2011

# Synthesis and Characterization o f Nucleoside Analogues and their Incorporation into PNA Monomers and Oligopeptides

Andre Henri St. Amant

# Synthesis and Characterization of Nucleoside Analogues and their Incorporation into PNA Monomers and Oligopeptides

(Spine Title: Synthesis of Novel PNA Monomers)
(Thesis Format: Monograph)

by

André H. St. Amant

Graduate Program in Chemistry

2

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

THE UNIVERSITY OF WESTERN ONTARIO
SCHOOL OF GRADUATE AND POSTDOCTORAL STUDIES

**CERTIFICATE OF EXAMINATION**

| *Supervisor* | *Examiners* |
|---|---|
| *Dr. Robert H. E. Hudson* | *Dr. Leonard Luyt* |
| *Supervisory Committee* | *Dr. James Wisner* |
| *Dr. James Wisner* | *Dr. Jin Zhang* |
| *Dr. Beth Gillies* | |

The thesis by

**André Henri St. Amant**

entitled:

# Synthesis and Characterization of Nucleoside Analogues and their Incorporation into PNA Monomers and Oligopeptides

is accepted in partial fulfillment of the
requirements for the degree of
**Master of Science**

Date_____

_____
Chair of the Thesis Examination Board

**Abstract**

The synthesis of peptide nucleic acid (PNA) is of interest for their binding to nucleic acids and their potential for gene-based diagnostics and therapy. This thesis describes the synthesis of Fmoc/Boc protected PNA monomers for thymine (T), 2,6-diaminopurine (DAP), and 2-aminopurine (2AP). The oligomerization and selectivity over mismatches in DNA were studied with 2AP showing a higher selectivity for T over DAP (11.5 °C vs 6 °C). Inclusion of 2AP allowed fluorometric monitoring of the denaturation of PNA:DNA hybrid duplexes.

The synthesis of an azide-containing PNA monomer was completed and successfully incorporated into PNA. A solid phase copper azide-alkyne cycloaddition was performed in quantitative yield. The synthesis of alkynes to be incorporated into the PNA is described. The syntheses of the monomers described will make unusual PNAs more accessible, allowing the study of their unique properties.

**Keywords**

Peptide Nucleic Acid, Thymine, 2,6-Diaminopurine, 2-Aminopurine, Azide, Copper Azide-Alkyne Cycloaddition, Solid Phase Click Reaction

**Acknowledgments**

I'd like to thank the Hudson lab members past and present for teaching me the ways of small molecule synthesis, analytical techniques, and working with PNA. I'd like to specifically thank Dr. Fil Wojciechowski, Dr. David Dodd and Dr. Mojmir Suchy for putting up with my endless string of questions.

Next I'd like to thank my friends. You guys have always been there for me and I'm lucky to have such good friends for life. No matter what happens, being able to spend time with you all has made everything worthwhile.

Dr. Hudson is next. You've been the best supervisor I could imagine. You've taught me so much, but you've also created a lab atmosphere that encourages learning and personal responsibility. Thank you for the opportunity to work in your lab and all of the help you've given me along the way.

Lastly and most importantly I'd like to thank my family. You have all been supportive of my academic choices. Back in the day when you guys bought me Yes Mag, when you bribed me to upgrade to enriched science classes with a CD player, and the years of New Scientist/Discover magazine you've provided for me. Whenever I've needed anything you guys have been there, and you continue to be supportive in whatever I end up doing after this degree. Thank you for providing me an amazing childhood, I wouldn't have changed a single thing in my life.

## Table of Contents

## List of Schemes

## List of Figures

## List of Abbreviations

2AP = 2-aminopurine

A = adenine

B = any nucleobase

Bhoc = (diphenylmethoxy)carbonyl

Bn = benzyl

Boc = tert-butyloxycarbonyl

Bz = benzoyl

C = cytosine

Cbz = carboxybenzyl

DAP = 2,6-diaminopurine

DCC = *N*,*N*'-dicyclohexylcarbodiimide

DCM = dichloromethane

DIPEA = *N*,*N*-diisopropylethylamine

DMAP = 4-dimethylaminopyridine

DMF = dimethylformamide

DMSO = dimethylsulfoxide

DNA = deoxyribonucleic acid

EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

EDTA = ethylenediaminetetraacetic acid

FCC = flash column chromatography

Fmoc = fluorenylmethyloxycarbonyl

G = guanine

HOBt = hydroxybenzotriazole

M = any metal

MeCN = acetonitrile

Pip = piperidine

PNA = peptide nucleic acid

Py = pyridine

RNA = ribonucleic acid

SPPS = solid phase peptide synthesis

T = thymine

THF = tetrahydrofuran

TLC = thin layer chromatography

TMS = trimethylsilyl

Tol = toluene

U = uracil

# Chapter 1

## Introduction

### 1.1 Nucleic Acid Chemistry

Nucleic acids contain all of the information needed for life to function and propagate. The information flow in the cell is represented by the central dogma of biology; DNA is transcribed into RNA, which is translated into proteins (**Fig. 1.1**.)

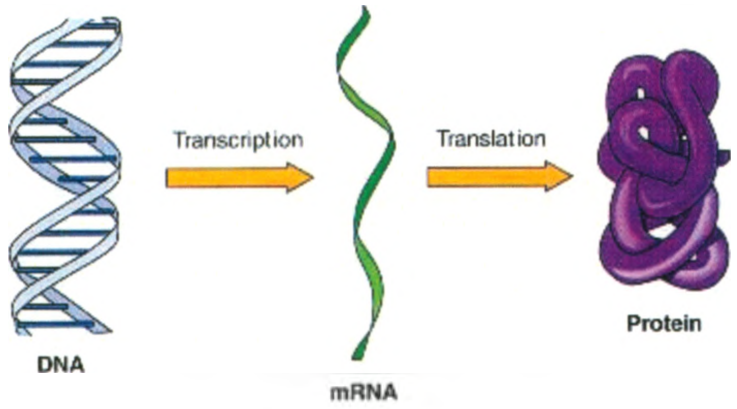



**Fig. 1.1** The central dogma of biology: information flow in biological systems.

The information in each step is amplified many times, making the integrity of the DNA so important to cells that they have evolved repair pathways. The cause of many diseases can be traced to mutations in the DNA, causing ineffective or detrimental proteins to be synthesized. Recently, nucleic acid chemistry has focused on developing technologies for the detection of disease, combined with treatment which can avert or delay disease symptoms.[1] Recent research in the field has focused on pro-active gene therapy, where an aberrant gene can be removed or repaired. Because natural nucleic acids are quickly broken down in cells and have poor pharmacodynamics, research has focused on the synthesis of nucleic acid analogues.[2]

### 1.2 Peptide Nucleic Acid Protecting Groups

Peptide nucleic acid (PNA) is a synthetic nucleic acid analogue of interest for diagnostic and therapeutic uses.[3] PNA is an oligomer consisting of repeating N-(2-aminoethyl)-glycine

units (**Fig. 1.2.**) PNA is of interest because: it can form proper base pairs to a complementary DNA or RNA sequence with good selectivity, it can bind tighter than natural nucleic acids because of its neutral backbone, and it is not degraded by cellular DNases, RNases, or proteases.[4]

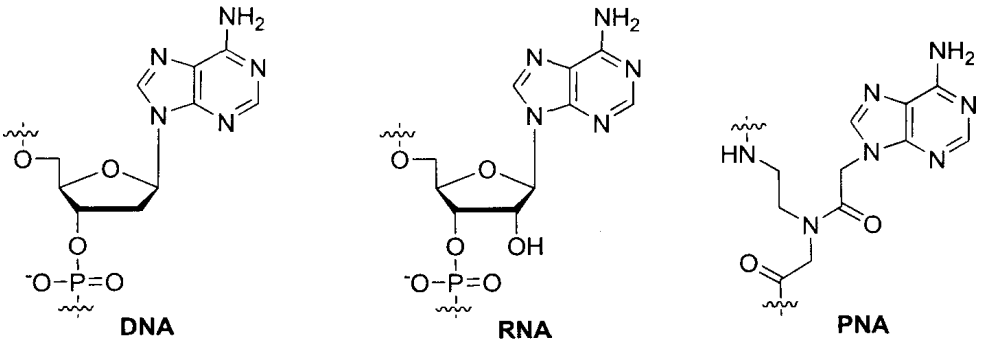



**Fig. 1.2** A comparison of DNA, RNA, and PNA units.

The synthesis of PNA is done through solid phase peptide synthesis (SPPS),[5] a technique used to synthesize short peptides (**Scheme 1.3**).

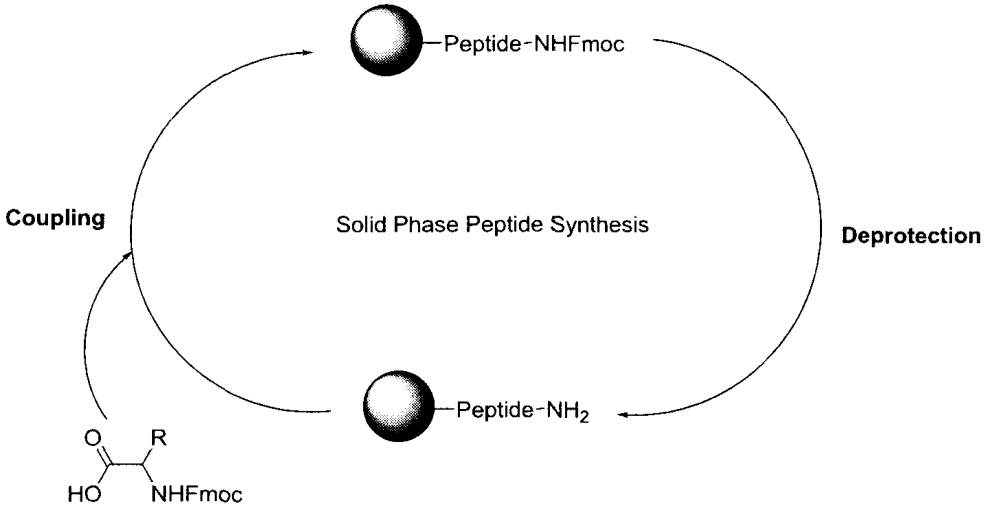



**Fig. 1.3** A cycle of solid phase peptide synthesis (SPPS).

The synthesis of peptides requires an orthogonal protection of the monomers. One protecting group is removed for each coupling step of the peptide synthesis (Fmoc (**Fig. 1.3**) in this case), and the second protecting group on the variable side chain is removed at the end (eg. Bhoc, which is compatible with Fmoc oligomerization chemistry).

Despite being reported over 15 years ago, the synthesis of PNA oligomers is not trivial, especially those containing unusual bases or repetitive sequences. The original protecting groups

(PGs) used in PNA oligomerization were Boc/Cbz[6] which fell into disfavour and gave way to the milder Fmoc/Bhoc protection strategy (**Fig. 1.4.**)[7]

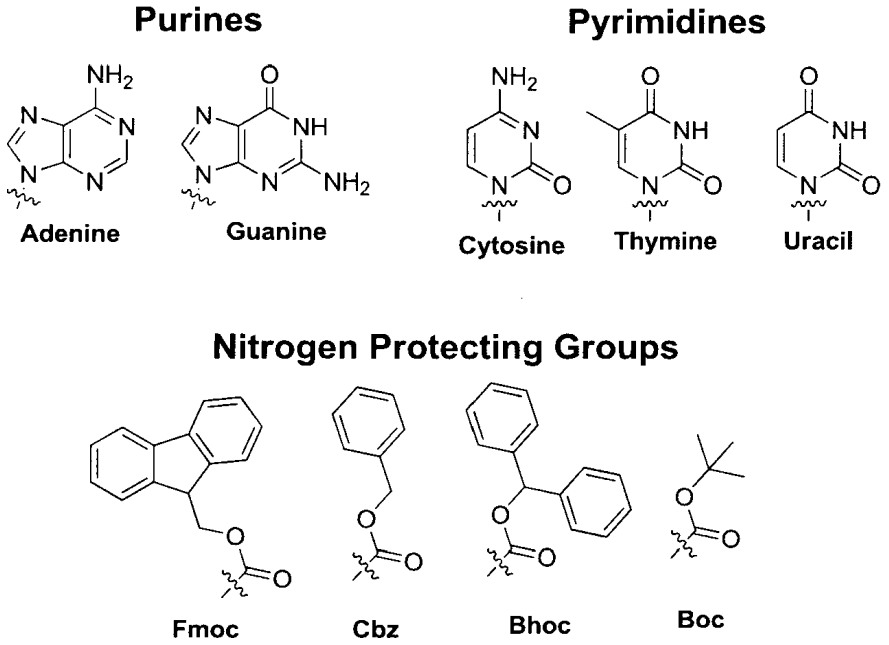



**Fig. 1.4** The natural nucleobases and the protecting groups used in PNA synthesis.

The conditions for Fmoc-based oligomerization are milder and the terminal Fmoc group provides a lipophilic handle that facilitates reversed-phase methods of purification of the oligomers. Our lab has developed an Fmoc/Boc PG strategy for the synthesis of PNA,[8] which has the following advantages: (i) commercially available Fmoc/Bhoc monomers are expensive; partly due to the costly reagents required compared to the Boc-strategy, (ii) the Bhoc group is sensitive to hydrogenation and mild acid conditions limiting its orthogonality to other PGs, (iii) the Boc group has comparable liability to Bhoc toward acidic cleavage, yet is stable to hydrogenolysis, (iv) the per-Boc protected nucleobase are lipophilic and do not possess H-bond donors, which will increase solubility while decreasing self-aggregation.

## 1.3 Fluorescent Nucleobase Analogues

The use of fluorescent reporter groups is ubiquitous in biological and medical research and diagnostics. Fluorescence-based techniques allow researchers to infer information about the

binding or structure of nucleic acids, and imaging can be used to determine position or concentration of sequences with a high specificity. The natural nucleobases (adenine, guanine, cytosine, and thymine/uracil) are not fluorescent, and therefore unnatural nucleobase must be used. The synthesis of base-discriminating fluorophores (BDF) (where the fluorophore is the nucleobase) is often desired, as the fluorescence properties change when properly base paired.[9]

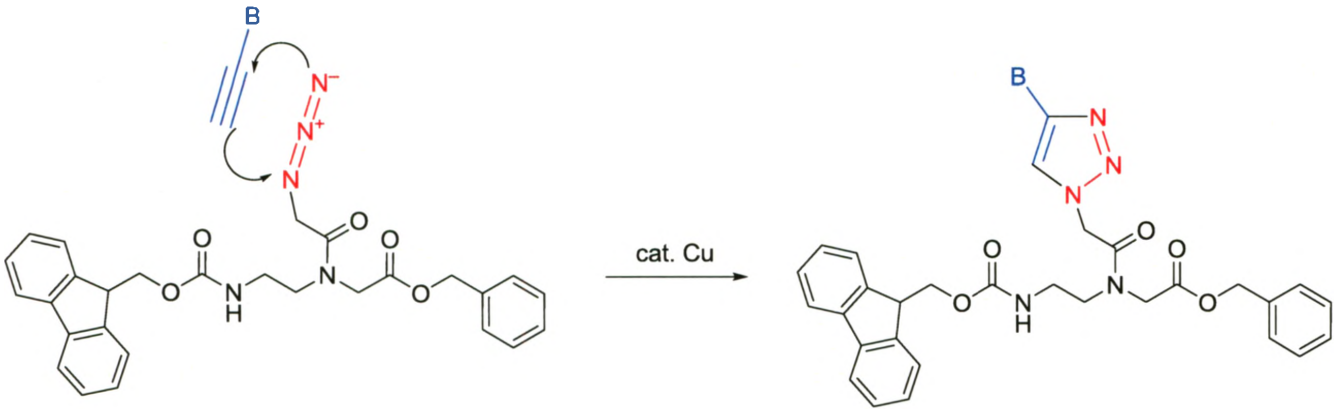
A second class of fluorescent reporter groups are nucleobases with a fluorophores appended. This moiety is often simpler to design, as many commercial fluorophores are available, but lack the local environment sensitivity of BDFs.

1.4 Unnatural Nucleic Acid Analogues

The design and synthesis of unnatural nucleoside analogues is a major goal of medicinal chemistry, with applications such as antiviral chemotherapeutics or in nucleic acid detection. The unnatural nucleobase 2,6-diaminopurine (DAP) has found interest for its strong and selective base pairing to uracil/thymine.[10] The replacement of adenine with DAP in PNA is one of the simplest ways to increase the binding to a target nucleic acid.

1.5 Azide Alkyne Huisgen Cycloaddition

"Click chemistry" is an emerging field important to researchers because it allows the synthesis of a library of compounds using efficient reactions. One such reaction is the Huisgen cycloaddition, where an azide and a terminal alkyne are conjugated to form a 1,2,3-triazole.[11] The reaction conditions are generally mild, and the reaction can be performed on a wide variety of alkynes and azides.[12] The reaction is of interest to our group as a way to create a variety of conjugated triazoles, using a modular approach for the use in PNA oligomers (**Scheme 1.1**.)




**Scheme 1.1** Copper-catalyzed azide alkyne Huisgen cycloaddition (CuAAC).

The CuAAC has found applications in the formation of difficult macrocycles, the appendage of fluorophores, the funtionalization of nano-materials, and many more.[13] The click reaction has been performed on PNA,[14,15] but never in the solid phase. A solid phase CuAAC has the potential to be included in the automated SPPS to form novel PNAs.

## 1.6 Fleximers

The term "fleximers" was coined by K.L. Seley[16] in 2001 to describe a nucleoside analogue where the purine nucleobase was split into its respective imidazole and pyrimidine heterocycles. The connection between the C-5 of the imidazole and the C-6 of the pyrimidine can rotate (**Fig. 1.5**,) adding a degree of flexibility to the purine nucleoside. These molecules have proposed uses as bioprobes, binding-site studies, or therapeutics.

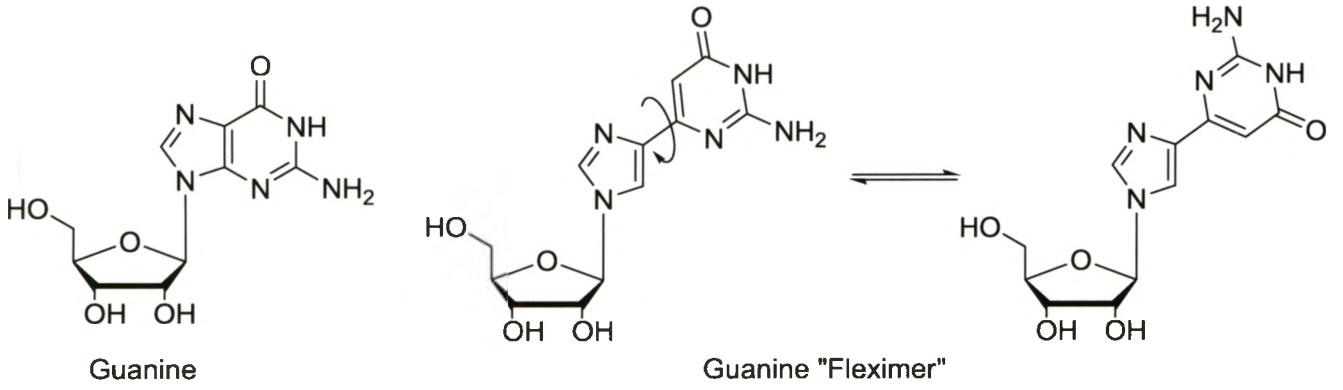



**Fig. 1.5** The first fleximer synthesized by Seley.

1.7 Abasic Sites

Abasic sites are a naturally occurring form of DNA damage that typically arises from depurination (**Fig 1.6.**)[17]

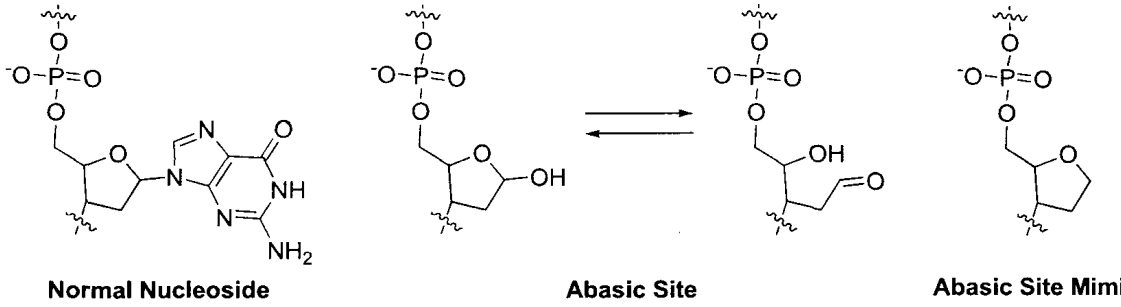



**Fig. 1.6** Depiction of a normal nucleoside compared to an abasic site in equilibrium between its cyclic and open-chain form, and the stable abasic site mimic used in abasic site detection studies.

The detection of abasic sites is often done using fluoresce response across from an abasic site surrogate.[18] The design we investigated uses a complementary PNA sequence with a click fluorophore that will fill the space of the missing base and intercalate within the adjacent bases. Many fluorophores display solvent-dependent intensity or wavelength, so it is theorized that our base going from a polar solvent ($H_2O$) to the non-polar environment within the DNA helix will provide a fluorescence response that can be measured.

1.8 Goals of this Research

The main goal of this thesis is to synthesize, characterize, and oligomerize two types of PNA monomers.

The Boc-nucleobase protection project (Chaper 2) involves developing a synthetic pathway to thymine, 2-aminopurine, and 2,6-diaminopurine PNA monomers using Fmoc/Boc protection. This first requires the synthesis of the per-Boc protected nucleobase acetic acid. This is coupled to an Fmoc-protected PNA backbone containing a benzyl-protected acid. After coupling, the benzyl ester is hydrogenated to produce the Fmoc/Boc PNA monomers ready for SPPS.

The oligomerization of the Fmoc/Boc thymine PNA monomer was examined in a poly-T tract, and its compatibility with commercial thymine PNA monomer. Two peptides were synthesized containing 2,6-diaminopurine or 2-aminopurine to examine their selectivity against mismatches. The fluorescence properties of 2-aminopurine-containing PNA was also examined.

The azide project (Chapter 3) involved the synthesis of an azide modified PNA monomer that was used in a click reaction to produce metal-binding, fluorescent, or fleximer-like nucleobase surrogates. The synthesis of an azide appended PNA monomer allowed its oligomerization and subsequent solid phase click reaction.

The second goal of this part of the thesis was the synthesis of a variety of alkynes to undergo the click reaction with the aforementioned PNA-azide monomer. These alkynes included a metal-binding pyridine, fleximer-like pyrimidines, and the fluorescent probe fluorene.

The final goal of this project was to examine the azide-appended PNA's ability to undergo a solid-phase CuAAC with alkynes. The binding and fluorescence properties of the fluorene and pyrene alkynes were of particular interest for abasic site detection.

## References

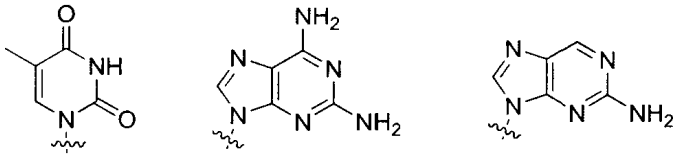

(1) Mulligan, R. *Science* **1993**, *260*, 926.
(2) Dean, D. A. *Advanced Drug Delivery Reviews* **2000**, *44*, 81.
(3) Nielsen, P.; Egholm, M.; Berg, R.; Buchardt, O. *Science* **1991**, *254*, 1497.
(4) Egholm, M.; Buchardt, O.; Christensen, L.; Behrens, C.; Freier, S. M.; Driver, D. A.; Berg, R. H.; Kim, S. K.; Norden, B.; Nielsen, P. E. *Nature* **1993**, *365*, 566.
(5) Christensen, L.; Fitzpatrick, R.; Gildea, B.; Petersen, K. H.; Hansen, H. F.; Koch, T.; Egholm, M.; Buchardt, O.; Nielsen, P. E.; Coull, J.; Berg, R. H. *Journal of Peptide Science* **1995**, *1*, 175.
(6) Dueholm, K. L.; Egholm, M.; Behrens, C.; Christensen, L.; Hansen, H. F.; Vulpius, T.; Petersen, K. H.; Berg, R. H.; Nielsen, P. E.; Buchardt, O. *The Journal of Organic Chemistry* **1994**, *59*, 5767.
(7) Thomson, S. A.; Josey, J. A.; Cadilla, R.; Gaul, M. D.; Fred Hassman, C.; Luzzio, M. J.; Pipe, A. J.; Reed, K. L.; Ricca, D. J.; Wiethe, R. W.; Noble, S. A. *Tetrahedron* **1995**, *51*, 6179.
(8) Wojciechowski, F.; Hudson, R. H. E. *The Journal of Organic Chemistry* **2008**, *73*, 3807.
(9) Dodd, D. W.; Hudson, R. H. E. *Mini-Reviews in Organic Chemistry* **2009**, *6*, 378.
(10) Haaima, G.; Hansen, H. F.; Christensen, L.; Dahl, O.; Nielsen, P. E. *Nucleic Acids Research* **1997**, *25*, 4639.
(11) *Proceedings of the Chemical Society* **1961**, 357.
(12) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angewandte Chemie International Edition* **2001**, *40*, 2004.
(13) Meldal, M.; Tornøe, C. W. *Chemical Reviews* **2008**, *108*, 2952.
(14) Chouikhi, D.; Barluenga, S.; Winssinger, N. *Chemical Communications* **2010**, *46*, 5476.
(15) Chouikhi, D.; S. Barluenga, Winssinger, N. *Chemical Communications* **2010**, *30*, 5476.
(16) Seley, K. L.; Zhang, L.; Hagos, A. *Organic Letters* **2001**, *3*, 3209.
(17) Srivastava, D. K.; Vande Berg, B. J.; Prasad, R.; Molina, J. T.; Beard, W. A.; Tomkinson, A. E.; Wilson, S. H. *Journal of Biological Chemistry* **1998**, *273*, 21203.
(18) Stivers, J. T. *Nucleic Acids Research* **1998**, *26*, 3837.

# Chapter 2

## An Fmoc/Boc Protection Strategy for PNA

### Introduction

The thymine PNA monomer is unique because it is the only natural nucleobase that does not possess a functional group that requires protection under normal circumstances. The methyl group at the 5-position imparts some organic solubility, and it lacks an exocyclic amine that would interfere with normal peptide coupling (**Fig. 2.1**.) The protection of the thymine PNA monomer is still of interest to increase the coupling yields of poly-T tracts, which have been noted to result in up to 70% of truncated products.[1] The synthesis of Fmoc/Boc thymine PNA monomer also completes the series of natural nucleobases previously reported by our lab.[2]




Thymine 2,6-Diaminopurine 2-Aminopurine

**Fig. 2.1** Nucleobases for which Boc protection has been developed.

The nucleobase 2,6-diaminopurine (DAP) is of interest for its ability to form an extra hydrogen bond with uracil or thymine. It has also been investigated for its ability as double duplex invasion agents when paired with thiouracil.[3] Although the Fmoc/Cbz monomer has been reported,[4] the highly lipophilic Fmoc/Boc protected monomer remains to be synthesized.

The nucleobase 2-aminopurine (2AP) is an important nucleobase used in fluorescence studies. It forms a proper base pair with thymine and can simply replace adenine within a sequence without compromising structure.[5] The fluorescent properties of 2-aminopurine have been used extensively for the probing of DNA interactions, but has only been incorporated once into PNA.[6] The 2AP monomer synthesized contained the unprotected nucleobase on a Boc PNA

monomer and the conditions or yields were not included. The key step in their synthesis relies on a Pd/C hydrogenation of the 6-position chlorine, which in our experience is not a trivial reaction. Leaving the exocyclic amine unprotected also leads us to believe the oligomerization would be low yielding from side reactions that can occur. Finally, their examination of a PNA containing 2-aminopurine in a PNA:DNA duplex only used adenine and thymine. 2-Aminopurine is known to form charge transfer complexes with guanine,[7] and this interaction in PNA warrants further investigation.

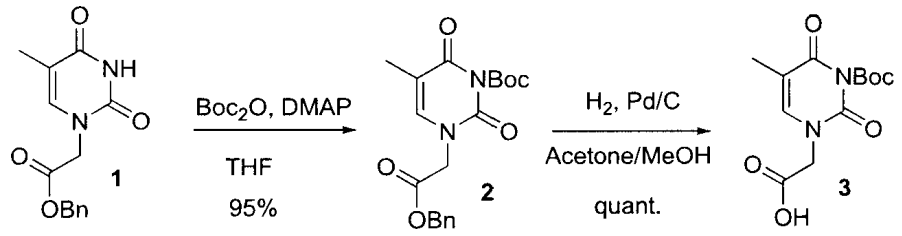
Our investigation into 2-aminopurine required the synthesis of its PNA monomer, and to make it compatible with our previously synthesized monomers, we decided to examine the Fmoc/Boc protection strategy.

The objective of this project is to complete a facile synthesis of Fmoc/Boc protected PNA monomers for unusual nucleobases and demonstrate their use in the synthesis of PNA. This will allow researchers access to PNA with unusual nucleobases.

**Results and Discussion**

2.1 Boc Protected Thymine PNA Monomer

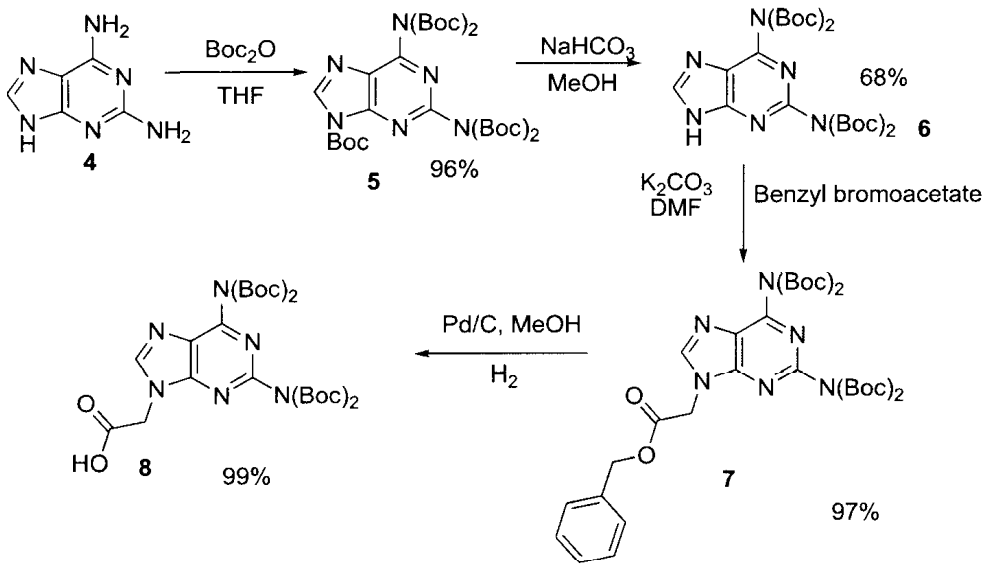
The synthesis of Boc-protected thymine was completed though the Boc-protection and subsequent hydrogenation of the readily prepared 2-thymine acetic acid benzyl ester[8] (**1**) in excellent yields (**Scheme 2.1**) ready to be coupled to a PNA backbone.




**Scheme 2.1** The synthesis of *N*-Boc-thyminyl-1-acetic acid.

2.2 Tetrakis-Boc Protected 2,6-Diaminopurine PNA Monomer

The synthesis of tetrakis-Boc protected 2,6-diaminopurine was accomplished by penta-Boc protection of the commercially available 2,6-diaminopurine to form **5**, which proceeded in excellent yield (**Scheme 2.2.**) 2,6-Diaminopurine is insoluble in THF, and as it reacts the product becomes fully organic soluble. The next step was a mono-deprotection using $NaHCO_3$ in MeOH to afford **6** in acceptable yields. The tetrakis-Boc protected nucleobase was subjected to an alkylation with benzyl bromoacetate, with the bulky di-Boc-6-amino group directing the reaction to the *N*-9 position. The hydrogenation of the benzyl ester **7** furnished the final tetra-Boc protected nucleobase acid **8** in excellent yield, ready to be coupled to a PNA backbone.

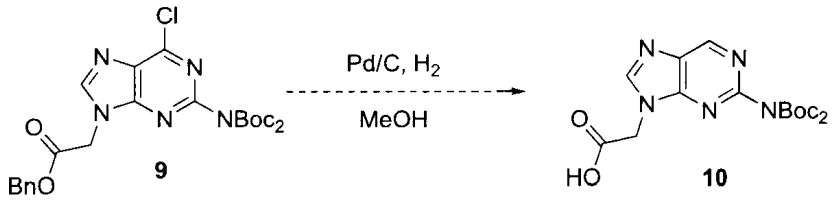



**Scheme 2.2** The synthesis of tetrakis-(Boc)-2,6-diaminopurin-9-yl acetic acid.

2.3 Bis-Boc Protected 2-Aminopurine PNA Monomer

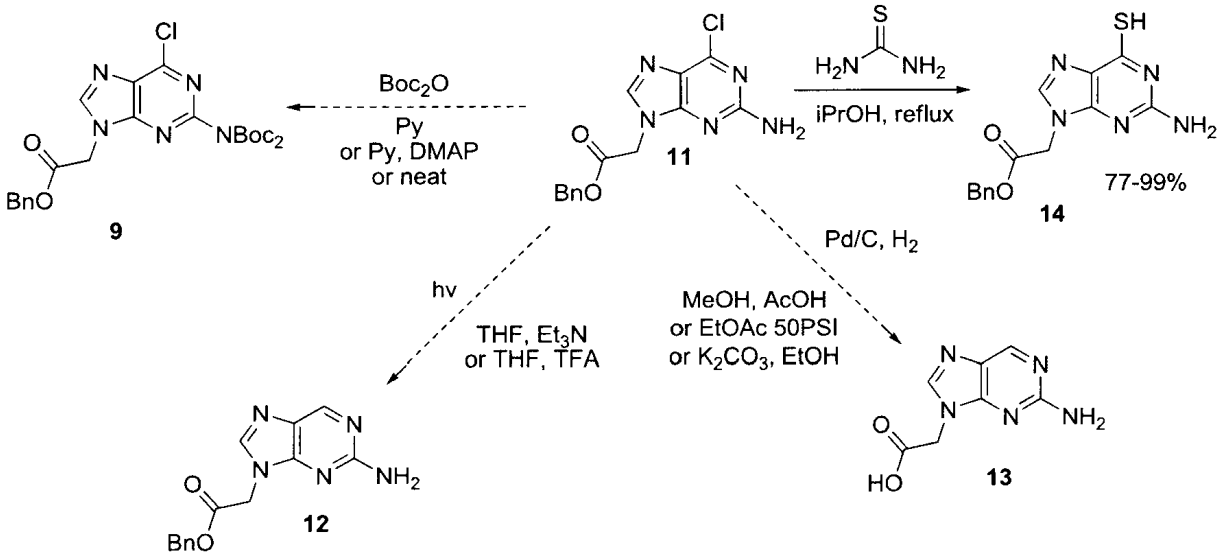
The initial synthetic approach began from the advanced intermediate **9** (**Scheme 2.3**,) which was synthesized in a one-step reaction from a literature compound (discussed later in this section.) This molecule was subjected to Pd/C under an atmosphere of hydrogen in an attempt to perform a simultaneous hydrogenation/hydrogenolysis to produce the diBoc-protected **10**. The reaction was run until the disappearance of the starting material (18 h) and produced an

intractable mixture of products. This initial failure led to the synthesis of a variety of purines, finally leading to a route relying on optimization of this key reaction.




**Scheme 2.3** The first attempted synthesis of di-(Boc)-2-aminopurin-9-yl acetic acid.

The alternate synthetic scheme proposed used the intermediate benzyl 2-amino-6-chloropurine-9-acetate **11** (**Scheme 2.4**,) which was synthesized in gram quantities from 2-amino-6-chloropurine following a literature procedure.[9]



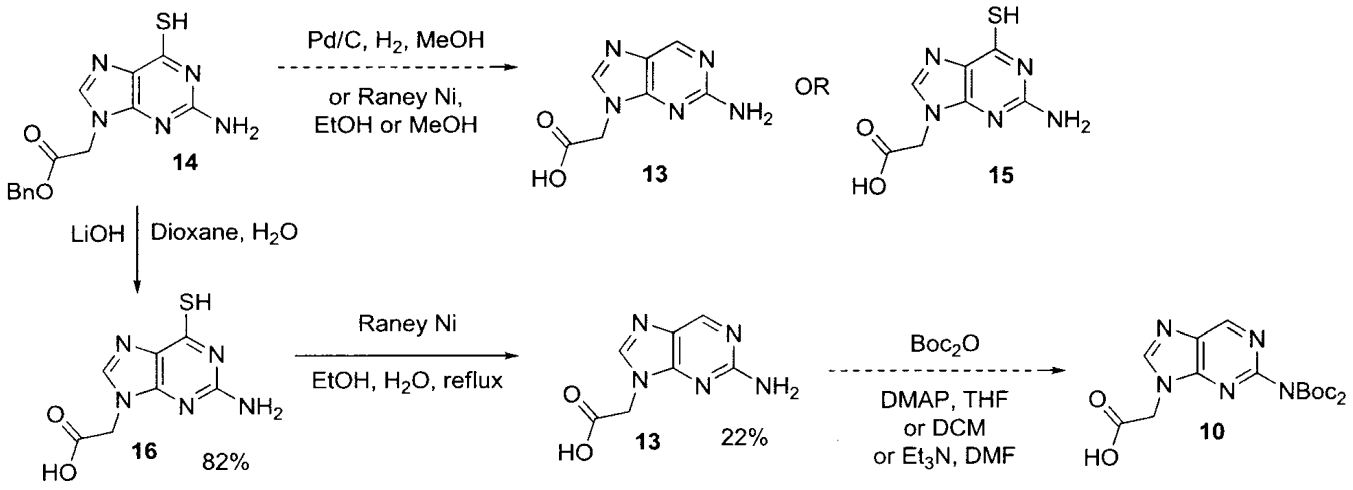

**Scheme 2.4** The chemistry of benzyl 2-amino-6-chloropurin-9-yl acetate.

The diBoc-protection of **11** proved futile under a variety of conditions. Using pyridine as a solvent, various temperatures, and using DMAP as a catalyst gave either no reaction or an intractable mixture. One interesting paper was published on the di-Boc protection of purine analogues in neat $Boc_2O$ using a ball mill grinder.[10] A ball mill was made using a peptide shaker in an attempt to synthesize **9**, but we were unable to reproduce the clean Boc protections reported for this analogue with our improvised equipment.

There are several reactions reported using photochemical or electrochemical reductive dehalogenation, with some literature precedence for 6-chloropurine. Our lab is not equipped for electrochemical reactions, but several photochemical reactions were performed in Dr. Workentin's laboratory at the University of Western Ontario. Photochemical reactions were attempted in basic, acidic, or neutral THF to produce **12**, but all conditions gave an intractable mixture of products.

The hydrogenation of **11** using Pd/C and hydrogen was attempted under acidic, basic and high pressure conditions in an attempt to synthesize **13**. All reaction conditions hydrogenated the benzyl ester and produced negligible amount of the desired product.

There is literature precedence for the facile conversion of 6-chloropurine to 6-thiopurine using thiourea.[11] The reaction worked well to produce **14** , and could give yields up to 99%. With the reaction able to be performed easily on gram scale, the new scheme would use a combination of diBoc protection, Raney Ni desulfurization, and ester hydrolysis/hydrogenation to yield the desired di-Boc acid **10** (**Scheme 2.5**.)

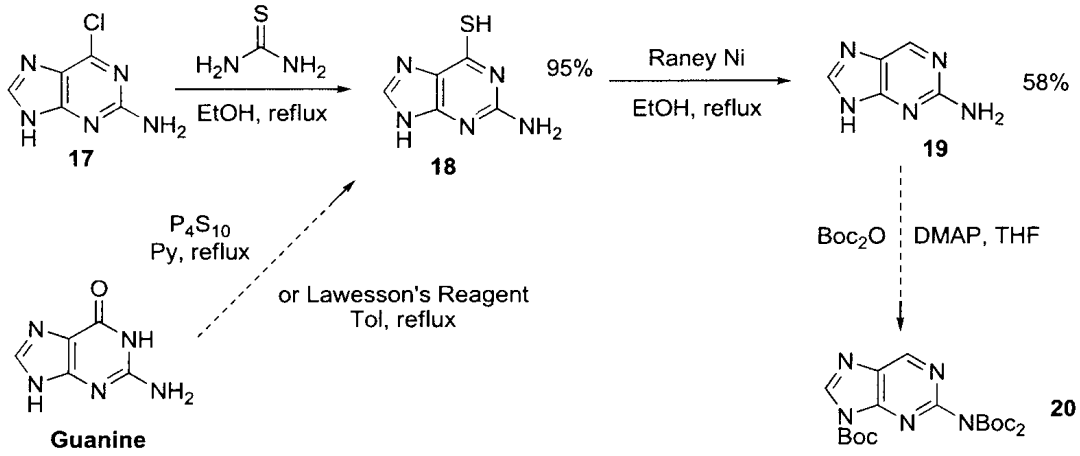



**Scheme 2.5** The chemistry of benzyl 2-amino-6-thiopurin-9-yl acetate.

The first reaction attempted with **14** was a simultaneous hydrogenation/desulfurization using Pd/C or Raney Ni which gave trace amounts of products **13** and **15** but could not be

performed in acceptable yield for either product. An ester hydrolysis could be performed using LiOH in a dioxane/water mix to yield **16**, which could be desulfurized in low yield to afford **13**. The di-Boc protection of **13** could not be performed under a variety of solvents and conditions and thus this route was abandoned.

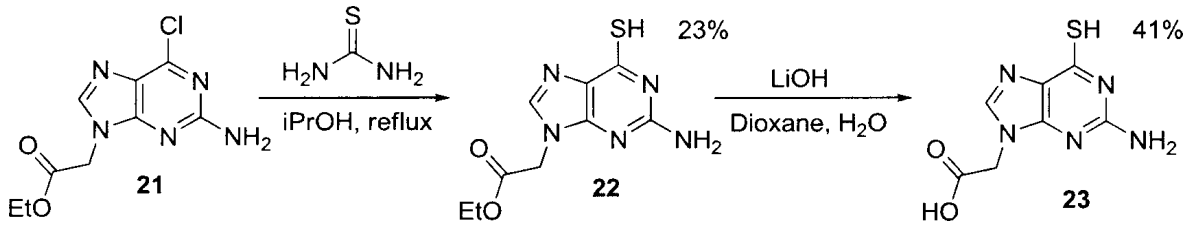
The moderate success of the Raney Ni desulfurization of a 6-thiopurine led to an interest in synthesizing 2-aminopurine (**19**) for a direct Boc protection (**Scheme 2.6.**)




**Scheme 2.6** The synthesis of 2-aminopurine.

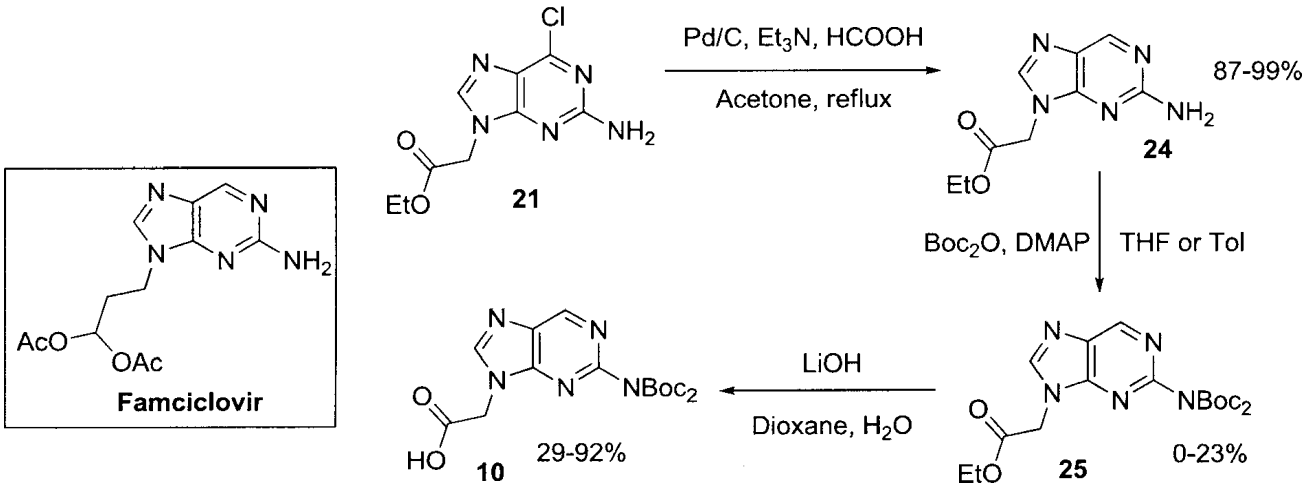
While 2-aminopurine is commercially available ($547/g),[12] its high price and the availability of its precursors in our laboratory prompted a synthesis from 2-amino-6-chloropurine (**17**.) The conversion of the 6-position chlorine to a thiol with thiourea proceeded in excellent yield to produce **18**, which underwent a desulfurization reaction with Raney Ni to afford 2-aminopurine (**19**) in acceptable yield. The thiol **18** can also be synthesized from the inexpensive starting material guanine, but this reaction could not be driven to completion and the product was contaminated with starting material. The Boc protection of **19** proved difficult, which was surprising because of the ease of Boc-protecting its analogues such as 2,6-diaminopurine or adenine.[13] Without being able to Boc protect 2-aminopurine, a different synthetic scheme had to be pursued.

One purine derivative available in our laboratory was ethyl 2-amino-6-chloropurine-9-acetate (**21**,) which would be converted into the acid derivative **13** (**Scheme 2.7**) to be used in discovering and optimizing a di-Boc protection procedure. The replacement of the 6-position chlorine with a thiol using thiourea produced **22** in very low yield. The subsequent ester hydrolysis to form **23** proceeded in low yield as well, which ultimately led to the abandonment of this pathway.



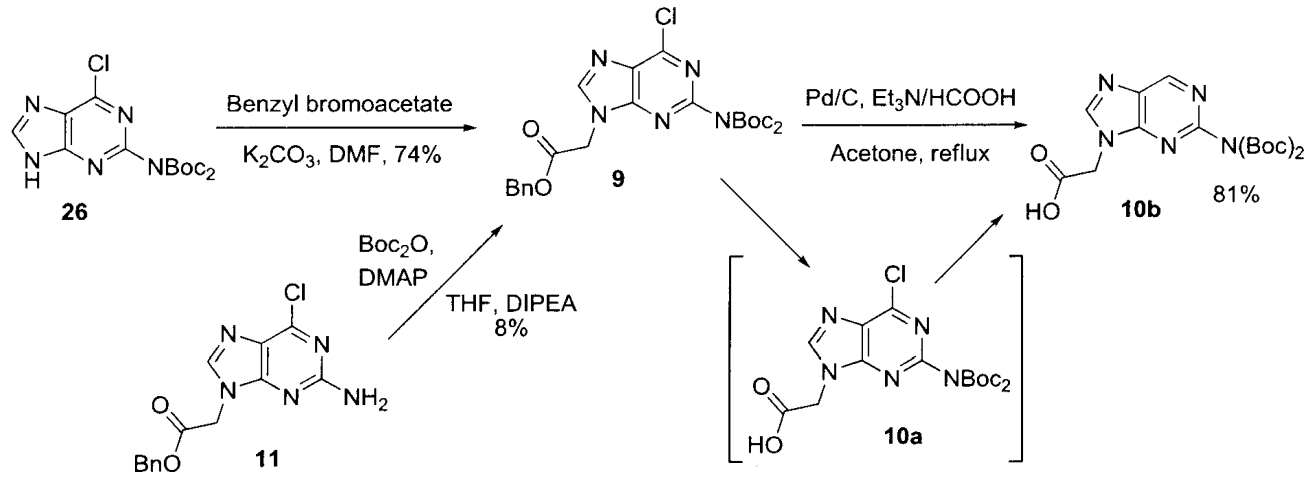

**Scheme 2.7** Alternate synthesis of 2-amino-6-thiopurin-9-yl acetic acid.

The 2-aminopurine moiety is used in the herpes simplex virus 2 drug Famciclovir (**Scheme 2.8**,) which has been synthesized in the literature[14] from its 6-chloro precursor using a Pd/C transfer hydrogenolysis reaction in a refluxing solution of $Et_3N$/HCOOH in acetone. The reaction worked in excellent yield to dechlorinate **21** yielding **24**. The subsequent diBoc protection gave low, variable yields and was difficult to optimize. Two small scale ester hydrolyses were performed with variable yields to yield the final product **10**, but lack of material prevented the synthesis of enough to couple to the PNA backbone.




**Scheme 2.8** Structure of Famciclovir and the unoptimized synthesis of bis-Boc-2-aminopurin-9-yl acetate.

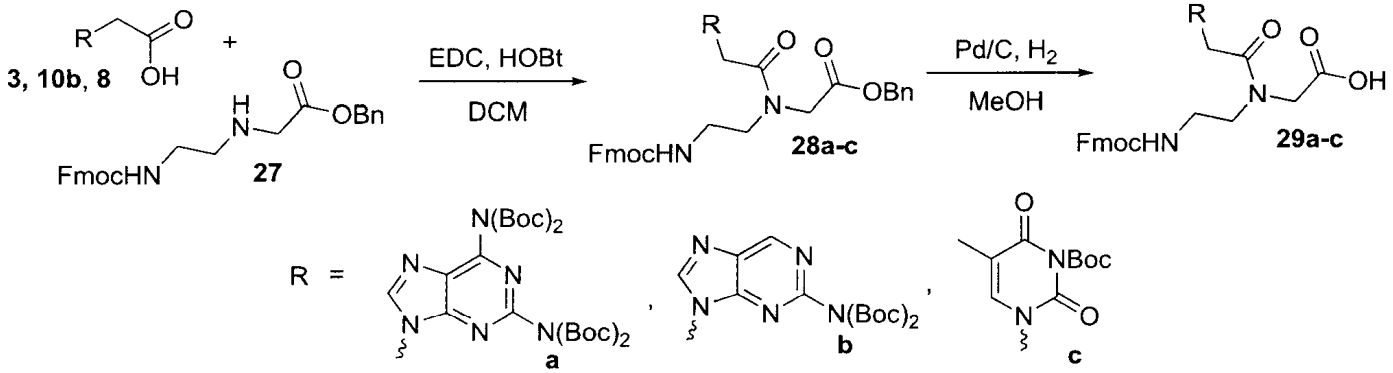
Armed with a reliable reaction to remove the 6-position chlorine, attention was turned to the advanced intermediate **9**. The original synthesis of **9** was through the *N*-9 alkylation of the literature compound **26**, which can be prepared from the commercially available 6-chloro-2-aminopurine in 99% yield.[15] A second route to synthesize **9** was attempted using **11** (**Scheme 2.9**.) The large scale di-Boc protection gave low yields as predicted, but provided enough material to be used in the new hydrogenation/hydrogenolysis reaction. Two pilot scale reactions were performed on **9**, and the material proved difficult to track by TLC due to the similar Rf values of **10a** and **10b**. One solution was to track the progress of the reaction by HPLC, adding more equivalents of the hydrogen transfer agent every 2 h. This scheme finally yielded enough **10b** to use in a coupling reaction with PNA backbone to produce the monomer.




**Scheme 2.9** The optimized synthesis of di-(Boc)-2-aminopurin-9-yl acetic acid.

## 2.4 Coupling of Boc-Protected Acids to PNA Backbone

The coupling of the Boc-protected acids to the Fmoc/Bn PNA backbone required a standard EDC/HOBt coupling in DCM and proceeded in typical coupling yields of 58-71% (**Scheme 2.10.**) The hydrogenation of the benzyl protecting group with Pd/C and $H_2$ gas in methanol proceeded in >95% yield to afford the PNA monomer ready for oligomerization.




**Scheme 2.10** The coupling of the Boc-protected acids to an Fmoc/Bn PNA backbone to form PNA monomers.

## 2.5 Oligomerization of Fmoc/Boc Protected PNA Monomers

The peptide synthesis was carried out using the PNA monomers **29a-c** in order to show that the Fmoc/Boc protection strategy was effective for the synthesis of PNA (**Fig. 2.2.**)

| | Sequence | Tm (°C) |
|---|---|---|
| **PNA1** | $^{N}$C-T-T-T-C-C-T-**DAP**-C-A-C-T-G-T-Lys$^{C}$ | - |
| **DNA1G** | $^{3'}$G-A-A-A-G-G-A- G -G-T-G-A-C-A$^{5'}$ | 49.5 |
| **DNA1A** | $^{3'}$G-A-A-A-G-G-A- A -G-T-G-A-C-A$^{5'}$ | 50.0 |
| **DNA1C** | $^{3'}$G-A-A-A-G-G-A- C -G-T-G-A-C-A$^{5'}$ | 56.5 |
| **DNA1T** | $^{3'}$G-A-A-A-G-G-A- T -G-T-G-A-C-A$^{5'}$ | 62.5 |
| **PNA2** | $^{N}$G-T-T-G-C-**2AP**-C-T-G-G-A-Lys$^{C}$ | - |
| **DNA2G** | $^{3'}$C-A-A-C-G- G -G-A-C-C-T$^{5'}$ | 45.5 |
| **DNA2A** | $^{3'}$C-A-A-C-G- A -G-A-C-C-T$^{5'}$ | 46.0 |
| **DNA2C** | $^{3'}$C-A-A-C-G- C -G-A-C-C-T$^{5'}$ | 51.0 |
| **DNA2T** | $^{3'}$C-A-A-C-G- T -G-A-C-C-T$^{5'}$ | 62.5 |

**Figure 2.2** The PNAs synthesized and their DNA complements where **DAP** = 2,6-diaminopurine, **2AP** = 2-aminopurine. The Tms were calculated at 2 μM in a buffer containing NaCl (100 mM), EDTA (0.1 mM), and $Na_2PO_4$ (10 mM, pH 7).

The HPLC traces of crude **PNA1** and **PNA2** showed comparable coupling yields and purity to previous PNA syntheses in our laboratory. Melting temperature determinations of the PNAs with their DNA complements were performed (**Fig. 2.2**) to measure the effect of DNA mismatches on duplex stability. Both 2,6-diaminopurine and 2-aminopurine prefer binding to thymine, forming 3 and 2 hydrogen bonds respectively.

The selectivity of DAP and 2AP for thymine was studied to see the effect of a mutation on the duplex stability. The **PNA1** demonstrated selectivity for at least 6 °C over mismatches, and the **PNA2** by 11.5 °C. For **PNA1** and **PNA2** the second most stable duplex is a mismatch with the pyrimidine C. This is because the purines are bicyclic, creating a large steric repulsion compared to the monocyclic pyrimidines.

Because of the extra hydrogen bond of a DAP:T match, this substitution was predicted to be more selective against mismatches compared to 2AP:T, but this was not the case. This is likely due sequence-dependent effects.

The **PNA2** sequence has the advantage of being fluorescent and an experiment was done to see if this property could be exploited to produce a melt curve with similar results to hyperchromicity-based UV-Vis melt curve (**Fig. 2.3**). The Tm of the melt curves gave results in line with the standard method of measuring the nucleobases hyperchromicity.

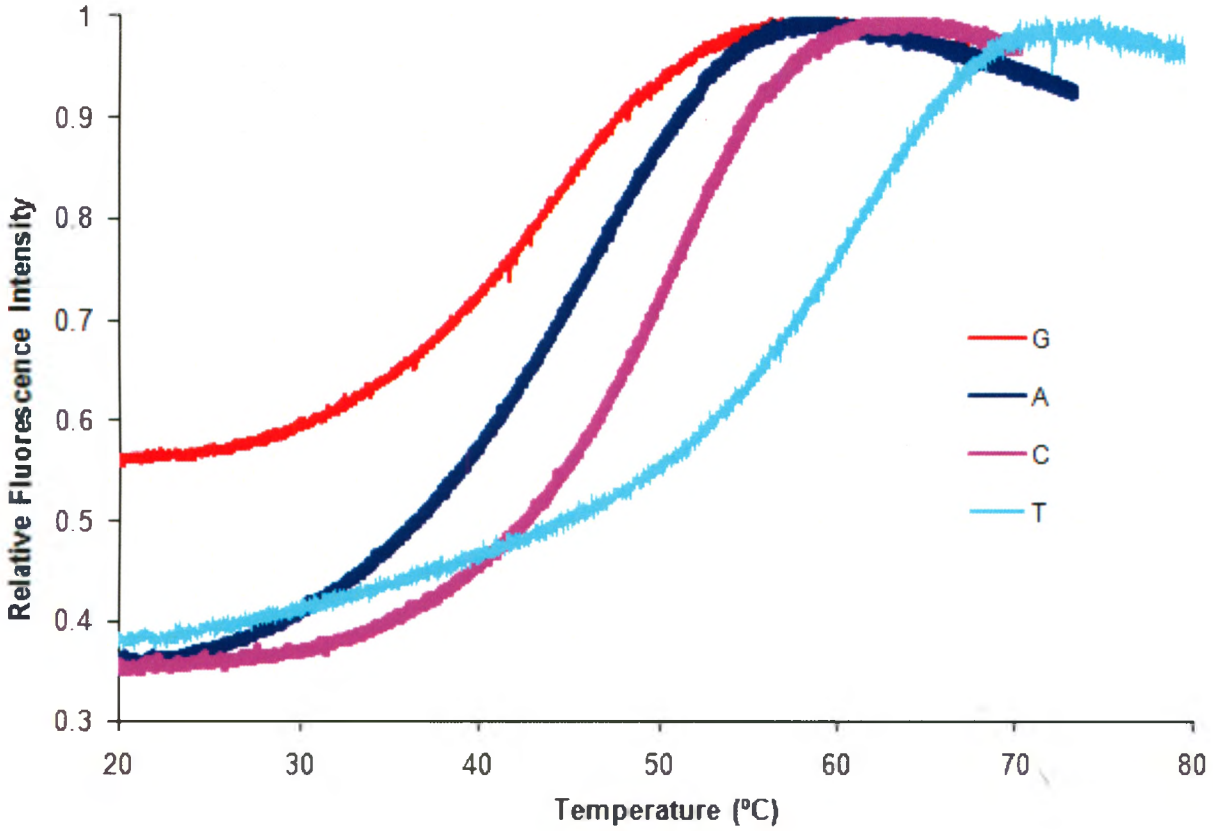



**Fig. 2.3** Fluorescence melting curve of **PNA2** in single-strand form and annealed to **DNA2B** (where **B** represents nucleobases G, A, C, and T) at 2 μM in a buffer containing NaCl (100 mM), EDTA (0.1 mM), and $Na_2PO_4$ (10 mM, pH 7). The fluorescence intensities were normalized to the maximum fluorescence of each run.

The fluorescence profile of the **PNA2** duplexes were measured at room temperature (**Fig. 2.4**) with the guanine mismatch giving the highest fluorescence. The single stranded **PNA2** gave the second highest fluorescence, with the remaining duplexes fluorescence showing a trend of decreasing as duplex stability increases.

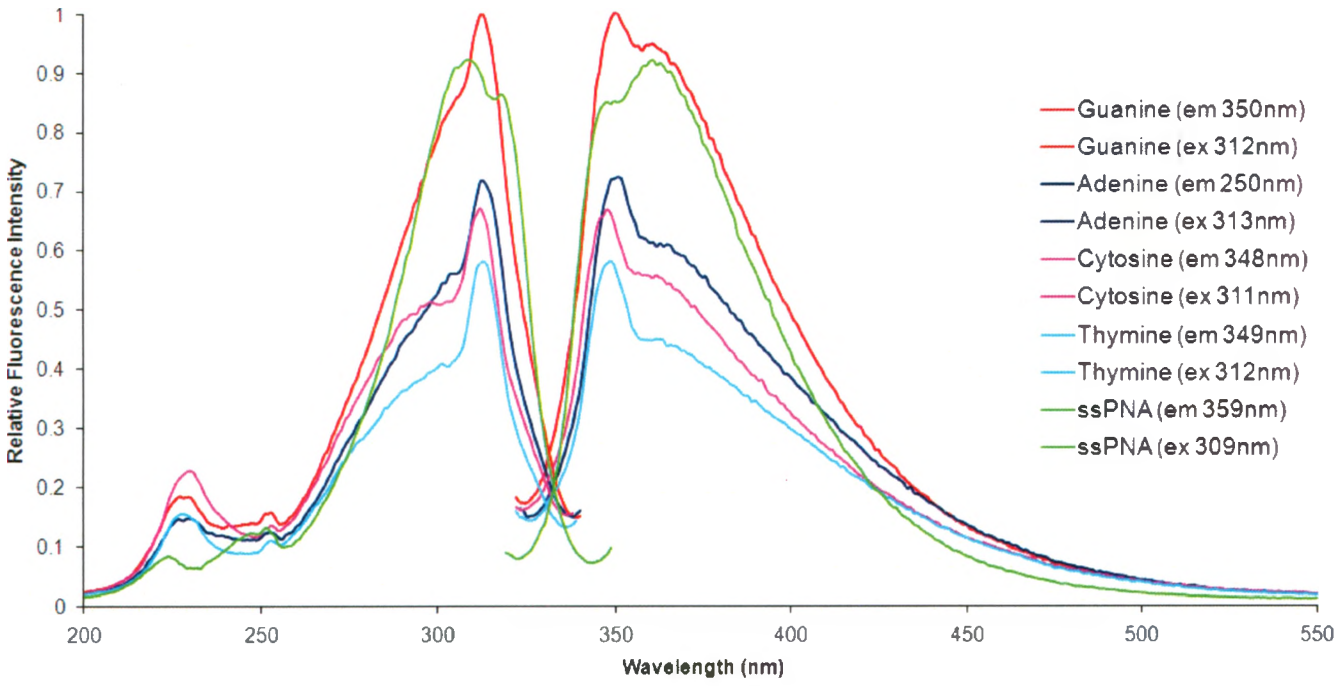



**Fig. 2.4** Fluorescence profile of **PNA2** in single-strand form and annealed to **DNA2B** (where **B** represents nucleobases G, A, C, and T) at 2 μM in a buffer containing NaCl (100 mM), EDTA (0.1 mM), and $Na_2PO_4$ (10 mM, pH 7).

## Conclusion

The synthesis of a Fmoc/Boc protected PNA monomers has been completed for thymine, 2,6-diaminopurine, and 2-aminopurine. The oligomerization of thymine has been completed with no evidence of increased truncation, and can be used in combination with the unprotected PNA monomer.

The DAP monomer has been incorporated into a PNA and its melting temperature against thymine vs. mismatches showed a selectivity of 6 °C over mismatches.

The 2-aminopurine monomer was also successfully incorporated into PNA, and its selectivity for thymine in this sequence was 11.5 °C. The fluorescence of the nucleobase can be used to perform a fluorescence binding curve that gives a comparable Tm to the standard hyperchromicity melt curve. The fluorescence profile of 2-aminopurine-containing single stranded PNA at room temperature gave the second highest fluorescence profile, the highest

being the guanine mismatch. The fluorescence was significantly quenched with the tighter binding mismatches, with the lowest fluorescence against thymine.

**Experimental**

**PNA1.** HRMS (ESI) calculated for $C_{155}H_{204}N_{74}O_{47}$: [M + $3H^+$] 1285.5361, Found 1285.8582; [M + $4H^+$] 964.4040, Found 964.6530.

**PNA2.** HRMS (ESI) calculated for $C_{125}H_{161}N_{67}O_{35}$: [M + $3H^+$] 1054.4371, Found 1054.7417; [M + $4H^+$] 791.0798, Found 791.3110.

**Benzyl *N*-Boc-thyminyl-1-acetate (2).** Benzyl thyminyl-1-acetate (3.0 g, 11 mmol), $Boc_2O$ (4.8 g, 22 mmol), and DMAP (2.7 g, 22 mmol) were added to THF (30mL) at 0 °C. After 0.5 h the solution was warmed to room temperature. After 18 h, the reaction mixture was concentrated in vacuo. The residue was dissolved in DCM (100 mL) and extracted with $H_2O$ (3x100 mL). The organic layer was dried over anhydrous $Na_2SO_4$ and the solvent removed. The residue was purified by FCC (EtOAc-Hexane = 2:3, v/v) to give **2** (3.9g, 95 % yield) as a light-yellow foam: $^1H$ NMR (400 MHz, $CDCl_3$) = 7.30 - 7.41 (m, 5 H), 6.90 (s, 1 H), 5.20 (s, 2 H), 4.47 (s, 2 H), 1.92 (s, 3 H), 1.56 - 1.62 (m, 9 H); HRMS (EI) calculated for $[C_{19}H_{22}N_2O_6]^+$: 374.1478, Found 374.1484.

***N*-Boc-thyminyl-1-acetic acid (3).** **2** (0.75 g, 2.0 mmol) was dissolved in minimal acetone:MeOH solution (1:1, v/v). Pd/C (80 mg) was added and the reaction flask was placed under an atmosphere of $H_2$. The suspension was stirred at room temperature for 2 h. The solvent was removed and the residue suspended in MeOH (100 mL). The suspension was filtered through Celite, and the solvent removed to yield **3** (5.7 g, quant. yield) as a glassy white solid: $^1H$ NMR (400 MHz, DMSO-$d_6$) = 7.66 (s, 1 H), 4.43 (s, 2 H), 1.81 (s, 3 H), 1.51 (s, 9 H); HRMS (EI) calculated for $[C_{11}H_{16}N_2O_6]^+$: 284.1008, Found 284.1021.

**Penta-Boc-2,6-diaminopurine (5).** 2,6-Diaminopurine (0.500 g, 3.33 mmol), $Boc_2O$ (6.12 g, 26.6 mmol), and DMAP (0.061 g, 0.50 mmol) were added to THF (25 mL) at 0 °C. The solution was stirred for 5 min then warmed to room temperature. After 18 h the solvent was removed. The residue was dissolved in DCM (150 mL) and washed with $H_2O$ (100 mL). The organic layer was dried over $Na_2SO_4$ and the solvent was removed. The residue was purified by FCC (EtOAc-

Hex = 1:2) to yield **5** (2.08 g, 96 % yield) as a white foam: $^{1}$H NMR (400 MHz, $CDCl_3$) = 8.52 (s, 1 H), 1.68 (s, 9 H), 1.43 (s, 18 H), 1.41 (s, 18 H); $^{13}$C NMR (101 MHz, $CDCl_3$) = 153.7, 152.9, 151.6, 150.6, 149.7, 145.5, 143.8, 127.8, 87.3, 83.9, 83.2, 41.9, 27.8, 27.7, 27.6; HRMS (EI) calculated for $[C_{30}H_{46}N_6O_{10}]^+$: 650.3275, Found 650.3262.

**Tetra-Boc-9*H*-2,6-diaminopurine (6).** **5** (1.93 g, 2.97 mmol) was suspended in mixture of MeOH (50mL) and saturated $NaHCO_3$ solution (25mL). The turbid solution was heated to 50 °C for 1 h and then cooled to room temperature. After 18 h the MeOH was removed by rotary evaporation and the solution diluted with $H_2O$ (100 mL) and extracted with DCM (2 x 100 mL). The combined organic layers were dried over $Na_2SO_4$ and the solvent was removed to yield **6** (1.12 g, 68 % yield) as a white foam: $^{1}$H NMR (400 MHz, DMSO-$d_6$) = 8.63 (s, 1 H), 5.05 (br. s., 2 H), 1.36 (s, 18 H), 1.33 (s, 18 H); $^{13}$C NMR (101 MHz, $CDCl_3$) = 151.4, 150.8, 149.9, 145.3, 109.9, 84.3, 83.4, 27.7, 27.6; HRMS (EI) calculated for $[C_{25}H_{38}N_6O_8]^+$: 550.2751, Found 550.2757.

**Benzyl tetra-Boc-2,6-diaminopurinyl-9-acetate (7).** **6** (0.500 g, 0.908 mmol) and $K_2CO_3$ (0.138 g, 0.999 mmol) were added to DMF (3mL) at 0 °C. Benzyl bromoacetate (0.16 mL, 1.0 mmol) was added dropwise over 5 min. The solution was stirred for 10 min then warmed to room temperature. After 18 h the reaction mixture was diluted with $H_2O$ (100 mL) and extracted with ether (2 x 100 mL). The organic layers were combined, washed with water (50mL), dried over $Na_2SO_4$, and the solvent removed to yield **7** (0.616 g, 97% yield) as a white foam: $^{1}$H NMR (400 MHz, $CDCl_3$) = 8.14 (s, 1 H), 7.31 - 7.40 (m, 5 H), 5.21 (s, 2 H), 5.05 (s, 2 H), 1.40 (s, 18 H), 1.39 (s, 18 H); $^{13}$C NMR (101MHz, $CDCl_3$) = 176.0, 167.0, 154.2, 152.2, 151.0, 150.6, 149.8, 145.8, 128.7, 128.4, 126.7, 83.7, 83.2, 67.9, 52.9, 44.3, 44.1, 27.7, 27.6; HRMS (ESI) calculated for $[C_{34}H_{47}N_6O_{10}]^+$: 699.3348, Found 699.2743.

**Tetra-Boc-2,6-diaminopurinyl-9-acetic acid (8).** **7** (0.509 g, 0.728 mmol) was dissolved in MeOH (5 mL). Pd/C (60mg) was added and the reaction vessel was placed under an atmosphere of $H_2$. The suspension was stirred for 2h at room temperature. The suspension was filtered through Celite with MeOH (100 mL) and the solvent removed to yield **8** (0.439 g, 99 % yield) as a glassy grey solid: $^{1}$H NMR (400 MHz, DMSO-$d_6$) = 8.63 (s, 1 H), 5.05 (br. s., 2 H), 1.35 (s, 18

H), 1.33 (s, 18 H); $^{13}$C NMR (101 MHz, MeOH-$d_4$) = 200.7, 156.1, 153.2, 152.4, 151.5, 150.0, 85.6, 85.1, 28.2, 28.1; HRMS (ESI) calculated for $[C_{27}H_{41}N_6O_{10}]^+$: 609.2879, Found 609.2688.

**Benzyl 6-chloro-2-di-Boc-aminopurinyl-9-acetate (9).** 6-Chloro-2-di-Boc-aminopurine (1.28 g, 3.45 mmol) and $K_2CO_3$ (0.524 g, 3.79 mmol) were suspended in DMF (15 mL) and cooled to 0 °C. Benzyl bromoacetate (0.60 mL, 3.8 mmol) was added dropwise over 10 min. The solution was stirred for 5min and then warmed to room temperature. After 18 h the suspension was filtered through Celite with EtOAc (100 mL) then concentrated in vacuo. The oil obtained was suspended in $H_2O$ (100 mL) and extracted with $Et_2O$ (2 x 200 mL). The organic layers were combined, dried over $Na_2SO_4$ and the solvent removed. The residue was purified by FCC (EtOAc-Hex = 1:1) to yield **9** (1.32 g, 74 % yield) as a white powder: $^1$H NMR (400MHz, $CDCl_3$) = 8.19 (s, 1 H), 7.31 - 7.41 (m, 5 H), 5.22 (s, 2 H), 5.05 (s, 2 H), 1.42 (s, 18 H); $^{13}$C NMR (101 MHz, $CDCl_3$) = 166.2, 152.7, 152.0, 151.1, 150.3, 146.4, 134.2, 129.4, 128.8, 128.6, 128.5, 83.6, 68.1, 44.4, 27.7; HRMS (ESI) calculated for $[C_{24}H_{29}ClN_5O_6]^+$: 518.1801, Found 518.1806.

**2-Di-Boc-aminopurinyl-9-acetic acid (10b). 9** (0.304 g, 0.587 mmol) was dissolved in a mixture of acetone (4 mL), $Et_3N$ (1.4 mL), and formic acid (0.2 mL). Pd/C (40mg) was added and the reaction mixture was brought to reflux. The two products formed were tracked by HPLC. Two further equivalents of Pd/C (40 mg), $Et_3N$ (1.4 mL), and formic acid (0.2 mL) were added after 2h and 4h. When only 1 product remained by HPLC, the mixture was filtered through Celite with acetone (100 mL) and the solvent removed. The residue was suspended in $H_2O$ (25 mL) and extracted with DCM (3 x 50 mL). The organic fractions were combined and washed with a 1M $KHSO_4$ solution (50 mL). The organic layer was dried over $Na_2SO_4$ and the solvent removed to yield **10b** (0.186 g, 81 % yield) as a glassy white solid: $^1$H NMR (400 MHz, MeOH-*d4*) = 9.10 (s, 1 H), 8.57 (s, 1 H), 5.17 (s, 2 H), 1.39 (s, 18 H); $^{13}$C NMR (101 MHz, MeOH-*d4*) = 170.0, 154.4, 153.9, 152.2, 150.3, 149.8, 133.3, 85.0, 45.3, 28.2; HRMS (ESI) calculated for $[C_{17}H_{24}N_5O_6]^+$: 394.1721, Found 394.1727.

**2-Aminopurinyl-9-acetic acid (13). 16** (1.74 g, 7.73 mmol) was dissolved in 95 % EtOH (170 mL) and $H_2O$ (170 mL) and the solution was heated to reflux. A Raney Ni slurry in $H_2O$ (20

mL) was added and the solution refluxed for 6.5 h. The solution was hot-filtered to remove the Raney Ni and the solvent evaporated. The slightly green solid remaining was dissolved in boiling $H_2O$ (350 mL) and hot-filtered again. The solvent was removed, and the residue was dissolved in a boiling $H_2O$:EtOH solution (100 mL, 1:1). The solution was hot-filtered and the mother liquor's solvent removed to yield **13** (0.327 g, 22 % yield) as a white powder: $^1$H NMR (400 MHz, DMSO-$d_6$) = 8.52 (br. s., 1 H), 7.93 (br. s., 1 H), 6.36 (br. s., 2 H), 4.37 (br. s., 2 H); HRMS (EI) calculated for $[C_7H_7N_5O_2]^+$: 193.0600, Found 193.0603.

**Benzyl 6-thio-2-aminopurinyl-9-acetate (14). 11** (3.19 g, 10.0 mmol) and thiourea (1.53 g, 20.0 mmol) were dissolved in iPrOH (150 mL) and heated to reflux for 0.5 h. The reaction mixture was cooled on an ice bath for 0.5 h then filtered. The filtrate was washed with cold iPrOH (50 mL), $Et_2O$ (50 mL), then dried under vacuum to yield **14** (3.11 g, 99 % yield) as a light-yellow powder: $^1$H NMR (400 MHz, DMSO-$d_6$) = 12.08 (s, 1 H), 8.05 (s, 1 H), 7.31 - 7.42 (m, 5 H), 6.96 (br. s., 2 H), 5.20 (s, 2 H), 5.00 (s, 2 H)

**6-Thio-2-aminopurinyl-9-acetic acid (16). 14** (3.11 g, 9.86 mmol) was dissolved in dioxane (90 mL) and cooled on an ice bath. LiOH (0.65 g, 27 mmol) dissolved in $H_2O$ (90 mL) was added and the solution was stirred for 2 h. A 1 M HCl solution was slowly added to adjust the pH to ~2.5 and the solution was filtered. The filtrate was washed with 0.25 M HCl (20 mL), $Et_2O$ (20 mL), then dried under vacuum to yield **16** (1.83 g, 82 % yield) as a light-yellow powder: $^1$H NMR (400 MHz, DMSO-$d_6$) = 11.99 (s, 1 H), 7.94 (s, 1 H), 6.89 (br. s., 2 H), 4.79 (s, 2 H); $^{13}$C NMR (101 MHz, DMSO-$d_6$) = 174.6, 169.1, 153.6, 148.0, 141.2, 126.7, 44.4

**6-Thio-2-aminopurine (18).** 6-Chloro-2-aminopurine (1.00 g, 5.90 mmol) and thiourea (0.457 g, 6.00 mmol) were dissolved in EtOH (75 mL) and heated to reflux for 1 h. The reaction mixture was put in the freezer overnight then filtered. The filtrate was washed with EtOH (50 mL), $Et_2O$ (50 mL), then dried under vacuum to yield **18** (0.937 g, 95 % yield) as a light-yellow powder: $^1$H NMR (400 MHz, DMSO-$d_6$) = 12.45 (br. s., 1 H), 8.60 (s, 1 H), 7.02 (br. s., 2 H)

**2-Aminopurine (19). 18** (0.602 g, 3.60 mmol) was dissolved in 95 % EtOH (50 mL) and $H_2O$ (50 mL) and the solution was heated to reflux. A Raney Ni slurry in $H_2O$ (7 mL) was added and

the solution refluxed for 9 h. The solution was hot-filtered through Celite and the solvent evaporated to yield **19** (0.281 g, 58 % yield) as a light yellow powder. $^1$H NMR (400 MHz, DMSO-$d_6$) = 12.54 (br. s., 1 H), 8.55 (s, 1 H), 8.04 (s, 1 H), 6.32 (s, 1 H); HRMS (EI) calculated for $[C_5H_5N_5]^+$: 135.0545, Found 135.0548.

**Ethyl 2-aminopurinyl-9-acetate (24). 21** (0.256 g, 1.00 mmol) was dissolved in a mixture of acetone (4 mL), $Et_3N$ (1.4 mL), and formic acid (0.2 mL). Pd/C (40mg) was added and the reaction mixture was brought to reflux. After 2 h, another equivalent of Pd/C (40 mg), $Et_3N$ (1.4 mL), and formic acid (0.2 mL) was added. After 2 h the mixture was filtered through Celite with acetone (100 mL) and the solvent removed. The residue was suspended in $H_2O$ (100 mL) and extracted with DCM (8 x 100 mL). The combined organic layers were dried over $Na_2SO_4$ and the solvent removed to yield **24** (0.192 g, 87 % yield) as a white solid. $^1$H NMR (400 MHz, $CDCl_3$) = 8.72 (br. s., 1 H), 7.82 (br. s., 1 H), 5.05 (br. s., 2 H), 4.81 - 4.93 (m, 2 H), 4.21 - 4.35 (m, 2 H), 1.21 - 1.39 (m, 3 H); HRMS (EI) calculated for $[C_9H_{11}N_5O_2]^+$: 221.0913, Found 221.0913.

**Ethyl di-Boc-2-aminopurinyl-9-acetate (25). 24** (0.10 g, 0.45 mmol), $Boc_2O$ (0.80 g, 3.6 mmol), and DMAP (0.165 g, 3.6 mmol) and $Et_3N$ (0.15 mL, 1.0 mmol) were added to THF (20 mL) and heated to reflux. After 3.5 h the solvent was removed and the residue dissolved in DCM (100 mL). A mixture of AcOH (5 mL) and $H_2O$ (100 mL) was added and the mixture extracted with DCM (2 x 100 mL). The combined organic layers were dried over $Na_2SO_4$ and the solvent was removed. The residue was purified by FCC (EtOAc-Hex = 1:1→10:0) to yield **25** (0.043 g, 23 % yield) as a white foam: $^1$H NMR (400 MHz, $CDCl_3$) = 9.15 (s, 1 H), 8.20 (s, 1 H), 5.04 (s, 2 H), 4.28 (q, $J$ = 7.0 Hz, 2 H), 1.43 (s, 18 H), 1.32 (t, $J$ = 7.2 Hz, 3 H); $^{13}$C NMR (101 MHz, $CDCl_3$) = 150.9, 149.5, 146.5, 110.1, 92.2, 83.2, 62.4, 44.0, 42.0, 27.7, 14.0; HRMS (EI) calculated for $[C_{19}H_{27}N_5O_6]^+$: 421.1961, Found 421.1953.

**Tetra-Boc-2,6-diaminopurinyl-9-acetate Fmoc/Bn PNA Backbone (28a).** Fmoc/Bn PNA backbone (0.061 g, 0.180 mmol), **8** (0.164 g, 0.270 mmol), EDC (0.056 g, 0.36 mmol) and HOBt hydrate (0.006 g, 0.04 mmol) were added to DCM (5mL) at 0 °C. The solution stirred for 20min then warmed to room temperature. The reaction was tracked by TLC. Upon completion, the

reaction mixture was diluted with DCM (100 mL) and washed against a mixture of a saturated $NaHCO_3$ solution (25 mL) and brine (50 mL). The organic layer was dried over $Na_2SO_4$, and the solvent removed. The residue was subjected to FCC (EtOAc-Hex = 2:1 to 1:0) to yield **28a** (0.131 g, 71 % yield) as a white powder: $^1$H NMR (400 MHz, DMSO-$d_6$) = 8.49 (s, min), 8.47 (s, maj.), 7.83 - 7.89 (m, 2 H), 7.66 (t, J = 6.3 Hz, 2 H), 7.25 - 7.49 (m, 9 H), 5.41 (br. s., 1 H), 5.23 (br. s., 2 H), 5.10 (s, 1 H), 4.50 (s, 1 H), 4.36 (d, J = 7.0 Hz, 1 H), 4.13 - 4.32 (m, 3 H), 3.50 - 3.60 (m, 1 H), 3.06 - 3.16 (m, 1 H), 1.28 - 1.37 (m, 36 H); $^{13}$C NMR (101 MHz, $CDCl_3$) = 154.6, 152.2, 151.1, 151.1, 150.2, 147.1, 144.0, 143.8, 141.5, 129.2, 129.1, 128.9, 128.8, 128.5, 128.0, 128.0, 127.3, 125.3, 125.1, 120.3, 84.0, 83.6, 67.6, 66.7, 49.4, 49.2, 47.4, 31.8, 28.0, 27.9, 22.9, 14.4; HRMS (ESI) calculated for $[C_{53}H_{65}N_8O_{13}]^+$: 1021.4671, Found 1021.4711.

**2-Di-Boc-aminopurinyl-9-acetate Fmoc/Bn PNA (28b).** Fmoc/Bn PNA backbone (0.276 g, 0.81 mmol), **10b** (0.264 g, 0.67 mmol), EDC (0.155 g, 1.00 mmol) and HOBt hydrate (0.026 g, 0.17 mmol) were added to DCM (5 mL) at 0 °C. The solution stirred for 40 min and then warmed to room temperature. After 2 days the reaction was diluted with DCM (150mL), washed with a saturated $NaHCO_3$ solution (100 mL), $H_2O$ (100 mL) and brine (100 mL). The organic layer was dried over $Na_2SO_4$ and the solvent removed. The residue was subjected to FCC (EtOAc-Hex = 4:1 to 1:0) to yield **28b** (0.313 g, 58 % yield) as a white foam: $^1$H NMR (400 MHz, DMSO-$d_6$) = 9.17 (s, 1 H), 8.47 (d, 1 H), 7.87 (d, J = 7.2 Hz, 2 H), 7.66 (t, J = 7.5 Hz, 2 H), 7.22 - 7.52 (m, 9 H), 5.40 (br. s., 1 H), 5.23 (br. s., 1 H), 5.09 (s, 1 H), 4.50 (br. s., 1 H), 4.10 - 4.40 (m, 4 H), 3.51 - 3.60 (m, 1 H), 3.07 - 3.15 (m, 1 H), 1.31 (s, 18 H); $^{13}$C NMR (101 MHz, $CDCl_3$) = 169.3, 156.6, 152.7, 151.0, 149.3, 147.4, 146.4, 143.7, 143.5, 141.2, 128.8, 128.6, 128.6, 128.2, 127.7, 127.7, 127.0, 124.8, 120.0, 109.9, 83.3, 67.4, 49.2, 47.1, 29.6, 27.8, 27.3; HRMS (ESI) calculated for $[C_{43}H_{48}N_7O_9]^+$: 806.3514, Found 806.3529.

**Tetra-Boc-2,6-diaminopurinyl-9-acetate Fmoc PNA Monomer (29a). 28a** (0.796 g, 0.78 mmol) was dissolved in MeOH (20 mL) and Pd/C (50mg) was added. The reaction vessel was placed under an atmosphere of $H_2$. The suspension was stirred for 3 h at room temperature. The reaction was filtered through Celite with MeOH (100 mL) and the solvent removed to yield **29a** (0.726 g, 96 % yield) as a flaky white solid: $^1$H NMR (400 MHz, Acetone-*d6*) = 8.31 (s, 1 H), 7.75 - 7.83 (m, 2 H), 7.63 (d, J = 7.4 Hz, 2 H), 7.22 - 7.39 (m, 4 H), 5.38 (s, 1 H), 5.26 (s, 1 H),

4.34 - 4.44 (m, 2 H), 4.09 - 4.29 (m, 3 H), 3.69 (t, J = 6.3 Hz, 1 H), 3.43 - 3.55 (m, 2 H), 3.25 - 3.33 (m, 1 H), 1.30 - 1.42 (m, 36 H); $^{13}$C NMR (101 MHz, Acetone-*d6*) = 171.0, 167.9, 167.3, 157.5, 155.8, 152.8, 151.7, 151.2, 151.0, 148.9, 145.1, 142.1, 128.6, 128.0, 128.0, 127.3, 126.2, 126.1, 120.9, 84.0, 83.5, 67.0, 48.1, 45.3, 40.0, 28.0, 27.9; HRMS (ESI) calculated for $[C_{46}H_{59}N_5O_{13}]^+$: 931.4196, Found 931.3406.

**2-di-Boc-aminopurinyl-9-acetate Fmoc PNA Monomer (29b). 28b** (0.313 g, 0.389 mmol) was dissolved in MeOH (10 mL) and Pd/C (100 mg) was added. The reaction mixture was placed under an atmosphere of $H_2$. The suspension was stirred for 12h at room temperature. The suspension was filtered through Celite with MeOH (100 mL) and the solvent removed to yield **29b** (0.266 g, 95% yield) as an off-white solid: $^1$H NMR (400 MHz, $CDCl_3$) = 8.99 - 9.21 (m, 2 H), 8.23 - 8.39 (m, maj.), 8.10 (br. s., min.), 7.62 - 7.81 (m, 2 H), 7.44 - 7.62 (m, 2 H), 7.11 - 7.41 (m, 4 H), 6.83 (br. s., maj), 6.05 (br. s., min.), 4.96 - 5.16 (m, 1 H), 4.85 (br. s., 1 H), 4.29 - 4.42 (m, 1 H), 4.11 - 4.27 (m, 2 H), 4.01 (br. s., 1 H), 3.77 (br. s., 1 H), 3.51 (br. s., 2 H), 3.26 - 3.42 (m, 1 H), 2.74 (br. s., 2 H), 1.44 (s, 18 H); $^{13}$C NMR (400 MHz, $CDCl_3$) = 171.6, 166.9, 166.3, 156.9, 152.7, 151.0, 148.9, 148.3, 143.6, 141.2, 131.4, 127.7, 127.1, 125.0, 124.3, 120.0, 83.6, 66.7, 50.6, 48.9, 47.1, 39.3, 27.8; HRMS (ESI) calculated for $[C_{36}H_{41}N_7O_9Na]^+$: 738.2863, Found 738.2877.

***N*-Boc-thyminyl-1-acetate Fmoc/Bn PNA Backbone (28c).** Fmoc/Bn PNA backbone (0.927 g, 2.13 mmol) and **3** (0.759 g, 2.67 mmol) were dissolved in DCM (10mL) and cooled to 0 °C. EDC (0.497 g, 3.20 mmol) and HOBt hydrate (0.081 g, 0.53 mmol) were added. The solution was stirred for 0.5 h then allowed to warm to room temperature. After 18 h a saturated $NaHCO_3$ solution (100 mL) was added and extracted with DCM (2 x 100 mL). The organic layer was dried over $Na_2SO_4$ and the solvent removed. The residue was purified by FCC (EtOAc-Hex = 8:2 to 9:1) to yield **28c** (1.14 g, 77 % yield) as a white foam: $^1$H NMR (400 MHz, DMSO-$d_6$) = 7.88 (d, J = 7.4 Hz, 2 H), 7.67 (d, J = 7.8 Hz, 2 H), 7.26 - 7.48 (m, 10 H), 5.21 (s, min.), 5.13 (s, maj.), 4.76 (s, 2 H), 4.58 (s, min.), 4.10 - 4.40 (m, 5 H), 2.99 - 3.61 (m, 5 H), 1.79 (s, 3 H), 1.48 (s, 9 H); $^{13}$C NMR (101 MHz, $CDCl_3$) = 170.7, 169.0, 168.8, 167.2, 166.9, 161.4, 161.3, 156.4, 156.3, 148.7, 148.6, 147.7, 143.5, 143.4, 140.8, 140.5, 140.3, 134.7, 134.4, 128.2, 128.1, 128.1, 127.9, 127.8, 127.4, 127.3, 126.7, 124.7, 119.6, 109.7, 109.5, 86.3, 86.2, 67.4, 66.8, 66.4, 66.2,

60.0, 49.8, 48.5, 48.2, 47.9, 47.6, 47.5, 46.7, 38.8, 38.4, 27.0, 20.6, 13.8, 11.9; HRMS (EI) calculated for $[C_{38}H_{40}N_4O_9Na]^+$: 719.2693, Found 719.2693.

***N*-Boc-thyminyl-1-acetate Fmoc PNA Monomer (29c). 28c** (0.251 g, 0.36 mmol) was dissolved in minimal acetone:MeOH solution (1:1, v/v). Pd/C (80 mg) was added and the reaction flask was placed under an atmosphere of $H_2$ at 0 °C. After 0.5 h the suspension was stirred at room temperature for a further 2 h. The solvent was removed and the residue suspended in MeOH (100 mL). The suspension was filtered through Celite, and the solvent removed to yield **29c** (0.212 g, 95 % yield) as an off-white powder: $^1$H NMR (400 MHz, DMSO-$d_6$) = 12.79 (s, 1 H), 7.89 (d, J = 7.4 Hz, 2 H), 7.68 (d, J = 7.4 Hz, 2 H), 7.10 - 7.52 (m, 5 H), 4.74 (s, 1 H), 4.55 (s, 1 H), 4.14 - 4.39 (m, 3 H), 4.00 (s, 1 H), 3.19 - 3.47 (m, 5 H), 3.03 - 3.17 (m, 1 H), 1.79 (s, 3 H), 1.49 (s, 9 H); $^{13}$C NMR (151 MHz, DMSO-$d_6$) = 171.0, 170.6, 167.8, 167.4, 166.8, 164.5, 161.4, 156.5, 156.3, 151.1, 148.6, 148.1, 144.0, 142.1, 140.8, 127.7, 127.2, 125.2, 120.2, 108.3, 86.2, 65.7, 47.9, 47.1, 46.9, 31.4, 27.1, 12.0; HRMS (ESI) calculated for $[C_{31}H_{34}N_4O_9Na]^+$: 629.2223, Found 629.2252.

## References


(1) Altenbrunn, F.; Seitz, O. *Organic & Biomolecular Chemistry* **2008**, *6*, 2493.
(2) Wojciechowski, F.; Hudson, R. H. E. *The Journal of Organic Chemistry* **2008**, *73*, 3807.
(3) Lohse, J.; Dahl, O.; Nielsen, P. E. *Proceedings of the National Academy of Sciences* **1999**, *96*, 11804.
(4) Jeon, J. W.; Son, S. J.; Yoo, C. E.; Hong, I. S.; Song, J. B.; Suh, J. *Organic Letters* **2002**, *4*, 4155.
(5) Rachofsky, E. L.; Osman, R.; Ross, J. B. A. *Biochemistry* **2001**, *40*, 946.
(6) P. Gangamani, B.; A. Kumar, V. *Chemical Communications* **1997**, 1913.
(7) O'Neil, M. A.; Barton, J. K. *Journal of the American Chemical Society* **2002**, *124*, 13053.
(8) Breeger, S.; von Meltzer, M.; Hennecke, U.; Carell, T. *Chemistry – A European Journal* **2006**, *12*, 6469.
(9) Pothukanuri, S.; Pianowski, Z.; Winssinger, N. *European Journal of Organic Chemistry* **2008**, *2008*, 3141.
(10) Sikchi, S. A.; Hultin, P. G. *The Journal of Organic Chemistry* **2006**, *71*, 5888.
(11) Bendich, A.; Russell, P. J.; Fox, J. J. *Journal of the American Chemical Society* **1954**, *76*, 6073.
(12) Sigma Aldrich Catalogue Vol. 2011.
(13) Dey, S.; Garner, P. *The Journal of Organic Chemistry* **2000**, *65*, 7697.
(14) Štimac, A.; Kobe, J. *Synthesis* **1990**, *1990*, 461.
(15) Porcheddu, A.; Giacomelli, G.; Piredda, I.; Carta, M.; Nieddu, G. *European Journal of Organic Chemistry* **2008**, *2008*, 5786.

# Chapter 3

## Azide Functionalized PNA

### Introduction

The synthesis of a PNA monomer with the ability to be functionalized after peptide synthesis is of interest to researchers to allow a combinatorial approach to a variety of PNAs from a single solid phase peptide synthesis (SPPS.) The introduction of an azide into a PNA monomer would allow us to introduce a variety of fluorophores, metal-binding ligands, or create expanded nucleobase "fleximers" similar to Seley's work in nucleoside chemistry.[1]

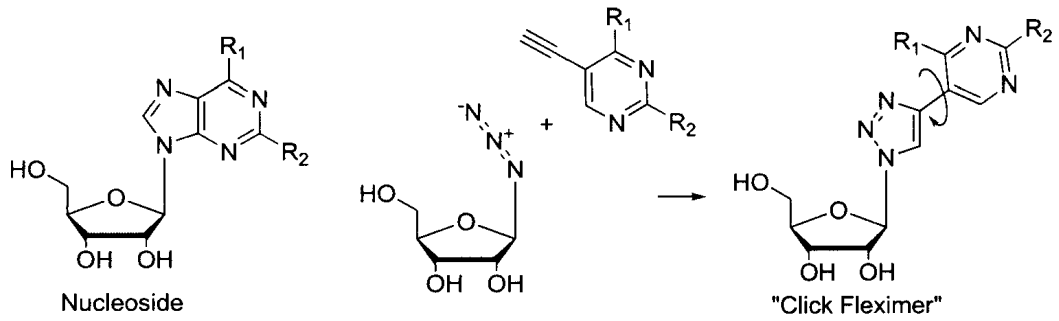
The introduction of fluorophores into PNA has been used for the detection of mutations in nucleic acids, detection of specific sequences through hairpin molecular beacons, and the detection of abasic sites.[2] Abasic sites are an indicator of cell damage, and our group is interested in the detection of these sites using fluorescent nucleobases.

Metal binding ligands have been introduced into PNA to allow them the bind tighter to nucleic acids, for metal detection, or to form highly organized structures.[3] To our knowledge, introduction of a metal-binding ligand into PNA has required the synthesis of a separate PNA monomer for each ligand. The use of a click reaction to form a ligand with the *N*-2 on the triazole helping coordinate a metal would allow a facile synthesis of different metal-binding PNAs from a single monomer.

One structural motif commonly used in nucleic acid chemistry is a hairpin, where two complementary ends are attached by a flexible linker. The arms can fold over and self-associate to form a complex that resembles a hairpin. This type of structure has been used for detection of nucleic acid complements as molecular beacons,[4] or the detection of metals.[5] Several PNA

hairpins have been synthesized,[6,7] and these structures have potential in nucleic acid detection or as molecular switches.

Fleximers are molecules in which a purine nucleobase has been separated to its imidazole and pyrimidine rings, and they have potential as medications and as probes for examining protein-nucleobase interactions. The synthesis of imidazole fleximers is reported to be laborious and synthetically challenging.[1] We envisioned synthesizing a single azide, then being able to perform the cycloaddition with a variety of alkynes to form a 1,2,3-triazole fleximer (**Scheme 3.1**.)




**Scheme 3.1** Modular approach to click fleximers.

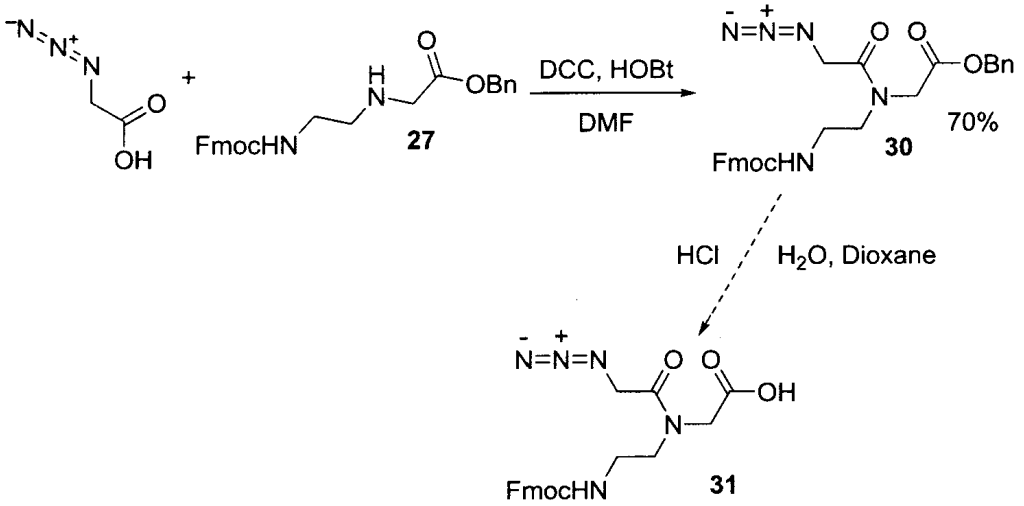
The goal of this project is to synthesize a PNA monomer containing an azide and a variety of alkynes to undergo the click reaction. These "click products" can contain a fluorophore for abasic site detection, a metal binding ligand, or a click fleximer to form expanded base pairs.

## Results and Discussion

### 3.1 Synthesis of a PNA Monomer Containing a Metal Ligand

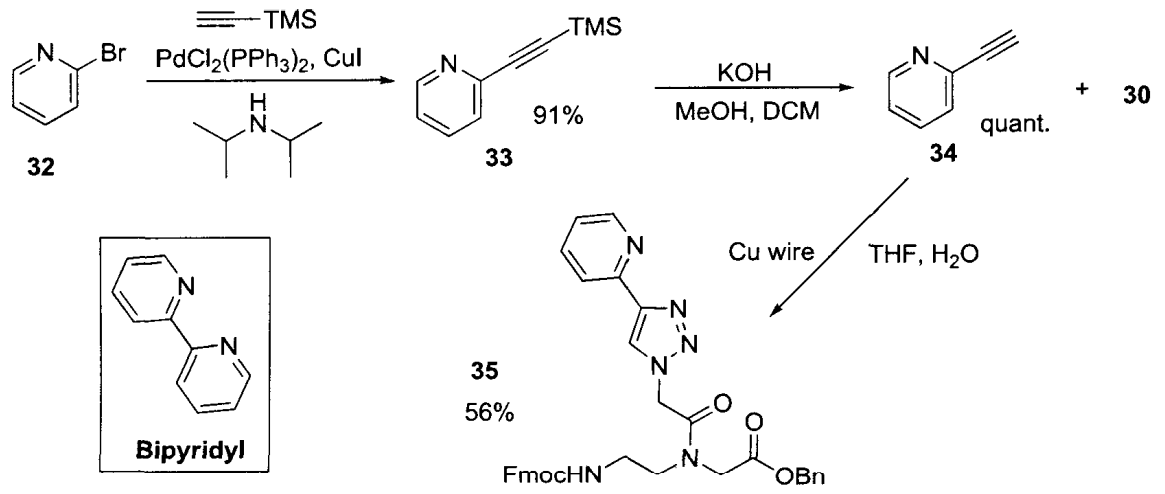
The first attempt at an azide-containing PNA monomer proceeded through a coupling of azido acetic acid with the Fmoc/Bn PNA backbone **27** to produce **30** (**Scheme 3.2**.) Attempts to yield the azide PNA monomer **31** through benzyl hydrogenation would yield the primary amine,

and a basic ester hydrolysis would remove the Fmoc protecting group. An acid ester hydrolysis was attempted, but did not give a practical amount of the product.



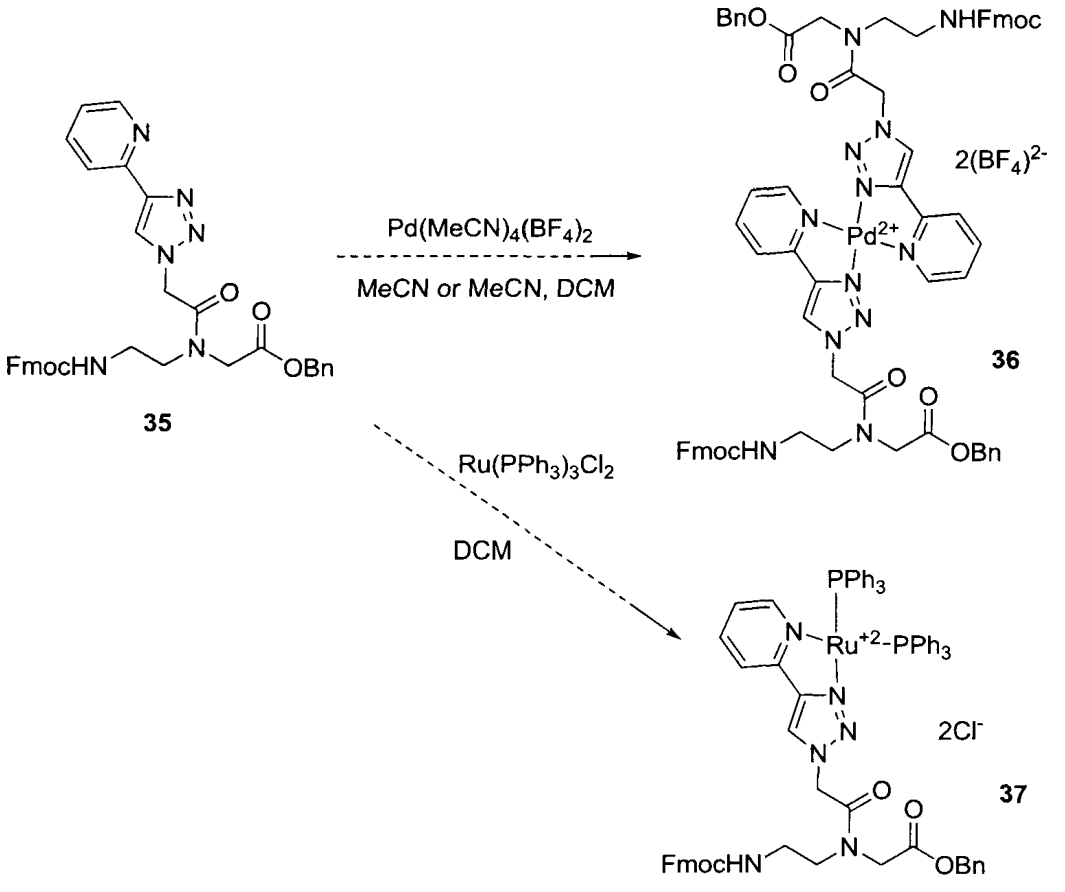

**Scheme 3.2** The coupling of azidoacetic acid to Fmoc/Bn PNA backbone to form azide-funtionalized PNA benzyl ester.

The alkyne 2-ethynylpyridine was synthesized (**Scheme 3.3**) to undergo the click reaction to form a bipyridinyl analogue. Bipyridine is a common bidentate ligand in inorganic chemistry and has been incorporated into PNA.[8] We predicted that the click product **35** would be able to bind ligands effectively. The synthesis began with 2-bromopyridine (**32**) following literature procedure.[9] The coupling of azide **31** with alkyne **34** could not be performed using $CuSO_4$ and NaAsc in a tBuOH-$H_2O$ solution, but proceeded very cleanly in THF-$H_2O$ using a clean piece of copper wire as catalyst to yield the metal-binding ligand **35**.



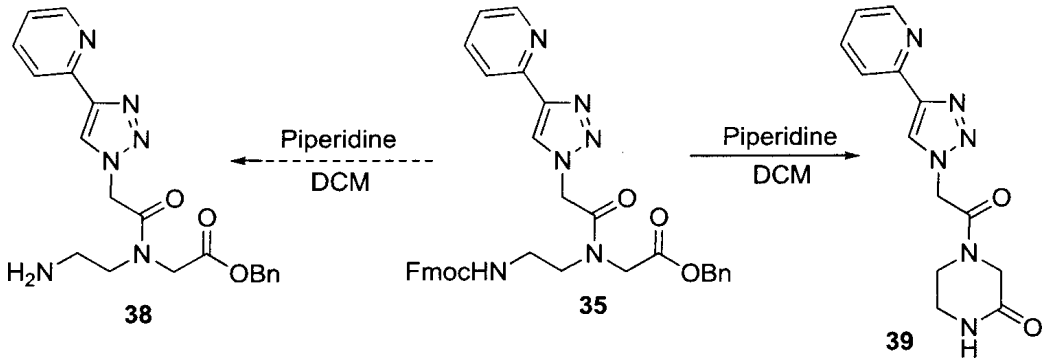

**Scheme 3.3** The synthesis of 2-ethynylpyridine and its coupling to azide-funtionalized PNA benzyl ester to form click PNA **35**.

Attempts were made to synthesize and crystallize metal complexes with our PNA ligand (**Scheme 3.4**) in order to characterize the metal binding coordination. Under a large variety of crystallization conditions, no crystals suitable for x-ray crystallography were produced.




**Scheme 3.4** Attempts to synthesize metal complexes of **35** with Pd(II) or Ru(II).

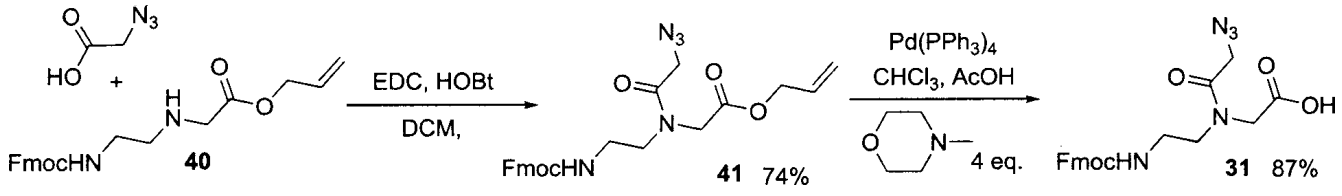
We theorized that the large, flexible, non-polar protecting groups may hinder crystal growth, so an attempt was made to remove the Fmoc group to increase the polarity of the ligand (**Scheme 3.5.**) This was met by a side-reaction[10] that forms the 6-membered lactam and was not pursued further.




**Scheme 3.5** Fmoc removal on a PNA benzyl ester monomer leads to formation of the γ-lactam.

The second attempt towards an azide-appended PNA would require an ester that could be cleaved without using base or hydrogenation conditions. PNA backbone submonomers with the t-butyl or allyl ester were available in our laboratory. An attempt to couple azido acetic acid to the PNA backbone t-butyl ester failed to give the desired product. The backbone was stored as the hydrochloride salt to protect the Fmoc group from the secondary amine, but this may have resulted in significant degradation.

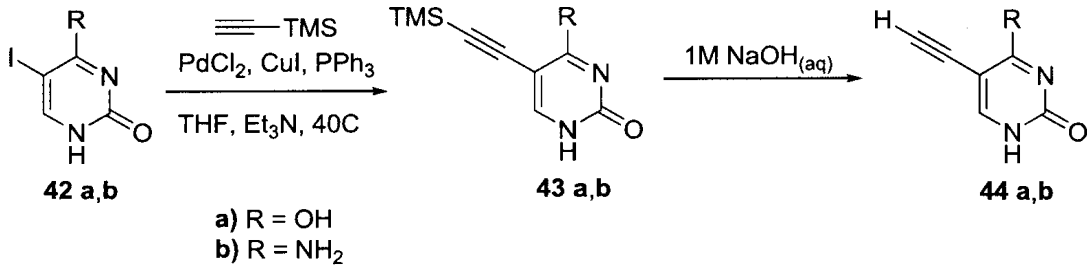
The third attempt to synthesize the azide PNA monomer **31** used the allyl ester backbone which was coupled to azido acetic acid in good yield (**Scheme 3.6.**) There is literature precedence for using Pd(0) to remove allyl esters without affecting azide functional groups,[11] and this reaction was found to be high yielding for the synthesis of the azide PNA monomer **31**, ready for use in peptide synthesis.




**Scheme 3.6** The coupling of azidoacetic acid to Fmoc/Bn PNA backbone to form azide-funtionalized PNA benzyl ester.

## 3.2 Synthesis of Substrates for Click Chemistry

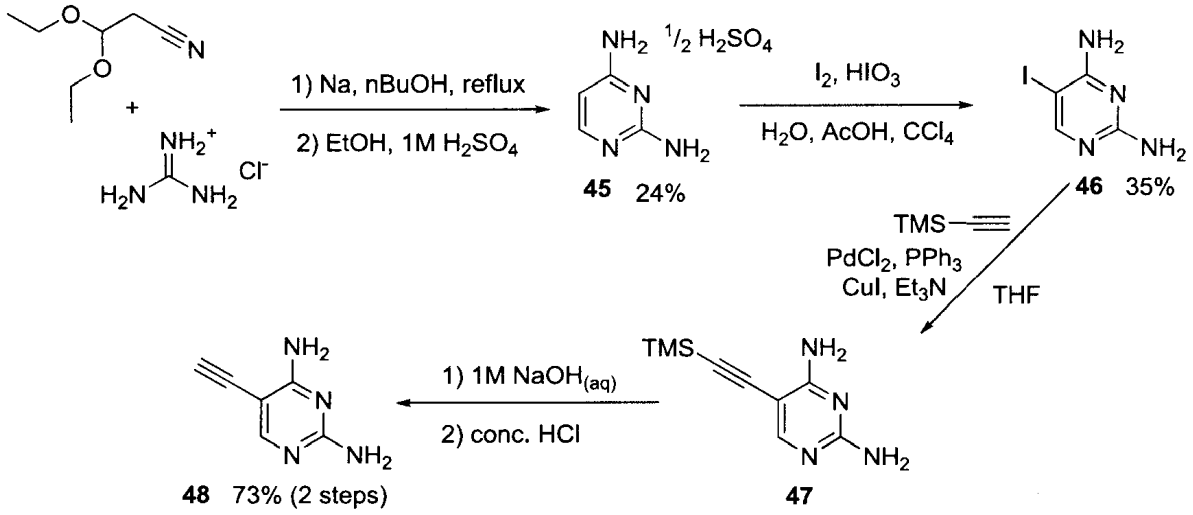
The synthesis of a variety of 5-ethynylpyrimidines was undertaken to act as candidates for the click reaction. The synthesis followed a simple two-step reaction starting from 5-iodo or 5-bromopyrimidines. A Sonogashira reaction between the aryl halide and TMS-acetylene generally proceeded smoothly, and the crude product could be used directly in the TMS removal step. The first pyrimidines examined were uracil (**44a**) for its acceptor-donor-acceptor moiety as a xanthine analogue, followed by cytosine (**44b**) an iso-guanine analogue (**Scheme 3.7.**) The one-pot reaction proceeded cleanly and avoided the need for purification by column chromatography.




**Scheme 3.7** The one-pot synthesis of 5-ethynyluracil and 5-ethynylcytosine.

The next alkyne examined was 2,4-diaminopyrimidine (**48**) for its ability to form a donor-acceptor-donor base pair similar to 2,6-diaminopurine (**Scheme 3.8.**) The nucleobase was synthesized through a condensation between 3,3-diethoxypropionitrile and guanidine hydrochloride, an often low-yielding reaction for pyrimidine. The product **45** was isolated as a sulphate salt, and neutralization to the free base proved troublesome. The salt was used directly

in an iodination reaction to provide the 5-iodo derivative **35** in low yield. The one-pot Sonogashira reaction then TMS removal required some changes compared to the uracil/cytosine derivatives. The previous purifications after TMS removal relied on a cyclic proton deprotonation rendering the product soluble in water. The absence of a ketone moiety for 2,4-diaminopyrimidine meant a different purification was required. The best results were obtained by performing the TMS removal with NaOH, then solubilising the product with HCl. The bis(trimethylsilyl)ether was filtered off, and upon neutralization with NaOH the product (**48**) could be extracted into an organic solvent.

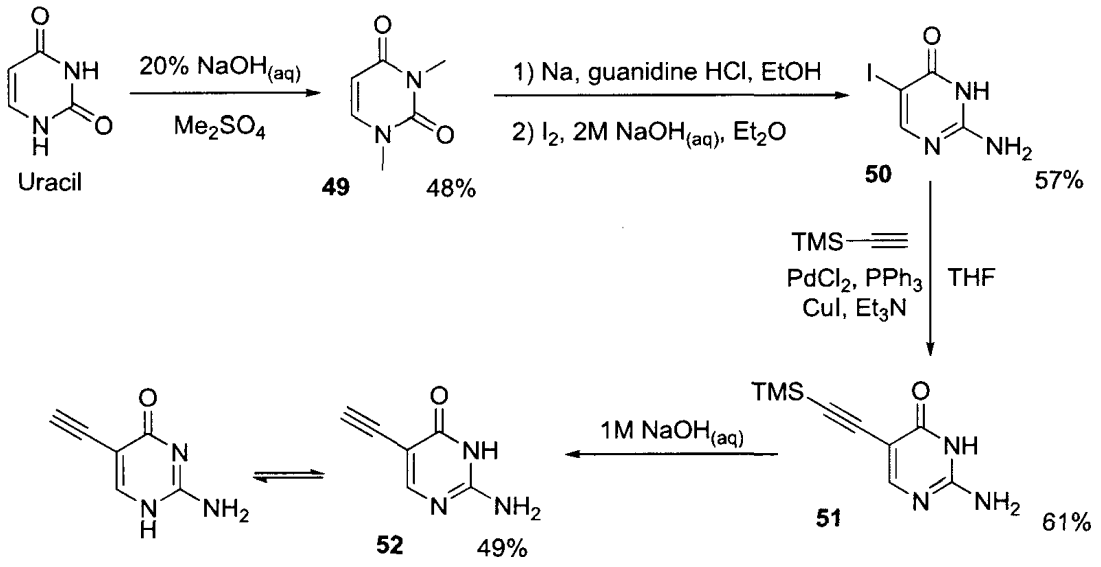



**Scheme 3.8** The synthesis of 5-ethynyl-2,4-diaminopyrimidine.

The fourth pyrimidine examined was isocytosine which possesses two semi-stable tautomers[12] (**Scheme 3.9.**) The tautomerization in base-pairing could introduce mutations into DNA replication, potentially finding use as an antibiotic or antiviral drug.

The synthesis began with the dimethylation of uracil to form **49** followed by the one-pot replacement of the dimethylurea moiety with guanidine and a 5-position iodination to give **50**. The Sonogashira reaction with TMS-acetylene provided the fluorescent nucleobase **51**, which was isolated rather than continuing the TMS-removal. Fluorescent nucleobases, especially minimally modified base discriminating fluorophores,[13] are of interest in our laboratory for

nucleic acid detection. Unfortunately fluorescence was quenched upon TMS removal to form the alkyne **52**, but fluorescence may be re-introduced through a click reaction with an appropriate azide.

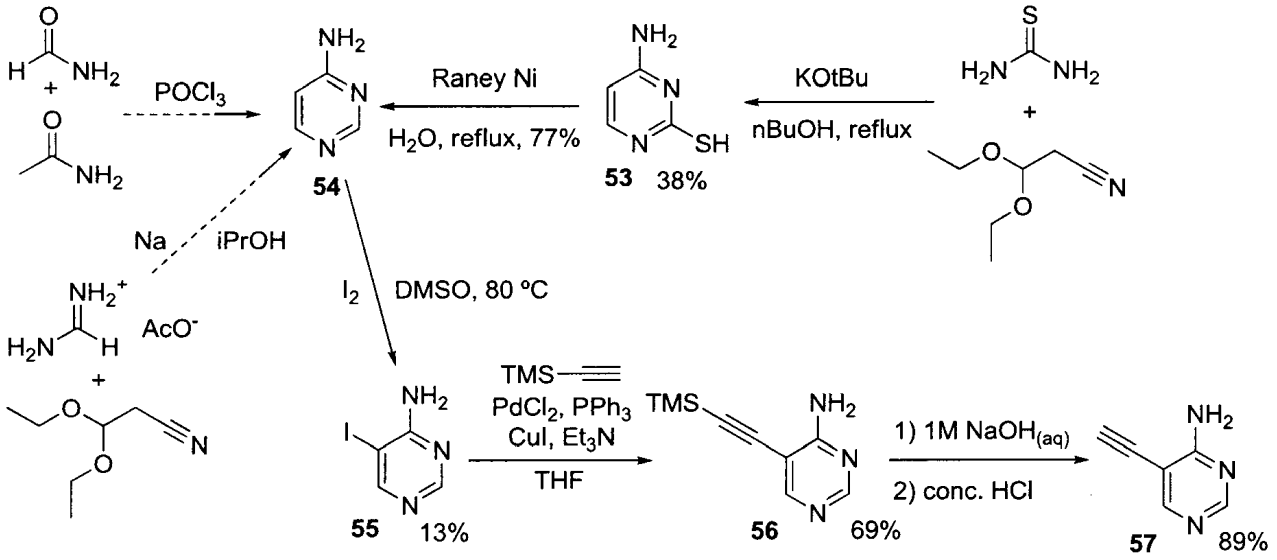



**Scheme 3.9** The synthesis of 5-ethynyl-isocytosine and its two tautomeric forms.

The next pyrimidine examined was 4-aminopyrimidine (**54**,) an analogue of adenine. The nucleobase is expensive (1g = $250,)[14] therefore a literature preparation was attempted using a condensation of formamide and acetamide using phosphoryl chloride (**Scheme 3.10**.) This dangerous reaction is performed in a pressure vessel at 120 °C, producing a large amount of hydrogen chloride as the reaction proceeds. After two failed attempts to repeat this literature procedure, a different route was examined using a condensation of 3,3-diethoxypropionitrile and formamidine acetate. Again, the literature procedure could not be reproduced and a different pathway had to be imagined.

The synthesis through a 4-amino-2-thiopyrimidine intermediate (**53**) has been reported,[15] and had the advantage of being able to be performed on a large scale (~5g), using relatively inexpensive starting materials, and no column chromatography. The condensation between thiourea and 3,3-diethoxypropionitrile provided a large amount of intermediate **53** to work with.

A portion of this material was used in a desulphurization reaction using Raney Ni to provide 4-aminopyrimidine (**54**) in good yield. Several iodination reactions were performed, and this pyrimidine proved especially unreactive and the product difficult to isolate. The only reaction to provide the 5-iodo derivative was elemental iodine in DMSO at elevated temperatures and this produced **55** in extremely low yield. Nevertheless, the Sonogashira was performed in moderate yield and the TMS derivative **56** was isolated. Analogous to the 2,4-diaminopyrimidine derivative, the TMS removal was performed using the product's aqueous solubility in low pH to afford the alkyne **57**.



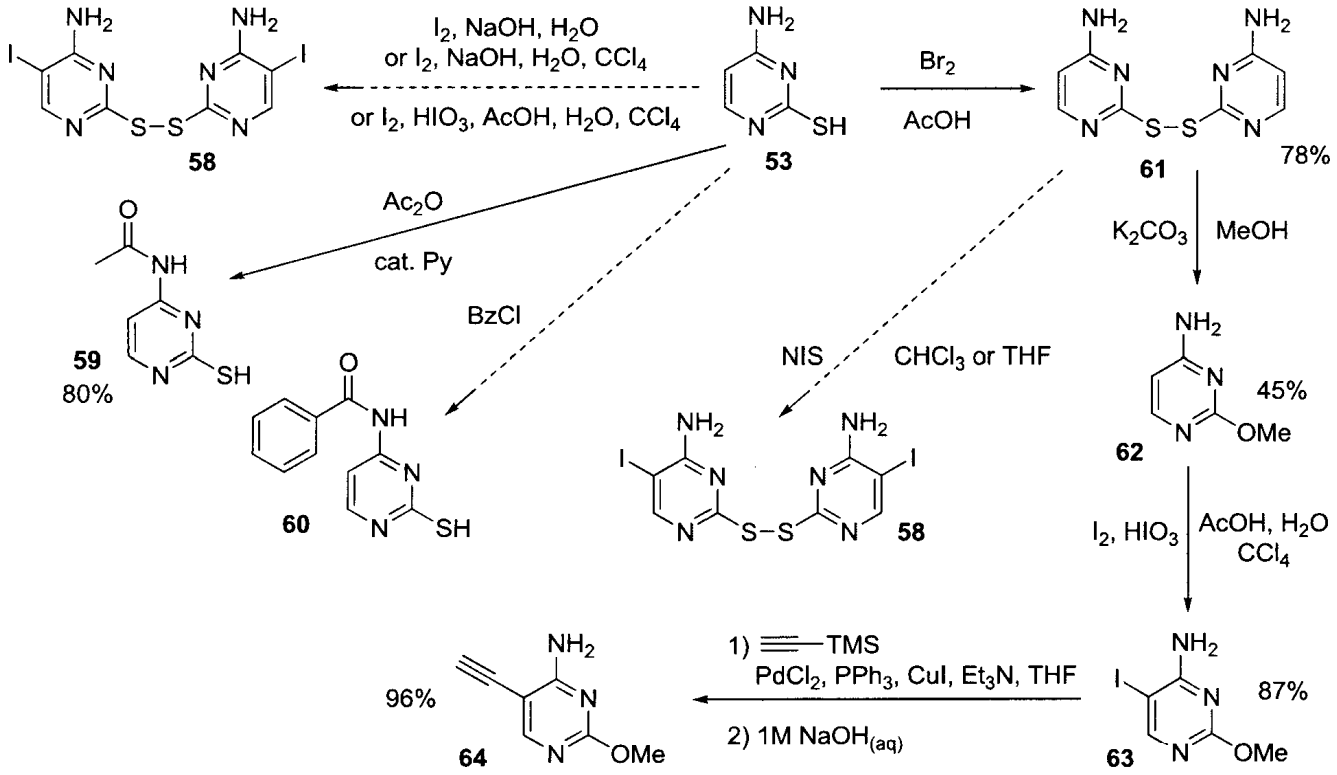

**Scheme 3.10** The synthesis of 5-ethynyl-4-aminopyrimidine.

The ease of synthesis of the 4-amino-2-thiopyrimidine (**53**) spurred an interest in synthesizing a thiocytosine alkyne (**Scheme 3.11.**) The thiol required protection, as we expected an unprotected thiol to quench the Pd (0) catalyst during a Sonogashira reaction. One protection strategy is to oxidize the thiol to a disulfide bridge, perform the necessary reactions, then reduce the compound back to the thiol. Elemental iodine is known to oxidize thiols to disulfides, so several attempts were made to simultaneously dimerize and iodinate the thiocytosine to **58** with no success.

The next attempt was to protect the thiol through either acetylation (**59**) or benzoylation (**60**.) The acetylation was the only reaction that gave a product that could be isolated in acceptable yields, and evidence pointed towards the acetylation occurring on the exo-cyclic nitrogen.

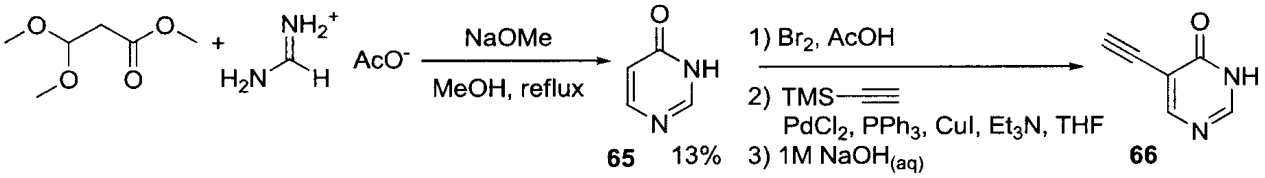
The failure of iodine as an oxidizing/iodinating agent prompted examination of bromine to perform the same reaction. Using 3 equivalents of elemental bromine, the reaction goes very cleanly to the disulfide derivative of thiocytosine (**61**.) Attempts to iodinate this intermediate to **58** were abandoned after several failed attempts.

A literature search for reactions that can be performed on disulfides of pyrimidine analogues produced a reaction where a sulphur bond is cleaved using base and methanol to produce *O*-methylcytosine **62** in moderate yield.[16] This compound can only form 2 hydrogen bonds with guanine, with one steric clash. The *O*-methylcytosine analogue synthesized could potentially form a proper base pair with hypoxanthine. The synthesis of the 5-iodo derivative **63** was performed, followed by the coupling to TMS acetylene and the TMS deprotection to form the alkyne **64**. The TMS deprotection step didn't require an acidic work-up because of the organic solubility afforded by the *O*-methyl group.




**Scheme 3.11** The synthesis of 5-ethynyl-2-methoxy-4-aminopyrimidine.

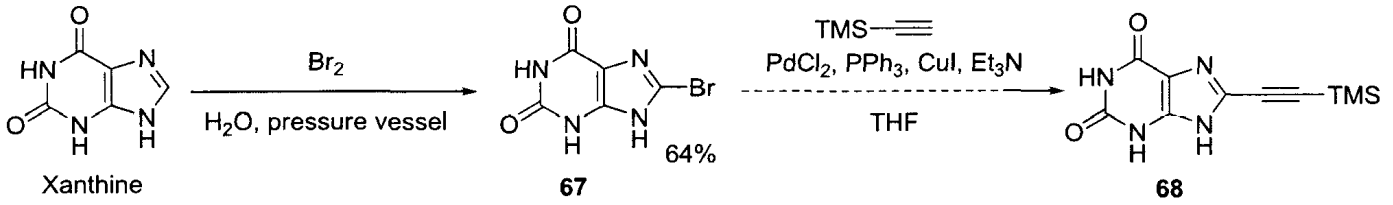
The next pyrimidine examined was 4-pyrimidone for its ability to base pair to adenine (**Scheme 3.12.**) The condensation of methyl 3,3-dimethoxypropionate with formamidine acetate gave 4-pyrimidone (**65**) in very low yield. The 5-position bromination was isolated as the hydrobromide salt, which was used directly in the Sonogashira reaction followed by the TMS removal to produce the alkyne **66** in low yield.




**Scheme 3.12** The synthesis of 5-ethynyl-4-pyrimidone.

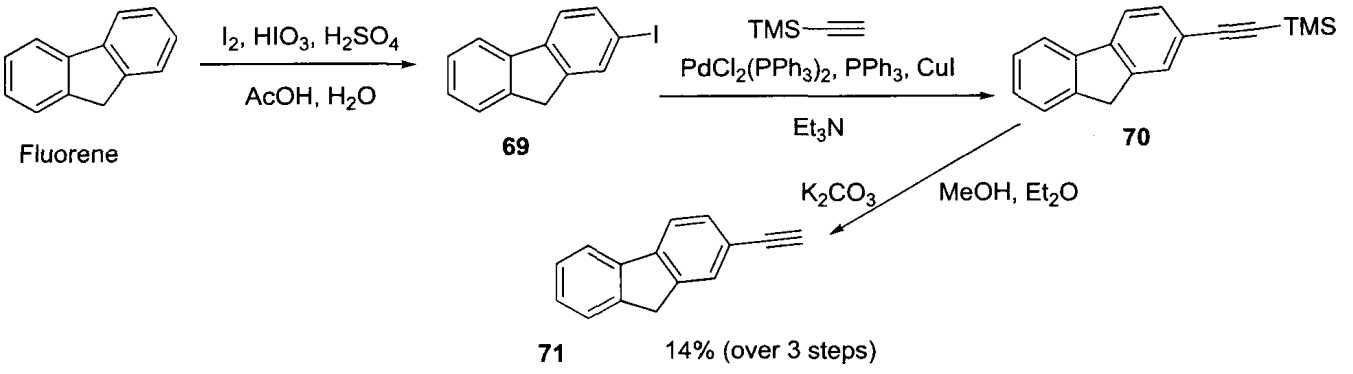
The next target was 8-ethynylxanthine, which is an interesting target for an expanded 3-ring nucleobase (**Scheme 3.13.**) A bromination was carried out following literature procedure[17] to give the 8-bromo product **67** cleanly. Two Sonogashira reactions were attempted, and they were run until complete disappearance of the starting material. No compounds could be isolated

from the complex mixture of products, with the most likely explanation being that the reaction produced an unstable/reactive intermediate and that perhaps **68** could not be synthesized using this strategy.




**Scheme 3.13** The attempted synthesis of 8-ethynylxanthine.

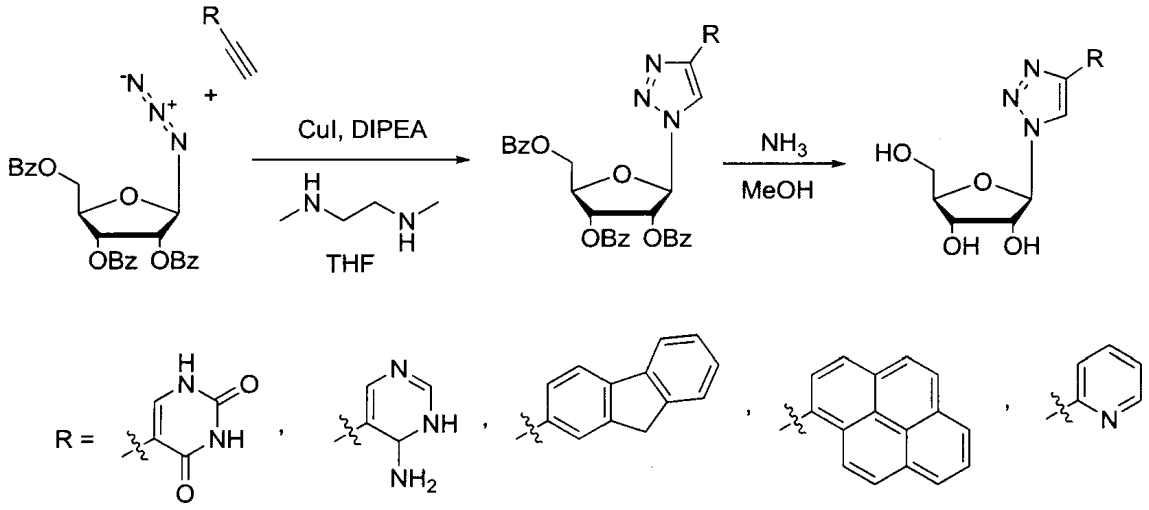
The 2-ethynylfluorene (**71**) moiety has been used by our lab as a fluorescent reporter group appended at the 5-position of uracil.[18] This project would use the alkyne in a click reaction, and with the 1,2,3-triazole in conjugation with the fluorene this would produce a new fluorophore. A synthesis was performed following a literature procedure[18] (**Scheme 3.14**) to synthesize **71** on a large scale for fluorescent reporting. The synthesis gave typical yields starting from the relatively inexpensive starting material fluorene to produce the alkyne **71**.




**Scheme 3.14** The synthesis of 2-ethynylfluorene.

## 3.3 Click Reactions of Alkynes to form "Click Fleximers"

Several of the alkynes were used in a click reaction by our undergraduate thesis student Leslie Bean on an azide-appended ribose (**Scheme 3.15.**) These fleximers are analogous to Seley's wherein the imidazole is replaced with a 1,2,3-triazole. The advantage to this method is that a single azide can be coupled to a variety of alkynes with ease.




**Scheme 3.15** "Click fleximers", "click fluorophores", and metal-binding ligand appended ribose synthesized by Leslie Bean.[19]

The compounds show promise as fluorophores and gelators, and examination of the base pairing, fluorescence, and metal binding properties are in progress. The "click fleximers" of pyrimidines may have potential as antiviral agents for their ability to introduce mutations in DNA replication.

3.4 Azide Containing PNA

With the azide containing PNA monomer **31** in hand, SPPS was performed to create two oligomers (**Fig. 3.1**) to examine the ability to do the "click" reaction on the resin.

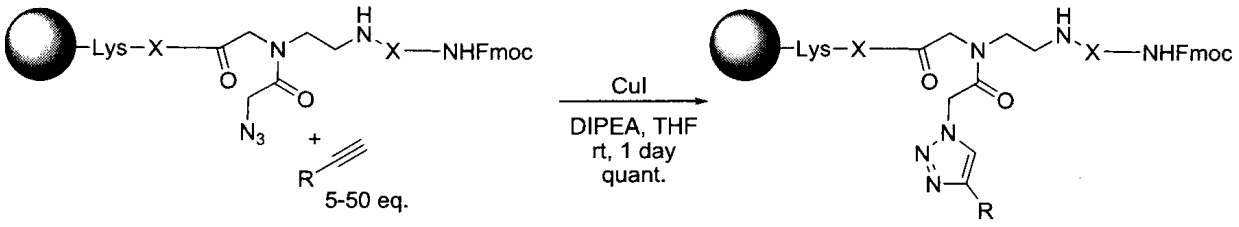
| | Sequence | Tm (°C) |
|---|---|---|
| **PNA3** | $^{N}$T-G-C- **Az**-C-T-G-Lys$^{C}$ | - |
| **PNA3F** | $^{N}$T-G-C- **F** -C-T-G-Lys$^{C}$ | 24.0 |
| **PNA3P** | $^{N}$T-G-C- **P** -C-T-G-Lys$^{C}$ | 20.5 |
| **DNA3** | $^{3'}$C-A-A-C-G- **S** -G-A-C-C-T$^{5'}$ | - |
| **PNA4** | $^{N}$Lys-**Az**-C-C-T-G-T-T-T-T-T-C-A-G-G-**Az**-Lys$^{C}$ | - |
| **PNA4M** | $^{N}$Lys-**M** -C-C-T-G-T-T-T-T-T-C-A-G-G-**M** -Lys$^{C}$ | - |

**Figure 3.1** The PNAs synthesized and the DNA complement where **Az** = azide, **F** = fluorenyl click, **P** = pyrenyl click, **S** = abasic site, and **M** = metal-binding. The Tms were calculated at 2 μM in a buffer containing NaCl (100 mM), EDTA (0.1 mM), and $Na_2PO_4$ (10 mM, pH 7).

The solid phase click reaction has several advantages:

1. Only a single monomer is synthesized to access a variety of "clicked bases."
2. An excess of alkyne can be used. After the reaction the unreacted starting material and the catalyst could be washed away.
3. The click reaction could be automated, allowing different alkynes to be clicked at different times during peptide synthesis.
4. The resin could be split and used to produce different clicked products from one peptide synthesis.

There is some literature precedence for azides that are α to a carbonyl acting as a leaving group in peptide synthesis, but our monomer was found to be stable to our Fmoc SPPS conditions. Several click conditions were explored, with the best results placing the resin in a vial with an excess of alkyne, CuI, and DIPEA in either THF or DMF (**Scheme 3.16**).

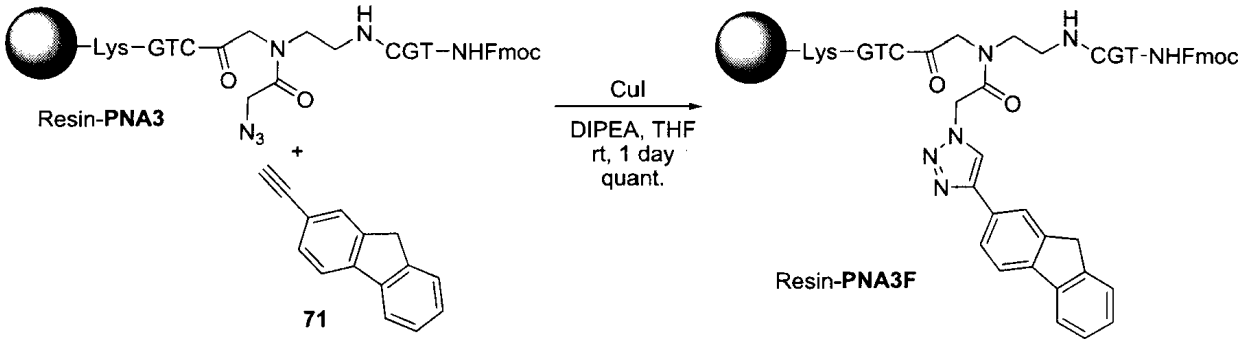



**Scheme 3.16** A solid phase click reaction on an azide-functionalized PNA.

After shaking the vial overnight, the unreacted starting materials and catalyst were removed by washing the resin with DMF, DCM, MeOH, and $H_2O$. Thus far, click reactions have been performed at the end of the peptide synthesis with the terminal Fmoc protecting group attached. The resin is then deprotected with 20% piperidine/DMF and the PNA cleaved from the resin using TFA (1mL) and one drop of $Et_3SiH$, and the "click bases" synthesized have been stable to these conditions.

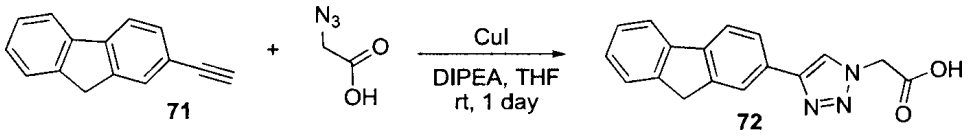
3.5 Synthesis of Fluorescent "Click" PNA

The first fluorophore examined was fluorene (**Scheme 3.17.**) The click reaction using 2-ethynylfluorene (**71**) proceeded quantitatively, with no detection of the unreacted azide by LC/MS.




**Scheme 3.17** The solid phase click reaction of 2-ethynylfluorene on an azide-functionalized PNA.

To quantify the concentration of **PNA3F**, the $\varepsilon_{260}$ of the individual components are summed, necessitating the synthesis of a fluorene click model compound (**Scheme 3.18.**)




**Scheme 3.18** The synthesis of "fluorene click" model compound **72**.

The second fluorophore appended was pyrene in the same fashion (**Fig. 3.2.**) The click reaction to form a model compound of pyrene analogous to **72** proved difficult to synthesize in sufficient purity, so the $\varepsilon_{260}$ of N-methyl-1-aminopyrene was used.[20]

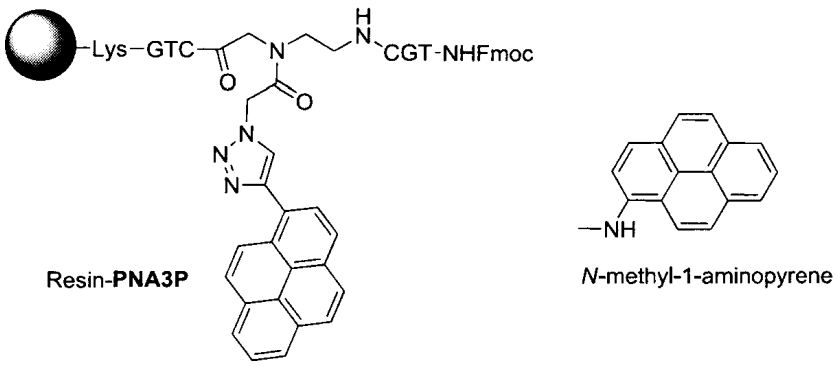



**Figure 3.2** The "pyrene click" **PNA3P** and the model compound used.

3.6 Binding/Fluorescence Studies of Fluorescent "Click" PNA

The 11-mer DNA sequence **DNA3** was purchased from the University of Calgary University Core DNA Services containing an abasic site in the middle position. The **PNA3**:**DNA3** duplexes were examined in a binding study. The Tm for the **PNA3F**:**DNA3** duplex was 24 °C, and for **PNA3P**:**DNA3**, 20.5 °C. The pyrene was slightly destabilizing, which could be related to the sterics of the fluorophores sticking into the abasic site. The fluorene was also destabilizing compared to literature 7mer PNA:DNA duplexes (Tm ~30 °C), indicating the size of the fluorophores may be too large to avoid a steric clash (**Fig. 3.3**.)

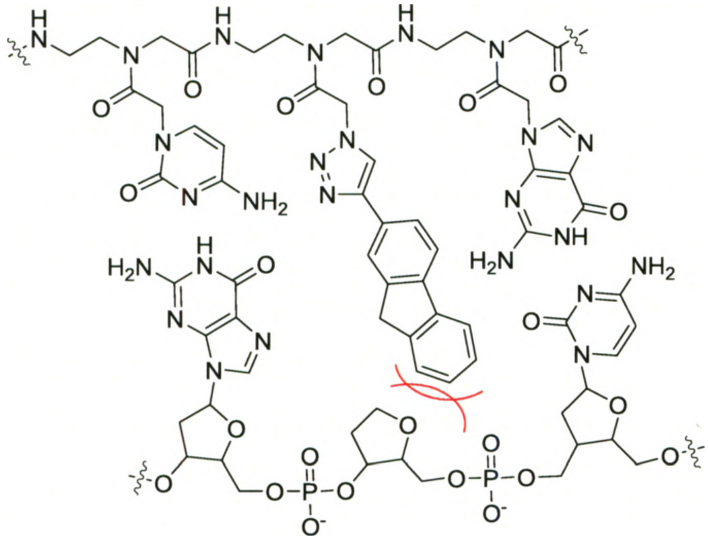
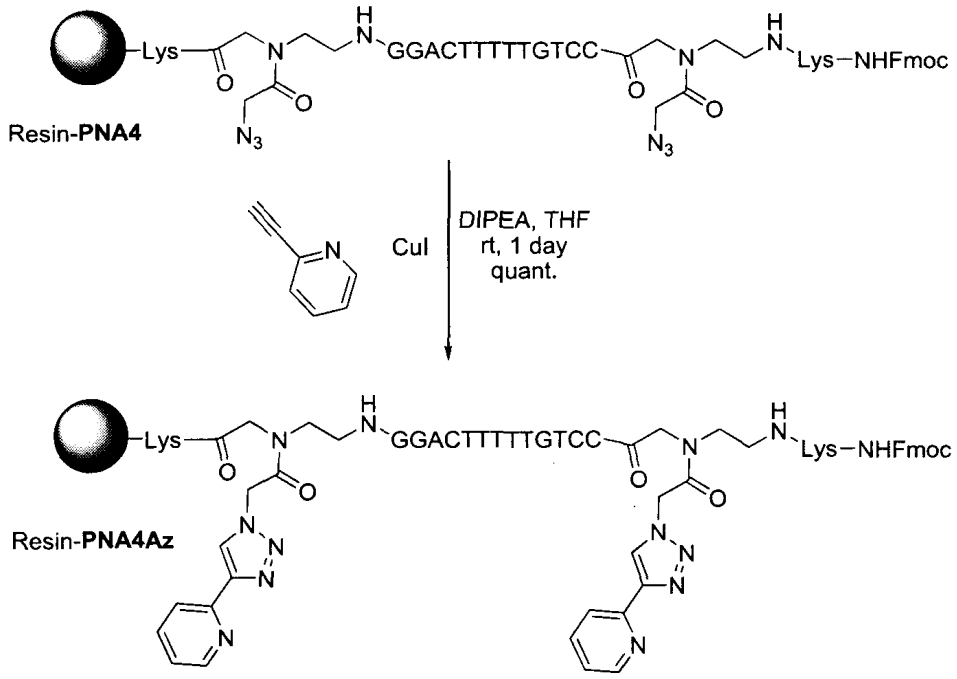


**Figure 3.3** The binding pocket of the fluorene-click nucleobase with a potential steric clash in red.

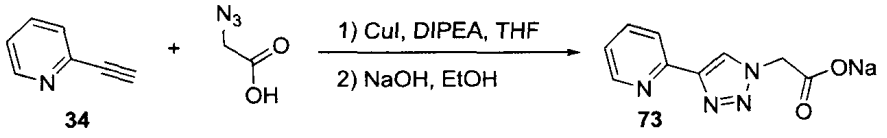
3.7 Metal-Dependent Hairpin PNA

The second PNA synthesized with the azide monomer **3** was **PNA4**, which underwent the click reaction with **34** (**Scheme 3.19**.)




**Scheme 3.19** The solid phase click reaction of **34** on an azide-functionalized PNA.

The synthesis of the pyridine-click model compound **73** (**Scheme 3.20**) was performed in order to quantify the concentration of PNA obtained.




**Scheme 3.20** The synthesis of "metal-binding click" model compound.

The goal of synthesizing **PNA4M** was to create a PNA hairpin structure that would bind a stabilizing square planar metal (**Fig. 3.4.**)

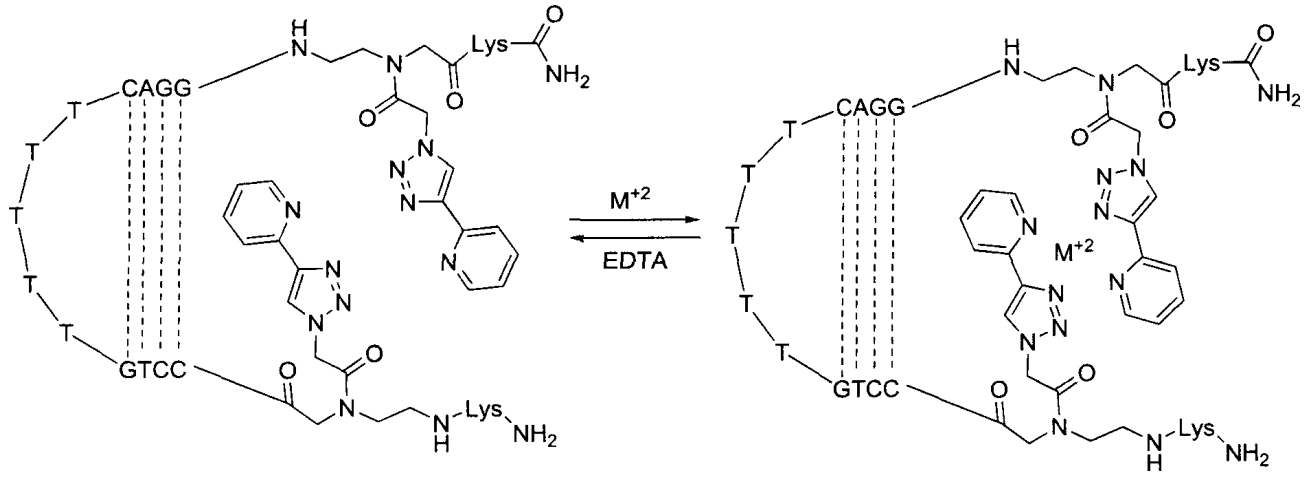
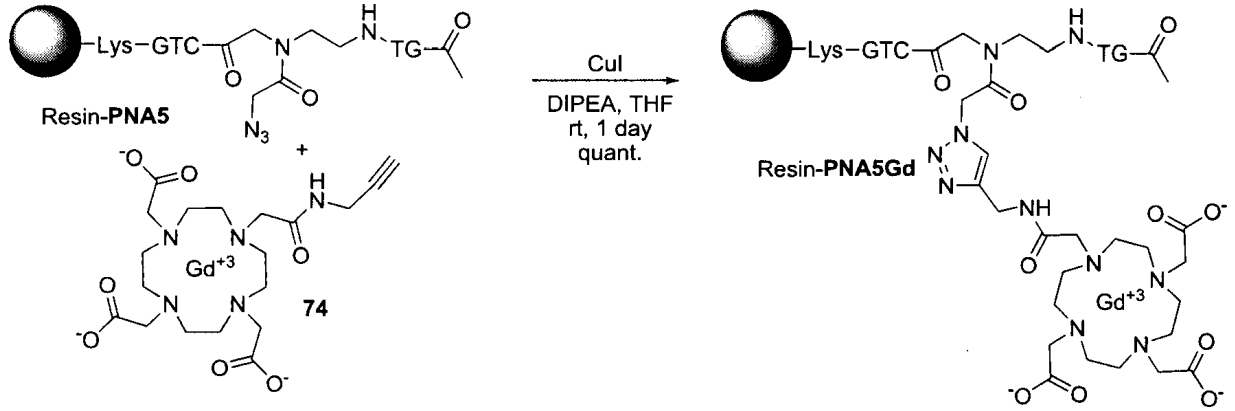



**Figure 3.4** The metal dependent stabilization of **PNA4M**'s hairpin structure

The results at 2 μM with square planar metals were promising, but the results could not be repeated at higher or lower concentrations, an indication that the interaction observed is not unimolecular. The characterization of PNA hairpins is more difficult than DNA hairpins because of other organized secondary structures that can be formed, so other studies will have to be performed.

3.8 MRI Imaging Agent Containing PNA

The two main areas of research in our group are nucleic acid analogues and MRI imaging agents, so a brief experiment was performed to examine the feasibility of combining these agents using the click reaction. DOTA-related contrast agents have been known to demetalate at low pH, so our concern was that the TFA cleavage of the PNA from the resin would destroy the contrast agent. An experiment was performed where a gadolinium contrast agent (**74**) underwent the solid phase click reaction with **PNA5** and cleaved from the resin to examine its stability after cleavage from the resin (**Scheme 3.21**.)




**Scheme 3.21** The solid phase click reaction of a gadolinium contrast agent on an azide-functionalized PNA.

After cleavage from the resin the LC/MS showed clean **PNA5Gd** with no trace of unreacted **PNA5** or demetalated products. This experiment showed the potential of the azide appended PNA to be functionalized with an MRI contrast agent, and future studies are underway.

## Conclusion

The synthesis of an azide appended Fmoc/Bn PNA monomer (**31**) was performed and used in a click reaction with 2-ethynylpyridine to form the metal-binding PNA **35**, which was unable to be crystallized with metals. The second approach to azide PNA used the allyl PNA backbone **40** to synthesize the azide PNA monomer **31**.

The synthesis of a variety of alkynes was undertaken for use in a click reactions to form "click fleximers," fluorophores and metal binding ligands. Undergraduate student Leslie Bean utilized several of these alkynes in a click reaction with azide-appended ribose.

Azide appended **PNA3** was synthesized and a solid-phase click reaction was performed on resin using 2-ethynylfluorene and 1-ethynylpyrene to examine the stability in a duplex with DNA containing an abasic site. **PNA3F** showed modest stability over **PNA3P** (24 and 20.5 °C respectively) suggesting that steric bulk is destabilizing these compounds.

**PNA4** was synthesized and underwent a two simultaneous click reactions with 2-ethynylpyridine to form a metal-binding PNA hairpin and studies on its properties are in progress.

The gadolinium containing contrast agent **74** successfully underwent the click reaction on the resin to form **PNA5Gd** and was able to be cleaved from the resin without degradation. Future studies will include the incorporation of contrast agents containing other lanthanides (eg. europium) into PNA designed for MRI studies.

## Experimental

**General procedure for solid phase click reaction.** The resin was placed in a vial with either THF or DMF (1 mL) containing alkyne (5-50 eq.) and DIPEA (50-100 eq.). CuI (0.1-1 eq.) was added, the vial flushed with $N_2$, sealed, and shaken overnight in the dark. The resin was added to a peptide vessel and the resin washed with DMF (3 mL), DCM (3 mL), MeOH (3 mL), $H_2O$ (3 mL), MeOH (3 mL), DCM (3 mL) and then dried. The resin was returned to the peptide synthesizer for Fmoc deprotection. The PNA was cleaved and purified following literature procedure.[21]

**PNA3.** HRMS (ESI) calculated for $C_{76}H_{104}N_{40}O_{23}$: [M + $2H^+$] 973.4177, Found 973.9149; [M + $3H^+$] 649.2811, Found 649.6036.

**PNA3F.** HRMS (ESI) calculated for $C_{91}H_{115}N_{40}O_{23}$: [M + $2H^+$] 1068.9608, Found 1068.9182; [M + $3H^+$] 712.9765, Found 712.9348.

**PNA3P.** HRMS (ESI) calculated for $C_{94}H_{114}N_{40}O_{23}$: [M + $2H^+$] 1086.4569, Found 1086.9707; [M + $3H^+$] 724.6405, Found 724.9683

**PNA4M.** HRMS (ESI) calculated for $C_{178}H_{230}N_{84}O_{50}$: [M + $4H^+$] 1086.9588, Found 1087.1980; [M + $5H^+$] 896.7686, Found 870.1615

**PNA5Gd.** HRMS (ESI) calculated for $C_{86}H_{121}N_{41}O_{27}Gd^{157}$: 2316.8595, Found 2316.8616.

**2-Azidoacetate Fmoc/Bn PNA (30).** A solution of azidoacetic acid (1.14 g, 11.2 mmol), DCC (3.48 g, 16.9 mmol) and HOBt (1.72 g, 11.2 mmol) in DMF (17 mL) was cooled on an ice bath and stirred for 10 min. A solution of Fmoc/Bn PNA backbone (2.42 g, 5.62 mmol) in DMF (10 mL) was added and stirred for 2 h. The reaction mixture was poured into DCM (200 mL) and washed with a saturated sodium bicarbonate solution (2 x 200 mL). The organic layer was dried over $Na_2SO_4$ and evaporated to dryness. The solution was triturated in acetone (5 mL) and filtered to remove excess DCU. The solvent was removed and the residue was purified by FCC (EtOAc-Hex = 1:1) to yield **31** (2.02 g, 70 % yield) as a waxy white solid: $^1$H NMR (400 MHz, $CDCl_3$) = 7.71 - 7.80 (t, $J$ = 6.8 Hz, 2 H), 7.58 (d, $J$ = 7.4 Hz, 2 H), 7.24 - 7.44 (m, 9 H), 5.65 (t, maj.), 5.27 (t, min.), 5.20 (s, maj.), 5.18 (s, min.), 4.41 (d, $J$ = 7.0 Hz, maj.), 4.36 (d, $J$ = 7.0 Hz, min.), 4.21 (t, $J$ = 5.3 Hz, 1 H), 4.08 (s, maj.), 4.03 (s, 1 H), 3.91 (s, maj.), 4.03 (s, 1 H), 3.91 (s, maj.), 3.79 (s, min.), 3.57 (t, $J$ = 6.1 Hz, maj.), 3.28-3.44 (m, 3 H), 2.76 (b. s., min.), 1.93 (m, 0.5

H), 1.67 (m, 0.5 H), 1.34 (m, 0.5 H), 1.10 (m, 0.5 H); HRMS (ESI) calculated for $[C_{28}H_{28}N_5O_5]^+$: 514.2085, Found 514.1880.

**2-Azidoacetate Fmoc PNA Monomer (31).** **41** (0.291 g, 0.628 mmol) was dissolved in a mixture of chloroform (8.5 mL), AcOH (0.5 mL), and *N*-methylmorpholine (0.26 mL, 4 eq.) and degassed. $Pd(PPh_3)_4$ (0.01 g, 0.009 mmol) was added and the solution stirred until complete reaction of the starting material (~7 days). The mixture was then poured into DCM (100 mL) and washed with 1M $KHSO_{4(aq)}$ (25 mL). The aqueous layer was back-extracted with DCM (100 mL), then the organic layers combined, dried over $Na_2SO_4$ and evaporated to dryness. The residue was purified by FCC (EtOAc-AcOH = 99:1) to yield **31** (0.231 g, 87 % yield) as a waxy white solid: $^1$H NMR (400 MHz, $CDCl_3$) = 9.57 (br. s., 1 H), 7.79 - 7.90 (m, 1 H), 7.60 - 7.72 (m, 1 H), 7.48 (br. s., 2 H), 7.34 - 7.45 (m, 2 H), 7.30 (d, $J$ = 7.4 Hz, 1 H), 4.79 (br. s., 1 H), 4.37 - 4.58 (m, 1 H), 4.28 (d, $J$ = 5.5 Hz, 1 H), 4.01 - 4.21 (m, 2 H), 3.94 (d, $J$ = 18.8 Hz, 1 H), 3.29 - 3.60 (m, 2 H), 2.82 (br. s., 1 H); $^{13}$C NMR (101 MHz, $CDCl_3$) = 171.9, 171.7, 171.0, 169.3, 169.0, 168.4, 157.3, 157.0, 156.8, 143.5, 141.1, 137.7, 132.4, 132.0, 131.9, 128.9, 128.6, 128.5, 128.1, 127.7, 127.6, 127.2, 126.9, 125.1, 125.0, 124.9, 124.6, 124.2, 119.8, 66.9, 66.5, 50.2, 49.8, 49.3, 48.5, 47.8, 46.9, 39.3, 38.9, 21.3; HRMS (ESI) calculated for $[C_{21}H_{22}N_5O_5]^+$: 424.1615, Found 424.1621.

**2-Pyridin-1-1,2,3-triazol-5-yl acetate Fmoc/Bn PNA (35).** A solution of **30** (0.90 g, 1.75 mmol) and **34** (0.30 g, 2.91 mmol) were dissolved in a mixture of THF (18 mL) and $H_2O$ (9 mL). Sanded copper wire (~0.25 g) was added and the solution heated to 40 °C for 48 h. EtOAc was added (250 mL) and the solution was filtered through Celite. The solvent was evaporated and the residue was taken up into EtOAc:hexane (1:1, 300 mL) and filtered through a plug of silica. The solution was evaporated to yield **35** (0.61 g, 56 % yield) as a waxy white solid: $^1$H NMR (400 MHz, $CDCl_3$) = 7.77 (t, 2 H), 7.59 (d, $J$ = 7.4 Hz, 2 H), 7.28 - 7.46 (m, 9 H), 5.67 (t, $J$ = 6.1 Hz, 1 H), 5.27 - 5.33 (m, 1 H), 5.21 (d, $J$ = 7.2 Hz, 2 H), 4.43 (d, $J$ = 6.6 Hz, 1 H), 4.37 (d, $J$ = 7.0 Hz, 1 H), 4.17 - 4.25 (m, 1 H), 4.10 (s, 1 H), 4.04 (s, 1 H), 3.93 (s, 1 H), 3.81 (s, 1 H), 1.34 (br. s., 1 H), 1.02 - 1.20 (m, 1 H); HRMS (ESI) calculated for $[C_{35}H_{33}N_6O_5]^+$: 617.2507, Found 617.2135.

**2-Azidoacetate Fmoc/Allyl PNA (41).** A solution of azidoacetic acid (1.7 g, 1.7 mmol) and **40** (0.32 g, 0.85 mmol) were combined in DCM (5 mL) and cooled on an ice bath. EDC (0.32 g, 1.7 mmol) and HOBt (0.038 g, 0.28 mmol) were added and stirred on the ice bath for 10 min, then an additional 18 h at room temperature. The reaction mixture was then poured into DCM (100 mL) and washed with a saturated sodium bicarbonate solution (25 mL) then brine (50 mL). The organic layer was dried over $Na_2SO_4$ and evaporated to dryness. The residue was purified by FCC (EtOAc-Hex = 3:2) to yield **41** (0.29 g, 74 % yield) as a waxy white solid: $^1$H NMR (400 MHz, $CDCl_3$) = 7.82 - 7.95 (m, 2 H), 7.61 - 7.78 (m, 2 H), 7.35 - 7.61 (m, 4 H), 5.91 - 6.17 (m, 1 H), 5.33 - 5.59 (m, 2 H), 4.65 - 4.91 (m, 2 H), 4.39 - 4.65 (m, 2 H), 4.28 - 4.36 (m, 1 H), 4.03 - 4.27 (m, 3 H), 3.88 - 3.99 (m, 1 H), 3.58 - 3.79 (m, 1 H), 3.32 - 3.58 (m, 3 H); $^{13}$C NMR (101 MHz, $CDCl_3$) = 212.2, 169.1, 168.5, 168.2, 156.4, 156.4, 143.6, 143.5, 141.0, 131.1, 130.7, 127.5, 127.4, 126.8, 124.8, 124.7, 119.7, 119.7, 119.4, 118.8, 108.5, 66.6, 66.4, 66.3, 65.9, 60.1, 53.3, 50.1, 49.6, 48.4, 48.4, 47.9, 46.8, 38.8, 38.6, 20.8, 13.9; HRMS (ESI) calculated for $[C_{24}H_{25}N_5O_5]^+$: 464.1928, Found 464.1923.

**5-Ethynyluracil (44a).** 5-Iodouracil (1.0 g, 4.2 mmol) was suspended in THF (20 mL) and degassed with $N_2$. $PdCl_2$ (0.4 mmol), $Ph_3P$ (0.8 mmol), and CuI (0.4 mmol) were added and the solution degassed with $N_2$. $Et_3N$ (8.6 mmol) and TMS-acetylene (7.0 mmol) was added and the solution degassed with $N_2$. The solution was stirred at 40 °C in the dark for 18 h. THF (200 mL) was added, the solution was filtered through Celite, and the solvent removed by rotary evaporation. The solid was suspended in DCM (20 mL), and cooled on ice. The suspension was filtered and washed with cold DCM (20 mL). The crude 5-TMS-ethynyluracil (**43a**) derivative was suspended in 1 M $NaOH_{(aq)}$ (40 mL) and stirred for 2 h. The suspension was diluted with $H_2O$ (40 mL) and filtered. Approximately 20 mL of the solvent was removed by rotary evaporation, and AcOH was added to adjust the pH to ~5. The solution was cooled on ice and filtered. The precipitate was washed with cold $H_2O$ (10 mL), cold acetone (10 mL) and $Et_2O$ (20 mL) to yield **44a** (0.377 g, 66 % yield) as a yellow/brown powder: $^1$H NMR (400 MHz, DMSO) = 11.34 (br. s., 1 H), 7.81 (s, 1 H), 4.03 (s, 1 H); HRMS (EI) calculated for $[C_6H_4N_2O_2]^+$: 136.0273, Found 136.0274.

**5-Ethynylcytosine (44b).** 5-Iodocytosine (1.0 g, 4.2 mmol) was subjected to the same conditions as **44a**. THF (200 mL) was added, the solution was filtered through Celite, and the solvent removed by rotary evaporation. The solid was suspended in EtOAc (20 mL), and cooled on ice. The suspension was filtered and washed with cold EtOAc (20 mL). The crude 5-TMS-ethynylcytosine (**43b**) derivative was suspended in 1 M $NaOH_{(aq)}$ (40 mL) and stirred for 2 h. The suspension was diluted with $H_2O$ (40 mL) and filtered. Approximately 20 mL of the solvent was removed by rotary evaporation, and AcOH was added to adjust the pH to ~5. The solution was cooled on ice and filtered. The precipitate was washed with cold $H_2O$ (10 mL), cold acetone (10 mL) and $Et_2O$ (20 mL) to yield **44b** (0.062 g, 11 % yield) as a grey powder: $^1H$ NMR (400 MHz, DMSO) = 10.96 (br. s., 1 H), 7.74 (s, 1 H), 7.57 (br. s., 1 H), 6.70 (br. s., 1 H), 4.30 (s, 1 H); HRMS (EI) calculated for $[C_6H_5N_3O]^+$: 135.0433, Found 135.0434.

**5-Iodo-2,4-diaminopyrimidine (46).** 2,6-Diaminopurine hemi-sulphuric acid (0.40 g, 1.9 mmol) was dissolved in a mixture of $H_2O$ (3 mL), AcOH (5 mL) and $CCl_4$ (2 mL). $I_2$ (0.39 g, 1.5 mmol) and $HIO_3$ (0.069 g, 0.38 mmol) were added sequentially and the vessel was sealed and heated to 50 °C for 18 h. The solvent was removed and the solid dissolved in hot $H_2O$ (40 mL), to which acetone (40 mL) and $Et_2O$ (20 mL) were added. The solid was filtered then suspended in 1M $NaOH_{(aq)}$ (12 mL) and stirred for 2 h. The solid was filtered and washed with $H_2O$ (4 mL) to yield **46** as white powder: $^1H$ NMR (400 MHz, DMSO) = 7.92 (s, 1 H), 6.35 (br. s., 2 H), 6.07 (br. s., 2 H); HRMS (ESI) calculated for $[C_4H_6IN_4]^+$: 236.9632, Found 236.9448.

**5-TMS-ethynyl-2,4-diaminopyrimidine (47). 46** (0.8 mmol) was subjected to the same conditions as **44a**. THF (200 mL) was added, the solution was filtered through Celite, and the solvent removed by rotary evaporation. The residue was subjected to a silica plug with EtOAc:Hex (1:1, 200 mL) as an eluent. The silica plug was then washed with EtOAc (300 mL), which was collected and the solvent removed by rotary evaporation. The crude **47** (113% theoretical yield) was used as is in the next step: $^1H$ NMR (600 MHz, $CDCl_3$) = 8.04 (s, 1 H), 5.45 (br. s., 2 H), 5.35 (br. s., 2 H), 0.23 (s, 9 H); $^{13}C$ NMR (151 MHz, $CDCl_3$) = 163.9, 161.6, 159.9, 101.1, 98.2, 92.5, 0.0; HRMS (EI) calculated for $[C_9H_{14}N_4Si]^+$: 206.0988, Found 206.0979.

**5-Ethynyl-2,4-diaminopyrimidine (48).** The crude **47** (0.8 mmol of **46**) was suspended in 1 M $NaOH_{(aq)}$ (20 mL) and stirred for 3 h. The solution was cooled on ice, and conc. HCl was added dropwise until the pH was ~3, then the solution was filtered. The mother liquor was neutralized with 1 M $NaOH_{(aq)}$ and extracted with EtOAc (150 mL). The organic layer was dried with $Na_2SO_4$, and the solvent was removed with rotary evaporation to yield **48** (0.078 g, 73 % yield from 5-iodo-diaminopyrimidine) as a yellow powder: $^1$H NMR (600 MHz, MeOH-$d_4$) = 7.88 (s, 1 H), 3.75 (s, 1 H); $^{13}$C NMR (101 MHz, MeOH-$d_4$) = 166.0, 163.4, 160.4, 92.2, 84.8, 78.0; HRMS (EI) calculated for $[C_6H_6N_4]^+$: 134.0592, Found 134.0599.

**5-TMS-ethynylisocytosine (51). 50** (1.0 g, 4.2 mmol) was subjected to the same conditions as **44a**. THF (200 mL) was added, the solution was filtered through Celite, and the solvent removed by rotary evaporation. The residue was subjected to FCC with EtOAc as an eluent. The relevant fractions were combined and the solvent removed by rotary evaporation to yield **51** (0.531 g, 61 % yield) as a yellow/orange powder: $^1$H NMR (400 MHz, DMSO-$d_6$) = 11.25 (br. s., 1 H), 7.81 (s, 1 H), 6.92 (br. s., 2 H), 0.16 (s, 9 H); HRMS (EI) calculated for $[C_9H_{13}N_3OSi]^+$: 207.0828, Found 207.0829.

**5-Ethynylisocytosine (52). 51** (0.435 g, 2.1 mmol) was suspended in 1 M $NaOH_{(aq)}$ (40 mL) and stirred for 18 h. The suspension was filtered and AcOH was added to adjust the pH to ~5. The solution was cooled on an ice bath and filtered. The filtrate was washed with cold $H_2O$ (20 mL), cold acetone (20 mL) and $Et_2O$ (20 mL) to yield **52** (0.14 g, 49 % yield) as a tan powder: $^1$H NMR (400 MHz, DMSO-$d_6$) = 11.23 (br. s., 1 H), 7.81 (s, 1 H), 6.92 (br. s., 2 H), 3.94 (s, 1 H); HRMS (EI) calculated for $[C_6H_5N_3O]^+$: 135.0433, Found 135.0437.

**2-Thiocytosine (53).** Sodium *t*-butoxide (9.0 g, 93 mmol) was dissolved in nBuOH (41 mL). 3,3-diethoxypropionitrile (14 mL, 93 mmol) and thiourea (7.53 g, 98.9 mmol) were added and the mixture heated to reflux for 5 h. The mixture was cooled on an ice bath then filtered. The filtrate was washed with hexane (50 mL), then suspended in $H_2O$ (150 mL). The pH was adjusted to ~6 using AcOH then filtered. The filtrate was washed with $H_2O$ (50 mL), $Et_2O$ (50 mL) then dried to yield **53** (4.5 g, 38 % yield) as a tan powder. $^1$H NMR (600 MHz, DMSO-$d_6$) =

11.96 (br. s., 1 H), 7.58 (br. s., 1 H), 7.50 (br. s., 1 H), 7.38 (d, $J$ = 7.0 Hz, 1 H), 5.91 (d, $J$ = 7.0 Hz, 1 H)

**4-Aminopyrimidine (54). 53** (3.38 g, 26.6 mmol) was suspended in $H_2O$ (55 mL). A Raney Ni solution in $H_2O$ was added (20 mL) and the mixture heated to reflux for 2 h. The mixture was hot-filtered to remove the Raney Ni and the filtrate washed with $H_2O$. The solvent was removed and the solid triturated with EtOAc then decanted (4 x 50 mL). The organic fractions were combined and the solvent removed to provide **54** (1.95 g, 77 % yield) as a yellow-green powder: $^1$H NMR (400 MHz, $CDCl_3$) = 8.57 (s, 1 H), 8.22 (d, $J$ = 5.9 Hz, 1 H), 6.44 (dd, $J$ = 1.4, 6.1 Hz, 1 H), 5.09 (br. s., 2 H)

**5-Iodo-4-aminopyrimidine (55). 54** (0.95 g, 10 mmol) and $I_2$ (5.1 g, 20 mmol) were dissolved in DMSO (20 mL). The solution was heated to 80 °C for 15 min. EtOAc (125 mL) was added and the solution was washed with a mixture of 5 % $Na_2S_2O_3$ (100 mL), a saturated $NaHCO_3$ solution (50 mL), $H_2O$ (50 mL) then the organic layer was washed again with 5 % $Na_2S_2O_3$ (100 mL). The organic layer's solvent was removed to provide **55** (0.29 g, 13 % yield) as a yellow powder: $^1$H NMR (400 MHz, DMSO-$d_6$) = 8.44 (s, 1 H), 8.30 (s, 1 H)

**5-TMS-ethynyl-4-aminopyrimidine (56). 55** (0.246 g, 1.11 mmol) was subjected to the same conditions as **44a**. THF (200 mL) was added, the solution was filtered through Celite, and the solvent removed by rotary evaporation. The residue was subjected to FCC (EtOAc:Hex 1:1→6:4). The relevant fractions were combined and the solvent removed by rotary evaporation to yield **56** (0.146 g, 69 % yield) as a tan powder: $^1$H NMR (400 MHz, $CDCl_3$) = 8.43 (s, 1 H), 8.29 (s, 1 H), 6.13 (br. s., 2 H), 0.14 - 0.30 (m, 9 H); $^{13}$C NMR (101 MHz, $CDCl_3$) = 162.8, 157.8, 156.8, 104.5, 101.9, 96.5, -0.3; HRMS (EI) calculated for $[C_9H_{13}N_3Si]^+$: 191.0879, Found 191.0886.

**5-Ethynyl-4-aminopyrimidine (57). 56** (0.139 g, 0.727 mmol) was suspended in 1 M $NaOH_{(aq)}$ (15 mL) and stirred for 2 h. The solution was cooled on ice, and conc. HCl was added dropwise until the pH was ~3. The solution was diluted with $H_2O$ (15 mL) and filtered. The mother liquor was basified with 1 M $NaOH_{(aq)}$ (5 mL) and extracted with EtOAc (2 x 150 mL). The organic

layer was dried with $Na_2SO_4$, and the solvent was removed with rotary evaporation to yield **57** (0.077 g, 89 % yield) as a tan powder: $^1$H NMR (600 MHz, MeOH-$d_4$) = 8.35 (s, 1 H), 8.23 (s, 1 H), 4.04 (s, 1 H); $^{13}$C NMR (151 MHz, MeOH-$d_4$) = 165.1, 158.5, 158.1, 102.3, 88.3, 76.5; HRMS (EI) calculated for $[C_6H_5N_3]^+$: 119.0483, Found 119.0484.

**4-Amino-2-[4-amino-pyrimidin-2-yl]dithiopyrimidine (61). 53** (1.2 g, 9.3 mmol) was dissolved in AcOH (20 mL) and $Br_2$ (1.4 mL, 28 mmol) was added dropwise. The solution was stirred for 18 h then diluted with $H_2O$ (20 mL). The solids go into solution then reprecipitate over ~5 min. The solution was cooled on an ice bath then filtered. The filtrate was washed with $H_2O$ (10 mL) and $Et_2O$ (100 mL) to yield **61** (0.92 g, 78 % yield) as a yellow powder: $^1$H NMR (400 MHz, DMSO-$d_6$) = 8.02 (br. s., 2 H), 7.94 (d, $J$ = 5.9 Hz, 2 H), 6.90 (br. s., 2 H), 6.51 (d, $J$ = 6.3 Hz, 2 H); HRMS (ESI) calculated for $[C_8H_9N_6S_2]^+$: 253.0325, Found 253.0474.

**4-Amino-2-methoxypyrimidine (62). 61** (0.41 g, 1.6 mmol) was dissolved in MeOH (20 mL) and $K_2CO_3$ (0.526 g, 3.81 mmol) was added and the solution was stirred at 50 °C for 2 days. The solution was poured into $H_2O$ (100 mL) and extracted with DCM (8 x 100 mL). The organic layers were combined, dried with $Na_2SO_4$ and evaporated to dryness to yield **62** (0.18 g, 45 % yield) as a yellow powder:[16] $^1$H NMR (400 MHz, DMSO-$d_6$) = 7.85 (d, $J$ = 5.9 Hz, 1 H), 6.85 (br. s., 2 H), 6.06 (d, $J$ = 5.9 Hz, 1 H), 3.74 (s, 3 H); MP 154-162 °C.

**4-Amino-5-iodo-2-methoxypyrimidine (63). 62** (0.178 g, 1.42 mmol) was dissolved in a mixture of $H_2O$ (2.25 mL), AcOH (3.75 mL) and $CCl_4$ (1.5 mL). $I_2$ (0.293 g, 1.15 mmol) and $HIO_3$ (0.051 g, 0.29 mmol) were added sequentially and the vessel was sealed and heated to 50 °C for 18 h. The solvent was removed and the solid suspended in 5% $Na_2S_2O_{3(aq)}$ (100 mL) and extracted with DCM (100 mL). The organic layer was dried with $Na_2SO_4$ and evaporated to dryness to yield **63** (0.32 g, 87 % yield) as yellow crystals: $^1$H NMR (400 MHz, $CDCl_3$) = 8.29 (s, 1 H), 5.35 (br. s., 2 H), 3.91 (s, 3 H); $^{13}$C NMR (101 MHz, $CDCl_3$) = 165.5, 163.5, 163.1, 67.3, 54.7; HRMS (ESI) calculated for $[C_5H_7IN_3O]^+$: 251.9628, Found 251.9370.

**5-Ethynyl-4-amino-2-methoxypyrimidine (64). 63** (0.31 g, 1.2 mmol) was subjected to the same conditions as **44a**. THF (200 mL) was added, the solution was filtered through Celite, and

the solvent removed by rotary evaporation. The solid was suspended in hexane (200 mL) and filtered through a silica plug. The silica was then washed with EtOAc (200 mL) and this fraction was collected and the solvent removed. The crude TMS-derivative was suspended in a mixture of 1 M $NaOH_{(aq)}$ (10 mL) and MeOH (10 mL) and stirred for 1 h. The solution was cooled on ice, and conc. HCl was added dropwise until the pH was ~3. The solution was and filtered and washed with $H_2O$ (10 mL). The mother liquor was basified with 1 M $NaOH_{(aq)}$ until the pH was ~9. The solvent was removed until ~5 mL remained and then diluted with $H_2O$ (100 mL). The solution was extracted with DCM (2 x 100 mL). The organic layers were combined, dried with $Na_2SO_4$, and the solvent was removed with rotary evaporation to yield **64** (0.17 g, 96.% yield) as a yellow/brown powder: $^1$H NMR (600 MHz, MeOH-$d_4$) = 8.06 (s, 1 H), 3.88 (s, 3 H), 3.85 (s, 1 H); $^{13}$C NMR (151 MHz, MeOH-$d_4$) = 167.0, 165.8, 161.0, 95.8, 86.4, 77.0, 55.3; HRMS (ESI) calculated for $[C_7H_8N_3O]^+$: 150.0662, Found 150.0503.

**4-Pyrimidone (65).** Sodium (0.53 g, 23 mmol) was dissolved in MeOH (10 mL) under $N_2$. Methyl 3,3-dimethoxypropionate (2.8 mL, 20 mmol) and formamidine acetate (2.06 g, 19.8 mmol) were added and the mixture heated to reflux for 2 h. The mixture was cooled on an ice bath and AcOH (1 mL) was added. The solvent was removed and the residue was subjected to FCC (MeOH:EtOAc 1:4). The relevant fractions were combined and the solvent removed by rotary evaporation to yield **65** (0.243 g, 13 % yield) as a yellow powder: $^1$H NMR (400 MHz, DMSO-$d_6$) = 8.33 (br. s., 1 H), 8.02 (d, $J$ = 5.9 Hz, 1 H), 6.87 (br. s., 1 H), 6.40 (d, $J$ = 5.9 Hz, 1 H)

**5-Ethynyl-4-pyrimidone (66). 65** (0.208 g, 2.16 mmol) was dissolved in AcOH (5 mL) and a solution of $Br_2$ (0.12 mL, 2.2 mmol in 0.2 mL AcOH) was added dropwise and the solution stirred for 4.5 h. EtOH (40 mL) was added and the solvent removed. Hot EtOH (10 mL) was added and the flask placed in the freezer overnight. The mixture was filtered and washed with $Et_2O$ (10 mL) to yield 5-bromo-4-aminopyrimidine hydrobromide, which was subjected to the same conditions as **44a**. THF (200 mL) was added, the solution was filtered through Celite, and the solvent removed by rotary evaporation. The residue was subjected to FCC (EtOAc:Hex 1:1). The relevant fractions were combined and the solvent removed by rotary evaporation to yield 5-TMS-ethynyl-4-pyrimidone, which was suspended in 1 M $NaOH_{(aq)}$ (10 mL) and stirred for 0.5

h. The solution was diluted with $H_2O$ (10 mL), cooled on an ice bath, and filtered. The mother liquor was acidified with AcOH to pH ~6, then extracted with DCM (2 x 50 mL). The organic layer was dried with $Na_2SO_4$, and the solvent was removed with rotary evaporation to yield **66** (0.052 g, 20 % yield) as a tan powder: $^1$H NMR (400 MHz, MeOH-$d_4$): = 8.37 (s, 1 H), 8.25 (s, 1 H), 4.05 ppm (s, 1 H); $^{13}$C NMR (101 MHz, MeOH-$d_4$) = 168.0, 158.6, 158.2, 88.2, 76.5, 39.7; HRMS (ESI) calculated for $[C_6H_5N_2O]^+$: 121.0396, Found 121.0661.

**2-(4-(Fluoren-2-yl)-1H-1,2,3-triazol-1-yl)acetic acid (72). 71** (0.100 g, 0.525 mmol) and azidoacetic acid (0.202 g, 2.00 mmol) were dissolved in THF (5 mL). CuI (0.10 g, 1.3 mmol) and DIPEA (1.75 mL) were added and the solution stirred for 18 h. The solvent was removed, the residue dissolved in 1M $NaHSO_4$ (100 mL), and extracted with $Et_2O$ (2 x 100 mL) and EtOAc (2 x 100 mL). The organic layers were combined, dried with $Na_2SO_4$, and the solvent was removed with rotary evaporation. The residue was dissolved in boiling EtOAc (30 mL), hot filtered, and allowed to cool. Hexane was added (60 mL) and the solution cooled on an ice bath. The solid was filtered off to yield **72** (0.048 g, % yield) as a grey powder: $^1$H NMR (400 MHz, DMSO-$d_6$) = 8.58 (br. s., 1 H), 8.34 (s, 1 H), 8.01 (d, $J$ = 7.4 Hz, 1 H), 7.87 (t, $J$ = 7.6 Hz, 1 H), 7.22 - 7.36 (m, 1 H), 4.67 (s, 2 H); $\varepsilon_{260}$ = 4870 ($A \cdot M^{-1} \cdot cm^{-1}$).

**Sodium 2-(4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl)acetate (73). 34** (0.326 g, 3.16 mmol) and azidoacetic acid (0.217 g, 2.15 mmol) were dissolved in THF (5 mL). CuI (0.025 g, 0.13 mmol) and DIPEA (1 mL) were added and the solution stirred for 2.5 h. The solution was diluted with THF (100 mL), filtered through Celite, then the solvent removed. The residue was dissolved in EtOH (10 mL) and concentrated $NaOH_{(EtOH)}$ was added until the pH ~9. The solid was filtered off and washed with EtOH (10 mL) to yield **73** (0.048 g, 31 % yield) as a tan powder: $^1$H NMR (400 MHz, DMSO-$d_6$) = 8.59 (s, 1 H), 8.09 (s, 1 H), 7.98 (d, $J$ = 7.8 Hz, 1 H), 7.86 - 7.96 (m, 2 H), 7.61 (d, $J$ = 7.4 Hz, 1 H), 7.40 (t, $J$ = 6.6 Hz, 1 H), 7.33 (t, $J$ = 7.4 Hz, 1 H), 5.32 (s, 2 H), 4.00 (s, 2 H); $\varepsilon_{260}$ = 7040 ($A \cdot M^{-1} \cdot cm^{-1}$).

**References**


(1) Seley, K. L.; Zhang, L.; Hagos, A. *Organic Letters* **2001**, *3*, 3209.
(2) Nielsen, P. E. *Current Opinion in Biotechnology* **2001**, *12*, 16.
(3) Popescu, D.-L.; Parolin, T. J.; Achim, C. *Journal of the American Chemical Society* **2003**, *125*, 6354.
(4) Li, Y.; Zhou, X.; Ye, D. *Biochemical and Biophysical Research Communications* **2008**, *373*, 457.
(5) Wang, H.; Kim, Y.; Liu, H.; Zhu, Z.; Bamrungsap, S.; Tan, W. *Journal of the American Chemical Society* **2009**, *131*, 8221.
(6) Armitage, B.; Ly, D.; Koch, T.; Frydenlund, H.; Ørum, H.; Schuster, G. B. *Biochemistry* **1998**, *37*, 9417.
(7) Thurley, S.; Röglin, L.; Seitz, O. *Journal of the American Chemical Society* **2007**, *129*, 12693.
(8) Franzini, R. M.; Watson, R. M.; Patra, G. K.; Breece, R. M.; Tierney, D. L.; Hendrich, M. P.; Achim, C. *Inorganic Chemistry* **2006**, *45*, 9798.
(9) Holmes, B. T.; Pennington, W. T.; Hanks, T. W. *Synthetic Communications* **2003**, *33*, 2447.
(10) Thomson, S. A.; Josey, J. A.; Cadilla, R.; Gaul, M. D.; Fred Hassman, C.; Luzzio, M. J.; Pipe, A. J.; Reed, K. L.; Ricca, D. J.; Wiethe, R. W.; Noble, S. A. *Tetrahedron* **1995**, *51*, 6179.
(11) Oertel, K.; Zech, G.; Kunz, H. *Angewandte Chemie International Edition* **2000**, *39*, 1431.
(12) Dračínský, M.; Jansa, P.; Ahonen, K.; Buděšínský, M. *European Journal of Organic Chemistry* **2011**, *2011*, 1544.
(13) Dodd, D. W.; Hudson, R. H. E. *Mini-Reviews in Organic Chemistry* **2009**, *6*, 378.
(14) Co., S.-A. 2011; Vol. 2011.
(15) Cavalieri, L. F.; Bendich, A. *Journal of the American Chemical Society* **1950**, *72*, 2587.
(16) HERMANN, P.; Lynch, F., Ed.; Dynamit Nobel AG: Germany, 1982; Vol. 4447609.
(17) Li, C. S. D.-d. O., CA), Ramtohul, Yeeman K. (Montreal, CA), Huang, Zheng (Kirkland, CA), Lachance, Nicolas (Pierrefonds, CA) United States, 2009.
(18) Wojciechowski, F.; Hudson, R. H. E. *Nucleosides, Nucleotides and Nucleic Acids* **2007**, *26*, 1199.
(19) Bean, L., University of Western Ontario, 2011.
(20) Kostenko, E.; Dobrikov, M.; Pyshnyi, D.; Petyuk, V.; Komarova, N.; Vlassov, V.; Zenkova, M. *Nucleic Acids Research* **2001**, *29*, 3611.
(21) Hudson, R. H.; Liu, Y.; Wojciechowski, F. *Canadian Journal of Chemistry* **2007**, *85*, 302.

# Chapter 4

## Conclusion & Outlook

The synthesis of a Fmoc/Boc protected PNA monomers has been completed for thymine, 2,6-diaminopurine, and 2-aminopurine. The oligomerization of a poly-T tract has been completed with no evidence of an increase in truncation products. The Boc-protected T PNA monomer has also been demonstrated to be compatible with commercial T-monomer and they can be used interchangeably during oligomerization. Future studies could investigate the extent of truncation in poly-T tracts with unprotected T PNA monomer, and the degree of Boc-protection necessary to afford the growing peptide enough solubility to reduce truncation.

The 2,6-diaminopurine monomer has been incorporated into a PNA and the melting temperature of T versus mismatches showed a preference of 6 °C. DAP has been shown to stabilize duplexes when replacing A, and a synthetically facile route to DAP-containing PNA could make studies on its stabilization more accessible to researchers. Should PNA one day be used as a medicine in gene therapy there will be a need for a short and selective knock-down of nucleic acid targets, and DAP could help increase the selectivity and the binding strength.

The 2-aminopurine monomer has been successfully incorporated into PNA, and its mismatch selectivity for T was 11.5 °C. The fluorescence properties of the nucleobase were exploited to perform a fluorescence melt curve that gave a comparable Tm value to the standard technique using uv-hyperchromicity. The fluorescence profile of the nucleobase against mismatches showed a higher fluorescence, with quenching upon hybridization with T. This report of the synthesis of the monomer is the first in full experimental detail, and this will make 2AP-containing PNA more accessible researchers to exploit its unique fluorescence properties.

Future studies using 2AP could include the fluorescence lifetime, fluorescence anisotropy, and charge transfer effects.

As a more facile approach to introducing modified bases into PNA, click chemistry with an azide monomer was investigated. The first approach to "click PNA" was through the synthesis of an azide appended Fmoc/Bn PNA monomer. Because of the limitations of the protecting group strategy, the click had to be performed before debenzylation. A click reaction with 2-ethynylpyridine was performed but unfortunately no metal complexes were able to be crystallized. The second approach to azide-containing PNA used the allyl PNA backbone, which afford the monomer in excellent yield. The allyl-ester can be selectively removed whilst preserving the azide functional group. This opened up the possibility of incorporating the azido-PNA into an oligomer prior to doing the click chemistry.

The synthesis of a variety of alkynes was performed for use in click reactions to form "click fleximers," fluorophores and metal binding ligands. Several of the 5-ethynylpyrimidines were used in the synthesis of ribose click fleximers by undergraduate student Leslie Bean. These compounds have potential as medicines or could be incorporated into RNA to study the binding moiety of these novel nucleobases.

PNA was synthesized incorporating the azide PNA monomer and a solid phase click reaction was performed on the resin using 2-ethynylfluorene and 1-ethynylpyrene. These fluorescent PNAs were used to measure the melting temperature in a duplex with DNA containing an abasic site. The duplexes were destabilized, with fluorene showing modest stability over pyrene (24 and 20.5 °C, respectively). We hypothesize that these compounds are too large to fit in the binding pocket and future studies should incorporate more compact fluorophores.

A PNA was designed to form a hairpin, with and azide on both ends. This underwent two simultaneous click reactions with 2-ethynylpyridine to form metal binding ligands. Studies are underway to determine if metals would stabilize the hairpin formation. If this design is successful, it would describe the first metal-dependent PNA hairpin and could have potential as molecular switches.

A gadolinium/alkyne-containing contrast agent successfully underwent a solid phase click reaction to an azide-containing PNA and was able to be cleaved from the resin without degradation of the complex. The growing field of customized contrast agents is of interest to our research group, and being able to customize the target of these compounds is being investigated by our group.

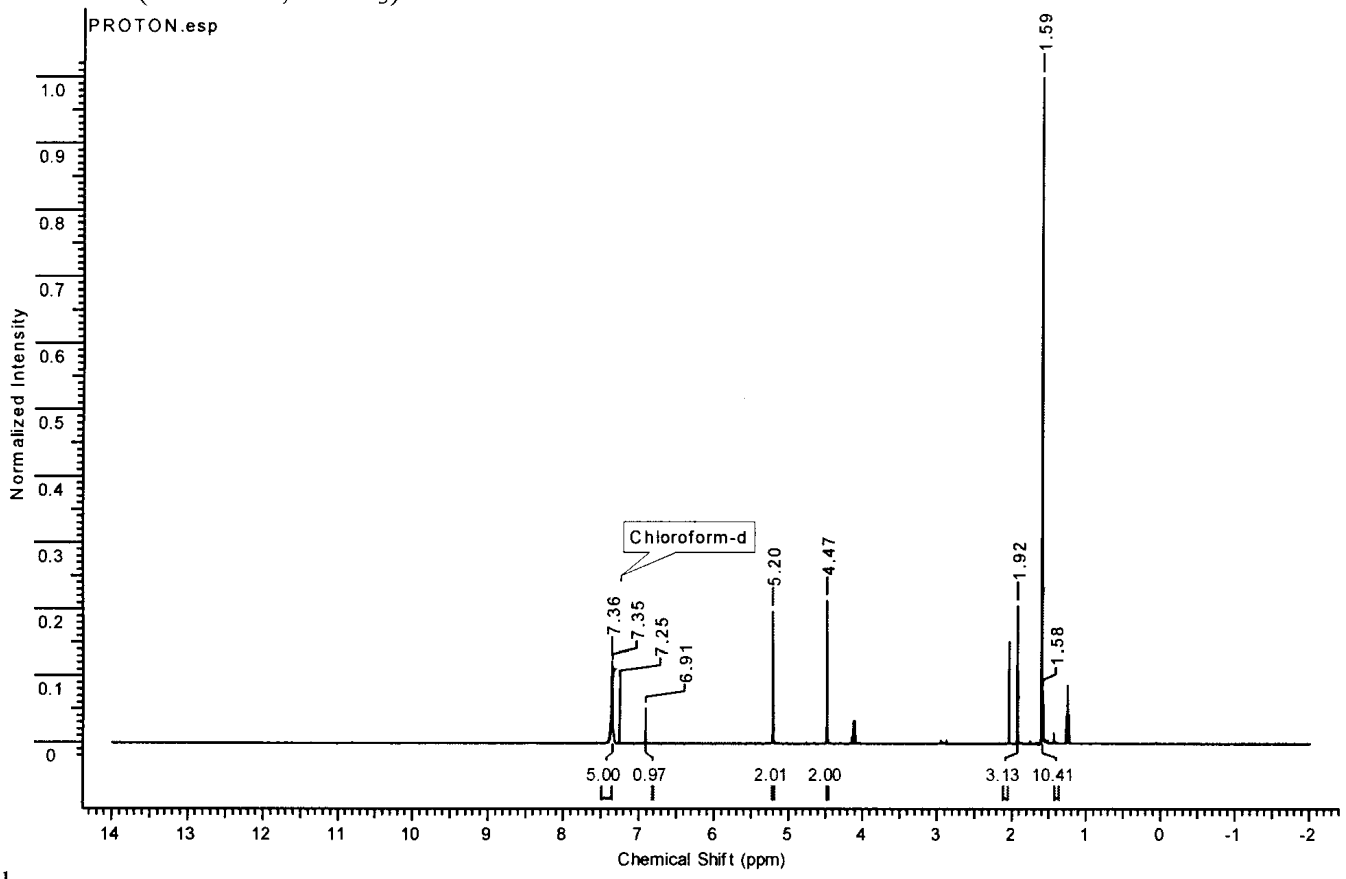
In conclusion, the description of the synthesis of these PNA monomers will facilitate the synthesis of PNA containing unique nucleobases or azides, thus increasing the accessibility to this promising nucleic acid analogue for further study.

# Appendix

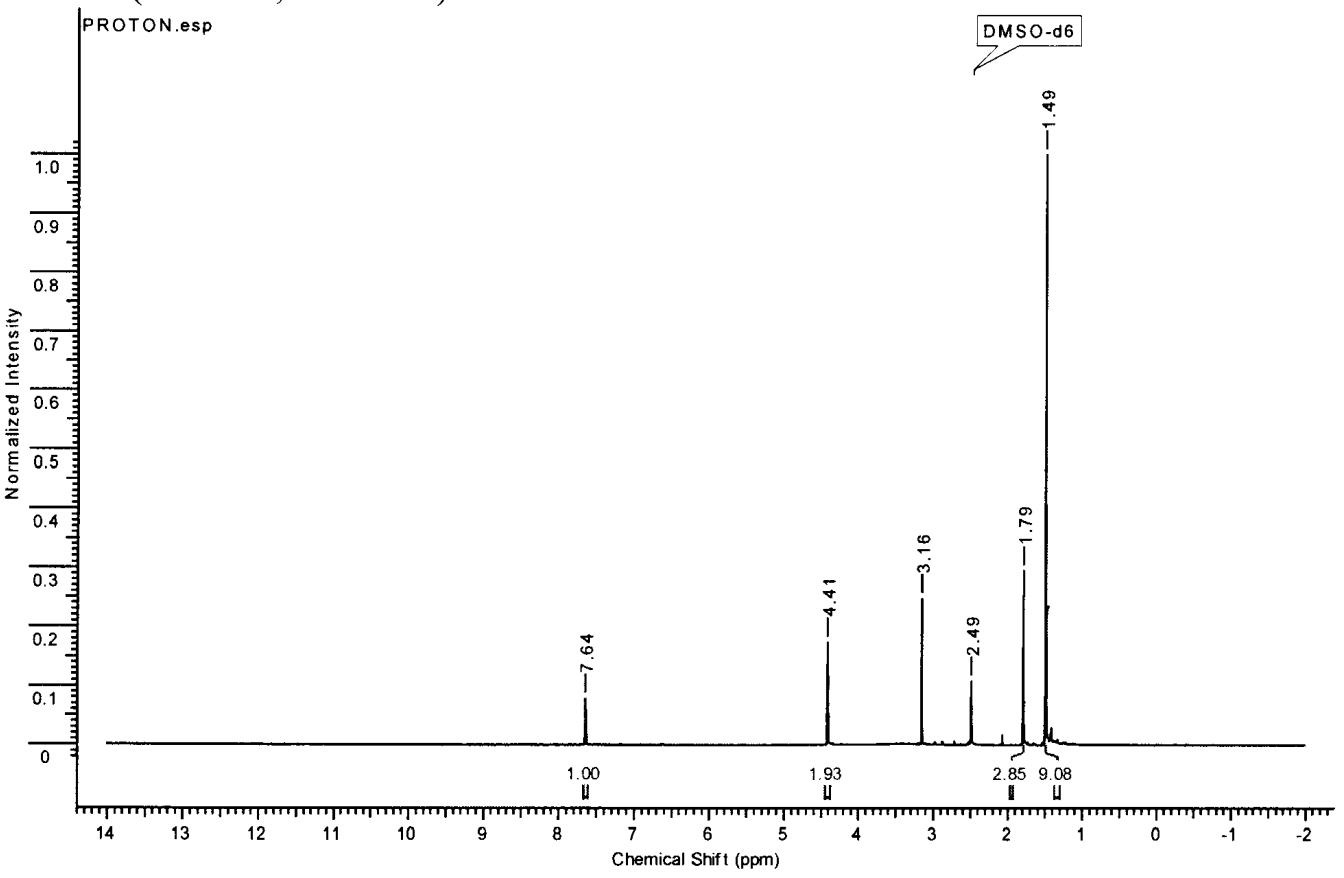
Table of Contents

**General Remarks.** All chemicals were obtained from commercial sources and were of ACS reagent grade or higher and were used without further purification. Solvents for solution-phase chemistry were dried by passing through activated alumina columns. Flash column chromatography (FCC) was performed on Merck Kieselgel 60, 230-400 mesh. Thin layer chromatography (TLC) was performed on Merck Kieselgel 60 TLC plates. Chemical shifts are reported in parts per million (δ), were measured from Tetramethylsilane (0 ppm) and are referenced to the solvent $CDCl_3$ (7.26 ppm), DMSO-*d6* (2.49 ppm), $D_2O$ (4.79 ppm) for $^1H$ NMR and $CDCl_3$ (77.0 ppm), DMSO-*d6* (39.5 ppm) for $^{13}C$ NMR. Multiplicities are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br s (broad singlet). Coupling constants (*J*) are reported in Hertz (Hz). Resonances due to restricted rotation around the amide bond (rotamers) are reported as major (maj.) and minor (min.). High resolution mass spectra (HRMS) were obtained using electron impact (EI) or electrospray (ESI).

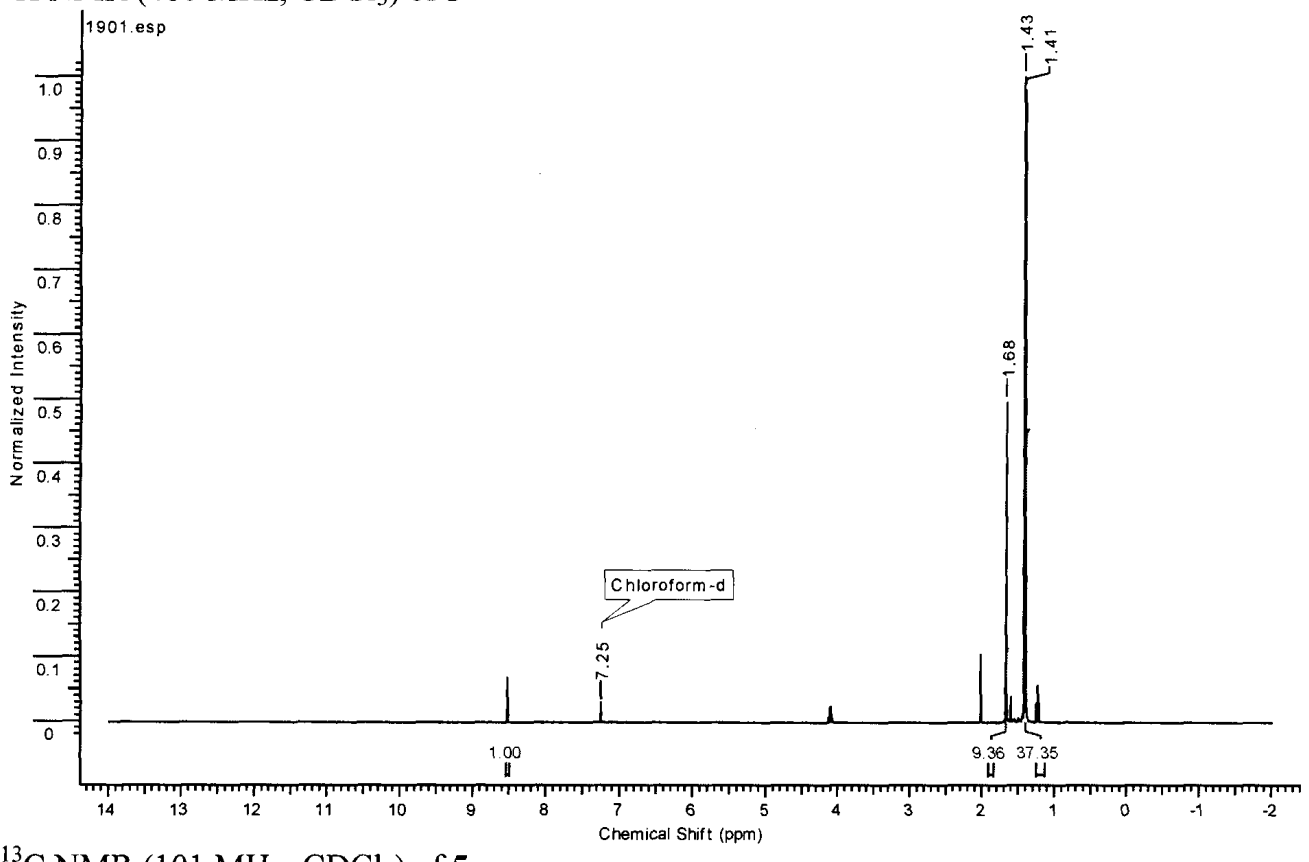
$^1$H NMR (400 MHz, $CDCl_3$) of **2**



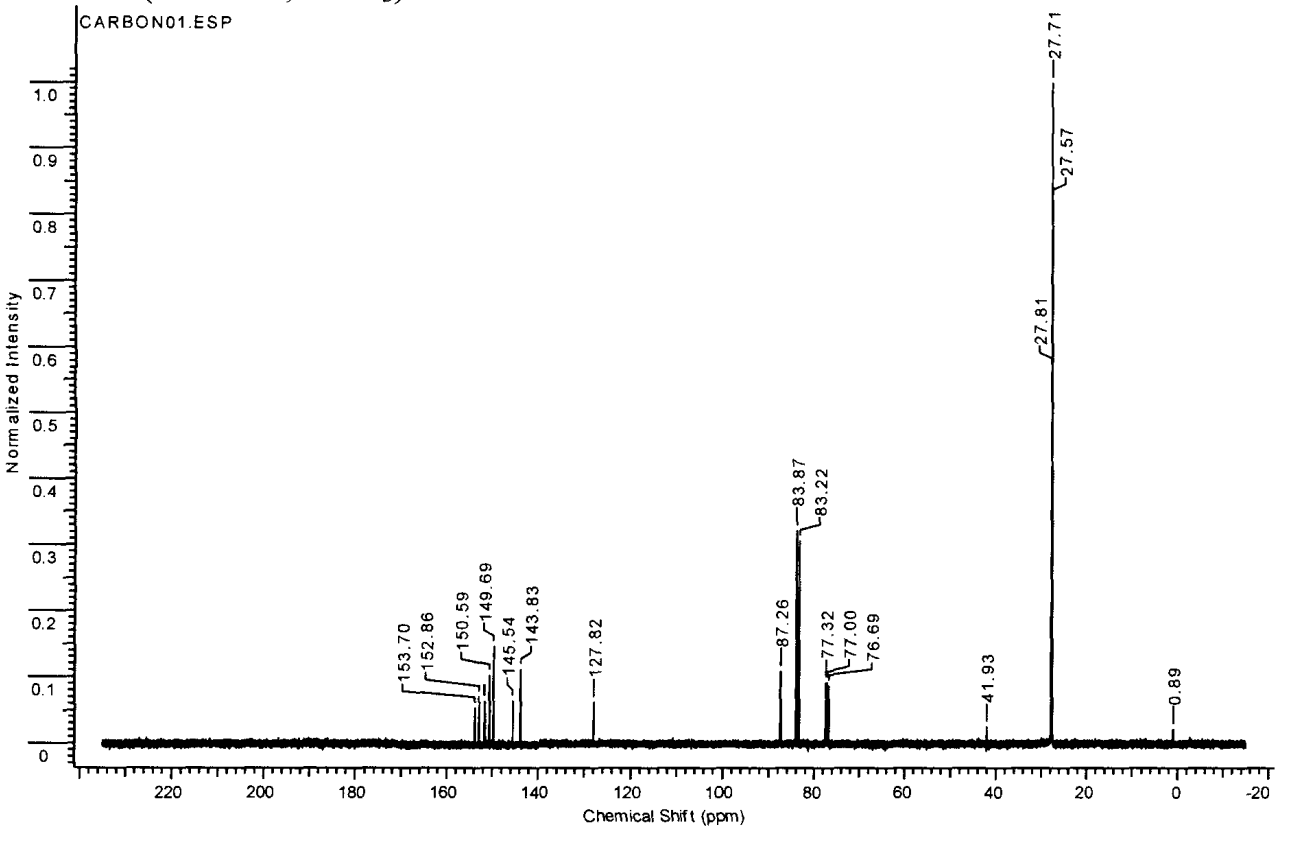

$^1$H NMR (400 MHz, DMSO-d6) of **3**



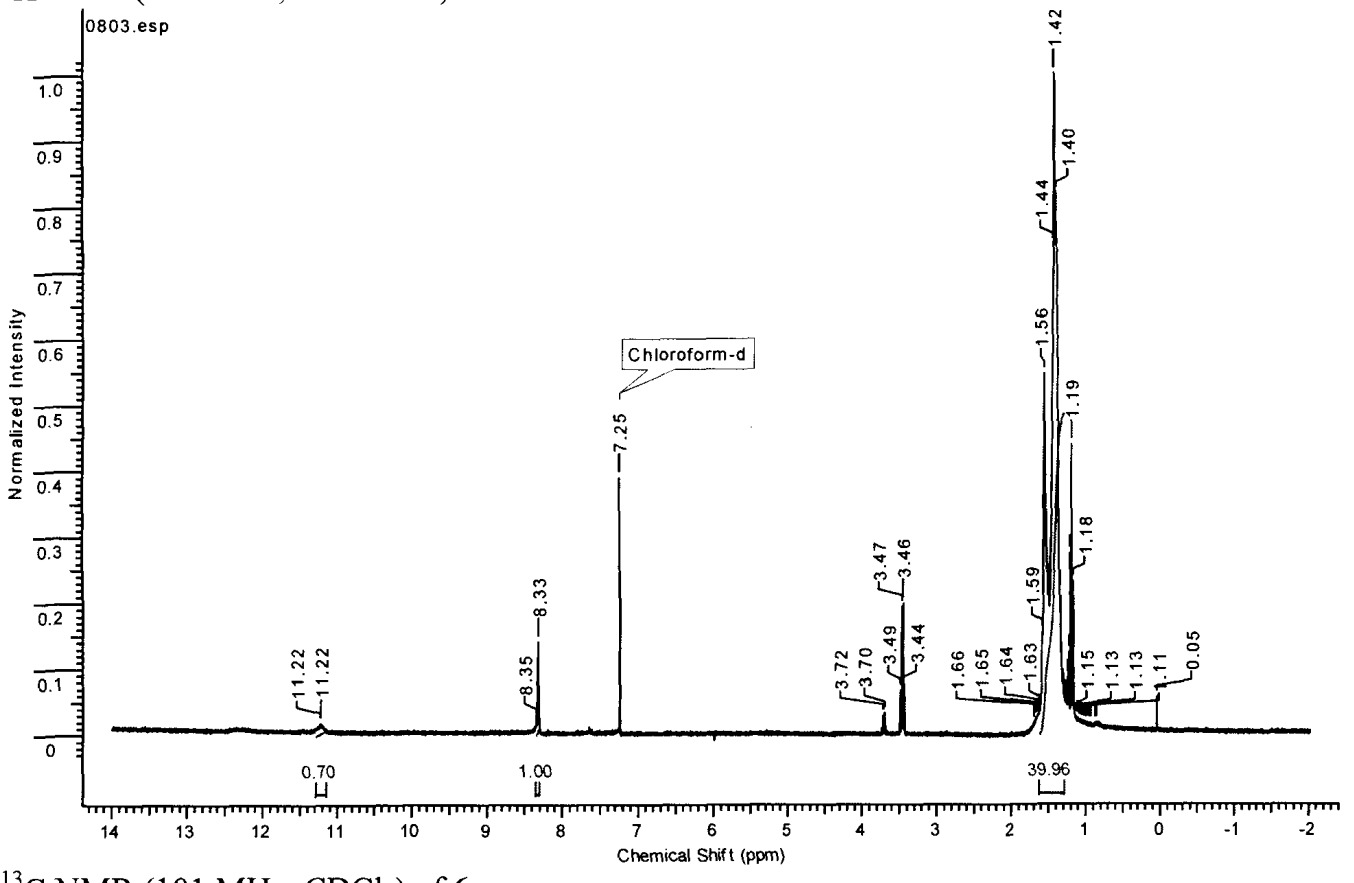
$^{1}$H NMR (400 MHz, $CDCl_3$) of **5**



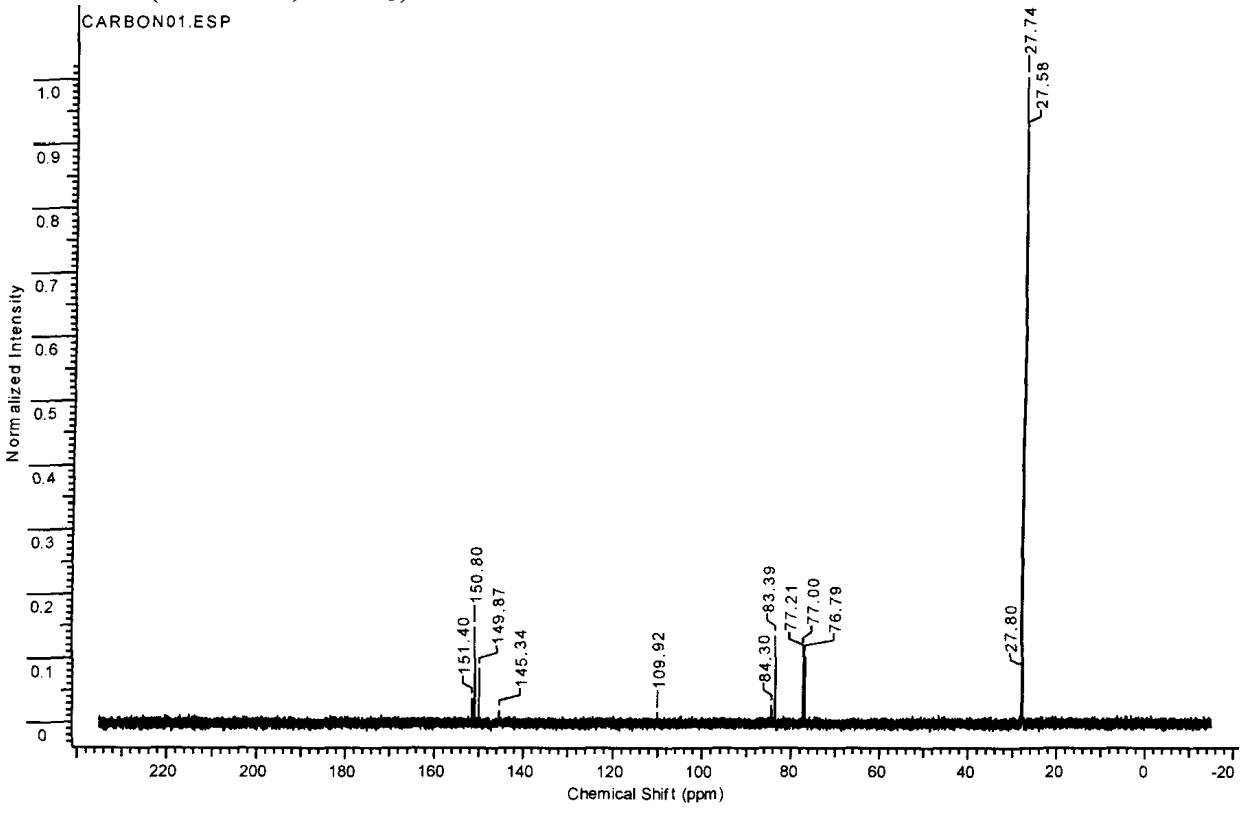

$^{13}$C NMR (101 MHz, $CDCl_3$) of **5**



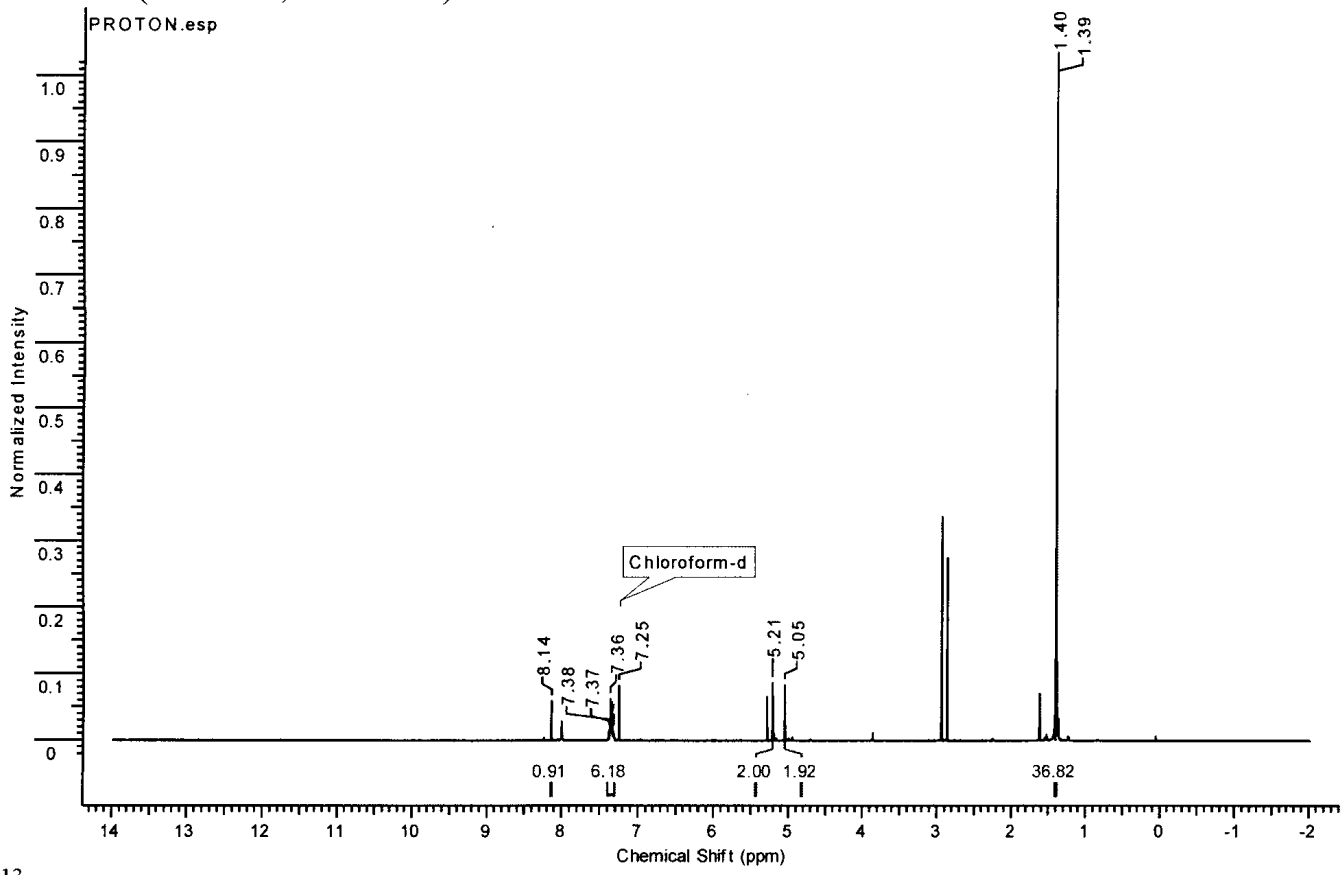
$^{1}$H NMR (400 MHz, DMSO-*d6*) of **6**



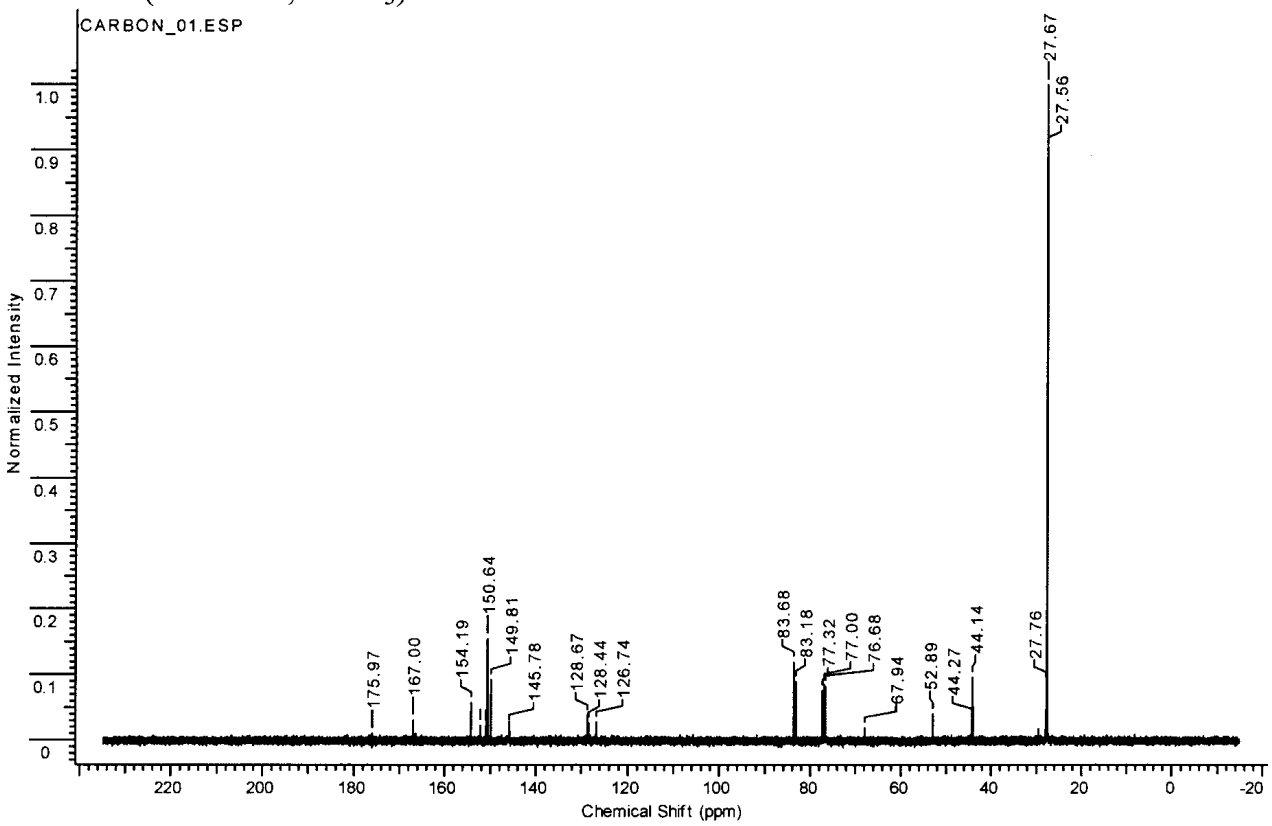

$^{13}$C NMR (101 MHz, $CDCl_3$) of **6**



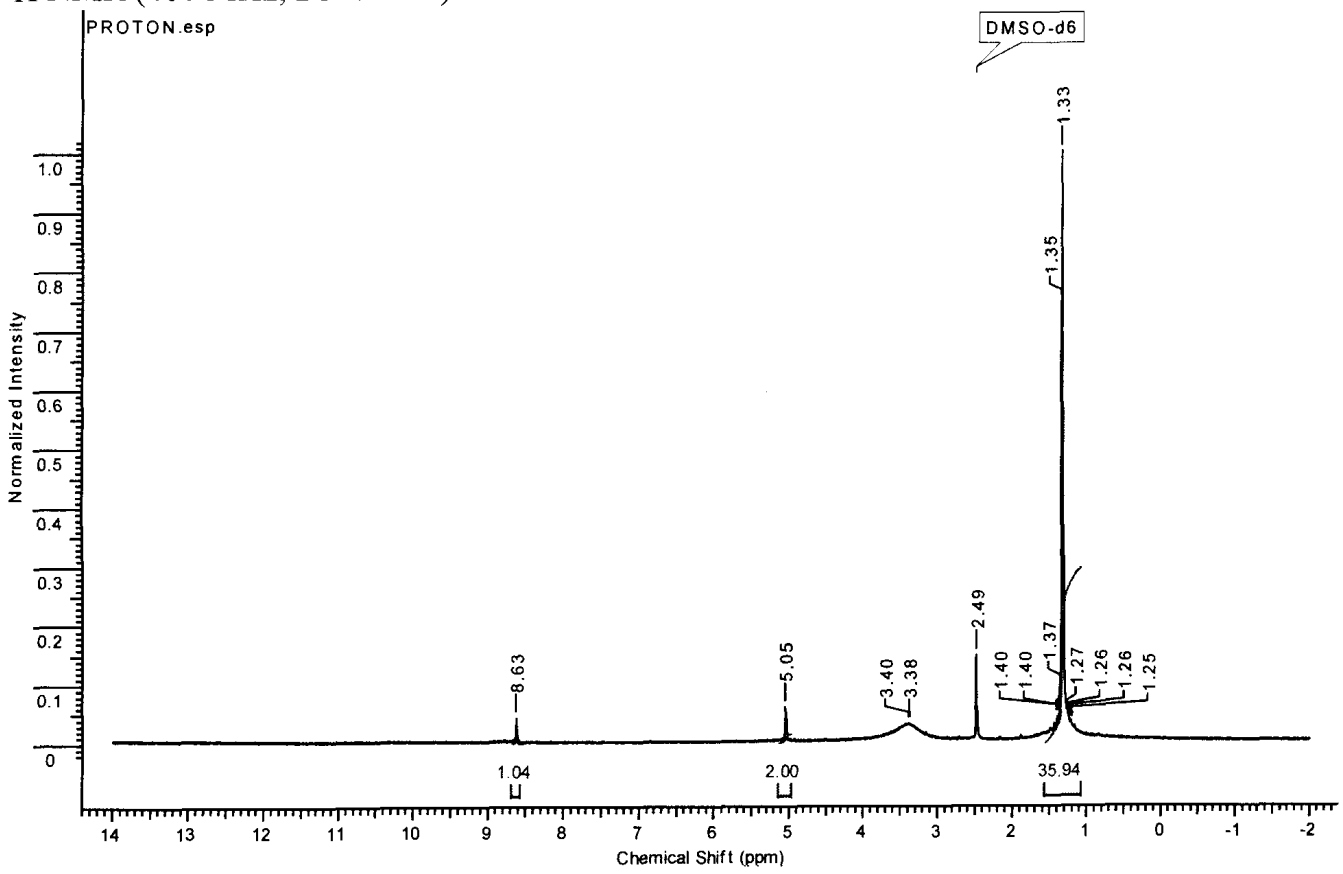
$^{1}$H NMR (400 MHz, DMSO-*d6*) of **7**



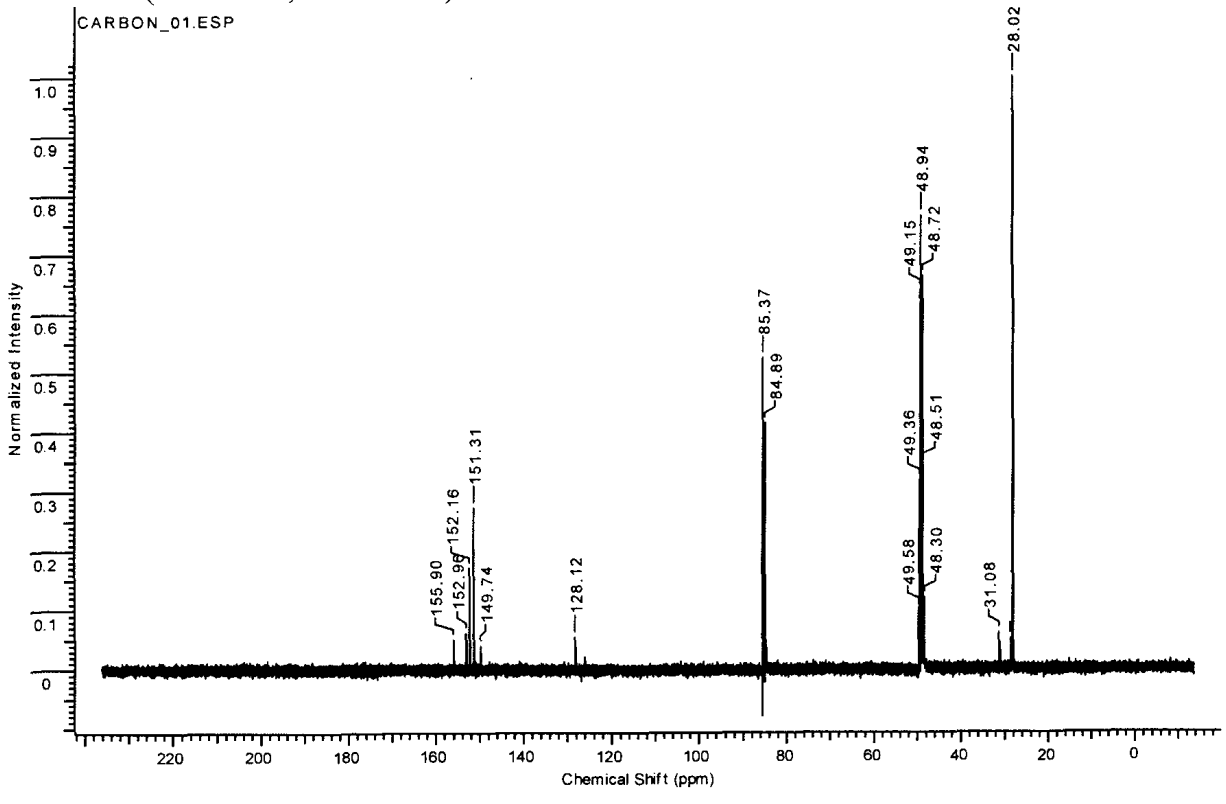

$^{13}$C NMR (101 MHz, $CDCl_3$) of **7**



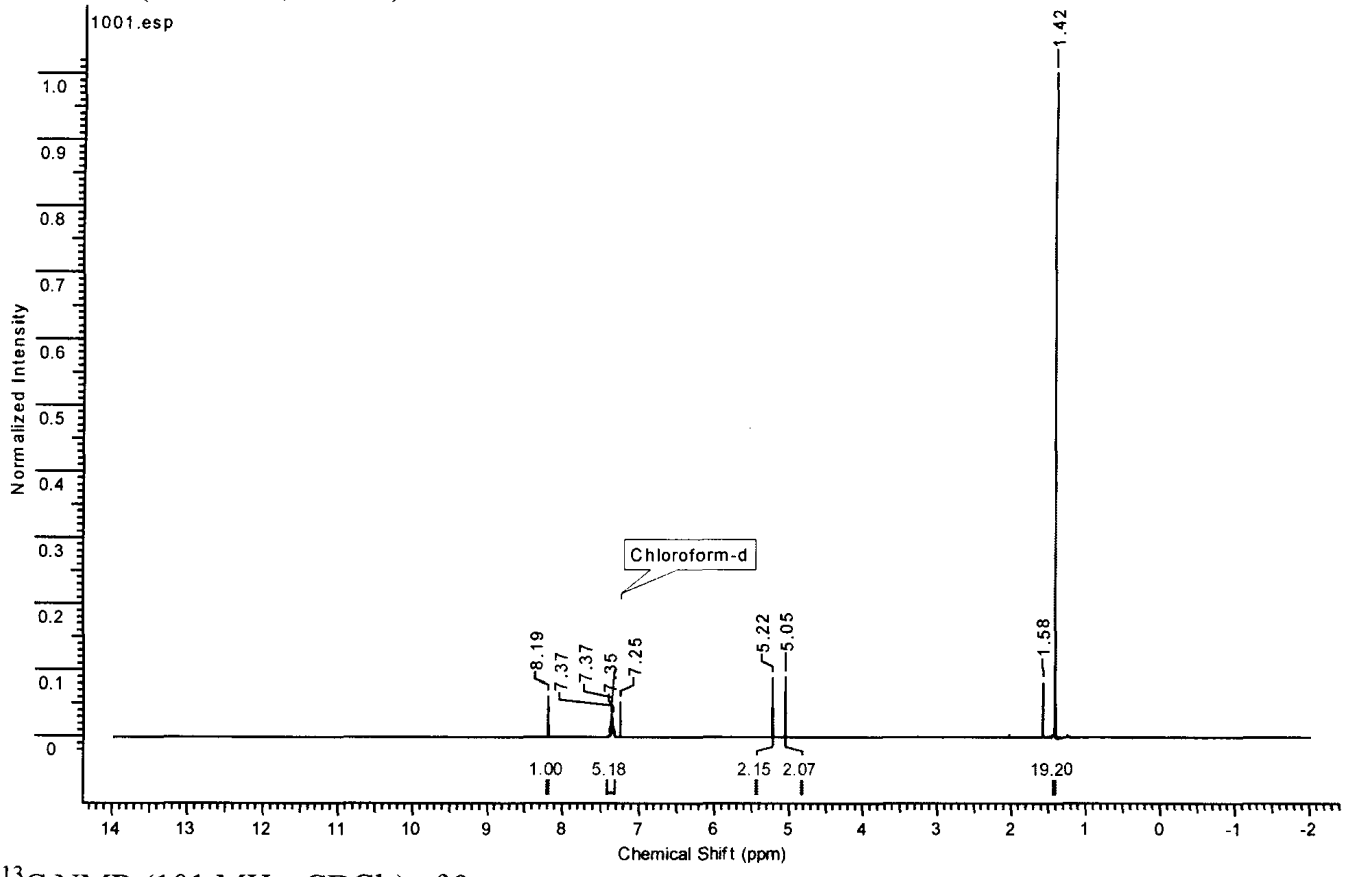
$^{1}$H NMR (400 MHz, DMSO-*d6*) of **8**



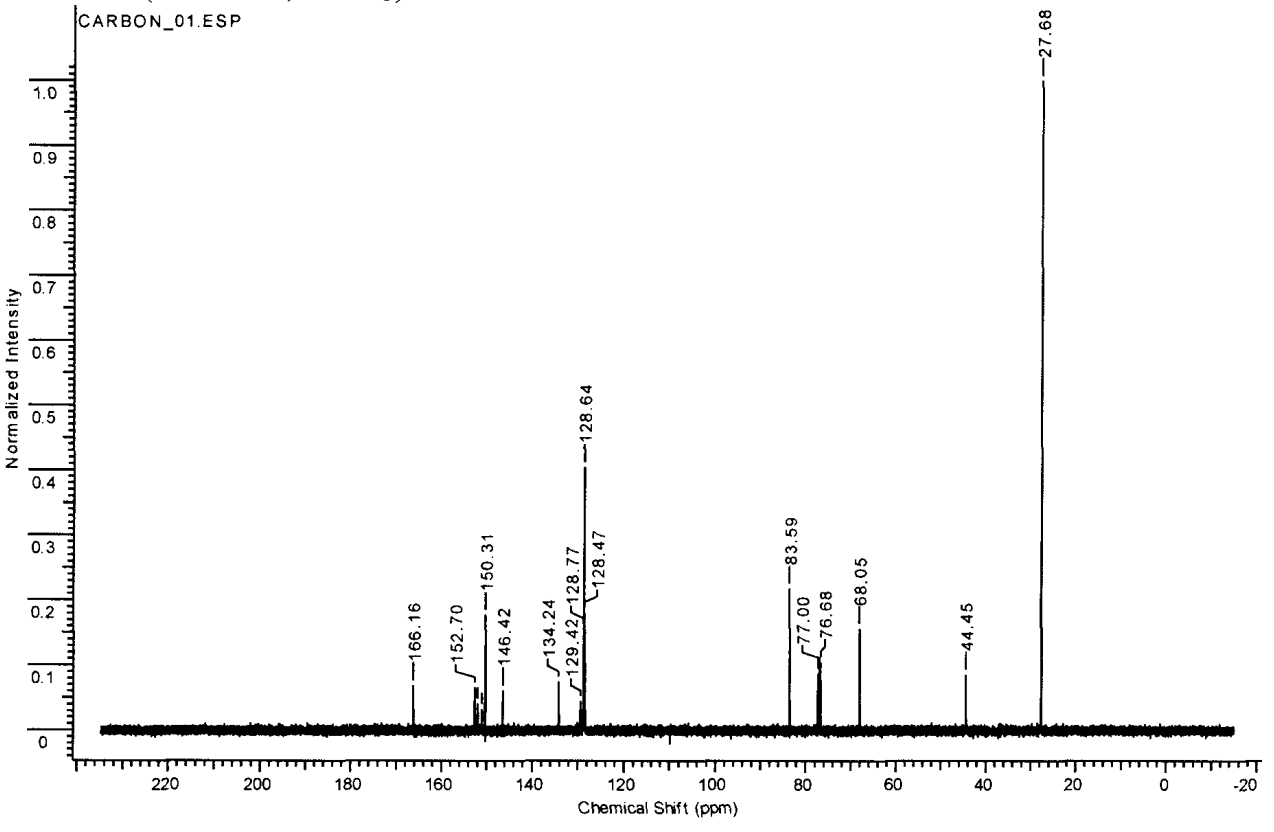

$^{13}$C NMR (101 MHz, MeOD-*d4*) of **8**



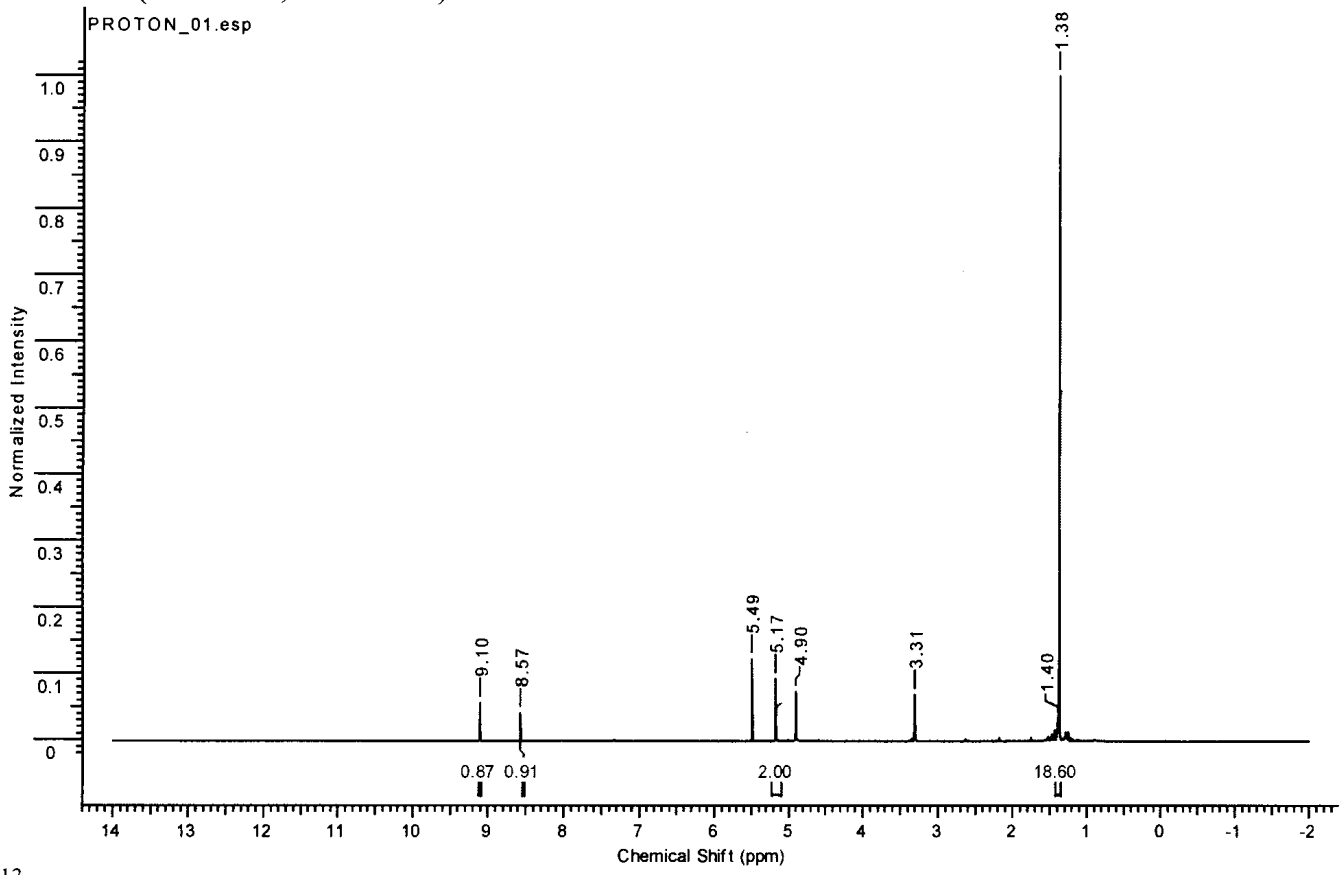
$^{1}$H NMR (400 MHz, $CDCl_3$) of **9**



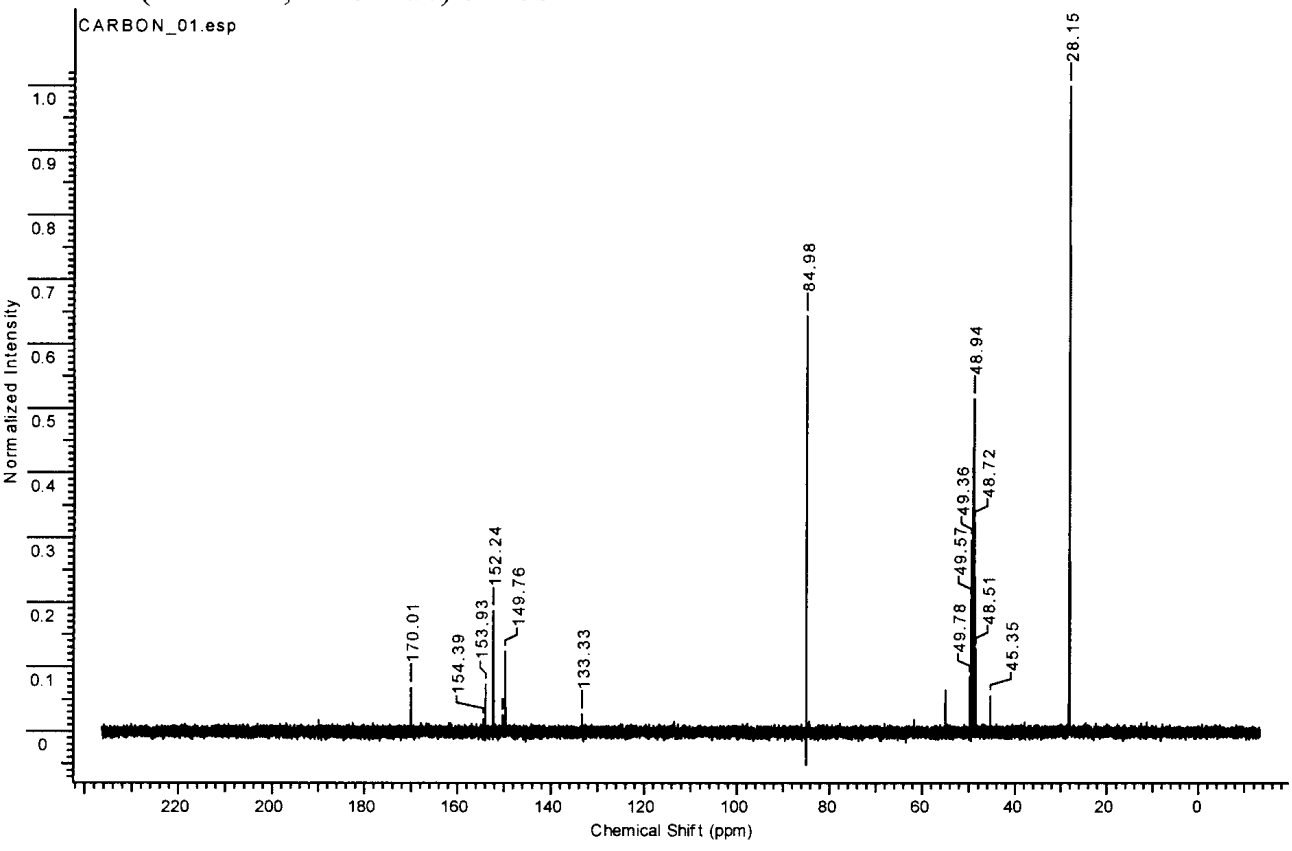

$^{13}$C NMR (101 MHz, $CDCl_3$) of **9**



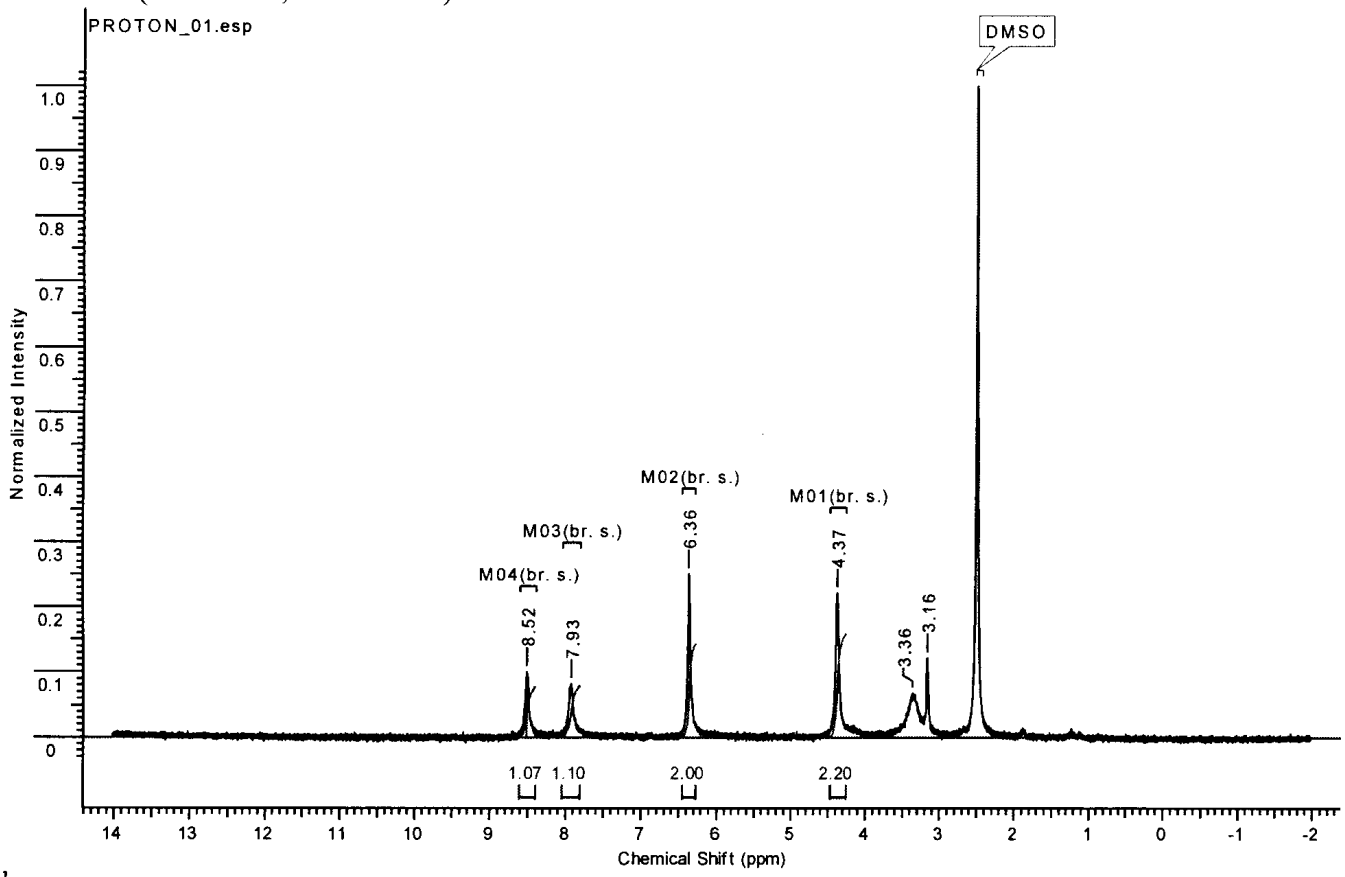
$^{1}$H NMR (400 MHz, MeOH-*d4*) of **10b**



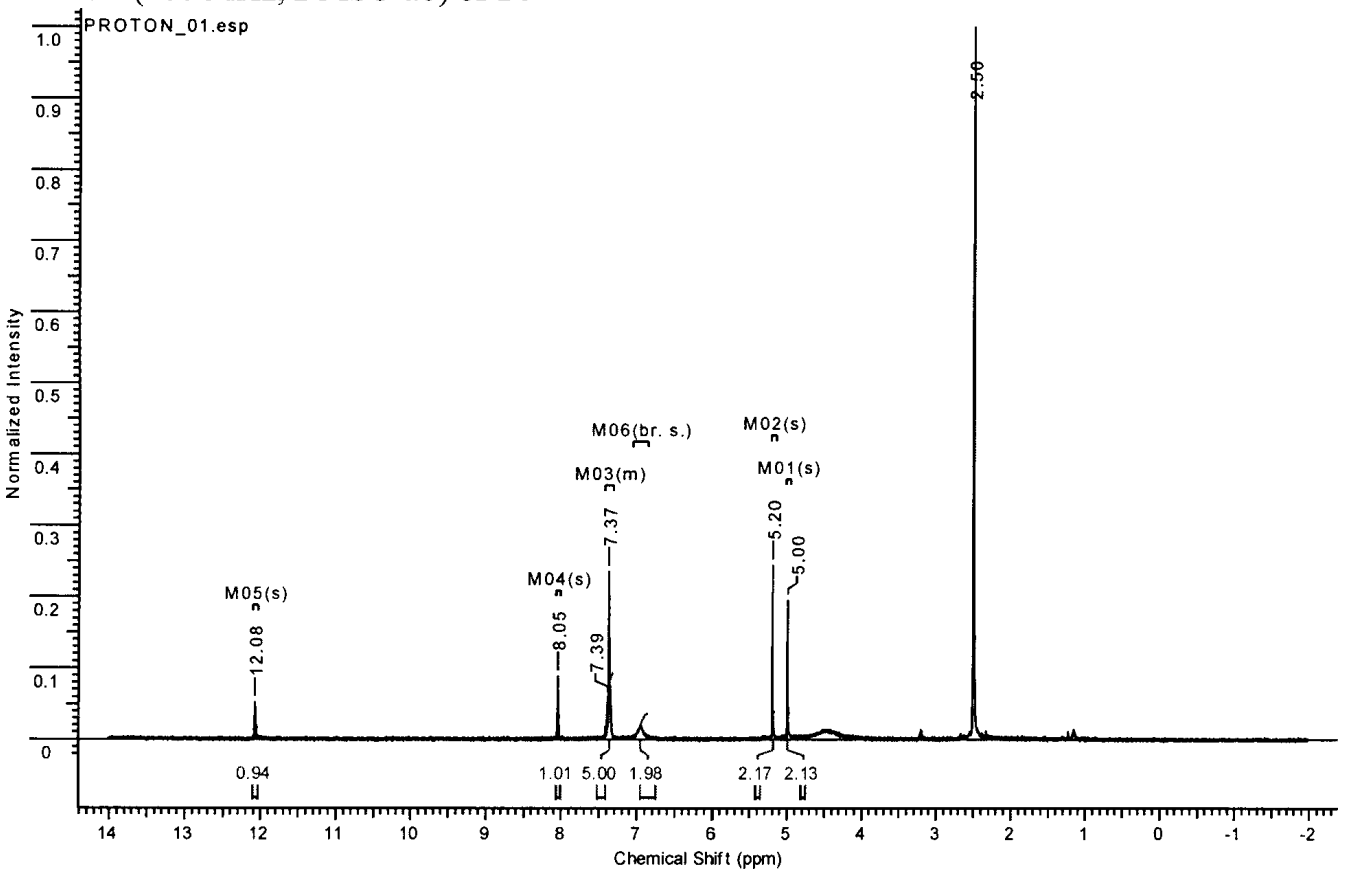

$^{13}$C NMR (101 MHz, MeOH-*d4*) of **10b**



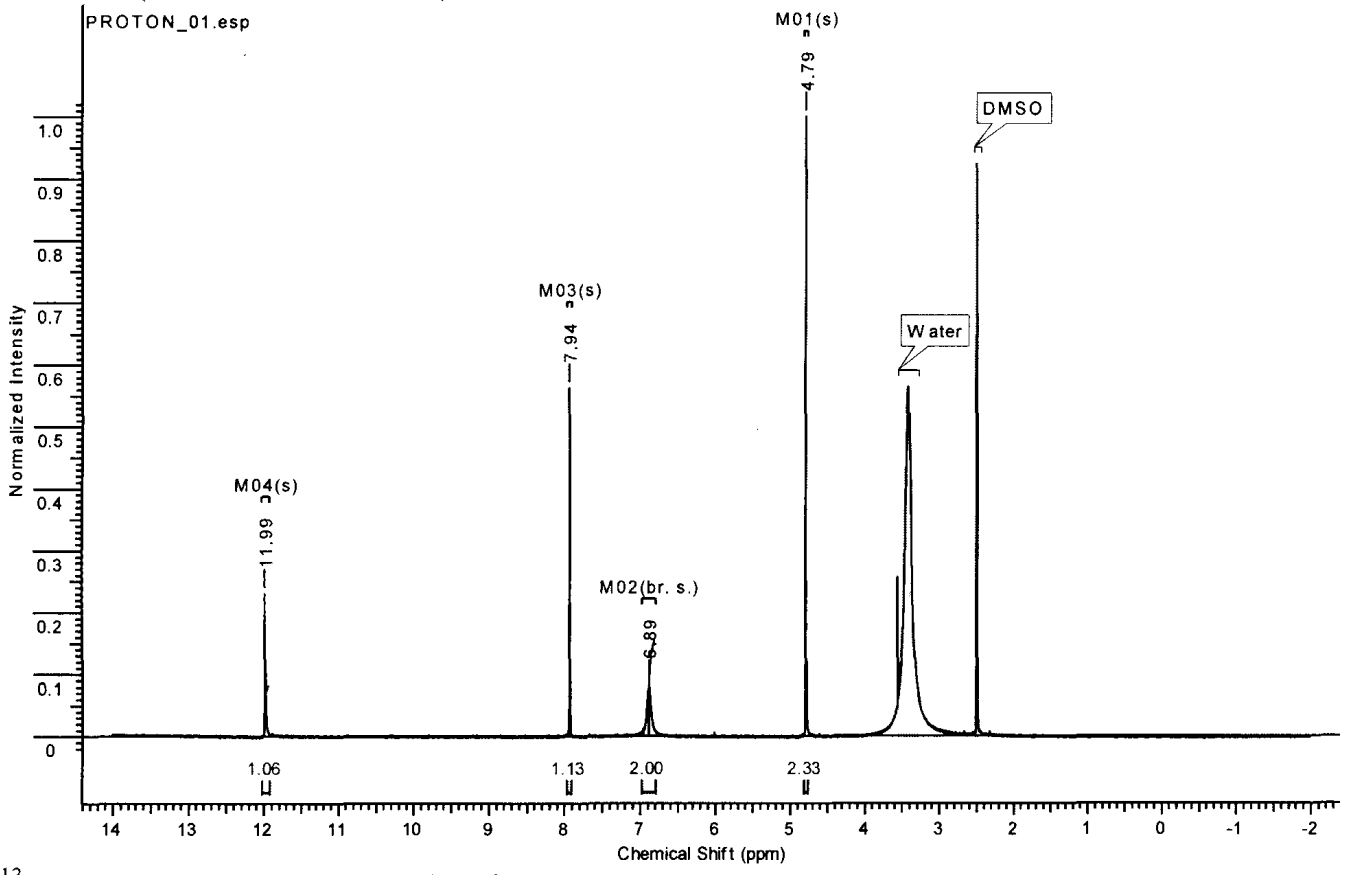
$^1$H NMR (400 MHz, DMSO-*d6*) of **13**



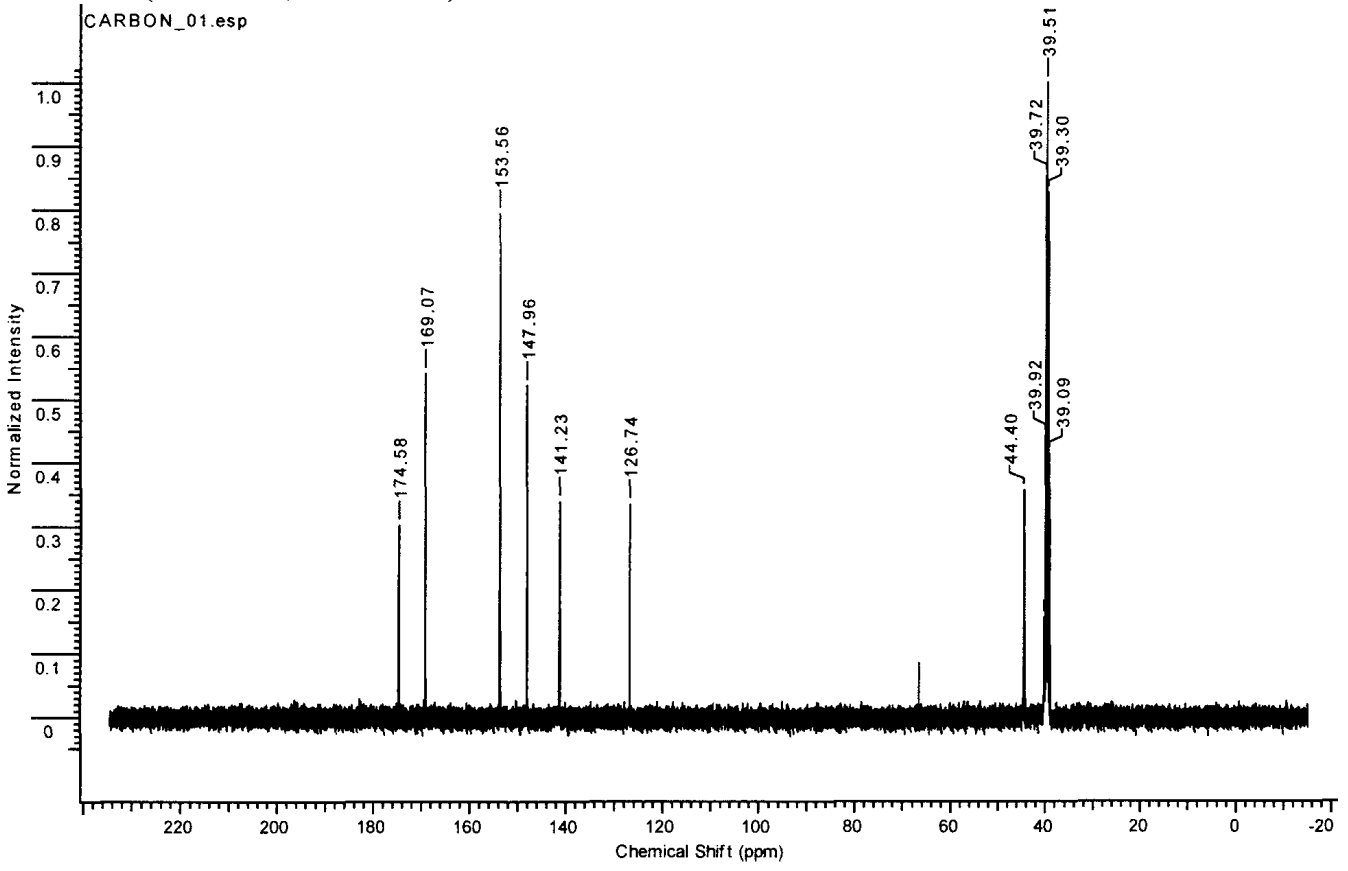

$^1$H NMR (400 MHz, DMSO-*d6*) of **14**



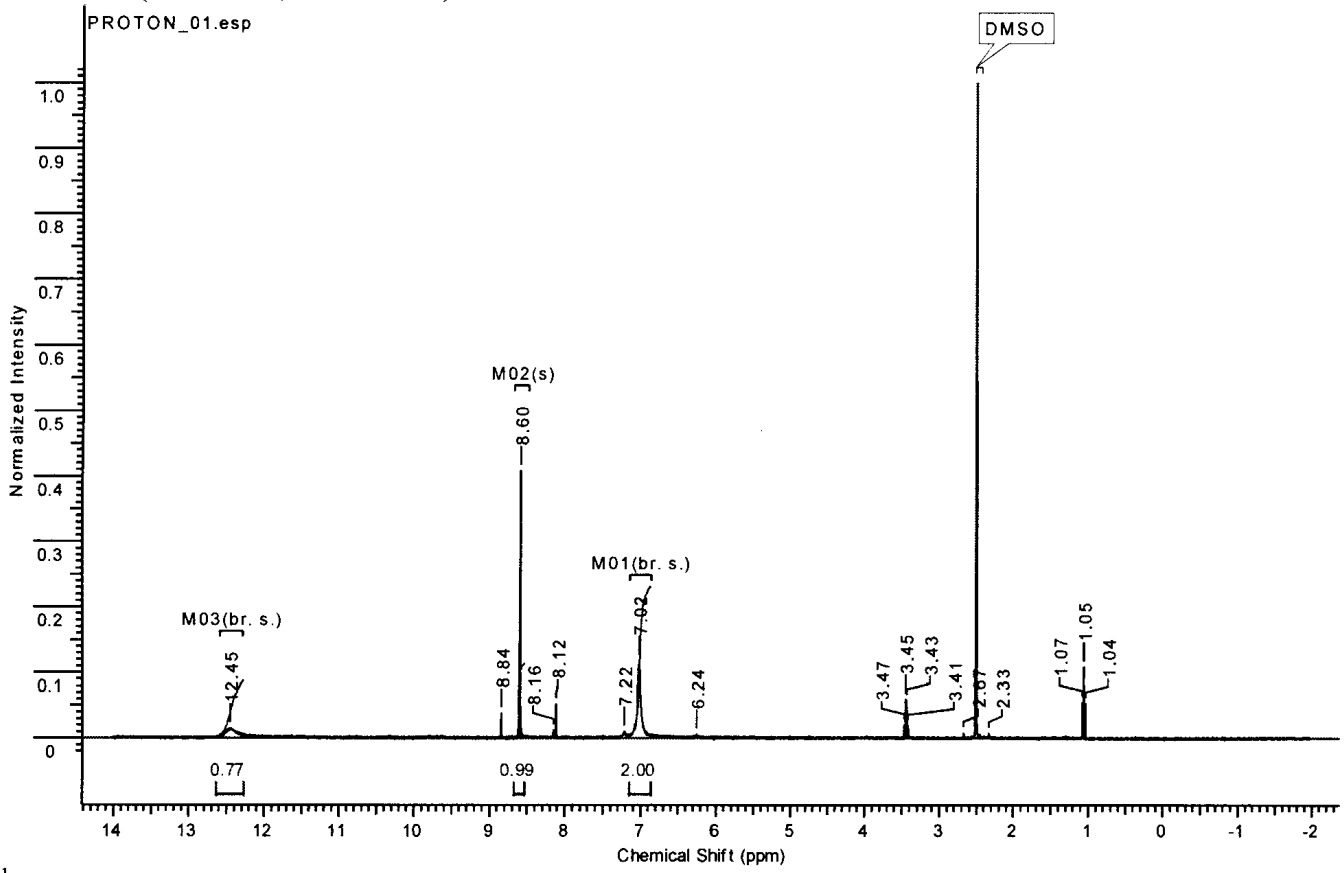
$^{1}$H NMR (400 MHz, DMSO-*d6*) of **16**



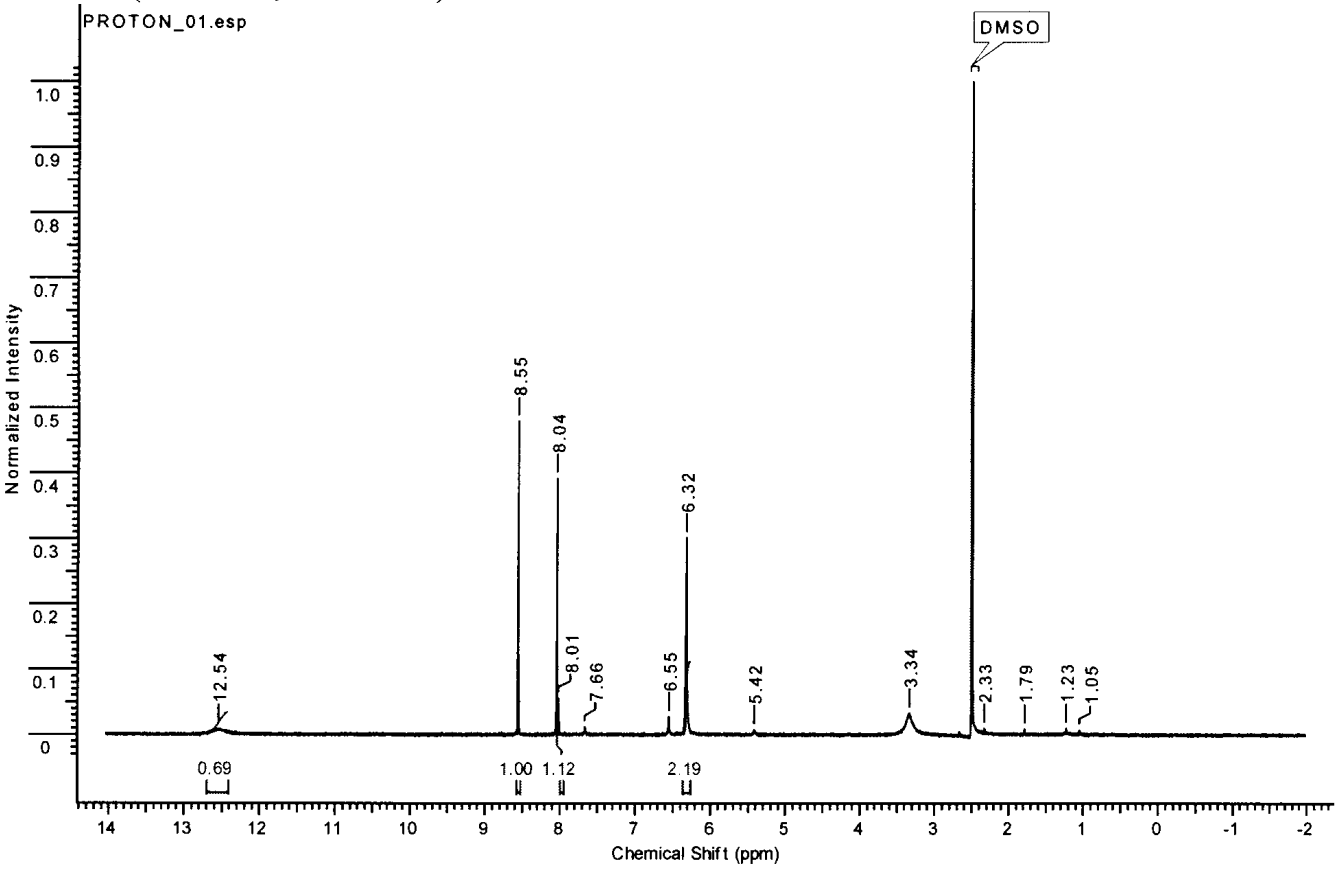

$^{13}$C NMR (101 MHz, DMSO-*d6*) of **16**



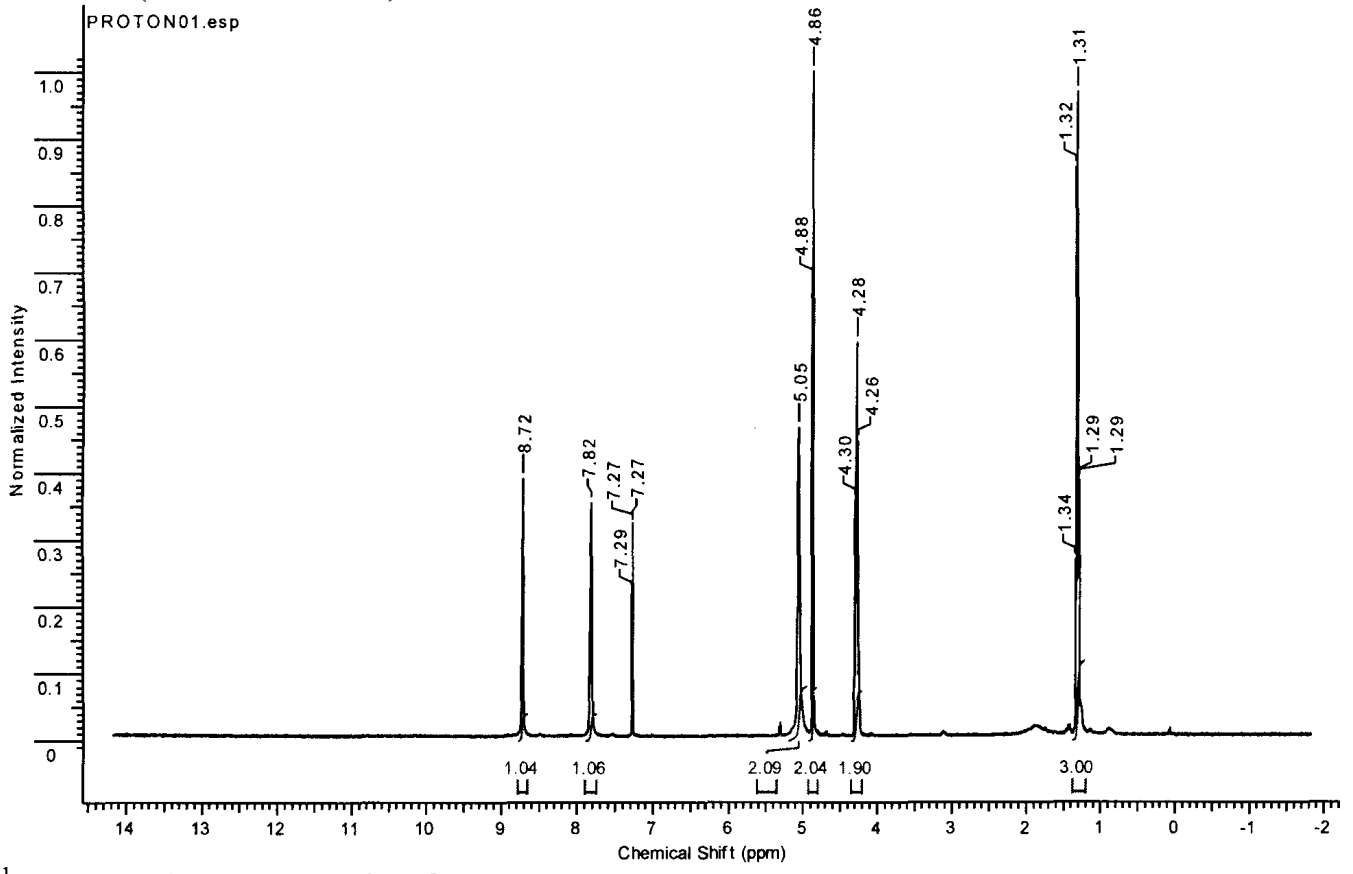
$^1$H NMR (400 MHz, DMSO-*d6*) of **18**



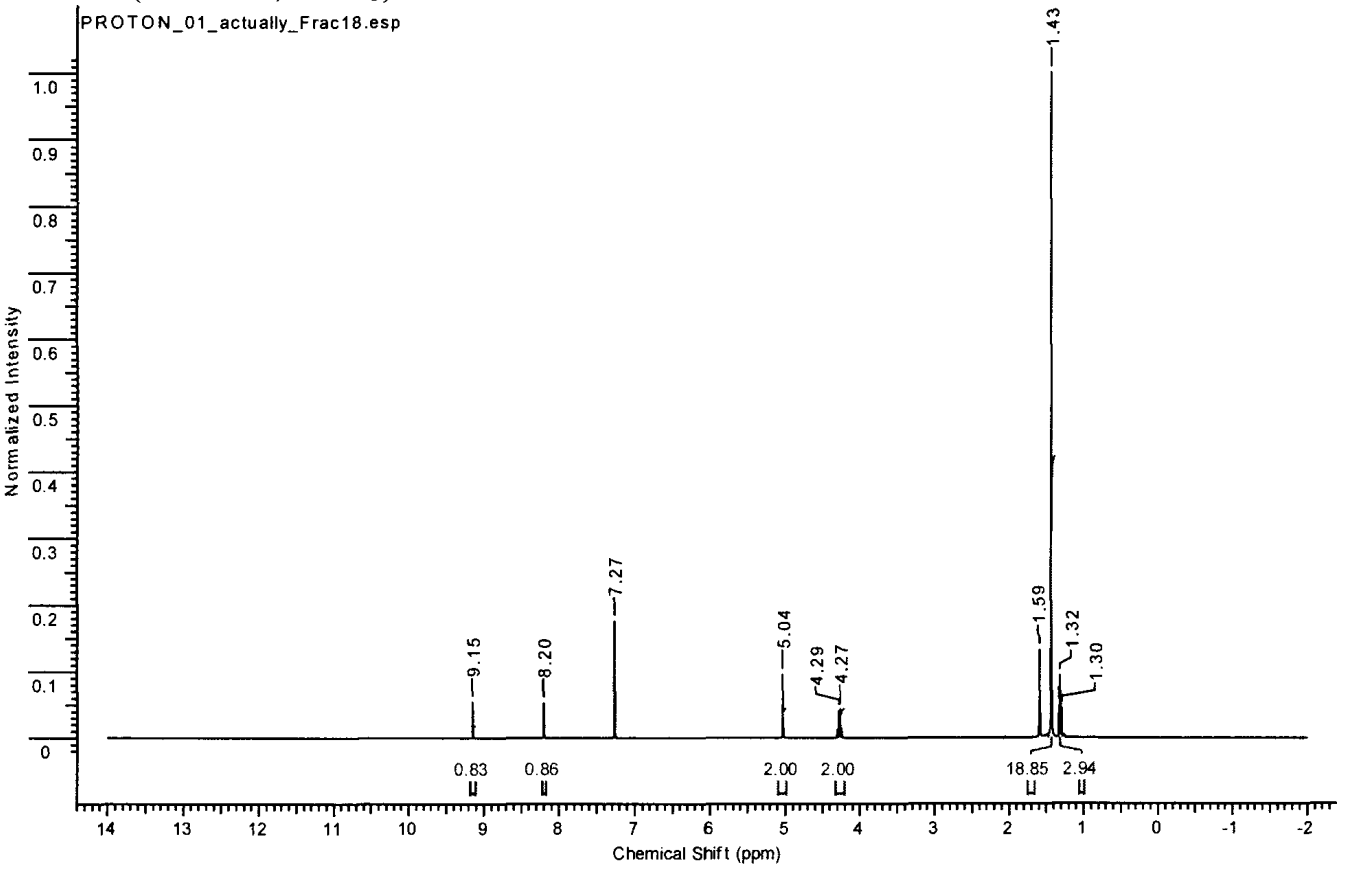

$^1$H NMR (400 MHz, DMSO-*d6*) of **19**



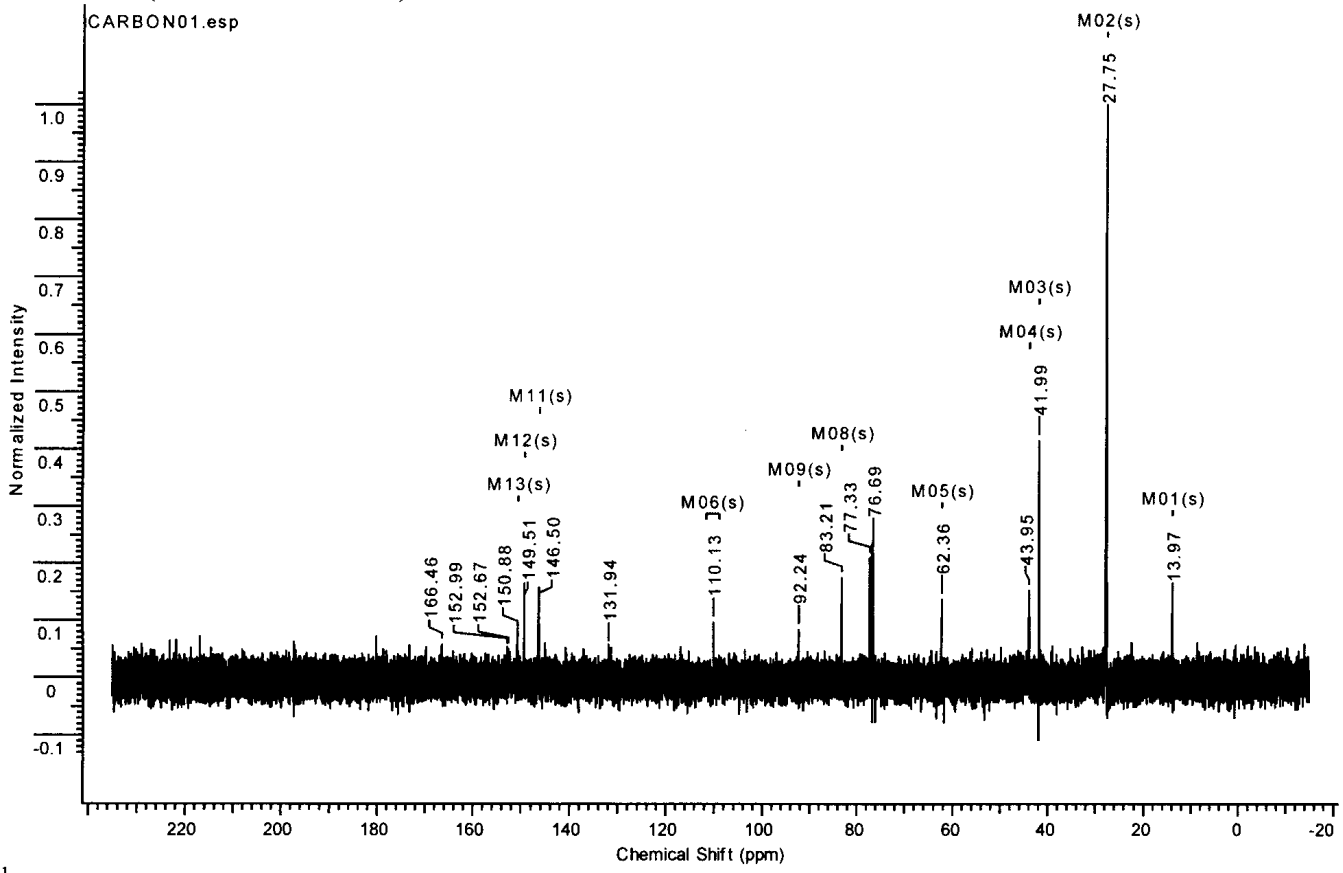
$^1$H NMR (400 MHz, $CDCl_3$) of **24**



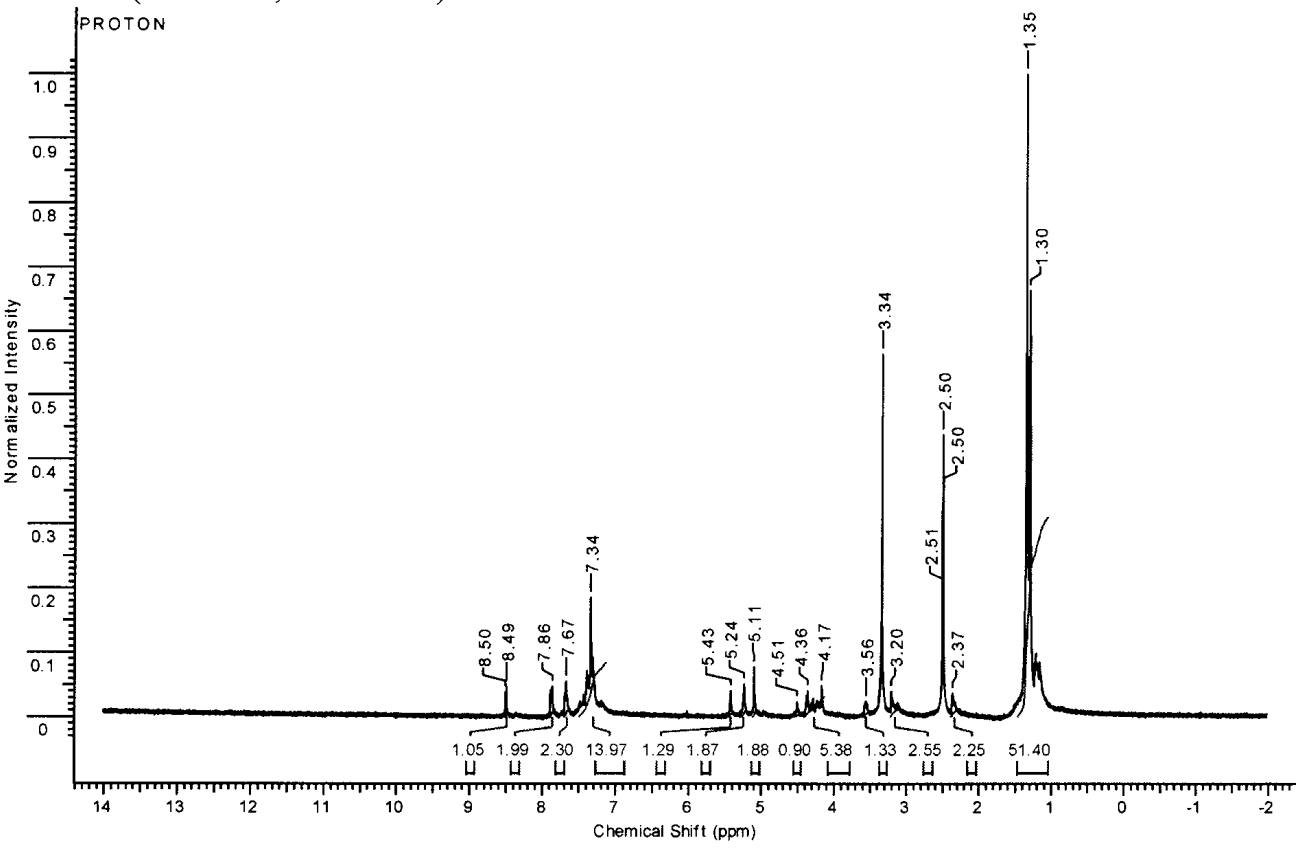

$^1$H NMR (400 MHz, $CDCl_3$) of **25**



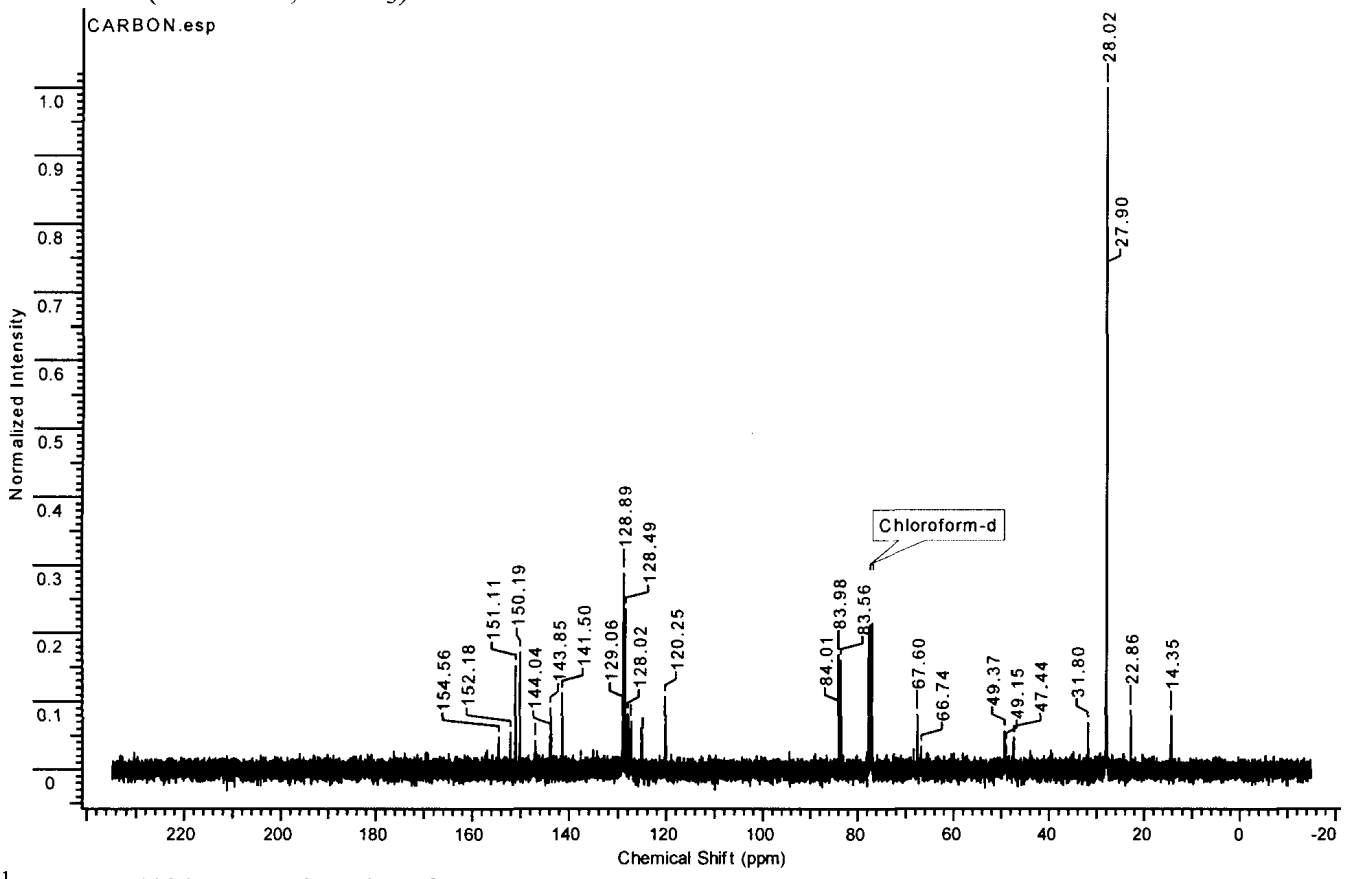
$^{13}$C NMR (101 MHz, $CDCl_3$) of **25**



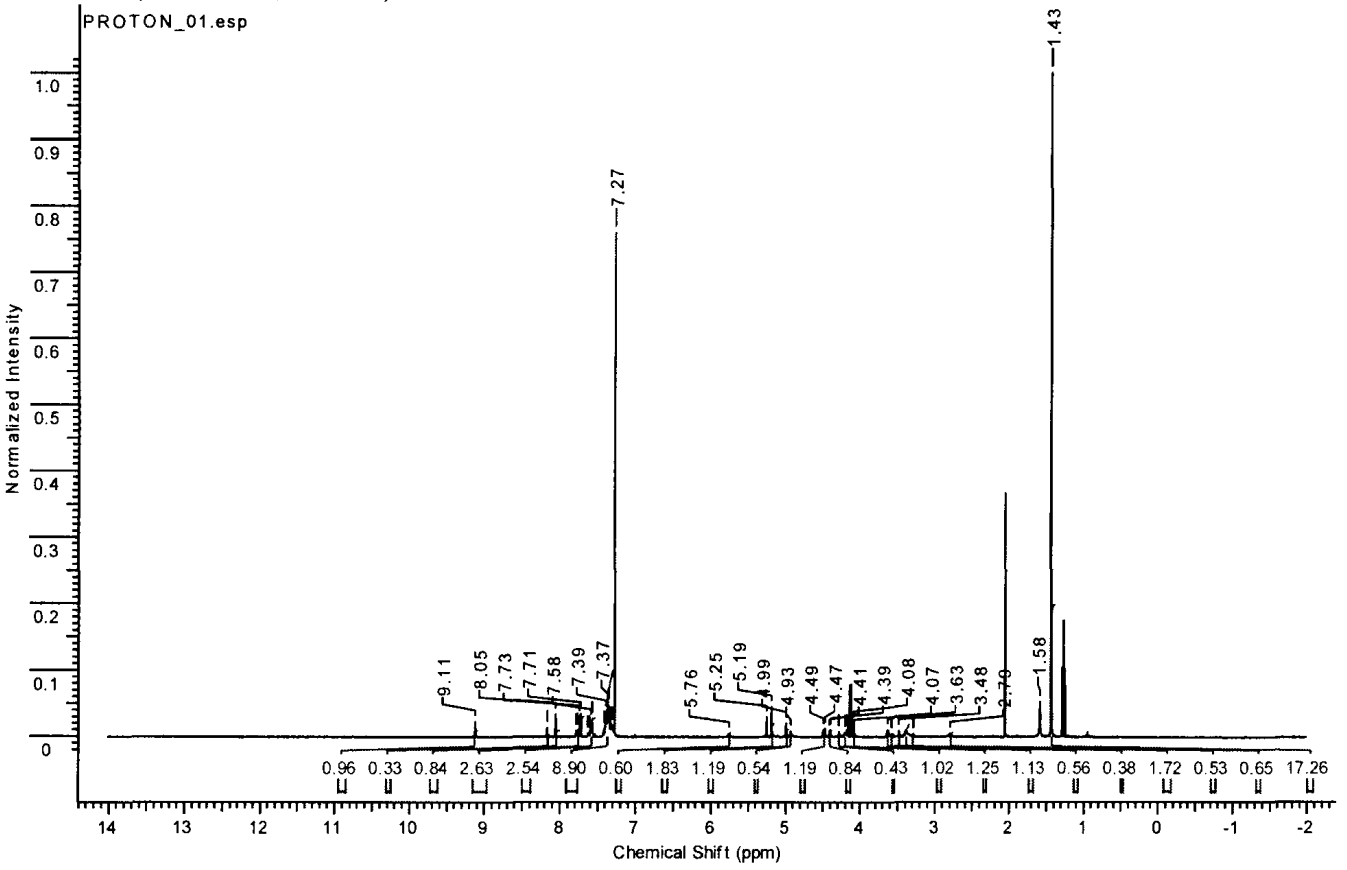

$^{1}$H NMR (400 MHz, DMSO-*d6*) of **28a**



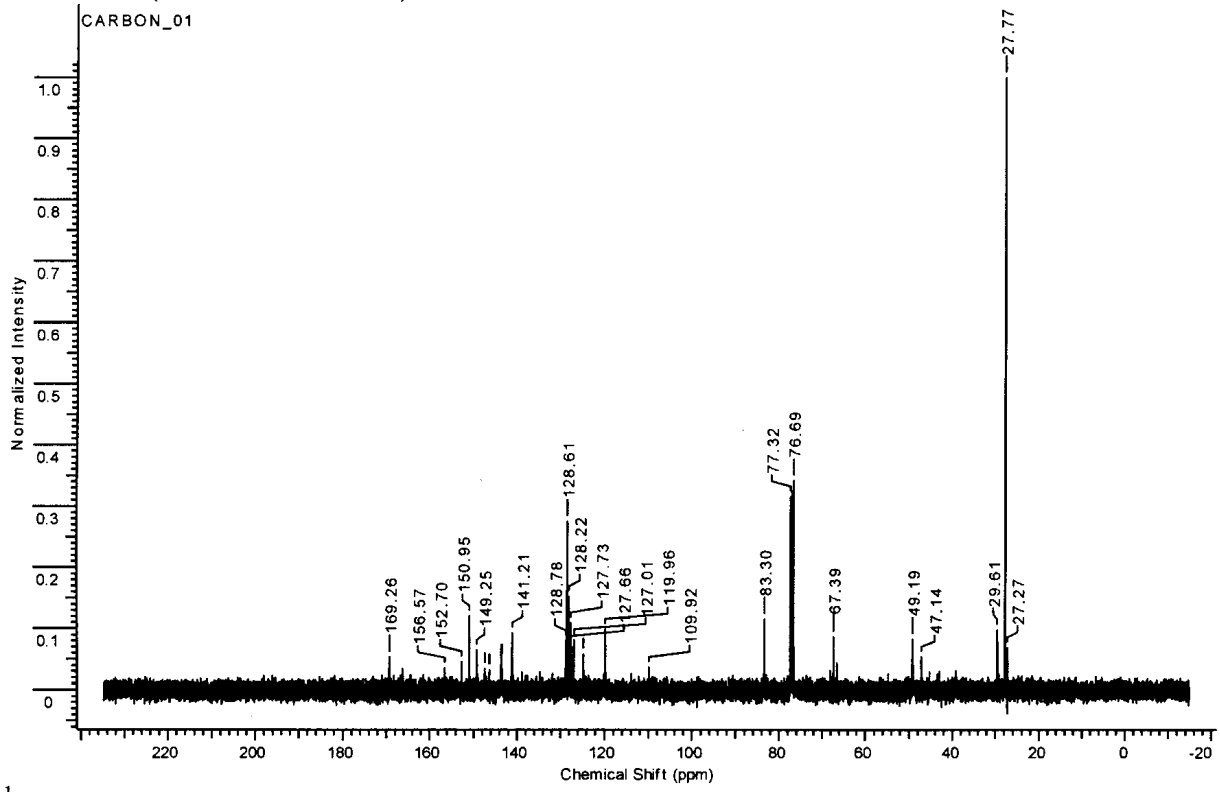
$^{13}$C NMR (101 MHz, $CDCl_3$) of **28a**



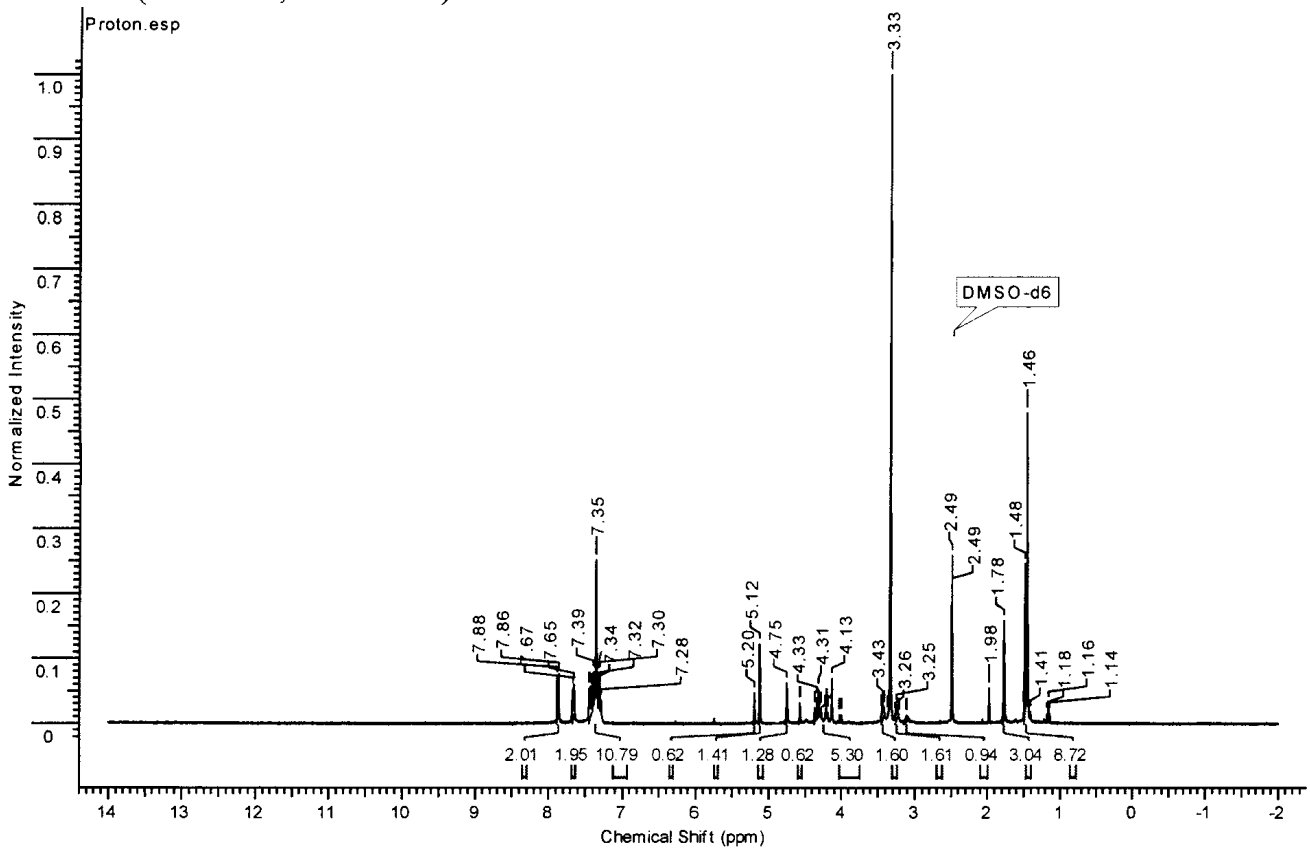

$^{1}$H NMR (400 MHz, $CDCl_3$) of **28b**



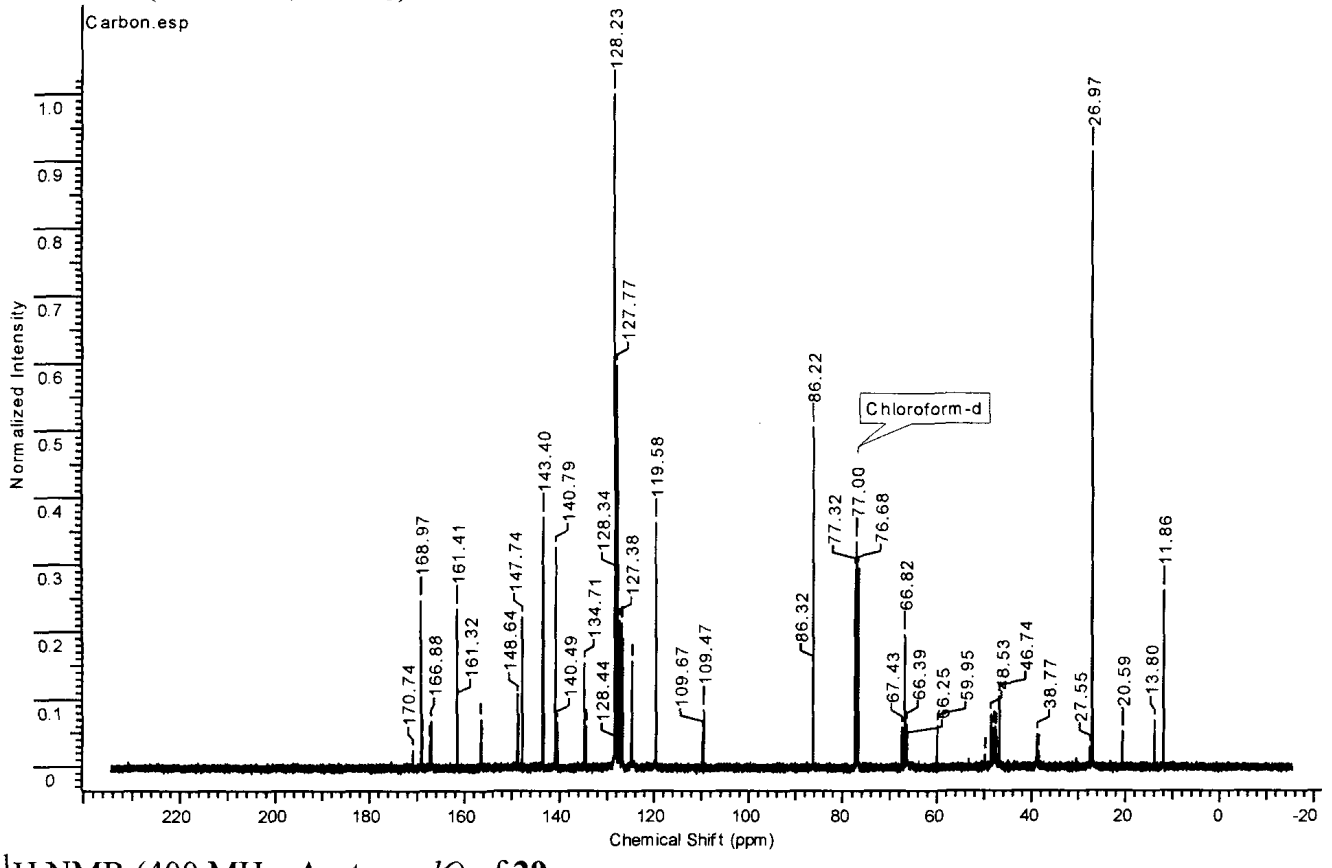
$^{13}$C NMR (101 MHz, $CDCl_3$) of **28b**



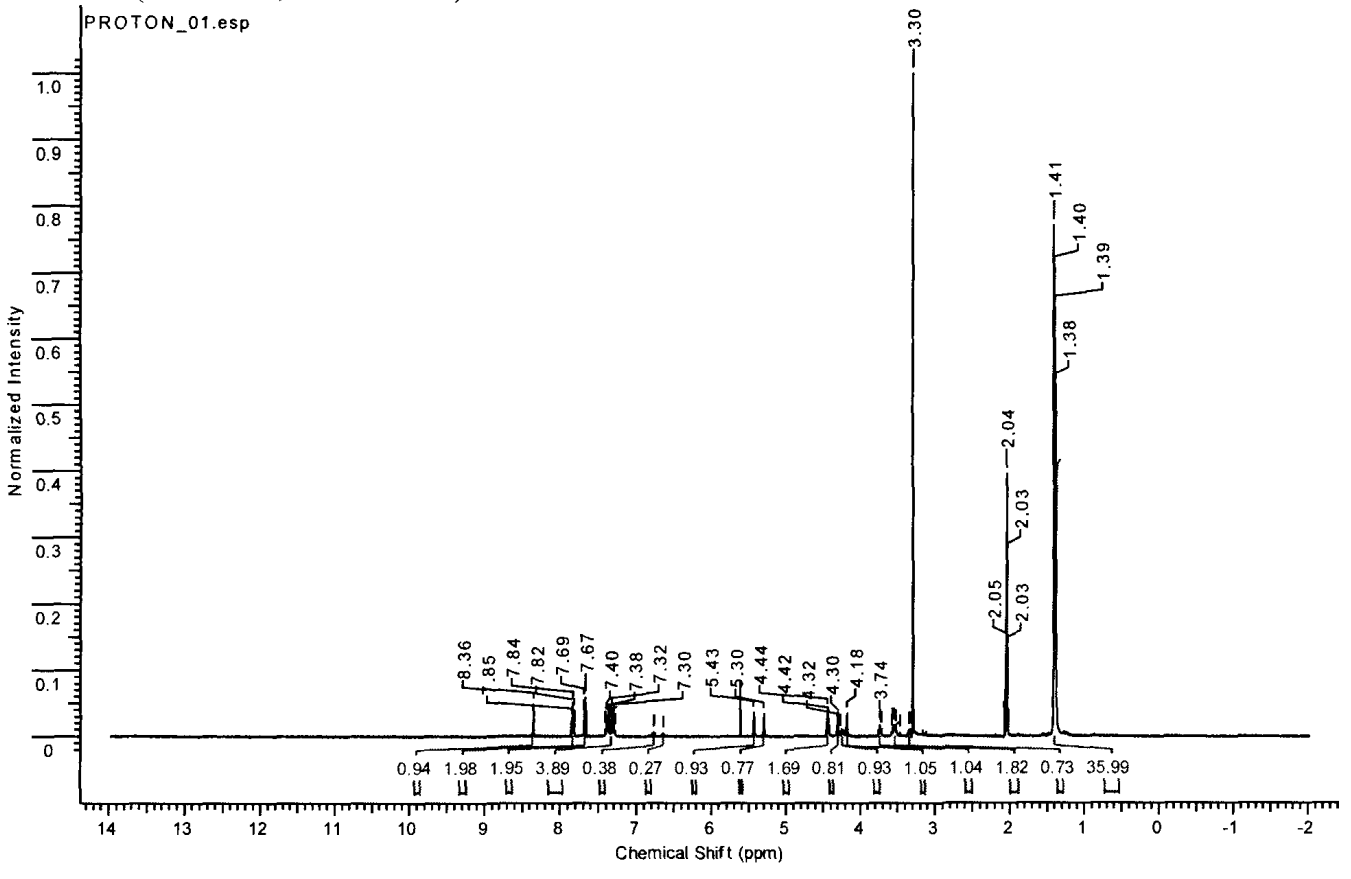

$^{1}$H NMR (400 MHz, DMSO-*d6*) of **28c**



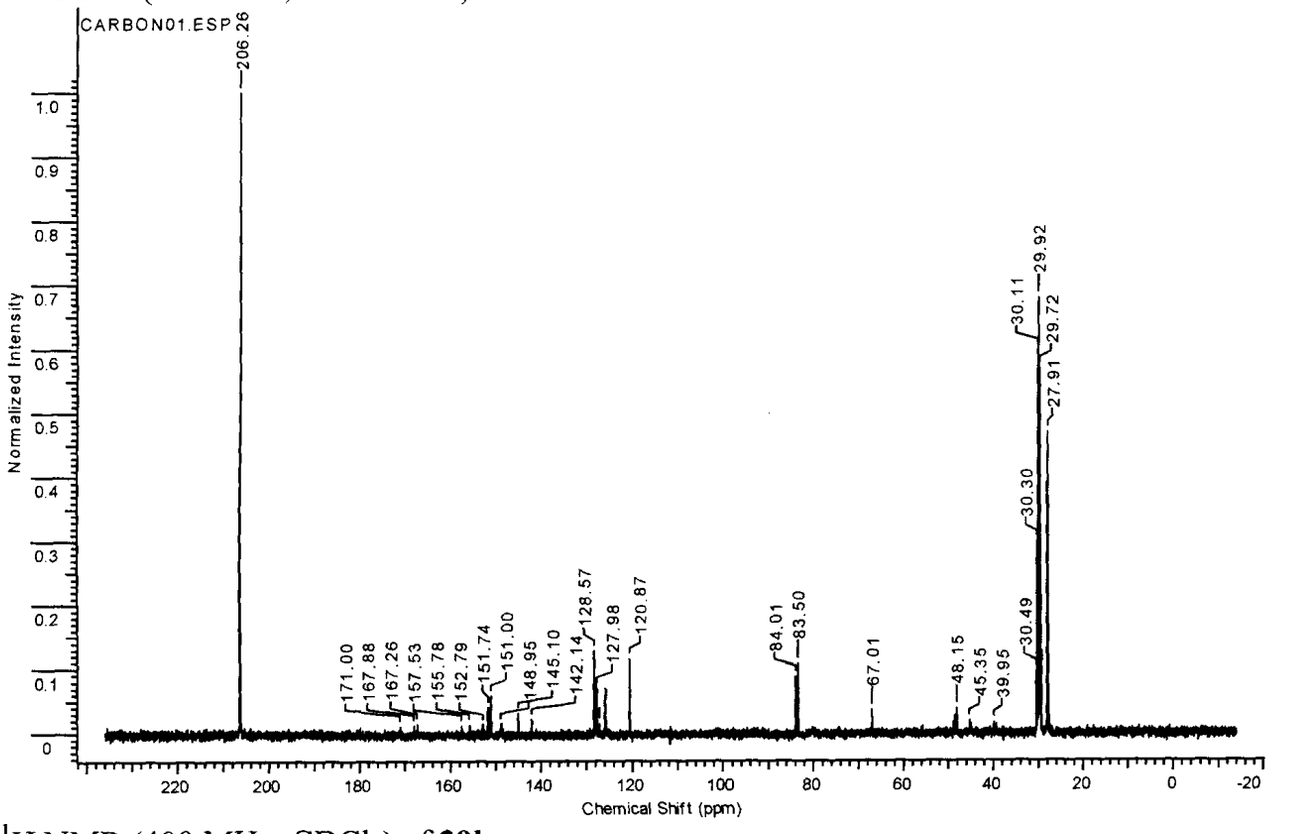
$^{13}C$ NMR (101 MHz, $CDCl_3$) of **28c**



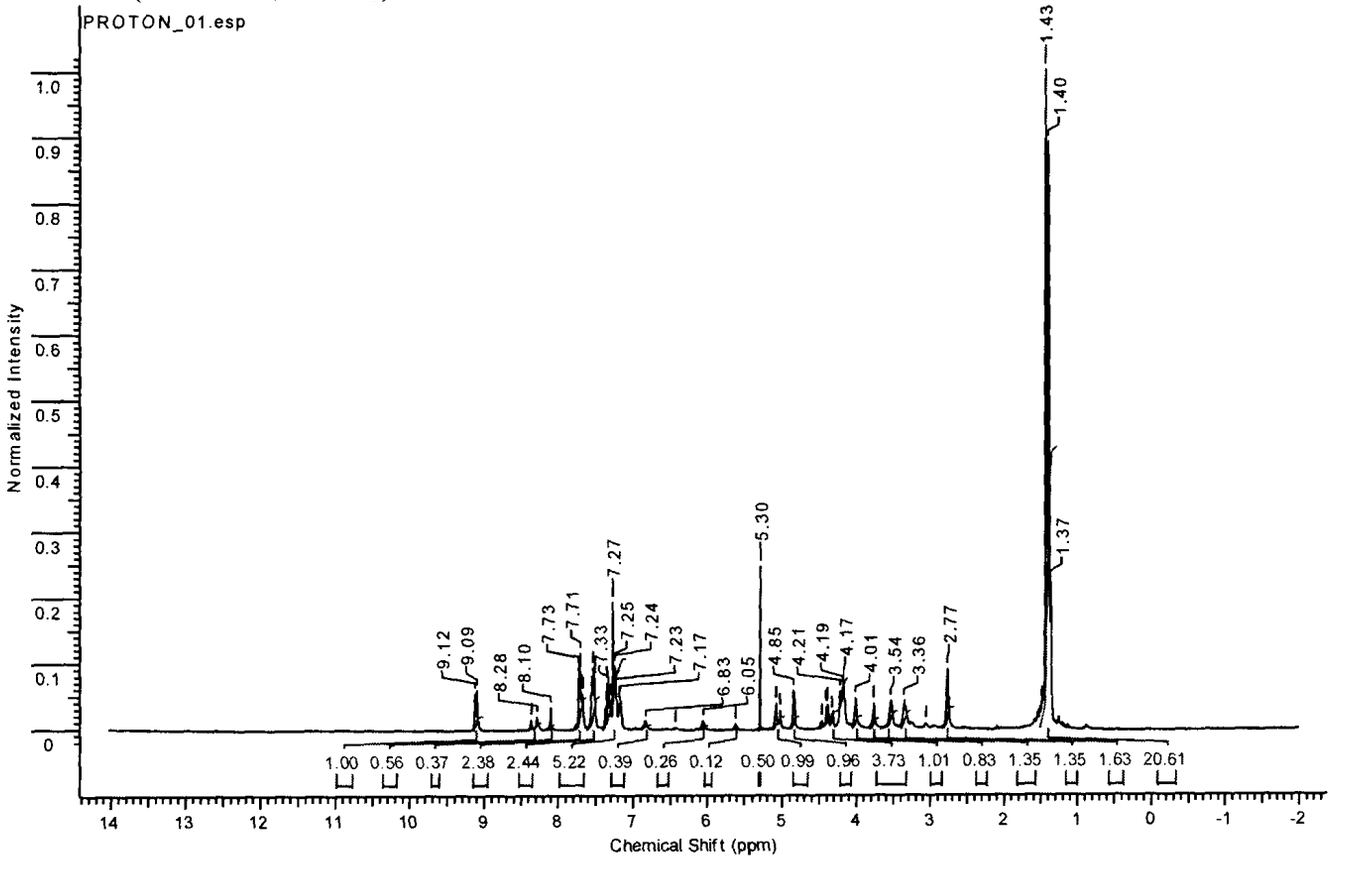

$^{1}H$ NMR (400 MHz, Acetone-*d6*) of **29a**



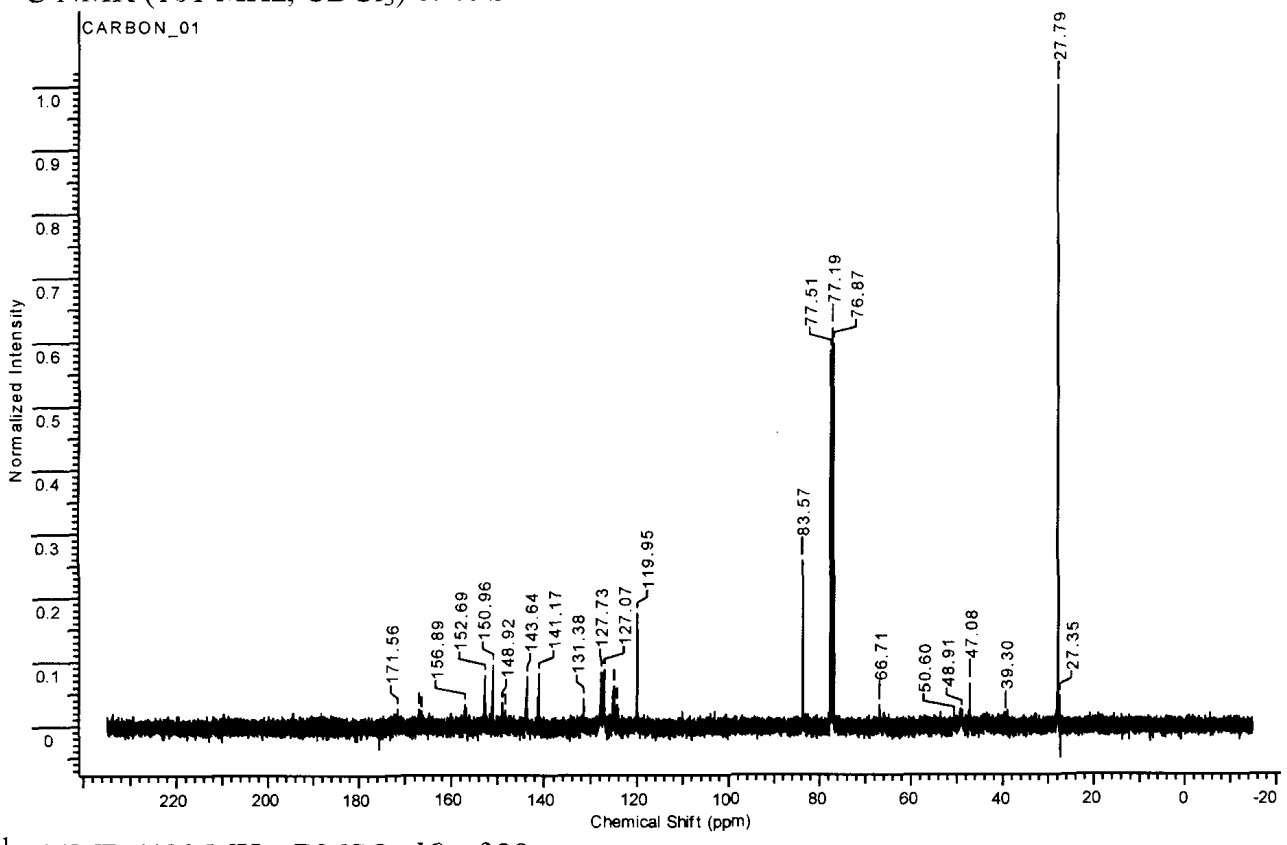
$^{13}$C NMR (101 MHz, Acetone-*d6*) of **29a**



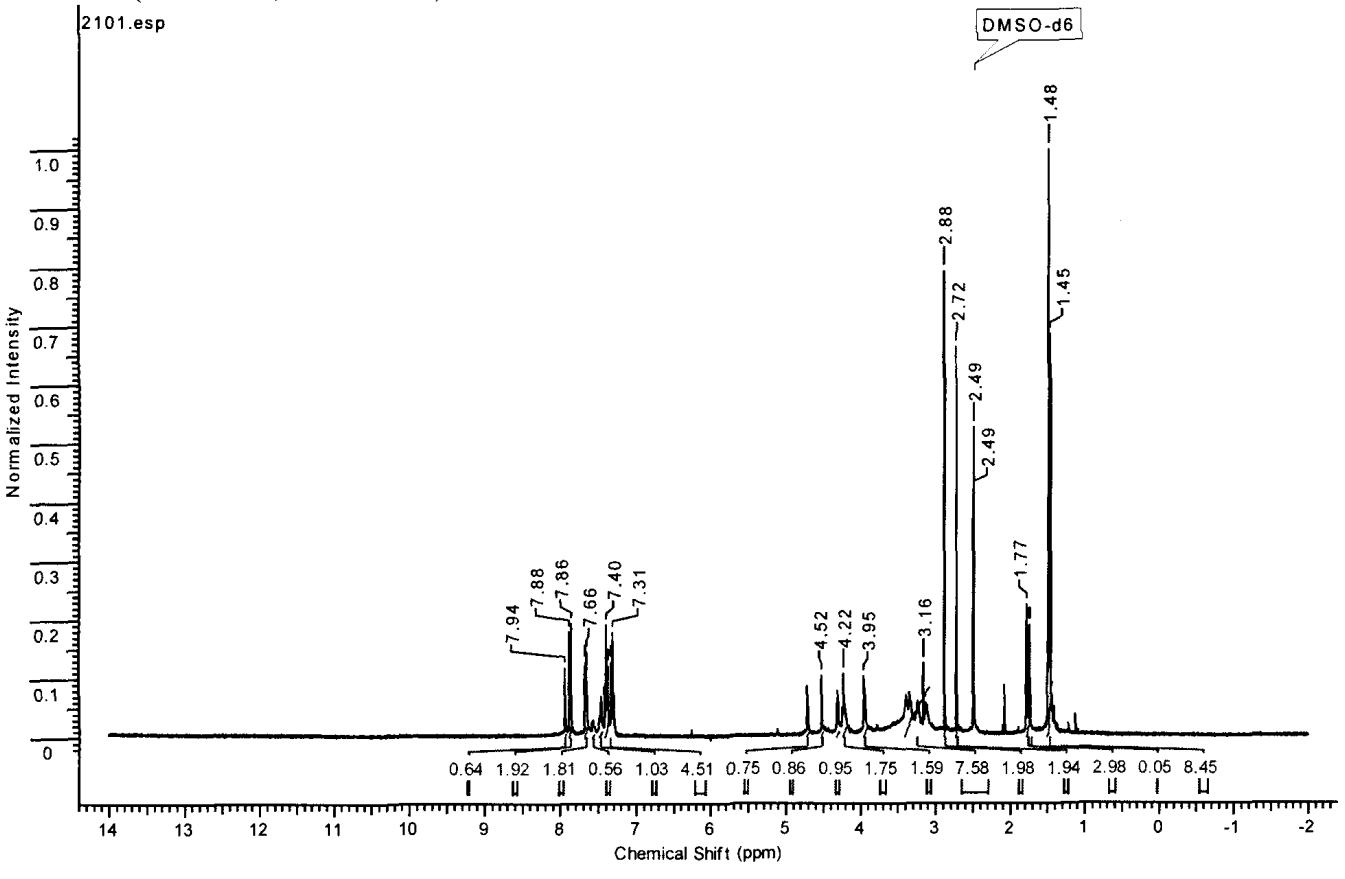

$^{1}$H NMR (400 MHz, $CDCl_3$) of **29b**



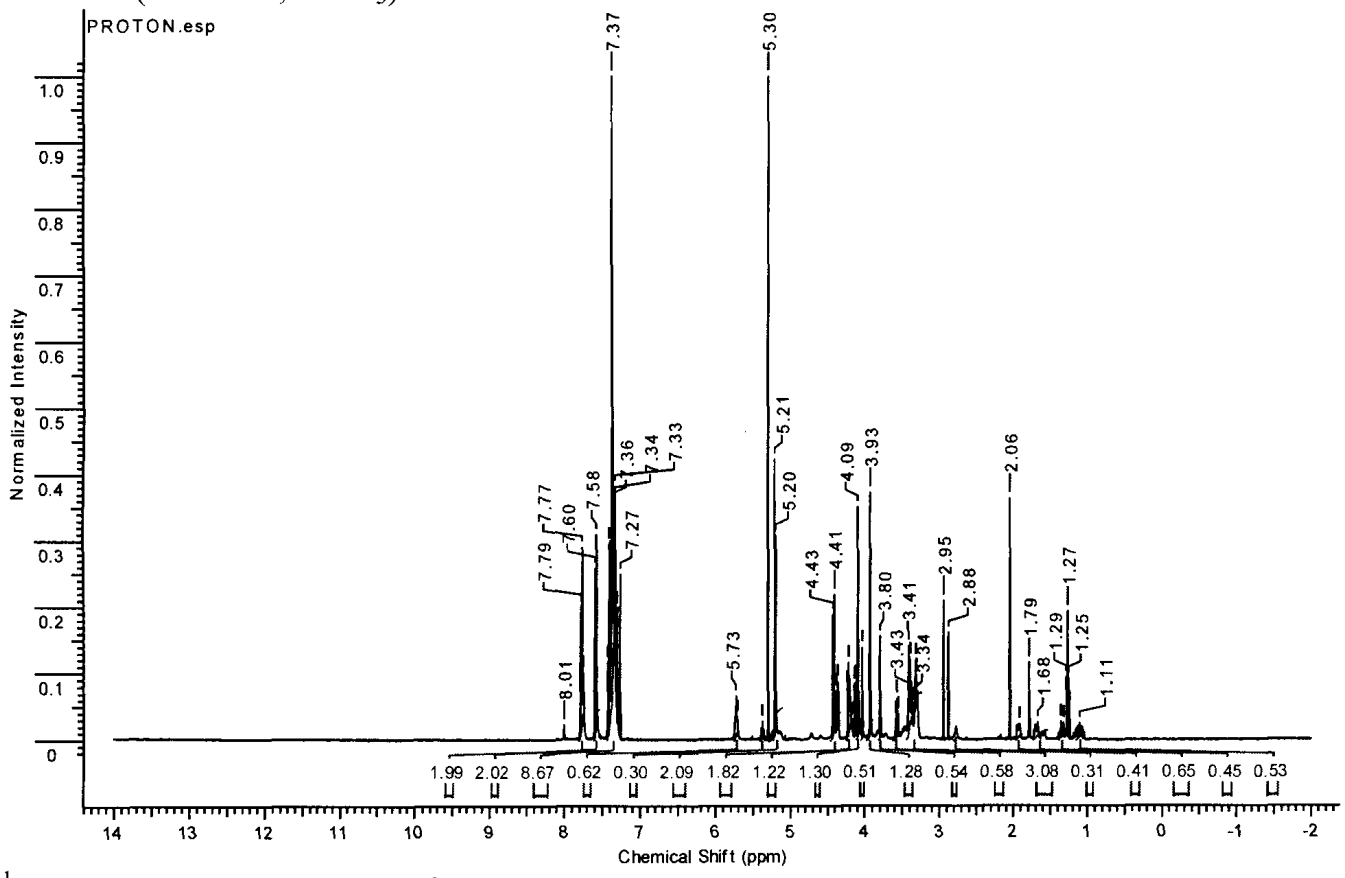
$^{13}C$ NMR (101 MHz, $CDCl_3$) of **29b**



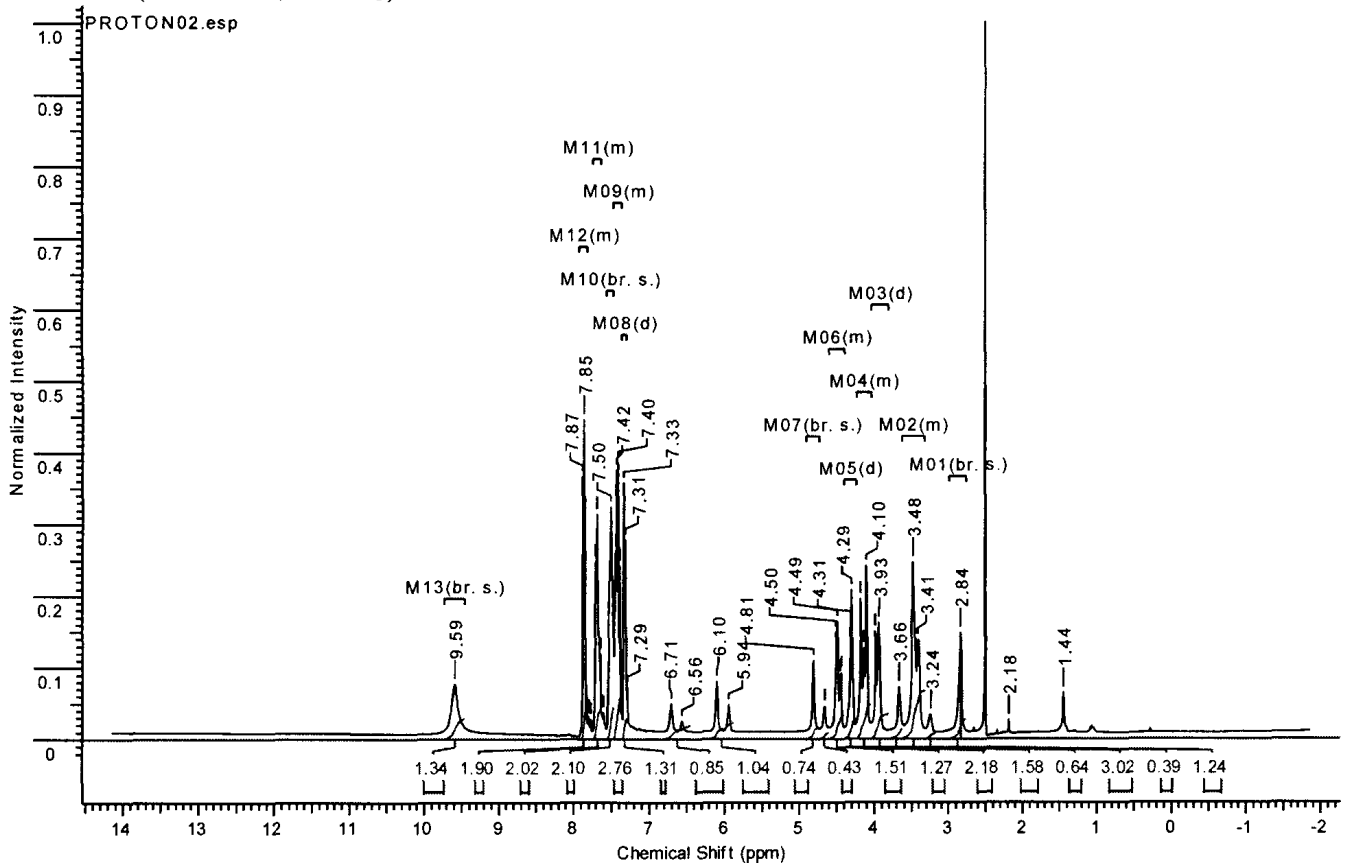

$^{1}H$ NMR (400 MHz, DMSO-*d6*) of **29c**



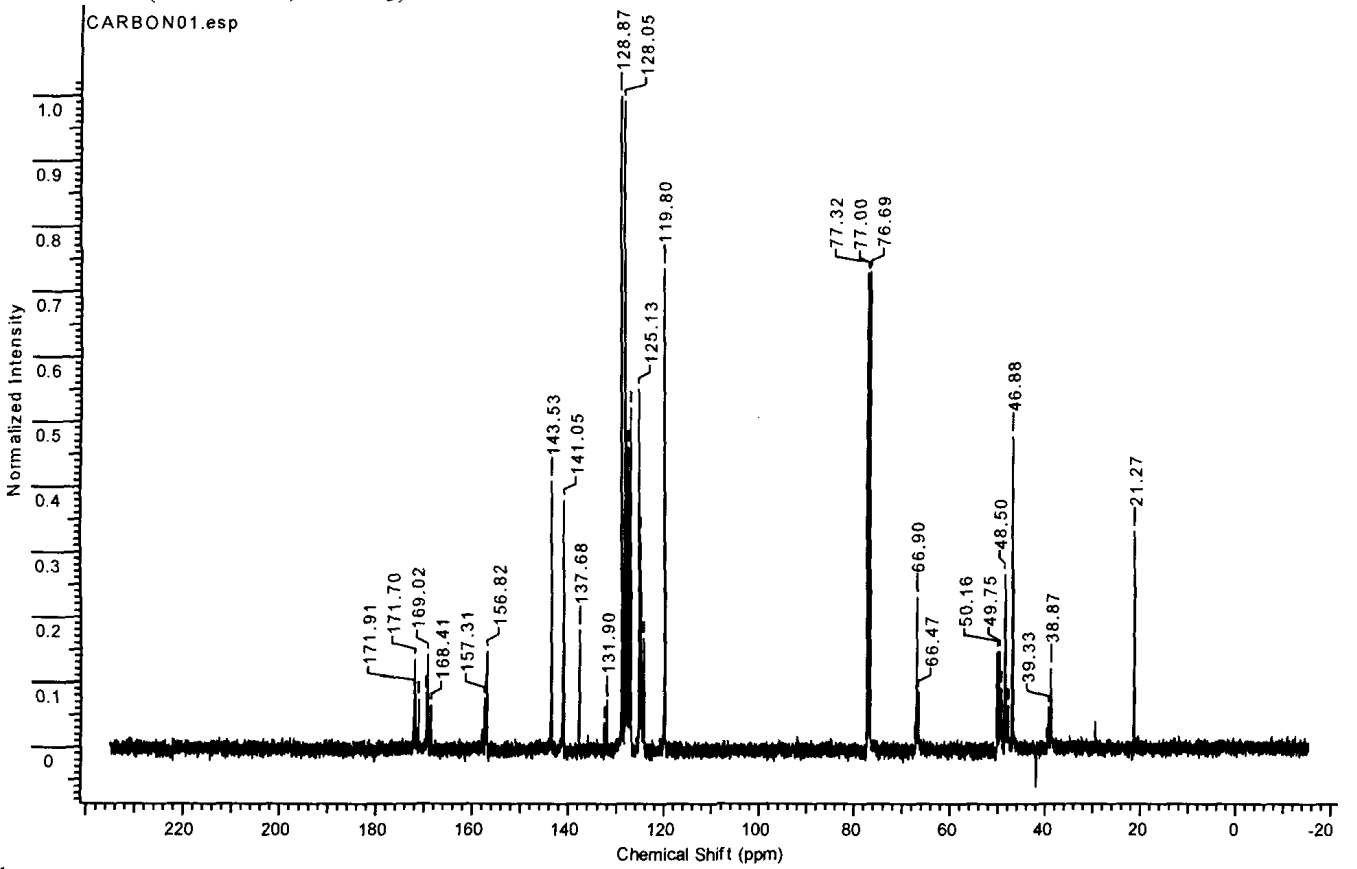
$^1$H NMR (400 MHz, $CDCl_3$) of **30**



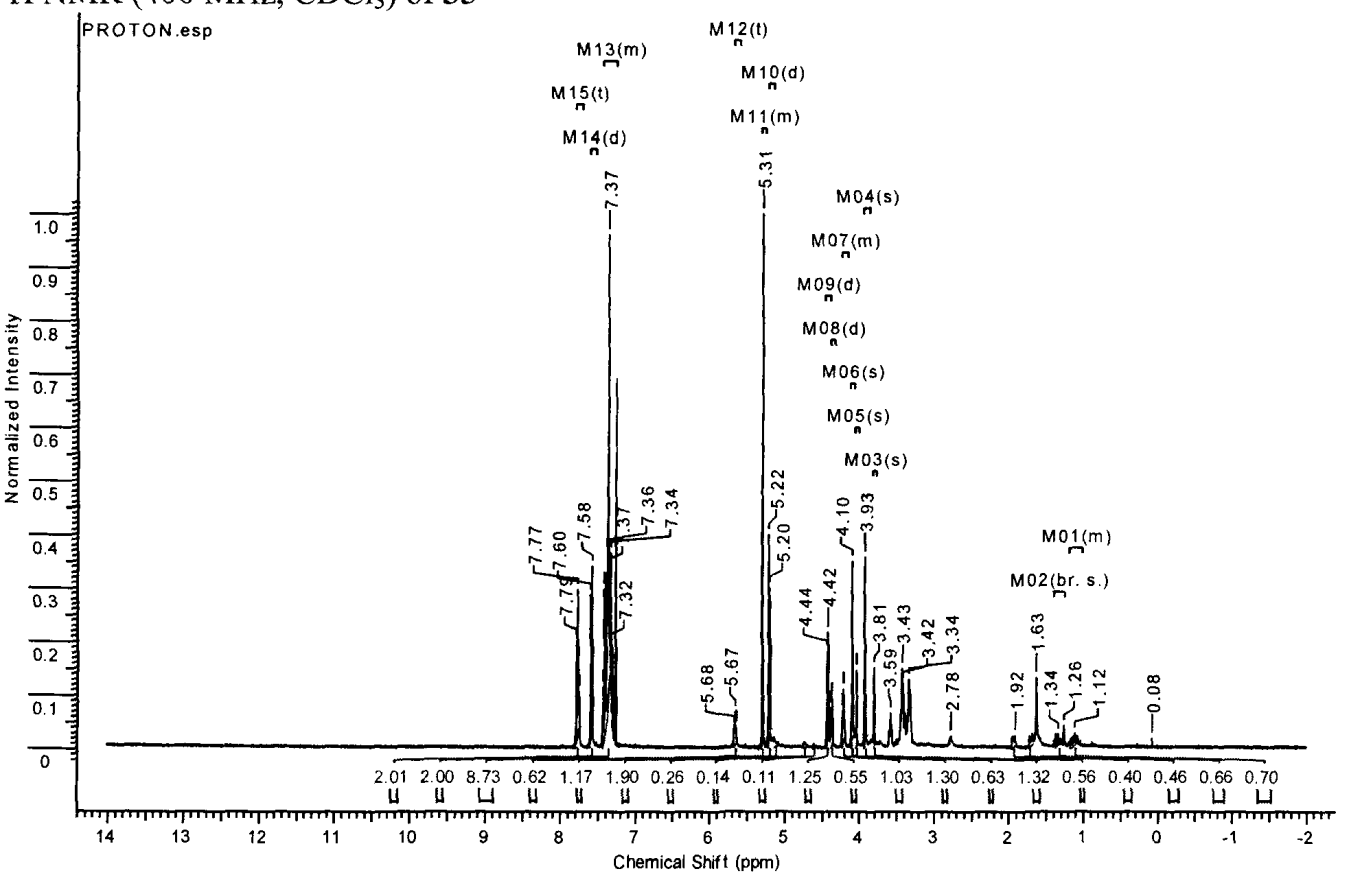

$^1$H NMR (400 MHz, $CDCl_3$) of **32**



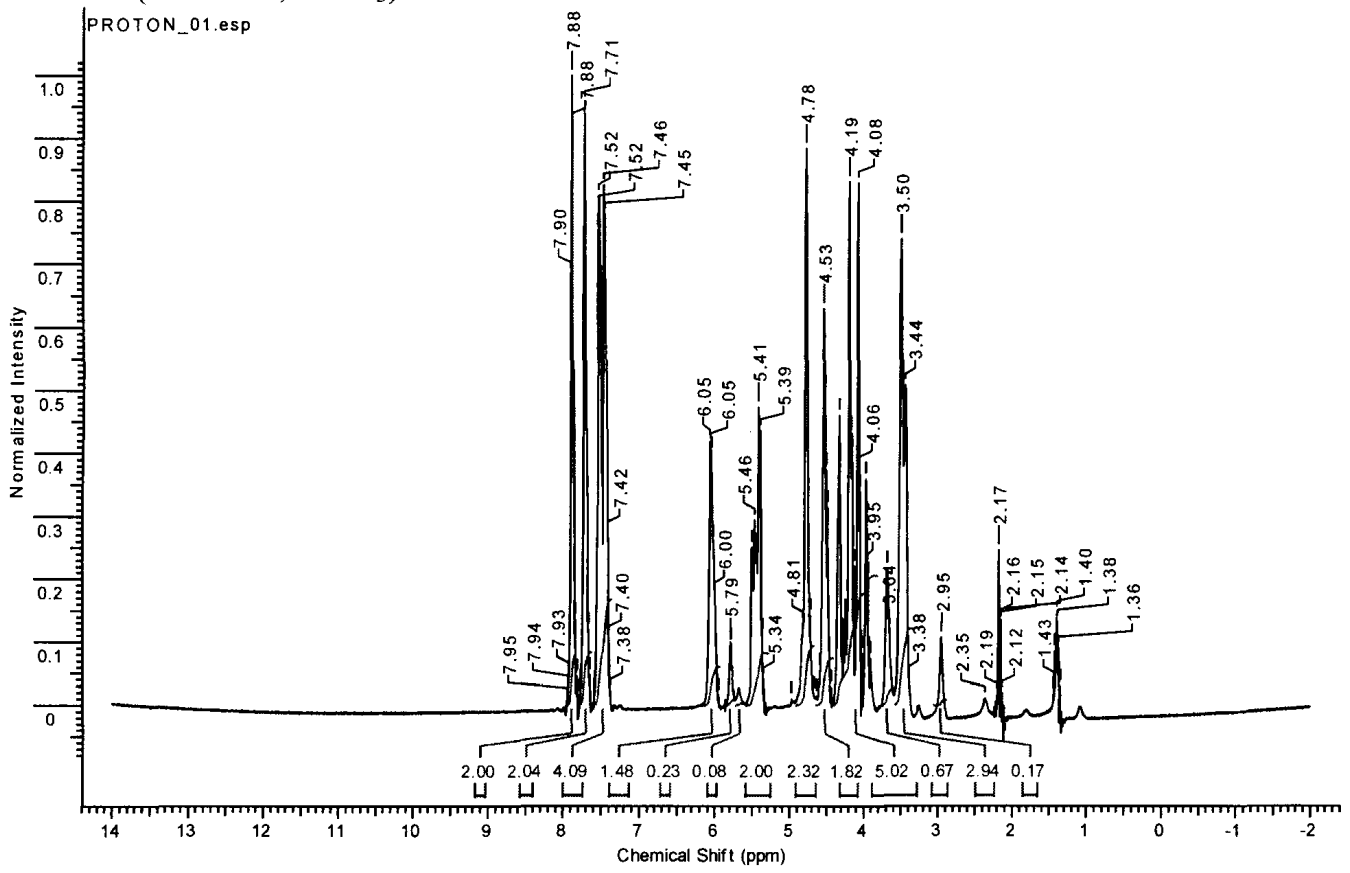
$^{13}$C NMR (101 MHz, $CDCl_3$) of **32**



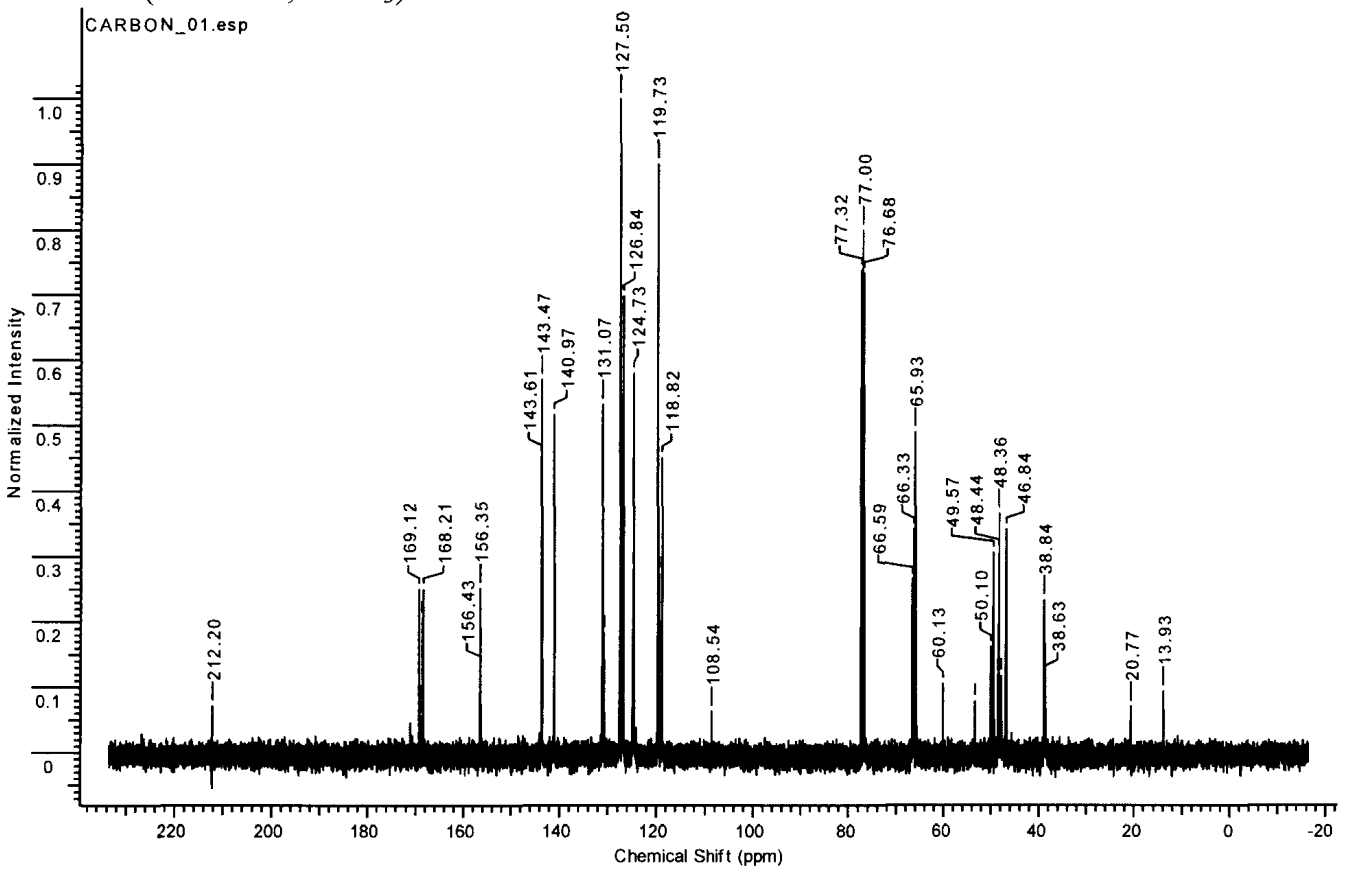

$^{1}$H NMR (400 MHz, $CDCl_3$) of **35**



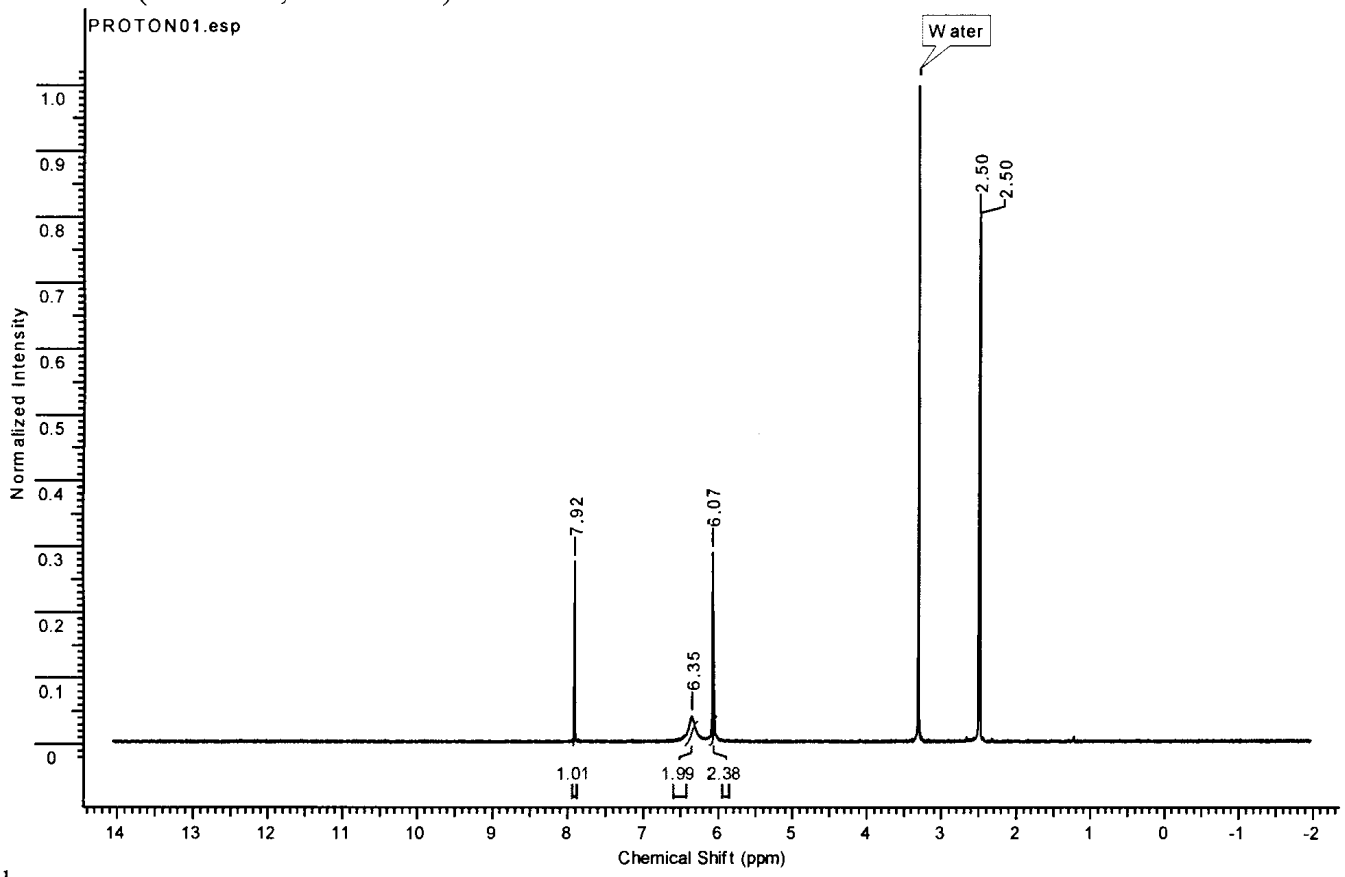
$^{1}$H NMR (400 MHz, $CDCl_3$) of **41**



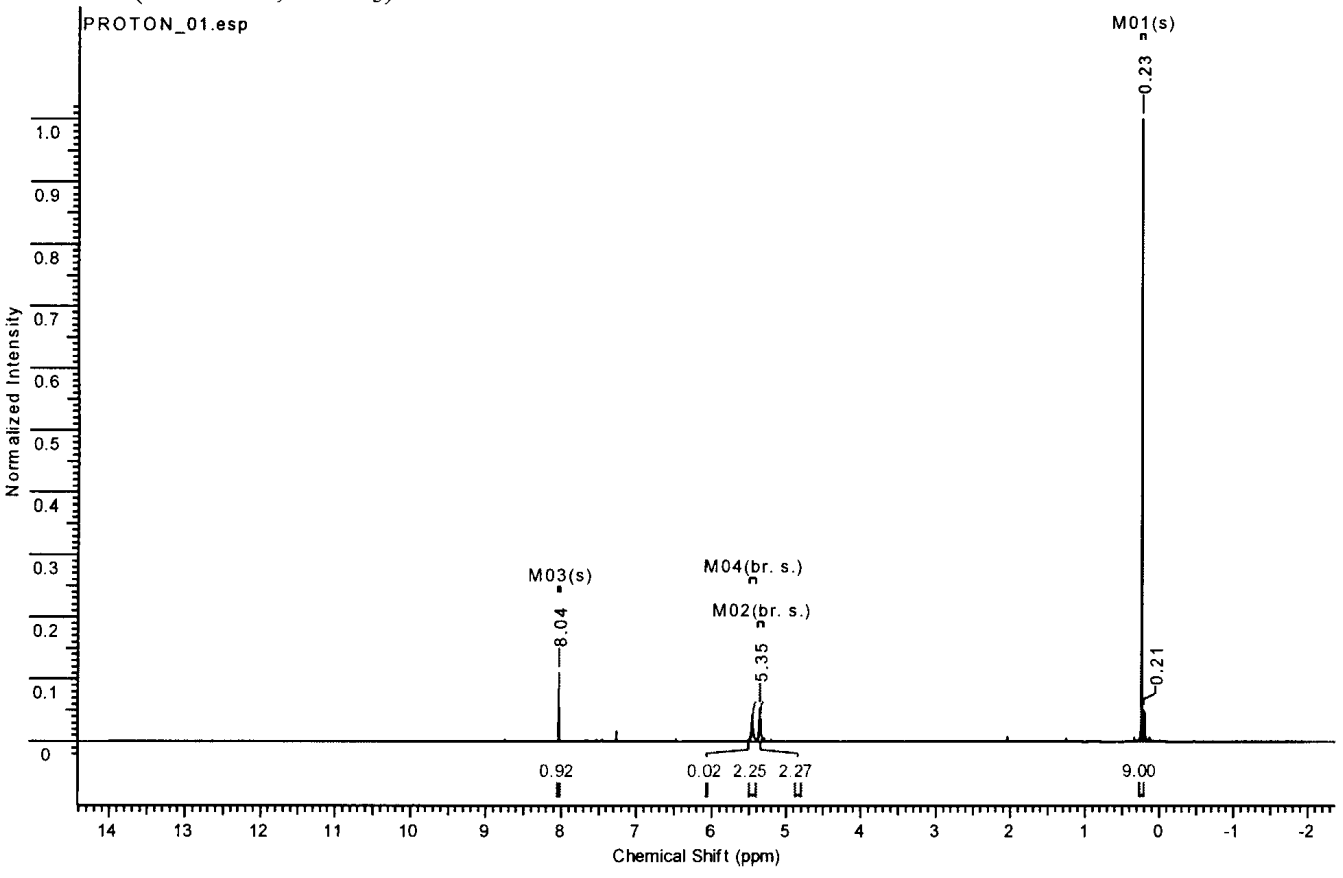

$^{13}$C NMR (101 MHz, $CDCl_3$) of **41**



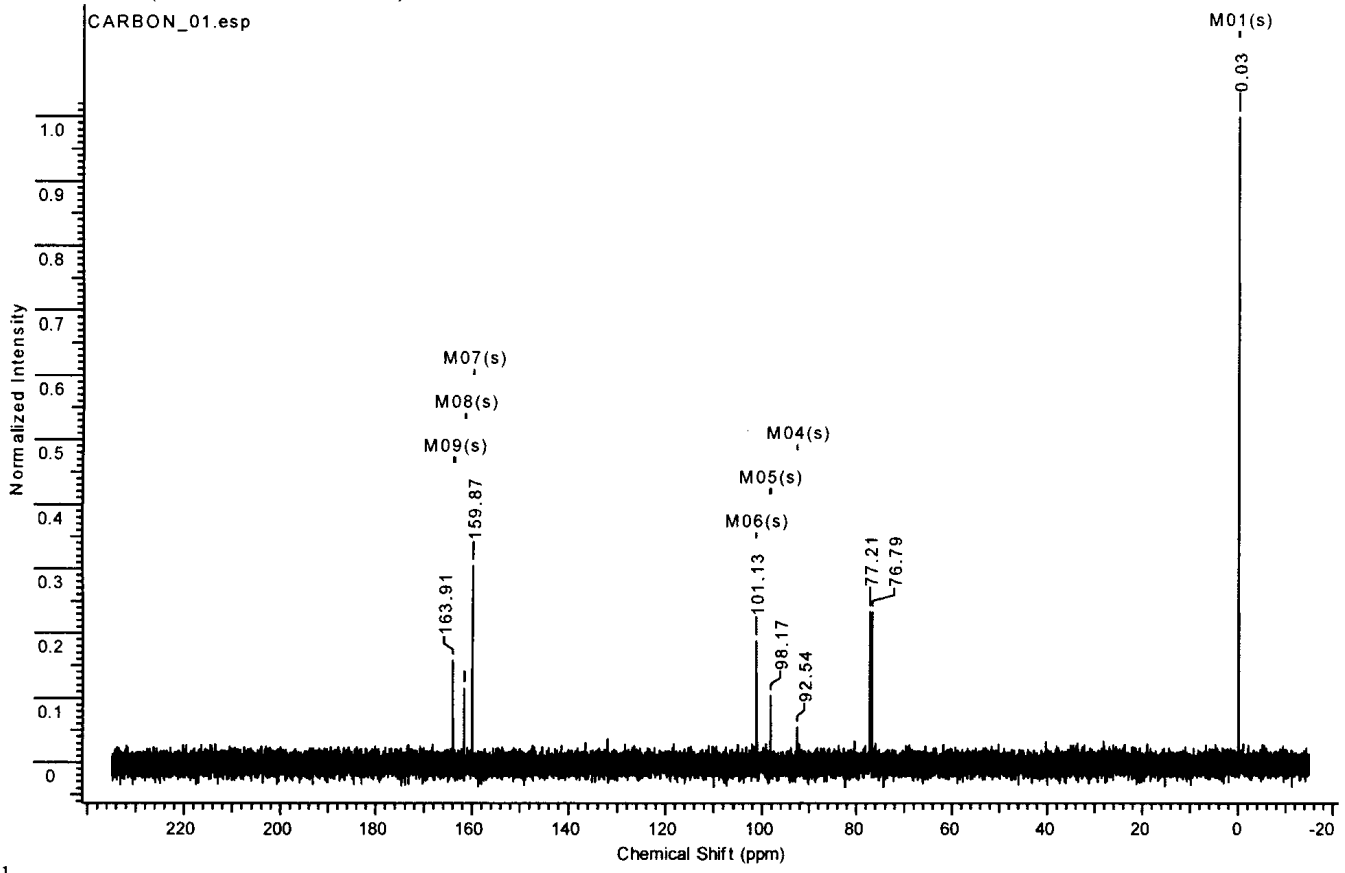
$^1$H NMR (400 MHz, DMSO-*d6*) of **46**



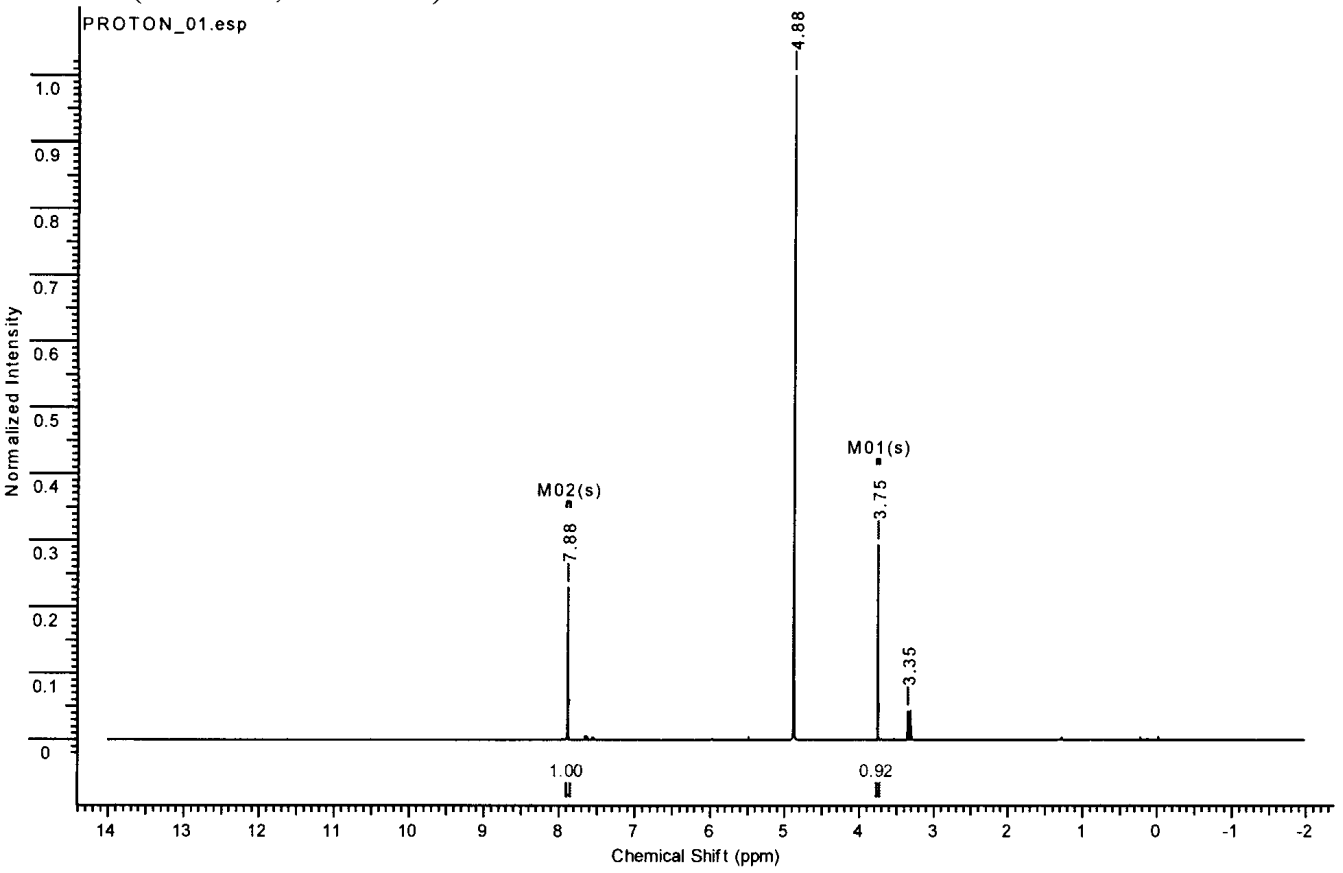

$^1$H NMR (400 MHz, $CDCl_3$) of **47**



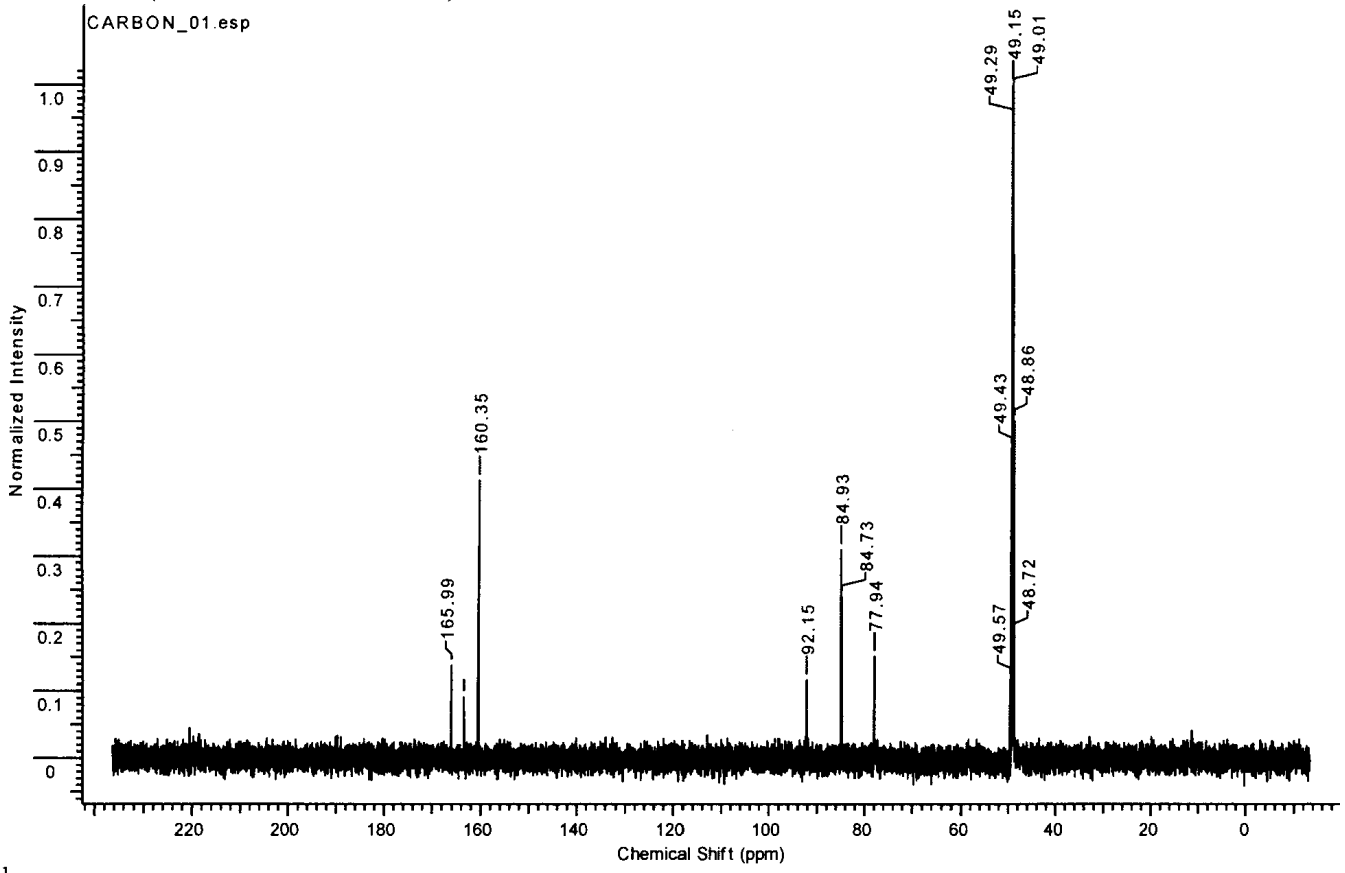
$^{13}$C NMR (101 MHz, $CDCl_3$) of **47**



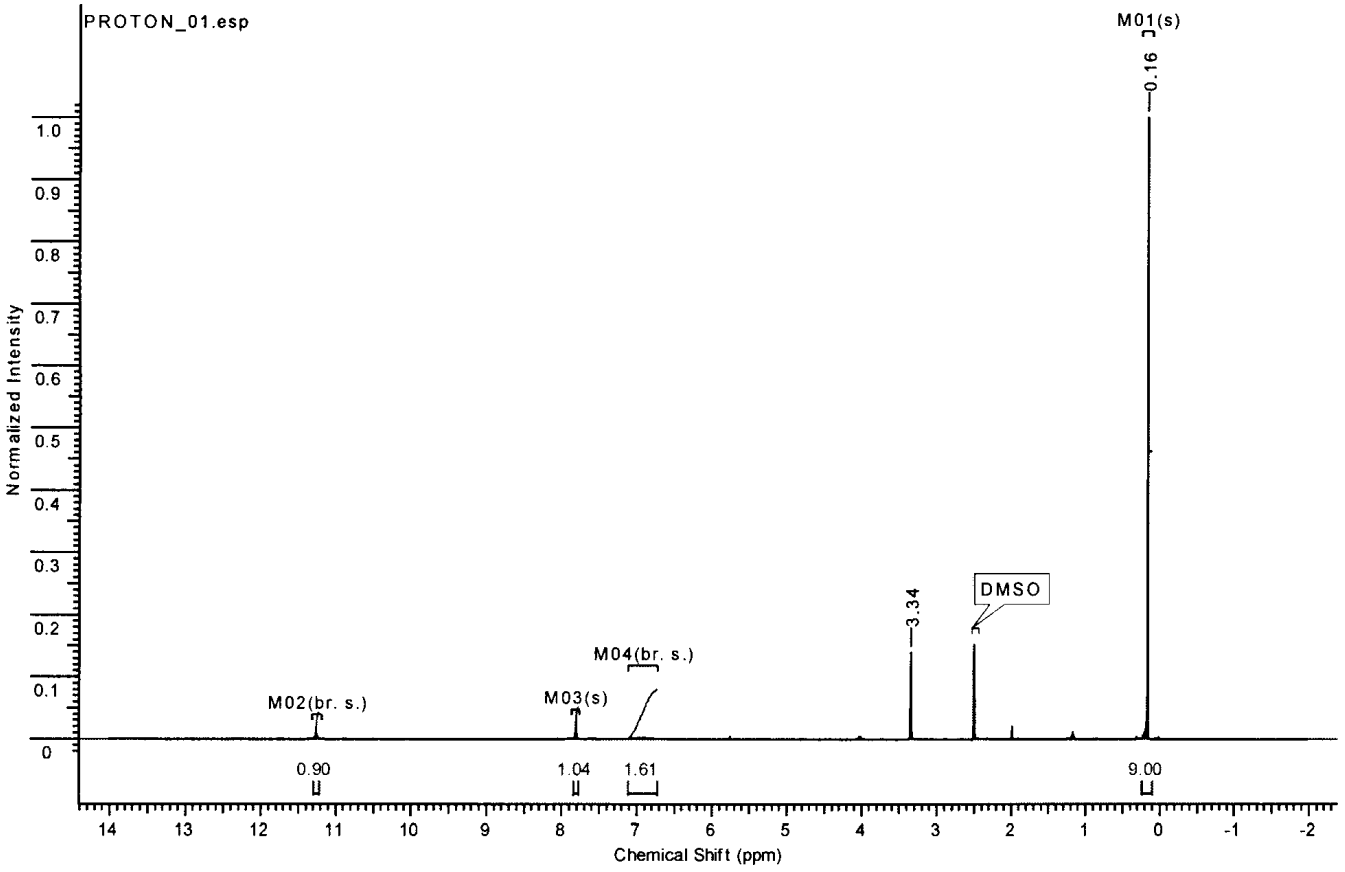

$^{1}$H NMR (400 MHz, MeOH-*d4*) of **48**



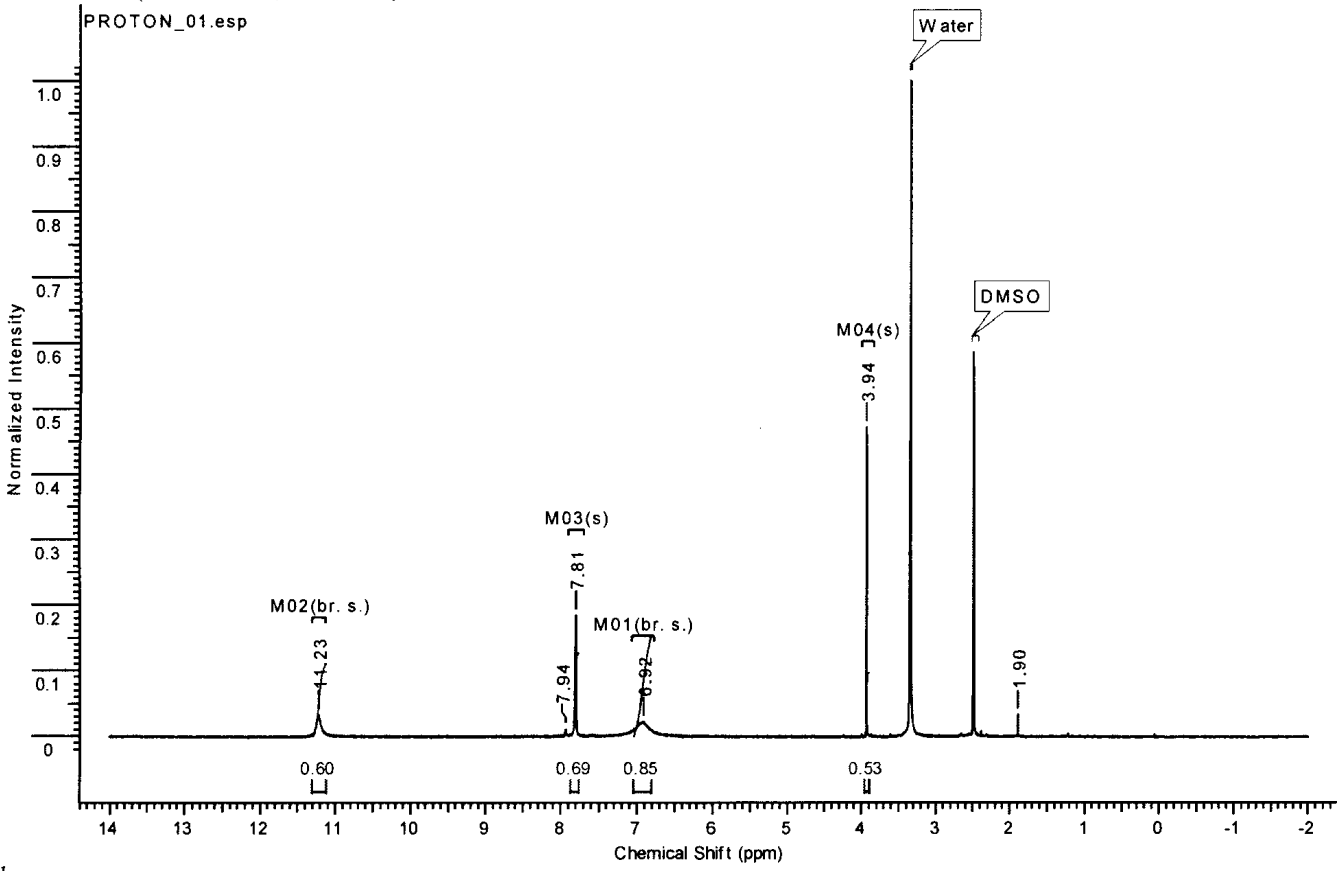
$^{13}$C NMR (101 MHz, MeOH-*d4*) of **48**



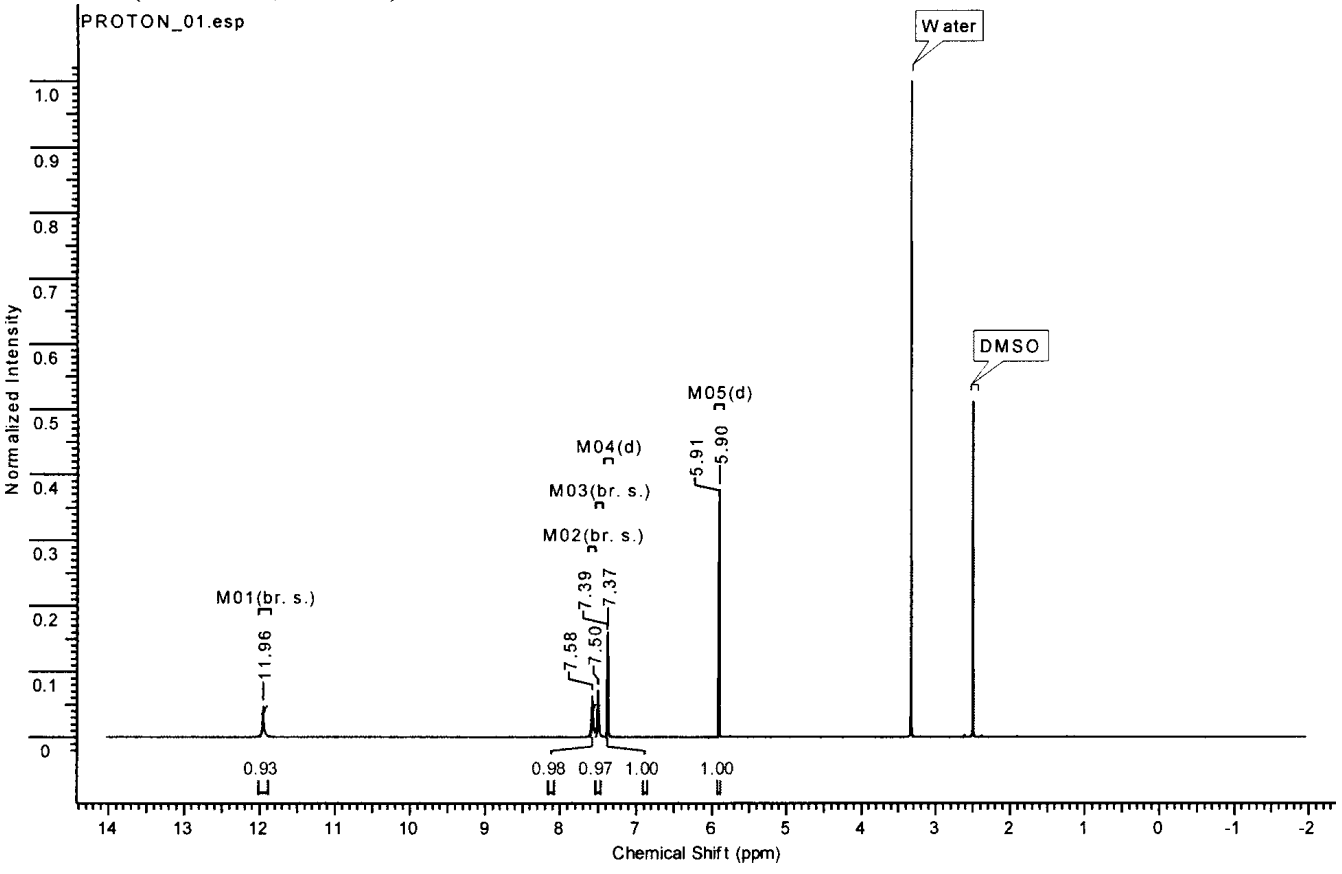

$^{1}$H NMR (400 MHz, DMSO) of **51**



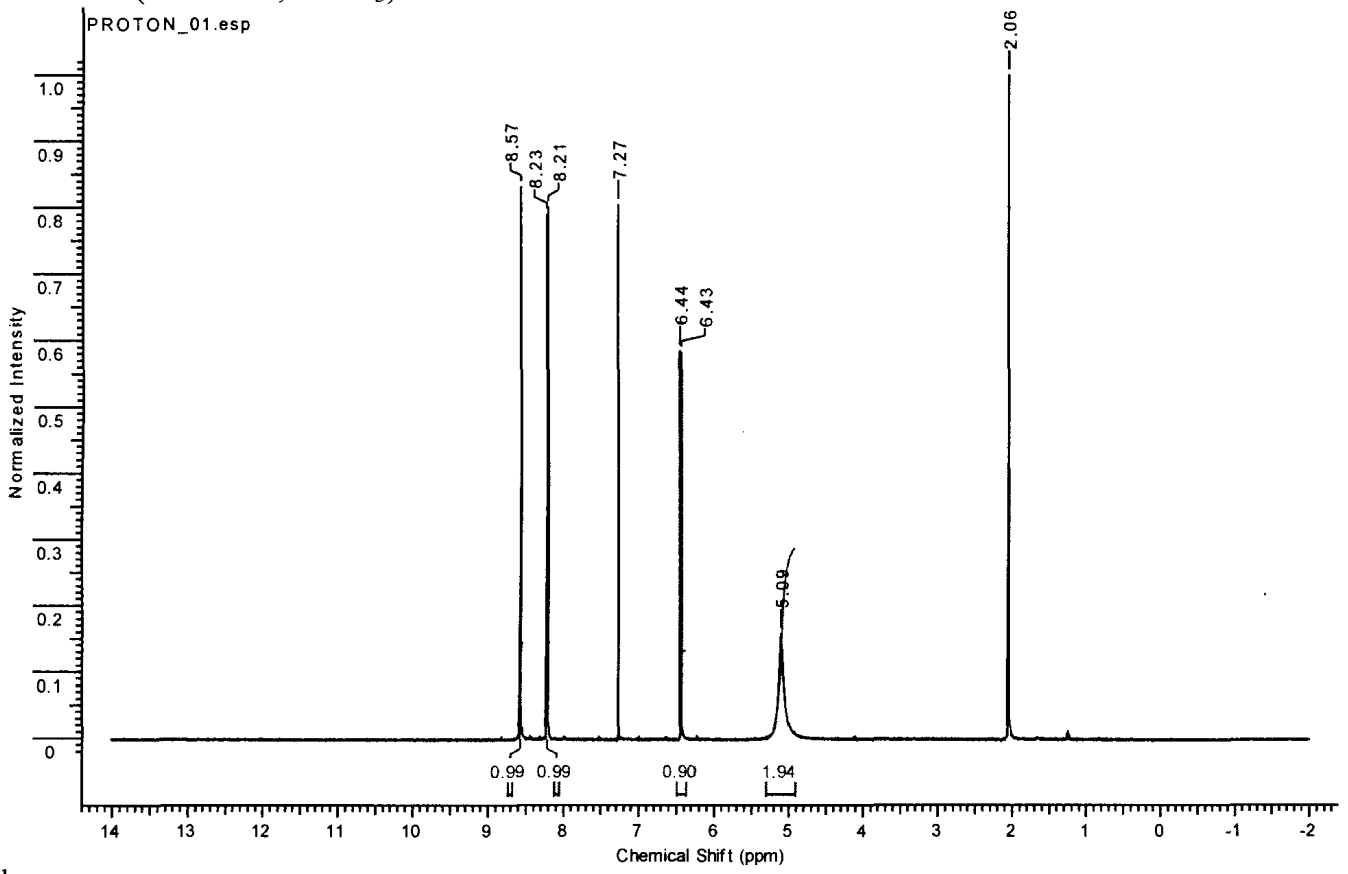
$^1$H NMR (400 MHz, DMSO) of **52**



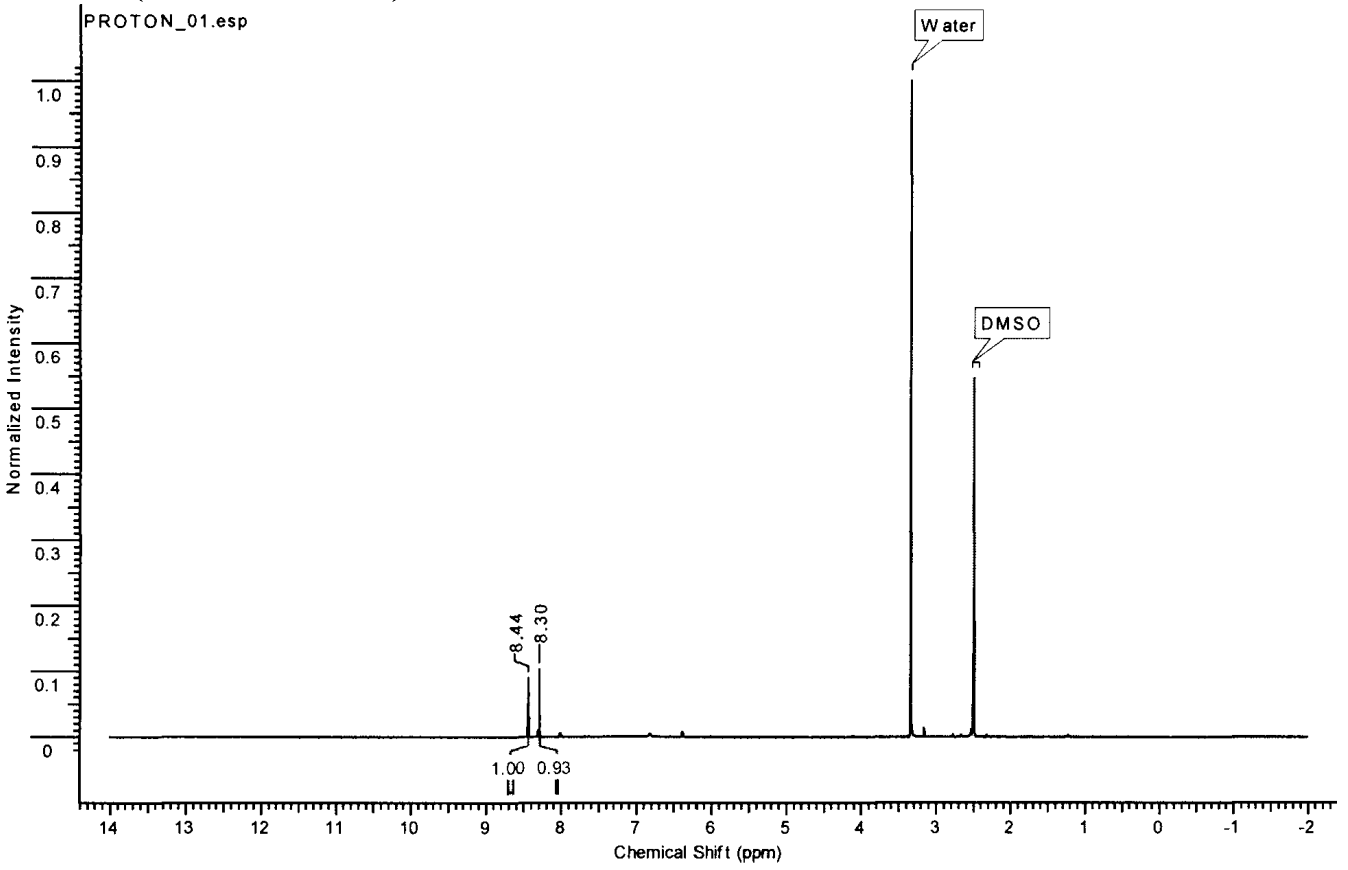

$^1$H NMR (600 MHz, DMSO) of **53**



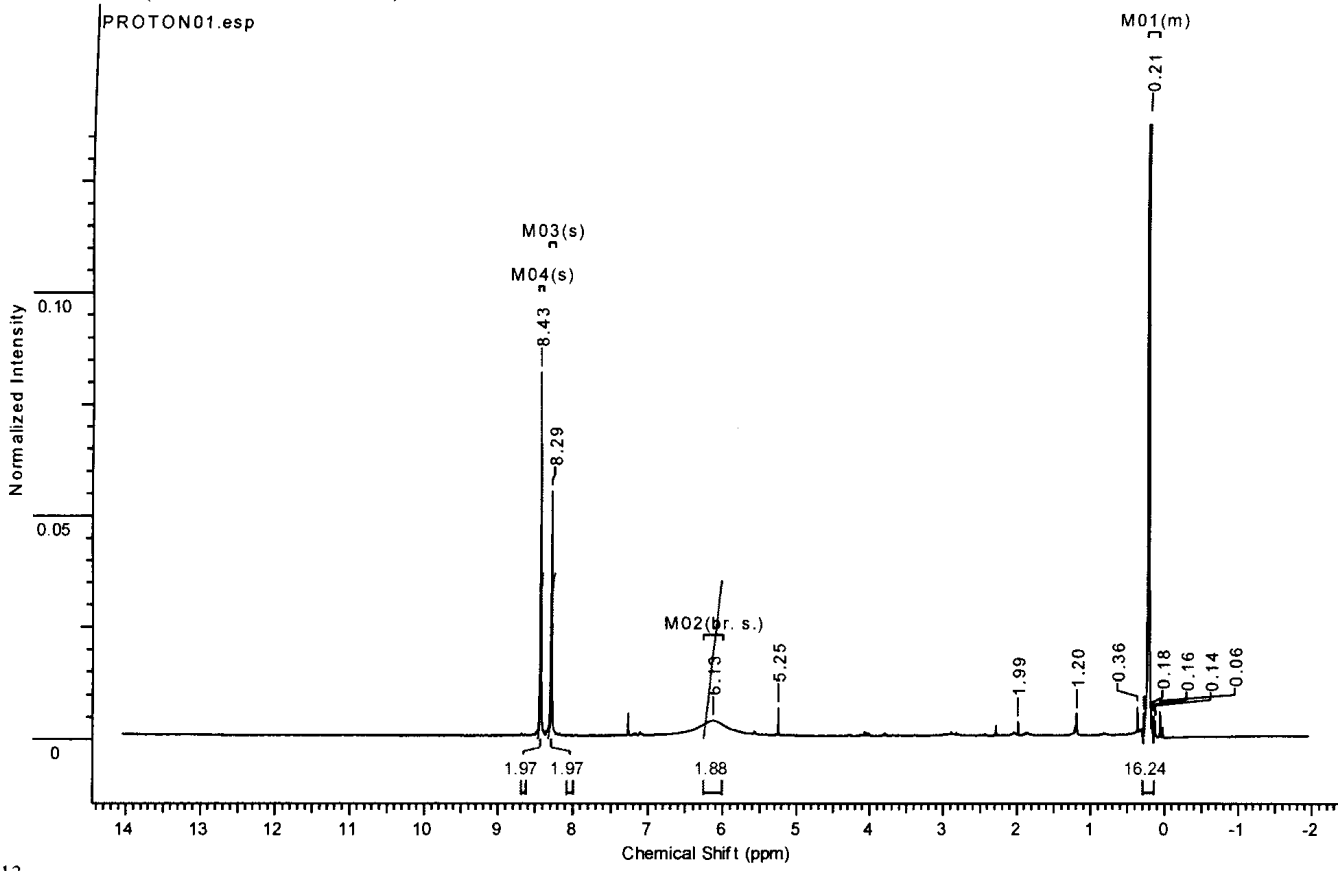
$^1$H NMR (400 MHz, $CDCl_3$) of **54**



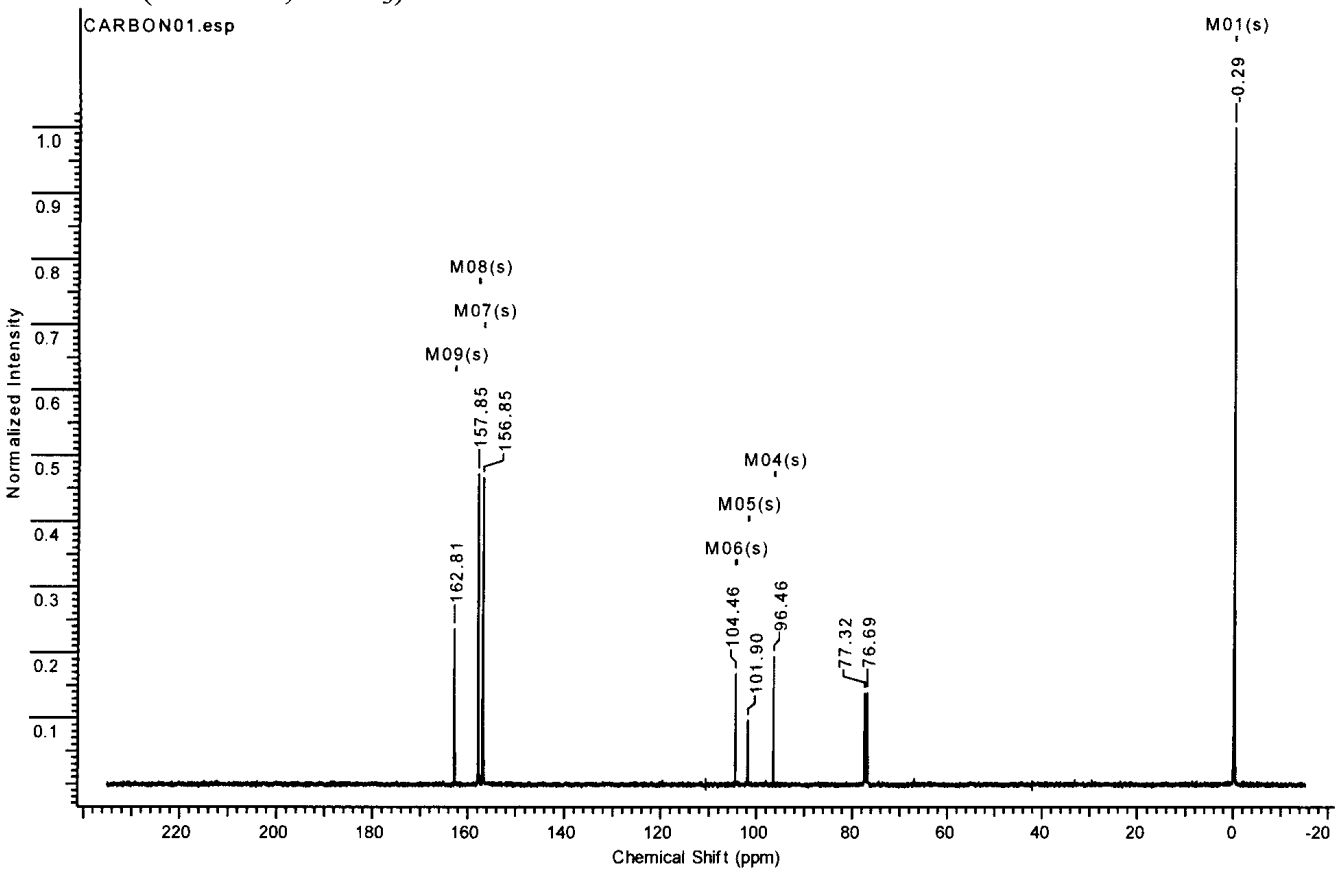

$^1$H NMR (400 MHz, DMSO) of **55**



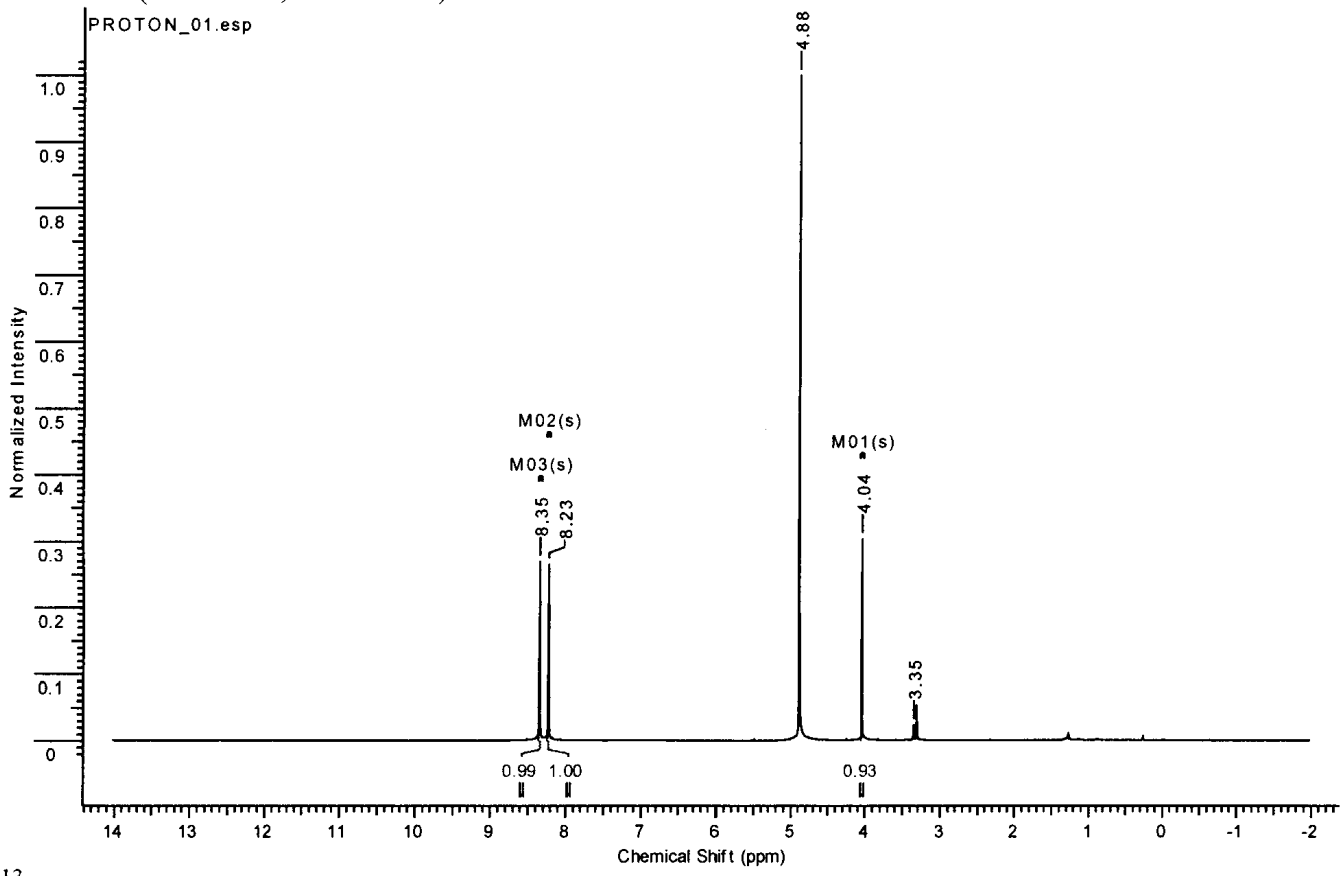
$^{1}$H NMR (400 MHz, $CDCl_3$) of **56**



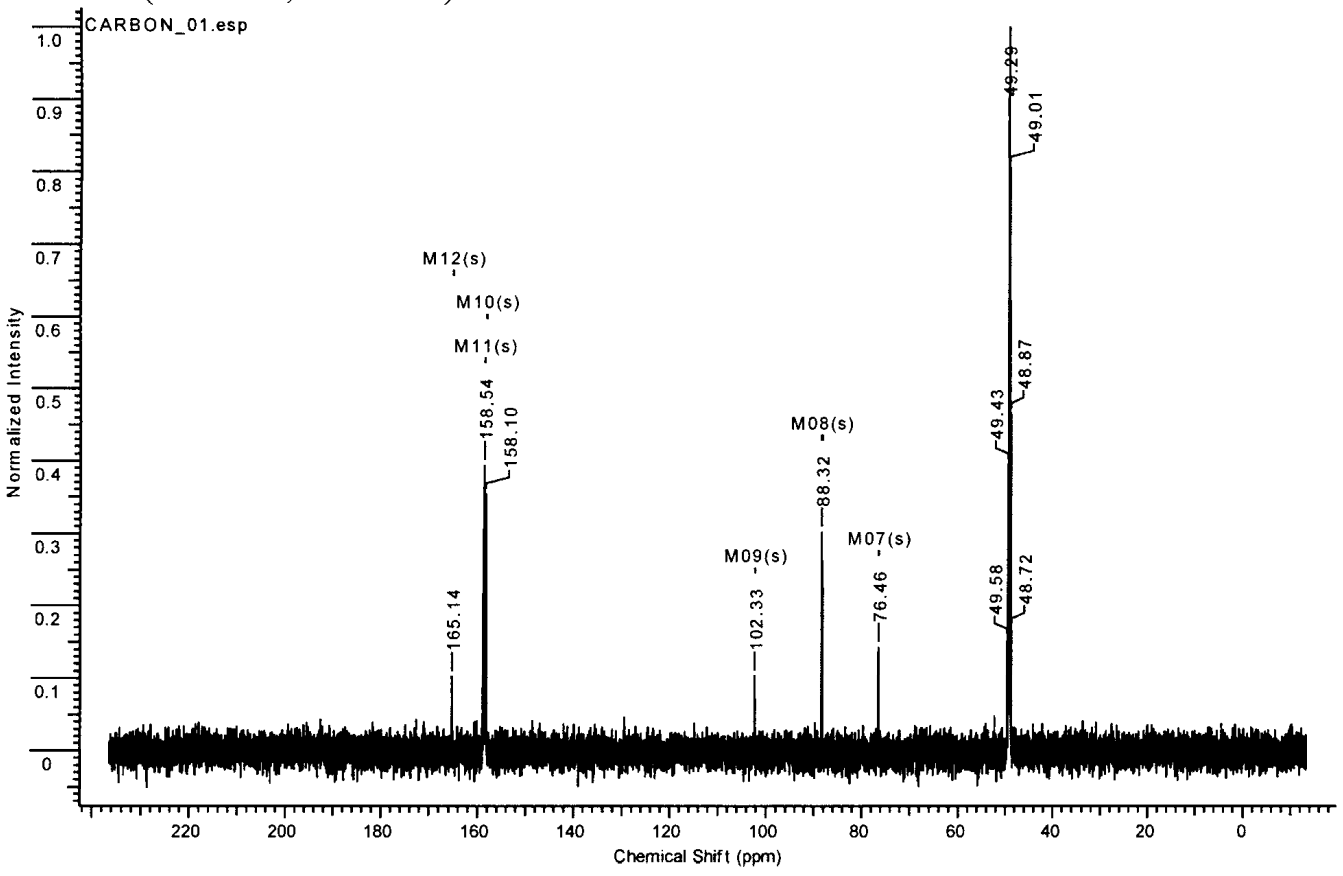

$^{13}$C NMR (101 MHz, $CDCl_3$) of **56**



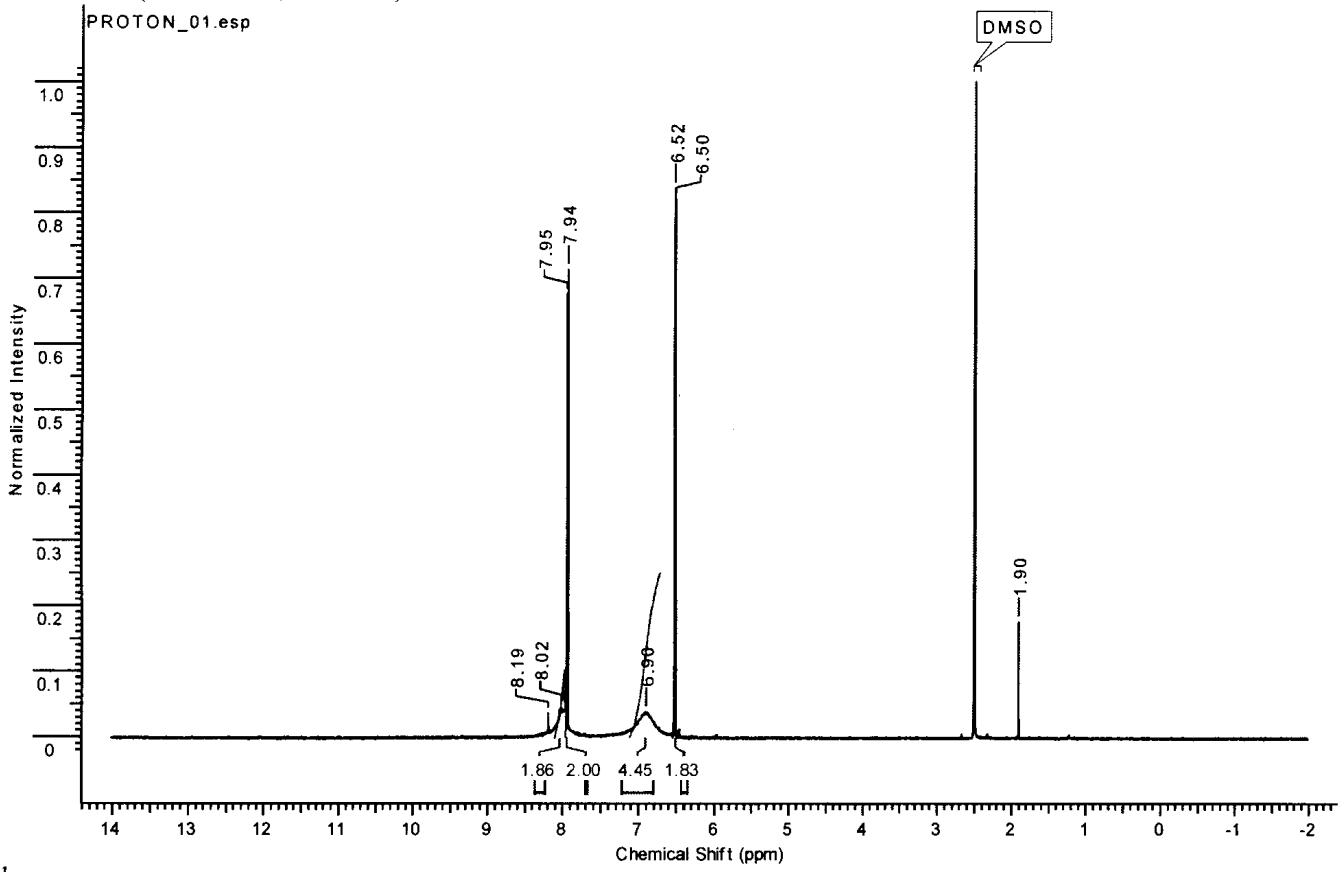
$^{1}$H NMR (400 MHz, MeOH-*d4*) of **57**



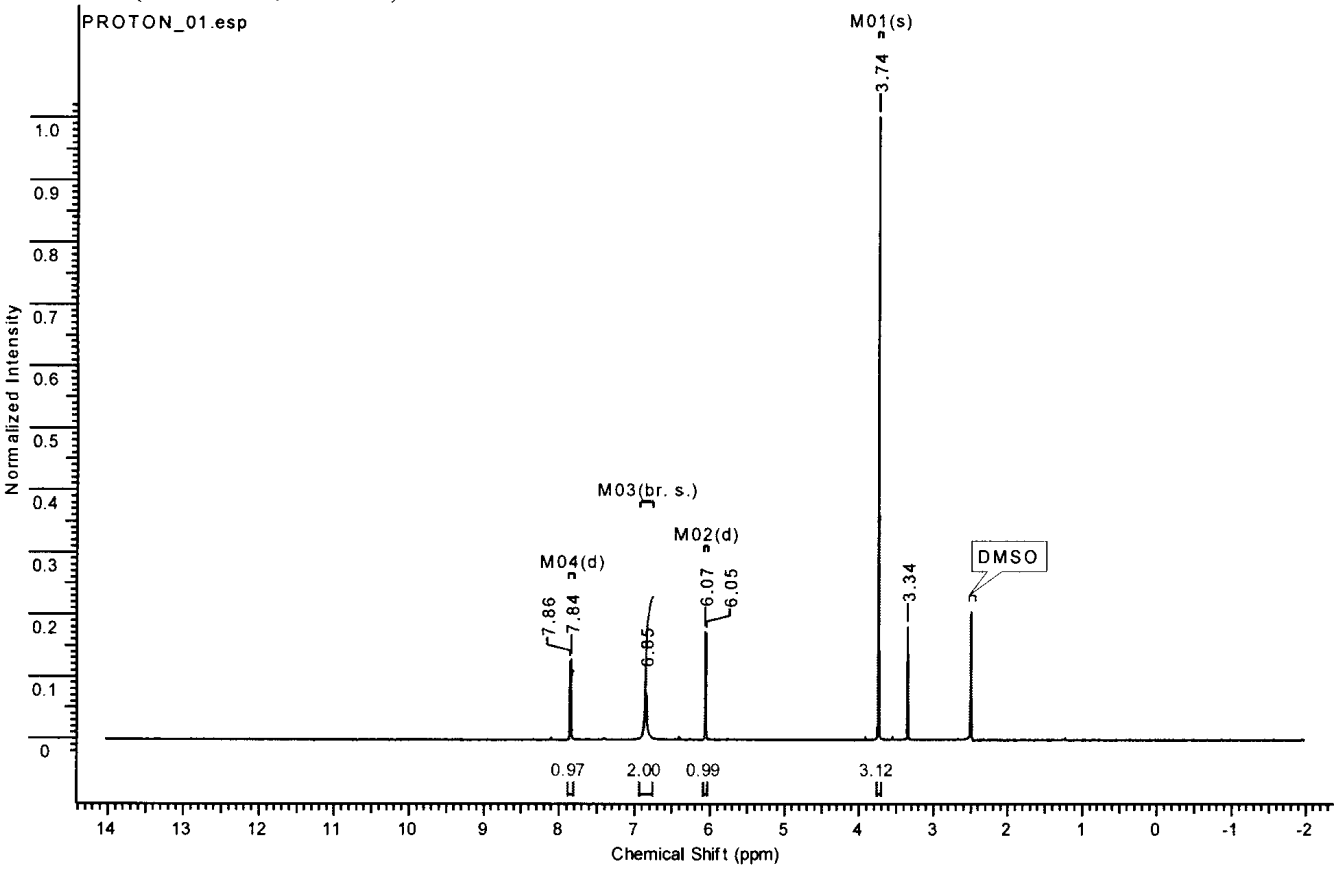

$^{13}$C NMR (101 MHz, MeOH-*d4*) of **57**



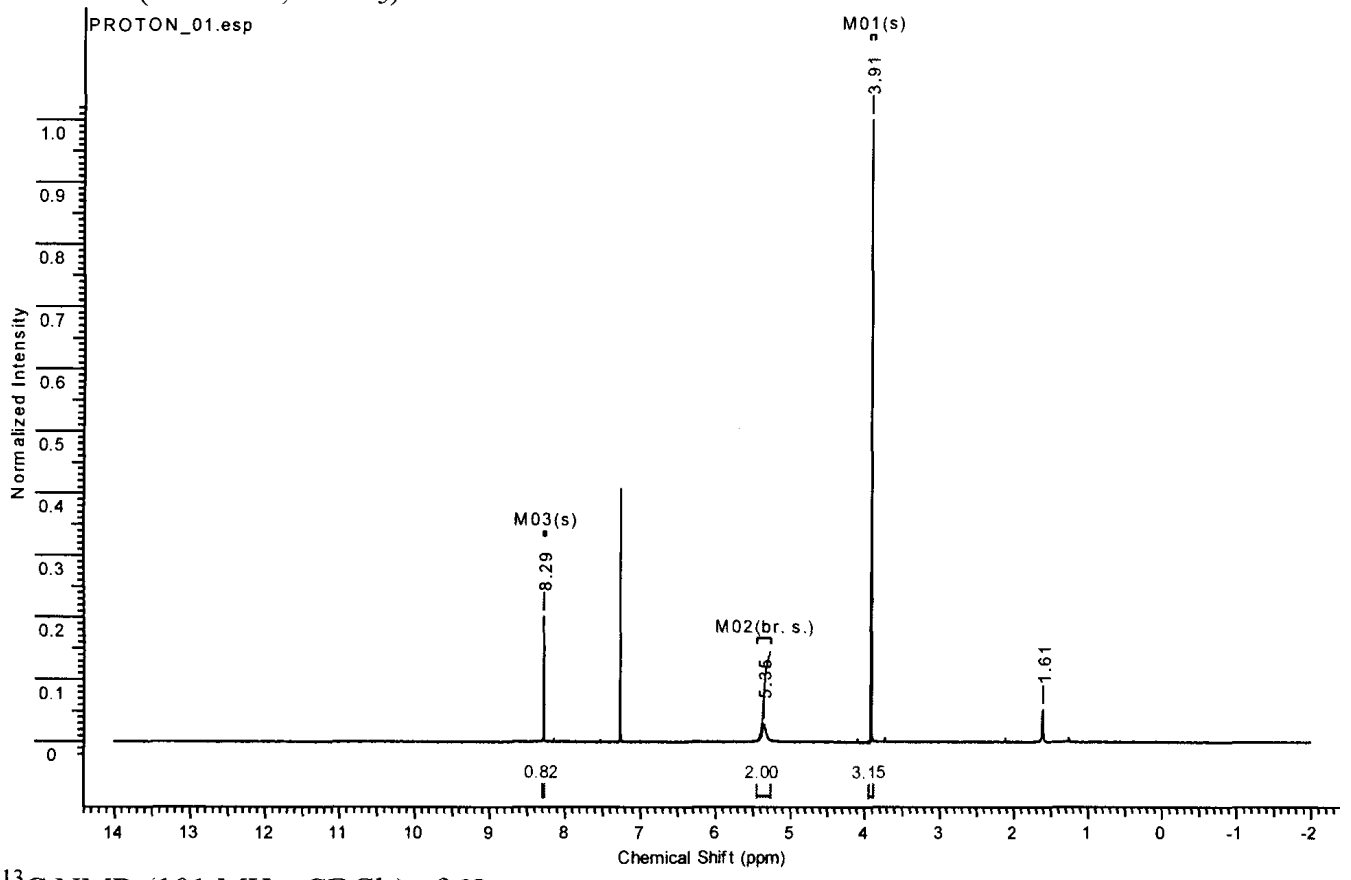
$^1$H NMR (400 MHz, DMSO) of **61**



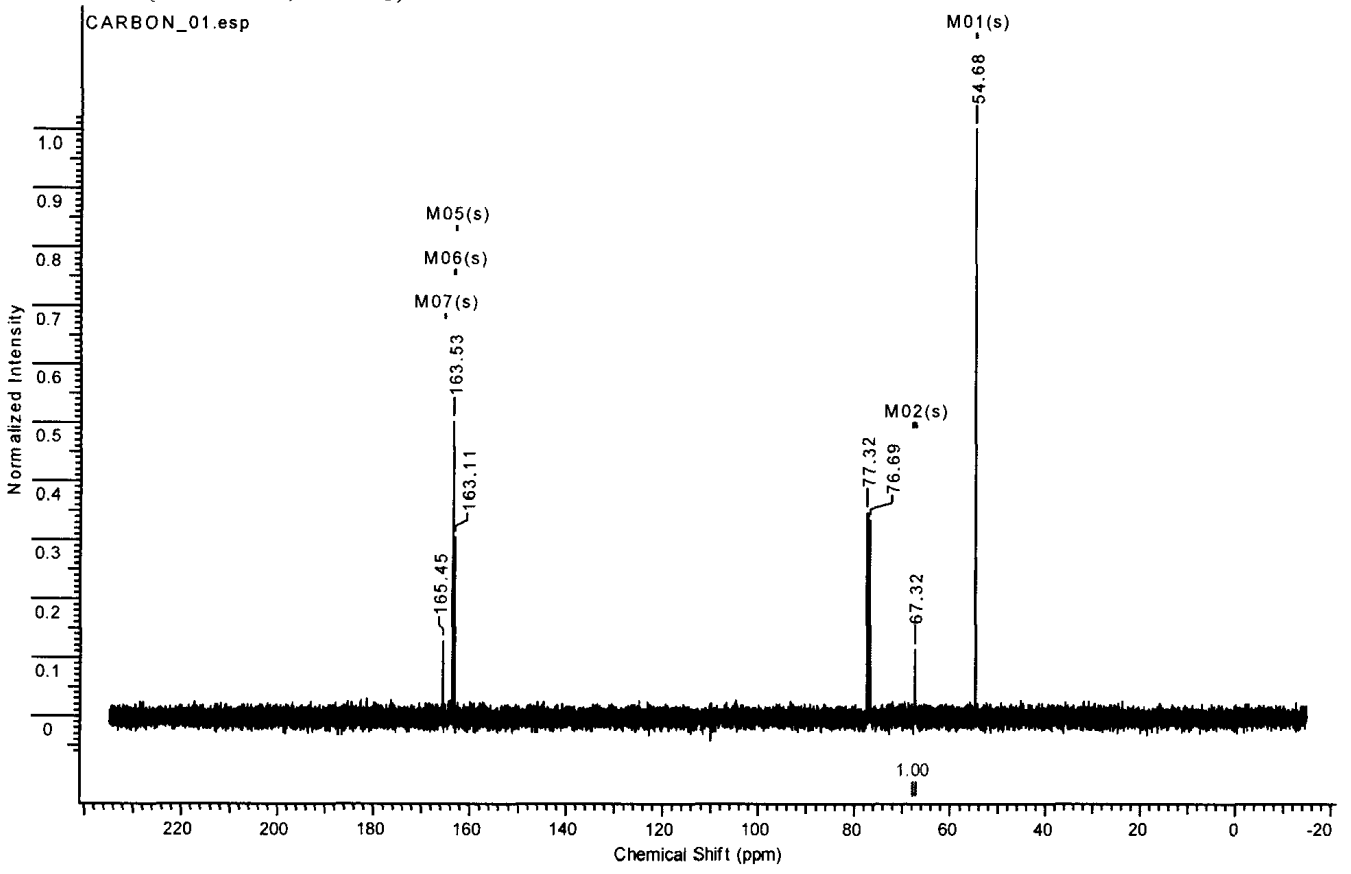

$^1$H NMR (400 MHz, DMSO) of **62**



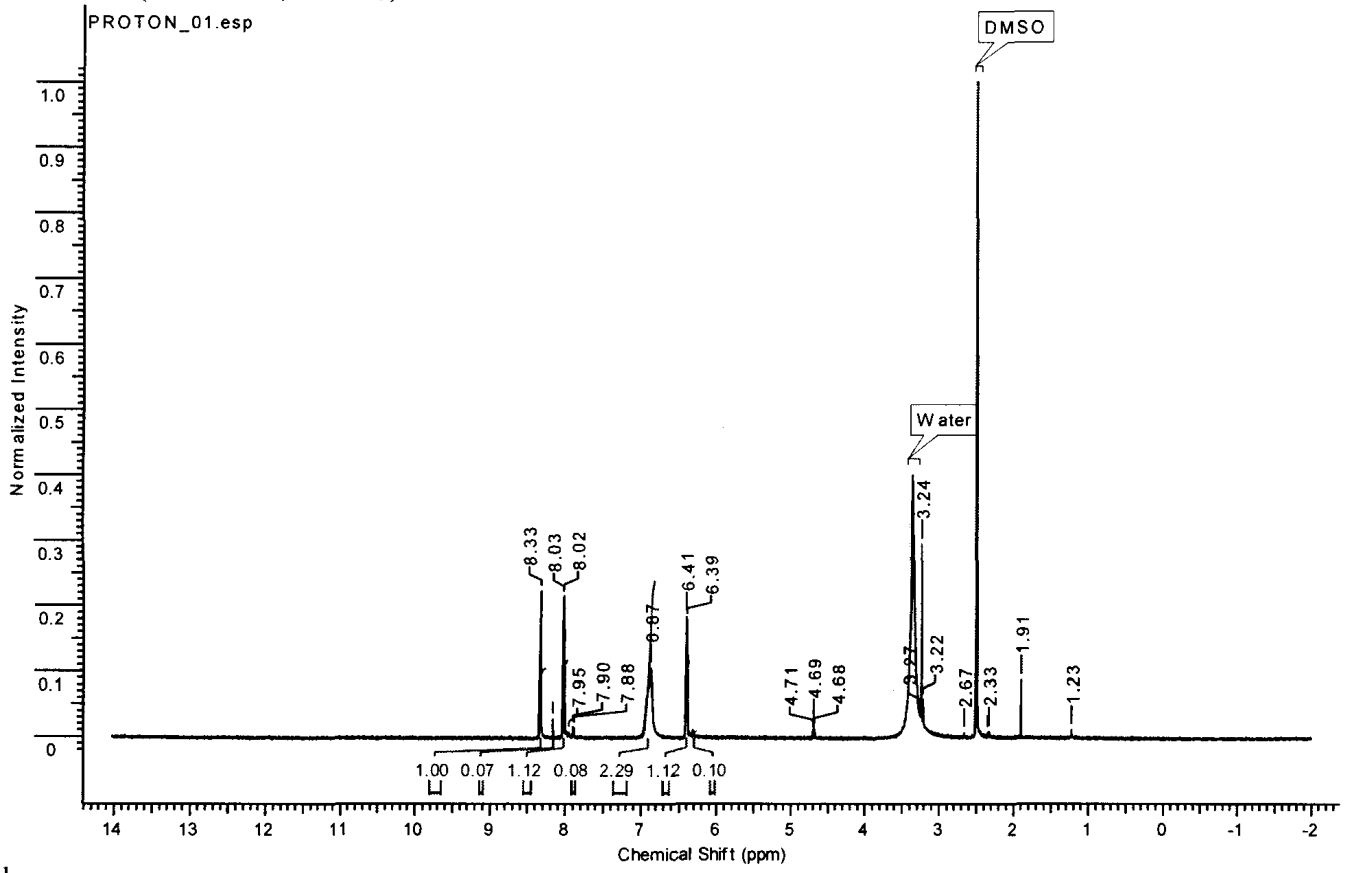
$^{1}$H NMR (400 MHz, $CDCl_3$) of **63**



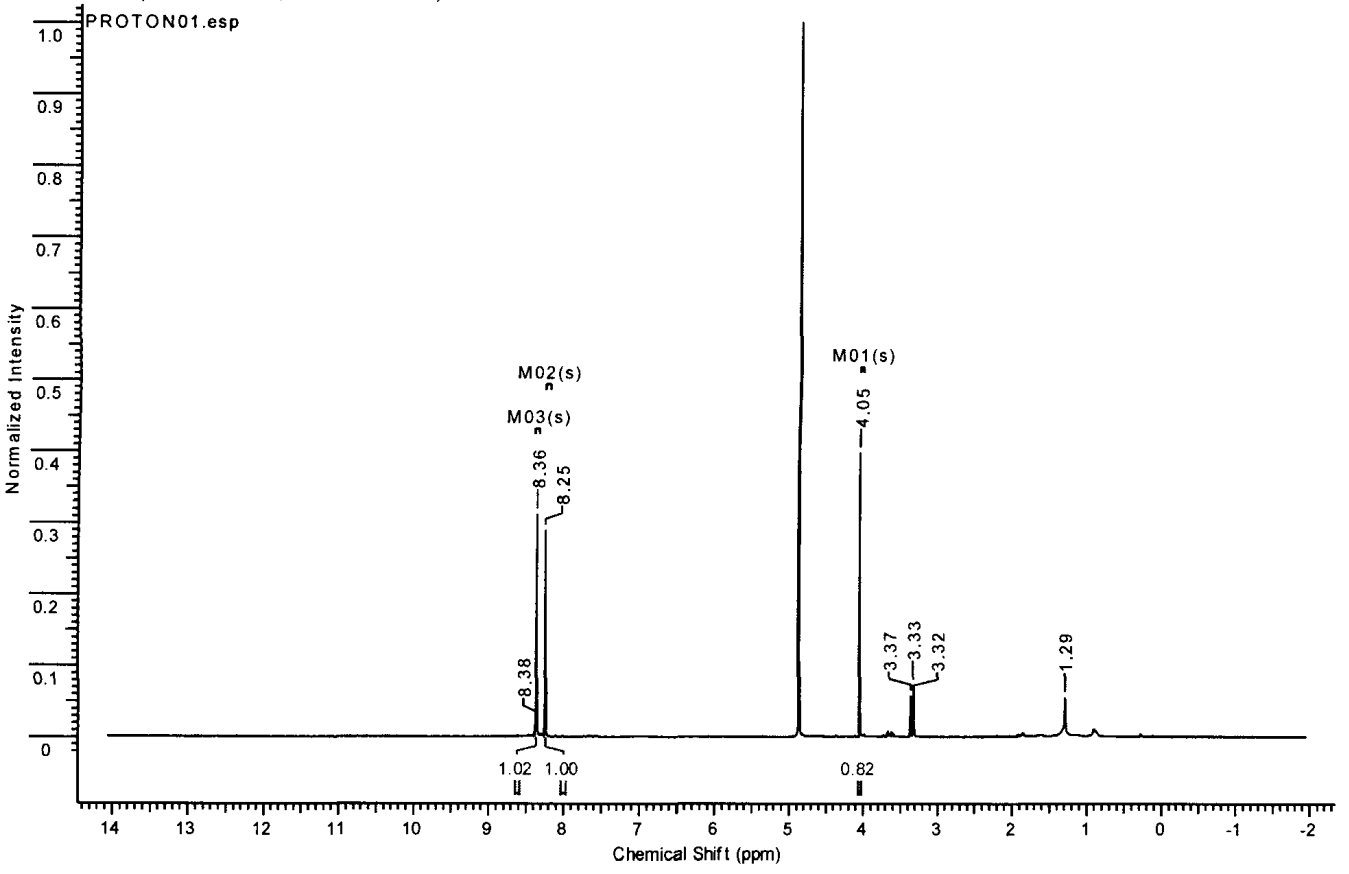

$^{13}$C NMR (101 MHz, $CDCl_3$) of **63**



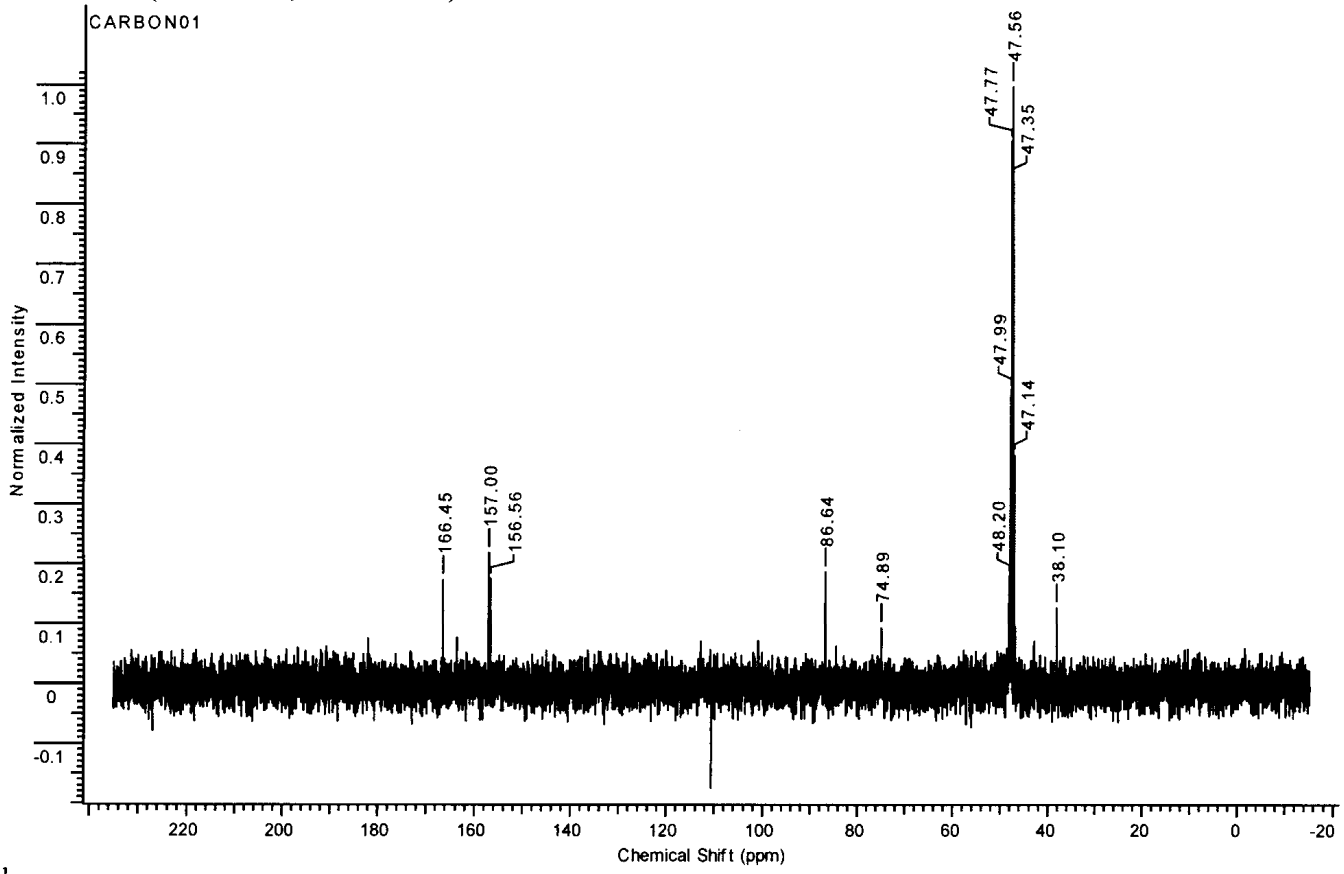
$^1$H NMR (400 MHz, $CDCl_3$) of **65**



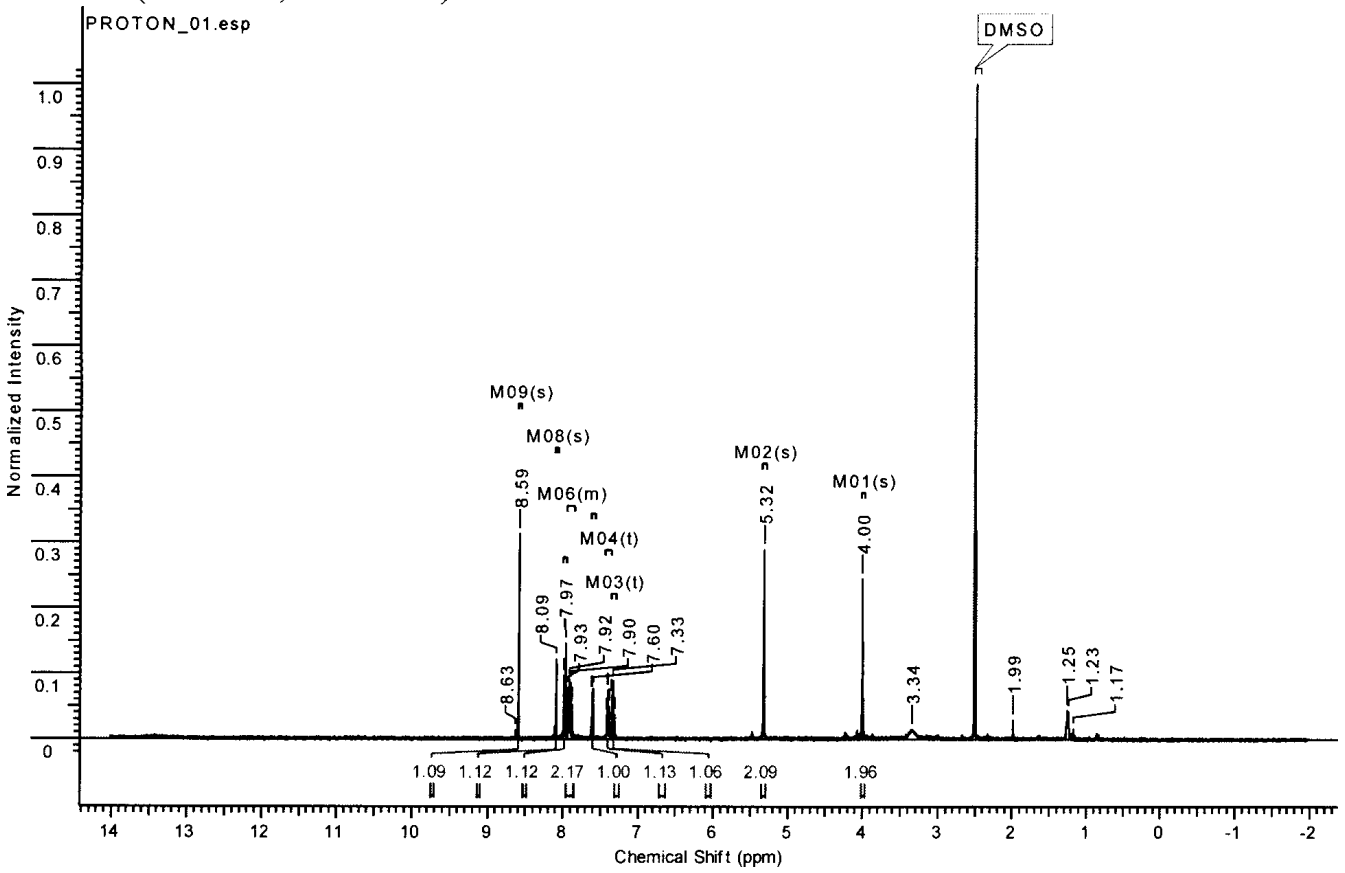

$^1$H NMR (400 MHz, MeOH-*d4*) of **66**



$^{13}$C NMR (101 MHz, MeOH-*d4*) of **66**




$^{1}$H NMR (400 MHz, DMSO-*d6*) of **72**



$^1$H NMR (400 MHz, DMSO-*d6*) of **73**