THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Master of Science (Chemistry)
DEGREE

Chemistry
FIELD

Chemistry
DEPARTMENT

TITLE: Synthesis of Acid Labile Reagent for the Purification of Specific tRNAs

NAME: Miss Suwimon Suebka

THIS THESIS HAS BEEN ACCEPTED BY

THESIS ADVISOR
(Mr. Pitak Chuawong, Ph.D.)

THESIS CO-ADVISOR
(Mr. Wanchai Pluempanupat, Ph.D.)

THESIS CO-ADVISOR
(Mr. Nonlawat Boonyalai, Ph.D.)

DEPARTMENT HEAD
(Assistant Professor Supa Hannongbua, Dr.rer.nat.)

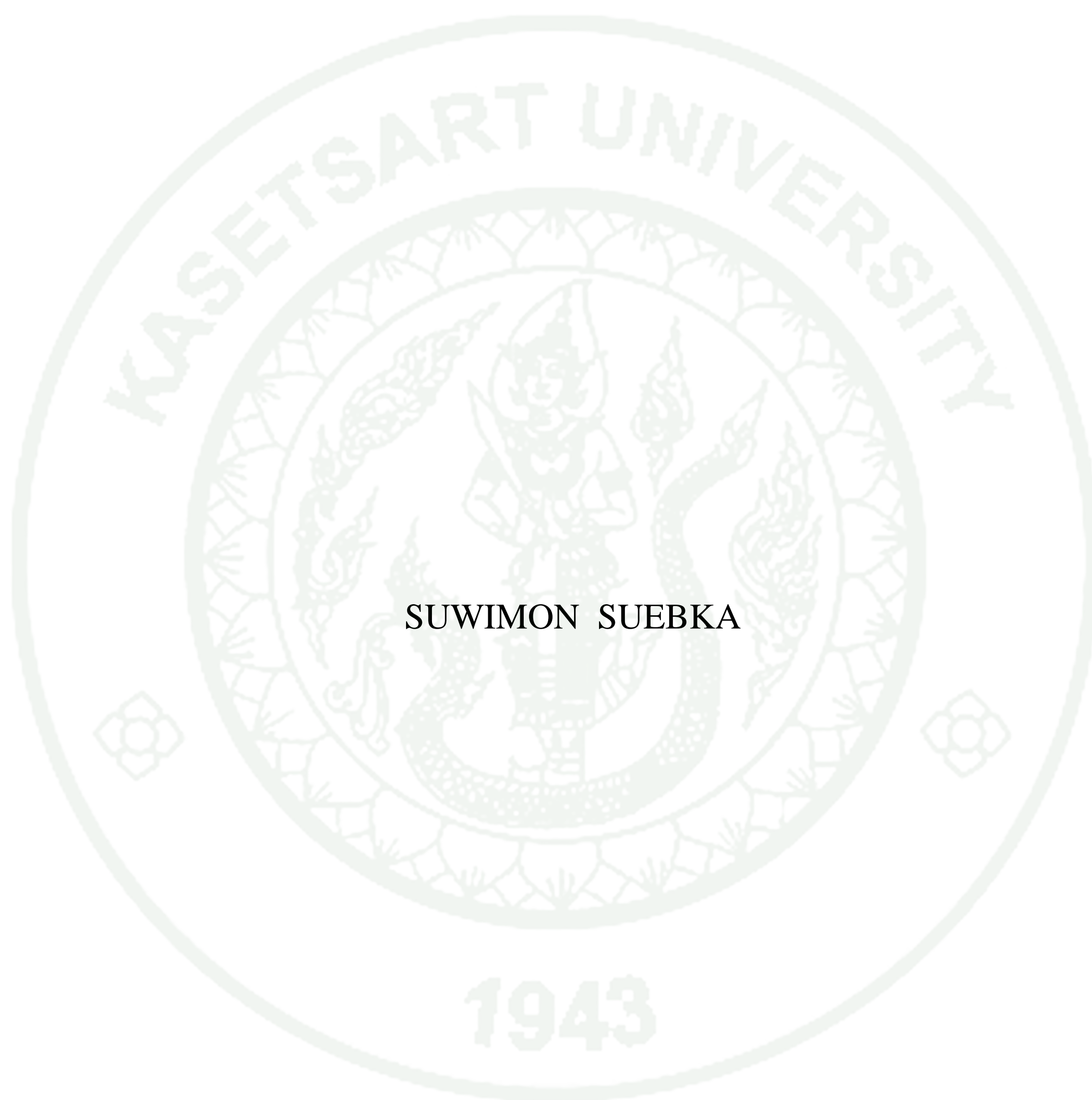
APPROVED BY THE GRADUATE SCHOOL ON

DEAN
(Associate Professor Gunjana Theeragool, D.Agr.)

Copyright by Kasetsart University All rights reserved

THESIS

SYNTHESIS OF ACID LABILE REAGENT FOR THE PURIFICATION OF SPECIFIC tRNAs



SUWIMON SUEBKA

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Chemistry)
Graduate School, Kasetsart University
2012

Copyright by Kasetsart University All rights reserved

Suwimon Suebka 2012: Synthesis of Acid Labile Reagent for the Purification of Specific tRNAs. Master of Science (Chemistry), Major Field: Chemistry, Department of Chemistry. Thesis Advisor: Mr. Pitak Chuawong, Ph.D. 80 pages.

Biomolecules are crucial building blocks for all living organisms on this planet. The purification of biomoleculs such as tRNAs is important for an investigation of their chemical properties and structural diversities. Consequently, it is necessary to develop an efficient purification technique as well as to look for novel bioconjugation systems in order to facilitate biomolecule manipulation. The study of aminoacyl-tRNAs is one of our main interests. The desired tRNA could be separated from a pool of tRNAs using a specific activity of an enzyme called aminoacyl-tRNA synthetase (AARS) in order to provide correctly charged aminoacyl-tRNA. The resulting system could be applied to immobilized-metal affinity chromatography.

In this study, acid labile reagent for the purification of specific tRNA was synthesized. The synthesis consisted of two parts; 1) synthesis of a conjugate molecule with terminal alkyne, 2) synthesis of a compound with terminal azido group, and 3) the utilization of click chemistry. Another terminus of azido conjugate molecule was coupled with histidylhistidine in order to facilitate the immobilized-metal affinity chromatography. The synthesis of terminal alkyne with active carbonate was proven to be problematic due to low stability of resulting carbonate functional group. The conjugate molecule with azido group was successfully synthesized with reasonable yield. The synthetic details as well as efforts toward the development of this acid labile reagent are presented herein.

_________________________ ______________________________ ___ /___ /___

Student's signature Thesis Advisor's signature

Copyright by Kasetsart University All rights reserved

ACKNOWLEDGEMENTS

I am overwhelmed with gratitude for my thesis advisor, Dr. Pitak Chuawong who has given me graduate study opportunity for his help, encouregement, careful guidance and his patience to me all time.

This thesis was supported by the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Office of the Higher Education Commission, Ministry of Education and The Kasetsart University Research and Development Institute (KURDI). The Science Research Fund (ScRF-E3/2551) from the Faculty of Science, Kasetsart University ais also acknowledged. I would like to thank The Development and Promotion of Science and Technology talent project (DPST) for financial support.

Finally, I would like to thank all members in PC's laboratory for kind help and encouragement. I am verry thankful to my family for every supports and understanding during my graduate study.

Suwimon Suebka
April, 2012

Copyright by Kasetsart University All rights reserved

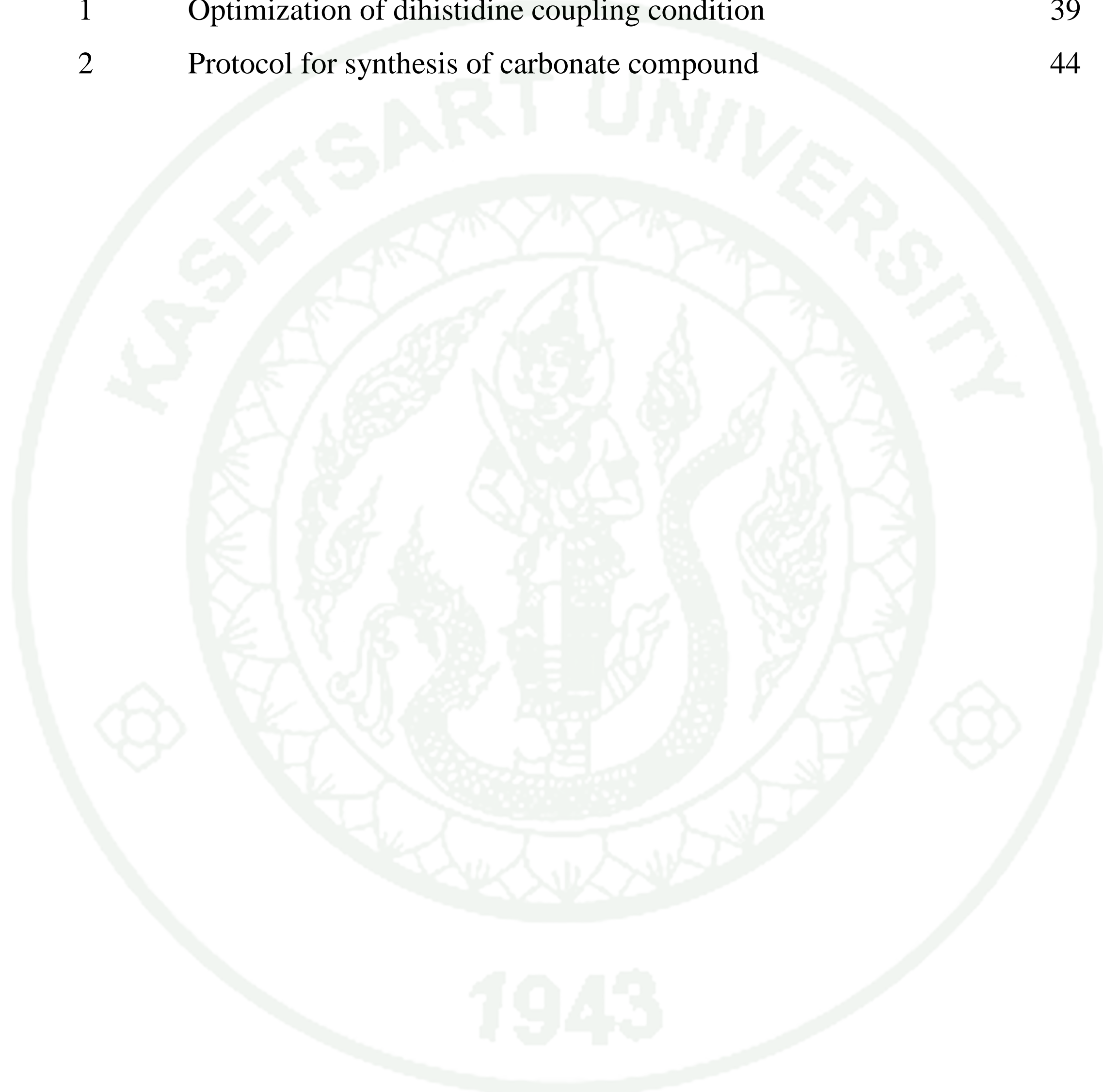
TABLE OF CONTENTS

	Page
TABLE OF CONTENTS	i
LIST OF TABLES	ii
LIST OF FIGURES	iii
LIST OF ABBREVIATIONS	v
INTRODUCTION	1
OBJECTIVES	4
LITERATURE REVIEW	5
MATERIALS AND METHODS	20
RESULTS AND DISCUSSIONS	32
CONCLUSION	46
LITERATURE CITED	47
APPENDIX	55
CURRICULUM VITAE	80

Copyright by Kasetsart University All rights reserved

LIST OF TABLES

Table		Page
1	Optimization of dihistidine coupling condition	39
2	Protocol for synthesis of carbonate compound	44



Copyright by Kasetsart University All rights reserved

LIST OF FIGURES

Figure		Page
1	Nucleosides in RNA	1
2	Purification of tRNA	2
3	Interaction between histidine and Ni-NTA matrix	3
4	The first generation of target molecule (compound 1)	9
5	The second generation of target molecule (compound 2)	13

Appendix Figure

1	^{1}H NMR of acetone cyanohydrin (3)	56
2	^{13}C NMR of acetone cyanohydrin (3)	57
3	^{1}H NMR of 2,2'-oxybis(ethane-2,1-diyl) bis(4-methylbenzene-sulfonate) (8a)	58
4	^{13}C NMR of 2,2'-oxybis(ethane-2,1-diyl) bis(4-methylbenzene-sulfonate) (8a)	59
5	^{1}H NMR of 2,2'-(ethane-1,2-diylbis(oxy))bis(ethane-2,1-diyl) bis-(4-methylbenzenesulfonate) (8b)	60
6	^{13}C NMR of 2,2'-(ethane-1,2-diylbis(oxy))bis(ethane-2,1-diyl) bis-(4-methylbenzenesulfonate) (8b)	61
7	^{1}H NMR of 1-azido-2-(2-azidoethoxy)ethane (13a)	62
8	^{13}C NMR of 1-azido-2-(2-azidoethoxy)ethane (13a)	63
9	^{1}H NMR of 1,2-bis(2-azidoethoxy)ethane (13b)	64
10	^{13}C NMR of 1,2-bis(2-azidoethoxy)ethane (13b)	65
11	^{1}H NMR of 2-(2-azidoethoxy)ethanamine (11a)	66
12	^{13}C NMR of 2-(2-azidoethoxy)ethanamine (11a)	67
13	^{1}H NMR of 2-(2-(2-azidoethoxy)ethoxy)ethanamine (11b)	68
14	^{13}C NMR of 2-(2-(2-azidoethoxy)ethoxy)ethanamine (11b)	69
15	^{1}H NMR of histidylhistidine (14)	70

Copyright by Kasetsart University All rights reserved

LIST OF FIGURES (Continued)

Appendix Figure **Page**

16	^{13}C NMR of histidylhistidine (14)	71
17	^{1}H NMR of methyl hexynoate (15)	72
18	^{13}C NMR of methyl hexynoate (15)	73
19	^{1}H NMR of benzyl hexynoate (18)	74
20	^{13}C NMR of benzyl hexynoate (18)	75
21	^{1}H NMR of 2-methylhept-6-yn-2-ol (16)	76
22	^{13}C NMR of 2-methylhept-6-yn-2-ol (16)	77
23	^{1}H NMR of benzyl 2-chloroacetate (21)	78
24	^{13}C NMR of benzyl 2-chloroacetate (21)	79

Copyright by Kasetsart University All rights reserved

LIST OF ABBREVIATIONS

ADP	=	adenosine diphosphate
AMP	=	adenosine monophosphate
anh.	=	anhydrous
ATP	=	adenosine triphosphate
BD-cellulose	=	benzyl-DEAE cellulose
CDMT	=	2-chloro-4,6-dimethoxy-1,3,5-triazine
DCC	=	*N,N'*-dicyclohexylcarbodiimide
DEAE	=	diethylaminoethanol
DMAP	=	4-dimethylaminopyridine
DMF	=	dimethylformamide
DMTMM	=	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleotide triphosphate
EF-Tu	=	elongation factor thermo unstable
ESI	=	electrospray ionization
EtOAc	=	ethyl acetate
EtOH	=	ethyl alcohol
g	=	gram
GTP	=	guanosine triphosphate
HOBt	=	hydroxybenzotriazole
hr	=	hour
Hz	=	hertz
L	=	liter
LRMS	=	low resolution mass spectrometry
M	=	molar
MeOH	=	methyl alcohol
mg	=	milligram
MHz	=	megahertz
mL	=	milliliter

Copyright by Kasetsart University All rights reserved

LIST OF ABBREVIATIONS (Continued)

mmol	=	millimol
MOMCl	=	chloromethyl methyl ether
mRNA	=	messenger ribonucleic acid
MS	=	mass spectrometry
MsCl	=	methanesulfonyl chloride
NHS	=	*N*-hydroxysuccinimide
Ni-NTA	=	nickel-nitrilotriacetic acid
NMM	=	*N*-methylmorpholine
NMR	=	nuclear magnetic resonance
PNP	=	*p*-nitrophenol
RNA	=	ribonucleic acid
RNase	=	ribonuclease
RPC	=	reversed-phase chromatography
rt	=	room temperature
RT-PCR	=	reverse transcription polymerase chain reaction
sat.	=	saturated
S_N2	=	bimolecular nucleophilic substitution
sRNA	=	soluble ribonucleic acid
TBAI	=	tetrabutylammonium iodide
TLC	=	thin layer chromatography
THF	=	tetrahydrofuran
tRNA	=	transfer ribonucleic acid
TsCl	=	4-toluenesulfonyl choride
°C	=	degree Celsius
⥮	=	reflux

Copyright by Kasetsart University All rights reserved

SYNTHESIS OF ACID LABILE REAGENT FOR THE PURIFICATION OF SPECIFIC tRNAs

INTRODUCTION

Biomolecules are organic compounds produced by living organisms. These classes of compounds including small molecules such as vitamins, amino acids, and nucleotides as well as large polymers such as proteins and nucleic acids (DNA and RNA) are crucial for all living organisms. Among the biomolecules mentioned above, we focus on transfer-ribonucleic acid (tRNA). The tRNA is a biopolymer of nucleotides linked together through phosphodiester bonds. These nucleotides contain ribose, nitrogenous base (adenine, cytosine, guanine, and uracil), and phosphate groups. Transfer RNA also contains a three base region called anticodon (Crick, 1968), which can pair with the corresponding codon on mRNA through hydrogen bonding. Each tRNA molecule can be aminoacylated by only one type of amino acid at the 3′ terminus (the acceptor stem) to form aminoacyl-tRNA. This covalent linkage formation is catalyzed by the aminoacyl-tRNA synthetase. However, the genetic code may contain multiple condons that correspond to the same amino acid. Therefore, tRNAs bearing different anticodons can carry the same amino acid.

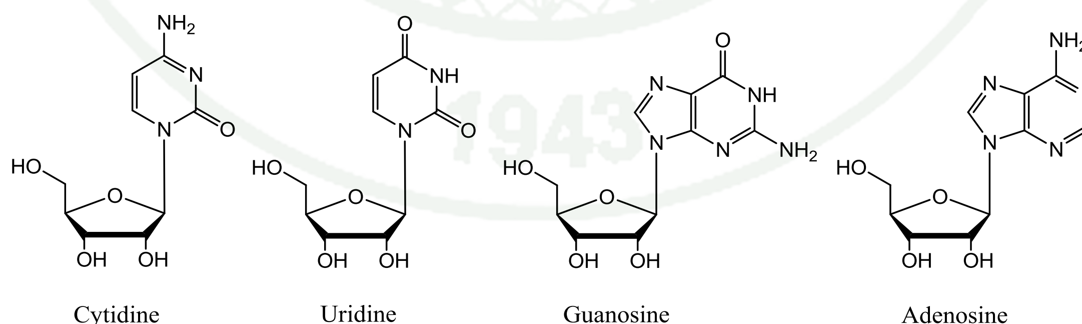


Figure 1 Nucleosides in RNA

Copyright by Kasetsart University All rights reserved

The purification of tRNAs is crucial for an investigation of their chemical properties and structural diversities. Consequently, it is necessary to develop an efficient purification technique as well as to look for novel bioconjugation systems. The study of aminoacyl-tRNAs (aminoacyl-transfer RNA) is one of our main interests. For our plan (Figure 2), the desired tRNA could be separated from a pool of tRNAs using a specific activity of enzymes called aminoacyl-tRNA synthetases (AARSs) in order to provide correctly charged aminoacyl-tRNA (1). The resulting system could be treated with bioconjugate molecule (2) and applied to immobilized-metal affinity chromatography, Ni-Nitrilotriacetic acid (Ni-NTA) (3). Treatment with mild acid condition will afford decomposition to give CO_2 and aminoacyl-tRNA (4). Finally, deacylation of aminoacyl-tRNA will provide pure tRNA.

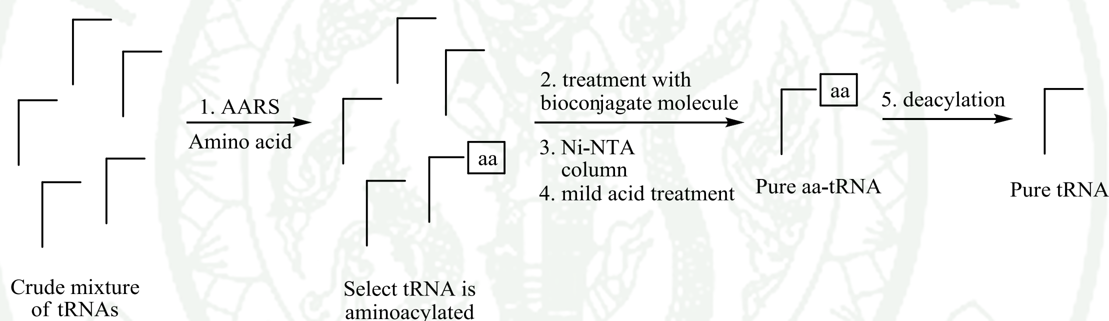


Figure 2 Purification of tRNA

Nitrilotriacetic acid (NTA) resin is a common reagent for the purification of histidine-tagged protein. It is also expected to be used for the purification of a molecule containing polyhistidine moiety. However, only two histidine units of histidine-tagged can bind to Ni-NTA resin (Figure 3). Consequently, the bioconjugate molecule was designed to consist of two histidine units. It could be conjugated with aminoacyl-tRNAs and also consists of carbamate group, which shows labile acid property (pH~4) and decomposes to CO_2 and aminoacyl-tRNA. To this, aminoacy-tRNA is obtained.

Copyright by Kasetsart University All rights reserved

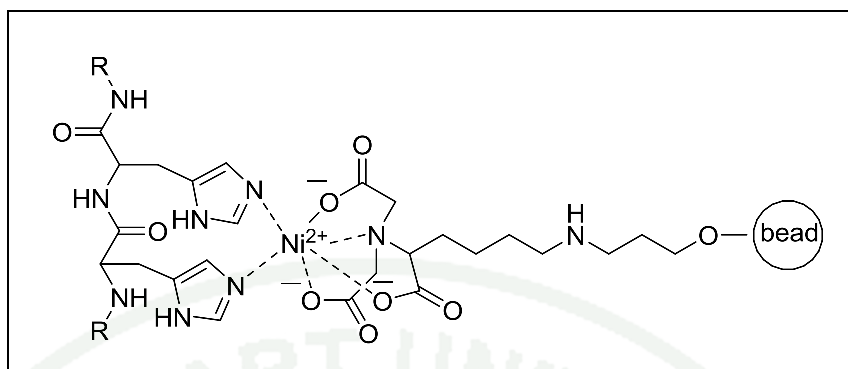
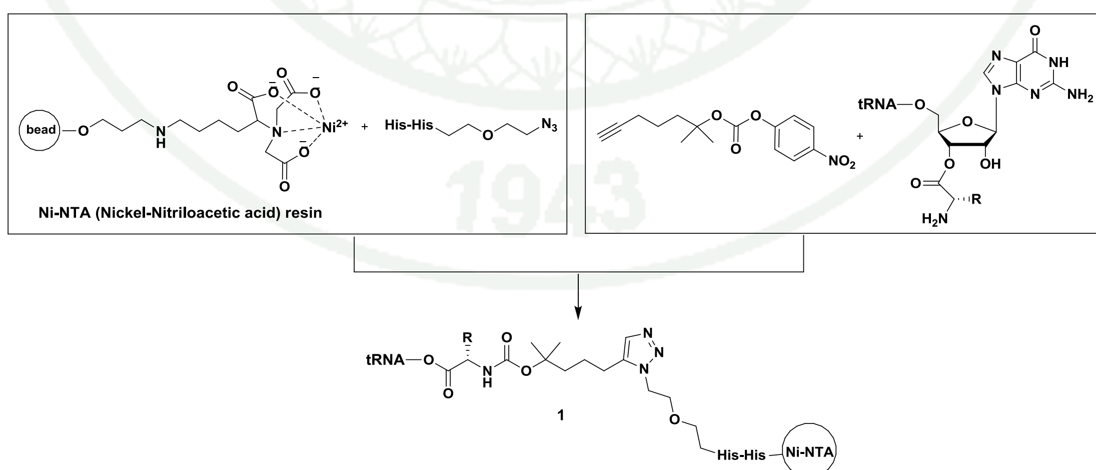


Figure 3 Interaction between histidine and Ni-NTA matrix

In this study, acid labile reagent for the purification of specific tRNA was designed to be constructed from two parts (scheme 1), a terminal alkyne and a compound with terminal azido group. They are linked by click chemistry. Another terminus of azido compound could be coupled with dihistidine in order to link with immobilized-metal affinity chromatography. A terminal alkyne containing carbonate group will be linked to aminoacyl-tRNA by formation of carbamate functional group. The acid labile functional group should readily decompose to liberate the desired aminoacyl-tRNA under acidic condition, liberating carbondioxide and aminoacyl-tRNA. The desired tRNA could be obtained from the final step via deacylation.



Scheme 1 Synthetic plan of the target bioconjugated molecule 1

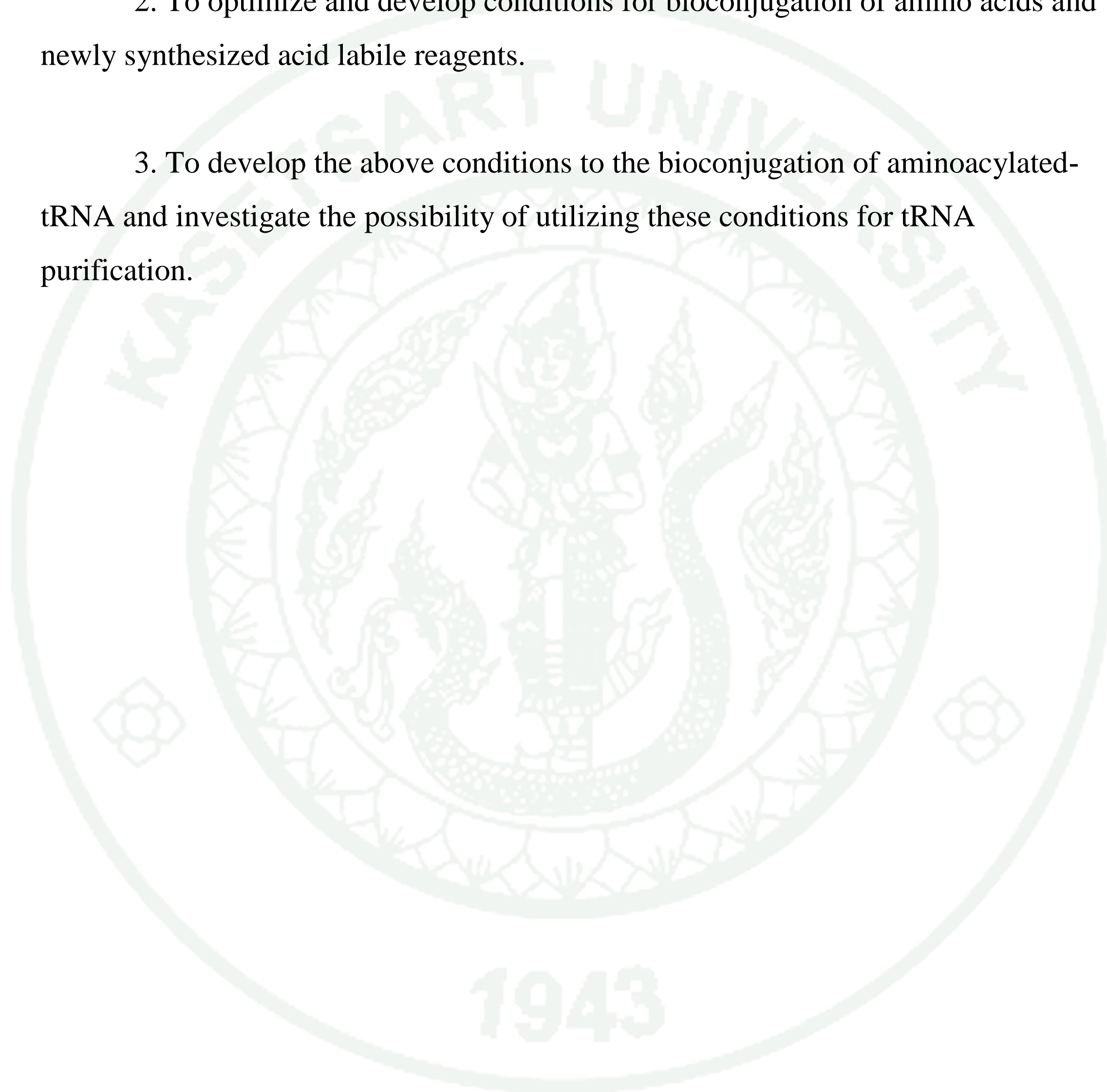
Copyright by Kasetsart University All rights reserved

OBJECTIVES

1. To design and synthesize acid labile reagents.

2. To optimize and develop conditions for bioconjugation of amino acids and newly synthesized acid labile reagents.

3. To develop the above conditions to the bioconjugation of aminoacylated-tRNA and investigate the possibility of utilizing these conditions for tRNA purification.



Copyright by Kasetsart University All rights reserved

LITERATURE REVIEW

1. Discovery of tRNAs and aminoacyl-tRNA synthetase

Transfer-ribonucleic acid (tRNA) was first discovered and researched when Mahlon Hagland in Paul Zamecnik's group studied a cell free protein synthesis system from rat liver in 1956. They found an enzyme in the pH 5-insoluble fraction (pH 5 enzyme) that activated amino acids in the presence of ATP to provide aminoacyl-adenylates (Hoagland *et al*., 1956; Zamecnik, 2005). The aminoacyl-adenylates were constituted using amino acid-dependent ATP-PPi exchange and by their reaction with neutral hydroxylamine to form amino acid hydroxamate. Then, the Zamecnik's group studied further the formation of aminoacyl-adenylate and found that the same enzyme in a pH 5 fraction transferred radio labeled amino acid (^{14}C-leucine) to an RNA acceptor and the ^{14}C-leucine attached to the RNA was subsequently transferred to protein in a protein synthesis system (Hoagland, *et al.*, 1957, 1958, 1996). This result indicated that RNA played as intermediate carrier of the amino acid in protein synthesis. At the same time, Ogata and Nohara (1957) obtained evidence for transfer of amino acid by the pH 5 enzyme to an RNA. Moreover, Holley (1957) exposed the pH 5 enzyme catalyzed alanine-dependent ATP-AMP exchange which was sensitive to ribonuclease. This result also supported the concept of amino acid transfer to RNA although the ribonuclease sensitive ATP-AMP exchange was observed only in the presence of alanine and no other amino acids. The RNA in the pH 5 enzyme fraction was named soluble RNA (sRNA) by Zamecnik and his colleagues. Then, this RNA is known as tRNA. Furthermore, it was demonstrated that the amino acid-activating enzyme in the pH 5 fraction also catalyzed the transfer of activated amino acid to tRNA (Berg and Ofengand, 1985; Lipmann *et al*, 1959). Presently, this enzyme is known as aminoacyl-tRNA synthetase (AARS).

After tRNA and aminoacyl-tRNA synthetase were discovered. It was quickly found that each aminoacyl-tRNA synthetase is specific for an amino acid and each

Copyright by Kasetsart University All rights reserved

enzyme attaches a specific amino acid onto cognate tRNA. Many reports including work from Potter, Canellahis, Herbert and Zamecnik concluded that tRNA contained a common sequence, -CCA, at the 3-end which is necessary for transfer of amino acid to tRNA (Canellakis, 1957; Hecht *et al*., 1958a, 1958b; Heidelberger *et al*., 1956). Then, Zachau's work in Lipmann's group established that amino acids were attached to the 3-terminal A residue of the tRNA via aminoacyl-ester linkages to 2- or 3-hydroxyl groups of ribose (Zachau *et al*., 1958) In conclusion, protein synthesis starts from activation of amino acid by aminoacyl-tRNA synthetases to from aminoacyl-adenylates. Then, the activated amino acids are transferred to tRNA acceptors and aminoacyl-tRNAs are formed. Finally, aminoacyl-tRNAs are transferred to the ribosome where translation begins. Consequently, tRNAs played an important role in translating the nucleotide sequence in mRNAs into amino acid sequences in protein. The first tRNA sequenced was alanine tRNA from yeast, which was discovered by Holley and coworkers in 1965 (Holley *et al*., 1965). They proposed three possible secondary structures for this tRNA, one of which is now known as cloverleaf structure. Sequences of several tRNAs were succeeded subsequently, serine tRNA by Zachua and coworkers (Zachau *et al*., 1966), tyrosine tRNA by Madison and coworkers (Madison *et al*., 1966) and phenylalanine tRNA by RajBhandary, Khorana and coworkers (RajBhandary *et al*., 1966, 1967). The revelation of several tRNAs sequences constituted the cloverleaf as the common secondary structure of tRNA. Moreover, it led to the identification of anticodon sequences of tRNAs. This knowledge led Crick to propose a set of base-pairing rules for codon-anticodon interactions in "The Wobble Hypothesis" (Crick, 1966, 1968). This hypothesis was confirmed by experiments showing that purified yeast phenylalanine tRNA with anticodon sequence G_mAA could read either of phenylalanine codons UUU and UUC in an *in vitro* protein synthesis system (Söll and RajBhadary, 1967).

2. Purification of tRNA

After the sequences of several tRNAs were obtained, scientists focusedr on the studies of structure-function of tRNAs. Method for the purification of tRNA was developed in order to obtain purified tRNA in large quantity from a variety of

Copyright by Kasetsart University All rights reserved

sources. Fortunately, several groups described effective methods based upon differences in the chromatographic properties of various tRNAs, including the use of benzoylated-DEAE-cellulose (BD-cellulose) by Tener and coworkers (Gillam *et al*., 1967), DEAE-Sephadex by Nishimura and coworkers (Nishimura *et al*., 1967), reversed-phase chromatography (RPC) by Kelmers, Novelli and coworkers (Pearson *et al*., 1971) and Sepharose 4B by Holmes, Reid, Hatfield and coworkers (Holmes *et al*., 1975)

The BD-cellulose column separates tRNA based on interaction of exposed hydrophobic bases to the matrix and affords an essentially one step purification of yeast phenylalanine tRNA starting from total yeast tRNA. This tRNA uniquely contains the hydrophobic base Y (Wybutine) which is located next to the anticodon sequence. Accordingly, the yeast phenylalanine tRNA binds tightly to the column even in 1 M NaCl and can be eluted off the column only in the presence of 10% ethanol. The easy purification of yeast phenylalanine tRNA and the fact that it contained a strongly fluorescent base led to its use in many of the early biophysical and functional studies on tRNAs by several laboratories including Zachau's group. Because of the facilitated purification, the tRNA also became commercially available. Not long after that, three-dimensional structure of tRNA was established by two laboratories working independently (Kim *et al*., 1974; Robertus *et al*., 1974). However, this tRNA remained the only RNA of known three-dimensional structure for several years. DEAE-Sephadex and Sepharose 4B columns also proved quite useful for large scale purification of many tRNAs. The RPC columns, which could be run at different pHs, temperatures and at high pressure, were quite versatile. Because the columns are run at high pressure, chromatography is quite rapid allowing for fractionation of not only tRNAs but also aminoacyl-tRNAs. They also provided excellent resolution of tRNAs on either small scale or large scale (RajBhandary *et al*., 2006).

In an alternative approach the desired amino acid is attached to its acceptor tRNA in the bulk tRNA mixture by aminoacyl-tRNA synthetase and then separated by the difference in chemical properties between aminoacylated and unesterified

Copyright by Kasetsart University All rights reserved

tRNAs. The affinity chromatography of aminoacyl-tRNAs, which was based on the specificity of the aminoacylation combined with the selectivity elongation factor Tu·GTP for the binding of aminoacyl-tRNA, was reported by Klyde and Bernfield (Klyde and Bernfield, 1973). Gel permeation chromatography was used to separate the aminoacyl-tRNA·EF-Tu·GTP ternary complex from non-aminoacylated tRNA. This method required the separation of aminoacyl-tRNA from the protein following the gel filtration. Such a step can be omitted when EF-Tu·GTP is immobilized. Then, Derwenskus and coworkers reported the immobilization of thermostable EF-Tu·GTP from *T. thermophilus* on cyanogen bromide activated Sepharose for isolation of specific tRNA isoacceptors (Derwenskus *et al*., 1984). Later, a very similar procedure was developed by Louie and coworkers, using immobilization of *E. coli* EF-Tu·GTP on 6-aminohexanoic acid-activated Sepharose (Louie *et al*., 1984). The main disadvantage of these methods was the deactivation of EF-Tu during its immobilization on cyanogen bromide-activated Sepharose. Only 5% of the EF-Tu was active in the aminoacyl-tRNA binding in the matrix-bound state when this procedure for immobilization was used.

In 1995, Ribeiro and coworkers reported a method for immobilization of *T. thermophilus* EF-Tu·GTP. The EF-Tu bearing a His-tag on its C-terminus was immobilized on Ni^{2+} -nitriloacetic acid (Ni-NTA) agarose column (Ribeiro *et al*., 1995). The bound EF-Tu·GTP completely retained its activity to bind aminoacyl-tRNA, allowing rapid isolation of substantial of specific tRNA isoacceptors in only one chromatographic step. However, these conditions for formation of aminoacyl-tRNA·EF-Tu·GTP ternary complex were limited because the variables need to be adjusted to the optimum. Afterwards, Pütz and coworkers reported a method to separate low amounts of aminoacyl-tRNAs from pools of inactive variants. The sulfo-*N*-hydroxysuccinimide biotin (sulfo-NHS-biotin) was used to capture aminoacyl-tRNA on streptavidine-coated magnetic beads (Pütz *et al*, 1997). Variants bound to the solid phase can be amplified by RT-PCR and transcription, providing tRNAs for subsequent selection rounds. In our study, new reagent for the capture of aminoacyl-tRNA was designed and synthesized. It will be investigated for tRNA purification by using affinity chromatography.

Copyright by Kasetsart University All rights reserved

3. Synthesis of functional linker for affinity chromatography

Compound (1) is the first generation of target molecule (Figure 4). It contains carbonate group, triazole ring and histidylhistidine.

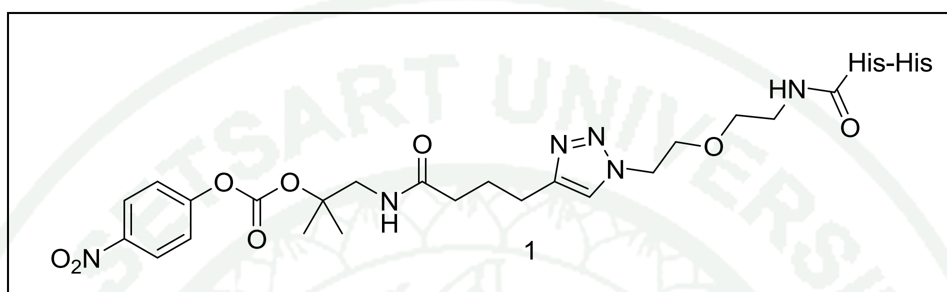
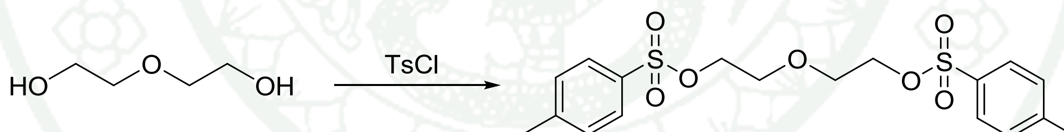
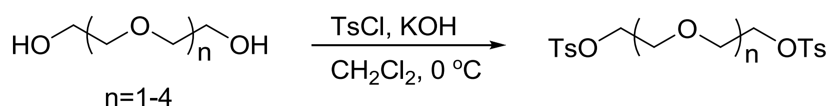


Figure 4 The first generation of target molecule (compound 1)

The triazole ring of compound (1) could be formed via click chemistry between an azide and an alkyne. The synthesis of this azide could be started from diethylene glycol converted to a ditosylated compound.



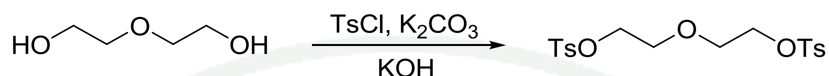
This reaction is related to the synthesis of ditosylated polyethyleneglycol (Bonger *et al*., 2007). In this report, *p*-toluenesulfonyl chloride was used as reagent and the reaction was kept at 0 °C. The desired product was obtained greater than 95% yield.



Another method was reported using solvent-free condition (Kazemi *et al*., 2007). In this synthesis, potassium carbonate was used as a base and the mixture was

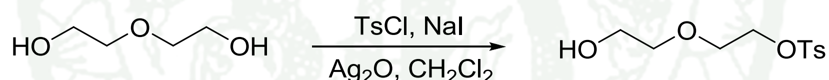
Copyright by Kasetsart University All rights reserved

grinded in a mortar. The remaining tosylchloride was removed by addition of potassium hydroxide and accelerated by dropping of *t*-BuOH. This method provided the desired product greater than 97% yield.

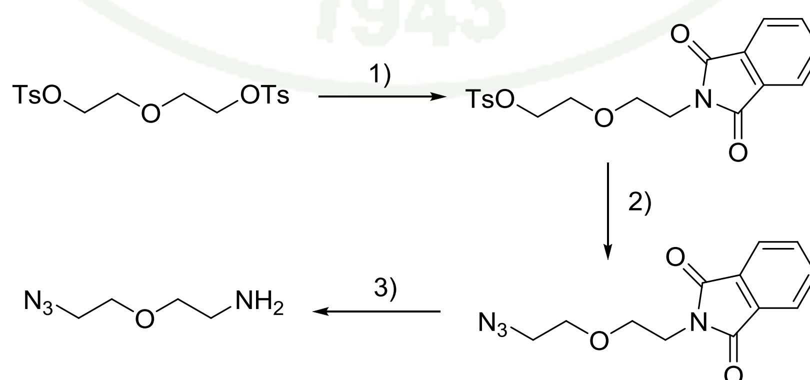


In order to obtain ditosylated product completely, the stoichiometry of tosylchloride was controlled to be over two fold compared to the mole of starting alcohol. There was a report using tosylchloride only 0.2 equivalent of tetra(ethyleneglycol) and this protocol gave monotosylated product in 75% yield (Xie *et al*., 2005). Moreover, selective monotosylation could be induced by silver (I) oxide (Bouzide and Sauvé, 2002). The reaction of symmetrical diols with tosylchloride (1:1 eq) in the presence of silver (I) oxide and potassium iodide provides monotosylated product greater than 93% yield (varies with type of diol)

.

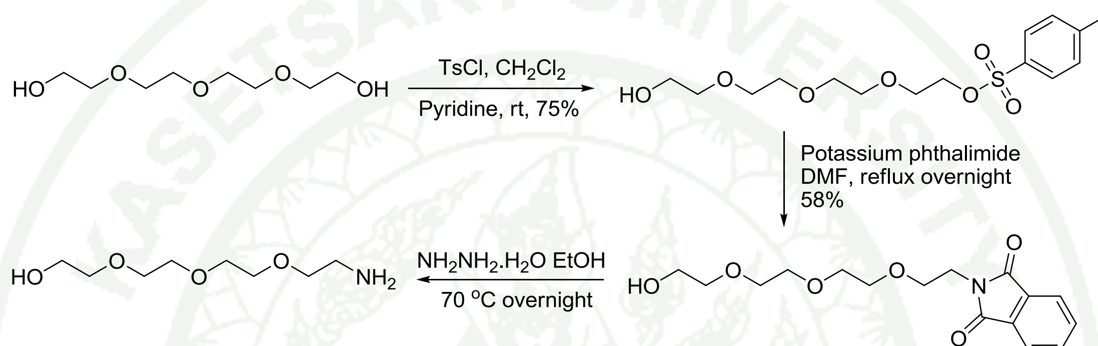


Ditosylated diethyleneglycol could be converted to amino azide compound via three steps 1) displacement of one tosyl group by phthalimide group 2) displacement of another tosyl group by azide group 3) conversion of phthalimide group to amino group.

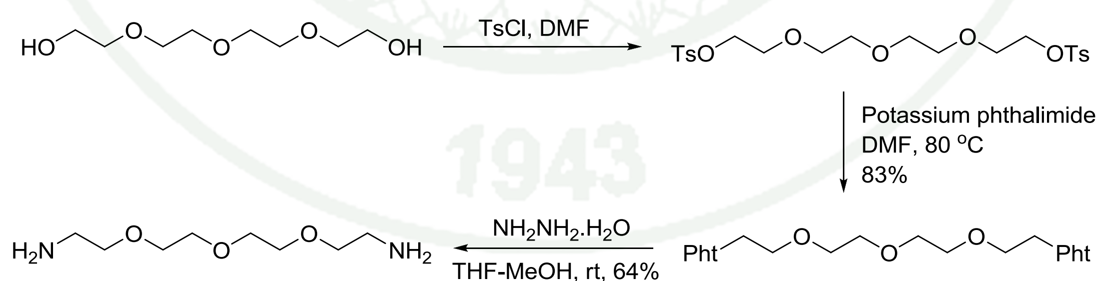


Copyright by Kasetsart University All rights reserved

This synthetic plan was related to the synthesis of 2-[2-[2-hydroxyethoxy]ethoxy ethylamine reported by Xie and coworkers (Xie *et al*., 2005). They used tetra(ethyleneglycol) as starting material converted to tosylated tetra(ethyleneglycol). Then, potassium phthalimide was used to convert tosylated compound to phthalimide compound. Finally, phthalimide was transformed to amine using hydrazine monohydrate.

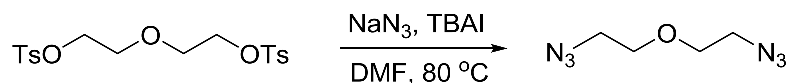


Furthermore, Numata and coworker reported another synthesis. Tetra(ethyleneglycol) was also used as stating material converted to ditosylated compound. Then, it was treated with potassium phthalimide to provide diphthalimide compound and also converted to diamino compound by using hydrazine monohydrate (Numata *et al*., 2005).

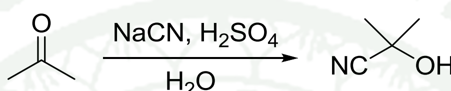


Foe the installation of azide group, there is a protocol reported by Bonger and coworker. Ditosylated ethyleneglycol was transformed to diazido compound using sodium azide as reagent in the presence of tetrabutylammonium iodide (TBAI) (Bonger *et al*., 2007). This method gave the product over 95% yield.

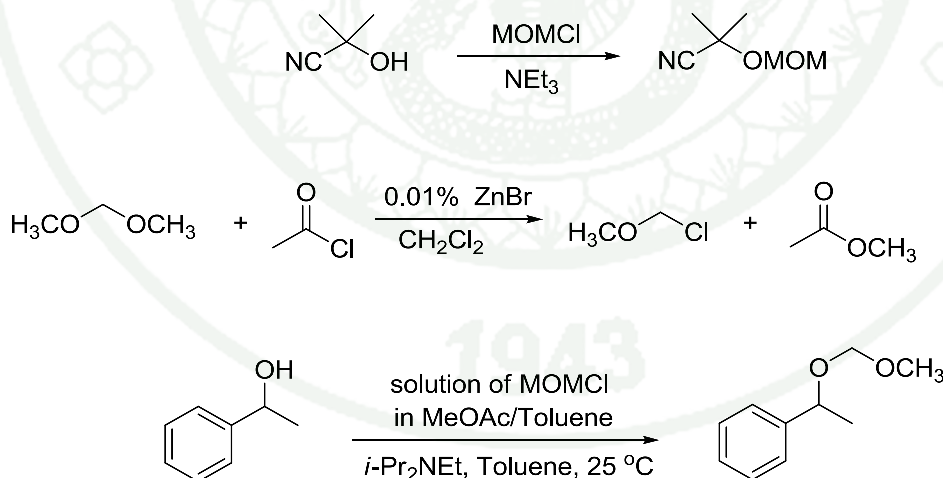
Copyright by Kasetsart University All rights reserved



For an alkyne moiety, the synthesis could be started from the formation of acetone cyanohydrin. Cox and Stormont reported the related reaction in 1943. Acetone cyanohydrin was generated from acetone and sodium cyanide in the presence of acid (Cox and Stormont, 1943)



Then, acetone cyanohydrin will be protected with chloromethyl methyl ether which could be generated in situ from dimethoxymethane and acetyl chloride in the presence of 0.01% anhydrous zinc bromide (Berliner and Belecki, 2005, 2007). Then, chloromethyl methyl ether will be used in protection step of alcohol without further manipulation.



In order to overcome the problematic synthetic route, compound (2) was designed to be the second generation of target molecule (Figure 5).

Copyright by Kasetsart University All rights reserved

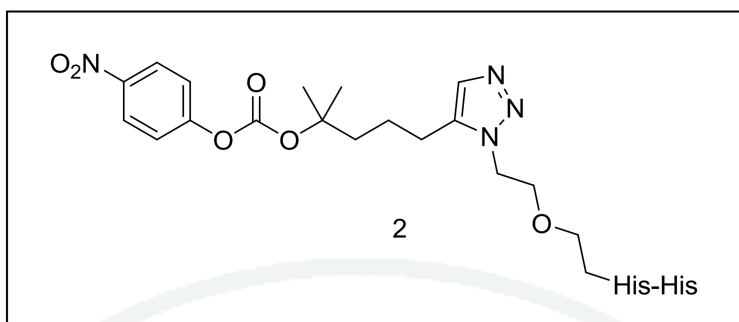
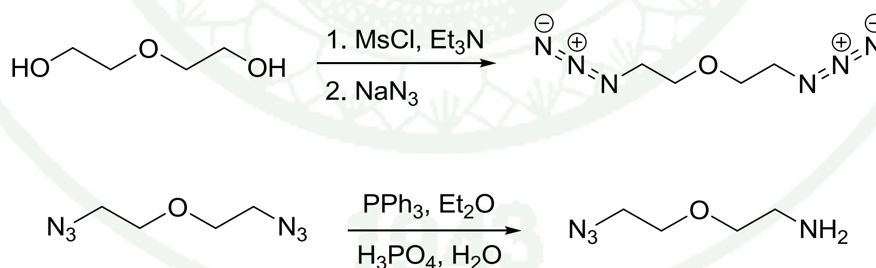


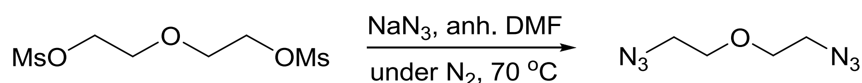
Figure 5 The second generation of target molecule (compound 2)

For the azide moiety, ditosylated diethyleneglycol will be converted to diazido diethyleneglycol and this diazide compound will be partially reduced to an amino azide compound.

This reaction is related to amino azide formation reported by Schwabacher (Schwabacher *et al*., 1998). In this synthesis, tetraethylene glycol was converted to dimesylate compound which was converted to diazide subsequently. To this step diazido compound was obtained in 67% yield. Then, the diazide was converted to the amino azide using Staudinger reaction and provided the product in 82% yield.

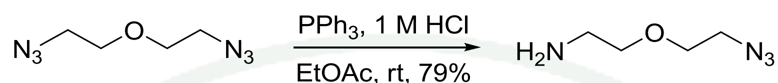


Another procedure for diazide formation was reported by Spangenberg and coworkers. Mesylated compound was reacted with sodium azide under dry condition and provided diazido product greater than 90% yield (Spangenberg *et al*., 2009).



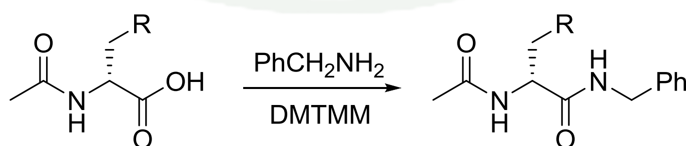
Copyright by Kasetsart University All rights reserved

Moreover, Tekeuchi and coworkwes reported a protocol of Staudinger reduction using triphenylphosphine in the presence of 1 molar hydrochloric acid (Tekeuchi *et al*., 2009).

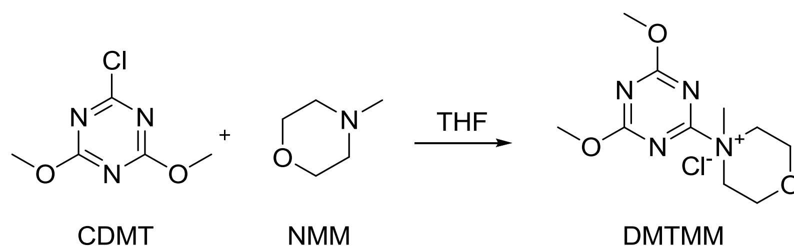


Finally, coupling of amino azide by polyhistidine will be the last step for the synthesis of this part of the molecule. Histidylhistidine was chosen to be our first candidate for polyhistidine moiety. It will be synthesized by coupling of Boc-His-OH and *L*-histidine. A general protocol was reported by Nashed and Mitra. They coupled acyclovir (ACV) (9-[(2-hydroxyethoxy)methyl]guanine) to Boc-Gly-OH using dicyclohexylcarbodiimide (DCC) and 4-(*N,N*-dimethylamino) pyridine (DMAP) as reagent (Nashed and Mitra, 2003). Dahiya and coworker also reported protocol for peptide formation of *L*-amino acid methyl ester hydrochlorides and Boc-amino acids. Boc-amino acids were activated using DCC to formed active ester and coupled with *L*-amino acid methyl ester hydrochlorides, respectively (Dahiya *et al*., 2008a, 2008b, 2008c, 2008d).

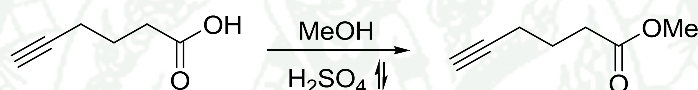
Alternatively, there is an interesting method to form peptide bond using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) (Morieux *et al*., 2008). This reagent could be generated from the reaction of 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) and *N*-methylmorpholine (NMM) in tetrahydrofuran (Kunishima *et al*., 1999).



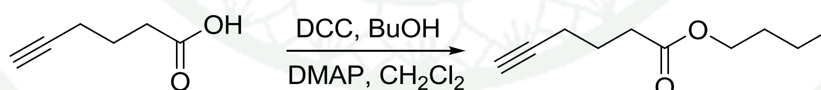
Copyright by Kasetsart University All rights reserved



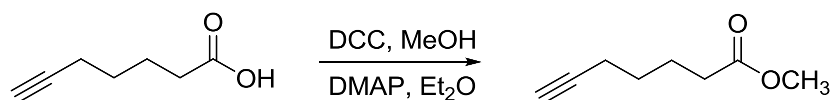
Another alkyne moiety of compound (2) could be synthesized from hexynoic acid. This alkyne-carboxylic acid will be converted to methyl hexynoate. This reaction is related to the synthesis of methyl hexynoate (Prasad *et al*., 2006). Hexynoic acid was treated with methanol in the presence of sulfuric acid to form the resulting ester.



Moreover, Harkat reported an alternative method in 2009. In this synthesis, dicyclohexylcarbodiimide (DCC) was used to activate an acid before it reacted with butanol (Harkat *et al*., 2009). Dichloromethane was used as solvent and the reaction was run at room temperature. This method provided the product in 70% yield.

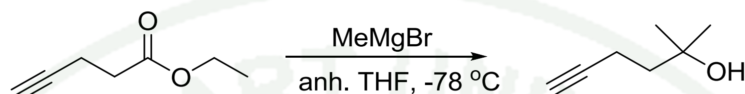


In addition, Suk and coworker reported a similar method for the synthesis of methyl 6-heptynoate using diethylether as solvent. The product was obtained in 69% yield. (Suk *et al*., 2008)

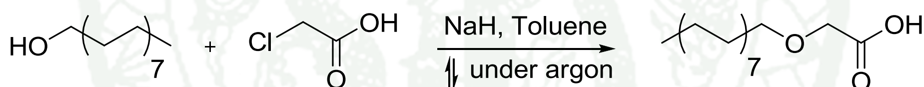


Copyright by Kasetsart University All rights reserved

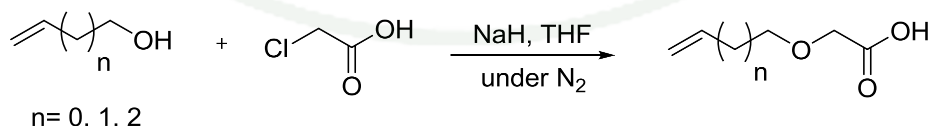
The acquired methyl hexanoate could be converted to gem-dimethyl alcohol by Grignard reagent. This reaction is related to Grignard reaction of ethyl pentynoate (Aponick *et al*., 2009). In this report, methylmegnesium bromide was used to convert an ester to gem-dimethyl alcohol. This reaction was kept at -78 °C.



Because of an expensive starting hexynoic acid, a similar acid with terminal alkyne could be synthesized from reaction of propagyl alcohol and chloroacetic acid. Many reports could be adapted to suit our reaction. Wissner and coworkers reported a synthesis of 1-(hexadecy1oxy)acetic using chloroacetic acid and 1-hexadecanol. Sodium hydride was used as base and toluene was used as solvent. The reaction was refluxed under argon and it provided the product in 68% yield (Wissner *et al*., 1985).



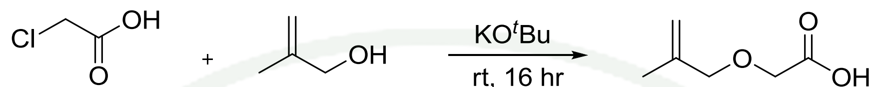
Cossy and coworkers also reported a synthesis of α-alkoxy acetic acids from chloroacetic acid and unsaturated alcohol using sodium hydride as base. Tetrahydrofuran was used as solvent and this reaction was stirred at room temperature under nitrogen. This protocol provided the product greater than 50% yield (Cossy *et al*., 2002).



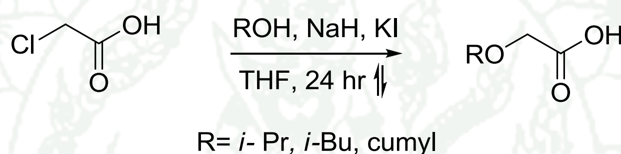
Similarly, Li and coworkers reported a coupling of chloroacetic acid and β-methallyl alcohol using *tert*-butoxide as base. The reaction was stirred at room temperature for 16 hr. An acid-base workup to be utilized and enabled the effective removal of excess methallyl alcohol. However, the major impurity, 2-*tert*-

Copyright by Kasetsart University All rights reserved

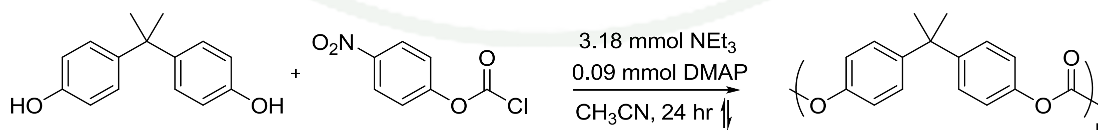
butyloxyacetic acid, was observed significantly. It resulted from the reaction between chloroacetic acid and potassium *tert*-butoxide. This impurity was minimized by the use of an excess of methallyl alcohol (Li *et al*., 2006).



In addition, Denmark and Chung reported a protocol to prepared α-protected lycolic acids from chloroacetic acid with various alcohols in refluxing THF. This method afforded the desired greater than 60% yield (Denmark and Chung, 2008).

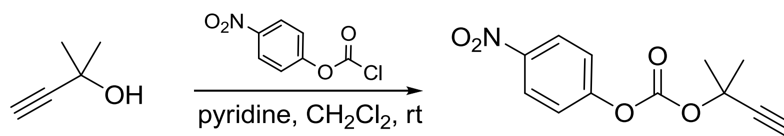


Then, this alcohol could be converted to a carbonate compound. This reaction is related to carbonate formation of ribonucleoside using *p*-nitrophenyl chloroformate in pyridine. The reaction was run at room temperature (Letsingear and Ogilvie, 1967). This method provided the desired product in 89% yield. In addition, Martin and Brittain reported carbonate formation of bisphenol. They used two equivalents of triethylamine and 6% stoichiometric equivalent of dimethylaminopyridine (DMAP). DMAP was used in addition to triethylamine to catalyze the condensation reaction (Martin and Brittain, 2002).

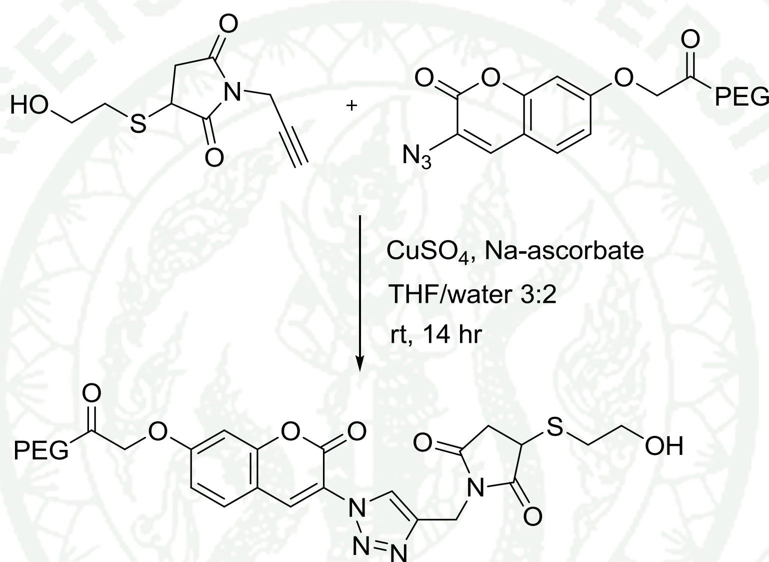


Then, Bertrand and Gesson reported a reaction which used starting material closely related to our alcohol (Bertrand and Gesson, 2007). The carbonated formation of 2-methylbut-3-yn-2-ol was carried on using pyridine as base in dichloromethane.

Copyright by Kasetsart University All rights reserved



When terminal alkyne and azide are obtained, the Click chemistry will be used for the construction of triazole ring. A similar reaction was reported using a 1,3-dipolar cycloaddition catalyzed by cupper (I) (Bertrand and Gesson, 2007).



A useful Cu(I) catalysed azide-alkyne 1,3-dipolar cycloaddition under neutral condition was reported by Dirks and coworkers. In this reaction, copper(II)sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$) and sodium ascorbate were used to induce the formation of triazole ring. The reaction was run at room temperature for 14 hr and strong fluorescence product was obtained in 47% yield (Dirks *et al*., 2009).

Affinity chromatography is a crucial step in the isolation of desired tRNA. The agarose beads bearing amine groups were fuctionalized to azide (Punna *et al*., 2005). The agarose-azide beads were treated with the complementary (alkyne containing amino acid), which is the derivative of the C-terminal sequence of HIV Protease. This method indicates that the Click chemistry could be successfully carried out on beaded agarose. In addition, Biotin-PEG-alkyne synthesized from Biotin-PEG-

Copyright by Kasetsart University All rights reserved

amine was also treated with agarose-azide beads. Finally, the oligopeptide was cleaved from the resin retaining its N-terminal with protecting group. This study demonstrated that the immobilization affinity chromatography by clickable agarose is useful and is of interest to our further studies.

Copyright by Kasetsart University All rights reserved

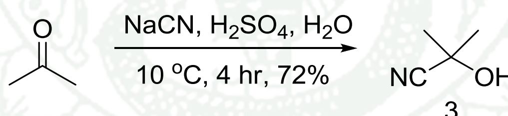
MATERAILS AND METHODS

1. Chemicals and instruments

All chemicals were purchased from Acros, Fluka, or Sigma-Aldrich. Solvents were laboratory grade and dried using Solvent Purification System: PURE SOLV MD-4. Basic apparatuses and glassware in chemistry laboratory were used. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) were recorded by a VARIAN^{UNITY} INOVA 400 MH_Z NMR spectrometer. Chemical shifts (δ) are given in parts per million (ppm) relative to a singlet peak of residue $CDCl_3$ (δ = 7.26) for ^{1}H NMR, and the central line of $CDCl_3$ (δ = 77.0) for ^{13}C NMR spectra. Mass spectra were recorded on a Variance, Agilent 1100 series by direct inlet ESI mode.

2. Synthetic procedure

2.1 Synthesis of Acetone cyanohydrin (3)

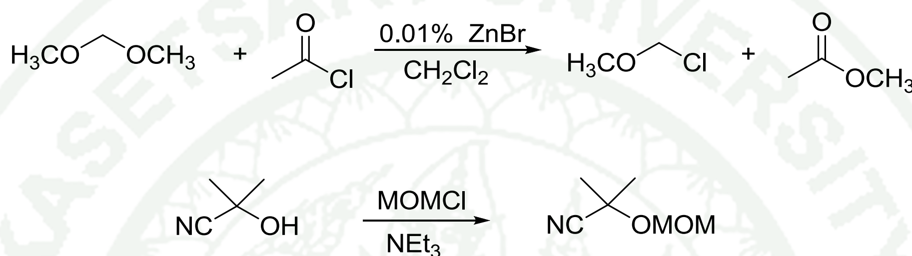


To the solution of powdered 95% NaCN (11.76 g, 0.24 mol,) in mixed solvent of water (27 mL) and acetone (21 mL), the flask was surrounded by an ice bath, and the solution was stirred vigorously. When the temperature downed to 15 °C, 40% H_2SO_4 (50 mL) was added over a period of three hours, the temperature being kept between 10 and 20 ˚C. The reaction was monitored by thin layer chromatography (20% EtOAc in hexane). After 4 hr, it was done. A layer of acetone cyanohydrin formed and was separated from aqueous layer. Then, the aqueous layer was filtrated and washed with three portions of acetone. The combined filtrate and acetone washings were added to the aqueous solution, which was then extracted three times with ether. The extracts were combined with the acetone cyanohydrin previously separated and dried with anhydrous sodium sulfate. After the removal of solvent, a

Copyright by Kasetsart University All rights reserved

product was obtained as colorless oil in 72% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.64 (s, 6H), 3.68 (br s, OH); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 29.0, 64.9, 122.6; LRMS (ESI, positive), MS *m/z* 100.2 [M-CN]

2.2 Synthesis of chloromethyl methyl ether and protection of acetone cyanohydrins



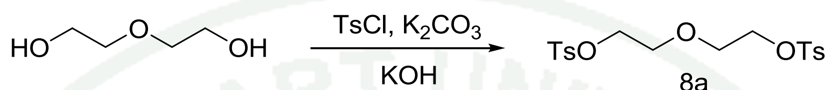
To a solution of anhydrous zinc bromide (0.001 g, 0.01% mol) and dimethoxymethane (4.4 mL, 0.05 mol) in dry CH_2Cl_2 (14 mL) was added acetyl chloride (3.6 mL, 0.05 mol) dropwise. The reaction was stirred under nitrogen and monitored by thin layer chromatography (3% MeOH in CH_2Cl_2). After 3 hr, the reaction was done. Then, chloromethyl methyl ether will be used in protection step without further manipulation.

Acetone cyanohydrins (1 mL, 0.01 mol, assumed the previous step was completed) and triethylamine (4.5 mL) were stirred at room temperature for 15 min and placed in the addition funnel. The reaction flask containing a dichloromethane solution of MOMCl prepared previously was immersed in an ice bath and cooled to 5-10 °C. Then, the solution of acetone cyanohydrin and triethylamine was added dropwise to the reaction and the temperature was kept below 25 °C during addition. After addition, the reaction was allowed to come to room temperature and then stirred for 12 hr (monitored by thin layer chromatography using 5% MeOH in CH_2Cl_2). After the reaction was complete, CH_2Cl_2 was added in order to dilute the reaction mixture and NH_4Cl solution was add and stirred for a minimum of 5 min to ensure all residual of MOMCl is decomposed. Then, the biphasic mixture was washed once with water,

Copyright by Kasetsart University All rights reserved

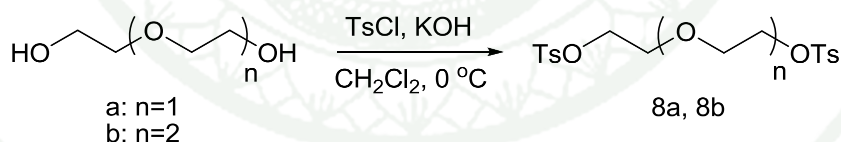
once with brine. The organic layer was separated, dried with $MgSO_4$ and concentrated by rotary evaporation. Finally, the product was purified by distillation.

2.3 Synthesis of ditosylated diethyleneglycol (8a) (Solvent free condition)



A mortar was charged with dry K_2CO_3 (2.5 g), diethyleneglycol (0.5 mL, 5 mmol), TsCl (1.43 g, 7.5 mmol) and grinded for 5 min. After the completion of tosylation, remaining TsCl was removed by addition of powdered KOH (2.5 g, 25 mmol) and grinded for 2 min. Crude was added a few drop of *t*-BuOH to accelerate the removal of TsCl. Then, the product was extracted by addition of ether (25 mL), filtered and removed the organic solvent. Further purification can be carried out on the crude solid tosylate by recrystallization in *n*-Hexane. The product was obtained in 11% yield as colorless crystal.

2.4 Synthesis of ditosylated di- and triethyleneglycol (8a and 8b)



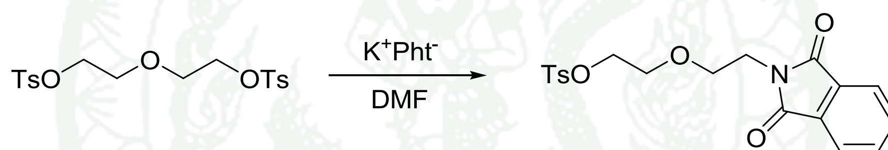
To a solution of di- or triethyleneglycol (3.5 mmol) in CH_2Cl_2 (5 mL) was added *p*-toluenesulfonyl chloride (TsCl) (1.4 g, 7.2 mmol) and cooled to 0 °C. Powdered KOH (1.6 g, 28 mmol) was carefully added in small portion and the reaction mixture was stirred at 0°C for 4 hr. After the reaction was done, dichloromethane and ice-water were added. The organic layer was separated, dried over Na_2SO_4 and evaporated to give white solid**.** Product can be recrystallized by petroleum ether.

Copyright by Kasetsart University All rights reserved

2,2'-oxybis(ethane-2,1-diyl) bis(4-methylbenzene-sulfonate) (8a) (2.6 g, 87%)**;** ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.38 (s, 6H), 3.54 (t, *J* = 4.8 Hz, 4H), 4.02 (t, *J* = 4.7 Hz, 4H), 7.28 (d, *J* = 8.0 Hz, 2H), 7.71 (d, *J* = 8.4 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 21.6, 68.8, 68.9, 127.9, 129.9; LRMS (ESI, positive), MS *m/z* 414.6 $[M+H]^+$

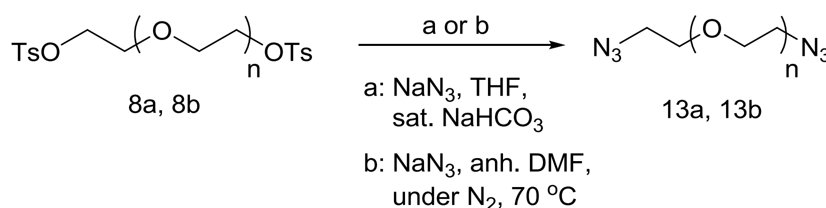
2,2'-(ethane-1,2-diylbis(oxy))bis(ethane-2,1-diyl)bis-(4-methylbenzenesulfonate) (8b) (2.67 g, 81%); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.44 (s, 6H), 3.52 (s, 4H), 3.65 (t, *J* = 4.8 Hz, 4H), 4.13 (t, *J* = 4.7 Hz, 4H), 7.33 (d, *J* = 8.0 Hz, 4H), 7.78 (d, *J* = 8.3 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 21.3, 68.7, 69.2, 70.6, 127.9, 129.8, 132.9, 144.8; LRMS (ESI, positive), MS *m/z* 458.9 $[M+H]^+$

2.5 Synthesis of monophthalimide diethyleneglycol (9)



Ditosylate diethyleneglycol (0.05 g, 0.12 mmol) and potassium phthalimide (0.01 g, 0.05 mmol,) were added in dry DMF (2.5 mL) and the reaction mixture was stirred at 80 °C for 12 hr. After the reaction was done (monitored by thin layer chromatography using 10% MeOH in CH_2Cl_2), DMF was removed under reduced pressure. Finally, the crude product was separated by column chromatography using 5-25% MeOH in CH_2Cl_2.

2.6 Synthesis of diazido di- and triethyleneglycol (13a and 13b)



Copyright by Kasetsart University All rights reserved

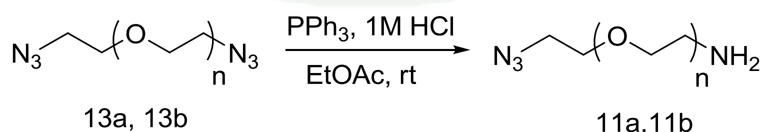
Method A. Ditosylated compound (7.3 mmol) was dissolved in THF and NaN_3 (0.94 g, 14 mmol) was then added. Sat. $NaHCO_3$ was gradually dropped until NaN_3 was completely dissolved. The reaction mixture was refluxed for 48 hr. After the reaction was done, the product was extracted by diethyl ether (20 mL, 3 times). Column chromatography was used to purify the crude product which was obtained in 40% yield.

Method B. In dry round bottomed-flask under N_2 was introduced the ditosylated compound (7.3 mmol), DMF (25 mL) and NaN_3 (1.35 g, 21 mmol). The reaction mixture was stirred at 70 °C for 48 hr. After the reaction was done, the mixture was diluted with water and extracted with diethyl ether (3 times). The organic layer was dried and evaporated. The residue was purified by column chromatography (5% EtOAc in hexane) to give colorless liquid.

1-azido-2-(2-azidoethoxy)ethane (13a) (0.93 g, 82%); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.39 (t, *J* = 5.0 Hz, 4H), 3.66 (t, *J* = 5.2 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 50.7, 70.0; LRMS (ESI, positive), MS *m/z* 100.6 $[M\text{-}2N_2]^+$

1,2-bis(2-azidoethoxy)ethane (13b) (1.25 g, 86%); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.34 (t, *J* = 5.2 Hz, 4H), 3.64 (t, *J* = 5.0 Hz, 8H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 50.6, 70.0, 70.6; LRMS (ESI, positive), MS *m/z* 173.1$[M\text{-}N_2]^+$

2.7 Synthesis of amino azide of diethyleneglycol (11a) and triethyleneglycol (11b)



To a solution of diazide compound (4.5 mmol) in EtOAc (35 mL) and 1 M HCl aqueous solution (7.2 mL) was added PPh_3 (1.2 g, 4.6 mmol) at room temperature. After the reaction was stirred for 4 hr, additional 1 M HCl (3 mL) was

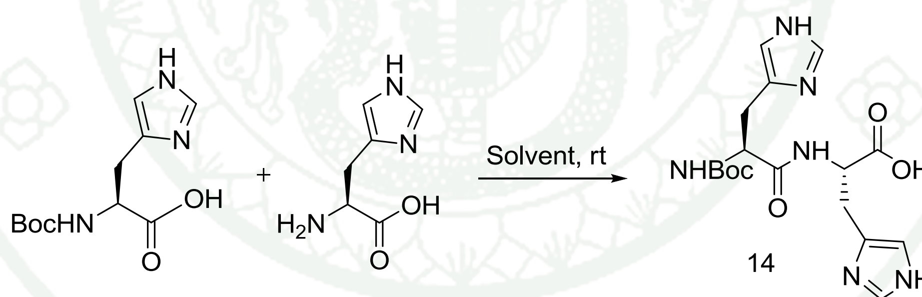
Copyright by Kasetsart University All rights reserved

added and the organic layer was extracted with water. The combined aqueous layer was washed with EtOAc and concentrated under reduced pressure. The residue was separated by reverse phase column chromatography.

2-(2-azidoethoxy)ethanamine (11a) (0.26 g, 45%); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 2.94 (dd, *J* = 11.2, 5.6 Hz, 2H), 3.45 (t, *J* = 5.1 Hz, 2H), 3.62 (t, *J* = 4.6 Hz, 2H), 3.66 (t, *J* = 5.4 Hz, 2H), 8.10 (br s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 49.8, 66.3, 66.8; LRMS (ESI, positive), MS *m/z* 131.0 $[M+H]^+$

2-(2-(2-azidoethoxy)ethoxy)ethanamine (11b) (0.3 g, 39%); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.25 (dd, *J* = 10.5, 5.4 Hz, 1H), 3.42 (t, *J* = 4.8 Hz, 2H), 3.6-3.8 (m, 6H), 3.79 (t, *J* = 5.0 Hz, 2H), 4.98 (s, 2H), 8.04 (br s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 39.6, 50.5, 66.6, 69.6, 70.0, 70.2; LRMS (ESI, positive), MS *m/z* 175.1 $[M+H]^+$

2.8 Synthesis of histidylhistidine (14).



Method A. A solution of Boc-His-OH (0.5 g, 2 mmol) and L-histidine (0.45 g, 3 mmol) in MeOH was stirred at room temperature for 10 min. 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) (0.65 g, 2.3 mmol) was added to the reaction mixture and stirred for 14 hr. Insoluble solid was filtered off and solvent was evaporated. The residue was purified by column chromatography (25-50% EtOAc in hexane

To generate DMTMM, *N*-methylmorpholine (NMM) (2.02 g, 20 mmol), was added to a solution of 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) (3.86 g, 22

Copyright by Kasetsart University All rights reserved

mmol) in THF (60 mL) at room temperature. A white solid appeared within several minutes. After stirring for 30 min at room temperature, the solid was collected by suction and washed with THF and dried to give DMTMM (5.52 g, 100%). Although the purity of DMTMM is good enough for condensation at this point, it can be recrystallized from methanol and ether. The product was obtained as a white solid.

Method B. Boc-His-OH (0.5 g, 2 mmol) and L-histidine (0.6 g, 4 mmol) were dissolved in DMF and stirred at room temperature for 10 min. DCC (0.6 g, 3 mmol), Et_3N (0.5 mL, 5 mmol) and DMAP (0.002 g, 0.02 mmol) were added to the reaction mixture and stirred for 24 hr. Insoluble solid was filtered off and solvent was evaporated. The residue was purified by column chromatography (25-50% EtOAc in hexane).

Method C. Boc-His-OH (0.5 g, 2 mmol) was dissolved in DMF. DCC (0.5 g, 2.4 mmol), PNP (0.3 g, 2.2 mmol), Et_3N (0.5 mL, 5 mmol) and DMAP (0.002 g, 0.02 mmol) were added to the reaction mixture and stirred for 4 hr. L-histidine (0.6 g, 4 mmol) was added to the reaction mixture and stirred for 20 hr. Insoluble solid was filtered off and solvent was evaporated. The residue was purified by column chromatography (25-50% EtOAc in hexane).

Method D. Boc-His-OH (0.5 g, 2 mmol) was dissolved in DMF. DCC (0.5 g, 2.4 mmol), HOBt (0.3 g, 2.2 mmol), Et_3N (0.5 mL, 5 mmol) and DMAP (0.002 g, 0.02 mmol) were added to the reaction mixture and stirred for 4 hr. L-histidine (0.6 g, 4 mmol) was added to the reaction mixture and stirred for 20 hr. Insoluble solid was filtered off and solvent was evaporated. The residue was purified by column chromatography (25-50% EtOAc in hexane).

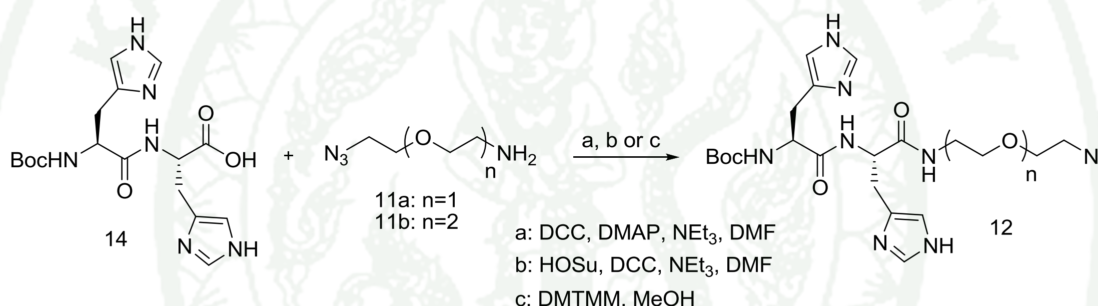
Method E. Boc-His-OH (0.5 g, 2 mmol) was dissolved in DMF. *N*-hydroxysuccinimide was added and stirred at room temperature. Solution of DCC (0.5 g, 2.4 mmol) in DMF was added to the reaction. Et_3N (0.5 mL, 5 mmol) and DMAP (0.002 g, 0.02 mmol) were added and the reaction mixture was stirred for 6 hr. L-histidine (0.6 g, 4 mmol) was added to the reaction mixture and stirred for 20 hr.

Copyright by Kasetsart University All rights reserved

Insoluble solid was filtered off and solvent was evaporated. The residue was purified by column chromatography (25-50% EtOAc in hexane).

The product was obtained as white solid (0.48 g, 61%); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.28 (s, 9H), 2.78 (s, 2H), 2.86 (s, 2H), 4.52 (d, J = 6.1 Hz, 1H), 5.90 (d, J = 4.9 Hz, 1H), 6.71 (s, 2H), 7.42 (s, 2H), 7.90 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 28.2, 38.6, 52.1, 53.4, 68.1, 79.8, 116.2, 128.7, 130.8, 132.3, 133.6, 135.2, 155.6, 167.7, 172.6; LRMS (ESI, positive), MS *m/z* 391.2 $[M+H]^+$

2.9 Coupling reaction of histidylhistidine and amino azide



Method A. Histidylhistidine (0.2 g, 0.5 mmol) and amino azide (0.08 g, 0.6 mmol) were dissolved in DMF (5 mL) and stirred at room temperature for 10 min. DCC (0.12 g, 0.6 mmol), Et_3N (0.2 mL, 2 mmol) and DMAP (0.002 g, 0.02 mmol) were added to the reaction mixture and stirred for 24 hr. Insoluble solid was filtered off and solvent was evaporated. The residue was purified by column chromatography (EtOAc in hexane).

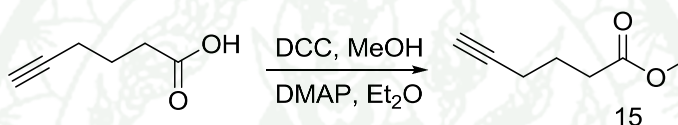
Method B. Histidylhistidine (0.1 g, 0.27 mmol) was dissolved in EtOAc (2 mL) and *N*-hydroxysuccinamide (0.04 g, 0.3 mmol) was added and stirred at room temperature. DCC (0.06 g, 0.3 mmol) in 1 mL of EtOAc was added to the reaction. Then, white precipitate was seen in the reaction. After the reaction was done, insoluble solid was filtered and the mixture was extracted with 5% $NaHCO_3$ until all excess NHS was washed to aqueous layer, H_2O and sat. NaCl. Organic layer was

Copyright by Kasetsart University All rights reserved

dried over Na_2SO_4 and evaporated. Crude product was separated by column chromatography.

Method C. A solution of histidylhistidine (0.2 g, 0.5 mmol) and amino azide (0.08 g, 0.6 mmol) in MeOH was stirred at room temperature for 10 min. DMTMM (0.17 g, 0.6 mmol) was added to the reaction mixture and stirred for 14 hr. Insoluble solid was filtered off and solvent was evaporated. The residue was purified by column chromatography.

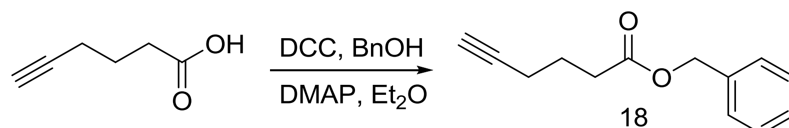
2.10 Synthesis of methyl hexylnoate (15)



To a solution of DCC (1.24 g, 6 mmol) and DMAP (0.06 g, 0.5 mmol,) in diethyl ether (10 mL) were added 5-hexylnoic acid (0.5 mL, 5 mmol) dropwise and the reaction was stirred for 5 min under nitrogen. Methanol (0.6 mL, 15 mmol) was then added dropwise to the reaction and stirred at room temperature under N_2. After 3 hr, the reaction was done (monitored by thin layer chromatography using 10% EtOAc in hexane). The reaction mixture was filtered and evaporated to give liquid crude product which was separated by column chromatography using 3-10% EtOAc in hexane. The product was obtained as colorless oil. (0.1 g, 16%); ^1H NMR (400 MHz, $CDCl_3$) δ: 1.84 (q, J = 7.0 Hz, 2H), 1.95 (t, J = 2.7 Hz, 1H), 2.25 (dt, J = 7.0, 2.7 Hz, 2H), 2.44 (t, J = 7.4 Hz, 2H), 3.67 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 18.1, 23.9, 32.9, 51.8, 69.4, 83.5, 173.8; LRMS (ESI, positive), MS *m/z* 149.1 $[M+Na]^+$

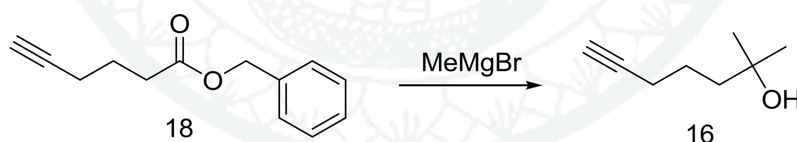
Copyright by Kasetsart University All rights reserved

2.11 Synthesis of benzyl hexynoate (18)



To a solution of DCC (1.24 g, 6 mmol) and DMAP (0.06 g, 0.5 mmol,) in diethyl ether (10 mL) were added 5-hexylnoic acid (0.5 mL, 5 mmol) dropwise and the reaction was stirred for 5 min under nitrogen. benzyl alcohol (1.6 mL, 15 mmol) was then added dropwise to the reaction and stirred at room temperature under N_2. Insoluble solid was filtered off and solvent was evaporated. The product was purified by column chromatography (3-10% EtOAc in hexane) to give colorless liquid (0.93 g, 92%); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.88 (m, 2H), 1.96 (t, J = 2.7 Hz, 1H), 2.27 (dt, J = 6.9, 2.6 Hz, 2H), 2.51 (t, J = 7.4 Hz, 2H), 5.13 (s, 2H), 7.36 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 17.8, 23.6, 32.9, 66.2, 69.1, 83.2, 128.2, 128.2, 128.5, 172.8; LRMS (ESI, positive), MS m/z 225.2 $[M+Na]^+$

2.12 Synthesis of 2-methylhept-6-yn-2-ol (16)



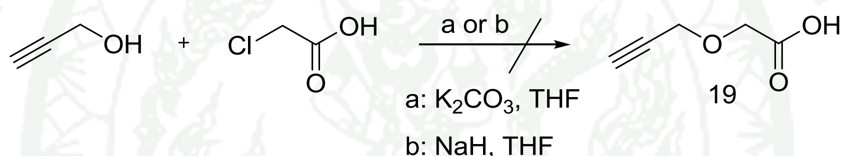
To a solution of methylmagnesium bromide (MeMgBr) was added a solution of benzyl hexynoate (7 g, 34 mmol) in THF dropwise at -78 °C. The mixture was kept at this temperature for 1 hr, then warm to room temperature slowly over 30 min. After the reaction was done, it was quenched with sat.NH_4Cl and then water. The aqueous layer was extracted with diethyl ether, dried and evaporated. The mixture was selective oxidized by stirring with excess MnO_2 (15 g) in CH_2Cl_2 for 12 hr. Crude product was separated by column chromatography (2-10% EtOAc in hexane) to give colorless liquid (3.3 g, 77%); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.21 (s, 6H), 1.57-1.60 (m, 4H), 1.95 (t, J = 2.6 Hz, 1H), 2.21 (dt, J = 6.7, 2.8 Hz, 2H); ^{13}C NMR (100 MHz,

Copyright by Kasetsart University All rights reserved

$CDCl_3$) δ: 19.1, 23.7, 29.5, 43.1, 68.8, 71.1, 84.7; LRMS (ESI, positive), MS *m/z* 109.2 [M-H_2O]

To generate MnO_2, a solution of $MnCl_2$ (200 g in H_2O 2 L) was stirred at 70 °C and a solution of $KMnO_4$ (160 g in H_2O 2 L) was stirred at 60 °C. The solution of $MnCl_2$ was added to the solution of $KMnO_4$ during 10 min. The suspension was stirred for 2 hr in hood to ensure with evolution of chloride. The reaction mixture was stirred at room temperature overnight. The precipitate was filtered off, washed, and dried at 120-130 °C for 18 hr.

2.13 Synthesis of 2-(prop-2-ynyloxy)acetic acid (19)

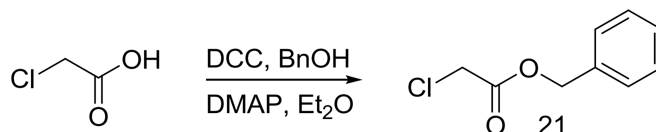


Method A. Propagyl alcohol (0.6 mL, 0.01 mol), Chloroacetic acid (1 g, 0.01 mol) and K_2CO_3 (3 g, 0.02 mol) were dissolved in THF (26 mL) and the reaction mixture was refluxed. After the reaction was stirred overnight, Insoluble solid was filtered off and solvent was evaporated. The reaction was monitored by thin layer chromatography using MeOH.

Method B. NaH (1.5 g, 0.06 mol) and propagyl alcohol (1.6 mL, 0.03 mol) were added dried THF (30 mL) and the reaction mixture was stirred at room temperature under N_2 for 15 min. Chloroacetic acid (1 g, 0.01 mol) was added to the reaction and the reaction was heated to 60 °C. After the reaction was stirred for 24 hr, it was cooled to room temperature. The reaction mixture was hydrolyzed with water (20 mL) and THF was removed by evaporation under reduced pressure. The aqueous layer was washed with diethyl ether (30 mL, 2 times), and acidified until pH 3–4 with concentrated HCl. The aqueous layer was extracted with diethyl ether and the combined organic extracts were washed with water, dried over $MgSO_4$, filtered, and concentrated under reduced pressure.

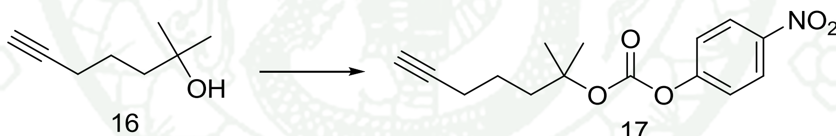
Copyright by Kasetsart University All rights reserved

2.14 Synthesis of benzyl 2-chloroacetate (21)



To a solution of chloroacetic acid (0.5 g, 5 mmol) and benzyl alcohol (1.6 mL, 15 mmol) in diethyl ether (25 mL) was added DCC (1.3 g, 6 mmol) and DMAP (0.06 g, 0.5 mmol). The reaction mixture was stirred at room temperature under N_2. Insoluble solid was filtered off and solvent was evaporated. The product was purified by column chromatography (3-10% EtOAc in hexane) to give colorless liquid (0.71 g, 76%); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.13 (s, 2H), 5.19 (s, 2H), 7.32-7.51 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 40.8, 67.8, 128.4, 128.6, 134.8, 167.1; LRMS (ESI, positive), MS *m/z* 183 [M-H]

2.15 Synthesis of 2-methylhept-6-yn-2-yl 4-nitrophenyl carbonate (17)



Method A. Alcohol (16) (5 mmol) was added to a solution of bis(p-nitrophenyl) carbonate (5 mmol) in CH_2Cl_2 (or THF) (25 mL), the mixture was stirred for 12 hr, and then the solvent was evaporated. The reaction was monitored by TLC (10% EtOAc in hexane).

Method B. To a solution of alcohol (16) (0.6 g, 5 mmol) in CH_2Cl_2 (or THF) (20 mL) was added 4-nitro-phenyl chloroformate (1.1 g, 51 mol) and pyridine (4.12 mL, 0.051 mol). After 24 hr stirring at ambient temperature, the reacting mixture was washed with saturated NaCl (2x100 mL), the organic layer dried with $MgSO_4$ and solvent removed under reduce pressure The reaction was monitored by TLC (10% EtOAc in hexane).

Copyright by Kasetsart University All rights reserved

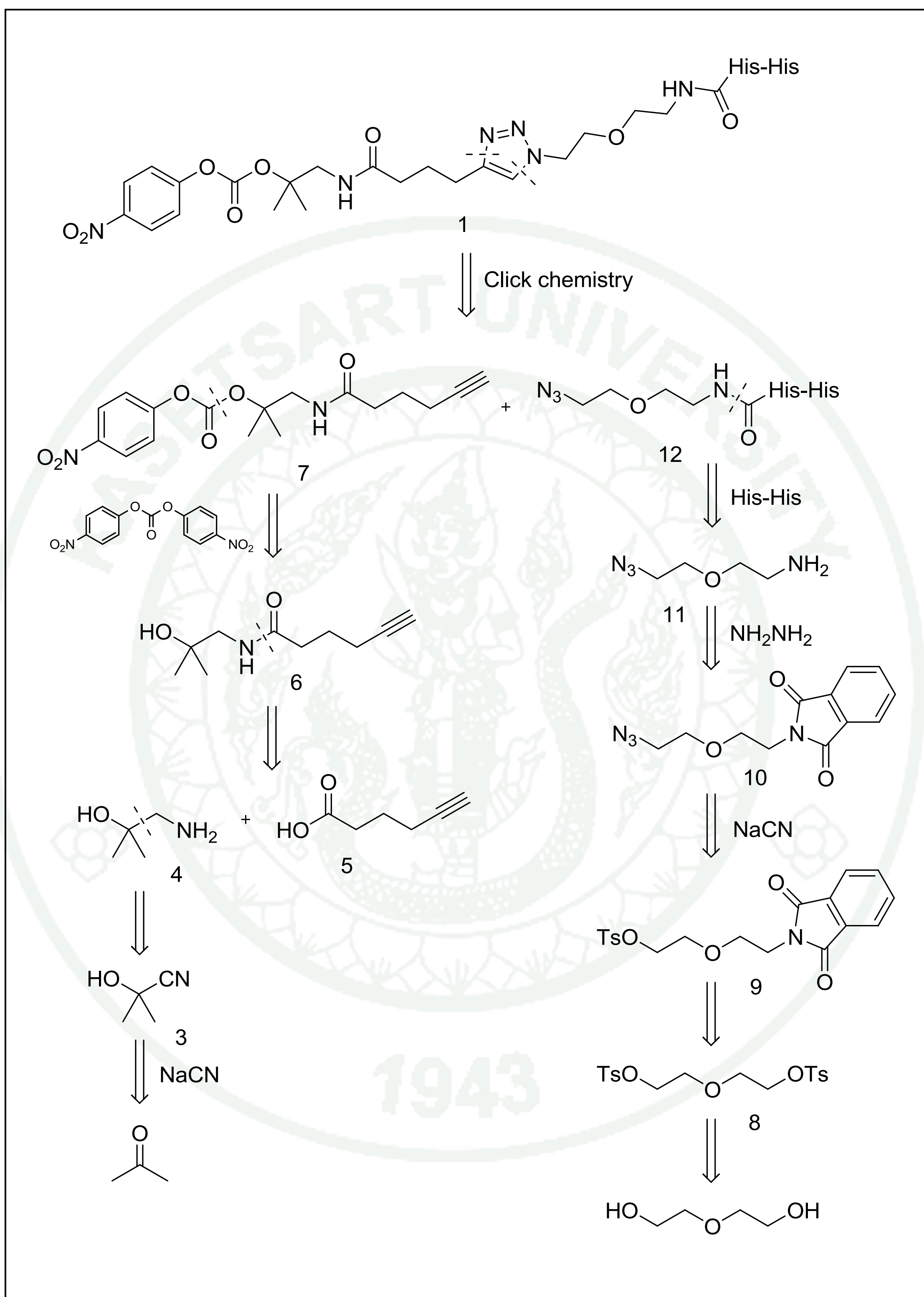
RESULTS AND DISCUSSIONS

In this study, acid labile reagent for the purification of specific tRNA was designed to consist of three main components; 1) triazole ring from click reaction of a terminal alkyne and a compound with terminal azido group, 2) histidylhistidine for binding with immobilized-metal affinity chromatography, 3) carbonate group for formation of carbamate functional group with aminoacyl-tRNA.

1. Synthesis of the first generation of target molecule (compound 1)

Compound (1) is the first generation of target molecule. The retrosynthetic analysis of compound (1) is shown in scheme 2. The triazole ring in compound (1) could be constituted from click reaction of terminal alkyne and terminal azido compound. The terminal alkyne (7) consisted of carbonate group could be established from reaction of alcohol (6) and bis-(4-nitrophenyl) carbonate. Alcohol (6) could be installed a terminal alkyne group from reaction of 5-hexynoic acid (5) and gem-dimethyl alcohol (4). An amino group of alcohol (4) was converted from nitrile group of acetone cyanohydrin (3) which will be protected at hydroxyl group before reduction step. The starting material of this synthesis could be acetone and it could be transformed to acetone cyanohydrin by reaction with sodium cyanide. For the azide moiety, an azido compound consisted of histidylhistidine (12) could be derived from amino azide compound (11). An amino group of compound (11) could be transformed from phthalimide group of azido compound (10). An azido group of compound (10) could be formed from substitution reaction of compound (9). Reaction of ditosylated diethyleneglycol (8) and potassium phthalimide could be provided compound (9). The first step of synthesis of terminal azide could be started from tosylation of diethylene glycol.

Copyright by Kasetsart University All rights reserved

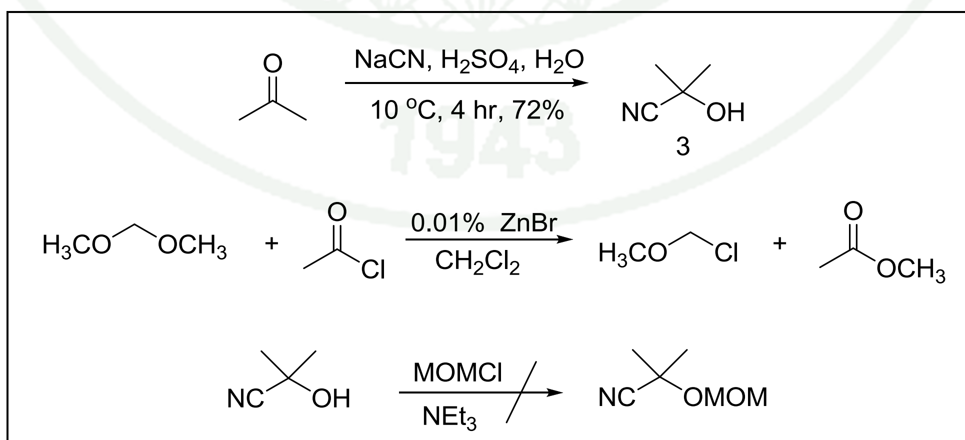


Scheme 2 Retrosynthetic analysis of compound 1

Copyright by Kasetsart University All rights reserved

1.1) Synthesis of an acid labile conjugate molecule with terminal alkyne

The synthesis was started from formation of acetone cyanohydrin (3) (scheme 3). Reaction of acetone and sodium cyanide in the presence of acid provided the desired product (3) in good yield. Then, acetone cyanohydrin was protected with chloromethyl methyl ether (MOMCl). Although this protecting reagent cannot be ordered and imported in Thailand, it can be generated in situ from reaction of dimethoxymethane and acetyl chloride in the presence of 0.01% anhydrous zinc bromide. In step of MOMCl preparation, TLC (3% MeOH in CH_2Cl_2) showed that new spot occurred. This new spot was believed to be MOMCl. Thus, the solution of acetone cyanohydrins and triethylamine in CH_2Cl_2 were then added to be protected with MOMCl. Unfortunately, although the reaction was stirred at room temperature over night or refluxed, the new product of this step monitored by TLC could not be observed. Change of leaving group to the better was chosen to solve this problem. Therefore, NaI was added to the solution of MOMCl for changing leaving group from Cl^- to I^-. However, the product was not observed. From this result, it can be concluded that the new spot on TLC appeared in MOMCl generating step may not be MOMCl. Moreover, the condition to generate MOMCl in high temperature was attempted and the product was purified by distillation. Nevertheless, the distillate product was obtained in small amount and it cannot protect acetone cyanohydrin.

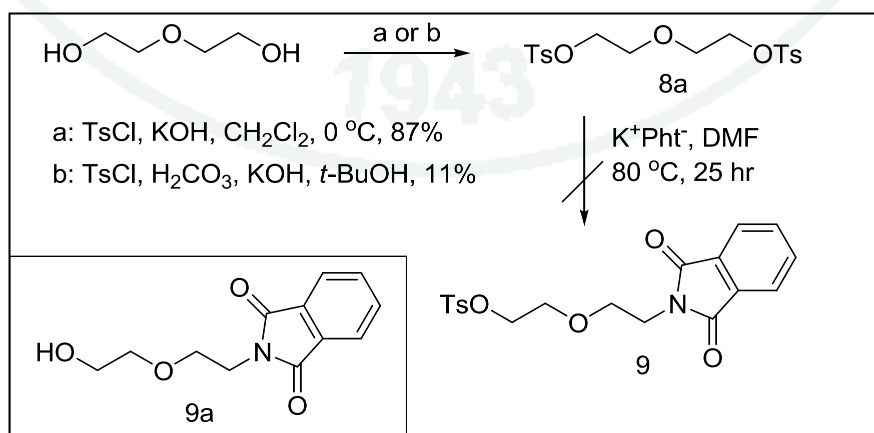


Scheme 3 Synthesis of acetone cyanohydrin and its protection

Copyright by Kasetsart University All rights reserved

1.2) Synthesis of bifunctional linker, amino-azido compounds

Because of our problematic synthetic route to terminal alkyne compound, the synthesis efforts were shifted to the bifunctional linkers, amino-azido compounds. Diethyleneglycol was used as a starting material. It was converted to ditosylated diethyleneglycol (8) (scheme 4). Two protocols were attempted in this step and gave ditosylated product in very different yield. For condition a, dichloromethane was used as solvent and potassium hydroxide played as a basic catalyst. This protocol provided the desired product in 87% yield. On the other hand, condition b was a solvent free-system, and potassium carbonate was used as base and the mixture was grinded in a mortar. The remaining tosylchloride was removed by addition of potassium hydroxide and accelerated by dropping of *t*-BuOH. Finally, the product was extracted by addtion of diethyl ether. This method provided the desired product in only 11% yield. Probable, the product was extracted by diethyl ether in small amoumt. Extraction in many times might improve quantity of product. However, condition a was more interested than condition b, so it was used for synthesis of other ditosylated compounds. Then, ditosylated diethyleneglycol (8a) was converted to compound (9) by displacement of tosyl group with phthalimide. The reaction was stirred over 24 hr and heated to 80 °C in DMF. Unfortunately, compound (9a) was only observed whereas the desired product (9) was not obtained. Removal of tosyl group to provide undesired compound (9a) was still not reasoned.

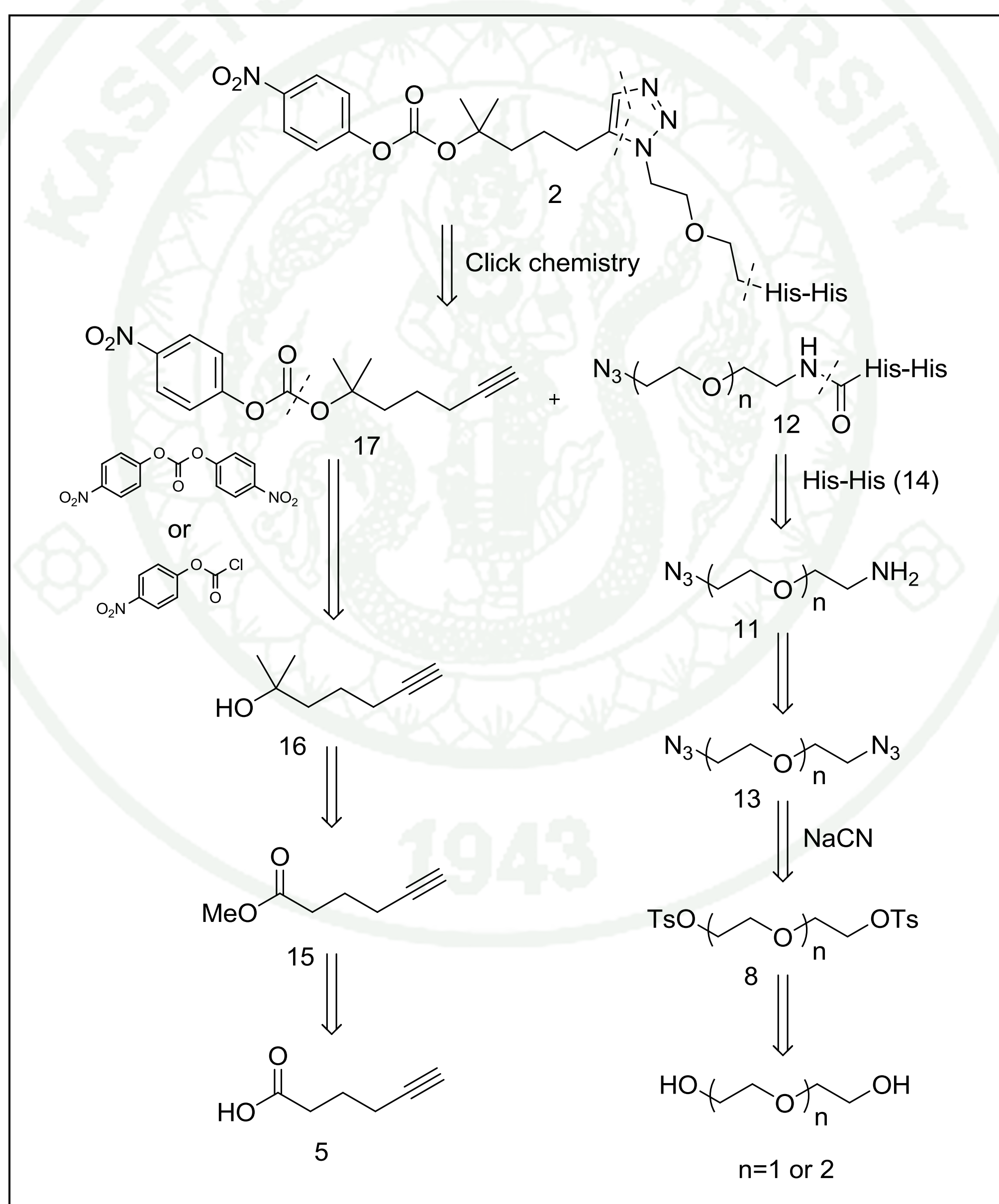


Scheme 4 Synthesis of tosylated compound

Copyright by Kasetsart University All rights reserved

2. Synthesis of the second generation of target molecule (compound 2)

Due to the complication and many problems in synthesis of compound (1), the acid labile reagent was adjusted to solve problematic synthetic route. Compound (2) is the second generation of target molecule. It was developed in order to solve problematic synthetic route for compound (1). The retrosynthetic analysis of compound (2) is shown in scheme 5.



Scheme 5 Retrosynthetic analysis of compound 2

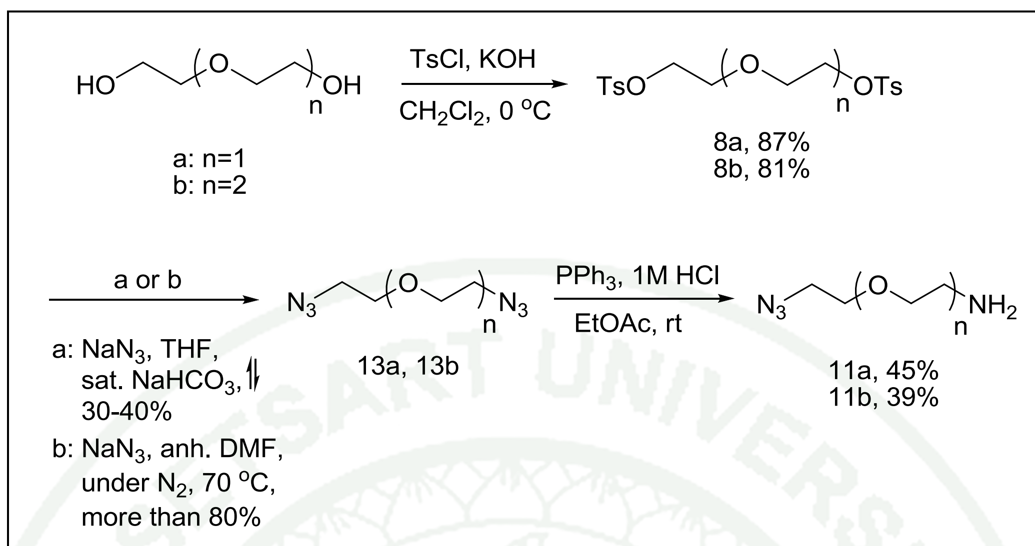
Copyright by Kasetsart University All rights reserved

To synthesize the azide moiety, histidylhistidine linker, compound (12), could be derived from amino azide compound (11). However, the synthesis of compound (11) was changed to consider partial reduction of diazido compound (13) by Staudinger reduction. Diazido compound (13) could be converted from ditosylated di- or triethyleneglycol which was transformed from di- or trietyleneglycol. Compound (16) was a new terminal alkyne improved from compound (7). It could be constructed from gem-dimethyl alcohol (15) reacted with and bis-(4-nitrophenyl) carbonate or 4-nitrophenyl chloroformate. Geminal-dimethyl alcohol (15) could be converted from Grignard reaction of methyl hexynoate (14). Ester (14) could be established from esterification of 5-hexynoic acid (5).

2.1) Synthesis of an acid labile conjugate molecule with amino azide

The di- and triethyleneglycol were used as starting materials (Scheme 6). Separately, these compounds were converted to the ditosylated alcohol 8a and 8b by the same protocol as mentioned above, followed by the S_N2 replacement with nucleophilic azide to give compound 13a and 13b. Two protocols of substitution reaction were attempted. In condition a, sodium azide was incompletely dissolved in THF. Therefore, saturated sodium hydrogen carbonate was dropped until sodium azide was completely dissolved. This condition provided the desired products in 30-40% yield. In the second, the reaction was run in DMF at 80 °C under N_2. The diazido products from this protocol were obtained over 80% yield. Thus, condition b was chosen to increase amount of product. Finally, the partial reduction of diazide compounds was accomplished under Staudinger reaction condition providing amino azide compound 11a and 11b. The residues were purified by reverse-phase column chromatography (MeOH-H_2O) and the product was obtained in moderated yield. From this step, amino azide compound was obtained and the last step of this part was installation of histidylhistidine into amino azide.

Copyright by Kasetsart University All rights reserved



Scheme 6 Synthesis of bifunctional linker, amino-azido compounds.

2.2) Synthesis of histidylhistidine

The peptide synthesis reported herein utilizes the traditional solution synthesis without the use of solid supports. The histidylhistidine was chosen to be our first candidate for polyhistidine moiety of our conjugate molecule due to the fact that the binding to the commercially available, Ni-NTA (Nickel-Nitriloacetic acid) resin only requires two histidine units. Therefore, the solution peptide synthesis for histidine coupling was investigated.

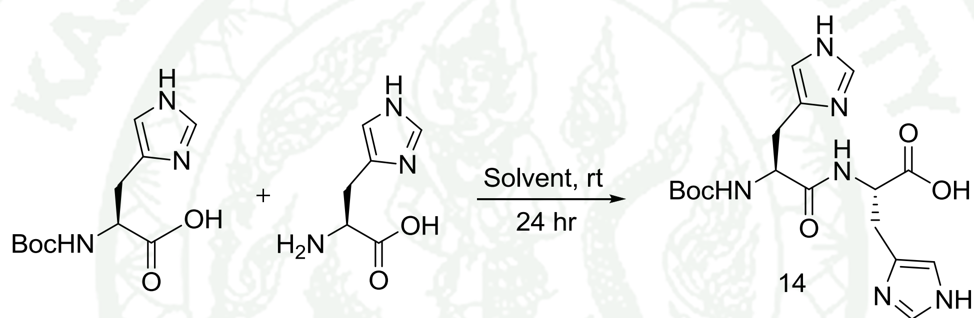
A general protocol with dicyclohexylcarbodiimide (DCC) and *N,N*-dimethylaminopyridine (DMAP) for the amide bond formation was used. The histidylhistidine product was obtained with an unpleasant yield (Table 1, entry 1 and 2). Therefore, we sought to employ a more effective activating reagent for our amino acid coupling reaction.

Formation of active ester by several reagents including *p*-nitrophenol (PNP), hydroxybenzotriazole (HOBt) and *N*-hydroxysuccinimide (HOSu), also provided the desired with an unpleasant yield. The use of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) as an activating agent in methanol

Copyright by Kasetsart University All rights reserved

provided the best result for histidine coupling (Table 1, entry 7). Gratefully, methanol could be used as solvent without ester bond formation. Although DMTMM was very expensive, fresh DMTMM could be generated from reaction of *N*-methylmorpholine (NMM) and 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT), which are more economical (Scheme 7). However, histidylhistine was obtained in low to moderate yield. A main factor causing unpleasant yield was the nucleophilicity of *N*-imidazole which competed α-NH_2 to attack an electrophile.

Table 1 Optimization of dihistidine coupling condition



Entry	Reagent	Solvent	% yield
1	DCC, DMAP, NEt_3	DCM	Trace
2	DCC, DMAP, NEt_3	DMF	35
3	PNP, DCC, NEt_3	DMF	32
4	HOBt, DCC, NEt_3	DMF	Trace
5	HOSu, DCC, NEt_3	EtOAc	No reaction
6	DMTMM	DMF	27
7	DMTMM	THF	No reaction
8	DMTMM	MeOH	61

Copyright by Kasetsart University All rights reserved

NMM + CDMT $\xrightarrow[100\%]{THF}$ DMTMM

Scheme 7 Generation of DMTMM

2.3) Coupling reaction of histidylhistidine and amino azide

Several methods were attempted including general protocol by DCC/DMAP, *N*-hydroxysuccinimide (HOSu) and DMTMM (Scheme 8). Unfortunately, the desired product was not observed. As well as the synthesis of histidylhistidine, a main factor causing unpleasant yield might be the competition of nucleophiles, α-NH_2 and *N*-imidazole. Moreover, steric factor of histidylhistidine was probably involved in unsuccessful synthesis.

14 + 11a: n=1, 11b: n=2 → 12

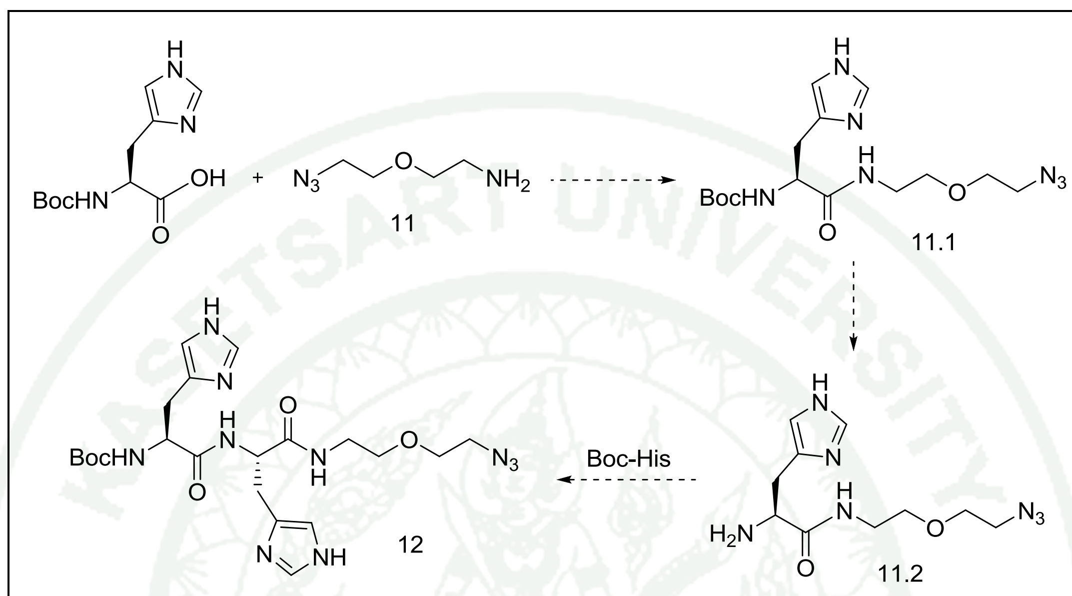
a: DCC, DMAP, NEt_3, DMF
b: HOSu, DCC, NEt_3, DMF
c: DMTMM, MeOH

Scheme 8 Coupling reaction of histidylhistidine and amino azide

In order to solve the unsuccessful route, sequence of the synthesis will be rearranged (Scheme 9). First, Boc-histidine will be coupled with aminoazide (11). Then, coupled product (11.1) will be treated with acid to remove Boc group and give

Copyright by Kasetsart University All rights reserved

compound (11.2). Finally, amine (11.2) will be coupled with Boc-histidine again to provide the product (12).



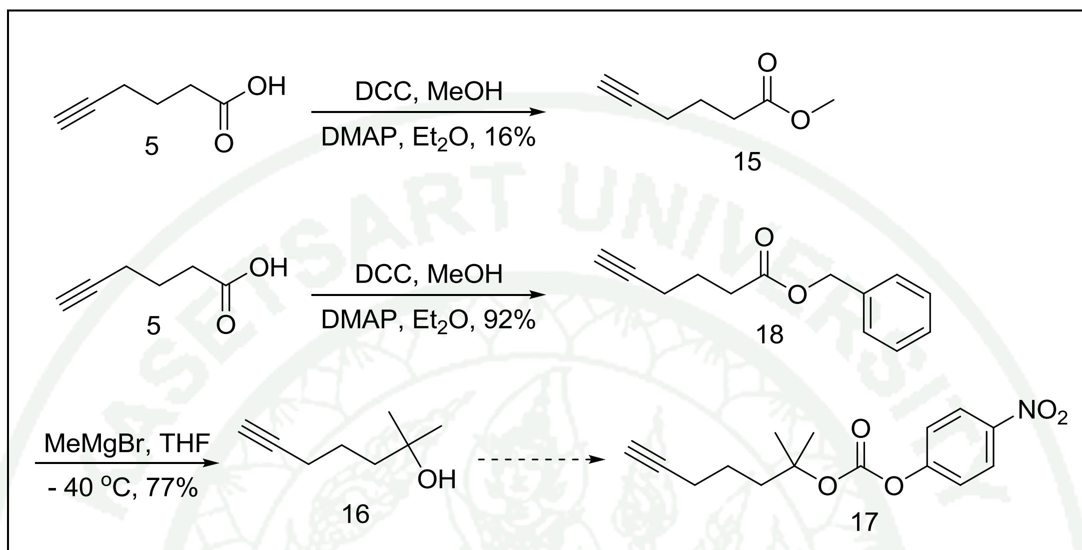
Scheme 9 New plan for synthesis of a compound with terminal azido group

2.4) Synthesis of an acid labile conjugate molecule with terminal alkyne

Esterification of 5-hexynoic acid (5) with methanol was the first step in order to obtain hexynoate ester. It was accomplished by using the traditional DCC/DMAP condition in diethyl ether to provide methyl hexanoate (15) (scheme 10). Unfortunately, methyl hexynoate was very volatile when it was evaporated to remove solvent. Thus, the product was obtained in low yield. Benzyl hexynoate was chosen instead of methyl hexynoate to improve the yield. Reaction of hexynoic acid with benzyl alcohol by the same protocol gave the desired product (18) over 80% yield. Therefore, benzyl hexynoate was more suitable for this step. The alcohol (16) was easily obtained by nucleophilic addition from methyl Grignard reagent to benzyl hexanoate. However, the desired geminal-dimethyl alcohol product appeared as an inseparable mixture with the by-product benzyl alcohol after the completion of this reaction. We decided to oxidize the by-product benzyl alcohol to benzaldehyde using MnO_2 in order to facilitate the chromatographic separation. Gratefully, the desired

Copyright by Kasetsart University All rights reserved

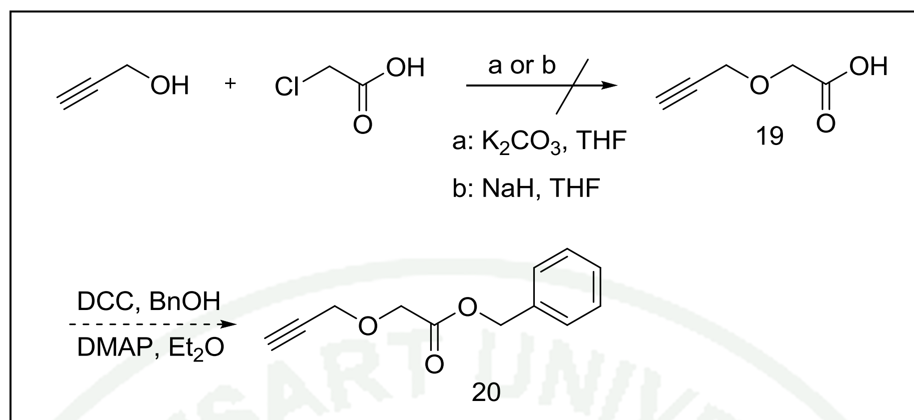
alcohol could be separated from the oxidized by-product by simple column chromatography.



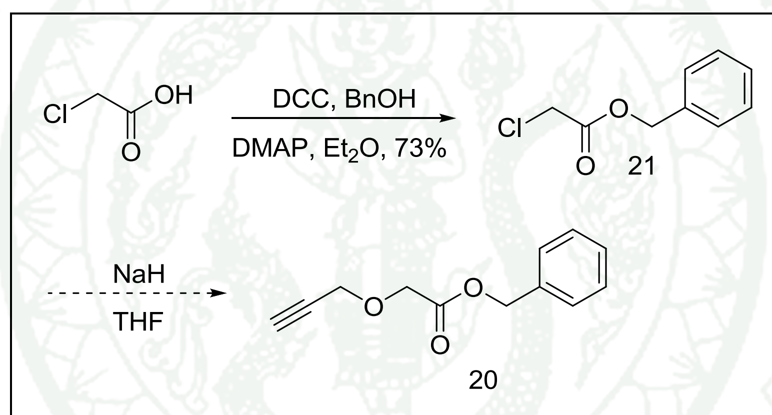
Scheme 10 Synthesis of an acid labile conjugate molecule with terminal alkyne

Before the oxidation of by-product was utilized in order to solve the separation problem, we tried to synthesize a new ester for this step. Benzyl 2-(prop-2-ynyloxy)acetate (20) was chosen to be our target. First, the terminal alkyne was installed from the reaction between propagyl alcohol and chloroacetic acid (scheme 11). Unfortunately, the desired product was not observed despite many attempts with various conditions including: 1) NaH, THF, refluxed, 2) NaH in toluene, reflux, 3) NaH, KI in THF, reflux and 4) K_2CO_3 in THF. Possibly, the problem was extraction because the product was very polar molecule. However, the product was not observed in organic phase although the reaction mixture was acidified until pH 3. Then, sequence of this synthesis was designed to rearrange. It was moved to construct benzyl ester moiety first. Chloroacetic acid was activated by DCC and formed the product (21) in good yield (scheme 12). Nevertheless, installation of terminal alkyne was still unsuccessful.

Copyright by Kasetsart University All rights reserved



Scheme 11 Synthesis of new benzyl ester (route 1)



Scheme 12 Synthesis of new benzyl ester (route 2)

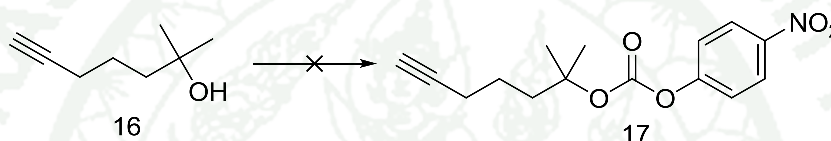
2.5) Synthesis of carbonate compound

The ultimate goal for synthesis of an acid labile conjugate molecule with terminal alkyne is to install the active carbonate moiety to our previously synthesized geminal-dimethyl alkynol (16). The reagent, bis-*p*-nitrophenyl carbonate was utilized (Table 2, Entry 1-4). Unfortunately, the carbonate formation appeared to be unsuccessful. Possibly, steric factor of this tertiary alcohol effected to decrease nucleophilicity, so bis-*p*-nitrophenyl carbonate cannot react with the alcohol. Consequently, the more reactive reagent, *p*-nitrophenyl chloroformate was attempted. Nonetheless, *p*-nitrophenyl chloroformate received from supplier appeared to be

Copyright by Kasetsart University All rights reserved

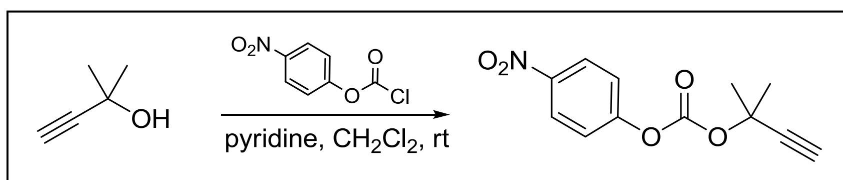
liquid while it was reported in MSDS (Material safety data sheet) to be solid at room temperature. Unluckily, NMR data or *m/z* was not enough to confirm reagent. However, this reagent was tried and it also cannot form the carbonate product (Table 2, Entry 5-8). In order to confirm that the reagent was impractical, the protocol reported by Bertrand and coworker which was used as reference will be repeated (Scheme 13) (Bertrand and Gesson, 2007). Moreover, the use of pyridine as solvent, which is stronger condition, is interesting to attempt.

Table 2 Protocol for synthesis of carbonate compound



Entry	Reagent	Temp.	Solvent	% yield
1	bis-*p*-nitrophenyl carbonate, Pyridine	rt	DCM	no reaction
2	bis-*p*-nitrophenyl carbonate, Pyridine	reflux	DCM	no reaction
3	bis-*p*-nitrophenyl carbonate, Pyridine	rt	THF	no reaction
4	bis-*p*-nitrophenyl carbonate, Pyridine	reflux	THF	no reaction
5	*p*-nitrophenyl chloroformate, Pyridine	rt	DCM	no reaction
6	*p*-nitrophenyl chloroformate, Pyridine	reflux	DCM	no reaction
7	*p*-nitrophenyl chloroformate, Pyridine	rt	THF	no reaction
8	*p*-nitrophenyl chloroformate, Pyridine	reflux	THF	no reaction

Copyright by Kasetsart University All rights reserved



Scheme 13 The carbonated formation of 2-methylbut-3-yn-2-ol by Bertrand and coworker

KASETSART UNIVERSITY
1943
Copyright by Kasetsart University All rights reserved

CONCLUSION

The new system for tRNA purification has been developed utilizing the azide-alkyne 1,3-dipolar cycloaddition reaction as the key method to connect the modified aminoacyl-tRNA with the Ni-NTA solid support. The conjugated molecules including amino azide compound and geminal-dimethyl alkynol were successfully synthesized. These molecules are key intermediates for the construction of our acid-labile bioconjugate system. The alternative activating groups for the construction of activated carbonate moiety of the terminal alkyne bioconjugate molecule are needed, and the selections as well as the utilization of new activating groups are being explored. Once completed, the system will be tested with aminoacyl-tRNAs for specific tRNA purification.

Copyright by Kasetsart University All rights reserved

LITERATURE CITED

Aponick, A., C. Li and J. A. Palmes. 2009. Au-catalyzed cyclization of monopropargylictriols: an expedient synthesis of monounsaturated spiroketals. **Org. Lett.** 11: 121-124.

Berg, P. and E. J. Ofengand. 1958. An enzymatic mechanism for linking amino acids to RNA. **Proc. Natl. Acad. Sci. USA.** 44: 78–86.

Berliner, M and K. Belecki. 2005. Simple, rapid procedure for the synthesis of chloromethyl methyl ether and other chloro alkyl ethers. **J. Org. Chem.** 70: 9618-9621.

Berliner, M and K. Belecki. 2007. Synthesis of alpha-halo ethers from symmetric acetals and *in situ* methoxymethylation of an alcohol. **Organic Syntheses** 84: 102.

Bertrand, P. and J. P. Gesson. 2007. Click chemistry with *o*-dimethylpropargylcarbamate for preparation of pH-sensitive functional groups. A case study. **J. Org. Chem.** 72: 3596-3599.

Bonger, K. M.,R. J.B.H.N. van den Berg, L. H. Heitman, A. P. IJzerman, J. Oosterom, C. M. Timmers, H. S. Overkleeft and G. A. van der Marel. 2007. Synthesis and evaluation of homo-bivalent GnRHR ligands. **Bioorg. Med. Chem.** 15: 4841–4856.

Bouzide, A. and G. Sauvé. 2002. Silver (I) oxide mediated highly selective monotosylation of symmetrical diols. application to the synthesis of polysubstituted cyclic ethers. **Org. Lett.** 4: 2329-2332.

Copyright by Kasetsart University All rights reserved

Canellakis, E. S. 1957. On the mechanism of incorporation of adenylic acid form adenosine triphosphate into ribonucleic acid by soluble mammalian enzyme systems. **Biochim. Biophys. Acta.** 25: 217–218.

Craig, L. C. 1952. Countercurrent distribution. **Methods Med. Res.** 5: 3–24.

Crick, F. H. C. 1966. Codon-anticodon pairing: The wobble hypothesis. **J. Mol. Biol.** 19: 548–555.

Crick, F. H. C. 1968. The origin of the genetic code. **J. Mol. Biol.** 38: 367-379.

Cossy, J., C. Taillier and V. Bellosta. 2002. Synthesis of 3-oxo oxacycloalkenes by ring closing metathesis. **Tetrahedron. Lett.** 43: 7263–7266.

Cox, R. F. B. and R. T. Stormont. 1943. Acetone cyanohydrin. **Organic Syntheses** 2: 7.

Dahiya R., A. Kumar and R. Yadav. 2008a. Synthesis and biological activity of peptide derivatives of iodoquinazolinones/nitroimidazoles. **Molecules** 13: 958-976.

Dahiya R. and A. Kumar. 2008b. Synthetic and biological studies on a cyclopolypeptide of plant origin. **J. Zhejiang. Univ. Sci. B**. 9:391-400.

Dahiya R. 2008c. Synthesis and biological activity of a cyclic hexapeptide from *Dianthussuperbus*. **Chemical Papers** 62: 527–535.

Dahiya R. 2008d. Synthesis and *in vitro* cytotoxic activity of a natural peptide of plant origin. **J. Iran. Chem. Soc.** 5: 445-452.

Copyright by Kasetsart University All rights reserved

Denmark, S. E. and W. Chung. 2008. Lewis Base Activation of Lewis Acids: Catalytic, Enantioselective Addition of Glycolate-Derived Silyl Ketene Acetals to Aldehydes. **J. Org. Chem.** 73: 4582–4595.

Dirks, A. J., J. J. L. M. Cornelissen and R. J. M. Nolte. 2009. Monitoring protein-polymer conjugation by a fluorogenic Cu(I)-catalyzed azide-alkyne 1,3-dipolar cycloaddition. **Bioconjugate Chem.** 20: 1129-1138.

Gillam, I., S. Millward, D. Blew, M. von Tigerstrom, E. Wimmer and G. M. Tener. 1967. The separation of soluble ribonucleic acids on benzoylated diethylaminoethylcellulose. **Biochemistry** 6: 3043–3056.

Harkat, H., A. Y.Dembelé, J. Weibel, A. Blanc and P. Pale. 2009. Cyclization of alkynoic acids with gold catalysts: a surprising dichotomy between Au^{I} and Au^{III}. **Tetrahedron** 65: 1871–1879.

Hecht, L. I., M. L. Stephenson and P. C. Zamecnik. 1958b. Dependence of amino acid binding to soluble ribonucleic acid on cytidine triphosphate. **Biochim. Biophys. Acta.** 29: 460–461.

Hecht, L. I., P. C. Zamecnik, M. L. Stephenson and J. F. Scott. 1958a. Nucleoside tri-phosphates as precursors of ribonucleic acid end groups in a mammalian system. **J. Biol. Chem.** 233: 954–963.

Heidelberger, C., E. Harbers, K. C. Leibman, Y. Takagi and V. R. Potter. 1956. Specific incorporation of adenosine-5'phosphate-^{32}P into ribonucleic acid in rat liver homogenates. **Biochim. Biophys. Acta.** 20: 445–446.

Hoagland, M. 1996. Biochemistry or molecular biology? The discovery of 'soluble RNA'. **Trends Biochem. Sci.** 21: 77–80.

Copyright by Kasetsart University All rights reserved

Hoagland, M. B., E. B. Keller and P. C. Zamecnik. 1956. Enzymatic carboxyl activation of amino acids. **J. Biol. Chem.** 218: 345–358.

Hoagland, M. B., M. L. Stephenson, J. F. Scott, L. I. Hecht and P. C. Zamecnik. 1958. A soluble ribonucleic acid intermediate in protein synthesis. **J. Biol. Chem.** 231: 241–257.

Hoagland, M. B., P. C. Zamecnik and M. L. Stephenson. 1957. Intermediate reactions in protein biosynthesis. **Biochim.Biophys. Acta.** 24: 215–216.

Holley, R. W. 1957. An alanine-dependent, ribonuclease-inhibited conversion of AMP to ATP and its possible relationship to protein synthesis. **J. Am. Chem. Soc.** 79: 658–661.

Holley, R. W., J. Apgar, G. A. Everett, J. T. Madison, M. Marquisee, S. H. Merrill, J. R. Penswick and A. Zamir. 1965. Structure of a ribonucleic acid. **Science** 147: 1462–1465.

Holmes, W. M., R. E. Hurd, B. R. Reid, R. A. Rimerman and G. W. Hatfield. 1975. Separation of transfer ribonucleic acid by sepharose chromatography using reverse salt gradients. **Proc. Natl. Acad. Sci. USA.** 72: 1068–1071.

Kazemi, F., A. R. Massah and M. Javaherian. 2007. Chemoselective and scalable preparation of alkyl tosylates under solvent-free conditions. **Tetrahedron** 63: 5083-5087.

Kim, S. H., F. L. Suddath, G. J. Quigley, A. McPherson, J. L. Sussman, A. H. Wang, N. C. Seeman and A. Rich. 1974. Three-dimensional tertiary structure of yeast phenylalanine transfer RNA. **Science** 185: 435–440.

Copyright by Kasetsart University All rights reserved

Klyde, B. J. and M. R. Bernfleld. 1973. Purification of chicken liver seryl transfer ribonucleic acid by complex formation with elongation factor EF-Tu:GTP. General micromethod of aminoacyl transfer ribonucleic acid purification. **Biochemistry** 12: 3752-3757.

Kunishima, M., C. Kawachi, J. Morita, K. Terao, F. Iwasaki, and S. Tani. 1999. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride: An efficient condensing agent leading to the formation of amides and esters. **Tetrahedron** 55: 13159-13170.

Letsinger, R. L., and K. K. Ogilvie. Use of p-nitrophenyl chloroformate in blocking hydroxyl groups in nucleosides. **J. Org. Chem.** 1967. 32: 296-300**.**

Li, W., G. S. Wayne, J. E. Lallaman, S. Chang and S. J. Wittenberger. 2006. An Improved Synthesis of Pyran-3,5-dione: Application to the Synthesis of ABT-598, a Potassium Channel Opener, via Hantzsch Reaction. **J. Org. Chem.** 71: 1725-1727.

Lipmann, F., W. C. Hülsmann, G. Hartmann, H. G. Boman and G. Acs. 1959. Amino acid activation and protein synthesis. **J. Cell Comp.Physiol.** 54: 75–88.

Louie, A., E. Masuda, M. Yoder and F. Jurnak. 1984. Affinity purification of aminoacyl-tRNA. **Anal. Biochem.** 141: 402-408.

Madison, J. T., G. A. Everett and H. Kung. 1966. Nucleotide sequence of a yeast tyrosine transfer RNA. **Science** 153: 531–534.

Martin, E. H. and J. Brittain. 2002. A convenient laboratory preparation of aromatic polycarbonate. **Polymer Bulletin** 47: 517-520.

Copyright by Kasetsart University All rights reserved

Morieux, P., J. P. Stables and H. Kohn. 2008. Synthesis and anticonvulsant activities of N-benzyl (2R)-2-acetamido-3-oxysubstituted propionamide derivatives. **Bioorg. Med. Chem.** 16: 8968–8975.

Nashed, Y.E. and A.K. Mitra. 2003. Synthesis and characterization of novel dipeptide ester prodrugs of acyclovir. **Spectrochimica Acta Part A.** 59: 2033-2039.

Nishimura, S., F. Harada, U. Narushima and T. Seno. 1967. Purification of methionine-, valine-, phenylalanine- and tyrosine-specific tRNA from *Escherichia coli*. **Biochim. Biophys. Acta.** 142: 133–148.

Numata, M., K. Koumoto, M. MiZu, K. Sakurai and S. Shinkai. 2005. Parallel *vs.* anti-parallel orientation in a curdlan/oligo(dA) complex as estimated by a FRET technique. **Org. Biomol. Chem.** 3: 2255-2261.

Ogata, K. and H. Nohara. 1957. The possible role of the ribonucleic acid (RNA) of the pH 5 enzyme in amino acid activation. **Biochim. Biophys. Acta.** 25: 659–660.

Pearson, R. L., J. F. Weiss and A. D. Kelmers. 1971. Improved separation of transfer RNA's on polychlorotrifuoroethylene-supported reversed-phase chromatography columns. **Biochim. Biophys. Acta.** 228: 770–774.

Prasad, J. V. N. V., F. E. Boyer, L. Chupak, M. Dermyer, Q. Ding, K. Gavardinas, S. E. Hagen, M. D. Huband, W. Jiao, T. Kaneko, S. N. Maiti, M. Melnick, K. Romero, M. Patterson and X. Wu. 2006. Synthesis and structure–activity studies of novel benzocycloheptanoneoxazolidinone antibacterial agents. **Bioorg. Med. Chem. Lett.** 16: 5392–5397.

Punna, s., E. Kaltgrad and M. G. Finn. 2005. "Clickable" Agarose for Affinity Chromatography. **Bioconjugate Chem.** 16: 1536-1541.

Copyright by Kasetsart University All rights reserved

Pütz, J., J. Wientges, M. Sissler, R. Giege, C. Florentz and A. Schwienhorst. 1997. Rapid selection of aminoacyl-tRNAs based on biotinylation of α-NH_2 group of charged amino acids. **Nucleic acids Res.** 25: 1862-1863.

RajBhandary, U. L., A. Stuart, R. D. Faulkner, S. H. Chang and H. G. Khorana. 1966. Nucleotide sequence studies on yeast phenylalanine sRNA. **Cold Spring Harb. Symp. Quant. Biol.** 31: 425–434.

Rajbhandary, U. L. and C. Köhrer. Early days of tRNA research: Discovery, function, purification and sequence analysis. 2006. **J. Biosci.** 31: 439–451.

RajBhandary, U. L., S. H. Chang, A. Stuart, R. D. Faulkner, R. M. Hoskinson and H. G. Khorana. 1967. Studies on polynucleotides. LXVIII. The primary structure of yeast phenylalanine transfer RNA. **Proc. Natl. Acad. Sci. USA.** 57: 751–758.

Ribeiro, S., S. Nock and M. Sprinzl. 1995. Purification of aminoacyl-TRNA by affinity chromatography on immobilized *Thermus thermophilus* EF-Tu·GTP. **Anal. Biochem.** 228: 330-335.

Robertus, J. D., J. E. Ladner, J. T. Finch, D. Rhodes, R. S. Brown, B. F. Clark and A. Klug. 1974. Structure of yeast phenylalanine tRNA at 3 Å resolution. **Nature (London)** 250: 546–551.

Schwabacher, A. W., J. W. Lane, M. W. Schiesher, K. M. Leigh and C. W. Johnson. 1998. Desymmetrization reactions: Efficient preparation of unsymmetrically substituted linker molecules. **J. Org. Chem.** 63: 1727-1729.

Spangenberg, T., B. Breit, A. Mann. 2009. Hydroformylation of Homoallylic Azides: A Rapid Approach toward Alkaloids. **Org. Lett.** 11: 261-264.

Copyright by Kasetsart University All rights reserved

Söll, D. and U. L. RajBhandary. 1967. Studies on polynucleotides. LXXVI. Specificity of transfer RNA for codon recognition as studied by amino acid incorporation. **J. Mol. Biol.** 29: 113–124.

Suk. H. K., M. J. Byung, J. K. Woo and Y. C. Ji. 2008. Embedding nanofibers in a polymer matrix by polymerization of organogels comprising heterobifunctional organogelators and monomeric solvent. **Chem. Mater.** 20: 5532-5540.

Takeuchi, T., N. Takahashi, K. Ishi, T. Kusayanagi, K. Kuramochi and F. Sugawara. 2009. Antitumor antibiotic fostriecin covalently binds to cysteine-269 residue of protein phosphatase 2A catalytic subunit in mammalian cells. **Bioorg. Med. Chem.** 17: 8113-8122.

Wissner, A., C. A. Kohler and B. M. Goldstein. 1985. Analogues of Platelet Activating Factor. 3.' Replacement of the Phosphate Moiety with a Sulfonylbismethylene Group. **J. Med. Chem.** 28: 1365-1367.

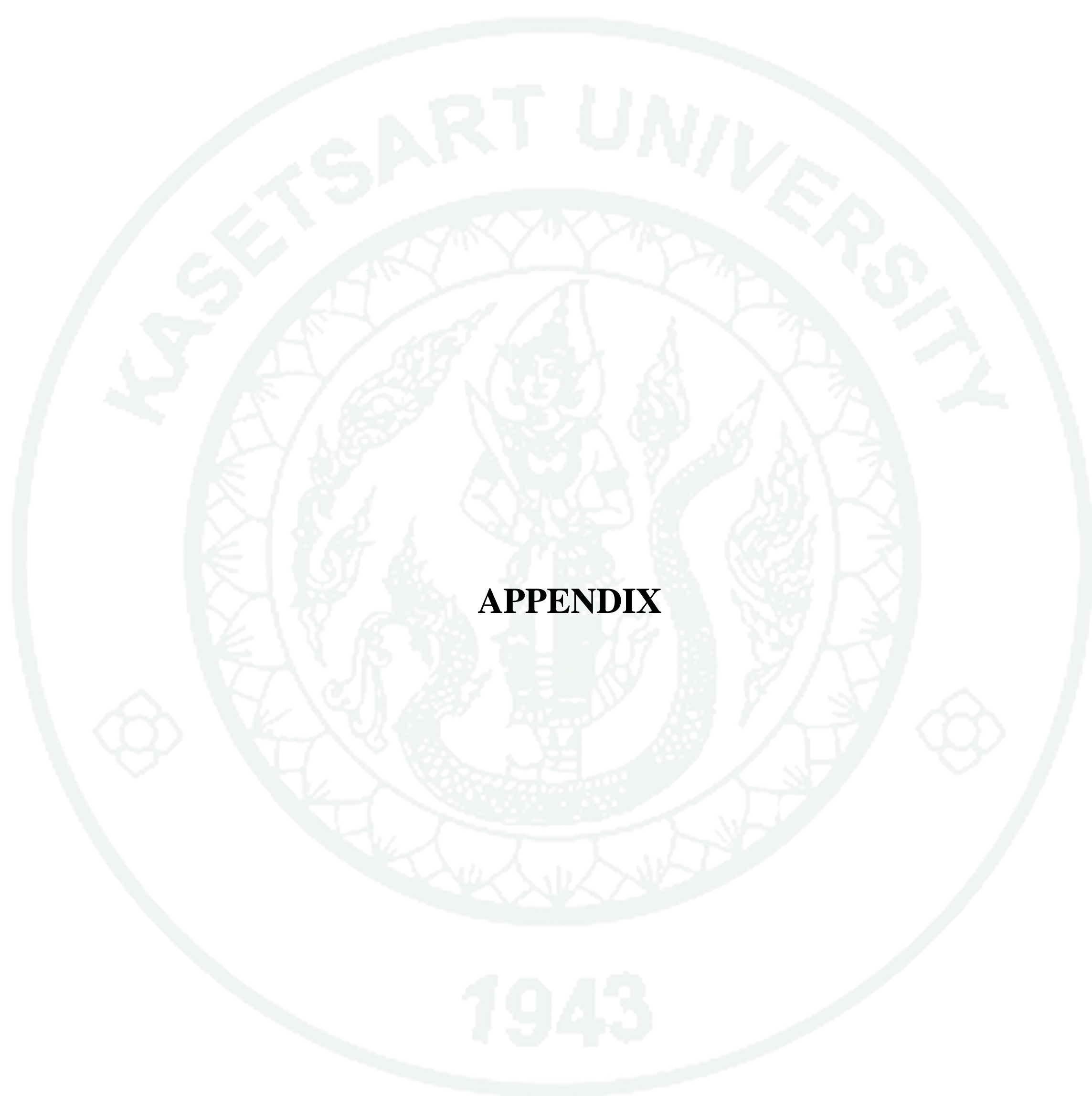
Xie, H., O. Braha, S. Cheley and H. Bayley. 2005. Single-molecule observation of the catalytic subunit of cAMP-dependent protein kinase binding to an inhibitor peptide. **Chemistry & Biology** 12: 109-120.

Zachau, H. G., D. Dütting and H. Feldmann. 1966. The structures of two serine transfer ribonucleic acids. **Hoppe Seylers Z. Physiol. Chem.** 347: 212–235.

Zachau, H. G., G. Acs and F. Lipmann. 1958. Isolation of adenosine amino acid esters from a ribonuclease digest of soluble, liver ribonucleic acid. **Proc. Natl. Acad. Sci. USA.** 44: 885–889.

Zamecnik, P. C. 2005. From protein synthesis to genetic insertion. **Annu. Rev. Biochem.** 74: 1–28.

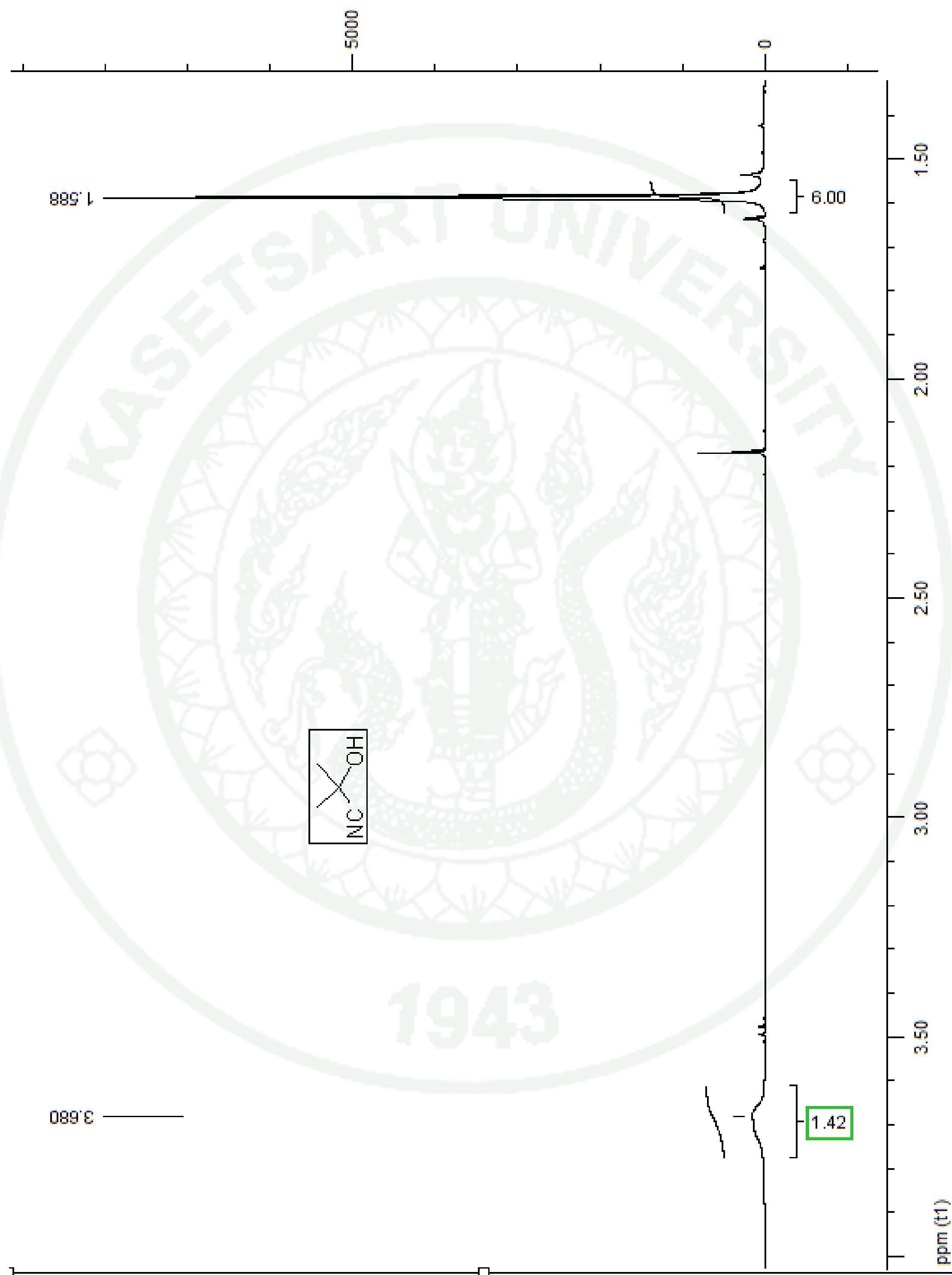
Copyright by Kasetsart University All rights reserved



APPENDIX

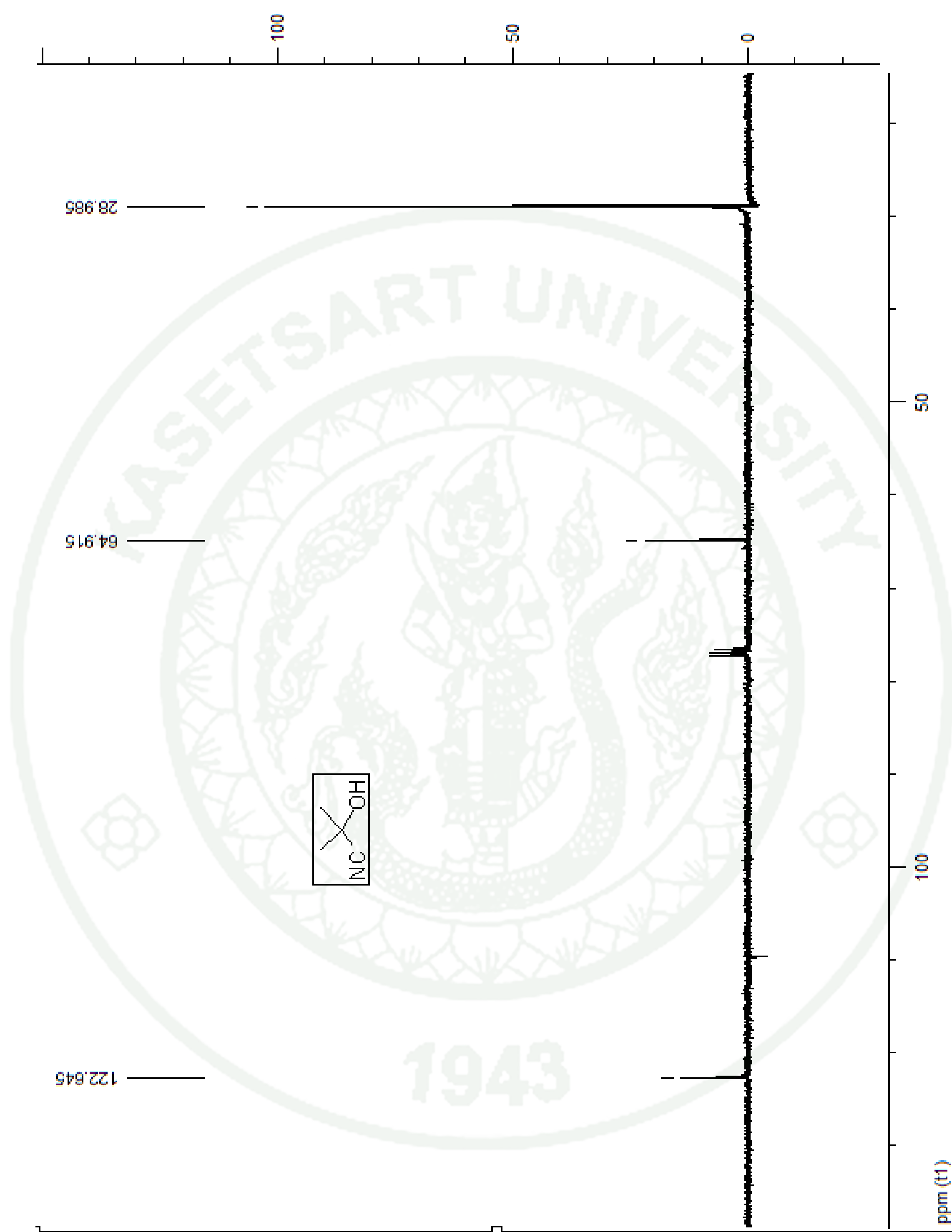
Copyright by Kasetsart University All rights reserved

NMR data



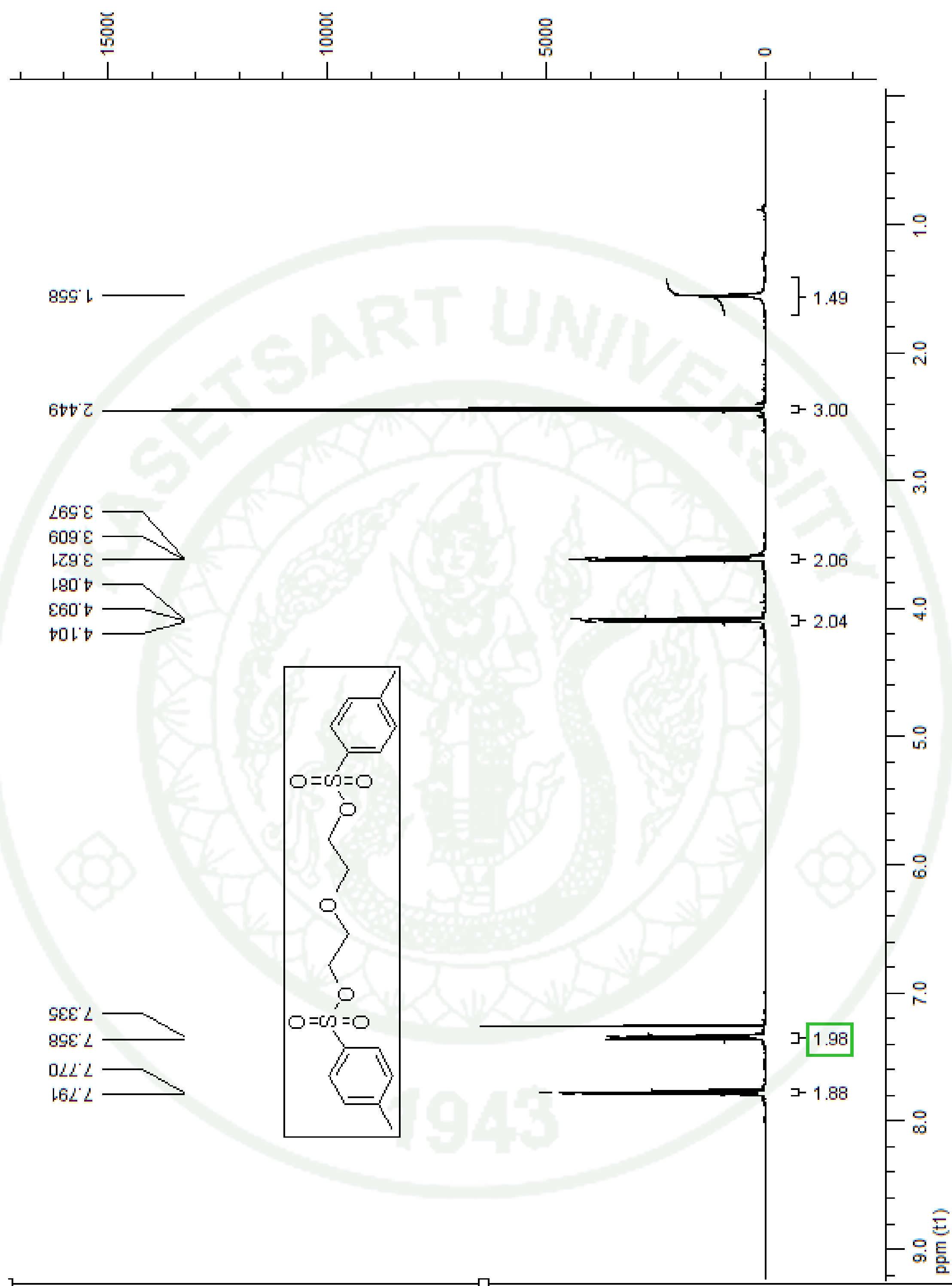
Appendix Figure 1 ¹H NMR of acetone cyanohydrin (3)

Copyright by Kasetsart University All rights reserved



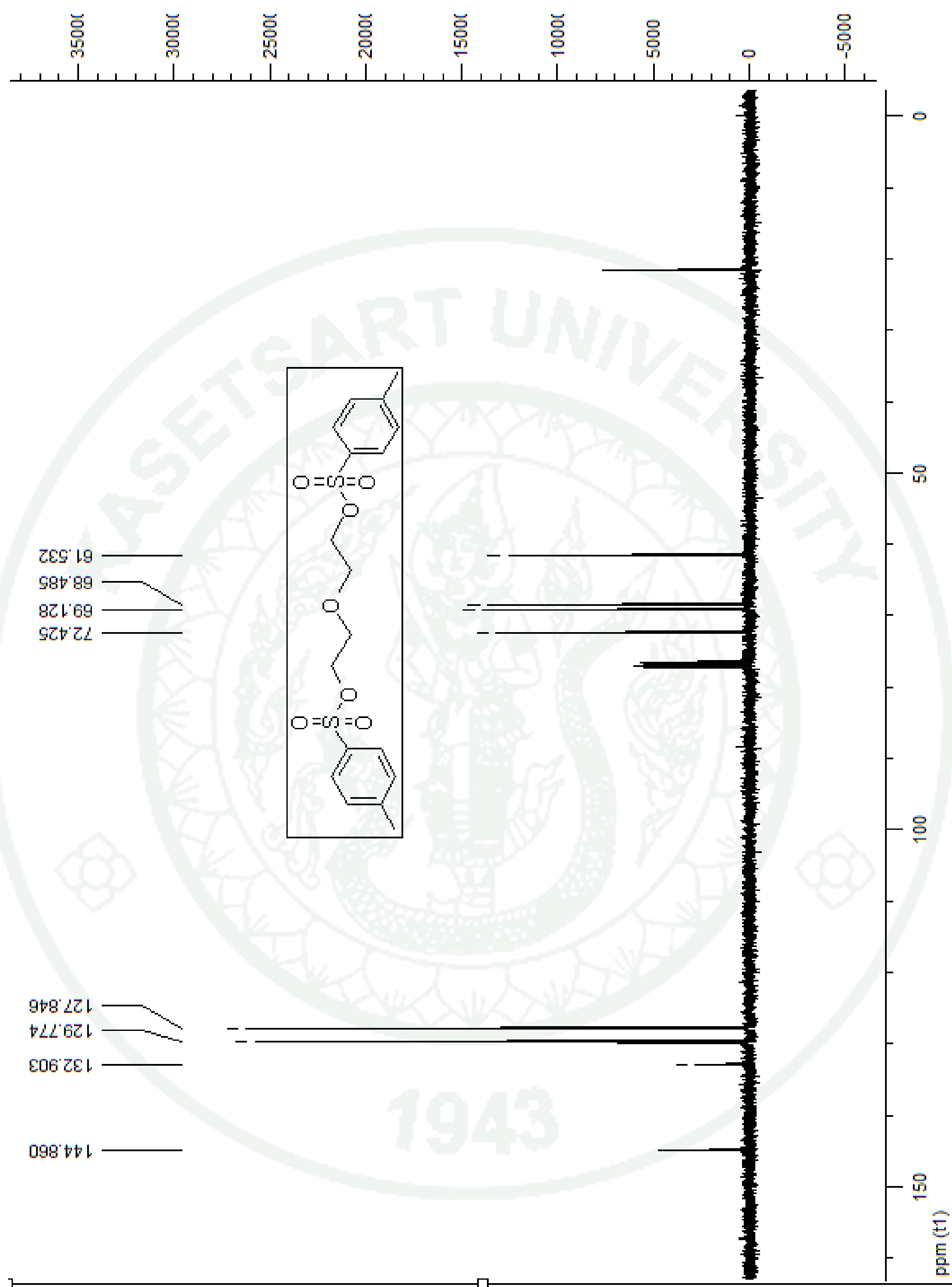
Appendix Figure 2 ^{13}C NMR of acetone cyanohydrin (3)

Copyright by Kasetsart University All rights reserved



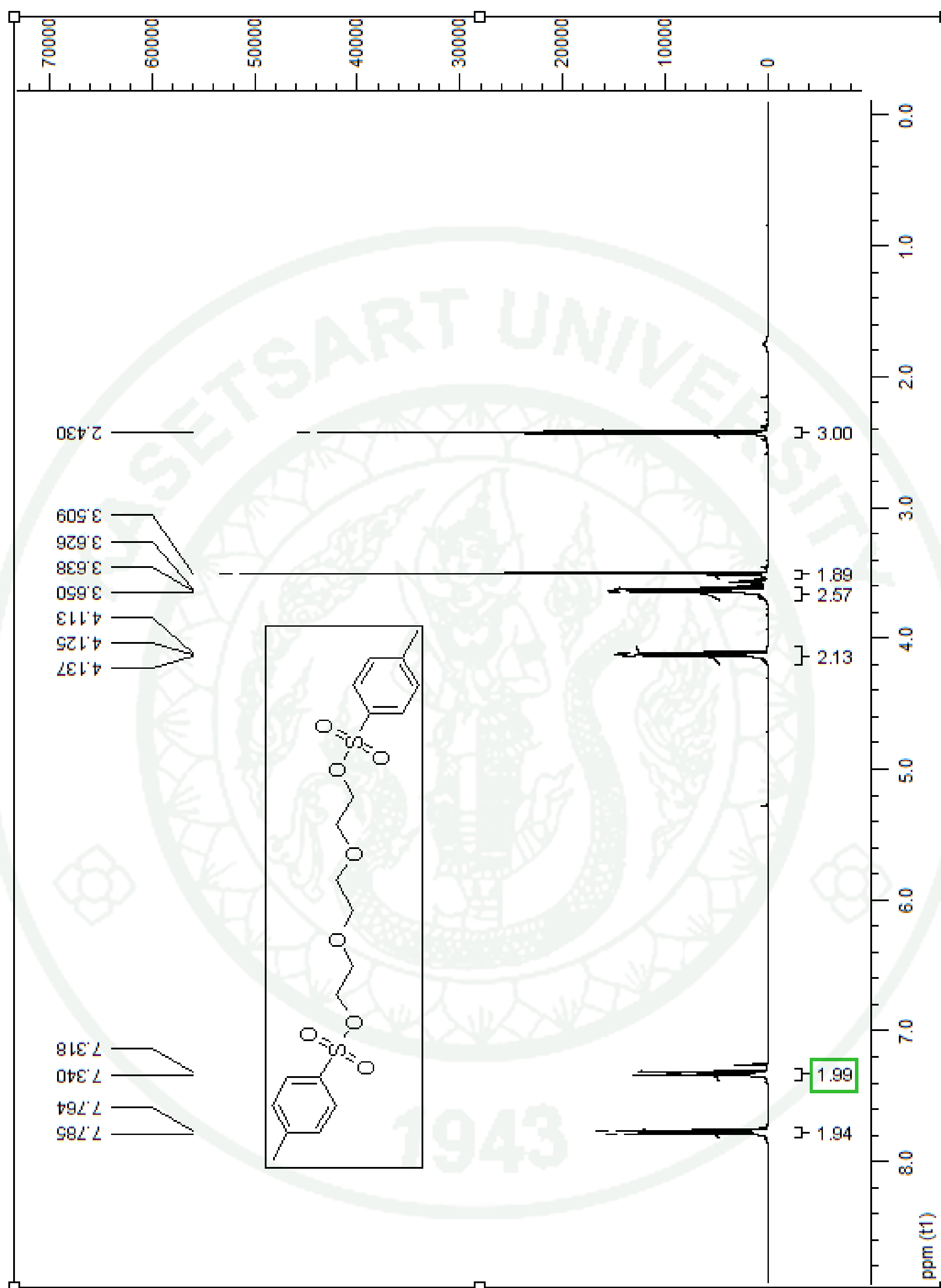
Appendix Figure 3 ^{1}H NMR of 2,2'-oxybis(ethane-2,1-diyl) bis(4-methylbenzene-sulfonate) (8a)

Copyright by Kasetsart University All rights reserved



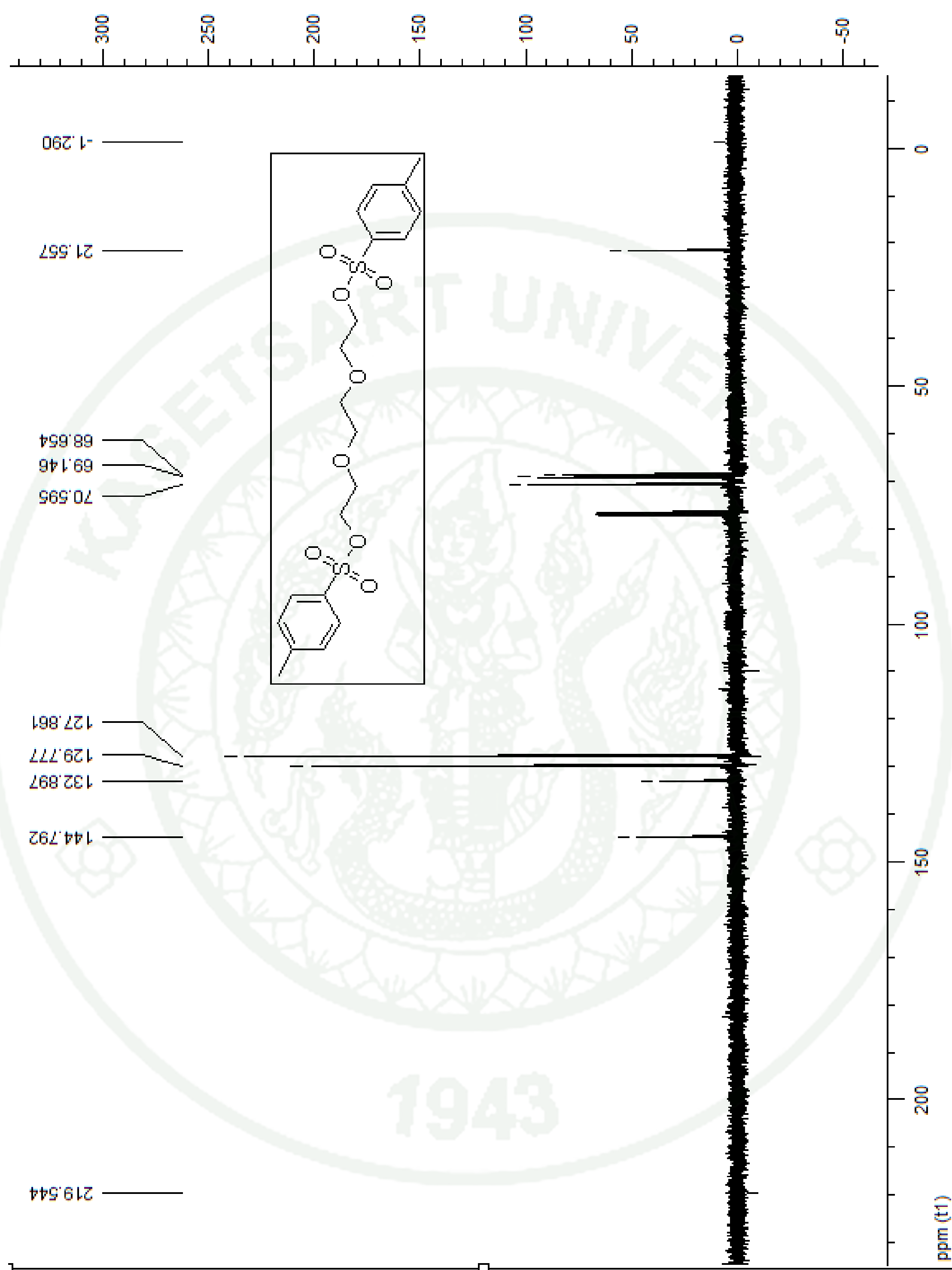
Appendix Figure 4 ^{13}C NMR of 2,2'-oxybis(ethane-2,1-diyl) bis(4-methylbenzene-sulfonate) (8a)

Copyright by Kasetsart University All rights reserved



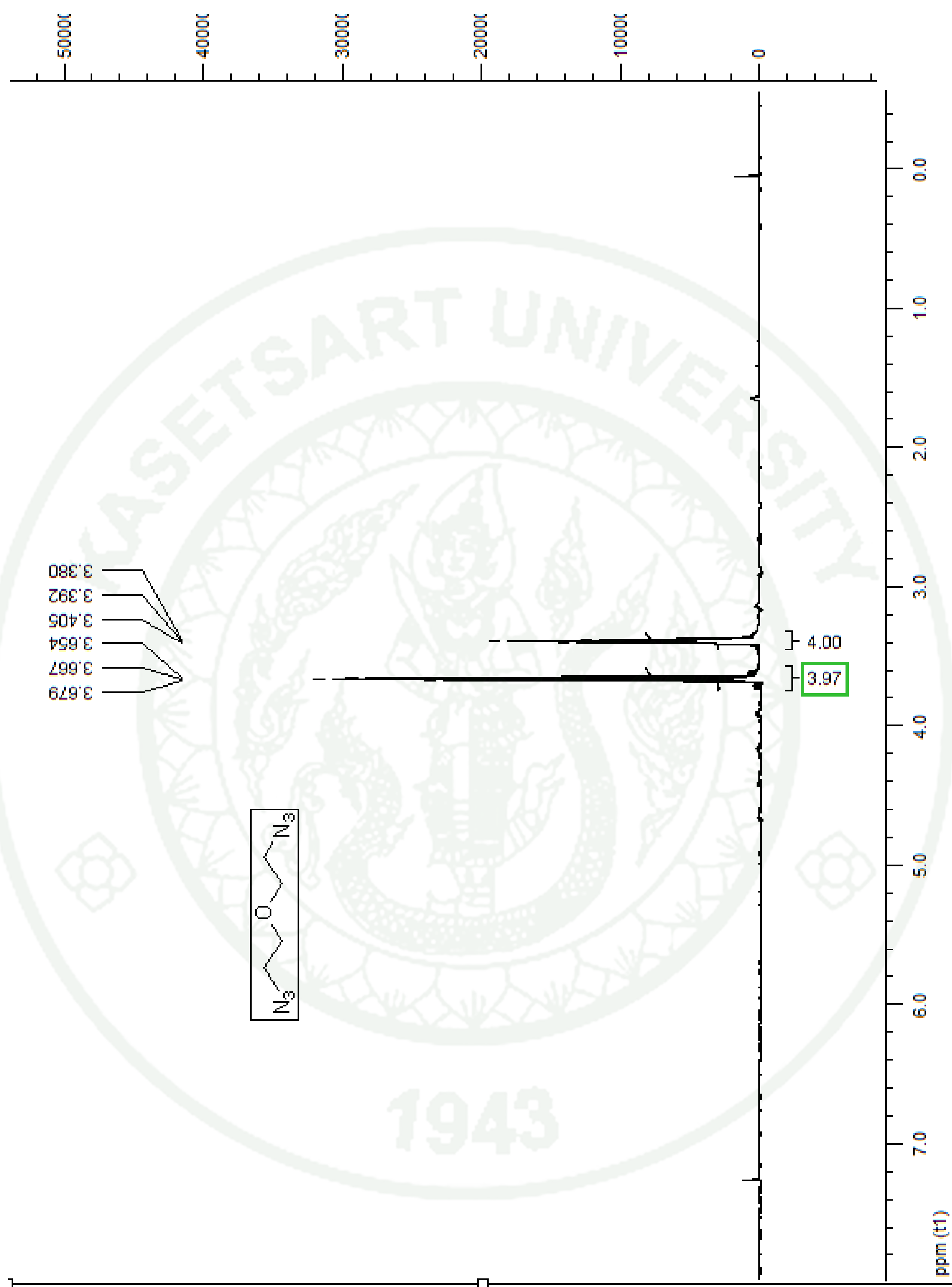
Appendix Figure 5 ^{1}H NMR of 2,2'-(ethane-1,2-diylbis(oxy))bis(ethane-2,1-diyl)bis-(4-methylbenzenesulfonate) (8b)

Copyright by Kasetsart University All rights reserved



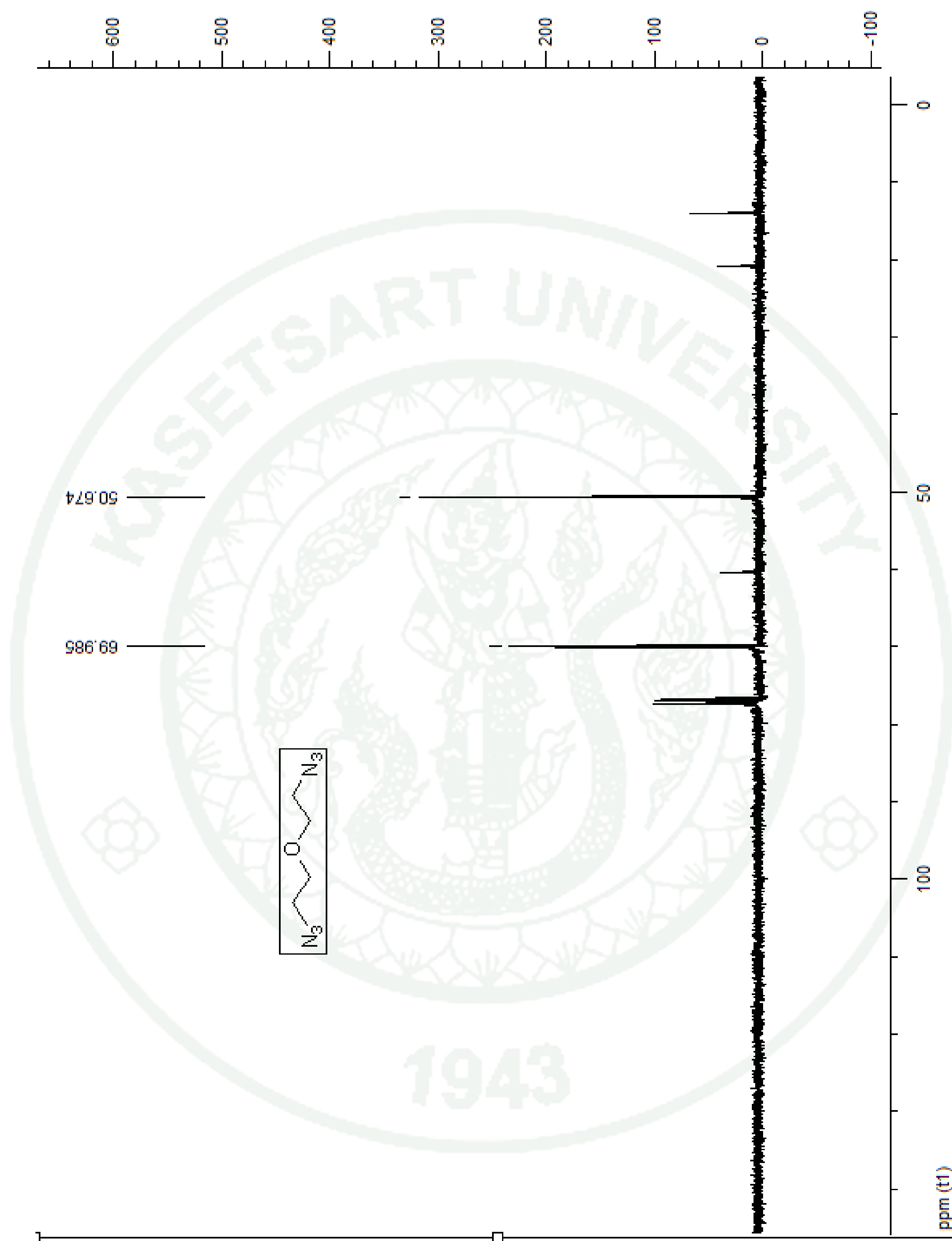
Appendix Figure 6 ^{13}C NMR of 2,2'-(ethane-1,2-diylbis(oxy))bis(ethane-2,1-diyl)bis-(4-methylbenzenesulfonate) (8b)

Copyright by Kasetsart University All rights reserved



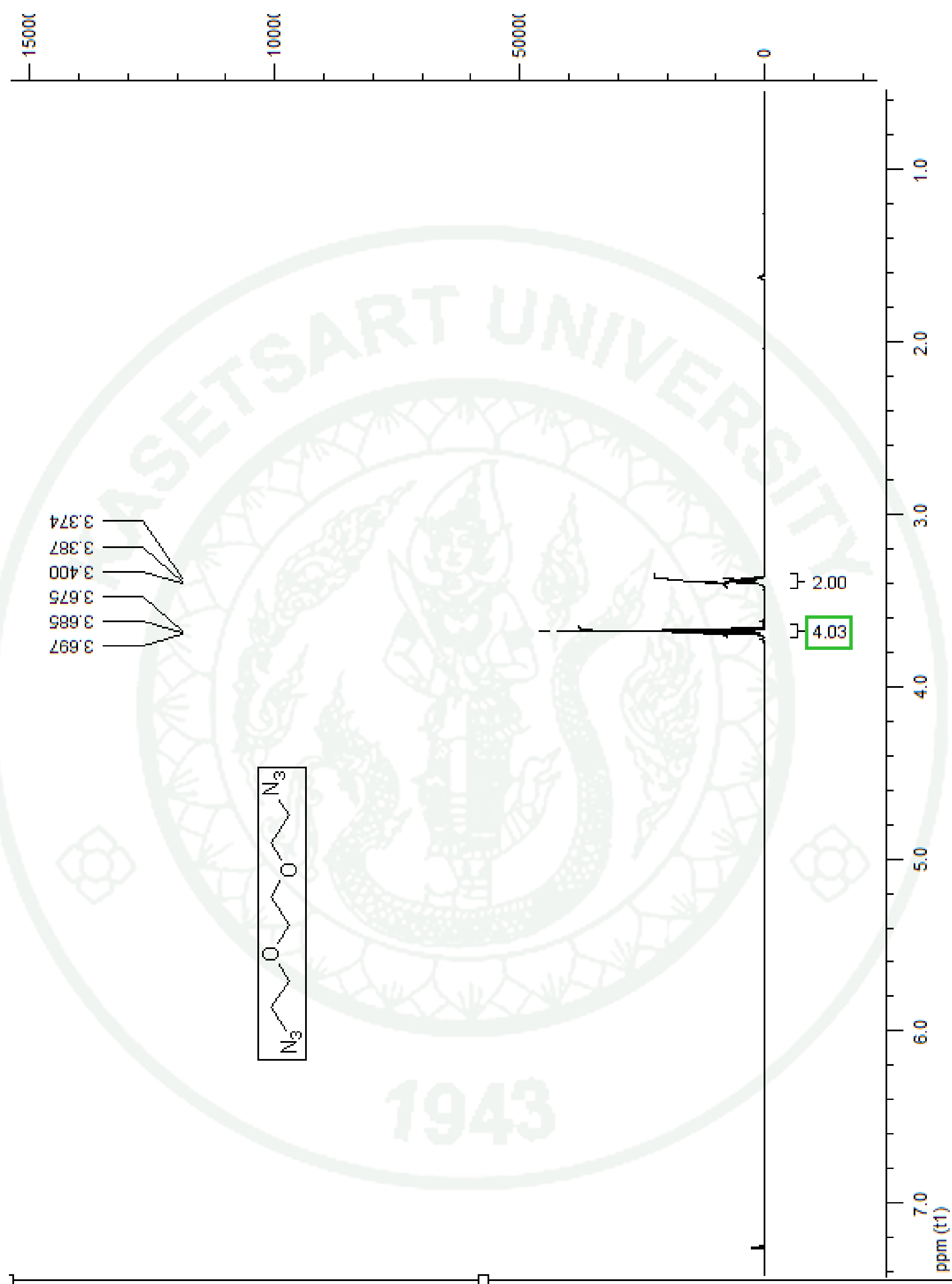
Appendix Figure 7 ^{1}H NMR of 1-azido-2-(2-azidoethoxy)ethane (13a)

Copyright by Kasetsart University All rights reserved



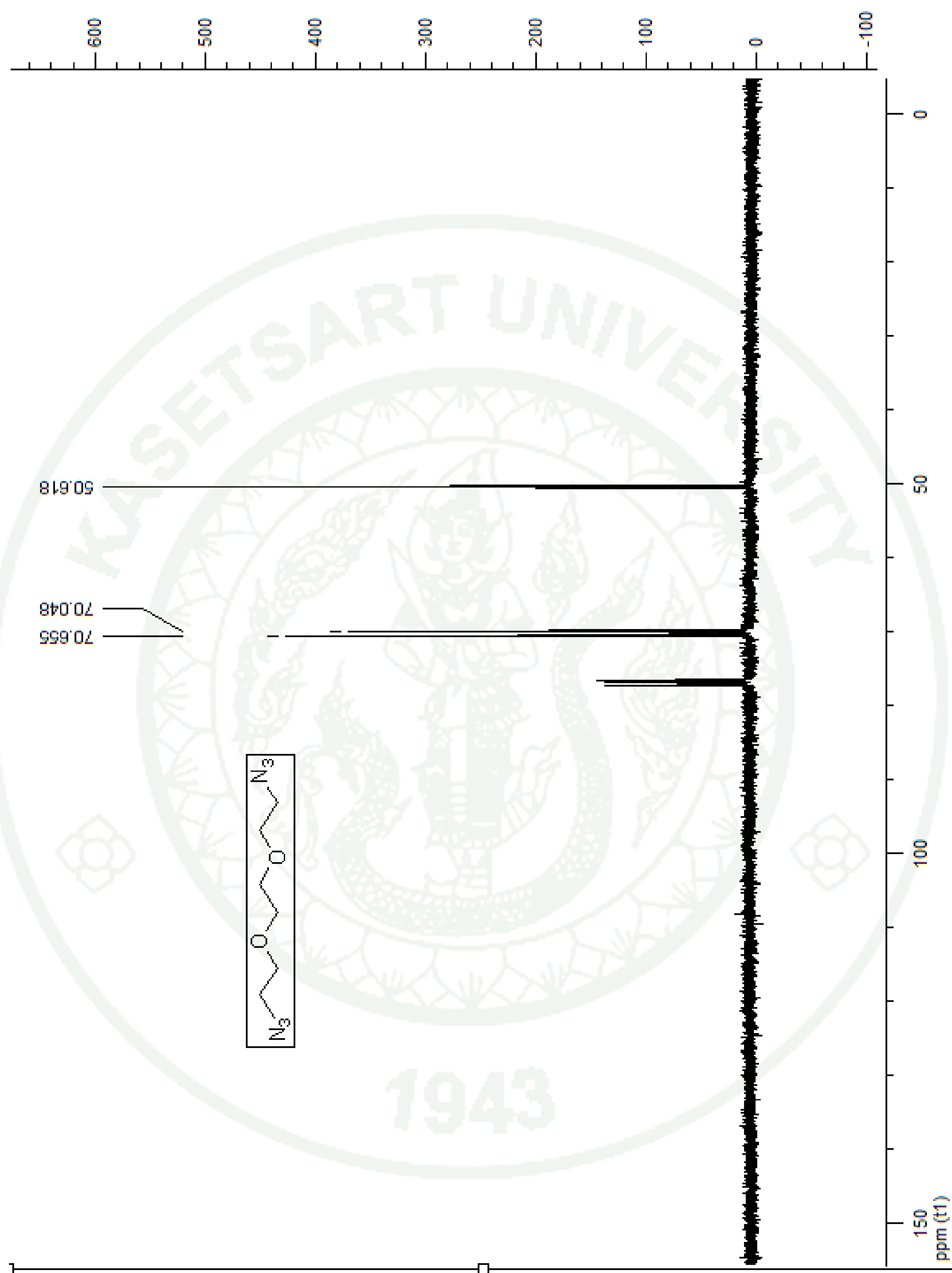
Appendix Figure 8 ^{13}C NMR of 1-azido-2-(2-azidoethoxy)ethane (13a)

Copyright by Kasetsart University All rights reserved



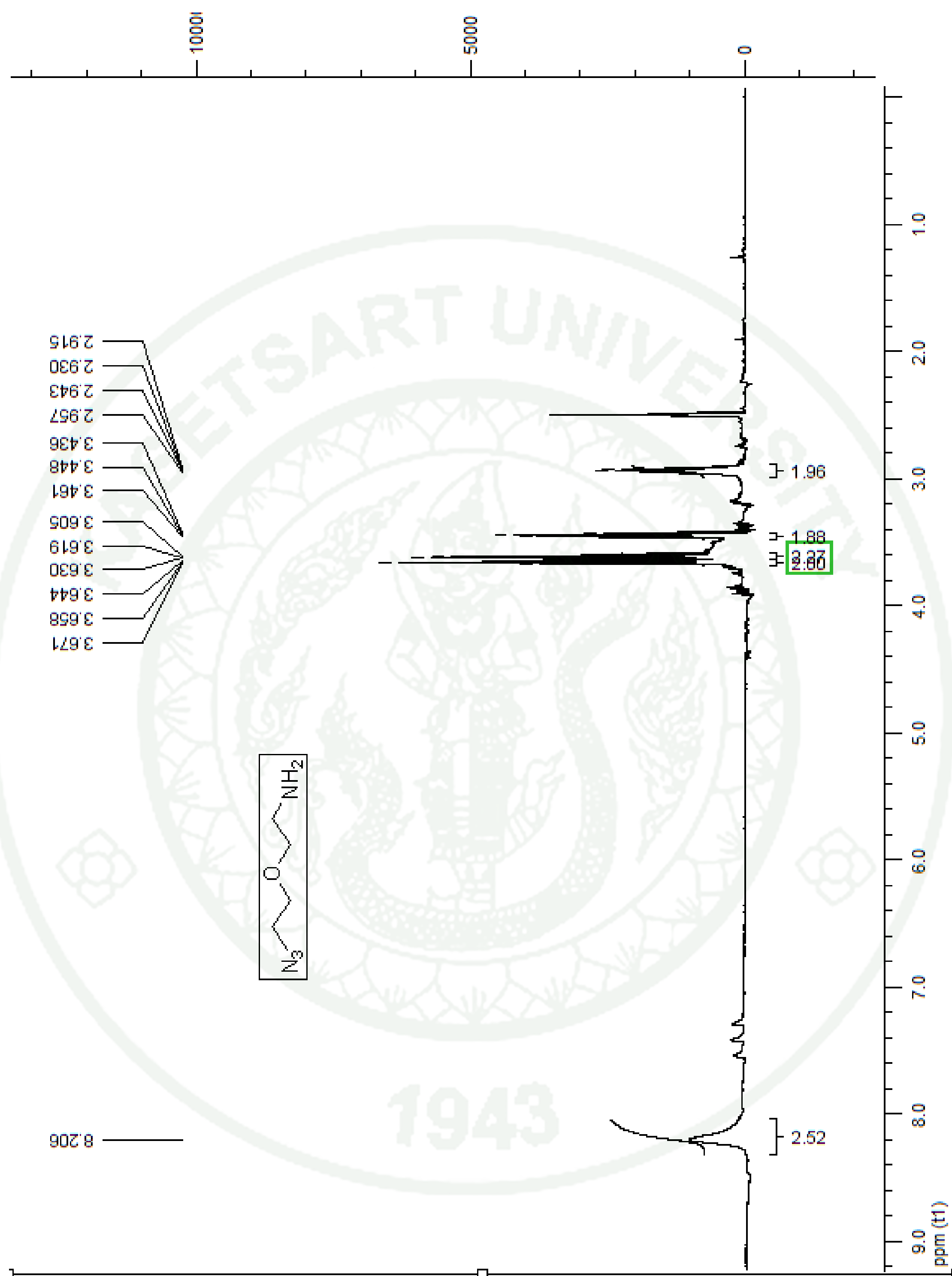
Appendix Figure 9 ^{1}H NMR of 1,2-bis(2-azidoethoxy)ethane (13b)

Copyright by Kasetsart University All rights reserved



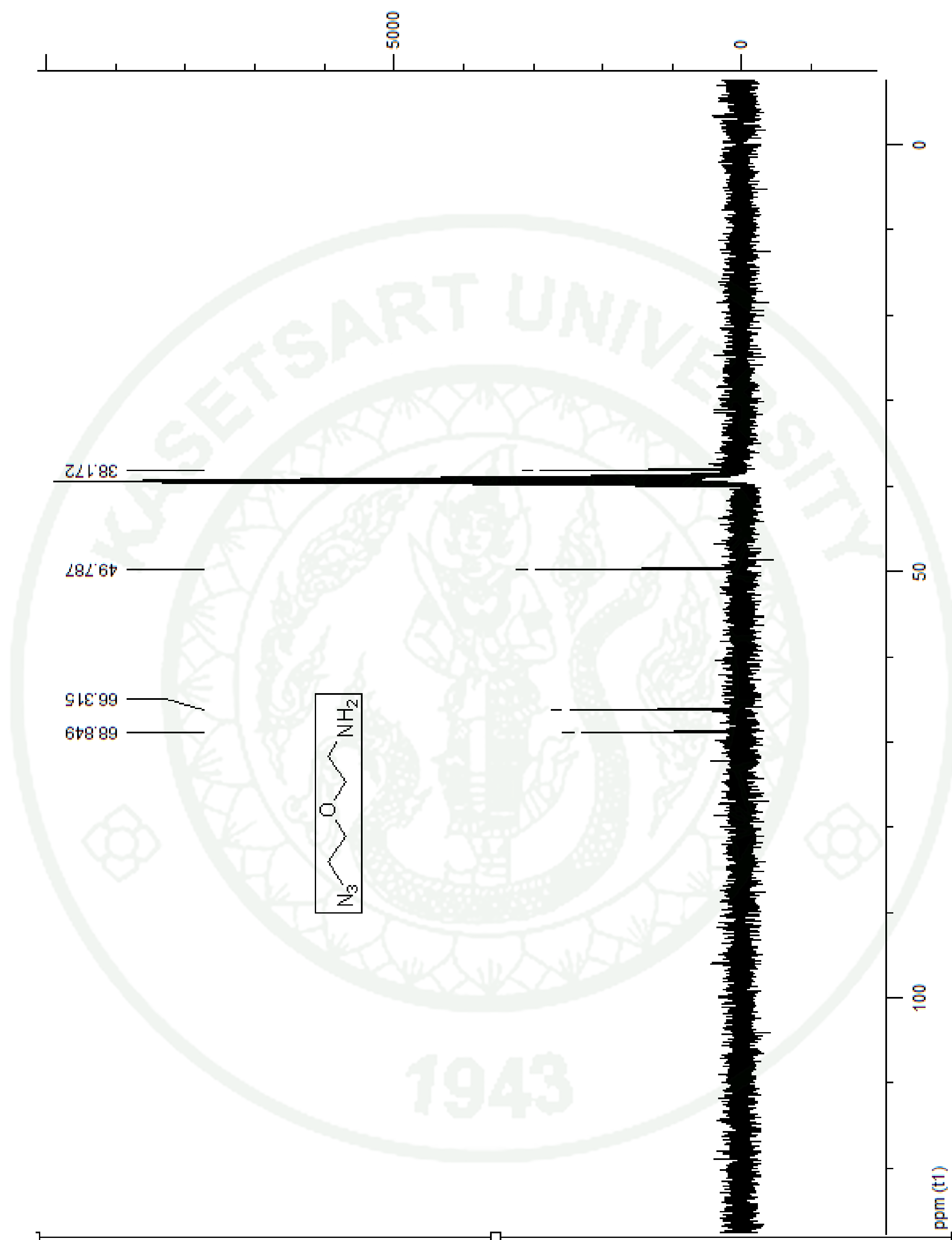
Appendix Figure 10 ^{13}C NMR of 1,2-bis(2-azidoethoxy)ethane (13b)

Copyright by Kasetsart University All rights reserved



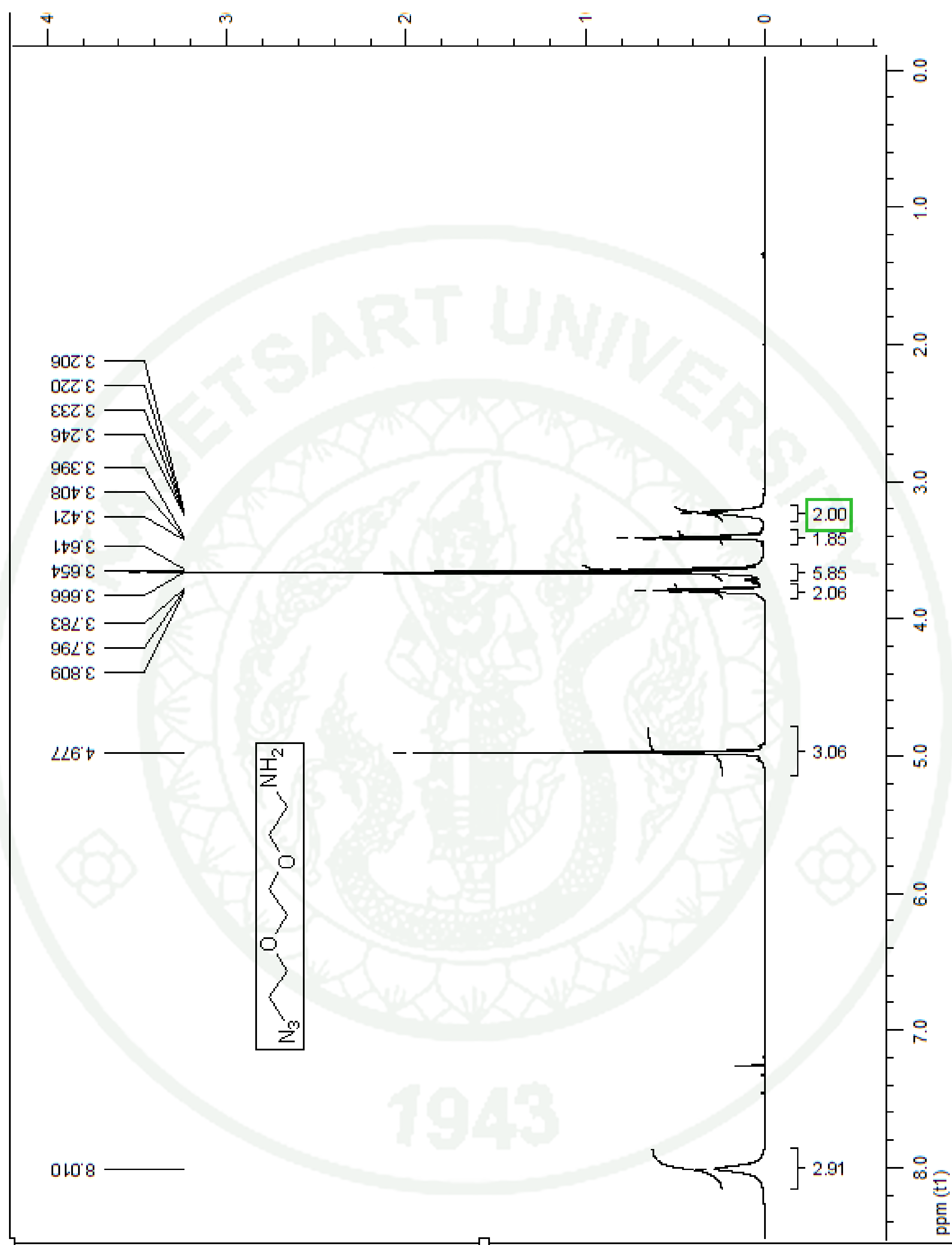
Appendix Figure 11 ^{1}H NMR of 2-(2-azidoethoxy)ethanamine (11a)

Copyright by Kasetsart University All rights reserved



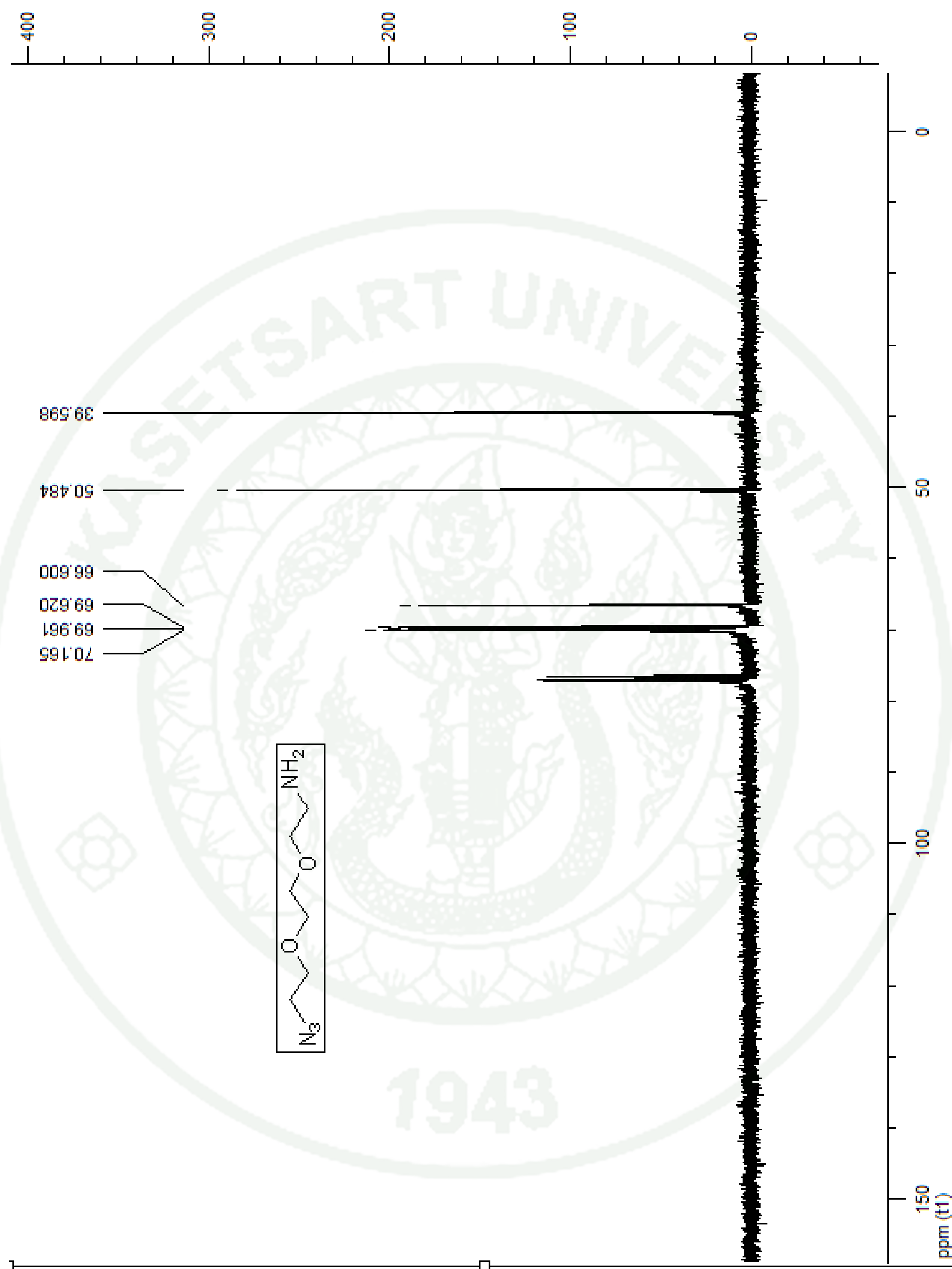
Appendix Figure 12 ^{13}C NMR of 2-(2-azidoethoxy)ethanamine (11a)

Copyright by Kasetsart University All rights reserved



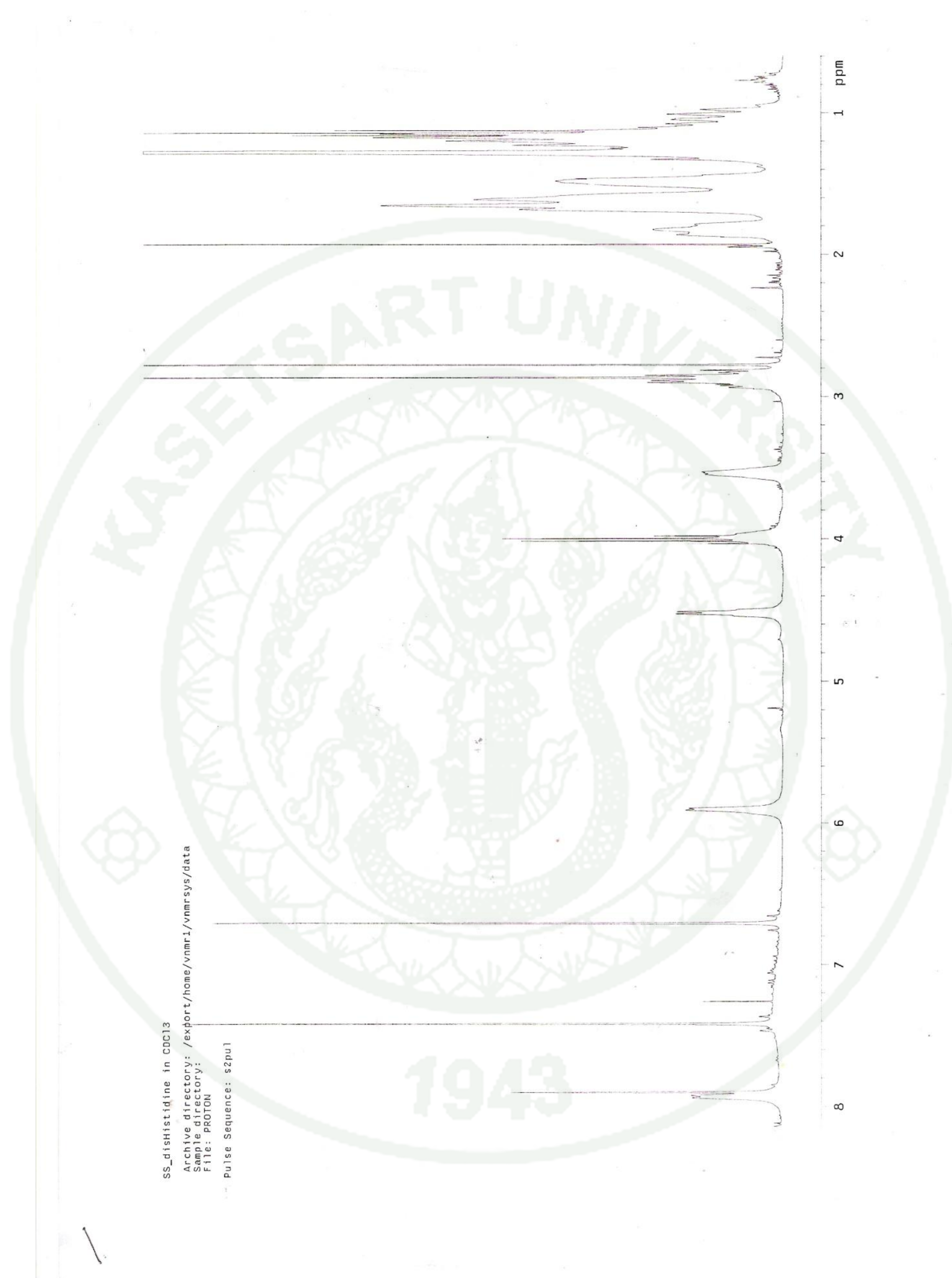
Appendix Figure 13 ^{1}H NMR of 2-(2-(2-azidoethoxy)ethoxy)ethanamine (11b)

Copyright by Kasetsart University All rights reserved



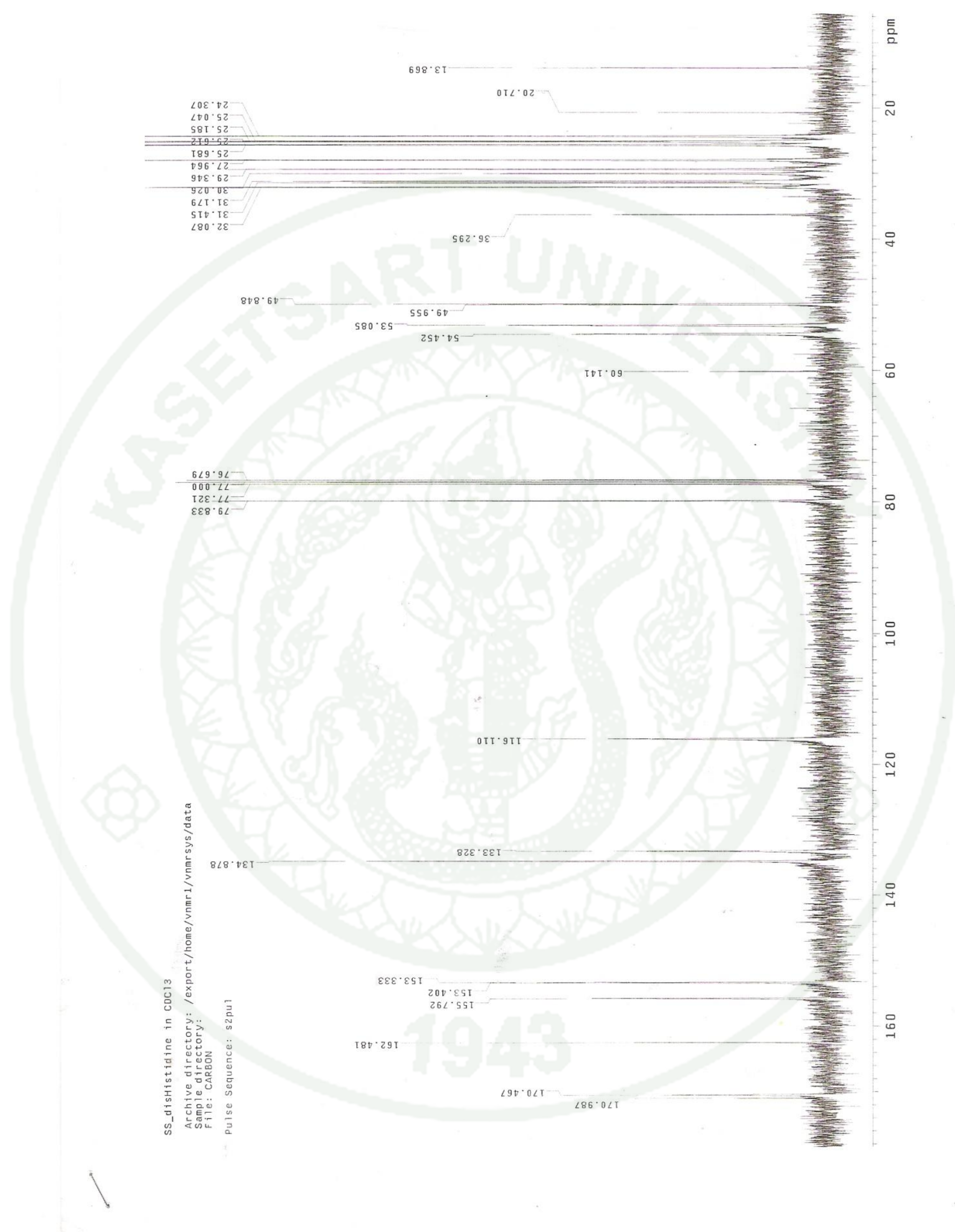
Appendix Figure 14 ^{13}C NMR of 2-(2-(2-azidoethoxy)ethoxy)ethanamine (11b)

Copyright by Kasetsart University All rights reserved



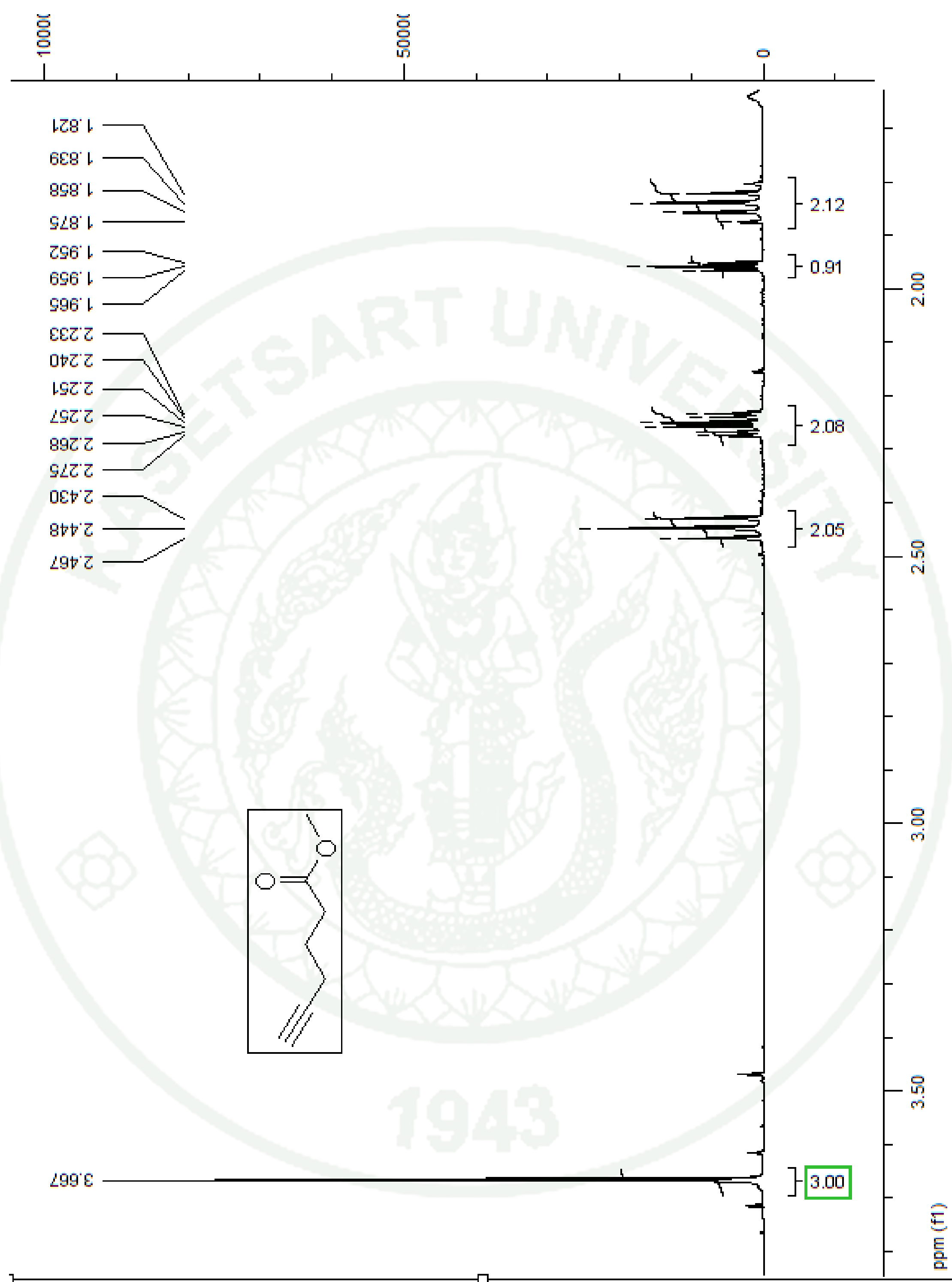
Appendix Figure 15 ^{1}H NMR of histidylhistidine (14)

Copyright by Kasetsart University All rights reserved



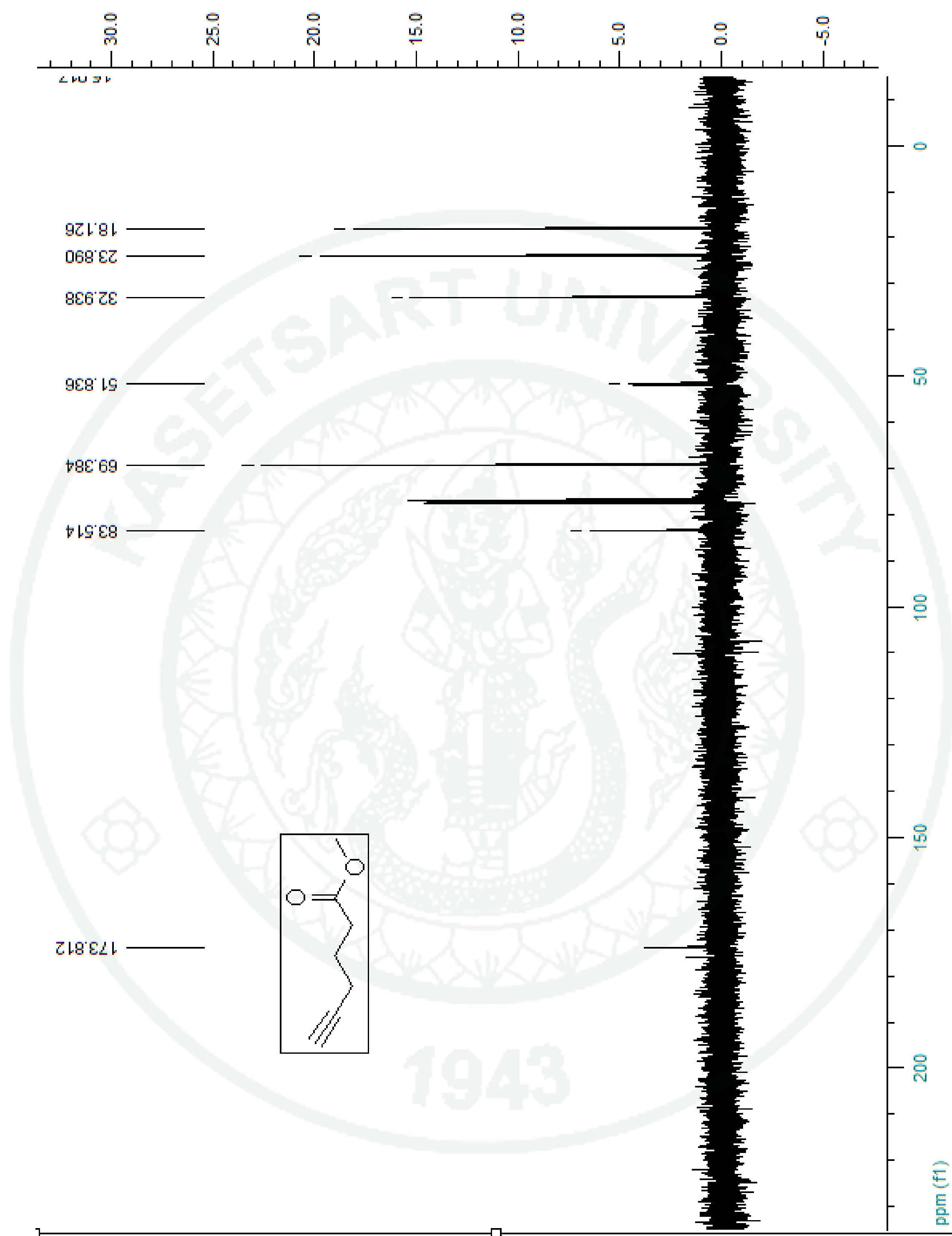
Appendix Figure 16 ^{13}C NMR of histidylhistidine (14)

Copyright by Kasetsart University All rights reserved



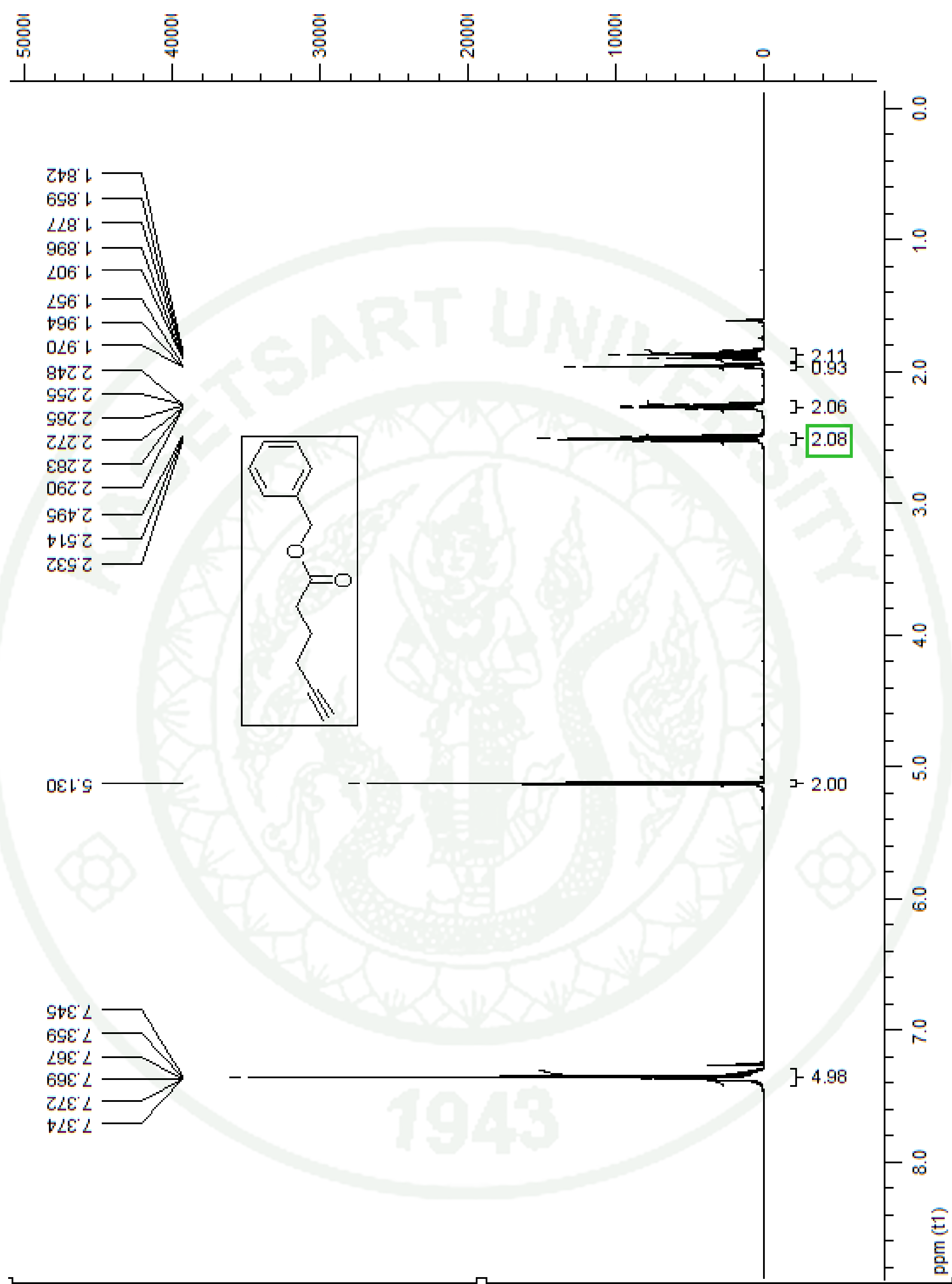
Appendix Figure 17 ^{1}H NMR of methyl hexynoate (15)

Copyright by Kasetsart University All rights reserved



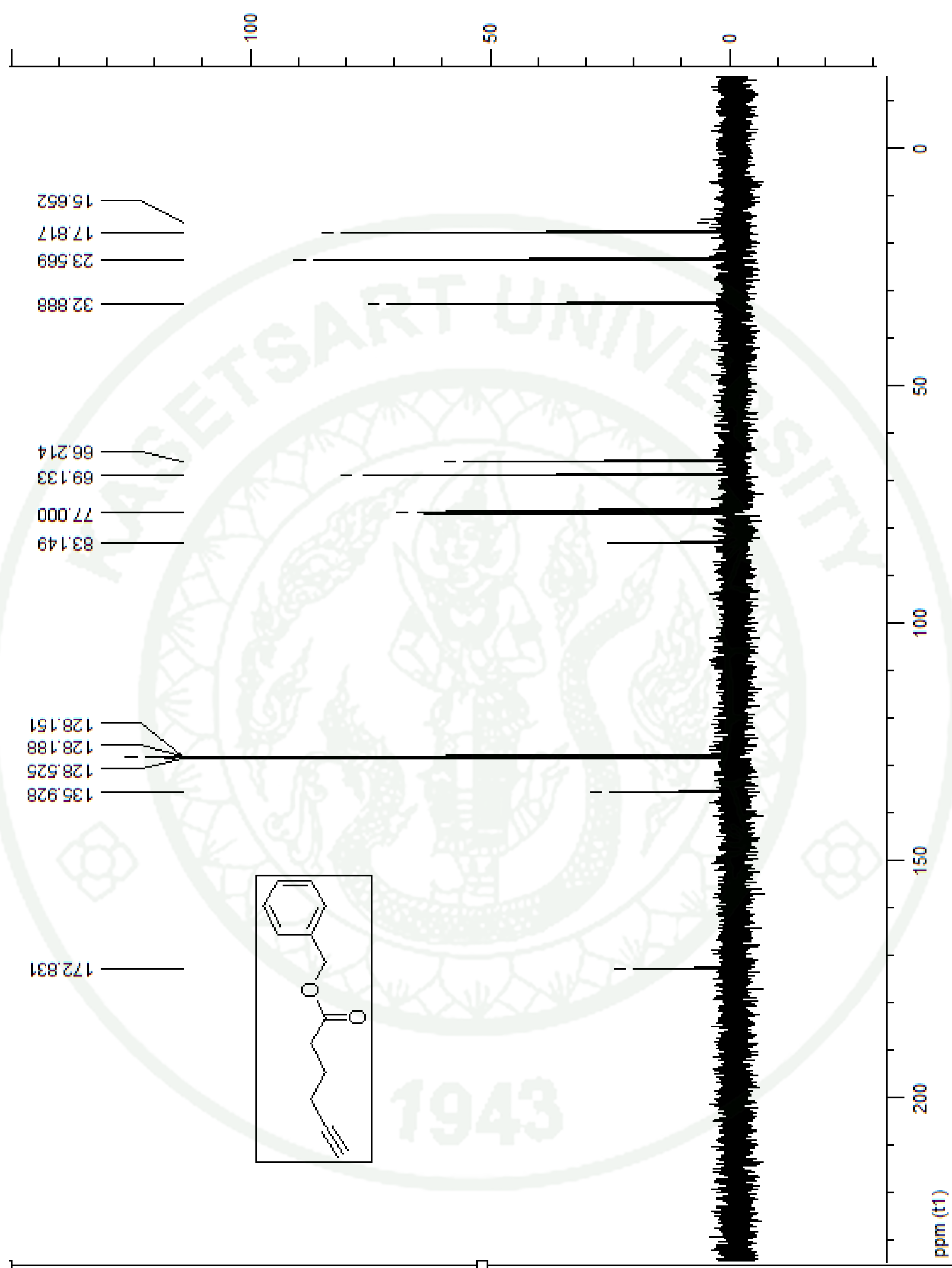
Appendix Figure 18 ^{13}C NMR of methyl hexynoate (15)

Copyright by Kasetsart University All rights reserved



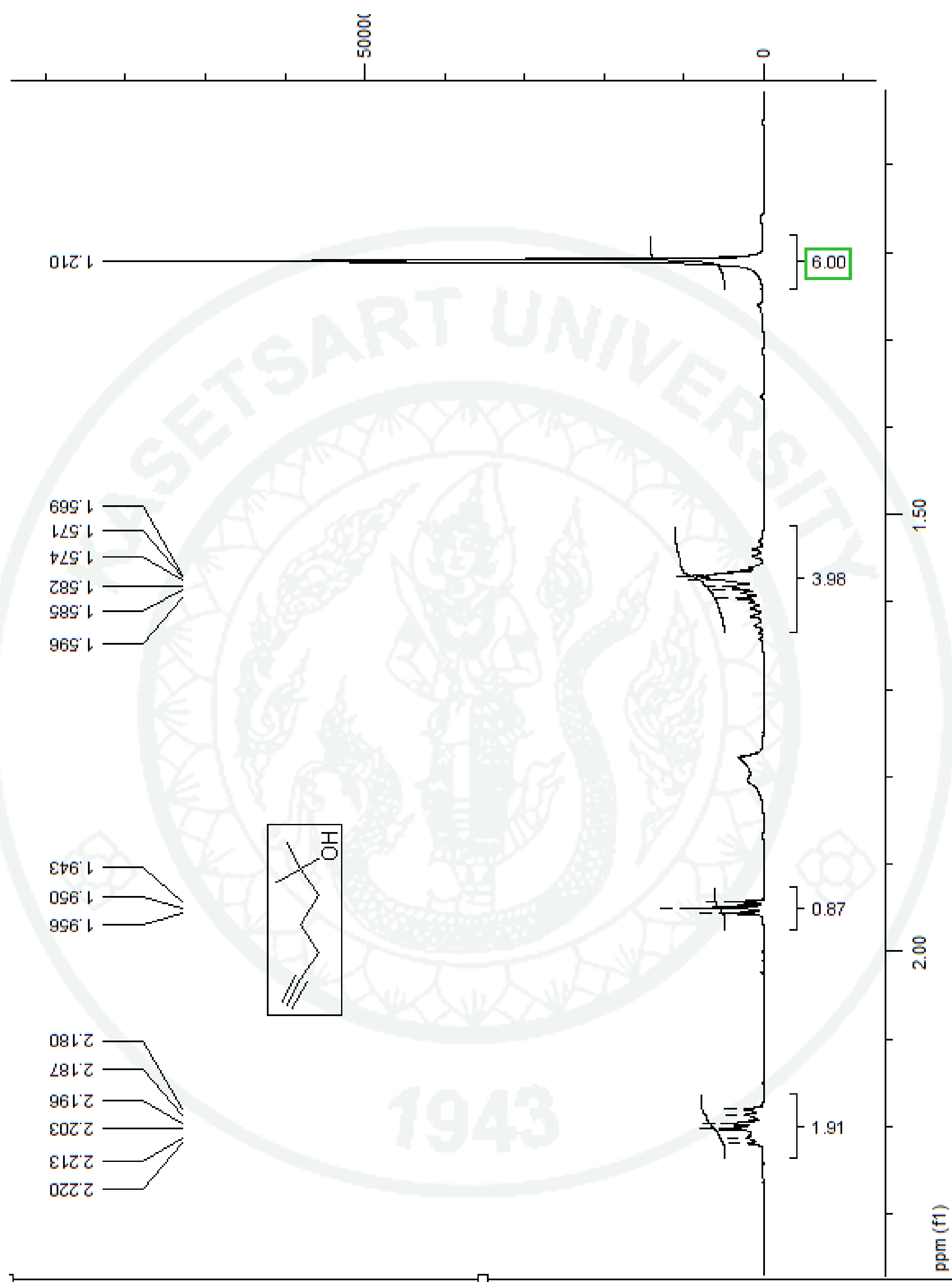
Appendix Figure 19 ^{1}H NMR of benzyl hexynoate (18)

Copyright by Kasetsart University All rights reserved



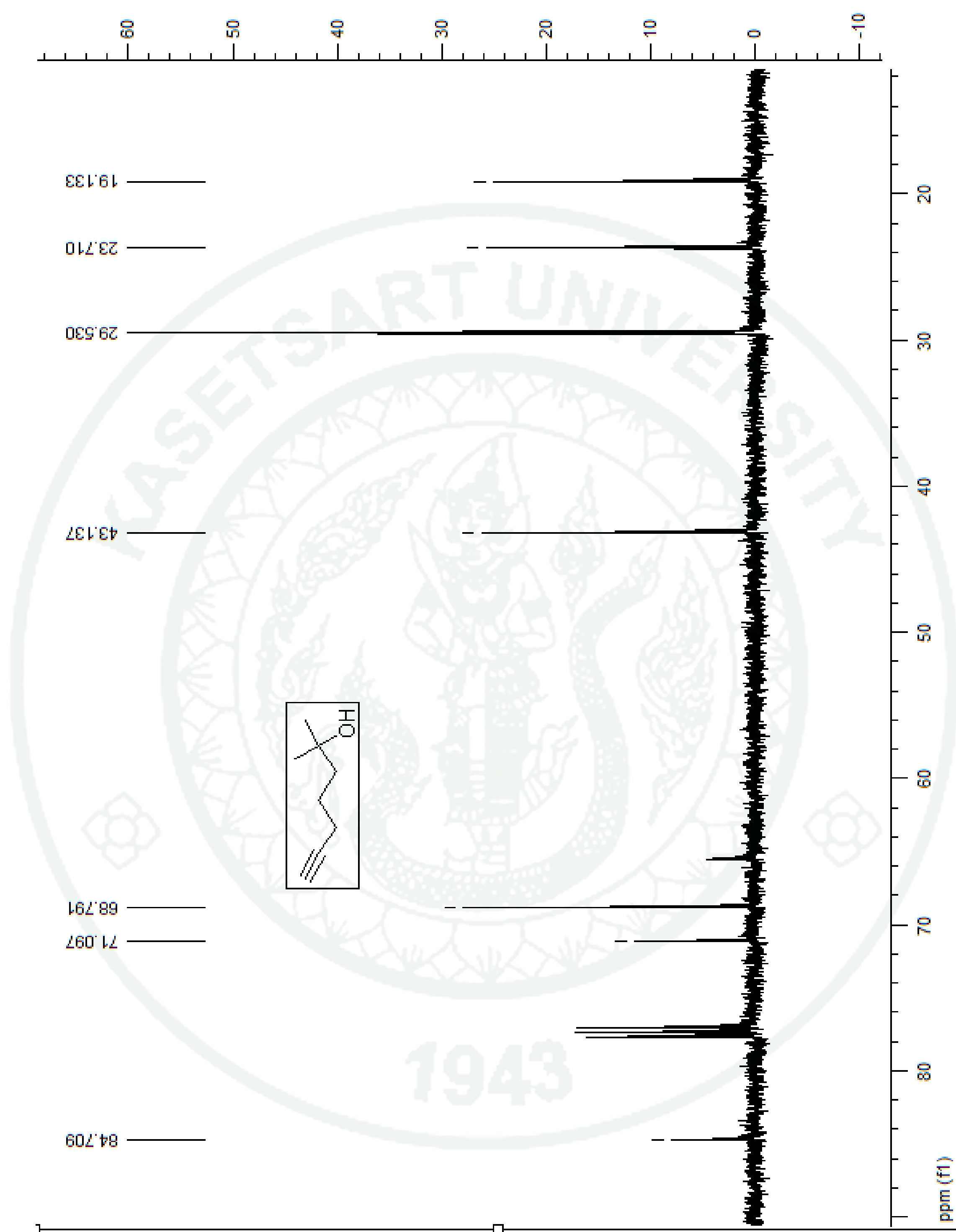
Appendix Figure 20 ^{13}C NMR of benzyl hexynoate (18)

Copyright by Kasetsart University All rights reserved



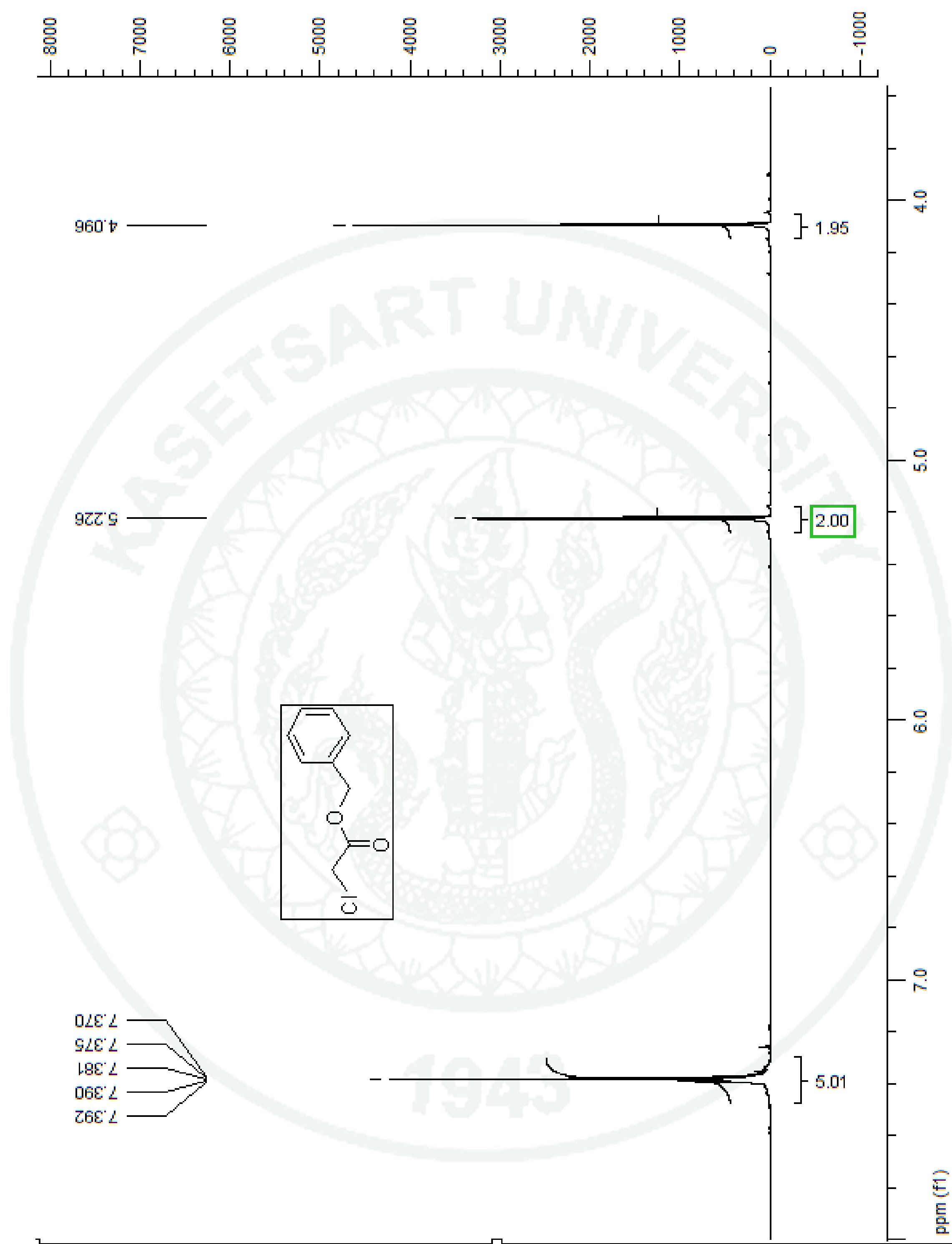
Appendix Figure 21 ^{1}H NMR of 2-methylhept-6-yn-2-ol (16)

Copyright by Kasetsart University All rights reserved



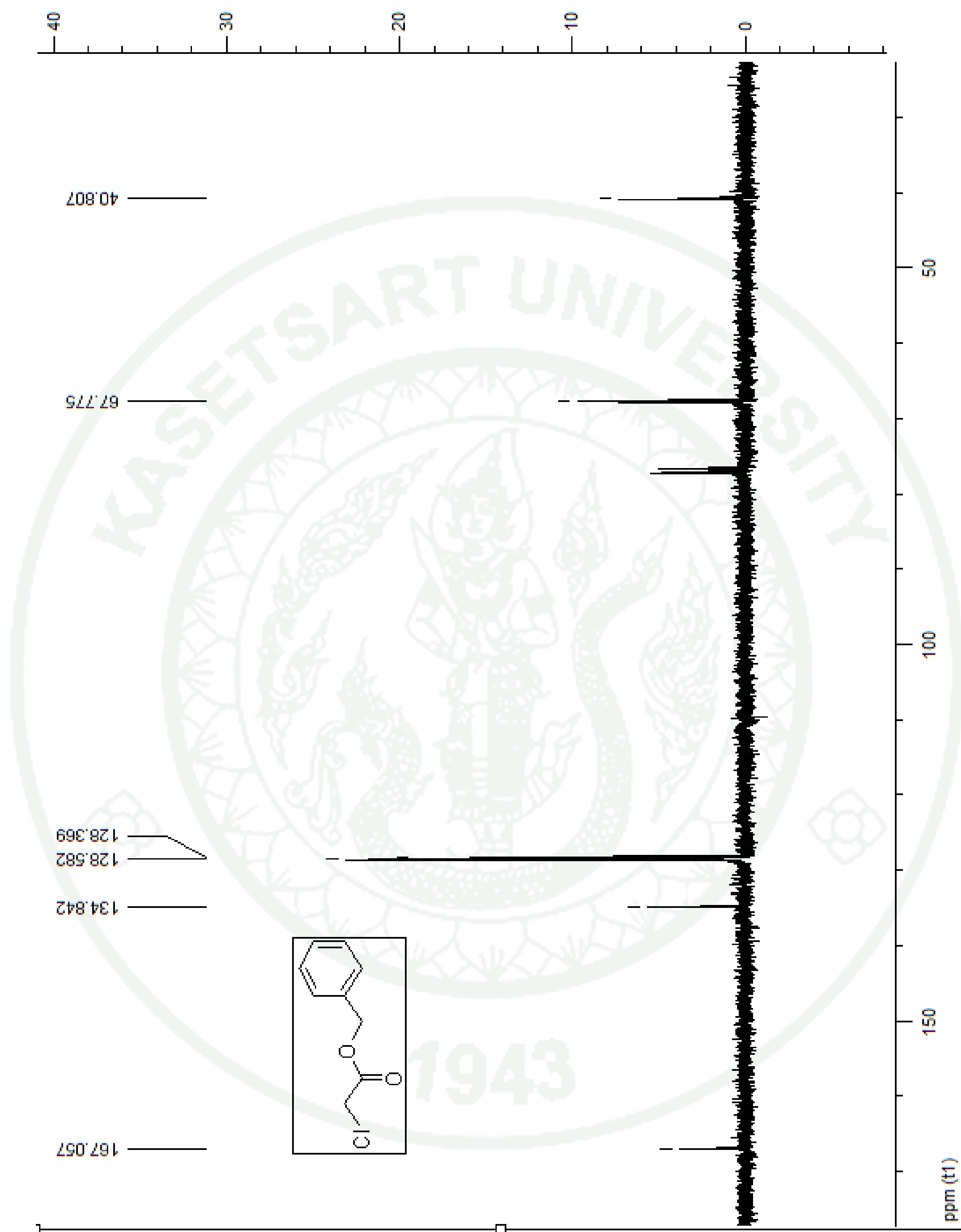
Appendix Figure 22 ^{13}C NMR of 2-methylhept-6-yn-2-ol (16)

Copyright by Kasetsart University All rights reserved



Appendix Figure 23 ^{1}H NMR of benzyl 2-chloroacetate (21)

Copyright by Kasetsart University All rights reserved



Appendix Figure 24 ^{13}C NMR of benzyl 2-chloroacetate (21)

Copyright by Kasetsart University All rights reserved

CURRICULUM VITAE

NAME : Miss. Suwimon Suebka

BIRTH DATE : February 2, 1986

BIRTH PLACE : Nakhon Ratchasima, Thailand

EDUCATION :

YEAR	INSTITUTE	DEGREE/DIPLOMA
2007	Kasetsart Univ.	B.Sc.(Chemistry)

WORK PLACE : Faculty of Science, Kasetsart University

SCHOLARSHIP/AWARDS : The Development and Promotion of Science and Technology talent project (DPST)

Copyright by Kasetsart University All rights reserved