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SYNTHESIS AND BIOLOGICAL STUDIES OF AMPHIPHILIC COMPOUNDS
DERIVED FROM SACCHARIDES AND AMINOGLYCOSIDES

by

Madher N. Alfindee

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Chemistry

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2019

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ABSTRACT

Synthesis and Biological Studies of Amphiphilic Compounds Derived from Saccharides
and Aminoglycosides

by

Madher N. Alfindee, Doctor of Philosophy

Utah State University, 2019

Major Professor: Dr. Cheng-Wei Tom Chang
Department: Chemistry and Biochemistry

For this dissertation research, two libraries of amphiphilic compounds were synthesized and biologically examined. The first library of compounds were amphiphilic aminoglycosides. Several aryl substituted kanamycin A derivatives were synthesized. This library of amphiphilic aminoglycosides was tested as antifungal agents and connexin inhibitors. Most of the compounds exhibited excellent fungal growth inhibition. The new amphiphilic kanamycins showed better connexin inhibition activity than the parent compound (kanamycin A) with less toxicity.

The second series of compounds was carbohydrate esters. A regioselective one-step esterification and scalable protocol of mono sugars was developed and used to synthesize a wide range of carbohydrate ester compounds. Among these compounds, mannose with a 14-carbon chain ester (**MAN014**) displayed antimicrobial activities and was easily produced in large quantity at relatively low cost. **MAN014** was highly active against bacterial strains, including *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) as well as several fungal species that included: *Fusarium graminearum*, *Candida albicans*, *Aspergillus flavus*, and *Cryptococcus neoformans*. **MAN014** was

shown to cause cell membrane permeabilization in *S. aureus* and *F. graminearum* and to have low toxicity toward mammalian cells.

(278 pages)

PUBLIC ABSTRACT

Synthesis and Biological Studies of Amphiphilic Compounds Derived from Saccharides
and Aminoglycosides

by

Madher N. Alfindee, Doctor of Philosophy

Utah State University, 2019

Adjacent cells communicate through gap junctions (GJs). These GJs are formed by head to head docking of two hemichannels (HCs) from two adjacent cells. HCs are connexin hexamer proteins. Connexin mutation is the most frequent cause of childhood hearing loss. This hearing impairment affects 2 in every 2000 children. Inhibition of the HCs might be the key factor to treat such disorders. A library of amphiphilic kanamycins was synthesized to be tested as HC inhibitors. These compounds showed excellent inhibition activity in comparison with the parent compound (kanamycin A) with less toxicity.

A library of monosaccharide esters with varying carbon chain lengths (acetyl (C2) to hexadecyl (C16)) were synthesized, characterized, and tested for bioactivity. Carbohydrate esters showed low toxicity while remaining active against bacteria and fungi. The compound 6-*O*-tetradecanoyl-D-mannopyranose (**MAN014**), a mannose ester with a fourteen-carbon chain, showed the greatest antibacterial and antifungal properties. A mode of action study was tested against *Staphylococcus aureus* (bacteria) and *Fusarium graminearum* (fungus) and found the compound perturbed the cell membrane.

DEDICATION

I would like to dedicate my work to my parents and my family for of their endless love, support, and sacrifice.

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LIST OF ABBREVIATIONS

AAG: Amphiphilic aminoglycosides

Ac: Acetyl

AG: Aminoglycoside

AHB: (S) 4-amino-2-hydroxyl-butyryl

Anhyd.: Anhydrous

ATP: Adenosine triphosphate

B.C.: Before Christ

Bn: Benzyl

BnBr: Benzyl bromide

CE: Carbohydrate esters

CNS: Central nervous system

COSY: Correlation spectroscopy

DCM: Dichloromethane

DMAP: 4-(*N,N*-dimethylamino) pyridine

DMEM: Dulbecco's Modified Eagle Medium

DMF: Dimethylformamide

DMSO: Dimethyl sulfoxide

EFSA: European food safety authority

Et₃N: Triethylamine

EtOAc: Ethylacetate

EtOH: Ethanol

Eq: Equivalent

FDA: The U.S. Food and drug administration

FHB: Fusarium head blight

G⁺: Gram-positive bacteria

G⁻: Gram-negative bacteria

GLC: Glucose

GRAS: Generally regarded as safe

GJC: Gap junction channels

HC: Hemichannels

HPLC: High-performance liquid chromatography

HRMS: High-resolution mass spectrometry

IC₅₀: Half maximal inhibitory concentration

kDa: kilodalton

KLM: Potassium liquid medium

MAN: Mannose

Me: Methyl

MeCN: Acrylonitrile

MRSA: Methicillin-resistant *Staphylococcus aureus*

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADH: Nicotinamide adenine dinucleotide

NAG: N-acetylglucosamine

NLM: Sodium liquid medium

OD: Optical density

PBS: Phosphate buffered saline

PI: Propidium iodide

PNS: Peripheral nervous system

Py: Pyridine

RT: Room temperature

SAR: Structure-activity relationship

TBAF: Tetrabutylammonium fluoride

TBAI: Tetrabutylammonium iodide

Tf₂O: Trifluoromethanesulfonyl acid anhydride

THF: Tetrahydrofuran

TIPSCl: Triisopropylsilyl chloride

TLC: Thin layer chromatography

TsOH: p-Toluene sulfonic acid

WFP: World food program

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C-9	¹ H NMR of 6- <i>O</i> -Decanoyl-D-mannopyranose (MAN010).....	201
C-10	¹³ C NMR of 6- <i>O</i> -Decanoyl-D-mannopyranose (MAN010).....	202
C-11	¹ H NMR of 6- <i>O</i> -Tridecanoyl-D-mannopyranose (MAN013).....	203
C-12	¹³ C NMR of 6- <i>O</i> -Tridecanoyl-D-mannopyranose (MAN013).....	204
C-13	¹ H NMR of a mixture of 2,6-Di- <i>O</i> -tetradecanoyl-D-mannopyranose and 3,6-Di- <i>O</i> -tetradecanoyl-D-mannopyranose (MAN014d)	205
C-14	¹ H- ¹ H COSY NMR of a mixture of 2,6-Di- <i>O</i> -tetradecanoyl-D- mannopyranose and 3,6-Di- <i>O</i> -tetradecanoyl-D-mannopyranose (MAN014d)	206
C-15	¹³ C NMR of a mixture of 2,6-Di- <i>O</i> -tetradecanoyl-D-mannopyranose and 3,6-Di- <i>O</i> -tetradecanoyl-D-mannopyranose (MAN014d)	207
C-16	Gated ¹³ C NMR of a mixture of 2,6-Di- <i>O</i> -tetradecanoyl-D- mannopyranose and 3,6-Di- <i>O</i> -tetradecanoyl-D-mannopyranose (MAN014d)	208
C-17	¹ H NMR of Methyl-6- <i>O</i> -tetradecanoyl-D-mannopyranose (MM014).....	209
C-18	¹³ C NMR of Methyl-6- <i>O</i> -tetradecanoyl-D-mannopyranose (MM014)	210
C-19	¹ H NMR of 6- <i>O</i> -Pentadecanoyl-D-mannopyranose (MAN015).....	211
C-20	¹³ C NMR of 6- <i>O</i> -Pentadecanoyl-D-mannopyranose (MAN015).....	212

C-21	^1H NMR of 2-Acetamido-2-deoxy-6- <i>O</i> -octanoyl- α -D-glucopyranose (NAG008)	213
C-22	^{13}C NMR of 2-Acetamido-2-deoxy-6- <i>O</i> -octanoyl- α -D-glucopyranose (NAG008)	214
C-23	^1H NMR of 2-Acetamido-2-deoxy-3,6-di- <i>O</i> -octanoyl- α -D-glucopyranose (NAG008d)	215
C-24	^{13}C NMR of 2-Acetamido-2-deoxy-3,6-di- <i>O</i> -octanoyl- α -D-glucopyranose (NAG008d)	216
C-25	^1H NMR of 2-Acetamido-2-deoxy-6- <i>O</i> -decanoyl- α -D-glucopyranose (NAG010)	217
C-26	^1H - ^1H COSY NMR of 2-Acetamido-2-deoxy-6- <i>O</i> -decanoyl- α -D- glucopyranose (NAG010)	218
C-27	^{13}C NMR of 2-Acetamido-2-deoxy-6- <i>O</i> -decanoyl- α -D-glucopyranose (NAG010)	219
C-28	^1H NMR of 2-Acetamido-2-deoxy-3,6-di- <i>O</i> -decanoyl- α -D-glucopyranose (NAG010d)	220
C-29	^1H - ^1H COSY NMR of 2-Acetamido-2-deoxy-3,6-di- <i>O</i> -decanoyl- α -D- glucopyranose (NAG010d)	221
C-30	^{13}C NMR of 2-Acetamido-2-deoxy-3,6-di- <i>O</i> -decanoyl- α -D-glucopyranose (NAG010d)	222
C-31	^1H NMR of 2-Acetamido-2-deoxy-6- <i>O</i> -dodecanoyl- α -D-glucopyranose (NAG012)	223

C-32	^{13}C NMR of 2-Acetamido-2-deoxy-6- <i>O</i> -dodecanoyl- α -D-glucopyranose (NAG012)	224
C-33	^1H NMR of 2-Acetamido-2-deoxy-3,6-di- <i>O</i> -dodecanoyl- α -D- glucopyranose (NAG012d)	225
C-34	^{13}C NMR of 2-Acetamido-2-deoxy-3,6-di- <i>O</i> -dodecanoyl- α -D- glucopyranose (NAG012d)	226
C-35	^1H NMR of 2-Acetamido-2-deoxy-6- <i>O</i> -tetradecanoyl- α -D-glucopyranose (NAG014)	227
C-36	^{13}C NMR of 2-Acetamido-2-deoxy-6- <i>O</i> -tetradecanoyl- α -D-glucopyranose (NAG014)	228
C-37	^1H NMR of 2-Acetamido-2-deoxy-3,6-di- <i>O</i> -tetradecanoyl- α -D- glucopyranose (NAG014d)	229
C-38	^1H - ^1H COSY NMR of 2-Acetamido-2-deoxy-3,6-di- <i>O</i> -tetradecanoyl- α -D-glucopyranose (NAG014d)	230
C-39	^{13}C NMR of 2-Acetamido-2-deoxy-3,6-di- <i>O</i> -tetradecanoyl- α -D- glucopyranose (NAG014d)	231
C-40	^1H NMR of 2-Acetamido-2-deoxy-6- <i>O</i> -hexadecanoyl- α -D- glucopyranose (NAG016)	232
C-41	^{13}C NMR of 2-Acetamido-2-deoxy-6- <i>O</i> -hexadecanoyl- α -D- glucopyranose (NAG016)	233
C-42	^1H NMR of 2-Acetamido-2-deoxy-3,6-di- <i>O</i> -hexadecanoyl- α -D- glucopyranose (NAG016d)	234

C- 43	^{13}C NMR of 2-Acetamido-2-deoxy-3,6-di- <i>O</i> -hexadecanoyl- α -D-glucopyranose (NAG016d)	235
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CHAPTER 1

GENERAL INTRODUCTION

1-1 Introduction to Aminoglycosides and Kanamycins

Since the discovery of the first aminoglycoside (AG), streptomycin (figure 1-1), in 1944, AGs have been one of the most effective treatments for bacterial infection.¹⁻² These compounds have one or more amino saccharide rings and a 2-deoxystreptamine ring connected together through a glycoside bond. The AGs are classified based on the 2-deoxystreptamine (figure 1-1) ring substitution positions. The first is neomycin class (4,5-disubstituted 2-deoxystreptamine, such as neomycin B, paromomycin I, ribostamycin), and the second is kanamycin class (4,6-disubstituted 2-deoxystreptamine, such as kanamycin A, kanamycin B, dibekacin, tobramycin, amikacin, and arbekacin) (figure 1-1). In addition to these two classes, there is a monosubstituted 2-deoxystreptamine AG such as streptomycin (figure 1-1).

Kanamycin was first isolated from *Streptomyces kanamyceticus* in 1957 by Hamao Umezawa.³⁻⁴ This discovery was recognized as a milestone in the field of antibiotics due to its antibacterial potency.⁵ A year later, kanamycin was approved to be clinically used as one of the most powerful antibacterial agents, saving a tremendous number of lives.⁴ Excessive clinical chemotherapy use of kanamycin caused bacterial resistance to be developed 8 years after its discovery.⁶ Unfortunately, kanamycin became clinically obsolete due to the bacterial resistance.⁷ Reviving kanamycin activity through chemical modification became a primary research focus for many chemists around the world.⁸⁻⁹

Despite the efforts to develop novel antibacterial kanamycin analogs, only two compounds, amikacin in 1972 and arbekacin in 1994, were synthesized.¹⁰⁻¹² Subsequently, it was found that introducing a hydrophobic group to aminoglycosides could repurpose the compounds from being antibacterial agents to being antifungal agents and/or connexin inhibitors.¹³⁻¹⁴

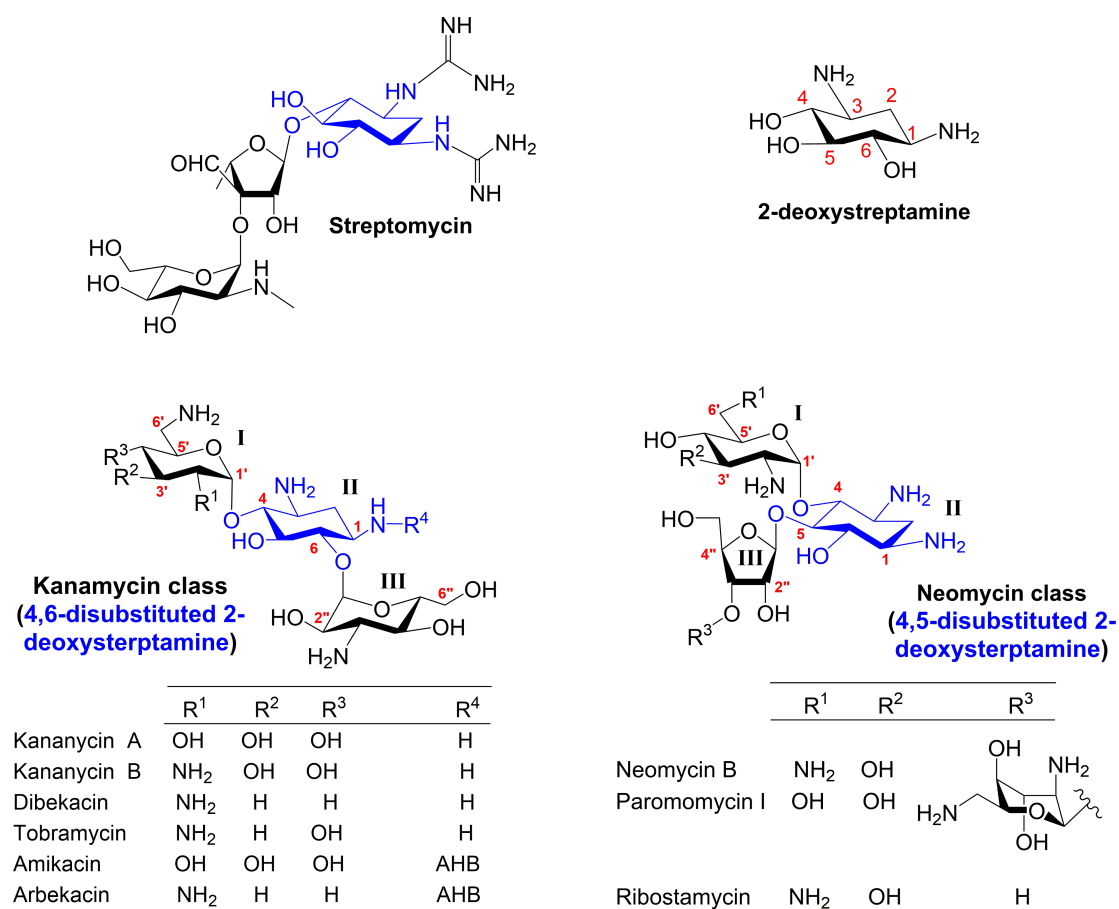


Figure 1-1. Structure of streptomycin, 2-deoxystreptamine, kanamycin, and neomycin classes

1-2 Project 1 Background and Rational: Antifungal amphiphilic kanamycins

The health threat of fungal infections is often underestimated by health agencies and the public due to the prevalence of viral and bacterial infections.¹⁵ Fungal infections mainly affect people with immunosuppressed systems because healthy immune systems are effective at clearing infections. Fungal infections are rapidly rising as a significant health threat in the world, since the population of immunosuppressed people is increasing, and fungal resistance toward antifungal agents is growing. Fungal infections are the leading cause of death for more than 50% of acquired immunodeficiency syndrome (AIDS) patients.¹⁶ Three fungal pathogens are responsible for the high mortality rates of AIDS patients: *Aspergillus fumigatus*, *Candida albicans*, and *Cryptococcus neoformans*.¹⁷ High mortality rates are strongly connected to fungal resistance and the limited number of antifungal compounds.¹⁸ To overcome this problem, new antifungal agents are urgently needed to help save lives.

In 2005, Lee *et al.* reported moderate antifungal activity of AGs (e.g., neomycin, paromomycin, ribostamycin, and streptomycin).¹⁹ Meanwhile, the Chang group had built a vast library of amphiphilic AGs with many kanamycin-based compounds.²⁰⁻²¹ The antifungal report of Lee *et al.* inspired us to screen our amphiphilic AG library against fungi. The screen identified a kanamycin B based compound, **FG08**²² (figure 1-2), a semi-synthetic compound that was active against fungi but not active against bacteria. **FG08** inhibited the growth of *Fusarium graminearum*²³, filamentous fungi that cause Fusarium head blight (FHB), a devastating disease in wheat and barley. Structurally, **FG08** is an amphiphilic kanamycin B analog with three distinctive characteristics in the

third ring: first, the presence of an octyl group at the 4'' position, second, the presence of a hydroxyl group at the 3'' site, and third, the absence of an oxygen at the 6'' location.

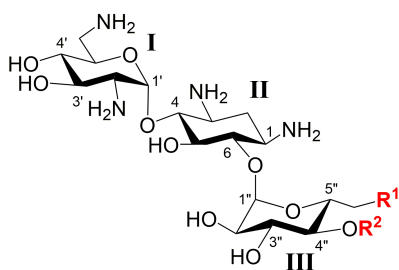
FG08 was synthesized via a difficult glycosylation reaction that requires extremely dry conditions and multiple protection steps for the amino and hydroxyl groups before the glycosylation as well as deprotection steps after.²⁴⁻²⁶

The success of repurposing **FG08** as an antifungal agent raised four questions regarding the effect of each distinctive characteristic on the antifungal activity: 1) the impact of the linear carbon chain length; 2) the effect of the deoxy group; 3) the impact of the alkyl group location; and 4) the effect of the 3'' hydroxyl group. These questions were answered through a structure-activity relationship (SAR) study.

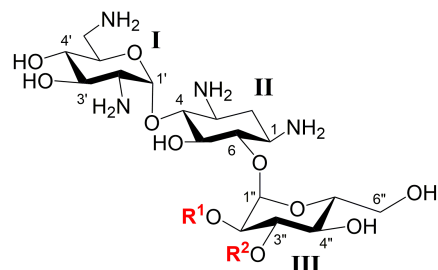
The carbon chain length impact was determined through the synthesis of three compounds: **FG01** with a butyl substitute, **FG02** with a dodecyl substitute, and **FG05** with a hexyl substitute (figure 1-2).^{22, 27} These compounds were biologically less active than **FG08**, which had an octyl substitute. This showed that the octyl substitution is optimal for antifungal activity.

The effect of the deoxy group at site 6'' was evaluated by adding a hydroxyl group at that location to produce **FG03** (figure 1-2). The antifungal activity of this compound was similar to **FG08**, which indicates that the deoxy group has a minor influence on antifungal activity.

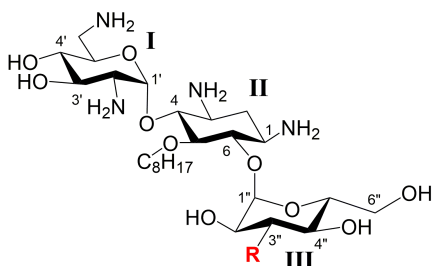
The third part of the SAR study was to examine the effect of the hydroxyl group at the 3'' position on antifungal activity by replacing the hydroxyl group with an amine group to produce **FG15** (figure 1-2). This compound had a comparable antifungal activity



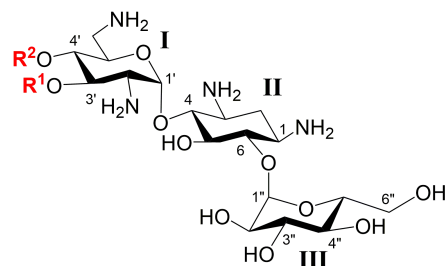
FG01, $R^1 = H$, $R^2 = C_4H_9$
FG02, $R^1 = H$, $R^2 = C_{12}H_{25}$
FG03, $R^1 = OH$, $R^2 = C_8H_{17}$
FG05, $R^1 = OC_6H_{13}$, $R^2 = H$
FG06, $R^1 = OC_8H_{17}$, $R^2 = H$
FG08, $R^1 = H$, $R^2 = C_8H_{17}$



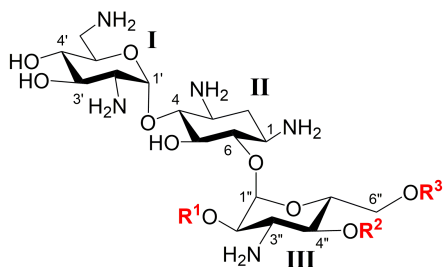
FG07, $R^1 = H$, $R^2 = C_8H_{17}$
FG09, $R^1 = C_8H_{17}$, $R^2 = H$



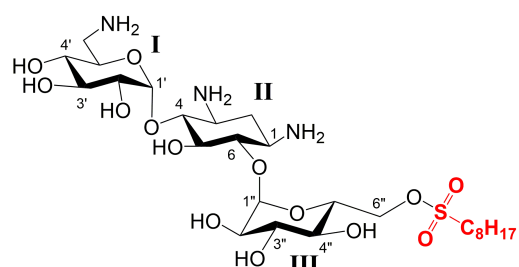
FG10, $R = OH$
FG11, $R = NH_2$



FG12, $R^1 = C_8H_{17}$, $R^2 = OH$
FG13, $R^1 = OH$, $R^2 = C_8H_{17}$



FG14, $R^1 = C_8H_{17}$, $R^2 = H$, $R^3 = H$
FG15, $R^1 = H$, $R^2 = C_8H_{17}$, $R^3 = H$
FG16, $R^1 = H$, $R^2 = H$, $R^3 = C_8H_{17}$
FG17, $R^1 = H$, $R^2 = C_8H_{17}$, $R^3 = C_8H_{17}$
FG18, $R^1 = C_8H_{17}$, $R^2 = C_8H_{17}$, $R^3 = H$



K20

Figure 1-2. Structures of **FG** compounds and **K20**.

to **FG08**, which indicates that the hydroxyl group has a minor influence on antifungal activity.

Finally, the effect of the alkyl chain location was evaluated by synthesizing **FG06** with an octyl substitute at the 4'' position, **FG07** with an octyl substitute at the 3'' position, **FG09** with an octyl substitute at the 2'' site, **FG12** with an octyl substitute at the 3' location, and **FG13** with an octyl substitute at the 4' position (figure 1-2). These compounds are less active than **FG08**, except compound **FG06**, which has comparable antifungal activity to **FG08**. The fungal growth inhibition of the **FG06** shows that the optimal substitution site is the 4'' position followed by the 6'' location. However, **FG08** can be synthesized only in a small quantity (mg scale) due to the complexity of the glycosylation reaction as well as the multiple protection and deprotection steps. The limited amount of **FG08** makes agricultural field studies and medical testing not practical.

Inspired by the antifungal results of **FG06** and the need to devise a large-scale synthesis of amphiphilic kanamycin compounds to make wide usage practical, we developed a direct modification protocol for the second generation of amphiphilic kanamycin analogs.²⁸ Using this protocol, we synthesized a library of kanamycin A analogs substituted at the 6'' position with various alkylsulfonyl groups. These compounds were tested against a panel of bacteria and fungi. Among them, **K20**, which has an octansulfonyl group (figure 1-2), was the most active compound against fungi but did not have any bacterial growth inhibition.

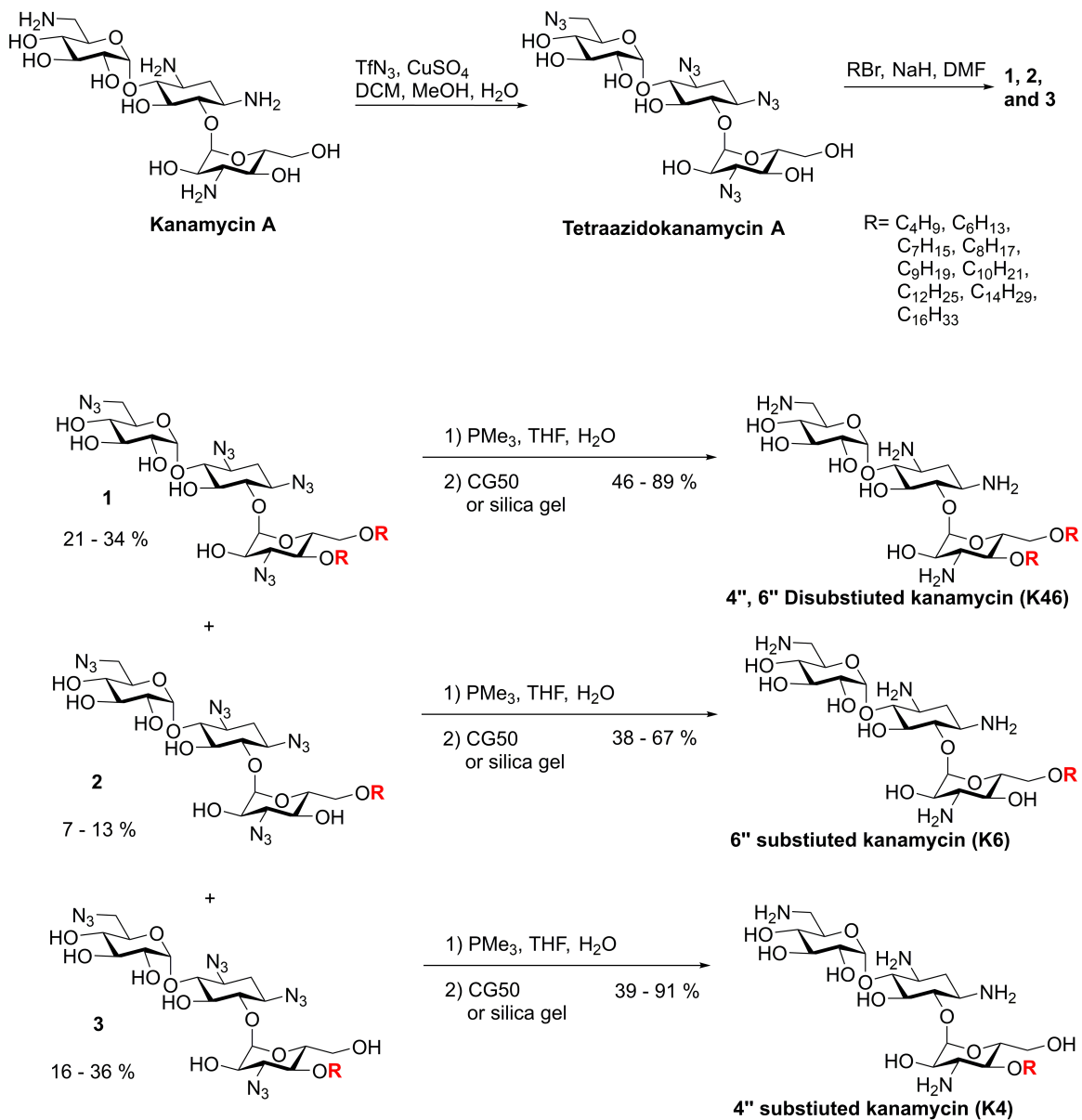
Our group previously studied the effect of four carbon chain lengths: butyl (C4) at the 4'' position, hexyl (C6) at 6'' the position, octyl (C8) at the 4'' position, and dodecyl

(C12) at the 4'' position on antifungal activity. We showed that the octyl group is the optimal carbon chain for an antifungal agent. However, we did not study the effect of other carbon chains. Therefore, we built a new library of amphiphilic kanamycins using a novel three product protocol, starting from a butyl (C4) up to a hexadecyl (C16) (scheme 1-1). In this protocol, a regioselective direct modification of tetraazidokanamycin A is performed using alkyl bromide and sodium hydride. The protocol outcomes are a 4'', 6'' dialkylated tetraazidokanamycin A (**K46**), a 4'' mono-alkylated tetraazidokanamycin A (**K4**), and a 6'' mono-alkylated tetraazidokanamycin A (**K6**).

Several studies, focused on cell membrane permeability, were performed to evaluate the mode of action for amphiphilic aminoglycosides.²⁹⁻³¹ Non-membrane permeable dyes, SYTOXTM green or propidium iodide (PI), were used in these studies. These dyes enter the cell only if the cell membrane integrity has been compromised by an agent, such as an amphiphilic aminoglycoside.³²⁻³³ Before entering the cell, these dyes are non-fluorescent, and upon entering the cell, these dyes will emit fluorescence after they bind to the nucleic acids.

Fosso *et al.* reported that kanamycin B amphiphilic analogs can affect the cell membrane integrity of *Candida albicans* ATCC 64124 by using PI dye. These analogs had a dodecyl (C12) and a tetradecyl (C14) carbon chains attached to kanamycin B at the 6'' position through thioether linkage.³¹ In another study, Subedi *et al.* reported that *Candida albicans* MYA 2876 membrane integrity can be affected by an amphiphilic kanamycin A analog. This compound had an octyl (C8) group substituted at 6' via amide linkage.³⁴

Scheme 1-1.



1-2.1 Project 1: Aims and Objectives for Amphiphilic Kanamycins as Antifungals

This project aims to test the antifungal activity of the new amphiphilic kanamycins against a panel of fungal strains and evaluate the mode of action of these compounds by using non-permeable SYTOXTM green and PI dyes through time-based kinetic membrane permeabilization studies and fluorescence images.

The project objectives are to answer questions regarding new amphiphilic kanamycins: 1) Do aryl substituted kanamycin compounds have similar antifungal and antibacterial profiles to alkyl substituted kanamycins? 2) How do the electric effects of the aryl substitution affect antifungal activity? 3) Does the degree of hydrophobicity affect antifungal activity?

1-3 Project 2 Background and Rationale: Amphiphilic Kanamycins as Connexin Inhibitors

Communication between the adjacent cells is crucial to regulate cell tasks such as growth, embryogenesis, and homeostasis and its accomplished by transferring ions, metabolites, and small molecules.³⁵ This communication is mediated by protein channels called gap junction channels (GJCs) (figure 1-3).³⁶⁻³⁷ Each GJC is formed by a head to head connection of two trans-membrane hemichannels (HCs) (figure 1-3) with one HC from each cell.³⁸⁻³⁹ An HC can pair with a neighbor cell HC to form a GJC or remain unpaired (free).⁴⁰⁻⁴¹ HC is a hexamer of connexins; each connexin (figure 1-4) is built from four helical subunits (i.e., M1, M2, M3, and M4).⁴² These subunits have two extracellular loops called extracellular loop 1 (EL1) and extracellular loop 2 (EL2)

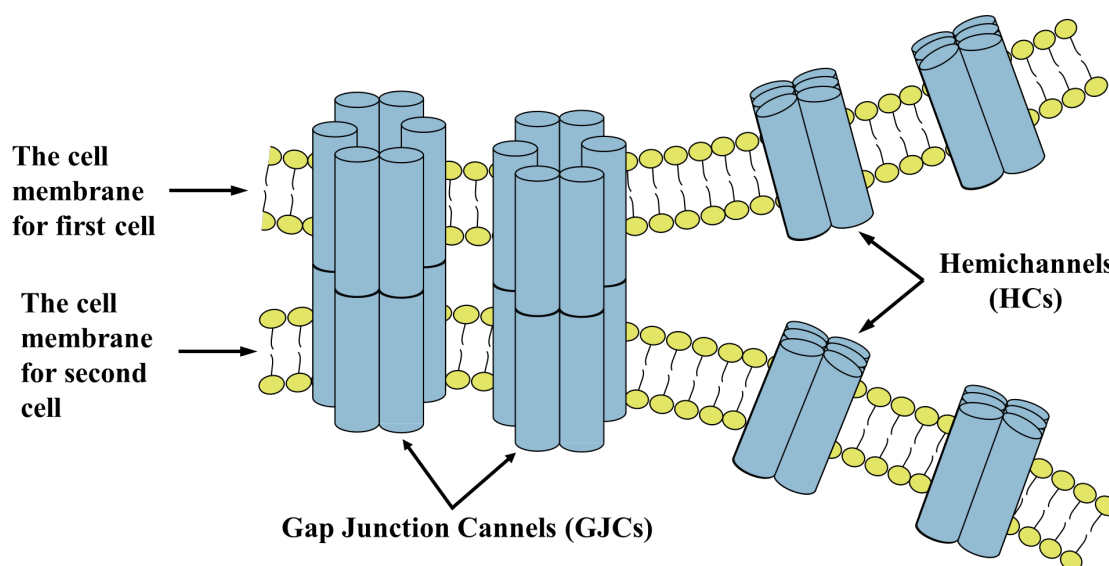


Figure 1-3. Gap junction channels (GJCs) and hemichannels (HCs).

with three cytoplasmatic domains (N-terminal, C-, and cytoplasmatic loop (CL)).⁴³ In mammals, there are more than 20 known connexins with variable functions and they are named based on the molecular wieght.⁴⁴⁻⁴⁷ For example, connexin with ~ 26 kDa is named Cx26.⁴⁸

Connexins are oligomerized to form HC at different areas in the cell depending on the connexin type; for instance, Cx32 is oligomerized in the endoplasmic reticulum/Golgi area while Cx43 is oligomerized in the Golgi network.⁴⁹ After oligomerization, the HC is

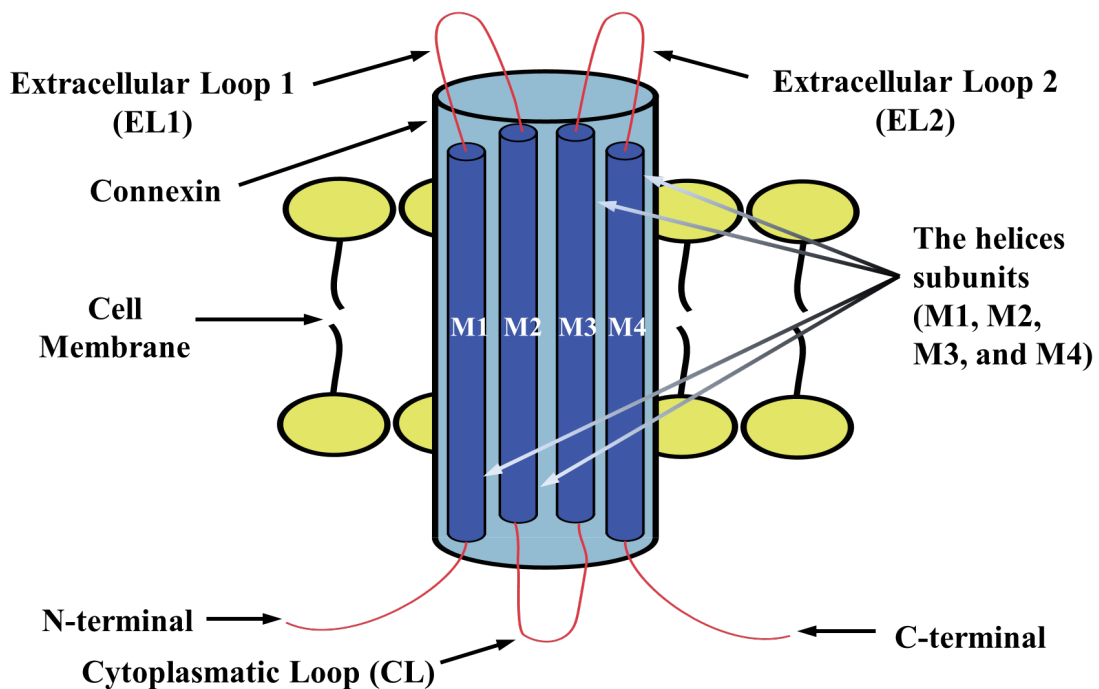


Figure 1-4. Connexin subunits

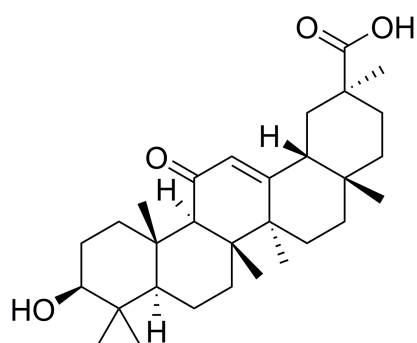
transferred and inserted into the cell membrane while it is in a closed configuration.^{48, 50} HC remains closed most of the time until it is paired with HC from another cell to build at GJC.⁵¹

Recent studies showed that closed HC could open to contribute in the exchange of essential molecules such as ATP, NAD⁺, and glutamate with the extracellular environment.^{40, 48, 50, 52-53} Uncontrolled opening of these free HCs causes cell fluids to leak, which might lead to cell damage and death.⁵⁴ The uncontrolled opening of undocked HCs is strongly believed to be one of the significant causes for non-syndrome human disorders such as child hearing loss.⁴¹

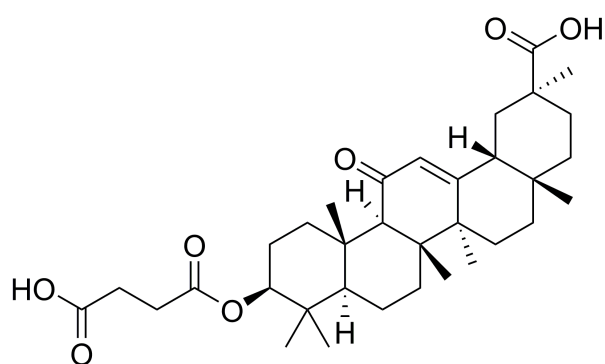
Hearing is a complex process that requires many cell types from many tissues inside the inner ear.⁵⁵ Communication between these cells through GJC is essential for the hearing process. Hearing is an essential sense for vertebrates and is necessary to develop and acquire language in young children.⁵⁶ In the US, newborn hearing loss afflicts almost 2 per 1000 children, of which 15 percent are due to connexin Cx26 mutations in the inner ear. Molecules that prevent the opening of HCs, hereafter called HC inhibitors, could be invaluable in prevent and treating disorders caused by connexin mutations.

Identifying HC inhibitors will be vital toward finding treatment for disorders caused by connexins mutations.⁵⁷ Currently, few compounds have been used as HC inhibitors in research (e.g., glycyrrhetic acid and its derived compound carbenoxolone, quinine, mefloquine, and 2-Aminophenoxyborate) (figure 1-5).⁵⁸⁻⁶¹ These compounds have been found to inhibit both HC and GJC with no selectivity for one over the other.⁶²⁻⁶³ Using peptides as inhibitors has shown to be more selective. However, their mechanism of

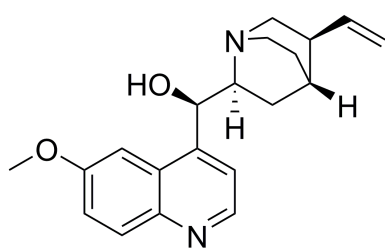
action and their activity in different settings still unclear.⁶⁴



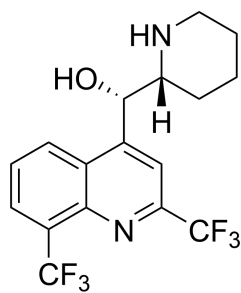
Glycyrrhetic acid



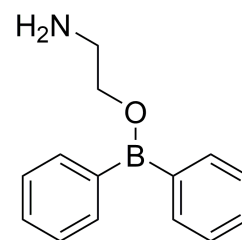
Carbenoxolone



Quinine



Mefloquine



2-Aminophenoxyborate

Figure 1-5. Structures of connexin inhibitors

In contrast, the AG gentamicin (figure 1-6) has shown good selectivity of inhibiting HC without affecting GJC while having good half maximal inhibitory concentration (IC_{50}) at $133.4 \mu M$.⁶⁵ Moreover, commercially available AGs [i.e., kanamycin A, kanamycin B, geneticin (figure 1-6), neomycin, and paromomycin] were tested as HC inhibitors using a recently developed protocol.^{37, 58, 66} These compounds were found to inhibit connexins ($IC_{50} = 0.44 \mu M - 42 \mu M$). Nevertheless, AGs still have toxicity, which has limited their use while amphiphilic aminoglycosides (AAG) have shown less toxicity making them more attractive connexin inhibitor candidates.

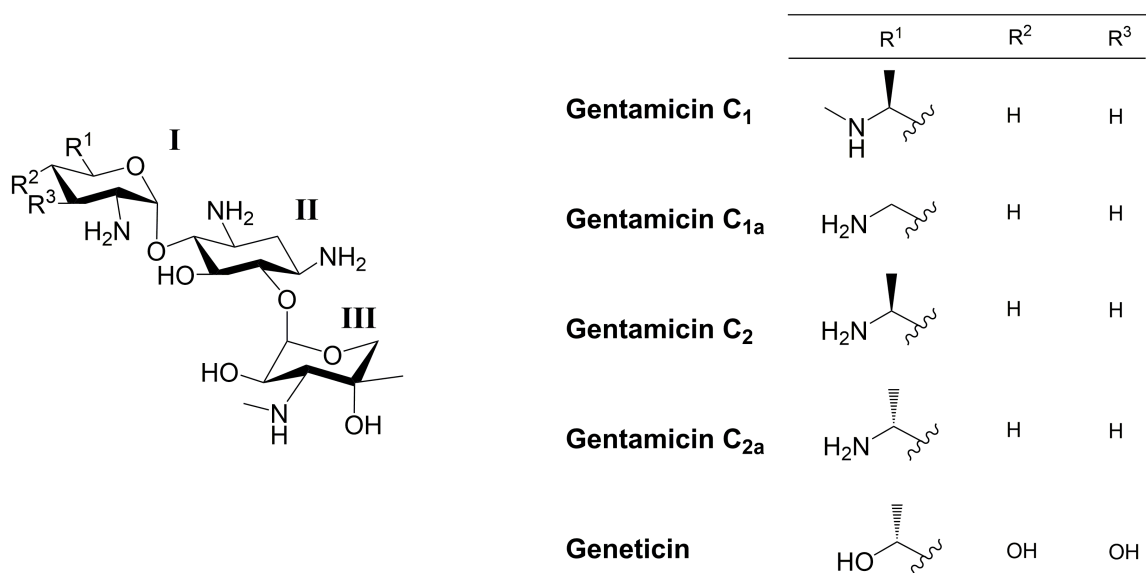


Figure 1-6. Gentamicin and geneticin structures

1-3.1 Project 2: Aims and Objectives for Amphiphilic Kanamycins as Connexin Inhibitors.

The aims of project 2 are to test the inhibitory effects of the 4'' and 6'' diarylated amphiphilic kanamycins on connexin Cx26 activity. Other aims are to evaluate the antibacterial activities against a panel of bacterial strains and to examine the cytotoxicity of these compounds.

The objectives of this project are to perform complete structure activity relationship study to elucidate the effect of two types of substituents, alkyl and aryl on the connexin inhibition activity.

1-4 Introduction to Carbohydrate Esters and Antimicrobial Food Additives.

1-4.1 Project 3 Background and Rational

Antimicrobial food preservatives are chemical compounds that are added to food to prevent spoilage and/or preventing food contamination with pathogenic microbes. Compounds such as sodium benzoate and sodium sorbate are used as antimicrobial food preservatives.

Sodium benzoate is listed as “generally regarded as safe” (GRAS) by the U.S. Food and Drug Administration (FDA).⁶⁷⁻⁶⁸ It is widely used as a food preservative especially for acidic foods such as soft drinks and jams, and is also common in medicine.⁶⁹ Nevertheless, this compound is not entirely safe. It has been reported that traces of carcinogenic benzene has been found in soft drinks, which indicates decarboxylation of sodium benzoate has occurred.⁷⁰⁻⁷²

Sodium sorbate is used as a preservative in ketchup, meat, cheese, and mayonnaise.⁷³ This compound has been banned by the European Food Safety Authority (EFSA) from being used as a food preservative because of its possible genotoxic properties.⁷³ Sodium sorbate is still permitted as a food preservative in the US, Australia, Canada, and New Zealand.⁷⁴ Due to the potential health issues of these common preservatives, there is growing interest in developing new antimicrobial food additives.⁷⁵

Carbohydrate esters (CE) are class of amphiphilic compounds that have a wide range of applications.⁷⁶⁻⁷⁹ These compounds are tasteless, odorless, non-toxic, and non-irritating and can be degraded by organisms into a sugar and a fatty acid, which are safe and nutritional.⁸⁰⁻⁸² Sucrose mono, di, and triesters are already approved by the FDA to be

used as emulsifiers in food and medicine.⁸³ The sucrose esters world annual production is 4000 tons, the highest among the CE.

Some CEs have been reported as possessing antimicrobial properties.⁸⁴⁻⁸⁵ A library of sucrose stearate (C18), sucrose laurate (C12), sucrose caprate (C10), maltose caprate (C10), fructose caprate (C10), and glucose caprate (C10) were reported.⁷⁹ These esters were tested against five food pathogens: *Escherichia coli* AS1.90, *Salmonella typhimurium* AS1.1174 (gram-negative bacteria), *Staphylococcus aureus* AS1.89, *Bacillus subtilis* AS1.1849, and *Bacillus cereus* AS1.1846 (gram-positive bacteria). Sucrose caprate was the most active compound against these strains.

In another study, a series of CEs derived from glucose and mannose with various fatty acids were synthesized.⁸⁶ Testing of the CEs toward different *S. aureus* bacterial strains [ATCC 25923, NCTC 1803, methicillin resistance *S. aureus* (MRSA) ATCC 33591, MRSA ATCC 33592, and MRSA ATCC 43300] showed that methyl 6-*O*-lauroyl- β -D-glucopyranoside was the most active compound. Furthermore, the mode of action study for glucose derived compounds showed that these CEs are membrane disrupters.

All the reported CEs are acylated at the primary hydroxyl group on the carbohydrate. Saccharides have multiple secondary hydroxyl groups with one or more primary hydroxyl groups. The primary hydroxyl group is slightly more reactive than the secondary hydroxyl groups, but selective acylation at that primary hydroxyl group is challenging through conventional chemical synthesis route, and it is not challenging through the enzyme-catalyzed reactions.⁸⁷ In conventional CE chemical synthesis, selective acylation can be achieved by protecting the secondary hydroxyl groups leaving

the primary hydroxyl group free for esterification then deprotection.^{84, 88} This process is not economic because it needs more time, chemical reagents, and labor.

In the industry, CEs are chemically synthesized using high temperatures (i.e., 100 to 185 °C).⁸⁹ Using high temperatures produce byproducts with multi degree of esterification. These byproducts require separation that add cost to the overall process.

Enzymes such as lipase have been used as catalysts for CE synthesis reactions via esterification of a carbohydrate with a fatty acid and transesterification of a sugar with a vinyl fatty acid ester. These are one step reactions with very high acylation regioselectivity at the primary hydroxyl group.⁸⁹ Enzyme catalyzed synthesis reactions need mild pH (6.3 to 8.0) and temperature (37 to 57 °C) conditions. However, this method of CE synthesis has three drawbacks that limit its application in synthesis of CEs: viable solvents, dry reaction conditions, and substrate specificity.

The solvent choice is a significant challenge. It should dissolve a polar carbohydrate and a nonpolar fatty acid or fatty acid vinyl ester as well as the enzyme itself. In many cases, solvents that can dissolve both of the substrates such as N,N-dimethylformamide (DMF) or pyridine, which might reduce the enzyme activity.⁹⁰

Lipase usually catalyzes ester hydrolysis to a fatty acid and an alcohol, but it also can catalyze ester formation (reversing the reaction) under anhydrous solvent conditions.⁹¹ Under these conditions, the lipase has high substrate specificity which can be seen in the variable reaction yields for variable sugar and variable carbon chain lengths.⁹²

1-4.2 Project 3: Aims and Objectives for CEs as Antimicrobial Food Additives

This work is aimed at finding and developing a protocol that combines advantages from both chemical and enzymatic CE synthesis. The protocol should be done in short reaction steps to be economical in terms of cost and labor, and it should be applicable for a wide range of sugars. In addition to that, it should have high selectivity for the primary hydroxyl group over the secondary hydroxyl groups and should be scalable for large scale syntheses.

The objectives are to study the structure-activity relationship (SAR) for different mono sugars with different acyl carbon chain lengths. The synthesized CE compounds can help to identify the optimal mono sugar and the optimal carbon chain (lead compound) after screening the biological activities of these CEs against a panel of bacterial and fungal strains. The lead compound can be further tested against mammalian cells to ensure it is not toxic and it might be an excellent candidate to be an antimicrobial food additive.

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CHAPTER 2

ANTIFUNGAL ACTIVITIES OF 4'',6'' -DISUBSTITUTED AMPHIPHILIC
KANAMYCINS ^a**2-1 Abstract**

Amphiphilic kanamycins derived from the classic antibiotic kanamycin have attracted interest due to their novel bioactivities beyond inhibition of bacteria. In this study, the recently described 4'',6''- diaryl amphiphilic kanamycins reported as inhibitors of connexin were examined for their antifungal activities. Nearly all 4'', 6''-diaryl amphiphilic kanamycins tested had antifungal activities comparable to those 4'', 6''- dialkyl amphiphilic kanamycins, reported previously against several fungal strains. The minimal growth inhibitory concentrations (MICs) correlated with the degree of amphiphilicity (cLogD) of the di-substituted amphiphilic kanamycins. Using the fluorogenic dyes, SYTOXTM green and propidium iodide, the most active compounds at the corresponding MICs or 2×MICs caused biphasic dye fluorescence increases over time with intact cells. Further lowering the concentrations to half MICs caused first order fluorescence increases. Interestingly, 4×MIC or 8×MICs levels resulted in fluorescence suppression that did not correlate with the MIC and plasma membrane permeabilization. The results show that 4'',6''-diaryl amphiphilic kanamycins are antifungal and that amphiphilicity parameter cLogD is useful for the design of the most membrane-active versions. A cautionary limitation of fluorescence suppression was revealed when using fluorogenic dyes to measure cell-permeation mechanisms with these antifungals at high concentrations. Finally, 4'',6''-diaryl amphiphilic kanamycins elevate the production of

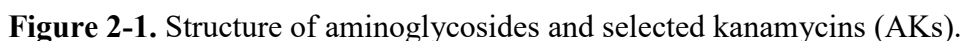
cellular reactive oxygen species as other reported for amphiphilic kanamycins.

^a Adapted from (AlFindee, M. N.; Subedi, Y. P.; Grilley, M.; Takemoto, J. Y.; Chang, C.-W. T. “Antifungal activities of 4'',6''-disubstituted amphiphilic kanamycins” *Molecules*. **2019**, *24*(10), 1882). Copyright © 2019, MDPI.

2-1 Introduction

Although commonly unappreciated, fungal diseases cause tremendous economic loss and health impacts globally. Annually, more than a billion people acquire fungal infections. The death rate from fungal diseases is equal to that of tuberculosis and malaria combined, with approximately 10% of those deaths due to cryptococcal meningitis ¹⁻². Immunosuppressed individuals, such as those infected with HIV or undergoing treatment for cancer or organ transplants, are especially susceptible to invasive fungal infections. Current treatment options for fungal infections include polyene-based compounds (e.g., amphotericin B) [3–5], cytosine-based compounds (e.g., flucytosine) [5,6], and azole-based compounds (e.g., itraconazole and fluconazole) [5,7,8]. However, as exemplified in the recent outbreak of *Candida auris* [9], drug resistance is a growing problem. Further research to develop new and effective treatments for fungal diseases is urgent.

Kanamycin belongs to a class of antibacterial compounds known as aminoglycosides that are active against both Gram-negative (G^-) and Gram-positive (G^+) bacteria, albeit its clinical use is limited due to the emergence of bacterial resistance (Figure 2-1) [10]. To overcome the problem of bacterial resistance, extensive research has been devoted to structural modifications of aminoglycosides that lead to the discovery of amphiphilic aminoglycosides [11,12]. In contrast to antibacterial kanamycin



AKs are known to show their antimicrobial activity by increasing the membrane permeability of microorganisms [17–21]. Fluorogenic dyes, such as SYTOX™ green and propidium iodide (PI), are commonly employed for the study of membrane

permeabilization (Figure 2-2). SYTOXTM green is non-fluorescent and cannot penetrate the plasma membrane of intact organisms. However, in the presence of agents that compromise membrane integrity, SYTOXTM green enters the cytoplasm, binds to nucleic acids, and emits fluorescence. Propidium iodide (PI) has similar properties and is widely used for evaluating membrane permeabilization of substances in fungi and bacteria.

We recently reported the synthesis of 4'',6''-diaryl AKs (compounds **1** – **8**) as connexin inhibitors [22,23] (Figure 2-3). Since these are structurally similar to the antifungal 4'',6''-dialkyl AKs (compounds **9** – **13**), we decided to investigate their antifungal activities and to conduct mode of action studies of both groups of compounds using fluorogenic dyes.

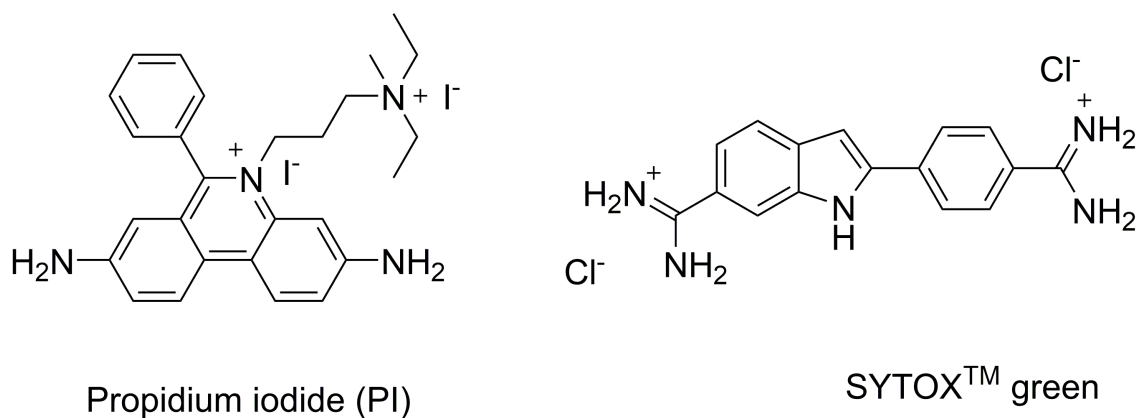


Figure 2-2. Structures of SYTOXTM green and propidium iodide (PI).

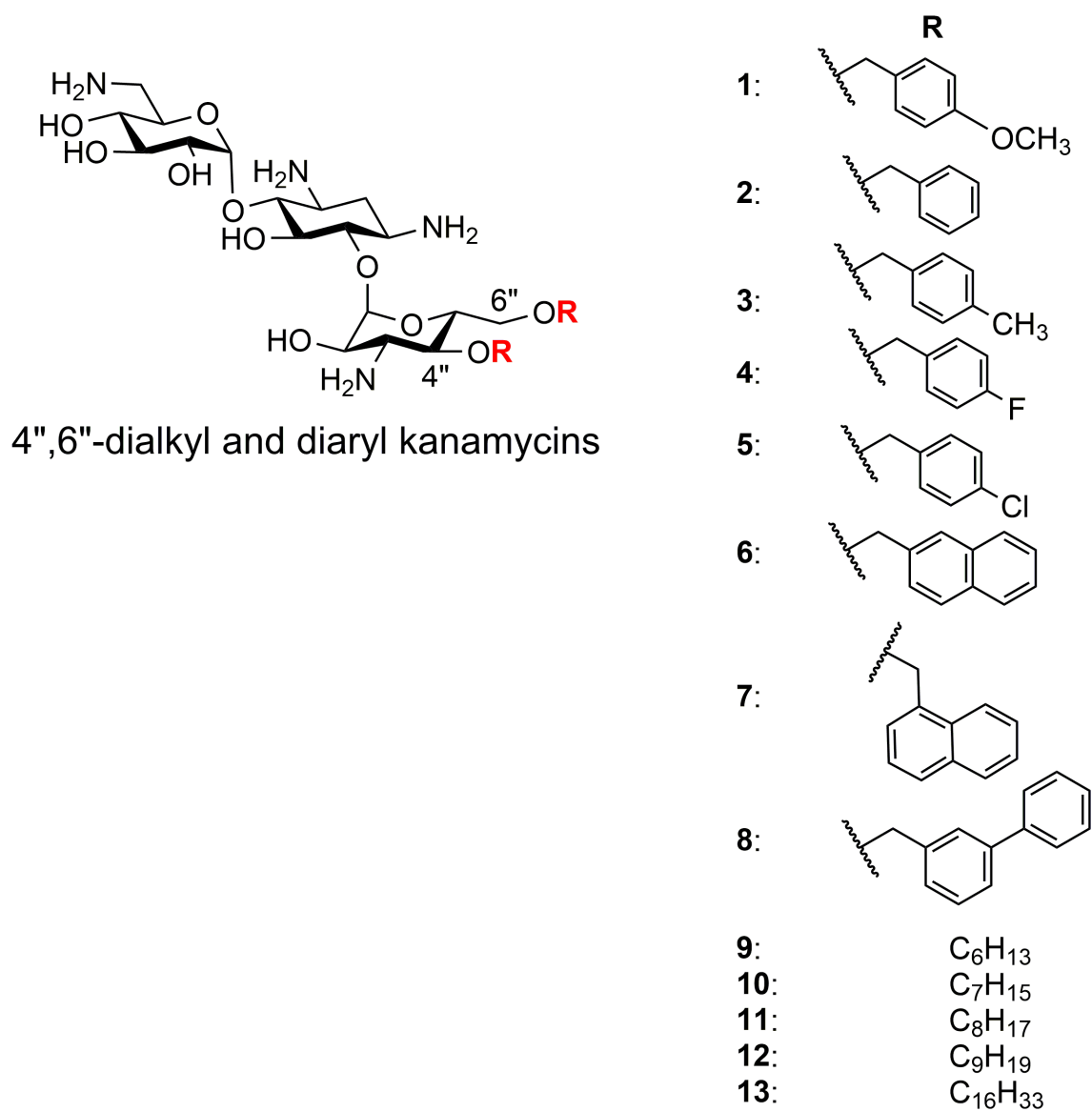


Figure 2-3. Structure of selected AKs.

2-2 Results and Discussion

2-2.1. Fungal Growth Inhibition by 4'',6''-Disubstituted AKs

Thirteen disubstituted AKs were examined for growth inhibition capabilities against a panel of fungi that included yeasts and the filamentous fungus *Fusarium graminearum* (Table 2-1). The 4'', 6''- diaryl compounds **6**, **7**, and **8** were strongly inhibitory toward *F. graminearum* (minimal growth inhibitory concentrations, MICs, 2 – 16 µg/mL) and compounds **1**– **5** had low (MICs, >32 µg/mL) to moderate (MICs, 16 – 32 µg/mL) inhibitory activities. Likewise, except with compounds **1** and **2**, *Cryptococcus neoformans* H99 and *C. neoformans* VR-54 were highly susceptible to the diaryl compounds (MICs, 2 – 16 µg/mL) as was *Rhodotorula pilimanae* (ATCC 26423) (MICs, 2 – 32 µg/mL). In contrast, except with compounds **4** and **6**, *Candida albicans* 64124 (azole-resistant) and *C. albicans* MYA2876 (azole sensitive) were not susceptible to the diaryl compounds (MICs, >32). The 4'', 6''-dialkyl compounds, except **13**, displayed strong inhibitory activities against the *C. neoformans* strains (MICs, 2 – 16 µg/mL), and moderate activities against *Candida* strains 64124 and MYA2876 (MICs, 8 – 128 µg/mL). Compound **13**, with a long linear alkyl chain (C16), had no antifungal activity (MICs, = or >256). Overall, except for compounds **1**, **2** and **13**, the 4'', 6''-disubstituted AKs (10 of 13) were strongly inhibitory to *F. graminearum*, *C. neoformans* and *R. pilimanae* (ATCC 26423), but less so or moderately with the *C. albicans* strains.

Table 2-1. Minimal growth inhibitory concentrations (MICs) of AKs against fungal strains

Compound	cLogD	Strains					
		A	B	C	D	E	F
1	-10.32	32-64	256	256	32	64	128
2	-9.81	256	256	>256	32	64	64
3	-8.88	64-128	>256	256	8	16	32
4	-9.53	16-32	32-64	32-64	4	8	16
5	-8.77	16-32	64	128	4-8	8	8
6	-7.81	4-16	32-64	64	2-4	8	8
7	-7.81	4-8	128	128	8-16	4	8
8	-6.44	2-8	128-256	256	8-16	4	2-4
9	-9.36	32	32	16-32	8-16	8	8
10	-8.57	32	ND	8-16	2	4	2
11	-7.78	16	ND	128	8-16	8	8-16
12	-6.99	ND	8	16-32	4	4	4
13	-1.44	>256	>256	>256	>256	256	>256
K20	-11.62	32	16	32	8-16	8	16

MIC unit: µg/mL; ND: Not determined; Strains; (**A**) *Fusarium graminearum* B4-5A, (**B**) *Candida albicans* 64124, (**C**) *Candida albicans* MYA-2876, (**D**) *Rhodotorula Pilimanae* (ATCC 26423), (**E**) *Cryptococcus neoformans* H99, (**F**) *Cryptococcus neoformans* VR-54.

2-2.2. Analysis of Correlation Between MIC and cLogD

Structurally diverse AKs have been synthesized for the purpose of elucidating structure-activity relationships (SAR) [7,20]. These AKs carry variations of hydrophobic moieties or linkages of the hydrophobic moieties to the kanamycin core. Several factors that may contribute to the differences of antimicrobial activity, such as the chain length of the hydrophobic moiety and the linkage, were deduced primarily on the revealed MICs of these AKs. Despite these labor-intensive efforts, contradictory SARs were noted, and these factors seemed to escalate the complexity in understanding the nature of the antimicrobial selectivity of AKs. Thus, we explored the use of a water/1-octanol distribution coefficient (cLogD) as a simplified means to evaluate the amphiphilicity of the disubstituted AK compounds and possible correlation with antifungal activity. The cLogD was calculated using Marvin Sketch (version 18.19) keeping a 0.1 molar concentration of Na⁺, K⁺, and Cl⁻ ions (Table 1). When plotting cLogD (x-axis) vs. MICs (y-axis) for all tested fungi (z-axis), a clearer SAR was observed (Figure 2- 4). Compounds with cLogD values between -9.5 and -6.4 had the lowest MIC values against *F. graminearum*, *C. neoformans* (two strains) and *R. pilimanae*. cLogD values above (e.g. compound **13**) or below (e.g., compounds **1** and **2**) this range corresponded to the highest MIC values. Such a trend was not observed with *C. albicans* (two strains) because of fluctuating MIC values. This analysis suggests that it is possible to use cLogD as a guideline for designing antifungal AK of the same class, which can drastically reduce the synthetic burden.

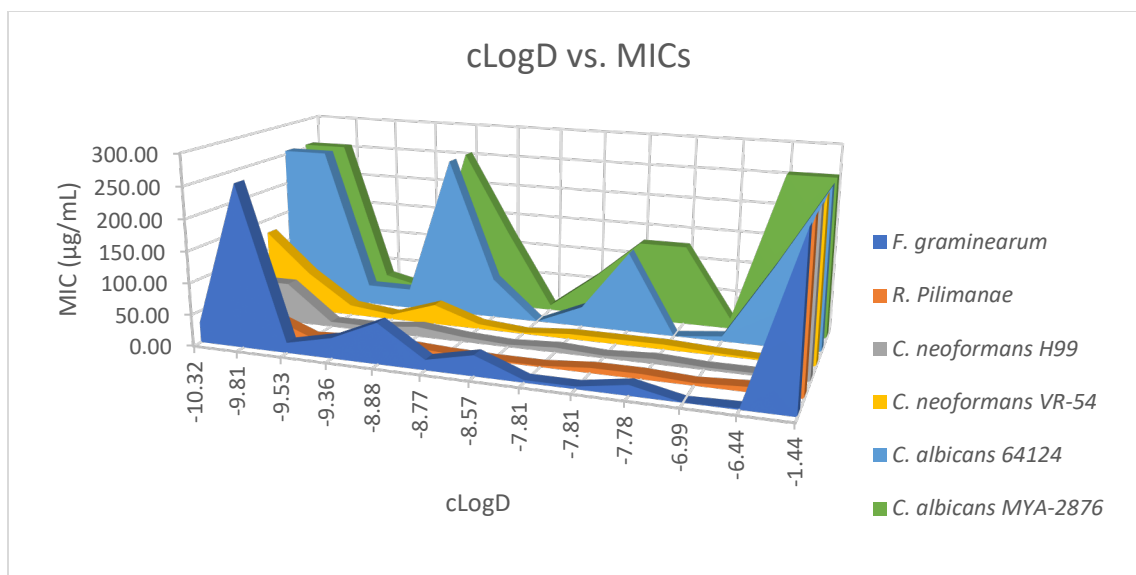


Figure 2-4. Relationship of (cLogD) VS. MIC

2-2.3. Plasma Membrane Permeabilization of 4'',6''-Disubstituted Kanamycins

Plasma membrane permeabilization studies were conducted by light microscopy using the dyes SYTOXTM green with *C. neoformans* H99 treated with diaryl compound **7** or the dialkyl compounds (**11** and **13**) at 1×MIC (Figure 2-5). Triton X-100 (1%), a non-fungal targeting agent, but known to cause membrane permeabilization of mammalian cells, was used for comparison. As expected, most fungal cells treated with compounds **7** and **11** showed fluorescence emitted from SYTOXTM green. In contrast, almost no cells emitted fluorescence when treated with compound **13** (256 µg/mL). A few fungal cells emitted fluorescence when treated with Triton X-100, suggesting that this agent at 1% concentration causes a small degree of membrane permeabilization.

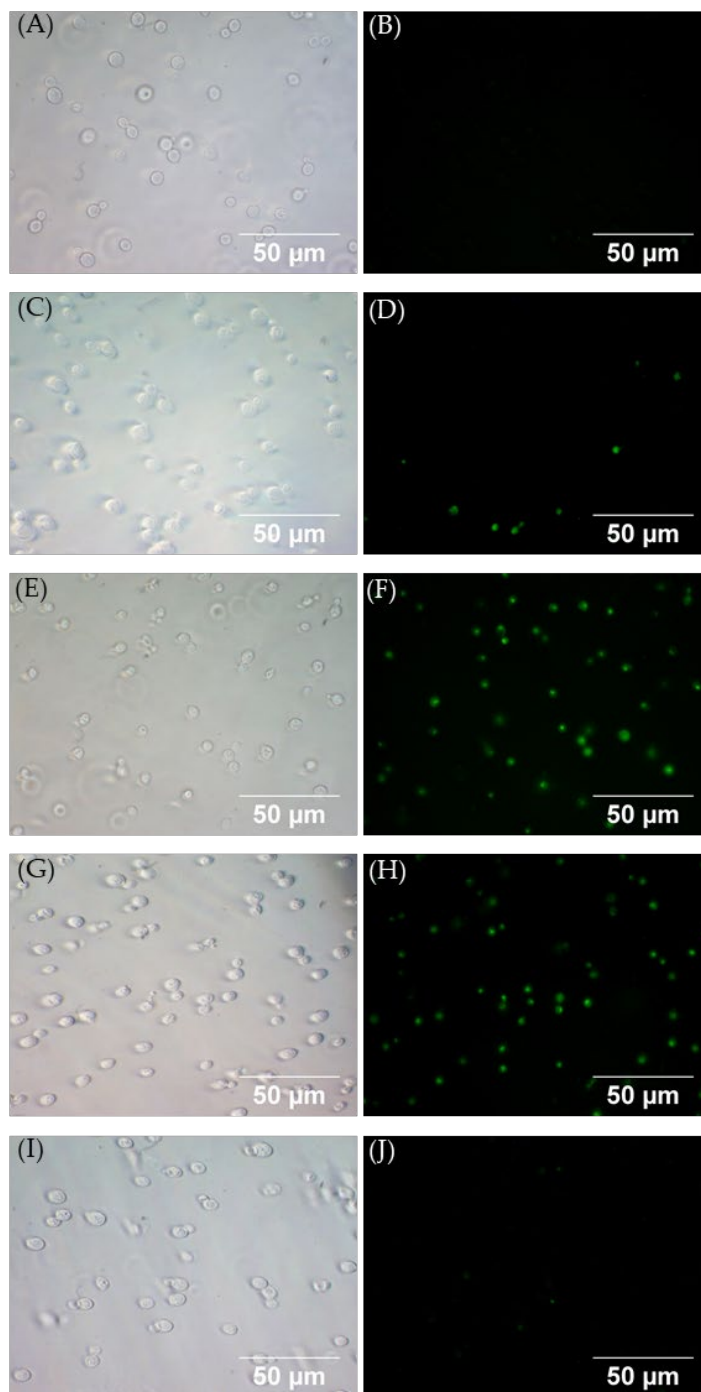


Figure 2-5. Phase contrast (left panels) and fluorescent images (right panels) of *C. neoformans* H99. (A) and (B): Images of cells treated with SYTOX™ green alone; (C) and (D): Images of cells treated with SYTOX™ green and 1% Triton X-100; (E) and (F): Images of cells treated with SYTOX™ green and compound **7** (1x MIC); (G) and (H): Images of cells treated with SYTOX™ green and compound **11** (1x MIC); (I) and (J): Images of cells treated with SYTOX™ green and compound **13** (256 μg/mL).

We previously reported that fungi treated with active AKs with hydrophobic groups attached at the 6' position displayed a fast increase of dye fluorescence observed in a time-dependent fashion [24]. To determine if the disubstituted AKs behave similarly, time-dependent kinetic membrane permeabilization experiments were performed using *C. neoformans* H99 fungi at 1×MIC of the AKs. The relative fluorescence unit (RFU) was monitored every 3 min for 4 h.

The most growth inhibitory 4'',6''-disubstituted AKs (compounds **5**, **6**, **7**, and **8**) caused the highest levels of SYTOXTM green fluorescence. For most of the tested compounds, the kinetics of the fluorescence signals revealed biphasic fluorescence increases over time: A fast membrane permeabilization within the first 15 min that quickly levels to various RFUs (Figure 2-6). The leveled units, in general, follow similar orders in all experiments using SYTOXTM green or PI. The less active AKs (**1**, **2** and **3**) showed profiles similar to Triton X-100. Despite the overall similarities of the biphasic fluorescence profiles, differences were observed between the profiles. First, different total RFU levels (at 4 h) were achieved with different AKs and with the two dyes despite using the same fungal cell densities, fluorogenic dye concentrations, and compounds at 1×MIC. Second, in three experiments (Figure 2-6B – D), several AKs caused total RFU levels lower than controls with no AK. Third, certain compounds displayed different RFU kinetic profiles using SYTOXTM green vs PI. For example, the RFU kinetic profiles of compounds **2** and **4** appeared flat or linear using PI (Figure 2-6B) but biphasic using SYTOXTM green (Figure 2-6A). For compounds **5** and **6**, different RFU profiles were obtained from the experiments using SYTOXTM green vs. PI. For compound **12**, the RFU profiles were linear using SYTOXTM green but biphasic using PI (Figure 2-6C, D).

Because the only variable parameters in these experiments were the individual AKs and their MICs, it was speculated that the fluorescence properties of the dyes reflected in the RFU and kinetic profiles were directly affected by individual AKs and their concentrations. To explore this hypothesis, we carried out further experiments using 2-fold adjusted concentrations of the MICs of the AKs.

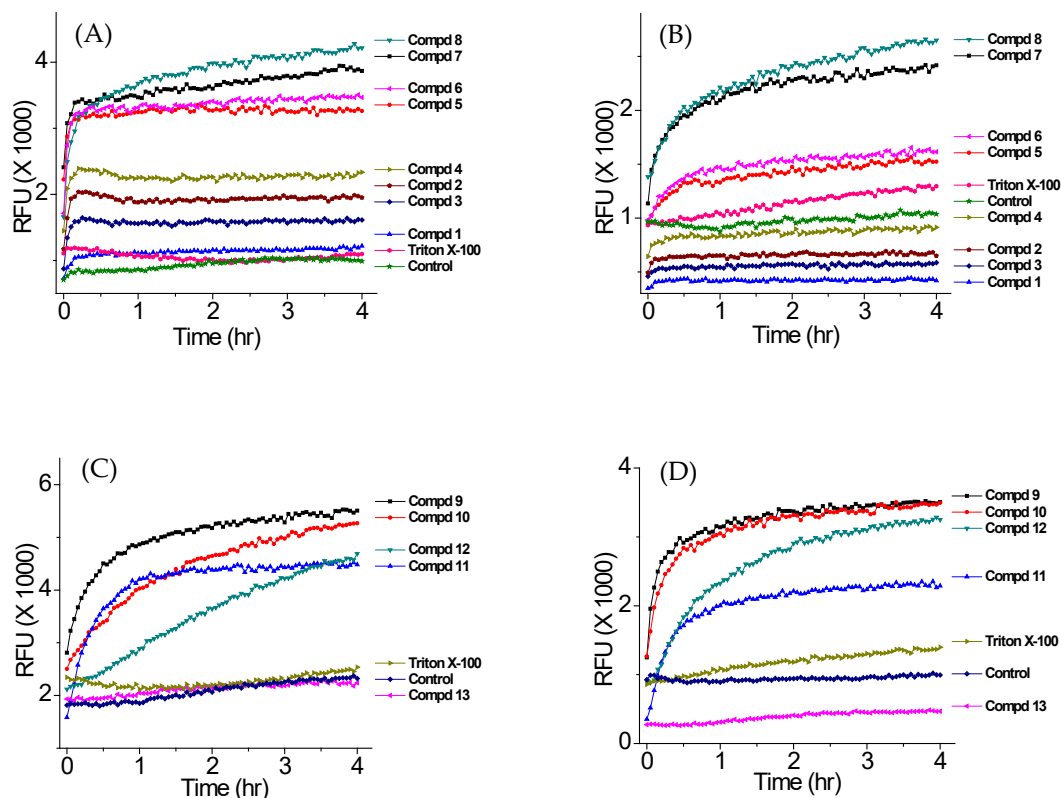


Figure 2-6. Kinetic membrane permeabilization of *C. neoformans* H99. (A) Cells treated with 4'',6''-diaryl AKs using SYTOXTM green; (B) cells treated with 4'',6''-diaryl AKs using PI; (C) cells treated with 4'',6''-dialkyl AKs using SYTOXTM green; (D) cells treated with 4'',6''-dialkyl AKs using PI. The figure with standard deviation is available in figure A-1.

Fluorescence kinetics and levels with selected 4'', 6'' disubstituted AKs at various concentrations were measured with *C. neoformans* H99 using SYTOXTM green and PI (Figure 2-7). It was observed that the AK concentration significantly influenced the degree of fluorescence independently of MIC and membrane permeabilization capabilities. For example, compound **7** showed linear fluorescence kinetics at 0.5×MIC, biphasic kinetics at 1×MIC, biphasic kinetics but leveled at lower RFUs at 2×MIC, and suppression at 4×MIC and 8×MIC (Figure 2-7A,B). The same trend was observed when using PI. For compound **10**, similar kinetic profiles were obtained at 1×MIC but not when 2×MIC was employed (Figures 2-7C,D). The kinetic RFU profiles of compound **11** behaved like those of compound **7** (Figure 2-7E,F). These data support our speculation that the fluorescence of dyes can be affected by the concentration of the tested AK compounds.

Images of fungi treated with compound **11** provide further evidence for the direct effect of the AK concentration on fluorescence (Figure 2-8). Cells treated with compound **11** and PI for 2 h, emit fluorescence at 0.5x and 1×MICs but little or no fluorescence at 8×MIC. A similar result was obtained with SYTOXTM green.

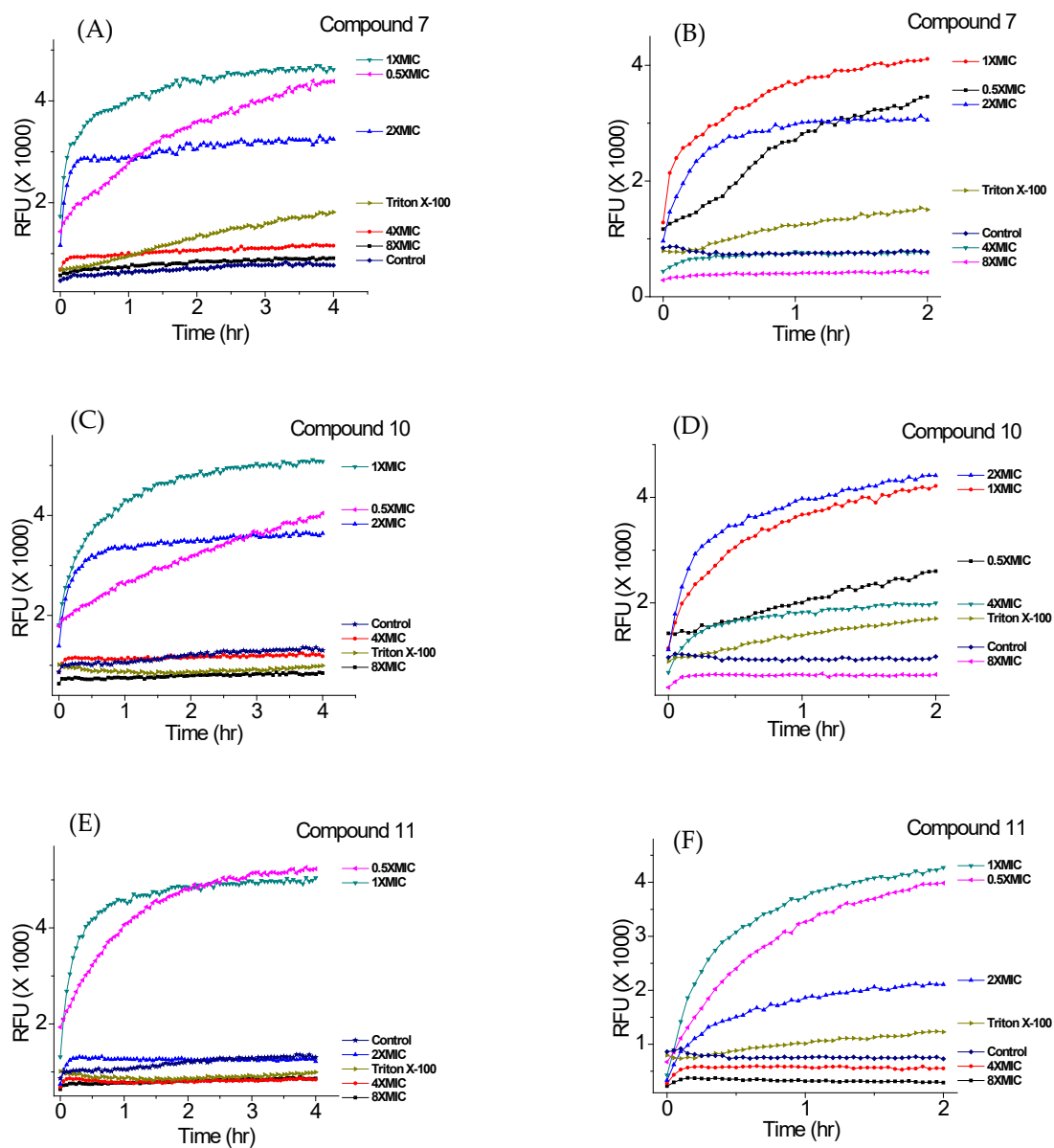


Figure 2-7. Kinetic membrane permeabilization using various concentrations of AKs. (A): Cells treated with compound **7** using SYTOXTM green; (B): Cells treated with compound **7** using PI; (C): Cells treated with compound **10** using SYTOXTM green; (D): Cells treated with compound **10** using PI; (E): Cells treated with compound **11** using SYTOXTM green; (F): Cells treated with compound **11** using PI. The figure with standard deviation is available in figure A-2.

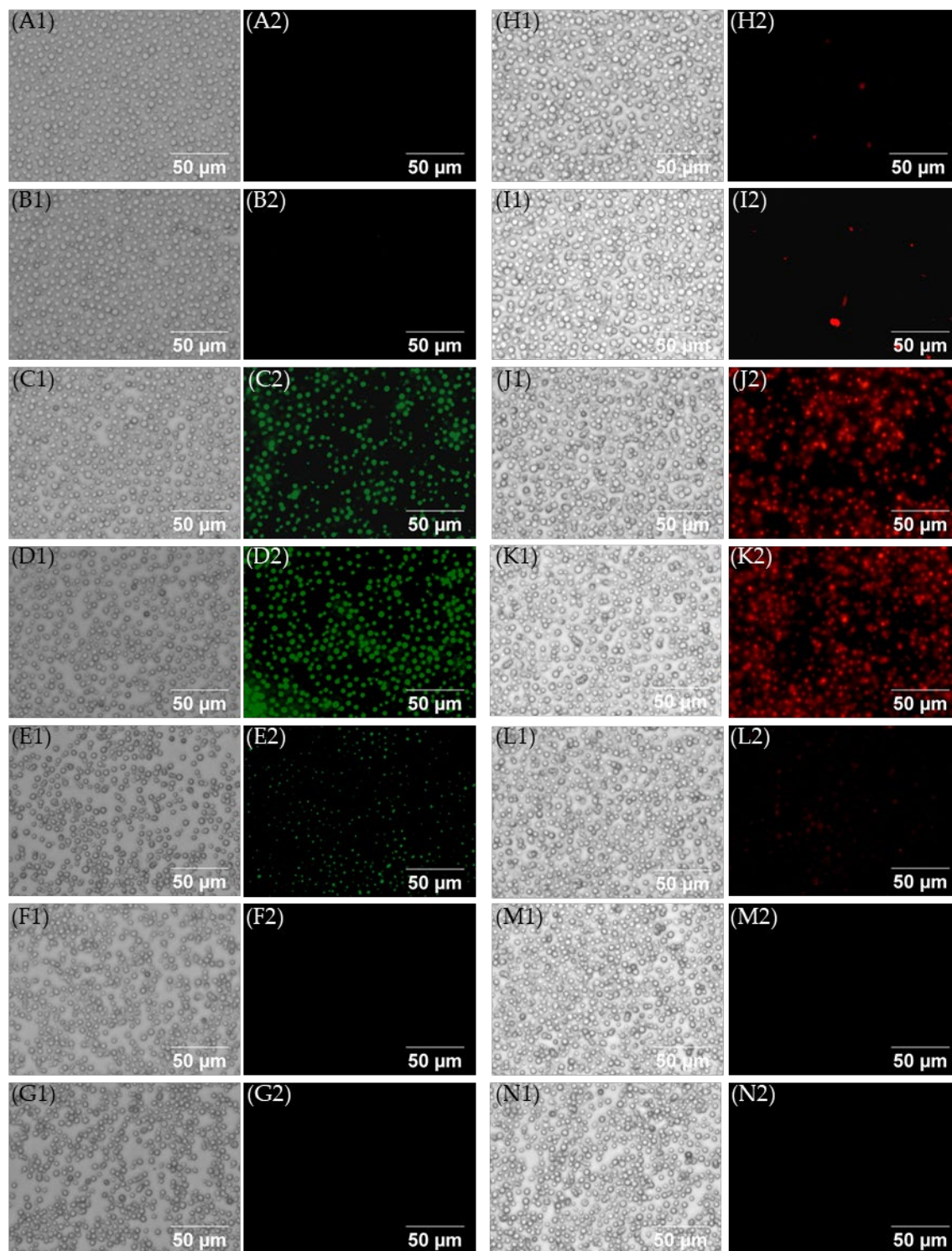


Figure 2-8. Images of *C. neoformans* treated with varied concentrations of compound **11**. (A1, A2) Cells treated with only SYTOX™ green; (B1, B2) cells treated with Triton X-100 and SYTOX™ green; (C1, C2) cells treated with 0.5×MIC of **11** and SYTOX™ green; (D1, D2) cells treated with 1×MIC of **11** and SYTOX™ green; (E1, E2) cells treated with 2×MIC of **11** and SYTOX™ green; (F1, F2) cells treated with 4×MIC of **11** and

SYTOXTM green; (G1, G2) cells treated with 8×MIC of **11** and SYTOXTM green; (H1, H2) cells treated with only PI; (I1, I2) cells treated with Triton X-100 and PI; (J1, J2) cells treated with 0.5×MIC of **11** and PI; (K1, K2) cells treated with 1×MIC of **11** and PI; (L1, L2) cells treated with 2×MIC of **11** and PI; (M1, M2) cells treated with 4×MIC of **11** and PI; (N1, N2) cells treated with 8×MIC of **11** and PI.

These results indicate that there is an optimal ratio of AK vs. fluorogenic dye when measuring plasma membrane permeabilization by AK compounds. By considering the MICs that gave rise to the maximum leveled RFU, the concentration fell in the region around 5 μ M of AK and 0.01 μ M of SYTOXTM green or 0.4 μ M of PI. Judging from the structures of the AKs, SYTOXTM green and PI (Figure 2-2), it is likely that ionic or hydrogen bonding contribute to the AK inhibiting effect toward these fluorogenic dyes. Therefore, caution needs to be taken when evaluating the cellular effect of AKs or other classes of compounds using fluorogenic probes. Finally, since high RFUs were observed with the use of AK levels at < 1x MIC, possibilities are opened for using such low AK concentrations to detect fungi without significant fungicidal effect.

2-2.4. Effect of 4'',6''-Disubstituted Kanamycins on the Production of Reactive Oxygen Species

It has been reported that a wide range of antimicrobials of different classes, albeit having various modes of action, will have a common effect of increased oxidative stress by promoting the production of reactive oxygen species (ROS), which can lead to cell death. We have also demonstrated that 6'-substituted AKs exert this common effect. Thus, several selective 4'',6''-disubstituted kanamycins, compounds **4**, **7** and **11**, were subjected to the investigation of induced ROS production. The experiments of ROS production were conducted using 1,1'-(hexane-1,6-diyl)bis(3-decyl-4,9-dioxo-4,9-

dihydro-1H-naphtho[2,3-d][1,2,3]triazol-3-ium triflate (**14**) (Figure 2-9) [25,26], known to produce ROS, as the positive control. Compound **4** showed almost no ROS generation as compared to control, while compound **11** showed moderate ROS generation, and compound **7** showed a much higher level of ROS generation (Figure 2-9).

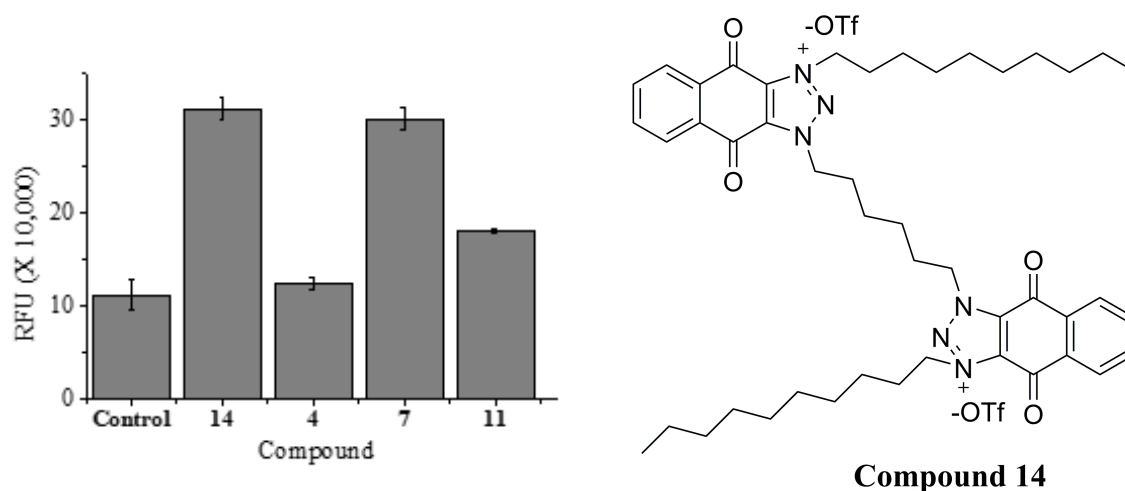


Figure 2-9. ROS study for 4'',6''-disubstituted AKs and compound **14** structure

Compound **7** contains a naphthalene motif that can better stabilize radicals. Hence, it explains why compound **7** promoted ROS production to the level of the positive control, compound **14**. Compound **11**, with the linear alkyl chain, exerted a moderate level of ROS production similar to the 6'-alkylated AKs as we have noted previously. Fluoro substituted molecules, such as in the case of compound **4**, are noted to inhibit the formation of radicals [27]. Therefore, it is not surprising that compound **4** displayed almost no elevation of ROS production as compared to the control (blank). However,

compound **4** showed similar antifungal activities as compounds **7** and **11**. The ROS measurement was conducted following 3 h of incubation of fungi with the AKs, a much shorter time compared to the determination of the MIC, which often requires 48 h of incubation. Thus, compound **4** may exert fast membrane permeabilization, as observed with the fast RFU increases. However, the presence of fluoro substituents deters the formation of ROS resulting in the lower ROS generation but similar antifungal activity. Hence, it is likely that the actual mode of the antifungal mode of action is a combination of two main factors: (1) The rate of membrane permeabilization and 2) the rate of ROS production

2-3. Materials and Methods

Procedure for the antifungal activity. The fungal MIC test was carried out similar to previously reported protocols [28]. In brief, fungi were grown in RPMI 1640 medium for 48 h at 37 °C. Cells, or spores in the case of *F. graminearum* were counted and diluted to 4×10^4 cells/mL in the growth medium. Fungi were added to the compounds dissolved in growth medium maintaining final concentrations of the compounds from 256 to 0.125 µg/mL. Fungi, treated with compounds, were incubated at 37 °C for 48 h to see the inhibitory effect of the compounds. The fungal MIC testing was done in triplicate trials.

Procedure for kinetic cell permeabilization using 1×MIC of compounds. The fungi cell membrane permeabilization study was performed in a 96-well cell culture plate. *C. neoformans* H99 was grown in PDB medium at 37 °C for 48 h. From the

growth, 4 mL of fungi were taken and washed with water twice and resuspended in 1 mL of water. Fungi cells were counted, and cells were diluted to 4×10^6 Cells/mL as the final confluence. Then, compounds and dye were added to the fungi, maintaining the final concentration of compounds to $1 \times \text{MIC}$, $0.01 \mu\text{M}$ for SYTOXTM green and $0.4 \mu\text{g/mL}$ for PI. The control cells were treated with dye only. The fluorescence intensity were measured in every 3 min for a period of four hours at 28°C with an excitation/emission wavelength of $480_{\text{Ex}}/525_{\text{Em}}$ nm for SYTOXTM green and $537_{\text{Ex}}/617_{\text{Em}}$ nm for PI using the Cytation 5 imaging reader. The images of the compounds **7**, **11**, **13**, and Triton X-100 were taken after 60 min of incubation using the green channel and bright field filter set in the Cytation 5 imaging reader and Olympus IX 71.

Procedure for kinetic cell permeabilization using multiple MIC of the compounds. This assay was performed similarly to the fungi cell membrane permeabilization study using $0.125 \times \text{MIC}$ to $8 \times \text{MIC}$ of compounds **2**, **7**, **10**, and **11**. The fluorescence intensity was measured every 3 min for a period of two or four hours. The images of the representative compound **11** were taken after the 60 min of incubation using the green channel for SYTOXTM green and after 120 min using the red channel for PI along with the bright field filter set.

Procedure for reactive oxygen species (ROS) generation study. The effect of the amphiphilic kanamycin to generate ROS species in the fungal cells was studied in the

C. neoformans H99 strain, using the previously reported ROS generating amphiphilic antifungal compound **14**, as the positive control [25,26]. The fungal cells were grown in PDB medium at 28 °C for 48 h with gentle shaking. The cells were washed with water twice before using for the assay by centrifugation at 10,000 rpm for 2 min. A volume of 0.5 mL of cells in water (final cell confluence, 4×10^7 cells/mL) was incubated with $1 \times \text{MIC}$ of the compounds for 3 h at 37 °C. Then, the cells were washed twice with 0.5 mL water and finally resuspended in 0.5 mL water. The cells were then incubated for another 30 min with 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) dye (25 mM final concentration) at 37 °C. After incubation, the cells were washed twice with the same volume of water and resuspended in water. The fluorescence of the cells was measured in the Cytation 5 imaging reader with an excitation and emission wavelength of 485 nm and 525 nm respectively. The experiment was performed at least in triplicate.

2-4. Conclusions

We have examined the antifungal activities of 4'',6''-diaryl kanamycins as well as the previously described 4'',6''-dialkyl kanamycins. These two classes of AKs show similar antifungal profiles against a panel of fungi despite having different hydrophobic groups. The amphiphilicity parameter cLogD can be useful for the structural design when no obvious SAR can be deduced. Using the fluorogenic dye (SYTOXTM green), 4'',6''-diaryl kanamycins were shown to exert their antifungal activities via increased plasma membrane permeability. It was shown, however, that the fluorescent properties of the

commonly used fluorogenic dyes SYTOXTM green and PI are drastically affected by the concentrations of the AKs. Therefore, to acquire meaningful results in such approaches, prudent practice is needed to establish the optimal ratio of AKs to fluorogenic dyes for the experiments. Finally, from a combination of studying kinetic membrane permeabilization, ROS production, cLogD and fungal growth MICs, we conclude that the mode of antifungal action of the 4", 6"disubstituted AKs is a combination of plasma membrane permeabilization and oxidative stress.

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CHAPTER 3

INHIBITION OF CONNEXIN HEMICHANNELS BY NEW AMPHIPHILIC
AMINOGLYCOSIDES WITHOUT ANTIBIOTIC ACTIVITY ^a**3-1 Abstract**

Connexins hemichannels (HCs) from adjacent cells form gap junctional channels that mediate cell-to-cell communication. Abnormal opening of “free” undocked HCs can produce cell damage and participate in the mechanism of disorders such as cardiac infarct, stroke, deafness, skin diseases, and cataracts. Therefore, inhibitors of connexin HCs have great pharmacological potential. Antibiotic aminoglycosides (AGs) have been recently identified as connexin HC inhibitors, but their antibiotic effect is an issue for the treatment of disorders where infections do not play a role. Herein, we synthesized and tested several amphiphilic AGs without antibiotic effect for their inhibition against connexin HCs, using a newly developed cell-based bacterial growth complementation assay. Several leads with superior potency than the parent compound, kanamycin A, were identified. Unlike traditional AGs, these amphiphilic AGs are not bactericidal and are not toxic to mammalian cells, making them better than traditional AGs as HC inhibitors for clinical use and other applications.

^a Adapted with permission from (AlFindee, M. N.; Subedi, Y. P.; Fiori, M. C.; Krishnan, S.; Kjellgren, A.; Altenberg, G. A.; Chang, C.-W. T. “Inhibition of Connexin Hemichannels by New Amphiphilic Aminoglycosides without Antibiotic Activity” *ACS Med. Chem. Lett.* **2018**, 9, 697-701). Copyright © 2018, American Chemical society

3-2 Introduction

Cell-to-cell communication is essential for transporting metabolites, ions, and signal molecules between adjacent cells. This occurs predominantly through gap-junction channels (GJCs) that are formed when two connexin hemichannels (HCs; connexin hexamers), one from the adjacent cells, dock head-to-head (Figure 3-1).^{1,2} There are 21 connexin isoforms that form a variety of GJCs and HCs that differ in permeability and regulation.¹⁻³ The importance of GJCs in physiology and pathophysiology is well established from several decades of research, but the role of plasma-membrane

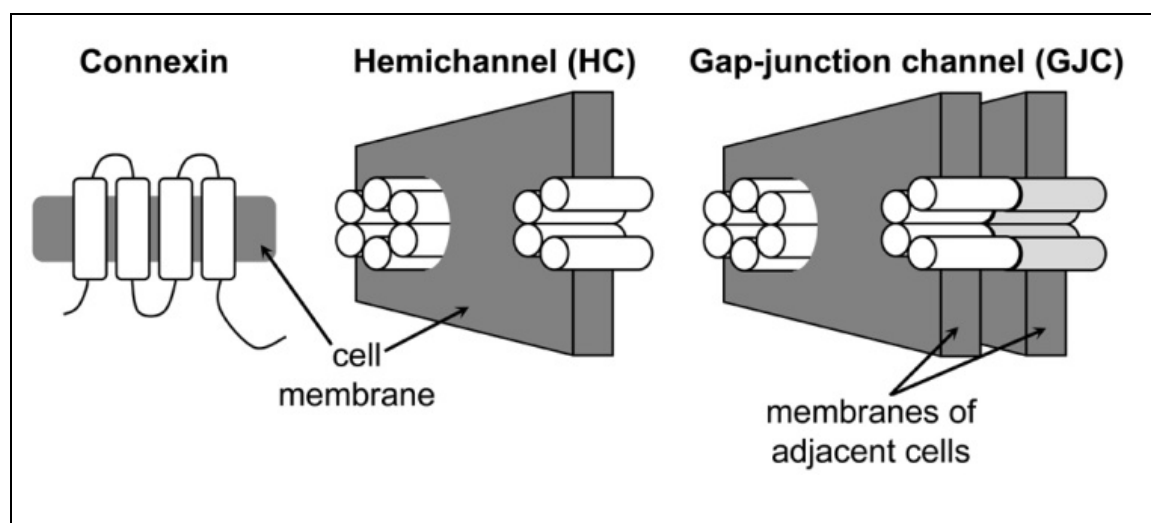


Figure 3-1. Schematic representation of a connexin monomer, a hemichannel (HC) and a gap-junction channel (GJC). Each cylinder in HC and GJC corresponds to a connexin subunit.

undocked HCs is more recent.^{2,4,5} Most HCs are closed under normal conditions, but they still participate in autocrine and paracrine signaling through the efflux of ATP, NAD^+ , glutamate, prostaglandins, and other mediators.⁶ HCs also seem to play a role in disorders of the inner ear (deafness), eye (cataracts), heart (infarcts), and brain (cerebrovascular accidents).^{2,5} Abnormal opening of the “large” nonselective HCs can damage cells by

depolarization, swelling, alterations in ionic gradients, and concentrations of metabolites and second messengers. There is interest in the finding and development of connexin HC inhibitors since they could offer insights on the role of HCs in normal function and disease, as well as serve as therapeutics in disorders associated with connexin HCs.

A variety of molecules have been investigated for their inhibitory effect toward connexin HCs, including 2-aminoethoxydiphenyl borate, ioxynil, carbenoxolone, 18- β -glycyrrhetic acid, antimalarial drugs (mefloquine), and n-alkanols⁷ (Figure 3-2). These compounds cannot be used as starting points for the development of clinically useful HC inhibitors because they are not selective for connexin HCs and/or they are toxic.⁷ Connexin peptide inhibitors are synthetic peptides corresponding to sequences of connexins' extracellular or intracellular loops.⁷⁻⁹ Some of the peptide-based connexin

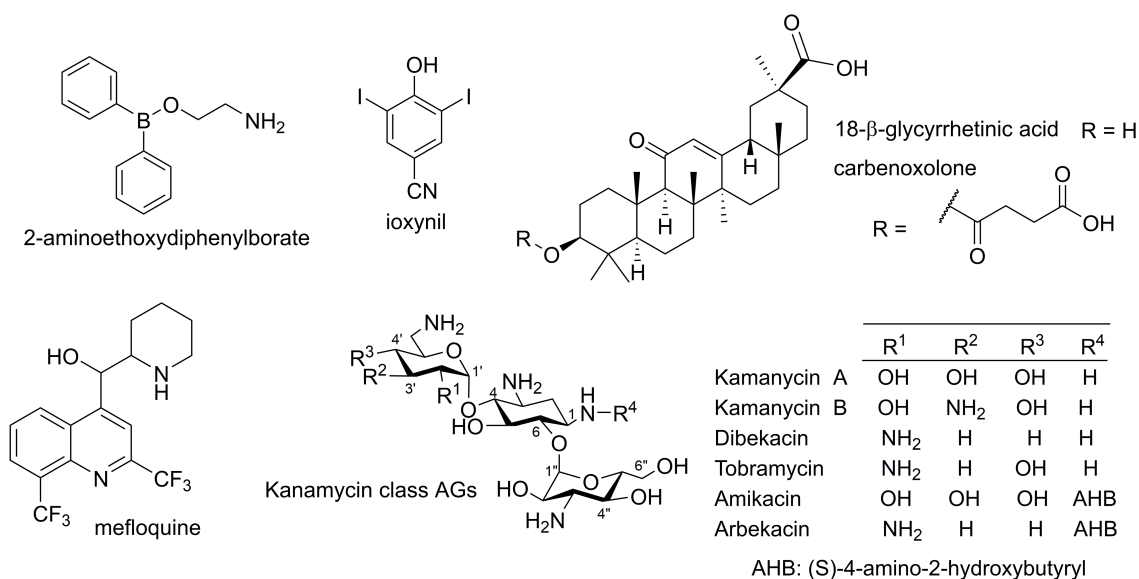
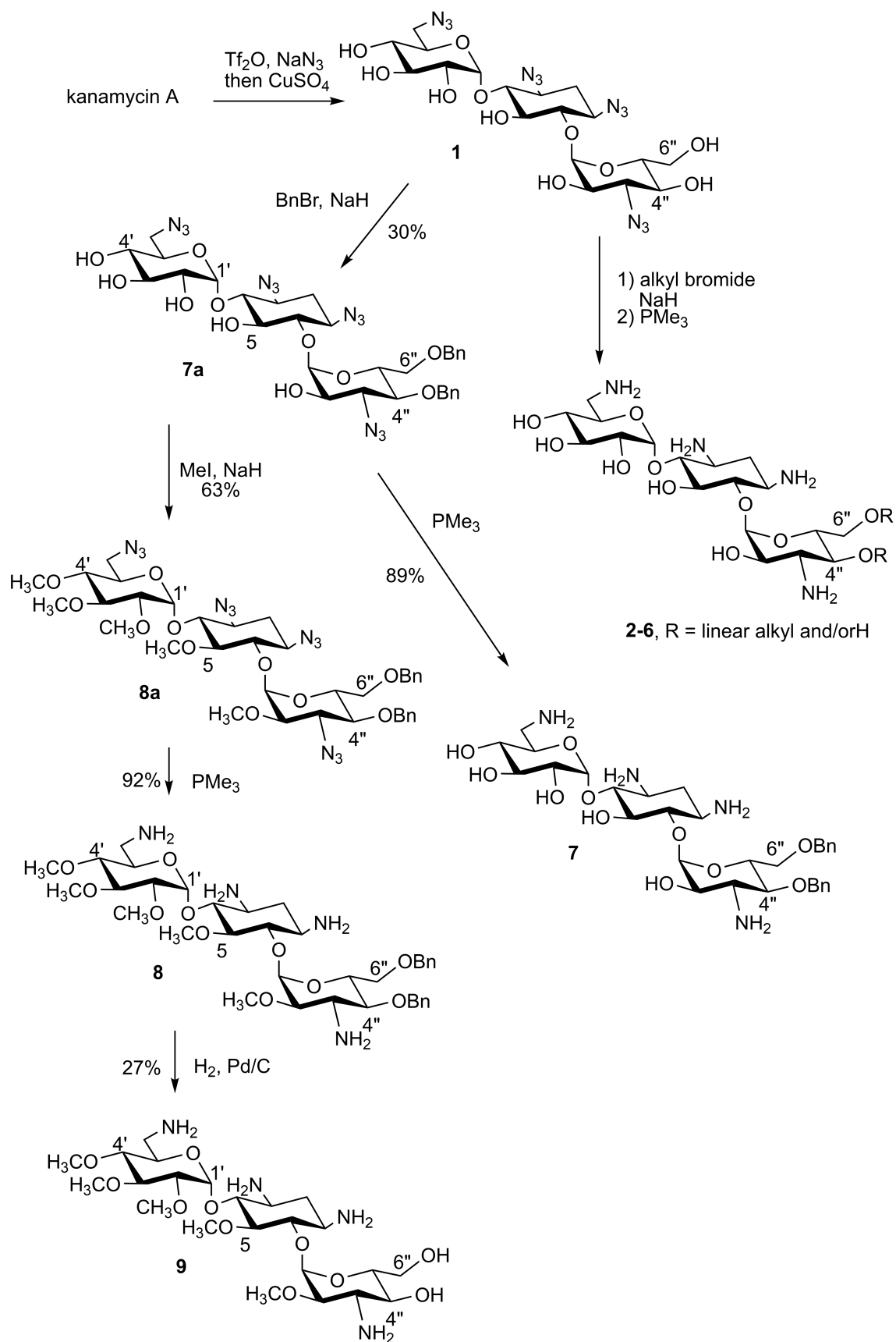


Figure 3-2. Examples of inhibitors of connexin HCs.

inhibitors have been used to treat arrhythmias and to accelerate wound healing.⁷⁻⁹ Most of these peptides act on GJCs and HCs, but a few seem to selectively inhibit HCs.⁷ However, their clinical potential is still unclear,¹⁰ and nevertheless, other avenues to develop clinically useful inhibitors for HC have been mostly unexplored. In this context, aminoglycosides (AGs) such as kanamycin and gentamicin have been recently identified as potent inhibitors of connexin HCs.¹¹⁻¹⁴ AGs have been used as antibacterial agents for over 60 years, and they are still among the most used antibiotics.^{15,16} Although nephrotoxicity and ototoxicity are relatively common complications of AGs treatment, they can be managed.¹⁷ We have discovered that chemically modified AGs, especially amphiphilic kanamycin derivatives, have biological activity while showing significantly reduced cytotoxicity.¹⁸⁻²³ With this in mind, we began to explore amphiphilic kanamycin derivatives as connexin HC inhibitors, using connexin 26 (Cx26) HCs as test targets.

We have reported a three-step synthesis of amphiphilic kanamycin derivatives bearing linear alkyl chains at the *O*-4'', *O*-6'', or *O*-4'' and *O*-6'' positions of kanamycin A (Scheme 3-1).¹⁹ These derivatives show moderate antifungal activity and no antibacterial activity, making them good candidates as Cx26 HC inhibitors without antibiotic effect; they could be used without the risk of promoting the generation of AG-resistant bacteria. Five amphiphilic kanamycin A derivatives were selected randomly for testing. To provide more information in structure-activity relationship (SAR), we also synthesized three additional derivatives: one with a benzyl (Bn) group attached at *O*-4'' and *O*-6'' positions (compound **7**) and two with or without a Bn group attached at *O*-4'' and *O*-6'' positions, and a methyl (Me) group at other hydroxyl groups (5, 2', 3', 4', and 2'')

Scheme 3-1



(compounds **8** and **9**, respectively). These derivatives were prepared by sequential benzylation and methylation followed by reduction of azido groups or hydrogenolysis of the Bn. To elucidate the role of amino groups, compound **1** (Scheme 3-1) was also tested. Please see the Appendix B for details on the synthesis and characterization of the new kanamycin derivatives, including the use of acetylation and ^1H - ^1H COSY for the confirmation of the sites of modifications.

Connexin HC inhibition was conducted using a bacteria-based assay of HC function.¹⁴ In this assay, Cx26 HCs are expressed in *E. coli* LB2003 cells, which lack three major K^+ uptake systems (Kdp, Kup, and Trk), and as a result, they cannot grow in low- $[\text{K}^+]$ media.^{14,24,25} However, growth of this *E. coli* strain in low- $[\text{K}^+]$ medium can be rescued by expression of K^+ -permeable channels such as connexin HCs.^{13,14,26} Under the conditions of the assay (LB2003 cells expressing HCs and grown in low- K^+ medium), inhibition of the HCs reduces or abolishes growth.^{13,14,26} This is a simple assay where bacterial growth in multi-well plates can be followed by measuring the absorbance at 600 nm (OD_{600}).¹⁴ Although not formally needed for most of the AGs tested here (they do not have antibiotic effect), we routinely transform the *E. coli* LB2003 with a plasmid that codes for the AG modifying enzyme aminoglycoside 3'-phosphotransferase, which makes the cells resistant to kanamycin A and its derivatives.¹⁴

3-3 Results and Discussions

The kanamycin derivative without a free amino group (compound **1**) was inactive, suggesting an essential role of this group. One of the disubstituted derivatives (compound **3**) displayed a lower IC_{50} for inhibition of growth dependent on Cx26 than the

monosubstituted compound **4** (Table 3-1). Compared to compound **3** (hexyl), the potency was reduced when the alkyl chain length was shortened to butyl in compound **2** or extended to octyl (compound **5**) or nonyl (compound **6**).

Table 3-1. Inhibition of Cx26-dependent cell growth by kanamycin A and synthetic AGs

Compound	Modifications	IC ₅₀ (μM)
Kanamycin A	-	9.4 ± 1.1
1	-	No effect ^a
2	butyl at <i>O</i> -4'' and <i>O</i> -6''	19.0 ± 2.8 ^b
3	hexyl at <i>O</i> -4'' and <i>O</i> -6''	6.2 ± 1.4
4	hexyl at <i>O</i> -4''	13.5 ± 1.8 ^b
5	octyl at <i>O</i> -4'' and <i>O</i> -6''	13.6 ± 0.5 ^b
6	nonyl at <i>O</i> -4'' and <i>O</i> -6''	18.8 ± 1.3 ^b
7	Bn at <i>O</i> -4'' and <i>O</i> -6''	7.6 ± 1.2
8	Me at 5, 2', 3', 4' and 2'', Bn at <i>O</i> -4'' and <i>O</i> -6''	8.4 ± 1.4
9	Me at 5, 2', 3', 4' and 2''	No effect ^a

^a No effect: the absence of inhibition at 100 μM. ^b Denotes P < 0.01 vs kanamycin A.

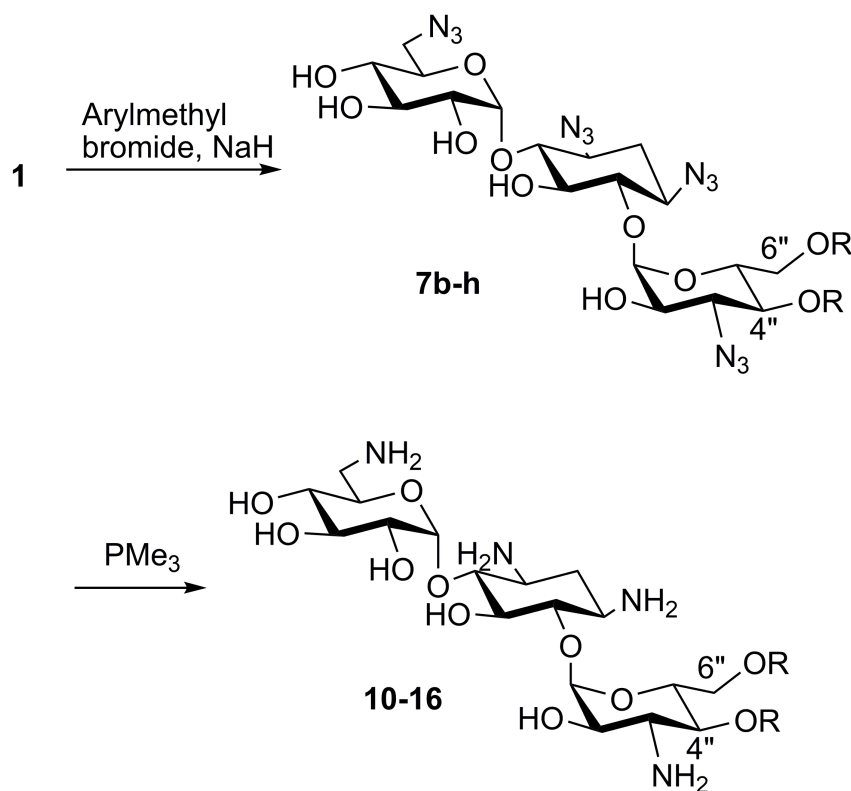
The potency of compounds **7** and **8** was similar, implying that the presence of hydroxyl groups, or the associated hydrogen bond interactions, is not necessary for the AG effect on HCs. However, the lack of inhibitory activity of compound **9** demonstrates that having hydrophobic groups at *O*-4'' and/or *O*-6'' positions is essential in the absence of hydroxyl

groups (compare compounds **8** and **9**). The maximal inhibition of growth dependent on Cx26 was similar to that of kanamycin A and was not statistically different among the new compounds with inhibitory effect (compounds **2–8**), averaging $99 \pm 3\%$.

From the data in Table 3-1, compounds **3**, **7**, and **8** were the most potent inhibitors. However, derivatization based on the hexyl group scaffold of compound **3** is difficult. Because of the ease of the chemical synthesis and purification process, we decided to focus on the derivatization bearing aryl-based substituents. Compounds **7** and **8**, with Bn groups at *O*-4'' and *O*-6'', can be readily modified with variations of substituents on the benzene ring or shape/size of the aromatic motif. Considering the simpler reaction scheme and the flexibility on the groups accepted at the *O*-4'' and *O*-6'' positions, and in the absence of an advantage of compound **8** over compound **7**, we selected the latter for further development.

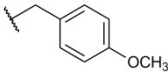
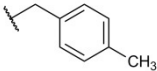
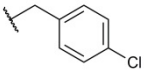
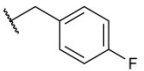
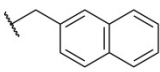
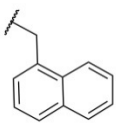
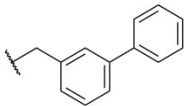
A library of kanamycin derivatives bearing arylmethyl substituents was synthesized to investigate two factors related to SAR (please see Appendix B), the electronic and steric (size) effects of the aryl group; see Scheme 3-2 and Table 3-2, and

Scheme 3-2



an expanded table (Table B-1). We selected four benzene derivatives with electron-donating and withdrawing substituents (methoxy, methyl, chloro, and fluoro) at the para position to explore the first factor. Three arylmethyl groups, 1-naphthalenemethyl, 2-naphthalenemethyl, and biphenyl, were selected for studying the second factor. The synthesis was conducted as described previously,¹⁹ using commercially available arylmethyl bromides. When alkylating compound **1**, we observed a significant amount of

Table 3-2. Second generation synthetic AGs.

Compound	R	IC ₅₀ (μM)
10		13.0 ± 0.7**
11		4.3 ± 0.4*
12		2.5 ± 0.6**
13		8.1 ± 0.5
14		4.9 ± 0.2*
15		6.6 ± 0.5
16		6.2 ± 0.7

* and ** denote $P < 0.05$ and $P < 0.01$ vs. compound 7, respectively.

monosubstituted adducts bearing arylmethyl groups at *O*-4'' or *O*-6'' positions. Attempts to improve the yields for disubstituted adducts by varying the equivalents of arylmethyl bromides or separating two monosubstituted adducts using flash chromatography were unsuccessful. Therefore, only the disubstituted adducts were isolated to proceed toward the completed synthesis. The IC₅₀ values for inhibition by these derivatives are summarized in Table 3-2. It appears that the relationship between the electronic effect and inhibitory potency is not strong, but rather subtle. Compounds with a moderate electron-donating group (CH₃-, compound **11**) or moderate electron-withdrawing group

(Cl-, compound **12**) showed better activities than those with a strong electron- donating (CH₃O-) group (compound **10**) or a strong electron- withdrawing (F) group (compound **13**). Compounds **11** and **12** had superior inhibitory potency than kanamycin A and the parent compound **7**, which has no substituent on the benzene ring. The inhibitory potency of the derivative with a bicyclic aromatic ring (compound **14**) was slightly higher than that of compounds **15** and **16**, but the differences were not major. Combining these results, it seems that compounds **11**, **12**, and **14** can all serve as leads for further development. The maximal inhibition of growth dependent on Cx26 was similar for compounds **10–16** and averaged $94 \pm 2\%$. Examples of growth inhibition by three of the new aminoglycosides, including compound **12**, are shown (Figure B-1).

All of the kanamycin derivatives were examined for their antibacterial activity against *E. coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923). Except for compounds **15** and **16**, which have minimum inhibitory concentrations (MICs) of 64 and 16 µg/mL against *S. aureus*, respectively, the derivatives did not show significant antibacterial activity, with MICs ranging from 128 to >256 µg/mL against both bacteria. The cytotoxicity of the newly synthesized disubstituted kanamycin derivatives on HeLa cells was also examined and is presented in (Figure B-2). Compounds **13**, **14**, and **16** showed moderate cytotoxicity (40–60% reduction of cell viability) at 100 µg/mL, which is at least 20-fold higher than the corresponding IC₅₀ for Cx26 HC inhibition. No significant reduction of cell viability was observed for the rest of the compounds at concentrations up to 100 µg/mL. The low cytotoxicity is advantageous for potential uses as therapeutics.

3-4 Conclusion

We have discovered a new application for amphiphilic kanamycin derivatives as connexin HC inhibitors. Previous studies have shown that the AG gentamicin inhibits HC, but not GJCs,¹² although indirect GJC inhibition, probably through the generation of free radicals, has also been reported.²⁷ Therefore, the issue of selective inhibition of HC vs. GJC by AGs remains to be clarified. In any case, the new amphiphilic kanamycin derivatives presented here represent a novel class of HC inhibitors that have the advantages of not having antibacterial activity and displaying low cytotoxicity. We have identified the preferred structural motifs for improving HC inhibitory potency. Development of suitable connexin HC inhibitors may pave the way for better understanding the mechanism of HC inhibition and the role of connexin HCs in human disorders.

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CHAPTER 4

ONE-STEP SYNTHESIS OF CARBOHYDRATE ESTERS AS ANTIBACTERIAL
AND ANTIFUNGAL AGENTS ^a**4-1 Abstract**

Carbohydrate esters are biodegradable, and the degraded adducts are naturally occurring carbohydrates and fatty acids which are environmentally friendly and non-toxic to human. A simple one-step regioselective acylation of mono-carbohydrates has been developed that leads to the synthesis of a wide range of carbohydrate esters. Screening of these acylated carbohydrates revealed that several compounds were active against a panel of bacteria and fungi, including *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus flavus*, and *Fusarium graminearum*. Unlike prior studies on carbohydrate esters that focus only on antibacterial applications, our compounds are found to be active against both bacteria and fungi. Furthermore, the synthetic methodology is suitable for scale-up production for a variety of acylated carbohydrates. The identified lead compound, **MAN014**, can be used as an antimicrobial in applications such as food processing and preservation and for treatment of bacterial and fungal diseases in animals and plants.

^a Adapted with permission from (AlFindee, M. N.; Zhang, Q.; Subedi, Y. P.; Shrestha, J. P.; Kawasaki, Y.; Grilley, M.; Takemoto, J. Y.; Chang, C.-W. T. "One-Step Synthesis of Carbohydrate Esters as Antibacterial and Antifungal Agents" *Bioorgan. Med. Chem.* **2018**, 26, 765–774). Copyright © 2018, Elsevier BV.

4-2 Introduction

Fungal and bacterial pathogens cause significant economic losses in agriculture and food industry. For example, it is estimated that fungal pathogens destroy more than 125 million tons/yr of the top five food crops (rice, wheat, maize, potatoes, and soybeans) that can feed more than 600 million people.³ Food recalls due to pathogen contamination or illness from foodborne diseases cause significant economic loss.⁴ Common disinfection practices in the food industry involve chemical disinfection and irradiation (e.g., X-ray).⁵⁻⁶ While chemicals, such as hypochlorites, iodophors, peroxyacetic acid, and quaternary ammonium compounds, have longer lasting effectiveness than observed following irradiation,⁷ these chemicals pose health and environmental hazards to humans and animals.⁸ Recently, there is a trend of using natural products (e.g., tea) in combating foodborne bacterial and fungal pathogens,⁹⁻¹⁰ however, the antimicrobial activities of most natural products are too low to enable their practical use. In the area of food preservatives, sodium benzoate and maleic acid are two common food preservatives. Nonetheless, sodium benzoate may undergo decarboxylation when used with acidic components, (such as vitamin C), leading to the formation of carcinogenic benzene, and maleic acid interrupts the cell energy metabolism.¹¹⁻¹² Potassium sorbate is a popular natural food preservative that is generally regarded as safe (GRAS) by the FDA. Nevertheless, potassium sorbate has been reported to be toxic and mutagenic to human blood cells.¹³ In short, there is still a great need for useful natural antimicrobial food additives that are GRAS.

Carbohydrate esters have attracted great interest due to their wide range of applications in industry and medicine.¹⁴⁻¹⁵ These compounds, mostly acylated at the primary hydroxyl

group, are biodegradable and non-toxic while they act as antimicrobials.¹⁶⁻¹⁷

Carbohydrate esters are synthesized by two general methods: chemical and enzyme-catalyzed synthesis.¹⁸ Both have advantages and disadvantages. Most of the syntheses of carbohydrate esters focus on enzyme-catalyzed esterification or transesterification. These latter methods have the advantage of selective incorporation of the acyl group at the primary hydroxyl group of carbohydrates without tedious protection and deprotection steps.¹⁹⁻²¹ However, this synthesis method has several drawbacks: First, the enzyme-catalyzed reactions often employ chemically synthesized vinyl esters as the substrates or require the use of organic solvents.²²⁻²³ Therefore, the synthetic conditions are not amenable to “green” processes. Secondly, many enzymes, such as lipases, exert a significant degree of substrate specificity and the yields or conversion efficiency may vary drastically among different carbohydrates and fatty acids of various chain length.²⁴ Third, many of these reported reactions were conducted at mg to gram scale.²⁵ The feasibility for scale-up production to kg scale remains to be demonstrated in most cases.

Chemical-based syntheses can generate a wide variety of carbohydrate esters without the limitation of substrate specificity. The regioselective chemical synthesis of carbohydrate esters is challenging. It is difficult to incorporate the acyl group selectively to the desired hydroxyl groups on carbohydrates. To circumvent this problem, many reported methods rely on multiple protection and deprotection steps to achieve the regioselective incorporation of the acyl group at the primary hydroxyl group of carbohydrates.²⁶⁻²⁸ This strategy not only increases the cost of synthesis but also reduces the prospects for green production processes and impedes the economical large scale production of carbohydrate esters. In short, the enzymatic process is regioselective but

can be challenging for scaled-up production while the chemical process is not region-specific but is more suitable for making libraries of carbohydrate esters allowing a facile identification of leads.

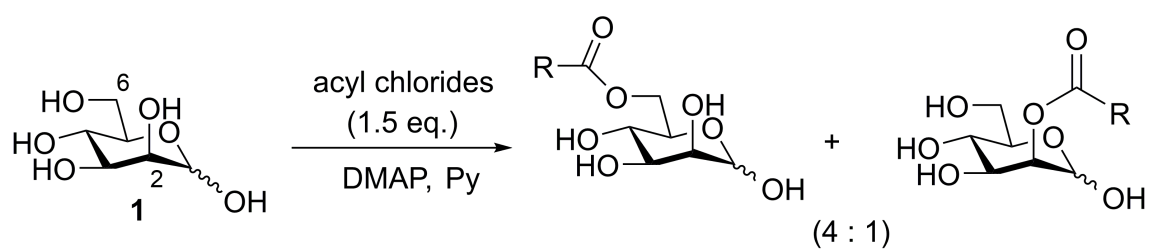
In light of the problems with traditional synthetic strategies of carbohydrate esters, our group began to explore a novel one-step chemical synthesis strategy for the regioselective production of carbohydrate esters. Our approach uses of the fact that most monosaccharides have one primary hydroxyl group which will be more reactive than secondary hydroxyl groups.²⁹ By controlling the reaction condition, we promote regioselectively incorporation of the acyl group at the primary hydroxyl group similar to the enzymatic method. With this strategy, we gain easy access to diverse carbohydrate esters made from natural constituents, and that are subject to detailed structure-activity relationship (SAR) studies. The lead compounds can be quickly identified and then synthesized on a large scale. The lead carbohydrate esters can be purified to provide bioactive compounds consist of natural components.

4-3 Materials and Methods

We selected four natural monosaccharides (mannose (1), glucose (2), *N*-acetylglucosamine (3) and galactose (4)) and ten acyl groups (acetyl (C2), butanoyl (C4), hexanoyl (C6), octanoyl (C8), decanoyl (C10), dodecanoyl (C12), tridecanoyl (C13), tetradecanoyl (C14), pentadecanoyl (C15), and hexadecanoyl (C16) for constructing libraries of carbohydrate esters. To accommodate the solubilities of the carbohydrates and acyl chlorides, pyridine was used as the solvent. The primary hydroxyl groups are more nucleophilic than the anomeric and the secondary hydroxyl groups. Therefore, the

regioselective acylation is expected to be achieved by controlling the equivalent of acyl chloride and the reaction temperature. In most of the cases, 1.5 equivalents of acyl chloride were employed to optimize the production of mono- over di-acylated carbohydrate esters. In some cases, 1.2 equivalents of the acyl chloride were used to obtain the monoacylated predominantly and 2.5 equivalents to yield predominantly the di-acylated products for comparison. All the reactions were conducted with 1-2 g of carbohydrates and *N,N*-dimethylaminopyridine (DMAP) was used as the catalyst.

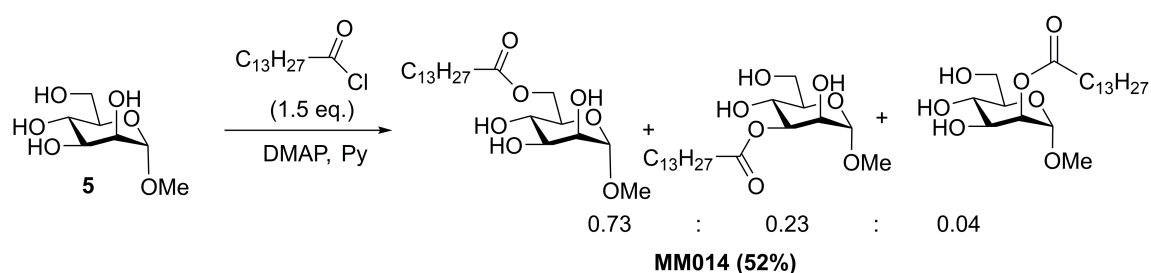
Eight mannose esters with carbon chain from 2, 4, 6, 8, 10, 12, 14, and 16 were synthesized, and 1.5 eq. of acyl chloride was found to be optimal for generation of mono-acylated mannose (Scheme 4-1). Despite numerous attempts, the mono-acylated



MAN002 (43%): R= CH₃
MAN004 (43%): R= C₃H₇
MAN006 (40%): R= C₅H₁₁
MAN008 (58%): R= C₇H₁₅
MAN010 (25%): R= C₉H₁₉
MAN012 (40%): R= C₁₁H₂₃
MAN013 (24%): R= C₁₂H₂₅
MAN014 (48%): R= C₁₃H₂₇
MAN015 (21%): R= C₁₄H₂₉
MAN016 (56%): R= C₁₅H₃₁

Scheme 4-1.

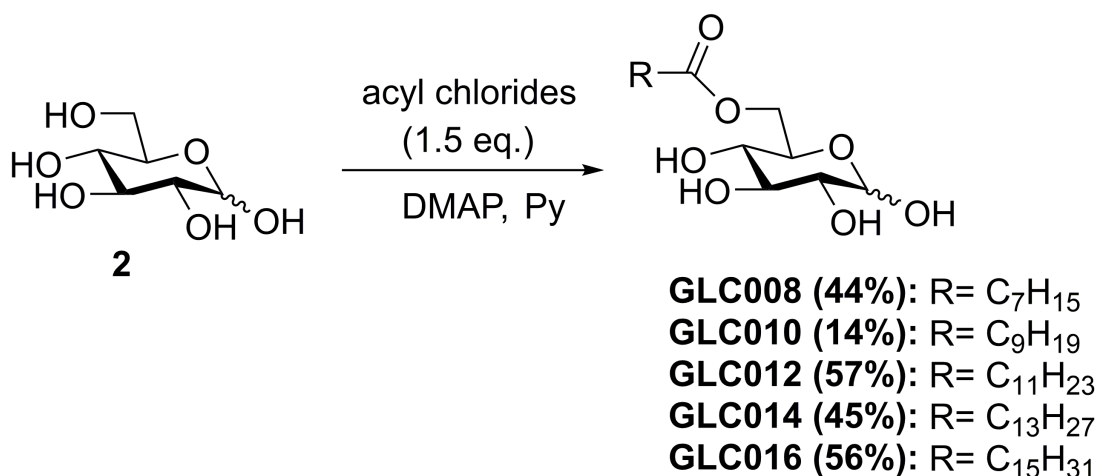
mannose was obtained as an inseparable mixture of 6-*O*-acylated mannose and 2-*O*-acylated mannose in 4:1 ratio (calculated from the ^1H NMR and the sites of acylation were confirmed by the characteristic downfield shift of the diastereotopic H-6 protons as well as the coupling constant of H-2). Chemical derivatization of 6-*O*-acylated mannose and 2-*O*-acylated mannose using trityl chloride also failed to achieve the separation of these two regioisomers. Therefore, these mono-acylated mannose derivatives were tested for their biological activity as a mixture. Following the initial testing of biological activity, we found that the mannose ester with fourteen carbon chain (**MAN014**) was the most active against bacteria and fungi. To optimize the carbon chain length and provide more detailed SAR, we decided to expand our library to include methylmannopyranoside, **5** with fourteen carbon chain ester (**MM014**) (Scheme 4-2). The 6-*O*-acylated adduct was obtained as the major product as expected. However, 3-*O*-acylated adduct was observed as the main by-product according to the integral ratio calculated from the ^1H NMR.



Scheme 4-2

For the same reason, mannose esters with acetyl (C2, **MAN002**), butanoyl (C4, **MAN004**), hexanoyl (C6, **MAN006**), tridecanoyl (C13, **MAN013**), and pentadecanoyl (C15, **MAN015**) groups were also prepared. A similar ratio of 6-*O*-acylated mannose and 2-*O*-acylated mannose was observed for all the reactions.

Acylation of glucose with acyl chlorides of C8, C10, C12, and C14 was conducted in a similar fashion. The acylation of glucose is more regioselective than mannose. Five 6-acylated glucose derivatives, **GLC008**, **GLC010**, **GLC012**, **GLC014**, and **GLC016** were synthesized, and site of acylation was confirmed again by the characteristic downfield shift of the diastereotopic H-6 protons (Scheme 4-3).^{30,20, 31-32}

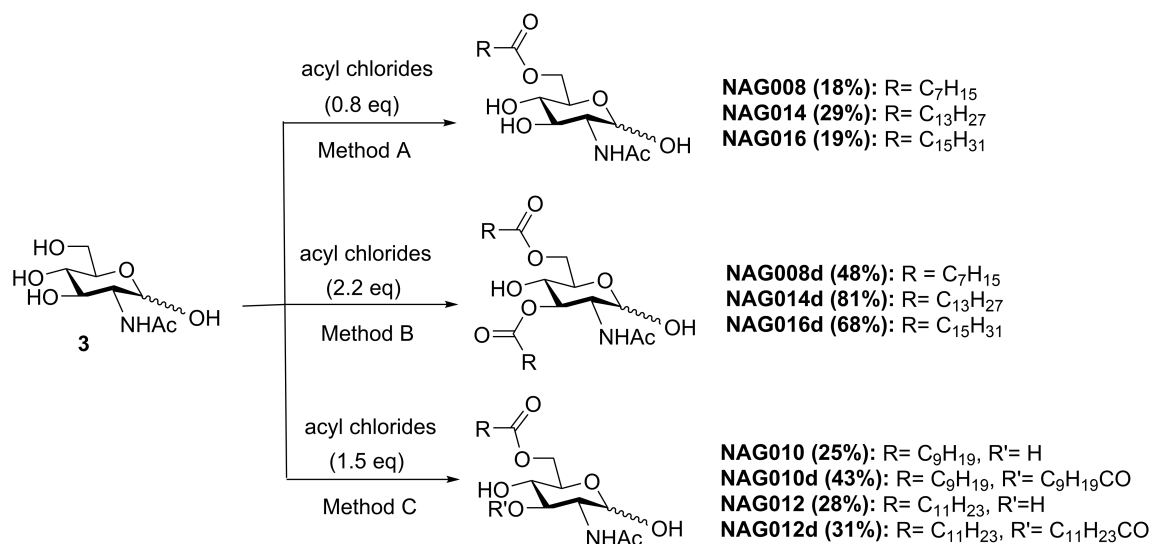


Scheme 4-3

Acylation of *N*-acetylglucosamine with acyl chlorides of C8, C10, C12, C14, and C16 was more challenging and offered a mixture of mono and diacetylated adducts (Scheme 4-4). To optimize the yields of both mono and diacetylated *N*-acetylglucosamine for biological study, we developed three different methods (Methods A, B, and C) for

their synthesis. Method A involves the use of 0.8 eq. of acyl chlorides to optimize the production of mono-acylated *N*-acetylglucosamine (NAG). In method C, 2.2 eq. of acyl chlorides were used to favor the production of di-acylated NAG. Finally, we discovered that using 1.5 eq. acyl chlorides (Method C) can generate mono- and di-acylated *N*-acetylglucosamine (NAG) in one pot with satisfactory yields for both products, and these two products can be separated using column chromatography. The acylation took place at the *O*-6 position for the mono-acylated products. The sites of acylation for the diacetylated products occurred at *O*-6 and *O*-3. In all the cases the sites of acylation were confirmed by ^1H and ^1H - ^1H COSY NMR.

Unfortunately, acylation of galactose provided a complex inseparable mixture with no distinct major products.



Scheme 4-4

4-4 Results and Discussion

4-4.1 Antibacterial activity

All of the carbohydrate esters were tested against Gram-positive (G⁺) *S. aureus* (ATCC 25923) and Gram-negative (G⁻) *Escherichia coli* (ATCC 25922) using vancomycin, kanamycin A, neomycin as the controls. The minimum inhibitory concentrations (MICs) for these compounds are summarized in Table 4-1. **MAN014** is the most active compound with MICs of 16 - 32 µg/mL and 128 µg/mL against *S. aureus*

Table 4-1. MIC values of carbohydrate esters against bacteria^a

Entry	Compound	<i>S. aureus</i> (ATCC 25923)	<i>E. coli</i> (ATCC 25922)	<i>S. aureus</i> (ATCC 33591) (MRSA)
1	MAN012	128	>256	128 - 256
2	MAN013	64 - 128	>256	ND
3	MAN014	16 - 32	128	16 - 32
4	MM014	128	>256	64 -128
5	MAN015	64	>256	ND
6	Vancomycin	2	>256	2
7	Kanamycin	1 - 2	2 - 4	256
8	Neomycin	2	2 - 4	>256

^a Unit: (µg/mL), ^b ND: Not determined.

and *E. coli*, respectively. **MAN012**, **MAN014**, and **MM014** were chosen to be further tested against methicillin-resistant *S. aureus* (MRSA, ATCC 33591, and ATCC 43300),

Pseudomonas aeruginosa (G⁻, ATCC 27853), *Klebsiella pneumoniae* (G⁻, ATCC 13883).

MAN014 is the most active compound against all of these bacterial strains with MICs of 16 - 32 µg/mL and 128 µg/mL against MRSA and G⁻ bacteria, respectively. Sodium myristate (C14) was tested, and no antibacterial activity was noted. Overall, the lead carbohydrate esters are more active against G⁺ than G⁻ bacteria. In conclusion, **MAN014** has superior antibacterial activity than aminoglycosides (kanamycin and neomycin) against MRSA, and better activity against G⁻ bacteria than vancomycin.

4-4.2 Antifungal activity

A panel of human and plant fungal pathogens was used to test the antifungal activity for the synthesized compounds using voriconazole (an azole-class antifungal agent) and **K20**³³ as controls (Table 4-2). In general, mannose esters, **MAN015**, **MAN014**, and **MAN013** are relatively active against all the tested strains. Other

Table 4-2. The MIC of carbohydrate esters against fungi ^a

Entry	Compound	<i>Aspergillus flavus</i>	<i>Fusarium graminearum</i> B4-5A	<i>Candida albicans</i> 64124 (azole-resistant)	<i>Candida albicans</i> MYA2876 (azole-susceptible)	<i>Cryptococcus neoformans</i> H99	<i>Rhodotorula pilimanae</i>
1	MAN012	128	128	256	256	256	128
2	MAN013	32 - 64	16 - 32	64	64 - 128	32 - 64	32 - 64
3	MAN014	32	32	64	64	8	8
4	MM014	> 256	4 - 16	> 256	> 256	256	> 256
5	MAN015	4 - 16	8	16 - 32	64	8 - 16	16
6	K20	> 256	8	256	64	4	4
7	Voriconazole	1	32	>256	0.125	0.125	8

^a Unit: (µg/mL).

compounds have MIC values $>256 \mu\text{g/mL}$ against the tested fungi. *Candida albicans* 64124 is a human pathogen that is resistant to many clinically used azole-based antifungal agents. *Aspergillus* species are typically recalcitrant to most recognized antifungal drugs. Thus, infectious mycoses of humans and animals caused by pathogenic *Aspergillus* species are often untreatable and serious and have high mortality rates. It is noted that all three mannose esters are more active than the controls against *C. albicans* 64124 and *Aspergillus flavus*. Based on the results of antibacterial and antifungal activities, we conclude that mannose plays an essential role in the biological activity of carbohydrate esters.

We have reported that amphiphilic antifungal kanamycin **K20** can exert strong synergism with azole-based antifungal agents.³⁴ Thus, we also conducted in vitro synergistic studies using **MAN014** and several azole-class antifungal agents using the checkerboard method. The fractional inhibitory concentration index (FICI) is summarized in Table C-1. In contrast to **K20** that shows strong synergism with most azoles, **MAN014** showed good synergy (FIC indices > 0.5) with all tested crop fungicides against *A. flavus*. On the other hand, **MAN014** did not show synergy with all azoles tested against other fungal strains (table1 C-1). **K20** is active against fungi but inactive against most bacteria while **MAN014** displays a broad-spectrum activity against both fungi and bacteria.

4-4.3 Mode of growth inhibitory action by MAN014

Carbohydrate esters are amphiphilic compounds and consequently are predicted to perturb cell membranes as their mode of action.³⁵⁻³⁷ Based on this assumption,

SYTOXTM green dye was used to examine **MAN014** effects on cell membrane permeability.³⁸ Normally non-fluorescent, SYTOXTM green becomes fluorescent when it enters cells through pores of permeable membranes and binds to nucleic acids. The capability of **MAN014** to form membrane pores was tested with bacterial strain *S. aureus* (ATCC 25923) and the filamentous fungal strain, *Fusarium graminearum* B-4-5A using the protocols described by Weerden et al.^{36, 39} Strains were incubated with and without **MAN014** for two hours. Strains were incubated with **MAN014** at 1×MIC, 2×MIC, and 4×MIC. **MAN014** at 1×MIC caused increases of green fluorescence by SYTOXTM green in cells of both *S. aureus* (ATCC 25923) and *F. graminearum* B-4-5A (Figures 4-1 and 4-2). Similar results were obtained with 2×MIC and 4×MIC of **MAN014**. These observations support membrane pore formation as the mode of growth inhibitory action of **MAN014** against bacteria and fungi.

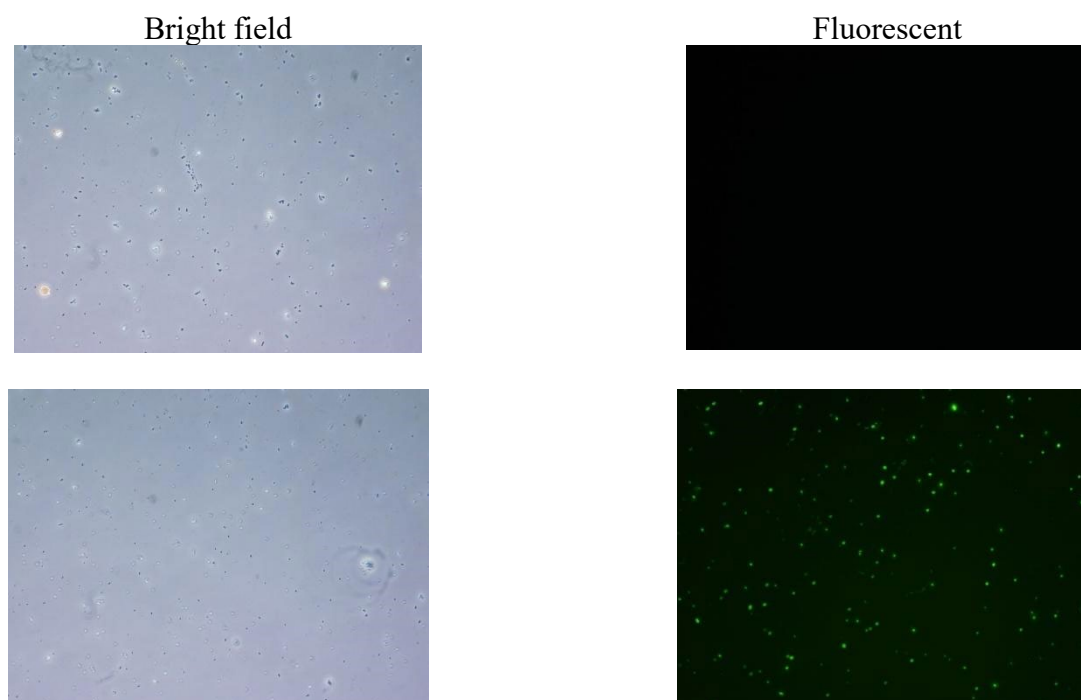


Figure 4-1. *S. aureus* (ATCC25923) single hypha experiment, blank control (top row), and bacteria incubated with **MAN014** at 1X MIC for 2h (bottom row).

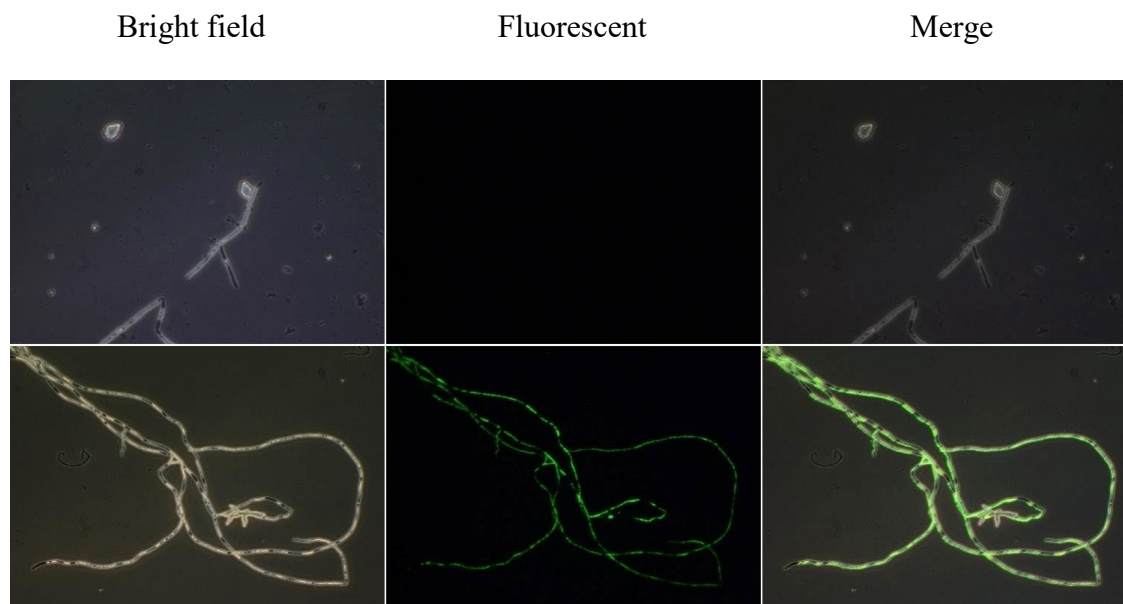


Figure 4-2. *Fusarium graminearum* single hypha experiment, blank control (top row), and fungi incubated with **MAN014** at 1X MIC for 2h (bottom row).

4-4.4 Mammalian cell cytotoxicity

A primary concern of using amphiphilic compounds in food, human, animal or other applications, is toxicity toward mammalian cells. The cytotoxicity of **MAN014** was evaluated using three mammalian cells, including A549 (cancer epithelial lung), Beas-2B (normal, epithelial lung) and HeLa (adenocarcinoma, epithelial cervix), and MTT assay (Figure 4-3). The IC_{50} values for **MAN014** were determined to be 107.65 ± 1.98 , 77.35 ± 6.14 and 71.76 ± 4.69 $\mu\text{g/mL}$ for A549, Beas-2B, and HeLa, respectively. At low concentrations (up to 10 $\mu\text{g/mL}$), **MAN014** appeared to have no cytotoxicity or even slightly promoted cell growth. Significant cytotoxicity of **MAN014** was observed with a concentration at 100 $\mu\text{g/mL}$, which is 2-8 fold higher than the MICs.

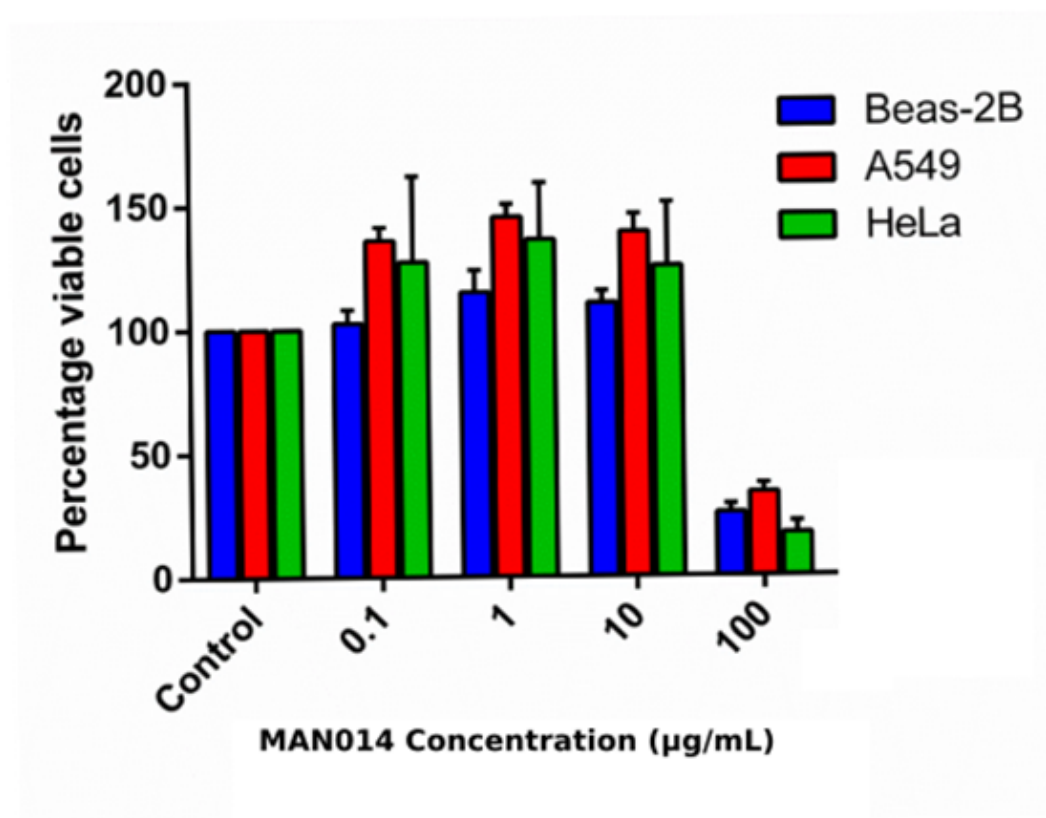


Figure 4-3. Cytotoxicity of MAN014

4-5 Large Scale Synthesis of MAN014

In an effort to demonstrate the feasible large-scale synthesis of lead compound. We carried out a 30g scale synthesis of **MAN014** using 1.5 eq. myristoyl chloride. The reaction mixture was quenched by adding 1 N HCl and the products, which contained both mono- and di-acylated mannose was collected by filtration. Further purification using flash column chromatography provided **MAN014** in 48% yield along with 28% of the di-acylated adduct. The purity of **MAN014** was examined by ^1H NMR, and no residual pyridine or DMAP can be observed. In contrast to the smaller scale synthesis, two types of di-acylated mannose derivatives were isolated as an inseparable mixture,

which consists of about 1/1 ratio of 2,6-di-*O*-acylated ($\alpha/\beta = 85/15$, confirmed by gated ^{13}C NMR and COSY) and 1,3 di-*O*-acylated ($\alpha/\beta = 60/40$) adducts. These di-acylated products are difficult to assay for biological activity due to poor solubility in aqueous media.

4-6 Conclusions

We have successfully established a one-step synthesis of a library of carbohydrate esters. In contrast to most of the studies in this area that focus on antibacterial activity, several of the currently described synthesized carbohydrate esters are active against both fungi and bacteria. A detailed SAR was conducted allowing the identification of a lead compound, **MAN014**. **MAN014** shows broad-spectrum activity against bacteria and fungi while having relatively low cytotoxicity toward animal cells. Mode of action studies confirms that MAN014 exerts its bioactivity via pore formation of the cell membrane. Finally, we have demonstrated that MAN014 can be synthesized in large scale with high purity. All of these features combined make **MAN014** a prominent and environment-friendly antibacterial and antifungal natural compound that can be used for applications in medicine, public health, and agriculture.

4-7 Experimental Section

4-7.1 General Procedures.

All chemicals were purchased from the commercially available resources without any further purification. Pyridine was dried over calcium hydride. Mass spectrometry was taken by high-resolution mass spectrometry (HRMS) using a TOF mass spectrometer at

University of California, Riverside mass spectrometry facility. Two NMR instruments were used JEOL300 (300 MHz) and Bruker Ascend (500 MHz) for the ^1H and 75 or 125 MHz for the ^{13}C Nuclei. CDCl_3 and CD_3OD were used as solvents. The parts per million (ppm) were used to express the chemical shifts on the δ scale. The peaks splitting pattern were expressed as (s; for the singlet), (d; doublet), (t; triplet), (q; quadrate), (m; multiplet), and (ddd; doublet of doublets of doublets). Coupling constants J were measured in Hertz (Hz).

4-7.2 General procedure for the synthesis of acylated mannose and glucose.

Synthesis of mono-acylated sugars, 0.5 g (1 equiv.) of sugar (glucose or mannose) and 1.5 equiv. of corresponding acyl chloride was stirred in 20 mL of pyridine for overnight. The formation of the product was confirmed by TLC ($\text{EtOAc}/\text{MeOH} = 9/1$). The reaction solution was concentrated under reduced pressure, and the crude oily reaction mixture was loaded to a silica gel column. The column chromatography was eluted by a gradient from pure EtOAc to $\text{EtOAc}/\text{MeOH} = 9/1$. After the column, the collected product was dried under reduced pressure to offer slight yellowish solid. The purity of the carbohydrates was confirmed by ^1H NMR (the final product is mainly α sugar mixed with a small amount of β sugar due to the natural constitution of the corresponding sugar).

4-7.3 General procedure for the synthesis of acylated *N*-acetylglucosamine.

Method A: 0.5 g (1 equiv.) of *N*-acetylglucosamine and 0.8 equiv. of corresponding acyl chloride was stirred in 20 mL of pyridine for overnight. The formation of the product was confirmed by TLC (Hexane: EtOAc = 7.5:2.5). The reaction solution was concentrated under reduced pressure, and the crude oily reaction mixture was loaded to a silica gel column. The column chromatography was eluted by a gradient from pure Hexane to Hexane: EtOAc = 7.5:2.5. After the column, the collected product was dried under reduced pressure to offer slight yellowish solid

Method B: 0.5 g (1 equiv.) of *N*-acetylglucosamine and 2.2 equiv. of corresponding acyl chloride was stirred in 20 mL of pyridine for overnight. The formation of the product was confirmed by TLC (Hexane: EtOAc = 7.5:2.5). The reaction solution was concentrated under reduced pressure, and the crude oily reaction mixture was loaded to a silica gel column. The column chromatography was eluted by a gradient from pure Hexane to Hexane: EtOAc = 7.5:2.5. After the column, the collected product was dried under reduced pressure to offer slight yellowish solid.

Method C: 0.5 g (1 equiv.) of *N*-acetylglucosamine and 1.5 equiv. of corresponding acyl chloride was stirred in 20 mL of pyridine for overnight. The formation of the product was confirmed by TLC (Hexane: EtOAc = 7.5:2.5). The reaction solution was concentrated under reduced pressure, and the crude oily reaction mixture was loaded to a silica gel column. The column chromatography was eluted by a gradient from pure Hexane to Hexane: EtOAc = 7.5:2.5. After the column, the collected product was dried under reduced pressure to offer slight yellowish solid.

4-7.4 6-*O*-Acetyl-D-mannopyranose (**MAN002**).

Please refer to the general procedure for synthesis of carbohydrates esters.

MAN002 was obtained as a light yellowish oil (0.27 g, 1.19 mmole, 43%) in a mixture of α/β anomers in a 3/1 ratio. The reported NMR is for the α anomer. ^1H NMR (CD_3OD , 500 MHz) δ 5.06 (s, 1H), 4.38 (dd, $J = 11.8$ Hz, $J = 2.0$ Hz, 1H), 4.22 (dd, $J = 11.9$ Hz, $J = 6.2$ Hz, 1H), 3.6 – 4.0 (m, 4H), 2.07 (s, 3H); ^{13}C NMR (CD_3OD , 125 MHz) δ 171.61, 94.51, 71.38, 70.78, 70.22, 67.43, 63.94, 19.37; ESI/APCI Calcd. for $\text{C}_8\text{H}_{13}\text{O}_6$ [$\text{M} - \text{OH}$] $^+$: 205.0712; Measured m/z : 205.0718.

4-7.5 6-*O*-Butanoyl-D-mannopyranose (**MAN004**).

Please refer to the general procedure for synthesis of carbohydrates esters.

MAN004 was obtained as a light yellowish oil (0.30 g, 1.19 mmole, 43%) in a mixture of α/β anomers in a 3/1 ratio. The reported NMR is for the α anomer. ^1H NMR (CD_3OD , 500 MHz) δ 4.38 (dd, $J = 11.8$ Hz, $J = 2.0$ Hz, 1H), 4.22 (dd, $J = 11.8$ Hz, $J = 6.2$ Hz, 1H), 3.6 – 4.0 (m, 4H), 2.34 (t, $J = 7.3$ Hz, 2H), 1.6 – 1.7 (m, 2H), 0.9 – 1.0 (m, 3H); ^{13}C NMR (CD_3OD , 125 MHz) δ 174.12, 94.84, 71.38, 70.80, 70.30, 67.46, 63.75, 35.48, 18.00, 12.59; ESI/APCI Calcd. for $\text{C}_{10}\text{H}_{17}\text{O}_6$ [$\text{M} - \text{OH}$] $^+$: 233.1025; Measured m/z : 233.1023.

4-7.6 6-*O*-Hexanoyl-D-mannopyranose (**MAN006**).

Please refer to the general procedure for synthesis of carbohydrates esters.

MAN006 was obtained as a light yellowish oil (0.31 g, 1.11 mmole, 40%) in a mixture of α/β anomers in a 3/1 ratio. The reported NMR is for the α anomer. ^1H NMR (CD_3OD , 500 MHz) δ 5.06 (s, 1H), 4.38 (dd, $J = 11.8$ Hz, $J = 2.0$ Hz, 1H), 4.22 (dd, $J = 11.8$ Hz, $J = 6.2$ Hz, 1H), 3.6 – 4.0 (m, 4H), 2.35 (t, $J = 7.5$ Hz, 2H), 1.6 – 1.78 (m, 2H), 1.3 – 1.4 (m, 4H), 0.9 – 1.0 (m, 3H); ^{13}C NMR (CD_3OD , 125 MHz) δ 174.29, 94.49, 71.38, 70.80, 70.30, 67.46, 63.78, 33.55, 31.04, 24.27, 21.99, 12.59; ESI/APCI Calcd. for $\text{C}_{12}\text{H}_{21}\text{O}_6$ $[\text{M} - \text{OH}]^+$: 261.1338; Measured m/z : 261.1360.

4-7.7 6-*O*-Octanoyl-D-mannopyranose (**MAN008**).

Please refer to the general procedure for synthesis of carbohydrates esters.

MAN008 was obtained as a light yellowish oil (0.49 g, 1.61 mmole, 58%) in a mixture of α/β anomers in a 3/1 ratio. The reported NMR is for the α anomer. ^1H NMR (CD_3OD , 500 MHz) δ 5.05 (s, 1H), 4.35 (dd, $J = 11.3$ Hz, $J = 2.0$ Hz, 1H), 4.1 – 4.2 (m, 1H), 3.4 – 4.0 (m, 4H), 2.3 – 2.4 (m, 2H), 1.5 – 1.7 (m, 2H), 1.2 - 1.4 (m, 8H), 0.88 (t, $J = 6.6$ Hz, 3H); ^{13}C NMR (CD_3OD , 125 MHz) δ 174.28, 94.49, 71.38, 70.81, 69.41, 67.47, 61.38, 33.59, 31.48, 28.81, 28.71, 24.60, 22.28, 13.05; ESI/APCI Calcd. for $\text{C}_{14}\text{H}_{25}\text{O}_6$ $[\text{M} - \text{OH}]^+$: 289.1651; Measured m/z : 289.1680.

4-7.8 6-*O*-Decanoyl-D-mannopyranose (**MAN010**).

Please refer to the general procedure for synthesis of carbohydrates esters.

MAN010 was obtained as a light yellowish oil (0.23 g, 0.69 mmole, 25%) in a mixture of α/β anomers in a 3/1 ratio. The reported NMR is for the α anomer. ^1H NMR (CD_3OD , 500 MHz) δ 5.03 (s, 1H), 4.35 (dd, $J = 11.5$ Hz, $J = 2.0$ Hz, 1H), 4.1 – 4.2 (m, 1H), 3.4 – 4.0 (m, 4H), 2.3 – 2.4 (m, 2H), 1.5 – 1.7 (m, 2H), 1.2 - 1.4 (m, 12H), 0.88 (t, $J = 6.6$ Hz, 3H); ^{13}C NMR (CD_3OD , 125 MHz) δ 174.28, 94.49, 71.37, 70.81, 70.30, 67.77, 63.81, 33.60, 31.67, 29.21, 29.06, 29.04, 28.87, 24.61, 22.35, 13.11; ESI/APCI Calcd. for $\text{C}_{16}\text{H}_{29}\text{O}_6$ $[\text{M} - \text{OH}]^+$: 317.1964; Measured m/z : 317.1990.

4-7.9 6-*O*-Dodecanoyl-D-mannopyranose (**MAN012**).

This compound has been reported previously.⁴⁰⁻⁴¹ Please refer to the general procedure for the synthesis of carbohydrates esters. **MAN012** was obtained as light yellowish oil (0.40 g, 1.11 mmole, 40%) in a mixture of α/β anomers in a 3/1 ratio. The reported NMR is for the α anomer. ^1H NMR (CD_3OD , 300 MHz) δ 5.0 – 5.1 (m, 1H), 4.35 (dd, $J = 11.6$ Hz, $J = 2.0$ Hz, 1H), 4.1 – 4.2 (m, 1H), 3.4 – 4.0 (m, 4H), 2.3 – 2.4 (m, 2H), 1.5 – 1.7 (m, 2H), 1.2 - 1.4 (m, 16H), 0.88 (t, $J = 6.5$ Hz, 3H).

4-7.10 6-*O*-Tridecanoyl-D-mannopyranose (**MAN013**).

Please refer to the general procedure for synthesis of carbohydrates esters.

MAN013 was obtained as a light yellowish oil (0.23 g, 0.61 mmole, 24%) in a mixture of

α/β anomers in a 3/1 ratio. The reported NMR is for the α anomer. ^1H NMR (CD_3OD , 500 MHz) δ 5.03 (s, 1H), 4.35 (dd, $J = 11.6$ Hz, $J = 2.0$ Hz, 1H), 4.1 – 4.2 (m, 1H), 3.4 – 4.0 (m, 4H), 2.3 – 2.4 (m, 2H), 1.5 – 1.7 (m, 2H), 1.2 - 1.4 (m, 18H), 0.88 (t, $J = 6.6$ Hz, 3H); ^{13}C NMR (CD_3OD , 125 MHz) δ 174.26, 94.51, 71.40, 70.81, 71.31, 67.47, 63.78, 33.58, 31.68, 29.39 (2 Carbons), 29.36 (2 Carbons), 29.34, 29.22, 29.08, 28.85, 22.33, 13.03; ESI/APCI Calcd. for $\text{C}_{19}\text{H}_{36}\text{O}_7\text{Na}$ $[\text{M} + \text{Na}]^+$: 399.2353; Measured m/z : 399.2339.

4-7.11 6-*O*-Tetradecanoyl-D-mannopyranose (**MAN014**).

This compound has been reported previously.⁴² Please refer to the general procedure for synthesis of carbohydrates esters. **MAN014** was obtained as a light yellowish solid (0.52 g, 1.33 mmole, 48%) in a mixture of α/β anomers in a 3/1 ratio. The reported NMR is for the α anomer. ^1H NMR (CD_3OD , 500 MHz) δ 5.03 (d, $J = 1.6$ Hz, 1H), 4.39 (dd, $J = 11.7$ Hz, $J = 2.0$ Hz, 1H), 4.22 (dd, $J = 11.7$ Hz, $J = 6.1$ Hz, 1H), 3.7 – 4.0 (m, 3H), 3.63 (t, $J = 9.6$ Hz, 1H), 2.35 (t, $J = 7.5$ Hz, 2H), 1.6 – 1.7 (m, 2H), 1.2 - 1.4 (m, 20H), 0.91 (t, $J = 6.9$ Hz, 3H).

4-7.12 2,6-Di-*O*-tetradecanoyl-D-mannopyranose (**MAN014d**).

Please refer to the general procedure for synthesis of carbohydrates esters. **MAN014d** was obtained as a white solid: mp 88.7 - 94.6 °C (0.38 g, 0.63 mmole, 28%) in a mixture of α/β anomers in a 8.5/1.5 ratio. The reported NMR is for the α anomer. ^1H

NMR (CDCl₃, 500 MHz) δ 5.26 (s, H1), 5.17 (dd, $J = 3.4$ Hz, $J = 1.6$ Hz, H2), 4.57 (dd, $J = 12.1$ Hz, $J = 4.1$ Hz, H6eq), 4.29 (dd, $J = 12.1$ Hz, $J = 2.1$ Hz, H6ax), 4.11 (dd, $J = 9.5$ Hz, $J = 3.4$ Hz, H3), 4.0 – 4.1 (m, H5), 3.64 (t, $J = 9.7$ Hz, H4), 3.4 – 4.0 (m, 4H), 2.3 – 2.4 (m, 4H), 1.6 – 1.7 (m, 4H), 1.2 - 1.4 (m, 40H), 0.90 (t, $J = 7.9$ Hz, 6H); ¹³C NMR (CDCl₃, 125 MHz) δ 174.86, 173.62, 93.78, 71.85, 70.71, 69.31, 67.85, 63.19, 34.34, 34.24, 31.92 (2 Carbons), 29.69 (2 Carbons), 29.66 (7 Carbons), 29.61, 29.51, 29.46, 29.36 (2 Carbons), 29.30, 29.28, 29.15, 29.10, 25.01, 24.93, 22.69 (2 Carbons); ESI/APCI Calcd. for C₃₄H₆₄O₈Na [M + Na]⁺: 600.4601; Measured m/z : 623.4494.

4-7.13 3,6-Di-*O*-tetradecanoyl-D-mannopyranose (**MAN014d**).

Please refer to the general procedure for synthesis of carbohydrates esters.

MAN014d was obtained as a white solid: mp 88.7 - 94.6 °C (0.38 g, 0.63 mmole, 28%) in a mixture of α/β anomers in a 6/4 ratio. The reported NMR is for the α anomer. ¹H NMR (CDCl₃, 500 MHz) δ 5.28 (s, H1), 5.21 (dd, $J = 9.8$ Hz, $J = 3.1$ Hz, H3), 4.57 (dd, $J = 12.2$ Hz, $J = 4.5$ Hz, H6eq), 4.37 (dd, $J = 12.3$ Hz, $J = 2.2$ Hz, H6ax), 4.0 – 4.1 (m, H2, H5), 3.87 (t, $J = 9.8$ Hz, H4), 3.4 – 4.0 (m, 4H), 2.3 – 2.4 (m, 4H), 1.6 – 1.7 (m, 4H), 1.2 - 1.4 (m, 40H), 0.90 (t, $J = 7.9$ Hz, 6H); ¹³C NMR (CDCl₃, 125 MHz) δ 174.86, 173.62, 92.54, 71.17, 69.68, 68.85, 65.89, 63.14, 34.23, 34.17, 31.92 (2 Carbons), 29.69 (2 Carbons), 29.66 (8 Carbons), 29.51, 29.45, 29.36 (2 Carbons), 29.30, 29.26, 29.20, 29.10, 24.95, 24.87, 22.69 (2 Carbons); ESI/APCI Calcd. for C₃₄H₆₄O₈Na [M + Na]⁺: 600.4601; Measured m/z : 623.4494.

4-7.14 Methyl 6-*O*-tridecanoyl- α -D-mannopyranose (**MM014**).

Please refer to the general procedure for synthesis of carbohydrates esters.

MM014 was obtained as a light yellowish oil (0.58 g, 1.44 mmole, 52%) in a mixture of α/β anomers in a 3/1 ratio. The reported NMR is for the α anomer. ^1H NMR (CD_3OD , 500 MHz) δ 4.62 (d, $J = 1.5$ Hz, 1H), 4.42 (dd, $J = 11.7$ Hz, $J = 1.7$ Hz, 1H), 4.22 (dd, $J = 11.7$ Hz, $J = 6.6$ Hz, 1H), 3.6 – 3.9 (m, 4H), 3.38 (s, 3H), 2.73 (t, $J = 7.4$ Hz, 2H), 1.6 – 1.7 (m, 2H), 1.3 - 1.4 (m, 20H), 0.92 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR (CD_3OD , 125 MHz) δ 174.08, 101.38, 71.13, 70.63, 70.49, 67.30, 63.69, 53.81, 33.63, 31.69, 29.41, 29.38 (2 Carbons), 29.35, 29.23, 29.10, 29.04, 28.84, 24.66, 22.35, 13.08; ESI/APCI Calcd. for $\text{C}_{21}\text{H}_{40}\text{O}_6$ $[\text{M}-\text{OH}]^+$: 387.2747; Measured m/z : 387.2782.

4-7.15 6-*O*-Pentadecanoyl-D-mannopyranose (**MAN015**).

Please refer to the general procedure for synthesis of carbohydrates esters.

MAN015 was obtained as a light yellowish oil (0.19 g, 0.47 mmole, 21%) in a mixture of α/β anomers in a 3/1 ratio. The reported NMR is for the α anomer. ^1H NMR (CD_3OD , 500 MHz) δ 5.05 (d, $J = 1.75$ Hz, 1H), 4.39 (dd, $J = 11.8$ Hz, $J = 2.0$ Hz, 1H), 4.22 (dd, $J = 11.8$ Hz, $J = 6.1$ Hz, 1H), 3.7 – 4.0 (m, 3H), 3.6 – 3.7 (m, 1H), 2.3 – 2.4 (m, 2H), 1.6 – 1.7 (m, 2H), 1.2 - 1.4 (m, 22H), 0.88 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR (CD_3OD , 125 MHz) δ 174.26, 94.51, 71.40, 70.80, 71.31, 67.47, 63.78, 33.58, 31.68, 29.39 (2 Carbons), 29.36 (2 Carbons), 29.34, 29.22, 29.08 (2 Carbons), 28.85, 24.58, 22.33, 13.03; ESI/APCI Calcd. for $\text{C}_{21}\text{H}_{40}\text{O}_6\text{Na}$ $[\text{M} + \text{Na}]^+$: 427.2666; Measured m/z : 427.2652.

4-7.16 6-*O*-Hexadecanoyl-D-mannopyranose (**MAN016**).

This compound has been reported previously.⁴¹ Please refer to the general procedure for the synthesis of carbohydrates esters. **MAN016** was obtained as a light yellowish solid (0.53 g, 1.26 mmole, 56%) in a mixture of α/β anomers in a 3/1 ratio. The reported NMR is for the α anomer. ¹H NMR (CD₃OD, 300 MHz) δ 5.05 (s, 1H), 4.35 (dd, $J = 11.7$ Hz, $J = 2.0$ Hz, 1H), 4.1 – 4.2 (m, 1H), 3.4 – 3.9 (m, 4H), 2.3 – 2.4 (m, 2H), 1.6 – 1.7 (m, 2H), 1.2 - 1.4 (m, 24H), 0.88 (t, $J = 6.8$ Hz, 3H).

4-7.17 2-Acetamido-2-deoxy-6-*O*-octanoyl- α -D-glucopyranose (**NAG008**).

Please refer to the method (A) for synthesis of mono and di-acylated *N*-acetylglucosamine. **NAG008** was obtained as a light yellowish oil (0.44 g, 1.27 mmole, 58%). ¹H NMR (CD₃OD, 500 MHz) δ 5.04 (d, $J = 3.4$ Hz, 1H), 4.35 (dd, $J = 11.8$ Hz, $J = 2.0$ Hz, 1H), 4.18 (dd, $J = 11.8$ Hz, $J = 5.1$ Hz, 1H), 3.95 (ddd, $J = 9.9$ Hz, $J = 5.1$ Hz, $J = 2.0$ Hz, 1H), 3.83 (dd, $J = 10.6$ Hz, $J = 3.4$ Hz, 1H), 3.67 (dd, $J = 10.6$ Hz, $J = 8.9$ Hz, 1H), 3.33 (dd, $J = 9.9$ Hz, $J = 8.9$ Hz, 1H), 2.32 (t, $J = 7.3$ Hz, 2H), 1.96 (s, 3H), 1.5 – 1.7 (m, 2H), 1.2 - 1.4 (m, 8H), 0.88 (t, $J = 6.1$ Hz, 3H); ¹³C NMR (CD₃OD, 125 MHz) δ 175.64, 173.79, 92.70, 72.64, 70.86, 70.01, 64.89, 55.94, 35.35, 32.99, 30.29, 30.22, 26.15, 23.80, 22.76, 14.55; ESI/APCI Calcd. for C₁₆H₃₀NO₇ [MH]⁺ : 348.2017; Measured m/z : 348.2025.

4-7.18 2-Acetamido-3,6-di-*O*-octanoyl-2-deoxy- α -D-glucopyranose (**NAG008d**).

Please refer to the method (B) for synthesis of mono and di-acylated *N*-acetylglucosamine. **NAG008d** was obtained as a light yellowish solid: mp 111.9 – 116.5 °C (0.51 g, 1.08 mmole, 48%). ¹H NMR (CDCl₃, 500 MHz) δ 6.37 (d, *J* = 9.2 Hz, 1H), 5.2 – 5.3 (m, 2H), 4.72 (d, *J* = 3.4 Hz, 1H), 4.43 (dd, *J* = 12.2 Hz, *J* = 3.6 Hz, 1H), 4.27 (dd, *J* = 12.0 Hz, *J* = 1.7 Hz, 1H), 4.0 - 4.2 (m, 2H), 3.5 – 3.6 (m, 1H), 3.24 (d, *J* = 6.1 Hz, 1H), 2.3 – 2.4 (m, 4H), 1.93 (s, 3H), 1.5 – 1.7 (m, 4H), 1.2 - 1.4 (m, 16H), 0.8 – 0.9 (m, 6H); ¹³C NMR (CDCl₃, 125 MHz) δ 175.55, 175.03, 170.91, 91.92, 73.17, 70.06, 68.95, 63.04, 52.35, 34.53, 34.34, 31.84 (2 Carbons), 29.29 (2 Carbons), 29.20, 29.10, 25.13, 25.06, 23.18, 22.78 (2 Carbons), 14.24 (2 Carbons); ESI/APCI Calcd. for C₂₄H₄₄NO₈ [MH]⁺ : 474.3061; Measured *m/z* : 474.3081.

4-7.19 2-Acetamido-2-deoxy-6-*O*-decanoyl- α -D-glucopyranose (**NAG010**).

Please refer to the method (C) for synthesis of mono and di-acylated *N*-acetylglucosamine. **NAG010** was obtained as a light yellowish oil (0.21 g, 0.57 mmole, 25%). ¹H NMR (CD₃OD, 500 MHz) δ 5.08 (d, *J* = 3.4 Hz, 1H), 4.40 (dd, *J* = 11.8 Hz, *J* = 2.1 Hz, 1H), 4.23 (dd, *J* = 11.8 Hz, *J* = 5.3 Hz, 1H), 3.9 – 4.0 (m, 1H), 3.87 (dd, *J* = 10.7 Hz, *J* = 3.5 Hz, 1H), 3.72 (dd, *J* = 10.6 Hz, *J* = 8.8 Hz, 1H), 3.38 (dd, *J* = 9.9 Hz, *J* = 8.9 Hz, 1H), 2.36 (t, *J* = 7.5 Hz, 2H), 2.00 (s, 3H), 1.5 – 1.6 (m, 2H), 1.2 - 1.4 (m, 12H), 0.88 (t, *J* = 6.8 Hz, 3H); ¹³C NMR (CD₃OD, 125 MHz) δ 174.14, 172.27, 91.19, 71.15, 71.13, 69.35, 63.37, 54.44, 33.59, 31.63, 29.17, 29.01 (2 Carbons), 28.80, 24.62, 22.31, 21.20, 13.02; ESI/APCI Calcd. for C₁₈H₃₄NO₇ [MH]⁺ : 376.2330; Measured *m/z* :

376.2331.

4-7.20 2-Acetamido-3,6-di-*O*-decanoyl-2-deoxy- α -D-glucopyranose (**NAG010d**).

Please refer to the method (C) for synthesis of mono and di-acylated *N*-acetylglucosamine. **NAG010d** was obtained as a white solid: mp 107.1 – 115.9 °C (0.51 g, 0.97 mmole, 43%). ¹H NMR (CDCl₃, 500 MHz) δ 6.37 (d, *J* = 9.2 Hz, 1H), 5.2 – 5.3 (m, 2H), 4.72 (d, *J* = 3.4 Hz, 1H), 4.43 (dd, *J* = 12.2 Hz, *J* = 3.6 Hz, 1H), 4.27 (dd, *J* = 12.0 Hz, *J* = 1.7 Hz, 1H), 4.0 - 4.2 (m, 2H), 3.5 – 3.6 (m, 1H), 3.24 (d, *J* = 6.1 Hz, 1H), 2.3 – 2.4 (m, 4H), 1.93 (s, 3H), 1.5 – 1.7 (m, 4H), 1.2 - 1.4 (m, 16H), 0.8 – 0.9 (m, 6H); ¹³C NMR (CDCl₃, 125 MHz) δ 175.55, 175.03, 170.91, 91.92, 73.17, 70.06, 68.95, 63.04, 52.35, 34.53, 34.34, 31.84 (2 Carbons), 29.29 (2 Carbons), 29.20, 29.10, 25.13, 25.06, 23.18, 22.78 (2 Carbons), 14.24 (2 Carbons); ESI/APCI Calcd. for C₂₈H₅₂NO₈ [MH]⁺ : 530.3687; Measured m/z : 530.3674.

4-7.21 2-Acetamido-2-deoxy-6-*O*-dodecanoyl- α -D-glucopyranose (**NAG012**).

Please refer to the method (C) for synthesis of mono and di-acylated *N*-acetylglucosamine. **NAG012** was obtained as a white solid (0.26 g, 0.63 mmole, 28%). ¹H NMR (CD₃OD, 500 MHz) δ 5.08 (d, *J* = 3.5 Hz, 1H), 4.39 (dd, *J* = 11.8 Hz, *J* = 2.0 Hz, 1H), 4.22 (dd, *J* = 11.8 Hz, *J* = 5.2 Hz, 1H), 3.9 – 4.0 (m, 1H), 3.86 (dd, *J* = 10.6 Hz, *J* = 3.4 Hz, 1H), 3.72 (dd, *J* = 10.6 Hz, *J* = 8.9 Hz, 1H), 3.37 (dd, *J* = 9.3 Hz, *J* = 9.3 Hz, 1H), 2.35 (t, *J* = 7.5 Hz, 2H), 2.00 (s, 3H), 1.5 – 1.6 (m, 2H), 1.2 - 1.4 (m, 16H),

0.91 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR (CD_3OD , 125 MHz) δ 174.12, 172.26, 91.19, 71.15, 71.13, 69.35, 63.37, 54.44, 33.59, 31.67, 29.33 (2 Carbons), 29.21, 29.06, 29.01, 28.81, 24.62, 22.33, 21.21, 13.03; ESI/APCI Calcd. for $\text{C}_{20}\text{H}_{37}\text{NO}_7$ $[\text{MH}]^+$: 403.2570; Measured m/z : 403.2649.

4-7.22 2-Acetamido-3,6-di-*O*-dodecanoyl-2-deoxy- α -D-glucopyranose (**NAG012d**).

Please refer to the method (C) for synthesis of mono and di-acylated *N*-acetylglucosamine. **NAG012d** was obtained as a white solid: mp 72.3 – 75.8 °C (0.51 g, 0.97 mmole, 43%). ^1H NMR (CDCl_3 , 500 MHz) δ 6.42 (d, $J = 9.2$ Hz, 1H), 5.2 – 5.3 (m, 2H), 4.44 (dd, $J = 12.2$ Hz, $J = 3.6$ Hz, 1H), 4.32 (dd, $J = 12.1$ Hz, $J = 1.7$ Hz, 1H), 4.1 – 4.2 (m, 1H), 4.0 – 4.1 (m, 1H), 3.59 (d, $J = 9.4$ Hz, 1H), 2.3 – 2.4 (m, 4H), 1.97 (s, 3H), 1.5 – 1.7 (m, 4H), 1.2 – 1.4 (m, 32H), 0.88 (t, $J = 6.9$ Hz, 6H); ^{13}C NMR (CDCl_3 , 125 MHz) δ 175.35, 174.80, 170.86, 91.69, 72.97, 69.90, 68.78, 62.85, 52.23, 34.35, 34.15, 31.90 (2 Carbons), 29.62 (2 Carbons), 29.61 (2 Carbons), 29.50 (2 Carbons), 29.33 (2 Carbons), 29.29 (2 Carbons), 29.09, 29.06, 24.96, 24.88, 22.95, 22.67 (2 Carbons), 14.09 (2 Carbons); ESI/APCI Calcd. for $\text{C}_{32}\text{H}_{60}\text{NO}_8$ $[\text{MH}]^+$: 586.4313; Measured m/z : 586.4336.

4-7.23 2-Acetamido-2-deoxy-6-*O*-tetradecanoyl- α -D-glucopyranose (**NAG014**).

Please refer to the method (A) for synthesis of mono and di-acylated *N*-acetylglucosamine. **NAG014** was obtained as a light yellowish solid: mp 149.1 – 153.2

°C (0.58 g, 1.33 mmole, 59%). ¹H NMR (CD₃OD, 500 MHz) δ 5.04 (d, *J* = 3.4 Hz, 1H), 4.37 (dd, *J* = 11.8 Hz, *J* = 2.2 Hz, 1H), 4.20 (dd, *J* = 11.7 Hz, *J* = 5.4 Hz, 1H), 3.9 – 4.0 (m, 1H), 3.84 (dd, *J* = 10.6 Hz, *J* = 3.4 Hz, 1H), 3.69 (dd, *J* = 10.4 Hz, *J* = 8.7 Hz, 1H), 3.35 (dd, *J* = 9.6 Hz, *J* = 8.5 Hz, 1H), 2.36 (t, *J* = 7.3 Hz, 2H), 1.96 (s, 3H), 1.5 – 1.6 (m, 2H), 1.2 - 1.4 (m, 20H), 0.88 (t, *J* = 6.7 Hz, 3H); ¹³C NMR (CD₃OD, 125 MHz) δ 175.50, 175.02, 170.71, 92.01, 73.13, 70.20, 68.96, 63.03, 52.31, 34.55, 32.12, 29.86, 29.71, 29.57, 29.50, 29.37, 29.31, 25.17, 25.10, 24.97, 23.28, 22.89, 14.32; ESI/APCI Calcd. for C₂₂H₄₂NO₇ [MH]⁺ : 432.2956; Measured m/z : 432.2945.

4-7.24 2-Acetamido-3,6-di-*O*-tetradecanoyl-2-deoxy-α-D-glucopyranose (**NAG014d**).

Please refer to the method (B) for synthesis of mono and di-acylated *N*-acetylglucosamine. **NAG014d** was obtained as a light yellowish solid: mp 80.4 – 86.5 °C (1.17 g, 1.83 mmole, 81%). ¹H NMR (CDCl₃, 500 MHz) δ 6.23 (d, *J* = 9.2 Hz, 1H), 5.2 – 5.3 (m, 2H), 4.46 (dd, *J* = 12.2 Hz, *J* = 3.6 Hz, 1H), 4.2 – 4.3 (m, 1H), 4.1 - 4.2 (m, 1H), 4.0 - 4.1 (m, 1H), 3.58 (dd, *J* = 9.6 Hz, 1H), 2.3 – 2.4 (m, 4H), 1.96 (s, 3H), 1.6 – 1.7 (m, 4H), 1.2 - 1.4 (m, 40H), 0.86 (t, *J* = 6.7 Hz, 6H); ¹³C NMR (CDCl₃, 125 MHz) δ 175.68, 173.81, 92.01, 73.13, 72.20, 68.96, 63.03, 52.31, 34.55, 34.36, 32.12, 29.86 (8 Carbons), 29.71 (2 Carbons), 29.57 (2 Carbons), 29.50 (2 Carbons), 29.37, 29.31, 25.17, 25.10, 24.97, 23.28, 22.89 (2 Carbons), 14.32 (2 Carbons); ESI/APCI Calcd. for C₃₆H₆₈NO₈ [MH]⁺ : 642.4939; Measured m/z : 642.4844.

4-7.25 2-Acetamido-2-deoxy-6-*O*-hexadecanoyl- α -D-glucopyranose (**NAG016**).

Please refer to the method (A) for synthesis of mono and di-acylated *N*-acetylglucosamine. **NAG016** was obtained as a light yellowish solid: mp 124.8 – 129.4 °C (0.59 g, 1.29 mmole, 57%). ¹H NMR (CD₃OD, 500 MHz) δ 5.08 (d, *J* = 3.5 Hz, 1H), 4.40 (dd, *J* = 11.8 Hz, *J* = 2.0 Hz, 1H), 4.23 (dd, *J* = 11.8 Hz, *J* = 5.3 Hz, 1H), 3.9 – 4.0 (m, 1H), 3.87 (dd, *J* = 10.6 Hz, *J* = 3.4 Hz, 1H), 3.72 (dd, *J* = 10.5 Hz, *J* = 8.9 Hz, 1H), 3.35 (dd, *J* = 9.6 Hz, *J* = 8.5 Hz, 1H), 2.36 (t, *J* = 7.4 Hz, 2H), 2.00 (s, 3H), 1.5 – 1.6 (m, 2H), 1.2 - 1.4 (m, 24H), 0.92 (t, *J* = 6.9 Hz, 3H); ¹³C NMR (CD₃OD, 125 MHz) δ 174.13, 172.26, 91.20, 71.15, 71.13, 69.35, 63.36, 54.44, 33.59, 31.67, 29.38 (3 Carbons), 29.36 (2 Carbons), 29.33, 29.21, 29.07, 29.02, 28.82, 24.63, 22.33, 21.20, 13.03; ESI/APCI Calcd. for C₂₄H₄₆NO₇ [MH]⁺ : 460.3269; Measured *m/z* : 460.3259.

4-7.26 2-Acetamido-3,6-di-*O*-tetradecanoyl-2-deoxy- α -D-glucopyranose (**NAG016d**).

Please refer to the method (B) for synthesis of mono and di-acylated *N*-acetylglucosamine. **NAG016d** was obtained as a white solid: mp 101.4 – 109.5 °C (1.07 g, 1.54 mmole, 68%). ¹H NMR (CDCl₃, 500 MHz) δ 5.88 (d, *J* = 9.2 Hz, 1H), 5.2 – 5.3 (m, 2H), 4.52 (dd, *J* = 12.3 Hz, *J* = 3.7 Hz, 1H), 4.1 – 4.3 (m, 3H), 4.0 - 4.1 (m, 1H), 3.5 – 3.6 (m, 1H), 3.11 (s, 1H), 2.82 (s, 1H), 2.3 – 2.4 (m, 4H), 1.94 (s, 3H), 1.6 – 1.7 (m, 4H), 1.2 - 1.4 (m, 48H), 0.88 (t, *J* = 6.5 Hz, 6H); ¹³C NMR (CDCl₃, 125 MHz) δ 175.15 (2 Carbons), 174.71, 91.91, 72.85, 70.24, 68.70, 62.73, 52.11, 34.35, 34.16, 34.14, 31.93 (2 Carbons), 29.71 (7 Carbons), 29.67 (5 Carbons), 29.63, 29.50, 29.37 (2 Carbons),

29.28, 29.17, 29.11, 25.00, 24.92, 23.17, 22.70 (2 Carbons), 14.13 (2 Carbons);
ESI/APCI Calcd. for $C_{40}H_{76}NO_8$ $[MH]^+$: 698.5565; Measured m/z : 698.5550.

4-7.27 Antibacterial MIC Determination.

A liquid culture of a selected bacterial strain was inoculated into Trypticase Soy broth at 35°C for 1-2 h. The bacteria cell concentration (no. of cells/mL and absorbance at 600 nm) was determined and diluted with broth, if necessary, to an absorbance value of 0.08 to 0.1 at 600 nm. The adjusted inoculated medium (100 μ L) was diluted with 10 mL of broth and then applied to a 96-well microtiter plate (50 μ L). A series of solutions (50 μ L each in 2-fold dilution) of the tested compounds was added to the testing wells. The 96-well plate was incubated at 35°C for 12 to 18 h. The minimum inhibitory concentration (MIC) is defined as the minimum concentration of compound needed to completely inhibit the growth of bacteria. The MIC determinations for each experiment were repeated at least three times.

4-7.28 Antifungal MIC and FIC Index Determinations.

In vitro growth inhibition of yeast strains by carbohydrate ester compounds were determined using MIC microbroth dilution assays in 96-well uncoated polystyrene microtiter plates (Corning Costar, Corning, NY, USA) as described in the M27-A2 reference methods of the Clinical and Laboratory Standards Institute (CLSI) (formerly the National Committee for Clinical Standards Laboratory Standards) (NCCLS).⁴³ Serial dilutions of compounds were made in uncoated polystyrene 96-well plates in the range of

0.25 to 512 µg/mL. For MIC determinations with filamentous fungi, previously described methods were used.⁴⁴ FIC index determinations were carried out according to previously described methods by checkerboard analysis.⁴⁵ Viable yeast cell or fungal spore concentrations were determined by agar medium plate colony counting. The final concentration was adjusted to 1×10^5 colony forming units/mL. The range of concentrations of drugs used for FIC index determinations was based on their corresponding MIC values. Fungal growth was examined visually. All experiments were done in duplicate.

4-7.29 Cytotoxicity assay:

Mammalian cells were grown in DMEM 1X (Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 µg/mL streptomycin, and 100 U/mL penicillin. Two-hundred µL of cells (25,000 cells/mL) were added to 96-well cell culture treated plates (Corning™) and incubated for 24 h at 37 °C with 5% CO₂. Different concentrations of MAN014 (1.0, 10.0, 100.0 µg/mL) were added and incubated for another 48 h. Triton X100 was added to a separate well as a positive control. For cell viability assay, 20 µL of MTT stock solution (5 mg/mL) was added to each well and incubated for 4 h. Upon completion of incubation, the media was carefully removed and washed twice with 100 mL of PBS buffer. The live cells will reduce MTT reagent to purple crystals, which was dissolved in 200 µL of DMSO. Then the absorbance was measured at 570 nm and 670 nm with a microplate reader (Synergy H4). The results are expressed as percentage viability compared with that of control. For each type of mammalian cell, the assay was repeated 3 times, and each assay was performed in triplicate. The standard deviation was

determined from data sets of three independent experiments.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

Two libraries of amphiphilic compounds were successfully synthesized, and their bioactivities were tested. The first reported library of compounds was aryl substituted kanamycin A analogs for a detailed structure activity relationship study. This library was prepared using a novel three products protocol, and it was tested in two different applications: as antifungal agents and as connexin Cx26 inhibitors.

Compounds **4** – **8** were able to strongly inhibit *Fusarium graminearum* B4-5A, *Rhodotorula Pilimanae*, *Cryptococcus neoformans* H99, and *Cryptococcus neoformans* VR-54 fungal growth with MIC rang 2-32 µg/mL. Most of the aryl substituted kanamycins did not have fungal growth inhibition against *Candida albicans* strains 64124 and MYA2876 except compounds **4** and **6**, which had good fungal growth with MIC = 32- 64 µg/mL. The mode of action study showed that these compounds might affect the cell membrane integrity.

The aryl substituted kanamycins displayed low cytotoxicity toward mammalian cells except compounds **13**, **14**, and **16**, which had moderate cytotoxicity at 100 µg/mL with 40 – 60 % reduced cell viability. Although compounds **13**, **14**, and **16** had moderate cytotoxicity, these compounds had at least 20-fold IC₅₀ higher than the connexin Cx26 inhibition counterpart.

The aryl substituted kanamycins showed excellent connexin Cx26 inhibition behavior. Compounds **11**, **12**, and **14** were the most active (i.e., IC₅₀ = 4.3, 2.5, and 4.9 µM respectively) while compound **1** did not have any connexin inhibition up to 100 µM.

A library of aryl substituted kanamycins was synthesized and successfully repurposed in two applications: as antifungal agents and connexin inhibitors. Most of the compounds showed very good antifungal activity as well as strong connexin inhibition effect compares with kanamycin A (the parent compound). Finally, these compounds had low toxicity toward mammalian cells.

The future directions for the antifungal agents are to study the nature of the dye compound interaction that diminished the fluorescence intensity when the compound concentration goes more than 2-fold of the MIC. This study will be done through 1) measuring the critical micelle concentration (CMC); 2) doing the same study with the compound being in neutral form. The future directions for aryl substituted kanamycins as connexin inhibitors are to further study the inhibition of these compounds against more connexin types that exist in the inner ear to see the selectivity of the amphiphilic kanamycins.

The second reported class of compounds was carbohydrate esters. A simple and regioselective one step carbohydrate esterification protocol was successfully developed. This protocol was applied to build the library from three monosaccharides (glucose, mannose, and N-acetylglucosamine), and various acyl carbon chain lengths [acetyl (C2) up to hexadecanoyl (C16)] to be studied in detailed structure activity relationship (SAR) study.

The carbohydrate ester compounds were tested as antibacterial and antifungal agents against a panel of bacteria (*Staphylococcus aureus*, methicillin-resistant *S. aureus* MRSA, and *Escherichia coli*) and fungi (i.e., *Aspergillus flavus*, *Fusarium graminearum*

B4-5A, *Candida albicans* 64124, *Candida albicans* MYA2876, *Cryptococcus neoformans*, and *Rhodotorula pilimanae*). The SAR study identified the compound **MAN014**, mannose acylated with tetradecanoyl, as the lead with wide-spectrum antibacterial and antifungal activities with MIC rang 16 – 128 µg/mL against bacteria and 4 – 64 µg/mL against fungi.

Carbohydrate esters are biodegradable that decompose to a saccharide and a fatty acid, which are non-toxic. **MAN014** showed low toxicity toward mammalian cells. This compound's mode of action was through affecting the cell membrane integrity. Finally, **MAN014** can be produced on large scales (i.e., 100g). These features all together, made environment friendly **MAN014** an excellent candidate to be used as antimicrobial in medicine and agriculture.

MAN014 was obtained as a mixture of two mono-acylated mannose at the 6 and the 3 positions. The future directions for this project are to separate these two products and test their antibacterial, antifungal activities, and cytotoxicity. This will tell whether there is one active compound and the other is less active, or they act in synergistic manner. In addition to that, development of a green synthesis routes should be pursued.

APPENDICES

APPENDIX A

CHAPTER II SUPPLEMENTRY MATERIALS

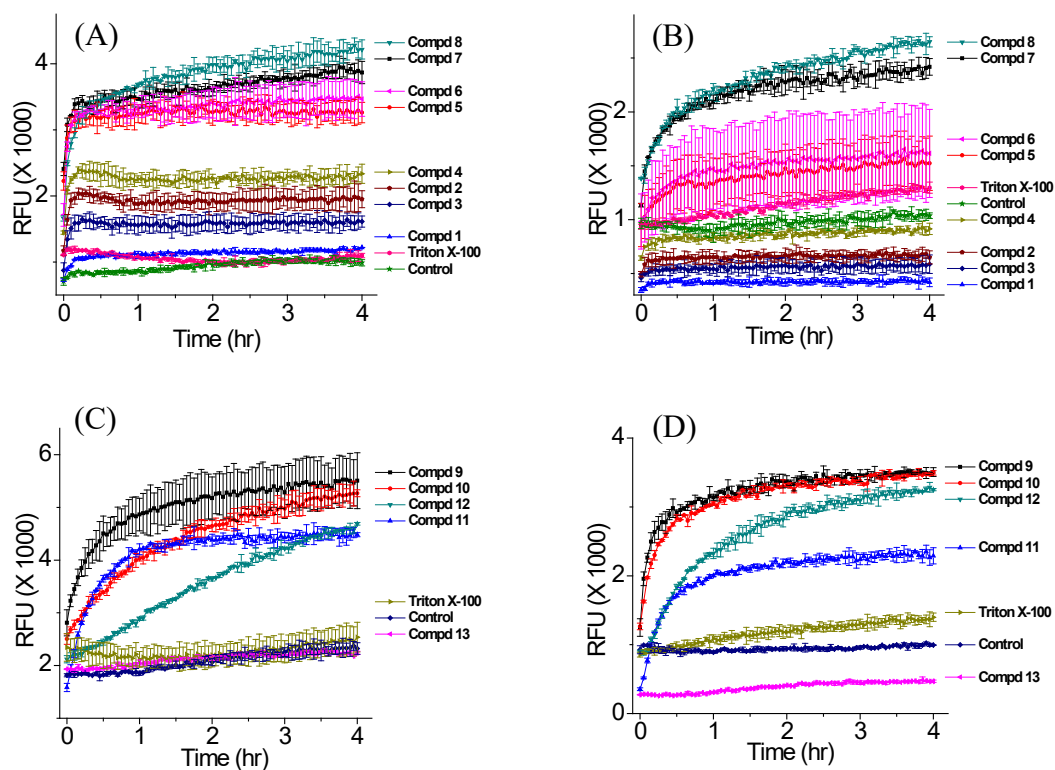


Figure A-1. Kinetic Membrane Permeabilization of *C. neoformans* treated with: (A) AKs attached with aryl groups and Sytox; (B) AKs attached with aryl groups and PI; (C) AKs attached with alkyl groups and SYTOXTM green; (D) AKs attached with alkyl groups and PI.

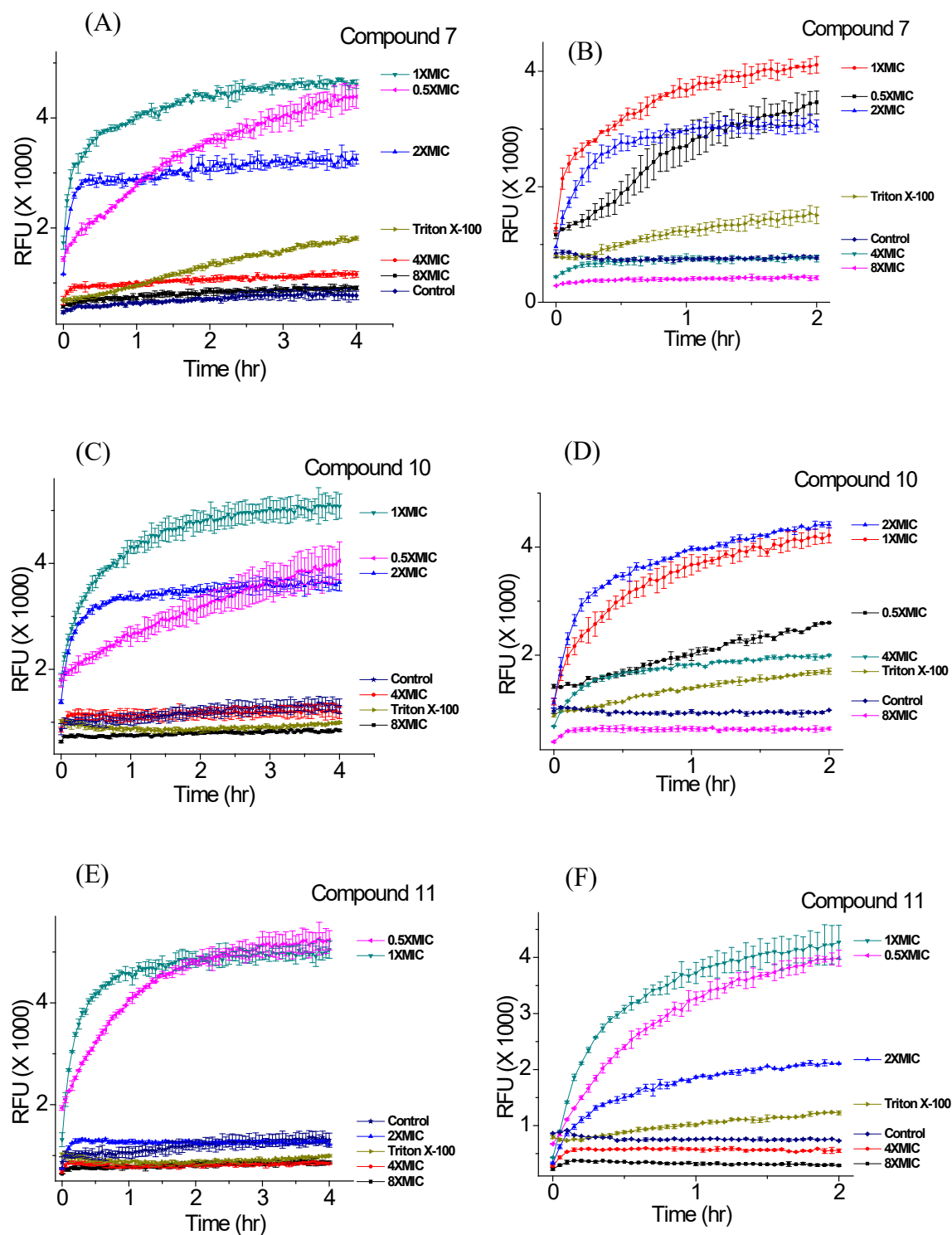


Figure A-2. Kinetic membrane permeabilization of *C. neoformans* treated with multiple MIC of (A) 2 and 0.01 μM SYTOXTM green; (B) 7 and 0.01 μM SYTOXTM green; (C) 7 and 0.4

$\mu\text{g/mL}$ PI; (D)) **10** and $0.01\ \mu\text{M}$ SYTOXTM green; (E) **10** and $0.4\ \mu\text{g/mL}$ PI; (F) **11** and $0.01\ \mu\text{M}$ SYTOXTM green; (G) **11** and $0.4\ \mu\text{g/mL}$ PI.

APPENDIX B

CHAPTER III SUPPLEMENTRY MATERIALS

B-1. Materials and Methods

Materials and Instrumentation

Chemicals were used directly from commercial sources without further purification. DMF was dried over molecular sieves (Fisher scientific, Grade 514). ^1H NMR, ^{13}C NMR, and ^1H - ^1H COSY spectra were acquired using JEOL300 (300 MHz) and Bruker Ascend (500 MHz) spectrometers at room temperature. A Shimadzu Prominence-i 2030C 3D LC system with an Agilent Zorbax RX-C18 5 μm 4.6 X 250 mm column was used for HPLC analysis of the compounds to confirm their purity.

Biological Experiments

Antibacterial assay: The minimum inhibitory concentration (MIC) of the newly synthesized compounds were tested against *E. coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC25923). The concentration of bacteria grown in Lysogeny Broth (LB) was adjusted to an absorbance at 600 nm (OD_{600}) of 0.08-0.1 using LB. A series of 2-fold dilutions was made for each compound in 96-well plates (USA Scientific CC7682-7596) starting from 256 $\mu\text{g/mL}$, and then, 50 μL of the diluted bacteria suspension was transferred to the wells. Bacteria were incubated for 18-24 h at 35°C before measuring OD_{600} to assess growth. Experiments for each compound were done at least in duplicates.

Connexin inhibition assay: We have found recently that functional human Cx26 HCs can be expressed in *E. coli*, and have developed and optimized a growth-complementation assay to assess HC function using LB2003 as host^{1,2}. LB2003 cells generously provided by Dr. E.P. Bakker (Osnabrück University, Osnabrück, Germany) were transformed with the plasmid pQE-Cx26 (human Cx26 into pQE60) and pREP4.

The plasmid pQE60 confers resistance to ampicillin, and pREP4 confers resistance to kanamycin and its derivatives. LB2003 cells cannot grow in low-[K⁺] media because of insufficient K⁺ uptake for growth due to the knockout of three key K⁺ transporters (Δ trk, Δ kup, Δ kdp strain)²⁻⁴. Details and validation of the growth complementation assay have been published recently². In brief, competent LB2003 containing the pREP4 plasmid were transformed with pQE-Cx26. These cells were grown overnight in Luria- Bertani medium (BD, Franklin Lakes, NJ, USA) supplemented with 100 mM KCl (to allow growth) and 0.4 mg/mL ampicillin, and then washed four times with NLM (see below), to remove residual K⁺ from the Luria-Bertani medium. The washed cells were resuspended in complementation growth medium to an OD₆₀₀ of 0.2. NLM contained 46 mM Na₂HPO₄, 23 mM NaH₂PO₄, 8 mM (NH₄)₂SO₄, 0.4 mM MgSO₄, 0.012 mM FeSO₄, 1 mM sodium citrate, 44 mM glucose and 0.006 mM thiamine hydrochloride, pH 7.0. KLM had the same composition that NLM except for the equimolar replacement of Na⁺ with K⁺. For growth complementation we used NLM + 4 mM KCl, and Cx26 expression was induced with 0.5 mM IPTG at the time of dilution to OD₆₀₀ = 0.2. For the assay, the cells were seeded in 96-well plates and were incubated at 30°C, with shaking at 500 rpm. OD₆₀₀ was measured after 18-h incubation in a plate reader. The initial OD₆₀₀ = 0.2 was subtracted for growth calculations. Data are presented as means \pm SEM. Statistically significant differences were calculated by the Student t-test for unpaired data. Data were obtained from at least 3 independent experiments, with 3 repeats per experiment.

Cytotoxicity assay: Cytotoxicity assays in HeLa cells were performed as described.⁵

Chemical Experiments

General procedure for *O*-Alkylation of 1,3,6',3''-tetraazidokanamycin A. 0.5 g of tetraazidokanamycin A was dissolved in 20 mL of dry DMF and a catalytic amount of tetrabutylammonium iodide (TBAI) was added. Then, 10 equivalents of NaH were added and the mixture was stirred for 15 min. After that, 1.5 equivalents of arylmethyl bromide was added and the reaction mixture was stirred overnight at room temperature. TLC (100% ethyl acetate elution) was used to confirm the completion of the reaction. The reaction was quenched by adding 5 mL of methanol, and the reaction mixture was concentrated and subjected to column chromatography (30:70 ethyl acetate: hexanes to 0:100). Three yellowish sticky oil products were identified, but unfortunately two of them were in an inseparable mixture.

General procedure for acetylation. 8 equivalents of Ac₂O and 16 equivalents of Et₃N were added to 0.02 g of the 4'',6''-Di-*O* alkylated tetraazidokanamycin dissolved in 20 mL of anhydrous DCM, along with a catalytic amount of DMAP. The reaction mixture was stirred overnight at room temperature. After completion of the reaction, monitored by TLC, solvent was removed by compressed air. The residue obtained was purified by flash column using 100% DCM. This procedure is employed to allow ¹H-¹H COSY experiments to confirm the sites of alkylation⁶. The acylated adducts are not included in the manuscript but can be found in this document.

General procedure for Staudinger reaction and preparation of final compounds.

Azide groups in alkyl substituted tetraazidokanamycin were reduced to amine by Staudinger reaction⁶.

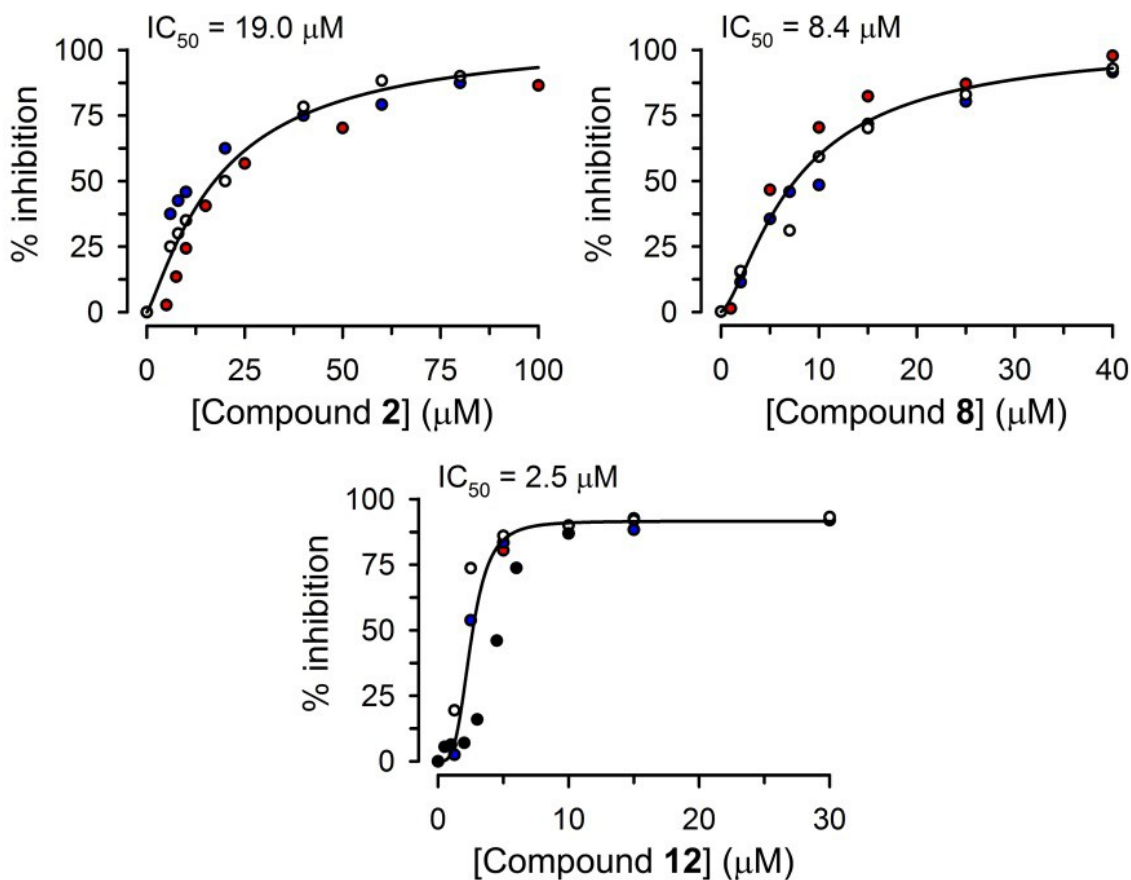


Figure B-1. Examples of inhibition of Cx26-dependent growth complementation by three of the new amphiphilic AGs. Each symbol corresponds to a triplicate average, and each color corresponds to an independent experiment. The lines are the fits of the Hill's equation to the data. Growth inhibition was significant for the three compounds ($P < 0.01$ vs. growth in the absence of drug).

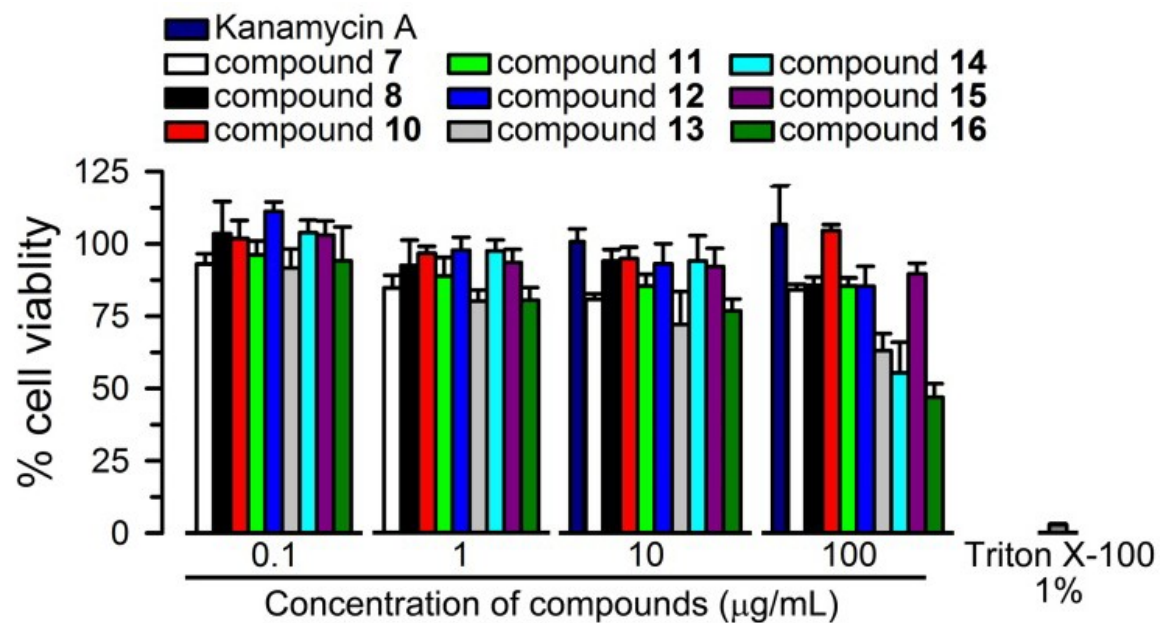


Figure B-2. Cytotoxicity of kanamycin A and amphiphilic kanamycin derivatives.

Table B-1. Yields of second generation synthetic AGs.

Compound	Yield (%)	Compound	Yield (%)
7b	32	10	54
7c	35	11	88
7d	41	12	58
7e	37	13	71
7f	41	14	72
7g	43	15	42
7h	26	16	74

B-2 Compound Characterization Data

4'',6''-Di-*O*-benzyl-1,3,6',3''-tetraazidokanamycin (7a). ¹H NMR (300 MHz, Methanol-D₃) δ 7.2 – 7.4 (m, 10H), 5.21 (d, *J* = 3.9 Hz, 1H), 5.20 (d, *J* = 4.2 Hz, 1H), 4.73 (d, *J* = 10.8 Hz, 1H), 4.56 (d, *J* = 12.0 Hz, 1H), 4.49 (d, *J* = 10.8 Hz, 1H), 4.44 (d, *J* = 12.0 Hz, 1H), 4.2 – 4.3 (m, 1H), 4.0 – 4.1 (m, 1H), 3.3 - 3.7 (m, 15H), 2.3 - 2.4 (m, 1H), 1.52 (ddd, *J* = 12.3 Hz 1H). ¹³C NMR (75. MHz, Methanol-D₃) δ 138.18 (2 Carbons), 128.24 (2 Carbons), 128.11 (2 Carbons), 128.03 (2 Carbons), 127.97 (2 Carbons), 127.60 (2 Carbons), 101.20, 98.43, 83.89, 80.05, 76.50 (2 Carbons), 74.39, 73.54, 73.18, 72.63, 72.23, 71.22, 70.81, 70.26, 68.35, 66.92, 61.09, 59.61, 51.49, 32.18. ESI/APCI calcd for C₃₂H₄₀N₁₂O₁₁Na [M+Na]⁺ *m/z* 791.2832; measured *m/z* 791.2858. Yield: 196 mg (255mmol, 30%).

4'',6''-Di-*O*-(4-methoxybenzyl)-1,3,6',3''-tetraazidokanamycin (7b). ¹H NMR (500 MHz, Methanol- D₃) δ 7.28 (d, *J* = 7.7 Hz, 2H), 7.11 (d, *J* = 7.7 Hz, 2H), 6.89 (d, *J* = 7.4 Hz, 2H), 6.85 (d, *J* = 7.4 Hz, 2H), 5.24 (d, *J* = 3.8 Hz, 1H), 5.23 (d, *J* = 3.5 Hz, 1H), 4.65 (d, *J* = 10.4 Hz, 1H), 4.55 (d, *J* = 11.6 Hz, 1H), 4.3 – 4.4 (m, 3H), 4.0 – 4.1 (m, 1H), 3.3 - 3.7 (m, 21H), 2.3 - 2.4 (m, 1H), 1.55 (ddd, *J* = 12.5 Hz 1H). ¹³C NMR (125 MHz, Methanol-D₃) δ 159.56, 159.49, 129.97, 129.81, 129.66 (2 Carbons), 129.52 (2 Carbons), 113.46 (2 Carbons), 113.25 (2 Carbons), 101.00, 98.23, 83.67, 79.95, 75.94, 74.19, 73.88, 73.37, 72.59, 72.45, 72.04, 70.96, 70.64, 70.05, 67.58, 66.72, 60.86, 59.44, 54.29, 54.27, 51.32, 31.99. ESI/APCI calcd for C₃₄H₄₄N₁₂O₁₃Na [M+Na]⁺: 851.3048 *m/z* ; measured 851.3096 *m/z*. Yield: 225 mg (272mmol, 32%).

4'',6''-Di-*O*-(4-methylbenzyl)-1,3,6',3''-tetraazidokanamycin (7c). ¹H NMR (300 MHz, Methanol-D₃) δ 7.0 – 7.2 (m, 8H), 5.20 (d, *J* = 2.7 Hz, 1H), 5.19 (d, *J* = 3.6 Hz, 1H), 4.65 (d, *J* = 10.8 Hz, 1H), 4.53 (d, *J* = 12.0 Hz, 1H), 4.2 – 4.4 (m, 3H), 4.0 – 4.1 (m, 1H), 3.3 - 3.7 (m, 15H), 2.3 - 2.4 (m, 7H), 1.52 (ddd, *J* = 12.3 Hz 1H). ¹³C NMR (75 MHz, Methanol-D₃) δ 137.42, 137.30, 134.96, 134.85 128.75 (2 Carbons), 128.56 (2 Carbons), 128.17 (2 Carbons), 128.03 (2 Carbons), 101.07, 98.43, 83.76, 79.92, 76.15, 74.24, 74.16, 73.41, 72.87, 72.50, 72.11, 71.05, 70.67, 70.08, 66.79, 60.96, 59.49, 51.37, 32.08, 29.43, 19.92 (2 Carbons). ESI/APCI calcd for C₃₄H₄₄N₁₂O₁₁Na [M+Na]⁺: *m/z* 819.3145; measured *m/z* 819.3158. Yield: 237 mg (298 mmol, 35%).

4'',6''-Di-*O*-(4-chlorobenzyl)-1,3,6',3''-tetraazidokanamycin (7d). ¹H NMR (500 MHz, Methanol-D₃) δ 7.3 – 7.4 (m, 6H), 7.2 (d, *J* = 7.8 Hz 2H), 5.24 (d, *J* = 3.4 Hz, 2H),

4.76 (d, $J = 11.3$ Hz, 1H), 4.58 (d, $J = 12.1$ Hz, 1H), 4.51 (d, $J = 11.1$ Hz, 1H), 4.46 (d, $J = 12.2$ Hz, 1H), 4.3 – 4.4 (m, 1H), 4.0 – 4.1 (m, 1H), 3.3 - 3.7 (m, 15H), 2.3 - 2.4 (m, 1H), 1.56 (ddd, $J = 12.2$ Hz 1H). ^{13}C NMR (125 MHz, Methanol-D₃) δ 138.87, 138.855, 133.16, 133.11, 129.30 (2 Carbons), 129.09 (2 Carbons), 128.14 (2 Carbons), 128.01 (2 Carbons), 100.98, 98.24, 83.68, 79.90, 76.36, 74.21, 73.35, 73.19, 72.45, 72.05, 72.02, 71.08, 70.64, 69.99, 69.18, 66.64, 60.91, 59.43, 51.31, 32.01. ESI/APCI calcd for $\text{C}_{32}\text{H}_{38}\text{Cl}_2\text{N}_{12}\text{O}_{11}\text{Na}$ $[\text{M}+\text{Na}]^+$: 859.2058 m/z ; measured 859.2072 m/z . Yield: 292 mg (349 mmol, 41%).

4'',6''-Di-*O*-(4-fluorobenzyl)-1,3,6',3''-tetraazidokanamycin (7e). ^1H NMR (300 MHz, Methanol-D₃) δ 7.2 – 7.4 (m, 4H), 6.9 – 7.1 (m, 4H), 5.25 (d, $J = 3.6$ Hz, 2H), 4.71 (d, $J = 11.1$ Hz, 1H), 4.53 (d, $J = 11.4$ Hz, 1H), 4.4 – 4.5 (m, 2H), 4.3 – 4.4 (m, 1H), 4.0 – 4.1 (m, 1H), 3.3 - 3.7 (m, 15H), 2.3 - 2.4 (m, 1H), 1.52 (ddd, $J = 12.3$ Hz 1H). ^{13}C NMR (75 MHz, Methanol-D₃) δ 162.50 (d, $J_{\text{CF}} = 243.1$ Hz), 162.46 (d, $J_{\text{CF}} = 244.2$ Hz), 134.12 (d, $J_{\text{CF}} = 3.0$ Hz), 134.07 (d, $J_{\text{CF}} = 3.0$ Hz), 129.77 (d, $J_{\text{CF}} = 8.1$ Hz, 2 Carbons), 129.65 (d, $J_{\text{CF}} = 8.2$ Hz, 2 Carbons), 114.72 (d, $J_{\text{CF}} = 17.6$ Hz, 2 Carbons), 114.55 (d, $J_{\text{CF}} = 17.6$ Hz, 2 Carbons), 101.06, 98.31, 83.74, 79.96, 76, 29, 74.27, 73.41 (2 Carbons), 72.51, 72.21, 72.12, 71.10, 70.69, 70.09, 68.14, 66.72, 60.99, 59.50, 51.37, 32.09. ESI/APCI calcd for $\text{C}_{32}\text{H}_{38}\text{N}_{12}\text{O}_{11}\text{F}_2\text{Na}$ $[\text{M}+\text{Na}]^+$: m/z 827.2643; measured m/z 827.2652. Yield: 253 mg (315 mmol, 37%).

4'',6''-Di-*O*-(2-naphthalenemethyl)-1,3,6',3''-tetraazidokanamycin (7f). ^1H NMR (500 MHz, Methanol-D₃) δ 7.7 – 7.8 (m, 5H), 7.63 (d, $J = 8.4$ Hz, 1H), 7.57 (d, $J = 7.9$

Hz, 1H), 7.3 – 7.5 (m, 6H), 7.57 (dd, $J = 8.4$ Hz, $J = 1.5$ Hz, 1H), 5.26 (d, $J = 3.7$ Hz, 1H), 5.22 (d, $J = 3.8$ Hz, 1H), 4.8 – 4.9 (m, 1H), 4.75 (d, $J = 12.8$ Hz, 1H), 4.5 – 4.6 (m, 2H), 4.3 – 4.4 (m, 1H), 4.0 – 4.1 (m, 1H), 3.3 – 3.7 (m, 15H), 2.3 – 2.4 (m, 1H), 1.51 (ddd, $J = 12.5$ Hz 1H). ^{13}C NMR (125 MHz, Methanol- D_3) δ 135.35, 135.33, 133.31, 133.19, 133.17, 133.01, 127.90, 127.60, 127.56, 127.53, 127.38, 127.22, 126.76, 126.35, 125.95, 125.85, 125.72, 125.67, 125.63, 125.55, 101.00, 98.28, 83.72, 79.97, 76.19, 74.18 (2 Carbons), 73.35, 73.04, 72.43, 72.04, 71.06, 70.62, 70.08, 68.02, 66.79, 60.85, 59.40, 51.32, 31.96. ESI/APCI calcd for $\text{C}_{40}\text{H}_{44}\text{N}_{12}\text{O}_{11}\text{Na}$ $[\text{M}+\text{Na}]^+$: 891.3150 m/z ; measured 891.3111 m/z . Yield: 302 mg (349 μmol , 41%).

4'',6''-Di-*O*-(1-naphthalenemethyl)-1,3,6',3''-tetraazidokanamycin (7g). ^1H NMR (500 MHz, Methanol- D_3) δ 8.13 (d, $J = 8.3$ Hz, 1H), 7.9 (d, $J = 7.7$ Hz, 1H), 7.7 – 7.8 (m, 4H), 7.4 – 7.5 (m, 6H), 7.29 (t, $J = 7.5$ Hz, 1H), 7.07 (d, $J = 6.6$ Hz, 1H), 5.25 (d, $J = 3.3$ Hz, 1H), 5.1 – 5.2 (m, 2H), 5.01 (d, $J = 12.2$ Hz, 1H), 4.6 – 4.7 (m, 2H), 4.3 – 4.4 (m, 1H), 4.0 – 4.1 (m, 1H), 3.3 – 3.7 (m, 14H), 2.9 (dd, $J = 13.2$ Hz, $J = 8.1$ Hz, 1H), 2.3 – 2.4 (m, 1H), 1.52 (ddd, $J = 12.5$ Hz 1H). ^{13}C NMR (125 MHz, Methanol- D_3) δ 133.93, 133.76, 133.45, 133.40, 131.74, 131.65, 128.43, 128.28, 128.23, 128.07, 126.53 (2 Carbons), 125.86, 125.80, 125.48, 125.32, 124.82, 124.80, 123.87, 123.71, 100.95, 98.23, 83.66, 79.83, 75.51, 74.14, 73.33, 72.40, 72.02, 71.86, 71.36, 71.23, 70.60, 70.07, 68.11, 66.95, 60.86, 59.38, 51.30, 31.95. ESI/APCI calcd for $\text{C}_{40}\text{H}_{44}\text{N}_{12}\text{O}_{11}\text{Na}$ $[\text{M}+\text{Na}]^+$: 891.3145 m/z ; measured 891.3164 m/z . Yield: 318 mg (366 μmol , 43%).

4'',6''-Di-*O*-(3-phenylbenzyl)-1,3,6',3''-tetraazidokanamycin (7h). ¹H NMR (500 MHz, Methanol-D₃) δ 7.3 – 7.6 (m, 17H), 7.21 (d, *J* = 7.6 Hz, 1H), 5.26 (d, *J* = 3.7 Hz, 1H), 5.17 (d, *J* = 3.8 Hz, 1H), 4.83 (d, *J* = 11.0 Hz, 1H), 4.68 (d, *J* = 12.0 Hz, 1H), 4.61 (d, *J* = 11.0 Hz, 1H), 4.53 (d, *J* = 12.1 Hz, 1H), 4.3 – 4.4 (m, 1H), 4.0 – 4.1 (m, 1H), 3.3 – 3.7 (m, 15H), 2.3 – 2.4 (m, 1H), 1.51 (ddd, *J* = 12.2 Hz 1H). ¹³C NMR (125 MHz, Methanol-D₃) δ 141.25, 141.18, 140.85, 140.79, 138.56 (2 Carbons), 128.55, 128.46 (2 Carbons), 128.43 (3 Carbons), 127.00, 126.96, 126.73, 126.67, 126.64 (4 Carbons), 126.42, 126.34, 126.10, 126.06, 100.99, 98.25, 83.70, 79.87, 76.39, 74.20, 74.16, 73.34, 72.83, 72.41, 72.02, 71.04, 70.61, 70.01, 68.10, 66.77, 60.90, 59.40, 51.30, 31.98. ESI/APCI calcd for C₄₄H₄₈N₁₂O₁₁Na [M+Na]⁺ 943.3463; *m/z* ; measured 943.3355 *m/z*. Yield: 204 mg (221 mmol, 26%).

5, 2', 3', 4', 2''- Penta-*O*-methyl-4'',6''-di-*O*-benzyl-1,3,6',3''-tetraazidokanamycin (8a). ¹H NMR (300 MHz, Chloroform-D) δ 7.2 – 7.4 (m, 10H), 5.59 (d, *J* = 3.3 Hz, 1H), 5.43 (d, *J* = 3.9 Hz, 1H), 4.78 (d, *J* = 10.8 Hz, 1H), 4.64 (d, *J* = 12.0 Hz, 1H), 4.4 – 4.5 (m, 2H), 4.0 – 4.2 (m, 2H), 3.4 – 3.8 (m, 28H), 3.1 – 3.3 (m, 2H), 2.3 – 2.4 (m, 1H), 1.5 – 1.6 (m, 1H). ¹³C NMR (75 MHz, Chloroform-D) δ 138.06, 137.89, 128.51 (2 Carbons), 128.39 (2 Carbons), 128.16 (2 Carbons), 128.01 (2 Carbons), 127.91, 127.83, 97.03, 95.53, 84.58, 83.51, 81.81, 80.53, 80.41, 77.83, 77.31, 76.15, 74.79, 73.63, 70.66, 70.24, 68.10, 65.19, 61.27, 61.02, 60.88, 60.71, 60.66, 59.29, 59.15, 51.33, 32.63. ESI/APCI calcd for C₃₇H₅₀N₁₂O₁₁Na [M+Na]⁺; *m/z* 861.3614; measured *m/z* 861.3640. Yield: 344 mg (410 mmol, 63%).

4'',6''-Di-*O*-benzylkanamycin (7). ^1H NMR (300 MHz, D_2O) δ 7.2 – 7.4 (m, 8H), 7.1 – 7.2 (m, 2H), 5.44 (d, $J = 3.9$ Hz, 1H), 5.01 (d, $J = 3.6$ Hz, 1H), 4.5 – 4.6 (m, 2H), 4.3 – 4.4 (m, 2H), 4.0 – 4.1 (m, 1H), 3.4 – 3.9 (m, 12H), 3.2 – 3.3 (m, 2H), 3.0 – 3.1 (m, 2H), 2.4 – 2.5 (m, 1H), 1.84 (ddd, $J = 12.9$ Hz 1H). ^{13}C NMR (75 MHz, D_2O) δ 136.68, 136.23, 128.98 (2 Carbons), 128.95 (3 Carbons), 128.89 (2 Carbons), 128.73 (2 Carbons), 128.66, 100.53, 96.31, 83.90, 78.10, 74.45, 73.33, 73.23, 72.76, 72.09, 71.01, 70.80, 68.69, 68.08, 67.49, 54.23, 49.89, 47.84, 44.75, 40.36, 27.61. ESI/APCI calcd for $\text{C}_{32}\text{H}_{49}\text{N}_4\text{O}_{11}$ $[\text{MH}]^+$: m/z 665.3392; measured m/z 665.3407. Yield: 99 mg (122 μmol , 89%).

5, 2', 3', 4', 2''- Penta-*O*-methyl-4'',6''-di-*O*-benzylkanamycin (8). ^1H NMR (300 MHz, D_2O) δ 7.1 – 7.4 (m, 10H), 5.43 (d, $J = 3.3$ Hz, 1H), 5.23 (d, $J = 3.0$ Hz, 1H), 4.4 – 4.6 (m, 4H), 3.1 – 3.9 (m, 32H), 2.3 – 2.4 (m, 1H), 1.74 (ddd, $J = 12.3$ Hz 1H). ^{13}C NMR (75 MHz, D_2O) δ 136.64, 136.26, 129.05 (2 Carbons), 128.96 (2 Carbons), 128.90, 128.82 (2 Carbons), 128.69 (2 Carbons), 128.63, 97.99, 95.10, 82.61, 81.44, 81.31, 79.85, 79.68, 78.56, 75.78, 74.50, 73.38, 73.16, 71.38, 68.29, 67.10, 60.19, 59.98, 59.86, 59.63, 58.36, 53.04, 49.68, 48.15, 40.09, 28.18. ESI/APCI calcd for $\text{C}_{37}\text{H}_{59}\text{N}_4\text{O}_{11}$ $[\text{MH}]^+$: m/z 735.4175; measured m/z 735.4196. Yield: 130 mg (148 μmol , 92%).

5, 2', 3', 4', 2''- Penta-*O*-methyl-kanamycin (9). ^1H NMR (300 MHz, D_2O) 5.46 (d, $J = 3.3$ Hz, 1H), 5.35 (d, $J = 3.3$ Hz, 1H), 4.00 (t, $J = 9.9$ Hz, 1H), 3.1 – 3.9 (m, 31H), 2.3 – 2.4 (m, 1H), 1.83 (ddd, $J = 12.3$ Hz 1H). ^{13}C NMR (75 MHz, D_2O) δ 98.30, 94.44, 81.57, 81.41, 80.60, 79.81, 79.67, 78.70, 74.80, 73.02, 68.45, 65.26, 60.19, 59.97 (2 Carbons),

59.59, 59.52, 57.11, 53.82, 49.73, 47.85, 40.10, 27.44. ESI/APCI calcd for $C_{23}H_{47}N_4O_{11}$ $[MH]^+$: m/z 555.3236; measured m/z 555.3234. Yield: 14 mg (20 mmol, 27%).

4'',6''-Di-*O*-(4-methoxybenzyl) kanamycin (10). 1H NMR (500 MHz, D_2O) δ 6.94 (d, J = 8.6 Hz, 2H), 6.63 (d, J = 8.6 Hz, 2H), 6.4 – 6.5 (m, 4H), 5.18 (d, J = 3.6 Hz, 1H), 4.68 (m, 1H, overlapped with H_2O), 4.24 (d, J = 11.8 Hz, 1H), 4.03 (d, J = 10.4 Hz, 1H), 3.97 (d, J = 11.5 Hz, 1H), 3.75 (d, J = 10.4 Hz, 1H), 2.7 - 3.7 (m, 23H), 2.1 - 2.2 (m, 1H), 1.51 (ddd, J = 12.4 Hz 1H). ^{13}C NMR (125 MHz, D_2O) δ 158.61, 158.48, 130.71 (2 Carbons), 130.01 (2 Carbons), 128.28, 128.13, 113.59 (2 Carbons), 113.54 (2 Carbons), 100.34, 96.00, 83.63, 77.57, 73.65, 72.41 (2 Carbons), 72.09, 71.53, 70.42 (2 Carbons), 70.36, 69.25, 67.70, 65.80, 54.79, 54.63, 53.75, 49.47, 47.36, 39.91. ESI/APCI calcd for $C_{34}H_{52}N_4O_{13}Na$ $[M+Na]^+$: 747.3423 m/z ; measured 747.3436 m/z . Yield: 173 mg (199 mmol, 54%).

4'',6''-Di-*O*-(4-methylbenzyl) kanamycin (11). 1H NMR (500 MHz, D_2O) δ 7.23 (d, J = 7.9 Hz, 2H), 7.13 (d, J = 7.4 Hz, 4H), 7.13 (d, J = 7.9 Hz, 2H), 5.49 (d, J = 4.0 Hz, 1H), 5.05 (d, J = 3.7 Hz, 1H), 4.54 (d, J = 11.6 Hz, 1H), 4.47 (d, J = 10.8 Hz, 1H), 4.36 (d, J = 11.6 Hz, 1H), 4.25 (d, J = 10.8 Hz, 1H), 4.0 – 4.1 (m, 1H), 3.8 – 4.0 (m, 4H), 3.6 - 3.7 (m, 4H), 3.4 - 3.5 (m, 5H), 3.2 - 3.3 (m, 2H), 3.1 - 3.2 (m, 1H), 2.4 - 2.5 (m, 1H), 2.25 (s, 3H), 2.18 (s, 3H), 1.88 (ddd, J = 12.5 Hz 1H). ^{13}C NMR (125 MHz, D_2O) δ 139.12, 138.86, 133.37, 133.08, 129.38 (4 Carbons), 129.19 (2 Carbons), 128.75 (2 Carbons), 100.44, 96.28, 83.83, 78.03, 74.21, 73.13, 72.88, 72.74, 72.02, 70.94, 70.79, 70.76, 68.63, 68.02, 66.93, 54.20, 49.83, 47.81, 40.33, 27.56, 20.30, 20.28.

ESI/APCI calcd for $C_{34}H_{53}N_4O_{11}$ $[MH]^+$: 693.3705 m/z ; measured 693.3731 m/z . Yield: 198 mg (236 mmol, 88%).

4'',6''-Di-*O*-(4-chlorobenzyl) kanamycin (12). 1H NMR (500 MHz, D_2O) δ 7.2 – 7.3 (m, 6H), 7.07 (d, J = 8.4 Hz 2H), 5.44 (d, J = 3.9 Hz, 1H), 5.44 (d, J = 3.6 Hz, 1H), 4.5 – 4.6 (m, 2H), 4.36 (d, J = 11.9 Hz, 1H), 4.27 (d, J = 11.3 Hz, 1H), 4.0 – 4.1 (m, 1H), 4.0 – 4.1 (m, 1H), 3.1 - 3.9 (m, 15H), 2.4 - 2.5 (m, 1H), 1.81 (ddd, J = 12.5 Hz 1H). ^{13}C NMR (125 MHz, D_2O) δ 135.06, 134.82, 133.73, 133.66, 130.50 (2 Carbons), 129.64 (2 Carbons), 128.72 (2 Carbons), 128.69 (2 Carbons), 100.40, 96.56, 84.09, 78.39, 73.58, 73.51, 73.02, 72.24, 71.99, 70.77 (4 Carbons), 68.61, 68.12, 67.09, 54.20, 49.87, 47.96, 40.32, 28.05. ESI/APCI calcd for $C_{32}H_{47}ClN_4O_{11}$ $[MH]^+$: 733.2613 m/z ; measured 733.2621 m/z . Yield: 53 mg (60 mmol, 58%).

4'',6''-Di-*O*-(4-fluorobenzyl) kanamycin (13). 1H NMR (500 MHz, D_2O) 7.3 – 7.4 (m, 2H), 7.1 – 7.2 (m, 2H), 7.0 – 7.1 (m, 4H), 5.47 (d, J = 3.9 Hz, 1H), 5.03 (d, J = 3.6 Hz, 1H), 4.5 – 4.6 (m, 2H), 4.41 (d, J = 11.6 Hz, 1H), 4.34 (d, J = 11.0 Hz, 1H), 4.0 – 4.1 (m, 1H), 3.3 - 3.9 (m, 16H), 2.3 - 2.4 (m, 1H), 1.82 (ddd, J = 12.5 Hz 1H). ^{13}C NMR (125 MHz, D_2O) δ 162.57 (d, J_{CF} = 244.3 Hz), 162.49 (d, J_{CF} = 244.3 Hz), 132.46 (d, J_{CF} = 2.8 Hz), 132.12 (d, J_{CF} = 2.8 Hz), 130.94 (d, J_{CF} = 8.4 Hz, 2 Carbons), 130.43 (d, J_{CF} = 8.4 Hz, 2 Carbons), 115.49 (d, J_{CF} = 21.6 Hz, 2 Carbons), 115.43 (d, J_{CF} = 21.6 Hz, 2 Carbons), 100.44, 98.51, 83.98, 78.40, 73.60, 73.31, 72.87, 72.34, 72.01, 70.83, 70.75, 70.70, 68.62, 68.07, 67.11, 54.17, 49.58, 47.87, 40.26, 27.88. ESI/APCI calcd for $C_{32}H_{47}F_2N_4O_{11}$ $[MH]^+$: 701.3204 m/z ; measured 701.3200 m/z . Yield: 66 mg (78 mmol,

71%).

4'',6''-Di-*O*-(2-naphthalenmethyl) kanamycin (14). ^1H NMR (500 MHz, D_2O) δ 7.2 – 7.7 (m, 13H), 7.00 (dd, $J = 8.4$ Hz, $J = 1.6$ Hz, 1H), 5.39 (d, $J = 3.9$ Hz, 1H), 5.06 (d, $J = 3.6$ Hz, 1H), 4.6 – 4.7 (m, 2H), 4.37 (d, $J = 11.9$ Hz, 1H), 4.28 (d, $J = 11.4$ Hz, 1H), 4.0 – 4.1 (m, 1H), 3.0 – 3.9 (m, 16H), 2.3 – 2.4 (m, 1H), 1.86 (ddd, $J = 12.5$ Hz 1H). ^{13}C NMR (125 MHz, D_2O) δ 133.79, 133.50, 132.84, 132.77, 132.69, 132.53, 128.47, 128.31, 127.99, 127.81 (2 Carbons), 127.61, 127.60, 127.01, 126.62, 126.55, 126.52 (2 Carbons), 126.47, 125.62, 100.43, 96.46, 83.87, 77.83, 73.24, 73.04, 72.98, 71.90, 70.90, 70.68, 70.65, 68.59, 68.03, 66.99, 54.24, 49.76, 47.92, 40.29, 27.54. ESI/APCI calcd for $\text{C}_{40}\text{H}_{53}\text{N}_4\text{O}_{11}$ $[\text{MH}]^+$: 765.3705 m/z ; measured 765.3755 m/z . Yield: 85 mg (94 μmol , 72%).

4'',6''-Di-*O*-(1-naphthalenmethyl) kanamycin (15). ^1H NMR (500 MHz, D_2O) δ 7.87 (d, $J = 8.3$ Hz, 1H), 7.7 – 7.8 (m, 4H), 7.5 – 7.6 (m, 2H), 7.2 – 7.5 (m, 6H), 7.06 (d, $J = 6.8$ Hz, 1H), 5.17 (d, $J = 3.9$ Hz, 1H), 5.05 (d, $J = 3.6$ Hz, 1H), 4.7 – 4.8 (m, 2H), 4.3 – 4.4 (m, 2H), 3.9 – 4.0 (m, 1H), 3.3 – 3.7 (m, 15H), 2.9 – 3.0 (m, 1H), 2.4 – 2.5 (m, 1H), 1.84 (ddd, $J = 12.5$ Hz 1H). ^{13}C NMR (125 MHz, D_2O) δ 133.49, 133.38, 131.85, 131.80, 131.35, 130.65, 129.34, 129.29, 128.75, 128.68, 127.94, 127.28, 126.92, 126.68, 126.29, 126.11, 125.46, 125.34, 123.65, 123.14, 100.07, 96.06, 83.59, 77.81, 73.81, 72.77, 72.11, 71.97, 71.09, 71.06, 70.71, 70.61, 68.46, 67.89, 67.70, 54.33, 49.68, 47.70, 40.21, 27.51. ESI/ APCI calcd for $\text{C}_{40}\text{H}_{53}\text{N}_4\text{O}_{11}$ $[\text{MH}]^+$: 765.3705 m/z ; measured 765.3704 m/z . Yield: 209 mg (229 μmol , 42%).

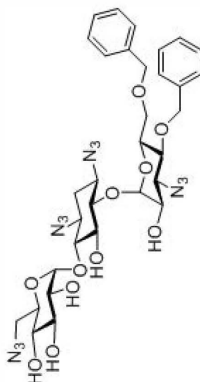
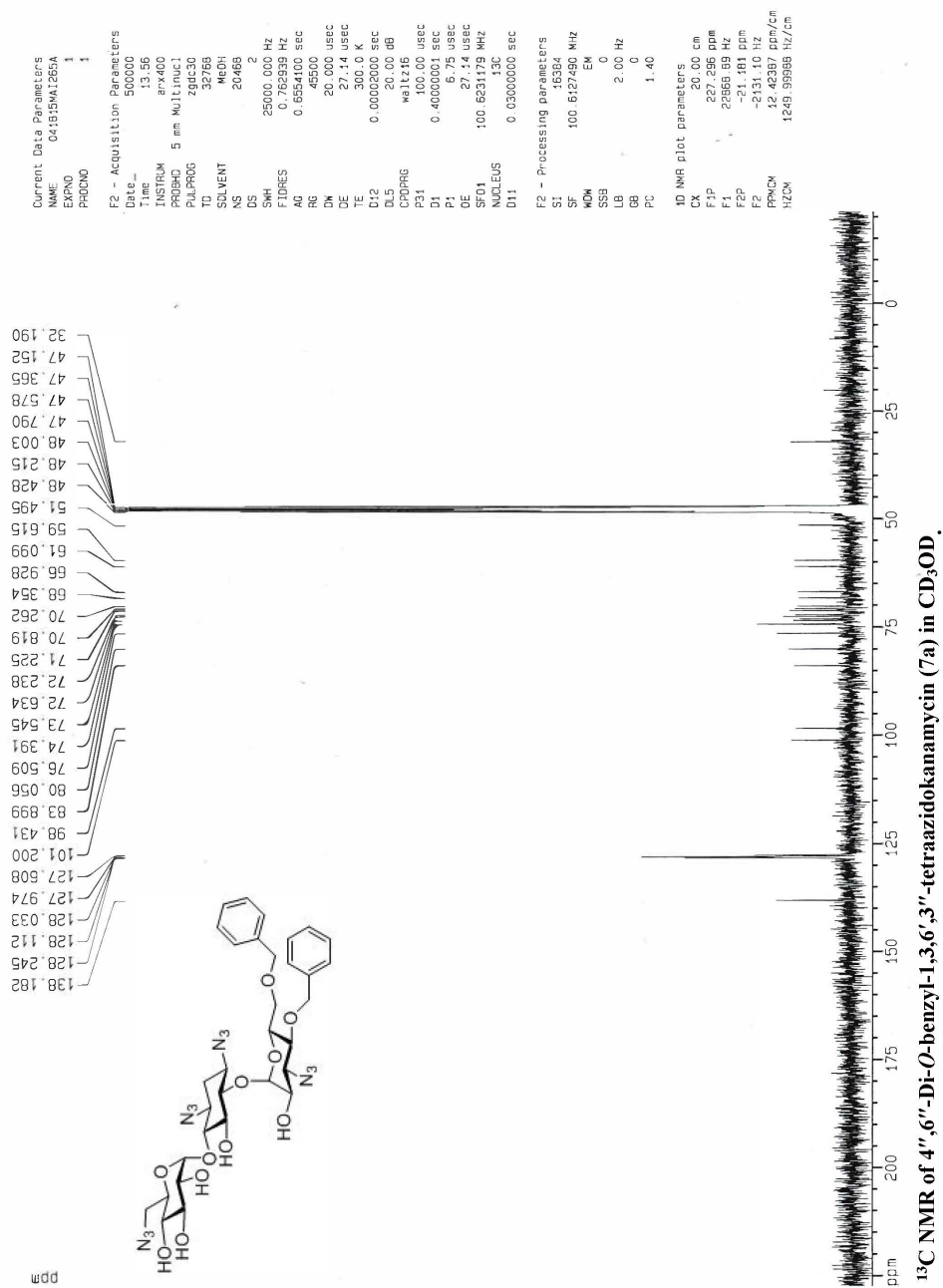
4'',6''-Di-O-(3-phenylbenzyl)-kanamycin (16). ^1H NMR (500 MHz, D_2O) δ 7.42 (s, 1H), 7.1 – 7.4 (m, 16H), 7.03 (d, J = 7.6 Hz, 1H), 5.49 (d, J = 3.9 Hz, 1H), 5.05 (d, J = 3.4 Hz, 1H), 4.59 (d, J = 12.0 Hz, 1H), 4.51 (d, J = 11.1 Hz, 1H), 4.2 – 4.3 (m, 2H), 4.0 – 4.1 (m, 1H), 3.0 – 3.9 (m, 16H), 2.4 - 2.5 (m, 1H), 1.86 (dd, J = 12.5 Hz, 1H). ^{13}C NMR (125 MHz, D_2O) δ 140.75, 140.68, 139.89, 139.78, 136.80, 136.78, 129.35, 129.25, 129.03 (2 Carbons), 128.92 (2 Carbons), 128.12, 127.76, 127.65, 127.23, 127.21, 126.98, 126.94, 126.84 (2 Carbons), 126.72 (2 Carbons), 126.50, 100.52, 96.86, 83.73, 78.25, 74.21, 73.28, 73.00 (2 Carbons), 71.96, 70.91, 70.76, 70.68, 68.66, 68.05, 66.91, 54.23, 49.81, 47.92, 40.26, 27.58. ESI/APCI calcd for $\text{C}_{44}\text{H}_{57}\text{N}_4\text{O}_{11}$ $[\text{MH}]^+$: 817.4024 m/z; measured 817.4005 m/z. Yield: 215 mg (223 mmol, 74%).

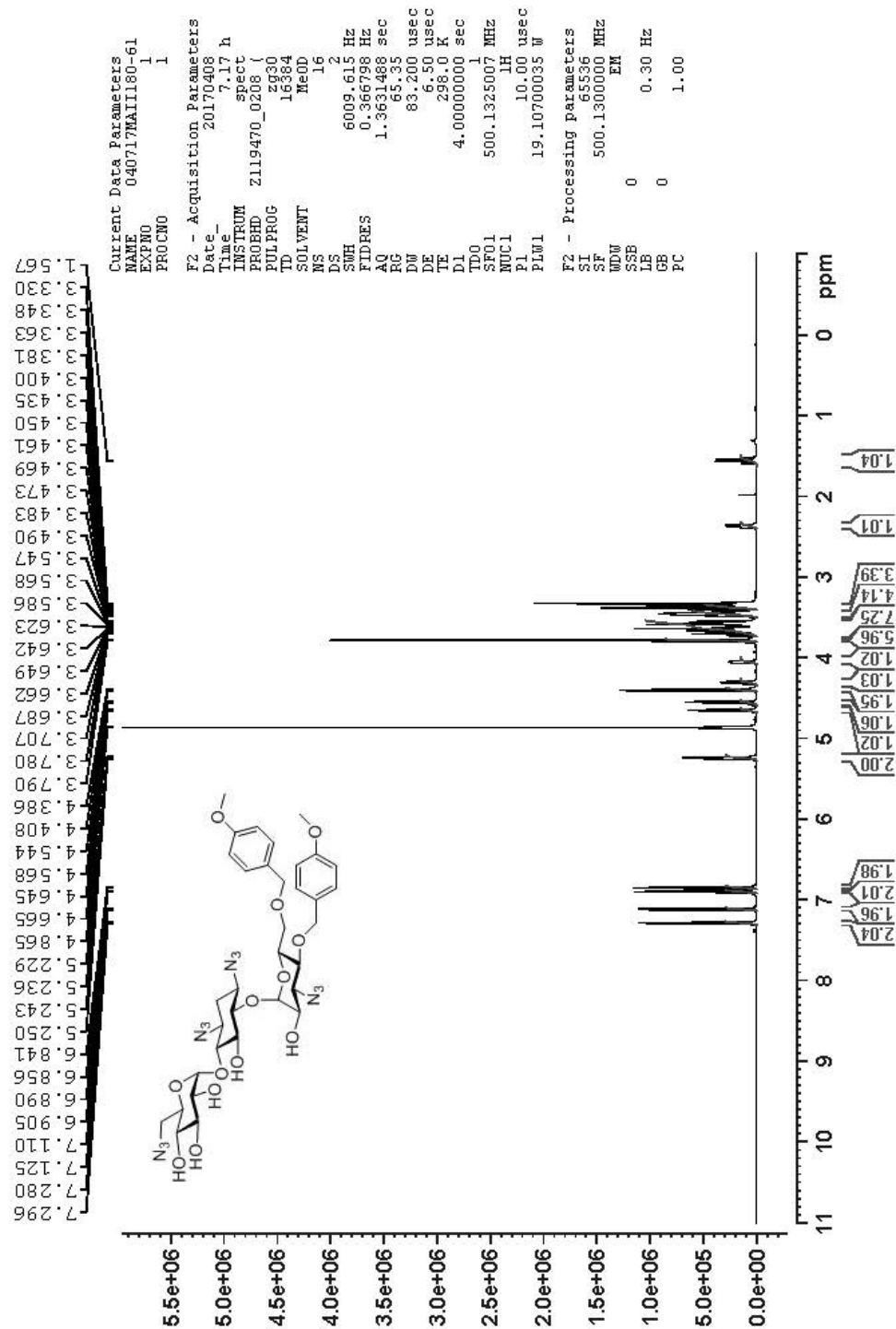
B-3 References

1. Fiori, M. C.; Krishnan, S.; Cortes, D. M.; Retamal, M. A.; Reuss, L.; Altenberg, G. A.; Cuello, L. G., *Biosci. Rep.* **2015**, 35 (2), e00177.
2. Krishnan, S.; Fiori, M. C.; Whisenant, T. E.; Cortes, D. M.; Altenberg, G. A.; Cuello, L. G., *SLAS Discov.* **2017**, 22 (2), 135-143.
3. Stumpe, S.; Bakker, E. P., *Arch. Microbiol.* **1997**, 167 (2-3), 126-136.
4. Buurman, E. T.; McLaggan, D.; Naprstek, J.; Epstein, W., *J. Bacteriol.* **2004**, 186 (13), 4238-4245.
5. Shrestha, J. P.; Baker, C.; Kawasaki, Y.; Subedi, Y. P.; de Paul, N. N. V.; Takemoto, J. Y.; Chang, C.-W. T., *Eur. J. Med. Chem.* **2017**, 126, 696-704.

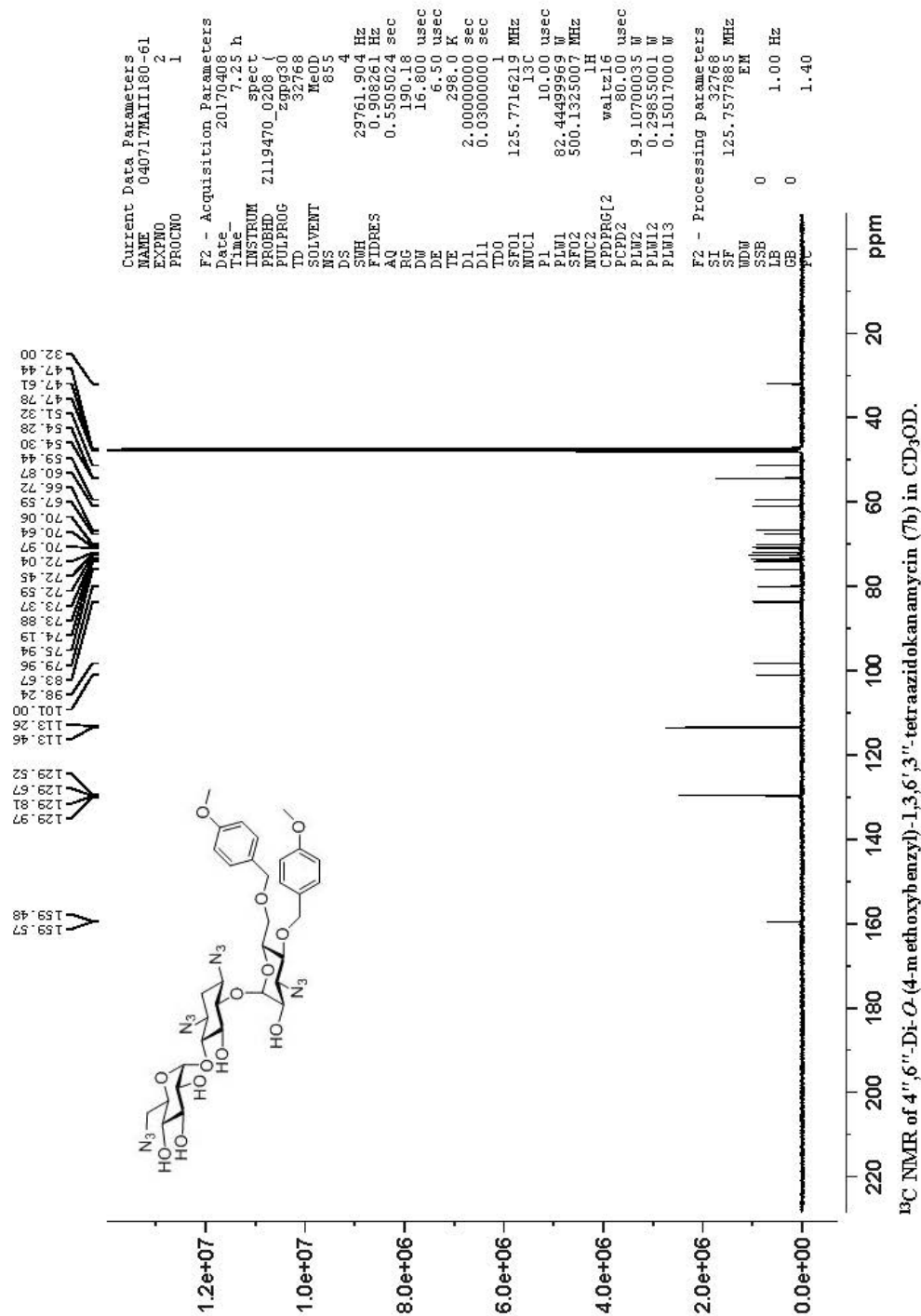
6. Zhang, Q.; Alfindee, M. N.; Shrestha, J. P.; Nziko, V. d. P. N.; Kawasaki, Y.; Peng, X.; Takemoto, J. Y.; Chang, C.-W. T. *J. Org. Chem.* **2016**, 81, 10651-10663.

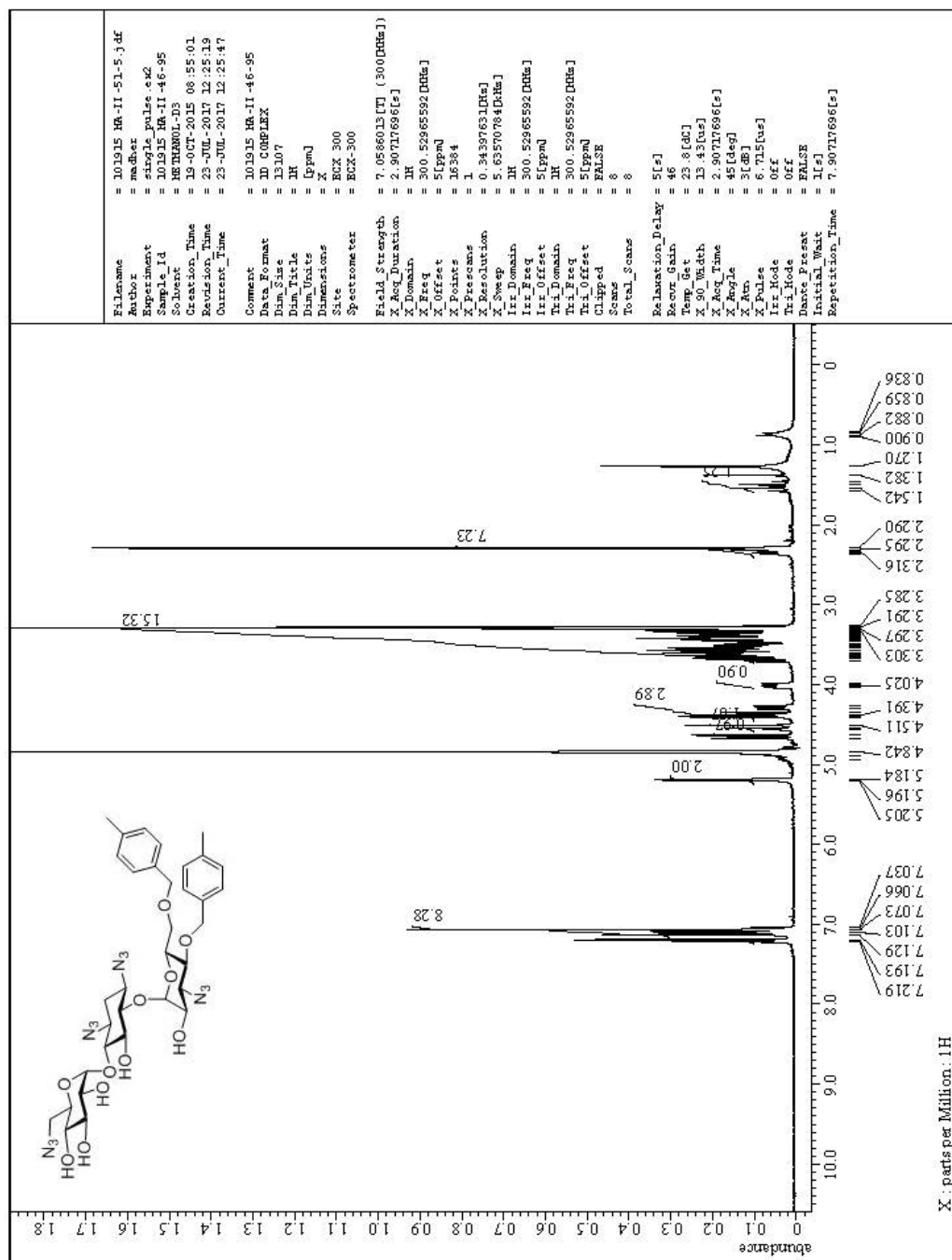
Standard	Experiment
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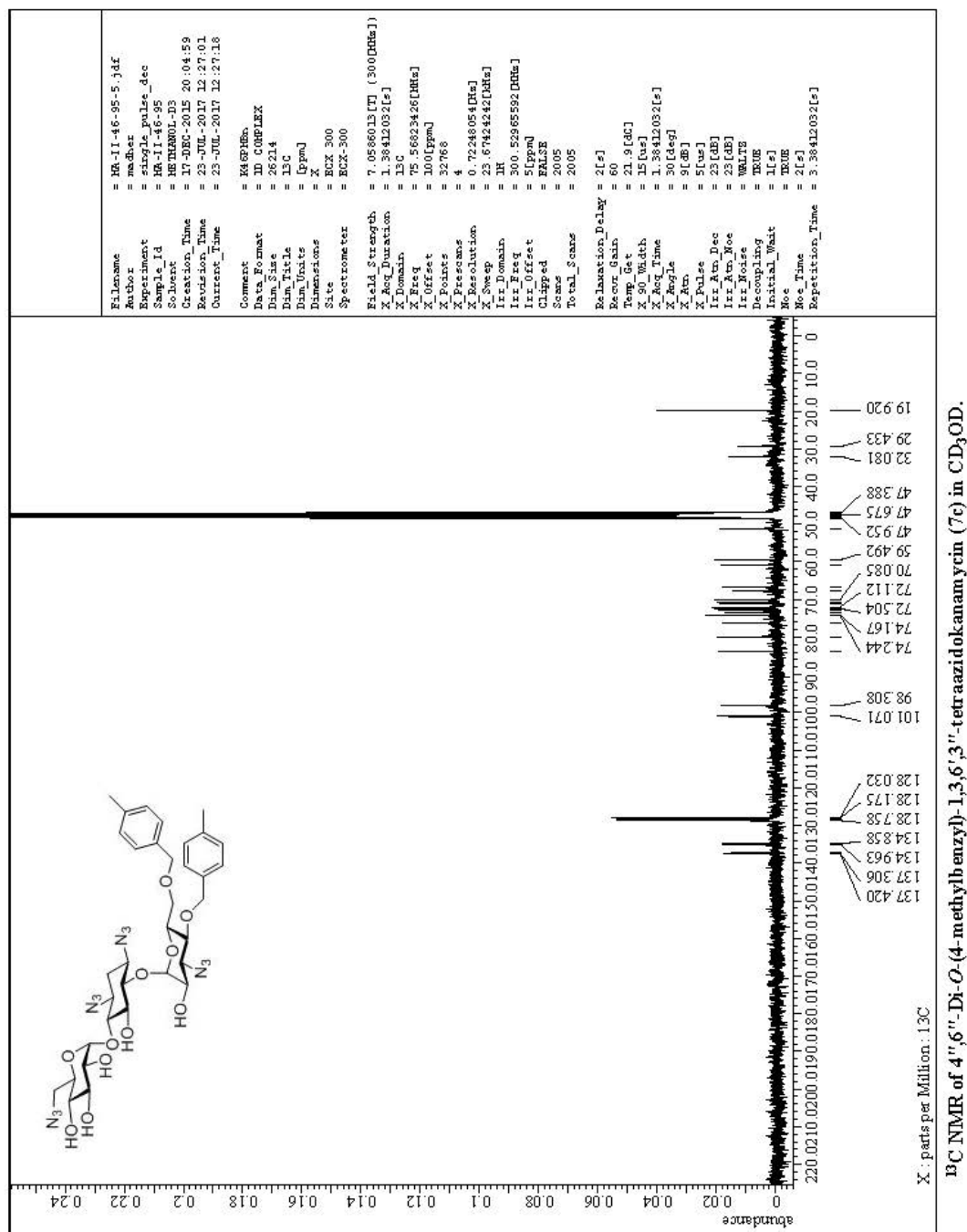


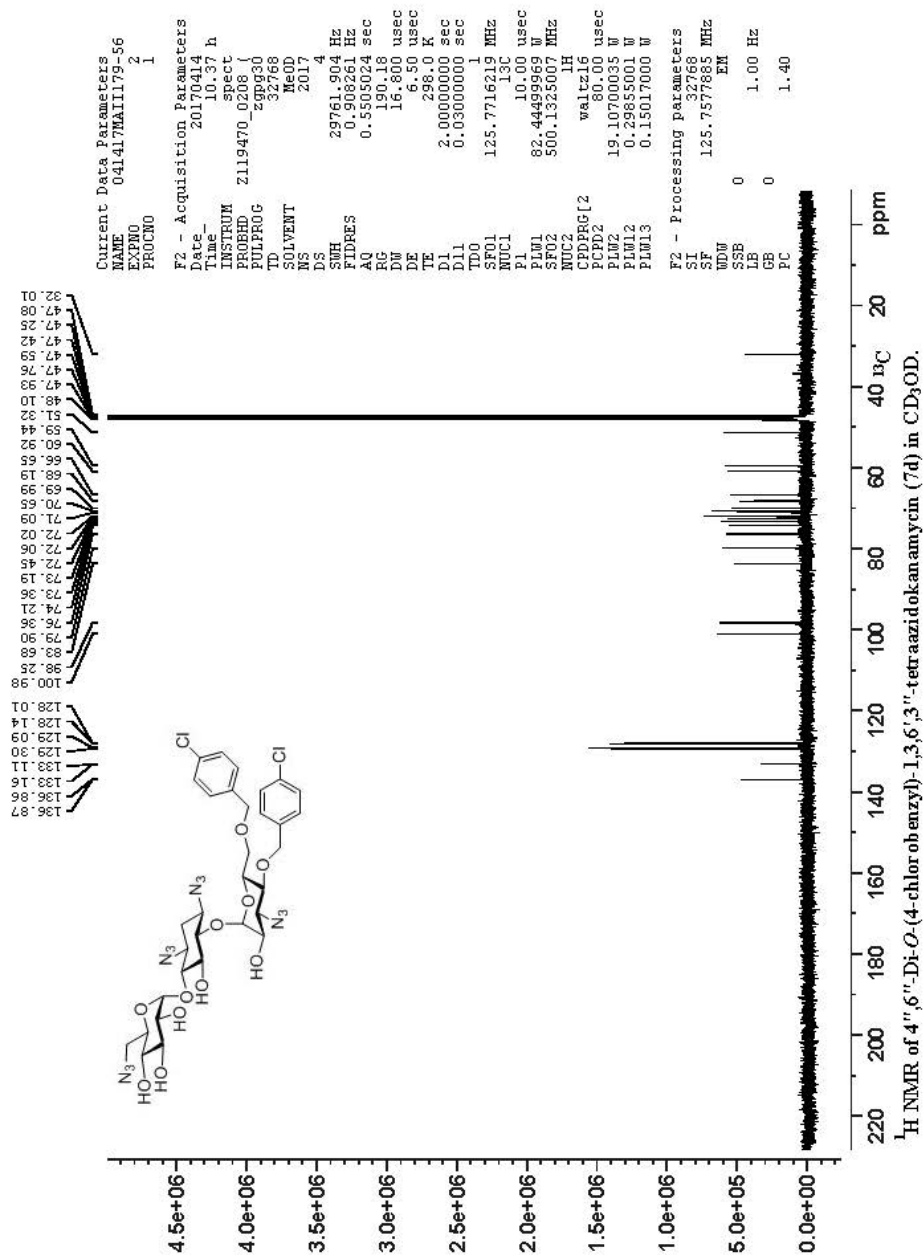
¹H NMR of 4''-Di-O-(4-methoxybenzyl)-1,3,6',3''-tetraazidokananyrin (7b) in CD₃OD.

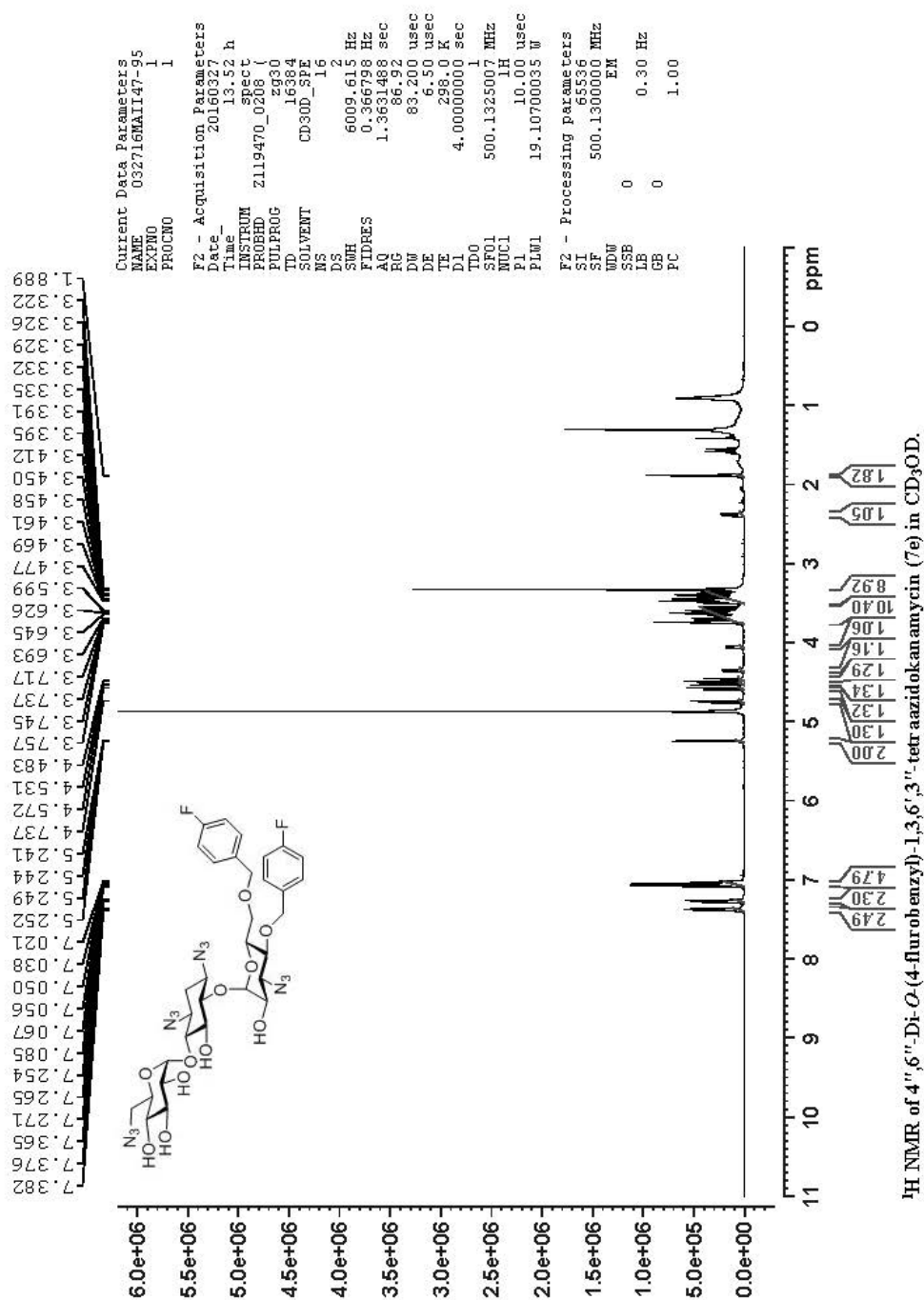


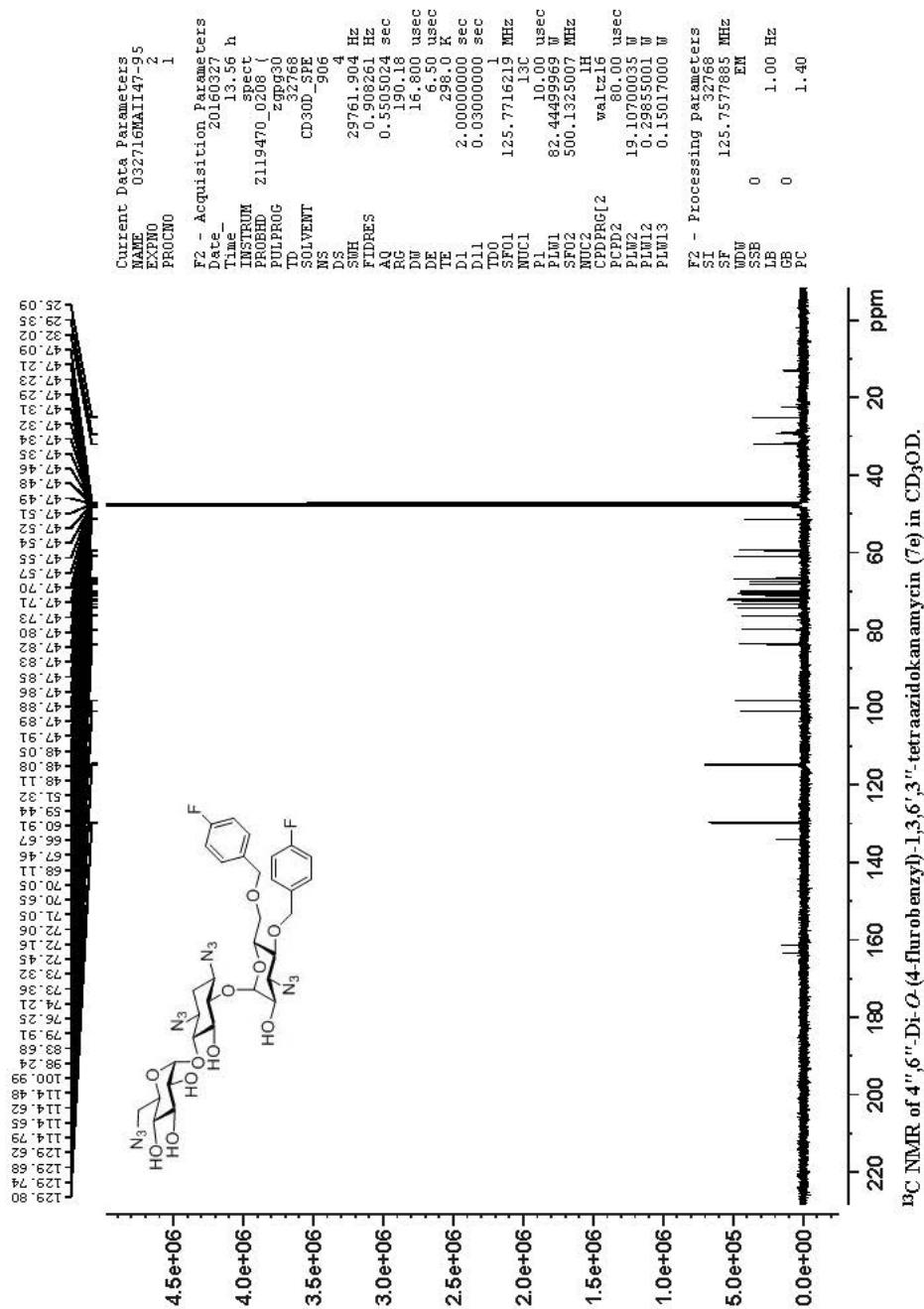


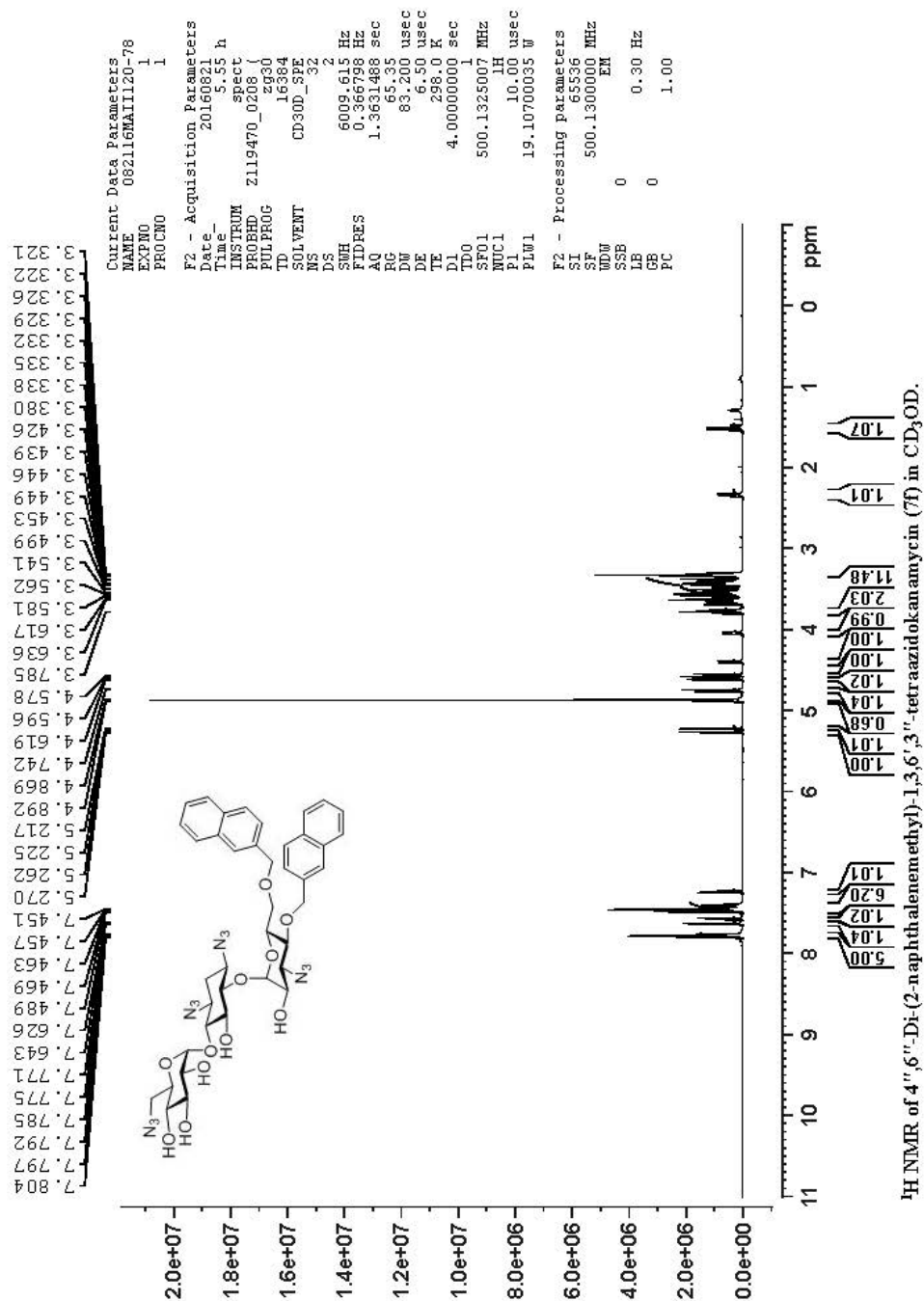
B-6

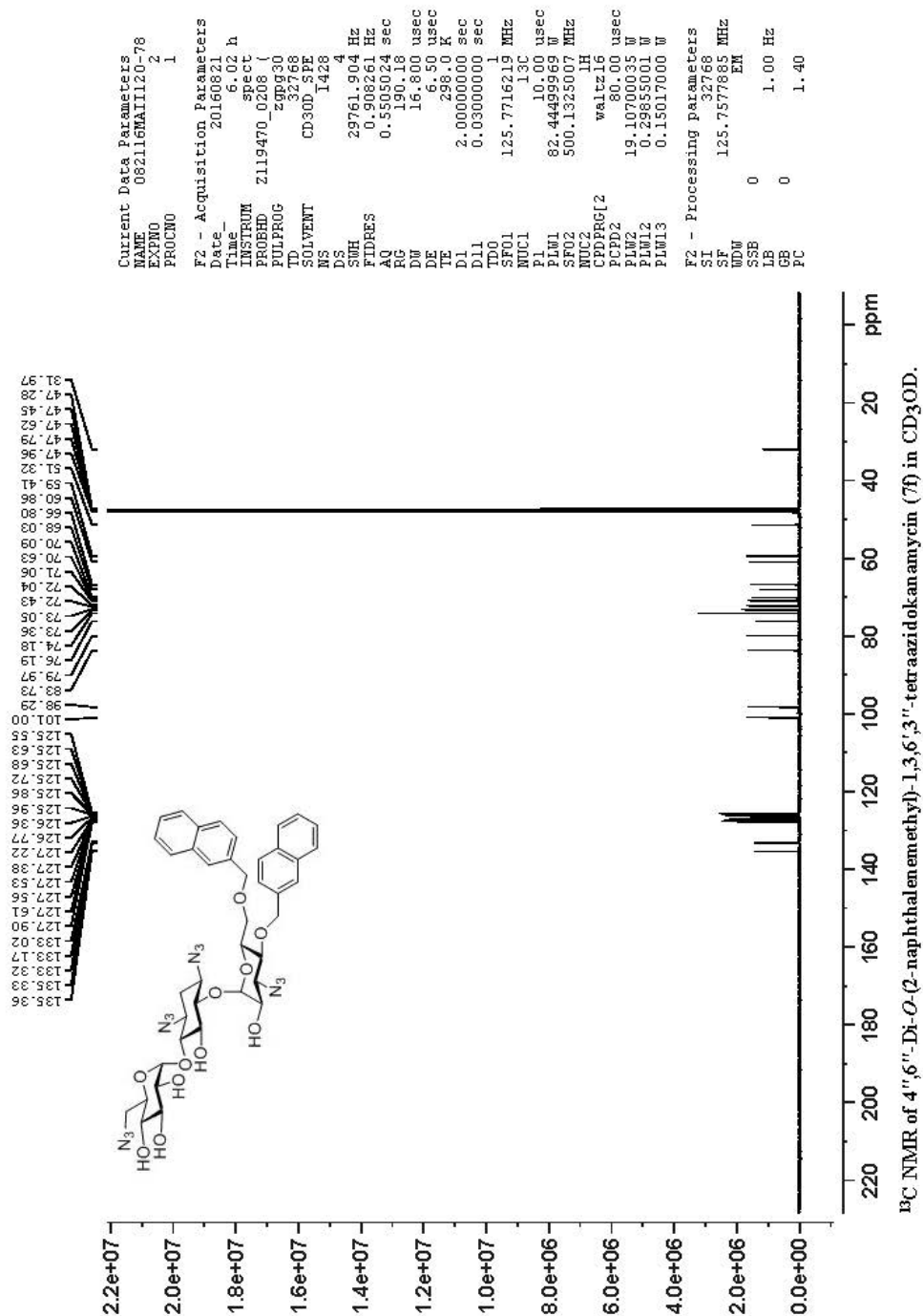


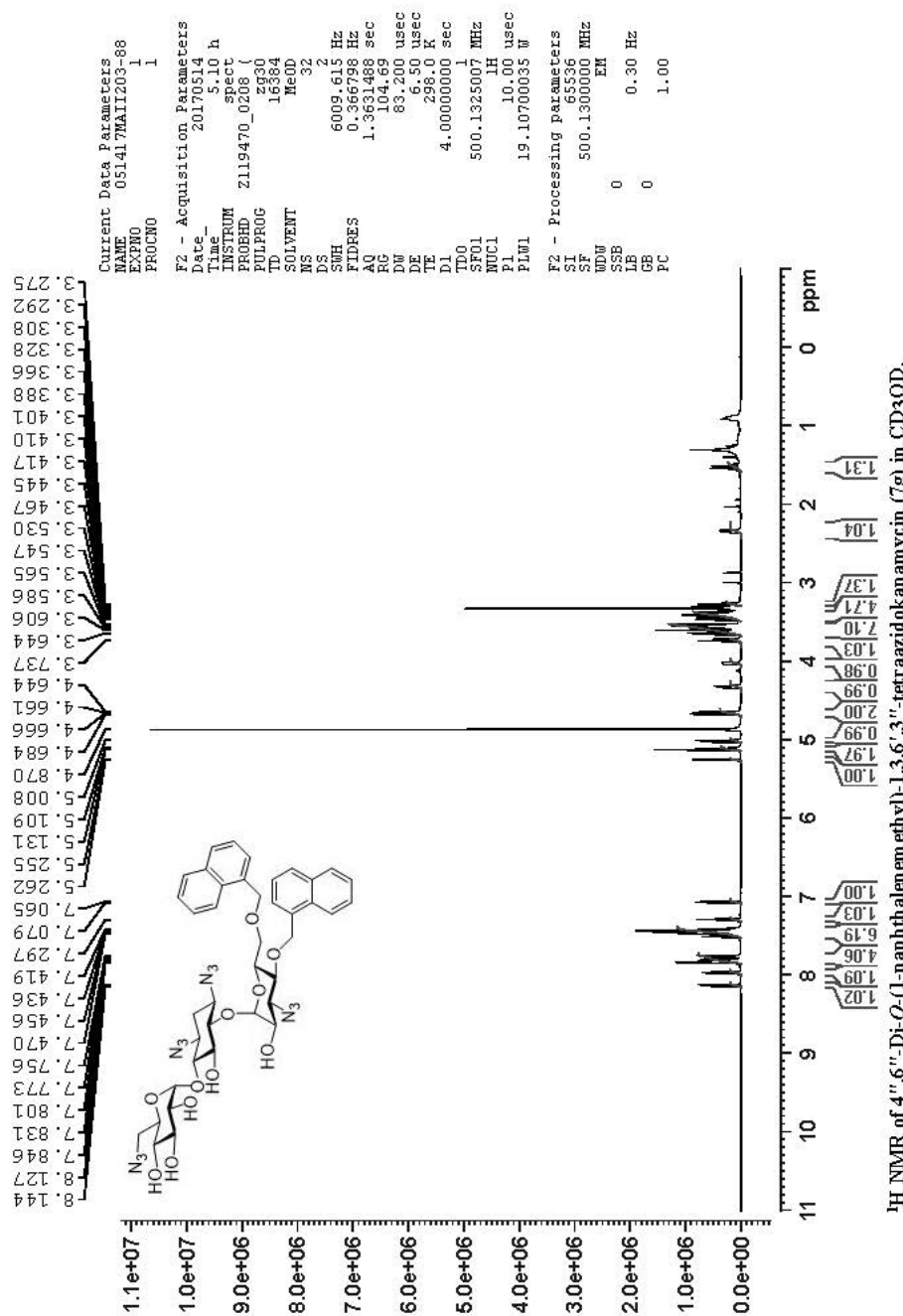


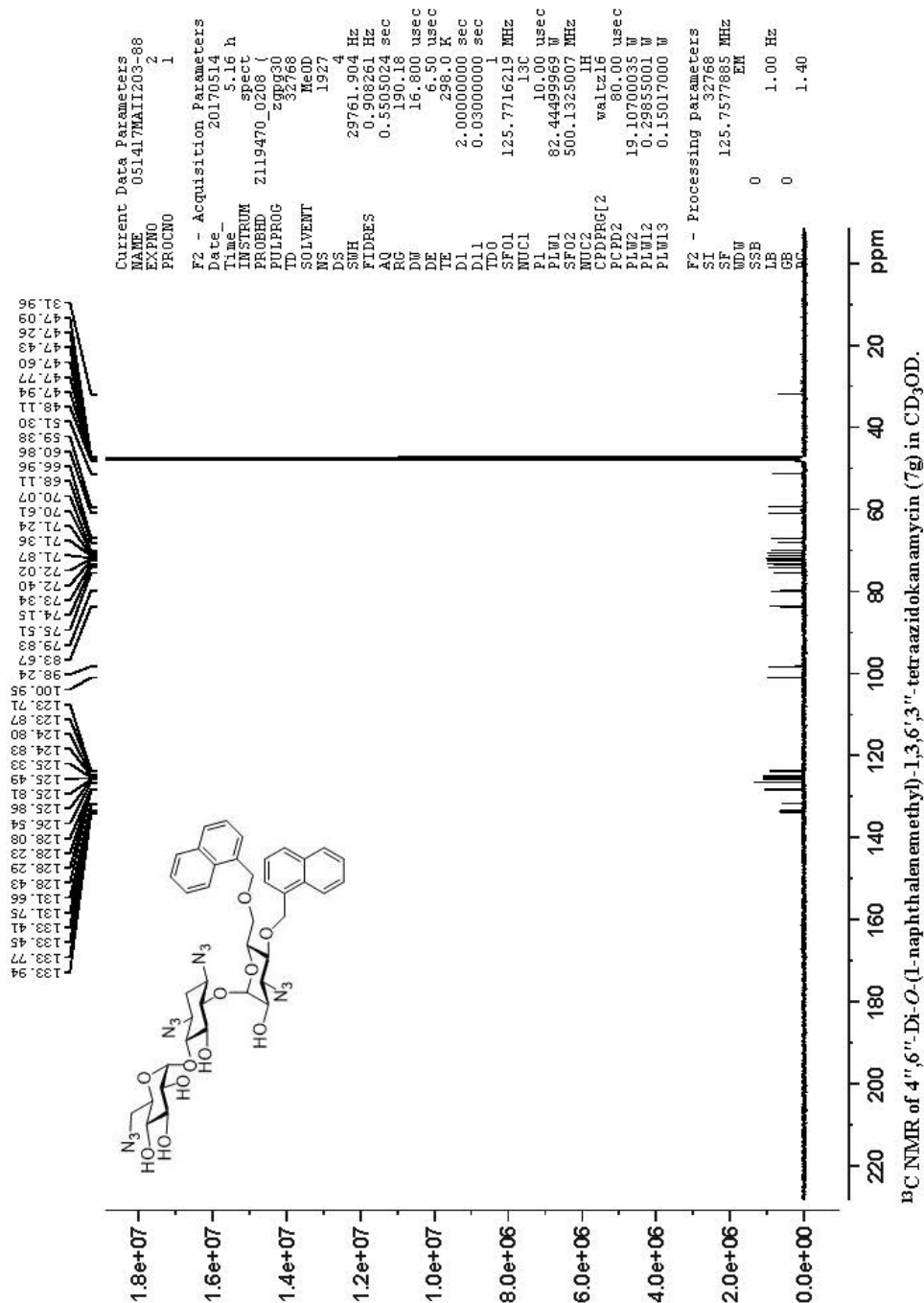


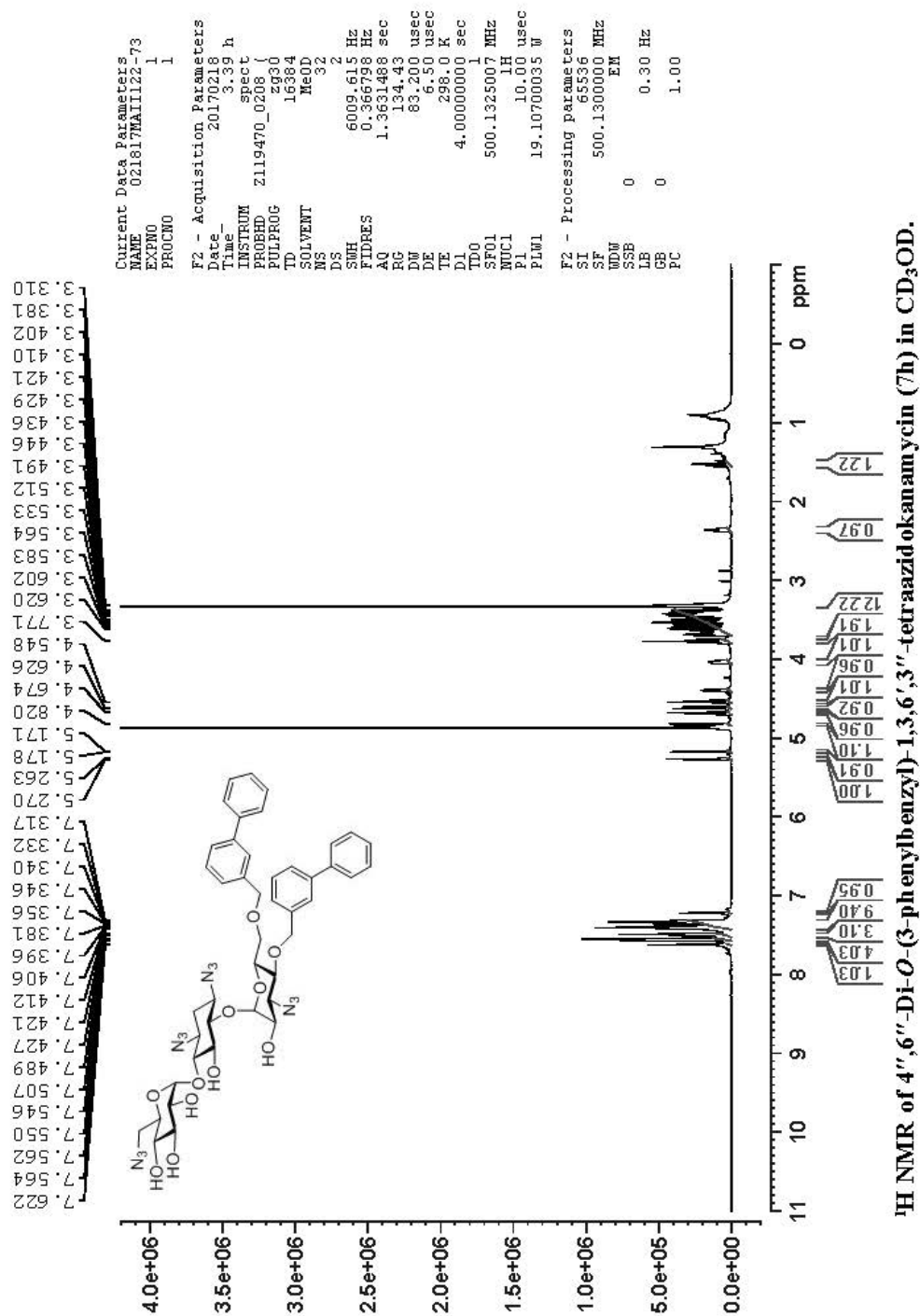




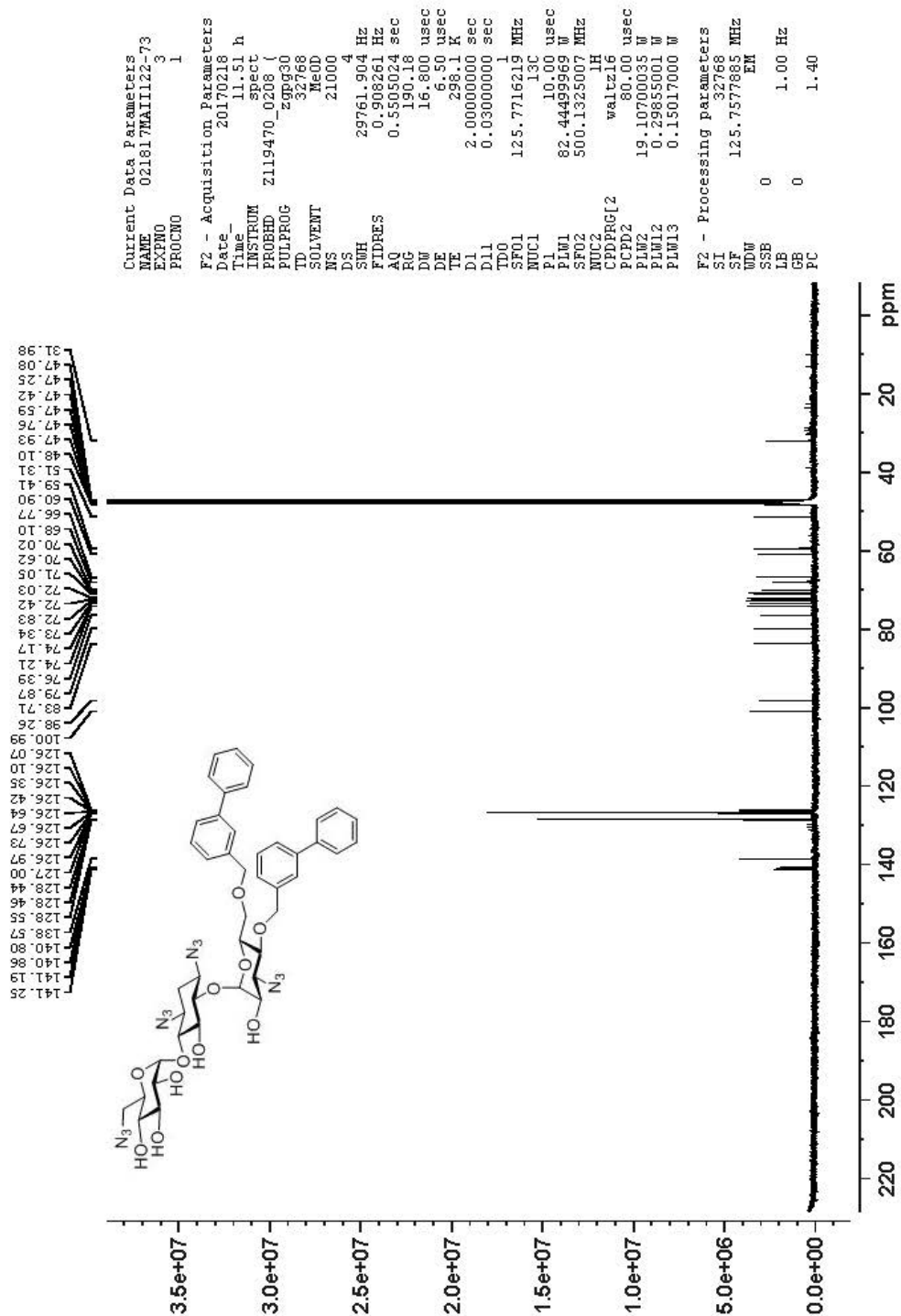


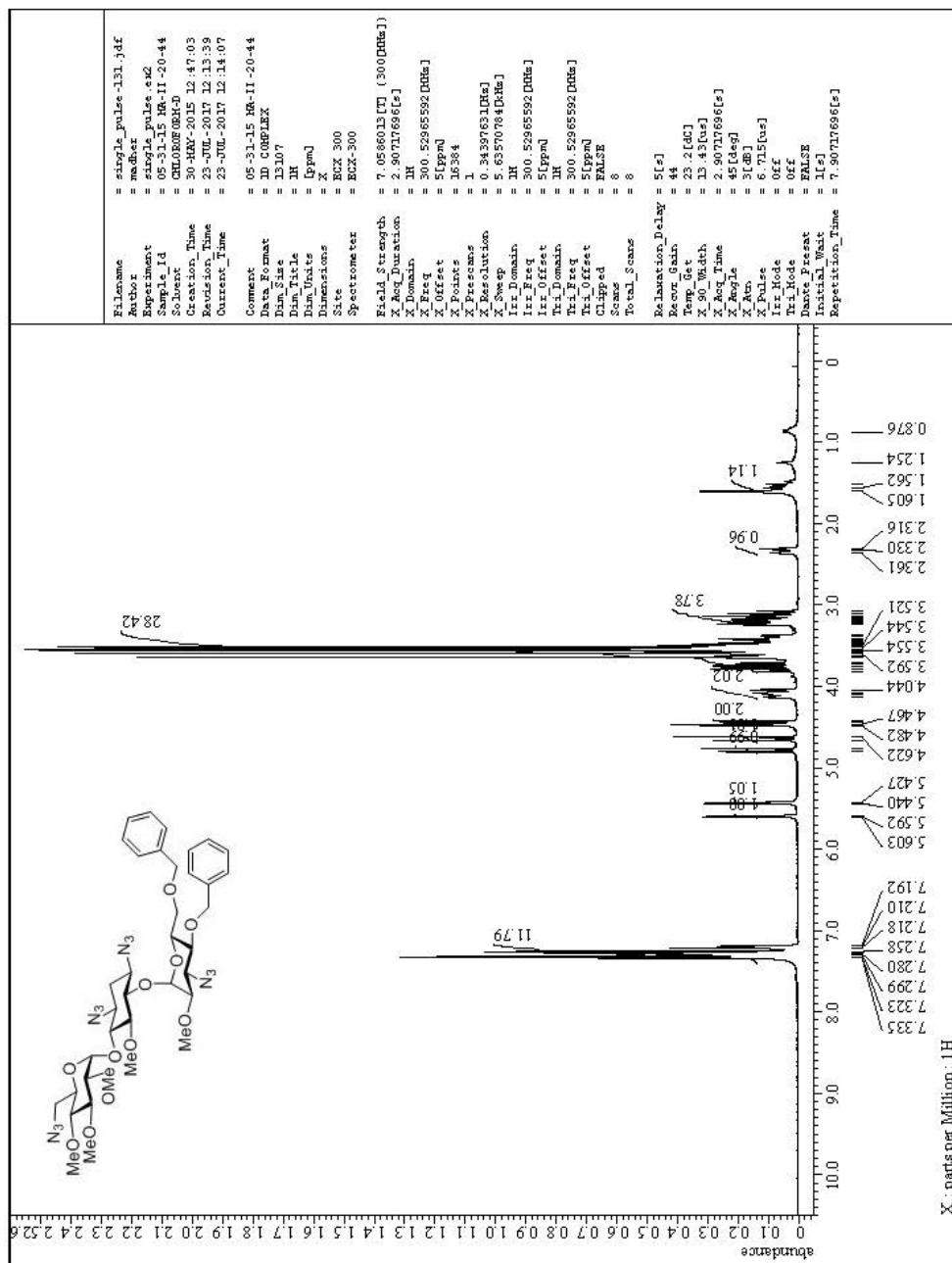


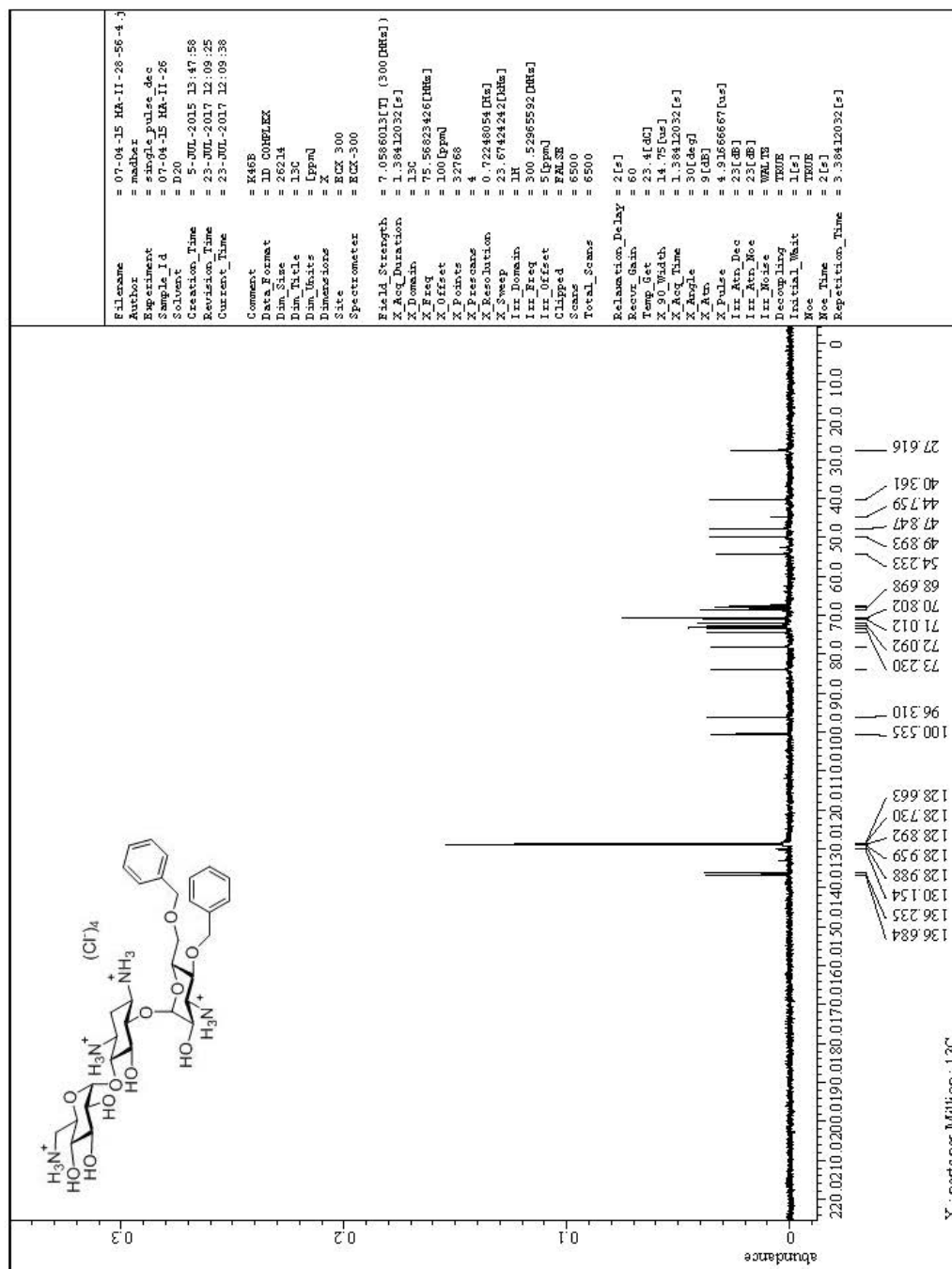


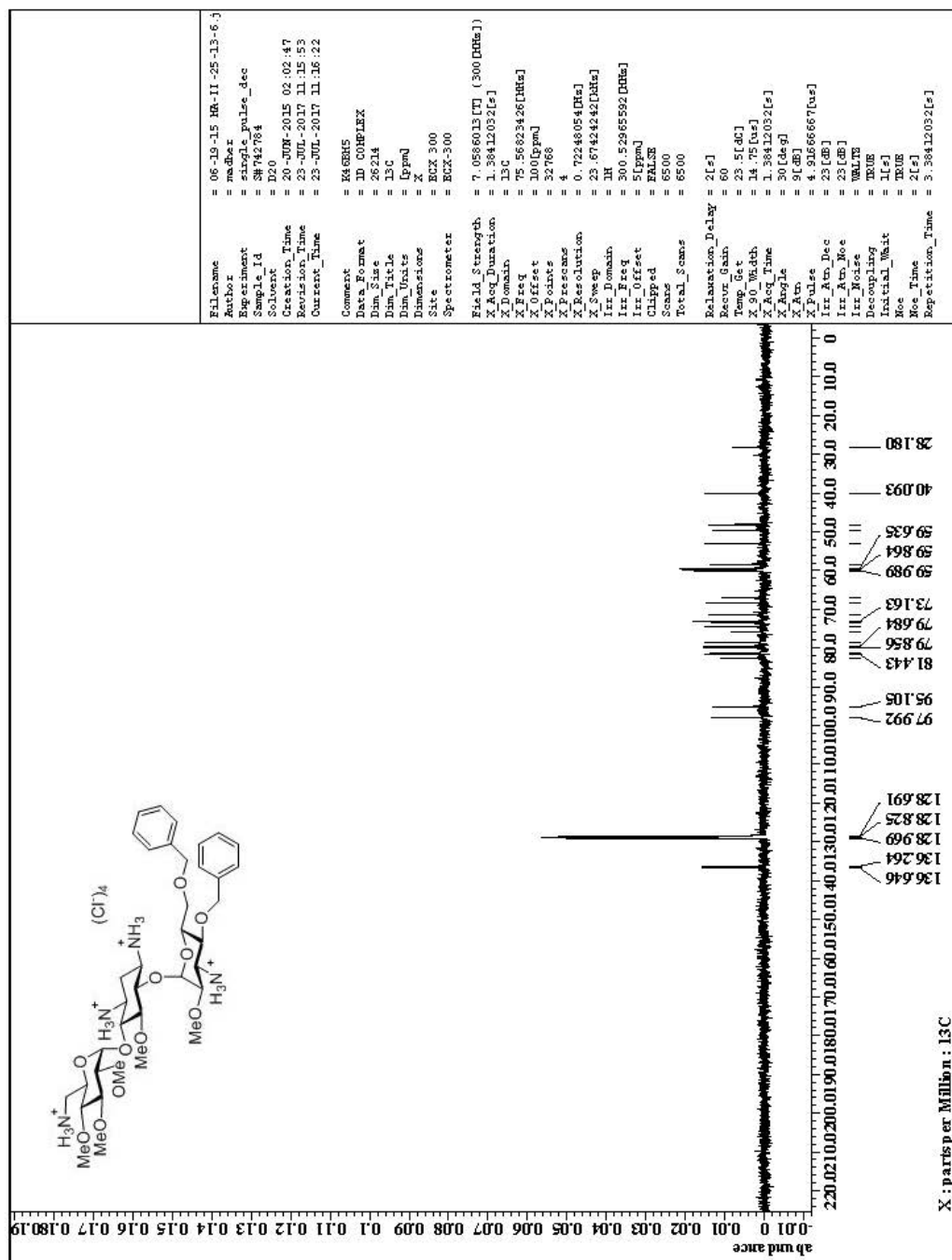


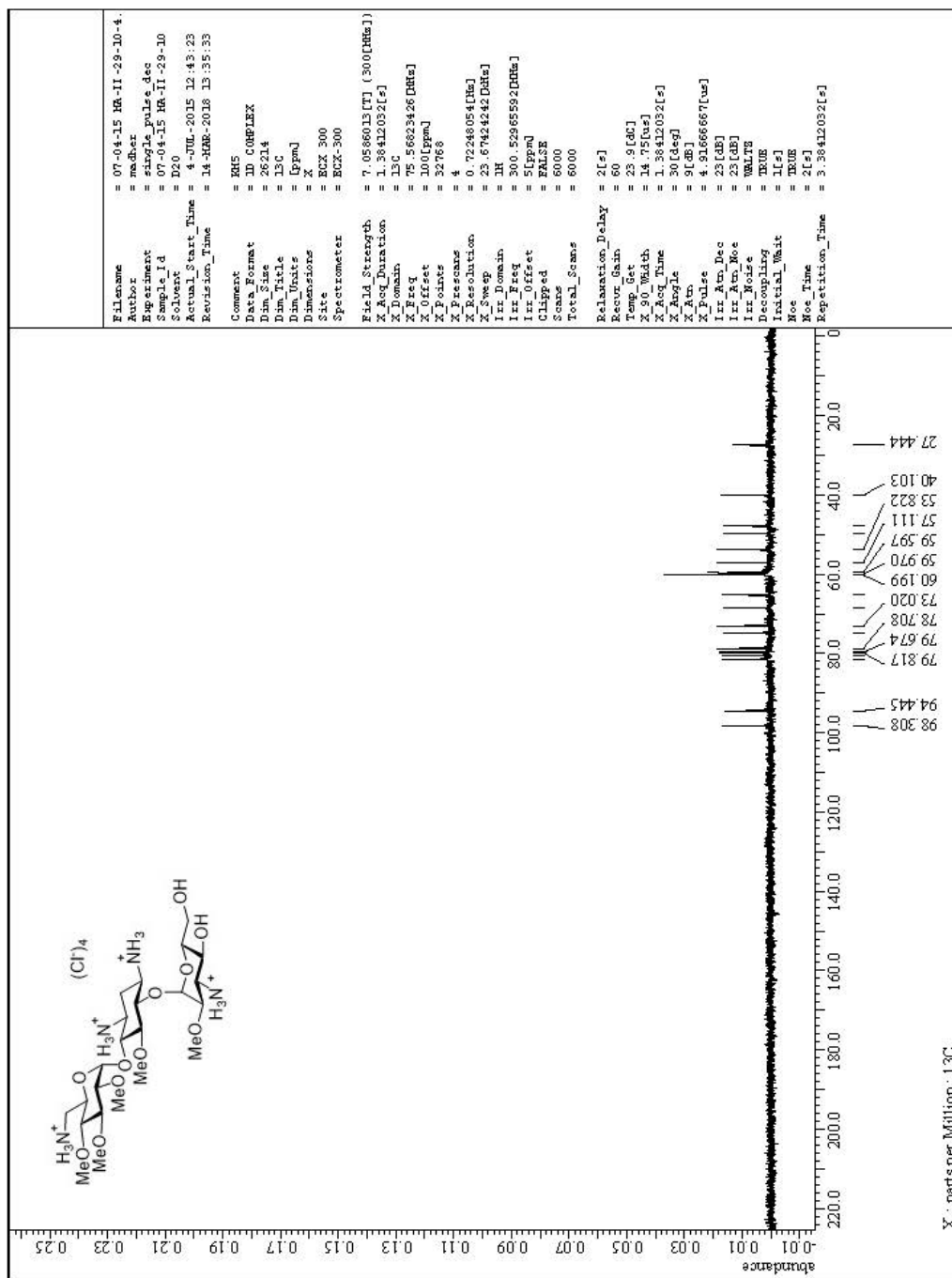
¹H NMR of 4'',6''-Di-O-(3-phenylbenzyl)-1,3,6',3''-tetraazido okanamylin (7h) in CD₃OD.

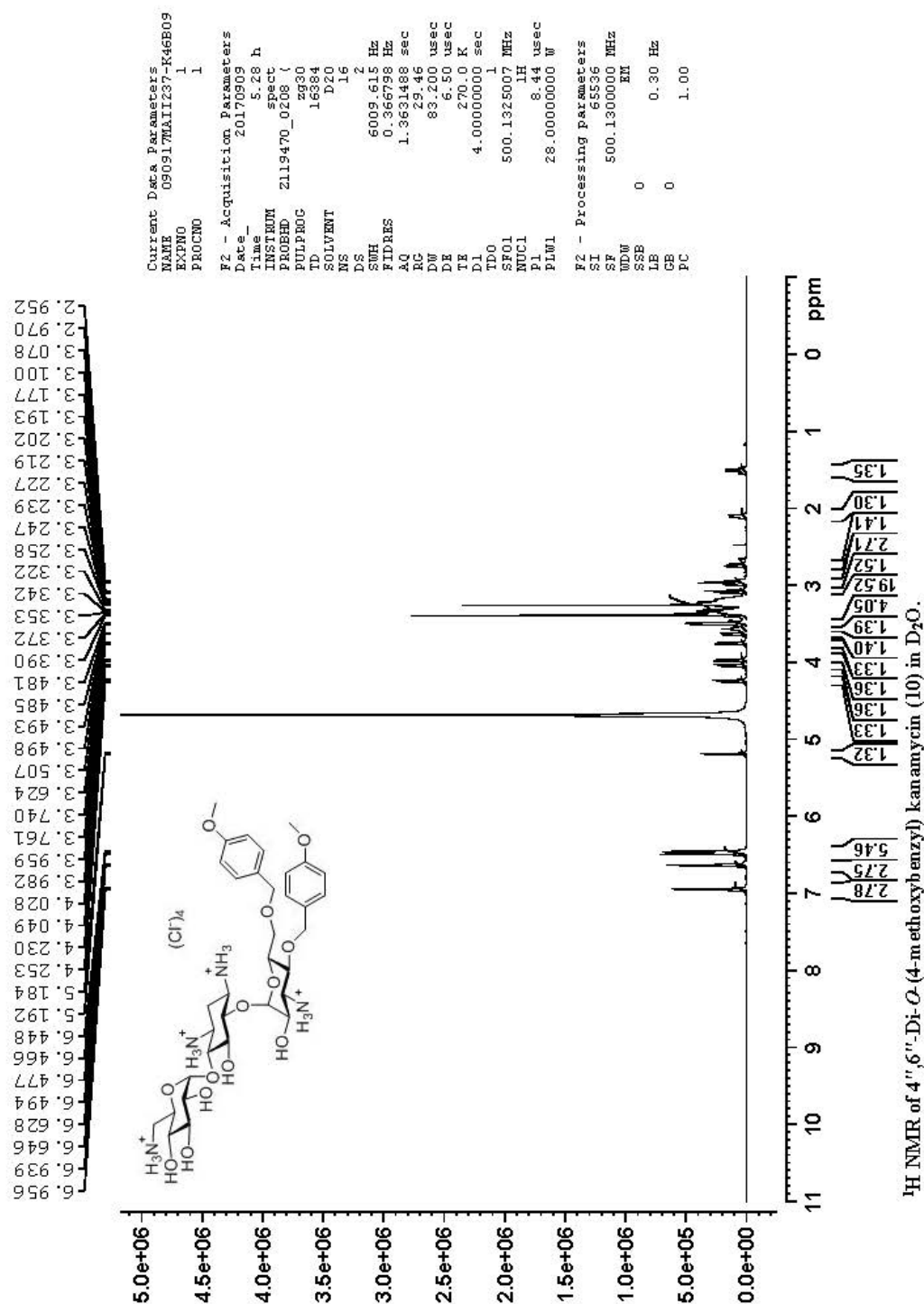


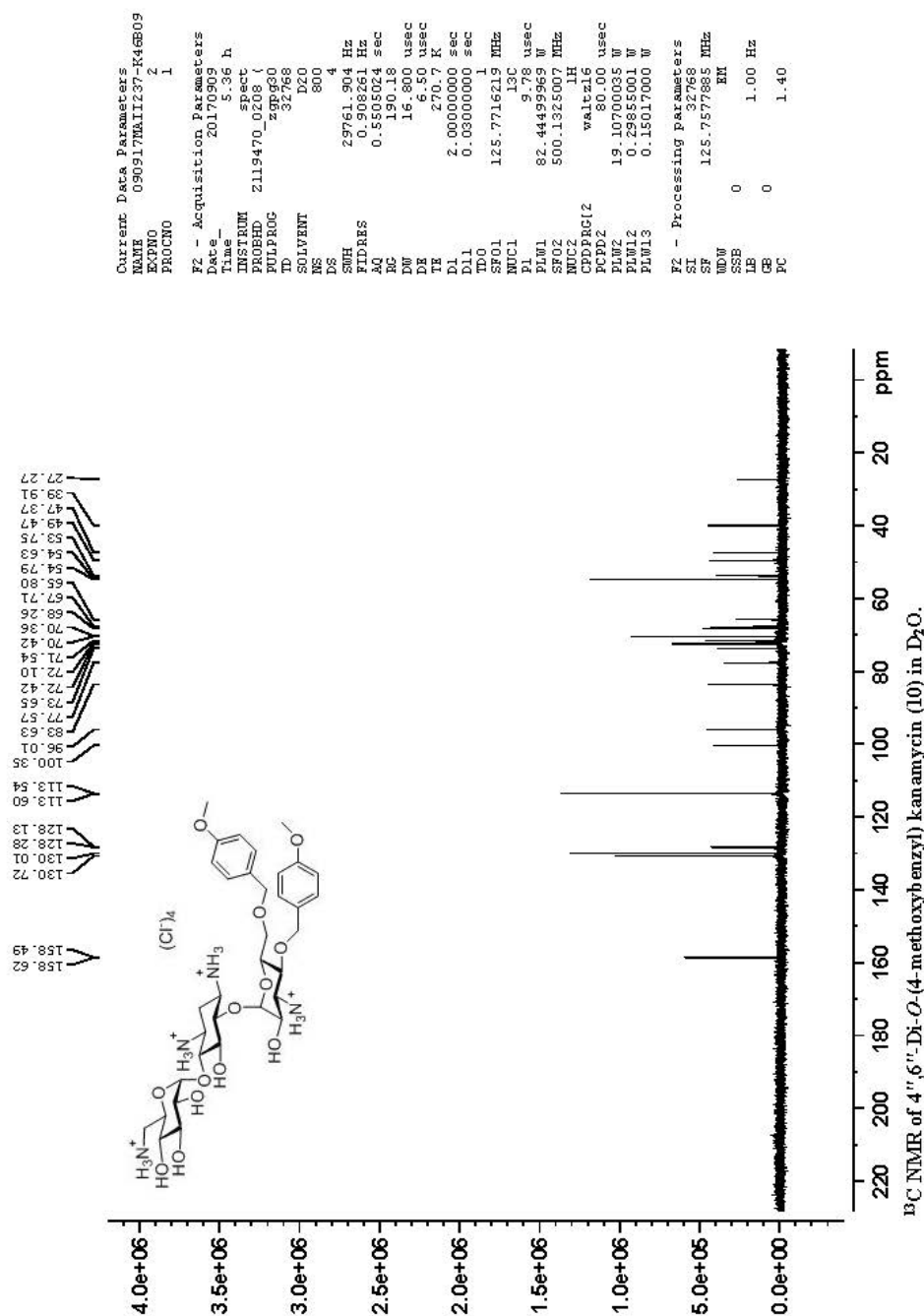
¹H NMR of 5, 2', 3', 4', 2''-Penta-*O*-methyl-4', 6'', di-*O*-benzyl-1, 3, 6', 3''-tetr aazidokanamycin (8a) in CDCl₃.



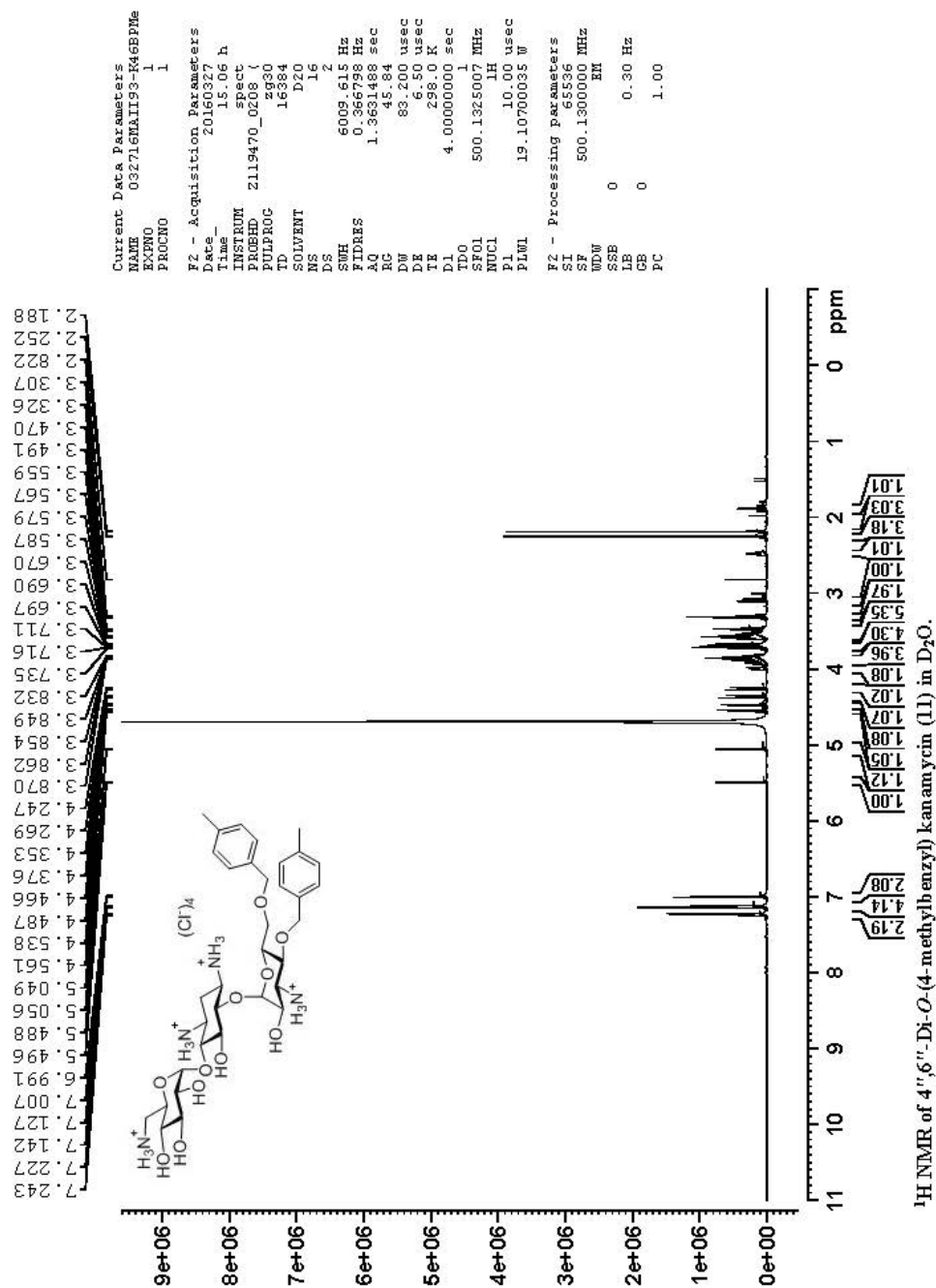


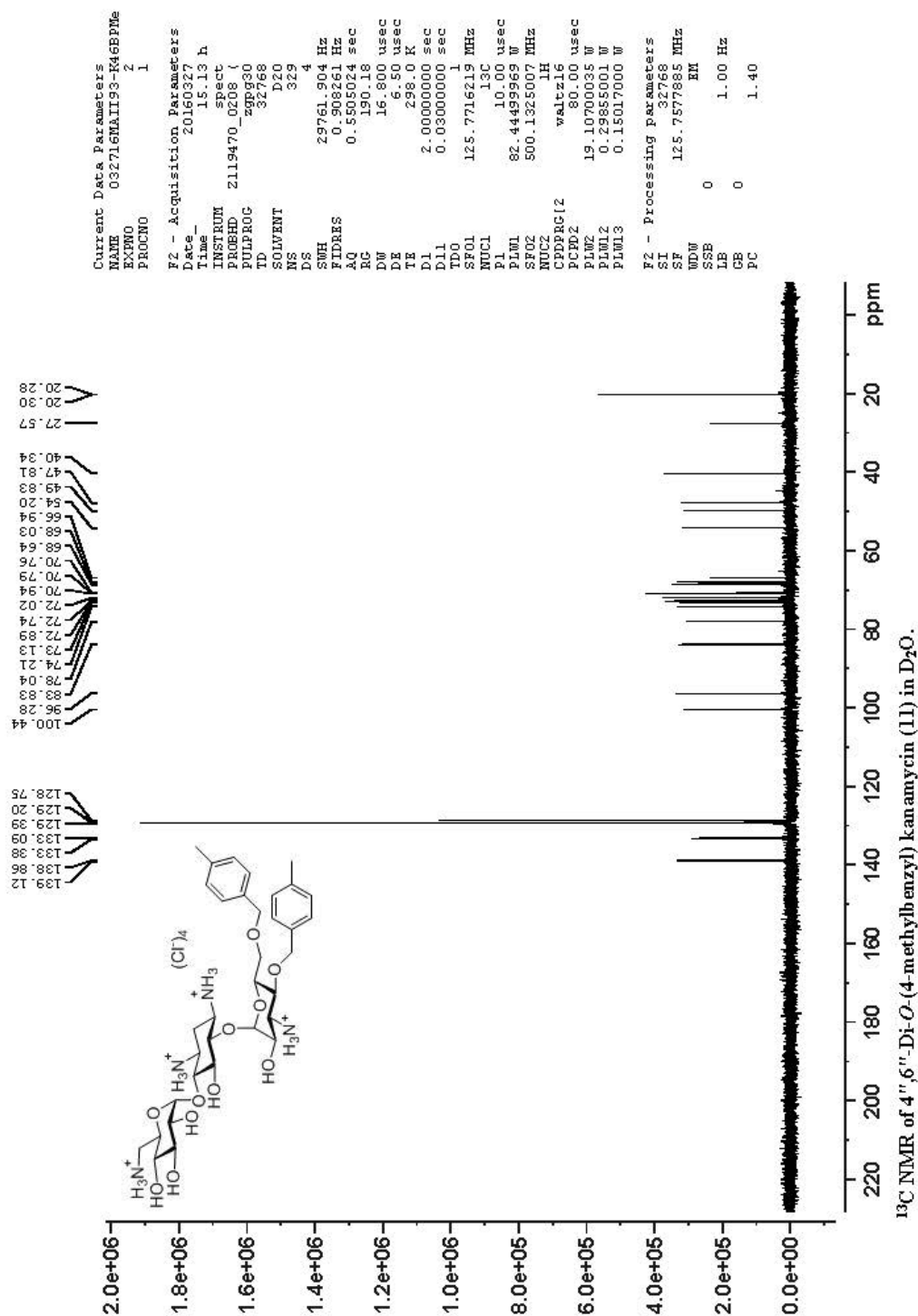


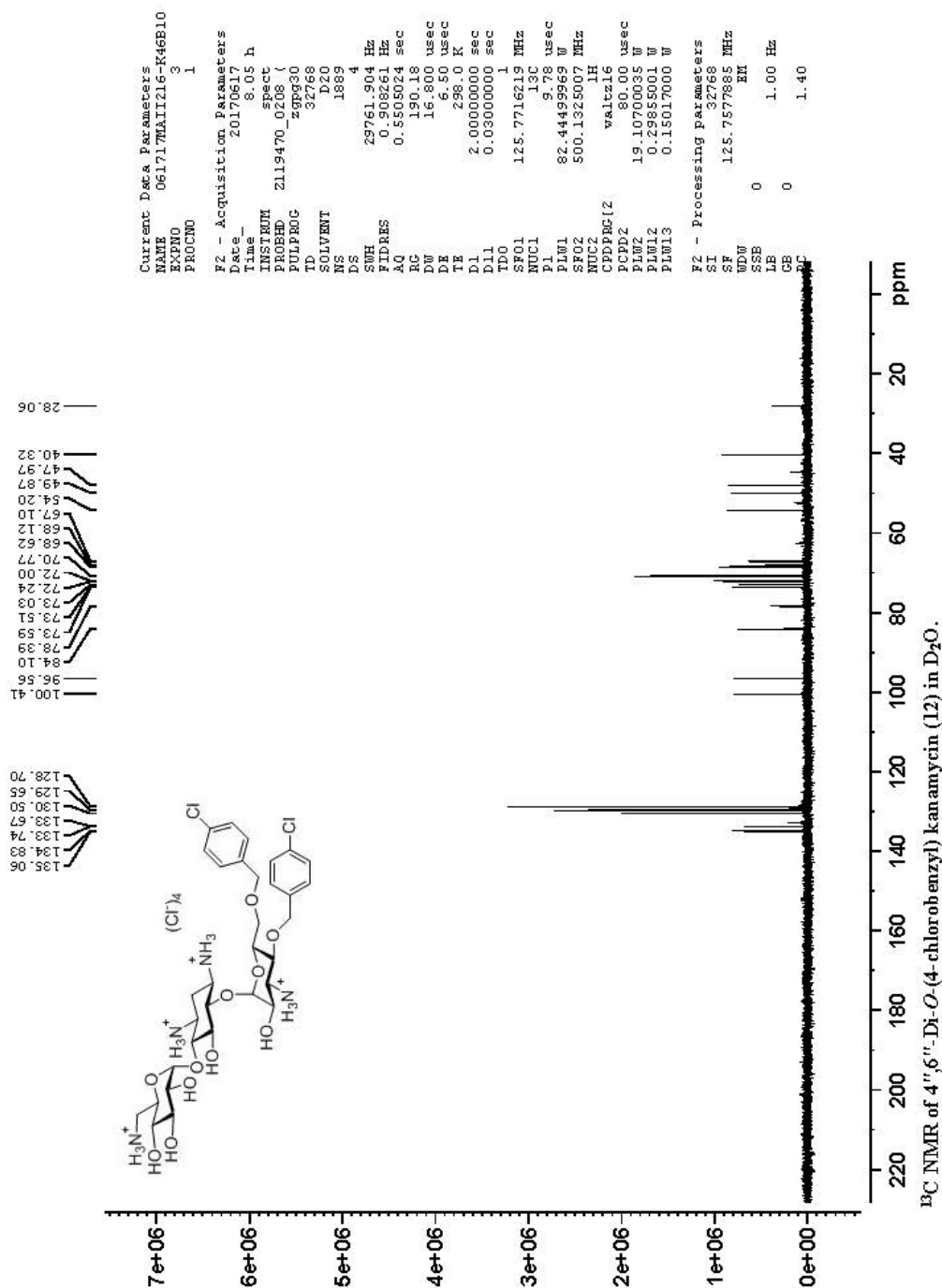


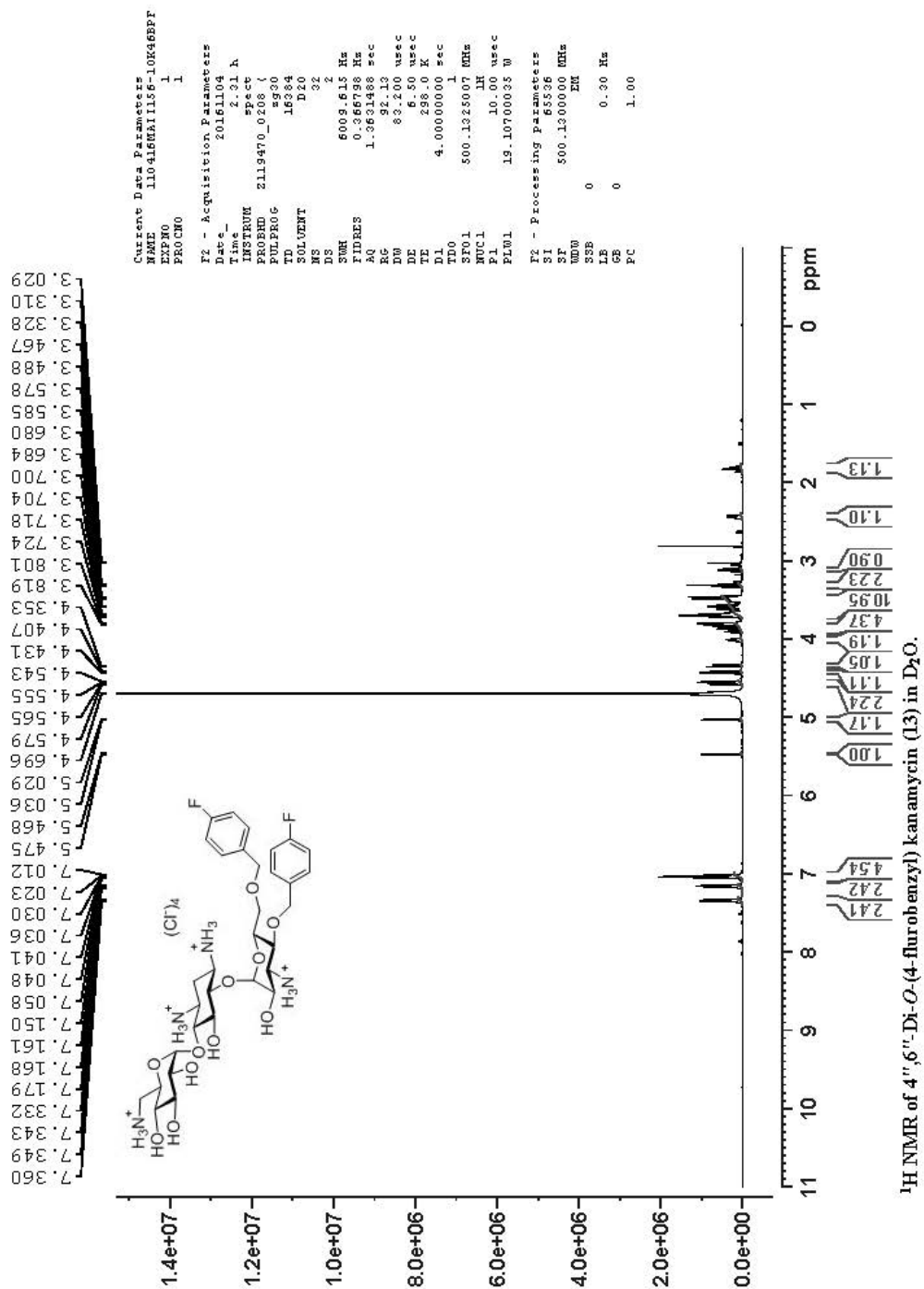


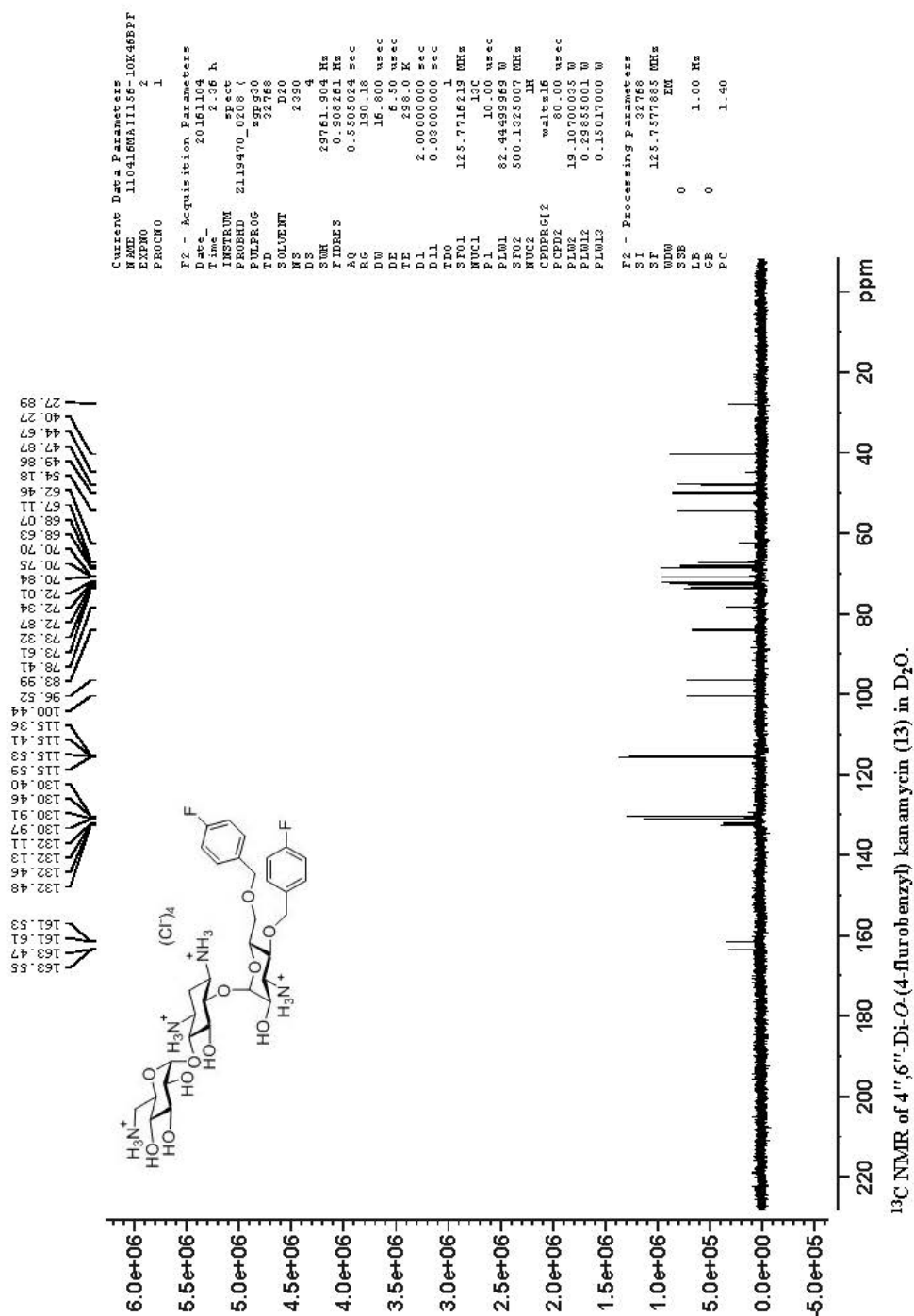
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		SOLVENT	D2O
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		DS	4
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		FIDRES	0.908261 Hz
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		RG	190.18
		REC	16.800 usec
		DEC	6.50 usec
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		DELTA	2.00000000 sec
		DELTA1	0.03000000 sec
		DELTA2	1
		SFO1	125.7716219 MHz
		NUC1	¹³ C
		PL1	9.78 usec
		PL2	82.444399697 MHz
		SFO2	500.1325007 MHz
		NUC2	¹ H
		PL12	wait: 1H
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		PLM13	0.15017000 W
WZ - Processing parameters			
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		SSB	0
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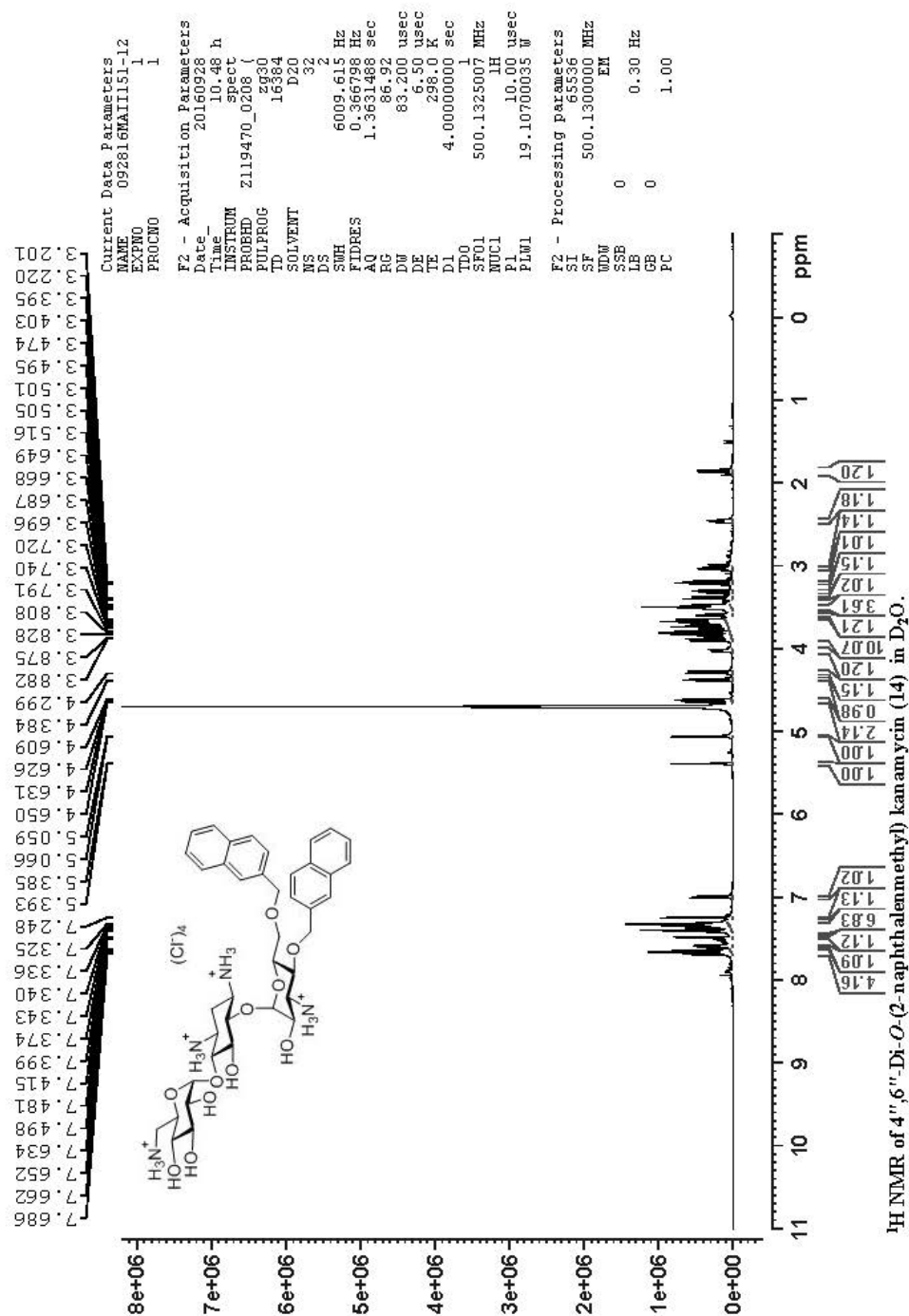


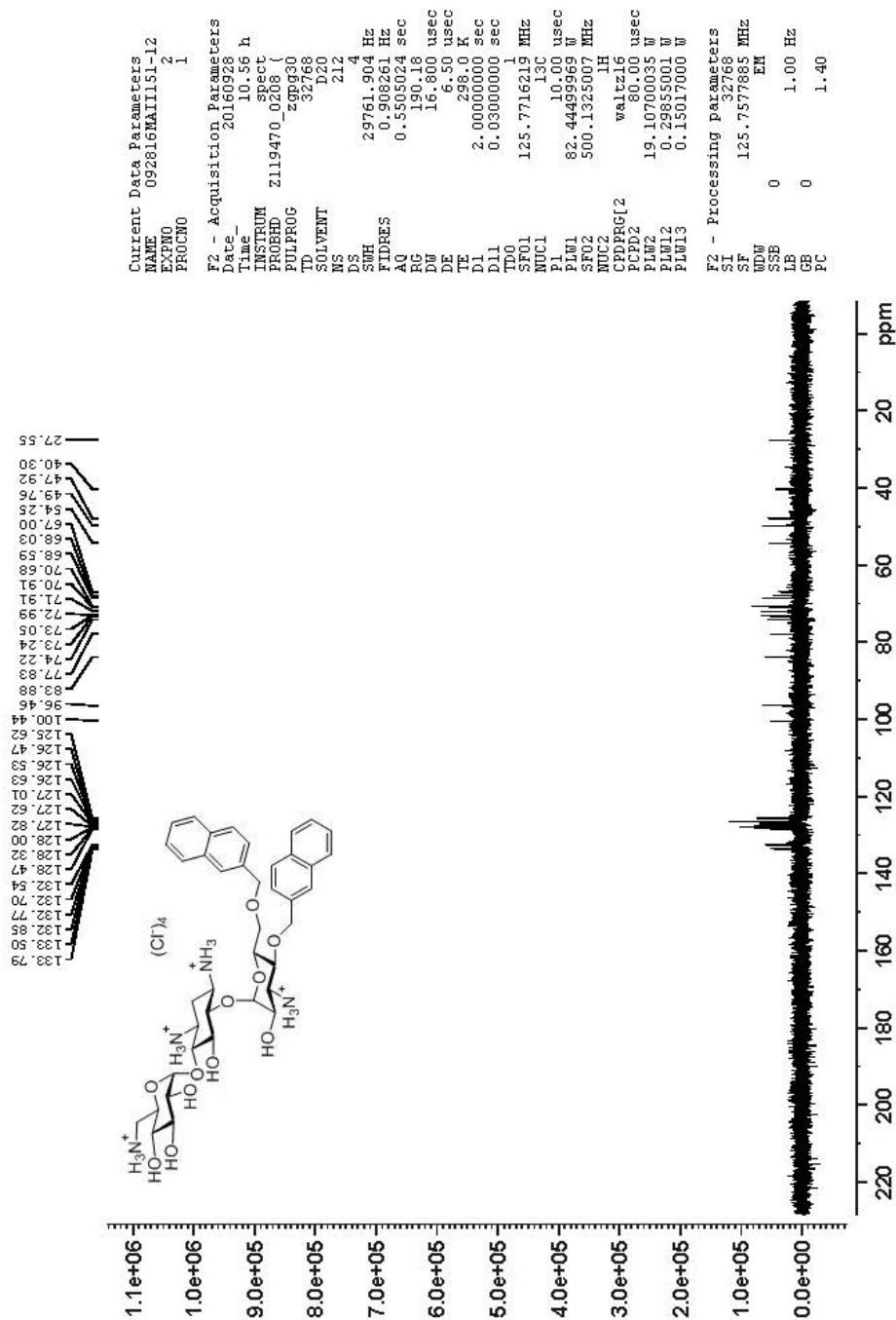


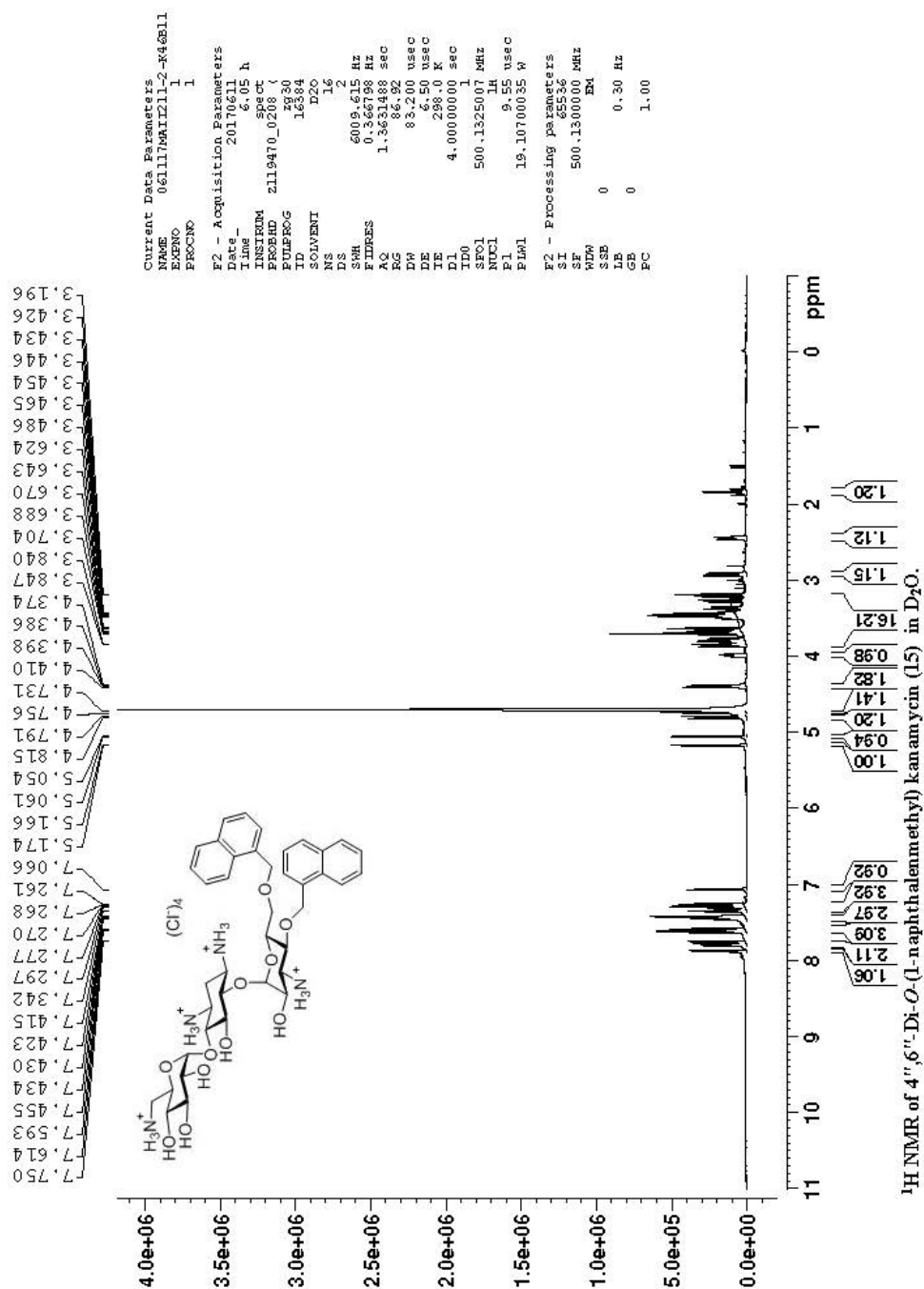


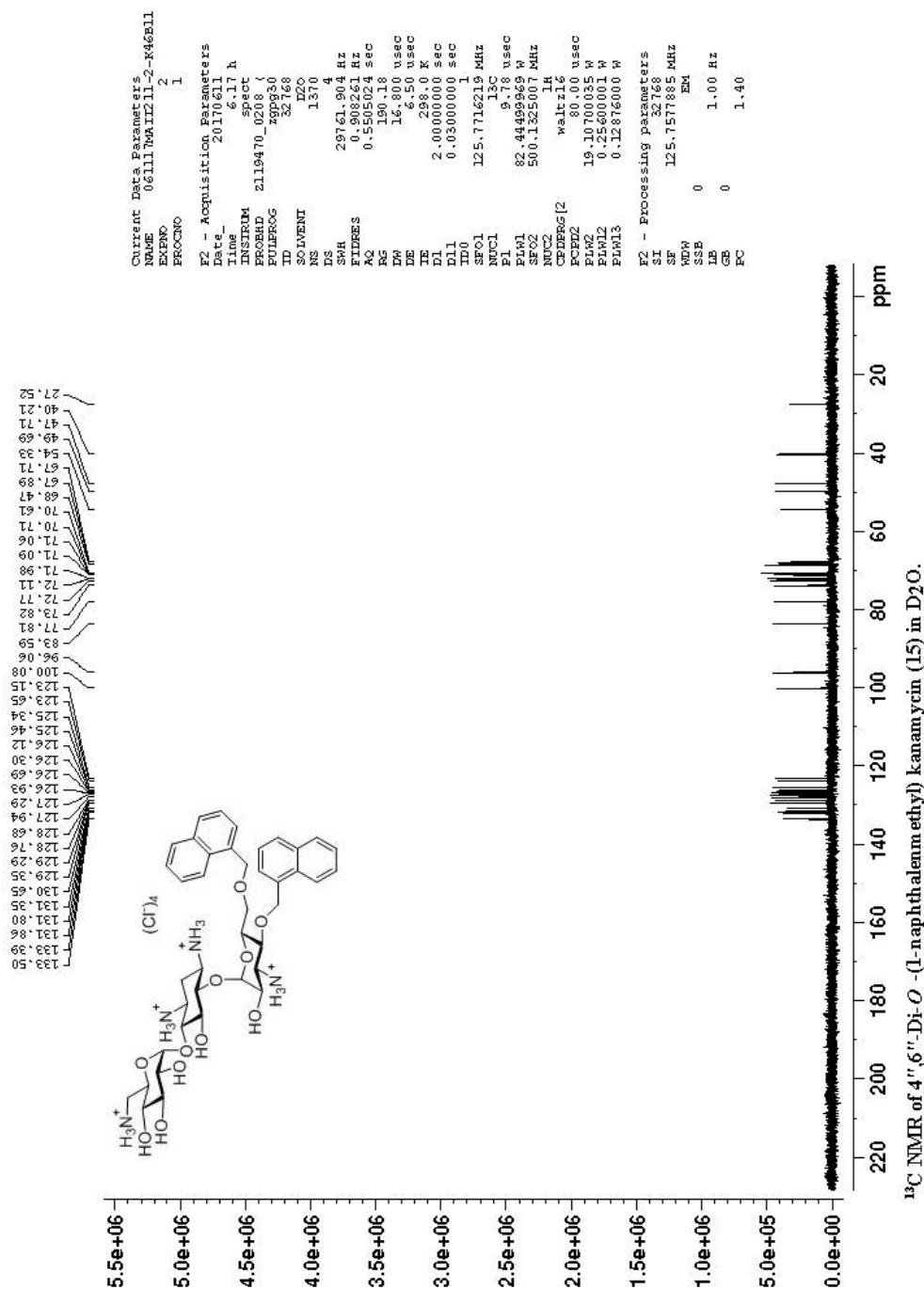


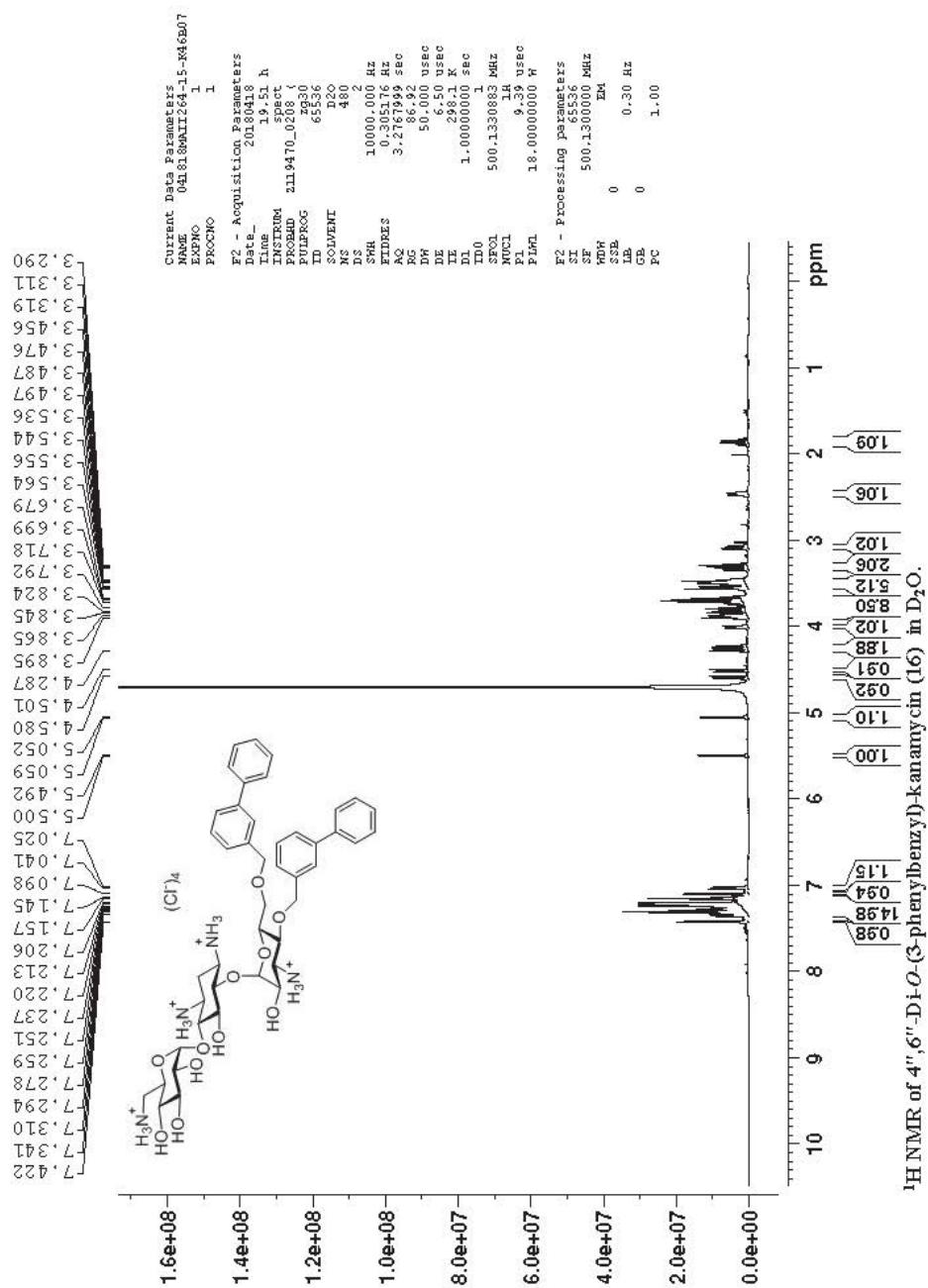


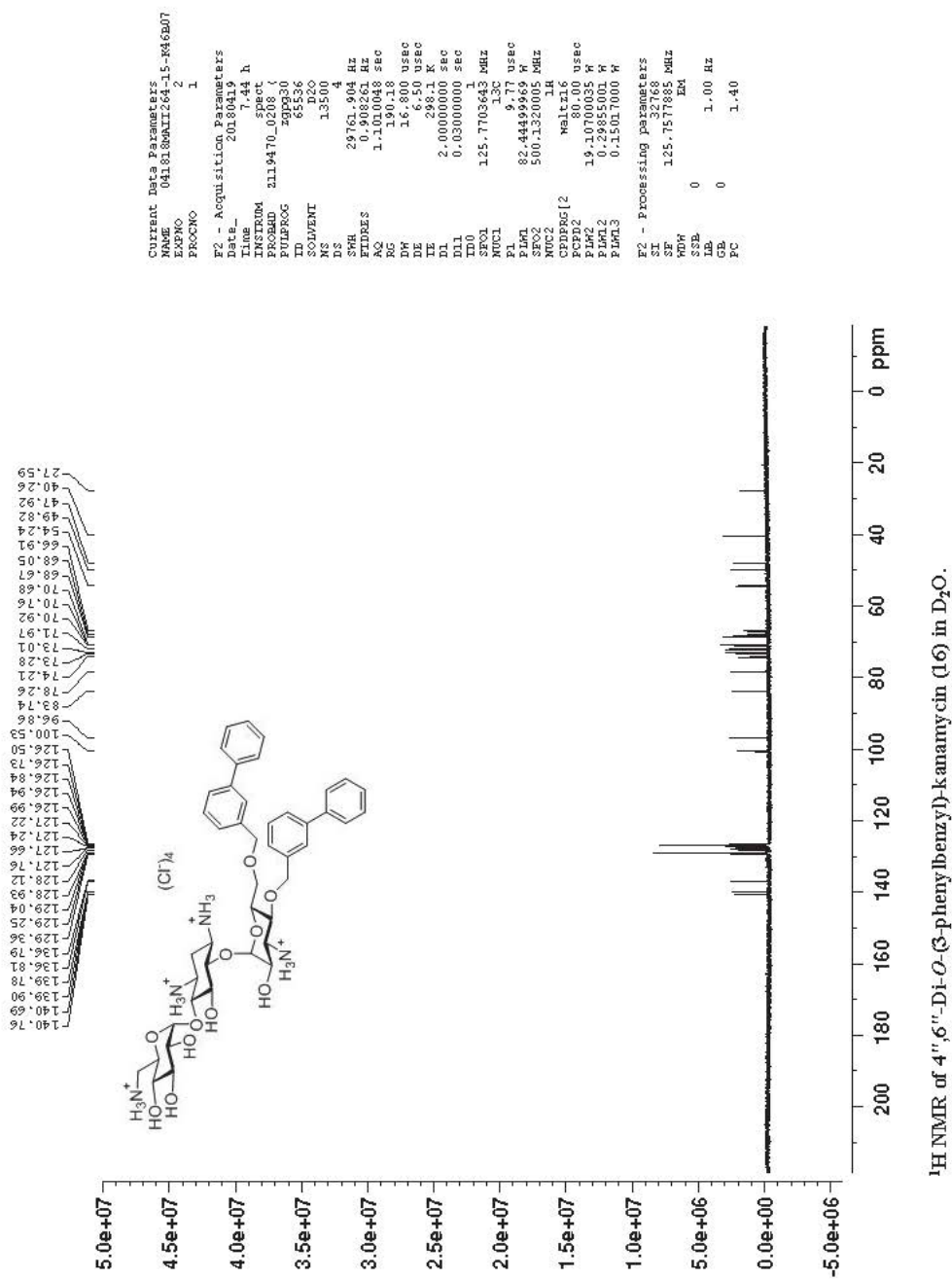


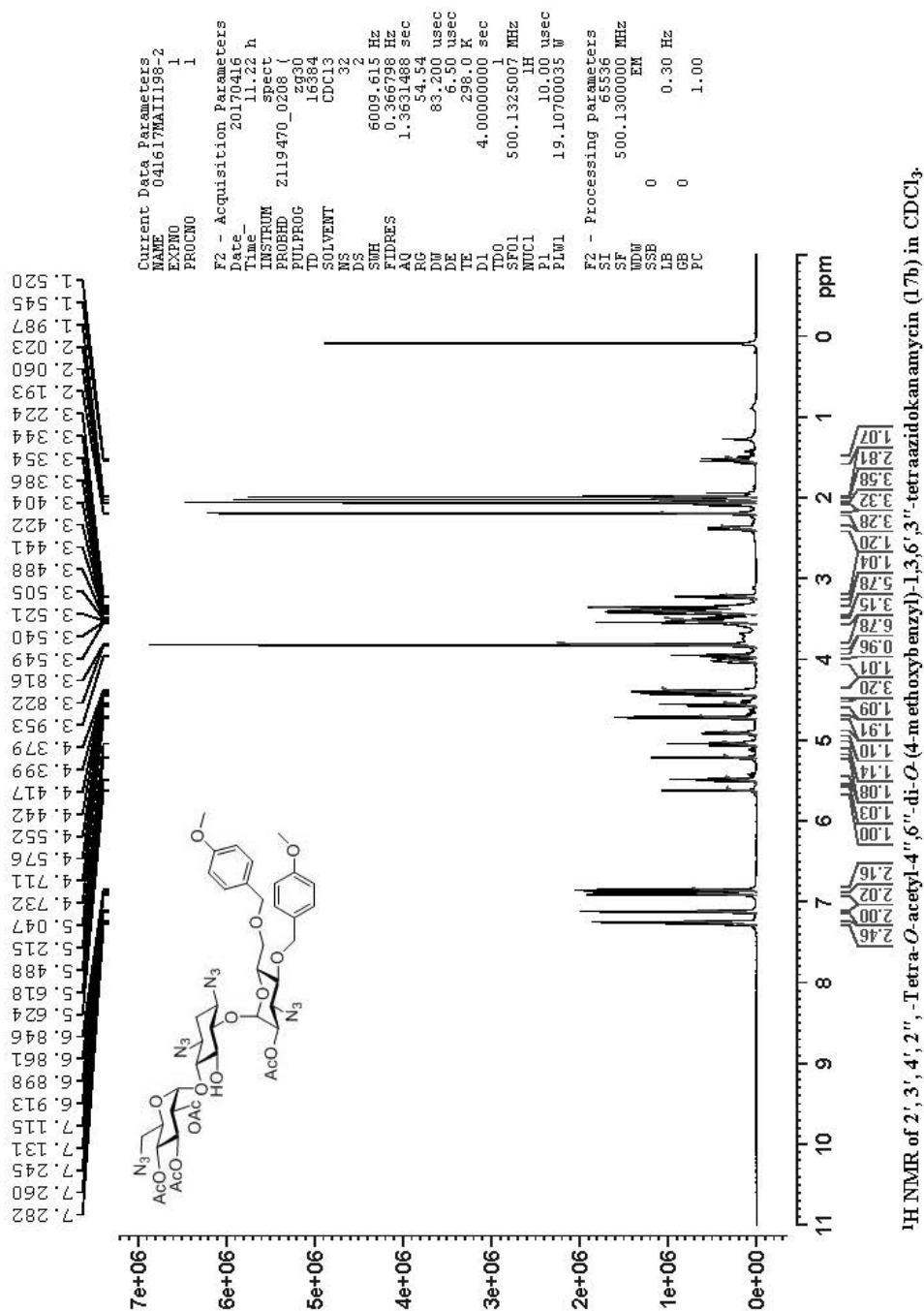




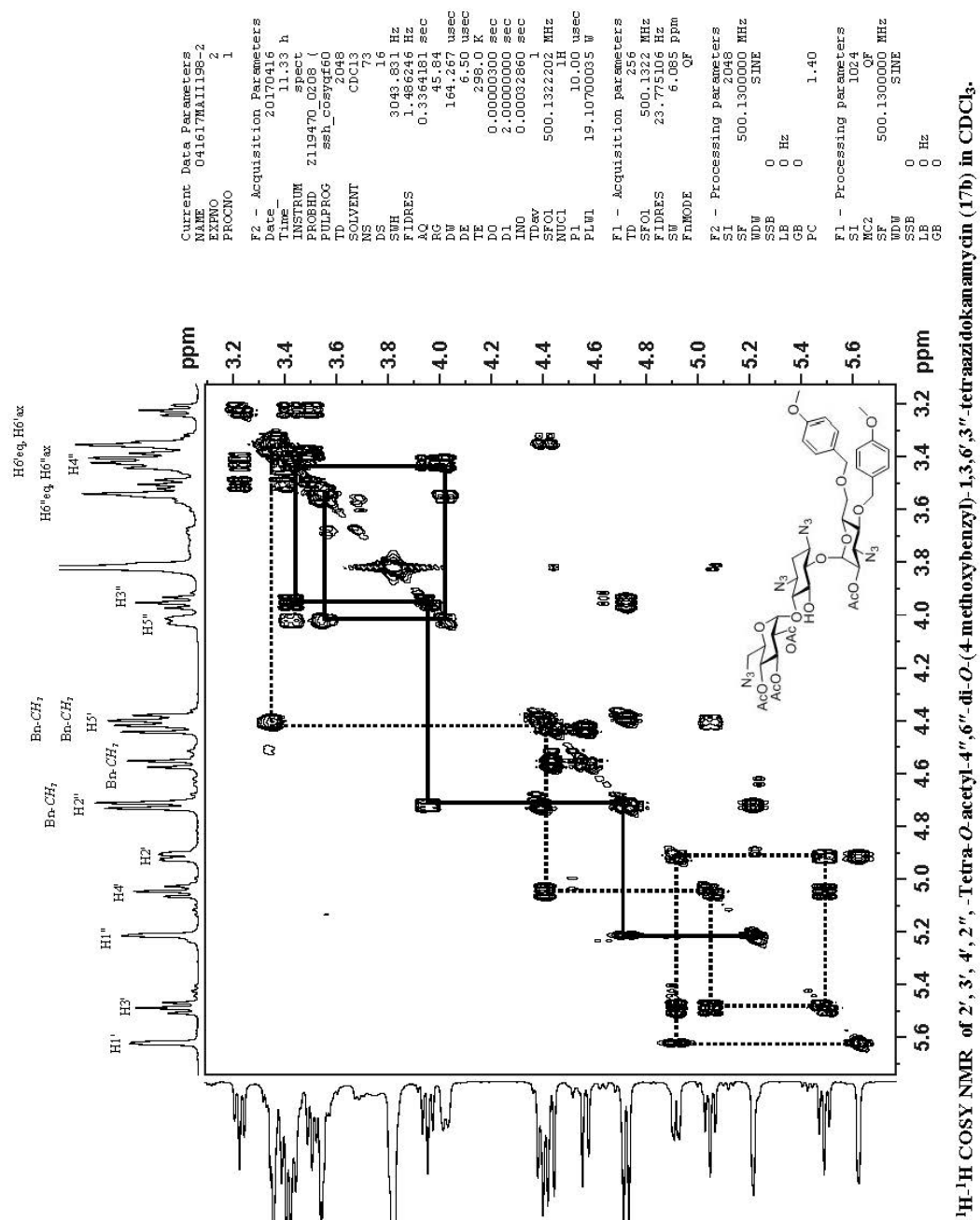


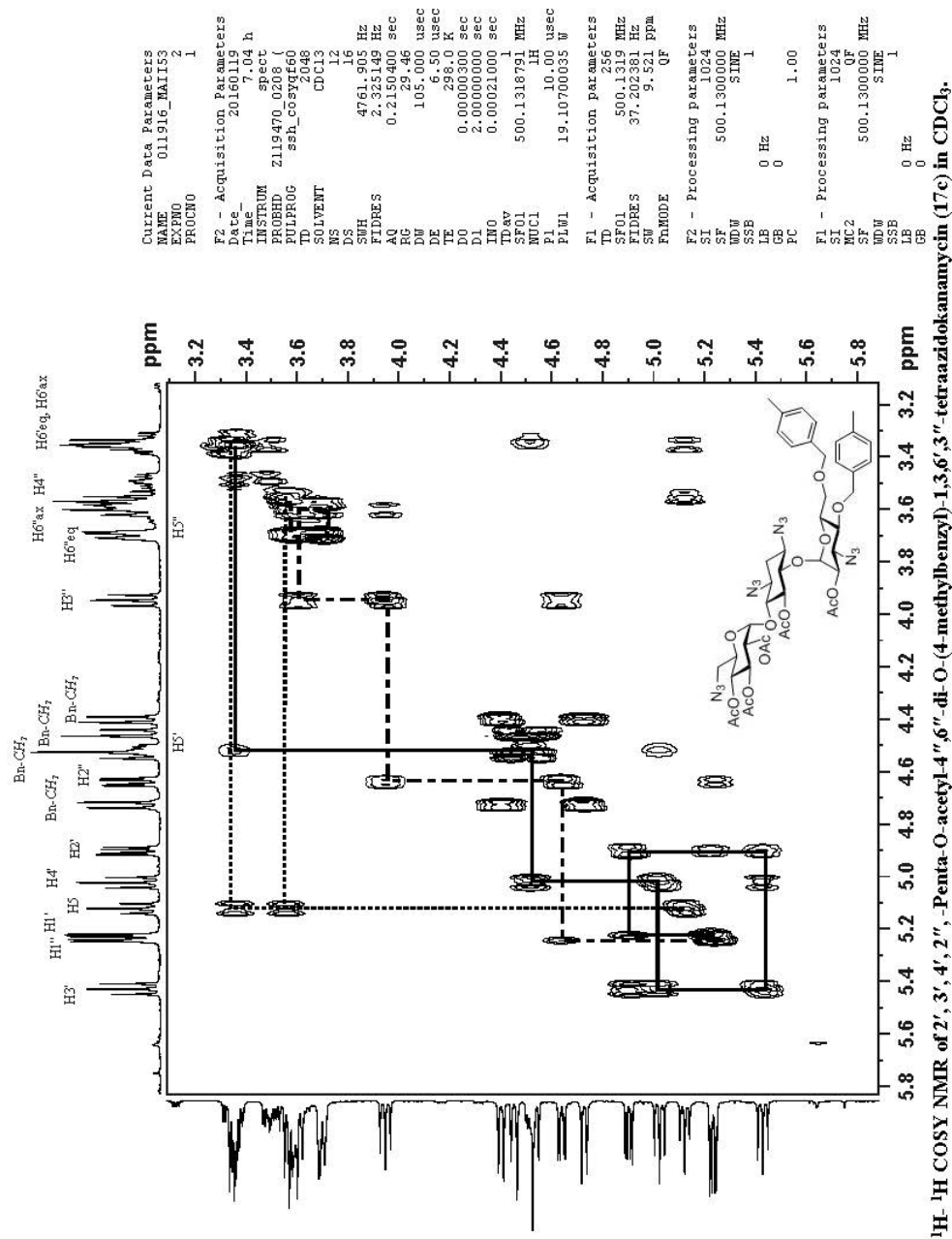


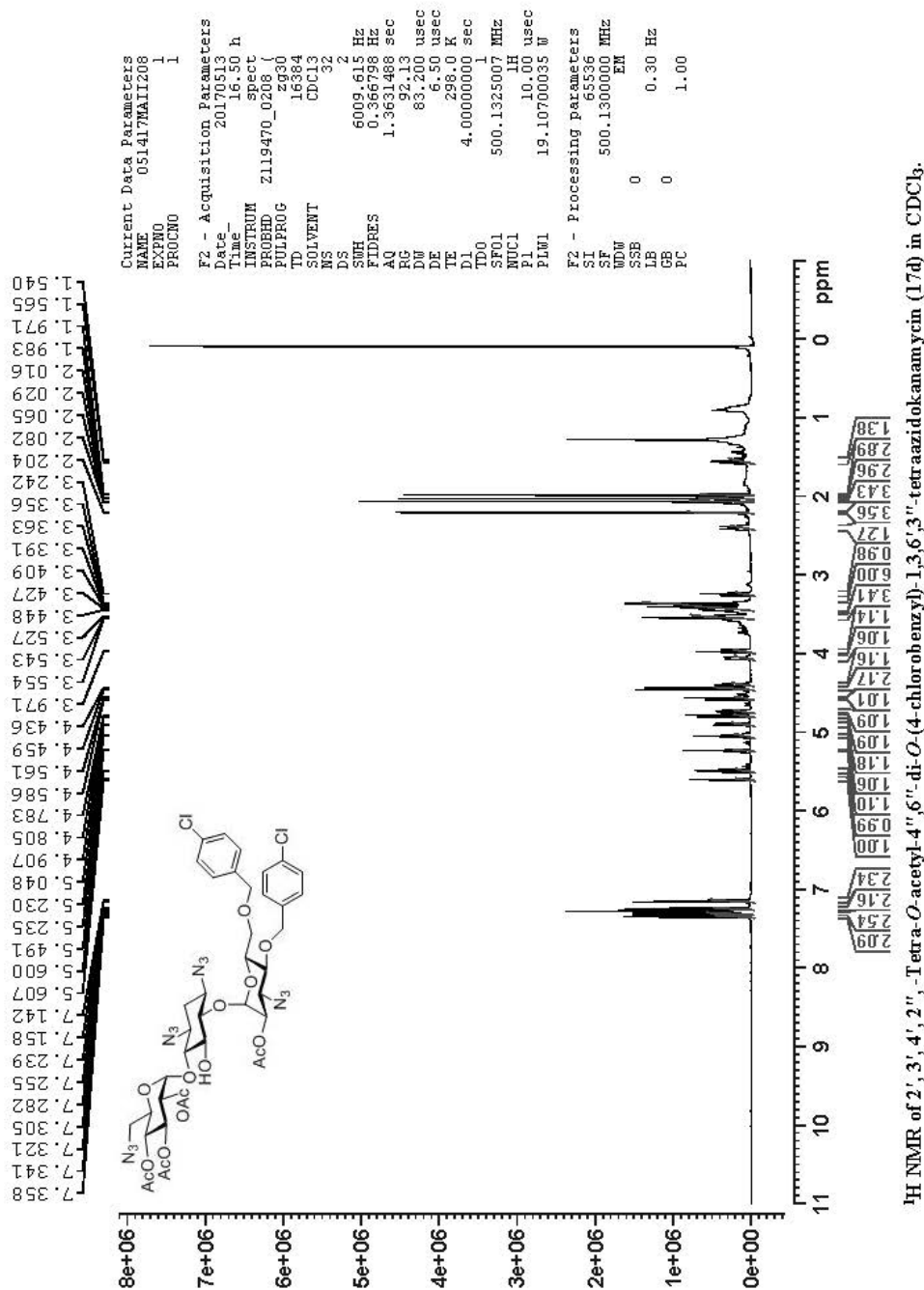




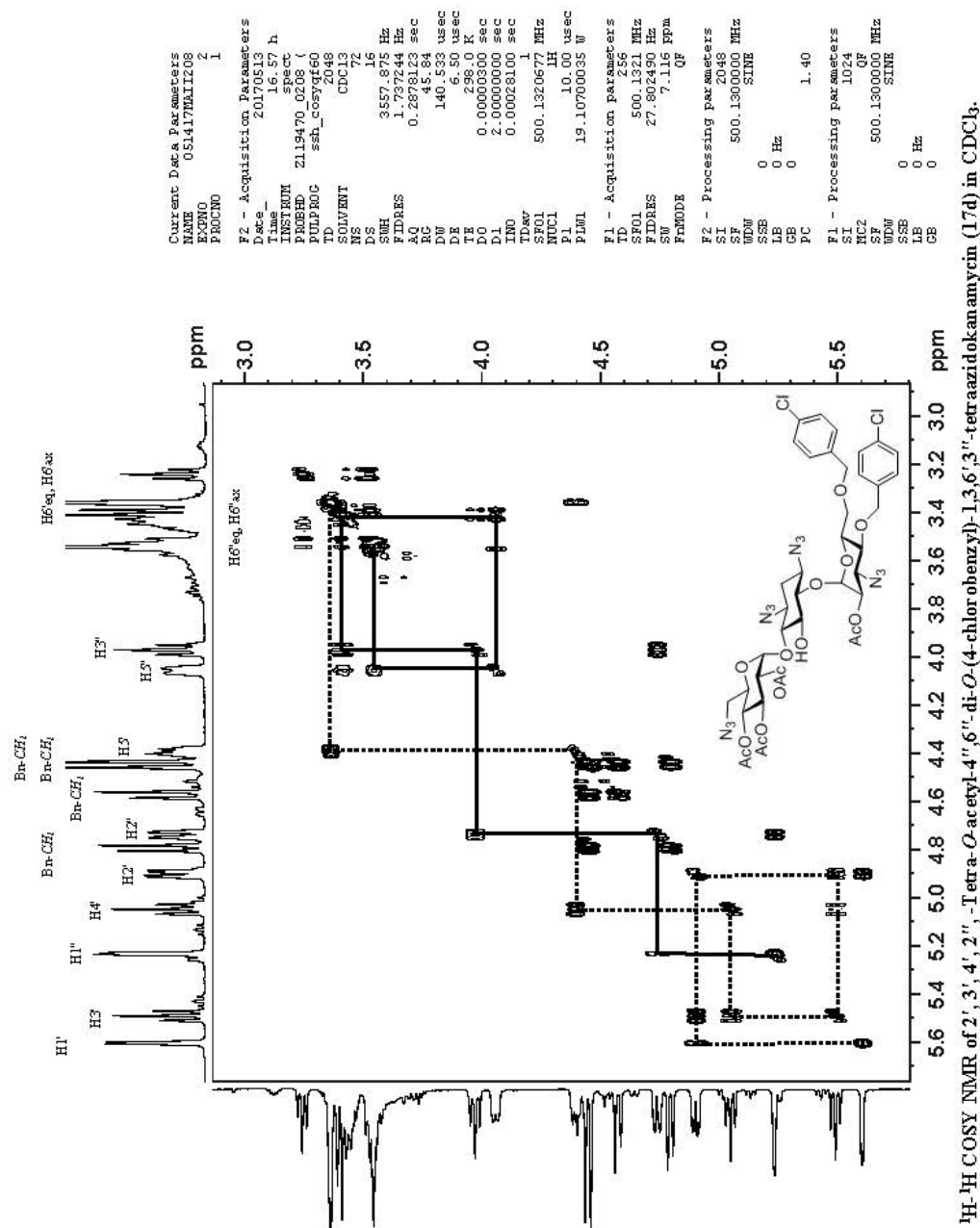
B-42

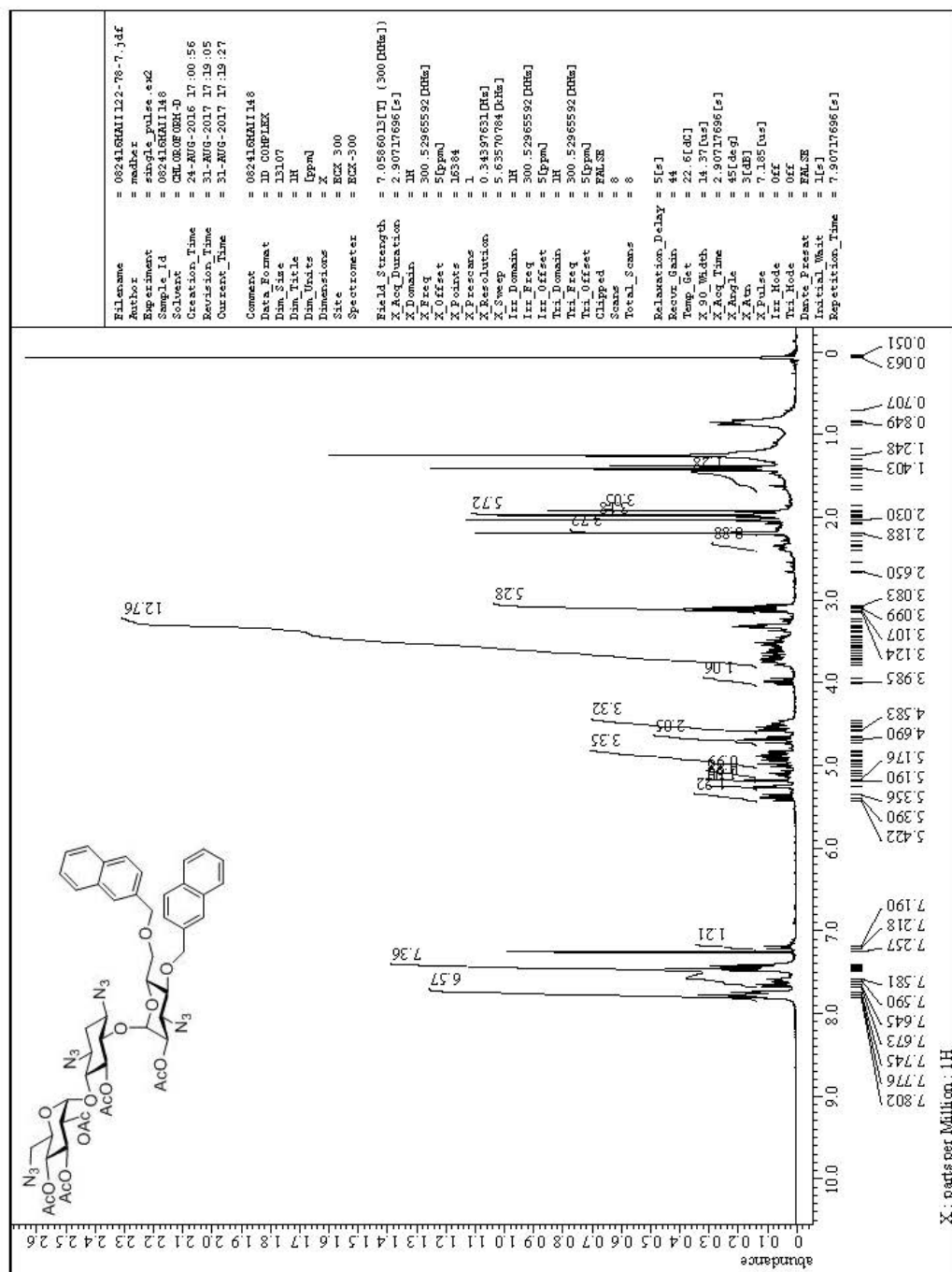


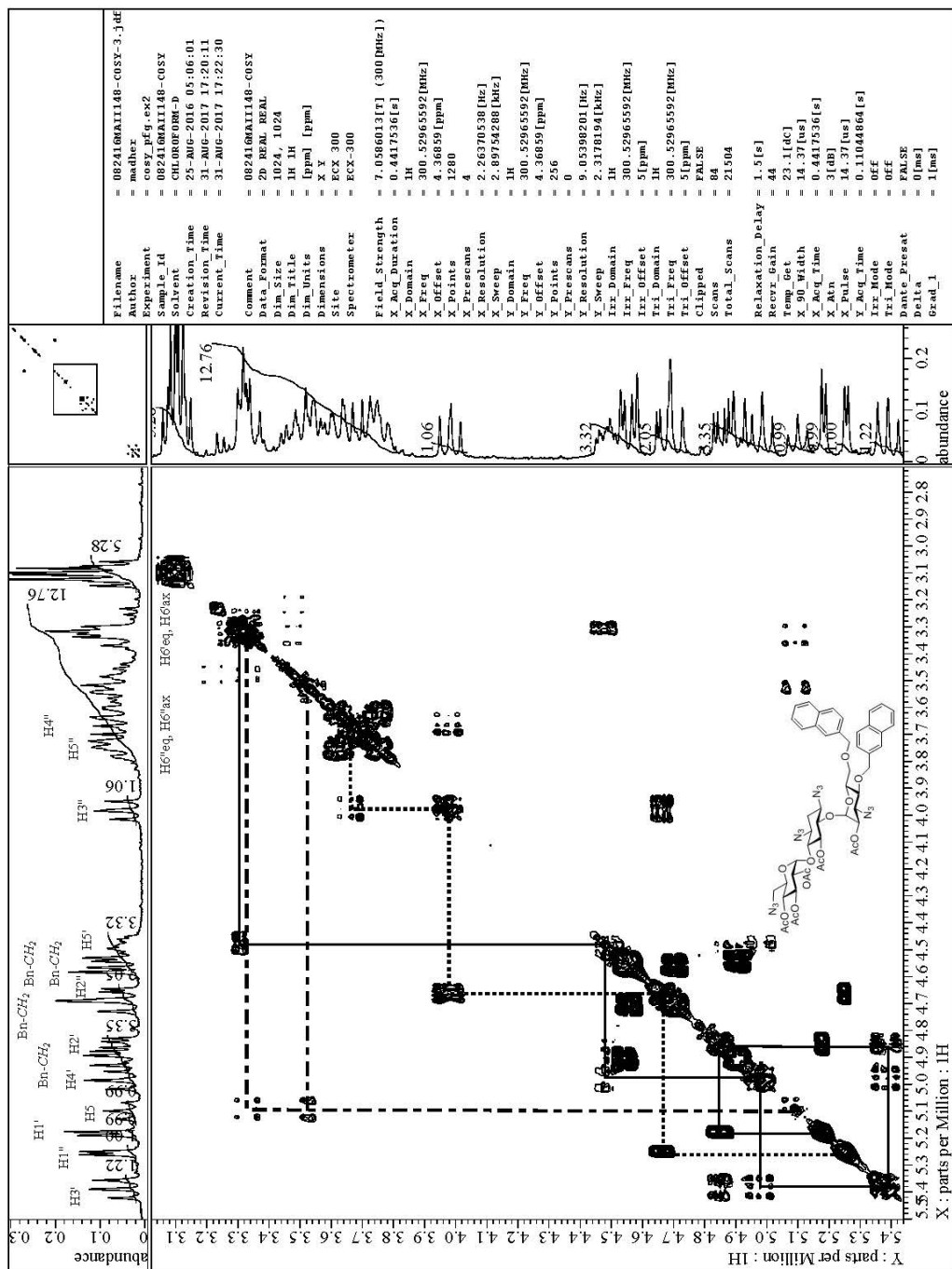


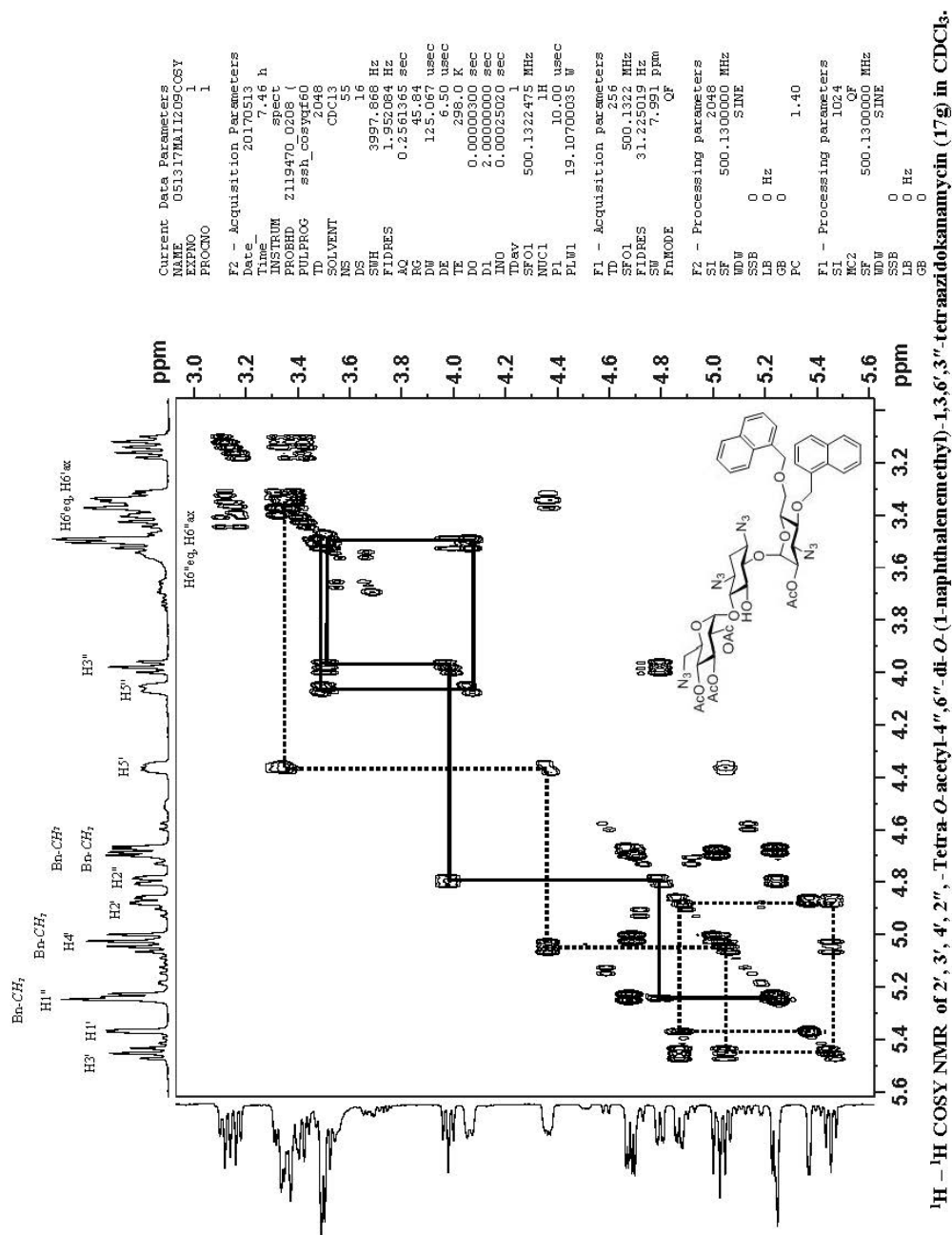


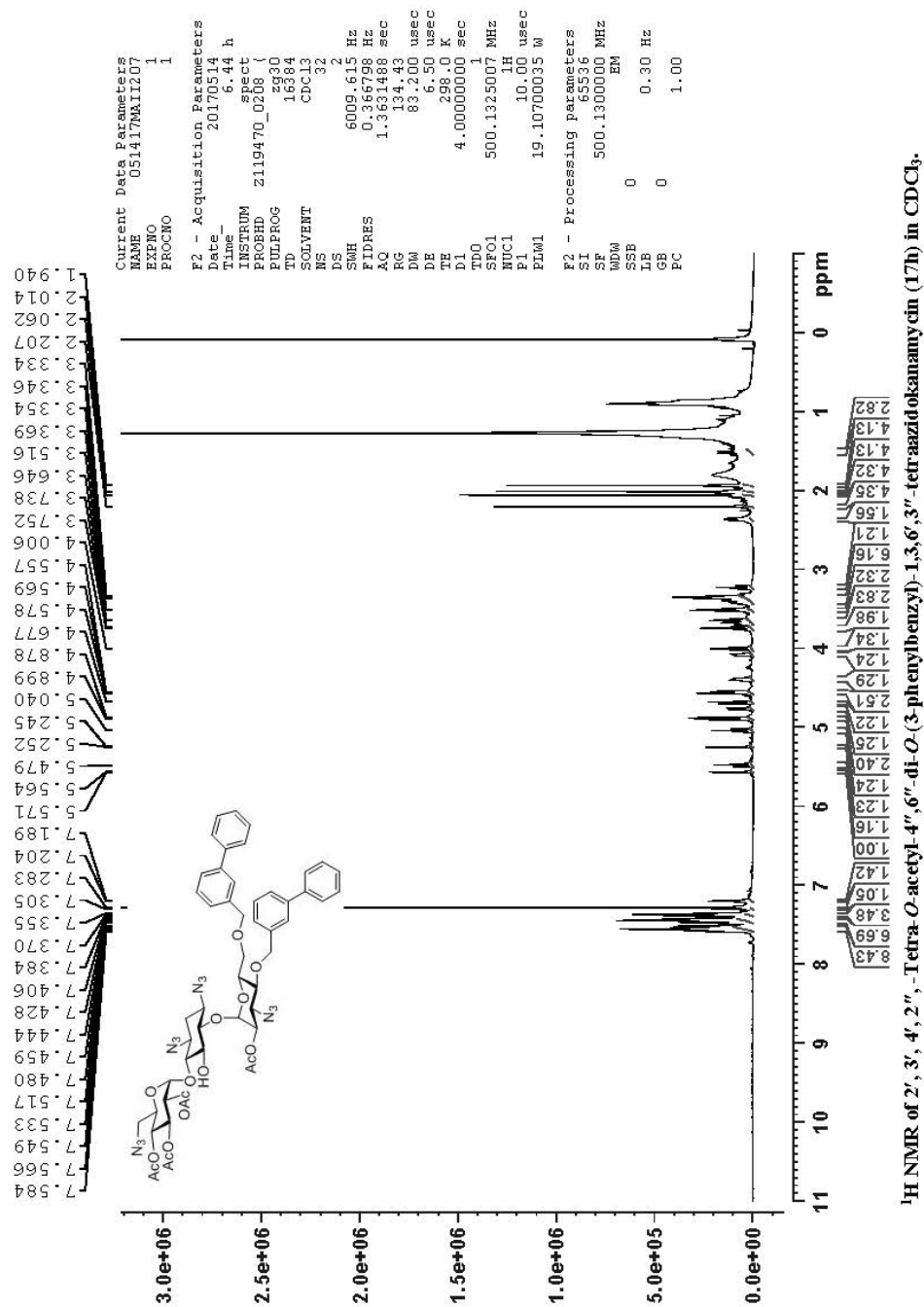
B-46



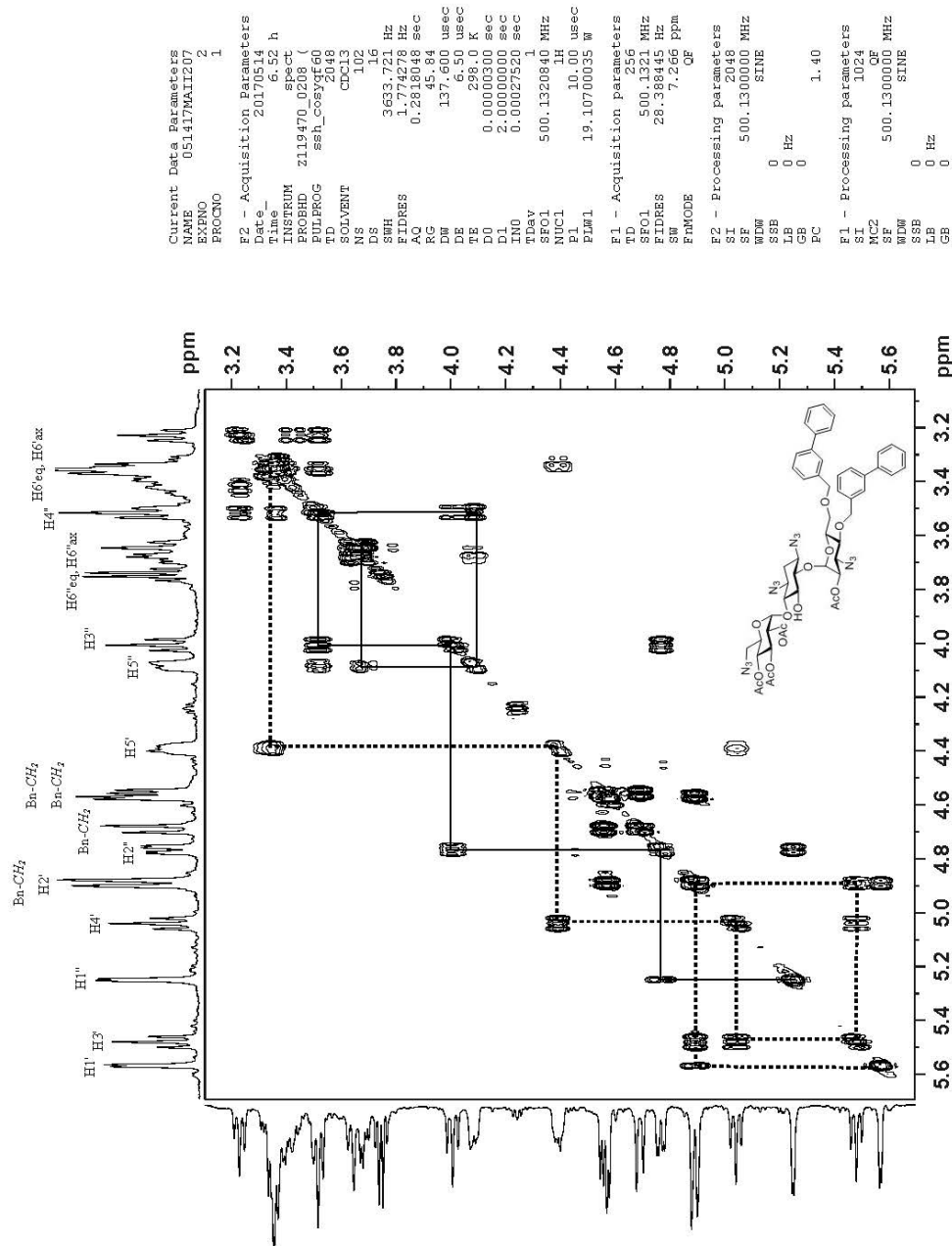


¹H-¹H COSY NMR of 2', 3', 4', 2'', -Tetra-*O*-acetyl-4'', 6''-di-*O*-(2-naphthalenemethyl)-1, 3, 6', 3''-tetraazidokanamycin (17f) in CDCl₃.





B-54


¹H-¹H COSY NMR of 2', 3', 4', 2'', -Tetra-*O*-acetyl-4'', 6'', -di-*O*-(3-phenylbenzyl)-1,3,6', 3''-tetraazidokanamycin (17h) in CDCl₃.

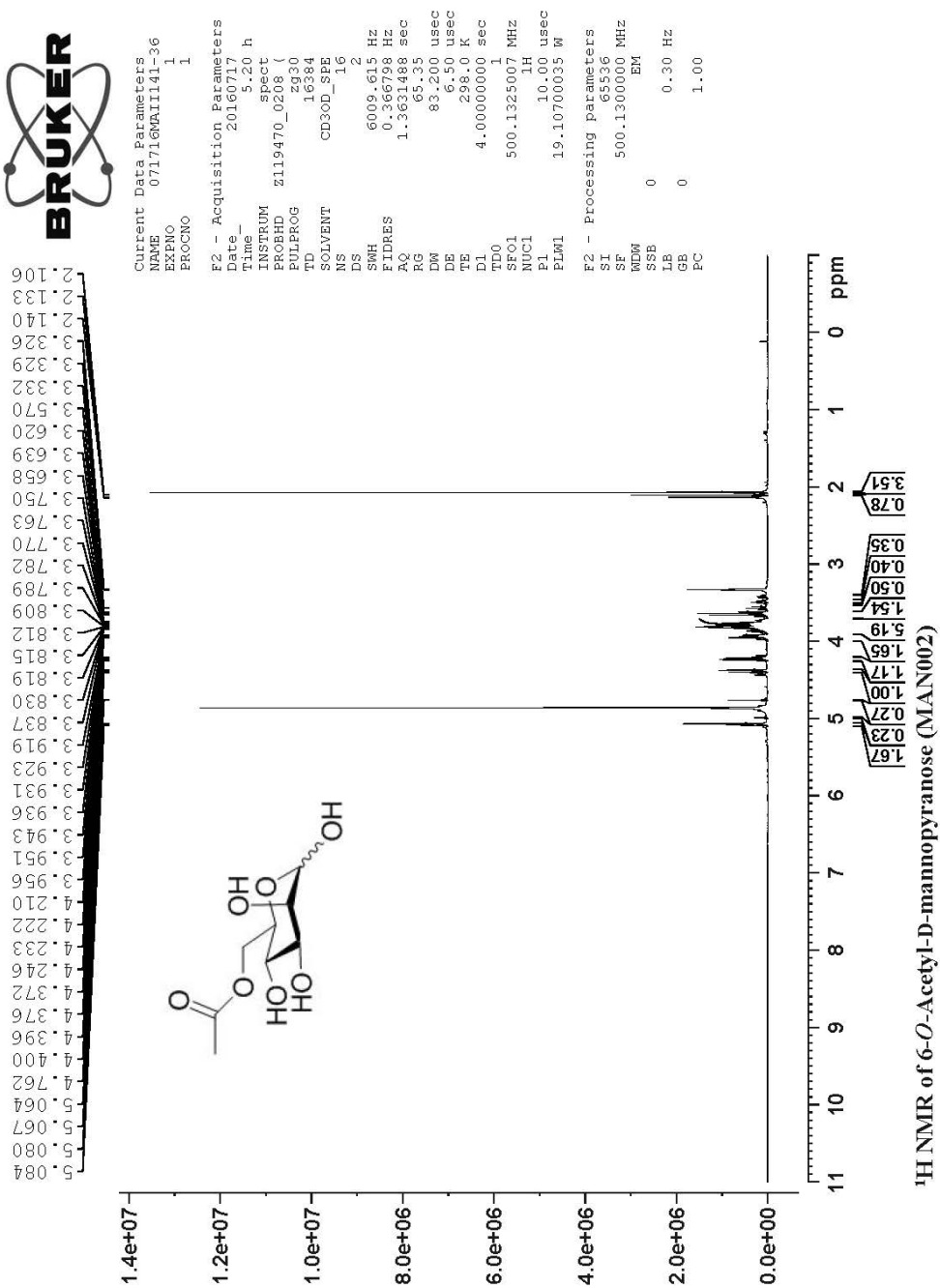
APPENDIX C

CHAPTER IV SUPPLEMENTRY MATERIALS

Table C-1. Antifungal synergistic activities of azoles combined with MAN014^a

Azole	FIC index		
	<i>Candida albicans</i> MYA2876 (azole susceptible)	<i>Cryptococcus</i> <i>neoformans</i> H99	<i>Rhodotorula</i> <i>pilimanae</i>
Clotrimazole	0.999	0.624	0.626
Fluconazole	0.250	0.515	0.563
Itraconazole	0.750	0.751	0.500
Posaconazole	0.624	0.626	0.751
Voriconazole	0.624	1.00	2.00

^a Combination interactions are classified as synergistically inhibitory if the FICI is ≤ 0.5 , indifferent if $>0.5 - 4$, and antagonistic if >4 .

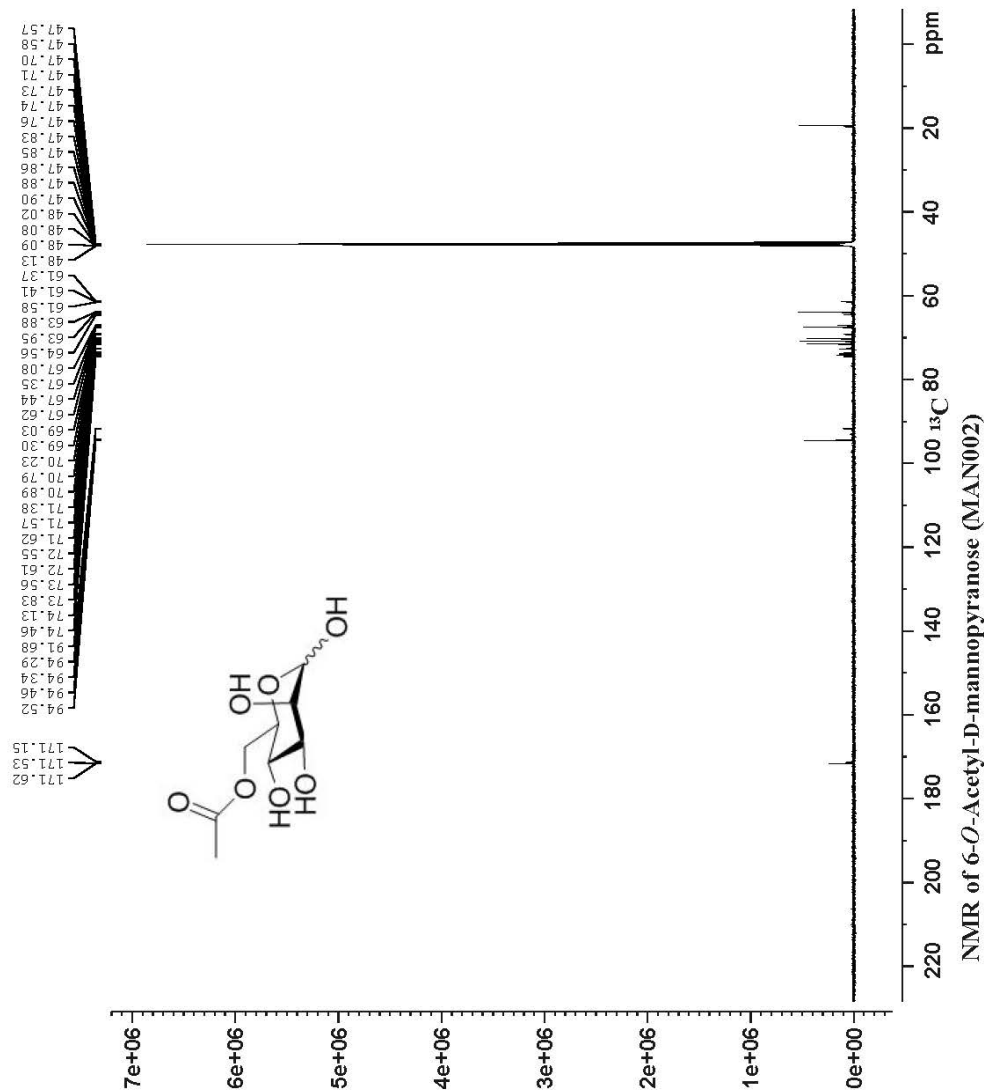


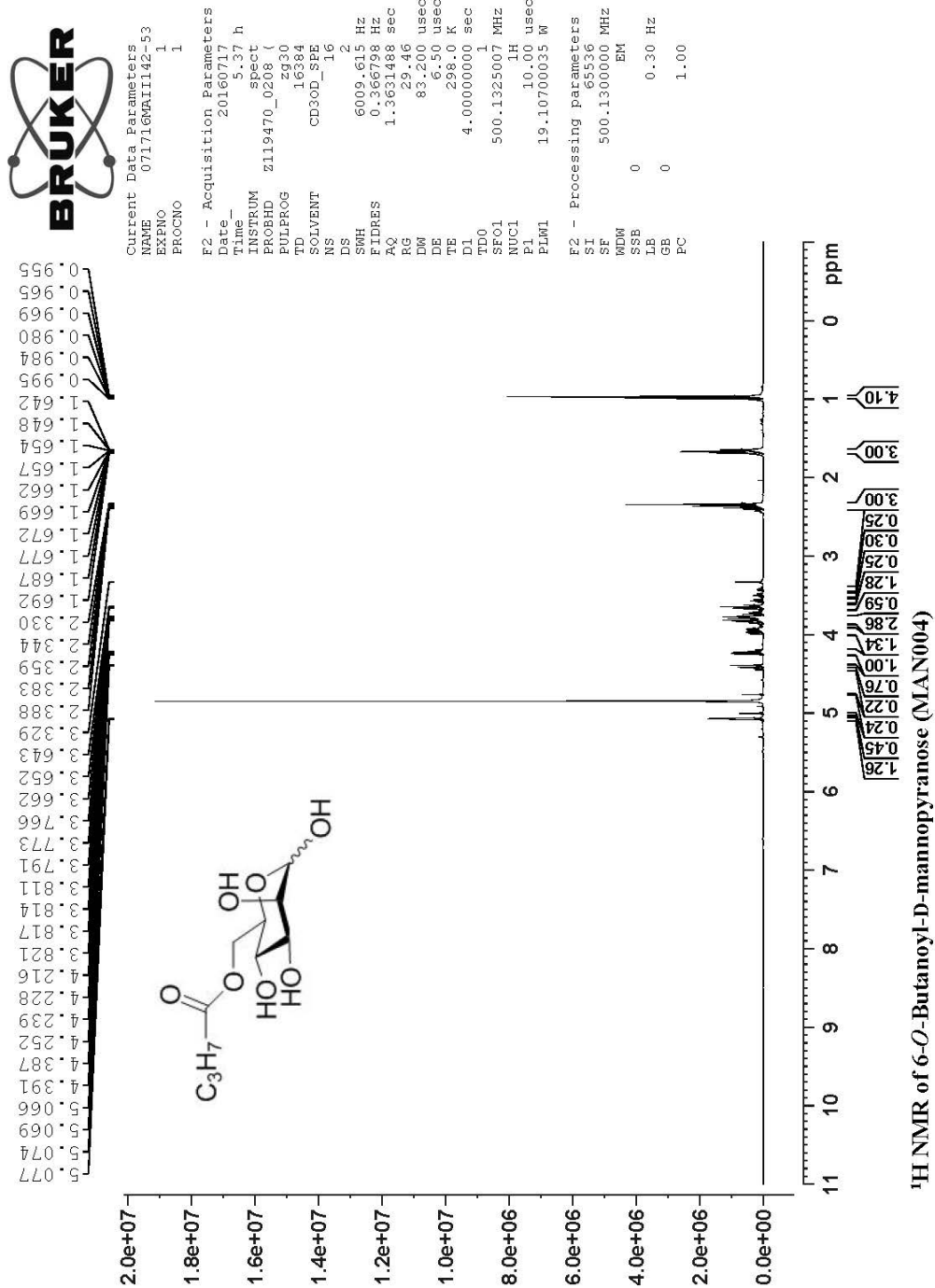


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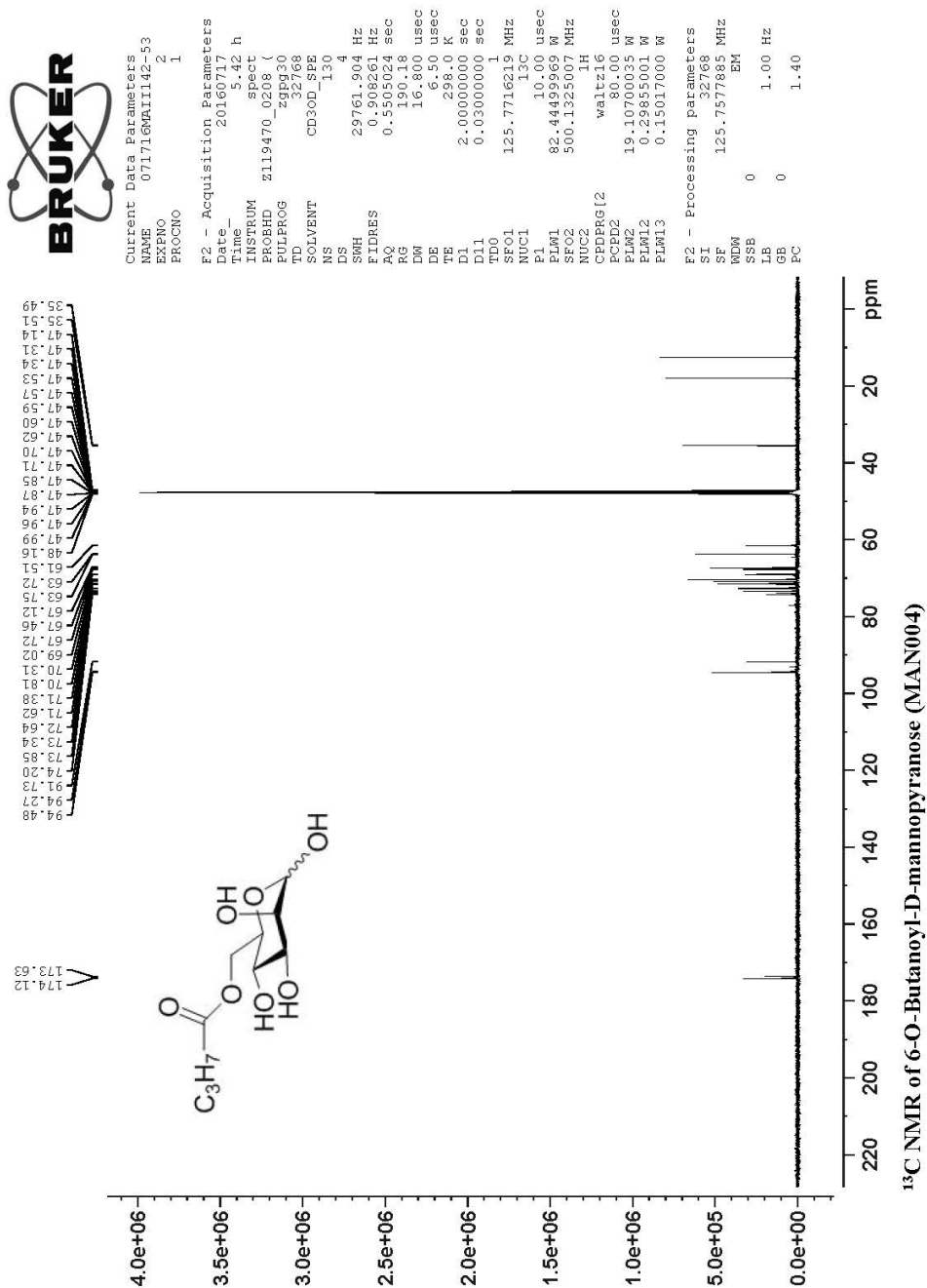
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 TD 200
 SOLVENT CD3OD_SPE
 NS 4
 DS 25761.904 Hz
 SWH 0.908261 Hz
 FIDRES 0.5505024 sec
 AQ 190.18
 RG 16.800 usec
 DE 298.0 K
 TE 2.00000000 sec
 D1 0.03000000 sec
 TD0 1
 SF01 125.7716219 MHz
 NUC1 13C
 P1 10.00 usec
 PLW1 82.44499969 W
 SFO2 500.1325007 MHz
 NUC2 1H
 CPDPRG2 waltz16
 PCPD2 80.00 usec
 PLW2 19.10700035 W
 PLW12 0.29855001 W
 PLW13 0.15017000 W

F2 - Processing parameters
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 WDW EM
 SSB 0
 LB 1.00 Hz
 GB 0
 PC 1.40





C-4

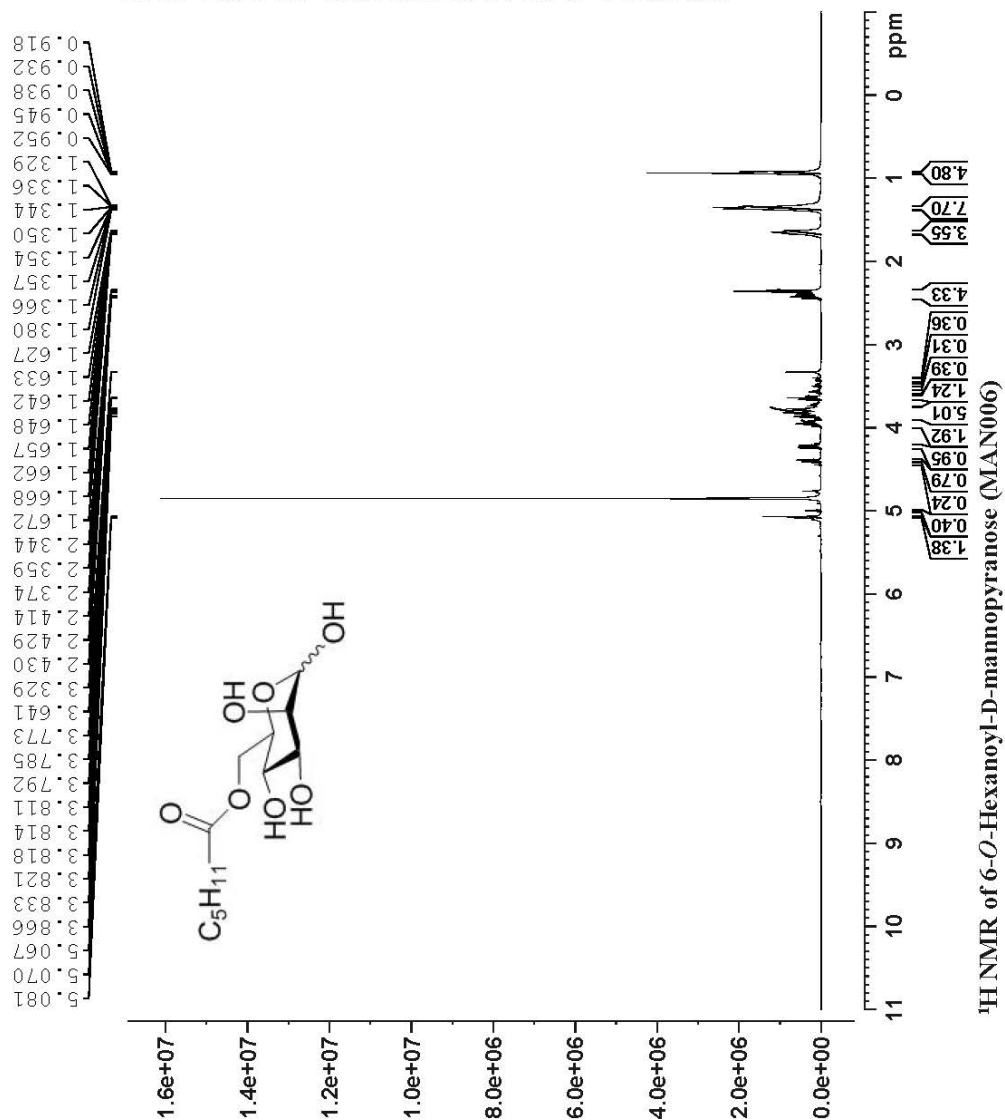


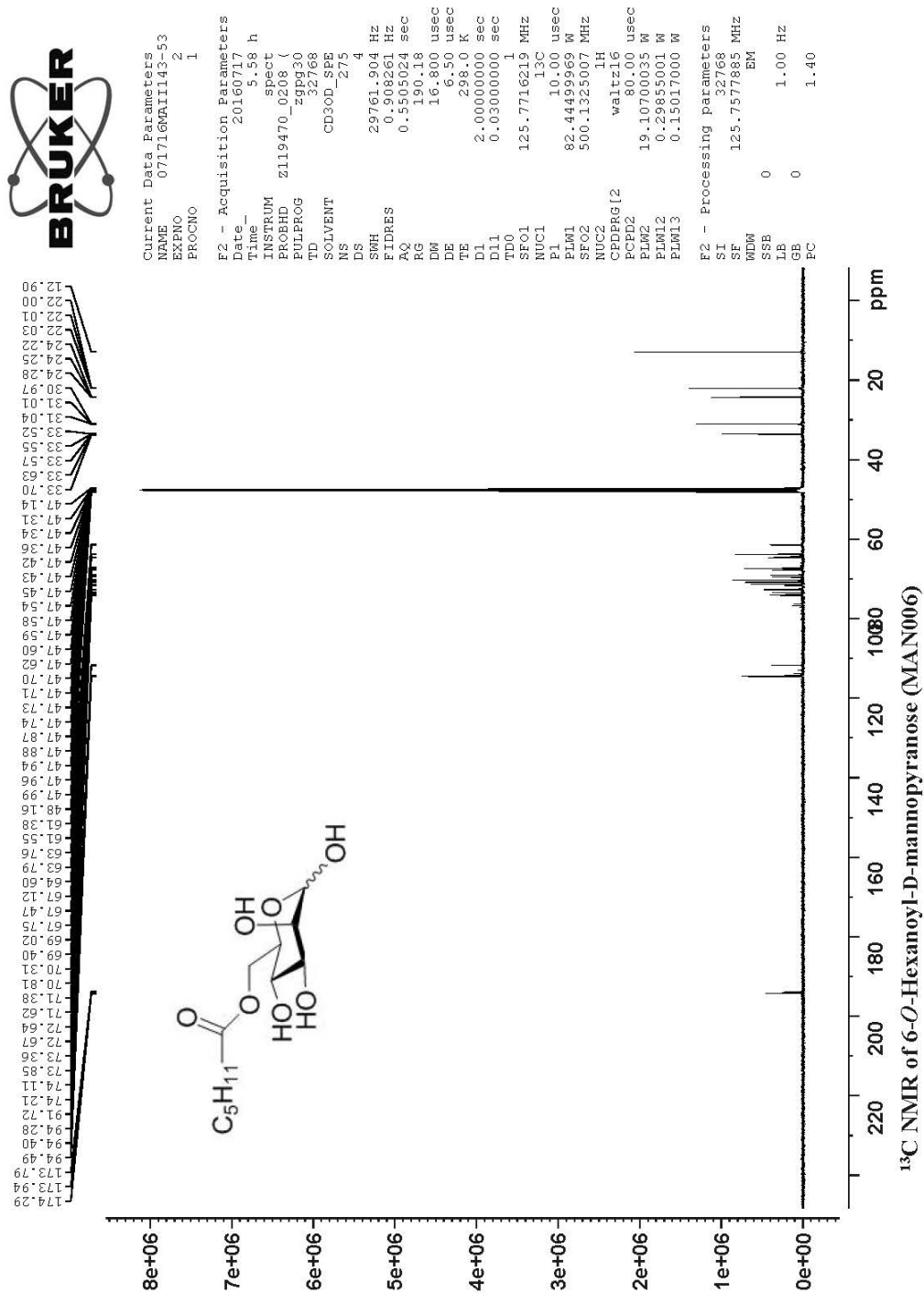


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 PROCNO 1

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 SOLVENT CD3OD_SFB
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 DS 2
 SWH 6009.615 Hz
 FIDRES 0.366798 Hz
 AQ 1.3631488 sec
 RG 29.46
 DW 83.200 usec
 DE 6.50 usec
 TE 298.0 K
 D1 4.00000000 sec
 D11 1
 SFO1 500.1325007 MHz
 NUC1 1H
 P1 10.00 usec
 PL1 19.10700035 W

F2 - Processing parameters
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Current Data Parameters
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 EXPNO 1
 PROCNO 1

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 TD 16384

SOLVENT CD3OD_SPE
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 DS 2

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 DE 6.50 usec
 TE 298.0 K

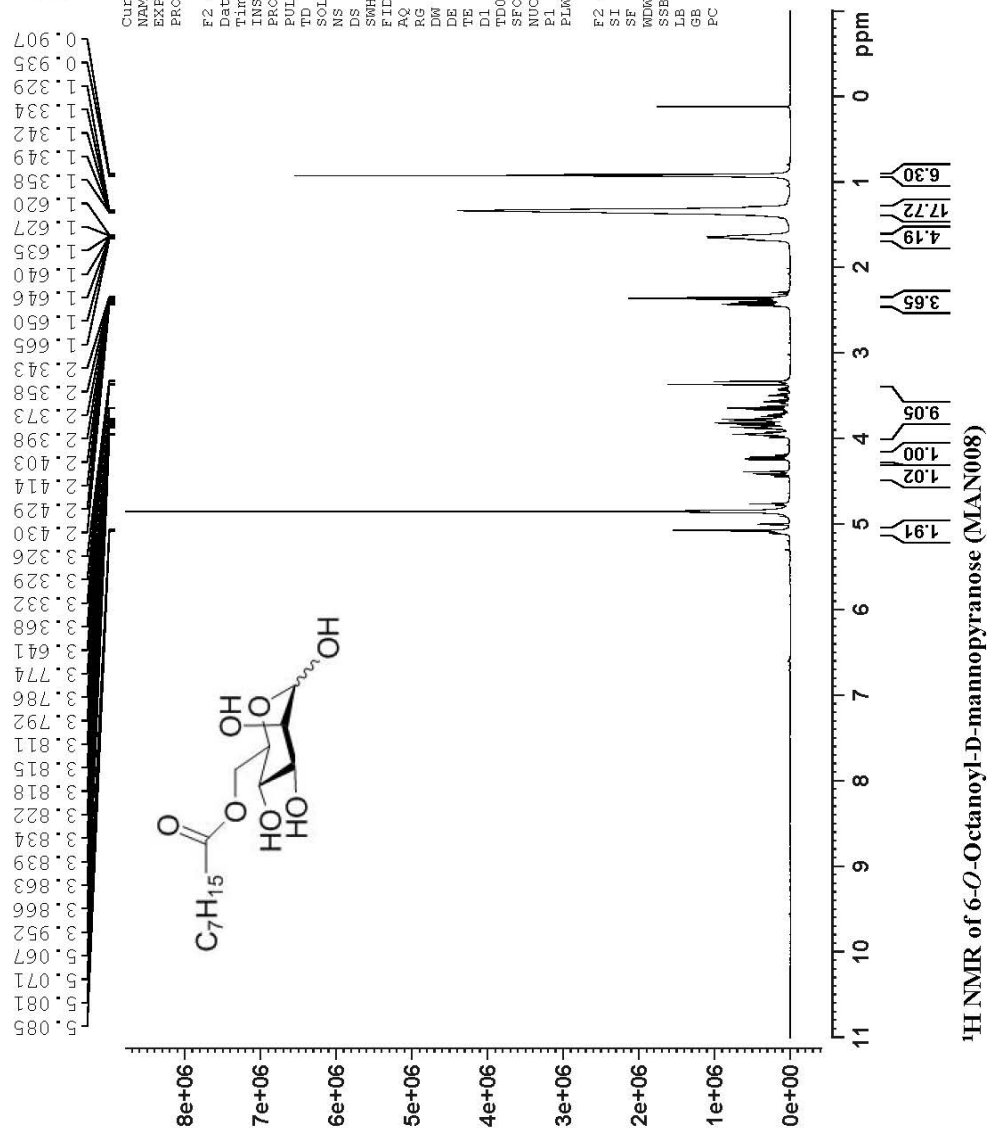
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 SFO1 500.1325007 MHz

NUC1 1H
 PL 10.00 usec
 PLW1 19.10700035 W

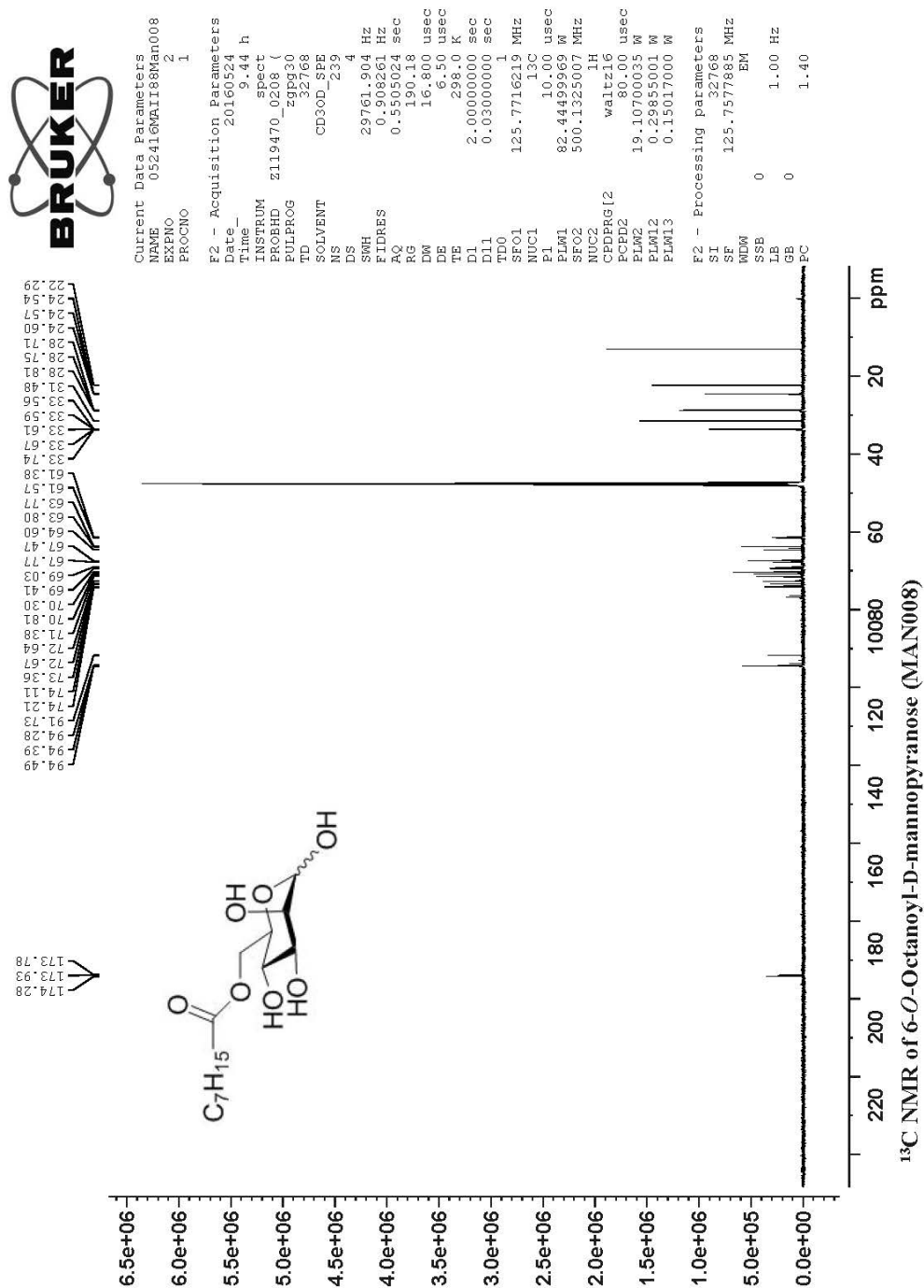
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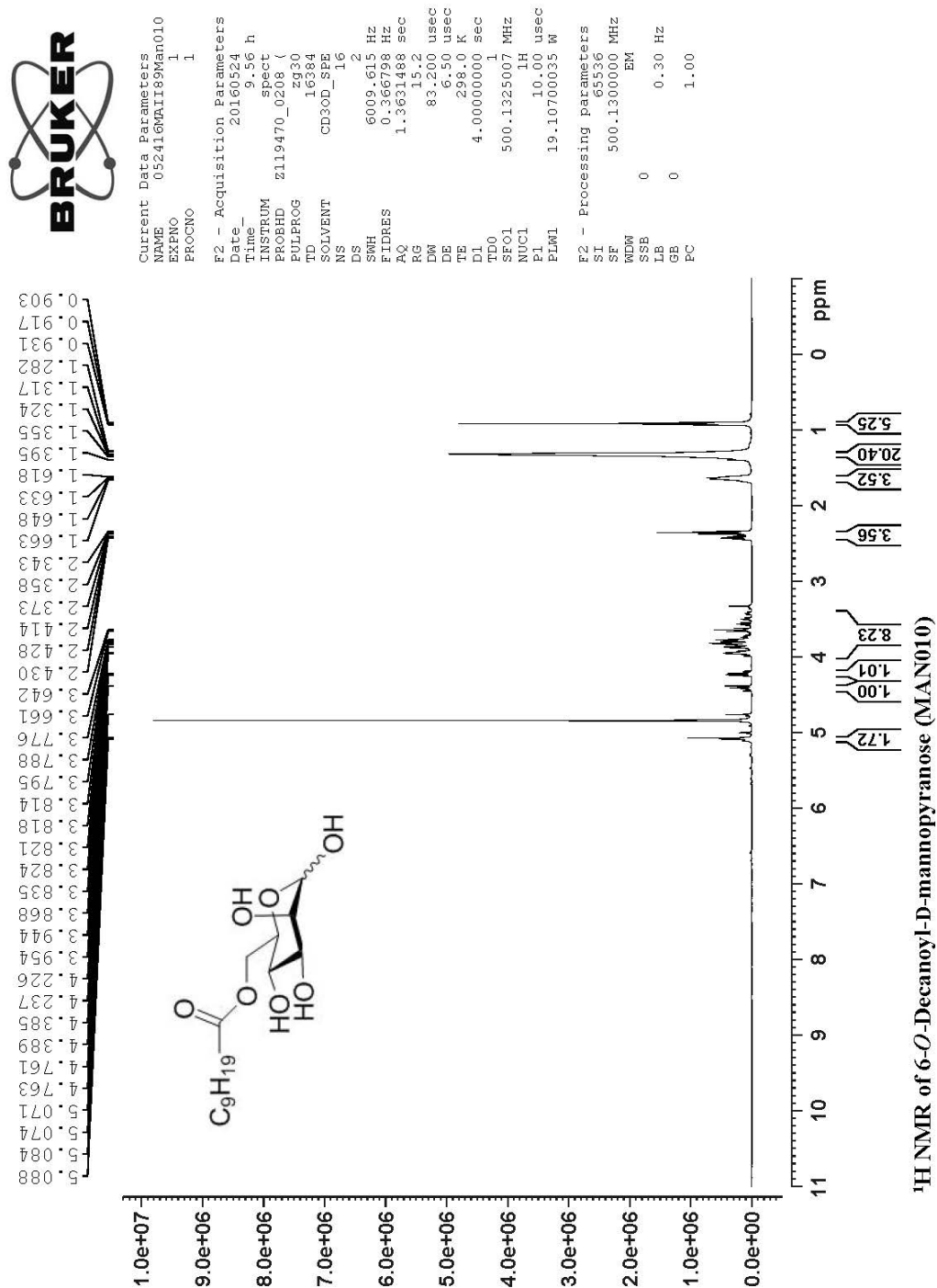
GB 0
 PC 1.00

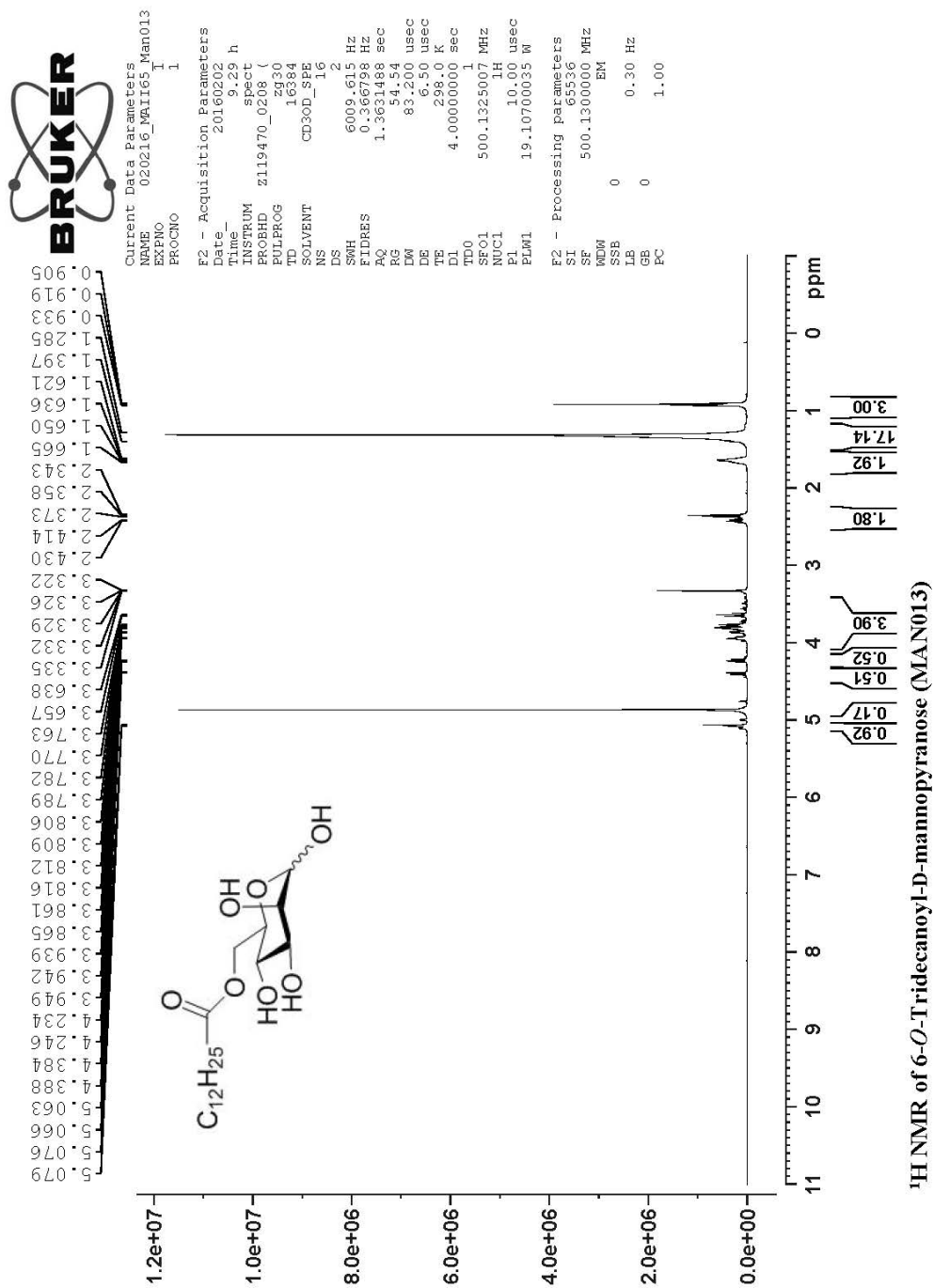


C-8



C-9





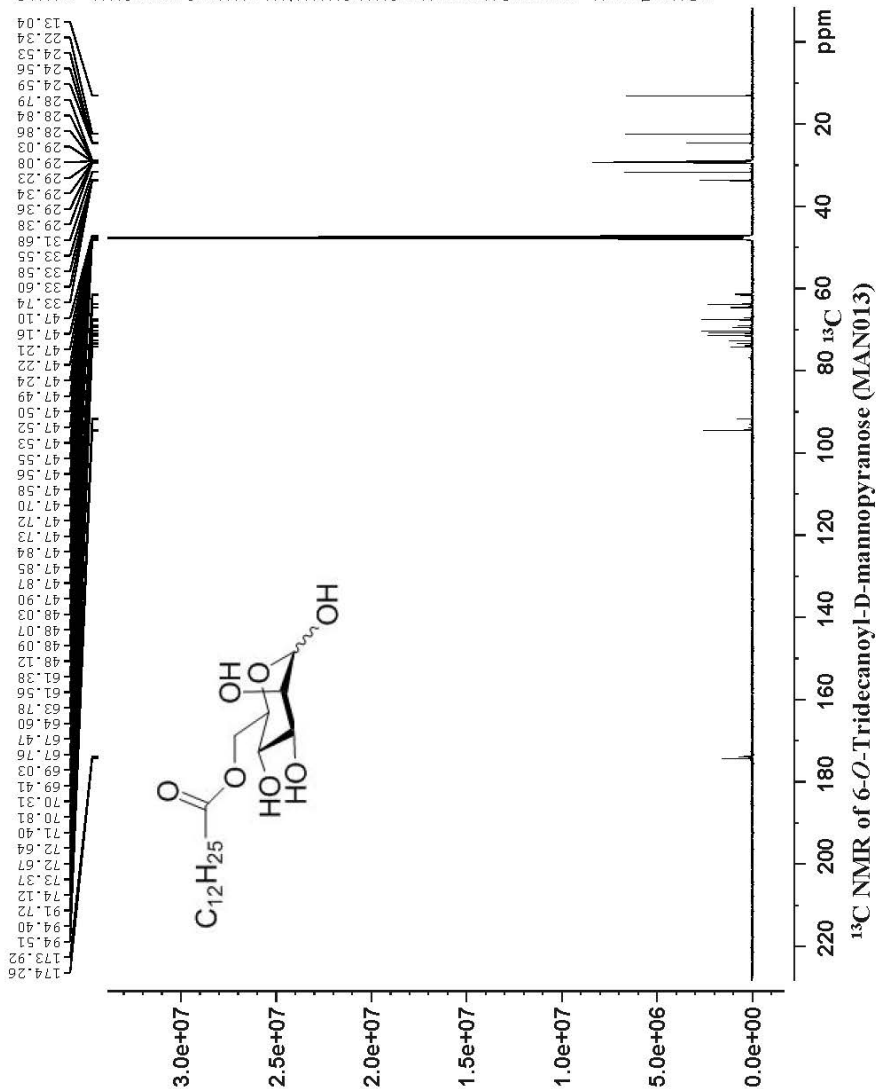


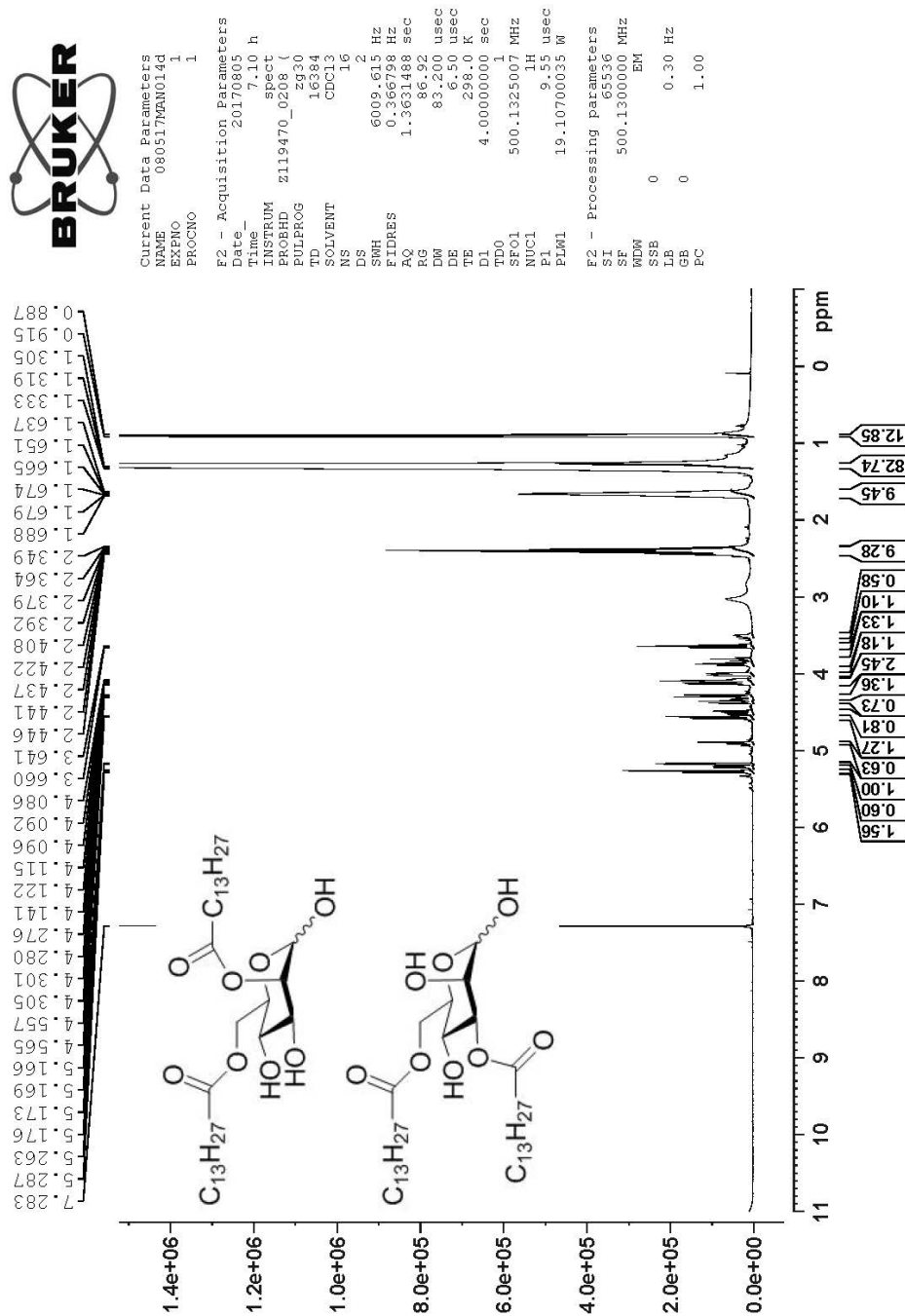
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 PROCNO 1

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 SOLVENT CD3OD_SPE
 NS 2382
 DS 4
 SWH 29761.904 Hz
 FIDRES 0.508261 Hz
 AQ 0.555024 sec
 RG 190.18
 DW 16.80 usec
 DE 28.50 usec
 TE 298.0 K
 D1 2.00000000 sec
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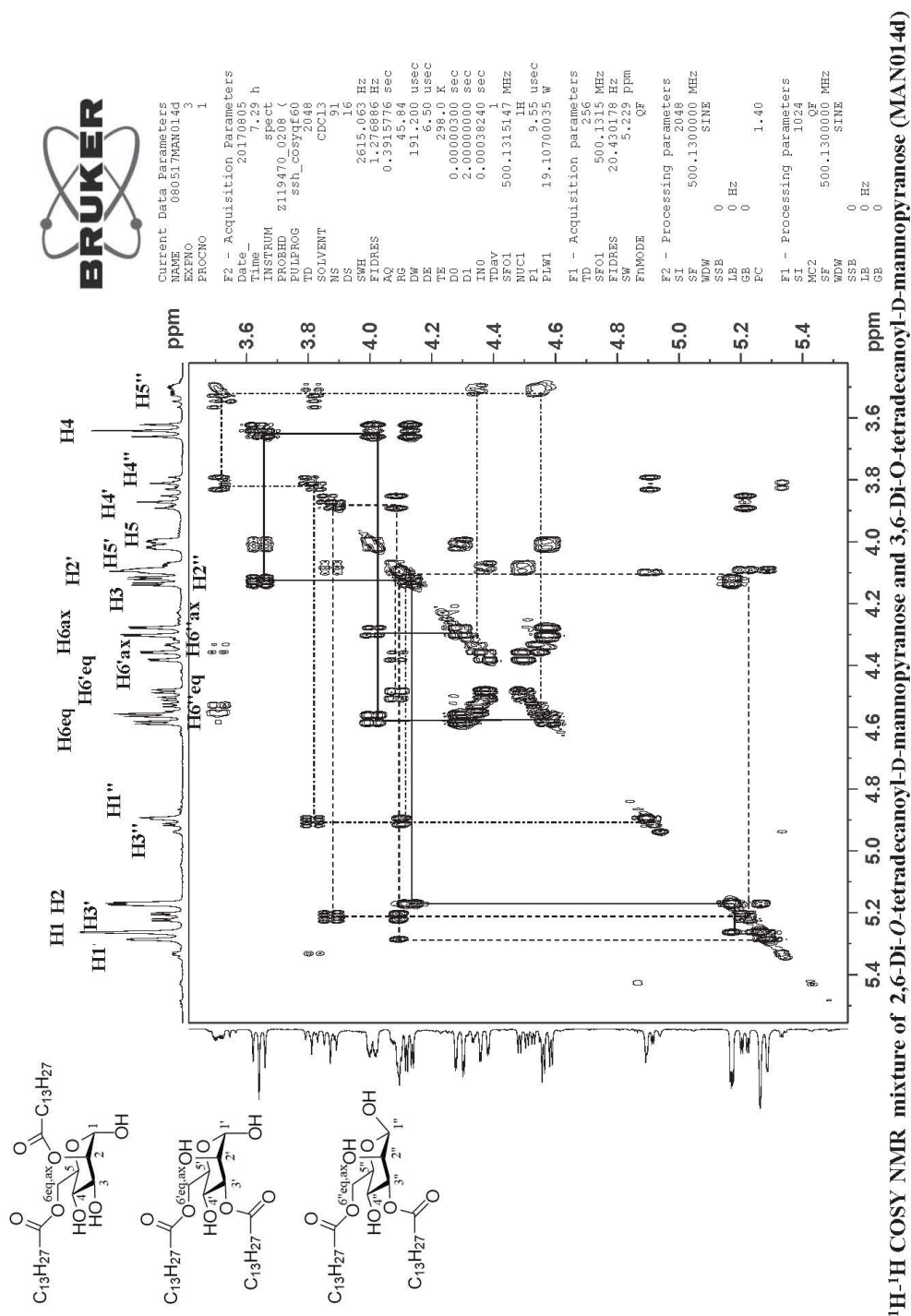
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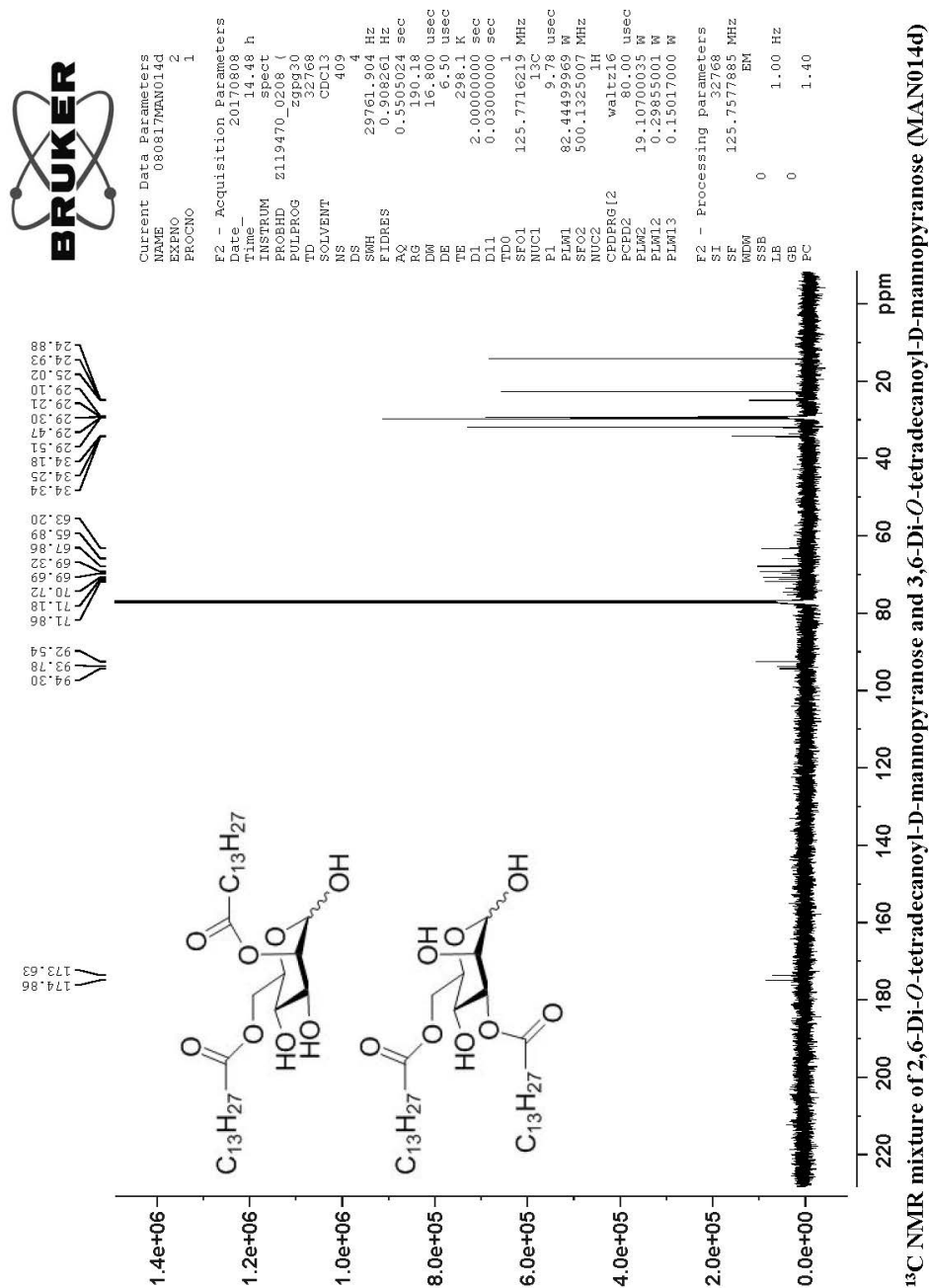


¹H NMR mixture of 2,6-Di-O-tetradecanoyl-D-mannopyranose and 3,6-Di-O-tetradecanoyl-D-mannopyranose (MAN014d)

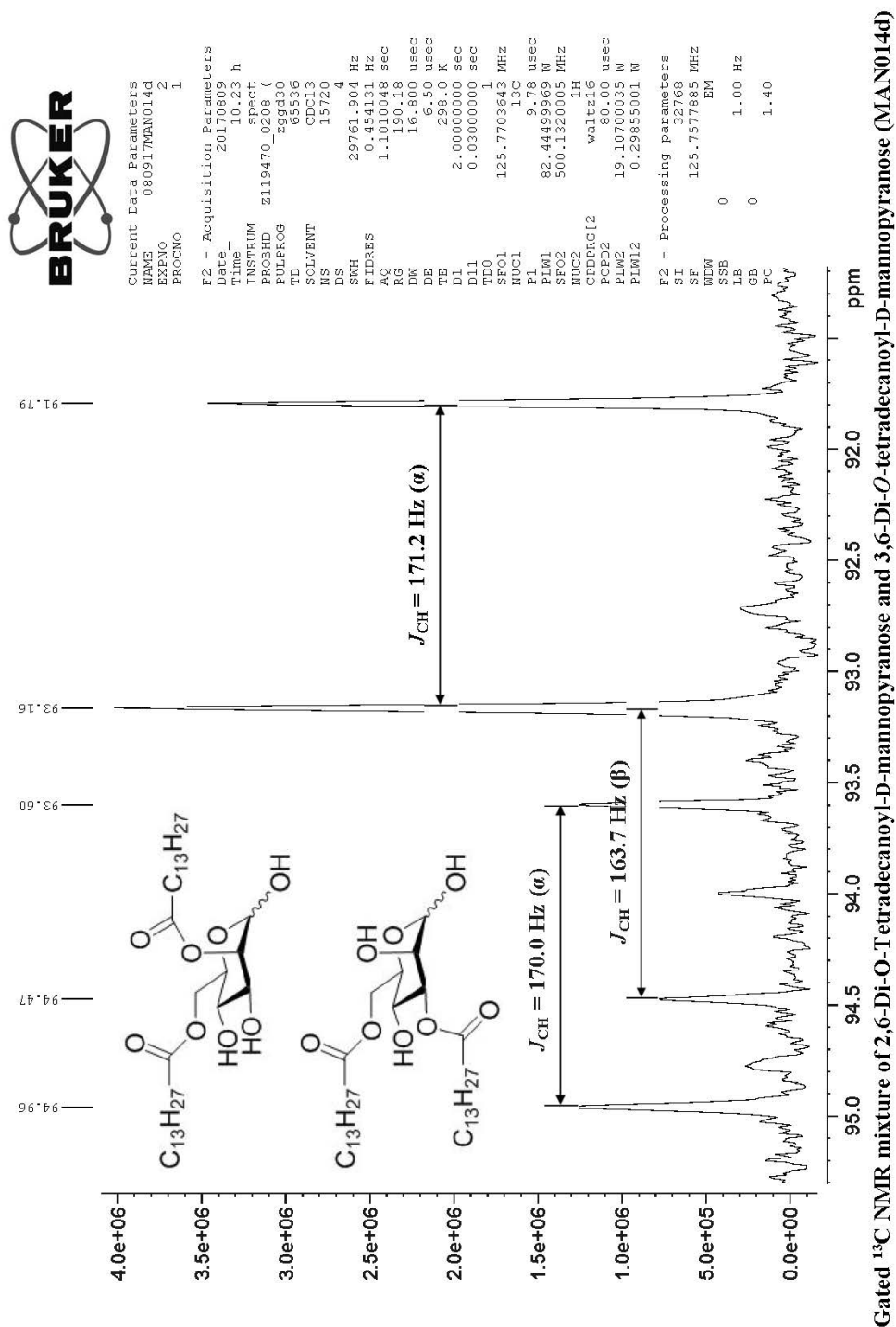
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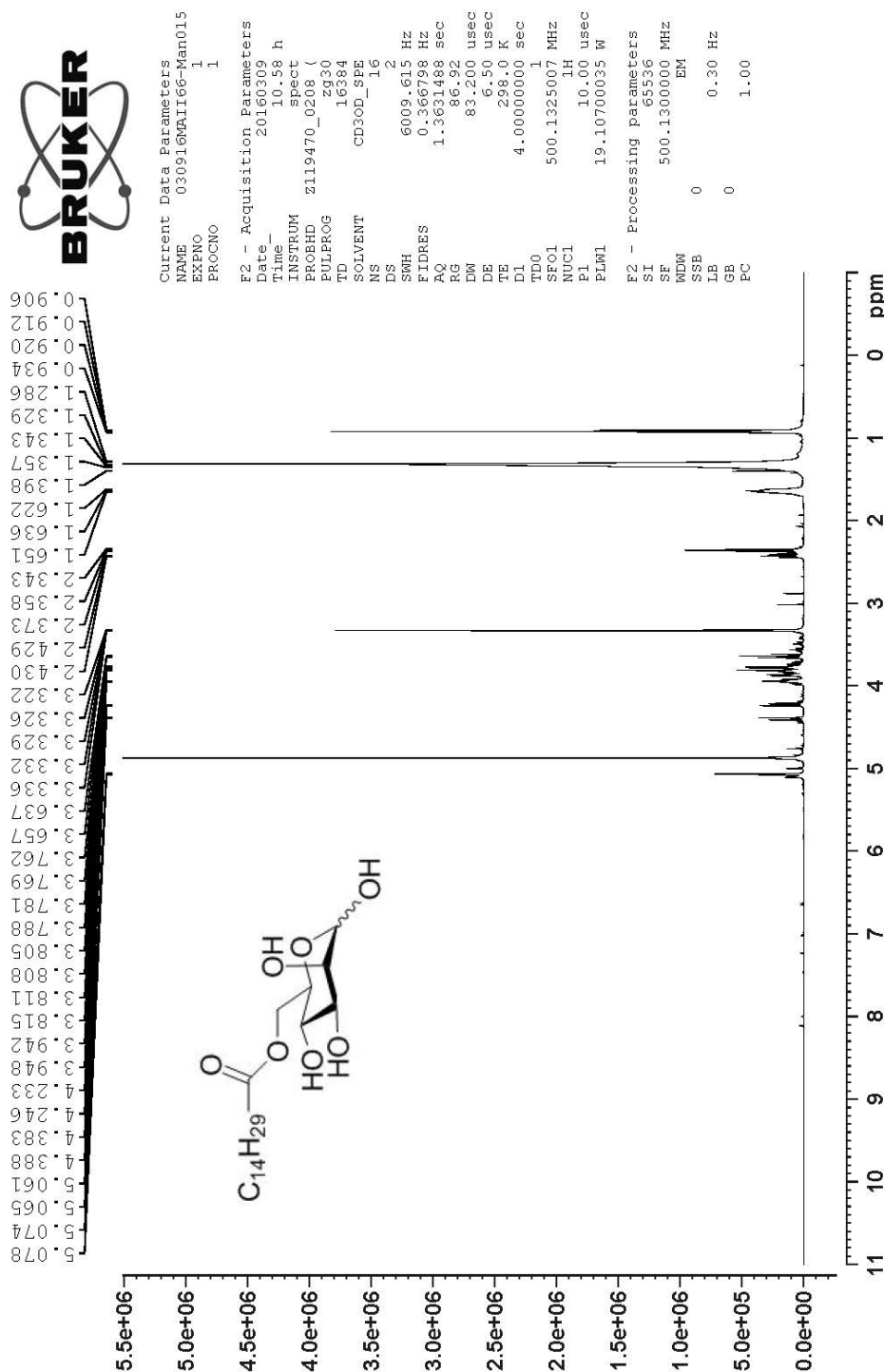


C-15

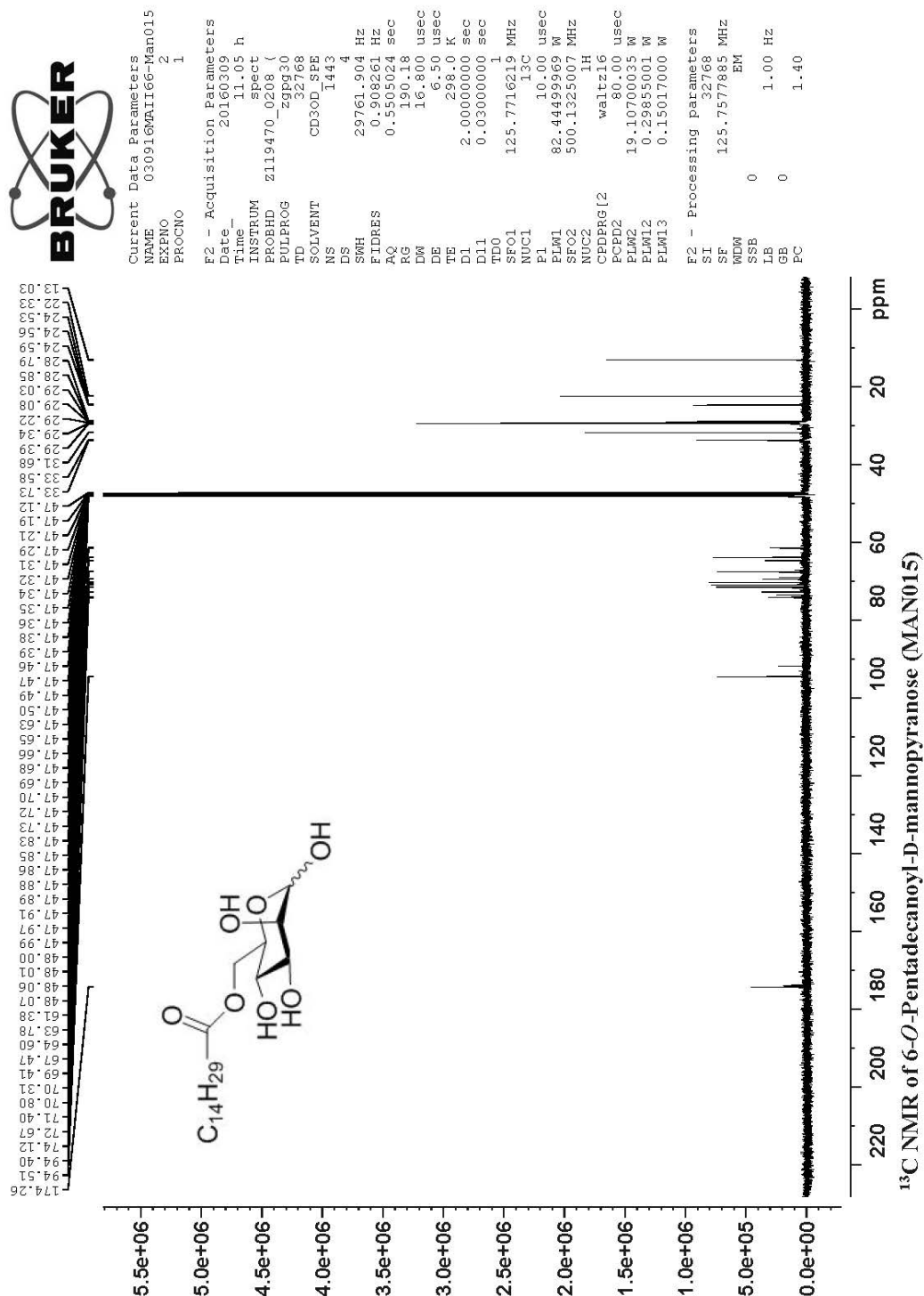


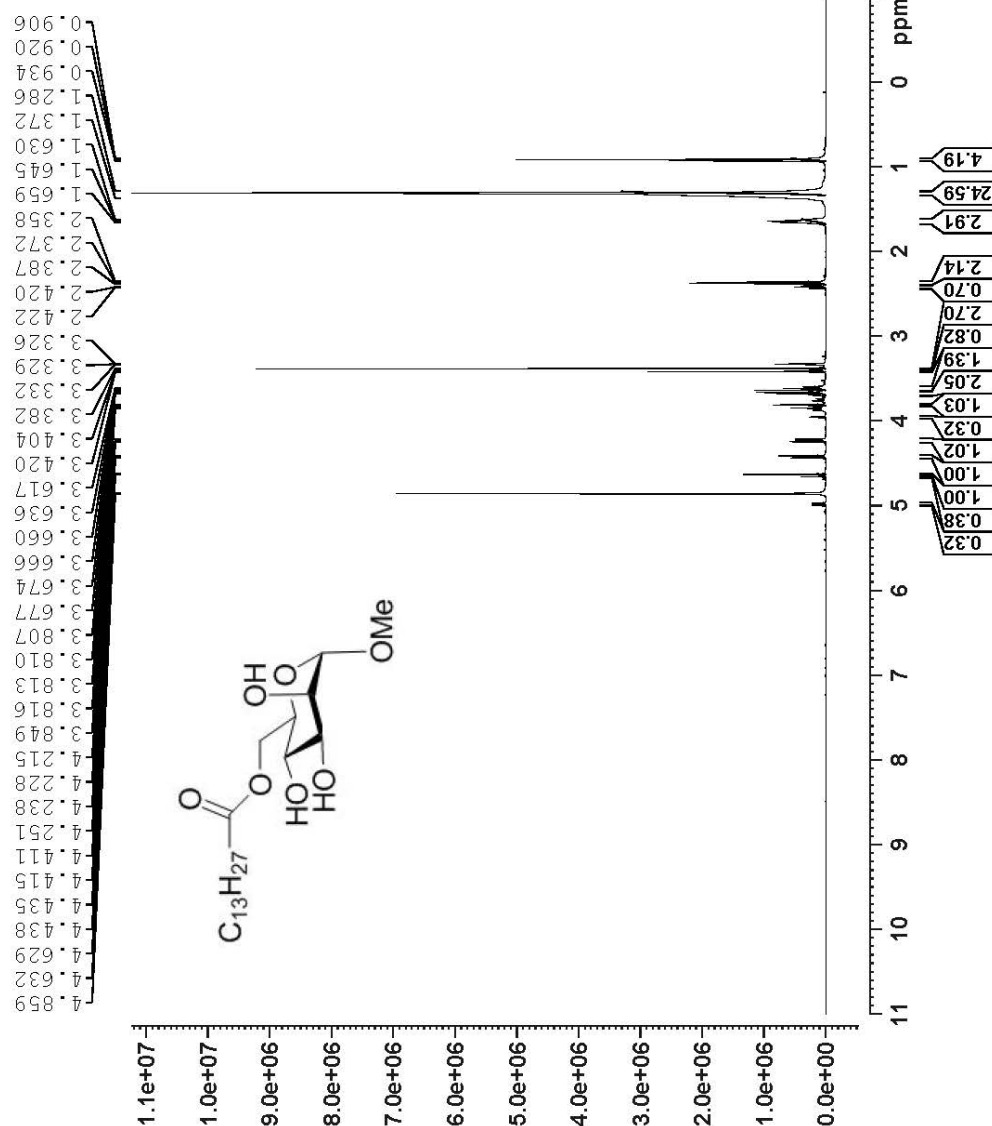
C-16





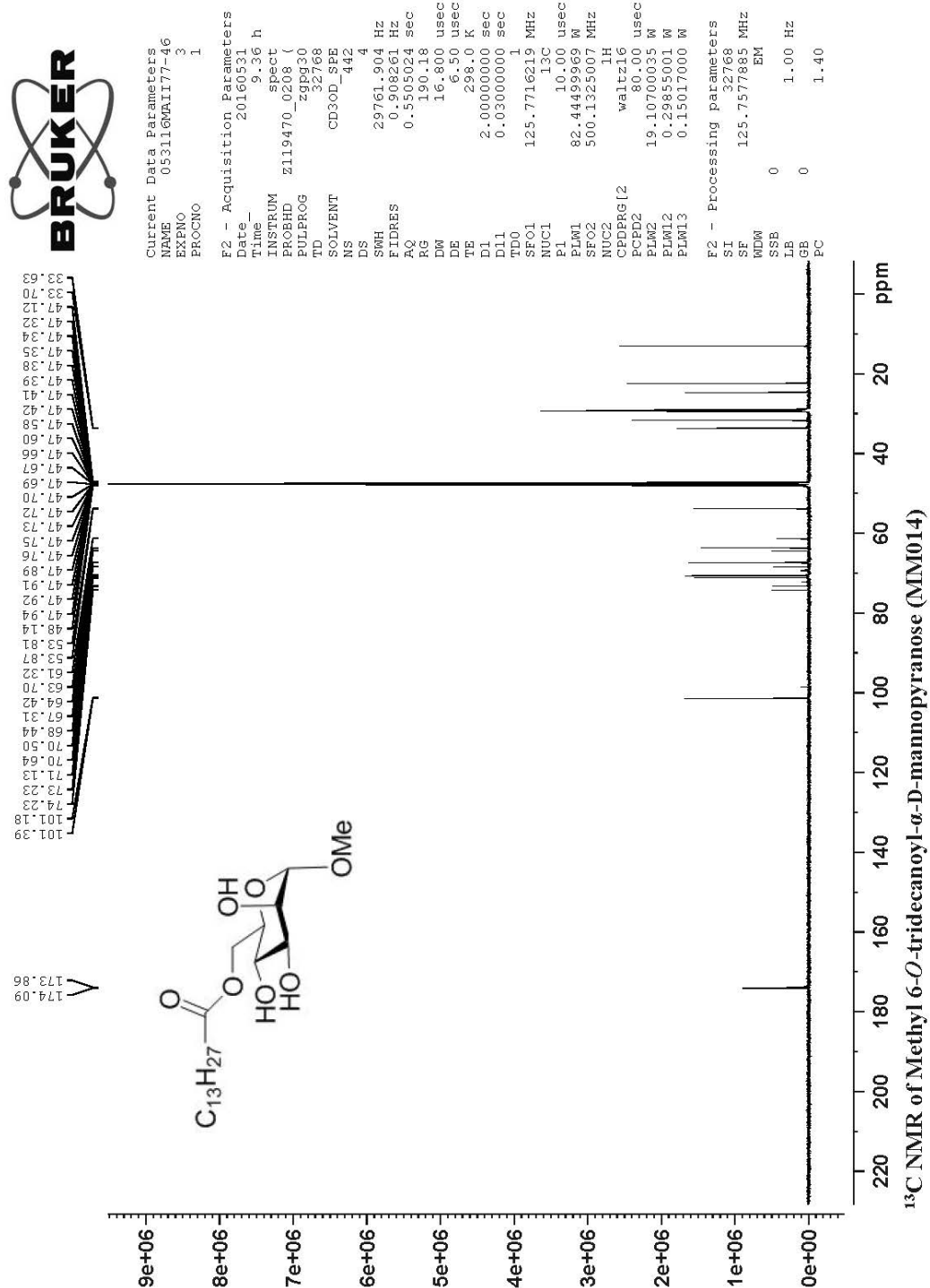
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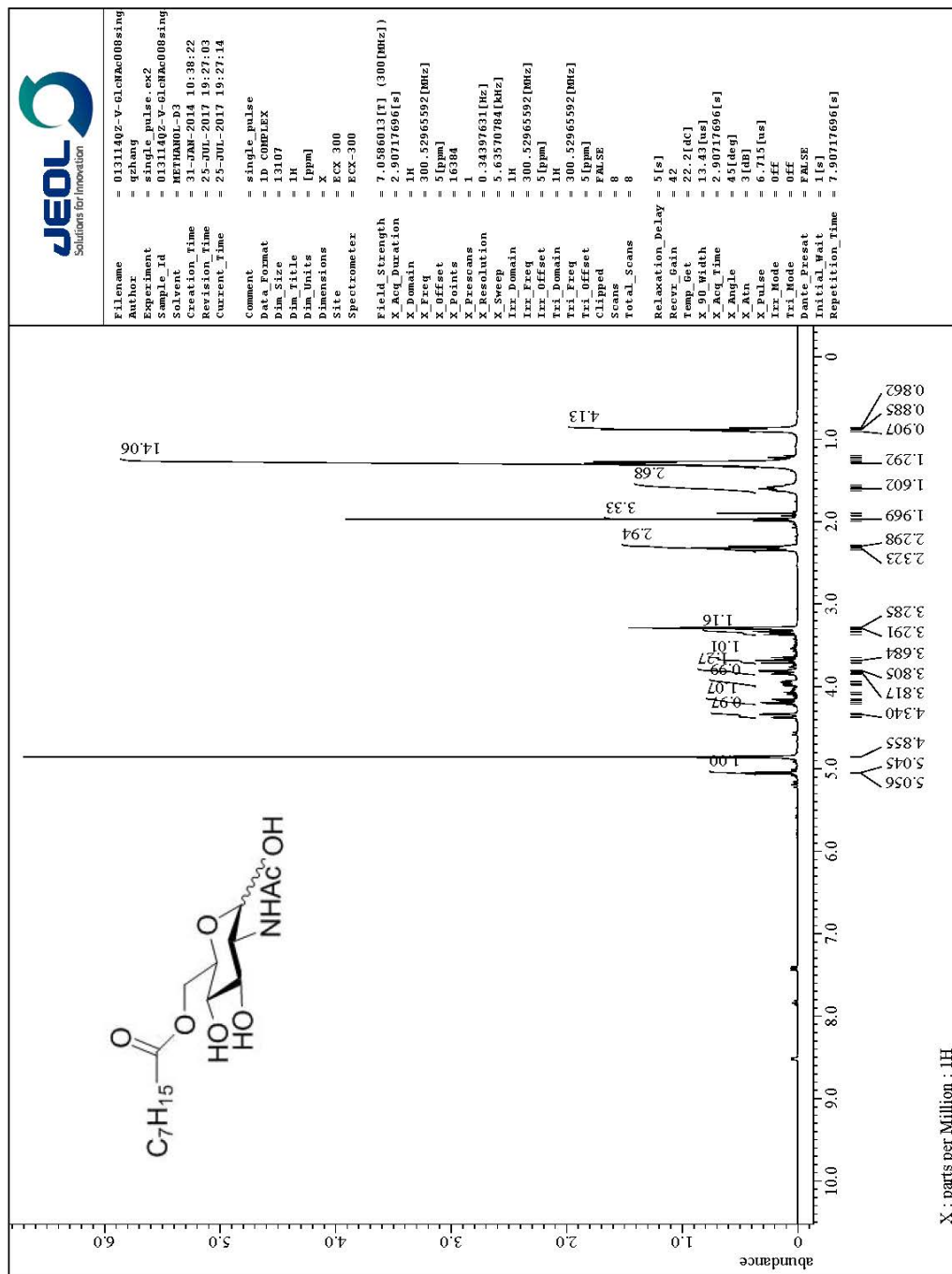


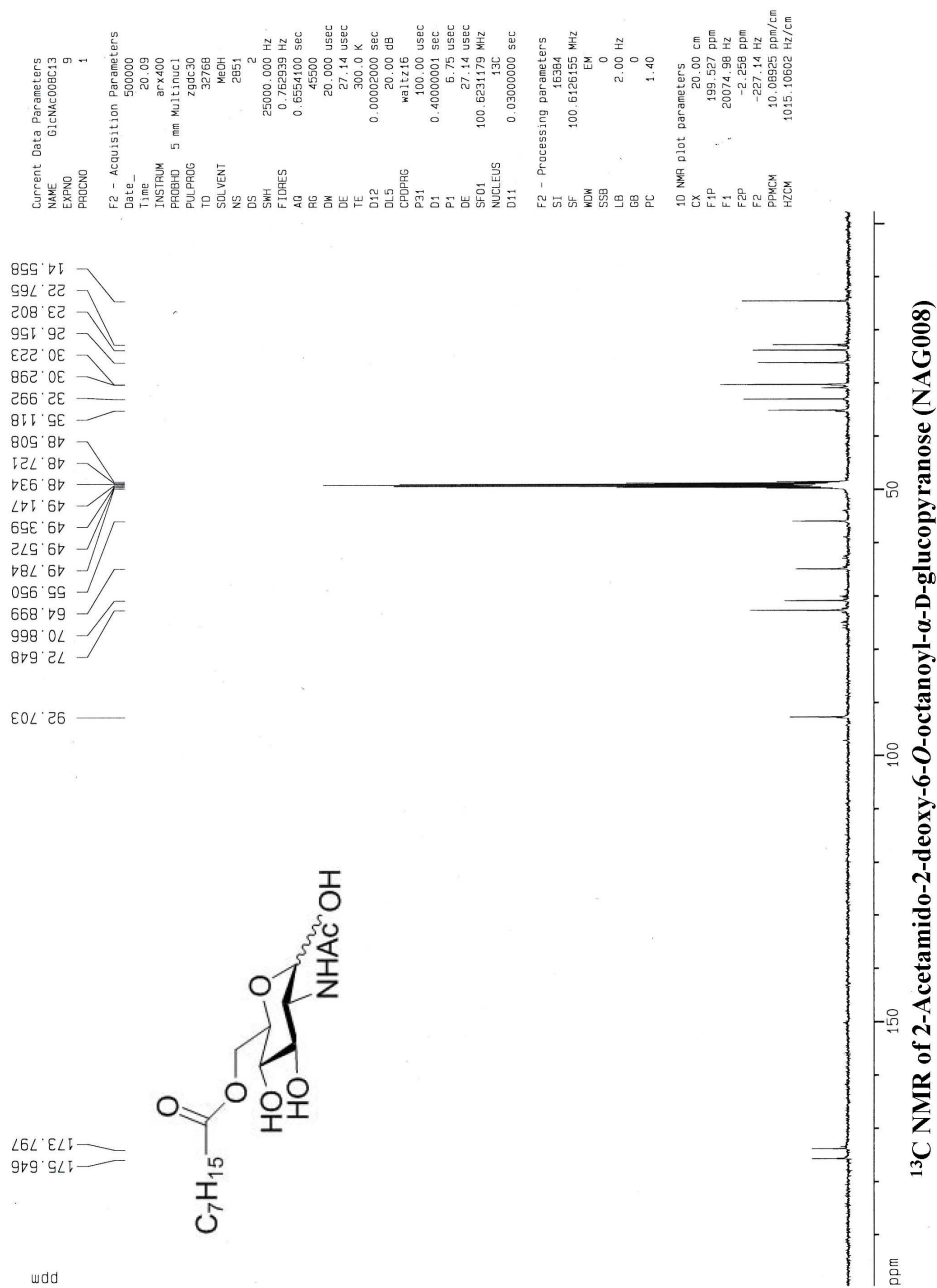


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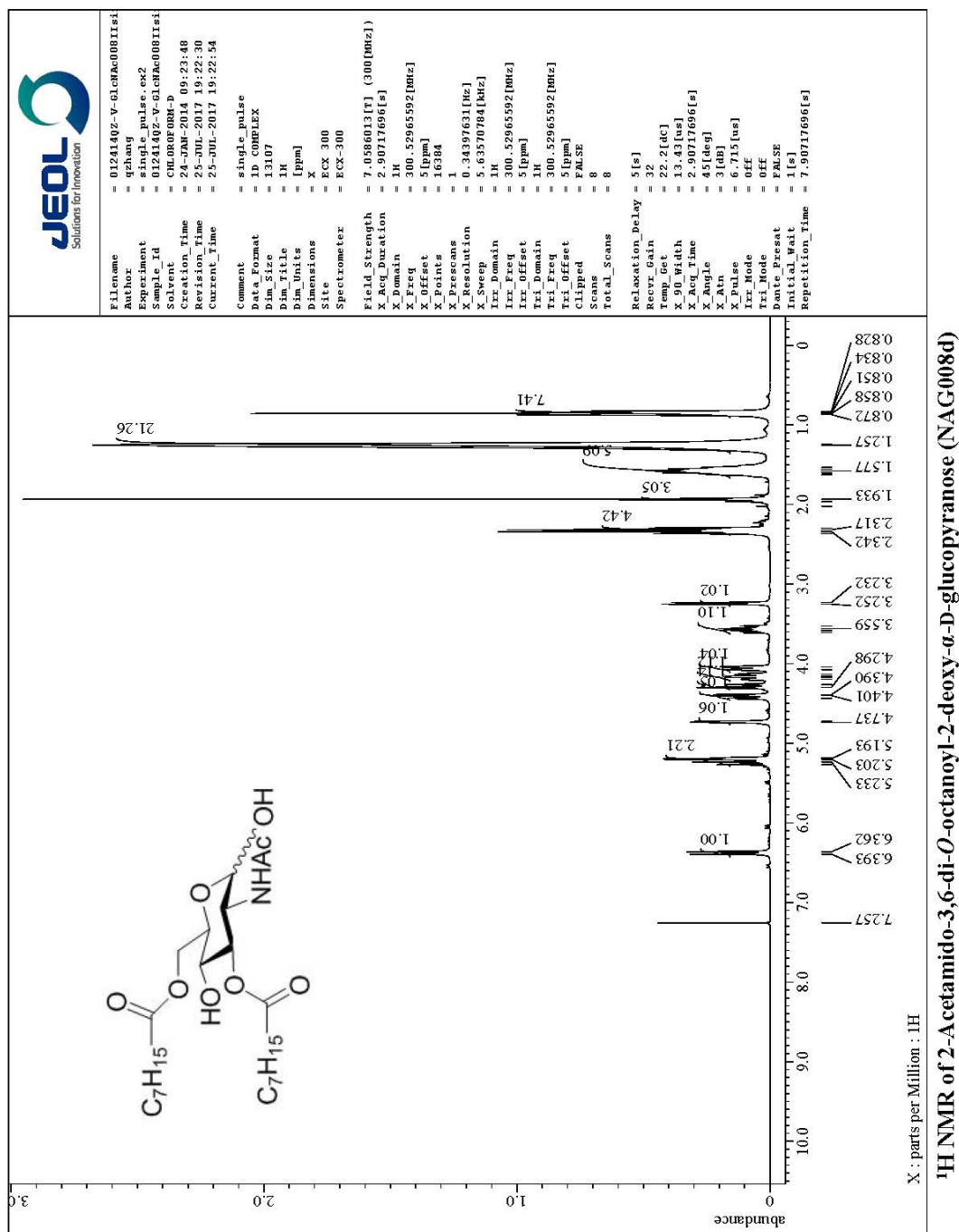
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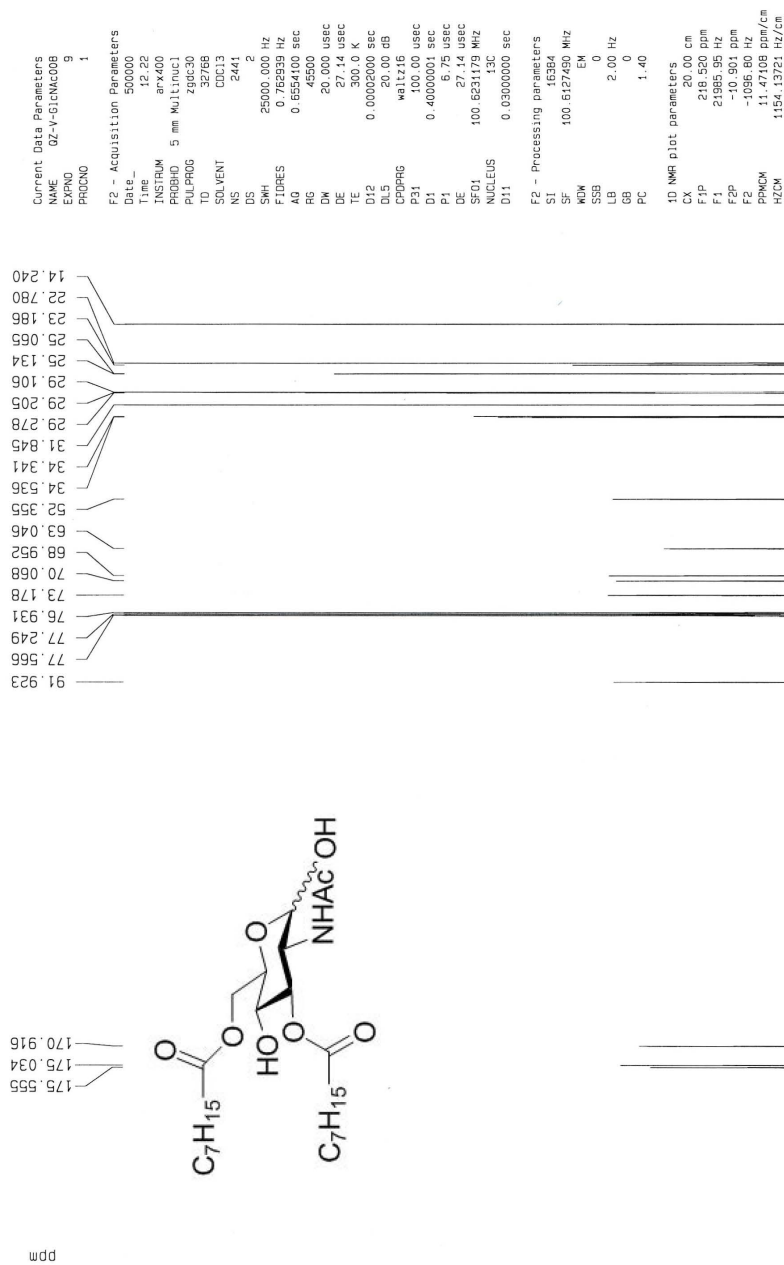




C-23



Standard
Experiment

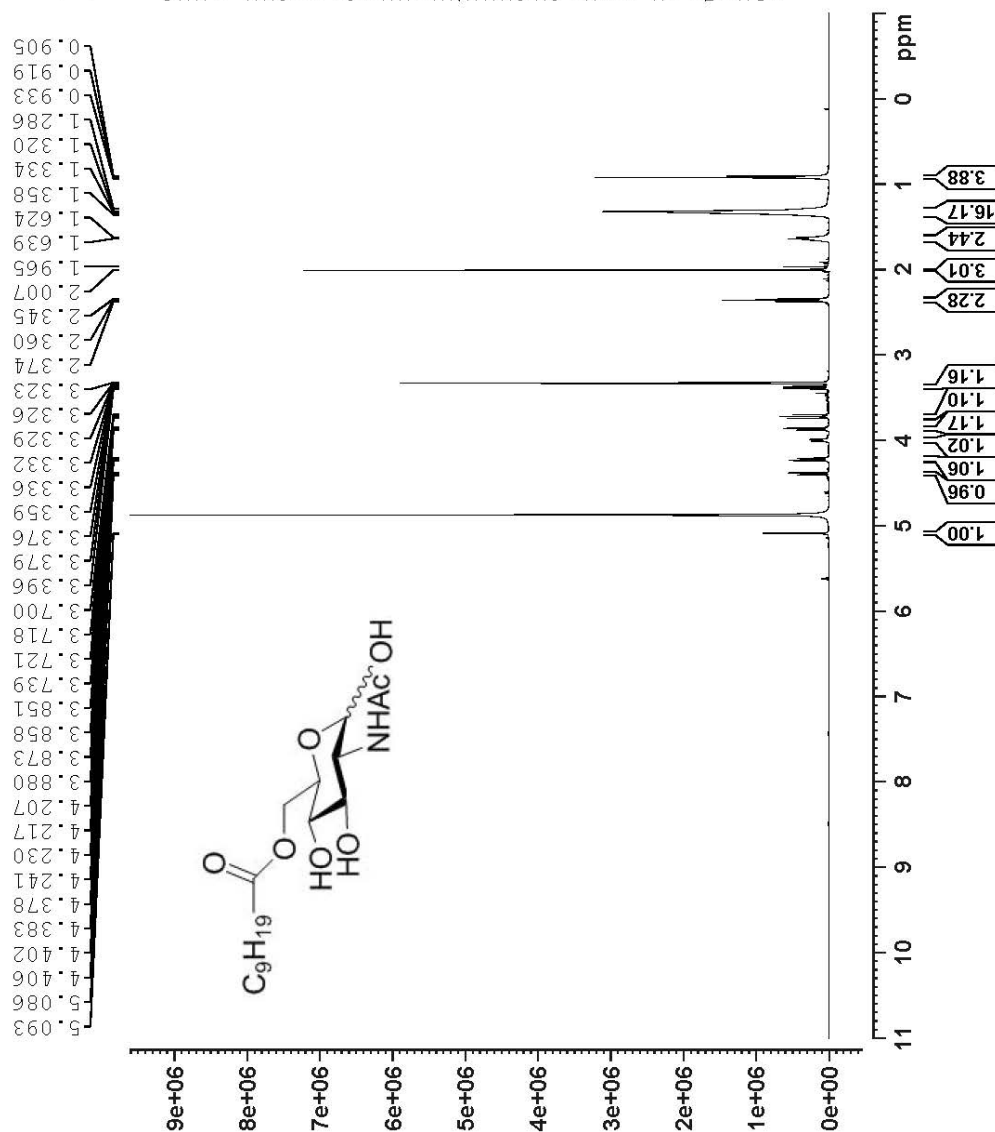




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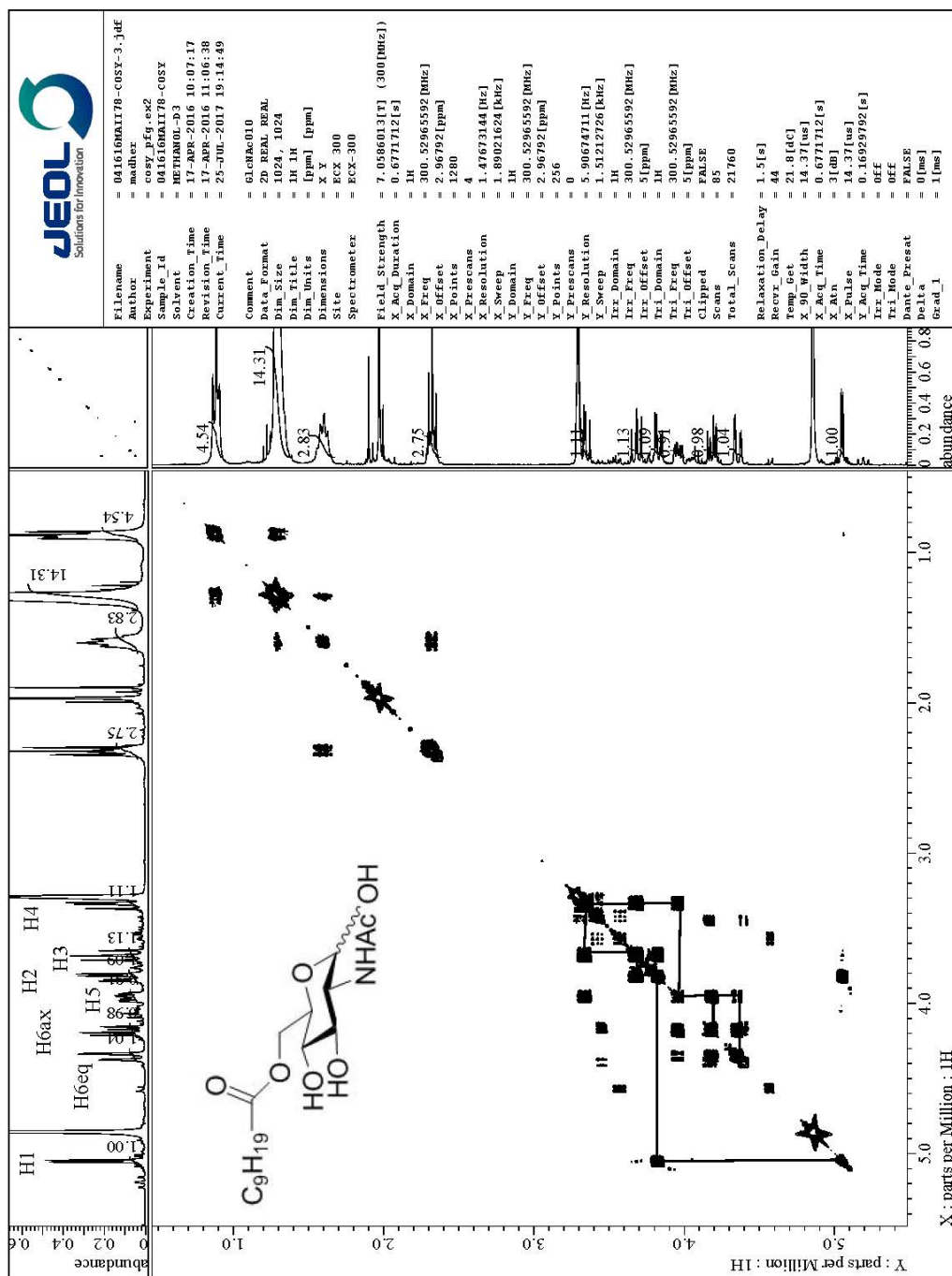
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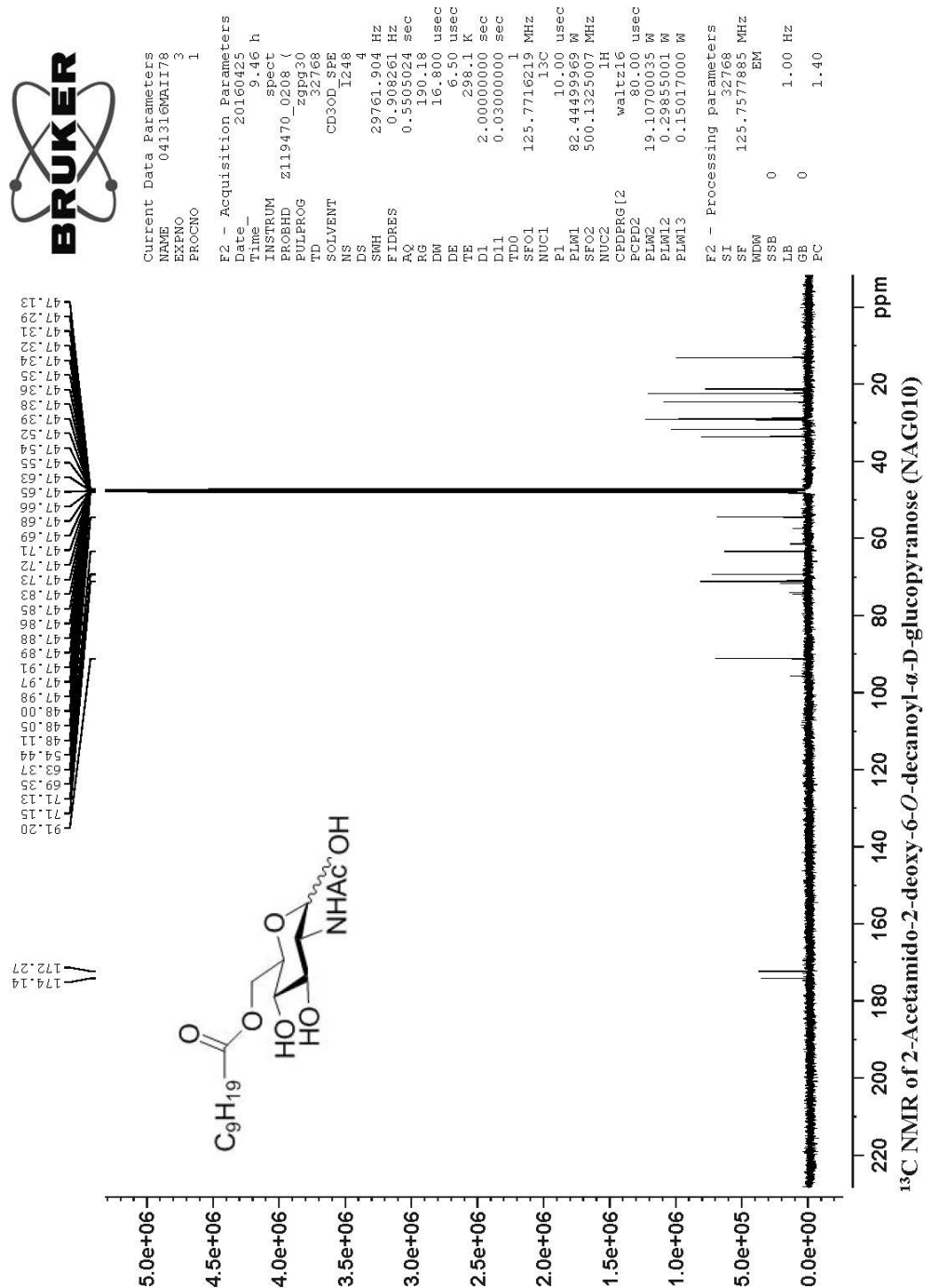


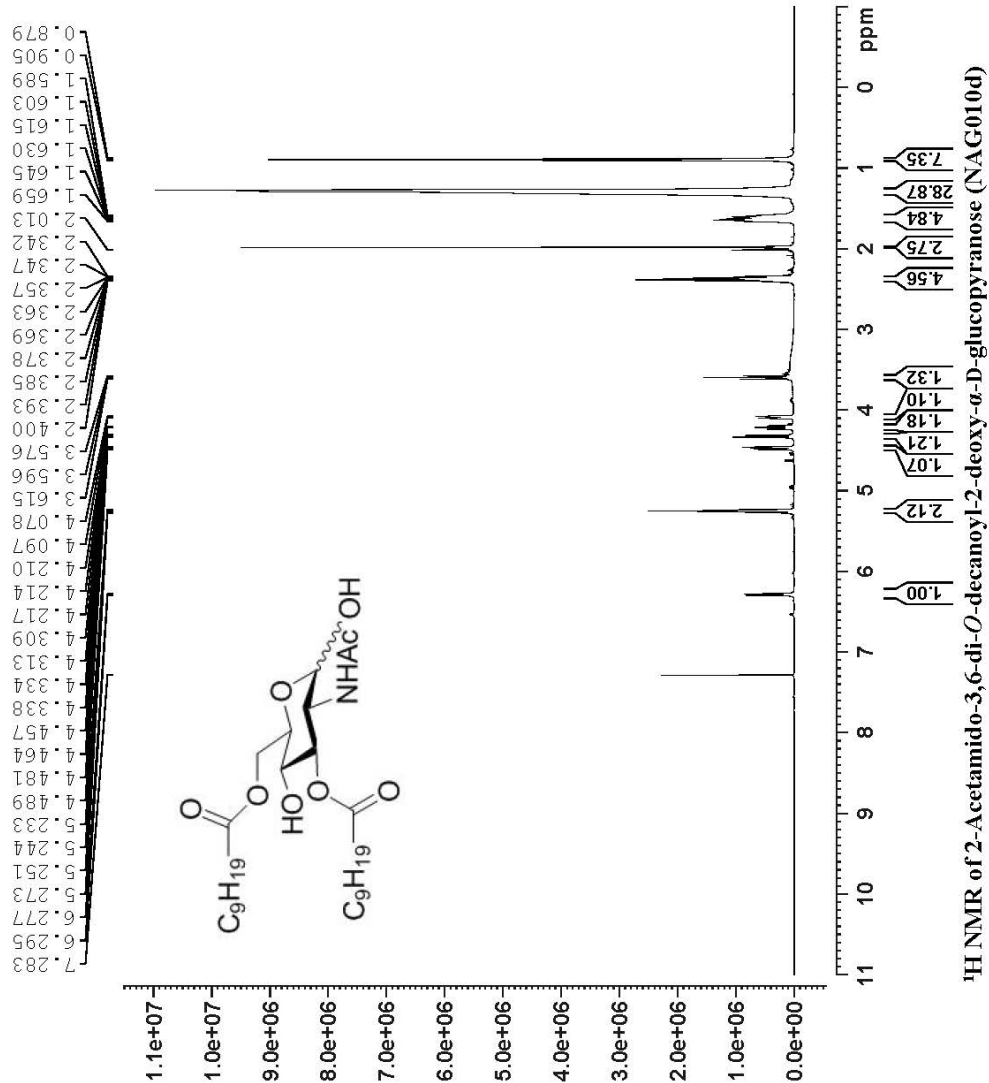
¹H NMR of 2-Acetamido-2-deoxy-6-O-decanoyl-α-D-glucopyranose (NAG-010)

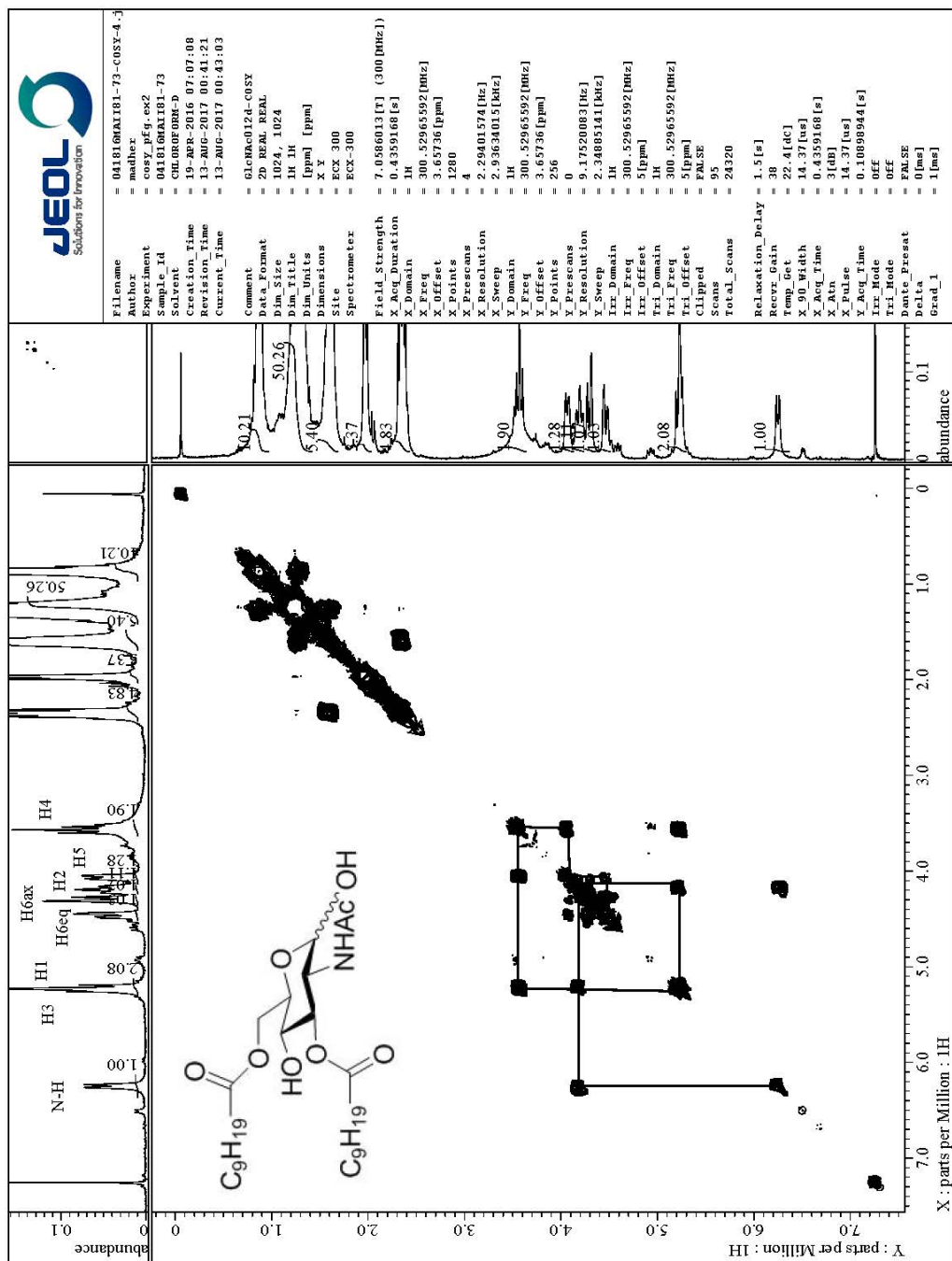
C-26

¹H-¹H COSY NMR of 2-Acetamido-2-deoxy-6-O-decanoyl-α-D-glucopyranose (NAG010)

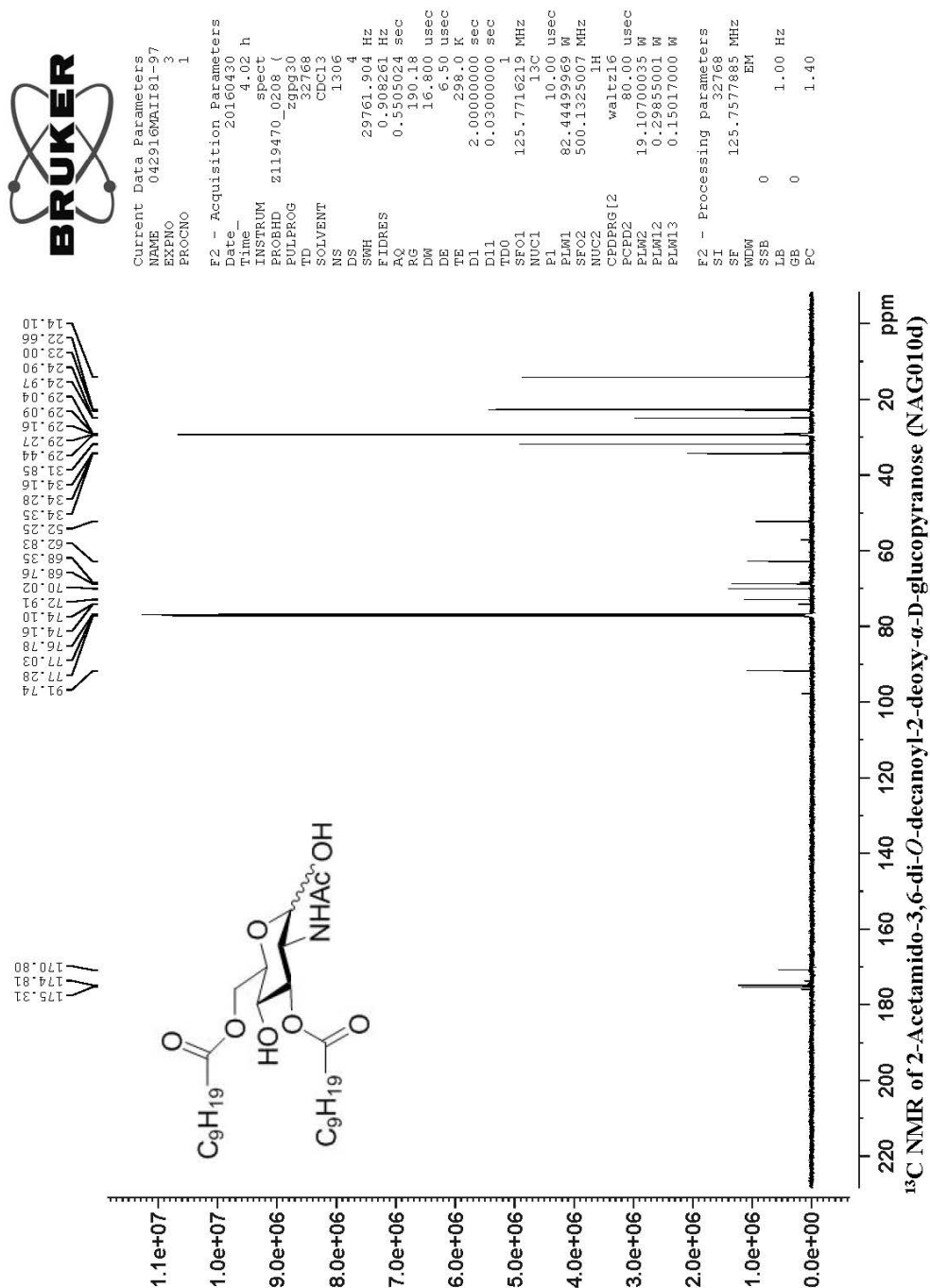
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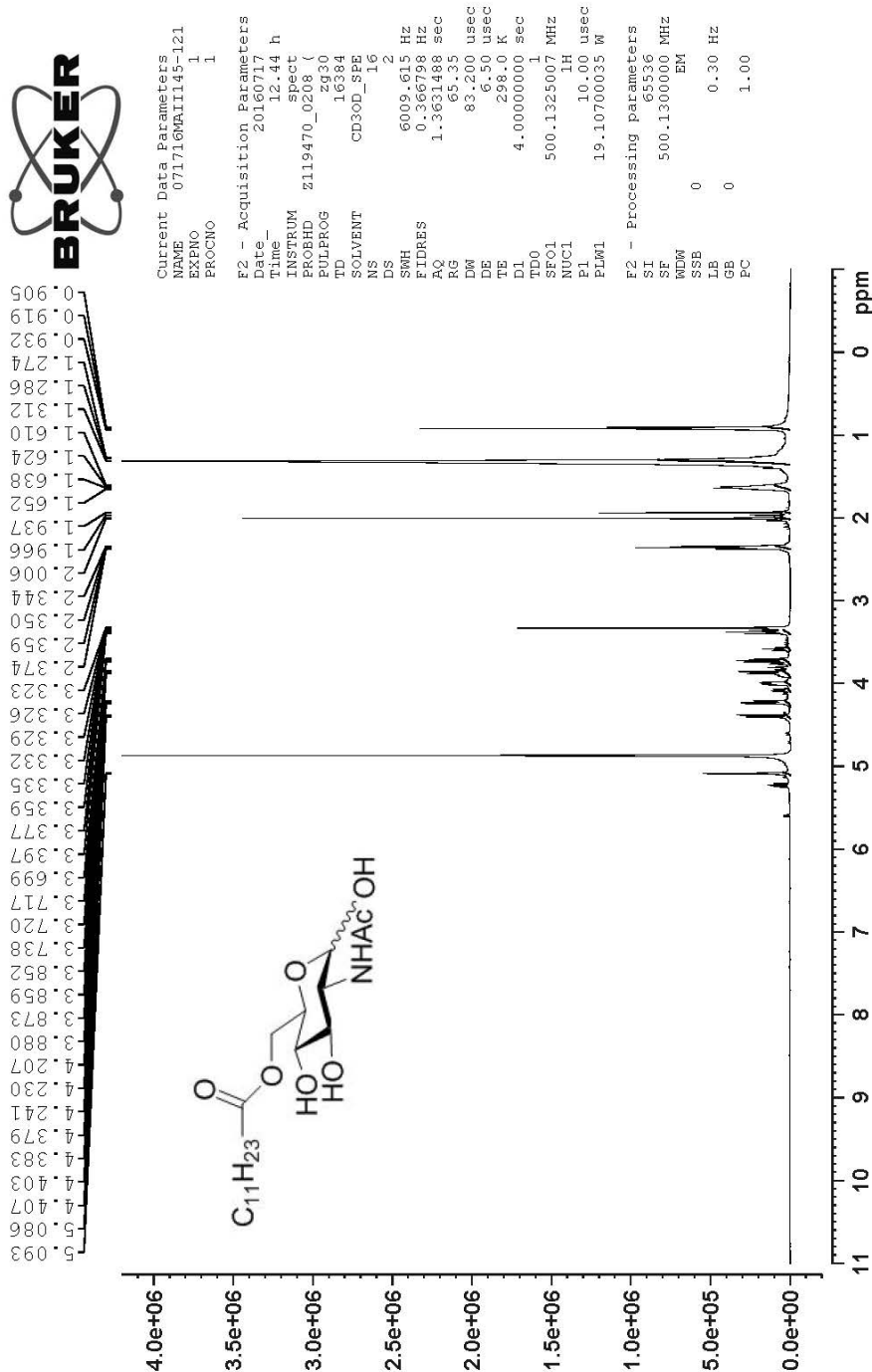




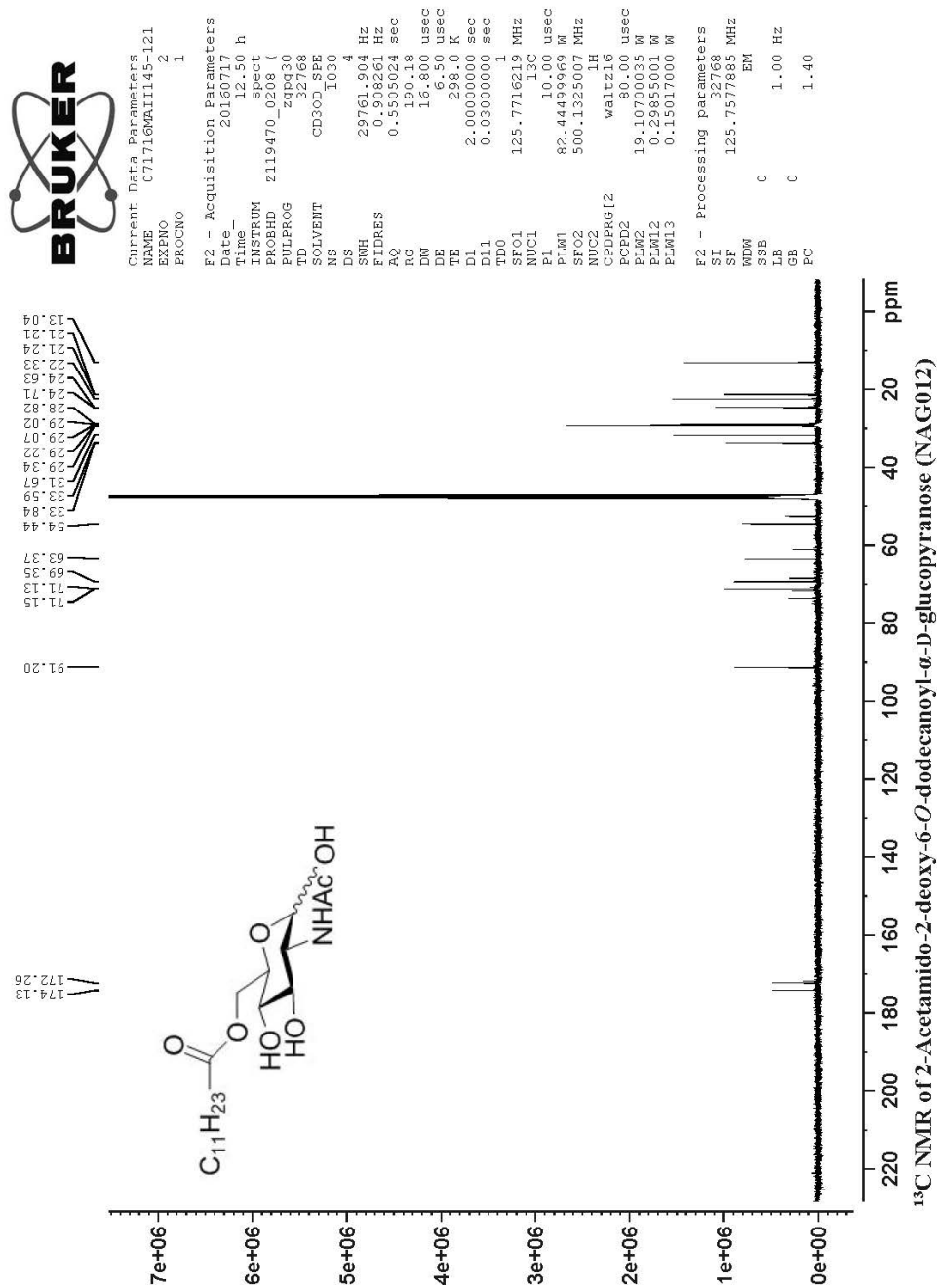


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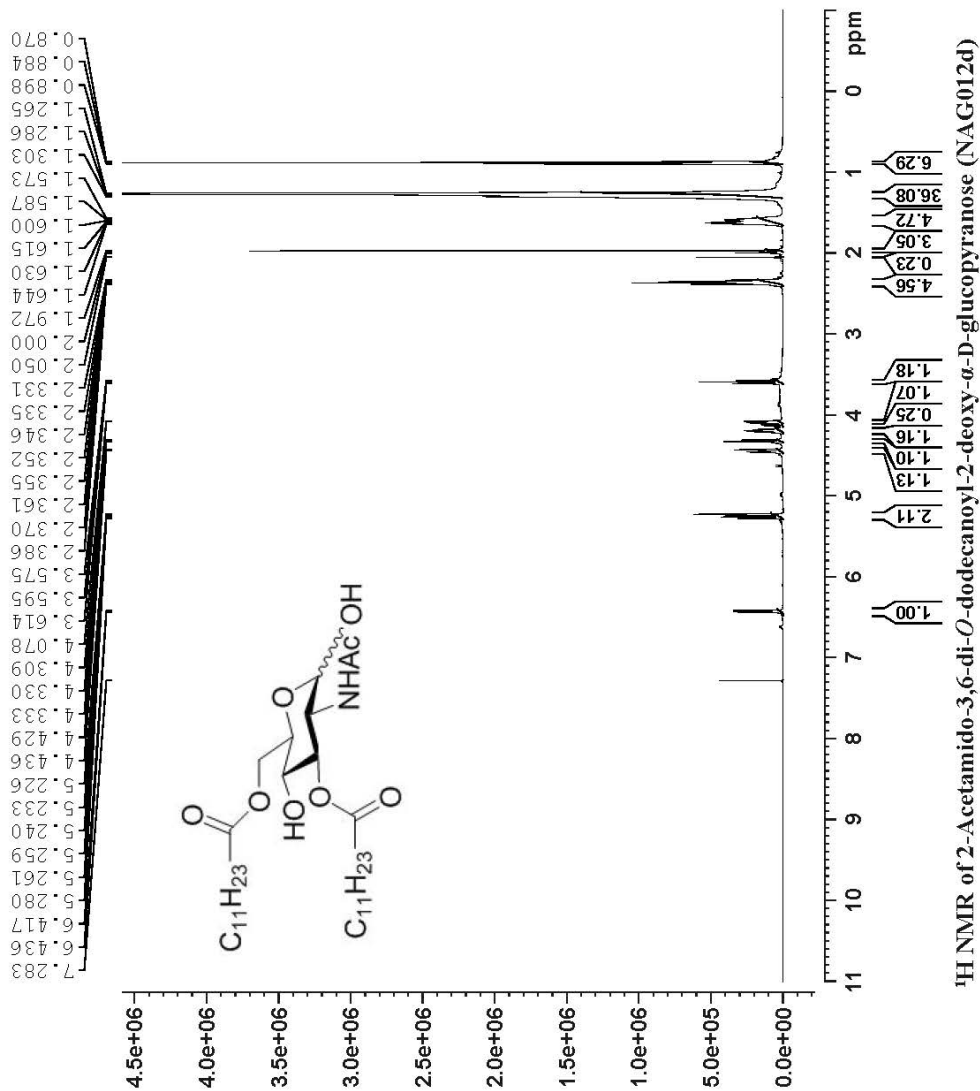


¹H NMR of 2-Acetamido-2-deoxy-6-O-dodecanoyl- α -D-glucopyranose (NAG012)





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 FIDRES 0.366798 Hz
 AQ 1.3631488 sec
 RG 16.56
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 DE 6.50 usec
 TE 298.0 K
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 SI 65536
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 SSB 0
 LB 0.30 Hz
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^1H NMR of 2-Acetamido-3,6-di-O-dodecanoyl-2-deoxy- α -D-glucopyranose (NAG012d)



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 EXPNO 2
 PROCNO 1

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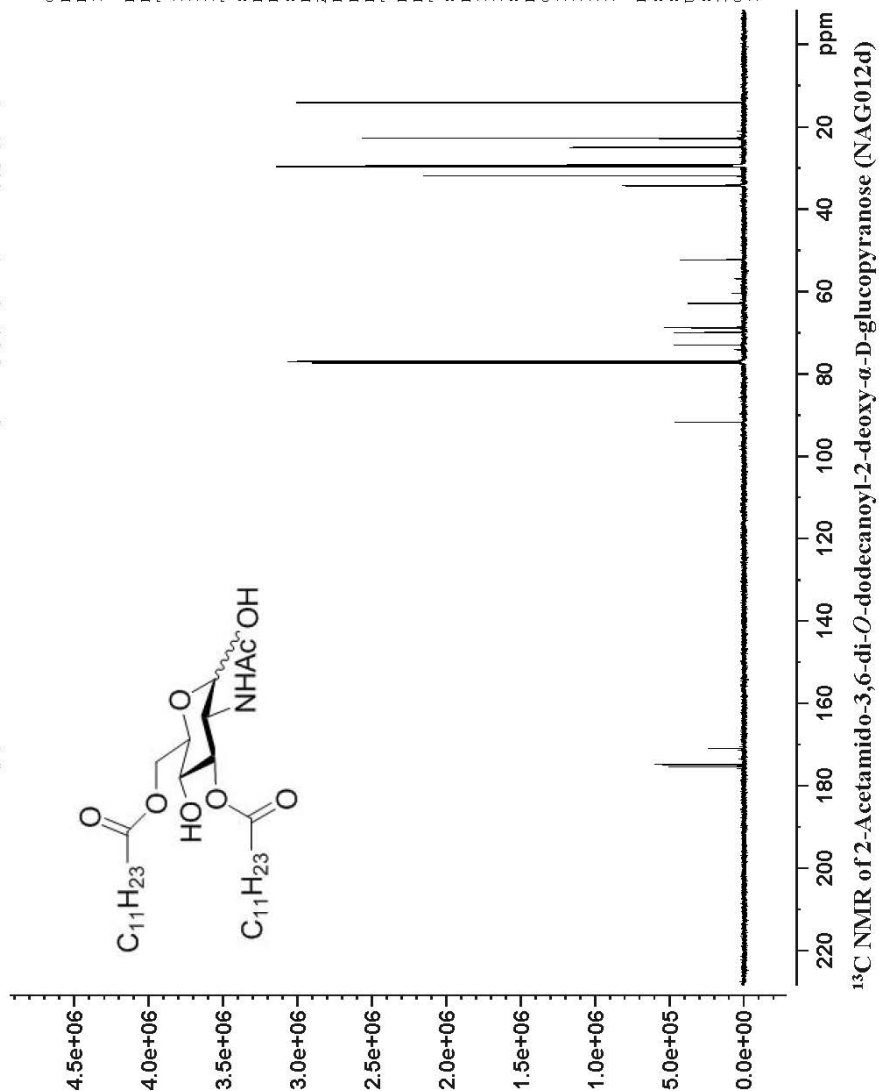
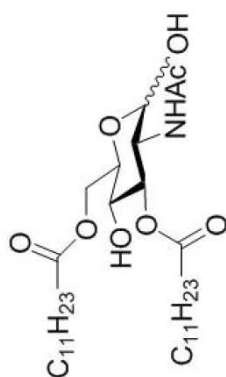
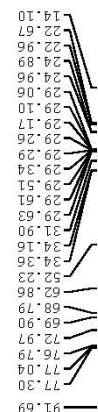
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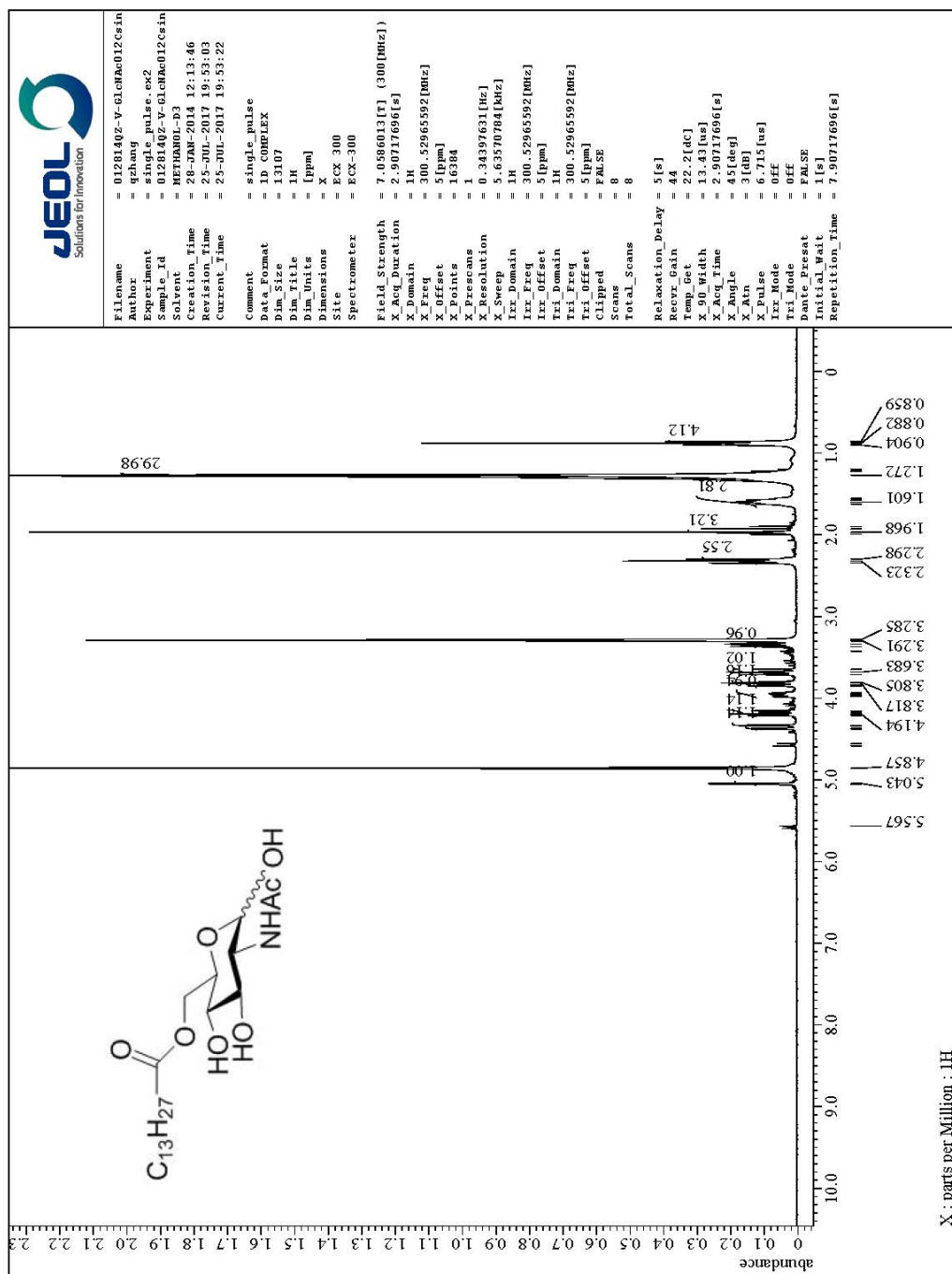
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S38

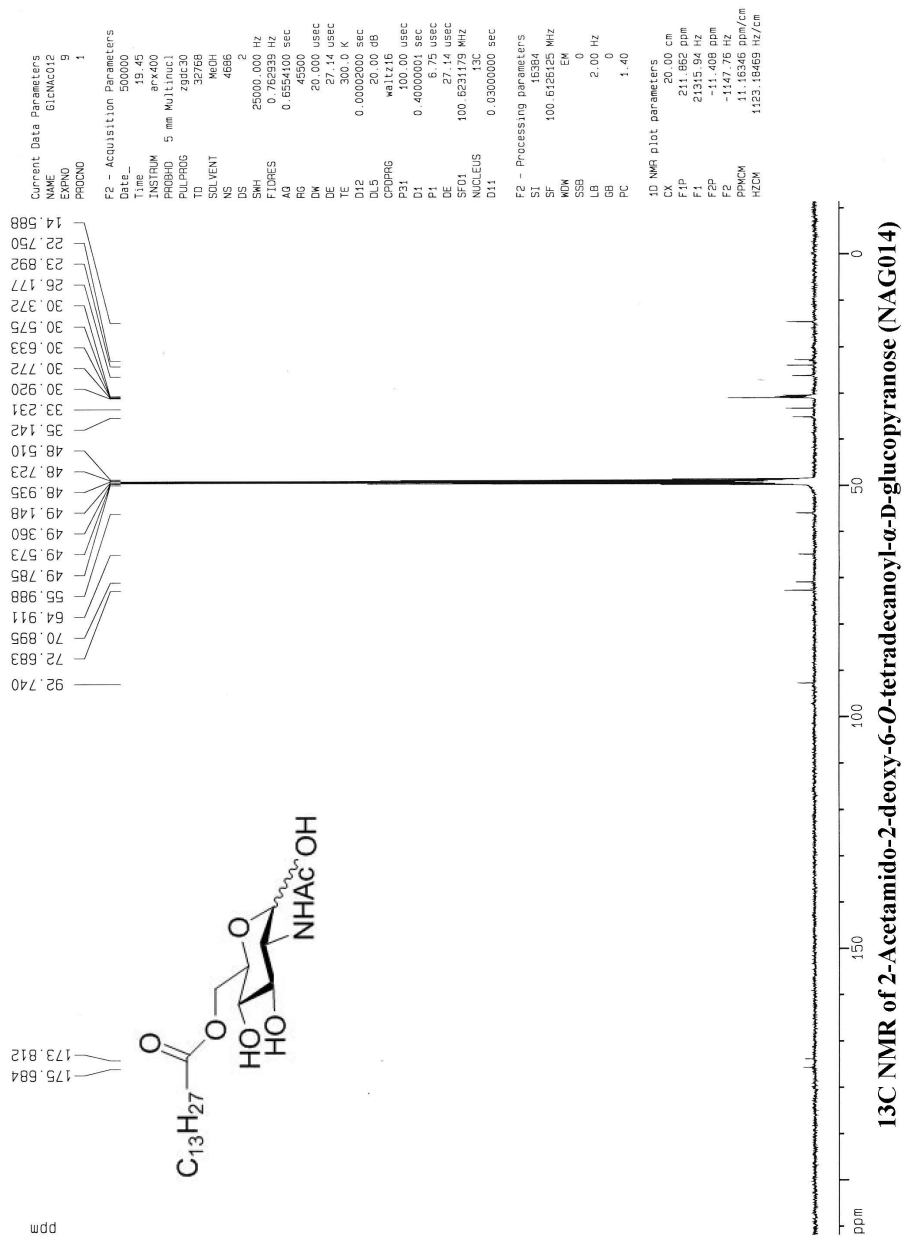
C-35



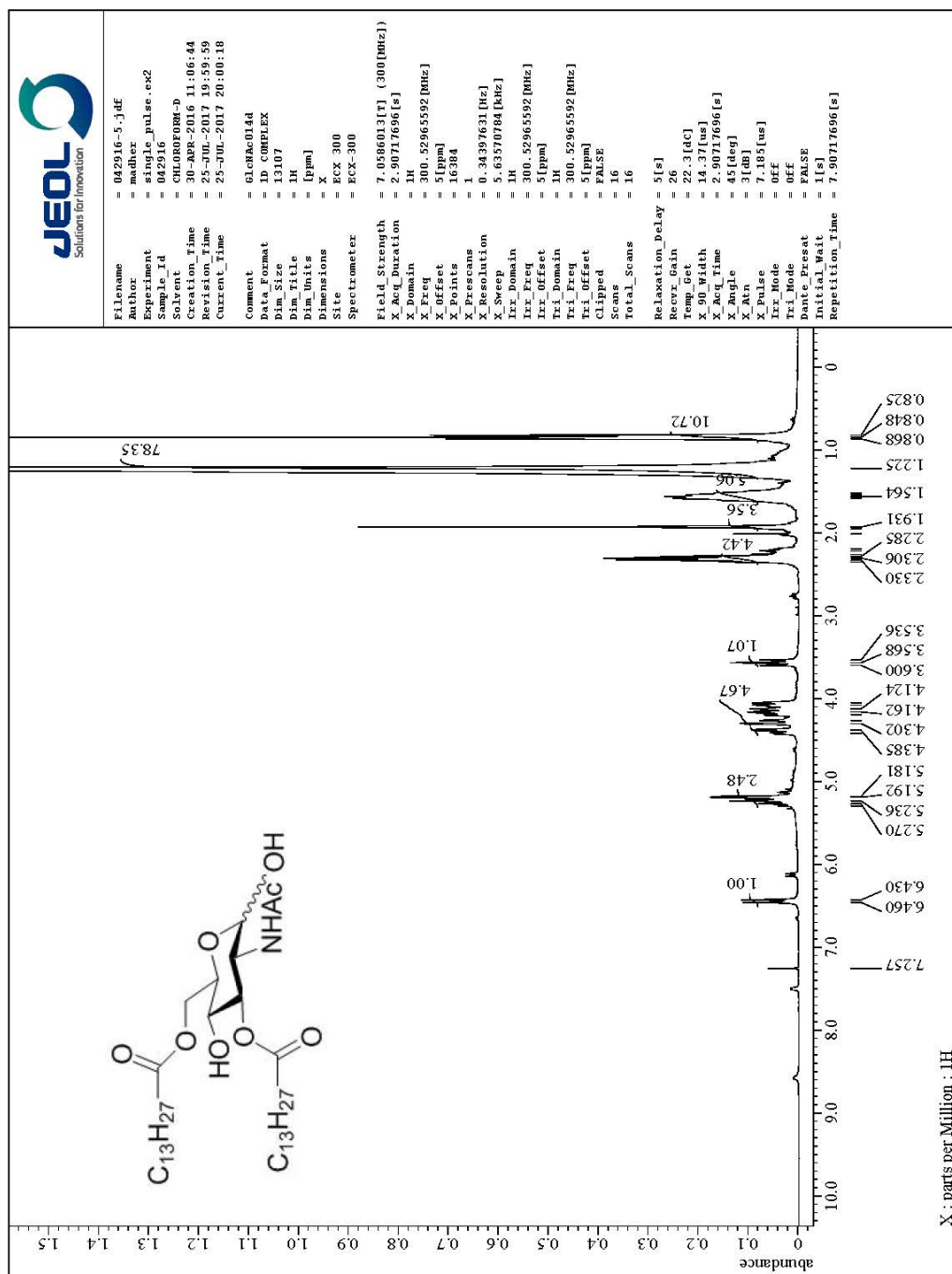
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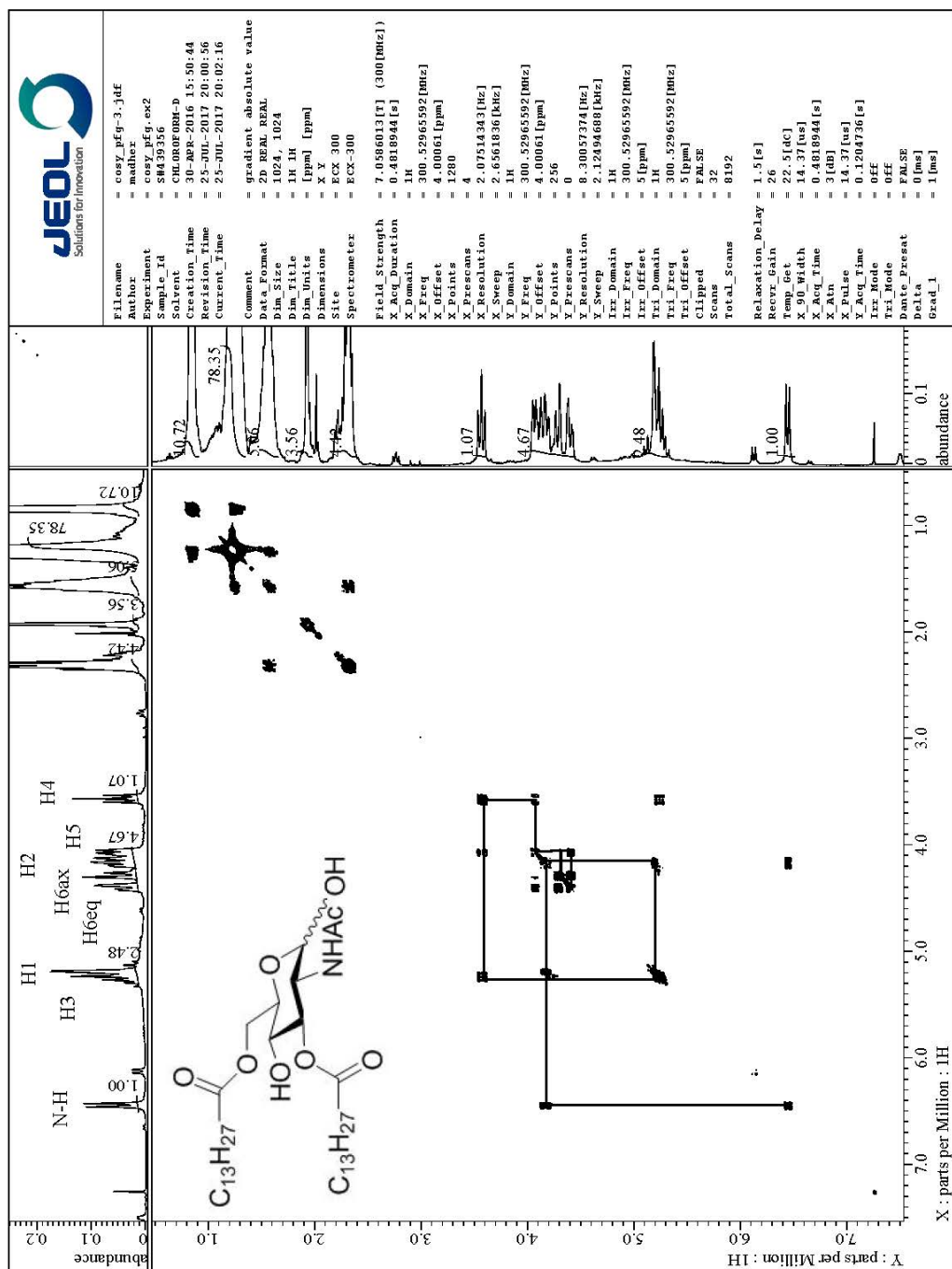
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Experiment



C-37

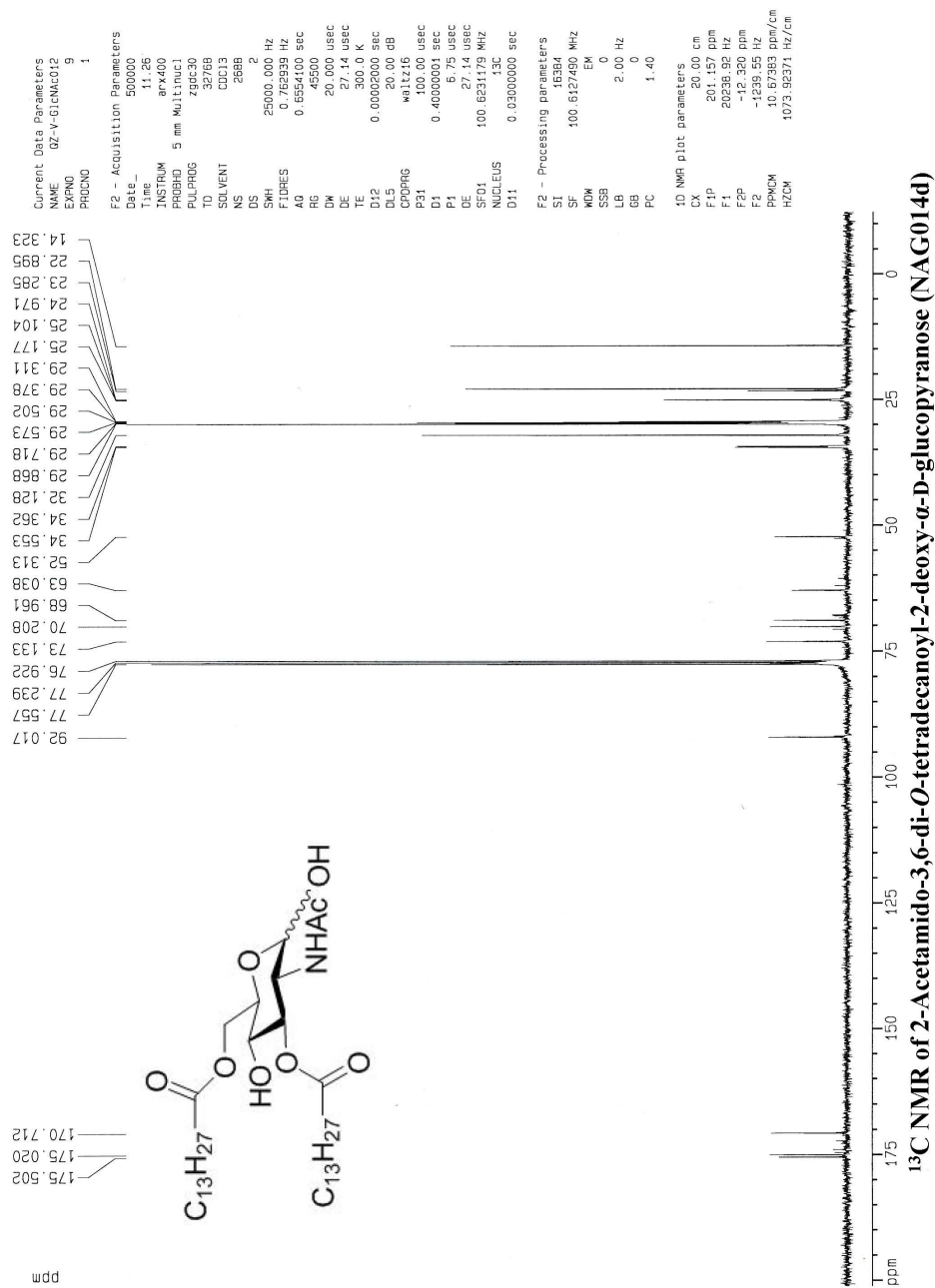


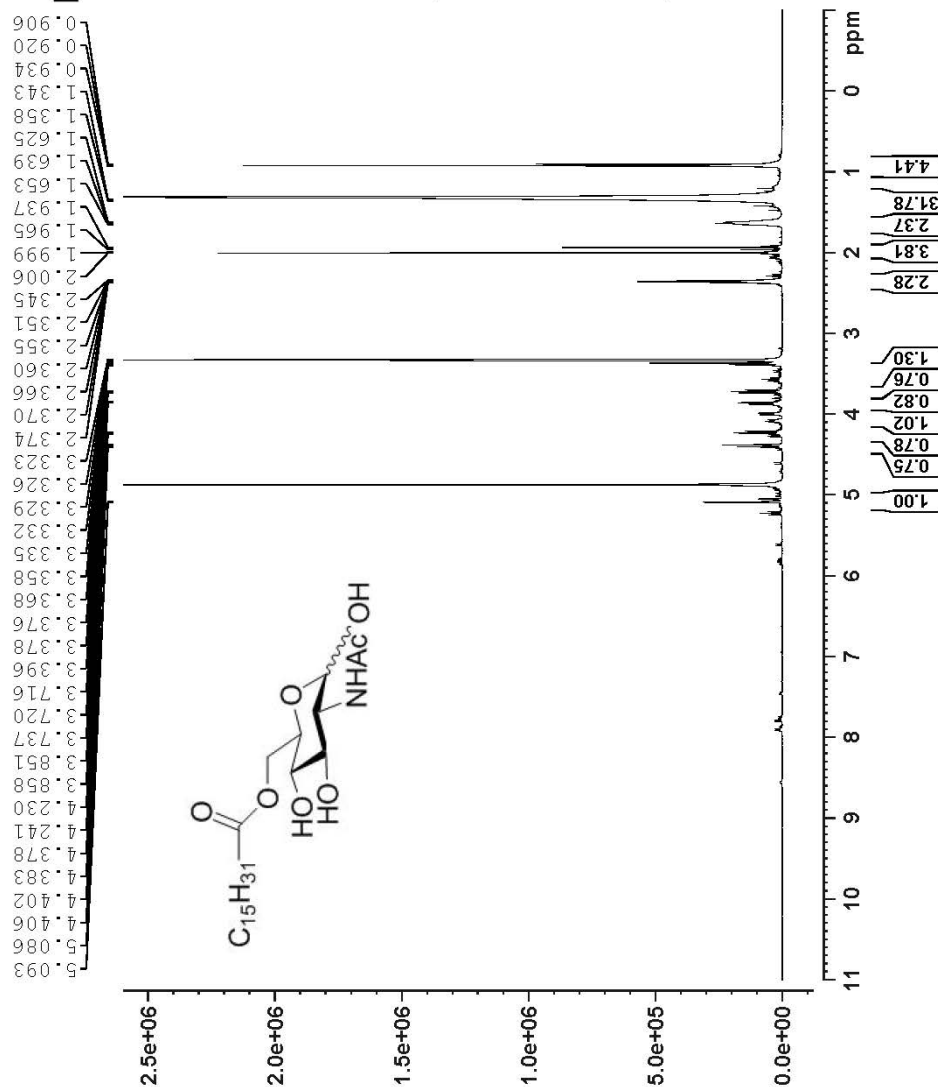
C-38



C-39

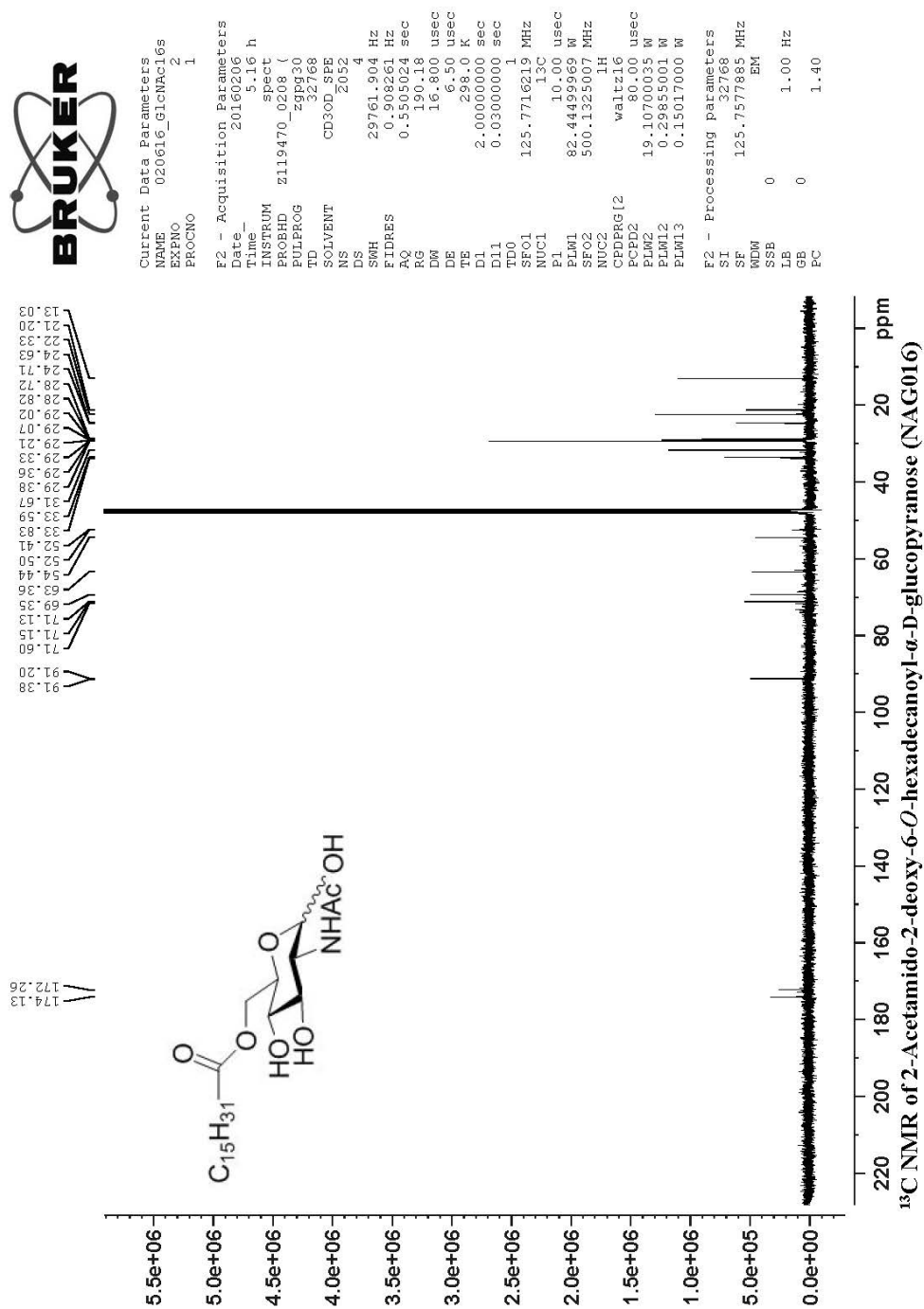
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Experiment



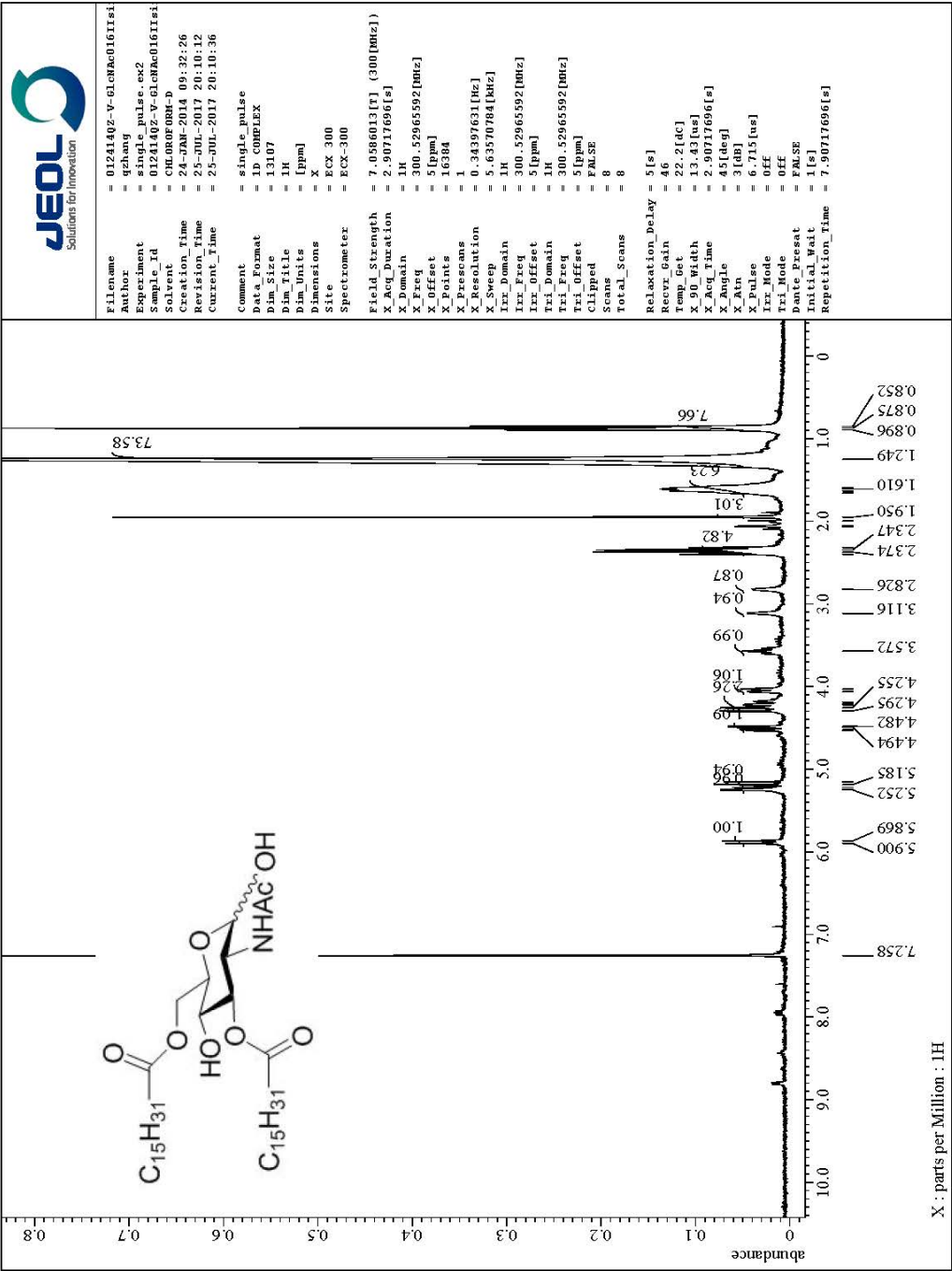


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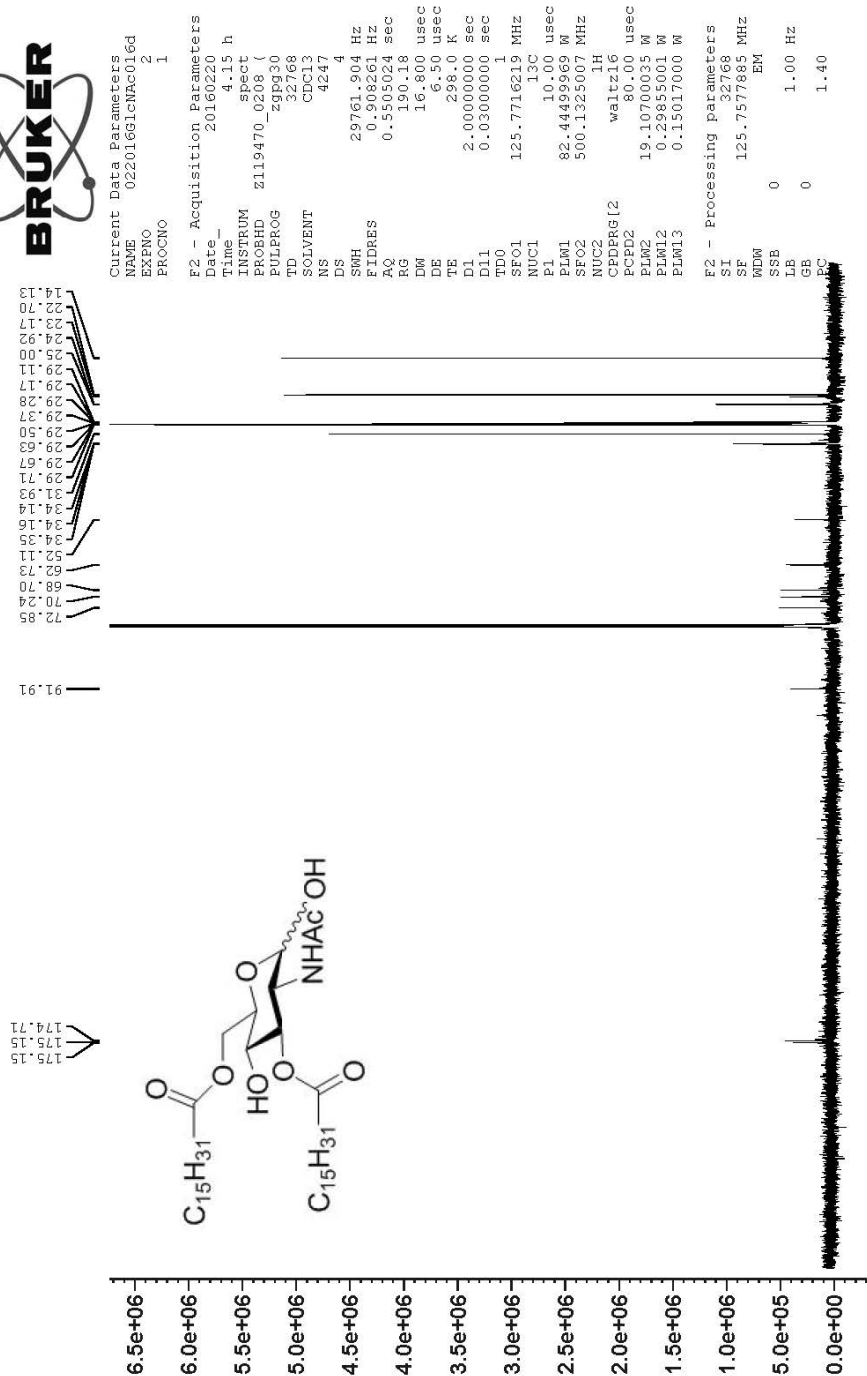
C-41



C-42



¹H NMR of 2-Acetamido-3,6-di-O-hexadecanoyl-2-deoxy- α -D-glucopyranose (NAG-016d)



APPENDIX D

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Title: Inhibition of Connexin Hemichannels by New Amphiphilic Aminoglycosides without Antibiotic Activity

Author: Madher N. AlFindee, Yagya P. Subedi, Mariana C. Fiori, et al

Publication: ACS Medicinal Chemistry Letters

Publisher: American Chemical Society

Date: Jul 1, 2018

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Title: One-step synthesis of carbohydrate esters as antibacterial and antifungal agents

Author: Madher N. AlFindee, Qian Zhang, Yagya Prasad Subedi, Jaya P. Shrestha, Yukie Kawasaki, Michelle Grilley, Jon Y. Takemoto, Cheng-Wei Tom Chang

Publication: Bioorganic & Medicinal Chemistry

Publisher: Elsevier

Date: 1 February 2018

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- 2- AlFindee, M. N.; Subedi, Y. P.; Grilley, M. M.; Takemoto, J. Y.; Chang, C.-W. T. "Antifungal Activities of 4",6"-Disubstituted Amphiphilic Kanamycins" *Molecules* **2019**, 24(10), 1882.

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- 2- AlFindee, M. N.; Subedi, Y. P.; Fiori, M. C.; Krishnan, S.; Kjellgren, A.; Altenberg, G. A.; Chang, C.-W. T. "Inhibition of Connexin Hemichannels by New Amphiphilic Aminoglycosides without Antibiotic Activity" *ACS Med. Chem. Lett.* **2018**, 9, 697-701.
- 3- AlFindee, M. N.; Subedi, Y. P.; Grilley, M. M.; Takemoto, J. Y.; Chang, C.-W. T. "Antifungal Activities of 4",6"-Disubstituted Amphiphilic Kanamycins" *Molecules* **2019**, 24(10), 1882.

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Date: _____

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- 2- AlFindee, M. N.; Subedi, Y. P.; Grilley, M. M.; Takemoto, J. Y.; Chang, C.-W. T. "Antifungal Activities of 4",6"-Disubstituted Amphiphilic Kanamycins" *Molecules* **2019**, *24*(10), 1882.

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Please, indicate your approval of this request by signing in the space provided, attaching any other form or instruction necessary to confirm permission.

Thank you for your cooperation,

Madher N. Alfindee

I, Dr. Michelle M. Grilley hereby give permission to Madher N. Alfindee to reprint the above listed in his dissertation.

Signed: _____

Date: 5/20/2019

Permission Letter

May 11, 2019

Madher N. Alfindee
Dept. of Chemistry and Biochemistry
Utah State University
0300 Old Main Hill
Logan, UT 84322-0300

Dear Dr. Srinivasan Krishnan:

I am in the process of preparing my dissertation in the Chemistry and Biochemistry department at Utah State University. I hope to complete my degree program in Summer of 2019.

I am requesting your permission to include the following manuscripts, as it is form, in my doctoral dissertation:

AlFindee, M. N.; Subedi, Y. P.; Fiori, M. C.; Krishnan, S.; Kjellgren, A.; Altenberg, G. A.; Chang, C.-W. T.
"Inhibition of Connexin Hemichannels by New Amphiphilic Aminoglycosides without Antibiotic Activity" *ACS Med. Chem. Lett.* **2018**, 9, 697-701.

A copy of this permission letter will be included in my dissertation appendix.

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Thank you for your cooperation,

Madher N. Alfindee

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Signed: _____ Date: 5/19/2019

CURRICULUM VITAE

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A. Education and Training**Education**

B.Sc. Basra University, Basra, IRAQ, 1997

M.Sc. Basra University, Basra, IRAQ, 2000

Ph.D. Student, Utah State University, Logan, USA, 2013-present.

B. Positions and Honors**Positions and Employment**

2002-2011 Assistant Lecturer, College of Pharmacy, University of Basra, Basra, IRAQ.

2011-present Lecturer, College of Pharmacy, University of Basra, Basra, IRAQ.

Fall 2015, CHEM 2315, Laboratory instructor, Organic chemistry laboratory I, Utah State University, Logan, Utah, USA.

Spring 2016, CHEM 2325, Laboratory instructor, Organic chemistry laboratory II, Utah State University, Logan, Utah, USA.

Fall 2016, CHEM 2315, Laboratory instructor, Organic chemistry laboratory I, Utah State

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October 1st, 2016 – June 1st, 2017 Department of Chemistry and Biochemistry NMR instrument manager.

Honors

Letter of thanks and appreciation from the college of pharmacy dean, university of Basra, 2005.

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Letter of thanks and appreciation from the college of pharmacy dean, university of Basra, 2009.

Letter of thanks and appreciation from the college of pharmacy dean, university of Basra, 2009.

Letter of thanks and appreciation from the university of Basra president, 2012.

Best teaching assistance award from the Chemistry and Biochemistry department, Utah State University, Logan, USA, 2019.

C. Publications

12. **Alfindee, M. N.**; Subedi, Y. P.; Grilley, M. M.; Takemoto, J. Y.; Chang, C.-W. T., Antifungal activities of 4",6"-disubstituted amphiphilic kanamycins. *Molecules* **2019**, *24* (10), 1882.
11. Kjellgren, A.; Fiori, M. C.; **Alfindee, M. N.**; Subedi, Y. P.; Krishnan, S.; Chang, C.-W. T.; Altenberg, G. A., Inhibition of connexion hemichannels by new aminoglycosides without antibiotic activity. *Biophys. J.* **2019**, *116* (3), 250a.
10. Subedi, Y. P.; **Alfindee, M. N.**; Shrestha, J. P.; Becker, G.; Grilley, M.; Takemoto, J. Y.; Chang, C.-W. T., Synthesis and Biological Activity Investigation of Azole and Quinone Hybridized Phosphonates. *Bioorg. Med. Chem. Lett.* **2018**, *28*, 18, 3034-3037.
9. **Alfindee, M. N.**, Subedi, Y. P., Fiori, M. C., Krishnan S., Kjellgren A., Altenberg G. A., Chang, C.-W. T., Inhibition of Connexin Hemichannels by New Amphiphilic Aminoglycosides without Antibiotic Activity *ACS Med. Chem. Lett.* **2018**, *9*, 7, 697-701.
8. Subedi, Y. P., **Alfindee, M. N.**, Shrestha, J. P., Chang, C.-W. T., Tuning the Biological Activity of Cationic Anthraquinone Analogues Specifically toward *Staphylococcus aureus* *E. J. Med. Chem.* **2018**, *157*, 5, 683-690.
7. Subedi, Y. P.; **Alfindee, M. N.**; Takemoto, J.; Chang, T., Antifungal Amphiphilic Kanamycins: New Life for an Old Drug *MedChemComm* **2018**, *9*, 909-919.
6. **Alfindee, M. N.**; Zhang, Q.; Subedi, Y. P.; Shrestha, J. P.; Kawasaki, Y.; Grilley, M.; Takemoto, J. Y.; Chang, C.-W. T., One-Step Synthesis of Carbohydrate Esters as Antibacterial and Antifungal Agents. *Bioorgan. Med. Chem.* **2018**, *26*, 765-774.
5. Zhang, Q.; **Alfindee, M. N.**; Shrestha, J. P.; Nziko, V. de P. N.; Kawasaki, Y.; Peng, X.; Takemoto, J. Y.; Chang, C.-W. T., Divergent Synthesis of Three Classes of Antifungal Amphiphilic Kanamycin Derivatives. *J. Org. Chem.* **2016**, *81* (22), 10651-10663.
4. Udumula, V.; Nazari, S. H.; Burt, S. R.; **Alfindee, M. N.**; Michaelis, D. J. Chemo- and Site-Selective Alkyl and Aryl Azide Reductions with Heterogeneous Nanoparticle Catalysts, *ACS Catal.* **2016**, *6*(7), 4423-4427.
3. Marina Fosso, **Alfindee, M. N.**, Qian Zhang, Vincent de Paul Nzuwah Nziko, Yukie Kawasaki, Sanjib K. Shrestha, Jeremiah Bearss., Rylee Gregory, Jon Y. Takemoto, and Cheng-Wei Tom Chang, Structure-Activity Relationships for Antibacterial to Antifungal Conversion of Kanamycin to Amphiphilic Analogs, *J. Org. Chem.*, **2015**, *80* (9), pp 4398-4411.
2. **Alfindee, M. N.**, Preparation and Analytical Study of New Chelating Resin Containing Tetracycline Drug, *Journal of Basrah Researches (Sciences)*, **2012**, *38*(3A), 78-88.

1. **Alfindee, M. N.**, Ali M. A. M, Kadhim R. T., Synthesis and Analytical Study of New Chelating Resin Containing Sulfadiazine Drug, *E-Journal of Chemistry*, **2010**, 7(3), 1095-1100.

D. Presentations

Oral Presentations:

- 1) “Amphiphilic Amino Glycosides”, Madher N. Alfindee, organic and inorganic chemistry division seminar, Utah State University, 2014.
- 2) “Structure-Activity Relationships for Antibacterial to Antifungal Conversion of Kanamycin to Amphiphilic Analogs”, Marina Fosso, Madher N. AlFindee, Qian Zhang, Vincent de Paul Nzuwah Nziko, Yukie Kawasaki, Sanjib K. Shrestha, Jeremiah Bearss,, Rylee Gregory, Jon Y. Takemoto, and Cheng-Wei Tom Chang, organic and inorganic chemistry division seminar, Utah State University, 2015.
- 3) “Proposed Total Synthesis of Aranciamycins 1 - 4, Antimycobacterial Anthracyclines from an Australian Marine-Derived Streptomyces sp” Madher N. AlFindee, organic and inorganic chemistry division seminar, Utah State University, 2016.
- 4) “Practical and Concise Synthesizes of Antifungal Amphiphilic Kanamycin Analogs”, Qian Zhang, Madher N. Alfindee, Jaya P. Shrestha, Vincent de Paul Nzuwah Nziko, Yukie Kawasaki, Xinrui Peng, Jon Y. Takemoto, and Cheng-Wei Tom Chang, organic and inorganic chemistry division seminar, Utah State University, 2017.
- 5) “One-step synthesis of carbohydrate esters as antibacterial and antifungal agents”, Madher N. AlFindee, Qian Zhang, Yagya Prasad Subedi, Jaya P. Shrestha, Yukie Kawasaki, Michelle Grilley, Jon Y. Takemoto, Cheng-Wei Tom Chang, organic and inorganic chemistry division seminar, Utah State University, 2018.

Poster Presentation:

- 1) SBI meeting, Utah State University, February 2014, “Structural Optimization of Kanamycin B Analogs”.
- 2) SBI meeting, Utah State University, February 2015, “Structure-Activity Relationship for Antibacterial to Antifungal Conversion of Kanamycin B”.
- 3) SBI meeting, Utah State University, February 2016, “Practical and Divergent Synthesis of Three Classes of Antifungal Amphiphilic Kanamycin Derivatives”.
- 4) 253rd ACS National Meeting, San Francisco, April 2017, “Practical and Divergent Synthesis of Three Classes of Antifungal Amphiphilic Kanamycin Derivatives”.