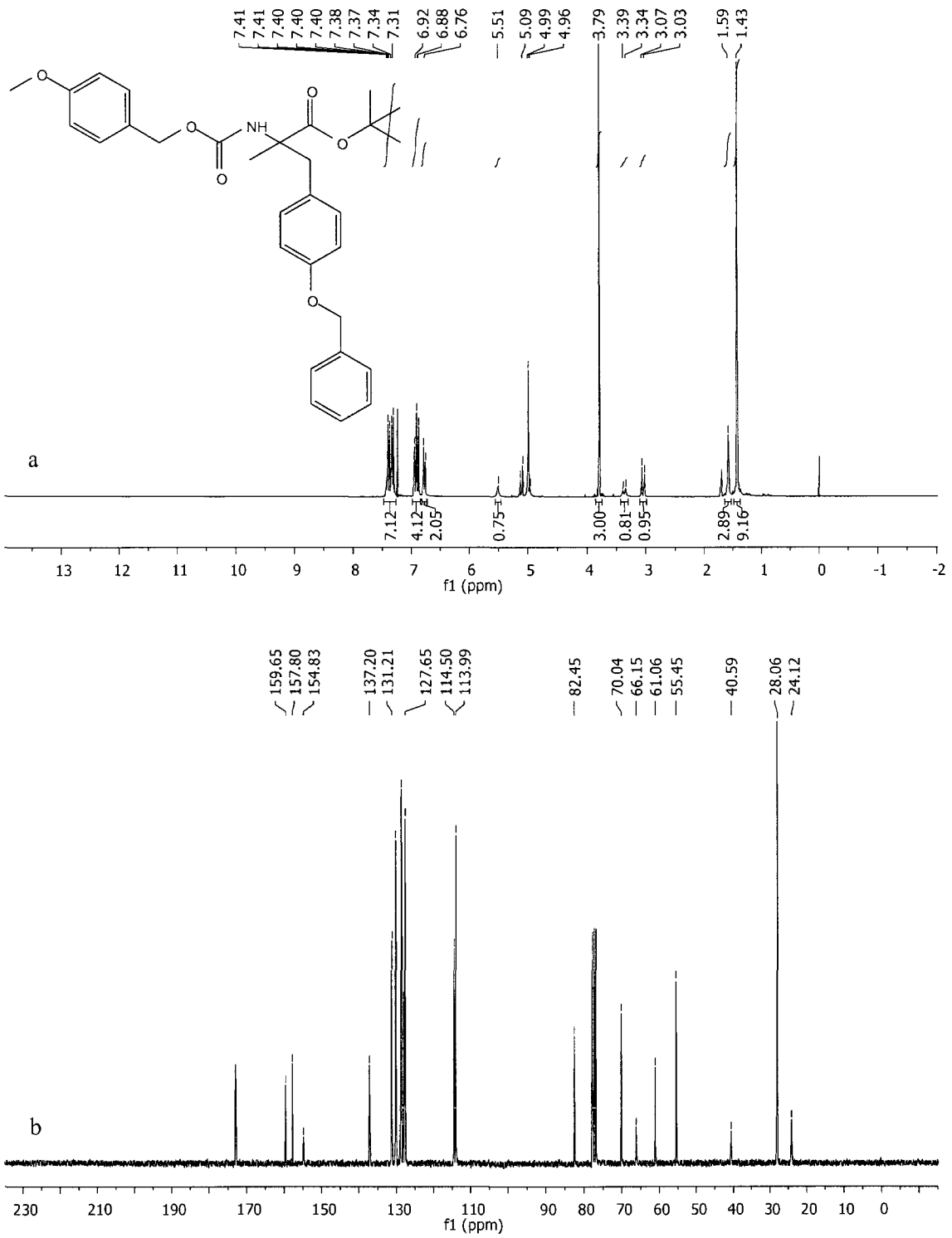
A.7. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **2.35b**



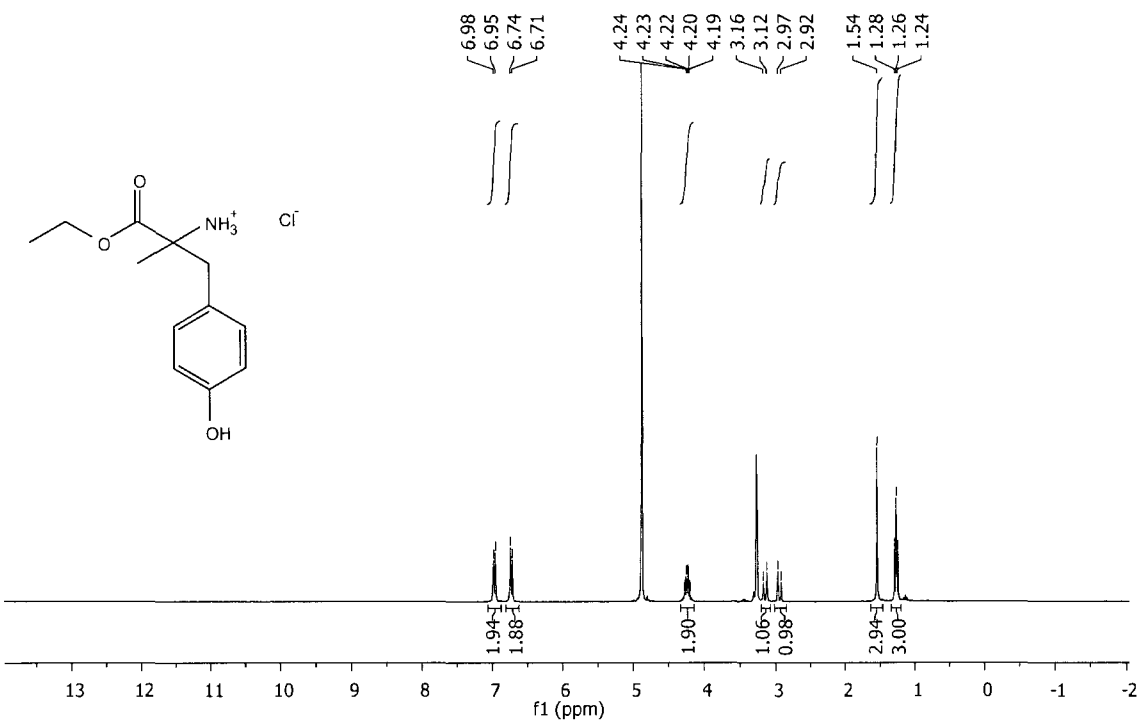
A.8. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **2.37b**



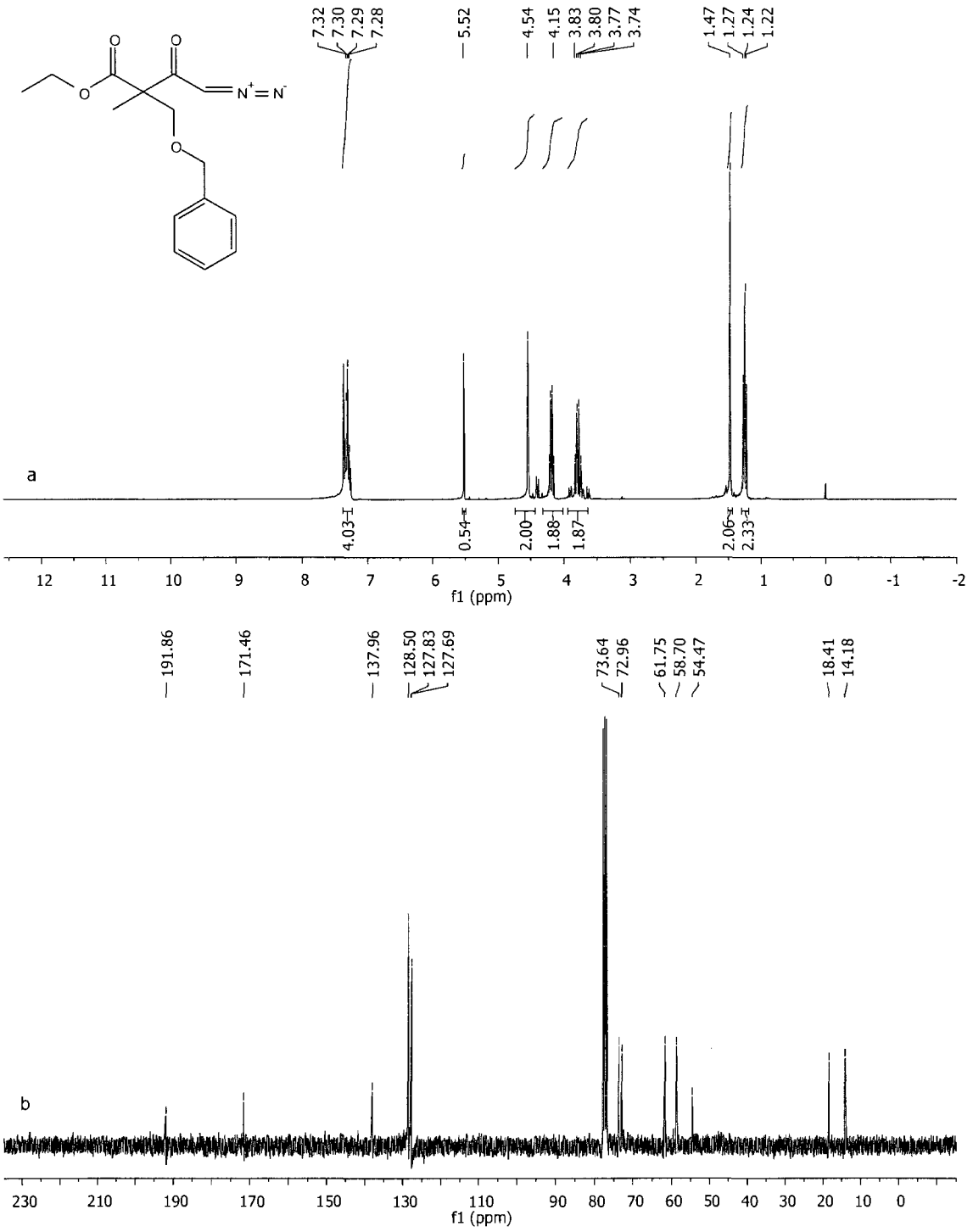
A.9. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **2.38b**



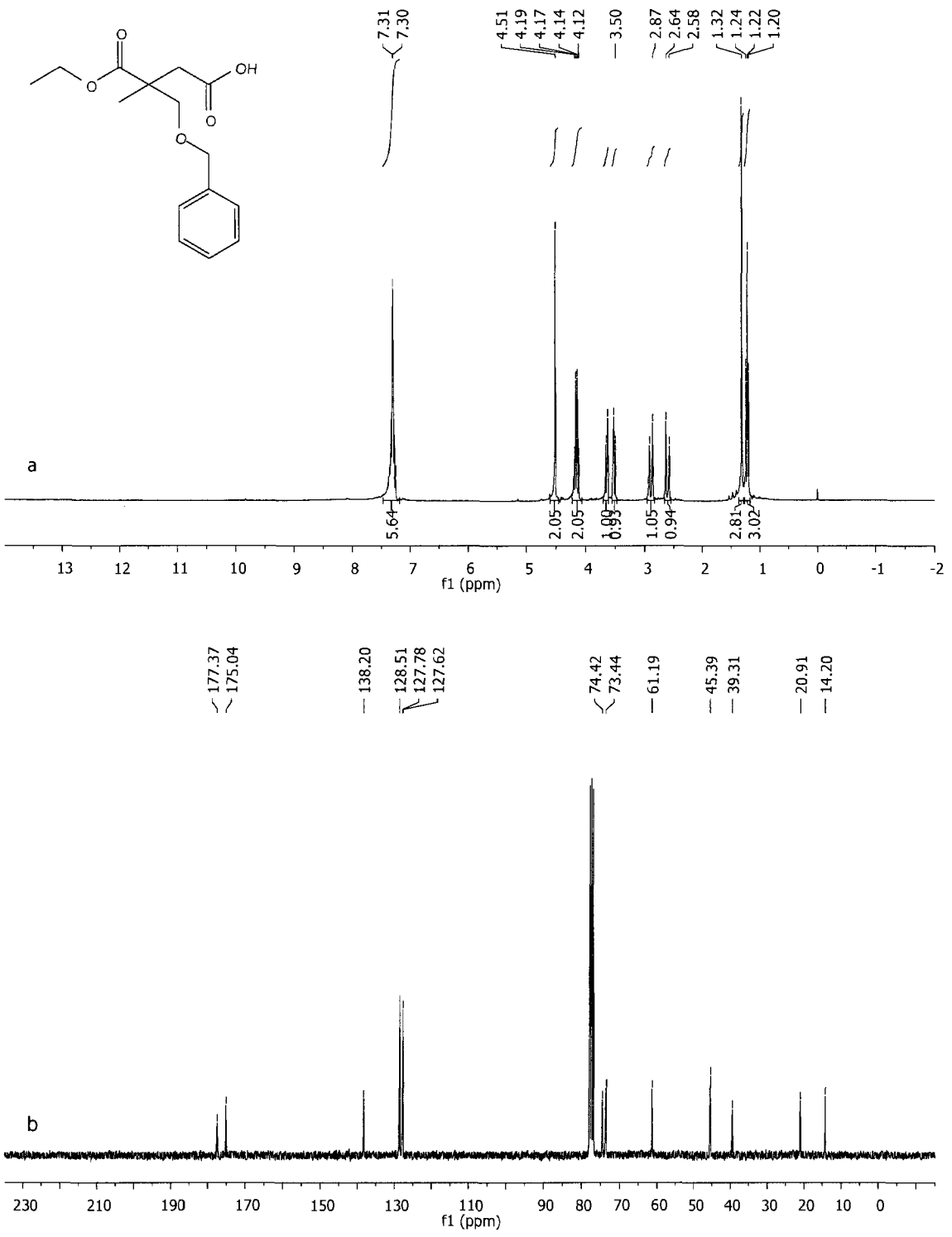
A.10. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **2.39b**



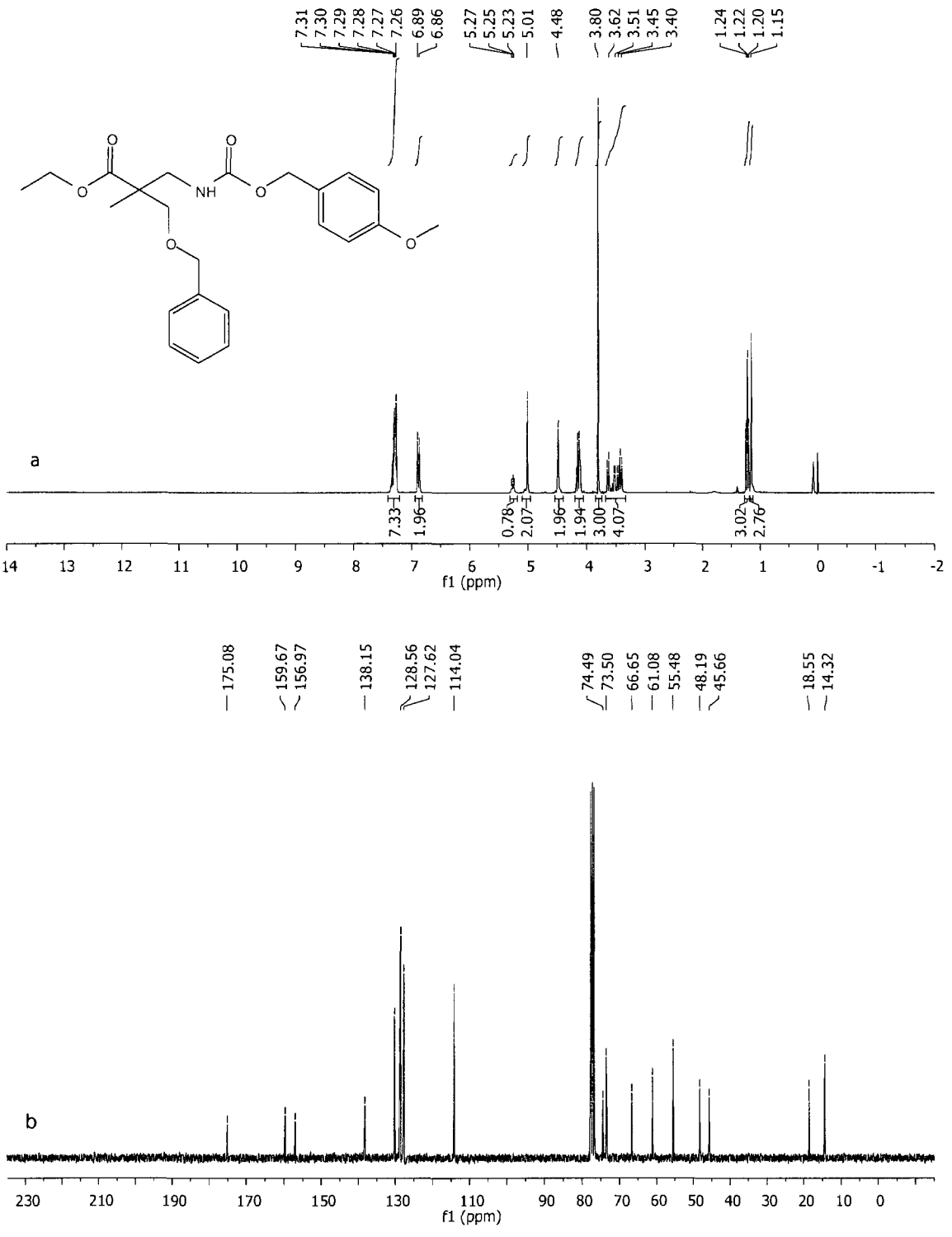
A.11. ^{1}H-NMR of compound **2.36b**



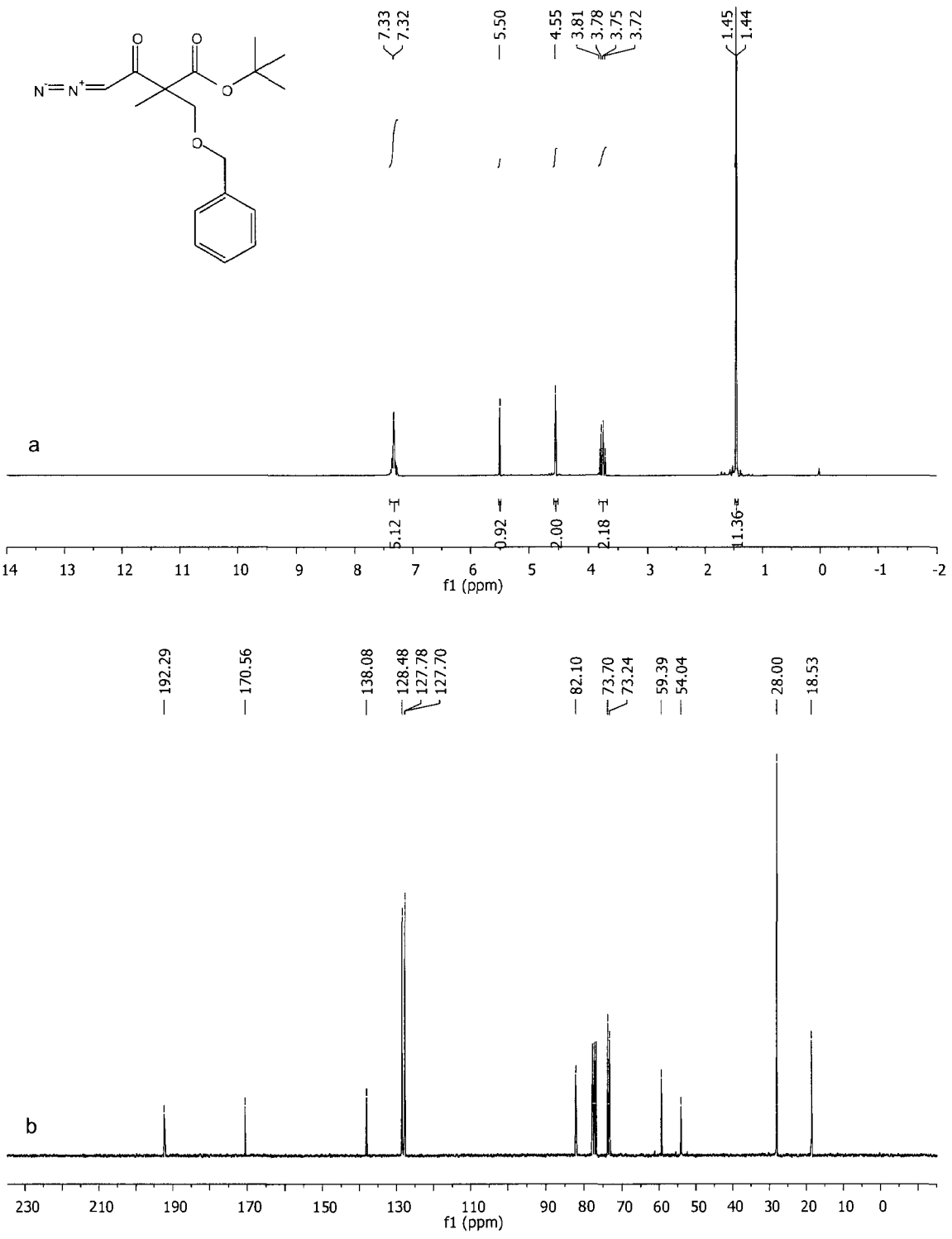
A.12. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.32a**



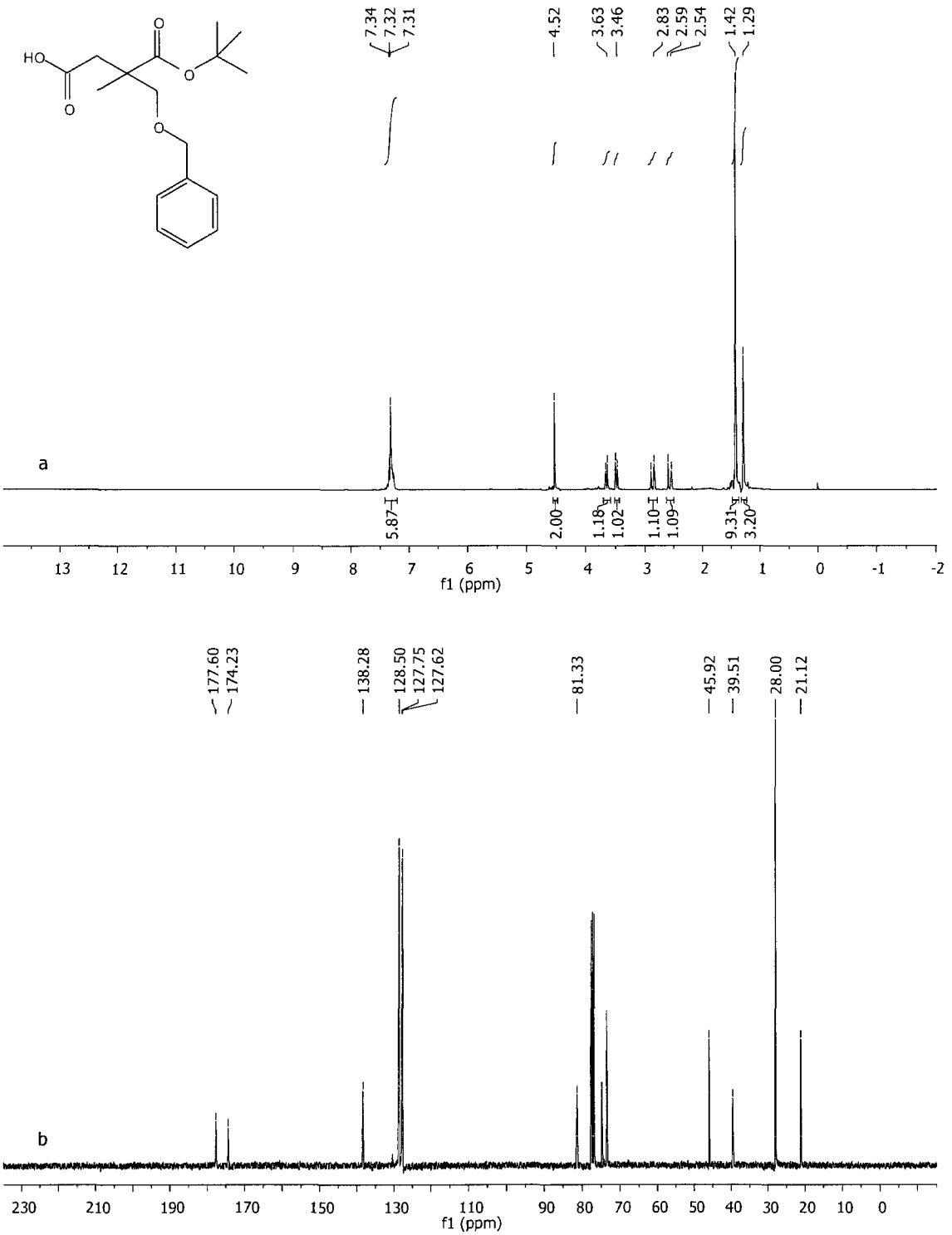
A.13. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.33a**



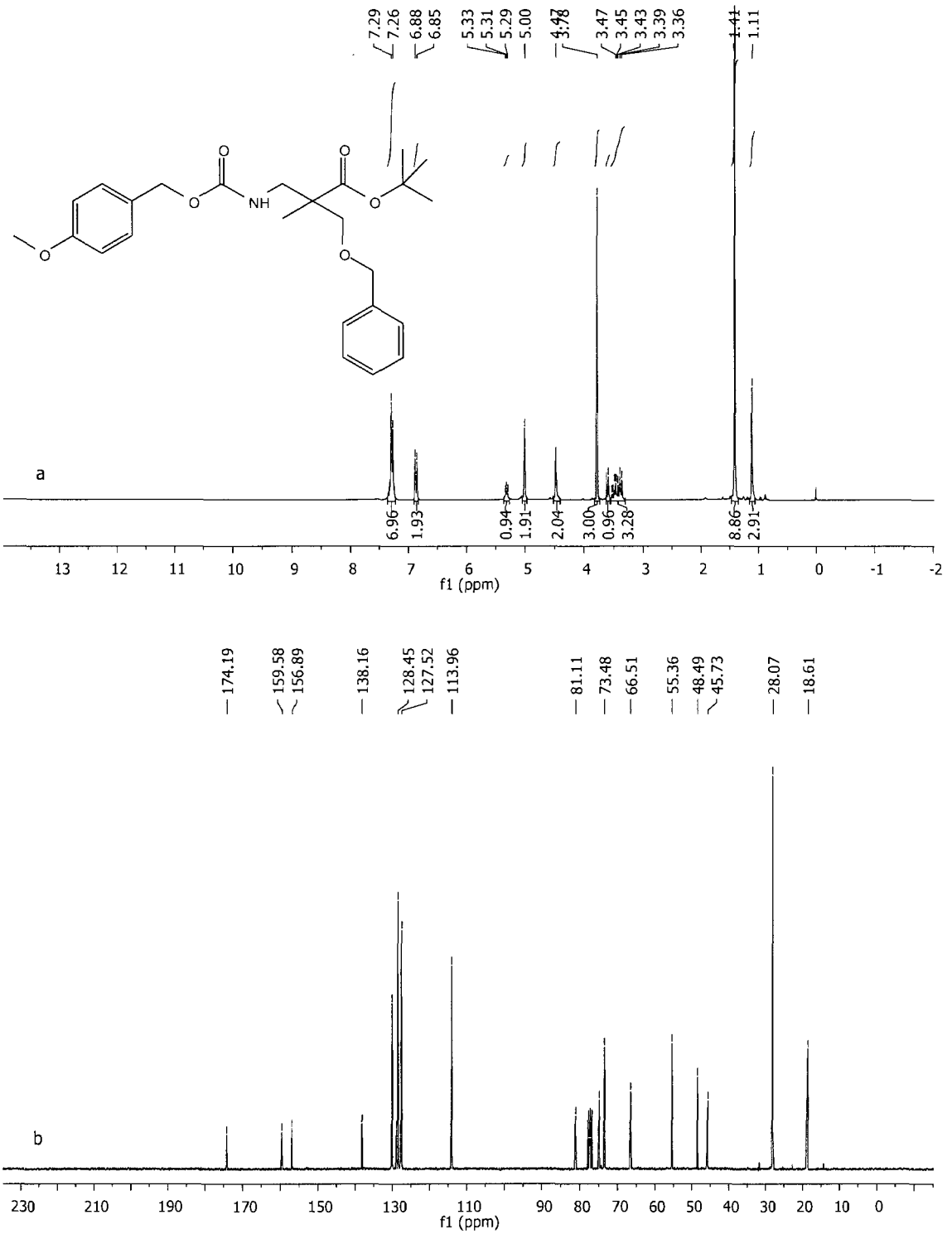
A.14. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.34a**



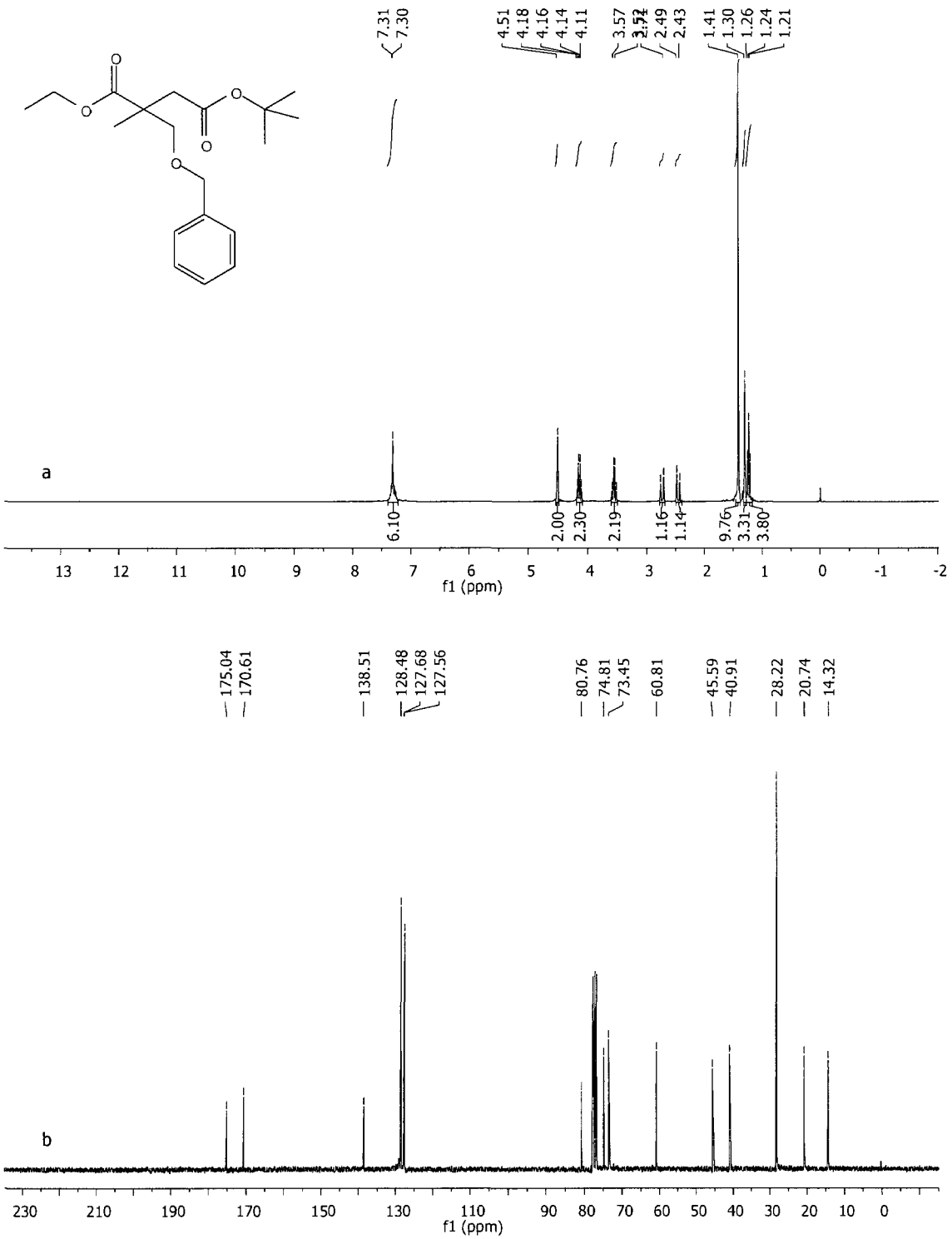
A.15. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.35a**



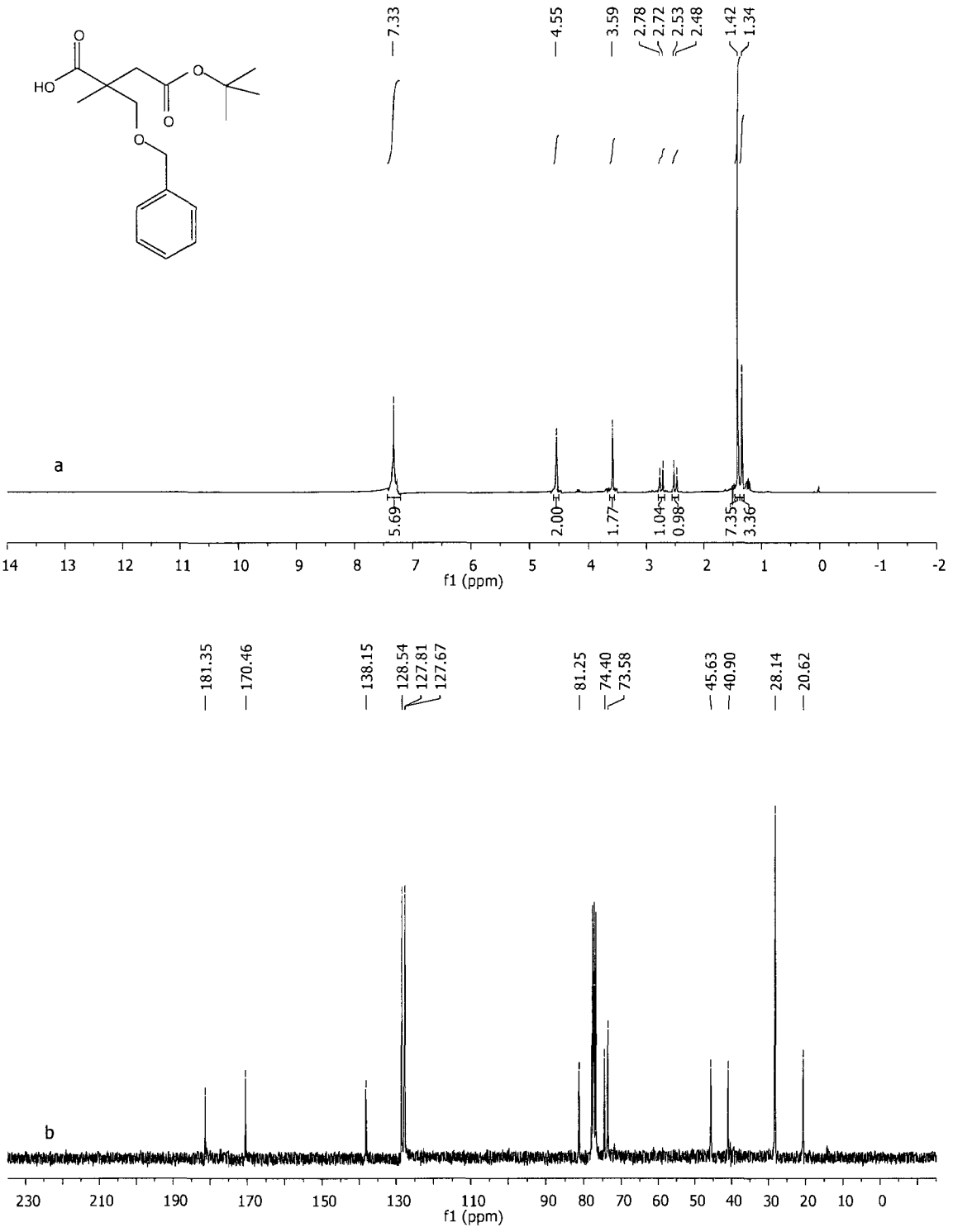
A.16. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.36a**



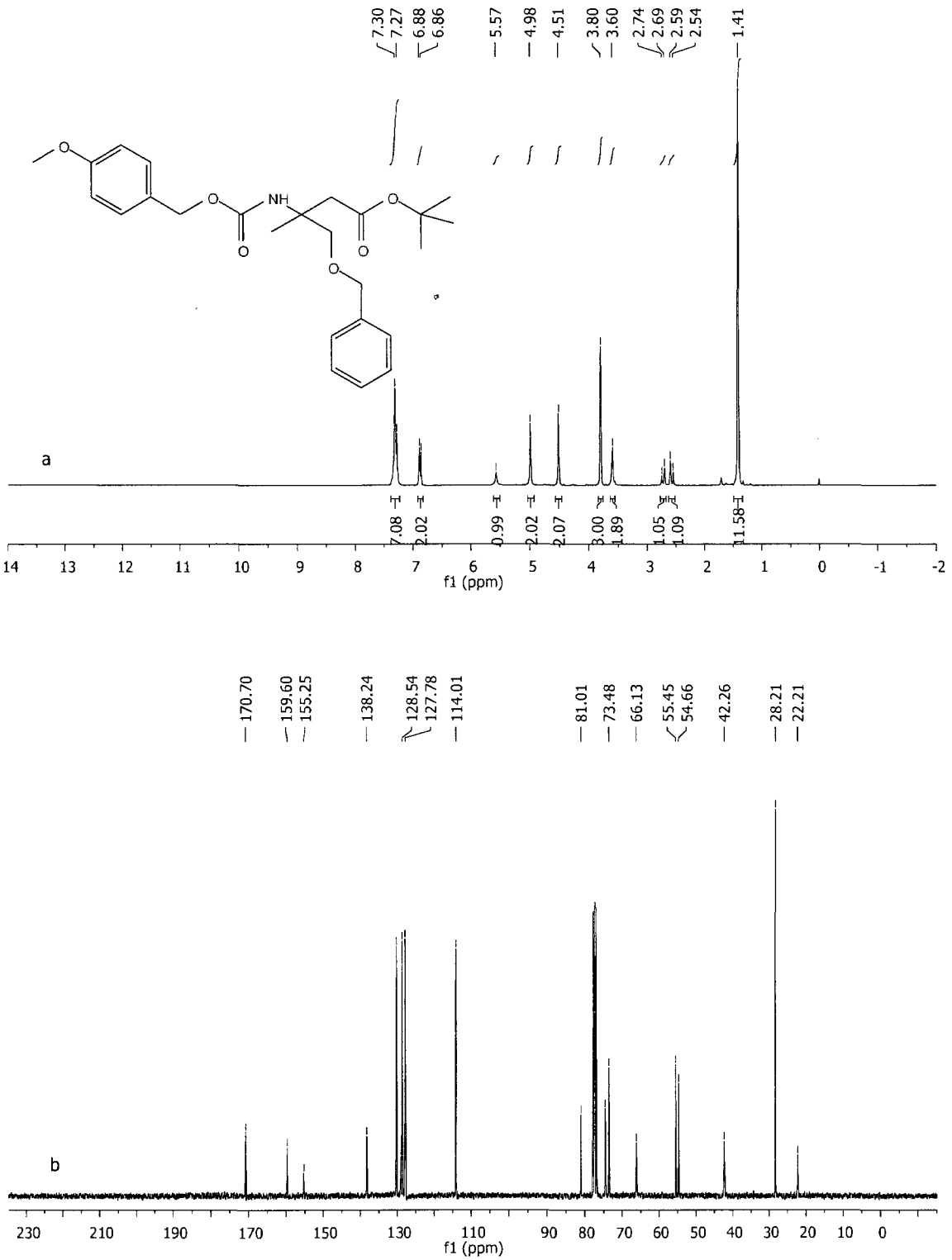
A.17. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.37a**



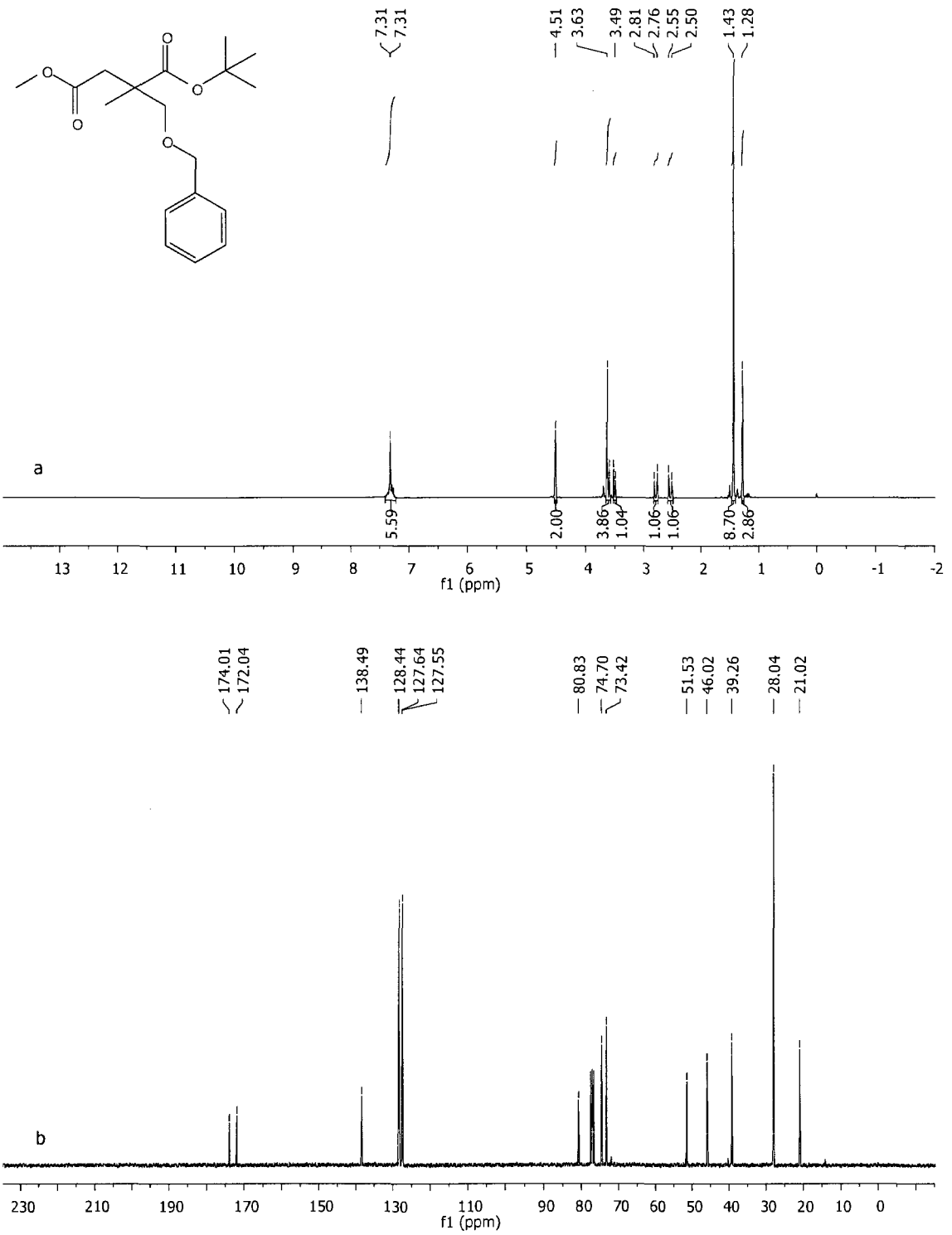
A.18. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.38a**



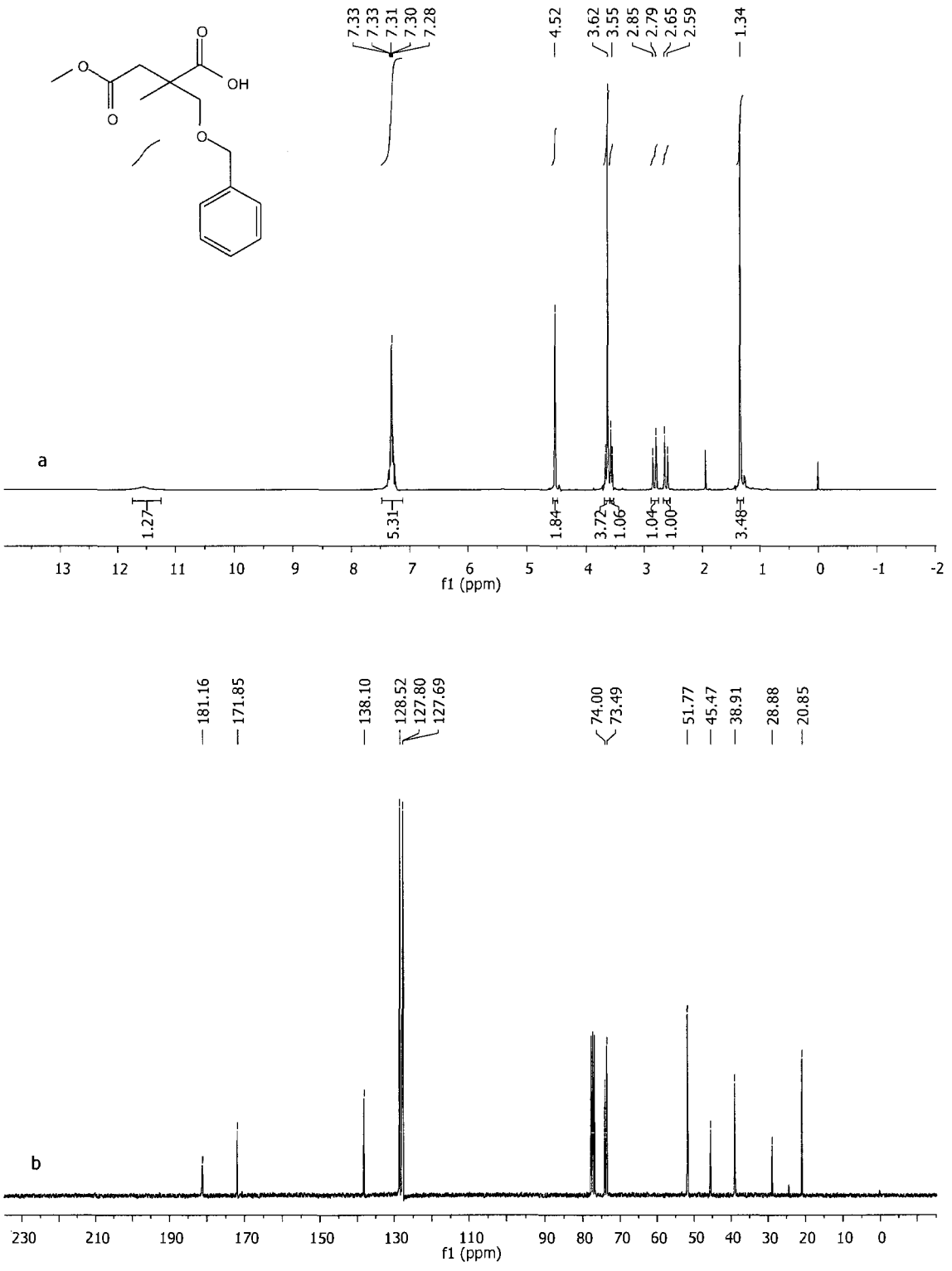
A.19. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.39a**



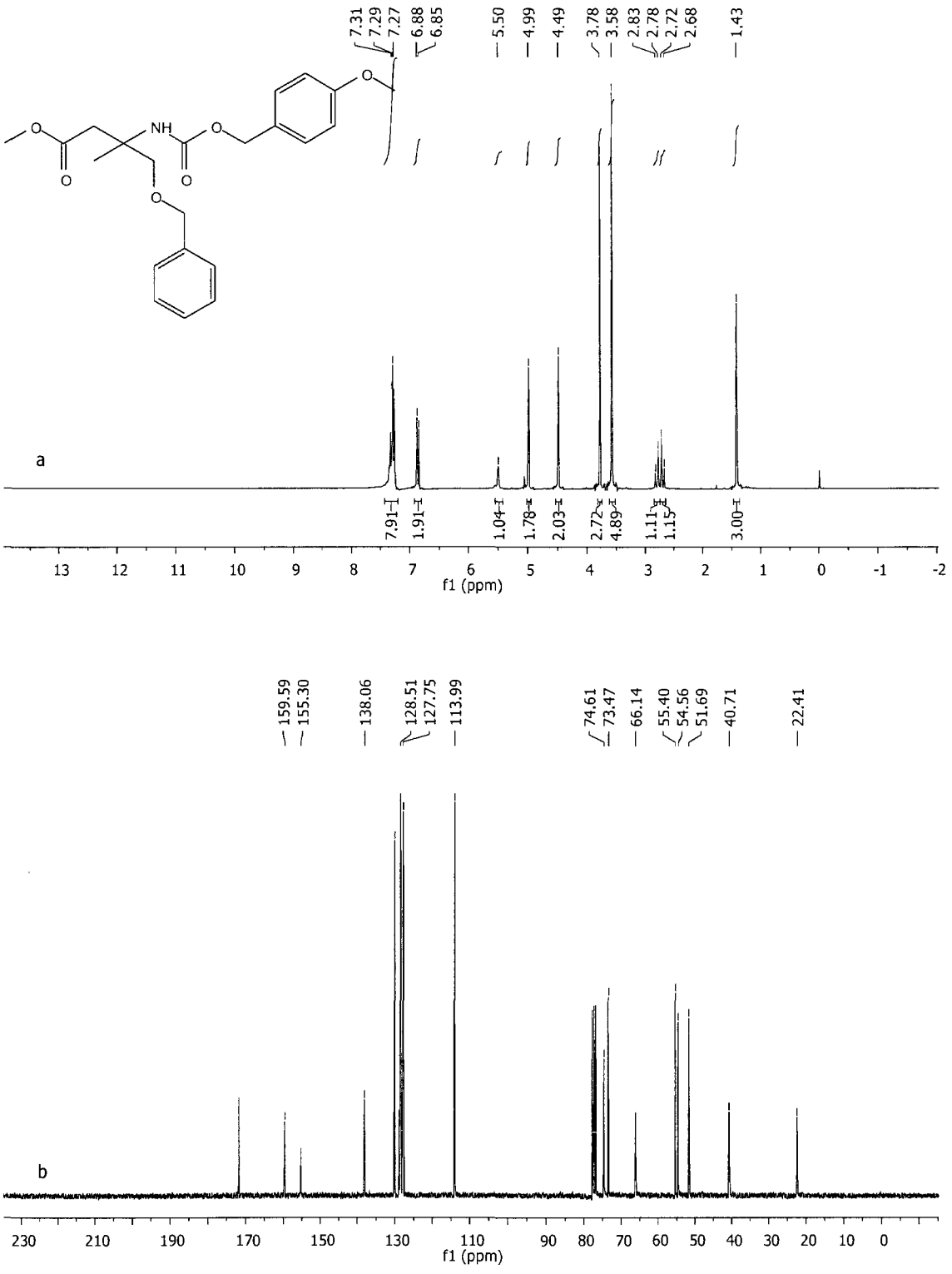
A.20. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.40a**



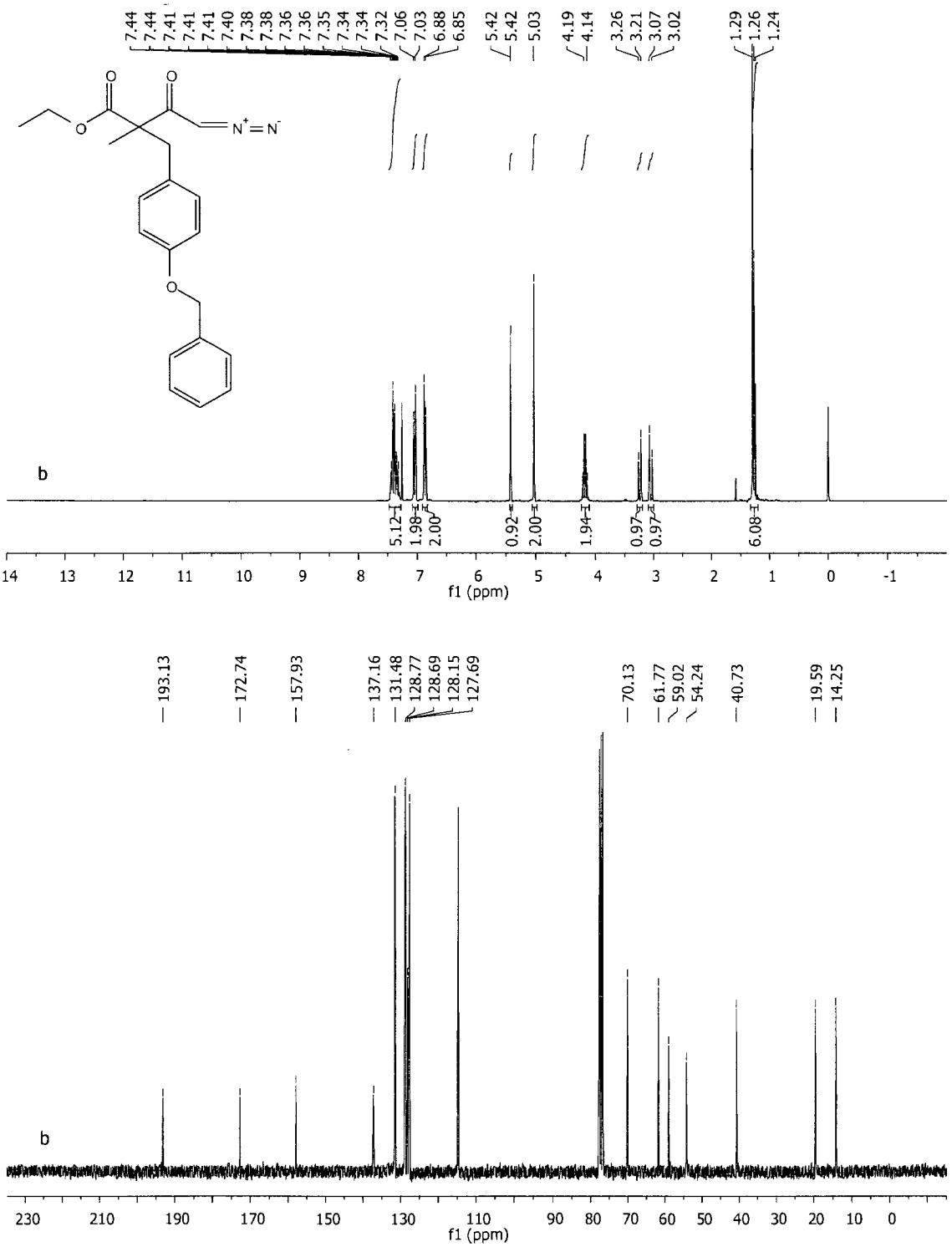
A.21. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.41a**



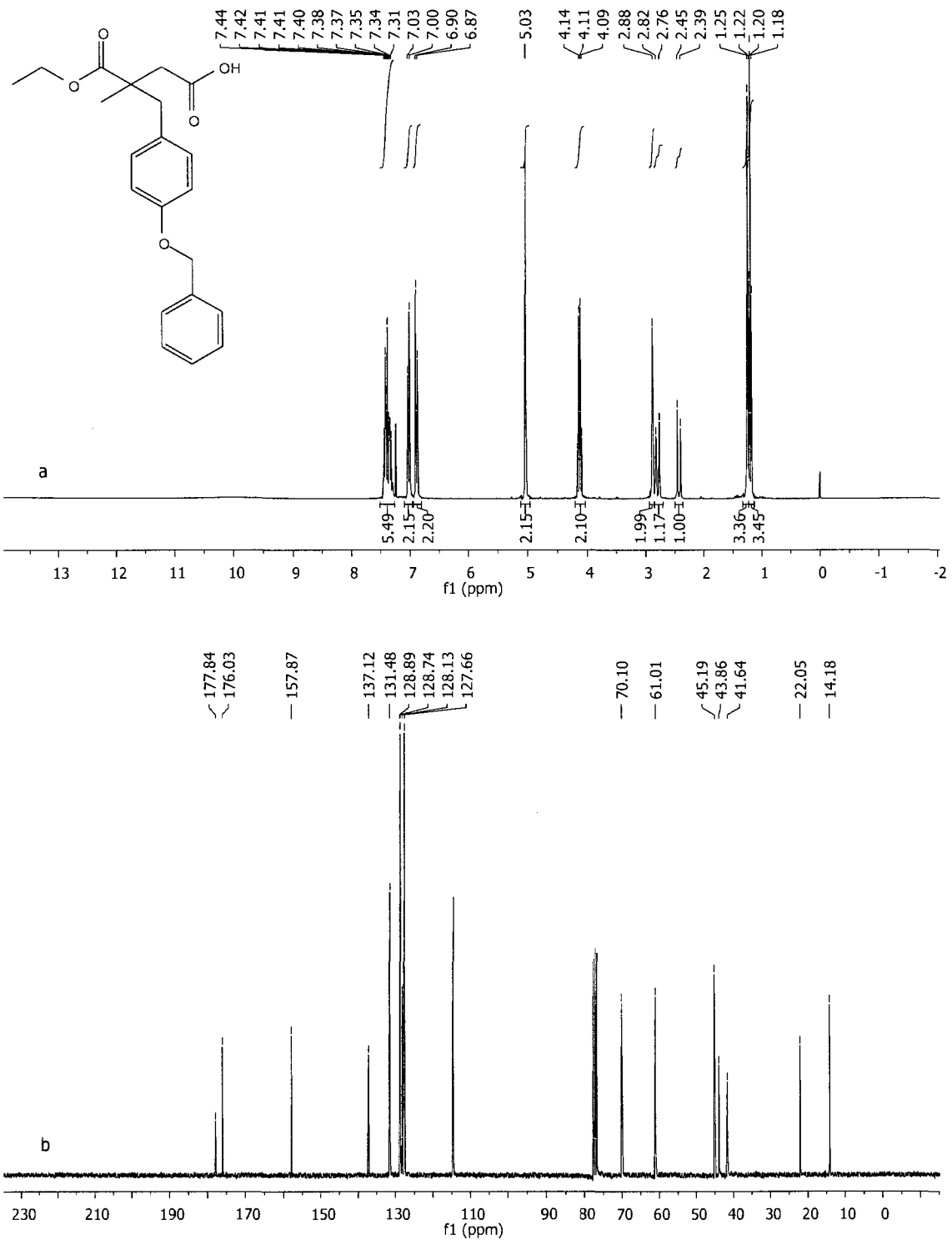
A.22. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.42a**



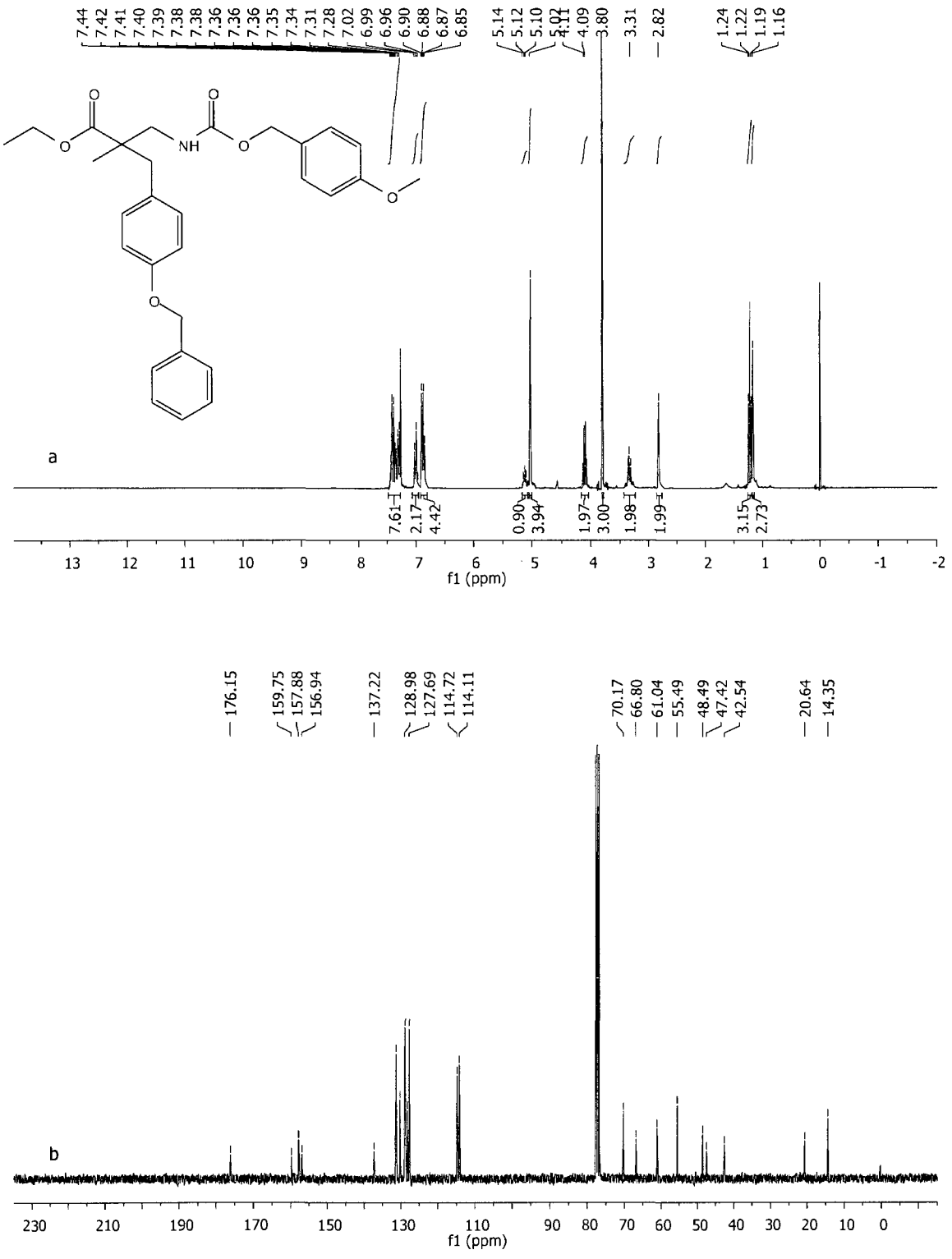
A.23. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.43a**



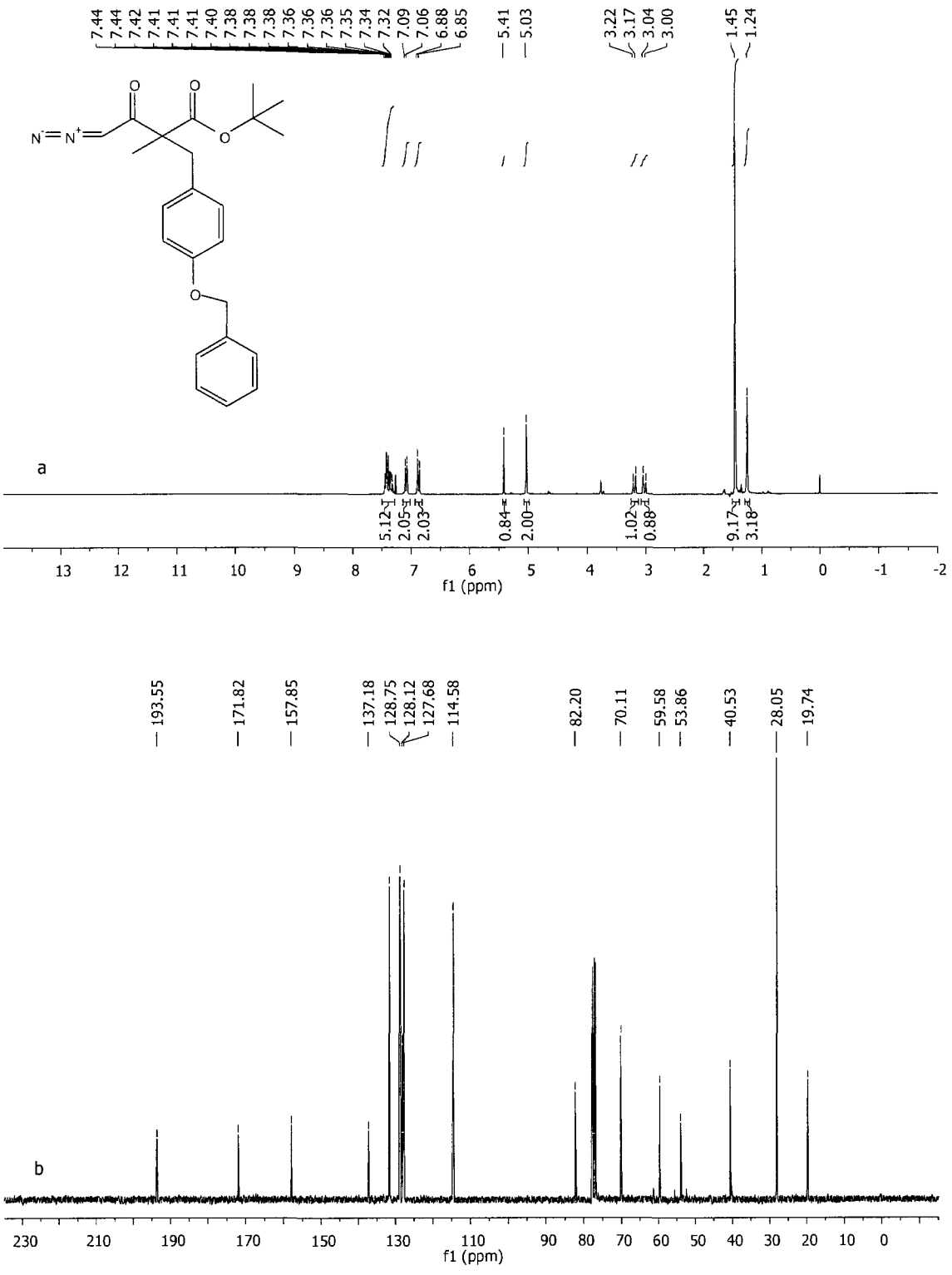
A.24. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.32b**



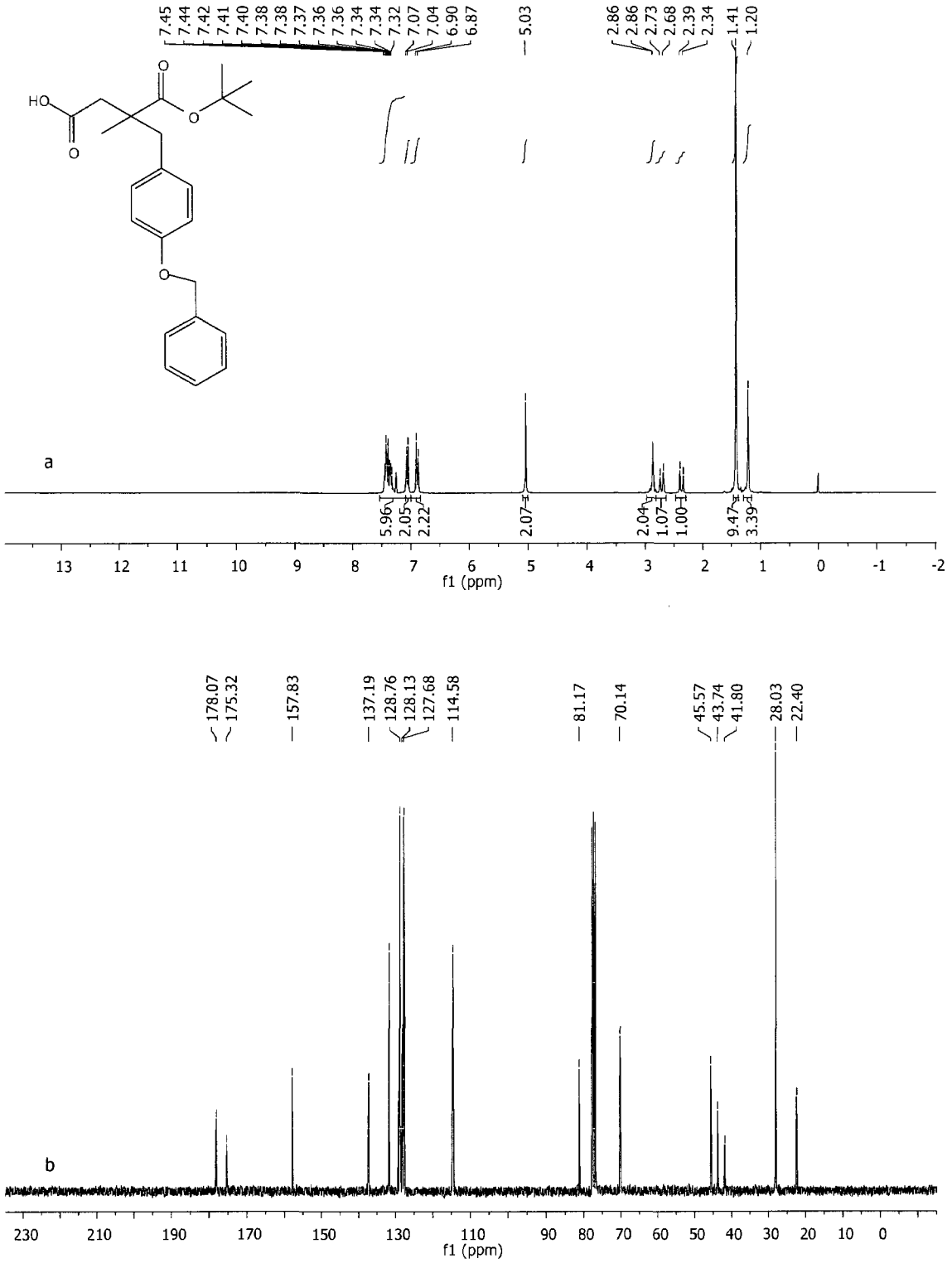
A.25. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.33b**



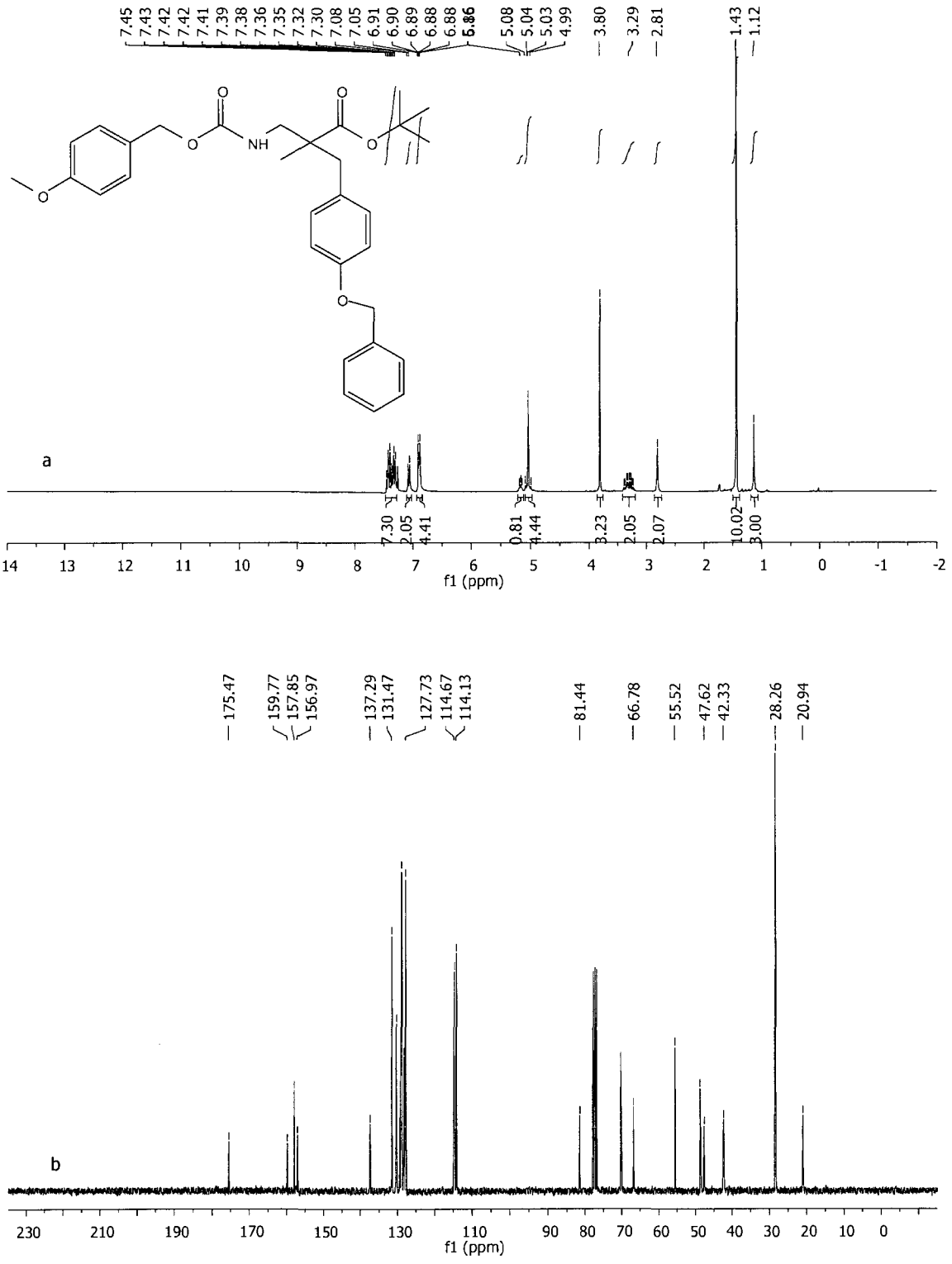
A.26. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.34b**



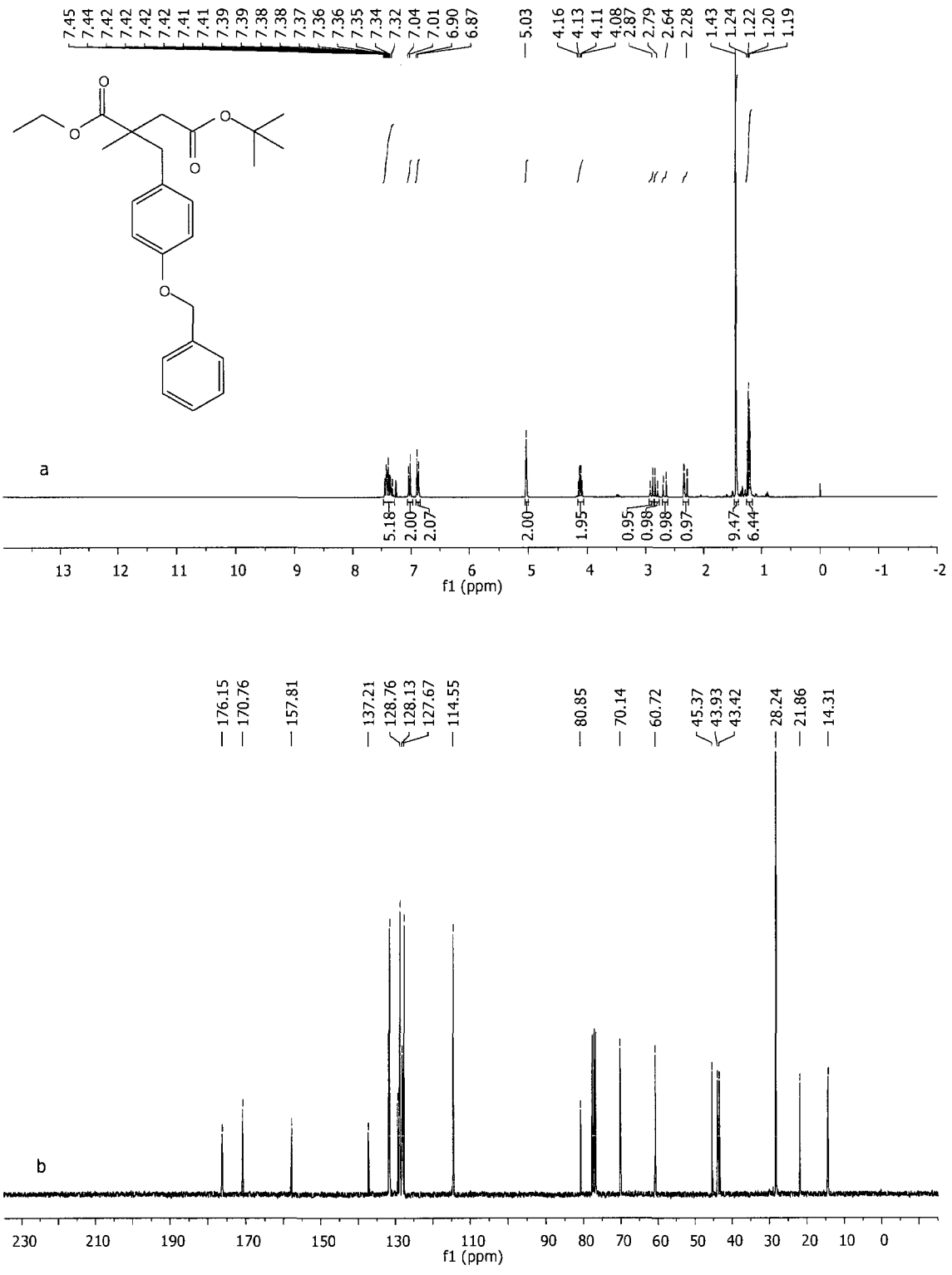
A.27. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.35b**



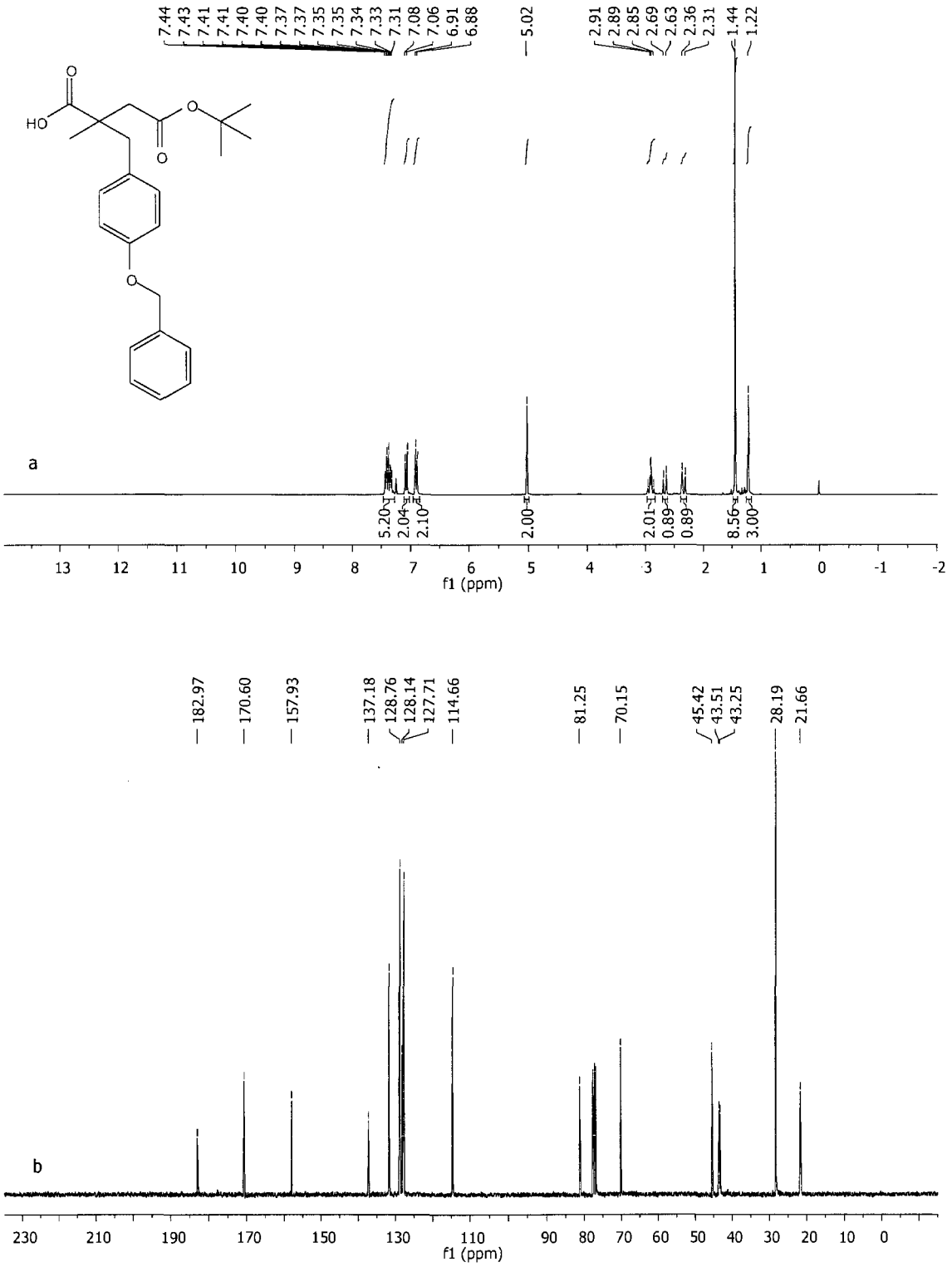
A.28. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.36b**



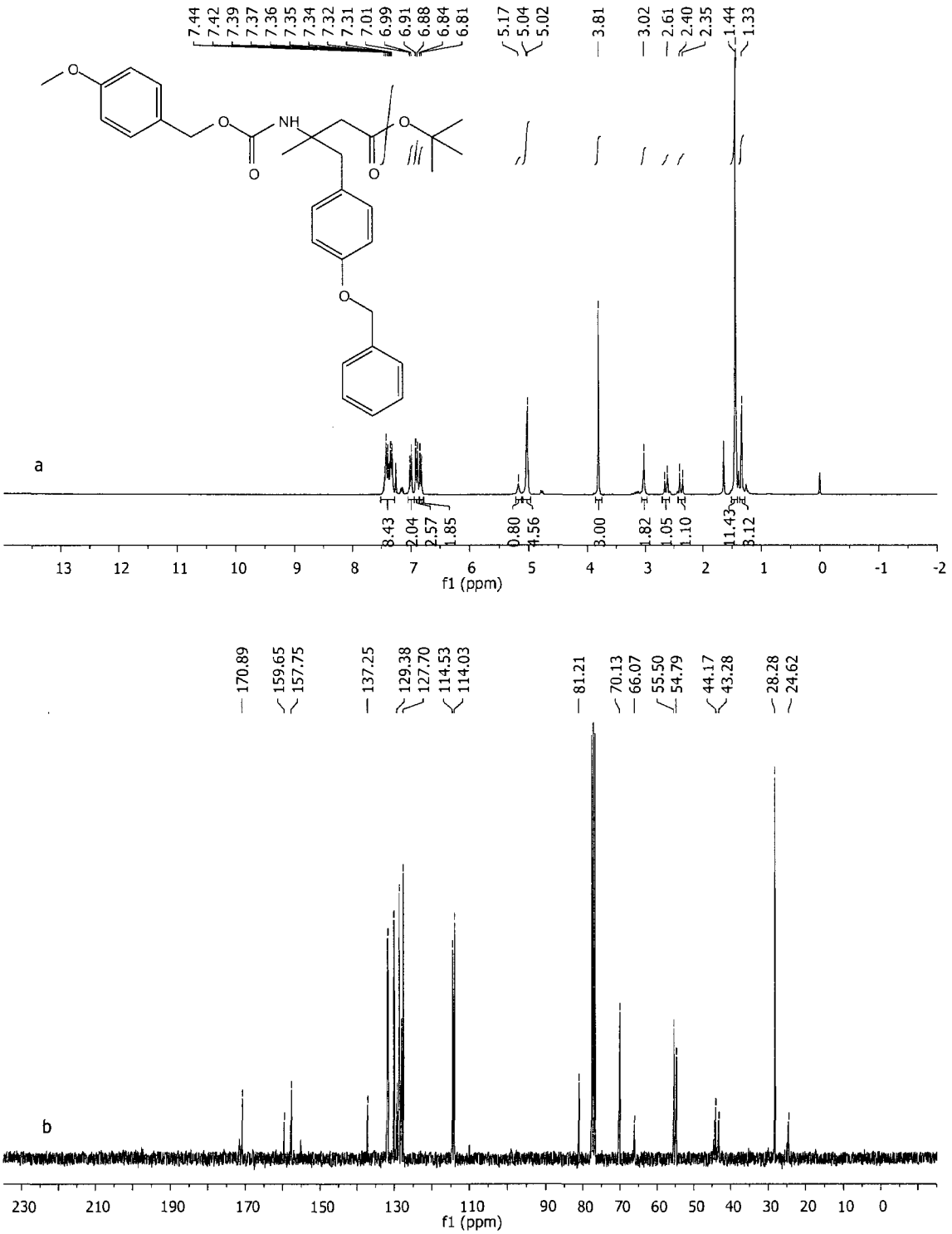
A.29. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.37b**



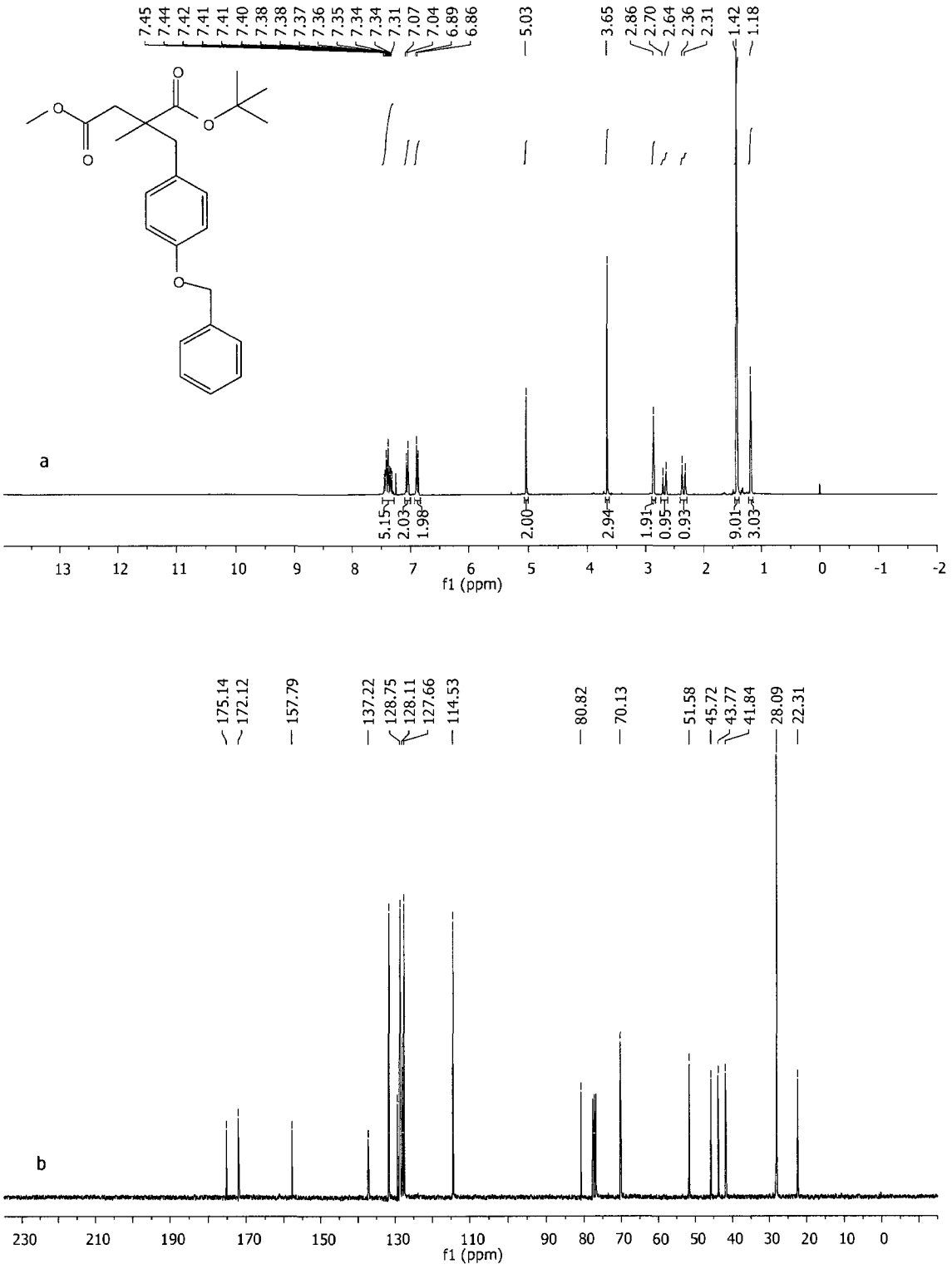
A.30. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.38b**



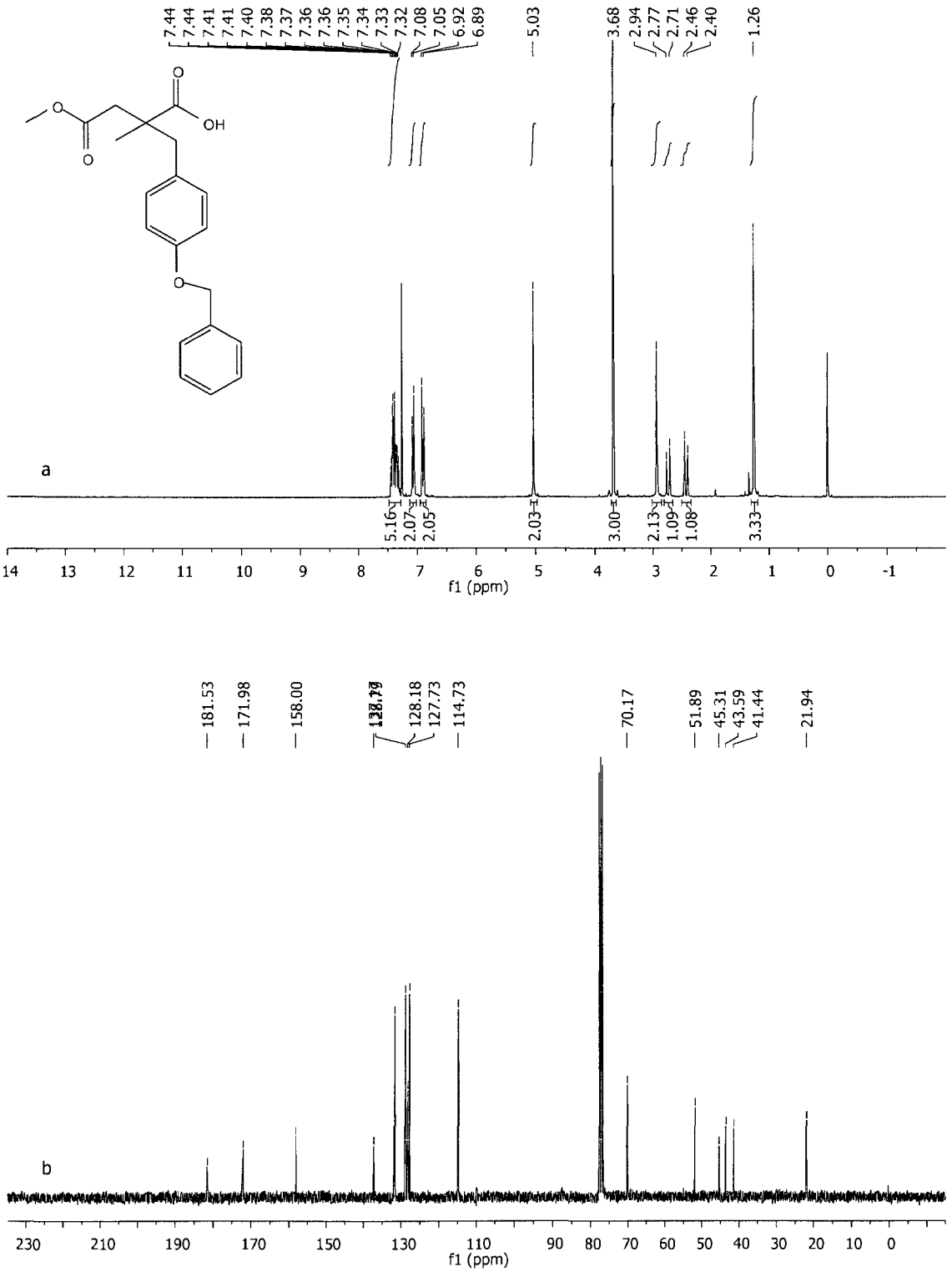
A.31. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.39b**



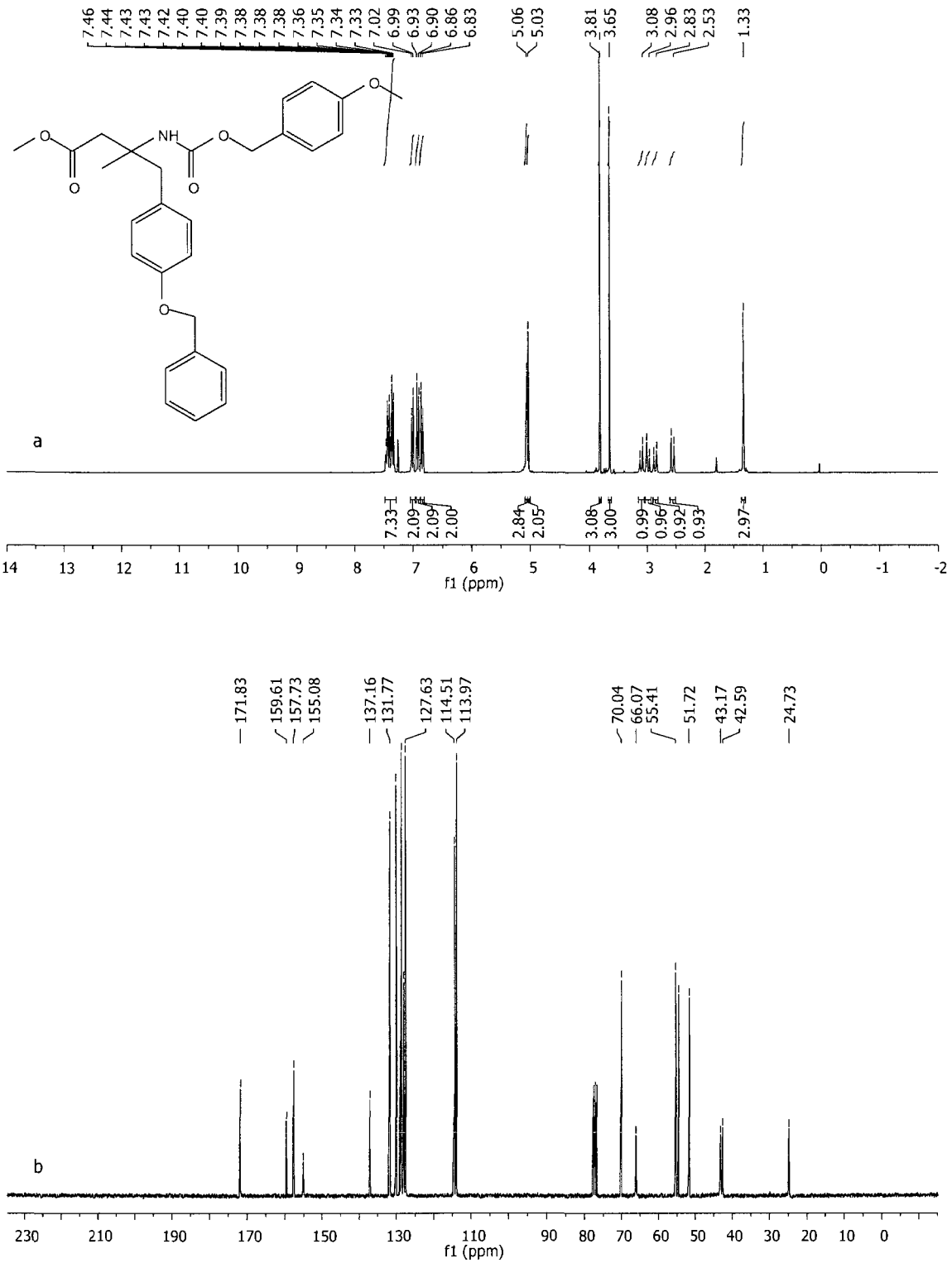
A.32. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.40b**



A.33. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.41b**

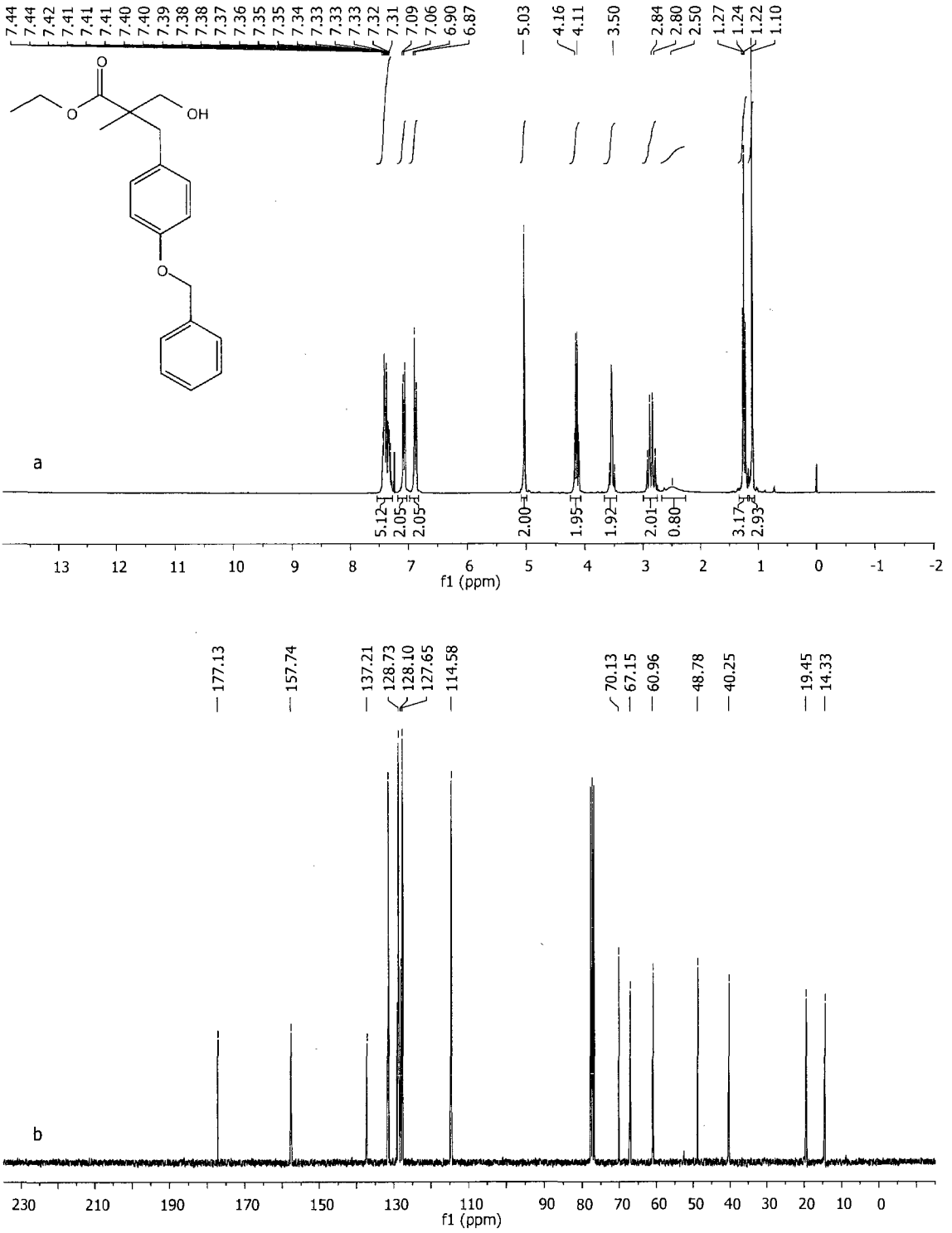


A.34. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.42b**

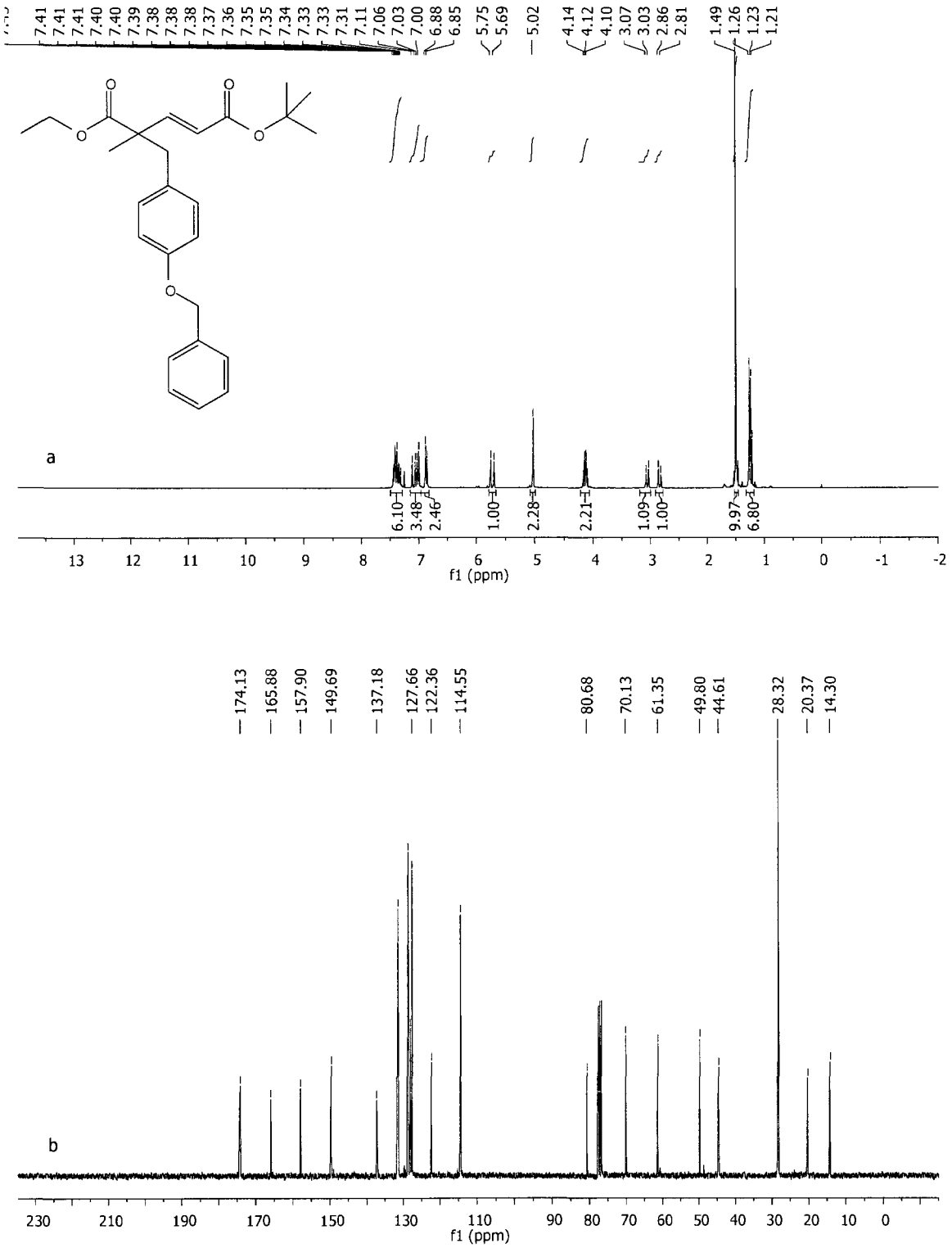


A.35. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **3.43b**

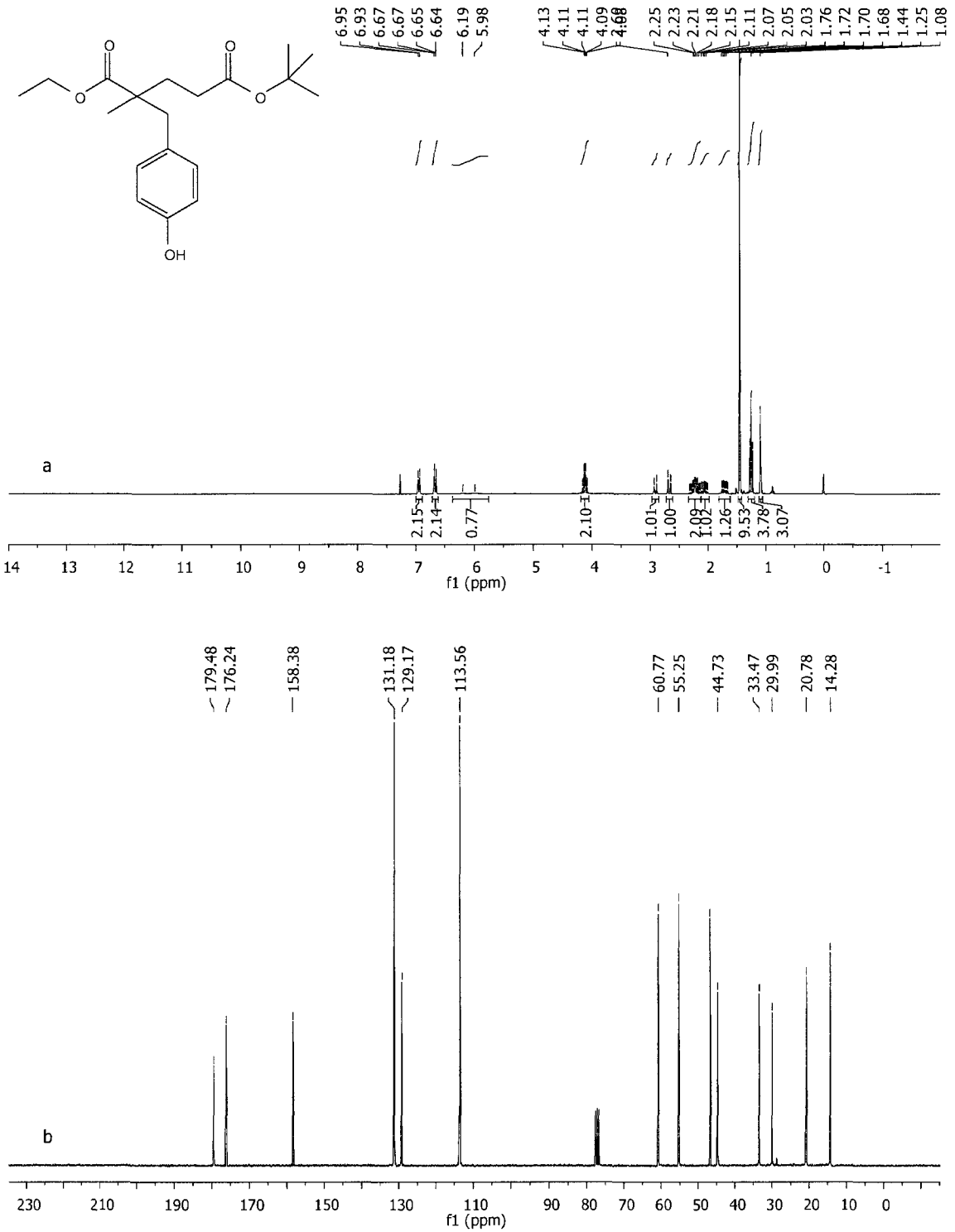
Chapter IV



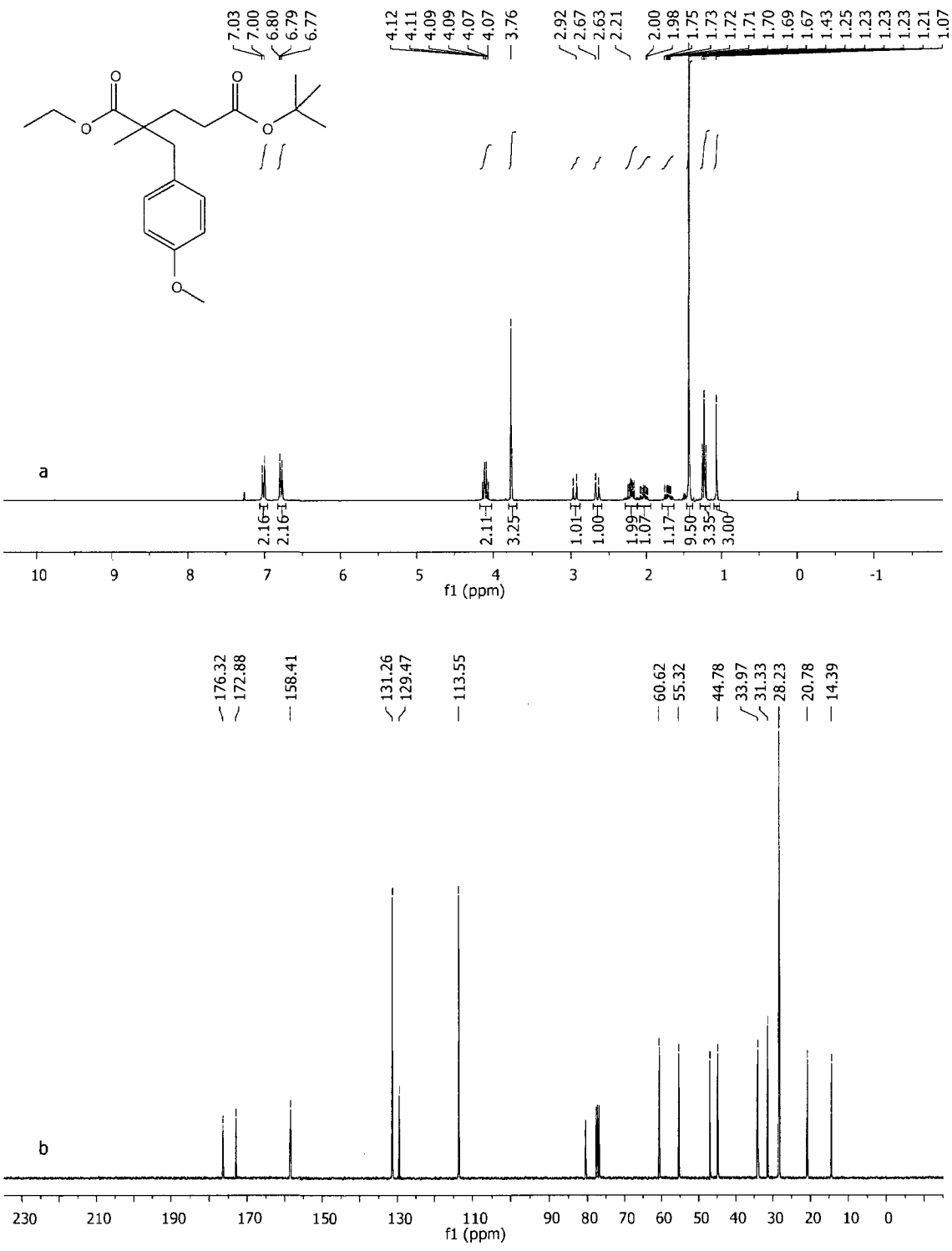
A.36. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **4.43**



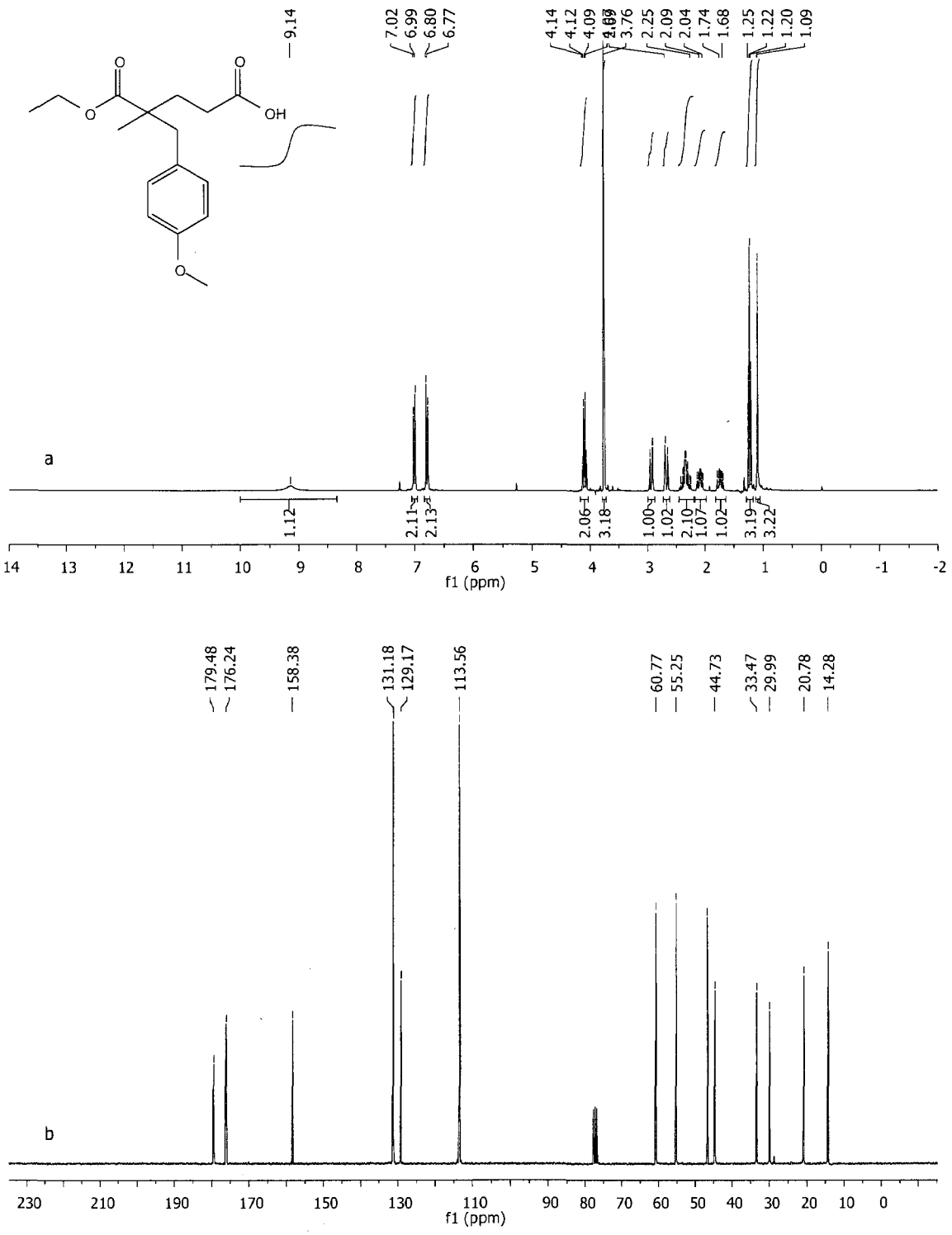
A.37. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **4.45**



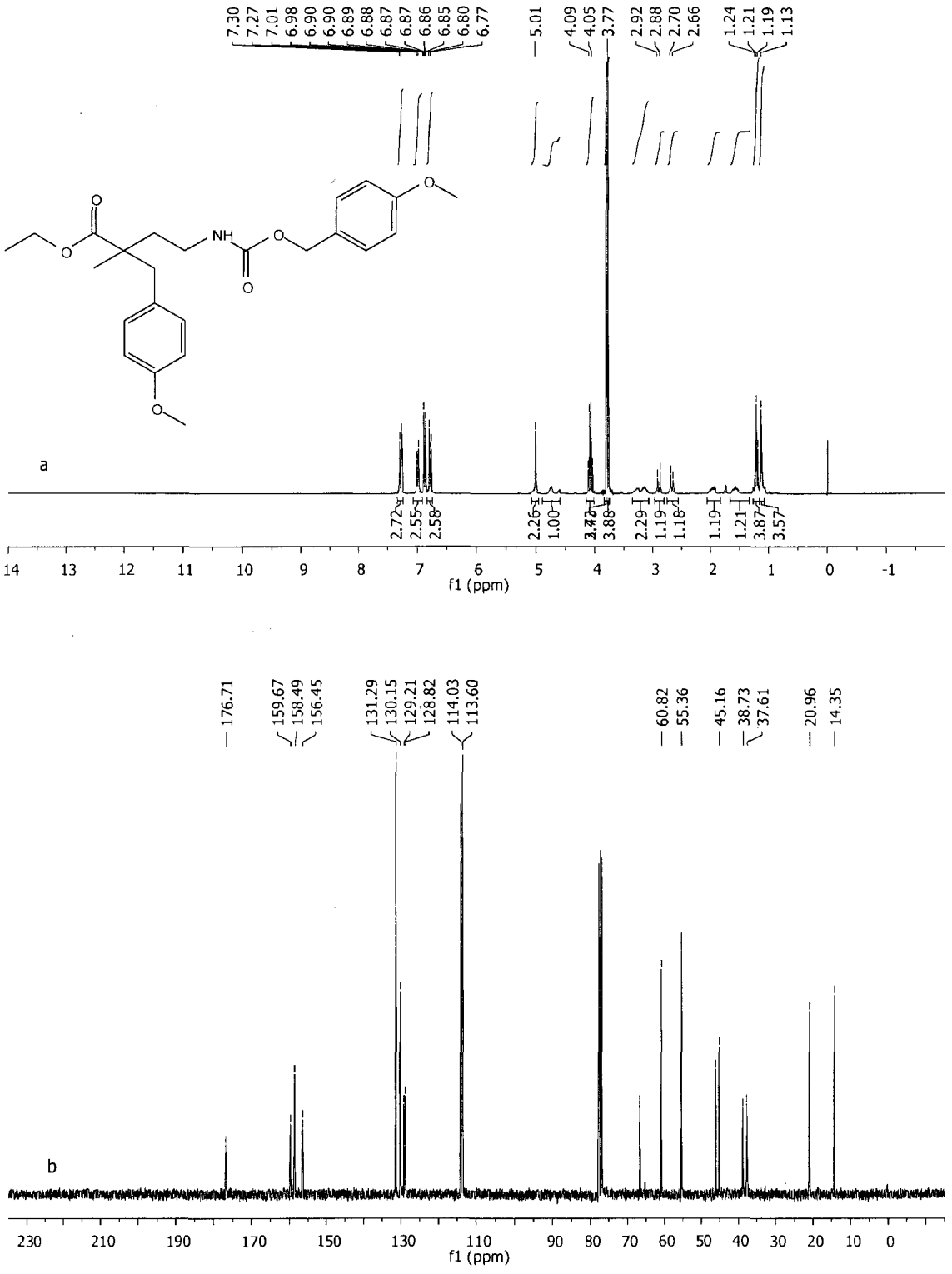
A.38. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **4.46**



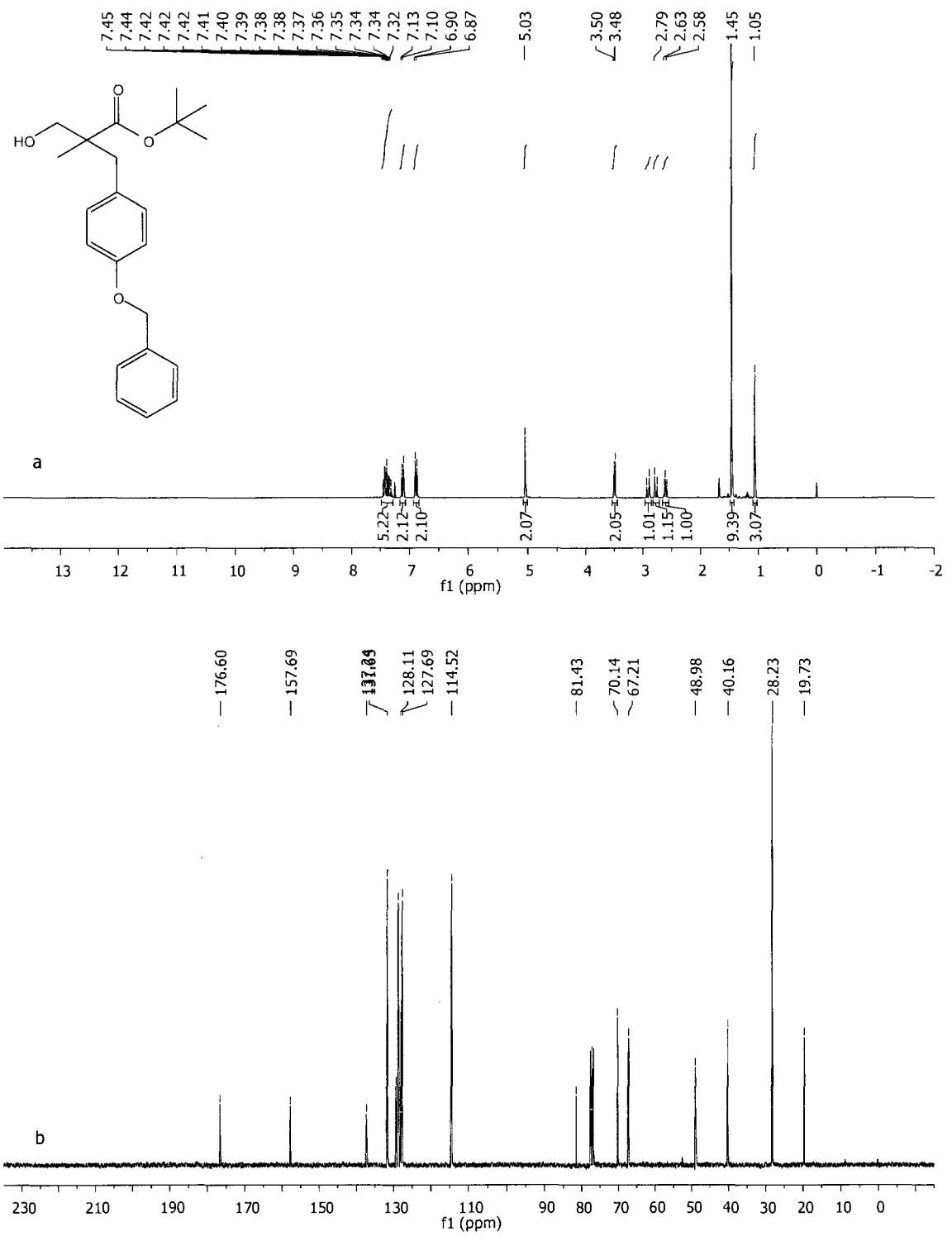
A.39. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **4.47**



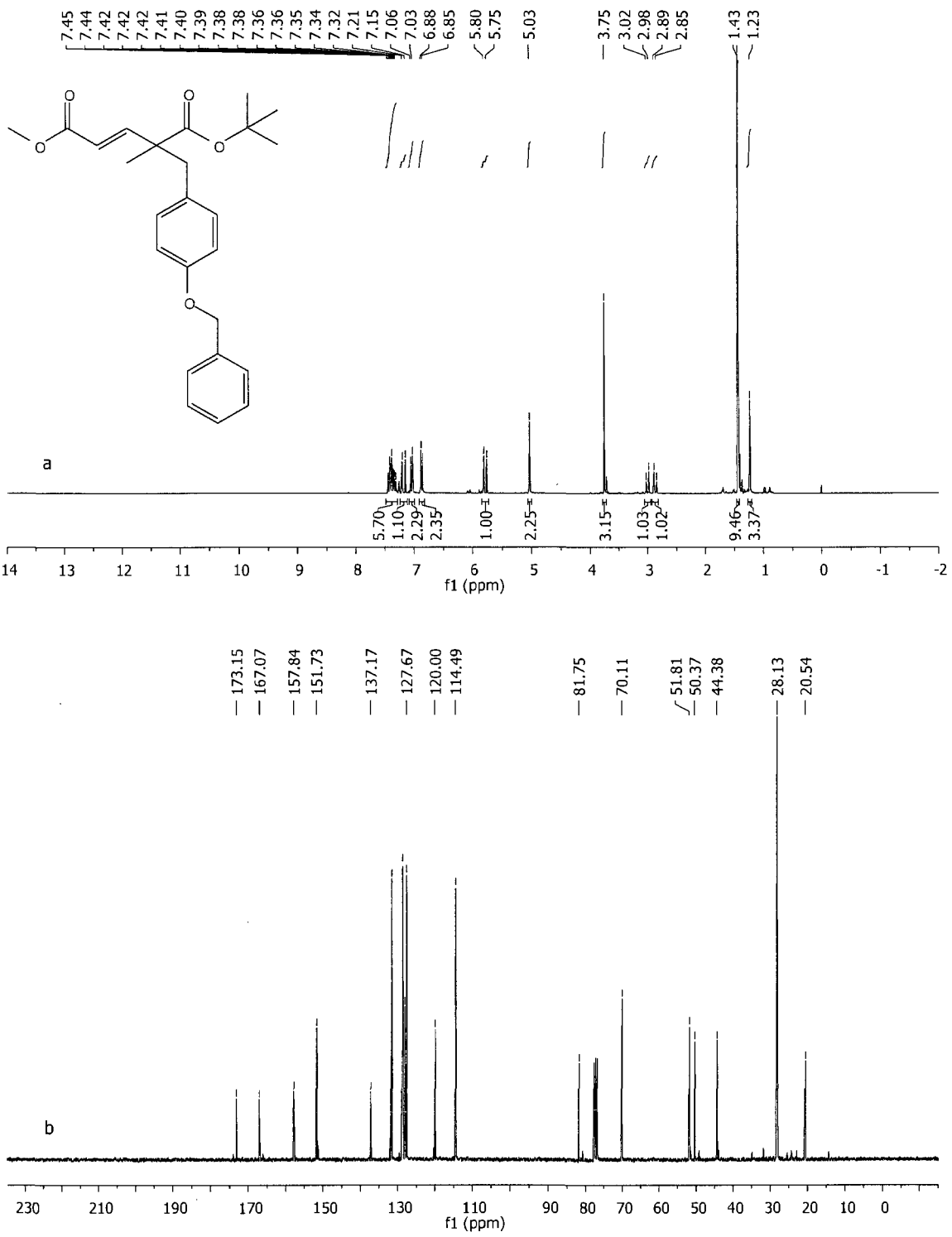
A.40. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **4.48**



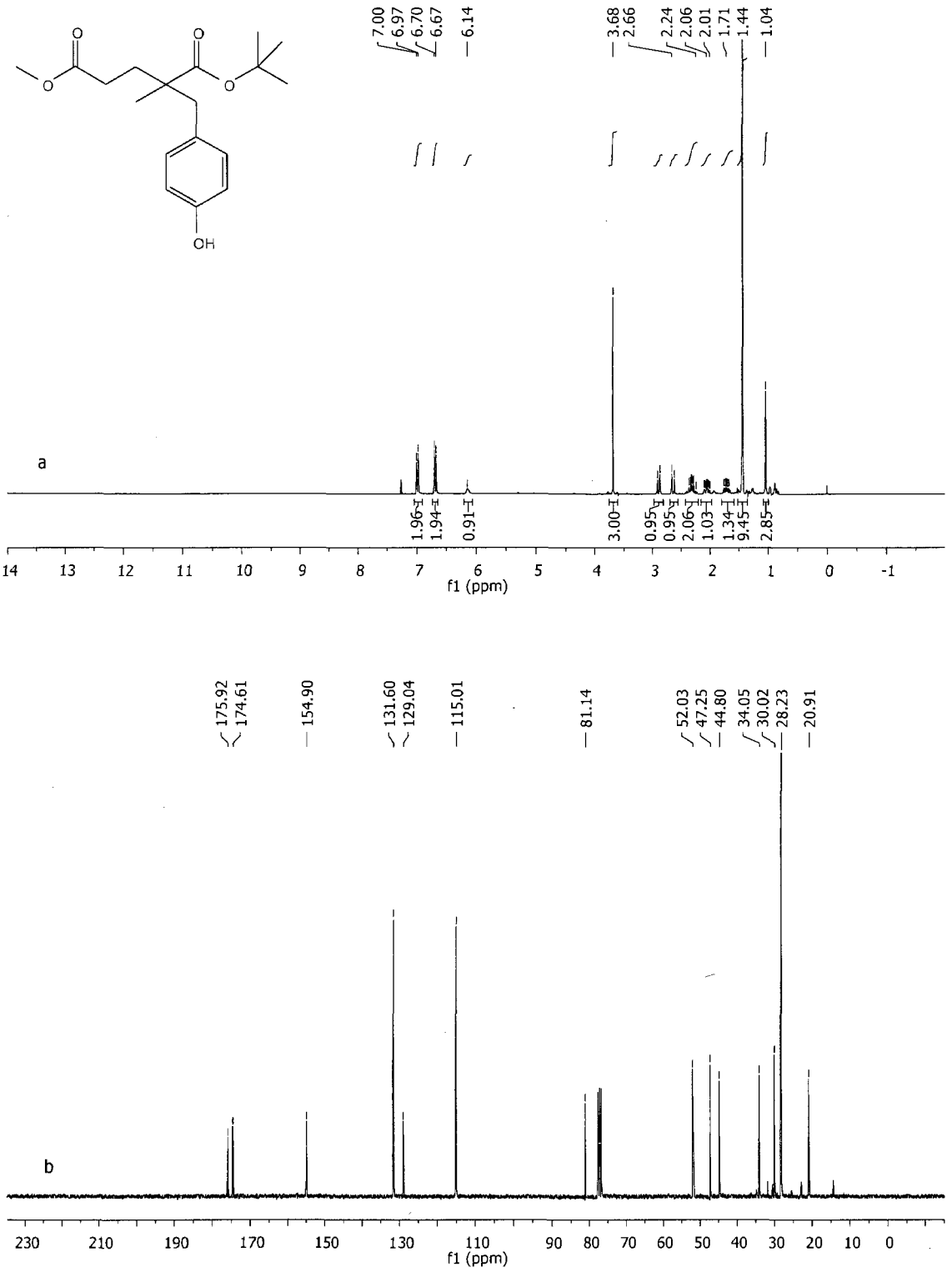
A.41. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **4.49**



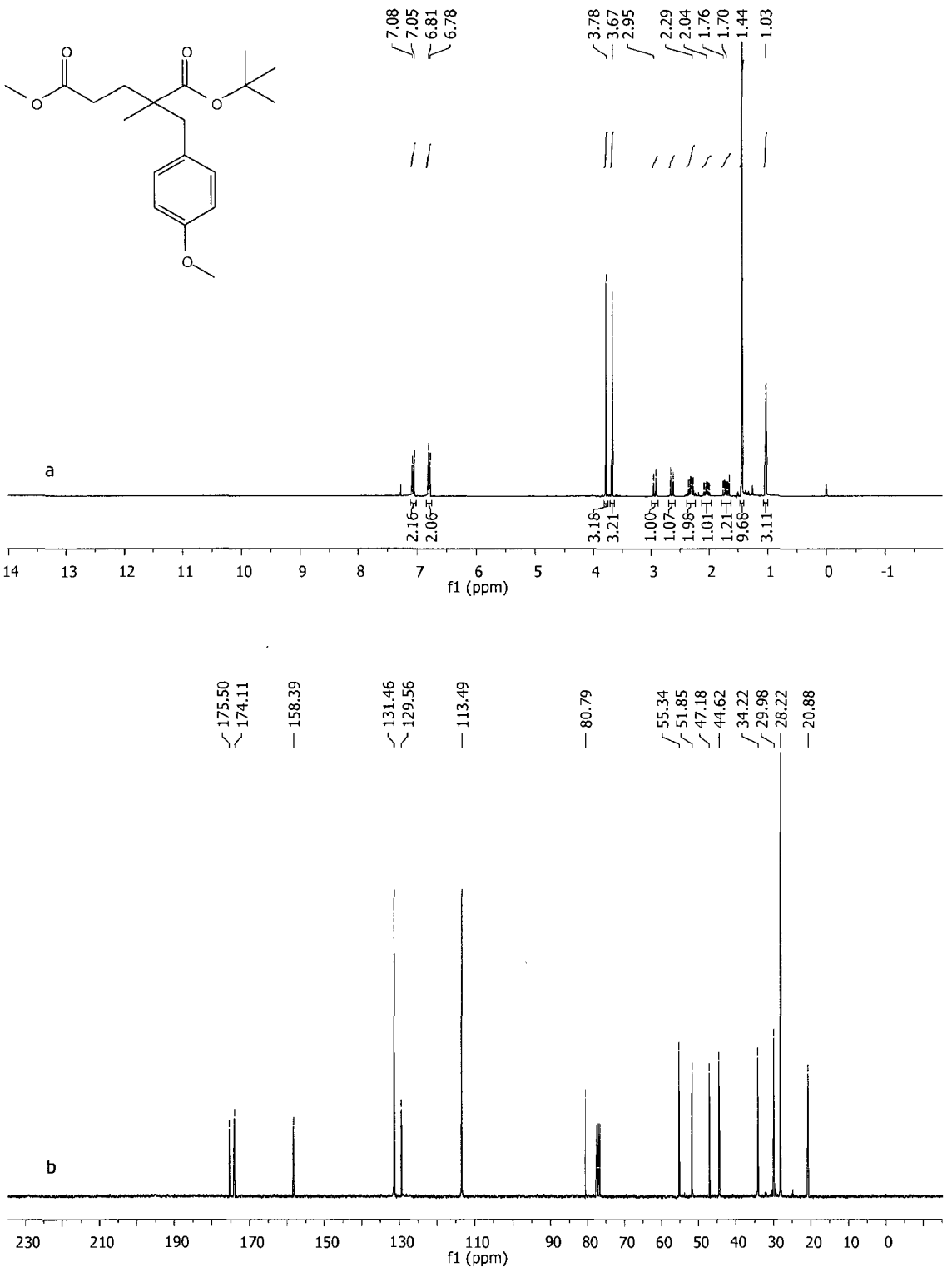
A.42. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **4.53**



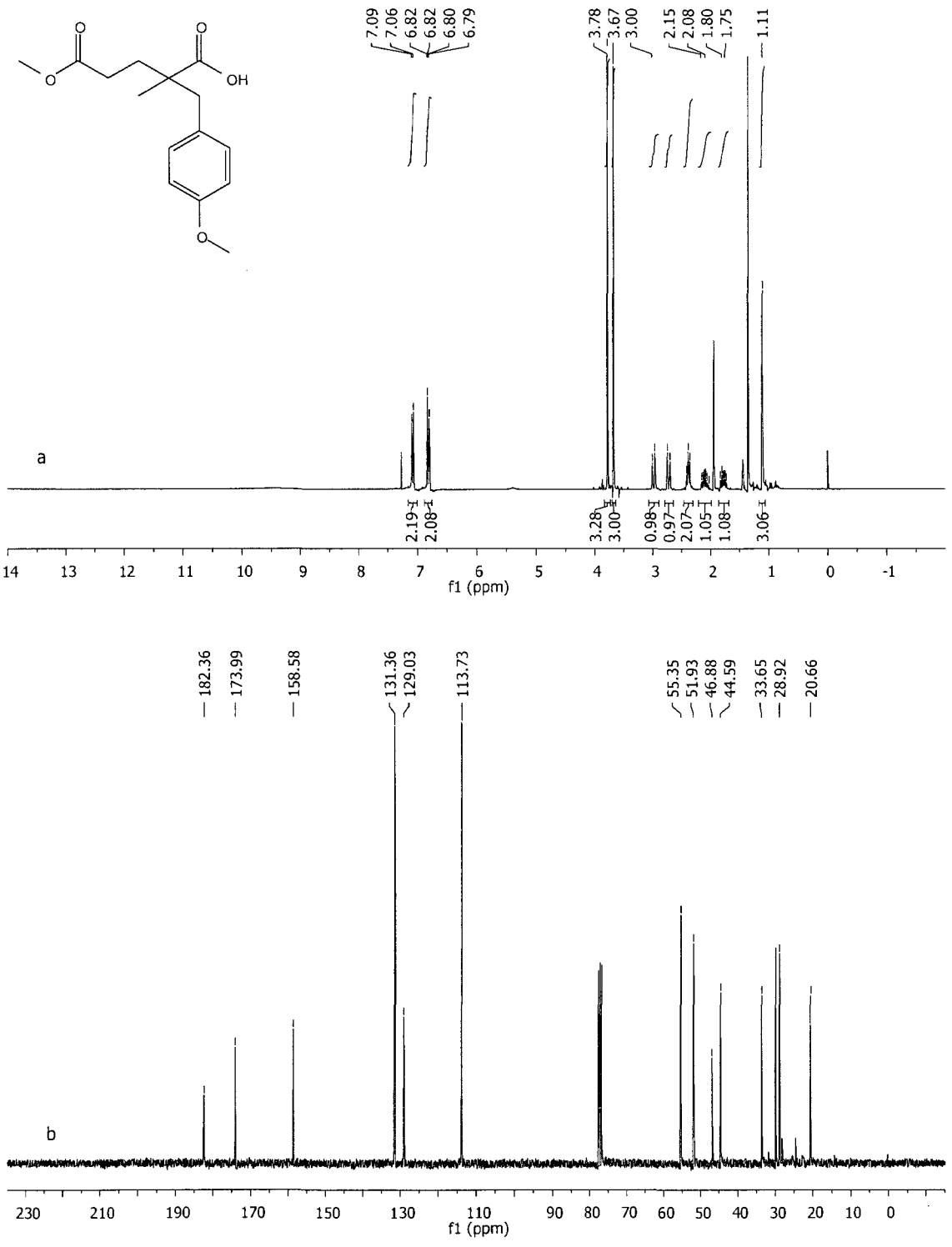
A.43. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **4.55**



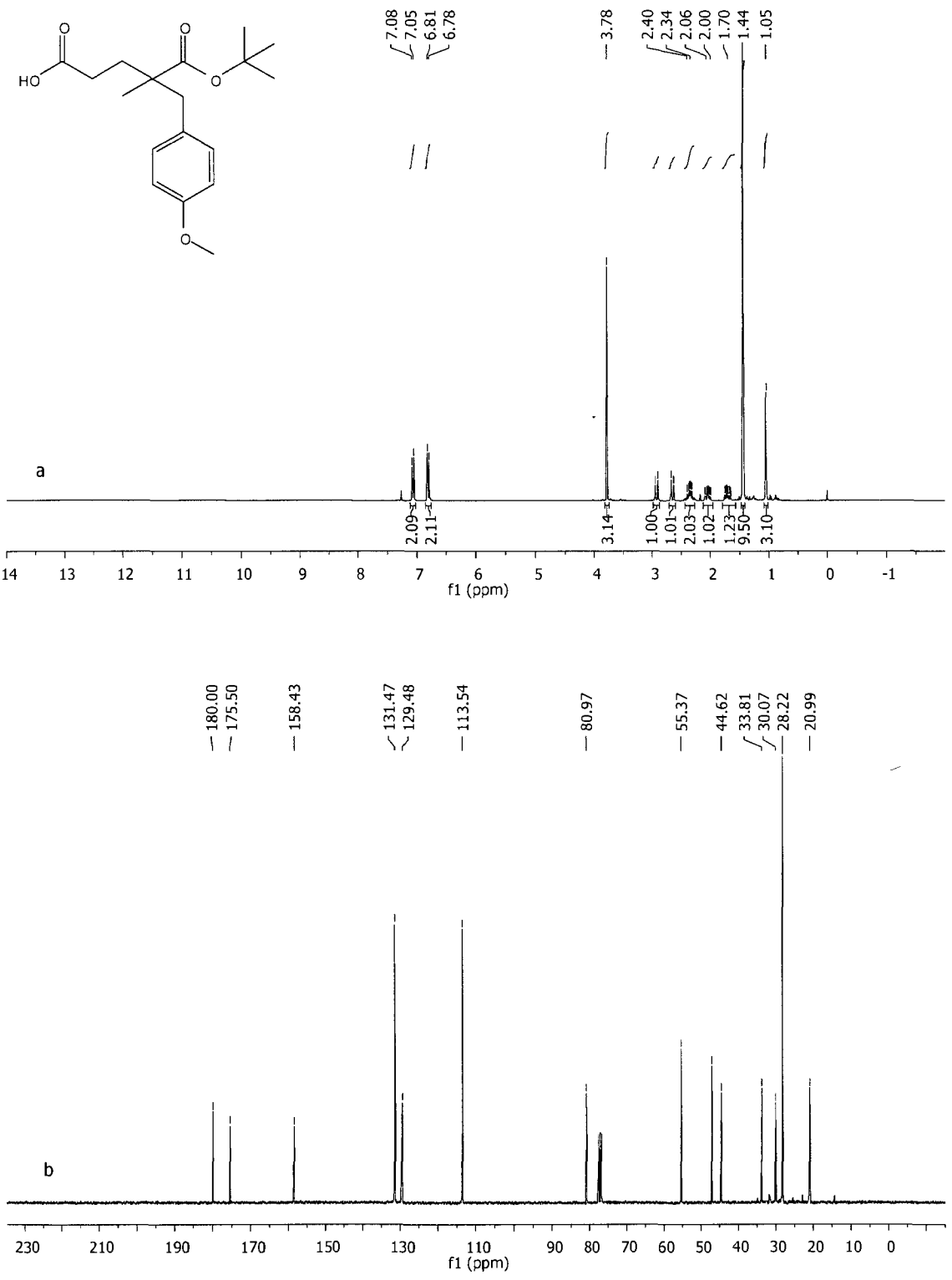
A.44. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **4.56**



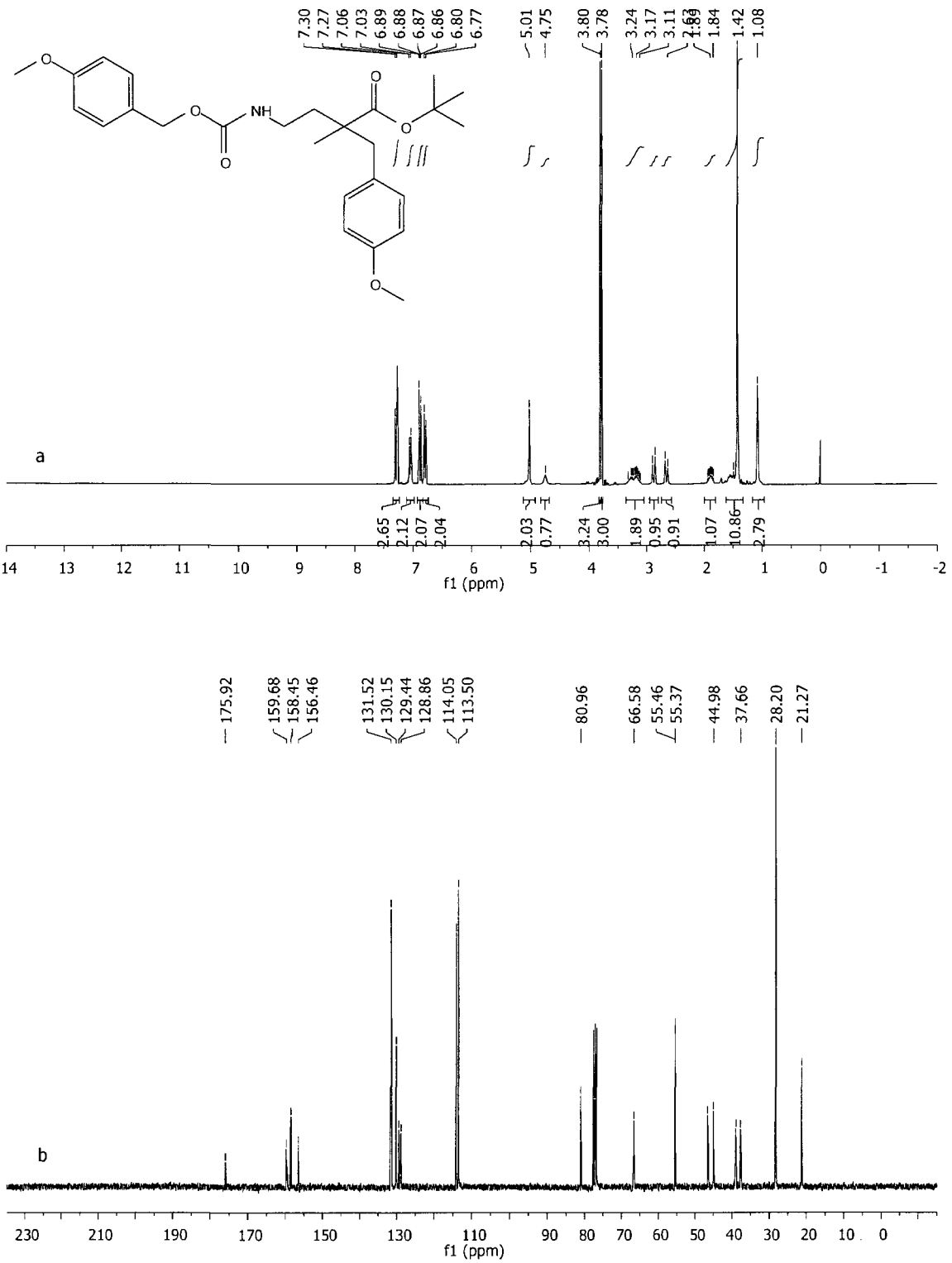
A.45. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **4.57**



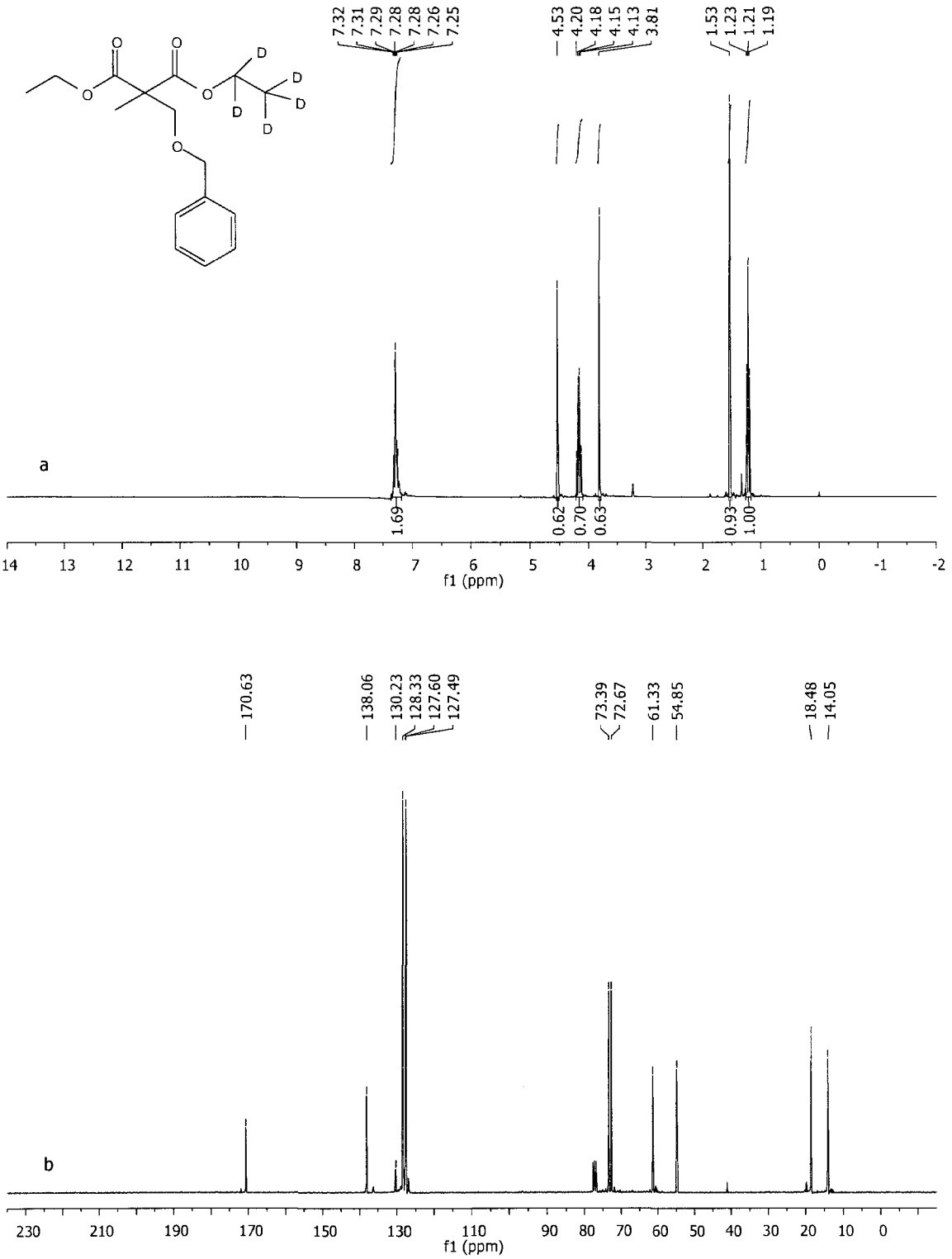
A.46. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **4.58**



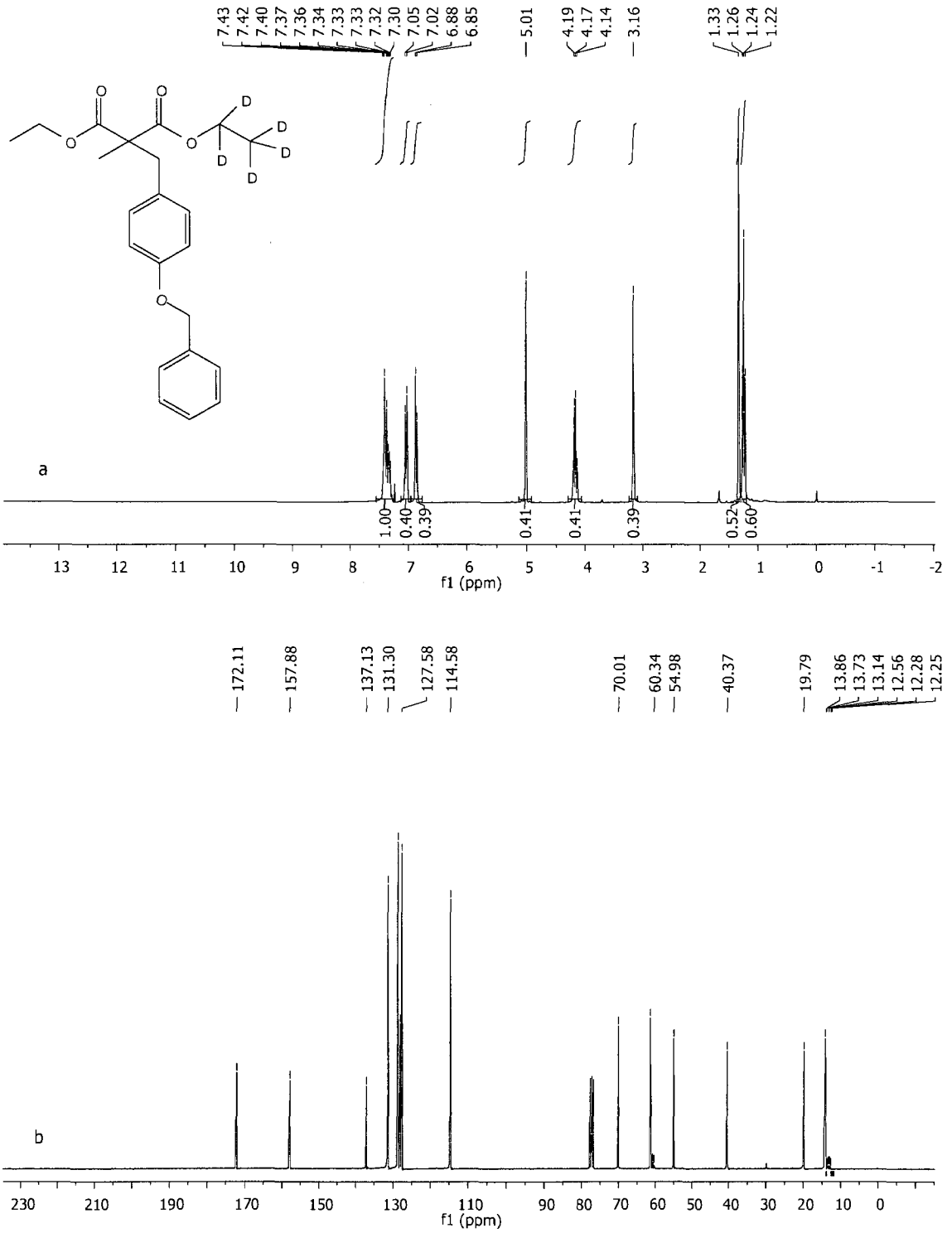
A.47. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **4.60**



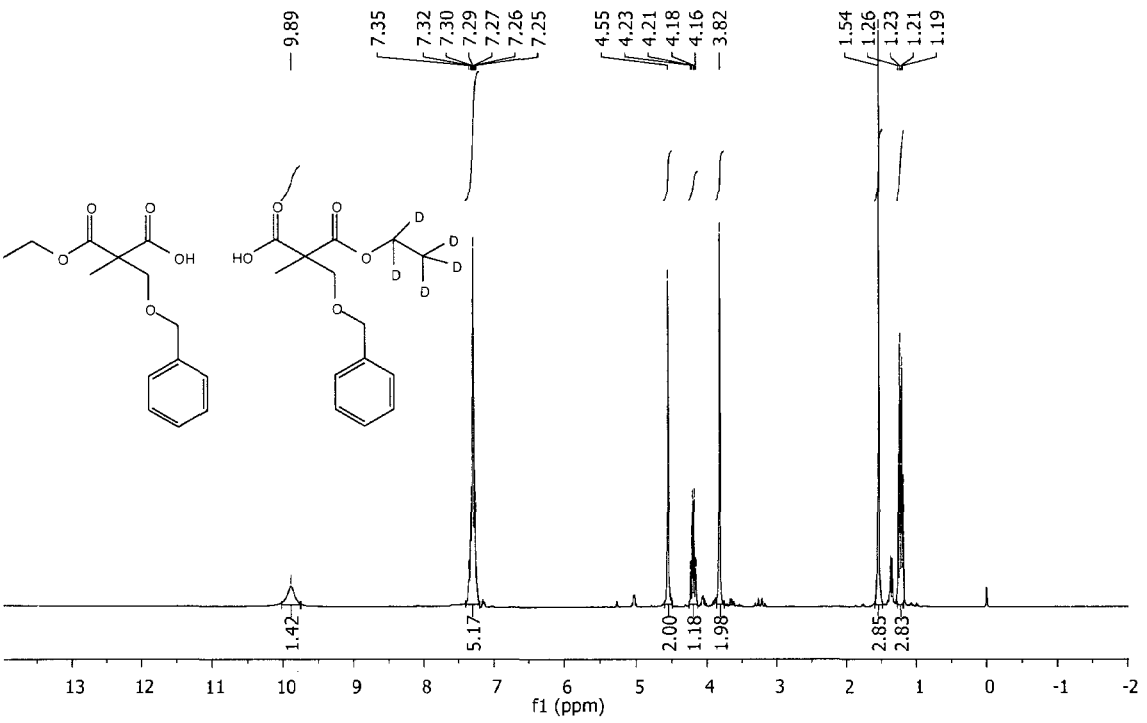
A.48. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **4.61**



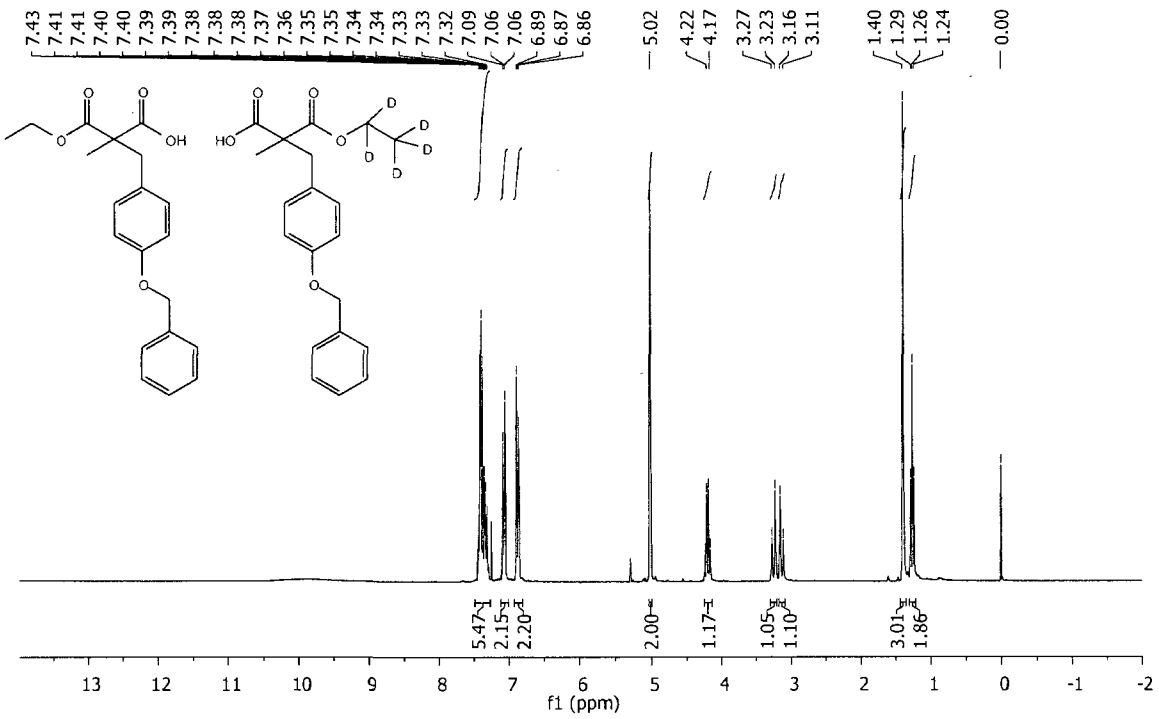
A.49. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **5.31a**



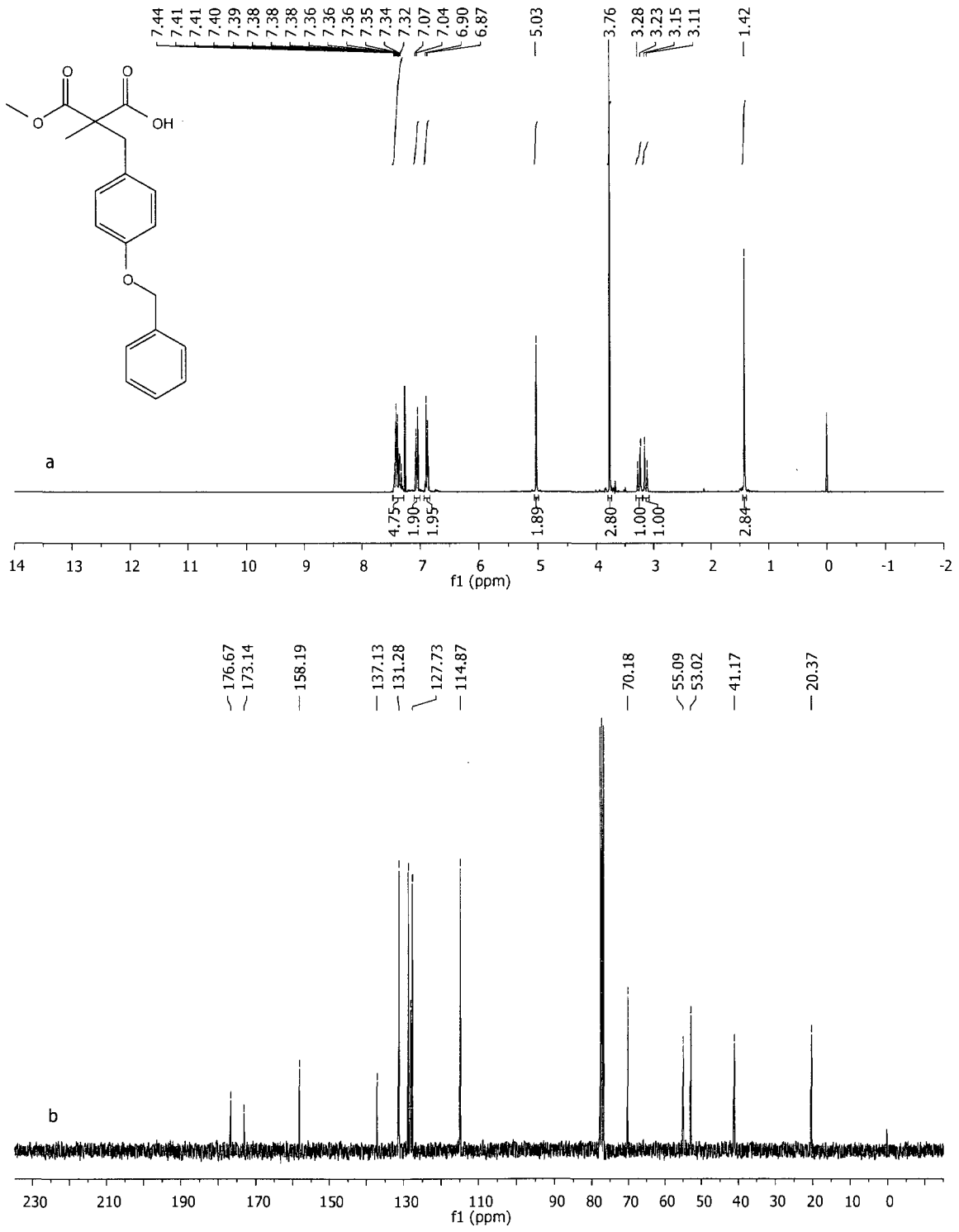
A.50. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **5.31b**



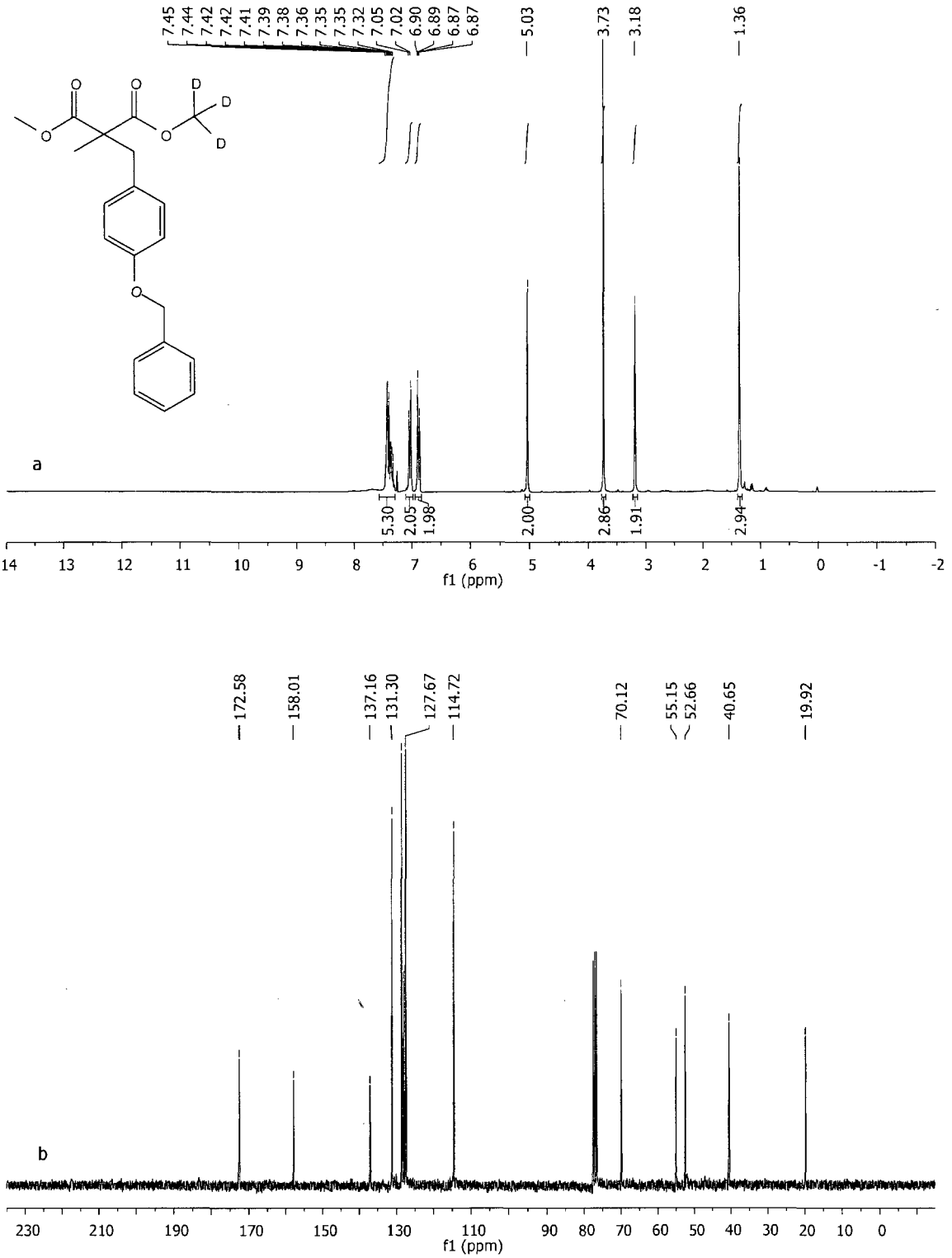
A.51. a) ^{1}H-NMR of compound +/- **5.32a**



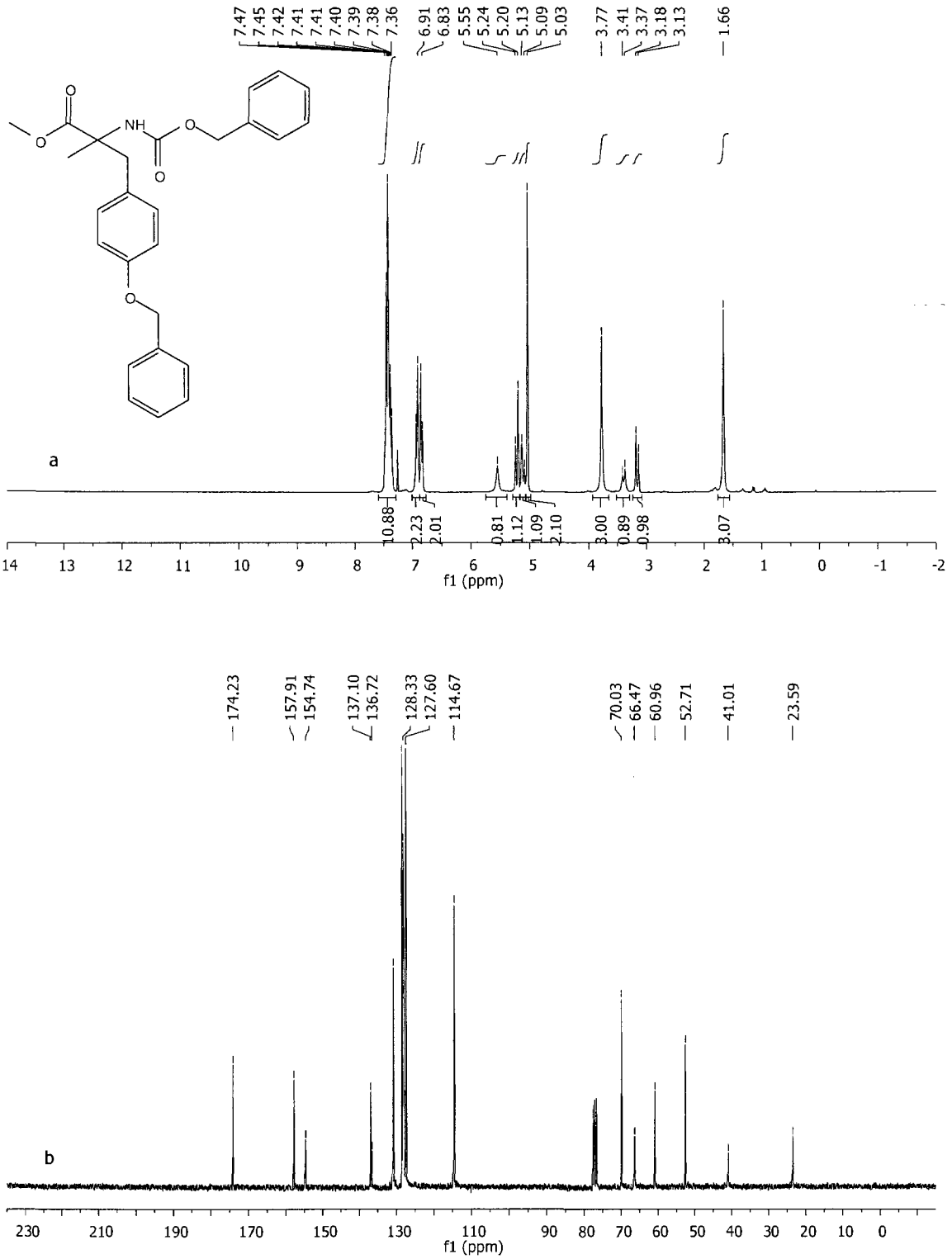
A.52. a) ^{1}H-NMR of compound **+/- 5.32b**



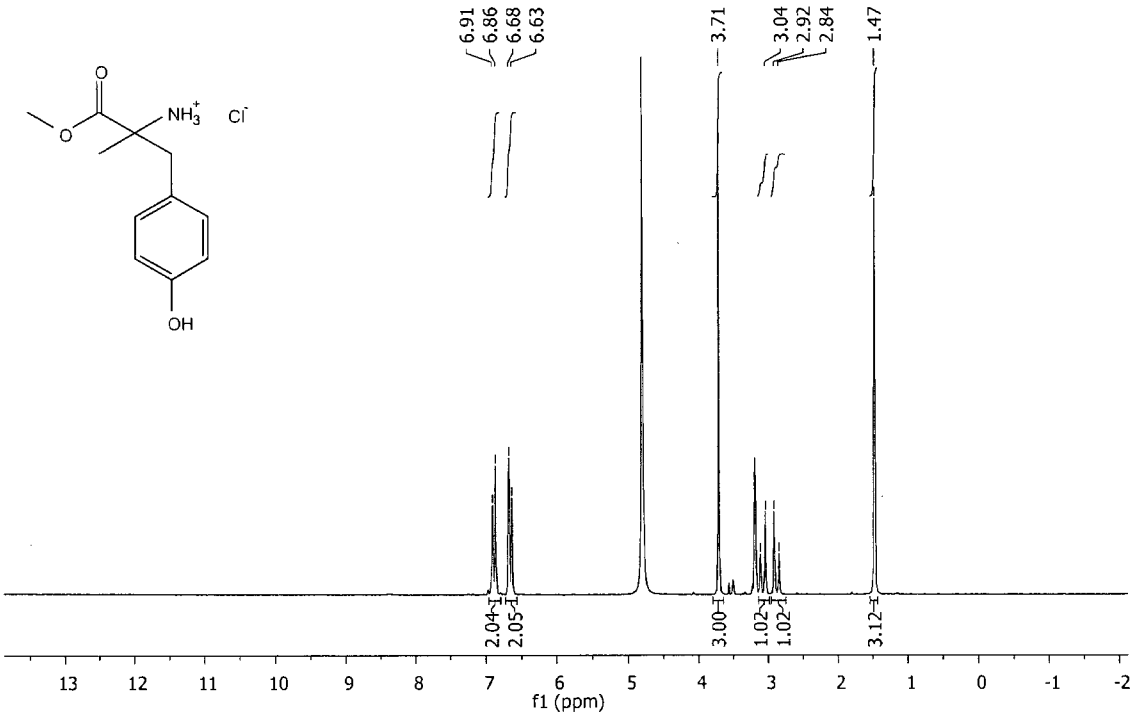
A.53. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **5.38**



A.54. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **5.39**



A.55. a) ^{1}H-NMR and b) ^{13}C-NMR of compound **5.40**



A.56. ^{1}H-NMR of compound **5.41**