ANDREW STEPHEN OSBORNE

I. Investigations in the Chemistry and Enzymology of L-Tryptophan
II. Kinetic Studies of Active Site Mutants C295L and C295V of Secondary Alcohol Dehydrogenase from *Thermoanaerobacter ethanolicus*
(Under the Direction of ROBERT S. PHILLIPS)

Chapter I of this dissertation investigates the efficient synthesis of ^{15}N-L-tryptophan, and its subsequent use as an NMR probe for the detection of conformational changes in wild type tryptophan synthase and mutants K87T, D305A, and E109D. ^{15}N-HSQC-NMR of enzyme-probe complexes with and without the presence of the α-ligand, α-glycerophosphate, were obtained. The wild type enzyme showed a weak cross peak at 10.25 ppm and 132 ppm in absence of α-glycerophosphate, and a signal six times stronger in the presence of glycerophosphate. Mutants K87T and E109D show a similar cross peak irrespective of α-glycerophosphate, indicating a closed conformation, but mutant D305A only shows a similar peak in the presence of α-glycerophosphate.

Chapter II of this dissertation is the report of the efficient and regioselective nitration of N_α, N_1-bis-trifluoroacetyl-L-tryptophan methyl ester. Aromatic nitration of L-tryptophan has been problematic in the past, and the methodology reported here is useful as the protected nitro-L-tryptophan products are versatile reagents for the synthesis of other aromatic derivatives of L-tryptophan via Sandmeyer chemistry. Both isomers are synthesized in high yield, and purified without chromatography.

Chapter III of this dissertation is the kinetic investigation of mutants C295L and C295V of secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus.* The mutants were evaluated for their efficiency in catalyzing the oxidation of the small chiral alcohols 2-butanol, 2-pentanol and 2-hexanol at 15º C, 25º C, 35º C, 45º C, and 55º C. Mutant C295L showed a temperature dependence in enantioselectivity for all alcohols, with the (R)-enantiomers being favored at low temperatures. Mutant C295V shows a trend reversal in temperature dependence for 2-butanol and 2-pentanol near 315 K.
INDEX WORDS: HSQC-NMR, Tryptophan Synthase, L-Tryptophan, Alcohol Dehydrogenase, *Thermoanaerobacter ethanolicus*, Asymmetric Reduction.

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BY

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The University of Georgia
December 2002

DEDICATION

To the Lord Jesus Christ

To my parents, my wife Jill, my brothers and sisters, and the rest of my family

ACKNOWLEDGMENTS

First and foremost, I would like to thank to my Major Professor, Professor Robert S. Phillips for his generous financial support, wisdom, guidance and patience. I would also like to thank my other committee members, Dr. Thomas Johnson and Dr. George Majetich for their training and teaching. I also owe a great deal of personal and professional gratitude to Professors Robert A. Scott, Richard K. Hill and Butch Atwood for their financial support, mentoring, and encouragement.

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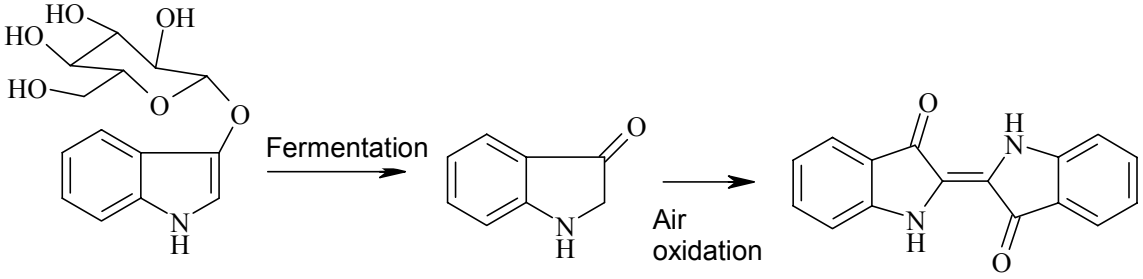
INTRODUCTION AND LITERATURE REVIEW

Section 1. History, Chemistry and Enzymology of L-Tryptophan[1]

"Illuminating by Fragmentation", from the Greek, is the literal meaning of the word tryptophan **(1)**.[2] In 1890, this name was given to a mysterious substance, known to be present in the putrefied tissues of various human organs, which produced a red compound upon reaction with aqueous chlorine or fuming nitric acid.[3-5] This in itself would be of limited curiosity if it weren't for the chemistry of Adolf von Baeyer.[6] Indigo[7] **(2)**, "The King of Dyestuffs", was the subject of much scientific investigation during the nineteenth century owing to the expense involved with the extraction of its natural product precursor, indican **(3)**, and with the actual process of vat dyeing with the reduced form of indigo, indoxyl **(4)** (Fig.1). The chemistry of Baeyer not only led to several syntheses of indigo, but to the elucidation of a pure compound that also produced a red substance upon treatment with fuming nitric acid, indole. Thusly, a correlation between indole and this mysterious color producing substance was established.



Indican **(3)** Indoxyl **(4)** Indigo **(2)**

Figure 1. Method for large-scale production of indigo

In 1841, Erdmann reported that oxidation of indigo by chromium trioxide afforded the brilliant orange compound, isatin **(5),**[8] and Baeyer and Emmerling reported that the reduction of isatin with zinc afforded indole **(6),**[9] a colorless compound with a melting point of 52º C. Indole was also prepared by treatment of *ortho*-nitrocinnamic

acid **(7)** with potassium hydroxide, and these two syntheses provided structural confirmation for this new compound (Fig.2).

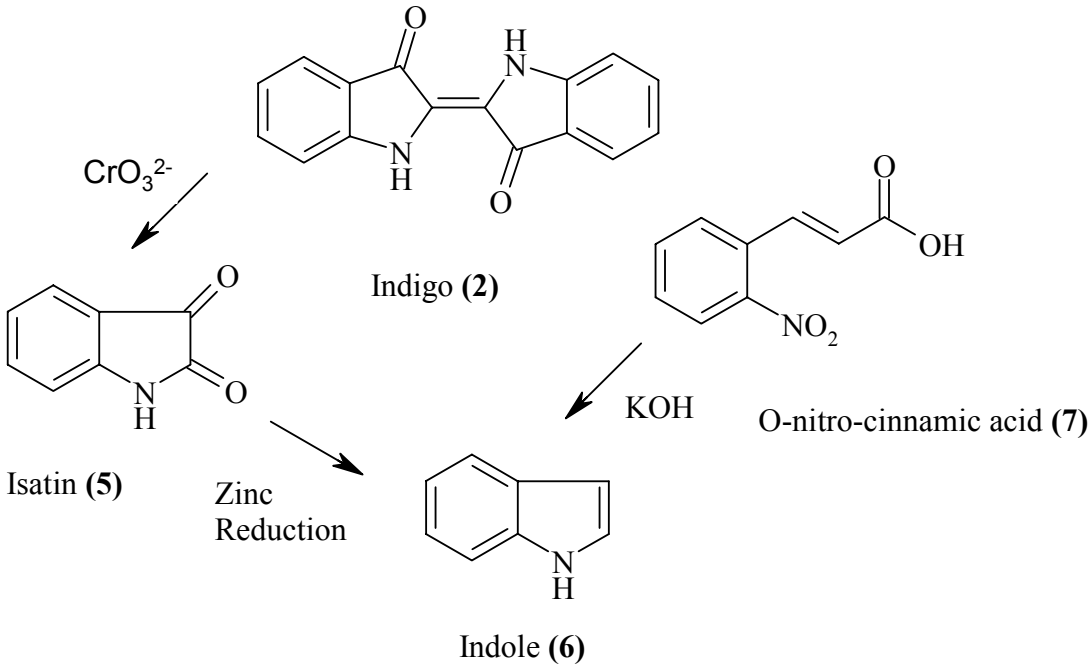


Figure 2. Synthesis and structure proof of indole.

The report of the red product produced by indole's reaction with fuming nitric acid, now known as the "colored product" and "colored reaction" respectively, garnered the attention of Nencki[10] and Kuhne,[11] working independently. Mutually convinced that this compound was related to, if not the identity of, the substance present in organ tissues, these scientists repeated the decade's old work of examining putrefied organ proteins and were able to isolate several substances which produced the colored compound via the colored reaction. Curiously, the substances isolated by Nencki or Kuhne in 1875 had several properties which differed from the indole prepared by Baeyer. The melting points of several of these compounds were over 90º C, and all of them had the characteristic fecal odor as reported in the decades old original work of Tiedemann,[3] Bopp,[12] and Bernard.[4] 3-Methyl indole, isolated from human feces by Brieger in 1877,[13] produced the

fecal odor characteristic of the putrification extracts, melted at 95-97º C and was unreactive to fuming nitric acid. It was given the name skatole **(8)** for obvious reasons.[13] Recognizing that indole contaminated with skatole could account for the inconsistencies between Baeyer's indole and the crystals isolated from human organs, Nencki separated indole, with identical properties to those of Baeyer, from this possible contaminant by recrystallization from ether and water.[14]

The presence of indole in protein was now confirmed, but several questions still needed to be answered. First, protein only afforded the colored compound after being putrefied extensively, or by other chemical modification. Secondly, some proteins provided a stronger indication with the colored reaction than others. For instance, putrification of gelatin failed to produce compounds positive to the colored reaction; additionally, it had been established that gelatin was also of marginal nutritional value. With this knowledge, a correlation between an indole containing molecule in protein and essential nutrition could be established. Even though the identity of the indole containing molecule had not been established, it was named, *en obscura,* according to the positive colored reaction obtained after its degradation.

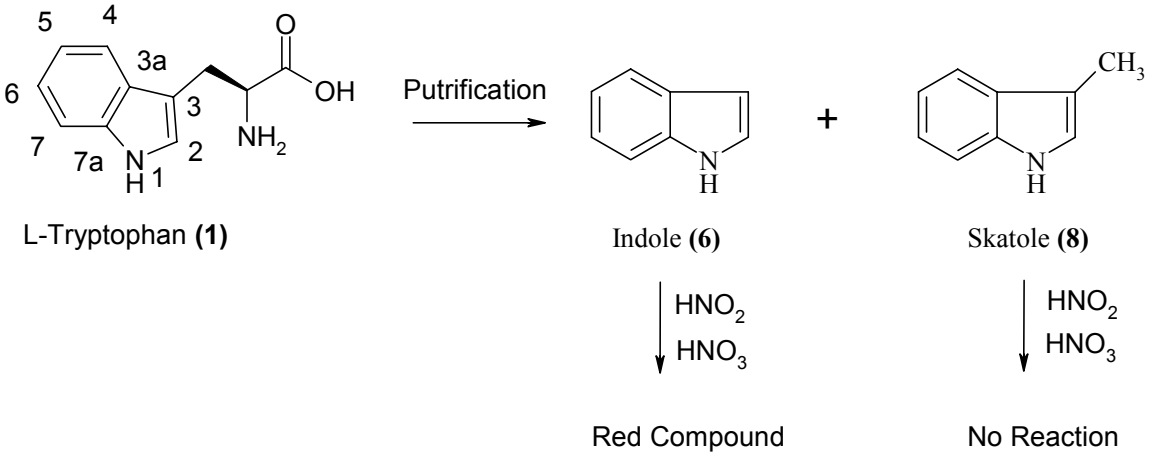


Figure 3. Degradation of L-tryptophan into indole and skatole

The structure of tryptophan would not be determined for another twelve years,[14] but an important fact was already apparent. The chemistry of L-tryptophan cannot be considered separate from the chemistry of indole, and that is just as true today.

L-tryptophan is present in essentially all protein, and plays many roles in biochemistry aside from as a structural element in peptides. L-tryptophan, one of eight essential amino acids in animals, is the precursor of several important hormones including serotonin **(9)** and melatonin **(10)** (Fig. 4). Serotonin, 5-hydroxytryptamine, is a neurotransmitter and has been the subject of much research for the development of drugs to combat depression and other ailments. Selective serotonin reuptake inhibitors (SSRI) such as Prozac™ and Paxil™ have recently seen widespread use for combating depression,[16] bipolar disorder and other conditions affecting sense of well-being. Melatonin is a pineal hormone which regulates daily and seasonal changes in reproductive activity, sleep patterns, appetite and energy metabolism.[17] It is not surprising that L-tryptophan, as a dietary supplement, has been reported to be very effective in treating mild cases of depression. However, in 1988, contaminants in commercially available L-tryptophan, from the Japanese company Showa-Denko, K.K., was reportedly responsible for an outbreak of 1300 cases of Eosinophilia Myalgia Syndrome which was responsible for more than 38 deaths.[18.19] Ondansetron **(11)**[20] is a selective 5-HT_3 antagonist and is used clinically as an anti-emetic to combat side effects of chemotherapy including nausea and vomiting. There are thousands of natural products containing L-tryptophan's functional group, indole, among them are the bis-indole anti-tumor agents vincristine **(12)** and vinblastine, and the noted hallucinogen, LSD, lysergic acid diethylamide **(13)** (Fig. 4).

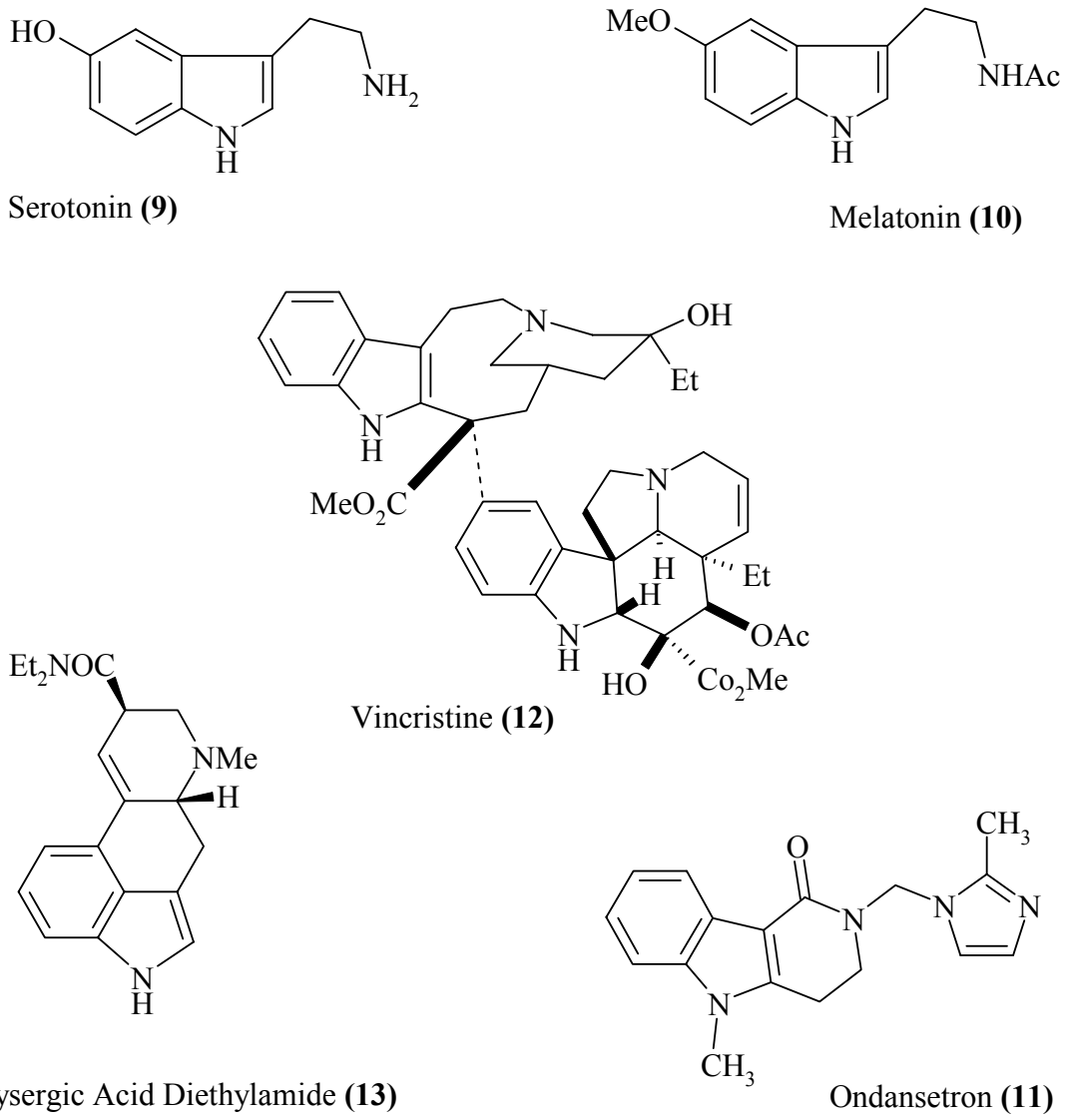


Figure 4. Indole containing molecules of natural origin.

L-tryptophan has been the subject of synthetic investigation since the elucidation of its structure by Ellinger in 1906.[21] Hopkins and Cole believed the structure of tryptophan to be skatole-α-aminoacetic acid;[15] however, Ellinger proved this structure to be incorrect by synthesizing a key oxidative cleavage product of tryptophan, indole-β-aldehyde **(14)**. This molecule proved to be a key intermediate as Ellinger reported the total synthesis of tryptophan the very next year with Flamand.[22] Starting with indole **(6)**,

indole-β-aldehyde **(14)** was synthesized by a Riemer-Tiemann chlorocarbene insertion. Azalactone **(15)** was made upon condensation with hippuric acid. Saponification of **(15)**, followed by reduction and hydrolysis of **(16)**, afforded tryptophan in less than 4% overall yield (Fig. 5). Modifications of this synthesis improved overall yield to about 30%, but the inefficiency of the Reimer-Tiemann reaction eventually led synthetic investigations of tryptophan in alternate directions.

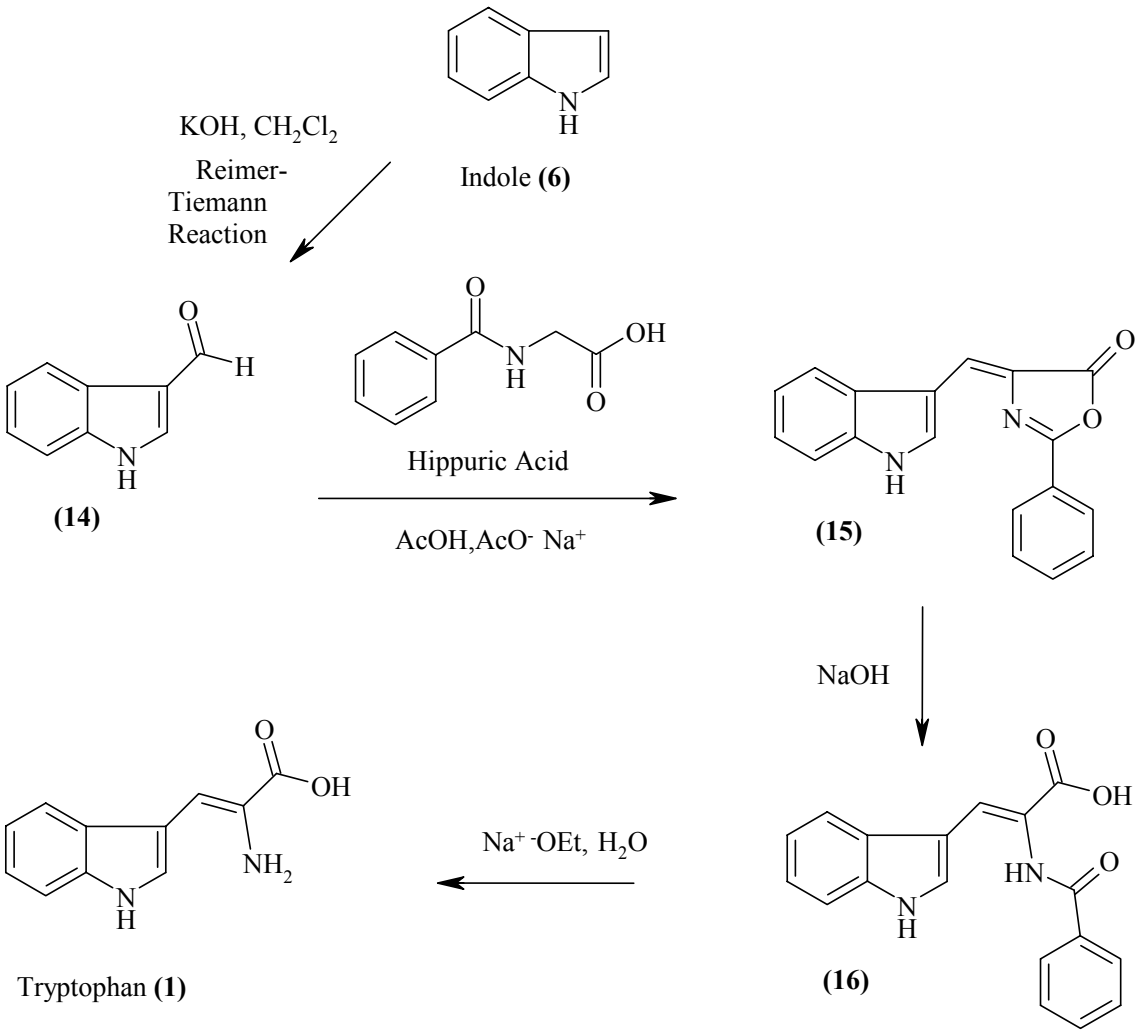


Figure 5. First total synthesis of tryptophan by Ellinger and Flamand.

The most common modern synthesis of L-tryptophan uses readily available gramine **(17)** as the starting material. Gramine itself is easily prepared in high yield from indole via Mannich reaction in acetic acid[23] or with Eschenmoser's salt **(18)** in toluene.[24,25] Substitution of the electrophilic β-methyl by nucleophilic acetamidomalonate **(19)**, followed by workup in aqueous acid at 80º C results in mono-decarboxylation and hydrolysis of the remaining ester and amide groups to afford racemic tryptophan **(1)** in 73% overall yield[26,27] (Fig.6).

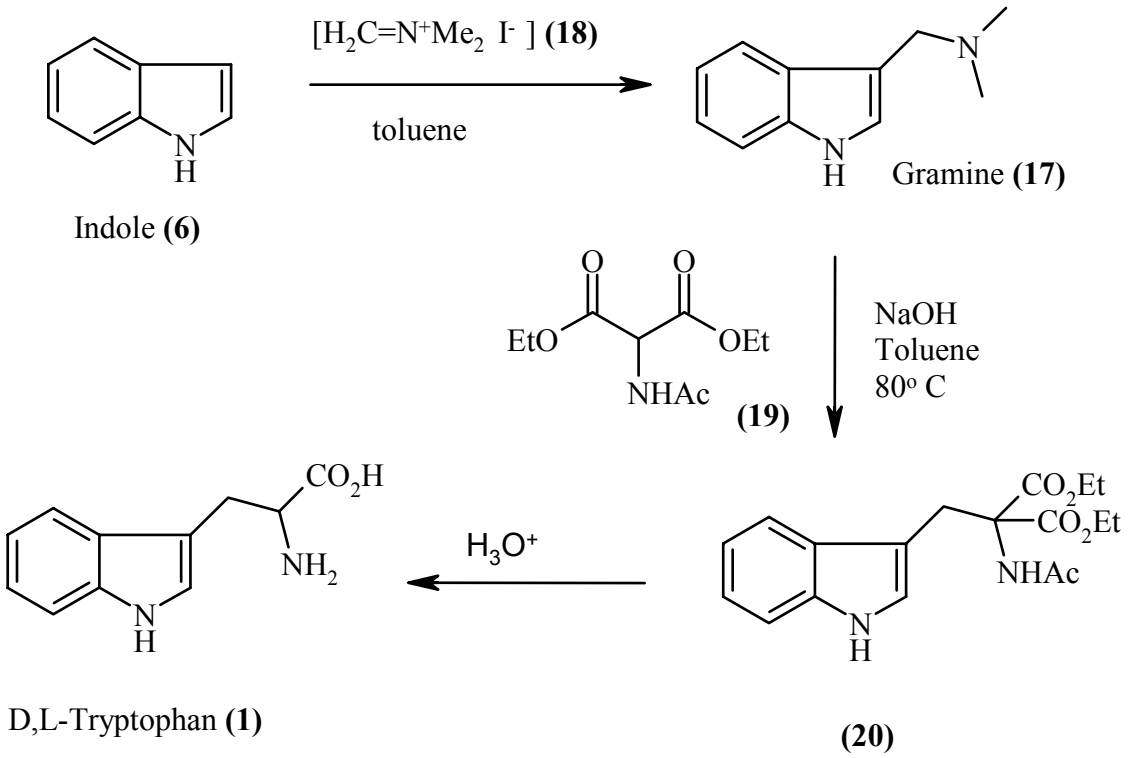


Figure 6. Efficient, racemic synthesis of L-tryptophan

In recent years, the most effective means for synthesizing optically pure L-tryptophan from indole has been by condensation with L-serine using the pyridoxal-5'-phosphate (vitamin B6, PLP) dependent enzyme, tryptophan synthase. This method is effective for the preparation of many L-tryptophan derivatives from the corresponding substituted

indoles. Isomers of L-tryptophan substituted by chlorine,[28] fluorine,[29] and methyl[30] have been synthesized in this manner.

Despite the utility of tryptophan synthase for the preparation of many L-tryptophan derivatives, this procedure is not effective for reactions involving bulky substrates such as bromo and nitro derivatives of indole.[31] Because of this, only a few isomers of L-tryptophan substituted with bromine or nitro have been reported in the literature. Bromo-L-tryptophan isomers that have been published include 2-bromo,[32,33] 5-bromo,[34] and 7-bromo.[35] Nitro-L-tryptophan isomers reported in the literature include 2-nitro,[36] 5-nitro,[37,38] and 6-nitro-L-tryptophan.[39-41] Direct methods for synthesizing bromo-L-tryptophan are absent from the literature as these compounds have only been prepared from brominated precursors or from the corresponding protected nitro-L-tryptophans.[42] Direct nitration of L-tryptophan affords 6-nitro-L-tryptophan in reported good yield,[40] but the complicated workup and purification hamper attainable yield and cost effectiveness significantly. The protected L-tryptophan derivative, N_α-trifluoroacetyl-L-tryptophan methyl ester, affords the 6-nitro product in 40% yield and the 2-nitro product in 6.8% yield from nitration with nitric acid in acetic acid. However, a complex mixture is obtained, requiring column chromatography for purification. Reaction of N_α-trifluoroacetyl-L-tryptophan methyl ester with N-bromo succinimide (NBS) in CCl_4 yields the 2-bromo derivative in 83% yield.[32]

Obviously the chemistry of tryptophan's functional group indole has been extensively studied for the past century. The association of indole with essential nutrition was established even before a fundamental understanding of protein and macronutrients had been formed, and its prevalence in natural products is second to none.[43] Synthetic methodology for indole syntheses covers a broad range of applications from the simple preparation of indole from a mono-substituted benzene, to pyrrole ring closure as a key step in the total syntheses of natural products. The Fischer indole cyclization[44-46] is the most widely used method for synthesizing the 2, 3-benzopyrrole heterocycle. Formally,

the reaction comprising the name is the cyclization of a phenyl hydrazone to form the pyrrole ring of indole. However, the transformation of aniline or a functionalized aniline to the corresponding indole derivative via the phenylhydrazine is called the Fischer synthesis.[44] The first step in a Fischer indole synthesis is the preparation of the phenylhydrazine (Fig. 7) of the aromatic amine starting material. Aniline **(21)** is a common Fischer synthesis starting material,[47] and here it is treated with HCl and sodium nitrite to form the diazonium salt **(22)**. Reduction of **(22)** with stannous chloride affords phenylhydrazine **(23)**.

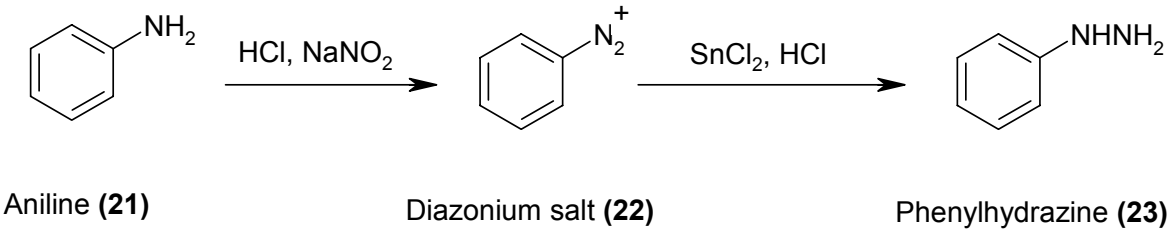


Figure 7. Preparation of phenylhydrazine

A classic example of the Fischer synthesis is the reaction of phenylhydrazine **(23)** with acetophenone **(24)**.[48] The phenylhydrazone **(25)** is formed by treatment with HCl and ring closure is catalyzed by zinc chloride to afford 2-phenylindole **(26)** (Fig. 8).

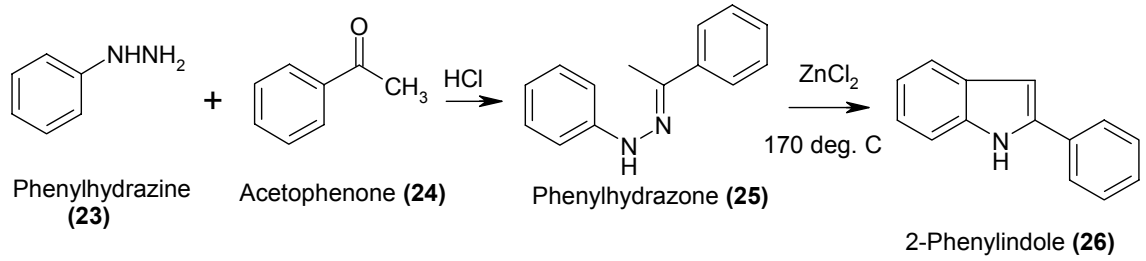


Figure 8. Fischer indole cyclization

The utility of the Fischer synthesis is showcased in R.B. Woodward's synthesis of strychnine[49,50] (Fig. 9). A Fischer cyclization of phenylhydrazine **(23)**, with 3,4-dimethoxyacetophenone **(27)** is the first synthetic step. Polyphosphoric acid-catalyzed ring closure of the phenylhydrazone produced 2-(3,4-dimethoxybenzyl)-indole **(28)**. Recognizing the utility of Gramine **(17)**, Woodward's next reaction was a Mannich alkylation at the 3 position to afford 2-veratrylindole **(29)** in 92% yield.

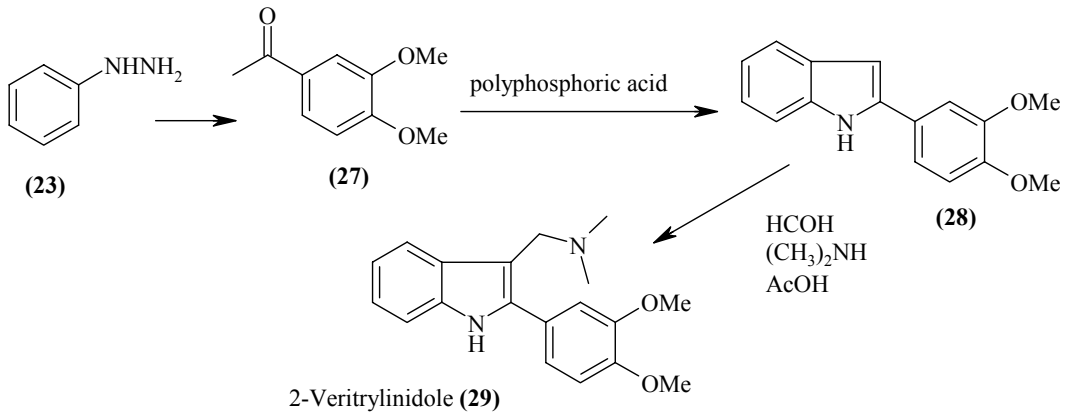


Figure 9. Fischer synthesis showcased in R.B. Woodward's synthesis of strychnine.

The Fischer synthesis is a versatile and efficient means to prepare functionalized indoles, but it is inefficient for synthesizing indole itself, or indole derivatives bearing electron-withdrawing groups or labile functionalities on the pyrrole ring. Difficulties in the preparation of less functionalized indoles by the Fischer method are due to lower yield in the formation of their respective phenylhydrazines, and separately, their failure to cyclize once formed. Formation of less functionalized phenylhydrazones can be accomplished via the Japp-Klingemann reaction.[51-53] In 1887, Japp and Klingemann reported the synthesis of the phenylhydrazone of ethyl pyruvate **(34)** upon reaction of aniline's diazonium salt **(22)** with ethyl 2-methylacetoacetate **(32)** (Fig. 10). This conversion has been accomplished in good yield,[53] but the lack of efficiency in cyclization to the benzopyrrole prevents this method from being viable for syntheses demanding high yield.

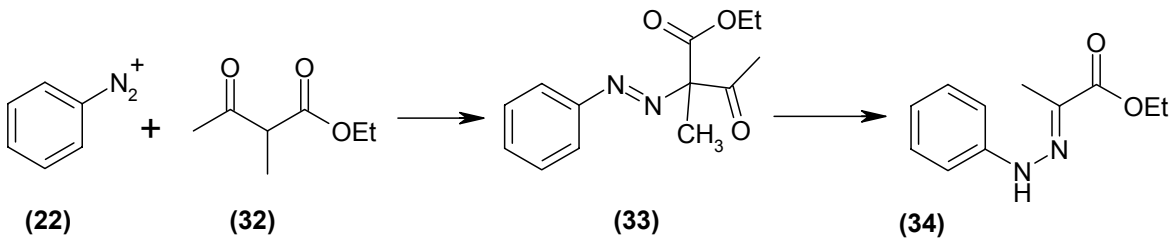


Figure 10. Japp Klingemann formation of the phenylhydrazone of ethyl pyruvate

The most efficient method for synthesizing unsubstituted indole from aniline is via isatin (Fig.11). Isatin **(5)** is prepared in good yield from aniline **(23)** by reaction with chloral hydrate.[54,55] The resulting isonitrosoacetanilide **(35)** is converted to isatin by refluxing in aqueous acid. Reduction of isatin is most efficiently accomplished by hydride reduction with 4 equivalents of LAH.[56]

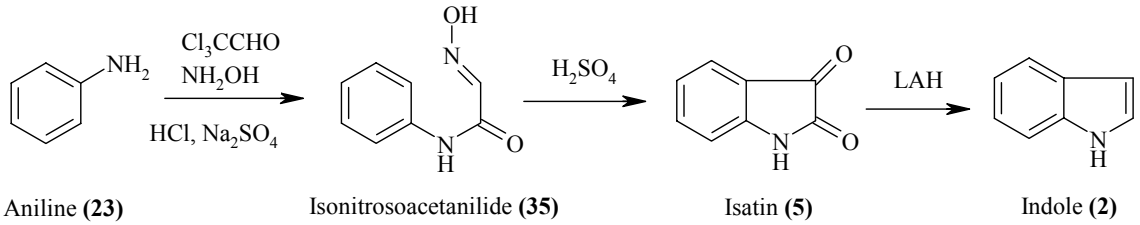
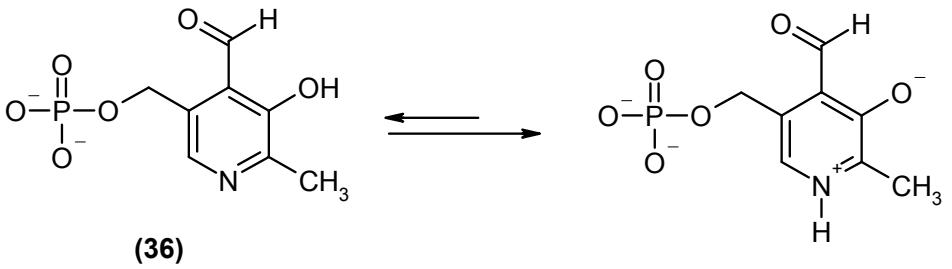


Figure 11. Sandmeyer synthesis of Isatin

Recent advances in chemoenzymology have brought enzyme catalysis to the bench of synthetic chemistry as routine methodology,[57,58] and this is certainly true for the synthesis of L-tryptophan and many of its derivatives.[28-30] Tryptophan synthase and tryptophan indole-lyase are two pyridoxal 5'-phosphate (vitamin B6, PLP) **(36)** dependent enzymes associated with the biosynthetic catalysis and degredation of L-tryptophan. PLP dependent enzymes themselves are prevalent in nature and catalyze a wide variety of reactions involving amino acids, including transaminations, α and β-decarboxylations, β and γ eliminations, racemizations and aldol reactions, where the

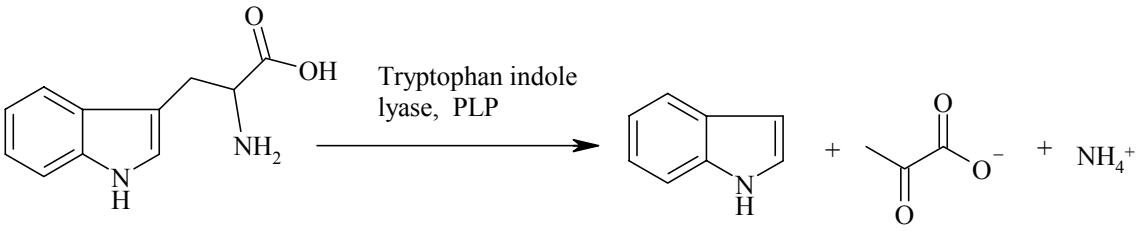
function of PLP is to act as an electron-withdrawing functionality to stabilize reaction intermediates and to form Schiff base complexes with the enzyme substrates. Structurally, PLP is represented as a pair of tautomers (Fig. 12).



(36)

Figure 12. Tautomers of pyridoxal 5'-phosphate

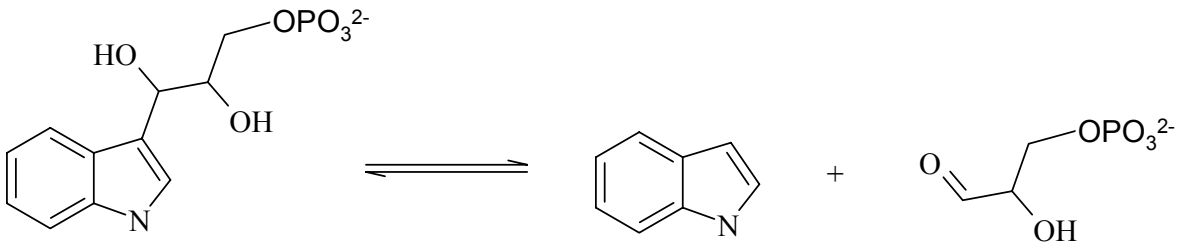
Tryptophan indole-lyase [EC 4.1.99.1] catalyzes the reversible hydrolytic cleavage of L-tryptophan to indole, pyruvate and ammonium (Fig. 13),[59] and has been applied to numerous other substrates as well.[60,61] Although catalysis equally favors the forward and reverse reactions, tryptophan indole-lyase has an increased tolerance for varying substrate structures compared to tryptophan synthase.



L-tryptophan **(1)** Indole **(6)** Pyruvate **(37)**

Figure 13. Reaction catalyzed by tryptophan indole-lyase

Tryptophan synthase (EC 4.2.1.20) is the most useful enzyme for synthesizing L-tryptophan from indole and certain derivatives thereof, perhaps because this conversion mimics its role in nature, where it catalyzes the final two steps of the biosynthesis of L-tryptophan. Structurally, the enzyme is a four subunit complex consisting of two identical α structures flanking a central β_2 dimer. Schematically, this can be accurately described as α-ββ-α.[63-66] Each α-subunit independently catalyzes the reversible cleavage of indole 3-glycerophosphate **(38)** to indole **(6)** plus glyceraldehyde-3-phosphate **(39)**; hence, this is described as the α-reaction. The β_2 complex, or simply β, catalyzes the irreversible conversion of indole **(6)** and L-serine **(40)** to L-tryptophan **(1)**, with PLP **(36)** as the cofactor (Fig. 14).

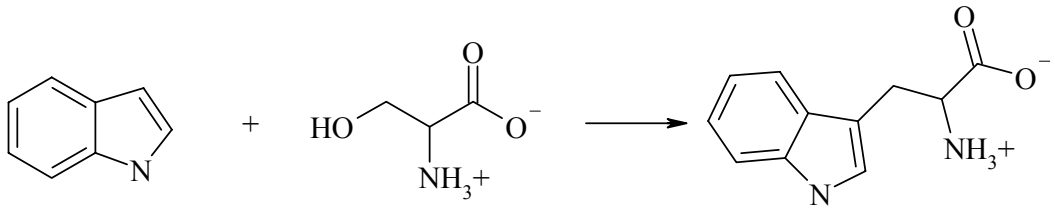


Indole 3-glycerophosphate **(38)**

Indole **(6)**

Glyceraldehyde-3-phosphate **(39)**

Alpha Reaction



Indole **(6)**

L-serine **(40)**

L-tryptophan **(1)**

Beta Reaction

Figure 14. Reactions catalyzed by tryptophan synthase

Interestingly, indole is not observed as an intermediate in kinetic studies, suggesting that it never leaves the tryptophan synthase structure. X-ray crystallographic structures have revealed a 25-30 Å interenzyme tunnel linking the α and β active sites whereby indole travels to the β-site directly upon α-cleavage (Fig. 15).[67-73] Moreover, intermediate complexes of the α and β-reactions allosterically regulate catalysis at the other.[74-76] A product of the α-reaction, glyceraldehyde-3- phosphate, binds with the α-complex to allosterically regulate the binding of L-serine to PLP, and serves as a steric inhibitor, blocking the entrance of indole into the interenzyme tunnel. In contrast, the reactive intermediate complex of the β-reaction, aminoacrylate, activates the α-reaction 27 fold. Together, these interactions promote the forward progress of the αβ-reaction.

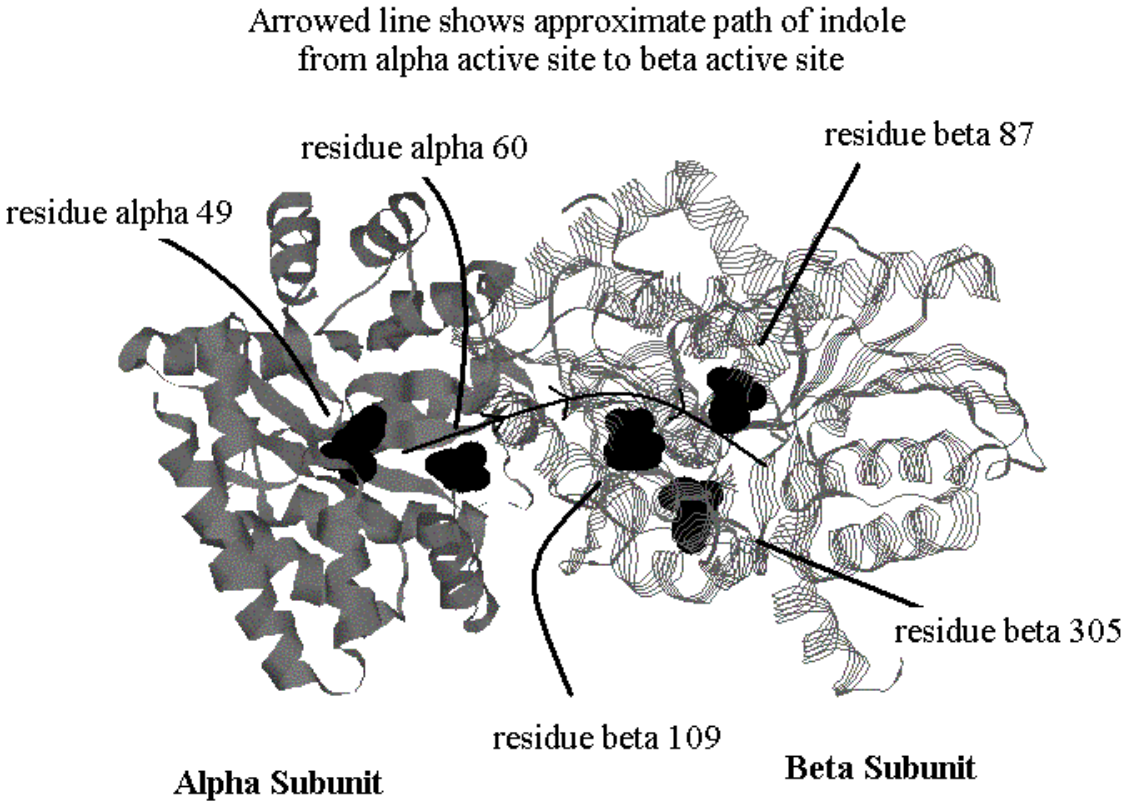


Figure 15. Tryptophan synthase: key residues and substrate path.

Mechanistic studies of the β-reaction of tryptophan synthase (Fig. 16) revealed that residue βK87 is the attachment point of PLP[77,78] and serves in several catalytic roles. Void of any substrate, PLP is bound to residue K87 in the form of the internal aldimine, a Schiff base complex (E).

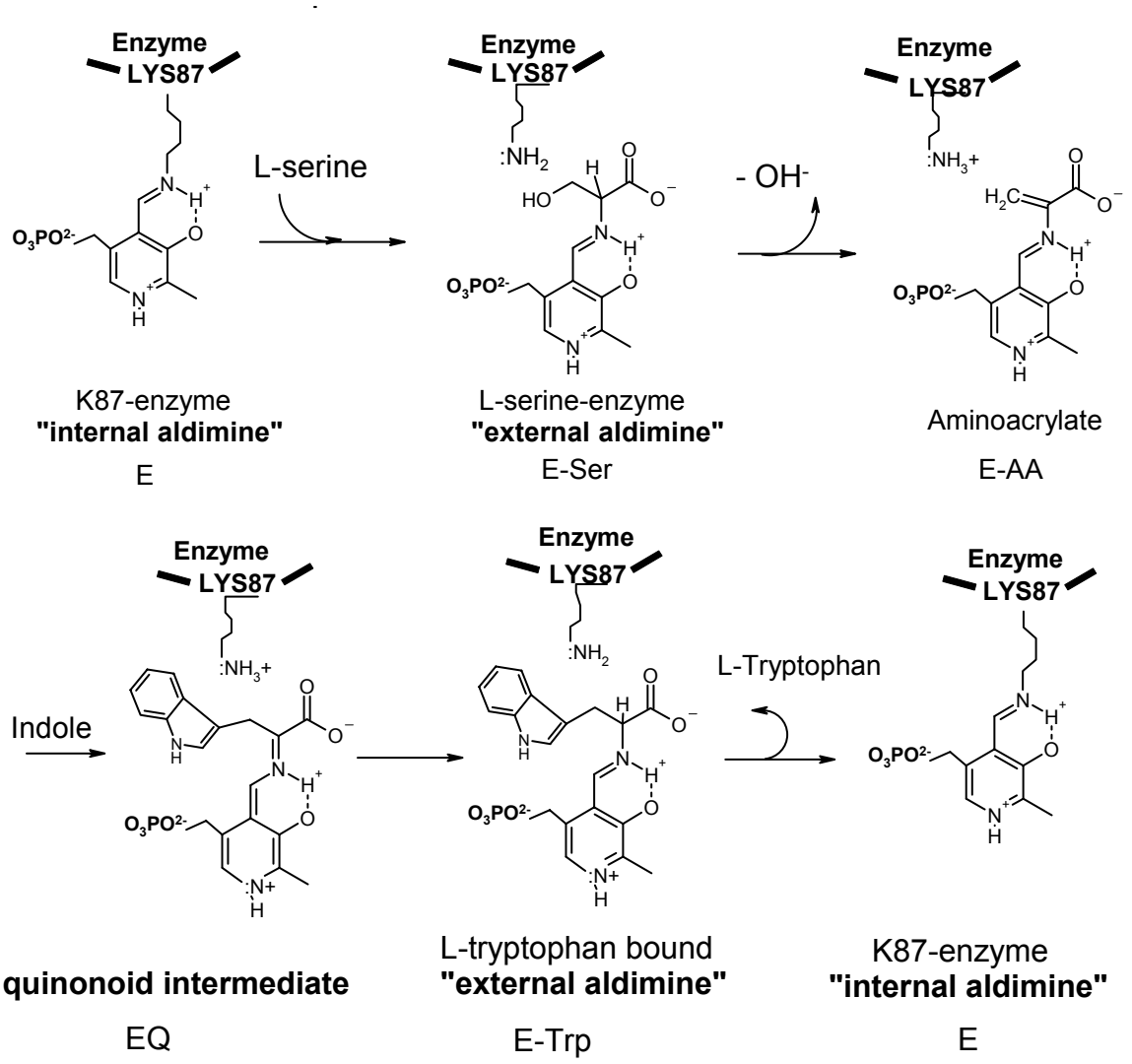


Figure 16. Mechanism of tryptophan synthase

Attachment of L-serine, promoted by the presence of G3P at the α active site, forms the external aldimine (E-ser) which is converted to the aminoacrylate (E-AA) upon loss of

water; this is the reactive intermediate for addition of indole. Addition of indole to the aminoacrylate affords the quinonoid intermediate (EQ). At this point, the amine residue of βK87 acts as a Bronsted-Lowry acid, and provides the proton for the conversion to the L-tryptophan external aldimine (E-Trp). The release of L-tryptophan leads to the regeneration of the original internal aldimine, ready to catalyze another synthesis of L-tryptophan.

Section 2. Synthetic Applications of Alcohol Dehydrogenases

The use of enzymes in the synthetic lab has expanded greatly in the last two decades.[57,58] Major contributors for this trend are the increased emphasis on stereo-control in methodology, the availability of enzymes with increased thermal and chemical stability, and the mild conditions under which they catalyze reactions. Additional benefits associated with the use of enzymes in organic synthesis include a reduced need for organic solvents and heavy metals, both of which are costly to purchase and dispose of.

Alcohol dehydrogenases (ADH) are among the most synthetically useful enzymes in organic chemistry. Reasons for their value to the organic chemist include the ability of ADHs to synthesize chiral alcohols in high enantiomeric excess under mild conditions,[57] and for the ubiquity of chiral alcohols as starting materials in natural products and pharmaceutical synthesis. The term alcohol dehydrogenase is a categorical descriptor for enzymes that catalyze the oxidation of primary or secondary alcohols to the corresponding aldehydes or ketones, or catalyze the reduction of carbonyls to alcohols.[82,83] Essentially all ADHs used in synthesis incorporate either nicotinamide adenine dinucleotide (NAD) **(41)** or its 2'-phosphate, NADP **(42)**, as an hydride donor/acceptor (Fig. 17). Owing to the mechanism of catalysis, ADHs are more

generally categorized as oxidoreductase enzymes. In addition to NAD(P) dependence, most ADHs share some structural and catalytic properties including dimeric or tetrameric subunit structures,[84] and the presence of active site Zn^{2+} as a Lewis acid catalyst. Common alcohol dehydrogenases of synthetic value

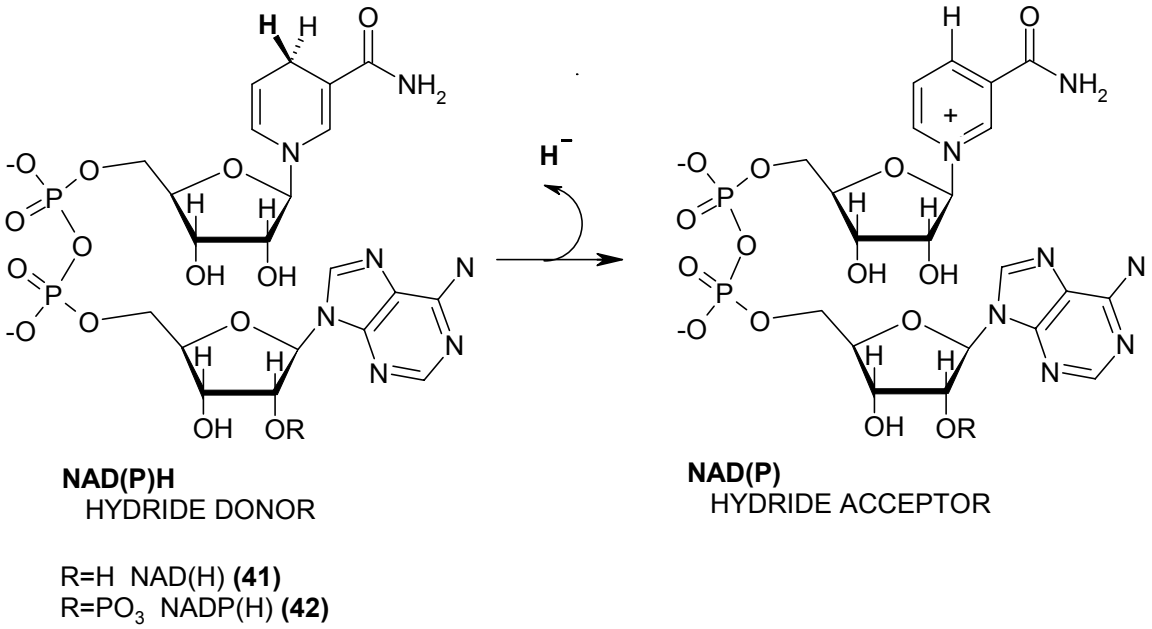


Figure 17. Structures of NAD(P) and NAD(P)H. Bold H indicates pro-R hydrogen.

are those from baker's yeast (YADH) [E.C.1.1.1.1],[57,85] horse liver (HLADH)[EC 1.1.1.1];[86] and two ADHs from thermophilic organisms[87-90] which are seeing increasing synthetic use: TBADH [EC 1.1.1.2] from *Thermoanaerobacter brockii*,[91-94] and SADH [EC 1.1.1.2] from *Thermoanaerobacter ethanolicus*.[95-97]

Figure 18 illustrates the catalytic mechanism[98-100] of 2-butanol formation by TBADH, and is similar to the catalysis by other ADHs. X-ray data[101] reveals that the Zn^{2+} ion is tetrahedrally coordinated by residues Cys37, His59, Asp150 and Glu60 and the acidic proton is furnished by water or a threonine. The pro-R hydride of the

nicotinamide is delivered to the *re* face of the substrate affording the (S)-alcohol; and this occurs when the smaller alkyl group is oriented in the small alkyl pocket and the larger alkyl group is in the large alkyl pocket.[93]

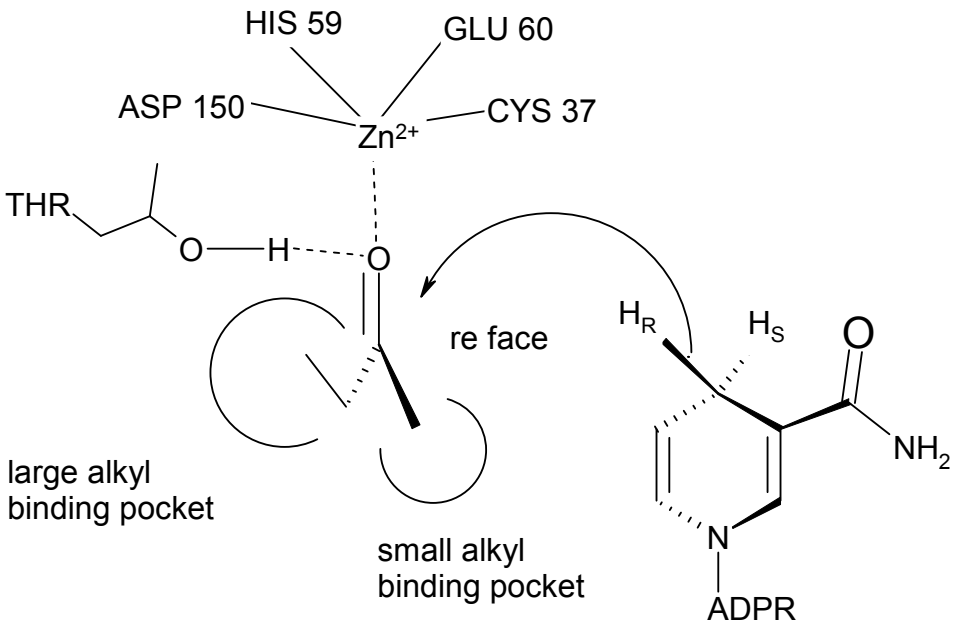


Figure 18. Delivery of pro-R hydride of the nicotinamide to the re-face of 2-butanone.

This specific stereochemical outcome is known as Prelog's rule,[102] and is a useful model for determining the stereochemistry of reductions catalyzed by YADH, HLDH, and TBADH. SADH also obeys Prelog's rule in most cases, and has demonstrated a temperature dependence on stereochemistry. (R)-butanol is preferentially oxidized above 26ºC, where the (S)-butanol is preferentially oxidized below 26ºC.[103,104]

The use of baker's yeast in organic synthesis represents the easiest and cheapest chemoenzymatic method available to the chemist,[57] and has several key advantages. Yeast suitable for synthetic chemistry is extremely cheap and readily available, reactions involving whole cells require no purification, and the very costly nicotinamide cofactor is already present. This method is useful for the oxidation and chiral reduction of small substrates, but YADH is wholly unsuitable for the reduction of cyclic ketones or

substrates bearing multiple alkyl substituents larger than methyl.[105,106] Other limitations to the use of bakers yeast for synthetic transformations include low enantiomeric excess, sensitivity to heat, organic solvents, and salt concentration. Reactions involving YADH purified from yeast cells show greatly increased enantiomeric excess and an increased tolerance for substrate variation over the reactions with whole cells; however, reactions with the pure enzyme still exhibit the main limitations of YADH, its inherent sensitivity to temperature and solvents which effect dissociation of the tetramer.

HLADH has seen widespread use in organic synthesis,[86] and is the best characterized of all oxidoreductases.[84] HLADH is a dimer composed of two identical 40 KDa, 374 amino acid subunits,[107] which contrasts with the tetrameric forms of YADH, TBADH, and SADH. HLADH is also unique as the active site Zn^{2+} is trigonally coordinated.[108] Like YADH, the periphery of the enzyme contains a large number of free cysteines which are believed to contribute their thermal and chemical sensitivity as thiol groups are prone to oxidation, alkylation, and complexation with heavy metals.[109] Despite these limitations, HLADH exhibits some remarkable catalytic properties including reported facial distinction in meso compounds and prochiral geometric isomers.[110] Enantioselective reductions of cyclic and bicyclic ketones are common, especially for three and six membered rings.[85] Reductions of non-nitrogen containing heterocyclic carbonyls are also accomplished in good enantiomeric excess.[102] Cyclic ketones with a heterocyclic nitrogen are poor substrates presumably due to coordination of the nitrogen to the active site zinc. Oxidations are also in HLADH's repertoire as the enantioselective, and regioselective oxidation of α-amino alcohols and *gem*-diols to afford chiral aldehydes[111] is reported.

Several NADP(H) dependent, tetrameric ADHs have been isolated from thermophilic bacteria which have been useful for the synthetic chemist.[87-90] TBADH from *Thermoanaerobacter brockii*[91-94] has shown increased tolerance to reaction conditions and substrate structural variability, including acyclic ketones.[92-94] It has also

shown a reversal in stereoselectivity based on substrate size.[92] TBADH has been used for the asymmetric synthesis of chiral alcohols[91,92,94] and in several total syntheses.[93,94]

Secondary alcohol dehydrogenase (SADH) is one of two ADHs isolated from *Thermoanaerobacter ethanolicus*.[88] It is structurally similar to TBADH, and consistent with its name, has a preference for secondary alcohols over primary alcohols. SADH has also been used in synthetically applicable oxidations and reductions,[95-97] and has demonstrated a reversal in enantiospecificity for alcohol oxidation. Although the selectivity for R-alcohol oxidation is limited, the potential for the use of SADH for the synthesis of both R and S chiral alcohols is compelling. Recently, several mutants of SADH have been studied.[112-116] Phillips and coworkers[115] have reported an increase in both activity and R-specificity with mutant S39T, whose mutant residue resides within the large alkyl binding domain[92] along with the bulk of the nicotinamide cofactor.[116] Asymmetric reduction of ethynyl ketones by SADH occurs with high yield and enantiomeric excess, but at the expense of enzyme deactivation, presumably from a nucleophilic active site residue.[96] TBADH shares a similar homology with SADH, and modeling studies revealed the presence of cysteine in the small alkyl binding domain. Substitution of the analogous cysteine in SADH, cysteine 295, by the smaller aliphatic and non-nucleophilic residue alanine did not alleviate the inactivation of the enzyme by ethynyl ketones, and the temperature correlation to enantioselectivity for 2-butanol and 2-pentanol was lost.[116]

Oxidations of small chiral alcohols by SADH mutants C295L and C295V are reported in chapter 3 of this dissertation, and results indicate that these mutations affect the enantioselectivity of catalysis significantly.[117] Further study of active site mutants of SADH and other ADHs should result in the development more accurate models for predicting the stereochemical outcome of reactions, and lead to mutants with improved enantioselectivity, increased flexibility for diverse substrates, and resistance to deactivation.

Bibliography

1. *Chemistry of the Amino Acids*, Greenstein, J.P., Winitz, M.H., John Wiley and Sons, N.Y. 1961
2. Neumeister, R., *Z. Biol.*, **26**, 324 (1890)
3. Tiedemann, F., Gmelin, L., *Die Verdauung nach Versuchen*, 2nd Ed., Heidelberg and Leipzig, **1**, 31, 358 (1831)
4. Bernard, C., *Compt. Rend. Suppl.*, **1**, 379 (1856)
5. Vickery, H.B., Schmidt, C.L.A., *Chem. Revs.*, **9**, 169 (1931)
6. Baeyer, A, Knop, C.A., *Ann.*, **140**, 295 (1866)
7. Remers, W.A., Brown, R.K., *Heterocyclic Compounds; Part One*; Houlihan, ed., Wiley-Interscience, NY, 1972
8. Erdmann, O.L., *J. Prakt. Chem.*, **24**, 1 (1841)
9. Baeyer, A., Emmerling, A., *Ber.*, **2**, 679 (1869)
10. Nencki, M., *Ber.*, **8**, 722 (1875)
11. Kuhne, W., *Ber.*, **8**, 206 (1875)
12. Bopp, F., *Ann.*, **69**, 16 (1849)
13. Breiger, L., *Ber.*, **10**, 1027 (1877)
14. Nencki, M., *Ber.*, **28**, 560 (1895)
15. Hopkins, F.G., Cole, S.W., *J. Physiol.*, **27**, 418 (1902)
16. Tracy, A.B., *Prozac: Panacea or Pandora?*, Cassia, NY, 1994
17. Hazlerigg, D.G., *J. Endocrinology*, **170**, 493-501 (2001)
18. Slutsker, L., Hoesly, F.C., Miller, L., *J. Am. Med. Assoc.*, **264**, 213-217 (1990)
19. Belongia, E.A., Hedberg, C.W., *N. Engl. J. Med.*, **323**, 357-365 (1990)
20. Sharma, S., Abdullah, N., *Singapore Med. J.*, **41**, 4, 147-150 (2000)
21. Ellinger, A., *Ber.*, **39**, 2515 (1906)
22. Ellinger, A., Flamand, C., *Ber.*, **40**, 3029 (1907)
23. Kuhn, H., Stein, O., *Ber.*, **70**, 567 (1937)
24. Kozikowski, A.P., Ishida, H., *Heterocycles*, **14**, 55 (1980)

25. Schreiber, J., Maag, H., Haschimoto, N., Eschenmoser, A., *Angew. Chem. Int. Ed. Engl.*, **10**, 330 (1970)

26. Howe, E.E., Zambito, A.J., Tishler, M., *J. Am. Chem. Soc.*, **67**, 38 (1945)

27. Albright, J.D., Snyder, H.R., *J.Am.Chem.Soc.*, **81**, 2239 (1959)

28. Lee, M, Phillips, R.S., *J. Heterocyclic Chem.*, **31**, 711 (1994)

29. Lee, M., Phillips, R.S., *Mag. Res. Chem.*, **30**, 1135-1040, (1992)

30. Lee, M., Phillips, R.S., *J. Heterocyclic Chem.*, **29**, 1181-1187 (1992)

31. Chapter II of this Dissertation

32. Phillips, R.S., Cohen, L.A., *Tetrahedron Lett.*, **24**, 5555-5558 (1983)

33. Phillips, R.S., Cohen, L.A., *J. Am. Chem. Soc.*, **108**, 2024-2030 (1986)

34. Nagarathnam, D., Johnson, M.E., *Synth. Commun.*, **23**, 2011-2017 (1993)

35. Allen, M.C., Brundish, D.E., Wade, R., *J.C.S. Perkin I*, 2025-2030 (1982)

36. Phillips, R.S., Cohen, L.A., *J. Heterocyclic Chem.*, **25**, 191-192 (1988)

37. Hino, T., Taniguchi, M., Gonsho, A., Nakagawa, M., *Heterocycles*, **12**, 1027-1030 (1979)

38. Hino, T., Tohru, T., *Tetrahedron*, **37**, 1487-1494 (1981)

39. Phillips, R.S., Marmorstein, R.Q., *Arch. Biochem. Biophys.*, **262**, 337-344 (1988)

40. De Fazi, R., Berti, G., Da Settimo, A., *Gazz. Chim. Ital.*, **89**, 2238-2257 (1959)

41. Melhado, L.L., Leonard, N.J., *J. Org. Chem.*, **48**, 5130-5133 (1983)

42. Ohno, M., Spande, T.F., Witkop, B., *J. Am. Chem. Soc.*, **90**, 6521-6522 (1968); ibid., **92**, 343-348 (1970)

43. Joule, J.A., Mills, K., Smith, G.F., *Heterocyclic Chemistry*, 3rd Ed., Chapman and Hall, NY (1995) p.305

44. Robinson, B., *The Fischer Indole Synthesis*, John Wiley and Sons, NY (1982)

45. Sundberg, R.J., *The Chemistry of Indoles*, Academic Press, NY (1970)

46. Fischer, E., Ber., **8**, 589 (1875)

47. Coleman, G.H., *Org. Syn. Coll. Vol. 1*, 432 (1932)

48. Schriner, R.L., Ashley, W.C., Welch, E., *Org. Syn. Coll. Vol. III*, 775 (1955)

49. Woodward, R.B., Cava, M.P., Ollis, W.D., Hunger, A., Daeniker, H.U., Schenker, K., *Tetrahedron*, **19**, 247-288 (1963)

50. Woodward, R.B., Cava, M.P., Ollis, W.D., Hunger, A., Daeniker, H.U., Schenker, K., *J. Am. Chem. Soc.*, **76**, 4749-4751 (1954)

51. Japp, F.R., Klingemann, F., Ber., **20**, 2942 (1887)

52. Japp, F.R., Klingemann, F., Ber., **21**, 549 (1888)

53. Phillips, R.R., Organic Reactions, **10**, 2, John Wiley and Sons, NY (1942)

54. Sandmeyer, T., Helvetica Chemica Acta, **2**, 234 (1919)

55. Marvel, C.S., Hiers, G.S., *Organic Syntheses*, Coll. Vol. 1, 327 (1944)

56. Giovanni, E., Lorenz, T., *Helvetica Chemica Acta*, **161**, 1552-1561 (1957)

57. Drauz, K., Waldmann, H., *Enzymatic Catalysis in Organic Synthesis*, **2**, VCH, NY (1995)

58. Wong, C.H., Whitesides, G.M., *Enzymes in Synthetic Organic* Chemistry, **12**, Pergamon, NY (1994)

59. Snell, E.E., *Adv. Enzymol*., **42**, 287 (1975)

60. Watanabe, T., Snell, E.E., *J. Biochem*., **82**, 733 (1977)

61. Ahmed, S.A., Martin, B., Miles, E.W., *Biochemistry*, **25**, 4233 (1986)

62. Phillips, R.S., Miles, E.W., Cohen, L.A., *Biochemistry*, **23**, 6228 (1984)

63. Yanofsky, C., Crawford, I.P. *The Enzymes* (Boyer, P.D., Ed.) 3rd. ed., Vol. VII, pp 1-31, Academic Press, New York (1972)

64. Miles, E.W., *Adv. Enzymol*., **49**, 127-186 (1979)

65. Miles, E.W., *Adv. Enzymol*., **64**, 93-172 (1991)

66. Miles, E.W. *Subcellular Biology*, vol. 24, pp 207-254, Plenum Press, New York (1995)

67. Hyde, C.C., Ahmed, S.A., Padlan, E.A., Miles, E.W., Davies, D.R. *J. Biol.Chem*., **263** (17857-17871)

68. Dunn, M.F., Aguilar, V., Drewe, W.F., Houben. K., Robustell, B., Roy, M., Ind. *J. Biochem. Biophys*., **24**, 44-51 (1987)

69. Dunn, M.F., Aguilar, V., Brzovic, P., Drewe, W.F., Houben, K., Leja, C.A., Roy, M., *Biochem*., **29**, 8598-8607 (1990)

70. Kirshner, K., Lane, A.N., Strasser, W.M., *Biochemistry*, **30**, 472-478 (1991)

71. Kirshner, K., Wiskocil, R.L., Foehn, M., Rezeau, L., *Eur. J. Biochem*., **60**, 513-523 (1975)

72. Anderson, K.S., Miles, E.W., Johnson, K.A., *J. Biol. Chem*., **266,** 8020-8033, (1991)

73. Brzovic, P.S., Kayastha, A.M., Miles, E.W., Dunn, M.F., *Biochem.*, **31**, 1180-1181 (1992)

74. Ahmed, S.A., Ruvinov, S.B., Kayastha, A.M., Miles, E.W., *J. Biol. Chem*., **266**, 21548-21557 (1991)

75. Peracchi, A., Bettati, S., Mozzarelli, A., Rossi, G.L., Miles, E.W., Dunn, M. F., *Biochem*, **35**, 1872-1880 (1996)

76. Schneider, T.R., Gerhardt, E., Lee, M., Liang, P-H., Anderson, K.S., Schlichting, I., *Biochem*., **37**, 5394-5406 (1998)

77. Rhee, S., Parris, K.D., Hyde, C.C., Ahmed, S.A., Miles, E.W., Davies, D.R., *Biochem*., **36**, 7664-7680 (1997)

78. Lu, E., Petsko, g., *Biochem*, **29**, 6619-6625 (1990)

79. Weyland, M., Schlichting, I*., Biochem*., **38**, 16469-16480 (1999)

80. Rhee, S., Parris, K.D., Ahmed, S.A., Miles, E.W., Davies, D.R*., Biochem*., **35**, 4211-4221 (1996)

81. Brzovic, P.S., Sawa, Y., Hyde, C.C., Miles, E.W., Dunn, M.F., *J. Biol. Chem*., **267**, 13028-13038 (1992)

82. Branden, C.I., Tooze, J., *Introduction to Protein Structure*, pp.142-182, Garland, NY (1991)

83. Bruice, T.C., Benkovic, S.J., *Bioorganic Mechanisms*, p.301, Benjamin, NY (1965)

84. Branden, C.I., Jornvall, H., Eklund, H., Furugren, B., *The Enzymes*, Boyer P.D., Ed., pp.103-190, Academic Press, New York (1975)

85. Faber, K. *Biotransformations in Organic Chemistry*, pp.145-173, Springer-Verlag, New York (1995)

86. Hammel, W., Kula, M.R., *Eur. J. Biochem*., **184**, 1-13 (1989)

87. Bryant, F.O., Wiegel, J., Ljungdahl,L.G., *Appl. Environ. Microbiol*., 460-465 (1998)

88. Bryant, F.O., Ljungdahl, L.G., *Biochem. Biophys. Res. Commun*., **100**, 793-799 (1981)

89. Lamed, R., Zeikus, J.G., *Biochem. J*.,**195**, 183 (1981)

90. Lamed, R. Zeikus, J.G*., J. Bacteriol*.,**141**, 1251 (1980)

91. Lamed, R.J., Keinan, E., Zeikus, J.G., *Enzyme Microb. Technol*., **3**, 144-148 (1981)

92. Keinan, E., Hafeli, F.V., Seth, K.K., Lamed, R.J., *J. Am. Chem. Soc*., **108**, 162 - 169 (1986)

93. Keinan, E., Seth, K.K., Lamed, R.J., *J. Am. Chem. Soc*., **108**, 3474 (1986)

94. De Amici, M., De Micheli, C., Carrea, G., Spezia, S., *J. Org. Chem*., **54**, 2646-2650 (1989)

95. Zheng, C., Pham, V.T., Phillips, R.S*., Bioorg. Med. Chem. Lett*., **2**, 619-622 (1992)

96. Heiss, C., Phillips, R.S., J. Chem. Soc., *Perkin Trans.1*., 2821 (2001)

97. Zheng, C., Pham, V.T., Phillips, R.S., *Catal. Today*, **22**, 607-620 (1994)

98. Ford, B.J., Askins, K.J., Taylor, K.B., *Biotechnol. Bioeng.*, **42**, 367-375 (1993)

99. Oestreicher, E.G., Pereira, D.A., Pinto, G.F., *J. Biotechnol*., **46**, 23-31 (1996)

100. Pereira, D.A., Pinto, G.F., Oestreicher, E.G*., J. Biotechnol*., **34**, 43-50 (1996)

101. Korkhin, Y., Kalb, A.J., Peretz, M., Bogin, O., Burstein, Y*., J. Mol. Biol*., **278**, 967-981 (1998)

102. Prelog, V., *Pure Appl. Chem*., **9**, 119-130 (1964)

103. Pham, V.T., Phillips, R.S., Ljungdahl, L.G., *J. Am.Chem.Soc.,* **111**, 1935-1936 (1989)

104. Pham, V.T., Phillips, R.S., *J. Am. Chem. Soc.,* **112**, 3629-3632 (1990)

105. Ferre, E., Gil, G., Barre, M., Bertrand, M., *Enz. Microb. Tech.*, **8**, 297 (1986)

106. Fan, F., Lorenzen, J.A., Flapp, B.V., *Biochemistry*, **30**, 6397 (1991)

107. Eklund, H., Nordstrom, B., Zeppezauer, E., Soderlund, G., Ohlsson, I., Boiwe, T., Branden, C.I., FEBS Lett., 44, 200 (1974)

108. Eklund, H., Nordstrom, B., Zeppezauer, E., Soderlund, G., Ohlsson, I., Boiwe, T., Soderberg, B.O., Tapia, O., Branden, C.I., *J. Biol. Chem*., **102**, 27-59 (1976)

109. Drum, D.E., Li, T.K., Vallee, B.L., *Biochemistry*, **8**, 3792 (1969)

110. Wong, C.H., Whitesides, G.M., *Enzymes in Synthetic Organic* Chemistry, **12**, Pergamon, NY (1994)

111. Matos, J.R., Smith, M.B., Wong, C.H., *Bioorg. Chem.*, **13**, 121 (1985)

112. Secundo, F., Phillips, R.S., *Enzyme Microb. Technol.,* **19**, 487-492 (1996)

113. Burdette, D.S., Vieille, C., Zeikus, J.G., *Biochem. J*., **316**, 115-122 (1996)

114. Burdette, D.S., Secundo, F., Phillips, R.S., Dong, J., Scott, R.A., Zeikus, J. G., *Biochem. J.,* **326**, 717-724 (1997)

115. Tripp, A.E., Burdette, D.S., Zeikus, J.G., Phillips, R.S., *J. Am.Chem.Soc*., **120**, 5137-5141 (1998)

116. Heiss, C., Laiveneiks, M., Zeikus, J.G., Phillips, R.S., *Bioorg. Med. Chem*., **9**, 1659-1666 (2001)

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CHAPTER I

^{15}N-HSQC-NMR ANALYSIS OF WILD TYPE, K87T, D305A, AND E109D MUTANTS OF TRYPTOPHAN SYNTHASE COMPLEXED WITH 1-^{15}N-L-TRYPTOPHAN.*

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* Osborne, Andrew S., Phillips, Robert S. and Teng, Q. To be submitted to *Journal of Biomolecular NMR*.

Abstract

1-^{15}N-L-tryptophan was synthesized and complexed with wild type tryptophan synthase and β-subunit mutants, K87T, D305A and E109D, in the presence or absence of the α-ligand, α-glycerophosphate. Enzyme-substrate complexes were observed by ^{15}N heteronuclear single-quantum coherence nuclear magnetic resonance spectroscopy (^{15}N-HSQC-NMR) for the presence of ^{15}N-L-tryptophan bound to the β active site. Experimental results are similar to those obtained by kinetic and x-ray methods. Wild type tryptophan synthase shows a weak cross peak at 10.25 ppm and 132 ppm in the absence of α-glycerophosphate. The addition of glycerophosphate produces a signal six times more intense, consistant with closure of the active site. Spectra of mutants, K87T and E109D, show a similar cross peak with and without the presence of α-glycerophosphate, indicating the preference for a closed conformation. In contrast, mutant D305A only shows a peak in the presence of α-glycerophosphate.

Introduction

Bacterial tryptophan synthase (EC 4.2.1.20) is a pyridoxal-5'-phosphate (PLP, vitamin B6) dependent enzyme that catalyzes the final two steps of the biosynthesis of L-tryptophan. Structurally, the enzyme is a four subunit complex consisting of two identical α-structures flanking a central β_2-dimer. Schematically, this can be accurately described as α-ββ-α.[1-4] Each α-subunit independently catalyzes the reversible aldol cleavage of indole 3-glycerophosphate to indole plus glyceraldehyde 3-phosphate; hence, this is described as the α-reaction. The β_2-complex, or simply β, catalyzes the irreversible conversion of indole and L-serine to L-tryptophan, with PLP as the cofactor (Fig. 1.1).

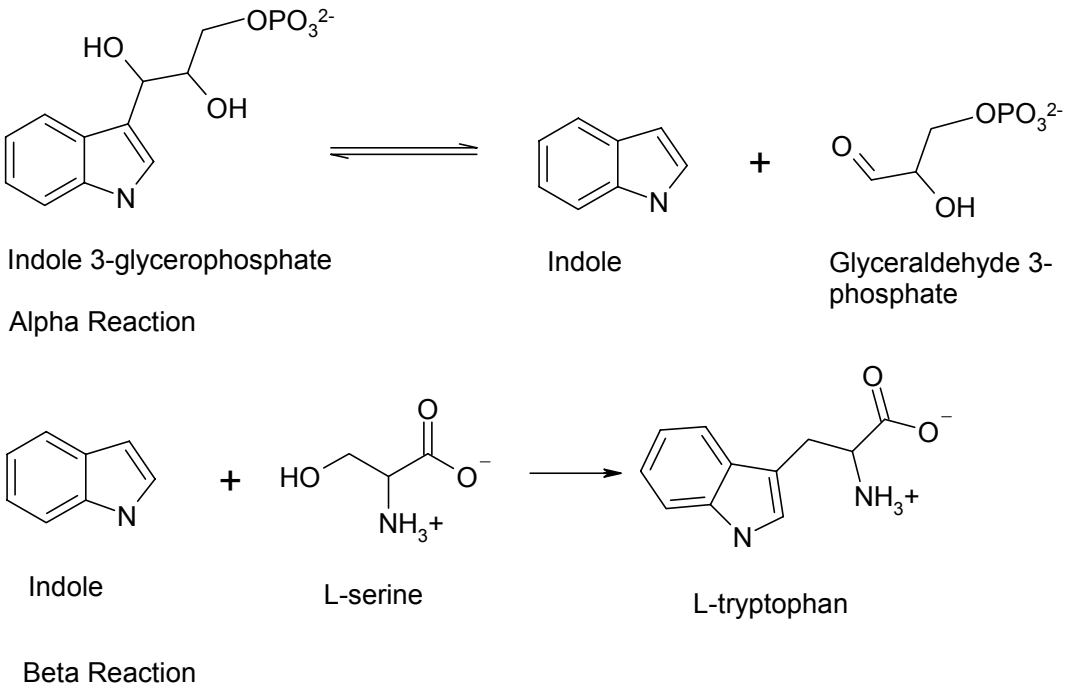


Figure 1.1 Reactions catalyzed by tryptophan synthase

Interestingly, indole is not observed as an intermediate in kinetic studies, suggesting that it never leaves the tryptophan synthase structure. X-ray crystallographic structures have

revealed a 25-30 Å interenzyme tunnel linking the α and β active sites whereby indole travels to the β-site directly upon α-cleavage (Fig. 1.2).[5-11] Moreover, intermediate complexes of the α and β-reactions allosterically regulate catalysis at the other.[12-14] A product of the α-reaction, glyceraldehyde-3-phosphate, binds with the α-complex to allosterically regulate the binding of L-serine to PLP, and serves as a steric inhibitor, blocking the entrance of indole into the interenzyme tunnel. In contrast, the reactive intermediate complex of the β-reaction, aminoacrylate, activates the α-reaction 27 fold. Together, these interactions promote the forward progress of the αβ-reaction.

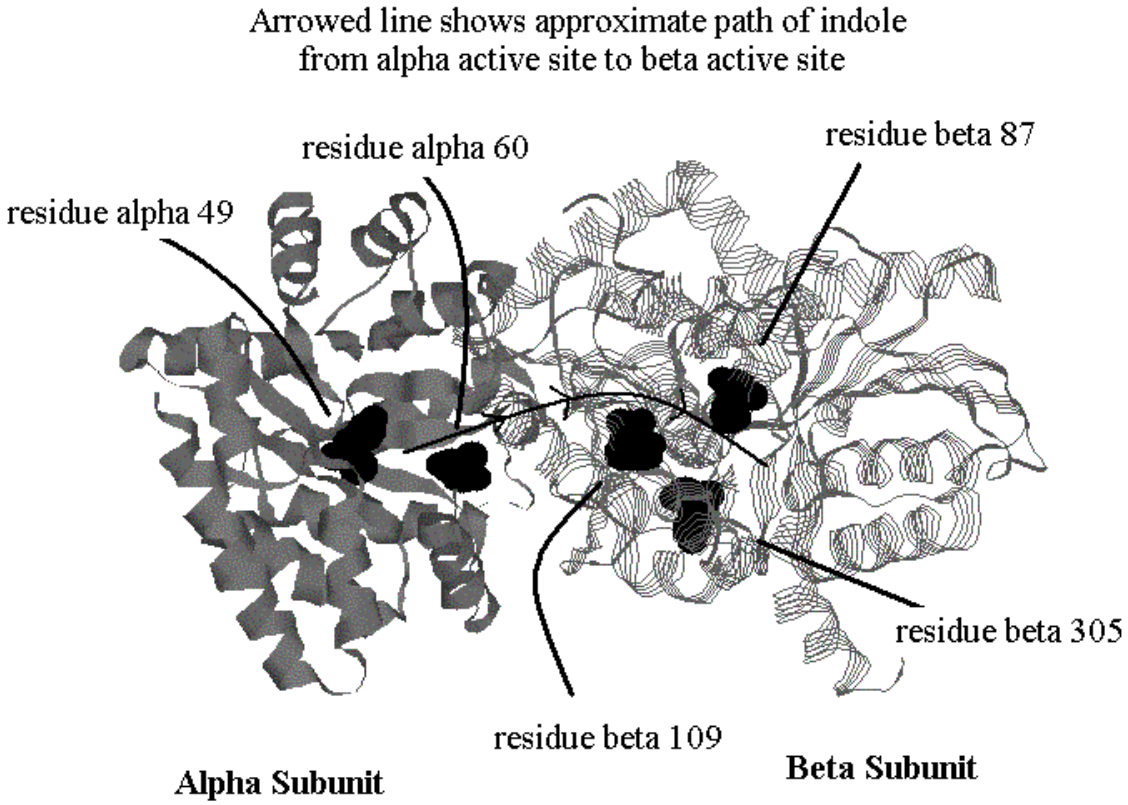


Figure 1.2. Tryptophan synthase: key residues and substrate path.

Although the mechanism of the β-reaction (Fig.1.3) has been studied extensively, there is much to be learned about the roles of individual residues in catalysis and subunit

intercommunication. Residue βK87 has been determined to be the attachment point of PLP[15,16] and serves in several catalytic roles. Void of any substrate, PLP is bound to residue K87 in the form of the internal aldimine, a Schiff base complex (E). Attachment of L-serine, promoted by the presence of G3P at the α active site, forms the external aldimine (E-ser) which is converted to the aminoacrylate (E-AA) upon loss of water. This is the reactive intermediate for addition of indole. Addition of indole to the aminoacrylate affords the quinonoid intermediate (EQ). At this point, the amine residue of βK87 acts as a Brönsted-Lowry acid, and provides the proton for the conversion to the L-tryptophan external aldimine (E-Trp). Release of L-tryptophan leads to the regeneration of the original internal aldimine, ready to catalyze another synthesis of L-tryptophan.

Evidence of allosteric regulation comes from both X-ray data[14,17,18] showing conformational changes associated with bound intermediates, and from kinetic data[12,13,19] of wild type and several key mutants of the enzyme, where key residues associated with allosteric regulation have been substituted with residues bearing less complementary side chains. Much direct evidence on the nature of enzyme conformational change and mechanistic proof has been acquired by direct study of mutants of this enzyme. Mutants of three different key residues in the β-active site have received particular attention and have demonstrated significant effect on reaction catalysis. βK87T, βE109D, and βD305A mutants of bacterial tryptophan synthase have demonstrated conformational and kinetic deviations from wild type.

In this experiment, L-tryptophan substituted by ^{15}N at the 1 position of the indole ring was synthesized and successfully used as an NMR probe to determine conformational state information of wild and β-active site mutants K87T, E109D and D305A tryptophan synthase with and without the presence of an analog of the α-reaction product, α-glycerol phosphate. This method permits the elucidation of important

conformational information without the difficulties associated with X-ray crystallography or lack of structural information provided by kinetic measurement.

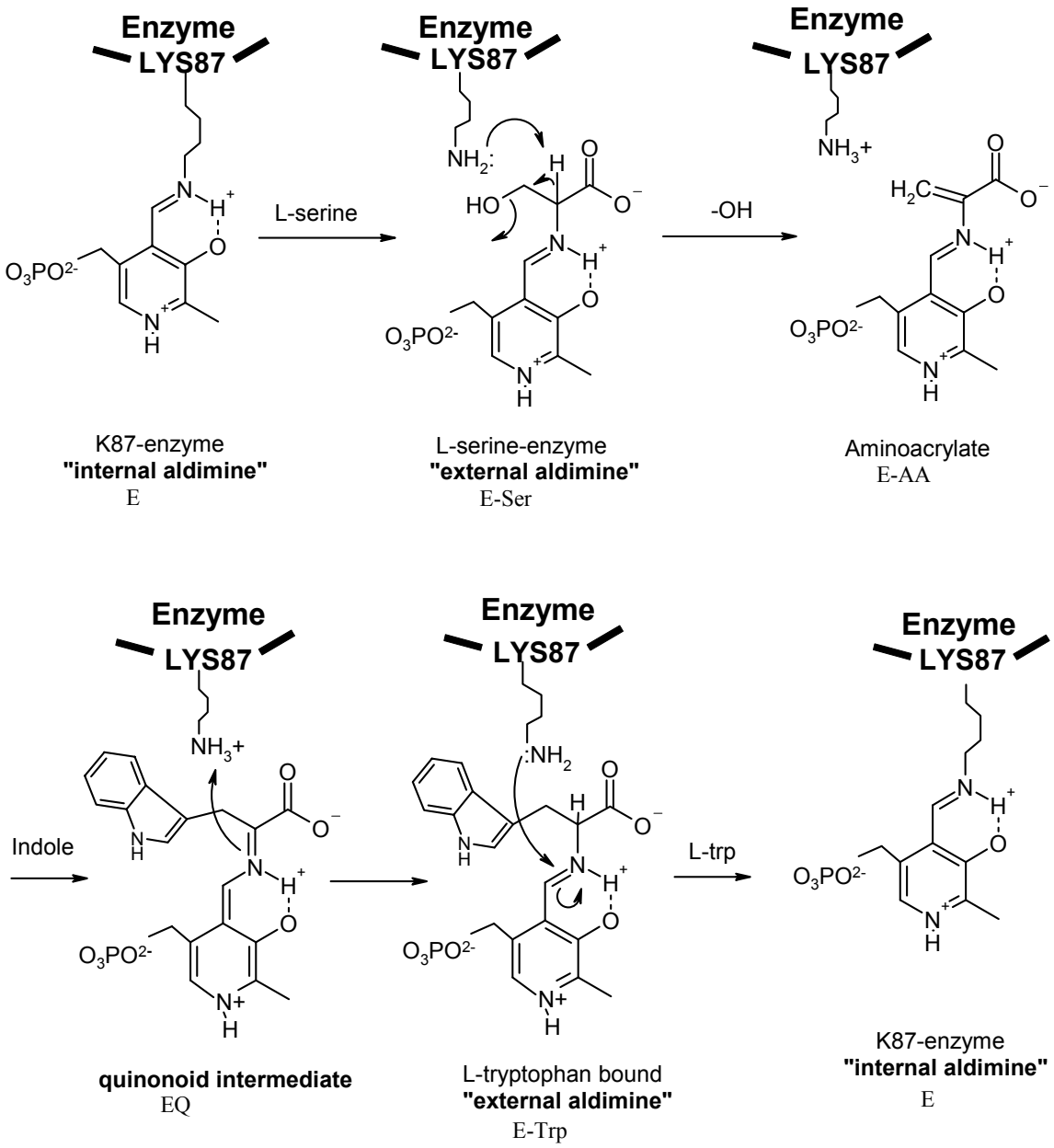


Figure 1.3. Mechanism of the β reaction of tryptophan synthase

Materials and Methods

Materials. Amino acids and other bioreagents were obtained from Sigma. ^{15}N-aniline was obtained from Isotec.

Synthesis of 1-^{15}N-L-tryptophan: [20]

Synthesis of ^{15}N Isonitrosoacetanilide:[21,22] ^{15}N-Aniline (500 mg, 0.0053 mol) was placed in a 100 ml round bottom flask with 5 ml of water and 0.5 ml concentrated HCl. To this solution, 1.0 g (0.0051 mol) of chloral hydrate in 15 ml water was added followed by 15 g sodium sulfate. A solution consisting of 1.2 g (0.05 mol) hydroxylamine hydrochloride in 5 ml water was added to this after 20 minutes of stirring at room temperature. The reaction mixture was heated at reflux, in an oil bath over the period of one hour under reflux. The flask containing the reaction mixture was immediately removed from heat and allowed to cool to room temperature and then placed in an ice bath. The product crystallized from the ice-cold solution where it was filtered *in vacuo*. The product, ^{15}N-isonitrosoacetanilide, was recovered as a white, crystalline powder, which was dried *in vacuo* to afford 770 mg (88%).

Synthesis of ^{15}N-isatin: Isonitrosoacetanilide (500 mg, 0.003 mol) was added to 5 ml of concentrated sulfuric acid at 40° C. The reaction mixture was slowly heated to 65° C and stired at that temperature for 1 hour. Upon completion of the reaction, the opaque, purple mixture was poured over 200 g crushed ice. The ice quickly melted to reveal a bright orange precipitate, which was collected by filtration to afford 412 mg isatin (92%).

Synthesis of ^{15}N-indole: ^{15}N-Isatin was converted to ^{15}N- indole by hydride reduction.[23] ^{15}N-isatin (350 mg, 0.00237 mol) was combined with 6 ml freshly distilled diethyl ether and cooled to –78° C. Four equivalents of lithium aluminum hydride (LAH) in 10 ml diethyl ether were cooled to -78° C and added dropwise to the ^{15}N-isatin solution with vigorous stirring. This mixture was allowed to warm to room temperature over a period of 6 hours. Upon reaction completion, ethanol was added until gas

evolution stopped. Water (5 ml) was then added to the reaction mixture, followed by 1.0 ml of 1.0 M HCl. The aqueous layer was separated and the ether layer was extracted with 5 ml of 0.5 M HCl. The ether layer was steam distilled, and ^{15}N-indole was isolated from the distillate following concentration by rotary evaporation. The product, ^{15}N-indole, was purified by cold-finger sublimation to afford 185 mg white solid (66%). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 6.31 (1H, t, 3-CH), 6.7 (1H, q, 2-CH), 7.1 (1H, t, NH), 7.3 (1H, d, Ar), 7.65 (1H, d, Ar), 7.82 (1H, t, Ar), 8.34 (1H, t, Ar).

Synthesis of ^{15}N-L-tryptophan: 1-^{15}N-L-tryptophan was synthesized from ^{15}N-indole and L-serine by enzymatic catalysis with tryptophan synthase.[24] ^{15}N-indole (137 mg) was placed in a 100 ml round bottom flask with 50 ml 0.1M potassium phosphate buffer, pH 7.8. L-serine, 200 mg, 200 µl tryptophan synthase solution (6.7 mg enzyme) and 0.5 mg of pyridoxal-5'-phosphate were added. The reaction solution was sealed, and wrapped in aluminum foil, and allowed to stir for 30 h. Upon reaction completion, the solution was heated to 80° C to denature the enzyme. The mixture was filtered through celite and the filtrate was concentrated by rotary evaporation. The concentrated filtrate was loaded onto a reverse phase column (2.5cmx30cm; Analtech bonded C_{18}, 35-75 micron, cat#53010). ^{15}N-L-Tryptophan was eluted in 5% to 20% methanol: water. 198 mg ^{15}N-L-tryptophan was recovered (83%). Characterization and complete ^{1}H and ^{13}C -NMR assignments were obtained (Figs. 1.6-1.10). ^{15}N-L-Tryptophan, ^{1}H NMR (400 Mhz, D_2O): δ 3.10 (1H, dd, $J_{\beta\beta'}$ = 15.6, $J_{\alpha\beta'}$ = 8.6 Hz, β'-H), 3.15 (1H, s, -OH), 3.28 (1H, dd, $J_{\beta\beta'}$ = 14.9, $J_{\alpha\beta}$ = 4.7 Hz, β-H), 3.83 (1H, dd, J = 8.6, 4.7 Hz, α-H), 6.99 (1H, t, J=7.0 Hz, C5-H), 7.08 (1H, t, J = 7.8 Hz, C6-H), 7.10 (1H, d, J = 4.7 Hz, C2-H), 7.31 (1H, d, J=7.8 Hz, C7-H), 7.51 (1H, d, J=7.8 Hz, C4-H); ^{13}C NMR (100 Mhz, D_2O): δ 26.5 (β-C), 55.1 (α-C), 107.5 (3-C), 112.0 (7-C), 118.5 (4-C), 119.5 (5-C), 122.2 (6-C), 125.1 (2-C), 126.7 (3'-C), 136.4 (7'-C), 174.6 (-COO-), ESMS, m/z 206 (M+1 calcd for $C_{11}H_{12}N_2O_2$, 206).

Growth and Expression of Tryptophan Synthase: Wild type tryptophan synthase used in chemoenzymatic reactions was expressed from *E. coli* containing plasmid pSTB7, and grown from an agar plate into 2 liters L-broth containing 50 mg/L ampicillin. Cells were lysed and enzyme was collected and purified as previously described.[25,26] All samples of tryptophan synthase, wild type and mutant, used in the NMR experiments were grown and purified in the lab of Dr. Edith Miles at the National Institutes of Health.

NMR sample preparation: Individual samples of the various tryptophan synthase derivatives were prepared as followed: Enzyme solution, 1.0 ml, was thawed and allowed to warm to room temperature where 0.5 mg of pyridoxal-5'-phosphate (PLP) was added and allowed to stir for 1 hour. The enzyme was then dialized on a Sephadex G-25M column rinsed with 10 mM triethanolamine (TEA·HCl), pH 8. The enzyme was collected by elution in 10 mM TEA·HCl and the eluent, 3 ml, was concentrated by ultrafiltration in an amicon to 0.5 ml.

Three NMR measurements were made for each enzyme: The initial measurements were made with samples containing 500 µl enzyme in 10 mM TEA·HCl, pH 8 plus 50 µl D_2O. The two subsequent measurements involved additions to the sample including, first, the addition of 25 µl solution containing 0.1 M ^{15}N-L-tryptophan for the second measurement, and addition of 25 µl 0.5 M glycerophosphate for the third.

Data collection: NMR data were collected with a Varian Inova 500 spectrometer (499.8 MHz, ^{1}H). Water signal suppression was done using flip-back pulses[27] and pulse field gradients.[28] HSQC Experiments[29] were carried out with sensitivity-enhanced gradient coherence selection[30] and the data were processed with NMRPipe.[31]

Results and Discussion

The methodology chosen for the synthesis of 1-^{15}N-L-tryptophan affords the highest possible yield of product starting from ^{15}N- aniline. Critically, this synthesis also incorporates the aniline nitrogen as the nitrogen at the 1 position of the L-tryptophan product. Many syntheses of L-tryptophan have been reported in the literature and many of these could be adapted to our needs. Conveniently, the enzyme of study, tryptophan synthase, is very useful in chemo-enzymatic synthesis. Conversion of indole to L-tryptophan can be carried out in very high yield producing the single product enantiomer. Several total syntheses of ^{15}N-L-tryptophan have been reported in the literature[20,32,33] and each incorporated the conversion of ^{15}N-indole to ^{15}N-L-tryptophan using tryptophan synthase either by reaction in cell cultures modified to express this enzyme in high yield, or by the free enzyme in buffer. None of the previously reported syntheses use ^{15}N aniline, either as a step or starting material. Of the various ways to synthesize indole from aniline, including the Fischer indole synthesis,[34,35] Japp-Klingeman synthesis[36] and Sandmeyer synthesis of isatin,[37] the latter was chosen for its overall efficiency.

1-^{15}N-L-Tryptophan was synthesized efficiently and cleanly from ^{15}N-aniline, obtained from Isotec (Fig.1.4). A Sandmeyer reaction of ^{15}N-aniline with chloral hydrate afforded ^{15}N-isatin, a brilliant orange powder, following ring closure with sulfuric acid. Subsequent reduction with four equivalents of lithium aluminum hydride (LAH) and purification by steam distillation produced ^{15}N-indole in high yield. ^{15}N-L-Tryptophan was chemoenzymatically synthesized from ^{15}N-aniline by reaction with tryptophan synthase with one equivalent of L-serine and a catalytic amount of PLP. TLC analysis of this transformation revealed rapid formation of product. The reaction was allowed to continue for twenty four additional hours to assure complete transformation, at which time TLC indicated no residual starting material, ^{15}N-indole.

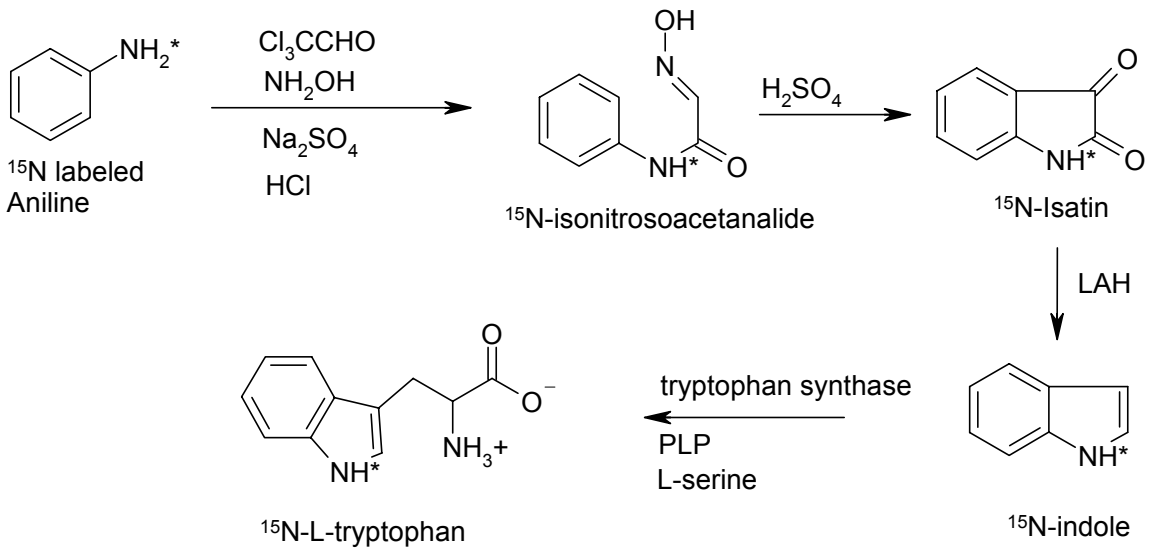


Figure 1.4. Synthesis of ^{15}N-L-tryptophan

NMR has much potential as a tool in the study of enzyme catalysis. Where X-ray crystallography is labor and time intensive and only capable of observing protein structure in a crystalline form, NMR observation of enzyme function via labeled substrate is quick and easy to perform and can provide novel information of the structural and electronic environment of an enzyme's active site and intermediate states. NMR is of particular use as a tool for the study of wild and mutant forms of an enzyme where proof of residue function is often proven by qualitative changes in enzyme catalysis afforded by site directed mutagenesis of that residue. Simple observation or non-observation of a single signal is often all that is needed to prove function.

Tryptophan synthase is an ideal enzyme for study by labeled substrate NMR of wild and mutant enzyme forms for several reasons. First, tryptophan synthase has been the subject of considerable study, and X-ray structures of wild and several mutant forms have been reported. Information provided by labeled substrate NMR can both be verified by known data and can provide new information as well. Secondly, various intermediate complexes of this enzyme allosterically regulate enzyme conformation, catalysis and

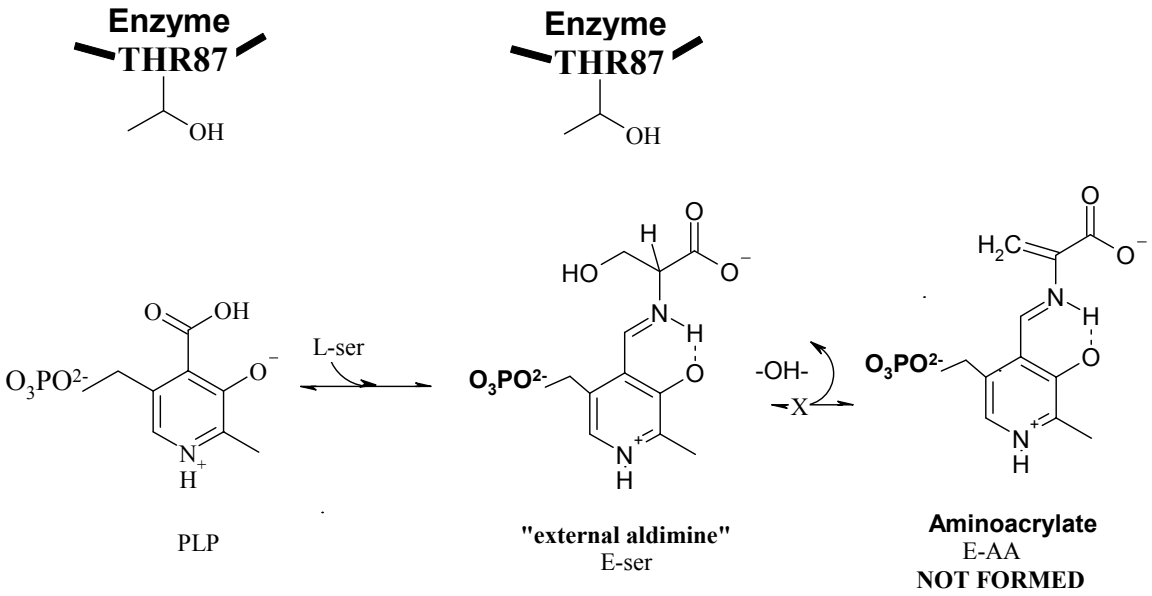
intermediate stability of disparate active sites, and NMR is a well-suited tool for measuring these subtle conformation and electronic changes. Finally, proof of key β residue function can be elucidated by simple observation of product binding in the β site. This is a simple measurement which serves well as an initial foray into this type of analysis.

The experimental method used in these experiments is ^{15}N-HSQC-NMR. ^{15}N-HSQC-NMR facilitates the observation of a ^{15}N signal indirectly by observing the amine proton.[28] The principal advantage to this technique is an enormous increase in detection capability versus direct ^{15}N measurement, which permits experimental observation under biologically and chemically relevant concentrations. Because this method observes the ^{15}N indirectly, the N-H proton must be present, and not exchanging rapidly, which necessitates performing the experiment in a slightly acidic environment. An additional benefit to ^{15}N-NMR is the sensitivity of the ^{15}N signal coupling constant to changes in chemical environment. This method has the potential of detecting minor changes in the electronic and steric environment of an enzyme active site. The great advantage of using an ^{15}N labeled probe with this technique is the ability to observe a single nitrogen atom in the enzyme-substrate complex due to the very low natural abundance of ^{15}N among the enzyme residues.

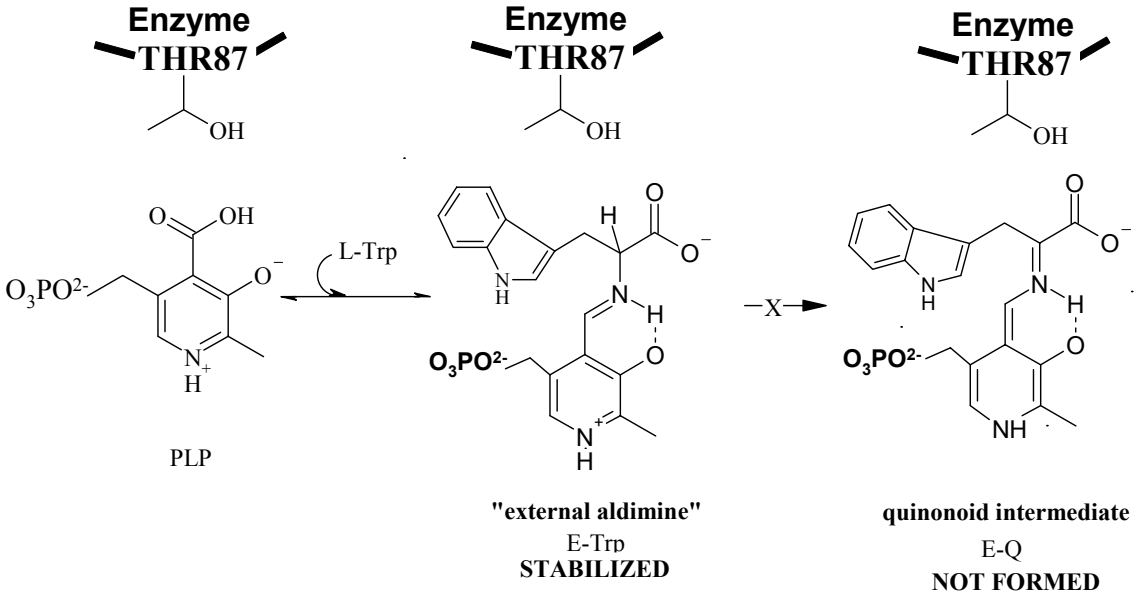
^{15}N-HSQC-NMR experiments were performed on wild type tryptophan synthase, and K87T, E109D and D305A mutants, with PLP and 1-^{15}N-L-tryptophan with and without the ligand, α- glycerophosphate. The wild type enzyme produced a signal with an intensity five times greater with the presence of added α-glycerophosphate (Fig. 1.12) than without (Fig.1.11). Enzymes K87T and E109D with 1-^{15}N-L-tryptophan produced signals equal in intensity with or without the presence of α-glycerophosphate (Figs. 1.13-1.15). Mutant D305A with 1-^{15}N-L-tryptophan only produced a signal in the presence of glycerophosphate (Figs. 1.16, 1.17)

L-Tryptophan bound to the active site of tryptophan synthase is stabilized with the α -active site in a closed conformation. This complex is observed by HSQC-NMR of 1-^{15}N-L-tryptophan as a signal approximately 130 ppm in the ^{15}N axis and 10.2 ppm in the ^{1}H axis. The observation of this signal is dependent on either the α active site adopting a closed conformation, itself dependent on the presence of the α–site ligand α-glycerophosphate or glyceraldehyde-3-phosphate in the wild type enzyme, or stabilization of an L-tryptophan covalent bond to either PLP or some other residue within the β–active site. ^{15}N-HSQC-NMR experiments of the wild type tryptophan synthase confirm this model. In the absence of α-glycerophosphate the NMR signal is observed at a very low signal strength. This indicates that the β-active site has not adapted a fully closed conformation; and the L-tryptophan NH is free to exchange from the β-active site to free solution. In the presence of α-glycerophosphate, the NMR signal is more than five times stronger, indicating a closed β conformation and confirming allosteric regulation of β-catalysis by an α-ligand. Control experiments show no ^{15}N signal from 1-^{15}N-L-tryptophan in the same buffer in the absence of tryptophan synthase.

Residue β lysine-87 is of central importance in the mechanism of tryptophan synthase, acting as the covalent attachment point for PLP, as Bronsted-Lowry acid and Bronsted-Lowry base. The side chain of this residue is responsible for binding PLP in the form of the internal aldimine, which is labile to attack by L-serine to form the external aldimine. At this point, the residual lysine amine acts as base to abstract the external aldimine's α-proton whereby the key reactive intermediate, aminoacrylate, is formed. Mutation of the β87 residue to threonine (K87T) causes failure of the enzyme to allow formation of the aminoacrylate (fig. 1.5), thus indole cannot add to form the quinonoid intermediate.



B-K87T tryptophan synthase does not catalyze the conversion of L-serine- external adimine to the amino- acrylate



B-K87T tryptophan synthase does not catalyze the conversion of L-tryptophan to the quinonoid intermediate in the reverse reaction

Figure 1.5. Catalytic mechanism of βK87T Tryptophan Synthase

Failure of this catalytic step proves the critical role of βK87 in enzyme function. Furthermore, addition of L-tryptophan to βK87T under typical reaction conditions leads to the stable formation of the L-tryptophan external aldimine. Further reverse reaction to form the quinonoid intermediate is not possible as the threonine hydroxyl group is neither basic enough nor close enough to the L-tryptophan α proton to allow extraction. Since there is no lysine present in K87T, the external aldimine is very stable, and the internal aldimine cannot form.

This property of βK87T reactivity is useful for the study of ligand-bound X-ray crystallography of tryptophan synthase. Currently, X-ray crystal structures of K87T have been solved for various configurations including L-serine bound to the β-site, L-ser bound to the β-site and indole-3-propanol phosphate bound to the α-site, L- serine bound to the β-site and α-glycerophosphate bound to the α-site, and of L-tryptophan bound to the β-site. NMR experiments with ^{15}N-L-tryptophan support the current model for the role of K87T. A strong ^{15}N signal is observed for ^{15}N-L-tryptophan with βK87T with and without the presence of α-glycerophosphate.

The second mutant of tryptophan synthase examined by substrate bound ^{15}N NMR is βE109D.[11] In this variant, the glutamate residue is replaced with aspartate. The location of this residue within the β active site is adjacent to the critical PLP aldehyde functionality. In this proximity, the glutamate carboxyl can serve several roles including as a Lewis base to hydrogen bond to the indole NH. Substitution of the glutamate residue by aspartate would reduce the reach and conformational freedom of the carboxyl functionality. Kinetic studies of βE109D have indicated that allosteric communication between the α and β active sites is unaffected by the mutation.[11] G3P bound to the α-site catalyzes the reaction of L-serine at the β-site as with the wild type enzyme. Furthermore, formation of the aminoacrylate is unaffected as well as its allosteric communication to the α-site to release glyceraldehyde-3-phosphate. ^{15}N-HSQC-NMR spectra of ^{15}N-L-tryptophan with E109D tryptophan synthase reveals a

strong signal with and without the presence of α-glycerophosphate. This indicates a clear deviation from the behavior of the wild type enzyme and most likely suggests that the L-tryptophan external aldimine is stabilized in a closed conformation, as with the K87T mutant. In this event, allosteric regulation of the α-site is moot.

Since formation of the aminoacrylate is essentially unaffected by the βE109D mutation, the critical role of this residue must involve the catalysis of indole binding to the aminoacrylate. Indeed, kinetic experiments reveal an accumulation of indole at the β active site for E109D whereas this accumulation does not occur with the wild type. Furthermore, the E109D mutant demonstrates a marked preference for indoline as the β -site nuclophile as opposed to indole, perhaps suggesting a change in the steric environment of the active site.

The third mutant of tryptophan synthase studied by ^{15}N-HSQC-NMR is βD305A.[38] Unlike mutants βK87T and βE109D, residue βD305 is not located in the β active site. X-ray data of different conformations of tryptophan synthase suggest residue D305 to be involved with allosteric communication between the α and β subunits via the monovalent cation binding site. The results of our experiments suggest that allosteric communication is similar to that of the wild type enzyme. Since the ^{15}N peak is not observed in the absence of α-glycerophosphate, but is observed in the presence of α-glycerophosphate, this supports the premise that an α-ligand still regulates the closure of the β active site. The absence of signal without the α-ligand suggests that the protein may adopt a more open conformation when α-ligands are absent.

Site-directed mutagenesis is a very powerful tool for proving residue function in enzyme catalysis. Complementary to ultraviolet spectroscopy and X-ray crystallography, NMR can now be applied to the study of enzyme catalysis via labeled substrate NMR. This technique can provide a very quick determination of enzyme catalysis and provide crucial structural information as well. Although this study is limited to the use of the 1-

^{15}N isotopomer of L-tryptophan, ^{13}C isotopomers would be ideal for study as would be isotopomers of L-serine, indole and PLP. This will be the focus of future work.

References

1. Yanofsky, C., Crawford, I.P. *The Enzymes* (Boyer, P.D., Ed.) 3rd. ed., Vol. VII, pp 1-31, Academic Press, New York (1972)
2. Miles, E.W., *Adv. Enzymol*., **49**, 127-186 (1979)
3. Miles, E.W., *Adv. Enzymol*., **64**, 93-172 (1991)
4. Miles, E.W. *Subcellular Biology*, vol 24, pp 207-254, Plenum Press, New York (1995)
5. Hyde, C.C., Ahmed, S.A., Padlan, E.A., Miles, E.W., Davies, D.R. *J. Biol.Chem*., **263** (17857-17871)
6. Dunn, M.F., Aguilar, V., Drewe, W.F., Houben. K., Robustell, B., Roy, M., Ind. *J. Biochem. Biophys*., **24**, 44-51 (1987)
7. Dunn, M.F., Aguilar, V., Brzovic, P., Drewe, W.F., Houben, K., Leja, C.A., Roy, M., *Biochem*., **29**, 8598-8607 (1990)
8. Kirshner, K., Lane, A.N., Strasser, W.M., *Biochemistry*, **30**, 472-478 (1991)
9. Kirshner, K., Wiskocil, R.L., Foehn, M., Rezeau, L., *Eur. J. Biochem*., **60**, 513-523 (1975)
10. Anderson, K.S., Miles, E.W., Johnson, K.A., *J. Biol. Chem*., **266,** 8020-8033, (1991)
11. Brzovic, P.S., Kayastha, A.M., Miles, E.W., Dunn, M.F*., Biochemistry*, **31**, 1180-1181 (1992)
12. Ahmed, S.A., Ruvinov, S.B., Kayastha, A.M., Miles, E.W*., J. Biol. Chem*., **266**, 21548-21557 (1991)
13. Peracchi, A., Bettati, S., Mozzarelli, A., Rossi, G.L., Miles, E.W., Dunn, M. F., *Biochem*, **35**, 1872-1880 (1996)
14. Schneider, T.R., Gerhardt, E., Lee, M., Liang, P-H., Anderson, K.S., Schlichting, I., *Biochem.*, **37**, 5394-5406 (1998)
15. Rhee, S., Parris, K.D., Hyde, C.C., Ahmed, S.A., Miles, E.W., Davies, D.R., *Biochem.*, **36**, 7664-7680 (1997)

16. Lu, E., Petsko, g., *Biochem*, **29**, 6619-6625 (1990)

17. Weyland, M., Schlichting, I., *Biochem.*, **38**, 16469-16480 (1999)

18. Rhee, S., Parris, K.D., Ahmed, S.A., Miles, E.W., Davies, D.R., *Biochem*., **35**, 4211-4221 (1996)

19. Brzovic, P.S., Sawa, Y., Hyde, C.C., Miles, E.W., Dunn, M.F., *J. Biol. Chem*., **267**, 13028-13038 (1992)

20. van den Berg, E.M.M., Baldew, A.U., de Goede, A.T.J.W., Raap, J., Lugtenburg, J., *Trav. Chim. Pays-Bas*., **107**, 73-81 (1988)

21. Sandmeyer, T., *Helvetica Chemica Acta*, **2**, 234 (1919)

22. Marvel, C.S., Hiers, G.S., *Organic Syntheses*, Coll. Vol. 1, 327 (1944)

23. Giovanni, E., Lorenz, T., *Helvetica Chemica Acta*, **161**, 1552-1561 (1957)

24. Lee, M., Phillips, R.S., *J. Heterocyclic Chem.*, **31**,711 (1994)

25. Miles, E.W., Kawasaki, H., Ahmed, S.A., Morita, H., Morita, H., Nagata, S., *J. Biol. Chem*., **264**, 6280-6287 (1989)

26. Yang, L.-H., Ahmed, S.A., Miles, E.W., *Prot. Expr. Purif*., **8**, 126-136 (1996)

27. Grzesiek, S., Bax, A, *J. Am. Chem. Soc.*, **115**, 12593 (1993)

28. Piotto, M., Saudek, V., Skilenar, V., *J. Biomol. NMR*, **2**, 661 (1992)

29. Vuister, G.W., Bax, A., *J. Magn. Reson*., **98**, 428 (1992)

30. Zhang, O.,Kay, L.E., Olivier, J.P., Forman-Kay, J.D., *J. Biomol. NMR*, **4**, 845 (1994)

31. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu,G., Pfeifer, J., Bax, A., *J. Biomol. NMR*, **6**, 277-293 (1995)

32. Yuan, S-S, Ajami, A.M., *Tetrahedron*, **38**, 2051-2053 (1982)

33. Unkefer, C.J., Siegfried, N.L., Silks, L.A-III., Hanners, J.L., Ehler, D.S., Gibson, R., *J. Labelled comp. Radiopharm.*, **29**, 1247-1256 (1991)

34. Robinson. B., *The Fischer Indole Synthesis*, John Wiley and Sons, N.Y. (1982)

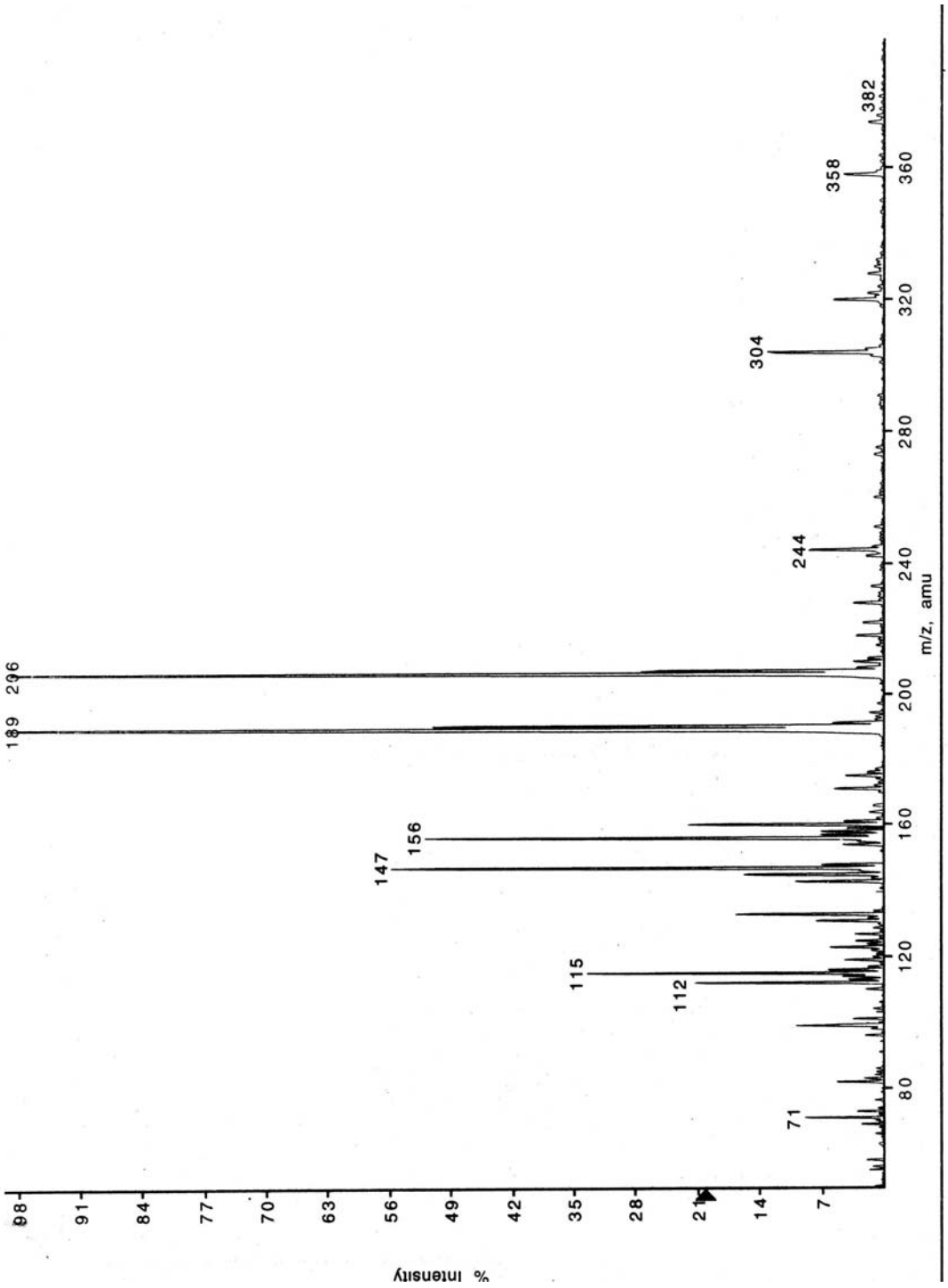
35. Heterocyclic Compounds, Houlihan, W.J., ed., Wiley-Interscience, N.Y. (1972)

36. Phillips, R.R. Organic Reactions, vol. 10: *The Japp-Klingemann Reaction* (Adams, R., Ed.) John Wiley and Sons, N.Y. (1959)

37. Popp, F.D., *Adv. Hetrocycl. Chem*., **18**, 1 (1975)

38. Ferrari, D., Yang, L.H., Miles, E.W., Dunn, M.F., *Biochem.*, **40**, 7421-7432 (2001)

Figure 1.6. Electrospray mass spectrum of ^{15}N-L-tryptophan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91
84
77
70
63
56
49
42
35
28
21
14
7
80
120
160
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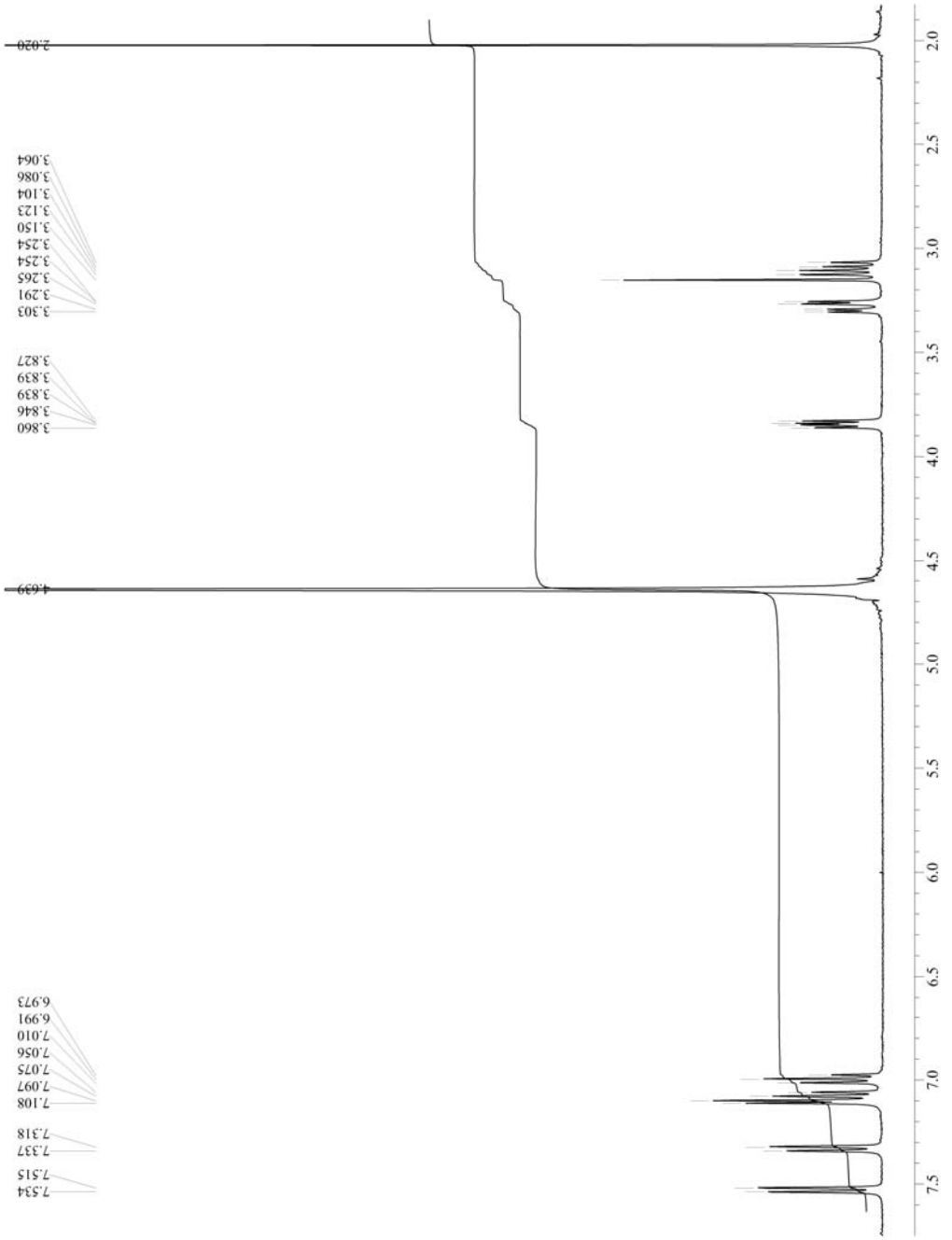
240
280
320
360
m/z, amu
189
296
147
156
115
112
71
244
304
358
382

Figure 1.7. ^{1}H-NMR spectrum of ^{15}N-Ltryptophan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3.150
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3.265
3.291
3.303
3.827
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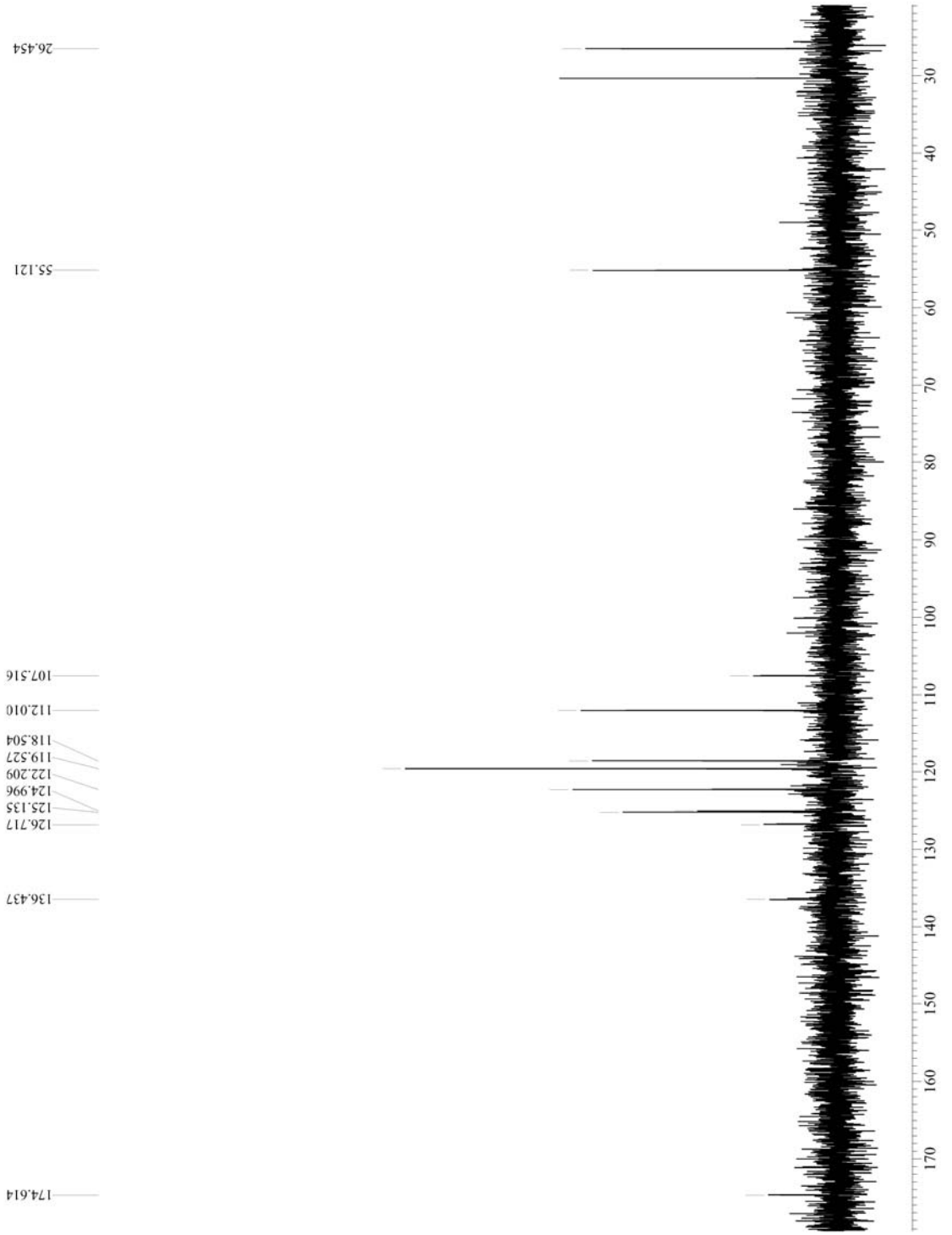
3.839
3.846
3.860
4.639
6.973
6.991
7.010
7.056
7.075
7.097
7.108
7.318
7.337
7.515
7.534
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5

Figure 1.8. ^{13}C-NMR spectrum of ^{15}N-Ltryptophan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125.135
126.717
136.437
174.614
30
40
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140
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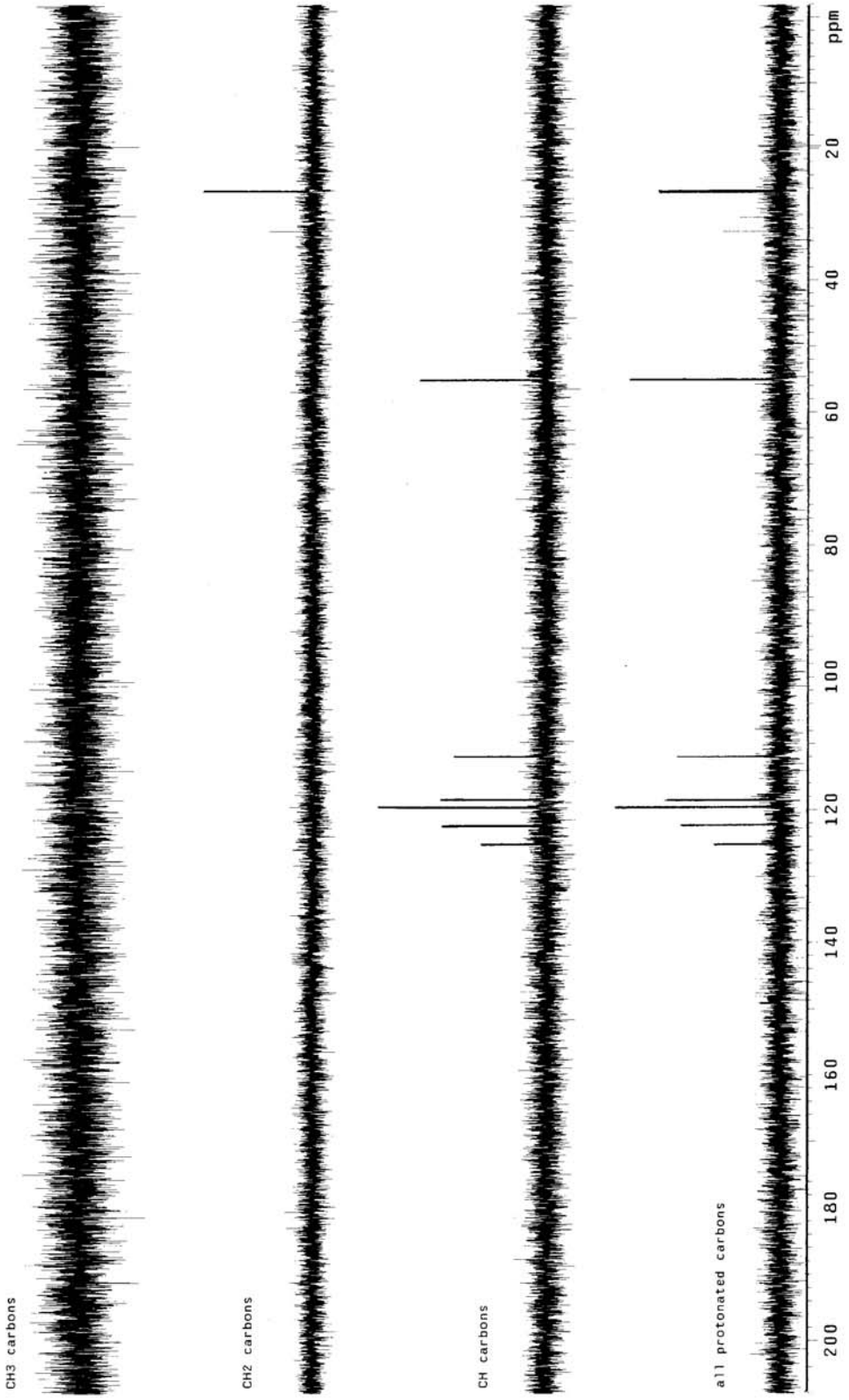
170

Figure 1.9. DEPT-NMR spectrum of ^{15}N-Ltryptophan


CH3 carbons
CH2 carbons
CH carbons
all protonated carbons
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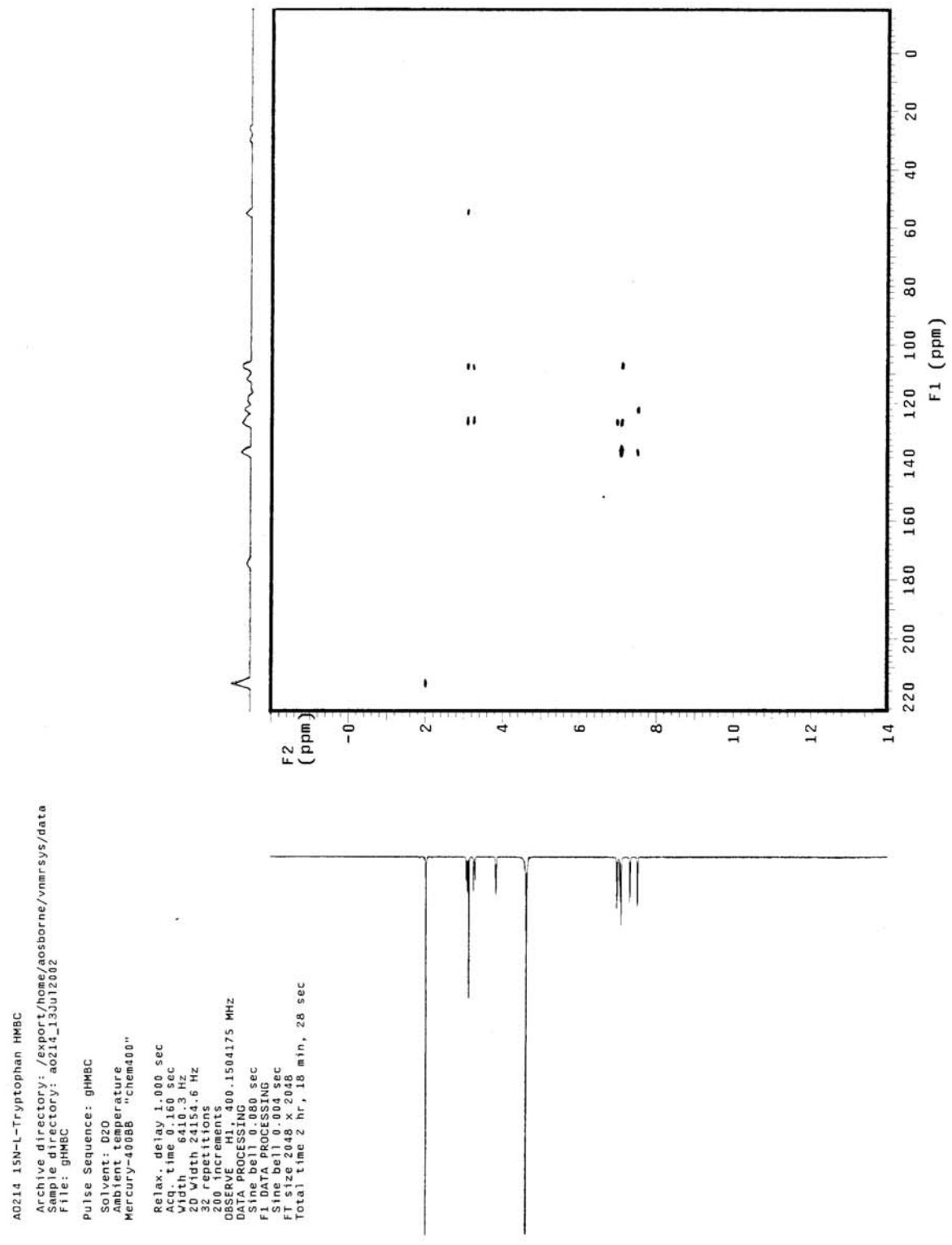
140
120
100
80
60
40
20
ppm

Figure 1.10. gHMBC-NMR spectrum of ^{15}N-Ltryptophan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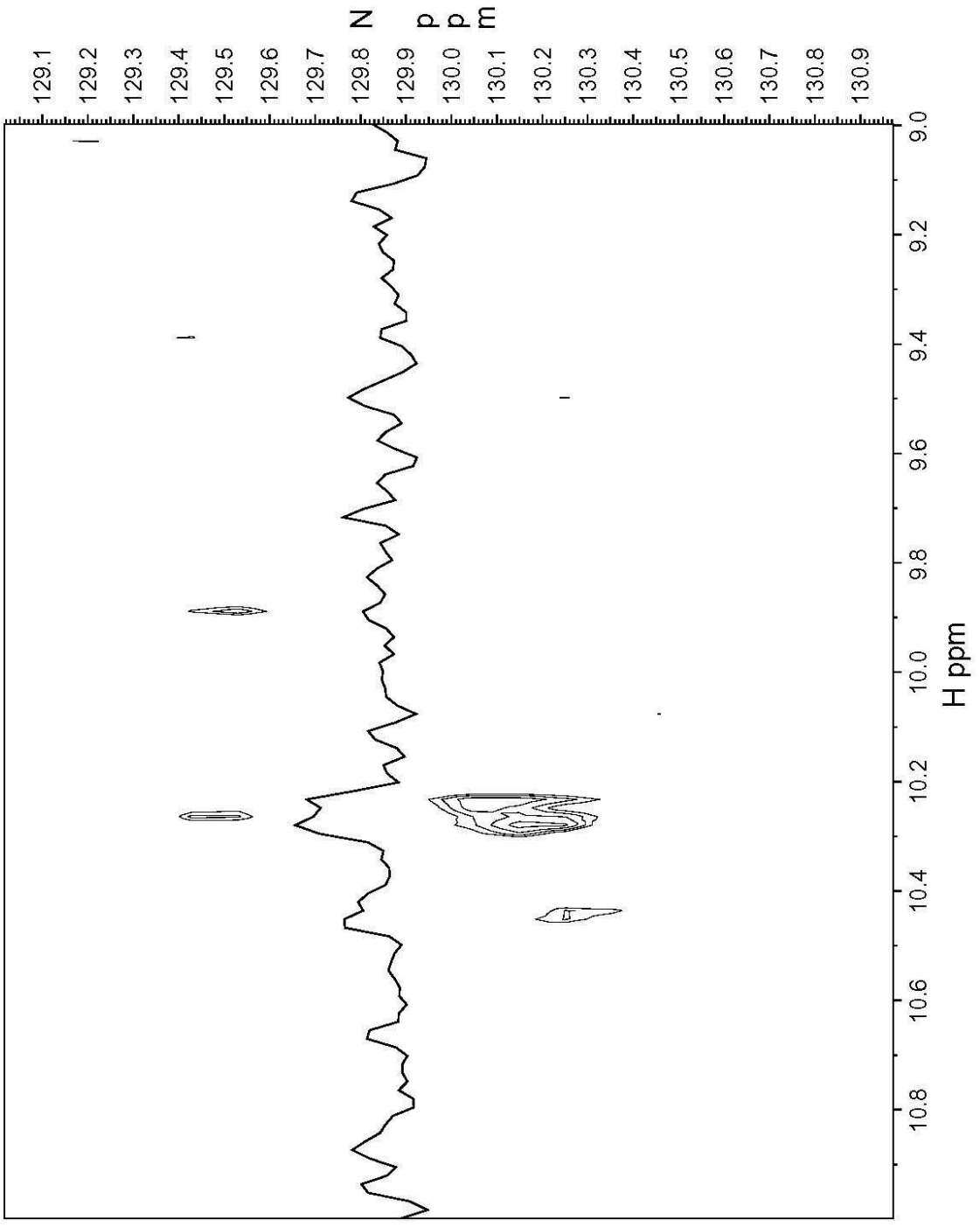
Mercury-400BB "chem400"
Relax. delay 1.000 sec
Acq. time 0.160 sec
Width 6410.3 Hz
2D Width 24154.6 Hz
32 repetitions
200 increments
OBSERVE H1, 400.1504175 MHz
DATA PROCESSING
Sine bell 0.080 sec
F1 DATA PROCESSING
Sine bell 0.004 sec
FT size 2048 x 2048
Total time 2 hr, 18 min, 28 sec
F2 (ppm)
F1 (ppm)

Figure 1.11. ^{15}N-HSQC-NMR spectrum of ^{15}N-L-tryptophan bound to wild type tryptophan synthase without the presence of glycerophosphate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129.3
129.4
129.5
129.6
129.7
129.8
129.9
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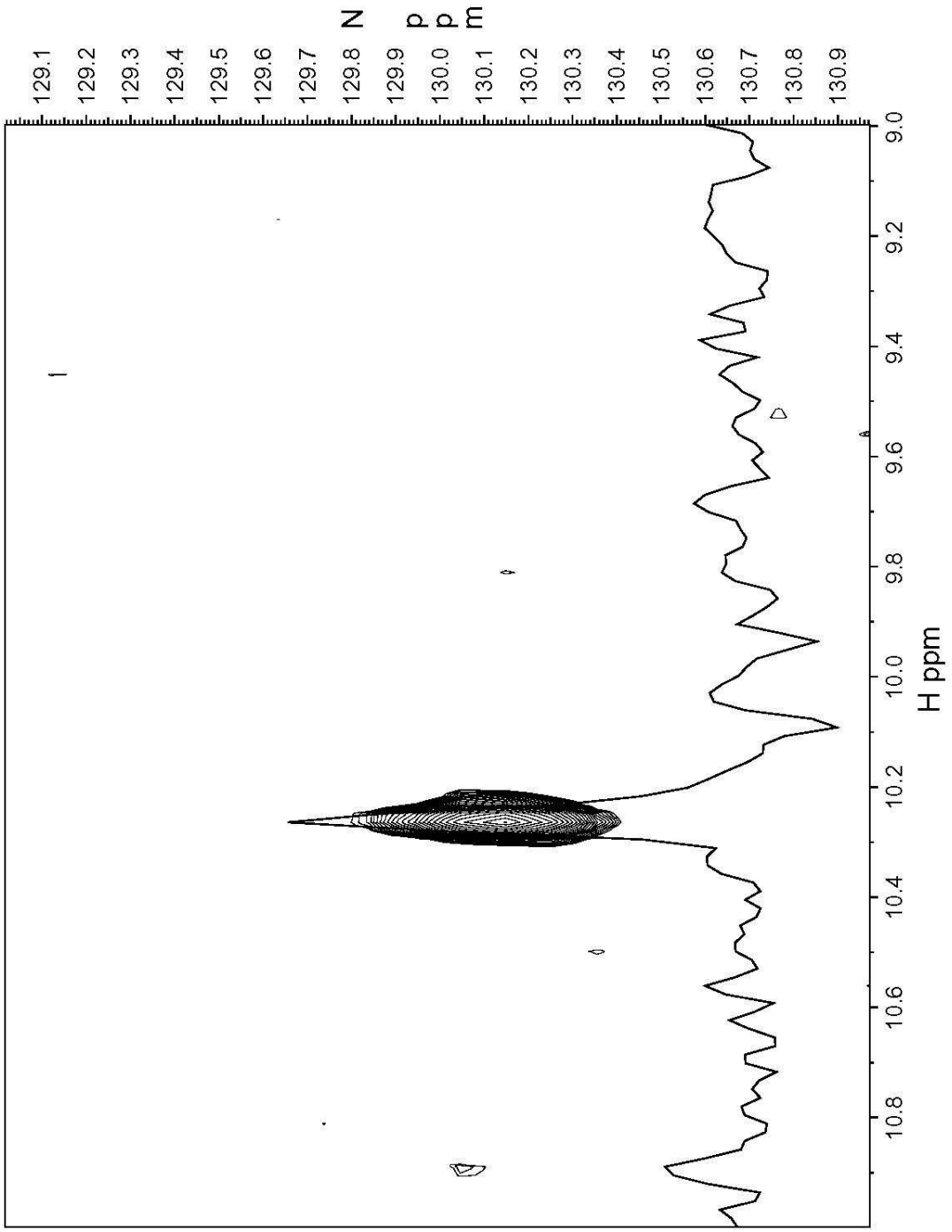
130.1
130.2
130.3
130.4
130.5
130.6
130.7
130.8
130.9
H ppm
9.0
9.2
9.4
9.6
9.8
10.0
10.2
10.4
10.6
10.8

Figure 1.12. ^{15}N-HSQC-NMR of ^{15}N-L-tryptophan bound to wild type tryptophan synthase with the presence of glycerophosphate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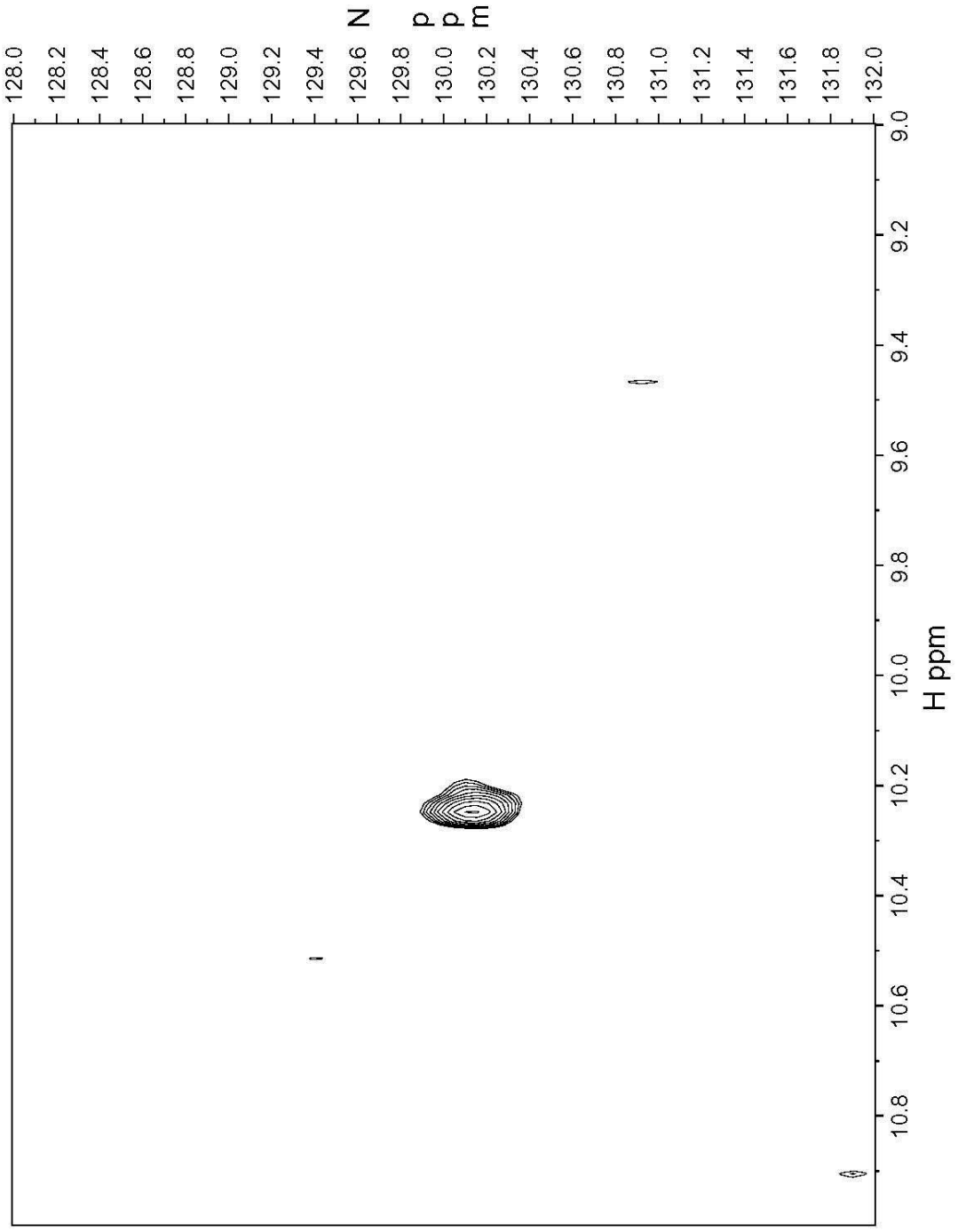
130.3
130.4
130.5
130.6
130.7
130.8
130.9
H ppm
9.0
9.2
9.4
9.6
9.8
10.0
10.2
10.4
10.6
10.8

Figure 1.13. ^{15}N-HSQC-NMR spectrum of ^{15}N-L-tryptophan bound to mutant βK87T tryptophan synthase without the presence of glycerophosphate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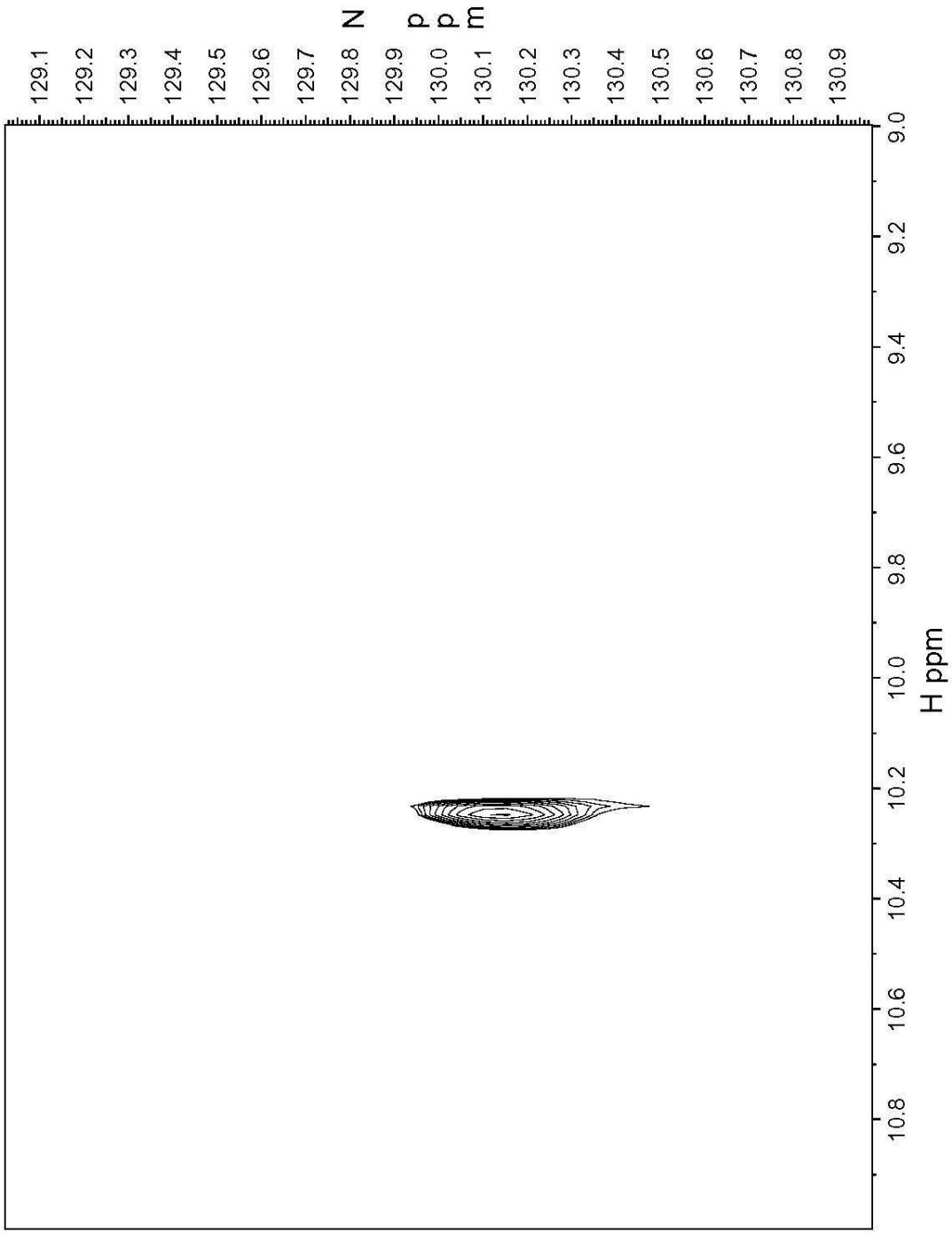
130.4
130.6
130.8
131.0
131.2
131.4
131.6
131.8
132.0
H ppm
9.0
9.2
9.4
9.6
9.8
10.0
10.2
10.4
10.6
10.8

Figure 1.14. ^{15}N-HSQC-NMR spectrum of ^{15}N-L-tryptophan bound to mutant βK87T tryptophan synthase with the presence of glycerophosphate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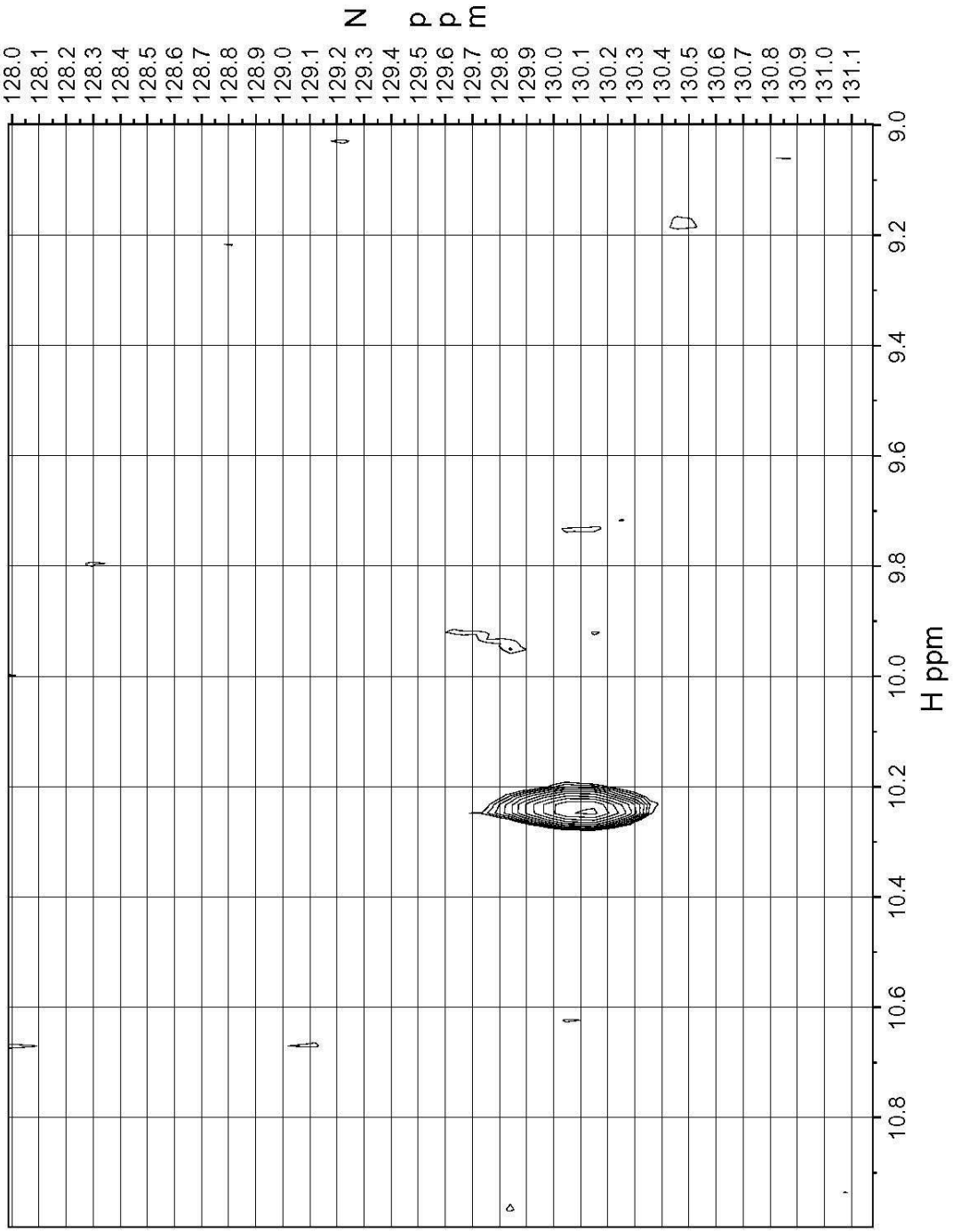
130.5
130.6
130.7
130.8
130.9
H ppm
9.0
9.2
9.4
9.6
9.8
10.0
10.2
10.4
10.6
10.8

Figure 1.15. ^{15}N-HSQC-NMR spectrum of ^{15}N-L-tryptophan bound to mutant βD305A tryptophan synthase with the presence of glycerophosphate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129.7
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131.0
131.1
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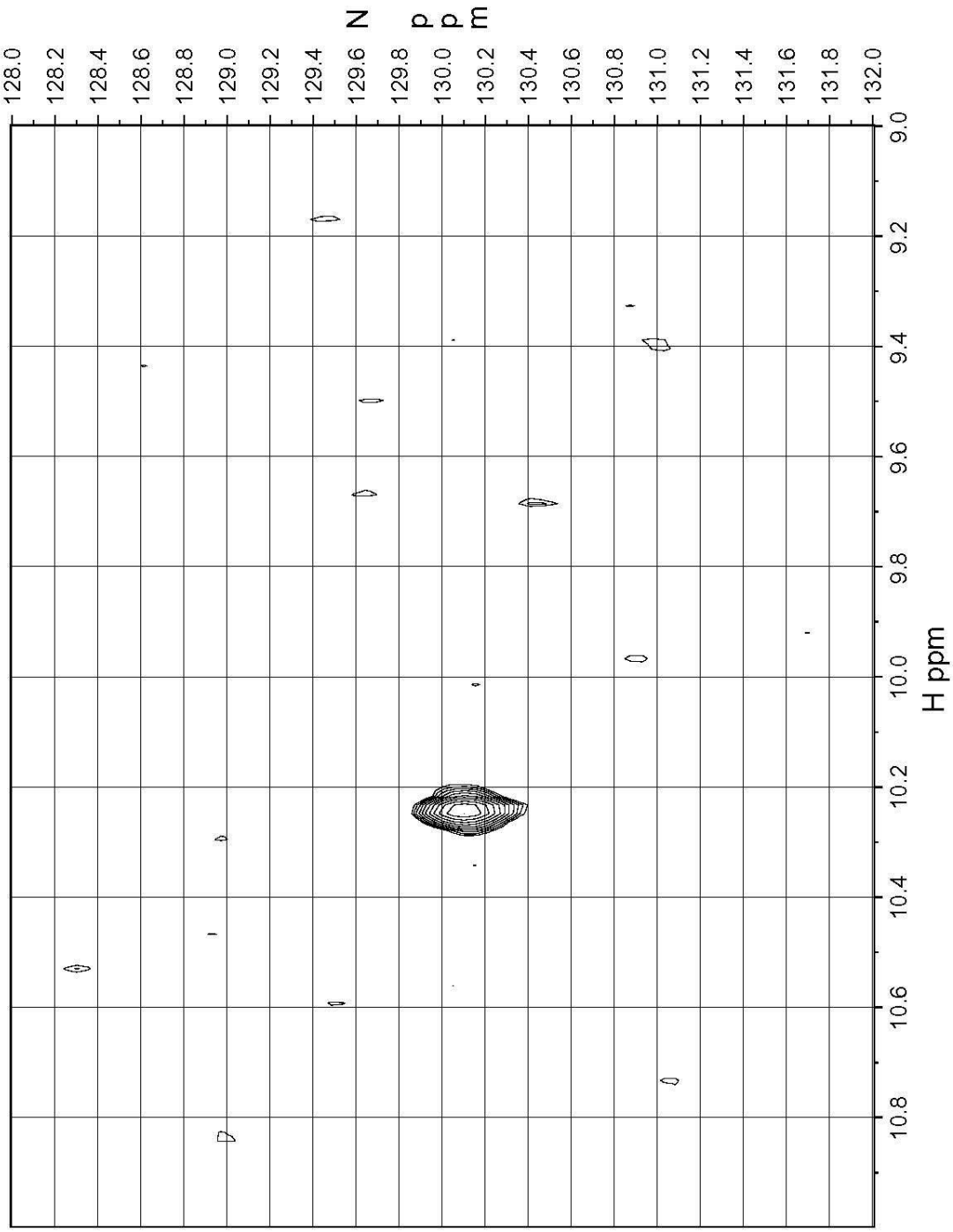
9.2
9.4
9.6
9.8
10.0
10.2
10.4
10.6
10.8
H ppm

Figure 1.16. ^{15}N-HSQC-NMR spectrum of ^{15}N-L-tryptophan bound to mutant βE109D tryptophan synthase with the presence of glycerophosphate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H ppm
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130.8
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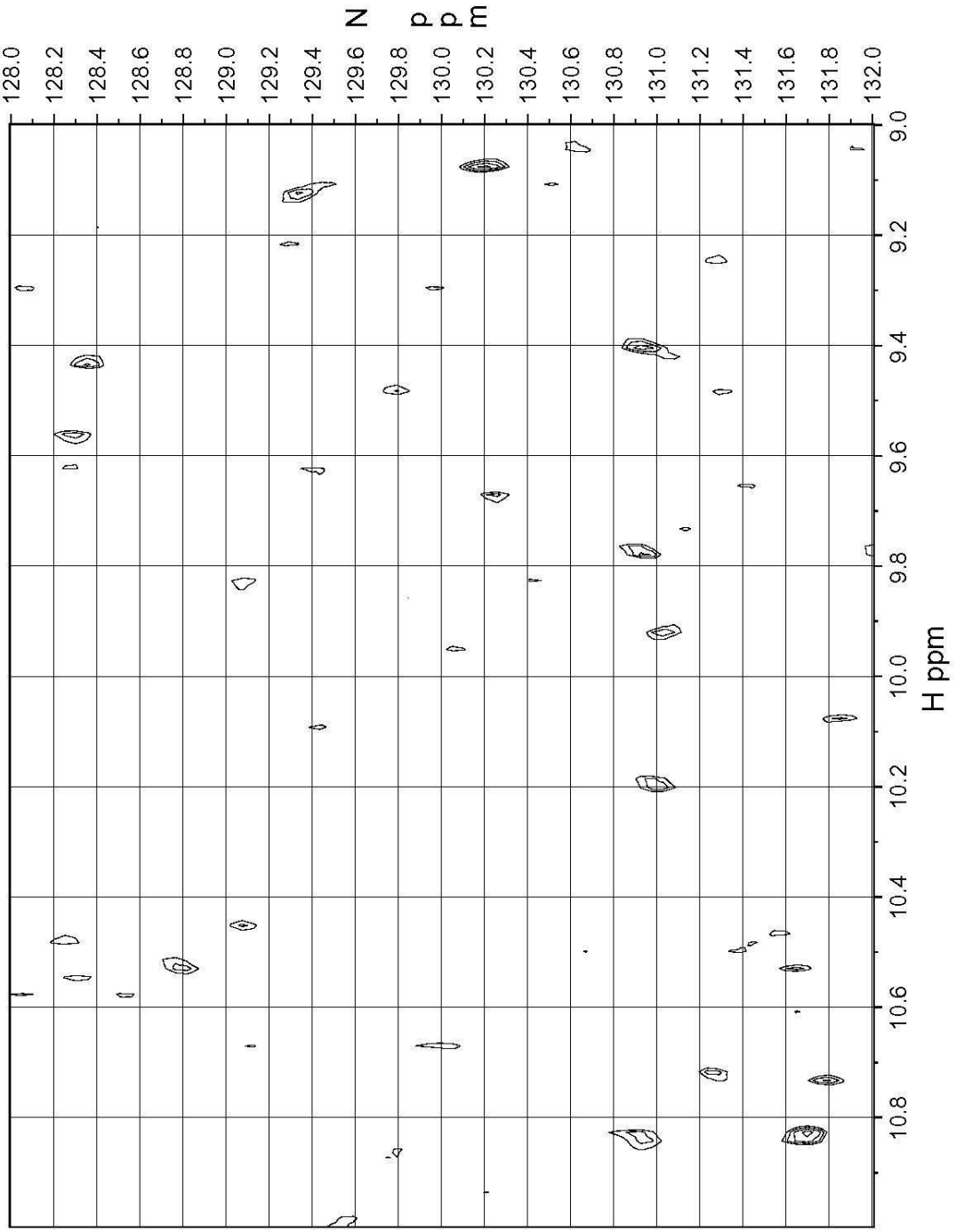
131.6
131.8
132.0
9.0
9.2
9.4
9.6
9.8
10.0
10.2
10.4
10.6
10.8

Figure 1.17. ^{15}N-HSQC-NMR spectrum of ^{15}N-L-tryptophan bound to mutant βD305A tryptophan synthase without the presence of glycerophosphate


N ppm
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128.2
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129.8
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130.4
130.6
130.8
131.0
131.2
131.4
131.6
131.8
132.0
9.0
9.2
9.4
9.6
9.8
10.0
10.2
10.4
10.6
10.8
H ppm

CHAPTER II

REGIOSELECTIVE, EFFICIENT AND SOLVENT CONTROLLED SYNTHESIS OF PROTECTED 2 AND 6-NITRO-L-TRYPTOPHANS

Introduction

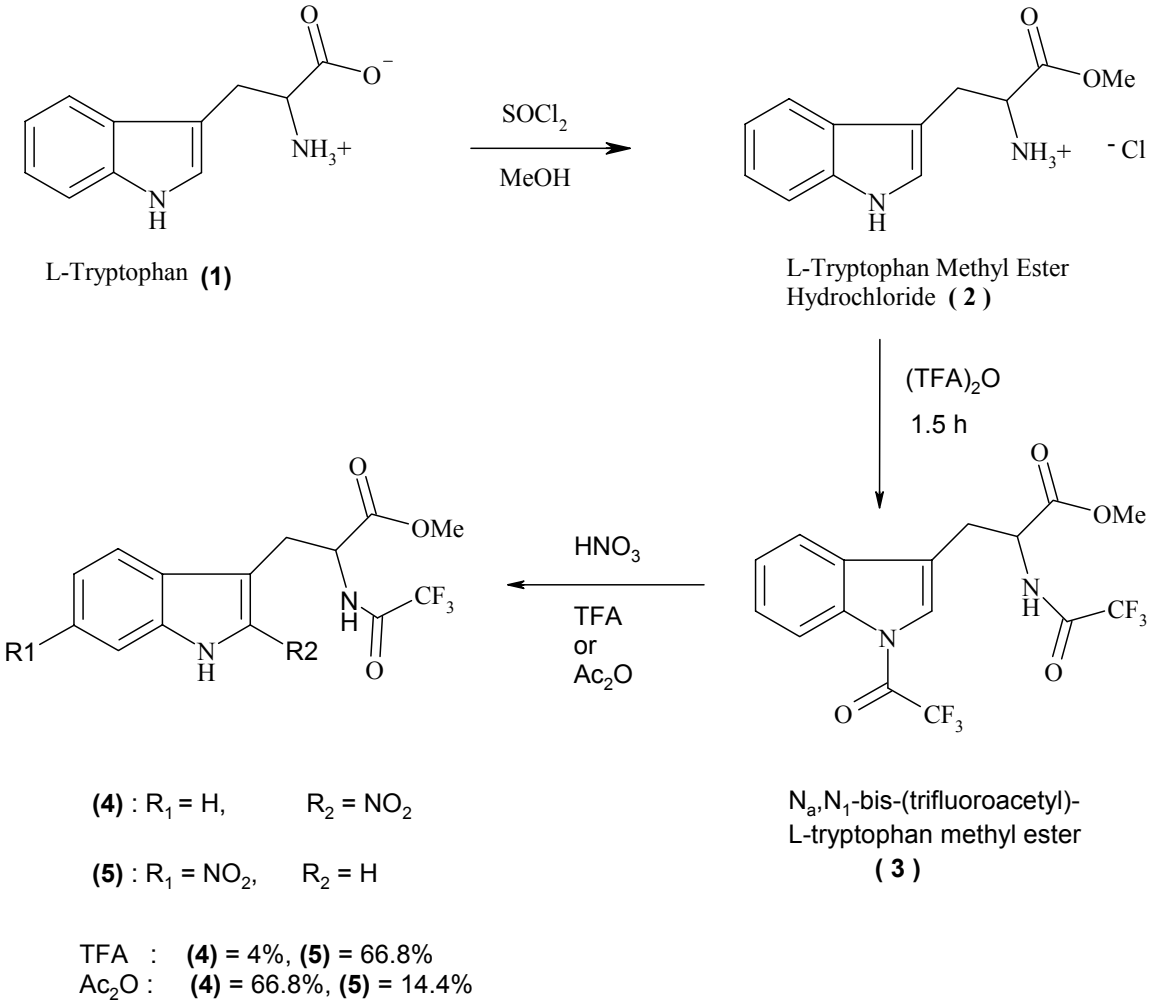
Derivatives of L-tryptophan have been the subject of considerable chemical and biological study, and several derivatives have received particular attention for their utility in a wide range of applications. 5-Bromo-L-tryptophan has shown promise in treating sickle cell anemia.[1] 6-Chloro-D-Tryptophan has been the subject of substantial research owing to its properties as a non-nutritive sweetner,[2] and several efficient syntheses of this molecule have been reported that improve upon the racemic synthesis reported by Tweddle, et.al.[3-5] 6-Azido-L-tryptophan is used as a photoinactivator and photoaffinity label for a variety of enzymes including tryptophan synthase and tryptophan indole lyase.[6-8] L-Tryptophan substituted by chlorine,[5] methyl,[9] and fluorine[10] has been prepared efficiently and characterized by ^{1}H, ^{13}C, and 2D NMR methods.

L-Tryptophan substituted by bromine and nitro has been more problematic to synthesize efficiently, mainly because chemo-enzymatic synthesis of the L-tryptophan derivative from the corresponding substituted indole has not been successful. Several isomers of L-tryptophan substituted with bromine have been reported in the literature including 2-bromo,[11-12] 5-bromo,[13] and 7-bromo.[14] Nitro-L-tryptophan isomers reported in the literature include 2-nitro,[15] 5-nitro,[16,17] and 6-nitro-L-tryptophan.[6,18,19]

Methods for bromination of L-tryptophan as the free amino acid are completely absent from the literature as these compounds have only been prepared from brominated precursors or from the appropriately protected nitro-L-tryptophans.[20] Nitration of L-tryptophan affords 6-nitro-L-tryptophan in reported good yield,[19] but complicated workup and purification hamper attainable yield and cost effectiveness significantly. In our hands, direct nitration of L-tryptophan under the reported conditions gives a very low yield. The protected L-tryptophan derivative, N_{α}-trifluoroacetyl-L-tryptophan methyl ester, affords the 6-nitro product in 40% yield and the 2-nitro product in 6.8% yield from nitration with nitric acid in acetic acid. However, a complex mixture is obtained,

requiring column chromatography for purification. Reaction of N_α -trifluoroacetyl-L-tryptophan methyl ester with NBS in CCl_4 yields the 2-bromo derivative in 83% yield.[11]

N_α,N_1-(bistrifluoroacetyl)-L-tryptophan methyl ester has been reported as a tryptophan derivative for gas chromatography analysis.[21] However, few studies on the use of this compound for the synthesis of L-tryptophan derivatives have been reported. We now report its use as an effective protected derivative for L-tryptophan substitution. The indole ring is highly reactive with electrophiles, so a protecting group would be useful to moderate its reactivity. The addition of a trifluoroacetyl group at the indole nitrogen changes the electronic environment of the aromatic system, allowing for efficient substitution of the benzene and pyrrole rings. Furthermore, recovery of the substituted product retaining one or both trifluoroacetyl protective groups is desirable for further synthesis, including Sandmeyer chemistry after the reduction of the nitro group. A clean and efficient, solvent controlled, regioselective synthesis of the 2-nitro **(4)** and 6-nitro **(5)** isomers of N_α-trifluoroacetyl-L-tryptophan methyl ester is reported here by a simple three step process, with regioselection occurring in the final step. Each isomer can be synthesized in excess of 60% isolated yield in the final step by selecting either trifluoroacetic acid (TFA) or acetic anhydride (Ac_2O) as the solvent/ Lewis acid catalyst (Scheme 2.1).

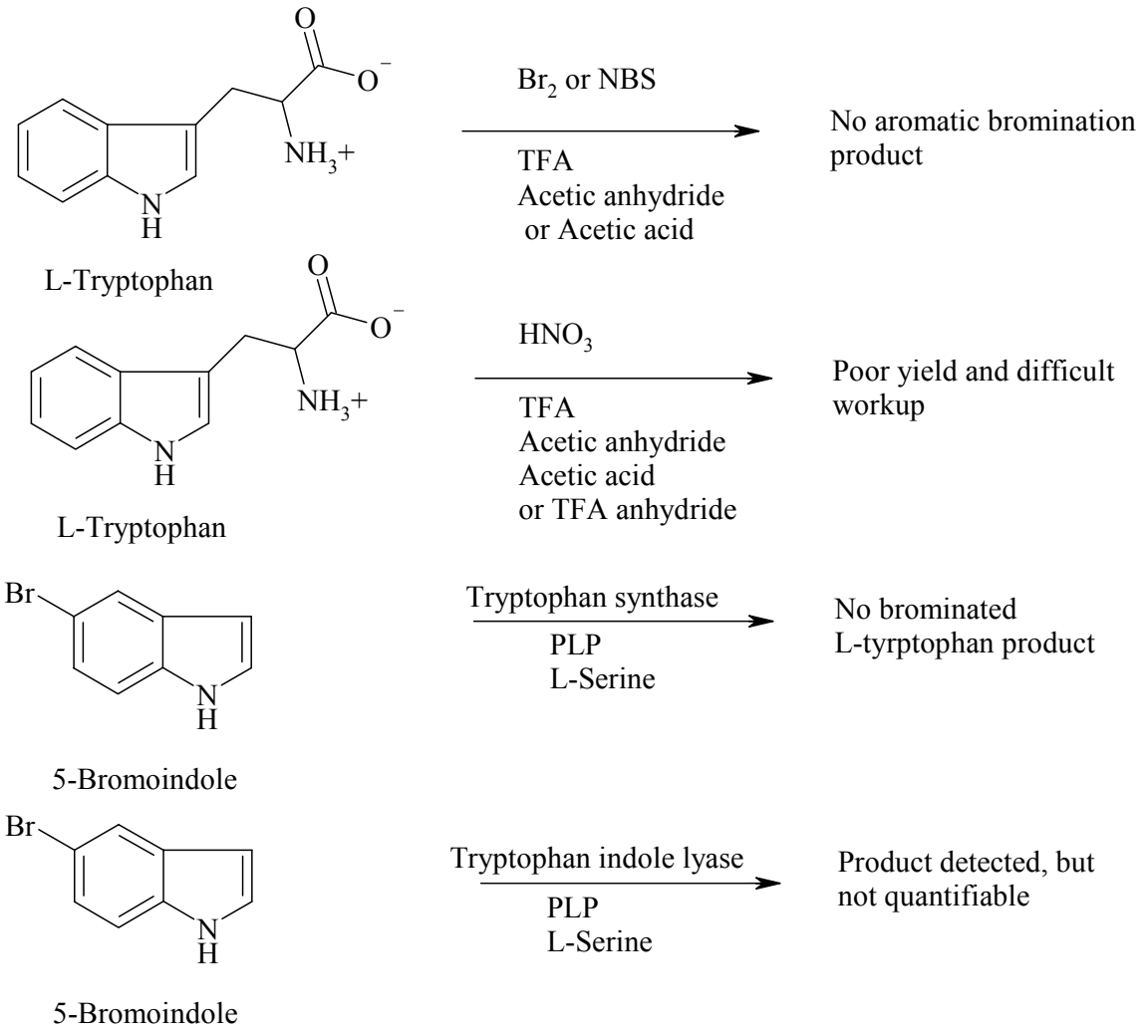


Scheme 2.1. Synthesis of protected 2-nitro or 6-nitro-L-tryptophan

Results

Efforts to synthesize bromo and nitro derivatives of L-tryptophan were attempted from three approaches: 1) chemo-enzymatic synthesis of substituted L-tryptophan from the corresponding substituted indole, 2) direct substitution of free L-tryptophan by a variety of nitrating and brominating agents and catalyst, and 3) substitution of L-tryptophan protected as the N_{α},N_1-(bistrifluoroacetyl) methyl ester. The synthesis of 5-bromo-L-tryptophan from 5-bromoindole and L-serine catalyzed by tryptophan synthase was unsuccessful, as no product was isolated. The same reaction was also tried with tryptophan indole-lyase, which, despite chromatographic evidence of catalysis, produced

essentially no quantifiable yield. Direct halogenation and nitration of L-tryptophan failed to produce acceptable results despite a literature precedence for the efficient synthesis of 6-nitro-D-tryptophan in this manner[19] (Scheme 2.2).



Scheme 2.2. Initial efforts to synthesize substituted L-tryptophan

Synthesis of N_α,N_1-(bistrifluoroacetyl)-L-tryptophan methyl ester was performed quickly and efficiently in a two step procedure similar to that reported by Saroff and Makisumi.[21] L-Tryptophan **(1)** was converted to the methyl ester hydrochloride **(2)** by treatment with $SOCl_2$ and methanol at -42º C. The product was then reacted with trifluoroacetic

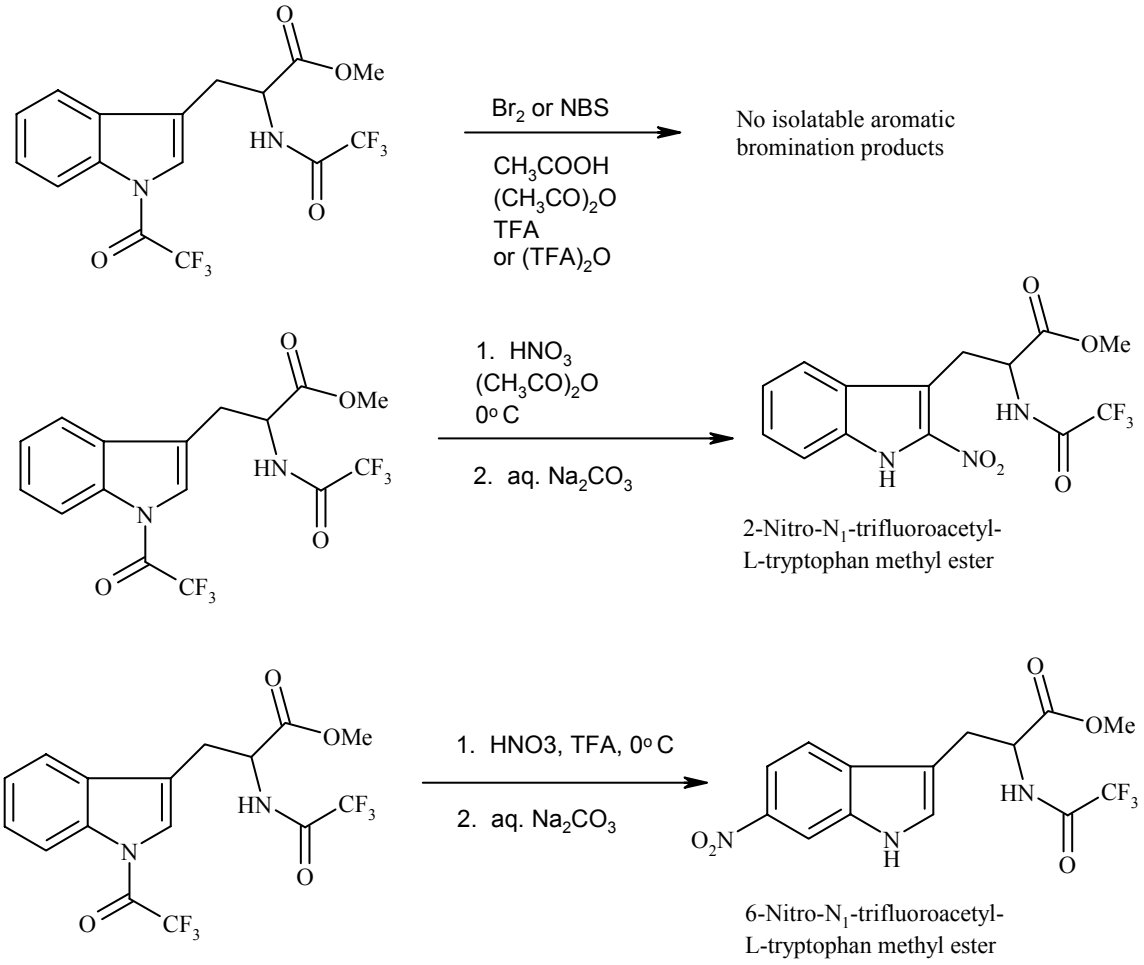
anhydride at room temperature for three hours to afford the product **(3)** in 44% overall yield. Bromination reactions of **(3)** with a variety of solvents and were attempted, but all reactions were unsuccessful. However, nitration of **(3)** occurred in high yield under a variety of conditions (Table 2.1), and a synthetic preference for either **(4)** or **(5)** was achieved by choice of solvents (Scheme 2.3).

Table 2.1. Nitration experiments for N_{α},N_1-(bistrifluoroacetyl)-L-tryptophan methyl ester

Experiment	Temp.	Solvent	Time	(3)	(4)	(5)	other
1	25°C	CH_3COOH	24 hours	58	0	0	42
2	25°C	$(CH_3CO)_2O$	15 min	40	33	15.4	11.6
3	25°C	TFA	15 min	0	11.8	66.8	21.4
4	25°C	$(TFA)_2O$	15 min	0	4	8	88
5	0°C	CH_3COOH	24 hours	45	2	3	50
6	0°C	$(CH_3CO)_2O$	1 hour	0	66.8	14.4	18.8
7	0°C	TFA	1 hour	0	4	68.8	27.2
8	0°C	$(TFA)_2O$	4 hours	9	7	10	74
9	60°C	$(CH_3CO)_2O$	4 hours	0	6	12	82
10	60°C	TFA	4 hours	0	0	10	90

Solvents were chosen for their ability to not only dissolve both reactants and products, but to also act as a Lewis acid in generating suitably electrophilic NO_2^+. Acetic acid, acetic anhydride, trifluoroacetic acid and trifluoroacetic anhydride were individually reacted with **(3)** in the presence of nitric acid at 0º C, 25º C, and 60º C. Nitration of **(3)** in trifluoroacetic acid produced the 6-nitro product **(5)** in over 66% yield at both 0º C and 25º C. Despite similar yields, the product afforded by the reaction at 0º C proved much easier to separate and purify. Nitration of **(3)** in acetic anhydride afforded the 2-nitro

derivative **(4)** in 68% yield (Figs. 2.1-2.17). Small scale reactions were worked up by diluting the reaction mixture in deionized water, and extracting into ethyl acetate, followed by GC-MS analysis.



Scheme 2.3. Substitution of N_{α},N_1-(bistrifluoroacetyl)-L-tryptophan methyl ester

This gentle method kept all protection groups largely intact. Workup in 5% sodium carbonate allowed for a higher yield, but with the cleavage of the N_1-trifluoroacetyl group. Multi-gram scale synthesis of **(4)** and **(5)** were achieved by quenching the reaction mixtures in 5% sodium carbonate and purification by recrystallization. No chromatography or additional purification efforts were required.

Discussion

Preparation of derivatives of L-tryptophan with aromatic substituents is an important endeavor as many such compounds have demonstrated great biological activity, have served as useful probes for examining the nature of enzyme catalysis, and have been a platform for studying the effect of substituents on aromatic systems. An extensive examination of nitro, bromo, and azido-L-tryptophan derivatives has not been performed due to difficulties in their synthesis. An efficient means of nitrating functionally protected L-tryptophan presents an attractive strategy not only for the preparation of nitro L-tryptophan derivatives, but to many other L-tryptophan derivatives as well. Reduction of aromatic nitro groups to amines is well established, as is the conversion of aromatic amines to a variety of functional groups, including bromo and azido groups by Sandmeyer methodology. A synthetic route that involves the protection of L-tryptophan in a manner that promotes the desired regioselective substitution of the nitro group as well as shielding the molecule from future degradation would be ideal.

Protection of L-tryptophan as the N_{α},N_1-(bistrifluoroacetyl) methyl ester has allowed for the exceptionally efficient, regioselective nitration of L-tryptophan at the 2 and 6 positions with solvent being the only variable. Furthermore, this transformation is accomplished without requiring chromatography or purification following recrystallization of the crude product cake. In both reactions, neutralization of the reaction mixture with 5% Na_2CO_3 resulted in the loss of the N_1-trifluoroacetyl group. This group can be retained by diluting the reaction product with water, followed by extraction into ethyl acetate, however, the bistrifluoroacetyl derivatives do not recrystallize efficiently, and the N_1-trifluoroacetyl groups cleave during flash chromatography with silica, resulting in further degradation of the product. If necessary, the N_1-trifluoroacetyl group may be reintroduced upon reaction with trifluoroacetic anhydride.

Experimental

Synthesis of L-Tryptophan methyl ester hydrochloride[22]

Methanol (200 ml) was placed in a 500 ml round bottom flask and cooled to -42° C in a dry ice-acetonitrile bath. Thionyl chloride (28 g, 235 mmol) was added dropwise via addition funnel and the reaction was purged with nitrogen. L-tryptophan (23.28 g, 114 mmol) was added quickly, and the reaction was again purged with nitrogen and mechanically stirred. The flask was removed from the dry ice-acetonitrile bath and allowed to warm to ambient temperature. After 4 hours total, the reaction was quenched 100 ml water. The product was extracted into ethyl acetate, dried over anhydrous Na_2SO_4, and evaporated to dryness. The resulting solid cake was recrystallized from methanol-water affording 19.6 g L-tryptophan methyl ester hydrochloride (77 mmol, 68%).

Synthesis of N_α,N_1-*bis*-(trifluoroacetyl)-L-tryptophan methyl ester[21]

L-Tryptophan methyl ester hydrochloride (1.2 g, 4 mmol) was added to 12 ml trifluoroacetic anhydride in a 50 ml round bottom flask. The solution was allowed to stir at ambient temperature for 90 min. Petroleum ether (20 ml) was added and the product crystallized as a white solid. The product was separated by vacuum filtration and dried *in vacuo* to afford 1.115 g N_α,N_1-*bis*(trifluoroacetyl)-L-tryptophan methyl ester (2.7 mmol, 67%). N_α,N_1-bis(trifluoroacetyl)-L-tryptophan methyl ester, (Figs. 2.18-2.22): ^{1}H NMR (400 MHz, acetone-d_6): δ 3.38 (1H, dd, $J_{\beta\beta'}$ = 14.9 Hz, $J_{\alpha\beta'}$ = 9.4 Hz, β'-H), 3.50 (1H, dd, $J_{\beta\beta'}$ = 14.9 Hz, $J_{\alpha\beta}$ = 4.70 Hz, β-C), 3.75 (1H, s, -OCH_3), 4.95 (1H, dd, J_α = 9.4 Hz, $J_{\alpha\beta}$ = 4.7 Hz, α-H), 7.44 (1H, t, J = 7.1 Hz, C5-H), 7.49 (1H, t, J = 7.49 Hz, C6-H), 7.67 (1H, d, J = 7.0 Hz, C2-H), 7.77 (1H, d, J = 7.0 Hz, C4-H), 8.37 (1H, d, J = 7.1 Hz, C7-H), 8.95 (1H, s, NH); ^{13}C NMR (100 MHz, acetone-d_6): δ 30.2 (β-C), 56.4 (α-C), 56.3 (OCH_3), 119.8 (CF_3, q, J = 287.2 Hz, 120.2), (CF_3, q, J = 287.1 Hz), 120.8 (C-7), 123.7 (C-4), 125.3 (C-3'), 126.8 (C-2), 129.8 (C-6), 130.6 (C-5), 134.7 (C-7'), 140.3 (C-3), 158.0 (amide C=O, q, J_{NF} = 39.8 Hz), 161.2 (indole amide C=O, q, J_{NF} = 37.4 Hz), 174.3 (-

COO-), GC-MS, m/z 359 (M+1 calcd for $C_{16}H_{12}F_6N_2O_4$, 359.26). Calcd for $C_{16}H_{12}F_6N_2O_4$: C, 46.84%; H, 2.95%; N, 6.83%; found: C, 46.93%; H, 2.97%; N, 6.80%.

General procedure for nitration of N_α,N_1-*bis*(trifluoroacetyl)-L-tryptophan methyl ester

N_α,N_1-*bis*(trifluoroacetyl)-L-tryptophan methyl ester, 100 mg, was placed in a 5 ml conical vial containing 1.0 ml acetic anhydride at 0º C and allowed to stir for 10 minutes. Acetic anhydride containing concentrated nitric acid (200 µl in 1.0 ml) was added dropwise over five minutes. The reaction was followed by thin layer chromatography and subsequently quenched in excess 5% Na_2CO_3 upon disappearance of starting material or emergence of side products. The aqueous layer was extracted in ethyl acetate (4 x 20 ml). The combined organic extracts were dried over Na_2SO_4 and the solvent removed by rotary evaporation. The products were separated and purified by flash chromatography (2.5 cm x 30 cm silica column, 20% ethyl acetate in hexanes). 2-Nitro-N_1-trifluoroacetyl-L-tryptophan methyl ester was isolated as the fastest moving compound by normal phase TLC (20% ethyl acetate in hexanes), with an $R_f = 0.85$. The fourth fastest moving compound was isolated and characterized as 6-nitro-N_1-trifluoroacetyl-L-tryptophan methyl ester, with an $R_f = 0.55$. Reactions involving other electrophile/ solvent/ temperature combinations were followed as described above and reactions were quenched according to TLC results, and purified likewise.

2-Nitro-N-trifluoroacetyl-L-tryptophan methyl ester, (Figs. 2.23-2.28) ^{1}H NMR (400 MHz, acetone-d_6): δ 3.70 (1H, dd, $J_{\beta\beta'} = 21.9$, $J_{\alpha\beta} = 12.2$ Hz, β-H), 3.72 (1H, s, OCH_3), 3.92 (1H, dd, $J_{\beta\beta'} = 21.9$, $J_{\alpha\beta'} = 9.4$ Hz, β'-H), 5.00 (1H, dd, J = 9.4, 12.2 Hz, α-H), 7.22 (1H, t, J = 8.1 Hz, C6-H), 7.46 (1H, t, J = 7.8 Hz, C5-H), 7.52 (1H, dd, $J_{45} = 8.4$, $J_{46} = 0.9$ Hz, C4-H), 7.83 (1H, d, J = 8.6 Hz, C7-H), 8.83 (1H, br d, NH), 11.74 (1H, br s, indole NH), ^{13}C NMR (100 Mhz, acetone- d_6): δ 26.4 (β-C), 52.3 (-OCH_3), 52.5 (α-C), 113.1 (4-C), 114.1 (3-C), 116.0 (-CF3, q, J= 288 Hz), 121.8 (7-C), 121.9 (6-C), 127.1 (C-3'), 128.4 (5-C), 134.5 (7'-C), 138.7 (2-C), 156.8 (q, $J_{CF} = 36.1$ Hz, amide C=O), 170.1 (-

COO-); GC-MS, m/z 359 (M+1 calcd for $C_{16}H_{12}F_6N_2O_4$, 359.26). Calcd for $C_{16}H_{12}F_6N_2O_4$: C, 46.61%; H, 3.37%; N, 11.70%; found: C, 46.92%; H, 3.37%; N, 11.56%. 6-Nitro-N_α-trifluoroacetyl-L-tryptophan methyl ester; (Figs. 29-34) ^{1}H NMR (400 Mhz, acetone- d_6): δ 3.38 (1H, dd, $J_{\beta\beta'}$ = 14.9, $J_{\alpha\beta}$ = 8.6 Hz, β-H), 3.52 (1H, dd, $J_{\beta\beta'}$ = 14.9, $J_{\alpha\beta}$' = 5.5 Hz, β-H), 3.73 (3H, S, -OCH3), 4.95 (1H, dd, J = 8.6, 5.5 Hz, α-H), 7.78 (1H, d, J=8.6 Hz, C4-H), 7.94 (1H, dd, J_{45} = 8.6, J_{56} = 1.6 Hz , C5-H), 7.68 (1H, d, J = 2.35 Hz, C2-H), 8.38 (1H, d, J = 2.3 Hz, C7-H), 8.75 (1H, br d, amide NH), 10.87 (1H, br s, indole N-H); ^{13}C NMR (100 Mhz, acetone- d_6): δ 26.88 (β-C), 52.38 (O-CH_3), 53.97 (α-C), 108.6 (C-7), 111.2 (C-3), 114.3 (C-5), 114.7 (q, J_{CF} = 287 Hz, CF_3), 118.6 (C-2), 130.6 (C-4), 132.3 (C-3'), 135.12 (C-6), 143.0 (C7'), 156.9 (q, J_{CF} = 36.8 Hz, amide C=O), 170.5 (-COO-); GC-MS, m/z 359 (M+1 calcd for $C_{16}H_{12}F_6N_2O_4$, 359.26). Calcd for $C_{16}H_{12}F_6N_2O_4$: C, 46.61%; H, 3.37%; N, 11.70%; found: C, 46.43%; H, 3.49%; N, 11.52%.

General procedure for the preparative scale synthesis of 6-nitro-N_α-bistrifluoroacetyl-L-tryptophan methyl ester

N_α,N_1-*bis*-(trifluoroacetyl)-L-tryptophan methyl ester (2.0 g, 4.84 mmol) was placed in a 50 ml round bottomed flask with 10 ml of acetic anhydride. The reaction mixture was cooled to -2º C. A solution of nitric acid in acetic anhydride (3 ml in 10 ml) was added drop-wise and the reaction was allowed to stir. After 30 minutes, the last traces of starting material dissolved and after 45 minutes, the last traces of starting material disappeared as observed by TLC, at which time the reaction was quenched by pouring it over 20 ml ice-cold 5% sodium carbonate. Additional sodium carbonate was added until the pH of the mixture was 7.5. A bright yellow precipitate was collected by suction filtration and the resulting cake was washed with cold water. The product was then dissolved in minimal ethyl acetate and a small volume of hexanes was added until the solution first indicated opacity. The mixture was then allowed to sit overnight at 4º C where a bright yellow crystalline precipitate was observed. This product was collected

by suction filtration, washed with a small volume of hexanes and dried *in vacuo.* GC-MS of the reaction product showed a single GC trace and the MS was consistent with the mass spectrum of pure 6-nitro-N_{α}-trifluoroacetyl-L-tryptophan methyl ester. The supernatant was concentrated by rotary evaporation and the residue was recrystallized in ethyl acetate-hexanes to afford an additional 195 mg of the 6-nitro product. The total yield of 6-nitro-N_{α}-trifluoroacetyl-L-tryptophan methyl ester was 1.18 grams (3.29 mmol, 68%).

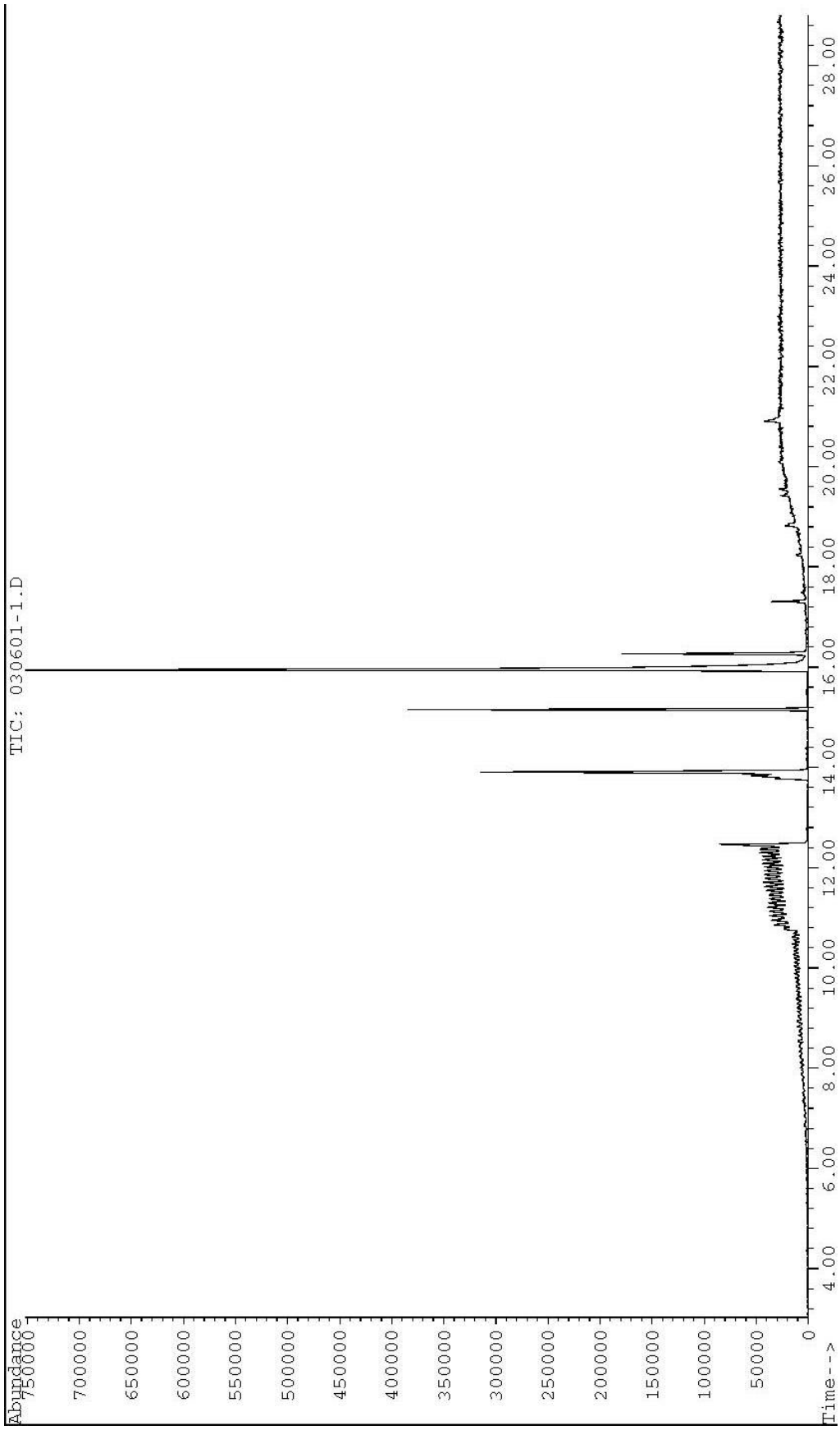
Preparative scale synthesis of 2-Nitro-N_{α}-trifluoroacetyl-L-tryptophan methyl ester

The preparative scale synthesis of 2-Nitro-N_{α}-trifluoroacetyl-L-tryptophan methyl ester was similar to the preparation of 6-Nitro-N_{α}-trifluoroacetyl-L-tryptophan methyl ester except for the substitution of trifluoroacetic acid in the place of acetic anhydride. Additionally, overnight crystallization resulted in the precipitation of the entire yield of the product, which was recrystallized in ethyl acetate-hexanes to afford 1.20 grams (3.30 mmol, 68%) product.

References

1. Manavalan, P., Prabhakaran, M., Johnson, M.E., *J. Mol. Biol*., **223**, 71-81 (1992)
2. Kornfeld, E.C., Sheneman, J.M., Suarez, T., *Chem. Abstr*., **72**, 30438c(1970)
3. Rydon, H.N., Tweddle, J.C., *J. Chem. Soc*., 3499 (1955)
4. Moriya, T., Hagio, K., Yoneda, N., *Bull. Chem. Soc. Japan*, **48**, 2217-2218 (1975)
5. Lee, M., Phillips, R.S., *J. Hetrocyclic Chem*., **31**, 711 (1994)
6. Melhado, L.L., Leonard, N.J., *J. Org. Chem*., **48**, 5130-5133 (1983)
7. Phillips, R.S., Miles, E.W., *Biochem.*, **24**, 4694-4707 (1985)
8. Brock, P.W., Myers, R., Baker, D.C., Hardman, J.K., *Arch. Biochem. Biophys*., **220**, 435-442 (1983)
9. Lee, M., Phillips, R.S., *J. Heterocyclic Chem*., **29**, 1181-1187 (1992)
10. Lee, M, Phillips, R.S., *Mag. Res. Chem*., **30**, 1135-1040 (1992)
11. Phillips, R.S., Cohen, L.A., *Tetrahedron Lett*., **24**, 5555-5558 (1983)
12. Phillips, R.S., Cohen, L.A., *J. Am. Chem. Soc*., **108**, 2024-2030 (1986)
13. Nagarathnam, D., Johnson, M.E., *Synth. Commun.,* **23**, 2011-2017 (1993)
14. Allen, M.C., Brundish, D.E., Wade, R., *J.Chem. Soc. Perkin I*, 2025-2030 (1982)
15. Phillips, R.S., Cohen, L.A., *J. Heterocyclic Chem*., **25**, 191-192 (1988)
16. Hino, T., Taniguchi, M., Gonsho, A., Nakagawa, M., *Heterocycles*, **12**, 1027-1030 (1979)
17. Hino, T., Tohru, T., *Tetrahedron*, **37**, 1487-1494 (1981)
18. Phillips, R.S., Marmorstein, R.Q., *Arch. Biochem. Biophys.,* **262**, 337-344 (1988)
19. De Fazi, R., Berti, G., Da Settimo, A., *Gazz. Chim. Ital*., **89**, 2238-2257 (1959)
20. Ohno, M., Spande, T.F., Witkop, B., *J. Am. Chem. Soc*., **90**, 6521-6522 (1968); ibid., **92**, 343-348 (1970)
21. Makisumi, S., Saroff, H.A., *J. of G.C*.,**1**, 23 (1965)
22. Benson, S.C., Lee, L., Yang, L., Snyder, J.K., *Tetrahedron*, **56**, 1165-1180 (2000)

Figure 2.1. GC-MS of Experiment 1 product mixture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Abundance
Time-->
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700000
650000
600000
550000
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00

Figure 2.2. GC-MS of Experiment 2 product mixture

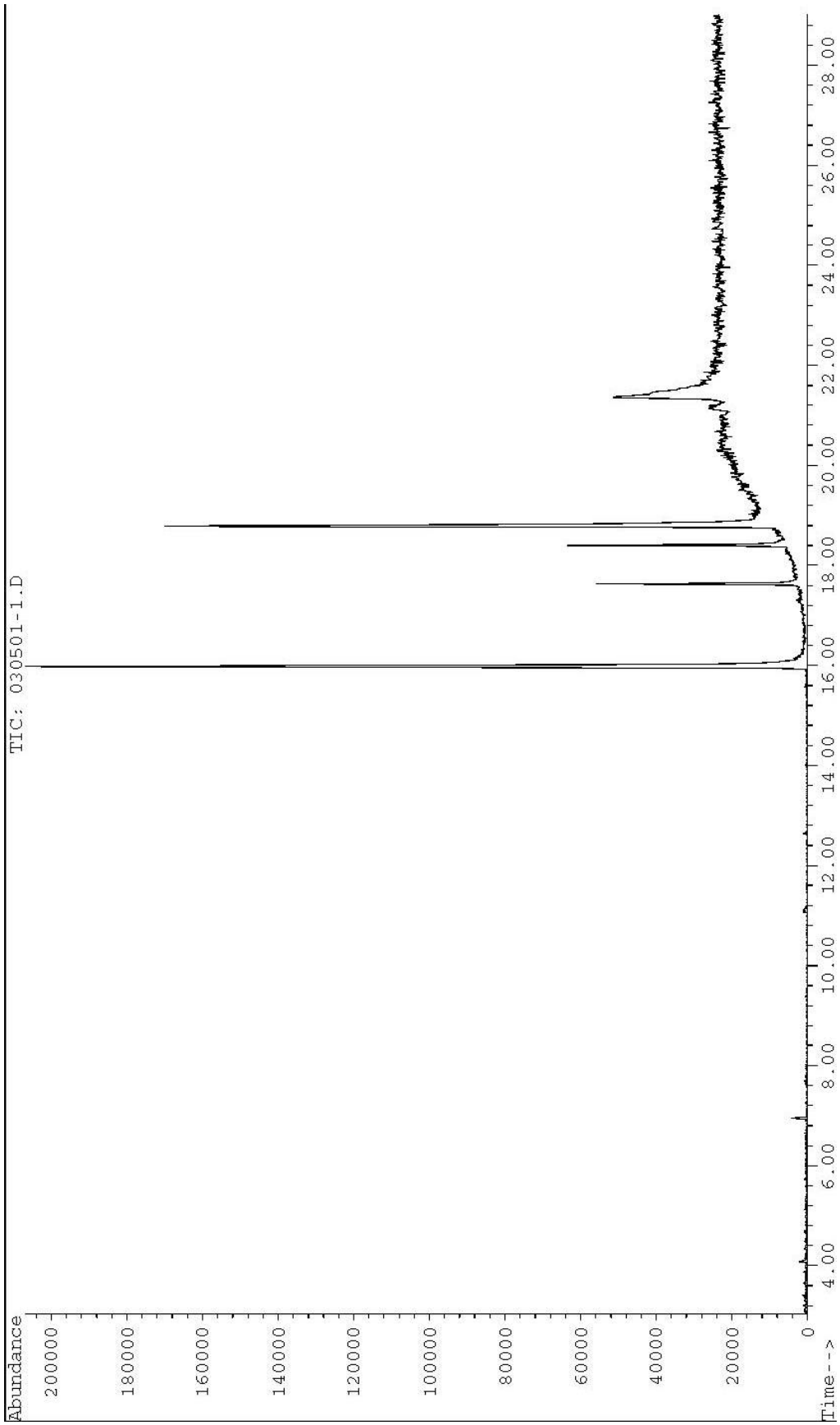
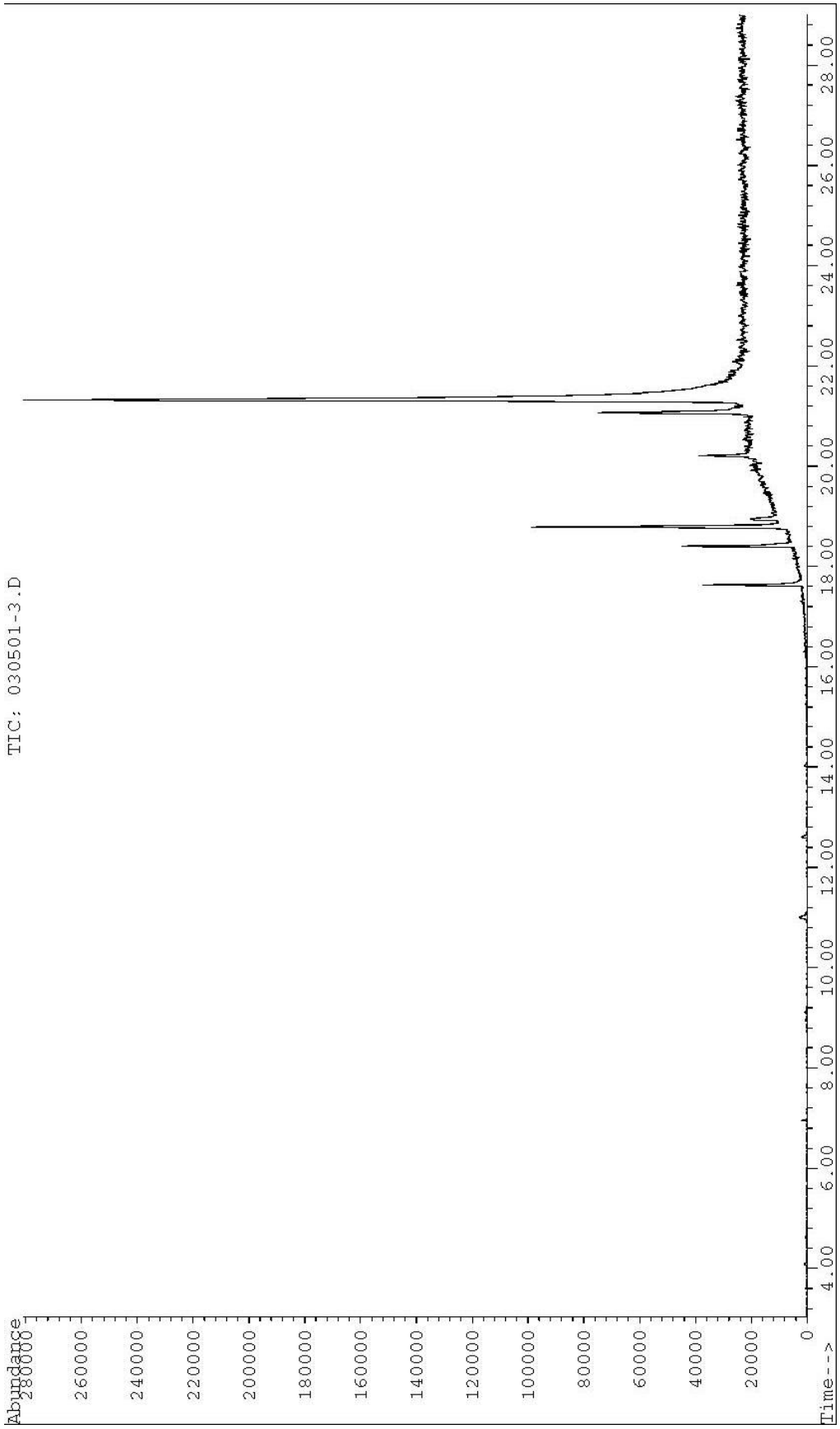

TIC: 030501-1.D
Abundance
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
Time-->
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00

Figure 2.3. GC-MS of Experiment 3 product mixture


TIC: 030501-3.D
Abundance
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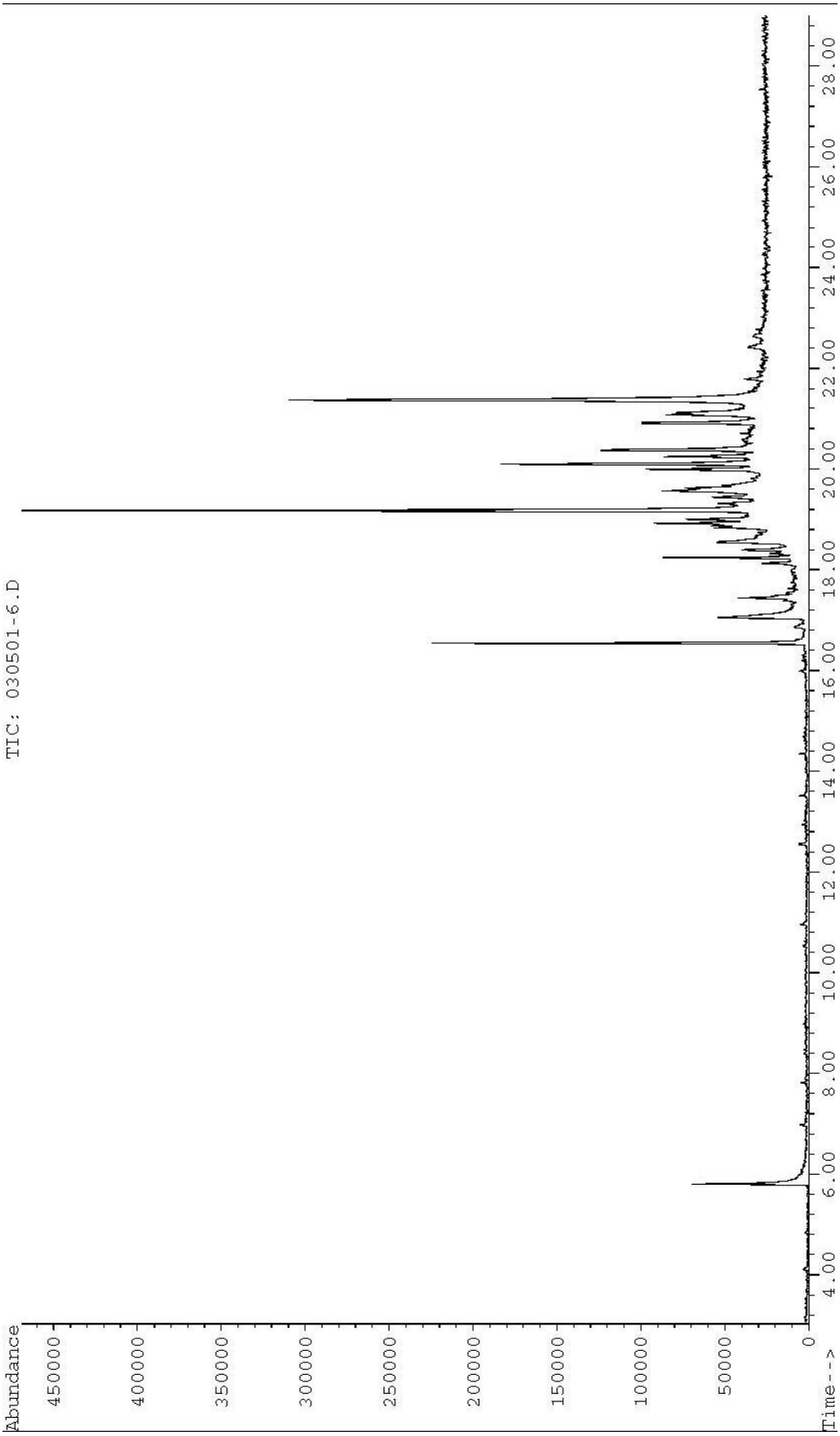
260000
240000
220000
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
Time-->
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00

Figure 2.4. GC-MS of Experiment 4 product mixture


TIC: 030501-6.D
Abundance
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400000
350000
300000
250000
200000
150000
100000
50000
0
Time-->
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00

Figure 2.5. GC-MS of Experiment 5 product mixture

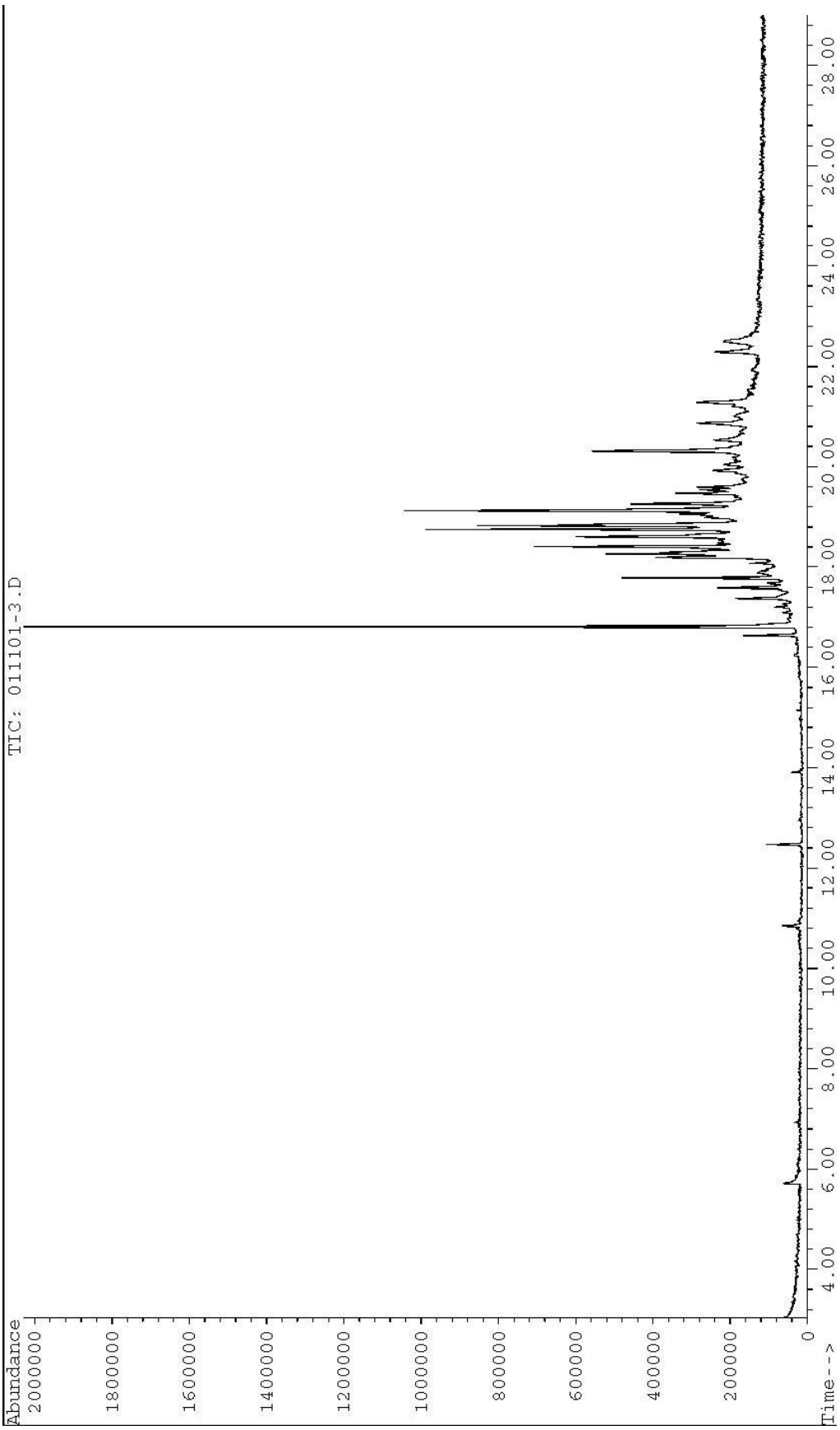

TIC: 011101-3.D
Abundance
2000000
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
Time-->
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00

Figure 2.6. GC of Experiment 6 product mixture, MS of first elution peak

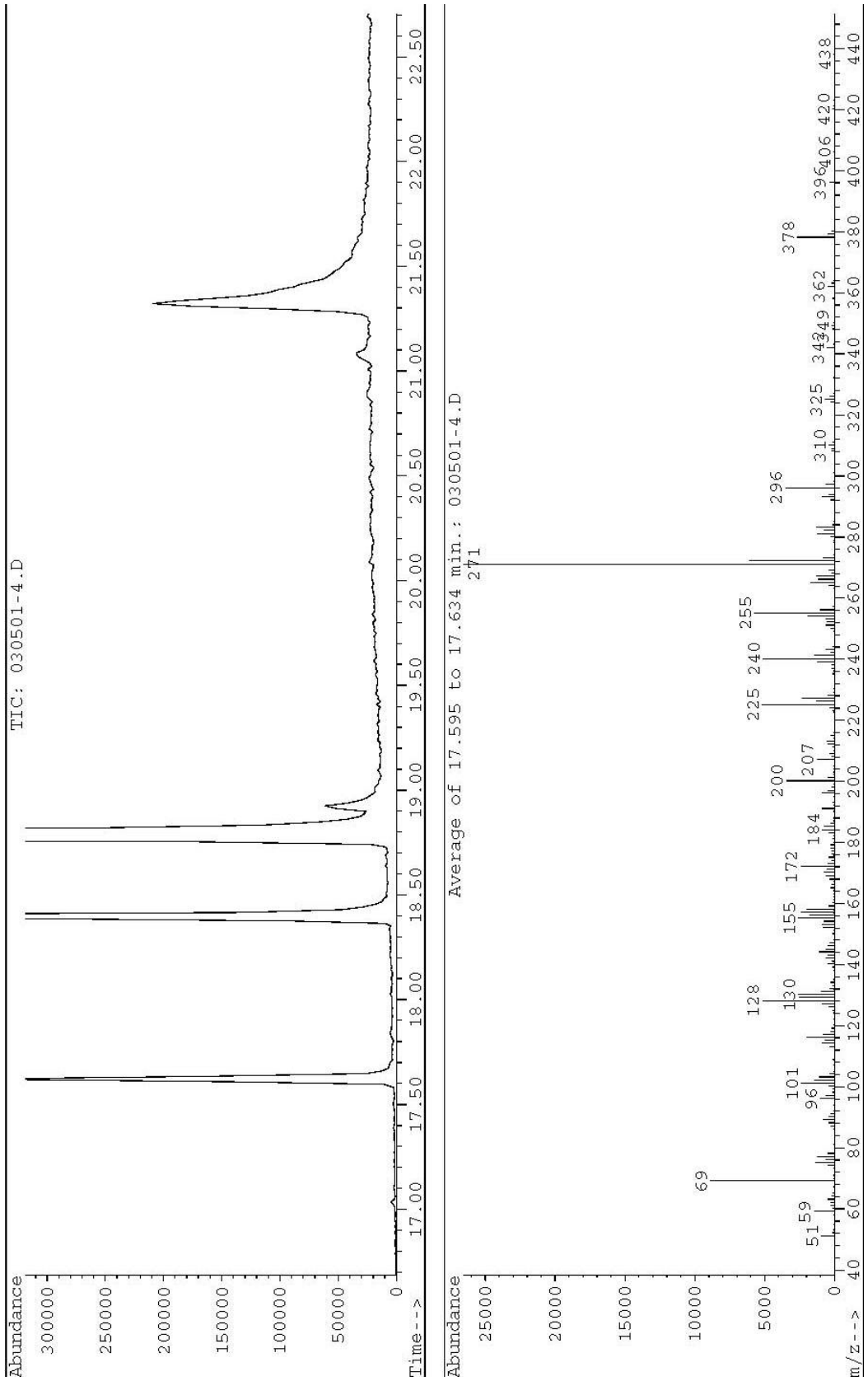

TIC: 030501-4.D
Abundance
Time-->
Average of 17.595 to 17.634 min.: 030501-4.D
Abundance
m/z-->

Figure 2.7. GC of Experiment 6 product mixture, MS of second elution peak

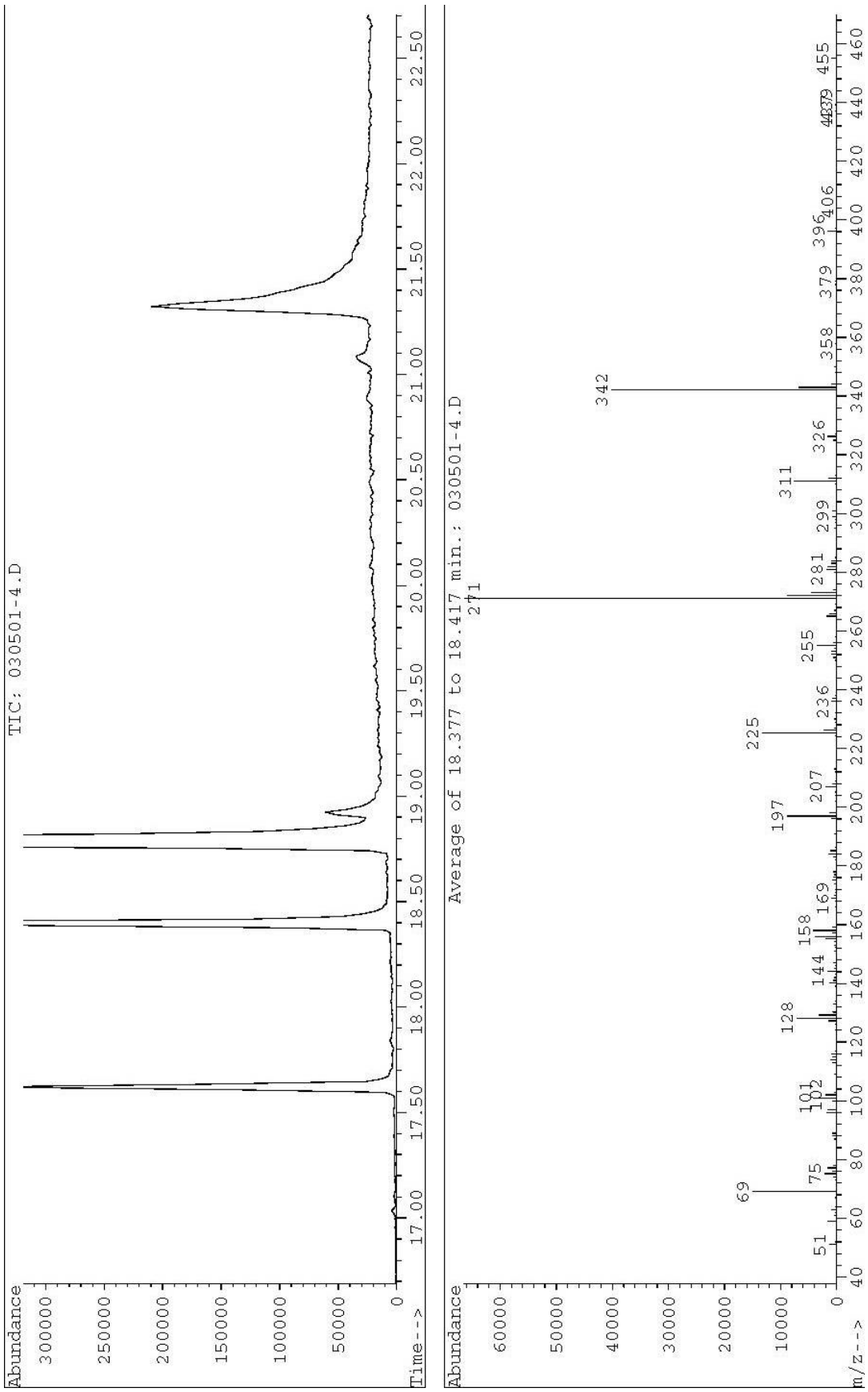

TIC: 030501-4.D
Abundance
Time-->
Average of 18.377 to 18.417 min.: 030501-4.D
Abundance
m/z-->

Figure 2.8. GC of Experiment 6 product mixture, MS of third elution peak

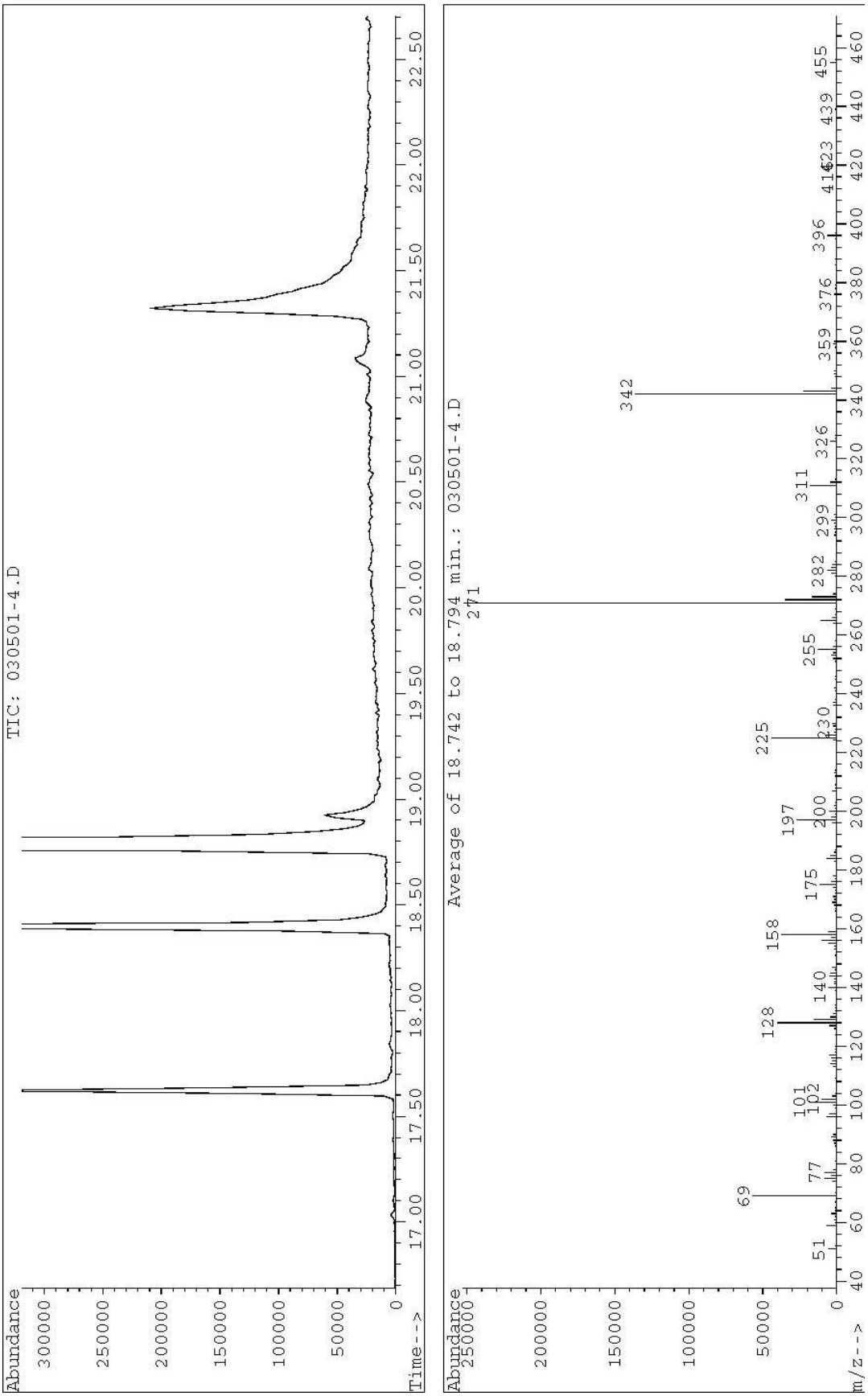

TIC: 030501-4.D
Abundance
Time-->
Average of 18.742 to 18.794 min.: 030501-4.D
Abundance
m/z-->

Figure 2.9. GC of Experiment 6 product mixture, MS of fourth elution peak

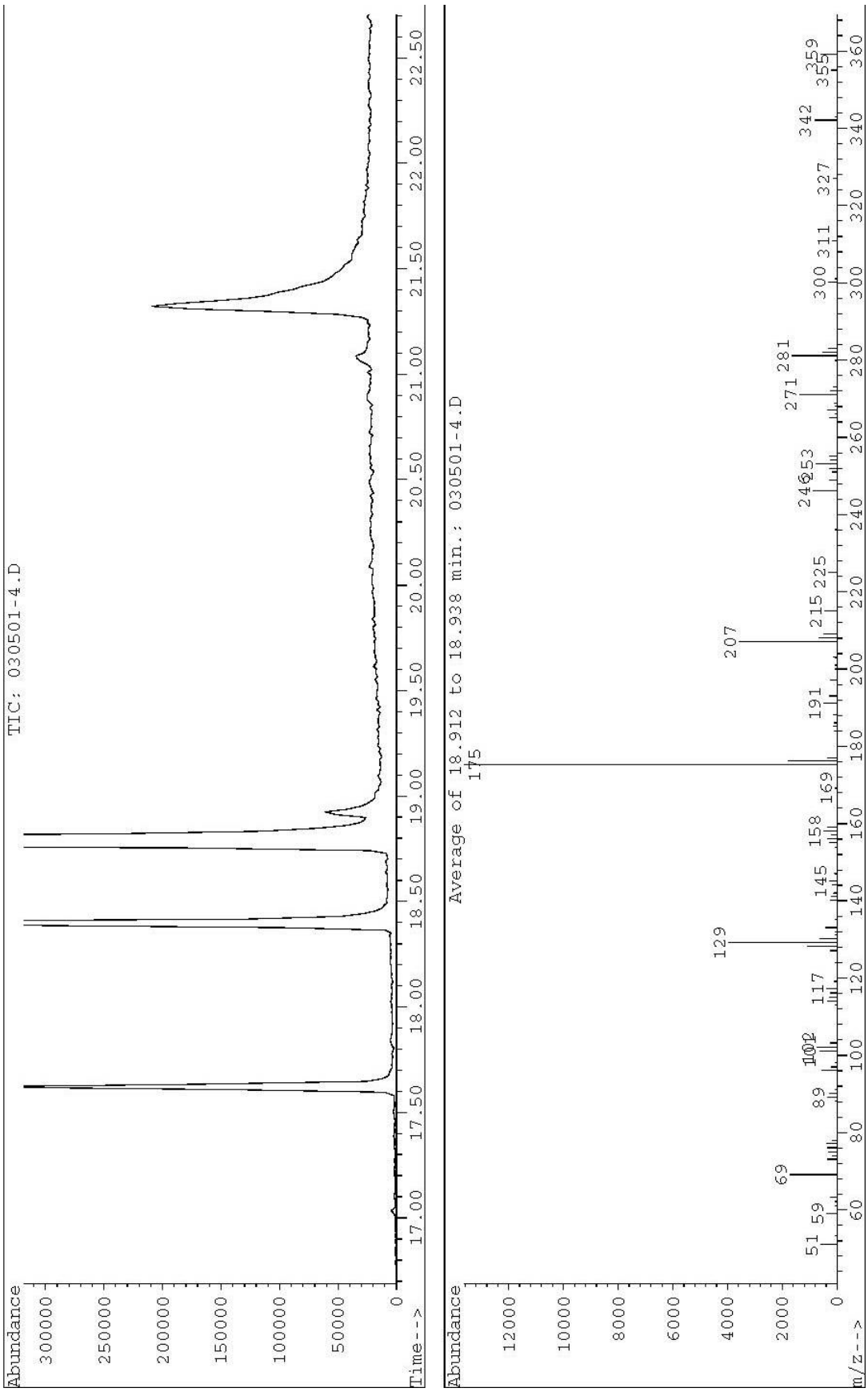

TIC: 030501-4.D
Abundance
Time-->
Average of 18.912 to 18.938 min.: 030501-4.D
Abundance
m/z-->

Figure 2.10. GC of Experiment 6, MS of fifth elution peak

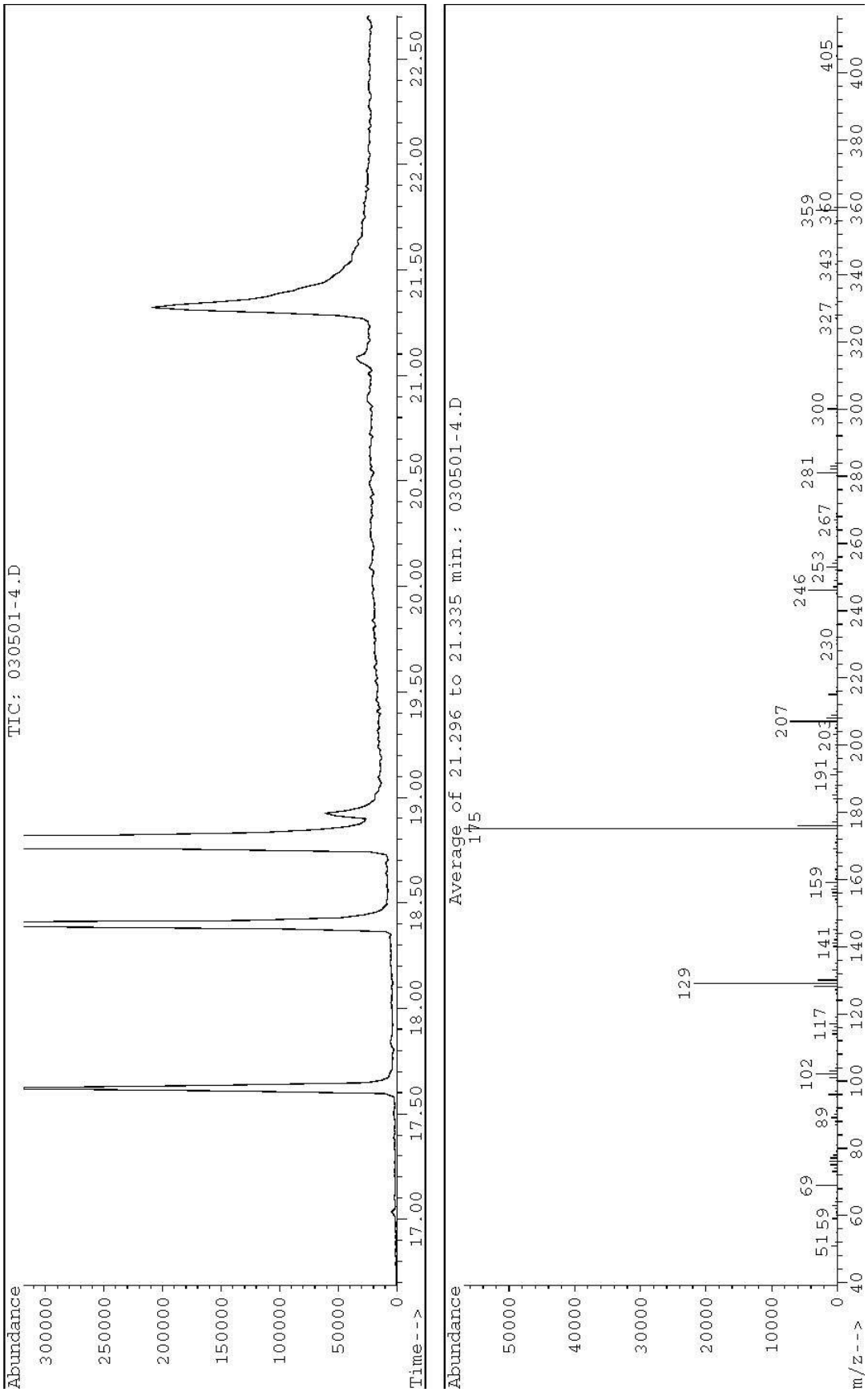

TIC: 030501-4.D
Abundance
Time-->
Average of 21.296 to 21.335 min.: 030501-4.D
Abundance
m/z-->

Figure 2.11. GC of Experiment 7, MS of first elution peak

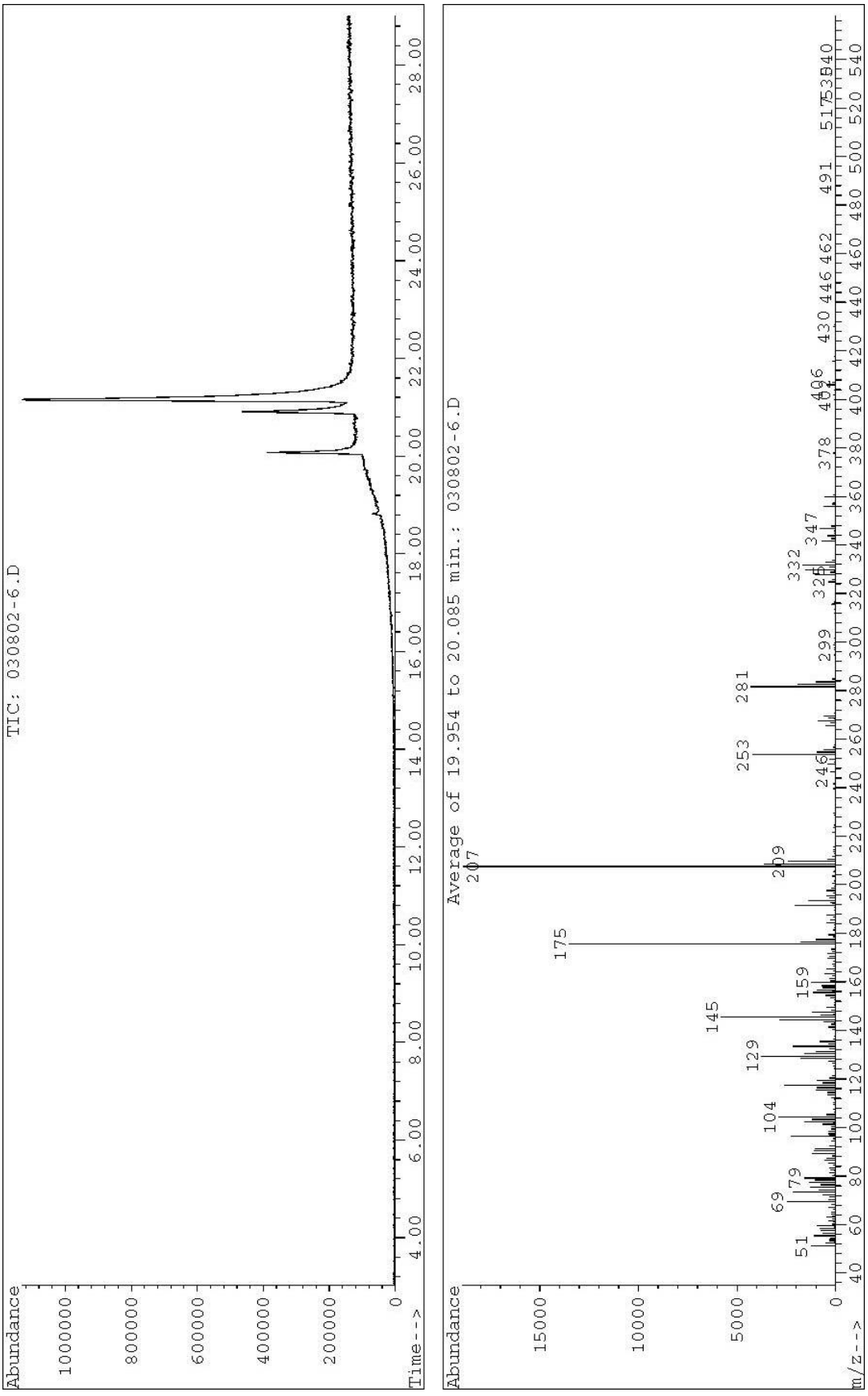

TIC: 030802-6.D
Abundance
Time-->
Average of 19.954 to 20.085 min.: 030802-6.D
Abundance
m/z-->

Figure 2.12. GC of Experiment 7, MS of second elution peak

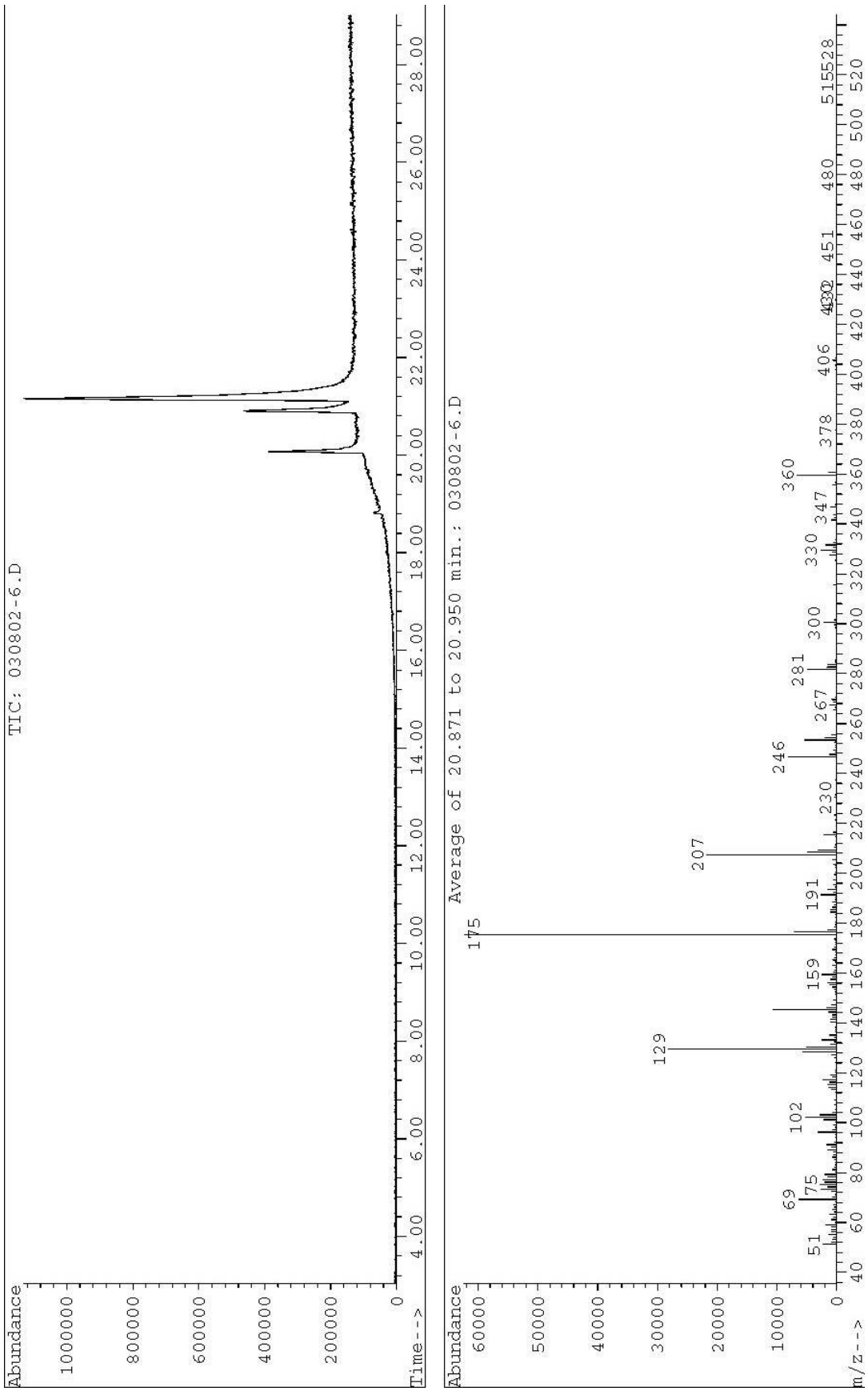

TIC: 030802-6.D
Abundance
Time-->
Average of 20.871 to 20.950 min.: 030802-6.D
Abundance
m/z-->

Figure 2.13. GC of Experiment 7, MS of third elution peak

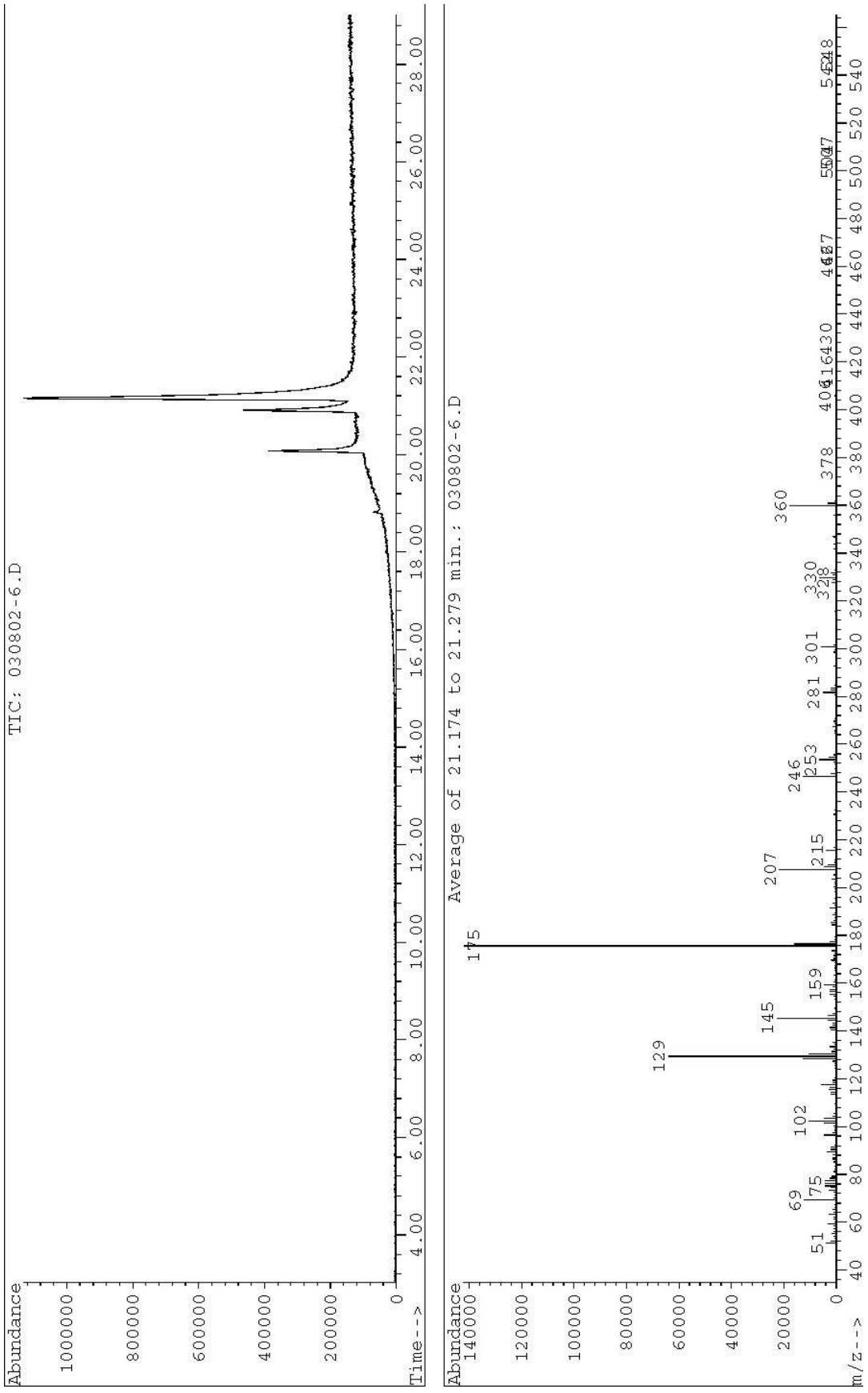

TIC: 030802-6.D
Abundance
Time-->
Average of 21.174 to 21.279 min.: 030802-6.D
Abundance
m/z-->

Figure 2.14. GS-MS of Experiment 8 product mixture

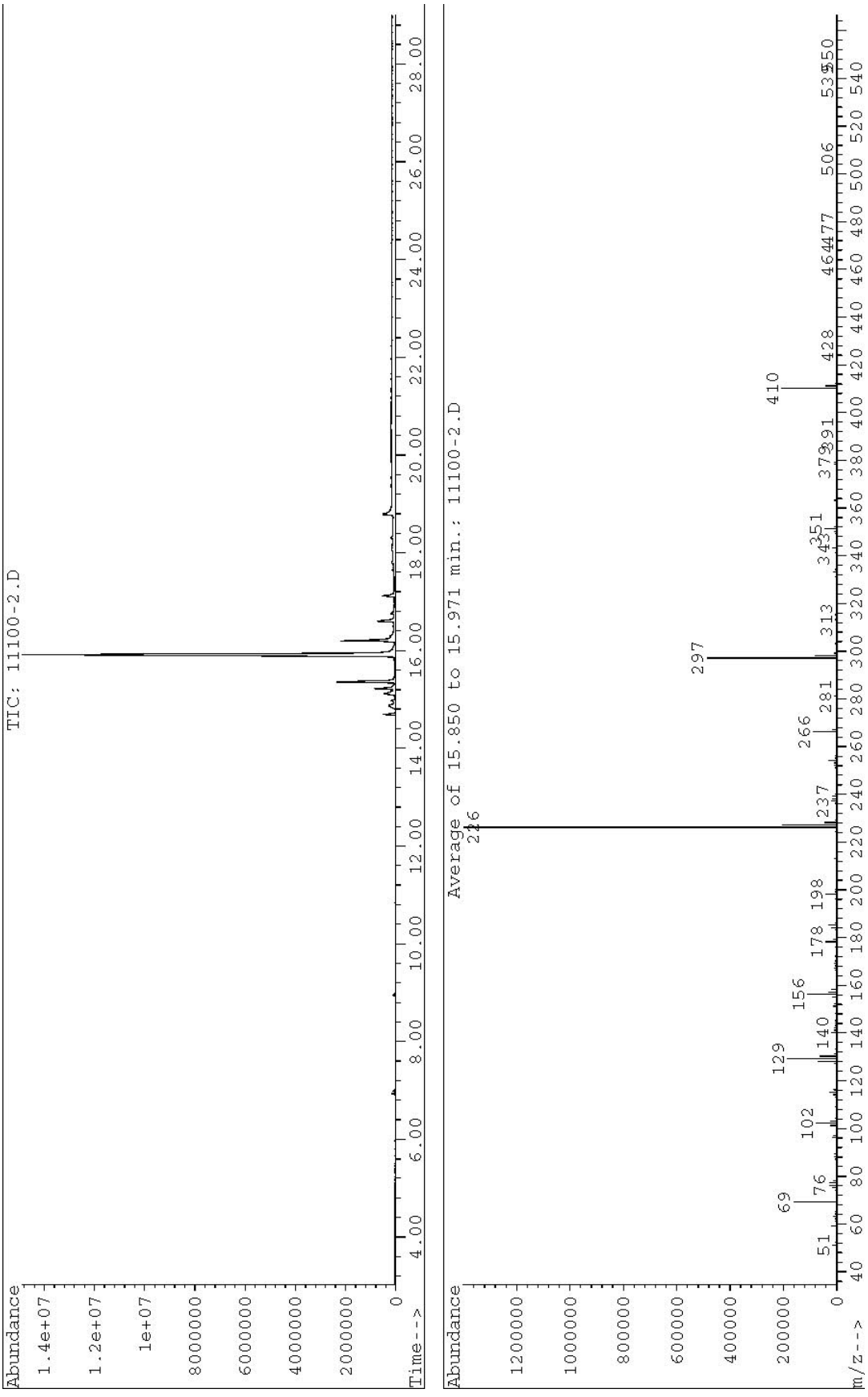
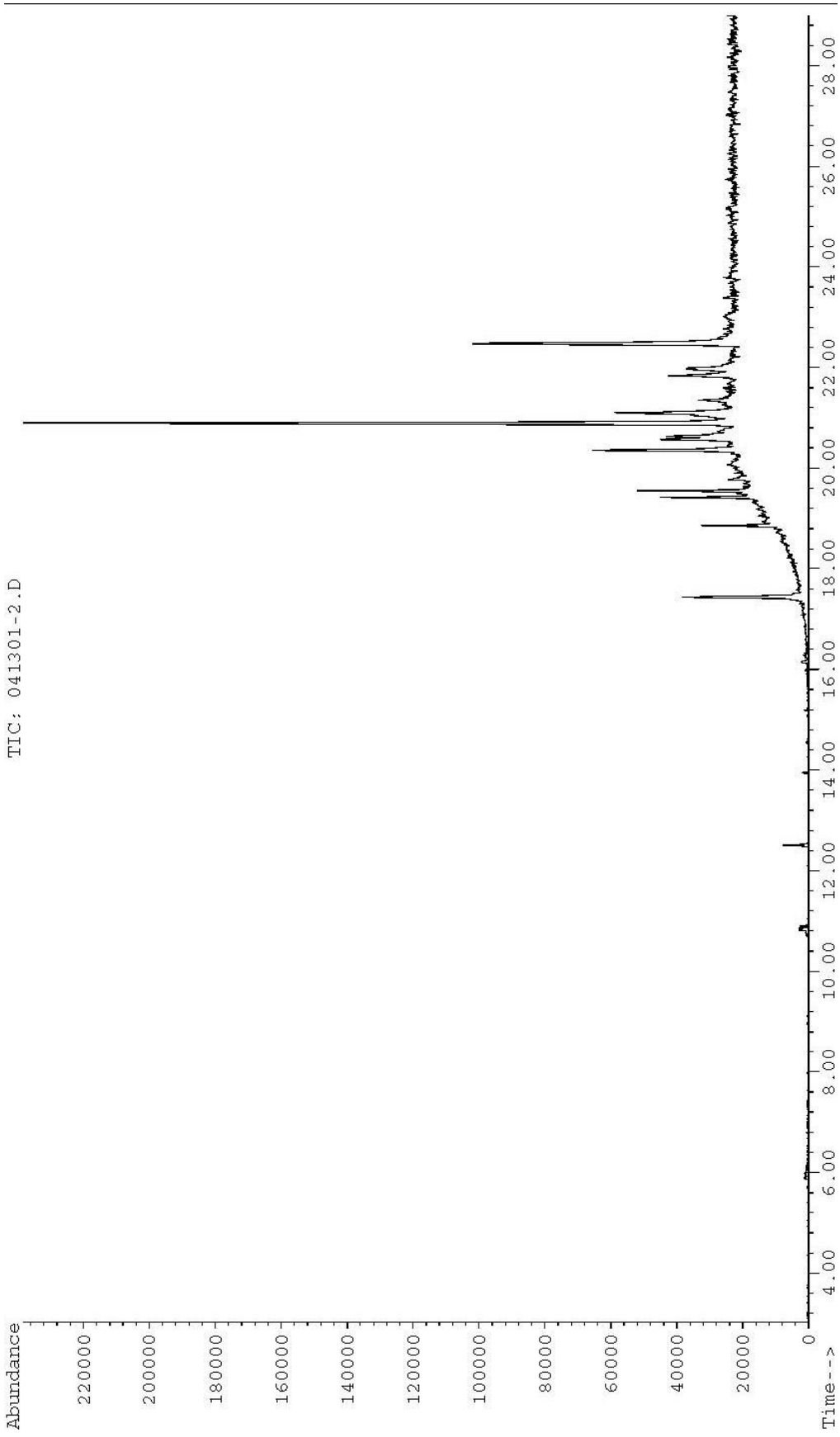

TIC: 11100-2.D
Abundance
Time-->
Average of 15.850 to 15.971 min.: 11100-2.D
Abundance
m/z-->

Figure 2.15. GC-MS of Experiment 9 product mixture


TIC: 041301-2.D
Abundance
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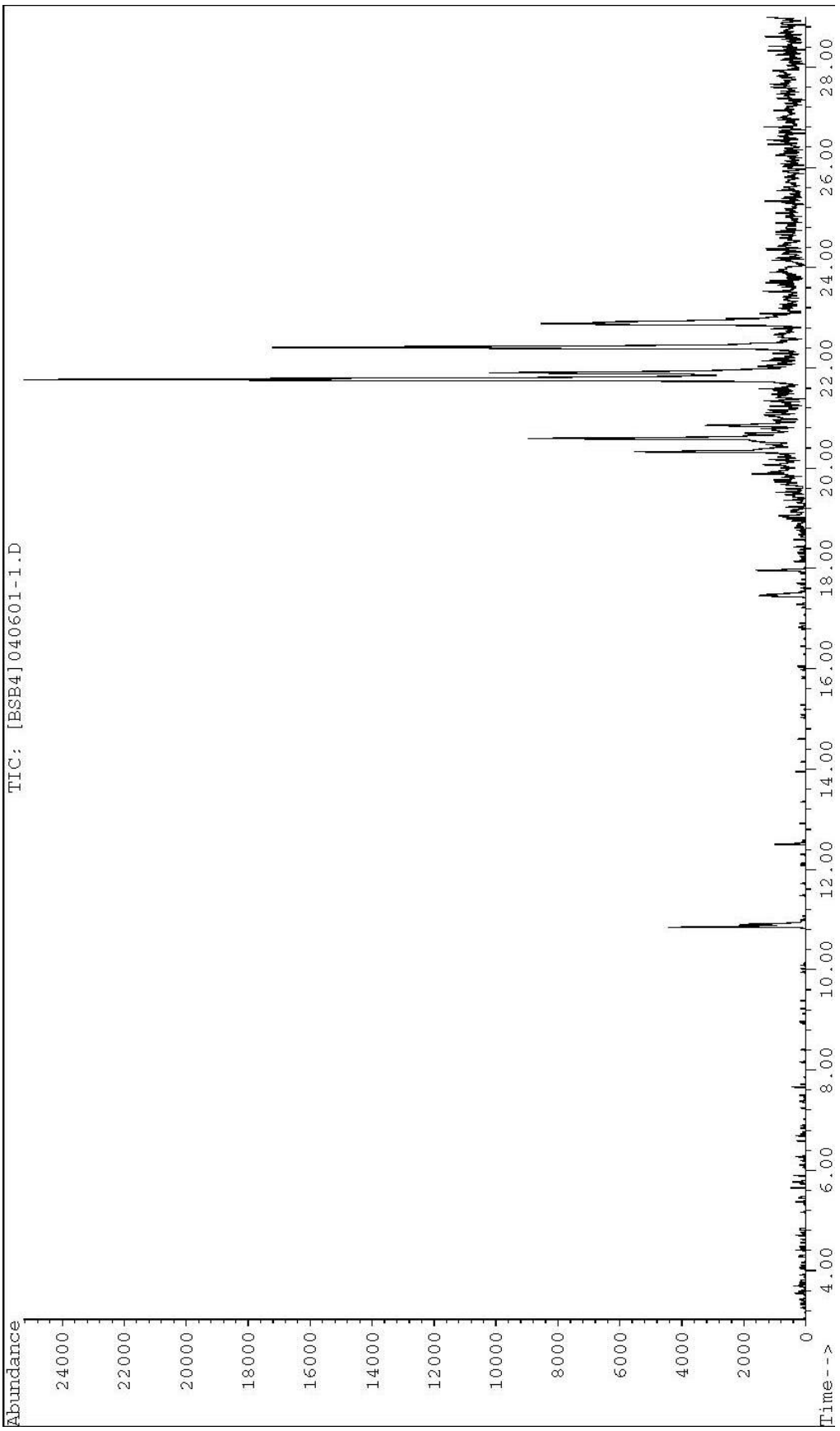
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
Time-->
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00

Figure 2.16. GC-MS of Experiment 10 product mixture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Time-->
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18000
16000
14000
12000
10000
8000
6000
4000
2000
0
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00

Figure 2.17. MS of N_{α},N_{1}-bis-trifluoroacetyl-L-tryptophan methyl ester

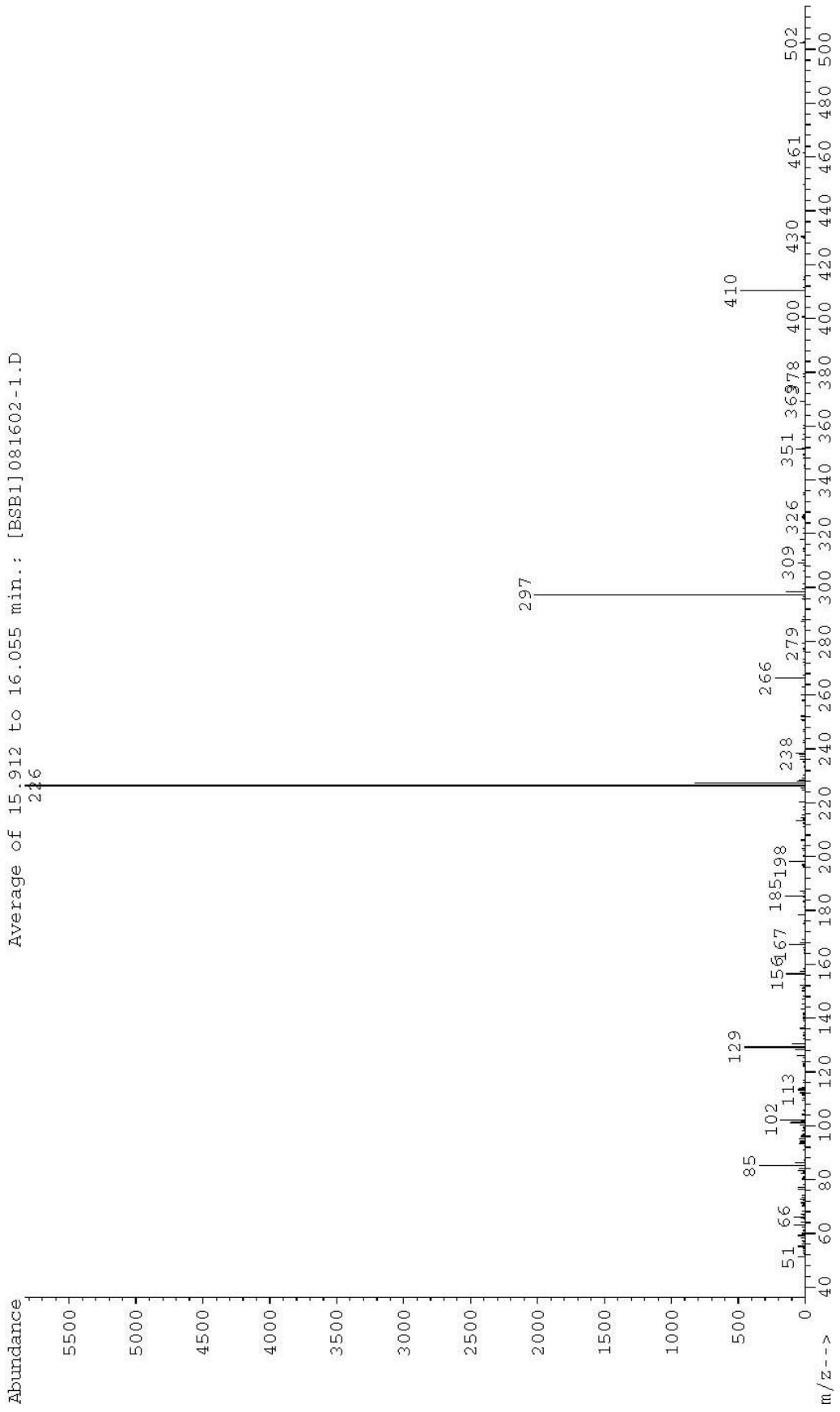

Average of 15.912 to 16.055 min.: [BSB1]081602-1.D
Abundance
m/z-->
226
297
410
129
85
51
66
102
113
156
167
185
198
238
266
279
309
326
351
369
378
430
461
502

Figure 2.18. ^{1}H-NMR spectrum of N_{α},N_{1}-bis-trifluoroacetyl-L-tryptophan methyl ester

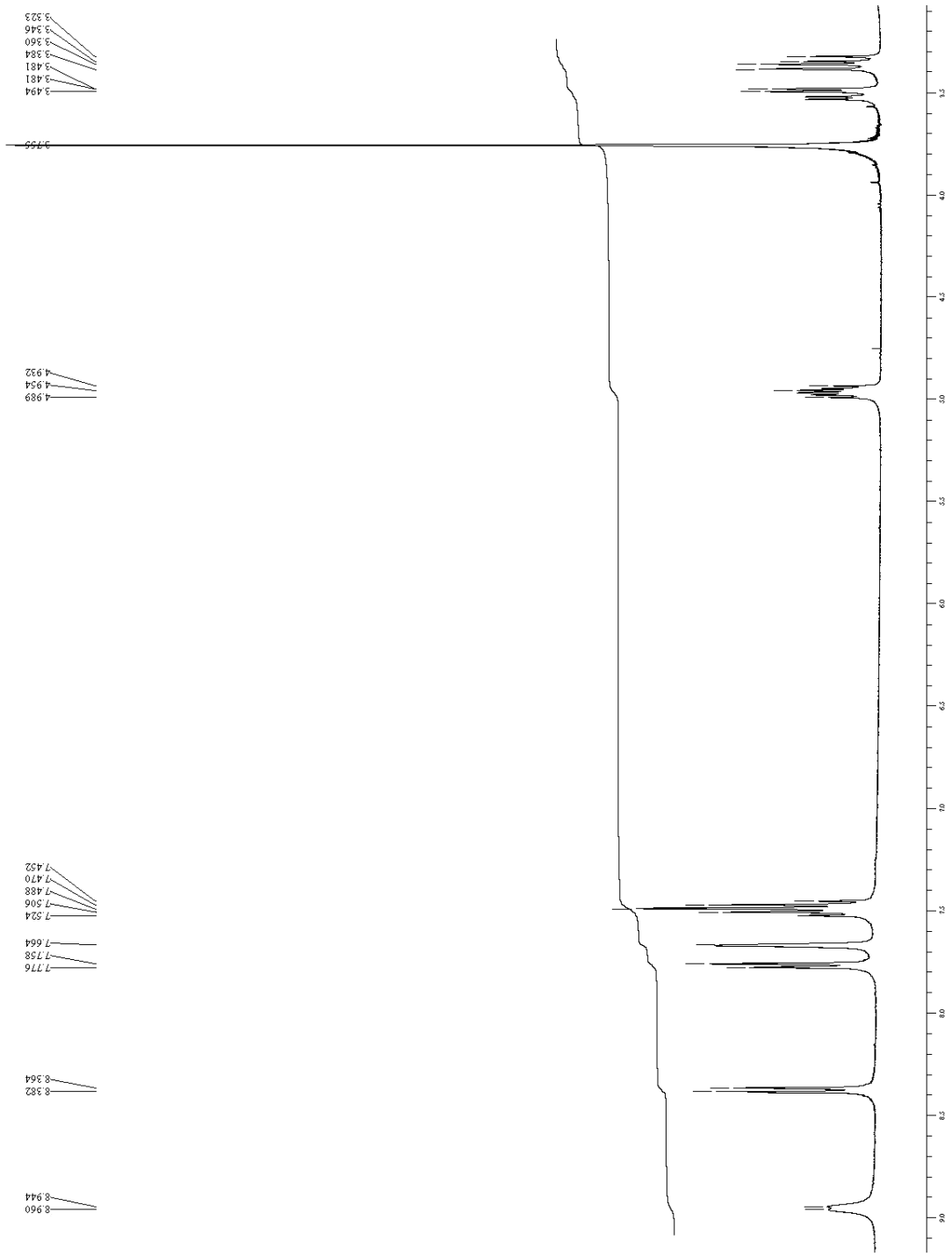

3.323
3.346
3.360
3.384
3.481
3.481
3.494
3.755
4.932
4.954
4.989
7.452
7.470
7.488
7.506
7.524
7.664
7.758
7.776
8.364
8.382
8.944
8.960

Figure 2.19. ^{13}C-NMR spectrum of N_{α},N_1-bis-trifluoroacetyl-L-tryptophan methyl ester

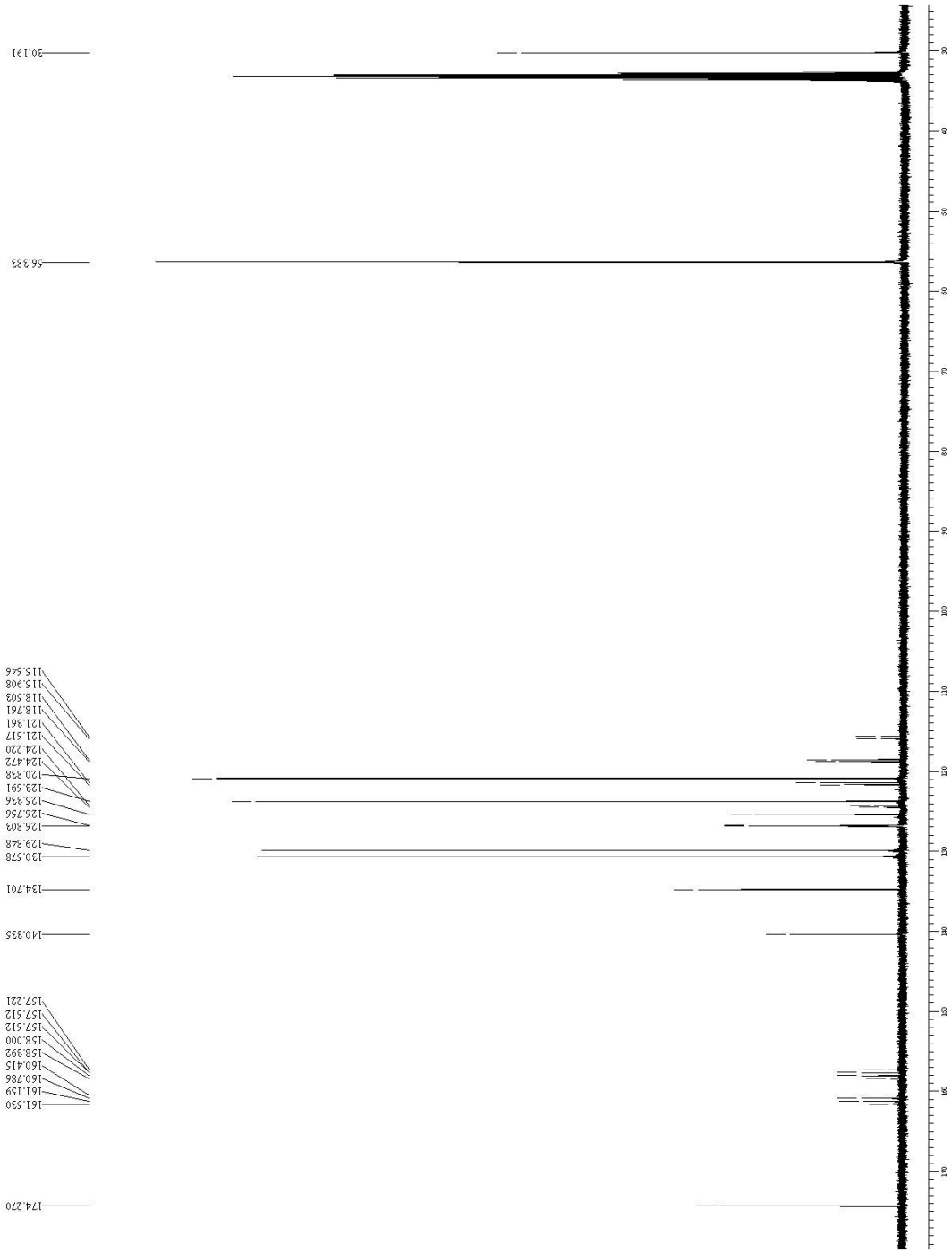

30.191
56.383
115.646
115.908
118.303
118.761
121.361
121.617
124.220
124.472
120.838
123.691
125.336
126.756
126.803
129.848
130.578
134.701
140.335
157.221
157.612
157.612
158.000
158.392
160.415
160.786
161.159
161.530
174.270

Figure 2.20. DEPT-NMR Spectra of N_{α},N_{1}-bis-trifluoroacetyl-L-tryptophan methyl ester

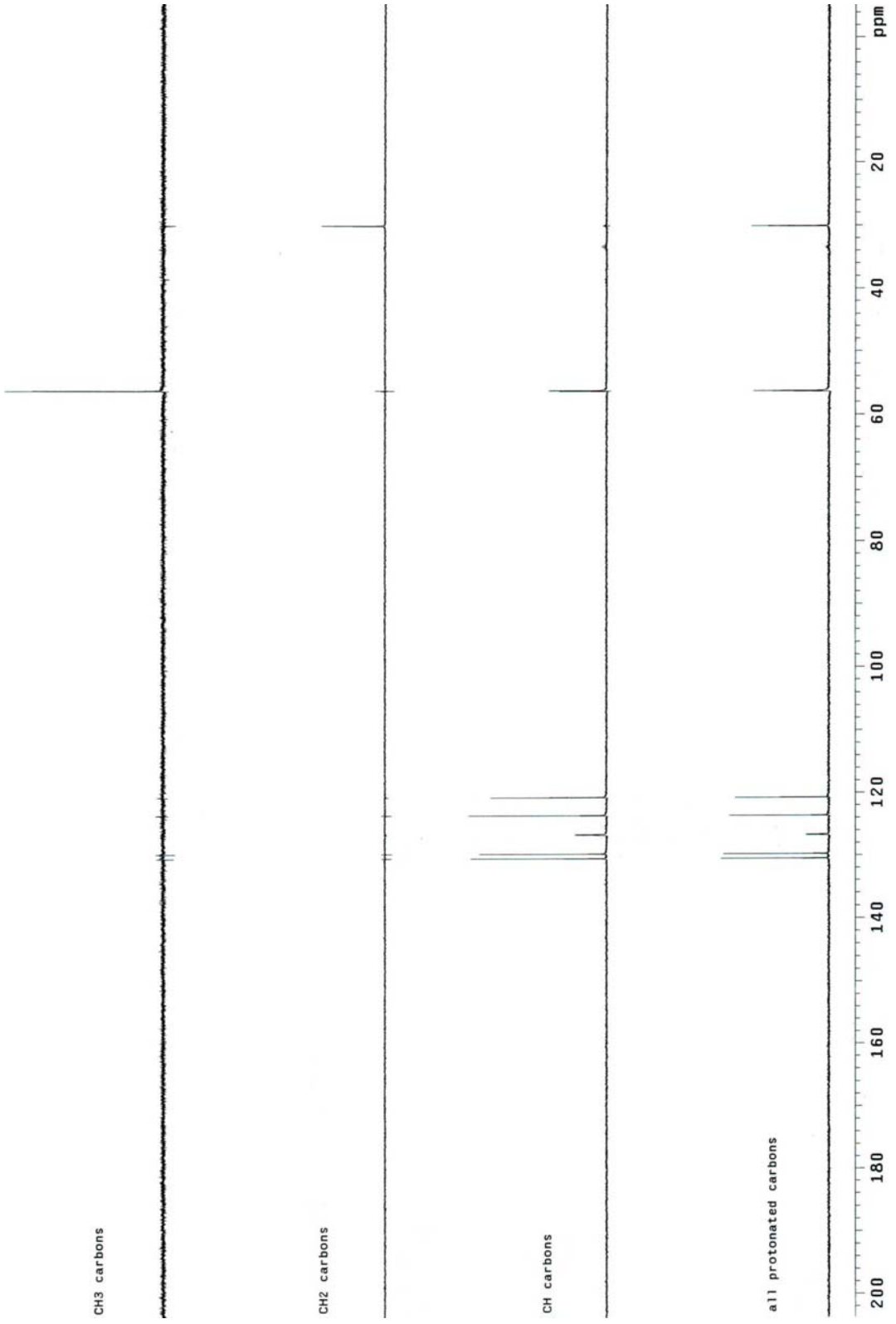

CH3 carbons
CH2 carbons
CH carbons
all protonated carbons
200
180
160
140
120
100
80
60
40
20
ppm

Figure 2.21. gHMBC-NMR spectrum of N_{α},N_{1}-bis-trifluoroacetyl-L-tryptophan methyl ester

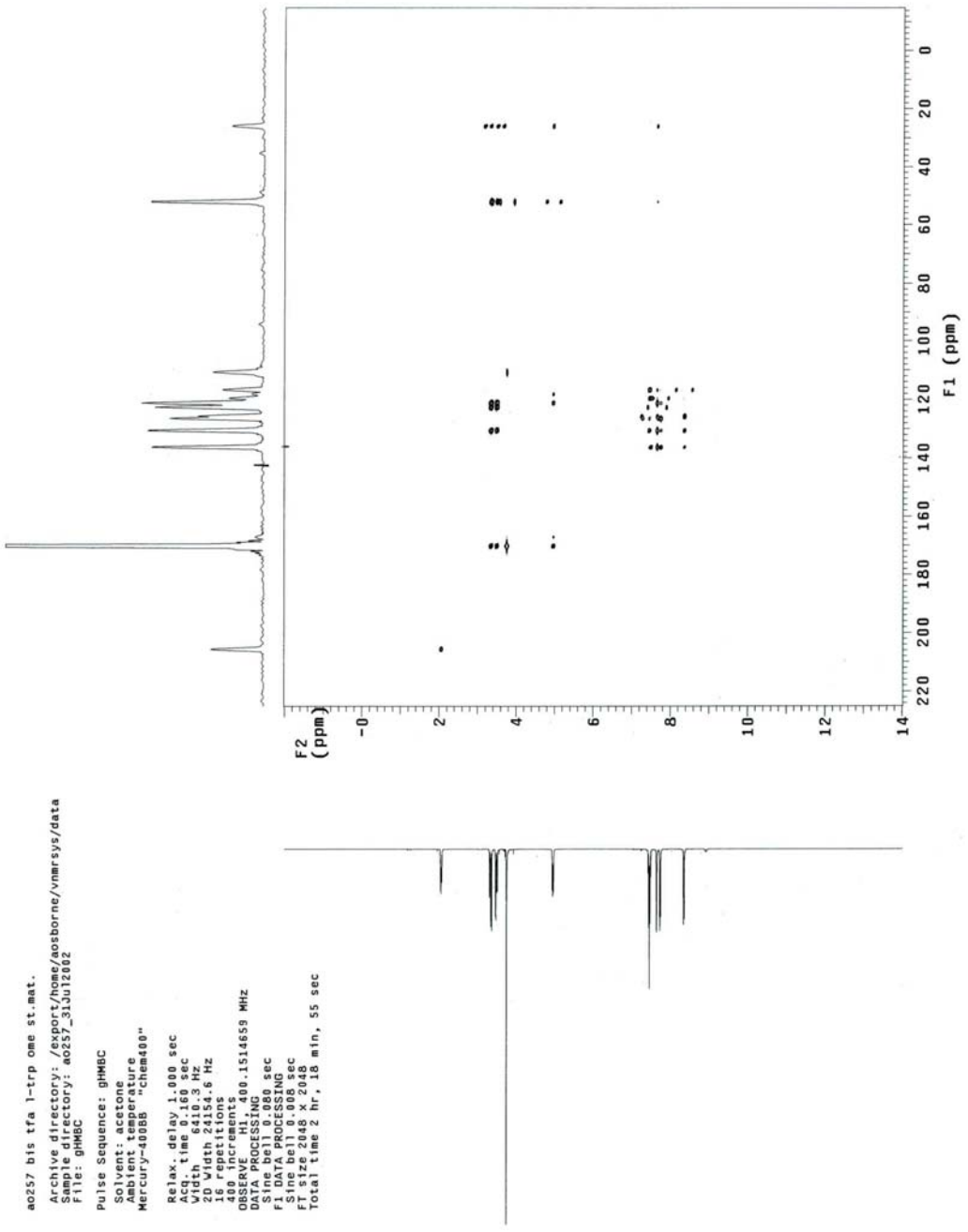

ao257 bis tfa l-trp ome st.mat.
Archive directory: /export/home/aosborne/vnmrsys/data
Sample directory: ao257_31Jul2002
File: gHMBC
Pulse Sequence: gHMBC
Solvent: acetone
Ambient temperature
Mercury-400BB "Chem400"
Relax. delay 1.000 sec
Acq. time 0.160 sec
Width 6410.3 Hz
2D Width 24154.6 Hz
16 repetitions
400 increments
OBSERVE H1, 400.1514659 MHz
DATA PROCESSING
Sine bell 0.080 sec
F1 DATA PROCESSING
Sine bell 0.008 sec
FT size 2048 x 2048
Total time 2 hr, 18 min, 55 sec
F2 (ppm)
F1 (ppm)

Figure 2.22. gHMQC-NMR spectrum of N_{α},N_1-bis-trifluoroacetyl-L-tryptophan methyl ester

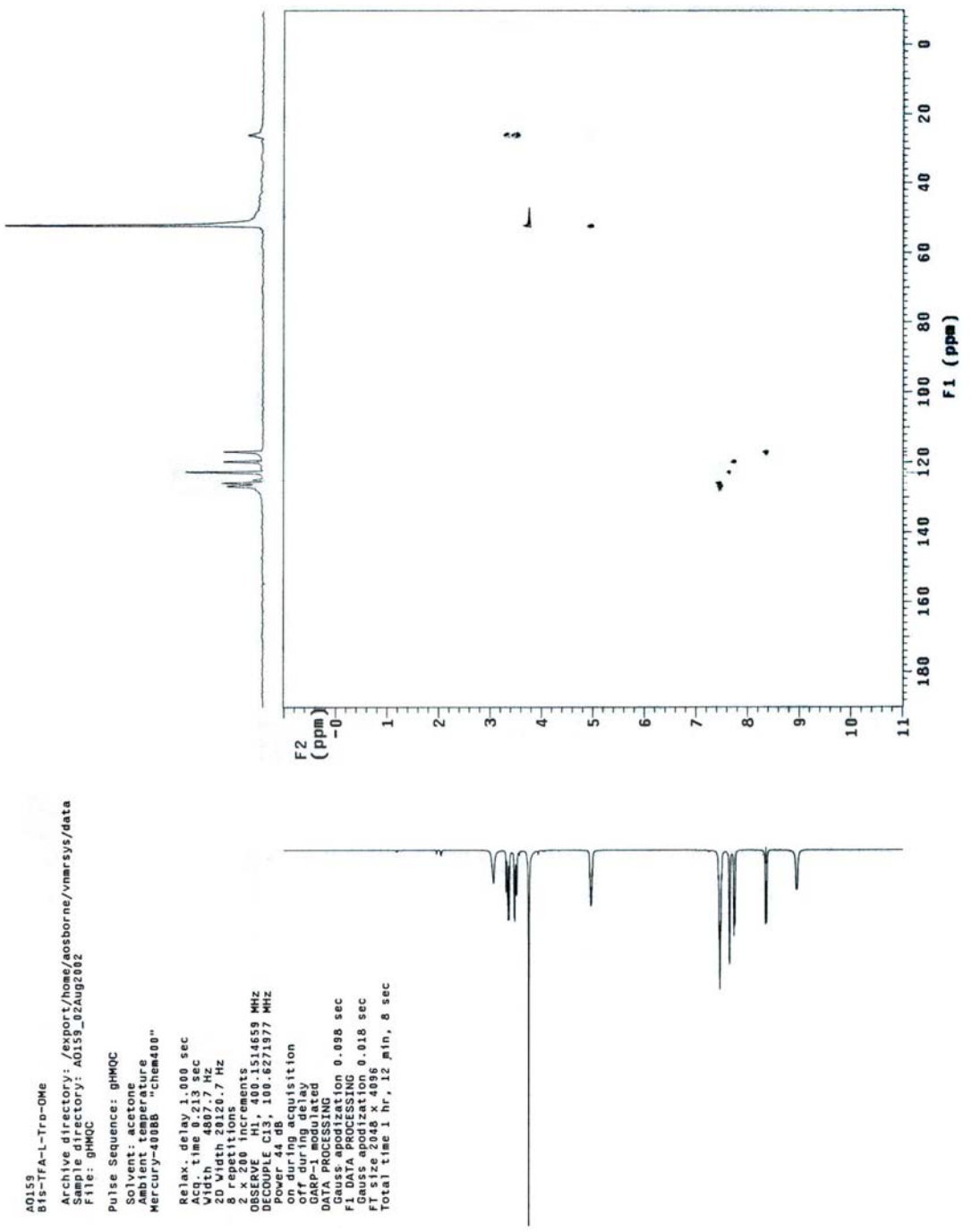

AO159
Bis-TFA-L-Trp-OMe
Archive directory: /export/home/aosborne/vnmrsys/data
Sample directory: AO159_02Aug2002
File: gHMQC
Pulse Sequence: gHMQC
Solvent: acetone
Ambient temperature
Mercury-400BB "chem400"
Relax. delay 1.000 sec
Acq. time 0.213 sec
Width 4807.7 Hz
2D Width 20120.7 Hz
8 repetitions
256 increments
OBSERVE H1, 400.1514659 MHz
DECOUPLE C13, 100.6271977 MHz
Power 44 dB
on during acquisition
off during delay
GARP-1 modulated
DATA PROCESSING
Gauss apodization 0.098 sec
FI DATA PROCESSING
Gauss apodization 0.018 sec
FT size 2048 x 4096
Total time 1 hr, 12 min, 8 sec
F2 (ppm)
F1 (ppm)

Figure 2.23. GC-MS of 2-nitro-N_{α}-trifluoroacetyl-L-tryptophan methyl ester

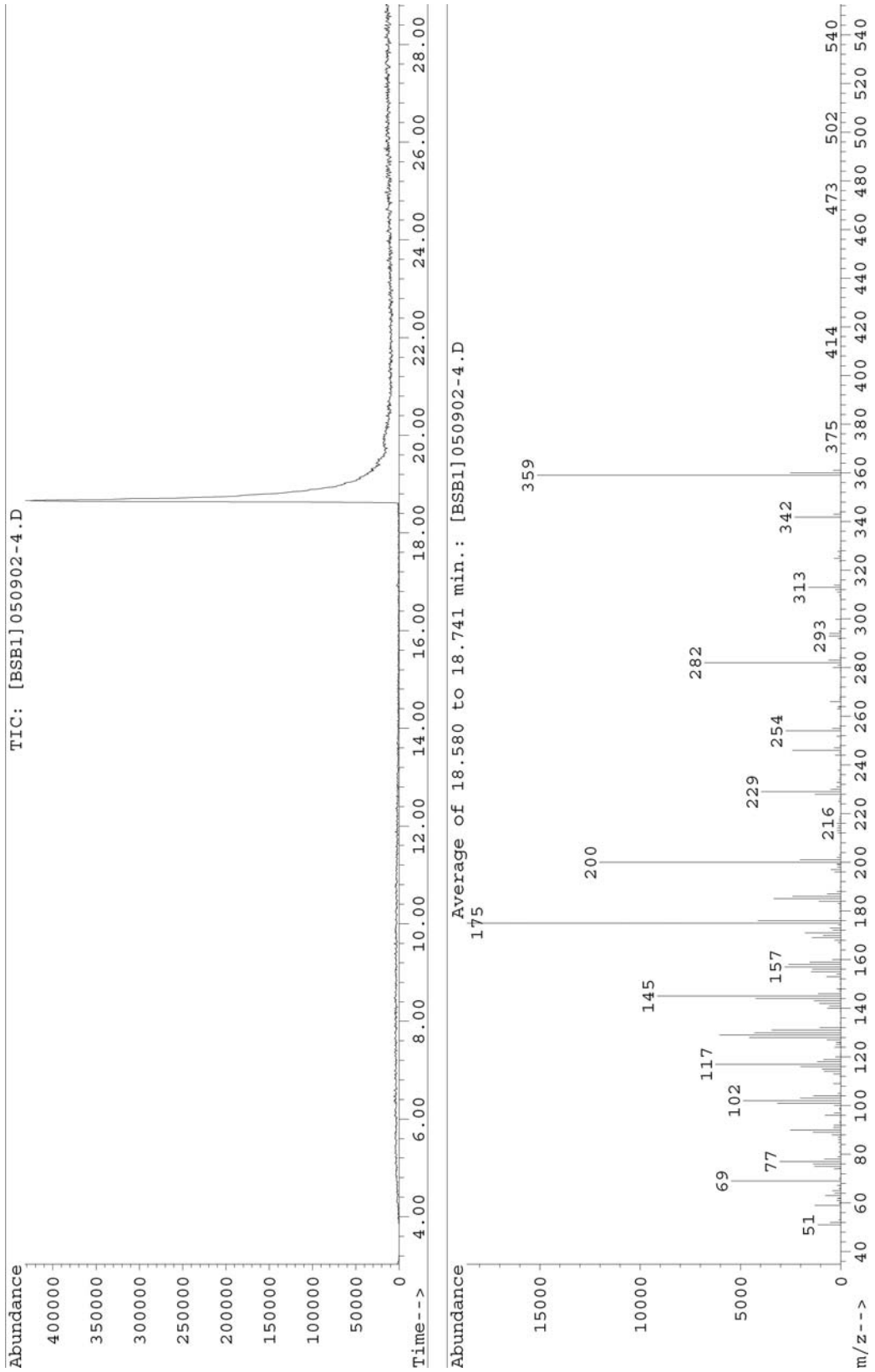

TIC: [BSB1]050902-4.D
Abundance
Time-->
Average of 18.580 to 18.741 min.: [BSB1]050902-4.D
Abundance
m/z-->

Figure 2.24. ^{1}H-NMR spectrum of 2-nitro-N_{α}-trifluoroacetyl-L-tryptophan methyl ester

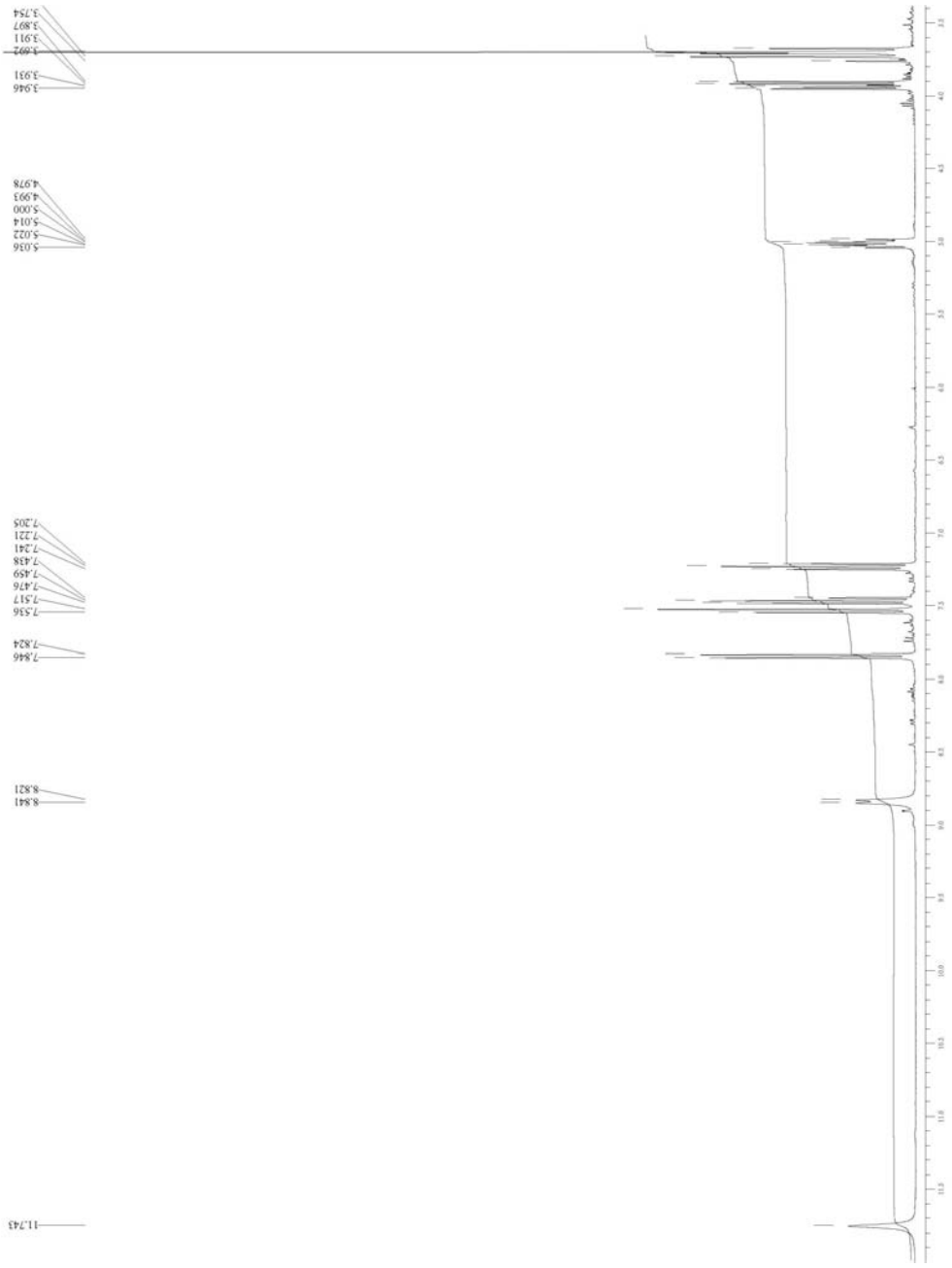

3.754
3.897
3.911
3.927
3.931
3.946
4.978
4.993
5.000
5.014
5.022
5.036
7.205
7.221
7.241
7.438
7.459
7.476
7.517
7.536
7.824
7.846
8.821
8.841
11.743

Figure 2.25. ^{13}C-NMR spectrum of 2-nitro-N_{α}-trifluoroacetyl-L-tryptophan methyl ester

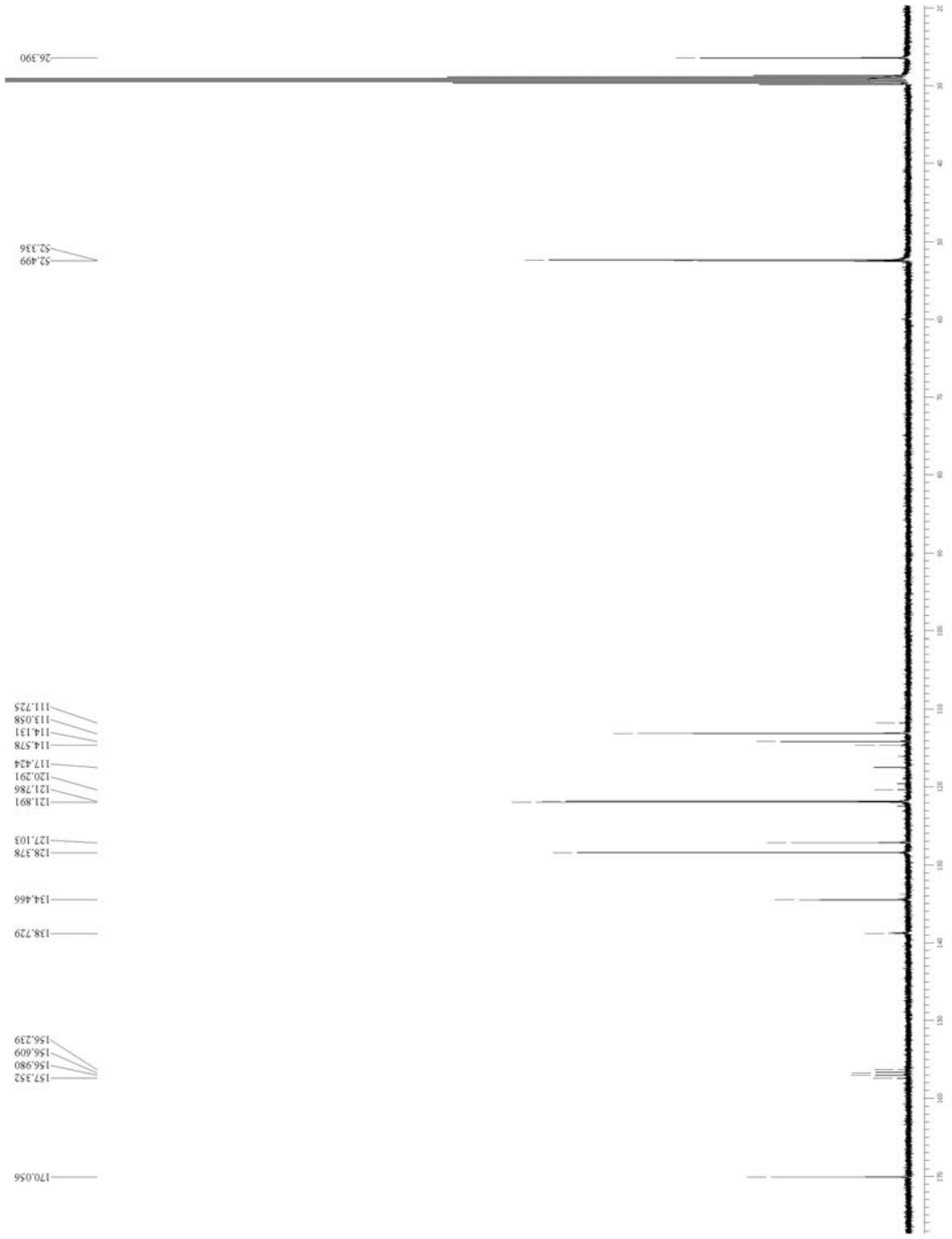

26.390
52.336
52.499
111.725
113.058
114.131
114.578
117.424
120.291
121.786
121.891
127.103
128.378
134.466
138.729
156.239
156.609
156.980
157.352
170.056

Figure 2.26. DEPT-NMR spectrum of 2-nitro-N_{α}-trifluoroacetyl-L-tryptophan methyl ester

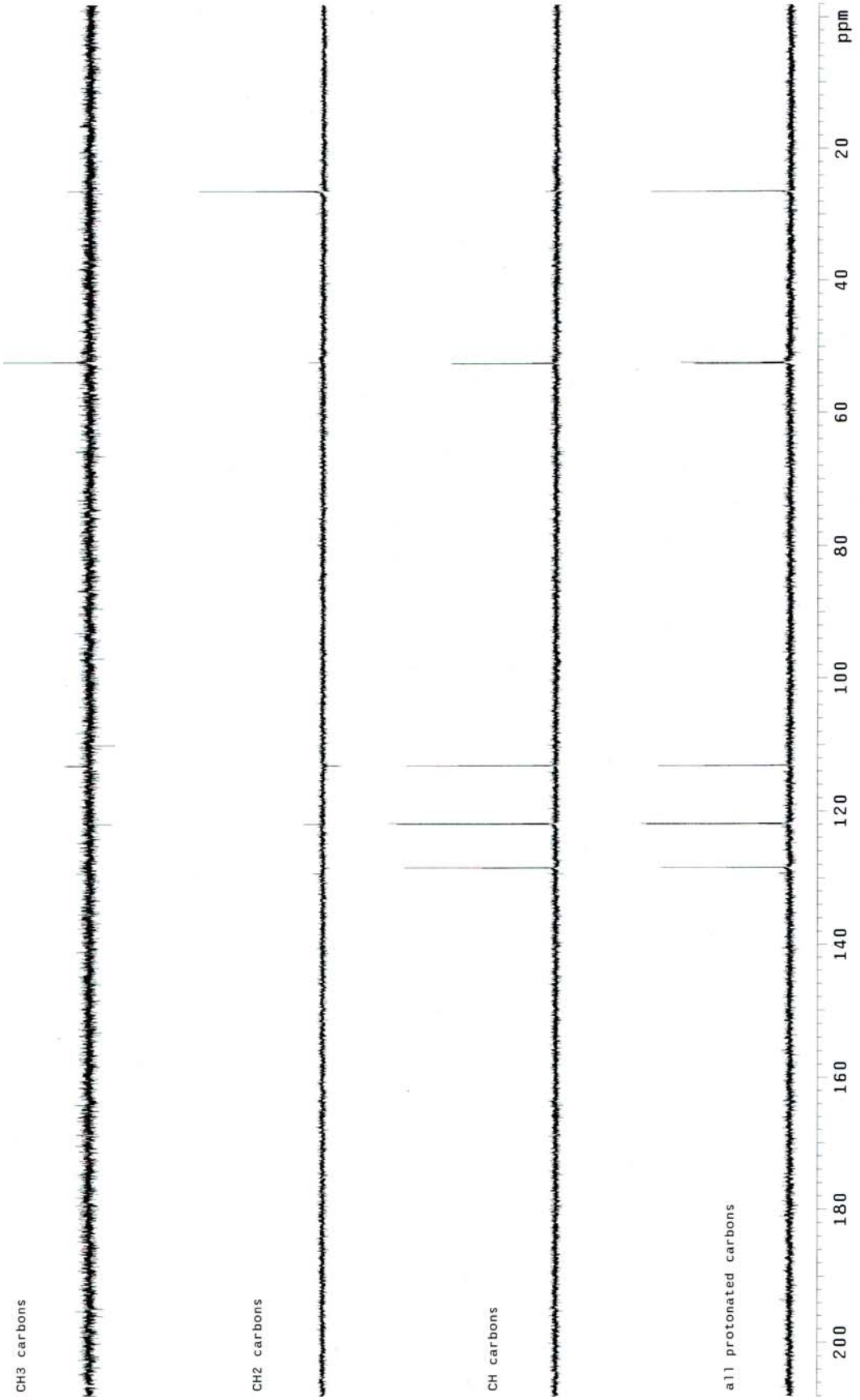

CH3 carbons
CH2 carbons
CH carbons
all protonated carbons
200
180
160
140
120
100
80
60
40
20
ppm

Figure 2.27. gHMBC-NMR spectrum of 2-nitro-N_{α}-trifluoroacetyl-L-tryptophan methyl ester

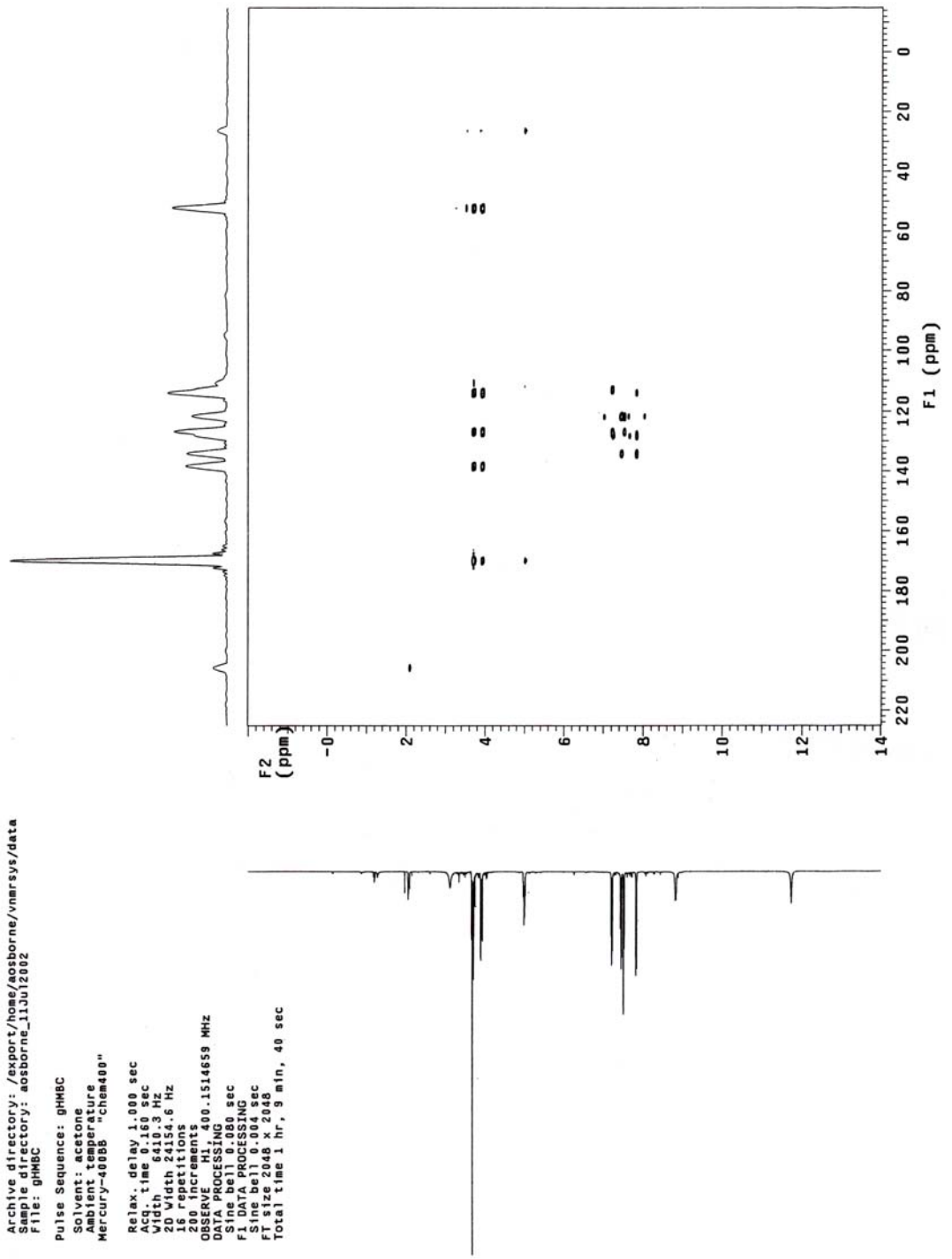

Archive directory: /export/home/aosborne/vnmrsys/data
Sample directory: aosborne_11Jul2002
File: gHMBC
Pulse Sequence: gHMBC
Solvent: acetone
Ambient temperature
Mercury-400BB "chem400"
Relax. delay 1.000 sec
Acq. time 0.160 sec
Width 6410.3 Hz
2D Width 24154.6 Hz
16 repetitions
200 increments
OBSERVE H1, 400.1514659 MHz
DATA PROCESSING
Sine bell 0.080 sec
F1 DATA PROCESSING
Sine bell 0.004 sec
FT size 2048 x 2048
Total time 1 hr, 9 min, 40 sec
F2 (ppm)
F1 (ppm)

Figure 2.28. gHMQC-NMR spectrum of 2-nitro-N_{α}-trifluoroacetyl-L-tryptophan methyl ester

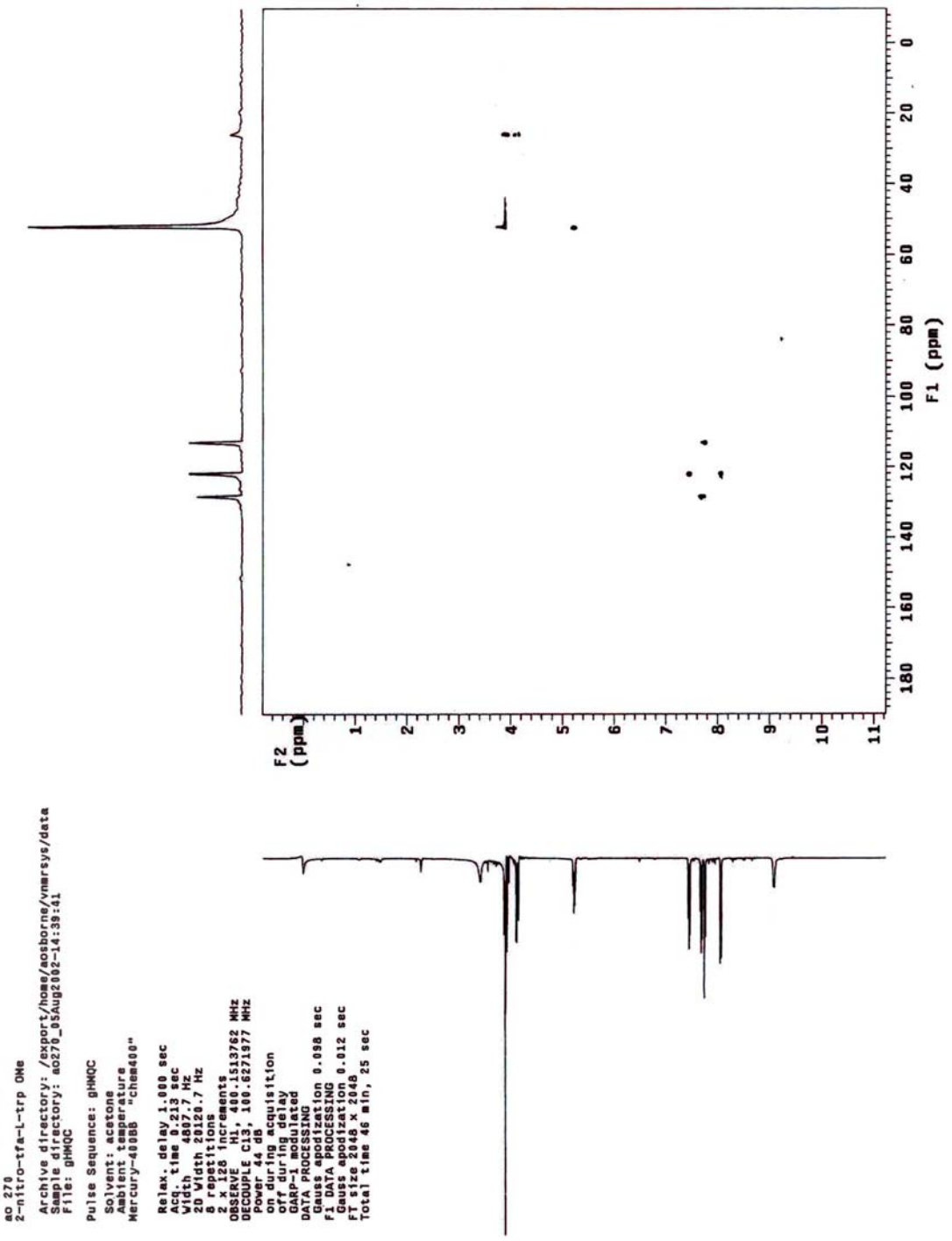
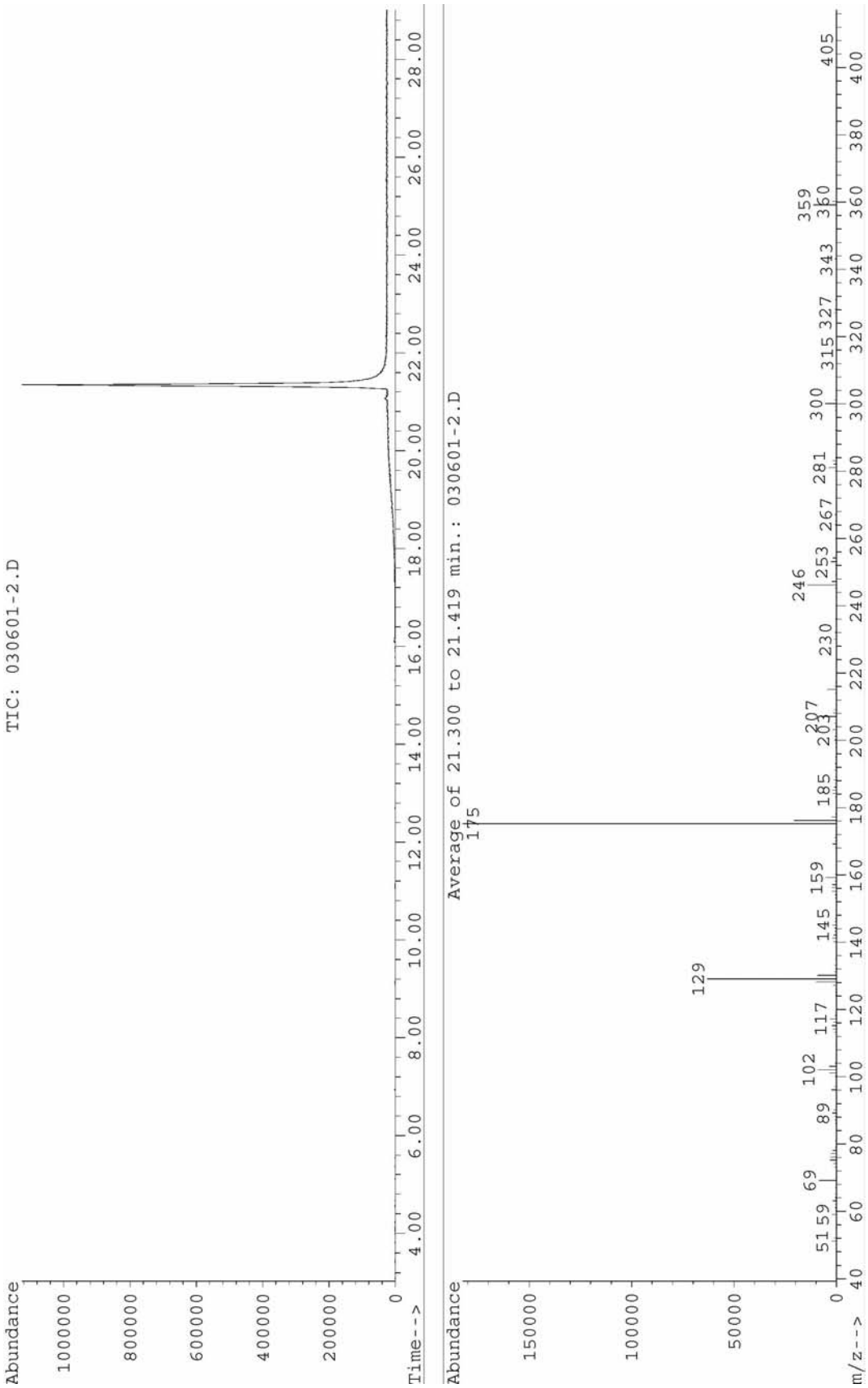

ao 270
2-nitro-tfa-L-trp OMe
Archive directory: /export/home/aosborne/vnmrsys/data
Sample directory: ao270_05Aug2002-14:39:41
File: gHMQC
Pulse Sequence: gHMQC
Solvent: acetone
Ambient temperature
Mercury-400BB "chem400"
Relax. delay 1.000 sec
Acq. time 0.213 sec
Width 4812.7 Hz
2D Width 28128.7 Hz
8 repetitions
2 x 128 increments
OBSERVE H1, 400.1513762 MHz
DECOUPLE C13, 100.6271977 MHz
Power 44 dB
on during acquisition
off during delay
GARP-1 modulated
DATA PROCESSING
Gauss apodization 0.098 sec
F1 DATA PROCESSING
Gauss apodization 0.012 sec
FT size 2048 x 2048
Total time 46 min, 25 sec
F2 (ppm)
F1 (ppm)

Figure 2.29. GC-MS of 2-nitro-N_{α}-trifluoroacetyl-L-tryptophan methyl ester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Abundance
Time-->
Average of 21.300 to 21.419 min.: 030601-2.D
Abundance
m/z-->

Figure 2.30. ^{1}H-NMR spectrum of 6-nitro-N_{α}-trifluoroacetyl-L-tryptophan methyl ester

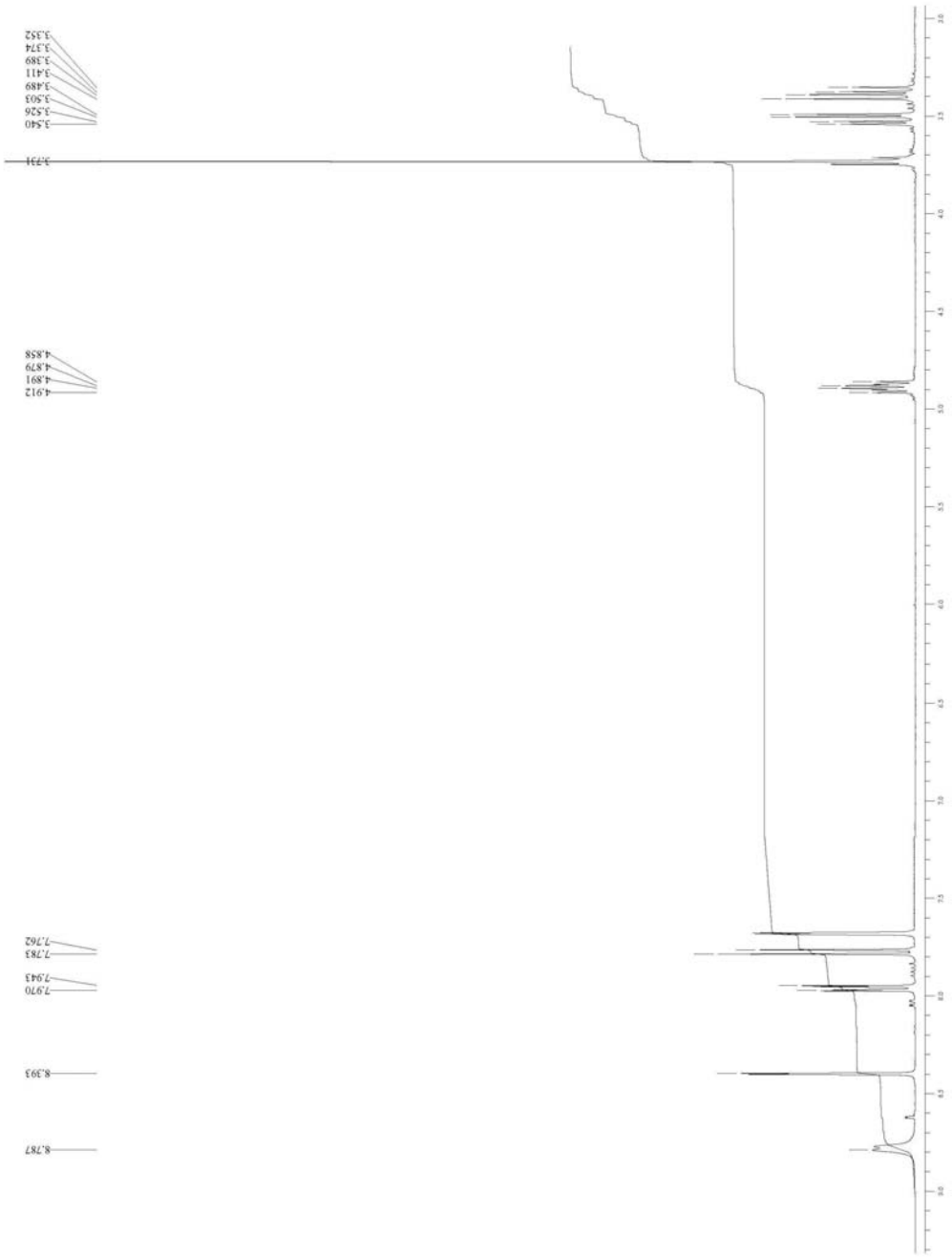

3.352
3.374
3.389
3.411
3.489
3.503
3.526
3.540
3.731
4.858
4.879
4.891
4.912
7.762
7.783
7.943
7.970
8.393
8.787

Figure 2.31. ^{13}C-NMR spectrum of 6-nitro-N_{α}-trifluoroacetyl-L-tryptophan methyl ester

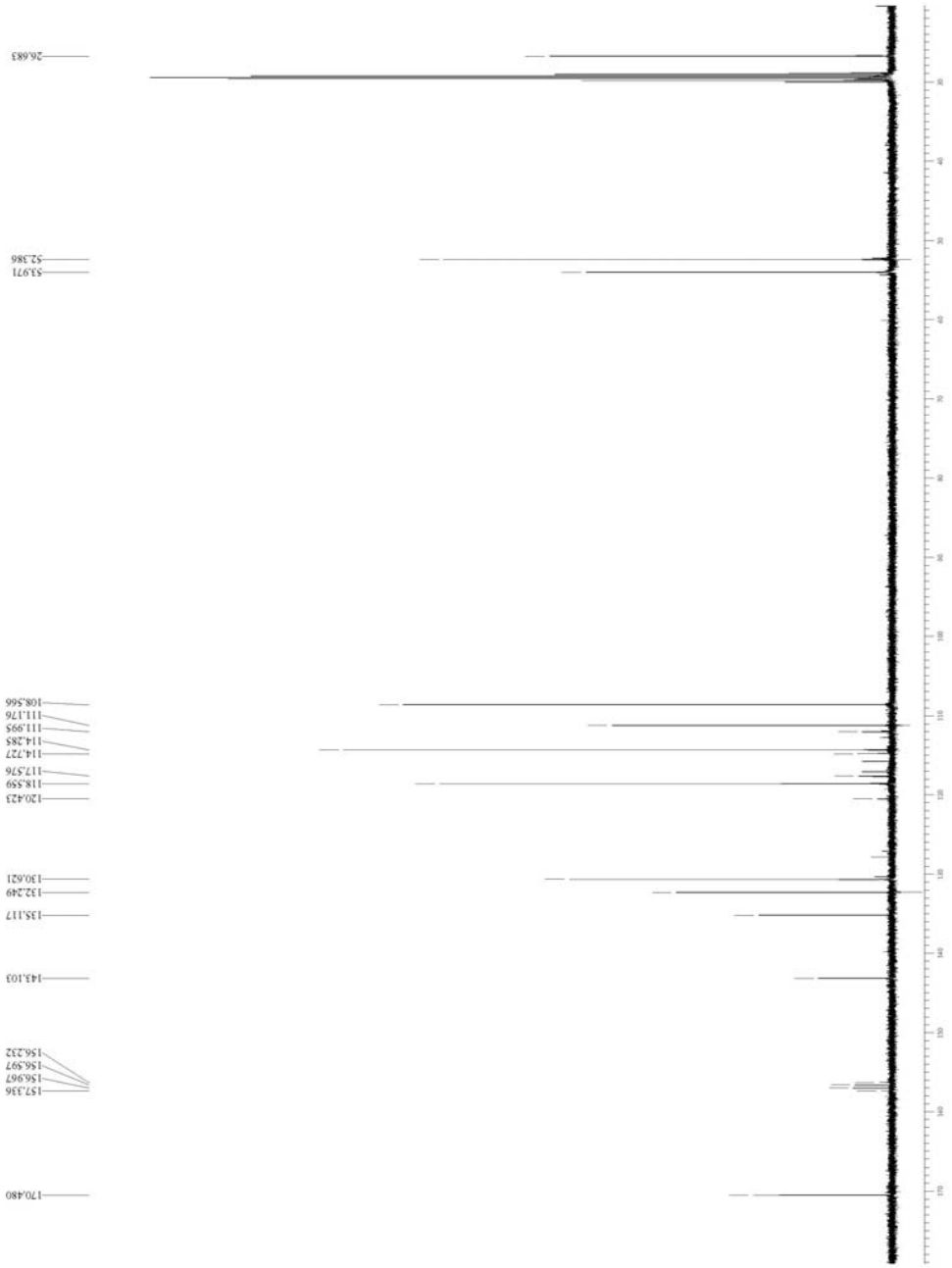

26.683
52.386
53.971
108.566
111.176
111.995
114.285
114.727
117.576
118.559
120.423
130.621
132.249
135.117
143.103
156.232
156.397
156.967
157.336
170.480

Figure 2.32. DEPT-NMR spectrum of 6-nitro-N_{α}-trifluoroacetyl-L-tryptophan methyl ester

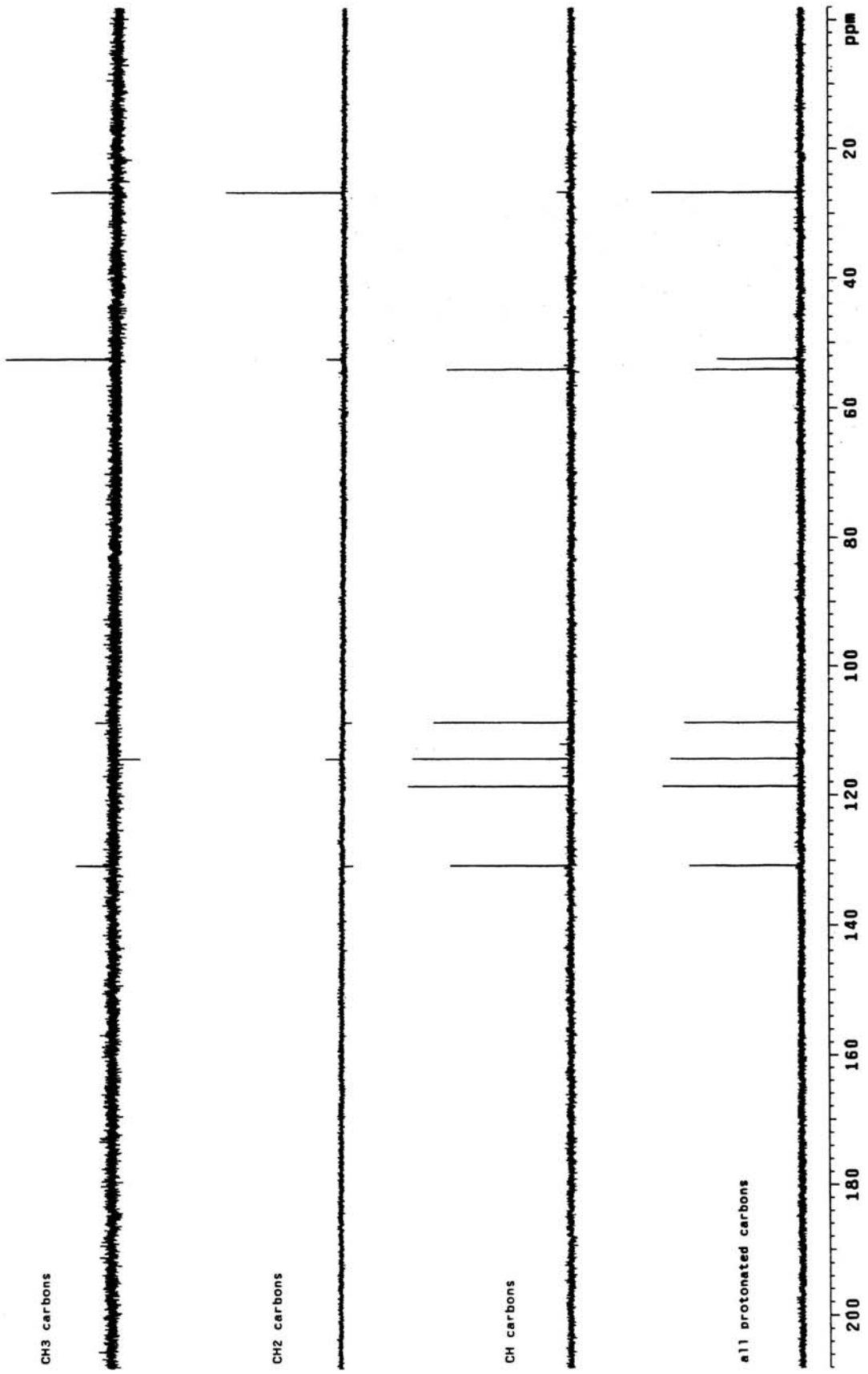
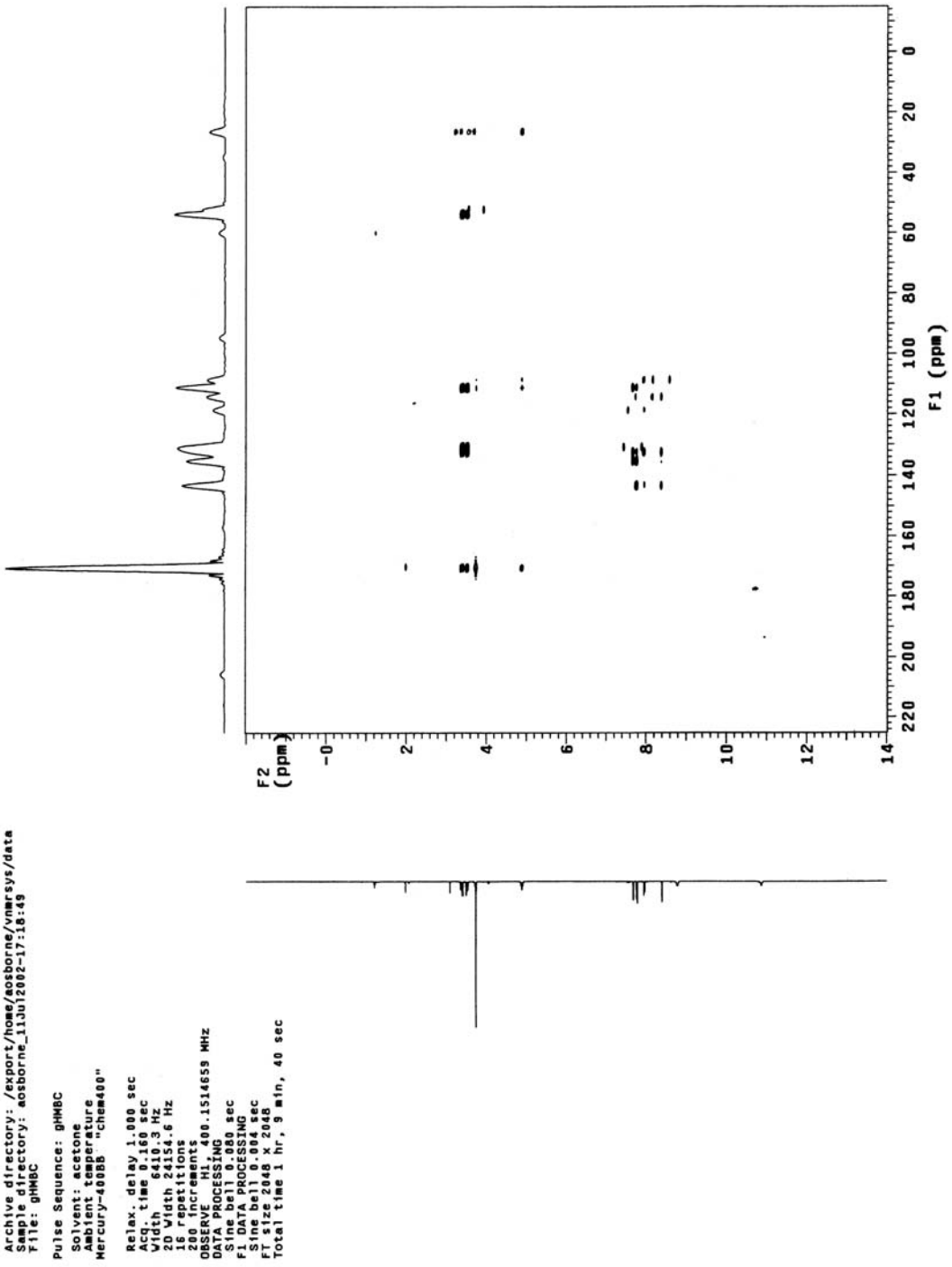

CH3 carbons
CH2 carbons
CH carbons
all protonated carbons
200
180
160
140
120
100
80
60
40
20
ppm

Figure 2.33. HMBC-NMR spectrum of 6-nitro-N_{α}-trifluoroacetyl-L-tryptophan methyl ester


Archive directory: /export/home/aosborne/vnmrsys/data
Sample directory: aosborne_11Jul2002-17:18:49
File: gHMBC
Pulse Sequence: gHMBC
Solvent: acetone
Ambient temperature
Mercury-400BB "chem400"
Relax. delay 1.000 sec
Acq. time 0.160 sec
Width 6410.3 Hz
2D Width 24154.6 Hz
16 repetitions
128 increments
OBSERVE H1, 400.1514659 MHz
DATA PROCESSING
Sine bell 0.080 sec
F1 DATA PROCESSING
Sine bell 0.004 sec
FT size 2048 x 2048
Total time 1 hr, 9 min, 40 sec
F2 (ppm)
F1 (ppm)

CHAPTER III

KINETIC INVESTIGATION OF CHIRAL ALCOHOL OXIDATION BY C295L AND C295V MUTANTS OF SECONDARY ALCOHOL DEHYDROGENASE FROM THERMOANAEROBACTER ETHANOLICUS

Introduction

Despite the enormous potential of enzymes in catalyzing organic reactions,[1,2] several drawbacks severely limit their usefulness. Most enzymes catalyze reactions over a very narrow range of temperatures, are limited to a narrow selection of solvents, and are usually effective for a narrow scope of substrate structure. Additionally, enzymes most often are only effective for the synthesis of a single enantiomer. Recent discoveries of organisms that thrive under diverse conditions, extremophiles,[3-5] have led to the isolation of enzymes that catalyze reactions under comparatively diverse conditions. Such enzymes have demonstrated a great increase in range of reaction conditions, both thermally and chemically, and some have demonstrated the ability to catalyze transformations of structurally diverse substrates.

Among the most synthetically useful enzymes are the alcohol dehydrogenases (ADHs). Several factors contribute to their synthetic importance, including the ubiquity and synthetic utility of chiral alcohols, and their ability to synthesize them from achiral precursors under mild reaction conditions.[1] Most ADHs share some structural and catalytic properties, including dimeric or tetrameric subunit structures,[7] presence of active site Zn serving as a Lewis acid, and a nicotinamide cofactor acting as an hydride donor/acceptor. Among the historically significant alcohol dehydrogenases are those from yeast (YADH)[2,8] and horse liver (HLADH);[9] however, these enzymes suffer thermo-sensitivity and limited effectiveness with structurally diverse substrates.[9]

Several NADP(H) dependent, tetrameric ADHs have been isolated from thermophilic bacteria[10-13] that have been useful for the synthetic chemist. TBADH from *Thermoanaerobacter brockii*[14-17] has shown increased tolerance to reaction conditions and substrate structural variability, including acyclic ketones.[14-18] It has also shown a reversal in stereoselectivity based on substrate size.[15] TBADH has been used for the asymmetric synthesis of chiral alcohols[14,15,17] and for several total syntheses.[16-18]

Secondary alcohol dehydrogenase (SADH) is one of two ADHs isolated from *Thermoanaerobacter ethanolicus,*[11] and preferentially oxidizes secondary alcohols versus

primary alcohols, hence its name. Incidently, the other ADH from *Thermoanaerobacter ethanolicus* prefers primary alcohols for oxidation. SADH is structurally similar to TBADH, and consistent with its name, has a preference for secondary alcohols over primary alcohols.

SADH has also been used for synthetically applicable oxidations and reductions[19-21] and has demonstrated a reversal in enantiospecificity for alcohol oxidation. SADH oxidizes (S)-2-butanol faster below 26º C, and (R)-2-butanol faster above 26º C.[22-23] Although the selectivity for R-alcohol oxidation is limited, the potential for the use of SADH for the synthesis of both R and S chiral alcohols is compelling. Recently, several mutants of SADH have been studied.[24-28] Phillips and coworkers[27] have reported an increase in both activity and R-specificity with mutant S39T SADH, whose mutant residue resides within the large alkyl binding domain[15] along with the bulk of the nicotinamide cofactor.[28] Asymmetric reduction of ethynyl ketones by SADH occurs with high yield and enantiomeric excess, but at the expense of enzyme deactivation, presumably from a nucleophilic active site residue.[20] Modeling studies of the homologically similar TBADH revealed the presence of cysteine in the small alkyl binding domain. Substitution of the analogous cysteine in SADH by the smaller aliphatic and non-nucleophilic residue, alanine, did not alleviate the inactivation of the enzyme by ethynyl ketones, and the temperature correlation to enantioselectivity for 2-butanol and 2-pentanol was lost.[27]

In this study, SADH mutants C295V and C295L were evaluated for their efficiency in catalyzing the oxidation of the chiral alcohols, 2-butanol, 2-pentanol, and 2-hexanol. Both mutants showed a ten-fold reduction in enzyme activity compared to the wild type for 2-butanol and 2-pentanol. SADH C295L showed a strong preference for (R)-2-butanol and (R)-2-pentanol with a minor temperature dependence for both. SADH C295L showed a trivial preference for the R-enantiomer of 2-hexanol at 295 K, and preference for the S-enantiomer increased sharply with temperature. The mutant SADH C295V was less efficient than the leucine mutant despite its smaller size. Interestingly,

this mutant shows a temperature trend reversal for enantioselectivity with 2-butanol and 2-pentanol near 315 K.

Materials and Methods

Materials. Bacto tryptone and yeast extract were purchased from Difco Laboratories Company. Casein hydrolysate, sodium chloride, kanamycin, ampicillin, and $NADP^+$ were obtained from United States Biochemical Corporation. Tris HCl, Red A agarose (R 0503, Reactive Red 120), sodium perchlorate, and ammonium sulfate were obtained from Sigma. Ethanol, (R)- and (S)-2-pentanol, (R)- and (S)-2-butanol, (R)- and (S)-2-hexanol, hydrochloric acid, and sodium hydroxide were obtained from Aldrich. BioRad assay reagent was obtained from BioRad, and dithiothreitol (DTT) was purchased from Fischer Biotech. *E. coli* expressing SADH mutants C295L and C295V were obtained from the lab of Professor J. G. Zeikus at Michigan State University.

Instrumentation. Enzymes were purified by Red Agarose column chromatography[26] using a Bio-Rad Biologic liquid chromatograph with fraction collector and UV detector and was controlled by Bio-Rad LP Dataview software. All UV-Vis measurements were recorded using a Varian- Cary 1E spectrophotometer equipped with a twelve cell, temperature controlled cell changer assembly. Kinetic calculations were performed using the non-linear Michaelis-Menten algorithm, HYPERO, written by Cleland.[29]

Growth of Cells. Mutants C295L and C295V were grown from *E. coli* DH5α, containing the recombinant *adhB* gene for mutant SADH, on an agar plate. Single colonies of cells were introduced to two separate flasks each containing 1 L complex medium composed of casein hydrolysate (20 g), yeast extract (10 g), sodium chloride (5 g), kanamycin (25 mg), and ampicillin (100 mg).[25] After 24 hours of shaking at 37º C, cells of *E. coli* were collected by centrifugation at 10,000 x g for 20 min. The cell pellets were suspended in 0.05M buffer A (tris HCl buffer (pH 7.8) containing dithiothreotol (DTT, 5 mmol)), and the suspension was frozen.

Purification of mutant SADH. The frozen cell suspension, containing *E. coli* cells expressing mutant SADH,[25] was thawed and sonicated 4 x 1 minutes at 5 minute intervals. The temperature of the suspension was kept at 0º C. The cell debris was removed by centrifugation for 25 minutes at 10,000 x g. The resulting supernatant was heated to 70º C for 60 min and the denatured, thermally sensitive protein was removed by centrifugation for 25 min at 10,000 x g. The supernatant containing SADH was combined with ammonium sulfate to achieve 20% saturation and was stirred for 60 minutes. Precipitated impurities were removed by centrifugation at 10,000 x g for 25 min. The supernatant was combined with ammonium sulfate to achieve 60% saturation and was stirred for 60 min. The suspension was centrifuged at 12,500 x g for 30 min and the resulting pellet was collected and re-dissolved in minimal buffer A, and dialyzed against 3L buffer A overnight. The enzyme solution was loaded onto a 2.5 cm diameter column containing 40 ml Red A Agarose, pre-washed and equilibrated with buffer A. The enzyme was purified by a programmed, gradient elution of 0-30 min buffer A, 30-270 min 1.0 mM sodium perchlorate in buffer A (Buffer B), 270-300 min in buffer B all at a rate of 1 ml/min. Fractions containing SADH were pooled into four separate fractions and each was concentrated by precipitation in 60% ammonium sulfate followed by dissolution in minimal buffer A.

Secondary Alcohol Dehydrogenase Assay. Enzyme concentration was determined by measuring the visible absorbance of an enzyme-assay reagent solution as performed by Bradford.[30] Bio-Rad protein-assay solution (200 µl), water (190 µl), and separate enzyme fractions (10 µl) were combined in a quartz cuvette, and absorbance at 595 nm was measured. Protein concentration was determined as a function of absorbance according to the equation A=0.04457*[E]-0.0015. A is absorbance, [E] is enzyme concentration, and the equation represents the best fit line of absorbance measured at several known concentrations of albumin. Enzyme specific activity was determined by measuring the production of NADPH ($\Delta\varepsilon_{340}$= 6.22 x 10^3 M^{-1} cm^{-1}) in a solution containing 10 µl 100x diluted enzyme and 590 µl assay mixture that was 200 mM in 2-

propanol, 1.25 mM in NADP in 100 mM tris HCl buffer (pH = 8.9). One unit of activity (U) is the amount of enzyme required to reduce 1 μmol of NADP per minute per milligram of enzyme.[22-23]

Kinetic Experiments. SADH mutants C295A and C295V were assayed for their ability to oxidize (R)- and (S)- 2-butanol, (R)- and (S)- 2-pentanol and (R)- and (S)- 2-hexanol to the corresponding ketones at 15 ºC, 25 ºC, 35 ºC, 45 ºC, and 55 ºC. Quartz cuvettes for each kinetic assay contained a total volume 600 μl, which consisted of 10 μl enzyme solution and 590 μl of an assay mixture composed of 1.00 mM $NADP^+$, 50 mM Tris HCl buffer adjusted to pH = 8.9 for each temperature, and geometrically increasing amounts of alcohol including a blank. Typical alcohol concentrations were: 0 mM, 12.5 mM, 25 mM, 50 mM, 100 mM, 200 mM. Six cuvettes for each assay, each containing 590 μl assay mixture, were allowed to equilibrate for 5 minutes at reaction temperature prior to the addition of 10 μl enzyme soltution. The rates of the reactions were determined by measuring the production of NADP (λ_{max} = 340 nm) as the reaction progressed. Values of K_{cat} and K_m were calculated for each assay, and these values were used to determine $\Delta\Delta G^{\ddagger}$. The values of $-RT\ln E$, where $E = (K_{cat})/(K_m)_R/(K_{cat}/K_m)_S$, were plotted for each alcohol as a function of temperature and the slope and y intercept of a best-fit line to these data were used to determine $\Delta\Delta S^{\ddagger}$ and $\Delta\Delta H^{\ddagger}$.

Results

Protein Concentration and Activity. Purified SADH mutants C295L and C295V in buffer A were evaluated for protein concentration and specific activity. Concentrations were determined by the Bradford[30] assay, by which the concentration of purified C295L was determined to be 31 mg/ml, and the concentration of mutant C295V was determined to be 24 mg/ml. The specific activities of the mutants were measured as a function of NADPH production using 2-propanol as the substrate.[22,23] The specific activity of mutant C295L was determined to be 31 U/mg, and the specific activity of

C295V was 24 U/mg. These values are lower than those for both the wild type (54 U/mg),[15] and C295A mutant (43 U/mg).[20]

Relative Enantiospecificities. The catalytic efficiencies (K_{cat}/K_m) of SADH mutants C295L and C295V were compared to that of the wild type at 55º C with (R)- and (S)-2-butanol and (R) and (S)-2-pentanol. Overall, the mutant enzymes showed an approximate ten-fold decrease in catalytic efficiency compared to the wild type. Enantiospecificity ratios (E) for the wild type enzyme were 2.818 favoring (R)-2-butanol and 0.669 favoring (S)-2-pentanol.[27] Mutant C295L favored (R)-2-butanol with an enantiospecificity ratio of 2.4717, and favored (R)-2-pentanol with an E value of 3.9625 where mutant C295V favored (R)-2-butanol with an E value of 2.590, and (S)-2-pentanol with an E value of 0.4436 (Table 3.1).

Table 3.1. Relative catalytic efficiencies of wild type and C295L and C295V mutants of SADH for enantiomers of 2-butanol and 2-pentanol at 55º C.

K_{cat}/k_m (x 10^4 $M^{-1}s^{-1}$)			
Substrate	**Wild type SADH[26]**	**C295L SADH**	**C295V SADH**
(R)-2-butanol	31.0 ± 3.0	2.62 ± 0.156	1.22 ± 0.183
(S)-2-butanol	11.0 ± 2.0	1.06 ± 0.0742	0.471 ± 0.0894
(R)-2-pentanol	8.7 ± 0.8	0.951 ± 0.0572	0.177 ± 0.0162
(S)-2-pentanol	13. 0 ± 2.0	0.240 ± 0.0967	0.399 ± 0.0550

The catalytic efficiencies of SADH mutants C295L and C295V were each evaluated with (R)- and (S)-2-butanol, (*R*)- and (S)- 2-pentanol and (R)- and (S)-2-hexanol at a constant pH of 8.9. K_{cat}/K_m values were determined for each enantiomer at each of five temperatures between 293 and 333 K. From these values, enantiospecificity ratios (E) were calculated where $E = (K_{cat}/K_m)_R/(K_{cat}/K_m)_S$. For each alcohol/ enzyme

combination, –RTlnE values were plotted as a function of temperature; and weighted, linear least squares lines were fit to these data (Figures 3.1-3.3). Since $-RT\ln E = \Delta\Delta G^{\ddagger}$, values for $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ were determined according to the linear function $\Delta\Delta G^{\ddagger} = \Delta\Delta H^{\ddagger} - T\Delta\Delta S^{\ddagger}$ where $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ are determined as the y intercept and slope of the fit lines respectively. The temperature where both enantiomers of a substrate are catalyzed equally is the racemic temperature, T_R, and is a constant for each alcohol/ enzyme combination. When $\Delta\Delta G^{\ddagger} = 0$, $T_R = \Delta\Delta H^{\ddagger}/\Delta\Delta S^{\ddagger}$. Values of $\Delta\Delta H^{\ddagger}$, $\Delta\Delta S^{\ddagger}$, and T_R for wild type and C295A are listed with those for C295L and C295V in Table 3.2.

Table 3.2. Values of $\Delta\Delta H^{\ddagger}$, $\Delta\Delta S^{\ddagger}$ and T_R for wild type, C295A, C295L and C295V SADH.

Enzyme/ Substrate		$\Delta\Delta H^{\ddagger}$ ((Kcal/mol)	$\Delta\Delta S^{\ddagger}$ (cal/ºC mol)	T_R (ºC)
Wild-type	2-butanol	8.2 ± 1.0	27.6 ± 3.4	297
	2-pentanol	2.8 ± 0.4	7.3 ± 1.2	383.6
	2-hexanol	5.8 ± 0.2	11.4 ± 0.7	508
C295A	2-butanol	-0.6 ± 0.5	-1.2 ± 1.5	500
	2-pentanol	2.5 ± 0.3	8.35 ± 1.2	298.5
	2-hexanol	0.3 ± 0.4	-0.5 ± 1.1	-600
C295L	2-butanol	-2.27 ± 0.01	-5.02 ± 0.02	451.6
	2-pentanol	1.81 ± 0.261	8.24 ± 1.72	219.9
	2-hexanol	-3.56 ± 1.42	-12.1 ± 4.9	294.5
C295V	2-butanol	-12.6 ± 1.8	-39.2 ± 6.0	321
	2-pentanol, K_1	3.74 ± 1.9	11.5 ± 6.4	325
	2-Pentanol, K_2	-6.78± 1.9	-21.9±6.1	309
	2-hexanol	4.78 ± 0.9	11.24 ± 2.9	425.4

Discussion

Deactivation of wild type SADH by the reduction of ethynyl ketoesters was the impetus for the preparation of mutant C295A. Modeling studies based on the *X*-ray structure of TBADH, which has a similar homology to SADH, revealed residue cysteine 295 to be located in the small alkyl binding pocket proposed by Keinan, et.al.[15] Phillips and Heiss[20] hypothesized that this nucleophilic residue is responsible for

enzyme inactivation; therefore, replacement of it by an inert residue should restore the catalytic properties with this class of molecules. Although the specific activity of C295A SADH was determined to be similar to that of the wild type SADH for small chiral alcohols, reaction catalysis of ethynyl ketones by C295A SADH was still accompanied by rapid, irreversible inactivation. Interestingly, the novel temperature dependant reversal in enantioselectivity observed with the wild type enzyme[22] is largely absent with this mutant. There are several possible contributors for this, including the loss of rotational entropy attributed to the cysteine 295 sulfhydryl group's replacement by alanine's methyl group. Additionally, X-ray data of TBADH reveals the presence of an active site water molecule with both the free enzyme and with bound (S)-2-butanol.[31] Expulsion of this water by (R)-alcohols with the wild type could account for a large portion of the entropy difference between wild and C295A enzymes with 2-butanols.[32] The smaller size of the alanine 295 residue apparently increases the capacity of the small binding pocket as the size based reversal in enantioselectivity is largely reduced as well. The small values of $\Delta\Delta H^{\ddagger}$ observed for all alcohols with C295A indicate that both binding pockets accommodate small alkyl groups similarly. Since the values of $\Delta\Delta H^{\ddagger}$ for the wild type SADH favor the (S)-configuration for these alcohols, this reduction of enthalpic selectivity with C295A suggests a net increase in steric capacity of the small alkyl binding pocket.[32] The small values for $\Delta\Delta S^{\ddagger}$ for 2-butanol and 2-hexanol support the hypothesis that water expulsion by the bound wild type enzyme is largely responsible for the increased entropy with the (R)- alcohols, as rotational restriction of cysteine 295 with the larger (R)-alcohols would reduce entropy for the bound state causing values for $\Delta\Delta S^{\ddagger}$ to decrease in value or become negative.

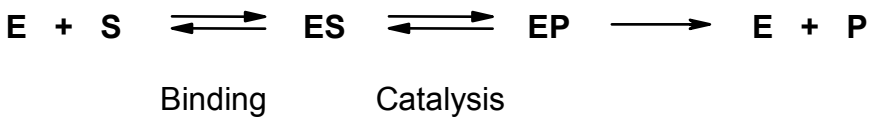
Proof that active site water, and not restriction of modal entropy, is the reason for the large values of $\Delta\Delta S^{\ddagger}$ with the wild type enzyme would be provided by mutation of cysteine 295 to a residue with a larger, bulkier alkyl chain. Residues with larger alkyl groups should have an effect on both size based dependence on substrate enantioselectivity due to increased small pocket steric hindrance, and on temperature

dependence on enantioselectivity due to the correlation between temperature and modal increases in side chain entropy. Additionally, the associated increase in small alkyl binding pocket hydrophobicity should expel active site water from the free enzyme thus eliminating water's contribution to the large entropy terms with small (R)-alcohols. Substitutions of cysteine 295 by leucine or valine are ideal mutants for testing this hypothesis, as leucine's alkyl chain is considerably bulkier than cysteine and has the potential to have a large relative entropy due to the many possible degrees of rotational freedom. Valine, on the other hand, has a large van der Waal's radius at the β-position due to the methyl group. Changes in rotational entropy due to steric interactions with substrate should be easily measurable.

Values of $\Delta\Delta H^{\ddagger}$ for 2-pentanol with mutants C295L and C295V are similar to those for the wild type and mutant C295A. A plausible explanation for this is that 2-pentanol's 3 carbon chain has a favorable steric fit in the large alkyl pocket and is repulsed by the side chain of residue 295. This is further supported by the value of $\Delta\Delta H^{\ddagger}$ for 2-pentanols with mutant S39T. Substitution of the large pocket residue serine 39 by the larger threonine results in a modest lowering of $\Delta\Delta H^{\ddagger}$ from 2.8 cal/ºC*mol for the wild type to 0.6 cal/ºC*mol for the mutant which would indicate a slight steric interaction. Values of $\Delta\Delta H^{\ddagger}$ for 2-butanol with both C295L and C295V are negative indicating a better steric fit with the ethyl group in the small pocket. The $\Delta\Delta H^{\ddagger}$ for 2-hexanol with mutant C295V is positive, which may be due to the steric bulk of valine's β-methyl, where the $\Delta\Delta H^{\ddagger}$ of 2-hexanol with C295L is negative which may be due to absence of active site water, and leucine's smaller van der Waal's radius at the β-position.

Mutant C295L shows negative values of $\Delta\Delta S^{\ddagger}$ for 2-butanol and 2-hexanol which supports our hypothesis that rotational restriction of a large side chain should result in a decrease in entropy for the (R)-enantiomer and increasingly favor the (S)-enantiomer with increasing temperature. Interestingly, the $\Delta\Delta S^{\ddagger}$ for 2-pentanol is positive and similar in value to those for wild type and C295A SADH.

Mutant C295V shows an inversion of temperature dependence for both 2-butanol and 2-pentanol. Below 35º C, the large negative value for $\Delta\Delta H^{\ddagger}$ for 2-butanol indicates a preference for the (R)-enantiomer and the very large negative value for $\Delta\Delta S^{\ddagger}$ indicating a sizable loss in entropy possibly associated with restriction of the C295V side chain. However, above 30º C, there is an apparent reversal in this trend, although values for $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ associated with this have yet to be established. The inversion temperature for 2-pentanol is similar to that for the 2-butanol, but the associated values for $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ are opposite in sign. The positive $\Delta\Delta H^{\ddagger}$ for 2-pentanol below 35º C indicates a better steric fit with the propyl group oriented in the large pocket and the positive value for $\Delta\Delta S^{\ddagger}$ indicates a favorable entropy for the (R)-enantiomer where the larger alkyl group is in the small pocket. Conversely, above 30º C the negative value for $\Delta\Delta H^{\ddagger}$ indicates a steric preference for the (R)-enantiomer where the alkyl group is in the small pocket, and the large negative $\Delta\Delta S^{\ddagger}$ indicates a loss of entropy with the alkyl group in the small pocket which is consistent with our hypothesis. A plausible explanation for the observed inversion in temperature dependence is described by the isoinversion principle,[33] which attributes the non-linearity of these data to a change in rate-limiting step in a two step mechanism. (Scheme 3.1).



Scheme 3.1 Catalysis according to the Isoinversion Principle. **E** is enzyme, **S** is substrate, **P** is product.

The first step in scheme 1 is binding, or formation of the enzyme-substrate complex. The second step is catalysis, or transformation of the enzyme-substrate

complex to the enzyme-product complex. These two steps favor opposite enantiomers, and have different activation energies.

The kinetic data for the 2-hexanol with C295A shows a consistent temperature dependence trend for enantioselectivity with a positive value for $\Delta\Delta H^{\ddagger}$ and a negative value for $\Delta\Delta S^{\ddagger}$. This indicates a preference of the larger alkyl group for the large binding pocket, and the entropy term suggests an increase in entropy with the alkyl group in the small pocket. A possible explanation for this within the confines of our hypothesis is that 2-pentanol's butyl group adopts a conformation within the small alkyl pocket which allows for free rotation of the valine's isopropyl group.

Bibliography

1. Wong, C., Whitesides, G.M., *Enzymes in Synthetic Organic Chemistry*; Tetrahedron Organic Chemistry Series, 1994 (12) Elsevier Science Series, Oxford. 1-40, 142-182
2. Drauz, K., Waldmann, H., *Enzyme Catalysis in Organic Synthesis: A Comprehensive Handbook Vol. II*, 1995, VCH Press, New York, 600-616
3. Brock, T.D., *Thermophiles, General, Molecular, and Applied Microbiology*, 1986, John Wiley and Sons, New York
4. Rothschild, L.J., Mancinelli, R.L., *Nature*, **409**,1092-1101 (2001)
5. Madigan, M.T., Marrs, B.L., *Scientific American*, April, 67-71 (1997)
6. Lamed, R.J., Keinan, E., Zeikus, J.G., *Enzyme Microb. Technol*., **3**, 144-148 (1981)
7. Branden, C.I., Jornvall, H., Eklund, H., Furugren, B., *The Enzymes*, Boyer P.D., Ed., 1975 (IX-A) Academic Press, New York, 103-190
8. Faber, K. *Biotransformations in Organic Chemistry*, 1995, Springer-Verlag, New York, 145-173
9. Hammel, W., Kula, M.R., *Eur. J. Biochem.,* **184**, 1-13 (1981)
10. Bryant, F.O., Wiegel, J., Ljungdahl,L.G., *Appl. Environ. Microbiol.,* 460-465 (1998)
11. Bryant, F.O., Ljungdahl, L.G., *Biochem. Biophys. Res. Commun.,* **100**, 793-799 (1981)
12. Lamed, R., Zeikus, J.G., *Biochem. J*., **195**, 183 (1981)
13. Lamed, R. Zeikus, J.G., *J. Bacteriol.,***141**, 1251 (1980)
14. Lamed, R.J., Keinan, E., Zeikus, J.G., *Enzyme Microb. Technol.,* **3**, 144-148 (1981)
15. Keinan, E., Hafeli, F.V., Seth, K.K., Lamed, R.J., *J. Am. Chem. Soc*., **108**, 162 - 169 (1986)
16. Keinan, E., Seth, K.K., Lamed, R.J., *J. Am. Chem. Soc.,* **108**, 3474 (1986)

17. De Amici, M., De Micheli, C., Carrea, G., Spezia, S., *J. Org. Chem*., **54**, 2646-2650 (1989)

18. Keinan, E., Sinha, S.C., Singh, S.P., *Tetrahedron*, **47**, 4631-4638 (1991)

19. Zheng, C., Pham, V.T., Phillips, R.S., *Bioorg. Med. Chem. Lett.,* **2**, 619-622 (1992)

20. Heiss, C., Phillips, R.S., *J. Chem. Soc., Perkin Trans.1*., 2821 (2001)

21. Zheng, C., Pham, V.T., Phillips, R.S., *Catal. Today*, **22**,607-620 (1994)

22. Pham, V.T., Phillips, R.S., Ljungdahl, L.G., *J. Am. Chem. Soc.,* **111**, 1935-1936 (1989)

23. Pham, V.T., Phillips, R.S., *J. Am. Chem. Soc.,* **112**, 3629-3632 (1990)

24. Secundo, F., Phillips, R.S., *Enzyme Microb. Technol.,* **19**, 487-492 (1996)

25. Burdette, D.S., Vieille, C., Zeikus, J.G., *Biochem. J*., **316**, 115-122 (1996)

26. Burdette, D.S., Secundo, F., Phillips, R.S., Dong, J., Scott, R.A., Zeikus, J. G., *Biochem. J.,* **326**, 717-724 (1997)

27. Tripp, A.E., Burdette, D.S., Zeikus, J.G., Phillips, R.S., *J. Am. Chem. Soc*., **120**, 5137-5141 (1998)

28. Heiss, C., Laiveneiks, M., Zeikus, J.G., Phillips, R.S., *Bioorg. Med. Chem*., **9**, 1659-1666 (2001)

29. Cleland, W.W., *Methods Enzymol*., **63**, 103-138 (1979)

30. Bradford, M., *Anal. Biochem*., **72**, 248-254 (1972)

31. Li, C., Heatwole, J., Saelaiman, S., Shoham, M., *Proteins*, **37**, 619 (1999)

32. Heiss, C., Laivenieks, M., Zeikus, J.G., Phillips, R.S., *J. Am. Chem. Soc*., **123**, 345-346 (2001)

33. Buschmann, H.H., Scharf, H.D., Hoffmann, N., Esser, P., *Angew. Chemie*, Intl. Ed., **30,** 477-515 (1991)

Figure 3.1. Temperature dependence of the enantiospecificity of wild-type and C295L SADH. Filled symbols and dotted fit lines are for the oxidation of alcohols by wild-type SADH. Open symbols with solid fit lines are for C295L. Diamonds are for 2-butanol, triangles are for 2-pentanol, and squares are for 2-hexanol. Temperature is in K, units for –RTlnE are cal/mol.

-RTlnE (cal/mol)
2300
1800
1300
800
300
-200
-700
-1200
285
295
305
315
325
335
345
Temperature (K)

Figure 3.2. Temperature dependence of the enantiospecificity of wild-type and C295V SADH. Filled symbols and dotted fit lines are for the oxidation of alcohols by wild-type SADH. Open symbols with solid fit lines are for C295V 2-butanol, K_2 2-pentanol, and 2-hexanol. The dashed line refers to 2-pentanol K_1. Diamonds are for 2-butanol, triangles are for 2-pentanol, and squares are for 2-hexanol. Temperature is in K, units for –RTlnE are cal/mol.

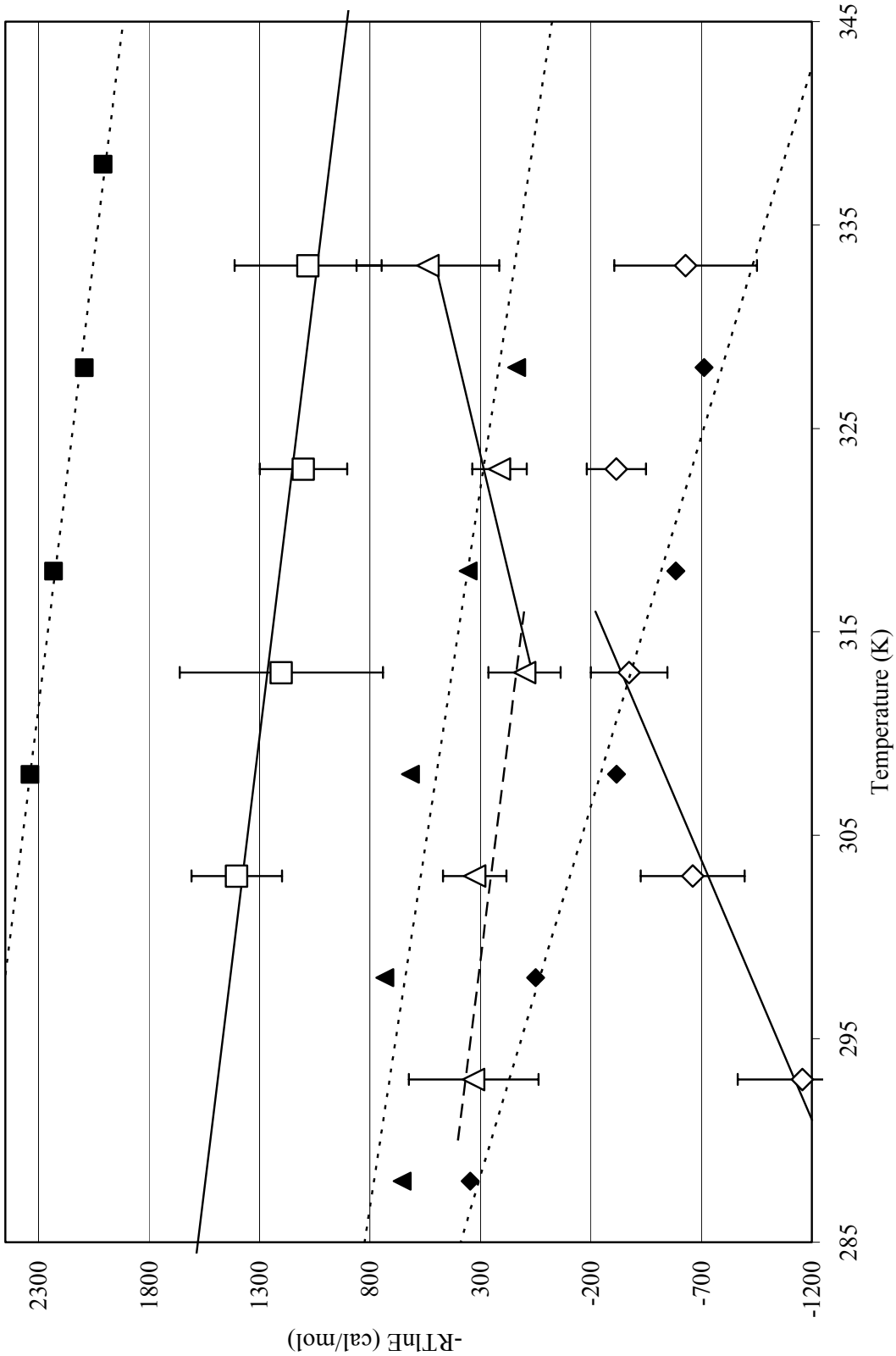
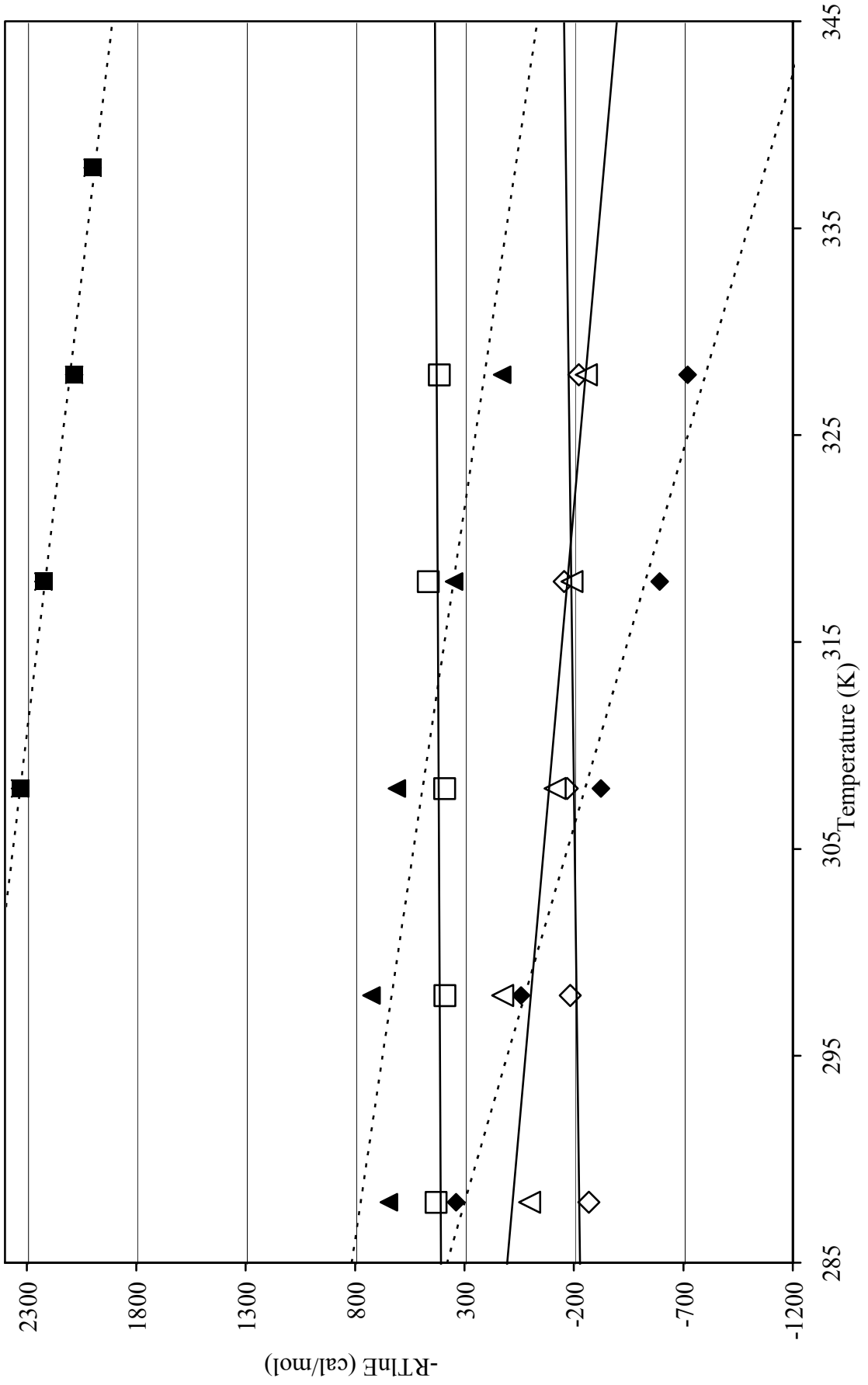

-RTlnE (cal/mol)
2300
1800
1300
800
300
-200
-700
-1200
285
295
305
315
325
335
345
Temperature (K)

Figure 3.3. Temperature dependence of the enantiospecificity of wild-type and C295A SADH. Filled symbols and dashed fit lines are for the oxidation of alcohols by wild-type SADH. Open symbols with solid fit lines are for C295A. Diamonds are for 2-butanol, triangles are for 2-pentanol, and squares are for 2-hexanol. Temperature is in K, units for –RTlnE are cal/mol.


-RTlnE (cal/mol)
2300
1800
1300
800
300
-200
-700
-1200
285
295
305
315
325
335
345
Temperature (K)

CONCLUSION

As chemistry is the central science, organic chemistry is the central chemistry. All disciplines of the chemical sciences converge with the study of carbon and its molecules, and this dissertation in organic chemistry reflects this wide scope. Subject matter addressed in the three chapters of this edition include: organic synthesis, organic spectroscopy, physical organic chemistry and chemoenzymology.

The use of isotopically labeled probes for the study of enzyme catalysis by NMR is a promising technique. With this method, a single atom of a substrate can be observed as it interacts with an enzyme containing hundreds of like atoms. In chapter I, ^{15}N-L-tryptophan was complexed with wild type tryptophan synthase and mutants K87T, E109D, and D305A with and without the presence of the α-subunit ligand, α-glycerophosphate. The NMR signals observed provide evidence confirming allosteric regulation, and provided specific information about the roles of specific residues. Future work will investigate the use of ^{13}C isotopomers of L-tryptophan, L-serine and PLP as NMR probes.

Derivatives of L-tryptophan with aromatic substituents are important targets for organic synthesis. Chapter II is the report of our preparation of protected 2- and 6-nitro-L-tryptophan in good yield. These synthons open the door for the preparation of many other derivatives via functional group interconversion by Sandmeyer chemistry. Future work will focus on the efficient transformation of the nitro group to the corresponding amino group, and on the preparation of other nitro-L-tryptophan isomers.

Secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* is the subject of chapter III. SADH is among the most versatile and effective biocatalytic agents available to the organic chemist. This enzyme exhibits increased tolerance to temperature and organic solvents compared to other enzymes of synthetic value, and is effective for the reduction and oxidation of a wide range of substrates. The results of our kinetic evaluation of small alkyl binding pocket mutants C295L and C295V indicate that

enantioselectivity and temperature dependence on enantioselectivity is partly determined by the polarity and steric effects of this residue. Further work will explore the catalytic effects of substituting cysteine 295 with other residues exhibiting branching at the β carbon, such as serine and isoleucine. The ultimate goal of this project is the development of a mutant of SADH that can accommodate a wide latitude of substrates, is not inactivated by other functional groups, and catalyzes transformations with high enantioselectivity.