MECHANISTIC STUDIES, OPTIMIZATION, AND APPLICATIONS OF THE STAUDINGER LIGATION

by

Matthew B. Soellner

A dissertation submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

(Chemistry)

at the

UNIVERSITY OF WISCONSIN–MADISON

2005

A dissertation entitled

MECHANISTIC STUDIES, OPTIMIZATION, AND
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submitted to the Graduate School of the
University of Wisconsin-Madison
in partial fulfillment of the requirements for the
degree of Doctor of Philosophy

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MATTHEW BRYAN SOELLNER

Date of Final Oral Examination: December 9, 2005

Month & Year Degree to be awarded: **December** 2005 **May** **August**

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MECHANISTIC STUDIES, OPTIMIZATION, AND APPLICATIONS OF THE STAUDINGER LIGATION

Matthew B. Soellner

Under the supervision of Professor Ronald T. Raines

At the University of Wisconsin–Madison

The ligation of synthetic peptides provides a convergent route for the total chemical synthesis of proteins. Currently, the most common ligation method is "native chemical ligation". A limitation of native chemical ligation is its intrinsic reliance on having a cysteine residue at the ligation juncture. Cysteine is uncommon, and most proteins cannot be prepared by any method that allows peptides to be coupled only at cysteine residues. This thesis describes work to overcome this restriction with a new chemical ligation method, the Staudinger ligation.

The Staudinger ligation provides a means to form a native amide bond between a peptide with a *C*-terminal phosphinothioester and a peptide with an *N*-terminal α-azido acid residue. The most efficacious phosphinothiol is shown to be (diphenylphosphino)methanethiol, which provides high isolated yields (>90%) for couplings involving a glycine at the ligation site. The peptide product of this reaction is free of racemization. Additionally, a high-yielding and rapid synthesis of (diphenylphosphino)methanethiol is reported.

An important feature of the Staudinger ligation with (diphenylphosphino)methanethiol thioesters is the limited reactivity of the azido group in biological systems. Because of

this high chemoselectivity, the Staudinger ligation is an ideal reaction for the immobilization of peptides or proteins. A general method is described for the site-specific attachment of biological anayltes and certified with the immobilization of fully functional ribonuclease A to a glass surface.

Although the Staudinger ligation remains an ideal reaction for couplings involving a glycine, it is desirable to determine reaction conditions that would allow for the ligation between any two residues. To facilitate further optimization, a sensitive and continuous assay based on ^{13}C NMR spectroscopy is developed and used to elucidate the reaction mechanism and kinetics of the Staudinger ligation. Based on these data, a novel class of phosphinothiol reagents is discovered that allows for the Staudinger ligation between two non-glycyl residues at the ligation site in high isolated yields (up to 84%).

Acknowledgements

Many individuals have contributed to the production of this thesis. I consider myself extremely fortunate in the associations I have enjoyed during my time here at the University of Wisconsin. On both a professional and personal level I have worked with people who have made my graduate school experience extremely enjoyable. I am profoundly grateful for the impact my associates have had on me.

I wish to especially acknowledge Professor Ron Raines. He has been a tremendous advisor. He gives his students a great deal of freedom to develop new ideas. His insight and creativity have taught me to be more imaginative in my own approach to science. In addition, his enthusiasm for science is very contagious and has made me even more excited and interested in chemistry during my time in the Raines lab.

Professor Laura Kiessling also deserves special acknowledgement. The original idea for a traceless form of the Staudinger ligation was hers. At the time I started working on this project meetings with her and her students were of great importance in our desire to optimize the Staudinger ligation reaction. Two of her students, Drs. Ron Hinklin and Yi He provided helpful suggestions in the early stages of the work presented in this thesis.

My interactions with all the faculty in the department of chemistry have been very positive. I particularly need to acknowledge Professor Hans Reich for sharing his expertise in dealing with phosphines and thiols with me.

Dr. Brad Nilsson deserves special acknowledgement and recognition for serving as a mentor to me while he was a fellow graduate student in the Raines lab. He was incredibly generous with his time and ideas. His help was of tremendous importance to me and enabled much of the work presented in this thesis.

Annie Tam joined the Staudinger ligation project in my third year and was a tremendous

help and asset to the project. Her hard work and determination are insipiring. She also became a good friend in my later years and for that I am very greatful. Dr. Jonathan Hodges, a postdoc in the Raines lab during much of my time as a graduate student was a very positive influence on my thesis work. Along with his helpful advice on synthetic routes, Jonathan introduced me to the world of computational chemistry. The power of this technique was used in Chapter 5 of this thesis for the optimization of Staudinger ligation reagents. Bryan Smith, a biochemist in the Raines lab, served as a great colleague and I was fortunate enough to be able to work with him on his OVS-RNase A projects. Luke Lavis was a wonderful person to share a lab with. His optimism and knowledge led to some promising collaborations which unfortunately never were successful.

The students and postdocs I've worked with in the Raines lab have given me stories to tell for a long time to come. I'm grateful to know them and for the impact they've had on my work, and for making my experience here as fun as it has been. I thank Ken, Cara, Josh, Luke, Jason, Eric, and, Frank (the chemists) for making the lab a great place to work. The Raines lab biochemists (too numerous to mention individually) also deserve recognition for their helpful scientific advice.

The scientific staff at the University of Wisconsin is also tremendous. I thank Dr. Milo Westler and Dr. Charlie Fry of the NMR facilities for their assistance with carrying out many of our NMR studies.

Thanks to Drs. Tim Jackson, Troy Stich, Tom Clark, Brad Steinhoff, and to Ryan Nelson and Rachel Weller for their friendships while in graduate school. I was lucky to have met each of these people and they were great friends whom I shared many wonderful times with.

Finally, I thank my parents for their profound impact on my life. Their support in all forms can never be repaid. Their love and encouragement have been a constant to me.

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List of Abbreviations

Ala	alanine
Ac	acetyl
Asn	asparagine
Bn	benzyl
Boc	*tert*-butoxycarbonyl
CBD	chitin binding domain
Cys	cysteine
DABCO	1,4-diazabicyclo[2.2.2]octane
DCC	dicyclohexylcarbodiimide
DCU	dicyclohexylurea
DIEA	diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	*N*,*N*-dimethylformamide
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol
ESI	electrospray ionization
EtOAc	ethyl acetate
Fmoc	9-fluorenylmethoxycarbonyl
GFP	green fluorescent protein
Glu	glutamic acid

Gly	glycine
HATU	(*N*-[dimethylamino)-1*H*-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene-*N*-methylmethanaminium hexafluorophosphate *N*-oxide
HOBt	*N*-hydroxybenzotriazole
HPLC	high performance (pressure) liquid chromatography
Leu	leucine
Lys	lysine
MALDI	matrix-assisted laser desorption ionization
MS	mass spectrometry
NMR	nuclear magnetic resonance
Phe	phenylalanine
Pro	proline
PyBOP	benzotrizole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
rDNA	recombinant DNA
RNase A	ribonuclease A
RNase S	ribonuclease S
*t*Bu	*tert*-butyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Trt	trityl
UV	ultraviolet
Val	valine

Chapter 1*

CHEMICAL SYNTHESIS OF PROTEINS

1.1 Introduction

The human genome contains 30,000 or so genes (Lander et al., 2001; Venter et al., 2001). Scientists from a broad range of disciplines are now working to reveal the structure and function of the proteins encoded by these genes. Their findings could lead to the solution of a multitude of problems in biology and medicine. In addition to structure—function analyses of extant proteins, protein chemists are working to create new proteins with desirable properties, either by *de novo* design or by altering natural frameworks.

The study of natural proteins and the creation of non-natural ones requires the ability to access and manipulate proteins. The isolation of proteins from their natural source is often tedious, idiosyncratic, and impractical. In contrast, the production of proteins with recombinant DNA (rDNA) technology, either in a heterologous host or *in vitro*, can provide access to large quantities of protein and allows for the exchange of one of the 20 common amino acid residues for another. Yet, aggregation often limits the yield of properly folded proteins produced with rDNA. Moreover, the restrictions of the genetic code severely limit the possible modifications.

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* This chapter has been published, in part, under the same title. Reference: Nilsson, B. L.; Soellner, M. B.; Raines, R. T. *Annu. Rev. Biophys. Biomol. Struct.* **2005**, *34*, 91-118.

The chemical synthesis and semisynthesis of proteins harbors the potential to overcome many of the disadvantages of current protein production methods (Borgia & Fields, 2000; Casi & Hilvert, 2003; Kent, 2003). In particular, chemical synthesis using established solid-phase techniques are rapid to effect and easily automated, and facilitate purification. Accordingly, the application of existing and emerging synthetic methods could facilitate research in all aspects of protein science.

Chemical synthesis enables the facile incorporation of non-natural functionality into proteins. The genetic code limits the components of natural proteins to 20 or so α-amino acids. Methods that overcome this limitation but still rely on the ribosome are similarly limited to a subset of α-amino acids and α-hydroxy acids. In marked contrast, the non-natural functionality made available by chemical synthesis is limited only by the constraints of the periodic table and the imagination of protein chemists.

The desire to synthesize proteins is not new. On December 12, 1902, Emil Fischer delivered his Nobel Prize lecture in Stockholm, Sweden, saying in part (Fischer, 1902):

> Of the chemical aids in the living organism the ferments—mostly referred to nowadays as enzymes—are so pre-eminent that they may justifiably be claimed to be involved in most of the chemical transformations in the living cell. The examination of the synthetic glucosides has shown that the action of the enzymes depends to a large extent on the geometrical structure of the molecule to be attacked, that the two must match like lock and key. Consequently, with their aid, the organism is capable of performing highly specific chemical transformations which can never be accomplished with the customary agents. To equal Nature here, the same means have to be applied, and I therefore foresee the day when physiological chemistry will not only make extensive use of the natural enzymes as agents, but when it will also prepare synthetic ferments for its purposes.

A century later, Fischer's vision is becoming reality. Enzymes and other proteins are not only accessible targets for synthetic chemistry, but are poised to become dominant targets

of the 21st century. Herein, we discuss current efforts toward preparing proteins synthetically, focusing on the development of powerful new methodologies for splicing peptide fragments in a convergent strategy for the total chemical synthesis of proteins.

1.2 Peptide Synthesis

The chemical synthesis of proteins is now possible because of the prodigious advances in peptide synthesis that have occurred over the last century. Fischer's 1901 synthesis of glycyl glycine is the first reported synthesis of a dipeptide and is also the first instance of the term "peptide" being used to refer to a polymer of amino acids (Fischer & Fourneau, 1901). His 1907 synthesis of an octadecapeptide consisting of 15 glycine and 3 leucine residues was a remarkable achievement, despite his inability to control its amino acid sequence (Fischer & Fourneau, 1901).

An important advance in peptide synthesis was Bergmann's 1932 introduction of reversible protection for the α-amino group (Bergmann & Zervas, 1932). With the emergence of protecting group strategies, it became possible to synthesize small peptide hormones. For example, in 1953 du Vigneaud and coworkers reported a solution-phase synthesis of the octapeptide hormone oxytocin (Du Vigneaud et al., 1953). Even though fifty years had passed since Fischer's first synthesis of a peptide, these types of syntheses were still only accomplished with considerable effort (Merrifield, 1996).

The advent of solid-phase methods heralded a revolution for peptide synthesis (Merrifield, 1986). In 1963, Merrifield described the first solid-phase synthesis of a peptide, a tetrapeptide (Merrifield, 1963). He attached an amino acid to an insoluble

support via its carboxyl group and then coupled the next amino acid, which had a protected amino group and an activated carboxyl group. The amino protecting group was removed, and the next amino acid was coupled in a like manner. Within a few years, Merrifield reported the development of an instrument for the automated synthesis of peptides (Merrifield et al., 1966). In short order, he and Gutte used this new strategy to achieve the first synthesis of an enzyme, ribonuclease A (RNase A), albeit in low overall yield (Gutte & Merrifield, 1969; Gutte & Merrifield, 1971). Concurrently, a team led by Hirschmann reported the chemical synthesis of RNase S (which consists of residues 21–124 of RNase A) by solution-phase segment condensation reactions (Hirschmann et al., 1969).

Automated solid-phase peptide synthesis is commonplace today. In the most common strategy (Figure 1.1), an amino acid with both α-amino group and side-chain protection is immobilized to a resin. The α-amino protecting group is typically an acid-sensitive *tert*-butoxycarbonyl (Boc) group or base-sensitive 9-fluorenylmethyloxycarbonyl (Fmoc) group (Wellings & Atherton, 1997). These α-amino protecting groups can be removed quickly and completely, a protected amino acid with an activated carboxyl group can then be coupled to the unprotected resin-bound amine. The coupling reactions are forced to completion by the use of an excess of the activated soluble amino acid. The cycle of deprotection and coupling is repeated to complete the sequence. Side-chain deprotection and cleavage the resin yields the desired peptide (Guy & Fields, 1997; Stewart, 1997).

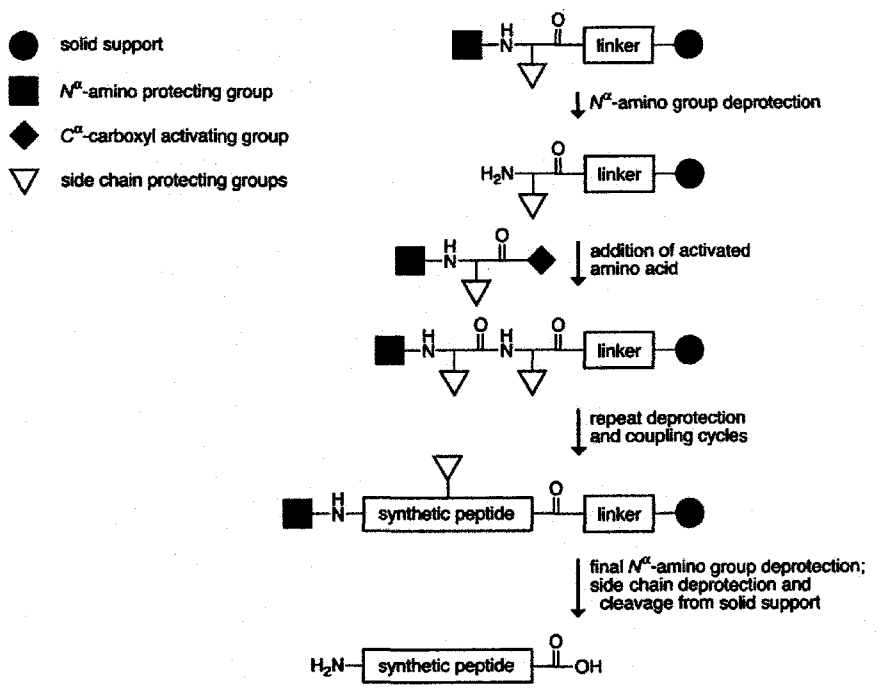


Figure 1.1 Solid-phase peptide synthesis.

The efficiency of solid-phase peptide synthesis continues to improve. New solid supports have increased the length of accessible peptides (Meldal, 1997). New linkers between the support and the peptide have diversified the conditions that can be used to liberate a synthetic peptide (Songster & Barany, 1997). New side-chain protection strategies have minimized deleterious side reactions. Finally, new carboxyl activating group have increased the speed and efficiency of amino acid couplings, while reducing the risk of epimerization (Albericio & Carpino, 1997; Miranda & Alewood, 1999).

Solid-phase peptide synthesis alone has enabled the total chemical synthesis of some proteins. Since the pioneering work of Merrifield, proteins that contain as many as 166 amino acid residues have been synthesized in this fashion (Merrifield, 1996). These syntheses have, in some cases, been critical to structure–function analyses of the target

proteins. Notably, the chemical synthesis of HIV-1 protease enabled the structural characterization of protease·inhibitor complexes (Miller et al., 1989; Wlodawer et al., 1989; Swain et al., 1990). In addition, an enantiomer of this protease was synthesized with D amino acids to demonstrate its chiral specificity for a peptide substrate derived from D amino acids (deL. Milton et al., 1992). Another notable example is a recent synthesis of a modified B1 domain of streptococcal G protein (Odaert et al., 1999). This synthesis incorporated a completely non-natural dibenzofuran-based β-turn mimic as a conformational probe. These studies highlight the true power and potential of the total chemical synthesis of proteins in enhancing our understanding of protein structure and function.

Despite the significant gains made in advancing the technology of solid-phase peptide synthesis, there remain limitations. Modern peptide synthesis is typically limited to peptides of ≤40 residues (Bray, 2003). Peptides and proteins of greater length can be prepared, although not routinely. Hence, most proteins cannot by synthesized by the stepwise assembly of amino acid monomers.

The convergent assembly of protected or partially protected peptide segments, both in solution and on solid-phase, is one approach used to access proteins that contain more than 40 residues (Lloyd-Williams et al., 1992; Albericio et al., 1997; Aimoto, 1999; Sato & Aimoto, 2003). The 238-residue precursor of the green fluorescent protein (GFP) was synthesized with this segment-condensation approach (Nishiuchi et al., 1998). A total of 26 peptide fragments corresponding to parts of the GFP were synthesized, assembled in solution, and deprotected. The resulting protein exhibited a fluorescence spectrum indistinguishable from that of the biosynthetic protein upon standing in solution. In

addition to producing a large, complex protein in its native form by chemical synthesis, this study also demonstrated unequivocally that the formation of the GFP fluorophore is not dependent on any external cofactors.

1.3 Protein Synthesis by Peptide Ligation

The formation of an amide bond between protected peptide fragments by the attack of an amino group on an activated carboxyl group can be problematic, especially with large peptides (Coltart, 2000). The problem is that both the intrinsic rate constant (k) for this reaction and the reactant concentrations are often too low for a substantial reaction velocity (v, where $v = k$[peptide 1][peptide 2]). In other words, the reactivity between an amino group and activated carboxyl group is not inherently high enough for acyl transfer to overcome the relatively low solubility of peptides.

In nature, peptide bond formation occurs by sequential transfer of the *C*-terminal acyl group of a nascent peptide chain to the α-amino group of an aminoacyl *t*RNA. The *C*-terminal acyl group is activated only as an ester with a *t*RNA, and the transfer is aided, in large part, simply by the reactants being held in close proximity by the ribosome. Proteases have been engineered to perform in a similar manner, and used for the convergent synthesis of proteins as large as RNase A.

New chemical strategies have emerged that take advantage of enforced proximity to couple peptides. These *peptide ligation* methods provide a practical and powerful means to assemble synthetic peptides into proteins. The peptides can be protected or unprotected, and coupling can occur in an aqueous or organic solvent, in solution or on a

solid support.

Peptide ligation strategies utilize three steps (Figure 1.2). An initial capture step links the peptides by a chemical reaction that is more rapid than is intermolecular acyl transfer to an amine and that uses functional groups with reactivity that is orthoganol to that in proteins. The pivotal acyl transfer step is thus rendered intramolecular, and hence can occur with maximal efficiency (Page & Jencks, 1971; Menger, 1985). In the last step, the capture moiety is released, either in a discrete chemical process or spontaneously.

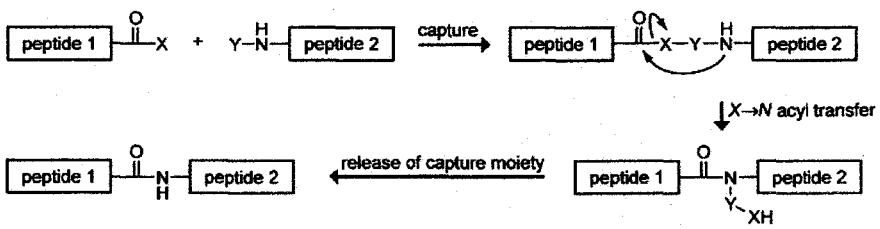


Figure 1.2 A general strategy for peptide ligation.

Below, we discuss current strategies for peptide ligation, along with their relative advantages and disadvantages. We also highlight some notable applications of these strategies. Our focus is on ligation strategies that yield native amide bonds. We do not discuss approaches that yield non-natural bonds between peptide fragments (Tam et al., 1999; Tam et al., 2001; Eom et al., 2003).

1.4 Peptide Ligation with Sulfur

1.4.1 Prior Thiol Capture

Modern methods of peptide ligation trace their origin to Kemp's *prior thiol capture* strategy (Figure 1.3). In this approach, 4-hydroxy-6-mercaptodibenzofuran is used as the association element to bring the peptide coupling partners together (Kemp & Galakatos, 1986; Kemp et al., 1986a; Kemp & Fotouhi, 1987; Fotouhi et al., 1989; Kemp & Carey, 1989). This strategy uses a highly efficient thiol–disulfide exchange reaction in a capture step prior to the acyl transfer reaction. Acyl transfer occurs with a half-life ranging from 0.1 to 50 h, depending on the bulk of the side chain of the *C*-terminal acyloxy-derived residue (Kemp et al., 1986b), and occurs without racemization of the coupled amino acids (McBride & Kemp, 1987). Upon completion of the acyl transfer reaction, phosphine reduction of the mixed disulfide yields the native peptide.

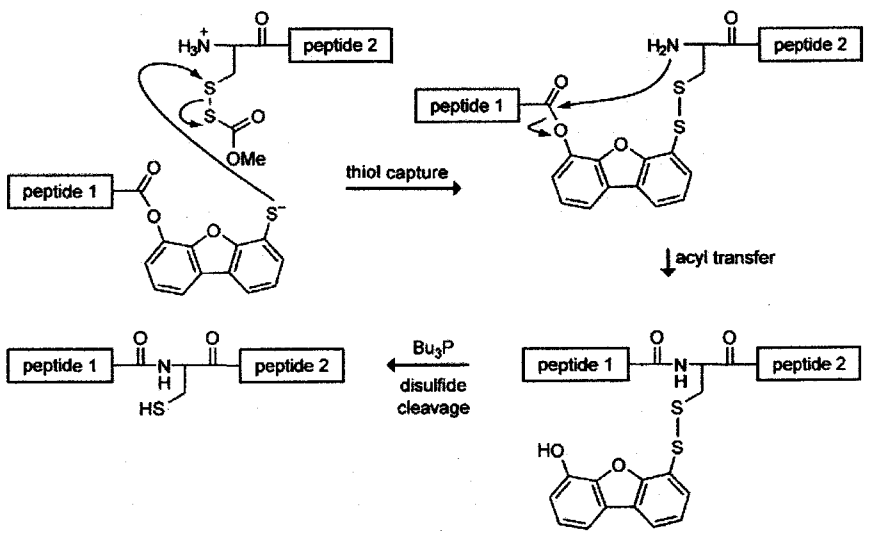


Figure 1.3 Prior thiol capture.

The utility of this approach was demonstrated by the ligation of a variety of peptide fragments. In one study, the *C*-terminal 29-residue fragment of BPTI was synthesized from four segments, each possessing an *N*-terminal cysteine residues and a *C*-terminal dibenzofuran (Fotouhi et al., 1989). The synthesis commenced with the two fragments at the extreme *C*-terminus and proceeded sequentially towards the *N*-terminus. The *N*-terminal cysteine residues were protected orthogonally until they were needed for coupling, when they were deprotected and derivatized as mixed disulfides. In a separate study, a 39-residue peptide and a 25-residue peptide were synthesized in high yield utilizing side-chain protection only at cysteine residues (Kemp & Carey, 1993). The ligation of fully protected peptides was 50-fold slower.

Kemp's prior thiol capture strategy is a seminal contribution to the development of peptide ligation concepts and methodology. It represents the first demonstration of the chemoselective ligation of unprotected peptide fragments. In addition, it represents the first systematic application of the use of proximity effects to evoke acyl transfer via an intramolecular reaction.

1.4.2 Native Chemical Ligation

Currently, the most common ligation method is *native chemical ligation* (Figure 1.4). The chemical foundation for this reaction was discovered by Wieland in 1953, when the reaction of ValSPh and CysOH in aqueous buffer was shown to yield the dipeptide: ValCysOH (Wieland et al., 1953). The reaction proceeded through the intermediacy of a thioester containing the sulfur of the cysteine residue. In the 1990's, Kent developed this

reaction into a practical method to ligate large peptide fragments (Dawson et al., 1994; Dawson & Kent, 2000; Kent, 2003). In native chemical ligation, the thiolate of an *N*-terminal cysteine residue of one peptide attacks the *C*-terminal thioester of a second peptide to effect a transthioesterification. An amide linkage forms after rapid *S*→*N* acyl transfer.

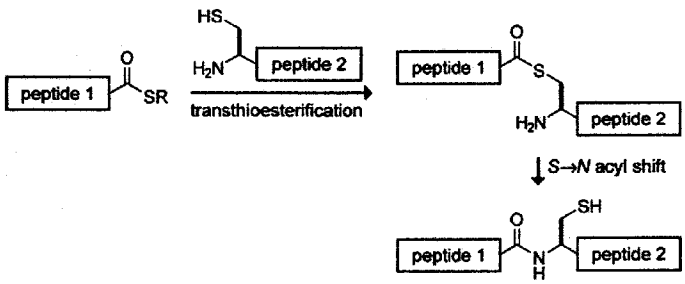


Figure 1.4 Native chemical ligation.

Native chemical ligation is a powerful approach for the total chemical synthesis of proteins. Ligations are effected in aqueous solution with unprotected peptides (Muir et al., 1997). A chaotrope such as guanidinium chloride or urea is added to discourage peptide aggregation and structure. Transthioesterification with embedded cysteine residues does not lead to amide bond formation and hence is reversible. The ligation rate can be modulated by the addition of exogenous thiols (Dawson et al., 1997). Ligation can also occur between cysteine and any of the other common amino acids, though ligation to hindered amino acids such as valine, isoleucine, and proline occurs relatively slowly (Hackeng et al., 1999). Although normally carried out in solution, native chemical ligation has been used to couple unprotected peptide fragments on a solid support

(Camarero et al., 1998; Canne et al., 1999; Brik et al., 2000).

The application of native chemical ligation in the synthesis of proteins is burgeoning. Some recent examples of target proteins include human matrix Gla protein (84 residues) (Hackeng et al., 2001), the anticoagulant microprotein S (116 residues) (Hackeng et al., 2000), and human neutrophil pro α-defensin-1 (75 residues) (Wu et al., 2003). Proteins with post-translational modifications that are inaccessible with rDNA techniques have been synthesized successfully with native chemical ligation. These include the glycoproteins diptericin (58 residues) (Shin et al., 1999) and lymphotactin (93 residues) (Marcaurelle et al., 2001). The prion protein has also been synthesized with appropriate mimics of its glycosylphosphatidyl inositol anchor (Ball et al., 2001).

A powerful aspect of the chemical synthesis of proteins is the ability to incorporate non-natural residues as probes of structure and function, or for therapeutic purposes. Native chemical ligation has yielded many important examples of modified synthetic proteins. The protooncogene H-Ras (166 residues) and its binding partner, the Ras-binding domain (81 residues), have been synthesized with the site-specific incorporation of fluorescent tags to probe the interactions between these two proteins (Becker et al., 2001; Becker et al., 2003). In a particularly noteworthy example, a synthetic polyethylene glycol-modified erythropoiesis protein (166 residues) has been synthesized by native chemical ligation (Kochendoerfer et al., 2003). The appended polymer chains serve to prolong its half-life in vivo and hence its potent biological activity.

A one-pot total synthesis of a crambin has been performed in high-yield by the sequential coupling of three synthetic polypeptide chains. This synthesis of crambin (46 residues) did not require purification of the intermediates and afforded high yields of

intact protein. Additionally, folding of the synthetic protein was performed in the reaction mixture prior to purification.

1.4.3 Expressed Protein Ligation

An intein is a natural splicing element that is analogous to the intron of nucleic acids. An intein mediates its own excision from a peptide sequence through a series of acyl transfer reactions that ultimately result in the splicing of its flanking peptides. In 1998, Muir, Evans, and their coworkers appropriated the chemistry of intein-mediated, post-transcriptional protein splicing for protein semisynthesis (Evans et al., 1998; Muir et al., 1998; Severinov & Muir, 1998). In their *expressed protein ligation* (Figure 1.5), the fusion of a target protein to a modified intein is used to install a thioester at the *C*-terminus of the target protein. The fusion protein can be bound to an affinity resin, where the incipient thioester can be trapped with a small-molecule thiol, thus eluting the target protein as a simple *C*-terminal thioester and leaving the remainder of the fusion protein bound to the affinity resin.

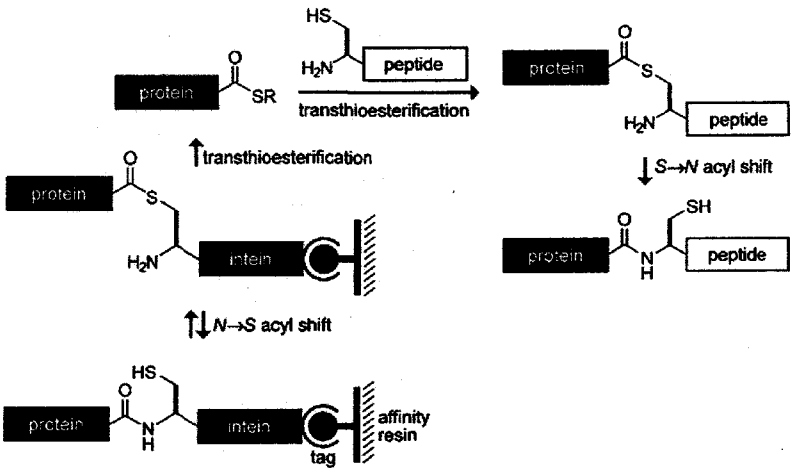


Figure 1.5 Expressed protein ligation.

The semisynthesis of proteins using expressed protein ligation represents a powerful union of chemistry and biology. In effect, the use of rDNA to produce protein fragments increases the size of proteins that are accessible to native chemical ligation (Figure 1.5, *cf*: Figure 1.4). The bulk of a target protein can be accessed by biosynthesis and then ligated to a peptide prepared by chemical synthesis.

Expressed protein ligation has achieved widespread use in protein structure–function analyses (Goody et al., 2002; Muir, 2003). In particular, the semisynthesis of proteins that are phosphorylated, glycosylated, or labeled with isotopes or fluorophores on specific residues is leading to notable insights. In addition, expressed protein ligation has been used to produce a functional single-chain antibody with a *C*-terminal thioester, which could be the basis of a new class of diagnostic and therapeutic agents (Sydor et al., 2002).

Expressed protein ligation has facilitated protein "prosthesis". In two examples, reverse turns in RNase A were replaced with synthetic reverse-turn mimics: dinipecotic acid (Arnold et al., 2002) and dimethylproline (Arnold et al., 2003) (Figure 1.6). The

incorporation of these turn mimics increased the thermal stability of the protein, and the variant containing dimethylproline folded more quickly.

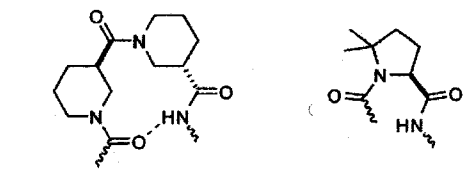


Figure 1.6 Prosthetic modules installed into RNase A by expressed protein ligation (Arnold et al., 2002; Arnold et al., 2003).

Intein-mediated protein splicing has been induced to occur in living cells, enabling new biological applications. One strategy involves splitting an intein into two fragments (Mootz & Muir, 2002; Giriat & Muir, 2003; Mootz et al., 2003). When these fragments associate noncovalently, the activity of the intein is regained and the ensuing splicing event can produce a functional protein. Fragment association can be modulated by a cell-permeable small molecule. The binding of a small molecule has also been used to activate an intact intein, which can then activate a target protein.

1.4.4 Chemical Modification of Ligated Peptides

The utility of the native chemical ligation approach has been expanded by chemical modification. In these strategies, an additional step allows ligation with a thiol-containing residue, but yields a protein that does not contain a thiol. To date, this approach has been used in only a few specific instances.

One such example is native chemical ligation at cysteine followed by desulfurization (Yan & Dawson, 2001). This strategy employs standard native chemical ligation using a cysteine residue. After the ligation step, chemical desulfurization of the cysteine residue gives alanine at the ligation junction (Figure 1.7). Desulfurization has been effected by hydrogen gas and a palladium catalyst. Three proteins have been synthesized with this strategy: streptococcal protein G B1 domain (56 residues), a variant of barnase (110 residues), and the cyclic antibiotic microcin J25 (21 residues). All thiols in a protein target will be reduced by known desulfurization conditions, but disulfide bonds are unaffected, making this strategy applicable to proteins that contain cystine but not cysteine residues.

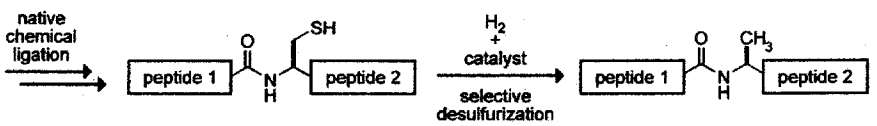


Figure 1.7 Ligation at an incipient alanine residue.

A second example yields methionine at the splice site by ligation with homocysteine followed by its chemical methylation to form methionine (Tam & Yu, 1998). This ligation proceeds initially in a nearly identical fashion to native chemical ligation (Figure 1.8, *cf*: Figure 1.4). Homocysteine effects *S*→*N* acyl transfer efficiently, despite having an additional methylene group in its side chain. After acyl transfer, sulfur methylation is effected with methyl *p*-nitrobenzenesulfonate. An excess of the methylating reagent is used, but the methylation of amino groups is avoided by limiting reaction times. Again,

this method is limited to targets that lack a cysteine residue.

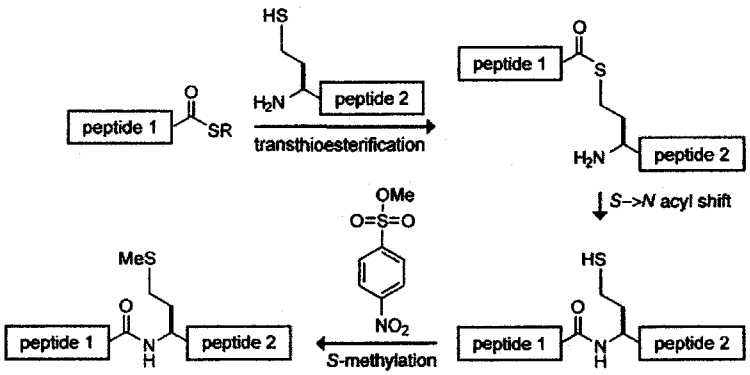


Figure 1.8 Ligation at an incipient methionine residue.

1.4.5 Acyl-Initiated Capture

Another original approach to peptide ligation is the *in situ* generation of a cysteine thioester by acyl-intiated rather than side-chain–initiated capture (Tam et al., 1995). In this method, Tam reacted a peptide having a *C*-terminal thioacid with a second peptide having *β*-bromoalanine as its *N*-terminal residue to generate a thioester analogous to that formed during native chemical ligation (Figure 1.9, *cf*: Figure 1.4). This intermediate then undergoes *S*→*N* acyl transfer, resulting in a native amide bond with a cysteine residue at the ligation junction. A disadvantage of this method is that bromoalanine is prone to aziridine formation and other undesirable side reactions.

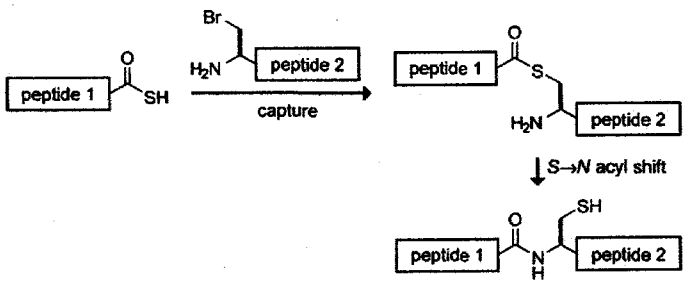


Figure 1.9 Acyl-initiated capture.

1.5 Peptide Ligation with Selenium

Selenocysteine is a natural amino acid residue with a low natural abundance. During the biosynthesis of selenoproteins, selenocysteine (Sec or U) is incorporated by the ribosomal translation of *m*RNA and has its own *t*RNA^{Sec} and codon, UGA (which is also the *opal* stop codon). Decoding a UGA codon for selenocysteine requires a unique structure in the 3′ untranslated region of the *m*RNA called a *s*elenocysteine *i*nsertion *s*equence (SECIS) element. The production of eukaryotic selenocysteine-containing proteins in prokaryotes is problematic because eukaryotic and prokaryotic cells use a different SECIS element (Arner et al., 1999).

The feasibility of using selenocysteine in native chemical ligation has been demonstrated with model systems (Gieselman et al., 2001; Hondal et al., 2001; Quaderer et al., 2001). pH–Rate profiles have demonstrated that a ligation with a selenocysteine residue occurs much more readily than does one with cysteine (Hondal et al., 2001), as expected from the lower pK_a of selenols and the somewhat higher reactivity of selenates. For example, the reaction with selenocysteine is 10^3-fold faster at pH 5.0 than that with cysteine.

Selenocysteine has been used to mediate expressed protein ligation (Hondal et al., 2001; Hondal & Raines, 2002). rDNA technology was used to prepare a fragment corresponding to residues 1–109 of RNase A with a *C*-terminal thioester. Standard solid-phase methods were used to synthesize a peptide corresponding to residues 110–124, but with selenocysteine rather than cysteine as residue 110. The thioester fragment and the peptide fragment were then ligated and the product was folded and purified. The integrity of the desired C110U variant was verified by mass spectrometry and its wild-type enzymatic activity. The data indicate that C110U RNase A is not only an intact protein, but a correctly folded enzyme with a selenosulfide (Se–S) bond between Sec110 and Cys56.

A semisynthesis of a selenocysteine-containing variant of azurin has also been achieved with expressed protein ligation (Berry et al., 2002). Cys112 was replaced with selenocysteine and fragment 112–128 was ligated to fragment 1–111. The variant azurin protein displayed unique spectral properties that were used to reveal the nature of the enzyme–copper interaction in the native protein.

The isomorphous replacement of sulfur with selenium can stabilize a protein. Specifically, proteins containing selenosulfide bonds should have greater conformational stability in a reducing environment than proteins with disulfide bonds, as the reduction potential of a selenosulfide bond is much less than that of the corresponding disulfide bond (Besse et al., 1997). This use of selenocysteine to stabilize an enzyme represents another form of protein prosthesis.

Selenomethionine has been incorporated into bovine pancreatic polypeptide with a strategy similar to that used for methionine (*cf*: Figure 1.8) (Roelfes & Hilvert, 2003).

Here, native chemical ligation of a peptide fragment having an *N*-terminal selenohomocysteine residue was used to effect native chemical ligation. Subsequent *Se*-methylation yielded a selenomethionine residue. This method could have general applicability, as the methylation of selenium should be chemoselective at acidic pH.

1.6 General Strategies for Peptide Ligation

A limitation of native chemical ligation is its intrinsic reliance on having a cysteine residue at the ligation juncture. Cysteine is the second least common amino acid, comprising only 1.7% of all residues in proteins (Figure 1.10). Hence, most proteins cannot be prepared in their native form by any method that allows for peptides to be coupled only at cysteine residues. Non-native cysteine residues have, however, been added to enable protein synthesis by native chemical ligation (Kochendoerfer et al., 2003) or semisynthesis by expressed protein ligation.

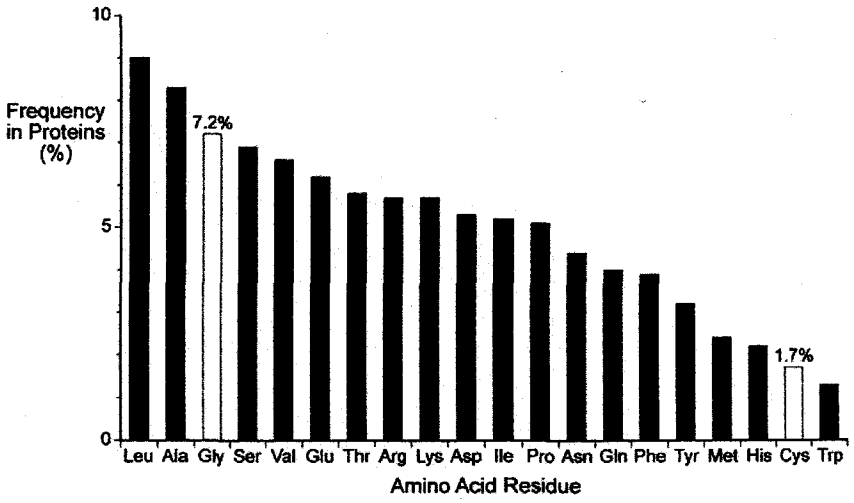


Figure 1.10 Frequency of occurrence of amino acid residues in the primary structures of 1021 unrelated proteins (McCaldon & Argos, 1988b). The most common residues at ligation junctions in synthetic proteins are cysteine (as in native chemical ligation) and glycine (*vide infra*).

The addition of non-native cysteine residues can incur risk. Of the functional groups in the 20 common amino acids, the thiolate of cysteine is by far the most reactive towards disulfide bonds, $O_2(g)$, and other electrophiles. In addition, cysteine can suffer base-catalyzed β-elimination to form dehydroalanine, which can undergo further deleterious reactions. The side chains of non-native cysteine residues have been alkylated (*e.g.*, with bromoacetate to form 4-thiahomoglutamate (Kochendoerfer et al., 2003)) to minimize these risks.

The removal of the cysteine limitation by applying a more general ligation technology (Figure 1.2) would extend greatly the utility of protein synthesis (Cotton & Muir, 1999). An ideal technique would enable complete and rapid ligation between any two amino

acid residues without detectable epimerization. Below, we describe three such strategies.

1.6.1 Conformationally Assisted Ligation

A peptide with a *C*-terminal thioester can be ligated with a second peptide that does not contain a cysteine residue at its *N*-terminus if there is a strong conformational preference for the association of the two fragments (Beligere & Dawson, 1999). For example, chymotrypsin inhibitor 2 (CI2) has been divided into two peptides, which correspond to residues 1–40 and 41–64. These fragments self-associate to form a native-like complex. When fragment 1–40 was synthesized as a *C*-terminal thioester, complete ligation to fragment 41–64 (residue 41 is methionine) was complete in under 2 h. This route was used to prepare CI2 labeled site-specifically with donor and acceptor dyes for studies of protein folding and stability (Deniz et al., 2000).

The S-peptide (residues 1–20) and S-protein (residues 21–124) fragments of RNase A associate strongly in solution. The incubation of S-peptide having a *C*-terminal thioester with S-protein induces a conformationally assisted ligation. The ligation was complete in 10 h to yield intact RNase A (Beligere & Dawson, 1999).

Conformationally assisted ligation has limitations. The method can be used only for the ligation of peptides that have an inherent propensity for self association. In addition, ligations to form an Xaa–Yaa peptide bond are slow when Xaa is a β-branched residue (Beligere & Dawson, 1999).

1.6.2 Removable Auxiliaries

An emerging strategy in protein synthesis has been the use of auxiliaries that act as

cysteine surrogates to mediate the chemical ligation of peptide fragments (Figure 1.11). These auxiliaries are appended synthetically to the *N*-terminus of a peptide. A second peptide is activated at its *C*-terminus in a manner that allows capture of the auxiliary. After the capture step, a *Y*→*N* acyl transfer reaction produces an amide. The final step is removal of the extraneous atoms of the auxiliary, which often involves an additional synthetic step.

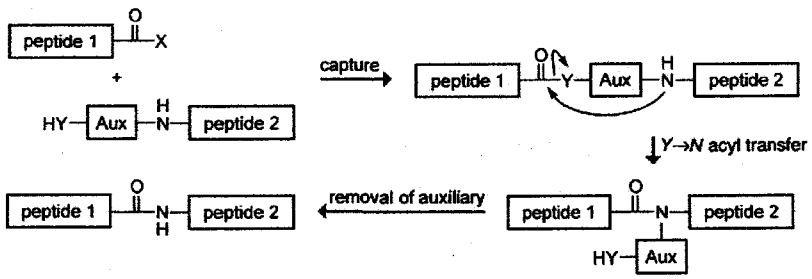


Figure 1.11 Auxiliary-mediated peptide ligation.

The first auxiliaries used for peptide ligation at non-cysteine residues were N^{α}-ethanethiol and N^{α}-oxyethanethiol (Figure 1.12) (Canne et al., 1996). Peptides containing these auxiliaries at their *N*-terminus were synthesized and coupled to peptides with a *C*-terminal thioester under conditions similar to those used in native chemical ligation. The most efficient couplings occurred when the *N*- and *C*-terminal residues were both glycine. Yields were lower if one of these residues was phenylalanine and the other was glycine. No ligation was detected between two non-glycyl residues. After ligation, the N^{α}-oxyethanethiol (but not the N^{α}-ethanethiol) auxiliary was removed efficiently by reduction with zinc under acidic conditions. The N^{α}-oxyethanethiol auxiliary has also

been used in the cyclization of synthetic peptides (Shao et al., 1998).

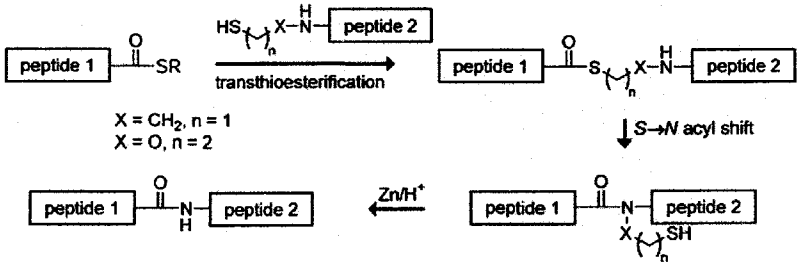


Figure 1.12 Native chemical ligation with ethanethiol and oxyethanethiol auxiliaries.

N^{α}-2-Mercaptobenzylamine is the basis for a second class of auxiliaries for peptide ligation (Figure 1.13). Peptides incorporating these auxiliaries at the *N*-terminus react with thioesters in a manner similar to native chemical ligation (*cf*: Figure 1.4). Acyl transfer occurs via a six-membered ring. The auxiliaries, with appropriate phenyl-ring substitution, can be removed under acidic conditions after acyl transfer.

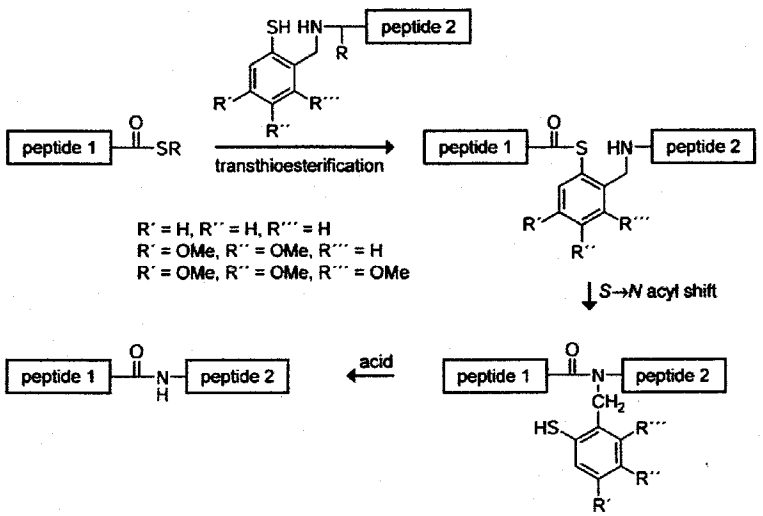


Figure 1.13 N^{α}-2-Mercaptobenzylamine-assisted chemical ligations.

Three N^{α}-2-mercaptobenzylamine auxiliaries have been reported by Dawson and others. The simple mercaptobenzylamine auxiliary (R′ = R″ = R‴ = H in Figure 1.13) was the first of this class to be used for peptide ligation (Offer & Dawson, 2000). This auxiliary enabled acyl transfer if a glycine residue flanked the ligation junction and a β-branched residue did not. The simple mercaptobenzylamine auxiliary could not be removed by acid treatment of the rearranged peptides. The dimethoxyl auxiliary (R′ = R″ = OMe and R‴ = H in Figure 1.13) was reported subsequently, although its use was demonstrated in only a single peptide ligation in which both of the residues flanking the ligation junction were glycine (Kawakami et al., 2001). Treatment with a trifluoromethanesulfonic acid/thioanisole/trifluoroacetic acid cocktail removed the dimethoxyl auxiliary completely from the acyl-transfer product to give a native peptide bond. The trimethoxyl auxiliary (R′ = R″ = R‴ = OMe in Figure 1.13) is the most

recently reported ligation agent of this class (Offer et al., 2002). The half-lives for acyl transfer were 0.2, 2.0, and 5.0 h for Gly–Gly, Lys–Gly, and Gly–Ala, respectively. No acyl transfer occurred between alanine residues. The auxiliary could be removed after acyl transfer using either TFA or HF. The 63-residue SH3 domain of α-spectrin was prepared by chemical ligation between Lys27 and Gly28 using the trimethoxyl auxiliary.

An N^{α}-(1-phenyl-2-mercaptoethyl) group represents a third class of auxiliary-assisted chemical ligation (Figure 1.14). These agents are used in a manner similar to that of the N^{α}-2-mercaptobenzylamine auxiliaries (*cf*: Figure 1.13). The N^{α}-(1-phenyl-2-mercaptoethyl) auxiliaries incorporate a more nucleophilic alkyl thiol in their design to enhance the efficiency of transthioesterification in the capture step, and mediate acyl transfer via a more favorable five-membered ring. Again, substitution on the phenyl ring affects the lability of the auxiliary after acyl transfer.

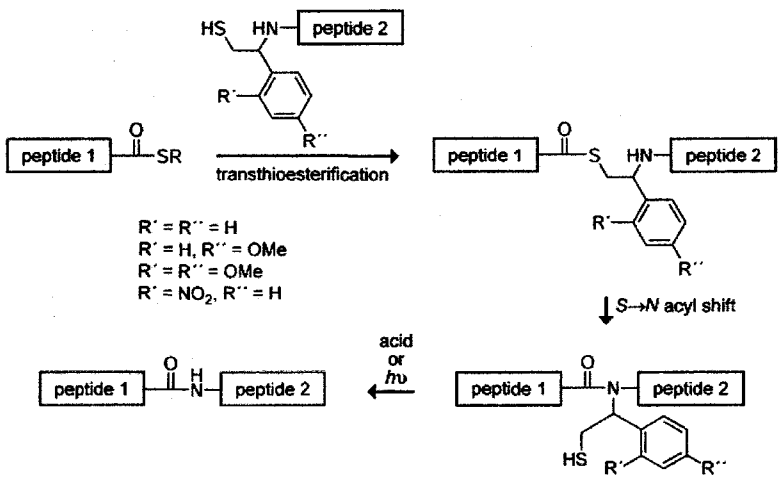


Figure 1.14 N^{α}-(1-Phenyl-2-mercaptoethyl)-assisted chemical ligation.

Initial studies of the N^{α}-(1-phenyl-2-mercaptoethyl) class of auxiliaries were undertaken using compounds with an unsubstituted ring (R′ = R″ = H in Figure 1.14) (Marinzi et al., 2001). Transthioesterification occurred efficiently regardless of sequence; thus acyl transfer limits the ligation rate. Half-lives for acyl transfer were 30–40 and 120–140 min for Gly–Gly and Ala–Gly coupling, respectively; acyl transfer could not be used to induce Gly–Ala and Ala–Ala coupling. No removal of the N^{α}-(1-phenyl-2-mercaptoethyl) auxiliary from rearranged products has been reported to date.

Two ring-substituted forms of the same auxiliary were reported concurrently. The N^{α}-(1-(4-methoxyphenyl)-2-mercaptoethyl) auxiliary (R′ = H and R″ = OMe in Figure 1.14) and the N^{α}-(1-(2,4-dimethoxyphenyl)-2-mercaptoethyl) auxiliary (R′ = OMe and R″ = OMe) were both shown to ligate efficiently at Xaa–Gly sites in which Xaa was Gly, His, Ala, or Lys. Acyl transfer yields were 76–98%, with the typical rearrangement being complete in 16 h. Ligations using the dimethoxyl auxiliary for Xaa–Gly (where Xaa ≠ Gly) required longer reaction times (40 h). The auxiliaries could be removed after acyl transfer using HF (methoxyl) or TFA (dimethyoxyl). These auxiliaries have been applied to the total chemical synthesis of cytochrome b562 (106 residues) (Low et al., 2001) and for the cyclization of 11-residue cyclic peptides (Cardona et al., 2003).

The N^{α}-(1-(2-nitrophenyl)-2-mercaptoethyl) auxiliary (R′ = NO_2 and R″ = H in Figure 1.14) is photolabile. Capture and acyl transfer was demonstrated by the ligation of two 10-residue peptides to produce a Gly–Gly peptide bond. Acyl transfer was complete after 24 h. The auxiliary was removed efficiently by photochemical irradiation at 365 nm for 6 h in an aqueous buffer, pH 7.4. Although the use of the nitro auxiliary is still subject

to the glycine limitation, it could be removed under far milder conditions than its analogs.

1.6.3 Staudinger Ligation

The Staudinger reaction has inspired a simple alternative to native chemical ligation with the promise of removing the cysteine limitation. In the Staudinger reaction, a phosphine is used to reduce an azide to an amine: $PR_3 + N_3R' + H_2O \rightarrow O{=}PR_3 + H_2NR' + N_2(g)$ (Staudinger & Meyer, 1919; Gololobov et al., 1981; Gololobov & Kasukhin, 1992). This reaction occurs via a stable intermediate, an iminophosphorane ($R_3P^+{-}\bar{N}R'$, which is also known, less precisely, as an "aza-ylide"). Vilarrasa and others have shown that the nitrogen of the iminophosphorane can be acylated, both in intermolecular (*i.e.*, three-component) and intramolecular (two-component; Figure 1.15) ligations (García et al., 1984; García et al., 1986; Bosch et al., 1993; Bosch et al., 1995; Mizuno et al., 1999b; Velasco et al., 2000). Hydrolysis of the resulting amidophosphonium salt gives an amide and phosphine oxide. By rearranging the functional groups in the two components, Bertozzi has shown that the phosphine itself can serve as the acyl donor (Figure 1.15) (Saxon & Bertozzi, 2000a). Her two-component ligation enables the formation of an amide bond between two molecules, but leaves a phosphine oxide in the amide product.

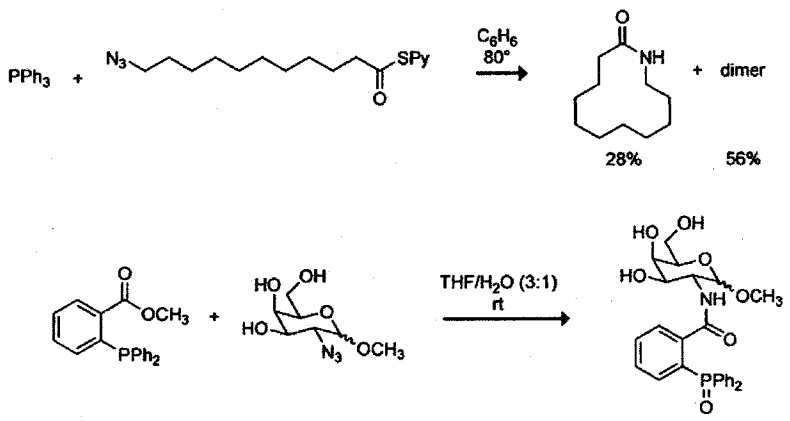


Figure 1.15 Precedents for the Staudinger ligation of peptides. Top: Vilarrasa and coworkers (Bosch et al., 1993); Bottom: Bertozzi and coworkers (Saxon & Bertozzi, 2000a).

A "traceless" *Staudinger ligation* has been developed for the chemical synthesis of proteins (Nilsson et al., 2000; Nilsson et al., 2001). In the capture step, a peptide having a *C*-terminal phosphinothioester reacts with a peptide having an *N*-terminal azide to form an iminophosphorane (Figure 1.16). Attack of the iminophosphorane nitrogen on the thioester leads to an amidophosphonium salt that is acyclic, which is the key to the ligation being traceless. Hydrolysis of the amidophosphonium salt produces an amide bond between the two peptides and releases a phosphine oxide. No extraneous atoms remain in the amide product. The reaction mechanism and kinetics of the Staudinger ligation are detailed in Chapter 4.

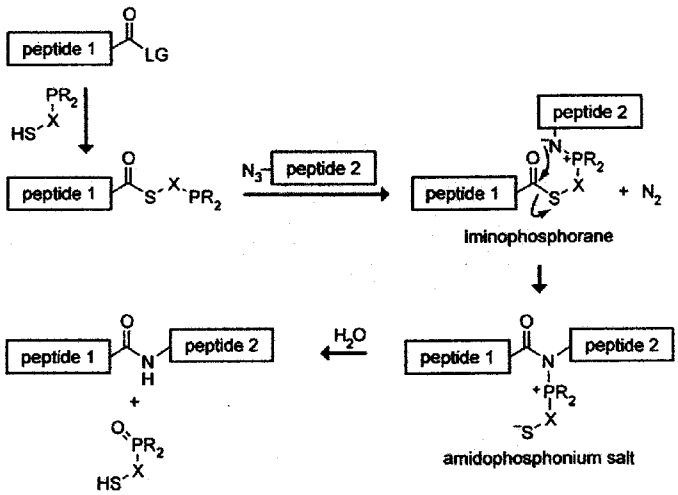


Figure 1.16 Staudinger ligation of peptides mediated by a phosphinothiol.

In essence, the Staudinger ligation of peptides using a phophinothiol couples the energetics of native chemical ligation with that of the Staudinger reaction. This coupling is apparent in a comparison of functional group interconversions during the two ligation reactions (Figure 1.17). The dissimilar groups are the reactants and products of the Staudinger reaction, which is exergonic (Gololobov et al., 1981; Gololobov & Kasukhin, 1992). Accordingly, the thermodynamic driving force of the Staudinger ligation exceeds that of native chemical ligation. The Staudinger ligation is being used increasingly in the chemical synthesis of proteins, as well as other areas of synthetic chemistry and chemical biology (Kohn & Breinbauer, 2004).

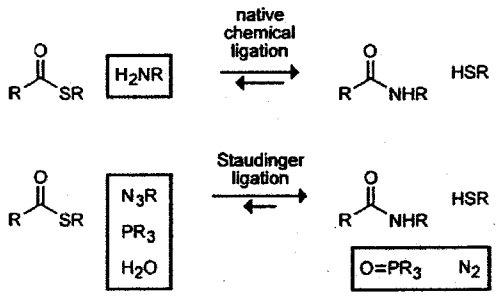


Figure 1.17 Comparison of functional group interconversions during native chemical ligation and phosphinothiol-mediated Staudinger ligation. Dissimilar groups are boxed.

Several phosphinothiol compounds have been assessed for the ability to mediate the Staudinger ligation. These reagents are fully characterized and compared in detail in Chapter 4. Compound **1.1** was the first phosphinothiol tested in this capacity (Figure 1.18) (Nilsson et al., 2000). Its coupling to N^{α}-acetyl amino acids and incubation of the resulting thioester with N_3GlyNHBn (where "N_3Gly" refers to a $N_3CH_2C(O)$ unit) resulted in dipeptide formation, albeit in low yields (≤35%). Still, phosphinothiol **1.1** mediated the first traceless Staudinger ligation (Nilsson et al., 2000).

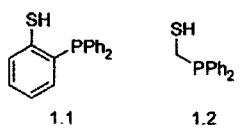


Figure 1.18 Phosphinothiols used in the Staudinger ligation of peptides.

Phosphinothiol **1.2** was a second generation phosphinothiol (Nilsson et al., 2001; Soellner et al., 2002; Nilsson et al., 2003; Soellner et al., 2003). Staudinger ligation with phosphinothiol **1.1** occurs through an intermediate with a six-membered ring. Reducing

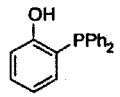
the size of this ring would bring the nucleophilic iminophosphorane nitrogen more proximal to the electrophilic thioester carbon and thus could improve the yields for the Staudinger ligation products. To access a transition state with a smaller ring, the *o*-phenyl group of phosphinothiol **1.1** was replaced with a single methylene group. Thioesters of phosphinothiol **1.2** with AcOH, AcGlyOH, and AcPheOH were prepared either by transthioesterification or by coupling with dicyclohexylcarbodiimide. These phosphinothioesters formed dipeptides with N_3GlyNHBn in >90% yields (Nilsson et al., 2001). Additionally, as described in detail in Chapter 2, AcGlySCH_2PPh_2 was coupled with the D and L enantiomers of N_3Ser(Bn)NHBn, N_3PheNHBn, and N_3Asp(OBn)NHBn (Soellner et al., 2002). These ligations, which likewise proceeded in >90% yield, confirmed that the Staudinger ligation retains the stereochemistry at the α-carbon of the azido acid. As with known auxiliary-assisted ligations (*vide supra*), the Staudinger ligation with phosphinothiols **1.1** and **1.2** is less efficient when a glycine residue is not at the ligation junction (Nilsson BN, Soellner MB, Raines RT, unpublished results).

The Staudinger ligation with phosphinothiol **1.2** has been exploited in the assembly of RNase A (Nilsson et al., 2003). After folding, this semisynthetic RNase A had a molecular mass and enzymatic activity that was indistinguishable from that of biosynthetic RNase A. Moreover, the semisynthesis incorporated a ^{13}C-enriched proline residue at position 114 for NMR spectroscopic analysis of protein conformation. This semisynthesis is unique in invoking four distinct chemical processes to create the peptide bonds of a protein: *m*RNA translation by the ribosome, solid-phase peptide synthesis, expressed protein ligation, and the Staudinger ligation (Table 1.1). Access to this arsenal greatly empowers modern protein chemists.

Table 1.1 Methods to form peptide bonds during the semisynthesis of RNase A (Nilsson et al., 2003).

Bond	Ligation Method
1-109	mRNA Translation by Ribosome (sequential)
109-110	Expressed Protein Ligation (convergent)
110-111	Solid-Phase Peptide Synthesis (sequential)
111-112	Staudinger Ligation (convergent)
112-124	Solid-Phase Peptide Synthesis (sequential)

Phosphinophenol **1.3** has been used to promote the Staudinger ligation of two non-glycyl amino acids (Figure 1.19) (Merkx et al., 2003a). Under specialized reaction conditions, Gly–Phe and Ala–Phe couplings occurred in 32–36% yields. The reaction conditions are not, however, readily adapted to unprotected peptides in aqueous solution, as amino groups (*e.g.*, *N*-terminal and lysyl side chain) are acylated by esters of phosphinophenol **1.3** (Merkx et al., 2003a). Moreover, thioester fragments generated by expressed protein ligation or other methods cannot be converted readily to esters with phosphinophenol **1.3**, which highlights an intrinsic advantage of phosphinothiols in mediating the Staudinger ligation (Figure 1.16). Still, the success with phosphinophenol **1.3** provides an important precedent in the development of the Staudinger ligation as a sequence-independent method for peptide ligation. Efforts directed at improving the yields of phosphinothiol **1.2** with couplings involving two non-glycyl residues is discussed in Chapter 5.



1.3

Figure 1.19 Phosphinophenol used in the Staudinger ligation of peptides at non-glycyl residues.

1.7 Prospectus

The development and refinement of peptide ligation methods for the convergent synthesis of proteins has entered an explosive phase of growth. Native chemical ligation and expressed protein ligation have already enabled the synthesis and semisynthesis of >100 proteins (Dawson & Kent, 2000; Muir, 2003). The junctions between peptides in these proteins must, however, be at the *N*-terminus of a cysteine residue. Removable auxiliaries and the Staudinger ligation provide the means to ligate peptides between any two residues in a protein sequence. The ligation of peptides on a solid support is likely to facilitate protein folding (by minimizing aggregation) and enable protein production to be automated from start to finish. Accordingly, synthetic chemistry is poised to make ever-increasing contributions to protein science for the foreseeable future.

Chapter 2*

STAUDINGER LIGATION OF α-AZIDO ACIDS RETAINS STEREOCHEMISTRY

Abstract: The Staudinger ligation of peptides with a C-terminal phosphinothioester and N-terminal azide is an emerging method in protein chemistry. Here, the first Staudinger ligations of non-glycyl azides are reported and shown to proceed both in nearly quantitative yield and with no detectable effect on the stereochemistry at the α-carbon of the azide. These results demonstrate further the potential of the Staudinger ligation as a general method for the total synthesis of proteins from peptide fragments.

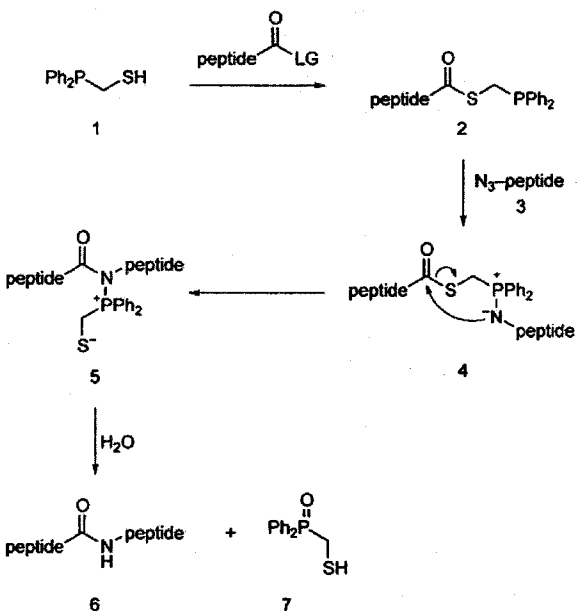
The chemoselective ligation of peptides can be used to effect the total chemical synthesis of proteins (Tam et al., 1999; Borgia & Fields, 2000; Dawson & Kent, 2000; Miranda & Alewood, 2000; Tam et al., 2001). The most common ligation method, native chemical ligation, relies on the presence of a cysteine residue at the *N*-terminus of each ligation junction (Wieland et al., 1953; Dawson et al., 1994). We have identified the "Staudinger ligation" as a peptide ligation method that has the potential to be universal—independent of the presence of any particular side chain (Nilsson et al., 2000; Nilsson et al., 2001; Kohn & Breinbauer, 2004). This method is based on the Staudinger reaction, wherein a phosphine reduces an azide via a stable iminophosphorane intermediate

* This chapter has been published previously under the same title. Reference: Soellner, M. B.; Nilsson, B. L.; Raines, R. T. *J. Org. Chem.* **2002**, *67*, 4993-4996.

(Staudinger & Meyer, 1919; Gololobov & Kasukhin, 1992). Acylation of this iminophosphorane yields an amide (García et al., 1984; García et al., 1986; Urpí & Vilarrasa, 1986; Bosch et al., 1993; Inazu & Kobayashi, 1993; Bosch et al., 1995; Shalev et al., 1996; Maunier et al., 1997; Afonso, 1998; Ariza et al., 1998; Tang & Pelletier, 1998; Mizuno et al., 1999a; Mizuno et al., 1999b; Boullanger et al., 2000; Malkinson et al., 2000; Saxon et al., 2000; Saxon & Bertozzi, 2000b; Velasco et al., 2000; Ariza et al., 2001; Kiick et al., 2002).

In our version of the Staudinger ligation (Scheme 2.1) (Nilsson et al., 2000; Nilsson et al., 2001; Kohn & Breinbauer, 2004), a peptide fragment having a *C*-terminal phosphinothioester (**2.2**) reacts with another peptide fragment having an *N*-terminal azide (**2.3**). The resulting iminophosphorane (**2.4**) leads, after an S- to N-acyl shift, to an amidophosphonium salt (**2.5**). The P–N bond of the amidophosphonium salt is hydrolyzed readily to produce the amide product (**2.6**) and a phosphine oxide (**2.7**). Importantly, no residual atoms remain in the amide product (Nilsson et al., 2000; Saxon et al., 2000; Nilsson et al., 2001; Kohn & Breinbauer, 2004). Previously, we showed that the ligation of glycyl and phenylalanyl thioesters of phosphinothiol **2.1** with glycyl azides proceeds in high yield (Nilsson et al., 2001).

Scheme 2.1 Staudinger ligation of peptide fragments.



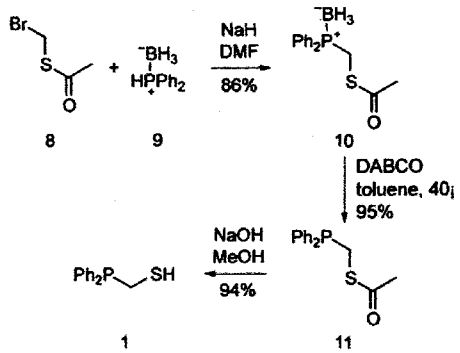
All natural α-amino acids except glycine have a stereogenic center at their α-carbon. To be an effective tool for the total chemical synthesis of proteins, a peptide ligation reaction must proceed without epimerization. The coupling of thioesters in native chemical ligation, which like the Staudinger ligation (Scheme 2.1) involves transthioesterification followed by an S- to N-acyl shift (Wieland et al., 1953; Dawson et al., 1994), is known to proceed without detectable racemization (Lu et al., 1996). Because the Staudinger ligation has been demonstrated previously only with glycyl azides, the propensity for epimerization of the α-azido acid has not been assessed. This issue is of concern because an α-carbanion could be stabilized by inductive or resonance effects in azide **2.3** and iminophosphorane **2.4** (Zaloom & Roberts, 1981).

Here, we report the first use of the Staudinger ligation to couple a peptide containing

a non-glycyl azide. We also search for epimerization during Staudinger ligation reactions. Finally, we report an improved synthesis of phosphinothiol **2.1** (Scheme 2.1), which is the most effective known phosphinothiol for effecting the Staudinger ligation of peptides.

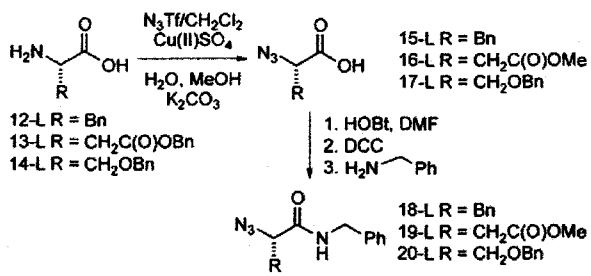
The previously reported synthesis of phosphinothiol **2.1** required four steps, two of which were problematic, with an overall yield of only 39%.[4b] We have developed an improved synthesis that uses air-stable borane protection of the phosphine (Scheme 2.2) (Brunel et al., 1998; Carboni & Monnier, 1999). The synthesis is based on the easily prepared alkylating agent **2.8** (Farrington et al., 1989) and the commercially available borane–diphenylphosphine complex **2.9**. Compound **2.9** is deprotonated by sodium hydride in DMF followed by addition of **2.8** to give borane-complex **2.10** (86% yield) (Imamoto et al., 1990). Complex **2.10** is stable to air and moisture, and can be stored on the shelf at room temperature for months without any sign of oxidation or decomposition. The borane complex is disrupted by mild heating with DABCO in toluene for 4 h (95% yield) (Brisset et al., 1993). The protecting group of the resulting acyl phosphinothiol **2.11** is removed as described previously (Nilsson et al., 2001) to give phosphinothiol **2.1** (94% yield). The overall yield for this three-step synthesis is 74%.

Scheme 2.2 Synthesis of diphenylphosphinomethanethiol.



We then prepared several non-glycyl α-azido acids to determine if epimerization occurs during the Staudinger ligation. The azido benzamides of both the D and L enantiomers of phenylalanine, serine, and aspartic acid were prepared (Scheme 2.3). The azido group was prepared by diazo transfer (Zaloom & Roberts, 1981); the benzamide was prepared by DCC/HOBt coupling with benzyl amine. Phenylalanine, aspartic acid, and serine were chosen as being representative of three distinct side chains and moderate (phenylalanine) to high (aspartate and serine) propensity to epimerize during standard peptide couplings (Romoff & Goodman, 1997).

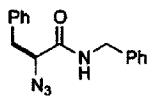
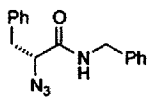
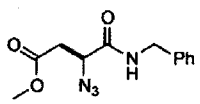
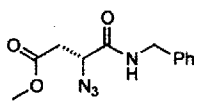
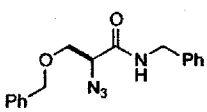
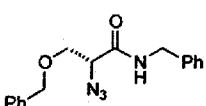
Scheme 2.3 Synthesis of α-azido benzamides.



Each of these azido acids was coupled with phosphinothioester **2.1** (Table 2.1). The couplings were carried out in THF/H_2O (3:1) for 12 h at room temperature with a 1:1 stoichiometry of starting materials. The resulting peptides were purified by flash chromatography to give a nearly quantitative yield of each product (Table 2.1). The high yield of this equimolar reaction of phosphinothiol **2.1** with non-glycyl azides is consistent with those observed previously with glycyl azides (Nilsson et al., 2001).

Table 2.1 Staudinger ligation of $AcGlySCH_2PPh_2$ (**2.21**) and Non-Glycyl α-Acido Acids

Table 1. Staudinger Ligation of $AcGlySCH_2PPh_2$ (21) and Non-Glycyl α-Azido Acids

α-azido acid	peptide	yield (%)[b]
 18-L	AcGly(L)PheNHBn 22-L	90
 18-D	AcGly(D)PheNHBn 22-D	93
 19-L	AcGly(L)Asp(OMe)NHBn 23-L	91
 19-D	AcGly(D)Asp(OMe)NHBn 23-D	95
 20-L	AcGly(L)Ser(Bn)NHBn 24-L	92
 20-D	AcGly(D)Ser(Bn)NHBn 24-D	99

[a]Reaction conditions THF/H_2O (3:1) at room temperature for 12 h.
[b]Isolated yield of product after purification by flash chromatography.

The chirality of the Staudinger ligation products from the reaction of the D and L α-azido acids was analyzed by HPLC using a D-phenylglycine chiral column. The chromatographic conditions enabled the baseline resolution of the two possible enantiomeric products (Figure 2.1). After reaction of the D epimer, there was no evidence of product containing the L epimer, and vice versa. Thus, the Staudinger ligation proceeds without detectable epimerization of the α-carbon of the azido acid.

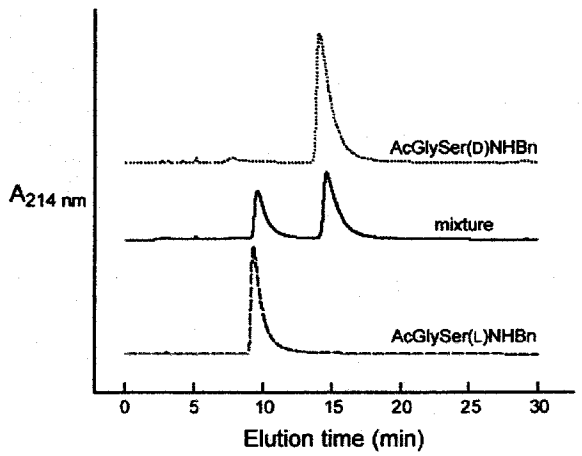


Figure 2.1 HPLC elution profile of AcGly(D)SerNHBn, AcGly(L)SerNHBn, and a mixture of the two enantiomers.

We conclude that the Staudinger ligation can be used to couple non-glycl azides in nearly quantitative yield and without detectable epimerization. Thus, the Staudinger ligation holds promise as a general method for the convergent assembly of proteins from peptide fragments.

Experimental Section

General Experimental

Amino acids were from NovaBiochem (San Diego, California) and all other chemicals and solvents were from Aldrich (Milwaukee, Wisconsin). Reactions were monitored by thin-layer chromatography with visualization by UV light or staining with ninhydrin or I_2. Silica gel used in flash chromatography had 230–400 mesh and 60 Å pore size. Chiral HPLC was performed with a D-phenylglycine analytical chiral column from MetaChem (Torrence, California). NMR spectra were obtained with a Varian INOVA-500 MHz spectrometer or a Bruker AC-300 300 MHz spectrometer at the University of Wisconsin nuclear magnetic resonance facility. Carbon-13 and phosphorus-31 NMR spectra were both proton-decoupled and phosphorus-31 spectra were referenced against an external standard of deuterated phosphoric acid (0 ppm). Mass spectra were obtained with electrospray ionization (ESI) techniques.

Borane-thioacetic acid S-[(diphenylphosphanyl)-methyl] ester complex (2.10). Borane–diphenylphosphine complex **2.9** (10.33 g, 51.6 mmol) was dissolved in dry DMF under Ar(g) and cooled to 0 °C. NaH (1.24 g, 51.6 mmol) was added slowly, and the mixture was stirred at 0 °C until bubbling ceased. Alkylating agent **2.8**(Farrington et al., 1989) (8.73 g, 51.6 mmol) was then added, and the mixture was allowed to warm to room temperature and stirred for 12 h. Solvent was removed under reduced pressure, and the residue was purified by flash chromatography (silica gel, 10% v/v EtOAc in hexanes). Compound **2.10** was isolated as a colorless oil in 86% yield. **Spectral data.** ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.74-7.67 (m, 4 H), 7.54-7.41 (m, 6 H), 3.72 (d, J = 6 Hz, 2 H), 2.23 (s, 3 H), 1.51-0.53 (broad m, 3 H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.94, 132.26 (d,

J = 9.2 Hz), 131.61 (d, J = 2.3 Hz), 128.71 (d, J = 10.2 Hz), 127.43 (d, J = 55.4 Hz), 29.87, 23.59 (d, J = 35.5 Hz) ppm; ^{31}P NMR (121 MHz, $CDCl_3$) δ 19.40 (d, J = 59.3 Hz) ppm; MS (ESI) *m/z* 311.0806 (MNa^+ [$C_{15}H_{18}BOPSNa$] = 311.0807).

Thioacetic acid S-[(diphenylphosphanyl)-methyl] ester (2.11). Compound **2.10** (4.00 g, 13.9 mmol) was dissolved in toluene (0.14 L) under Ar(g). DABCO (1.56 g, 13.9 mmol) was added, and the mixture was heated at 40 °C for 4 h. Solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2 and washed with both 1 N HCl and saturated brine. The organic layer was dried over $MgSO_4$(s), and the solvent was removed under reduced pressure. Compound **2.11** was isolated in 95% yield, and was used without further purification. **Spectral Data.** As reported previously.(Nilsson et al., 2001)

2(*S*)-Azido-N-benzyl-3-phenyl-propionamide (2.18-L). N_3(L)PheOH (**2.15-L**) was synthesized from L-phenylalanine essentially by the procedure of Lundquist and Pelletier.(Lundquist & Pelletier, 2001) N_3(L)PheOH (1.08 g, 5.7 mmol) was dissolved in anhydrous DMF (40 mL). HOBt (0.87 g, 5.7 mmol) was then added, followed by DCC (1.17 g, 5.7 mmol). Once precipitate was observed in the reaction, benzylamine (0.62 mL, 5.7 mmol) was added. The reaction was allowed to stir under Ar(g) for 3 h. The resulting precipitate (DCU) was removed by filtration, and the filtrate was concentrated under reduced pressure to give a yellow oil. This oil was purified by flash chromatography (silica gel, 35% v/v ethyl acetate in hexanes). N_3(L)PheNHBn (**2.18-L**) was isolated as an off-white solid in 90% yield. The procedure was repeated with D-phenylalanine to give N_3(D)PheNHBn (**2.18-D**) as a white solid in 92% yield. **Spectral data.** The spectral data for both N_3PheNHBn products (D and L enantiomers) are

identical. **N_3(L)PheNHBn (2.18-L)** ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.31-7.12 (m, 8 H), 7.11 (m, 2 H), 6.55 (bs, 1 H), 4.38 (m, 2 H), 4.22 (dd, J = 7.8, 4.6 Hz, 1 H), 3.34 (dd, J = 14.0, 4.5 Hz, 1 H), 3.07 (dd, J = 14.1, 7.5 Hz, 1 H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.30, 137.35, 135.96, 129.51, 128.61, 128.66, 128.61, 128.55, 127.70, 127.68, 127.66, 127.57, 127.16, 65.40, 43.41, 38.41 ppm; MS (ESI) *m/z* 303.1235 (MNa^+ [$C_{16}H_{16}N_4ONa$] = 303.1222).

3(*S*)-Azido-*N*-benzyl-succinamic acid methyl ester (2.19-L). Benzyl-protected L-aspartate was used in the procedure of Lundquist and Pelletier (Lundquist & Pelletier, 2001) to give N_3(L)Asp(OMe)OH (**2.16-L**). Under these conditions, we observed transesterification to give the methyl ester product as opposed to the benzyl ester. N_3(L)Asp(OMe)OH (**2.16-L**) was produced as a yellowish oil in 78% yield. N_3(L)Asp(OMe)OH (**2.16-L**) was then coupled with benzylamine as above to give N_3(L)Asp(OMe)NHBn (**2.19-L**) as a yellowish oil in 90% yield (70% overall, two steps). The procedure above was repeated with benzyl-protected D-aspartate to give N_3(L)Asp(OMe)NHBn (**2.19-D**) as a yellowish oil in 67% overall yield. **Spectral Data.** The spectral data for both N_3Asp(OMe)OH (D and L enantiomers) and both N_3Asp(OMe)NHBn (D and L enantiomers) products are identical. **N_3(L)Asp(OMe)OH (2.16-L)** ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.24 (bs, 1H), 4.47 (dd, J = 7.4, 5.3 Hz, 1 H), 3.76 (s, 3 H), 2.91 (dd, J = 16.9, 5.1 Hz, 1 H), 2.79 (dd, J = 16.8, 7.6 Hz) ppm; ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.68, 170.12, 50.09, 52.44, 35.84 ppm; MS (ESI) *m/z* 196.0340 (MNa^+ [$C_5H_7N_3O_4Na$] = 196.0334). **N_3(L)Asp(OMe)NHBn (2.19-L)** ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38-7.27 (m, 5 H), 6.83 (bs, 1 H), 4.54 (m, 3 H), 3.75 (s, 3 H), 3.18 (dd,

J = 17.1, 3.7 Hz, 1 H), 2.75 (dd, J = 17.3, 8.7 Hz, 1 H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.77, 167.90, 137.35, 128.81, 127.80, 127.77, 60.32, 52.24, 43.71, 37.00 ppm; MS (ESI) *m/z* 285.0953 (MNa^+ [$C_{12}H_{12}N_4O_3Na$] = 285.0964).

2(*S*)-Azido-N-benzyl-3-benzyloxy-propionamide (2.20-L). Benzyl-protected L-serine was used in the procedure above to give N_3(L)Ser(Bzl)NHBn (**2.20-L**) as a yellowish oil in 93% yield. The procedure was repeated with benzyl-protected D-serine to give N_3(D)Ser(Bzl)NHBn (**2.20-D**) as a yellowish oil in 90% yield. **Spectral Data.** The spectral data for both N_3Ser(Bzl)NHBn products (D and L enantiomers) are identical. **N_3(L)Ser(Bzl)NHBn (2.20-L)** ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36-7.23 (m, 10 H), 6.86 (bs, 1 H), 4.57 (s, 2 H), 4.43 (m, 2 H), 4.25 (dd, J = 6.9, 3.5 Hz, 1 H), 4.01 (dd, J = 10.3, 3.5 Hz, 1 H), 3.83 (10.1, 6.7 Hz, 1 H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.81, 137.40, 137.22, 128.65, 128.40, 127.81, 127.59, 127.54, 73.45, 70.54, 63.28, 43.38 ppm; MS (ESI) *m/z* 333.1337 (MNa^+ [$C_{17}H_{18}N_4O_2Na$] = 333.1327).

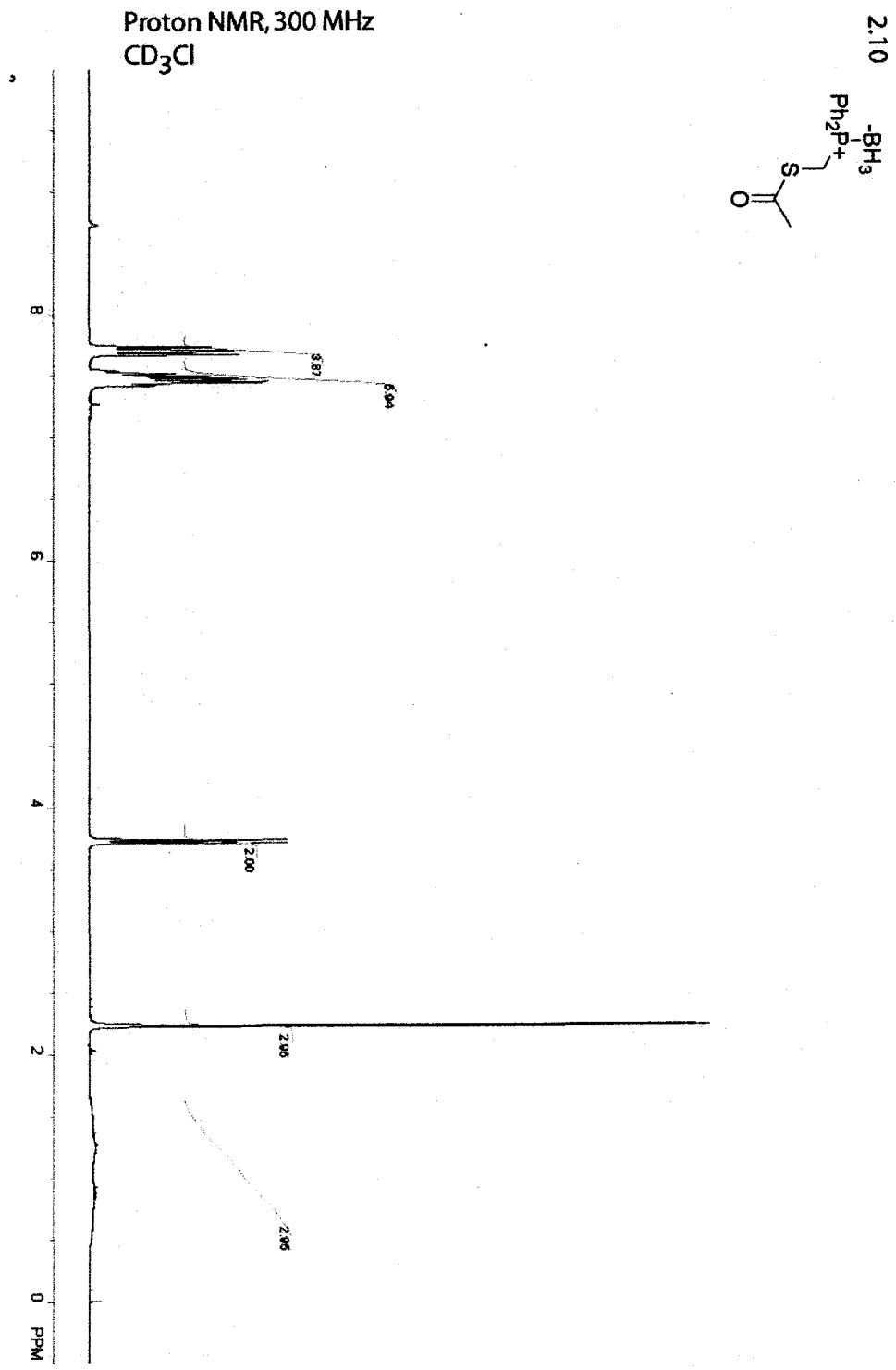
Acetylamino-thioacetic acid S-[(diphenylphosphanyl)-methyl] ester (2.21). N-Acetylglycine (1.90 g, 16.2 mmol) was dissolved in anhydrous DMF (75 mL). HOBt (2.48 g, 16.2 mmol) was added to the resulting solution followed by DCC (3.34 g, 16.2 mmol). Once precipitate (DCU) was observed, phosphinothiol **2.1** was added (3.77 g, 16.2 mmol). The reaction mixture was allowed to stir under Ar(g) for 3 h. The precipitate was removed by filtration, and the filtrate was concentrated under reduced pressure to give a white solid. This solid was dissolved in ethyl acetate and purified by flash chromatography (silica gel, ethyl acetate). Compound **2.21** was isolated in 96% yield. **Spectral Data.** As reported previously (Nilsson et al., 2001).

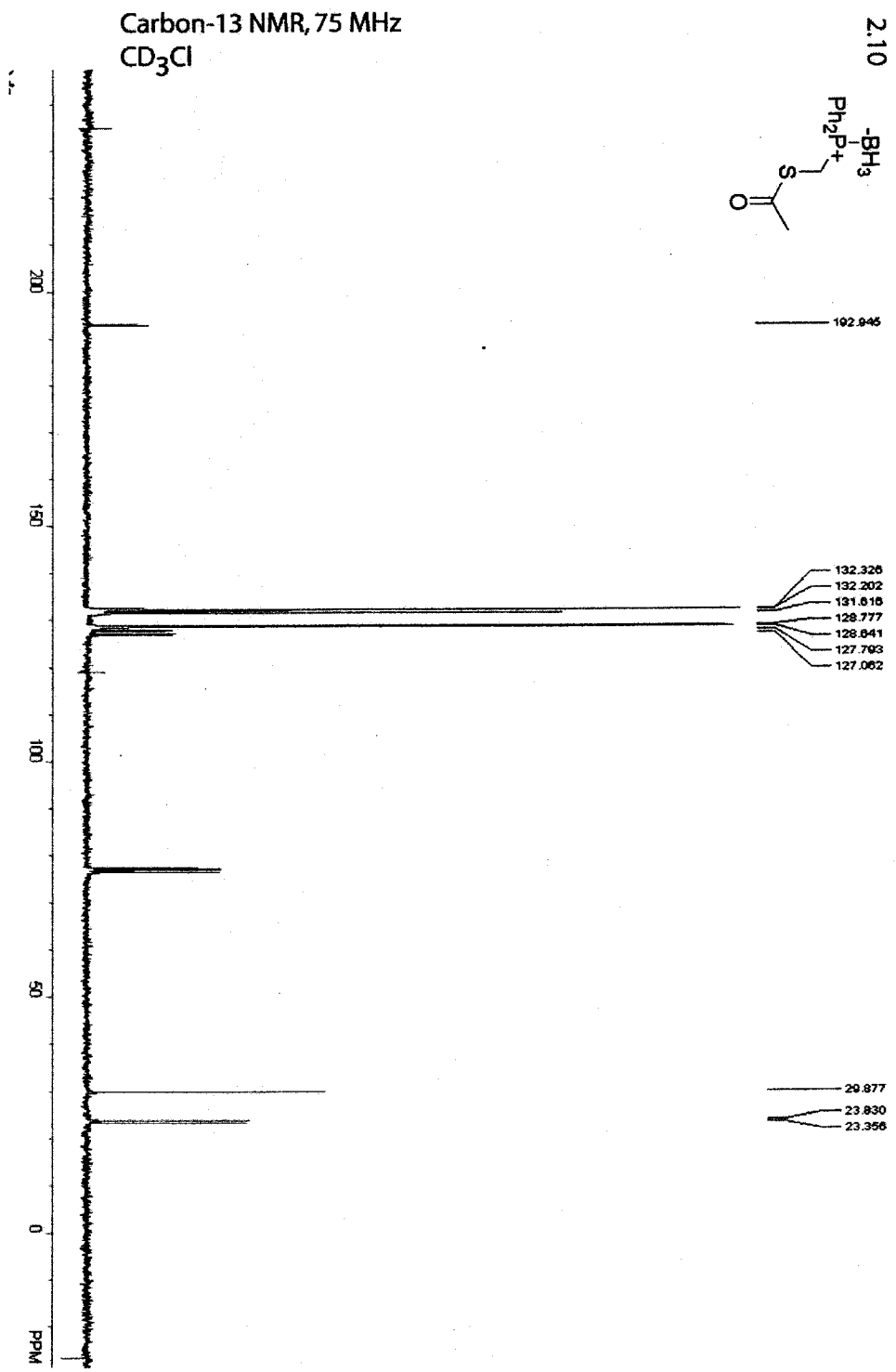
2(*S*)-(2-Acetylamino-acetylamino)-N-benzyl-3-phenyl-propionamide (2.22-L). N-Acetylglycylphosphinothioester **2.9** (0.166 g, 0.5 mmol) and N_3(L)PheNHBn (**2.18-L**) (0.140 g, 0.5 mmol) was dissolved in THF/H_2O (3:1, 4 mL), and the mixture was stirred at room temperature for 12 h. Solvent was removed under reduced pressure, and the residue was purified by flash chromatography (silica gel, 5% v/v methanol in dichloromethane). AcGly(L)PheNHBn (**2.22-L**) was obtained in as a white solid in 90% yield. The procedure was repeated with N_3(D)PheNHBn (**2.18-D**) to give AcGly(D)PheNHBn (**2.22-D**) in 93% yield. **Spectral Data.** The spectral data for both dipeptide products (D and L enantiomers) are identical. **AcGly(L)PheNHBn (2.22-L)** ^{1}H NMR (300 MHz, $CDCl_3$:CD_3OD 1:1) δ 7.30-7.22 (m, 6 H), 7.19-7.16 (m, 2 H), 7.16-7.11 (m, 2H), 4.63 (t, J = 7.3 Hz, 1 H), 4.33 (dd, J = 31.1, 14.6 Hz, 2 H), 3.79 (dd, J = 33.1, 16.7 Hz, 2 H), 3.12 (dd, J = 13.8, 7.2 Hz, 1 H), 2.98 (dd J = 13.7, 7.2 Hz, 1 H), 1.98 (s, 3 H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$:CD_3OD 1:1) δ 171.98, 170.93, 169.33, 137.29, 136.00, 128.76, 128.05, 127.97, 127.03, 126.75, 126.41, 54.16, 42.79, 42.37, 37.52, 21.56 ppm; MS (ESI) *m/z* 376.1624 (MNa^+ [$C_{20}H_{23}N_3O_3Na$] = 376.1637).

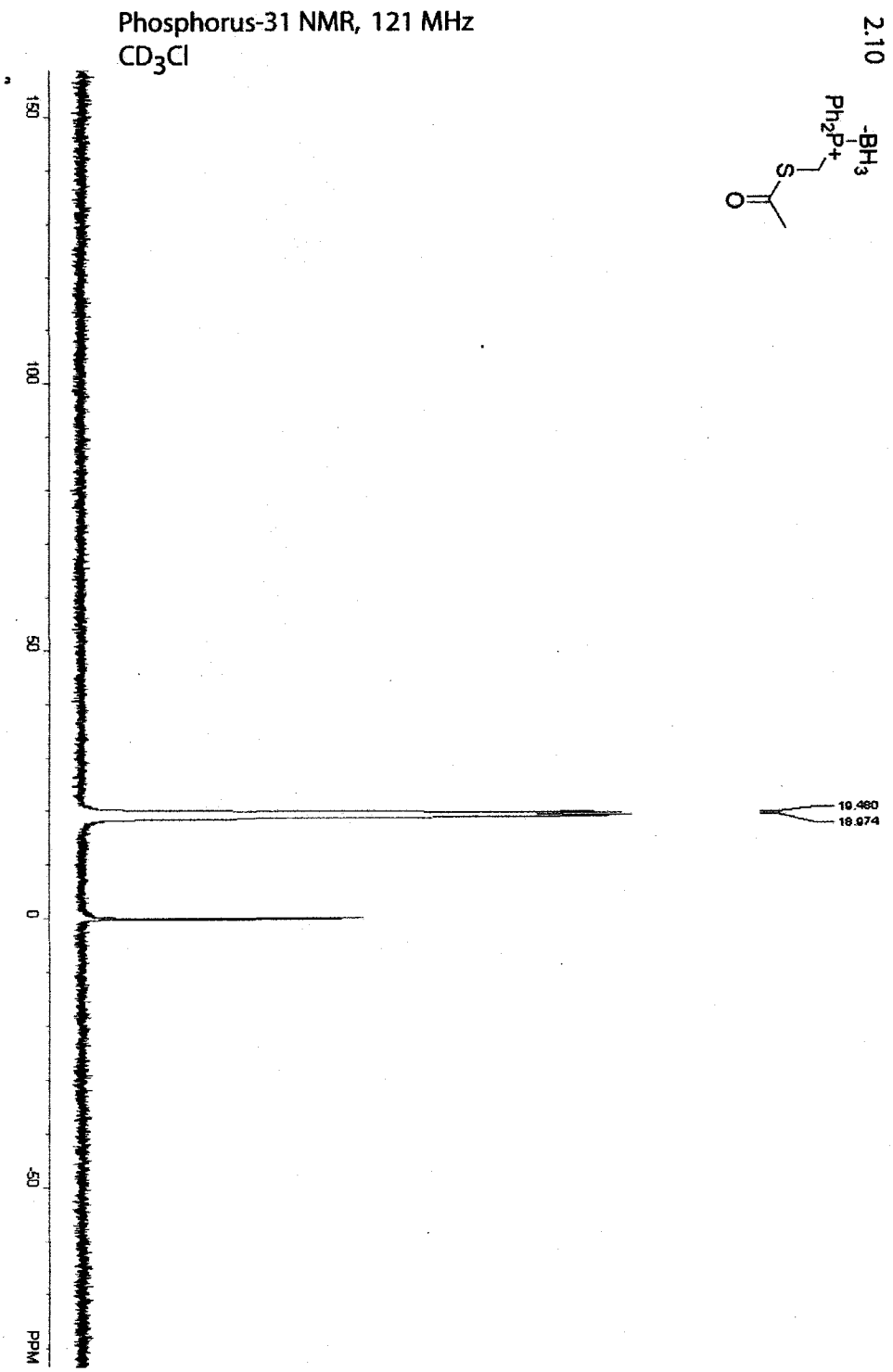
3(*S*)-(2-Acetylamino-acetylamino)-N-benzyl-succinamic acid methyl ester (2.23-L). N_3(L)Asp(OMe)NHBn (**2.19-L**) was used in the procedure above to give AcGly(L)Asp(OMe)NHBn (**2.23-L**) as a white solid in 91% yield. The procedure was repeated with N_3(D)Asp(OMe)NHBn (**2.19-D**) to give AcGly(D)Asp(OMe)NHBn (**2.23-D**) as a white solid in 95% yield. **Spectral Data.** The spectral data for both AcGlyAsp(OMe)NHBn products (D and L enantiomers) are identical. **AcGly(L)Asp(OMe)NHBn (2.23-L)** ^{1}H NMR (500 MHz, $CDCl_3$:CD_3OD 1:1) δ 7.34-7.23 (m, 5 H), 4.84 (t, J = 5.7 Hz, 1 H), 4.34 (s, 2 H), 3.84 (q, J = 16.6 Hz, 2H), 3.69 (s, 3

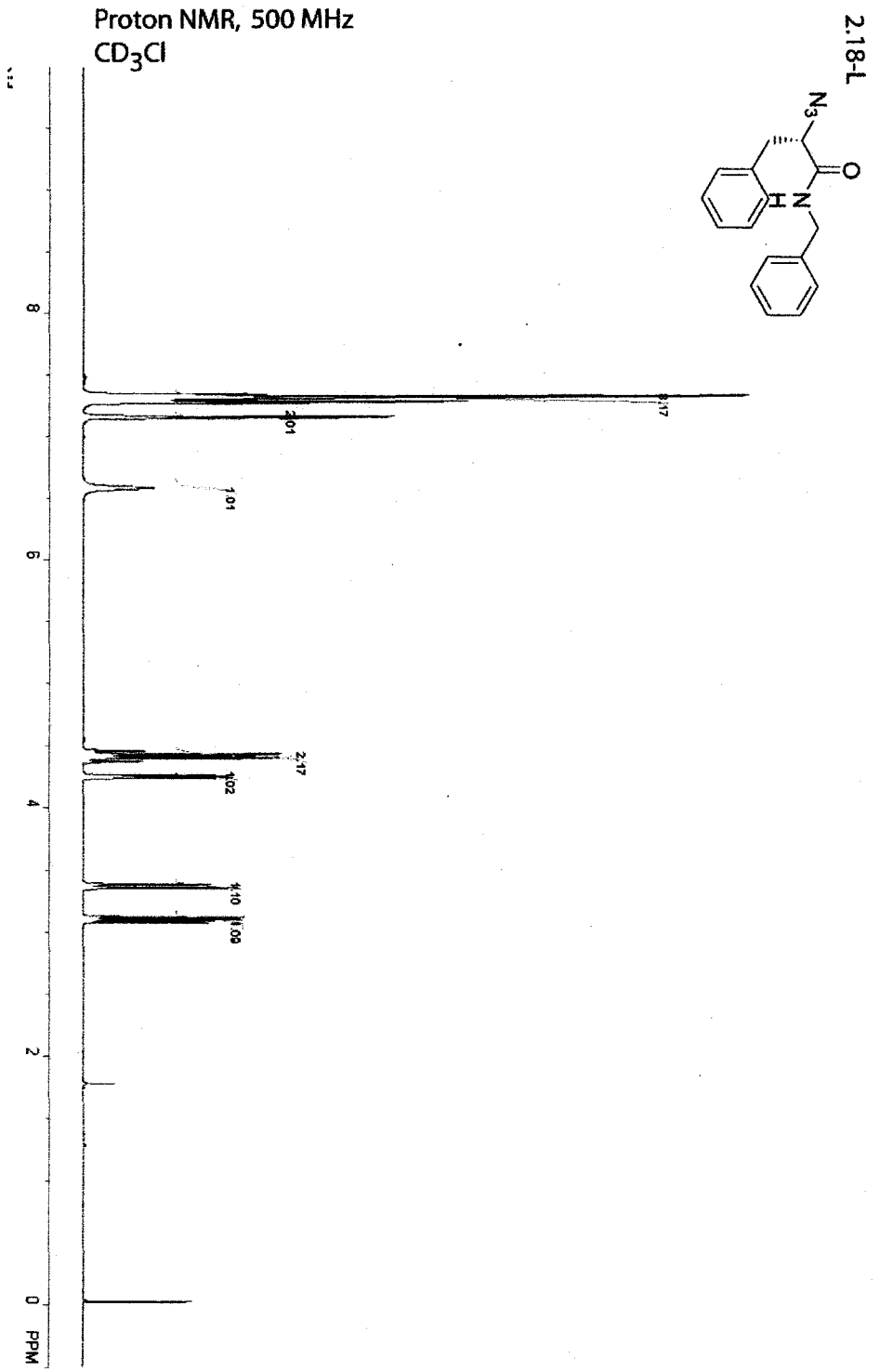
H), 2.87 (m, 2 H), 2.01 (s, 3 H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$:CD_3OD 1:1) δ 177.24, 176.43, 175.18, 174.58, 142.56, 133.17, 133.03, 131.93, 131.78, 56.46, 54.13, 47.91, 47.81, 47.67, 40.11, 26.59 ppm; MS (ESI) *m/z* 358.1388 (MNa^+ [$C_{16}H_{21}N_3O_5Na$] = 358.1379).

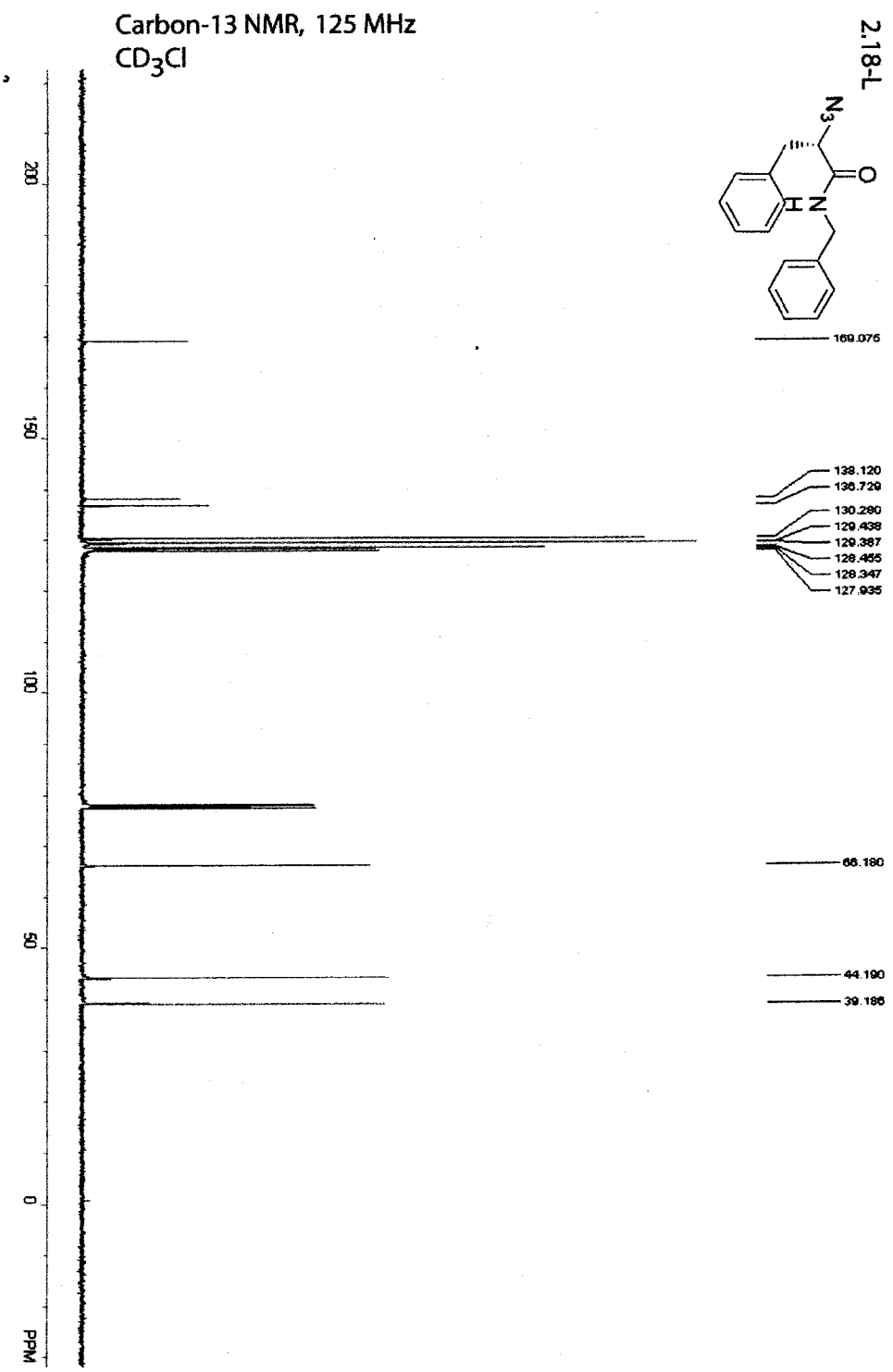
2(*S*)-(2-Acetylamino-acetylamino)-N-benzyl-3-benzyloxy-propionamide (2.24-L). N_3(L)Ser(Bzl)NHBn (**2.20-L**) was used in the procedure above to give AcGly(L)Ser(Bzl)NHBn (**2.24-L**) as a white solid in 92% yield. The procedure was repeated with N_3(D)PheNHBn (**2.20-D**) to give AcGly(D)Ser(Bzl)NHBn (**2.24-D**) as a white solid in 99% yield. **Spectral Data.** The spectral data for both AcGlySer(Bzl)NHBn products (D and L enantiomers) are identical. **AcGly(L)Ser(Bzl)NHBn (2.24-L)** ^{1}H NMR (500 MHz, $CDCl_3$:CD_3OD 1:1) δ 7.34-7.21 (m, 10 H), 4.60 (t, *J* = 4.4 Hz, 1 H), 4.43 (dd, *J* = 23.9, 14.9 Hz, 2 H), 3.85 (m, 3 H), 3.69 (dd, *J* = 9.6, 4.6 Hz, 1 H), 1.98 (s, 3 H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$:CD_3OD 1:1) δ 172.19, 169.86, 169.61, 137.49, 127.87, 127.31, 127.23, 127.76, 126.59, 72.88, 69.07, 52.93, 42.71, 42.49, 21.38 ppm; MS (ESI) *m/z* 406.1750 (MNa^+ [$C_{21}H_{25}N_3O_4Na$] = 406.1743).

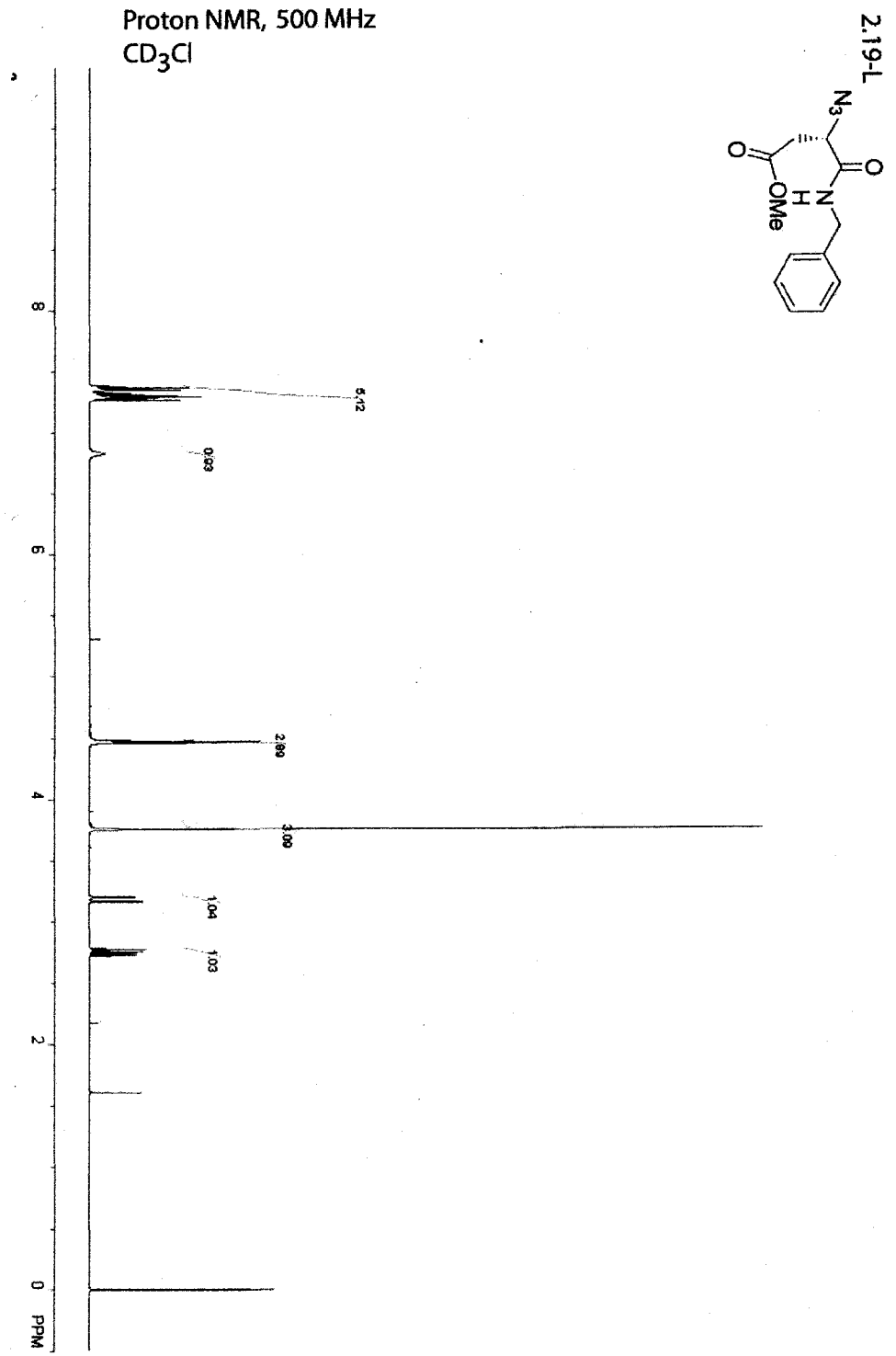


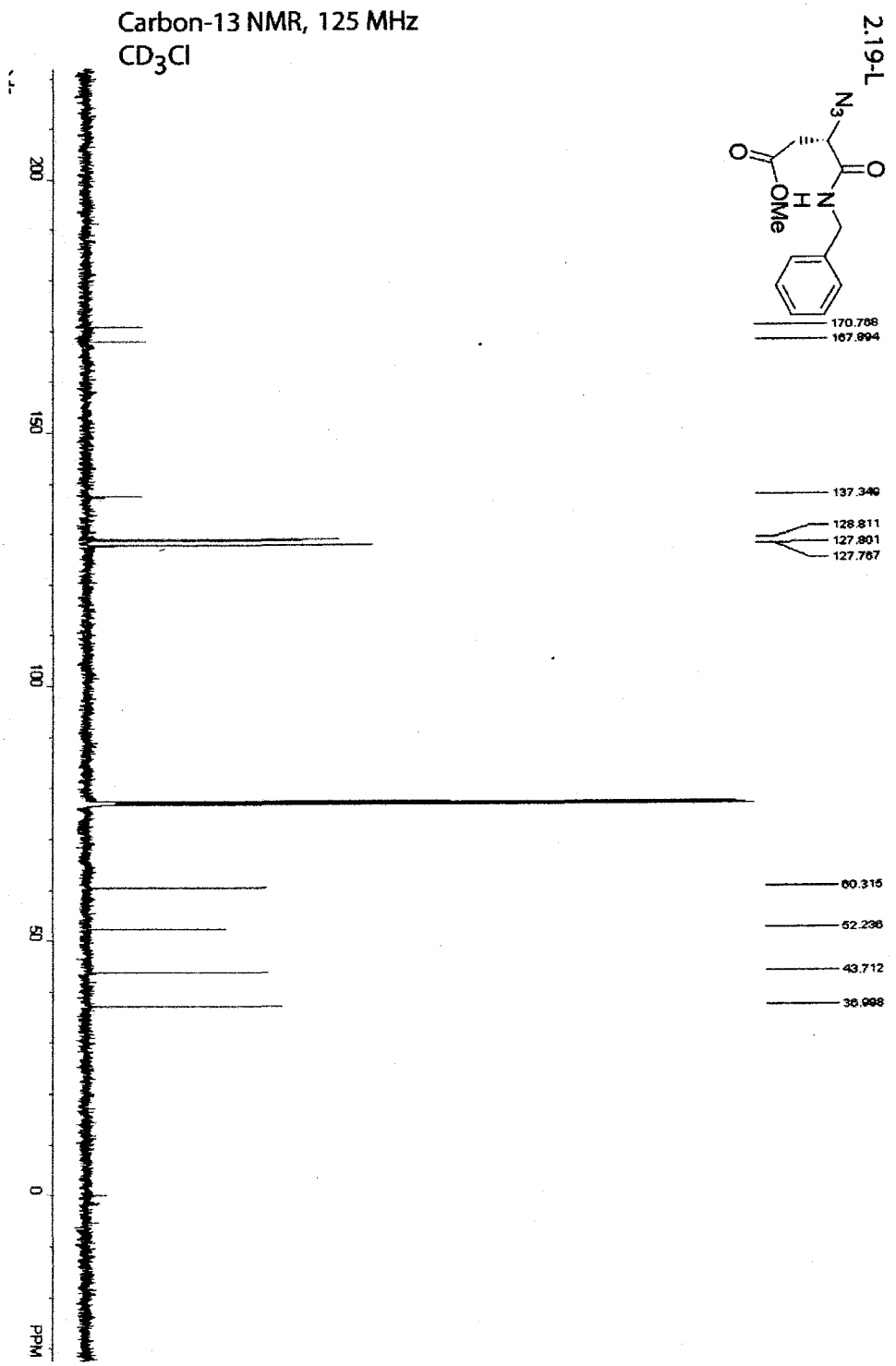


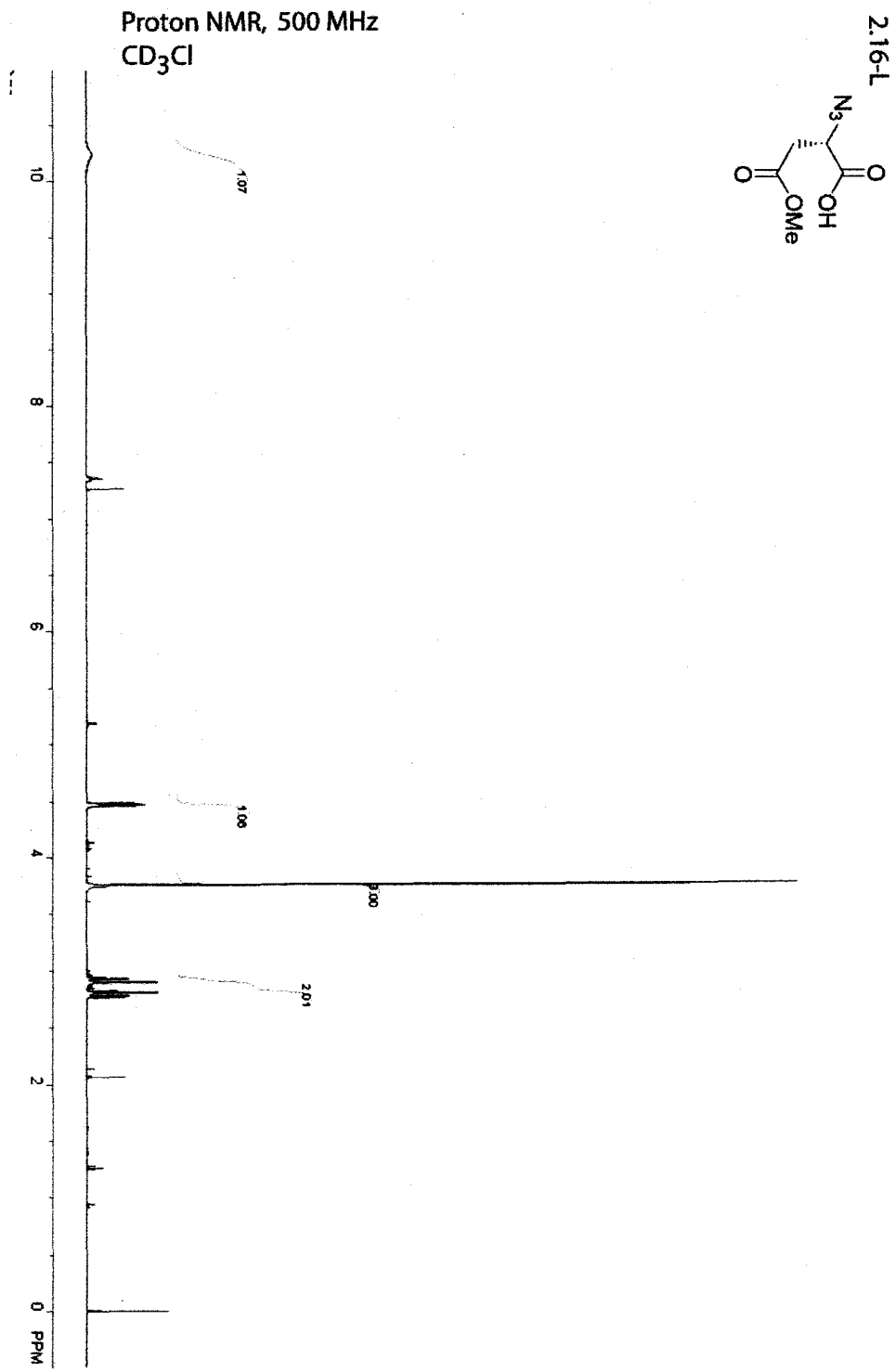


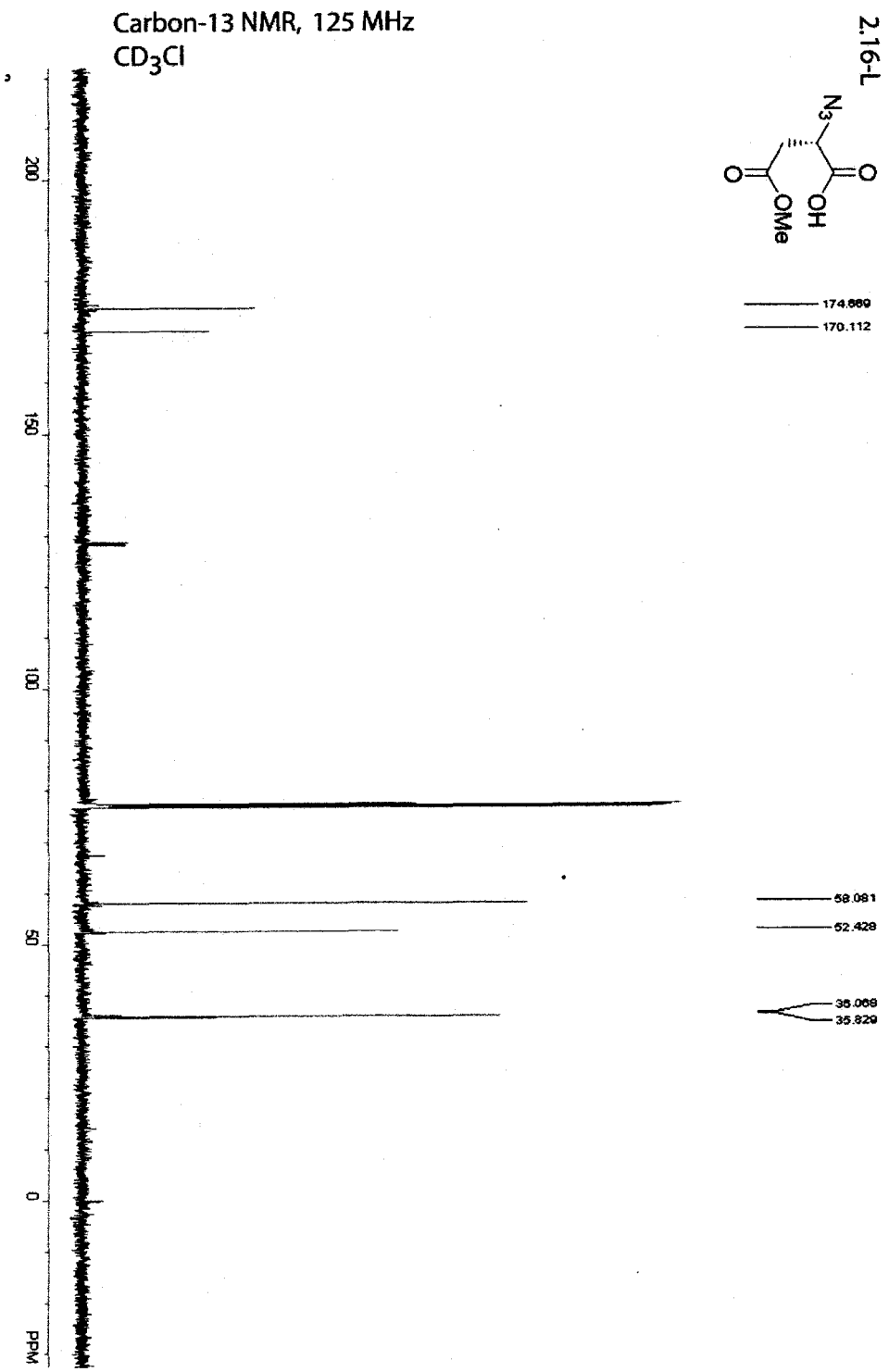


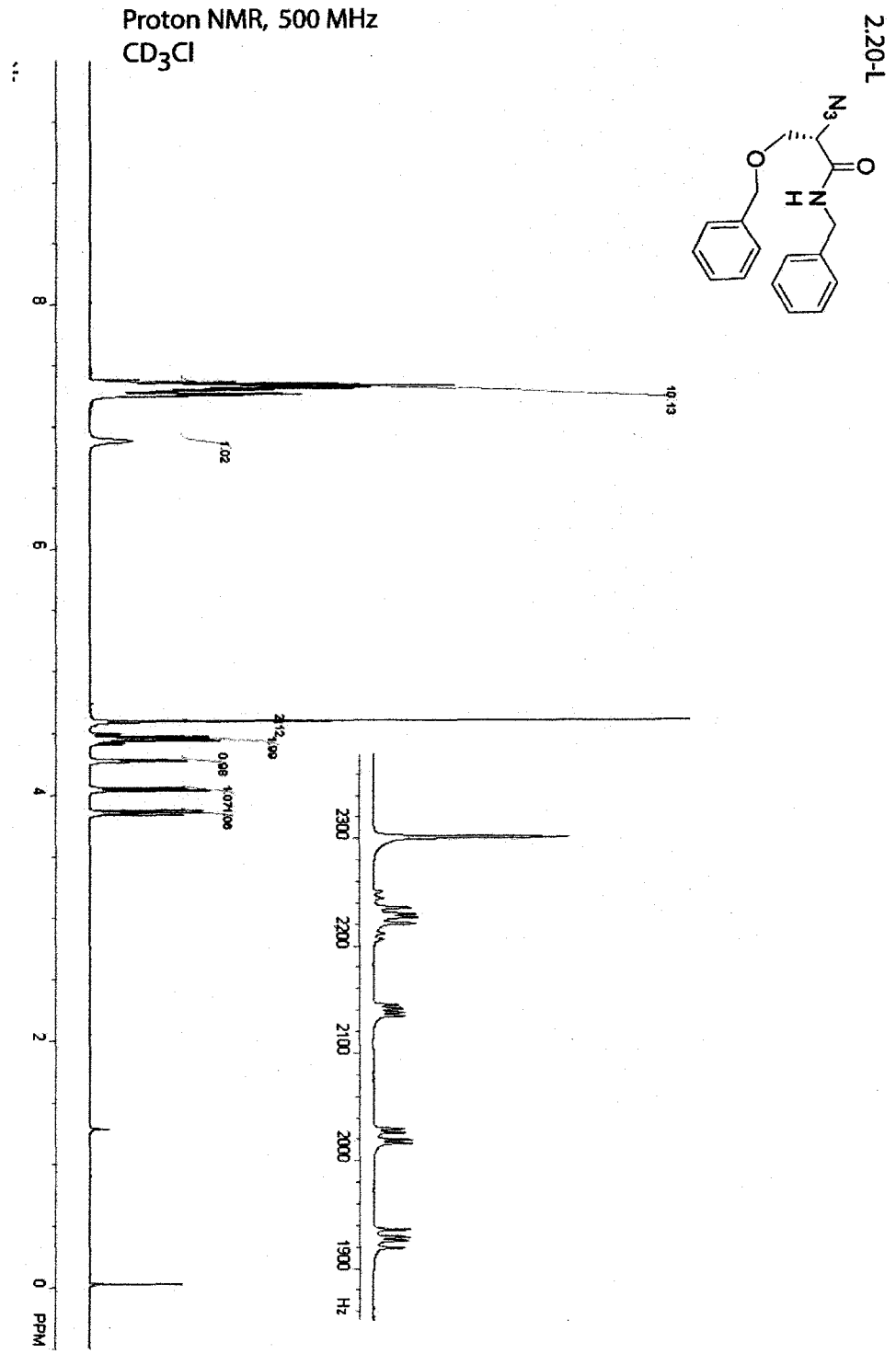


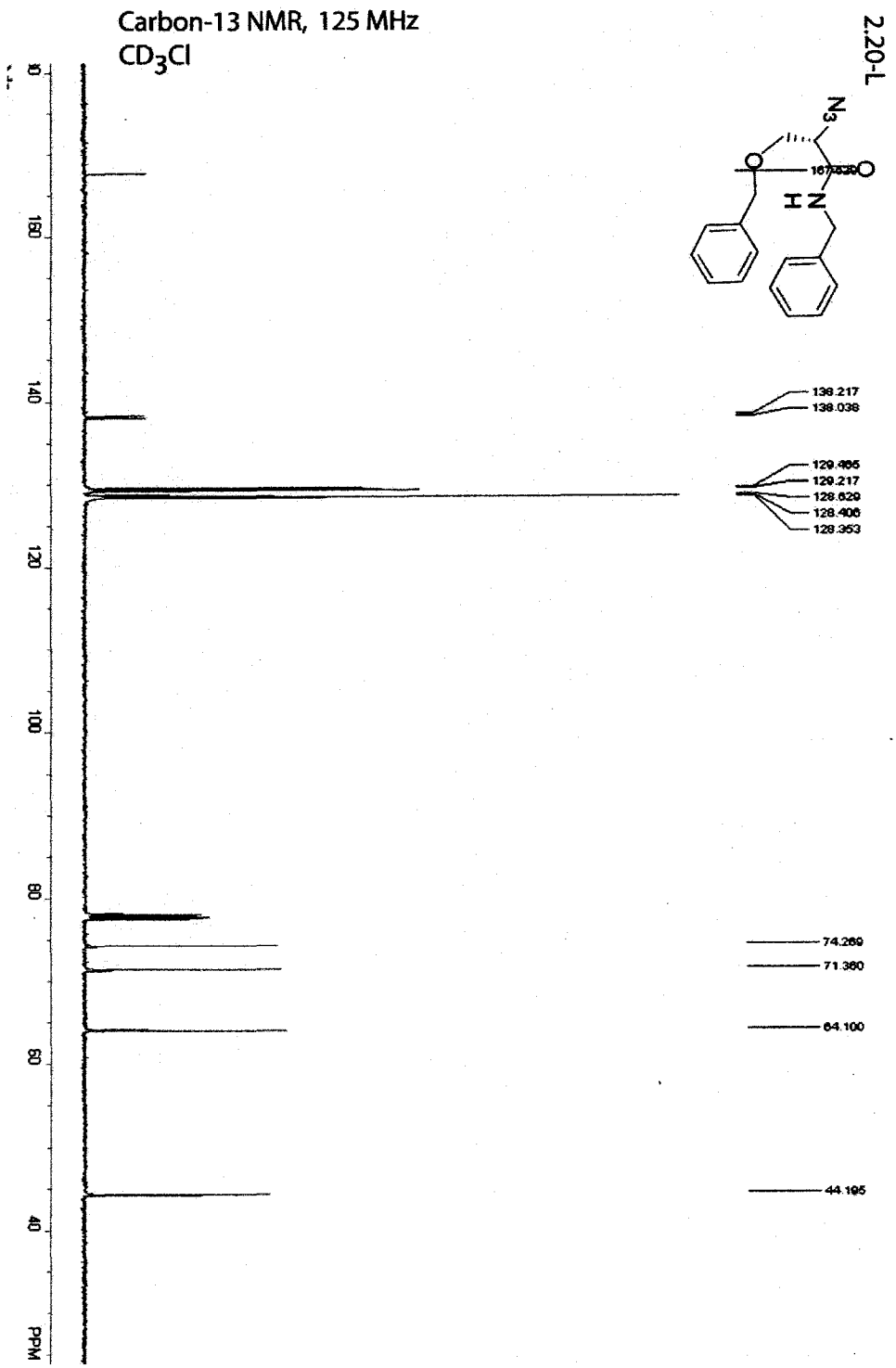


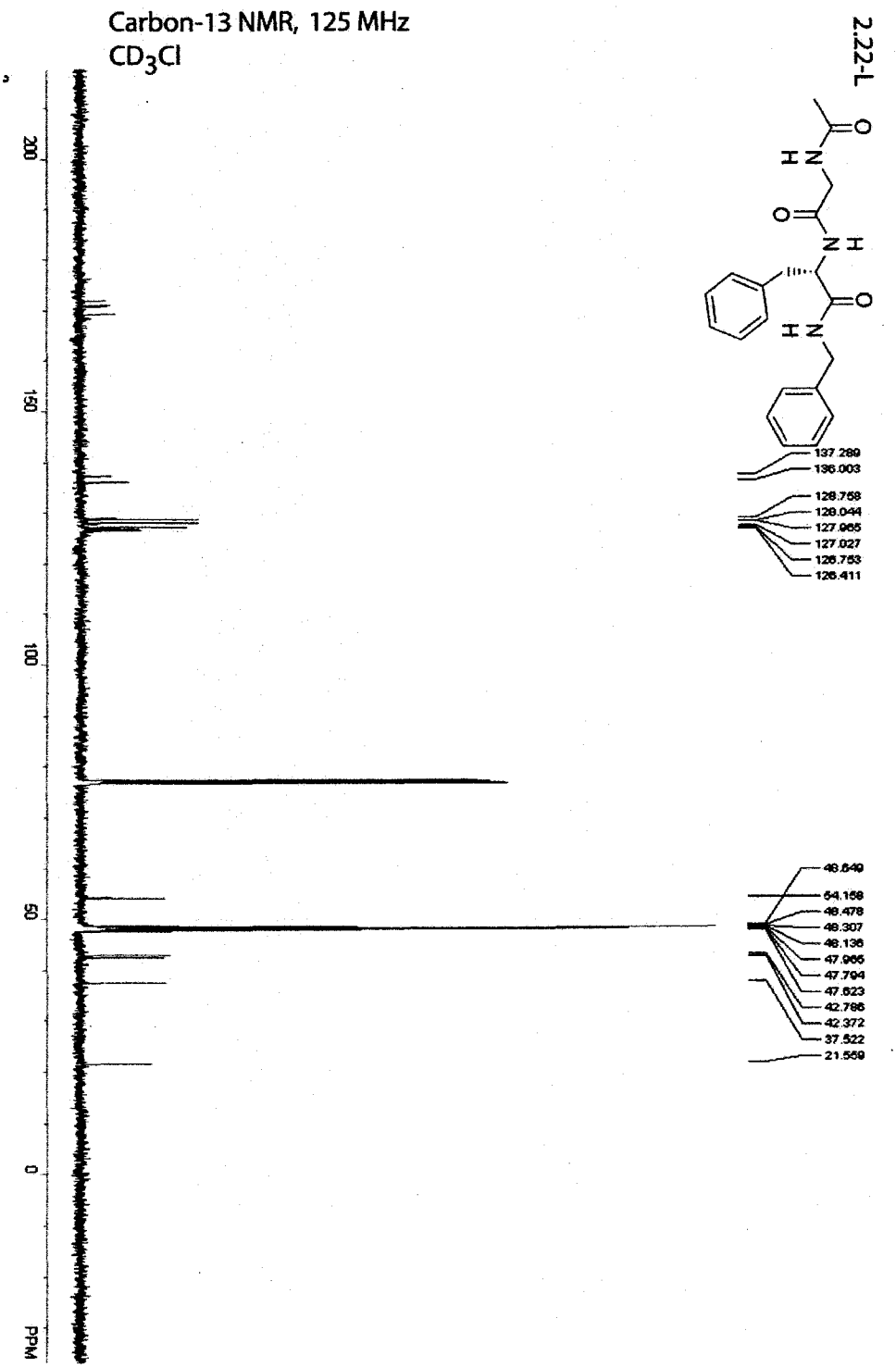


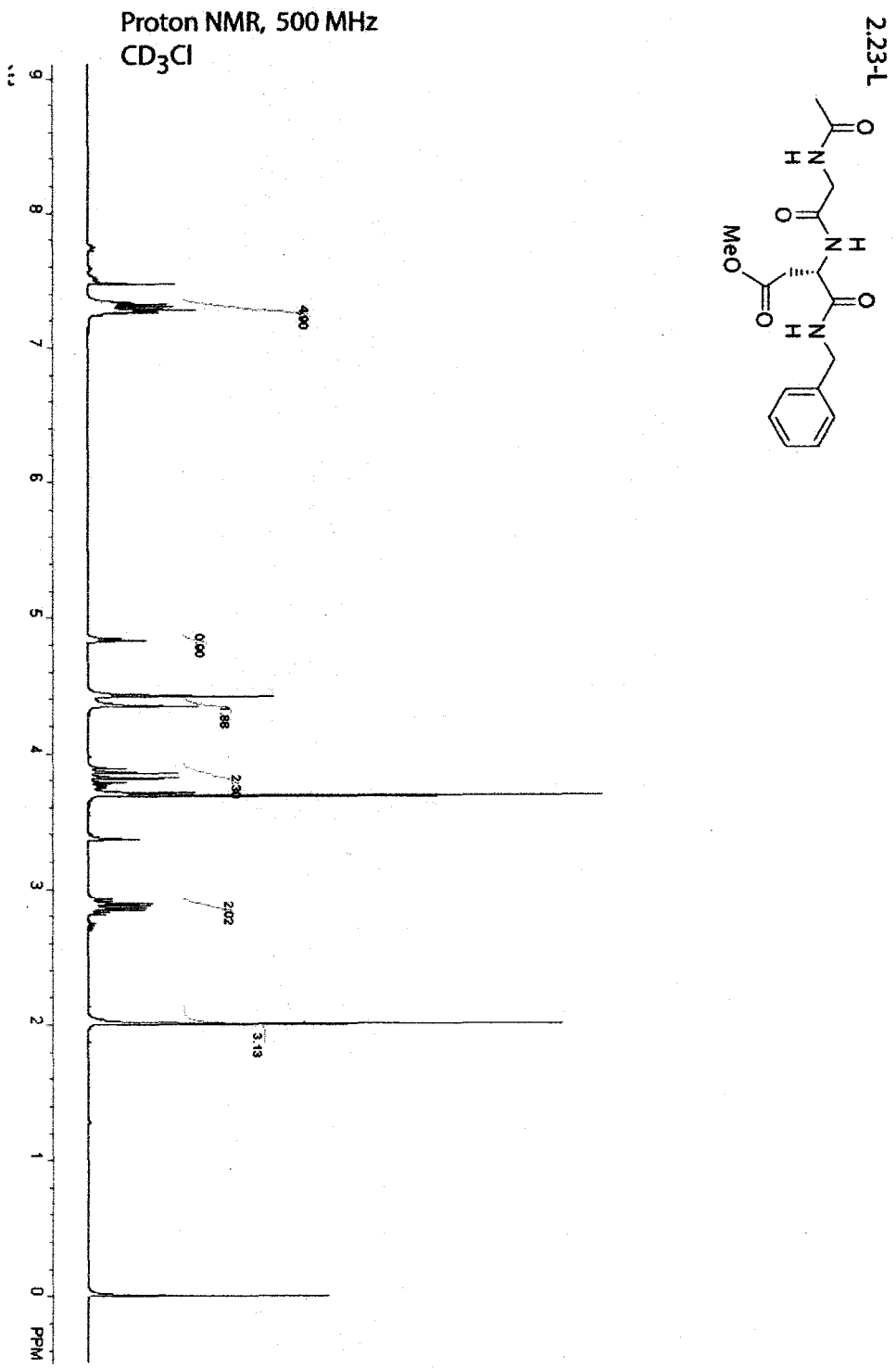




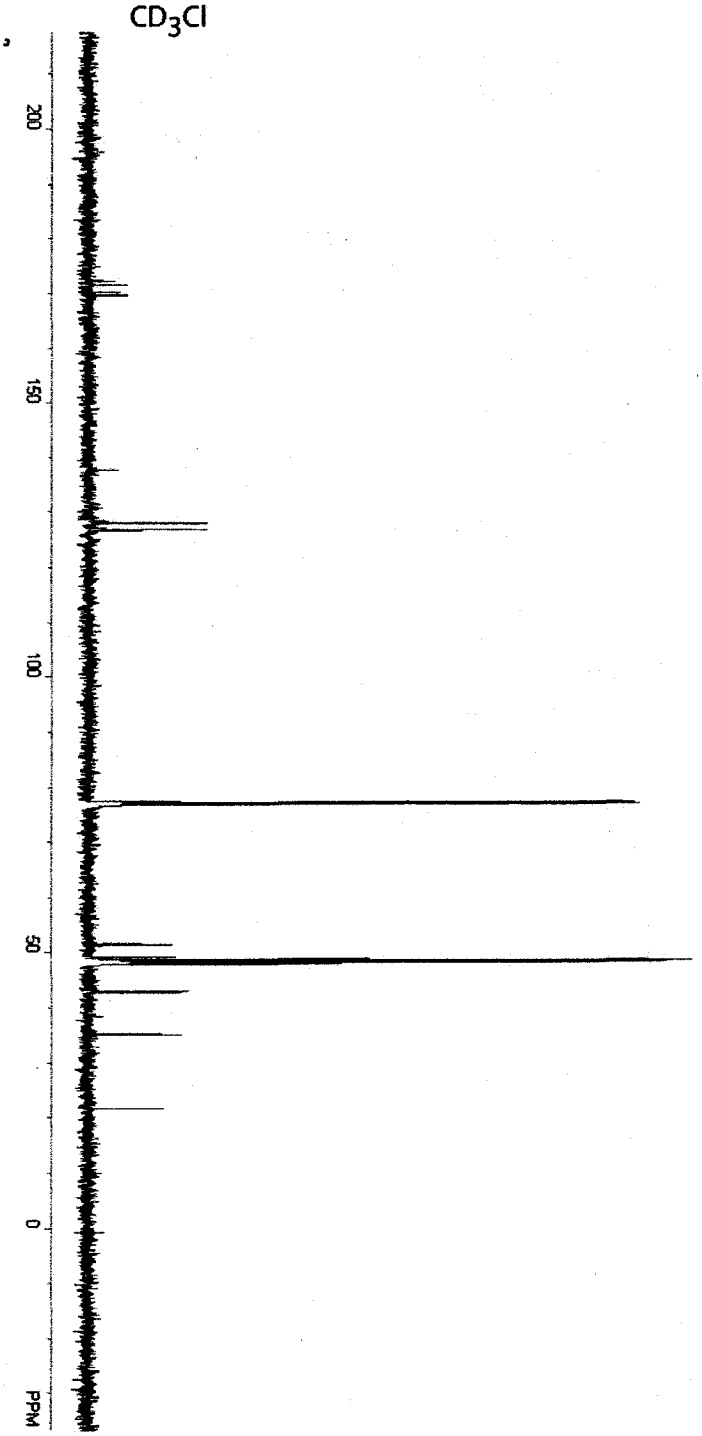




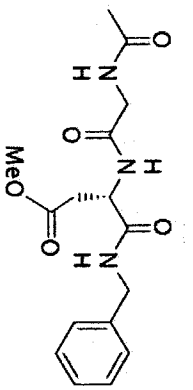


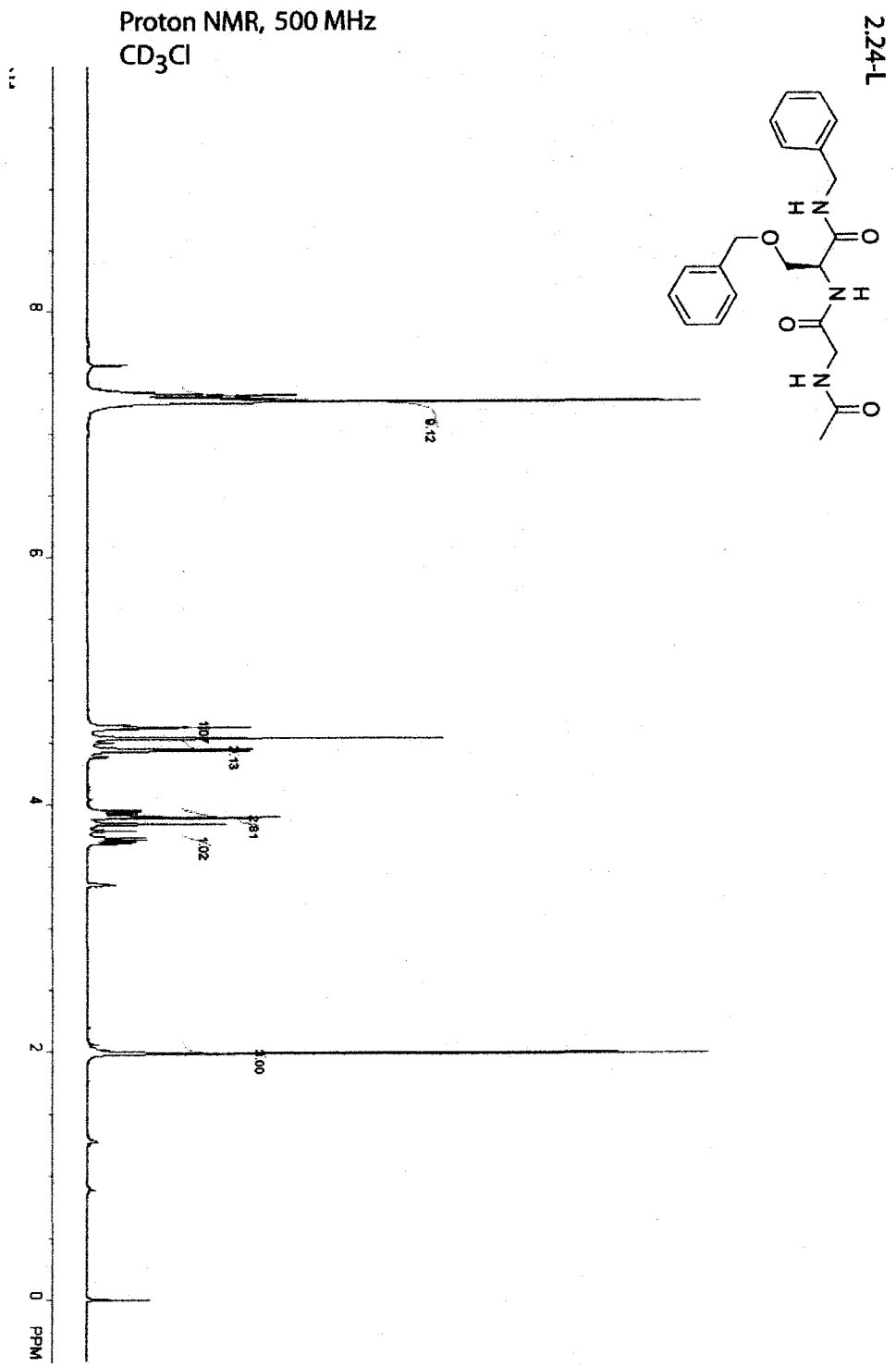


Carbon-13 NMR, 125 MHz
CD_3Cl



2.23-L





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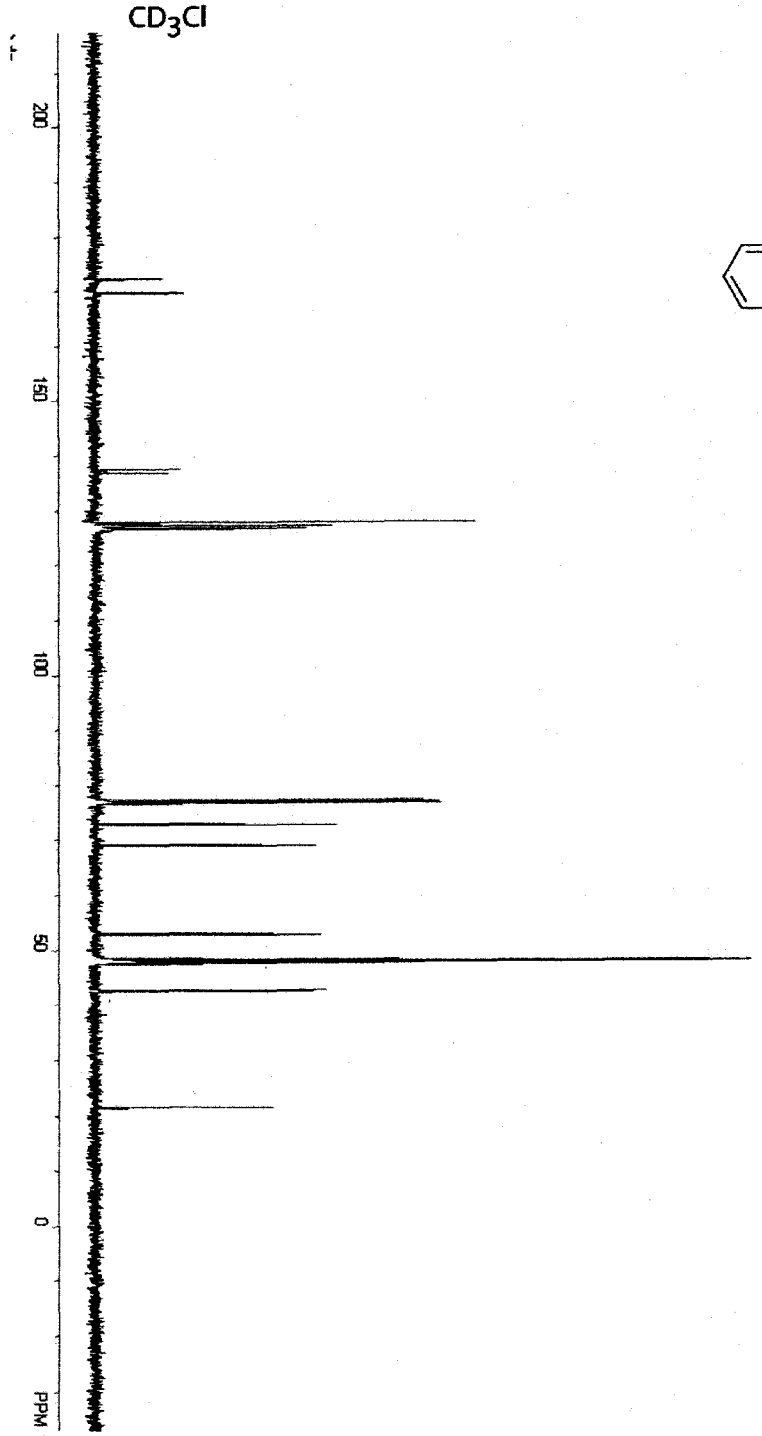
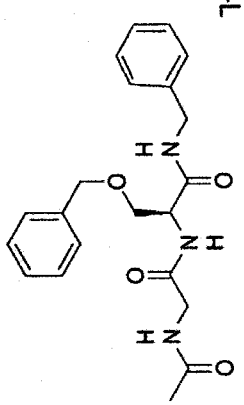
2.24-L





Chapter 3*

SITE-SPECIFIC PROTEIN IMMOBILIZATION BY STAUDINGER LIGATION

Microarrays in which proteins are immobilized to a surface enable high-throughput experiments that require only small amounts of analyte (Kodadek, 2001; Zhu & Snyder, 2001; Lee & Mrksich, 2002). Such protein "chips" can be used to detect protein–ligand, protein–protein, and antibody–antigen interactions. Attaching proteins covalently (MacBeath & Schreiber, 2000; Houseman et al., 2002; Lesaicherre et al., 2002; Toepert et al., 2003) rather than noncovalently (Zhu et al., 2001) produces more robust surfaces. Attaching proteins in a uniform (that is, specific) (Zhu et al., 2001; Houseman et al., 2002; Lesaicherre et al., 2002; Toepert et al., 2003) rather than random (MacBeath & Schreiber, 2000)manner can provide a substantial advantage in assay sensitivity (Peluso et al., 2003). Here, we report a general method for the covalent, uniform immobilization of peptides and proteins that is both rapid and high-yielding. The method relies on a traceless version of the Staudinger ligation in which an azide and phosphinothioester react to form an amide (Scheme 3.1) (Soellner et al., 2002; Nilsson et al., 2003). The reaction is known to occur in high yield at room temperature in aqueous or wet organic solvents, and is compatible with the unprotected functional groups of proteinogenic

* This chapter has been published previously under the same title. Reference: Soellner, M. B.; Dickson, K. A.; Nilsson, B. L.; Raines, R. T. *J. Am. Chem. Soc.* **2003**, *125*, 11790-11791.

amino acids.

As a model protein to immobilize, we chose ribonuclease S′ (RNase S′), which is a truncated form of RNase S. The protease subtilisin prefers to cleave a single peptide bond in RNase A. The product of this cleavage, RNase S, consists of two tightly associated fragments: S-peptide, which derives from residues 1–20 of RNase A, and S-protein, which derives from residues 21–124 (Raines, 1998). Although neither fragment alone has any enzymatic activity, RNase S is as active as intact RNase A. The first 15 residues of S-peptide (S15) bind to S-protein with affinity similar to that of S-peptide. We reasoned that the immobilization of S15 followed by incubation with S-protein would generate an active RNase S′ tethered to a surface.

The S15 peptide was synthesized with an azido group in either a side chain or its main chain. To install an azido group into a side chain, the ε-amino group of Lys1 was replaced with an azido group to generate (N_3)Lys1 S15. To install an azido group into the main chain, a polyethyleneglycol (PEG) fragment with a terminal azido group was attached to the α-amino group of Lys1 to generate N_3–PEG–S15 (Figure 3.1).

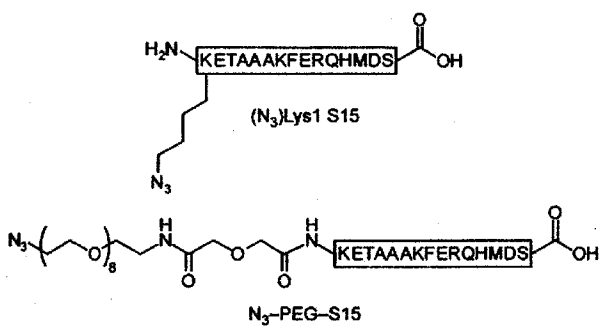
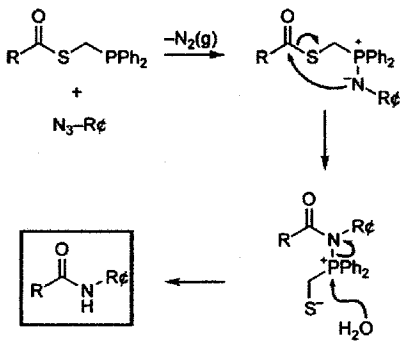


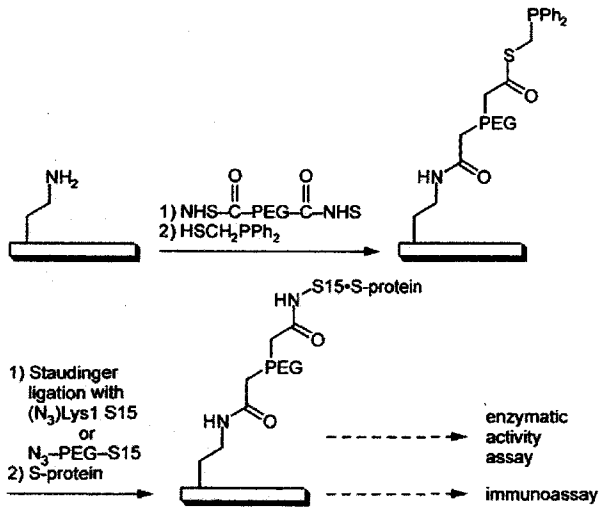
Figure 3.1 (N_3)Lys1 S15 and N_3–PEG–S15.

As a surface, we chose glass slides because of their compatibility with standard array and detection equipment. An amine-derivatized slide was treated with an excess of PEG having succinimidyl ester termini (NHS–C(O)–PEG–C(O)– NHS) in DMF for 2 h, and diphenylphosphinomethanethiol (Soellner et al., 2002) in DMF for 2 h, resulting in a surface-bound phosphinothioester (Scheme 3.2). The PEG layer (M_r = 3400) prevented the nonspecific attachment of protein to the glass surface. Serial dilutions of (N_3)Lys1 S15 or N_3–PEG–S15 were spotted on this phosphinothioester-derivitized slide(peptide concentrations were 5.64 μM–56.4 pM for (N_3)Lys1 S15 (M_r = 1773.8), and 4.38 μM–43.8 pM for N_3–PEG–S15 (M_r = 2284.4)). S-Protein in 0.10 M sodium phosphate buffer (pH 7.2) was spotted on the slide, which was washed thoroughly with buffer.

Scheme 3.1 The Staudinger ligation.



Scheme 3.2 Immobilization scheme for azide analytes.



Two types of assays were used to detect the presence of immobilized RNase S′. First, the ribonucleolytic activity of each spot was determined with a fluorogenic substrate (Kelemen et al., 1999). This assay revealed the amount of active RNase S′ on the surface. After activity assays were performed, the slide was washed thoroughly, and immobilized RNase S′ was visualized by immunostaining. This assay revealed the total amount of RNase S′ on the surface and, hence, the overall yield of the immobilization reaction. Together, the assays indicated that coupling via a side chain (as with (N_3)Lys1 S15) proceeded in 51% yield, and that the enzyme coupled in this manner had 85% activity. Coupling via the main chain (as with N_3–PEG–S15) proceeded in 67% yield, and the enzyme coupled in this manner had 92% activity.

Control experiments indicate that binding occurred only by Staudinger ligation (Figure 3.2A, row 3). First, S15 without an azido group was spotted on a phosphinothioester-derivitized slide. After incubation of the slide with S-protein, no immobilized peptide was detected with the activity assay or immunoassay. Second, immobilized peptide was

necessary for S-protein to bind to the surface, indicating that the phosphinothioester does not react with the functional groups of proteinogenic amino acids. Finally, azido-peptides did not react with a phosphinothioester-derivitized slide that had been treated with NH_2OH, which rapidly cleaves thioesters.

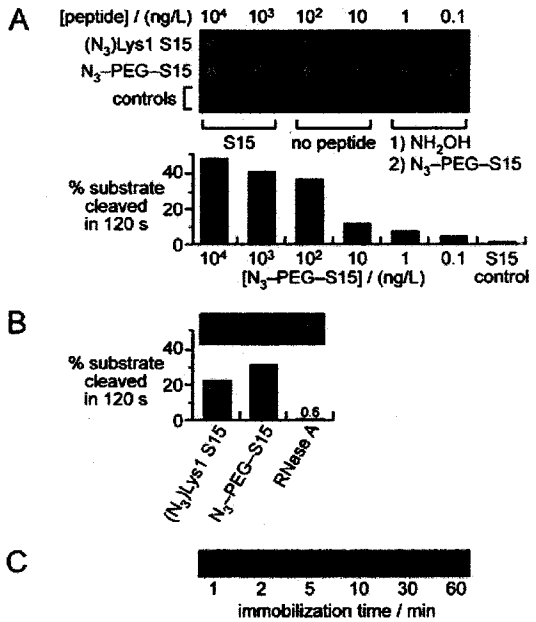


Figure 3.2 Site-specific protein immobilization by Staudinger ligation. **A.** Yield and activity of immobilization by Staudinger ligation. A phosphinothioester-derived slide was spotted for 8 h at room temperature with dilutions of an azido-S15 in DMF/H_2O (50:1). The slide was washed thoroughly with DMF, and then with 0.10 M sodium phosphate buffer (pH 7.2). As controls, wild-type S15 (5 μM) was spotted for 8 h, no peptide was spotted, or the slide was treated with NH_2OH before spotting for 8 h with N_3–PEG–S15. After incubation with S-protein (10 μg/μL) for 8 h, each spot was assayed for ribonucleolytic activity and fluorescence after staining with a primary antibody (to RNase A) and a secondary antibody conjugated to Alexa Fluor® 488. **B.** Uniform versus random immobilization of protein. A slide displaying NHS-esters was spotted with ribonuclease A (5.0 nM) or phosphinothioester, an azido-S15 (5.0 nM), and S-protein (10 μg/μL). Each spot was assayed as in panel A. **C.** Timecourse of immobilization by Staudinger ligation. A phosphinothioester-derived slide was spotted with N_3–PEG–S15 (5.6 nM) for 1–60 min, and then quenched with NH_2OH. After incubation with S-protein (10 μg/μL), each spot was subjected to immunoassay as in panel A.

Uniform immobilization of a protein analyte yields higher activity than does random immobilization. Amine-derivatized slides were treated with NHS–C(O)–PEG–C(O)–NHS in DMF for 2 h, and then intact RNase A (5.0 nM) in buffer for 8 h. Such immobilization will occur randomly via enzymic amino groups. Coupling proceeded in 45% yield, but the coupled RNase A had only 6% activity (Figure 3.2B). This low activity is likely due to RNase A being attached through amino groups that are important for function (Raines, 1998), which is an intrinsic disadvantage of random immobilization.

Immobilization by Staudinger ligation is remarkably rapid. A sub-saturating concentration of N_3–PEG–S15 was spotted for 1–60 min. After addition of S-protein, an immunoassay showed that the same amount of peptide became attached to the slide in 1 min as in 1 h (Figure 3.2C) or 8 h (data not shown). Accordingly, the immobilization reaction has $t_{1/2} < 1$ min.

The Staudinger ligation is the most efficacious method known for the site-specific, covalent immobilization of a protein. No other approach enables more rapid immobilization or a higher yield of active protein (*cf.*: ref 2–5). Azido-peptides (Soellner et al., 2002) and azido-proteins (Kiick et al., 2002) are readily attainable, and the reactivity of the azido group is orthoganol to that of biomolecules. Accordingly, the Staudinger ligation could be of unsurpassed utility in creating microarrays of functional peptides and proteins.

Experimental Section.

General Experimental. Chemicals and solvents were from Aldrich with the exception of Fmoc-protected amino acids (Novabiochem). Amine-derivatized slides were from CEL Associates (ArrayIt.com). Anhydrous THF, DMF, and CH_2Cl_2 were from a CYCLE-TAINER® solvent delivery system (Baker). Other anhydrous solvents were obtained in septum-sealed bottles. Reaction progress was monitored by thin-layer chromatography and visualized by illuminating with UV light or staining with I_2. Flash chromatography was performed with silica gel 60, 230–400 mesh (Silicycle). Peptide synthesis was performed using standard Fmoc-protection strategies with HATU activation on an Applied Biosystems Pioneer automated synthesizer. Preparative HPLC was performed with a Varian Dynamax C-18 reversed-phase column. Analytical HPLC was performed with a Vydac C-18 reversed-phase column using a linear gradient of H_2O and CH_3CN, both containing TFA (0.1% v/v). NMR spectra were obtained using Bruker AC-300 or Varian UNITY-500 spectrometers. Mass spectra were obtained using electrospray ionization (ESI) or matrix-assisted laser desorption ionization (MALDI) techniques.

Synthesis of Boc–Lys(N_3)OH (3.1). Boc–Lys(N_3)OH was synthesized from Boc–LysOH by using procedures for Cu(II)-catalyzed diazo-transfer to amines. Boc–LysOH (4.38 g, 17.8 mmol), $CuSO_4 \cdot 5H_2O$ (44.7 mg, 178.2 μmol) and K_2CO_3 (3.69 g, 26.8 mmol) were dissolved in H_2O (57 mL) and MeOH (114 mL). N_3Tf (17.8 mmol in 150 mL CH_2Cl_2) was added, and the reaction mixture was stirred overnight. MeOH was removed under reduced pressure. The resulting aqueous solution (50 mL) was diluted with H_2O (0.10 L), and the pH was adjusted to 6.0 with HCl. Sodium phosphate buffer

(150 mL, 0.25 M, pH 6.2) was added, and the solution was washed with EtOAc (3×) to remove the triflic amine byproduct. The pH of the washed aqueous layer was adjusted to 2.0 with HCl, and the resulting solution was washed with EtOAc (4×) to extract the desired product. The organic layer was then dried over $MgSO_4$(s) and filtered, and the solvent was removed under reduced pressure to yield Boc–Lys(N_3)OH as a clear oil (50%). **Spectral data.** ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.93 (broad s, 1H), 5.11 (d, J = 8.3 Hz, 1H), 4.34 (m, 1H), 3.30 (d, J = 7.0 Hz, 2H), 1.80 (m, 2H), 1.65 (m, 2H), 1.49 (m, 2H), 1.46 (s, 9H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$) δ 179.96, 156.30, 97.44, 55.79, 53.79, 34.66, 31.07, 30.96, 25.20 ppm; MS (ESI) m/z 295.1383 (MNa^+ [$C_{11}H_{20}N_4O_4Na$] = 295.1382).

Synthesis of (N_3)Lys1 S15. S-Peptide residues 2–15 was synthesized on a 0.2-mmol scale using pre-loaded Fmoc–Ser–PEG–PS (Applied Biosystems) as the solid support. Boc–Lys(N_3)OH (218 mg, 0.8 mmol) was dissolved in DMF (15 mL), and PyBOP (416 mg, 0.8 mmol) was added to the resulting solution. The solution was placed under Ar(g), and DIPEA (206.8 mg, 1.6 mmol) was added. The resulting mixture was added to a peptide synthesis vessel containing S-peptide residues 2–15 on a solid support. The coupling reaction was allowed to proceed for 2 h with mixing via bubbling Ar(g). The resin was then filtered and washed with DMF followed by CH_2Cl_2. Cleavage cocktail (9.5 mL TFA, 0.5 mL TIS, and 0.5 mL H_2O) was then added to the resin. The cleavage reaction was allowed to proceed for 2 h with mixing via bubbling Ar(g). The filtrate was then added dropwise to ice-cold diethyl ether (30 mL), and the deprotected peptide was collected by centrifugation. The pellet was washed (2×) in ice-cold diethyl ether and

dissolved in H_2O/CH_3CN (90:10). The peptide was purified by reversed-phase HPLC on a C-18 column. **Spectral data.** MS (MALDI) *m/z* 1775.0 (MH^+ = 1774.8).

Synthesis of N_3–PEG–S15. S15 was synthesized on the 0.2-mmol scale using pre-loaded Fmoc-Ser-PEG-PS (Applied Biosystems) as the solid support. N_3–PEG–CO_2H (444 mg, 0.8 mmol, Novabiochem) was dissolved in 15 mL DMF and PyBOP (416 mg, 0.8 mmol) was added to the resulting solution. The reaction was placed under Ar(g) and DIPEA (206.8 mg, 1.6 mmol) was added. The resulting mixture was added to a peptide synthesis vessel containing S15 on a solid support. The coupling reaction was allowed to proceed for 2 h with mixing via bubbling Ar(g). The resin was then filtered and washed with DMF followed by CH_2Cl_2. Cleavage cocktail (9.5 mL TFA, 0.5 mL TIS, and 0.5 mL H_2O, premixed) was then added to the resin. The cleavage reaction was allowed to proceed for 2 h with mixing via bubbling Ar(g). The filtrate was then added dropwise to ice-cold diethyl ether (30 mL) and centrifuged to collect the deprotected peptide. The pellet was washed twice in ice-cold diethyl ether and dissolved in H_2O/CH_3CN (90:10). The peptide was purified by reversed-phase HPLC on a C-18 column. **Spectral data.** MS (MALDI) *m/z* 2285.8 (MH^+ = 2285.4).

Preparation of Phosphinothioester-Derivatized Surface. PEG (M_r 3400) disuccinimidyl propionate (NHS–C(O)–PEG–C(O)–NHS, Shearwater Polymers, 0.1 M in anhydrous DMF) was added to a glass microscope slide derivastized with 3-aminopropyltriethoxysilane. The reaction was allowed to proceed for 2 h under Ar(g). The reaction mixture was washed extensively with anhydrous DMF. To generate a surface-bound phosphinothioester, phosphinomethanethiol (0.10 M in anhydrous DMF) was added to the slide and allowed to react for 2 h under Ar(g). The slide was then

washed with anhydrous DMF (20 × 10 mL) and allowed to dry under a stream of Ar(g).

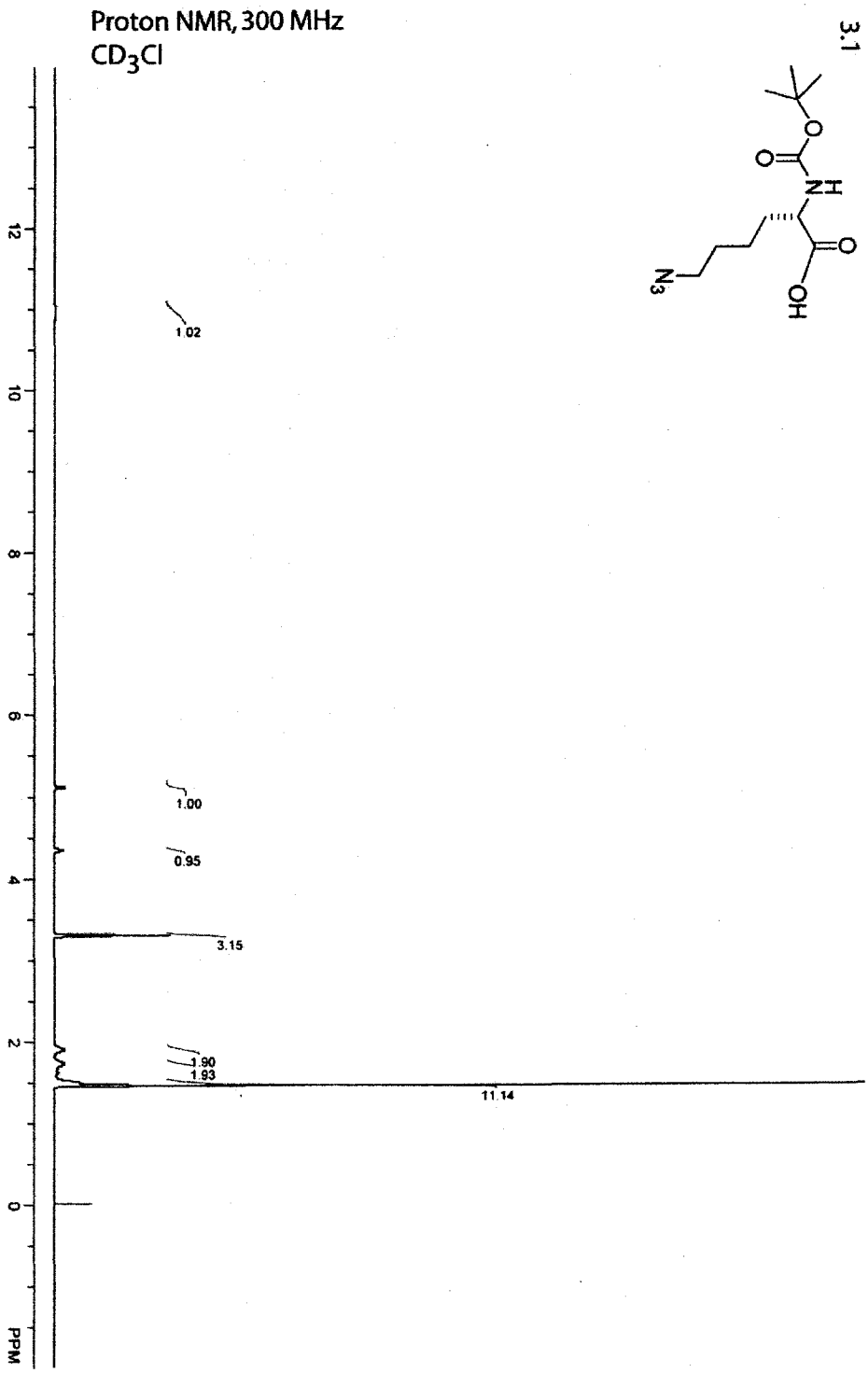
Staudinger Ligation on Phosphinothioester-Derivatized Surface. An azido-peptide ((N_3)Lys1 S15 or N_3–PEG–S15) in DMF/H_2O (50:1) was added to a phosphinothioester-derivatized slide at a desired concentration. (Peptide concentrations were spotted with a micropipettor in 0.8-mm spots. Peptide concentrations ranged from ~5 μM to 50 pM, see text.) The Staudinger ligation was allowed to proceed for 8 h in an enclosed chamber saturated with DMF. The slide was then washed with DMF (20 × 10 mL) and 0.10 M phosphate buffer (pH 7.2, 20 × 10 mL).

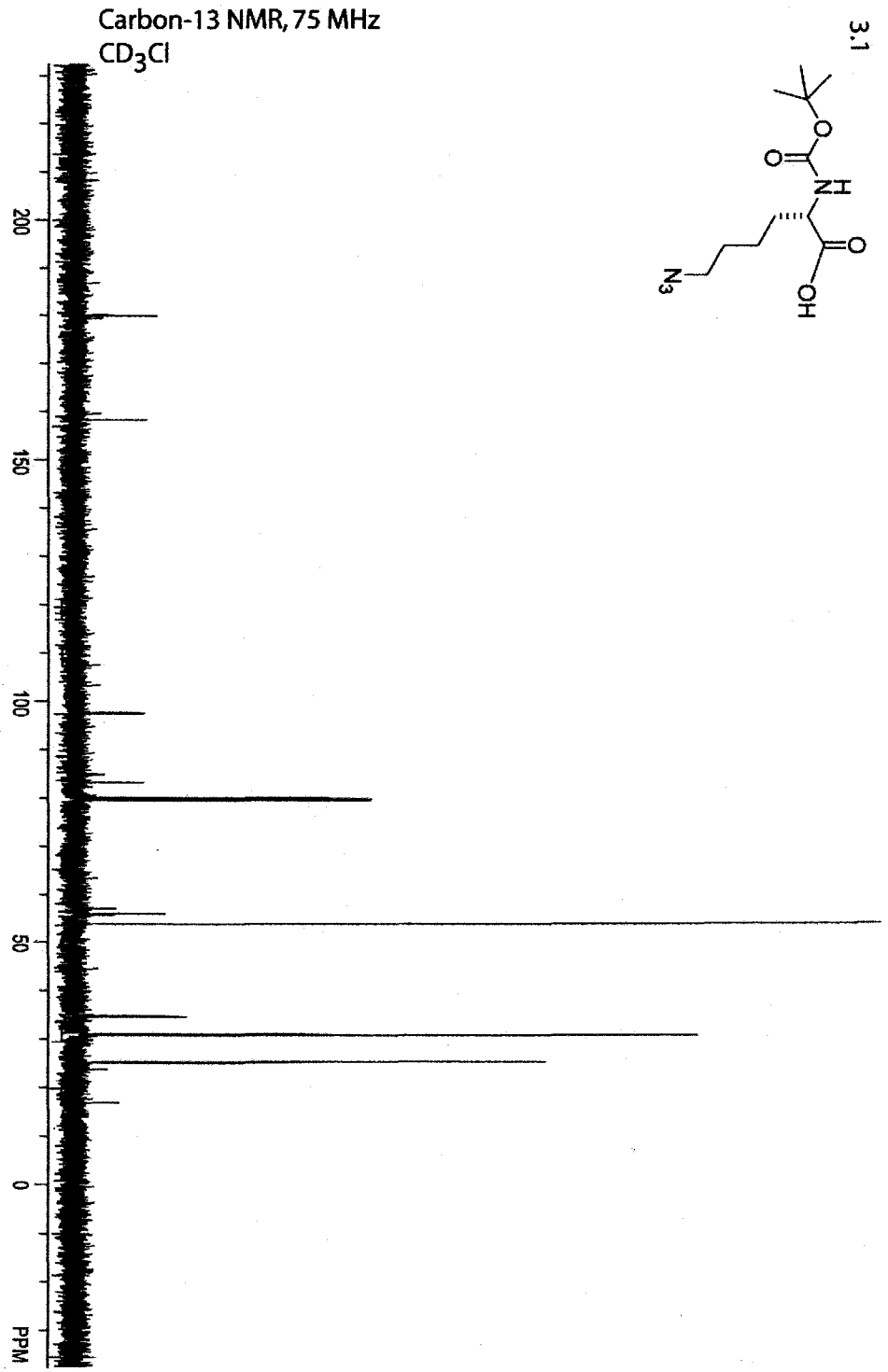
Generation of Immobilized Ribonuclease S′. RNase A and RNase S were removed from commercial S-protein (Sigma Chemical) by reversed-phase HPLC on a Vydac C-4 column. Purified S-protein (10 mg/mL in 0.10 M sodium phosphate buffer, pH 7.2) was incubated for 8 h on a slide presenting immobilized S15 (see above). The slide was then was thoroughly with buffer (30 × 10 mL). Slides were stored in 0.10 M sodium phosphate buffer, pH 7.2.

Activity Assay of Immobilized Ribonuclease S′. The ribonucleolytic activity of each spot was determined using the fluorogenic substrate, 6-FAM–(dA)rU$(dA)_2$–6-TAMRA. Cleavage of this substrate results in a *ca.* 200-fold increase in fluorescence intensity (excitation at 494 nm; emission at 515 nm). Assays were performed by adding 0.10 M MES–NaOH buffer (pH 6.0) containing 0.10 M NaCl and 6 μM 6-FAM–(dA)rU$(dA)_2$–6-TAMRA to each spot. At various times, an aliquot was removed from the plate and its fluorescence was recorded. The concentration of RNase S′ in each spot was determined by using the equation $[\text{RNase S}'] = (\Delta I/\Delta t)/[(I_f - I_0)(k_{cat}/K_M)]$, where $\Delta I/\Delta t$ is the initial

velocity of the reaction, I_0 is the fluorescence intensity prior to exposure to the plate, I_f is the fluorescence intensity after complete hydrolysis of the substrate with excess RNase A, and $k_{cat}/K_M = 3.6 \times 10^7\ M^{-1}s^{-1}$.

Immunoassay of Immobilized Ribonuclease S′. RNase S′ attached to the plate was visualized by immunostaining. Rabbit IgG raised against RNase A (Biodesign International) was used at a concentration of 2 μg/mL. Each spot was incubated with primary antibody for 30 min at 23 °C. After exposure to primary antibody, the entire slide was washed with PBS (4 × 20 mL). Secondary antibody conjugated to Alexa Fluor® 488 (Molecular Probes) was diluted in PBS to 1 μg/mL and incubated as described for primary antibody. The slide was then washed with PBS (4 × 20 mL). RNase S′ was detected by using a FluorImager SI fluorescence scanner (Molecular Dynamics). The quantity of immobilized protein was determined with IMAGEQUANT densitometry software (Molecular Dynamics) and a standard curve generated by spotting various concentrations of Alexa Fluor® 488-conjugated secondary antibody.





Chapter 4

REACTION MECHANISM AND KINETICS OF THE STAUDINGER LIGATION

Abstract: The Staudinger ligation enables the formation of an amide bond between a peptide with a *C*-terminal phosphino(thio)ester and a peptide with an *N*-terminal azide. In the Staudinger ligation reaction mediated by phosphinoalcohols, the iminophosphorane is known to proceed by an aza-Wittig reaction that yields a stable imidate. Here, the reaction mediated by phosphinothiols, the iminophosphorane is shown to proceed by *S*→*N* acyl transfer. The development of a quantitative NMR assay enabled the collection of high-quality data for the observation of reaction intermediates and kinetic analyses. Less efficacious coupling reagents and reaction conditions lead to the formation of an amine byproduct, which results from a Staudinger reduction reaction, or a phosphonamide byproduct, which results from an aza-Wittig reaction. No intermediates were found to accumulate during the Staudinger ligation of glycyl residues mediated by (diphenylphosphino)methanethiol, which proceeded with a second-order rate constant of $k_2 = 7.7 \times 10^{-3}\ M^{-1}s^{-1}$ that was unchanged in the presence of exogenous nucleophiles. In comparison to (diphenylphosphino)methanethiol, Staudinger ligation reactions mediated by phosphinoalcohols have low rate constants or chemoselectivity. Accordingly, (diphenylphosphino)methanethiol is the most efficacious known reagent for effecting the traceless Staudinger ligation.

4.1 Introduction

The advent of methodology for the chemoselective ligation of peptide fragments has made proteins accessible targets for total chemical synthesis (Nilsson et al., 2005). Many proteins have already been assembled from synthetic peptides using prior capture strategies. "Native chemical ligation"—the coupling of a peptide (or protein) containing a *C*-terminal thioester with another peptide containing an *N*-terminal cysteine residue—has been the most widely applied of such strategies (Dawson & Kent, 2000; Tam et al., 2001; Kent, 2003). "Expressed protein ligation" is a variation of native chemical ligation in which the *C*-terminal thioester is accessed by using recombinant DNA technology (Muir, 2003). While powerful, these methods are limited by their requirement for cysteine, which is the second least common residue (McCaldon & Argos, 1988a).

Emerging strategies for protein assembly avoid the need for a cysteine residue at the ligation junction. The Staudinger ligation is one such strategy (Kohn & Breinbauer, 2004; Brase et al., 2005). In one form of the Staudinger ligation, a peptide with a *C*-terminal phosphinothioester is coupled with a second peptide having an *N*-terminal azido acid to form a native amide bond without any residual atoms (Nilsson et al., 2000; Nilsson et al., 2001). The overall reaction occurs without racemization (Soellner et al., 2002). The Staudinger ligation has been used in the orthogonal assembly of a fully functional enzyme and for the site-specific immobilization of peptides and small molecules to a surface (Nilsson et al., 2003; Soellner et al., 2003). A variety of phosphines have been explored in applying the Staudinger ligation to other problems, including glycopeptides synthesis (Bianchi & Bernardi, 2004; He et al., 2004) and biomolecular labeling (Saxon et al., 2000; Saxon & Bertozzi, 2000b; Kiick et al., 2002; Saxon et al., 2002; Ovaa et al.,

2003; Restituyo et al., 2003; Vocadlo et al., 2003; Wang et al., 2003; Hosoya et al., 2004; Kho et al., 2004; Rose et al., 2005; Sprung et al., 2005).

Two distinct phosphinothiols have been used specifically for the Staudinger ligation of peptides. The first, a phosphinothiophenol, was able to provide dipeptides from phosphinothioesters and azido acids, albeit in modest yield (≤35%) (Nilsson et al., 2000). The second was a phosphinomethanethiol that effected the Staudinger ligation in high yield (>90%) (Nilsson et al., 2001; Soellner et al., 2002). These couplings were performed in mixed THF/water or DMF/water solvents with a stoichiometric ratio of reagents. To form an Xaa–Yaa junction, however, either Xaa or Yaa (or both) must be glycine residue for the ligation reaction to be efficient (B. L. Nilsson, M. B. Soellner, and R. T. Raines, unpublished results).

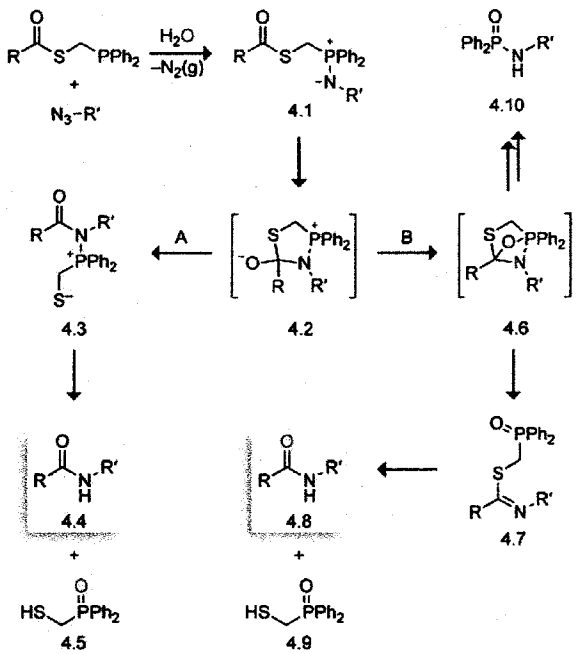
Herein, we investigate the mechanism and kinetics of the Staudinger ligation reaction between a variety of phosphino(thio)esters and azides. To do so, we develop and use a continuous NMR-based assay for the Staudinger ligation reaction. This assay provides detailed insight as well as the means to optimize this versatile synthetic reaction.

4.2 Results and Discussion

Mechanism of the Staudinger Ligation Reaction. Our putative mechanism for the Staudinger ligation reaction is shown in Scheme 4.1 (Nilsson et al., 2001). The nitrogen atom of an iminophosphorane such as **4.1** has intrinsic nucleophilicity and can be acylated by a thioester (Scheme 4.1, Path A) (García et al., 1984; García et al., 1986; Urpí & Vilarrasa, 1986; Bosch et al., 1993; Bosch et al., 1995; Bosch et al., 1996; Ariza et al., 1998; Ariza et al., 2001). This acylation likely proceeds via tetrahedral intermediate **4.2**

to form amidophosphonium salt **4.3**. Finally, the P–N bond of the amidophosphonium salt is hydrolyzed to form amide **4.4** and phosphine oxide **4.5**.

There is considerable literature precedent to indicate that the iminophosphorane formed from a phosphinoester (as opposed to a phosphinothioester) tends to undergo an aza-Wittig reaction (Molina & Vilaplana, 1994) to produce a stable imidate (Scheme 4.1, Path B) (Restituyo et al., 2003; Rose et al., 2005). Indeed, Bertozzi and coworkers recently used ^{31}P NMR to demonstrate that a Staudinger ligation reaction entailing a phosphinoester proceeds through an oxazaphosphetane (Saxon et al., 2002), an intermediate in the aza-Wittig reaction of an iminophosphorane and ester to form an imidate. In Scheme 4.1, the intramolecular aza-Wittig reaction of iminophosphorane **4.1** would also result in the formation of an oxazaphosphetane, here **4.6**, which could lead to thioimidate **4.7**. In contrast to the stable imidate formed during Staudinger ligation reactions mediated by phosphinoalcohols, a thioimidate has not been observed as a product of the reaction mediated by phosphinothiols (David et al., 2003; He et al., 2004; Grandjean et al., 2005). Nonetheless, the failure to observe thioimidate **4.7** does not preclude its existence on the reaction pathway. Accordingly, we did an experiment to search for evidence of the aza-Wittig reaction of iminophosphorate **4.1**.



Scheme 4.1. Putative Mechanisms for the Staudinger Ligation Reaction with (Diphenylphosphino)methanethiol

In our proposed mechanism for the Staudinger ligation reaction mediated by a phosphinothiol (Scheme 4.1, Path A) (Nilsson et al., 2000; Nilsson et al., 2001), the oxygen of phosphine oxide **4.5** derives from water during hydrolysis. In the alternative aza-Wittig mechanism (Scheme 4.1, Path B), this oxygen originates from the thioester oxygen. To distinguish between these two mechanisms, we reacted azide **4.11** (R = $AcNHCH_2$ in Scheme 4.1) and phosphinothioester **4.12** (R′ = $CH_2C(O)NHBn$) in $[^{18}O]H_2O$ and examined the products with mass spectrometry. We found that amide **4.4** contained exclusively ^{16}O, while phosphine oxide **4.5** contained only ^{18}O (Spectral data. Amide **4.4,** MS (ESI) *m/z* 286.1149 (MNa^+ $[C_{13}H_{17}N_3O_3Na^+]$ = 286.1162); phosphine oxide **4.5**, MS (ESI) *m/z* 273.0366 (MNa^+ $[C_{13}H_{13}{}^{18}OPSNa^+]$ = 273.0359)). These data demonstrate that an aza-Wittig reaction does not occur to an appreciable extent in the

traceless Staudinger ligation reaction to form a Gly–Gly junction using (diphenylphosphino)methanethiol as the coupling reagent.

NMR Assay to Monitor the Staudinger Ligation. We next sought to obtain detailed mechanistic and kinetic information about the Staudinger ligation reaction. To do so, we needed an assay. NMR spectroscopy offers the ability to observe a chemical reaction continuously and without its perturbation. The resulting data can be used to identify reaction intermediates, as well as obtain quantitative data on the rate of their appearance and disappearance. Perhaps most importantly, NMR spectroscopy enables the revelation of a product distribution profile that is unperturbed by the vagaries of chemical purification. Finally, an assay based on NMR spectroscopy is facile, allowing many coupling reagents and reaction conditions to be surveyed rapidly.

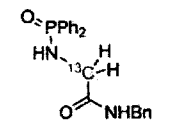
We developed the means to monitor the Staudinger ligation by using ^{13}C NMR spectroscopy with an azido acid enriched with ^{13}C at its α-carbon. Detection using ^{13}C NMR spectroscopy was chosen after attempts to follow the reaction with ^{15}N-labeled azido acids were unsuccessful due to low sensitivity. The use of ^{31}P NMR spectroscopy was also investigated, but, of course, reports not on amide **4.4** but on phosphine oxide **4.5**, which can form disulfides that complicate analyses. DMF was chosen as the solvent, as this solvent had been shown previously to be conducive to the Staudinger ligation reaction (Nilsson et al., 2003; Soellner et al., 2003). In addition, DMF has a high boiling point (153 °C), which allows for monitoring overnight reactions with no significant change in solute concentrations due to solvent evaporation.

^{13}C NMR spectroscopy enabled us to observe each intermediate in the traceless Staudinger ligation of acetylglycyl phosphinothioester **4.11** and an equimolar amount of

^{13}C-labeled azide **4.12** (Figure 4.1). To correlate the observed shifts with discrete intermediates along the reaction pathway, mimics of the intermediates were synthesized and characterized further by ^{13}C NMR spectroscopy.

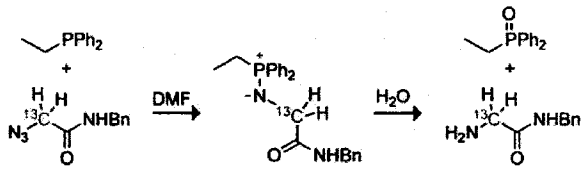
To obtain a mimic for the iminophosphorane intermediate, the reaction of diphenylethylphosphine and the ^{13}C-labeled azide **4.12** was followed by ^{13}C NMR spectroscopy, both in the presence and absence of water (Scheme 4.2). The iminophosphorane **4.13** was shown to have a shift of 49.54 ppm, which indicated that the signal at 49.20 ppm in Figure 4.1 was due to iminophosphorane **4.15** in the Staudinger ligation reaction. This model reaction also provided information for the shift of the amine **4.14** byproduct, found at 44.45 ppm. During the Staudinger ligation reaction, the chemical shift of amine **4.14** changed from 44.05 to 44.58 ppm, which is likely due to a decrease in solution pH and hence amine protonation during the course of the reaction. To obtain chemical shift information on the amidophosphonium salt intermediate, the reaction was monitored in anhydrous DMF. Although the reaction in this anhydrous solvent did provide amide **4.18** (thereby suggesting that amidophosphonium salt **4.3** in Scheme 1 can fragment to form amide **4.4** and a thiaphosphiranium salt), it also produced a marked accumulation of a species with a chemical shift of 45.08 ppm, which was likely amidophosphonium salt **4.16**. Likewise, Staudinger ligation reactions mediated by (diphenylphosphino)ethanethiol, which has an additional methylene group between its phosphorus and sulfur atoms, implicated the chemical shift at 45.08 as arising from amidophosphonium salt **4.16** (*vida infra*). The compound conferring the signal at 43.69 ppm was isolated from the completed reaction and found to be a previously unknown byproduct, phosphonamide **4.17**. Finally, the chemical shift of isolated amide **4.18** was

found to be 52.52 ppm. Use of this NMR assay data revealed that the Staudinger ligation of phosphinothioester **4.11** and azide **4.12** proceeds without significant buildup of any intermediate (Figure 4.1). Moreover, the reaction proceeded rapidly, with a half-life of 7 min.



4.17, δ = 43.70 ppm

Scheme 4.2. ^{13}C Chemical Shifts of Mimics of Reaction Intermediates



4.12 δ = 51.58 ppm

4.13 δ = 49.54 ppm

4.14 δ = 44.45 ppm

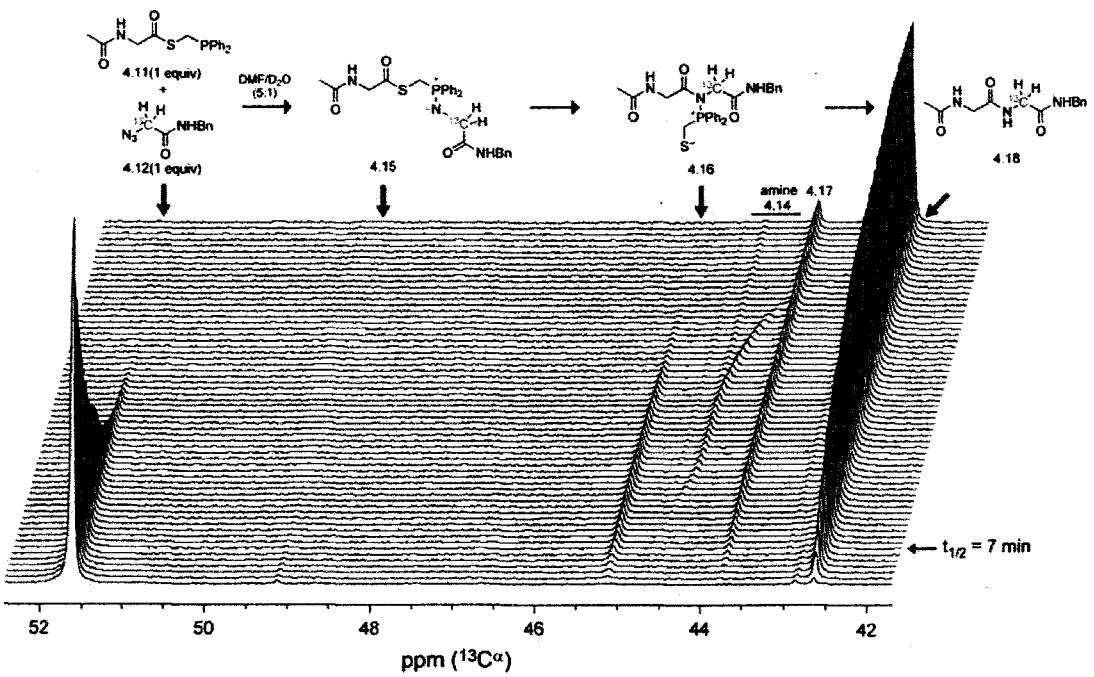
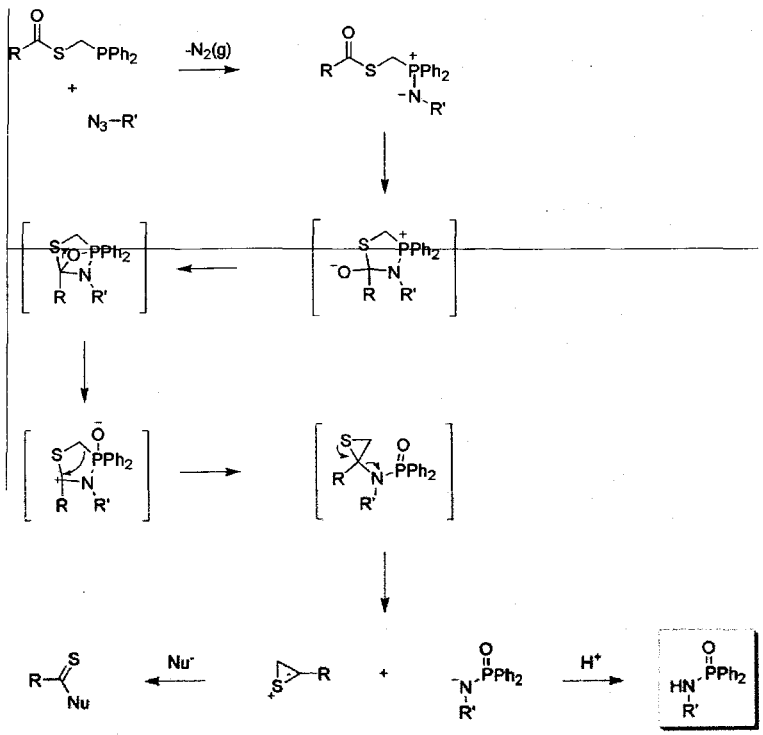


Figure 4.1. ^{13}C NMR Assay of the Staudinger Ligation Reaction. Reactions were performed at room temperature with reagent concentrations of 0.175 M. Ninety spectra were acquired over a scaled 12-h time course. The experimental design allowed for the collection of data for initial rate calculations, as well as quantitative determination of reaction yields. Peak integrations were calibrated with a standard curve generated with purified ^{13}C-labeled reactant, product, or intermediate mimic.

Mechanism for Phosphonamide Byproduct Formation. To reveal the source of the phosponamide **4.17** byproduct, phosphinothioester **4.11** and azide **4.12** were reacted in the presence of $[^{18}O]H_2O$. The phosphonamide **4.17** byproduct produced during this reaction was isolated and found to contain exclusively ^{16}O (Spectral data. MS (ESI) *m/z* 388.1251 (MNa^+ $[C_{20}{}^{13}CH_{21}N_2O_2PNa^+]$ = 388.1266)). This result indicates that phosphonamide byproduct forms via an aza-Wittig reaction of the iminophosphorane (Scheme 4.1). The oxazaphosphetane **4.6** is then transformed into phosphonamide **4.10** by an (as yet) unknown mechanism. A proposed mechanism for this transformation is

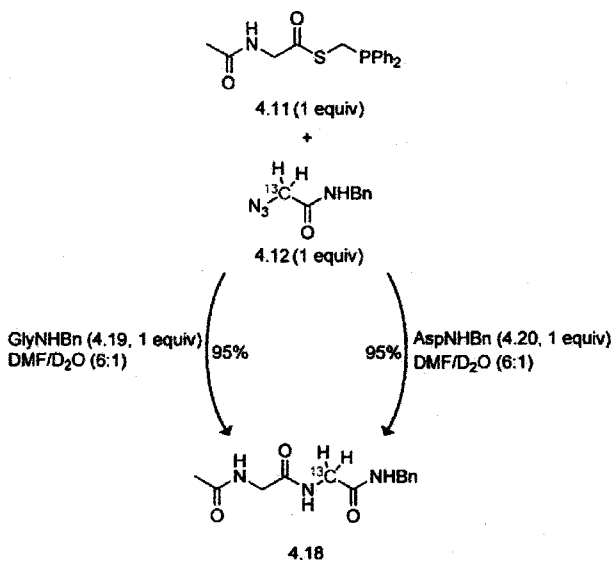
detailed in Scheme 4.3

Scheme 4.3. Proposed mechanism for phosphonamide byproduct formation via aza-Wittig reaction.



Chemoselectivity of the Staudinger Ligation. To examine its chemoselectivity, the Staudinger ligation reaction of equimolar amounts of phosphinothioester **4.11** and azide **4.12** in DMF/D_2O (6:1) was examined by ^{13}C NMR spectroscopy in the presence of compounds containing functional groups present in proteinogenic amino acids (Scheme 4.3). No significant changes in the spectra were observed upon addition of stoichiometric amounts of $GlyNH_2$ (**4.19**) or $AspNH_2$ (**4.20**). These data confirm the chemoselectivity of the reaction using (diphenylphosphino)methanethiol as a coupling reagent (Soellner et al., 2003).

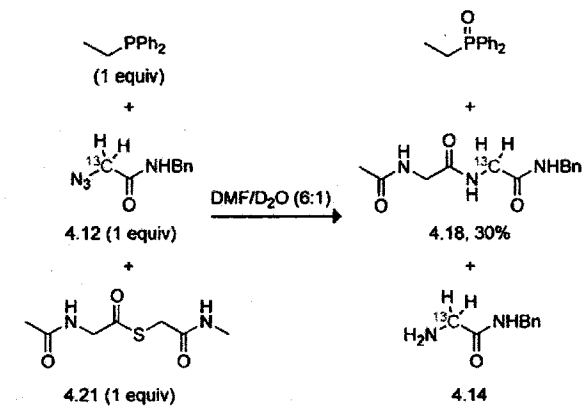
Scheme 4.3. Staudinger Ligation Reaction in the Presence of Amino Acids



Rate of Glycyl Couplings using (Diphenylphosphino)methanethiol. We used our NMR assay to examine the coupling of phosphinothioester **4.11** with azido **4.12** (Figure 4.1). The data from this experiment were used to calculate a second-order rate constant for the appearance of amide **4.18** of $k_2 = (7.7 \pm 0.3) \times 10^{-3}$ $M^{-1}s^{-1}$. This value is similar to that reported previously for the Staudinger reduction of ethyl(2-azido)acetate by triphenylphosphine in benzene ($k_2 = 0.01$ $M^{-1}s^{-1}$) (Leffler & Temple, 1967). Recent work by Bertozzi and coworkers on a non-traceless Staudinger ligation with an oxygen ester revealed a similar rate constant (Lin et al., 2005). As is the case with the Staudinger reduction (Leffler & Temple, 1967), the absence of intermediate accumulation indicates that the encounter of the azide and phosphine is the rate-determining step in the Staudinger ligation reaction.

To quantify the effect of tethering the phosphine functionality to the thioester functionality, we used our NMR assay to examine an intermolecular version of the traceless Staudinger ligation (Scheme 4.4). These reactions proceed in low yield relative to their intramolecular counterparts (Bosch et al., 1995). Based on the derived rate constants (Figure 3), we calculate that the effective concentration of the nitrogen nucleophile of iminophosphorane **4.1** was $EC = (7.7 \times 10^{-3}\ M^{-1}s^{-1}) / (6.5 \times 10^{-4}\ M^{-2}s^{-1}) =$ 12 M.

Scheme 4.4. Intermolecular Staudinger Ligation Reaction



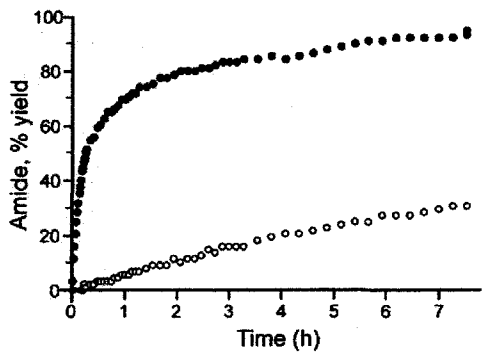
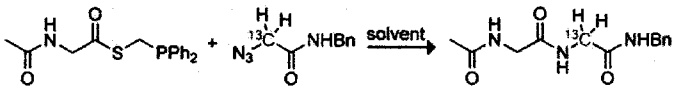


Figure 4.2. Amide formation during intramolecular (•) and intermolecular (○) Staudinger ligation reaction. The intramolecular reaction was between phosphinothioester **4.11** and azide **4.12**. The intermolecular reaction was between (diphenylethyl)phosphine, azide **4.12**, and thioester **4.21** (Scheme 4.4). Reactions were performed at room temperature with reagent concentrations of 0.175 M, and were monitored by ^{13}C NMR spectroscopy as in Figure 1. The initial rate constants for amide formation were $(7.7 \pm 0.3) \times 10^{-3}$ $M^{-1}s^{-1}$ (intramolecular) and $(6.5 \pm 0.1) \times 10^{-4}$ $M^{-2}s^{-1}$ (intermolecular).

Effect of Solvent Polarity on Reaction Rates. The Staudinger ligation of azide **4.12** and phosphinothioester **4.11** was assayed in three solvents of different polarity. The observed rate constants are listed in Table 4.1. In general, the reaction was more rapid in

more polar solvents and correlated well with the solvent polarity–polarizability (SPP) scale (Catalan et al., 1995). These data indicate that the rate-determining step involves a polar transition state that is stabilized by polar solvents. This transition state is likely that for the formation of the initial $R_3–P^+–N=N–N^-–R'$ phosphazide intermediate (Gololobov et al., 1981; Gololobov & Kasukhin, 1992).

Table 4.1. Effect of Solvent Polarity on the Rate of the Staudinger Ligation Reaction

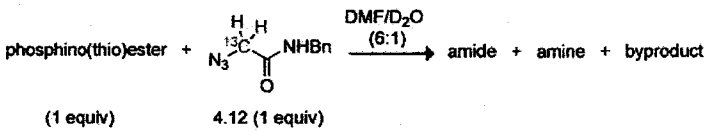


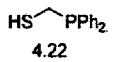
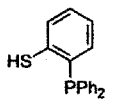
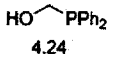
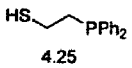
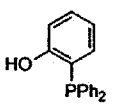
4.11 (1 equiv) 4.12 (1 equiv) 4.18

solvent	SPP	k_{obs} (10^{-3} $M^{-1}s^{-1}$)
DMF	0.954	7.7
CH_3CN	0.895	4.7
THF	0.838	1.3

Comparison of Coupling Reagents in the Staudinger Ligation. Several reagents other than phosphinomethanethiol **4.22** been reported for use in the traceless Staudinger ligation, with varied success (Saxon et al., 2000; Merkx et al., 2003b; Han & Viola, 2004). These compounds include phosphinothiophenol **4.23**,(Nilsson et al., 2000) phosphinomethanol **4.23** (Saxon et al., 2000), phosphinoethanethiol **4.25** (Han & Viola, 2004), and phosphinophenol **4.26** (Saxon et al., 2000) (Figure 4.4). We used our NMR assay to compare the efficacy of these reagents for the reaction of the appropriate phosphino(thio)ester of acetylglycine and azide **4.12** in DMF/D_2O (6:1). The results are listed in Table 4.2.

Table 4.2. Effect of Phosphino(thio)ester on the Rate and Product Distribution of the Staudinger Ligation Reaction



phosphino(thio)ester	k_{obs} (10^{-3} $M^{-1}s^{-1}$)	% amide	% amine	% byproduct
 4.22	7.7	95	3	2
 4.23	1.0	38	62	0
 4.24	0.12	11	0	89
 4.25	0.65	39	61	0
 4.26	7.4	99	0	0

The first traceless Staudinger ligation used phosphinothiophenol **4.23** as the coupling reagent (Nilsson et al., 2000). The yield of this reaction, as determined herein by our NMR assay, was 38%. This yield is similar to the 35% yield determined previously by product isolation (Nilsson et al., 2000). No buildup of intermediates was observed during the course of the reaction. After 12 h, the reaction consisted solely of amide **4.18** product and amine **4.17** byproduct. The rate constant for the reaction of azide **4.12** and the $AcGlySC_6H_4$-*o*-PPh_2 was found to be $k_2 = (1.04 \pm 0.05) \times 10^{-3}$ $M^{-1}s^{-1}$. This rate constant is 14% lower than that for the same Staudinger ligation reaction mediated by phosphinothiol **4.22**. The lower reaction rate mediated by phosphinothiophenol **4.23** has another consequence—an increase in the production of amine **4.14** byproduct,

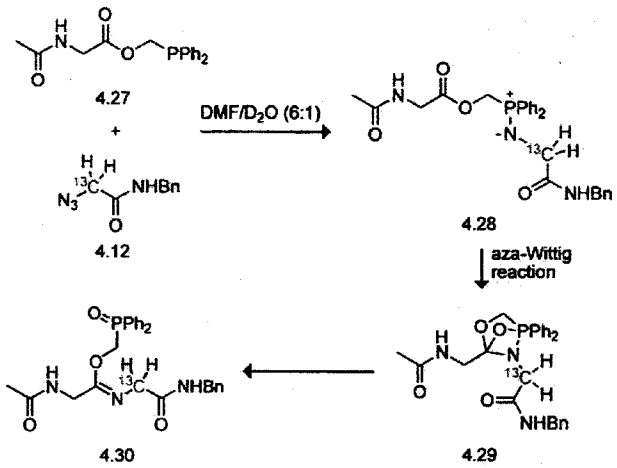
presumably because hydrolysis of iminophosphorane **4.1** is able to compete more successfully with *S*→*N* acyl transfer.

The next coupling reagent examined was (diphenylphosphino)methanol (**4.24**). This reagent was described by Bertozzi and coworkers and differs from (diphenylphosphino)methanethiol (**4.22**) only in its bridging oxygen (Saxon et al., 2000), enabling a direct comparison of an ester and thioester reactants for a traceless Staudinger ligation. Bertozzi and coworkers reported that the Staudinger ligation mediated by phosphinomethanol yield only the amine byproduct.(Saxon et al., 2000) In contrast, we observe nearly exclusively aza-Wittig byproducts with our NMR assay. The reaction of azide **4.12** and phosphinoester **4.27** resulted in a poor yield of amide product (11%). Mass spectrometric analysis indicates that the aza-Wittig adduct is likely to be oxazaphosphetane **4.29** or imidate **4.30**, which have identical mass. (Spectral data. MS (ESI) *m/z* 501.1731 (MNa^+ [$C_{25}{}^{13}CH_{28}N_3O_4PNa^+$] = 501.1743)). The presence of a crosspeak (^{13}C–^{31}P ^3J = 20.2 Hz) in the ^{13}C–^{31}P two-dimensional COSY NMR spectrum provides additional support for the presence of oxazaphosphetane **4.29**. Attempted purification of this adduct led to its decomposition.

The inability of phosphoinoalcohol **4.24** to mediate the Staudinger ligation indicates that the nature of the leaving group plays an important role in the partitioning of tetrahedral intermediate **4.2** towards a Staudinger ligation reaction (Scheme 4.1, Path A) or aza-Wittig reaction (Scheme 4.1, Path B). With a poor leaving group, such as an alkoxide, the oxyanion of the tetrahedral intermediate is long-lived enough to react with the oxophilic phosphorus to form bicyclic compound **4.29** (Scheme 4.5). In contrast, a good leaving group, such as a thiolate, is displaced quickly, leading to the formation of

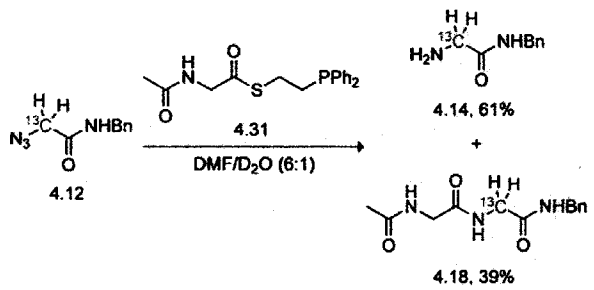
amidophosphonium salt **4.3**. These results are consistent with our proposed mechanism (Scheme 4.1), and highlight a key advantage of using a thioester rather than an oxygen ester in the traceless Staudinger ligation reaction.

Scheme 4.5. Staudinger Ligation Reaction with (Diphenylphosphino)methanol



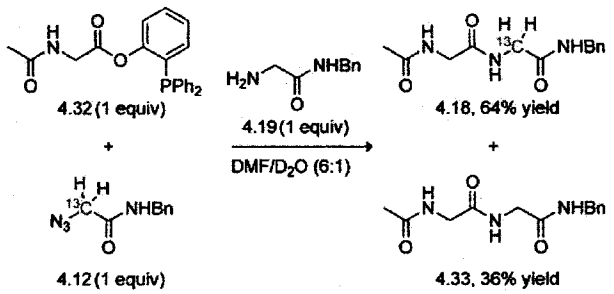
Recently, Viola and coworkers examined the Staudinger ligation mediated by (diphenylphosphino)ethanethiol (**4.25**) (Han & Viola, 2004). This reaction was reported to proceed in quantitative yield as monitored by TLC. With our NMR assay, however, we found that the reaction of azide **4.12** and phosphinothioester **4.31** in DMF/D_2O (6:1) yields mostly the amine byproduct (Scheme 4.6, 61% amine **4.14**, 39% amide **4.18**). The high level of amine is likely due to the increased size of the ring (6 atoms versus 5) during the nucleophilic attack of the iminophosphorane nitrogen on the thioester. The rate constant for amide formation was $k_2 = (6.5 \pm 0.1) \times 10^{-4}$ $M^{-1}s^{-1}$, which is only 8% that with (diphenylphosphino)methanethiol **4.22**.

Scheme 4.6. Staudinger Ligation Reaction with (Diphenylphosphino)ethanethiol



Finally, we examined (*o*-diphenylphosphino)phenol (**4.26**). This coupling reagent was first described by Bertozzi and coworkers and later used by Liskamp and coworkers in the synthesis of tetrapeptides with non-glycyl residues (Saxon et al., 2000; Merkx et al., 2003b). Bertozzi and coworkers obtained a yield of >95% using this reagent for acyl transfers to an azido nucleotide (Saxon et al., 2000). Likewise, our yield for the reaction of azide **4.12** with phosphinoester **4.32** was nearly quantitative (97%) with no observable formation of amine byproduct or reaction intermediates (Scheme 4.7). The rate constant for amide formation was $k_2 = (7.43 \pm 0.03) \times 10^{-3}\ M^{-1}s^{-1}$, which is indistinguishable from that with (diphenylphosphino)methanethiol **4.22** as the coupling reagent [$k_2 = (7.7 \pm 0.3) \times 10^{-3}\ M^{-1}s^{-1}$].

Scheme 4.7. Staudinger Ligation Reaction with (o-Diphenylphosphino)phenol

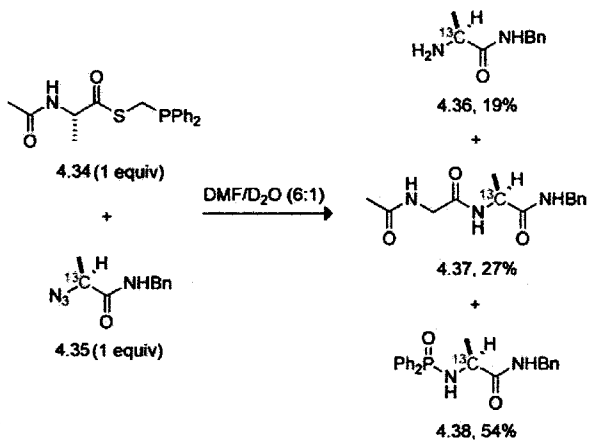


As phosphinophenol **4.26** was the most efficacious reagent examined aside from (diphenylphosphino)methanethiol **4.22** for effecting the traceless a Staudinger ligation, its reactivity was investigated further. The presence of a stoichiometric amount of $GlyNH_2$ **(4.19)** during the reaction of azide **4.12** and phosphinoester **4.32** decreased the yield of amide **4.18** from 97% to 64% (Scheme 4.8). This result is in contrast to that with phosphinothioester **4.11**, which produced no decrease in amide **4.18** product in the presence of a stoichiometric amount of $GlyNH_2$ (*vida supra*). Presumably, the diminished yield with phosphinoester **4.32** was due to the greater electrophilicity of the aryl ester of **4.32** than the alkyl thioester of **4.11**. This result correlates well with findings by Liskamp and coworkers, who found that both *N*-terminal and lysyl ε-amino groups can undergo non-specific reaction with esters derived from phosphinophenol **4.26** (Merkx et al., 2003b). Thus, phosphinophenol **4.26** can be an excellent reagent for mediating the traceless Staudinger ligation, but its intrinsic lack of chemoselectivity constrains its use. These data do not vitiate the utility of reagents such as phosphinophenol **4.26** in mediating the Staudinger ligation, but indicate that such aryl esters can behave simply as activated acyl groups that are prone to attack by biomolecular nucleophiles.

Evaluation of non-Glycyl Couplings. As described earlier, all reported traceless Staudinger ligations involving (diphenylphosphino)methanethiol **4.22** require a glycine residue at either the *N*- or *C*-terminus of the ligation junction. Couplings involving non-glycyl residues provide low yields (20–50%). Upon consideration of the proposed mechanism (Scheme 4.1), the rationale for this is not readily apparent. We used our NMR assay to examine reactions involving non-glycyl azides and non-glycyl phosphinothioesters. The results are listed in Table 4.3.

High yields (>90%) were always obtained when either the *N*-terminal azido acid or the *C*-terminal phosphinothioester residue was a glycine (Table 4.3). As a model coupling for non-glycyl residues, azide **4.35** (which is derived from alanine) and alanyl phosphinothioester **4.34** were reacted in DMF/D_2O (6:1). At room temperature, the reaction resulted in 27% amide **4.37** product, 54% phosphonamide **4.38** byproduct, and 19% amine **4.36** byproduct (Scheme 4.8). When the reaction was run in the presence of [^{18}O]H_2O, mass spectrometry confirmed that the phosphonamide **4.38** byproduct contained exclusively ^{16}O, indicating again that this byproduct results from an aza-Wittig reaction.

Scheme 4.8. Staudinger Ligation Reaction of non-Glycyl Residues



The distribution of products during non-glycyl couplings depends on the solvent. In anhydrous DMF, the reaction of azide **4.35** and alanyl phosphinothioester **4.34** no longer produces any Staudinger reduction byproduct, amine **4.36**, but results in 36% amide **4.37** product and 64% phosphonamide **4.38** byproduct. In the presence of water, the amine **4.36** byproduct is more prevalent than in glycyl couplings, perhaps due to the greater steric hindrance in the *S*→*N* acyl transfer reaction of the iminophosphorane intermediate. Indeed, the rate constant for the reaction was found to be $k_2 = (3.7 \pm 0.3) \times 10^{-3}$ $M^{-1}s^{-1}$, which is half that for the Gly–Gly coupling. Increasing the temperature of the reaction increased the ratio of amide **4.37** to byproduct **4.38**. Still, at the highest temperature tested (50 °C), the yield of amide **4.37** remained a moderate 48%. These yields for a non-glycyl coupling with (diphenylphosphino)methanethiol **4.22** are comparable to those obtained with phosphinophenol **4.26**, as reported previously (Merkx et al., 2003b).

To acertain whether side-chain stereochemistry of the α-carbon plays a role in the decreased yields of a non-glycyl coupling, the reaction of azide **4.35** and D-alanyl

phosphinothioester (**4.39**) was examined. The yield for this reaction was essentially identical to that for the L-alanyl phosphinothioester, indicating that the stereochemistry of the amino acid residues does not play a role in the moderate yields obtained from such couplings.

4.3 Conclusions

We have elucidated the mechanism for the Staudinger ligation reaction mediated by phosphinothiols. By developing and using a sensitive and continuous assay based on ^{13}C NMR spectroscopy, we were able detect reaction intermediates and effect kinetic analyses. In addition, we were able to compare reagents for their efficacy in mediating the Staudinger ligation reaction. Based on its high rate constant and chemoselectivity, (diphenylphosphino)methanethiol (**4.22**), which was described first in 2001 (Nilsson et al., 2001), was shown herein to be the most efficacious of known reagents for mediating the coupling of glycyl residues. Efforts are underway in our laboratory to use the extant understanding of the mechanism and kinetics of the Staudinger ligation reaction to develop new reagents and reaction conditions for the high-yielding coupling of non-glycyl residues and other chemical transformations.

4.4 Experimental Procedures

General. Reactions were monitored by thin-layer chromatography with visualization by UV light or staining with ninhydrin or I_2. Compound purification was carried out with an automated chromatography system. Silica gel used in flash chromatography had 230–400 mesh and 60 Å pore size. Reagent chemicals were obtained from commercial

suppliers, and reagent grade solvents were used without further purification. NMR spectra were obtained with a 500 or 400 MHz spectrometer at the National Magnetic Resonance Facility at Madison or the University of Wisconsin nuclear magnetic resonance facility, respectively. Carbon-13 and phosphorus-31 spectra were both proton-decoupled, and phosphorus-31 spectra were referenced against an external standard of deuterated phosphoric acid (0 ppm). Mass spectra were obtained with electrospray ionization (ESI) techniques.

General Procedures for Kinetics Experiments using ^{13}C NMR Spectroscopy. The desired phosphino(thio)ester (0.105 mmol in 300 μL of DMF) was mixed with the appropriate ^{13}C-labeled azide (0.105 mmol in 300 μL of DMF/D_2O (2:1)) in a vial. After mixing, the solution was transferred to an NMR tube. (Due to the evolution of $N_2(g)$, NMR tubes were not capped during experiments.) The NMR tube containing the reaction mixture was then added inserted into an NMR spectrometer that had been pre-locked on a sample containing ^{13}C-labeled azide in 600 μL of DMF/D_2O (5:1). After an initial delay of 45 s, the acquisition of NMR spectra was initiated. Ninety spectra were acquired over a scaled time course of 16 h (fifteen spectra during the first 15 min, fifteen spectra during the next 30 min, fifteen spectra during the next 60 min, fifteen spectra during the next 120 min, fifteen spectra during the next 240 min, and fifteen spectra during the final 480 min). Each time point was designed to consume 44 s, with the remaining time being a pre-acquisition delay before the next scan. An appropriate flip angle (30° pulse) and relaxation delay (10 s) were chosen to obtain fully quantitative spectra at each time point and for each intermediate. In addition, the decoupler was turned on solely during the acquisition to prevent any NOE buildups. To confirm that the NMR assay provided

quantitative results, a standard curve was made for each starting material and available intermediate and shown to correlate well with the spectral integration during a reaction.

General Procedures for Staudinger Ligation Reactions. Unless noted otherwise, Staudinger ligation reactions were performed at room temperature with equimolar amounts of phosphino(thio)ester and azide (0.105 mmol) in DMF/D_2O (6:1; 600 μL).

AcGlySCH$_2$PPh$_2$ (4.11). *N*-Acetylglycine (1.90 g, 16.2 mmol) was dissolved in anhydrous DMF (75 mL). HOBt (2.48 g, 16.2 mmol) was added to the resulting solution, followed by DCC (3.34 g, 16.2 mmol). Once precipitate (DCU) was observed, phosphinothiol **4.26** was added (3.77 g, 16.2 mmol). The reaction mixture was allowed to stir under Ar(g) for 3 h. The precipitate was removed by filtration, and the filtrate was concentrated under reduced pressure to give a white solid. The solid was dissolved in ethyl acetate and purified by flash chromatography (silica gel, ethyl acetate). Phosphinothioester **4.11** was isolated in 96% yield. **Spectral data.** Spectral data were as reported previously (Nilsson et al., 2001).

[2-^{13}C]-2-Azido-*N*-benzyl-acetamide (4.12). Azide **4.12** was synthesized from 2-^{13}C bromoacetic bromide via methods reported previously (Nilsson et al., 2001) and isolated in 98% yield. **Spectral data.** ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.39–7.27 (m, 5H), 6.71 (bs, 1H), 4.47 (d, J = 5.7 Hz, 2H), 4.00 (s, 2H) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.66, 137.39, 128.43, 127.45,127.35, 52.06, 43.08 ppm; MS (ESI) m/z 214.0789 (MNa^+ [$C_8{}^{13}CH_{10}N_4ONa^+$] = 214.0780).

[^{13}C$^{\alpha}$]GlyNHBn (4.14). Azide **4.12** (951 mg, 5.0 mmol) was dissolved in anhydrous THF (30 mL) in a flame-dried flask under Ar(g). Ethyl diphenyl phosphine (1.29 g, 6.0

mmol) was added to the resulting solution, which was then allowed to stir under Ar(g) for 8 h. Water (3.3 mL, to 10% v/v) was added, and the reaction mixture was stirred for an additionall h. The solvent was then removed under reduced pressure, and the resulting oil was purified by flash chromatography (silica gel, 3% v/v MeOH in CH_2Cl_2). Amine **4.14** was isolated in 97% yield as a clear oil. **Spectral data.** Spectral data were the same as reported previously (Balboni et al., 2002).

$Ph_2P(O)[^{13}C^{\alpha}]$GlyNHBn (4.17). Phosphonamide **4.17** was isolated from a Staudinger ligation reaction of phosphinothioester **4.11** and azide **4.12**, performed as described above. The solvent of the reaction was removed under reduced pressure, and the resulting oil was washed with CH_2Cl_2. The suspension was filtered, and phosphonamide **4.17** was isolated in 1% yield.

Phosphonamide **4.17** was also synthesized directly by the reaction of diphenylphosphinic chloride and GlyNHBn (**4.14**). Diphenylphosphinic chloride (1.0 g, 4.2 mmol) was dissolved in anhydrous THF (20 mL). DMAP (60 mg, 0.5 mmol) was added to the resulting solution, followed by GlyNHBn (700 mg, 4.2 mmol). The reaction mixture was allowed to stir under Ar(g) for 8 h. The solvent was then removed under reduced pressure, and the resulting oil was purified by flash chromatography (silica gel, 3% v/v MeOH in CH_2Cl_2). Phosphonamide **4.17** was isolated in 81% yield.

The spectral data for the isolated and synthesized compounds were indistinguishable.

Spectral data. ^{1}H NMR (CD_3OD, 400 MHz) δ 7.91–7.86 (m, 5 H), 7.55–7.50 (m, 5H), 7.34–7.26 (m, 5H), 4.42 (s, 2H), 3.94 (s, 2H) ppm; ^{13}C NMR (CD_3OD, 125 MHz) δ 168.63, 138.19, 134.06, 132.75, 131.63, 131.60, 131.42, 131.32, 128.26, 128.18,

128.12, 127.22, 126.94, 51.71, 42.69 ppm; ^{31}P NMR (CD_3OD, 161 MHz) δ24.55 ppm; MS (ESI) *m/z* 388.1251 (MNa^+ [$C_{20}{}^{13}CH_{21}N_2O_2PNa^+$] = 388.1266).

AcGly[$^{13}C^{\alpha}$]GlyNHBn (4.18). Amide **4.18** was synthesized by using the general procedures for Staudinger ligation reactions (*vide supra*) **Spectral data.** Spectral data were the same as reported previously (Nilsson et al., 2001).

GlyNHBn (4.19) and AspNHBn (4.20). Amines **4.19** (Balboni et al., 2002) and **4.20** (van Leeuwen et al., 2002) were prepared according to procedures reported previously. **Spectral data.** Spectral data were as reported previously (Balboni et al., 2002; van Leeuwen et al., 2002).

AcGlySCH$_2$C(O)NHMe (4.21). *N*-Acetylglycine (4.0 g, 34.2 mmol) was dissolved in anhydrous DMF (100 mL). DCC (7.06 g, 34.2 mmol) was then added. Once precipitate (DCU) was observed, *N*-methyl mercaptoacetamide was added (3.6 g, 34.2 mmol). The reaction mixture was allowed to stir under Ar(g) for 18 h. The precipitate was removed by filtration, and the filtrate was concentrated under reduced pressure to give a white solid. The solid was dissolved in ethyl acetate and purified by flash chromatography (silica gel, ethyl acetate/hexanes). Thioester **4.21** was isolated as an off-white solid in 91% yield. **Spectral data.** ^{1}H NMR ($CDCl_3$, 400 MHz) δ6.09 (bs, 1H), 4.26 (bs, 1H), 3.59 (s, 2H), 2.82 (s, 2H), 2.18 (s, 3H), 2.10 (s, 3H) ppm; ^{13}C NMR ($CDCl_3$/CD_3OD (1:1), 125 MHz) δ196.99, 172.23, 169.07, 31.43, 31.40, 26.04, 25.90. 21.65 ppm; MS (ESI) *m/z* 227.0457 (MNa^+ [$C_7H_{12}N_2O_3SNa^+$] = 227.0461).

(Diphenylphosphino)methanethiol (4.22), (*o*-diphenylphosphino)benzenethiol (4.23), (diphenylphosphino)methanol (4.24), (diphenylphosphino)ethanethiol (4.25),

and (*o*-diphenylphosphino)phenol (4.26). Compounds **4.22** (Soellner et al., 2002), **4.23** (Nilsson et al., 2000), **4.24** (Saxon et al., 2000), **4.25** (Han & Viola, 2004), and **4.26** (Saxon et al., 2000) were prepared according to reports published previously. **Spectral data.** Spectral data were as reported previously (Nilsson et al., 2000; Saxon et al., 2000; Soellner et al., 2002; Han & Viola, 2004).

AcGlyOCH$_2$PPh$_2$ (4.27). *N*-Acetylglycine (1.12 g, 10.0 mmol) was dissolved in anhydrous CH_2Cl_2 (30 mL). DMAP (0.12 g, 1.0 mmol) was added to the resulting solution, followed by DCC (2.06 g, 10.0 mmol). Once precipitate (DCU) was observed, (diphenylphosphino)methanol (**4.24**; 2.16 g, 10.0 mmol) was added, and the reaction mixture was allowed to stir under Ar(g) for 18 h. The precipitate was removed by filtration, and the filtrate was concentrated under reduced pressure to give a clear oil. The oil was dissolved in ethyl acetate and purified by flash chromatography (silica gel, ethyl acetate/hexanes). Phosphinoester **4.27** was isolated as a colorless oil in 56% yield. **Spectral data.** ^{1}H NMR ($CDCl_3$, 400 MHz) δ7.47–7.42 (m, 4H), 7.39–7.37 (m, 6H), 5.89 (bt, 1H), 4.92 (d, J = 6.2 Hz, 2H), 3.98 (d, J = 5.0 Hz, 2H), 2.00 (s, 3H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ185.35, 169.59, 132.65, 132.50, 128.85, 128.27, 128.22, 48.13, 33.27, 25.17, 24.47 ppm; ^{31}P NMR ($CDCl_3$, 161 MHz) δ–20.02 ppm; MS (ESI) m/z 338.0920 (MNa^+ [$C_{17}H_{18}NO_3PNa^+$] = 338.0916).

AcGlySCH$_2$CH$_2$PPh$_2$ (4.31). *N*-Acetylglycine (475 mg, 4.06 mmol) was dissolved in anhydrous DMF (20 mL). HOBt (549 mg, 4.06 mmol) was added to the resulting solution, followed by DCC (838 mg, 4.06 mmol). Once precipitate (DCU) was observed, (diphenylphosphino)ethanethiol (**4.25**; 1.0 g, 4.06 mmol) was added. The reaction mixture was allowed to stir under Ar(g) for 3 h. The precipitate was removed by

filtration, and the filtrate was concentrated under reduced pressure to give a clear oil. The oil was dissolved in ethyl acetate and purified by flash chromatography (silica gel, 70% v/v ethyl acetate in hexanes). Phosphinoester **4.31** was isolated as a white solid in 92% yield. **Spectral data.** ^{1}H NMR ($CDCl_3$, 400 MHz) δ7.47–7.43 (m, 4H), 7.37–7.36 (m, 6H), 6.06 (bs, 1H), 4.20 (d, J = 5.6 Hz, 2H), 3.00 (m, 2H), 2.46 (m, 2H), 2.07 (s, 3H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ196.78, 170.65, 137.23, 137.14, 132.68, 132.52, 128.81, 128.53, 128.47, 126.33, 125.53, 49.15, 28.27 (d, J = 15.4 Hz), 25.60 (d, J = 23.1 Hz), 22.81 ppm; ^{31}P NMR ($CDCl_3$, 161 MHz) δ–19.88 ppm; MS (ESI) m/z 368.0852 (MNa^+ [$C_{18}H_{20}NO_2PSNa^+$] = 368.0845).

AcGlyO-C$_6$H$_4$-*o*-PPh$_2$ (4.32). *N*-Acetylglycine (562 mg, 4.8 mmol) was dissolved in anhydrous CH_2Cl_2 (50 mL). DMAP (59 mg, 0.5 mmol) was added to the resulting solution, followed by DCC (990 mg, 4.8 mmol). Once precipitate (DCU) was observed, (*o*-diphenylphosphino)phenol (**4.26**; 2.16 g, 10.0 mmol) was added, and the reaction mixture was allowed to stir under Ar(g) for 4 h. The precipitate was removed by filtration, and the filtrate was concentrated under reduced pressure to give a clear oil. The oil was dissolved in ethyl acetate and purified by flash chromatography (silica gel, ethyl acetate/hexanes (1:1)). Phosphinoester **4.32** was isolated as a white solid in 36% yield. **Spectral data.** ^{1}H NMR ($CDCl_3$, 400 MHz) δ7.42–7.28 (m, 12H), 7.19–7.17 (m, 2H), 6.87 (bt, 1H), 4.00 (d, J = 5.0 Hz, 2H), 1.98 (s, 3H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 169.98, 168.03, 135.24, 135.14, 133.99, 133.82, 130.12, 129.18, 128.73, 128.67, 126.63, 122.37, 41.27, 22.88 ppm; ^{31}P NMR ($CDCl_3$, 161 MHz) δ–19.07 ppm; MS (ESI) m/z 400.1071 (MNa^+ [$C_{22}H_{20}NO_3PNa^+$] = 400.1073).

AcGlyGlyNHBn (4.33). Amide **4.33** was synthesized using the general procedures for Staudinger ligation reactions (*vide supra*). **Spectral data.** Spectral data were the same as that reported previously (Nilsson et al., 2001).

AcAlaSCH$_2$PPh$_2$ (3.34). *N*-Acetylalanine (557 mg, 4.25 mmol) was dissolved in anhydrous DMF (20 mL). HOBt (527 mg, 3.90 mmol) was added to the resulting solution, followed by DCC (805 mg, 3.90 mmol). Once precipitate (DCU) was observed, (diphenylphosphino)methanethiol (**4.22**; 900 mg, 3.87 mmol) was added, and the reaction mixture was allowed to stir under Ar(g) for 4 h. The precipitate was removed by filtration, and the filtrate was concentrated under reduced pressure to give a clear oil. The oil was dissolved in ethyl acetate and purified by flash chromatography (silica gel, 70% v/v ethyl acetate in hexanes). Phosphinothioester **4.34** was isolated as a white solid in 83% yield.

This procedure was repeated with Ac-D-AlaOH to give Ac-D-AlaSCH$_2$PPh$_2$ (**4.39**) as a white solid in 92% yield. The spectral data for AcAlaSCH$_2$PPh$_2$ and Ac-D-AlaSCH$_2$PPh$_2$ were indistinguishable.

Spectral data. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.44–7.37 (m, 10 H), 5.89 (bt, 1H), 4.71 (bt, 1H), 3.53 (s, 2H), 2.02 (s, 3H), 1.64 (m, 1H), 1.32 (d, J = 5.8 Hz, 3H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 199.85, 169.70, 136.51, 132.74, 132.59, 132.57, 129.10, 128.51, 128.46, 54.83, 25.29 (d, J = 24.3 Hz), 22.99, 18.66 ppm; ^{31}P NMR ($CDCl_3$, 161 MHz) δ –17.97 ppm; MS (ESI) m/z 368.0853 (MNa^+ [$C_{18}H_{20}NO_2PSNa^+$] = 368.0845).

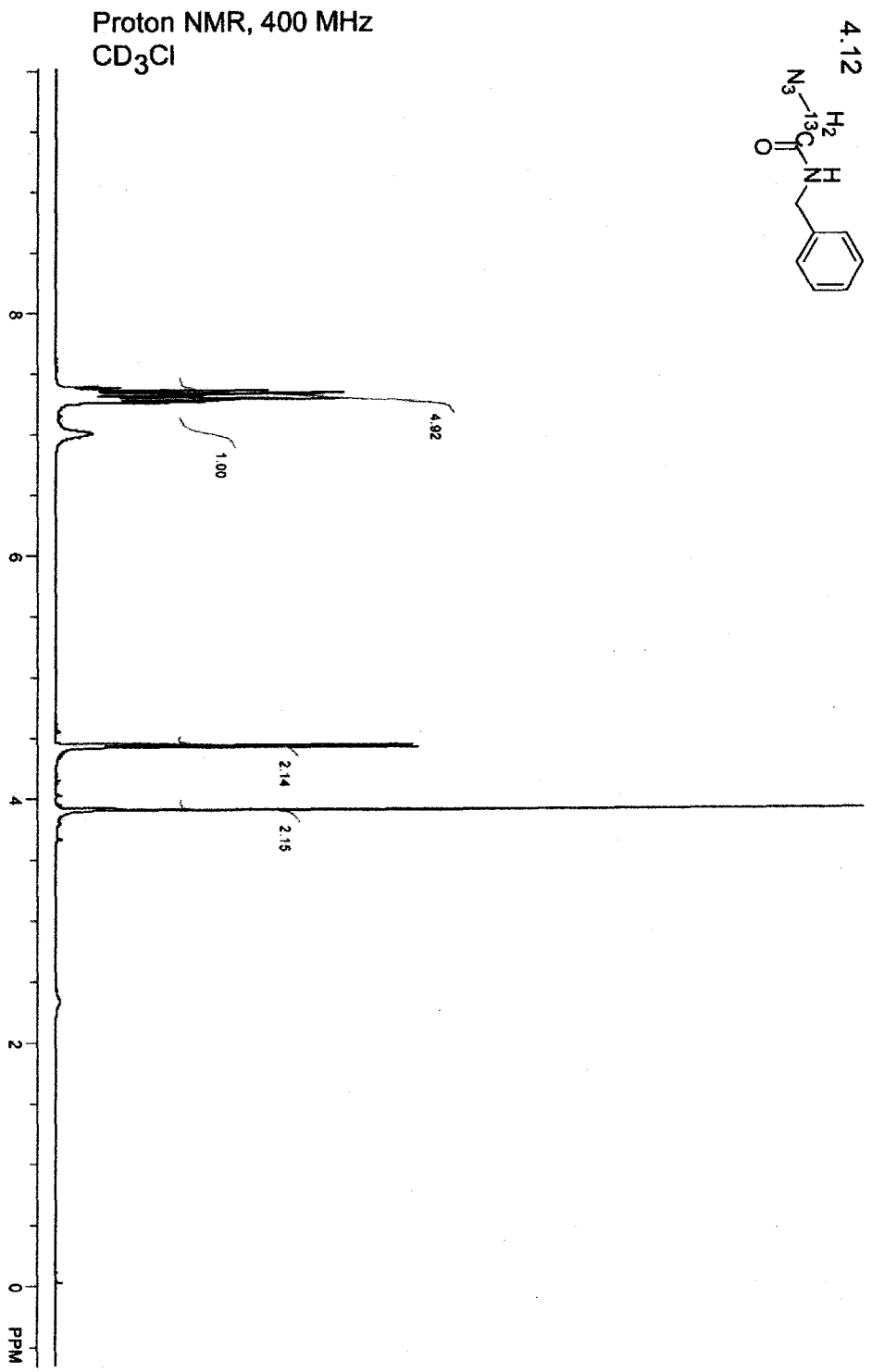
[2-^{13}C]-(2*S*)-2-Azido-*N*-benzyl-1-propionamide (4.35). [2-^{13}C]-(2*S*)-2-Azido-1-propionic acid was synthesized from [$^{13}C^{\alpha}$]-(2*S*)-alanine by the procedure of Lundquist

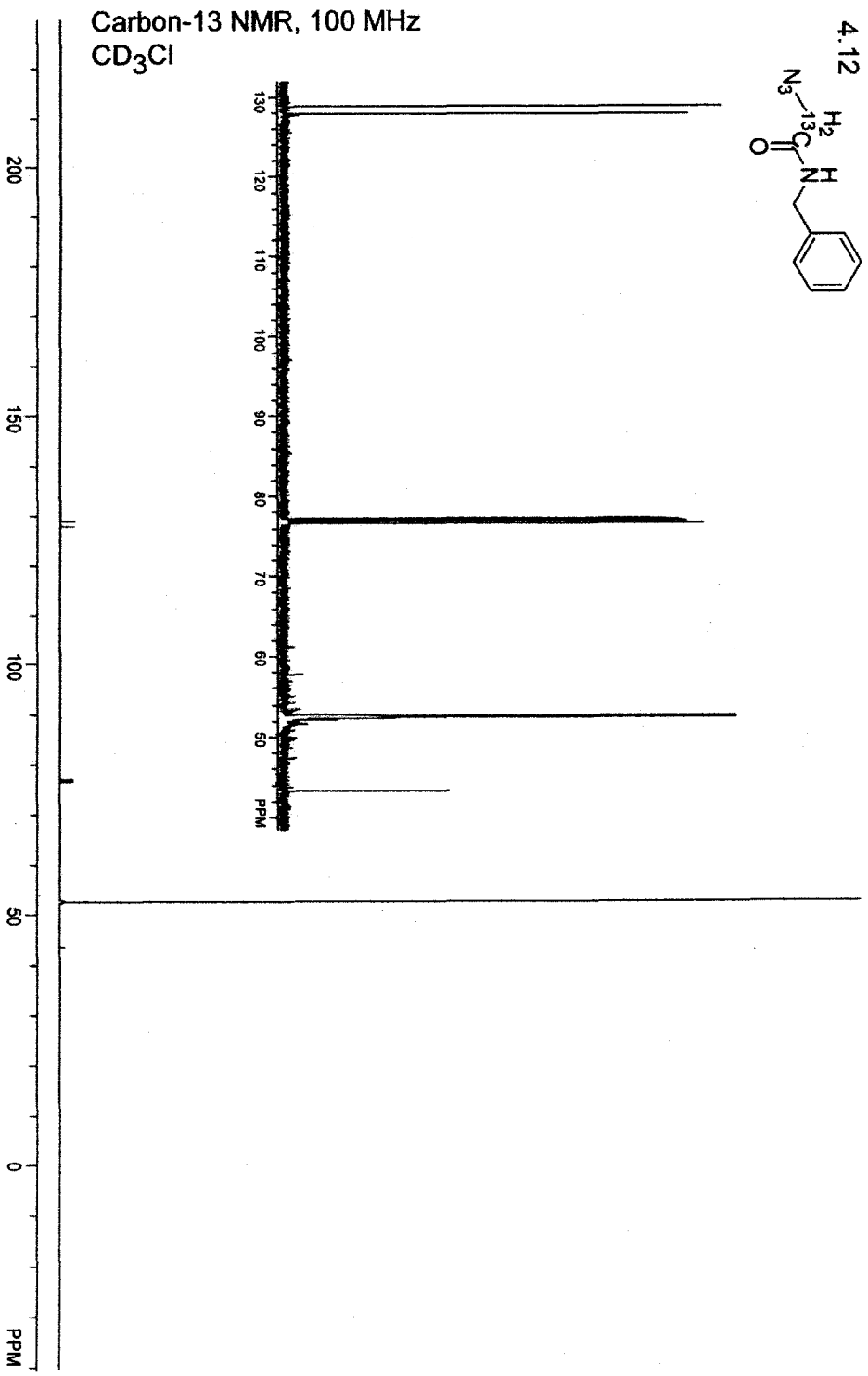
and Pelletier.(Lundquist & Pelletier, 2001) [2-^{13}C]-(2*S*)-2-Azido-1-propionic acid (280 mg, 2.8 mmol) was dissolved in anhydrous DMF (15 mL). Hydroxybenzotriazole (397 mg, 2.9 mmol) was then added, followed by DCC (607 mg, 2.9 mmol). Once precipitate (DCU) was observed, benzylamine (0.370 mL, 3.4 mmol) was added, and the reaction mixture was allowed to stir under Ar(g) for 3 h. The precipitate was removed by filtration, and the filtrate was removed under reduced pressure to yield a yellow oil. This oil was dissolved in 35% v/v ethyl acetate in hexanes and purified by flash chromatography (silica gel, 35% v/v ethyl acetate in hexanes). Azide **4.35** was isolated in 90% yield as an off-white solid. **Spectral data.** ^{1}H NMR ($CDCl_3$, 400 MHz) δ7.37–7.28 (m, 5 H), 6.67 (bs, 1H), 4.46 (s, 2H), 4.13 (dt, J = 144.7, 6.9 Hz, 1H), 1.60 (d, J = 2.3 Hz, 3H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ128.78, 127.74, 59.29, 43.52 ppm; MS (ESI) *m/z* 228.0935 (MNa^+ [$C_9{}^{13}CH_{12}N_4ONa^+$] = 228.0937).

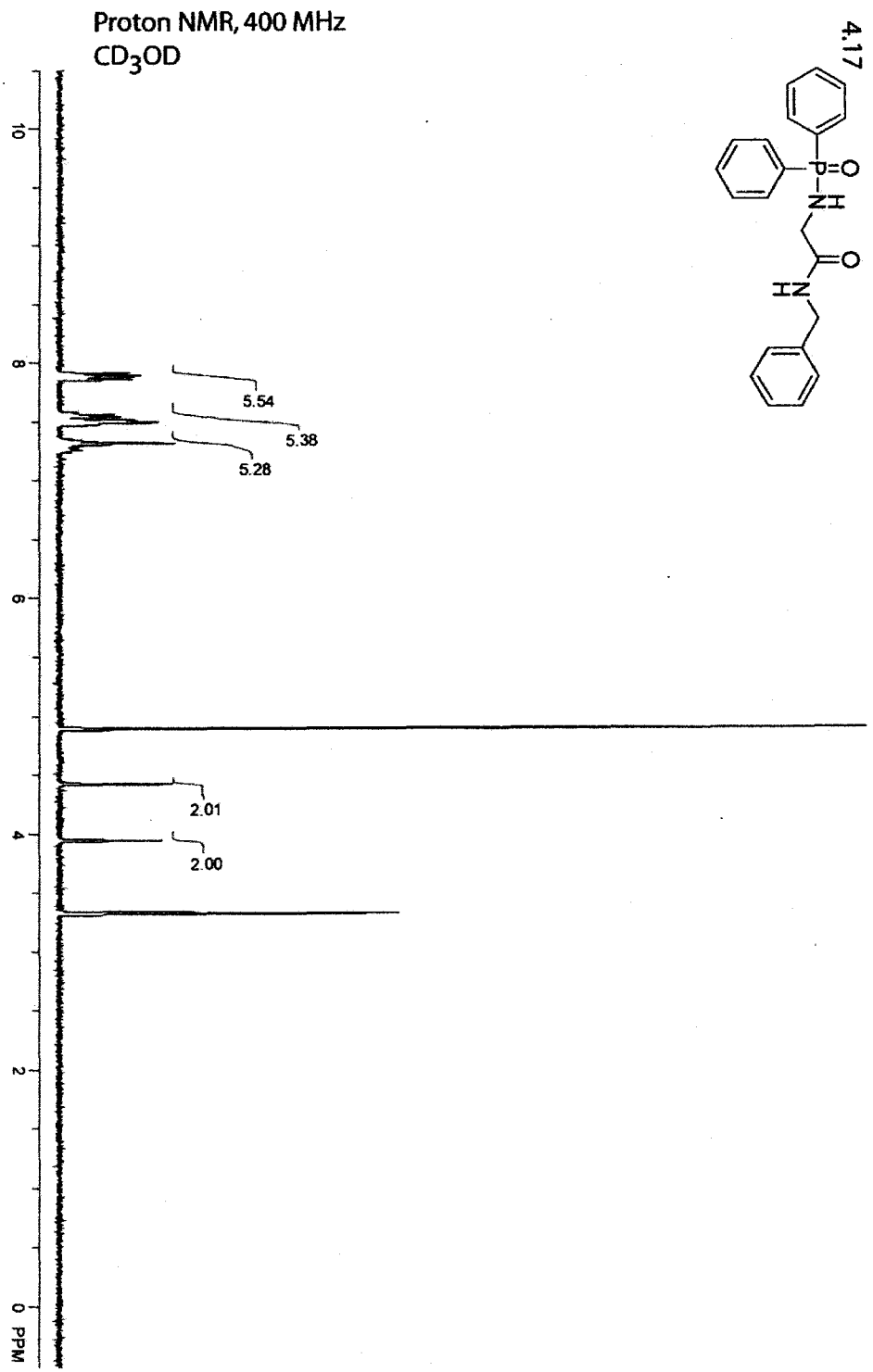
AcGlyAlaNHBn (4.37). Amide **4.37** was prepared by using the general procedures for Staudinger ligation reactions (*vide supra*). **Spectral data.** ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$ (1:1)) δ7.32–7.35 (m, 5 H), 4.51–4.22 (m, 4H), 1.98 (1.87) (s, 3H), 1.41 (d, J = 7.5 Hz, 3H), 1.34 (d, J = 6.9 Hz, 3H) ppm; ^{13}C NMR (75 MHz, $CDCl_3/CD_3OD$ (1:1), numbers in parentheses indicate doubling due to rotational isomerism) δ173.27 (172.78), 172.67 (172.54), 171.44, 137.59 (137.51), 127.39 (127.49), 126.28 (126.36), 126.02 (126.17), 48.92 (48.70), 48.59 (48.34), 41.99 (42.06), 20.50 (20.76), 15.99 (16.23), 15.45 ppm; MS (ESI) *m/z* 315.1511 (MNa^+ [$C_{14}{}^{13}CH_{21}N_3O_3Na^+$] = 315.1509).

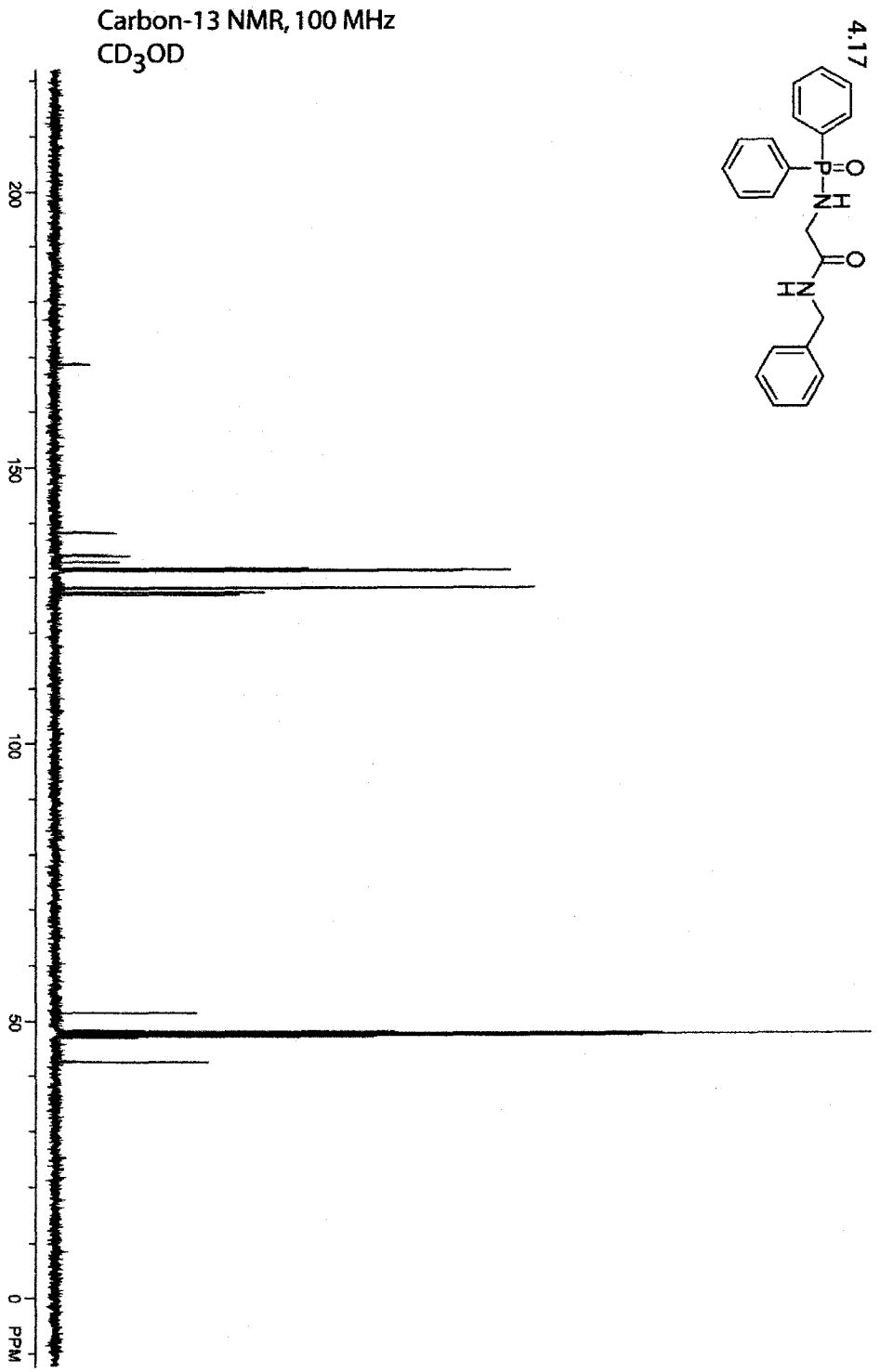
$Ph_2P(O)[^{13}C^{\alpha}]$AlaNHBn (4.38). Phosphonamide **4.38** was isolated from a Staudinger ligation reaction of phosphinothioester **4.34** and azide **4.35**, performed as described

above. The solvent of the reaction was removed under reduced pressure, and the resulting oil was washed with CH_2Cl_2. The suspension was then filtered, and the phosphonamide **4.38** was isolated in 54% yield. **Spectral data.** ^{1}H NMR (CD_3OD, 400 MHz) δ 7.92–7.86 (m, 5H), 7.58–7.48 (m, 5H), 7.35–7.26 (m, 5H), 4.41 (s, 2H), 4.00 (q, J = 7.1 Hz, 1H), 1.47 (d, J = 7.1 Hz, 3H) ppm; ^{13}C NMR (CD_3OD, 125 MHz) δ 171.58, 138.26, 134.07, 132.76, 131.63, 131.60, 131.42, 131.32, 128.26, 128.18, 128.13, 127.12, 126.92, 58.23, 42.64, 15.97 ppm; ^{31}P NMR (CD_3OD, 161 MHz) δ 24.63 ppm; MS (ESI) m/z 402.1432 (MNa^+ [$C_{21}{}^{13}CH_{23}N_2O_2PNa^+$] = 402.1423).

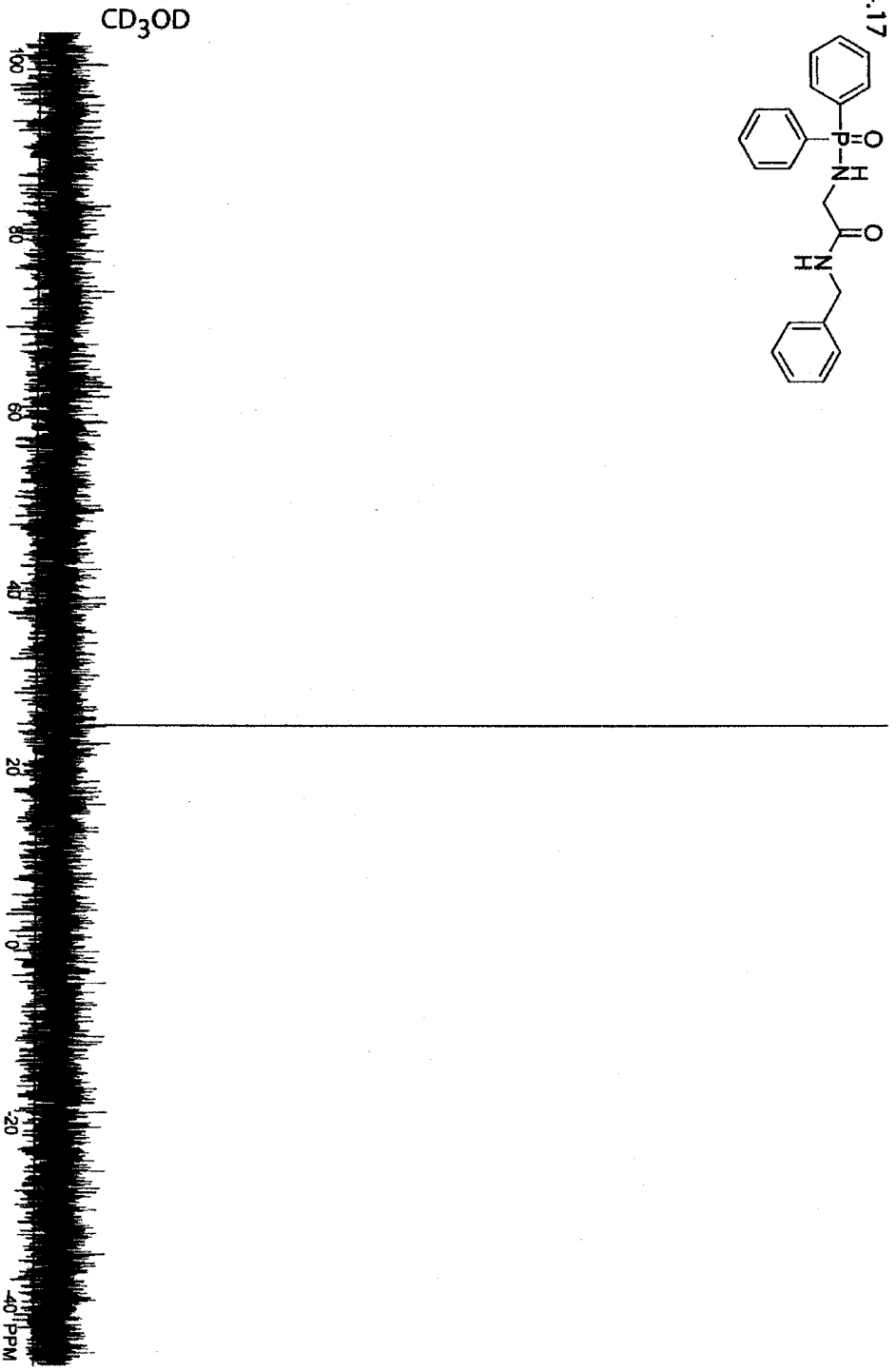








Phosphorus-31 NMR, 161 MHz
CD_3OD



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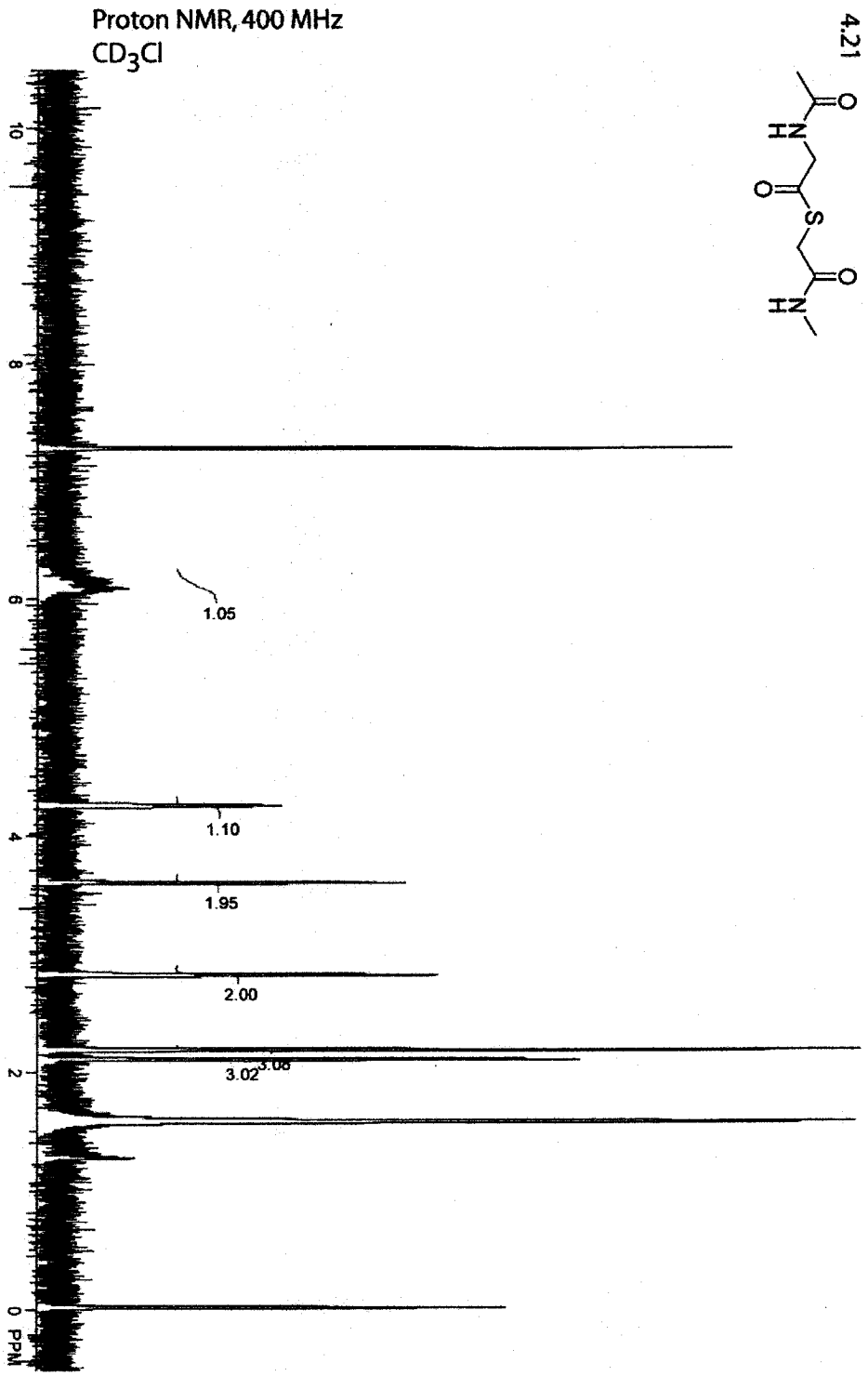
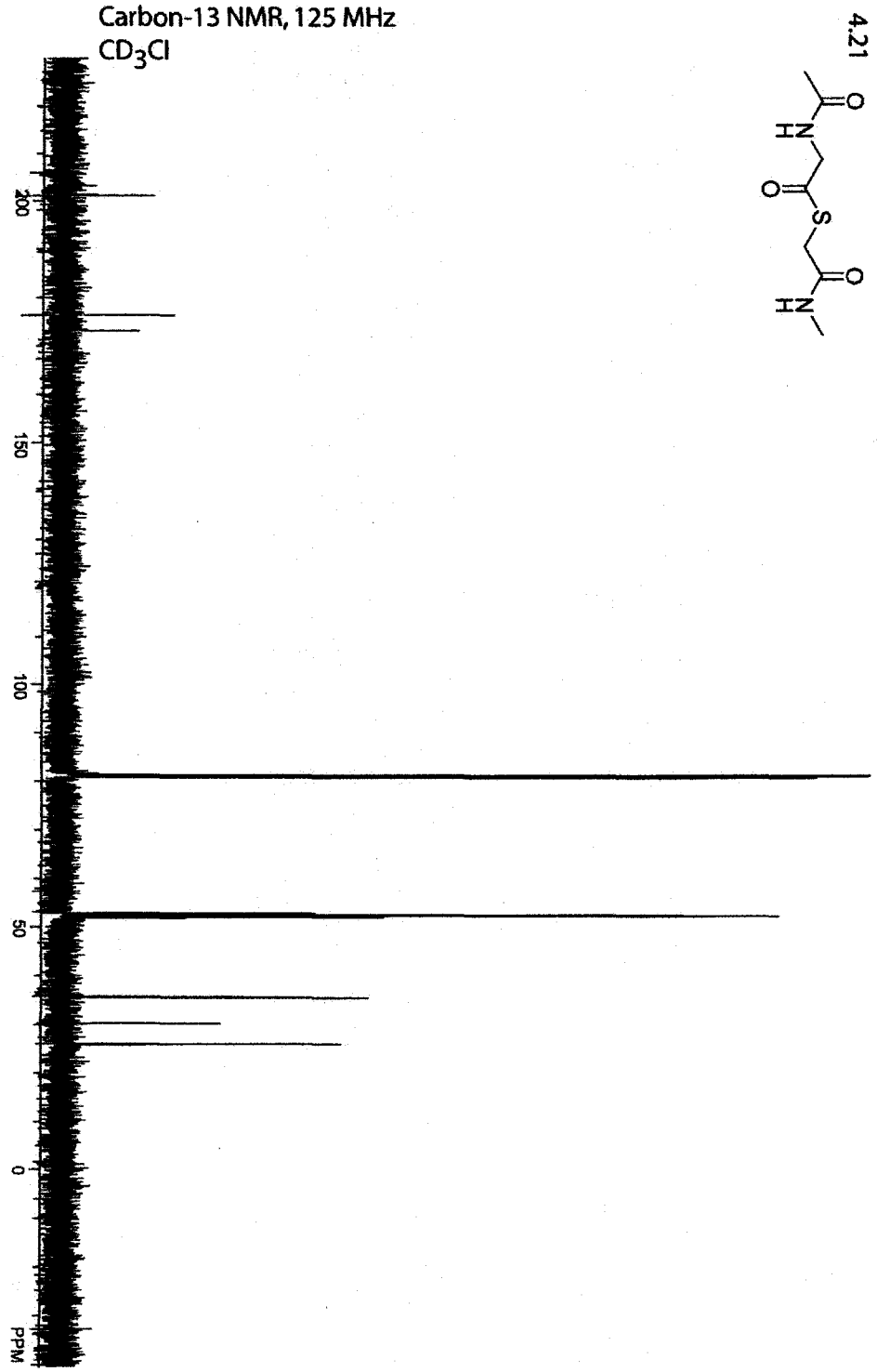
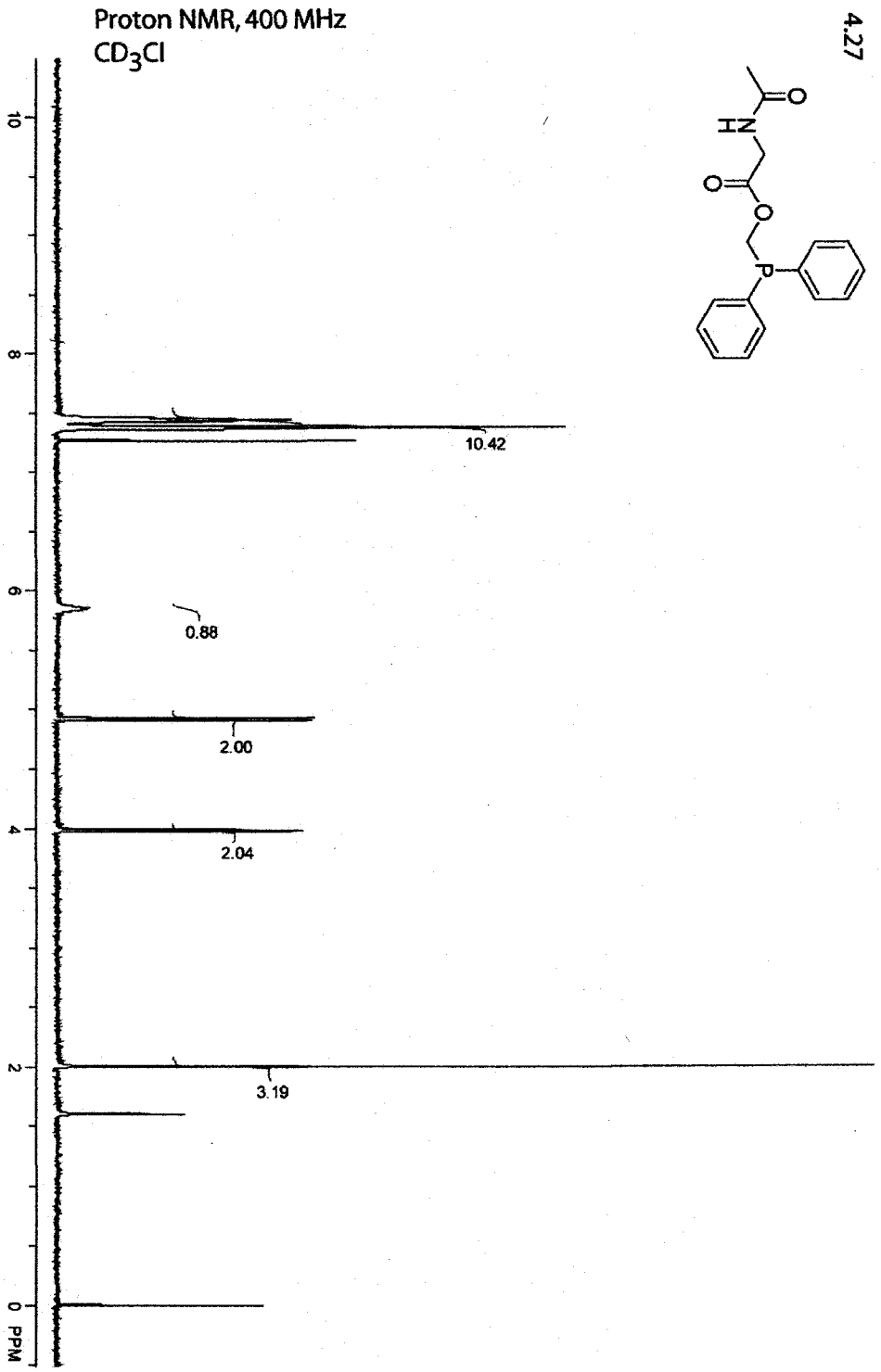
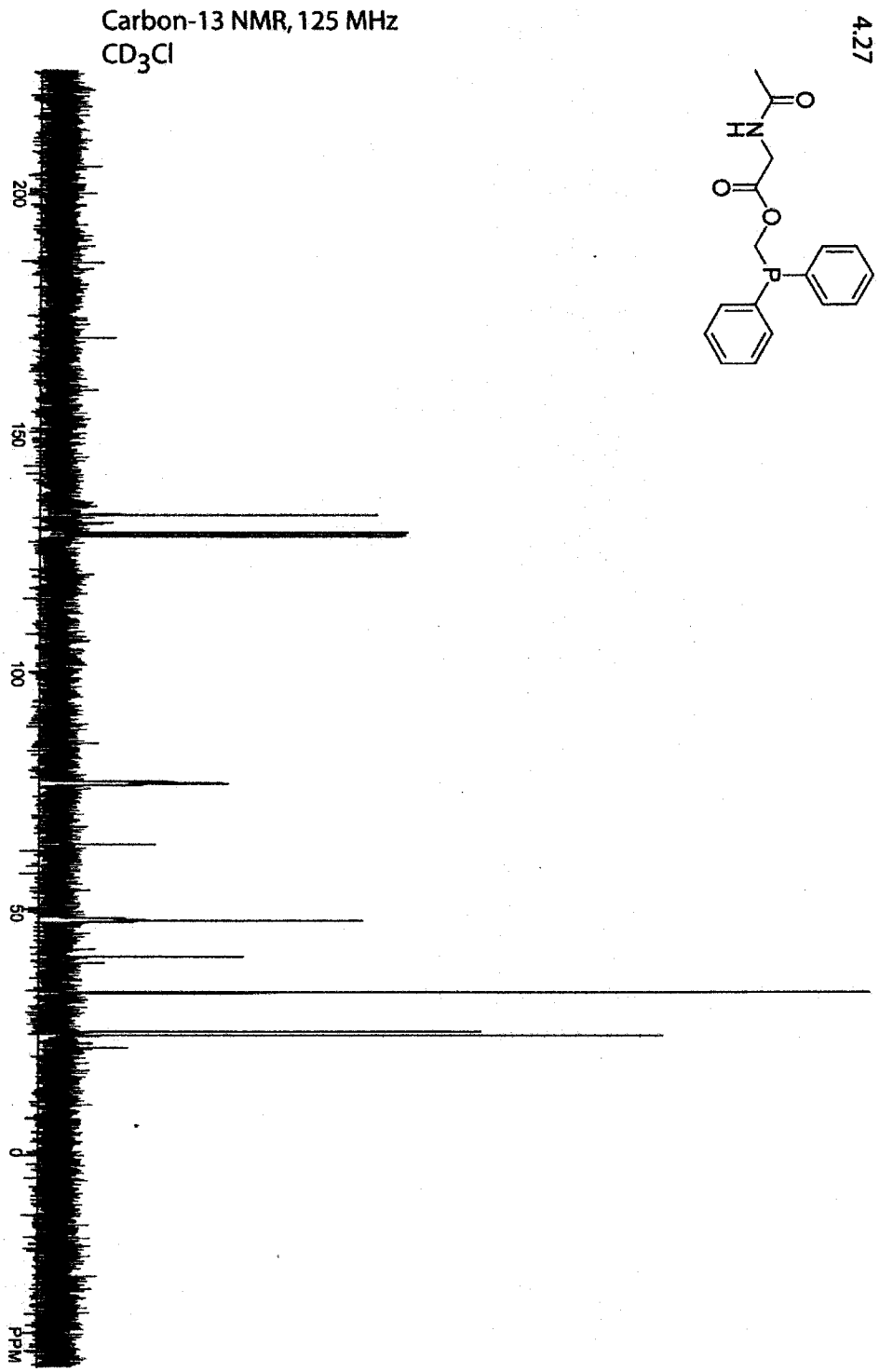
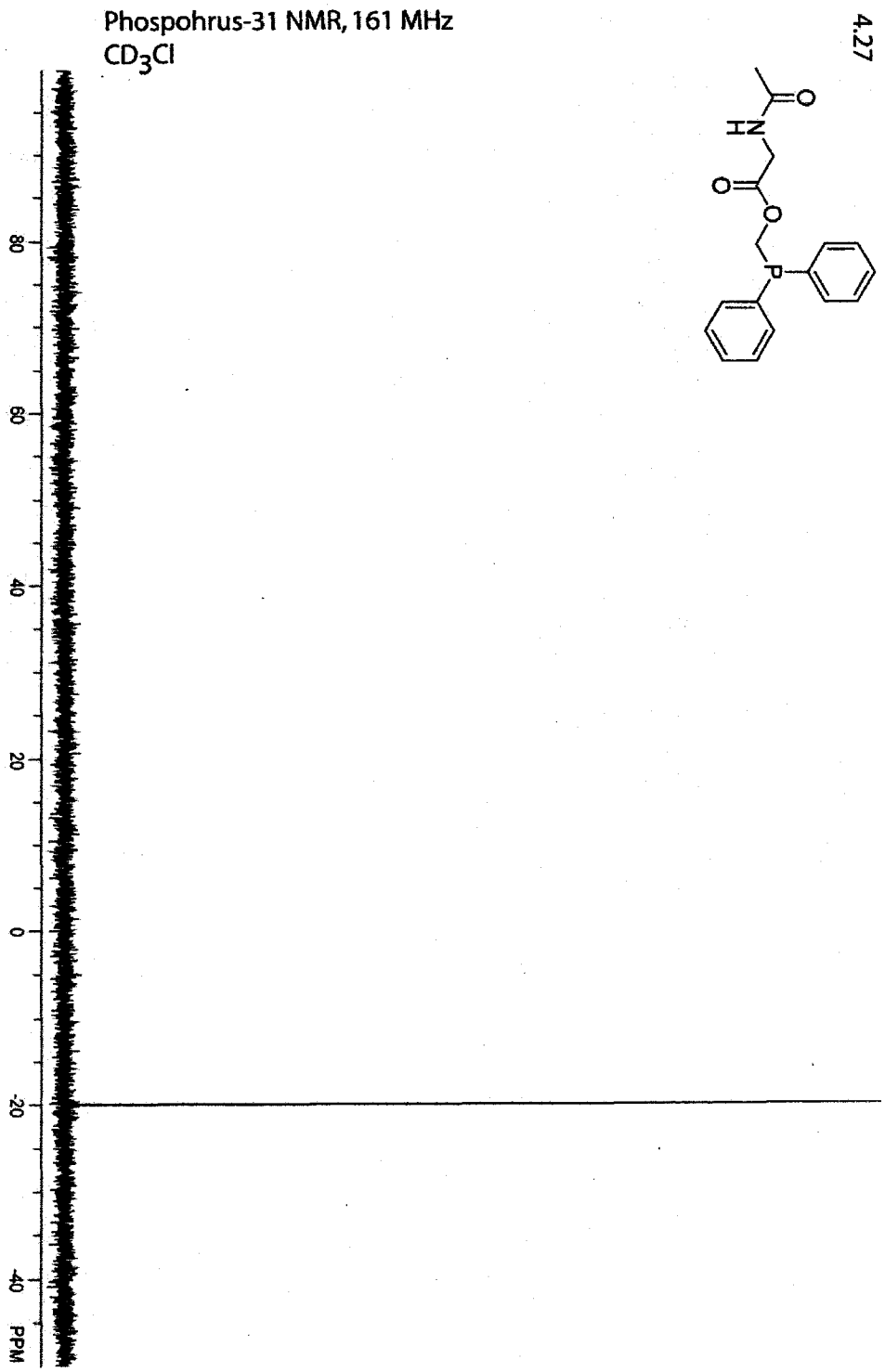
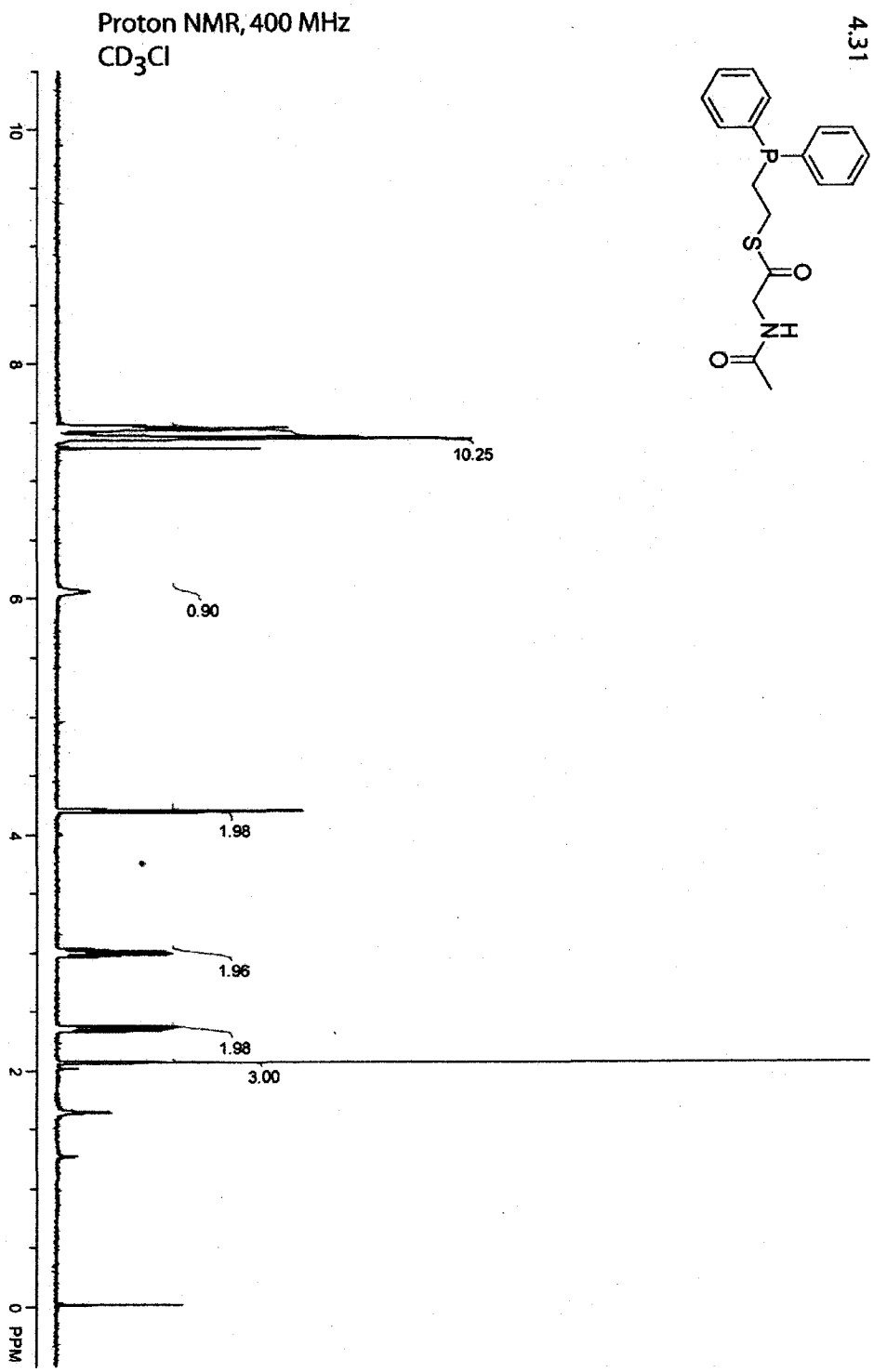


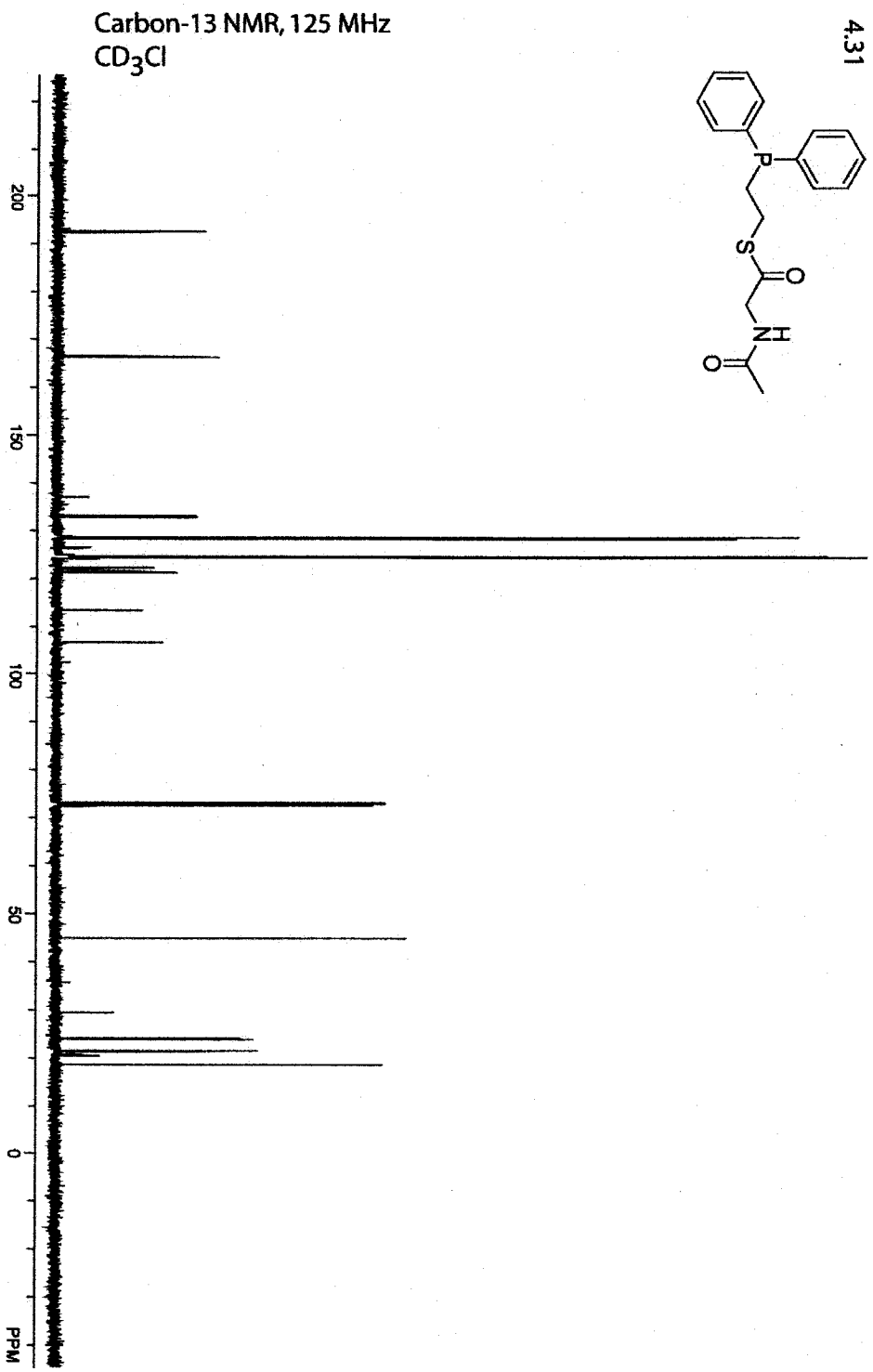




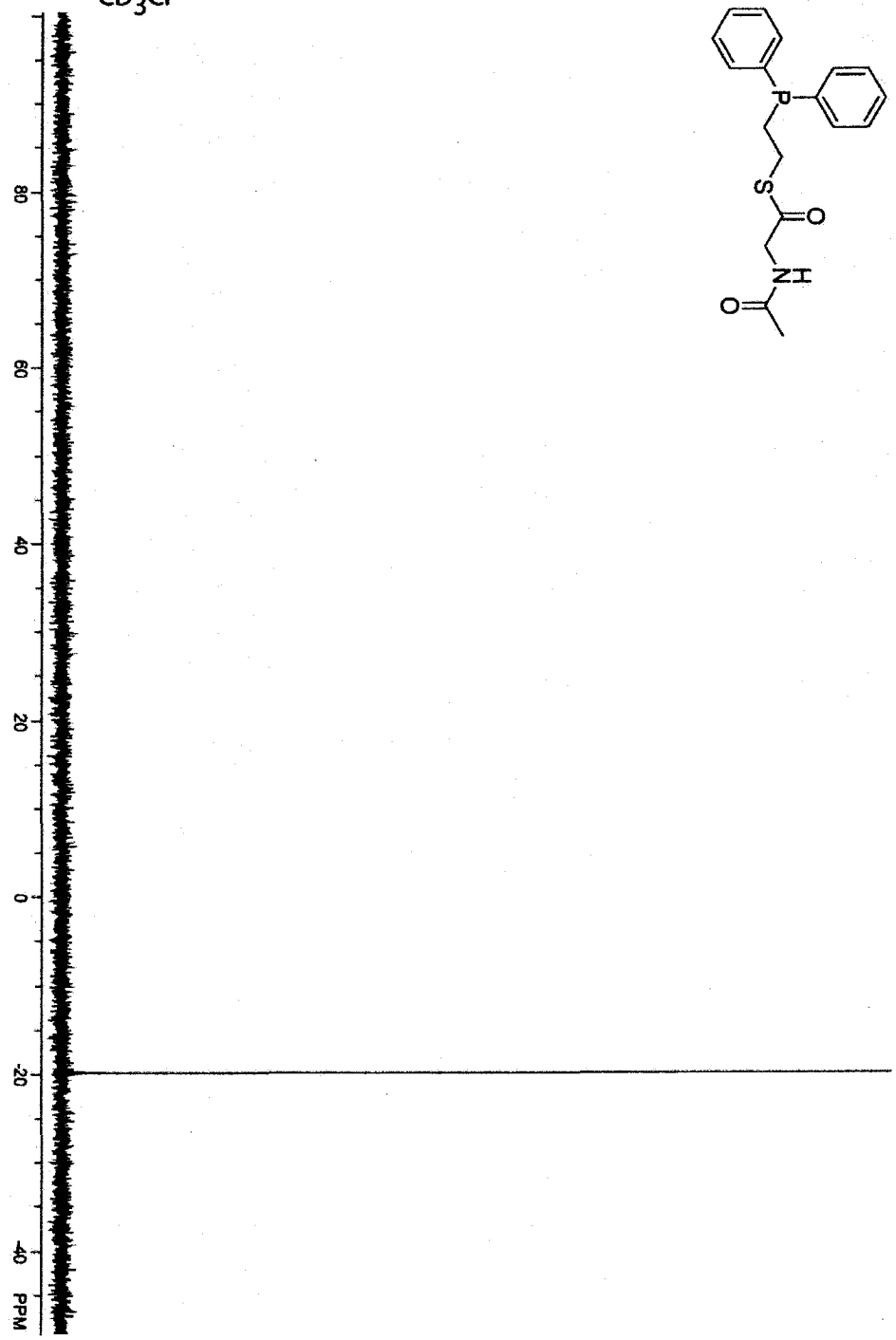




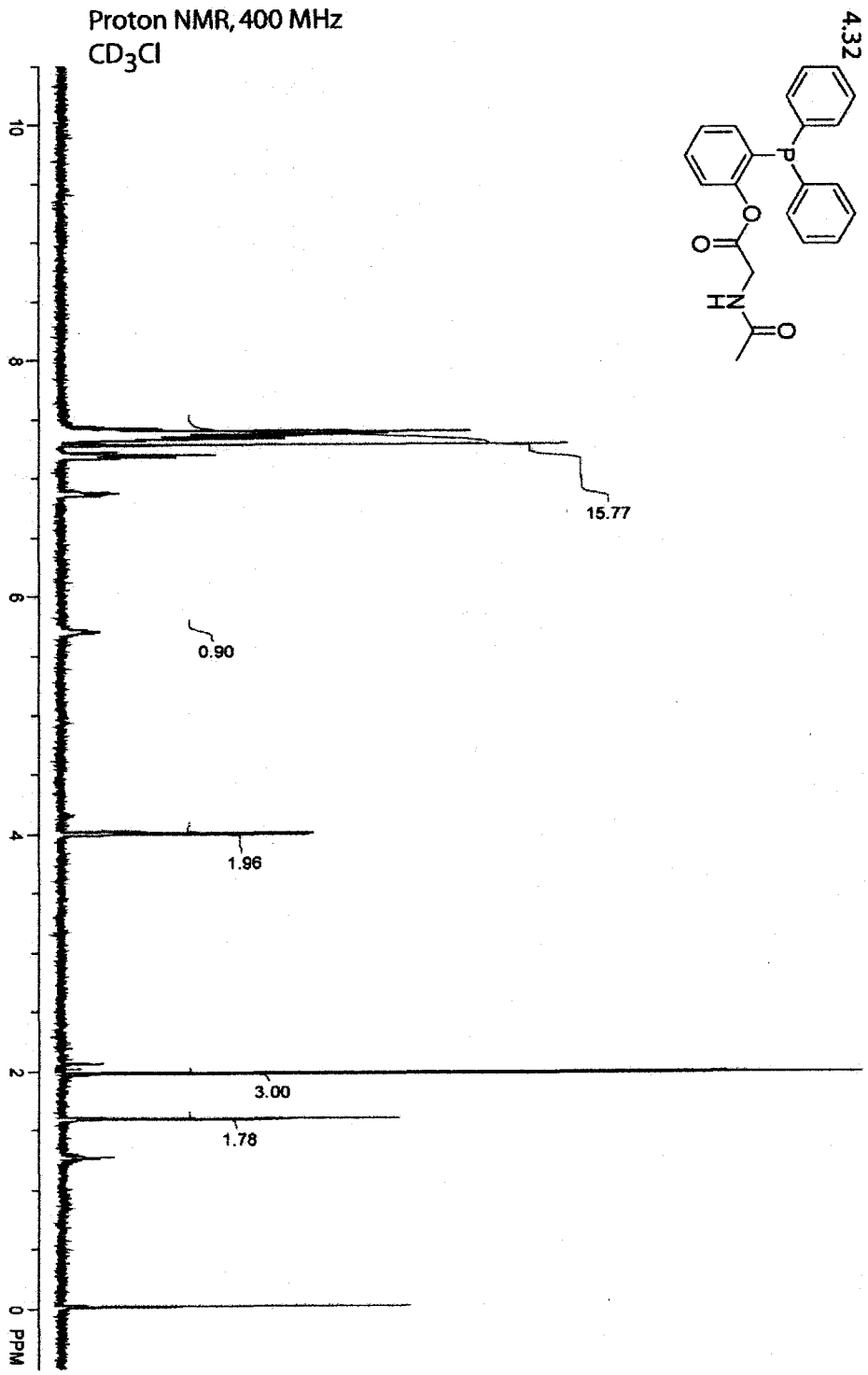


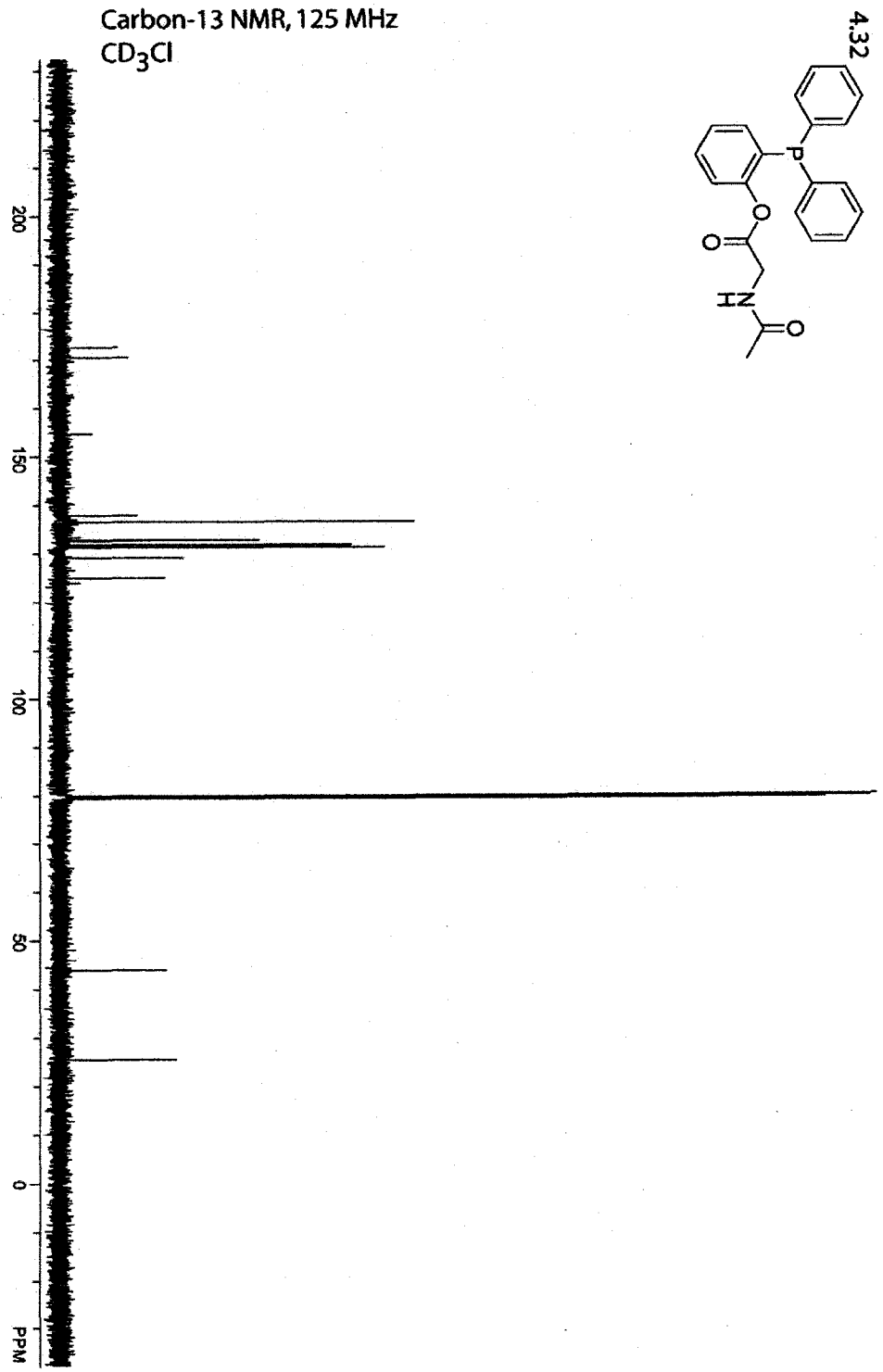


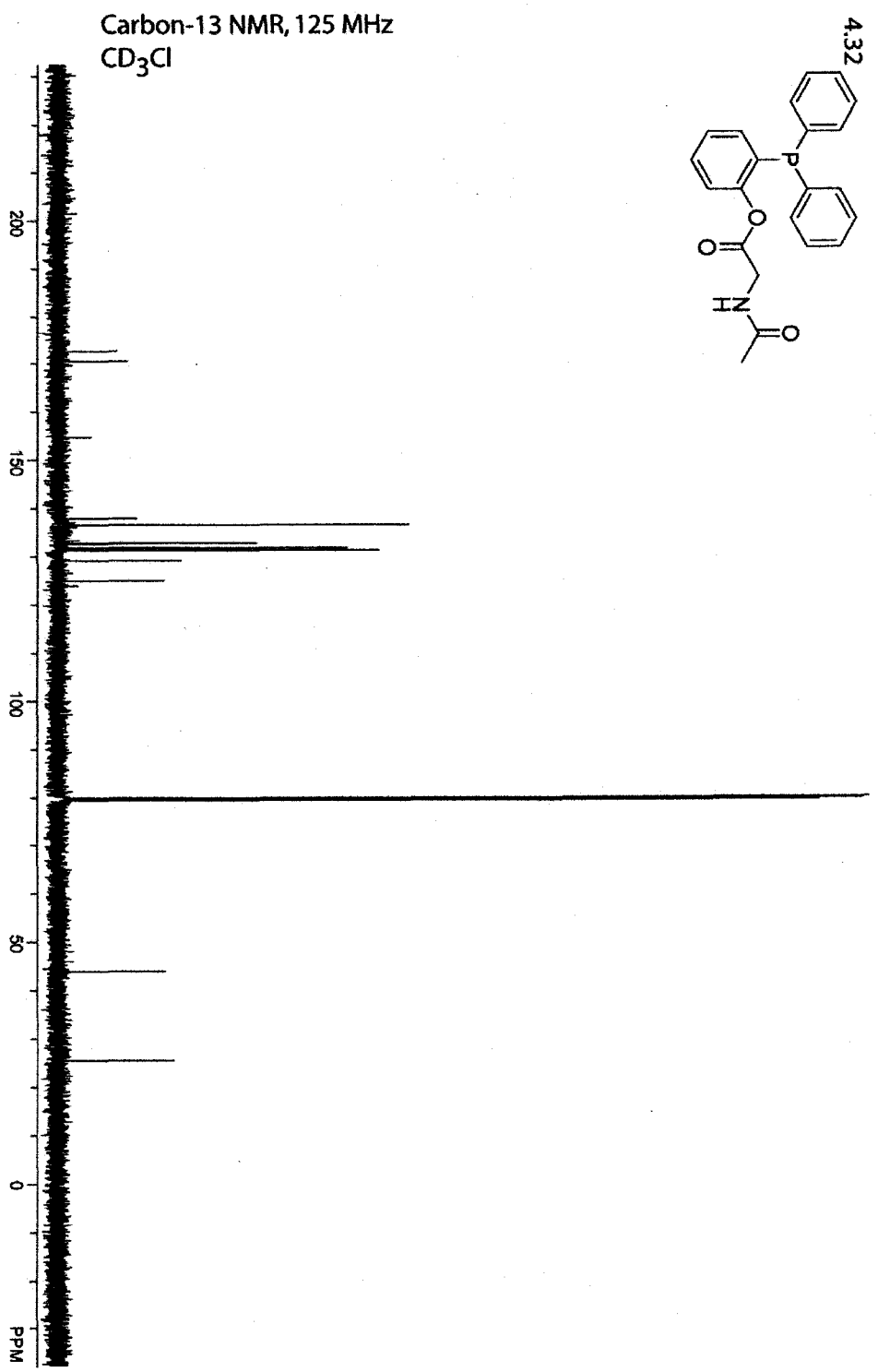
Phosphorus-31 NMR, 161 MHz
CD_3Cl

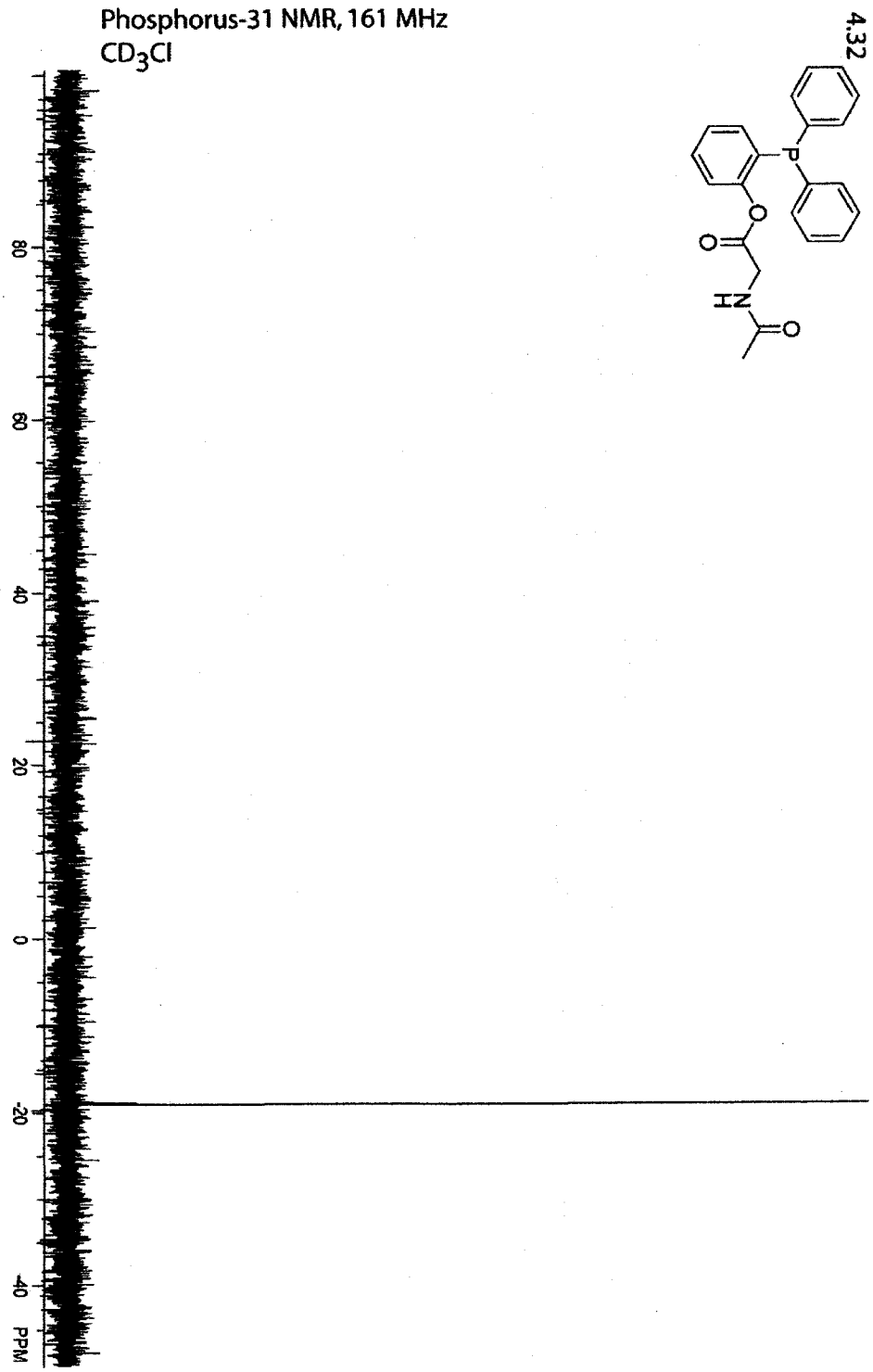


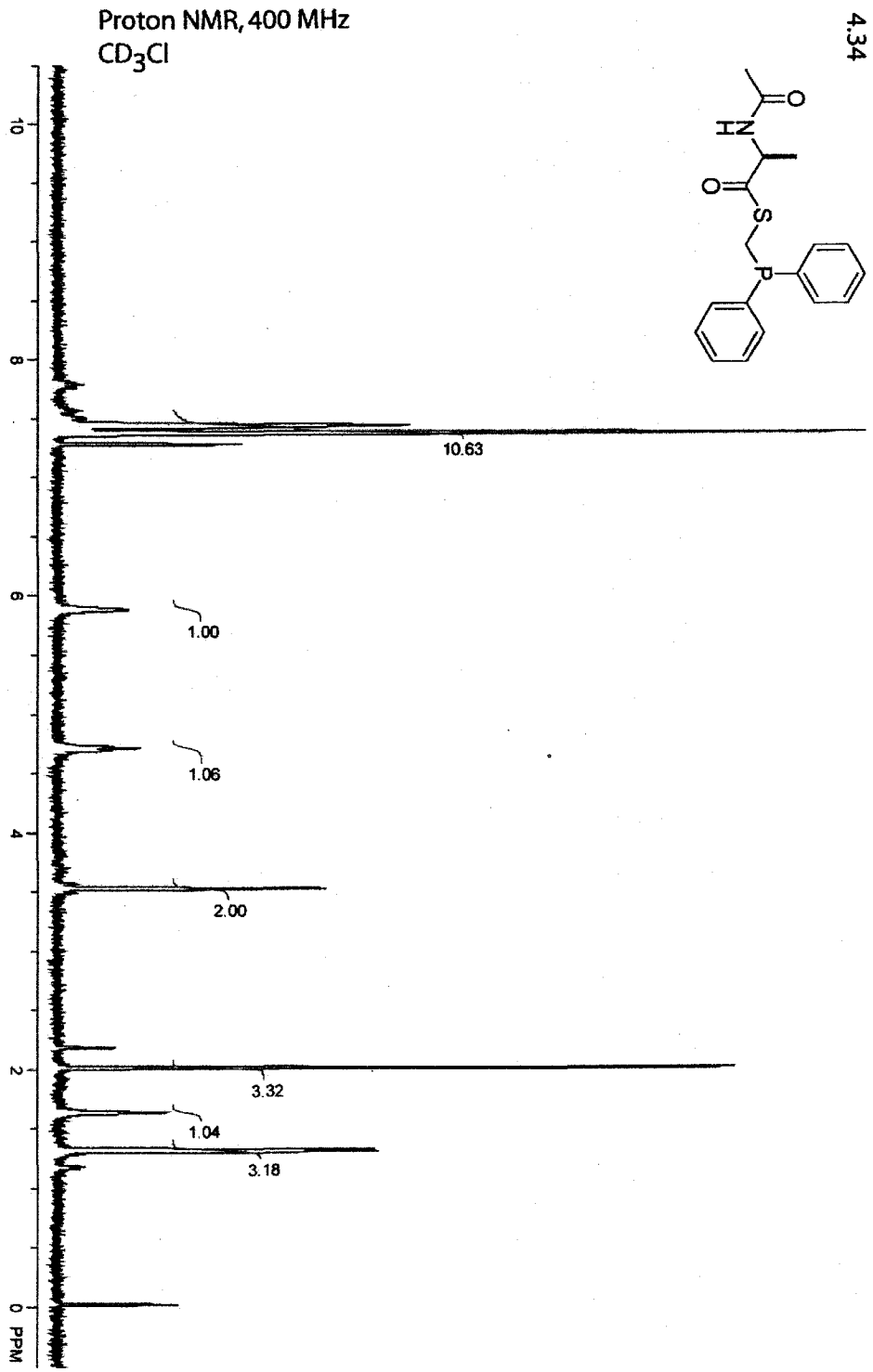
4.31

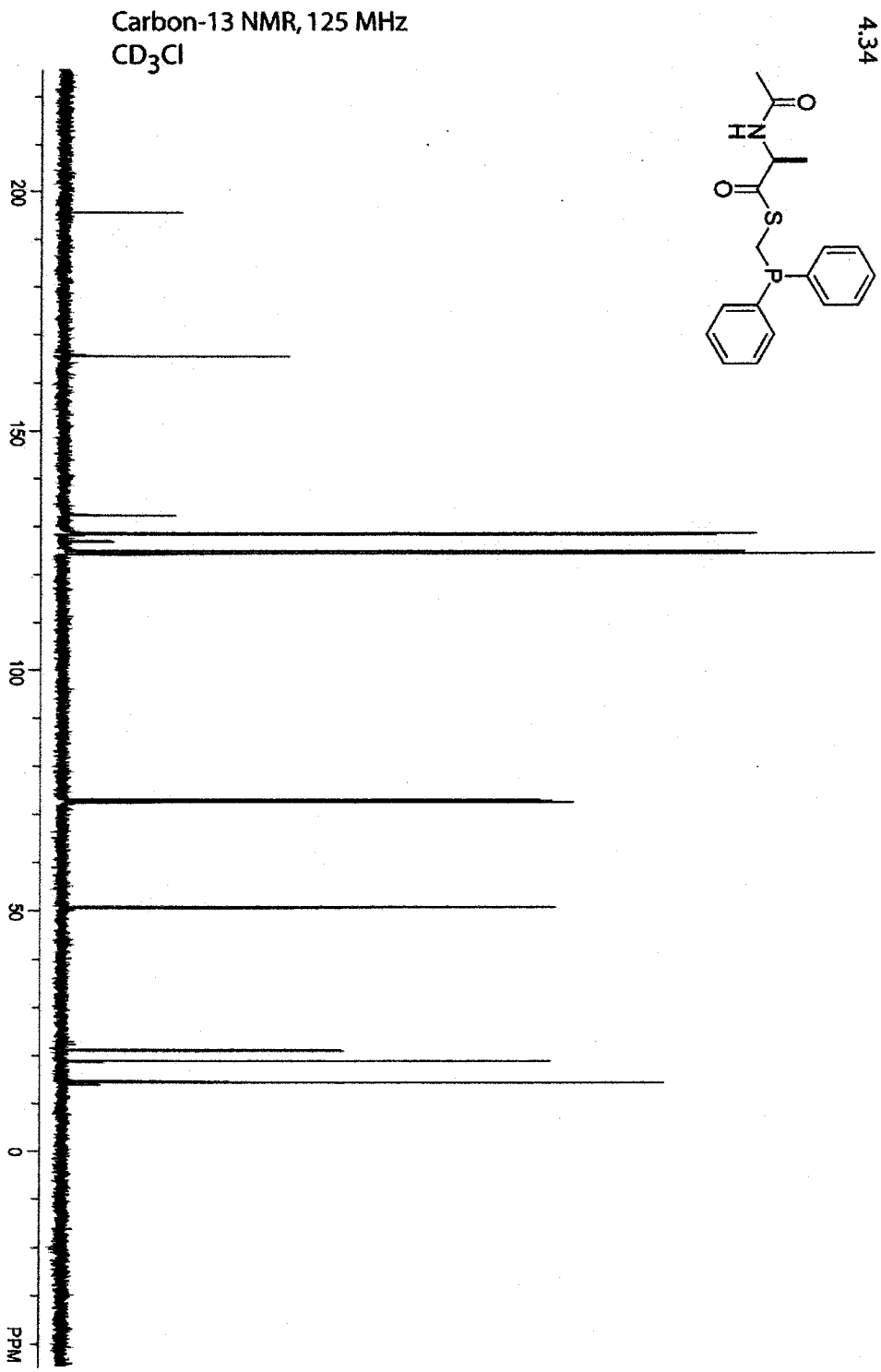






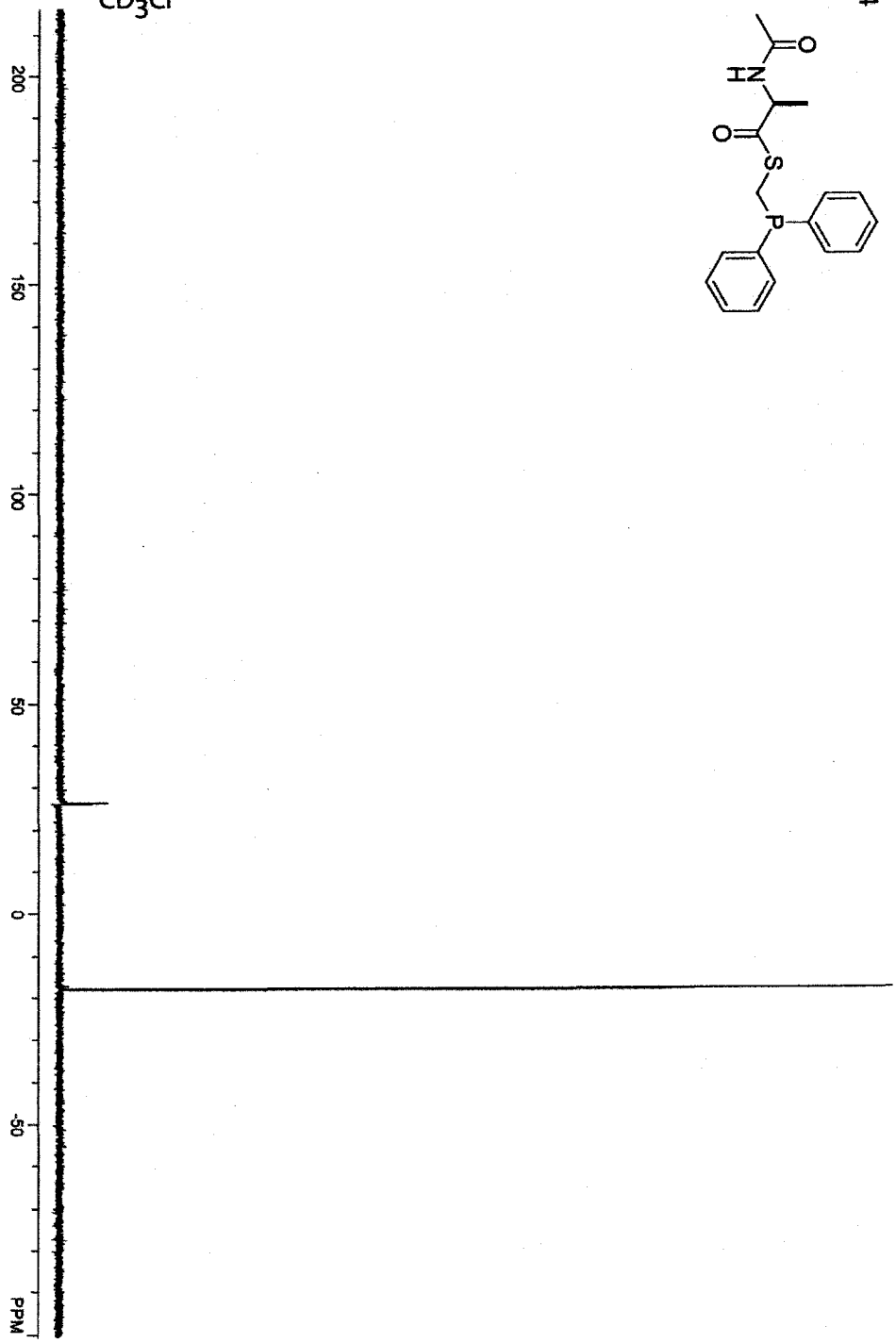


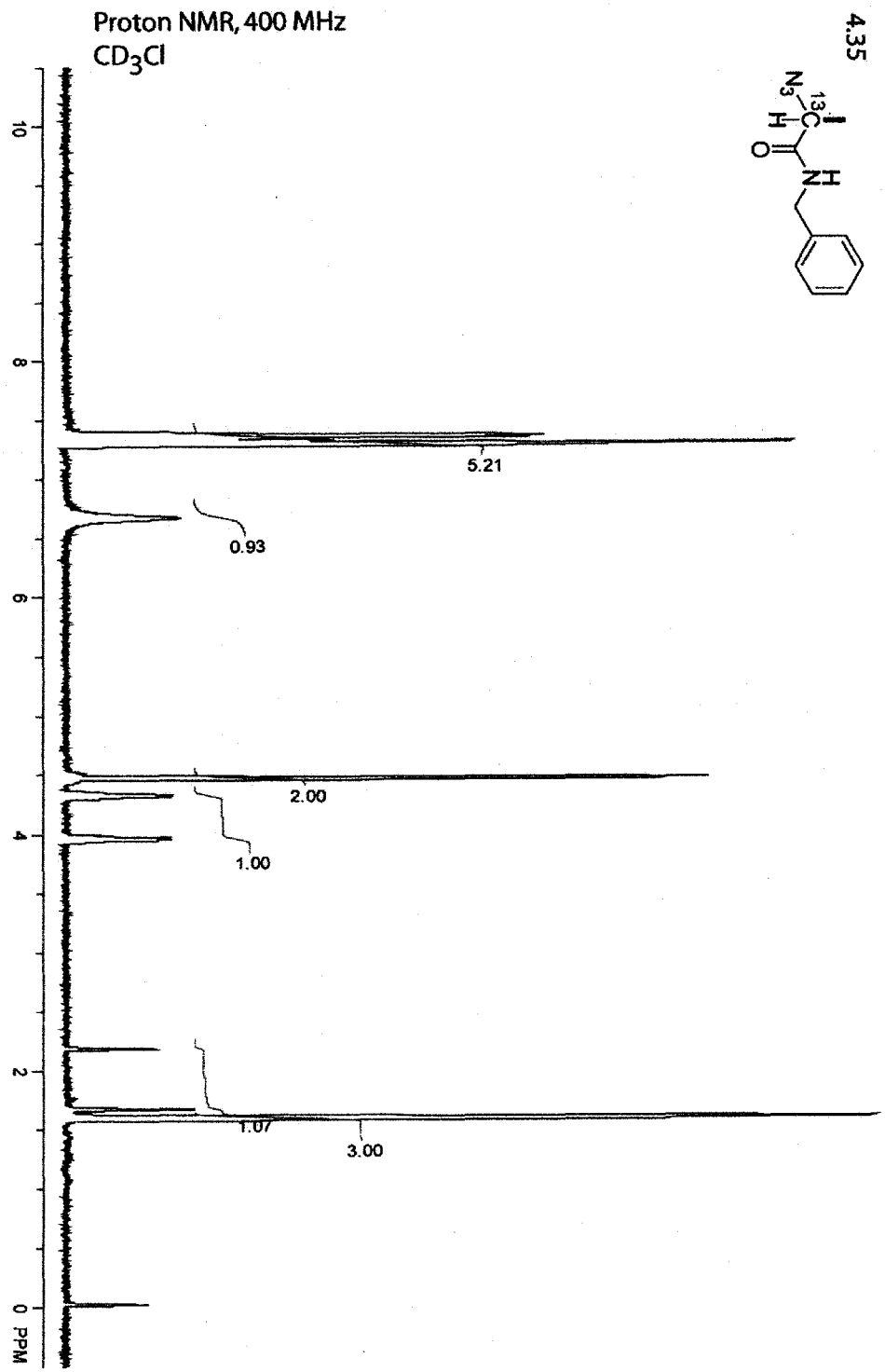


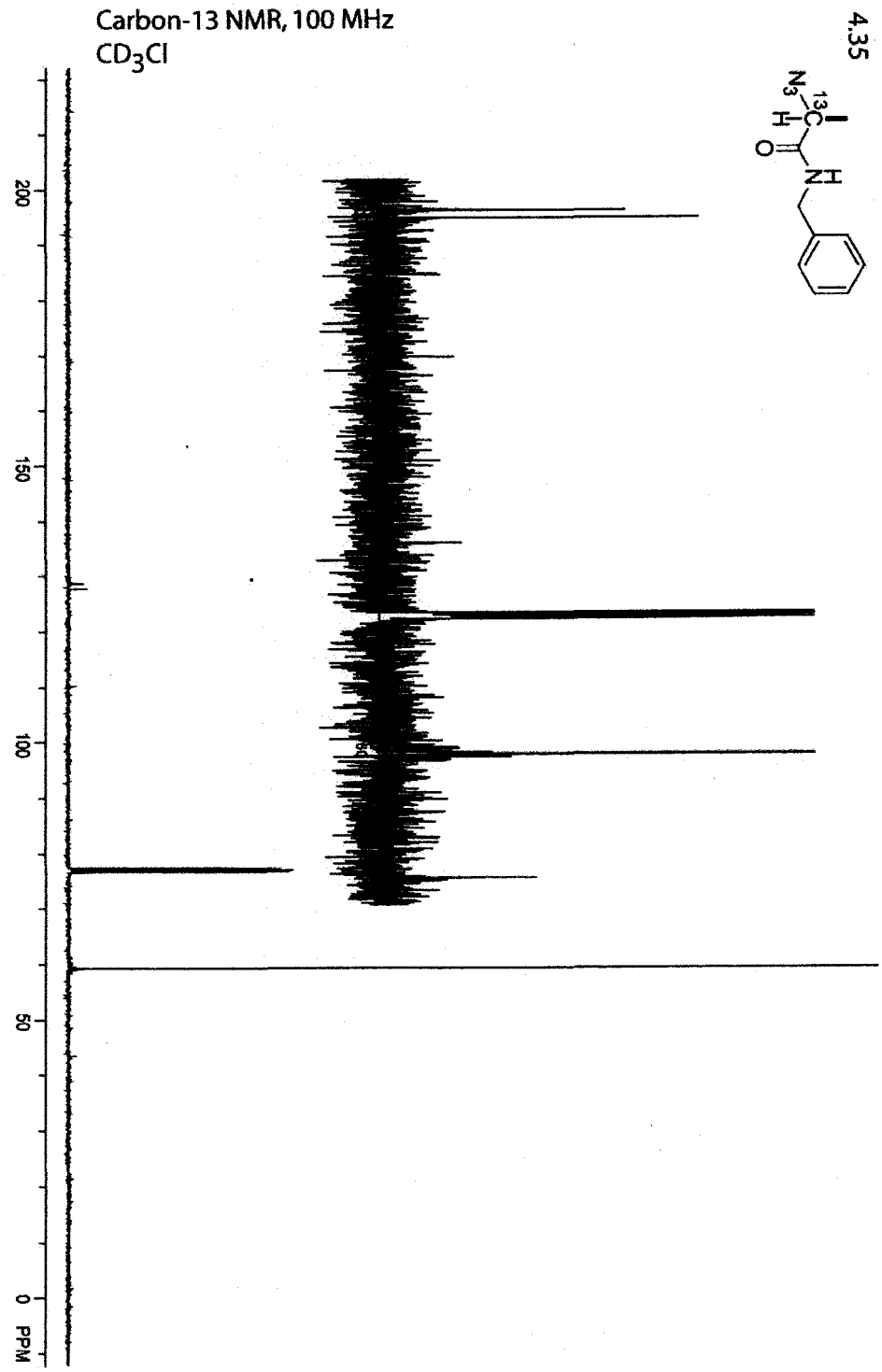


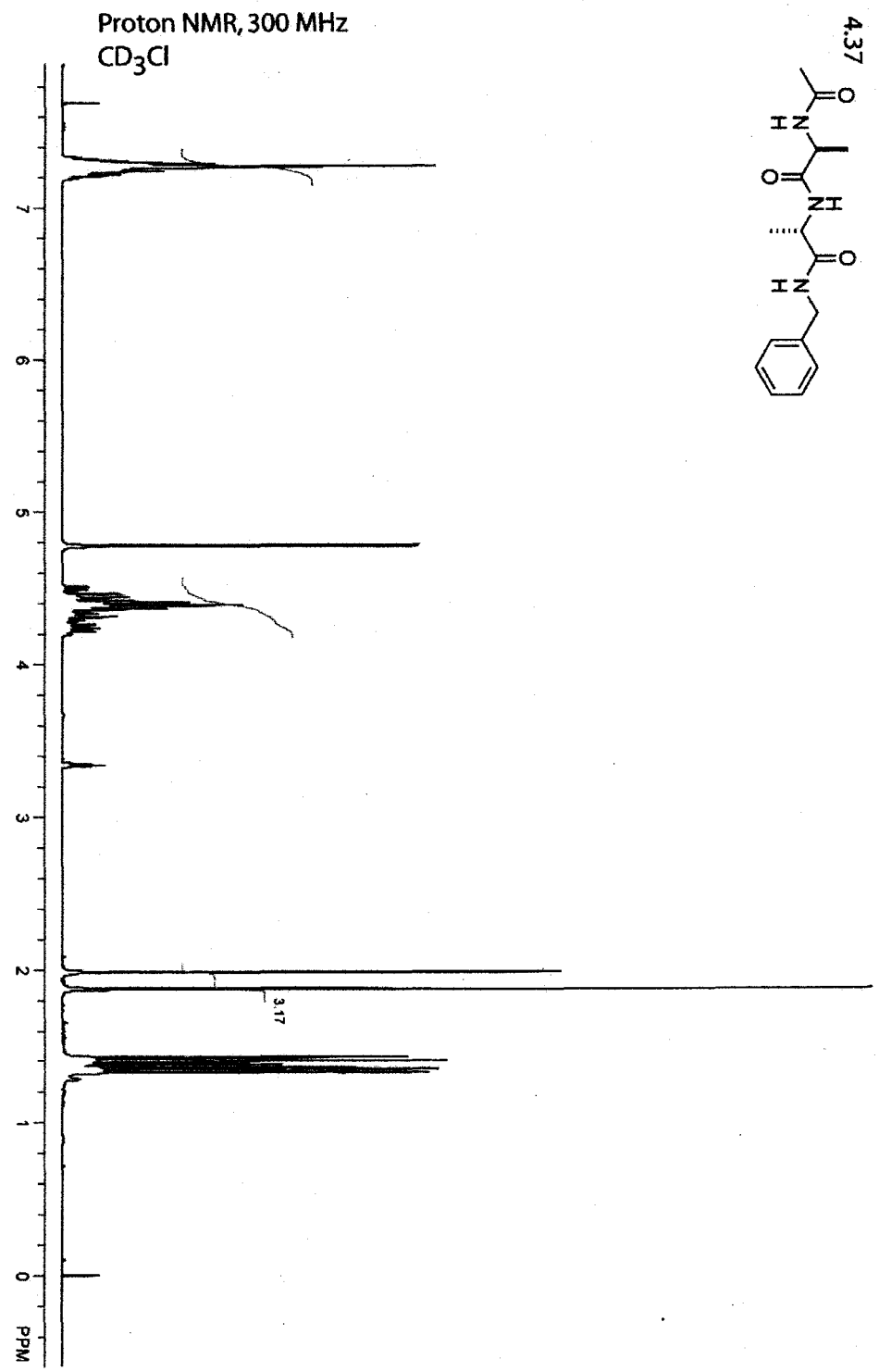
Phosphorus-31 NMR, 201 MHz
CD_3Cl

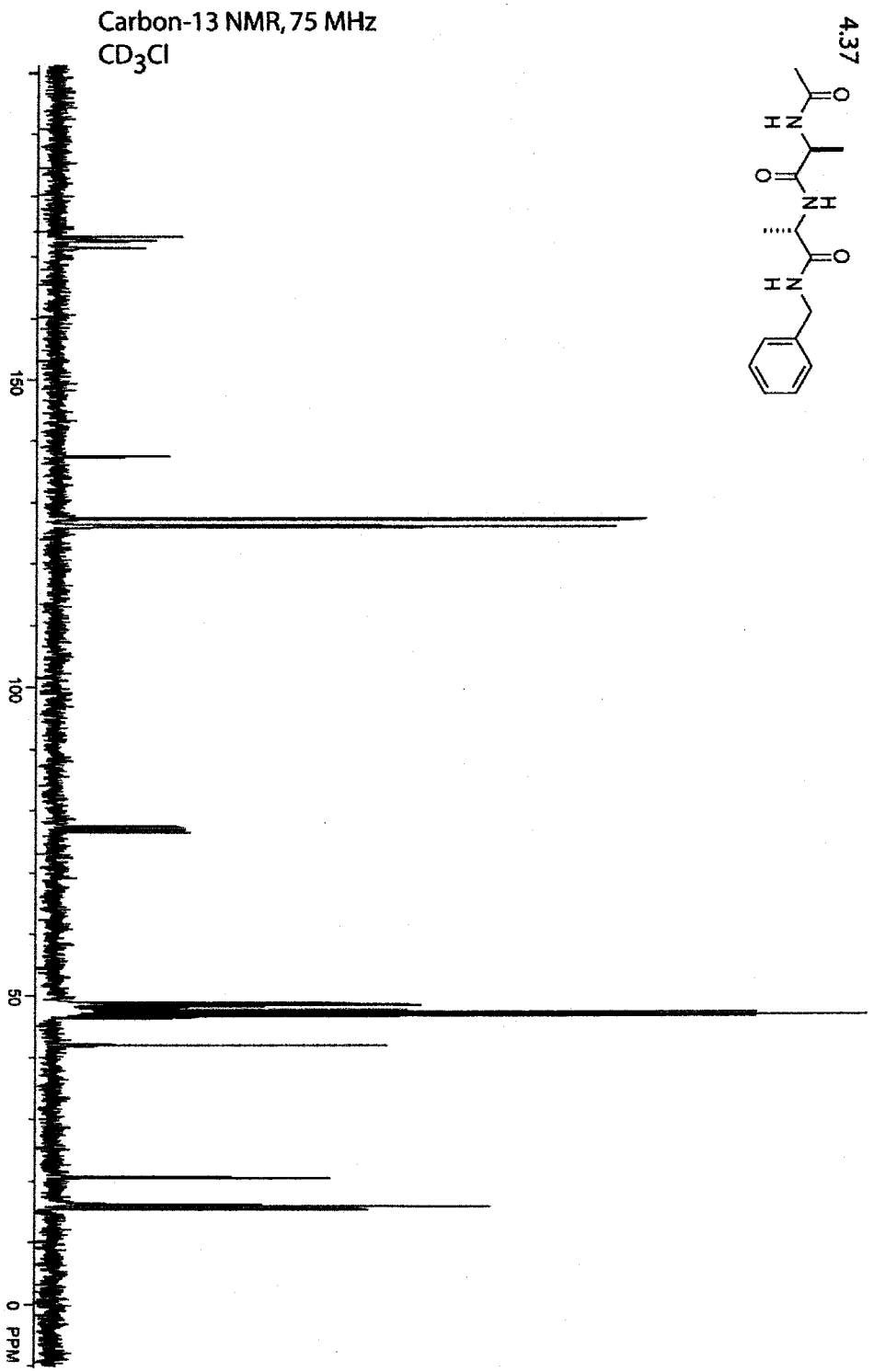
4.34

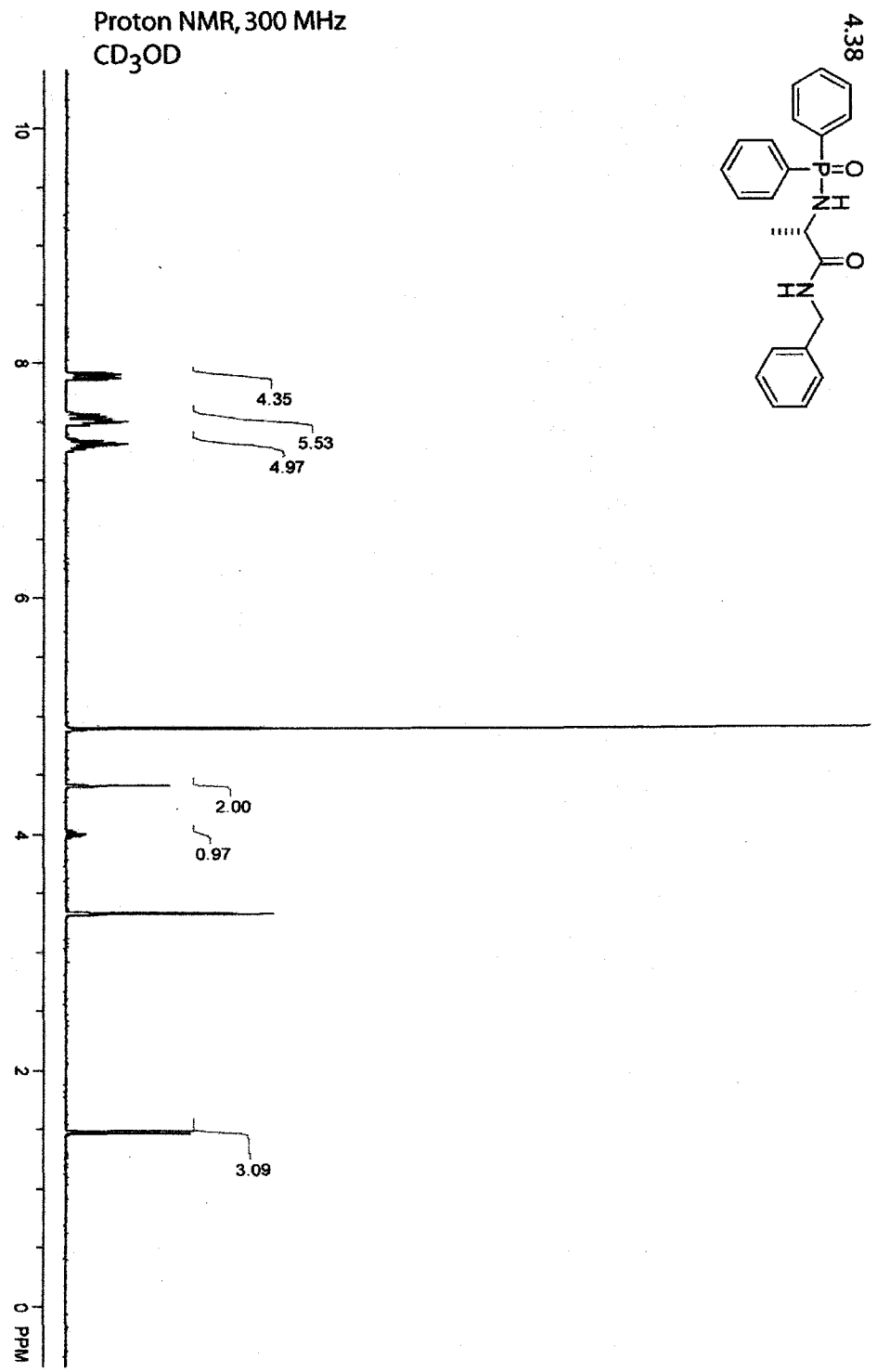


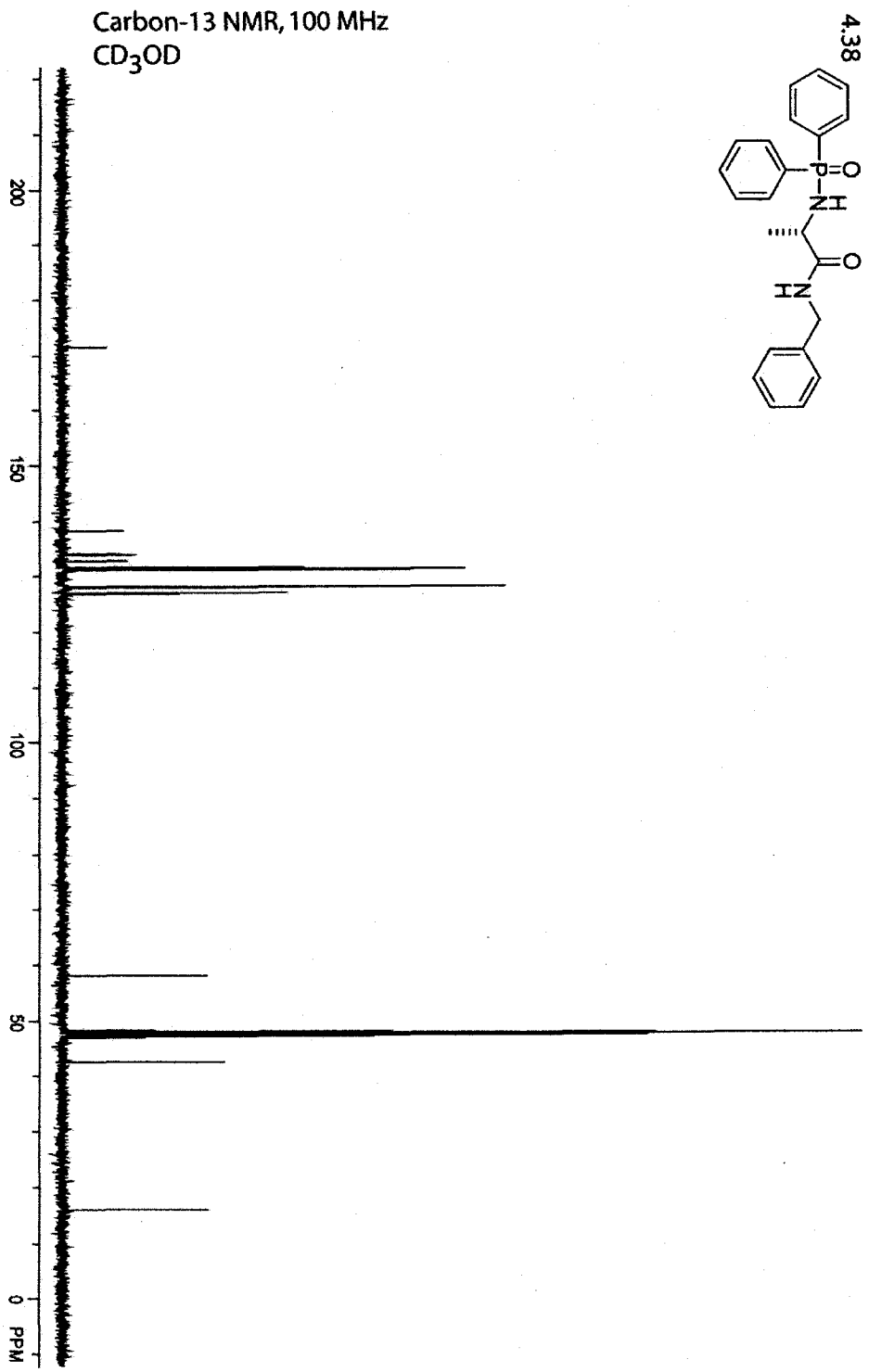




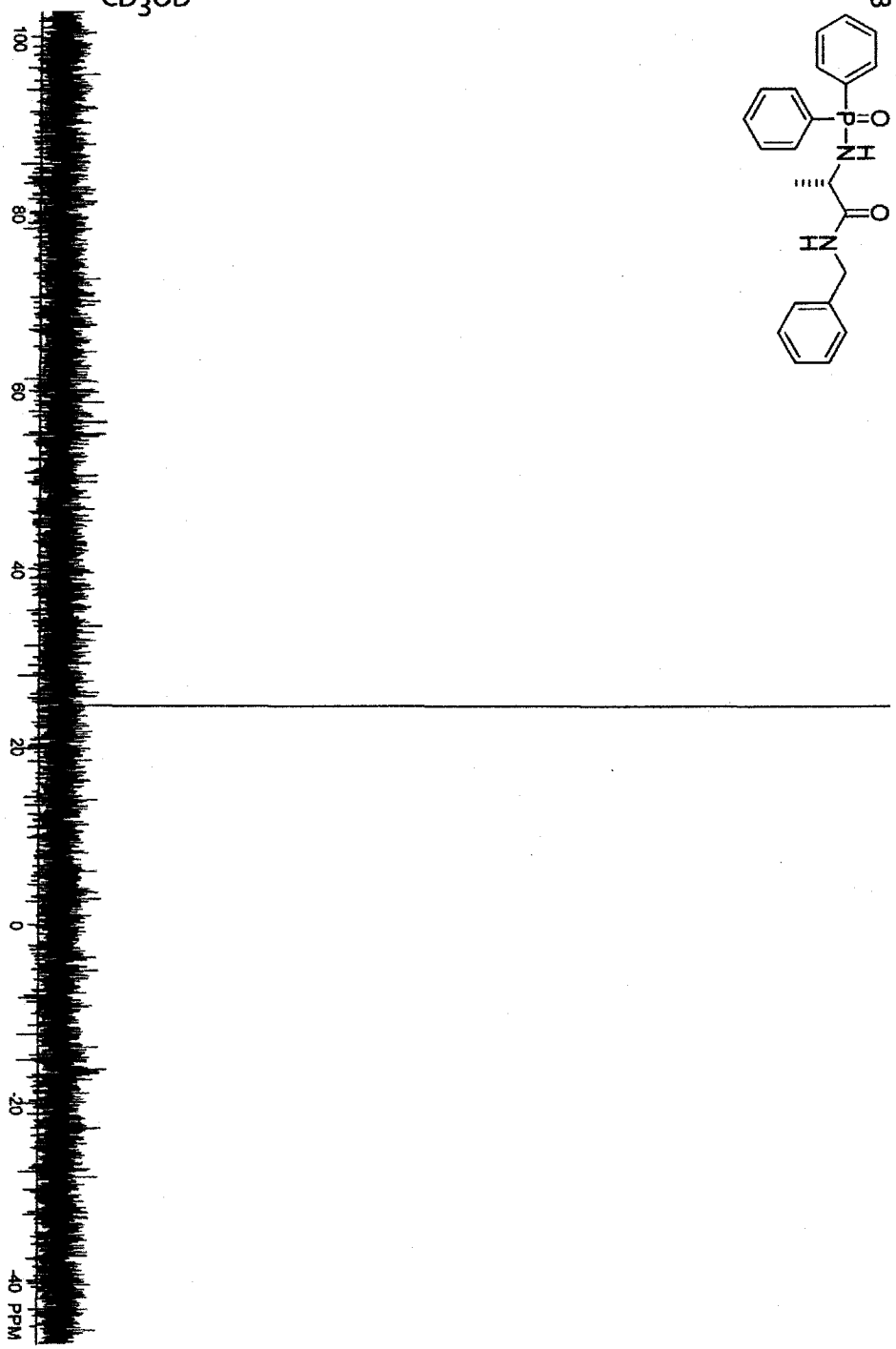








Phosphorus-31 NMR, 161 MHz
CD_3OD



Chapter 5

STAUDINGER LIGATION OF PEPTIDES AT NON-GLYCYL RESIDUES

5.1 Introduction

Total chemical synthesis is beginning to provide ready access to natural proteins, as well as enable the creation of nonnatural ones (Nilsson et al., 2005). Many proteins have already been assembled from synthetic peptides. "Native chemical ligation"—the coupling of a peptide (or protein) containing a *C*-terminal thioester with another peptide containing an *N*-terminal cysteine residue has been especially powerful (Dawson & Kent, 2000; Kent, 2003). "Expressed protein ligation" is a method by which *C*-terminal thioesters for native chemical ligation can be accessed using recombinant DNA techniques (Muir, 2003).

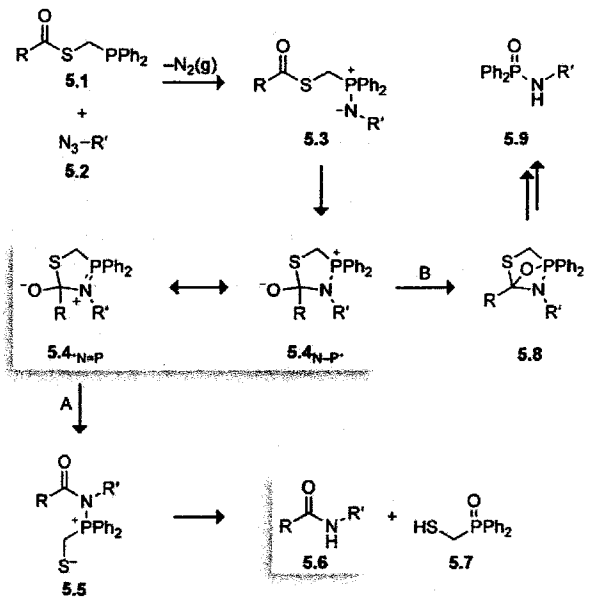
A limitation of native chemical ligation is its intrinsic reliance on having a cysteine residue at the ligation juncture. Cysteine is uncommon, comprising only 1.7% of all residues in proteins (McCaldon & Argos, 1988b). Modern peptide synthesis is typically limited to peptides of $\leq$40 residues (Bray, 2003), whereas most proteins contain hundreds of residues. Hence, most proteins cannot be prepared by any method that allows for peptides to be coupled only at cysteine residues.

An emerging strategy has been the use of auxiliaries that act as cysteine surrogates to mediate the chemical ligation of peptide fragments (Canne et al., 1996; Offer & Dawson, 2000). After the ligation, the auxiliary is removed to reveal the native amide bond.

Efforts toward these auxiliaries have revealed a requirement for one of the glycine residues to be present at the ligation junction (Marinzi et al., 2001; Offer et al., 2002). Although these methods are not sequence independent, they do relax significantly the cysteine limitation, as glycine is among the most common amino acids in proteins (McCaldon & Argos, 1988b).

Another ligation strategy devoted to eliminating the cysteine requirement at the ligation site is the traceless Staudinger ligation (Scheme 5.1) (Nilsson et al., 2000; Nilsson et al., 2001; Soellner et al., 2002; Nilsson et al., 2003; Soellner et al., 2003; Kohn & Breinbauer, 2004). In our version of the Staudinger ligation, a peptide with a C-terminal phosphinothioester (**5.1**) is coupled with a second peptide having an N-terminal azido acid (**5.2**) through the intermediacy of an iminophosphorane (**5.3**). The iminophosphorane can rearrange to give an amidophosphonium salt (**5.4**) as an acyl product that is hydrolyzed in the presence of water. The final ligation product contains a native amide bond (**5.5**) without any residual atoms (Nilsson et al., 2000; Nilsson et al., 2001). The ligation occurs without racemization (Soellner et al., 2002). The Staudinger ligation has been used in the orthogonal assembly of a protein (RNase A) (Nilsson et al., 2003) and for the site-specific immobilization of peptide to a surface (Soellner et al., 2003). Different reagents and methods have also been explored in applying the Staudinger ligation to peptide synthesis and *in vivo* labeling experiments (Saxon et al., 2000; Saxon & Bertozzi, 2000a; Kiick et al., 2002; Saxon et al., 2002; Vocadlo et al., 2003; Kohn & Breinbauer, 2004).

Scheme 5.1. Proposed mechanism of Staudinger ligation.

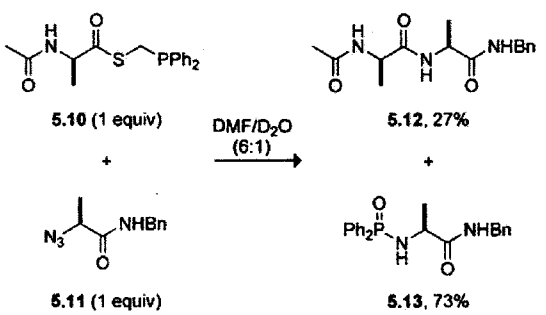


The mechanism for the Staudinger ligation appears to be indifferent to amino acid substitution at the ligation sites, yet, as with the auxiliary-mediated ligations, all reported traceless Staudinger ligations that proceed with a high (i.e., >50%) yield have have a glycine residue at either the *N*- or *C*-terminal residue at the ligation junction. Herein, we report the optimization of the traceless Staudinger ligation for the coupling at non-glycyl residues. By the rational manipulation of the electronic structure of a key intermediate, using both substitutions on the aryl substituents of the phosphine and solvent effects, reagents and conditions are presented that allow for the Staudinger ligation between non-glycyl resiudes.

5.2 Results and Discussion

Electronic Tuning Using Phosphine Substituents. As a model reaction for studying couplings involving two non-glycyl residues, Ala–Ala couplings were examined. Using the best conditions reported previously, coupling alanylphosphinothioester **5.10** with alanyl azide **5.11** results in moderate yields of dipeptide product **5.12** (Scheme 5.2, 27–48%) (Chapter 4). This moderate yield is due largely to the presence of a byproduct that results from aza-Wittig reaction of the iminophosphorane, phosphonamide **5.13** (Chapter 4). The key transformation in the partitioning of the iminophosphorane **5.3** between Staudinger ligation (Path A) product **5.6** and aza-Wittig (Path B) byproduct **5.9** results from the tetrahedral intermediate **5.4** (Scheme 5.1).

Scheme 5.2. Reaction of alanylphosphinothioester **5.10** with alanyl azide **5.11**.



In the reaction pathway (Scheme 5.1), the Staudinger ligation (Path A) requires that an electron pain on the oxygen of the tetrahedral intermediate **5.4** displaces sulfur atom to

form amidophosphonium salt **5.5**. Meanwhile, in the aza-Wittig reaction, an electron pair on the oxygen forms a bond with the oxophilic phosphorus atom, resulting in oxazaphosphetane **5.8**.

We hypothesized that the tuning of the electronic properties of the phosphorus atom in intermediate **5.4** could dictate whether amide **5.6** or phosphonamide **5.9** was formed from the Staudinger ligation or aza-Wittig process, respectively. The ability to reduce partitioning towards the aza-Wittig byproduct reaction would presumably allow for the coupling of two non-glycyl residues with high yields.

To test this hypothesis, compounds **5.14** and **5.15** were synthesized and evaluated relative to compound **5.10** for effecting the Staudinger ligation between non-glycyl couplings (Figure 5.1). It was believed that the *p*-chloro groups of compound **5.14** would decrease the electron density of the phosphorus atom in the tetrahedral intermediate **5.4** via electron withdrawal through the chlorine substitution. Based on our hypothesis, this decreased electron density on the phosphorus would lead to greater attraction between the carbonyl oxygen and the phosphorus atom, and subsequent partitioning the reaction toward the aza-Wittig process (Path B, Scheme 5.1). Conversely, the *p*-methoxy groups of compound **5.15** should increase the electron density of the phosphorus atom in intermediate **5.4** and make the Staudinger ligation more favorable, through decreased attraction between the carbonyl oxygen and the phosphorus atom. Compounds **5.10**, **5.14**, and **5.15** were synthesized via modifications of previous published syntheses for related phosphinothioesters (Scheme 3) (Nilsson et al., 2001; Grandjean et al., 2005).

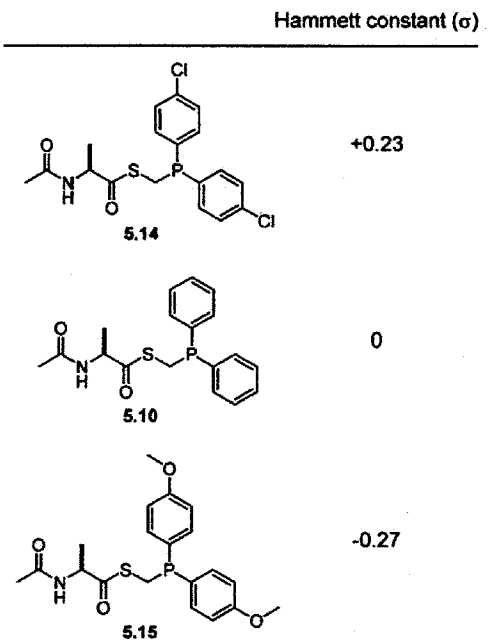
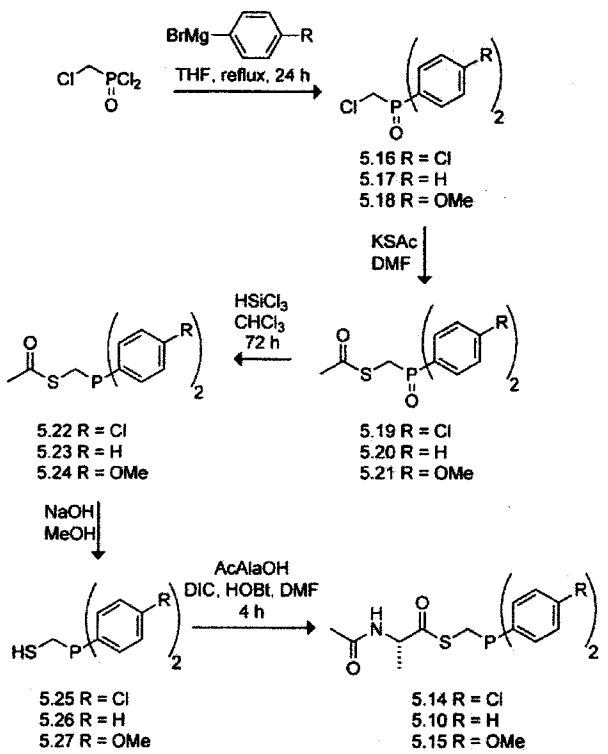


Figure 5.1. Proposed compounds for effecting the Staudinger ligation at non-glycyl residues.

Scheme 5.3. Synthesis of phosphinothiols **5.25**, **5.26**, and **5.27**.



The reaction of ^{13}C-labeled alanyl azide **5.11** with each alanyl phosphinothioester **5.10**, **5.14**, and **5.15** was performed in DMF and yields were obtained by ^{13}C NMR spectroscopy, using previously reported quantitative NMR methods (Chapter 4). Under these conditions, alanyl phosphinothioester **5.10** provided the desired compound in 47% yield. In agreement with our hypothesis, *p*-chloro compound **5.14** afforded diminished yield of amide product **5.12** (34%). The *p*-methoxy compound **5.15** also followed the expected trend, yielding 61% of amide product upon reaction with alanyl azide **5.11**. This represents a substantial increase from the previously reported yields for non-glycyl Staudinger ligation couplings (Merkx et al., 2003a). Figure 5.2 shows a graph of Hammett σ value versus yield of amide product. The yields obtained showed very high correlation to the Hammett σ value of the substituent on the phenyl ring ($r^2 = 0.9997$).

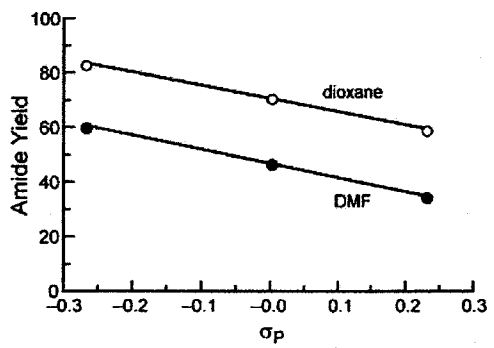
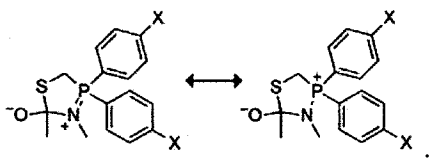


Figure 5.2. Graph of Hammett σ value versus yield of Ala-Ala amide product in DMF.

In an effort to quantify the effect of electron tuning of the phosphine substituents in the tetrahedral intermediate **5.4**, DFT calculations were performed using Gaussian 98. These calculations confirmed that the electron density of the phosphorus atom in the

tetrahedral intermediate increased considerably as the substiuent became more electron donating (Figure 5.3). As was observed experimentally, these calculations implicate that the weakening of the carbonyl oxygen—phosphorus interaction in the tetrahedral intermediate **5.4** is responsible for the decreased amount of aza-Wittig phosphonamide byproduct observed.



X	Cl	H	OMe
σ_P	+0.23	0.00	–0.27
P. charge	+0.131	+0.062	+0.044

Figure 5.3. Calculated electron density of the phosphorus atom in the tetrahedral intermediate.

Solvent Polarity Effects on Staudinger Ligation Reagents. Previous efforts aimed at optimizing the Staudinger ligation showed that the reaction proceeded more rapidly in polar solvents, presumably due to favorable stabilization of a polar transition state in the rate-determining step (Chapter 4). To determine the effect of solvent on the partitioning of iminophosphorane **5.3** between Staudinger ligation (Path A) product **5.6** and aza-Wittig (Path B) byproduct **5.9** (Scheme 5.1), the reaction of *p*-methoxy phosphinothioester **5.15** and alanyl azide **5.11** was performed in a variety of anhydrous solvents (acetonitrile, acetone, tetrahydrofuran, methylene chloride, methanol, nitromethane, dimethylformamide, dioxane and toluene). These solvents were chosen to represent a range of differing solvent dipoles, dielectric constants, and SPP (Catalan et al., 1995) values. The correlation of solvent characteristics (dipole, dielectric constant, and SPP value) is depicted in Figure 5.4. While little correlation is found for dipole and

dielectric constant versus yield of amide product, there is a good correlation of SPP value versus amide yield.

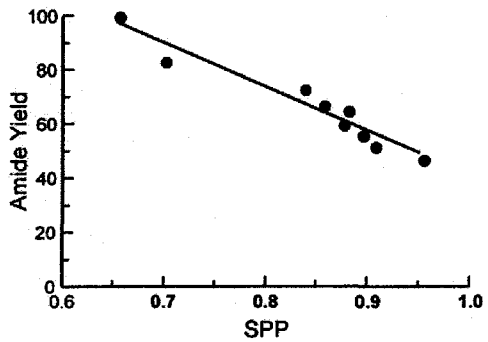


Figure 5.4. Graph of solvent polarity polarizibility (SPP) constant versus yield of Ala-Ala amide product.

Surprisingly, the highest yields were obtained with nonpolar solvents (toluene, dioxane, THF). The yield obtained of AcAlaAlaNHBn product is high (83%) in dioxane and nearly quantitative (99%) in toluene. These yields are a substantial increase from previously published yields for peptide ligation at two non-glycyl residues. Each of the previously described alanyl phosphinothioesters (*p*-chloro phenyl **5.14**, *p*-methoxy phenyl **5.15**, and phenyl **5.10**) were reacted with alanyl azide **5.11** in dioxane. Once again, the yields obtained correlated well with the Hammett σ constant for the substituent (Figure 5.5).

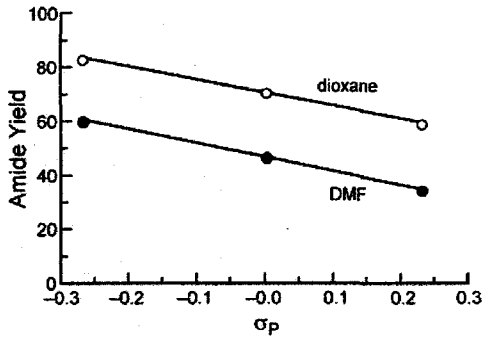
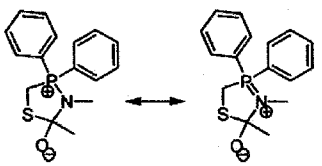


Figure 5.5. Graph of Hammett σ value versus yield of Ala-Ala amide product in DMF and dioxane.

As a potential explanation for non-polar solvents affording higher yields of the desired peptide product, we performed DFT calculations of the key tetrahedral intermediate in different solvents using Gaussian98. The calculations showed that the electron density on the phosphorus atom in the tetrahedral intermediate increases in non-polar solvents (Figure 5.6). This is likely due to the enhanced stability of charge separation in polar solvents. By experiment, lowering the electron density on the phosphorus atom leads to increased byproduct formation via aza-Wittig reaction.

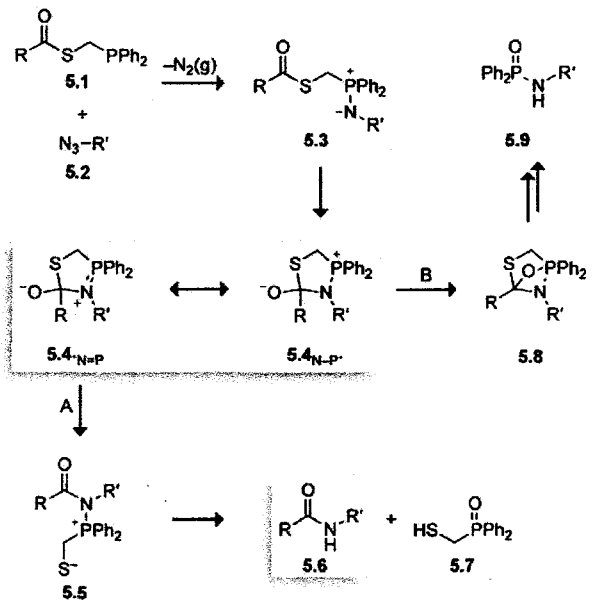


	polar solvent	non-polar solvent
Solvent	H_2O	THF
P, charge	+.082	+.028
N, charge	-.348	-.335

Figure 5.6. The effect of solvent polarity on the calculated electron density of the phosphorus atom in the tetrahedral intermediate.

The experimental and theoretical results can be explained by the relative contribution of $\mathbf{5.4}_{+P\text{-}N}$ and $\mathbf{5.4}_{P=N+}$ to the structure of tetrahedral intermediate **5.4**. This relative contribution is affected by both solvent polarity and substituents on the phosphine and leads to the observed distributions of Staudinger ligation (Path A) and aza-Wittig (Path B) products.\

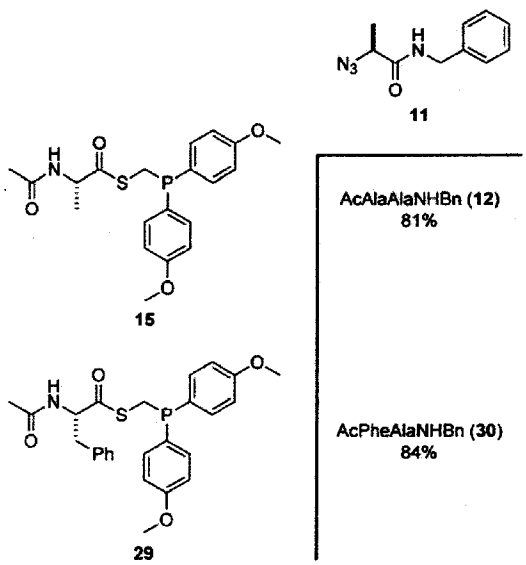
Scheme 5.4. Refined mechanism of Staudinger ligation involving resonance of the tetrahedral intermediate **5.4**.



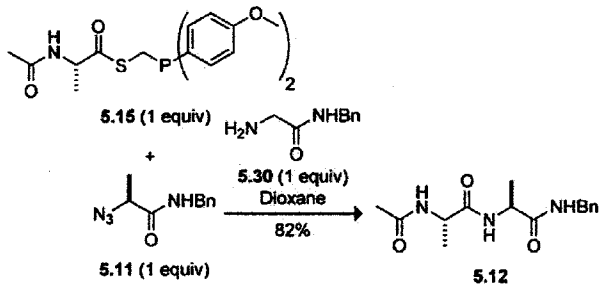
Model Couplings Involving non-Glycyl Residues. Utilizing the conditions most favorable for non-glycyl ligations, a series of model ligations were performed to determine the scope of these reactions (Table 5.1). Dioxane was chosen as the solvent, because many starting materials and products were insoluble in the most nonpolar solvents tested, such as toluene. Alanyl azide **5.11** was reacted (dioxane, 50 °C, 12 h)

with both alanyl *p*-methoxy phosphinothioester **5.15** and phenylalanyl *p*-methoxy phosphinothioester **5.28** to form AcAlaAlaNHBn (**5.12**, 81%), and AcPheAlaNHBn (**5.29**, 84%), respectively. After chromatography using silica gel, each of these couplings afforded the desired dipeptide in high isolated yields (>80%). These couplings represent a substantial improvement upon the yields previously reported for traceless Staudinger ligations at two non-glycyl amino acids (<50%) (Chapter 4) (Merkx et al., 2003b). To confirm that the thioester functionality retains its high chemoselectivity during the ligations, the coupling of alanyl azide **5.11** and alanyl *p*-methoxy phosphinothioester **5.15** was performed in the presence of glycyl amine **5.30** (Scheme 5.5) under conditions identical to the previous couplings. Gratifyingly, no decrease in the AcAlaAlaNHBn **5.12** product was observed, indicating that this new phosphinothioester retain the high chemoselectivity found in previously reported reagents (Chapter 4).

Table 5.1. Isolated yields of model reactions involving phosphinothioesters **5.15** and **5.28** with azide **5.11**.



Scheme 5.5. Chemoselectivity of phosphinothioester **5.15** with azide **5.11** under optimized conditions.



5.3 Conclusions

This work has elucidated the factors critical for the optimization of the Staudinger ligation at non-glycyl ligation sites. The synthesis and evaluation of novel phosphinothiols for effecting the Staudinger ligation have been presented and evaluated under a variety of experimental conditions. No longer a shortcoming of the Staudinger ligation, couplings involving two non-glycyl amino acids have now been performed in high yields. The Staudinger ligation is now poised to enable the iterative ligation of unprotected peptides toward the chemical synthesis of proteins.

5.4 Experimental Prodedures

General. Reactions were monitored by thin-layer chromatography with visualization by UV light or staining with ninhydrin or I_2. Compound purification was carried out with an automated chromatography system. Silica gel used in flash chromatography had 230–400 mesh and 60 Å pore size. Reagent chemicals were obtained from commercial suppliers, and reagent grade solvents were used without further purification. NMR spectra were obtained with a 500 or 400 MHz spectrometer at the National Magnetic Resonance Facility at Madison or the University of Wisconsin nuclear magnetic resonance facility, respectively. Carbon-13 and phosphorus-31 spectra were both proton-decoupled, and phosphorus-31 spectra were referenced against an external standard of deuterated phosphoric acid (0 ppm). Mass spectra were obtained with electrospray ionization (ESI) techniques.

Procedures for calculations using Gaussian98. A global minimum-energy structure for the tetrahedral intermediate **5.4** was determined by Monte Carlo calculations using the program Macromodel® and the Merck Molecular Force Field (MMFF®). The minimum-energy structure thus obtained was imported into Gaussian98 and minimized further using DFT calculations at the B3LYP/6-31+g(d,p) level of theory. (If a negative frequency was found, then the structure was optimized again at the same level of theory.) Single-point energy calculations were then performed in a variety of solvents by using CPCM techniques. Mulliken charges were obtained from the single point energy calculations.

2-Acetylamino-thiopropionic acid S-[(diphenylphosphanyl)-methyl] ester (5.10). N-Acetylalanine (557 mg, 4.25 mmol) was dissolved in anhydrous DMF (20 mL). HOBt (527 mg, 3.90 mmol) was then added to the resulting solution, followed by DCC (805 mg, 3.90 mmol). Once precipitate (DCU) was observed, phosphinothiol **5.26** was added (900 mg, 3.87 mmol). The reaction mixture was allowed to stir under Ar(g) for 4 h. The precipitate was removed by filtration, and the filtrate was concentrated under reduced pressure to give a clear oil. The oil was dissolved in ethyl acetate and purified by flash chromatography (silica gel, 70% ethyl acetate/hexanes). Phosphinoester **5.10** was isolated as a white solid in 83% yield. **Spectral data.** ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.44–7.37 (m, 10H), 5.89 (bt, 1H), 4.71 (bt, 1H), 3.53 (s, 2H), 2.02 (s, 3H), 1.64 (m, 1H), 1.32 (d, J = 5.8 Hz, 3H) ppm; ^{13}C NMR (CDCl3, 125 MHz) δ 199.85, 169.70, 136.51, 132.74, 132.59, 132.57, 129.10, 128.51, 128.46, 54.83, 25.29 (d, J = 24.3 Hz), 22.99, 18.66 ppm; ^{31}P NMR (CDCl3, 161 MHz) δ -17.97 ppm; MS (ESI) m/z 368.0853 (MNa^+ $[C_{18}H_{20}NO_2PSNa^+]$ = 368.0845).

N_3AlaNHBn (5.11). Following the protocol published by Lundquist and Pelletier (Lundquist & Pelletier, 2001), Azidoalanine was synthesized from alanine. L-Alanine (280 mg, 2.8 mmol) was dissolved in anhydrous DMF (15 mL). Hydroxybenzotrizole (397 mg, 2.9 mmol) was then added followed by addition of DCC (607 mg, 2.9 mmol). Once precipitate (DCU) was observed in the reaction, benzylamine (0.370 mL, 3.4 mmol) was added. Reaction was allowed to stir under argon for 3h. The resulting precipitate (DCU byproduct) was filtered away and resulting filtrate was evaporated under reduced

pressure to yield a yellow oil. This oil was dissolved in 35% v/v ethyl acetate in hexanes and purified by flash chromatography (silica gel, 35% v/v ethyl acetate in hexanes). Azide **5.11** was isolated in 90% yield as an off-white solid. **Spectral data.** ^{1}H NMR (CDCl3, 400 MHz) δ7.37–7.28 (m, 5H), 6.67 (bs, 1H), 4.46 (s, 2H), 4.13 (dt, J = 144.7, 6.9 Hz, 1H), 1.60 (d, J = 2.3 Hz, 3H) ppm; ^{13}C NMR (CDCl3, 125 MHz) δ128.78, 127.74, 59.29, 43.52 ppm; MS (ESI) *m/z* 227.0909 (MNa^+ [$C_{10}H_{12}N_4ONa^+$] = 227.0903).

AcAlaAlaNHBn (5.12). Azide **5.10** (36.2 mg, 0.105 mmol) and phosphinothioester **5.11** (21.4 mg, 0.105 mmol) were added to a reaction vessel and dissolved in DMF/H_2O (6:1, 1 mL). The resulting reaction mixture was allowed to stir 12 h. Amide **5.12** was isolated as a white solid in 27% yield (3% v/v MeOH in CH_2Cl_2). **Spectral data.** ^{1}H NMR (300 MHz, $CDCl_3$:CD_3OD (1:1)) δ7.32-7.35 (m, 5H), 4.51–4.22 (m, 4H), 1.98 (1.87) (s, 3H), 1.41 (d, J = 7.5 Hz, 3H), 1.34 (d, J = 6.9 Hz, 3H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$:CD_3OD (1:1), numbers in parentheses indicate doubling due to rotational isomerism) δ173.27 (172.78), 172.67 (172.54), 171.44, 137.59 (137.51), 127.39 (127.49), 126.28 (126.36), 126.02 (126.17), 48.92 (48.70), 48.59 (48.34), 41.99 (42.06), 20.50 (20.76), 15.99 (16.23), 15.45 ppm; MS (ESI) *m/z* 315.1511 (MNa^+ [$C_{14}{}^{13}CH_{21}N_3O_3Na^+$] = 315.1509).

$Ph_2P(O)$AlaNHBn (5.13). Azide **5.10** (36.2 mg, 0.105 mmol) and phosphinothioester **5.11** (21.4 mg, 0.105 mmol) were added to a reaction vessel and dissolved in DMF/H_2O (6:1, 1 mL). The resulting reaction mixture was allowed to stir 12 h. Phosphonamide **5.13** was isolated as a white solid in 73% yield (3% v/v MeOH in CH_2Cl_2). **Spectral data.** ^{1}H NMR (CD_3OD, 400 MHz) δ7.92–7.86 (m, 5H), 7.58–7.48 (m, 5H), 7.35–7.26 (m, 5H),

4.41 (s, 2H), 4.00 (q, J = 7.1 Hz, 1H), 1.47 (d, J = 7.1 Hz, 3H) ppm; ^{13}C NMR (CD_3OD, 125 MHz) δ 171.58, 138.26, 134.07, 132.76, 131.63, 131.60, 131.42, 131.32, 128.26, 128.18, 128.13, 127.12, 126.92, 58.23, 42.64, 15.97 ppm; ^{31}P NMR (CD_3OD, 161 MHz) δ 24.63 ppm; MS (ESI) m/z 401.1395 (MNa^+ [$C_{22}H_{23}N_2O_2PNa^+$] = 401.1389).

AcAlaSCH$_2$P(C$_6$H$_4$-*p*-Cl)$_2$ (5.14). N-Acetylalanine (5157 mg, 1.2 mmol) was dissolved in anhydrous DMF (8 mL). HOBt (148 mg, 1.1 mmol) was then added to the resulting solution, followed by DCC (138 mg, 1.1 mmol). Once precipitate (DCU) was observed, phosphinothiol **25** was added (330 mg, 1.09 mmol). The reaction mixture was allowed to stir under Ar(g) for 4 h. The precipitate was removed by filtration, and the filtrate was concentrated under reduced pressure to give a clear oil. The oil was dissolved in ethyl acetate and purified by flash chromatography (silica gel, 50% v/v ethyl acetate/hexanes). Phosphinoester **5.14** was isolated as a white solid in 93% yield. **Spectral data.** ^{1}H NMR (CDCl3, 400 MHz) δ 7.36–7.32 (m, 8H), 5.28 (bt, 1H), 4.70 (bt, 1H), 3.46 (d, J = 6.3 Hz, 2H), 2.03 (s, 3H), 1.62 (m, 1H), 1.32 (d, J = 6.3 Hz, 3 H) ppm; ^{13}C NMR (CDCl3, 125 MHz) δ 199.55, 169.65, 135.78, 134.11, 133.93, 128.96, 128.88, 54.91, 25.19 (d, J = 24.6 Hz), 23.12, 18.77 ppm; ^{31}P NMR (CDCl3, 161 MHz) δ –16.94 ppm; MS (ESI) m/z 436.0059 (MNa^+ [$C_{18}H_{18}Cl_2NO_2PSNa^+$] = 436.0065).

AcAlaSCH$_2$P(C$_6$H$_4$-*p*-OMe)$_2$ (5.15). N-Acetylalanine (1.13 g, 8.70 mmol) was dissolved in anhydrous DMF (40 mL). HOBt (1.05 g, 7.80 mmol) was then added to the resulting solution, followed by DCC (984 mg, 7.80 mmol). Once precipitate (DCU) was observed, phosphinothiol **5.27** was added (2.30 mg, 7.86 mmol). The reaction mixture was allowed to stir under Ar(g) for 4 h. The precipitate was removed by filtration, and the

filtrate was concentrated under reduced pressure to give a clear oil. The oil was dissolved in ethyl acetate and purified by flash chromatography (silica gel, 70% ethyl acetate/hexanes). Phosphinoester **5.10** was isolated as a white solid in 94% yield. **Spectral data.** ^{1}H NMR (CDCl3, 400 MHz) δ 7.39–7.35 (m, 4H), 6.92–6.90 (m, 4H), 5.96 (bt, 1H), 5.70 (bt, 1H), 3.83 (s, 6H), 3.45 (m, 1H), 2.02 (s, 3H), 1.32 (d, J = 6.4 Hz, 2H), 1.15 (d, J = 6.7 Hz, 3H) ppm; ^{13}C NMR (CDCl3, 125 MHz, numbers in parentheses indicate doubling due to rotational isomerism) δ 199.96, 170.19, 160.49, 134.26, 127.59, 126.56, 125.89, 117.46, 114.25, 114.19, 110.98, 55.19 (54.99), 42.25, 26.01 (d, J = 23.2 Hz), 23.30 (23.04), 18.76 ppm; ^{31}P NMR (CDCl3, 161 MHz) δ –19.02 ppm; MS (ESI) *m/z* 428.1065(MNa^{+} [$C_{20}H_{24}NO_4PSNa^{+}$] = 428.1056).

$ClCH_2P(O)(C_6H_4$-*p*-Cl)$_2$ (5.16), $ClCH_2P(O)(C_6H_5)_2$ (5.17). Phosphine oxides **5.16** (Grandjean et al., 2005) and **5.17** (Nilsson et al., 2001) were prepared according to a previously published reports. **Spectral data.** Spectral data were as reported previously. **Spectral data.** Spectral data was the same as that reported previously (Nilsson et al., 2001; Grandjean et al., 2005).

$ClCH_2P(O)(C_6H_4$-*p*-OMe)$_2$ (5.18). Chloromethylphosphonic dichloride (20 g, 120 mmol) was dissolved in anyhdrous THF (120 mL). A solution of 4-methoxyphenylmagnesium bromide (0.5 M) in THF (480 mL, 240 mmol) was added dropwise over 1 h. The resulting mixture was stirred at reflux for 24 h. The reaction was then quenched by the addition of water (20 mL), and solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 and the resulting solution was washed once with water (50 mL) and once with brine (50 mL). The organic layer was dried over anhydrous $MgSO_4$(s) and filtered, and solvent was removed under reduced pressure. The

residue was purified by flash chromatography (silica gel, 3% v/v methanol in methylene chloride). Phosphine oxide **5.18** was isolated as a white solid in 63% yield. **Spectral data.** ^{1}H NMR (CDCl3, 400 MHz) δ 7.77–7.72 (m, 4H), 7.04–7.00 (m, 4H), 4.00 (d, J = 7.0 Hz, 2H), 3.88 (s, 6H) ppm; ^{13}C NMR (CDCl3, 125 MHz) δ 162.93, 133.54, 133.45, 121.46, 120.37, 114.37, 114.23, 55.36, 38.07 (d, J = 73.2 Hz) ppm; ^{31}P NMR (CDCl3, 161 MHz) δ 27.38 ppm; MS (ESI) m/z 310.0530 (MNa^+ [$C_{15}H_{16}ClO_3PNa^+$] = 310.0526).

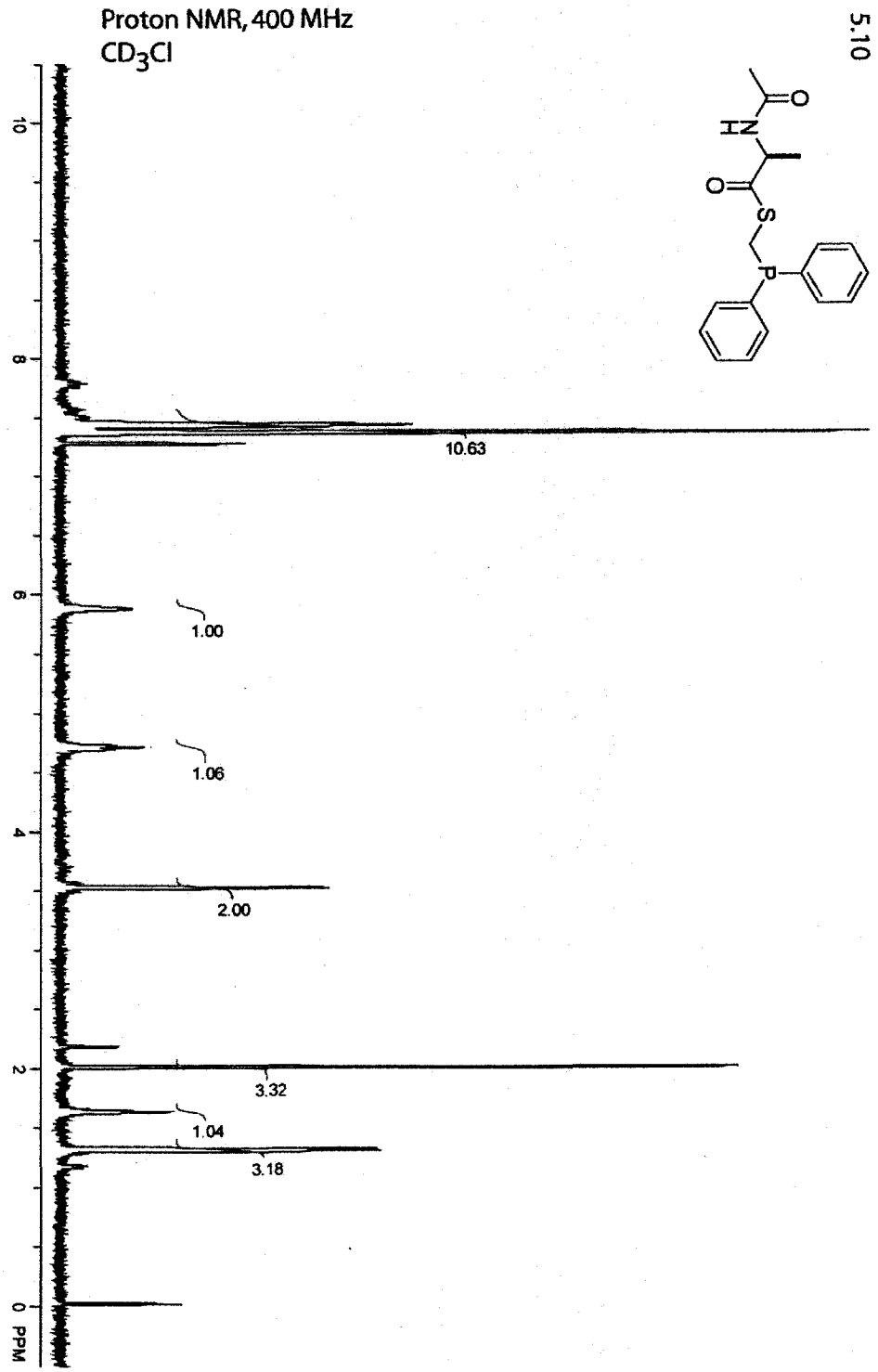
$AcSCH_2P(O)(C_6H_4$-*p*-$Cl)_2$ (5.19) and $AcSCH_2P(O)(C_6H_5)_2$ (5.20). Phosphine oxides **5.19** (Grandjean et al., 2005) and **5.20** (Nilsson et al., 2001) were prepared from phosphine oxides **5.16** and **5.17** according to previously published reports. **Spectral data.** Spectral data were as reported previously (Nilsson et al., 2001; Grandjean et al., 2005).

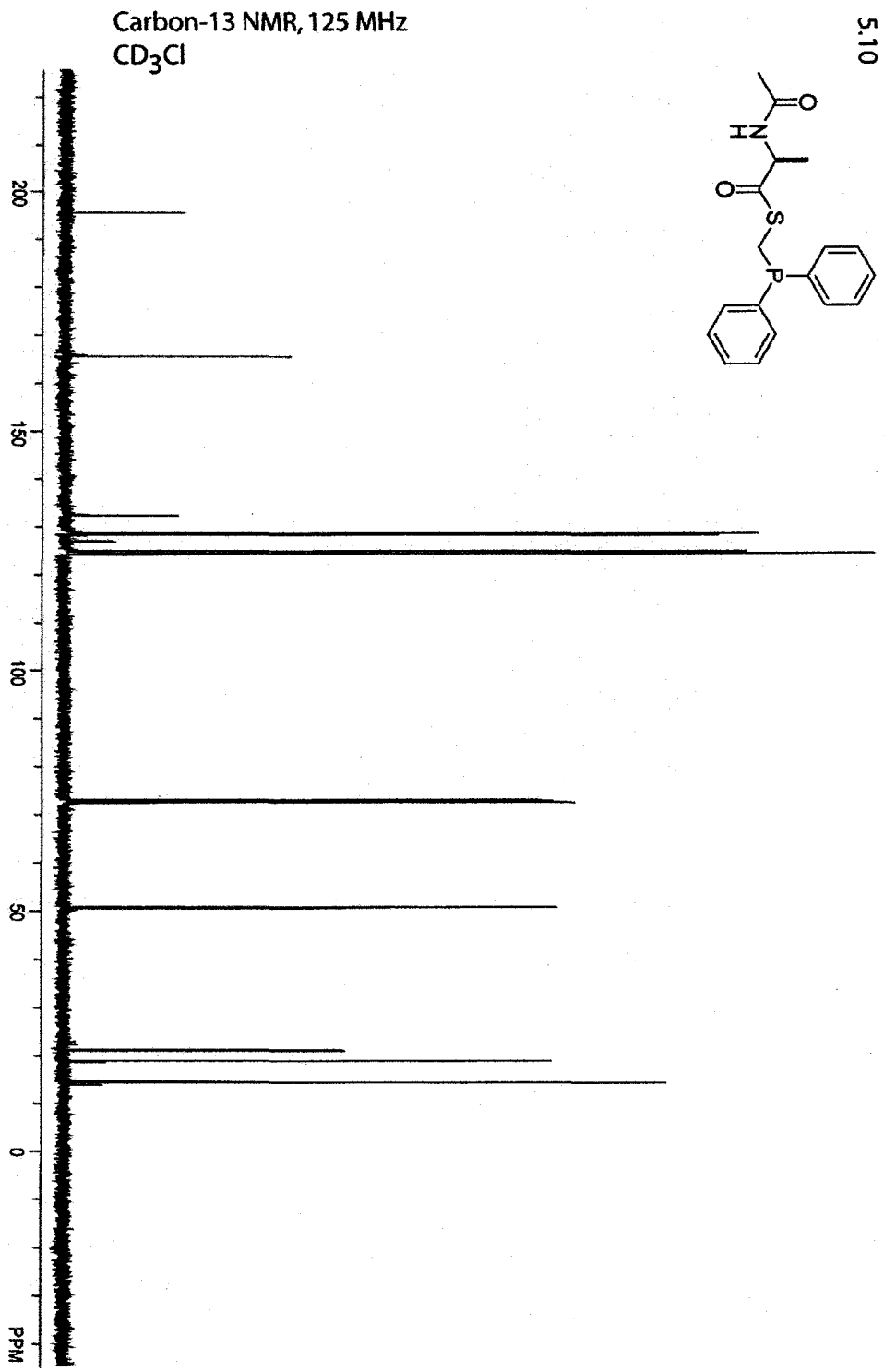
$AcSCH_2P(O)(C_6H_4$-*p*-$OMe)_2$ (5.21). Phosphine oxide **5.18** (5.8 g, 18.2 mmol) was dissolved in DMF (50 mL). Potassium thiolacetate (2.5 g, 21.8 mmol) was then added, and the reaction mixture was stirred under Ar(g) for 18 h. The solvent was removed under reduced pressure, and the resulting oil was purified by flash chromatography (3% v/v MeOH in CH_2Cl_2). Phosphine oxide **5.21** was isolated in 87 % yield. **Spectral data.** ^{1}H NMR (CDCl3, 400 MHz) δ 7.70-7.65 (m, 4H), 6.99-6.97 (m, 4H), 3.85 (s, 6H), 3.70 (d, J = 8.7 Hz, 2H), 2.28 (s, 3H) ppm; ^{13}C NMR (CDCl3, 125 MHz) δ 162.90, 133.20, 123.41, 123.32, 114.41, 55.59, 30.35, 28.00 (d, J = 70.6 Hz) ppm; ^{31}P NMR (CDCl3, 161 MHz) δ 29.19 ppm; MS (ESI) m/z 373.0627 (MNa^+ [$C_{17}H_{19}O_4PSNa^+$] = 373.0634).

$AcSCH_2P(C_6H_4$-*p*-$Cl)_2$ (5.22) and $AcSCH_2P(C_6H_5)_2$ (5.23). Phosphines **5.22** (Grandjean et al., 2005) and **5.23** (Nilsson et al., 2001) were prepared from phosphine oxides **5.19** and **5.20** according to previously published reports. **Spectral data.** Spectral

data were as reported previously (Nilsson et al., 2001; Grandjean et al., 2005).

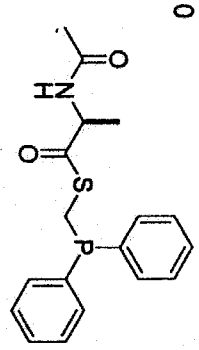
$AcSCH_2P(C_6H_4$-*p*-$OMe)_2$ (5.24). Phosphine oxide **5.21** (1.06 g, 2.95 mmol) was dissolved in anhydrous chloroform (10 mL). To this solution was added trichlorosilane (8 mL, 79 mmol), and the resulting solution was stirred under Ar(g) for 72 h. Solvent was removed under reduced pressure. (CAUTION: Excess trichlorosilane in the removed solvent was quenched by the slow addition of saturated sodium bicarbonate in a well-ventilated hood.) The residue was purified by flash chromatography (silica gel, 3% v/v methanol in methylene chloride). Phosphine **5.24** was isolated as a white solid in 92% yield. **Spectral data.** ^{1}H NMR (CDCl3, 400 MHz) δ 7.38–7.36 (m, 4H), 6.93–6.89 (m, 4H), 3.85 (s, 6 H), 3.47 (bs, 2H), 2.32 (s, 3H) ppm; ^{13}C NMR (CDCl3, 125 MHz) δ 194.38, 160.46, 134.25, 134.04, 127.91, 127.79, 114.24, 114.18, 55.20, 30.34, 26.43 (d, J = 20.4 Hz) ppm; ^{31}P NMR (CDCl3, 161 MHz) δ –19.71 ppm; MS (ESI) *m/z* 357.0679 (MNa^+ [$C_{17}H_{19}O_3PSNa^+$] = 357.0685).

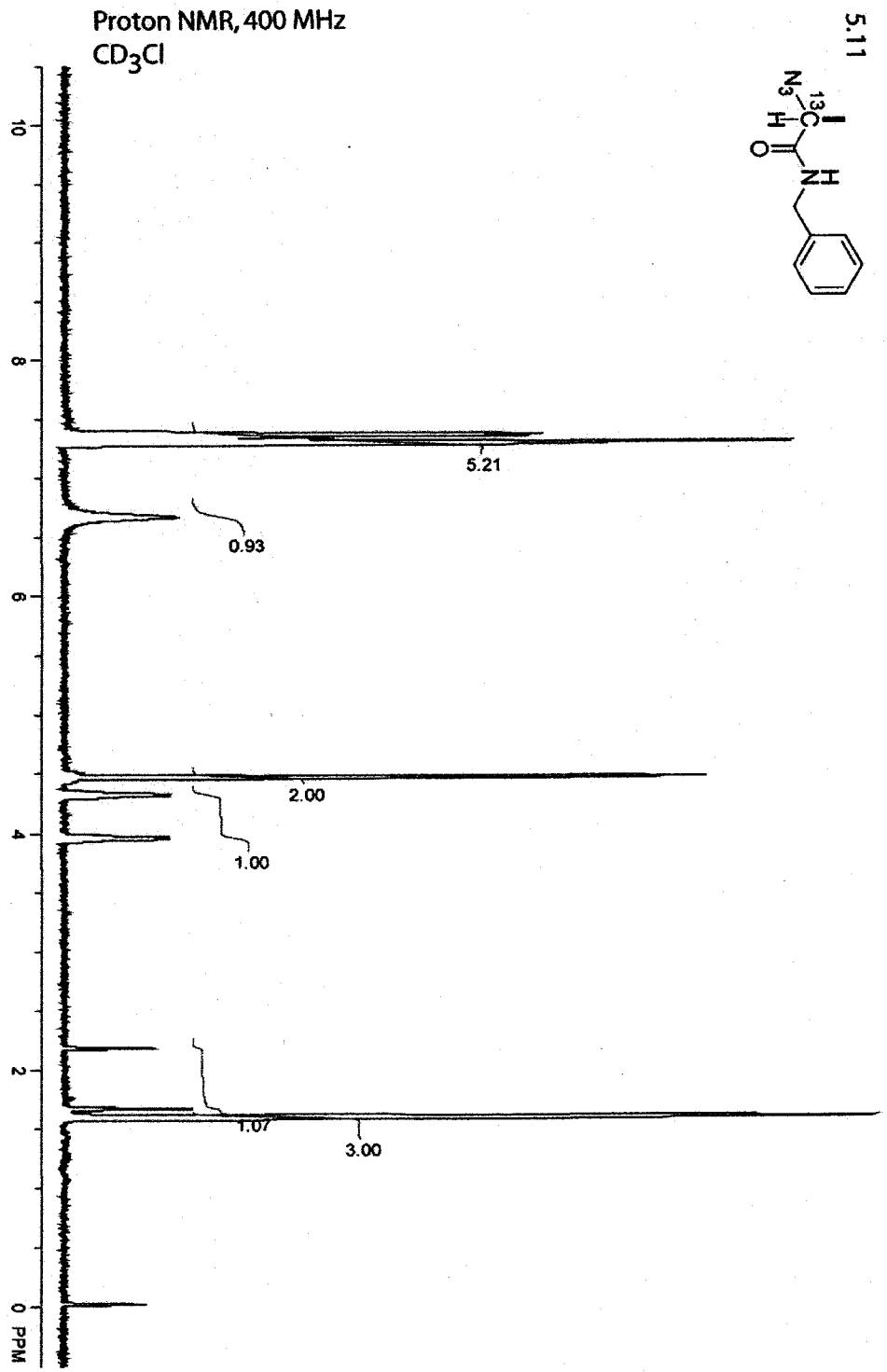


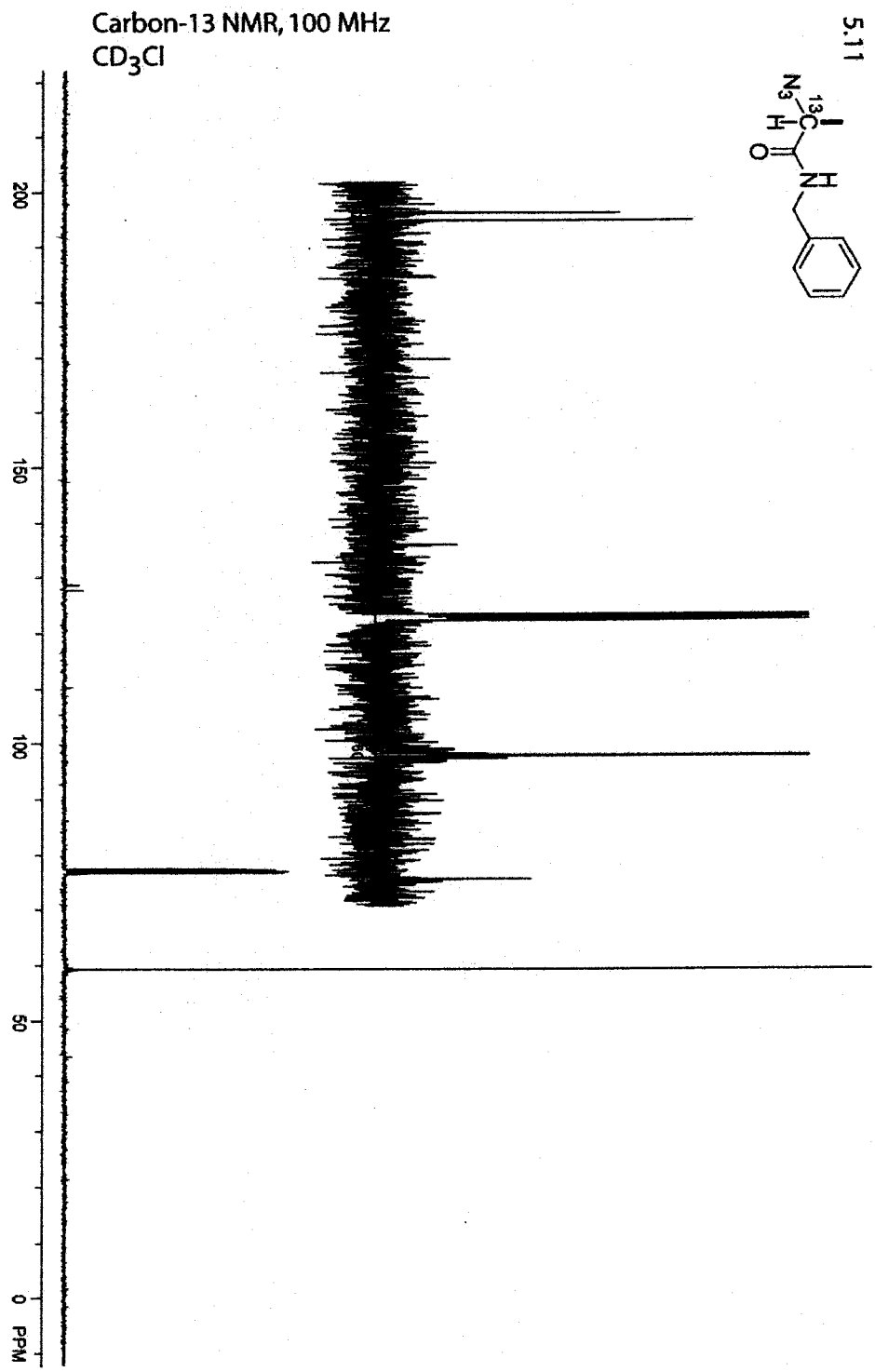


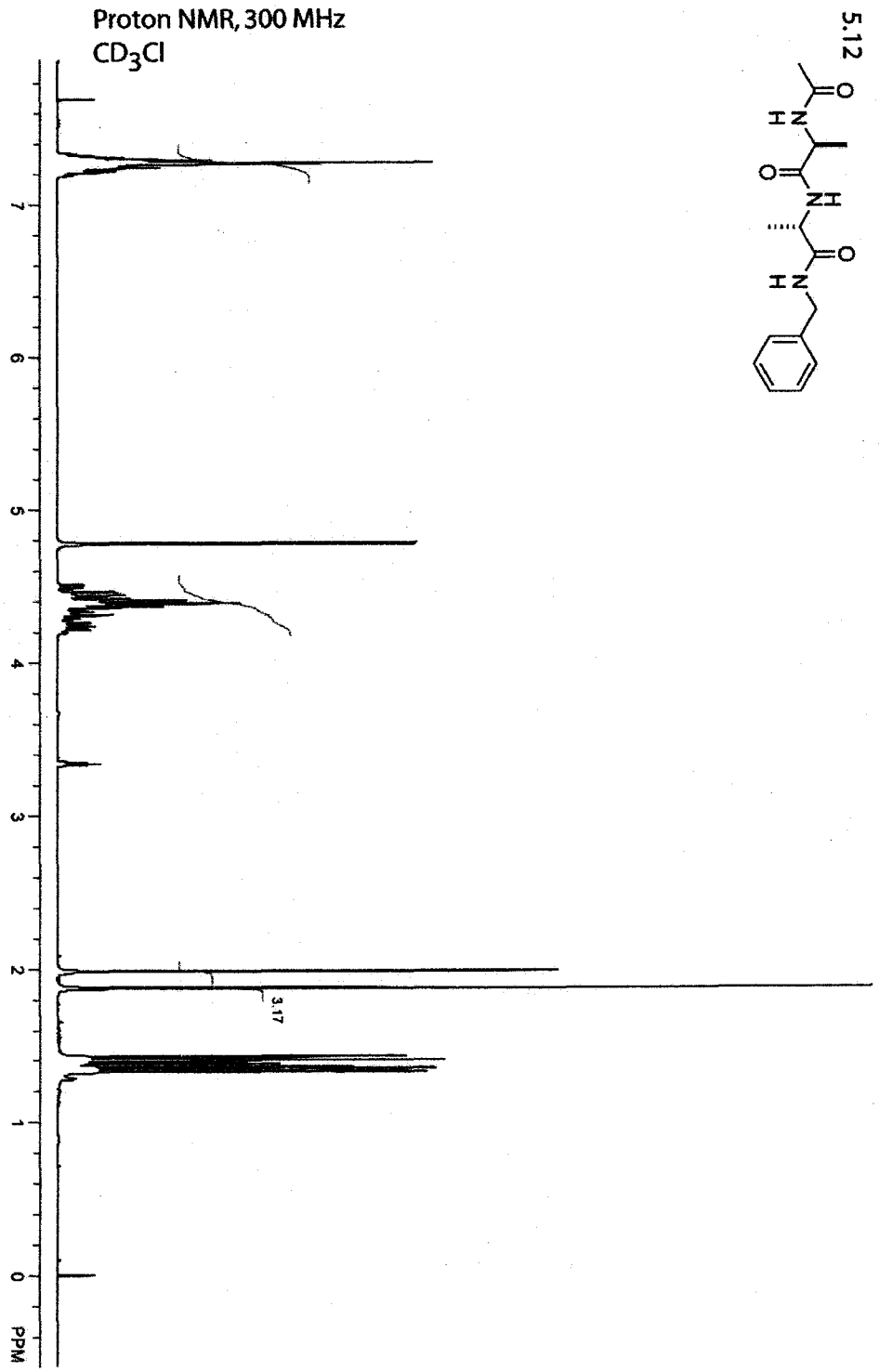
Phosphorus-31 NMR, 201 MHz
CD_3Cl

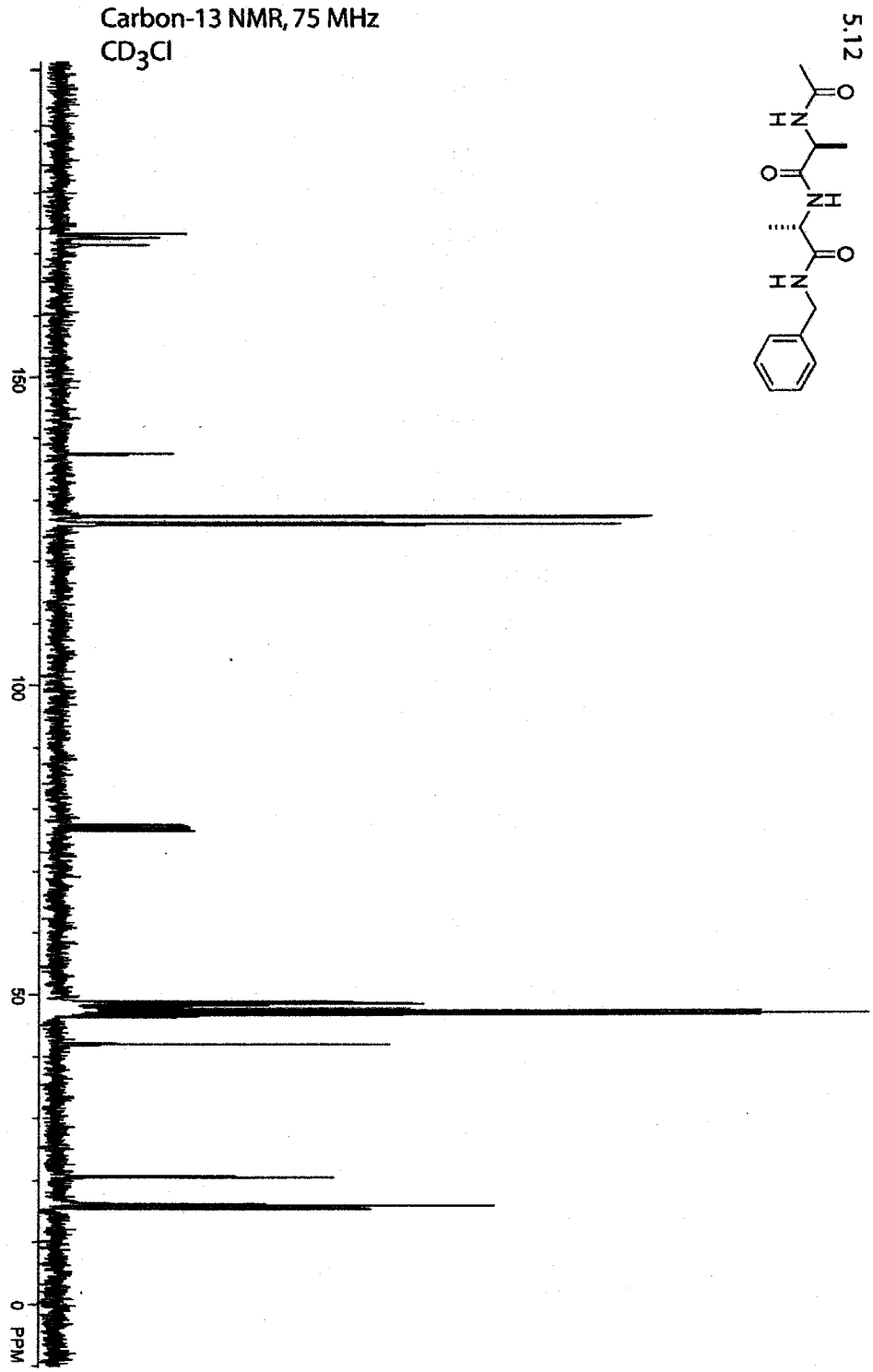
5.10

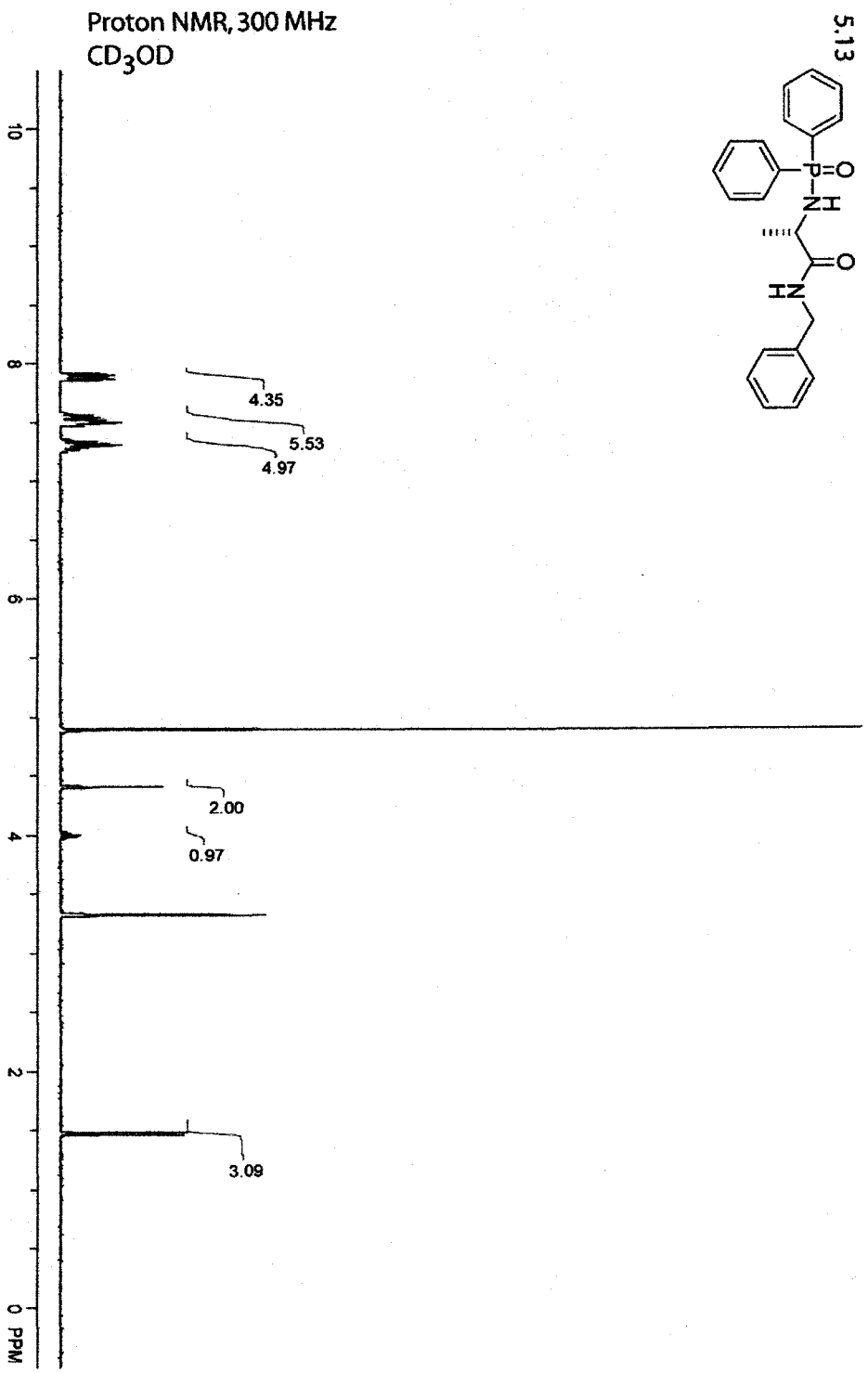


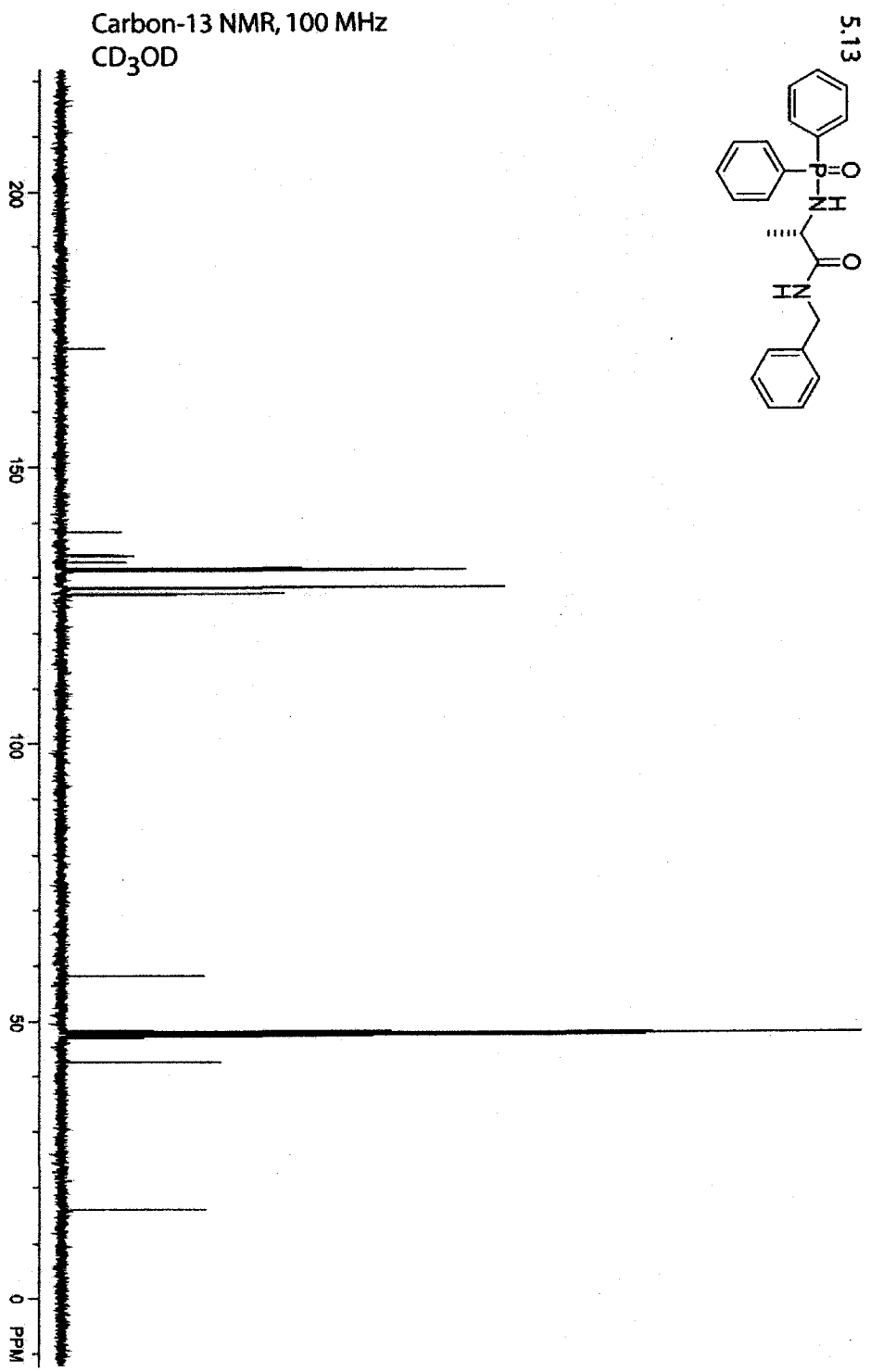




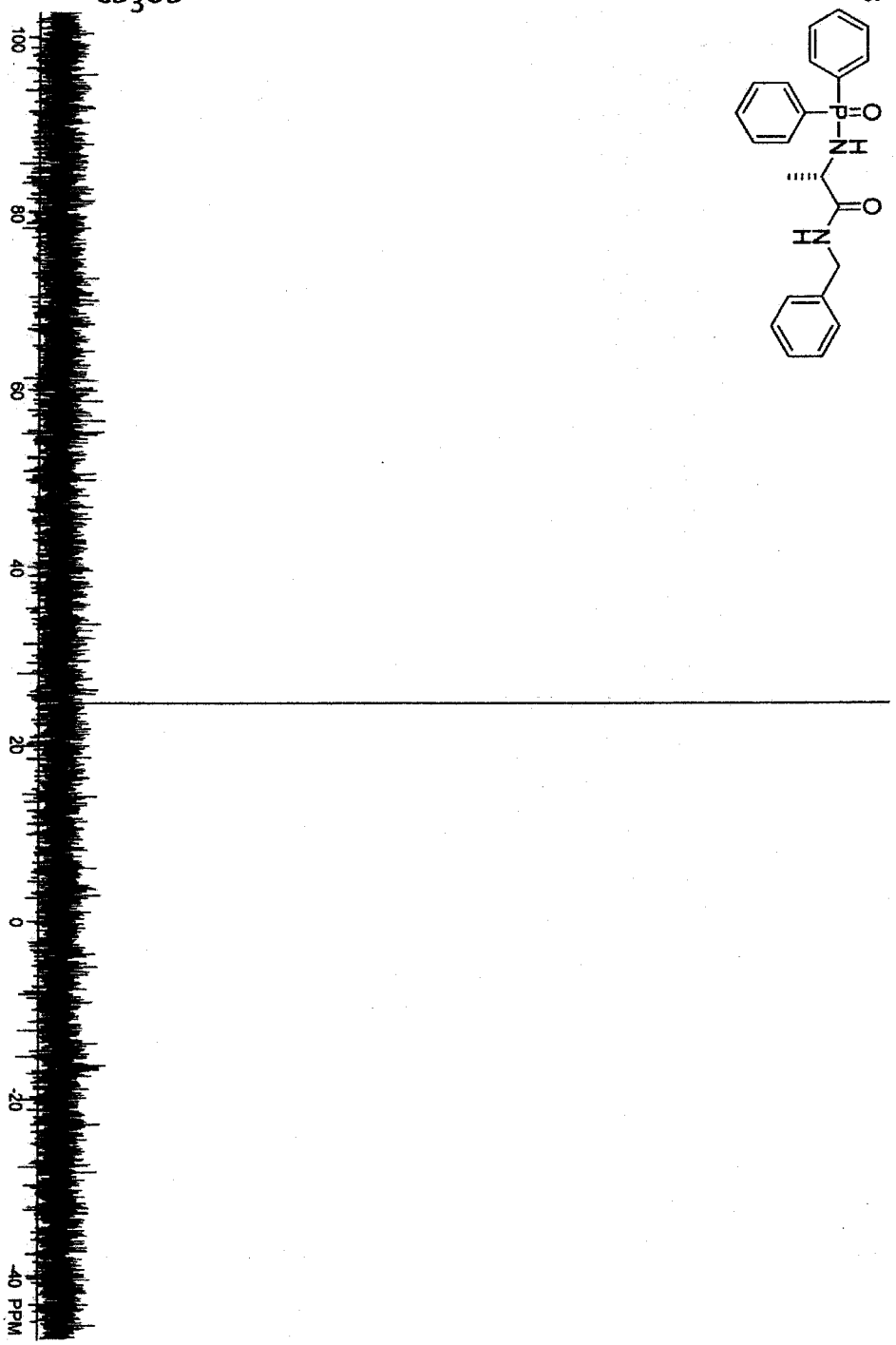








Phosphorus-31 NMR, 161 MHz
CD_3OD



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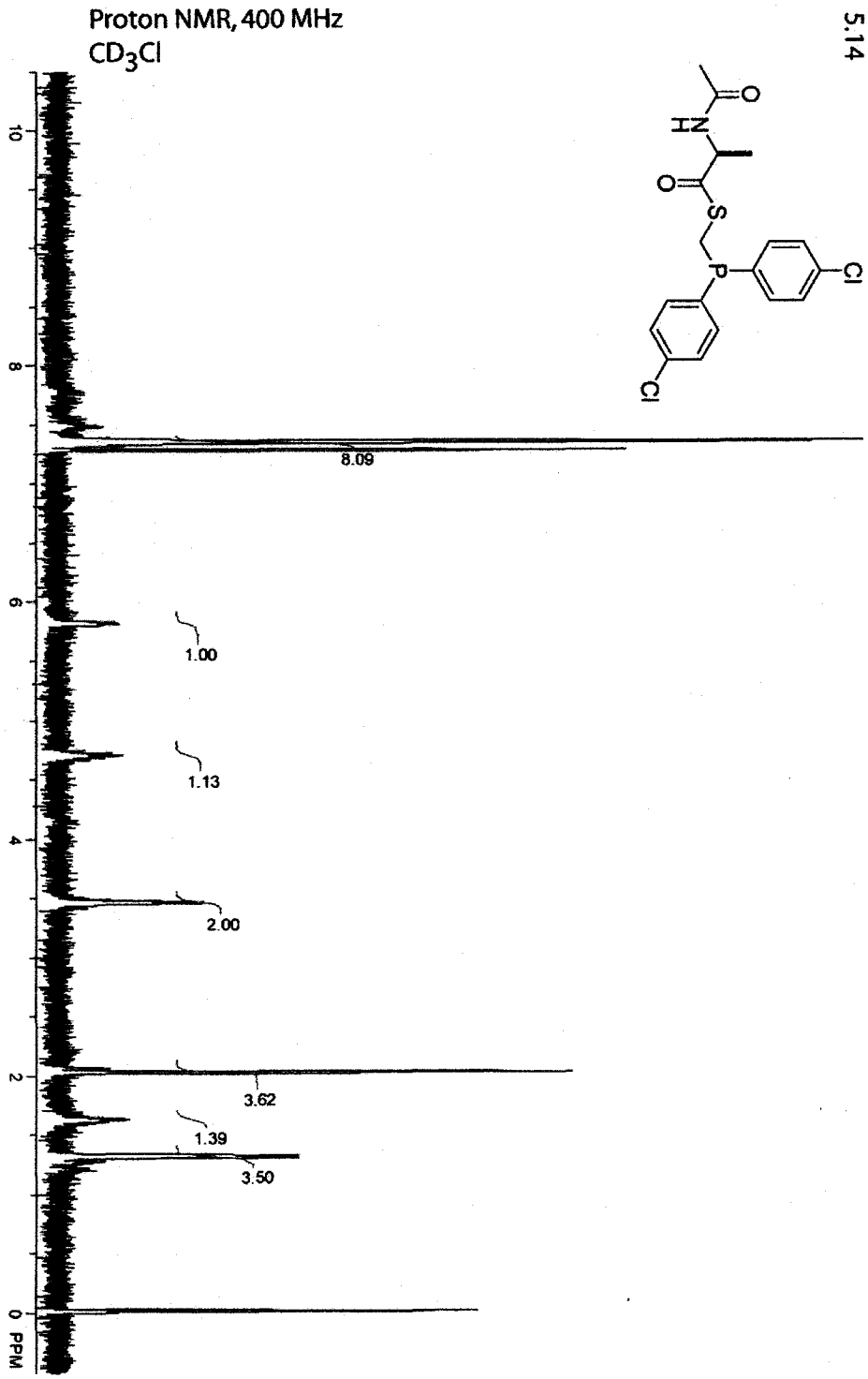
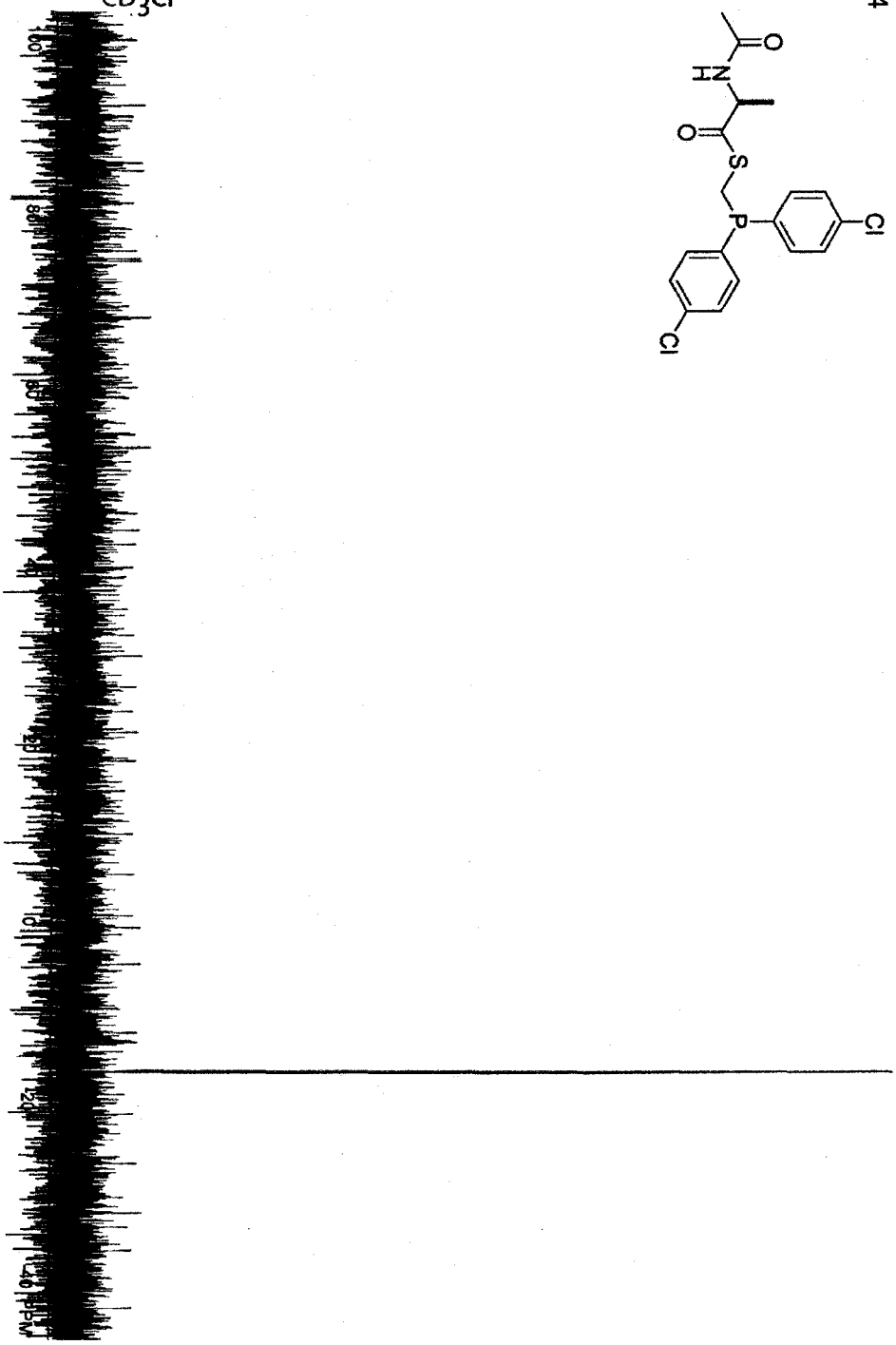
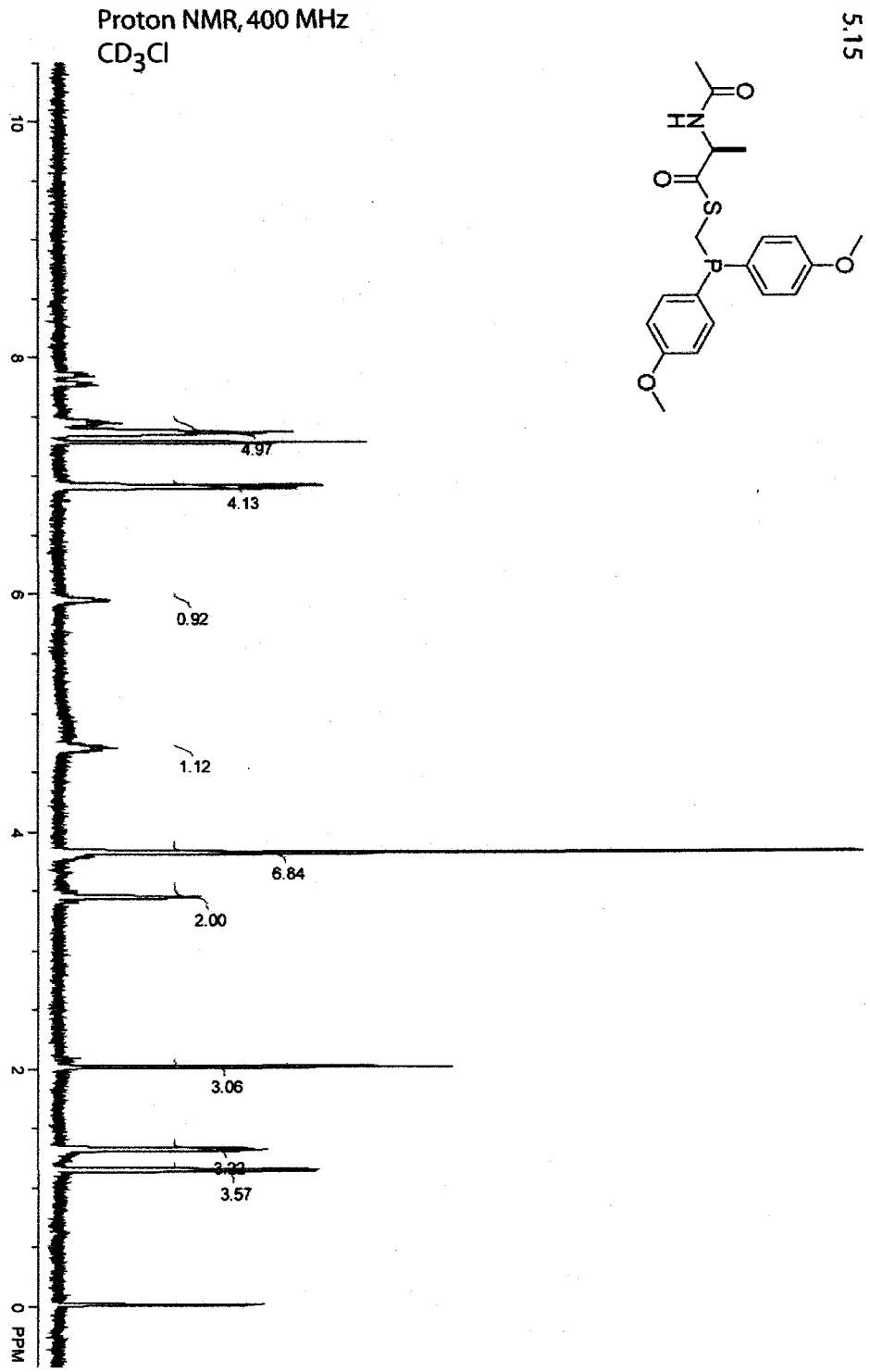
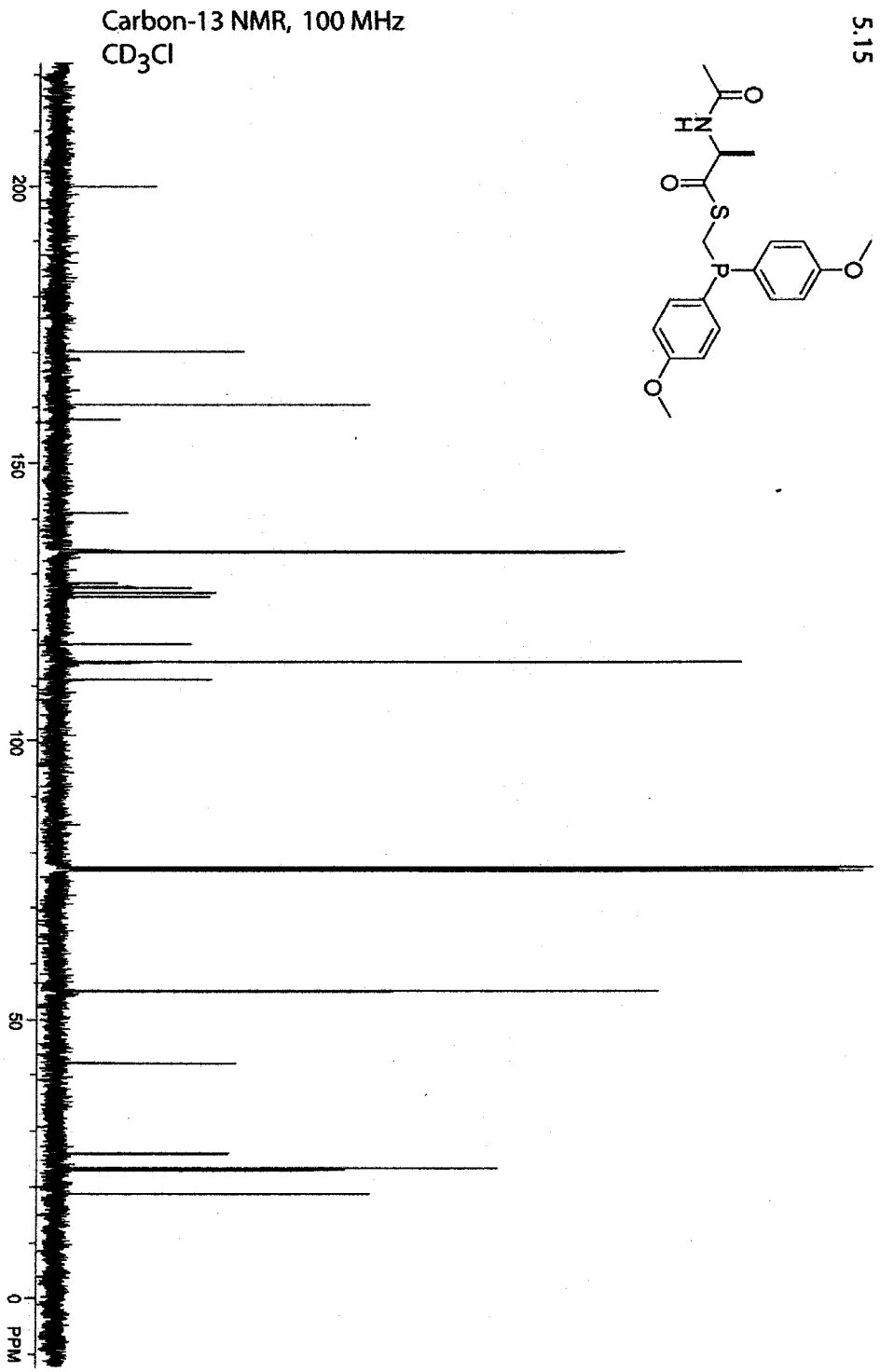


Phosphorus NMR, 161 MHz
CD_3Cl

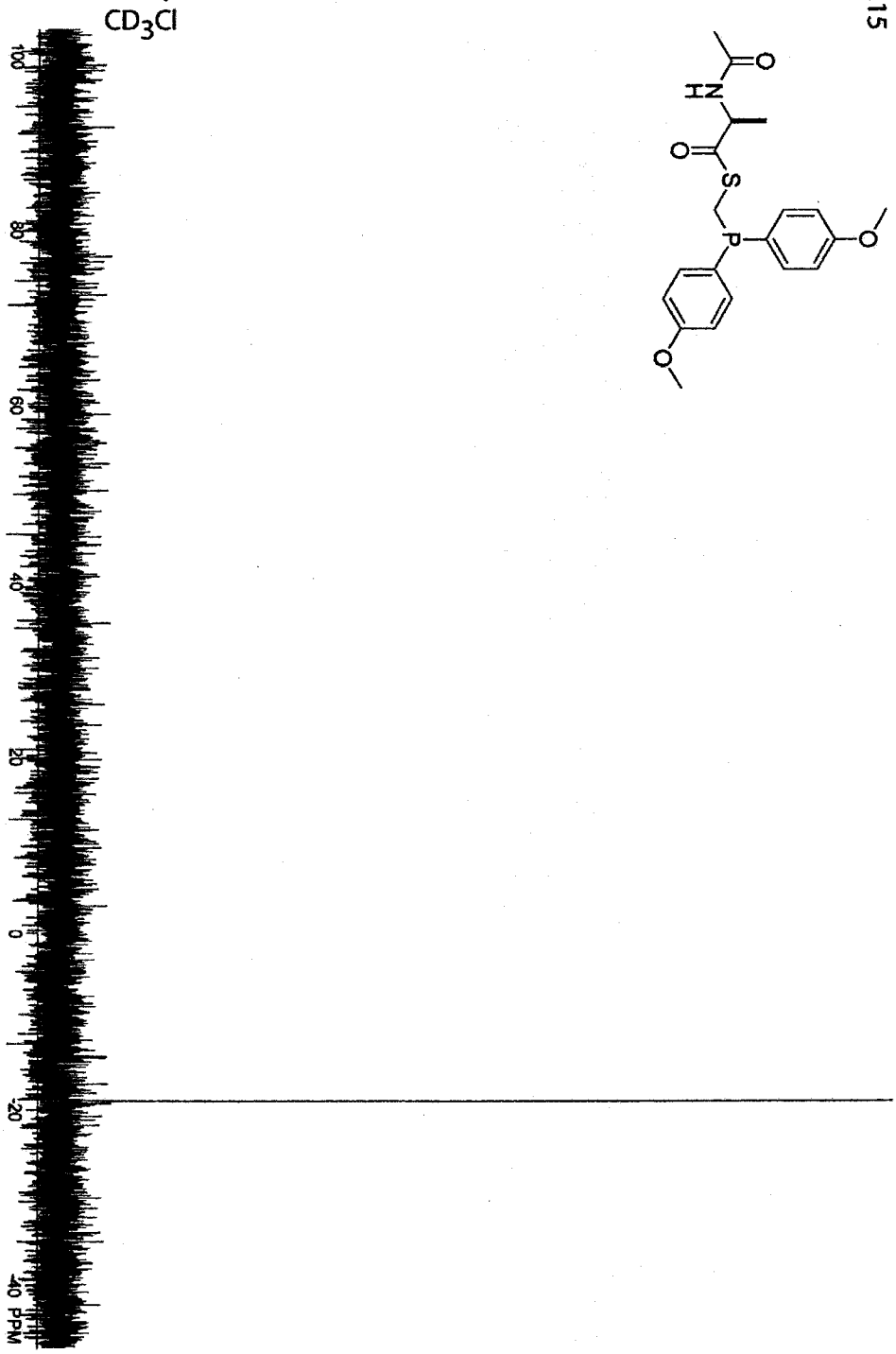


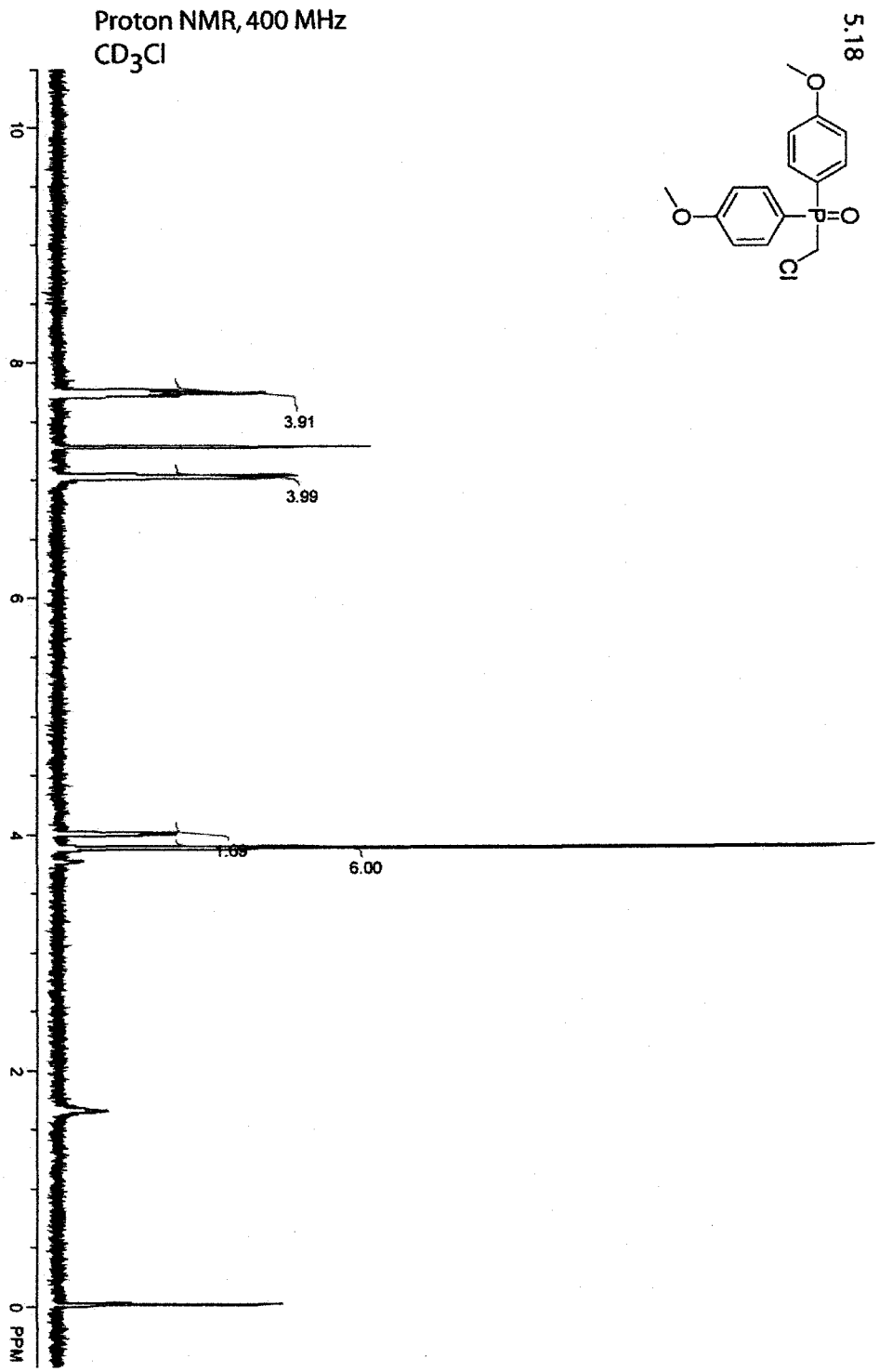


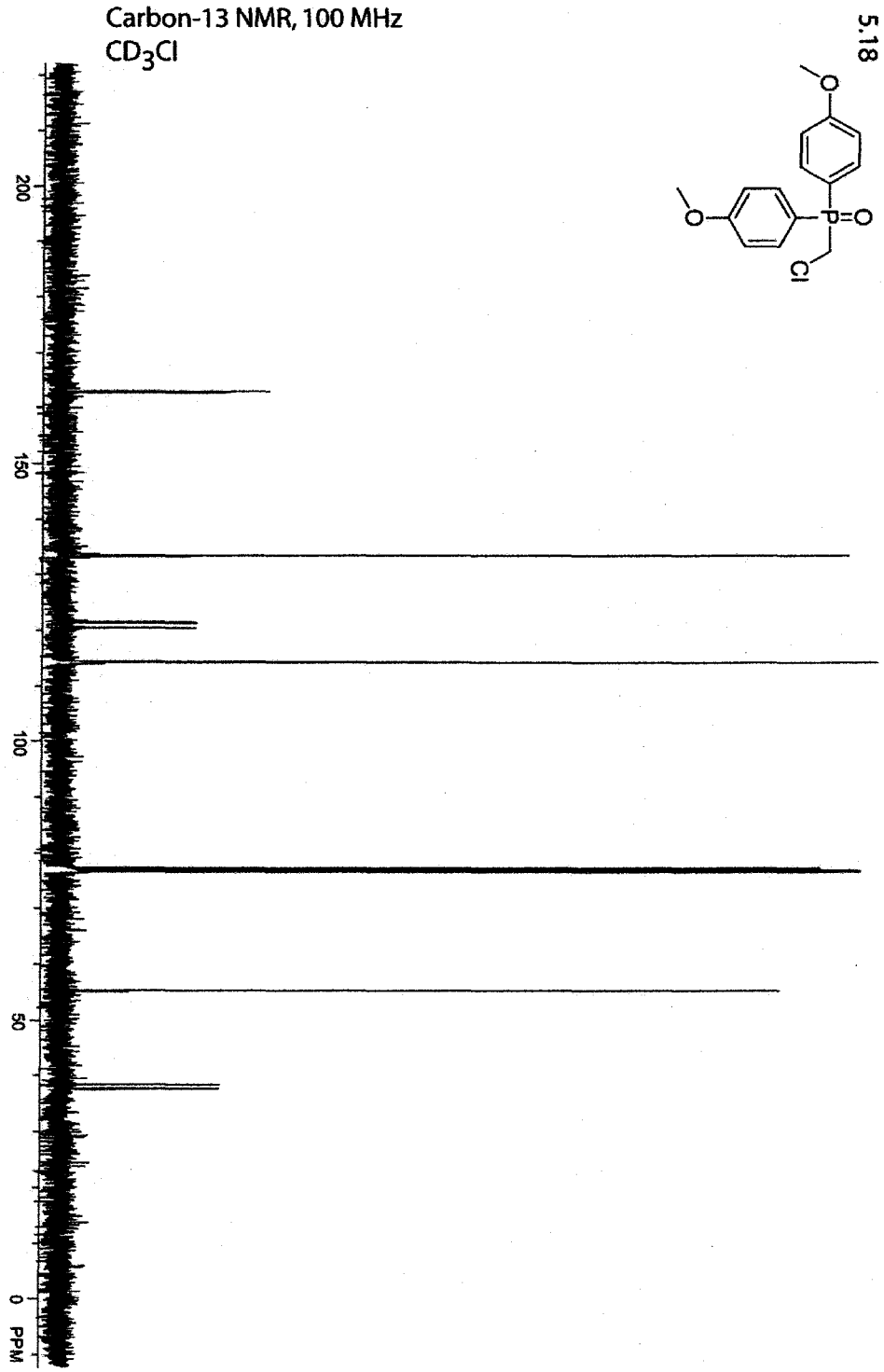


Phosphorus-31 NMR, 161 MHz
CD_3Cl

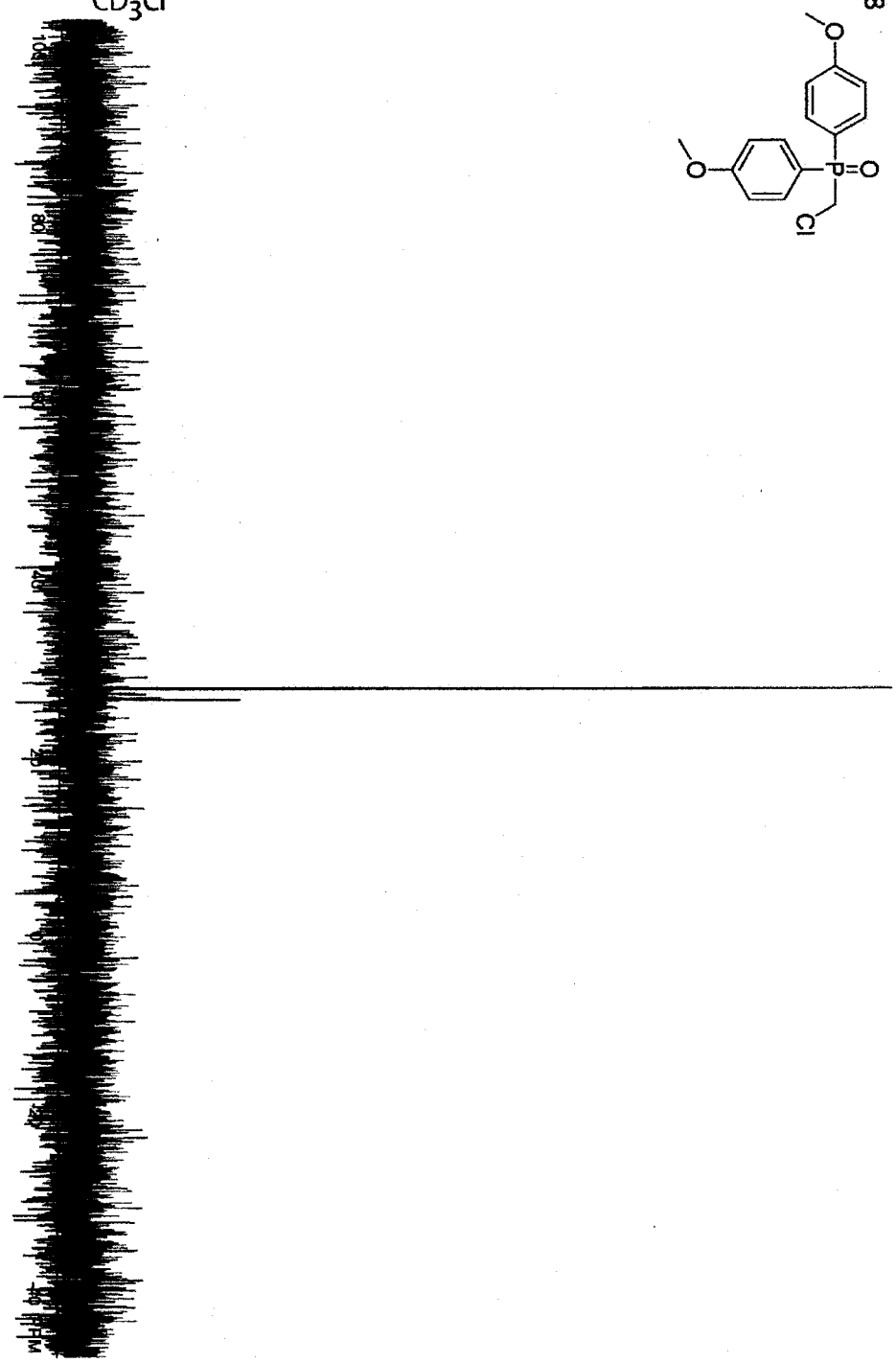
5.15







Phosphorus-31 NMR, 161 MHz
CD_3Cl



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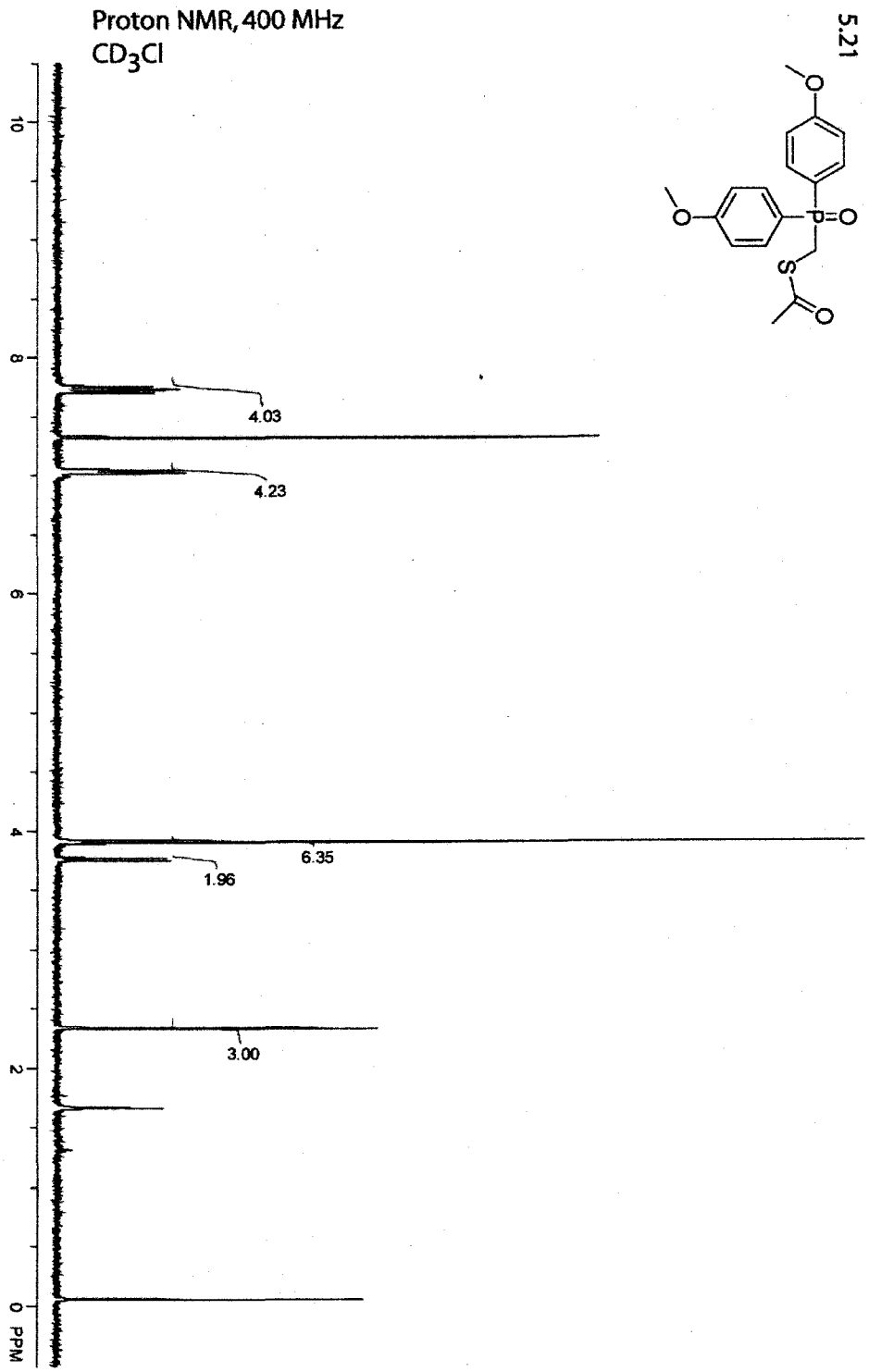

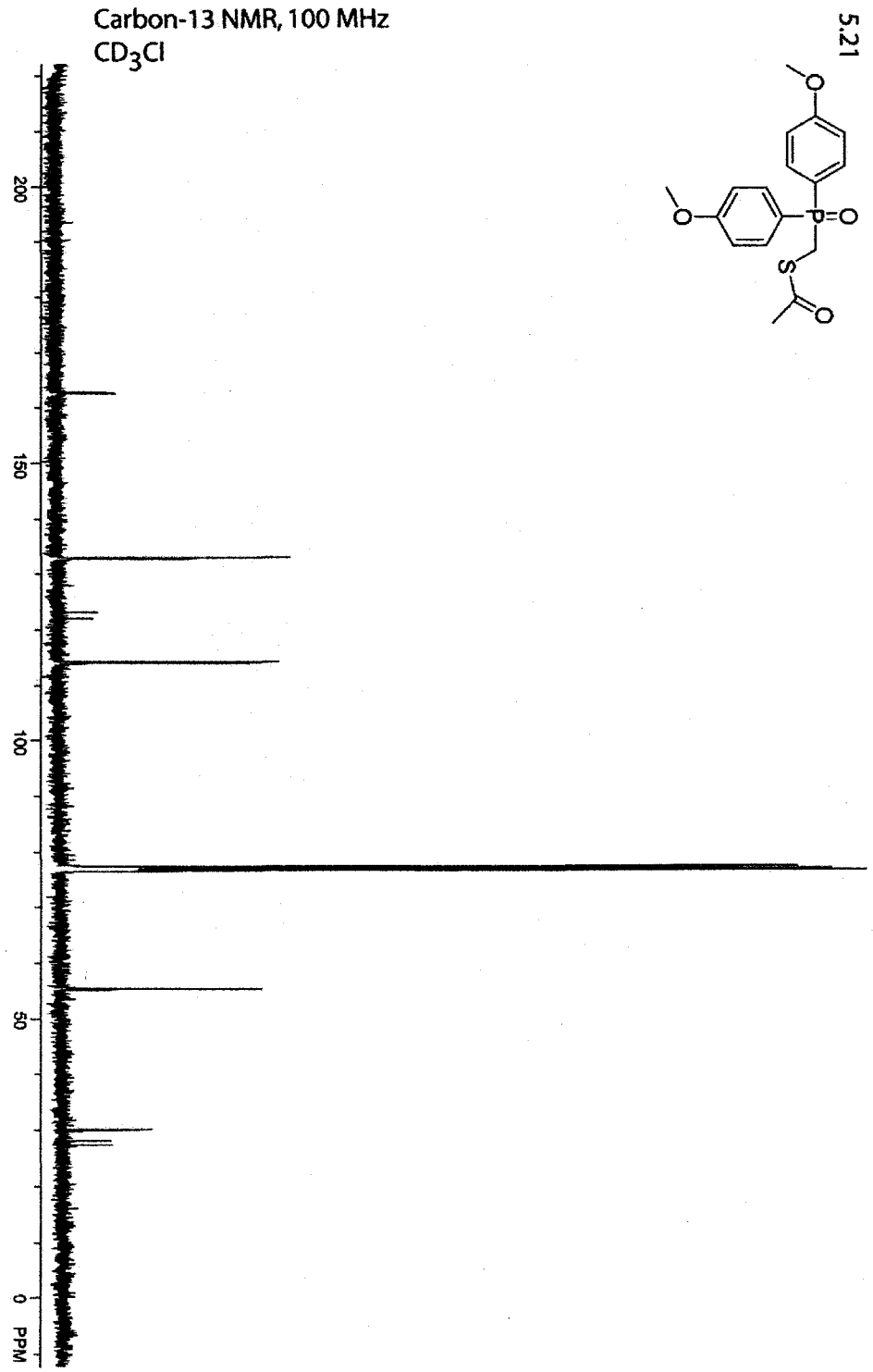

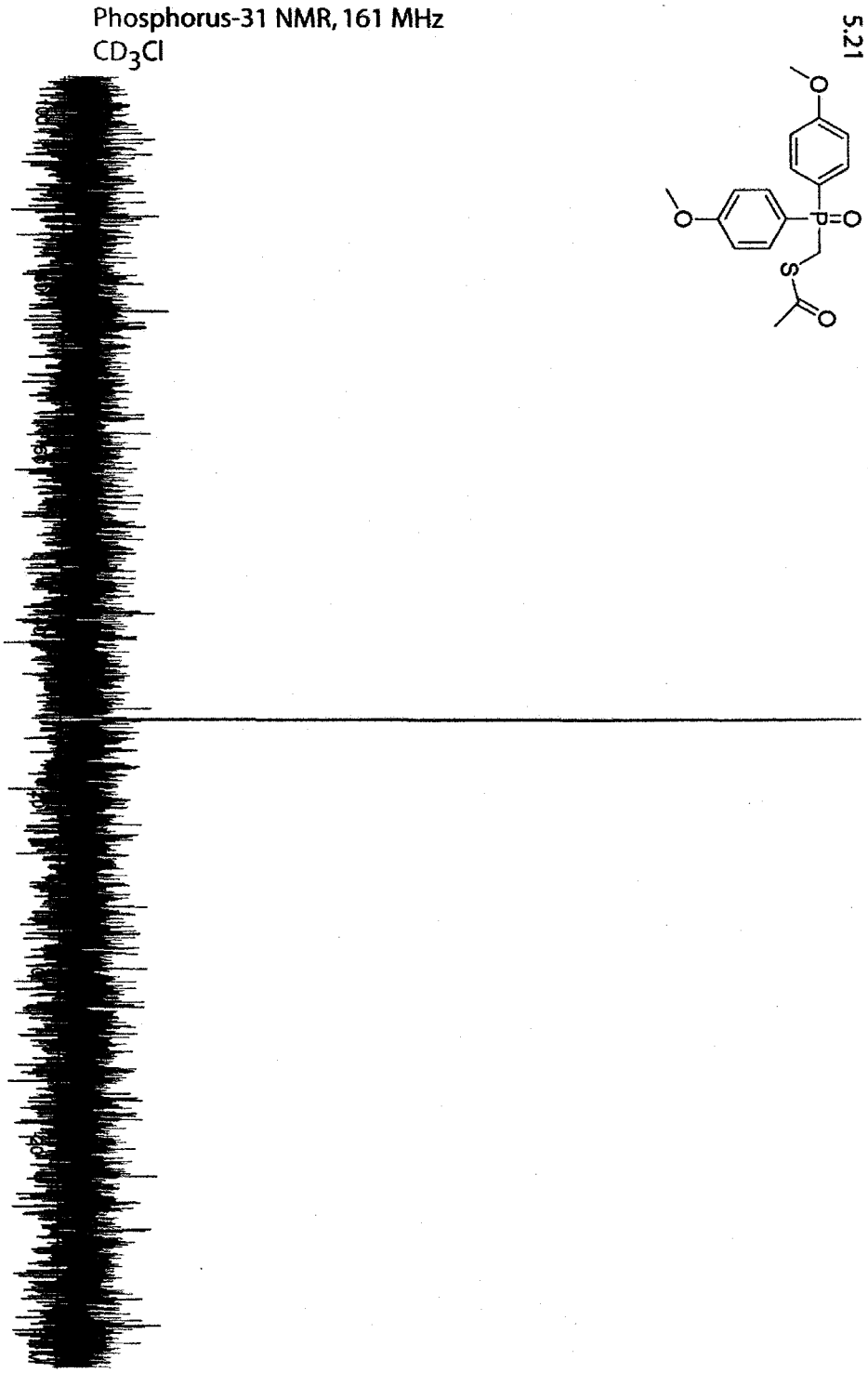

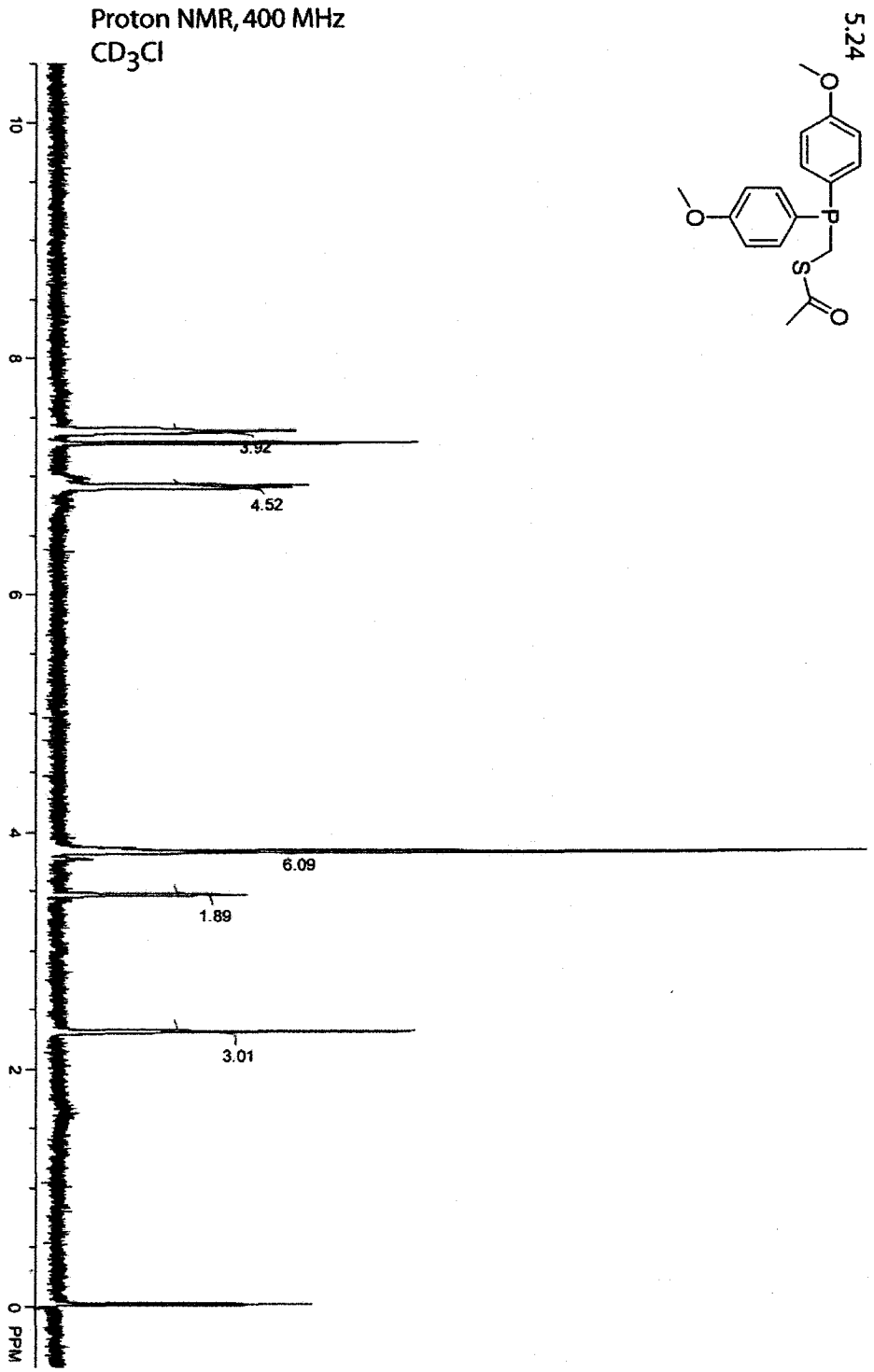

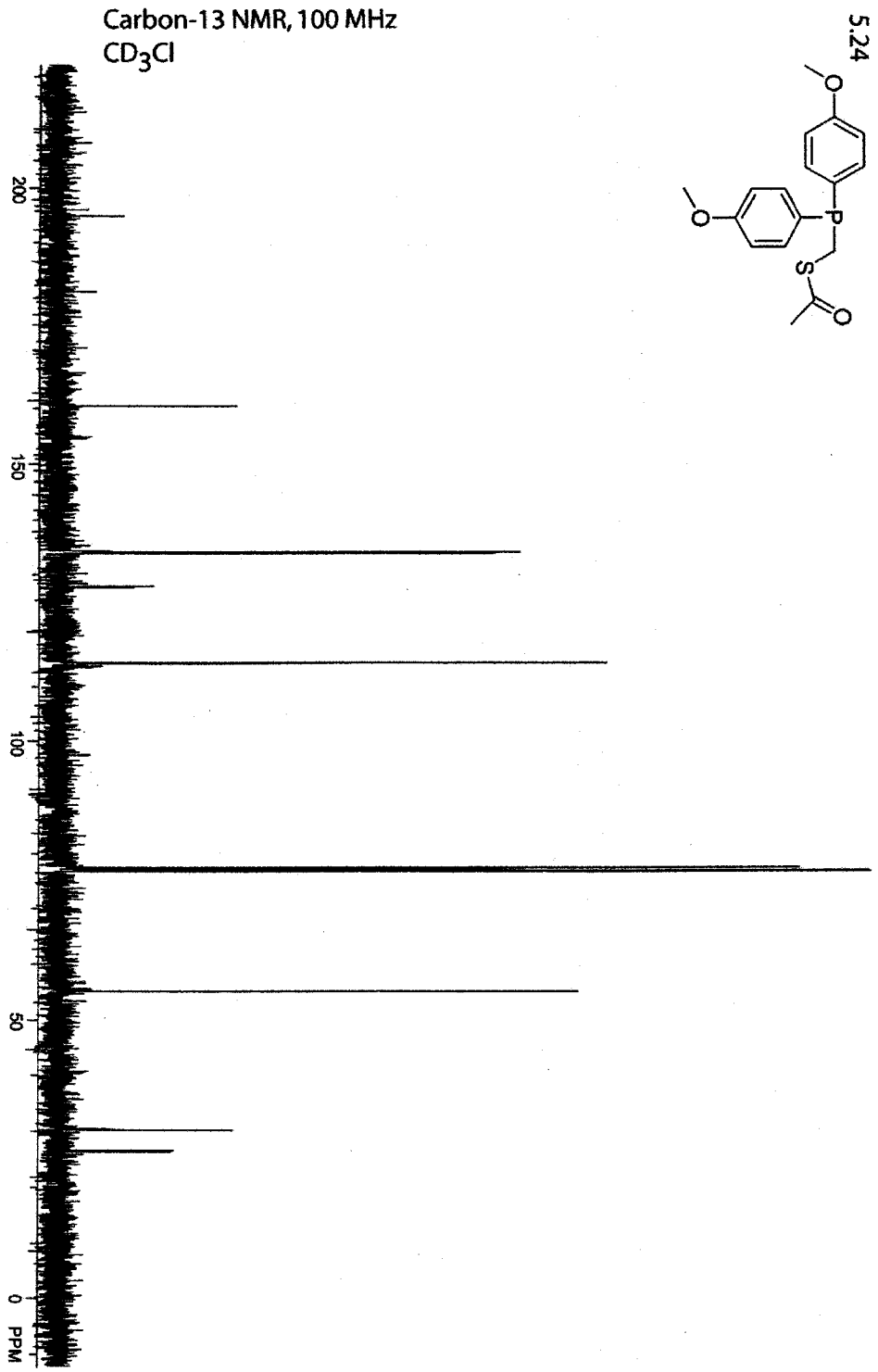

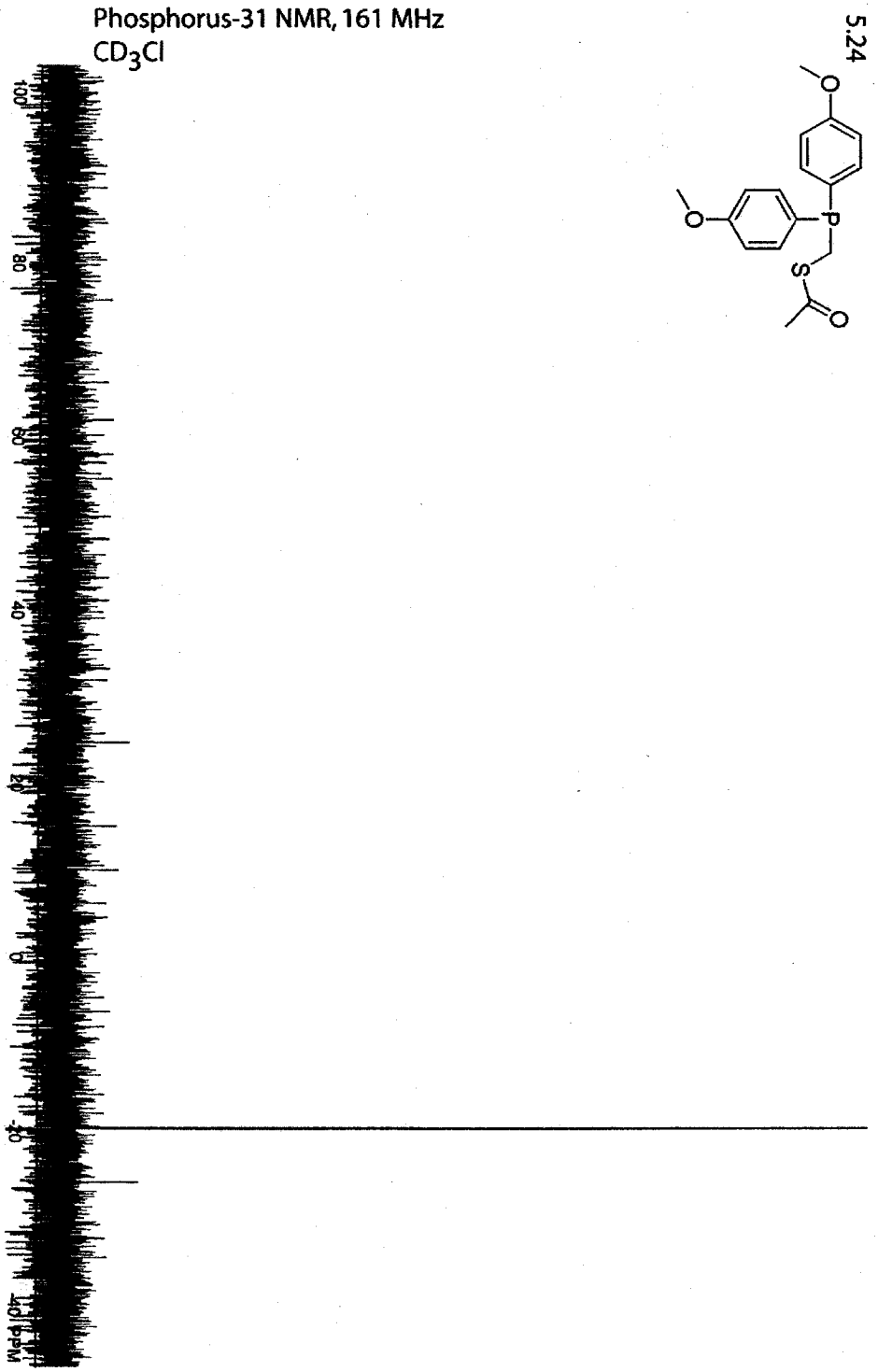

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